

Insulin-like growth factor 1 inhibits phagocytosis of alveolar epithelial cells in asthmatic mice

MIMI MU^{1*}, FENGJIAO WU^{1*}, JING HE^{1*}, XU TANG², HUA MA¹, SHUJUN GUO¹ and CHUANWANG SONG¹

¹Department of Immunology, Anhui Provincial Key Laboratory of Infection and Immunity;

²Department of Clinical Laboratory Medicine, Bengbu Medical College, Bengbu, Anhui 233030, P.R. China

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Abstract. The phagocytosis of apoptotic cells by alveolar epithelial cells helps to eliminate airway inflammation. Insulin-like growth factor 1 (IGF-1) regulates cell metabolism and proliferation, and promotes cell survival, while it may also promote the proliferation and differentiation of alveolar epithelial cells during the repair of lung injury. The present study investigated the effect of IGF-1 on the phagocytic activity of alveolar epithelial cells, a nonprofessional phagocyte. IGF-1 was elevated in lung tissue and bronchoalveolar lavage fluid obtained from mice with ovalbumin-induced asthma. IGF-1 was reduced by 50% in the lung tissue and by nearly 100% in the bronchoalveolar lavage fluid in asthmatic mice established by depletion of alveolar macrophages using 2-chloroadenosine. In addition, interleukin-33 induced IGF-1 production in primary alveolar macrophages. It was also observed that IGF-1 inhibited the phagocytosis of fluorescent microspheres and apoptotic cells by MLE-12 alveolar epithelial cells. Antibody blocking of IGF-1 enhanced the phagocytosis of fluorescent microspheres and apoptotic cells, and significantly reduced inflammatory cell infiltration in airway and perivascular tissues. The elevated IGF-1 level in the lungs of asthma model mice was mainly produced in alveolar macrophages. Taken together, the current study demonstrated that IGF-1 inhibited phagocytosis by alveolar epithelial cells, and that IGF-1 blockade enhanced the phagocytic activity and alleviated airway inflammation. These results support the potential use of IGF-1 as a target in the treatment of asthma.

Introduction

Phagocytosis, first described by Metchnikoff >100 years ago, is the engulfing and internalizing of particles with a size of $\geq 0.5 \mu\text{m}$ by cells. Unlike micropinocytosis, phagocytosis is initiated by the recognition and binding of cell surface receptors to target particles (1,2). Professional phagocytes, including macrophages, neutrophils and dendritic cells, participate in the first line of defense against infection by clearing pathogens from the sites of infection (3). Nonprofessional phagocytes, such as epithelial and endothelial cells, and fibroblasts, do not internalize pathogens, but clear senescent cells by engulfing apoptotic bodies (4,5). Therefore, nonprofessional phagocytes avoid the release of inflammatory contents of apoptotic cells in this way, and thus serve an important role in maintaining tissue homeostasis. Recently, Juncadella *et al* (6) reported that the function of airway epithelial cells to phagocytose apoptotic cells was attenuated in asthmatic mice, thereby forming interleukin (IL)-33-dependent allergic airway inflammation. This indicates that the engulfment of apoptotic cells by airway epithelial cells is an important mechanism for the regression of airway inflammation.

Insulin-like growth factor 1 (IGF-1) is a small 7.6-kDa peptide that exhibits 50% homology with insulin. It is a hormone with local activity following autocrine or paracrine secretion, and regulates cell survival, metabolism, proliferation and differentiation (7,8). IGF-1 is predominantly produced in the liver, while it is also produced in certain other organs, such as the lungs, where it is elevated under conditions of acute lung injury, pulmonary fibrosis and asthma (9). IGF-1 promotes the proliferation and differentiation of alveolar epithelial cells during the repair of hypoxia-induced lung injury (10). Although IGF-1 has been demonstrated to promote the phagocytic activity of mouse macrophages (11), its effects on nonprofessional phagocytes, such as alveolar epithelial cells, have not been described to date. Recently, it has been reported that the concentration of IGF-1 was elevated in the lung tissues of asthmatic mice (12).

As the engulfment of apoptotic cells by the airway epithelium affects the progression of airway inflammation in asthmatic mice, the effect of IGF-1 on the phagocytic function of alveolar epithelial cells was investigated in the present study. The study revealed that IGF-1 inhibited the phagocytosis of fluorescent microspheres and apoptotic cells by MLE-12

Correspondence to: Dr Chuanwang Song, Department of Immunology, Anhui Provincial Key Laboratory of Infection and Immunity, Bengbu Medical College, 2600 Songhai Avenue, Bengbu, Anhui 233030, P.R. China
E-mail: chuanwangsong@163.com

*Contributed equally

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alveolar epithelial cells and by mouse alveolar epithelial cells from primary cultures. IGF-1 was elevated in the lung tissue and bronchoalveolar lavage fluid (BALF) of asthmatic mice, whereas IGF-1 blockade promoted phagocytosis by alveolar epithelial cells and reduced airway inflammation.

