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

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Summary

Background: Nasal polyposis (NP) is a chronic inflammatory disease of the nasal cavity and sinuses regulated by T cells. Regulatory T (Treg) cells are involved in controlling immune responses and inhibiting the allergen-specific effector cell response. The aim of this study was to evaluate whether NP patients had defects in Treg cells after specific allergen exposure and the possible correlation between atopy and Treg cells.

Methods: Peripheral blood mononuclear cells (PBMCs), isolated from NP patients and controls, were cultured with allergen+phytohemagglutinin (PHA) or PHA stimulation for 48h. The frequency of CD4+CD25+Foxp3+ cells was measured by flow cytometry. The level of Foxp3 was measured by Real-time PCR. Concentrations of Interferon-γ (IFN-γ), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) in culture supernatants were determined by ELISA.

Results: Both atopic and non-atopic NP patients had a significantly decreased frequency of Treg cells and Foxp3 level in allergen stimulated PBMCs, also significantly decreased TGF-β level in culture supernatants. The decrease was even more striking in the atopic group. Also, there were significantly negative correlations between Treg cells and IFN-γ, IL-4, IL-5. Moreover, in the atopic group, allergen stimulation downregulated Treg cells and increased IFN-γ, IL-4, IL-5 levels, while upregulating Treg cells and decreasing IFN-γ, IL-4, IL-5 levels in controls.

Conclusions: Patients with NP have a defective Treg cell response after allergen stimulation which is related to excessive Th1 and Th2 responses to specific allergens. Atopy may increase the impairment of Treg and exacerbate NP through the defective suppression of Treg on Th1 and Th2. *(Asian Pac J Allergy Immunol 2014;32:300-7)*

Introduction

Nasal polyposis (NP) is a chronic inflammatory disease of the nasal cavity and sinuses regulated by T cells. Usually, T cell infiltration in NP presents a Th2 skewed eosinophilic pattern, with high levels of Interleukin-5 (IL-5), eosinophile cationic protein (ECP), eotaxin and Immunoglobulin E (IgE).[1,2] Although insights into the pathophysiology of NP have greatly expanded over the last two decades, the aetiology and pathogenesis of NP is still unclear and the role of atopy in NP is still a controversial issue. Further details still need to be understood.

CD4+CD25+regulatory T (Treg) cells expressing the forkhead/winged helix transcription factor (Foxp3) as a specific marker of Treg cells are believed to play important roles in maintaining immunological tolerance and in controlling immune responses.[3,4] Treg cells are considered to be important for the regulation of allergen-specific responses.[5] They can suppress allergen-induced specific T-cell activation and also suppress effector cell of allergic inflammation such as mast cells, basophils, and eosinophils.[6]

However, several reports suggest that the suppressive activity of CD4+CD25+ Treg cells appears to be affected by various factors, including the type of allergen, allergen exposure and

individual's allergic status.[7, 8] In addition, Wang et al. reported that patients with allergic asthma had an insufficient CD4+CD25+ Treg cell response and an excessive Th2 response to specific allergens.[9] Schaub et al. demonstrated that in the offspring of atopic mothers, Treg cell numbers, expression, and function were impaired at birth.[10] Our previous study also demonstrated that the number of Treg cells was downregulated in both the local tissues and peripheral blood of NP patients, especially in atopic NP patients.[11] Nevertheless, what is the response of Treg after allergen stimulation in NP patients? Is it defective? Are there any correlations between Treg cells and the atopic status of NP patients? Therefore, it is interesting to further explore the function of Treg cells by *in vitro* research.

For a better understanding of the pathology involved in NP, the present study aimed to confirm the decrease in Treg cells in NP patients *in vitro* and to investigate the response of Treg after allergen stimulation in both atopic and non-atopic patients. We also aimed to explore the possible relationship between Tregs and allergen stimulated Th1 and Th2-cytokine production.

