

C1q/TNF-related protein-9 alleviates airway inflammation in asthma

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ABSTRACT

Backgrounds: Asthma is characterized as inflammatory disorder in the respiratory system with increasing tendency. Most of the asthma patients suffered from the disease since childhood. Thus, developing novel therapeutic targets of childhood asthma is necessary. Here, we conducted the present study to investigate the effects of CTRP9 (C1q tumor necrosis factor-related protein 9), a newly identified anti-inflammatory factor, on asthma.

Methods: Sixty asthmatic children (30 moderate and 30 mild) were recruited. The mRNA level of CTRP9 in peripheral blood mononuclear cells (PBMCs) and protein level of CTRP9 in serum and induced sputum (IS) samples from asthma patients and healthy controls (HCs) were measured by qPCR and ELISA, respectively. The anti-inflammatory effects of CTRP9 was determined *in vitro* and potential therapeutic effect on asthma was evaluated in mouse model.

Results: The mRNA and protein levels of CTRP9 was significantly down-regulated in asthmatics than HCs. Furthermore, the expression level of CTRP9 was negatively correlated with the expression of TNF- α , IL-1 β , and IL-6 in PBMCs. The CTRP9 significantly suppressed the expression of pro-inflammatory factors in PBMCs and sputum cells from asthma patients *in vitro*. And delivering CTRP9 into mouse model of asthma showed disease alleviation.

Conclusion: Our data here indicated that CTRP9 may alleviate airway inflammation and remodeling in asthma.

1. Introduction

Asthma is characterized as a chronic inflammatory disorder in the respiratory system with increasing tendency [1]. Most of the asthma patients suffered from the disease since childhood [1]. Thus, developing novel therapeutic targets of childhood asthma is necessary [2].

Type 1, 2 and 17 T helper lymphocyte (Th1, Th2, Th17) are the major contributors to asthma development, along with hyper-responsiveness of the bronchi, obstruction of the airflow and remodeling of the airway [3,4]. Pro-inflammatory cytokines, such as IL-4, IL-5, IL-13, IL-33, and TNF- α , are also positively related to the disease activities of asthma [3,5–8]. Furthermore, it has been demonstrated that the IL-37, an anti-inflammatory cytokine, has a protective role in the mouse model of asthma [9]. Thus, targeting the inflammation pathway might be a promising candidate for asthma treatment.

CTRP9 (C1q tumor necrosis factor-related protein 9) is a newly discovered adipokine, the closest paralog of adiponectin. After proteolytic cleavage, it can release the globular domain (gCTRP9) that serves as the major circulatory isoform. Upon binding to adiponectin

receptor 1 (AdipoR1) and N-cadherin, CTRP9 can activate a variety of signaling pathways to regulate the metabolism of glucose and lipid, and also suppress the inflammation [10]. It has been demonstrated that the CTRP9 could activate the AMPK/ACC (acetyl-CoA carboxylase) signaling, leading to inhibition of NF- κ B nuclear translocation and pro-inflammatory cytokine secretion [11–16].

Here, we conducted the present study to investigate the effects of CTRP9 with anti-inflammation functions on asthma. And our data here indicated that CTRP9 may alleviate airway inflammation and remodeling in asthma through suppressing the activation of NLRP3 inflammasome.

2. Methods and materials

2.1. Patients

Sixty asthmatic children (30 moderate and 30 mild) were recruited from the The Second Hospital of Jilin University, followed the Global Initiative for Asthma guidelines [17]. This study was approved by the

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Table 1
Clinical characteristics.

Characteristics	Patients		Healthy controls
	Mild	Moderate	
Number	30	30	30
Gender (female/male)	16/14	15/15	16/14
Height (cm)	138.5 (103–160)	136.8 (101–163)	134.9 (100–162)
Body weight (kg)	22.6 (15–38)	21.9 (13–36)	24.2 (18–36)
Age (range), years	8 (4–13)	8 (4–12)	7.5 (4–14)
Positive skin test (%)	66.8	68.2	–
FVC (%)	78.4(55–123)	81.8(58–129)	–
FEV1(%)	93.7(51–122)	96.9(55–128)	–
FEV1/FVC	0.9(0.8–1.0)	0.9(0.8–1.0)	–
Total IgE (kU/L)	296.7 ± 197	385 ± 227*	–

FVC: forced vital capacity; FEV1: forced expiratory volume in 1 s; * $P < 0.05$.

