

Decreased expression of interleukin-37 and its anti-inflammatory effect in allergic rhinitis

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Abstract. Interleukin-37 (IL-37), a novel member of the IL-1 cytokine family has been identified as a natural suppressor of innate immunity and inflammatory responses. The present study aimed to determine the expression of IL-37 in peripheral blood mononuclear cells (PBMCs) from patients with allergic rhinitis (AR), and examine the possible immunosuppressive effect of IL-37 on inflammatory mediators and CD4⁺ T cells in the pathogenesis of AR. The expression levels of IL-37 were determined in PBMCs from 39 patients with AR and 43 controls using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis and flow cytometry. Cytokines in the supernatants of the PBMCs and CD4⁺ T cells, which were stimulated with lipopolysaccharide in the presence or absence of IL-37, were assayed using enzyme-linked immunosorbent assays and RT-qPCR analysis. The results showed that the patients with AR exhibited significantly decreased expression of IL-37, and increased expression levels of interleukin (IL)-1 β and IL-6 in PBMCs. Recombinant IL-37 (rIL-37) inhibited the production of IL-1 β and IL-6, and enhanced the production of IL-27 in PBMCs from the patients with AR and the control individuals. rIL-37 also markedly decreased the expression of IL-17 by CD4⁺ T cells in the patients with AR and controls. These results suggested that IL-37 may be an important cytokine in the pathogenesis of AR. It may have a protective role in AR by inhibiting the production of proinflammatory cytokines and through suppressive regulation of the Th17 response.

Introduction

Allergic rhinitis (AR) is an immunoglobulin E-mediated type of inflammation of the upper airway, which is induced by allergens and regulated by T cells. AR has an estimated worldwide incidence rate of 10-20% (1). The authors' previous epidemiological investigations demonstrated that, in western China, the prevalence of self-reported AR was 32.30% in Chongqing, 34.3% in Chengdu, 37.9% in Urumqi, and 30.3% in Nanning (2). AR has a major effect on quality of life by causing symptoms of sneezing, nasal congestion, nasal pruritus, rhinorrhea and obstruction of the nasal passages. Furthermore, AR is a known risk factor for comorbid conditions, including asthma, rhinosinusitis, nasal polyposis and sleep disorders, resulting in important medical and social problems (3,4). Over the last 20 years, the pathogenesis of AR has been widely investigated and the majority of studies have focused on proinflammatory cytokines. However, anti-inflammatory cytokines in AR have received less attention.

Interleukin (IL)-37, a novel member of the IL-1 family and originally termed IL-1F7, has been shown to be a natural suppressor of innate immunity and inflammatory responses (5,6). IL-1F7b is the largest variant, and is referred to as IL-37 in the present study. IL-37 interacts with the cell surface receptors, IL-18Ro and IL-18-binding protein (BP), and has five splice variants (IL-1F7a-e) (7). It is expressed in human peripheral blood mononuclear cells (PBMCs) and various tissues at low levels, and can be induced by inflammatory stimulation, including Toll-like receptor agonists. A previous study demonstrated that the expression of IL-37 in dendritic cells (DCs) promoted the generation of semimature tolerogenic DCs and suppressed antigen-specific immune responses of the skin, which revealed IL-37 as an inhibitor of the adaptive immune response (8). The abnormal expression of IL-37 has been reported in several inflammation-related diseases, including Vogt-Koyanagi-Harada disease (9-11), inflammatory bowel disease (12), systemic lupus erythematosus (13), Graves' disease (14), ankylosing spondylitis (15) and rheumatoid arthritis (16,17). In addition, Lunding *et al* (18) demonstrated that IL-37 ablated a Th2 cell-directed allergic inflammatory response and the hallmarks of experimental asthma in mice, suggesting that IL-37 may be critical for the pathogenesis of asthma. It has also been reported that IL-37 is an important cytokine in the control of asthma by suppressing

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the production of inflammatory cytokines, tumor necrosis factors, IL- β , IL-6 and thymic stromal lymphopoietin (19,20). These findings indicate that IL-37 may be involved in inflammatory responses, and may have an immunosuppressive role in allergic disease. However, whether there are any correlations between IL-37 and AR remain to be elucidated, and the function of IL-37 in AR warrants further investigation.

