

Interleukin-2 levels in exhaled breath condensates, asthma severity, and asthma control in nonallergic asthma

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ABSTRACT

There was evidence that interleukin (IL)-2 and IL-10 in the airways play roles in regulating the asthmatic inflammatory response. The purpose of this study was to measure the levels of these cytokines in exhaled breath condensates (EBCs) from asthmatic airways and their correlation with a clinical assessment of asthma severity. The levels of IL-2 and IL-10 in EBC, Asthma Control Test (ACT) score, and the forced expiratory volume in 1 second (FEV₁) were studied in 28 steroid-free asthmatic patients and 10 healthy volunteers. The results were analyzed according to their allergic status, asthma severity, and body weight. The correlations between IL-2 and IL-10 levels, percent predicted FEV₁, ACT score, and body mass index were also determined. Both IL-2 and IL-10 levels in EBC significantly increased in asthmatic patients, especially in patients with moderate-to-severe persistent asthma, compared with those in normal controls. However, the significant correlations between IL-2 levels and ACT ($r = -0.684$; $p = 0.007$), as well as with percent predicted FEV₁ ($r = -0.671$; $p = 0.009$), were established only in patients with nonallergic asthma. The elevation of IL-2 levels in EBC in obese subjects was observed but was probably related to asthma severity. The levels of IL-2 and IL-10 in EBC increase in asthmatic patients but only IL-2 levels significantly correlate with the ACT score and percent predicted FEV₁ in nonallergic asthma. Additional studies should be explored to confirm the reliability of ACT score as a predictor of inflammatory response in asthmatic airways. Clinical trial NCT01246414, www.clinicaltrials.gov.

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Airway inflammation is one of the main characteristic features of asthma. The interaction between proinflammatory and anti-inflammatory cytokines in the airways could affect the severity of asthma and its phenotypes. The activation of T lymphocytes *via* interleukin (IL)-2 and IL-2 receptor plays an important role in sustaining asthma symptoms, particularly during moderate and severe asthma attacks.^{1,2} Previous studies have indicated that individuals with nonallergic asthma had elevated levels of IL-2 and IL-5, whereas those with allergic asthma had elevated IL-4 and IL-5 in bronchoalveolar lavage (BAL) and bronchial biopsy specimens.^{3,4} Evidence suggested that the concentrations of IL-2 in BAL fluid were higher in symptomatic asthma compared with those of normal subjects and asymptomatic patients. Levels of measurable IL-2 in

BAL fluid inversely correlated with the forced expiratory volume in 1 second (FEV₁) and were associated with eosinophilic infiltration in asthmatic airways.²

In contrast, IL-10 has been proposed as an anti-inflammatory cytokine in the airways. There was evidence that reduced IL-10 production significantly contributed to the inflammatory response in asthma and the expression of IL-10 resulted in an inhibition of airway inflammation.^{5,6} Several studies confirm the ability of IL-10 to regulate the inflammatory responses by reducing T-helper 2 cytokine production,⁶ as well as inhibiting functions of monocytes,⁷ neutrophils,⁸ and eosinophils.⁹ The decrease of IL-10 levels in sputum was reported in patients with asthma or chronic obstructive pulmonary disease.¹⁰ In addition, there was evidence that the secretion of IL-10 in BAL fluid and in peripheral blood mononuclear cells in asthmatic patients was lower than those in nonasthmatic subjects both at baseline and after stimulation.¹¹

Several tools are currently in use to evaluate the severity of asthma and the quality of asthma care. The Asthma Control Test (ACT), a symptom-based questionnaire, has been developed and validated as a “clinical” tool for assessing asthma control in addition to using the Global Initiative for Asthma (GINA) guidelines to assess asthma severity in clinical practice.^{12–14} The correlation data between asthma severity based on a symptom-based questionnaire, pulmonary function test, and airway inflammation, however, are not con-

