Peripheral Blood Th17 Cells and Neutrophils in *Dermatophagoides pteronyssinus*-induced Early- and Late-Phase Asthmatic Response

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Key Words: *allergic asthma; early-phase asthmatic response; late-phase asthmatic response; dual asthmatic response; Th17 cells; neutrophil functions.*

Summary. *Background and Objective. Biphasic cellular immune reactions, which follow allergen inhalation, are a specific feature of inflammation in allergic asthma. The aim of this study was to determine the changes in the percentage of peripheral blood Th17 cells and neutrophil functions after Dermatophagoides pteronyssinus-induced early- and late-phase asthmatic response in patients with allergic asthma.*

Material and Methods. A total of 19 patients with allergic asthma were examined. Eleven patients developed an isolated early-phase asthmatic response (EAR), whereas 8 developed both early- and late-phase (dual) asthmatic responses (DAR) after the bronchial challenge with Dermatophagoides pteronyssinus. The control group included 15 healthy subjects. Peripheral blood collection was performed 24 hours before as well as 7 and 24 hours after the bronchial challenge. The percentage of Th17 cells, and chemotaxis and apoptosis of neutrophils were analyzed by flow cytometry. The serum IL-8 and IL-17 levels were determined by ELISA.

Results. After the bronchial challenge, the percentage of Th17 and IL-17 levels increased considerably 7 and 24 hours after the challenge in both groups of patients. Moreover, 24 hours after the challenge, the percentage of Th17 cells and IL-17 levels were significantly higher in the patients with the DAR than those with the EAR or healthy controls. Seven and 24 hours after the challenge, neutrophil chemotaxis was greater in the patients with the DAR as compared with those with the EAR and healthy controls as well. The apoptotic activity of neutrophils was lower 24 hours after the challenge in the patients with the DAR than those with the EAR.

Conclusions. Dermatophagoides pteronyssinus-induced early- and late-phase asthmatic response in patients with allergic asthma was found to be accompanied by an increased percentage of peripheral blood Th17 cells and elevated serum IL-17 levels as well as altered neutrophil functions.

Introduction

Allergic asthma is a chronic inflammatory disease of the airways associated with the response of predominant type 2 helper T (Th2) cells to an inhaled allergen (1). The activation of antigen-specific Th2 cells with a subsequent release of interleukin 4 (IL-4) and interleukin 5 (IL-5) leads to immunoglobulin E (IgE)-mediated mast cell activation and eosinophilic inflammation in the airways (2). This mechanism is believed to be crucial in the pathogenesis of allergic asthma. Allergen-induced early-phase asthmatic response (EAR) occurs within minutes after allergen inhalation and is considered a result of IgE-mediated mast cell activation and release of various mediators (histamine, leukotrienes, prostaglandin D2, bradykinin, and platelet-activating factor), which induce an acute fall in lung function and symptoms of acute asthma (3). In part of patients with allergic asthma, EAR is followed by a secondary fall in lung function 2–7 hours after initial allergen exposure (4). This late-phase asthmatic response (LAR) is characterized by an influx of inflammatory cells, which are predominantly eosinophils, as well as neutrophils, basophils, and T cells (5). The LAR is associated with an increase in airway hyperresponsiveness and leads to the airway remodeling, which develops as the disease progresses (6). It is supposed that individuals who develop both the EAR and the LAR (dual asthmatic response, DAR) after inhaled allergen challenge also have more marked local inflammation as well as changes in systemic inflammation compared with those who develop only the EAR. The differences between the pathways leading to the isolated EAR or DAR have not been known.

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There is an increasing interest in the role played by the neutrophils, which are thought to contribute to the development of allergic inflammation. It has been reported that the neutrophil count in the airways of patients with asthma is increased and further increases during asthma exacerbations (7, 8). Neutrophil influx in the airways is related to the activation of these cells. They produce leukotrienes (LT) A_2, LTB_2, thromboxane (TX) A_2, cytokines (IL-1β, IL-6, IL-8, tumor necrosis factor a (TNF-α), transforming growth factor β [TGF-β]), proteases (elastase, matrix metalloproteinases 2 and 9 [MMP2 and MMP9], myeloperoxidase [MPO]), adhesion molecules, reactive oxygen species (ROS), and eosinophil cationic protein (ECP) (2, 9). Activated neutrophils may prolong the inflammatory process and lead to persistent airway inflammation, as well as changes in airway function.

Recently it has been demonstrated that allergen inhalation leads to an enhanced expression of IL-17 in the airways, and this mediator constituted the link between the activation of allergen-induced T cells and recruitment of neutrophils (10). IL-17 can activate neutrophil recruitment indirectly via the induction of cytokines by resident airway structural cells (epithelial cells, fibroblast, smooth muscle cells) (11). Moreover, IL-8 that is known as the most important chemoattractant for neutrophils is synthesized by many cells, including neutrophils themselves and activated Th17 cells (12). Despite clear evidence demonstrating the importance of IL-8 in neutrophil migration, IL-17 seems to be most important not for the early phase, but for the durable phase of neutrophil mobilization in host defense in the lungs (13). The mechanisms underlying neutrophil recruitment into the airways and persistent airway neutrophilia in patients with allergic asthma are still under investigations (14).

In asthma, reduced cell death of different inflammatory cells is one of the most important mechanisms for cell accumulation in inflamed airways. Delayed neutrophil apoptosis exacerbates and prolongs inflammation as well as prevents a spontaneous resolution of allergic inflammation (15). Recently performed studies have reported a direct effect of IL-17 on neutrophils as well as macrophages via IL-17 receptors, but the mechanism how macrophages control the neutrophil turnover has to be elucidated yet (16, 17). According to this, IL-17 may be involved in controlling the total turnover of neutrophils not only in the recruitment and accumulation of these cells. The exact role of neutrophils in asthma still remains unknown; however, a greater amount of neutrophils in inflamed airways or altered neutrophil functions and delayed neutrophil apoptosis could enhance the proinflammatory potential of these cells.

