

## Original Article



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### Disclosure

There are no financial or other issues that might lead to conflict of interest.

# The Role of Macrophage Migration Inhibitory Factor (MIF) in Asthmatic Airway Remodeling

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## ABSTRACT

**Purpose:** Recent studies have demonstrated that macrophage migration inhibitory factor (MIF) is of importance in asthmatic inflammation. The role of MIF in modulating airway remodeling has not yet been thoroughly elucidated to date. In the present study, we hypothesized that MIF promoted airway remodeling by intensifying airway smooth muscle cell (ASMC) autophagy and explored the specific mechanisms.

**Methods:** MIF knockdown in the lung tissues of C57BL/6 mice was conducted by instilling intratracheally adeno-associated virus (AAV) vectors (MIF-mutant AAV9) into mouse lung tissues. Mice genetically deficient in the autophagy marker ATG5 (ATG5<sup>-/-</sup>) was used to detect the role of autophagy in ovalbumin (OVA)-asthmatic murine models. Moreover, to block the expression of MIF and CD74 *in vitro* models, inhibitors, antibodies and lentivirus transfection techniques were employed.

**Results:** First, MIF knockdown in the lung tissues of mice showed markedly reduced airway remodeling in OVA murine mice models. Secondly, ASMC autophagy was increased in the OVA-challenged models. Mice genetically deficient in the autophagy marker ATG5 (ATG5<sup>-/-</sup>) that were primed and challenged with OVA showed lower airway remodeling than genetically wild-type asthmatic mice. Thirdly, MIF can induce ASMC autophagy *in vitro*. Moreover, the cellular source of MIF which promoted ASMC autophagy was macrophages. Finally, MIF promoted ASMC autophagy in a CD74-dependent manner.

**Conclusions:** MIF can increase asthmatic airway remodeling by enhancing ASMC autophagy. Macrophage-derived MIF can promote ASMC autophagy by targeting CD74.

**Keywords:** Macrophage migration inhibitory factor; airway remodeling; autophagy; Smooth muscle

## INTRODUCTION

Asthma is a major health burden worldwide and approximately 300 million people are suffering from it at present.<sup>1</sup> Previous studies have proved that asthma was a chronic airway inflammatory disease which is characterized by the participation of mast cells, eosinophils and T lymphocytes.<sup>2</sup> As the development of bronchoscopy techniques, the importance of airway remodeling has been noticed.<sup>3</sup> Recent studies identified that airway remodeling is the main cause of airway hyperresponsiveness and airway narrowing in asthma.<sup>4</sup> Airway remodeling is closely associated with subepithelial fibrosis, goblet cell (GC) hyperplasia and metaplasia, and the increase of airway smooth muscle (ASM) mass.<sup>5</sup> The proliferation and migration of airway smooth muscle cells (ASMCs) remarkably affect the pathological process of airway remodeling in asthma.<sup>6-8</sup>

Autophagy is a highly conserved catabolic pathway by referring to the sequestration and degradation of cytoplasmic contents. The major role of autophagy is to protect cells by removing the damaged organelles.<sup>9,10</sup> Recent studies have demonstrated that autophagy plays a pivotal role in the pathological process of asthma.<sup>11</sup> The autophagy of various structural cells, such as airway epithelial cells and ASMCs, has proved to increase in asthma.<sup>12</sup> Another study showed that autophagy can promote transforming growth factor (TGF)- $\beta$ 1-induced airway remodeling and reduce lung function in asthma.<sup>13</sup> Interestingly, simvastatin can ameliorate airway remodeling by increasing autophagy in mouse asthma model.<sup>14</sup>

Macrophage migration inhibitory factor (MIF) is a crucial pro-inflammatory cytokine and multi-functional immune regulator.<sup>15,16</sup> MIF is mainly produced by monocytes/macrophages and lymphocytes. Structural cells, including epithelial cells and ASMC, can also secrete MIF.<sup>17,18</sup> It has been proved that MIF primarily functions to promote inflammatory response, antagonize glucocorticoid effect and mediate the release of other pro-inflammatory factors.<sup>19</sup> MIF plays a nonredundant role in asthma. MIF levels has been found to be enhanced in bronchoalveolar lavage fluid (BALF), serum, and sputum in asthmatic patients.<sup>20</sup> MIF-deficient mice revealed less pulmonary inflammation and lower airway hyperresponsiveness in an ovalbumin (OVA)-induced asthma model.<sup>21</sup> MIF may promote the proliferation and migration of ASMC by triggering ERK1/2 and FAK pathways.<sup>22</sup> (S,R)3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1), an MIF antagonist, can inhibit airway remodeling in *murine* OVA-induced asthma model.<sup>23</sup> Interestingly, MIF knockout can exacerbate cardiac remodeling by reducing the loss of autophagy in the heart.<sup>24</sup> Although data have been generated in cardiac remodeling, the role of MIF in asthmatic airway remodeling is unknown. Here, we investigated the role of MIF in airway remodeling and precise regulation mechanisms in different models of experimental asthma *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Patients

A total of 15 patients with stable asthma and 15 control subjects were recruited. Asthma was diagnosed by a pulmonary or allergy specialist from Affiliated Hospital of Guilin Medical University according to the criteria of the Global Initiative for Asthma.<sup>25</sup> Control subjects did not have asthma in their history. The experiments were approved by the Ethics Committee of Affiliated Hospital of Guilin Medical University (GLMUIA2019024), and written informed consent was obtained from each patient. Subject characteristics are shown in **Table**.

**Table.** Characteristics of the control subjects and the asthmatic patients

Characteristics	Control subjects	Asthmatic patients
Sex (male/female)	8/7	13/2
Age	41.06 ± 7.44	30.22 ± 12.76
FEV1% predicted	-	85.45 ± 10.05
Total IgE levels (IU/mL)	46.50 ± 29.66	344.89 ± 107.13*
Eotaxin-2 (pg/mL)	103.56 ± 33.34	335.53 ± 122.89*

Data are expressed as means ± standard deviation.

FEV1% predicted, forced expiratory volume in 1 second/predicted value ratio; IgE, immunoglobulin E; Eotaxin-2, eosinophil chemokine-2.

\* $P < 0.05$  compared with control.

Heparinized peripheral venous blood was collected from each participant. Serum samples were collected, frozen at  $-80^{\circ}\text{C}$  and thawed immediately before analysis. Freeze-thaw cycles did not exceed 3 times.

### Mice

Mice were from Shanghai Model Organisms Center or were bred at the Animal Center of Shanghai University of Traditional Chinese Medicine (PZSHUTCM18120712). ATG5<sup>+/-</sup> mice in the C57BL/6 genetic background were used at generation N8. The mice were age-matched females (6–9 weeks of age) and feeded on OVA-free diet in a pathogen-free environment. All the mouse experiments were approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine.

### Proteins, antibodies and other reagents

Recombinant mouse MIF was purchased from R&D Systems (Minneapolis, MN, USA). OVA was purchased from Sigma (Darmstadt, Germany). Recombinant murine interleukin (IL)-4 was purchased from Peprotech (Rocky Hill, CT, USA). The MIF inhibitor ISO-1 was purchased from medChemExpress (Monmouth Junction, NJ, USA). Anti-TGF- $\beta$ 1, anti- $\alpha$ -SMA, anti-LC3A/B, anti- $\beta$ -actin, anti-ATG5 and anti-P62 were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Collagen-I, anti-MIF and anti-Becclin1 were purchased from Abcam (Cambridge, UK). Anti-F4/80<sup>+</sup> was purchased from R&D Systems. The secondary antibodies were anti-rabbit immunoglobulin G (IgG) (Alexa Fluor<sup>®</sup> 488 Conjugate) and anti-mouse IgG (Alexa Fluor<sup>®</sup> 647 Conjugate) from Cell Signaling Technology. The neutralizing anti-mouse CD74 antibody (clone LN-1) was bought from BD Pharmingen (Santiago, MN, USA).

### Sensitization and challenge with OVA

To build an OVA asthmatic mouse model, mice were sensitized by intraperitoneal (i.p.) injection with 100  $\mu\text{g}$  of OVA and 2 mg of aluminum hydroxide in 200  $\mu\text{L}$  of saline on days 0, 7 and 14, followed by intranasal administration of 20 mg/mL OVA in 50  $\mu\text{L}$  saline from days 15 to 28 for 2 weeks. Non-OVA-challenged mice were sensitized and challenged with saline alone.

In the MIF-knockdown mouse model, female C57BL/6 mice (20–25 g) were randomly assigned to 4 groups ( $n = 5$ –6/group). In the control and solely OVA-challenged groups, 50  $\mu\text{L}$  of saline were instilled intratracheally every day. The wild-type (WT) capsid AAV9 group or the capsid MIF-mutant AAV9 group ( $10^{10}$  vg in 50  $\mu\text{L}$  of saline/mouse/day) containing the DNA of enhanced green fluorescence protein was delivered to the mouse lungs. AAV9 or saline was administered during the OVA-challenged process from days 15 to 28 for 2 weeks.