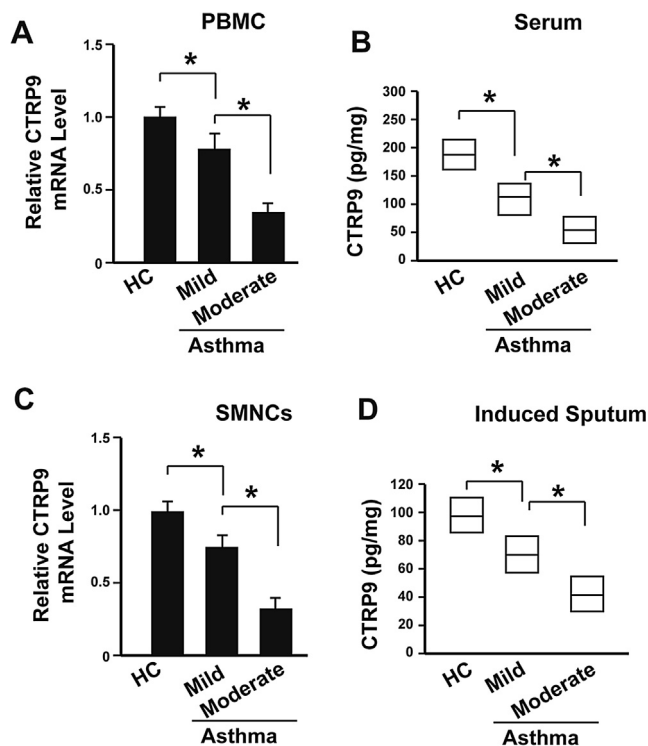


Fig. 1. Down-regulated CTRP9 in asthmatic pediatric patients. (A) Relative mRNA levels of CTRP9 in PBMCs from mild ($n = 30$), moderate ($n = 30$) asthmatic patients and also the healthy controls (HC, $n = 30$) were determined by qPCR. (B) Serum levels of CTRP9 in mild ($n = 30$), moderate ($n = 30$) asthmatic patients and also the healthy controls (HC, $n = 30$) were determined by ELISA. (C) Relative mRNA levels of CTRP9 in induced sputum cells from mild ($n = 30$), moderate ($n = 30$) asthmatic patients and also the healthy controls (HC, $n = 30$) were determined by qPCR. (D) Protein levels of CTRP9 in induced sputum from mild ($n = 30$), moderate ($n = 30$) asthmatic patients and also the healthy controls (HC, $n = 30$) were determined by ELISA. "Asthma" group indicated the total asthmatic patients ($n = 60$) including the mild and moderate asthmatic patients. * indicates $P < 0.05$.

ethics committees of the The Second Hospital of Jilin University, and informed consent was obtained from all participants. Children with asthma were treated with inhaled glucocorticoids (Table 1). Thirty healthy children (4–13 years old) without any respiratory allergic manifestations were recruited as controls.

2.2. Sputum induction and collection

Patients firstly inhaled 200 mg salbutamol, and then 5% NaCl solution. The sputum was collected and mixed with 0.1% dithiothreitol

(1:1 in volume) completely. The mixture was then centrifuged at 1500g for 30 min, and the supernatant was subjected to cytokine measurement.

2.3. Cell isolation and culture

SMNCs (sputum mononuclear cells) and PBMCs (peripheral blood mononuclear cells) were isolated with Ficoll density gradient centrifugation and cultured with RPMI1640 plus 10% FBS and 1% penicillin/streptomycin. Cells were plated into 24-well plates at a concentration of 5×10^4 cells/well and stimulated with 100 ng/ml lipopolysaccharides (LPS) (Sigma) for 24 h, followed by LPS plus different concentration of CTRP9 (R&D Systems) treatment for another 48 h. Cell culture supernatants were subjected to cytokine secretion analysis with ELISA. And cells were lysed to analysis the mRNA levels of the cytokines with qPCR.

