CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Semaphorin 3E Inhibits House Dust Mite—Induced Angiogenesis in a Mouse Model of Allergic Asthma

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Increased angiogenesis is a characteristic feature of remodeling in asthmatic airways and stems from the imbalance between pro-angiogenic and anti-angiogenic factors. Surprisingly, the factors regulating this process in allergic asthma are poorly defined. Previously, we showed an important role of semaphorins 3E (Sema3E) in growth factor–induced airway smooth muscle proliferation and migration *in vitro*, and in down-regulating airway inflammation, T helper 2/T helper 17 cytokine response, mucus cell hyperplasia, and airway hyperresponsiveness *in vivo*. However, the role of Sema3E in airway angiogenesis is not fully understood. Here, we investigated the role of Sema3E in airway angiogenesis using a house dust mite (HDM) murine model of allergic asthma. Intranasal treatment with recombinant Sema3E significantly reduced the expression of angiogenesis markers within the airways of HDM-challenged mice compared with untreated mice. HDM-induced expression of vascular endothelial growth factor (VEGF) and VEGF receptor 2 protein were diminished substantially on Sema3E treatment. Interestingly, Sema3E-treated mice showed an enhanced expression of the negative regulator of angiogenesis, soluble VEGF receptor 1, compared with the untreated mice. These events were reversed in Sema3E-deficient mice at baseline or on HDM challenge. Taken together, this study provides the first evidence that Sema3E modulates angiogenesis in allergic asthmatic airways via modulating pro- and anti-angiogenic factors. (*Am J Pathol* 2019, 189: 762–772; <https://doi.org/10.1016/j.ajpath.2019.01.008>)

Asthma is a heterogeneous atopic disease, characterized by airway hyperresponsiveness, inflammation, and remodeling.^{1–3} Airway remodeling refers to the alteration of normal airway structural features including epithelial changes and subepithelial fibrosis, airway smooth muscle (ASM) cell hypertrophy/hyperplasia, goblet cell hyperplasia, and increased angiogenesis.^{4,5}

Angiogenesis, the sprouting of new blood vessels from pre-existing ones,⁵ is a vital process in physiological conditions. However, imbalance between pro- and anti-angiogenic molecules leads to increased angiogenesis in some pathologic conditions. Angiogenesis is one of the hallmarks of airway remodeling and is considered a key determinant of chronic inflammatory disorders including asthma and chronic obstructive pulmonary disease.⁶ It is believed that this pathology arises via activation of several pro-angiogenic growth factors in which vascular endothelial growth factor (VEGF) has

been recognized as a master regulator of blood vessel growth in asthmatic airways. In fact, VEGF is a mitogen for endothelial cells and promotes their proliferation and differentiation through the tyrosin kinase receptor VEGF receptor 2 (VEGFR2).⁷ VEGF–VEGFR2 signaling in endothelial cells promotes degradation of the extracellular matrix, migration, proliferation, lumen formation, and vessel stabilization.⁸

Emerging evidence suggests that a family of axon guidance cues, semaphorins, play a critical role in the development of the nervous system.⁹ However, recently semaphorins have received extensive attention because of their importance in

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processes other than neuronal guidance, including cell proliferation, migration, differentiation, and angiogenesis.^{10,11} Among eight classes of semaphorins, class 3 is the only secreted form in vertebrates that transduce signals through their receptor neuropilins and plexins.¹² Among class 3 semaphorins (Semas), Sema3A has been shown previously to play an essential role in the immunopathogenesis of atopic disorders such as asthma, allergic rhinitis, and atopic dermatitis.^{13–16} Furthermore, Sema3E is considered a unique semaphorin because of its ability to signal through the single-pass transmembrane receptor, plexin D1, independently of neuropilins. This characteristic turns it into a repulsive cue; however, the interaction of Sema3E/plexinD1 by Nrp1 or VEGFR2 reverts the function of Sema3E from repulsion to attraction.^{17,18}

Because of the dynamic expression of plexinD1 on endothelial cells at the front of actively sprouting blood vessels, the axis of Sema3E/plexinD1 has emerged as a fundamental pathway in angiogenesis.^{19–21} Sema3E inhibits human ASM cell proliferation and migration, suggesting an important role of this pathway in airway remodeling.¹⁰ Sema3E is also critical in neutrophil migration *in vitro* and *in vivo*.²² Furthermore, *Sema3e*-deficient mice showed an enhanced airway hyper-responsiveness, airway inflammation, and mucus cell hyperplasia,²³ suggesting a key role of this axis in allergic asthma. However, the role of Sema3E in airway angiogenesis is not known. Here, we investigated the role of Sema3E in allergen-induced angiogenesis using a mouse model of allergic asthma.

