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Bevacizumab reduced auto-phosphorylation of VEGFR2 to protect HDM-induced asthma mice

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a major angiogenic factor involved in both normal physiological processes, such as embryonic development and wound healing, and in diseases, like cancer. Recent studies have revealed the functions of VEGF in inflammation and immunoregulation. Asthma is a chronic inflammation of the airways characterized by airway epithelial barrier dysfunction and imbalance in T-helper (Th) 1/Th2 during immunoregulation. We hypothesized that VEGF plays an important role in asthma. Utilizing a house dust mite extract (HDM)-induced murine model of asthma, we investigated whether bevacizumab, a humanized *anti*-VEGF monoclonal antibody, could protect the epithelial barrier in murine airways. We found that bevacizumab reduced airway hyper-responsiveness (AHR) and airway inflammation induced by HDM. In addition, HDM exposure promoted expression of VEGF, and caused AHR, disruptions of the epithelial barrier, and airway inflammation. Bevacizumab ameliorated AHR and the release of Th2 cytokines, thereby protecting the epithelial barrier. Our data suggest that bevacizumab may be a new therapeutic strategy for asthma.

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1. Introduction

Asthma is a chronic disease involving allergic inflammation and studies on asthma are usually focused on dysfunction of airway epithelium, inflammation, and T-helper (TH) 1/Th2 immunoregulation [1]. The loss of epithelial barrier, disruption of epithelium, infiltration of inflammation, and excessive Th2 cytokine release leads to airway hyper-responsiveness (AHR) and ultimately, airway remodeling [1]. During this process, airway epithelium acts as a barrier to various stimuli and direct activation of the antigen-presenting cells. Excessive Th2-driven inflammatory response and intensive allergic stimulation synergistically aggravate cell injury and shedding, AHR, and dysregulation of inflammatory mediators [2].

Vascular endothelial growth factor (VEGF) is a crucial mediator

of angiogenesis that is released from many different types of cells. VEGF is a major transcriptional target for hypoxia-inducible factor (HIF)-1 and the VEGF/VEGF receptor (VEGFR) signaling pathway helps recovery from tissue damage and inflammation [3]. Studies also found VEGF expression in many lung diseases such as cancer, asthma, chronic obstructive pulmonary disease, lung injury, to name a few [4,5]. VEGF mediates cell permeability, cell migration, immune-surveillance, and angiogenesis in these pathological processes. Modulation of VEGF expression and *anti*-VEGF therapy are already utilized in the treatment of lung cancer; however, lack of VEGF causes endothelial cells to die [6]. The exact function of VEGF in different diseases is still not fully understood. Investigating the changes in the VEGF/VEGFR pathway and the effect of bevacizumab, a humanized *anti*-VEGF monoclonal antibody, in asthma may help clarify the role of VEGF in asthma and provide additional therapeutic strategies to treat conditions like asthma that involve chronic airway inflammation.

Recent studies have showed that VEGF promotes airway remodeling and causes epithelial barrier dysfunction by affecting zonula occludens proteins in ovalbumin (OVA)-induced BALB/c mice, and that an *anti*-VEGF monoclonal antibody alleviated the

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airway remodeling and restored epithelial barrier dysfunction [7,8]. However, in both studies [7,8], airway remodeling did not occur at an early time. VEGF/VEGF-mediated P38/mitogen-activated protein kinase (MAPK) activation had also been seen in endothelial cells [9]. In our previous studies, we showed that VEGF caused the dysfunction of epithelial permeability in 16HBE cells and exposure to toluene diisocyanate, a common and established cause of occupational asthma, caused the secretion of VEGF [7,10]. The HIF-1-VEGF pathway also interacts with the cytokines network, including cytokines interleukin (IL)-1 β , nuclear factor (NF)- κ B, and monocyte chemoattractant protein (MCP) -1, to mediate inflammation [11–13].

The role of VEGF in asthma prior to induction of airway remodeling is still unknown; therefore, in this study, we addressed the role of VEGF using a house dust mite-extract (HDM)-induced murine model of asthma.

