

T Helper 17 Cells and Related Cytokines after Allergen Inhalation Challenge in Allergic Asthmatics

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Key Words

T helper 17 cells · IL-17 · Allergic asthma

Abstract

Background: T helper (Th)17 cells may play a role in allergic asthma. This study assessed the effect of allergen inhalation challenge on circulating Th17 cells and related cytokines in allergic asthmatics. **Methods:** Peripheral blood mononuclear cells were collected from 16 atopic asthmatics before and 24 h after allergen challenge, as well as from 10 atopic non-asthmatics and 10 normal controls. Cells were stained for Th17 cytokines and their receptors (IL-17A, IL-17F, IL-21, IL-22, IL-17R, and IL-23R) using flow cytometry. Cytokine concentrations from cell culture supernatants were quantified using a multiplex assay for IL-17A, IL-17F, IL-21, IL-22, and IL-23. **Results:** At baseline, asthmatics had a higher percentage of circulating Th17 cells ($1.2 \pm 0.5\%$) compared to normal controls ($0.9 \pm 0.66\%$, $p < 0.001$) but not compared to atopic nonasthmatics ($1.13 \pm 0.5\%$). There was a significant increase in Th17 cells in asthmatics after allergen challenge to $1.55 \pm 0.4\%$ ($p < 0.05$) and a trend toward significance in IL-17R expression from 3.4 ± 4.3 to $6.86 \pm 6.84\%$ after allergen challenge ($p = 0.06$). There was also a significant reduction in IL-21-positive cells following allergen challenge from 3.46 ± 1.85 to $2.33 \pm 1.37\%$ ($p < 0.001$). There were no significant differences in IL-17F, IL-22 and IL-23R expression. The concentration of IL-17A in culture supernatant was significantly

higher in asthmatics compared to normal controls and IL-17A significantly increased 24 h after allergen challenge. **Conclusions:** The increase of Th17 cells and IL-17A in atopic asthma after allergen inhalation challenge suggests a possible role for Th17 in allergen-induced airway responses.

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Introduction

Asthma is characterized by variable airflow obstruction, airway hyperresponsiveness, airway inflammation and remodeling, thought to be mediated through the release of proinflammatory cytokines, chemokines and lipid mediators [1]. The airway inflammation is often eosinophilic but can be neutrophilic, particularly in asthmatics who do not respond well to inhaled corticosteroids [2].

T lymphocytes play a major role in the pathogenesis of asthma. It is believed that cytokines and chemokines released from T helper (Th)2 lymphocytes predominantly drive eosinophilic inflammation. More recently, another subset of T cells has been identified which produce IL-17 and other members of this family of cytokines, which have been labeled as Th17 cells [3]. These cells have been implicated in various immune and inflammatory processes [4] by releasing proinflammatory cytokines, chemokines, adhesion molecules and growth factors, which

Table 1. Subject characteristics

	Normal controls	Atopic nonasthmatics	Atopic asthmatics
Number of subjects	10	10	16
Sex (M/F)	4/6	5/5	8/8
Age, years	31±12	36.5±11	38.5±15.5
FEV ₁ , % predicted	100±9.0	98±10.0	89.8±10.2 ¹
Methacholine PC ₂₀ , mg/ml	>16	>16	5.74 (2.4) ²
IgE, kU/l	21.6±22.3	72.2±65.5*	119±131.9 ¹
FEV ₁ (EAR/LAR), % fall			30.9±7.0/23.0±9.5

Data are presented as means ± SD, or geometric mean ± geometric SD for methacholine PC₂₀.

¹ Significantly different ($p < 0.05$) from normal controls. ² Significantly different from both normal controls and atopic nonasthmatics.

stimulate T cell priming, antibody production, eosinophil and neutrophil chemoattraction, and fibroblast proliferation. The IL-17 family of cytokines includes IL-17A, IL-17F, IL-21, IL-22, IL-23, and IL-25 [5].

