Expression of Semaphorin 3E in Asthma and its Role in Allergic Airway Disease

By

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THESIS ABSTRACT

Asthma is a chronic condition characterized by variable airflow obstruction, bronchial hyper-responsiveness, airway inflammation and remodeling. In spite of tremendous advances, the regulatory mechanisms controlling these pathological features have not yet been completely addressed. From an immunological perspective, type 2 inflammation and eosinophilic infiltration are the most striking hallmarks of asthma. At physiological level, structural changes such as increase in smooth muscle mass take the center stage which is usually associated with clinical measures of asthma. There might be some regulatory mediators capable of tuning airway inflammation and remodeling under homeostatic conditions but abrogated in asthmatic conditions.

Semaphorin 3E (Sema3E) is an axon guidance molecule that is ubiquitously expressed and plays diverse roles in structural and inflammatory cells such as regulation of cell migration, proliferation and angiogenesis. However, its role in clinical and experimental asthma remains unclear. In this thesis, I have set out to uncover the expression and function of Sema3E in allergic asthma. It is geenrally hypothesized that Sema3E is down-regulated in allergic asthma which orchestrates the function of inflammatory (dendritic cells and neutrophils) and structural (airway smooth muscle) cells. Replenishment of Sema3E, which is suppressed under asthmatic conditions, could confer protection against allergic asthma by modulation of cellular functions.

I began by comparing the expression of Sema3E between allergic asthmatics and healthy subjects. A remarkable down-regulation of Sema3E under asthmatic patients was observed which was further confirmed in a mouse model of the disease. Decreased expression of Sema3E

was specifically demonstrated on bronchial epithelial cells obtained from asthmatic patients at both mRNA and protein levels.

To address the function of Sema3E in allergic asthma *in vivo*, I extended my studies to mouse models of the disease and demonstrated that *Sema3e* gene deletion results in exacerbated allergic asthma pathology induced by allergen exposure. To investigate the translational relevance of my findings, I performed treatment of an asthmatic mouse model with exogenous Sema3E in which its intranasal administration attenuated airway inflammation, remodeling and hyper-responsiveness. The mechanism underlying Sema3E's role in pathogenesis of allergic asthma was extensively studied indicating a crucial role of this mediator in modulation of dendritic cells and neutrophils functions. Our data demonstrated that both dendritic cells and neutrophils express the Sema3E high affinity receptor, PlexinD1, which makes them responsive to Sema3E treatment. Then, I studied expression and function of PlexinD1 on human airway smooth muscle (ASM) cells. I found that PlexinD1 surface expression was reduced on ASM cells from asthmatic patients. Treatment of ASM cells with Sema3E inhibited their proliferation and migration as the characteristic feature of airway remodeling. Suppression of Rac1 GTPase activity and phosphorylation of Akt/PI3K and ERK/MAPK were found as signaling mechanisms underlying Sema3E's inhibitory effects. Together, these findings show that Sema3E thereby appears as a novel regulatory mediator, upstream of pro-allergic events, suggestive of a new approach to attenuate allergic asthma deficits.

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DEDICATIONS

I dedicate this thesis with a special feeling of gratitude to my wife, Forough Khadem, who never left my side during the hard times of my studies.

And it is also dedicated to all researchers who spend their life to make the world a better place to live through science!

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LIST OF ABBREVIATIONS

AHR: Airway Hyperresponsiveness

ALI: Acute Lung Injury

APC: Antigen Presenting Cell

ASM: Airway Smooth Muscle

BAL: Bronchoalvelar lavage

BMDC: Bone-Marrow-derived Dendritic Cell

CBD: Chronic Beryllium Disease

CD: Cluster of Differentiation

cDC: Conventional Dendritic Cell

COPD: Chronic Obstructive Pulmonary Disease

DAMP: Danger-Associated Molecular Patterns

DC: Dendritic cell

Der f1: *Dermatophagoides farina* group 1

Der p2: *Dermatophagoides pteronyssinus* group-2

ECM: Extracellular Matrix

EdU: 5-ethynyl-2'-deoxyuridine

EGF: Epidermal Growth Factor

ErbB2: Erythroblastic leukaemia viral oncogene homologue 2

ERK: Extracellular Signal-Regulated Kinase

FcεRI: Fc Epsilon Receptor 1

FEV1: Forced Expiratory Volume in 1 second

FGF2: Fibroblast Growth Factor 2

Flt3: Fms-like tyrosine kinase 3

FOX: Forkhead box

GATA3: GATA box 3

GEF: Guanidine Exchange Factor

GM-CSF: Granulocyte Monocyte-Colony Stimulating Factor

GPI: Glycophosphatidylinositol

GSK3: Glycogen Synthase Kinase 3

GTPase: Guanosine triphosphatase

HASMC: Human Airway Smooth Muscle Cells

HDM: House Dust Mite

HMGB1: High Mobility Group Box 1

IFN γ: Interferon γ

IgE: Immunoglobulin E

IL: Interleukin

ILC: Innate Lymphoid Cells

JAK: Janus Kinase

JNK: Jun Amino Terminal Kinase

LO: Lipoxygenase

LT: Leukotriene

MAPK: Mitogen-Activated Protein Kinase

MBP: Major Basic Protein

MDC: Macrophage-derived chemokine

MICAL: Molecule Interacting with Cas Ligand

MLN: Mediastinal Lymph Nodes

MPO: Myeloperoxidase

mTOR: mammalian Target Of Rapamycin

MUC: Mucin gene

NF-κB: Nuclear Factor Kappa B

Nrp: Neuropilin

PAR: Protease-Activated Receptors

pDC: Plasmacytoid Dendritic Cell

PDGF: Platelet-Derived Growth Factor

PI3K: Phosphatidylinositide 3-kinase

PTX3: Pentraxin 3

Rac1: Ras-related C3 botulinum toxin substrate 1

RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted

Rap: Ras-related proteins

Ras: Rat Sarcoma

RBD: Rho GTPase Binding Domain

ROS: Reactive Oxygen Species

SelR: Sulfoxide Reductase

Sema: Semaphorin

SLPI: Secretory Leukocyte Protease Inhibitor

STAT: Signal Transducer and Activator of Transcription

TAPVC: Total Anomalous Pulmonary Venous Connection

TARC: Thymus and Activation Regulated Chemokine

Tf: T Follicular

TGF-β: Transforming Growth Factor Beta

Th: T helper

TLR: toll-like receptor

TNF: Tumor Necrosis Factor

Treg: Regulatory T cells

TSLP: Thymic Stromal Lymphopoietin

VCAM-1: Vascular Cell Adhesion Molecule -1

VEGF: Vascular Endothelial Growth Factor

VEGFR2: Vascular Endothelial Growth Factor Receptor 2

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1 CHAPTER 1: INTRODUCTION

1.1 Clinical overview of asthma

Asthma is broadly defined as a chronic inflammatory disease of the conducting airways which is pathophysiologically characterized by bronchial hyper-reactivity, airway wall remodeling and airway narrowing. From a clinical point of view, asthma is associated with repetitive episodes of airflow obstruction, wheezing, breathlessness and chest tightness in predisposed individuals (1). It is estimated that up to 300 million people are affected by asthma worldwide with at least 250,000 deaths attributed to the disease each year which makes it the most common chronic lung disease (2). It is highly prevalent in affluent societies, wherein approximately 1 in 10 children and 1 in 12 adults is diagnosed with asthma with a large socioeconomic burden (1). In addistion, there is a remarkable increase of asthma along with urbanization in developing countries (3). Asthma could be controlled by a combination of current therapies including an anti-inflammatory inhaled corticosteroid (4) and a broncho-constricting β2-adrenergic agonist (5, 6). Despite effective treatment, asthma is considered as a significant cause of morbidity and mortality because the disease is refractory to inhaled corticosteroid treatment in almost 5-10% of asthmatic patients that require oral administration of corticosteroid and hospitalization (7).

Although asthma is considered as a chronic condition, acute exacerbations are an important clinical feature of the disease in some patients. In fact, asthma exacerbations occur for a period of hours or days and correspond to the major cause of hospital admission in asthmatic

patients. Viral infections are the leading cause of asthma exacerbations, though it could be induced by allergens, irritants and stress (8).

The airway inflammation in most of asthmatic patients is eosinophil-rich that corresponds to a dominant type 2 inflammatory response. In addition, asthma is usually associated with structural alterations in the airways, e.g. smooth muscle hyperplasia and hypertrophy, mucus hyper-secretion and deposition of extracellular matrix proteins; collectively called airway remodeling (9, 10). Deterioration of lung function is another hallmark of asthma pathology that could develop over time as a consequence of airway remodeling. However, asthma severity determined by lung function decline might not necessarily correspond to the measures of airway remodeling in some patients (11, 12). The precise mechanism of airway inflammation and remodeling in asthma will be discussed in details later.

1.2 Asthma phenotypes

Although "asthma" is a term extensively used to describe a single airway disease, it practically refers to a heterogeneous group of clinical symptoms with reversible expiratory airflow limitation. In fact, asthma could identify different phenotypes rather than a single disease (13). Categorizing asthma based on its origin into two major forms atopic (extrinsic) vs. non-atopic (intrinsic) is a traditional but still valid classification. The most asthmatic children and approximately half of asthmatic adults suffer from atopic form, in which the disease is associated with allergic sensitization defined by the presence of elevated level of serum immunoglobulin E (IgE) and/or a positive skin-prick test to the common allergens such as house dust mite (HDM), animal dander, fungal spores, pollen, etc. Non-atopic asthma,

frequently observed in adults, is developed independent of allergen-specific IgE response. Interestingly, this form of the disease is more common in women and is usually very difficult to treat (14).

The most widely used stratification of asthma is based on severity of the disease in which patients are categorized as mild, moderate and severe asthmatics according to the clinical measures such as forced expiratory volume in 1 second (FEV1) (15, 16). In mild to moderate patients, airway inflammation is restricted mostly to the large conducting airways, but in contrast severe asthmatics usually suffer from inflammatory complications in their small airways (17, 18). There are further sub-categories of severe asthma such as difficult, refractory, steroid-resistant, and brittle asthma where the latter is distinguished from all other types by recurrent, severe attacks (19, 20).

Although it seems very applicable and clear classifications, but categorizing such a heterogeneous syndrome based on severity or origin is an oversimplification and stratification of this condition as distinct phenotypes or endotypes according to updated clinical and biological criteria is of great importance to develop efficient strategies aiming to improved management decision (21). An endotype is a specific biological pathway which explains the observable properties of a phenotype (21). According to the “natural history, consistent clinical and physiological characteristics, an underlying pathobiology with identifiable biomarkers and genetics and a predictable response to general and specific therapies”, asthma could be categorized into early-onset allergic, late-onset eosinophilic, exercise-induced, obesity-related and neutrophilic pheno/endo-types (22). In asthmatic patients who smoke, there is a substantial clinical overlap with chronic obstructive pulmonary disease (COPD) (23, 24). Although each asthma endotype has separate features, there is a dynamic link between these forms and some environmental factors

such as infection, occupational exposure, smoking, hormonal status and obesity can alter the pathophysiology underlying each form of the disease (22). Exploring unknown pathways involved in the pathobiology of asthma might result in development of better approaches to stratify asthma endotypes based on novel biomarkers with higher sensitivity than current methods.

1.3 Allergic airway inflammation

Airway inflammation in allergic asthma is triggered by aeroallergen exposure that involves a dynamic interaction between respiratory epithelium, the innate and adaptive immune systems leading to a chronic response associated with remodeling of the airways. The first encounter with allergen induces an acute early-phase reaction, type I immediate hypersensitivity, which is followed by a late-phase reaction in many cases. Persistent exposure to the allergen(s) in a continuous manner leads to chronic allergic inflammation and tissue alterations (25).

Early-phase reactions occur within seconds to minutes of allergen challenge due to the release of mediators from mast cells which express high-affinity IgE receptors (FcεRI). IgE cross-linking by allergens induces secretion of three types of inflammatory mediators that contribute to the acute signs of reaction (26). It includes: 1) Preformed mediators stored in cytoplasmic granules which are released upon degranulation; 2) newly synthesized mediators such as cytokines, chemokines and growth factors; and 3) lipid-derived mediators (27, 28). Late-phase reactions mediated by both resident cells and recruited leukocytes upon allergen exposure develop during 2-6 hours (29). The persistent chronic airway inflammation induced by continuous allergen exposure is associated with perpetuation of the inflammatory response and also induction of a series of structural alterations of the airways, airway remodeling (25). Fig. 1-1

is a schematic representation of allergic airway inflammatory events (25) that will be explained in details later.

