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Original Article

Aberrant Th2 Immune Responses Are Associated With a Reduced Frequency of IL-35-Induced Regulatory T Cells After Allergen Exposure in Patients With Allergic Asthma

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There are no financial or other issues that might lead to conflict of interest.

ABSTRACT

Purpose: Allergen exposure induces aberrant T helper (Th) 2 immune responses in patients with allergic asthma, but not in sensitized asymptomatic and nonallergic subjects. Interleukin (IL)-35-induced regulatory T (iTr35) cells are a new subset of regulatory T cells with immunoregulatory properties. These cells can significantly suppress Th2 responses in seasonal allergic rhinitis. However, it remains unknown whether iTr35 cells are involved in the immunoregulation of allergic asthmatic individuals after specific allergen exposure.
Methods: The iTr35 cell frequency in peripheral blood mononuclear cells (PBMCs) was measured in patients with allergic asthma as well as in asymptomatic and healthy subjects. The difference in naïve $CD4^+$ T cell conversion to iTr35 cells *in vitro* during allergen stimulation was also investigated. The effects of iTr35 cells on naïve $CD4^+$ T cell differentiation into Th2 cells, $CD4^+CD25^-$ T (Teff) cell proliferation and Th2 cytokine production *in vitro* were assessed.
Results: Significantly reduced iTr35 cell frequencies and IL-35 expression levels were found in asthmatic patients with Derp1 allergy compared with asymptomatic and healthy subjects. Moreover, the circulating iTr35 cell proportion and IL-35 expression level in asthmatic patients gradually decreased with disease severity. Patients with allergic asthma had reduced transformation of naïve $CD4^+$ T cells into iTr35 cells and IL-35 production after allergen exposure compared with asymptomatic and healthy subjects. Most importantly, iTr35 cells inhibited allergen-driven differentiation of naïve $CD4^+$ T cells into Th2 cells, Teff cell proliferation and Th2 cytokine production in an IL-35-dependent manner.
Conclusions: The results of our study suggest that iTr35 cells may play an important role in preventing Th2 responses to allergens by secreting IL-35 and that iTr35 cells may be a potential new immune regulator of allergic asthma.

Keywords: Asthma; allergens; Th2 cells; IL-35; regulatory; immunomodulatory

INTRODUCTION

Allergic asthma is a disease of chronic airway inflammation.[1] T helper (Th) 2 cells predominate and induce allergic airway inflammation in asthmatic patients by producing type 2 cytokines including interleukin (IL)-4, IL-5 and IL-13.[2,3] A variety of allergens, such

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as house dust mite allergens, grass pollen and cockroach allergens, play a crucial role in the pathogenesis of allergic asthma triggered by Th2 cells.[4] However, aberrant Th2-cell responses to allergen exposure are present only in patients with allergic disorders, but not in sensitized asymptomatic or healthy subjects.[5,6] One possible explanation for this is that there are some mechanisms that prevent Th2-cell responses to allergens and mediate immune tolerance in these individuals.

Regulatory T cells (Tregs) play a crucial role in maintaining immune self-tolerance by suppressing the aberrant or excessive function of other cells.[7] Many inhibitory mechanisms are described in Tregs, including the release of suppressive cytokines, such as transforming growth factor-β (TGF-β) and IL-10, and the regulation of cell maturation or function via mechanisms, such as the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA4).[8,9] To date, there have been 2 broad categories of Tregs: natural thymic-derived $CD4^+CD25^+Foxp3^+$ Tregs (nTregs) and peripheral antigen-induced regulatory T cells (iTregs).[10] These iTregs are mainly generated from peripheral naïve T cells in response to specific antigens or cytokine exposure.[11]

Recently, it has been found that a novel inhibitory cytokine secreted by nTregs, IL-35, is a member of the IL-12 family and is a heterodimer comprised of Epstein-Barr virus-induced gene 3 (EBI3) and IL-12p35, which induces the conversion of naïve T cells to IL-35-induced Tregs, called iTr35 cells. These cells are generated by antigenic stimulation both *in vivo* and *in vitro*, and mediate inhibition by producing IL-35.[12] It has been reported that iTr35 cells significantly inhibit Th2 and Th17 responses in patients with seasonal allergic rhinitis.[13,14] Additionally, a decreased proportion of iTr35 cells has been observed in allergic rhinitis patients.[14] These studies have suggested that iTr35 cells effectively regulate immune responses and maintain immune tolerance to allergens in healthy individuals and that their quantities and functions might be defective in patients with allergic disorders. However, it is unknown whether iTr35 cells are involved in the immunoregulation of allergic asthmatic individuals after allergen exposure. In the present study, we enrolled a cohort of allergic asthmatics (with age-/sex-matched sensitized asymptomatic and healthy individuals) and investigated (1) the difference in naïve $CD4^+$ T cell conversion into iTr35 cells after allergen exposure in allergic asthmatic, sensitized asymptomatic and healthy subjects, and (2) the effect of iTr35 cells on *Th2* cell responses during allergen stimulation in patients with allergic asthma.

MATERIALS AND METHODS

Subjects

This research was approved by the Medical Ethics Committee of Zhongnan Hospital (approval number: 2017001), and all donors provided written informed consent. There were 76 volunteers who were recruited for participation between January 2017 and May 2017 (32 allergic asthmatic patients, 19 sensitized asymptomatic individuals, and 25 healthy controls (**Table 1**). All subjects underwent skin prick tests for *Dermatophagoides pteronyssinus* 1 (Derp1) and common environmental allergens. Allergic asthmatic patients were chosen according to the Global Initiative for Asthma criteria[15]: (1) having allergic asthma symptoms, (2) meeting the pulmonary function test criteria of asthma, (3) being monosensitized to *Dermatophagoides pteronyssinus* 1 (Derp1, Indoor Biotechnologies, Charlottesville, VA, USA) (wheal diameter ≥ 3 mm),[16] (4) having no other atopic diseases, and (5) having not used oral or intravenous steroids in the previous 4 weeks. The severity of allergic asthma was assessed on the basis

Table 1. Clinical characteristics of the study subjects

Characteristics	Allergic asthmatic patients (n = 32)	Asymptomatic sensitized subjects (n = 19)	Healthy individuals (n = 25)
Age (years)	33 (28.8–37.0)	31 (28.0–34.5)	32 (26.0–35.0)
Sex (male/female)	15/17	8/11	11/14
FEV1 (% of predicted)	73 (62.8–85.0)*	102 (98.0–110.0)	109 (103.0–116.0)
Derp1-specific IgE (kU/L)	32.9 (21.3–67.6)	33.2 (18.7–40.3)	0*
Total IgE (kU/L)	581.4 (491.3–808.4)	421.9 (379.1–585.2)	42.4 (28.3–65.9)*

FEV1, forced expiratory volume in one second; Derp 1, *Dermatophagoides pteronyssinus* 1; IgE, immunoglobulin E.
*$P < 0.05$.

of the Global Initiative for Asthma criteria.[15] Sensitized asymptomatic subjects were chosen according to the following criteria: (1) having no allergy symptoms of allergic rhinitis, asthma or other atopic diseases, and (2) being monosensitized to Derp1 (wheal diameter ≥ 3 mm). Healthy controls had no allergic diseases and had negative reactions to Derp1 and common environmental allergens.

Assay of total immunoglobulin E (IgE) and Derp1-specific IgE

The serum levels of total IgE and specific IgE (sIgE) to Der1 were quantified using fluorescence enzyme immunoassay (ImmunoCAP immunoassay system, Thermo Fisher, Waltham, MA, US). The sIgE levels of less than 0.35 kU/L were considered negative.[17]

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were separated from the peripheral blood samples of donors by standard density gradient centrifugation. The PBMCs isolated were washed twice in phosphate-buffered saline and then resuspended in RPMI-1640 medium. Cell viability was examined using trypan blue assay (more than 95%). Plasma samples of all subjects were harvested and stored at −70°C for measurement.

