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Expression of Interleukin-35 in Children with Acute Allergic Asthma

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ABSTRACT

Background: Allergic asthma is believed to be a T helper 2 cell (Th2) preponderant response caused by airway hyper-responsiveness. Interleukin-35 (IL-35) is a newly discovered anti-inflammatory cytokine.

Objective: To determine whether the expression of IL-35 is associated with type-2 inflammation in children with asthma exacerbations.

Methods: Thirty children (6-12 years old) with acute allergic asthma and twenty healthy controls were enrolled. Sputum was collected from lower airways. IL-35 and type 2 cytokines expression from serum and sputum were measured at mRNA and protein level by real-time PCR and enzyme-linked immunosorbent assay (ELISA), respectively. The sampling and the test were repeated eight weeks after the asthma exacerbation.

Results: At the time of exacerbation, IL-35 expression decreased significantly in induced sputum and serum than in the controls. The expression of IL-35 was negatively correlated with IL-4, IL-5 and IL-13 expression. The IL-35 from induced sputum increased significantly, whereas type-2 cytokines decreased significantly eight weeks after the exacerbation.

Conclusion: Our results showed that decreased IL-35 was associated with type-2 cytokines in asthma exacerbations in children, suggesting that IL-35 may be a potential future drug target for asthma exacerbations.

Keywords: Asthma, Exacerbation, Interleukin-35, Th2 Cytokines

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INTRODUCTION

Asthma in children is a worldwide public health concern with death rates ranging from 0 to 0.7 per 100,000 children (1). It also ranks among the top 20 diseases worldwide for disability-adjusted life years in children (2). Acute asthma exacerbations are very common in asthmatic children, and even the best-managed asthma patients will have acute asthma exacerbations. For example, 2.1% and 10.7% of children with asthma in the United States reported at least one hospitalization and one emergency department visit, respectively (3). Although most asthmatic children received good efficacy from the daily administration of low-to-medium-dose inhaled corticosteroid, about 50% of children experienced at least one episode of asthma exacerbation (4, 5). Allergic asthma is one of the most common

subtypes of asthma in children, which is believed to be a type 2 inflammation caused by allergens (6). However, its mechanism and regulation are not fully understood.

Interleukin-35 (IL-35) is a cytokine consisting of IL-12 p35 and EBV-induced gene 3 (EBI3). IL-35 is mainly produced from naturally regulated T cells and depends upon the level of Foxp3 (7). It is mainly found in bone marrow, thyroid, blood and et al., (8). The IL-35 receptor (IL-35R) is composed of IL-12R β2 and glycoprotein (gp130). The binding of IL-35 and its receptors activates the signal transducer or activator of transcription (STAT) 1 and STAT4 pathway (8). IL-35 can regulate B and T cells, induce regulatory T cells (iTr35) proliferation, inhibit T cell proliferation and Th17 differentiation (8-10). Therefore, IL-35 has been shown to have a significant inhibitory effect on a number of autoimmune disorders. (11). Animal experiments suggested that IL-35 could significantly relieve the specific respiratory response induced by allergen and intramuscular injection IL-35 inhibited the level of cyclic allergen specificity and total IgE in the long term (12). These studies showed that targeting IL-35 may be a promising treatment for allergic asthma.

In the present study, we explored the correlation between IL-35 and Th2 inflammation during asthma exacerbations in children.

METHODS

Patients

Thirty school-aged children (6-12-year old) with acute exacerbation of allergic asthma and 20 healthy controls matched for age and gender between June 2018 and August 2018 in the emergency department of our hospital were recruited in our study. We enrolled patients that had positive skin prick test or specific IgE (Phadia, Uppsala, Sweden) for common inhalational allergen when they compromise with the allergic asthmatic attack such as repeated sharp breaths, expiratory wheezing of both lungs with prolonged expiratory time during the onset, and bronchodilator relieved the symptom. Children with other respiratory diseases were excluded. All children were examined and sampled within 24 hours of admission (after the necessary treatment) and again after 8 weeks. All the children were new cases without drug usage. The municipal ethics committee gave us their blessing. All participants signed a written informed consent form. None of the healthy controls had any allergic or respiratory diseases or experienced asthma-like syndromes.

All children were treated with salbutamol/ipratropium and methylprednisolone on admission for asthma control. Then oral prednisone 37.5 mg was provided for 9 consecutive days.