Th17 cells have been implicated in causing neutrophilic airway inflammation in asthma [6]. However, Th17 cells have also been demonstrated to mediate eosinophilic inflammation in murine models of allergic airway responses [7, 8]. In addition, increases in Th17 cells and IL-17 expression in sputum [9, 10], bronchoalveolar lavage [11], lung tissue [12], and peripheral blood [13], as well as correlations between IL-17 mRNA and IL-5 mRNA have been demonstrated in patients with asthma [10]. Circulating Th17 cells and IL-17 levels have been shown to be significantly higher in the peripheral blood of subjects with atopic asthma following allergen inhalation challenge to house dust mites (HDM) [14] and in response to in vitro stimulation with HDM extract [15] compared to nonatopic asthmatics and normal controls. These data have suggested a role for Th17 cells as regulators of allergic inflammation in asthma and provided evidence of an association between eosinophilic airway inflammation mediated by Th17 and Th2 cells. Higher levels of IL-17A are also considered an independent risk factor for severe asthma [16] and correlate with the severity of asthma [13] and airway hyperresponsiveness [9].

There have been limited data examining the role of Th17 cells and related cytokines in allergen-induced airway responses in asthma. The aim of the present study was to explore the changes in circulating Th17 cells and related cytokines before and after allergen challenge subjects with mild atopic asthma and to compare these changes to levels in atopic nonasthmatics and normal controls.

Material and Methods

Subjects

A total of 16 atopic asthmatics who exhibited both early and late asthmatic responses to allergen inhalation challenge, 10 atopic nonasthmatics and 10 healthy controls were recruited into the study (table 1). All subjects provided signed informed consent and the study was approved by the Hamilton Health Science Research Ethics Board.

The atopic asthmatics were nonsmoking adults with mild stable asthma, with a methacholine provocative concentration causing a 20% fall (PC₂₀) in the forced expired volume in 1 s (FEV₁) ≤16 mg/ml, baseline FEV₁ ≥80% predicted, positive allergy skin test to common aeroallergens (requiring only as needed short-acting β₂-agonists less than daily), and no requirement for regular inhaled corticosteroids. Atopic nonasthmatics were categorized by positive skin test to 1 or more aeroallergens, no history of asthma and a methacholine PC₂₀ >16 mg/ml. Normal controls had no history of respiratory diseases, a methacholine PC₂₀ >16 mg/ml and negative skin test. Subjects were excluded if they had upper respiratory tract infection or asthma exacerbation within the previous 4 weeks or used inhaled or oral corticosteroids within 4 weeks of the study onset. Antihistamines were withheld 1 week before allergen and methacholine challenge. Short-acting β₂-agonists were withheld 8 h before bronchial provocation tests.

Study Design

Subjects attended the laboratory on two (for normal controls and atopic nonasthmatics) or three occasions (for atopic asthmatics). At an initial visit, a full medical history, skin prick tests, pulmonary function test, and methacholine challenge were performed. Blood samples were obtained from normal and atopic subjects at a second visit, within 1 week of their initial visit. Asthma subjects had allergen inhalation challenge within 1 week of their initial visit and blood sample was withdrawn both before (second visit) and 24 h after (third visit) allergen challenge.

Spirometry

Lung function was carried out using a Vmax[®] SensorMedics[®] 6200 Autobox DL. The FEV₁, forced vital capacity (FVC) and FEV₁/FVC ratio were measured and recorded.

Skin Prick Test and Methacholine Challenge

Skin prick testing to 14 common aeroallergens was conducted. The allergens used were dog epidermis, cat epidermis, horse epidermis, feather mix, Dictyoptera, HDM (*Dermatophagoides farina* and *D. pteronyssinus*), Alternaria, Hormodendrum, Aspergillus, tree mix, grass mix, ragweed, and weed mix, plus a positive control (histamine) and negative control (buffer and glycerol). The methacholine challenge was undertaken using the method described by Juniper et al. [17] by 2 min tidal breathing of doubling concentrations of methacholine. The test was terminated when the FEV₁ fell by 20%, and the provocative concentration of methacholine causing 20% fall in FEV₁ (PC₂₀) was calculated.