Materials and Methods

Animals

Six- to 8-week-old immunocompetent female Bagg albino/c mice, *Sema3e*^{-/-} mice (129P2 B1/6; kindly provided by Dr. Fanny Mann, Developmental Biology Institute of Marseille Luminy, Université de la Méditerranée, Marseille, France), and their littermate wild-type control were used in this study.²⁴ All animals were maintained at the Centre of Animal Care Services of the University of Manitoba under specific pathogen-free conditions and used in accordance with the Canadian Council for Animal Care guidelines.

House Dust Mite Exposure Model

Induction of allergic asthma was performed as described in Figure 1A. In brief, mice were sensitized and challenged intranasally with HDM resuspended in sterile saline (25 µg) (*Dermatophagoides pteronyssinus*; Greer Laboratories Inc., Lenoir, NC) for 5 consecutive days for 2 weeks. Murine Sema3E—fragment crystallizable region (Fc region) recombinant protein was produced as fusion protein N-terminal to a functional mouse γ2c Fc domain using Chinese hamster ovary cells. Secreted Sema3E-Fc protein was purified by protein A-affinity chromatography as previously described.²⁴ Sema3E-Fc (10 µg/kg) or Fc-Ig control was administered intranasally 1 hour before each HDM exposure

(25 µg). Saline-treated mice were used as a control group. In different experiments, HDM was intranasally administered to either *Sema3e*^{-/-} mice or wild-type littermates according to Figure 1A. Control mice received saline instead of HDM.

Methacholine Challenge Test

Lung mechanics were measured using the FlexiVent ventilator (SCIREQ, Montreal, Quebec, Canada) as previously described.²⁵ In brief, HDM or saline-sensitized and challenged mice underwent a tracheotomy. Then, an increasing gradient of methacholine dose (0, 3, 6, 12, 25, and 50 mg/mL) was administered intratracheally with a 5-minute interval between the doses and lung function parameters were performed including airway resistance, tissue resistance, and tissue elastance.

Bronchoalveolar Lavage Fluid Characterization

The total number of leukocytes in bronchoalveolar lavage fluid and differential cell counts was performed as described previously.²⁶

Histologic Examination

Formalin-fixed tissues were paraffin-embedded, and 5-µm-thick sections were cut and stained with hematoxylin and eosin for evaluating the level of cell infiltration.²⁷

Immunofluorescence Staining

Tissue sections were stained as previously described.¹⁰ Briefly, deparaffinized 5-µm tissue sections were washed and incubated with blocking solution (1% bovine serum albumin, 0.1% cold fish skin gelatin, and 5% second animal serum in Tris-buffered saline) for 45 minutes at room temperature. Sections were incubated for 3 hours at room temperature with rabbit anti-mouse von Willebrand factor (vWF) polyclonal antibody (1:400; Dako, Santa Clara, CA), rabbit anti-mouse CD31 polyclonal (1:50; Abcam, Toronto, ON, Canada), goat anti-mouse VEGF polyclonal antibody (1:20; R&D Systems, Minneapolis, MN), goat anti-mouse VEGFR1 polyclonal antibody (1:20; R&D Systems), rabbit anti-mouse VEGFR2 polyclonal antibody (1:200; Biorbyt, San Francisco, CA), mouse anti-human neutrophil elastase monoclonal antibody (1:20; Dako), and rabbit anti-mouse major basic protein polyclonal antibody (1:40; Cloud-Clone Corp., Katy, TX). Tissue sections were incubated with Alexa Fluor—568 goat anti-rabbit IgG, Alexa Fluor—488 chicken anti-goat IgG, or Alexa Fluor—488 goat anti-mouse IgG (heavy and light) (Life Technologies, Burlington, ON, Canada) for 1 hour at room temperature. Slides then were washed extensively for 3 × 5 minutes with Tris-buffered saline and counterstained with ProLong Gold Antifade mountant with DAPI (Life Technologies). Images were acquired using a digital Zeiss Axioskop 2 mot plus microscope using AxioVision Rel software version 4.8 (Carl Zeiss Microscopy, LLC, Thornwood, NY).

