Interleukin-17 Producing T Cells could be a Marker for Patients with Allergic Rhinitis

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ABSTRACT: **Background:** Interleukin-17A (IL-17A)-producing CD4+T helper cells have been implicated in allergic inflammation; however, the role of IL-17A in allergic rhinitis (AR) patients with different degrees of atopy and airway reactivity to methacholine (Mch) has not been examined.

Objectives: To explore IL-17A-producing CD3+CD4+T cells in peripheral blood of patients with persistent AR and assess the degree of atopy, eosinophil count (Eo count), and bronchial hyper-responsiveness (BHR) to methacholine.

Methods: The study involved 61 patients and 30 controls. The percentage of CD3+CD4+IL-17A+T cells in peripheral blood was measured by flow cytometry, bronchial challenges with Mch were performed, as were skin prick tests with standard inhalant allergens, and Eo count was measured. Atopic status was determined by the number of positive SPT results and wheal mean diameter.

Results: A statistically significant difference in Th17 cell percentage was found in the AR and control groups (2.59 ± 1.32% and 1.24 ± 0.22% respectively, $P = 0.001$). Forty-one patients (67.2%) were polysensitized to indoor and outdoor allergens, while 20 (32.8%) had positive skin prick tests to indoor allergens. CD4+T cells were significantly higher in the patient group compared to the control group (2.91 ± 1.5% versus 1.91 ± 0.62%, $P = 0.005$), as was Eo count (4.48 ± 2.13 vs. 2.32 ± 1.83) ($P = 0.0001$). Forty-one in the AR group (67%) and 7 (23%) in the control group were Mch-positive ($P = 0.001$). The percentage of IL-17A-producing CD4+T cells was significantly higher in males compared to females (3.15 ± 1.8% versus 2.31 ± 0.9%, $P = 0.02$)

Conclusions: Polysensitized AR patients exhibited higher IL-17A-producing CD4+T cell levels and eosinophil counts. Male patients displayed a higher frequency of IL-17A-producing T cells.

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KEY WORDS: Th17, interleukin-17 (IL-17), allergic rhinitis (AR), flow cytometry, atopy, bronchial hyper-responsiveness, gender, eosinophils, methacholine

Sensitization to inhalant allergens is a specific feature of allergic rhinitis. An association between atopy and bronchial hyper-responsiveness was found in patients with allergic rhinitis: namely, exposure to multiple foreign substances increases BHR in atopic patients. Recently, interleukin 17A-producing T helper cells have challenged the classic Th1/Th2 paradigm, and have been implicated in a growing number of inflammatory conditions. Recent data have provided new insights into the pathogenesis of atopic diseases [1].

Current data suggest a central role of Th17 in orchestrating adaptive immune responses. Furthermore, there is growing evidence that IL-17A plays an important role in autoimmune diseases [2,3]. Consistent with the broad expression pattern of its receptor, IL-17A acts on a variety of cells that respond by up-regulating the expression of pro-inflammatory cytokines, chemokines and metalloproteinases (MMP1, MMP3, MMP13). Emerging evidence suggests active involvement of IL-17A in a range of pathologic conditions in humans, ranging from common asthma and allergic rhinitis to certain autoimmune diseases [2]. Indeed, one of the major questions in immunology remains: Why does an individual develop an autoimmune disease or allergy [3].

The number of studies on molecular mechanisms of the most prevalent allergic diseases – allergic rhinitis and bronchial asthma – are increasing. Over the past decades, the clinical symptoms in patients with allergic respiratory diseases tend to overlap those of upper and lower airways. Although the concept of combined upper and lower respiratory diseases is unanimously accepted, the border between clinical conditions relating to allergy of upper airways and those of latent immune inflammation of lower airways is not the focus of the latest research.

Bronchial hyper-responsiveness to methacholine in AR is a risk factor for the development of asthma [4]. Among rhinitis

BHR = bronchial hyper-responsiveness
Th = T helper cells
IL-17A = interleukin-17A
MMP = metalloproteinase
AR = allergic rhinitis

patients, those who are allergic have BHR more frequently [5]. Recently, IL-17A was shown to be associated with response to allergens and is probably an allergy biomarker [6].

Positive skin tests correlate with BHR in asthmatics and AR patients. The latest data suggest that sensitization to allergens could be a major factor determining the degree of hyper-responsiveness; however, the findings in published studies are controversial [5,7]. The role of IL-17A in the development of AR is not clearly established. While some studies have revealed its possible role in response to seasonal allergens [6], an association between Th17 cells and perennial allergens was not observed. The aim of the present study was to investigate a relationship of sensitization to various allergen, BHR and expression of IL-17A by T cells in peripheral blood of AR patients.