Detection of iTr35 cells among PBMCs

Separated PBMCs (1×10^6 cells/mL) from each subject were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin (Sigma-Aldrich, St. Louis, MO, USA) for 4 hours at 37°C in an atmosphere of 5% carbon dioxide. Activated PBMCs were cultured with 3 µg/mL brefeldin A (eBioscience, San Diego, CA, USA) for 3 hours. Cell viability was assessed by trypan blue staining (more than 95%) before staining with mAbs, and cells were collected for flow cytometry. Briefly, iTr35 cells were defined as $CD4^+Foxp3^-EBI3^+p35^+$ T cells.[12] Cells were surface immunostained with FITC-anti-human CD4 (11-0049-42; eBioscience, San Diego, CA, USA) for 30 minutes and then further fixed and permeabilized (00-5123-43 and 00-8333-56; eBioscience). Intracellular staining was performed with Foxp3-APC, EBI3-PE and IL-12p35-PerCP (17-4776-42, 12-7358-42 and MA5-23622; eBioscience). All stained PBMCs were detected by a FACSCanto II (BD, Oxford, United Kingdom).

Cell sorting

Human naïve $CD4^+$ T cells, effector T (Teff) cells and dendritic cells (DCs) were further selected from PBMCs using the EasySep cell isolation kit (StemCell Technologies, Vancouver, British Columbia, Canada). Naïve $CD4^+$ T and Teff cells were defined as the $CD4^+CD45RA^+$ T cells and $CD4^+CD25^-$ T cells, respectively. The cell purity was greater than 96%.

Generation of iTr35 cells *in vitro*

The iTr35 cell lineage was generated as previously described.[12] Isolated naïve $CD4^+$ T cells (1×10^5 cells/mL) were cultured with recombinant Derp1 (5 µg/mL) and rhIL-35 (10 ng/mL;

Chimerigen, San Diego, CA, USA). RPMI-1640 medium was half-changed on the fourth day by adding Derp1 and rhIL-35 to maintain the initial concentration. Subsequently, to determine the percentage of iTr35 cells ($CD4^+Foxp3^-EBI3^+p35^+$ T cells), cultured naïve $CD4^+$ T cells were harvested 6 days later and then stained with the same fluorescent antibodies as PBMCs for flow cytometry. Finally, generated iTr35 cells were isolated using a cell enrichment and detection kit (130-092-122, Miltenyi Biotec, Bergisch Gladbach, Germany) and detection antibody EBI3-PE (eBioscience).[18] Freshly generated iTr35 cells were washed twice with complete culture medium to remove rhIL-35, adjusted for cell number (5×10^4 cells/mL), and further expanded with recombinant Derp1 stimulation *in vitro.* After 3 days, cells and culture supernatants were harvested for IL-35 expression level detection and subsequent functional assays.

Carboxyfluorescein succinimidyl ester (CFSE)-based suppression assays

Freshly isolated autologous responder T cells (naïve $CD4^+$ or Teff cells) were labeled using fluorescent 5,6-CFSE. The CFSE-labeled responder T cells were then cultured with autologously generated iTr35 cells under different conditions *in vitro*. CFSE-based suppression assays were performed using flow cytometry to measure the proliferation of responder cells and were assessed by calculating the division index (DI).[19]

Effect of iTr35 cells on naïve $CD4^+$ T-cell proliferation and Th2 differentiation

CFSE-naïve $CD4^+$ T cells (5×10^4 cells/well) were cocultured with autologous dendritic cells (5×10^3 cells/well). Derp1 (5 μg/mL) and recombinant human thymic stromal lymphopoietin (TSLP, 15 ng/mL; R&D Systems, Minneapolis, MN, USA) were used for stimulation in the presence or absence of autologously generated iTr35 cells (5×10^3 cells/well) for 72 hours. The neutralizing isotype antibody (20 μg/mL; Clone # 11711, R&D Systems) or anti-IL-35 Ab (10 μg/mL or 20 μg/mL; Clone # 607201 and 27537, R&D Systems) was added to the culture. The division index (DI) of CFSE-labeled naïve $CD4^+$ T cells was analyzed by flow cytometry and FlowJo VX (Ashland, OR, USA). The percentage of Th2 cells and the GATA-3 mRNA expression level were measured by flow cytometry and quantitative polymerase chain reaction (qPCR), respectively. Th2 cells were defined as the $CD4^+IL\text{-}4^+$ T cells. The culture supernatants were harvested for the detection of Th2 cytokines.

Effect of iTr35 cells on Teff-cell proliferation and cytokine production

CFSE-labeled Teff cells (1×10^5 cells/well) from patients with allergic asthma were cultured with autologous DCs (5×10^3 cells/well) and different concentrations of autologous iTr35 cells under Derp1 stimulation for 5 days. Anti-IL-35 antibody (10 μg/mL or 20 μg/mL, R&D Systems) or isotype antibody was used in neutralization assays. The suppression of Derp1-mediated Teff-cell proliferation *in vitro* by iTr35 cells was measured according to the division index (DI). The culture supernatants were harvested for the detection of Th2, Th17 and Th1 cytokines.

RNA isolation and qPCR

Total RNA was extracted using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using the ReverTra Ace qPCR RT kit protocol. (Toyobo, Osaka, Japan) and amplified using SYBR Premix Ex Taq™ (Takara Bio, Inc., Otsu, Japan). Target gene expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase using the $2^{(-\Delta\Delta Ct)}$ method.[20] The primer sequences are described in **Table 2.**[21-23]

Detection of cytokines

The levels of IL-35, IL-10, IL-4, IL-5, IL-13, IL-17 and interferon (IFN)-γ were determined using enzyme-linked immunosorbent assay according to the manufacturer's protocol (eBioscience).

Table 2. Primer sequences for real-time qPCR

Genes	Primer sequences (5′–3′)
EBI3	
Forward	TCCTTCATTGCCACGTACAG
Reverse	GCTCTGTTATGAAAGGCACG
IL-12p35	
Forward	TGCAAAGCTTCTGATGGATCC
Reverse	AAAATCCGGTTCTTCAAGGGA
GATA-3	
Forward	GAACCGGCCCCTCATTAAG
Reverse	ATTTTTCGGTTTCTGGTCTGGAT
GAPDH	
Forward	GGTGTGAACCATGAGAAGTATGACA
Reverse	GTCCTTCCACGATACCAAAGTTGT

qPCR, quantitative polymerase chain reaction; EBI3, Epstein-Barr virus-induced gene 3; IL, interleukin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

All statistical analyses were performed using Prism version 7 (GraphPad Software, La Jolla, CA, USA). The data are presented as the median (interquartile range). A P value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of the study subjects

Thirty-two allergic asthmatic patients, 19 sensitized asymptomatic individuals, and 25 healthy controls were recruited (**Table 1**). The 3 groups were age- and sex-matched. Asthmatic patients had significantly decreased FEV_1% values in the pulmonary function test compared with those of asymptomatic individuals and healthy controls ($P < 0.05$). The serum levels of total IgE and sIgE to Derp1 were significantly higher in asthmatic patients and asymptomatic individuals than in healthy controls ($P < 0.05$ for each).

Allergic asthmatic patients have reduced iTr35-cell frequencies and IL-35 expression levels

The iTr35-cell frequency in PBMCs was measured in allergic asthmatic patients, asymptomatic individuals and healthy controls (**Fig. 1A**). As shown in **Fig. 1B and C**, asthmatic patients had significantly reduced iTr35-cell frequencies and IL-35 protein levels in peripheral blood compared with asymptomatic individuals and healthy controls ($P < 0.001$ for each). In contrast, the iTr35-cell frequency and the concentration of IL-35 in peripheral blood were similar to those of asymptomatic individuals and healthy controls. Moreover, we analyzed the iTr35-cell frequencies and IL-35 expression levels in asthmatic patients with different disease severities. The results revealed that the proportion of circulating iTr35 cells and the concentration of IL-35 in asthmatic patients gradually decreased in mild, moderate and severe asthmatic patients according to severity (**Fig. 1D and E**). We further studied the relationship between IL-35 protein levels and iTr35-cell frequencies in the 3 groups. The results revealed that the IL-35 protein level was positively correlated with the proportion of iTr35 cells in each group (**Fig. 1F-H**).