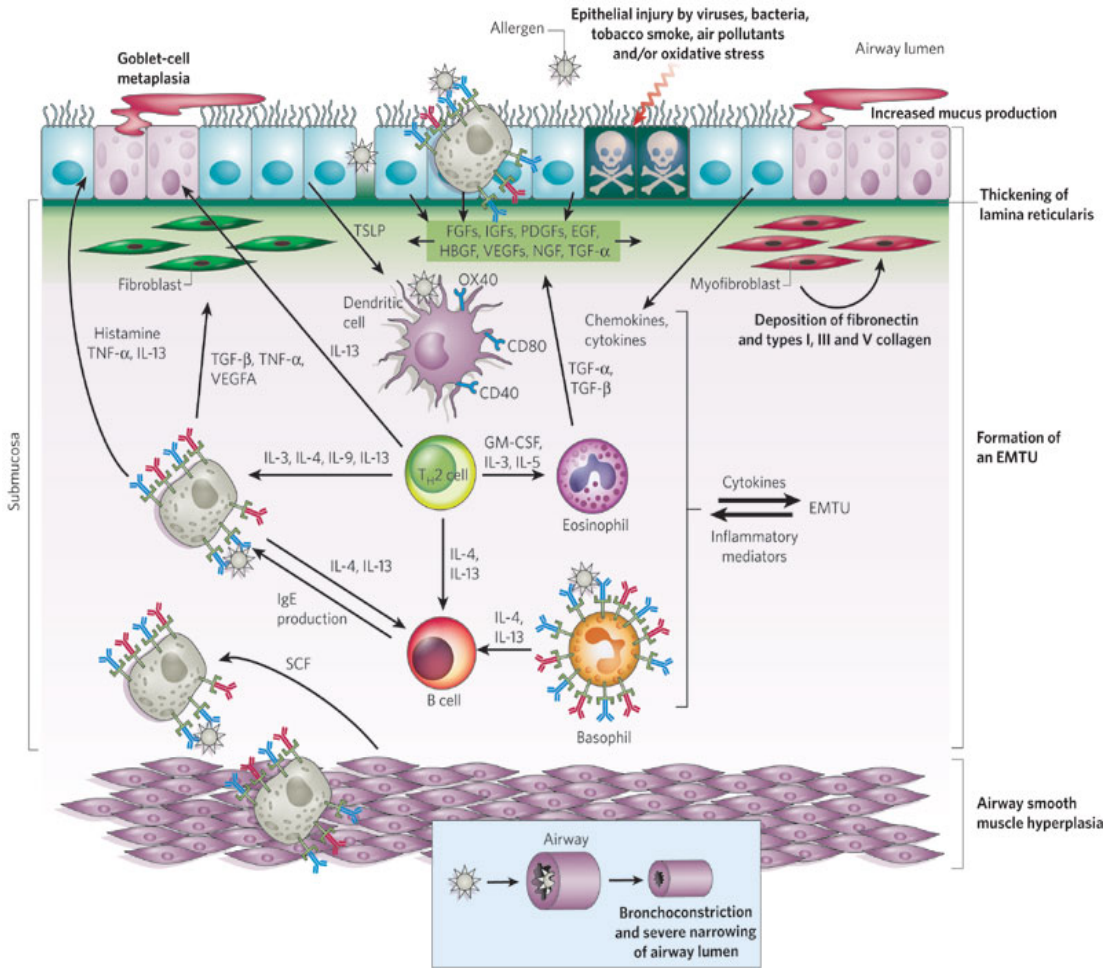

Figure 1-1 ***Chronic stage of allergen-induced airway inflammation.***
Innate recognition of innocuous aeroallergens by airway epithelial or dendritic cells leads to an unnecessary activation of immune system. It results in induction of Th2 response and IgE synthesis by B cells as the characteristic hallmarks of airway inflammation in allergic asthma. In addition, chronic exposure with the allergens causes formation of an epithelial-mesenchymal trophic unit (EMTU) in submucosal area where airway inflammation is perpetuated. Structural changes such as goblet cell metaplasia responsible for overproduction of mucus, thickening of lamina reticularis and more importantly for this project airway smooth muscle hyperplasia is developed during chronicity of allergic asthma as a consequence of chronic airway inflammation or in parallel with inflammatory response.

1.4 Eosinophilic vs. neutrophilic allergic asthma

Airway inflammation in allergic asthma is mainly characterized by increased number of eosinophils in both peripheral blood and airways of most patients which correlates with disease severity in some studies (30-32). However, there are non-eosinophilic cases of allergic asthma which should be distinguished from eosinophilic ones because of different clinical and pathological manifestations that may require differential treatment (33, 34). Anatomically, eosinophils could be found in the large or central airways as well as peripheral parts of the lungs in asthmatic patients (35). Terminal differentiation of eosinophils in asthmatics takes place not only within the bone marrow, but also in the inflamed airways locally (36). Interleukin (IL)-5 is a key mediator involved in several aspects of eosinophil biology such as differentiation, maturation, activation, survival, and their recruitment (37) that will be explained later. Eosinophils exert their functions by release of preformed granular cytotoxic mediators such as eosinophil peroxidase and major basic protein (MBP) which contribute to epithelial damage in the asthmatic patients. In addition, secretion of oxygen radicals and lipid mediators such as leukotriene (LT)B_4 as well as various cytokines and chemokines by eosinophils aggravates allergen-induced airway inflammation and remodeling (37).

Neutrophils are another subset of granulocytes observed in the airways of allergic asthmatic patients. Several studies have demonstrated that neutrophil number is increased in severe but not mild and moderate asthmatics (38-40). In addition, neutrophilic inflammation is found in asthma exacerbations (41), fatal asthma (41-43), occupational asthma (44), nocturnal asthma (45, 46), and childhood asthma (47-49). Neutrophilic asthma is specifically characterized by “basement-membrane thickening, relative glucocorticoid resistance, a positive smoking history, increased airflow obstruction, frequent viral exacerbations, increased numbers of

neutrophils in airway secretions during acute exacerbations, and increased sputum levels of IL-8" (50). Despite increased airway neutrophilia in some asthmatics, it is not clearly established whether it is attributable to corticosteroid therapy or an unknown consequence of alteration in airway microbiome (51). One of the crucial common features of severe asthmatic patients with high airway neutrophilia is that they are usually refractory to treatment with corticosteroids. In these cases, targeting neutrophils has been proposed as an alternative strategy (50). Unfortunately, this approach is limited by the lack of effective, safe and specific interventions directed against neutrophil recruitment or functions. For instance, TNF (52), IL-17A (53, 54), and leukotrienes (55) which are involved in neutrophil recruitment have been previously targeted to treat severe neutrophilic asthma. The results have not been clinically promising probably because of redundancy, tolerability, adverse effects or other issues (51). In fact, the mechanisms underlying the refractory phenotype in neutrophilic asthma remain elusive. Considering the redundancy of mediators involved in development, recruitment and function of neutrophils into the airways *in vivo*, targeting a specific pathway to inhibit neutrophil recruitment could not be an effective strategy aiming to treat severe corticosteroid-insensitive neutrophilic asthma. This could be the reason to explain why blocking IL-17, TNF and other mediators did not end up with successful outcomes in clinical trials. In addition, the current knowledge about this issue is surprisingly limited which necessitates more in depth mechanistic studies to precisely investigate the neutrophil functions in severe asthmatic patients. Deciphering intrinsic immunomodulatory mediators/pathways impaired in neutrophilic asthma would be a key step towards development of efficient strategies to control this condition.

Based on correlation between airway neutrophilia and severity of asthma, there should be a mechanistic link between enhanced airway neutrophils and chronic airway narrowing which has not been completely addressed (56). Neutrophils may play a role in the pathophysiology of chronic severe allergic airway inflammation via releasing an arsenal of inflammatory mediators including reactive oxygen species (ROS), cytokines, lipid mediators, defensins, elastase, myeloperoxidase (MPO), and cathepsin G (57, 58). Furthermore, studies on accumulation of neutrophils in acute near fatal severe asthma exacerbations suggest that they could be involved in initiation or even resolution phase of asthma attacks for instance by digestion of airway mucus plugs via neutrophil proteases (59).

It should be mentioned that neutrophils may undergo "phenotype switching" during airway inflammation in severe asthma. Apart from extended longevity of neutrophils in asthmatic airways, pro-survival epithelial-derived mediators (LTB4, IL-8, IL-1β, and IL-17A) can also induce phenotype switching into hybrid effector cells with antigen presenting cell (APC)-like properties (50). New insights into emerging concepts of enhanced neutrophil migration (60, 61) and survival (62-64), and phenotype switching (50) in severe asthmatic inflammation could open a new avenue to target the right neutrophil phenotype at the right time, at the right place, and in the right asthmatic patient.

1.5 Dendritic cells

Dendritic cells (DCs) are professional APC that form an interface between innate and adaptive immunity and play diverse roles in health and disease (65, 66). Although DCs are found throughout the respiratory tree, they are mainly located in the conducting airways of epithelium where approximately 500 of them are distributed per mm^2 as a contiguous network with those of

parenchymal lung (67, 68). The kinetic of DC response to aeroallergen challenge is rapid and almost similar to those of neutrophils (69). Typically DCs migrate to the lymph nodes upon sampling the antigens where they present processed ones to T cells. The consequent outcome is usually induction of a protective Th1 response. However, in atopic predisposed individuals, the Th2-biased response is primed by DC (70). The precise mechanism underlying DC-mediated Th2 deviation is not clear. It has been shown that allergen inhalation increases DC in the sputum obtained from human asthmatic patients (71, 72) suggestive of a functional role in disease pathophysiology. Studies on mouse models have revealed that injection of DC in mouse models induces leukocyte activation and recruitment into the airways. On the other hand, DC depletion abrogates bronchial hyperresponsivness as the cardinal feature of asthma pathology (73). These interesting observations collectively suggest that DCs could be an appropriate cell target to control allergic asthma manifestations such as airway hyperresponsiveness. However, the translational relevance of these experimental studies carried out on mouse models needs to be addressed in clinical settings.

According to studies in early 1990s (74, 75), there is a remarkable functional heterogeneity in respiratory DC subsets. More recent studies have revealed that DCs are, not only functionally but also, phenotypically, anatomically and developmentally different from each other (76, 77). Anatomically DC could be found in “most tissue compartments, including the large extrathoracic- and intrathoracic-conducting airways, the lung parenchyma accessible by lung tissue digestion, the alveolar compartment accessible by bronchoalveolar lavage, the pleura, the perivascular space, as well marginating inside the pulmonary vessels” in mouse lungs (76, 78, 79).

In fact, there is a “division of labor” between five lung DC subsets that have been described so far (54, 76). Conventional DC (cDC) and plasmacytoid DC (pDC) are considered as the major lung DC subsets with distinct functions in which an integrin surface marker, CD11c, is highly expressed on cDC. This subset could be further subdivided into $CD11b^+$ or $CD11b^-$ populations wherein the latter express langerin and CD103 (80-82). Pulmonary $CD11c^+$ $CD11b^+$ $CD103^-$ cDC is an extremely important subset in asthma studies because of the ability of priming effector $CD4^+$ Th cells in both homeostatic and asthmatic conditions (83, 84). On the other hand, $CD11c^+$ $CD11b^-$ $CD103^+$ cDC play a crucial role in development of tolerogenic protective responses upon allergen inhalation in mice (85).

From a developmental point of view, $CD11b^+$ and $CD103^+$ DC originate from distinct circulating monocyte progenitors. $CD103^+$ pulmonary DC preferentially derive from Ly-$6C^{hi}CCR2^{hi}$ monocytes, while conversely from Ly-$6C^{lo}CCR2^{lo}$ monocytes repopulate $CD11b^+$ cDC subset (86). Both tracheal and interstitial lung subsets have been shown to be from a single circulating progenitor cell derived from the bone marrow (87). The mechanisms regulating development of cDC subsets at homeostatic or inflammatory/allergic conditions has not been fully understood. The potential developmental defects of these cells in a non-inflammatory milieu could lead to heighten the outcome of exposure with specific inflammatory insults. For instance, deregulated development of $CD11b^+$ cDC at the baseline, might make the individuals prone to allergic inflammation wherein Th2 response is dominant. Therefore, understanding these defects is of great importance to provide novel therapeutic strategies for allergic asthma.

Lung pDC represent a smaller population compared to cDC characterized by intermediate expression of CD11c. In addition, pDC are PDCA-1^+ Siglec-H^+ $Ly6C^+$ $B220^+$ (88, 89). Depletion and adoptive transfer of pDC abrogates and induces inhalation tolerance, respectively

(88) which suggests a key, but not well-known, role of these cells in maintaining airway homeostasis. One of the mechanisms that has been proposed to explain how the inhalation tolerance is broken and Th2 response is induced under asthmatic conditions is based on triggering Toll-like receptor TLR4 signaling on lung DC via low doses of lipopolysaccharide (LPS) existing in typical allergens such as dust mite (90). Therefore, determining the level of LPS contamination in experimental models of allergic asthma is very important.