The aim of this study was analyze the percentage of peripheral blood Th17 cells, serum IL-17 levels, and the activity of peripheral blood neutrophils in patients with *Dermatophagoides pteronyssinus* (*D. pteronyssinus*)-induced early- and late-phase asthmatic response.

Material and Methods

Subjects. A total of 34 nonsmoking adults (16 men and 18 women; mean age, 31±2 years) were examined: 19 patients with intermittent- or mild-to-moderate persistent allergic asthma, defined according to the Global Initiative for Asthma (GINA) criteria (18), and 15 healthy subjects who comprised the control group. The patients were recruited from the Department of Pulmonology and Immunology, Hospital of the Lithuanian University of Health Sciences, Kaunas. The study protocol was approved by the Regional Ethics Committee for Biomedical Research, Lithuanian University of Health Sciences, and each participant gave his/her informed written consent.

Patients with allergic asthma had a clinical history of the disease for ⩾1 year, current symptoms, and positive results of skin prick test (⩾3 mm) with *D. pteronyssinus*. All the patients were instructed to refrain from using inhaled as well as oral steroids at least 1 month before visits, short-acting β2 agonists at least 12 hours and long-acting β2 agonists at least 48 hours before the lung function test, and antihistamines and antileukotrienes, 7 days before skin prick test. None of the patients had a history of smoking. Baseline forced expiratory volume in one second (FEV_1) was more than 70% of the predicted value in all patients. None of the patients had any clinically significant allergy to other allergens such as pollen or cat and dog dander. All healthy subjects were nonsmokers, without symptoms of asthma, with normal findings of spirometry, and all showed negative results of skin prick test.

Skin Prick Testing. All patients were screened for allergy by the skin prick test using the standardized allergen extracts (Stallergenes S.A., France) for the following allergens: *D. pteronyssinus*, *D. farinae*, cat and dog dander, mixed grass pollen, birch pollen, mugwort, cockroach, *Alternaria*, *Aspergillus*, and *Cladosporium*. Phenolated glycerol saline was used for a negative control, and histamine hydrochloride (10 mg/mL) was used for a positive control. The results of skin prick testing were evaluated after 15 minutes. The results of skin prick test were considered positive if the mean wheal diameter was ⩾3 mm (19).

Study Design. On a screening visit (V0), all subjects were informed about participation in the study, informed written consent was obtained, inclusion/exclusion criteria were verified, and also physical examination, spirometry, responsiveness to methacholine, and skin prick test were performed. Twen-

ty-four hours before bronchial challenge with *D. pteronyssinus* (V1), spirometry was performed and peripheral blood was collected; these data were used as baseline values.

A bronchial challenge with *D. pteronyssinus* was performed at 8:00 AM, and spirometry was reassessed every 10 minutes within the first hour and later on every hour for subsequent 6 hours. Physical examination and peripheral blood collection were repeated 7 hours and 24 hours after bronchial challenge with *D. pteronyssinus* (V2).

Lung Function Testing. Pulmonary function was tested using a pneumotachometric spirometer CustovitM (Custo Med, Germany). The best measurements of baseline FEV_1, forced vital capacity (FVC), and FEV_1/FVC ratio from 3 reproducible measurements were recorded. The results were compared with the predicted values matched for age, body height, and sex according to the standard methodology (20).

Measurement of Airway Responsiveness to Methacholine. Airway responsiveness was assessed as changes in airway function after the challenge with inhaled methacholine using a reservoir method (21). Methacholine was nebulized into a 10-L reservoir with a pressure nebulizer (Pari Provocation I; Pari, Starnberg, Germany). Aerolized methacholine was inhaled through a one-way valve at 5-minute intervals starting with methacholine at a dose of 15 μg and doubling it until a 20% decrease in FEV_1 from the baseline or the total cumulative dose of 3.87 mg was achieved. The bronchoconstricting effect of each dose of methacholine was expressed as a percentage of decrease in FEV_1 from the baseline value. The provocative dose of methacholine causing a $\geqslant$20% fall in FEV_1 (PD_{20}) was calculated from the log dose-response curve by linear interpolation of 2 adjacent data points.

Bronchial Challenge With Allergen. A bronchial challenge was performed with a *D. pteronyssinus* allergen (Stallergenes SA, France) at different concentrations (0.1 IR/mL, 1.0 IR/mL, 10 IR/mL, and 33.3 IR/mL) using a KoKo DigiDoser nebulizer (Sunrise Medical, Somerset, Pa, USA) (22). A freeze-dried lyophilized allergen was diluted with nonphenol saline. The isolated EAR to *D. pteronyssinus* was considered when a $\geqslant$20% fall in FEV_1 from baseline within the first hour after allergen challenge was achieved. The DAR to *D. pteronyssinus* was defined as a $\geqslant$20% decrease in FEV_1 from baseline within the first hour of allergen inhalation (EAR) and subsequently a $\geqslant$15% fall in FEV_1 between 2 and 7 hours after the allergen challenge (LAR).

