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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.

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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- β 1 [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 studies 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 pre-operative 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% CO₂ 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 °C in a 5% CO₂ 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'-GCTGTGATCTTGCCAGAAC-3', R: 5'-CTGCCATCATTCGTGTAATCC-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

in atopic group than in non-atopic group ($p < 0.05$). However, no statistically significant differences were found between two groups from the standpoint of symptom score, age, sex, duration of disease or recurrence ($p > 0.05$), and asthma was only noted in atopic group.

3.2. Effect of allergen on the frequency of Th17 cells in PBMCs from NP patients

The prevalence of Th17 cells in stimulated PBMCs was expressed by a ratio of CD3 + CD8-IL-17+/CD3 + CD8- cells. The frequency of CD3 + CD8-IL-17 + cells in PHA stimulated and HDM + PHA stimulated cultures was $1.88 \pm 0.41\%$ and $2.27 \pm 0.46\%$ in atopic NP patients, $1.59 \pm 0.39\%$ and $1.66 \pm 0.41\%$ in non-atopic NP patients, and $1.30 \pm 0.35\%$ and $1.35 \pm 0.31\%$ in controls, respectively. The cytometric analysis showed that the frequency of CD3 + CD8-IL-17 + cells in HDM + PHA stimulated PBMCs was significantly upregulated in atopic NP patients than in non-atopic NP patients and controls ($p < 0.01$). However, the differences were not significant between non-atopic NP patients and controls ($p > 0.05$) (Fig. 1). Our previous research showed that the frequency of peripheral CD3 + CD8-IL-17 + cells was significantly increased at baseline in both atopic and non-atopic groups, and a significant difference was found between atopic and non-atopic groups.

We next investigated if there were any significant changes in the frequencies of CD3 + CD8-IL-17 + cells between HDM + PHA and PHA stimulation in all three groups respectively. The frequency of CD3 + CD8-IL-17 + cells was significantly increased in response to HDM only in atopic NP patients ($p < 0.01$), and was statistically unchanged in non-atopic group and controls. (Fig. 1)

3.3. RORc expression after allergen stimulation

Consistent with the results of Th17 population in PBMCs, Fig. 2 reported that mRNA levels of RORc in HDM + PHA stimulated PBMCs were significantly higher in atopic NP patients compared with non-atopic NP patients and the control group ($p < 0.01$), while the differences were not significant between non-atopic NP patients and controls ($p > 0.05$).

In atopic group, the levels of RORc in HDM + PHA stimulated PBMCs were significantly increased when compared with PHA stimulated PBMCs ($p < 0.01$). However, RORc expression was not significantly modified by the addition of HDM in non-atopic and control group. (Fig. 2)

3.4. Th17-, Th1-, Th2-related cytokine levels in the culture supernatants

The concentrations of IL-17A, IFN- γ , IL-4 and IL-5 in culture supernatants from activated PBMCs were measured by ELISA. As shown in Fig. 3, in comparison with control subjects, IL-17A levels in HDM + PHA stimulated supernatants were significantly higher in atopic NP patients ($p < 0.01$), whereas there were no significant differences of IL-17A expression between non-atopic group and controls

($p > 0.05$). In addition, in atopic group, IL-17A levels were markedly enhanced in HDM + PHA stimulated supernatants versus that seen in their respective PHA stimulated ones ($p < 0.01$). However, IL-17A levels were not significantly changed in response to HDM in non-atopic group and controls ($p > 0.05$). Our previous research showed that the IL-17A levels were significantly increased at baseline in both atopic and non-atopic groups, and a significant difference was found between atopic and non-atopic groups [21].

Fig. 3 showed that increased IFN- γ , IL-4, and IL-5 concentrations in HDM + PHA stimulated supernatants were observed in both atopic and non-atopic NP patients compared with controls. In addition, in atopic group, productions of these cytokines were higher in culture supernatants of HDM + PHA stimulated PBMCs than in supernatants of HDM + PHA stimulated ones.

3.5. Correlations between Th17 population and Th1, Th2 cytokine productions in NP

To figure out the possible interactions between Th17 and Th1, Th2 which were altered in NP, the relationships of Th17 cells population with IFN- γ , IL-4, and IL-5 levels were analyzed by Spearman test. However, as reported in Fig. 4, we found no correlations between the frequency of Th17 and IFN- γ , IL-4, and IL-5 levels ($p > 0.05$).