Determination of Asthma

Fractional exhaled nitric oxide (FeNO) was detected by NIOX® (Aerocrine AB, Sweden). Pulmonary function was assessed by Spirometer (Medical Technologies, Switzerland).

The sputum induction from asthmatic children was performed by EasyNeb 3 (Flaem Nuova, Italy) after being treated with terbutaline as described previously (13, 14). In brief, every child was treated with 5 mg nebulized albuterol. Then the sputum was induced by nebulized normal saline (0.9%) with 9-cm corrugated tubing at intervals of 30 s, 1 min, 2 min, and 4 min. The pulmonary function and the peak expiratory flow (PEF) was measured 1 min after every administration. Enough sputum was expectorated by cough at each interval. Supplemental β-agonist was provided if PEF decreased by more than 10% from baseline between consecutive measurements. In normal subjects, the sputum was induced by aerosol inhalation. The sputum samples were treated with dithiothreitol (0.2%, Calbiochem, USA) and PBS. Supernatant and cell particles were mixed with RNAlater® (Ambion, USA) under −80 °C for further analysis after

centrifugation at 1,000 g for 5 min.

Real-time PCR

Total RNA extraction was achieved using TRIzol (Life Technologies, California). The cDNA was synthesized by oligo(dT) primer and M-MLV reverse transcriptase (TAKARA, Japan). Real-time PCR was conducted via ABI PRISM 7300 Detection System (Applied Biosystems, California). The primers are: IL-4 sense, 5'-GATTTGCAGTG ACAATGTGAG-3', antisense, 5'-TCCTATGCTGAAACTTTGTAG-3'. IL-5 sense, 5'-AGCTGCCTAC GTGTATGCCA-3', antisense, 5'-GCAGTGCCAAGG TCTCTTTCA-3'. IL-13 sense, 5'-GATTC CAGGGCTGCACAGTA-3', antisense, 5'-GGTCAACATCACCCAGAACC-3'. IL-35 sense, 5'-CTCGGATCCGACA TGTCCAAGCTGCTCTTC-3', antisense, 5'-GCCACCGCCGCTTCCACCGC CAC CGGG CTTATGGGG TGC-3', β-actin sense, 5′-CGAAACTACCT TCAACTCCATC-3′, antisense, 5′-AGTGATCTCCTTCTGCAT CCT-3′. Reaction conditions included: heating to 95°C for 10 minutes, denatured at 95°C for 10 s with 40 cycles, annealed at 60°C for 60 s. The relative expression of cytokines was determined employing the 2-ΔΔCt method.

Determination of Th2 Cytokines Expression

The serum or sputum concentrations of IL-35, IL-4, IL-5, and IL-13 were determined by ELISA kits (R&D Systems, USA) as described by the instructions. Every experiment was repeated three times.

Statistical Analysis

SPSS version 22 (IBM, USA) was made use of. All data are shown as the mean and SD. Levene test was applied to test the normality of data. Kruskal-Wallis H test, nonparametric Mann-Whitney U test, and Spearman rank correlation analysis were performed. A p-value less than 0.05 was considered significant.

RESULTS

Baseline Information of Patients

The baseline information of study subjects is summarized in Table 1. We found that the eosinophil number, neutrophils number, total IgE were significantly higher in the asthma children than in the controls, particularly during the asthma exacerbation.

Protein Levels of IL-35 and the Th2 Cytokines during Exacerbation

We found that both mRNA and protein levels of serum and sputum IL-35 significantly decreased in asthma children than in the normal controls, whereas the sputum and serum Th2 cytokines expression significantly upregulated in asthma children than in the normal control (Figure 1, 2, Table 2). Moreover, the sputum and serum IL-35 protein levels negatively correlated with Th2 cytokines levels (Table 3).

Protein Levels of IL-35 and the Th2 Cytokines 8 Weeks after Exacerbation

Eight weeks after the exacerbation, both

Table 1. Baseline characteristic of allergic rhinitis and the control children

Groups	Asthma group	Control
Number	30	20
Sex (Male: Female)	16:14	12:8
Age (years)	8.8 (6-12)	9.3 (6-12)
Eosinophil (count/mm^3)	156 (89-1750)*	32 (7-110)
Neutrophil (count/mm^3)	14500 (8000-21900)*	6300 (1200-10100)
IgE (IU/ml)	183.2 (65.3-1750.0) **	24.0 (4.1-62.0)
FeNO (ppb)	62 (33-78)	11 (8-21)
FEV1(% of predicted)	98.1±1.8*	51.6±5.8

* Compared with the control group, P<0.05. ** Compared with the control group, P<0.01.

