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Anti-inflammatory Mechanisms of IL-38 in Chinese Patients with Allergic Rhinitis

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ABSTRACT

Background: T-helper 17 (Th17) cell response is engaged in the onset of allergic rhinitis (AR). Moreover, interleukin (IL)-38 is thought to be involved in inhibiting cytokine secretion in the Th17 pathway.

Objective: To evaluate the regulatory function of IL-38 on abnormal Th17 responses in Chinese patients with AR.

Methods: Forty-five participants, divided into an AR group (n=25) and a control group (n=20), were recruited for the study. In addition, the expression of IL-38 and Th17-related cytokines was measured as well as the Th17 cell count in participants. By implementing recombinant IL-38 (rIL-38), the intervention of human peripheral blood mononuclear cells (PBMCs) was performed. Then, flow cytometry, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA) were used to detect theTh17 milieu.

Results: The expression of IL-38 in the AR group notably reduced compared with that in the control, whereas Th17 cell frequency and the expression levels of its transcription factor (*RORC*) and cytokines (IL-17A and IL-23) increased. The differentiation and immune function of Th17 cells in PBMCs were inhibited by rIL-38.

Conclusion: Th17 responses are inhibited by IL-38 in patients with AR. Therefore, the obtained findings indicate that IL-38 is a potential therapeutic target for Chinese patients with AR.

Keywords: Allergic Rhinitis, Interleukin-17, Interleukin-38, Peripheral Blood Mononuclear Cells, Th17 Cells

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INTRODUCTION

As a frequently occurring ailment in China, allergic rhinitis (AR) is characterized by nasal mucosal inflammation that involves a variety of immunocompetent cells and cytokines [1]. Patients with AR can experience symptoms such as nasal itching, sneezing, hypersecretion, and swelling of the nasal mucosa, which can result in a decline in adult work energy, an increase in learning disabilities, and school absenteeism in children [2, 3]. Moreover, it may worsen secondary disorders, such as asthma, sinusitis, or teeth development disorders, under uncontrolled conditions [4]. Therefore, research on the pathogenesis of AR has become a crucial topic in academic circles.

Patients with AR have shown infiltration of cells expressing interleukin (IL)-17, which is localized in the nasal mucosa, and an abnormal increase in IL-17 expression in peripheral circulation [5]. Li et al. reported the pivotal role of IL-17 during the development process in a mouse AR model [6]. However, the relevant regulatory mechanism of the T-helper 17 (Th17) cell response in Chinese patients with AR has not been fully elucidated.

As part of the IL-1 superfamily, the IL-36 cytokine family is composed of multiple cytokines (IL-36α, IL-36β, and IL-36γ), which can also activate the IL-36 receptor (IL-36R) [7]. On the contrary, IL-38 plays an antagonistic function through competitive binding to IL-36R [8]. Chi et al. found that Th17 cell differentiation could be enhanced by IL-36 signaling, with further research suggesting that IL-17 also promotes IL-36 expression, which could form an auto-amplification loop as IL-17 is a cytokine involved in the Th17 pathway [9, 10].

As a cytokine with an antagonistic function, IL-38 is involved in inhibiting Th17 cytokine production and angiogenesis in a mouse model of arthritis [11]. Furthermore, secukinumab, an antibody that can antagonize IL-17A, could upregulate serum IL-38 expression in patients with psoriasis [12]. The above findings confirm the inverse antagonistic function of IL-38 in Th17-related inflammatory diseases. Thus, we hypothesize that the anti-inflammatory role of IL-38 may also exist in the inflammatory disease AR, in which the function of Th17 is critical. In this study, we investigate whether IL-38 is abnormally expressed in Chinese AR patients, and evaluate its regulatory function in the Th17 milieu.

MATERIALS AND METHODS

Subjects

On the whole, twenty-five Chinese patients with AR, all with a history of allergy for at least two years, as well as 20 patients with simple nasal septum deviation without AR, were recruited for this study as the experimental group and the healthy controls (HCs), respectively. Specimen collection was performed from October 2020 to January 2021 at the Department of Otolaryngology, the First Affiliated Hospital of Chongqing Medical University. The diagnostic criteria for the disease were based on the criteria of the Initiative on Allergic Rhinitis and its Impact on Asthma [13]. To assess the allergic status, the skin prick test was used, and the results were defined as positive based on criteria used in a previous study [14]. The exclusion criteria were presented: patients with chronic diseases of the heart, lung, and digestive system, as well as other chronic diseases including asthma, malnutrition, cystic fibrosis, drug-induced rhinitis, or occupational rhinitis. Patients undergoing any additional complications, and those with a long history of using corticosteroids, antihistamines, or leukotriene receptor antagonists, were also excluded from the study. The controls were expected to have both an allergy-free history and a negative skin prick test.

The study design (approval number: 2020-202055) was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University , ensuring that it was executed in compliance with the Declaration of Helsinki. Before being enrolled in the current work, all subjects signed an informed consent form. In addition, no adverse reactions were found in any of the participants.

