

Interleukin 17 and RANTES levels in induced sputum of patients with allergic rhinitis after a single nasal allergen challenge

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Background: Interleukin 17 (IL-17) is produced by T_H17 cells and was recently implicated in the development of the T_H2 cell response. RANTES (regulated on activation of normal T cells expressed and secreted), among other chemokines, plays a crucial role in chemotaxis of eosinophils into airway mucosa. According to the “united airway” hypothesis, markers of inflammation in allergic diseases are elevated in the upper and lower airways.

Objective: To assess the impact of a single nasal allergen challenge on IL-17 and RANTES levels in induced sputum of patients with allergic rhinitis (AR).

Methods: Eighteen patients with a history of AR due to grass pollen confirmed by positive skin prick test results and 10 control subjects entered the study. Initially, all the patients underwent sputum induction. A single nasal placebo challenge was performed 24 hours later, with repeated sputum induction 24 hours after challenge. After 4 weeks of washout, these procedures were repeated with allergen challenge. Differential cell counts in sputum were determined, and concentrations of IL-17 and RANTES were measured by means of enzyme-linked immunosorbent assay.

Results: Levels of IL-17 and RANTES significantly increased in sputum of patients with AR after allergen (but not placebo) challenge ($P = .03$ and $P = .007$, respectively). Postallergen levels of both cytokines in sputum were positively correlated ($r = 0.570$, $P = .02$). Allergen challenge led to increased total inflammatory cell ($P = .005$) and eosinophil ($P = .03$) counts in induced sputum of patients with AR.

Conclusions: Nasal allergen challenge induces the enhanced secretion of IL-17 and RANTES in the lower airways of nonasthmatic patients with AR.

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INTRODUCTION

Allergic rhinitis (AR) is an inflammatory disease in which local overproduction of T_H2 cytokines, mucosal eosinophilia, and local upregulation of adhesion molecules are well defined.¹ Accumulating evidence underlines the importance of AR in the development of lower airway inflammation.² Markers of allergic inflammation in the lower respiratory tract have been found in induced sputum, bronchoalveolar lavage fluid (BALF), and bronchial biopsy specimens of individuals with AR without bronchial hyperresponsiveness (BHR) or asthma.^{3–6} It is now recognized that allergic processes in the upper and lower parts of the respiratory tract are closely related and do not constitute distinct disease categories, which led to the generally accepted concept of “one airway, one disease.” Allergen exposure of a sensitized nasal mucosa is associated with immunopathologic changes at the level of the lower respiratory tract, suggesting that direct contact

between allergen and mucosa is not necessary to elicit allergic inflammation.⁷

T lymphocytes play a crucial role in the development and maintenance of allergic diseases of the respiratory tract through the production of an array of proinflammatory mediators.¹ Recently, a separate T-cell lineage (T_H17 cells or inflammatory T cells) producing interleukin 17A (IL-17A) has been identified.^{8,9} IL-17 plays an essential role in the recruitment of neutrophils through the release of CXC chemokines from stromal cells.¹⁰ However, results of recent studies performed in mice indicate that IL-17 also has a systemic effect on the development of the T_H2 response by the activation of allergen-specific T cells, which leads to BHR and eosinophilic inflammation.

RANTES (regulated on activation of normal T cells expressed and secreted), among other chemokines, plays a crucial role in chemotaxis of eosinophils into airway mucosa.^{12,13} Increased RANTES messenger RNA and protein levels in nasal secretions of patients with AR have been observed during the pollen season and after specific nasal provocation testing (NPT).^{14–16}

To date, few authors have studied AR in the context of lower airway inflammation. After specific NPT, increased sputum eosinophil cationic protein and intercellular cell adhesion molecule 1 (ICAM-1) levels and decreased IL-10

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levels,¹⁷ as well as upregulation of adhesion molecules (ICAM-1, vascular cell adhesion molecule 1 [VCAM-1], and E-selectin) on bronchial microvasculature,³ have been found in the lower airways of nonasthmatic patients with AR.