Allergen Inhalation Challenge

The allergen challenge was performed on atopic asthmatics as previously described [18]. Briefly, the type of allergen was identified from the skin prick testing. The concentration of allergen causing 20% fall in FEV₁ was predicted using the methacholine (PC₂₀), and the titration of allergen was determined from the skin prick testing as previously described [19]. The early asthma response (EAR) was defined by >20% fall in FEV₁ between 0 and 3 h after allergen, while the late asthma response (LAR) was defined by >15% fall in FEV₁ between 3 and 7 h after allergen inhalation. All asthmatic subjects enrolled in the study had both EAR and LAR (dual responders).

Cell Separation and Culture

Peripheral venous blood samples (15 ml) were withdrawn into lithium heparin tubes (BD Dickenson) at baseline in all subjects and at 24 h after the allergen inhalation challenge in the atopic asthmatics.

Peripheral Blood Mononuclear Cell Preparation

Complete RPMI (Gibco, Germany) was prepared in a 50-ml tube filled with RPMI media to 20 ml and then supplemented with 1.25 ml HEPES (25 mM), 2.5 ml fetal calf serum (5%) and 50 µl penicillin and streptomycin (1%) and filled to 50 ml with RPMI.

PBMCs were separated from peripheral blood using Ficoll-Paque density gradient centrifugation (Sigma, UK). Cells were resuspended in complete RPMI medium and separated into 3 tubes at 1.5 million cells per tube. The cells were then stimulated with 50 ng/ml (10 µl) of phorbol myristate acetate, 1 µg/ml (100 µl) of Ionomycin and 10 µg/ml (10 µl) of Brefeldin (protein transport inhibitor; eBioscience), cultured for 4 h and then incubated at 37°C before final staining. A cell stimulation cocktail and protein transport inhibitor was used for activation and subsequent intracellular detection of Th17 cytokines [20].

Flow Cytometry and Intracellular Staining

Prepared PBMC were harvested from the wells after an incubation period of 4 h. The cells were then washed, FC blocked and incubated for 15 min. They were then stained for surface markers with FITC anti-human CD3 and V450 anti-human CD4 and with APC anti-human IL-17RA and PE anti-human IL-23R for 30 min at 4°C, washed twice with the staining buffer and fixed with 100 µl fixation buffer for 20 min at 4°C. The cells were then permeabilized twice with 2 ml of permeabilization buffer and resuspended in 100 µl of permeabilization buffer. They were then stained with 5 µl of V450 anti-human CD4 and FITC anti-human IL-17A, PE anti-human IL-17F, PerCP anti-human IL-22, and eFlour 660 anti-human IL-21 for 20 min at 4°C.

The cells were washed twice in permeabilizing solution, resuspended with 400 µl of FACS staining buffer, and finally measured using 15-color LSR II flow cytometer sorter equipped with 3 lasers (BD Biosciences). Isotype-matched monoclonal antibodies were used as controls. Data analysis was performed using the FACS Diva software program (BD Biosciences). The gating strategy is demonstrated in figure 1.

Th17 Multiplex Assay

The levels of IL-17A, IL-17F, IL-21, IL-22, and IL-23 were analyzed in duplicate from PBMC culture supernatant using BIO-Plex Pro™ human Th17 cytokine assay (Bio-Rad Laboratories) according to the manufacturer's instructions.

The assay working range is the interval between the lower and the upper limit of quantification in which both intra-assay precision and standard curve accuracy are demonstrated. The Th17 assays are designed to meet an intra-assay precision of ≤10% and a standard curve recovery of 80–120%. The lower and the upper limit of quantification are as follows: IL-17A (1.2–19,682 pg/ml), IL-17F (3.0–18,667 pg/ml), IL-21 (9.0–147,023 pg/ml), IL-22 (3.9–11,916 pg/ml), and IL-23 (7.4–120,386 pg/ml).