Sample and Blood Collection

Inferior turbinate tissue was collected from patients with AR and HCs, each with nasal obstruction refractory to medical therapy and hypertrophy of the inferior turbinate after turbinectomy, respectively. Before paraffin embedding, nasal mucosa specimens were soaked in 10% buffered formalin and prepared for immunohistochemical analysis.

From 06:00 a.m. to 08:00 a.m., fasting

venous blood samples (8 mL) were gathered from the research subjects for experimental analysis. Heparin anticoagulant tubes were used for the collection of the experimental sample, which was separated after centrifugation (1,000 *g*, 4 °C) for a quarter and subsequently frozen for follow-up analysis.

Separation of Peripheral Blood Mononuclear Cells (PBMCs)

At a temperature of 4 °C, the partial blood sample in the heparin anticoagulant tube was stratified using density gradient centrifugation on a lymphocyte isolation medium (MP Biomedical, LLC, Santa Ana, CA, USA) within the period of half an hr. Then, we performed PBMC collection at the interface between the plasma and lymphocyte separation fluid. In addition, PBMC was cryopreserved at 2×10^6 cells/mL in RPMI-1640 media (Sigma-Aldrich, St. Louis, MO, USA) with a 10% concentration of dimethyl sulfoxide (Sigma-Aldrich) and 40% concentration of fetal bovine serum (Biowest, Nuaillé, France) and also preserved at −160 °C until flow cytometry analysis was carried out.

Th17 Cell Detection by Flow Cytometry in PBMCs

The viability of PBMC was shown to be over 99% after thawing. Then the concentration of PBMC was adjusted to 1.5×10^6 cells/mL in RPMI 1640 medium (Sigma-Aldrich), added with 10% inactivated fetal bovine serum (Biowest) as well as 1% Antibiotic/Antimitotic Solution 100× (Sigma-Aldrich). Cells were seeded in 24 well plates, then activated with phorbol myristate acetate (PMA, 50 ng/ml; Alexis Biochemicals, San Diego, CA) and ionomycin (1 μM; Sigma, USA) to promote the intracellular production of IL-17 with GolgiStopTM (BD Bioscience, San Jose, CA, USA) for 5 hrs. In line with the instruction of the manufacturer, we stained cells for surface markers and intracellular cytokines. The cells were subject to incubation for half an hr. at 4 °C with the surface marker antibodies including APC (Allophycocyanin)-anti-human CD4 monoclonal antibodies from BioLegend (San Diego, CA, USA). Besides, the cells were rinsed twice with 0.9% saline solution (SS) and also fixed with 4% paraformaldehyde for 20 min. Subsequently, the cells were washed twice with SS and permeabilized with Perm/Wash Buffer (BD Bioscience) for a quarter and stained with PE (phycoerythrin)-anti-human IL-17 monoclonal antibodies from BioLegend (San Diego, CA, USA). Moreover, appropriate isotype controls were contained in all experiments. After resuspension of the cells in 300 μL of phosphate buffer saline, quantitative analysis of Th17 cells was performed using a fluorescence-assisted cell sorting flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and CellQuest software (Becton-Dickinson, Franklin Lakes, NJ, USA). In addition, $CD4^+$ $IL\text{-}17^+$ cells were adopted for representing Th17 cells [15].

Th17 Cell Differentiation and Cytokine Production in PBMCs After Treatment

The steps indicated in section 2.4, before the addition of anti-CD3 and anti-CD28, were first performed. Subsequently, to differentiate Th17 cells, rhIL-6 (20 ng/mL, Peprotech, East Windsor, NJ, USA), rhTGF-β (1 ng/mL, R&D), anti-IL-4 (10 μg/mL, BioLegend, San Diego, CA, USA), and anti-IFN-γ (10 μg/mL, BioLegend, San Diego, CA, USA) were added for five days. Finally, recombinant IL-38 or IL-17A (1 mg; R&D Systems, Minneapolis, MN, USA), which had varying concentrations, were added to the medium. Moreover, NF-κB inhibitor (ProSpec, East Brunswick, NJ, USA) and SB203580 (MAPK inhibitor) (Beyotime, Shanghai, China) were also added [16]. To identify IL-23 and IL-17A expression in the supernatant, we conducted an enzyme-linked immunosorbent assay (ELISA) in this study. Simultaneously, the Th17 percentage after treatment was estimated using flow cytometry.

Quantitative Real-time Polymerase Chain Reaction (PCR)

Using the Trizol reagent (Life

Technologies, Carlsbad, CA, USA), the total RNA was isolated from PBMCs. By adopting a cDNA kit (Qiagen), cDNA was acquired. PCR amplification was completed with the use of SYBR Green Universal PCR Master Mix (Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed to normalize the results. The primers for IL-38, RORC, and GAPDH were presented including IL-38 sense, 5'- CCC CAT GGC AAG ATA CTA C-3'; antisense, 5'-CCT CTT CTG TCT CCA CAC AT-3'; *RORC* sense, 5'-GGC TTT AAG TTA TTT ATG TA-3'; antisense, 5'-CCT GTA TTT ATT TGA GCT ATT-3'; GAPDH sense, 5'-AGC CAC ATC GCT CAG ACAC-3, antisense, 5'-GCC CAA TAC GAC CAA ATCC-3' [16, 17].