2. Materials and methods

2.1. Animals

BALB/c mice (male, 6-week old, 20–24 g) were purchased from Southern Medical University and housed in specific pathogen-free housing maintained at room temperature (24 °C), humidity range 40–70%, with a 12-h light/dark cycle). Sterilized water and food were provided ad libitum. The mice used in our study were treated according to an experimental plan approved by the committee of Southern Medical University on the use and care of animals. All procedures were complied with the institutional animal ethics committee for the care and use of laboratory animals.

2.2. Treatment with HDM and/or bevacizumab and assessment of airway function and inflammation

HDM was purchased from ALK-Abello A/S (Alutard, Denmark), methacholine was purchased from Sigma-Aldrich (Shanghai, China) and the humanized anti-VEGF monoclonal antibody (bevacizumab) was purchased from Roche (Switzerland).

In this study, sevoflurane-anesthesia (Maruishi Pharmaceutical Co. Ltd.) was administered intranasally to the BALB/c mice, followed by nasal instillation of 10 μ L phosphate-buffered saline (PBS), and either HDM (400 U per mouse) alone, bevacizumab (5 mg/kg body weight) alone, or bevacizumab (5 mg/kg body weight) + HDM (400 U per mouse), for 5 consecutive days, for a total of 8 weeks. In the last group, mice were pretreated with bevacizumab for 30 min before administration of HDM.

Twenty-four hours after the last administration, lung resistance (R_L) was assessed to evaluate the AHR. Mice were placed in a barometric plethysmo-graphic chamber (Buxco Electronics, Troy, NY) and provoked with vehicle (PBS), followed by increasing concentrations of methacholine (6.25, 12.5, 25, or 50 mg/mL) via a nebulizer (Buxco Electronics, Inc., Troy, NY) for 3 min. The R_L values and other data were monitored at the same time. After finishing the test, the mice were sacrificed with an overdose of sodium pentobarbitone (administered intraperitoneally at a concentration of 100 mg/kg body weight; Sigma-Aldrich, Shanghai, China).

2.3. Western blotting analysis

Tissue lysates of right lungs were collected and boiled in standard SDS sample buffer. The VEGF (Cell Signaling Technology [CST]), VEGFR-2 (CST), phosphorylated (p)-VEGFR-2 (CST), p-AKT (Thr 308; CST), pan-AKT (CST), E-cadherin (Santa Cruz), β -catenin (Santa Cruz), occludin (Santa Cruz), and claudin1/2 (Santa Cruz.) antibodies were used to detect the respective proteins.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from the retro-orbital sinus, and serum samples were obtained after centrifugation at 13000 rpm for 10 min. The chest cavity was exposed, the trachea was carefully intubated, and the catheter secured with ligatures. Lung lavage was performed twice in situ with 0.8 mL sterile saline, which was slowly infused into the lungs and withdrawn. The bronchial alveolar lavage fluid (BALF) was collected after centrifugation at 3000 rpm for 10 min. IgE (BD Biosciences) in serum samples, and VEGF-A, IL-4, IL-5, IL-13, IL-33, IFN- γ (all from the Affymetrix product line; eBiosciences) in BALF samples, were measured by ELISA following the manufacturer's instructions.

2.5. Pathological staining

The left lungs were infused with 4% neutral paraformaldehyde (0.2 mL), then removed and immersed in 4% neutral paraformaldehyde for preparing pathological sections. Lung sections (4 mm) were cut with a Leica microtome 2030 (Leica, Nussloch, Germany). Lung tissues were stained with hematoxylin and eosin to assess pathological changes.