Because IL-17 and RANTES are implicated in the development of the T_H2 response, we hypothesized that they might be involved in a cross-talk between nasal and bronchial mucosa after NPT. To test this hypothesis, we measured prechallenge and postchallenge levels of both cytokines in induced sputum of nonasthmatic patients with AR vs controls.

MATERIALS AND METHODS

Study Population

The study protocol was approved by the bioethical committee of the Medical University of Silesia. Written informed consent was obtained from all the participants before enrollment.

Fifty-five patients from the Outpatient Allergology Unit, Pulmonology Department, Medical University of Silesia, were screened for AR symptoms. Of these, 18 patients (8 women and 10 men; age range, 21–31 years; mean [SD] age, 24.9 [4.2] years) fulfilled all the entry criteria and were eligible for randomization. All the patients had symptoms suggestive of AR during the grass pollen season for at least 3 consecutive years and positive skin prick test (SPT) reactions to grass pollen. Patients with concurrent allergy to tree pollen were excluded from the study to avoid priming of the nasal mucosa. Patients with any asthma symptoms or in whom BHR (in the methacholine challenge test) was present were excluded from the study.

Study participants had not received systemic or local medication that might affect the response to NPT. Other exclusion criteria for entry were (1) younger than 18 years and older than 35 years, (2) smoking history of more than 10 pack-years, (3) history of infection for 4 weeks before the test day, (4) anatomical anomalies or previous nasal or sinus surgery, (5) nonallergic nasal or sinus disease, (6) chronic inflammatory processes or allergic diseases other than AR, (7) history of specific immunotherapy, (8) pregnancy, (9) forced expiratory volume in 1 second (FEV₁) less than 80% of predicted, and (10) poor patient compliance.

Ten control subjects (5 women and 5 men; age range, 21–30 years; mean [SD] age, 26.3 [3.0] years) volunteered to take part in the study. They had no symptoms and no history of AR or any other chronic disease. The SPT results for a panel of common aeroallergens were negative in all the subjects. The exclusion criteria were the same as those for patients with AR.

Lung Function and SPT

Lung function was measured using a dry spirometer (MasterLab, Jaeger, Wurzburg, Germany). The SPTs were performed, according to European Academy of Allergology and Clinical Immunology guidelines,¹⁸ on the volar part of the forearm using standardized lancets with 1-mm tips. A standard battery of 12 common allergens, including grass and tree

pollens, molds, feathers, and house dust mites (Allergopharma, Reinbeck, Germany), was used. The negative control was allergen diluent, and the positive control was histamine dihydrochloride solution at 1 mg/mL. A wheal of 3 mm or greater was considered positive.

Study Design

The study was designed as a 2-stage, crossover challenge trial. During the first phase, baseline sputum induction was undertaken. A single nasal placebo challenge (with diluent solution) was performed 24 hours later, followed by repeated sputum induction 24 hours after challenge. After a washout of 30 days, these procedures were repeated, and specific allergen challenge was performed.

NPT Procedure

Specific, unilateral NPT was performed according to current recommendations.^{19–21} Challenges were performed during the pollen-free winter months in a room with constant temperature and humidity. Participants underwent provocation between 8 AM and noon, and before starting the procedure, they were acclimatized for 30 minutes. All the tests were performed by the same investigator (A.S.-O.). All the patients were asymptomatic at the time of testing and had normal spirometry findings (FEV₁ >80% of predicted). No decongestants were used during NPT.

The same batch of a standardized nasal extract of grass pollen and the diluent solution of the allergen extract (Allergopharma) was used throughout the study. The allergen solution containing 5,000 standardized biological units/mL of grass pollen mixture was delivered through the nostril into the inferior turbinate region using a nasal pump sprayer, insufflating 1 puff (0.04–0.05 mL) into the challenged nasal cavity. During insufflation, patients held their breath and kept their head in a forward prone position to avoid swallowing or aspiration of allergen to the lower respiratory tract.

