

ORIGINAL ARTICLE

FOXP3-marked IL-17a-producing regulatory T cells are increased in patients with allergic rhinitis

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Abstract

Conclusion: The deficient regulatory T cells may play a role in the development of allergic rhinitis. **Objective:** A subpopulation of regulatory T cell that produce IL-17a were proved to show an apparent duality of regulatory T cell and effector T helper-17 cells. We studied this subpopulation in patients with allergic rhinitis. **Methods:** Fresh whole blood from 20 patients with allergic rhinitis and 19 healthy donors was used to investigate the frequencies of CD4⁺IFN- γ ⁺ T cells (effector T helper-1), CD4⁺IL-4⁺ T cells (effector T helper-2) and CD4⁺IL-17a⁺ T cells (effector T helper-17) by flow cytometry after stimulation for 4–6 h. Simultaneously, CD4⁺ T lymphocytes were isolated from peripheral blood mononuclear cells and then the frequencies of regulatory T cells and IL-17a-producing regulatory T cells in the allergic rhinitis group were compared with healthy controls after T-cell antigen receptor stimulation for 48 h. The suppressive capacity of CD4⁺CD25^{high}CD127^{low} regulatory T cells that were stimulated by Dermatophagoides pteronyssinus in both groups was isolated and assessed. **Results:** The frequencies of effector T helper-17 and effector T helper-2 cells were higher in the allergic group compared with healthy controls. Regulatory T cells were similar in both groups, but IL-17a-producing regulatory T cells were increased in the allergic group. In addition, the capacity of regulatory T cells to suppress effector T helper-17 cytokine production was significantly decreased in the allergic group.

Keywords: Th1, Th2, Th17, Treg

Introduction

In the last two decades, the regulatory T cell (Treg) has been identified as an essential subset in immune tolerance. The mechanism of allergic rhinitis was shown to be connected to effector T helper (Th) lymphocytes and Treg [1]. In the context of allergy, peripheral blood mononuclear cells (PBMCs) display an expansion of interleukin (IL)-4-producing Th2 cells and decreased interferon (IFN)- γ -producing Th1 cells [2,3]. Treg cells play a predominant role in allergen-specific immunotherapy (allergen-SIT) through inhibition of the development of allergic Th2 responses [4]. Besides, IL-17a-producing Th17, a distinct lineage of CD4⁺

T cells, was found to be a pro-inflammatory helper T cell in an asthmatic mouse model [5]. In human peripheral blood, a subpopulation of Treg has the capacity to produce IL-17a, sharing phenotypic characteristics of both Th17 and Treg cells [6]. This double-faced subpopulation still displays suppressive activity in vitro [7]. IL-17a-producing Treg cells may play critical roles in antimicrobial defense, autoimmune diseases, and inflammation [8].

The levels of various T-cell subsets in allergic diseases have been investigated previously. To further explore the mechanism of allergy, we investigated the distribution of IFN- γ -secreting CD4⁺ helper T (Th1) cells, IL-4-secreting CD4⁺ helper T (Th2)

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cells, IL-17a-secreting CD4⁺ helper (Th17) cells, and FOXP3-marked regulatory T (Treg) cells with or without IL-17a production. We isolated and assessed the suppressive capacity of CD4⁺CD25^{high}CD127^{low} Tregs that were stimulated by *Dermatophagoides pteronyssinus* in both groups.

Material and methods

Study participants

Twenty adults with a clinical diagnosis of persistent allergic rhinitis and 19 age- and gender-matched healthy controls without allergic history were enrolled in this study. The patients' clinical characteristics are shown in Table I. They were diagnosed according to the criteria of the Initiative on Allergic Rhinitis and its Impact on Asthma (ARIA) [9]. All the patients were recruited from the Allergy Clinic of Beijing Tongren Hospital. They all had experienced mite-induced rhinoconjunctivitis varying from moderate to severe of at least 2 years' duration. Their symptom scores were at least 6 for more than 4 days during the run-in period, as assessed by the Total 5 Symptom Score (T5SS, including rhinorrhea, sneezing, nasal congestion, and nasal and ocular pruritus) [10].