Correlations between serum cytokine levels and iTr35-cell frequency in peripheral blood

To investigate the potential role of iTr35 cells in driving Th2 immunity in allergic asthmatic patients, the correlations between the iTr35 cell frequency and serum Th2 cytokine levels

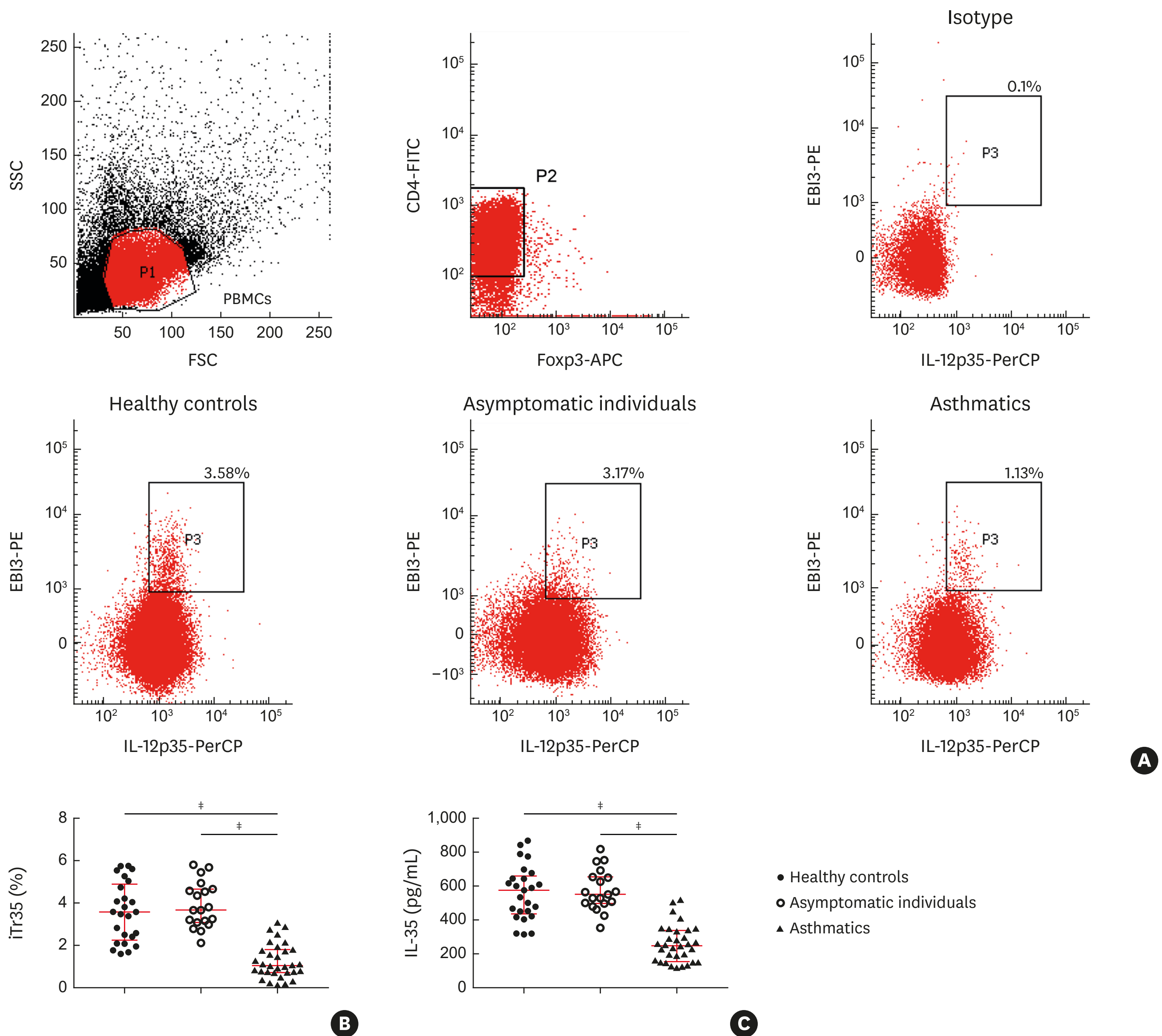

Fig. 1. Reduced iTr35 cell counts and IL-35 levels in allergic asthmatic patients. (A) Flow cytometric gating strategy and representative flow cytometry plots of iTr35 cells in PBMCs from healthy controls, asymptomatic subjects and asthmatic patients. (B) The frequency of iTr35 cells in PBMCs and (C) serum IL-35 protein levels from healthy controls (n = 25), asymptomatic subjects (n = 19) and asthmatic patients (n = 32).
SSC, side scatter; FSC, forward scatter; FITC, fluorescein isothiocyanate; EBI3, Epstein-Barr virus-induced gene 3; iTr35, IL-35-induced regulatory T; IL, interleukin; PBMC, peripheral blood mononuclear cell.
*P < 0.05, †P < 0.01, ‡P < 0.001.

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were investigated in three groups. The results showed that IL-4, IL-5, and IL-13 levels were inversely correlated with the iTr35 cell frequency in asthmatic, asymptomatic and healthy individuals (**Fig. 2A-C**). Moreover, serum Derp1-specific IgE levels were inversely correlated with the percentage of iTr35 cells in asthmatic individuals. However, there was no significant correlation between serum total IgE levels and the iTr35 cell frequency in the asthmatic group (**Fig. 2D and E**).

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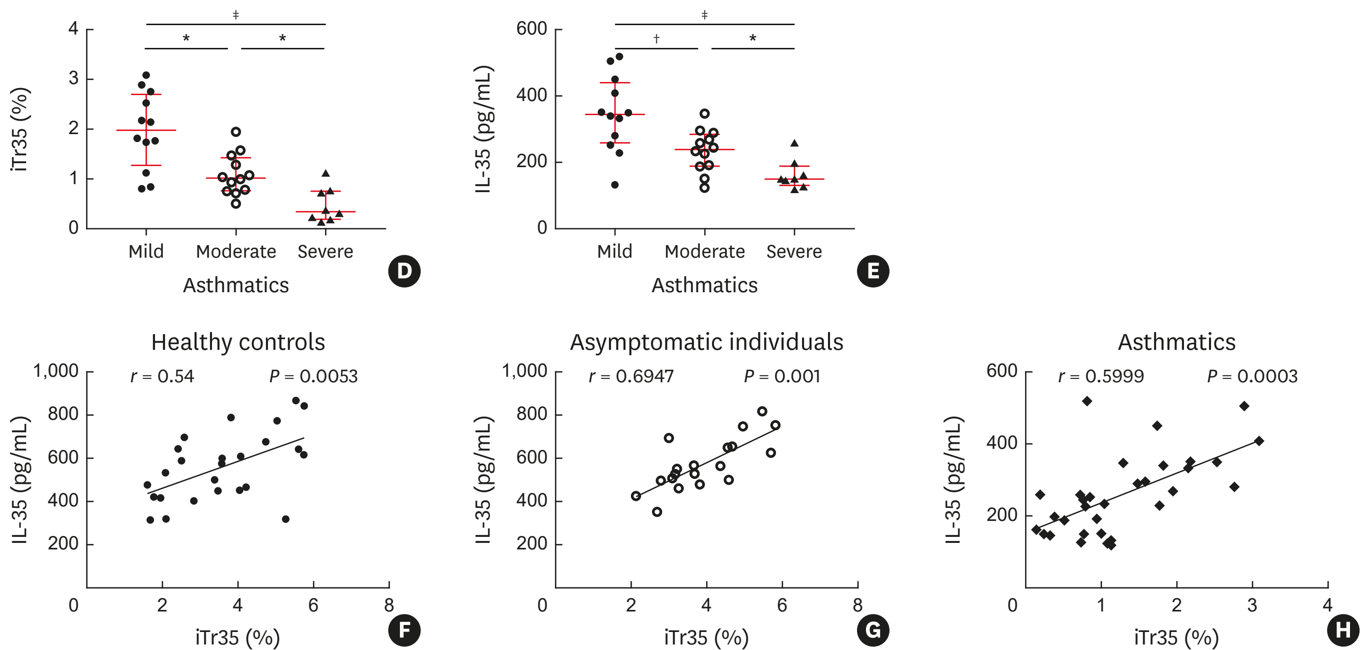