Sputum Induction and Cellular Evaluation

Sputum was induced by means of hypertonic saline inhalation (3%, 4%, and 5% for 7 minutes) using an ultrasonic nebulizer device (Thomex MB; Medbryt, Warsaw, Poland) and was processed as previously reported.²² Centrifugation (10 minutes at 1,000 rpm) separated cell pellet and supernatant. The supernatant samples were stored at –70°C until assayed. The obtained sediment was washed with sterile phosphate-buffered saline (Sigma-Aldrich Corp, St Louis, Missouri) and then was suspended in 1 mL of RPMI (Sigma-Aldrich Corp). After staining the cells using the Kimura method, the total number of nonsquamous cells was counted using a Neubauer hemocytometer, allowing determination of the number of cells in 1 g of sputum. The sample was then transferred onto a cytospin slide and was further centrifuged at 500 rpm, air-dried, and stained using the Giemsa method. A minimum of 300 cells were counted on each slide and were further classified as epithelial cells, eosinophils, neutrophils, lymphocytes, and macrophages.

Measuring Cytokine Levels

Sputum IL-17 and RANTES concentrations were assayed using sandwich enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, Minnesota) according to the manufacturer's protocol. The minimum detectable IL-17 and RANTES level was 15.6 pg/mL for both cytokines.

Statistical Analysis

Statistical evaluation was performed using a software package (Statistica 6.0; StatSoft Inc, Tulsa, Oklahoma). The Kolmogorov-Smirnov test was used to test variables for normal distribution. The *t* and Wilcoxon matched pairs tests were used to determine pre-NPT and post-NPT differences. Intergroup comparisons were performed using the Mann-Whitney test. The Spearman rank correlation test was used to evaluate correlations between variables. $P < .05$ was accepted as statistically significant.

RESULTS

Baseline characteristics of the included patients are summarized in Table 1. Both groups were comparable regarding age, sex (both groups included a higher percentage of men), FEV₁, and smoking status. Allergen challenge was safe and well-tolerated by all the participants. Spirometric observation registered no significant changes in FEV₁ after placebo or allergen administration at 15 minutes and during the 24 hours after challenge. All 18 patients with AR had a positive response to NPT with allergen (but not placebo) 15 minutes after challenge (increase of nasal airway resistance measured using rhinomanometry of $\geq 60\%$ compared with baseline or a clinical symptom score > 3 points). None of the 10 control subjects had a positive response to NPT with allergen or placebo.

Prechallenge and postchallenge sputum samples were obtained from 18 patients with AR and 10 controls. Cell viability did not differ between the studied groups ($P > .05$) and was higher than 72%. The total cell count and sputum cell differential count, expressed as the absolute cell number, are presented in Table 2. Compared with controls, induced sputum of patients with AR was characterized by a significantly higher absolute number of total cells, eosinophils, neutrophils, and macrophages, at baseline and after placebo and allergen challenge ($P < .05$ for all intergroup comparisons).

Nasal allergen (but not placebo) challenge in patients with AR induced further significant influx of inflammatory cells ($P = .005$), including eosinophils ($P = .03$), into the lower airways (Table 2). No significant changes in induced sputum cellularity were observed in the control group after allergen and placebo challenge compared with baseline values ($P > .05$ for all prechallenge-postchallenge comparisons).