The present study aimed to determine the mRNA and protein levels of IL-37 in PBMCs from patients with AR, in order to evaluate the expression of IL-37 in AR. This was followed by examination of the possible immunosuppressive effect of IL-37 on inflammatory mediators and CD4⁺ T cells in the pathogenesis of AR.

Materials and methods

Subjects. Overall, 39 patients (23 women and 16 men) between 12 and 52 years of age were recruited, between April 2015 and September 2015. All patients were identified by and treated at the outpatient clinic of the Department of Otolaryngology, Head and Neck Surgery at the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). The diagnosis of AR was based on the patients' medical history, symptoms and the presence of a positive skin prick test (SPT; Allergopharma, Hamburg, Germany) in response to a panel of common allergens defined by the Allergic Rhinitis and its Impact on Asthma 2008 guidelines (21). The SPT results were diagnosed in accordance with these commendations of the Subcommittee on Allergen Standardization and Skin Tests of the European Academy of Allergy and Clinical Immunology (22). A positive SPT result was defined as the formation of a wheal measuring $\geq 50\%$ of the diameter of the histamine control wheal, and ≥ 3 mm larger than the diameter of the negative control wheal. A total of 18 inhaled allergens were assessed, including house dust, grass, tree, mold, food, and cat and dog dander. Patients with accompanying systemic disease were excluded from the study. An additional 43 healthy volunteers of the same ethnicity as the patients were recruited as a control group.

Ethics statement. The local Ethics Committee of the First Affiliated Hospital of Chongqing Medical University provided permission and assisted in obtaining informed consent from all participants. Written informed consent was obtained from all participants. Informed consent was obtained from the next of kin, caretakers or guardians of any minors involved in the study.

Cell isolation and culture. The PBMCs were obtained using standard Ficoll-Hypaque density centrifugation (TBD Science, Tianjin, China) at 800 x g for 25 min at room temperature within 1 h of collection. The cells were then washed twice with phosphate-buffered saline (PBS) and resuspended at 1×10^6 /ml in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin, 100 ium supplemented with 10 mmol/l L-glutamine. To investigate the effect of IL-37 on the production of proinflammatory cytokines by the PBMCs, the PBMCs from four controls were stimulated with 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich; Merck Millipore,

Darmstadt, Germany) in the presence of recombinant IL-37 (rIL-37) at different concentrations (0, 50, 100 and 200 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA) for 72 h at room temperature. Following incubation, the cells and culture supernatants were collected to analyze the RNA and protein levels of cytokines. The duration of 72 h was selected according to a previous study; therefore, the cytokines were not measured at different time-points (10). Subsequently, the PBMCs from seven patients with AR and six controls were re-suspended at a concentration of 1×10^6 cells/ml and stimulated with or without 100 ng/ml rIL-37 in the presence of 100 ng/ml LPS for 72 h at room temperature, following which the culture supernatants were harvested and frozen at -80°C for cytokine analysis using an enzyme-linked immunosorbent assay (ELISA). The cells in the control groups were stimulated with the same volume of PBS alone.