In the ATG5<sup>+/-</sup> autophagy-deficient mouse model, WT and ATG5<sup>+/-</sup> mice were randomly divided into 4 groups (n = 5–6/group) according to whether the mice would undergo the sensitization and challenge with OVA.

In the ISO-1-mediated MIF blockage model, female mice were randomly assigned to 3 groups (n = 5–6 /group). In the ISO-1 group, mice were given 35 mg/kg of ISO-1 by i.p. injection 0.5 hours before each OVA challenge for 2 weeks.

All the mice were sacrificed 24 hours after the last challenge. BALF and lung tissues were harvested for further experiments.

### **BALF and cellular analysis**

BALF was collected by lavaging the lung 3 times with 0.5 mL of warm saline via a tracheal catheter. The BALF was immediately centrifuged (10 minutes, 4°C, 1,000 g). Cell pellets were resuspended in 1 mL of phosphate-buffered saline (PBS) for total and differential cell counting, and the supernatants were rapidly collected and frozen for further investigation. Differential cell counting was performed on hematoxylin and eosin (H&E)-stained cytopins.

### **Measurement of cytokine expression in human and mouse samples**

The concentrations of cytokines in human serum and mouse BALF supernatants were assayed by enzyme-linked immunosorbent assay (Boster Biological Technology, Wuhan, China; R&D Systems). All analyses and calibrations were performed in triplicate. Optical densities were determined using an absorbance microplate reader at 450 nm.

### **mRNA analysis**

Total RNA was isolated from cells using TRIzol<sup>®</sup> reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using a Takara reverse transcriptase kit. Polymerase chain reaction was performed by denaturation (5 minutes, 94°C), followed by 36 cycles of denaturation (45 seconds, 94°C), annealing (45 seconds, 53°C) and extension (1 minute, 72°C), then ended with final extension (10 minutes, 72°C). The primer sequences used were as follows. MIF (mouse): 5'-CCAGAACCGCAACTACAGTAAGC-3' (forward) and 5'-TTGGCAGCGTTCATGTCGTA-ATAG-3' (reverse),  $\alpha$ -SMA (mouse): 5'-GCGTGGCTATTCCTTCGTGACTAC-3' (forward) and 5'-CGTCAGGCAGTTCGTAGCTCTTC-3' (reverse), Beclin1 (mouse): 5'-AGGCAGTGGCG-GCTCCTATTC-3' and 5'-TGAGGACACCCAGGCAAGACC-3' (reverse), ATG5 (mouse): 5'-CG-CCCCTGAAGATGGAGAGA-3' (forward) and 5'-CAATCTGTTGGCTGGGGGAC-3' (reverse),  $\beta$ -actin (mouse): 5'-ATCACTATTGGCAACGAGCGGTTC-3' (forward) and 5'-CAGCACT-GTGTGGCATAGAGGTC (reverse), ATG5 (rat): 5'-CTCAGCTCTGCCTTGGAAACATCAC-3' (forward) and 5'-AAGTGAGCCTCAACTGCATCCTTG-3' (reverse),  $\beta$ -actin (rat): 5'-CTGAGAG-GGAAATCGTGCGTGAC-3' (forward) and 5'-AGGAAGAGGATGCGGCAGTGG-3' (reverse).

### **Histological analysis**

After sacrificed, the lungs of mice were removed and fixed in paraformaldehyde and embedded in paraffin. H&E staining was used to evaluate inflammatory cell infiltration. For the enumeration of mucin-positive GCs, Alcian blue and periodic acid-Schiff (AB-PAS) stains were used. Sections were stained with Masson stain to evaluate collagen deposition.

### **Immunofluorescence**

Sections were dewaxed and rehydrated, and antigen retrieval was performed with 10 mM sodium citrate (pH 6.1). Next, the sections were blocked with 5% bovine serum albumin for

30 minutes at 37°C. The slides were incubated with anti-TGF- $\beta$ 1, anti- $\alpha$ -SMA, anti-LC3A/B, anti-P62, anti-Collagen-I, anti-MIF and anti-F4/80<sup>+</sup> (1:200) overnight at 4°C and then processed with the corresponding secondary antibodies (1:400) for 2 hours at room temperature. The slides were stained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) to visualize nuclei. Finally, a fluorescence microscope with  $\times$  20 or  $\times$  40 objective lens (Nikon, Tokyo, Japan) was used to view the immunolabeled slides in 5 fields of view. Immunofluorescence quantification was performed using ImageJ v5.0 software (National Institutes of Health, Bethesda, MD, USA).

### Western blot analysis

Cells were lysed in ice-cold NP40 lysis buffer (Sigma). Protein concentrations were measured with the Protein BCA Assay Kit (Beyotime, Beijing, China). Protein samples (30  $\mu$ g of total protein each) were boiled at 100°C for 5 minutes and loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride; the membranes were probed using a standard immunoblotting protocol. The following primary antibodies were used: anti-LC3A/B (1:1,000), anti-P62 (1:1,000), anti-Beclin1 (1:1,000), anti-ATG5 (1:1,000), anti-Collagen I (1:1,000), anti- $\alpha$ -SMA (1:1,000) and anti- $\beta$ -actin (1:1,000). The quantification of protein expression was performed by densitometry using ImageJ v5.0 software (National Institutes of Health).

### Cell culture

The immortalized mouse airway smooth muscle cell line (MASMC) was purchased from Otwo Biotech (Shenzhen, China). The rat alveolar macrophage cell line NR8383 was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd (Shanghai, China). The peritoneal macrophage cell line RAW264.7 was purchased from Shanghai Institutes for Biological Sciences. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cells were grown at 37°C in humidified air with 5% CO<sub>2</sub>. For ASMC stimulation, we exposed 100 ng/mL MIF to ASMC for 12 or 24 hours in the MIF exposure group, and the same volume of PBS was used in the control group. For macrophage stimulation, in the IL-4+ISO-1 group, macrophages were pre-incubated with ISO-1 (100 nM) for 30 minutes and stimulated with IL-4 (10 ng/mL) for 24 hours. In the IL-4 group, the same volume of PBS was used to replace ISO-1, and then stimulated with IL-4 (10 ng/mL) for 24 hours. We used the same volume of PBS in the control group. After 24 hours, we collected the supernatant, which was used for culturing ASMC for 24 hours. In the Short hairpin RNA (shRNA)-MIF-knockdown model, RAW264.7 cells, shRNA-negative control (NC) transfected RAW264.7 cells and shRNA-MIF transfected RAW264.7 cells were incubated with IL-4 (10 ng/mL) for 24 hours, respectively. The same volume of PBS was used in the control group.

### Cell proliferation assay

Proliferation of immortalized ASMC was assessed by colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis (Cell Proliferation Elisa; Abcam) according to the manufacturer's instructions. Briefly, cells starved for 12 hours in DMEM without FBS and were stimulated with 100 ng/mL MIF for 24 hours. After preincubation, ASMCs were labeled with BrdU for 2 hours. BrdU incorporation was detected by adding an anti-BrdU-POD antibody and subsequent substrate reaction.

### Virus transfection

The shRNA sequence targeting mouse MIF and CD74 genes were inserted into PGMLV-hU6-MCS-CMV-ZsGreen1-PGK-Puro-WPRE vector to generate MIF-si plasmid and CD74-si plasmid. The sequence silencing MIF expression was 5'-GGGTCTACATCAACTATTACG-3'.

The sequence silencing CD74 expression was 5'-GGCTCTGTTTGAGATGAGCA-3'. Plasmid transfected with scramble siRNA (5'-TTCTCCGAACGTGTCACGT-3') was used as a NC (synthesized by Genomeditech Co. Ltd, Shanghai, China). Lentiviral particles targeting MIF and CD74 were transfected into RAW264.7 cells and MASCs, respectively. Virus inoculation was conducted according to the previous study.<sup>26</sup> Briefly, the cells were seeded in 24 well culture plates at a density of  $5 \times 10^4$  cells/well and cultured with DMEM supplemented with 10% FBS. Before transfection, lentivirus stocks were diluted with culture medium (portion: 1:10) and polybrene (Sigma) was added to a final concentration of 6  $\mu\text{g}/\text{mL}$ . When cell density reached approximately 50% confluence, the medium was replaced with the diluted lentivirus stock solution. After the cells were cultured for 24 hours, the solution was exchanged with DMEM supplemented with 10% FBS, the cells were then incubated for another 24 hours at 37°C, 5% CO<sub>2</sub>. Then the cells were seeded in 10 cm culture dishes at a density of about 500 cells/dish. The medium containing 5  $\mu\text{g}/\text{mL}$  puromycin was exchanged every 3 days. After continuous selection with puromycin for approximately 3 weeks, the surviving cell colonies were selected using cloning rings, and then were expanded and subcloned using the limiting dilution method. Adeno-associated virus (AAV) targeting MIF and GFP-RFP-LC3-transfected MASCs were also obtained from Genomeditech Co. Ltd. The transfection efficiency was confirmed by fluorescence microscopy and Western blotting (**Supplementary Fig. S1**).

