THE EFFECT OF SEMAPHORIN 3E ON ANGIOGENESIS IN MURINE MODEL OF ALLERGIC ASTHMA

By

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ABSTRACT

Increased angiogenesis is an important characteristic of remodeling in asthmatic airways which stems from the imbalance between pro-angiogenic and anti-angiogenic factors. Surprisingly, the factors regulating this process in allergic asthma are poorly defined. The focus of this thesis is to investigate the effect of Semaphorin 3E (Sema3E) on angiogenesis events within the airways of murine model of allergic asthma.

The role of Sema3E in asthma angiogenesis was tested in wild type and *Sema3e*$^{-/-}$ mice exposed to House Dust Mite (HDM) and monitored for changes in blood vessels number in the lungs. In addition, the potential of Sema3E, in reversing features of allergen inflammation, was tested in mouse model. In both cases immunohistochemistry and immunofluorescence staining of lung tissues used to assess the changes in the level of angiogenesis. Moreover, the expression of pro- and anti-angiogenic factors in total lung homogenate was assessed by ELISA and Real-Time PCR.

The results showed that WT and *Sema3e*$^{-/-}$ mice both developed the HDM induced allergic asthma phenotype, but, the lung sections of HDM exposed *Sema3e*$^{-/-}$ mice had enhanced number of blood vessels compared to WT mice. The enhanced angiogenesis in *Sema3*$^{-/-}$ mice was coupled with increased level of angiogenesis driving factors VEGF and it receptor VEGFR-2. However, in WT mice the level of soluble VEGFR-1 secretion increased significantly which inhibited VEGF / VEGFR-2 binding.

Besides, Sema3E treatment reduced the level of angiogenesis and inhibited HDM-induced secretion of VEGF and the expression of its receptor VEGFR-2 while increased the level of soluble VEGFR-1. Analyzing the ratio of VEGF / soluble VEGFR-1 revealed that in the presence of Sema3E in both models, soluble VEGFR-1 is the dominant factor which has an inhibitory role on angiogenic effect of VEGF.

Taken together, this study provided the first evidence that Sema3E can modulate angiogenesis in allergic asthmatic airways.

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DEDICATIONS

This dissertation is dedicated to:

The memory of my father (Farhad)! I miss him every day but I am glad to know I am progressing in a way he always dreamed about.

My mom (Fahimeh) and brother (Aydin), who were with me all the way and supported me in every way possible during the hardest phase of our life. I would not have been able to achieve so much without your incredible support and sacrifice.

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LIST OF ABBREVIATIONS

AHR: Airway Hyper Responsiveness

ANG: Angiopoietin

ANOVA: Analysis of variance

ASM: Airway Smooth Muscle

ASMC: Airway Smooth Muscle Cells

BALF: Broncho-Alveolar Lavage Fluid

BCA: bicinchoninic Acid

BCL-2: B-cell lymphoma 2

bFGF: basic Fibroblast Growth Factor

BSA: Bovine serum albumin

CACS: Centre of Animal Care

CCL: chemokine ligand

CD: Cluster of Differentiation

Cdc42: Cell division control protein 42

cDNA: Complementary Deoxyribonucleic acid

CDR: Cysteine Rich Domains

CXCL: Chemokine Ligand

CXCR: Chemokine Receptor

DAPI: 4',6-Diamidino-2-Phenylindole

ddH2O: Double-distilled water

DIVAA: Directed in vivo Angiogenesis Assay

Dll4: Delta-Like 4

ECM: Extracellular Matrix

ECP: Eosinophil Cationic Protein

ECs: Endothelial Cells

EGF: Epidermal Growth Factor

EGFL: Epidermal Growth Factor Like Protein

EGFR: Epidermal Growth Factor Receptor

ELISA: Enzyme-Linked Immunosorbent Assay

eNOS: Endothelial Nitric Oxide Synthase

ERK: Extracellular Signal Regulated Kinases

FA: Focal Adhesion

FAK: Focal Adhesion Kinase

Fc: Fraction crystallizable

FGF: Fibroblast Growth Factor

GAP: GTPase Activating Domain

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GDP: Guanosine Diphosphate

GEF: Guanine Nucleotide Exchange Factors

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

GPCR: G-protein-Coupled Receptors

GPI: Glycophosphatidylinositol

GTP: Guanosine Triphosphate

HDM: House Dust Mite

HIF: Hypoxia Inducible Factor

HRP: Horseradish peroxidase

H & E: Hematoxylin and Eosin

H2O2: Hydrogen peroxide

Ig: Immunoglobulin

IGF-1: Insulin-like Growth Factor 1

IL: Interleukin

i.p: Intraperitoneal

IPT: Ig-like Plexins and Transcription Factors

JAK / STAT: Janus Kinase / Signal Transducer and Activator of Transcription

KO: Knockout

mAb: Monoclonal Antibody

MAPK: Mitogen-Activated Protein Kinases

MBP: Major Basic Protein

MCh: Methacholine

MCP-1: Monocyte Chemoattractant Protein-1

MMP: Matrix Metallopeptidase

NK cell: Natural Killer cell

NRP: Neuropilin

OD: Optical Density

PBS: Phosphate Buffered Saline

PDGF: Platelet Derived Growth Factor

PDGFR: Platelet Derived Growth Factor Receptor

PECAM-1: Platelet Endothelial Cell Adhesion Molecule-1

PH Domain: Pleckstrin Homology Domain

PGE2: Prostaglandin E2

PIGF: Placenta Growth Factor

PI3K: Phosphoinositide 3-Kinase

PI (4,5) P2: Phosphatidylinositol 4,5-Bisphosphate

PMSF: Phenylmethylsulfonyl Fluoride

PSI: Plexin Semaphorin Integrin

Real-Time PCR: A real-time polymerase chain reaction

RPMI: Roswell Park Memorial Institute medium

RQ: Relative quantitative

RTK: Receptor Tyrosine Kinase

Sema: Semaphorin

sFLT-1: Soluble FLT-1

TBS: Tris-buffered saline

TGFβ1: Transforming Growth Factor beta 1

Th cells: T helper cell

TIM: T-cell Immunoglobulin and Mucin Domain

TIMP: Tissue Inhibitors of Metalloproteinases

TNF-α: Tumor Necrosis Factor-α

VE-Cadherin: Vascular Endothelial-Cadherin

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

VSMCs: Vascular Smooth Muscle Cells

vWF: Von Willebrand factor

WT: Wild Type

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1. INTRODUCTION

1.1 Chapter 1: Asthma

Asthma is one of the most common chronic diseases affecting 300 million people of all ages with 250,000 annual deaths [1]. Several epidemiological studies have shown that the prevalence of asthma is higher in high income countries in comparison to low and middle income countries. It has been suggested that this increase is related to many factors including the hygiene hypothesis paradigm [2, 3].

The clinical symptoms of asthma are repeated episodes of wheezing, shortness of breath, chest tightness, and nighttime or early morning coughing [4, 5]. From the pathological point of view, asthma is defined as a chronic inflammatory disease of the airways which is characterized by airflow obstruction, airway inflammation, airway hyperresponsiveness and tissue remodeling [6-9].

1.1.1 Pathogenesis of asthma

The key pathophysiological changes in asthma include airway inflammation, airway remodeling and airway hyperresponsiveness. Inflammatory as well as structural cells play a concerted role in these events.

Airway inflammation

Many inflammatory cells infiltrate the asthmatic airways, particularly eosinophils, neutrophils, basophils, macrophages, mast cells, T lymphocytes and dendritic cells [10]. The role of these cells in the progression of inflammation in asthma is explained further:

T cells: It has been indicated that T cells, particularly T helper type 2 (Th2) cells have a major role in allergic asthma by producing high levels of Th2 cytokines including IL-4, IL-5, IL-13 [11]. In addition, overexpression of GATA3, a key transcription factor of Th2, in asthmatic airways is another evidence which shows that Th2 cells play a significant role in asthma pathogenesis [12]. The increased level of Th2 cytokines in the asthmatic patient's lung leads to elevated levels of airway inflammation by affecting IgE overproduction (by IL-4), recruiting eosinophils (by IL-5 and IL-13) and mast cells to the site of inflammation.

Eosinophils: Eosinophils are the major inflammatory cells which contribute to the exacerbation of inflammation and tissue damage in asthmatic airways [13, 14]. The maturation, activation, survival and recruitment of these cells in the airway wall have been recognized as the crucial pathological event that develops asthma phenotype. In this condition they are able to produce leukotrienes, inflammatory enzymes and cytokines including eosinophil cationic protein (ECP), eosinophil peroxidase, major basic protein (MBP) and eosinophil-derived neurotoxin which enhance airway inflammation [15-17].

Eosinophilic asthma mainly arises from Th2 lymphocytes and its cytokines including IL-5, IL-4, and IL-13. In addition, innate immune responses including activation of dendritic cells, bronchial epithelial cells and innate lymphoid cells play a crucial role in inducing eosinophilic asthma [18, 19].

Eosinophil infiltration into the airways leads to mild and moderate asthma. However, severe disease is the result of eosinophils and neutrophils activation.

Neutrophils: According to previous studies, neutrophil recruitment and activation in the airways has a crucial role in asthma inflammation [20-22]. In fact, the first leucocytes that enter to the site of inflammation are neutrophils. They act as a destructive element by releasing proteases and inflammatory mediators including MMP-9, neutrophil elastase, myeloperoxidase and reactive oxygen species which ultimately cause injury to the surrounding tissue [23]. Neutrophils recruit to the inflammatory zone as a result of IL-17 production from activated Th17 cells [24]. In addition, IL-8 serves as a most potent neutrophil chemoattractant and has a significant role in recruitment of these cells. Pro-inflammatory cytokines and tumour necrosis factor-alpha (TNF-α) intensify IL-8 production and subsequently neutrophil recruitment to the inflammatory site. IL-8 also can be produced by neutrophils in response to inflammatory mediators which in turn recruit more neutrophils to the site of inflammation. The ultimate result of all these phenomena is enhancement of inflammation level in the asthmatic airways [23, 25].

Mast cells: Mast cells have a pivotal importance in inflammatory processes of allergic asthma. Following activation of mast cells by IgE receptor cross linking, they produce several cytokines, chemokines and growth factors including TNF-a, IL-4, and IL-13, histamine, tryptase and chymase, which results in inflammatory responses. Among them, TNF-α is recognized as the most important cytokine which causes inflammation in asthmatic airways by the activation of adhesion molecules on endothelial cells and the subsequent transmigration of inflammatory leucocytes. In addition to cytokine production, they have a significant role in activating lymphocytes, eosinophils, neutrophils and monocytes/macrophages. Through this way, mast cells contribute to airway inflammation and remodeling [21, 26].

Natural killer (NK) cells: NK cells are another effector inflammatory cell in the lung of asthmatic patients. In the asthmatic condition, the number and activation level of NK cells increase dramatically which leads to production of large amounts of Th1 (IFNγ) and Th2 cytokines [27]. It results in increased levels of inflammation by recruitment of dendritic cells, T cells and eosinophils which ultimately develops and maintains allergic airway disease [27-29].

Macrophages and dendritic cells: Dendritic cells act as key antigen-presenting cells to stimulate Th2 cell production. Moreover, macrophages which are recognized as the most frequent cells in the airways, release inflammatory mediators and cytokines after getting activated in the presence of allergens through low-affinity IgE receptors [21].

With reference to the above mentioned subject, it can be concluded that the airway inflammation play a dominant role in the pathophysiology of asthma. In fact, airway inflammation is an important element that can trigger and enhance the other pathological aspects of allergic asthma including airway remodeling and airway hyperresponsiveness.

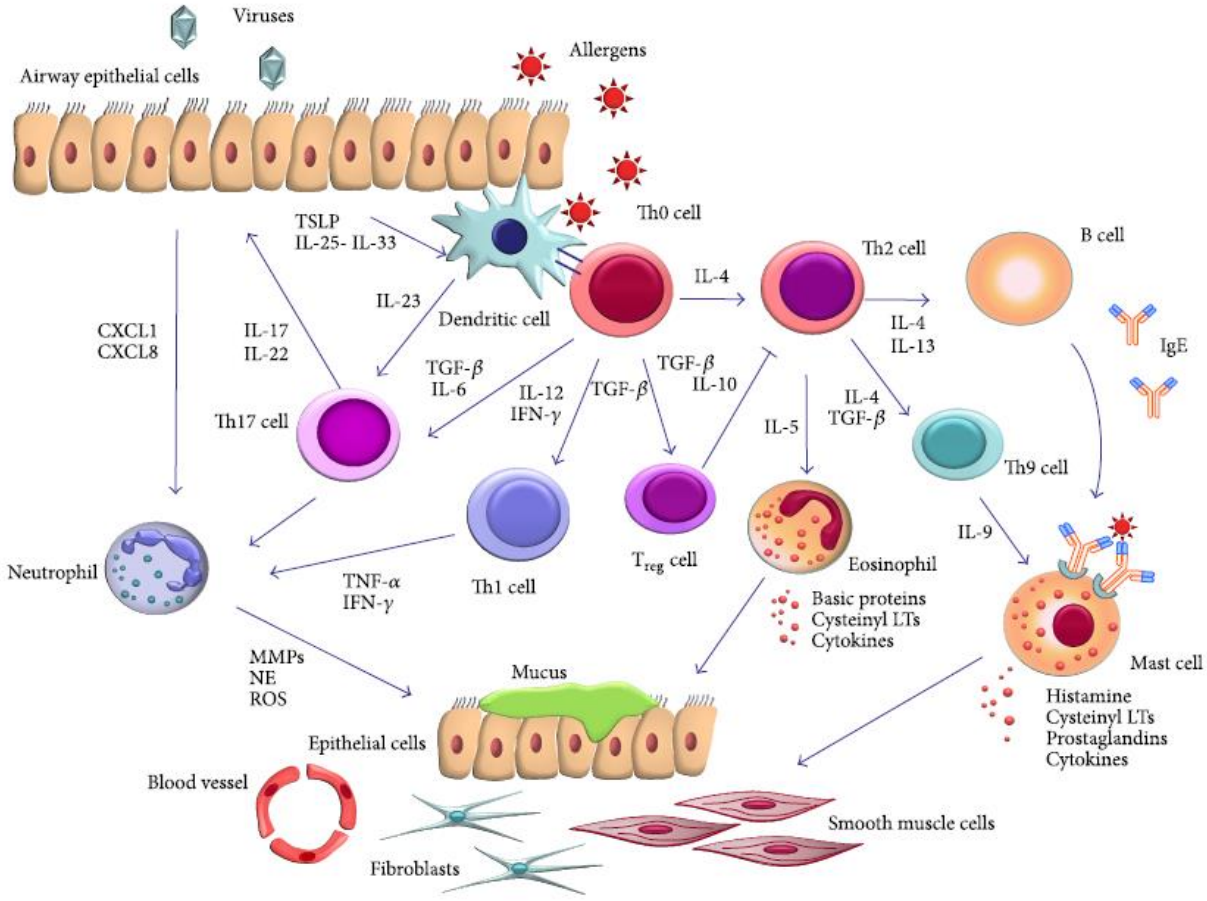


Figure 1.1. Pathobiology of airway inflammation in asthma. Asthma originates from complex interactions between genetic factors and environmental agents such as aeroallergens and respiratory viruses. In particular, within the airway lumen allergens can be captured by dendritic cells, which process antigenic molecules and present them to naïve (Th0) T helper cells. The consequent activation of allergen-specific

Th2 cells is responsible for the production of IL-4 and IL-13 that promote B-cell operated synthesis of IgE antibodies; moreover, Th2 cells also release IL-5, which induces eosinophil maturation and survival. These events are noticeably favoured by a functional defect of IL-10/TGF-β producing T regulatory (Treg) cells that normally exert an immunosuppressive action on Th2 cell-mediated responses. In addition to Th2 cells, IL-9 releasing Th9 cells can also undergo activation, thus leading to the growth and recruitment of mast cells, which upon IgE-dependent degranulation release both preformed and newly synthesized mediators. Other important T lymphocytes contributing to asthma pathobiology are Th17 cells, producing IL17A and IL-17F which cause neutrophil recruitment and expansion. Furthermore, IL-12-dependent and IFN-γ releasing Th1 cells can be activated, especially as a result of airway infections sustained by respiratory viruses. Finally, many mediators, cytokines, and growth factors produced by several different cells involved in chronic asthmatic inflammation may also affect the functions and proliferation rates of airway structural cellular elements including epithelial cells, fibroblasts, smooth muscle cells, and endothelial vascular cells.

Airway remodeling: Airway remodeling is defined as the structural changes of the airway wall which affect the composition and organization of cellular and molecular components of the airway [30, 31]. These changes are a consequence of repeated injury and repair processes in response to inflammation and persistent airflow limitation [30, 32]. Airway structural changes include thickness of the airway wall which is a result of epithelial changes, subepithelial fibrosis, smooth muscle hypertrophy and hyperplasia, increased fibroblast and myofibroblast, mucus metaplasia or overproduction of mucus by goblet cells, collagen deposition in basement membrane zone and neovascularization [33, 34]. These changes lead to airway hyperractivity (AHR) and cause the loss of lung function over time [32, 35]. In addition, chemokines (e.g. CCL3, CCL11, MMP9), cytokines (e.g. IL-1, IL-4, IL-5, IL-9, IL-13, IL-17, IL-25) and growth factors (e.g. TGFβ1, EGF, FGF, VEGF), are released by structural and inflammatory cells, which strengthen these structural changes [32, 36].

Airway hyperresponssiveness (AHR): One of the fundamental features of asthma is Airway hyperresponsiveness, which is defined as an increased sensitivity of the airways to different stimulants. AHR is mostly related to abnormalities of airway smooth muscles (ASM), airway inflammation and airway remodeling, and it can lead to bronchoconstriction [37, 38]. In fact, in asthmatic conditions, smooth muscles which surround the airways become sensitive to stimuli, and as a result smooth muscle contraction will occur after exposure to stimuli including allergens or

irritants. In this condition, because of narrowing the airways, the air flow is blocked and results in bronchoconstriction and AHR [10, 39].

1.1.2 Pathological aspects of airway remodeling

1.1.2.1 Epithelial changes

Superficial airway epithelium acts as a defensive barrier and protects the body against environmental stress. Airway epithelial damage and dysregulated repair processes (epithelial cell proliferation and cell death at a higher rate) play a significant role in various respiratory diseases, including asthma. In fact, dysregulated epithelium, which is in a key position to coordinate responses, delivers the signals that affect the underlying mesenchyme and result in epithelial–immune cell interaction. This process intensifies the inflammatory and remodeling responses by production and secretion of various cytokines and growth factors, including PGE2, IL-8, MCP-1, RANTES, eotaxin, TNF-α, IL-1β, IL-6, oncostatin-M, IL-11, IL- 10, IL-16, IL-18, GM-CSF, b-FGF, TGF-β1 and TGF-β2, IGF-1, PDGF, and IL-5 and IL-13 as Th2 cytokines [40]. The connection between the epithelium and its underlying mesenchyme causes remodeling responses and is defined as subepithelial fibrosis. It has a significant role in airway wall thickening and subsequently chronic airflow obstruction and airway hyperresponsiveness. This pathology can be considered as one of the leading causes of asthma [37, 41, 42].

1.1.2.2 Increased myofibroblast, fibroblast and collagen deposition in subepithelial zone

Another aspect of remodeling in asthmatic airways is the increased number of myofibroblasts under the bronchial epithelial basement membrane [43]. In fact, in asthmatic condition increased myofibroblasts and fibroblasts produce immunomodulatory cytokines and chemokines in addition to cell surface receptors expression. These elements play a vital role in several processes including cell adhesion, leukocyte activation and subsequently development of airway inflammation [44]. In addition, the increased level of myofibroblasts deposits the high level of collagens I, III and V, fibronectin and tenascin in the subepithelial zone, the juxtaposition of the smooth muscle layer and the lamina reticularis [45]. This pathological alteration is another important cause of increasing the density and thickening of basement membrane which leads to airways narrowing [46, 47].

1.1.2.3 Airway smooth muscle cell (ASM) hypertrophy and hyperplasia:

One of the most prominent alterations of airway remodeling in allergic asthma is increasing the thickness of the airway smooth muscle (ASM) layer. This event occurs as a consequence of airway smooth muscle cells hyperplasia (increase in ASM cell number) and hypertrophy (increase in ASM cell size) and differentiation or migration of mesenchymal cells to ASM bundles [48, 49].

ASM hyperplasia occurs as a result of increased rates of division or decreased rates of apoptosis [50]. It has been reported that the proliferative rate of ASM cells is under the influence of several factors and mediators including cytokines, the components of extracellular matrix (ECM), matrix metalloproteinase-2 (MMP-2), mechanical stress and reactive oxygen species. The elevated level of ASM cells proliferation leads to airway wall thickening, chronic obstruction, and bronchial hyperresponsiveness [34]. In addition, according to previous studies, it has been identified that the increased level of stretch, presence of TGF-β and IL-1β have potential hypertrophic effect on ASM cells [50]. Furthermore, it has been shown in *in vivo* studies that the intrinsic abnormalities of smooth muscle contractility, which leads to hypercontractile phenotype, have a considerable influence on smooth muscle cell hypertrophy, bronchial hyperresponsiveness and airflow limitation [51].

Moreover, airway smooth muscle cells hyperplasia occurs in response to chemical compounds that stimulate cell division and trigger mitosis. These compounds are named mitogens and categorized in 3 main groups [36, 50]:

1. Growth factors including PDGF, EGF, FGF-2 and insulin-like growth factor that activate receptor tyrosine kinase (RTK) activity [50, 52, 53].
2. Contractile agonists including α-thrombin, serotonin, thromboxanes, endothelin-1, leukotriene-D4 that signal through G-protein-coupled receptors (GPCR) [54].
3. Pro-inflammatory cytokines (IL-1β, TNF-α, IL-6), which signal through cytokine receptors [54]. These receptors are non-RTKs such as Src family members,

mitogen-activated protein kinase (MAPK) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling systems [36, 50].

Also, phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) signaling pathways are significantly involved in ASM cells proliferation. In fact, activation of PI3K and ERK pathways results in activation of transcription factors which phosphorylate D-type cyclins and facilitate cell cycle progression [55-58]. The above mentioned events lead to ASM cells hypertrophy and hyperplasia and exacerbation of airway structural changes which results in persistent airway inflammation, bronchial hyperresponsiveness and airflow limitation in allergic asthma.

1.1.2.4 Goblet cell hyperplasia and mucus overproduction

Goblet cells are located in the epithelium of respiratory tract and act toward host defense. They are known as an important source of mucin glycoproteins secretion in the respiratory system. In the asthmatic condition, the number of goblet cells increases significantly due to elevated level of goblet cell hyperplasia or metaplasia which refers to conversion of non-granulated secretory cells to goblet cells. In this condition, mucus overproduction will be initiated due to accumulation of high amount of mucus results in airway obstruction [34, 59]. This aspect of pathology was

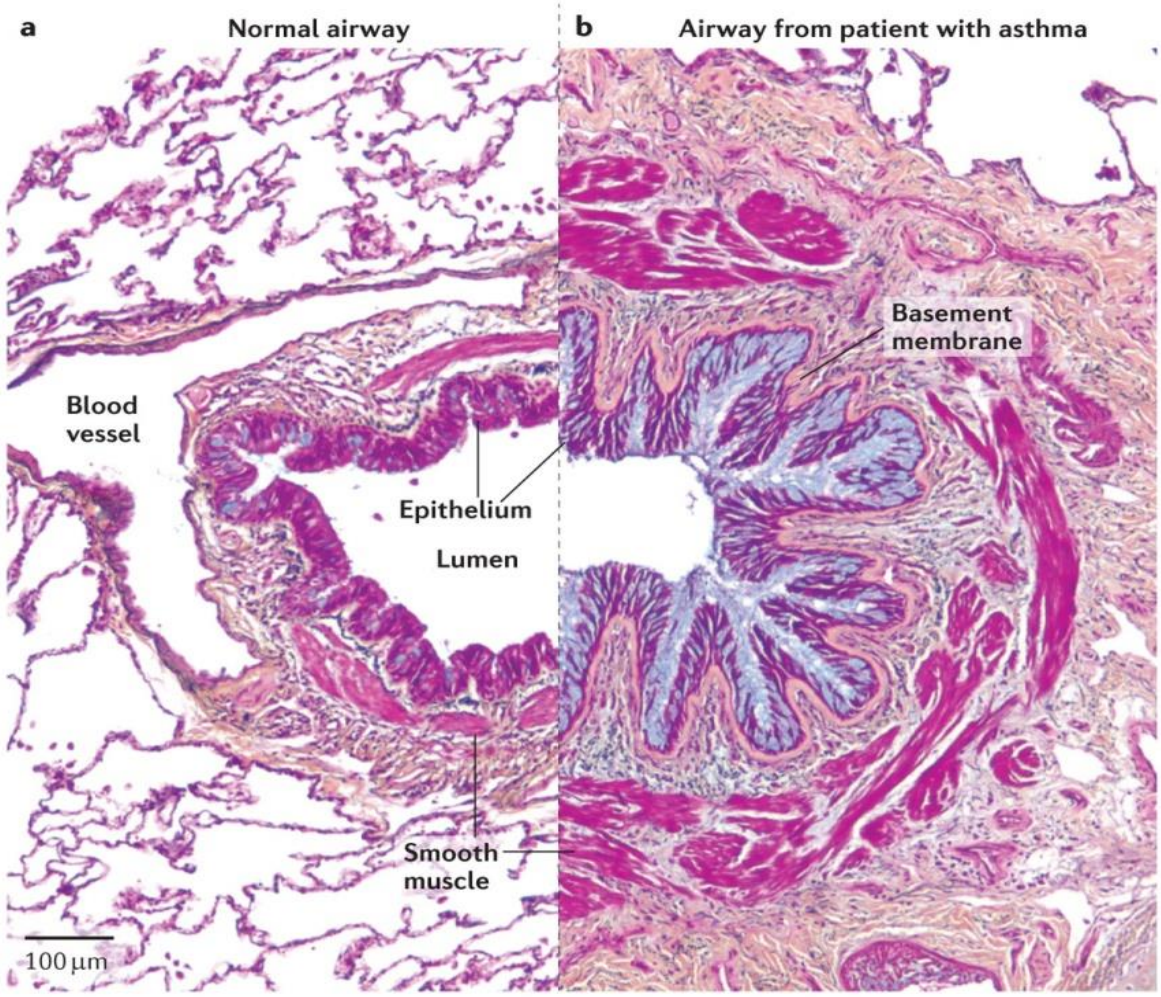
also confirmed by observing the increased level of MUC5AC gene expression in asthmatic subjects compared to healthy individuals [59].

The mechanism of goblet cell hyperplasia is not completely understood, however it has been shown that T helper 2 (Th2) cytokines including IL-4 and IL-13 play a key role in induction of goblet cell hyperplasia, mucin gene upregulation and mucus overproduction [60, 61]. In addition, Epidermal growth factor receptors (EGFR) signaling has a significant role in goblet cell metaplasia [62]. It has been indicated that in the presence of proinflammatory cytokine including TNF-α, the level of EGFR expression will be up-regulated in the epithelium. The stimulation of EGFR by EGF or oxidative stress results in mucus overproduction. Oxidative stress applies its effect via activating p44/42mapk pathway [62-64]. Moreover, activated neutrophils which are recruited by IL-13 to the airways release oxygen-free radicals which cause mucus synthesis via EGFR activation [61, 65]. Therefore, this aspect of airway remodeling also leads to exacerbation of asthma.

1.1.2.5 Vascular remodeling and pathological angiogenesis

Vascular remodeling or angiogenesis is a significant characteristic of allergic asthmatic airways and plays an important role in asthma pathogenesis as one of the major aspect of structural airway wall alteration [30, 66].

Angiogenesis is defined as a multiple complex process in which new vessels form from pre-existing ones [48-50]. This is a vital process in homeostatic condition to provide the oxygen and nutrients; however, increased angiogenesis, which is a consequence of imbalance between pro-angiogenic and anti-angiogenic molecules, occurs in many chronic inflammatory diseases including asthma [51]. Therefore, this phenomenon deserves a full consideration from every conceivable angle.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Lumen
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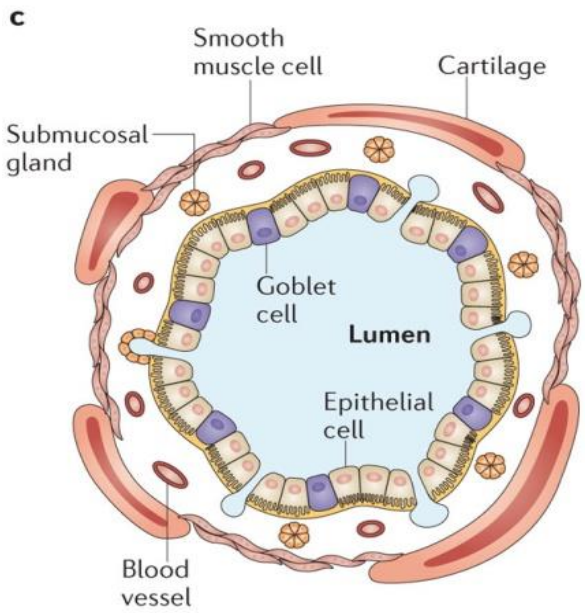
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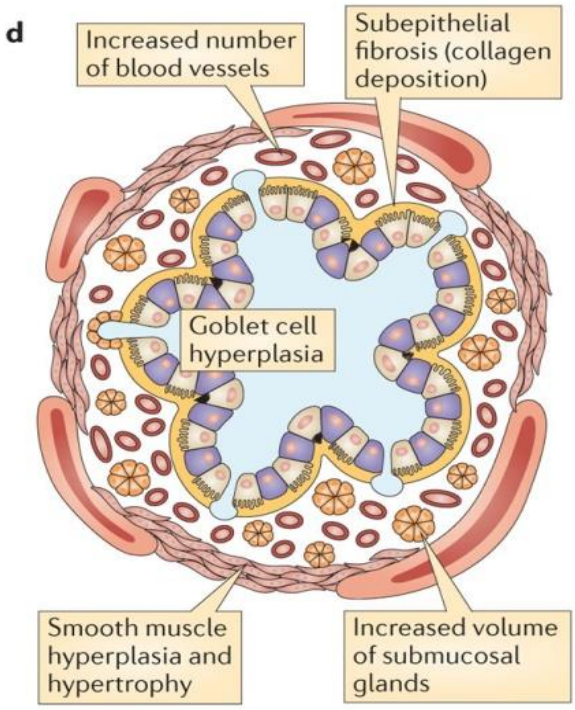

d
Increased number of blood vessels
Subepithelial fibrosis (collagen deposition)
Goblet cell hyperplasia
Smooth muscle hyperplasia and hypertrophy
Increased volume of submucosal glands

Figure 1.2. Airway pathology in asthma. Airway structures in medium-sized healthy airways (part a; a schematic representation is depicted in part c) and in a patient with asthma (part b; a schematic representation is depicted in part d). The airways in asthma show considerable structural remodeling, including goblet cell hyperplasia, subepithelial fibrosis, increases in smooth muscle volume and blood vessels.