Evaluation of Cytokine Production in $CD4^+$ T Cells. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood on Ficoll-Paque gradient and resuspended in RPMI 1640 (Sigma-Aldrich, USA) containing L-glutamine autologous plasma. Two million cells per mL were activated for 5 hours at 37°C with phorbol-12-myristate-13-acetate (PMA) (50 ng/mL; Sigma-Aldrich, USA) and ionomycin (1 μg/mL; Invitrogen, USA) in the presence of protein transport inhibitor (BD GolgiStop™, BD Biosciences, USA) to avoid cytokine secretion. After the activation, the cells were washed 2 times with stain buffer (fetal bovine serum [FBS], BD Biosciences) and fixed with cold BD Cytofix™ fixation buffer (BD Biosciences, USA) for 10 minutes at room temperature. After the fixation, the cells were washed twice. Permeabilization of the fixed cells was performed by adding BD Perm/Wash™ buffer (BD Biosciences, USA) and incubating the cells for 15 minutes at room temperature. The cells were stained by adding 20 μL of human Th1/Th2/Th17 phenotyping cocktail (BD Biosciences, USA) containing human CD4 PERCP-CY5.5, human IL-17A PE, human IFN-GMA FITC, and human IL-4 APC in each tube and incubating for 30 minutes at room temperature in the dark. Finally, the cells were washed twice with Perm/Wash™ buffer and suspended in stain buffer (FBS) before flow cytometric analysis. Flow cytometry was performed on a FACSCanto flow cytometer (BD Biosciences, USA). Results are reported as the percentages of $CD4^+$ T cells producing IL-17. Antimouse Ig, κ/negative control (FBS) compensation particles (BD Biosciences) were used as an isotype control.

Evaluation of Neutrophil Chemotaxis in Vitro. Neutrophils from peripheral blood were isolated by density gradient centrifugation. The whole blood was layered on Ficoll-Paque PLUS (GE Healthcare, Finland) and centrifuged at 1000*g* for 30 minutes at room temperature. Neutrophil population was separated by hypotonic lysis of erythrocytes. Isolated neutrophils were diluted in cell culture RPMI 1640 media (Biological Industries, Israel) at a final concentration of 2×10^6/mL. The viability of neutrophils was checked by flow cytometry, and it always was >95%.

Neutrophil chemotaxis in vitro was performed in a 10-well cell transmigration chamber (Neuro Probe, USA). The lower and upper wells of the chamber were separated by a polyvinylpyrrolidone (PVP)-treated polycarbonate track-etched membrane, containing 2×10^6 3-μm/mm^2 pores (Neuro Probe, USA). The lower wells were prefilled with isotonic Percoll (GE Healthcare, Finland), and chemotactic factors at different concentrations (IL-8 at 10, 30, or 100 ng/mL or IL-17 at 10, 30, or 100 ng/mL); RPMI 1640 was used as a negative control. The upper wells were filled with 2×10^6/mL neutrophil culture suspension and incubated for 2 hours (37°C,

5% CO_2). After the incubation, the suspensions of upper and lower wells were resuspended into flow cytometry tubes. Nonmigrated neutrophils remained in the upper wells. The number of migrated neutrophil was calculated by flow cytometry using the reference cells (BD Biosciences, USA), according to the manufacturer's recommendations. The amount of migrated neutrophils was expressed in percentages.

Apoptosis Assay. Isolated peripheral blood neutrophils were resuspended in the annexin-binding buffer (pH 7.4) containing 50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$ (Invitrogen, USA) and incubated with fluorescein isothiocyanate-labeled (FITC)-annexin V (Invitrogen, USA) and propidium iodide (PI) for 15 minutes at room temperature in the dark. After the incubation, apoptosis was analyzed by flow cytometry using the CellQuest software (BD Biosciences, USA). Apoptotic cells were quantified as the percentage of the total population that was positive for FITC, but negative for PI. Necrotic cells were positive for both FITC and PI.

Detection of Cytokine in Serum. The serum IL-17 and IL-8 levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BioSource S.A., Belgium, and BD Biosciences, USA, respectively). The minimum detectable dose was 2 pg/mL and 0.8 pg/mL, respectively, and was defined as the lowest cytokine concentrations that could be differentiated from zero.

The peripheral blood cell analysis was performed on an automated hematology analyzer (Sysmex XE-5000, Japan).

Statistical Analysis. Statistical analysis was performed using the Statistical Package for Social Sciences, version 17.0 for Windows (SPSS 17.0). All data are presented as mean±SEM (standard error of the mean). The Mann-Whitney *U* test was used to evaluate statistical differences between the patients' and control groups, and the Wilcoxon test was used for paired analyses. A *P* value of <0.05 was considered statistically significant.

Results

Characteristics of Studied Subjects. There were no significant age and gender differences comparing the groups (Table 1). The patients with allergic asthma (n=19) were divided into 2 groups depending on the results of bronchial challenge with *D. pteronyssinus:* patients who developed the isolated EAR to inhaled *D. pteronyssinus* (n=11) and patients who developed the DAR to inhaled *D. pteronyssinus* (n=8). All patients were sensitized to *D. pteronyssinus* detected by the skin prick test. The mean wheal diameter induced by *D. pteronyssinus* was similar in both groups of patients. The baseline FEV_1 (% of predicted) was similar in both patients' groups. The maximum percentage fall in FEV_1 0–1 hour after the bronchial challenge in the patients with the EAR and DAR was 28%±4% and 30%±2%, respectively. The maximum percentage fall in FEV_1 2–7 hours after the bronchial challenge was 33%±8% in the patients with allergic asthma who developed the DAR. No clinically severe late reactions were recorded.

The percentage of eosinophils in peripheral blood was significantly higher in both groups of patients with allergic asthma 24 hours before and 24 hours after the bronchial challenge as compared with the healthy subjects (Table 2). There were no significant differences in the peripheral blood leukocyte, lymphocyte, and neutrophil counts before and after the bronchial challenge in all the groups of studied subjects.