To investigate the effect of IL-37 on effector cytokine production by CD4⁺ T cells, the CD4⁺ T cells were separated from the PBMCs obtained the AR patients and controls using magnetic microbeads (CD4⁺ cell purity $\geq 98\%$; Miltenyi Biotec, Inc., Cambridge, MA, USA). The CD4⁺ T cells were re-suspended at a concentration of 1×10^6 cells/ml and stimulated with anti-CD3/CD28 (eBioscience; Thermo Fisher Scientific, Inc.) in the presence or absence of 100 ng/ml rIL-37 for 72 h to detect the levels of IL-10 and IL-17 in the supernatants.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The mRNA levels of IL-37 and proinflammatory cytokines from the PBMCs were determined using RT-qPCR analysis. Total RNA was extracted from the isolated PBMCs using TRIzol extraction (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and was reverse transcribed to cDNA using random hexamer primers and RNase H-reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). The expression levels of mRNA were determined using the ABI Prism 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR Premix Taq (Takara Biotechnology Co., Ltd., Dalian, China). The PCR primer sequences are summarized in Table I. PRISM samples contained 1X SYBR Green Master Mix, 1.5 μl , 5 μM primers, and 25 ng synthesized cDNA in a 25 μl volume. Reaction mixtures were heated to 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec, and annealing extension at 60°C for 60 sec. All PCR reactions were performed in duplicate. The PCR products were verified by melting curve analysis. Relative mRNA levels of target genes were calculated by the $2^{-\Delta\Delta\text{Ct}}$ method (23).

ELISA analysis. The concentrations of IL-1entrations of and IL-27 in the culture supernatants of the PBMCs were assayed using specific ELISA kits (R&D Systems, Inc.) according to the manufacturer's instructions. All assays were performed in duplicate. The results are expressed in pg/ml. In preliminary experiments, the serum level of IL-37 was determined using ELISA, however, the level of IL-37 was too low to be detected. Therefore, in the serum level of IL-37 was not examined in the present study.

Flow cytometry. For analysis of the expression of IL-37, the PBMCs were fixed with BD Cytofix™ fixation buffer

Table I. List of the sequences of human gene primers.

Primer, length (bp)	Sequence (5'-3')
IL-37, 25	F: AGTGCTGCTTAGAAGACCCGG R: AGAGTCCAGGACCAGTACTTTGTGA
IL-1, 20	F: ACCAAACCTCTTCGAGGCAC R: AGCCATCATTTCACTGGCGA
IL-27EBI3, 10	F: TTACAAGCGTCAGGGAGCTG R: TTCCCCGTAGTCTGTGAGGT
IL-27P28, 22	F: CTGGACCAACATGGAGAGGATG R: TAAGACGAGCTCCAAGGAGTG
IL-6, 21	F: AGCCACTCACCTCTTCAGAAC R: ACATGTCTCCTTTCTCAGGGC
IL-10, 20	F: TACGGCGCTGTCATCGATTT R: TAGAGTCGCCACCCTGATGT
β-actin, 22	F: CCTGACTGACTACCTCATGAAG R: GACGTAGCACAGCTTCTCCTTA

IL, interleukin; F, forward; R, reverse.

(BD Biosciences, Franklin Lakes, NJ, USA) at 37°C for 10 min and permeabilized in BD Phosflow™ Perm Buffer III (BD Biosciences) at 4°C for 30 min. The cells were then incubated with anti-IL-37 antibody (cat. no. ab116282, 1:200; Abcam, Cambridge, MA, USA) for 30 min at 22°C. Subsequently, the cells were stained with PE goat anti-mouse immunoglobulin (cat. no. 1010-09, 1:100; 4A Biotech Co., Ltd., Beijing, China), which was incubated with the cells for 30 min at 22°C according to the manufacturer's instructions. The fluorescence profiles were analyzed using a FACScan cytometer equipped with CellQuest software 4.0 (BD Biosciences) in terms of the mean fluorescence intensity (MFI). The results were calculated as increments relative to the isotypic control (IC) using the following formula: (MFI of sample - MFI of IC)/MFI of IC. The data were processed using FlowJo software 10 (Treestar, Inc., Ashland, OR, USA).