Patients with asthma or other diseases were excluded. None of the participants had taken any medical treatment for at least 1 week or oral steroids for 3 months before undergoing the skin prick test (SPT). The SPT results were positive for *D. pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f) (ALK-Abello, Hørsholm, Denmark) with the diameter of the wheal equal to or greater than their positive controls. Specific immunoglobulin E (IgE) (Pharmacia CAP System, Pharmacia Diagnostics, Uppsala, Sweden) to Der p and Der f was positive, with a RAST (radioallergosorbent test) value of at least 2.43 kU/ml (grade 2). The blood samples were obtained within 15 min after SPT. None of the healthy donors had any symptoms of rhinoconjunctivitis or positive SPT result and their IgE was all grade 0.

Informed consent was obtained from all the subjects in the study. The procedures followed were in accordance with our institutional review board for human studies and with the Helsinki Declaration.

Stimulation and flow cytometry measurements of blood cells for Th1/Th2/Th17

For intracellular cytokine detection, 500 µl of freshly drawn whole blood was added to an isopycnic reaction system, which was composed of RPMI 1640 medium, 25 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), 1 µg/ml ionomycin

Table I. Clinical characteristics of study participants.

Characteristic	Allergic rhinitis (n = 20)	Controls (n = 19)
Age (>18 years)	26.35 ± 7.47	26.74 ± 3.14
Gender		
Female	45.00% (9/20)	36.84% (7/19)
Male	55.00% (11/20)	63.16% (12/19)
Symptom score (>6)	12.07 ± 2.92	0
SPT d1 grade (≥+++)		
+++	20.00% (4/20)	–
++++	80.00% (16/20)	–
d1 IgE grade (1–6)		
1	0.00% (0/20)	100.00% (19/19)
2	20.00% (4/20)	00.00% (0/19)
3	40.00% (8/20)	00.00% (0/19)
4	15.00% (3/20)	00.00% (0/19)
5	15.00% (3/20)	00.00% (0/19)
6	10.00% (2/20)	00.00% (0/19)
SPT d2 grade (≥+++)		
+++	30.00% (6/20)	–
++++	70.00 (14/20)	–
d2 IgE grade (0–6)		
1	0.00% (0/20)	100.00% (19/19)
2	20.00% (4/20)	00.00% (0/19)
3	25.00% (5/20)	00.00% (0/19)
4	20.00% (4/20)	00.00% (0/19)
5	25.00% (5/20)	00.00% (0/19)
6	10.00% (2/20)	00.00% (0/19)

Data are mean ± SD or number of participants.

(Sigma-Aldrich), and 1.7 µg/ml monensin (Alexis Biochemicals). The system was incubated for 4–6 h at 37°C, 5% CO₂ before the staining procedure. All the tubes were stained with APC-conjugated CD3 monoclonal antibodies (mAbs), Percp-conjugated CD8 mAbs. According to the manufacturer's instructions for the intracellular cytokine stain (BD Biosciences), one portion was stained with PE-conjugated IL-17a mAbs and the other portion with PE-conjugated IL-4 mAbs along with FITC-conjugated IFN-γ mAb (BD Biosciences). Respective matched isotype controls were used. The cells were analyzed by BD FACSaria flow cytometry (BD Biosciences).