In addition to the indispensable role of lung cDC and pDC in regulation of primary allergic response (sensitization phase), they are also crucial during the effector phase of allergic asthma (76). Upon allergen challenge, DC overexpress several co-stimulatory factors including CD80 and CD86 that contribute to T cell activation (91, 92). Th2 attracting chemokines such as CCL17 (thymus and activation regulated chemokine, TARC) and CCL22 (Macrophage-derived chemokine, MDC) as well as eosinophil-selective chemokines produced by $CD11b^{+}$ cDC after allergen challenge is another functional contribution of lung DC in the effector phase of asthma (93, 94). It has been shown that expression of these chemokines in pulmonary DC subsets is tightly regulated by some innate epithelial-derived mediators such as thymic stromal lymphopoietin (TSLP) (95) and IL-33 (96-98). In general, there is a dynamic interaction between airway epithelial cells and DC wherein TSLP (99), IL-25 (100), and IL-33 (101) play a key role as innate instructing signals for lung DC and consequently induction of Th2 response. There are other airway epithelial mediators involved in regulation of lung DC functions including endogenous danger-associated molecular patterns (DAMP) or alarmins, uric acid, ATP, high mobility group box 1 (HMGB1), and S100 proteins (102, 103). Besides indirect epithelial cell-mediated activation of lung DC (104), allergens can also directly affect lung DC function in asthma. For instance, Derp1, as a potent allergic component in HDM with cysteine protease

activity, has been previously shown to activate human cDC and pDC to induce type 2 response via regulation of polarizing cytokines, co-stimulatory molecules, and cell surface receptors (105, 106). A C-type lectin called dectin-2 expressed on bone-marrow-derived DC (BMDC) has also been shown to directly detect HDM that induces leukotriene synthesis in a spleen tyrosine kinase (Syk)-dependent manner in mouse model (107). These studies indicate that several pathways contribute to regulation of airway DC functions as a complex network

In addition to Th2 cells, it has been shown that group 2 innate lymphoid cells (ILC2) play major role in initiation and perpetuation of type 2 inflammatory responses (108-110). Other cell types such as eosinophils, neutrophils, mast cells, basophils, γδ T cells, and IgE-producing B cells are also involved in the orchestration of Th2 cell responses. Therefore, the term "type 2", instead of so-called "Th2", has been suggested to describe the overall response (111). However, other T cell subsets are crucial to determine the outcome of airway inflammation in allergic asthma. For example, Th17 cells affect neutrophilic inflammation, as a hallmark of severe asthma, by regulating neutrophil survival. Moreover, T follicular (TF) cells promote differentiation of memory B cells at the germinal centers; and regulatory T cells (T_{reg}) suppress Th1, Th2, and Th17 responses (111) which are all crucial to determine the outcome of airway inflammation in allergic asthma.

The mechanism underlying how DC induce a type 2 response upon migration to lung draining mediastinal lymph nodes (MLN) has remained to be addressed. Although DC are believed to be localized in T cell zone of lymph nodes to activate naive cells in a type 2-biased manner, a helminth infection mouse model (*Heligmosomoides polygyrus*) has shown that $CD4^+$ T cells and DC co-localize in areas outside of the T cell zone (112, 113). Surprisingly, IL-4, known as a key factor driving Th2 differentiation, is not expressed by DC, while they produce IL-12 (involved in

Th1 priming), as well as transforming growth factor beta (TGF-β), IL-23 and IL-6 (which contribute to Th17 differentiation) in different inflammatory conditions. Therefore, it has been suggested that type 2 immunity is the "default pathway" when DC fail to promote Th1 or Th17 differentiation (111).

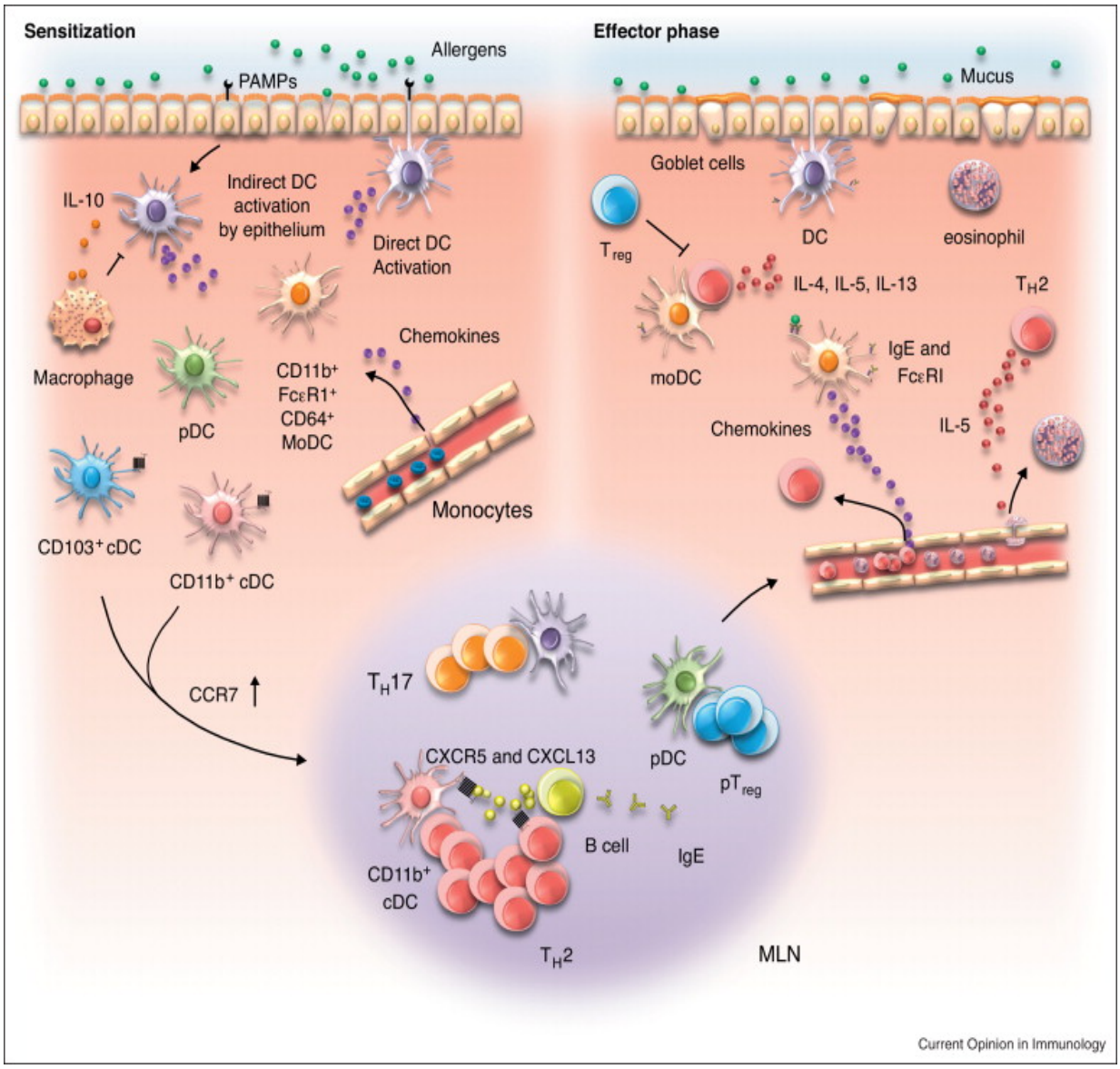

Figure 1-2 ***Pulmonary dendritic cell subset heterogeneity and functions in allergic asthma***
In the homeostatic conditions interstitial macrophages negatively regulates DC activation via IL-10 production. However, activation of DCs occurs by direct inhalation of allergens through DC protrusion or indirect through innate epithelial-derived cytokines and danger signals during sensitization phase. Both CD103$^+$ and CD11b$^+$ cDC subsets upregulate CCR7 surface expression which enables them to migrate into draining MLN where Th2 differentiation takes place. pDCs interact with peripheral T_{reg} cells in MLN to induce tolerance. Monocyte-derived (Mo)DCs perpetuate Th2 inflammatory response especially in effector phase.

Allergen-induced type 2 inflammatory features are abrogated in ovalbumin (OVA) and papain models, wherein $CD11c^+$ DC are conditionally abrogated (46, 114). More specifically, the $Fc\varepsilon R1^+$ $CD11c^+$ inflammatory DC subset, not basophils, is essential to induce type 2 response in HDM mouse model of allergic asthma (73). These studies demonstrate the crucial role of DC as "necessary and sufficient" players to develop type 2 inflammation. However, according to other studies basophils might also cooperate with DC in promoting type 2 response by releasing histamine, leukotrienes, IL-4, and IL-13 upon activation and prior to development of Th2 cells (115).

The crucial question is how allergen exposure induces deviation of immune response towards a type 2 pattern? The first potential mechanism could be sensing of allergens by pattern recognition receptors expressed on DC (116) or structural cells (117). As mentioned earlier, LPS contamination of HDM induces toll-like receptor (TLR) 4 signaling on airway structural cells to produce cytokines such as TSLP, IL-25, and IL-33 (90, 117). The structural and functional homology of *Dermatophagoides pteronyssinus* group-2 (Der p2), a major allergen in HDM, to the LPS binding element of TLR4 (MD2) which induces type 2 response further supports this notion (118). In addition, serine or cysteine protease activity of HDM (Der p1 and Der f1), papain, and allergens from *Aspergillus* and ragweed is a key determinant of type 2 response as their inactive forms do not induce type 2 inflammatory response (119, 120). These enzymes can target protease-activated receptors (PAR) expressed on airway epithelial cells (121) which have been shown to induce TSLP expression, as an innate Th2 inducer, in these cells upon stimulation with a fungal aeroallergen, *Alternaria in vitro* (122). In contrast, studies on cat and cockroach allergens, without any protease activity, have suggested that allergic type 2 response could be promoted via other potential mechanisms independent of protease activity (111). Therefore,

protease activity of allergens might explain the mechanism of Th2 induction in certain cases and there should be other unknown mechanisms apart from pattern recognition and protease activity. Interrupting the intrinsic mechanisms of allergen sensing by the immune system in predisposed individuals could help design novel immunotherapies for allergic asthma.

Recognition of tissue damage and metabolic changes caused by allergens has been proposed as alternative mechanisms underlying DC-induced type 2 biased immunity. For instance, alum administration was shown to cause tissue damage and cell death leading to the release of uric acid crystals (123) and also host DNA (124) which both products program DC to induce type 2 responses. In fact, type 2 response is considered as a rapid repair response to tissue damage which might be protective in the case of helminthic infections (125, 126), but not in allergic asthma. Finally, metabolic changes, e.g. amino acid starvation or oxygen deprivation leading to "integrated stress response", could induce DC-mediated type 2 response (127) as shown for papain in which protease activity causes tissue damage and the stress response (111).

As another layer of complexity, allergic asthma is not a pure type 2 inflammatory disease in all cases. The role of Th17-deviated response in refractory cases of the disease which is associated with neutrophilic airway inflammation has been previously addressed (53). Very recently, the percentage of (interferon γ) $IFN\gamma^{+}CD4^{+}$ T cells and $IL\text{-}17^{+}CD4^{+}$ T cells was shown to be higher in the airways of severe asthmatics than mild to moderate patients and IFNγ levels was higher in severe asthmatics, though IL-17 was not significantly different. Surprisingly, the level of Th2 cytokines, IL-5 and IL-13, was elevated only in mild to moderate but not in severe asthmatic patients (128). Using computer-assisted pathway analysis tools, it has been shown that high IFNγ levels in the airways promote AHR via suppression of secretory leukocyte protease inhibitor (SLPI) in both humans and mouse model of severe asthma (128). Therefore, in severe refractory

asthma a complex inflammatory response is involved which is characterized by an enhanced level of IFNγ and also TNF (52) along with increased Th2 and Th17 responses in the airways (129). These findings further highlight the heterogeneity of the disease and variation in current therapies as well as diverse roles of cells and mediators underlying asthma immunobiology. Therefore, a comprehensive knowledge is required to explain various aspects of the disease and effective treatment of each endotype caused by dysregulation of specific pathway(s) in personalized manner.

1.6 Cytokine network in allergic asthma

As a key feature of allergic asthma a certain set of mediators, categorized as Th2 cytokines, are overexpressed which include IL-3, IL-4, IL-5, IL-9, IL-13 and granulocyte–macrophage colony-stimulating factor (GM-CSF). The majority of these cytokines are encoded on the long arm of chromosome 5 (5q31-33) in human (130, 131) which are tightly regulated by p38 MAPK-mediated phosphorylation of a key transcription factor called GATA box 3 (GATA-3) (132). In addition, there are other mediators such as Th1, Th17 and epithelial-derived cytokines as well as chemokines which contribute to allergic asthma pathogenesis. Although each of these mediators has distinct properties, they have some common features such as the cellular sources, target cells, signaling pathways, and functional overlap among their own category and also between other inflammatory mediators as a dynamic network of interactions determining the mechanism of asthma pathology in terms of airway inflammation, remodeling, and AHR (37, 133).

IL-4 is the major cytokine promoting the Th2 fate that signals through IL-4Rα and γ common chains. Upon binding of IL-4 to type 1 IL-4R (IL-4Rα/γc) on naïve $CD4^+$ T cells, receptor is dimerized which leads to the phosphorylation of the tyrosine residues by Janus Kinases (JAK).