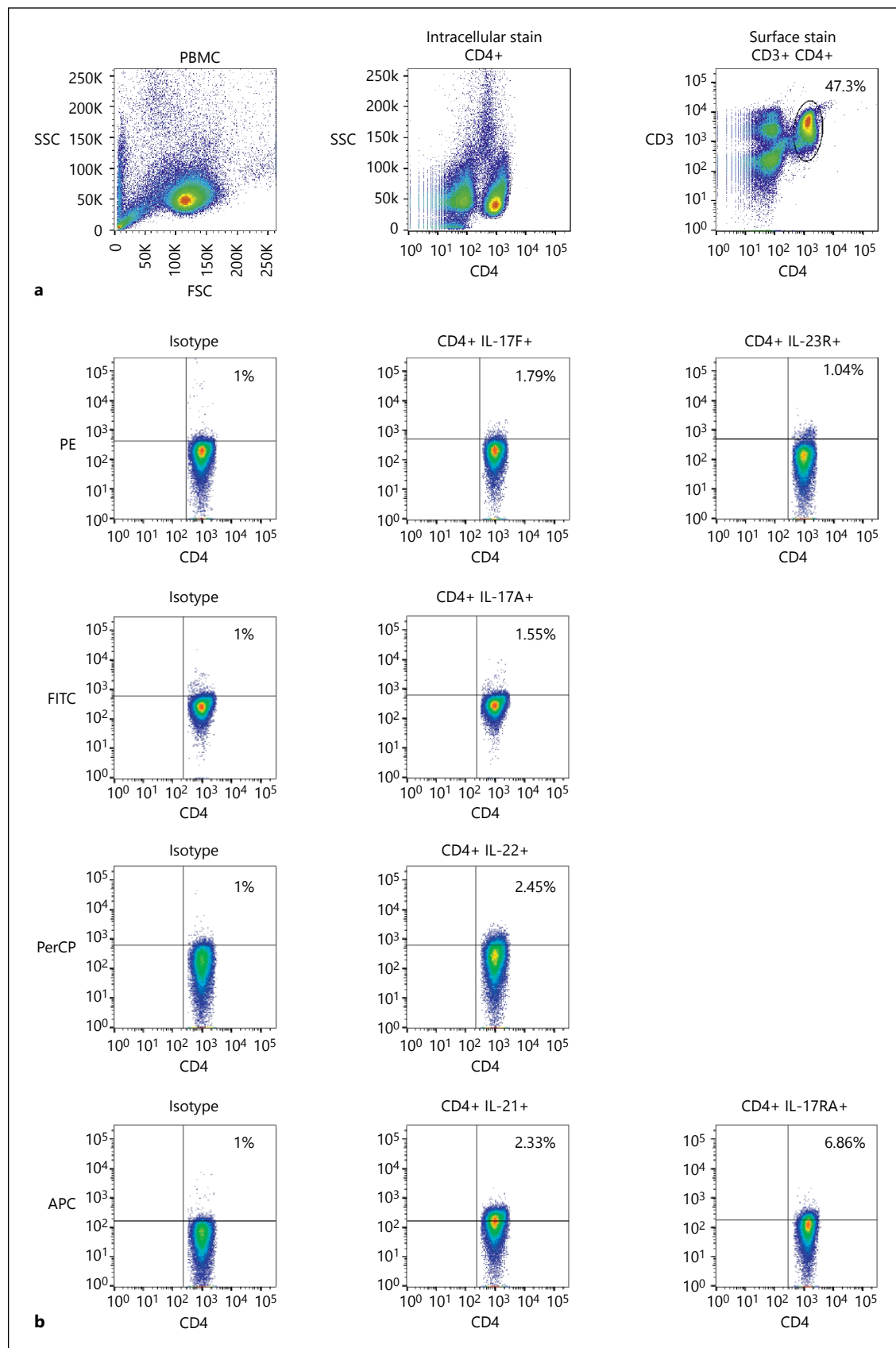
A total of 5 million PBMCs were centrifuged at 500 g for 10 min at 4°C. The culture supernatant was poured off and the cells were resuspended in 1 ml of complete RPMI. Two wells were used at 0.5 ml per well and were cultured and stimulated with 5 µl of phytohemagglutinin (1/100) per well. The cells were then incubated at 37°C, 5% CO₂ and high humidity for 24 h. After 24 h of incubation time, the cells were harvested from the wells, resuspended and centrifuged at 500 g for 10 min at 4°C. The supernatant was removed and aliquoted into 500-µl Eppendorf tubes and stored at –80°C for multiplex analysis.