Measurable quantities of IL-17 were detected in sputum samples taken from 17 of 18 patients with AR and in 8 of 10 controls. RANTES was detected in all sputum samples assayed. The reproducibility of the assays was good. The overall coefficient of variation was calculated to be 5.2% (range, 1.9%–7.6%) for IL-17 and 4.0% (range, 3.2%–5.6%) for RANTES. Baseline and postchallenge RANTES and IL-17 levels are presented in Table 2. Baseline sputum concentrations of both cytokines were comparable between AR and control patients before placebo and allergen challenge ($P > .05$ for all intergroup comparisons). Levels of IL-17 and RANTES significantly increased in induced sputum of patients with AR 24 hours after allergen (but not placebo) challenge compared with baseline values ($P = .03$ and $P = .007$, respectively) (Fig 1). Increased postchallenge levels of both cytokines were positively correlated ($r = 0.570$, $P = .02$). Postallergen IL-17 and RANTES sputum levels were also higher in patients with AR compared with controls ($P = .04$ and $P = .002$, respectively). Sputum IL-17 and RANTES concentrations in controls remained unaffected after allergen and placebo challenge.

DISCUSSION

Although it is clear that the condition of the nose has an impact on lower airway physiology, only a few studies have been performed that aimed to explain the contribution of inflammatory mediators in nasobronchial interactions in AR. Increased numbers of CD4⁺, CD8⁺, and CD45RO⁺ lymphocytes and IL-5⁺ cells have been found in bronchial biopsy specimens obtained from nonasthmatic patients with AR during the pollen season.²³ Beeh et al¹⁷ demonstrated decreased levels of IL-10 in induced sputum of nonasthmatic patients with AR after NPT. In the study by Braunstahl et al,³ NPT in patients with AR led to the upregulation of ICAM-1,

Table 1. Characteristics of the Study Participants

Characteristics	Patients with allergic rhinitis (n = 18)	Control group (n = 10)
Age, mean (SD) [range], y	24.9 (4.2) [21–3]	26.3 (3.0) [21–30]
Sex, F/M, No. (%)	8/10 (44/56)	5/5 (50/50)
FEV ₁ , median (range), % of predicted	109.6 (86.2–152.3)	107.7 (94.7–118.3)
Wheal diameter to grass pollen in SPT, median (range), mm	9.5 (6.0–19.0)	0.0 (0.0–1.5)
Cigarette smoking N/Ex/Y, %	17/1/0	9/0/1

Abbreviations: Ex, former smokers; FEV₁, forced expiratory volume in 1 second; N, nonsmokers; SPT, skin prick testing; Y, current cigarette smokers.

Table 2. Changes in Sputum Cellularity and Cytokine Concentrations Induced by Allergen and Placebo Challenge in Patients With Allergic Rhinitis and Controls^a

	Patients with allergic rhinitis						Control group					
	Allergen			Placebo			Allergen			Placebo		
	Prechallenge	Postchallenge	Prechallenge	Postchallenge	Prechallenge	Postchallenge	Prechallenge	Postchallenge	Prechallenge	Postchallenge	Prechallenge	Postchallenge
Total cell count, $\times 10^6/\text{g}$ of sputum	1.36 (0.56–5.60)	1.73 (0.57–6.20) ^b	1.70 (0.65–3.87)	1.33 (0.43–3.52)	0.91 (0.27–1.40)	0.95 (0.29–3.50)	0.75 (0.31–1.61)	0.70 (0.21–1.16)				
Eosinophils, $\times 10^6/\text{g}$ of sputum	0.05 (0.00–0.50)	0.08 (0.00–0.75) ^b	0.05 (0.00–0.55)	0.04 (0.00–0.29)	0.01 (0.00–0.03)	0.01 (0.00–0.02)	0.01 (0.00–0.02)	0.01 (0.00–0.02)				
Neutrophils, $\times 10^6/\text{g}$ of sputum	0.54 (0.13–2.68)	0.71 (0.15–3.17)	0.54 (0.12–1.29)	0.47 (0.09–1.21)	0.29 (0.05–0.48)	0.32 (0.07–1.07)	0.3 (0.09–0.58)	0.26 (0.07–0.52)				
Macrophages, $\times 10^6/\text{g}$ of sputum	0.80 (0.16–2.58)	0.91 (0.18–2.57)	0.89 (0.14–2.57)	0.90 (0.18–2.37)	0.55 (0.22–0.89)	0.57 (0.17–2.06)	0.43 (0.21–1.23)	0.48 (0.09–0.81)				
Lymphocytes, $\times 10^6/\text{g}$ of sputum	0.01 (0.00–0.06)	0.01 (0.000–0.08)	0.01 (0.00–0.04)	0.01 (0.00–0.06)	0.01 (0.00–0.01)	0.00 (0.00–0.02)	0.01 (0.00–0.02)	0.00 (0.00–0.02)				
IL-17, pg/mL	31.3 (17.4–115.8)	40.7 (26.6–149.2) ^b	28.9 (15.6–52.9)	26.6 (15.4–50.4)	32.4 (19.9–60.4)	24.8 (22.1–55.4)	35.5 (16.5–70.6)	33.1 (17.8–50.4)				
RANTES, pg/mL	36.6 (15.8–73.7)	53.7 (26.5–89.4) ^b	35.1 (18.1–71.1)	31.1 (16.8–75.7)	30.5 (19.0–43.0)	28.3 (22.0–46.1)	29.6 (21.4–49.1)	26.3 (18.0–52.0)				

