Decreased Circulating Interleukin-35 Levels Are Related to Interleukin-4-Producing CD8+ T Cells in Patients with Allergic Asthma

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ABSTRACT

Interleukin (IL)-35 is a newly discovered suppressive cytokine and has been shown to alleviate inflammatory and autoimmune diseases. The purpose of this study was to investigate immunomodulatory capacity of IL-35 in patients with allergic asthma. IL-35 mRNA expression levels in peripheral blood mononuclear cells (PBMCs) were detected by quantitative real-time PCR (qPCR). The frequencies of cytotoxic T cells (Tc)1, Tc2 and Tc17 cells were measured by flow cytometry. Plasma levels of IL-35, interferon (IFN)-γ, IL-4, and IL-17 were examined by enzyme-linked immunosorbent assay (ELISA). The correlations between plasma IL-35 levels and Tc1, Tc2, and Tc17 cytokine production in allergic asthmatics (n = 25) and healthy controls (n = 12) were analyzed by Pearson’s test.

IL-35 protein and mRNA expression levels were down-regulated in allergic asthmatics compared with healthy controls. The frequencies of Tc2 and Tc17 cells were significantly increased in patients with asthma, and the frequency of Tc1 cells did not differ between asthmatic patients and healthy controls. Similarly, plasma levels of IL-4 and IL-17 were significantly increased in asthmatic patients, while there was no difference in IFN-γ levels between allergic asthma patients and healthy controls. More importantly, plasma IL-35 protein levels were negatively correlated with the frequency of IL-4-producing CD8+ T (Tc2) cells and with the IL-4 level in patients with allergic asthma.

Our results suggest that decreased circulating IL-35 levels could contribute to the pathogenesis of allergic asthma by regulating CD8+ T cells.

Keywords: Allergic asthma; CD8+ T cells; Interleukin-4; Interleukin-35

INTRODUCTION

Allergic asthma is a chronic airway inflammation characterized by infiltration of many inflammatory cells, including mast cells, eosinophils, lymphocytes, basophils, and monocytes. Over the past decades, it has been demonstrated that CD4+ T lymphocytes, such as T helper (Th)1, Th2, and Th17 cells, orchestrate atopic asthmatic inflammation by secreting many cytokines. Apart from CD4+ T cells, CD8+ T lymphocytes (Tc) are also reported to be involved in the
regulation of allergic inflammation.\textsuperscript{3,4} CD8\(^{+}\) T cells are classified as Tc1, Tc2, or Tc17 cells according to their distinct cytokine secretion patterns. Tc1 and Tc2 cell subsets mainly secrete interferon (IFN)-\(\gamma\) and interleukin (IL)-4, respectively. Tc17 cells, on the other hand, may produce IL-17, IL-21 and IL-22.\textsuperscript{5} A potential role for CD8\(^{+}\) T cells in allergic asthma is mediating airway inflammation and airway hyperresponsiveness.\textsuperscript{6}

IL-35, which acts as a novel immunosuppressive cytokine produced by regulatory T cells (Tregs), belongs to the IL-12 cytokine family.\textsuperscript{7} IL-35 is a heterodimeric protein composed of two subunits, Epstein-Barr virus-induced gene 3 (EBI3) and IL-12p35. In contrast with other IL-12 family members, IL-35 shows uniquely inhibitory properties.\textsuperscript{8} It has been reported that IL-35 can suppress the function of CD4\(^{+}\) T cells. Recently, IL-35 has been reported to be involved in the pathogenesis of many inflammatory and autoimmune diseases by mediating the suppression of CD4\(^{+}\) effector T cells.\textsuperscript{9,12} Studies have demonstrated that IL-35 might suppress Th17 cell development and differentiation in autoimmune inflammation.\textsuperscript{13} In addition, IL-35 has also been found to be critical to the development of Th2 cells and to attenuate allergen-specific Th2 responses and production of Th2 cytokines of allergic airways disease.\textsuperscript{14} However, whether IL-35 is involved in the regulation of CD8\(^{+}\) T cells in allergic asthmatic patients is not well established. In this study, we investigated IL-35 expression levels in patients with allergic asthma and compared it to the healthy controls and analyzed the correlations between circulating IL-35 levels and cytokine production by CD8\(^{+}\) T cells.

