

Alveolar Macrophage-Derived Vascular Endothelial Growth Factor Contributes to Allergic Airway Inflammation in a Mouse Asthma Model

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Abstract

Vascular endothelial growth factor (VEGF) is a potent proangiogenic factor that correlates with vascular permeability and remodelling in asthma. Recently, alveolar macrophages (AM) were shown to be an important source of VEGF during lung injury. Our previous studies demonstrated that AM are an important subset of macrophages in the initiation of asthmatic symptoms. Here, we further investigated whether AM-derived VEGF was required for allergic airway inflammation in asthma. In this study, we reported that the expression of VEGF in AM was significantly increased after allergen challenge. Depleting AM or neutralizing VEGF in alveolus prevented ovalbumin (OVA)-induced asthma-related inflammation by inhibiting the infiltration of inflammatory cells in the lung, reduced the level of the cytokines, IL-4, IL-5, and IL-13, in the bronchoalveolar lavage fluid (BALF) and decreased airway hyper-responsiveness (AHR). Moreover, the inhibition of miR-20b increased the protein level of VEGF in normal AM; conversely, increasing miR-20b in asthmatic AM resulted in decreased VEGF protein levels. These findings suggest that AM-derived VEGF is necessary for allergic airway inflammation in asthmatic mice and miR-20b negatively regulates this expression.

Introduction

Asthma is a heterogeneous disease that is broadly defined as a clinical syndrome characterized by reversible airway obstruction, peribronchial inflammation and airway hyperresponsiveness (AHR) [1, 2]. The incidence of asthma has increased markedly in recent years, with the greatest prevalence observed in developed countries [3]. Some of the most common changes in the airway wall of asthmatics are epithelial desquamation, goblet cell hyperplasia, smooth muscle hypertrophy-hyperplasia, as well as growth and proliferation of new vessels [4]. Vascular endothelial growth factor (VEGF) is one of the most potent proangiogenic factors; it stimulates endothelial cell migration and proliferation and is widely expressed in highly vascularized organs and tissues, including the lung [5, 6]. Increased VEGF levels in induced sputum and bronchoalveolar lavage fluid (BALF) and VEGF-positive cells in bronchial biopsies have been found in asthmatic patients compared with healthy controls [7]. Recent studies have demonstrated that overexpression of

VEGF in the murine lung induces an asthma-like phenotype including parenchymal and vascular remodelling, oedema, mucus metaplasia, myocyte hyperplasia, AHR and augmented Th2 inflammation [8]. These findings suggest that VEGF plays an important role during the pathophysiological processes of allergic asthma.

Although epithelial cells have been considered as the primary producer of VEGF in the lung, other cell types can produce VEGF, including smooth muscle cells, endothelial cells, CD4⁺ T cells and macrophages [9–13]. Our previous study proved that alveolar macrophages (AM) are involved in the initiation of asthmatic symptoms [14]. As AM may produce VEGF [15], we therefore hypothesized that AM-derived VEGF was required for allergic airway inflammation in asthma. In this study, we demonstrate that the expression of VEGF was significantly increased in AMs from asthmatic mice. This macrophage-derived VEGF was necessary for allergic airway inflammation in a mouse model mimicking allergic asthma and miR-20b negatively regulated VEGF expression in AM.

Materials and methods

Mice. Female BALB/c mice (6–8 weeks old) were purchased from the Center of Medical Experimental Animals of Hubei Province (Wuhan, China) for studies approved by the Animal Care and Use Committee of Bengbu Medical College.

Induced allergic asthma mouse model. BALB/c mice were sensitized by intraperitoneal injection of 0.2 ml solution containing 100 µg ovalbumin (OVA; Sigma-Aldrich, St Louis, MO, USA) in Imject® Alum (Pierce, Rockford, IL, USA) on d0, d7 and d14. The sensitized mice were then challenged with the administration of aerosolized 1% OVA for 30 min, 5 days/week (once a day) from d17 to d49. Age- and gender-matched naive mice were used as controls. The protocol is illustrated in Fig. 1. For blocking experiments, mice received aerosolized anti-VEGF-neutralizing Ab (0.02 µg/ml) or the isotype control Ab 12 h prior to each OVA challenge.