2.6. Immunohistochemistry

For immunohistochemical detection, sections of lung were dewaxed with xylene, alcohol, and PBS, followed by submersion and boiling in citrate buffer (pH 6.0) for antigen retrieval. Then, to block the endogenous peroxidase after natural cooling, sections were immersed in 5% H₂O₂ for 10 min, followed by incubation with the anti-VEGF antibody, anti-E-cadherin antibody and anti- β -catenin antibody respectively overnight at 4 °C. After washing with PBS, sections were incubated with anti-rabbit secondary antibody for 30 min at room temperature. Stained sections were developed with a DAB-peroxidase substrate kit. Finally, the sections were stained with hematoxylin.

2.7. Statistical analysis

Statistical analysis was performing using the SPSS (version 19.0) software package. The variables were expressed as mean $\pm$ standard deviation. One-way ANOVA accompanied by Bonferroni's post hoc test for multiple comparisons were utilized to compare differences among groups. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. VEGF is released in asthmatic mice and bevacizumab alleviates AHR

To confirm the role of VEGF and the effect of bevacizumab in the murine asthma model, we evaluated lung tissue pathology and AHR first. The injury to the epithelium and infiltration of inflammatory cells in HDM-treated mice were observed using hematoxylin staining; bevacizumab treatment resulted in a protective effect, based on the results of histological analysis (Fig. 1A). Bevacizumab did not increase murine AHR by itself; but, AHR in HDM-treated mice was significantly increased and the R_L values were partly restored after pretreatment with bevacizumab (Fig. 1B). Immunohistochemical staining data showed that HDM induced VEGF expression, whereas bevacizumab reduced VEGF expression (Fig. 1C). The trend of VEGF-A expression in the BALF was similar to the changes in VEGF expression detected in the immunohistochemical analysis, suggesting that VEGF and VEGF-A participate in

