

Interleukin-36 receptor antagonist alleviates airway inflammation in asthma via inhibiting the activation of Interleukin-36 pathway

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ABSTRACT

Backgrounds: Asthma is characterized as an 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 pediatric asthma is necessary. Here, we conducted the present study to investigate the effects of IL-36RN (Interleukin-36 receptor antagonist), a newly identified anti-inflammatory factor, on asthma.

Methods: Sixty asthmatic children (30 moderate and 30 mild) were recruited. The levels of IL-36RN in peripheral blood mononuclear cells (PBMCs), serum and induced sputum (IS) samples from asthma patients and healthy controls (HCs) were measured by qPCR and ELISA. The anti-inflammatory effects of IL-36RN were determined *in vitro* and potential therapeutic effect on asthma was evaluated in the mouse model of asthma.

Results: The mRNA and protein levels of IL-36RN were significant down-regulated in asthmatics than HCs. The IL-36RN significantly suppressed the expression of pro-inflammatory factors in PBMCs and sputum cells from asthma patients *in vitro*. And delivering IL-36RN into the mouse model of asthma showed disease alleviation. Pathway analysis showed that the IL-36RN may alleviate airway inflammation in asthma through suppressing the activation of IL-36 pathway.

Conclusion: Our data here indicated that IL-36RN may alleviate airway inflammation in asthma through suppressing the activation of IL-36 pathway.

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 17T helper lymphocytes (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 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.

Here, we conducted the present study to investigate the effects of IL-36RN (Interleukin-36 receptor antagonist), a newly identified anti-

inflammatory factor [10], on asthma. And our data here indicated that IL-36RN may alleviate airway inflammation in asthma through suppressing the activation of IL-36 pathway.

2. Materials and methods

2.1. Patients

Sixty asthmatic children (30 moderate and 30 mild) were recruited from the Department of Pediatrics, First Peoples' Hospital of Shangqiu City, followed the Global Initiative for Asthma guidelines [11]. This study was approved by the ethics committees of the First Peoples' Hospital of Shangqiu City (FPHSC2015DP085), and informed consent was obtained from all participants (both childrens' assent and the permission of their parents). Children with asthma were treated with inhaled glucocorticoids (Table 1). Thirty healthy children (4–12 years old) without any respiratory allergic manifestations were recruited as

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Table 1
Clinical characteristics.

Characteristics	Patients	Healthy controls
Number	60	30
Gender (female/male)	32/28	16/14
Age (range), years	8 (4–14)	7.5 (4–12)
Positive skin test (%)	67.5	–
FVC (%)	81.5(56–126)	–
FEV1(%)	96.5(52–126)	–
FEV1/FVC	0.9(0.8–1.0)	–

FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s.

