

Interleukin-20 Promotes Airway Remodeling in Asthma

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Abstract—Previous studies have demonstrated that interleukin-20 (IL-20) is a pro-inflammatory cytokine, and it has been implicated in psoriasis, lupus nephritis, rheumatoid arthritis, atherosclerosis, and ulcerative colitis. Little is known about the effects of IL-20 in airway remodeling in asthma. The aim of our study was to demonstrate the function of IL-20 in airway remodeling in asthma. To identify the expression of IL-20 and its receptor, IL-20R1/IL-20R2, in the airway epithelium in bronchial tissues, bronchial biopsy specimens were collected from patients and mice with asthma and healthy subjects and stained with specific antibodies. To characterize the effects of IL-20 in asthmatic airway remodeling, we silenced and stimulated IL-20 in cell lines isolated from mice by shRNA and recombinant protein approaches, respectively, and detected the expression of α -SMA and FN-1 by Western blot analysis. First, overexpression of IL-20 and its receptor, IL-20R1/IL-20R2, was detected in the airway epithelium collected from patients and mice with asthma. Second, IL-20 increased the expression of fibronectin-1 and α -SMA, and silencing of IL-20 in mouse lung epithelial (MLE)-12 cells decreased the expression of fibronectin-1 and α -SMA. IL-20 may be a critical cytokine in airway remodeling in asthma. This study indicates that targeting IL-20 and/or its receptors may be a new therapeutic strategy for asthma.

KEY WORDS: interleukin-20; airway remodeling; α -SMA; fibronectin-1; asthma.

INTRODUCTION

Asthma is a chronic disease of the airways, and the key pathophysiologic abnormalities of asthma include airway hyperresponsiveness, chronic inflammation, and airway remodeling [1, 2]. A significant limitation of current asthma therapies is their lack of effect on any features of airway remodeling, even though these structural changes contribute to disease

chronicity. Many factors contribute to asthmatic airway remodeling, such as transforming growth factor- β (TGF- β) and granulocyte-macrophage colony-stimulating factor (GM-CSF). A prominent feature of asthmatic airway remodeling is subepithelial fibrosis, which consists of the thickening of the subepithelial reticularis basement membrane. The thickened basement membrane is the result of extracellular matrix deposition, primarily collagens I, III, and V, and noncollagenous matrix deposition, including elastin, fibronectin-1, tenascin, lumican, and proteoglycans [3, 4]. Interleukin (IL)-20 is a member of the so-called IL-10 family of cytokines, which also includes IL-19, IL-22, IL-24, and IL-26. IL-10 is generally considered an anti-inflammatory cytokine, while IL-20 seems to possess pro-inflammatory attributes. A study showed that IL-20 was produced by monocytes early after lipopolysaccharide (LPS) stimulation and then rapidly decreased. These interactions indicated that IL-20 may be an early pro-inflammatory cytokine [5–7]. IL-20 was shown to target keratinocytes that express the specific heterodimeric receptor IL-

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20R1/IL-20R2. Both receptor chains were found in keratinocytes, endothelial cells, and certain mononuclear cells in psoriatic lesions [8].

Previous studies demonstrated that IL-20 was a pro-inflammatory cytokine and have implicated it in psoriasis, lupus nephritis, rheumatoid arthritis, atherosclerosis, and ulcerative colitis [9–14]. No previous studies have explored the function of IL-20 in asthma. The aim of our study was to demonstrate the function of IL-20 in airway remodeling in asthma.

MATERIALS AND METHODS

Ethics Statement

This research was approved by the ethics review committee for human studies at Qilu Hospital, Shandong University. All biopsy specimens were obtained from all participants with their informed consent.

The animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of Shandong University.

The protocol was approved by the Committee on the Ethics of Animal Experiments of Shandong University. Each mouse was anesthetized with intraperitoneal ketamine (35 mg/kg). Cervical dislocation was used to kill the mice. All efforts were made to minimize pain and discomfort.

Patients

The study was approved by the Ethics Review Committee for Human Studies at Qilu Hospital of Shandong University. Each subject provided written informed consent. The airway epithelial biopsy specimens from 10 asthma patients were obtained from Shandong Provincial Chest Hospital (Jinan, Shandong, China), and 10 healthy control biopsy specimens were obtained from Qilu Hospital of Shandong University (Jinan, Shandong, China). The asthmatic specimens were taken by bronchoscope, and the control specimens were taken by pneumoresection. The characteristics of the subjects are shown in Table 1.

