



LncRNA-MEG3 functions as a competing endogenous RNA to regulate Treg/Th17 balance in patients with asthma by targeting microRNA-17/ ROR γ t

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ARTICLE INFO

Keywords:

Asthma
LncRNA
microRNA
Treg
Th17
ROR γ t
Foxp3

ABSTRACT

Background: Treg/Th17 imbalance plays an essential role in the pathogenesis of asthma. Disordered LncRNAs were observed in asthma, however, whether LncRNAs can regulate the Treg/Th17 balance and its mechanism still needs to be investigated.

Methods: Microarrays were performed to identify the differentially expressed LncRNAs and microRNAs in peripheral blood CD4 + T cells from patients with asthma and healthy controls. Bioinformatical evidence was used to select candidate LncRNAs and microRNAs which may involve in regulation of Treg/Th17 balance. The function of LncRNA-MEG3 and microRNA-17 on the alteration of the CD4 + T cell population were determined in vitro experiments. Meanwhile, the regulatory effect of LncRNA-MEG3 and microRNA-17 on ROR γ t or Foxp3 was estimated. The interaction of LncRNA-MEG3 with microRNA-17 was confirmed by dual luciferase reporter assay and RNA pull-down.

Results: 25 LncRNAs and 19 microRNAs were selected as candidate genes which differentially expressed in CD4 + T cells from patients with asthma compared with healthy controls and had potential to control Treg/Th17 balance by regulating ROR γ t or Foxp3. Alternation of LncRNA-MEG3 changed the function and increased the percentage of Th17. LncRNA-MEG3 could regulate the ROR γ t mRNA and protein level. LncRNA-MEG3 could inhibit the level of microRNA-17 as a competing endogenous RNA (ceRNA). microRNA-17 suppressed Th17 though targeting ROR γ t directly.

Conclusion: LncRNA-MEG3 can sponge microRNA-17 as a ceRNA, thereby regulating ROR γ t and ultimately affecting Treg/Th17 balance in asthma. The LncRNA/microRNA axis may have potential application in clinical treatment and diagnosis of the disease.

1. Introduction

Asthma is a chronic airway inflammation characterized by increased airway responsiveness, airway remodeling, and reversible airway obstruction [1]. It is currently believed that abnormal release of cytokines caused by imbalance of CD4 + T lymphocytes is the main cause of airway damage, increased responsiveness and dysfunction [2,3]. CD4 + T cells can be classified into helper T cells (Th cells) and regulatory T cells (Treg cells) basing their different functions. Th cells mainly include Th1 cells, Th2 cells and Th17 cells, whose main function is to secrete cytokines [4]. Th17 cell is a subtype of CD4 + T cell newly identified in recent years in addition to Th1 and Th2 cell. Th17 cells expand inflammatory responses by promoting the release of other inflammatory factors and by recruiting neutrophils [5]. Th17 cells exert a strong pro-inflammatory effect by secreting IL-17 [6,7]. Treg cells are

mainly involved in regulation of immune response and function of other immune cells, thus limiting immune response that involves T cells to a suitable range to avoid damage to the body [8]. There are several subtypes of Treg cells, among which CD4 + CD25 + Treg cells has been found to inhibit Th cells and is now been extensively studied in the area of asthma research [9]. Studies have shown that Treg and Th17 cells, antagonizing each other, are in equilibrium under normal circumstances. Accumulating evidence suggest that Treg/Th17 imbalance play an important role in the development of asthma [10]. Zissler et al found that in peripheral blood of patients with allergic asthma, both the proportion of Th17 cells and the level of IL-17 increased, while the proportion of CD4 + CD25 + Treg cells decreased [11]. Some studies have pointed out that the degree of Treg/Th17 imbalance is correlated with the severity of symptoms in asthmatic mice: the greater Treg/Th17 imbalance, the stronger airway inflammatory response in mice. After

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<https://doi.org/10.1016/j.bioph.2018.12.080>

Received 6 November 2018; Received in revised form 15 December 2018; Accepted 17 December 2018

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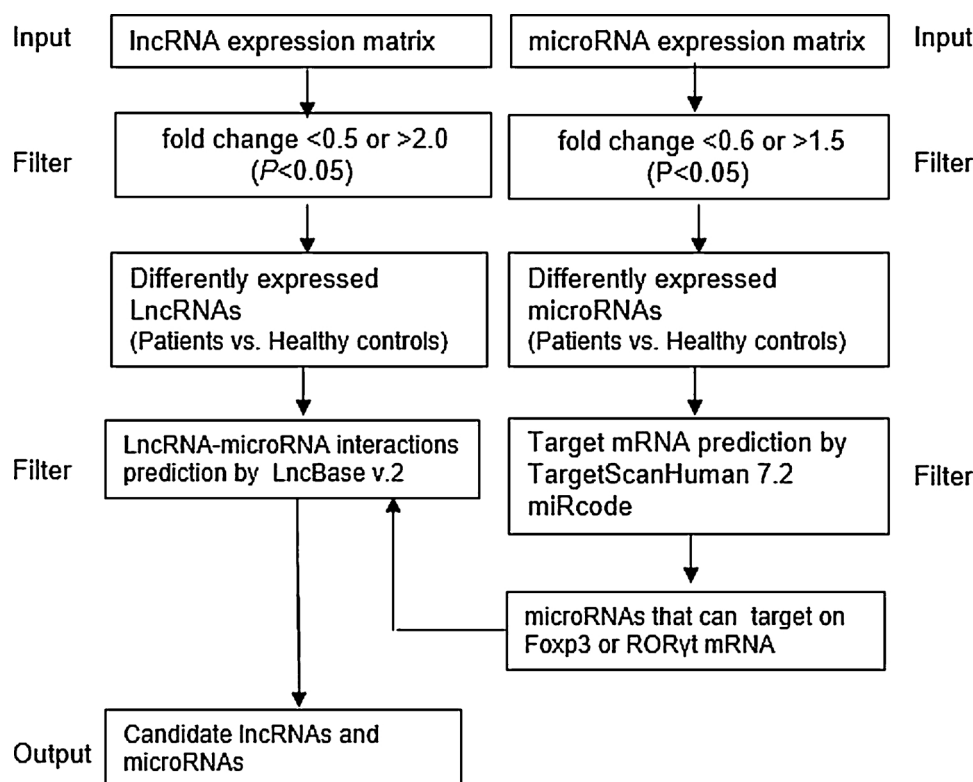


Fig. 1. The workflow for selection of candidate lncRNAs and microRNAs. Differentially expressed lncRNAs/microRNAs in CD4 + T cells (patients vs. *Healthy control*) were listed out. Then microRNAs which could not target RORγ or Foxp3 were excluded. lncRNAs which could not interact with candidate microRNAs were not included.