Cytosolic signal transducer and activator of transcription 6 (STAT6) binds to this phosphorylated site that results in phosphorylation of STAT6 by JAK. Then, dimerized pSTAT6 is translocated to the nucleus (134) where it binds to target DNA and regulates gene expression (135). Interestingly, airway eosinophilic inflammation, chemokine production, and goblet cell hyperplasia is abrogated in STAT6 knockout mice (136). More importantly, STAT6 regulates the expression of GATA3 which is considered as the master transcription factor in Th2 development (137). Furthermore, a recent study in mouse model of allergic asthma indicates that STAT3 plays opposing roles in Th2 response wherein its absence is associated with lower Th2 differentiation in bronchial lymph nodes but higher in the airways (138). IL-4 has been shown to promote expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells which increases recruitment of inflammatory cells to the airways (37). It is also able to induce IgE class switching in B cells as well as upregulation of FcεR2 (CD23) on airway inflammatory cells (139). It has been demonstrated that IL-4 also induces Stat3 and Stat5 signaling particularly in human suggestive of multifunctional properties for this cytokine.

IL-13 is structurally and functionally homologous to IL-4 and it binds a heterodimer composed of the IL-4Rα and IL-13Rα subunits. Similar to IL-4, the concentration of IL-13 is elevated in allergic asthma which is associated with IgE synthesis, mucus hyper-secretion and eosinophilia (140). Although IL-4 and IL-13 are both produced by $CD4^+$ T cells, only IL-13 is produced by ILC2 (141, 142). As a unique feature, blocking of IL-13 has been demonstrated to protect mice from AHR upon allergen exposure which could be attributed via direct effects on airway epithelial and smooth muscle cells not the inflammatory cells (143-145). However, blocking IL-13 signaling by targeting its receptor (IL-13Rα) did not improve FEV1 in clinical trials on

moderate to severe patients (146) suggestive of considerable differences between humans and animal models and presence of a potential unknown compensatory mechanism in humans.

IL-5 is a crucial cytokine involved in maturation, terminal differentiation, survival, activation, and migration of eosinophils (37, 147, 148). *In* vivo studies by using mouse models of allergic asthma clearly support a role for this cytokine; wherein IL-5 monoclonal antibodies has been found to be effective as an effective experimental therapy (149). According to the results of studies on humans, blocking either IL-5 (150) or IL-5 receptor (151) may reduce local and systemic eosinophilic inflammation but does not efficiently improve the allergen-induced late airway response, nor the baseline AHR in asthmatic patients (152).

IL-9 is another important type 2 cytokine in allergic asthma which is produced by different inflammatory cells. In 1998, it was revealed that lung-specific over-expression of IL-9 results in AHR and airway inflammation without any allergen exposure (153) via induction of IL-5 and IL-13 release by non-T cells (154). In addition, anti-IL-9 antibody treatment inhibits the development of allergen-induced airway eosinophilic inflammation and AHR (155). Unlike other type 2 cytokines, IL-9 is able to directly promote migration of mast cell progenitors to the airways (156). It is important to mention that a distinct IL-9 producing $CD4^+$ T cell subset (Th9 phenotype) has been described in which allergic airway inflammation is controlled by the transcription factor PU.1 independent of the typical Th2 response in mice (157).

IL-10 is a typical anti-inflammatory cytokine which is significantly reduced in asthmatic individuals (158). It can inhibit eosinophil survival and migration by preventing the release of chemattractants such as RANTES (regulated on activation, normal T cell expressed and secreted) and IL-8 from human ASM cells (159). In contrast to IL-4 and IL-13, it can downregulate isotype switching of activated B cells and IgE synthesis (37). Exogenous

recombinant IL-10 inhibits allergen-mediated airway inflammation, but surprisingly via an unknown mechanism it is able to augment AHR (160).

IL-12 promotes Th1 cell differentiation via stimulating production of IFNγ in T and natural killer (NK) cells; whereas it inhibits Th2 cell differentiation (161). It is involved in reduction of HDM-specific IgE sythesis, AHR, and airway eosinophilia in a time dependent manner in mouse models (162-164). Despite a remarkable reduction in blood and sputum eosinophilia, administration of recombinant human IL-12 does not improve lung function (165). Similar to IL-10, in asthmatic patients, IL-12 level is significantly reduced (166) suggesting a key role in pathogenesis of the disease.

IFNγ is considered as a typical Th1 cytokine which was previously shown to prevent allergen-induced airway eosinophilia and AHR in mice (167). However, the precise protective versus pathogenic role of IFNγ in allergic asthma is not clear. On one hand, T-bet deficient mice, which are unable to mount an IFNγ response, spontaneously develop asthma-like symptoms in the airways. On the other hand, IFNγ-overexpressing mice have increased airway type 2 eosinophilic inflammation (168). This discrepancy could be explained in part via potential different effects of IFNγ at different concentrations. It has been suggested that low levels of IFNγ might promote, while high levels inhibit the allergic airway inflammation (169).

The Th17 population and IL-17 levels are enhanced in asthmatic conditions especially in severe cases of the disease (170). IL-17A promotes airway inflammation by recruiting neutrophils and increasing their survival. Exogenous IL-17A increases allergen-induced eosinophilia and AHR whereas neutralization of IL-17 by using specific monoclonal antibody decreases pulmonary neutrophilia in mice (171, 172).

IL-25 (IL-17E), IL-33, and TSLP are the major epithelial-derived cytokines that play crucial roles especially in initiation and propagation of allergic response in which they induce the production of type 2 cytokines from ILC2 and also promote Th2 differentiation via DC (102, 173, 174). Blockade of IL-33 ameliorates allergic airway eosinophilic type 2 inflammation (175). HDM-induced airway inflammation and mucus production is diminished in TSLP receptor deficient mice (176). IL-25 is involved in IgE-mediated responses, airway angiogenesis, and eosinophilia by stimulating the release of type 2 cytokines in experimental models of allergic asthma (177).

Collectively, allergic asthma is distinguished by an altered level of a specific set of cytokines which play crucial roles in immune response to the aeroallergens and function as a dynamic network together. Emerging cytokines, chemokines and other mediators released from different cellular sources to the airways will provide a better understanding of allergic asthma immunopathogenesis and also novel therapeutic targets considering their redundancy and pleotropic effects.

1.7 Airway remodeling

In chronic asthmatic patients, airway remodeling involves alterations in structural cells in all of the layers of the airway wall which includes epithelial injury and repair, increased number of mucus-producing (goblet) cells, increased deposition of extracellular-matrix molecules such as collagen in the lamina reticularis, increased development of myofibroblasts, increased vascularity (angiogenesis) and increased thickness of the muscle bundles with increased size (hypertrophy), number (hyperplasia) and function of the airway smooth muscle cells.

The bronchial mucosa in healthy individuals is composed of “columnar ciliated epithelial cells, intermixed mucus secreting goblet cells, and a pool of basal cells responsible for epithelial regeneration”. Goblet cell hyperplasia/metaplasia is a critical remodeling event in the airway epithelium (178, 179). Expression of genes encoding mucin glycoproteins (MUC), specifically MUC5AC, is upregulated in asthmatic patients. It has been shown that IL-13, as a typical type 2 cytokine, indirectly induces goblet cell hyperplasia and mucus production via TGF-β2, forkhead box 2 (FOXA2), and 15- lipoxygenase (LO)1 signaling pathways (143, 180, 181). Neutrophil elastase is another important inflammatory mediator which promotes mucus secretion with a potential contribution to pathogenesis of severe asthma where extensive luminal mucus plugging is observed in both large and small airways (182, 183).

Bronchial epithelial cell hyperplasia could lead to mucosal thickening in asthma. In fact, airway epithelial cells obtained from asthmatic patients show increased Ki-67 and decreased Bcl-2 expression representing higher levels of proliferation and lower levels of apoptosis in these cells (184, 185). In addition, studies on severe asthmatics indicate the activation of autophagy in their airway epithelium (186). Decreased expression of occludins and claudins in the airway epithelium of severe asthmatic patients makes their epithelium more permeable (187). It is associated with downregulation of adherens junction proteins such as E-cadherin leading to more vulnerability to the epithelial injury (188) as evidenced by over-expression of epidermal growth factor (EGF) receptor, a marker of epithelial injury (189). Cigarette smoke is a potent injurious stimulus for asthmatic epithelial cells because of enhanced mucus production, epithelial thickness and proliferation in current smokers versus ex- and never-smokers (190).

Sub-epithelial fibrosis of the conducting airways is a common feature of airway remodeling in asthma which includes thickening in the basement membrane which is restricted to the lamina

reticularis and increased deposition of extracellular matrix (ECM) components such as collagen in the submucosal space (191, 192). There is no study to address whether there is a correlation between asthma severity and sub-epithelial fibrosis. Enhanced sub-mucosal vascularity in terms of increased endothelial cell number and vasodilation is a feature of airway remodeling in asthmatics patients (192, 193). Different animal models by using HDM have shown increased pulmonary angiogenesis (194-196) suggesting a direct effect of HDM on airway structural cells independent of the inflammatory cells. However, there are controversial studies that failed to address the increased angiogenesis feature of allergic asthma in patients with different severities (197). The typical features of airway remodeling in asthma and its comparison with normal airways have been shown in Fig. 1-3.

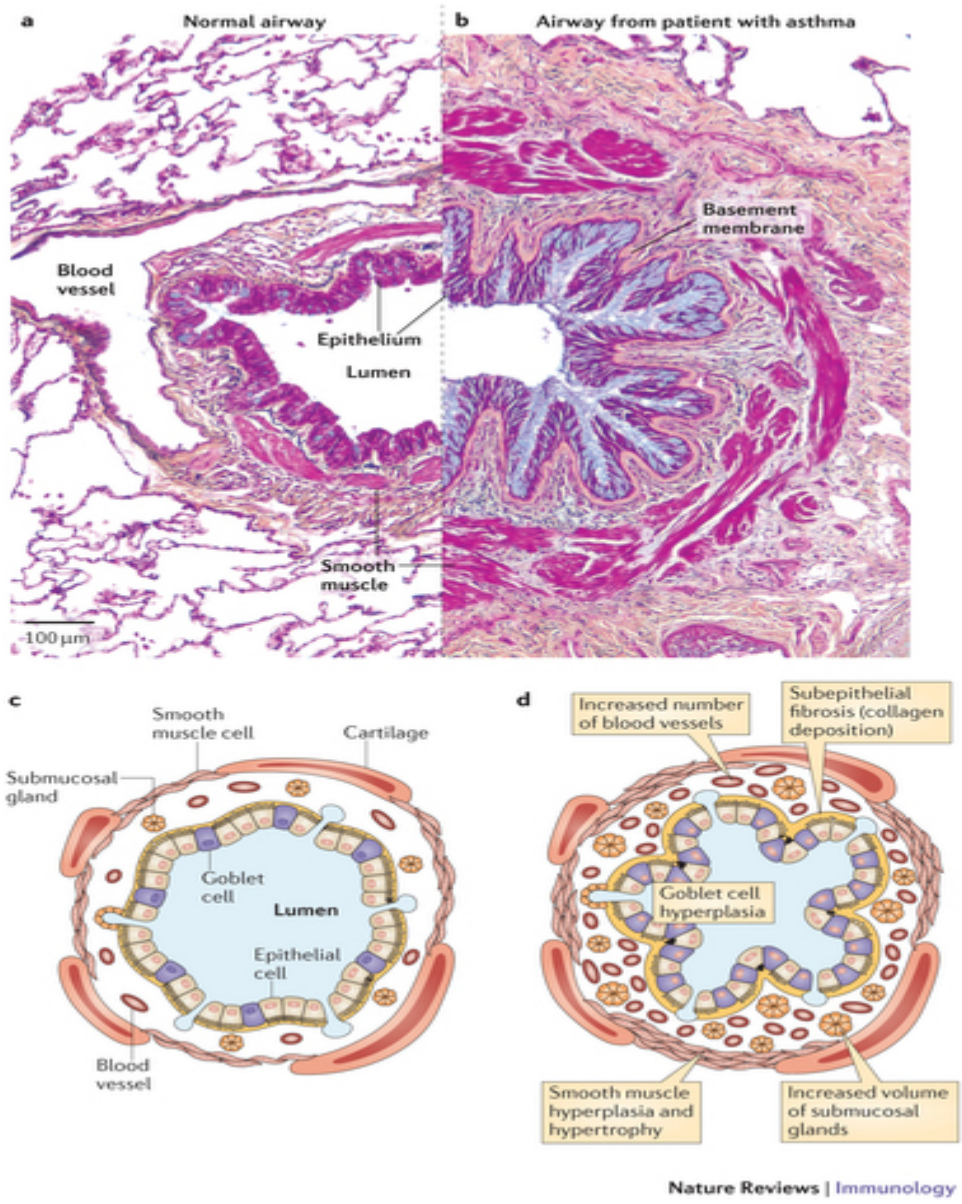

Figure 1-3 ***Airway pathology in asthma***

Airway structures in medium-sized healthy (a and c) compared to asthmatic (b and d) airways. The airways in asthma show considerable structural remodeling, including goblet cell hyperplasia, angiogenesis, sub-epithelial fibrosis and bronchial smooth muscle hyper-plasia/trophy.