Animal Model

Twenty female BALB/c mice (8 to 10 weeks old, 20±2 g) were obtained from the Animal Experiment Center of Shandong University and were randomly divided into 2 groups: the ovalbumin (OVA) group and the control group.

Table 1. Characteristics of the Subjects

	Healthy control subjects	Asthmatic patients
Sex (M/F)	5/5	6/4
Age (years)	27.2±5.31	29.7±4.14
FEV1 (L)	4.17±0.69	3.68±0.84
FEV1 (%)	99.6±7.15	91.8±19.85
PC20 (mg/mL)	145.17±20.12	1.99±0.84

Mice in the OVA group were sensitized with OVA as follows [15]: 100 mg/ml OVA (Sigma, St. Louis, MT, USA) was emulsified with 200 mg/ml aluminum hydroxide (Fuchen Chemistry Inc., Tianjin, China) in saline (Hualu Inc., Shandong, China) before intraperitoneal injection of 0.1 mL per mouse at days 1 and 8. From days 15 to 21, the mice were nebulized with a 5 % (w/v) OVA solution for 30 min in a 30 cm×24 cm×50 cm chamber daily. The control group received intraperitoneal injections of saline followed by nebulization with saline.

Histological Analysis

Immunohistochemical staining was performed as described previously [16]. The following primary antibodies were used: anti-IL-20 antibody (Santa Cruz, USA) (1:100), anti-IL-20R1 antibody (Santa Cruz, USA) (1:100), anti-IL-20R2 antibody (Santa Cruz, USA) (1:100), anti-fibronectin-1 antibody (Santa Cruz, USA) (1:200), and anti- α -SMA antibody (Santa Cruz, USA) (1:300).

Western Blotting Analysis

Lung tissue was homogenized in the presence of protease inhibitors to extract the lung protein. The lysate protein concentration was determined using a BCA protein assay kit (Thermo Rad Laboratories, USA). For Western blotting analysis, an equal amount of protein (30 μ g) from each group was loaded on an SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon; Millipore Corp, Bedford, MA, USA). The membrane was then blocked by incubation in 5 % skim milk in Tris-buffered saline (TBS, 50 mM Tris-Cl pH 7.5, 150 mM NaCl) for 60 min at room temperature and probed with anti-IL-20 antibody (Santa Cruz, USA) (1:500), anti-IL-20R1 antibody (Santa Cruz, USA) (1:500), anti-IL-20R2 antibody (Santa Cruz, USA) (1:500), anti-fibronectin-1 antibody (Santa Cruz, USA) (1:500), and anti- α -SMA antibody (Santa Cruz, USA) (1:500) overnight

at 4 °C. Following incubation with the primary antibodies, the membrane was incubated with secondary antibodies for 1 h at room temperature. The membrane was exposed to a high performance autoradiography film (Fuji XR film, Fuji Film Corporation, Tokyo, Japan) and visualized using the ECL system (Santa Cruz, CA, USA).

The integrated density values (IDV) of the band intensities from the film were analyzed by IMAGEQUANT 5.2 software (Molecular Dynamics, Sunnyvale, CA, USA).

Enzyme-Linked Immunosorbent Assay

The IL-20 concentrations in bronchoalveolar lavage fluid (BALF) was determined by the enzyme-linked immunosorbent assay (ELISA) using ELISA kits according to the manufacturer's instructions (KYM, Beijing, China).

Cell Culture and Transfection

Mouse lung epithelial (MLE)-12 cell was purchased from cell bank (ATCC cell bank, USA) and cultured in DMEM/F12 complete medium supplemented with 10 % fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37 °C under conditions of 5 % CO₂. After 48 h in culture, 90 % of the adherent cells were of epithelial morphology. The cells were seeded at 1×10^5 cells per well in 6-well culture plates.

Transient Transfection

Transient transfections were performed using shRNA Plasmid Transfection Reagent: (sc-108061 Santa Cruz, USA) and IL-20-shRNA plasmid (sc-108062, Santa Cruz, USA) according to the manufacturer's instructions. After 48 h of incubation, the cells were harvested for analysis of IL-20 α -SMA and fibronectin-1, and

then, the cells were collected to analyze protein expression by Western blotting analysis.