Preparation of BALF and isolation of AM. Twenty-four hours after the last antigen challenge, animals were anesthetized using pentobarbital and the lungs were lavaged with saline (4 × 0.8 ml) via tracheal cannulation. The obtained BALF from each animal was immediately centrifuged at 400×g for 5 min, and the supernatant was stored at –70 °C until used in an enzyme-linked immunosorbent assay (ELISA). After red blood cell lysis, cell pellets from BALF were washed twice with PBS and then cells were incubated in a six-well plate for 2 h; the adherent cells harvested were confirmed to be >96% pure AM as assessed using Wright–Giemsa staining (Fig. 2).

Assay for cytokines. For mouse cytokine detection, the BALF was collected as described earlier. The levels of IL-4, IL-5, IL-13 and VEGF in the BALF supernatant and VEGF in the culture supernatant of AM were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Determination of airway responsiveness. Airway responsiveness was assessed as changes in airway function after challenge with aerosolized methacholine (Sigma-Aldrich) as described previously [16]. Briefly, mice were anesthetized with pentobarbital sodium (80 mg/kg). The trachea was then surgically exposed, cannulated (18G), and

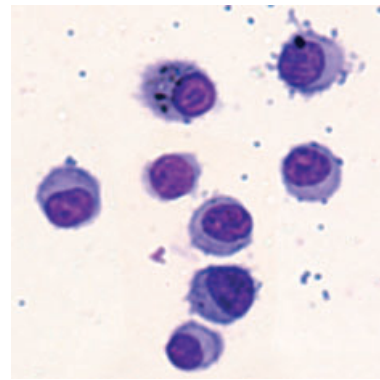


Figure 2 Morphological properties of alveolar macrophages (AM). Bronchoalveolar lavage fluid (BALF) was collected by aspiration via tracheal cannulation. After lysis of RBC, the cells in BALF were incubated in a six-well plate for 2 h and the adherent cells were stained with a Wright–Giemsa staining solution (magnification = ×1000).

connected to a specialized ventilator (flexiVent SCIREQ; Montreal, QC, Canada). Ventilation was achieved at 160 breaths/min at a tidal volume of 0.15 ml with a positive end-expiratory pressure of 2–4 cm H₂O. Lung resistance was continuously computed by fitting flow, volume and pressure. Mice were challenged with methacholine aerosol in increasing concentrations (6.25–50 mg/ml in saline). Aerosolized agent was administered for 10 s with a tidal volume of 0.5 ml. From 20 s up to 3 min after each aerosol challenge, the data of lung resistance were continuously collected. Maximum values of lung resistance were selected to express changes in airway function, which was represented as a percentage change from baseline after administration of the saline aerosol.

Alveolar macrophage depletion. Sensitized mice were given aerosolized 5 mM 2-chloroadenosine (2-CA; Sigma-Aldrich) for 20 min, 12 h before OVA challenge. More than 75% of AM were depleted, as evaluated by counting AM in BALF from treated and untreated mice; other cell types were not depleted.

Lung histology. For histopathological assessment, the lungs of mice were excised, fixed in 4% buffered formalin for 12 h and embedded in paraffin. The tissues were sliced into 5-µm sections and stained with haematoxylin and eosin.

Western blot analysis. Western blot was performed as described previously [17]. Briefly, AMs lysates and prestained molecular weight markers were separated by SDS-PAGE followed by transfer onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline with 0.5% of Triton X-100) and probed with respective antibodies, according to the manufacturer's instructions. After incubation with the secondary antibody conjugated with HRP, membranes were extensively washed, and the blots were visualized by enhanced chemiluminescence according to the

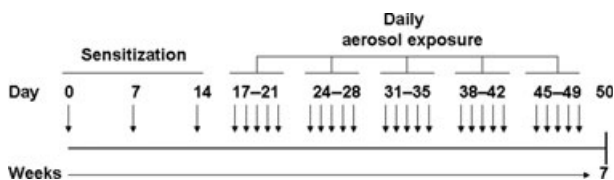


Figure 1 Experimental model procedure. BALB/c mice were sensitized by i.p. injection of ovalbumin (OVA) bound to Imject® alum and challenged with aerosolized OVA (see Materials and methods for the detailed protocol).

manufacturer's protocol (ECL kit; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Antibodies to VEGF were purchased from Abcam.

