Upregulated IGF-1 in the lungs of asthmatic mice originates from alveolar macrophages

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Abstract. Asthma is characterized by inflammation and remodeling of the airways. Insulin-like growth factor-1 (IGF-1) serves an important role in the repair of lung tissue injury and airway remodeling by elevating collagen and elastin content, increasing the thickness of smooth muscle and promoting the proliferation of lung epithelial and interstitial cells, as well as fibroblasts; however, the content of IGF-1 and its cellular origin in the lungs of patients with asthma remain unknown. In the present study, a mouse model of asthma was constructed. Following isolation of alveolar macrophages (AMs), the content of IGF-1 in lung tissue and bronchoalveolar lavage fluid (BALF) was detected by ELISA. The proliferation and phagocytosis of alveolar epithelial cells (AECs) stimulated by IGF-1 were detected by Cell Counting Kit-8 method and flow cytometry, respectively. In the present study, IGF-1 was upregulated in the lung tissues of asthmatic mice, and the content of IGF-1 in BALF was also elevated. Depletion of AMs by treating mice with 2-chloroadenosine via nose dripping reversed the increase of IGF-1 by 80% in lung tissues and by ~100% in BALF of asthmatic mice, suggesting that elevated IGF-1 in asthmatic mice predominantly originated from AMs. As IGF-1 promotes the proliferation and phagocytosis of AECs, AM-derived IGF-1 may serve an important role in the regulation of airway inflammation and remodeling in asthmatic mice.

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Introduction

Asthma is one of the most common types of respiratory diseases; ~300,000,000 asthma cases are reported worldwide and 250,000 people succumb to mortality from this disease annually (1). An epidemiological study revealed that the incidence and mortality of asthma have increased in the past decade, and has become a global public health problem (2). Asthma is a chronic inflammatory disease involving numerous cells and cytokines, and is characterized by inflammation and hyper-responsiveness of the airway (3). Recurrent airway inflammation results in inflammatory injury and fibrotic proliferation of the airway epithelium, consequently stimulating airway remodeling (4), which is also an important feature of asthma.

Insulin-like growth factor-1 (IGF-1) is a metabolism-associated growth factor that is structurally and functionally similar to insulin (5). IGF-1 serves an important regulatory role in the proliferation, differentiation, metabolism and survival of cells (6). IGF-1 is predominantly synthesized and secreted in the liver; additionally, IGF-1 is also produced by other tissues, such as the lungs, where it functions as an autocrine and paracrine hormone (7). In addition, IGF-1 exhibits numerous biological functions in a variety of organs, and it is associated with the pathogenesis of various diseases (8). IGF-1 stimulates the proliferation of lung epithelial cells and serves an important role in tissue repair in response to lung injury (9). Furthermore, IGF-1 promotes the proliferation of pulmonary fibroblasts via the IGF-1 receptor-mediated upregulation of FOS proto-oncogene activator protein 1 transcription factor subunit, early growth response protein (EGR)1 and EGR2 (10). IGF-1 can also stimulate the differentiation of fibroblasts into myoblast cells and serves an important role in pulmonary fibrosis (11). Chetty and Nielsen (12) reported that hyperoxia-induced IGF-1 promoted the proliferation of pulmonary interstitial cells, indicating that IGF-1 signaling may be involved in the repair of hyperoxia-induced lung injury. Vieira *et al* (13) revealed that creatine supplementation aggravated airway remodeling by increasing the thickness of smooth muscle, and upregulating the expression levels of collagen and elastin in the airway via IGF-1. Therefore, IGF-1 is associated with tissue repair and airway remodeling in the lungs; however, the cellular origin of IGF-1 in asthma remains unknown.

In the present study, the levels of IGF-1 in the lung tissues and bronchoalveolar lavage fluid (BALF) of asthmatic mice, as well as its cellular origin were examined. In addition, whether IGF-1 affected the proliferation and phagocytosis of alveolar epithelial cells (AECs) was investigated.