PATIENTS AND METHODS

The study population comprised 61 patients with clinical data for persistent AR and 30 controls. A detailed evaluation was conducted, including clinical history and physical examination, in patients and healthy controls. The patients were diagnosed with persistent allergic rhinitis according to ARIA criteria. The inclusion criteria were: clinical history of nasal obstruction and/or itching, rhinorrhea and sneezing most of the year, for 2 previous years, and positive (≥ 3 mm) skin prick test to at least one perennial allergen. Exclusion criteria were: bronchial asthma, chronic rhinosinusitis, nasal polyposis, excessive septal deviation, and current smoking. The patients were not under pharmacological treatment (antihistamines) at least 10 days before testing. Exclusion criteria for the healthy subjects were clinical history of rhinitis and positive reaction to any of the allergens from the test panel. All the patients and controls underwent a bronchial provocation test. Airway responsiveness to methacholine was evaluated, with FEV1 higher than 70% of predicted. Blood samples for the assessment of peripheral eosinophil counts and Th17 cell percentage were obtained from patients and controls. The study was approved by the local ethics committee and informed consent was obtained from all subjects.

SKIN PRICK TEST

To assess the atopic status a skin prick test was performed using the method of Pepys [8] with the following allergens (Stallergenes): house dust mites (*Dermatophagoides pteronyssinus, Dermatophagoides farinae*), animal feather, animal dander (cat, dog), 3 grasses, 5 grasses, 4 cereals, 12 grasses, trees (birch, beech, willow), *Ambrosia elatior*, *Artemisia vulgaris*, *Alternaria alternata*, Aspergillus mix, *Fusarium solani*, Penicillium mix, and cockroach. Histamine solution (10 mg/ml) and saline served as positive and negative control. The largest size of the wheal was measured after 15 minutes. A diameter of ≥ 3 mm was considered a positive reaction.

BHR = bronchial hyper-responsiveness

BRONCHIAL METHACHOLINE CHALLENGE

Spirometer Spirovit SP-10™ (Schiller, Switzerland) was used, meeting the criteria of the European Respiratory Society with predicted values and consistent with the European Community for Coal and Steel [9]. A methacholine inhalation test was carried out to measure the bronchoprovocation concentration causing a fall in FEV1 of 20% (PC20). This was followed by 2 min tidal breathing standard dosimeter protocol with nine methacholine dilutions: 0.03, 0.06, 0.125, 0.5, 1, 2, 4, 8, and 16 mg/ml, used for PC20 calculations [10].

ASSESSMENT OF EOSINOPHIL COUNTS

Whole blood (2 ml) with EDTA for eosinophil count evaluation was collected. The number of eosinophils higher than 3% was considered abnormal.

FLOW CYTOMETRIC ANALYSES OF IL-17A-EXPRESSING CD3+CD4+T CELLS

Blood samples were obtained from all patients and healthy subjects and were processed according to the methodology guidelines of Becton-Dickinson. T lymphocytes were activated for better Th17 expression.

The flow cytometer FACSort model (Becton-Dickinson) was used. PMA (phorbol ester), BFA, MAB FITS anti-human CD4 and anti-human CD3 PerCP were from Becton-Dickinson, as was MAB PE anti-human IL-17A. Peripheral blood mononuclear cells were obtained from Ficoll-Hypaque gradient of heparinized venous blood (3 ml), diluted with RPMI 1640 (invitrogen) and activated by 50 ng/ml phorbol myristate acetate at 37°C for 5 hours. During 5 hours incubation, after the first hour protein transport inhibitior GolgiPlug (Becton-Dickinson) was added. After incubation and washing were completed, MAB FITC-anti-human CD4 and anti-human CD3 PerCP were added, followed by subsequent fixation and permeabilization with Cytofix/Cytoperm. The intracellular cytokine staining was performed by adding MAB PE anti-human IL-17A. After washing with phosphate-buffered saline and 1% formaldehyde fixation, blood samples were stored at 4°C prior to flow cytometry. Isotype control was included in each assay. Results were expressed as percentages.

STATISTICAL ANALYSIS

Data were processed with software statistical computer applications EpiInfo 2008, Statgraphics v 3.5.1 and SPSS for Windows v.19.1. The results were presented as mean ± standard deviation. *P* value < 0.05 was considered statistically significant. For analysis of data requiring non-parametric statistics and for comparison of two independent samples with non-parametric distribution the Mann-Whitney test was applied. Fisher's t-test

was used for comparison of two independent variables with parametric distribution.

RESULTS

AR patients and controls were distributed by gender: in the group with allergic rhinitis the female/male ratio was 41 females/20 males; a similar proportion was observed in the control group: 18 females/12 males ($P = 0.01$) [Table 1]. The mean age of the patients and controls was similar, 36.25 ± 5 and 35.73 ± 6 years, respectively.

EOSINOPHIL COUNT

As shown in in Table 1 the eosinophil count was higher in the patients as compared to the healthy control subjects ($P = 0.0001$).