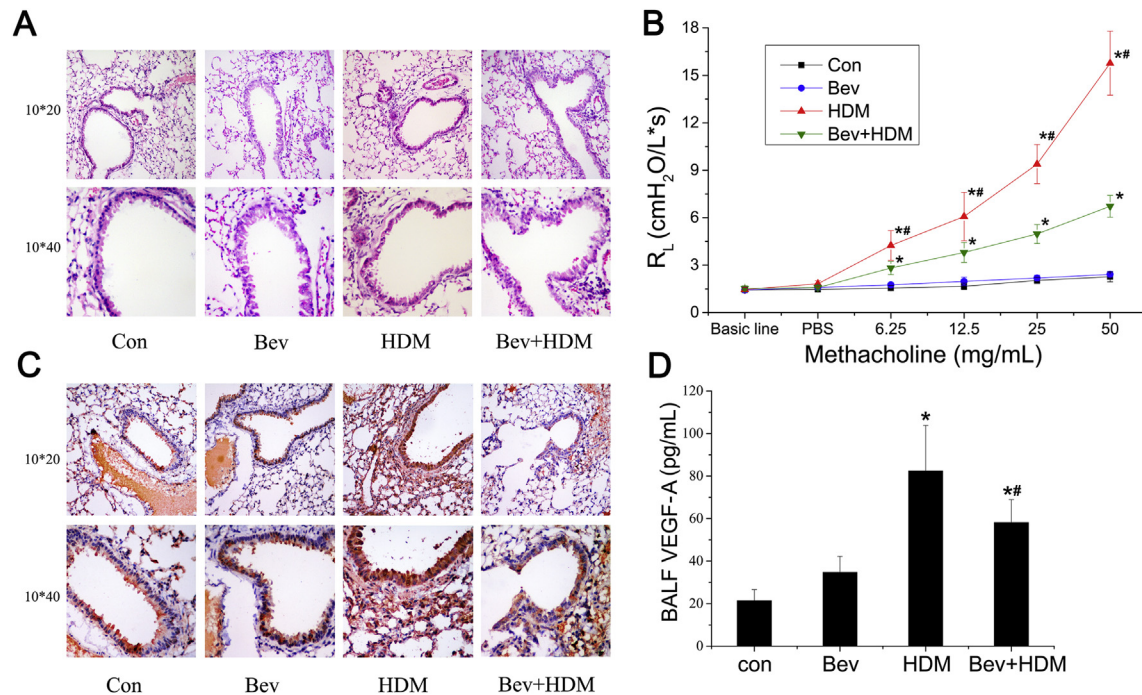


Fig. 1. The expression of VEGF in HDM-induced murine model of asthma and the effect of bevacizumab on the asthmatic pathological process. **A:** The effect of bevacizumab on HDM-treated mice in hematoxylin and eosin staining. Original magnification were 200 $\times$ and 400 $\times$. **B:** AHR (R_L) was assessed 24 h after the administration of the last treatment. Increasing concentrations of methacholine caused higher R_L values in the all groups, especially in the HDM-treated group. Mice pretreated with bevacizumab displayed reduced R_L (* P < 0.05 vs. the control group; ** P < 0.05 vs. the HDM group). **C:** Immunohistochemical analysis of VEGF (brown) with hematoxylin counterstaining (blue) in mice lungs under 200 $\times$ and 400 $\times$ magnification. **D:** ELISA of VEGF-A in BALF. Compared to the control (con) group, HDM-treated mice showed increased VEGF expression, whereas those treated with bevacizumab had reduced VEGF in BALF (* P < 0.05 vs. the control group; ** P < 0.05 vs. the HDM-treated group). Con, controls treated with vehicle alone; Bev, treated with bevacizumab alone; HDM, treated with house dust mite extract; Bev + HDM, treated with a combination of bevacizumab and HDM; PBS, phosphate-buffered saline. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HDM-induced murine asthma and that HDM promotes AHR, injury of epithelium, and airway inflammation via a VEGF-increase-dependent mechanism.

3.2. Bevacizumab inhibits HDM-induced disruption and dislocation of E-cadherin and β -catenin in mice

The integrity of epithelial barrier proteins is an essential first defense that deters permeability to a variety of factors from the outside environment. Therefore, changes in expression of these proteins, including E-cadherin and β -catenin, are also likely to be a central feature of the restoration of epithelial function by bevacizumab in the murine asthma model. We evaluated the changes in expression of E-cadherin and β -catenin in our HDM-induced asthma model using western blotting. Our results showed that HDM exposure disrupted E-cadherin and β -catenin, whereas bevacizumab protected expression of both proteins (Fig. 2 A). HDM exposure caused E-cadherin and β -catenin disruption and dislocation, and bevacizumab partly restored these deleterious alterations (Fig. 2B and C). Neither HDM nor bevacizumab affected the expression of occludin and claudin1/2 (Fig. 2 A). These results show that VEGF alters the expression and integrity of the adherence junction proteins β -catenin and E-cadherin to maintain epithelial barrier function in the asthmatic murine model.

3.3. HDM promotes, while bevacizumab inhibits, the release of Th2 cytokines

Imbalance in Th1/Th2 immunoregulation is a central characteristic in asthma, concomitant with airway inflammation. We detected the classical Th2 cytokines IL-4 and IL-5, the Th1 cytokine

IFN- γ in BALF, and the eosinophilic inflammatory marker, serum IgE, to distinguish which inflammatory type was augmented in our murine asthma model. HDM promoted release of IL-4, IL-5, and IL-33 in BALF and increase in serum IgE (Fig. 3A–C), whereas the expression of IFN- γ remained unchanged (Fig. 3 D), indicating that HDM-induced asthma results in Th2-driven inflammation. The protective effect of bevacizumab on alleviating airway inflammation and disruption of Th1/Th2 balance inflammation may, therefore, occur via suppression of the release of Th2 cytokines.

3.4. Bevacizumab protects against HDM-induced changes in the murine asthma model by reducing the expression of VEGFR-2, p-VEGFR-2 and p-AKT

VEGFR is the major receptor of VEGF and VEGFR-2 is considered to be more relevant to inflammation [14]. We addressed the relationship between VEGF and VEGFR-2 expression and also focused on the downstream p-AKT/AKT signaling pathway using western blotting analysis. Our data showed that HDM treatment promoted expression of p-VEGFR-2 and p-AKT, while it did not affect VEGFR-2 and pan-AKT expression (Fig. 4). The changes in p-AKT were in accordance with those observed in our previous study [15]. Bevacizumab reduced the expression of these proteins, suggesting that bevacizumab alters not only VEGF expression, but also the phosphorylation of its receptor and downstream phosphorylation of AKT. These changes in signaling proteins partly reveal the mechanism involved in the protective effect of bevacizumab in asthma.