4. Discussion

In the present study, we demonstrated for the first time that the frequency of Th17 cells in allergen stimulated PBMCs was significantly higher in atopic NP patients versus that in controls. Meanwhile, in atopic group, HDM + PHA stimulation induced significant increase of IL-17A production in culture supernatants versus that in PHA stimulated ones. These results indicated that the population and function of peripheral Th17 cells from atopic NP patients were significantly upregulated in response to HDM, suggesting that Th17 immunity is involved in the systemic immune responses to allergen in atopic NP. Thus we speculate that atopy may aggravate NP by stimulating the increase of Th17 population and IL-17A production.

Th17 is a novel CD4 + effector T cell lineage characterized by the production of some distinct cytokines, especially IL-17A. Previous studies showed that Th17 functions locally, as well as systemically, in promoting allergic airway inflammation, such as allergic asthma [22,23], allergic rhinitis [24,25]. The major effects of Th17 immunity in allergic airway inflammation are mediated by Th17 cytokines such as IL-17A and IL-22. IL-17A acts on airway epithelial cells, lung fibroblasts and other types of inflammatory cells to trigger the production of pro-inflammatory cytokines, chemokines and matrix metalloproteinases, promotes the recruitments of neutrophil and macrophage, and leads to tissue inflammation. In this study, we paid close attention on the role of Th17 cells in NP, especially in NP with atopy, which was also an upper airway inflammatory disease commonly encountered in clinical otorhinolaryngology. Our in vitro research showed a significant enhancement of Th17 population and IL-17A production in HDM + PHA stimulated PBMCs from atopic NP patients. This fact underlines that the response of Th17 cells to allergen is upregulated only in atopic NP patients, while not in non-atopic NP patients and control subjects. These indicate the important role of Th17 cell in the sensitized patients with NP and suggest that atopy may participate in the development of NP as an enhancer through the Th17 immunity.

In this study, we showed that PBMCs from atopic NP patients elicited an increased response to HDM allergen in terms of Th17 cells for the first time. Our results conformed to previous research. On the one hand, Giorgio Ciprandi et al. [24] reported that patients with allergic rhinitis had higher frequencies of IL-17 producing T cells than normal subjects. Tomomi Hashimoto et al. [26] found that IL-17 production was significantly induced in response to *Dermatophagoides farinae* (Df)

Table 1
Clinical features of patients with NP.

	Atopic NP	Non-atopic NP	Control	p value
Age (y), median (range)	41(22–64)	43(25–58)	32(24–45)	n.s.*
Sex (female:male)	11:13	8:10	5:5	n.s.
Asthma	2/24	0/18	0/10	n.s.*
Recurrence	5/24	3/18	0	n.s.*
Duration of NP (y)	5(3–10)	4(2.7)	0	n.s.
Symptom score	11(9–15)	12(10–14)	0	n.s.
Endoscopy score	9(6–10)	5(3–7)	0	$p < 0.01$
CT score	12(9–17)	9(8–11)	0	$p < 0.01$

Data are expressed as medians and interquartile ranges. The level of significance (p) was obtained by means of Student's t test or the Fisher exact test (marked with *), and the significance level equals a p value of 0.05. n.s., not significant.

