Clinical and Experimental Immunology, 2023, **214**, 260–274
https://doi.org/10.1093/cei/uxad099
Advance access publication 16 August 2023
Research Article

Clinical & Experimental IMMUNOLOGY

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Research Article

IL-38 alleviates airway remodeling in chronic asthma via blocking the profibrotic effect of IL-36γ

Min Zhang[1], Jian-Xia Zhou[1], Chu-Qin Huang[2], Kang-Ni Feng[1], Xiao-Ling Zou[1], Jie-Mei Cen[1], Ping Meng[1], Hong-Tao Li[1], and Tian-Tuo Zhang[*,1]

[1]Department of Pulmonary and Critical Care Medicine, The Third Affiliated Hospital of Sun Yat-Sen University, Institute of Respiratory Disease of Sun Yat-Sen University, Guangzhou, Guangdong, China
[2]State Key Laboratory of Respiratory Disease, National Clinical Research Center for Respiratory Disease, Guangzhou Institute of Respiratory Health, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong, China

[*]Correspondence: Tian-Tuo Zhang, MD, PhD, Department of Pulmonary and Critical Care Medicine, The Third Affiliated Hospital of Sun Yat-sen University, Institute of Respiratory Disease of Sun Yat-sen University, Guangzhou, Guangdong, China. Email: zhtituli@163.com

Abstract

Airway remodeling is a major feature of asthma. Interleukin (IL)-36γ is significantly upregulated and promotes airway hyper-responsiveness (AHR) in asthma, but its role in airway remodeling is unknown. Here, we aimed to investigate the role of IL-36γ in airway remodeling, and whether IL-38 can alleviate airway remodeling in chronic asthma by blocking the effects of IL-36γ. IL-36γ was quantified in mice inhaled with house dust mite (HDM). Extracellular matrix (ECM) deposition in lung tissues and AHR were assessed following IL-36γ administration to mice. Airway inflammation, AHR, and remodeling were evaluated after IL-38 or blocking IL-36 receptor (IL-36R) treatment in asthmatic mice. The effects of lung fibroblasts stimulated with IL-36γ and IL-38 were quantified *in vitro*. Increased expression of IL-36γ was detected in lung tissues of HDM-induced asthmatic mice. The intratracheal instillation of IL-36γ to mice significantly enhanced the ECM deposition, AHR, and the number of activated lung fibroblasts around the airways. IL-38 or blocking IL-36R treated asthmatic mice showed a significant alleviation in the airway inflammation, AHR, airway remodeling, and number of activated fibroblasts around airways as compared with the HDM group. *In vitro*, IL-36γ promoted the activation and migration of human lung fibroblasts (HFL-1). The administration of IL-38 can counteract these biological processes induced by IL-36γ in HFL-1cells. The results indicated that IL-38 can mitigate airway remodeling by blocking the profibrotic effects of IL-36γ in chronic asthma. IL-36γ may be a new therapeutic target, and IL-38 is a potential candidate agent for inhibiting airway remodeling in asthma.

Keywords: asthma, airway remodeling, interleukin-36γ, interleukin-38, lung fibroblasts

Abbreviations: AHR: airway hyper-responsiveness; α-SMA: alpha smooth muscle actin; BALF: bronchoalveolar lavage fluid; DAPI: 4,6-diamidino-2-phenylindole; ELISA: enzyme-linked immunosorbent assay; FBS: fetal bovine serum; HDM: house dust mite; IL: interleukin; IL-36R: interleukin-36 receptor; IL-36Ra: interleukin-36 receptor antagonist.

Introduction

Asthma is characterized as a heterogeneous chronic inflammatory airway disease that an estimated 300 million people suffer from it around the world, and it costs billions of dollars in medical expenses and causes 250 000 annual fatalities [1, 2]. A variety of cells are involved in the process of asthma and cause airway inflammation, airway hyper-reactivity (AHR), airway remodeling, and other pathological changes [3]. A long course of inflammation may lead to a suite of structural changes in the airway called airway remodeling. Airway remodeling is the pathological basics of fixed airflow restriction in asthma patients. In the past, persistent inflammation was believed to be the cause of airway remodeling. Severe asthma patients experience irreversible or partially reversible airflow limitation and endangering their lives. However, there is accumulating evidence that airway remodeling occurs concurrently with inflammation [4, 5]. Current therapeutic about airway inflammation do little for airway remodeling.

Interleukin (IL)-36 cytokines belong to IL-1 family and consist of three agonists IL-36 alpha (IL-36α), IL-36beta (IL-36β), and IL-36 gamma (IL-36γ) and the natural antagonist IL-36 receptor antagonist (IL-36Ra) and IL-38 [6]. IL-36 receptor agonists genes are localized to human and mouse chromosome 2 [7]. IL-36 receptor agonists exert the pro-inflammatory function by binding to the heterodimeric receptor compound of IL-36R and IL-1-receptor-affiliated protein IL-1RAcP1 and stimulating downstream signaling cascade including MyD88 adaptor protein complex, NF-kB, and MAPK pathways to promote inflammation [8]. Recently, a series of studies showed that IL-36α, IL-36β, and IL-36γ participate in allergic disease and were significantly upregulated, such as allergic rhinitis (AR), especially with asthma and allergic contact dermatitis (ACD) [9–12]. IL-36γ has been found to be upregulated in primary bronchial epithelial cells from asthma patients and is further increased upon infection with rhinovirus [13]. The mRNA level

Received 18 February 2023; Revised 30 June 2023; Accepted for publication 11 August 2023

and the protein concentration of IL-36γ are both increased in the lung tissues of HDM-induced mouse asthma mode [7]. Several pieces of evidence have shown that IL-36 has a promoting effect on tissue fibrosis. For example, Zhang et al. demonstrated that IL-36γ promotes HFL-1 cells releasing IL-6 and CXCL8 [14]. Scheibe et al. have indicated that IL-36 ligands can stimulate colonic fibroblasts releasing the type IV collagen and inhibiting IL-36R can reduce the tissue fibrosis in the chronic intestinal inflammation mice [15]. But the relationship between IL-36γ and airway remodeling in asthma is still unknown.

IL-38 is a 17–18 kDa protein which belongs to IL-1 family and shares 40% sequence similar to IL-1Ra and IL-36Ra. Thus, it is thought to be a typical antagonist of the IL-1 family along with IL-1Ra and IL-36Ra [16]. Three candidate receptors, IL-36R, IL-1R1, and IL-1 receptor accessory protein-like 1 (IL-1RAPL1), have been proposed for IL-38 and IL-38 exerts anti-inflammatory activities by binding to these receptors and blocking intracellular signaling [17–19]. The protective effect of IL-38 in immune diseases has been widely studied, such as RA [20], SLE [21], psoriasis [22], and inflammatory bowel disease [23]. The mRNA level of IL-38 was decreased in the lung tissues of ovalbumin-induced mice [24]. Sun et al. have shown that airway inflammation, AHR, and Th2 cytokines are reduced in asthmatic mice upon the injection with IL-38 [25]. However, the effect of IL-38 in allergic asthma remains largely unknown, including the potential on airway remodeling.

In this study, we aimed to investigate the effect of IL-36γ on airway remodeling in experimental mice models of asthma and *in vitro*, and then further elucidated whether IL-38 could inhibit airway remodeling in HDM-induced mice by blocking the effect of IL-36γ.

Materials and methods

Animals

SPF BALB/c mice (female, 6–8 weeks) were purchased from Guangdong Yao Kang Biotechnology Co. Ltd (Guangdong, China) and allowed to acclimate for 1 week. All mice were placed in a specific sterile level (SPF) environment with temperature 23 ± 1°C, humidity 60 ± 5%, and light–dark cycles of 12 hours. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Third Affiliated Hospital of Sun Yat-Sen University (number [2019]02-148-01).

HDM-induced asthma in mice

Twenty-five micrograms of house dust mite (cat. No. XPB82D3A2.5, Greer, USA) protein were resuspended in 25 μl phosphate-buffered saline (PBS) at a concentration of 1.0 mg/ml. Moreover, mice in the control group were injected with 25 μl of PBS meanwhile as a negative control group. All mice were euthanized 24 hours after last induced.

Intratracheal IL-36γ induction

One microgram of mouse recombinant IL-36γ was dissolved in 20 μl PBS and intratracheally instilled into mouse lungs for 2 weeks at a total of three times a week. The control group were received 20 μl of PBS. Lung tissues were collected for analysis 24 hours after the last exposure.