Statistical analysis. The software used for statistical analysis was SPSS for Windows (version 20.0; SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. Differences between the values were determined using an independent samples t-test or paired sample t-test. Grouped data were analyzed using a one-way analysis of variance, followed by the Student-Newman-Keuls test. When the equal variance test failed, a Mann-Whitney Rank Sum test was used. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Decreased expression of IL-37 in PBMCs from patients with AR. To investigate the role of IL-37 in AR, the mRNA expression levels of IL-37 were determined in PBMCs from patients with AR and controls using RT-qPCR analysis. The

results showed that the mRNA expression of IL-37 was significantly decreased in the PBMCs from patients with AR, compared with the normal controls ($P = 0.038$). The protein levels of IL-37 in the PBMCs were also determined using flow cytometry. The results showed that the protein level of IL-37 in the PBMCs from patients with AR was significantly lower, compared with that in the controls ($P < 0.001$), which was consistent with the obtained mRNA data (Fig. 1A-C).

IL-37 inhibits the production of proinflammatory cytokines by PBMCs from patients with AR and controls. To investigate the effect of IL-37 on the production of proinflammatory cytokines in PBMCs, the PBMCs from healthy controls were stimulated with LPS in the presence of different concentrations of rIL-37 (0, 10, 100 and 200 ng/ml) for 72 h. The mRNA expression levels of IL-1β and IL-6 in the PBMCs were measured using RT-qPCR analysis, and the protein expression levels of these two cytokines in the supernatants were measured using ELISA. The results demonstrated that the mRNA and protein levels of IL-1β and IL-6 were decreased when the PBMCs were stimulated with rIL-37 at 100 and 200 ng/ml (Fig. 2A-D).

The PBMCs from patients with AR and controls were then stimulated with LPS in the presence or absence of 100 ng/ml of rIL-37. The results showed that the expression levels of IL-11 and IL-6 in culture supernatants from the patients with AR were significantly higher than those from the controls ($P = 0.04$ and $P = 0.031$; Fig. 3A and B). In addition, the levels of IL-11 and IL-6 in the rIL-37-stimulated PBMC culture supernatants from the patients with AR were significantly decreased compared with those in the unstimulated PBMC culture supernatants ($P = 0.002$ and $P = 0.001$). In the healthy controls, the same significant differences were observed in the production of IL-1p and IL-6 between the rIL-37-stimulated and unstimulated PBMCs ($P = 0.005$ and $P = 0.028$; Fig. 3A and B).

IL-37 enhances the production of IL-27 in PBMCs from patients with AR and normal controls. To investigate the effect of IL-37 on the immunosuppressive production of IL-27 in PBMCs, the concentrations of IL-27 in the culture supernatants from the PBMCs were measured using ELISA. As exhibited in Fig. 4, compared with the control subjects, the level of IL-27 in the LPS-stimulated supernatants was significantly decreased in the patients with AR ($P = 0.042$). In addition, the levels of IL-27 in the AR and control groups were markedly enhanced in the rIL-37+LPS-stimulated supernatants, compared with the levels in the respective LPS-stimulated supernatants ($P < 0.001$ and $P < 0.001$; Fig. 4).

IL-37 reduces the production of IL-17A by CD4⁺ T cells from patients with AR and normal controls. To investigate the effect of IL-37 on CD4⁺ T cells, which are important in the pathogenesis of AR, the effect of IL-37 on cytokine production by CD4⁺ T cells was examined. CD4⁺ T cells from the patients with AR and controls were stimulated with anti-CD3/CD28 or anti-CD3/CD28+rIL-37. The levels of effector cytokines in the supernatants were then determined using ELISA. IL-37 significantly reduced the production of IL-17A by CD4⁺ T cells ($P < 0.001$ and $P < 0.001$). Compared with the control subjects, the level of IL-17A in the LPS stimulated