Peripheral Blood Th17 Cells. The percentage of Th17 cells was significantly higher in the patients with EAR and DAR compared with healthy subjects 24 hours before as well as 7 and 24 hours after the bronchial challenge (Fig. 1). The representative flow cytometry dot plots and gating strategy are shown in Fig. 2A–C. The percentage of Th17 cells in the both

Table 1. Demographic and Clinical Characteristics of the Study Population

Characteristic	Healthy Subjects n=15	Patients With Allergic Asthma n=19	
		EAR n=11	DAR n=8
Age, years	31±2 (22–45)	36±4 (21–50)	29±4 (21–42)
Sex (male/female), n/N	6/9	7/4	3/5
Wheal diameter induced by *D. pteronyssinus*, mm	0	5.6±0.4 (4–7)	6.6±1.0 (4–11)
FEV_1, % of predicted	105±13 (79–119)	104±4 (92–118)	95±4 (82–104)
PD_{20}, mg	–	0.43±0.10 (0.06–0.96)	0.42±0.10 (0.18–0.68)
Maximum fall in FEV_1 after bronchial challenge with *D. pteronyssinus* during the first hour, %	5±4 (2–9)	28±4 (20–33)	30±2 (22–32)
Maximum fall in FEV_1 after bronchial challenge with *D. pteronyssinus* after 2–7 hours, %	5±2 (4–7)	7±2 (5–9)	33±8 (15–62)

Values are mean±standard error of the mean (range) unless otherwise stated.
FEV_1, forced expiratory volume in the first second; PD_{20}, provocative dose of methacholine causing a 20% fall in FEV_1.

Table 2. Changes in Number of Peripheral Blood Cells Before and After Bronchial Challenge with *D. pteronyssinus* in Patients With Early-Phase, Dual Asthmatic Response, and Healthy Subjects

Characteristic	24 h Before	7 h After	24 h After
Patients with early-phase asthmatic response (n=11)			
Leukocytes, $\times 10^9$/L	5.9±0.4	7.1±0.4	6.0±0.3
Eosinophils, %	5.9±1.2*	4.6±0.7	6.3±0.7*
Eosinophils, $\times 10^9$/L	0.33±0.05*	0.25±0.05	0.50±0.15*
Neutrophils, %	54.4±3.3	53.0±3.2	51.1±2.0
Neutrophils, $\times 10^9$/L	3.10±0.38	4.0±0.51	3.0±0.19
Lymphocytes, %	33.0±2.3	31.5±2.1	32.3±1.7
Lymphocytes, $\times 10^9$/L	2.05±0.12	2.25±0.15	2.10±0.21
Patients with dual asthmatic response (n=8)			
Leukocytes, $\times 10^9$/L	4.8±0.2	5.6±0.4	6.1±0.7
Eosinophils, %	5.8±1.2*	5.1±0.7	6.4±0.9*
Eosinophils, $\times 10^9$/L	0.34±0.10*	0.32±0.11	0.47±0.07*
Neutrophils, %	53.4±3.2	51.0±3.8	53.1±2.5
Neutrophils, $\times 10^9$/L	3.35±0.59	4.33±0.84	3.17±0.46
Lymphocytes, %	32.0±1.9	33.5±2.7	30.3±1.1
Lymphocytes, $\times 10^9$/L	1.57±0.09	2.14±0.15	1.79±0.05
Healthy subjects (n=15)			
Leukocytes, $\times 10^9$/L	4.9±0.7	6.4±0.5	5.6±0.6
Eosinophils, %	3.3±1.6	2.4±0.6	2.7±1.3
Eosinophils, $\times 10^9$/L	0.2±0.03	0.3±0.1	0.2±0.08
Neutrophils, %	53.1±4.5	56.2±1.0	57.2±5.3
Neutrophils, $\times 10^9$/L	3.6±1.0	3.1±0.9	3.4±1.1
Lymphocytes, %	33.9±3.4	32.1±1.1	30.9±3.9
Lymphocytes, $\times 10^9$/L	1.7±0.4	1.6±0.5	1.6±0.8

Values are mean±standard error of the mean.
*$P<0.05$, versus healthy subjects.

groups of patients significantly increased 24 hours after the bronchial challenge as compared with the baseline values, but no significant increase in the percentage of Th17 cells was documented after 7 hours. Seven and 24 h after the bronchial challenge, the percentage of Th17 cells was significant higher in the patients with the DAR than those with the EAR. No significant difference in the percentage of Th17 cells was observed in the healthy subjects after the bronchial challenge.

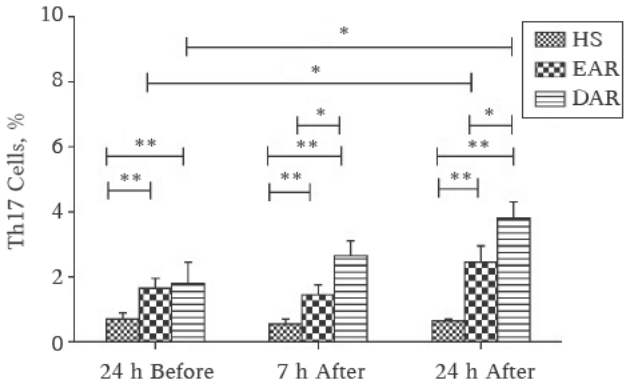

Fig. 1. The percentage of peripheral blood Th17 cells in patients with early-phase or dual asthmatic response and healthy subjects before and after bronchial challenge with *D. pteronyssinus*
HS, healthy subjects (n=15); EAR, patients with early-phase asthmatic response (n=11); DAR, patients with dual asthmatic response (n=8). *$P<0.05$; **$P<0.001$.

Serum IL-17 and IL-8 Levels. The serum IL-17 and IL-8 levels in both patients' groups with allergic asthma were significantly higher as compared with the healthy subjects 24 hours before as well as 7 and 24 hours after the bronchial challenge (Figs. 3 and 4). The patients with the DAR had the considerably higher serum IL-17 and IL-8 levels 7 and 24 hours after the bronchial challenge than those with the EAR. A significant increase in the serum IL-17 and IL-8 levels was observed 24 hours later after the bronchial challenge as compared with the baseline values in both groups of patients with allergic asthma. No significant differences in the levels of these cytokines were observed in the healthy subjects after the bronchial challenge.