Isolation, stimulation, and flow cytometry measurements of CD4⁺ T lymphocytes

PBMCs were separated from the freshly drawn blood by standard density gradient centrifugation with

Ficoll Paque. According to the manufacturer's instructions for the human CD4⁺ T cell isolation kit II (Miltenyi Biotec, Germany), CD4⁺ T cells were isolated from PBMCs by positive selection. CD4⁺ T cells were cultured in 24-well U-bottomed plates (Costar, Corning; Lowell, MA, USA) in RPMI 1640 medium supplemented with 10% autologous serum and CD3/CD28 Anti-Biotin MACSi Bead Particles (T Cell Activation/Expansion Kit, human, Miltenyi Biotec) [11]. The number of CD4⁺ T cells in every well was 2.5×10^6 . The 24-well U-bottomed plates were incubated at 37°C in a fully humidified incubator with 5% CO₂ for 2 days.

The activated CD4⁺ T cells were stained with Percp-cy5.5-conjugated CD25 mAbs (BD Biosciences) for 20 min at room temperature. For FOXP3 and IL-17a staining, we followed the manufacturer's instructions for FOXP3 Fix/Perm Buffer (4×) and FOXP3 Perm Buffer (10×) (Biolegend). The CD4⁺ T cells were analyzed using BD FACS Aria Flow cytometry (BD Biosciences), and 20 000 cells were acquired in every acquire time.

Purification of CD4⁺CD25^{high}CD127^{low} Treg and test for suppressive capacity of CD4⁺CD25^{high}CD127^{low} Treg

For Treg isolation and functional analysis, we obtained 50 ml of heparinized blood from each of five recruited patients with allergic rhinitis and six healthy controls. Non-CD4⁺ T and CD127^{high} T cells were depleted from the PBMCs by negative selection, then CD4⁺CD25⁺CD127^{low} T cells were positively selected from these CD4⁺ T and CD127^{low} T cells using a human CD4⁺CD25⁺CD127^{low} regulatory T-cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec). Only cells with the highest CD25 expression (CD25^{high}) were selected through incubation with a limiting quantity of anti-CD25 antibody beads.

The suppressive capacity of CD4⁺CD25^{high}CD127^{low} Treg was determined by cytokine analysis. We cultured isolated CD4⁺CD25⁺ T cells alone or mixed CD4⁺CD25⁺ T cells and CD4⁺CD25^{high}CD127^{low} Treg cells (5:1) in a final volume of 200 µl RPMI 1640 medium for 5 days with 10 µg/ml Der p at 37°C. We added 2×10^5 autologous irradiated CD3-depleted PBMCs as antigen-presenting cells to CD4⁺CD25⁺ T cell cultures. At day 5, we removed 100 µl of supernatant for Th1, Th2, and Th17 cytokine analysis (IFN-γ, IL-4, and IL-17a) with cytometric bead arrays (human Th1/Th2/Th17 cytokine kit; BD Biosciences).

Statistical analysis

A standard two-tailed *t* test or nonparametric tests for two independent samples was used in the statistical analysis. Means were reported. Spearman correlation was used to analyze relationships between these variants. *p* values of < 0.05 were considered significant. All analyses were performed with statistical software SPSS version 16.0 (SPSS Inc., USA).

Results

Increased Th17 and Th2 in patients with allergic rhinitis

Cytometric analysis showed that both Th17 (CD4⁺IL-17a⁺T cell) (AR, 1.71%; HD, 0.39%; *p* = 0.000) and Th2 (CD4⁺IL-4⁺ T cell) (AR, 1.81%; HD, 0.87%; *p* = 0.034) were increased in allergic patients compared with levels in healthy controls. Nevertheless, the means of Th1 (CD4⁺IFN-γ⁺ T cell) (AR, 16.24%; HD, 19.26%; *p* = 0.266) in these two groups were similar. Th17 and Th2 were also positively correlated (*r* = 0.379, *p* = 0.017). In addition, Th1 and Th2 were inversely correlated (*r* = -0.370, *p* = 0.020).