Image Analysis

Immunofluorescence staining of vWF and CD31 was analyzed quantitatively by counting the number of blood vessels positively stained for vWF and CD31 in five randomly chosen high-power fields of four tissue sections from each mouse.²⁸ The result was reported as an average of

the number of red blood vessels in each group.²⁸ Moreover, the result of VEGF, VEGFR1, and VEGFR2 staining was analyzed quantitatively by calculating the signal mean intensity of 20 randomly chosen high-power fields from each group using ImageJ software version 1.48v (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>). The result was reported as the mean intensity for each group.

Figure 1 Sema3E treatment attenuates house dust mite (HDM)-induced airway inflammation in mice. **A:** The protocol of an acute HDM mouse model of allergic inflammation. Female Bagg albino/c mice (age, 6 to 8 wk) were pretreated intranasally with HDM (2.5 mg/mL), Fc Ig, or Sema3E-Fc Ig (10 μ g/kg) 1 hour before the HDM (2.5 mg/mL) intranasal challenge. **B–F:** Each group contained four mice. Sema3E-Fc treatment reduced the total cell count (**B**) and the number of BAL eosinophils (**C**), but not neutrophils (**D**), macrophages (**E**), or lymphocytes (**F**) in comparison with untreated HDM-challenged mice. **G:** Reduced peribronchial inflammation is seen in Sema3E-Fc–treated compared with HDM-exposed animals. **H:** Lung sections were stained using neutrophil elastase and eosinophil major basic protein (MBP) antibodies to detect lung neutrophil and eosinophil infiltration. Statistical analysis was performed by one-way analysis of variance. $*P < 0.05$. Data are expressed as means $\pm$ SEM, $n = 4$. Scale bars = 50 μ m (**G** and **H**). Original magnification, $\times 200$ (**G** and **H**). BALF, bronchoalveolar lavage fluid; Fc, fragment crystallizable; Sema, semaphorin.

Lung Homogenate Preparation and ELISA

Lung samples were homogenized in cold homogenization buffer containing RPMI (Gibco, Life Technologies, Burlington, ON, Canada), Protease Inhibitor Cocktail (Sigma-Aldrich, Life Science, Oakville, ON, Canada), and phenylmethylsulfonyl fluoride (Sigma-Aldrich, Life Science). After centrifugation of the homogenates at $10,000 \times g$ for 15 minutes, supernatants were collected. The protein concentration of each sample was determined using the bicinchoninic acid protein assay kit, according to the manufacturer's instructions (EMD Millipore). The concentration of VEGF-A and soluble VEGFR1 (sVEGFR1) were quantitated using mouse VEGF and mouse sVEGFR1 DuoSet Enzyme-Linked Immunosorbent Assay (ELISA) kits (R&D Systems). The sensitivity limits of VEGF-A and the sVEGFR1 assay were 15.6 pg/mL and 150 pg/mL, respectively.

RNA Extraction, Reverse Transcription, and Real-Time PCR

Total RNA was extracted from the whole lung by using TRIzol (Invitrogen, Life Technologies, Burlington, ON, Canada) according to the manufacturer's protocol. RNA (2 µg) was subjected to MultiScribe Reverse Transcriptase to synthesize cDNA (Applied Biosystems, Foster City, CA) in a total volume of 20 µL. cDNA of each sample and sequence-specific *Vegfr1* primer (forward: 5'-TCACAGATGTGCCGAATG-3', reverse: 5'-CGTAGCAGAATCCAGGTAATG-3') and *Gapdh* primer (forward: 5'-AACTTTGGCATTGTGGAAGG-3', reverse: 5'-ACACATTGGGGGTAGGAACA-3') (10 µmol/L) were added to SYBR Select Master Mix (Applied Biosystems). The amplification of target genes was calculated by normalizing to the amplification of *Gapdh* and then normalizing to control groups. Then the normalized values were expressed as fold increase/decrease of relative quantitative over the values calculated with other groups.