Analysis of microRNAs using RT-PCR and quantitative RT-PCR. Total microRNAs (miRNAs) were isolated from AM using miRNA isolation kits (Ambion, Austin, TX, USA). Reverse transcription primers for miR-20b were designed using RNA mfold version 2.3 server; their specificity was identified according to previous study [18]. cDNA synthesis was performed using 100 ng enriched miRNA. A 67-bp cDNA product was amplified using PCR and the following primers: miR-20b, 5'-CCCAAAGTGCTCATAGTG-3' (sense); antisense primer, 5'-GACTGTTTCCTC TCTTCCTC-3'. For real-time PCR, the above-mentioned primers and the Taqman probe [6-FAM] TTGCGACTACACACACACACA [BHQ1a-6FAM] were mixed with TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min using the Stratagene QRT-PCR instrument.

Transfection. The transfection assay was performed as previously described [19]. Briefly, miR-20b (Dharmacon, Lafayette, CO, USA), miR-20b inhibitor (Ambion) and the corresponding control oligonucleotides were purchased. For transient transfection, 200 pmoles synthesized oligonucleotide or 2 µg plasmid was mixed with 100 µl Nucleofector solution (Amaxa, Gaithersburg, MD, USA); 2×10^6 AM were transfected using electroporation and a Nucleofector instrument. After transfection, the cells were incubated for 4 h at 37 °C prior to use in assays.

Statistical analysis. The data were analysed using SPSS 10.0 statistics software package (SPSS Inc., Chicago, IL, USA). Results were expressed as mean value $\pm$ SD and interpreted by Student *t*-test or one-way ANOVA followed by LSD *post hoc* test. Differences were considered to be statistically significant when $P < 0.05$.

Results

The expression of VEGF is upregulated in AM from asthmatic mice

Vascular endothelial growth factor is a potent mediator of vascular remodelling and angiogenesis in the lung. Increased levels of VEGF have been suggested to exist within asthmatic airways [7], and a previous study suggested AM may produce VEGF [15]. Therefore, the expression of VEGF in AM was analysed in control and asthmatic mice. A severe increase in VEGF protein expression was observed in freshly isolated AM from asthmatic mice compared with cells from control mice (Fig. 3A). Additionally, we measured the level of VEGF in the supernatants of cultured AM. The result showed that asthmatic AM secreted a higher level of VEGF compared with control AM (Fig. 3B). These data suggested that AM may produce increased amounts of VEGF in an asthmatic microenvironment.

AM-derived VEGF is necessary for allergic airway inflammation in the lung

Next, we investigated the role of AM-derived VEGF in the mouse allergic inflammation model that mimics asthma. Following exposure to aerosolized 2-CA, 75% of alveolar macrophages and 95% elevated VEGF were depleted, as assessed by counting the cells and measuring VEGF in BALF (Fig. 4). Thus, sensitized mice were challenged with OVA aerosol with or without macrophage depletion. Alternatively, the mice sensitized without macrophage depletion were first treated, or not, with aerosolized VEGF-neutralizing Ab 12 h prior to being challenged with OVA aerosol. Lung pathology of asthma was evident in the OVA-challenge group. Additionally, either macrophage depletion or