ELISA

The detection of IL-38 and Th17-related cytokine (IL-17A and IL-23) levels in serum, which was performed in line with the instructions of the manufacturer, was completed with the application of an ELISA kit (R&D Systems, Minneapolis, MN, USA).

Immunohistochemical Analysis

For immunohistochemical examination of IL-38 protein, all paraffin sections were mounted onto microscope slides, routinely dewaxed, and dehydrated with gradient alcohol. Then, endogenous peroxidase was removed with 3% H_2O_2. Finally, the sections were sealed with 10% normal sheep serum. Further experimental operations were performed according to the technical manual of the Histostain IHC SP kit (Invitrogen, Carlsbad, CA, USA), including the steps for DAB staining, hematoxylin re-staining, dehydration, xylene transparent, and medium gum sealing. The working solution concentration of the primary antibody (1 mg/mL polyclonal rabbit anti-IL-38; Abcam, Cambridge, England) and secondary antibody (biotinylated goat anti-rabbit secondary antibody, 2 mg/mL; Abcam, Cambridge, England) was 1:100. Moreover, phosphate buffer solution was used as the negative control primary antibody. To observe the results after staining, a cell counting grid was installed in the eyepiece on one side of a microscope (Olympus Corporation, Tokyo, Japan), and the five high-power fields (400× magnification) with the most positive cells were selected; the mean value was then recorded.

Statistical Analysis

For the analysis of all the data from this study, the one-way analysis of variance or t-test was used, depending on whether statistical significance needed to be calculated between three or more samples or between two samples, respectively. For comparison between each treatment and the control, Dunnett's multiple comparisons test was performed. Research data was denoted to be the mean±standard error of the mean. In addition, statistical Product and Service Solutions 22.0 was used for all statistical analyses. To analyze the correlations between different indices, Spearman's rank correlation analysis was employed. Correlations were defined as either negative or positive according to the r-value. The r-value was also used to define the strength of the correlations as follows: 0.0–0.3, weak; 0.3–0.6, moderate; 0.6–1.0, strong. In addition, the level of statistical significance was shown to be $P<0.05$.

RESULTS

Clinical Characteristics of the Study Participants

Table 1 presents the demographic features of the patients. Compared with the scores in the control group, the symptom scores of the AR group presented significant elevation ($P<0.05$). There existed no obvious difference in average age or sex distribution between the two groups ($P>0.05$).

Th17 Cell Levels and Their Transcription Factor in AR

Compared with the levels in HCs

Table 1. Demographic characteristics of patients with allergic rhinitis (AR)

	AR group	Control group
Number	25	20
Sex (male/female)	14/11	11/9
Age (years)	28.1±4.3	27.9±3.1
Allergen category (%)		
House dust mites	15 (60)	0
Pollen	6 (24)	0
Mixed allergens	4 (16)	0
Symptom score (T5SS)	8.2±1.4*	2.1±0.7

*Compared with the controls, P<0.05

(0.63±0.22%), Th17 cell levels significantly increased in patients with AR (1.61±0.30%) (P<0.05, Figure 1). Moreover, the expression of *RORC* mRNA in the AR group showed a trend similar to that of Th17 (3.06±0.58 *vs* 1.43±0.41). (P<0.05, Figure 2a).

mRNA and Protein Expression Levels of IL-38 and Th17 Cytokines in AR

According to the obtained results, the levels of IL-38 mRNA and protein in the AR group (2.68±0.47 and 116.30±12.90 pg/mL, respectively) notably lowered compared with those in the HC group (6.21±0.94 and 252.66±44.95 pg/mL, respectively; P<0.05, Figure 2b). The levels of Th17 cytokines (IL-17A and IL-23) in the AR group were notably higher (104.34±22.35 and 65.76±17.83 pg/mL, respectively), than those in the HC group (68.07±15.31 and 15.22±4.22 pg/mL, separately; P<0.05, Figure 2c). A similar downward trend was discovered for local