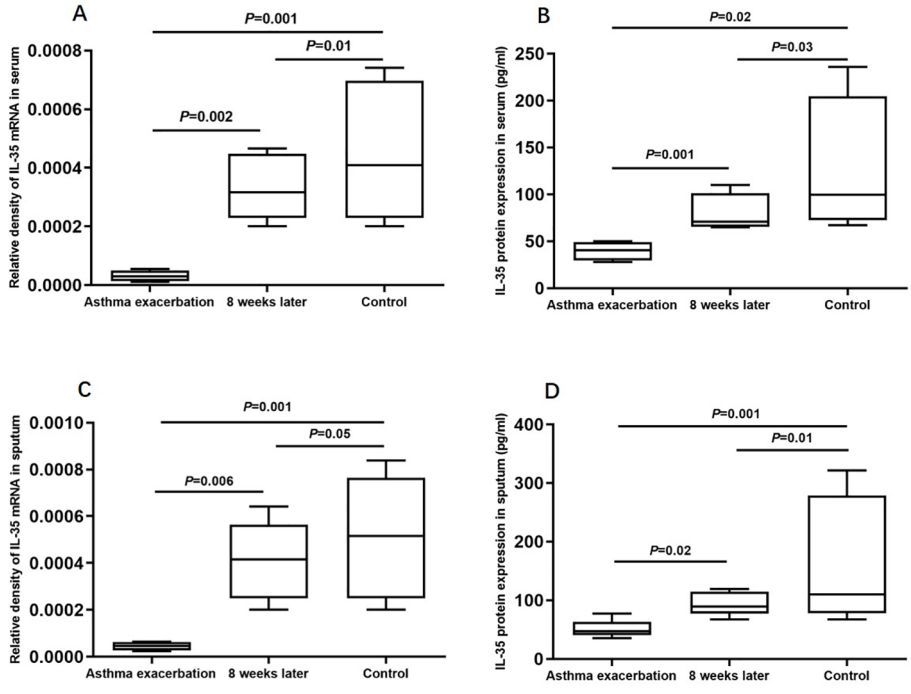

Figure 1. The mRNA and protein concentrations of IL-35 in the serum and sputum from asthma children with asthma exacerbation, 8 weeks after the exacerbation and the normal controls.