Statistical Analysis

For most variables, the results are presented as means ± standard deviations (SD). The methacholine PC₂₀ values are expressed as geometric means ± geometric SD. Statistical analysis was performed using Prism software. Student t tests were used to compare pre- and postallergen challenge values, and one-way ANOVA with Tukey posttest was used to compare all three groups (atopic asthmatics, atopic nonasthmatics and normal controls). Statistical significance was assumed if $p < 0.05$.

Results

There were no differences in age and gender between the groups (table 1). As expected from the recruitment criteria, the FEV₁ predicted was significantly lower in atopic asthmatics (89.75 ± 10.2%) compared to normal subjects (100 ± 9.0%, $p < 0.02$) and atopic nonasthmatics (98 ± 10.0%, $p = 0.05$). In addition, IgE levels were higher in atopic asthmatics (119.0 ± 131.9 × 1,000 U/l, $p < 0.007$) and atopic nonasthmatics (72.7 ± 65.5 × 1,000 U/l, $p < 0.002$) compared to normal controls (21.6 ± 22.3 × 1,000 U/l). The methacholine PC₂₀ was significantly lower in atopic asthma subjects (5.74 ± 2.4 mg/ml) compared to atopic nonasthmatics and normal controls (>16 mg/ml;



(For legend see next page.)

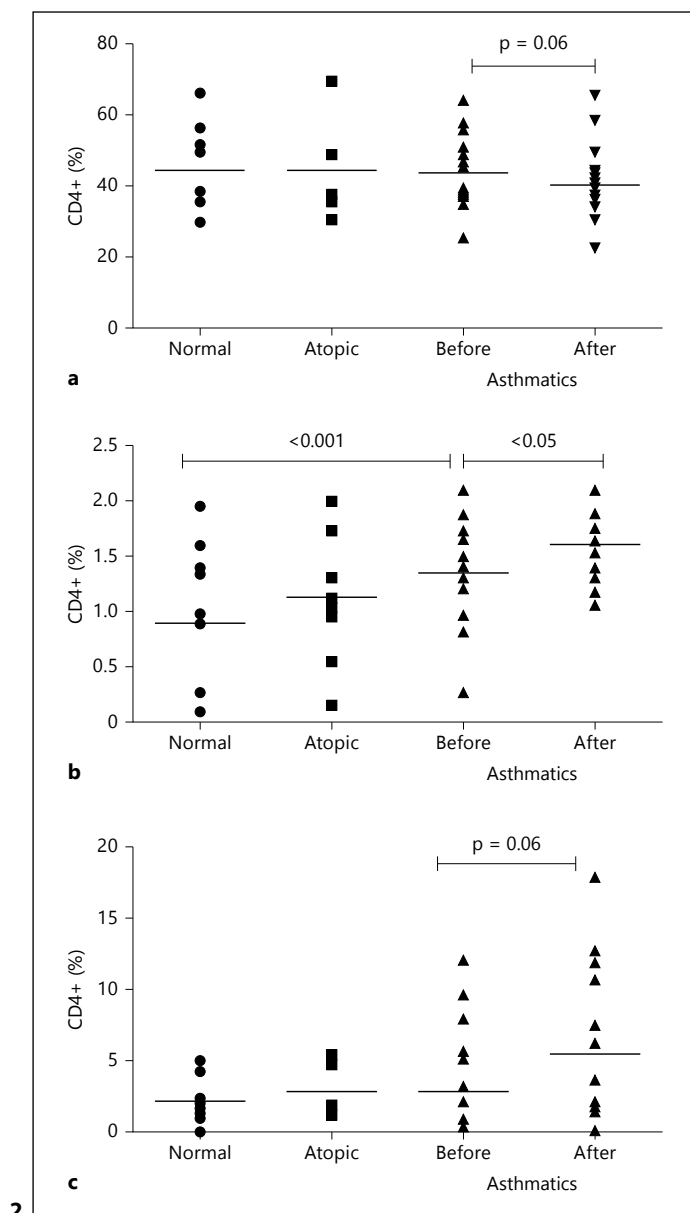


Fig. 1. Gating strategy for CD4+, IL-17A CD4+, IL-17F CD4+, IL-17RA CD4+, IL-21 CD4+, IL-22 CD4+, and IL-23 CD4+ cells (a) and appropriate isotype controls from the granulocyte layer of peripheral blood (b).