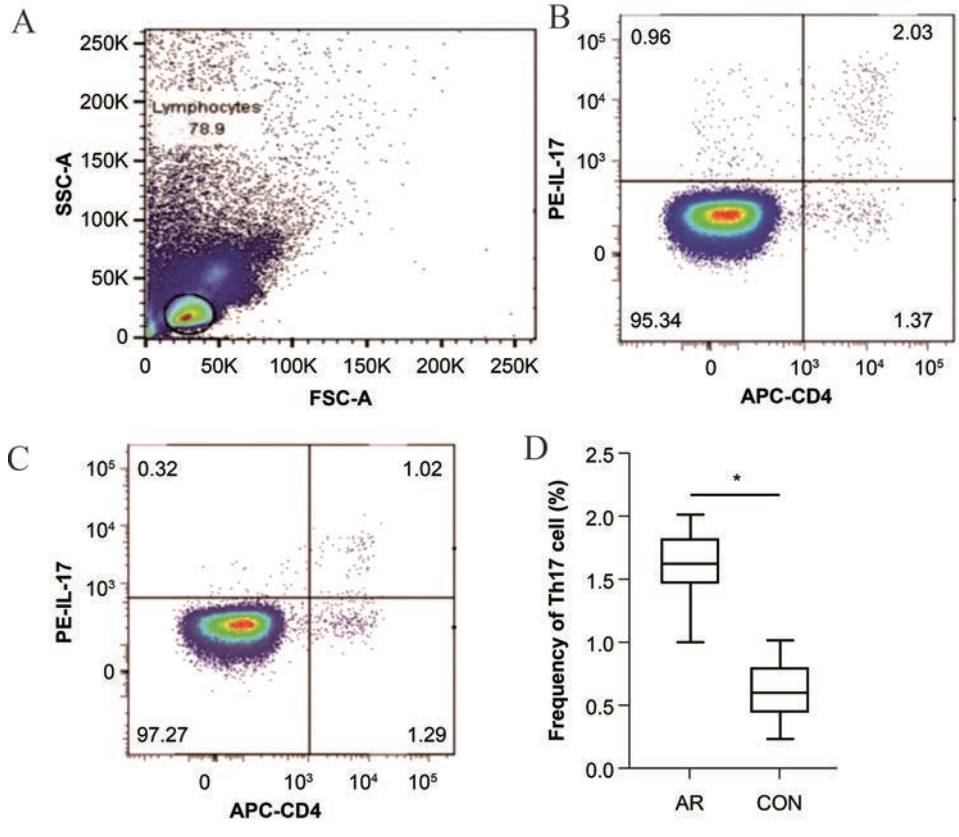

Figure 1. Level of Th17 cells in peripheral blood mononuclear cells (PBMCs) for each group. A: Lymphocytes were gated from PBMCs, B: Th17 cells ($CD4^+IL\text{-}17^+$ T lymphocytes) in the AR group were further evaluated by flow cytometry, C: Th17 cells ($CD4^+IL\text{-}17^+$ T lymphocytes) in the healthy controls (HCs), D: The proportion of Th17 cells in each group. *P<0.05 vs. HCs

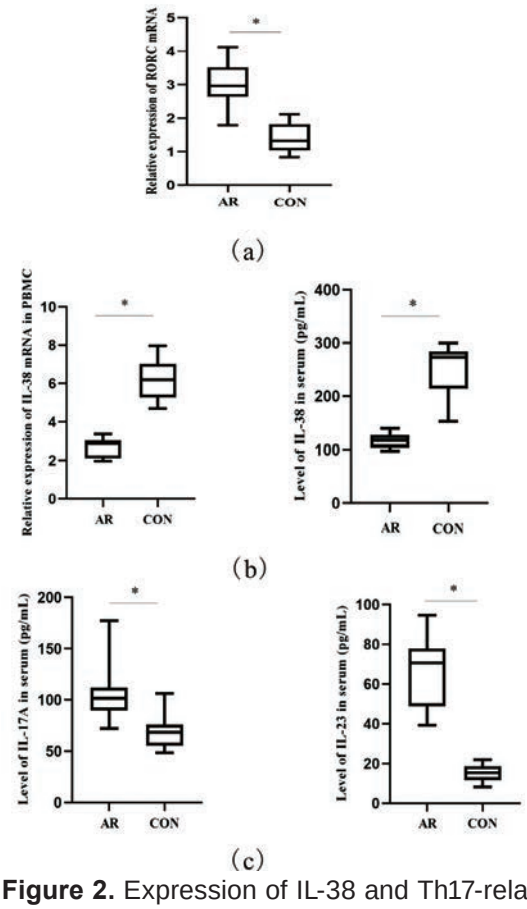

Figure 2. Expression of IL-38 and Th17-related indices. (a) Level of *RORC* mRNA in the PBMCs of each group; (b) Levels of IL-38 in each group; (c) Th17 cytokines levels in the serum of each group. *P<0.05 vs. HCs

expression of IL-38 in the nasal mucosa (4.78±1.17 *vs* 14.35±3.73; P<0.05, Figure 3). Moreover, we observed a negative correlation between the levels of IL-38 and Th17-related indices (Th17 cell, *RORC*, and cytokines) in patients with AR (P<0.01, Figure 4). This finding suggests that IL-38 may have a negative regulatory function on Th17.