FOXP3-marked regulatory T cells secreted higher levels of IL-17a in patients with allergic rhinitis

The frequencies of CD4⁺FOXP3⁺IL-17a⁺ T cell (Figure 1A, B1, B2), CD4⁺CD25⁺IL-17a⁺ T cells (Figure 1A, C1, C2) and CD4⁺CD25⁺FOXP3⁺IL-17a⁺ T cells (Figure 1A, D1, D2, E1, E2) were significantly higher in the patient group. However, no significant differences were observed between healthy donors and patients in Treg that were marked as CD4⁺FOXP3⁺, CD4⁺CD25⁺FOXP3⁺ or CD4⁺CD25⁺ T cells (Figure 1A, D1, D2).

Th2 was correlated with IL-17a-producing Treg (CD4⁺FOXP3⁺Treg) (*r* = 0.590, *p* = 0.000). However, Th17- and IL-17a-producing Treg (marked as CD4⁺FOXP3⁺Treg, CD4⁺CD25⁺ Treg, CD4⁺CD25⁺FOXP3⁺ Treg) were not related (*p* > 0.05).

Suppressive capacity of CD4⁺CD25^{high}CD127^{low} Treg cells after allergen stimulation

We observed that CD4⁺CD25⁺ T cells obtained from allergic individuals showed significantly increased cytokine production of both IL-17a (Figure 2A) and IL-4 compared with non-allergic controls (Figure 2B). In contrast, the Th1 cytokine IFN-γ production was not strongly suppressed by CD4⁺CD25^{high}CD127^{low} Treg cells in either of the groups (*p* = 0.177) (Figure 2C). Additionally, CD4⁺CD25^{high}CD127^{low} Treg cells