1.2 Chapter 2: Angiogenesis in Asthma

For the first time in 1960, Dunnill and colleague showed that the abnormalities and changes in vascular network happens in the airways of asthmatic patients [67]. Also, Leonardo da Vinci was the first to report the pathological angiogenesis in the lung [68]. According to current studies in both conditions, *ex vivo* and *in vivo*, it is proved that the level of formation of new blood vessels increases significantly in the sub-epithelial zone particularly in the mucosa of the lung of asthmatic patients. [69-71]. Moreover, it has been reported that the elevated level of blood flow and vascular permeability in the lung is a consequence of abnormal bronchial vascular alteration in the asthmatic airways which results in airway inflammation, airflow limitation, bronchial hyperresponsiveness and ASMCs remodeling [72-74]. These pathological events which are known as vascular remodeling will be persistent in asthmatic airways, by altering airway's components and intensifying the asthma pathogenesis [75].

1.2.1 Steps of angiogenesis in asthma

As mentioned earlier, angiogenesis is a complicated multistep process which is preceded by involvement of several factors [66]. Blood vessels are forming by closely assembled endothelial cells (ECs) in the inner lining of vessels and their role is providing oxygen and nutrients for organs [76]. In healthy condition, there is a

balance between signaling of proangiogenic and angiostatic growth factors which leads to maintenance of ECs in a quiescent phase [77, 78]. Moreover, in this condition, blood vessels adjust the blood flow and provide the tissue with adequate amounts of oxygen using oxygen and hypoxia-induced receptors or sensors [79]. However, in hypoxic or inflammatory condition, the level of pro-angiogenic growth factors and cytokines production will be up-regulated which promotes the growth of new blood vessels from pre-existing ones.

Angiogenesis process is divided into two phases: I) activation (sprouting) phase and II) resolution phase.

Activation phase has several steps including vessel wall's endothelial cells disassembly and ECs activation, degradation of basement membrane as a result of matrix metalloproteinase (ECM) activity, ECs migration and ECM invasion, ECs proliferation and ultimately formation of vessel's lumen.

Resolution phase is in the opposite way of activation phase and encompasses the following steps: Maturation of complexes at the junction between endothelial cells, re-formation of basement membrane and ECM, inhibition of ECs proliferation and migration, differentiation and recruitment of vessel's smooth muscle cells and pericytes to make the mature blood vessels lumen. Ultimately, angiogenesis process will be completed by establishment of blood flow in the newly formed blood vessels [80, 81].

The initial step of angiogenesis process in pathological condition is activation of endothelial cells which occurs according to the following description:

Vessel-destabilizing molecule, angiopoietin-2 (ANG-2), detaches the pericytes from endothelial cells and simplifies the activities of other pro-angiogenic cytokines [82-87]. Afterwards, under the effect of various pro-angiogenic factors, including Vascular Endothelial Growth Factor (VEGF), basic Fibroblast Growth Factor (b-FGF), IL-8 and activation of hypoxia-inducible factors in response to low level of oxygen, endothelial cells will be activated [79, 83, 88]. Activated endothelial cells then detach from each other by losing integrin and adhesion molecules particularly Vascular Endothelial cadherin (VE-cadherin) [89]. VE-cadherin is the most important and crucial molecule at the junctions between ECs which keeps endothelial cells connected to each other [90]. This molecule specifically expresses on ECs and contributes in transducing the signals from extra cellular matrix (ECM) to the cells by recruiting molecular complexes of cytoskeleton and signaling molecules at focal adhesion sites. However, the function of this molecule is impaired in activated ECs [91].

The second step of sprouting angiogenesis as an invasive process is production of matrix metalloproteinases (MMPs) by activated ECs [83]. In fact, the filopodia of endothelial tip cells express some receptors which can sense the gradient of guidance cues in the environment. As a result of activation of Neuropilin-1, VEGF-R2, Jagged-

1 and Notch-1 signaling on ECs tip cells by attractant cues, they initiate releasing the MMPs [91].

Matrix metalloproteinases (MMPs) are a family of proteases including collagens, laminins and elastin that can cleave peptide bonds between the proteins of ECM and eventually degrade the endothelial basement membrane [92]. It is an essential step in elimination of barrier proteins and generation space in ECM for activated endothelial cells to migrate [92-94]. The most important MMPs produced by activated ECs are MMP-1 (interstitial collagenase), MMP-2 (gelatinase A/type IV collagenase), MMP-9 (gelatinase B/type IV collagenase), and MT-1-MMP which can promote the process of angiogenesis [95, 96]. Subsequently, selected and activated tip cells will be polarized which leads to extension of several filopodia to the ECM. These extended filopodia are mostly involved in migratory activities [83]. Following tip cells migration and filopodia extension into the ECM, the proliferation of stalk cells will be initiated which results in vessel tube formation.

Stalk cells are recognized as the following part of ECs tip cells with fewer capabilities in filopodia production and migratory activities compared to tip cells; however, they are more proliferative after stimulation with pro-angiogenic factors. [97-99].

Following completion of activation phase of angiogenesis process, resolution phase will be started.

Resolution phase is a transition stage in which actively growing vasculature turn to a quiescent and completely formed functional blood vessels network [100].

At the first step of this phase, proliferating stalk cells reform the adherent complexes between endothelial cells to establish the junction and preserve their integrity. Consequently, newly formed sprouts join the adjacent sprouts to form a continuous lumen [97, 101]. EGFL7 plays a significant role in this step. This molecule is a secreted angiogenic factor which particularly expresses by proliferating ECs and applies its effect on the same cells. It acts as chemoattractant for endothelial cells and interacts with the ECM's components. The function of this molecule is important in formation of vascular lumens and maintaining the integrity of basement membrane [102]. Following lumen formation, the fusion will occur between adjacent branches after two endothelial tip cells encounter each other. These connections will be established by contribution of VE-cadherin and Angiopoietin-1 (Ang-1) to make continues lumen.

Ang-1 plays a vital role in maintaining the vascular integrity and ECs survival by signaling through Tie-2 receptor. The activation of this signaling pathway results in phosphorylation of phosphatidylinositol 3-kinase and subsequently activation of AKT pathway which leads to ECs survival. The consequence of all these changes is inhibition of vascular leakage, vascular inflammation and ultimately vascular damage [103]. In addition, Ang-1 is significantly involved in strengthening the ECs connection by regulating and localizing the PECAM-1 and VE-cadherin at the

junction between ECs [104] which leads to a reduction in vascular endothelial cell permeability [105, 106].

Beside Ang-1, VE-cadherin has been recognized as a specific and crucial adhesion molecule at the junction between ECs. This molecule has a pivotal role in maintaining ECs contact and vascular integrity as well as other cellular processes including cell proliferation and apoptosis. It is also involved in controlling the functions of vascular endothelial growth factor receptors (VEGFRs) [89].

In addition, in sprout formation and tube stabilization, integrin-ECM interactions play a crucial role [107]. For instance, contribution of β1 integrins, αv integrins, αvβ3 and αvβ5, is appreciated in this process which leads to activation of Src, FAK kinases and Rho GTPases (Cdc42 and Rac1) as downstream signaling pathways of integrin [101, 108, 109]. Moreover, integrins and other adhesion receptors including PECAM-1, E-selection, and VE-cadherin have the ability of making the connection between internal cytoskeleton of cells and ECM in order to transduce the signals between them. Integrins are involved in these processes by engaging the signaling molecules and cytoskeletal molecules at focal adhesion sites. Focal adhesion kinase (FAK) is a Non-receptor tyrosine kinase and plays a considerable role in cell migration and proliferation in angiogenesis. The activation of FAK is mostly dependent on integrin clustering at focal adhesion sites and results in cell migration, proliferation and other crucial processes in angiogenesis [91, 110]. Consequently, all these processes lead to vascular lumen formation and maintenance of their integrity.

Immediately after completion the lumen formation process, ECM starts to form to generate a new basement membrane. Tissue inhibitor of metalloproteinases (TIMP) family plays the main role in re-formation of ECM. They apply their effect by generating the MMPs inhibitory complexes. This family consists of 4 different subgroups including TIMP1, TIMP2, TIMP3, TIMP4 and each of them has a unique way to inhibit angiogenesis and re-establish the ECM. For instance, TIMP-1 blocks the ECs migration toward pro-angiogenic factors; however, TIMP-2 acts through inhibition of the MMP function and also reduction in endothelial tube formation level on the ECM [96]. In addition, TIMP-3, an ECM-bound molecule, blocks vascular endothelial cells chemotaxis toward VEGF and bFGF. Based on these functions, they re-establish the new membrane for newly formed blood vessels.

Furthermore, another prominent feature of resolution phase and blood vessels maturation step is differentiation and recruitment of mural cells including vascular smooth muscle cells (vSMCs) and pericytes to make the mature blood vessels lumen. Pericytes are mostly localized on ECs, capillaries and immature blood vessels, however, vSMCs act on mature and larger vessels. In addition, mural cells are involved in maintaining the vascular integrity by generating Ang-1 which acts as an endothelial-mural cell adhesion molecule to support blood vessels [111, 112]. Moreover, PDGF-B/PDGFRβ interactions play a significant role in pericytes proliferation, migration and localization to the vascular wall [100, 113, 114].

Blood flow establishment is the final step of angiogenesis process. In this condition, the presence of oxygen and nutrients leads to reduction in pro-angiogenic factors. Therefore, endothelial cell oxygen sensors will be inactivated, ECs proliferation and migration will be inhibited by contribution of angiostatin and endostatin [115-117] and ultimately these cells will return to the quiescent state [118-120]. Consequently, all above mentioned complicated processes lead to formation of mature blood vessels network.

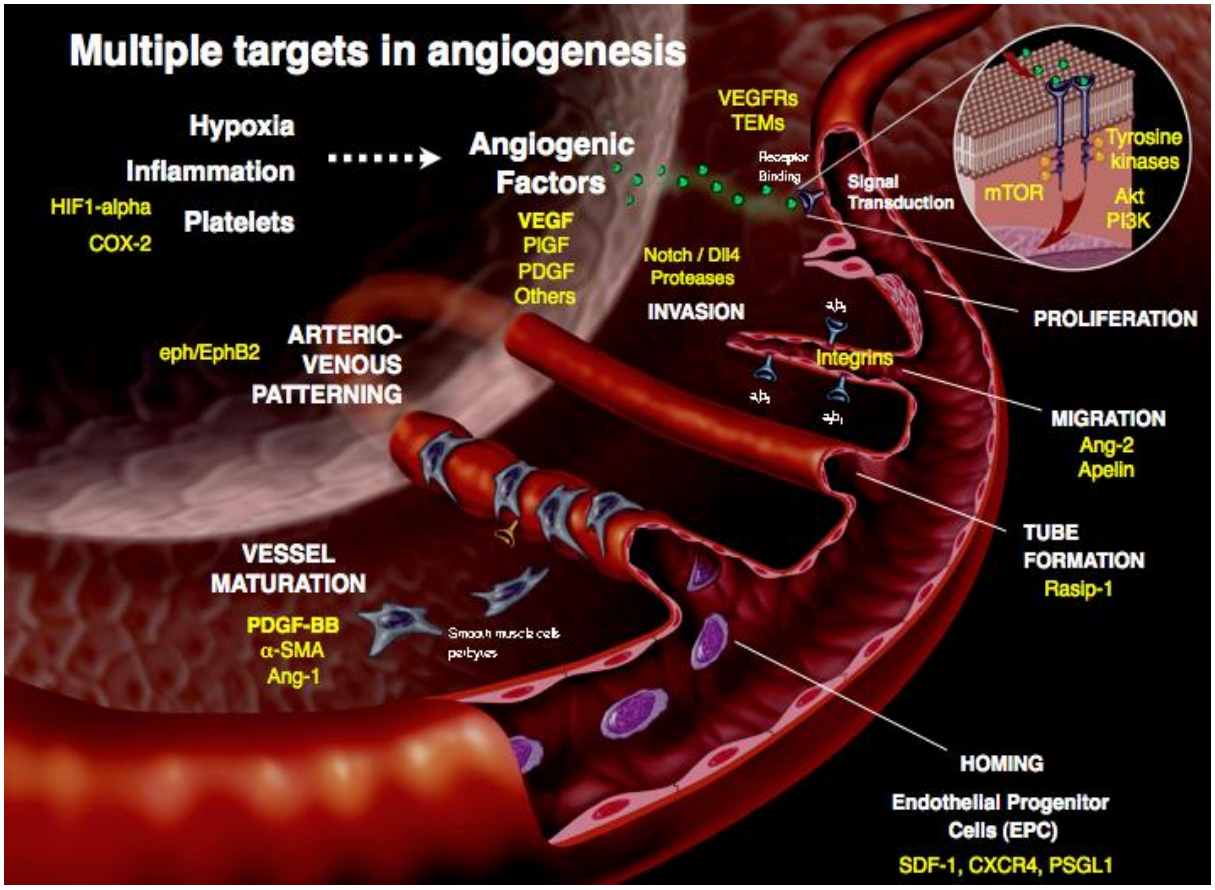


Figure 1.3. The process of angiogenesis. Blood vessels are comprised of endothelial cells ensheathed by pericytes, embedded in the stromal compartment. Hypoxia or other endogenous signals activate ECs and induce the release signaling factors (such as VEGF, Ang-2, FGF and chemokines) to promote the growth of new blood capillaries from pre-existing vessels. Pericytes detach from the vessel (Ang-2 signaling), and endothelial cells are activated and lose their close contact as the vessel dilates (VE-cadherin signaling). In sprout formation, a tip cell is selected (selection influenced by Neuropilin, VEGF/VEGFR and NOTCH / DLL4 and JAGGED1 signaling) which releases matrix metalloproteases (MT1-MMP) to degrade the basement membrane and remodel the extracellular matrix. Tip cells are polarized and extend numerous filopodia to guide sprout migration (via semaphorins, ephrins, and integrins guidance signals) toward angiogenic stimuli (VEGF gradient). Tip cells are primarily migratory and do

not proliferate. Stalk cells follow the tip cell and proliferate, extending the sprout. Proliferating stalk cells establish junctions with neighboring endothelial cells and release molecules such as EGFL7 that bind to extracellular membrane components and regulate vascular lumen formation. Fusion of neighboring branches occurs when 2 tip cells encounter each other, establish EC-EC junctions (VE-cadherin, Ang-1) and form a continuous lumen. Extracellular matrix is deposited to establish a new basement membrane (TIMPs), endothelial cell proliferation ceases, and pericytes are recruited to stabilize the new vessel (PDGFR/PDGF-B, Ang-1). Once blood flow is established, the perfusion of oxygen and nutrient reduces angiogenic stimuli (VEGF expression) and inactivates endothelial cell oxygen sensors, re-establishing the quiescent state of the blood vessel.

1.2.2 Pro-angiogenic factors and molecules in asthma

1.2.2.1 The role of hypoxia in angiogenesis in asthma

Numerous pro-angiogenic factors are involved in progression of pathological angiogenesis in allergic asthma [66]. The most important initiator of vascular formation in asthmatic airways is unavailability of sufficient oxygen as a result of airflow limitation, airway inflammation, airway wall thickening due to increase in ASM cell mass and sub-mucosa fibrosis [121, 122]. In fact these structural and cellular alterations in the lung lead to severe hypoxia because the increased components of airway wall require more oxygen; however, the oxygen supplier system is not enough to provide the sufficient amount of nutrients and oxygen. Therefore, as a result of regulatory responses, more number of blood vessels will be generated to compensate the shortage of oxygen and nutrient in the lung [123].

Activation of hypoxia inducible factor (HIF) plays a key role in hypoxic condition. HIF is a transcription factor which is consists of 2 subunits: HIF1-α and HIF1-β. In normoxia (normal oxygen condition) HIF1-α is hydroxylated and subsequently degraded in proteasome. However, in hypoxic condition, hydroxylation of HIF1-α is impaired which leads to elevation of HIF1-α level. It results in translocation of HIF1-α to the nucleus, dimerization with HIF1-β and binding to hypoxia response element in the promoter of target genes. The consequence of these processes is activation of genes involved in hypoxia.

Hypoxia has a significant role in exacerbation of asthma pathogenesis as well as angiogenesis through several ways. For instance, it has been shown that HIF transcription factors function is associated with airway inflammation [124]. Moreover, they play a significant role in every step of angiogenesis process by contributing in ECM degradation (by upregulation of MMP-2 expression), formation of vascular tubes, generation of basement membrane and vessels maturation (by participating in formation of ECM, including fibronectin). Additionally, HIF transcription factors are clearly involved in genes expression of pro-angiogenic growth factors, cytokines and chemokines including VEGF, Angiopoietin-1/2, FGF, TGF-β, IGF, MMPs, IL-1, IL-6, IL-8, CXCL3 and CXCR2 in the airways [79, 125, 126].

1.2.2.2 Vascular Endothelial Growth Factor (VEGF) and its receptors

Hypoxia has a considerable effect on regulating Vascular Endothelial Growth Factor (VEGF) expression, as it is a transcriptional target of HIF1-α [79]. VEGF, which is a highly specific angiogenic factor is recognized as a master regulator of blood vessels growth in asthmatic airways [127]. This growth factor acts as a specific mitogen for endothelial cells and causes detachment of endothelial cells from mother vessels and migration to the adjacent stroma to generate the new branches [128].

VEGF family consists of 7 different members, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PIGF. Among them, VEGF-A which denotes as VEGF, is recognized as the most potent angiogenic factor [129-131]. Alternative splicing of VEGF gene results in production of 5 molecular variant such as VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189} and VEGF-A_{206} and between them, VEGF-A_{165} is considered as the most dominant isoform [130].

VEGF family applies its effect via signaling through related high affinity tyrosine kinase receptors. There are 3 groups of VEGF receptors including VEGFR-1 (FLT-1), VEGFR-2 (KDR / FLK-1), and VEGFR-3 (FLT-4). In addition to these main receptors, VEGFs can also interact with Neuropilin-1 and Neuropilin-2 as their co-receptors [118, 132, 133]. VEGFR-1 and VEGFR-2 are mostly expressed on endothelial cells, epithelial cells, ASMCs, and some inflammatory cells including macrophages and T cells. However, VEGFR-3 is dominantly found on lymphatic epithelium [134]. Similar to VEGF, the expression of VEGFR-1 and VEGFR-2 are upregulated in the hypoxic condition [135, 136].

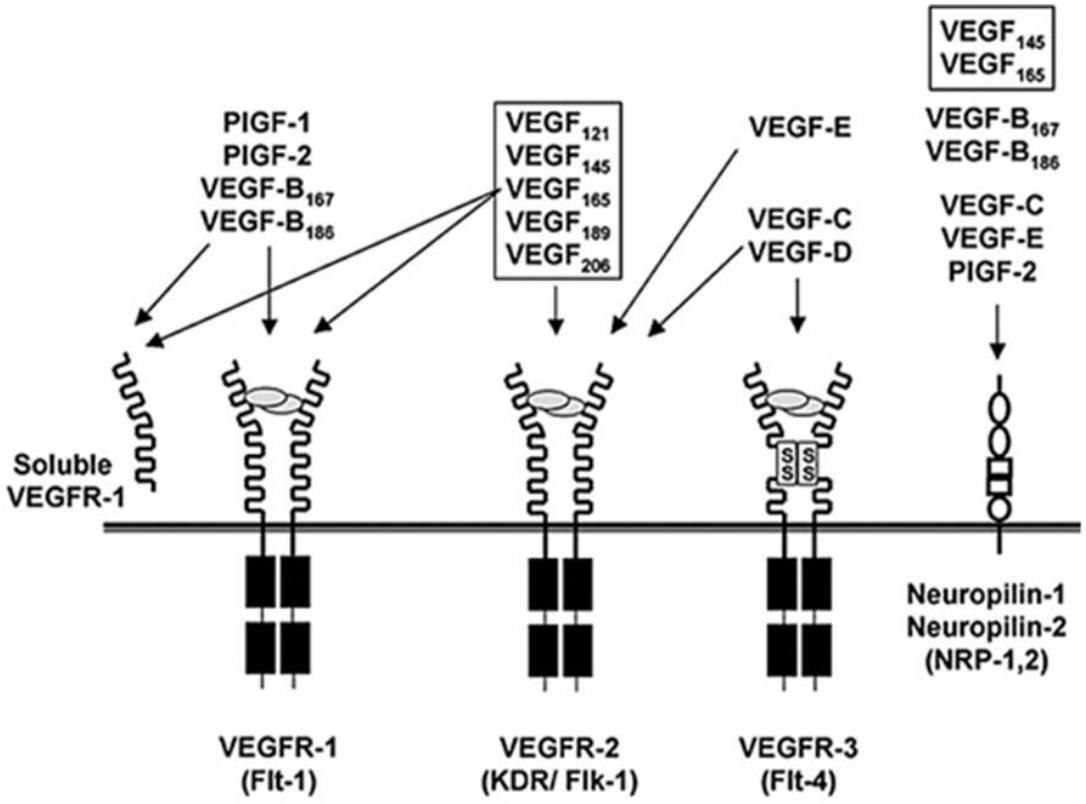


Figure 1.4. Interactions of VEGF family members with their receptor tyrosine kinases. VEGF tyrosine kinase receptors are related to the platelet-derived growth factor (PDGF) subfamily of receptor protein tyrosine kinases. VEGF shows high affinity binding to both VEGFR-2 and VEGFR-1 receptors. Placenta growth factor (PlGF) and VEGF-B bind with high-affinity binding to VEGFR-1 only. VEGF-C and -D are VEGF-related factors that bind to a related receptor VEGFR-2 and VEGFR-3. Neuropilin-1 and Neuropilin-2 are also novel non-RTK receptors and in some cases they perform as a coreceptor for VEGF family members.

As mentioned earlier, VEGF (VEGF-A) is a potent multifunctional growth factor with several angiogenic properties [137]. It applies its angiogenic effect by signaling through VEGFR-2. In fact VEGFR-2 is the most important mediator of angiogenic and mitogenic effect of VEGF [130, 133]. Binding of VEGF to VEGFR-2 on ECs activates several signaling pathways including Akt, FAK, p38MAPK, eNOS, Src and PI3K. Each of these pathways in turn, plays a significant role in ECs proliferation, migration and differentiation [129]. VEGF enhances angiogenesis by being involved in every step of this process. For instance, this growth factor can degrade ECM and basement membrane of the vessels by stimulating ECs toward MMP and other proteases production. This process facilitates ECs migration through surrounding tissue [129]. It also acts as a survival factor for ECs via preventing apoptosis through activating PI3-kinase / AKT pathway and expressing Bcl-2 protein in endothelial cells [138]. Moreover, VEGF / VEGFR-2 signaling has a significant role in filopodia formation and extension by activating Cdc42 on EC tip cells which results in vessels enlargement and branching [139-141]. From the above mentioned mechanisms, it can be concluded that VEGF and VEGFR-2 activation and function is the most important factor in angiogenesis process.

VEGF also affect angiogenesis indirectly by changing the environmental condition. To explain more, we can point to its ability in recruiting inflammatory cells including eosinophils, mast cells, macrophages, T cells and B cells and also promoting monocyte chemotaxis [142-145]. These cells produce pro-inflammatory cytokines

which in turn, can generate a pro-angiogenic condition by inducing VEGF, VEGFR-2 and other pro-angiogenic factors expression. In addition, VEGF can induce vascular hyperpermeability which results in extravasation of plasma proteins and inflammatory cells to extravascular area and ultimately elevation of inflammation level [146]. Endothelial cell-derived nitric oxide is another effect of VEGF on ECs which leads to vasodilatation [130].

In addition to VEGFR-2, there are other receptors and co-receptors for VEGF, all of which yield different result than VEGFR-2 when activated. For instance:

Neuropilins (NRP-1 and NRP-2) which are a receptor for collapsing-sema protein family act as a co-receptor for VEGFR-2, but they can also signal independently. When NRPs co-express with VEGFR-2, they elevate the activity of VEGFR-2 by enhancing the binding of $VEGF_{165}$ to this receptor. In fact they present $VEGF_{165}$ to VEGFR-2 in a manner that increases the effectiveness of VEGFR-2 signaling [129, 130, 147].

VEGFR-1 is also another receptor for VEGF which has different function than VEGFR-2. It mostly expresses on stalk cells as a result of notch signaling and reduces VEGF availability and prevents tip cells formation and migration [148, 149]. As a result of alternative splicing on the VEGFR-1 gene, another form of receptor is produced, which is called soluble VEGFR-1 (soluble FLT-1). It acts as a decoy receptor for VEGF and inhibits the activity and signaling of VEGF through VEGFR-2. It has been shown that VEGFR-1 has less tyrosine kinase activity due to weak

tyrosine phosphorylation and its activation negatively regulates VEGFR-2 functions such as ECs proliferation and migration [150]. Therefore, it can be concluded that VEGFR-1 inhibits sprouting and branching and through this way regulates angiogenesis negatively [130].

1.2.2.3 The sources of VEGF in asthmatic airways

There are numerous sources in asthmatic airways with the ability of generating angiogenic condition. Structural cells including ASMCs, epithelial cells, endothelial cells and inflammatory cells such as mast cells, eosinophils, basophils, neutrophils and macrophages are counted as the most important sources of pro-angiogenic factors in asthmatic condition that can elevate angiogenesis through direct or indirect way [131]. For instance activated macrophages increase angiogenesis by production of pro-angiogenic cytokines [151, 152]. Beside them, eosinophils also contribute in enhancement of angiogenesis in asthma by synthesizing and releasing several pro-angiogenic mediators such as VEGF, FGF-2, TGF-β, TNF-α and IL-8 and promoting ECs proliferation [153]. Basophil and mast cells are also considered as one of the potent sources of VEGF in asthmatic airways [154, 155]. In addition to VEGF, mast cells produces FGF-2, IL-8, TNF-α and TGF-β which have a direct effect on enhancing angiogenesis. Moreover, pro-inflammatory cytokines including TNF-α, IL-6 and IL-1β play a significant role in this process by inducing VEGF and

VEGFR-2 expression [156, 157]. In addition, IL-5 acts as the most important survival factor for eosinophils, therefore, enhances angiogenesis indirectly [158].

In addition to above mentioned sources of pro-angiogenic factors, airway smooth muscle cells are recognized as another potent source of VEGF in asthmatic airways. Initially, airway smooth muscle cells were merely considered as structural cells in the airways. However, it is now known that in addition to this characteristic, they have several significant roles in inducing airway inflammation, airway remodeling and angiogenesis [159]. They are involved in angiogenesis process as one of the main sources of VEGF in asthmatic condition. In fact, they interact with ECs and secrete VEGF, FGF, PDGF, IL-6, IL-8 and so many other pro-angiogenic factors which lead to angiogenic responses [160-162].

Moreover, Th2 cells play a considerable role in producing pro-angiogenic mediator in airway inflammation. They regulate production and release of VEGF by generating pro-inflammatory and Th2 cytokines. In fact, they produce IL-4, IL-5, and IL-13 in the allergic asthmatics condition which in turn, enhances VEGF production [78].

Therefore, it can be concluded that inflammatory condition and different steps of remodeling process have a considerable effect on induction of angiogenesis.

1.2.2.4 Other pro-angiogenic growth factors and cytokines

There are several angiogenic factors in addition to master regulator of blood vessels growth, VEGF that play an important role in induction of angiogenesis in asthmatics airway.

Basic Fibroblast growth factor (bFGF / FGF-2): It is known as the first identified pro-angiogenic factor [163]. FGF consist of 23 members and they signal trough different receptors including FGFR1 to FGFR5 [164, 165]. In fact, this mitogenic and angiogenic mediator directly enhances the level of angiogenesis by activating FGF tyrosine kinase receptors on the surface of ECs. For instance, FGF-2 / FGFR interaction on ECs leads to ECs proliferation and migration [166]. Furthermore, this interaction causes enlargement of vessels in response to increased blood flow [167]. In addition, FGFs can increase the formation of new blood vessels indirectly by inducing other cell types to produce angiogenic factors [129, 168].

Angiopoietin: Blood vessels are equipped with angiopoietin system which is a crucial binary system in keeping the balance between the quiescent and active state of blood vessels. This family consists of 3 ligands including ANG-1, ANG-2, ANG-3 and 2 receptors, TIE-1 and TIE-2. Among them, ANG-2 has a mitogenic and pro-angiogenic properties and releases from endothelial tip cells in the presence of angiogenic stimulant. It applies its effect by signaling trough TIE-2 receptors. ANG-2 / TIE-2 interaction results in enhancement of angiogenesis by affecting mural cell detachment, increasing vascular permeability and vascular branching. However,

ANG-1 maintains ECs in the quiescent state [86, 169]. In the asthmatic airways, macrophages, ECs, and lymphocytes, are known as the main source of ANG-2 production [170, 171].

Pro-angiogenic cytokines: In addition to all pro-angiogenic growth factors, there are several cytokines and chemokines that contribute in angiogenesis process. IL-8 is one of the most important pro-angiogenic cytokines produced by monocytes, macrophages and mast cells. It increases angiogenesis via stimulating ECs proliferation and subsequently protease production, inducing ECs chemotaxis and survival and preventing ECs apoptosis [172]. Moreover, IL-17, the main cytokine of TH17 cells, has a significant effect on angiogenesis in allergic asthmatic airway. IL-17 mediates angiogenesis by stimulating ECs migration, blood vessels lumen formation and regulating pro-angiogenic factors production including VEGF and FGF-2 [173-175].

The activation and function of all these pro-angiogenic mediators increase the level of blood vessels formation as one of the most important features of airway remodeling in the asthmatic patients. Ultimately, this process leads to asthma exacerbation.

1.3 Chapter 3: Semaphorin

1.3.1 Semaphorin molecules

Guidance cues have repulsive or attractive effect on growth cones and determine their direction toward or away from a specific region. Some of these cues apply their effect in diffusible form; however, the other groups are membrane bound. One of the important guidance cues is semaphorin glycoprotein family which was initially referred to as the collapsin family. They act as inhibitory or permissive guidance molecules in different systems [176]. Initially, they were recognized as axon guidance molecules, due to their importance in development of the nervous system and axonal guidance [177, 178]. However, recently they have received an immense attention due to their importance in other systems including, respiratory, cardiovascular and immune systems and as well as various processes such as angiogenesis, embryogenesis, tumor formation and progression [178, 179].

Semaphorins are classified into eight main classes, 1-7 and V. Classes 1 and 2 are found in invertebrate, while classes 3-7 exist in vertebrate and class V found only in viruses [179]. The differences between these classes are related to their sequence and structure. However, all members are considerably conserved in their extracellular domain, which consist of 500 amino acids and called semaphorin (sema) domain. This portion of semaphorin molecules is characterized as a cysteine-rich semaphorin protein domain by

which semaphorins mediate their effect. This domain is also a crucial component for determining the receptor binding specificity [178].