Airway hyper-responsiveness measurement

Invasive lung function assessment of mice was operated by using a BuxcoÒ FinePointe™ RC system (DSI, St. Paul, MN, USA). In brief, mice were anesthetized with 1% pentobarbital sodium (70 mg/kg, intraperitoneal injection) 24 hours after the last HDM induction. Mice were endotracheally intubated with a 24-gauge cannula and connected to a ventilator at a rate of 120 breaths per minute. Then, the airway resistance (RL, cm H_2O s/ml) of mice to 20 μl PBS or different doses of inhaled methacholine (Mch, 6.25, 12.5, 25, and 50 mg/ml, Sigma–Aldrich) were recorded for 3 minutes at each dose [26].

Bronchoalveolar lavage fluid analyses

Bronchoalveolar lavage fluid (BALF) was obtained by endotracheal intubation after AHR measurement. After lysis of RBCs, the total number of inflammatory cells in BALF was determined using a hemocytometer. For the differential counts, the cells (5×10^4 cells/slide) in the BALF were centrifuged onto slides. Giemsa staining was used for differential cell counting. At least 400 cells per sample were examined.

Cytokines quantification by ELISA

The concentrations of IL-36γ, IL-38, IL-5, IL-6, IL-13, IL-17, TSLP, active TGF-β1, and TNF-α were measured by ELISA kit according to the manufacturer's specifications.

Determination of lung hydroxyproline

For determination of hydroxyproline (HYP) contents, 50 mg wet tissue from the frozen lung of mice was hydrolyzed and boiled for 20 minutes. After adjusting the pH to 6.5, the HYP levels in individual hydrolysates were determined using the HYP Test Kit according to the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, China). Briefly, each hydrolysate sample was reacted with activated carbon (20 mg), and after centrifuging, the absorbance of the supernatant was measured at 550 nm by ultraviolet spectrophotometer. The data were expressed as micrograms of HYP per milligram of lung tissue.

Lung histopathology

The lung tissues were fixed in 4% paraformaldehyde and then embedded in paraffin fixation. The paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Masson trichrome, and toluidine blue to evaluate the airway inflammation, the production of mucus, collagen deposition, and mast cells recruitment in mice lung tissues. The airway inflammation score and airway wall thickness were quantified according to previously published methods [27]. The quantification of PAS staining and Masson trichrome staining was performed by ImageJ software. Mast cells were counted at least in three fields per section.

Flow cytometric analyses

The lungs tissues were minced and subsequently incubated with shaking for 30 minutes at 37°C with the collagenase type IV solution. After red blood cell lysis, cell suspensions were filtered through a 70 μm cell strainer. The Fc-gamma receptors were blocked with an anti-CD16/32 (clone: W17231A, BioLegend, USA) antibody for 30 minutes to avoid nonspecific staining. The cells were then stained with anti-mouse CD3 (clone: 145-2c11, BioLegend) and anti-mouse

CD4 (clone:RM4-5, BioLegend) antibodies for cell surface staining. Next, the cells were fixed with the Intracellular Fixation and Permeabilization kit at 4℃ for 1 hour and then stained with anti-mouse interferon (IFN)-γ (clone: XMG1.2, BioLegend) and anti-mouse IL-4 (clone: 11B11, BioLegend) antibodies for 30 minutes in the dark. Stained cells were analyzed using a flow cytometer. Data were analyzed using FlowJo software.

Immunohistochemistry

Antigenic repair was performed on the 5 μm paraffin sections, and endogenous peroxidase was wiped off. The IL-36γ (1:100, Affinity, China), α-SMA (1:200, Cell Signaling Company, USA), Fibronectin (1:200, Santa Cruz Company, USA), CD31 (1:40, Abcam, USA), and p-ERK (1:200, Cell Signaling Company) antibodies were incubated overnight at 4℃. Next day, the tissue sections were incubated with goat anti-rabbit antibody for 1 hour at room temperature, and then incubated with DAB for 5 minutes. Finally, the sections were dehydrated with a grade alcohol series as well as transparency with xylene, and photographed under the microscope. Protein expression was evaluated using ImageJ software.

Immunofluorescence

The lung tissue paraffin sections or HFL-1 cells were fixed with 4% paraformaldehyde and then permeated with 0.5% Triton X-100 (Solaibio, China) for 30 minutes. The paraffin sections were incubated with the antibodies of α-SMA (1:100, Cell Signaling Company), Vimentin (1:100, Cell Signaling Company), Collagen I (1:250 Santa Cruz), and Fibronectin (1:250, Santa Cruz) at 4°C overnight. The following day, the tissue sections were stained with Alexa Fluor goat anti-rabbit and DAPI in the dark. Finally, the sections were photographed with a fluorescence microscope (Nikon, Japan).

RNA isolation and quantitative real-time PCR

Total RNA of lung tissues and cells was extracted with Trizol reagent (CWBIO, China) and reversely transcribed into cDNA using Prime Script RT Reagent Kit (TaKaRa, Japan). The Light Cycler 480 System and ChamQ SYBR Green PCR kit (Vazyme, China) were used for real-time quantitative PCR. The relative expression of mRNA was normalized and evaluated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences are provided in Supplementary Table S1.

Cell culture and treatment

A normal human lung fibroblast cell line (HFL-1) was obtained (Procell, Wuhan, China) and cultured in Ham's F-12K containing 10% FBS and stimulated with rhIL-36γ and rhIL-38.

Isolation of total protein and western blot

Cultured HFL-1 cells were washed with PBS and were subjected to protein extraction by using a lysis buffer (KeyGEN, China). A BCA protein assay kit (Ding guo, China) was used to measure the concentration of protein. The samples were loaded with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore Company, USA). The membranes were blocked with 5% skimmed milk for 1 hour. The primary antibodies Fibronectin (1:500, Santa Cruz Company), Collagen I (1:500, Santa Cruz Company), α-SMA (1:1000, Cell Signaling Company), p-ERK (1:1000, Cell Signaling Company), t-ERK (1:1000, Cell Signaling Company), and GAPDH (1:2000, Cell Signaling Company) were incubated at 4°C overnight after washing with TBST solution. The PVDF membranes were washed with TBST and then incubated with HRP-conjugated goat anti-rabbit IgG for 1 hour. The PVDF membranes were washed three times with TBST and then visualized using an enhanced chemiluminescence reagent.

Wound healing and transwell assay

Cell migration ability was evaluated by using wound healing assay and transwell assay. A horizontal line was drawn with the pipette tip at the bottom behind the 6-well plate. 1×10^5 cells in each group were seeded into the six-well plate and incubated with PBS, rhIL-36γ, and rhIL-38 for 24 hours. The cells were cultured with serum-free medium and were treated with PBS, rhIL-36γ, and rhIL-38, respectively. Images of six randomly fields in each group were captured using a microscope at 40× magnification (0 and 24 hours) [28].

1×10^4 cells were suspended in 100 μl serum-free medium and cultured in the upper transwell chamber with an 8 μm pore polycarbonate membrane filter. 600 μl medium containing 10% FBS was placed in the bottom chamber. PBS and rhIL-36γ were added in the bottom chamber, respectively, incubated with or without rhIL-38. After 24 hours, the migrated cells on the membrane filter were fixed with 4% paraformaldehyde for 20 minutes, stained with 0.1% crystal violet for 30 minutes. After washing and drying, the migrated cells were photographed in five random fields using a 100× inverted microscope [28].

Statistical methods

All statistical analyses were performed by using GraphPad Prism 8. Unpaired Student's *t*-test was used to analyze the differences between the two groups. One-way ANOVA was used to compare multiple groups to the designated control group. Data were expressed as mean ± standard deviation. Results were considered statistically significant at $P < 0.05$.

Results

IL-36γ was significantly upregulated and IL-38 was downregulated in the lungs of HDM-induced asthma mice

IL-36 receptor agonist IL-36γ gene, a quantitative trait locus on mouse chromosome 2, is associated with allergen-induced bronchial hyper-responsiveness [7]. To investigate whether IL-36γ was upregulated and participate in the process of chronic asthma, we successfully established a mice model of chronic asthma according to the protocol of our previous research (Fig. 1A) [29] and analyzed. Continuous induction with HDM elicited inflammatory cells infiltration (Fig 1B–D; Supplementary Fig. S1) and collagen deposition around the airways in the lungs of mice (Fig. 1E–G). Immunohistochemical staining of lung tissue sections revealed that the expression of IL-36γ was significantly increased in the HDM group as compared with the PBS control (Fig. 1H and I). The mRNA level of IL-36γ was also upregulated in the lung tissues of mice in HDM group (Fig. 1J). Meanwhile, the concentration of IL-36γ was remarkably increased in BALF and lung homogenate of chronic asthma mice as compared to that in PBS group, which were the same with the previous research (Fig.