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clusive. ACT and the Childhood ACT (ACT) scores were found to correlate with the FEV₁ in asthmatic adults and children, respectively.^{15–17} A reverse correlation between IL-2 concentrations in BAL fluid and FEV₁ in symptomatic asthma was reported.² Some researchers, nevertheless, have not been able to show the correlation between clinical severity of asthma and sputum or serum IL-2 and IL-10 levels in atopic asthmatic patients.¹⁸ Furthermore, there was a study showing no association between ACT score and sputum eosinophil or neutrophil counts.¹⁹ Additionally, other studies have indicated poor correlations between FEV₁ and the severity assessed by a symptom-based questionnaire.^{20,21} Several factors influencing inflammatory responses in the airways probably contribute to this conflicting result. The diverse inflammatory patterns in allergic and nonallergic asthma should be taken into consideration.^{3,22,23} Obesity, which was recently found to be associated with systemic inflammation, may worsen the severity of asthma as well.^{24–26}

Although the direct measurement of cytokine levels from bronchial biopsy specimens should be the ideal method for analyzing inflammatory responses in the airways, this approach is not feasible in clinical practice. Recently, there has been increased interest in the ability to monitor airway inflammation using noninvasive methods such as the measurement of fractional exhaled nitric oxide or biomarkers in exhaled breath condensate (EBC).²⁷ Fluid collecting from EBC contains aerosolized airway lining fluid and volatile compounds and could be analyzed to study ongoing inflammatory responses in the lungs and airways as well.^{28,29} Several biomarkers, including cytokines, are present in EBC fluid from asthmatic patients.^{30–32} Therefore, the evaluation of EBC cytokines in different types of asthma and the association with asthma severity in clinical practice should be explored. The purposes of this study were (1) to measure the levels of IL-2 and IL-10 in EBC from different phenotypes of asthma based on allergic status, GINA classification, and body weight status and (2) to investigate the correlation among the levels of IL-2 and IL-10 in EBC, FEV₁, and ACT score in asthmatic patients in the absence of corticosteroids.

METHODS

Asthmatic patients (aged 15–65 years) visiting the Allergy Clinic, King Chulalongkorn Memorial Hospital, in 1 year-period who treated themselves with only inhaled short-acting β_2 -agonist on an as-needed basis and who had not used oral or inhaled corticosteroids in the 4 weeks before their clinic visit were recruited into this study. A skin-prick test was performed on the volar aspect of the forearm in these patients with a panel of 16 common aeroallergens in Thailand includ-

ing house dust, *Dermatophagoides pteronyssinus*, American cockroach, *Alternaria tenuis*, *Aspergillus mix*, mold mix, standardized *Bermuda* grass, standardized *Timothy* grass, standardized cat hair, dog epithelia (mixed breeds), kapok seeds, mixed feathers, *Acacia* spp., orris root, natural weed mix, and *Pyrethrum* (Greer Laboratories, Lenoir, NC). A wheal diameter >3 mm at 20 minutes after the skin-prick test was considered positive. Patients with a positive test for at least one allergen were classified as being allergic. Current cigarette smokers and subjects with a history of respiratory tract infection within 4 weeks were excluded.

Pulmonary function tests were assessed using the diagnostic spirometer (KoKo PFT; nSpire Health, Inc., Denver, CO) on the same day as the EBC collection after an inhaled short-acting β_2 -agonist had been withheld for at least 8 hours. Asthma severity was categorized according to the GINA classification.¹⁴ The ACT score was used to assess the asthma control of all patients in the 4 weeks before the study. Volunteers were stratified by body mass index as normal weight, overweight, and obese according to the recommended cutoff points for Asian populations.³³ Ten nonsmokers who are free of respiratory diseases were recruited as healthy control subjects.