Fig. 2. a Percentage of CD4+ cells from normal controls, atopic nonasthmatic subjects and atopic asthmatic subjects before and 24 h after inhaled allergen challenge. **b** IL-17A+ cells as a percentage of CD4+ cells from normal controls, atopic nonasthmatic subjects and atopic asthmatic subjects before and 24 h after inhaled allergen challenge. **c** IL-17RA+ cells as a percentage of CD4+ cells from normal controls, atopic nonasthmatic subjects and atopic asthmatic subjects before and 24 h after inhaled allergen challenge.

table 1). All of the atopic asthmatics experienced an EAR and LAR after inhaled allergen. The mean fall in FEV₁ was $30.9 \pm 7.0\%$ during the EAR and $23.0 \pm 9.5\%$ during the LAR.

Circulating Th17 Cells

There were no significant differences in circulating CD4+ cells as a percentage of all T cells among the three groups, being $43.6 \pm 9.15\%$ in atopic asthmatic, $44.4 \pm 11.3\%$ in atopic nonasthmatics and $44.2 \pm 12.2\%$ in normal controls (fig. 1a). The percentage of CD4+ cells fell slightly, but not significantly, to $40.2 \pm 10.2\%$ cells in the atopic asthmatics 24 h after allergen challenge ($p = 0.06$; fig. 2a).

Circulating Th17 cells expressed as a percentage of CD4+ cells were significantly higher in atopic asthmatics ($1.2 \pm 0.5\%$) compared to normal subjects ($0.9 \pm 0.66\%$, $p < 0.001$) but not between atopic asthmatics and atopic nonasthmatics ($1.13 \pm 0.5\%$; fig. 2b). The percentage of circulating Th17 cells increased significantly in atopic asthmatic subjects 24 h following allergen challenge to $1.55 \pm 0.40\%$ ($p < 0.05$; fig. 2b).

There was a nonsignificant increase in the expression of IL-17RA between atopic asthmatics ($3.4 \pm 4.3\%$ of CD4+ cells) compared to atopic nonasthmatics ($2.8 \pm 1.93\%$) and normal controls ($2.23 \pm 1.64\%$; fig. 1b, 2c). In addition, there was a nonsignificant increase in atopic asthmatics 24 h after allergen challenge to $6.86 \pm 6.84\%$ ($p = 0.06$; fig. 2c).

There were no differences in the percentage of IL-17F CD4+, IL-21 CD4+, IL-22 CD4+, or IL-23 CD4+ cells between atopic asthmatics, atopic nonasthmatics and normal controls. In addition, the percentage of IL-17F CD4+, IL-22 CD4+ or IL-23 CD4+ cells did not change after allergen challenge (table 2). By contrast, there was a significant reduction in the percentage of IL-21 CD4+ cells 24 h after allergen challenge from 3.46 ± 1.85 to $2.33 \pm 1.37\%$ ($p < 0.001$; table 2).

Th17 Cytokines

There was a significant increase in IL-17A levels in atopic asthmatics (151 ± 166 pg/ml) compared to normal controls (35.6 ± 19.9 pg/ml) and a further significant increase in atopic asthmatics 24 h after allergen challenge to 217 ± 227.7 pg/ml ($p < 0.01$). There were no significant differences between atopic asthmatics and atopic nonasthmatics (fig. 3a).

There were no statistically significant differences in levels of IL-17F, IL-21 and IL-22 between normal individuals, atopic nonasthmatics and asthmatics before