1.8 Airway smooth muscle

Asthma was first defined as “paroxysmal dyspnoea of a peculiar character with intervals of healthy respiration between attacks” in 1860 by Sir Henry Hyde Salter (198). In this classical definition, asthma is considered as a consequence of anomalous airway smooth muscle (ASM) contraction suggestive of asthma as a disease of ASM. Nowadays, the inflammatory nature of asthma has been challenged by emerging evidence indicating development of the disease independent of inflammatory circumstance which further highlights the role of ASM in asthma pathophysiology (199-201). ASM cells function as the effector cells to regulate airways relaxation or constriction. In fact, contraction of ASM cells is responsible for airflow obstruction, airway narrowing and bronchoconstriction, as the cause of the most severe symptom of an asthma attack (193). It has been revealed that the consequent symptoms of ASM cell contraction in allergic asthma is mediated by several mediators and signaling pathways (143, 202-205).

Nevertheless, the precise mechanism of ASM contractility has remained to be elucidated. In addition, secretory function of ASM cells is drastically enhanced under asthmatic conditions in which they produce a variety of inflammatory mediators including GM-CSF, IL-1β, IL-2, IL-5, IL-6, IL-8, and eotaxin which lead to amplified and/or perpetuated inflammation without the involvement of immune cells (206, 207). It is believed that ASM thickening in asthma, as the most striking feature of airway remodeling, is a consequence of both hypertrophy (increased size of ASM cells) and hyperplasia (increased number of ASM cells) (208-213). Importantly, both of these abnormalities have been shown to correlate with asthma severity (18, 214). Despite several *in vitro* studies on ASM cells, there is not tremendous *in vivo* evidence to support this correlation and directly connecting asthma severity to increased ASM mass.

In fact, contribution of enhanced ASM hyperplasia and hypertrophy was one of the first findings to be described in relation to the pathology of bronchial asthma (215, 216). Despite several studies aiming to understand the role of ASM cells on airway remodeling, summarized in Fig. 1-4, the precise mechanism underlying regulation of ASM hyperplasia and hypertrophy has remained to be fully understood.

It has been previously demonstrated that ASM cell number, but not size, is nearly twofold higher in subjects with asthma than the healthy control group associated with 50-83% increase of muscle mass in the submucosa (217). ASM cell hyperplasia could be mediated by several mechanisms under asthmatic conditions. Increased ASM cell proliferation has been suggested as the primary mechanism underlying ASM hyperplasia.

Nonetheless, the massive accumulation of ASM cells in asthmatic patients could not be exclusively explained through increased ASM cell proliferation considering the doubling time of these cells. Therefore, enhanced migration of ASM cells or their progenitors from beyond the muscle towards the epithelium has been suggested as another mechanism which could lead to ASM thickening in asthmatic airways (192, 218). Moreover, reduced apoptosis of ASM cells (219-221) and recruitment of (myo)fibroblasts to the sub-epithelium (222-224) are the potential alternative mechanisms which might be induced in asthmatic conditions and implicate in enhanced ASM mass. Along with anti-inflammatory effects, new therapeutic interventions to treat allergic asthma should be able to modulate human ASM cell proliferation and migration; induce ASM cell apoptosis or reduce their trans-differentiation from fibroblast.

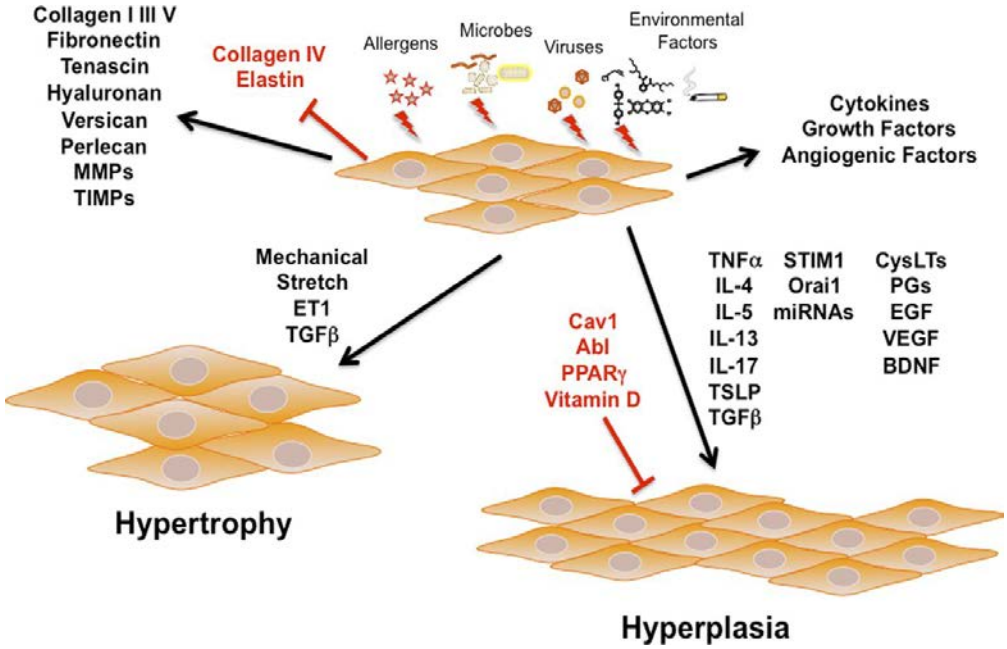

Figure 1-4 ***Role of ASM in airway remodeling***
Exposure with environmental allergens and other factors such as smoking and infections induces release of several mediators from ASM cells involved in different aspects of airway remodeling. Some of these mediators could promote hyperplasia and hypertrophy of ASM in an autocrine manner. ASM cells also respond to mediators produced by other cells in a paracrine fashion. There are some specific factors, e.g. VitaminD and Cav1, which are able to inhibit ASM hypeplasia.

Several lines of evidence have revealed that ASM cell proliferation is induced by numerous growth factors, cytokines, chemokines, and contractile agonists suggesting an important role of these factors in ASM cell hyperplasia and airway remodeling. For example, the mitogenic effect of platelet-derived growth factor (PDGF) (225), epidermal growth factor (EGF) (226), fibroblast growth factor 2 (FGF2) (227), TNF (228), leukotrienes (229), IL-8 and eotaxin (230) on ASM cells has been previously revealed. Eosinophils (231) isolated from asthmatic patients were also shown to induce ASM proliferation which was mediated via the release of cysteinyl leukotrienes from eosinophils in their co-culture as a direct contact-dependent manner (231). In addition, Naureckas and colleagues demonstrated that bronchoalvelar lavage (BAL) obtained from the fluid lining asthmatic airways contains excess mitogenic activity for ASM cells which is interestingly augmented upon allergen exposure in terms of cell number, $^{(3)}$[H]thymidine incorporation, extracellular signal-regulated kinase (ERK) phosphorylation, and cyclinD1 expression (232). Recently, Tiran and colleagues assessed the role of HDM on ASM cell proliferation indirectly via epithelial cells in the context of severe asthma by using an culture model combining epithelium layer in air-liquid interface (ALI) interacting with ASM cells. They have found that HDM stimulation of airway epithelial cells specifically enhances asthmatic, but not non-asthmatic, ASM cell proliferation via PAR2-mediated release of leukotrienes C4 as well as inducing expression of leukotrienes receptor CysLTR1 by ASM cells (233). On the other hand, potential therapeutic effect of vitamin D (234) and statins (235, 236) on airway remodeling could be mediated in part through inhibition of ASM cell proliferation as previously shown for current asthma therapies such as short-acting β2-adrenergic receptor agonist such as salbutamol (237).

Major molecular signaling pathways implicated in ASM cell proliferation include mitogen-activated protein kinases (MAPK), Ras-dependent phosphatidylinositide 3-kinases (PI3K), protein kinase C, reactive oxygen species, Rho-Rho kinase signaling, integrin-mediated signaling, and nuclear factor kappa B (NF-κB) signaling (238). MAPK superfamily is structurally divided into three groups: ERKs, JNKs (Jun amino terminal kinases), and p38s. Activation of the ERK (p42/44) signaling is essential for cell cycle regulation and is necessary for DNA synthesis in ASM cells (239). p38 is another MAPK involved in ASM cell proliferation in a stimulus-dependent manner (240). PI3K has been shown to be involved in ASM cell proliferation via phosphorylating phosphoinositides (PIP) (241) which leads to Akt activation as a crucial inhibitor of the glycogen synthase kinase 3 (GSK-3) and an activator of mammalian target of rapamycin (mTOR) (242). PI3K-dependent activation of small GTPases including Rac1 and Cdc42 induces cyclin D1 promoter activity in ASM cells in an ERK-independent fashion (243).

As explained earlier, increased ASM cell migrations is considered as an alternative mechanism of ASM hyperplasia in asthmatic airways. Previous studies have demonstrated pro-migratory effects of different mediators including PDGF (244, 245) (245), TGFβ (245), IL-1β (246), IL-8 (247) and IL-17 (248) on ASM cells. Furthermore, as shown by our group, emerging mediators such as pentraxin 3 (PTX3) (249) and TSLP (250) are involved in orchestration of human ASM cell migration. We have previously demonstrated that TSLP promotes migration of human myocytes by induction of actin polymerization and activation of a small GTPase called Ras-related C3 botulinum toxin substrate 1 (Rac1) (250). In contrast, β2-adrenergic agonists and glucocorticoids (218, 251) inhibit ASM cell migration *in vitro*. However, there is no *in vivo* evidence to support anti-migratory role of current asthma therapeutics.

A growing body of evidence suggests that alteration in the actin cytoskeleton plays a major role in migratory function of ASM cells which is tightly controlled by multiple signaling transduction pathways. Two types of receptors including RTK and GPCR are involved in promoting ASM cell migration. When they are activated upon binding to their ligands, downstream signaling events are immediately triggered which includes monomeric small GTPases (RhoA, Rac, Cdc42) and trimeric G-proteins. Activated G proteins, Ca^{2+}, and phospholipids such as PIP2 activate lipid kinases, ROCK, and MAPK. Then, protein kinases phosphorylate other protein kinases (e.g., MAPKAP kinase, LIM kinase) or proteins that regulate the final effector proteins (e.g., WAVE, WASP) which ultimately regulate actin polymerization and activation of motor proteins (238).

Understanding the precise signaling mechanisms underlying ASM cell proliferation and migration will enable us to design more targeted therapies for chronic airway disorders such as allergic asthma where smooth muscle remodeling is a common feature. There might be mediators with unknown functions in respiratory tracts or even beyond the airways which could signal through pathways already impaired in ASM cells under asthmatic conditions. Therefore, exploring such mediators is an essential step for novel anti-asthma therapeutics.

1.9 Airway hyperresponsiveness

Airway hyperresponsiveness (AHR), the most clinical characteristic feature of asthma, is defined as “excessive bronchial narrowing and manifests itself as an exaggerated bronchoconstrictor response of the airways to various inhaled stimuli” (252). Developed for the first time at 1946 (253), a bronchial challenge test is used to measure AHR in which methacholine or other constictors promote bronchospasm in healthy individuals, but with a lower threshold in

asthmatic patients (254). Methacholine challenge test results indicate that there are two different components of AHR: The first is baseline or persistent AHR observed in the majority of chronic asthmatics as an indicative of airway remodeling events; the second component is variable or episodic AHR reflecting airway inflammation and asthma severity (255).

It is believed that AHR is basically triggered by allergic chronic inflammation of the airways. This classical notion has been challenged by observing persistent AHR in the absence of inflammation mainly via functional changes in effector cells of AHR including ASM cells and neurons (256). Furthermore, airway obstruction could induce remodeling which may consequently contribute to the development of AHR (254). In fact, the modest effect of potent new anti-inflammatory drugs on AHR in asthmatic patients could be explained by the presence of remodeling mechanism underlying AHR. Novel therapeutic strategies should be able to modulate both structural and inflammatory changes in the airways, especially refractory severe asthmatic patients aiming to treat both episodic and persistent AHR (257, 258).

AHR is experimentally studied in mouse models of allergic asthma by measuring gold standard parameters of lung function including airway resistance and dynamic compliance upon the challenge of tracheotomized endotracheally intubated mice with a bronchial constrictor as an invasive approach (259, 260). There are 3 parameters to measure AHR: “Airway resistance” represents the resistance of the central or conducting airways and quantitatively assesses the level of constriction; “tissue resistance” reflects the energy dissipation in the alveoli and captures the ease with which the lungs can be inflated; and “tissue elastance” reflects the energy conservation in the alveoli and captures the elastic rigidity or the stiffness of the lungs (261).

1.10 Semaphorins

1.10.1 General overview of semaphorin family: History, classification, structure

Semaphorins were described for the first time in 1992 and 1993 by Alex Kolodkin and colleagues from the Howard Hughes Medical Institute, Berkeley, University of California. In an attempt to identify mediators involved in fasciculation of nerve axons in Grasshoppers, they found a molecule first called Fasciclin IV, homologous to semaphorin 3A in other species, with repulsive activity in neurons of developing insect limp buds (262). Another study by Yuling Luo et al at University of Pennsylvania in 1993 led to isolation of a new protein termed Collapsin-1 from chicken brain with growth cone collapsing activity which was in fact the chicken counterpart of human semaphorin 3A (263). Identification of these novel sequences and their unique functions suggested to categorize them in a specific family known as semaphorin/ collapsin (264).