At present, several commercial EBC collectors such as ECoScreen 2 (FILT GmbH, Berlin, Germany), ECoScreen Turbo (Carl Reiner GmbH, Vienna, Austria), and RTube (Respiratory Research, Inc., Austin, TX) are available. In this study, we used the custom-made device and followed the technique suggested by Effros *et al.*³⁴ EBC was collected using a double-walled glass condenser (2.2 cm in diameter and 58 cm long). This collecting vessel was cooled with a cooling unit consisting of a countercurrent of circulating ice water and a salt pump to maintain the temperature at $\sim 0^\circ\text{C}$. Subjects were asked to breathe tidally through a mouthpiece connected to a one-way valve and the condenser for 20 minutes while wearing a nose clip. The mouthpiece and condenser were connected by a 450 $\times$ 22-mm ID ventilator tube (Corr-a-Flex 2; Hudson RCI, Temecula, CA) inclined upward to minimize salivary contamination. The condensate dripped into polycarbonate collection tubes. Samples were snap-frozen at -20°C , and then stored at -70°C immediately after collection. Amylase concentrations in EBC samples were measured using dry chemistry (a detection limit of 30 $\mu\text{g}/\text{mL}$) and the ones with detectable amylase were considered contaminated with saliva and excluded from this study.

Two milliliters of EBC fluid were lyophilized with an evaporator centrifuge (CHAIST alpha 1–4; Martin Christ GmbH, Osterode, Germany) and resuspended in 60 μL of deionized water before the measurement of cytokines. Twenty-five microliters of each sample were analyzed using a bead-based immunoassay (cytomet-

Table 1 Baseline characteristics of asthmatic patients and healthy subjects

	Allergic Asthma <i>n</i> = 14	Nonallergic Asthma <i>n</i> = 14	Healthy Subjects <i>n</i> = 10
Gender (M/F)	3/ 11	7/ 7	4/ 6
Age (yr)	38.2 ± 3.8	37.6 ± 3.6	31.2 ± 1.8
Weight (kg)	63.9 ± 4.4	62.4 ± 4.7	61.2 ± 4.2
BMI (kg/m ²)	25.3 ± 1.6	24.4 ± 1.4	22.2 ± 1.2
Intermittent asthma	2	1	NA
Mild persistent asthma	4	8	
Moderate persistent asthma	6	4	
Severe persistent asthma	2	1	

Data are expressed as mean ± SE.

BMI = body mass index; M = male; F = female; NA = not applicable.

ric bead array; Bender Medsystems GmbH, Vienna, Austria). Cytokines in test samples and recombinant standards bound to capture beads were detected by PE-conjugated detection antibodies for IL-2 and IL-10 (the lower detection limits were 16.2 and 1.9 pg/mL, respectively) and measured in a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA) before being analyzed with Flowcytomix Pro software (Bender Medsystems GmbH). Measurement of the cytokine levels was performed in duplicate and the average of those values was reported. The laboratory investigator was blinded to the clinical data of the subjects. S. Boonpiyathad and P. Pornsuriyasak contributed equally to this work. The study was approved by the Research Ethics Committee, Faculty of Medicine, Chulalongkorn University, and all subjects gave written informed consent.

Statistical Analysis

All statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL). Results are expressed as means ± SE. Differences of cytokine levels among the groups were tested by one-way ANOVA with LSD *post hoc* analysis. The correlations between cytokine levels, percent predicted FEV₁, and ACT score were assessed using Pearson's linear regression after adjustment for the GINA asthma severity, obesity status, and allergic status. Values of *p* < 0.05 were considered statistically significant.

RESULTS

Demographic and Clinical Characteristics of Asthmatic Patients and Healthy Controls

Thirty-one asthmatic patients completed the study. The results from EBC samples in three asthmatic patients were excluded because of salivary contamination (detectable amylase in EBC fluid). Data from the remaining 28 asthmatic patients (14 allergic and 14 non-

allergic subjects) and 10 healthy individuals are shown in Table 1. There was no statistical difference in baseline clinical characteristics among the three groups. The severity of asthma in allergic and nonallergic asthmatic groups was comparable in terms of symptoms and FEV₁ assessment.