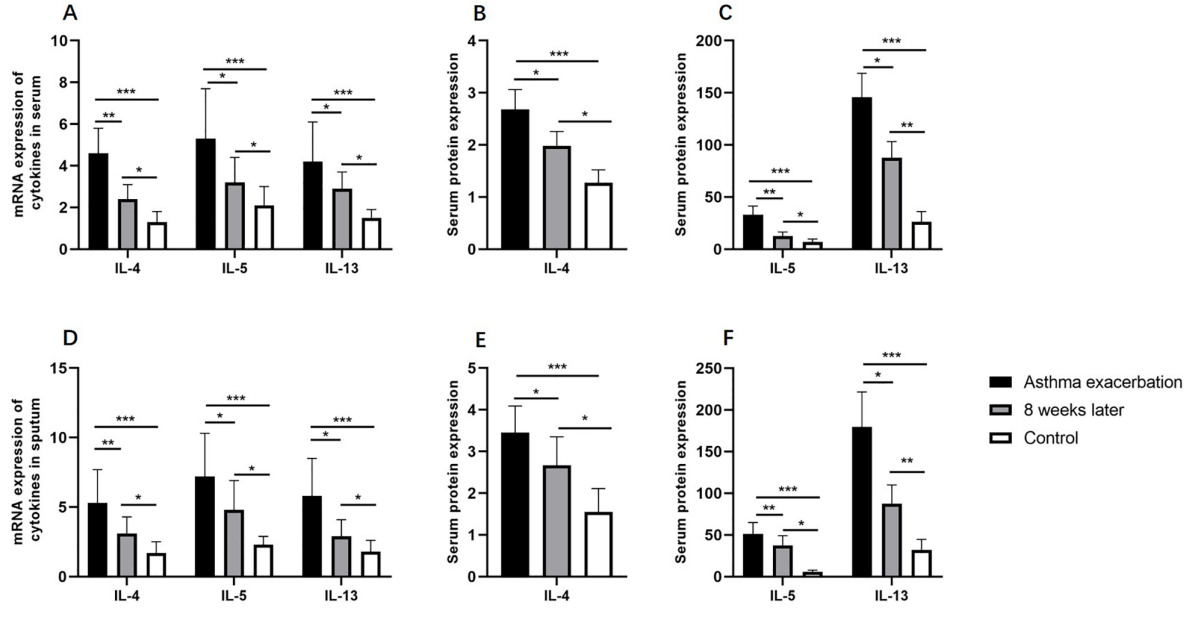

Figure 2. The mRNA and protein expression (pg/mL) of Th2 cytokines in the serum and sputum of asthma children with the asthma exacerbation, 8 weeks after the exacerbation and the normal controls. pg/mL, * compared with the control, P<0.05. ** Compared with the control, P<0.01. *** Compared with the control, P<0.001.

mRNA and protein expression of the sputum IL-35 significantly increased compared with the asthma exacerbation despite the fact that the level was still significantly lower than in the controls (Figure 1). On the other hand, Th2 cytokines levels significantly downregulated compared with asthma exacerbation despite the fact that the level was still significantly higher than in the controls (Figure 2, Table 4). The correlation between the sputum IL-35

Table 2. The mRNA and protein expression of IL-35 and Th2 cytokines at asthma exacerbation

	Asthma exacerbation	Control
IL-35 expression		
Serum IL-35 mRNA relative expression	0.000033±0.000012***	0.00051±0.00026
Serum IL-35 (pg/ml)	39.7±12.8*	125.5±46.8
Sputum IL-35 mRNA relative expression	0.000047±0.000019***	0.00062±0.00031
Sputum IL-35 (pg/ml)	49.6±221.8***	164.6±55.3
IL-4 expression		
Serum IL-4 mRNA relative expression	4.6±1.2***	1.3±0.5
Serum IL-4 (pg/ml)	2.68±0.38***	1.27±0.25
Sputum IL-4 mRNA relative expression	5.3±2.4***	1.7±0.8
Sputum IL-4 (pg/ml)	3.45±0.64***	1.55±0.56
IL-5 expression		
Serum IL-5 mRNA relative expression	5.3±2.4***	2.1±0.9
Serum IL-5 (pg/ml)	33.14±8.3***	6.88±2.79
Sputum IL-5 mRNA relative expression	7.2±3.1***	2.3±0.6
Sputum IL-5 (pg/ml)	51.38±13.46***	5.91±1.88
IL-13 expression		
Serum IL-13 mRNA relative expression	4.2±1.9***	1.5±0.4
Serum IL-13 (pg/ml)	145.78±23.1***	26.23±9.76
Sputum IL-13 mRNA relative expression	5.8±2.7***	1.8±0.8
Sputum IL-13 (pg/ml)	179.65±41.9***	32.11±12.48

* Compared with the control group, P<0.05. ** Compared with the control group, P<0.01. *** Compared with the control group, P<0.001.

Table 3. Relationship between IL-35 and Th2 cytokines protein expression at asthma exacerbation

	Serum IL-35		Sputum IL-35	
	r	P	r	P
Serum IL-4	-0.621	0.01	-	-
Serum IL-5	-0.535	0.01	-	-
Serum IL-13	-0.653	0.03	-	-
Sputum IL-4	-	-	-0.743	0.01
Sputum IL-5	-	-	-0.819	0.03
Sputum IL-13	-	-	-0.863	0.02

and Th2 cytokines has still existed (Table 5). However, the serum IL-35 and Th2 cytokines did not change.

DISCUSSION

We first provided evidence that IL-35 in the sputum during asthma exacerbation in children decreased significantly and negatively correlated with Th2 cytokines expression. These results implied that IL-35 might play important roles during the asthma exacerbation.

Despite hints of progress in comprehending the problem pathophysiology of asthma and its phenotypes, there lacked reliable biomarkers to predict severe asthma exacerbations. IL-35, mainly secreted by Tregs, has been recently proved to be a negative regulator in a variety of immune diseases. Therefore, we explored the expression of IL-35 during asthma exacerbation in children. As expected, the sputum and the serum IL-35 expression during asthma exacerbation decreased significantly more than in the controls. Decreased sputum and serum IL-35 expression was negatively related to up-regulated Th2 cytokines,

Table 4. The mRNA and protein expression of Th2 cytokines 8 weeks after the asthma exacerbation