4. Discussion

As a crucial factor involved in normal and pathological

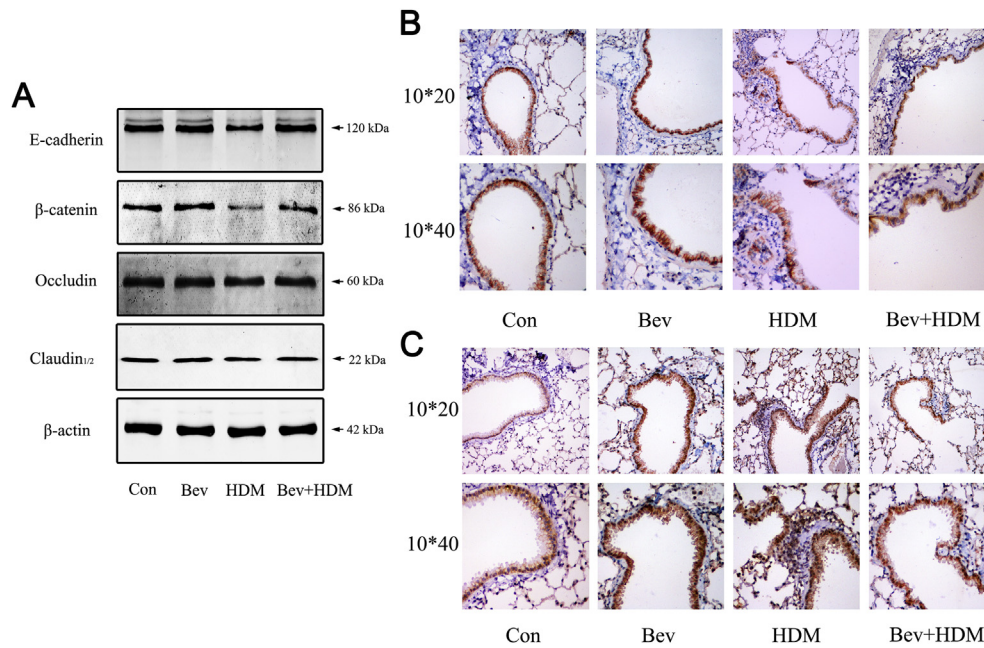


Fig. 2. The effect of bevacizumab on HDM-induced barrier protein disruption and expression. **A:** The expression of barrier proteins in the murine asthma model, as detected by western blotting. β -actin served as the internal control. **B:** Immunohistochemical analysis of β -catenin (brown) with hematoxylin counterstaining (blue) in murine lung sections under 200 $\times$ and 400 $\times$ magnification. **C:** Immunohistochemical analysis of E-cadherin (brown) with hematoxylin counterstaining (blue) in murine lung sections under 200 $\times$ and 400 $\times$ magnification. Con, controls treated with vehicle alone; Bev, treated with bevacizumab alone; HDM, treated with house dust mite extract; Bev + HDM, treated with a combination of bevacizumab and HDM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