Materials and methods

Cell culture. Primary alveolar epithelial cells and MLE-12 alveolar epithelial cells (Shanghai Jining Shine Biotechnology Corporation) were cultured in Dulbecco's modified Eagle's medium with 10% FBS (HyClone; GE Healthcare Life Sciences) containing 1% penicillin and streptomycin (Beyotime Institute of Biotechnology) in a 5% CO₂ incubator at 37°C. When the cultures reached 80-90% confluency, the cells were harvested with 0.25% pancreatin for subculturing MLE-12 cells. The cells were passaged every 1-2 days.

Animal model and treatment protocol. A total of 30 female BALB/c mice were purchased from the Experimental Animal Center of Bengbu Medical College. The mice (4-weeks-old) were housed in pathogen-free grade conditions with 60-70% humidity under a 12 h light/dark cycle at 25°C with free to access food and water. The process for establishing an asthma model is illustrated in Fig. 1. Briefly, mice weighing 15-30 g were intraperitoneally injected with 200 µl of a sensitizing solution containing 50 mg ovalbumin (Sigma-Aldrich; Merck KGaA) and 2 mg aluminum hydroxide (Thermo Fisher Scientific, Inc.) on days 0, 7 and 14. Beginning on day 21, the mice were placed in an inhalation chamber and treated with aerosolized 5% ovalbumin for 30 min once a day for 1 week. Control mice received a mock challenge with phosphate-buffered saline (PBS). To deplete alveolar macrophages, asthma model mice received 2-chloroadenosine (2-CA; Sigma-Aldrich; Merck KGaA) administered in four doses of 1 µg/20 µl once every 3 days, beginning on day 20. For IGF-1 blockade, asthma model mice were administered anti-IGF-1 antibody (1:10; cat. no. ab9572; Abcam) by intranasal drip in a dose of 1 µg/20 µl once every 3 days, beginning on day 20. Animal experiments in the present study were approved by the Ethics Committee of the Bengbu Medical College (Bengbu, China).

Collection of BALF and alveolar macrophages. Mice were anesthetized with 1% chloral hydrate prior to tracheal intubation, and bronchoalveolar lavage with 0.6 ml PBS was performed for six consecutive times. The BALF samples obtained from each lavage were centrifuged at 233 x g for 5 min at 4°C. The supernatant was collected for cytokine assays. Next, the red blood cells were lysed, and the pellet was resuspended in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences) and then seeded onto 6-well plates at a final density of 5x10⁵ cells/well. The cells were allowed to adhere for at least 2 h before washing away the nonadherent cells to expose the adherent alveolar macrophages. The purity of the isolated macrophages was >95%.

Isolation of primary alveolar epithelial cells. Mice were anesthetized with chloral hydrate prior to tracheal intubation and injection of 10-15 ml of air directly to the lungs. The pulmonary artery was lavaged with PBS, and bronchoalveolar

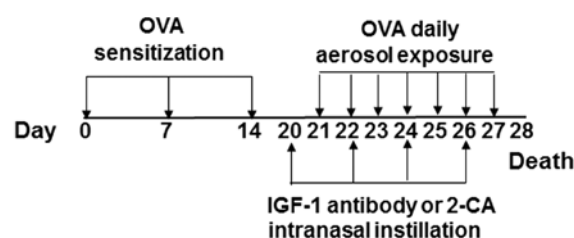


Figure 1. Flow chart of the study procedures. BALB/c mice were sensitized by intraperitoneal injection of OVA bound to aluminum hydroxide and challenged with aerosolized 5% OVA. 2-CA or IGF-1 blocking antibody was administered by intranasal instillation. OVA, ovalbumin; 2-CA, 2-chloroadenosine; IGF-1, insulin-like growth factor 1.

lavage was performed through the trachea until the lavage fluid was clear and transparent. Intrapulmonary digestion was performed by two successive injections of 15 ml 0.5% trypsin into the lungs for 10 min each. Digestion was stopped with RPMI-1640 medium containing 20% fetal calf serum (FCS). The trachea and bronchi were removed, following which the lung tissue was dissected and placed in RPMI-1640 medium. The lung tissue was cut into sections of <1 mm³ in size, ground between frosted glass sheets, and passed through 200 mesh and 400 mesh screens to obtain a suspension of primary alveolar epithelial cells.