2.4. Quantitative PCR

Total RNA was extracted with Trizol Reagent (Thermo Scientific). The cDNA was prepared by All-in-One™ First-Strand cDNA Synthesis Kit (Genecopoeia, USA) according to the manufacturer's instructions. Quantitative PCR was performed with SYBR Green qPCR Mix (Takara). The relative gene expression level was determined by $2^{-\Delta\Delta Ct}$ method and the GAPDH was used as the internal control. Primers sequences were as follows: CTRP9, forward: 5'-TGAAGCCAGTGCCTGTCTGT-3'; reverse: 5'-AGCCGACTCGAAGCTGGAGGT-3'; IL-1 β , forward: 5'-GCAATGAGGATGACTTGTCTTTG-3'; reverse: 5'-CAGAGGTCCAG GTCCTGGAA-3'; TNF- α , forward: 5'-ACCTCTCTCTAATCAGCCCTCT-3'; reverse: 5'-GGGTTTGCTACAACATG GGCTA-3'; IL-17, forward: 5'-ACCAATCCAAAAGGTCCTC-3'; reverse: 5'-TGGATGGGGACAGAGTTCAT-3'; IL-6, forward: 5'-AGCCACTCACCTCTTCAGAAC-3'; reverse: 5'-ACATG TCTC TTTTCTCAGGGC-3'; IL-17, forward: 5'-CCGGGACTGTGATGGTCAAC-3' and reverse: 5'-GCACCTTGCTCCAGATCA-3'; GAPDH forward: 5'-CATCAAGAAGGTGGTGA-3'; GAPDH reverse: 5'-TGTTGAA GTCAGAGGAGA-3'.

2.5. Animal studies

BALB/c mice (female, 4–6-week-old) were obtained from South Laboratory Animal Center, China. The mice were kept in specific pathogen free regions. Mouse model of asthma was established by intraperitoneal injection of 100 μ g OVA (Ovalbumin, Sigma) plus 1 mg aluminum hydroxide per mouse on days 1 and 14, followed by intranasal instillation of OVA (150 μ g/mouse) once per day from day 21 to 28 [18]. Recombinant human (rh) CTRP9 protein (1 μ g per mouse, R&D Systems) was administrated by tail-vein injection every two days from day 14–28. The animal experimental procedures were approved by the Institutional Animal Care and Use Committee of The Second Hospital of Jilin University.

2.6. Airway hyper-responsiveness measurement

The AHR (airway hyper-responsiveness) was assessed at day 29 with whole-body plethysmography (Buxco Electronics). The mice were treated with different concentration of aerosolized methacholine (0, 6.25, 12.5, 25, and 50 mg/mL) for 1 min, followed by 3 min of recording of enhanced pause (Penh).

2.7. Cell number assessment in bronchoalveolar lavage fluid

Mice were sacrificed after the AHR assessment. The lungs were perfused with 1 mL cold PBS, and the cell pellet re-suspended in PBS for cell number counting after centrifugation (400g for 5 min) and Wright's staining (Sigma).