TH17 CELL PERCENTAGE IN PERIPHERAL BLOOD

The flow cytometric analysis, presented in Figure 1, shows that the average values for Th17 in healthy subjects were 0.57–1.84% vs. 1.34–6.84% in patients. IL-17A+ CD4 Th17 was higher in patients with rhinitis than in controls, 2.59 ± 1.32% vs. 1.24 ± 0.22% respectively ($P = 0.001$) [Figure 1]. IL-17-producing T cells were significantly higher in male compared to female subjects, 3.15 ± 1.8% vs. 2.31 ± 0.9%, respectively ($P = 0.02$) [Table 1].

ASSESSMENT OF ATOPY

The immune response of the patients to skin prick tests with indoor allergens to house dust mites (*Dermatophagoides pteronyssinus, Dermatophagoides farinae*) was higher, followed by molds (Alternaria and Aspergillus). The number of patients sensitized to Fussarium, cat, animal feather and dog was similar [Table 2]. Table 3 presents the results of sensitization to different pollen allergens. The number of patients reacting to grass mixes was higher than the number of patients sensitized to tree and weed pollens.

Based on the results of the skin prick test, patients were classified as sensitized to indoor allergens (group I) or sensitized to indoor and outdoor irritants (group II). Group I comprised 20 subjects (32.8%) and Group II 41 (67.2%) ($P = 0.001$). Mean values of Th17 in these groups were 1.91 ± 0.62% (indoor) and 2.91 ± 1.5% (indoor, outdoor) ($P = 0.005$). The data analysis on wheal size and its relation to CD3+CD4+T cell levels did not show a significant correlation.

TH17 AND BHR

Forty-one AR patients (67%) and 7 healthy subjects (23%) were methacholine-positive ($P = 0.001$). The mean levels of Th17 cells among the 61 patients were not significantly different between methacholine-positive and negative subjects (2.59 ± 1.5% vs. 2.59 ± 0.9%) ($P = 0.9$). IL-17A expression was significantly higher in methacholine-positive patients as compared to

Table 1. Data on gender, age, Eo count, CD3+CD4+T cell IL-17 in patients and controls

	Controls		AR patients		Total	*P*
N	30		61		91	
Gender (female/male)	18	12	41	20	59/32	0.01
Age (yr)	35.73 ± 6		36.25 ± 5		36.08 ± 6	
Eo count (%)	2.32 ± 1.84		4.48 ± 2.13			0.0001
CD3+CD4+IL-17 A (%)	1.24 ± 0.22		2.59 ± 1.32			0.001
CD3+CD4+IL-17 A (%) by gender (female/male)	1.25 ± 0.24	1.22 ± 0.19	2.31 ± 0.9	3.15 ± 1.8		0.02
BHR CD3+CD4+IL-17 A (%)	7 (Mch+) 1.38 ± 0.3*	23 (Mch-) 1.15 ± 0.26**	41 (Mch+) 2.59 ± 1.5*	20 (Mch-) 2.59 ± 0.9**	48/43	0.001
CD3+CD4+IL-17 A (%) by number of sensitised patients			group I 1.91 ± 0.62	group II 2.91 ± 1.5		0.005

*$P = 0.040$, **$P = 0.015$

Mch- = methacholine-negative, Mch+ = methacholine-positive

Figure 1. Flow cytometric analysis of CD3+CD4+IL-17-producing T cells in **[A]** control patients and **[B]** allergic rhinitis patients

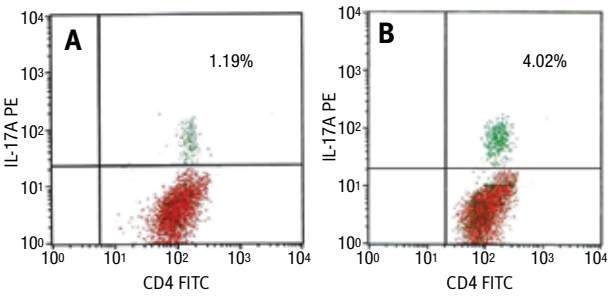

Table 2. Ratio of SPT with indoor allergens and corresponding number of sensitized patients measured by wheal mean diameter

Allergen	No. of patients	WMD in patients (n=61)	WMD in (controls n=30)	*P* value
Dermatophagoides pteronyssinus	48	3.85	0.13	0.0001
Dermatophagoides farinae	40	2.84	0.23	0.0001
Animal feather	7	0.46	0.10	NS
Cat	8	0.70	0.20	NS
Dog	7	0.59	0.20	NS
Alternaria alternata	13	0.95	0.23	0.02
Aspergillus mix	9	0.66	0.00	NS
Fusarium solani	8	0.56	0.10	0.03
Penicillium mix	5	0.31	0.00	NS
Cockroach	3	0.15	0.13	0.9