Methods

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 computed tomography (CT) scans, according to the current European EAACI Position Paper on Rhinosinusitis and Nasal Polyps and American guidelines.[12,13] Clinical data about patients included age, sex, duration of disease, history of asthma and recurrence of polyps. Symptom scores were assessed according to a visual analog scale (VAS).[14] The preoperative CT scans were graded according to the classification of Lund and Mackay.[15] The preoperative nasal endoscopy scores were graded according to the classification of Lanza and Kennedy.[16] 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.

Determination of atopy

The atopy test was based on SPTs (Allergopharma, Hamburg, Germany). The SPT results were assessed in accordance with the recommendations of the Subcommittee on Allergen Standardization and Skin Tests of the European Academy of Allergy and Clinical Immunology.[17] 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 had a positive SPT response with house dust mite.

Isolation and antigen stimulation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were obtained by standard Ficoll-Hypaque density centrifugation (Company Tianjin TBD, China) 400g for 20 min within 1hour 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, 100u/ml penicillin, 100μg/ml streptomycin and 2mmol/l L-glutamine. Cell viabilities were more than 95% as examined by trypan blue-exclusion assays.

Then, PBMCs were cultured with phytohemagglutinin (PHA, Sigma Chemicals, St Louis, MO, USA), a mitogen which can produce mitosis and specific T-cell activation. For antigen stimulation, PBMCs were incubated with house dust mite extracts (HDM, Allergopharma, Hamburg, Germany).Thus, PBMCs were cultured with HDM (20μg/ml) and PHA (5μg/ml) for 48h at 37℃ 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 were divided into two parts. One part was harvested for flow cytometry assays. The other part was immediately snap frozen and stored at -80℃ and supernatants were stored at -20℃ for further use in real-time PCR and ELISA testing.

Flow cytometric analysis of Treg

For the CD4+CD25+Foxp3+ cells analysis, cells were incubated with FITC-conjugated anti-human CD4 and PE-conjugated anti-human CD25 antibodies. After surface staining, the cells were re-

suspended in fixation and permeabilization solution according to the manufacturer's instructions (BD Biosciences, San Diego, CA) and then stained with PE-Cy5 anti-human Foxp3 antibodies. PE-conjugated mouse PE-Cy5 conjugated rat IgG 2a antibodies were used as isotype controls. All of the antibodies were from eBioscience. Fluorescence profiles were analysed using a FACScan cytometer equipped with CELLQUEST software (BD). The prevalence of Treg cells was expressed by a ratio of CD4+CD25+Foxp3+∕CD4+ T cells. The results was expressed as a percentage of positive cells.

Real-time PCR analysis for Foxp3

mRNA levels of the transcription factor Foxp3 were determined by means of real-time PCR. Total RNA was extracted from tissue samples using TRIzol extraction (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and 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 Foxp3: F: 5¢-GAGAAGCTGAGTGCCATGCA-3¢, R:5¢-AGGAGCCCTTGTCGGATGAT-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.

ELISA analysis for cytokines.

Supernatants were collected after centrifuging cell cultures at 1811 g for 5min at 4°C and stored at -20℃ until analysis. The levels of Interferon-γ (IFN-γ), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) in the supernatants were assayed 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.

Statistical analysis

The software used for statistical analysis was SPSS for Windows ver. 17.0 (SPSS, Chicago, IL, USA). A sample size of 42 NP patients was estimated to give 80% power and the type I error was set to 0.05 (1-sided). The estimations were supported by prior studies from our research group and external data published to assist sample size calculations.[11,18] Data are presented as mean ± standard deviation or medians and interquartile ranges depending on the distribution. For parametric data, 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 non-parametric Mann–Whitney test was used to evaluate statistical significance. The Wilcoxon matched pairs test was used to analyse differences in Treg population and cytokine expression in the HDM+PHA and PHA stimulated cultures. Correlation analysis was performed using Spearman's rank correlation for nonparametric data. Significance was accepted at $P < 0.05$.

Results

Clinical characteristics

The study subjects' clinical characteristics are summarized in Table 1. The endoscopy score and CT score were significantly higher in the atopic group than in the 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.

Table 1. Clinical features of patients with NP

	Atopic NP	Nonatopic 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.