Induction and assay of apoptotic cells. MLE-12 and primary alveolar epithelial cells (4x10⁵ cells/well) were stimulated with 0.25 µM curcumin (Sigma-Aldrich; Merck KGaA) for 72 h. Subsequently, the cells were stained for 20 min with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide using an assay kit (Beyotime Institute of Biotechnology), following the manufacturer's protocol. Apoptosis was assayed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) within 1 h of staining.

Phagocytosis assay. MLE-12 and freshly isolated alveolar epithelial cells (5x10⁴ cells/well) were stimulated for 48 h with 100, 200, 300, 500 and 1,000 ng/ml IGF-1 (Abcam). The cells were then incubated with 1 µl of a suspension of 4.55x10⁷ fluorescent microspheres (1 µm in diameter) for 2 h before washing twice in PBS with 5% FCS and fixing in 4% paraformaldehyde (PFA). Cells that had induced phagocytosis of fluorescent microspheres were detected with a FACSCalibur flow cytometer.

Furthermore, to assay the phagocytosis of apoptotic cells, activated MLE-12 or primary alveolar epithelial cells (5x10⁴ cells/well) were seeded into 6-well plates, and cocultured for 4 h with FITC-stained apoptotic MLE-12 cells. The cells were then washed twice with PBS containing 5% FCS and fixed with 4% PFA. The phagocytosis of FITC-labeled apoptotic cells was detected by flow cytometry.

Western blot assay. Isolated mouse lung tissue was washed with PBS, and protein extracts were prepared with NP-40 cell lysis buffer (Beyotime Institute of Biotechnology). The protein concentration of the cell lysates was measured using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Following separation by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the protein

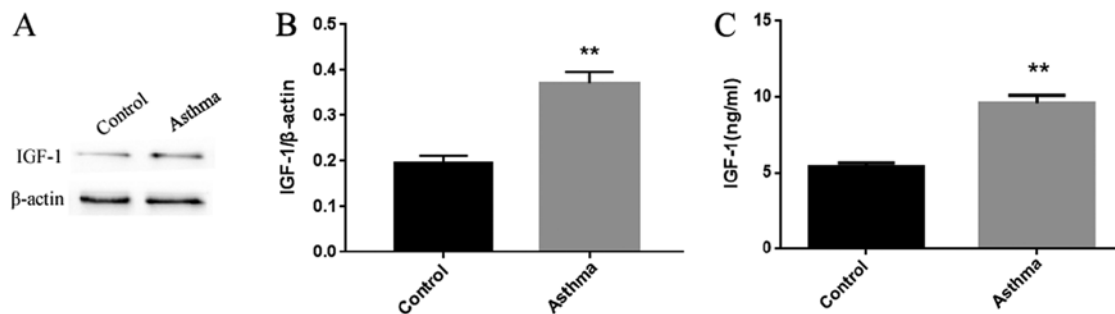


Figure 2. Increased IGF-1 protein in the lungs of asthmatic mice. (A) Representative western blot and (B) quantified levels of IGF-1 expression in lung tissues obtained from normal control and asthmatic mice. Densitometric analysis was conducted, and the expression of IGF-1 is shown relative to that of β -actin. (C) ELISA of IGF-1 level in the bronchoalveolar lavage fluid of normal control and asthmatic mice. Data are expressed as the mean \pm standard deviation of three independent experiments. ** $P < 0.01$ vs. control group. IGF-1, insulin-like growth factor 1.

samples were transferred to polyvinylidene difluoride (PVDF) membranes. Next, the PVDF membranes were blocked in Tris-buffered saline and Tween 20 (TBST) with 5% skim milk powder at 25°C for 2 h. The membranes were washed with TBST solution and incubated overnight at 4°C with IGF-1 (1:1,000; cat. no. ab9572; Abcam) and β -actin (1:1,000; cat. no. AF0003; Beyotime Institute of Biotechnology) primary antibodies. Membranes were washed and then incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) at 25°C for 2 h. Subsequently, the protein bands were read by enhanced chemiluminescence using a BeyoECL Plus kit, and strip density analysis was performed with ImageJ software 6.0 (<https://imagej.nih.gov/ij/>).

Enzyme-linked immunosorbent assay (ELISA). The content of IGF-1 and IL-33 in the BALF supernatants, as well as IGF-1 in lung tissues were assayed using commercially available ELISA kits (cat. nos. K02016571 and M27131086; Cusabio Biotech Co., Ltd.), following the manufacturer's protocol.

Lung histology. The mouse lungs were surgically obtained from mice 24 h after final antigen or mock challenge. Lung tissue was fixed at 25°C for 1 week in 4% PFA, dehydrated in a graded alcohol series, embedded in paraffin and sectioned at 5 μ m. Following dewaxing and rehydration, the sections were stained with hematoxylin-eosin, and observed and photographed under a light microscope.