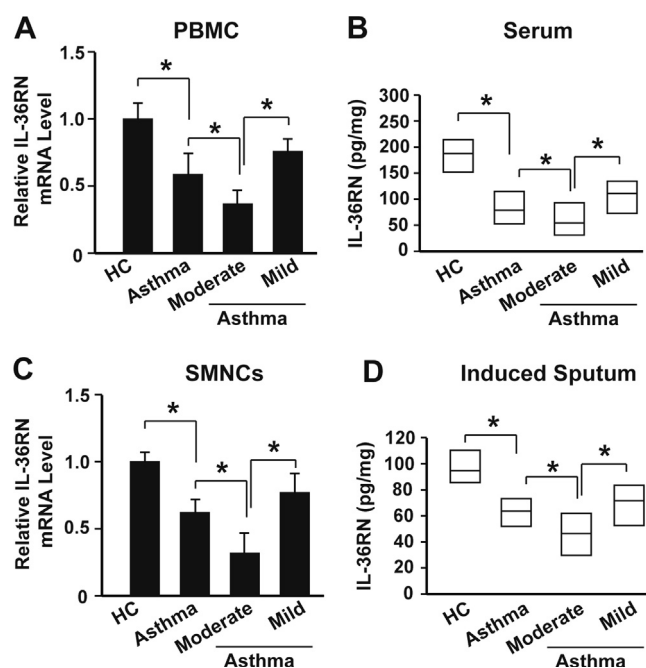


Fig. 1. Down-regulated IL-36RN in asthmatic pediatric patients. (A) Relative mRNA levels of IL-36RN in PBMCs from mild ($n = 30$), moderate ($n = 30$) asthmatic patients and also the healthy controls (HC, $n = 30$) were determined by qPCR. (B) Serum levels of IL-36RN in mild ($n = 30$), moderate ($n = 30$) asthmatic patients and also the healthy controls (HC, $n = 30$) were determined by ELISA. (C) Relative mRNA levels of IL-36RN 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 IL-36RN 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 $P < 0.05$.

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 1500 g 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 IL-36RN (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. Primer sequences were as follows: IL-36RN, forward: 5'-TGAAGCCAGTGCCTGTCATGT-3'; reverse: 5'-AGCCGACTCGAAGCTGGAGGT-3'; IL-1 β , forward: 5'-GCAATGAGGAT GACTTGTCTTTG-3'; reverse: 5'-CAGAGGTCCAG GTCCTG GAA-3'; TNF- α , forward: 5'-ACCTCTCTCTAATCAGCCCTCT-3'; reverse: 5'-GGGTTTGC TACAACATG GGCTA-3'; IL-17, forward: 5'-ACCAATCC CAAAAGGTCC TC-3'; reverse: 5'-TGGATGGGGACAGATTCAT-3'; IL-6, forward: 5'-AGCCA CTCACCTCTTCAAG-3'; reverse: 5'-ACATGTCTC CTTTCTCAGGGC-3'; IL-17, forward: 5'-CCCGGACTGTGATGGTCAAC-3' and reverse: 5'-GCACT TTGCCTCCAGATCA-3'; GAPDH forward: 5'-CATCAAGAAGGTGGTGAA-3'; GAPDH reverse: 5'-TGTGAAGTCAG AGGAGA-3'.

2.5. Enzyme-Linked immuno sorbent assay (ELISA)

ELISA was performed with ELISA Kits (Abcam) according to the manufacturer's instructions.

2.6. 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. A 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 [12]. Recombinant human (rh) IL-36RN protein (1 μ g per mouse, R&D Systems) was administrated by tail-vein injection every two days from day 14 to day 28. The animal experimental procedures were approved by the Institutional Animal Care and Use Committee of First Peoples' Hospital of Shangqiu City.

2.7. 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).

2.8. 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 was re-suspended in PBS for cell number counting after centrifugation (400 g for 5 min) and Wright's staining (Sigma).