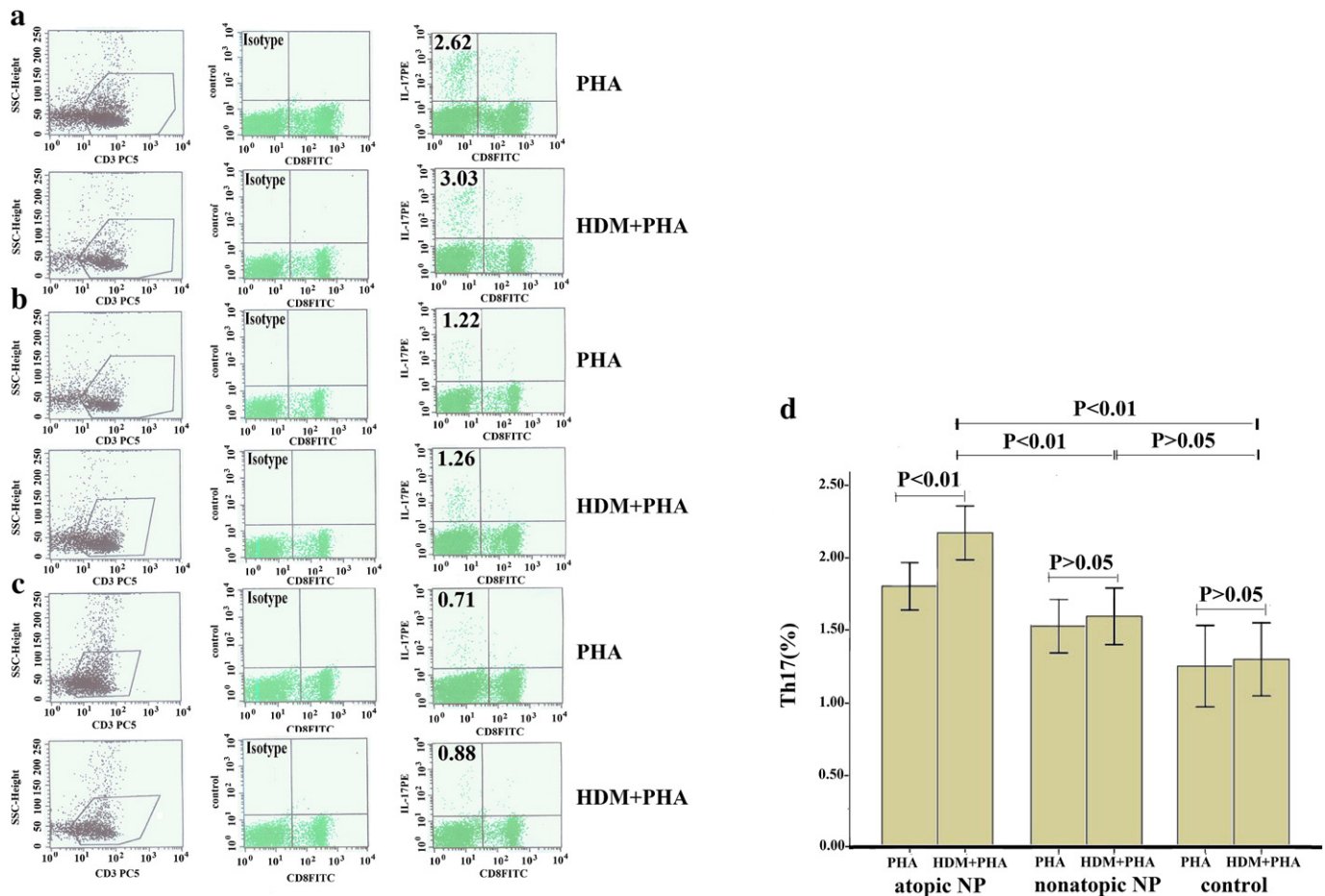


Fig. 1. Flow cytometric analysis of CD3 + CD8-IL-17 + cells in PHA stimulated and HDM + PHA stimulated PBMCs from atopic NP patients (a), non-atopic NP patients (b) and controls (c). (d): The frequency of Th17 cells in stimulated PBMCs. The percentage of CD3 + CD8-IL-17 + cells in PBMCs with HDM stimulation was significantly higher in atopic NP patients, while the differences were not significant between non-atopic NP patients and controls. In addition, the frequency of CD3 + CD8-IL-17 + cells was significantly increased in response to HDM only in atopic NP patients.

extract in atopic asthma. On the other hand, Lenarczyk et al. [27] reported that a type 0 antigen, tetanus toxoid, a type 1 antigen, purified protein derivatives from *Mycobacterium tuberculosis*, a potential type 2

rye grass antigen (Lol I), and an autoantigen SS-B (La) did not induce IL-17 production by PBMC of healthy controls at a protein level, which was consistent with our finding that HDM allergen did not induce IL-17A