Statistical analysis. The results are expressed as the mean \pm standard deviation. Similar results were obtained from three independent experiments. Statistical analysis was performed with SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was conducted to evaluate multiple group comparisons, while Student's *t*-tests were used to evaluate comparisons between two groups. *P*-values of < 0.05 were considered to denote statistically significant differences.

Results

Increased IGF-1 protein expression in the lungs of asthmatic mice. As shown in Fig. 2A, IGF-1 protein expression was significantly higher in the lung tissue of asthmatic mice as

compared with that in normal control mice. IGF-1 protein expression was also markedly higher in the BALF of asthmatic mice compared with that in normal mice (Fig. 2B).

Elevated IGF-1 expression in the lungs of asthmatic mice primarily results from synthesis in alveolar macrophages. IL-33, a cytokine that promotes airway inflammation, is elevated in the serum of asthma patients, and is associated with disease severity (13). In the current study, IL-33 was significantly elevated in the BALF of asthma model mice (Fig. 3A). IGF-1 expression was also significantly increased in primary alveolar macrophages stimulated by IL-33 *in vitro* (Fig. 3B). Following the depletion of alveolar macrophages by 2-CA, the elevation of IGF-1 expression in the lung tissues of asthmatic mice decreased by 50%, while the elevation of IGF-1 in the BALF almost completely disappeared (Fig. 3C and D). The results of Fig. 3C and D indicated that the increase in IGF-1 in the lung tissues of asthma model mice primarily resulted from synthesis in alveolar macrophages.

IGF-1 inhibits the phagocytic activity of alveolar epithelial cells. The phagocytosis of apoptotic cells by alveolar epithelial cells has been demonstrated to reduce airway inflammation, and IGF-1 significantly inhibited the phagocytosis of MLE-12 and primary alveolar epithelial cells. The results of the treatment of MLE-12 cells with 100-1,000 ng/ml IGF-1 on the phagocytosis of fluorescent microspheres are displayed in Fig. 4A and B. After 12 h of treatment, the phagocytic activity of MLE-12 cells significantly decreased at doses of 200, 300 and 500 ng/ml, with the inhibitory effect reaching a peak at 300 ng/ml IGF-1. Following stimulation of MLE-12 cells with 100 ng/ml curcumin for 24 h, the percentage of cells in late apoptosis was 97.8% compared with that of 2.95% for unstimulated MLE-12 cells (Fig. 4C). Upon treatment with 300 ng/ml IGF-1 for 4 h, the phagocytosis decreased to 32.6% (Fig. 4D and E). However, when treatment with 300 ng/ml IGF-1 was performed for 12 h, it resulted in a significant reduction in the phagocytosis of fluorescent microspheres and apoptotic cells by primary alveolar epithelial cells (Fig. 4F-I).

Antibody blocking of IGF-1 antibody promotes the phagocytic activity of primary alveolar epithelial cells and reduces lung inflammation in asthma model mice. Anti-IGF-1 blocking antibody was administered by nasal drops during the