Fig. 1. (Continued) Reduced iTr35 cell counts and IL-35 levels in allergic asthmatic patients. (D) The frequency of iTr35 cells and (E) serum IL-35 levels in asthmatic patients with different disease severities (mild: n = 12, moderate: n = 12, severe: n = 8). The data combined from at least 5 independent experiments. *P*-values were determined by the Kruskal-Wallis *H*-test. (F-H) Correlations between the frequency of iTr35 cells and IL-35 levels among the 3 groups (healthy: n = 25, asymptomatic: n = 19, asthmatic: n = 32). *P*-values were determined by Spearman's test.
SSC, side scatter; FSC, forward scatter; FITC, fluorescein isothiocyanate; EBI3, Epstein-Barr virus-induced gene 3; iTr35, IL-35-induced regulatory T; IL, interleukin; PBMC, peripheral blood mononuclear cell.
$^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$.

Correlations between serum cytokine levels and the levels of serum IL-35 and IL-10 in asthmatic patients

The correlations between serum cytokine levels and the levels of serum IL-35 and IL-10 were investigated in asthmatic patients. The results indicated that Th2 cytokines (IL-4, IL-5, and IL-13) and sIgE levels were inversely correlated with the serum IL-35 levels in asthmatic patients (**Fig. 3A**). We also measured the serum IL-10 levels in asthmatic patients, but we did not find any inverse correlations of Th2 cytokines or sIgE with serum IL-10 levels (**Fig. 3B**).

Decreased transformation of naïve CD4⁺ T cells into iTr35 cells after allergen stimulation in asthmatic patients *in vitro*

It has been reported that iTr35 cells can be induced from naïve $CD4^+$ T cells after allergen stimulation and have an IL-35-dependent suppressive function. Therefore, the difference in naïve $CD4^+$ T cell conversion to iTr35 cells *in vitro* during allergen stimulation in asthmatic patients, asymptomatic individuals and healthy controls was investigated. As shown in **Fig. 4A and B**, iTr35 cells generated from naïve $CD4^+$ T cells after Derp1 stimulation were observed in asthmatic patients, asymptomatic individuals and healthy controls. However, the proportion of generated iTr35 cells showed a significant difference among the 3 groups ($P < 0.001$ for all). Allergic asthmatic patients had a significantly reduced proportion of iTr35 cells compared with asymptomatic individuals and healthy controls, while asymptomatic individuals and healthy controls showed the same percentage as iTr35 cells after Derp1 stimulation (**Fig. 4B**). In addition, IL-35 expression levels in Derp1-stimulated cultures from the 3 groups were assessed. Allergic asthmatic patients had lower IL-35 protein expression in culture supernatants than asymptomatic individuals and healthy controls (**Fig. 4C**). IL-35 mRNA levels were also significantly lower in allergic asthmatic patients than in asymptomatic individuals and healthy

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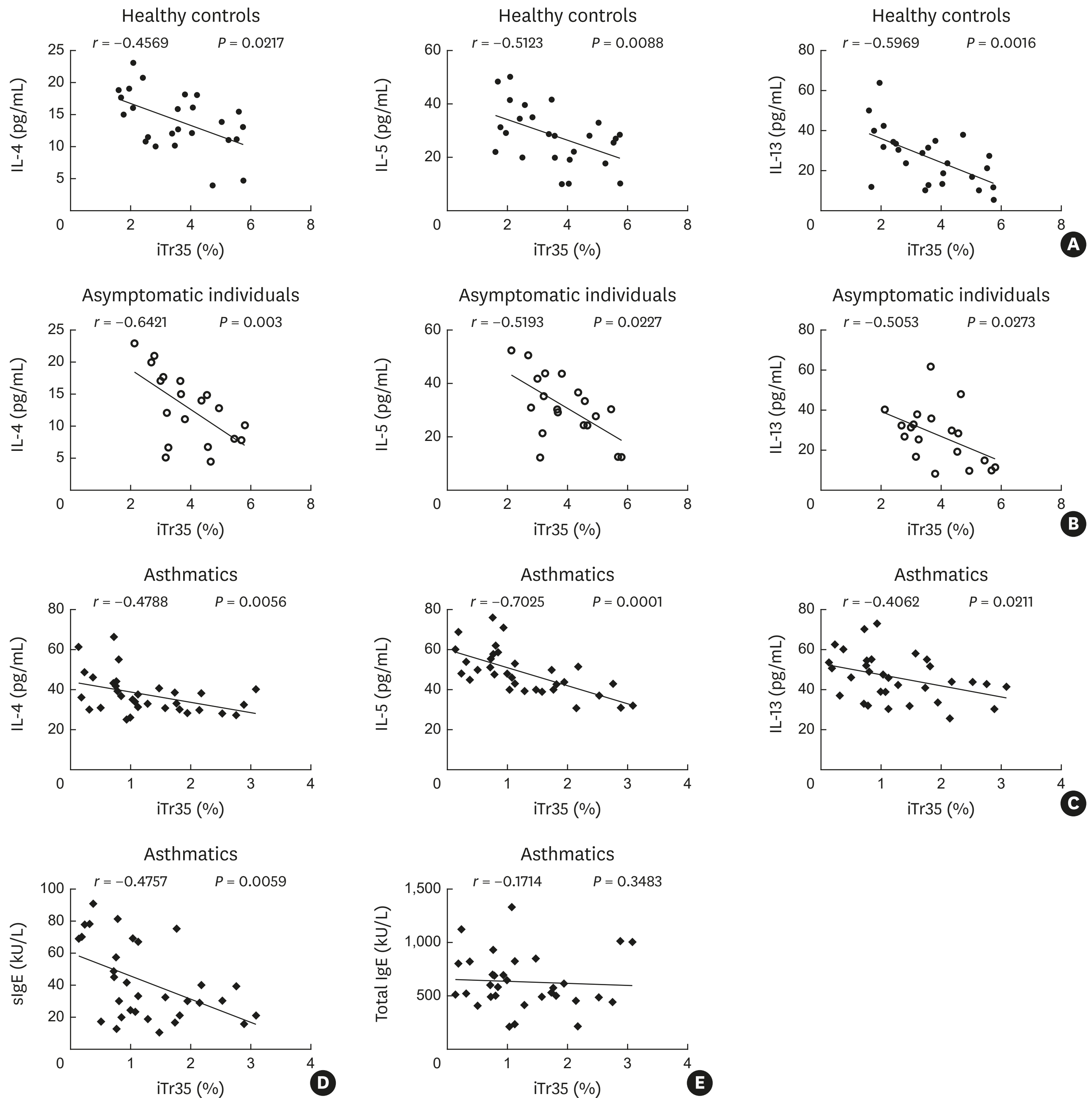

Fig. 2. Correlations between serum cytokine levels and the frequency of iTr35 cells in peripheral blood. IL-4, IL-5 and IL-13 levels were inversely correlated with the frequency of iTr35 cells in the (A) healthy controls (n = 25), (B) asymptomatic subjects (n = 19) and (C) asthmatic patients (n = 32). (D) Serum Derp1-specific IgE levels were inversely correlated with the frequency of iTr35 cells in asthmatic patients. (E) There was no correlation between serum total IgE levels and the frequency of iTr35 cells in asthmatic patients. *P*-values were determined by Spearman's test.
iTr35, IL-35-induced regulatory T; IL, interleukin; IgE, immunoglobulin E; sIgE, specific immunoglobulin E.

controls (**Fig. 4D and E**). Transformation of naïve $CD4^+$ T cells into iTr35 cells and IL-35 production were reduced after allergen exposure in asthmatic patients.