Chemotaxis of Neutrophils in Vitro. Chemotaxis of peripheral blood neutrophils isolated from the patients with allergic asthma and stimulated with different IL-17 concentrations (30 and 100 pg/mL) in vitro was significantly greater compared with the healthy subjects 24 hours before as well as 7 and 24 hours after the bronchial challenge (Fig. 5). Twenty-four hours after the bronchial challenge, chemotaxis of neutrophils stimulated with IL-17 at the concentrations of 30 and 100 pg/mL was significant greater in the patients with the DAR than those with the EAR. A significantly greater chemotaxis of neutrophils stimulated with 30 and 100 pg/mL of IL-17 was observed in the patients with the DAR

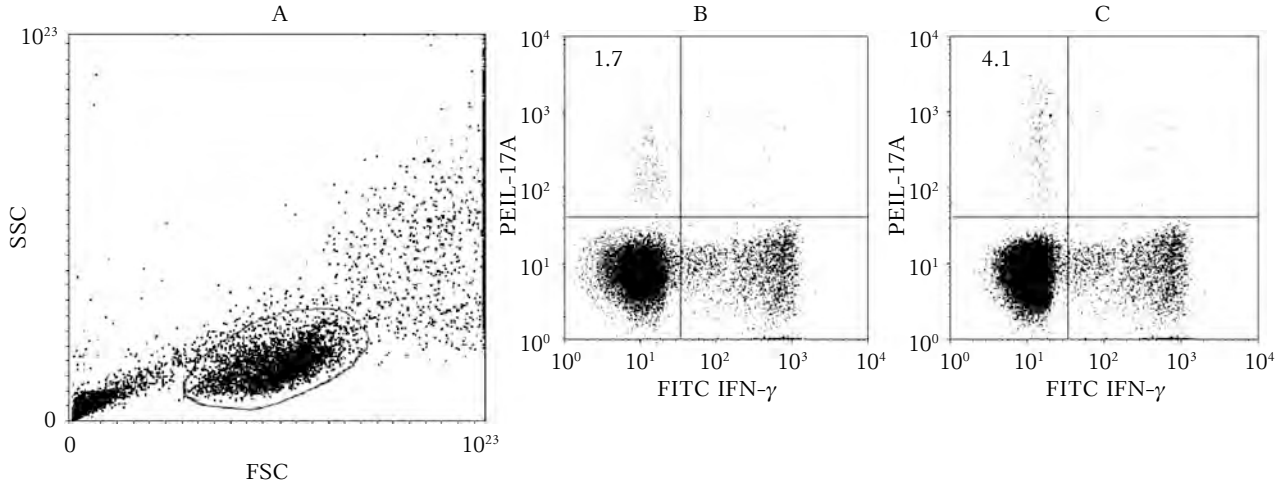

Fig. 2. Representative dot plots of peripheral blood Th17 cells in one patient with allergic asthma before and after the bronchial challenge

Lymphocytes were isolated from the peripheral blood of a patient with allergic asthma. After the stimulation in vitro with PMA and ionomycin for 5 hours, the cells were permeabilized, stained for CD4, IFN-γ and IL-17, and analyzed by FACS. Representative dot plots gated on CD4+ cells are shown (A). Numbers indicate the percentage of Th17 cells in the quadrants 24 h before (B) and 24 h after (C) bronchial challenge.

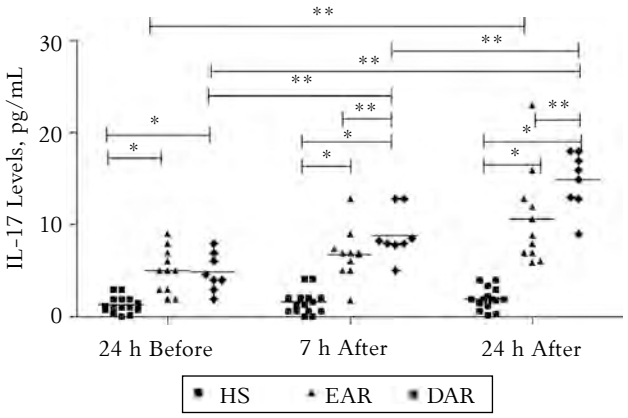

Fig. 3. Serum IL-17 levels in patients with the early-phase or dual asthmatic responses and healthy subjects before and after the bronchial challenge with *Dermatophagoides pteronyssinus*

EAR, patients with early-phase asthmatic response (n=11);
DAR, patients with dual asthmatic response (n=8);
HS, healthy subjects (n=15).
Data are expressed as mean±SEM. *P<0.05; **P<0.001.

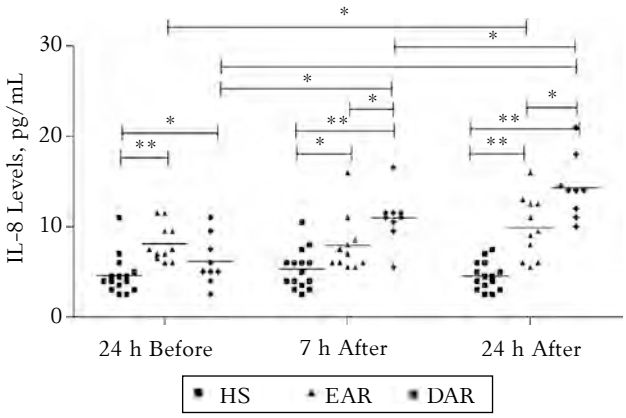

Fig. 4. Serum IL-8 levels in patients with the early-phase or dual asthmatic responses and healthy subjects before and after the bronchial challenge with *Dermatophagoides pteronyssinus*

EAR, patients with early-phase asthmatic response (n=11);
DAR, patients with dual asthmatic response (n=8);
HS, healthy subjects (n=15).
Data are expressed as mean±SEM. *P<0.05; **P<0.001.

7 and 24 hours later after the bronchial challenge as compared with the baseline values. The lowest concentration of IL-17 (10 ng/mL) did not stimulate the chemotaxis of neutrophils in both groups of patients 24 hours before as well as 7 and 24 hours after the bronchial challenge. No significant difference in the chemotaxis of peripheral blood neutrophils stimulated with different IL-17 concentrations was documented in the healthy subjects after the bronchial challenge.