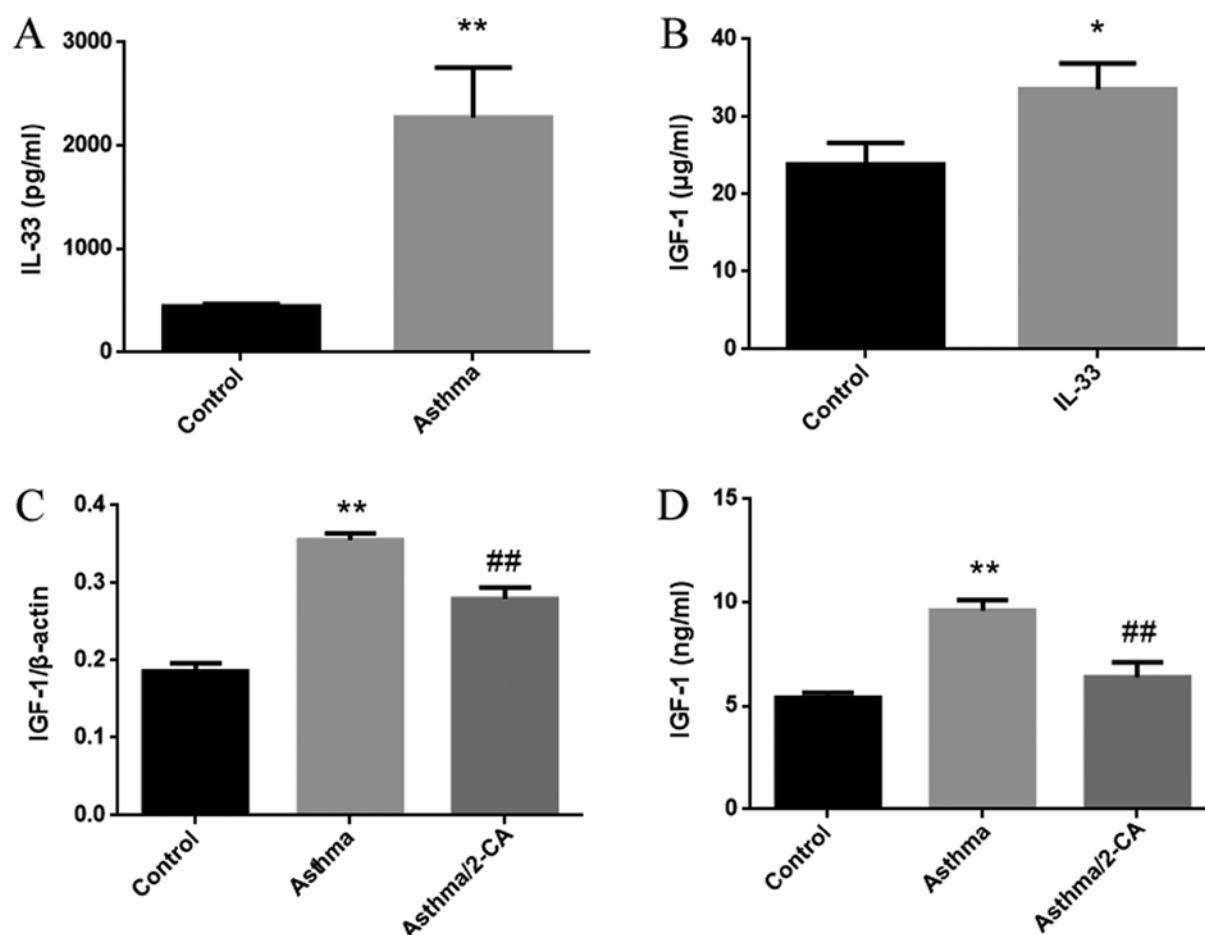


Figure 3. Increase in IGF-1 expression in the lung tissues of asthmatic mice primarily resulted from synthesis in alveolar macrophages. (A) ELISA of IL-33 expression in the BALF of normal control and asthmatic mice. (B) Primary alveolar macrophages from normal control mice were seeded into 6-well plates and stimulated with IL-33 (100 ng/ml) for 48 h; next, IGF-1 expression in the supernatant was determined by ELISA. The asthmatic mice were treated by intranasal instillation of 2-CA, and IGF-1 expression in (C) lung tissues and (D) BALF of asthmatic mice were examined by western blot and ELISA. Data are expressed as the mean ± standard deviation of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control group; ## $P < 0.01$ vs. asthma model mice. IGF-1, insulin-like growth factor 1; IL-33, interleukin-33; BALF, bronchoalveolar lavage fluid.

development of the asthma model. Subsequently, primary alveolar epithelial cells were isolated from mouse lung tissue, and their phagocytic activity was assayed with fluorescent microspheres and apoptotic cells. Blocking IGF-1 in the asthma model mice significantly enhanced the phagocytosis of fluorescent microspheres and apoptotic cells by primary alveolar epithelial cells (Fig. 5A and B). Compared with the normal mice, the asthmatic mice exhibited extensive lung inflammation with distinct perivascular and peribronchial cuffing. Intranasal instillation of IGF-1 blocking antibody significantly decreased the infiltration of inflammatory cells around the airways and blood vessels, and decreased the thickness of the bronchial mucosa and airway secretions (Fig. 5C). Hence, IGF-1 blockade also significantly reduced lung airway inflammation in asthma model mice.

Discussion

In the mouse model established in the present study, IGF-1 levels were found to be significantly elevated in the lung tissues and BALF of asthmatic mice compared with the normal controls. IGF-1 is a peptide of approximately 70 amino acids containing four domains; it mediates cell growth, metabolism,

proliferation and differentiation following binding to the IGF-1 receptor (IGF-1R) (8,14). It is active in lung development and various disease states, such as inflammation, fibrosis and tumors (9). Numerous studies have reported increased IGF-1 mRNA expression in intrabronchial biopsy tissue obtained from asthmatic patients and patients with tracheal epithelial fibrosis (15). In addition, Yao *et al* (12) reported increased IGF-1 protein expression, assayed by immunohistochemistry, in lung tissues from asthma model mice. The results of the present study are consistent with previous reports, all of which support further study of IGF-1 as a therapeutic target in asthma.