Statistical Analysis

The study results are presented as the means $\pm$ standard error of the mean (SEM). The statistical evaluation performed were nonparametric tests (Kruskal Wallis, Mann-Whitney *U* test), and $P < 0.05$ was considered significant.

RESULTS

Expression of IL-20, IL-20R1, and IL-20R2 Are Upregulated in Human Asthmatic Airway Epithelial Tissue

To identify the expression of IL-20 and its receptors in asthma patient epithelia, we examined the expression of IL-20, IL-20R1, and IL-20R2 in asthma epithelia samples. Increased expression levels of IL-20, IL-20R1, and IL-20R2 were found in asthma patient epithelia (Fig. 1a). The immunostaining score showed a significant difference between the asthma and control groups ($p < 0.05$, $p < 0.05$, $p < 0.05$, for IL-20, IL-20R1, and IL-20R2, respectively) (Fig. 1b).

IL-20, IL-20R1, and IL-20R2 Expression Are Upregulated in the Airways of OVA-Induced Mice

To identify the expression of IL-20 and its receptors in the lungs of OVA-induced mice, we detected the expression of IL-20, IL-20R1, and IL-20R2 in the epithelium by immunohistochemistry. We found that IL-20, IL-20R1, and IL-20R2 were highly expressed in epithelial cells of the airway in OVA-induced mice ($p < 0.05$, $p < 0.05$, $p < 0.05$, respectively) (Fig. 2a, b). As expected, the airway remodeling markers fibronectin-1 and α -SMA were

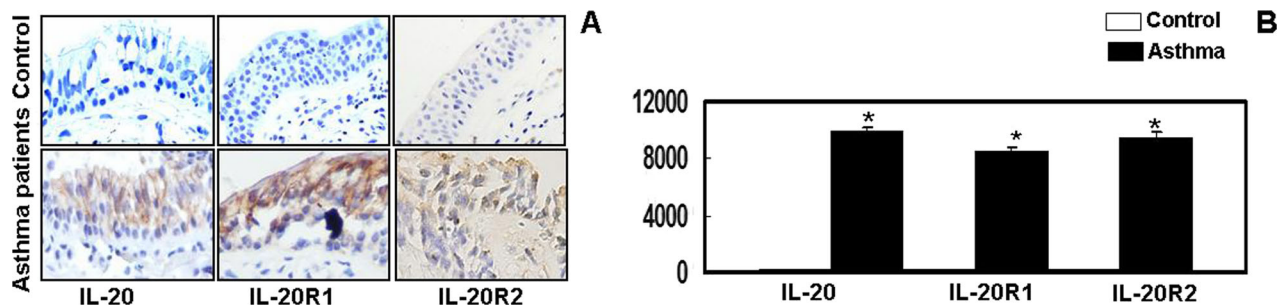


Fig. 1. Expression of IL-20, IL-20R1, and IL-20R2 in airway epithelium. IL-20, IL-20R1, and IL-20R2 staining were upregulated in airway epithelium in patients with asthma (a). Quantitation of IL-20, IL-20R1, IL-20R2 ($p < 0.01$, $p < 0.01$, $p < 0.01$, respectively) compared with control groups (b).

increased in the bronchial epithelial cells of asthmatic mice ($p<0.05$, $p<0.05$, respectively) (Fig. 2a, b). We also detected the expression of IL-20, IL-20R1, and IL-20R2 in the lung and BALF (Fig. 3a, b), and, as expected, the expression levels of fibronectin-1 and α -SMA were significantly elevated in OVA mice. These data are consistent with those of Fig. 2. These results suggest that IL-20 plays a vital role in asthmatic airway remodeling.

IL-20 Promotes Airway Remodeling in Airway Epithelial Cells *In Vitro*

To further explore the role of IL-20 in airway remodeling in asthma, we tested whether IL-20 can induce airway remodeling in mice airway epithelial cells. We used IL-20 to treat MLE-12 (mouse lung epithelial cell line) cells and detected the fibronectin-1 and α -SMA protein levels. We found that IL-20-induced fibronectin-1 and α -SMA upregulation occurred in an IL-20 dose-dependent manner (Fig. 4a). Upregulation of fibronectin-1 and α -SMA was observed from 6–24 h after IL-20 (1.5 ng/ml) stimulation (Fig. 4b). These results suggest that IL-20 promotes airway remodeling in asthma. To confirm these findings, IL-20 was silenced with IL-20-shRNA. Both fibronectin-1 and

α -SMA were inhibited when IL-20 was silenced. The results demonstrated that IL-20 promotes airway remodeling in asthma *in vitro* (Fig. 4c).

