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Allergen induced Th17 response in the peripheral blood mononuclear cells (PBMCs) of patients with nasal polyposis

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ABSTRACT

Background: Nasal polyposis (NP) is a chronic inflammatory disease of the nasal cavity and sinuses. Th17 cells have been considered to play roles in allergic airway diseases and various chronic inflammatory disorders.
Aim of the study: This study aimed to investigate the population and function of peripheral Th17 cells in response to house dust mite extracts (HDM) allergen in NP patients, and evaluate the possible correlation between Th17 cells and atopy, to explore the role of atopy in the pathogenesis of NP.
Methods: Peripheral blood mononuclear cells (PBMCs) obtained from atopic NP patients, non-atopic NP patients, and controls were stimulated by phytohemagglutinin (PHA) or HDM plus PHA. The resulting frequency of Th17 cells was detected by flow cytometry and the expression of RORc was measured by real-time PCR. Then the concentrations of IL-17A, INF-γ, IL-4 and IL-5 in the supernatants were assayed by specific ELISAs.
Results: The population and function of Th17 cells in allergen stimulated PBMCs were significantly higher in atopic NP patients. In addition, in atopic group, HDM + PHA stimulation induced significant increase of Th17 population and IL-17A production versus those in PHA stimulated ones. However, the frequency of Th17 cells was not correlated with Th1, Th2 cytokine productions.
Conclusion: Th17 immunity is involved in the systemic immune responses to allergen in atopic NP and atopy may aggravate NP by stimulating the increase of Th17 population and IL-17A production. The mechanism of Th17 cells response to allergen may be regulated differently from the regulation of Th1 and Th2 immunity in NP.

1. Introduction

Nasal polyposis (NP) is a chronic inflammatory disease of the mucosa of the nose and paranasal sinuses, characterized by a Th2 skewed eosinophilic inflammation with high levels of IL-5 and IgE and low TGF-β1 [1,2]. Histology shows the selective accumulation of numerous eosinophils, neutrophils, lymphocytes, plasma cells, and mast cells, as well as the formation of nasal polyps [3]. Research reveal that the prevalence and incidence of NP is still increasing and NP remains a significant health problem with a considerable socioeconomic burden [4,5].Over the last 2 decades, NP has been widely studied, whereas its pathogenesis is still not fully understood and the role of atopy in the aetiology and pathogenesis of NP is still a controversial issue [6].

Th17 cell, a new subset distinct from Th1 and Th2 cell, characterized by its preferential production of interleukins (IL)-17A and F and require retinoid orphan nuclear receptor (RORc) as a key transcription factor for its differentiation in humans [7,8]. IL-17A has an important and unique role in host protection against specific pathogens. Both IL-17A and IL-17F have pro-inflammatory properties and act on a broad range of cell types to induce the expression of cytokines (IL-6, IL-8, GM-CSF, and G-CSF), chemokines (CXCL1, CXCL10) and metalloproteinases. Also IL-17A and IL-17F are key cytokines for the recruitment, activation and migration of neutrophils. The inappropriate production of IL-17 by Th17 cells is thought to contribute to the pathology of a range of inflammatory diseases, and a growing list of chronic inflammatory disorders has been linked to Th17 [9–11].

In addition, Th17 cells and IL-17 cytokine family are considered to play roles in the development of various allergic disorders that have classically been considered to be Th2-mediated disorders, such as allergic asthma, allergic rhinitis, allergic dermatitis and allergic conjunctivitis [12]. Meanwhile, Yang Zhao et al. [13] showed that besides predominant Th2 immunity, abnormal Th17 immunity may be also involved in the pathogenesis of allergic asthma. Recently, the meaning of Th17 cells and IL-17 cytokine in NP has been paid close attention, which showed novel expression of Th17 cell and IL-17A in NP [14,15]. However, there were no in vitro studied that investigated the role of Th17 cells in NP and focused on the possible correlation between Th17 cells and atopy in the pathogenesis of NP.

Therefore, the present study aimed to investigate the population and function of peripheral Th17 cells from both atopic and non-atopic

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NP patients in response to HDM allergen, in order to evaluate in vitro characteristics of Th17 subsets from NP patients at the cellular level. Then, we investigated the possible correlation between Th17 cells and atopy, in order to explore the role of atopy in the pathogenesis of NP.

2. Methods

2.1. Patients

42 patients (23 men, 19 women; 24 atopic, 18 non-atopic) between 24 and 62 years of age were included. The diagnosis of NP was based on clinical history, anterior rhinoscopy, nasal endoscopy, and paranasal CT scan, according to the current European EAACI Position Paper on Rhinosinusitis and Nasal Polyps [5] and American guidelines [16]. Clinical data about patients included age, sex, duration of disease, history of asthma and recurrence. Symptom scores were assessed according to a visual analog scale (VAS) [17]. The preoperative CT scans were graded according to the classification by Lund and Mackay [18]. The preoperative nasal endoscopy scores were graded according to the classification by Lanza and Kennedy [19]. Patients with antrochoanal polyps, cystic fibrosis, fungal sinusitis, primary ciliary dyskinesia or systemic diseases were excluded. Ten patients with a deviated septum were recruited as a control group. Controls had no history of respiratory disease or atopy, and their skin prick test (SPT) results were negative. This study was approved by the ethical committee of Chongqing Medical University and informed consent was obtained from all subjects. Oral and topical applications of corticosteroids or antihistamines were withheld for a minimum of 4 weeks before the study.