**MATERIALS AND METHODS**

**Subjects**

Twenty-five allergic asthmatic patients from the outpatient department of Zhongnan Hospital of Wuhan University and 12 age-matched healthy controls were enrolled in this study. The criteria for allergic asthma were based on the Global Initiative for Asthma (GINA):\textsuperscript{15} (a) having recurrent episodes of cough, wheezing, and chest tightness; (b) having reversible airway obstruction; (c) having atopy with positive skin prick tests to at least one of 10 common allergens (*Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, mixed tree pollens, mixed grass pollens, cat fur, dog hair, fluffed cotton, cockroach, feather, and *Alternaria*); (d) having no other allergic diseases, autoimmune diseases or neoplastic diseases; and (e) not having received systemic steroids in the past 4 weeks (Treatment with inhaled steroids was allowed). Table 1 shows the clinical characteristics of the subjects. The study was approved by the Medical Ethics Committee of Zhongnan Hospital (2013-006). All subjects were fully informed about the purpose of the study and signed informed consent.

**Cell Separation**

Fifteen milliliters of heparinized venous blood was collected from all subjects. The blood was diluted by adding equal volumes of phosphate-buffered saline (PBS), laid on Ficoll-Hypaque solution (d=1.077 g/ml; Haoyang, Tianjin, China), and then centrifuged at 2000xrpm for 20 min. The peripheral blood mononuclear cell (PBMC) layer was removed and washed twice in PBS. Cell viability was examined by trypan blue dye exclusion. Plasma samples were collected and stored at −70°C for detection of cytokine concentrations.

**Cell Culture**

PBMCs were seeded in RPMI 1640 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) in six-well plates at 1 x 10\(^{6}\) cells/ml. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) and ionomycin (500 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37°C in a 5% CO\(_2\) atmosphere and then incubated for an additional 4 h in the presence of brefeldin A (3 μg/ml; eBioscience, San Diego, CA, USA). After treatment, cells were harvested for flow cytometry analysis and mRNA detection.

**Flow Cytometry**

The frequencies of IFN-\(\gamma\) (Tc1)-, IL-4 (Tc2)-, and IL-17 (Tc17)-producing CD8\(^{+}\) T cells were measured by surface molecule and intracellular cytokine staining. CD3 and CD8 surface molecules of CD8\(^{+}\) T cells were stained with PE-Cy5- and FITC-conjugated antibodies (eBioscience), respectively, for 30 min in the dark at 4°C. To analyze the intracellular production of the cytokines IFN-\(\gamma\), IL-4, and IL-17 in CD8\(^{+}\) T cells stimulated with PMA and ionomycin, cells were fixed, permeabilized, and then stained with PE-conjugated
anti-human monoclonal antibodies (against IFN-γ, IL-4, and IL-17, eBioscience) for 30 min in the dark at room temperature. Finally, all stained cells were resuspended in PBS and analyzed by a flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with Expo32 software (Beckman Coulter).

**Quantitative Real-Time PCR (qPCR)**

Total RNA was extracted from activated PBMCs using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized by reverse transcription using the ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. Expression levels of target genes were assessed by qPCR using SYBR Premix Ex Taq™ (TaKaRa, Shiga, Japan). Amplification was performed according to the following conditions: initial denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, at 58°C for 15 s, and at 72°C for 15 s. The expression levels of target genes in each sample were calculated using the 2^(-delta delta CT) method relative to glyceraldehyde phosphate dehydrogenase (GAPDH) expression. The sequences of primers used were as described previously. GAPDH forward: 5'-gggtgtaaccatgagatgaca-3', reverse: 5'-gtcttcagatccaaagtgt-3'; EB13 forward: 5'-tctccatgcacagtacag-3', reverse: 5'-gcctctgttatgaaaggcacg-3'; IL-12p35 forward: 5'-tcaaatctgctcttacacgg-3', reverse: 5'-aaatcgcggcttccagagga-3'.