drug intervention, the Treg/Th17 imbalance was corrected and respiratory symptoms were alleviated [12,13]. It has been clarified that fork-like transcription factor 3 (Foxp3) is not only an important molecular marker of Treg cells but also a key transcription factor that affects Treg function and development. Foxp3 deficiency results in insufficient differentiation of CD4 + T cells into Treg cells [14]. On the other hand, differentiation of Th17 cells is specifically regulated by the nuclear orphan receptor γt (RORγt), and differentiation of CD4 + T cells into Th17 cells is accompanied by an increase in RORγt [15]. Therefore, regulating Foxp3 or RORγt will affect the Treg/Th17 balance. However, pathways upstream of Foxp3 and RORγt in the process of asthma development has not been fully clarified.

lncRNAs are non-coding RNAs that are more than 200 nucleotides in length. They are involved in the development of multiple diseases, including asthma, through transcriptional or post-transcriptional regulation of protein expression through various mechanisms [16,17]. One of these mechanisms can be explained by the theory of competing endogenous RNA (ceRNA). The theory supposes that lncRNA can act as a ceRNA to competitively adsorb some microRNAs through base complementation, thereby reducing binding of these microRNAs to their target genes, indirectly leading to changes in expression of the target genes of these microRNAs [18]. However, during asthma development, whether some abnormal lncRNAs regulate specific microRNAs as ceRNAs, indirectly affects the levels of Foxp3 and RORγt and ultimately leads to Treg/Th17 imbalance is still unknown. In this study, we analyzed lncRNA and microRNA profiles of CD4 + T cells in peripheral blood of asthma patients with microarrays, and through a series of bioinformatic screening, we selected a series of lncRNA and microRNA that may be involved in Treg/Th17 imbalance. Through in vitro studies, we found that lncRNA-MEG3 may act as a ceRNA to regulate RORγt expression via microRNA-17, thereby affecting the Treg/Th17 balance in asthma.

2. Materials and methods

2.1. Patients and healthy control

A total of 52 patients with severe asthma, including 32 males and 20 females with a median age of 42, were recruited from the Nanjing Drum Tower Hospital. Patients were diagnosed as asthma according to the revised version of Chinese guideline for the prevention and management of bronchial asthma. All patients underwent inhaled corticosteroid treatment.

Asthma severity was evaluated by a respiratory physician based on a combination of symptoms, lung function, and amount of medication according to the guideline mentioned above. Forty-five healthy subjects (32 males and 13 females, with a median age of 35) were served as healthy controls. All participants provided written informed consent. Ethical approval was given by the Medical Research Ethics Committee of Nanjing Drum Tower Hospital. The clinical information of subjects was shown in **table S2**.

2.2. CD4 + T cells collection and culture

Peripheral blood samples collected from subjects were preserved in tubes with pretreatment of heparin sodium. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Ficoll-Hypaque solution (Pharmacia, Uppsala, Sweden). Then CD4 + T cells were isolated using a positive selection magnetic isolation system. Technical details could be found in our previous study [19]. The purity of isolated CD4 + T cells was evaluated by flow cytometry. CD4 + T cells with a purity of more than 90% could be used in further analysis. Isolated CD4 + T cells were routinely maintained in human T cell culture medium (OpTmizer™ CTS™ T-Cell Expansion SFM, Gibco, Rockville, MD, USA) at 37 °C, 5% CO₂.

2.3. RNA extraction and microarray analysis

Total RNA samples of CD4+ cells from asthmatic patients and

healthy controls were extracted using TRIzol reagent (Thermo Fisher Scientific Inc., USA) in accordance with the manufacturer's protocol. To assess the lncRNA expression profiling in the CD4 + T cells from subjects a microarray analysis was performed using Human LncRNA Array v2.0 (8 × 60 K, Arraystar). The miRNA expression profiling was obtained using Agilent Human miRNA Microarray V 21.0 (8 × 60 K, Agilent). The results of microarray analysis were analyzed using R software with related packages to obtain differentially expressed lncRNA or microRNA between patients and healthy controls.

2.4. Selection for candidate lncRNA and microRNA based on bioinformatics analysis

The workflow of selection for candidate lncRNAs and microRNAs was shown in Fig. 1. LncRNAs with a fold change < 0.5 or > 2.0 ($P < 0.05$) were defined as differentially expressed lncRNAs. For microRNA, the threshold was fold change < 0.6 or > 1.5 ($P < 0.05$). Differentially expressed lncRNA or microRNA between patients and healthy control were filtered according to bioinformatics evidence. In brief, target mRNAs of differentially expressed microRNA were predicted by TargetScanHuman 7.2 and miRcode software. microRNAs that has no potential to target on Foxp3 or RORγt mRNA were excluded. The differentially expressed lncRNAs were analyzed by LncBase v.2 that designed for showing interactions of lncRNA-microRNA based on experimental and prediction modules. LncRNAs or microRNAs that could not constitute a interaction were excluded.

2.5. Quantitative real-time PCR (qRT-PCR)

Extracted total RNA was reverse transcribed by a PrimeScript® RT reagent Kits (Takara, China) and followed with amplified using SYBR® Premix Ex Taq™ II kits (Takara, China) for quantifying of lncRNA-MEG3, Foxp3, and RORγt mRNA. For detecting microRNA-17, cDNA was synthesized by a miScript II RT Kit (QIAGEN, USA) and quantitated by miScript SYBR® Green PCR Kits (QIAGEN, USA).

For lncRNA-MEG3, Foxp3 and RORγt mRNA detection, GAPDH was used as a reference. SNORD-48 was served as a reference gene for microRNA-17 detection. The relative level changes of target genes were calculated by 2^{-ΔΔCT} method. The sequences of primers were shown in Table S1.

2.6. Cell transfection

To overexpress the lncRNA-MEG3 level in cells, pcDNA3.1-MEG3 vectors were transfected into cells. Before transfection CD4 + T cells were seeded into 6-well and cultured for 24 h. According to Li's study [20], Lipofectamine 2000 reagent (Invitrogen, USA) was used to transfected plasmids into T cells. For each well 3000 ng pcDNA3.1-MEG3 vectors mixed with 6 μL Lipofectamine 2000 reagent (Invitrogen, USA).

pcDNA3.1 empty vectors were served as negative control for the lncRNA-MEG3 overexpression. To down-regulate the level of lncRNA-MEG3 in CD4 + T cells, 12 μL MEG3-siRNA (10 μM) was transfected into cells with 9 μL Lipofectamine RNAiMAX reagent (Invitrogen, USA). To up-regulate microRNA-17 level of CD4 + T cells, 1 μL microRNA-17 mimics (10 μM) were transfected using Lipofectamine RNAiMAX reagent. For siRNA and mimic transfection, Allstar sequence (QIAGEN, USA) was used as negative control.

2.7. Cytokine detection by Enzyme-linked immunosorbent assay (ELISA)

The culture supernatant of CD4 + T cells was collected. The concentration of IL-17, IL-22, and IL-10 were measured by ELISA kits (R&D, Minneapolis, MN, USA). According to the manufacturer's protocols, The OD value at 450 nm wavelength was detected using a BioTek Microplate Reader System (BioTek, Winooski, VT, USA).