Statistical Analysis

Results are expressed as the means $\pm$ SEM. Differences between the groups were analyzed by one-way analysis of variance first to determine if any significant differences may exist among various experimental groups, and then Bonferroni post-tests were performed to detect statistically significant differences in each pair of experimental groups using GraphPad Prism 5.04 (Graphpad Software Inc., San Diego, CA). *P* values <0.05 were considered statistically significant.

Results

Sema3E-Fc Treatment Decreases Airway Hyperresponsiveness and Inflammation in HDM-Induced Allergic Inflammation

Sema3E recombinant protein reduces HDM-induced airway hyper-responsiveness and airway inflammation.²⁹ In brief,

Bagg albino/c mice were pretreated with Sema3E-Fc or Fc fragment Ig control (Fc Ig, 10 µg/kg) 1 hour before intranasal instillation of HDM as described in Figure 1A, and response to methacholine challenge was recorded. The level of different features of airway hyper-responsiveness including airway and tissue resistance and tissue elastance were increased in the HDM-challenged, Fc-pretreated mice and was attenuated significantly in the Sema3E-pretreated group (Supplemental Figure S1). Similarly, the level of inflammation in bronchoalveolar lavage fluid was examined by performing a cytologic examination on cytospin preparations by differential quick staining. The total bronchoalveolar lavage fluid cell count was reduced significantly in Sema3E-Fc-treated mice compared with HDM-challenged and Fc-treated controls (Figure 1B). In particular, Sema3E-Fc treatment reduced the level of granulocytes, mainly eosinophils (Figure 1C), and, to a lesser extent, neutrophils (Figure 1D). It did not significantly change the level of macrophages/mononuclear cells and lymphocytes in the airways (Figure 1, E and F). Photomicrographs of stained sections show an increased level of peribronchial inflammation in HDM-challenged mice. However, the infiltration of inflammatory cells was reduced in the Sema3E-treated group (Figure 1G), which was confirmed further by decreased immunoreactivity of neutrophil elastase and major basic protein (Figure 1H). Taken together, these data suggest that Sema3E reduces the levels of HDM-induced airway inflammation, particularly eosinophils.

Sema3E Regulates Expression of Angiogenesis Markers vWF and CD31

Previous studies have shown that the number of blood vessels is increased significantly in the airways of asthmatic patients in comparison with healthy controls.^{30–32} To examine the effect of Sema3E on HDM-induced angiogenesis in mice, immunofluorescence staining was performed using anti-vWF antibody. vWF is considered the most specific marker for lung endothelial cells.³³ CD31, a marker characterizing endothelial cells, also was used to stain blood vessels.³⁴ The number of newly formed blood vessels, as shown by vWF and CD31 staining, was reduced significantly in bronchial sections of the Sema3E-Fc treatment group compared with the Fc-Ig-treated HDM-challenged mice (Figure 2A and Supplemental Figure S2).

To ascertain the importance of Sema3E in airway neoangiogenesis, *Sema3e*^{-/-} and littermate control mice were subjected to HDM challenge (Figure 1A). A significant increase in immunoreactivity of vWF in *Sema3e*^{-/-} mice was detected at baseline and after HDM challenge compared with the wild-type littermates (Figure 2B). These data suggest that Sema3E reduces new blood vessel formation in the lung in a mouse model of HDM-induced airway inflammation.

Figure 2 von Willebrand factor (vWF) expression is regulated by semaphorin 3E (Sema3E) in a house dust mite (HDM) model of allergic inflammation. **A:** Bronchial sections were stained for vWF by immunofluorescence staining to assess the number of blood vessels per lung tissue area before and after HDM challenge. **B:** Enhanced vWF immunostaining in *Sema3e*^{-/-} compared with wild-type (WT) littermate upon saline or HDM exposure. The number of blood vessels per lung tissue area was determined as described in [Materials and Methods](#). Statistical analysis was performed by one-way analysis of variance. Data are expressed as means $\pm$ SEM, $n = 4$. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Scale bars = 50 μ m (**A** and **B**). Original magnification, $\times 100$ (**A** and **B**). Fc, fragment crystallizable; HPF, high-power field.