DISCUSSION

Our study demonstrated that IL-20 promotes airway remodeling in asthma. First, we found that the expression of IL-20 and its receptors was increased in the bronchial epithelial cells of asthma patients. We further confirmed the function of IL-20 in airway remodeling.

IL-20 belongs to the IL-10 family, which also includes IL-19, IL-22, IL-24, IL-26, IL-28, and IL-29 [17]. Previous studies have demonstrated that the IL-20 cytokine subfamily enhances tissue remodeling and wound-healing activities, which help to maintain tissue integrity and restore homeostasis of epithelia during infection and inflammatory responses [18]. In asthma patients, the bronchial epithelium is significantly abnormal, with structural changes and functional changes. These functional changes include increased expression and release of mediator-generating enzymes, growth factors, and pro-inflammatory cytokines. Disruption to the injury-repair cycle of damaged

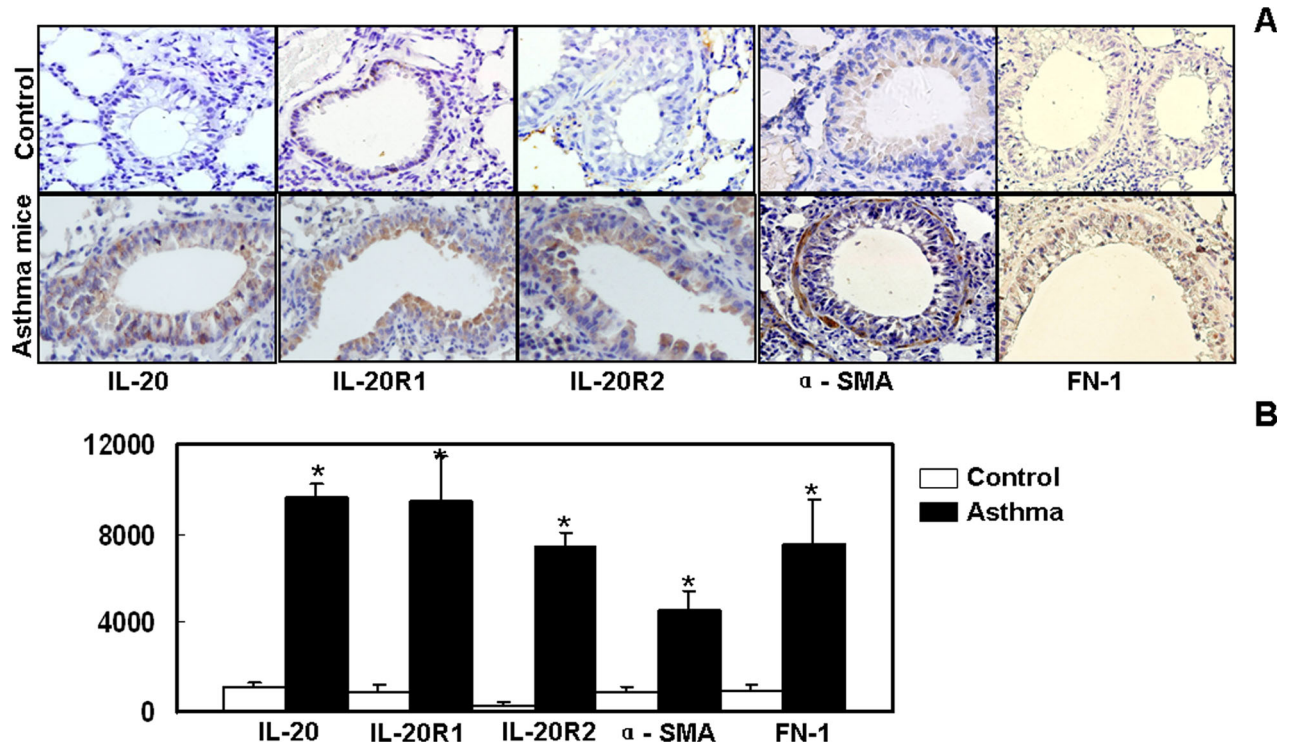


Fig. 2. a IL-20, IL-20R1, IL-20R2, α -SMA, and FN-1 expressions in asthmatic mice airways. $P<0.01$, $P<0.01$, $P<0.01$, $P<0.01$, and $P<0.01$, respectively. (IOD) quantitation (b).