Materials and methods

Preparation of the asthma model. Female BALB/c mice (6-weeks-old) were purchased from the Animal Center of Bengbu Medical College. The mice (18-20 g) were housed in specific pathogen-free grade conditions under a 12 h light-dark cycle at 20-26˚C with free to access food and water. BALB/c mice were sensitized by an intraperitoneal injection of 200 μl sensitizing solution [50 μg ovalbumin (OVA) and 2 mg $Al(OH)_3$] on days 0, 7 and 14. Sensitized mice were challenged by the administration of aerosolized 5% OVA solution from day 21, with 30 min of atomization every day and continuous atomization for 5 weeks. Control mice received a mock challenge with PBS. The present study was approved by the Ethics Committee of the Bengbu Medical College (Bengbu, China).

Preparation of BALF and acquisition of alveolar macrophages (AM). Mice were placed in a supine position following anesthesia with an intraperitoneal injection of chloral hydrate (400 mg/kg), and tracheal intubation was performed using a $12^{\#}$ needle. Mice underwent lavage using 0.8 ml saline via intubation for a total of 6 times. BALF was isolated by centrifugation at 1,230 x g for 5 min at 4˚C, and the supernatant was stored at -70˚C prior to cytokine analysis. Following erythrocyte lysis using Red Blood Cell Lysis Buffer (Beyotime Institute of Biotechnology, Shanghai, China), the pellet was resuspended in RPMI-1640 complete medium (containing 10% fetal bovine serum, HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and then seeded in a 6-well plate followed by incubation at 37˚C for 2 h. The adherent cells were identified as AMs and the purity of macrophages was determined to be >95%.

AM depletion. AM depletion was performed as described previously (14). Briefly, sensitized mice were treated with 2-chloroadenosine (2-CA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) via the nose one day prior to atomization. Each mouse received 10 μg 2-CA in 20 μl PBS, once every 3 days until the 54th day of the experimental process. Asthma model mice received PBS instead of 2-CA as the control. After obtaining the bronchoalveolar lavage, slides were prepared and stained with Wright-Giemsa staining solution. Specifically, 95% ethanol (1 ml) was added to each sample and cells were fixed for 10 min at room temperature. The ethanol was removed and Wright-Giemsa staining solution was added. The cells were stained for 5 min at 25˚C; the staining solution was discarded, the cells were rinsed with water and observed using a IX71 light microscope (magnification, x400; Olympus Corporation, Tokyo, Japan). Differential cells counts were performed and at least 400 cells were counted per sample. Post-treatment assessment by cell counting revealed that 75% of AMs were depleted, whereas the other cells in BALF were not affected.

Enzyme-linked immune sorbent assay (ELISA). The mice were sacrificed and the lung homogenate supernatant and BALF were collected. The levels of IGF-1 in the BALF and supernatant were determined using an ELISA kit (cat. no. CSB-E04581m; CUSABIO, Wuhan, China) according to the manufacturer's protocols.

Cell proliferation assay. Alveolar epithelial cells (MLE-12; Jining Shiye Biotechnology Co., Ltd., Shanghai, China) in complete Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum, HyClone; GE Healthcare Life Sciences) were seeded on 12-well plates and cultured to 70-80% confluence at 37˚C prior to stimulation using IGF-1 (10 ng/ml) the following day. Cells were divided into the control group and IGF-1-stimulated group. Following stimulation with IGF-1 for 12, 24, 48, 72 and 96 h at 37˚C, $3x10^3$ MLE-12 cells were seeded on 96-well plates. Then, 10 μl Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology) reagent was added into each well followed by incubation at 37˚C for 2 h. The absorbance at 450 nm was measured using a microplate reader.

Flow cytometry. MLE-12 cells were stimulated using 50 ng/ml IGF-1 (Abcam, Cambridge, UK) for 48 h at 37˚C, treated with 1 μl YELLOW GREEN (YG; excitation=441 nm and emission=486) fluorescent microspheres (1 μm in diameter, Polysciences, Inc., Warrington, PA, USA), and then incubated at 37˚C for 2 h. The control was treated with medium only. MLE-12 cells were washed twice using phosphate buffer and fixed for 30 min using 1% paraformaldehyde at 4˚C. Then, cell phagocytosis of fluorescent microspheres was detected using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, CA, USA). MLE-12 cells with fluorescent signals were analyzed using FlowJo 7.6.1 (FlowJo LLC, Ashland, OR, USA).