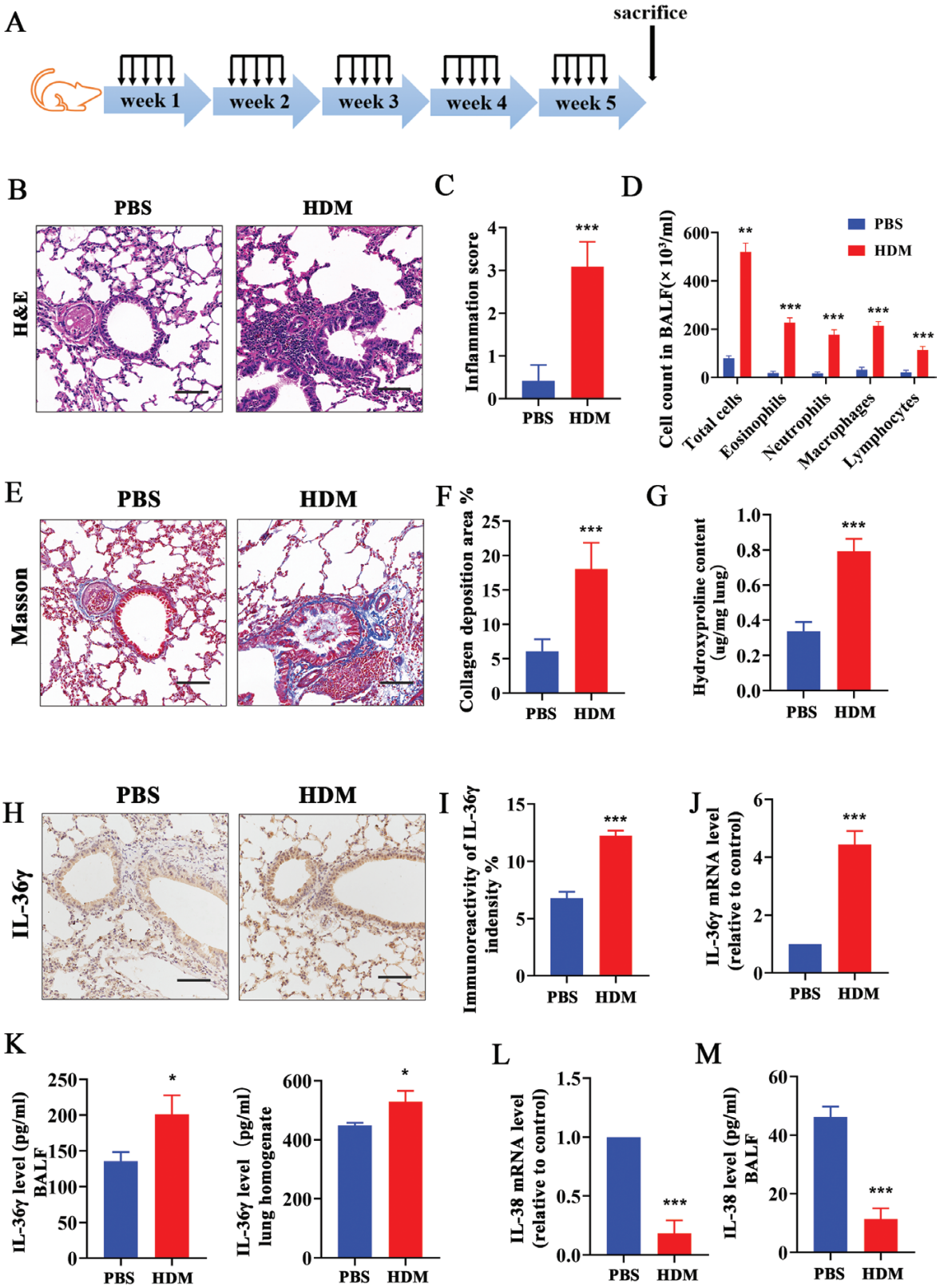

Figure 1. The expression of IL-36γ and IL-38 in HDM-induced chronic asthmatic mice. (A) The protocol of chronic asthma animal experimental model (n = 6 per group). (B and C) Representative histopathological images and quantification of the lung tissues in mice were performed with H&E staining. Scale bar = 50 μm (×200). (D) The total and differential inflammatory cell counts in BALF were determined by Giemsa staining. (E and F) Representative histopathological images and quantification of the lung tissues in mice were performed with Masson staining. Scale bar = 50 μm (×200). (G) The HYP level in lung of each group mice was measured by HYP assay kit. (H and I) Representative immunoreactivity images and quantification of IL-36γ staining in lung tissues from each group. (J) The mRNA level and (K) concentration of IL-36γ in lung tissue and BALF in each group mice. (L) The mRNA level and (M) concentration of IL-38 in lung tissue in each group mice. Bar diagrams and data are presented as the mean ± standard deviation (SD). * vs. PBS. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

1K) [7]. In addition, we found that both mRNA level of IL-38 in the lung tissues (Fig. 1L) and the concentration of IL-38 in BALF were downregulated in the HDM group mice compared with the PBS group (Fig. 1M).

Continues exposure of IL-36γ increased extracellular matrix deposition and AHR in chronic asthma mice

Increased deposition of extracellular matrix (ECM) is the main characteristic of airway remodeling [30]. In order to assess whether IL-36γ can contribute to airway remodeling, intra-nasal IL-36γ was administrated to BALB/C mice for 2 weeks at three times a week (Fig. 2A). Pathological staining results showed that the inflammatory cells infiltration around the airway and inflammation score were significantly increased after administration of IL-36γ at week 2 as compared with the PBS group and at week 1 (Fig. 2B and E). The collagen deposition level around the airway (Fig. 2C, F, and G) and the mRNA level of elastin (Fig. 2H) were remarkably upregulated after IL-36γ treatment compared to the PBS group. At the same time, the expression of Fibronectin was also obviously enhanced after 2 weeks of IL-36γ treatment by immunohistochemistry analysis (Fig. 2D and I). In addition, AHR was drastically increased after exposure of IL-36γ compared to the control group and was significantly higher at week 2 than week 1 (Fig. 2J).

Fibroblasts are key structural cells involved in airway remodeling in asthma. Activated fibroblasts can induce subepithelial fibrosis and produce large amounts of ECM [31]. In this study, we also found that the number of α-SMA and vimentin double positive activated lung fibroblasts around the airway was increased after IL-36γ stimulated as compared with the PBS group by immunofluorescence assay (Fig. 3A and B). The mRNA level of α-SMA and vimentin in the lung tissues was also upregulated after IL-36γ-administrated than the PBS group (Fig. 3C and D). All these findings suggested that IL-36γ may play an important role in airway remodeling.

IL-36γ promoted the activation of HFL-1 cells by ERK1/2 signaling pathway

Activated lung fibroblasts can produce amount of ECM which is a characteristic of airway remodeling [31]. Other research has demonstrated that IL-36 receptor agonists can create colonic fibroblasts activation by stimulating IL-36R [15]. To investigate the effect of IL-36γ on lung fibroblasts, rhIL-36γ was added to HFL-1 cells and analyzed. Cells were treated with 100 ng/ml IL-36γ [14]. As shown in Fig. 4A, the mRNA level of Fibronectin, Collagen I, and α-SMA was significantly increased in IL-36γ treated group as compared with the PBS group. Likewise, the protein expression of these three markers was also confirmed by immunofluorescence staining (Fig. 4B). Simultaneously, we found that IL-36γ enhanced the expression of Fibronectin, Collagen I, and α-SMA with a time-dependent manner by western blot (Fig. 4C).

It has been verified that IL-36 receptor agonists trigger downstream signaling cascade including MyD88 adaptor protein complex, NF-kB, and MAPK by binding to the heterodimeric receptor compound of IL-36R and IL-1 receptor-affiliated protein IL-1RAcP1 [7]. Previous studies have reported that phospho (p)-ERK1/2 is involved in the proliferation and activation of airway smooth muscle cells (ASMCs) and regulate airway remodeling of asthma [32]. But whether ERK1/2 signaling pathway participated in the activation of fibroblasts induced by IL-36γ is unknown. In this study, we found that IL-36γ can induce ERK1/2 phosphorylation in a time-dependent manner (Fig. 4D and E). PD98059 (a specific inhibitor of ERK1/2) can inhibit the expression of Fibronectin, Collagen I, and α-SMA in HFL-1 cells induced by IL-36γ (Fig. 4F). In general, we considered that IL-36γ regulates the activation of lung fibroblasts via an ERK1/2-dependent mechanism.