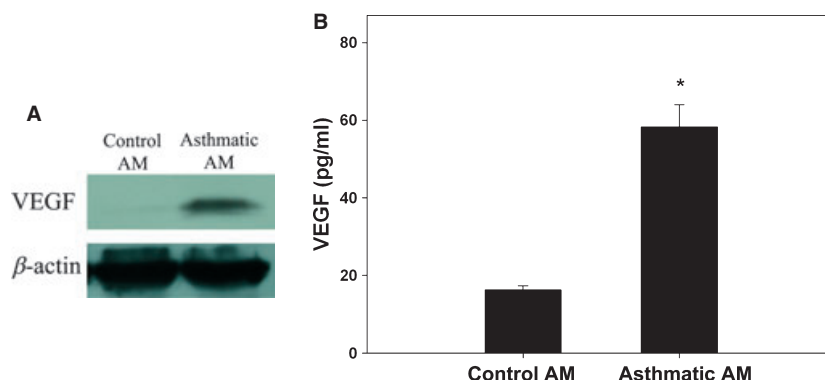


Figure 3 Enhanced expression and secretion of vascular endothelial growth factor (VEGF) in asthmatic alveolar macrophages (AM). (A) The expression of VEGF in freshly isolated AM from asthmatic and control mice was detected using Western blot. (B) AM (4×10^5 cells) from asthmatic mice or control mice were incubated in a 96-well plate for 48 h. The level of VEGF in the supernatants was determined using ELISA. * $P < 0.01$, compared with AM of control mice group.

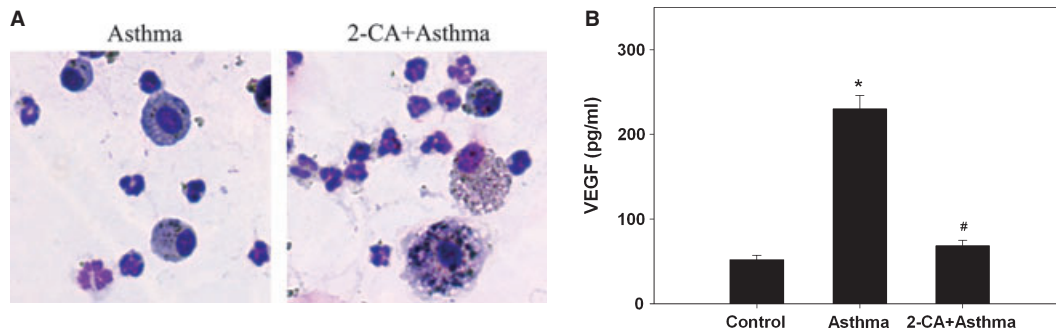


Figure 4 Depletion of alveolar macrophages (AM) using 2-chloroadenosine (2-CA). Sensitized mice were challenged with aerosolized ovalbumin (OVA) with or without 2-CA pretreatment (see Materials and methods for the detailed protocol). Twenty-four hours after the last OVA challenge, the cells from bronchoalveolar lavage fluid (BALF) were prepared using cytopsin techniques and stained with the Wright–Giemsa solution (A, magnification = $\times 1000$), and the level of vascular endothelial growth factor in BALF was determined using ELISA (B). * $P < 0.01$, compared with control group, # $P < 0.01$, compared with asthma group.