	Asthma exacerbation	8 weeks after exacerbation
IL-35 expression		
Serum IL-35 mRNA relative expression	0.000033±0.000012**	0.00031±0.00014
Serum IL-35 (pg/ml)	39.7±12.8***	79.2±21.3
Sputum IL-35 mRNA relative expression	0.000047±0.000019**	0.00038±0.00022
Sputum IL-35 (pg/ml)	49.6±221.8*	94.4±31.8
IL-4 expression		
Serum IL-4 mRNA relative expression	4.6±1.2**	2.4±0.7*
Serum IL-4 (pg/ml)	2.68±0.38*	1.98±0.28*
Sputum IL-4 mRNA relative expression	5.3±2.4**	3.1±1.2*
Sputum IL-4 (pg/ml)	3.45±0.64*	2.67±0.68*
IL-5 expression		
Serum IL-5 mRNA relative expression	5.3±2.4*	3.2±1.2*
Serum IL-5 (pg/ml)	33.14±8.3**	12.77±3.65*
Sputum IL-5 mRNA relative expression	7.2±3.1*	4.8±2.1*
Sputum IL-5 (pg/ml)	51.38±13.46**	37.64±11.39*
IL-13 expression		
Serum IL-13 mRNA relative expression	4.2±1.9*	2.9±0.8*
Serum IL-13 (pg/ml)	145.78±23.1*	87.65±15.88*
Sputum IL-13 mRNA relative expression	5.8±2.7*	2.9±1.2*
Sputum IL-13 (pg/ml)	179.65±41.9*	87.65±22.36*

* Compared with the control group, P<0.05. ** Compared with the control group, P<0.01. *** Compared with the control group, P<0.001.

Table 5. Relationship between IL-35 and Th2 cytokines protein expression 8 weeks after the asthma exacerbation

	Serum IL-35		Sputum IL-35	
	r	P	r	P
Serum IL-4	-0.638	0.01	-	-
Serum IL-5	-0.578	0.02	-	-
Serum IL-13	-0.669	0.01	-	-
Sputum IL-4	-	-	-0.721	0.01
Sputum IL-5	-	-	-0.801	0.01
Sputum IL-13	-	-	-0.823	0.02

proving that IL-35 was involved in the Th2 response both locally and systematically.

Consistently, Wang reported similar results that the serum IL-35 downregulated in asthmatic subjects, which contributed to up-regulated IL-4-producing CD8+ T cells (15). They also found that IL-35 can significantly inhibit IL-4 secretion from activated CD4+CD25+ T cells in allergic asthma (16). However, Wong's study showed opposite results, namely, that IL-35 levels in patients with allergic asthma elevated compared with the non-allergic controls. Their data suggested that IL-35 expression positively correlated with symptoms scores of patients (17). Moreover, Khoshkhui's study found no relationship between childhood asthma and serum IL-35 levels (18). These inconsistencies may be due to the heterogeneity of inflammation patterns and different physiopathology in the subtypes of studied asthmatic subjects. The other reasons included different detection systems, different sensitivity of methods, and the small

numbers of study subjects. In this study, we only recruited allergic asthma children to eliminate the influence of other subtypes of asthma on the cytokine expression.

In animal models of asthma, the roles of IL-35 in suppressing airway inflammation and airway high reaction (AHR) are consistent and assured. To exemplify, mice lacking EBI3 or p53 showed more severe airway inflammation and AHR than wild-type mice after ovalbumin (OVA)-lipopolysaccharide (LPS) sensitization and challenge (19). Previous studies also showed that administration of adenovirus-mediated IL-35 or recombinant IL-35 inhibits both AHR and allergic response in OVA-induced asthma mice (20, 21).

After standard treatment, we detected the mRNA or protein in levels of IL-35 and Th2 cytokines eight weeks later and found that both the sputum and serum were restored partially despite the fact that the levels were still significantly lower than in the controls. These results suggested that asthma exacerbation presented as both local and systematical inflammation.

Our research has certain drawbacks as well. First, the number of cases is modest, necessitating larger-scale studies. Second, no in vitro research was done to substantiate IL-35's direct participation in Th2 inflammation. Third, the follow-up period was insufficient.

CONCLUSIONS

At the time of asthma exacerbation, IL-35 expression in the induced sputum and serum decreased significantly than in the controls. However, the IL-35 levels increased significantly eight weeks after the exacerbation. Moreover, we found that IL-35 was associated with Th2 inflammation during asthma exacerbations. In sum, our study provided evidence that decreased IL-35 during asthma exacerbation may contribute to enhanced Th2 response, suggesting that IL-35 is a potential target for AR.

Conflicts of Interest: None declared.

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