Chemotaxis of neutrophils stimulated in vitro with IL-8 (10, 30, and 100 pg/mL) was significantly greater in the patients with allergic asthma than the healthy subjects 24 hours before as well as 7 and 24 hours after the bronchial challenge (Fig. 6). Seven and 24 hours after the bronchial challenge, the chemotaxis of neutrophils stimulated with 30 and 100 pg/mL of IL-8 was significantly greater in the patients with the DAR than those with the EAR. A significant increase in the migration of neutrophils stimulated with IL-8 at the concentrations of 30 and 100 pg/mL was observed 7 and 24 hours after the bronchial challenge as compared with the baseline values in the patients with the DAR. No significant differences in the chemotaxis of peripheral blood neutrophils stimulated with different IL-8 concen-

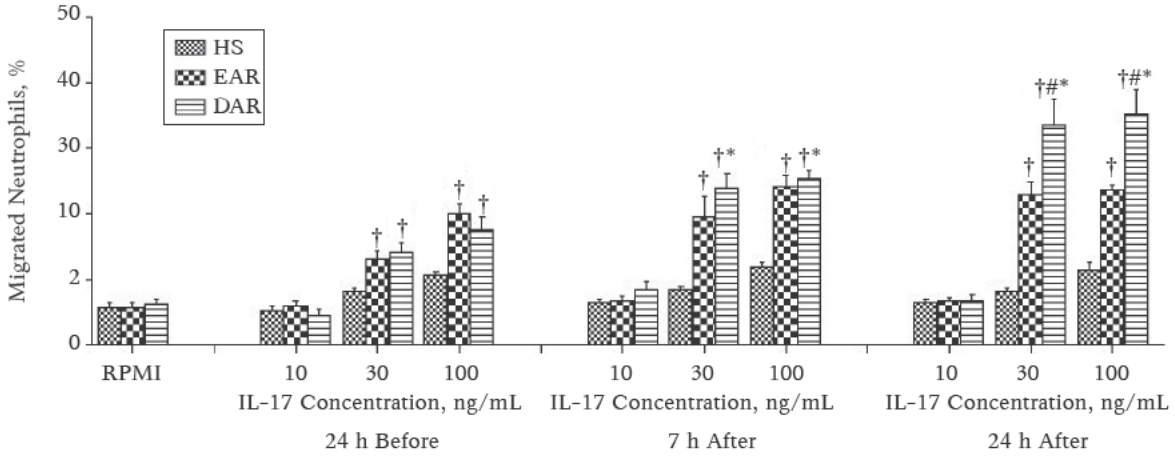

Fig. 5. Chemotaxis of neutrophils isolated from peripheral blood of patients with early-phase or dual asthmatic response and healthy subjects stimulated in vitro with different IL-17 concentrations

EAR, patients with early-phase asthmatic response (n=11); DAR, patients with dual asthmatic response (n=8); HS, healthy subjects (n=15).

Data are expressed as mean±SEM. **P*<0.05, versus baseline values; †*P*<0.001, versus healthy subjects; #*P*<0.05, versus EAR.

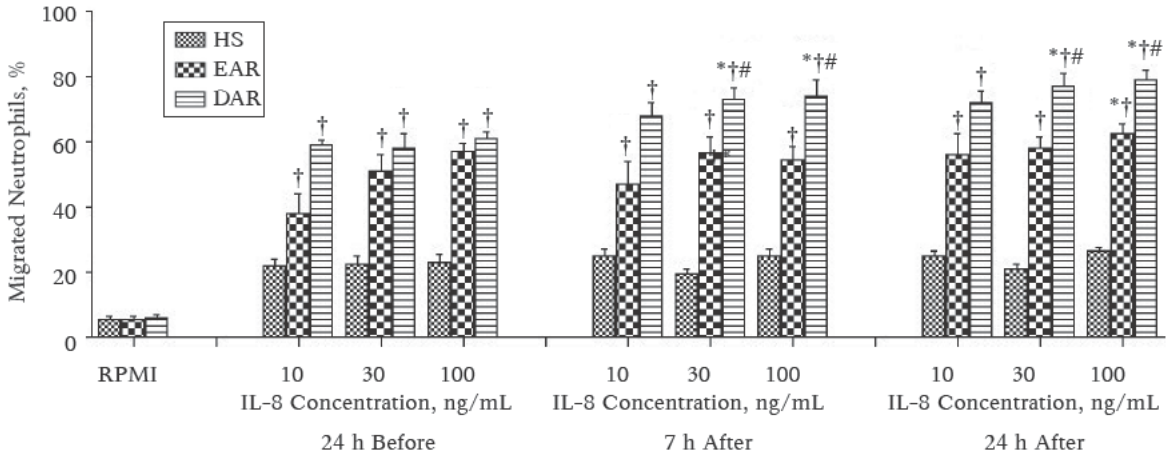

Fig. 6. Chemotaxis of neutrophils isolated from peripheral blood of patients with the early-phase or dual asthmatic responses and healthy subjects stimulated in vitro with different IL-8 concentrations

EAR, patients with early-phase asthmatic response (n=11); DAR, patients with dual asthmatic response (n=8); HS, healthy subjects (n=15).

Data are expressed as mean±SEM. **P*<0.05, versus baseline values; †*P*<0.001, versus healthy subjects; #*P*<0.05, versus EAR.

trations were recorded in the healthy subjects after the bronchial challenge.

Apoptosis of Neutrophils. The percentage of apoptotic neutrophils was significantly higher in the healthy subjects 24 hours before as well as 7 and 24 hours after the bronchial challenge compared with the patients with the EAR or DAR (Fig. 7). The representative dot plots from one patient with allergic asthma illustrating the method for the identification of apoptosis of neutrophils are shown in Fig. 8A and B. The percentage of apoptotic cells significantly decreased 24 hours after the bronchial challenge compared with the baseline values in the patients with the DAR, although no changes in the percentage of apoptotic neutrophils were documented in the EAR group. No significant difference in the percentage of apoptotic neutrophils was observed in the healthy subjects after the bronchial challenge.