2.8. Western blot

Total protein samples of CD4 + T Cells were isolated by RIPA lysis buffer with 1% protease inhibition (Beyotime, China). The concentration of protein samples was measured using a BCA method (Beyotime, China). Protein samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to NC Membranes. After blocking with 5% non-fat milk, NC Membranes incubated with Foxp3 (Santa Cruz Biotechnology, USA), RORγt (Merck Chemicals, Germany), Ago2 (Abcam plc. UK), and GAPDH (Merck Chemicals, Germany) primary antibodies. After washed by TBS-T buffer 3 time the membranes were incubated with the goat anti-rabbit secondary antibodies for 12 h at -4 °C. The protein bands were visualized using an enhanced chemiluminescence reagent (Beyotime, China) and the intensity was quantified using ImageJ software. 1.48 (National Institutes of Health, USA).

2.9. Flow cytometry

To identify the percentage of Th17, collected CD4 + T cells were incubated with ionomycin (1.0 μg/mL, Sigma-Aldrich, USA), phorbol ester (500.0 ng/mL, Sigma-Aldrich, USA) in the presence of monensin (0.1 mg/mL, Sigma-Aldrich, USA) for 4 h at 37 °C (5% CO₂) for quickly inducing Th17 cells. Then cells were washed two times using PBS and were incubated with FITC labeled anti-CD4 at 4 °C for 45 min. Cells were fixed and permeabilized, and then stained with PE-labeled anti-IL-17 monoclonal antibodies (BD Biosciences, USA). To identify the percentage of Treg cells, cells were incubated with PE-Cy7-labeled anti-CD4, FITC-labeled anti-CD25- at 4 °C for 30 min. Then cells were stained with PE-labeled anti-Foxp3 monoclonal antibodies (BD Biosciences, USA). The Flow cytometry was performed in a Guava easyCyte™ Cytometer system (Merck Millipore, Germany). The data were analysed by the software provided by Cytometer system.

2.10. Luciferase reporter assay

Luciferase reporter assay was performed using Promega Luciferase Assay System (Promega, USA) to confirm the direct binding ability of lncRNA-MEG3/microRNA-17 and microRNA-17/RORγt. Firstly the wildtype (wt) and mutant sequence of lncRNA-MEG3 were sub-cloned into PsiCHECK2 luciferase reporter vectors, respectively (Promega, USA). Then cells were co-transfected with PsiCHECK2 vectors and microRNA-17mimic or miR negative. The fluorescence signals were detected 48 h after transfection according to the manufacturer's instruction and relative fluorescence value (Luciferase/Renilla) was calculated. For confirmation of microRNA-17/ RORγt binding, The wildtype (WT) 3'-UTR sequence of RORγt containing microRNA-17 binding sites were sub-cloned into PsiCHECK2 luciferase reporter vector. The similar co-transfection and fluorescence measurement protocols were performed.

2.11. RNA pull-down

To prove that lncRNA-MEG3 regulated microRNA-17 as a ceRNA, the association of lncRNA-MEG3 with the RISC complex was determined using RNA pull-down. lncRNA-MEG3 was *in vitro* transcribed and biotin-labeled using a biotin RNA labeling mix (Roche, USA). Antisense RNA sequence of lncRNA-MEG3 was used as negative control. Biotin-labeled products of lncRNA-MEG3 and its antisense were treated with RNase-Free DNase I (Roche, USA) and purified with the RNeasy Mini Kit (Qiagen). CD4 + T cell extracts were incubated with Biotin-labeled products at 4 °C for 1 h. Then streptavidin agarose beads (Invitrogen) were added and incubated at room temperature for 1 h. Ago 2 proteins in each reaction were detected by Western blot. The microRNA-17 level in pulled down pellets was detected by qRT-PCR.

2.12. Statistical analysis

Statistical analysis was performed using R software (Version 3.4.1). Data was presented as a mean $\pm$ standard deviation. To compare the difference between two groups or multi-groups, *t*-test and one-way ANOVA were performed, respectively. A difference was considered significant at $P < 0.05$.

3. Results

3.1. Differential expression of lncRNA and microRNA in peripheral blood CD4 + T cells of asthma patients

Using microarrays, we obtained lncRNA and microRNA expression profiles in peripheral blood CD4 + T cells from asthma patients and healthy controls. Compared with healthy controls, 225 lncRNAs in peripheral blood CD4 + T cells from asthma patients showed a fold change < 0.5 or > 2.0 with a P value < 0.01 , among which 112 were upregulated and 113 were downregulated. And 213 microRNAs showed a fold change < 0.6 or > 1.5 with a P value < 0.05 , including 128 upregulated ones and 85 downregulated ones. Next, we further screened these differentially expressed lncRNAs and microRNAs according to the workflow mentioned in the method section (Fig. 1). Based on the bioinformatic evidence, 19 microRNAs were included as candidate microRNAs for their potentials to target Foxp3 or ROR γ t. Meanwhile, 25 lncRNAs were selected as candidate lncRNAs for their potential to interact with at least one of the above 19 microRNAs (basing on sequence complementarity). Compared with healthy controls, 15 of the 25 lncRNAs were up-regulated and 10 down-regulated; 9 of the 19 microRNAs were up-regulated and 10 down-regulated, as shown in Fig. 2A and B. These candidate lncRNAs may regulate the level of Foxp3 or ROR γ t via the candidate microRNAs as ceRNA, thereby affecting Treg/Th17 balance. After referring previous literature, we selected lncRNA-MEG3/microRNA-17 for validation.

3.2. Expression of lncRNA-MEG3 and microRNA-17 in CD4 + T cells of asthma patients

First, we confirmed the changes in lncRNA-MEG3 and microRNA-17 expression by qRT-PCR. As shown in Fig. 3, consistent with microarrays results, level of lncRNA-MEG3 in CD4 + T cells of asthma patients significantly increased compared with healthy controls ($P < 0.05$), while microRNA-17 decreased ($P < 0.05$).

3.3. Effect of lncRNA-MEG3 down-regulation on CD4 + T cells from asthma patients

Level of lncRNA-MEG3 in CD4 + T cells from asthma patients can be effectively reduced by siRNA (Fig. 4A), which was followed by a significant decrease in Th17-associated cytokines IL-17 and IL-22 (Fig. 4B and C) ($P < 0.05$ compared to negative transfection control). However, reducing lncRNA-MEG3 level showed no effects on Treg-associated cytokine IL-10 (Fig. 4D). Flow cytometry results showed that lncRNA-MEG3 inhibition resulted in a decrease in the percentage of Th17 cells ($P < 0.05$), but showed no effect on the percentage of Treg cells (Fig. 4E–F and Figure S1 A). After the lncRNA-MEG3 knockdown, mRNA and protein levels of ROR γ t significantly decreased ($P < 0.05$) but mRNA and protein levels of Foxp3 showed no changes (Fig. 4G and H).