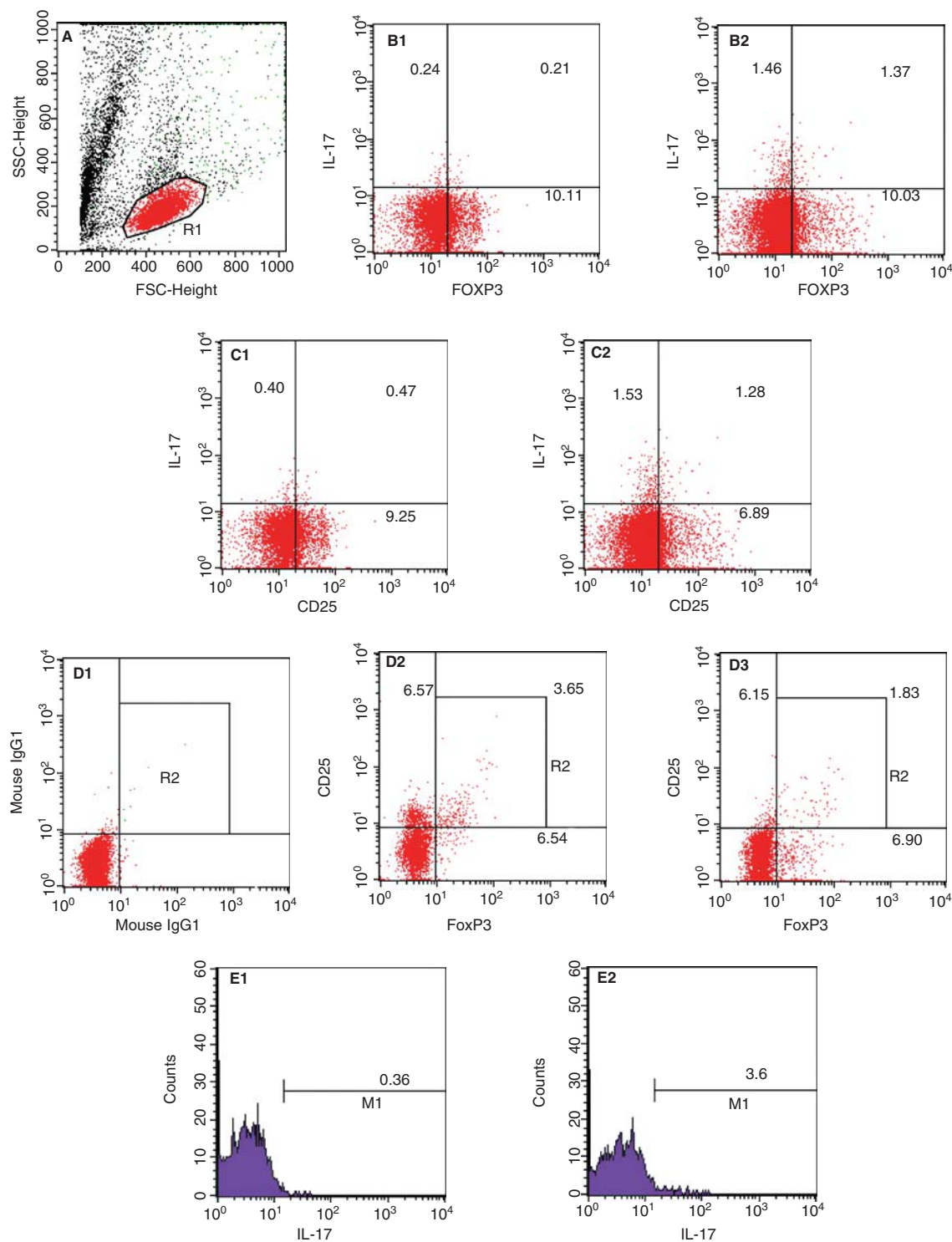


Figure 1. Flow cytometric analysis of Treg and IL-17a-producing nTreg in isolated CD4⁺ T cells from patients with allergic rhinitis (AR) ($n = 20$) and healthy controls ($n = 19$), which were stimulated with CD3/CD8 anti-biotin for 2 days. Values in density dot plots indicate percentages of different cell subsets in total CD4⁺ T cells. (A) CD4⁺ T cells (marked as R1) were gated in peripheral blood mononuclear cells (PBMCs). (B) Percentage of CD4⁺FOXP3⁺IL-17a⁺ T cells (right upper quadrant: B1, control; B2, AR); (C) Percentage of CD4⁺CD25⁺IL-17a⁺ T cells (right upper quadrant: C1, control; C2, AR). (D) Percentages of CD4⁺CD25⁺FOXP3⁺ T cells (marked as R2; in the right upper quadrant: D1, isotype; D2, control; D3, AR), CD4⁺CD25⁺ T cells (in the right upper and the left upper quadrant), CD4⁺FOXP3⁺ T cells (in the right upper and left lower quadrant). (E) Percentage of CD4⁺CD25⁺FOXP3⁺IL-17a⁺ T cells (in the histogram: E1, control; E2, AR). Cells in B1, B2, C1, C2, and E1, E2 were gated in R1, and cells in E1, E2 were gated in R1 and R2. Data are means of all participants; $p < 0.05$ is significant.

obtained from both the non-allergic and allergic group suppressed IFN- γ production (Figure 2C) of CD4⁺CD25⁻ T cells stimulated by Der p1. The suppression ratio of Th17 cytokine production (IL-17a secreted in CD4+CD25⁻ and CD4+CD25^{high}CD127^{low} T well/IL-17a secreted in CD4+CD25⁻ T well) was significantly higher in the allergic group (Figure 2D).

Discussion

With an implication that a portion of regulatory T cells are induced to express IL-17a [6,7,12], we stained IL-17a on purified CD4⁺ lymphocytes along with CD25 and FOXP3 after 48 h of TCR activation [8].