Discussion

Overall, our results demonstrated that inflammatory patterns in patients with allergic asthma who developed the EAR or the DAR later after the bronchial challenge with *D. pteronyssinus* were different. The DAR is accompanied by the increased percentages of Th17 cells and serum IL-17 levels, enhanced chemotaxis, and delayed apoptosis of peripheral blood neutrophils.

The results of this study also indicated that peripheral blood lymphocytes and neutrophils might

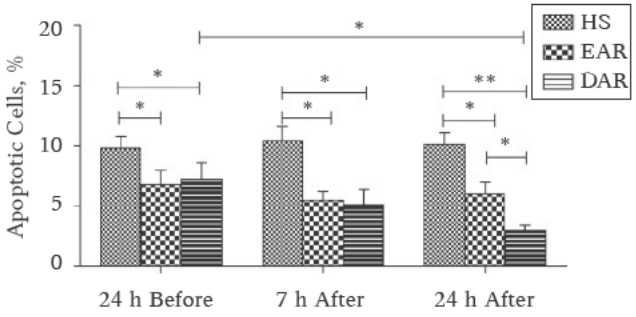

Fig. 7. Apoptosis of neutrophils isolated from peripheral blood of patients with the early-phase or dual asthmatic responses and healthy subjects before and after bronchial challenge with *Dermatophagoides pteronyssinus*

EAR, patients with early-phase asthmatic response (n=11); DAR, patients with dual asthmatic response (n=8); HS, healthy subjects (n=15).
Data are expressed as mean±SEM. **P*<0.05; ***P*<0.001.

represent a mirror event in human lungs. Some studies examined IL-17-expressing cells in the lungs by employing endobronchial biopsy and induced sputum. Some human studies have revealed the infiltration of Th17 lymphocytes in the submucosa of airways in patients with chronic lung inflammatory disorders (cystic fibrosis, chronic obstructive pulmonary disease, severe asthma) (23–25). The studies that assessed Th17 cell immunity in the local as well as systemic allergic inflammation are scarce; only few studies documented the infiltration of Th17 cells in patients with mild and moderate asthma (26). Nevertheless, there are some data on humans referring the importance of circulating Th17 cells in allergic asthma, especially in a more severe form of the disease (27). In our study, a significantly higher percentage of Th17 cells in peripheral blood of patients with allergic asthma were found, and it tended to increase after the allergen challenge in both groups of the patients with allergic asthma. Indeed, a maximum increase in Th17 cells and IL-17 levels occurred 7 and 24 hours after the allergen challenge with *D. pteronyssinus* in allergic asthma subjects with the DAR. The bronchial allergen challenge performed in this study was found to be a useful model of asthma exacerbation highlighting the changes in Th17 response, especially in the development of DAR.

Activated Th17 cells have been regarded to be the major source of IL-17 (12). It *is* known that IL-17 can also be produced by other T-lymphocyte subsets, neutrophils, and macrophages. Our findings demonstrated a rapid increase in serum IL-17 levels of patients with allergic asthma following the bronchial challenge. This explanation is consistent with the observation that in patients with allergic asthma, a higher percentage of Th17 cells, which are the principal source of IL-17, were found. Serum IL-17 levels were found to be elevated in allergic rhinitis and allergic asthma of different severity (28). In this study, the elevated IL-17 levels in the patients with allergic asthma, who developed the DAR after the bronchial challenge, were found, and the same tendency for Th17 cells was observed. All these data suggest an important role of Th17 response and an existing difference between asthma patients with the EAR and the DAR.

Mouse models of allergic asthma have demonstrated that IL-17 expression in the airways is regulated upon allergen inhalation and represents an association between T-cell activation induced by an allergen and neutrophilic influx. Treatment with anti-IL-17 monoclonal antibodies reduced neutrophilic influx in the airways (29, 30). Increased infiltration of the airways with neutrophils characterizes lung inflammation in severe asthma and is associ-

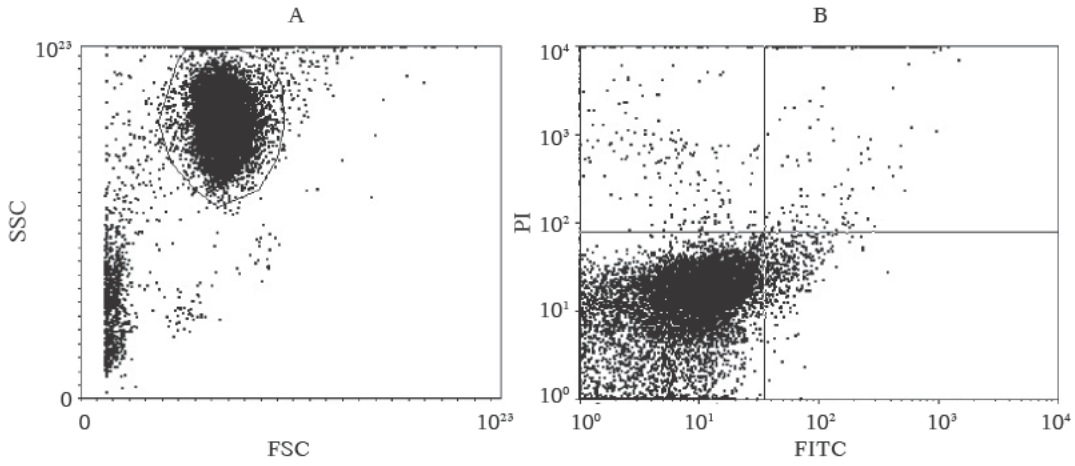

Fig. 8. Representative dot plots of neurophil population isolated from peripheral blood from one patient with allergic asthma