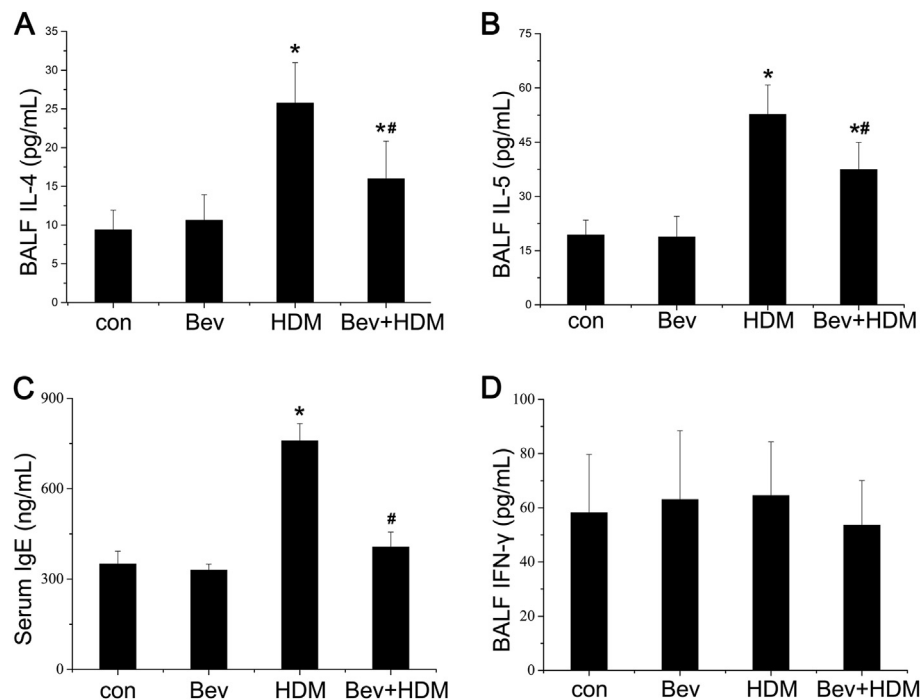


Fig. 3. HDM promotes, while bevacizumab inhibits, the release of Th2 cytokines. Imbalance in Th1/Th2 immunoregulation is a central characteristic in asthma, concomitant with airway inflammation. We detected the classical Th2 cytokines IL-4 and IL-5, the Th1 cytokine IFN- γ in BALF, and the eosinophilic inflammatory marker, serum IgE, to distinguish which inflammatory type was augmented in our murine asthma model. HDM promoted release of IL-4, IL-5, and IL-33 in BALF and increase in serum IgE (A–C), whereas the expression of IFN- γ remained unchanged (D), indicating that HDM-induced asthma results in Th2-driven inflammation. The protective effect of bevacizumab on alleviating airway inflammation and disruption of Th1/Th2 balance inflammation may, therefore, occur via suppression of the release of Th2 cytokines.

angiogenesis, VEGF has been proven to be an important molecule in mediating downstream signaling pathways such as the cAMP response element-binding protein, P38/MAPK and src pathways, in addition to serving as a target for cancer therapy and some lung

diseases [16]. Studies have shown the role of VEGF in promoting OVA-induced airway remodeling and epithelial barrier dysfunction in BALB/c mice [8,17]. Here, we report for the first time, to our knowledge, the role of VEGF in an HDM-induced murine asthma