2.2. Determination of atopy

The atopy test was based on skin prick test (SPT) (Allergopharma, Hamburg, Germany). The SPT results were diagnosed in accordance with the recommendations of the Subcommittee on Allergen Standardization and Skin Tests of the European Academy of Allergy and Clinical Immunology [20]. Patients were considered prick test positive if at least one allergen elicited a papule diameter that was as large as or larger than that produced by the positive control (histamine). A total of 18 inhaled allergens were tested, including house dust mite, grass, tree, mould, food, and cat and dog dander. Actually, every atopic subject included in this study got a positive SPT response with house dust mite.

2.3. Isolation and antigen stimulation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were obtained by standard Ficoll–Hypaque density centrifugation (Company Tianjin TBD, China) 400 g for 20 min within 1 h of collection. Then, the cells were washed twice with phosphate-buffered saline (PBS) and resuspended at 2×10^6/ml in RPMI-1640 medium supplemented with 10% calf serum, 100 unit/ml penicillin, 100 μg/ml streptomycin and 2 mmol/l L-glutamine. Cell viabilities were more than 95% as examined by trypan blue-exclusion assays. For polyclonal activation, cells were cultured with phytohemagglutinin (PHA, Sigma). For antigen stimulation, PBMCs were incubated with house dust mite extracts (HDM, Allergopharma, Hamburg, Germany).Thus, PBMCs were cultured with HDM (20 μg/ml) and PHA (5 μg/ml) for 48 h at 37 °C in a 5% CO_2 humidified atmosphere and compared with PHA stimulated PBMCs.

After each incubation time, the cells were centrifuged at 1811 g for 5 min. The cell pellet was individual into two parts. One part was harvested for flow cytometry assays. The other part was immediately snap frozen and stored at −80 °C and supernatants were stored at −20 °C for further use in real-time PCR and ELISA testing.

2.4. Flow cytometric analysis of Th17

PBMCs stimulated with antigen for 48 h were activated with phorbol myristate acetate (PMA, 50 ng/ml; Alexis Biochemicals, San Diego, CA) and ionomycin (1 μM; Sigma, USA) in the presence of monensin (1 μl/ml; BD, USA) for 4 h at 37 °Cin a 5% CO_2 atmosphere. Because of the downregulation of surface CD4 molecules after stimulation with PMA and ionomycin, we defined Th17 cells as CD3 + CD8-IL-17 + cells. For the CD3 + CD8-IL-17 + cells analysis, cells were incubated with PE-Cy5-conjugated anti-human CD3 and FITC-conjugated anti-human CD8 antibodies for 20 min at 4 °C in the dark, but not anti-CD4. After surface staining, cells were re-suspended in fixation and permeabilization solution according to the manufacturer's instructions (BD), and then stained with PE anti-human IL-17A antibodies. PE-conjugated mouse IgG1 antibodies were used as isotype controls. All of the antibodies were from eBioscience. Fluorescence profiles were analyzed using a FACScan cytometer equipped with CellQuest software (BD). The results are expressed as a percentage of positive cells. In our previous research, we demonstrated the basal values of peripheral CD3 + CD8-IL-17 + cells were ranged from 0.13% to 0.74% in control subjects, compared with 0.65% to 1.82% in non-atopic NP patients and 0.77% to 2.34% in atopic NP patients [21].

2.5. Real-time PCR analysis for RORc

For assaying the mRNA levels for the transcription factor RORc, we first isolated cell total RNA using TRIzol extraction (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and total RNA was reverse-transcribed to cDNA with random hexamer primers and RNase H-reverse transcriptase (Invitrogen). Expression of mRNA was determined using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Taq (TaKaRa Biotechnology, Dalian, China). The following primer pairs were used for RORc: F: 5′-GCTGTGATCTTGCCCAGAACC-3′, R: 5′-CTGCCCATCATTGCTGTTAATCC-3′. All PCRs were performed in duplicate. Relative gene expression was calculated by using the comparative CT method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization, and a no template sample was used as a negative control.

2.6. ELISA analysis for cytokines

The resulting supernatants were harvested and frozen at −20 °C until use. They were assayed for IL-17A, INF-γ, IL-4 and IL-5 using specific ELISA kits according to the manufacturer's instructions (all ELISA kits from eBioscience, San Diego, CA, USA). All assays were performed in duplicate. The results are expressed in pg/ml.

2.7. Statistical analysis

The software used for statistical analysis was SPSS for Windows ver. 17.0 (SPSS, Chicago, IL, USA). Data are presented as mean ± standard deviation or medians and interquartile ranges. Differences between the values were determined using Student's *t*-test. Grouped data were analyzed using a one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. When the equal variance test failed, a Mann–Whitney rank sum test was used. The Wilcoxon matched pairs test was used to analyse differences in Th17 population and cytokine expression in the HDM + PHA and PHA stimulated cultures. The Spearman test was used to determine correlations. Significance was accepted at $p<0.05$.

3. Results

3.1. Clinical characteristics

The study subjects' clinical characteristics are summarized in Table 1. Endoscopy scores, and CT scores were significantly higher