Abbreviations: IL-17, interleukin 17; RANTES, regulated on activation of normal T cells expressed and secreted.

^a Data are expressed as median (range).

^b $P < .05$ for prechallenge–postchallenge differences, Wilcoxon matched pairs, signed rank test.

VCAM-1, and E-selectin on nasal and bronchial microvasculature.

In the present study, we showed increased sputum IL-17 and RANTES levels in nonasthmatic patients with AR after NPT. To our knowledge, this is the first study to investigate postchallenge levels of these 2 cytokines in the lower airways of nonasthmatic patients with AR.

Previous studies proved that IL-17A was significantly expressed in asthmatic patients²⁴ and that its sputum levels correlated positively with the degree of BHR.²² We decided to study the contribution of IL-17 in nasobronchial interactions because a recent study¹¹ showed that this cytokine is involved in the activation of allergen-specific T cells. Given the broad proinflammatory effects of IL-17, its ability to sustain the T_H2 response,¹¹ and increased levels of IL-17 in induced sputum,^{22,24} BALF,²⁴ and bronchial biopsy samples²⁵ from asthmatic patients, we expected higher baseline IL-17 levels in sputum of patients with AR than in controls. In the present study baseline IL-17 sputum concentrations in patients with AR were more variable but were comparable with those of control subjects and slightly lower than its levels in induced sputum of asthmatic patients found in a previous study.²² Comparable baseline sputum IL-17 levels in both studied groups might, therefore, reflect the fact that inflammatory processes in the lower airways of nonasthmatic patients with AR outside the pollen season are not so enhanced as in asthmatic patients. In the present study, sputum IL-17 levels in patients with AR were significantly higher 24 hours after specific NPT compared with baseline. To our knowledge, there is only 1 study²⁶ of IL-17 concentrations in nasal secretions of patients with AR and viral rhinitis, which reported that detectable levels of this cytokine are found in nasal secretions from patients with viral rhinitis but not AR. Increased serum levels of IL-17 have been detected in patients with severe seasonal AR outside the pollen season.²⁷ Considering the fact that until now no other authors have measured either baseline or postchallenge levels of IL-17 in induced sputum (or any other material obtained from the lower airways) of patients with AR, we cannot relate this finding to the results of other studies. However, these observations clearly indicate that nasal exposure to allergen induces enhanced secretion of IL-17 in the lower airways of patients with AR. This finding is consistent with the results of previous studies in which NPT caused an upregulation of inflammatory markers¹⁷ and adhesion molecules³ in the lower respiratory tract, suggesting that systemic inflammatory response is triggered in response to local allergen exposure. It is possible that single exposure of unprimed nasal mucosa to allergen during NPT performed in this study caused temporary upregulation of IL-17 in the lower airways through transitory enhanced systemic IL-17 secretion. During prolonged natural exposure to allergen, such processes might consolidate, leading to the increased IL-17 serum levels present in severe AR.^{27,28}