IL-38 counteracted the activation and migration of lung fibroblasts induced by IL-36γ

IL-38 is considered to be a partial receptor antagonist of IL-36 and can exert its anti-inflammatory action by binding to IL-36 receptor [17]. It has been indicated that IL-38 can inhibit peripheral blood mononuclear cells (PBMCs) secreting IL-8 stimulated by IL-36γ [17]. To verify whether IL-38 can block the effect of IL-36γ on lung fibroblasts, IL-38 was treated to HFL-1 cells before IL-36γ stimulated. It showed that IL-38 not only remarkably decreased the elevated phosphorylation level of ERK1/2, similar to PD98059 (Fig. 5A and B), but also inhibited the expression of Fibronectin, Collagen I, and α-SMA induced by IL-36γ in HFL-1 cells (Fig. 5C and D). Other than blocking the activation of fibroblasts, we also found that there is an enhancement in the migratory ability of HFL-1 cells after IL-36γ stimulated through wound healing and transwell assays, and pretreatment of IL-38 can reverse this effect (Fig. 5E and F). Collectively, all these data supported the conjecture that IL-38 could restrain IL-36γ-induced activation and migration of HFL-1 cells.

IL-38 or blocking IL-36R ameliorates airway inflammation and AHR in HDM-induced asthma mice

To further confirm the effect of IL-38 on asthmatic airway inflammation and AHR *in vivo*, we used an HDM-induced asthma model in BALB/c mice, which could better mimic the key characteristics of asthma [25]. Mice were randomly divided into six groups: PBS group ($n = 6$), HDM group ($n = 6$), HDM + IL-36Ra group ($n = 6$), HDM + IL-38 group ($n = 6$), HDM + IL-36γ group ($n = 6$), and HDM + IL-36γ + IL-38 group ($n = 6$). The protocol of establishing the asthmatic model is shown in Fig. 6A. For intervention experiments, mice were intraperitoneally injected with 1 μg recombinant murine IL-38 (aa3–152) (Adipogen Life Sciences, USA) PBS solution and 1 μg recombinant murine IL-36Ra (PeproTech, USA) PBS solution 30 minutes before HDM exposure beginning at the third week, three times a week accompanied with 1 μg recombinant murine IL-36γ (NovoProtein, China) PBS solution intratracheally instilled into lungs. Our data showed that both IL-38 and IL-36Ra treatment can remarkably inhibit peribronchial inflammatory cells infiltration in the lung and the thickness of bronchial wall compared with experimental allergic asthma mice (Fig. 6B–D). Meanwhile, AHR in response to methacholine was also decreased after the administration of IL-38 or IL-36Ra (Fig. 6E). Moreover, the number of inflammatory cells (Fig. 6F; Supplementary Fig. S2) and the concentrations of inflammatory cytokines including IL-5, IL-13, IL-6, IL-17, TSLP, active TGF-β1, and TNF-α in the BALF were observed to decrease after IL-38 or IL-36Ra treated as compared with the HDM group (Fig. 6G). The increased recruited mast cells (Supplementary Fig. S3) and

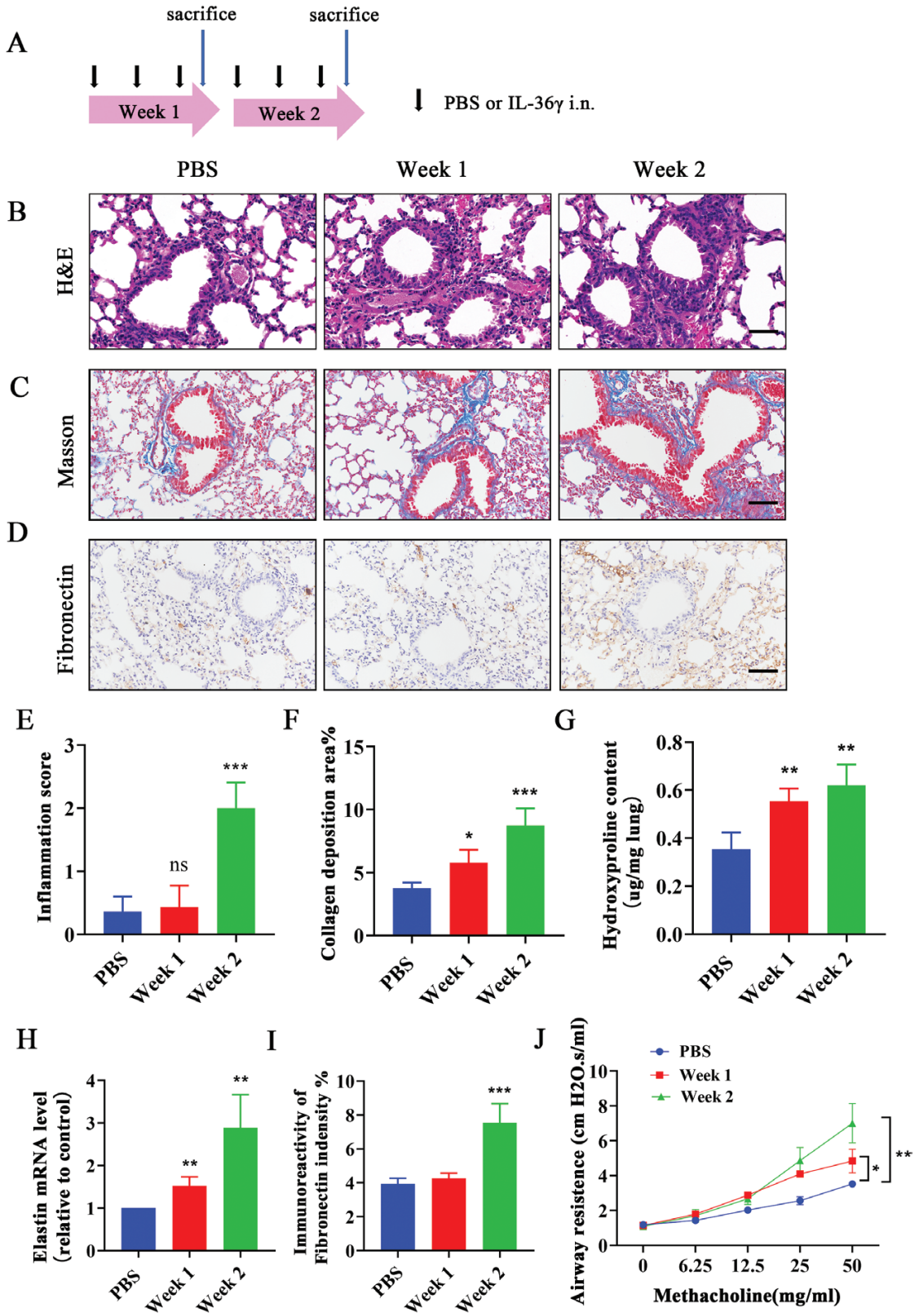

Figure 2. The effect of IL-36γ stimulation to airway inflammation, AHR and ECM deposition *in vivo*. (A) Intranasal administration of IL-36γ to mice for 2 weeks ($n = 6$ per group). (B and C) Representative histopathological images of airway and lung tissues from each group mice were performed with H&E staining and Masson staining. Scale bar = 50 μm (×200). (D) Representative immunohistochemical images of Fibronectin in mice lung tissues from each group. Scale bar = 50 μm (×200). Quantifications of the lung tissues were performed with H&E staining (E) and Masson staining (F). (G) The HYP level in lung of each group mice was measured by HYP assay kit. (H) Quantification of elastin mRNA level in the mice lung tissues from each group. (I) Quantification of Fibronectin expression in the lung of each group mice. (J) Airway resistance response to various doses of methacholine was tested within 24 hours after the final IL-36γ stimulated. Bar diagrams and data are presented as the mean ± standard deviation (SD). * vs. PBS. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, no significant differences