IL-33 was found to be elevated in the BALF of asthma model mice in the current study (Fig. 3A). It is known that IL-33 promotes systemic T helper 2 (Th2) cell responses and is constitutively expressed in a variety of tissues, including the airways of asthmatic patients, particularly those with severe disease (16,17). IL-33 activity is mediated by binding to its ST2 receptor, which is present on macrophages (18). IL-33 binding to alveolar macrophages can increase the expression of the mannose receptor IL-4Ra, as well as the production of C-C motif chemokine ligand 24 (CCL24) and CCL17, thus contributing to allergic inflammation (19,20). In the asthma

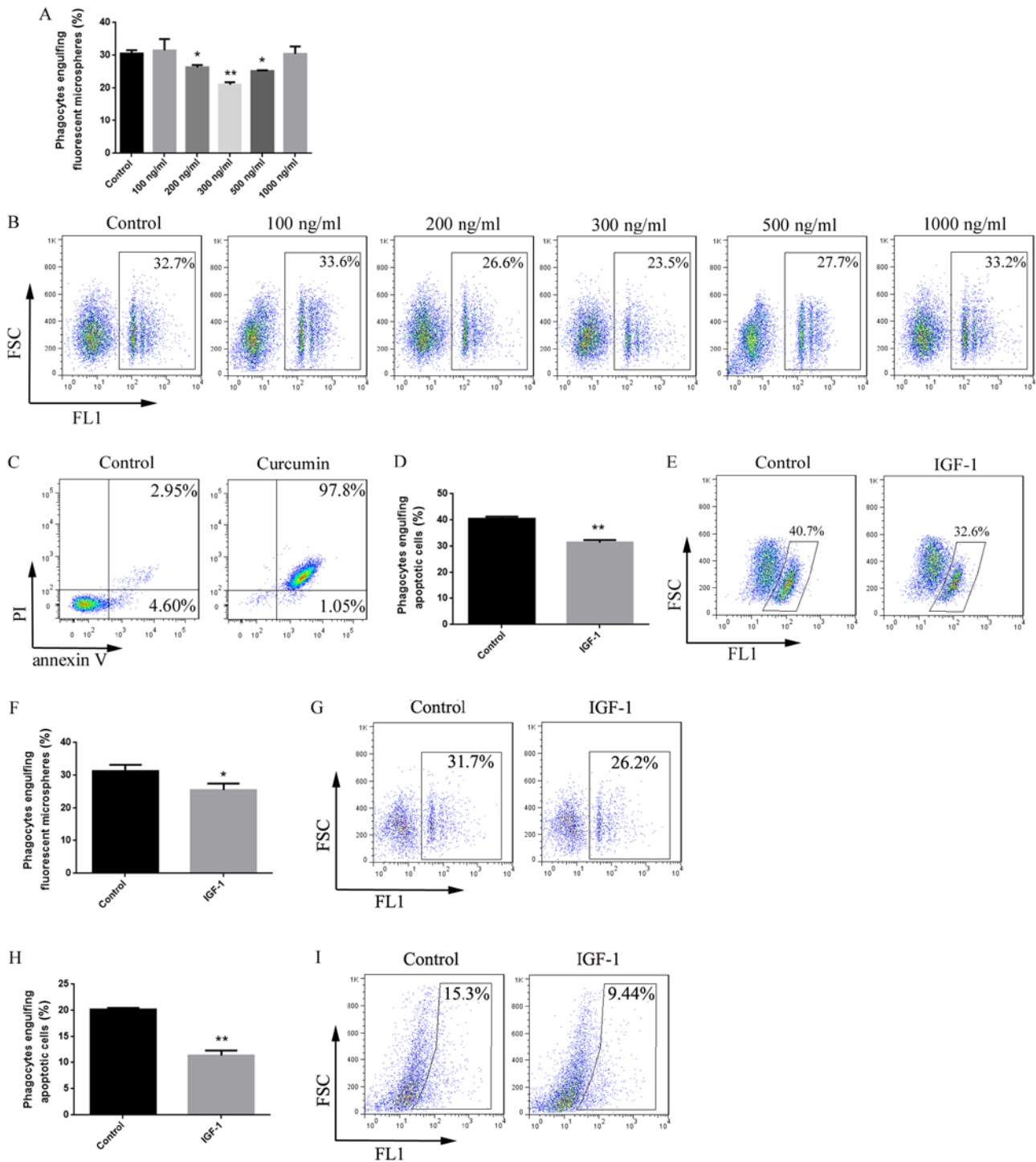


Figure 4. IGF-1 inhibits phagocytosis of MLE-12 and primary alveolar epithelial cells. (A) Percentage of phagocytosis of fluorescent microspheres, and (B) the corresponding flow cytometry results. MLE-12 cells were stimulated for 48 h with 100, 200, 300, 500 or 1000 ng/ml IGF-1, incubated with fluorescent microspheres (4.55×10^7 particles) for 12 h and then examined by flow cytometry to assess the phagocytosis. (C) MLE-12 cells were stimulated with curcumin for 72 h, stained with Annexin V-FITC and propidium iodide, and then apoptosis was assayed by flow cytometry. (D) Phagocytosis of apoptotic cells, and (E) the corresponding flow cytometry results of MLE-12 cells stimulated for 6 h with 300 ng/ml IGF-1. Following treatment, the cells were cocultured with FITC-stained apoptotic MLE-12 cells for 4 h, and then apoptosis was assayed by flow cytometry. (F) Phagocytosis of fluorescent microspheres and (G) corresponding flow cytometry results, as well as the (H) phagocytosis of apoptotic cells and (I) corresponding flow cytometry results, of primary alveolar epithelial cells treated with 300 ng/ml IGF-1 for 12 h. Following treatment, the cells were cocultured with fluorescent microspheres or apoptotic MLE-12 cells for 4 h, and then assessed by flow cytometry. Data are expressed as the mean \pm standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. unstimulated control group. IGF-1, insulin-like growth factor 1.

model mice investigated in the present study (Fig. 3B), IL-33 promoted IGF-1 expression by alveolar macrophages. IGF-1 production has been reported in endothelial cells, epithelial

cells, fibroblasts and macrophages, among others (21,22). In addition, Wang *et al* (23) reported that epidermal T cells can produce IGF-1. In the present study, the increase in IGF-1

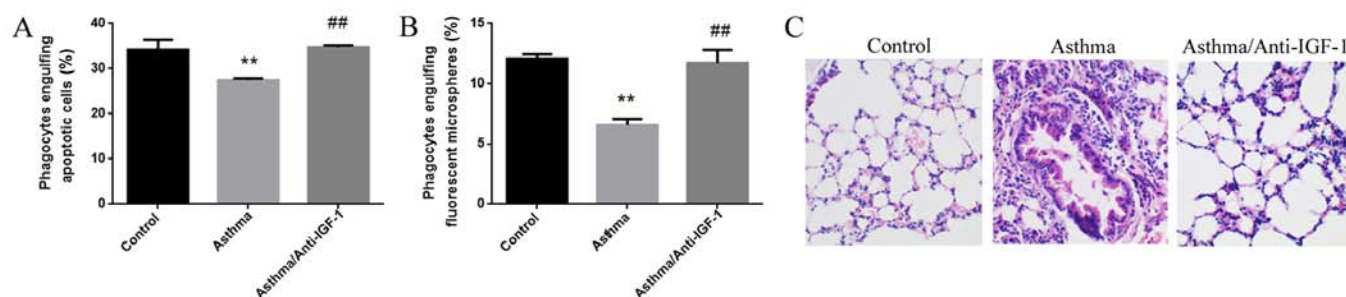


Figure 5. Antibody blocking of IGF-1 enhanced the phagocytic activity of alveolar epithelial cells and reduced airway inflammation in asthma model mice. Subsequent to intranasal instillation of anti-IGF-1 blocking antibody, primary alveolar epithelial cells were isolated and seeded into 6-well plates. Fluorescent microspheres or FITC-stained apoptotic MLE-12 cells were added, and the phagocytosis of (A) fluorescent microspheres or (B) apoptotic cells was assayed by flow cytometry. (C) Representative images of hematoxylin-eosin staining of lung tissue from mice in the treatment groups (magnification, $\times 200$). A representative image from three experiments is displayed. ** $P < 0.01$ vs. normal controls; ## $P < 0.01$ vs. asthma model mice. IGF-1, insulin-like growth factor 1.