We also measured the impact of NPT on sputum RANTES concentrations because several studies^{13–16} have stressed the

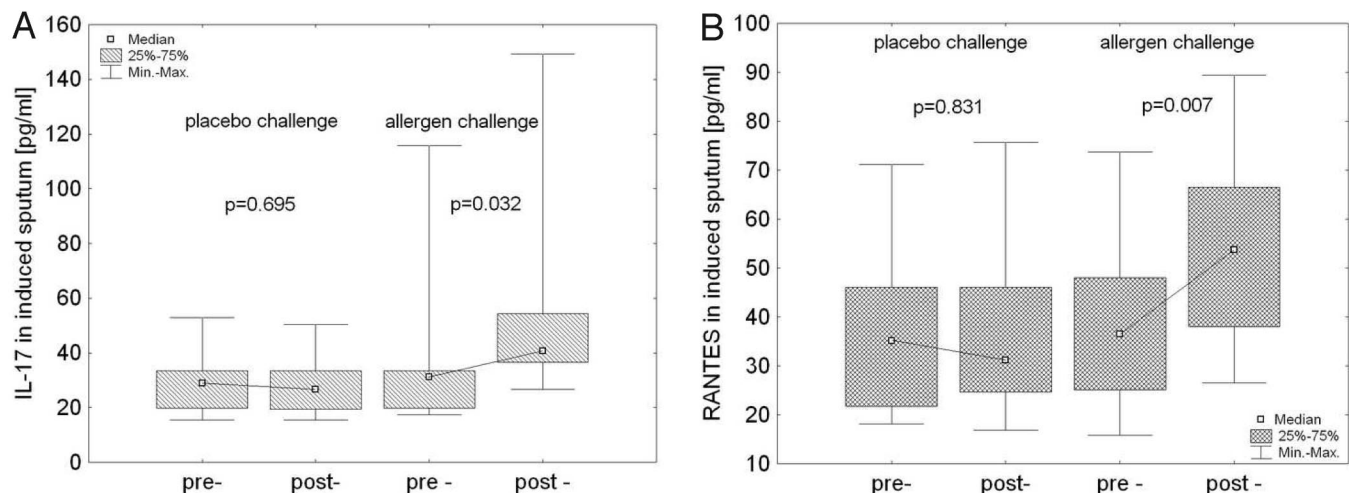


Figure 1. Evolution of mean levels of interleukin 17 (IL-17) (A) and RANTES (regulated on activation of normal T cells expressed and secreted) (B) in sputum of patients with allergic rhinitis after single nasal allergen or placebo challenge. Allergen challenge induced a significant increase in IL-17 (A) and RANTES (B) levels in induced sputum of patients with allergic rhinitis ($P < .05$). Levels of neither cytokine changed after placebo challenge ($P > .05$).