2.9. Histological analysis

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

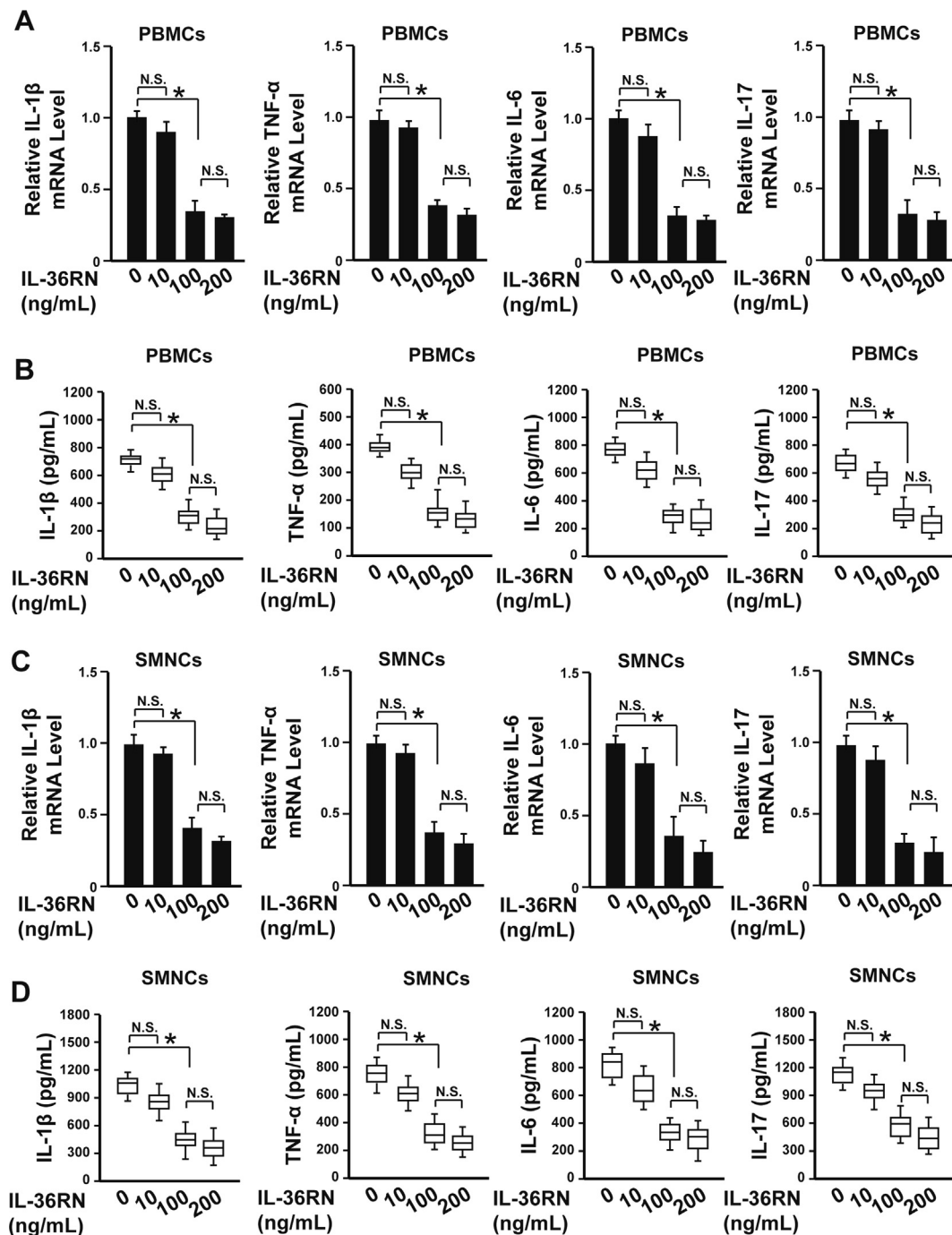


Fig. 2. IL-36RN suppressed the expression of pro-inflammatory cytokines in PBMCs and SMNCs. PBMCs and SMNCs were treated with LPS for 24 h, followed by LPS plus different concentration of IL-36RN treatment for 48 h. (A) Relative mRNA levels of pro-inflammatory genes in PBMCs from moderate asthmatic patients ($n = 10$) treated with IL-36RN were determined by qPCR. (B) The levels of pro-inflammatory cytokines in cell culture medium of PBMCs from moderate asthmatic patients ($n = 10$) treated with IL-36RN were determined by ELISA ($n = 10$). (C) Relative mRNA levels of pro-inflammatory genes in SMNCs from moderate asthmatic patients ($n = 10$) treated with IL-36RN were determined by qPCR. (D) The levels of pro-inflammatory cytokines in cell culture medium of SMNCs from moderate asthmatic patients ($n = 10$) treated with IL-36RN were determined by ELISA. PBMCs: peripheral blood mononuclear cells; SMNCs: sputum mononuclear cells; N.S. indicates no significant difference; * indicates $P < 0.05$.

2.10. 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 were used to assess the statistical significant of difference. P values under 0.05 were considered statistically significant.