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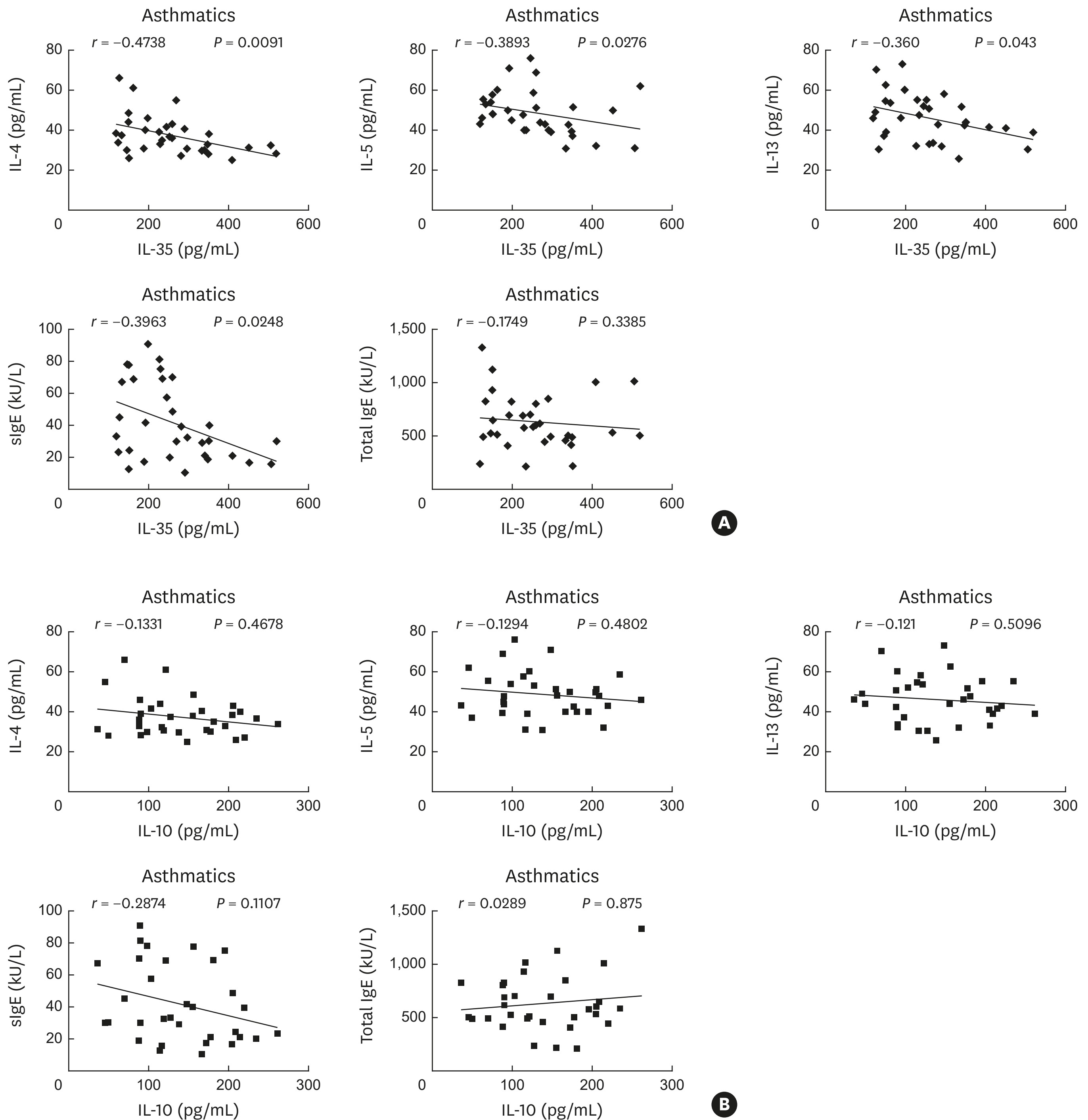

Fig. 3. Correlations between serum cytokine levels and the levels of serum IL-35 and IL-10 in asthmatic individuals. (A) Serum IL-4, IL-5, IL-13, and sIgE levels were inversely correlated with the levels of serum IL-35. (B) There was no correlation between Th2 cytokines or sIgE and serum IL-10 levels. *P*-values were determined by Spearman's test.
IL, interleukin; sIgE, specific immunoglobulin E; Th, T helper; IgE, immunoglobulin E.

Inhibition of naïve CD4⁺ T-cell proliferation by iTr35 cells

Previous studies have indicated that TSLP is involved in dendritic cell maturation and priming of Th2-immune responses in patients with allergic asthma.[14,24] Therefore, the effect of generated iTr35 cells on naïve $CD4^+$ T-cell proliferation and differentiation was assessed. The

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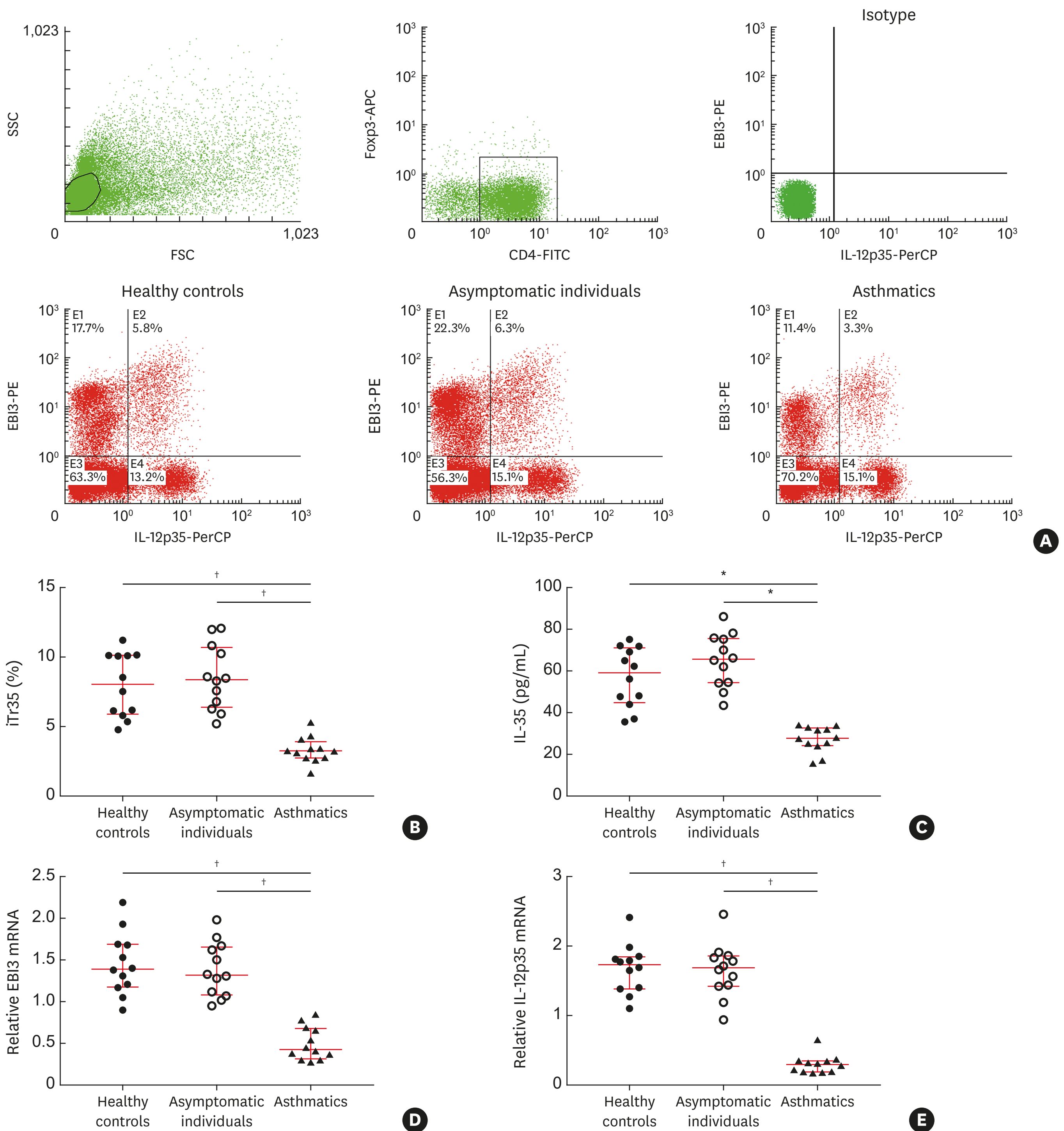