### Flow cytometric analysis

Immortalized mouse ASMCs were stained with fluorochrome-conjugated antibodies for CD44, CD182 and CD74 (all FITCconjugated; Abcam), CD184 (PE-conjugated; Bioss, Beijing, China). Unstained cells were used as controls. Cells were subjected to flow cytometry analysis using a CytoFLEX flow cytometer (Beckman Coulter). Data were analyzed using CytExpert software.

### Transmission electron microscopy

ASMCs were fixed with 4% glutaraldehyde and 1% OsO<sub>4</sub> in 0.1 M cacodylate buffer for 2 hours at 4°C. Ultrathin sections were stained with 4% uranyl acetate and lead citrate, and images were captured using a Hitachi 7700 electron microscope (Hitachi, Tokyo, Japan).

### Statistical analysis

The data are presented as the mean  $\pm$  standard deviation. Student's *t*-test and one-way analysis of variance were applied to calculate statistical significance among the groups. Significance was defined as  $P < 0.05$ .

## RESULTS

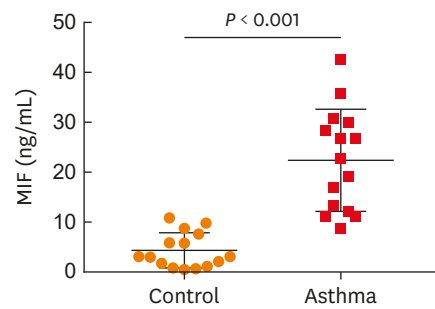
### MIF was up-regulated in asthmatic patients

To determine whether MIF is implicated in stable asthma, we measured the plasma level of MIF. We found that MIF expression was increased in the stable asthma group when compared to the control group (**Fig. 1**).

### MIF knockdown in the lung tissues of mice displays reduced airway remodeling in experimental asthma

To assess the relationship between MIF and airway remodeling, we established MIF-knock-down (MIF-mutant AAV9) mice and the mice were primed to OVA challenged to build a chronic murine asthma model. OVA-challenged mice deficient in MIF in the lung tissues showed less inflammation infiltration, decreased subepithelial collagen deposition and



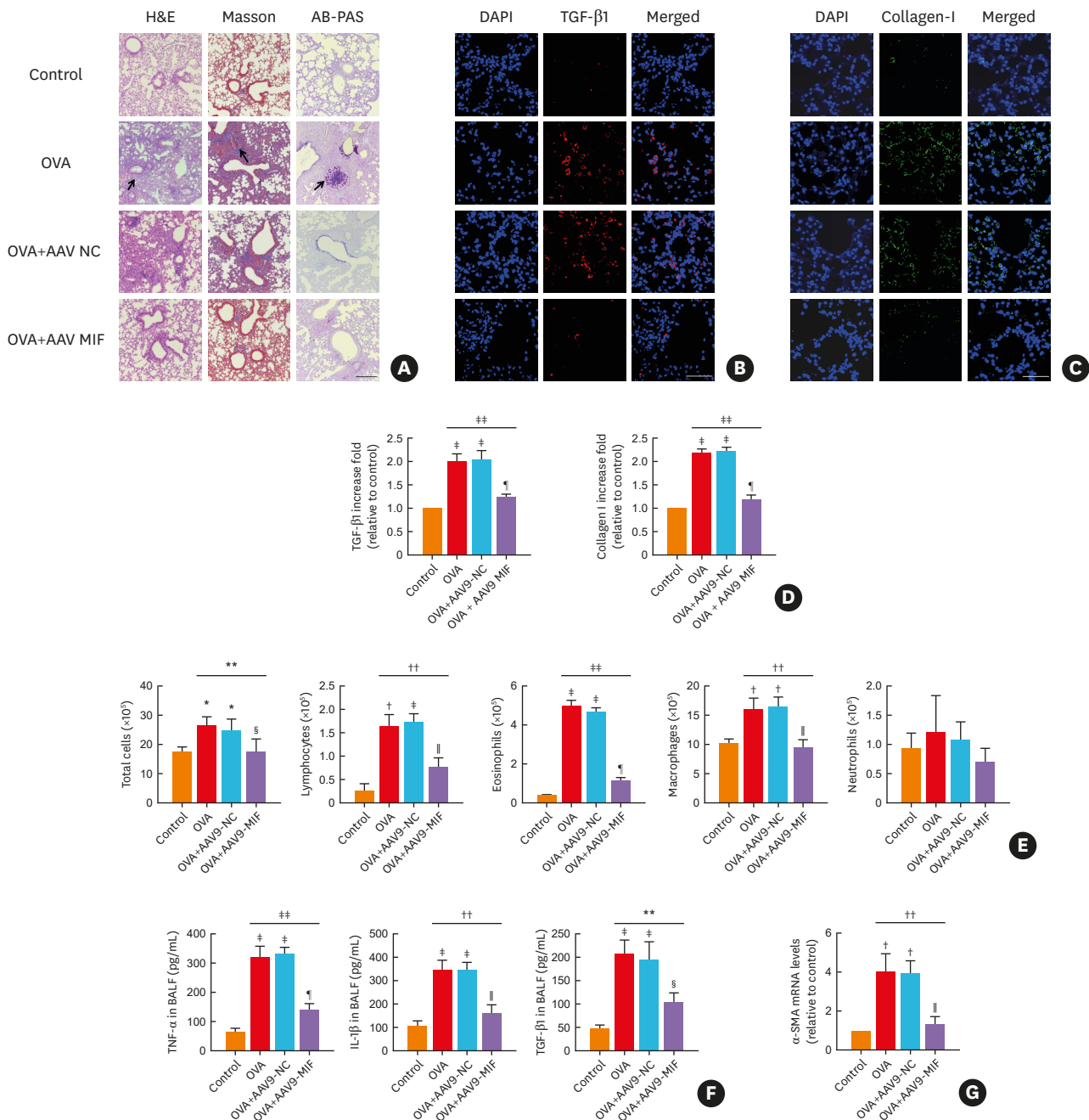


**Fig. 1.** Increased level of MIF in the serum of stable asthmatic patients. The serum level of MIF in the asthma group significantly increased compared with the control group.  $P < 0.001$  vs. control. MIF, migration inhibitory factor.

reduced GC hyperplasia-metaplasia than WT AAV9 asthmatic mice or solely OVA-challenged mice (**Fig. 2A**). The expression of airway remodeling-associated markers in the lung tissues, TGF- $\beta$ 1 and Collagen-I, were also reduced in the OVA+AAV9-MIF group compared with the OVA+AAV9-NC group or OVA group (**Fig. 2B-D**). We performed the measurement of tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$  and TGF- $\beta$ 1 protein contents as well as the counts of macrophages, lymphocytes, eosinophils and total cells in BALF, which also showed down-regulated expression in the OVA+AAV9-MIF group (**Fig. 2E and F**). No significant changes in BALF neutrophil amounts were found in the OVA+AAV9-MIF group (**Fig. 2E**). We also discovered the decreased mRNA expression of  $\alpha$ -SMA, the biomarker of ASMC, in MIF knock-down mice primed with OVA (**Fig. 2G**). The results of airway remodeling in the OVA+AAV9-NC group had no difference those in the OVA group, but airway remodeling in these 2 groups was much higher than in the control group. Moreover, we detected reduced MIF expression in the lung tissues and BALF of the OVA+AAV9-MIF group (**Fig. 3A**). We discovered that the results of macrophage localization were also consistent with MIF production in the lung tissues by applying confocal fluorescence to assess the coexpression of F4/80<sup>+</sup> cells and MIF (**Fig. 3B and C**).