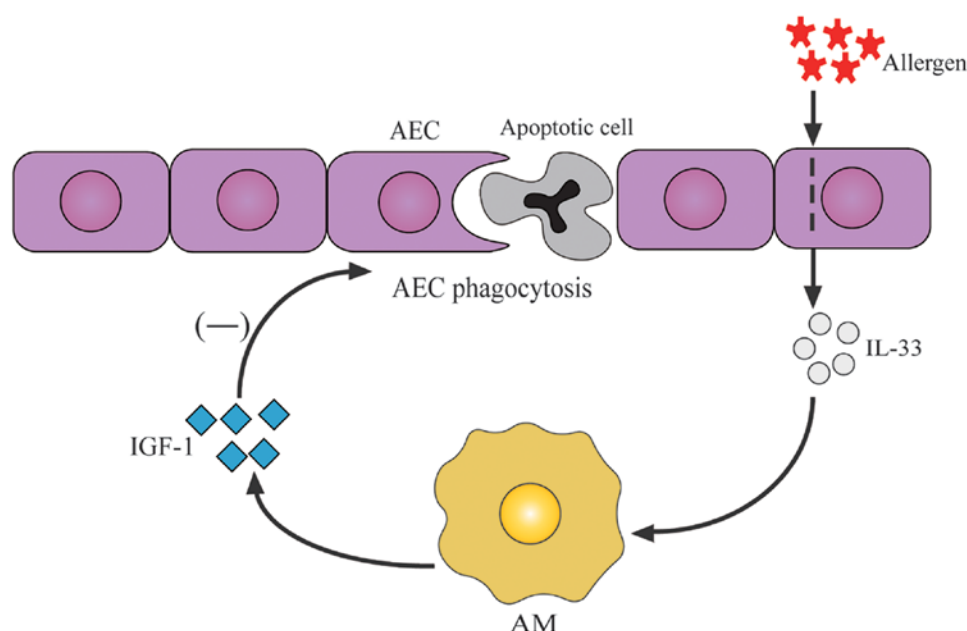


Figure 6. Schematic illustrating how AM-derived IGF-1 is thought to promote the phagocytosis of apoptotic cells in the airway of asthmatic mice. Allergen stimulation increases IL-33 secretion in the airways of asthmatic mice, which in turn induces IGF-1 production by AM. IGF-1 then inhibits phagocytosis of apoptotic cells by the surrounding AEC. IGF-1, insulin-like growth factor 1; AM, alveolar macrophages; AEC, alveolar epithelial cells.

that was detected in the asthma model animals was mainly produced in alveolar macrophages. This result is in line with the findings of Fritz *et al* (24), who demonstrated that alveolar macrophage-derived IGF-1 induced the proliferation of lung epithelial cells.

In the asthma model established in the current study, epithelial cells were nonprofessional phagocytes that helped to maintain tissue homeostasis by clearing apoptotic bodies. Allergens can stimulate the apoptosis of airway epithelial cells, whereas the surrounding intact epithelial cells can phagocytose the apoptotic cells and secrete anti-inflammatory cytokines. Airway epithelial cells, thus, regulate inflammation by phagocytosis (6). In the established model, IGF-1 prevented the phagocytosis of apoptotic cells by alveolar epithelial cells, thus increasing the release of inflammatory contents from apoptotic cells, thereby aggravating airway inflammation. Therefore, blocking IGF-1 improved airway inflammation and lung histopathology in asthmatic mice. IGF-1R-deficient mice

are less susceptible to skin inflammation in comparison with normal mice, indicating that IGF-1 signaling promotes inflammation (25). A previous study has reported reduced airway hyperresponsiveness, mucus secretion and eosinophil infiltration of airway tissues in IGF-1R-deficient asthmatic mice (26). These observations are consistent with the involvement of IGF-1 in the development of asthma.

Aerobic training is recommended as an adjuvant therapy for asthma patients, since it reduces the expression of proinflammatory signals, including IGF-1 and peribronchial leukocyte activation, leading to reduced airway inflammation and Th2 responses (27,28). The results of the mouse model described in the current study add to the evidence obtained from other asthma models that IGF-1 is a proinflammatory signal. Such evidence suggested that creatine supplementation promotes goblet cell proliferation, and upregulates the expression levels of IL-5, inducible nitric oxide synthase and proinflammatory mediators, such as IGF-1, in epithelial cells (29,30).

To date, few studies have investigated the association of IGF-1 signaling with phagocytosis. A study by Xiao *et al* (31) did not identify an effect of IGF-1 on the phagocytosis of peritoneal macrophages. Furthermore, Dos Santos Reis *et al* (11) reported that IGF-1 promoted phagocytosis by J774 macrophage cells. By contrast, in the current study model, IGF-1 inhibited phagocytosis by MLE-12 alveolar epithelial cells and primary alveolar epithelial cells. The differences in the findings of these studies may be the result of using different target cells.

In conclusion, the effects of IGF-1 on the phagocytosis of alveolar epithelial cells may represent a novel regulatory mechanism of airway inflammation, which is illustrated in Fig. 6. The results suggested that IL-33 was elevated in the airway of asthmatic mice and induced IGF-1 production by alveolar macrophages. IGF-1 then prevented the phagocytosis of apoptotic cells by alveolar epithelial cells and increased the release of inflammatory contents of apoptotic cells, thus leading to increased airway inflammation. Recurrent airway inflammation leads to airway hyperresponsiveness and airway remodeling, which exacerbates asthma. Nevertheless, in order to further explain the role of IGF-1 in the pathogenesis of asthma, it is necessary to construct an asthma model using IGF-1-deficient mice, and then observe airway inflammation and airway remodeling, which will be the focus of our future work. These efforts will support IGF-1 as a potential therapeutic target for the treatment of asthma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MM, FW, JH and XT performed the experiments. HM, SG and CS conducted data interpretation and analysis. CS wrote the manuscript. All authors have read and approved the final 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 there is no conflict of interest.

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