Western blot analysis. Total protein was extracted from lung tissue homogenates of mice using NP-40 Lysis Buffer (Beyotime Institute of Biotechnology). Extracted protein concentrations were measured using a Bicinchoninich Acid protein assay kit (Beyotime Institute of Biotechnology); 30 μg protein were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Then the membranes were blocked using 5% milk at room temperature for 2 h and incubated using the primary antibodies against IGF-1 (1:1,000; cat. no. ab9572; Abcam) or β-actin (1:1,000; cat. no. AF0003; Beyotime Institute of Biotechnology) at 4˚C overnight. Membranes were washed in Tris-buffered saline with 0.05% Tween-20 and incubated with horseradish peroxidase-labeled Goat Anti-Rabbit IgG (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) at room temperature for 2 h. The bands were visualized using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology). Relative protein expression was determined by densitometric analysis using Image Lab 4.1 (Bio-Rad Laboratories, Hercules, CA, USA). β-actin was used as the reference protein and the experiment was conducted three times.

Statistical analysis. The data were presented as mean ± standard deviation. Statistical comparison between two groups was performed using a Student's t-test. Comparisons between multiple groups were performed using a one-way analysis of variance followed by a Least Significant Difference post hoc

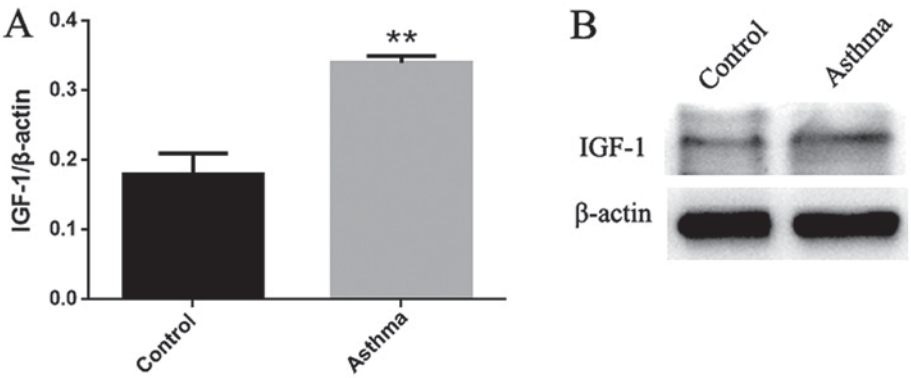

Figure 1. Elevated expression levels of IGF-1 in the lung tissues of asthmatic mice. Expression levels of IGF-1 in the lung tissue of asthmatic and control mice were determined by western blotting. (A) Densitometric analysis of blots from three independent experiments. (B) Representative western blotting from the triplicates. **P<0.01 vs. the control group. IGF-1, insulin-like growth factor 1.

test. All experiments were repeated at least three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of IGF-1 in lung tissues and BALF are increased in asthmatic mice. A mouse model of allergic asthma was constructed as described previously (14). In asthmatic mice, the number of eosinophils in BALF was increased and the expression levels of Th1 cytokine interferon-γ was decreased, while those of Th2 cytokines interleukin-4 (IL-4) and IL-13 were increased (15,16). The expression levels of IGF-1 in the lung tissues were significantly upregulated in asthmatic mice compared with in normal mice (Fig. 1). In addition, the expression levels of IGF-1 in BALF were significantly increased in asthmatic mice compared with in normal mice (P<0.01; Fig. 2).

Elevated IGF-1 in the lungs of asthmatic mice predominantly originates from AMs. As 2-CA effectively depletes AMs (17), it was applied as nasal drops to deplete AMs in the present study. Treatment with 2-CA depleted 75% of AMs and significantly reversed the increase in IGF-1 by ~100% in the BALF (P<0.01) and 85% in lung tissues of asthmatic mice (P<0.01; Fig. 3). This indicated that AMs are the main source of IGF-1 in the airways.

IGF-1 promotes the proliferation of AECs. IGF-1 can regulate the proliferation of numerous types of cell (9). As AMs are in close proximity to AECs in the alveolar microenvironment, whether AM-derived IGF-1 affects the proliferation of AECs was investigated in the present study. MLE-12 cells were stimulated with IGF-1 (10 ng/ml), and cell proliferation was determined using the CCK-8 method. As presented in Fig. 4, the optical density in IGF-1 stimulated groups at 24, 48, 72 and 96 h was significantly increased compared with in the respective controls (P<0.01), indicating that IGF-1 promoted the proliferation of AECs.