### OVA-challenged mice deficient in autophagy (ATG5<sup>-/-</sup>) show decreased airway remodeling

Numerous studies have demonstrated the pivotal role of autophagy in asthma,<sup>12,14</sup> but no consistent data have been achieved at present. Therefore, we detected the mRNA expression of the 2 important autophagy markers ATG5 and Beclin1 in lung (**Fig. 4A**). We also assessed fluorescence coexpressions of ASMC and autophagy ( $\alpha$ -SMA+LC3,  $\alpha$ -SMA+P62) in the lung tissues (**Fig. 4B and C**). The results showed that autophagy, especially ASMC autophagy, was increased in the OVA murine asthma model compared with the control group. After obtaining mice genetically deficient in autophagy (ATG5<sup>-/-</sup>), we subjected ATG5<sup>-/-</sup> and WT mice to OVA-challenged asthma model to further explore the function of autophagy in airway remodeling. The amounts of total cells, lymphocytes and eosinophils as well as the expression of TNF- $\alpha$ , IL-1 $\beta$  and TGF- $\beta$ 1 in BALF were decreased in OVA-challenged ATG5<sup>-/-</sup> mice compared with OVA-challenged WT mice (**Fig. 5A and B**). H&E, Masson and AB-PAS stains in the lung tissues also displayed the decrease in inflammation infiltration, collagen deposition and GC hyperplasia-metaplasia in ATG5<sup>-/-</sup> mice than in WT mice after OVA challenged (**Fig. 5C**). Immunofluorescence of airway remodeling markers ( $\alpha$ -SMA, TGF- $\beta$ 1 and Collagen-I) was less in ATG5<sup>-/-</sup> mice primed with OVA than in WT mice primed with OVA (**Fig. 5D-G**). Notably, no significant difference in airway remodeling results was found between WT mice challenged with saline and ATG5<sup>-/-</sup> mice challenged with saline.



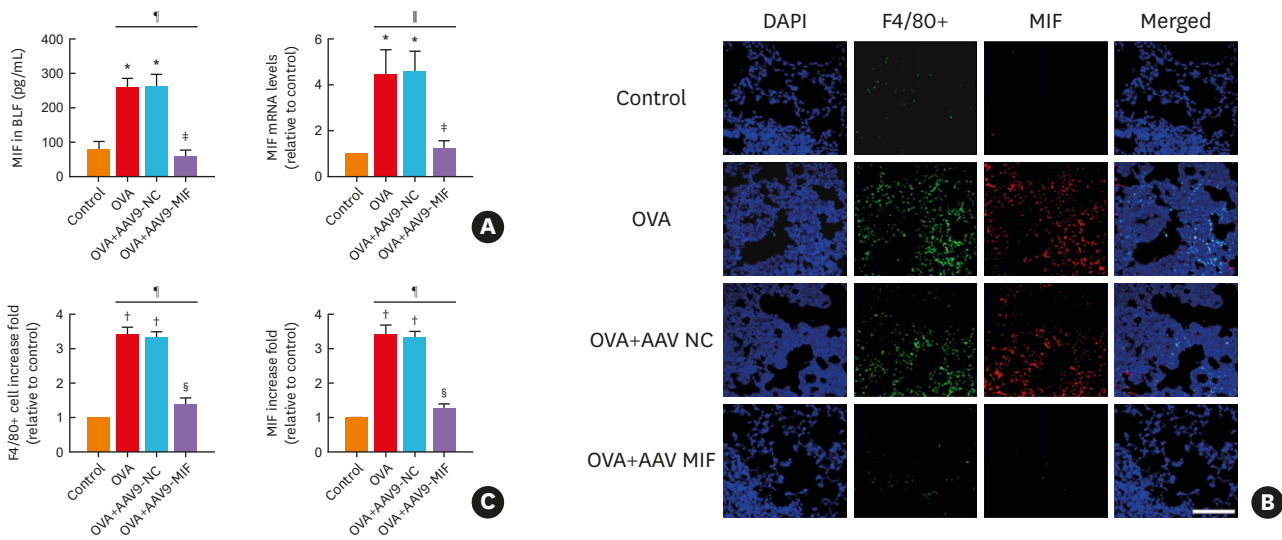
**Fig. 2.** Decreased airway remodeling in MIF-knock-down asthmatic mice. (A) The histological findings of the lung tissues 24 hours after challenge with OVA. The lung tissues were stained with H&E, Masson and AB-PAS (200 $\times$ , scale bar, 100  $\mu$ m). (B-D) The expression of representative airway remodeling-associated cytokines TGF- $\beta$ 1 and Collagen-I in the lungs was assessed by immunofluorescence (400 $\times$ , scale bar, 50  $\mu$ m). Decreased airway remodeling was verified by quantification of the fluorescence area of TGF- $\beta$ 1 and Collagen-I in the OVA+AAV9-MIF group compared with the OVA group or the OVA+AAV9-NC group. No difference was found between the OVA+AAV9-NC group and the solely OVA-challenged group. (E, F) Cell counts (total cells, lymphocytes, eosinophils and macrophages) and cytokine (TNF- $\alpha$ , IL-1 $\beta$  and TGF- $\beta$ 1) detection in BALF showed decreases in the OVA+AAV9-MIF group compared with the OVA group or OVA+AAV9-NC group. The amounts of neutrophils in BALF had no obvious changes among the groups. (G) Similarly, the mRNA expression of  $\alpha$ -SMA, an important airway remodeling marker, was also decreased in the OVA+AAV9-MIF group. Results are presented as the mean  $\pm$  SD for each group from 3 independent experiments using 5–6 mice per group.

MIF, migration inhibitory factor; OVA, ovalbumin; H&E, hematoxylin and eosin; AB-PAS, alcian blue and periodic acid-Schiff; TGF, transforming growth factor; AAV, adeno-associated virus; NC, negative control; SD, standard deviation; H&E, hematoxylin and eosin; AB-PAS, alcian blue and periodic acid-Schiff; DAPI, 4',6-diamidino-2-phenylindole; TGF, transforming growth factor; TNF, tumor necrosis factor; IL, interleukin.

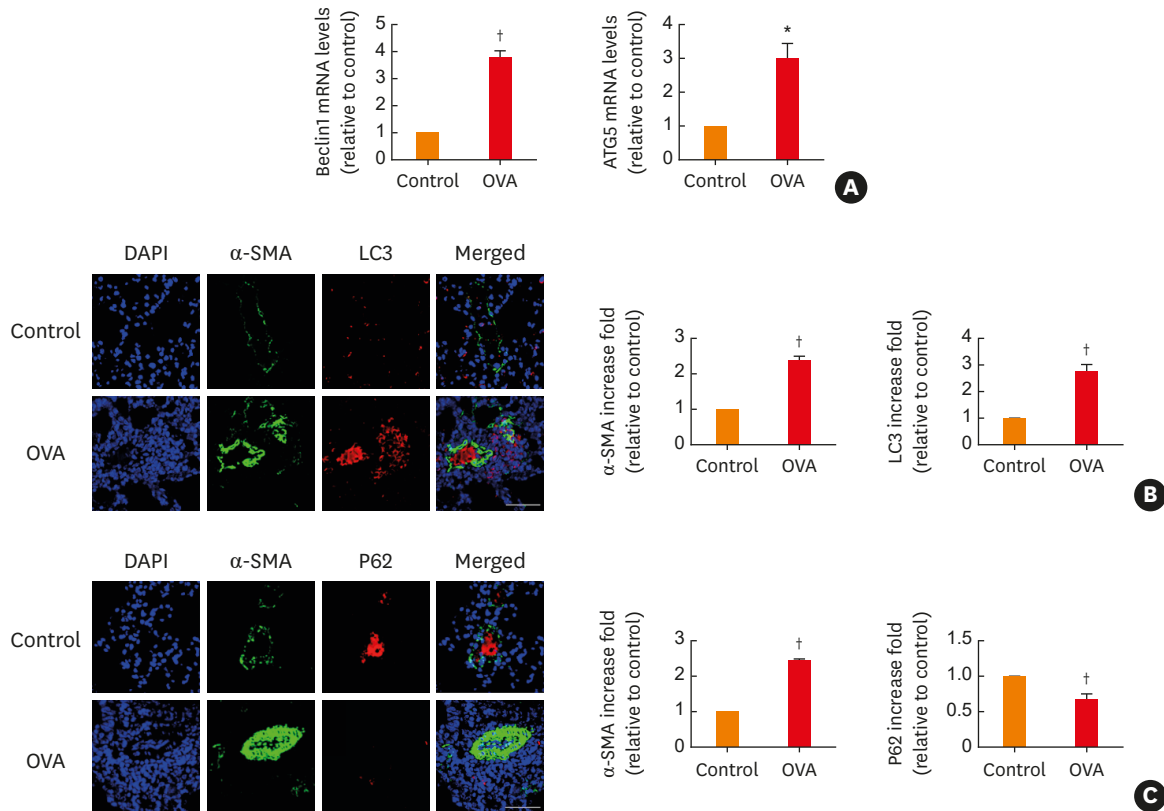
\* $P < 0.05$  vs. control; † $P < 0.01$  vs. control; ‡ $P < 0.001$  vs. control; § $P < 0.05$  vs. OVA+AAV9-NC group; ¶ $P < 0.01$  vs. OVA+AAV9-NC group; †† $P < 0.001$  vs. OVA+AAV9-NC group; \*\* $P < 0.05$  vs. OVA group; ††† $P < 0.01$  vs. OVA group; †††† $P < 0.001$  vs. OVA group.