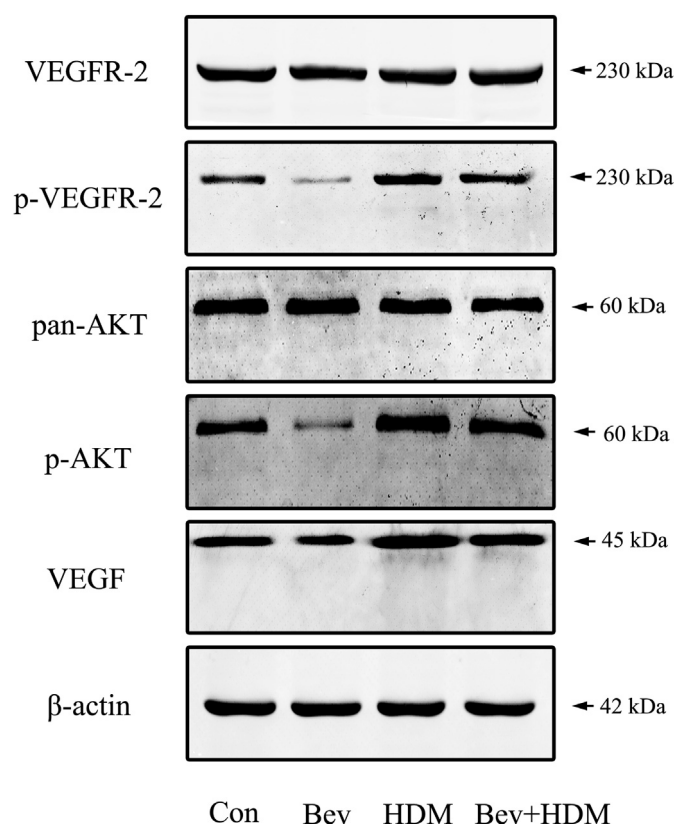


Fig. 4. The effect of bevacizumab on the VEGF receptor and downstream signaling pathway proteins in HDM induced murine model. The expressions of p-VEGFR-2 and p-AKT (Thr 308) were up-regulated in asthmatic mice, bevacizumab reduced expressions of these proteins. Both HDM and bevacizumab did not affect the expressions of VEGFR-2 and pan-AKT.

model. Our data demonstrate the relationship between VEGF and VEGFR-2/p-VEGFR-2 and we further investigated the exact function and underlying mechanism of VEGF-mediated alterations in asthma [18].

VEGF gene expression is limited in normal tissues, whereas VEGF family factors (VEGF-A, -B, -C, -D and placental growth factor) and its receptors are expressed much in significant amounts in structural and inflammatory cells in lung [19]. The receptors of VEGF are receptor tyrosine kinases, including VEGFR-1, VEGFR-2, and VEGFR-3. Although the amino acid compositions of the three receptors are highly similar, different VEGFRs have disparate and non-overlapping functions in signal transduction and pathological processes; moreover, the binding sites or types of VEGFRs combined with different VEGF are not alike in their functions [20,21]. VEGF-A is mainly induced by hypoxia and inflammation, and it activates VEGFR-2 to promote its auto-phosphorylation to generate p-VEGFR-2 [22]. VEGFR-2 is activated by VEGF-A primarily, and it affects differentiation, proliferation, and migration, along with specific effects on angiogenesis and regulation of permeability [23]. The expression of VEGFR-2 is regulated by the cytokine IL-6 [24], suggesting that the VEGF-A/VEGFR-2 pathway and the inflammatory process interact.

VEGFR-1 mainly mediates vascular development [25] and VEGFR-3 seems to regulate lymphangiogenesis [26], while VEGFR-2 is closely involved with inflammation. Therefore, we focused on the expression changes in VEGF, VEGFR-2, and p-VEGFR-2 in this study. We confirmed that HDM exposure induces expression of VEGF and VEGF-A, and that HDM and bevacizumab affect the amount of p-VEGFR-2, but not VEGFR-2, indicating that bevacizumab protects

the sensitized mice by inhibiting the activation, and not expression, of VEGFR-2 or by neutralizing VEGF-A/VEGF. One observation that does not fit our model for the role of VEGF in asthma is that we found that HDM promotes IL-6 release (data not shown) in the absence of VEGFR-2 upregulation. We intend to address this effect and its significance in detailed mechanistic studies of VEGF-cytokine interconnections in asthma in the future. Bevacizumab is a humanized monoclonal antibody which had been approved by the US Food and Drug Administration as a treatment agent in lung cancer [27]. We found that intranasal administration of bevacizumab could also function as a protective agent in asthmatic mice.