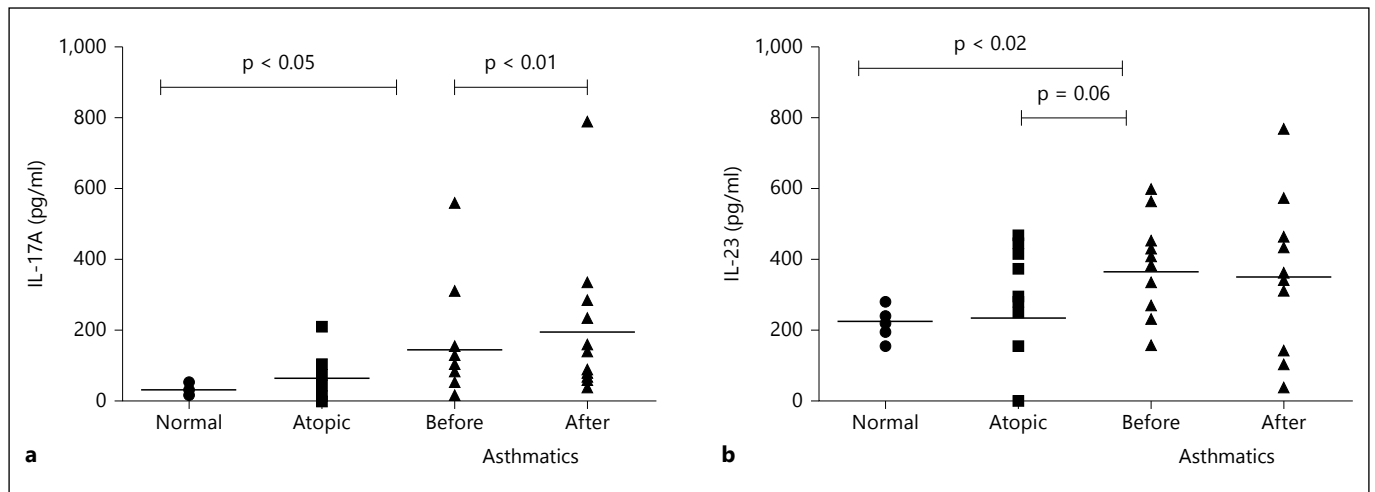


Fig. 3. Levels of IL-17A (**a**) and IL-23 (**b**) from PBMC culture supernatants from normal controls, atopic nonasthmatic subjects and atopic asthmatic subjects before and 24 h after inhaled allergen challenge.

Table 2. Flow cytometry and multiplex analysis demonstrating the percentage of CD4 and Th17 cells and Th17-related cytokine levels

	Normal controls	Atopic nonasthmatics	Asthmatics	
			before allergen	after allergen
<i>Cell percent</i>				
CD4+ cells	44.2±12.2	44.4±11.3	43.6±9.2	40.2±10.2
IL17A cells	0.90±0.66	1.13±0.53	1.20±0.5 ²	1.55±0.4 ¹
IL17RA+ cells	2.23±1.64	2.85±1.92	3.4±4.3	6.86±6.84
IL17F cells	0.8±1.15	0.53±1.04	0.77±0.66	1.79±1.03
IL21 cells	1.91±0.66	2.12±0.88	3.46±1.85	2.33±1.37 ¹
IL22 cells	5.37±4.16	4.27±3.26	2.38±1.33	2.45±1.2
IL23 cells	1.95±2.16	1.31±2.89	3.26±7.34	1.04±0.6
<i>Cytokine levels, pg/ml</i>				
IL17A	35.57±19.9	67.15±66.8	151.65±166.5 ²	217.22±227.7 ¹
IL17F	1,277.6±1,442.4	2,375.2±5,872.9	2,274.98±2,972.2	2,625.49±5,448.1
IL21	473.92±46.7	377.17±229.2	503.13±100.9	453.79±132.1
IL22	466.87±123	466.95±532.4	814.81±962.3	863.70±880.5
IL23	196.76±86.3	238.48±188.5	396.76±122 ²	416.83±197.2

All values are shown as means ± SD.

¹ Significantly different compared to before allergen. ² Significantly different from normal controls.

and after allergen challenge (table 2). There was, however, a significant increase in IL-23 levels between normal controls (196.7 ± 86.2 pg/ml) and asthmatics (396.76 ± 122 pg/ml, $p < 0.02$) and a trend toward significance compared to atopic nonasthmatics (238.5 ± 188.5 pg/ml, $p = 0.06$). However, there was no significant difference in the IL-23 level following allergen challenge (fig. 3b).