**MIF Promotes Airway Remodeling**

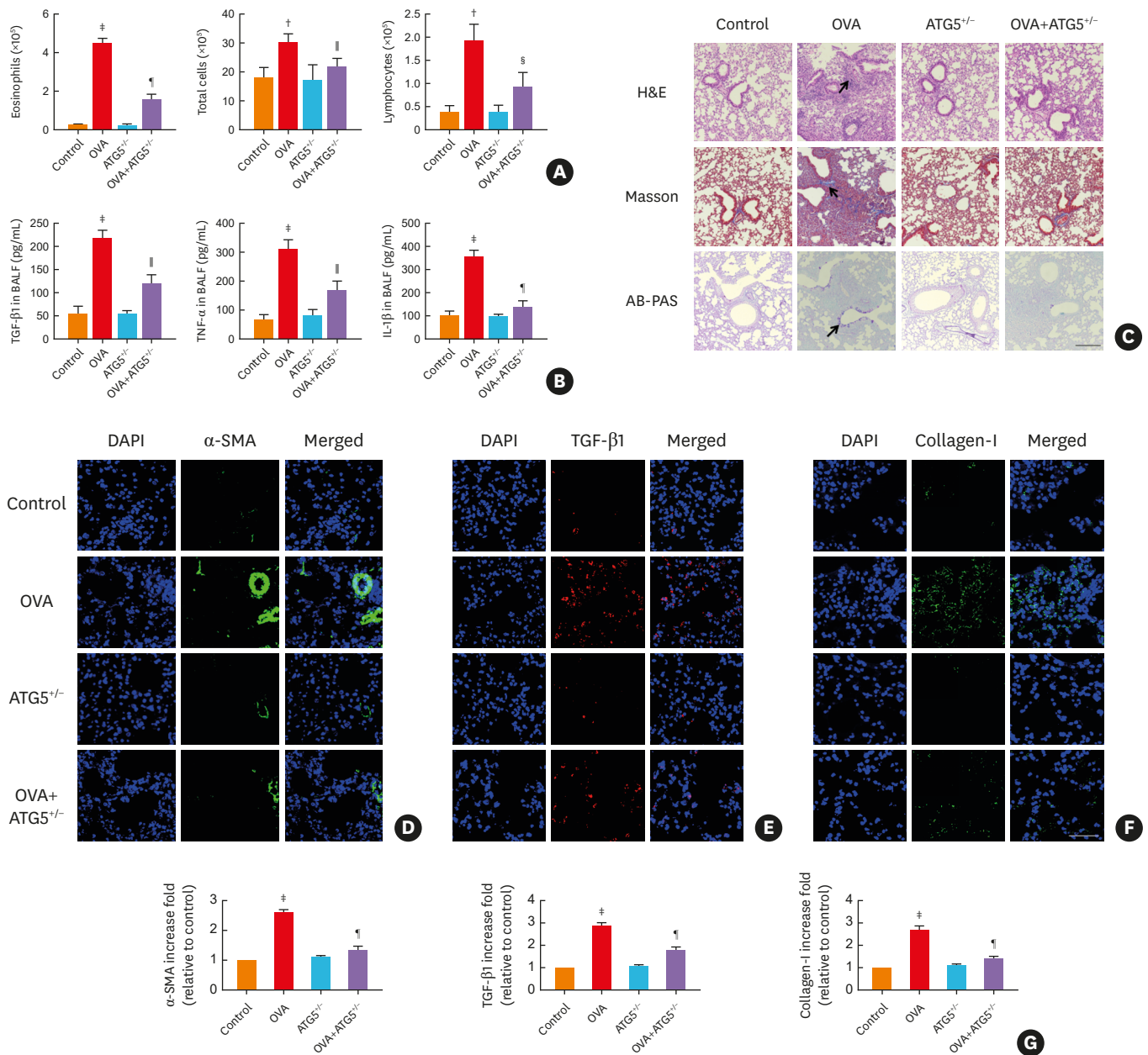


**Fig. 3.** MIF expression and macrophage amount in the MIF-knockdown asthmatic model. (A) The protein and mRNA expression of MIF increased in the OVA+AAV9-NC group and MIF knock-down attenuated the increase. (B, C) Immunofluorescence for F4/80+ and MIF and quantification of the fluorescence area indicated the consistence of macrophage accumulation and MIF production in the lung tissues (400 ×, scale bar, 50 μm). Results are presented as the mean ± SD for each group from 3 independent experiments using 5–6 mice per group. MIF, migration inhibitory factor; OVA, ovalbumin; AAV, adeno-associated virus; NC, negative control; SD, standard deviation; DAPI, 4',6-diamidino-2-phenylindole. \**P* < 0.01 vs. the control group; †*P* < 0.001 vs. the control group; ‡*P* < 0.01 vs. the OVA+AAV9-NC group; §*P* < 0.001 vs. the OVA+AAV9-NC group; ¶*P* < 0.01 vs. the OVA group; ¶*P* < 0.001 vs. the OVA group.



**Fig. 4.** ASMC autophagy increased in the OVA-challenged mice model. (A) Enhanced mRNA levels of autophagy markers, Beclin1 and ATG5, in the lung tissues of the OVA group. (B, C) The autophagy of ASMCs in the lung tissues was assessed by immunofluorescence (400 ×, scale bar, 50 μm) staining of α-SMA (ASMC marker) + LC3 (autophagy marker) and α-SMA + P62 (autophagy marker). Quantification showed the increase in ASMC autophagy in the OVA group. Results are presented as the mean ± SD from the 3 independent experiments using 5–6 mice per group. ASMC, airway smooth muscle cell; OVA, ovalbumin; AAV, adeno-associated virus; SD, standard deviation; DAPI, 4',6-diamidino-2-phenylindole. \**P* < 0.01 vs. the control group; †*P* < 0.001 vs. the control group.

**MIF Promotes Airway Remodeling**



**Fig. 5.** Reduced airway remodeling in ATG5<sup>-/-</sup> asthmatic mice. (A, B) Decreased allergen-induced inflammatory cells and cytokines production in the BALF in the OVA+ATG5<sup>-/-</sup> group compared with the OVA group. No increase in inflammatory findings in BALF was found in the ATG5<sup>-/-</sup> group compared with the control group. (C) Histological findings displayed a less degree of inflammatory cell infiltration (H&E), collagen deposition (Masson) and GC metaplasia (AB-PAS, 200 $\times$ , scale bar, 100  $\mu$ m) staining in the OVA+ATG5<sup>-/-</sup> group. (D-G). The immunofluorescence (400 $\times$ , scale bar, 50  $\mu$ m) of  $\alpha$ -SMA, TGF- $\beta$ 1 and Collagen-I also proved that airway remodeling can be inhibited after autophagy depletion in the experimental asthma model. Results are presented as the mean  $\pm$  SD from the 3 independent experiments using 5–6 mice per group.

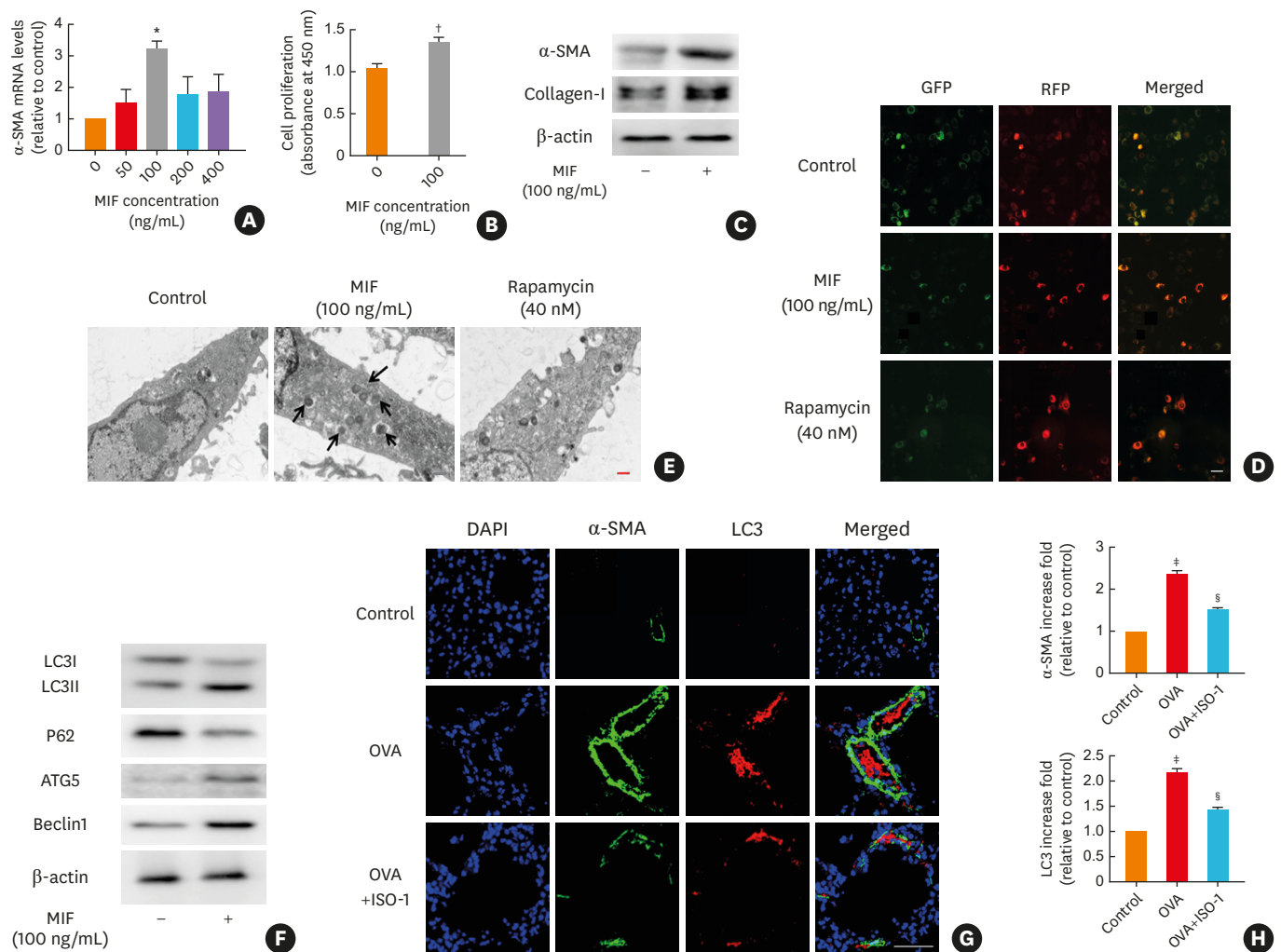
BALF, bronchoalveolar lavage fluid; OVA, ovalbumin; H&E, hematoxylin and eosin; GC, goblet cell; AB-PAS, alcian blue and periodic acid-Schiff; TNF, tumor necrosis factor; SD, standard deviation; DAPI, 4',6'-diamidino-2-phenylindole.