IGF-1 promotes the phagocytosis of AECs. IGF-1 signaling is involved in the inflammatory response (18); the role of non-professional phagocytosis in the inflammatory response has been reported (19). Thus, whether IGF-1 affects the phagocytosis of AECs, non-professional phagocytes, was investigated in the present study. Stimulation of MLE-12 cells with IGF-1 (50 ng/ml) significantly increased the digestion of fluorescent microspheres compared with the control (P<0.01; Fig. 5). This suggested that IGF-1 may inhibit airway inflammation by promoting the phagocytosis of AECs.

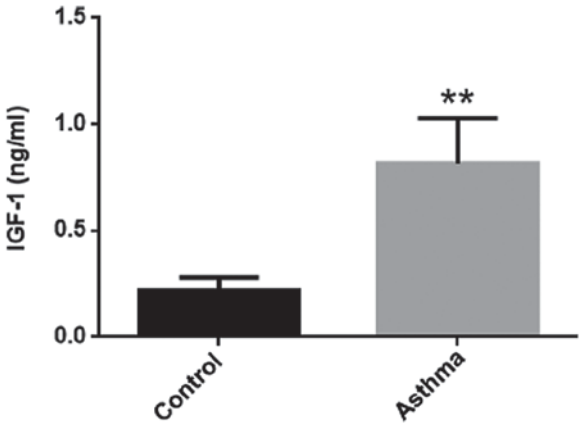

Figure 2. Expression levels of IGF-1 are increased in the BALF of asthmatic mice. Expression levels of IGF-1 in the BALF of asthma and control mice were determined by ELISA. IGF-1 was significantly upregulated in the BALF of asthmatic mice compared with in normal mice. **P<0.01 vs. the control group. BALF, bronchoalveolar lavage fluid; IGF-1, insulin-like growth factor 1.

Discussion

In the present study, the IGF-1 content in lung tissues and BALF of asthmatic mice was significantly increased, and elevated IGF-1 was predominantly derived from AMs. IGF-1 serves an important role in the development of normal lung tissue and is also associated with the pathology of numerous lung diseases (20). For example, patients with systemic sclerosis, severe skin lesions and pulmonary fibrosis exhibited increased serum levels of IGF-1, and the mRNA levels of IGF-1 in the skin of these patients were notably elevated compared with in normal controls (21). In a model of bleomycin-induced pulmonary fibrosis, the expression levels of IGF-1 in lung tissues were increased by 3-4-fold compared with in the control group (22). In addition, IGF-1 was upregulated in the lung tissue of acute respiratory distress syndrome (ARDS) patients with fibrous