There is another system for classifying semaphorin molecules which is based on the form of their localization. According to this system, Classes 1, 4, 5, and 6 are known as membrane-bound proteins, whereas classes 2 and 3 are characterized as secreted proteins, and class 7 is identified as glycosyl–phosphatidyl–inositol (GPI)-linked proteins [180].

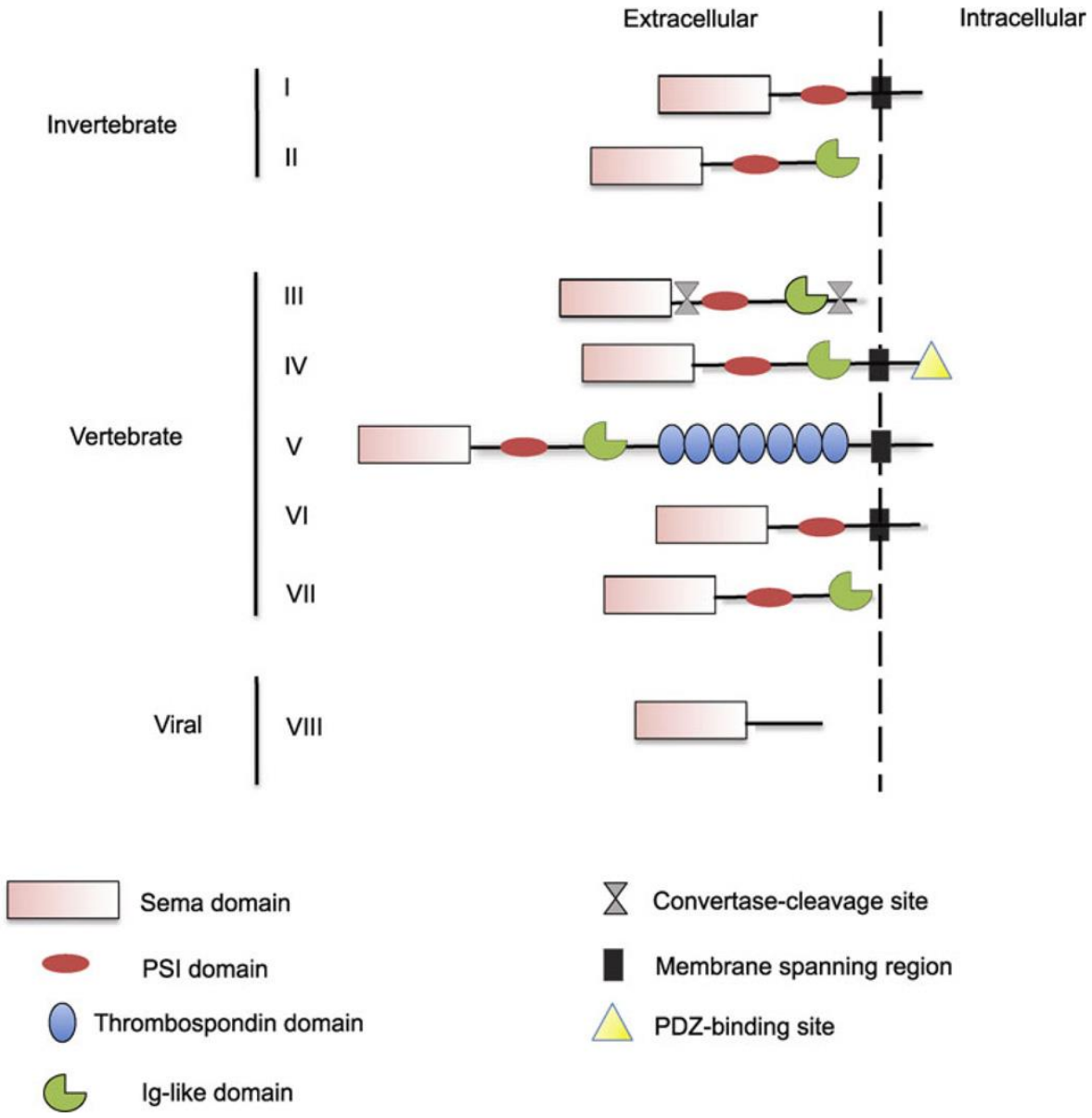


Figure 1.5. Semaphorins. There are eight classes of semaphorins. Classes I and II are found in invertebrates, classes III-VII are found in vertebrates and class VIII semaphorins are encoded by viruses. All semaphorins are characterized by sema domains which are followed by plexin semaphorin integrin (PSI), thrombospondin, and Ig-like domains. They can be either secreted or membrane-bound.

Mechanisms of semaphorin molecules:

The mechanism of semaphorin molecules is based on changing the cytoskeleton and organization of actin filaments and microtubule networks [181]. They make these alterations by F-actin polymerization and depolymerization, F-actin translocation and microtubule dynamic. They also play a significant role in directing the tissue morphogenesis by regulating cell migration, proliferation, adhesion and cytoskeletal organization [178].

Several post-translational modifications including proteolytic processing, oligomerization and so many others affect the function of transmembrane and secreted semaphorins. These alterations lead to semaphorin- mediated repulsive axon guidance, cell migration, invasive growth and growth cone collapse [182, 183].

The alteration of semaphorin's function in each organ leads to several abnormalities. For instance, if semaphorins alter in nervous system, it leads to some problems such as epilepsy, alzheimer, parkinson and schizophrenia [184, 185].

In addition, semaphorins play a considerable role in immune system by contributing in various phases of immune response. In fact, their alteration changes the proper functioning of the immune system [186].

Also, semaphorins contribute to cancer progression or suppression by affecting angiogenesis, metastasis, chemotaxis and tumorogenesis [187, 188].

Therefore, it can be concluded that semaphorins play a crucial roles in maintaining physiological homeostasis in many organs.

1.3.3 Semaphorin receptors and signaling

Semaphorins signal through two main classes of receptors including plexins and neuropilins [189, 190]. The majority of semaphorin molecules mediate their effect by signaling through plexins alone. But, class III semaphorins, except Sema3E, require neuropilin co-receptors for functioning and signaling properly [191, 192]. Beside these receptors, semaphorins can transmit their signals through other receptors including CD72 and T-cell immunoglobulin and mucin domain-containing protein 2 (TIM-2) [179]. In their signaling process, several proteins including G proteins, kinases, and regulators of the actin cytoskeleton are involved [178].

Plexins: These are conserved family of large proteins which act as single pass transmembrane receptors [193]. Plexins have 4 sub classes including A, B, C and D. In invertebrates, plexin-A and plexin-B play a vital role while in vertebrates all the four classes of plexins including class A (Plexin-A1, A2, A3, A4), class B (Plexin-B1, B2, B3), class C (C1), and class D (D1) contribute in transmitting the signals [194]. The extracellular part of plexins contains a sema domain which is an important portion for binding to semaphorins [195, 196]. Sema domin is also an autoinhibitory element which limits the activation of plexin molecules [197]. Following sema

domain, three PSI domains (plexin-semaphorin-integrin) and three IPT domains (immunoglobulin, plexin and transcription factors) are located. PSI domain is a small cystine-rich domain (CDR) which is crucial for protein-protein interactions [198]. Also, IPT domains are required for proper ligand binding to plexins [199]. The intracellular domain or cytoplasmic tail of plexin molecules, is highly conserved and plays a crucial role in transmitting the signals following ligand binding [200]. It contains a putative tyrosine phosphorylation sites, but doesn't show any kinase activity [201]. GTPase-binding domain and a segmented GTPase-activating protein (GAP) domain are known as the most important characteristics of intracellular domain of plexin which regulates many responses following plexin activation [194]. Plexins activation and signaling has a crucial role in cell movement, cytoskeleton rearrangement and synapse formation [202] by controlling R-Ras and RhoA activity [203].

Neuropilin: As mentioned earlier, most of the semaphorins signal through plexins. However, class 3 semaphorins, except Sema3E, bind to neuropilin receptors [191, 192]. These groups of receptors are transmembrane proteins (~900 a.a.) with a short cytoplasmic tail. Due to this characteristic, they lack intrinsic signaling capabilities [204]. Two members of this family of receptors are recognized, including NRP-1 and NRP-2 which form co-receptor complexes with other cell surface molecules to mediate signal transduction [194]. For instance, VEGF uses NRP-1 as a co-receptor of VEGFR-2 on the surface of ECs.

CD72: CD72 receptor is a member of C type lectin family and mostly expressed on lymphoid tissues and serves as a receptor for sema4D molecules [178].

Tim-2: T cell immunoglobulin and mucin (Tim) domain protein family is expressed on activated T cells and is considered as a receptor for Sema4A ligands [178].

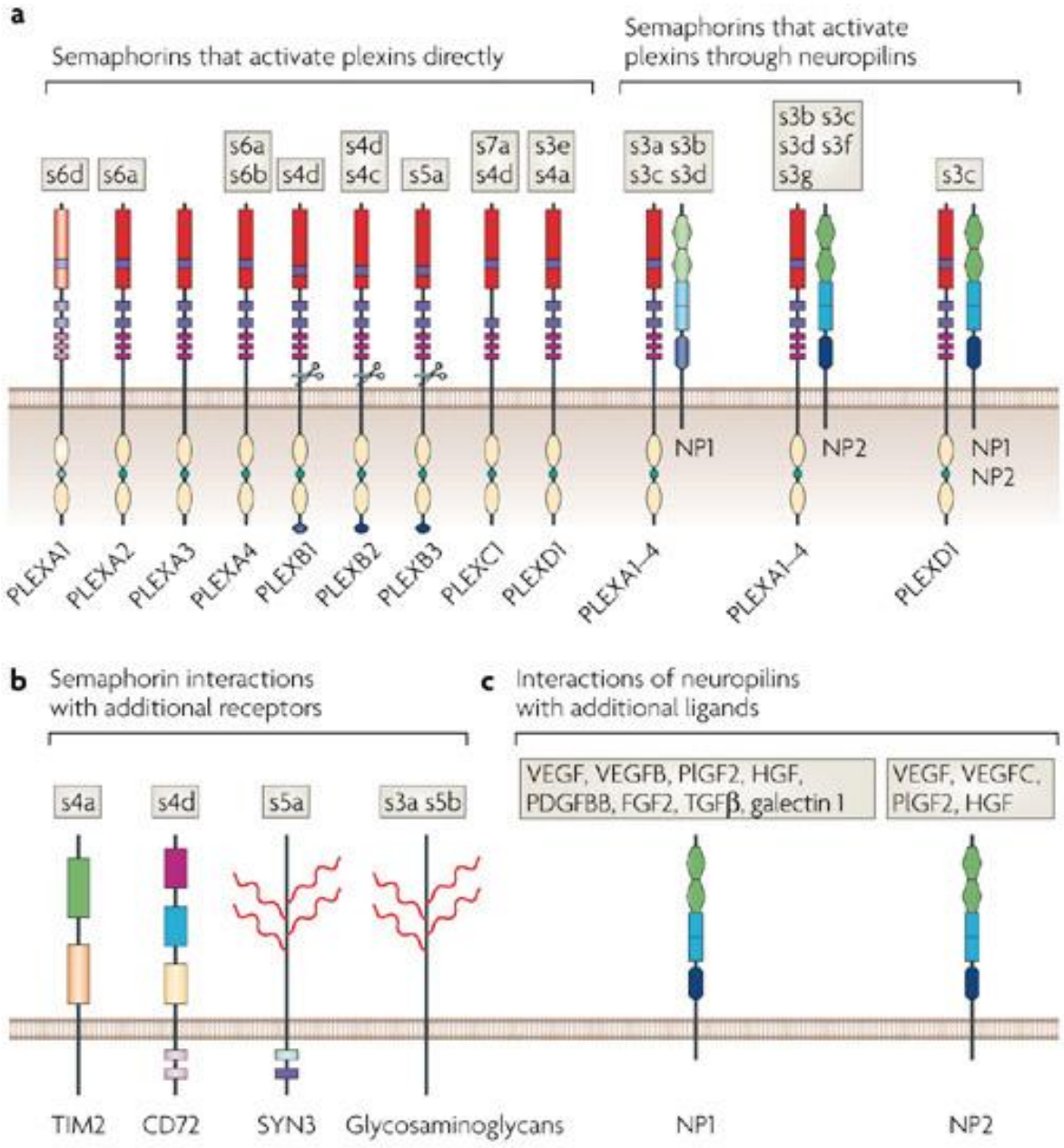


Figure 1.6. The interaction of semaphorins with various types of semaphorin receptors. a) The specific interactions between individual semaphorins and either single plexins (PLEX) or specific neuropilins (NRP) are shown. In the case of the neuropilins, the plexins that have been found to form complexes with these neuropilins and to serve as the signal-transducing elements in such complexes in response to the binding of the shown semaphorins are also indicated. **b)** The interactions of specific semaphorins with additional receptors such as the T-cell receptor TIM2, the lymphocyte receptor CD72 and proteoglycans. **c)** Interactions of neuropilins with ligands that do not belong to the semaphorin family, such as the members of the vascular endothelial growth factor (VEGF) gene family as well as hepatocyte growth factor (HGF) and platelet-derived growth factor B homodimer (PDGFBB), are shown.

1.3.5 Semaphorin 3E and its receptor

As mentioned earlier, there are 8 main classes of semaphorins [205]. Among them Class 3 semaphorins, which are secreted proteins, play a significant role in various processes including axon guidance, angiogenesis, tumor growth and metastasis [206]. They consist of 7 different subgroups (sema3A to sema3G) [178].

This group of semaphorins mainly signals through neuropilin receptors. However, the interaction between neuropilins and plexins is crucial for class 3 semaphorin signaling [206]. Among 7 different subclasses of semaphorin 3 family, Sema3E has been recognized as a unique one, due to its ability to signal trough plexinD1 independently of neuropilin receptors. In fact, the high affinity binding of Sema3E to plexinD1 on endothelial tip cells results in controlling of ECs positioning and patterning for forming vasculatures [192, 207].

Sema3E is an 85 – 90 kDa protein which is synthesized as a full-length precursor molecule. The furin-sensitive site of the precursor is subject to proteolytic maturation to produce p61 fragment, which is known as the main species of endogenous Sema3E. Furine proprotein-convertases is responsible for proteolytic maturation of full-length Sema3E [208].

As mentioned previously, Sema3E signals specifically through plexinD1 and in this condition it acts as a repellant molecule. However, the coexpression of neuropilin with plexinD1 changes the function of Sema3E and turns it into an attractant

molecule [191]. This shows that Sema3E can mediate different functions based on the receptors arrangement [191]. Collectively, the existing evidence suggests that Sema3E / plexinD1 signaling can affect several biological systems via the application of their regulatory role.

1.4 Chapter 4: Semaphorin3E and angiogenesis

It is clearly known that Sema3E / plexinsD1 interaction has a crucial role in many biological systems including vascular development [209]. In addition, according to previous studies, Sema3E / plexinD1 signaling has an inhibitory influence on cell migration and proliferation [210, 211]. The most important inhibitory effect of Sema3E / plexinD1, is on endothelial cells which leads to inhibition of vessel growth and branching through several processes, including:

A) Suppression of Delta-like 4 Notch signaling: As mentioned previously, VEGF, which is the master regulator of blood vessels formation, transforms endothelial cells to tip cells. Following tip cells formation, the expression of Delta-like 4 (Dll4) is initiated on their cell membrane. Delta-like 4 (Dll4) is a transmembrane ligand for notch receptors. These receptors are expressed on sprouting ECs and their function is critical for negatively regulating endothelial tip cells. The signaling pathway of Dll4 / notch, is known as one part of VEGF downstream signaling pathways and plays a significant role in determining the cell fate between stalks and tip cells [212, 213]. In fact, Dll4 activates Notch-1 receptors, which are expressed on the adjacent stalk cells [214]. This phenomena leads to suppression of tip cells differentiation into stalk cells. Consequently, the cells that initially were differentiated into a tip cells will stay tip cells; however, the adjacent endothelial cells remain undifferentiated or stalk cells [91]. The implication of

these sophisticated molecular pathways on endothelial cells is enhancing the efficiency of sprouting angiogenesis.

However, Sema3E / plexinD1 signaling changes this pattern. In fact, VEGF induces the expression of plexinD1 at the front of actively sprouting endothelial tip cells [215]. Sema3E then binds to plexinD1 which leads to suppression of Dll4 / Notch signaling pathway [212]. In fact, the negative feedback mechanism of Sema3E / plexinD1 on ECs, results in disruption of vascular network due to suppression of VEGF and Dll4 - Notch signaling pathway [212].

B) Soluble VEGFR-1 (sFlt-1) production: Another mechanism of decreasing the level of angiogenesis is reduction in the amount or the effect of pro-angiogenic factors on endothelial cells. Sema3E which is known as a repulsive cue, have the ability to reduce the effect of VEGF on ECs by signaling through plexinD1.

In some developmental studies in zebrafish, it has been shown that the loss of Plexin-D1 induces sprouting of new blood vessels; however, activation of Sema3E / plexin-D1 signaling suppresses VEGF-induced angiogenesis. In fact, Sema3E / plexin-D1 acts as an antagonist of VEGF signaling by elevating the level of soluble Flt-1 (sFlt-1) expression. sFlt-1 is VEGFR2 decoy receptor in endothelial cells and inhibits the signaling of VEGF. This phenomenon leads to negative regulation of ECs proliferation and migration [202, 216-218].

C) Actin filaments and microtubules network alteration: Cell migration is one of the most important steps of angiogenesis and is dependent on actin cytoskeleton alterations. Actin is one of the most abundant proteins in cells and contributes to several cellular processes, including cell motility, cell attachment, cell signaling and so many others [215, 219].

In the presence of Sema3E, the regular function and organization of actin filaments will be affected which has a direct influence on ECs migration and subsequently angiogenesis. In fact, Sema3E influences actin cytoskeletons and focal adhesions (FA), which are known as the main target of Sema3E on ECs. FA is an adhesive structure between cell and ECM which is formed upon integrin engagement. Sema3E / plexinD1 signaling has a significant influence on focal adhesion assembly / disassembly. In fact, this signaling pathway induces cytoskeletal remodeling, and as a result changes cell type, cell mobility, cell migration and cell attachment to ECM [220].

D) Endothelial cell signaling alterations: Another effect of Sema3E on angiogenesis process is changing the ECs cell signaling. As mentioned earlier, in angiogenic condition, VEGF induces filopodia extension by activating Cdc42, a member of Rho GTPases. GTPases are small and monomeric hydrolase enzymes that are involved in controlling various cellular processes [221]. They can bind to GTP and switches the active form of GTP to inactive form of GDP which leads to a variety of cellular signaling events. These small GTPases, are categorized into five main

groups including Ras, Rho, Rab, Arf and Ran and among them, Rho family is further divided into RhoA, Rac1 and Cdc42 [222].

Following Sema3E / plexinD1 interaction, anti-angiogenic signaling pathway will be initiated by activation of RhoJ signaling pathway. This phenomenon leads to retraction of filopodia in endothelial tip cells and suppression of vascular formation. Additionally, Sema3E / plexinD1 interaction inhibits Akt and ERK signaling pathway that results in inhibition of ECs proliferation and migration [209, 223].

Moreover, the intracellular domain of plexinD1 contains GTPase activating domain (GAP) which shows activity toward R-Ras. Activation of R-Ras promotes cell adhesion processes by activating the integrins [220, 224, 225]. However, Sema3E / plexin-D1 activation induces the association of GAP domain of plexinD1 with R-Ras, which leads to inactivation of R-Ras and alteration of cell/ECM adhesion features. Subsequently, R-Ras inactivation results in Arf-6 activation following several phenomena. Initially, phosphatidylinositol-4-phosphate-5-kinase become activated, which generates $PI(4,5)P_2$ locally. $PI(4,5)P_2$ then binds to the Arf6 GEF (guanine nucleotide exchange protein 100) PH domain which results in Arf6 activation [226]. The ultimate consequence of all these phenomena is inactivation of β1 integrin and disruption of adhesive structures, which inhibits ECs adhesion to the ECM, and leads to filopodia retraction in endothelial tip cells. In addition, Arf6 activation induces rapid focal adhesion (FA) disassembly, integrin internalization, and Ecs collapse,

which inhibits angiogenesis. Additionally, these processes provide a repulsive cue for ECs which inhibit growth and sprouting of new blood vessels [227].

By gathering these findings, it can be concluded that Sema3E molecules act as an effective chemorepellent for ECs by applying their effect through Plexin-D1 which ultimately result in angiogenesis inhibition.

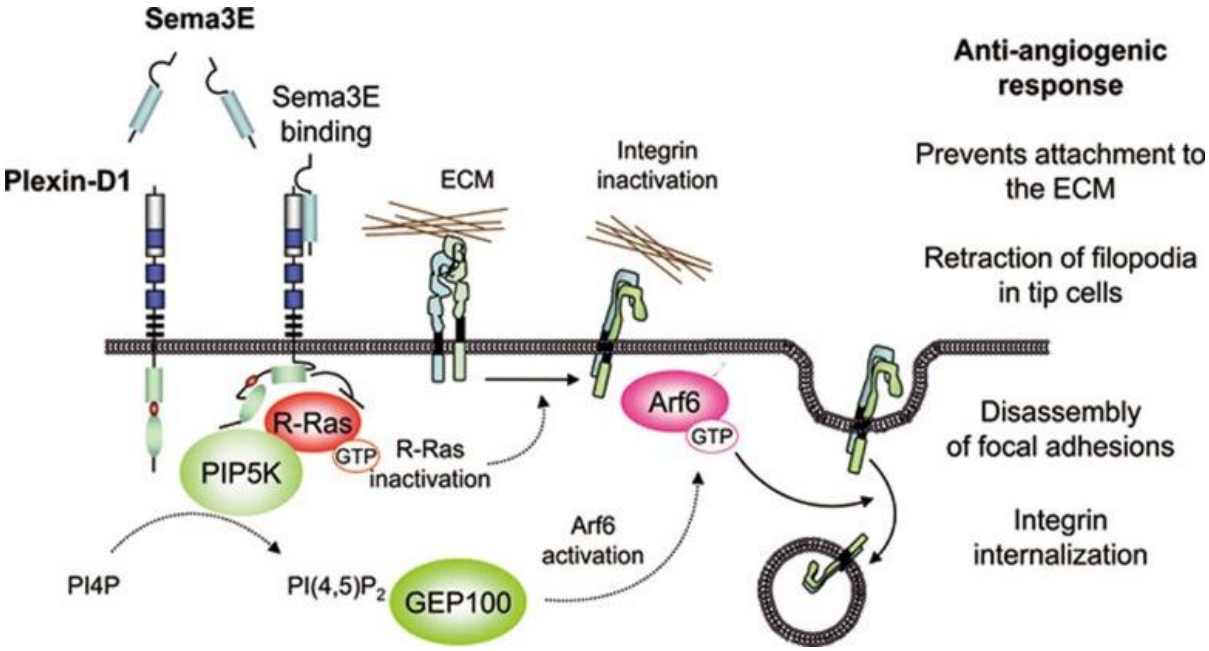


Figure 1.7. Anti-angiogenic signaling by Sema3E / plexinD1 in endothelial cells. The activation of plexinD1 by Sema3E induces the association of the Ras GAP domain of plexinD1 with R-Ras. This inactivates integrins and enables their subsequent internalization by the plexinD1-mediated activation of Arf6, thus inhibiting endothelial cell adhesion to the extracellular matrix (ECM) by disrupting integrin-mediated adhesive structures, and causing filopodial retraction in endothelial tip cells. The pathway by which Sema3E stimulation of plexinD1 leads to Arf6 activation involves phosphatidylinositol-4-phosphate-5-kinase β activation, which generates $PI(4,5)P_2$ locally. $PI(4,5)P_2$ then binds to the PH domain of an Arf6 GEF, guanine nucleotide exchange protein 100, resulting in Arf6 activation. Sema3E-induced Arf6 activation induces rapid focal adhesion (FA) disassembly, integrin internalization, and endothelial cell collapse, thereby inhibiting angiogenesis.

2. RATIONALE, HYPOTHESIS AND AIMS

2.1 Rationale

Asthma is a chronic inflammatory disease of the airways which is associated with airway hyperresponsiveness and airway remodeling. Increased angiogenesis is recognized as one of the prominent features of airway remodeling. However, the factors regulating this process in allergic asthma are poorly defined. Semaphorin 3E (Sema3E) is an axon-guidance secreted protein in neuronal system that has emerged as an essential mediator involved in cell migration and proliferation which are known to play a key role in angiogenesis. Moreover, our previous study revealed that Sema3E acts as a chemorepellent for human airway smooth muscle cells and inhibits the proliferation and migration of these cells in asthmatic condition suggesting an important role of this molecule in airway remodeling. However, no study has investigated the role of Sema3E in angiogenesis in allergic asthma. Therefore, we aim to investigate the effect of Sema3E treatment and deletion on angiogenesis events within the airways of murine model of allergic asthma.

2.2 Hypothesis

Semaphorin 3E inhibits house dust mite induced angiogenesis in mouse model of allergic asthma.

2.3 Aims

2.3.1 To investigate the effect of Sema3E treatment and deletion on angiogenesis in murine model of allergic asthma.

2.3.1.1 Study the effect of Sema3E on airway mechanics and inflammation.

2.3.1.2 Assess the effect of Sema3E on the level of angiogenesis in lung.

2.3.1.3 Determine the effect of Sema3E on pro-angiogenic factors in allergic asthmatic condition.

2.3.2 To study the mechanisms by which Sema3E-Plexin D1 affects pathological angiogenesis

2.3.2.1 Investigate the role of Sema3E on the level of soluble VEGFR1 (s-flt1) production as a modulating decoy of VEGF.

2.3.2.2 Study the effect of Sema3E on VEGFR2 expression as the most important mediator of angiogenic effect of VEGF.

3. MATHERIAL AND METHODS

3.1 Animal model

To generate Sema3E treated vs. Sema3E non-treated mouse model, immunocompetent female BALB/c mice (6-8 wk old) were used for House Dust Mite (HDM) or Saline challenge and Sema3E treatment. In addition, to investigate the role of Sema3E on angiogenesis in allergic asthma, Age-matched (6-8 wk old) Sema3E Knock out (*Sema3e*$^{-/-}$) and wild type (WT) littermate was generated using female 129p2 mice. All animal procedures were approved by the Centre of Animal Care Services (CACS) of University of Manitoba.

3.2 HDM sensitization and Sema3E treatment

Induction of allergic asthma was performed as described below. For this purpose, female BALB/c Mice were challenged intranasally with HDM-Fc Ig (25 μg) (D.PTERONY SSINUS, item#: XPB70D3A2.5, Greer, NC, USA) for 5 consecutive days for 2 weeks. In Sema3E treatment model, Sema3E-Fc (10 μg/kg per mouse) was administered intranasally 1h before each HDM challenge (25 μg) (Fig 3.1 A) (Mouse recombinant Sema3E-Fc protein was gifted from Dr. Jonathan Duke-Cohan, Dana Farber Cancer Institute, Harvard Medical School.)

Also, *Sema3e*$^{-/-}$ and WT mice, were subjected to HDM challenged intranasally (25 μg) for 5 consecutive days for 2 weeks as described in Fig 3.1 B. Saline treated mice were used as the control group. Each group contained four mice.

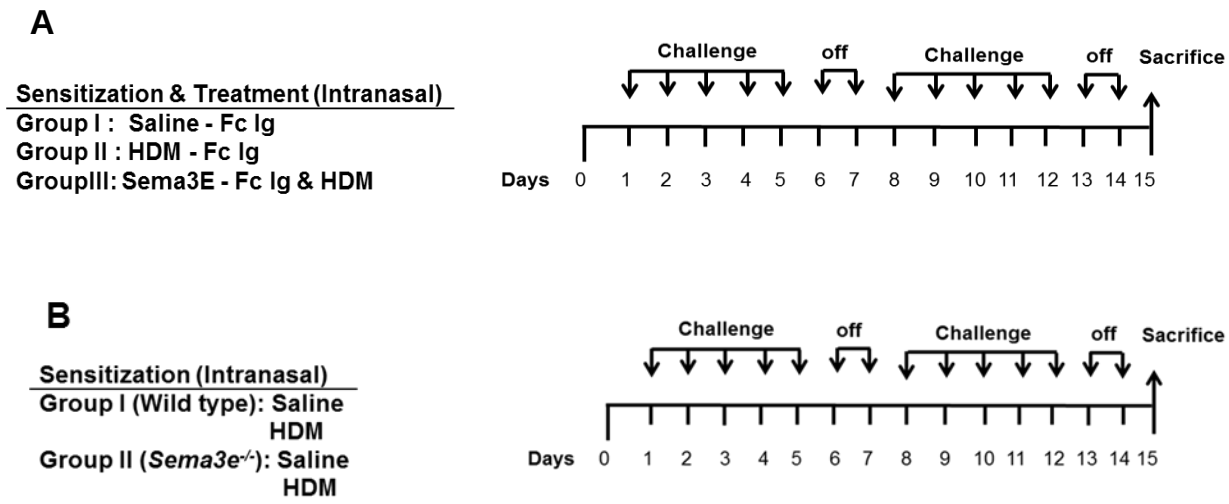


Figure 3.1. Mouse model system for investigating the effects of Sema3E on angiogenesis in allergic asthma.

After 2 weeks mice were anesthetized by i.p injection of pentobarbital (90 mg/kg ip injection) to measure the lung mechanics. They tracheotomized with a 20-gauge catheter which was connected to a FlexiVent small animal ventilator (Scireq Montreal, PQ, Canada). Mice were then ventilated with a tidal volume of 10 ml/kg body wt, 150 times/min and lung mechanics was measured after exposing mice to 50 mg/ml nebulized methacholine (MCh) using flexiVent ventilator [228]. To investigate the effects of MCh challenge on respiratory mechanics, low-frequency forced-oscillation technique was used. In this experiment, the mechanical ventilation was interrupted during low-frequency forced oscillation and then a volume perturbation signal was applied. Following these processes, flexiVent software calculated Newtonian resistance (Rn), peripheral tissue damping (G), and tissue elastance (H) and ultimately all mentioned parameters normalized to animal's body weight [228].

Afterwards, following the washing of airways with sterile saline, bronchoalveolar lavage fluid (BALF) was collected, mice were sacrificed and lung specimens were collected for further investigation.

3.3 Bronchoalveolar lavage fluid collection and characterization

Following the lung mechanics measurement, BALF was collected after washing airways lumina with 2 x 1 ml sterile saline. Typical 1000 - 1500 µl BALF was collected per mouse. The number of leukocytes was determined using

hemocytometer, by performing cytologic examination on cytospin preparations (Thermo Shandon CytoSpin 3 Cytocentrifuge, UK) by Diff Quick staining using modified Wright-Giemsa stain (HEMA 3 STAT PACK) (Fisher Scientific, Kalamazoo, MI, Cat #: 123-869). Differential cell counts were performed by counting at least 200 cells (eosinophils, lymphocytes, macrophages, and neutrophils) using a light microscope (Primo Star Digital Microscope and INFINITY camera by using infinity capture software) at x400 magnification in blind manner by three individuals and by considering standard morphologic criteria [229].