Effect of allergens on the frequency of Treg cells in PBMCs from NP patients

We investigated the effect of allergens on the frequency of Treg cells in PBMCs in both study groups. The percentage of CD4+CD25+Foxp3+ Treg cells in cultural PBMCs with allergen stimulation was significantly lower in both atopic and non-atopic patients with NP (7.65 ± 2.35, 12.11 ± 2.85, $P < 0.01$) as compared with the control group (18.17 ± 1.87). Moreover, the percentage was lower in the atopic group than in the non-atopic group ($P < 0.01$). (Figure 1)

Furthermore, in the atopic group, we detected significantly decreased CD4+CD25+Foxp3+ Treg cells in HDM+PHA stimulated PBMCs (7.65 ± 2.35) as compared with Tregs in PHA stimulated PBMCs (8.33 ± 2.77, $P < 0.01$). By contrast, the frequency of CD4+CD25+Foxp3+ Treg cells in the control group was significantly increased after HDM+PHA stimulation (PHA stimulated: 16.98 ± 1.21; HDM+PHA stimulated: 18.17 ± 1.87, $P=0.012$). However, no significant differences were observed in the non-atopic group, although the percentage of Treg cells was higher after HDM+PHA stimulation (PHA stimulated: 11.70 ± 2.38; HDM+PHA stimulated: 12.11 ± 2.85, $P > 0.05$). (Figure 1)

Foxp3 expression after allergen stimulation

Consistent with the results for the Treg population in PBMCs, Foxp3 expression was significantly downregulated in both the atopic and non-atopic groups ($P < 0.01$), versus control subjects. Furthermore, compared to the non-atopic group, the atopic group showed markedly decreased levels of Foxp3 ($P < 0.01$). (Figure 2)

In the atopic group, the level of Foxp3 in HDM+PHA stimulated PBMCs was significantly decreased as compared with that for PHA stimulated PBMCs ($P = 0.031$). However, Foxp3 expression in the control group was significantly increased after HDM+PHA stimulation ($P = 0.013$). No statistically significant differences were found in the non-atopic group ($P > 0.05$). (Figure 2)