Fig. 4. The difference in conversion of naïve CD4+ T cells to iTr35 cells *in vitro*. (A) Flow cytometric gating strategy and representative flow cytometry plots of iTr35 cells in naïve CD4+ T cells from healthy controls, asymptomatic subjects and asthmatic patients. (B) The proportion of iTr35 cells in naïve CD4+ T cells and (C) IL-35 protein levels in the culture supernatants of naïve CD4+ T cells from healthy controls, asymptomatic subjects and asthmatic patients that were co-cultured with Derp1 (n = 12 per group). *P*-values were determined by the Kruskal-Wallis *H*-test. (D, E) IL-35 mRNA expression levels in naïve CD4+ T cells with Derp1 stimulation in the healthy controls, asymptomatic subjects and asthmatic patients (n = 12 per group). The data are combined from at least eight independent experiments. *P*-values were determined by the Kruskal-Wallis *H*-test.
SSC, side scatter; FSC, forward scatter; FITC, fluorescein isothiocyanate; EBI3, Epstein-Barr virus-induced gene 3; iTr35, IL-35-induced regulatory T; IL, interleukin; Der p1, *Dermatophagoides pteronyssinus* 1.
*$P < 0.01$, †$P < 0.001$.

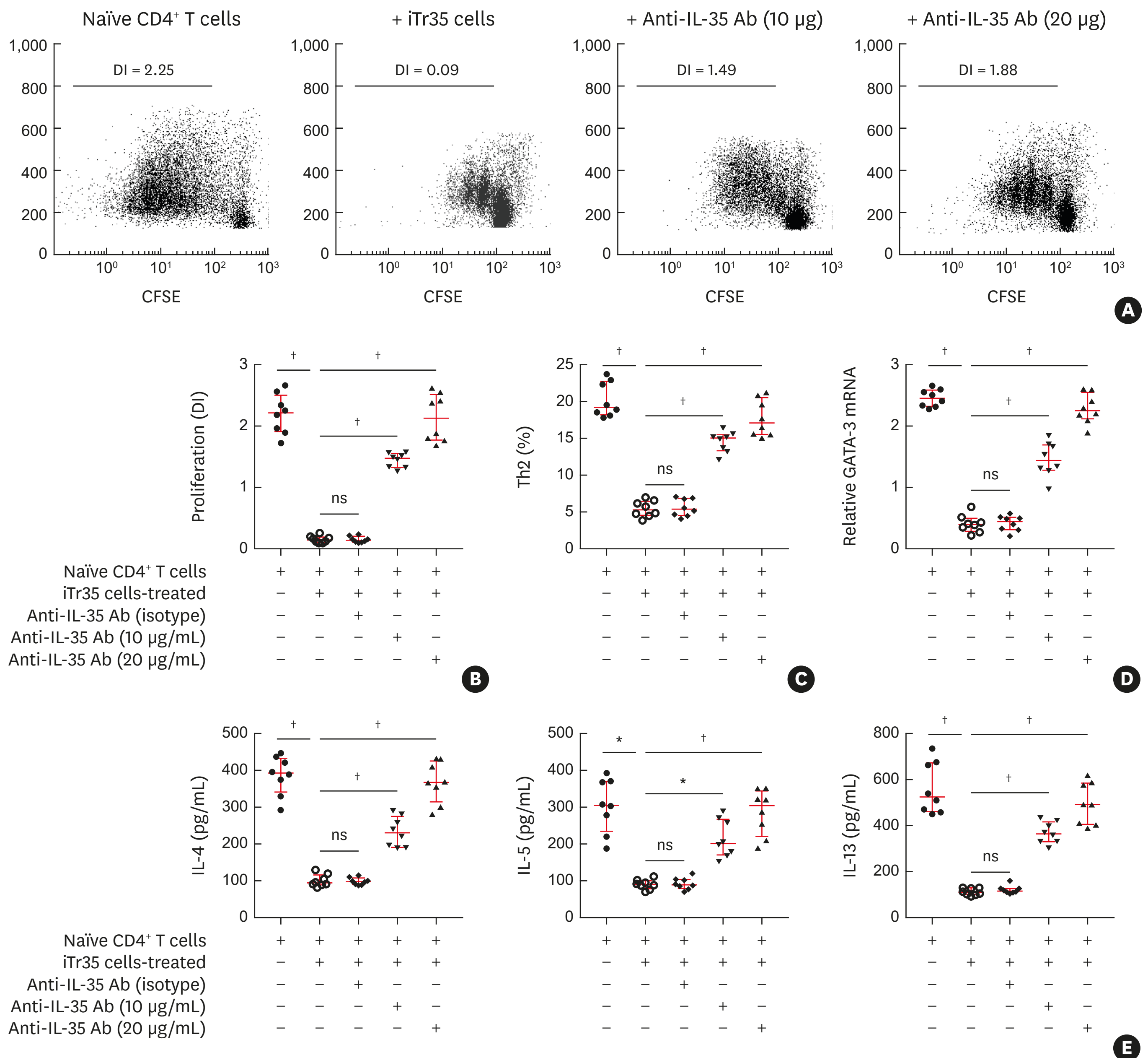

Fig. 5. Effect of iTr35 cells on naïve CD4+ T-cell proliferation and Th2 differentiation. Naïve CD4+ T cells were obtained from PBMCs of allergic asthmatic patients and cocultured with dendritic cells for 72 hours. Derp1 allergen and TSLP were used for stimulation in the presence or absence of autologous iTr35 cells that were supplemented with neutralizing anti-IL-35 antibodies. (A) Representative dot plots of naïve CD4+ T-cell proliferation under different conditions. (B) Naïve CD4+ T-cell proliferation was analyzed by calculating the division index. (C) The percentage of Th2 cells (CD4+IL-4+ T cells) and (D) the GATA-3 mRNA level. (E) IL-4, IL-5, and IL-13 production. The data are combined from at least 10 independent experiments (n = 8 per group). *P*-values were determined by the Friedman test.
iTr35, IL-35-induced regulatory T; Th, T helper; PBMC, peripheral blood mononuclear cell; Derp1, Dermatophagoides pteronyssinus 1; TSLP, thymic stromal lymphopoietin; IL, interleukin; ns, not significant; DI, division index.
*$P < 0.01$, †$P < 0.001$.

results showed that activated DCs that were stimulated with Derp1 and TSLP induced robust proliferation of naïve CD4+ T cells and that iTr35 cells suppressed naïve CD4+ T cell proliferation in an IL-35-dependent manner (**Fig. 5A and B**). Moreover, iTr35 cells also inhibited the proportion of Th2 cells (**Fig. 5C**), the GATA-3 mRNA expression level (**Fig. 5D**) and Th2 cytokine production (**Fig. 5E**) in an IL-35-dependent manner. These results collectively revealed that iTr35 cells inhibited the proliferation of naïve CD4+ T cells and their differentiation into Th2 cells.