importance of RANTES in the influx of eosinophils from the bloodstream into the airway mucosa. The baseline sputum RANTES levels of patients with AR did not differ from those of controls. This is consistent with the results of a previous study²⁹ in which increased RANTES levels have been found in induced sputum of chronic asthmatic patients but not in AR. To date, to our knowledge, no authors have studied the impact of NPT on RANTES levels in the lower airways of patients with AR. In the study by Teran et al,³⁰ increased levels of this cytokine were found in the BALF of atopic asthmatic patients after segmental bronchial provocation. Measurement of RANTES by means of enzyme-linked immunosorbent assay in $10\times$ -concentrated BALF in this study showed 5.75-fold higher levels of this cytokine at the site of provocation compared with placebo challenge at the control site. Postchallenge RANTES levels reported in this study are 3.5-fold higher than in the present study, but it must be stressed that the allergen solution in the study by Teran et al³⁰ was applied directly onto the bronchial wall of asthmatic patients (in whom the inflammatory process in the lower airways is more enhanced than in patients with AR) and that it was measured in $10\times$ -concentrated BALF (which makes comparison with induced sputum difficult). Rajakulasingam et al¹⁵ found that RANTES levels are significantly increased in the nasal lavage fluid (NLF) of patients with AR after NPT compared with baseline and the postplacebo values. The median maximum increase in NLF RANTES levels in patients with AR in this study was 192%. In the study by Erin et al,¹⁶ the increase in RANTES levels in NLF 2 hours after challenge was approximately 2-fold compared with its baseline values. In the present study, NPT induced a significant, but less than 1.5-fold, increase in RANTES levels evaluated in induced sputum of patients with AR. This increase was not so enhanced as in the previously mentioned studies.^{15,16} This

is probably because although NPT induces the secretion of RANTES in the upper and lower airways, this increase is more enhanced in nasal tissue (which represents the primary target organ in nonasthmatic AR patients with AR subjected to NPT) than in distant, secondary target tissues (eg, bronchi). These differences might also result from the different time points at which RANTES levels were evaluated in the upper and lower airways in the previously mentioned study (0–6 hours after the challenge) and the present study. The single end point of 24 hours after NPT used in the present study might not have been sufficient to detect maximum changes in levels of cytokines studied. On the basis of the present and previous²⁹ studies, we conclude that the secretion of RANTES in the lower airways of patients with AR outside the pollen season is comparable with that of healthy individuals. NPT induces further significant secretion of RANTES in bronchial mucosa of patients with AR, providing additional evidence of upregulation of pulmonary inflammatory response after upper airway exposure to allergen.

Positive correlations found between increased sputum levels of IL-17 and RANTES might reflect their cooperation in sustaining allergic inflammation. Although we lack direct proof, it is possible that IL-17A induces RANTES through mechanisms similar to the previously described relationship between IL-17 and IL-8,³¹ being, therefore, an important link connecting activated memory T cells with the influx of eosinophils. In a previous study,²² we observed a positive correlation between IL-17 levels and the sum of the percentages of neutrophils and eosinophils in induced sputum of asthmatic patients, supporting the potential association between IL-17 and the recruitment of granulocytes to bronchial mucosa in those patients. Another potential explanation of the positive correlation between sputum IL-17 and RANTES is that both cytokines are secreted by the same cell type. How-

ever, confirming this hypothesis requires additional studies aimed at identifying such cell lineage.

The significant postchallenge influx of eosinophils into the bronchial lumen observed in this study is consistent with the observations of other authors,^{3,32} who additionally found concurrent increased expression of adhesion molecules on bronchial microvasculature of patients with AR.³ However, we did not observe any correlations between increased sputum total cell count or eosinophil count and elevated IL-17 or RANTES levels. This is inconsistent with the results of other studies that found correlations between eosinophil number and RANTES in BALF³⁰ and induced sputum³³ of asthmatic patients 4 to 7 hours after SBP. We suspect that these conflicting results are based on different methodological approaches to evaluate cytokine levels in the lower airways. We were aware that the single end point of 24 hours after NPT might naturally limit the likelihood of detecting significant changes and correlations between variables studied. However, it was not only supported by former studies^{3,17,33} investigating inflammatory responses induced by NPT but was also related to the observation that levels of inflammatory markers in sputum have not been reproducible when induction with hypertonic saline was repeated at short intervals (<24 hours).³⁴

These results indicate that NPT in patients with AR is associated with increased sputum IL-17 and RANTES levels, pointing to their significance in enhancing lower airway allergic inflammation. It is possible that IL-17A and RANTES constitute an important link that connects activated memory T cells with the recruitment of eosinophils to the site of allergic inflammation.

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