Treg related cytokine levels in culture supernatants

The levels of IL-10, TGF-β were measured from the culture supernatants with allergen stimulation collected from the above experiments. The concentration of TGF-β was significantly lower in both atopic and non-atopic NP patients ($P < 0.01$, $P = 0.01$) than in control subjects. The difference was also significant between the atopic and non-atopic groups ($P = 0.046$). Nevertheless, the same
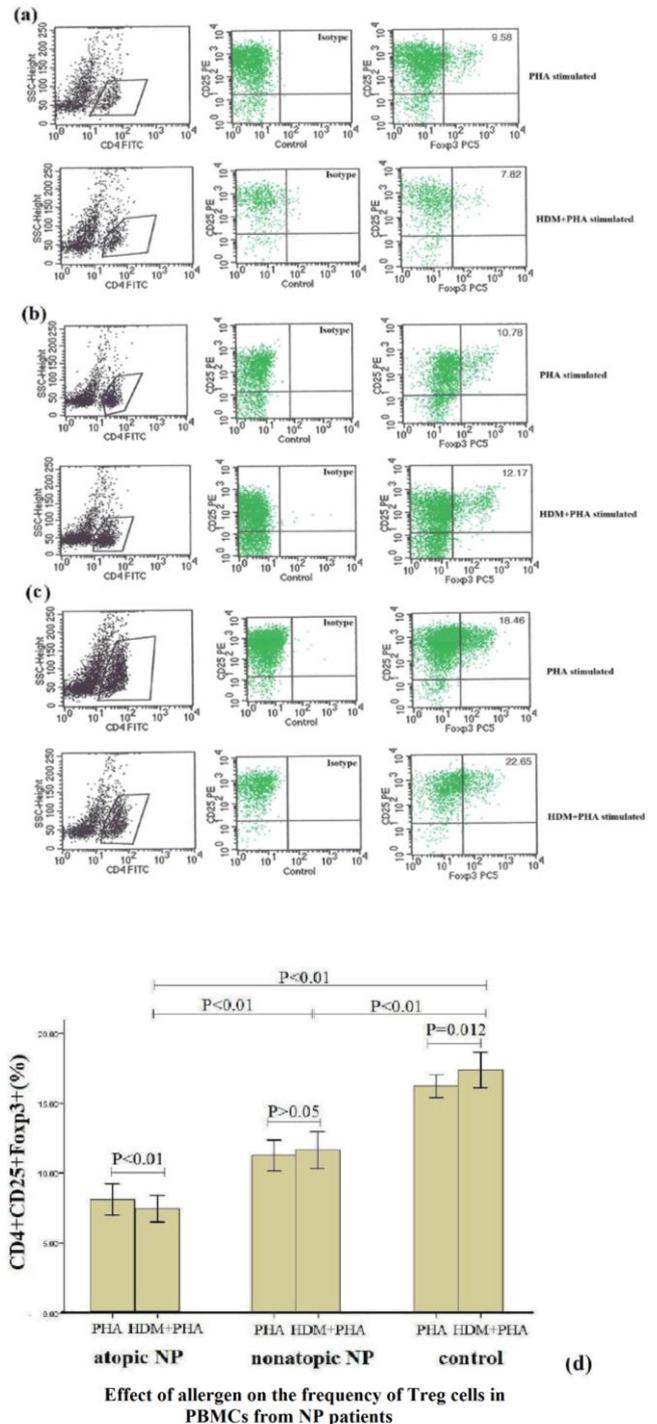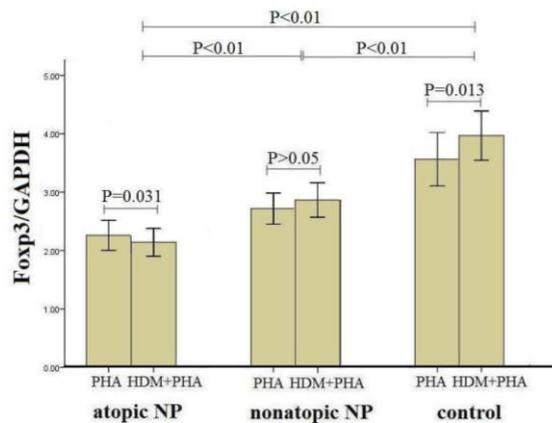

Figure 1. Flow cytometric analysis of CD4+CD25+ Foxp3+ cells in PHA stimulated and HDM+PHA stimulated PBMCs from atopic NP patients (a), non-atopic NP patients (b) and controls (c). (d): The frequencies of Treg cells in stimulated PBMCs. Error bars represent the standard error of the mean.
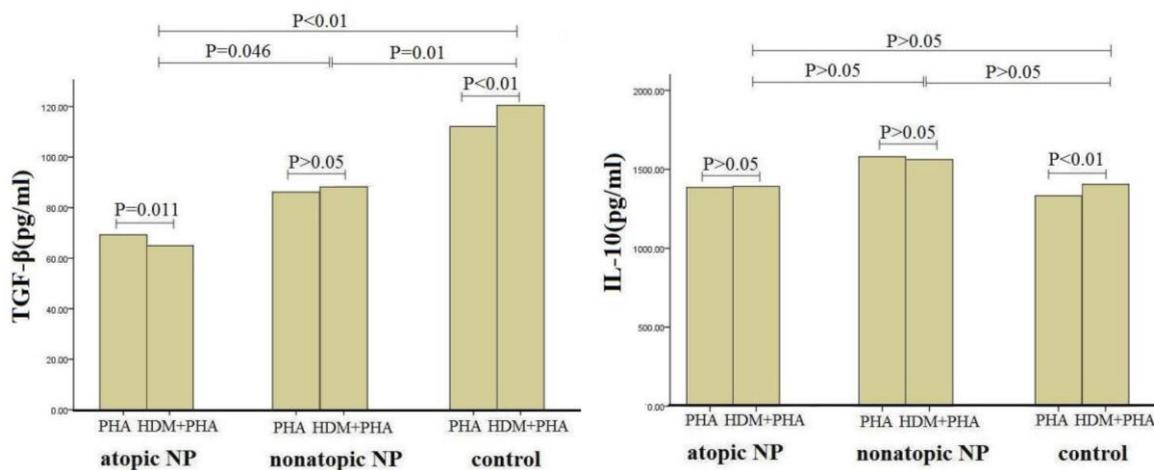
Figure 2. Real-time PCR analysis of Foxp3 levels in stimulated PBMCs from atopic NP patients, non-atopic NP patients and controls. Error bars represent the standard error of the mean.