WMD = wheal mean diameter

Table 3. Ratio of SPT with pollens and corresponding number of sensitized patients measured by wheal mean diameter

Allergen	No. of patients	WMD in patients (n=61)	WMD in controls (n=30)	*P* value
3 grasses	24	2.39	0.10	0.0001
5 grasses	23	2.13	0.10	0.0001
4 cereals	21	2.25	0.10	0.0001
12 grasses	7	0.52	0.10	NS
Birch	21	1.89	0.10	0.0001
Beech	16	1.31	0.00	NS
Willow	6	0.52	0.00	NS
Ragweed	3	0.23	0.00	NS
Artemisia vulgaris	3	0.25	0.00	NS

SPT = skin prick test, WMD = mean wheal diameter

methacholine-positive controls (2.59 ± 1.5% vs. 1.38 ± 0.3%) ($P = 0.040$). Values of Th17 cells in methacholine-negative subjects were significantly different between patients and controls respectively (2.59 ± 0.9% vs. 1.15 ± 0.26) ($P = 0.015$) [Table 1].

DISCUSSION

It has been reported that IL-17A-producing cells play a significant role in atopic diseases. The process of sensitization in allergic rhinitis is T cell-dependent [11,12]. Exposure to allergens in combination with genetic susceptibility induces T cell proliferation, resulting in allergic inflammation [13]. Until recently the major interest was directed at Th2 cytokine response to allergens. However, new data provide evidence for the pathogenic role of IL-17A in asthma [14,15]. Similar associations have been reported in AR [6,16] although the role of IL-17A in AR has not yet been defined. A recent study on ex vivo evaluation demonstrated higher frequencies of IL-17-producing T cells in AR patients with pollen allergy [6], suggesting the possible role of Th17 cells in the allergen response.

An important finding from our study was the established relationship between IL-17A-producing Th17 cells and polysensitization to different inhalant allergens in rhinitis patients. It is recognized that repeated allergen exposure may result in persistent airway inflammation [12]. Marinho et al. [7] investigated the influence of wheal and flare diameter on atopy and found a certain correlation between skin prick tests and lung function [7]. In an experimental model, Albrecht and colleagues [17] showed that IL-17A has a role in ongoing allergic inflammation and in priming toward new antigens influenced by polysensitization. The authors conclude that Th17-mediated airway inflammation determines a higher risk for allergic sensitization. While the role of IL-17A in pulmonary pathology is widely investigated, information on Th17-mediated inflammation in AR with evidence for polysensitization is rather scarce.

AR patients frequently develop BHR [5]. In atopic subjects the sensitization level determines airway responsiveness. During the pollen season BHR is also influenced by perennial allergens and environment factors. Despite the conflicting results of a number of studies, the prevailing opinion is that atopy and BHR are closely associated [5]. Interesting data are presented by Whitehead et al. [18] on the relationship [18] between IL-17 and BHR in asthmatics. They demonstrate that allergic sensitization through the airways initially leads to IL-17-dependent BHR. In our study we found an increased prevalence of BHR in patients with allergic rhinitis. However, no correlation was found between wheal mean diameter, BHR and percentage of CD3+CD4+ IL-17A+Th17 cells. Another finding of the present study was the influence of gender on Th17 expression. Male subjects with AR showed a higher percentage of Th17 compared to female subjects, despite the higher prevalence of females in the AR and control groups. Indeed, new evidence on the role of gender in inflammatory cytokine production from T helper cells was suggested [19], particularly in Th17 and Th9. The gender-specific immune response to repeated allergen exposure and allergic inflammation should be borne in mind.

A recent study by Dias and Banerjee [20] provide data on synergistic eosinophilic activity and IL-17. Nonetheless, the correlation between eosinophils and Th17 lymphocytes has not been fully established. Th17 cells have been shown to play an important role in hypereosinophilic systemic inflammation. Generally, AR is associated with mild peripheral blood eosinophilia, although eosinophils in the nasal secretion could be much higher than in blood levels [21]. In the present study the increased eosinophil counts were found in AR patients, as compared to controls. However, no significant correlation with IL-17A-producing T cells was established. It seems that despite the large panel of secreted cytokines and mediators, the production of cytotoxic proteins is likely the major effector function of eosinophils. The cytotoxic function of eosinophil proteins is expressed more in the peripheral blood of patients with AR.

CONCLUSIONS

Genetically predisposed individuals who are constantly exposed to allergens and environmental factors may produce a Th-17 cell-dependent immune response, factor IL-17, which could be used as a biomarker of polysensitization in AR patients. A significant gender-related Th17 expression was found, namely, male patients displayed a higher frequency of IL-17A-producing T cells. In conclusion, Th17 cells may contribute to clinical phenotyping of subjects with AR.

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