Our frequency study shows that levels of IL-17a-producing Th and Treg are higher in the peripheral

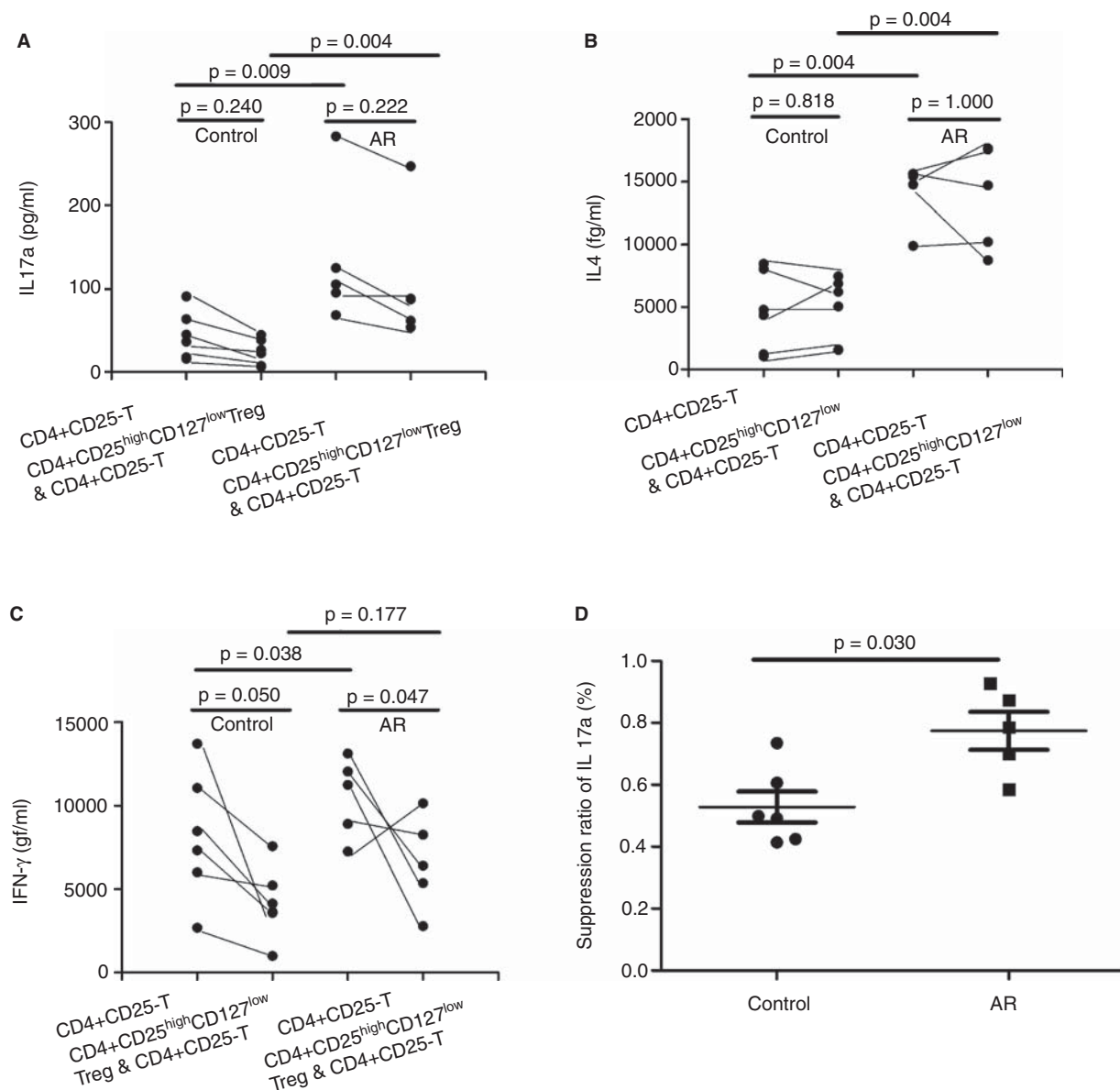


Figure 2. Suppressive capacity of CD4⁺CD25^{high}CD127^{low} Treg cells from patients with allergic rhinitis (AR) ($n = 5$) and healthy controls ($n = 6$) after stimulation by allergen Der p1 for 5 days. Cytokine production of both IL-17a (A) and IL-4 (B) were significantly increased in AR. (C) Th1 cytokine IFN- γ production was not strongly suppressed by CD4⁺CD25^{high}CD127^{low} Treg cells in the two groups ($p = 0.177$). CD4⁺CD25^{high}CD127^{low} Treg suppressed Th1 cytokine IFN- γ production in both groups. (D) The suppression ratio of Th17 cytokine production (IL-17a secreted in CD4+CD25⁻ and CD4+CD25^{high}CD127^{low} T well/IL-17a secreted in CD4+CD25⁻ T well) was increased in AR. $p < 0.05$ is significant.