3. Results

3.1. Down-regulated IL-36RN in asthmatic pediatric patients

To study the role of IL-36RN 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 IL-36RN were

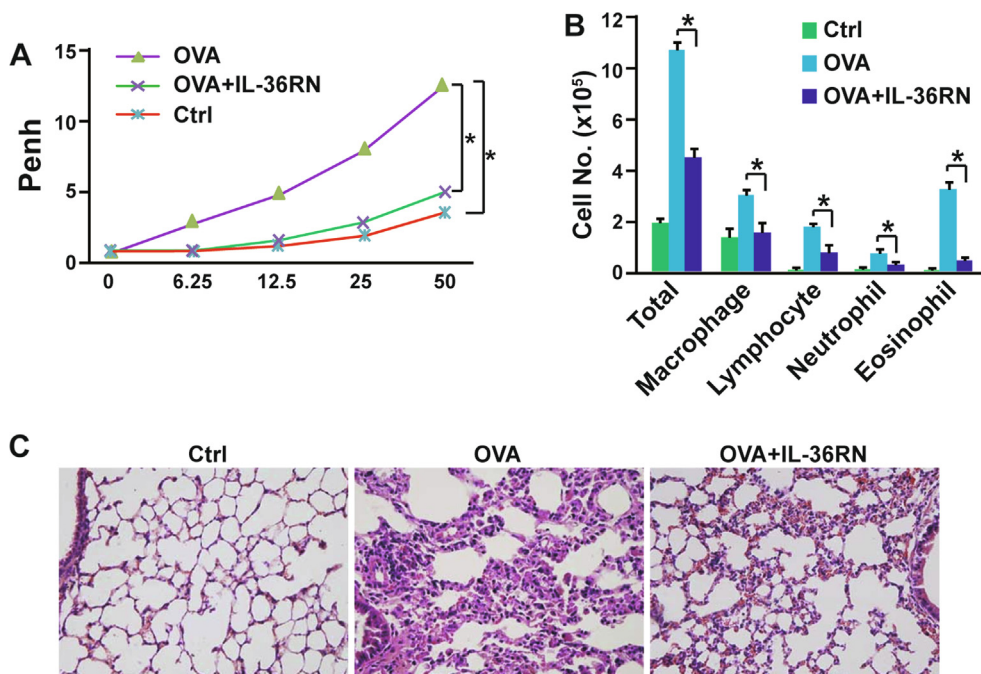


Fig. 3. IL-36RN alleviated asthmatic symptoms in the mouse model of asthma. The mice model of allergic asthma was established by OVA treatment. The mice were treated with tail-vein injection of recombinant human IL-36RN protein ($n = 8$ for each group). (A) AHR (airway hyper-responsiveness) in response to inhaled methacholine was analyzed. (B) IL-36RN suppressed OVA-induced inflammatory cell recruitment in BALF. (C) Representative images of HE staining of lung tissues. * indicates $P < 0.05$; Ctrl, control; OVA, Ovalbumin.