3.4. Effect of lncRNA-MEG3 up-regulation on normal CD4 + T cells from healthy controls

On the other hand, we also up-regulated lncRNA-MEG3 in CD4 + T cells from healthy controls by transfecting the pcDNA3.-MEG3 plasmid (Fig. 5 A). As shown in Fig. 5 B and C, lncRNA-MEG3 overexpression increased levels of IL-17 and IL-22 produced by CD4 + T cells from healthy controls ($P < 0.05$), but did not alter the level of cytokine IL-10 (Fig. 5 D). lncRNA-MEG3 overexpression also increased the percentage of Th17 in CD4 + T cells but did not affect the percentage of Treg cells (Fig. 5 E–F and Figure S1 B). lncRNA-MEG3 overexpression increased mRNA and protein levels of ROR γ t, but had no effect on

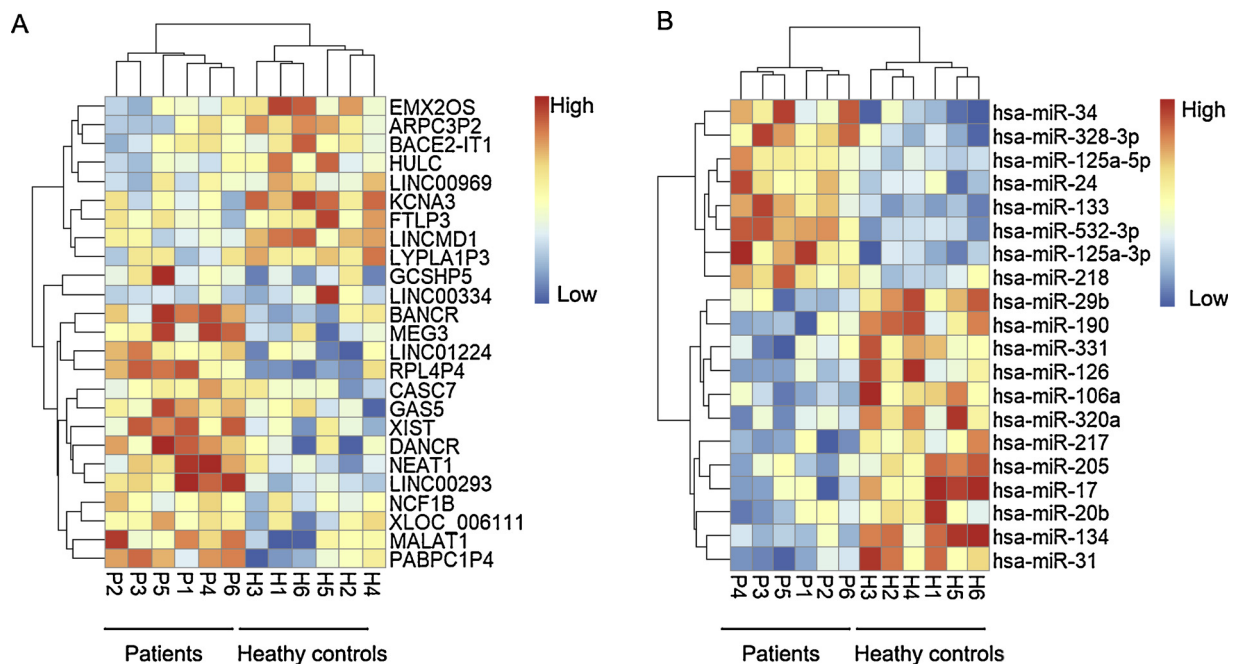


Fig. 2. The expression of selected lncRNAs and microRNAs in CD4 + cells from patients and healthy control were shown in heatmaps. (A) candidate lncRNAs, with a fold change < 0.5 or > 2.0 , which may interact with candidate microRNAs; (B) candidate microRNAs, with a fold change < 0.6 or > 1.5 , which may target on ROR γ or Foxp3.

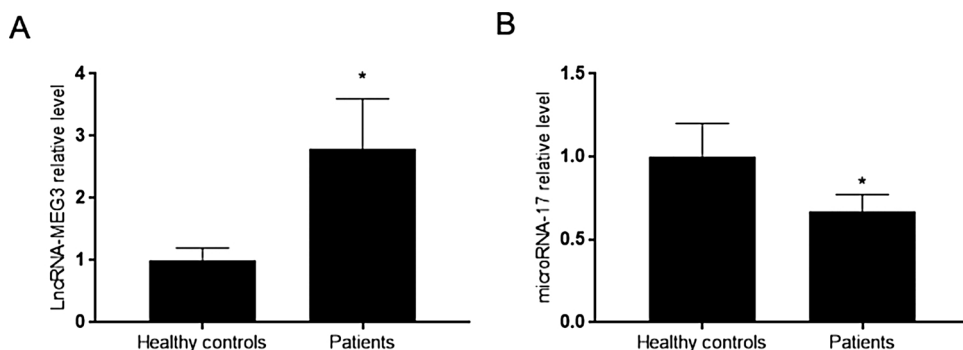


Fig. 3. lncRNA-MEG3 and microRNA-17 levels in CD4 + T cells from patients and healthy controls. (A) the lncRNA-MEG3 level was higher in CD4 + T cells collected from patients with asthma; (B) a lower microRNA-17 level was detected in the CD4 + T cells collected from patients with asthma. * $P < 0.05$ compared with CD4 + T cells collected from healthy controls.