IL-38 and Related Signal Pathway Inhibition of Th17 Cell Differentiation and Cytokine Expression by PBMCs

After the intervention of rIL-38, the frequency of Th17 cells decreased more than that after the PBS intervention (1.61±0.30 , P<0.05, Figure 5) and the frequency of Th17 cell after the intervention of high concentration rIL-38 (10 μg/mL) was significantly lower than that after the intervention of low concentration rIL-38 (1 μg/mL) (0.61±0.34 *vs* 1.03±0.34; P<0.05, Figure 5). Moreover, the frequency of Th17 cell after the intervention of rIL-38 (1 μg/mL) plus NF-κB inhibitor or SB203580 (MAPK inhibitor) (1.46±0.28 and 1.49±0.27) was found to be not notably lower than that after the PBS intervention (P>0.05, Figure 5). In the meantime, the expression of

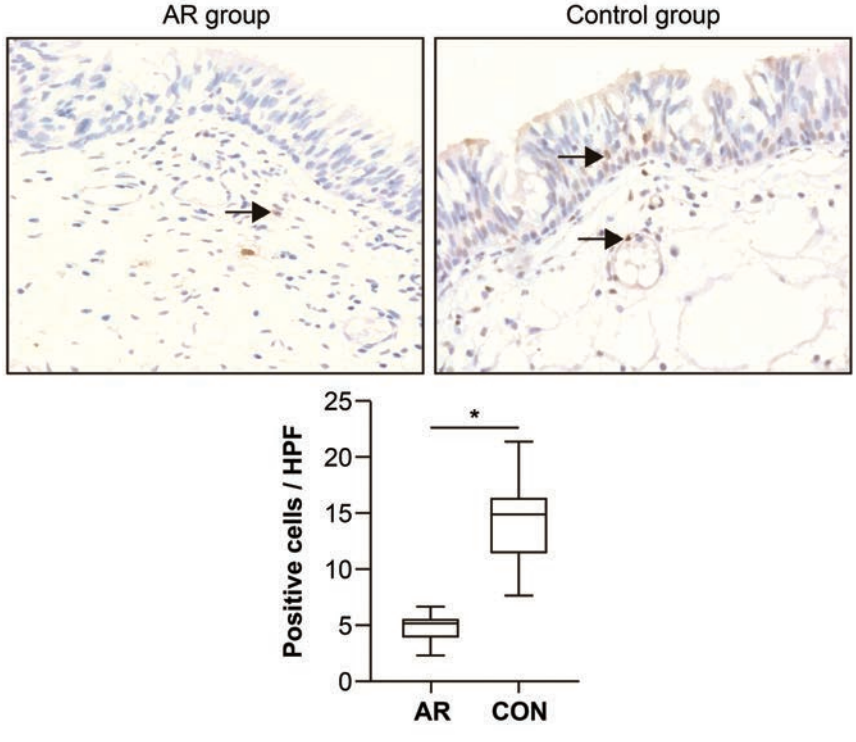

Figure 3. Levels of IL-38 in the local mucosa of each group. Arrows refer to positive cells. *P<0.05 vs. HCs