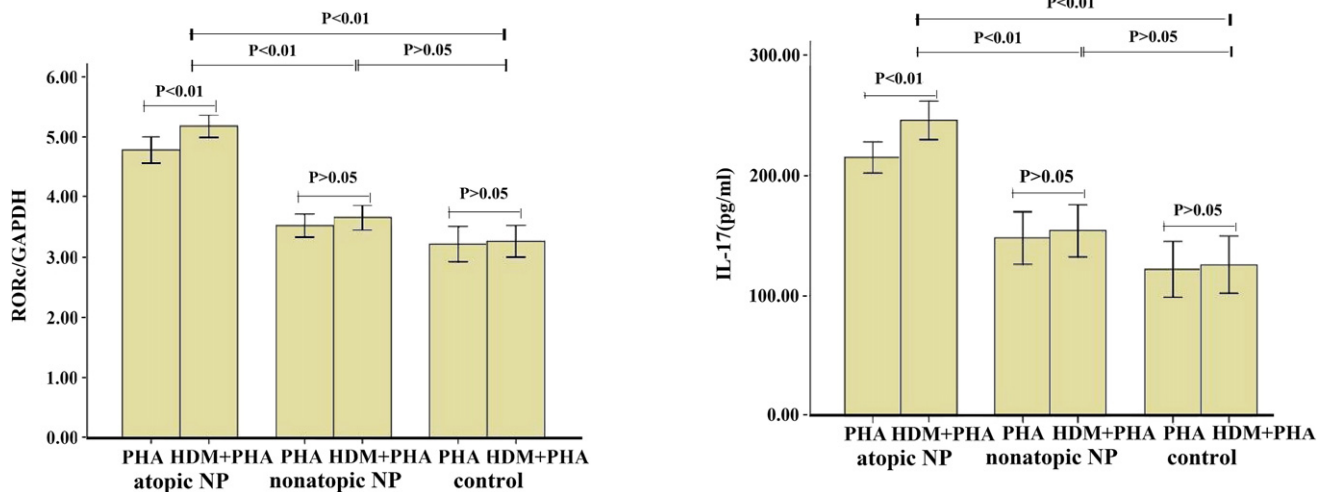


Fig. 2. Real-time PCR analysis of RORc in peripheral blood. mRNA levels of RORc in HDM + PHA stimulated PBMCs were significantly higher in atopic NP patients, whereas the differences were not significant between non-atopic NP patients and controls. In addition, the expression of RORc was significantly increased in response to HDM only in atopic NP patients.

Fig. 3. The concentrations of IL-17A in culture supernatants were detected by ELISA. IL-17A levels in HDM + PHA stimulated supernatants were significantly higher in atopic NP patients, whereas there were no significant differences of IL-17A expression between non-atopic group and controls. In addition, in atopic group, IL-17A levels were markedly enhanced in HDM + PHA stimulated supernatants versus that seen in their respective PHA stimulated ones.