<sup>†</sup>P < 0.01 vs. the control group; <sup>#</sup>P < 0.001 vs. the control group; <sup>§</sup>P < 0.05 vs. the OVA group; <sup>‡</sup>P < 0.01 vs. the OVA group.

**MIF induces ASMC autophagy**

Since MIF and ASMC autophagies are of significance in modulating airway remodeling, the relationship between MIF and ASMC autophagy was investigated. First, we studied the effects of MIF on ASM remodeling. We assessed ASMC proliferation as well as  $\alpha$ -SMA and Collagen-I

**MIF Promotes Airway Remodeling**



**Fig. 6.** MIF-induced autophagy in ASMCs. (A) Increased  $\alpha$ -SMA expression in ASMCs at a 100 ng/mL MIF exposure condition (12 hours). (B) The proliferation of ASMCs was measured by BrdU assay after treatment with 100 ng/mL MIF for 24 hours. (C) Western blot analysis showed the production of  $\alpha$ -SMA and Collagen-I in ASMCs enhanced after exposed to 100 ng/mL MIF (24 hours). (D) Since decreased green fluorescence spots in GFP-RFP-LC3 tagged cells implied more autophagy in cells, our results showed more autophagy occurred in the 100 ng/mL MIF exposure group. Rapamycin (40 nM) was used as the positive control (200  $\times$ , scale bar, 10  $\mu$ m). (E) TEM examination revealed more autophagy accumulation in the MIF (100 ng/mL)- or rapamycin (40 nM)-treatment group (5,000  $\times$ , scale bar, 2  $\mu$ m). (F) Autophagy-associated protein levels were assessed by western blot assay. (G, H) The OVA-challenged asthma mice treated with the MIF inhibitor ISO-1, and co-expression of  $\alpha$ -SMA and LC3 in the lung tissues decreased (400  $\times$ , scale bar, 50  $\mu$ m). All the experiments were independently repeated 3 times. MIF, migration inhibitory factor; ASMC, airway smooth muscle cell; TEM, transmission electron microscope; OVA, ovalbumin; ISO-1, (S,R)3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; DAPI, 4',6-diamidino-2-phenylindole.

\* $P < 0.05$  vs. MIF 0 ng/mL; † $P < 0.001$  vs. MIF 0 ng/mL; ‡ $P < 0.001$  vs. control; § $P < 0.001$  vs. the OVA group.

expression in ASMCs. The mRNA expression of  $\alpha$ -SMA in the ASMCs was significantly increased at 100 ng/mL MIF-exposure concentration (Fig. 6A). The proliferation of ASMC and protein levels of  $\alpha$ -SMA and Collagen-I in ASMCs were also found higher after MIF exposure (Fig. 6B and C). By referring to autophagy detection, we used GFP-RFP-LC3-tagged ASMCs using a transmission electron microscope (TEM). In the GFP-RFP-LC3-reporting system, mRFP is stable, and GFP is sensitive to pH. At autophagy stage II, more autolysosomes were formed and resulted in a low pH environment, which caused GFP quenching. Therefore, our GFP-RFP-LC3 results showed less green fluorescence after MIF exposure, which indicated more complete autophagy process occurred in ASMCs (Fig. 6D). After exposing MIF to ASMC, TEM examination showed autophagy accumulation (Fig. 6E). Rapamycin was used as a positive control. Protein

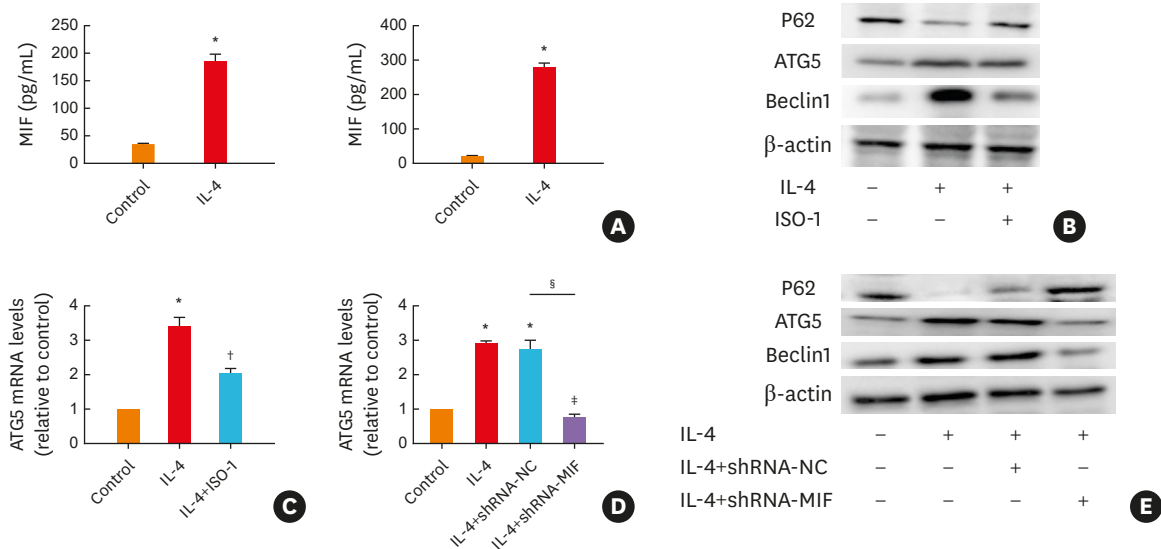
detection of a series of autophagy-associated markers (LC3II/I, P62, ATG5 and Beclin1) also revealed increased autophagy in MIF-exposed ASMCs (Fig. 6F). Moreover, we verified the association between MIF and ASMC autophagy *in vivo*. After MIF inhibition in mice by ISO-1, co-expression of  $\alpha$ -SMA and LC3 was reduced in OVA-challenged mice (Fig. 6G and H).

**The cellular source of MIF which mediates ASMC autophagy is macrophage**

First, we found that MIF was up-regulated in IL-4-induced peritoneal and alveolar macrophages supernatant (Fig. 7A). To assess whether MIF produced by macrophages is able to promote ASMC autophagy, we subjected IL-4-induced macrophage supernatants to ASMC after MIF in the supernatant was blocked and then assessed autophagy changes in ASMC. GFP-RFP-LC3 fluorescence indicated that autophagy was decreased in activated macrophage supernatant-induced ASMCs after MIF expression was inhibited by ISO-1 (Fig. 8A). Autophagy-associated mRNA (ATG5) and protein (P62, ATG5, Beclin1) expressions were also reduced in the IL-4+ISO-1 exposure group (Fig. 7B and C). We used MIF shRNA lentivirus to infect macrophages to establish another MIF blockage model. GFP-RFP-LC3 system and autophagy-associated mRNA (ATG5), and protein (P62, ATG5, Beclin1) detection also proved that autophagy was decreased in the IL-4+shRNA-MIF group (Fig. 7D and E, Fig. 8B).