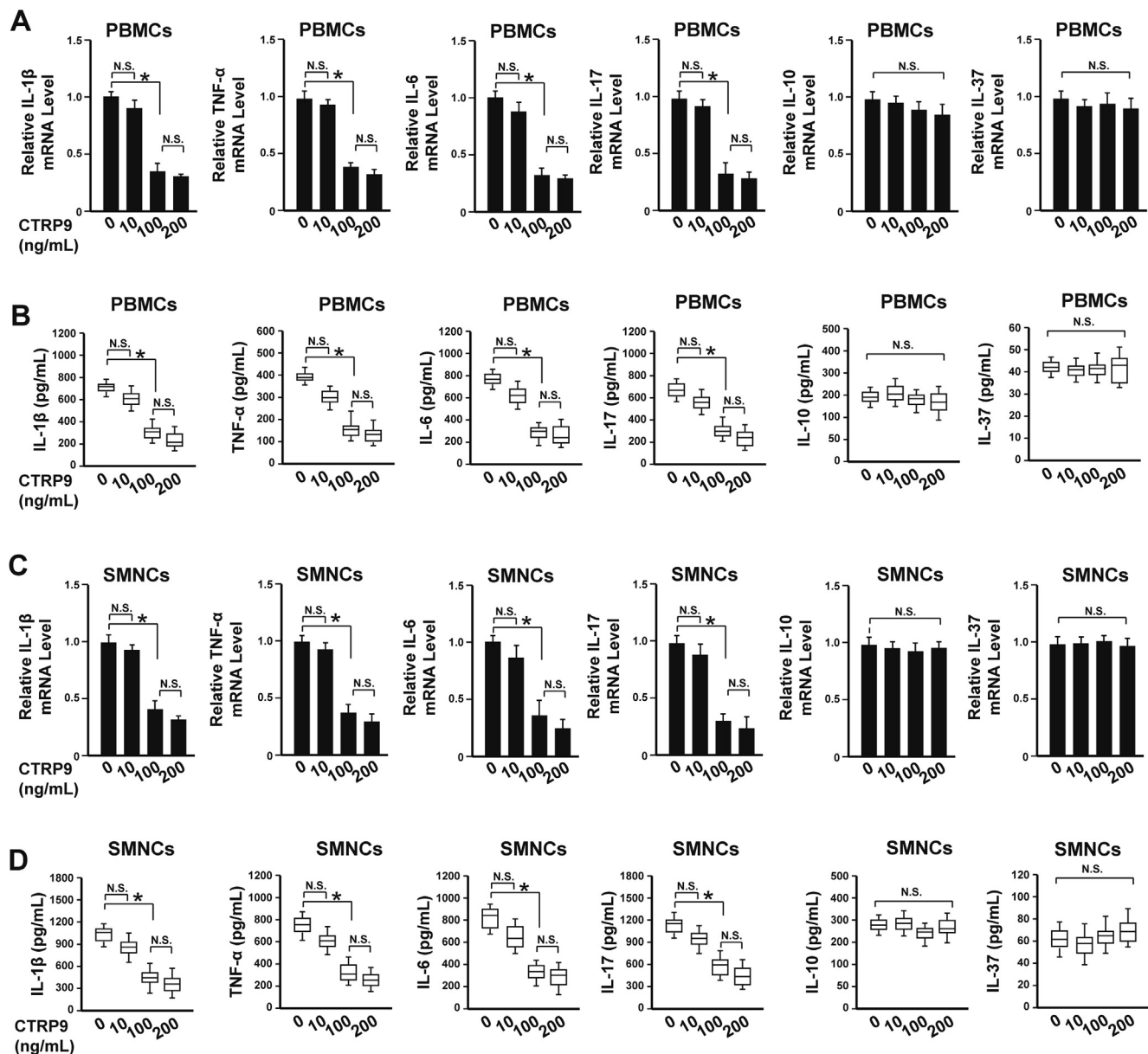


Fig. 2. CTRP9 suppressed the expression of pro-inflammatory cytokines in PBMCs and SMNCs. PBMCs and SMNCs were treated with LPS for 24 h, followed by LPS plus different concentration of CTRP9 treatment for 48 h. (A) Relative mRNA levels of pro- and anti-inflammatory genes in PBMCs from moderate asthmatic patients ($n = 10$) treated with CTRP9 were determined by qPCR. (B) The levels of pro- and anti-inflammatory cytokines in cell culture medium of PBMCs from moderate asthmatic patients ($n = 10$) treated with CTRP9 were determined by ELISA ($n = 10$). (C) Relative mRNA levels of pro- and anti-inflammatory genes in SMNCs from moderate asthmatic patients ($n = 10$) treated with CTRP9 were determined by qPCR. (D) The levels of pro- and anti-inflammatory cytokines in cell culture medium of SMNCs from moderate asthmatic patients ($n = 10$) treated with CTRP9 were determined by ELISA. PBMCs: peripheral blood mononuclear cells; SMNCs: sputum mononuclear cells; N.S. indicates no significant difference; * indicates $P < 0.05$.

2.8. Histological analysis

The lungs were fixed with 10% formalin, dehydrated, embedded in paraffin, sectioned and stained with Hematoxylin and Eosin (H&E) kit (Abcam).

2.9. Statistics

Data were expressed as mean ($\pm$ SE) and analyzed by SPSS software package (SPSS Inc). Unpaired Student's t test and one way ANOVA with Post Hoc (Tukey) test were used as appropriate to assess the statistical significant of difference. P values under 0.05 were considered statistically significant.