Suppression of the proliferation of Derp1-driven Teff cells and Th2-cytokine production by iTr35 cells

$CD4^+CD25^-$ T (Teff) cells were cultured with DCs and different concentrations of autologously generated iTr35 cells in the presence of Derp1. We investigated the proliferation of Derp1-stimulated CFSE-labeled Teff cells by flow cytometry. The results showed that iTr35 cells inhibited the proliferation of Derp1-stimulated Teff cells (**Fig. 6A and B**). Additionally, the inhibitory effects of iTr35 cells on Teff cell proliferation were dose-dependent. Interestingly, an anti-IL-35 antibody also significantly reversed this suppression in a dose-dependent manner (**Fig. 6C-F**). Derp1-stimulated Th2 and Th17 cytokines contribute to allergic asthma. Therefore, the levels of IL-4, IL-5, IL-13, IL-17 and (IFN)-γ were measured in the culture supernatants of activated Teff cells. The results suggested that iTr35 cells inhibited IL-4, IL-5, IL-13 and IL-17 production, and this suppression was also neutralized by an anti-IL-35 antibody (**Fig. 7A and B**). In contrast, iTr35 cells induced IFN-γ production in Teff cell supernatants (**Fig. 7C**).

DISCUSSION

It has been known that iTr35 cells, a newly discovered subset of induced regulatory T cells, have strong suppressive and regulatory functions by secreting IL-35 *in vivo*.[12] These cells express no Foxp3 and require no inhibitory cytokines (IL-10 or TGF-β). Previous studies have suggested that iTr35 cells generate an important mediator of infectious tolerance and contribute to tumor progression.[25,26] In addition, iTr35 cells has also been identified as a novel important immune regulator in patients with seasonal allergic rhinitis.[14] However, the role of iTr35 cells in patients with allergic asthma remains to be elucidated. In this study, we observed decreases in the number of circulating iTr35 cells and serum IL-35 levels in asthmatic patients compared with asymptomatic subjects and healthy controls, which was partly consistent with our previous findings.[27] We have shown that the ability to generate iTr35 cells is dramatically reduced and that iTr35 cells suppress Th2-immune responses to allergens in an IL-35-dependent manner in asthmatic patients. These results collectively demonstrate that iTr35 cells are involved in the immunopathogenesis of allergic asthma and play an important protective role by secreting IL-35 after allergen stimulation.

Previous studies demonstrated that IL-35 effectively inhibits activated Teff cell proliferation and that decreased IL-35 levels are involved in asthmatic patients.[27-29] However, the reason for reduced IL-35 levels in allergic asthma is unclear. It has been reported that IL-35 is mainly secreted by nTregs.[30] Recently, iTr35 cells were also found to possess strong suppressive functions by producing IL-35 and could be generated by naïve $CD4^+$ T cells via a positive feedback loop,[12] which provides us with new insight into the reason for decreased IL-35 in allergic asthma. Interestingly, our study offered indirect evidence that the transformation the naïve $CD4^+$ T cells into iTr35 cells is reduced in allergic asthmatic patients and that IL-35 is produced after Derp1 stimulation *in vitro* compared with those of asymptomatic subjects and healthy controls. Therefore, the decreased IL-35 levels are possibly a result of the reduced transformation of naïve $CD4^+$ T cells into iTr35 cells in asthmatic patients after allergen stimulation. Sensitized asymptomatic subjects may display different immune responses to allergen stimulation from allergic asthmatic patients. Asymptomatic subjects showed a significant increase in allergen-specific iTr35 cells and IL-35 production after allergen stimulation as in healthy controls. However, asthmatic patients had a lower percentage of iTr35 cells than sensitized asymptomatic subjects with Derp1 stimulation. These findings are in agreement with those of previous studies,[31] which can partly explain the immune characteristics of asymptomatic subjects after allergen exposure.

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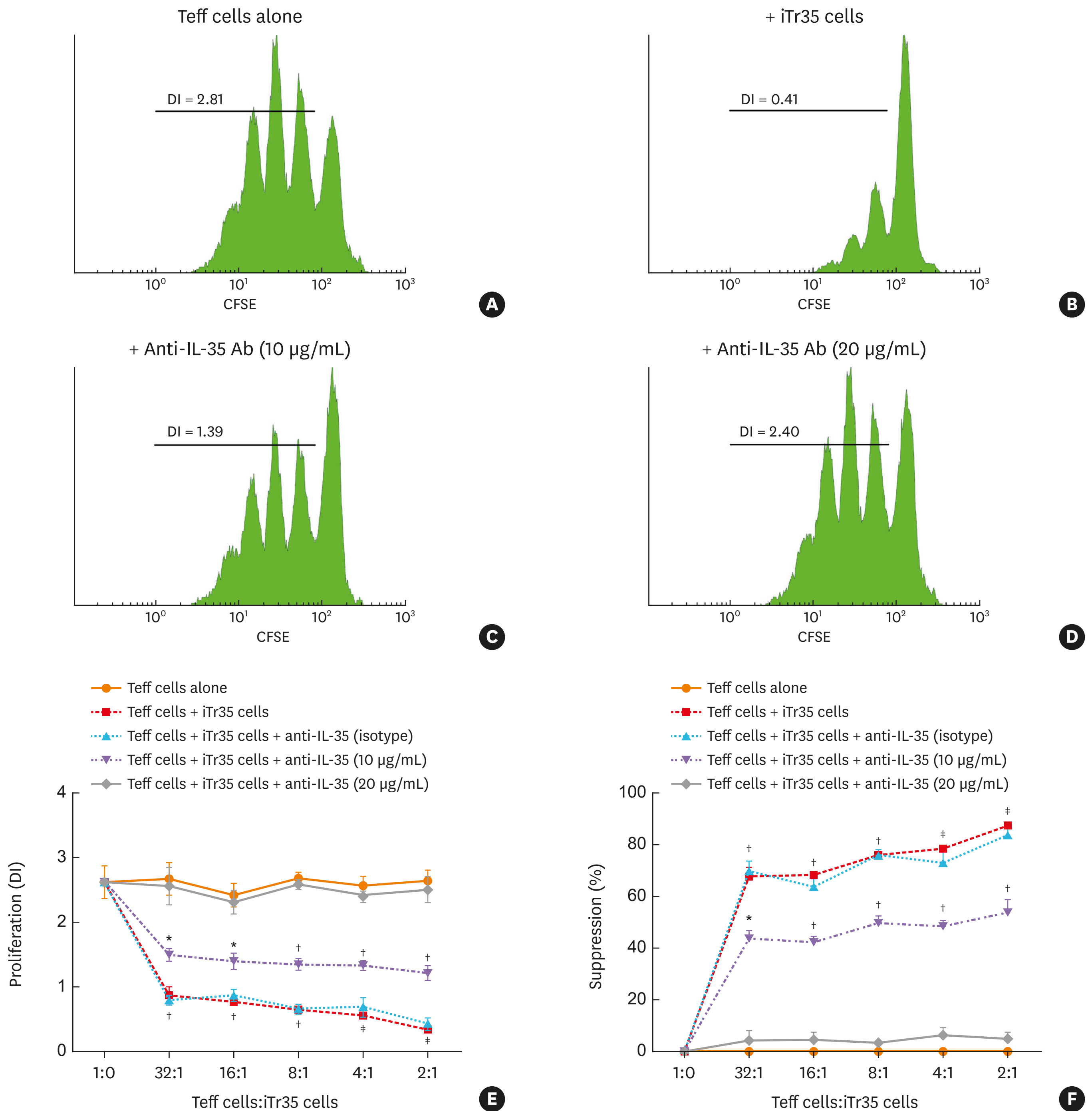

Fig. 6. Effect of iTr35 cells on allergen-driven Teff cell proliferation. Teff cells from patients with allergic asthma (n = 8) were cultured with DCs and different concentrations of autologous iTr35 cells under stimulation with Derp1 in the presence or absence of neutralizing anti-IL-35 antibodies. (A-D) Representative histograms of CFSE$^+$ Teff cell proliferation under different conditions (Teff cells:iTr35 cells = 2:1). (E) Derp1-driven CFSE$^+$ Teff cell proliferation was analyzed by calculating the division index, and (F) the suppression induced by iTr35 cells was measured. The data are combined from at least 4 independent experiments (n = 8 per group). *P*-values were determined by 2-way repeated analysis of variance.

iTr35, IL-35-induced regulatory T; Teff, effector T; DC, dendritic cell; Derp1, Dermatophagoides pteronyssinus 1; IL, interleukin; CFSE, carboxyfluorescein succinimidyl ester; DI, division index.