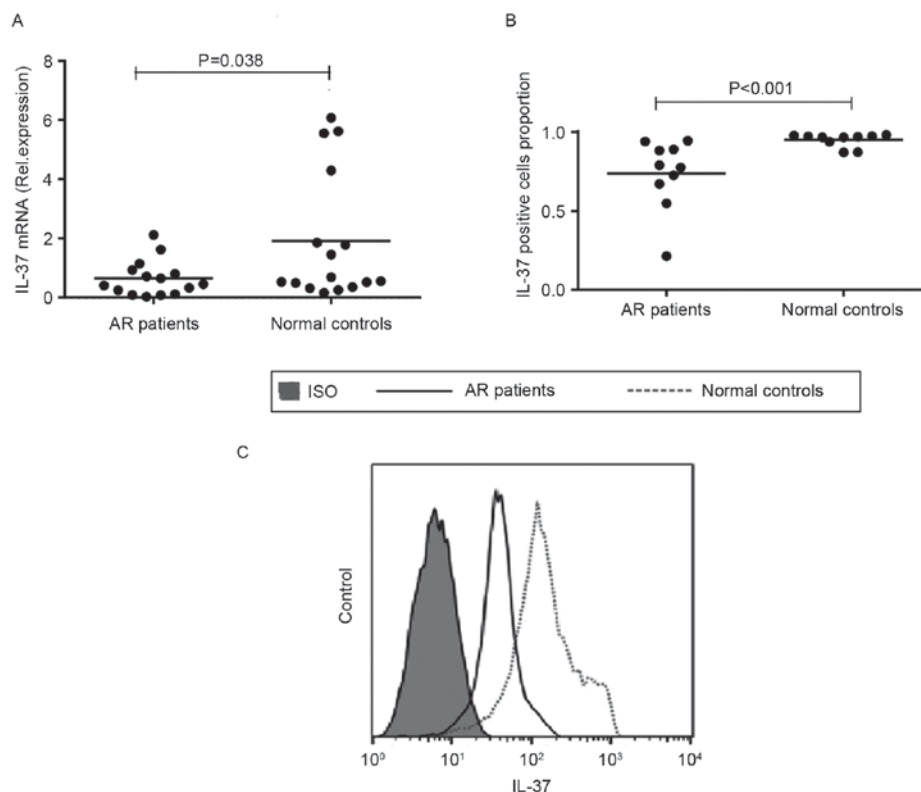


Figure 1. Patients with AR have lower levels of IL-37 than normal controls. (A) mRNA expression of IL-37 was decreased in patients with AR, compared with that in normal controls. Reverse transcription-quantitative polymerase chain reaction analysis of mRNA expression levels of IL-37 in PBMCs from untreated patients with AR (n=15) and sex- and age-matched normal controls (n=16). Levels were normalized to β -actin. (B) PBMCs from patients with AR (n=10) and normal controls (n=10) were analyzed for expression of IL-37 using flow cytometry. The statistical results are expressed as the proportion of positive cells. (C) Representative histograms from two independent experiments are shown, each from one patient and one healthy individual. The results of the protein levels of IL-37 were in line with those of the mRNA levels. AR, allergic rhinitis; IL-37, interleukin-37; PBMCs, peripheral blood mononuclear cells.