Although semaphorins were originally discovered as axon guidance molecules in the central nervous system (263, 265), emerging evidence suggest that they are ubiquitously expressed in other systems such as cardiovascular, endocrine, gastrointestinal, musculoskeletal, immune, and respiratory systems (266). Widely present in many tissues, semaphorins contribute to regulation of morphogenesis, angiogenesis, differentiation, cell adhesion, proliferation, and migration (267). In 1999, a standard nomenclature system was proposed for semaphorins and they were phylogenetically categorized into 8 classes in which classes 1 and 2 are found in invertebrates exclusively; whereas classes 3, 4, 6, and 7 semaphorins are specifically expressed in vertebrates. Class 5 semaphorins are found in both and class V is specific to viruses. Vertebrate secreted semaphorins are all known as class 3 semaphorins while classes 4, 5 and 6 are transmembrane and class 7 is glycophosphatidylinositol (GPI)-anchored (264).

The structural hallmark of all semaphorins as a common characteristic feature is the N-terminal “sema domain” which consists of ~500 amino acids with a seven-blade β-propeller fold conformation. The sema domain is tightly coupled to one or more cysteine-rich domain named the “PSI domain” which stands for plexin–semaphorin–integrin and IPTs (immunoglobulin domains shared by plexins and transcription factors) in their extracellular regions. All mammalian semaphorins contain “immunoglobulin (Ig)-like domains” or “thrombospondin type 1 repeats” except class 6. According to structural and functional studies, semaphorins exert their roles in homodimer forms and there is no evidence for functional semaphorin monomers or heterodimers, so far. The sema domain has been shown to be responsible for homodimerization and also interaction with the receptors (Shown in Fig. 1-5 and reviewed in (268, 269)).

1.10.2 Semaphorin receptors and co-receptors

Semaphorins function through binding to their receptors called plexins which are classified in 4 groups in mammalians (268). In 1995, a cell surface axonal antigen B2 tyrosine kinase receptor was identified in Xenopus (270) which was renamed as plexin for the first time “to highlight its role in organizing the plexiform layers of the neural retina” (271). The cloning of human plexins was completed in 1999 and these transmembrane proteins were classified in 4 subfamilies based on the sequence similarity of their ectodomains (272). Plexins are extremely important in axon guidance and also vascular patterning and their loss of functions has been shown to be associated with several diseases such as congenital and autoimmune disorders as well as different types of cancer (269).

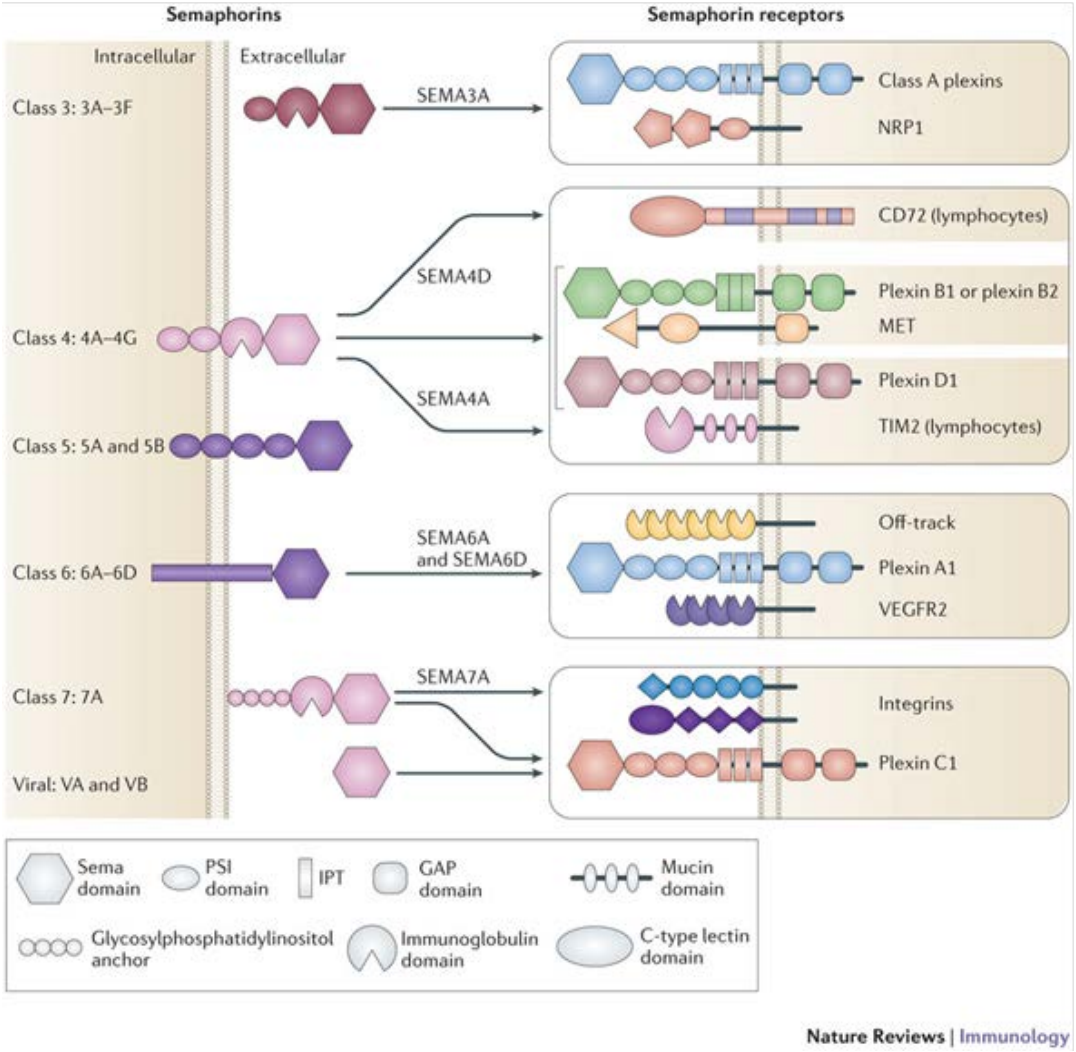

Figure 1-5 ***The structure of semaphorins and their receptors***

Semaphorins are characterized by an extracellular amino-terminal Sema domain followed by one or more cysteine-rich PSI domains. Plexins, which are the most common receptors of semaphorins, consist of an N-terminal Sema domain, followed by a combination of PSI domains and IPTs in their extracellular regions. Semaphorins and plexins interact through their Sema domains. Class 3 secreted semaphorins typically require the co-receptor Nrp1 to interact with the class A plexin receptor complex. However, Sema3E can bind to plexinD1 in a Nrp-independent manner. Class 4 transmembrane semaphorins bind to class B and class D plexin receptors. In lymphocytes, Sema4A also binds TIM2 and Sema4D binds CD72. Sema7A signals are mediated through β1 integrin receptors in both the nervous system and the immune system, and Sema7A also binds to plexin C1.

Interestingly, the structure of plexins is similar to semaphorins including an N-terminal sema-PSI domain that binds to semaphorins. Unlike the sema domain of semaphorins, sema domain in plexins does not undergo dimerization (273). In addition, other transmembrane proteins have been revealed to function as semaphorin co-receptors such as neuropilin (Nrp) 1 and Nrp2 (274). The extracellular portion of neuropilins contains two CUB (complement C1r/C1s) domains binding class 3 semaphorins, two coagulation factor V/VIII homology domains binding vascular endothelial growth factor (VEGF), and a MAM (meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu) domain involved in dimerization of neuropilins (274).

All class 3 semaphorins require Nrp1 as the co-receptor to interact with class A plexins (275) except Sema3E that directly binds to plexinD1 (276). Other semaphorin co-receptors include neural cell adhesion molecule L1 (L1CAM) which has been shown to interact with Nrp1 (277), and the receptor tyrosine kinases erythroblastic leukaemia viral oncogene homologue 2 (ERBB2) and MET which can interact with PlexinD1 (276) as well as class B plexins (278). Semaphorin 7A (Sema7A) can bind to PlexinC1 and also interact with β1 integrin receptors (279), in the nervous, immune and cardiovascular systems. Immune semaphorins, Sema4A and Sema4D, have been shown to interact with TIM2 (T-cell immunoglobulin and mucin domain-containing protein 2) and CD72, on the surface of lymphocytes, respectively. However, their binding affinity is much lower than those of corresponding plexin receptors from classes B and class D (280). Since the functional outcome of semaphorins in target cells is determined partly through interaction with their specific (co)receptors, a comprehensive knowledge to address the binding and signaling partners of each semaphorin is of great importance. It should be noted that semaphorin-holoreceptor interactions are context specific and might vary in different systems throughout the body.

1.10.3 Semaphorin functions

The first finding in the field of semaphorins were all suggestive of a repulsive role for these proteins in the process of axon guidance within the nervous system in which they guide migrating axonal growth cones during development. However, the first report in 1999 introducing a semaphorin as an attractive guidance cue totally revolutionized the notion. Today, Semaphorins are considered as bidirectional factors which could induce either repelling or attracting signal depending on the expression of their receptors and co-receptors and also the type of responding cells in a context-dependent manner.

From a functional point of view, semaphorins are more likely to provoke repulsion of target cells than attraction. For instance, semaphorin (Sema) 3A and Sema4D induce axonal collapse in growth cone region of neuronal cells (281, 282). Sema3F has been shown to inhibit tumor growth by reducing endothelial cell migration and adhesion (283). Conversely, Sema5A is able to promote angiogenesis via increasing endothelial cell proliferation, migration, and decreasing apoptosis (284). Even an individual semaphorin could exert bifunctional propeties and induce either repulsive or attractive responses. The functional outcome of a semaphorin, inhibition vs. activation, is determined by encountered biological milieu in terms of presence of repulsive or attractive signal in the context (285). For example, stimulation of neurons with cyclic nucleotides converts Sema3A repulsive activity into attraction (286). In fact, an individual semaphorin may exert different and even opposite functions depending on cell type or encountered biological conditions which highlights the complexity of semaphorins effects and necessitates understanding of their function in each specific cell, system and disease. Therefore, previous studies focused on the role of a semaphorin in a specific disorder or cell type could not be necessarily expected in a different disorder or cell type which needs to be separately investigated.

1.10.4 Semaphorin downstream signaling

The mechanism underlying how binding of a semaphorin induces plexin-dependent downstream signaling is not fully understood. However, it is believed to include a conformational change in cytoplasmic portions of the inactive plexin to a functionally active form upon semaphorin interaction (285). In general, semaphorin signaling mainly affects cytoskeleton compartments via reorganization of actin filaments and microtubule network (287). Molecular mechanisms which underlie semaphorin-plexin signaling include regulation of actin by Ras homolog (e.g. Rac-1) or modulation of integrin-mediated cell adhesion via Rat Sarcoma (Ras) proteins (e.g. R-Ras).

Monomeric p21 GTP-binding proteins, known as small GTPases, are early signaling components that regulate cellular functions through hydrolysis of GTP and cycling from the GDP to the GTP-bound state with the aid of guanidine exchange factors (GEFs). When bound to GTP, small GTPases are activated and attach to effector proteins to carry out a cascade of events including cell migration, proliferation, and angiogenesis (285, 287, 288). The cytoplasmic part of plexins contains GAP domains separated into two segments by a Rho GTPase binding domain (RBD). The plexin GAP domains directly interact with Ras-related proteins (RAP) which have been shown to play a crucial role in cell proliferation, migration, adhesion, and cell-cell junctions (289, 290).

Multifaceted functions of semaphorins are mediated by affecting not only small GTPases, but also through regulation of other pathways such as MAPK, PI3K, and STAT signaling (285, 291). Furthermore, emerging evidence has revealed that a family of cytosolic flavoprotein monooxygenases called “molecule interacting with Cas ligand” (MICAL) is both necessary and sufficient for semaphorin-plexin mediated F-actin reorganization in neurons (292). However,

more recent developmental studies have shown that a specific methionine sulfoxide reductase enzyme (SelR) opposes MICAL redox activity leading to neutralization of semaphorin–plexin repulsion (293). Emerging signaling pathways involved in semaphorin functions should be certainly addressed in order to develop semaphorin-based new therapeutic interventions.

1.10.5 Semaphorins in airway development, homeostasis and disease

As mentioned before, semaphorins are involved in regulation of different cellular events such as angiogenesis, cell adhesion, survival, proliferation and migration under developmental, physiological and pathological conditions. Since dysregulation of these processes has a key role in pathogenesis of airways diseases, semaphorins could be considered as novel regulators of airways dysfunction. However, the precise role of semaphorins in the pathogenesis of airway diseases has not been extensively investigated. Understanding the precise mechanism of semaphorins functions in healthy and unwell airways may open a new avenue to design new therapeutic approaches against airway diseases. New studies indicate that different members of semaphorin family contribute to airway physiology or pathology. Here, we review the findings on the role of semaphorins in various aspects of airway biology studied so far.