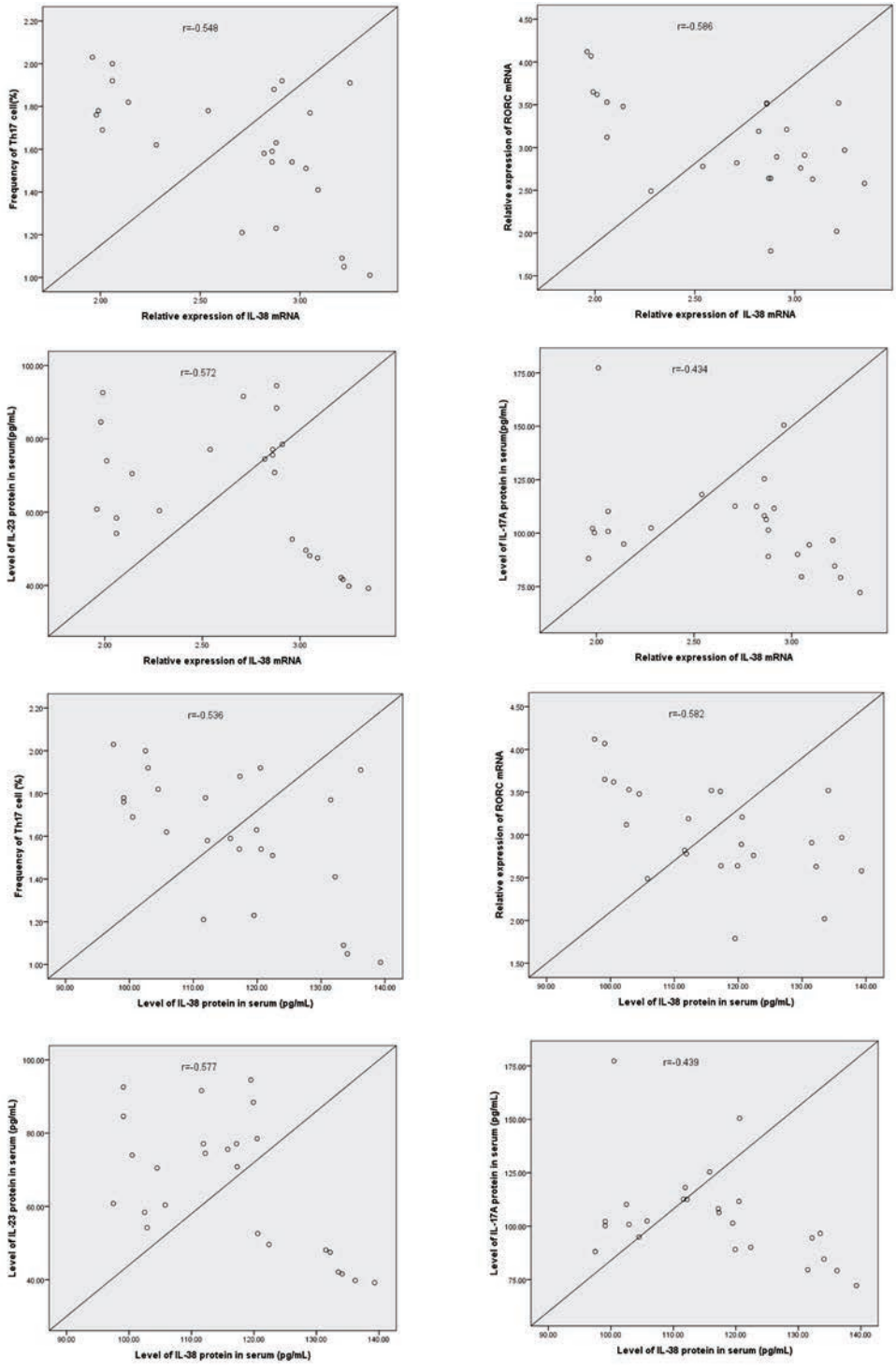

Figure 4. Correlation analysis between IL-38 and Th17-related indices in patients with AR

Th17-related indices (RORC, IL-17A and IL-23) in PBMCs from the AR group showed the same trend after the intervention (Figure 6a). The results also indicate that the intervention of IL-17A could induce IL-38 production, and the intervention results showed dose-response relationship (186.61±25.22 pg / mL *vs* 152.71±20.29 pg / mL *vs* 116.3±12.90 pg/mL; $P<0.05$, Figure 6b).

DISCUSSION

AR refers to an immune inflammatory disease characterized by adaptive and innate immune system activation; thus, Th cells are closely