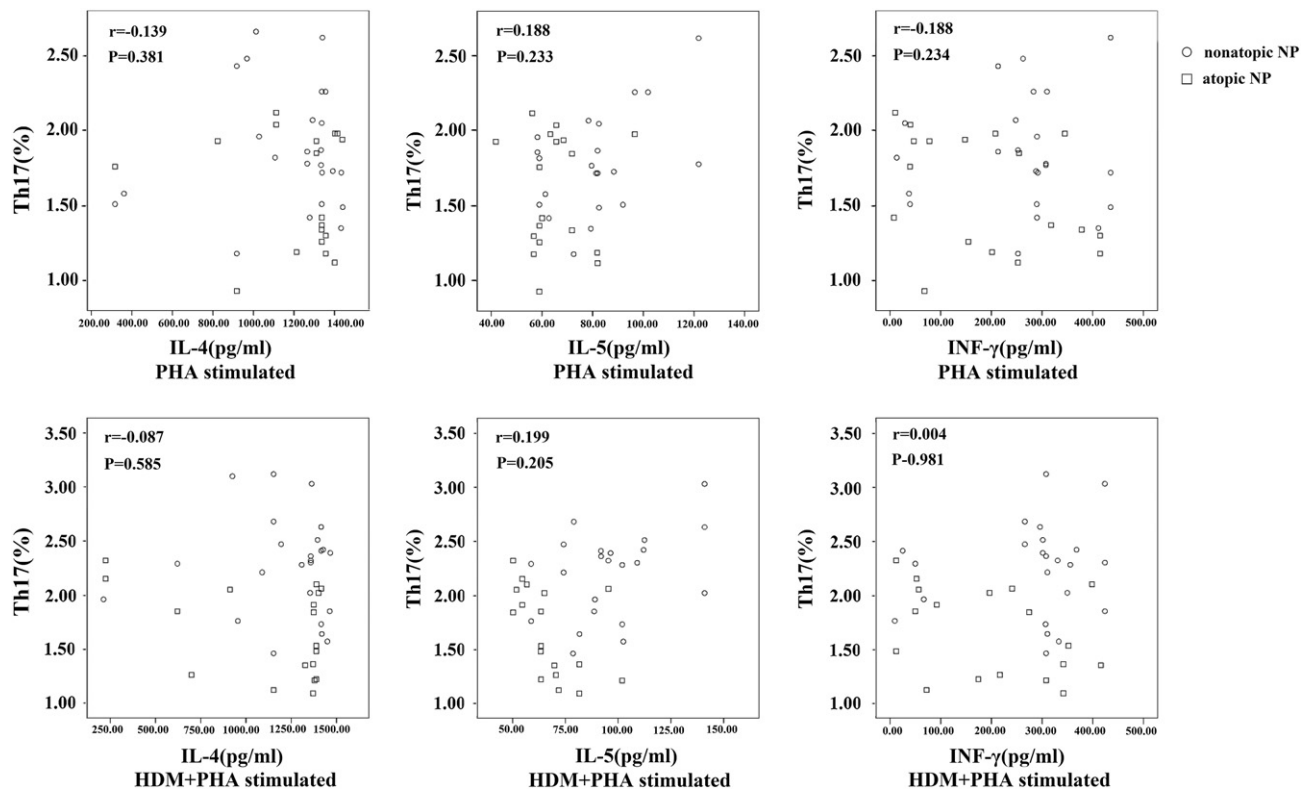


Fig. 4. Spearman analysis of Th17 cells and Th1, Th2 related cytokines. No significant correlations were found between the frequency of Th17 and Th1/Th2 cytokine productions in NP patients.

production by PBMC of unsensitized subjects. Moreover, Albanesi et al. [28] reported that IL-17A was produced by nickel-specific CD4+ T cell clones isolated from the lesional skin of allergic contact dermatitis patients upon stimulation with NiSO₄ in the presence of irradiated autologous PBMC. In addition to a type IV-allergic response, our study showed the Th17 cell immunity in a type I-allergic response.

RORc, an essential transcription factor involved in the generation of Th17 cells, regulates the differentiation and function of Th17 cells in humans [8]. Both the maintenance of cytokine production in human Th17 cells in vitro and human Th17 cell-mediated inflammatory diseases in vivo require the induction of RORc. Here we found that NP patients with atopy had higher expression of RORc at a mRNA level in HDM stimulated PBMCs compared with controls, which supported the higher response of Th17 cells to allergen in the sensitized patients with NP at the transcriptional level. However, the mechanisms that allergen initiates the aberrant transcription of RORc in atopic NP still need further study.

In addition, our results showed that HDM-induced immune response in PBMCs from NP patients was characterized by an increase of both Th1 and Th2 cytokine productions, which was in agreement with the Th1/Th2 imbalance theory and confirmed a predominance of Th2 immunity response in the pathogenesis of NP. To explore the possible interactions between Th17 and Th1/Th2, we analyzed the relationship between Th17 population and Th1, Th2 cytokines. Nevertheless, the frequency of Th17 was not correlated with the levels of Th1, Th2 cytokines. In addition, the response of Th17 cells to allergen was not consistent with the response of Th1 and Th2 cells. Thus, we think that the mechanism of allergen-induced Th17 cells response may be regulated differently from the regulation of Th1 and Th2 immunity in NP.

However, in the present study, our data showed that the frequency of Th17 cells and levels of IL-17A were not significantly increased after allergen stimulation in non-atopic NP patients. This is partially inconsistent with some previous study which demonstrated higher

Th17 cells population and IL-17A production in NP and other chronic inflammatory diseases [14,15,29]. Possibly, this discrepancy is caused by that HDM allergen induces enhanced allergen-specific Th17 response only in the sensitized patients, and Th17 cell plays a more important role in atopic inflammation.

IL-6, a key factor for the generation of Th17 cells from naïve T cells, induces Th17 cell differentiation together with TGF-β. It is also interesting to detect the response of IL-6 after allergen stimulation. Recently, Li Y et al. showed that in renal transplantation, the transplantation recipient with renal dysfunction had the highest level of Th17 cells compared with stable recipient and healthy control, with increased serums IL-6 and IL-17 [30]. We speculate that IL-6 levels in culture supernatants may be significantly increased after allergen stimulation in atopic NP patients, consistent with the results of IL-17A and we will verify this speculation in our future research. In addition, one limitation of our study is that the role of histamine in the context of Th17, Th1 and Th2 regulatory responses was not investigated, which is one of the major mediators of degranulation in allergic reaction. We believe that some more intensive studies will be taken to focus on it.

In conclusion, this study showed significantly increased population and function of peripheral Th17 cells from atopic NP patients in response to HDM. It demonstrates that HDM allergen may induce upregulated Th17 response in NP patients with atopy and atopy may participate in the development of NP as an enhancer through the Th17 immunity. Therefore, we think adequate management of atopy may support the disease management and control in these patients.

Disclosure statement

The authors declare that no financial or other conflicts of interest exist in relation to the content of the article.

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