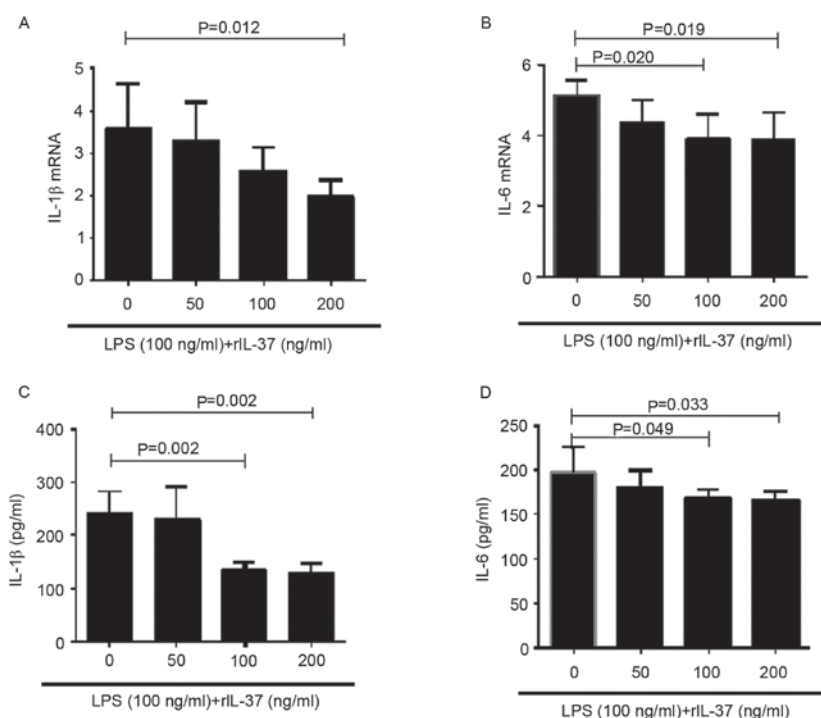


Figure 2. IL-37 inhibits the production of proinflammatory cytokines in PBMCs in a dose-dependent manner. PBMCs were isolated from healthy volunteers (n=4) and stimulated with LPS in the presence of different concentrations of rIL-37 (0, 50, 100 and 200 ng/ml) for 72 h. The mRNA expression levels of IL-1 and IL-6 in PBMCs were measured using reverse transcription-quantitative polymerase chain reaction analysis and their protein expression the supernatants were measured using enzyme-linked immunosorbent assays. rIL-37 inhibited the mRNA levels of (A) IL-1 β and (B) IL-6, at the protein levels of (C) IL-1 β and (D) IL-6 at 100 and 200 ng/ml. Data are expressed as the mean \pm standard deviation. IL, interleukin; PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; rIL-37, recombinant IL-37.

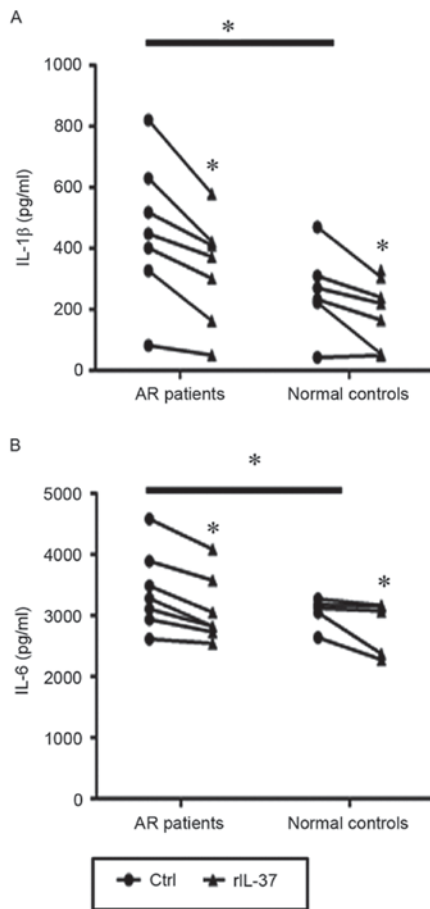


Figure 3. IL-37 inhibits the production of proinflammatory cytokines in PBMCs from patients with AR and normal controls. PBMCs from patients with AR (n=7) and normal controls (n=6) were stimulated with LPS or LPS+rIL-37 for 72 h. Expression levels of IL- β and IL-6 in the supernatants were measured using an enzyme-linked immunosorbent assays. The expression levels of (A) IL- β and (B) IL-6 were higher in the patients with AR, compared with those in the normal controls. IL-37 inhibited the production of IL- β and IL-6 in the patients with AR and normal controls. * P <0.05. AR, allergic rhinitis; IL, interleukin; PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharides; rIL-37, recombinant IL-37; Ctrl, control.