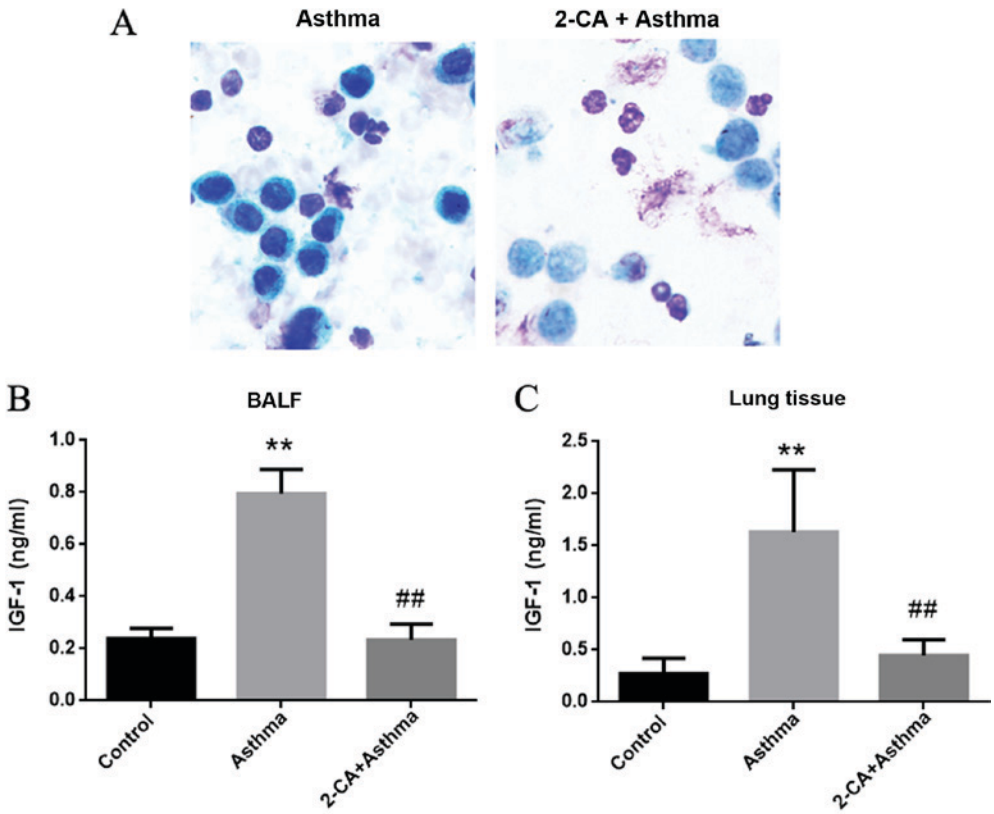

Figure 3. Treatment with 2-CA reduces the content of IGF-1 in the BALF and lung tissues of asthmatic mice. Cell acquisition via bronchoalveolar lavage was presented by (A) Wright staining, and the concentration of IGF-1 was determined in (B) BALF and (C) lung tissues of asthmatic mice with or without 2-CA treatment using ELISA. **P<0.01 vs. the control group; $^{##}$P<0.01 vs. the asthma group. BALF, bronchoalveolar lavage fluid; IGF-1, insulin-like growth factor 1; 2-CA, 2-chloroadenosine.

hyperplasia (23). Another study also revealed that patients with ARDS at the early stage exhibited increased IGF-1 in BALF (24). The results of the present study demonstrated that the content of IGF-1 in lung tissue and BALF was significantly upregulated in mice with chronic asthma compared with the control group.

IGF-1 is produced in numerous types of cell, including endothelial cells, epithelial cells, macrophages, adipose-derived stem cells, nucleus pulposus cells and dental pulp stem cells (25-28). Recently, Wang *et al* (29) reported that epidermal T cells also produced IGF-1. The results of the present study suggested that IGF-1 may derive from AMs in the lungs of asthmatic mice. Fritz *et al* (30) revealed that mouse AMs produced IGF-1, which is consistent with the findings of the present study. IGF-1 can promote the proliferation and differentiation of various types of cell. Yu *et al* (31) reported that IGF-1 may promote the proliferation of myoblast cells and serves an important role in the growth of skeletal muscle. In addition, IGF-1 was involved in the differentiation of mesenchymal stem cells into neural progenitor-like cells by promoting the proliferation and inhibiting apoptosis (32). Weng *et al* (33) demonstrated that IGF-1 may induce the proliferation of human retinal pigment epithelial cells and was considered to be the mitotic origin of these cells. IGF-1 also stimulated the growth of tumor cells of lung, liver and breast cancer, as well as osteosarcoma (34-37). Narasaraju *et al* (9) reported that IGF-1 served an important role in the proliferation and differentiation of alveolar epithelial cells in rats. In

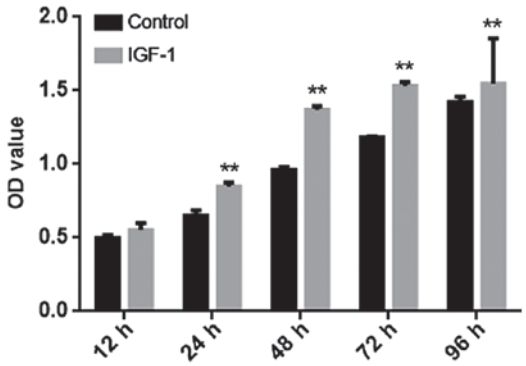

Figure 4. IGF-1 promotes the proliferation of alveolar epithelial cells. MLE-12 cells ($3x10^3$) were stimulated with IGF-1 (10 ng/ml) for 12, 24, 48, 72 and 96 h, and cell proliferation was determined using a Cell Counting Kit-8 assay. **P<0.01 vs. the control group. IGF-1, insulin-like growth factor 1; OD, optical density.

the present study, IGF-1 promoted the proliferation of mouse AECs. As AMs and AECs are located adjacently in the alveolar microenvironment, IGF-1 derived from AMs may regulate pulmonary remodeling by affecting AECs.