3. Results

3.1. Down-regulated CTRP9 in asthmatic pediatric patients

To study the role of CTRP9 in asthmatic pediatric patients, its expression levels were determined in PBMC and serum from the asthmatic patients (30 moderate and 30 mild) and healthy controls (HC). Data showed that the mRNA and protein levels of CTRP9 were significantly reduced in the asthmatic patients when comparing with healthy controls (Fig. 1 and B). Furthermore, the patients with moderate asthma had lower level of CTRP9 than patients with mild asthma (Fig. 1A and B). And this was further confirmed in the induced sputum cells (Fig. 1C and D). Thus the CTRP9 was down-regulated in asthmatic pediatric patients.

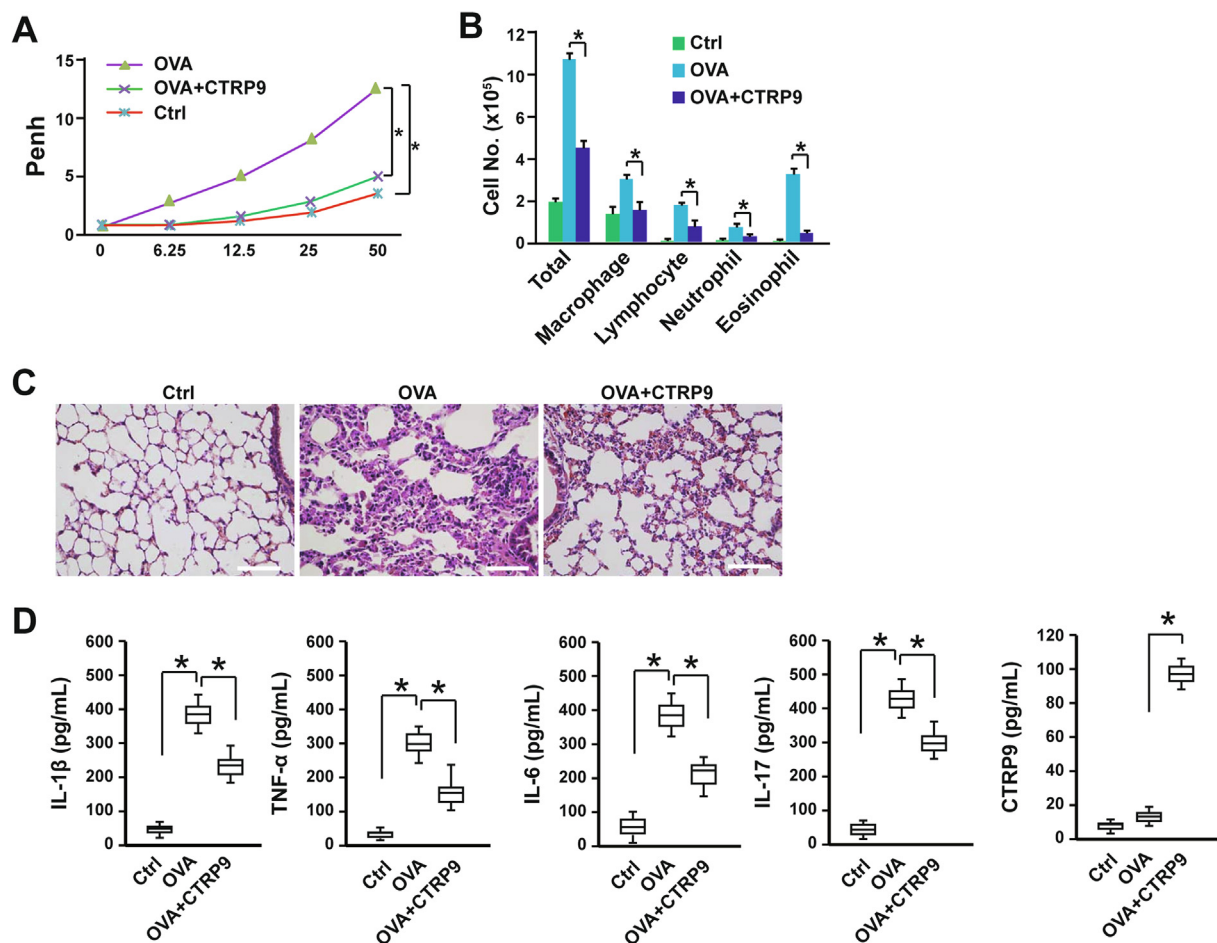


Fig. 3. CTRP9 alleviated asthmatic symptoms in the mouse model of asthma. The mice model of allergic asthma was established by OVA treatment. The mice were treated with tail-vein injection of recombinant human CTRP9 protein ($n = 8$ for each group). (A) AHR (airway hyper-responsiveness) in response to inhaled methacholine was analyzed. (B) CTRP9 suppressed OVA-induced inflammatory cell recruitment in BALF. (C) Representative images of HE staining of lung tissues. (D) The serum levels of pro-inflammatory cytokines were determined by ELISA ($n = 8$). * indicates $P < 0.05$; Ctrl: control; OVA: Ovalbumin; Scale bar 200 μm .