differences were not found for IL-10 ($P > 0.05$). (Figure 3)

In the atopic group, we detected significantly decreased TGF-β levels in HDM+PHA stimulated supernatants as compared with those in PHA stimulated ones ($P = 0.011$). By contrast, the level of TGF-β was significantly increased in the control group ($P < 0.01$). However, the differences in the non-atopic group were not significant ($P > 0.05$). Moreover, the levels of IL-10 in the control group were found to be significantly increased ($P < 0.01$). (Figure 3)

Th1/Th2-related cytokine levels in culture supernatants

In atopic NP patients, the stimulation of HDM+PHA induced significant increases of IFN-γ, IL-4, IL-5 levels in culture supernatants when compared with those in PHA stimulated ones ($P = 0.045$, $P < 0.01$, $P < 0.01$). By contrast, the stimulation of HDM+PHA induced significant decreases of all three cytokines in controls ($P = 0.037$, $P = 0.037$, $P = 0.028$). However, the PBMCs from non-atopic NP patients responded only with a statistical increase of IL-5 levels ($P = 0.028$). (Figure 4)

Correlations between Treg and Th1/Th2 cytokine secretion

In order to assess possible interactions between Treg cells and Th1/Th2 in NP patients, we examined the correlations for Treg cells with Th1/Th2 cytokines in both the HDM+PHA stimulated group and the PHA stimulated group. As shown in Figure 5, there were significant negative correlations between the frequency of Treg cells and the concentrations of IFN-γ, IL-4, IL-5($r = -0.525$, $r = -0.670$, $r = -0.519$, $P < 0.01$) in PHA stimulated group. Negative correlations were also found in HDM+PHA group ($r = -0.445$, $r = -0.722$, $r = -0.574$, $P < 0.01$).

Discussion

This study extended our previous study on Treg in NP. We demonstrated that the frequency of CD4+CD25+Foxp3+ Tregs in allergen stimulated
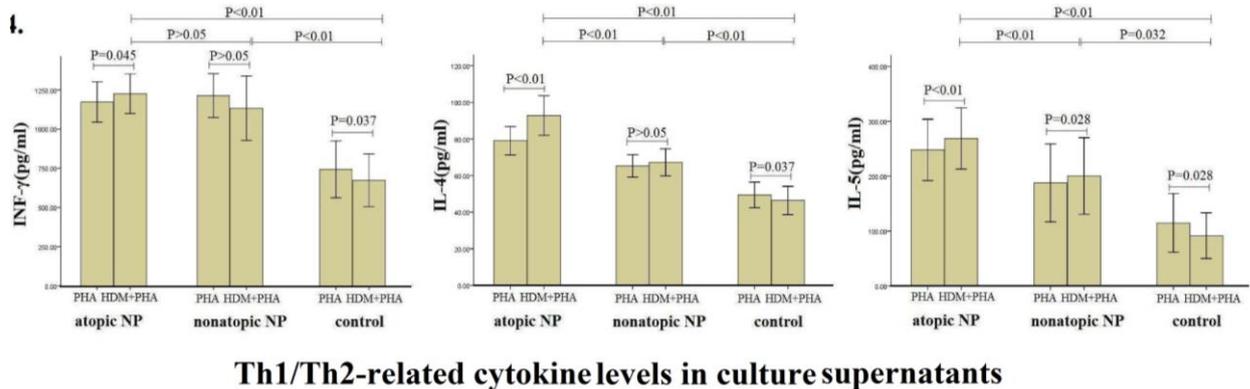

Figure 3. The levels of IL-10, TGF-β in the culture supernatants with allergen stimulation were measured by ELISA. HDM stimulation downregulated the level of TGF-β in atopic group, but upregulated the levels of TGF-β, IL-10 in controls.