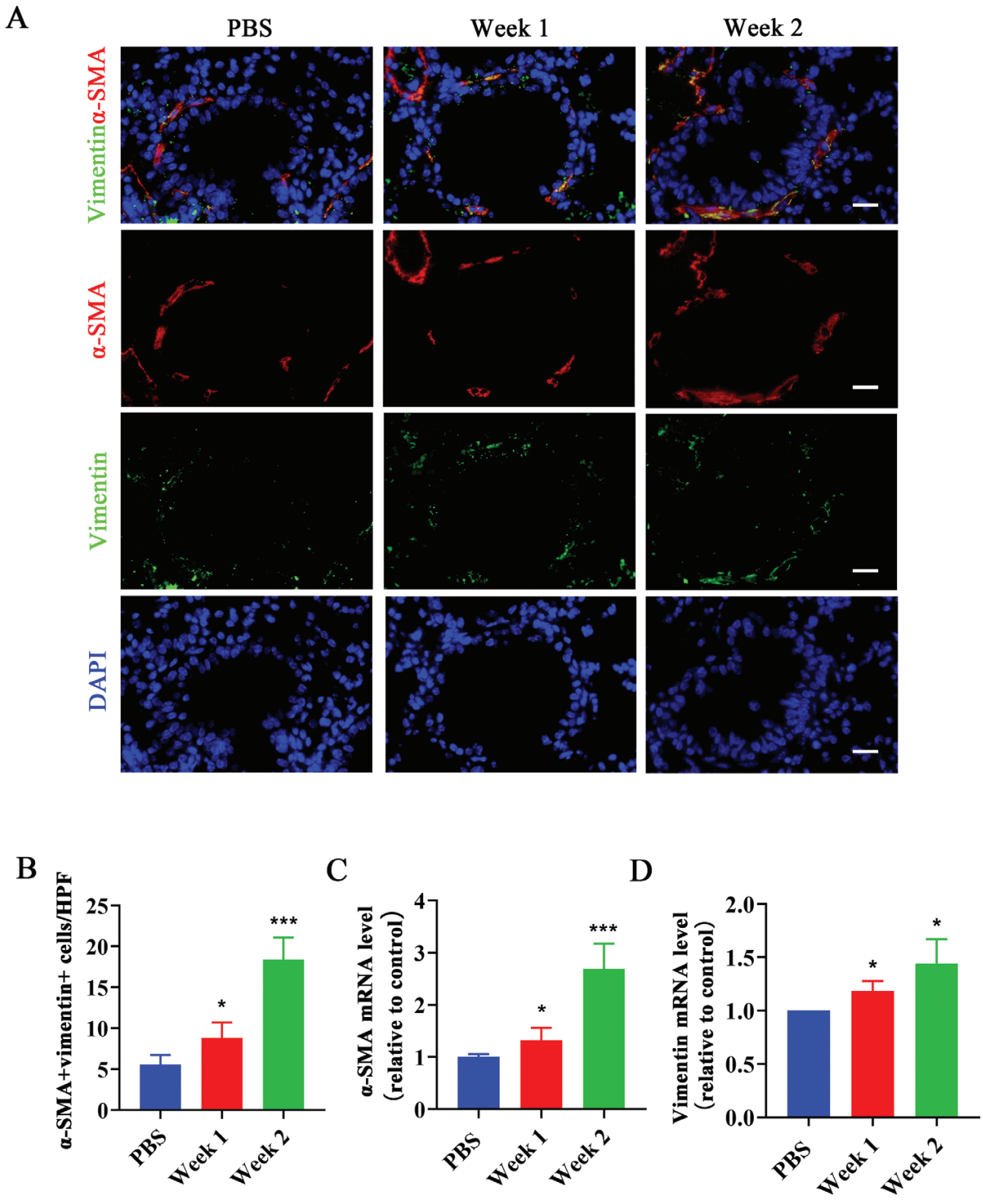

Figure 3. The effect of IL-36γ on the activation of lung fibroblast around airway. (A and B) Representative immunofluorescence images and quantification of α-SMA and vimentin double-positive activated fibroblast cells around the airways in each group. Scale bar = 20 μm (×400). (C and D) Quantification of α-SMA and vimentin mRNA level in the mice lung tissues from each group. Bar diagrams and data are presented as the mean ± standard deviation (SD). * vs. PBS. *$P < 0.05$; ***$P < 0.001$

proportion of Th2 lymphocytes (Fig. 6H) in the lung tissues of HDM group were also significantly attenuated after IL-38 or IL-36Ra treated. In addition, we found that inflammation cells and Th2 lymphocytes infiltration, the thickness of bronchial wall, and AHR were obviously enhanced in the mice from HDM + IL-36γ group accompanied with the increased

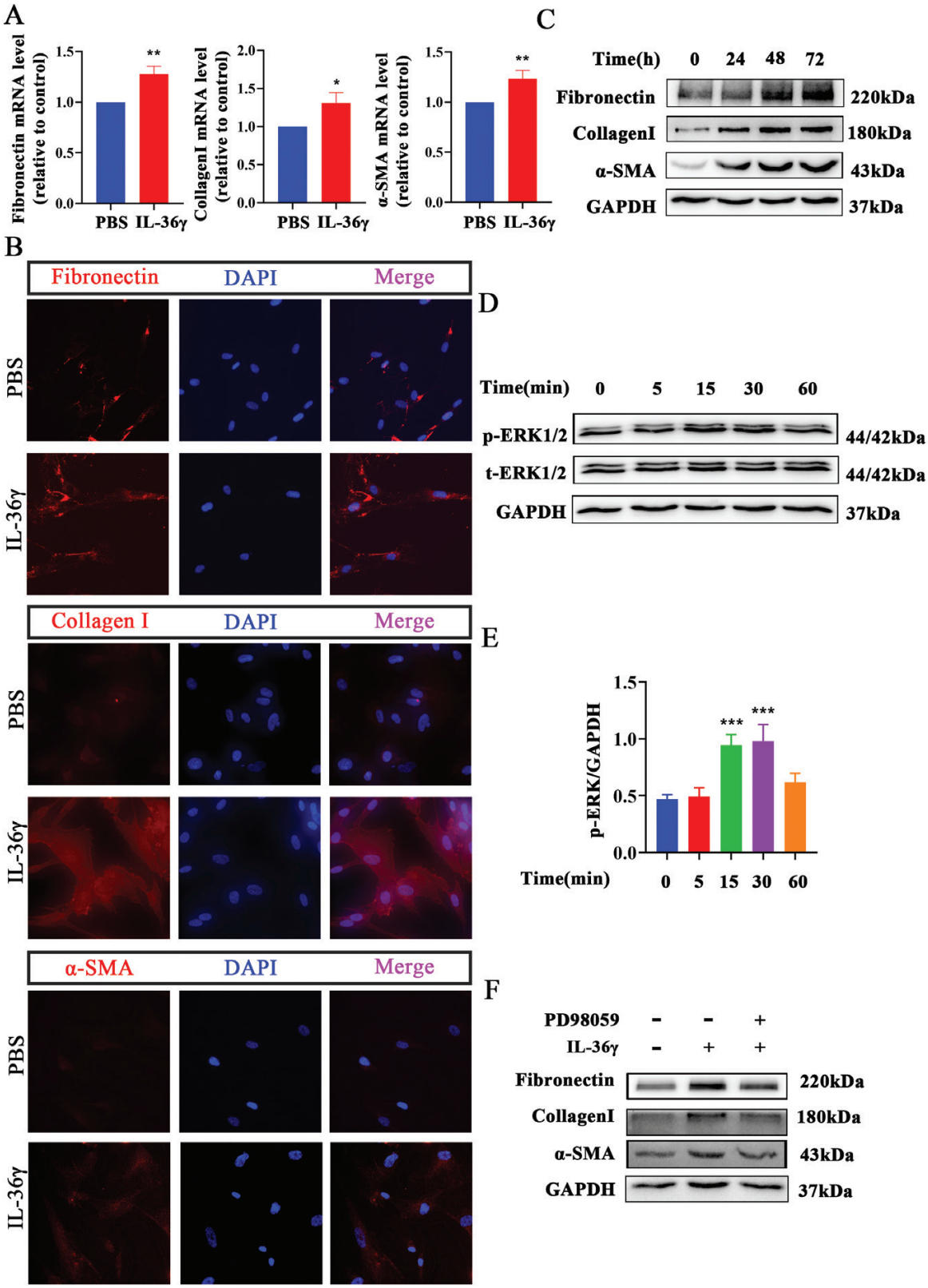

Figure 4. IL-36γ induced the activation of HFL-1 cells by ERK1/2 pathway. (A) Quantification of Fibronectin, Collagen I, and α-SMA mRNA level in HFL-1 cells treated with 100 ng/ml IL-36γ by RT–qPCR. (B) Representative immunofluorescence images of Fibronectin, Collagen I, and α-SMA protein expression in HFL-1 cells treated with 100 ng/ml IL-36γ. (C) Western blot analysis of Fibronectin, Collagen I, and α-SMA protein expression in HFL-1 cells treated with 100 ng/ml IL-36γ at different time. (D and E) Representative immunoblot analysis and quantification of the activation of ERK1/2 signaling pathway in HFL-1 cells treated with100 ng/ml IL-36γ at different time. (F) Representative immunoblot analysis of Fibronectin, Collagen I, and α-SMA in HFL-1 cells exposed to IL-36γ with or without PD98059 treated. Bar diagrams and data are presented as the mean ± standard deviation (SD). * vs. PBS group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