3.4 Histologic examination

Formalin-fixed tissues were paraffin embedded, and 5-µm-thick sections were cut, mounted on slides and stained with hematoxylin and eosin staining (H&E) for evaluating the level of cell infiltration and the level of inflammation and structural changes [230]. Images were taken at x200 under a digital Zeiss Axioskop 2 mot plus microscope using a Carl Zeiss AxioCam MRc 5 camera and AxioVision Rel 4.8 software.

3.5 Immunohistochemistry

Formalin-fixed tissues were paraffin embedded, and 5-µm-thick sections were prepared, deparaffinized in xylene for 2 X 5 min, and rehydrated through graded

concentrations of alcohol to water (2 x 100% ethanol, 95% ethanol, 70% ethanol and ddH_2O). Then tissues were boiled with microwave for 10 min in sodium citrate buffer (pH 6.0). Sections were washed 3 x 5 min with TRIS-buffered saline [TBS 1x] and then incubated with blocking solution (1% BSA and 5% second animal serum in TBS) for 45 min at room temperature. Rabbit anti-mouse CD31 poly clonal antibody (1:50) (Abcam, Cambridge, UK, Cat #: ab28364) as primary antibody or control rabbit IgG as a negative control and Rabbit anti human vWF (Von Willebrand factor) poly clonal antibody (1:400) (Dako, Santa Barbara, Calif, Cat #: A 0082) as primary antibody or rabbit IgG as an isotype control were added, and sections were incubated overnight at 4°C. Slides were then washed 3 x 5 min with TBS, followed by incubation for 2 h at room temperature with biotin-conjugated goat anti-rabbit IgG as secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, Cat #: 111-065-003). Slides were then washed 3 x 5 min with TBS and incubated with streptavidin-alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA, Cat #: 016-050-084) for 30 min at room temperature. Afterwards, slides were washed 3 x 5 min with TBS and developed by using Fast Red TR/Napthol AS-MX Alkaline Phosphatase Substrate Tablets Set (Sigma-Aldrich Missouri, USA, Cat #: F4648) and counterstained with Harris Modified Hematoxylin (Fisher Scientific, Fair Lawn, NJ, USA Cat #: SH26-500D). Positive cells were stained red after development with Fast Red. Isotype-matched control mAb was used for negative control. Images were taken at x100 using a digital Zeiss Axioskop 2 mot plus microscope and Carl Zeiss AxioCam MRc 5 camera by using AxioVision Rel 4.8

software. The result of staining was quantitatively analyzed by counting the number of red blood vessels (any cluster of endothelial cells that was clearly stained in red color) which were positive for CD31 or vWF. In fact, 4 tissues from each group (n=4) were stained and the number of blood vessels in each tissue were counted in five randomly chosen high power field and the result was reported as an average of the number of red blood vessels in each group [231, 232]. The analysis was performed according to the above-mentioned manner to investigate and compare the level of blood vessels formation in each bronchial section.

3.6 Immunofluorescence

Formalin-fixed paraffin embedded sections were prepared for Immunofluorescence staining as described previously in Immunohistochemistry section. Briefly, Formalin-fixed paraffin embedded tissues, were cut in 5-μm-thick sections and deparaffinized in xylene and rehydrated through graded concentrations of alcohol to water. Then, tissues were boiled with microwave for 10 min in sodium citrate buffer (pH 6.0). Sections were washed with TBS and then incubated with blocking solution (1% BSA and 5% second animal serum in TBS) for 45 min at room temperature. Sections were stained for Von Willebrand factor (vWF), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 1 (VEGFR-1), vascular endothelial growth factor receptor 2 (VEGFR-2), neutrophil elastase and eosinophil major basic

protein (MBP). The antibodies and isotype control antibodies that were used for the above-mentioned staining's, were according to table 3.1 description:

Table 3.1. Immunofluorescence staining antibodies

Antibody	Dilution	Isotype Control	Company and Cat #
Rabbit anti human vWF (Von Willebrand factor) poly clonal antibody	1:400	Rabbit IgG	Dako, Santa Barbara, Calif. Cat #: A 0082
Goat anti mouse VEGF polyclonal antibody	1:20	gout IgG	R & D Systems, MN, USA, Cat #: DY493
Goat anti mouse VEGFR-1 polyclonal antibody	1:20	gout IgG	R & D Systems, MN, USA, Cat #: DY471
Rabbit anti mouse VEGFR-2 polyclonal antibody	1:200	Rabbit IgG	Biorbyt, SF, USA, Cat #: orb11557
Mouse anti human neutrophil elastase monoclonal antibody	1:20	mouse IgG	Dako, Denmark, Cat #: M075201-2
Rabbit anti mouse major basic protein (MBP) polyclonal antibody	1:40	rabbit IgG	Cloud-Clone Crop, TX, USA, Cat #: PAB650Mu02

After adding primary antibodies, sections were incubated at room temperature for 2 hours. Then, slides were washed with 3 X 5 min with TBS, followed by incubation for 1 h at room temperature with secondary antibody according to the following description:

The secondary antibody that was used in vWF, VEGFR-2 and MBP staining was Alexa Flour 568 goat anti rabbit IgG (H+L) (Life technologies, OR, USA, Cat #: A-11011) and in VEGF and VEGFR-1 staining the secondary antibody was Alexa Flour 488 chicken anti goat IgG (H+L) (Life technologies, OR, USA, Cat #: A-21467). In addition, Alexa Flour 488 goat anti mouse IgG (H+L) (Life technologies, OR, USA, Cat #: A-11001) were used for neutrophil elastase staining. Slides were then washed extensively 3 X 5 min with TBS and counterstained with prolong gold anti-fade mountant with DAPI (Molecular Probes, Life technologies, USA Cat #: P36935). Images were taken at x100, x200, and x400 magnifications using a digital Zeiss Axioskop 2 mot plus microscope and Carl Zeiss AxioCam MRc 5 camera by using AxioVision Rel 4.8 software.

Image analysis: The result of vWF staining was quantitatively analyzed by counting the number of blood vessels which were positively stained for vWF. The number of blood vessels in five randomly chosen high power field of 4 tissues from each group (n=4) were counted and the result was reported as an average of the number of red blood vessels in each group [231]. The result of VEGF, VEGFR-1 and VEGFR-2 staining was quantitatively analyzed by calculating the mean intensity of 20 pictures

from each group using Image J 1.48v software downloaded from the NIH website (http://rsb.info.nih.gov/ij). To do the analysis, after opening the TIFF file of the picture in the Image J 1.48v, set measurement was chosen under analyze section and the threshold was adjusted to highlight the area of interest in the red color. Afterwards, under analyze section, measure was selected which allowed us to calculated the intensity measurements in the thresholded area of interest. The result was reported as mean intensity for each group [233].

3.7 Lung homogenate preparation for ELISA and BCA protein assay

Lung samples were homogenized in ice cold homogenization buffer containing RPMI (Gibco, Life technologies, ON, Canada, Cat #: 11875-093), Protease Inhibitor Cocktail (Sigam-Aldrich, Life science, St. Louis, MO, USA, Cat #: P2714-1BTL) and PMSF (Sigam-Aldrich, Life science, St. Louis, MO, USA, Cat #: P 7626). Following centrifugation of the homogenates at 10,000 g for 15 min, supernatants were collected. The protein concentration of each sample was determined using the BCA protein assay kit standardized to BSA, according to manufacturer's instruction (San Diego, CA, USA, Cat #: 71285-3).

3.8 Enzyme Linked Immunosorbent Assay (ELISA)

The concentration of VEGF-A and soluble VEGFR-1 / Flt-1 were quantitated using commercially available mouse VEGF DuoSet ELISA kit (R & D Systems, MN, USA, Cat #: DY 493) and Mouse sVEGFR-1 / Flt-1 DuoSet ELISA kit (R & D Systems, MN, USA, Cat #: DY 471) according to manufacturer's instruction, respectively. The sensitivity limit of VEGF and soluble VEGF R1 assay is 15.60 pg/ml and 150 pg/ml respectively as mentioned by the manufacturer's instruction. The experiment was performed in duplicate condition.

96 well plates were coated by 100 µl of Mouse VEGF or sVEGFR-1 capture antibody and for maximum sensitivity incubated over night at 4°C. Plates were tightly wrapped and kept in a humidified chamber to reduce the level of evaporation and variability in wells. At the second day, plates were washed four times manually with washing buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) and plates were dried by hitting them three times on paper towels. 200 µl blocking solution (1% BSA in PBS, pH 7.2-7.4) was added to each well and plates were incubated at room temperature for 2 hours. Following washing plates, 50 µl of each sample (lung homogenate) and standard were added to the wells and plates were incubated over night at 4°C for maximal sensitivity. The standard curve was generated using recombinant mouse VEGF or sVEGFR-1 standard in dilution buffer. The titration curves ran from 10000 to 78 pg/mL for testing VEGF and sVEGFR-1 levels in experimental samples and each

sample was titrated in dilution buffer from 1:2 to 1:4 and from 1:10 to 1:20 respectively.

At the third day, after washing and drying the plates according to above mentioned manner, 100 µl of biotinylated gout anti mouse VEGF or biotinylated gout anti mouse soluble VEGFR-1 detection antibody was added and plates were incubated at room temperature for 2 hours. Following washing and drying plates, they were incubated 30 to 45 minutes with 50 µl streptavidin- horseradish-peroxidase (HRP) (1:1000 dilution) at room temperature and developed with 50 µl substrate solution (1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine), R&D Systems, Cat #: DY999). Progress of assay was observed visually until the blue color appeared. Immediately, the reactions were stopped following adding 100 µl stop solution (2N H2SO4) to each well. OD was measured at 450nm with Spectra Max 190 ELISA reader (Molecular Devices, CA, USA). A standard curve was generated using the data achieved by serial dilution of the standard, and the concentration of VEGF and sVEGFR-1 in samples were calculated on the x axis and the OD on the y axis to generate a four parameter curve using SoftMax Pro 5.4.1 software [234].

3.9 Quantitative Real-Time PCR analysis

Total RNA was extracted from whole lung by using TRIzol (Invitrogen, Life Technologies, Cat #: 15596026, CA, USA) according to manufacturer's protocol and RNA concentration was measured using BioPhotometer (Eppendorf AG, Hamburg,

Germany). To synthesize cDNA, reverse transcription was perfumed with 2 μg of total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA, Cat #: 4368814) in a total volume of 20 μL according to the manufacturer's protocol. cDNA of each sample and sequence specific VEGFR1 and GAPDH primers (10 μM) were added to SYBR Select Master Mix (Applied Biosystems, USA, Cat #: 4472908). Real-Time PCR was performed in 96-well optical plate with an initial 1 cycle denaturation step for 10 min at 95°C, 40 cycles of PCR (95°C for 15 s, 60 °C for 35 s and 72°C for 35 s), 1 cycle of melting and 1 cooling cycle (Applied Biosystems 7500 Real-Time PCR system). Average data collection and detection of fluorescent product was performed at the end of the 72°C extension period. Products specificity was assessed by performing melting curve analysis and examining the quality of amplification curves. 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 (RQ) over the values calculated with other groups.

Table 2.2. Real-Time PCR primers (forward and reverse) and amplicon size used in analysis of gene expression assay

Gene	Forward (5′- 3′)	Reverse (5′- 3′)	Size (bp)
VEGFR-1	5′-TCACAGATGTGCCGAATG-3′	5′-CGTAGCAGAATCCAGGTAATG-3′	124
GAPDH	5′-AACTTTGGCATTGTGGAAGG-3′	5′-ACACATTGGGGGTAGGAACA-3′	217

3.10 Statistics

Results are expressed as the mean ± SEM. Differences between the groups was analyzed by one-way ANOVA first to determine if any significant differences may generally exist among various experimental groups then Bonferroni post tests were performed to detect statistically significant differences in each pair of experimental groups by GrapPad Prism 5.04. *P* values <0.05 was considered statistically significant.

4 RESULTS

4.1 Sema3E-Fc treatment decreases airway hyperresponsiveness in HDM-induced allergic inflammation.

To assess the effect of the Sema3E molecule on HDM-induced airway hyperresponsiveness, BALB/c mice were pre-treated with Sema3E-Fc (10μg/kg) or Fc fragment Ig control one hour prior to intranasal administration of HDM as described in figure 4.1A.

Our data revealed that in response to MCh the level of airway resistance, tissue resistance and tissue elastance were increased in HDM challenged Fc pretreated mice. However, in Sema3E pretreated group all these features were significantly reduced (**** $P < 0.0001$, *** $P < 0.001$) (Fig 4.1B). Together, these data revealed that Sema3E prevents the development of airway hyperresponsiveness in HDM-challenged mice.

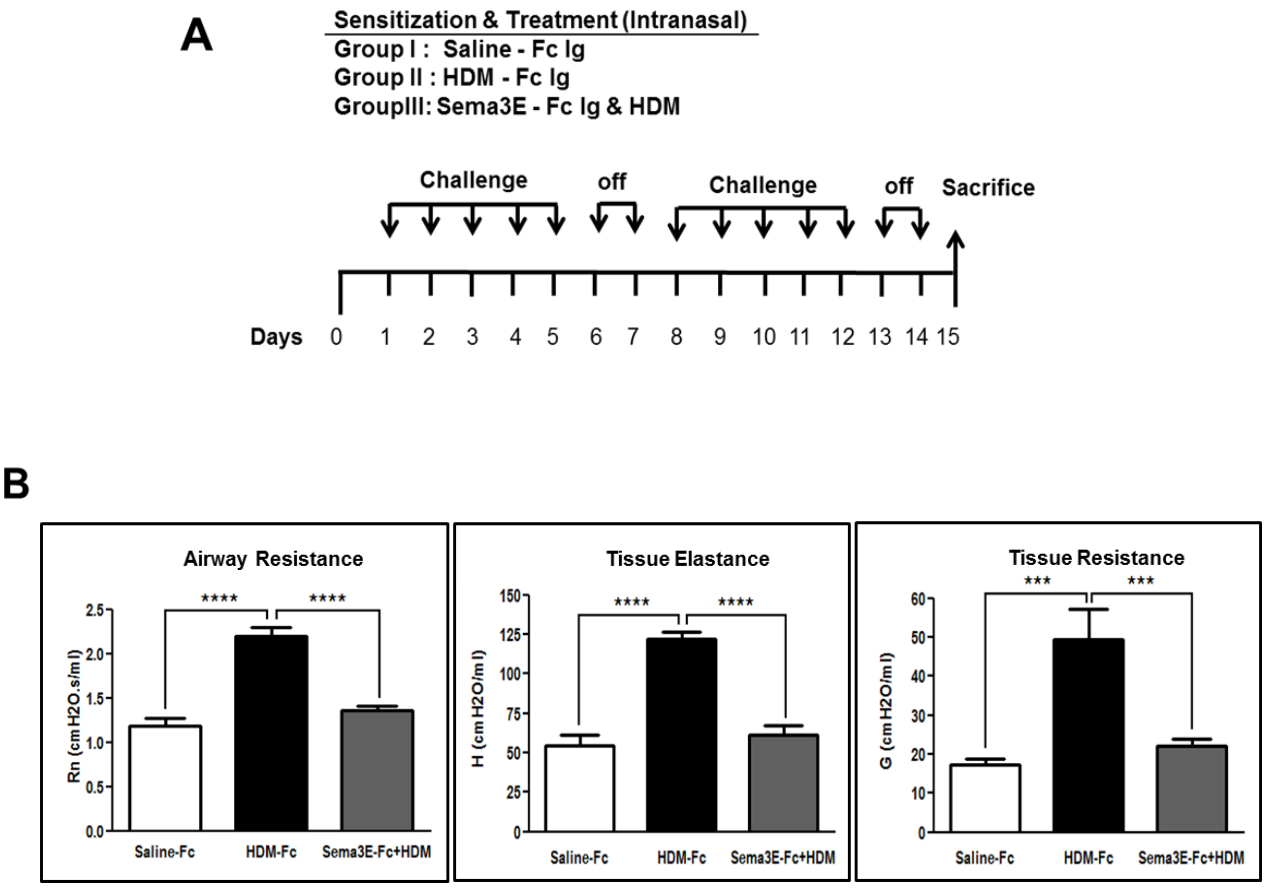
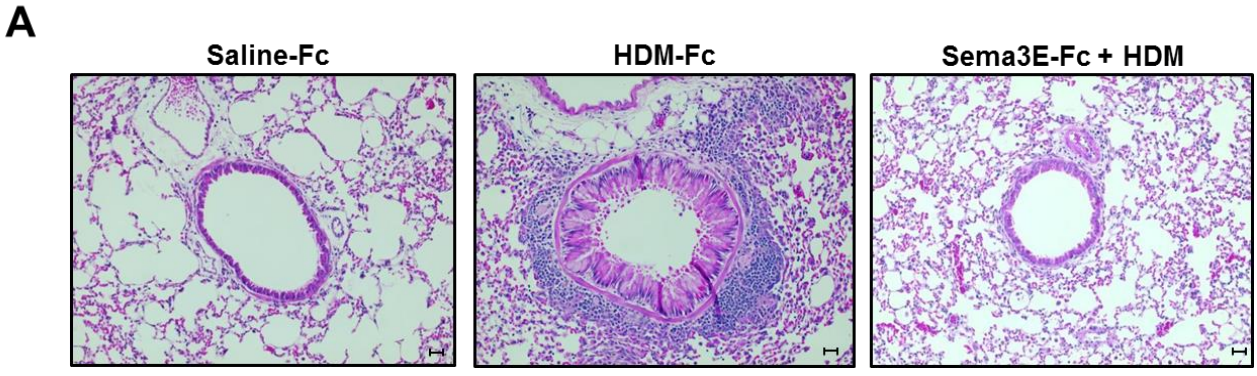


Figure 4.1. Sema3E-Fc treatment reduced the level of airway hyperresponsiveness in acute mouse model of HDM-induced allergic inflammation. (A) Acute mouse model of Sema3E treated vs. non-treated designed for investigating the effects of Sema3E on angiogenesis in allergic asthma. Female BALB/c mice (6–8 wk old) were challenged intranasally with House Dust Mite (HDM - Fc Ig 2.5mg/ml) or vehicle and Sema3E-Fc Ig (10 µg/kg per mouse) 1h before each HDM challenge. Each group contained four mice. (B) Sema3E treated mice shows attenuated level of airway hyperresponsiveness (Airway resistance [Rn], tissue elastance [H] and tissue resistance [G]) in response to methacholine (MCh) compared to HDM challenged mice. Statistical analyses was performed by one-way ANOVA, *** $P < 0.001$ and **** $P < 0.0001$. Error bars represent Mean ± SEM.

4.2 Sema3E-Fc treatment attenuates airway inflammation in HDM-challenged mice.

The level of inflammation in BALF and lung tissues was examined by performing cytologic examination on cytospin preparations by differential quik staining and H&E staining, respectively. Photomicrographs of stained sections showed increased level of peri-bronchial inflammation in HDM-challenged mice. However, the infiltration of inflammatory cells was significantly reduced in Sema3E treated group (Fig 4.2A). Concomitantly, total BALF cell count was significantly reduced in Sema3E-Fc treated mice compared to HDM-challenged and Fc treated control. In particular, Sema3E treatment reduced the level of granulocytes mainly eosinophils and neutrophils and re-establish to certain extend the level of macrophages mononuclear cells as well as B and T lymphocytes (Figure 4.2B). All together, these data reveal that Sema3E reduces the levels of HDM-induced airway inflammation particularly granulocytes and lymphocytes which are considered as one of the main sources of pro-angiogenic factors.



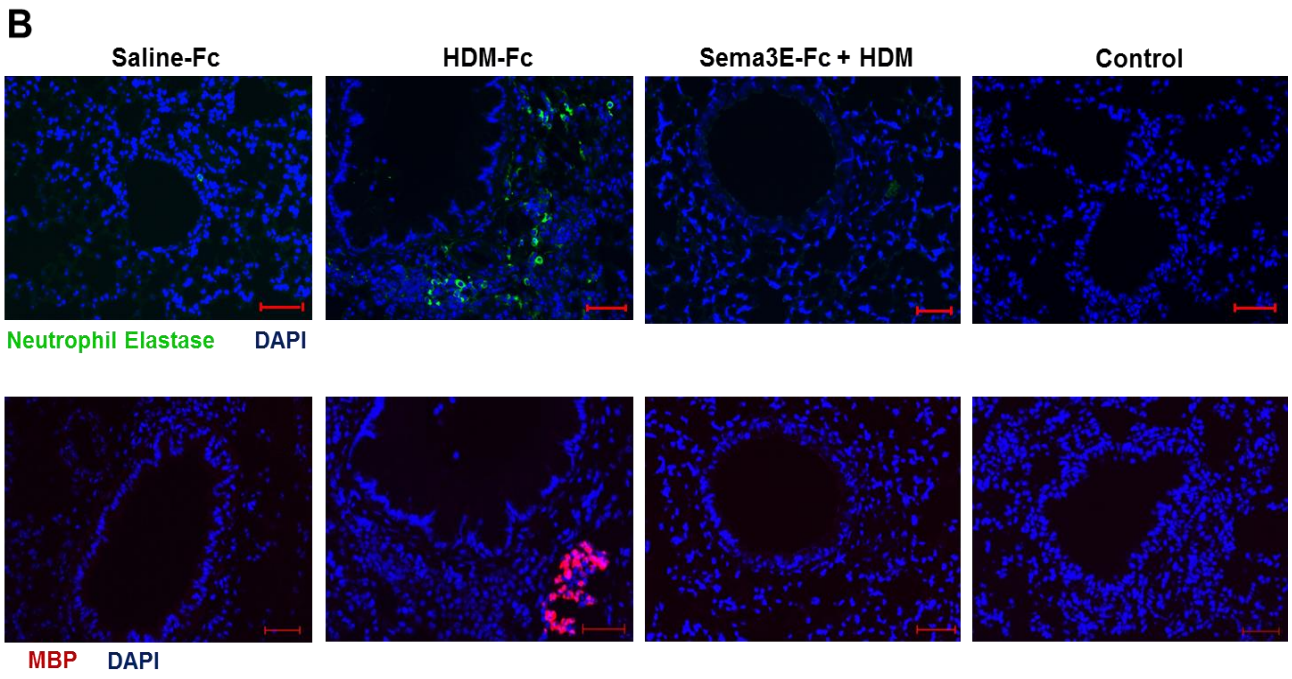
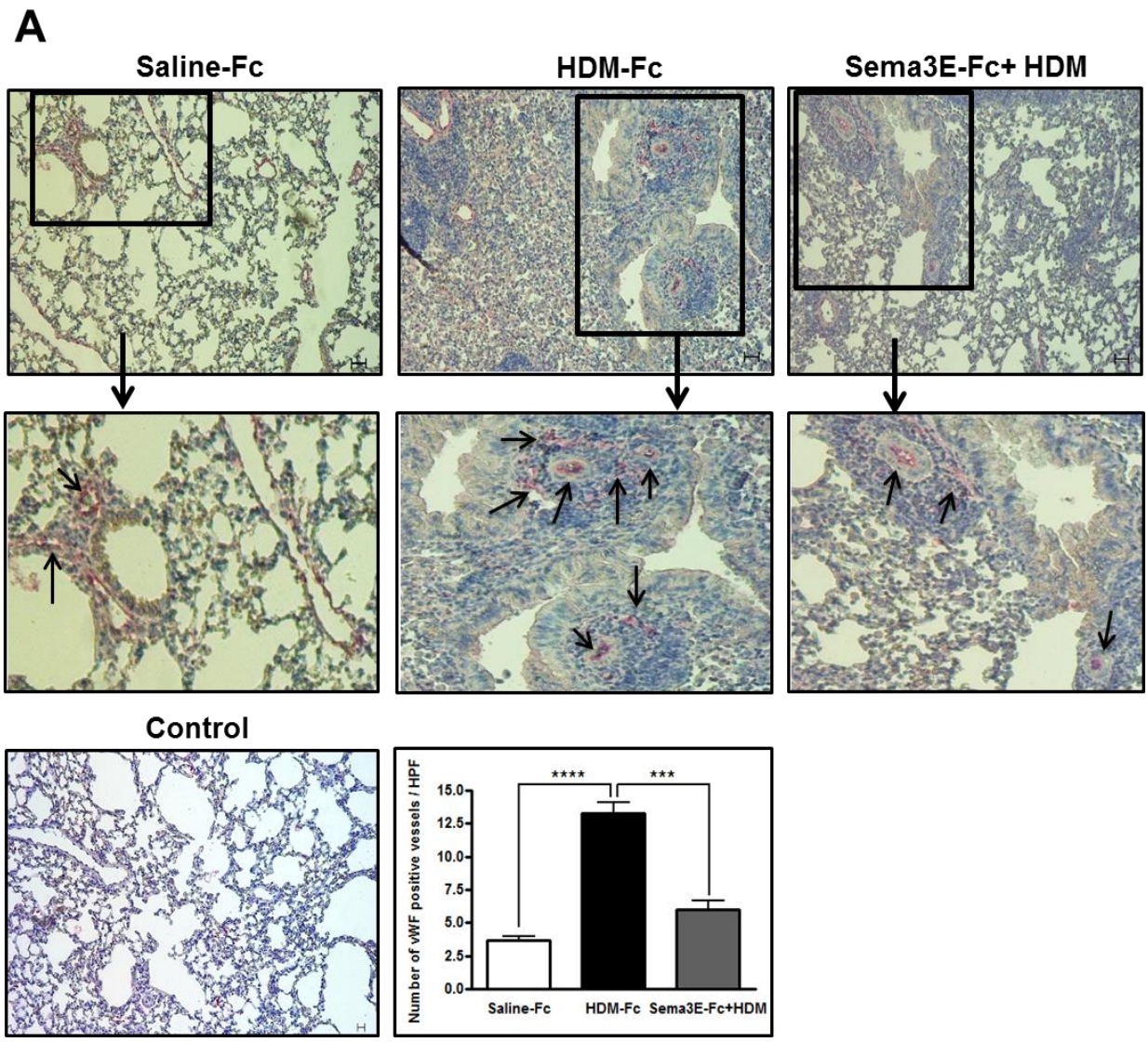
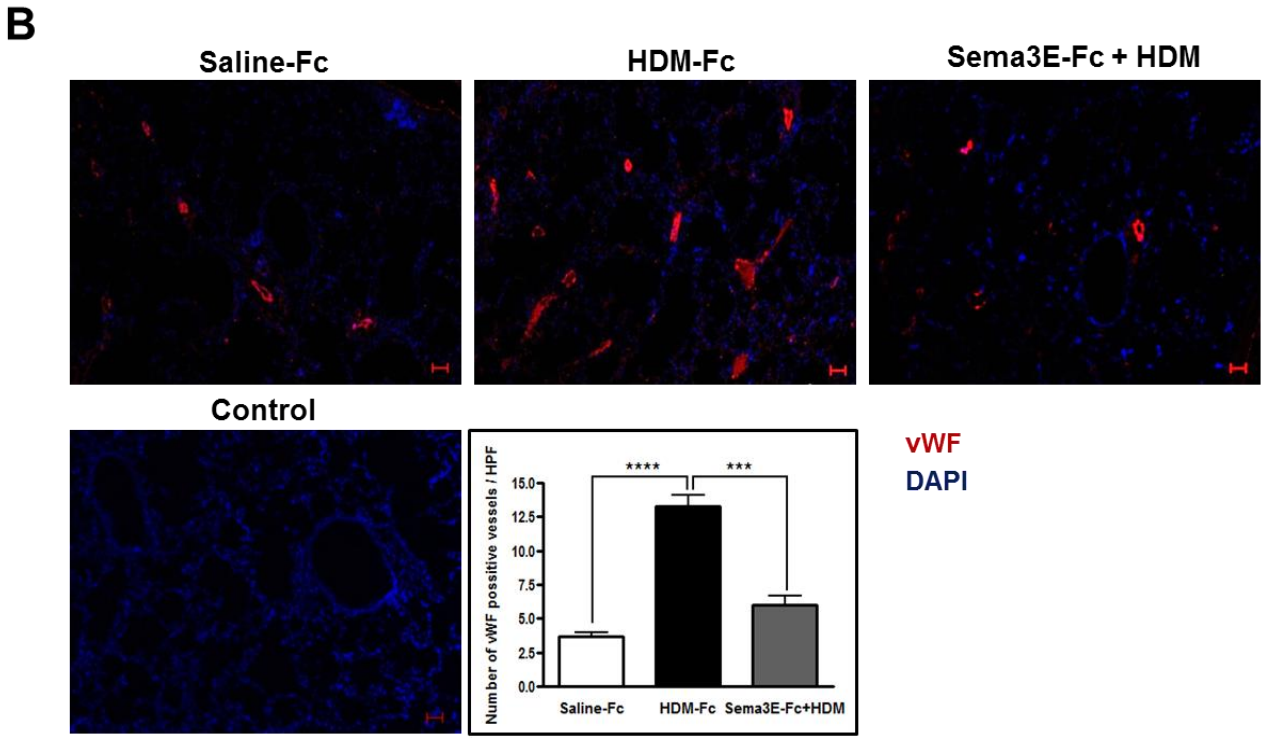


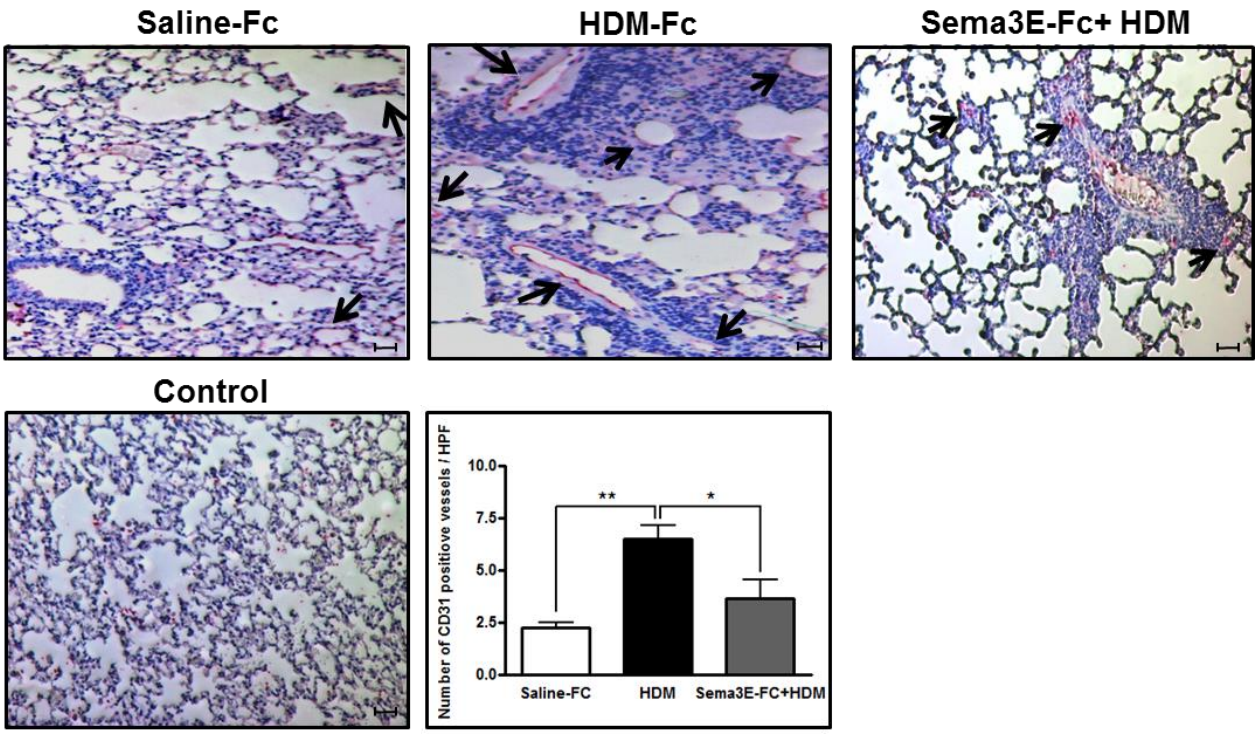
Figure 4.2. Sema3E decreases the level of inflammation in the airway of HDM-challenged mice. (A) Hematoxylin and eosin (H&E) staining of peri-bronchial sections shows reduced level of inflammation in Sema3E-Fc compared to HDM exposed animals. All Images were taken at x200. Scale Bar: 50 μm. (B) Lung sections were stained for neutrophil elastase and eosinophil major basic protein (MBP) to stain neutrophil and eosinophils for assessing the level of inflammation per lung tissue area. Scale bar, 50 μm.