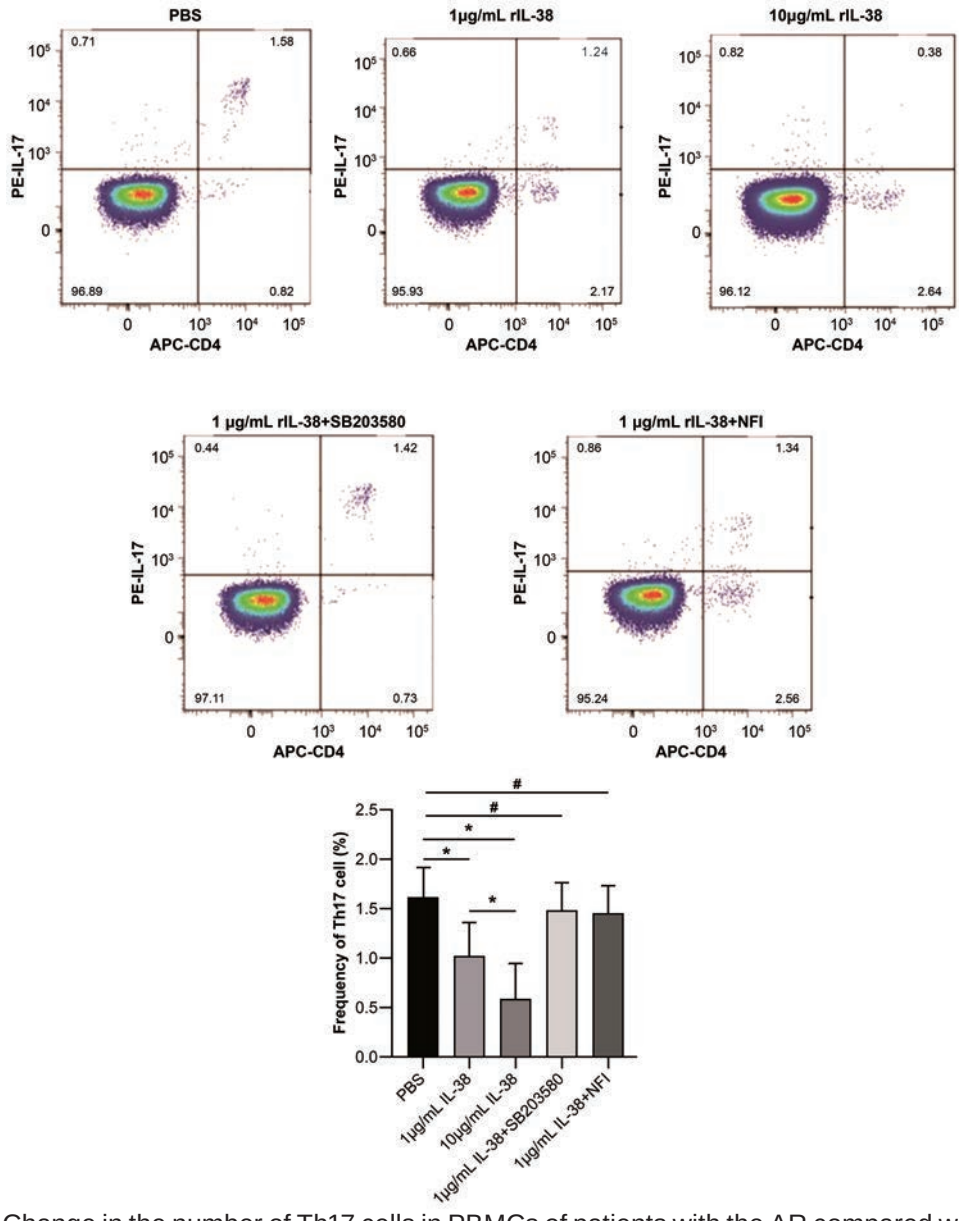

Figure 5. Change in the number of Th17 cells in PBMCs of patients with the AR compared with the HCs after intervention. *P<0.05 vs. phosphate-buffered saline (PBS), #P>0.05 vs. PBS

engaged in the pathogenesis of the disease [18, 19]. Th17 is one of the immune cells related to the pathogenesis of AR [20]. Its main effector, IL-17, which can impact airway epithelial cells as well as other inflammatory cells, can promote the recruitment of macrophages and neutrophils in local inflammation by stimulating these cells to secrete inflammatory cytokines, chemokines, as well as matrix metalloproteinases [21]. As a key mediator, the IL-23/IL-17 axis has been revealed as the main pathway for Th17 cells in the pathogenesis of AR [22]. Furthermore, IL-17 knock-down mice cannot be induced to have AR and show no nasal mucosal symptoms or histopathological changes [23], suggesting the crucial pathogenic role of IL-17 during the occurrence and development stage of AR. In this study, we observed abnormal expression of Th17 cells and effector factors in patients with AR, which confirmed an increased Th17 response in AR. Moreover, the IL-23/IL-17 axis, as a direct effect pathway, exerts a vital pro-inflammatory role in the pathogenesis of AR. However, to the best of our knowledge, the specific regulatory mechanism of the high expression of Th17 cells and IL-17 in AR is still unclear.