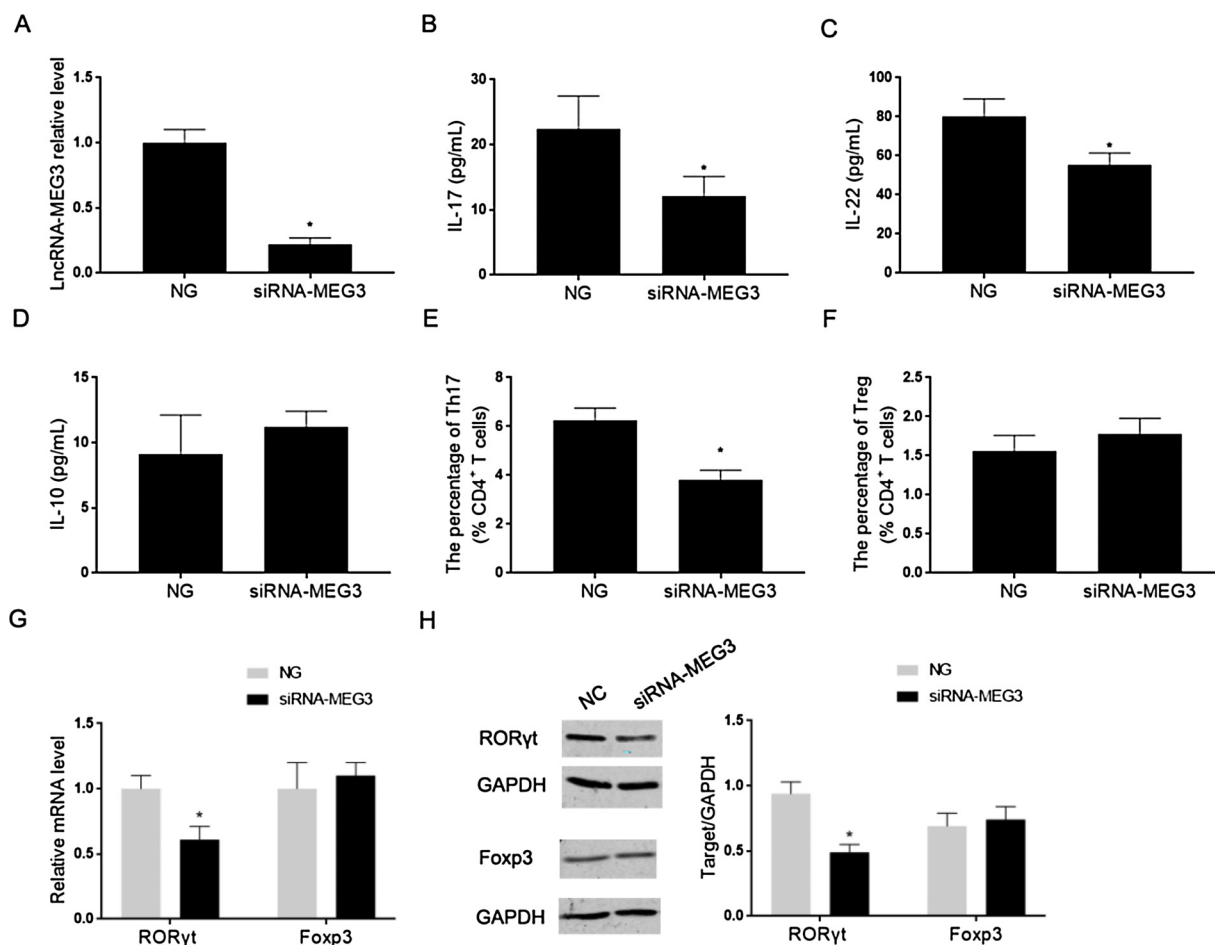


Fig. 4. The effect of lncRNA-MEG3 down-regulation on the alteration of CD4 + T cells from patients with asthma. siRNA effectively silenced the expression of lncRNA-MEG3 in cells (A); After the down-regulation of lncRNA-MEG3, IL-17 and IL-22 levels in the culture supernatant were decreased (B&C), but the level of IL-10 was not changed (D); siRNA-MEG3 transfection reduced the percentage of Th17 but had no effect on Treg ratio (E&F); siRNA-MEG3 reduced the mRNA and protein level of RORγt (G & H). * $P < 0.05$ compared with negative transfection control (NC).

Fox3p (Fig. 5 G and H).

3.5. Interaction between lncRNA-MEG3 and microRNA-17

Since both overexpression and knockdown of lncRNA-MEG3 altered the level of microRNA-17 (Fig. 6 A and B), and bioinformatic analysis also predicted a targeted relationship between them, we further confirmed such interaction by dual luciferase reporter assay and RNA pull-down assay. In dual luciferase reporter assay, only cells co-transfected with lncRNA-MEG3-WT and microRNA-17mimic showed a significant decrease in fluorescence (Fig. 6 C), which confirmed that lncRNA-MEG3 directly targets microRNA-17. In addition, in the RNA pull-down

assay, a clear protein band of Ago2 can be detected in the mixture of biotin-labeled MEG3 and CD4 + T cells extracts by western blot (Fig. 6 D), suggesting an association between MEG3 and the RISC complex. QRT-PCR detected a high level of microRNA-17 in MEG3-pulled-down pellets (Fig. 6 E). These results suggest that lncRNA-MEG3 can regulate microRNA-17 as a ceRNA.

3.6. The effect of microRNA-17 on Treg/Th17 balance of CD4 + T cells

To provide more evidence to prove that the function of lncRNA-MEG3 on Th17 cells was achieved via microRNA-17, we analyzed the effect of microRNA-17 on CD4 + T cells from asthma patients. As our