4.3 Sema3E reduces angiogenesis in HDM challenged mice.

According to what have been shown in previous studies, the number of blood vessels significantly increases in the airways of asthmatic patients in compare to healthy controls [128, 235, 236]. In fact, angiogenesis, which is define as a process of formation of new blood vessels from pre-existing ones, is an important pathophysiological phenomena in airway remodeling in allergic asthma [66, 76, 128, 129, 237]. Several endothelial cell markers are used to characterize blood vessels. Among them, von Willebrand Factor (vWF), which is expressed specifically on ECs, is considered as the most specific marker for endothelial cells [238]. Also, CD31 is another important marker for characterizing ECs [239]. To examine the effect of Sema3E on pathological angiogenesis in asthma, immunohistochemistry and immunofluorescence staining, were performed using anti-vWF and anti-CD31 antibody on bronchial sections of HDM challenged Sema3E-Fc or Fc-Ig control pre-treated mice. According to figure 4.3 A-C the number of newly formed blood vessels was significantly reduced in Sema3E-Fc treatment group compared to HDM challenged mice (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$). These data suggest that Sema3E reduces lung blood vessels formation in mouse model of HDM induced airway allergic inflammation.


A
Saline-Fc
HDM-Fc
Sema3E-Fc+ HDM
Control
Number of vWF positive vessels / HPF
15.0
12.5
10.0
7.5
5.0
2.5
0.0
****
***
Saline-Fc
HDM-Fc
Sema3E-Fc+HDM


B
Saline-Fc
HDM-Fc
Sema3E-Fc + HDM
Control
vWF
DAPI
Number of vWF possitive vessels / HPF
15.0
12.5
10.0
7.5
5.0
2.5
0.0
****
***
Saline-Fc
HDM-Fc
Sema3E-Fc+HDM


C
Saline-Fc
HDM-Fc
Sema3E-Fc+ HDM
Control
Number of CD31 positiove vessels / HPF
10.0
7.5
5.0
2.5
0.0
**
*
Saline-FC
HDM
Sema3E-FC+HDM

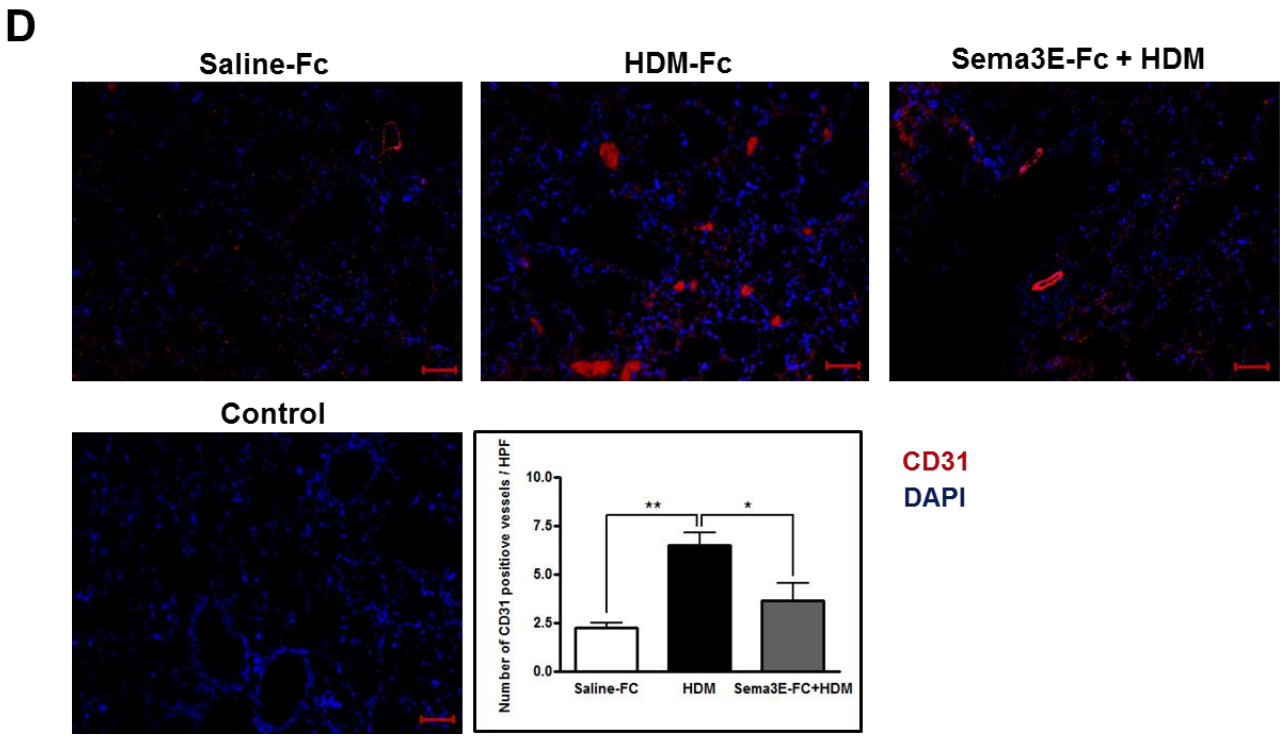


Figure 4.3: Sema3E-Fc treatment reduces angiogenesis in HDM induced allergic asthmatic mice. (A, C) Lung sections were stained by immunohistochemistry for Von Willebrand factor (vWF) and CD31 to assess the number of blood vessels per lung tissue area (B, D). Bronchial sections stained for von Willebrand factor and CD31 by Immunofluorescence staining to further confirm immunohistochemical staining. The experiment performed to visualize blood vessels and quantify the number of vessels per lung tissue area. All Images were taken at x100 magnification. Scale Bar: 50 μm. (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001). Statistical analyses was performed by one-way ANOVA, Error bars represent Mean ± SEM.

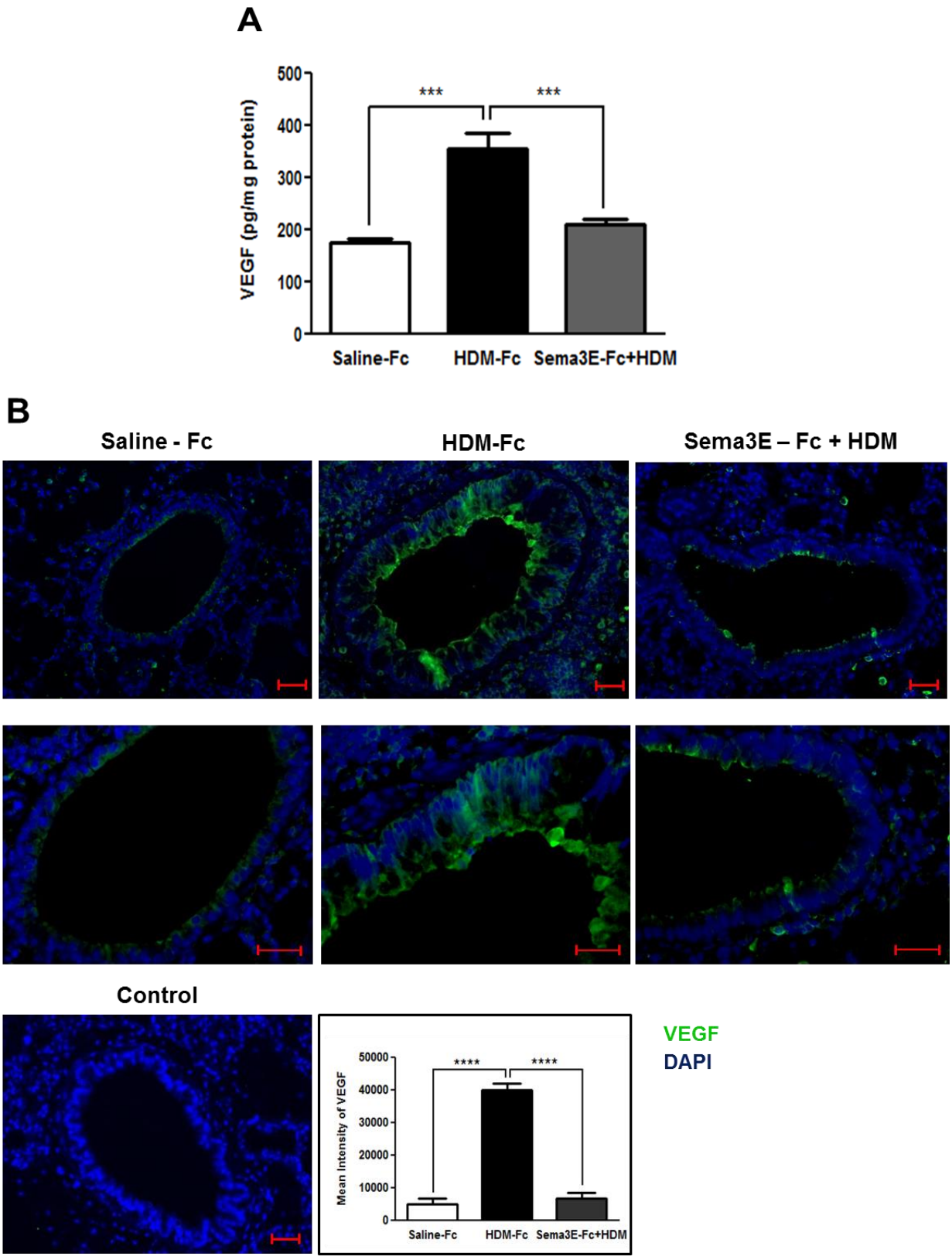
4.4 Sema3E decreases angiogenesis via reducing VEGF production and VEGFR-2 expression in the lung of HDM exposed mice.

Previous studies have obviously indicated a mitogenic role of VEGF for endothelial cells. This growth factor has a key role in increasing the proliferation and differentiation of endothelial cells via high affinity tyrosine kinase receptor, VEGFR-2, which culminates increased level of angiogenesis [130, 211, 240-242].

To investigate the role of Sema3E on VEGF production in asthmatic condition, ELISA and immunofluorescence staining was performed using lung homogenate and lung sections, respectively. According to our result (Fig 4.4 A), the level of VEGF production was significantly reduced in lung homogenate of HDM-challenged and Sema3E treated mice compared to HDM challenged alone (*** $P < 0.001$).

Furthermore, immunofluorescence staining showed reduced level of VEGF in lung sections of HDM-challenged and Sema3E treated mice in compare to HDM challenged alone. VEGF staining was predominantly positive in epithelial cell layers, airway smooth muscle cells and inflammatory cells (Fig 4.4B). These cells have been previously recognized as the main source of VEGF production in the airways [236, 243]. Taken together, our data suggest that Sema3E can decrease the level of angiogenesis in allergic asthmatic airways by reducing the level of VEGF expression in the airways. The angiogenic effect of VEGF is mediated through binding to high affinity tyrosine kinase receptor VEGFR-2. In fact, VEGFR-2 has been recognized as a major mediator of angiogenic and mitogenic effect of VEGF [130, 244]. VEGFR-2 immunofluorescence

staining was performed on lung sections to study the effect of Sema3E on expression of VEGFR-2 on blood vessels. According to our result, the level of VEGFR-2 staining was decreased in lung vessels of HDM-challenged and Sema3E treated mice in compare to HDM challenged alone (** $P < 0.0025$) (Fig 4.4 C). Overall, these data suggest that Sema3E plays an important role in reducing the level of angiogenesis in allergic asthmatic airways by decreasing the level of VEGFR-2 expression on vessels in addition to reducing the level of VEGF production as the most important pro-angiogenic factor.


A
VEGF (pg/mg protein)
500
400
300
200
100
0
***
***
Saline-Fc
HDM-Fc
Sema3E-Fc+HDM
B
Saline - Fc
HDM-Fc
Sema3E – Fc + HDM
Control
Mean Intensity of VEGF
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HDM-Fc
Sema3E-Fc+HDM
VEGF
DAPI

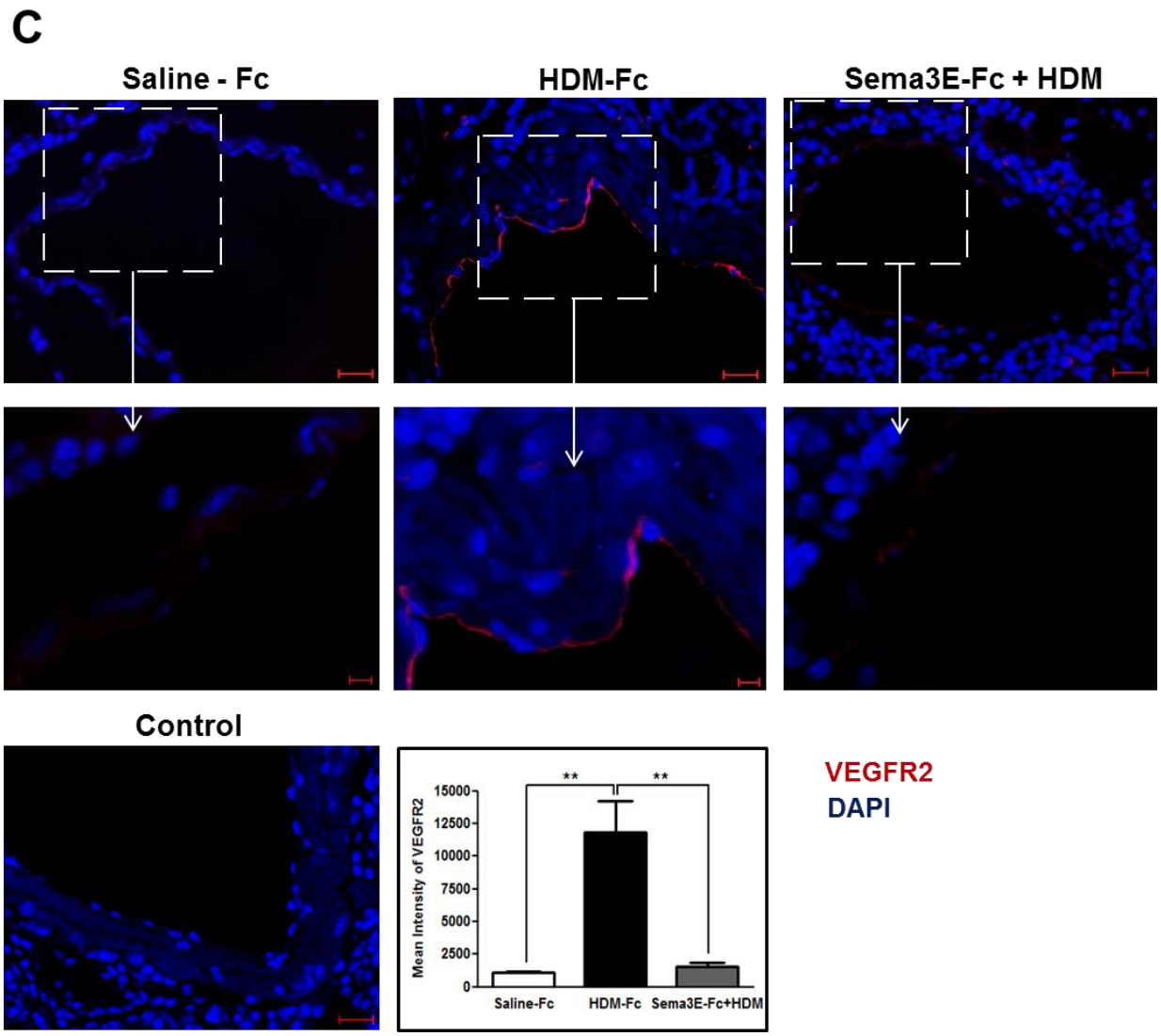


Figure 4.4: Sema3E-Fc treatment reduces the level of VEGF and VEGFR-2 expression in the airways of HDM exposed mice (A) The level of VEGF was measured in lung homogenate by performing ELISA and normalized to total protein. (B) Immunofluorescence staining was performed on lung sections using anti VEGF antibody to compare the level of VEGF production in the airway of Sema3E treated and HDM exposed mice. Low and high magnification (x200 and x400) pictures of Lung tissues were taken and mean intensity was measured per lung tissue area (***$P < 0.001$ and ****$P < 0.0001$). (C) Lung sections were stained for VEGFR-2 to assess the level of

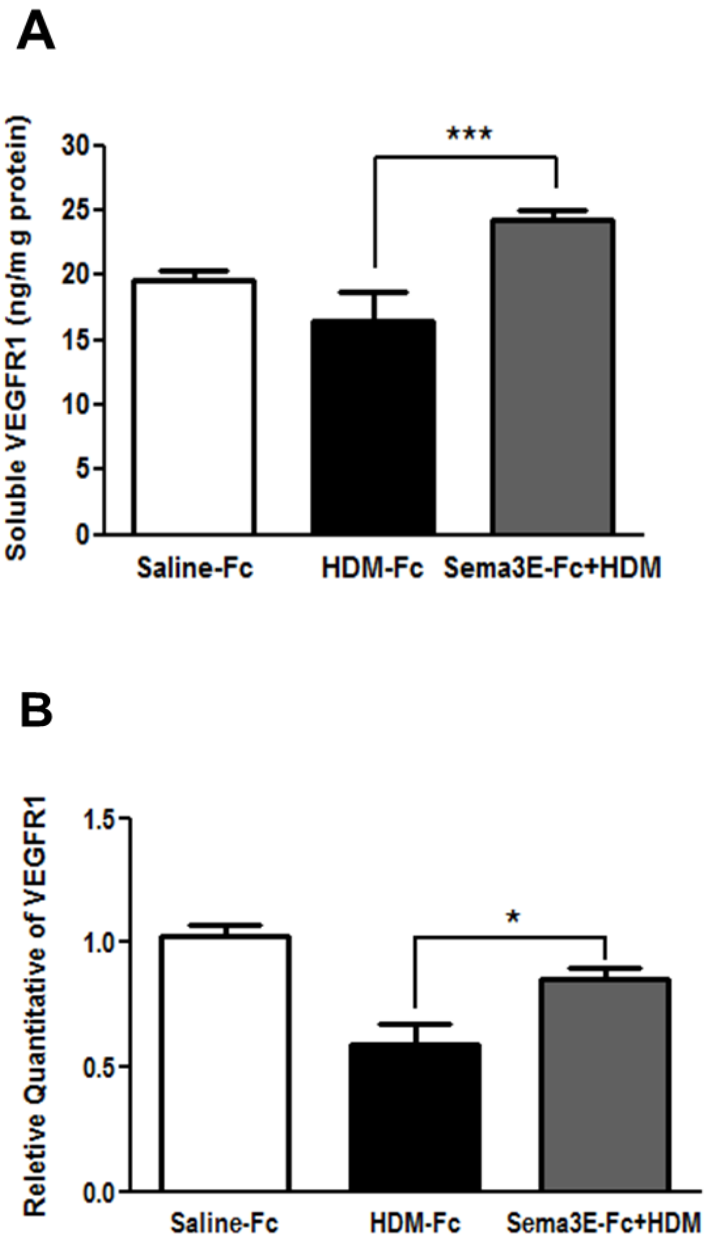
VEGFR-2 expression on the endothelial cell of vessels in the airway of mice. Low and high magnification (x400 and x1000) pictures of lung tissue were taken and mean intensity was measured per lung tissue area (**$P < 0.0025$). Statistical analyses was performed by one-way ANOVA, Error bars represent Mean ± SEM.

4.5 Anti-angiogenic role of Sema3E is mediated via increasing the level of soluble VEGFR-1

VEGFR-1 is another receptor for VEGF which has different function than VEGFR-2. Soluble VEGFR-1 (soluble Flt-1) is produced as a result of alternative splicing of the VEGFR-1 gene and act as a decoy receptor. It inhibits the binding of VEGF to VEGFR-2 and negatively regulates the activity of VEGF on the vascular endothelium [76, 130, 245-247]. In developmental study in zebra fish, it is shown that activation of PlexinD1 signaling on endothelial cells, after binding to Sema3E, induces soluble Flt-1 production which negatively controls VEGF signaling [209].

To investigate the effect of Sema3E on soluble Flt-1 production in mouse model of HDM induced allergic asthma ELISA was performed. According to our result, in the HDM-challenged and Sema3E treated mice the level of soluble Flt-1 was increased in compare to HDM challenged alone (*** $P < 0.001$) (Fig 4.5 A). In addition, gene expression study and immunofluorescence staining confirmed our results about the effective role of Sema3E in inducing soluble Flt-1 production. In fact, according to these data, the level of soluble Flt-1 increased in HDM-challenged and Sema3E treated mice compared to HDM alone challenged group (* $P < 0.05$, Fig 4.5 B and *** $P < 0.001$, Fig 4.5 C). Altogether, our data suggest that Sema3E reduces angiogenesis via enhancing soluble Flt-1 decoy receptor expression in Sema3E treated HDM induced allergic asthma. It conveys this message that as a result of binding of Sema3E to its receptor PlexinD1on endothelial tip cells, soluble Flt-1 will be produced and binds to VEGF. As a consequence, the amount

of VEGF that can bind to VEGFR2 on endothelial tip cells decreases which leads to inhibition of angiogenesis [209].



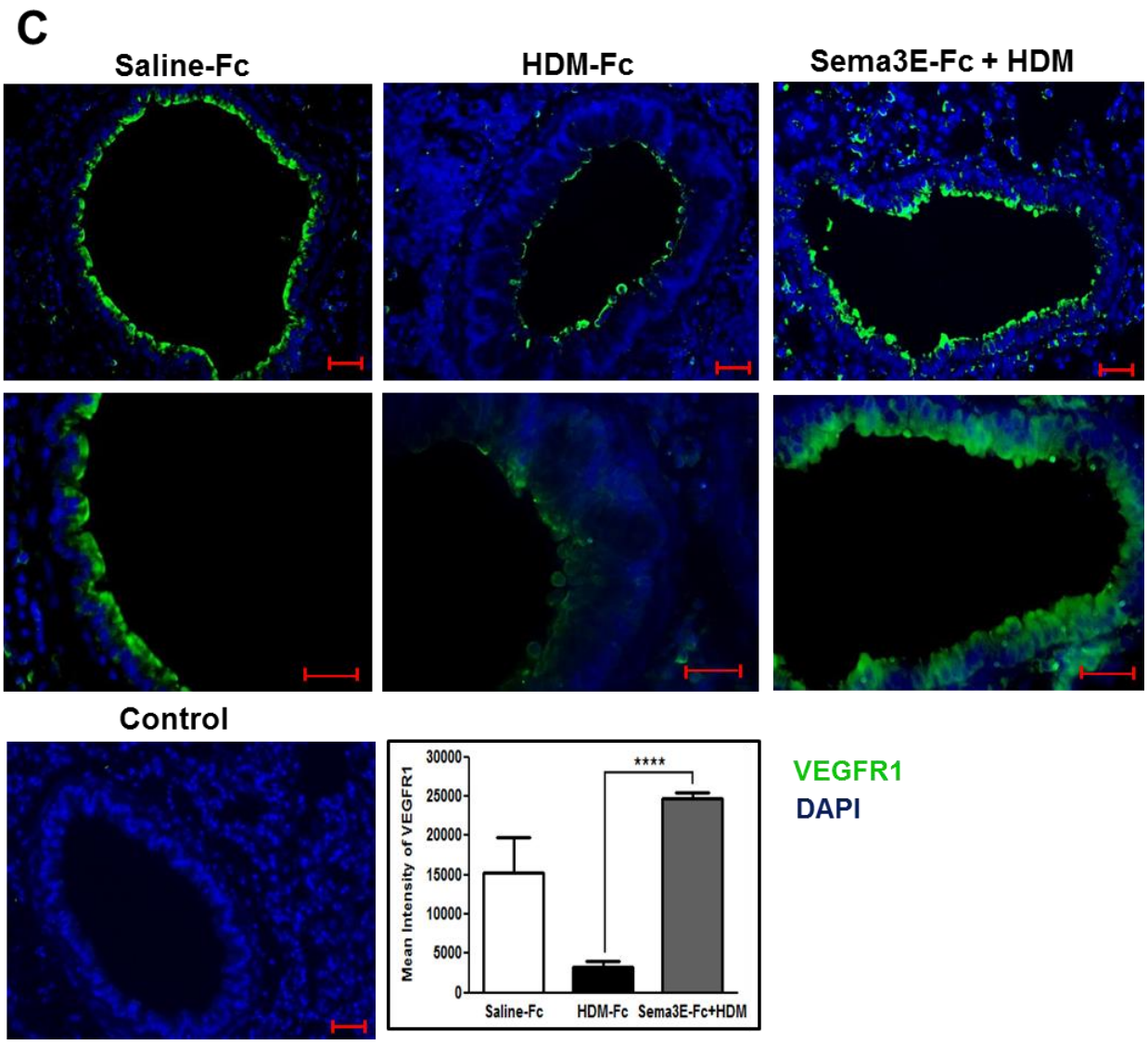


Figure 4.5: Sema3E acts as an anti-angiogenic molecule by stimulating the production of soluble VEGFR-1, the decoy receptor of VEGF (A) ELISA was performed to measure the level of Soluble VEGFR-1 in lung homogenate (*** $P <$ 0.001). (B) Real-Time PCR was done to study the level of soluble VEGFR-1 gene expression (* $P < 0.05$). (C) Lung sections were stained by anti-Soluble VEGFR-1 antibody to study the level of Soluble VEGFR-1 production in the airway of mice. Low and high magnification (x200 and x400) pictures of lung tissues were taken and mean intensity was calculated per lung tissue area (***$P <$ 0.001). Statistical analyses was performed by one-way ANOVA, Error bars represent Mean ± SEM.

4.6 Sema3E reduces angiogenesis by changing the ratio of VEGF to soluble VEGFR-1

The ratio of VEGF / soluble VEGFR-1 was measured after performing ELISA to recognize the dominant element before and after Sema3E treatment. Based on statistical analysis, the ratio of VEGF / soluble Flt-1 decreased significantly in HDM-challenged and Sema3E treated mice in compare to HDM challenged alone (** $P < 0.0045$ and * $P < 0.05$) (Fig 4.6). It shows that VEGF is the dominant factor in HDM challenged mice. However in HDM-challenged and Sema3E treated mice soluble Flt-1 acts as a dominant factor and reduces the level of angiogenesis by preventing the binding of VEGF to VEGFR-2.

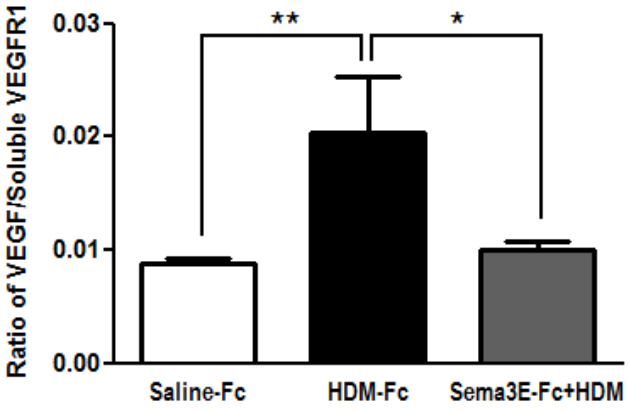


Figure 4.6: Sema3E-Fc treatment increased the level of soluble VEGFR-1 production. After determining the level of VEGF and Soluble VEGFR1 by ELISA using lung homogenate, the ratio of VEGF / Soluble VEGFR1 was calculated (** $P < 0.0045$ and * $P < 0.05$). Statistical analyses was performed by one-way ANOVA, Error bars represent Mean ± SEM.

4.7 Airway hyperresponsiveness increases significantly in *Sema3e*$^{-/-}$ animals.

To further confirm our result regarding the effective role of Sema3E on inhibiting the angiogenesis in HDM induced allergic asthma, we aimed to investigate the importance of Sema3e gene on angiogenesis in allergic asthmatic mouse model. To conduct our research, age-matched (6-8 wk old) Sema3e Knock out (*Sema3e*$^{-/-}$) and wild type (WT) littermate was generated using female 129p2 mice. 2 groups were designed in this model as depicted in Fig 4.7 A including acute model of WT and *Sema3e*$^{-/-}$ HDM challenged mice (HDM 2.5 mg/ml), WT and *Sema3e*$^{-/-}$ saline control group.

According to our result, the level of airway resistance, tissue resistance and tissue elastance were elevated dramatically in *Sema3e*$^{-/-}$ HDM challenged mice in response to MCh compared to WT littermates (* $P < 0.05$ and **** $P < 0.0001$) (Fig 4.7B). Together, these data showed that the presence of *Sema3e* gene plays a key role in preventing the development of airway hyperresponsiveness in WT animals compared to *Sema3e*$^{-/-}$.