blood of patients with allergic rhinitis. Treg are most often defined as CD4⁺ T cells expressing high levels of CD25 and FOXP3. We used them to mark Treg and found that AR patients have increased Th2, Th17, and IL-17a-producing Treg, but comparable Th1 and Treg. Flow cytometry measurements and the CBA test showed that FOXP3-marked regulatory T cells secrete higher levels of intracellular IL-17a and the suppression ratio of effector Th17 cytokine production is higher in patients with allergic rhinitis. There are more deficient Treg in allergic rhinitis. All this suggests that the deficiency of Treg cells may play a role in the development of allergic rhinitis. The defects of Treg in allergic diseases are not only quantitative but also functional.

Inflammatory Th17 and Th2 are increased in allergic rhinitis. Allergic rhinitis was caused by the imbalance of Th1/Th2. Recently, it has been reported that serum IL-17a levels and Th17 are higher in allergic rhinitis patients from children to adults [13–16]. In the present study, Th17 and Th2 were significantly increased in patients with allergic rhinitis and they were also positively related. Since the level of IL-17a was reported to be significantly higher in asthmatic lungs in a previous publication [13], we hypothesize that the high level of Th17 is responsible for the pathogenesis of allergic rhinitis. Unlike an earlier report [15], the present study shows that these two allergic inflammatory Th subsets (Th17 and Th2) may be facilitating impacts in the course of allergic rhinitis. We favored the unbalanced theory of Th1/Th2 but evidenced similar Th1 in these two groups, indicating that effector T helper cells may work as a team.

The deficiency of Treg cells may play a role in the development of allergic rhinitis. Previous research reported increased, similar or decreased amounts of Treg in the peripheral blood of patients with allergic rhinitis [16–18]. In comparison, the present study supports the result of similar amounts of Treg. In addition, HLA-DR⁺ Treg, which specially include IL-17a-producing Treg, are numerably and functionally similar in patients with allergic rhinitis and controls, but there is higher gene expression of molecules associated with contact-dependent cell activation and cytotoxicity in allergic rhinitis [7,19].

Furthermore, a special subpopulation of Treg that produces IL-17a was identified that only exists in peripheral blood; it was proved to sustain suppressive capacity but weaker than Treg, which shows apparent duality of Treg and Th17 [6–8]. In the present study, IL-17a-producing Treg proved to have a higher frequency in patients with allergic rhinitis. Previous studies have also shown that there

are more IL-17-producing CD4⁺FOXP3⁺ T cells in patients with allergic rhinitis or with inflammatory bowel diseases [8,12]. Our test for the suppressive capacity of CD4⁺CD25^{high}CD127^{low} Treg showed that cytokines of Th2 and Th17 were significantly increased in the allergic rhinitis group, but not the cytokine of Th1. This might suggest that Tregs in allergic rhinitis are not as capable as those in healthy controls in suppressing Th17 from producing IL-17a. Moreover, the increased Th17 suppression ratio in allergic rhinitis also supports this point.

IL-17a-producing Treg was also found to be responsible for the pathogenesis of cancer [20]. IL-17a⁺ Tregs are less suppressive and IL-17a expression on Treg is a sign of a weak suppressor. Therefore, it is conceivable that the deficiency of Treg cells may play a role in the development of allergic rhinitis. In the future, expression of IL-17a may be a new way to evaluate the suppressive function of Treg.

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