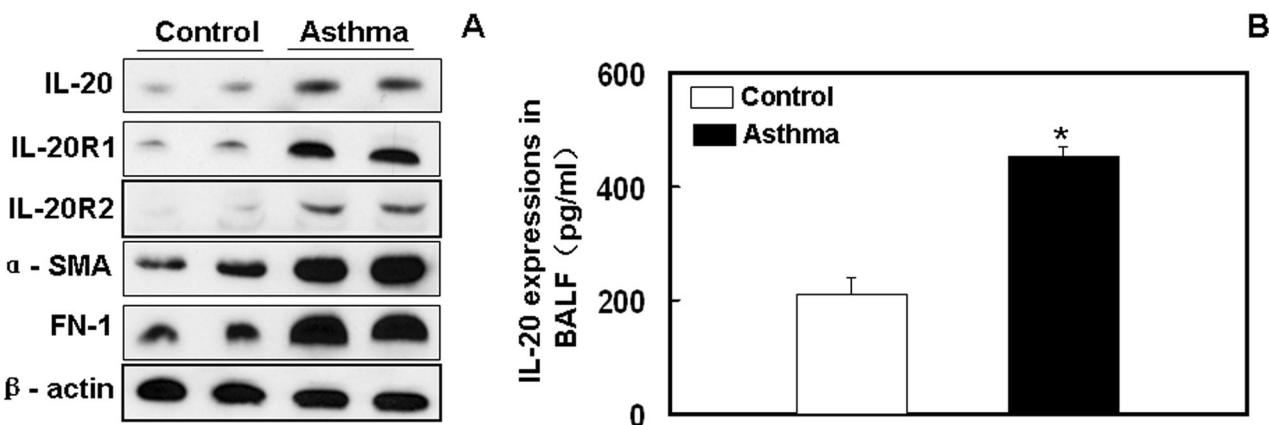


Fig. 3. Higher expression of IL-20, IL-20R1, IL-20R2, α -SMA, and FN-1 in the lungs of the asthmatic mouse model $P<0.01$, $P<0.01$, $P<0.01$, $P<0.01$, $P<0.01$, respectively (a). The concentration of IL-20 in BALF in asthma mice was higher than in the control group ($P<0.01$) (b).

epithelial cells is very important in the development of abnormal epithelium-mesenchymal interactions [19]. Thus, the overexpression and enhanced production of IL-20 in damaged epithelial cells may play a role in airway remodeling in asthma.

Signal transducer and activator of transcription factor 3 (STAT3) is essential in allergic inflammation. STAT3 is induced by a wide array of cytokines, such as IL-6, IL-10, and IL-13, and has been implicated in the regulation of cell growth, early embryonic development, immune tolerance, and inflammation. Previous studies have demonstrated that STAT3-dependent pathways induced by IL-13 in lung myofibroblasts are inhibited by the administration of the inhaled corticosteroid fluticasone. This suggests a role of STAT3 in airway inflammation and remodeling in asthmatics, which may reduce lung function level [20]. Overexpression of IL-20 in the epidermis triggered abnormal epidermal proliferation and differentiation in transgenic mice. Finally, it caused STAT3 activation [10]. IL-20 specifically targets KCs and activates epidermal thickening in the RHE. It also induces a number of other features that are

characteristic of the psoriatic epidermis, such as activated STAT3, expression of CK16, S100A7, and other S100 family members, and upregulation of many proteases involved in skin mobility and remodeling. We hypothesized that IL-20 may affect airway inflammation and airway remodeling in asthma through STAT3 pathways. Furthermore, as IL-10 signal transduction includes both STAT3-dependent and STAT3-independent pathways, it is possible that IL-20 signal transduction is similarly complex. Another study of IL-20 and airway inflammation is underway.