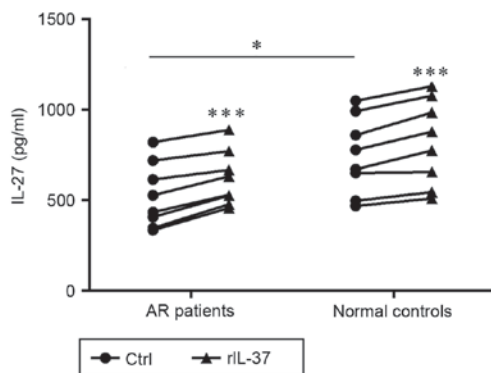


Figure 4. IL-37 enhances the production of IL-27 cytokines in peripheral blood mononuclear cells from patients with AR and normal controls. The expression of IL-27 in patients with AR was lower than that in the normal controls. * P <0.05; *** P <0.001. AR, allergic rhinitis; IL-37, interleukin-37; rIL-37, recombinant IL-37; Ctrl, control.

supernatant was significantly increased in the patients with AR (P =0.04). However, IL-37 did not affect the production of

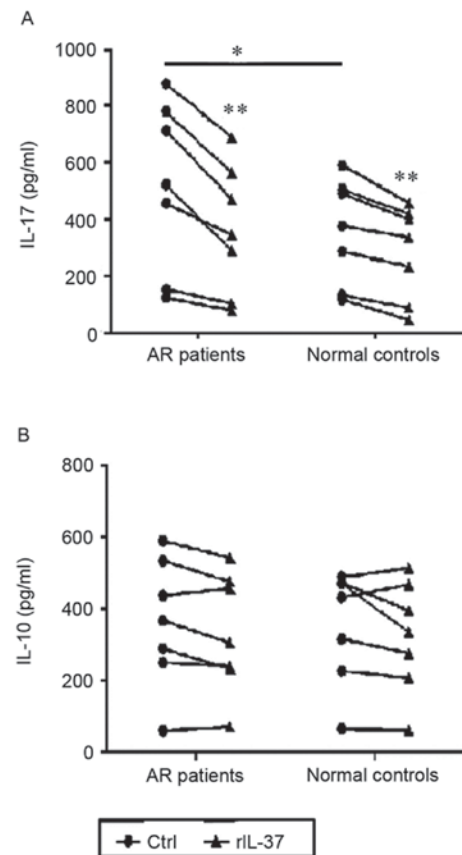


Figure 5. IL-37 suppresses the production of IL-17 by CD4⁺ T cells. CD4⁺ T cells were isolated from patients with AR (n=7) and healthy volunteers (n=7), and stimulated with anti-CD3/CD28 in the presence or absence of rIL-37 for 72 h. Levels of (A) IL-17 and (B) IL-10 were determined. IL-37 had no effect on the production of IL-10 by CD4⁺ T cells. * P <0.05 vs. AR patients; ** P <0.001 vs. Ctrl. AR, allergic rhinitis; IL, interleukin; rIL-37, recombinant IL-37; Ctrl, control.

IL-10 by stimulated CD4⁺ T cells in the AR or healthy control group (P =0.222, and P =0.070; Fig. 5A and B).

Discussion

In the present study, significant decreases in the expression of IL-37 were found at the mRNA and protein levels in PBMCs from patients with AR. In addition, significantly decreased levels of IL-1r and IL-6, and an increased level of IL-27 were found in rIL-37-stimulated PBMC culture supernatants from patients with AR. Finally, it was demonstrated that IL-37 reduced the production of IL-17 by stimulated CD4⁺ T cells in the patient group. These results indicated that, in AR patients, IL-37 significantly inhibited the production of proinflammatory cytokines from PBMCs and efficiently suppressed the release of IL-17 by CD4⁺ cells. Therefore, it was hypothesized that IL-37 acts not only as a negative regulator, but that it also has anti-inflammatory properties in an allergic immune response in the airways.