$^*P < 0.05$, $^†P < 0.01$, $^‡P < 0.001$.

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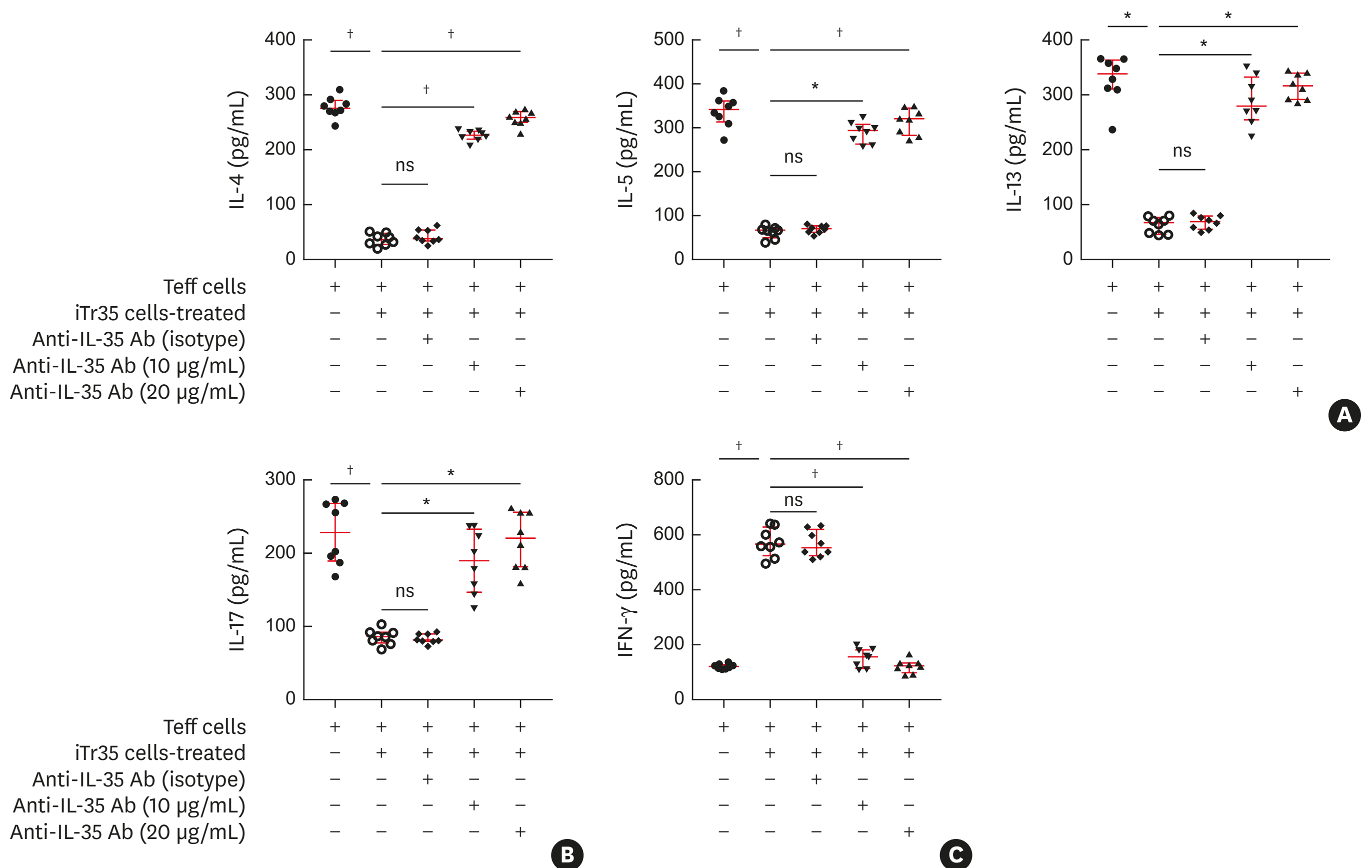

Fig. 7. Effect of iTr35 cells on allergen-stimulated Teff cell cytokine production. Teff cells from patients with allergic asthma (n = 8) were cultured with DCs and autologous iTr35 cells (Teff cells:iTr35 cells = 2:1) under stimulation with Derp1 for 5 days. Anti-IL-35 antibodies were used in neutralization assays. The concentrations of (A) IL-4, IL-5, IL-13, (B) IL-17 and (C) IFN-γ in culture supernatants. The data are combined from at least 5 independent experiments (n = 8 per group). *P*-values were determined by the Friedman test.
iTr35, IL-35-induced regulatory T; Teff, effector T; DC, dendritic cell; Derp1, Dermatophagoides pteronyssinus 1; IL, interleukin; IFN, interferon; ns, not significant.
$^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$.

Type 2 cytokines (IL-4, IL-5, and IL-13) are vital in stimulating B cells to secrete allergen-specific IgE and eosinophil infiltration, which promotes the classical Th2 allergic response.[2,32,33] Interestingly, our study showed that the number of iTr35 cells and the IL-35 level were inversely correlated with type 2 cytokines (IL-4, IL-5, and IL-13) and sIgE levels in asthmatic patients. Therefore, it is likely that excessive Th2-immune responses are associated with reduced allergen-specific iTr35 cells or IL-35 levels in allergic asthmatic patients. It has been demonstrated that GATA-3 is a key transcription factor for Th2-cell differentiation.[34] In addition, TSLP promotes DC maturation and stimulates naïve $CD4^{+}$ T cells to proliferate and differentiate into Th2 cells.[24] In the present study, we observed that iTr35 cells suppressed naïve $CD4^{+}$ T-cell proliferation and differentiation into Th2 cells in an IL-35-dependent manner, leading to suppression of GATA-3 expression and allergen-induced Th2 cytokine production. These findings suggest that allergen-specific iTr35 cells inhibit Th2-immune responses at the transcriptional and differentiation levels.

Allergen exposure leads Th2 cells to produce cytokines such as IL-4, IL-5, and IL-13,[35,36] whereas Th1 cells secrete IFN-γ and neutralize aberrant Th2-immune responses in allergic asthma.[37] Th17 cells have also been shown to contribute to allergic airway disease by producing IL-17.[38] It is not clear whether iTr35 cells can regulate Teff cell proliferation

and cytokine secretion in allergic asthma. As previously described,[14] we also found that iTr35 cells inhibited Derp1-stimulated Teff cell proliferation and cytokine production in allergic asthmatic patients. Moreover, these suppressive effects were IL-35-dependent, and neutralizing IL-35 antibody reversed this suppression. Our findings highlight that iTr35 cells contribute to suppressing effector T-cell proliferation and Th2-cytokine production by IL-35 after allergen exposure in asthmatic patients. However, exact mechanisms and signaling pathways required for iTr35-cell-mediated suppression remain unclear. Further studies are needed to confirm our results.

In conclusion, reduced transformation of naïve $CD4^+$ T cells into iTr35 cells was found after allergen exposure in allergic asthmatic patients. Increased allergen-specific iTr35 cells play a crucial role in preventing excessive Th2-cell responses to allergens by secreting IL-35 in sensitized asymptomatic subjects. These findings demonstrate that iTr35 cells, a novel type of induced Tregs, may be a potential immune mediator and have the potential to exert anti-allergic effects and promote allergen tolerance, which provides new evidence for the pathogenesis and treatment of allergic asthma.

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