Semaphorins and their receptors are coordinately expressed in the lung and affect branching morphogenesis in both positive and negative manners during development. It has been shown that Sema3C and Sema3F stimulate branching morphogenesis and increase proliferation of terminal epithelial cells. Interestingly, treatment with soluble Nrp1 or Nrp2 reverses the stimulating effect of these semaphorins. In contrast, Sema3A inhibits lung branching morphogenesis (294). Another study demonstrated the inhibitory effect of Sema3A on alveolar septation in fetal lung explants (295). Controversially, a more recent study revealed the non-

essential postnatal role of Sema3-Nrp1 signaling in alveolar development or vascular function; despite the key role during fetal pulmonary development (296), which needs to be further investigated. However, prenatal genetic ablation of Sema3-Nrp1 leads to respiratory distress, alveolar hemorrhaging, abnormally dilated capillaries, and disintegrating alveolar septa (296). PlexinB1, Sema4D receptor, has diverse roles in lung development and homeostasis during adulthood. However, deletion of the gene encoding plexinB1 did not cause major developmental defects in mouse addressing a redundant role for this molecule or an unknown compensatory mechanism during development (297).

The differential expression and effects of semaphorins and their receptors on development of respiratory system suggest that they should play regulatory roles depending on the circumstance. It may provide us with some clues to understand the mechanisms of pre- and post-natal airway defects probably leading to discover some new treatment options.

Semaphorins are crucial regulators of morphogenesis in developing lung and their dysfunction may contribute to some congenital deficiencies (294). For example, loss of Sema3A-Nrp1 signaling has been shown to cause “dysmorphic vascularization reminiscent of alveolar capillary dysplasia” as a key element for treatment of neonatal airway diseases (298).

“Total anomalous pulmonary venous connection” or TAPVC is a lethal congenital disorder of the airways in which pulmonary veins cannot connect normally to the left atrium, allowing mixing of pulmonary and systemic blood (299). Sequencing of Sema3D gene in TAPVC patients revealed a phenylalanine-to-leucine substitution that adversely affects Sema3D function as a cardinal repellent guidance mediator which regulates normal pulmonary venous patterning (300). According to emerging experimental evidence, the precise role of semaphorins in various cases

of airway and also cardiovascular congenital disorders should be further elucidated where there is an unmet need to develop novel more efficient therapeutic strategies.

It has been reported that pulmonary expression of two immune semaphorins, Sema4A and Sema4D, and their receptors including plexinB1, plexinD1, CD72 and Tim2 is enhanced by allergen exposure in an animal model of allergic airway inflammation (301). Sema4A treatment reduces the severity of allergen-induced Th2-type responses in mouse airways (302, 303). However their precise role and mechanism of action in pathogenesis of allergic airways disease has remained to be addressed.

Interestingly, expression of Sema3A is reduced in allergic rhinitis and intranasal administration of exogenous recombinant Sema3A leads to alleviation of the allergic signs in mice (304). More recently, increased expression of Sema3A and its receptor was shown in human asthmatic individuals and mouse model of the disease compared to controls suggesting a potential role in the pathogenesis of allergic asthma (305).

Differential expression of semaphorins in allergic vs. healthy airways suggests them as promising biomarkers in these disorders. The key unanswered question here is that whether semaphorins alleviate allergic manifestations through immune vs. neuronal mechanisms or a complex network of events underlies their therapeutic outcome. The other important issue that should be addressed in the case of allergic asthma is that whether structural changes, namely airway remodeling, as well as airway hyper-reactivity are influenced by semaphorin treatment or not.

Since glycosylphosphatidylinositol (GPI)-anchored semaphorin, Sema7A, is highly expressed on human eosinophils and induces alpha smooth muscle actin (α-SMA) production in fibroblasts, it has been suggested as a plausible modulator of eosinophil-mediated pulmonary

fibrosis as a hallmark of airway remodeling in patients with chronic asthma (306). More recently, Mizutani *et al.* have shown the critical role of Sema7A in experimental IgE-mediated neutrophilic airway inflammation *in vivo*. In fact, blocking Sema7A using specific antibody reduced pulmonary recruitment of inflammatory cells, e.g. neutrophils, in the lungs after IgE sensitization and during the allergen challenge which surprisingly did not change the level of IL-4, IL-5, IL-6, IL-13, and IL-17A. In addition, anti-Sema7A antibody inhibited AHR and neutrophilic inflammation synergistically induced by IL-33 and IL-17A in normal mice (307).

Chronic beryllium disease or CBD is a "granulomatous disorder characterized by an influx of beryllium-specific $CD4^+$ T cells into the lung" (308). PlexinA4 peptides have been recently identified as the first ligand for a $CD4^+$ T cell involved in metal-induced hypersensitivity. Based on this study, plexinA4-derived peptides have been suggested to play an exclusive role in metal ion coordination and the generation of common antigen specificity in CBD patients. In fact, plexinA4 is the first complete ligand for a metal-specific T cell receptor (TCR) and demonstrate the requirement for additional acidic amino acids contributed by the peptide for beryllium capture and presentation. This finding is important because it explains how a human disease is generated in a genetically susceptible host exposed to an environmental antigen (308).

Chronic obstructive pulmonary disease (COPD) is estimated to be the third leading cause of death in 2030 worldwide. Cigarette smoking is the most prevalent risk factor for the development of COPD. However, only %20 of smokers develops COPD suggesting that genetic predisposition may influence disease development. Pulmonary gene expression of Nrp1 has been previously shown (309, 310) which is reduced at protein level in COPD smokers compared with healthy individuals or non-COPD smokers (311). Deletion of Nrp1 in pulmonary epithelium enhances development of emphysema by promoting epithelial cell death in response to cigarette

smoke exposure (312). It suggests that loss of pulmonary epithelial Nrp1 enhances apoptosis of type I and II epithelial cells and airspace enlargement in response to chronic cigarette smoke exposure.

Sema3A-Nrp1 signaling pathway is also considered as a key axis controlling airspace enlargement which contributes to development of emphysema as an important feature of COPD. Environmental factors, e.g. cigarette smoke, may trigger emphysema by deregulation of this essential developmental signaling pathway in the fully mature lung which counterbalances cell injury and repair processes (295).

“Acute lung injury (ALI) develops in response to pneumonia, major surgery, or prolonged mechanical ventilation” (313) associated with a high mortality (314). Migration of neutrophils into the alveolar space is a cardinal initial feature of acute pulmonary inflammation during lung injury (315). Sema7A receptor, plexinC1 is expressed on alveolar neutrophils and induced during ALI. Functional inhibition of plexinC1, using specific mAb, reduces neutrophil migration *in vitro* and improves survival in mouse model. Therefore, blockade of Sema7A-plexinC1 signaling has been suggested as a novel therapy for pulmonary inflammation capable of improving overall outcome in experimental ALI (316).

Semaphorins are essential mediators in development/progression of lung cancer. Different members of class 3 semaphorins were previously reported to be involved in pathogenesis of lung cancer via affecting signaling pathways regulating angiogenesis (317, 318). Sema3A inhibits lung tumor angiogenesis and metastasis (317). It overcomes cancer hypoxia and dissemination (319). Loss of the 3p21.3-encoded semaphorins, Sema3B and F, induces development of lung cancer development (320) via multiple signaling pathways (318). Lung cancer cell growth is inhibited upon re-expression of Sema3B gene (321). Sema3B is a potential tumor suppressor that

induces apoptosis in Sema3B-inactivated lung tumor cells through the Nrp-1 receptor by inactivating the Akt signaling pathway (322). cDNA array experiments on non-small-cell lung cancer (NSCLC) have shown reduced expression of gene encoding Sema3B as a tumor suppressor gene (323). Sema3B exerts unexpected functions in cancer progression by fostering a prometastatic environment through elevated IL-8 secretion and recruitment of macrophages coupled to the suppression of tumor growth by activating the p38-mitogen-activated protein kinase pathway in an Nrp1-dependent manner (324). Loss of Sema3F genes occurs frequently in lung cancer and correlates with advanced stage of disease (318) and selective suppression of tumorigenicity by Sema3F in lung cancer cells has been shown *in vivo* (320). Expression of Sema4D is a frequently used strategy by which a wide variety of carcinomas including lung carcinoma may promote angiogenesis, and therefore is a possible therapeutic target for the treatment of these malignancies (325). Sema5A has emerged as a prognostic biomarker for non-small cell lung carcinoma (326).

Sema7A is a critical regulator of tissue remodeling and has an important role in TGF-β-induced lung fibrosis as well as scleroderma-related interstitial lung disease. It mediates its role via affecting myofibroblast hyperplasia, alveolar remodeling, and apoptosis (327). $Sema7a^{+}$ T_{reg} cells are associated with progressive idiopathic pulmonary fibrosis (328). Sema7A downregulation improves autoimmune fibrosis (329). It has been reported that Sema7A promotes neutrophil migration during hypoxia (330).

Considering the important role of semaphorins in airway biology, this family of neuronal chemorepellents could be considered as novel players in regulation of crucial cellular processes in the airways. Semaphorin signaling pathways significantly contribute to inhibit pathological processes in airway diseases. Addressing pathways predominant in the semaphorin signaling

may lead to design novel approaches to recapitulate the effects of semaphorins in a feasible, safe and translational manner. Semaphorins may also serve as potential biomarkers for airway diseases. Further investigations will determine the role of semaphorins in airway disorders and their use as therapeutic targets.

1.11 Semaphorin 3E

1.11.1 General overview

Semaphorin 3E (Sema3E) was previously called M-semaH, coll-5, M-SEMAH, M-SemaK, SEMAH. The official name of this gene is “sema domain, immunoglobulin domain (Ig), short basic domain, secreted (semaphorin) 3E.” SEMA3E is the official gene's symbol (http://www.ncbi.nlm.nih.gov/gene/9723). By sequencing clones obtained from a size-fractionated brain cDNA library, Nagase and colleagues cloned SEMA3E which shares 87.4% identity with mouse *Sema3e* (331).

In 1998, Christensen *et al.* sequenced SEMA3E gene, M-SEMAH, for the first time in tumor cell lines and cloned two cDNA transcripts as spliced variants for this gene. According to sequence analysis they predicted a protein of 775 amino acids with high homology to the family of semaphorin and collapsin proteins. The prediction of structural characteristics, such as the Sema domain and the immunoglobulin-like domain, identifies the molecule as a bona fide new member of the family. Other features, such as glycosylation sites, an NH2-terminal signal sequence, and a positively charged COOH terminus, suggest that M-SemaH could be a glycosylated and secreted molecule, possibly attached to the cell surface through the COOH terminus as proposed for Collapsin 1 (332). Cytogenetic studies revealed that the human

SEMA3E gene is located on the long (q) arm of chromosome 7 at position 21.11 (7q21.11) encompassing base pairs 83,363,905 to 83,649,162 in human.

"Both variants encode the same 775-amino acid protein, which contains an N-terminal signal sequence followed by a large semaphorin domain, a C2 immunoglobulin-like domain, and a positively charged C terminus. Semah also has 13 conserved cysteines and 3 potential N-glycosylation sites. Northern blot analysis detected transcripts of 7.0, 4.5, and 4.0 kb expressed at highest levels in adult mouse brain and lung. In addition, RT-PCR determined that the 5-prime ends of the 4.5- and 4.0-kb transcripts were identical" (332).

Screening of patients with CHARGE syndrome for mutations in the SEMA3E gene revealed a *de novo* mutation. CHARGE syndrome is a sporadic condition that is characterized by "choanal atresia, coloboma of the eye, cranial nerve dysfunction, characteristic external and inner ear abnormalities, cardiac defects, genitourinary abnormalities, and growth retardation" (333). In a patient originally described with CHARGE syndrome and a *de novo* balanced translocation involving chromosomes 2 and 7, Lalani and colleagues mapped the translocation breakpoints and identified the SEMA3E gene within 200 kb of the breakpoint on 7q21.11 (333).

Recently, exome sequencing analysis and computational modeling have shown that dysfunctional Sema3E signaling underlies gonadotropin-releasing hormone neuron deficiency in Kallmann syndrome. In that study, a point mutation in SEMA3E shared between two brothers with Kallmann syndrome was identified to cause inherited GnRH deficiency. Interestingly GnRH neuron deficiency in male mice was accompanied by impaired testes growth, a characteristic feature of Kallmann syndrome (334).

Besides non-redundant abovementioned role of Sema3E-PlexinD1 axis in nervous and cardiovascular systems, it also implicates in negative regulation of immune system. Sema3E

modulates the migration of $CD4^+CD8^+$ (double positive) T cells in murine thymus via inhibition of CCL25-CCR9 chemokine signaling which is a critical inducer of thymocyte migration from cortex into the medulla (335). Mechanistically, Sema3E-PlexinD1 axis mediates this function in thymocytes by a dynamic control of β1 integrin adhesion (336). Interestingly, PlexinD1 expression is downregulated in leukemic thymocytes which further highlights the important critical regulatory role of Sema3E-PlexinD1 in clinical settings (337).

1.11.2 The phenotype of Sema3E knockout mouse

Since majority of *in vivo* data presented in this thesis results from studies on mice with genetic deletion of Sema3E encoding gene, the effect of this deficiency on their phenotype is explained in details. *Sema3e*$^{-/-}$ mouse was generated by targeted disruption of the gene using a homologous recombination strategy in embryonic stem cells (338). These mice were shown to be viable, fertile and born at the frequency predicted for Mendelian transmission without any gross abnormality (338). However, the precise effect of Sema3E genetic deletion on the immune system which might make the mice predisposed to allergic or other types of inflammation has not been demonstrated. The function of airway structural cells such as ASM, epithelial and endothelial cells in the absence of Sema3E has not been neither investigated. Therefore, a comprehensive study aiming to address the potential difference of respiratory and immune systems between *Sema3e*$^{-/-}$ and WT mice is necessary.