IGF-1 signaling is involved in the inflammatory response (18). IGF-1 inhibits inflammatory responses by suppressing the release of inflammatory cytokines, including IL-1β and tumor necrosis factor-α (38). Alternatively, IGF-1 also stimulates the production of anti-inflammatory cytokines, including IL-4 and IL-10 to inhibit inflammatory

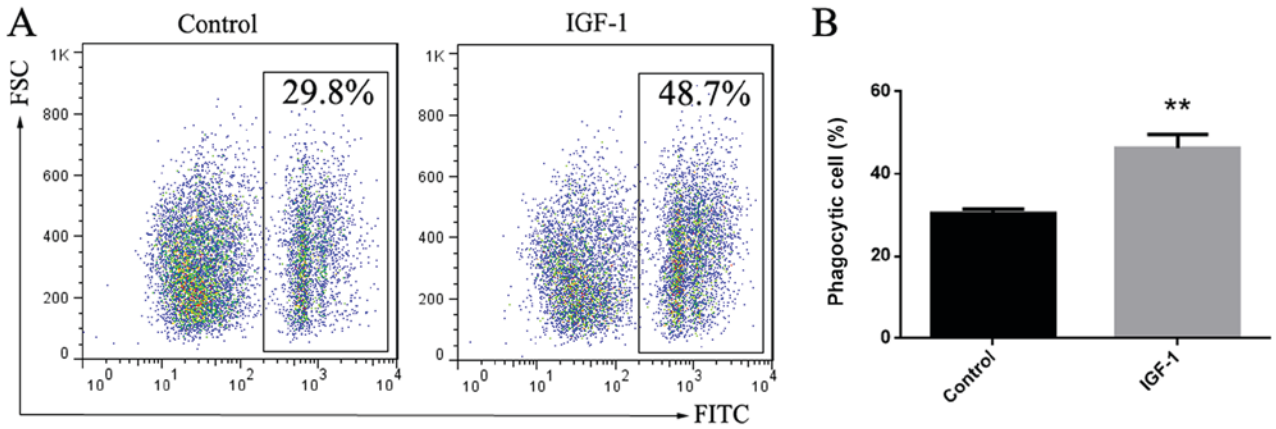

Figure 5. IGF-1 promotes the phagocytosis of alveolar epithelial cells. MLE-12 cells stimulated with IGF-1 (50 ng/ml) for 48 h were incubated with FITC-labeled microspheres for 2 h. The percentage of FITC-positive cells was determined by flow cytometry. (A) Representative flow cytometry plots. (B) Statistical analysis of the results from flow cytometry. **P<0.01 vs. the control group. FSC, forward scatter; FITC, fluorescein isothiocyanate; IGF-1, insulin-like growth factor 1

responses (39). Additionally, the removal of endotoxins and apoptotic bodies is promoted by phagocytosis to inhibit the inflammatory response and maintain the balance of the internal environment (40). A recent study revealed that IGF-1 served an important role in inflammatory responses by promoting the phagocytosis of dendritic cells (41). The present study indicated that IGF-1 induced the phagocytosis of AECs, suggesting that IGF-1 may inhibit pulmonary inflammation by affecting the alveolar cells required for structure, consequently maintaining the balance of alveolar microenvironment.

In summary, the expression levels of IGF-1 were significantly increased in the lungs of asthmatic mice; elevated IGF-1 was predominantly originated from AMs. The results of the present study indicated that AM-derived IGF-1 may serve an important role in the regulation of airway inflammation and remodeling in asthmatic mice. Further studies should be conducted to identify the functions of IGF-1 and its downstream molecules in allergic airway inflammation, which may provide novel insight into the treatment of this disease.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Authors' contributions

QF, SG and CS made substantial contributions to the design of the present study. JH, MM, HW, HM and XT performed the experiments. CS wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Bengbu Medical College (Bengbu, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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