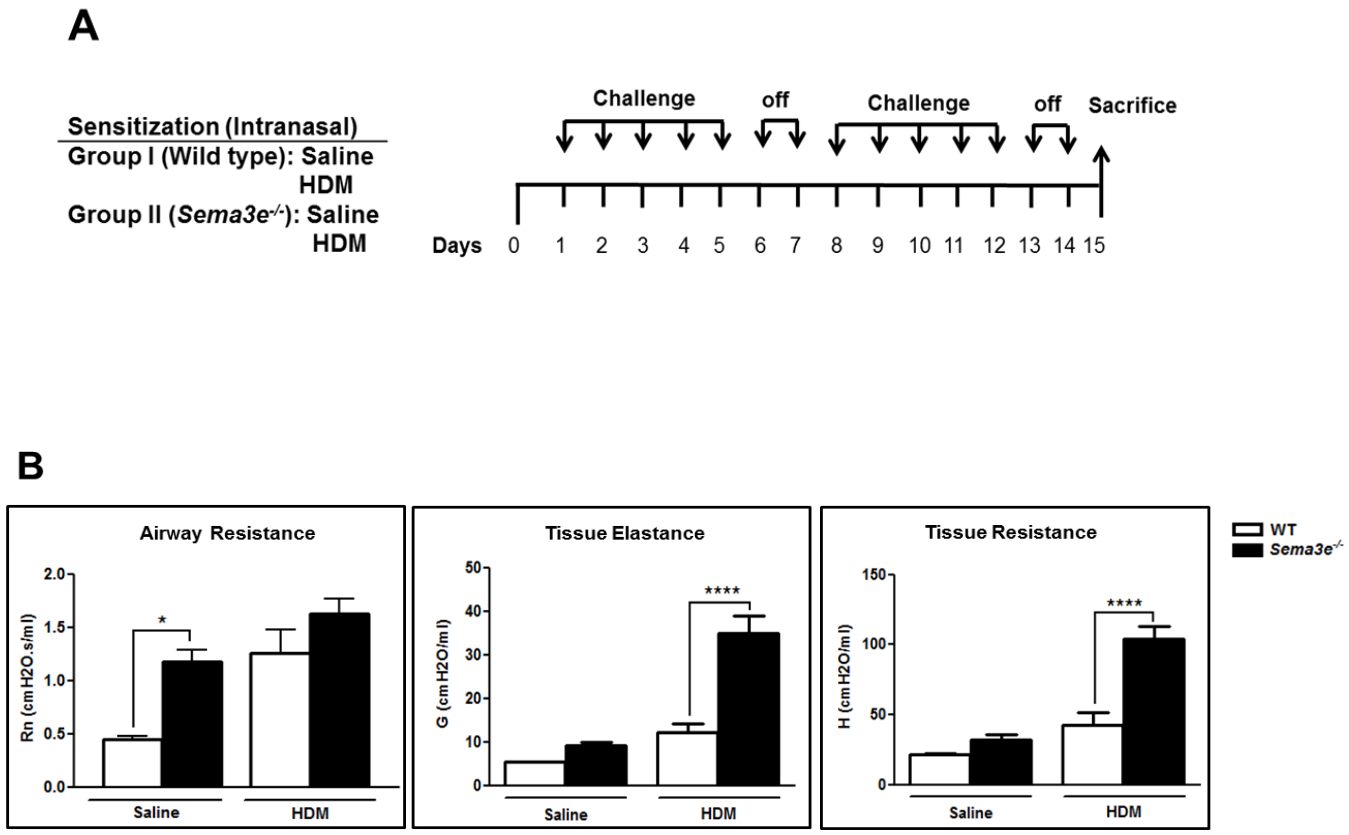
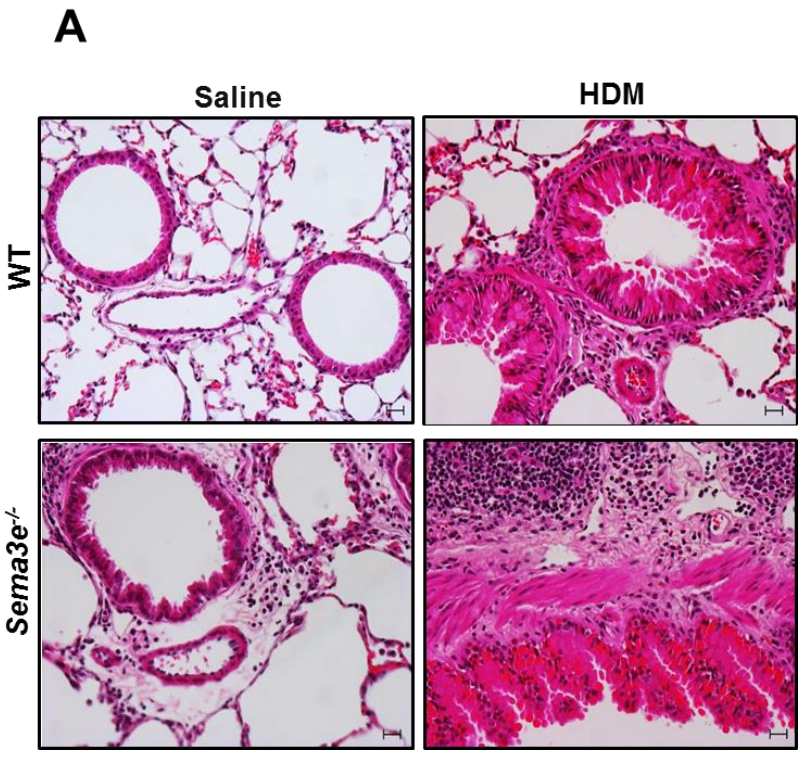


Figure 4.7: Acute mouse model of *Sema3e*$^{-/-}$ indicates increased level of airway hyperresponsiveness. (A) Acute mouse model of wild type (WT) vs. Sema3e Knock out (*Sema3e*$^{-/-}$) were designed to investigate the importance of *Sema3e* gene on angiogenesis in allergic asthma. Female 129P2 mice (6–8 wk old) were challenged intranasally with HDM (2.5 mg/ml) or vehicle. Each group contained four mice. (B) *Sema3e*$^{-/-}$ mice indicated the higher level of airway hyperresponsiveness in response to methacholine (MCh). Airway resistance (Rn), tissue elastance (H) and tissue resistance (G) of *Sema3e*$^{-/-}$ mice were elevated in compare to WT animals (* $P < 0.05$ and **** $P < 0.0001$). Statistical analyses was performed by one-way ANOVA, Error bars represent Mean ± SEM.

4.8 The level of airway inflammation is significantly higher in the absence of *Sema3e* gene (*Sema3e*$^{-/-}$)

Cytologic examination by differential quik staining on BALF cytospin preparations and H&E staining revealed the higher level of peri-bronchial inflammation in *Sema3e*$^{-/-}$ mice compared to WT littermates (Fig 4.8 A). Alongside, total BAL cell count was significantly increased in the absence of *Sema3e* gene. According to staining, the level of granulocytes, including eosinophils and neutrophils increased significantly in *Sema3e*$^{-/-}$ animals in both saline and HDM challenged mice in compare to WT littermates (Fig 4.8 B). Taken together, our results suggest that presence of Sem3e gene is pivotal in attenuating the levels of airway inflammation.



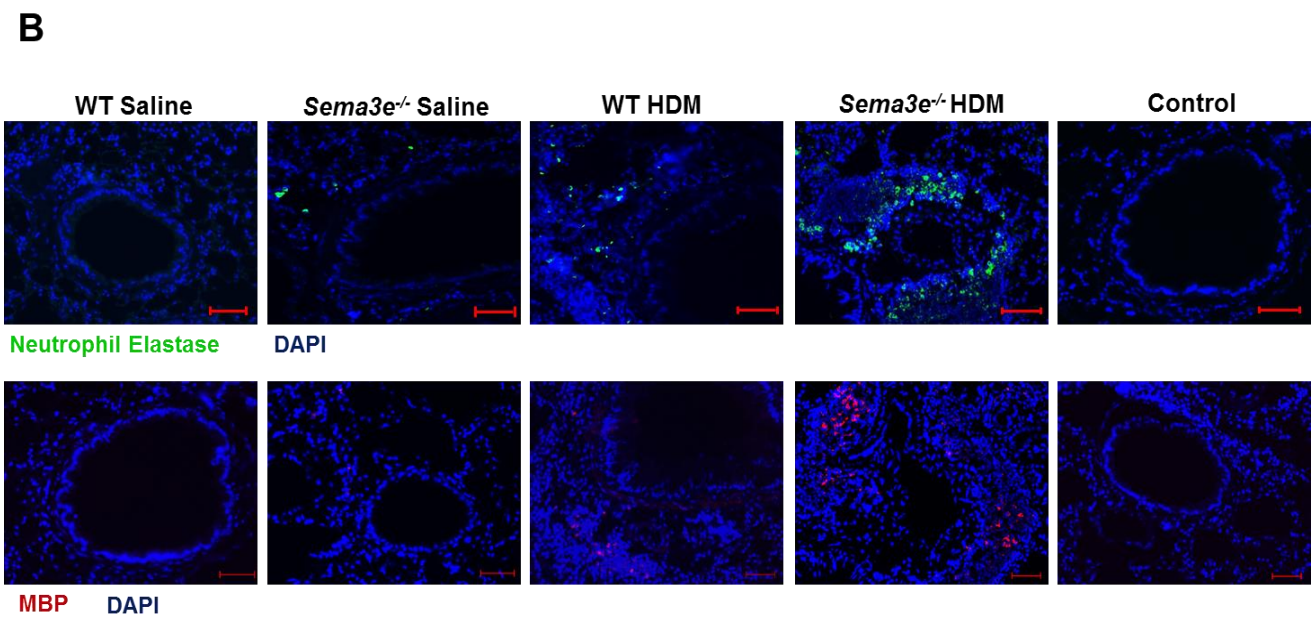
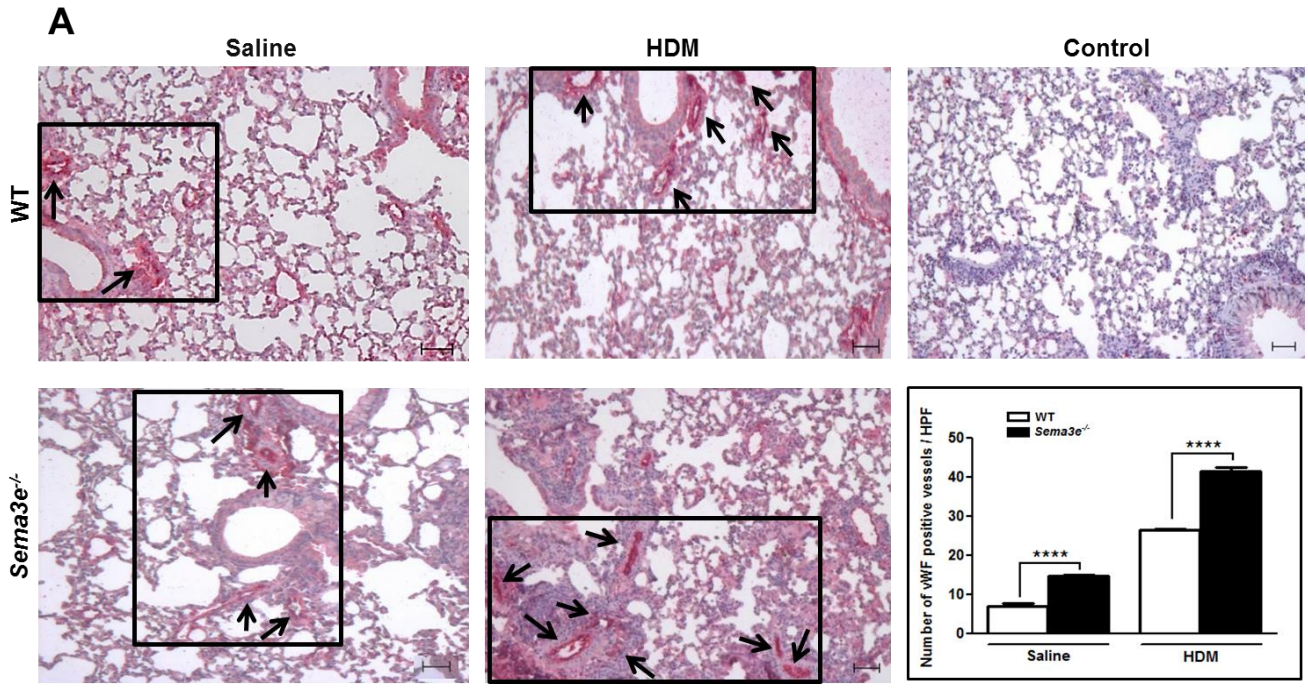


Figure 4.8. *Sema3e*$^{-/-}$ mice reveal higher level of airway inflammation compared to WT littermates (A) Hematoxylin and eosin (H&E) staining of peri-bronchial sections illustrates increased level of inflammation in HDM challenged and saline treated *Sema3e*$^{-/-}$ mice compared to WT animals. All Images were taken at x200. Scale Bar: 50 μm. (B) Bronchial sections were stained for neutrophil and eosinophil by targeting neutrophil elastase and major basic protein (MBP) to assess the level of inflammation per lung tissue area. Scale bar, 50 μm.

4.9 The level of angiogenesis increased significantly in the airways of *Sema3e*$^{-/-}$ mice.

As mentioned earlier, the number of newly formed blood vessels increases in the airways of asthmatic patients as an important pathophysiological feature of airway remodeling [66, 76, 128, 129, 237]. To investigate the differences between the level of angiogenesis in the presence and absence of Sema3e gene, Immunohistochemistry and Immunofluorescence staining was performed on bronchial sections. Anti-vWF antibody was used for targeting vWF as a most important marker for characterizing endothelial cells [238, 239]. According to figure 4.9 the number of newly formed blood vessels was significantly increased in the absence of *Sema3e* gene (** $P < 0.01$ and **** $P < 0.0001$). These data suggest that *Sema3e* gene has a regulatory role on formation of new blood vessels in both HDM exposed animals and control groups.


A
Saline
HDM
Control
WT
Sema3e-/-
WT
Sema3e-/-
Number of vWF positive vessels / HPF
50
40
30
20
10
0
****
****
Saline
HDM

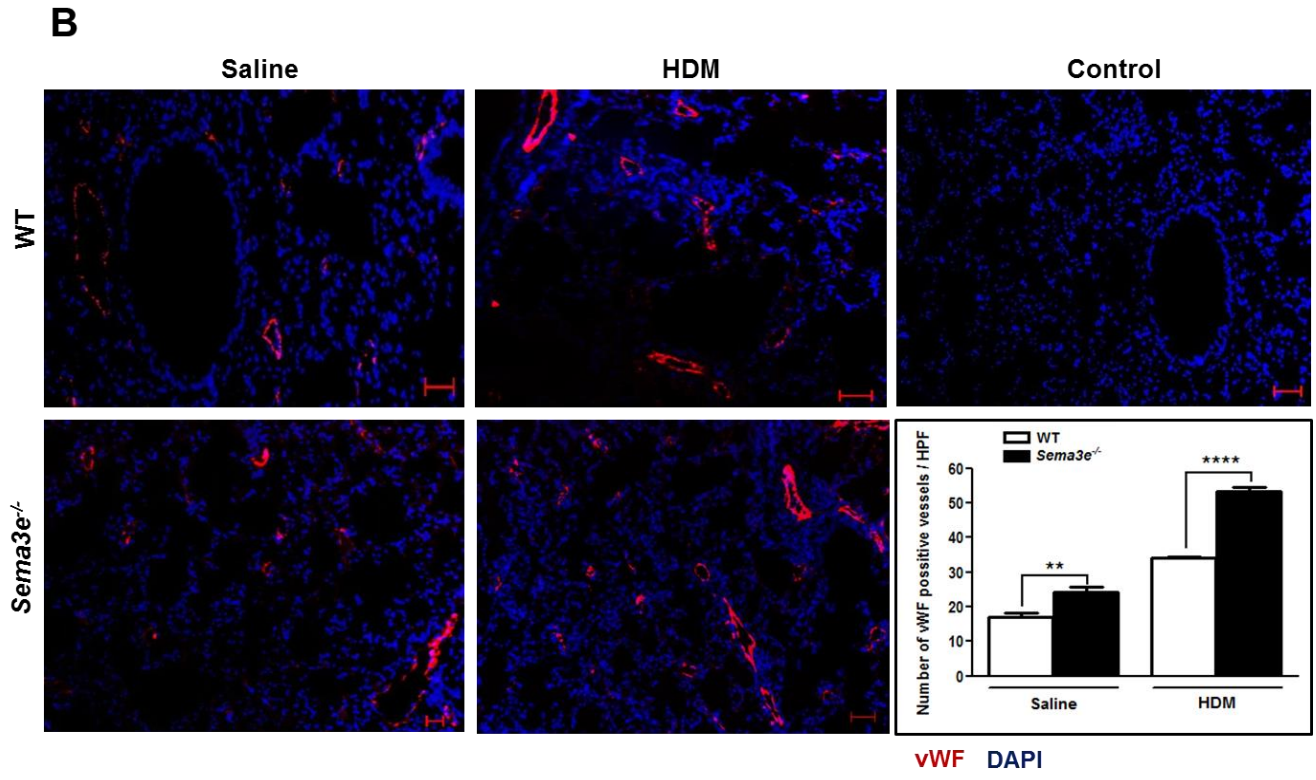

B
Saline
HDM
Control
WT
Sema3e-/-
WT
Sema3e-/-
Number of vWF possitive vessels / HPF
60
50
40
30
20
10
0
**
****
Saline
HDM
vWF DAPI

Figure 4.9: *Sema3e*$^{-/-}$ mice shows significantly more number of newly formed blood vessels in the airways compared to WT littermates. (A) Immunohistochemistry was performed on lung sections using vWF antibody to visualize and quantify the number of blood vessels per lung tissue area. (B) In another part of experiment, lung tissue sections were used for immunofluorescence staining for vWF to quantify and compare the level of angiogenesis in the absence and presence of *Sema3e* gene. Scale bar, 50 μm. Pictures were taken with x100 magnification. Statistical analyses was performed by one-way ANOVA (** $P < 0.01$ and **** $P < 0.0001$). Error bars represent Mean ± SEM.

4.10 Elevated level of VEGF and VEGFR-2 in *Sema3e*$^{-/-}$ mice can increase the level of angiogenesis.

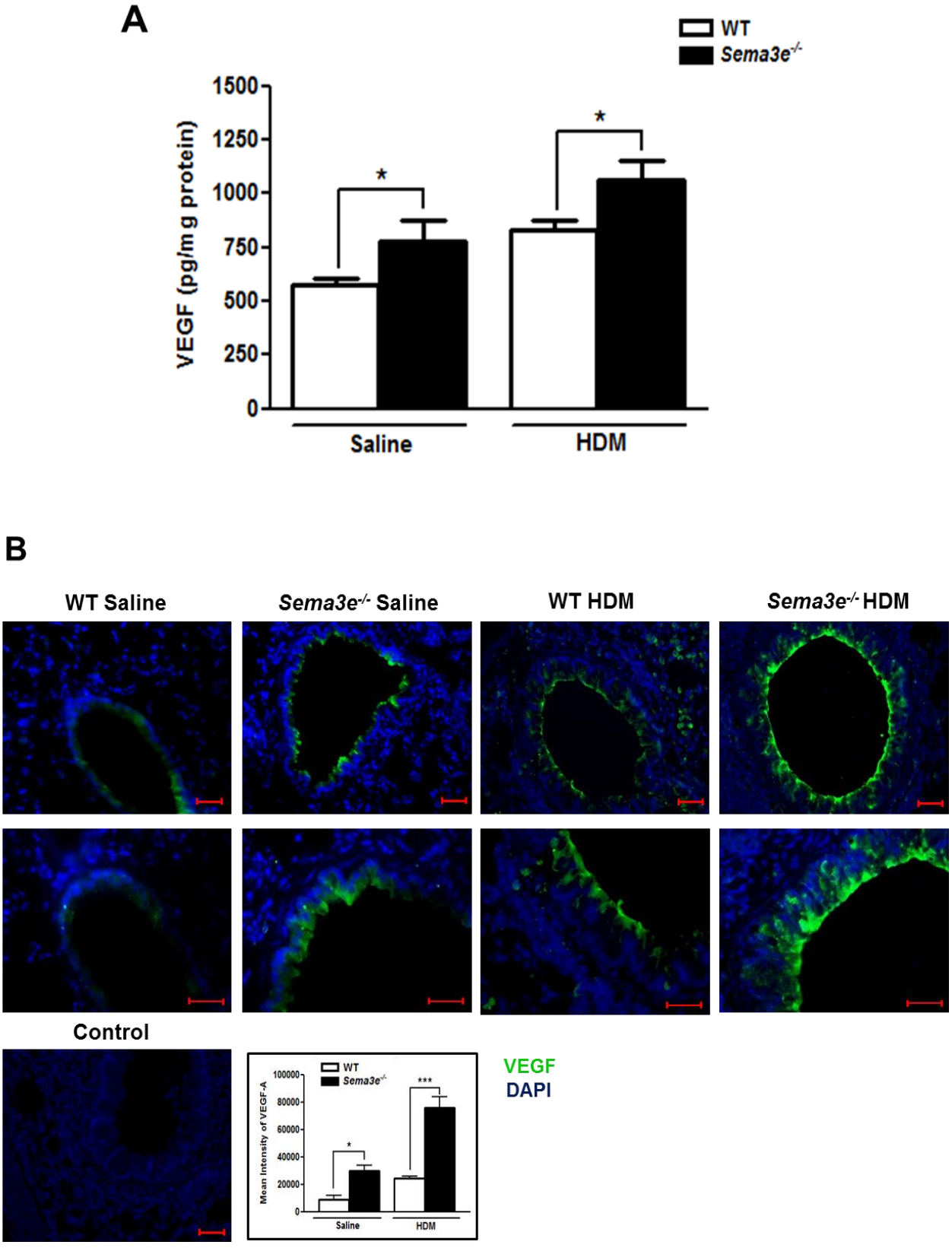
As mentioned previously, VEGF has been recognized as a most important pro-angiogenic factor in the asthmatic airways. It acts as a mitogen for endothelial cells and induces endothelial cells proliferation and differentiation via high affinity tyrosine kinase receptor, VEGFR-2 [130, 211, 240-242].

To investigate the role of Sema3e gene on regulating VEGF production in asthmatic condition, ELISA and immunofluorescence staining were performed on *Sema3e*$^{-/-}$ and WT lung homogenate and lung sections, respectively. According to Fig 4.10 A, *Sema3e*$^{-/-}$ animals show higher level of VEGF production compared to WT littermates (*** $P < 0.001$).

In addition, the increased level of VEGF was observed on lung sections of *Sema3e*$^{-/-}$ animals by performing immunofluorescence staining. As shown in Fig 4.10 B, Epithelial cell layers, airway smooth muscle cells and inflammatory cells, which are the main source of VEGF in asthmatic airways [236, 243], were mainly positive for VEGF staining in *Sema3e*$^{-/-}$ mice. These data were a further confirmation of the possible regulatory role of *Sema3e* on production of pro-angiogenic factor, VEGF, in allergic asthmatic airways.

In the next part of our study we focused on VEGFR-2 which has been recognized as the main mediator of angiogenic effect of VEGF [130, 244]. Immunofluorescence staining

was performed to assess VEGFR-2 expression on pre-bronchial sections of *Sema3e*$^{-/-}$ and WT littermates to study the level of VEGFR-2 expression on endothelial cells. This experiment revealed the increased level of VEGFR-2 expression in *Sema3e*$^{-/-}$ compared to WT animals (**$P < 0.01$) (Fig 4.10 C). Altogether, our data suggest that Sema3e may play a crucial role in inhibiting increased angiogenesis in allergic asthmatic airways by regulating the level of VEGF and VEGFR-2 as the two most important elements in promoting the angiogenesis process.


A
WT
Sema3e-/-
VEGF (pg/m g protein)
1500
1250
1000
750
500
250
0
*
*
Saline
HDM
B
WT Saline
Sema3e-/- Saline
WT HDM
Sema3e-/- HDM
Control
Mean Intensity of VEGF-A
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40000
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WT
Sema3e-/-
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Saline
HDM
VEGF
DAPI

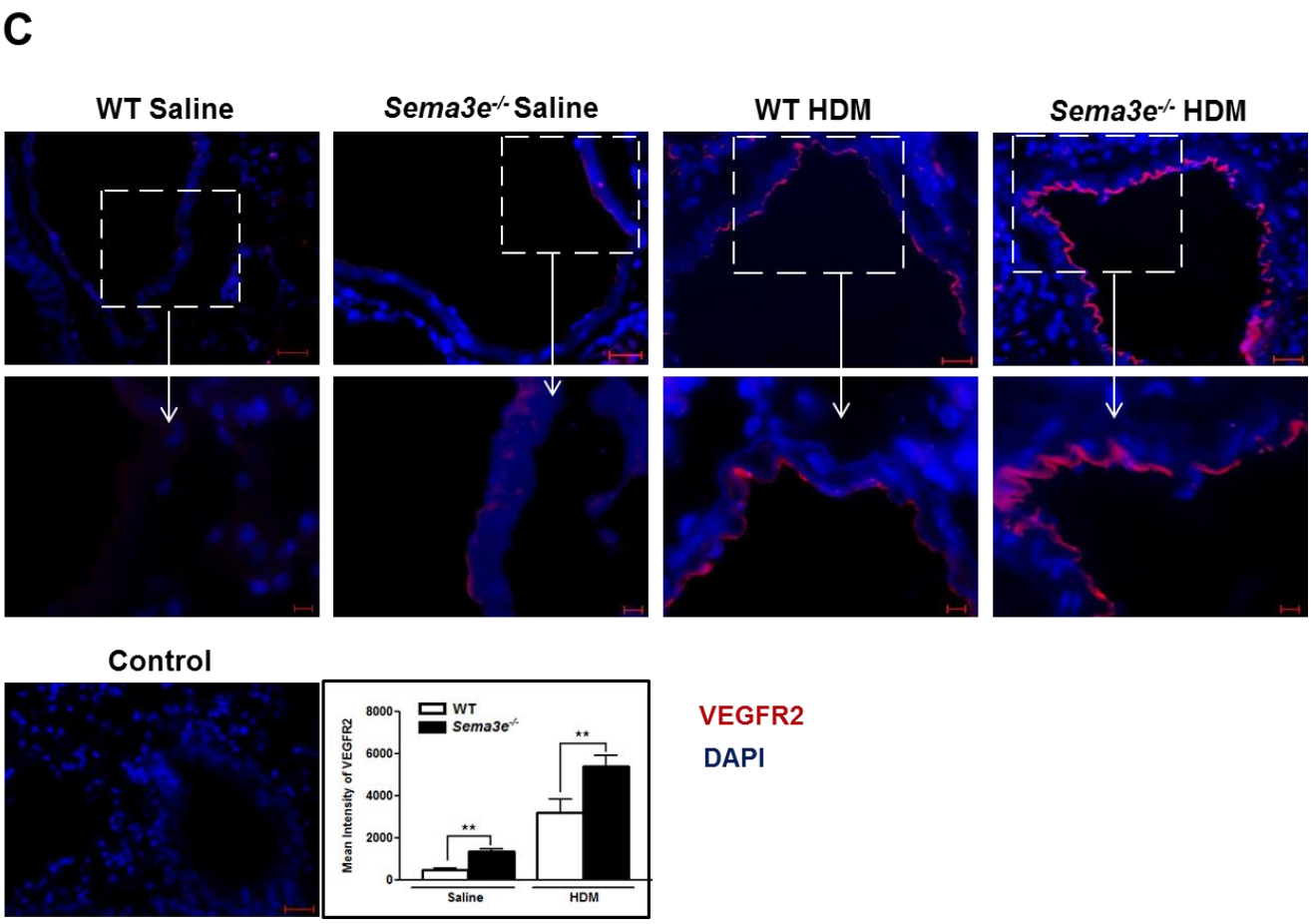


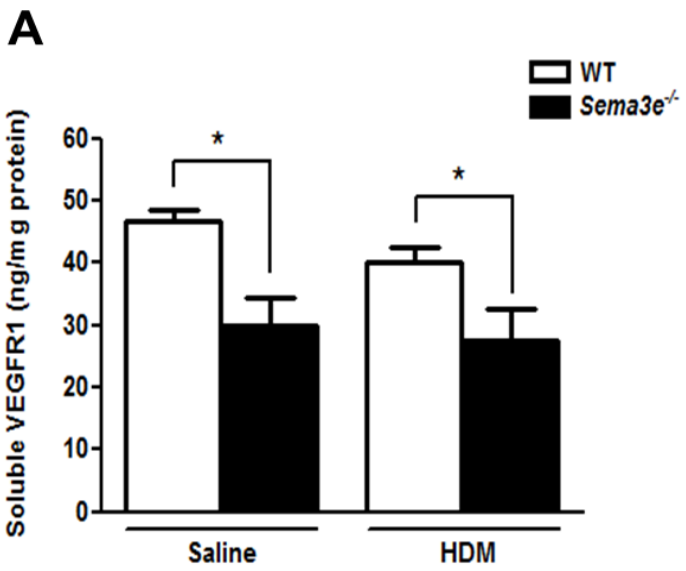
Figure 4.10: *Sema3e*$^{-/-}$ mice shows higher level of VEGF production and VEGFR-2 expression compared to WT animals. (A) ELISA was performed to measure the level of VEGF in lung homogenate of *Sema3e*$^{-/-}$ and WT littermates (*$P < 0.05$). (B) Immunofluorescence staining was done on lung sections to compare the level of VEGF production in the presence and absence of *Sema3e* in the airways of mice. Low and high magnification (x200 and x400) pictures of lung tissue were taken and mean intensity was measured per lung tissue area (***$P < 0.001$). (C) VEGFR-2 immunofluorescence staining was performed on pre-bronchial sections to study the level of VEGFR-2 expression on endothelial cells of the airway of mice in *Sema3e*$^{-/-}$ and WT littermates. Low and high magnification (x400 and x1000) images of lung sections were taken and mean intensity was measured per lung tissue area (**$P < 0.01$). Statistical analyses was performed by one-way ANOVA, Error bars represent Mean ± SEM.

4.11 Presence of *Sema3e* gene has a key role in inducing soluble VEGFR-1, a decoy receptor of VEGF.

According to previous studies, soluble VEGFR-1 (soluble Flt-1) has an inhibitory role on angiogenesis by preventing the binding of VEGF to VEGFR-2 [130]. It has been shown in developmental study in zebrafish that after binding of Sema3E to Plexin-D1 on endothelial tip cells, soluble Flt-1 will be produced and acts as a modulating decoy of VEGF [209].

To study the importance of Sema3e on soluble Flt-1 production in the mouse model of allergic asthma, the level of soluble Flt-1 was measured by ELISA using lung homogenate of *Sema3e*$^{-/-}$ and WT animals. Based on our data, the level of soluble flt-1 was significantly higher in WT animals compared to *Sema3e*$^{-/-}$ (* $P < 0.05$) (Fig 4.11 A). In addition, for further confirmation, gene expression study and immunofluorescence staining were performed and elevated level of soluble Flt-1 expression was observed in the presence of Sema3e in WT animals compared to *Sema3e*$^{-/-}$. (* $P < 0.05$, Fig 4.11B and **** $P < 0.0001$, Fig 4.11C).