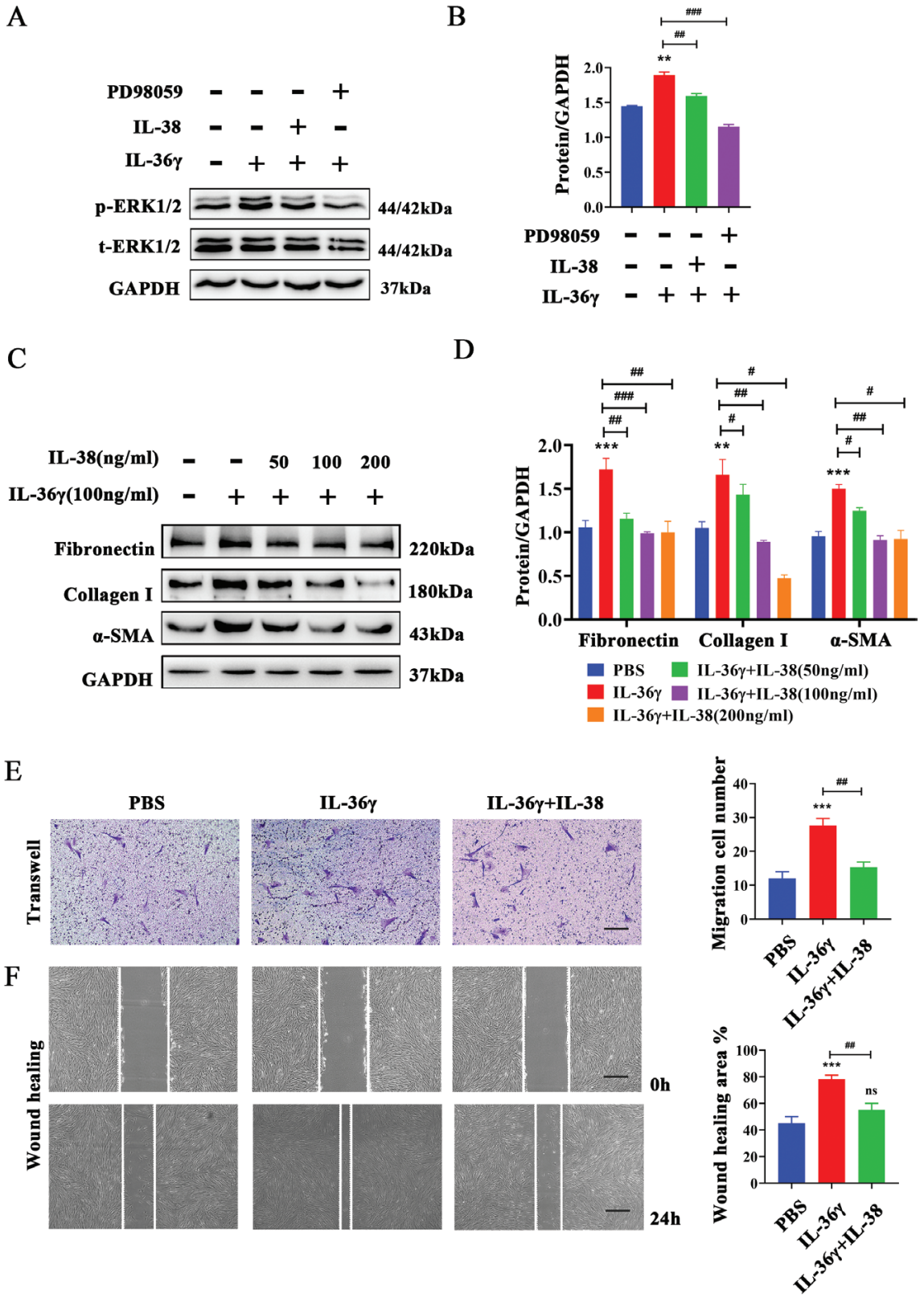

Figure 5. IL-38 inhibited IL-36γ-induced activation and migration in HFL-1 cells. (A and B) Western blotting analysis and quantification of the ERK1/2 pathway treated with IL-36γ with or without IL-38 or PD98059. (C and D) The protein expression of Fibronectin, Collagen I, and α-SMA in HFL-1 cells treated with IL-36γ, with or without different dose of IL-38 (50–200 ng/ml) by immunoblots analysis. (E and F) Representative images and quantification of cells' migrated ability induced by IL-36γ, with or without IL-38 through transwell assay (scale bar = 50 μm (×100)) and wound healing assay (scale bar = 20 μm (×40)). Bar diagrams and data are presented as the mean ± standard deviation (SD). * vs. PBS group; # vs. IL-36γ group. *,#$P < 0.05$; **,##$P < 0.01$; ***,###$P < 0.001$. Abbreviation: ns: no significant differences

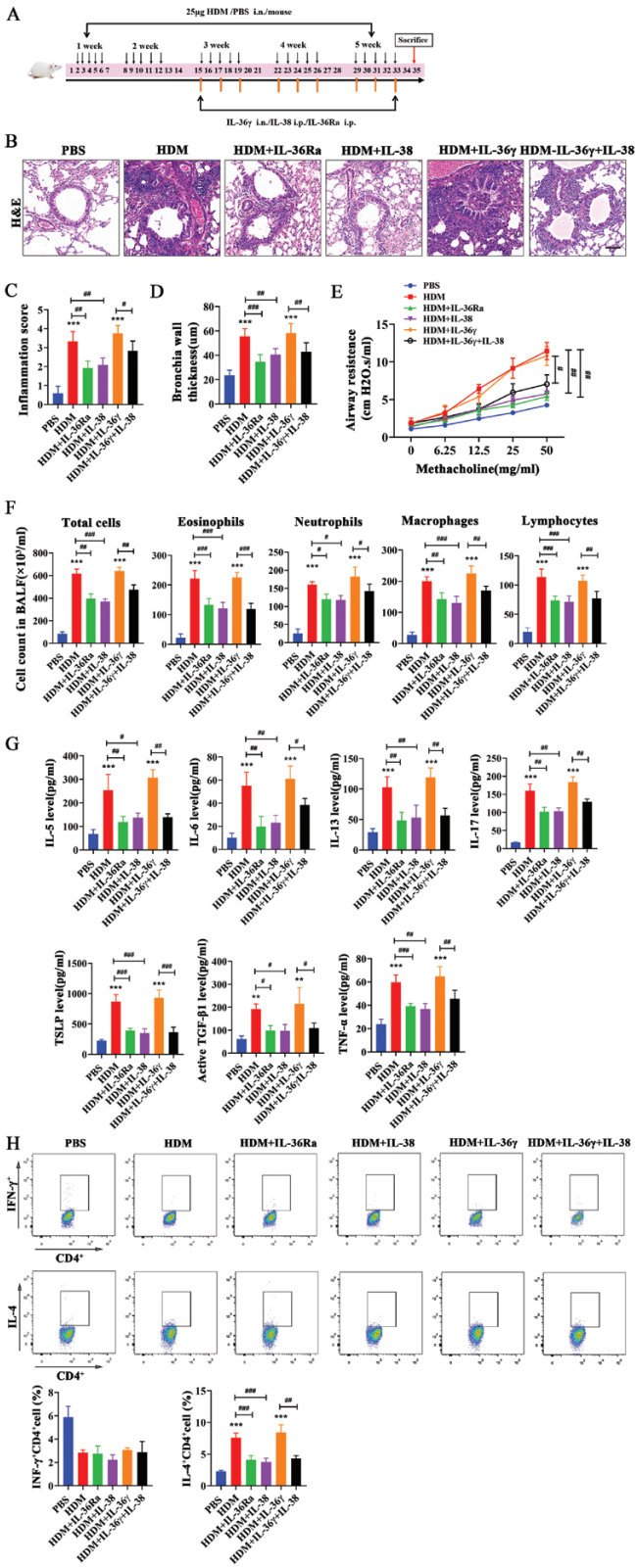

Figure 6. IL-38 or blocking IL-36R alleviated airway inflammation and AHR in asthma mice model. (A) Schematic diagram of the experimental protocol for the chronic asthma (n = 6 mice per group). (B) Representative histopathological image of lung tissues was performed with H&E staining. Scale bar = 50 μm (×200). Quantification was performed with H&E staining (C and D) in each group. (E) Airway resistance response to methacholine (0–50 mg/ml) was determined within 24 hours after the final HDM induced. (F) The total and differential inflammatory cell counts in the BALF from each group were determined by Giemsa staining. (G) Analysis of the concentrations of IL-5, IL-6, IL-17, IL-13, TSLP, active TGF-β1, and TNF-α in BALF from each group. (H) The frequencies of Th1 ($CD4^+$ $IFN\text{-}\gamma^+$) and Th2 ($CD4^+$ $IL\text{-}4^+$) cells in lung tissue were analyzed by using flow cytometry. Bar diagrams and data are presented as the mean ± standard deviation (SD). * vs. PBS group; # vs. the indicated group. *,#$P < 0.05$; **,##$P < 0.01$; ***,###$P < 0.001$

concentrations of cytokines in BALF. After treating with IL-38, all these changes were reversed.