Figure 4. The levels of IFN-γ, IL-4, IL-5 in the culture supernatants with allergen stimulation were measured by ELISA. HDM stimulation unregulated the levels of IFN-γ, IL-4, IL-5 in the atopic group, but downregulated them in controls. Error bars represent the standard error of the mean.

PBMCs was significantly lower in both atopic and non-atopic NP patients. In the atopic group, the decrease was even more striking. In addition, we showed that PBMCs from NP patients had an unregulated ability to generate both Th1 and Th2 cytokines and allergen stimulation increased the levels of IFN-γ, IL-4 and IL-5 in culture supernatants. Furthermore, there were significantly negative correlations between the frequency of Treg and the concentrations of IFN-γ, IL-4, IL-5 in NP patients. These results indicate that the response of Treg after allergen stimulation is defective in NP patients, which may play a crucial role in the pathogenesis of NP. Moreover, atopy is an important factor in NP, which may increase the impairment of Treg and finally aggravate NP through the defective suppression of Treg on Th1 and Th2.

Treg cells are believed to maintain immunological tolerance and to control immune responses.[19] Several studies in humans have demonstrated that in healthy individuals, if an immune response to common environmental allergens is detectable, allergen specific Treg cells represent the dominant subset.[20-22] Pérez Novo et al. showed that staphylococcal superantigens induced both Th1 and Th2 pro-inflammatory responses in patients with NP and asthma, and the nature of this response might be linked to a basal deficiency of Foxp3.[23] Li et al. demonstrated that Foxp3 is downregulated in NP and intranasal steroids attenuate the chronic inflammatory response by enhancing the expression and function of Foxp3 in NP.[24] In addition, Lin et al. found that

peripheral blood CD4+CD25+ T cells from house dust mite-allergic asthmatic children had a reduced suppression capacity.[25] In the present study, our data demonstrated that the frequency of Treg cells in HDM stimulated PBMCs was significantly lower in both NP patient groups. Allergens induced a significant decrease of Treg cells in PBMCs from atopic NP patients and an insufficient increment in the non-atopic group. However, allergen stimulation significantly increased the frequency of Treg in controls. These indicate that induction of Treg cells may have an important role in regulating the immune response to allergens in healthy subjects and this mechanism is impaired in NP patients, especially in atopic NP patients.