**MIF induced ASMC autophagy via CD74**

Overall, the receptors CD44, CD182, CD184 and CD74 have been demonstrated the predominant role of MIF *in vivo* and *in vitro*.<sup>27</sup> We therefore determined the expression of each of these receptors on ASMCs. FACS analysis showed that CD74 was the most abundant MIF receptor on ASMCs. CD44 and CD182 were present at lower expression levels and we can

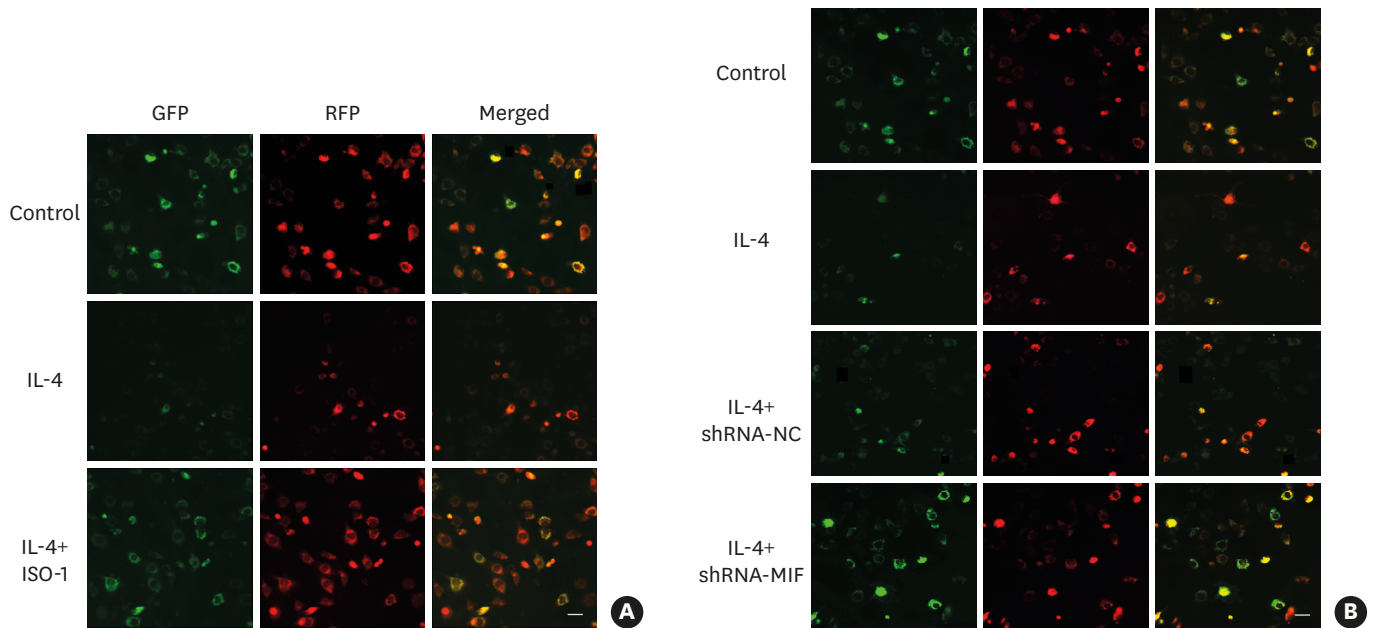


**Fig. 7. Macrophage-derived MIF promotes ASMC autophagy.** (A) The protein levels of MIF in the supernatant of 2 macrophage cell lines (NR8383 and RAW264.7) were measured by ELISA. (B) IL-4 (10 ng/mL) was used to activate macrophages and ISO-1 (100 nM) was used to inhibit MIF expression in macrophages. After pre-treatment with ISO-1, IL-4-induced macrophage supernatant was collected and exposed to ASMC for 24 hours. Autophagy-related protein expression in ASMCs was determined by western blot assay. (C) q-PCR analysis was used to assess mRNA expression of ATG5 in ISO-1-induced MIF inhibition model. (D) The RAW264.7 cells, shRNA-NC-transfected RAW264.7 and shRNA-MIF-transfected RAW264.7 cells were stimulated with IL-4 for 24 hours. Then ASMCs were incubated with collected macrophage supernatant for 24 hours. The mRNA expression of ATG5 in ASMC was reduced in the IL-4+shRNA-MIF group by q-PCR assays. (E) In the shRNA-MIF-mediated MIF blockage model, western blot assay analyzed the expression of P62, ATG5 and Beclin1 in ASMCs. All the experiments were independently repeated at least 3 times.

MIF, migration inhibitory factor; ASMC, airway smooth muscle cell; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; NC, negative control; ISO-1, (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; q-PCR, quantitative polymerase chain reaction; shRNA, short hairpin RNA.

\*P < 0.001 vs. the control group; †P < 0.01 vs. the IL-4 group; ‡P < 0.001 vs. the IL-4 group; §P < 0.01 vs. the IL-4+shRNA-NC group.





**Fig. 8.** The promotive effects of macrophage-derived MIF on ASMC autophagy determined by immunofluorescence. (A) In the ISO-1-mediated MIF inhibition model, the images of GFP-RFP-LC3-transfected cells showed that ASMC autophagy was decreased in the IL-4+ISO-1 group compared with the IL-4 group (200 ×, scale bar, 10 μm). (B) In the MIF-knock-down model, the IL-4+shRNA-MIF group displayed decreased ASMC autophagy (GFP-RFP-LC3 reporting system) compared with the IL-4 or IL-4+shRNA-NC group (200 ×, scale bar, 10 μm).

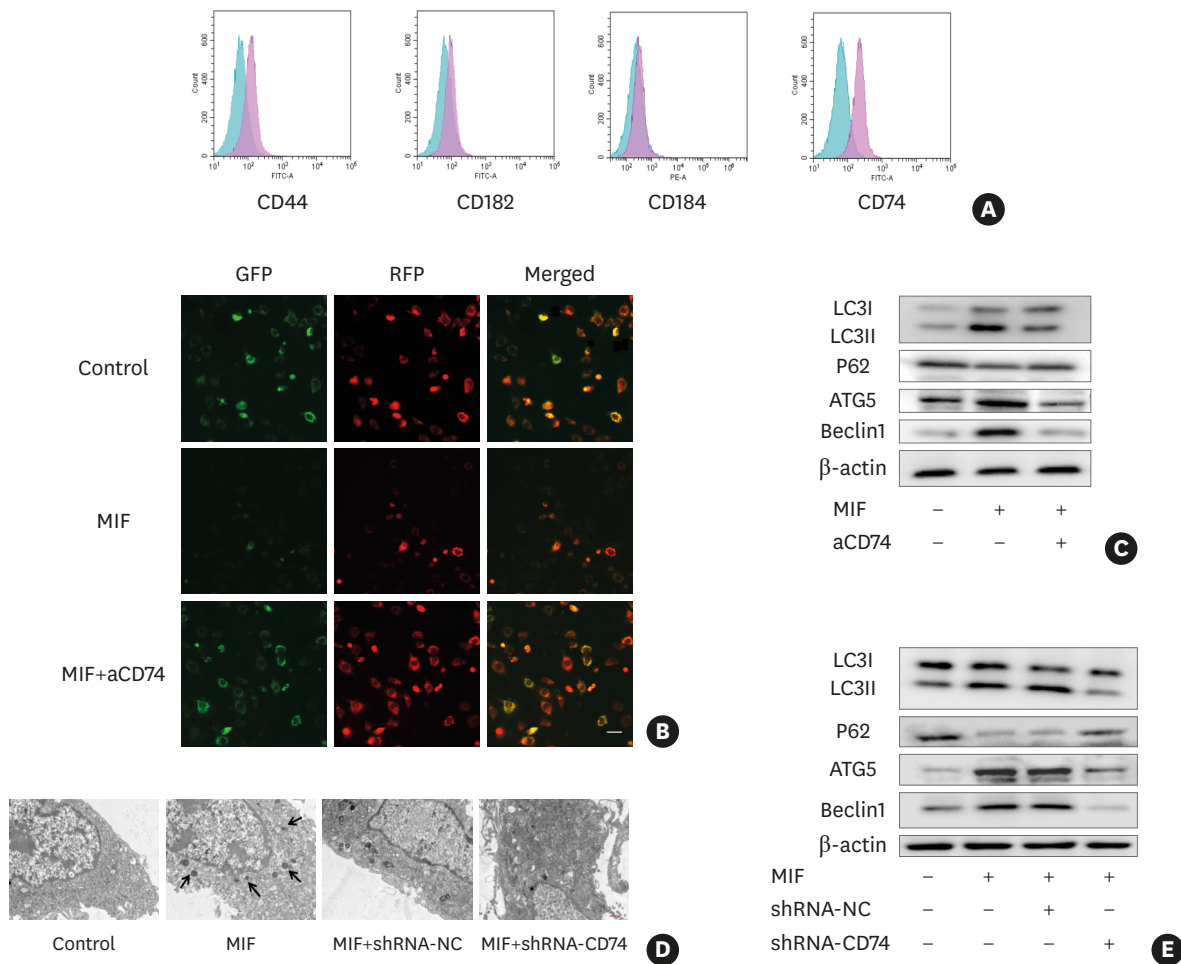
barely detect the expression of CD184 (**Fig. 9A**). As CD74 seems to have the main promotive effects on the ASMC autophagy of MIF, we used anti-CD74 antibody to block the expression of CD74 *in vitro*. GFP-RFP-LC3-transfected ASMCs displayed that MIF-induced autophagy was evidently reduced after anti-CD74 antibody exposure (**Fig. 9B**). The protein levels of autophagy markers (LC3, P62, ATG5 and Beclin1) were also decreased in the anti-CD74 antibody exposure group (**Fig. 9C**). We also used shRNA-CD74 lentivirus to knock-down CD74 in ASMCs. TEM analysis and protein detection of autophagy markers (LC3, P62, ATG5 and Beclin1) discovered less autophagy in the MIF+shRNA-CD74 group compared with the MIF+shRNA-NC group (**Fig. 9D and E**).