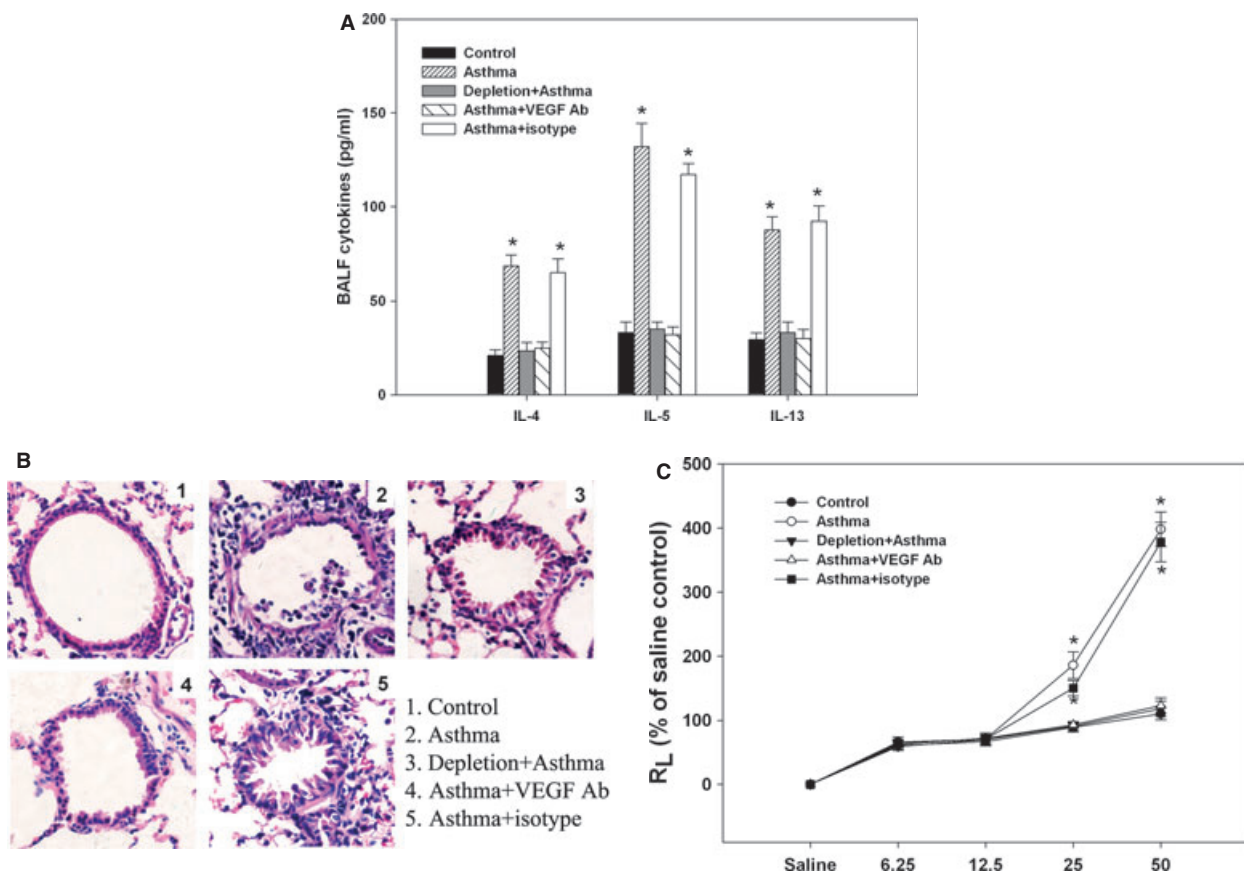


Figure 5 Alveolar macrophages (AM)-derived vascular endothelial growth factor (VEGF) is necessary for asthma-related allergic inflammation. Sensitized mice were challenged with ovalbumin (OVA) aerosol with or without macrophage depletion, or challenged with ovalbumin (OVA) aerosol and aerosolized VEGF-neutralizing Ab or control isotype Ab. The normal mice were used as control. The mice were analysed as follows: (A) IL-4, IL-5 and IL-13 levels in the bronchoalveolar lavage fluid were detected using ELISA kits. * $P < 0.05$, compared with control group. (B) Representative photomicrographs of H&E staining of lung sections from the different group are shown. (C) Airway hyperresponsiveness was measured using lung resistance in response to inhaled methacholine. * $P < 0.05$, compared with control group.

treatment with aerosolized VEGF-neutralizing Ab suppressed the production of the cytokines, IL-4, IL-5, and IL-13, in BALF (Fig. 5A), reduced the infiltration

of inflammatory cells and perivascular and peribronchial cuffing (Fig. 5B) in the lung and decreased airway hyperresponsiveness (Fig. 5C). Thus, these data suggest

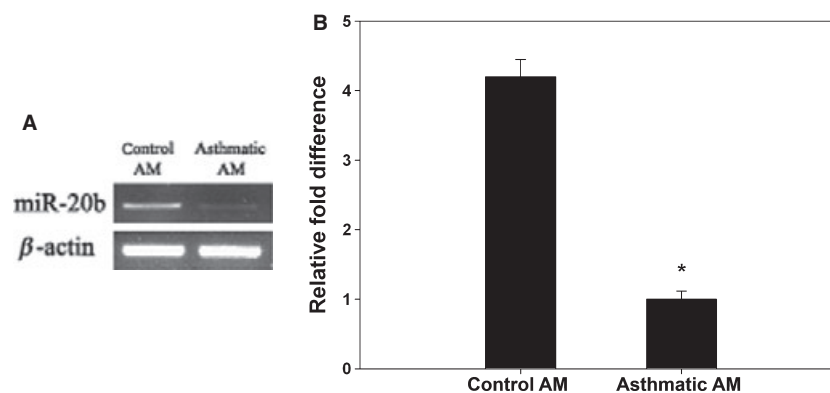


Figure 6 Alveolar macrophages (AM) expression of miR-20b. (A) AM expression of miR-20b from control and asthmatic mice was determined using RT-PCR. (B) AM expression of miR-20b was also determined using real-time RT-PCR. The relative gene expressions of miR-20b to let-7a were calculated by the $2^{-\Delta\Delta CT}$ methods. The miR-20b level in the asthmatic groups is designated as 1. * $P < 0.01$ versus control mice group.