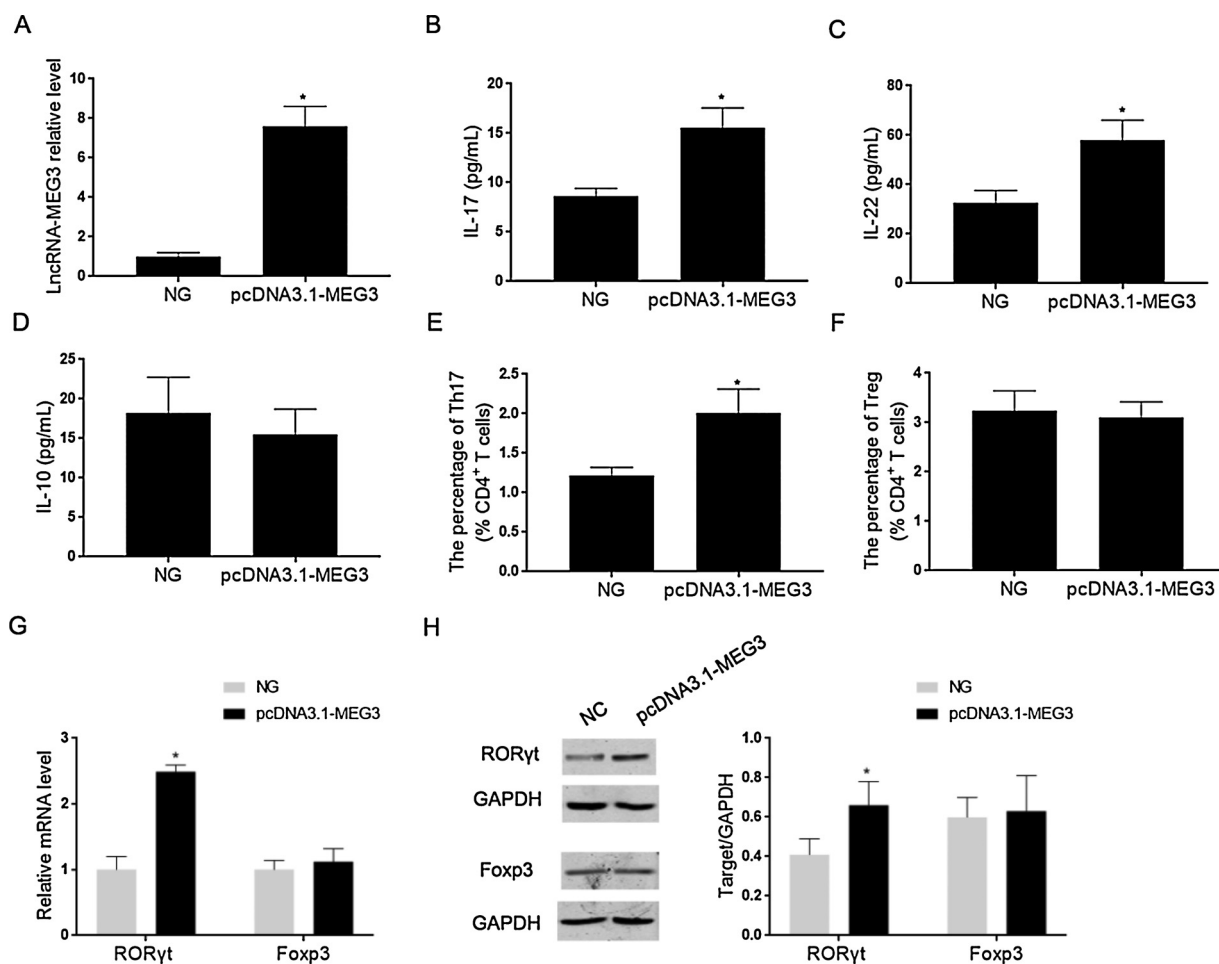


Fig. 5. The effect of lncRNA-MEG3 overexpression on the alteration of CD4⁺ T cells from healthy control. lncRNA-MEG3 was overexpressed by pcDNA3.1-MEG3 transfection (A); After the up-regulation of lncRNA-MEG3, IL-17 and IL-22 levels in the culture supernatant were increased (B&C), but the level of IL-10 was not changed (D); Overexpression of lncRNA-MEG3 increased the percentage of Th17 but had no effect on Treg ratio (E&F); mRNA and protein level of RORγt was enhanced by pcDNA3.1-MEG3 transfection (G & H). *P < 0.05 compared with negative transfection control (NC).

results showed that lncRNA-MEG3 had no effect on the Treg and Foxp3 level, and bioinformatic analysis did not support microRNA-17 targeting Foxp3, in this part, we only explored the effect of microRNA-17 on Th17. microRNA-17 mimic resulted in a similar effect with lncRNA-MEG3 down-regulation. Treatment of CD4⁺ T cells from asthma patients with microRNA-17 mimic (Fig. 7 A) significantly reduced the Th17-associated cytokines IL-17 and IL-22 (Fig. 7 B and C, $P < 0.05$). Th17 percentage was decreased after microRNA-17 overexpression (Fig. 7 D and Figure S1 C, $P < 0.05$). After transfection with microRNA-17 mimic, RORγt was downregulated in both mRNA and protein levels (Fig. 7 E and F). Dual luciferase reporter assay suggested that microRNA-17 can bind directly to the 3' UTR of RORγt mRNA (Fig. 7 G). This evidence suggests that microRNA-17 regulates Treg/Th17 ratio of CD4⁺ T cells in asthma patients by downregulating RORγt.

4. Discussion

Asthma is a disease characterized by uncontrolled airway inflammatory response. Therefore, immune imbalance is the central link in the pathophysiological mechanism of asthma. As an important cell that secretes cytokines and regulates immune function, CD4⁺ T cells play an important role in the development of asthma [21]. Earlier studies on asthma mechanisms focused on Th cells. The Th1/Th2 imbalance has long been recognized as the immunological pathogenesis of asthma, that is, Th1 inhibition and Th2 hyperfunction are the direct cause of airway hyperresponsiveness and chronic inflammation [22].

Briefly, Th1 mainly secrete interferon-γ (INF-γ) that inhibits expansion of inflammatory response and is considered a protective factor for asthma; while Th2 cells mainly secrete IL-4, IL-5, IL-9, etc, and Th2 activation is thought to increase the adverse effects of asthmatic inflammatory responses [23,24]. Recent studies have found phenomena contrary to this theory, and Th1/Th2 imbalance can't explain all phenomena in the development of asthma. For example, Th1 is not always served as a protective factor in all condition. Increased Th1 activity may aggravate asthma symptoms under certain circumstances [9]. Moreover, in some clinical and animal studies, measures that corrected Th1/Th2 imbalance by increasing Th1 ratio failed to achieve the desired therapeutic effect [25]. Recent studies have updated our understanding on the immunological pathogenesis of asthma: in the development of asthma, Treg/Th17 imbalance is more important than Th1/Th2 imbalance and is more closely related to asthma progression and severity [26]. This view has been confirmed in both animal models and asthma patients. These evidence suggest an important role of Treg/Th17 imbalance in the pathogenesis of asthma and its potential as a therapeutic target [27]. However, much remains to be clarified about the mechanism of Treg/Th17 imbalance before implementing clinical applications. Foxp3 is currently known as a key transcription factor that affects Treg differentiation and function. Differentiation of Th17 is specifically regulated by the nuclear orphan receptor-γt (RORγt) [28]. Multiple pieces of evidence have indicated abnormal expression of Foxp3 and RORγt in asthma, but the upstream regulatory mechanisms have not been fully understood. Studies have shown that lncRNA-MEG3