## DISCUSSION

In this study, we discovered the role of MIF in mediating airway remodeling in asthma. MIF can induce airway remodeling by promoting ASMC autophagy. The primary cellular source of MIF which induced ASMC autophagy was possibly macrophages. MIF mediated ASMC autophagy by binding to its receptor CD74 on ASMCs. Our data suggested a close link between MIF and airway remodeling, and supported the hypothesis that MIF-induced airway remodeling depended on its ability to activate ASMC autophagy.

Recent studies have reported the role of MIF in asthma from the perspective of regulating allergic inflammatory responses.<sup>28-30</sup> MIF can participate in antigen-induced inflammatory cell accumulation in the lung.<sup>28</sup> Blockade of MIF can mitigate airway inflammation in both house dust mite- and OVA-induced asthma models.<sup>29,30</sup> The modulation of MIF in airway inflammation was probably associated with eosinophil biology.<sup>31</sup> By referring to regulating airway remodeling, data only showed that the MIF inhibitor ISO-1 can suppress airway remodeling in a murine





**Fig. 9.** MIF induces ASMC autophagy by CD74. (A) Expression of the MIF receptors on immortalized ASMCs. FACS analysis revealed marked expression of CD74 and low-to-moderate expression of CD44, CD182 and CD184 on ASMCs. (B) GFP-RFP-LC3 fluorescence (200 $\times$ , scale bar, 10  $\mu$ m) results showed that the promotive effect of MIF (100 ng/mL) on ASMC autophagy can be blocked by aCD74 (5  $\mu$ g/mL). (C) Western blot assay of autophagy-related proteins also displayed decreased autophagy happened when treatment with aCD74 in MIF-induced ASMCs. (D) In the shRNA-CD74 knockdown model, MIF-induced ASMC autophagy (TEM analysis, 5,000 $\times$ , scale bar, 2  $\mu$ m) was inhibited in the MIF+shRNA-CD74 group. (E): The protein levels of LC3II/I, P62, ATG5 and Beclin1 were significantly reduced in the MIF+shRNA-CD74 group. All the experiments were independently repeated at least 3 times. MIF, migration inhibitory factor; ASMC, airway smooth muscle cell; aCD74, anti-CD74 antibody; shRNA, short hairpin RNA; TEM, transmission electron microscope.

model.<sup>23</sup> In the present study, up-regulated expression of MIF in stable asthmatic patients indicated that MIF was of importance in asthma remodeling. Our results in OVA-challenged mice support an intrinsic role of MIF in airway remodeling. When compared with WT mice, MIF knock-down mice showed a reduction in ASM production, collagen deposition and TGF- $\beta$ 1 accumulation. Of note, our results displayed the role of MIF in an OVA-induced chronic inflammatory model, unlike in other allergens-induced asthma models. The alteration in macrophages amounts in BALF was consistent with that of MIF expression in BALF. Immunofluorescence co-expressions of F4/80<sup>+</sup> (macrophage marker) and MIF in the lungs also indicated that MIF can be secreted by macrophages. More studies are needed to explore whether there are other cellular sources of MIF in regulating airway remodeling.

Previous studies have found that autophagy may be a new target for asthma therapy.<sup>32,33</sup> Autophagy was increased in asthmatic patients, and the autophagy inhibitor chloroquine decreased airway inflammation, AHR and airway remodeling in a mouse allergic asthma

model.<sup>34</sup> Paradoxically, simvastatins may ameliorate airway inflammation and airway remodeling in a murine asthma model by increasing autophagy.<sup>14</sup> There is currently no satisfactory conclusion about the role of autophagy in asthma. Our study indicated that ASMC autophagy was increased in an OVA-challenged mice model. We used ATG5 genetically knock-out mice to verify the effect of autophagy on the key features of asthma. Reductions in airway inflammation and airway remodeling were found in ATG5<sup>-/-</sup> asthmatic mice compared with WT asthmatic mice.

Several studies demonstrated that MIF can modulate cellular autophagy.<sup>24,35,36</sup> It has been proved that MIF can exacerbate aging-induced cardiac remodeling by increasing autophagy.<sup>24</sup> MIF can induce the autophagy of hepatoma cells by reactive oxygen species generation.<sup>34</sup> MIF can increase the autophagy of human hepatoma cells by regulating the STAT3-MIF-BNIP3-dependent pathway.<sup>36</sup> After exposure of ASMCs to 100 ng/mL MIF for 12 hours, the mRNA expression of  $\alpha$ -SMA in ASMCs was significantly increased. In the 200 ng/mL- and 400 ng/mL- MIF exposure groups,  $\alpha$ -SMA expression also enhanced, which was statistically not significant. The mRNA level may alter in a dose-dependent manner if we apply for different exposure times to ASMCs, which needs more studies. We first explored the regulation function of MIF on ASMC autophagy. Our findings demonstrated that MIF could promote ASM remodeling and increase ASMC autophagy. MIF can be secreted by immune cells, such as macrophages, T cells, B cells and dendritic cells.<sup>17</sup> To determine the cellular source of MIF which induced ASMC autophagy, we exposed MIF-blocked macrophage supernatant to ASMCs and evaluated autophagy-related markers. In our study, we used the rat alveolar macrophages NR8383 cell line and the murine peritoneal macrophages RAW264.7 cell line in an ISO-1-induced MIF inhibition model and a shRNA-MIF-knock-down model, respectively. These models using a different macrophage cell line showed that ASMC autophagy was obviously blocked after MIF inhibition in macrophages. As one of the key receptors on MIF, CD74 participated in modulating lung inflammation, chronic obstructive pulmonary disease and pulmonary hypertension.<sup>37-39</sup> Recent reports have proved that CD74 is involved in proliferative effects of MIF on various target cells.<sup>40,41</sup> No data were found about the role of MIF/CD74 in ASMC biology. Our study showed that MIF-induced ASMC autophagy was dependent on CD74. Further studies are needed to explore whether the function of MIF can be regulated by other receptors.

Emerging data have emphasized the role of MIF as an upstream regulator of inflammatory responses.<sup>19,42</sup> MIF exerts an important effect in eosinophilic inflammation, including asthma. Recent studies have proved that OVA-sensitized MIF<sup>-/-</sup> deficient mice have a less degree of lung inflammation and airway hyperresponsiveness, compared with WT mice. MIF deficiency also resulted in the decrease in specific IgE, IgG<sub>1</sub>, IgG<sub>2a</sub> and T helper 2 cytokines levels in the lung.<sup>21</sup> The role of MIF in airway remodeling is still unclear. One study indicated that the MIF inhibitor ISO-1 can ameliorate airway remodeling in OVA-sensitized mice.<sup>23</sup> A strength of this study is that MIF can increase airway remodeling by ASMC autophagy. We also first proved that MIF regulated ASMC autophagy by binding to its receptor CD74. In summary, we describe herein unexpected role of MIF in airway remodeling through CD74-mediated activation of ASMC autophagy. These results underscore the importance of autophagy in asthma and imply unique therapeutic targets for the treatment of chronic inflammatory diseases.

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## SUPPLEMENTARY MATERIAL

### Supplementary Fig. S1

(A) The transfection efficacy of AAV9 (5 mice/group) was determined by immunofluorescence (400 ×, scale bar, 50 μm) of eGFP. (B) Western blot assay showed that ATG5 expression was dramatically decreased in ATG5<sup>-/-</sup> mice (5 mice/group). (C) western blot assay proved that MIF expression was effectively blocked in shRNA-MIF transfected RAW264.7 cells. (D) The knock-down of CD74 in ASMCs was also determined by western blot assay.

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