IL-38 or blocking IL-36R alleviated airway remodeling in HDM-induced chronic asthma mice

To better understand the IL-38 potential on airway remodeling, we analyze the airway remodeling markers by histopathology. PAS-stained and Masson trichrome-stained sections demonstrated remarkable goblet cell hyperplasia and high collagen deposition around the bronchioles in the HDM group. The HYP content and mRNA level of elastin of lung tissues were also upregulated in asthmatic mice. These changes could be partly inhibited after the administration of IL-38 and IL-36Ra (Fig. 7A–F). Immunohistochemical staining of tissue sections revealed that around the bronchia, the expressions of α-SMA (Fig. 7G and J) and Fibronectin (Fig. 7H and K) were significantly increased in HDM group as compared with the PBS control. IL-38 and IL-36Ra treatment can dramatically inhibit the expression of these two markers. Meanwhile, immunohistochemistry staining for CD31 showed that the HDM-induced mice exhibited increased area of microvessels in the sub-epithelium when compared with the control groups, while IL-38 or IL-36Ra treatment stunted these increases (Supplementary Fig. S4). In addition, IL-38 can inhibit the expression of p-ERK1/2 which was upregulated in HDM group, and it was similar to the treatment with IL-36Ra (Fig. 7I and L). In HDM + IL-36γ group, all the above pathogeny structure were remarkably upregulated while they can be attenuated after the treatment of IL-38.

At the same time, the number of α-SMA and vimentin double-positive lung fibroblasts around the airway was remarkably increased in the HDM group compared with the PBS group, while IL-38 or IL-36Ra treatment group showed a decrease in the number by immunofluorescence assay (Fig. 8A and B). The mRNA level of α-SMA and vimentin in the lung tissues of mice in the HDM group was also increased and diminished after IL-38 or IL-36Ra treatment (Fig. 8C and D). Likewise, IL-36γ co-administration HDM increased the number of activated fibroblasts around the airways and treatment with IL-38 can recover these changes.

Discussion

Asthma is a chronic inflammatory disease of the airways. Airway remodeling is considered to be an important and independent pathologic feature of asthma. The main features include destruction of epithelial cells, abnormal deposition of ECM, airway smooth muscle hyperplasia/hypertrophy, and angiopoiesis [33]. Any of these pathologies can occur alone or in combination during the process of asthma [34]. In our research, we found that *in vivo*, IL-36γ was upregulated in chronic asthma mice lung tissues and continues intratracheally administration of IL-36γ can induce ECM deposition, AHR, and increased number of activated fibroblasts around mice

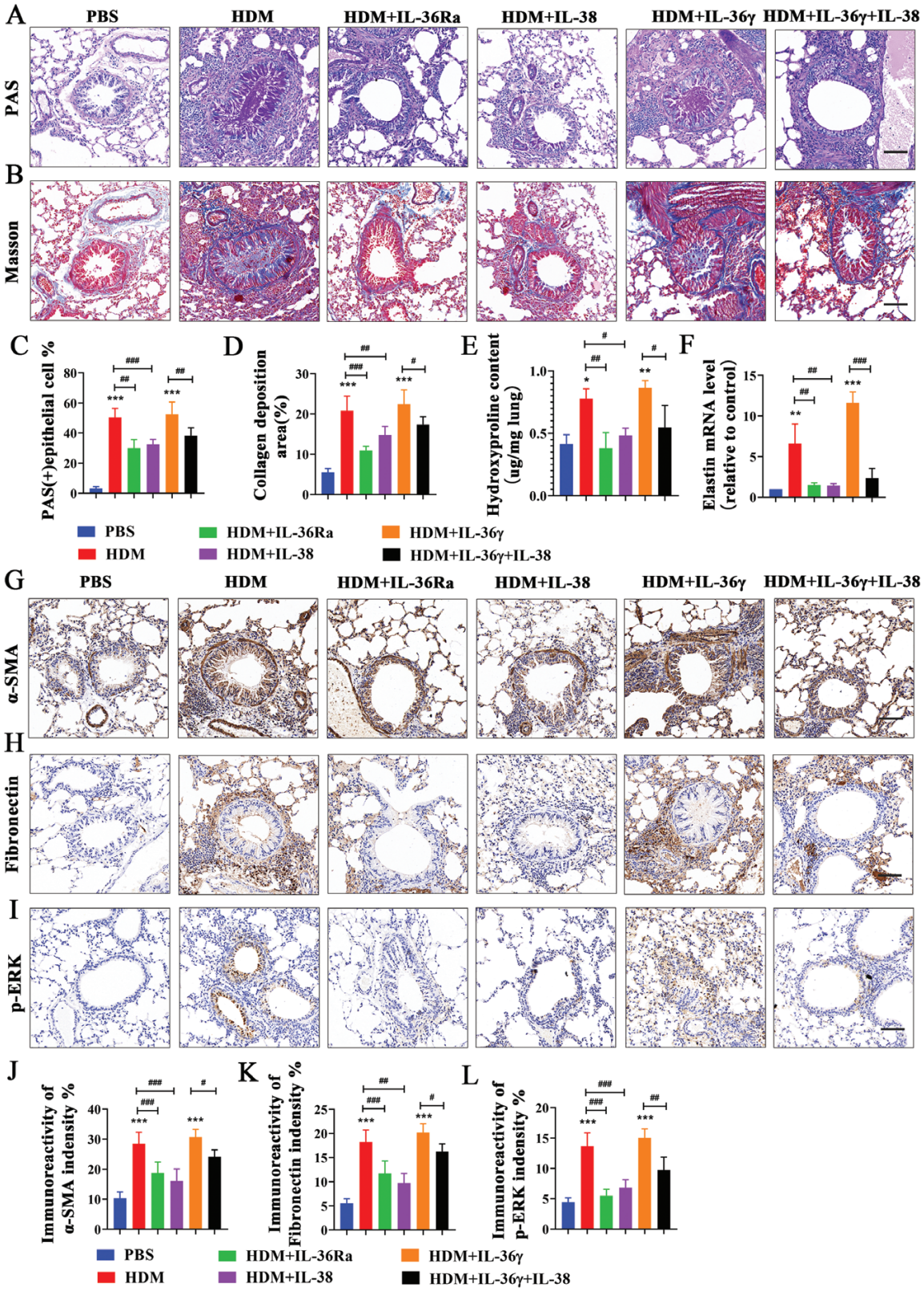

Figure 7. IL-38 or blocking IL-36R alleviated airway remodeling and the expression of p-ERK1/2 in an HDM-induced asthma murine model. (A and B) Representative images of PAS staining and Masson staining in lung tissues from each group. Scale bar = 50 μm (×200). (C and D) Quantification of PAS staining and Masson staining in each group. (E) The HYP content in lung of each group mice was measured by HYP assay kit. (F) Quantification of elastin mRNA level in the mice lung tissues of each group. (G–I) Representative immunoreactivity images of α-SMA, Fibronectin and p-ERK1/2 in lung tissues from each group. Scale bar = 50 μm (×200). (J–L) Quantification of the expression of α-SMA, Fibronectin , and p-ERK1/2 in each group. Bar diagrams and data are presented as the mean ± standard deviation (SD). * vs. PBS group; # vs. the indicated group. *,#$P < 0.05$; **,##$P < 0.01$; ***,###$P < 0.001$