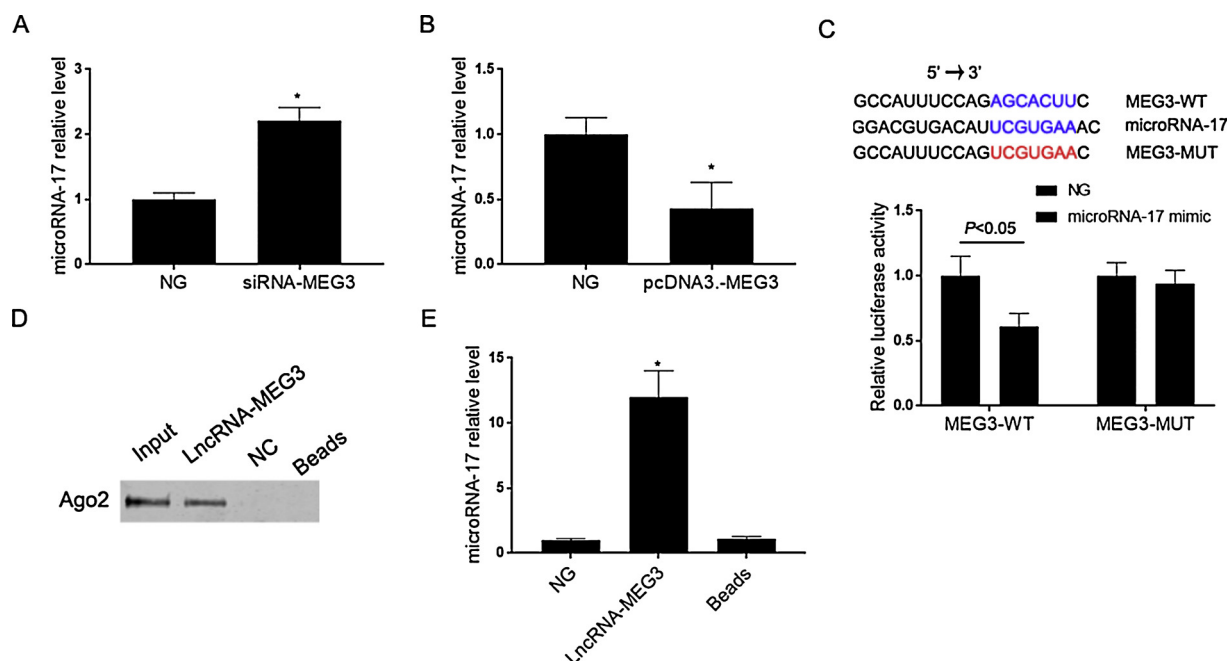


Fig. 6. LncRNA-MEG3 could inhibit the level of microRNA-17 as a ceRNA. Alteration of LncRNA-MEG3 induced the level changes of microRNA-17 (A & B); Dual-luciferase reporter assay was used to confirm the binding of LncRNA-MEG3 and microRNA-17 (C). Ago protein bands were detected in CD4 + T cells extracts incubated with LncRNA-MEG3 (D) and the higher microRNA-17 level was detected in LncRNA-MEG3-pulled-down pellets (E). * $P < 0.05$ compared with negative transfection control (NC).

regulates miR-125a-5p as a ceRNA in thrombocytopenic purpura, which affects the expression of Foxp3 and ROR γ t, and ultimately regulates Treg/Th17 balance [20]. Inspired by this study, we hypothesized that some lncRNAs in asthma may regulate Treg/Th17 balance in asthma through similar mechanisms.

lncRNA is involved in the transcriptional, post-transcriptional and epigenetic regulation of gene expression, and is thus involved in cell development, differentiation and growth. Recent studies indicate that lncRNA is involved in the development and activation of CD4 + T cells and may be involved in the maintenance of immune homeostasis [29]. Qiao reported that LncRNA-DQ786243 can regulate Foxp3 and affect the differentiation of Treg cells [30]. Spurlock et al. demonstrated the changes in lncRNA expression and function during T helper cell differentiation [31]. Abnormal expression of lncRNAs in asthma has been confirmed, and more importantly, these abnormally expressed lncRNAs are involved in the regulation of CD4 + T cells. For example, Zhu et al analyzed lncRNA expression profile of whole blood in patients with eosinophilic asthma and found some abnormally expressed lncRNAs in patients, some of which were believed to be involved in regulation of early differentiation of T-helper cells [17]. Some other evidence comes from studies on microRNAs. Wu et al. reported that miR-16 can affect Th17/Treg imbalance in rheumatoid arthritis [32]. Yao et al. found that MicroRNA-155 regulates differentiation of Treg and Th17 by targeted inhibition of SOCS1 [33]. Another study pointed out microRNA-21 is closely related to Th17/Treg ratio in peripheral blood of mice with hepatocellular carcinoma [34]. Although none of this evidence was obtained in asthma, they all suggest that microRNA does participate in the regulation of Th17/Treg balance. If some lncRNAs are capable of adsorbing these microRNAs (ceRNA theory), it's quite possible that these lncRNAs can indirectly regulate Th17/Treg balance.

To verify our hypothesis, we first used microarrays to obtain lncRNA and microRNA expression profiles in peripheral blood CD4 + T cells from asthma patients and healthy individuals. Several previous studies have indicated that lncRNA and microRNA in patients with milder asthma have little difference compared with healthy controls [35]. Therefore, this study only enrolled patients with severe asthma. Using microarrays, differences in lncRNA and microRNA levels have

been detected between peripheral blood CD + T cells from asthma patients and healthy individuals. These lncRNAs and microRNAs may play a role in asthma, but not necessarily all involved in Treg/Th17 regulation. So, we selected microRNAs that can potentially target Foxp3 or ROR γ t, and lncRNAs that can potentially interact with these microRNAs basing on bioinformatic evidence. Finally, we chose LncRNA-MEG3/microRNA-17 for further validation because bioinformatic evidence suggested a possible interaction between the lncRNA-MEG3/microRNA-17 and ROR γ t to be a potential target gene of microRNA-17. Moreover, previous studies have reported regulation of Treg/Th17 balance by LncRNA-MEG3 [20]. Firstly, expression of LncRNA-MEG3 and microRNA-17 in peripheral blood CD4 + T cells of asthma patients was verified by qRT-PCR, and it was found that the level of LncRNA-MEG3 increased and the level of microRNA-17 decreased. This result was in line with microarray results. Then we artificially regulated expression of LncRNA-MEG3 and found that proportion of Th17 cells, level of ROR γ t, and the level of Th17-associated cytokines in CD4 + T cells were all affected. These results suggest that LncRNA-MEG3 can tilt Treg/Th17 balance in the Th17 direction in asthma. We also verified the targeted effect of LncRNA-MEG3 on microRNA-17, the targeted effect of microRNA-17 on ROR γ t, and the effect of microRNA-17 on the function of Th17 cells. Based on this evidence, we believe that LncRNA-MEG3 can act as a ceRNA to inhibit microRNA-17 and consequently upregulated ROR γ t, leading to Th17 hyperfunction and decreased Treg/Th17 ratio. But unlike the findings by Li et al. [20], we found no significant effects of LncRNA-MEG3 on Treg cells. Li et al found that lncRNA-MEG3 can affect both Th17 and Treg in thrombocytopenic purpura. Firstly, this difference was not surprising. Bioinformatic analysis predicted that microRNA-17 does not target on Foxp3. Hence the regulatory effect of microRNA-17 on Treg was not expected. Actually, we did not find any candidate microRNA in this study that targets on both ROR γ t and Foxp3. Based on the theory of ceRNA we do not expect that lncRNA in our candidate list can regulated Treg and Th17 simultaneously. This difference may reflect some disease-specific features. The global genomic changes in CD4 + T cells are different in asthma and thrombocytopenic purpura. The regulatory networks of lncRNA are intricate and variant in different diseases. Any of change in