Both IL-2 and IL-10 Levels in EBC Were Increased in Asthmatic Patients Compared with Healthy Controls

The levels of IL-2, IL-10, and IL-2/IL-10 in EBC from asthmatic patients and healthy controls were comparatively analyzed (Table 2). The results showed a significant increase of IL-2 and IL-10 levels in EBC from asthmatic patients regardless of their allergic status (*p* = 0.043 and 0.026 for allergic asthma and *p* = 0.035 and 0.01 for nonallergic asthma, respectively) compared with those in healthy nonasthmatic controls.

Patients were also categorized into (1) intermittent asthma and mild persistent asthma and (2) moderate and severe persistent asthma, based on the GINA classification.¹⁴ IL-2 and IL-10 levels in the EBC from both asthmatic groups were significantly higher than those from nonasthmatic controls even after adjusting for body index and allergic status. The ACT score was also lower in the moderate and severe persistent asthma group compared with that of the intermittent and mild persistent asthma group (11.1 ± 0.8 pg/mL versus 16.4 ± 1.4 pg/mL; *p* < 0.05). IL-2 and IL-10 levels were comparable between patients with allergic and nonallergic asthma. The IL2/IL10 ratios in EBC were also comparable in both asthmatic patients and healthy control subjects.

Obese Individuals Had Higher IL-2 Levels in EBC but Possibly Related to Asthma Severity

Levels of IL-2, IL-10, and IL-2/IL-10 in EBC, as well as percent predicted FEV₁ in all volunteers were strat-

Table 2 Levels of IL-2, IL-10, and IL-2/IL-10 ratio in the EBCs of asthmatic patients categorized by allergic status and GINA classification compared with those in healthy subjects

	Allergic Asthma <i>n</i> = 14	Nonallergic Asthma <i>n</i> = 14	Total Asthma <i>n</i> = 28	Healthy Subjects <i>n</i> = 10	Intermittent–Mild Persistent Asthma <i>n</i> = 15	Moderate–Severe Persistent Asthma <i>n</i> = 13
IL-2 (pg/mL)	28.2 ± 2.4*	28.6 ± 2.4*	28.4 ± 1.7*	18.9 ± 4.6	26.0 ± 2.2	31.2 ± 2.4*
IL-10 (pg/mL)	5.1 ± 0.8*	6.5 ± 0.7*	5.8 ± 0.5*	2.4 ± 0.9	6.1 ± 0.6*	5.5 ± 0.9*
IL-2/IL10	5.7 ± 0.7	5.2 ± 0.7	5.4 ± 0.5	4.0 ± 1.2	5.0 ± 0.6	6.0 ± 0.8
FEV ₁ (percent predicted)	74.9 ± 4.4*	80.5 ± 4.1*	77.7 ± 3.0*	95.8 ± 2.7	89.1 ± 2.5	64.5 ± 2.8**
ACT score	13.4 ± 1.4	14.4 ± 1.3	13.9 ± 1.0	NA	16.4 ± 1.4	11.1 ± 0.8**

Data are expressed as mean ± SD.

*The value of $p < 0.05$ compared with healthy subjects; ** $p < 0.05$ compared with the intermittent–mild persistent asthma group after adjusting for allergic status and obesity status.

NA = not applicable; ACT = Asthma Control Test; EBC = exhaled breath condensates; GINA = Global Initiative for Asthma; FEV₁ = forced expiratory volume in 1 s.