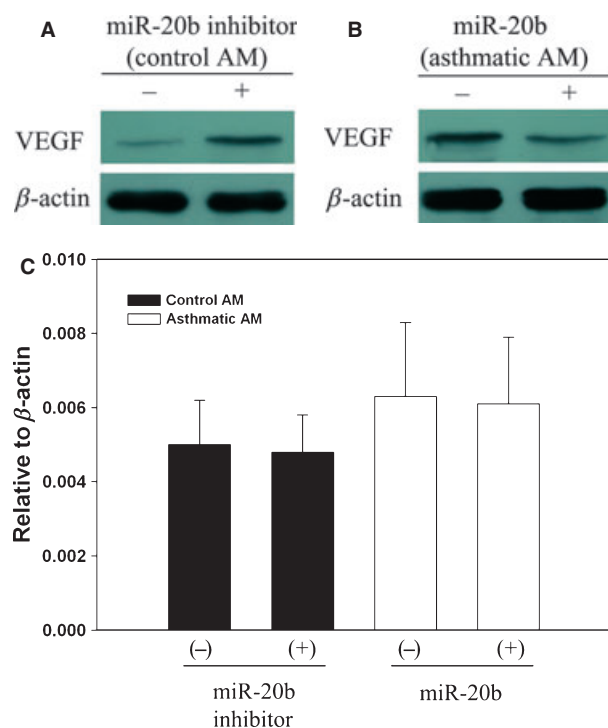


Figure 7 miR-20b negatively regulates the alveolar macrophages (AM) expression of vascular endothelial growth factor (VEGF). (A) miR-20b inhibitor increased VEGF protein in control AM. The miR-20b inhibitor or control oligonucleotide was transfected into control AM, and 48 h later, VEGF expression was determined using Western blot. (B) miR-20b decreased VEGF protein in asthmatic AM. The miR-20b or control oligonucleotide was transfected into asthmatic AM, and 48 h later, VEGF expression was determined using Western blot. (C) miR-20b inhibitor or miR-20b did not affect the mRNA levels of VEGF. The cells from (A) and (B) were examined using real-time RT-PCR to detect VEGF mRNA expression.

that VEGF-producing macrophages in alveolus are responsible for the airway inflammation observed in asthmatic mice.

MiR-20b negatively regulates VEGF expression in alveolar macrophages

Recently, microRNAs (miRNAs) have been implicated in the pathogenesis of asthma [20]. A previous study reported that miR-20b is an important regulator of VEGF [21]. Here, we hypothesized that AM-derived VEGF was regulated by miR-20b. To test this, we first determined the expression pattern of miR-20b in AM from control and asthmatic mice. Quantitative RT-PCR demonstrated that miR-20b was about fourfold lower in AM of asthmatic mice compared with control mice (Fig. 6). This expression pattern is inversely correlated with the expression of VEGF, which was very low in control mice, but very high in asthmatic mice. To verify that VEGF was targeted by miR-20b, a blocking strategy was adapted here by introducing miR-20b inhibitor to control AM, which indeed increased the protein level of VEGF (Fig. 7A). Next, asthmatic AM were transfected with miR-20b. This resulted in a significantly decreased level of VEGF protein compared with control AM (Fig. 7B). Nevertheless, the mRNA level of VEGF was not affected by either miR-20b inhibitor or miR-20b (Fig. 7C). Taken together, these data suggested that miR-20b negatively regulates VEGF protein expression in AM.

Discussion

In this study, we demonstrate that the AM expression of VEGF is markedly upregulated in asthmatic mice. Either depleting AMs or neutralizing VEGF in alveolus effectively inhibits OVA-induced allergic inflammation related in an induced asthma mouse model.