Altogether, our data suggest that the presence of *Sema3e* and its signaling trough PlexinD1 on endothelial tip cells could be the reason of soluble Flt-1 production and according to previous studies [209] in this situation Flt-1 can regulate angiogenesis by preventing VEGF and VEGFR-2 binding.


A
WT
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Soluble VEGFR1 (ng/mg protein)
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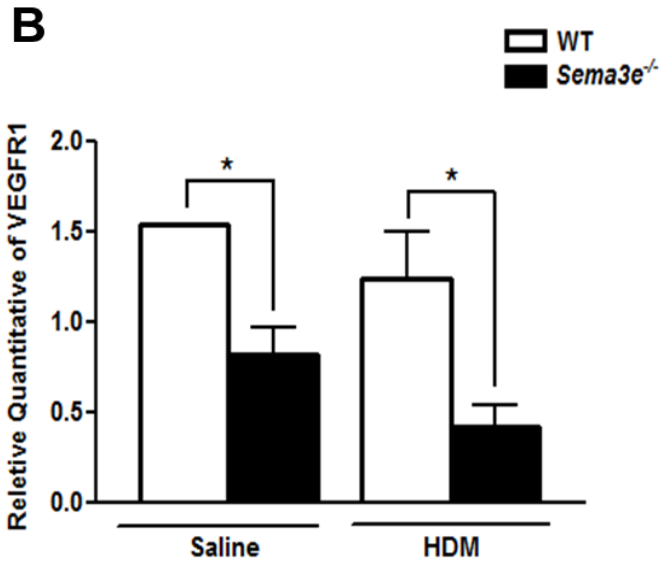
B
WT
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Reletive Quantitative of VEGFR1
2.0
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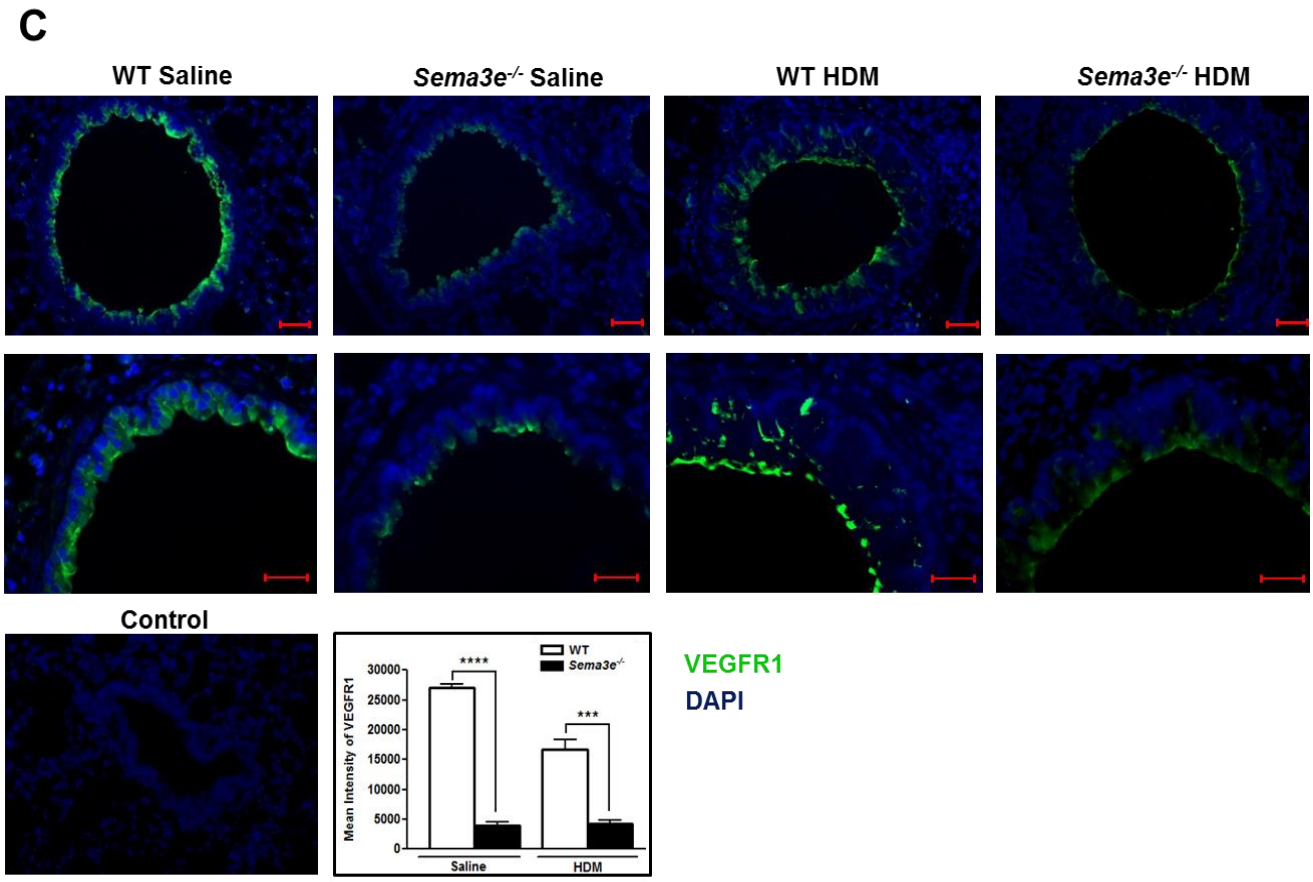

Figure 4.11: VEGFR-1 is produced in the presence of *Sema3e* gene. (A) ELISA was performed to measure the level of Soluble VEGFR-1 using lung homogenate of WT and *Sema3e*$^{-/-}$ animals (* $P < 0.05$). (B) The expression level of soluble VEGFR-1 gene was studied by real-time PCR (* $P < 0.05$). (C) Soluble VEGFR-1 immunofluorescence staining was performed on the bronchial sections to investigate the differences between the level of Soluble VEGFR-1 production in the airway of *Sema3e*$^{-/-}$ and WT animals. Images were taken at x200 and x400 magnification and mean intensity was measured per lung tissue area (***$P < 0.001$ and ****$P < 0.0001$). Statistical analyses was performed by one-way ANOVA, Error bars represent Mean ± SEM.

4.12 The ratio of VEGF to soluble VEGFR-1 changed significantly after knocking out *Sema3e* gene.

Following measurement of the level of VEGF and soluble Flt-1 by ELISA in lung homogenate of *Sema3e*$^{-/-}$ and WT animals, the ratio of VEGF / soluble Flt-1 was calculated. According to the result, this ratio was significantly higher in *Sema3e*$^{-/-}$ mice which show that VEGF is the dominant factor in this condition. However, in WT animals, the ratio was lower which indicates that the level of soluble Flt-1 is higher in these mice (* $P < 0.05$) (Fig 4.12). This statistical analysis suggests that in the absence of *Sema3e*, VEGF can play the main role and increase the level of angiogenesis. However, in the presence of *Sema3e*, the decreased level of formation of new blood vessels maybe related to production of soluble Flt-1 as a modulating decoy of VEGF.

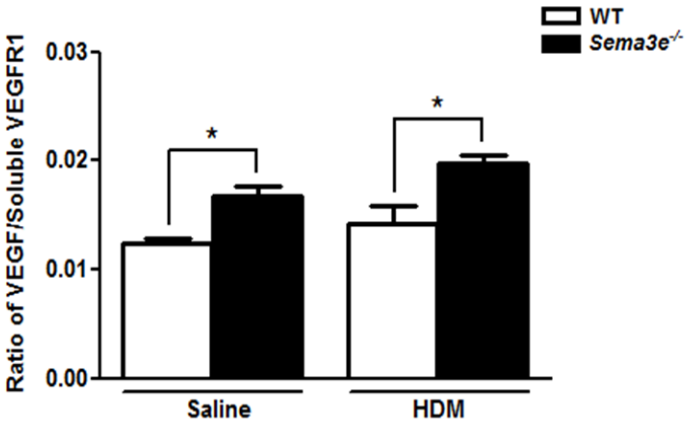

Figure 4.12: VEGF is the dominant factor in *Sema3e*$^{-/-}$ mice compared to soluble Flt-1. After determining the level VEGF and VEGFR-1 in lung homogenate by ELISA, the ratio of VEGF / Soluble VEGFR-1 was calculated (* $P < 0.05$). Statistical analyses was performed by one-way ANOVA, Error bars represent Mean ± SEM.

5. DISCUSSION

More than fifty years ago, Dunnill and colleagues 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 [67]. Therefore, finding a strategy to overcome this pathological aspect of asthma deserves a full consideration from every conceivable angle. This study is the first description of the inhibitory role of neuronal chemorepellant semaphorin 3E molecule on pathological angiogenesis in allergic asthma. The inhibitory effect of Sema3E molecule is mostly associated with the reduction of inflammation and pro-angiogenic factors in asthmatic airways which may provide a new strategy for treating and attenuating airway diseases.

Semaphorin molecules are known as multifunctional proteins involved in the pathogenesis of several diseases including autoimmune diseases, various cancers, neurological disorders, and so on.[205, 248-250]. However, their function in allergic asthmatic conditions remains unclear.

As mentioned previously, class 3 semaphorins bind to neuropilin receptors; but, among them, Sema3E molecules transduce their signals by a direct interaction with plexinD1. This characteristic, turn them into repulsive cues; however, the interaction of Sema3E / plexinD1 by neuropilin 1 or VEGFR-2 changes the function of Sema3E from repulsion to attraction [191, 251].

Previously we published that Sema3E inhibits human airway smooth muscle cells migration and proliferation in asthmatic condition, by inducing actin depolymerization and suppressing Rac1 GTPase activity and phosphorylation of PI3K/Akt and MAPK/ERK1/2 in human airway smooth muscle cells [210]. In addition, it has been shown that Sema3E has a regulatory role on angiogenesis in cancerous condition by blocking VEGFR-2 activation and its downstream signaling pathways [205, 211, 220]. However, the effect of Sema3E on angiogenesis in allergic asthma had not been previously described.

We hypothesized that, due to the repulsive feature of Sema3E and its inhibitory effect on ASMCs and ECs proliferation and migration, this molecule can be effective at reducing the level of blood vessels formation in asthmatic airways. In this study, we found that Sema3E can reduce asthma symptoms including airway hyperresponsiveness, inflammation and remodeling. Consistent with our hypothesis, analyzing the level of angiogenesis as an important feature of airway remodeling revealed that formation of blood vessels is limited in the airways of allergic asthmatic animals which were treated with Sema3E in compare to the groups which were just exposed to allergen (HDM). Moreover, in the absence of Sema3e gene in S*ema3e*$^{-/-}$ mice, the level of angiogenesis was significantly higher suggesting the pivotal role of Sema3e in inhibition of angiogenesis.

To a certain extent, this effect of Sema3E was associated with the reduction of inflammation and different aspects of airway remodeling which directly influences airway angiogenesis.

In fact, one of the prominent features of airway remodeling is a significant increase in ASMCs mass and number as a result of ASMCs hypertrophy and hyperplasia [252]. In addition, epithelial cells undergo changes in airway remodeling [42, 60]. In this condition, they produce a large amount of pro-inflammatory and pro-angiogenic growth factors and cytokines which elevates the level of formation of new blood vessels [161]. VEGF is the dominant pro-angiogenic factor that is produced by ASMCs and epithelial cells in asthmatic airway and acts as a strong mitogen for ECs and promotes angiogenesis process [99, 161]. However, according to our previous study Sema3E inhibits the proliferation of ASMCs [210]. Due to reduction in the number of ASMCs, we speculated that one of the main sources of pro-angiogenic factors including VEGF is blocked. Our studies have confirmed this speculation and suggested that Sema3E is able to reduce the level of VEGF in the lung of allergic asthmatic animals. According to previous studies, this condition ultimately can lead to disruption of blood vessels network due to suppression of Dll4 / Notch signaling pathway and losing the control of cell fate decision between tip and stalk cells [209, 212].

In addition, we found that the level of VEGFR-2 expression on endothelial cells decreases significantly in the presence of Sema3E.

Thus, these findings suggest that Sema3E may play a dual role in inhibition of pathological angiogenesis in allergic asthma. In fact, besides reducing the level of VEGF, they may decrease the expression VEGFR-2, which is known as the most important mediator of angiogenic effect of VEGF [253]. Therefore, as a result of decreased surface expression of VEGFR-2 on endothelial cells and its ligand, VEGF, the activation of angiogenic signaling pathways might be limited which reduces blood vessels formation.

In an effort to figure out the mechanism of action of Sema3E on endothelial cells, we investigated the role of soluble VEGFR-1 in Sema3E-mediated blood vessels disruption. Soluble VEGFR-1 which is a specific variant of VEGFR-1 receptor has an inhibitory role on angiogenesis by acting as VEGF decoy receptor [216]. It has been shown in developmental studies in zebrafish, that Sema3E has the ability to produce sVEGFR-1 following binding to plexinD1 [209]. As a novel finding in asthma field, we indicated that Sema3E is able to stimulate the production of sVEGFR-1 in asthmatic airways. Following this phenomena, the produced sVEGFR-1 inhibits VEGF/ VEGFR-2 signaling pathway by binding to VEGF which may results in blocking the main angiogenic pathway and disruption of blood vessels network.

Moreover, analyzing the ratio of VEGF to sVEGFR-1 in the airways of different groups of animals confirmed that, Sema3E has a significant role in producing sVEGFR-1.

According to our findings, in the presence of Sema3E, sVEGFR-1 plays the dominant role in the airways and inhibits VEGF / VEGFR-2 interaction. However, in the absence

of Sema3E, this phenomenon is abrogated and VEGF overcomes sVEGFR-1 which leads to activation of VEGF/ VEGFR-2 signaling and formation of more blood vessels.

To recapitulate, our research provided the first evidence regarding the role of semaphorin molecules in asthma pathology by influencing angiogenesis as an important feature of airway remodeling. As a novel finding, our results indicated that Sema3E molecules have the ability to inhibit new blood vessels formation in the allergic asthmatic airways by their repulsive activity which can be considered as novel way to overcome airway remodeling in allergic asthma. In fact, reduced level of angiogenesis in allergic asthmatic airways plays a significant role in controlling different aspect of asthma pathogenesis including airway inflammation, remodeling and ultimately airway hyperresponsiveness.

Although this research has reached its aims, there were few limitations which restricted us to assess the effect of Sema3E on ECs migration and proliferation as important phenomena in angiogenesis progression in allergic asthmatic airways. To achieve this aim, investigating the effect of Sema3E on VEGFR-2 downstream signaling pathways on ECs was crucial which was possible by isolating the primary endothelial cells from the lung of mice. However, in this study, primary endothelial cells isolation from the lung of mice and passaging them was a challenge which didn't allow us to study the effect of Sema3E on ERK, AKt, p38MAPK pathways and also the activity of small GTPases as VEGFR-2 downstream signaling pathways. In addition, due to time limitation this research was conducted only on acute mouse models. However, studying the effect of Sema3E on angiogenesis in chronic mouse models of allergic asthma could be added to this project to study the exact time of angiogenesis inhibition by sema3E.

6. Future Direction

1) Studying the effect of Sema3E on endothelial cells migration. As mentioned previously, Cell migration is one of the important steps of angiogenesis which is dependent on actin cytoskeleton alterations [215, 219]. It has been shown that semaphorin 3E molecule is able to affect microtubule networks and the organization of actin filaments by contributing in F-actin polymerization and depolymerization and through this way it can change the migratory activity of cells [178, 181]. Therefore to investigate the role of Sema3E on endothelial cells migration in allergic asthmatic airways, primary endothelial cells should be isolated from the lung of different groups of mice. It allows us to study the effect of Sema3E on F-actin alteration and ultimately ECs migration as an important step in blood vessels formation in allergic asthmatic condition.

2) Investigating the effect of Sema3E on β1 integrin and focal adhesions (FA). According to previous studies, Sma3E influences β1 integrin and focal adhesions (FA) on ECs which are known as the most important adhesive structure between endothelial cell and ECM. In fact, Sema3E / plexinD1 signaling has a significant influence on focal adhesion assembly / disassembly and in this way they change the normal function of adhesive structures which leads to detachment of ECs from each other and from ECM. In addition Sema3E / plexinD1 signaling changes the normal signal transduction from ECM to ECs by affecting focal adhesions and β1 integrin internalization which eventually results in blood vessels disruption [220]. However, still there is no clear study regarding the effect

of Sema3E on the β1 integrin and focal adhesions position in allergic asthmatic airways. Isolating primary endothelial cells form the lung of different groups of mice allows us to assess the effect of Sema3E on blood vessels integrity and signaling machinery of endothelial cells in angiogenesis process in our model.

3) Performing kinetic study at day 5, 10 and 14 to figure out the exact time point of starting angiogenesis and effective role of Sema3E on this process in acute mouse model of allergic asthma.

4) Real time monitoring of new airway vessel formation in the HDM challenged mice or HDM ± Sema3E treated mice by direct *in vivo* angiogenesis assay (DIVAA) or fluorescence labeling in mouse model. This technique allows quantifying the angiogenic responses *in vivo* and in this condition we will be able to quantify the level of angiogenesis *in vivo* in the presence and absence of Sema3E.

5) Studying angiogenesis in allergic asthmatic Plexin D1 knockout mice to confirm the importance of Sema3E / Plexin D1 interaction in controlling pathological angiogenesis in allergic asthma. In fact, by knocking out the gene of Plxnd1we will be able to investigate and confirm the pivotal role of sema3E signal transduction on angiogenesis allergic asthmatic airways.

7. REFERENCES

1. World Health Organization, *Global surveillance, prevention and control of chronic respiratory diseases: a comprehensive approach.* 2007.
2. Romagnani, S., *The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both?* Immunology, 2004. 112(3): p. 352-63.
3. American Academi of Allergy Ashtma and Immunology, *Asthma Statistics.* 2015.
4. Asthma Society of Canada, *What is Asthma* 2015.
5. National Heart, L.a.B.I. *What Are the Signs and Symptoms of Asthma*. 2014; Available from: http://www.nhlbi.nih.gov/health/health-topics/topics/asthma/signs.
6. Barnes, P.J., *Immunology of asthma and chronic obstructive pulmonary disease.* Nat Rev Immunol, 2008. 8(3): p. 183-92.
7. Hamid, Q., et al., *Inflammatory cells in asthma: mechanisms and implications for therapy.* J Allergy Clin Immunol, 2003. 111(1 Suppl): p. S5-S12; discussion S12-7.
8. Murdoch, J.R. and C.M. Lloyd, *Chronic inflammation and asthma.* Mutat Res, 2010. 690(1-2): p. 24-39.
9. Maddox, L. and D.A. Schwartz, *The pathophysiology of asthma.* Annu Rev Med, 2002. 53: p. 477-98.
10. Holgate, S.T., *Pathogenesis of asthma.* Clin Exp Allergy, 2008. 38(6): p. 872-97.
11. Kudo, M., Y. Ishigatsubo, and I. Aoki, *Pathology of asthma.* Front Microbiol, 2013. 4: p. 263.
12. Kay, A.B., *The role of T lymphocytes in asthma.* Chem Immunol Allergy, 2006. 91: p. 59-75.

13. **Lambrecht, B.N. and H. Hammad, *The immunology of asthma.* Nat Immunol, 2015. 16(1): p. 45-56.**

14. **Brusselle, G.G., T. Maes, and K.R. Bracke, *Eosinophils in the spotlight: Eosinophilic airway inflammation in nonallergic asthma.* Nat Med, 2013. 19(8): p. 977-9.**

15. **Possa, S.S., et al., *Eosinophilic inflammation in allergic asthma.* Front Pharmacol, 2013. 4: p. 46.**

16. **Bousquet, J., et al., *Eosinophilic inflammation in asthma.* N Engl J Med, 1990. 323(15): p. 1033-9.**

17. **Lee, J.J. and N.A. Lee, *Eosinophil degranulation: an evolutionary vestige or a universally destructive effector function?* Clin Exp Allergy, 2005. 35(8): p. 986-94.**

18. **Yu, Y. and G. Shi, *[Innate lymphoid cells and asthma].* Zhonghua Jie He He Hu Xi Za Zhi, 2014. 37(2): p. 122-5.**

19. **Erle, D.J. and D. Sheppard, *The cell biology of asthma.* J Cell Biol, 2014. 205(5): p. 621-31.**

20. **Jatakanon, A., et al., *Neutrophilic inflammation in severe persistent asthma.* Am J Respir Crit Care Med, 1999. 160(5 Pt 1): p. 1532-9.**

21. **Guidelines for the Diagnosis and Management of Asthma, *Section 2, Definition, Pathophysiology and Pathogenesis of Asthma, and Natural History of Asthma.***

22. **Kamath, A.V., et al., *Is the neutrophil the key effector cell in severe asthma?* Thorax, 2005. 60(7): p. 529-30.**

23. **Monteseirin, J., *Neutrophils and asthma.* J Investig Allergol Clin Immunol, 2009. 19(5): p. 340-54.**

24. **Lukacs, N.W., et al., *Mast cells produce ENA-78, which can function as a potent neutrophil chemoattractant during allergic airway inflammation.* J Leukoc Biol, 1998. 63(6): p. 746-51.**

25. **Linden, A., *Role of interleukin-17 and the neutrophil in asthma.* Int Arch Allergy Immunol, 2001. 126(3): p. 179-84.**

26. **Hart, P.H., *Regulation of the inflammatory response in asthma by mast cell products.* Immunol Cell Biol, 2001. 79(2): p. 149-53.**

27. **Wei, H., et al., *Involvement of human natural killer cells in asthma pathogenesis: natural killer 2 cells in type 2 cytokine predominance.* J Allergy Clin Immunol, 2005. 115(4): p. 841-7.**

28. **Karimi, K. and P. Forsythe, *Natural killer cells in asthma.* Front Immunol, 2013. 4: p. 159.**

29. **Akbari, O., et al., *CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma.* N Engl J Med, 2006. 354(11): p. 1117-29.**

30. **Halwani, R., S. Al-Muhsen, and Q. Hamid, *Airway remodeling in asthma.* Curr Opin Pharmacol, 2010. 10(3): p. 236-45.**

31. **Bergeron, C., M.K. Tulic, and Q. Hamid, *Airway remodelling in asthma: from benchside to clinical practice.* Can Respir J, 2010. 17(4): p. e85-93.**

32. **Al-Muhsen, S., J.R. Johnson, and Q. Hamid, *Remodeling in asthma.* J Allergy Clin Immunol, 2011. 128(3): p. 451-62; quiz 463-4.**

33. **Elias, J.A., et al., *Airway remodeling in asthma.* J Clin Invest, 1999. 104(8): p. 1001-6.**

34. **Woodruff, P.G. and J.V. Fahy, *Airway remodeling in asthma.* Semin Respir Crit Care Med, 2002. 23(4): p. 361-7.**

35. **Sumi, Y. and Q. Hamid, *Airway remodeling in asthma.* Allergol Int, 2007. 56(4): p. 341-8.**

36. **Shifren, A., et al., *Mechanisms of remodeling in asthmatic airways.* J Allergy (Cairo), 2012. 2012: p. 316049.**

37. **Berend, N., C.M. Salome, and G.G. King, *Mechanisms of airway hyperresponsiveness in asthma.* Respirology, 2008. 13(5): p. 624-31.**

38. O'Byrne, P.M. and M.D. Inman, *Airway hyperresponsiveness.* Chest, 2003. 123(3 Suppl): p. 411S-6S.

39. National Asthma, E. and P. Prevention, *Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007.* J Allergy Clin Immunol, 2007. 120(5 Suppl): p. S94-138.

40. Holgate, S.T., et al., *Epithelial-mesenchymal interactions in the pathogenesis of asthma.* J Allergy Clin Immunol, 2000. 105(2 Pt 1): p. 193-204.

41. Cohen, L., et al., *Epithelial cell proliferation contributes to airway remodeling in severe asthma.* Am J Respir Crit Care Med, 2007. 176(2): p. 138-45.

42. Davies, D.E., *The role of the epithelium in airway remodeling in asthma.* Proc Am Thorac Soc, 2009. 6(8): p. 678-82.

43. Zhang, S., et al., *Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma.* Lab Invest, 1999. 79(4): p. 395-405.

44. Descalzi, D., et al., *Importance of fibroblasts-myofibroblasts in asthma-induced airway remodeling.* Recent Pat Inflamm Allergy Drug Discov, 2007. 1(3): p. 237-41.

45. Brewster, C.E., et al., *Myofibroblasts and subepithelial fibrosis in bronchial asthma.* Am J Respir Cell Mol Biol, 1990. 3(5): p. 507-11.

46. Hoshino, M., Y. Nakamura, and J.J. Sim, *Expression of growth factors and remodelling of the airway wall in bronchial asthma.* Thorax, 1998. 53(1): p. 21-7.

47. Madame Curie Bioscience Database, S.M. *Tissue Repair in Asthma: The Origin of Airway Subepithelial Fibroblasts and Myofibroblasts*. 2015,08]; Available from: http://www.ncbi.nlm.nih.gov/books/NBK6013/.

48. **James, A.L., et al., *Airway smooth muscle hypertrophy and hyperplasia in asthma.* Am J Respir Crit Care Med, 2012. 185(10): p. 1058-64.**

49. **Bentley, J.K., et al., *Airway smooth muscle hyperplasia and hypertrophy correlate with glycogen synthase kinase-3(beta) phosphorylation in a mouse model of asthma.* Am J Physiol Lung Cell Mol Physiol, 2009. 296(2): p. L176-84.**

50. **Hirst, S.J., et al., *Proliferative aspects of airway smooth muscle.* J Allergy Clin Immunol, 2004. 114(2 Suppl): p. S2-17.**

51. **Noble, P.B., et al., *Airway smooth muscle in asthma: linking contraction and mechanotransduction to disease pathogenesis and remodelling.* Pulm Pharmacol Ther, 2014. 29(2): p. 96-107.**

52. **Freyer, A.M., S.R. Johnson, and I.P. Hall, *Effects of growth factors and extracellular matrix on survival of human airway smooth muscle cells.* Am J Respir Cell Mol Biol, 2001. 25(5): p. 569-76.**

53. **Howarth, P.H., et al., *Synthetic responses in airway smooth muscle.* J Allergy Clin Immunol, 2004. 114(2 Suppl): p. S32-50.**

54. **Zhou, L. and M.B. Hershenson, *Mitogenic signaling pathways in airway smooth muscle.* Respir Physiol Neurobiol, 2003. 137(2-3): p. 295-308.**

55. **Tagaya, E. and J. Tamaoki, *Mechanisms of airway remodeling in asthma.* Allergol Int, 2007. 56(4): p. 331-40.**

56. **Page, K., et al., *Regulation of cyclin D(1) expression and DNA synthesis by phosphatidylinositol 3-kinase in airway smooth muscle cells.* Am J Respir Cell Mol Biol, 2000. 23(4): p. 436-43.**

57. **Orsini, M.J., et al., *MAPK superfamily activation in human airway smooth muscle: mitogenesis requires prolonged p42/p44 activation.* Am J Physiol, 1999. 277(3 Pt 1): p. L479-88.**

58. Chen, G. and N. Khalil, *TGF-beta1 increases proliferation of airway smooth muscle cells by phosphorylation of map kinases.* Respir Res, 2006. 7: p. 2.

59. Ordonez, C.L., et al., *Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression.* Am J Respir Crit Care Med, 2001. 163(2): p. 517-23.

60. Fahy, J.V., *Remodeling of the airway epithelium in asthma.* Am J Respir Crit Care Med, 2001. 164(10 Pt 2): p. S46-51.

61. Shim, J.J., et al., *IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils.* Am J Physiol Lung Cell Mol Physiol, 2001. 280(1): p. L134-40.

62. Takeyama, K., et al., *Epidermal growth factor system regulates mucin production in airways.* Proc Natl Acad Sci U S A, 1999. 96(6): p. 3081-6.

63. Takeyama, K., et al., *Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils.* J Immunol, 2000. 164(3): p. 1546-52.

64. Takeyama, K., J.V. Fahy, and J.A. Nadel, *Relationship of epidermal growth factor receptors to goblet cell production in human bronchi.* Am J Respir Crit Care Med, 2001. 163(2): p. 511-6.

65. Park, J.A., et al., *Human neutrophil elastase-mediated goblet cell metaplasia is attenuated in TACE-deficient mice.* Am J Physiol Lung Cell Mol Physiol, 2013. 304(10): p. L701-7.

66. Ribatti, D., et al., *Angiogenesis in asthma.* Clin Exp Allergy, 2009. 39(12): p. 1815-21.

67. Dunnill, M.S., *The pathology of asthma, with special reference to changes in the bronchial mucosa.* J Clin Pathol, 1960. 13: p. 27-33.

68. Cudkowicz, L., *Leonardo de Vinci and the bronchial circulation.* Br J Tuberc Dis Chest, 1953. 47(1): p. 23-5.

69. **Vrugt, B., et al., *Bronchial angiogenesis in severe glucocorticoid-dependent asthma.* Eur Respir J, 2000. 15(6): p. 1014-21.**

70. **Wilson, J.W. and C.F. Robertson, *Angiogenesis in paediatric airway disease.* Paediatr Respir Rev, 2002. 3(3): p. 219-29.**

71. **Tanaka, H., et al., *Increased airway vascularity in newly diagnosed asthma using a high-magnification bronchovideoscope.* Am J Respir Crit Care Med, 2003. 168(12): p. 1495-9.**

72. **Kumar, S.D., et al., *Airway mucosal blood flow in bronchial asthma.* Am J Respir Crit Care Med, 1998. 158(1): p. 153-6.**

73. **Keglowich, L.F. and P. Borger, *The Three A's in Asthma - Airway Smooth Muscle, Airway Remodeling & Angiogenesis.* Open Respir Med J, 2015. 9: p. 70-80.**

74. **Kanazawa, H., K. Hirata, and J. Yoshikawa, *Involvement of vascular endothelial growth factor in exercise induced bronchoconstriction in asthmatic patients.* Thorax, 2002. 57(10): p. 885-8.**

75. **Harkness, L.M., A.W. Ashton, and J.K. Burgess, *Asthma is not only an airway disease, but also a vascular disease.* Pharmacol Ther, 2015. 148: p. 17-33.**

76. **Zanini, A., et al., *The role of the bronchial microvasculature in the airway remodelling in asthma and COPD.* Respir Res, 2010. 11: p. 132.**

77. **Mariscalco, G., et al., *Imbalance between pro-angiogenic and anti-angiogenic factors in rheumatic and mixomatous mitral valves.* Int J Cardiol, 2011. 152(3): p. 337-44.**

78. **Meyer, N. and C.A. Akdis, *Vascular endothelial growth factor as a key inducer of angiogenesis in the asthmatic airways.* Curr Allergy Asthma Rep, 2013. 13(1): p. 1-9.**

79. **Krock, B.L., N. Skuli, and M.C. Simon, *Hypoxia-induced angiogenesis: good and evil.* Genes Cancer, 2011. 2(12): p. 1117-33.**

80. **Pepper, M.S., *Manipulating angiogenesis. From basic science to the bedside.* Arterioscler Thromb Vasc Biol, 1997. 17(4): p. 605-19.**

81. Dr. Thomas H. Adair, D.J.-P.M., *Angiogenesis, Chapter 1: Overview of Angiogenesis*.

82. Felcht, M., et al., *Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signaling*. J Clin Invest, 2012. 122(6): p. 1991-2005.