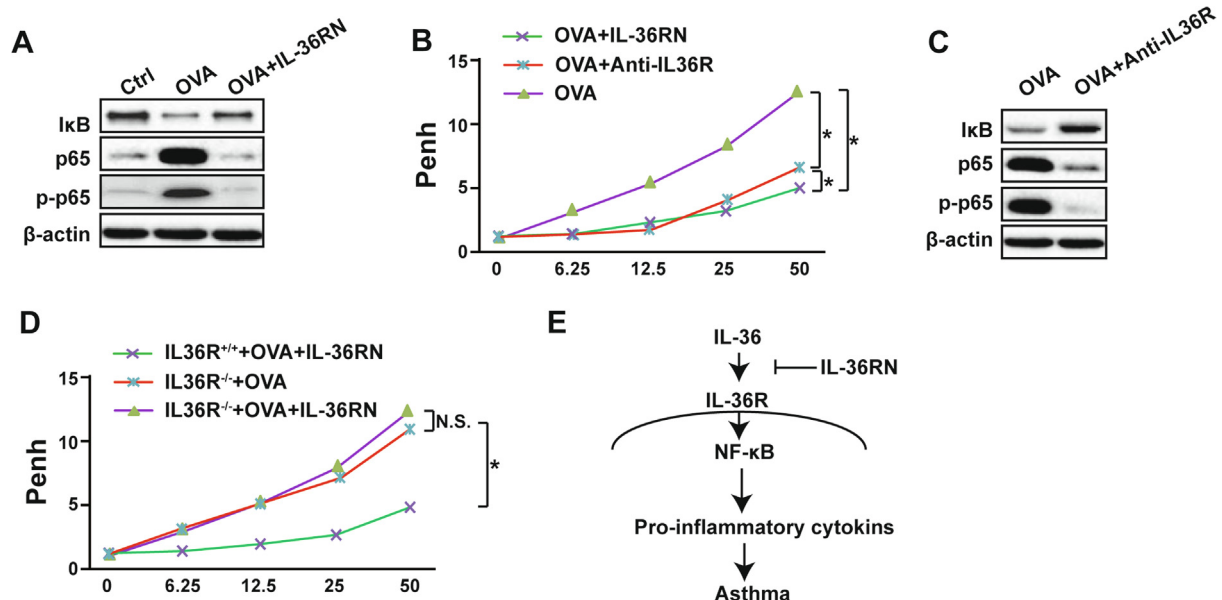


Fig. 4. IL-36RN suppressed NF-κB pathway through IL-36R blockade. The mice model of allergic asthma was established by OVA treatment. The mice were treated with tail-vein injection of recombinant human IL-36RN protein or anti-IL36R ($n = 8$ for each group). (A), (C) The bronchoalveolar lavage fluid cells were isolated from the mouse treated with OVA or OVA plus IL-36RN and subjected to detecting the protein levels of IκB, p65 and p-p65 (phospho-p65) by western blot. (B), (D) AHR (airway hyper-responsiveness) in response to inhaled methacholine was analyzed. (E) Proposed mechanism of IL-36RN on asthma suppression. * indicates $P < 0.05$; N.S. indicates no significant differences.

significantly reduced in the asthmatic patients when comparing with healthy controls (Fig. 1A–1B). Furthermore, the patients with moderate asthma had lower levels of IL-36RN than patients with mild asthma (Fig. 1A–1B). And this was further confirmed in the induced sputum cells (Fig. 1C–1D). Thus the IL-36RN was down-regulated in asthmatic pediatric patients.

3.2. IL-36RN suppressed the expression of pro-inflammatory cytokines

To investigate the potential role of IL-36RN 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 concentrations of recombinant IL-36RN were administrated. Data showed that the recombinant IL-36RN protein could significantly suppress the expression of pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6 and IL-17 (Fig. 2A). Furthermore, the secretion of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6 and IL-17) were also inhibited by IL-36RN protein (Fig. 2B). This was further confirmed in SMNCs (sputum mononuclear cells) (Fig. 2C–2D). Thus, the IL-36RN suppressed the expression of pro-inflammatory cytokines.

3.3. IL-36RN alleviated asthmatic symptoms in the mouse model of asthma

To study the potential therapeutic application of IL-36RN, the mouse model of allergic asthma was established by OVA treatment. Data showed that tail-vein injection of recombinant human IL-36RN protein could significantly suppress the OVA-induced AHR (airway hyper-responsiveness) (Fig. 3A). Furthermore, the IL-36RN reduced the number of inflammatory cells in the mouse bronchoalveolar lavage fluid (BALF) (Fig. 3B). And this was further confirmed by HE staining (Fig. 3C). Therefore, the IL-36RN could alleviate OVA-induced asthmatic symptoms in the mouse.