Asthma is a chronic airway inflammatory disease associated with airway wall remodelling [1]. Structural alteration of airway walls is an essential feature of asthma and

includes hypertrophy and hyperplasia of airway smooth muscle, mucous gland hyperplasia and qualitative and quantitative changes of airway blood vessels [22]. VEGF is an endothelial cell-specific mitogenic peptide and plays a central role in angiogenesis. VEGF also increases vascular permeability so that plasma proteins can leak into the extravascular space, which leads to oedema and profound alterations in the extracellular matrix [23]. VEGF is widely expressed in a variety of highly vascularized organs, including the lung [6]. In asthma, VEGF has been implicated in airway vascular remodelling either by increasing vascular permeability or stimulation of new blood vessel growth. Both effects may lead to increased airway wall thickness and airflow limitation [24]. A mouse model of toluene diisocyanate (TDI)-induced asthma suggested a contribution of VEGF in airway obstruction, migration of inflammatory cells and airway hyperresponsiveness development [25]. However, in the TDI-induced asthma patients, VEGF levels showed no significant changes in both sputum and serum [26]. These results suggested that VEGF might have a minor role in immediate bronchoconstriction after TDI exposure in human asthma. Several investigators suggested contribution of VEGF in chronic asthma and airway remodeling in chronic asthma patients. Hoshino *et al.* [27] demonstrated that VEGF expression was increased in the asthmatic airway of chronic asthma patients, which was correlated with airway calibre and airway hyperresponsiveness. Moreover, a recent study reported that in induced sputum from asthmatic patients, the measurable level of VEGF was increased compared with normal control subjects. VEGF mediates clinically relevant functions on the microvasculature in the airway of asthmatic subjects [28]. Epithelial cells, smooth muscle cells, endothelial cells, CD4⁺ T cells and macrophages can produce VEGF [9–13]. Recently, Medford *et al.* [15] demonstrated that AM were an important source of VEGF during lung injury. AM are the predominant resident immune effector cells in the alveolar spaces and conducting airways [29]. During the asthmatic process, AM may be activated by Th2 cytokines, leading to Th2 polarization and inflammation [30, 31]. After stimulation, they produce a range of inflammatory mediators and promote asthmatic development. Our results showed that AM are main producers of VEGF in the BALF from asthmatic mice. AM-derived VEGF is necessary for allergic airway inflammation in asthmatic mice, which is consistent with previous reports demonstrating that VEGF plays a role in the OVA-induced airway hypersensitivity response.

Until recently, the cellular mechanisms regulating VEGF expression were poorly understood. The expression of VEGF is regulated by multiple factors, including miRNAs [21, 32, 33]. Emerging evidence has demonstrated that miRNAs are important regulators of gene expression in the pathogenesis of asthma [34]. miRNAs are endoge-

nous non-coding small RNAs, 19–23 nucleotides in length [35, 36]. miRNAs are functionally involved in numerous cellular processes, including proliferation, differentiation, metabolism and motility [37–39]. Hua *et al.* [21] showed that miR-20b was an important regulator of VEGF in CNE cells under hypoxia; however, the role of miR-20b in VEGF regulation in the asthma microenvironment was unclear. In this study, we found that miR-20b was very highly expressed in AM from control mice, but very low in asthmatic mice, which was the inverse of VEGF expression. Additionally, we revealed that inhibition of endogenous miR-20b by an miR-20b inhibitor resulted in the upregulation of VEGF protein in control AM. Conversely, asthmatic AM transfected with miR-20b downregulated the expression of VEGF protein. These observations indicated that AM expression of VEGF was negatively regulated by endogenous miR-20b. In asthmatic mice, downregulation of miR-20b lead to upregulation of VEGF in AM, resulting in an augmentation of chronic inflammation characteristic of allergic asthma. What factors promote the reduction in miR-20b in AM from asthmatic mice? This is currently being explored.

Our findings identify a new role for alveolar macrophage in the pathogenesis of allergic asthma via their production of VEGF, which may be negatively regulated by miR-20b. Collectively, these results suggest that VEGF is a therapeutic target for asthma therapy, and the combination of targeting miR-20b and VEGF might be an even better choice.

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