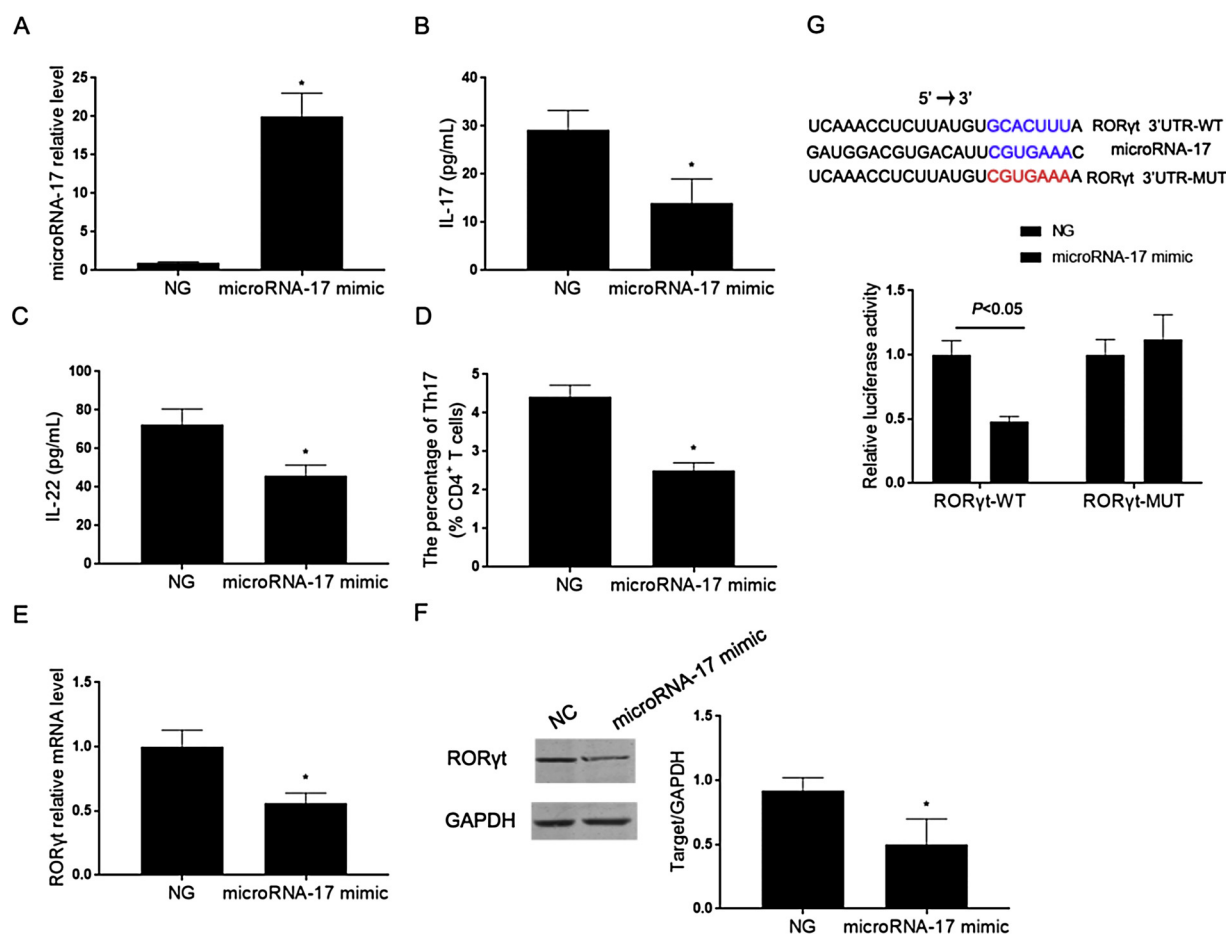


Fig. 7. microRNA-17 regulated Treg/Th17 balance of CD4⁺ T cells from patients via targeting RORγt. (A) Transfection of microRNA-17 mimics induced a high level of microRNA-17 in CD4⁺ T cells collected from patients. up-regulated of microRNA-17 boosted the level of IL-17 and IL-22 (B & C). Meanwhile, the percentage of Th17 decreased (D). microRNA-17 could inhibit the mRNA and protein expression of RORγt (E&F). Dual-luciferase reporter assay indicates that microRNA-17 could target on RORγt mRNA directly. **P* < 0.05 compared with negative transfection control (NC).

this network could induce different phenotype alteration during disease. It should be noted that although Li et al. found that lncRNA-MEG3 affected RORγt and Foxp3 expression via microRNA-125a-5p, they didn't prove direct targeting of RORγt and Foxp3 mRNA by microRNA-125a-5p, which means the effect of microRNA-125a-5p on RORγt and Foxp3 may be indirect in thrombocytopenic purpura. In this study, when screening for candidate genes, the microRNAs that directly target RORγt or Foxp3 were retained. And microRNAs that were regulated by lncRNAs but had no direct effect on RORγt or Foxp3 were ignored. it means we missed some microRNA which can regulated Treg/Th1 in a RORγt- or Foxp3-independent manner. We only designed our experiment based on ceRNA theory, it is the limitation of our study. In addition, besides lncRNA-MEG3 and microRNA-17, some other lncRNAs and microRNAs also found to conform to the regulatory pattern proposed in this study. For example, it was found that lncRNA-HULC is reduced in peripheral blood CD4⁺ T cells from asthma patients, while one of its potential target miR-218 is upregulated, and miR-218 targets mRNA of Foxp3, such lncRNA-miRNA pairs are also worthy of validation in future studies. Another weaknesses of our study includes that we did not design experiment in vivo (animal model) and we did not considered whether anti-asthmatic agents could regulate lncRNA-MEG3/microRNA-17.

In conclusion, this study found abnormalities in lncRNA and microRNA in peripheral blood CD4⁺ T cells from asthma patients. lncRNA-MEG3 can inhibit microRNA-17 as a ceRNA, thereby regulating RORγt and ultimately affecting Treg/Th17 balance in asthma. The lncRNA/microRNA axis may reveal the immunological mechanisms of asthma and have potential application in clinical treatment and

diagnosis of the disease.

Acknowledgement

This work was funded by the Fundamental Research Funds for The Central Universities (Grant number: YG1805010) and the Funds for Medical Science Development of Nanjing (Grant number: YKK17068).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2018.12.080>.

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Table S1 The sequences of used primers in qRT-PCR

Primers	5'-3'
MEG3 forward	GCTCTACTCCGTGGAAGCAC
MEG3 reverse	CAAACCAGGAAGGAGACGAG
Foxp3 forward	GGGTAGCCATGGAAACAGCA
Foxp3 reverse	TCGCATGTTGTGGAAGTTGAAGTAG
RORyt forward	CTGCAAGACTCATCGCCAAAG
RORyt reverse	TTCCACATGCTGGCTACACA
GAPDH forward	CCTGCACCACCAACTGCTTA
GAPDH reverse	GGCCATCCACAGTCTTCTGAG
microRNA-17 forward	CGGCGGCAAAGTGCTTACAG
microRNA-17 reverse	GTGCAGGGTCCGAGGT
SNORD-48 forward	TGATGATGACCCCAGGTAAGTCTGAGTG
SNORD-48 reverse	GTCAGAGCGCTGCGGTGATGGCATCAGC

Table S2 Clinical characteristics of the subjects included in this study

	Patients with asthma	Healthy control
Sex (Male/Female)	32/20	32/13
Median Age	42	35
BMI (kg/m ²)	26.1±3.9	23.4±6.6
FEV ₁ (%)	57.8±4.6	96.3±4.4
FEV ₁ /FVC ration (%)	59.9±4.1	81.3±5.6
Reversibility (%)	15.5±8.9	-
Median inhaled corticosteroid dose (µg/d)	2300	-
Smoking status (nonsmoker/ex-smoker)	45/7	35/10