Sema3E Plays an Essential Role in the Expression of VEGF and VEGFR2 in Asthma Mouse Model

Previous studies have shown a key role of VEGF in the proliferation and differentiation of endothelial cells via binding to the high-affinity tyrosine kinase receptor, VEGFR2, which culminates in an increased level of angiogenesis.^{7,35–38} To investigate whether the effect of Sema3E on reducing blood vessels in the HDM mouse model of allergic asthma is mediated via VEGF, ELISA and immunofluorescence staining were performed to probe for VEGF level in lung homogenate and lung sections, respectively. The level of VEGF secretion was reduced

significantly in lung homogenate of HDM-challenged and Sema3E-treated mice compared with HDM-challenged alone (Figure 3A). Furthermore, immunofluorescence staining showed a reduced level of VEGF in lung sections of HDM-challenged and Sema3E-treated mice compared with HDM-challenged alone. VEGF staining was predominantly positive in epithelial cell layers, and to a lesser extent in myofibroblasts and inflammatory cells (Figure 3B). Conversely, a significant increase in pulmonary VEGF immunoreactivity was observed in *Sema3e*^{-/-} mice compared with the WT littermates (Figure 3C), which was confirmed further by ELISA both at baseline and on HDM exposure (Figure 3D).

VEGF
DAPI

Figure 3 Regulation of vascular endothelial growth factor (VEGF) expression in the mouse airway by semaphorin 3E (Sema3E). **A** and **D**: The level of VEGF was measured in lung homogenate by performing ELISA and normalized to total protein. **B** and **C**: Immunofluorescence staining was performed on lung sections using anti-VEGF antibody to compare the level of VEGF production in the airways and blood vessels. Low- and high-magnification images of lung tissues were captured and the mean intensity was measured per lung tissue area. $*P < 0.05$, $***P < 0.001$, and $****P < 0.0001$. Scale bars = 50 μ m. Original magnification: $\times 200$ (**B**, top row, and **C**, top row); and $\times 400$ (**B**, bottom row, and **C**, middle row and bottom row). Fc, fragment crystallizable; HDM, house dust mite; WT, wild-type.

Figure 4 The role of semaphorin 3E (sema3E) in the expression of vascular endothelial growth factor receptor 2 (VEGFR2) in a house dust mite (HDM) mouse model of allergic inflammation. **A:** Lung sections were stained to assess the level of VEGFR2 expression on the airway vascular endothelium. **B:** VEGFR2 immunoreactivity in *Sema3e*^{-/-} and control mice with or without HDM challenge. Low- and high-magnification images of lung tissue were taken. Mean fluorescence intensity of VEGFR2 was measured ($n = 10$ areas of four lung tissue sections per group). Statistical analysis was performed by one-way analysis of variance. Data are expressed as means $\pm$ SEM, $n = 4$. $**P < 0.01$. Scale bars: 20 μ m (top rows); 5 μ m (middle rows). Original magnification: $\times 400$ (top rows); $\times 1000$ (middle rows and bottom rows). Fc, fragment crystallizable; WT, wild-type.

VEGFR2 is recognized as a major mediator of angiogenic and mitogenic effects of VEGF.^{35,39} The effect of *in vivo* Sema3E treatment on VEGFR2 expression was studied on blood vessels by performing immunofluorescence staining on lung sections. The expression of VEGFR2 staining was

down-regulated significantly in lung vessels of HDM-challenged and Sema3E-treated mice in comparison with HDM-challenged alone (Figure 4A). Genetic deletion of *Sema3e* was associated with basal and HDM increased expression of VEGFR2 (Figure 4B). Overall, Sema3E plays

an important role in negative regulation of angiogenesis in the HDM model of asthma by controlling VEGF and VEGFR2 expression within the airways.

Anti-Angiogenic Role of Sema3E Is Mediated via Regulating the Level of sVEGFR1

sVEGFR1, also known as soluble Flt-1, is produced upon alternative splicing of the VEGFR1 gene. sVEGFR1 acts as a decoy receptor by inhibiting the binding of VEGF to VEGFR2 and negatively regulates the activity of VEGF on

the vascular endothelium.^{35,40–43} To investigate whether the effect of Sema3E on reducing new blood vessels in our mouse model is related to sVEGFR1 production; ELISA, quantitative PCR, and immunofluorescence staining were performed. The level of sVEGFR1 was increased significantly in lung homogenate of HDM-challenged and Sema3E-Fc-treated mice in comparison with HDM-challenged alone as detected by ELISA (Figure 5A). In addition, gene expression study and immunofluorescence staining confirmed our results on the role of Sema3E in inducing sVEGFR1 production. In fact, the level of