The disruption of epithelium and barrier proteins and airway inflammation are the classical features characteristic of the early stages of asthma, and they influence each other. Loss of barrier protein expression/function and epithelium result in exacerbation of asthma and acute inflammation [1]. In this study, we focused on E-cadherin and β-catenin in adherence junctions, because they not only play an important role in defense, but also act as signaling molecules in inflammation. E-cadherin mediates immune regulation and activation of NF-κB-mediated transcription [28]; concomitantly, the VEGF/VEGFR signaling pathway causes the disruption of E-cadherin and promotes inflammation [12]. β-catenin functions in airway remodeling and the Wnt/β-catenin signaling pathway promotes the transcription of NF-κB and VEGF [29]. Aberrant Wnt/β-catenin signaling has been implicated in many pathologic processes such as vascular remodeling, lung diseases and organ differentiation, and the disruption of β-catenin had been detected in a different murine asthma model. The Wnt/β-catenin signaling pathway plays a role in vascular development by regulating VEGF availability [30,31]. In our previous study, we showed that VEGF causes dysregulation of epithelial permeability in 16HBE cells [7], which are consistent with results shown here and our model for the role of VEGF in barrier function dysregulation in asthma.

In this study, our data showed that VEGF expression correlates with disruptions in expression/function of adherence junction proteins, consistent with data from our previous studies [7,10]. Moreover, bevacizumab restored the expression and proper cellular localization of the junction proteins. Bevacizumab does not penetrate into the cell and then impact the intracellular signaling pathway, neither does it change the activity or conformation of VEGFR, suggesting that the protective effect of bevacizumab is mediated through its competition with VEGFR-2 to bind VEGF and therefore, indirectly reducing activated VEGFR-2. Based on these results and those from other recent studies [7,8,10,32], we infer that disruptions of β-catenin play an important role in promoting VEGF expression in HDM-induced asthma in mice.

Studies have revealed that upregulation of cytokines like IL-6 and IL-1β lead to enhanced VEGF expression; meanwhile, VEGF also mediates the activation of NF-κB-mediated expression of cytokines, creating a positive feedback loop [11–13]. Inherent expression of VEGF in inflammatory cells is considerably high and the expression is easily induced by inflammation and hypoxia [11,33]. As a chronic airway inflammatory disease, in asthma, the inflammation of bronchial mucosa and increased release of cytokines likely acts as a continual stimulus that promotes secretion of VEGF [34]. VEGF also promotes inflammation and release of IL-1β by activating NF-κB in a murine asthma model [35]. For instance, MCP-1 could directly induce VEGF, at the same time VEGF could also induces MCP-1 by activating the NF-κB signaling pathway [13]. The cytokine IL-1β activates the NF-κB-cyclooxygenase-2 axis to stabilize HIF-1, subsequently inducing VEGF, altogether constituting a feedback cycle [11]. Similarly, tumor necrosis factor-α promotes the toll-like receptor4-NF-κB signaling pathway, thereby

mediating inflammation [12]. These data suggest that the HIF- α -VEGF signaling pathway and cytokines network intersect and crosstalk to mediate inflammation and immunoregulation, particularly in asthma. Further studies are necessary to tease out the context(s) in which each pathway/mechanism is invoked in asthma and the consequences thereof.

In this study, bevacizumab and HDM did not stimulate the release of IFN- γ in BALF. However, bevacizumab reduced the HDM-induced BALF IL-4, IL-5 and serum IgE levels. IL-4 and IL-5 are classical Th2 cytokines in asthma, while IgE has a positive correlation with eosinophil inflammation. These data indicate that VEGF participates in promoting the release of Th2 cytokines and that bevacizumab counteracts the effects of VEGF and alleviates airway inflammation to protect sensitized mice. We did not further investigate the exact signaling pathway involved in cytokine release and immunoregulation in this study. Further investigation to elucidate the effect of VEGF/VEGFR-2 on these pathologic processes in asthma is warranted.

In summary, intranasal administration of bevacizumab has a protective effect in restoring epithelial barrier function and alleviating airway inflammation in mice, which may occur through VEGF-A neutralization and inhibition of VEGFR-2 activation. VEGF plays an important role in asthma and *anti*-VEGF agents may be a feasible future therapeutic strategy for asthma management.

Conflict of interest

All authors declare no conflicts of interest associated with this manuscript.

Acknowledgments

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