Table 3 Levels of IL-2, IL-10, and IL-2/IL-10 ratio in the EBCs stratified by BMI

	Normal Weight <i>n</i> = 16	Overweight <i>n</i> = 14	Obese <i>n</i> = 8
IL-2 (pg/mL)	21.77 ± 3.14	27.34 ± 2.44	31.82 ± 3.30*
IL-10 (pg/mL)	4.28 ± 0.67	5.47 ± 0.94	5.16 ± 1.25
IL-2/IL10	4.97 ± 0.82	5.39 ± 0.78	5.29 ± 0.78
FEV ₁ (percent predicted)	84.38 ± 4.14	84.79 ± 4.46	74.50 ± 5.19

BMI group was classified as normal weight (BMI, <23 kg/m²), overweight (BMI, 23–27.5 kg/m²), and obese (BMI, >27.5 kg/m²). Data are expressed as mean ± SD.

*The value of $p < 0.05$ compared with groups with normal weight; however, p value was no longer significant after adjusting for asthma severity.

BMI = body mass index; EBCs = exhaled breath condensates; IL = interleukin; FEV₁ = forced expiratory volume in 1 s.

ified by body mass index (Table 3). IL-2 and IL-10 levels and percent predicted FEV₁ in subjects with normal and overweight body weights were not statistically significant. In contrast, obese subjects had significantly higher IL-2 levels in EBC (31.82 ± 3.30 pg/mL) than those with normal weight (26.05 ± 2.06 pg/mL) or overweight (25.15 ± 7.81 pg/mL; $p < 0.05$). However, after adjusting for asthma severity, the correlation between IL-2 levels and obesity was no longer observed. No significant differences in IL-10 and IL2/IL-10 ratios were found.

IL-2 Levels in EBC Were Significantly Correlated with ACT Score and Percent Predicted FEV₁, Particularly in Patients with Nonallergic Asthma

The correlations of IL-2 and IL-10 levels with percent predicted FEV₁ and ACT score in asthmatic patients are shown in Table 4. IL-2 levels in EBC had significant reverse correlations with ACT score ($r = -0.464$; $p = 0.013$) and with percent predicted FEV₁ ($r = -0.466$ $p = 0.012$). Interestingly, the significant correlations be-

tween IL-2 levels in EBC and ACT score, as well as percent predicted FEV₁ were observed only in nonallergic asthmatic patients (Fig. 1). IL-10 levels in EBC had marginal correlation with percent predicted FEV₁ in allergic asthma.

DISCUSSION

The discovery of clinical parameters reflecting airway inflammation would be beneficial for asthma management. Our study shows that IL-2 and IL-10 levels in EBC of asthmatic patients were higher than those of healthy controls regardless of their allergic status. Interestingly, the levels of IL-2 were significantly elevated in patients with moderate and severe asthma compared with patients with milder asthma phenotypes. These findings agree with the earlier report that serum IL-2 receptors are elevated during moderate or severe asthmatic attacks.¹ Our study indicates that the measurement of IL-2 levels in EBC has a better diagnostic value than the measurement in serum and induced sputum because there was no association

Table 4 Correlations between levels of IL-2 and IL-10 with ACT score and percent predicted FEV₁ in asthmatic patients

Asthma severity parameters	IL-2			IL-10		
	All Asthma	Allergic	Nonallergic	All Asthma	Allergic	Nonallergic
ACT score						
Pearson Correlation	-0.464*	-0.261	-0.684**	-0.042	-0.279	0.210
Sig. (two tailed)	0.013	0.367	0.007	0.834	0.335	0.471
Percent predicted FEV ₁						
Pearson Correlation	-0.466*	-0.291	-0.671**	-0.122	-0.544*	0.312
Sig. (two tailed)	0.012	0.313	0.009	0.537	0.044	0.278

*Correlation is significant at the 0.05 level (two tailed).

**Correlation is significant at the 0.01 level (two tailed).

ACT = Asthma Control Test; FEV₁ = forced expiratory volume in 1 s; IL = interleukin.