Figure 5 Semaphorin 3E (Sema3E) regulates soluble vascular endothelial growth factor receptor 2 (sVEGFR1) airway expression in house dust mite (HDM)-induced allergic inflammation. **A:** Enzyme-linked immunosorbent assay (ELISA) was performed to measure the level of sVEGFR1 in lung homogenate. **B:** Real-time PCR was performed to study the level of sVEGFR1 gene expression. **C:** The ratio of VEGF/sVEGFR1 obtained by ELISA using lung homogenate was calculated. **D:** Lung sections were stained by anti-sVEGFR1 antibody to study the level of sVEGFR1 production in the airway of mice. Low- and high-magnification images of lung tissues were captured and the mean intensity was calculated per lung tissue area. **E** and **F:** ELISA (**E**) and real-time PCR (**F**) were performed to determine the expression of sVEGFR1 in *Sema3E*^{-/-} and wild-type (WT) mice. **G:** The ratio of VEGF/sVEGFR1 was determined in saline or HDM-exposed *Sema3E*^{-/-} versus WT mice. Statistical analysis was performed by one-way analysis of variance. Data are expressed as means ± SEM, *n* = 4. **P* < 0.05, ****P* < 0.001, and *****P* < 0.0001. Scale bars: 50 mm (**top row**); 20 mm (**bottom row**). Original magnification: ×200 (**top row**); ×400 (**bottom row**). Fc, fragment crystallizable.

sVEGFR1 increased in *Sema3E*-pretreated, HDM-challenged mice compared with HDM-alone challenged group (Figure 5, B and D). Altogether, these data suggest that *Sema3E* reduces angiogenesis via enhancing sVEGFR1 decoy receptor expression in *Sema3E*-treated, HDM-induced allergic asthma.

Interestingly, analysis of the ratio of VEGF/sVEGFR1 showed an effect of *Sema3E* pretreatment on enhancing the balance toward sVEGFR1. In fact, the ratio of VEGF/sVEGFR1 decreased significantly in *Sema3E*-pretreated and HDM-challenged mice in comparison with HDM-challenged alone (Figure 5C). In line with these findings, a significant decrease in expression of VEGFR1 and the ratio of VEGF/sVEGFR1 was observed in *Sema3e*^{-/-} mice both at baseline and after HDM exposure (Figure 5, E–G). In addition, sVEGFR1 immunoreactivity was reduced in the airways of *Sema3e*^{-/-} mice compared with the WT littermates before and after HDM challenge (Supplemental Figure S3). Collectively, these data suggest that sVEGFR1 is induced by *Sema3E* treatment and acts as a dominant factor by preventing the binding of VEGF to VEGFR2 and reduces the level of angiogenesis.

Discussion

More than 50 years ago, Dunnill⁴⁴ reported that the density of blood vessels increases significantly in the airways of asthmatic patients, leading to asthma exacerbation by intensifying airway inflammation and remodeling. Therefore, finding a strategy to overcome this pathologic aspect of asthma deserves full consideration. *Sema3E* inhibits human ASM cell migration and proliferation by inducing actin depolymerization, suppressing Rac1 GTPase activity, and phosphorylation of phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2.¹⁰ *Sema3E* deficiency in mice leads to an exaggerated allergic airway inflammation, remodeling, and hyper-responsiveness,⁴⁵ suggesting that *Sema3E*/PlexinD1 axis is dysregulated in allergic asthma. To support this notion further, recent findings showed a significant down-regulation of *Sema3E* in severe asthmatic patients⁴⁵ and in both acute and chronic mouse models of asthma.²⁹ The regulatory role of *Sema3E* in allergic asthma is mediated by regulating the recruitment of pulmonary dendritic cells⁴⁵ and neutrophils.⁴⁶ Here, we show an inhibitory effect of this neuronal chemorepellant on pathologic angiogenesis in allergic asthma. Specifically, the inhibitory effect of *Sema3E* molecules on these events is associated with the reduction of inflammation and pro-angiogenic factors in asthmatic airways.