The IL-20 subfamily signals through the IL-20R1/IL-20R2 (type I receptor complex) and/or the IL-22R1/IL-20R2 (type II receptor complex) complex. The IL-20 subfamily and IL-22 can induce STAT3 phosphorylation [21]. All of these receptors are expressed in monocytes, mesangial cells, maturing dendritic cells, keratinocytes, synovial fibroblasts, and intestinal macrophages. Sharing receptor subunits is a well-known feature within class I cytokine receptors. The IL-20R2 subunit could associate with IL-20R1, generating a functional receptor for IL-19, IL-20, and mda-7 (type I receptor complex). IL-20R2 also

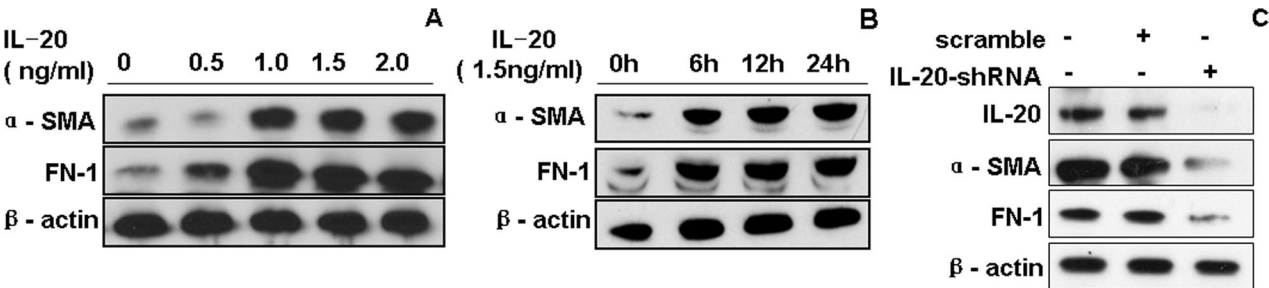


Fig. 4. IL-20 promotes the expression of α -SMA and FN-1 in MLE-2 cells in a time and dose-dependent manner (a, b). Blocking IL-20 can reduce α -SMA and FN-1 expression obviously (c).

could associate with the IL-22R subunit, generating a functional receptor for IL-20 and mda-7 but not for IL-19 (type II receptor complex) [7].

Our study showed that IL-20 and its receptors IL-20R1 and IL-20R2 are upregulated in the airway epithelium of asthma patients (Fig. 1), which indicates that IL-20 may act on airway epithelial cells in an autocrine manner.

In our *in vivo* animal experiments, for the asthmatic mice, IL-20 and its receptor, IL-20R1/IL-20R2, were overexpressed, and this was accompanied with increased synthesis of fibronectin-1 and α -SMA in asthma mice (Fig. 2). Meanwhile, the expression of IL-20 and its receptors in the lung and the concentration of IL-20 in BALF were upregulated in the asthma animal model (Fig. 3). These results suggest that IL-20 plays a vital role in airway remodeling in asthma.

To further examine whether IL-20 promotes airway remodeling in asthma, MLE-12 cells were treated with IL-20 recombinant protein. As expected, the expression of fibronectin-1 and α -SMA was significantly elevated. This result suggests that IL-20 may be an inducing agent in promoting airway remodeling. We also found that the expression levels of fibronectin-1 and α -SMA were inhibited after silencing IL-20 (Fig. 4). These data suggest that IL-20 promotes airway remodeling *in vitro*.

Since it is hard for us to get epithelium biopsy from asthma patients, we cannot analyze the relationship between IL-20 and asthma severity. In addition, due to the limitation of experimental conditions, we did not apply primary cell culture to detect the effect of IL-20 on asthma patients, for it is much closer to human pathophysiology; we just used the cell line to mimic the function of IL-20 in asthmatic airway remodeling.

Previously, the presence of IL-20 and its receptors in asthma has not been thoroughly discussed. Our research helps describe the process of airway remodeling in asthma. However, the mechanism of IL-20 in the complex pathogenesis of airway remodeling is unclear. For example, the downstream signaling pathway, biological activity, expression of related inflammatory molecules, and function of IL-20 in the pathogenesis of airway are unknown. Thus, more comprehensive studies are necessary.

CONCLUSION

IL-20 is a critical cytokine in airway remodeling in asthma. This study indicates that targeting IL-20 and its receptors or its downstream targets, α -SMA and fibronectin-1, is a new potential therapeutic strategy for asthma.

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Conflict of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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