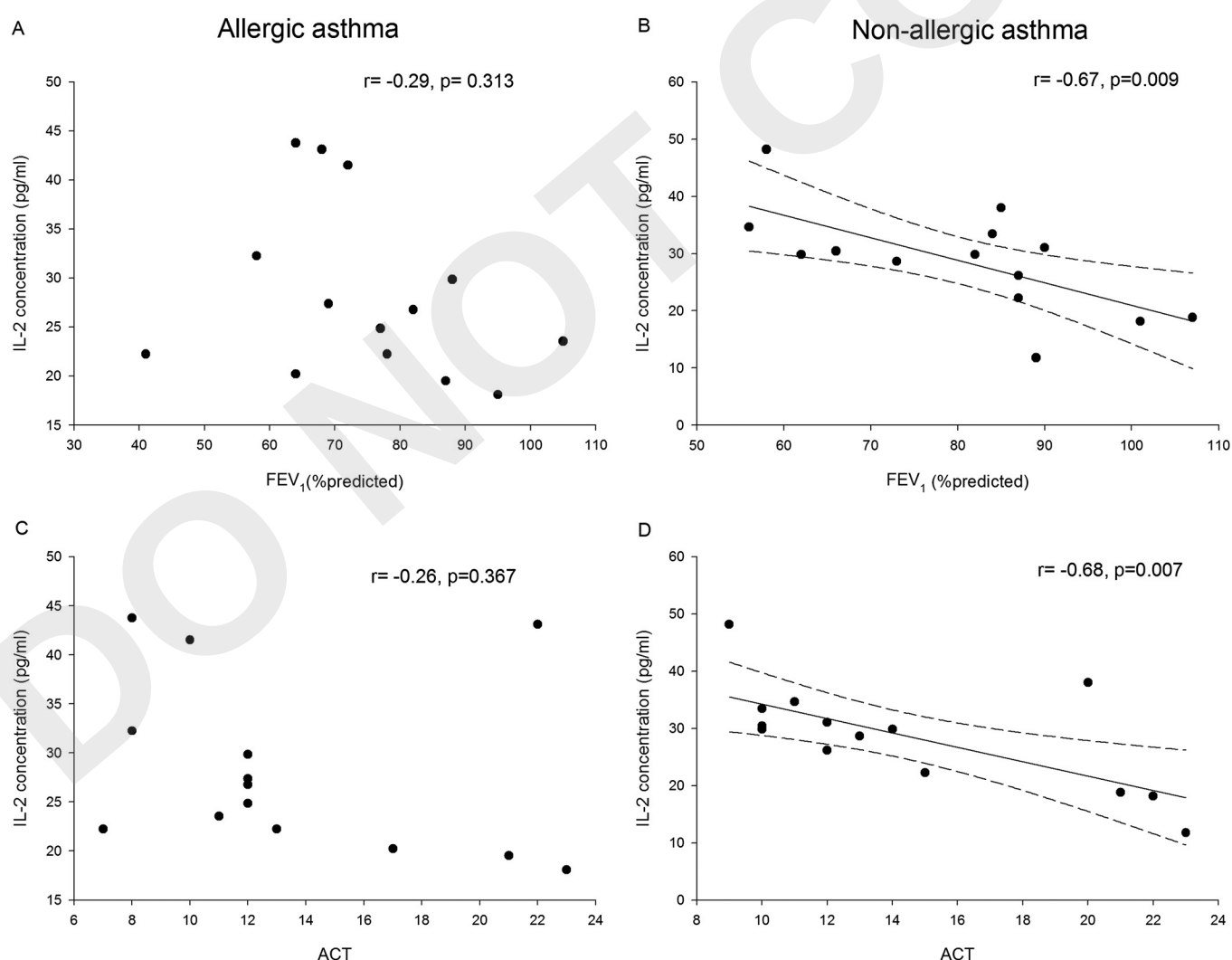


Figure 1. Regression graphs with 95% confidence interval showing the correlations between interleukin (IL)-2 concentrations in EBCs and percent predicted forced expiratory volume in 1 second (FEV₁) and the Asthma Control Test (ACT) score in asthmatic patients. Correlations between IL-2 concentrations and percent predicted FEV₁ in (A) allergic asthma and (B) nonallergic asthma. IL-2 concentration showed a significant inverse correlation with percent predicted FEV₁ in nonallergic asthma. ($r = -0.67$; $p = 0.009$). Correlations between IL-2 concentrations and ACT score in (C) allergic asthma and (D) nonallergic asthma. A significant inverse correlation between IL-2 concentrations and ACT score was observed in nonallergic asthma ($r = -0.68$; $p = 0.007$).