Herein, it was found that *Sema3E* mediates the reduction of granulocytic airway inflammation. In fact, neutrophils are known sources of pro-angiogenic factors including VEGF^{46,47} and also are targets of VEGF-A both in humans and mice.⁴⁸ Because the *Sema3E* receptor plexinD1 is

expressed in mouse and human neutrophils and *Sema3E* inhibits their migration *in vivo* and *in vitro*,²² it is reasonable to suggest that the effect of *Sema3E* on airway angiogenesis in this mouse model is owing partially to the inhibition of neutrophil migration. In concert with this presumption, both prophylactic and therapeutic treatment with *Sema3E* reduces C-X-C motif chemokine ligand 1 (CXCL1; alias KC), the murine equivalent of human IL-8, considered one of the most chemoattractant chemokines for neutrophils.²²

Similarly, *Sema3E* pretreatment diminished airways and tissue eosinophil recruitment upon HDM challenge. Eosinophils are producers of VEGF⁴⁹ and their recruitment is mediated mainly through eotaxin 1 and 2. Eotaxin 1 and 2 are considered major angiogenic factors that affect new vessel formation. Previous studies have shown that human and murine endothelial cells express the eotaxin receptor CCR3.^{50,51} Functionally, the binding of eotaxin on endothelial cells induces proliferation and migration.⁵¹ Taken together, these data suggest that inhibition of new vessel formation in the airways involves inhibition of eosinophil recruitment via inhibition of their chemoattractants, which also hamper endothelial cell proliferation and migration. It is enticing to suggest that the inhibitory effect of *Sema3E* on angiogenesis could be mediated by directly interfering with endothelial cell functions^{11,52} or indirectly by suppressing inflammation. Along the same line, we recently showed that the effect of *Sema3E* on reducing lung inflammation is mediated by decreasing lung dendritic cell accumulation.²⁹ Lung dendritic cell maturation has been shown to depend on VEGF effect,^{53–55} suggesting potential cross-talk between inflammation and angiogenesis.

VEGF is the dominant pro-angiogenic factor that is produced by ASM cells and epithelial cells in the asthmatic airway and acts as a strong mitogen for endothelial cells and promotes the angiogenesis process.^{56,57} Furthermore, *Sema3E* inhibits the proliferation of airway smooth muscle cells.¹⁰ As such, it is possible to envisage that the reduced level of VEGFs in the lung of HDM challenge mice pretreated with *Sema3E* is related to some extent to the reduced number of ASM cells, considered as one of the main sources of pro-angiogenic factors including VEGF.^{56,57}

We also found that not only *sema3E*, but also the level of VEGFR2 expression on endothelial cells, affects new vessel formation in the airways through reduction of VEGF protein expression. VEGFR2 is known as the most important mediator of the angiogenic effect of VEGF.⁵⁸ Therefore, as a result of decreased surface expression of VEGFR2 on endothelial cells and its ligand, VEGF, the activation of angiogenic signaling pathways might be limited, which may account for reduced blood vessel formation, which is in accordance with the *Sema3E* regulatory role on angiogenesis in cancerous conditions.^{21,37,59} Furthermore, previous studies have shown that *Sema3E* mediates the disruption of the blood vessel network via suppression of the delta-like 4/Notch signaling pathway and loss of the control of cell fate decisions between tip and stalk cells.^{20,52} Whether the same

mechanism is operating in airway endothelial cells needs to be investigated.

sVEGFR1 is a specific variant of the VEGFR1 receptor and has an inhibitory role on angiogenesis by acting as a VEGF decoy receptor.⁶⁰ In developmental studies using zebra fish as a model, Sema3E induced sVEGFR1 expression after binding to plexinD1.²⁰ These data are in agreement with our current study, in which pretreatment with Sema3E stimulated the production of sVEGFR1 in the airways of HDM-challenged mice. Conceptually, the sVEGFR1 produced by binding to VEGF inhibits VEGF/VEGFR2 interaction and consequently the signaling pathway, which may result in blocking the main angiogenic pathway and disruption of the blood vessel network. Moreover, analyzing the ratio of VEGF to sVEGFR1 in the airways of different groups of animals confirmed that Sema3E pretreatment has a significant role in inducing sVEGFR1.

This study provides the first evidence regarding the role of Sema3E in influencing angiogenesis as an important feature of asthmatic airway remodeling. Sema3E have the ability to inhibit new blood vessel formation in allergic asthmatic airways by affecting VEGF/VEGFR2 and sVEGFR1 production, and vWF and CD31 expression, the latter considered surrogate markers of new endothelial cell formation.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpathol.2019.01.008>.

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