Side light scatter (SSC) represents the granularity and complexity of the cells, and forward light scatter (FSC) represents the size of the cells (A). Fluorescein isothiocyanate-labeled (FITC)-annexin V versus propidium iodide (PI) dot plot showing viable, apoptotic, and necrotic neutrophils isolated from one patient with asthma 24 hours before the allergen challenge (B).

ated with chronic airway narrowing. Some studies confirmed that neutrophils are the predominant cells in the airways of patients with acute asthma exacerbation and in cases of fatal asthma (7, 8). In this study, we decided to investigate neutrophil chemotaxis in vitro using the different concentrations of IL-17, a potent stimulator of neutrophils, and IL-8, a classical chemoattractant. First, it is important to note that the neutrophil count in the peripheral blood of patients with asthma did not change after the bronchial challenge with *D. pteronyssinus*. Although there was no difference in the neutrophil count, the changes in some their functions were observed. The results demonstrated increased chemotaxis of neutrophils in the patients with allergic asthma before and after the allergen challenge. The comparison of patients with the EAR and the DAR revealed that changes in neutrophil chemotaxis were more expressed in the patients with the DAR 7 and 24 hours after the allergen challenge. It should be noted that intensive chemotaxis of neutrophils was induced by stimulating only with high IL-17 concentrations (30 and 100 ng/mL). There are conflicting data on the impact of IL-17 on neutrophil chemotaxis in vivo, and this should be considered while interpreting the results. Some data demonstrated that IL-17 stimulated neutrophil chemotaxis in the airways indirectly via the release of IL-8 in bronchial structural cells (13). A recent experimental animal study by Lemos et al. suggested a mechanism by which the IL-23/IL-17 axis induced neutrophil migration and hence the pathogenesis of articular inflammation (31). It was shown in vivo that IL-17-induced neutrophil migration to the knee joint was dependent on leukotrienes, TNF-α, CXCL1, and CXCL5, released by joint structural and resident cells (e.g., chondrocytes, synovial fibroblasts, and macrophages). In the same study, the authors reported that IL-17-mediated neutrophil migration in vitro could depend not only on the abovementioned chemokines and cytokines, but also on a direct effect of IL-17 on neutrophils (31). Explaining our results, we take into account the above observations that neutrophil migration in vivo can be influenced by a direct IL-17 effect on neutrophils and mainly depends on the concentration gradient. On the other hand, on activation with different mediators during the allergen challenge in vivo in patients with asthma, neutrophils can be activated and can synthesize and release a greater amount of cytokines and chemokines, including IL-8, potentially implicated in the migration of neutrophils in vitro. We suggest that the activity of neutrophil migration, stimulating it with IL-17 in vitro, is depended on the ability of neutrophils to produce chemokines, thus to induce their chemotaxis. However, whether neutrophils respond directly to IL-17 remains controversial. Future studies are needed for the investigation of the complex interaction between neutrophils and molecules involved in the processes that lead to neutrophil migration to an allergic inflammation area.

Apoptosis is the most common form of physiologic cell death and a necessary process to maintain cell numbers in organisms. In chronic diseases, such as allergic asthma, reduced apoptosis of neutrophils is one of the most important mechanisms for cell accumulation and prolongation of inflammation (32). Our study revealed a reduced percentage of apoptotic peripheral blood neutrophils in the patients with allergic asthma as compared with the healthy subjects. Moreover, the patients with allergic asthma who developed the *D. pteronyssinus*-induced DAR showed more decreased neutrophil apoptosis 24 hours after the challenge compared with those who developed the EAR. Various mechanisms have been implicated in delaying neutrophil apoptosis in the blood. An enhanced production of granulocyte-macrophage colony-stimulating factor is thought to mediate the suppression of neutrophil apoptosis. The data show that IL-8, known as the main chemoattractant, not only promotes the inflammatory response by recruiting neutrophils, but also acts to suppress apoptosis mainly through the IL-8RA (33). The impact of IL-17 on neutrophil apoptosis has not been elucidated yet. There are data suggesting that IL-17 can control the accumulation of neutrophils at the site of inflammation not only by the impact on their migration, but also by attenuating the delay in neutrophil apoptosis induced by inflammatory cytokines (16). Other researchers reported that IL-17 capability to stimulate neutrophil apoptosis is accompanied by other very important factor leading to a reduced neutrophil count at the site of inflammation, i.e., IL-17 impact on macrophages. They confirmed that IL-17 could stimulate macrophage phagocytosis of apoptotic neutrophils, thus controlling the total turnover of neutrophils. However, it remains to be examined whether this effect of IL-17 is also valid in humans in vivo (17).

Our study showed an increased IL-17 level and reduced neutrophil apoptosis in patients with asthma and after the bronchial asthma. Of note, all these changes were more pronounced in the patients who developed the DAR. This can be explained by the fact that in humans, IL-17 is found in very small quantities, and it could not directly affect neutrophil apoptosis. Moreover, many factors have an impact on neutrophil functions, and to prove this, more studies are needed.

Thus, IL-17 can be considered as a one of the most important factors having an impact on neutrophils and their functions: migration to and accumulation at the site of inflammation, apoptosis, and phagocytosis, i.e., neutrophil turnover, in the development of response to the antigen and control of inflammatory process.

Conclusions

The inhalation of *Dermatophagoides pteronyssinus* allergen in the patients with allergic asthma resulted in an enhanced Th17 cell response and had an impact on neutrophil functions: activated chemotaxis and delayed apoptosis. Furthermore, these changes were more pronounced in patients with early- and late-phase allergen-induced asthmatic response 24 hours after the bronchial challenge. Our findings support the hypothesis that Th17 cells and neutrophils are involved in the development of late-phase allergen-induced inflammation in allergic asthma.

Acknowledgments

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Statement of Conflict of Interest

The authors state no conflict of interest.

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