**Detection of Cytokines**

The concentrations of IL-35, IFN-γ, IL-4, and IL-17 in plasma were measured by enzyme-linked immunosorbent assay (ELISA) using kits according to the manufacturer’s protocols (IL-35 ELISA kit, BioLegend, San Diego, CA, USA; IFN-γ, IL-4, and IL-17 ELISA kits, eBioscience). The minimal detectable levels were 0.12 ng/ml for IL-35, 0.99 pg/ml for IFN-γ, 1.3 pg/ml for IL-4, and 0.5 pg/ml for IL-17. All samples were measured in duplicate.

**Statistical Analysis**

The statistical analysis was performed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). All data were expressed as means ± standard deviation (SD). Differences between groups were assessed using a nonparametric Mann-Whitney U test for independent samples. Correlation analysis was analyzed by Pearson’s test. Differences were considered significant at p<0.05.

**RESULTS**

**Characteristics of the Subjects**

Twenty-five allergic asthmatic patients and 12 healthy controls were enrolled in this study. The detailed clinical characteristics of the subjects are summarized in Table 1. The IgE level was significantly higher in asthmatics compared to healthy controls. The FEV₁% of predicted value was decreased in allergic asthmatic patients compared with healthy controls (Table 1).

**Decreased IL-35 Expression Levels in Allergic Asthmatics**

IL-35 expression levels in allergic asthmatic patients and healthy controls were measured by ELISA and qPCR. The results showed that the concentration of IL-35 in plasma from allergic asthmatics was significantly lower than that from healthy controls (Figure 1A). Similarly, the mRNA levels of the IL-35 subunits were significantly decreased in activated PBMCs from asthmatics compared with healthy controls (Figure 1B).

**Frequencies of IFN-γ, IL-4, and IL-17-producing CD8⁺ T cells in PBMCs**

CD8⁺ T cells play an important role in the pathogenesis of allergic asthma. Therefore, the frequencies of IFN-γ (Tc1)-, IL-4 (Tc2)-, and IL-17 (Tc17)-producing CD8⁺ T cells in PBMCs were...
Figure 1. IL-35 expression levels in healthy controls and allergic asthmatic patients. A: IL-35 protein levels in plasma from allergic asthmatic patients and healthy controls. B: mRNA expression levels of the IL-35 subunits, EBI3 and IL-12p35 in allergic asthmatics and healthy controls. Data are expressed as mean ± SD. *P < 0.05.

detected by flow cytometry. Representative cytometric profiles of IFN-γ, IL-4, and IL-17-positive CD3^+CD8^+ T cells from patients with allergic asthma and healthy controls are shown in Figure 2A. The percentages of Tc2 and Tc17 cells in PBMCs were higher in asthmatic patients compared to the healthy controls. In contrast, the frequency of IFN-γ-positive CD8^+ T (Tc1) cells in PBMCs did not differ significantly between the two groups (Figure 2B).

Figure 2. Frequencies of Tc1, Tc2, and Tc17 cells in PBMCs from allergic asthmatic patients and healthy controls. To analyze the frequencies of Tc1, Tc2, and Tc17 cells, PBMCs were stimulated with PMA and ionomycin in vitro and then harvested for flow cytometry analysis. A: Representative dot plots of Tc1, Tc2, and Tc17 cells from an asthmatic patient and a healthy control. B: The percentages of Tc1, Tc2, and Tc17 cells in PBMCs from asthmatic patients and healthy controls. Data are expressed as mean ± SD. *P < 0.05; **P < 0.01; NS, not significant.
levels of IFN-γ, IL-4, and IL-17 were detected using ELISA kits according to the manufacturer’s protocols. The results showed that the levels of IL-4 and IL-17 in plasma were significantly increased in the asthmatic group compared with the control group. On the other hand, there was no difference in the concentration of IFN-γ in plasma between the two groups (Figure 3).