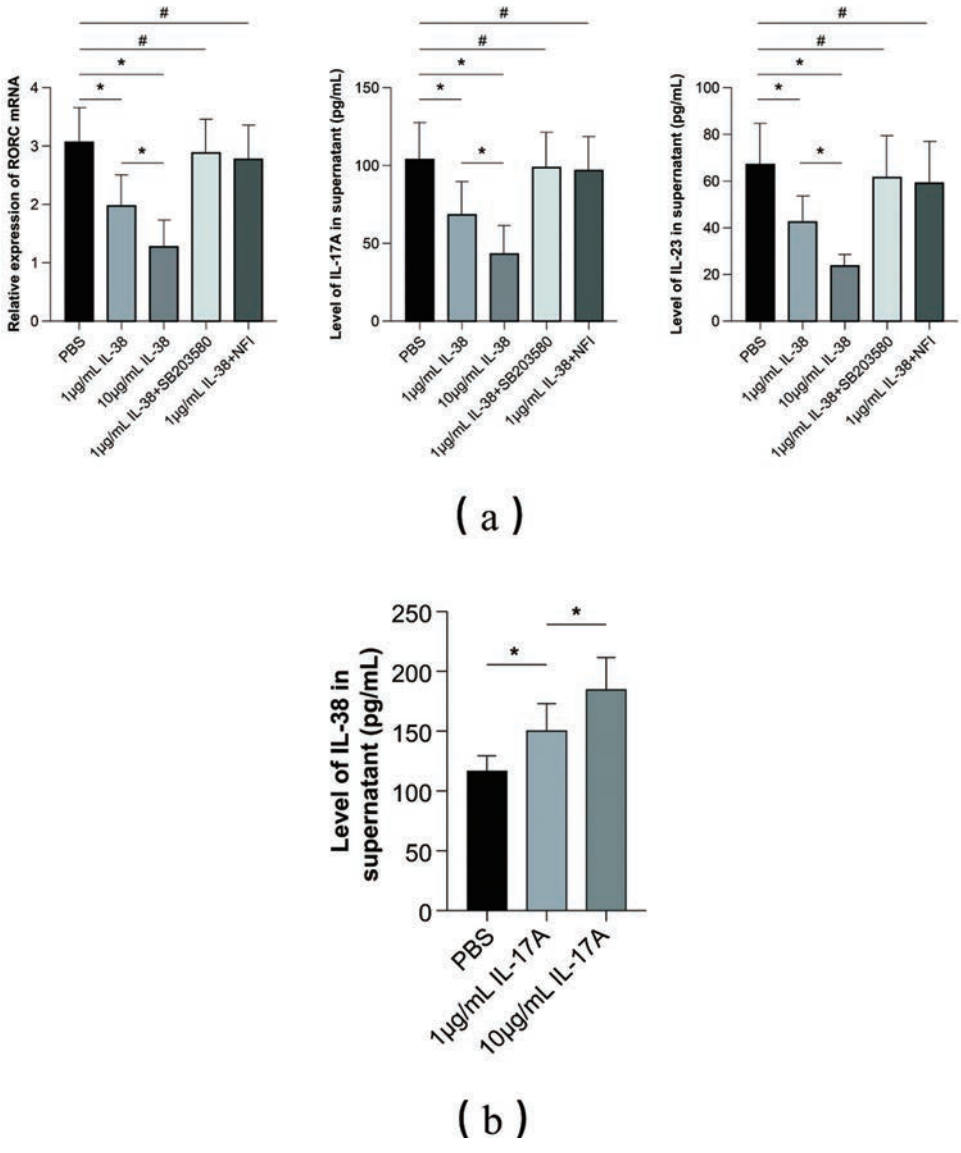

Figure 6. Expression of experimental indexes in the intervention experiment. (a) Changes in Th17-related indices in patients with the AR after intervention; (b) Level of IL-38 in the supernatant of patients with the AR after the intervention with rIL-17. *P<0.05 vs PBS, #P>0.05 vs. PBS

As a newly discovered member of the IL-1F superfamily, IL-38 is present in various tissues, and its competitive antagonism to IL-36R can further block the effect of the IL-36R-related signaling pathway [24]. A previous study found that the IL-36R pathway participates in the activation process of the IL-23/IL-17 axis [25]. Based on the above results, it is reasonable to suppose that weakening of the antagonistic effect of IL-38 on the IL-23/IL-17 axis results in the abnormal differentiation of Th17 cells in AR and subsequent disease occurrence.

Our experimental data indicated that the low expression of IL-38 in both serum and local nasal mucosa, conforming to the relevant research findings regarding Behcet's disease [26]. These results confirmed a corresponding decrease in the anti-inflammatory effect caused by the reduced expression of IL-38 in the entire body, as well as the local environment of AR. In contrast, previous research has demonstrated the overexpression of IL-38 in the tissue of inflammatory bowel diseases or systemic lupus erythematosus [27, 28]. These differences in results demonstrate that different IL-38-related balance and evolution processes may exist in different

tissues and diseases. However, the detailed mechanism requires further clarification. In addition, this study not only revealed the elevated expression of Th17 milieu-related indicators (Th17 cells, RORC, and cytokines) in patients with AR, which is consistent with data from previous studies [29, 30], but also indicated that the expression of IL-38 and Th17 milieu-related indicators was negatively correlated in AR. These findings confirmed that IL-38 may have a negative regulatory impact on Th17, the weakening of which directly leads to the abnormal activation of Th17 response in patients with AR. To confirm this effect, we conducted an *in vitro* study, which showed that IL-38 inhibits the differentiation of Th17 cells in PBMCs from the AR group, thereby preventing the overexpression of effector cytokines, suggesting that the inhibitory impact of IL-38 on diseases mainly involves the regulation of Th17 inflammation. This further confirmed the negative regulatory function of IL-38 on Th17 cells. Consistent with our findings, IL-38 possesses an inhibitory effect on chronic inflammation that is dependent on IL-17 [31]. Through its effect on IL-1 receptor helper protein-like 1, secretion of mature IL-38 by apoptotic cells completes the inhibition of IL-17 production by *in vitro* co-cultured human T cells [32]. Furthermore, owing to its antagonistic effect on IL-36R, IL-38 could attenuate the production of Th17 cytokine in PBMCs [33]. These results demonstrated that, during the onset and development stages of AR, the decreased level of IL-38 causes a loss of Th17 inhibition, triggering the corresponding inflammatory response and resulting in the onset of the disease. We also found that, as a normal downstream signaling pathway of IL-1 family members, inhibition of NF-κB and MAPK signaling pathways caused the cancellation of the negative regulatory response of Th17 by IL-38 [34]. These results could provide a target for further studies on AR-related signaling pathway mechanisms.

Interestingly, it was also shown in this study that IL-17 can trigger the production of IL-38, suggesting the presence of a negative feedback loop between IL-38 and the Th17 response. Thus, the Th17 milieu may have a self-adjustment function, which could prevent the unlimited amplification of inflammation in AR. However, this study only preliminarily analyzed the expression of cytokines; thus, the specific mechanism requires further study.

CONCLUSION

In summary, the findings of this study suggest that IL-38 can inhibit Th17 cell differentiation and block its pro-inflammatory effect by the regulation of NF-κB and MAPK signaling pathways in AR. Moreover, the obtained findings explain the anti-inflammatory mechanism of IL-38 in AR as well as offer a novel potential target for treating AR.

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Conflict of Interest: None declared.

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