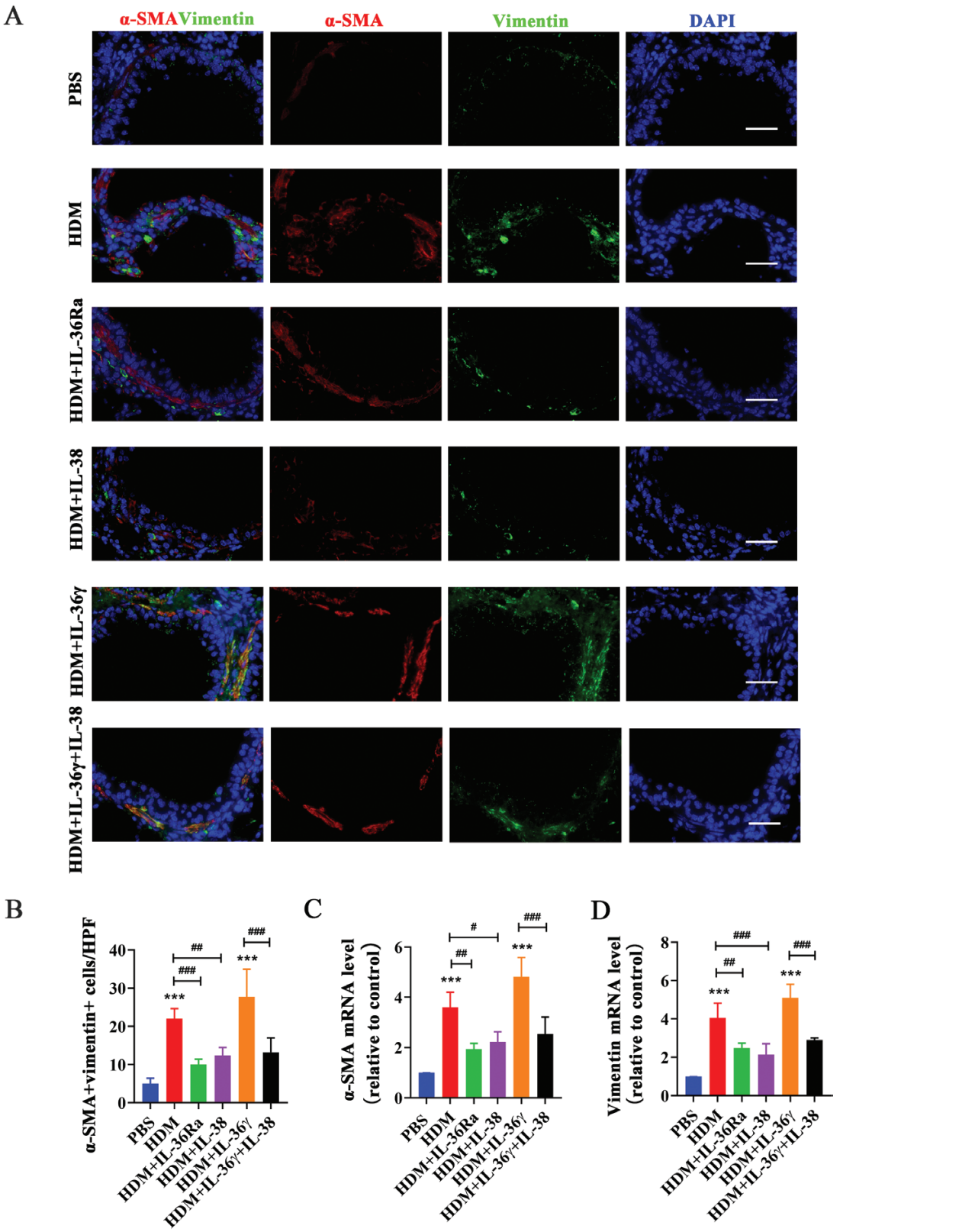

Figure 8. IL-38 or blocking IL-36R inhibits activation of lung fibroblast around airway in asthma mice. (A and B) Representative immunofluorescence images and quantification of α-SMA and vimentin double positive cells around the airways in the lung tissues from each group. Scale bar = 50 μm (×400). (C and D) Quantification of α-SMA and vimentin mRNA level in the mice lung tissues each group. Bar diagrams and data are presented as the mean ± standard deviation (SD). * vs. PBS group; # vs. the indicated group. *,#$P < 0.05$; **,##$P < 0.01$; ***,###$P < 0.001$

airways. IL-38 or blocking IL-36R treatment can alleviate the airway inflammation, AHR, airway remodeling, and the increased number of activated lung fibroblasts in chronic asthmatic mice. Meanwhile, *in vitro*, IL-36γ can promote the activation and migration of lung fibroblasts, which can be inhibited by IL-38. All these evidences can provide that IL-36γ may participate in asthma airway remodeling by upregulating profibrotic function on lung fibroblasts, and IL-38 can alleviate the airway remodeling by inhibiting this effect of IL-36γ.

Activated fibroblasts in the airways of asthmatic patients can produce large amounts of ECM and abnormal accumulation of ECM results in changes of tissue structure and function which contribute to airway remodeling and airflow limitation [35–37]. In this study, we revealed that intratracheal administration of IL-36γ to mice can promote deposition of collagen, upregulate the mRNA level of elastin and expression of Fibronectin, and increase the number of activated fibroblasts around the airway *in vivo*. It also enhanced AHR compared with the controls. Treatment with IL-36Ra can alleviate AHR and airway remodeling in chronic asthma mice. *In vitro*, IL-36γ can upregulate the expression of Fibronectin, Collagen I, and α-SMA in HFL-1 cells through ERK1/2 phosphorylation pathway which is involved in lung fibroblasts to myofibroblasts transition [38] and highly activated in chronic asthma model [39]. Meanwhile, IL-36γ also enhance the migratory ability of HFL-1 cells. All these evidences hint that IL-36γ may contribute to airway remodeling in HDM-induced asthma mice. In fact, many studies have confirmed that the IL-36 cytokines are associated with tissue remodeling in other diseases. For example, it has been demonstrated that IL-36 receptor agonists can activate intestinal fibroblasts and epithelial cells [15, 40]. Strong expression of IL-36α was found in samples from IBD patients with intestinal fibrous stenosis and correlated with the severity of inflammation [15]. IL-36R KO mice had a lower fibrosis score and reduced submucosal thickening in mice with inflammatory bowel disease, along with a reduction in the number of activated fibroblasts compared with WT mice [15]. Chi et al. confirmed that compared with WT mice, IL-36 receptor agonist IL-36α was upregulated in UUO mice, and IL-36R KO mice exhibited ameliorative renal function and inhibited fibrosis [41]. In addition, it has been found that IL -36γ and IL-36α were highly expressed in serum of IPF patients, and the increased levels are correlated with disease severity [42]. Collectively, connecting with our findings suggested that IL-36γ, as one of IL-36 receptor agonists, may be a potential target for airway remodeling in asthma.

IL-38 is an IL-1 family anti-inflammatory factor that can exert its function by binding the receptors, IL-36R, IL-1R1, or IL-1RAPL1 [17–19]. Sun et al. have demonstrated that IL-38 can alleviate airway obstruction, reduced eosinophil aggregation and proliferation of Th2 and Th7 cells, and promoted Treg-cell differentiation while inhibiting the release of Th2-type cytokines in asthma mice [25]. However, there was no evidence that IL-38 alleviates airway remodeling in chronic asthma. But the relation between IL-38 and tissues remodeling has been verified in other diseases. For example, IL-38 can ameliorate the pathological manifestations of bleomycin-induced idiopathic pulmonary fibrosis [43]. Wei et al.'s research showed that IL-38 may protect against ventricular remodeling after MI by regulating the function of dendritic cells (DCs) [44]. Shi et al. have indicated that different concentrations of IL-38 exert anti-inflammatory and antifibrotic effects in Thyroid-Associated Ophthalmopathy *in vitro* [45]. In this study, we have verified that IL-38 can inhibit the activation and migration of lung fibroblast induced by IL-36γ *in vitro*, and *in vivo*, with the treatment of IL-38, the airway inflammation, hyper-responsiveness, and remodeling in chronic asthma mice were significantly alleviated, similar to treatment of IL-36Ra. The high expression of p-ERK1/2 in the lung tissues of asthmatic mice was inhibited after IL-38 treatment. The number of activated fibroblasts around airway was also decreased after IL-38 treated compared with the asthma group. Collectively, all these data indicated that IL-38 has a promising therapeutic effect on chronic asthma airway remodeling, and the mechanism may be related to blocking the profibrotic effect of IL-36γ.

In this present study, we also tested the mRNA level of IL-36α and IL-36β in the mice lung tissues. IL-36α mRNA level was mildly elevated, and no IL-36β mRNA was detected in asthmatic mice lungs (data not shown). Although it has demonstrated the fibrotic effect of IL-36α on renal fibrosis and inflammatory bowel disease, the role of IL-36α on chronic asthma airway remodeling is worth to further in-depth research. Moreover, animal models can only mimic partial characteristics of human disease, and we need to collect more tissue specimens from asthma patients in subsequent studies to further analyze the relationship between IL-36γ and airway remodeling in asthma.

Conclusion

In summary, we demonstrated that IL-36γ may participate in promoting airway remodeling in chronic asthma by upregulating profibrotic functions of lung fibroblasts. Furthermore, we also revealed that IL-38 has a protective role on airway remodeling in chronic asthma through inhibiting the profibrotic effect of IL-36γ. IL-38 may be a promising target for the treatment of chronic asthma.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

Acknowledgement

We sincerely thank Tian-Yun Lan (Central Laboratory, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China) for assistance with flow cytometry analysis.

Ethics approval

The study was approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-Sen University (number [2019]02-148-01). The authors confirm that the animal research adhere to the ARRIVE guidelines.

Conflict of interests

None declared.

Funding

This study was supported by grants from the National Natural Science Foundation of China (grant nos. 81970017, 81973984).

Data availability

All data included in this study are available upon request by contact with the corresponding author.

Author contributions

Conceptualization: M.Z. and T.Z. Data curation: M.Z., J.Z., and T.Z. Formal analysis: M.Z., J.Z., C.H., X.Z., and T.Z. Funding acquisition: T.Z. and H.L. Investigation: M.Z., J.Z., K.F., and P.M. Methodology: M.Z., J.Z., C.H., K.F., and J.C. Validation: T.Z. Project administration: T.Z. Writing—original draft preparation: M.Z. Visualization: X.Z. All authors have read and agreed to the published version of the manuscript.

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