83. The angiogenesis foundation, D.W.L. *How is Angiogenesis Important for Health?* 2015, 08, 21]; Available from: https://www.angio.org/learn/angiogenesis/.

84. Gale, N.W., et al., *Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1.* Dev Cell, 2002. 3(3): p. 411-23.

85. Fiedler, U., et al., *Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation.* Nat Med, 2006. 12(2): p. 235-9.

86. Augustin, H.G., et al., *Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system.* Nat Rev Mol Cell Biol, 2009. 10(3): p. 165-77.

87. Huang, H., et al., *Targeting the ANGPT-TIE2 pathway in malignancy.* Nat Rev Cancer, 2010. 10(8): p. 575-85.

88. Palgan, K. and Z. Bartuzi, *Angiogenesis in bronchial asthma.* Int J Immunopathol Pharmacol, 2015. 28(3): p. 415-20.

89. Vestweber, D., *VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation.* Arterioscler Thromb Vasc Biol, 2008. 28(2): p. 223-32.

90. Dejana, E., F. Orsenigo, and M.G. Lampugnani, *The role of adherens junctions and VE-cadherin in the control of vascular permeability.* J Cell Sci, 2008. 121(Pt 13): p. 2115-22.

91. le Noble, F., et al., *Neural guidance molecules, tip cells, and mechanical factors in vascular development.* Cardiovasc Res, 2008. 78(2): p. 232-41.

92. van Hinsbergh, V.W. and P. Koolwijk, *Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead.* Cardiovasc Res, 2008. 78(2): p. 203-12.

93. **Handsley, M.M. and D.R. Edwards, *Metalloproteinases and their inhibitors in tumor angiogenesis.* Int J Cancer, 2005. 115(6): p. 849-60.**

94. **Stetler-Stevenson, W.G., *Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention.* J Clin Invest, 1999. 103(9): p. 1237-41.**

95. **Zucker, S., et al., *Vascular endothelial growth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: conversion of prothrombin to thrombin results in progelatinase A activation and cell proliferation.* Int J Cancer, 1998. 75(5): p. 780-6.**

96. **Sang, Q.X., *Complex role of matrix metalloproteinases in angiogenesis.* Cell Res, 1998. 8(3): p. 171-7.**

97. **Phng, L.K. and H. Gerhardt, *Angiogenesis: a team effort coordinated by notch.* Dev Cell, 2009. 16(2): p. 196-208.**

98. **Potente, M., H. Gerhardt, and P. Carmeliet, *Basic and therapeutic aspects of angiogenesis.* Cell, 2011. 146(6): p. 873-87.**

99. **Gerhardt, H., et al., *VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia.* J Cell Biol, 2003. 161(6): p. 1163-77.**

100. **Adams, R.H. and K. Alitalo, *Molecular regulation of angiogenesis and lymphangiogenesis.* Nat Rev Mol Cell Biol, 2007. 8(6): p. 464-78.**

101. **Iruela-Arispe, M.L. and G.E. Davis, *Cellular and molecular mechanisms of vascular lumen formation.* Dev Cell, 2009. 16(2): p. 222-31.**

102. **Nichol, D. and H. Stuhlmann, *EGFL7: a unique angiogenic signaling factor in vascular development and disease.* Blood, 2012. 119(6): p. 1345-52.**

103. **Kim, I., et al., *Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-Kinase/Akt signal transduction pathway.* Circ Res, 2000. 86(1): p. 24-9.**

104. **Gamble, J.R., et al., *Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions.* Circ Res, 2000. 87(7): p. 603-7.**

105. **Nawroth, R., et al., *VE-PTP and VE-cadherin ectodomains interact to facilitate regulation of phosphorylation and cell contacts.* EMBO J, 2002. 21(18): p. 4885-95.**

106. **Fukuhara, S., et al., *Tie2 is tied at the cell-cell contacts and to extracellular matrix by angiopoietin-1.* Exp Mol Med, 2009. 41(3): p. 133-9.**

107. **Avraamides, C.J., B. Garmy-Susini, and J.A. Varner, *Integrins in angiogenesis and lymphangiogenesis.* Nat Rev Cancer, 2008. 8(8): p. 604-17.**

108. **Bayless, K.J., R. Salazar, and G.E. Davis, *RGD-dependent vacuolation and lumen formation observed during endothelial cell morphogenesis in three-dimensional fibrin matrices involves the alpha(v)beta(3) and alpha(5)beta(1) integrins.* Am J Pathol, 2000. 156(5): p. 1673-83.**

109. **Barry, D.M., et al., *Cdc42 is required for cytoskeletal support of endothelial cell adhesion during blood vessel formation.* Development, 2015.**

110. **Tavora, B., et al., *Endothelial FAK is required for tumour angiogenesis.* EMBO Mol Med, 2010. 2(12): p. 516-28.**

111. **Armulik, A., A. Abramsson, and C. Betsholtz, *Endothelial/pericyte interactions.* Circ Res, 2005. 97(6): p. 512-23.**

112. **Bergers, G. and S. Song, *The role of pericytes in blood-vessel formation and maintenance.* Neuro Oncol, 2005. 7(4): p. 452-64.**

113. **Winkler, E.A., R.D. Bell, and B.V. Zlokovic, *Pericyte-specific expression of PDGF beta receptor in mouse models with normal and deficient PDGF beta receptor signaling.* Mol Neurodegener, 2010. 5: p. 32.**

114. **Carmeliet, P. and R.K. Jain, *Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases.* Nat Rev Drug Discov, 2011. 10(6): p. 417-27.**

115. O'Reilly, M.S., et al., *Endostatin: an endogenous inhibitor of angiogenesis and tumor growth.* Cell, 1997. 88(2): p. 277-85.

116. O'Reilly, M.S., et al., *Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma.* Cell, 1994. 79(2): p. 315-28.

117. Sim, B.K., N.J. MacDonald, and E.R. Gubish, *Angiostatin and endostatin: endogenous inhibitors of tumor growth.* Cancer Metastasis Rev, 2000. 19(1-2): p. 181-90.

118. Carmeliet, P., *Mechanisms of angiogenesis and arteriogenesis.* Nat Med, 2000. 6(4): p. 389-95.

119. Lucitti, J.L., et al., *Vascular remodeling of the mouse yolk sac requires hemodynamic force.* Development, 2007. 134(18): p. 3317-26.

120. Jain, R.K., *Molecular regulation of vessel maturation.* Nat Med, 2003. 9(6): p. 685-93.

121. Li, X. and J.W. Wilson, *Increased vascularity of the bronchial mucosa in mild asthma.* Am J Respir Crit Care Med, 1997. 156(1): p. 229-33.

122. Orsida, B.E., et al., *Vascularity in asthmatic airways: relation to inhaled steroid dose.* Thorax, 1999. 54(4): p. 289-95.

123. Tuder, R.M., et al., *Hypoxia and chronic lung disease.* J Mol Med (Berl), 2007. 85(12): p. 1317-24.

124. Huerta-Yepez, S., et al., *Hypoxia inducible factor promotes murine allergic airway inflammation and is increased in asthma and rhinitis.* Allergy, 2011. 66(7): p. 909-18.

125. Keglowich, L., et al., *Hypoxia exerts dualistic effects on inflammatory and proliferative responses of healthy and asthmatic primary human bronchial smooth muscle cells.* PLoS One, 2014. 9(2): p. e89875.

126. Lee, S.Y., et al., *Expression of vascular endothelial growth factor and hypoxia-inducible factor in the airway of asthmatic patients.* Ann Allergy Asthma Immunol, 2006. 97(6): p. 794-9.

127. **Asai, K., et al.,** ***Increased levels of vascular endothelial growth factor in induced sputum in asthmatic patients.*** **Clin Exp Allergy, 2003. 33(5): p. 595-9.**

128. **Hoshino, M., M. Takahashi, and N. Aoike,** ***Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis.*** **J Allergy Clin Immunol, 2001. 107(2): p. 295-301.**

129. **Cross, M.J. and L. Claesson-Welsh,** ***FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition.*** **Trends Pharmacol Sci, 2001. 22(4): p. 201-7.**

130. **Ferrara, N., H.P. Gerber, and J. LeCouter,** ***The biology of VEGF and its receptors.*** **Nat Med, 2003. 9(6): p. 669-76.**

131. **Detoraki, A., et al.,** ***Angiogenesis and lymphangiogenesis in bronchial asthma.*** **Allergy, 2010. 65(8): p. 946-58.**

132. **Deindl, E. and W. Schaper,** ***The art of arteriogenesis.*** **Cell Biochem Biophys, 2005. 43(1): p. 1-15.**

133. **Carmeliet, P. and R.K. Jain,** ***Molecular mechanisms and clinical applications of angiogenesis.*** **Nature, 2011. 473(7347): p. 298-307.**

134. **Shibuya, M., N. Ito, and L. Claesson-Welsh,** ***Structure and function of vascular endothelial growth factor receptor-1 and -2.*** **Curr Top Microbiol Immunol, 1999. 237: p. 59-83.**

135. **Gerber, H.P., et al.,** ***Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia.*** **J Biol Chem, 1997. 272(38): p. 23659-67.**

136. **Tuder, R.M., B.E. Flook, and N.F. Voelkel,** ***Increased gene expression for VEGF and the VEGF receptors KDR/Flk and Flt in lungs exposed to acute or to chronic hypoxia. Modulation of gene expression by nitric oxide.*** **J Clin Invest, 1995. 95(4): p. 1798-807.**

137. **Yancopoulos, G.D., et al.,** ***Vascular-specific growth factors and blood vessel formation.*** **Nature, 2000. 407(6801): p. 242-8.**

138. **Gerber, H.P., V. Dixit, and N. Ferrara,** ***Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells.*** **J Biol Chem, 1998. 273(21): p. 13313-6.**

139. **Stockmann, C., et al.,** ***Deletion of vascular endothelial growth factor in myeloid cells accelerates tumorigenesis.*** **Nature, 2008. 456(7223): p. 814-8.**

140. **Bentley, K., et al.,** ***Tipping the balance: robustness of tip cell selection, migration and fusion in angiogenesis.*** **PLoS Comput Biol, 2009. 5(10): p. e1000549.**

141. **De Smet, F., et al.,** ***Mechanisms of vessel branching: filopodia on endothelial tip cells lead the way.*** **Arterioscler Thromb Vasc Biol, 2009. 29(5): p. 639-49.**

142. **Detmar, M., et al.,** ***Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice.*** **J Invest Dermatol, 1998. 111(1): p. 1-6.**

143. **Gruber, B.L., M.J. Marchese, and R. Kew,** ***Angiogenic factors stimulate mast-cell migration.*** **Blood, 1995. 86(7): p. 2488-93.**

144. **Clauss, M., et al.,** ***Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration.*** **J Exp Med, 1990. 172(6): p. 1535-45.**

145. **Hattori, K., et al.,** ***Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells.*** **J Exp Med, 2001. 193(9): p. 1005-14.**

146. **Dvorak, H.F., et al.,** ***Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis.*** **Am J Pathol, 1995. 146(5): p. 1029-39.**

147. **Soker, S., et al.,** ***Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor.*** **Cell, 1998. 92(6): p. 735-45.**

148. **Chappell, J.C., et al.,** ***Local guidance of emerging vessel sprouts requires soluble Flt-1.*** **Dev Cell, 2009. 17(3): p. 377-86.**

149. **Chappell, J.C. and V.L. Bautch,** ***Vascular development: genetic mechanisms and links to vascular disease.*** **Curr Top Dev Biol, 2010. 90: p. 43-72.**

150. **Roberts, D.M., et al.,** ***The vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation.*** **Am J Pathol, 2004. 164(5): p. 1531-5.**

151. **Kerjaschki, D.,** ***The crucial role of macrophages in lymphangiogenesis.*** **J Clin Invest, 2005. 115(9): p. 2316-9.**

152. **Mayumi Ono,** ***Molecular links between tumor angiogenesis and inflammation: inflammatory stimuli of macrophages and cancer cells as targets for therapeutic strategy.***

153. **Puxeddu, I., et al.,** ***Human peripheral blood eosinophils induce angiogenesis.*** **Int J Biochem Cell Biol, 2005. 37(3): p. 628-36.**

154. **Abdel-Majid, R.M. and J.S. Marshall,** ***Prostaglandin E2 induces degranulation-independent production of vascular endothelial growth factor by human mast cells.*** **J Immunol, 2004. 172(2): p. 1227-36.**

155. **de Paulis, A., et al.,** ***Expression and functions of the vascular endothelial growth factors and their receptors in human basophils.*** **J Immunol, 2006. 177(10): p. 7322-31.**

156. **Hoeben, A., et al.,** ***Vascular endothelial growth factor and angiogenesis.*** **Pharmacol Rev, 2004. 56(4): p. 549-80.**

157. **Huggenberger, R. and M. Detmar,** ***The cutaneous vascular system in chronic skin inflammation.*** **J Investig Dermatol Symp Proc, 2011. 15(1): p. 24-32.**

158. **Horiuchi, T. and P.F. Weller, *Expression of vascular endothelial growth factor by human eosinophils: upregulation by granulocyte macrophage colony-stimulating factor and interleukin-5.* Am J Respir Cell Mol Biol, 1997. 17(1): p. 70-7.**

159. **McKay, S. and H.S. Sharma, *Autocrine regulation of asthmatic airway inflammation: role of airway smooth muscle.* Respir Res, 2002. 3: p. 11.**

160. **Knox, A.J., et al., *Human airway smooth muscle cells secrete vascular endothelial growth factor: up-regulation by bradykinin via a protein kinase C and prostanoid-dependent mechanism.* FASEB J, 2001. 15(13): p. 2480-8.**

161. **Simcock, D.E., et al., *Induction of angiogenesis by airway smooth muscle from patients with asthma.* Am J Respir Crit Care Med, 2008. 178(5): p. 460-8.**

162. **Alagappan, V.K., et al., *Angiogenesis and vascular remodeling in chronic airway diseases.* Cell Biochem Biophys, 2013. 67(2): p. 219-34.**

163. **Shing, Y., et al., *Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor.* Science, 1984. 223(4642): p. 1296-9.**

164. **Coumoul, X. and C.X. Deng, *Roles of FGF receptors in mammalian development and congenital diseases.* Birth Defects Res C Embryo Today, 2003. 69(4): p. 286-304.**

165. **Kim, I., et al., *A novel fibroblast growth factor receptor-5 preferentially expressed in the pancreas(1).* Biochim Biophys Acta, 2001. 1518(1-2): p. 152-6.**

166. **Folkman, J. and M. Klagsbrun, *Angiogenic factors.* Science, 1987. 235(4787): p. 442-7.**

167. **Singh, T.M., et al., *Basic fibroblast growth factor expression precedes flow-induced arterial enlargement.* J Surg Res, 1998. 77(2): p. 165-73.**

168. **Beenken, A. and M. Mohammadi, *The FGF family: biology, pathophysiology and therapy.* Nat Rev Drug Discov, 2009. 8(3): p. 235-53.**

169. **Saharinen, P., et al.,** ***Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts.*** **Nat Cell Biol, 2008. 10(5): p. 527-37.**

170. **Geerts, L., et al.,** ***Natural inhibitors of neutrophil function in acute respiratory distress syndrome.*** **Crit Care Med, 2001. 29(10): p. 1920-4.**

171. **Bhandari, V., et al.,** ***Hyperoxia causes angiopoietin 2-mediated acute lung injury and necrotic cell death.*** **Nat Med, 2006. 12(11): p. 1286-93.**

172. **Li, A., et al.,** ***IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis.*** **J Immunol, 2003. 170(6): p. 3369-76.**

173. **Numasaki, M., et al.,** ***Interleukin-17 promotes angiogenesis and tumor growth.*** **Blood, 2003. 101(7): p. 2620-7.**

174. **Numasaki, M., et al.,** ***IL-17 enhances the net angiogenic activity and in vivo growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis.*** **J Immunol, 2005. 175(9): p. 6177-89.**

175. **Takahashi, H., et al.,** ***Interleukin-17 enhances bFGF-, HGF- and VEGF-induced growth of vascular endothelial cells.*** **Immunol Lett, 2005. 98(2): p. 189-93.**

176. **Wong, J.T., W.T. Yu, and T.P. O'Connor,** ***Transmembrane grasshopper Semaphorin I promotes axon outgrowth in vivo.*** **Development, 1997. 124(18): p. 3597-607.**

177. **Vadasz, Z. and E. Toubi,** ***Semaphorins: their dual role in regulating immune-mediated diseases.*** **Clin Rev Allergy Immunol, 2014. 47(1): p. 17-25.**

178. **Yazdani, U. and J.R. Terman,** ***The semaphorins.*** **Genome Biol, 2006. 7(3): p. 211.**

179. **Roney, K., E. Holl, and J. Ting,** ***Immune plexins and semaphorins: old proteins, new immune functions.*** **Protein Cell, 2013. 4(1): p. 17-26.**

180. **Siebold, C. and E.Y. Jones, *Structural insights into semaphorins and their receptors.* Semin Cell Dev Biol, 2013. 24(3): p. 139-45.**

181. **Huber, A.B., et al., *Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance.* Annu Rev Neurosci, 2003. 26: p. 509-63.**

182. **Adams, R.H., et al., *The chemorepulsive activity of secreted semaphorins is regulated by furin-dependent proteolytic processing.* EMBO J, 1997. 16(20): p. 6077-86.**

183. **Klostermann, A., et al., *The chemorepulsive activity of the axonal guidance signal semaphorin D requires dimerization.* J Biol Chem, 1998. 273(13): p. 7326-31.**

184. **Pasterkamp, R.J. and A.L. Kolodkin, *Semaphorin junction: making tracks toward neural connectivity.* Curr Opin Neurobiol, 2003. 13(1): p. 79-89.**

185. **Eastwood, S.L., et al., *The axonal chemorepellant semaphorin 3A is increased in the cerebellum in schizophrenia and may contribute to its synaptic pathology.* Mol Psychiatry, 2003. 8(2): p. 148-55.**

186. **Takegahara, N., A. Kumanogoh, and H. Kikutani, *Semaphorins: a new class of immunoregulatory molecules.* Philos Trans R Soc Lond B Biol Sci, 2005. 360(1461): p. 1673-80.**

187. **Neufeld, G., et al., *Semaphorins in cancer.* Front Biosci, 2005. 10: p. 751-60.**

188. **Autiero, M., et al., *Role of neural guidance signals in blood vessel navigation.* Cardiovasc Res, 2005. 65(3): p. 629-38.**

189. **Rizzolio, S. and L. Tamagnone, *Multifaceted role of neuropilins in cancer.* Curr Med Chem, 2011. 18(23): p. 3563-75.**

190. **Zhou, Y., R.A. Gunput, and R.J. Pasterkamp, *Semaphorin signaling: progress made and promises ahead.* Trends Biochem Sci, 2008. 33(4): p. 161-70.**

191. **Chauvet, S., et al., *Gating of Sema3E/PlexinD1 signaling by neuropilin-1 switches axonal repulsion to attraction during brain development.* Neuron, 2007. 56(5): p. 807-22.**

192. **Gu, C., et al., *Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins.* Science, 2005. 307(5707): p. 265-8.**

193. **Takahashi, T., et al., *Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors.* Cell, 1999. 99(1): p. 59-69.**

194. **Kruger, R.P., J. Aurandt, and K.L. Guan, *Semaphorins command cells to move.* Nat Rev Mol Cell Biol, 2005. 6(10): p. 789-800.**

195. **Shields, J., Gottesman, II, and E. Slater, *Kallmann's 1946 schizophrenic twin study in the light of new information.* Acta Psychiatr Scand, 1967. 43(4): p. 385-96.**

196. **Antipenko, A., et al., *Structure of the semaphorin-3A receptor binding module.* Neuron, 2003. 39(4): p. 589-98.**

197. **Takahashi, T. and S.M. Strittmatter, *Plexina1 autoinhibition by the plexin sema domain.* Neuron, 2001. 29(2): p. 429-39.**

198. **Ohta, K., et al., *Plexin: a novel neuronal cell surface molecule that mediates cell adhesion via a homophilic binding mechanism in the presence of calcium ions.* Neuron, 1995. 14(6): p. 1189-99.**

199. **Aravind, L. and E.V. Koonin, *Gleaning non-trivial structural, functional and evolutionary information about proteins by iterative database searches.* J Mol Biol, 1999. 287(5): p. 1023-40.**

200. **Rohm, B., et al., *Plexin/neuropilin complexes mediate repulsion by the axonal guidance signal semaphorin 3A.* Mech Dev, 2000. 93(1-2): p. 95-104.**

201. **Maestrini, E., et al., *A family of transmembrane proteins with homology to the MET-hepatocyte growth factor receptor.* Proc Natl Acad Sci U S A, 1996. 93(2): p. 674-8.**

202. **Torres-Vazquez, J., et al., *Semaphorin-plexin signaling guides patterning of the developing vasculature.* Dev Cell, 2004. 7(1): p. 117-23.**

203. **Casazza, A., et al.,** ***Sema3E-Plexin D1 signaling drives human cancer cell invasiveness and metastatic spreading in mice.*** **J Clin Invest, 2010. 120(8): p. 2684-98.**

204. **Takagi, S., et al.,** ***Specific cell surface labels in the visual centers of Xenopus laevis tadpole identified using monoclonal antibodies.*** **Dev Biol, 1987. 122(1): p. 90-100.**

205. **Neufeld, G. and O. Kessler,** ***The semaphorins: versatile regulators of tumour progression and tumour angiogenesis.*** **Nat Rev Cancer, 2008. 8(8): p. 632-45.**

206. **Franco, M. and L. Tamagnone,** ***Tyrosine phosphorylation in semaphorin signalling: shifting into overdrive.*** **EMBO Rep, 2008. 9(9): p. 865-71.**

207. **Roodink, I., et al.,** ***Plexin D1 expression is induced on tumor vasculature and tumor cells: a novel target for diagnosis and therapy?*** **Cancer Res, 2005. 65(18): p. 8317-23.**

208. **Christensen, C., et al.,** ***Proteolytic processing converts the repelling signal Sema3E into an inducer of invasive growth and lung metastasis.*** **Cancer Res, 2005. 65(14): p. 6167-77.**

209. **Oh, W.J. and C. Gu,** ***The role and mechanism-of-action of Sema3E and Plexin-D1 in vascular and neural development.*** **Semin Cell Dev Biol, 2013. 24(3): p. 156-62.**

210. **Movassagh, H., et al.,** ***Neuronal chemorepellent Semaphorin 3E inhibits human airway smooth muscle cell proliferation and migration.*** **J Allergy Clin Immunol, 2014. 133(2): p. 560-7.**

211. **Moriya, J., et al.,** ***Inhibition of semaphorin as a novel strategy for therapeutic angiogenesis.*** **Circ Res, 2010. 106(2): p. 391-8.**

212. **Kim, J., et al.,** ***Semaphorin 3E-Plexin-D1 signaling regulates VEGF function in developmental angiogenesis via a feedback mechanism.*** **Genes Dev, 2011. 25(13): p. 1399-411.**

213. **Suchting, S., et al.,** ***The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching.*** **Proc Natl Acad Sci U S A, 2007. 104(9): p. 3225-30.**

214. **Hellstrom, M., et al.,** ***Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis.*** **Nature, 2007. 445(7129): p. 776-80.**

215. **Fukushima, Y., et al., *Sema3E-PlexinD1 signaling selectively suppresses disoriented angiogenesis in ischemic retinopathy in mice.* J Clin Invest, 2011. 121(5): p. 1974-85.**

216. **Zygmunt, T., et al., *Semaphorin-PlexinD1 signaling limits angiogenic potential via the VEGF decoy receptor sFlt1.* Dev Cell, 2011. 21(2): p. 301-14.**

217. **Rahimi, N., *VEGFR-1 and VEGFR-2: two non-identical twins with a unique physiognomy.* Front Biosci, 2006. 11: p. 818-29.**

218. **Krueger, J., et al., *Flt1 acts as a negative regulator of tip cell formation and branching morphogenesis in the zebrafish embryo.* Development, 2011. 138(10): p. 2111-20.**

219. **Tsakiridis, T., et al., *Role of the actin cytoskeleton in insulin action.* Microsc Res Tech, 1999. 47(2): p. 79-92.**

220. **Sakurai, A., C.L. Doci, and J.S. Gutkind, *Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer.* Cell Res, 2012. 22(1): p. 23-32.**

221. **Nobes, C.D. and A. Hall, *Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia.* Cell, 1995. 81(1): p. 53-62.**

222. **Etienne-Manneville, S. and A. Hall, *Rho GTPases in cell biology.* Nature, 2002. 420(6916): p. 629-35.**

223. **Zhang, W. and H.T. Liu, *MAPK signal pathways in the regulation of cell proliferation in mammalian cells.* Cell Res, 2002. 12(1): p. 9-18.**

224. **Oinuma, I., et al., *The Semaphorin 4D receptor Plexin-B1 is a GTPase activating protein for R-Ras.* Science, 2004. 305(5685): p. 862-5.**

225. **Uesugi, K., et al., *Different requirement for Rnd GTPases of R-Ras GAP activity of Plexin-C1 and Plexin-D1.* J Biol Chem, 2009. 284(11): p. 6743-51.**

226. **Dunphy, J.L., et al., *The Arf6 GEF GEP100/BRAG2 regulates cell adhesion by controlling endocytosis of beta1 integrins.* Curr Biol, 2006. 16(3): p. 315-20.**

227. **Sakurai, A., et al., *Semaphorin 3E initiates antiangiogenic signaling through plexin D1 by regulating Arf6 and R-Ras.* Mol Cell Biol, 2010. 30(12): p. 3086-98.**

228. **Ryu, M.H., et al., *Chronic exposure to perfluorinated compounds: Impact on airway hyperresponsiveness and inflammation.* Am J Physiol Lung Cell Mol Physiol, 2014. 307(10): p. L765-74.**

229. **Asosingh, K., et al., *Nascent endothelium initiates Th2 polarization of asthma.* J Immunol, 2013. 190(7): p. 3458-65.**

230. **Duan, W., et al., *Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model.* J Immunol, 2004. 172(11): p. 7053-9.**

231. **Lee, L.T., et al., *Quantitative physiology and immunohistochemistry of oral lesions.* Biomed Opt Express, 2013. 4(11): p. 2696-709.**

232. **Movassagh, H., et al., *Neuronal chemorepellent Semaphorin 3E inhibits human airway smooth muscle cell proliferation and migration.* J Allergy Clin Immunol, 2013.**

233. **Christine Labno, U.o.C., Integrated Light Microscopy Core,. *Basic Intensity Quantification with ImageJ*. [cited 2015 August, 06]; Available from: https://digital.bsd.uchicago.edu/resources_files/Basic%20image%20quantification.pdf.**

234. **Stinson, M., et al., *Quantification of human chemokine production in TLR-stimulated and antigen-specific recall responses.* Methods Mol Med, 2008. 138: p. 121-31.**

235. **Hoshino, M., et al., *Increased immunoreactivity of stromal cell-derived factor-1 and angiogenesis in asthma.* Eur Respir J, 2003. 21(5): p. 804-9.**

236. **Hoshino, M., Y. Nakamura, and Q.A. Hamid, *Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma.* J Allergy Clin Immunol, 2001. 107(6): p. 1034-8.**

237. **Nissim Ben Efraim, A.H. and F. Levi-Schaffer, *Tissue remodeling and angiogenesis in asthma: the role of the eosinophil.* Ther Adv Respir Dis, 2008. 2(3): p. 163-71.**

238. **Middleton, J., et al., *A comparative study of endothelial cell markers expressed in chronically inflamed human tissues: MECA-79, Duffy antigen receptor for chemokines, von Willebrand factor, CD31, CD34, CD105 and CD146.* J Pathol, 2005. 206(3): p. 260-8.**

239. **Pusztaszeri, M.P., W. Seelentag, and F.T. Bosman, *Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues.* J Histochem Cytochem, 2006. 54(4): p. 385-95.**

240. **Ribatti, D. and E. Crivellato, *"Sprouting angiogenesis", a reappraisal.* Dev Biol, 2012. 372(2): p. 157-65.**

241. **Dimmeler, S. and A.M. Zeiher, *Endothelial cell apoptosis in angiogenesis and vessel regression.* Circ Res, 2000. 87(6): p. 434-9.**

242. **Ferrari, G., et al., *VEGF, a prosurvival factor, acts in concert with TGF-beta1 to induce endothelial cell apoptosis.* Proc Natl Acad Sci U S A, 2006. 103(46): p. 17260-5.**

243. **Lee, C.G., et al., *Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung.* Nat Med, 2004. 10(10): p. 1095-103.**

244. **Zhang, N., et al., *Tracking angiogenesis induced by skin wounding and contact hypersensitivity using a Vegfr2-luciferase transgenic mouse.* Blood, 2004. 103(2): p. 617-26.**

245. **Bernatchez, P.N., S. Soker, and M.G. Sirois, *Vascular endothelial growth factor effect on endothelial cell proliferation, migration, and platelet-activating factor synthesis is Flk-1-dependent.* J Biol Chem, 1999. 274(43): p. 31047-54.**

246. **Cai, J., et al.,** ***Vascular endothelial growth factor-induced endothelial cell proliferation is regulated by interaction between VEGFR-2, SH-PTP1 and eNOS.*** **Microvasc Res, 2006. 71(1): p. 20-31.**

247. **Kendall, R.L. and K.A. Thomas,** ***Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor.*** **Proc Natl Acad Sci U S A, 1993. 90(22): p. 10705-9.**

248. **Gaur, P., et al.,** ***Role of class 3 semaphorins and their receptors in tumor growth and angiogenesis.*** **Clin Cancer Res, 2009. 15(22): p. 6763-70.**

249. **Makino, N., et al.,** ***Involvement of Sema4A in the progression of experimental autoimmune myocarditis.*** **FEBS Lett, 2008. 582(28): p. 3935-40.**

250. **Mann, F., S. Chauvet, and G. Rougon,** ***Semaphorins in development and adult brain: Implication for neurological diseases.*** **Prog Neurobiol, 2007. 82(2): p. 57-79.**

251. **Bellon, A., et al.,** ***VEGFR2 (KDR/Flk1) signaling mediates axon growth in response to semaphorin 3E in the developing brain.*** **Neuron, 2010. 66(2): p. 205-19.**

252. **Bentley, J.K. and M.B. Hershenson,** ***Airway smooth muscle growth in asthma: proliferation, hypertrophy, and migration.*** **Proc Am Thorac Soc, 2008. 5(1): p. 89-96.**

253. **Shibuya, M. and L. Claesson-Welsh,** ***Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis.*** **Exp Cell Res, 2006. 312(5): p. 549-60.**