In the present experiment, our data showed that the allergen-induced immune response in PBMCs from NP patients was characterized by an upregulation of both Th1 and Th2 pro-inflammatory cytokine production. Furthermore, Treg cells from PBMCs of NP patients were found to be negatively correlated with Th1 and Th2 cytokine secretion. These data suggest that the increased secretion of IFN-γ, IL-4 and IL-5 may be mainly caused by the defective suppression of Treg cells on Th1/Th2 balance. Consistent with this theory, Reefer et al. reported that CD4+CD25+ cells isolated from atopic dermatitis patients which expressed Foxp3 failed to suppress Th2 cytokine production *ex vivo*.[26] Thunberg et al. showed that Treg cells from non-allergic controls were able to suppress birch pollen stimulated effector cell proliferation; whereas CD4+CD25+ cells from allergic patients failed to
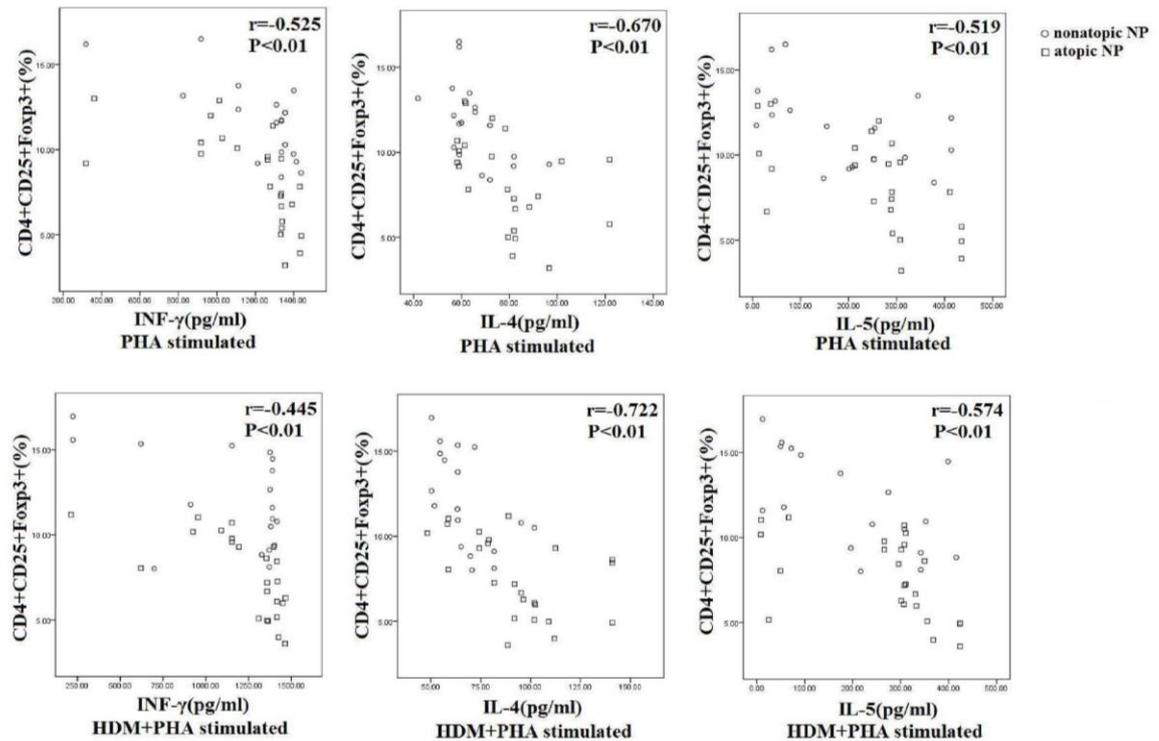
Figure 5. Spearman correlations of Treg cells and Th1, Th2 related cytokines. IFN-γ, IL-4 and IL-5 concentrations negatively correlated with the level of peripheral Treg cells in both the HDM+PHA stimulated group and the PHA stimulated group. ○: nonatopic NP, □: atopic NP.

suppress birch pollen stimulated proliferation and Th2 cytokine production.[27]

However, our results regarding IFN-γ concentration partially conflict with those from previous studies. Thunberg et al. demonstrated that CD4+CD25+ Treg cells from both allergic patients and controls could suppress the production of Th1 cytokines.[28] Yamanishi et al. found that in pollen-allergic patients, Treg cells predominantly suppressed Th1 responses rather than Th2 responses.[29] Our results showed that the IFN-γ level in supernatants of both atopic and non-atopic NP patients was significantly increased after allergen stimulation, indicating that impaired Tregs from NP patients fail to suppress not only Th2 but also Th1 cytokine production.

One limitation of our study is that a control group without the stimulation of PHA and allergen was not evaluated. Furthermore, the local specific response in polyp tissue was not evaluated. This may help to provide more complete and comprehensive results. We believe that more intensive *in vitro* studies are needed to focus on them.

In summary, we have demonstrated that allergen stimulation can decrease the frequency for Treg cells in PBMCs from both atopic and non-atopic NP patients and increase the levels of Th1 and Th2 cytokines in culture supernatants, which was more pronounced in NP patients with atopy. Furthermore, there were significant negative correlations between Treg cells and Th1 and Th2 cytokines. These data support a critical role for Treg cells in immune responses to allergens and confirm the defective suppression of Th1/Th2 by Treg cells from NP patients *in vitro*. In addition, these data indicate that atopy may aggravate NP by promoting the impaired suppression of Treg cells. Therefore, we think adequate management of allergies and atopy may promote disease control in NP patients.

Conflicts of interest

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

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