3.4. IL-36RN suppressed NF- κ B pathway through IL-36R blockage

IL-36RN is an IL-36 receptor (IL-36R) antagonist, which could bind to the IL-36R without activation of NF- κ B pathway [10]. To investigate whether the therapeutic role of IL-36RN in alleviating asthmatic symptoms in the mouse is through suppressing the NF- κ B pathway via IL-36R blockage, the bronchoalveolar lavage fluid cells isolated from the mouse treated with OVA or OVA plus IL-36RN were subjected to detecting the protein levels of I κ B, p65 and p-p65 (phospho-p65) by western blot. Data showed that the I κ B, the inhibitor of NF- κ B pathway, was up-regulated by IL-36RN, which was suppressed by OVA (Fig. 4A). And the important components of NF- κ B pathway, p65 and p-p65, were down-regulated by IL-36RN, which were induced by OVA (Fig. 4A). Interestingly, blocking the IL-36R with blocking antibody had comparable therapeutic effects on asthma with IL-36RN (Fig. 4B), which was also through suppressing the NF- κ B pathway (Fig. 4C). However, the therapeutic effects of IL-36RN was abolished in the IL-36R double knockout mice (Fig. 4D), indicating that the major mechanism of IL-36RN on asthma suppression was through IL-36R/ NF- κ B pathway (Fig. 4E).

4. Discussions

Asthma is a chronic inflammatory disorder in the respiratory system with increasing tendency [1,13]. For pediatric asthma treatment, corticosteroid inhalation is the only effective therapy. However, corticosteroids would induce bone density reduction, affecting the children growth [13]. 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 [3–8,14,15]. 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 IL-36RN, a newly identified anti-inflammatory factor [10], could alleviate airway inflammation in asthma through suppressing the activation of IL-36 pathway.

Our data here demonstrated that the mRNA and protein levels of IL-36RN were significant down-regulated in asthmatics than HCs. The IL-36RN significantly suppressed the expression of pro-inflammatory factors in PBMCs and sputum cells from asthma patients *in vitro*. And delivering IL-36RN into the mouse model of asthma showed disease alleviation through suppressing the activation of IL-36 pathway. Previous studies showed that IL-37, another important anti-inflammatory cytokine, was also decreased in pediatric patients with asthma and had therapeutic effects on the mouse model of asthma [12,16].

Furthermore, deficiency of IL-36RN, which is a rare disease in children, induces severe systemic symptoms of inflammation [17–21], indicating the important role of IL-36RN during inflammation suppression process. Our data here demonstrated that the levels of IL-36RN were significantly down-regulated in asthmatics. However, whether the down-regulation of IL-36RN is the inducer or stimulator of asthma development needs further investigations.

Targeting receptors with pro-inflammation functions might be a promising strategy to treat inflammation related diseases. IL-36R

(formerly IL-1Rrp2) is the most promiscuous primary receptor with three agonists, IL-36 α , IL-36 β , and IL-36 γ , and one antagonist, IL-36R antagonist (IL-36RN). After binding to their cognate primary receptor IL-36R, the agonists recruit the IL-1R accessory protein (IL-1RAcP), inducing the dimerization of the primary receptor with the accessory protein and producing signaling through engagement of cytoplasmic Toll/IL-1 receptor domains, ultimately leading to NF- κ B or MAPK activation [22,23]. Furthermore, the IL-36R activation could induce the expression of other pro-inflammatory cytokines, such as IL-17 and IL-1 β [24–26]. It has been demonstrated that the blocking antibody to IL-1R3, the receptor of IL-1, IL-33 and IL-36, could suppress the activities of inflammation related diseases [27]. However, natural receptor inhibitors, such as IL-36RN, might have more therapeutic potentials, because they are immunological tolerant. Nevertheless, the advantages and disadvantages of the blocking antibodies and natural receptor inhibitors need further investigation.

In summary, our data here indicated that IL-36RN may alleviate airway inflammation in asthma through suppressing the activation of IL-36 pathway.

Disclosure of potential conflicts of interest

The authors declare that they have no competing interests.

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

Xin-Guang Liu: Conceptualization, Resources, Supervision. **Jing Li:** Methodology, Data curation. **Lu-Jun Zheng:** Formal analysis, Validation. **Bo Han:** Methodology, Data curation. **Fang Huang:** Methodology, Data curation.

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