**Correlations between Plasma IL-35 Levels and Tc1, Tc2, and Tc17 Cytokine Production in Patients with Allergic Asthma**

To further assess the possible interactions between IL-35 and Tc1, Tc2, and Tc17 cytokines in asthmatic patients, we analyzed the correlations between IL-35 concentration and Tc1, Tc2, and Tc17 cytokine production in allergic asthmatics by Pearson’s test. As shown in Figure 4, IL-35 protein expression levels were negatively correlated with the frequency of IL-4-producing CD8⁺ T (Tc2) cells and with plasma IL-4 concentration ($r=-0.4556$, $p=0.0221$ and $r=-0.4745$, $p=0.0165$, respectively). However, we found no correlations between IL-35 protein levels and IFN-γ or IL-17 production.
DISCUSSION

Allergic asthma is one of the most common chronic airway diseases. CD8+ T lymphocytes play an important role in airway inflammation by releasing cytokines. It has been reported that IL-35 is a novel anti-inflammatory and suppressive cytokine. The aim of this study was to measure circulating IL-35 levels and to investigate its relationships with the production of cytokines by CD8+ T cells in patients with allergic asthma. In our study, we demonstrated that IL-35 protein and mRNA levels were dramatically decreased in allergic asthmatic patients compared with healthy controls. Meanwhile, we also observed that the frequencies of IL-4- and IL-17-producing CD8+ T cells, as well as the concentrations of Tc2- and Tc17-related cytokines in plasma were higher in asthmatic patients than in healthy controls. Particularly, plasma IL-35 protein levels were negatively correlated with the frequency of IL-4-positive CD8+ T (Tc2) cells and with IL-4 concentration in plasma, implying that IL-35 may be involved in the pathogenesis of allergic asthma by regulating the IL-4 production of CD8+ T cells.

It has been well demonstrated that CD8+ T cells are also critical for the initiation and persistence of chronic airway inflammation in allergic asthma.3,4 Tc2 cells can exacerbate allergic asthma in a similar manner to Th2 cells that secrete type 2 cytokines IL-4 and IL-5.5 On the other hand, Tc1 CD8+ T cells can act to antagonize a Th2 response and suppress airway inflammation by secreting IFN-γ.6 Consistent with a previous study reporting an increased frequency of Tc2 in CD8+ T cells in asthmatic patients,21 our results showed that the frequency of Tc2 cells and IL-4 levels increased in asthmatic patients, but the frequency of Tc1 cells and IFN-γ levels did not differ between allergic asthmatic patients and healthy controls, which demonstrated that an imbalance of Tc1/Tc2 cells contributed to the pathogenesis of allergic asthma. Furthermore, a functional disequilibrium of Tc17 CD8+ T cells may also contribute to the airway inflammation in asthma by secretion of cytokine IL-17.22 IL-17 can activate the release of neutrophil chemotactic factors and then induce acute and severe airway inflammation.23 Consistent with these findings,22,23 our results showed an increased percentage of Tc17 cells and IL-17 cytokine levels in asthmatic patients.

IL-35 is a newly discovered anti-inflammatory and suppressive cytokine that is mainly secreted from Tregs and is required for their immunosuppressive function in mice.7 It has been reported that IL-35 has been involved in the pathogenesis of inflammatory and autoimmune diseases by mediating CD4+ T cell responses.10-12,14,24 However, the immunomodulatory capacity of IL-35 on CD8+ T cells in allergic asthmatic patients remains to be investigated. Accordingly, we detected the IL-35 expression levels in patients with allergic asthma. Our results showed decreased IL-35 protein and mRNA levels in asthmatic patients, which was in accord with the results of Ma et al.25 To assess the clinical significance of IL-35 in allergic asthma, we further analyzed the correlations of plasma IL-35 levels with Tc1, Tc2, and Tc17 cytokine production in allergic asthmatics. Importantly, we found that there was a negative correlation between IL-35 protein levels and the frequency of Tc2 cells and plasma IL-4 concentration. Our data suggest that the deficiency in IL-35 may contribute to the destruction of immune response in allergic asthmatic patients. Decreased IL-35 levels could therefore lead to the increased frequency of Tc2 cells and IL-4 level in plasma. However, some other confirming experiments with more samples by using the antagonists of IL-35 are necessary in future works. Besides, the increase in Tc17 cells and IL-17 concentration in allergic asthma may be related to another pathogenic mechanism and also needs to be further investigated.

In conclusion, we found decreased circulating IL-35 levels in patients with allergic asthma, and plasma IL-35 levels were negatively correlated with the frequency of IL-4-positive CD8+ T cells and with IL-4 concentration. These findings suggest that IL-35 could be involved in the pathogenesis of allergic asthma and may be a novel immunotherapy for asthma in the future.

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IL-35 in Allergic Asthmatic Patients

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