between clinical severity of asthma and IL-2 levels in those specimens.¹⁸ It is possible that the measurement of serum IL-2 in asthmatic patients lacks sensitivity because it could be affected by other systemic inflammatory responses and not specifically to inflammation in the airways. Our study suggests that the elevated IL-2 levels in obese asthmatic patients might be secondary to asthma severity in this patient group. Until now, the cause and effect relationship between asthma and obesity has remained under debate.³⁵ According to our study, the measurement of IL-10 and IL-2/IL-10 ratio in EBC is not really helpful for predicting the severity of asthma because they were indistinguishable between mild and severe asthmatic patients.

Only a few studies so far have reported on the association between ACT and the levels of cytokines in EBC in asthmatic patients.³⁶ Our findings show an interesting result that the degree of correlation between airway inflammations, represented by IL-2 levels, and ACT score was comparable, if not better than its correlation with percent predicted FEV₁. The patients in this study were a population of asthmatic patients who had never used inhaled corticosteroids or discontinued oral and inhaled corticosteroids for at least 1 month; causing low ACT scores. The trustworthiness of the correlation between ACT and cytokines in EBC in this study lies in the fact that this correlation was not intervened by concurrent corticosteroid treatment. The good correlation between ACT score and inflammatory parameters in asthmatic airways suggesting that the use of ACT for the assessment of asthma control is at least as useful as percent predicted FEV₁ to monitor airway inflammation in clinical practice. In fact, ACT scoring, which represents asthma control status in the past 4 weeks, could be a better reflection of ongoing airway inflammation than the percent predicted FEV₁, which is a cross-sectional evaluation of lung function at a specific point in time.

It is noteworthy that the significant correlations between IL-2 levels in EBC with the ACT score and percent predicted FEV₁ were observed only in nonallergic asthmatic patients. It is possible that T-cell activation by IL-2 may play an important role in the pathogenesis of nonallergic asthma contributing to asthma severity, whereas Th2 cytokines may contribute to the severity of allergic asthma as well. The correlation between ACT score, percent predicted FEV₁, and other inflammatory cytokines should be explored to find the potential biomarker with the best correlation with clinical severity of allergic asthma and whether the ACT score is suitable to monitor the inflammation in allergic airways.

There are some points of concern regarding the study of EBC, particularly when using custom-made devices. Gross salivary contamination should be minimized by measuring amylase activity in acquired fluid or using a

saliva trap. Even using commercially available devices, the reported values of measured biomarkers still remain a concern because of variations of the EBC collecting system, respiratory droplet dilution, and intra-subject variability. The analysis of biomarkers in terms of relative change from baseline or the ratio between multiple biomarkers may be more useful.³⁷ Whether the correlation between clinical parameters and IL-2 levels is still reliable in patients receiving inhaled corticosteroids is still to be determined and additional studies with larger sample size are required to confirm these data. The comparison of cytokine concentrations among different studies is difficult because of the use of various EBC collection techniques and the protocol to process EBC fluid for cytokine measurement is not standardized. The possibility to step-up and step-down asthma medications based on ACT score and inflammatory cytokines in EBC should also be investigated.

CONCLUSION

We showed the increased IL-2 and IL-10 levels in EBC from asthmatic airways compared with the healthy controls. The levels of IL-2 in EBC correlate well with ACT score and percent predicted FEV₁ in nonallergic asthma. Additional studies should be explored to confirm the reliability of the ACT score as a predictor of inflammatory response in allergic asthmatic airways.

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