3.2. CTRP9 suppressed the expression of pro-inflammatory cytokines

To investigate the potential role of CTRP9 on asthma development, its anti-inflammatory effect was assessed in PBMCs (peripheral blood mononuclear cells) from moderate asthmatic patients ($n = 10$). The PBMCs were firstly stimulated with LPS, and then different concentration of recombinant CTRP9 was administrated. Data showed that the recombinant CTRP9 protein could significantly suppress the expression of pro-inflammatory cytokines, including IL-1 β , TNF- α , IL-6 and IL-17 (Fig. 2A). Furthermore, the secreted pro-inflammatory cytokines (IL-1 β , TNF- α , IL-6 and IL-17) were also inhibited by CTRP9 protein (Fig. 2B). This was further confirmed in SMNCs (sputum mononuclear cells) (Fig. 2C and D). Thus, the CTRP9 suppressed the expression of pro-inflammatory cytokines.

3.3. CTRP9 alleviated asthmatic symptoms in the mouse model of asthma

To study the potential therapeutic application of CTRP9, the mouse model of allergic asthma was established by OVA treatment. Data showed that tail-vein injection of recombinant human CTRP9 protein could significantly suppress the OVA-induced AHR (airway hyper-responsiveness) (Fig. 3A). Furthermore, the CTRP9 reduced the number of inflammatory cells in mouse bronchoalveolar lavage fluid (BALF) (Fig. 3B). And this was further confirmed by HE staining (Fig. 3C). Furthermore, the serum levels of pro-inflammatory cytokines (IL-1 β , TNF- α , IL-6 and IL-17) were also reduced by CTRP9 (Fig. 3D).

Therefore, the CTRP9 could alleviate OVA-induced asthmatic symptoms in the mouse.

4. Discussion

Asthma is a chronic inflammatory disorder in the respiratory system with increasing tendency [1,19]. For pediatric asthma treatment, corticosteroids Inhalation is the only effective therapy. However, corticosteroids would induce bone density reduction, affecting the children growth [19]. Thus, developing more effective therapy for pediatric asthma is urgent and necessary. Different types of immune cells and pro-inflammatory cytokines are involved into the asthma development [3–8,20,21]. Thus, immune suppression might be a promising strategy for asthma treatment. It has been demonstrated that the anti-inflammatory cytokine IL-37 could suppress the asthma development [9]. And we demonstrated here that CTRP9, a newly identified anti-inflammatory factor, could alleviate airway inflammation in asthma.

Our data here demonstrated that the mRNA and protein levels of CTRP9 was significant down-regulated in asthmatics than HCs. The CTRP9 significantly suppressed the expression of pro-inflammatory factors in PBMCs and sputum cells from asthma patients *in vitro*. And delivering CTRP9 into mouse model of asthma showed disease alleviation. Previous studies showed that the IL-37, another important anti-inflammatory cytokine, was also decreased in pediatric patients with asthma and had therapeutic effects on mouse model of asthma [18,22].

In summary, our data here indicated that CTRP9 may alleviate

airway inflammation in asthma.

5. Disclosure of potential conflicts of interest

The authors declare that they have no competing interests.

6. Data accessibility statement

All data have been presented in the figures. And other related information are available under request to the corresponding author.

CRedit authorship contribution statement

Min Qian: Formal analysis, Investigation, Writing - original draft. **Qingxiao Yang:** Methodology, Formal analysis, Validation, Software. **Jindong Li:** Methodology, Validation. **Bin Zhao:** Data curation, Resources. **Yunfeng Zhang:** Methodology, Validation, Resources. **Yinghao Zhao:** Investigation, Supervision, Project administration, Funding acquisition, Writing - review & editing.

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