IL-37 is a novel molecule of the IL-1 family with anti-inflammatory effects. On examining the role of IL-37 in allergic respiratory disease, previous data have demonstrated that IL-37 is able to ablate a Th2 cell-directed allergic inflammatory response and the hallmarks of experimental asthma

in mice, suggesting that IL-37 may be important in allergic pathogenesis (18). Decreases in the expression and production of IL-37 have also been recorded in restimulated PBMCs of children with allergic bronchial asthma (24), and a decreased level of IL-37 in induced sputum was found to correlate with disease severity, indicating that IL-37 may be an important cytokine in the control of asthma by suppressing the production of inflammatory cytokines (20). A study by Liu *et al* (25) reported significantly decreased expression of IL-37b in serum and nasal lavage from children with AR. Consistent with these findings, the experiments in the present study showed that PBMCs from patients with AR expressed a lower level of IL-37, compared with those from healthy controls. Taken together, the above findings indicate that IL-37 may be involved in the development of allergic respiratory disease.

On the basis of the above findings, it was hypothesized that a decrease in the production of IL-37 may be implicated in the pathogenesis of AR by a reduced capacity to counterbalance an ongoing allergic inflammatory response in the airways. To confirm this hypothesis, the present study examined the effect of rIL-37 on the production of inflammatory cytokines in PBMCs from patients with AR and healthy controls. The results showed that the expression levels of IL-1 ϵ and IL-6 were significantly increased in the PBMCs from patients with AR, and IL-37 effectively decreased the production of these proinflammatory cytokines. These findings indicated that IL-37 may have an immunosuppressive effect in the pathogenesis of AR through a potential anti-inflammatory function.

IL-27, a novel member of the IL-12 family, is reported to prevent the development of Th2 cells and Th17 cells in various inflammatory settings (26). The authors' previous study indicated that IL-27 gene polymorphisms were likely to be involved in susceptibility to AR (27). In the present study, the potential association between IL-37 and IL-27 in AR was examined. The results showed that the production of IL-27 in PBMCs of patients with AR was significantly decreased, and IL-37 downregulated the expression of IL-27 in PBMCs from patients with AR and normal controls. This demonstrated that IL-27 acted in synergy with IL-37 in the anti-inflammatory process, which had an immunosuppressive effect in the pathogenesis of AR.

As is already known, the function of CD4⁺ T cells is critical in immune responses. Our previous study demonstrated that the imbalance of Treg/Th17 cells was important in the pathogenesis of AR, and allergy may aggravate chronic sinusitis by promoting the imbalance of Th17/Treg cells (28). In the present study, the results showed that IL-37 reduced the production of IL-17 by CD4⁺ T cells, which indicated that IL-37 had a suppressive function in the immune regulation of AR and was able to inhibit Th17 cell responses. However, owing to limitations in time and funding, the present study included only 39 patients and 43 healthy volunteers. In further investigations, an increased sample size is planned to confirm this conclusion, and to detect the expression of IL-6, IL-1 β , IL-27, IL-10 and IL-17A in the PBMCs of patients with AR and controls. Future experiments also aim to examine the expression of IL-37 receptor, IL-1R8 and additional detailed investigations are required to clarify the exact mechanisms involved.

In conclusion, the results of the present study demonstrated that the expression of IL-37 in PBMCs from patients with AR was significantly reduced; rIL-37 negatively regulated the production of proinflammatory cytokines and induced the production of IL-27 by PBMCs. In addition, IL-37 reduced the production of IL-17 by CD4⁺ T cells. These findings indicate that IL-37 may be an important cytokine involved in the pathogenesis of AR. It may have a protective effect on AR by inhibiting the production of proinflammatory cytokines and through suppressive regulation of the Th17 response. However, further investigations are required to clarify the exact mechanism and to confirm whether IL-37 may be a potential target for the prevention and treatment of AR.

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