Discussion

This study has demonstrated increases in circulating Th17 cells and their associated cytokines IL-17A and IL-23, but not IL-17F or IL-21, in allergic asthmatic subjects compared to normal controls. However, no differences were demonstrated between allergic asthmatics and allergic nonasthmatic subjects. The levels of both Th17 cells

and IL-17A increased significantly 24 h after allergen challenge in allergic asthmatics, with a trend towards an increase in IL-17RA expression on CD4+ cells compared to before challenge. To our knowledge, this is the first study to compare and contrast IL-17 cells and their family of cytokines before and after an inhaled allergen challenge.

Previous studies have demonstrated that Th17 cells are increased in the peripheral blood and in the airways in patients with allergic asthma [10, 11, 15], and the current study is consistent with these results. In addition, Bajoriuniene et al. [21] demonstrated a significant increase in circulating Th17 cells and serum IL-17 (but not its subtypes) in subjects with allergic asthma and allergic rhinitis compared to normal controls and a further increase after allergen inhalation challenge to HDM. Our study has identified that IL-17A, but not IL-17F, is increased in expression between asthmatics and normal controls and between pre- and postallergen challenge. This is consistent with a previous study which showed increased numbers of IL-17A-producing cells and cytokine expression in bronchial biopsies and sputum of mild asthmatics compared to normal controls, whereas IL-17F was only slightly increased in mild asthma but significantly increased in moderate-to-severe asthma [22].

Several studies have demonstrated an increase in Th2-related cytokines (such as IL-5 and IL-13) after allergen inhalation and in response to in vitro stimulation of T cells with HDM extracts [23, 24]. Similar increases in Th17 cells and IL-17 levels have been demonstrated with HDM extract stimulation [15, 21]. In addition, levels of IL-17 mRNA in sputum from allergic asthmatics were demonstrated to correlate with levels of IL-5 mRNA [10].

IL-21 has been reported to have a role in regulating B cell switching to IgE producing cells and to suppress IgE expression in allergic diseases [25]. Another study demonstrated that the administration of recombinant IL-21 attenuated allergic responses in a murine model of allergic rhinitis [26], an effect attributed to the inhibition of IL-4, IL-5, IL-13, and eotaxin, and inhibition of eosinophil infiltration into the nasal mucosa.

An increase of IL-17, IL-22 and IL-23 has been demonstrated in asthmatic subjects of various severities compared to normal controls, as well as an increase in IL-25 in mild asthma compared to moderate and severe asthma [13]. IL-22 has both pro- and anti-inflammatory properties. Th17 cells are the main producers of IL-22, which acts together with IL-17A and IL-17F to enhance the host defense mechanism against microbial peptides and to enhance the pro-inflammatory properties of IL-17A in experimental allergic

asthma [27]. IL-23 is essential for the differentiation, regulation and development of fully functioning Th17 cells. IL-17 and IL-23 expression has also been shown to be necessary for eosinophilic airway inflammation in mice [7]. Our data showed a significant difference in IL-23 between asthma subjects and normal controls and a nonsignificant trend in IL-23 in asthmatics compared to allergic nonasthmatics. However, there were no significant changes in asthmatics following allergen challenge.

The current study has limitations as it used PBMC and culture supernatants and did not investigate the expression and behavior of Th17 cells in the airways. Efforts were made to measure cytokines associated with IL-17 in induced sputum from the study subjects, but cytokine levels were either below the level of detection or measurements were not reproducible, even with attempts to concentrate the sputum samples, as has been reported in previous studies [9, 22]. Others have also reported similar difficulty in measuring IL-17 in sputum, which is thought to be related to the effects of DTT used to solubilize sputum and/or the sensitivity of ELISA assays used for IL-17 analysis. The studies which have reported measuring IL-17 cytokines in sputum have reported contradictory results, where some reported higher levels of IL-17A in asthmatics compared to normal controls [9], while others reported higher levels of IL-17A in mild-to-moderate compared to severe asthma and normal controls [22].

In summary, this study has demonstrated increases in circulating IL-17 cells in atopic asthmatics compared to normal controls, but no differences were found between atopic nonasthmatic and asthmatic subjects. In addition, IL-17 cells and IL-17A (but not IL-17F, IL-21, IL-22, or IL-23) were increased in atopic asthmatics after allergen challenge. These results are consistent with the previously reported roles for IL-17 cells and IL-17A in murine models of allergen-induced airway responses.

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