Previous studies indicate that, due to crucial role of Sema3E, the mice lacking this protein display tremendous developmental, neurological and cardiovascular defects. First of all, deletion of Sema3E leads to inability of the intersomitic vessels to be excluded from the normally Sema3E-expressing caudal region of the somite and consequently overgrowth with a loss of the

normal segmented pattern (339). Moreover, development of an abnormal branched aortic plexus associated with a significantly narrowed avascular midline is evident in *Sema3e*$^{-/-}$ compared to normal embryos (338). Abnormal vasculature is a characteristic feature of *Sema3e*$^{-/-}$ mice not only during the development but also in disease models such as different types of cancer. Since formation of new blood vessels is one of the prominent facets of airway remodeling in allergic asthma, it could be speculated that the mice lacking Sema3E, as an anti-angiogenic factor, develop increased vasculature upon chronic allergen exposure. However, this key issue has not been addressed previously and requires rigorous mechanistic studies to elucidate what is the impact of knocking out Sema3E on both basal and allergen-induced airway angiogenesis.

From a neurological point of view, genetic ablation of Sema3E in mice is associated with considerable defects at both embryonic and postnatal stages. The performance of *Sema3e*$^{-/-}$ mice in the Morris water maze to study their spatial memory indicates that these mice are similar to WT littermates in swimming ability, motivation and retrieval of spatial reference memory. *Sema3e*$^{-/-}$ mice exhibit 30% longer latency; whereas WT mice learned from their initial placement and performed correctly on the first daily trial. In fact, the absence of Sema3E leads to intact spatial reference memory but a moderately impaired spatial working memory. The plus maze studies have further suggested reduced anxiety levels along with other emotional alterations in *Sema3e*$^{-/-}$ mice (340). Considering the importance of neuronal compartment in pathogenesis of asthma, the Sema3E could play an essential neuroimmunological role in the airways which has no been investigated so far.

It has been proposed that the reduced growth of subiculo-mammillary axons in *Sema3e*$^{-/-}$ embryos results in an adult behavioral pattern consistent with dysfunction of the mammillary bodies. Subiculo-mammillary tract in brain sections of *Sema3e*$^{-/-}$ embryos and also adult mice is

atypically thin compared to WT controls. This axonal projection defect could partly explain the abovementioned alteration of phenotypic behavior in the absence of Sema3E expression (340). Furthermore, in the nervous system of *Sema3e*$^{-/-}$ mice, glutamatergic synaptogenesis is enhanced which is accompanied by increased synaptic density suggestive of a restricting role for Sema3E to control the number of these synapses (341).

The effect of Sema3E genetic ablation on the immune system has been investigated to some extents. Surprisingly, the localization of thymocytes from *Sema3e*$^{-/-}$ mice is different than those of WT littermates. In the absence of Sema3E, $CD4^{+}CD8\alpha^{+}CD69^{+}$ thymocytes localize outside the medulla but they are mostly found inside or adjacent to the corticomedullary junction the medulla of thymus in WT control mice. It suggests that Sema3E could play a crucial role in directed migration of activated thymocytes. The lack of a clear delineation of the corticomedullary junction of thymus *in vivo* is a considerable immunological consequence of Sema3E gene deletion which might account for lymphocyte development (335). However, the functional outcome of this aberrant phenotype in specific inflammatory conditions such as allergic asthma has remained to be understood. In another study aiming to address the role of Sema3E in adipose tissue inflammation, it has been demonstrated that there is no difference in body weight, food intake, or visceral fat mass between *Sema3e*$^{-/-}$ and WT mice. Nonetheless, Sema3E deficiency is associated with a significant reduction of macrophages and also production of pro-inflammatory cytokines without any effect on vascularity in adipose tissue (342). This indicates a potential role of Sema3E in pulmonary macrophages considering the context-dependent manner of semaphorin effects.

Collectively, it might be implied that because of versatile and to some extents unknown roles of Sema3E in different systems/organs the precise and complete phenotype of *Sema3e*$^{-/-}$ mouse

model has not been explored. It would be of great importance to determine the potential baseline differences of specific cell functions between *Sema3e*$^{-/-}$ and WT mice prior to investigating its role in an allergic airway inflammation and remodeling.

1.11.3 Sema3E proteolytic processing

Growth-repelling properties of class 3 semaphorins depends on expression and function of full-size dimeric forms (343, 344). But, furin enzymes known as PACE (paired basic amino acid cleaving enzyme), which belong to the subtilisin-like proprotein convertase family, can target class 3 semaphorins and cleave them on specific sites leading to reduced repulsive activity as a post-translational effect (345). In the case of Sema3E, it was previously revealed that *in vivo* promotion of lung metastasis as well as *in vitro* induction of tumor cell growth and motility are the functional consequences of furin-dependent cleavage of full length (87.5 kDa) protein. In fact, studies on human cancer cell lines and experimental lung metastasis assay demonstrate that extracellular cleavage of full-length Sema3E to a truncated isoform (p61-Sema3E) reverses Sema3E role from a repelling cue into an invasive pro-metastatic factor (346).

A mutated, uncleavable recombinant variant of Sema3E (Uncl-Sema3E) binds to PlexinD1 like p61-Sema3E, but does not promote the association of PlexinD1 with ErbB2 nor activates the ensuing signaling cascade leading to metastatic spreading. Furthermore, Uncl-Sema3E competes with endogenous p61-Sema3E produced by tumor cells, thereby hampering their metastatic ability. Uncl-Sema3E also acts independently as a potent anti-angiogenic factor. It activates a PlexinD1-mediated signaling cascade in endothelial cells that leads to the inhibition of adhesion to extracellular matrix, directional migration and cell survival. The putative therapeutic potential of Uncl-Sema3E was validated in multiple orthotopic or spontaneous tumor models, where either

local or systemic delivery of Uncl-Sema3E-reduced angiogenesis, growth and metastasis, even in the case of tumors refractory to treatment with a soluble vascular endothelial growth factor trap. In summary, Uncl-Sema3E could be considered as a novel inhibitor of tumor angiogenesis and growth that concomitantly hampers metastatic spreading. Increased number of active-caspase 3-positive apoptotic endothelial cells upon treatment with Uncl-Sema3E has been shown as part of the mechanism underlying Uncl-Sema3E effects *in vivo.* (347). Studies using both orthotopic and spontaneous tumor models demonstrated a therapeutic potential of Uncl-Sema3E in which its local or systemic delivery decreases angiogenesis and metastasis, importantly in tumors refractory to treatment with a soluble VEGF trap. (347). The potential cleavage of Sema3E in the contexts other than cancer has not been investigated and should be considered as an essential issue in translational studies.

1.11.4 Sema3E holoreceptor complex and signaling

PlexinD1, shown as the direct binding partner of Sema3E, is dynamically expressed in many embryonic tissues and also after development particularly in endothelial cells of the vasculature. It is expressed in several tissues, though the function is not fully understood. For example, Plxnd1 gene expression has been revealed in the podocytes, adrenal and mammary glands, osteoblastic cells and bone tissues, the lung mesenchyme, the smooth muscle of the small intestine and immune cells (337).

PlexinD1 expression is essential for cardiovascular development wherein the mice with conventional genetic deletion of its gene will succumb two days postnatal because of cardiovascular defects (348). On the other hand, *Sema3e* deficient mice are viable after birth and developmental cardiovascular defects are recapitulated suggestive of additional PlexinD1

ligand(s) (338, 340). Sema3E is recognized as the canonical ligand for PlexinD1 (338) and it does not binds to other plexins which is an exception to the typical pattern of class 3 semaphorin interaction with Nrp-Plexin complexes (275). Sema3E-PlexinD1 binding is an Nrp-independent process which leads to PlexinD1-mediated endothelial cell repulsion independent of Nrp1 (338). It has been shown that Sema3E-PlexinD1 signaling could be affected by ErbB2 in tumor models (276, 347). However, Nrp1 (340) and VEGFR2 (349) are the only known co-receptors which could be associated with PlexinD1. Gating of Sema3E by these co-receptors is functionally crucial because it switches the repulsive effect of Sema3E-PlexinD1 signaling into an attractive outcome. In fact, Nrp1 expression determines the functional pattern of Sema3E in neuronal cells in axon guidance. Absence of Nrp1 in the neurons insures repelling function of Sema3E which is evident in the cortifugal and striatonigral tracts; whereas subiculo-mamillary tract neurons express Nrp1 which leads to an attracting phenotype exerted by Sema3E (340).

As another layer of complexity, it has been shown that chemo-attraction of Sema3E is mediated by “PlexinD1/Nrp1/VEGFR2 ternary complex” in which PlexinD1 functions as the ligand-binding partner and VEGFR2 as the signal transducing element (349). However, the co-receptor gating scenario does not occur in endothelial cells wherein Sema3E interaction with PlexinD1 retracts those cells leading to inhibition of angiogenesis (338, 350-352).

Although Sema3E can bind only to plexinD1, the receptor does not show specificity and could be ligated by Sema3A (353), Sema3C (354, 355) and Sema4A (356) with or without Nrp1 association in mice. However, PlexinD1 serves as the Sema3E high affinity receptor (338, 356). Specifically, the extracellular region of PlexinD1 containing Sema domain has been shown to be necessary for Sema3E binding which induces a complex network of signaling events inside the target cell (338, 357).

Approximately 630 amino acid intracellular portion of PlexinD1 contains a “Sex and Plexins” SP domain that harbors two highly conserved C1 and C2 regions (272) named RasGAP domain because of sequence similarity to GAPs and specificity for R-Ras family of small GTPases (358). In fact, Plexins are the only transmembrane receptors interacting directly with small GTPases (359, 360). T-segment is a ~40–60 C-terminal stretch of plexin receptors located right after C2 region which is highly conserved. This segment does not have any similarity to known protein domains and considered as a potential candidate region for providing signaling specificity to each plexin. In PlexinD1, BLAST analysis indicates that T-segments from mammals, birds, amphibians and fish, are “94–100% identical” but it has very low similarity with other plexin family members of the same organism (337, 361).

The GAP activity of PlexinD1 retracts integrin-mediated cell adhesion to the ECM and also downregulates MAPK and PI3K signaling, as the key pathways involved in cell survival, proliferation and migration (351, 362). Therefore, interaction of Sema3E with ectodomain of PlexinD1 could potentially prime the receptor by inducing an intracellular conformational change as depicted in Fig. 1-6. Consequently, activated small GTPases, e.g. Rac1, Cdc42 and Rnd, bind to the RBD which leads to disruption of the inhibitory association between the C1 and C2 regions and activation of the GAP (337).

Sema3E treatment of endothelial cells decreases the phosphorylation of focal adhesion kinase (FAK), a key molecule that regulates the turnover of integrin-containing focal adhesions. Rnd1, 2 and 3 have been shown to physically interact with the intracellular domain of PlexinD1 through RBD. These GTPases are required for the activation of *in vivo* RasGAP activity of PlexinD1. Therefore, they could be considered as the potential signaling components mediating the early signaling events upon Sema3E-PlexinD1 interaction regardless of the functional

repulsive vs attractive outcome. Another possibility is that PlexinD1 antagonizes the R-Ras GTPase activity merely via sequestering these enzymes a without catalytic intervention (351). Alteration in cytoskeletal and ECM compartments such as actin polymerization and integrin localization have been demonstrated to be the final targets of semaphorin signaling in various contexts which determine the functional outcome of semaphorins (Fig. 1-6). However, the precise mechanism underlying semaphorin effects has remained to be fully addressed. Based on the above observations the following model has been proposed: “Upon Sema3E stimulation, PlexinD1 in pre-existing PlexinD1-Rnd2/RLG complexes undergoes an Rnd2/RLG-dependent intracellular conformation change that translates the concentration and distribution of extracellular Sema3E cues into an intracellular gradient of distinct PlexinD1 activities”.

It is impotent to note that the plexin family does not show endogenous kinase activity though they are phosphorylated at cytosolic tyrosine residues *in vivo* (272) as an essential process regulating several cellular functions. Intracellular conserved tyrosine residues exist in all plexins which undergo phosphorylation. However, the localization of them is different between PlexinD1 (SP domain) and others (RBD). VEGFR2 as a tyrosine kinase receptor has been suggested to phosphorylate PlexinD1 to modify its activity (337) though no experimental evidence is available. The furin cleavage product p61 induces the formation of a PlexinD1-ErbB2 complex in a lung cancer model in which both of them are phosphorylated on their tyrosine residues and kinase activity of EebB2 might induce phosphorylation of PlexinD1 (276). Altogether, the precise mechanism underlying Sema3E-PlexinD1 signaling is not completely understood and the ultimate functional outcome more likely follows a cell and context-dependent fashion, wherein co-receptor ligation, intracellular conformational modifications on receptor

domains and post-translational proteolytic processing should be extensively studied in each cell/disease model.

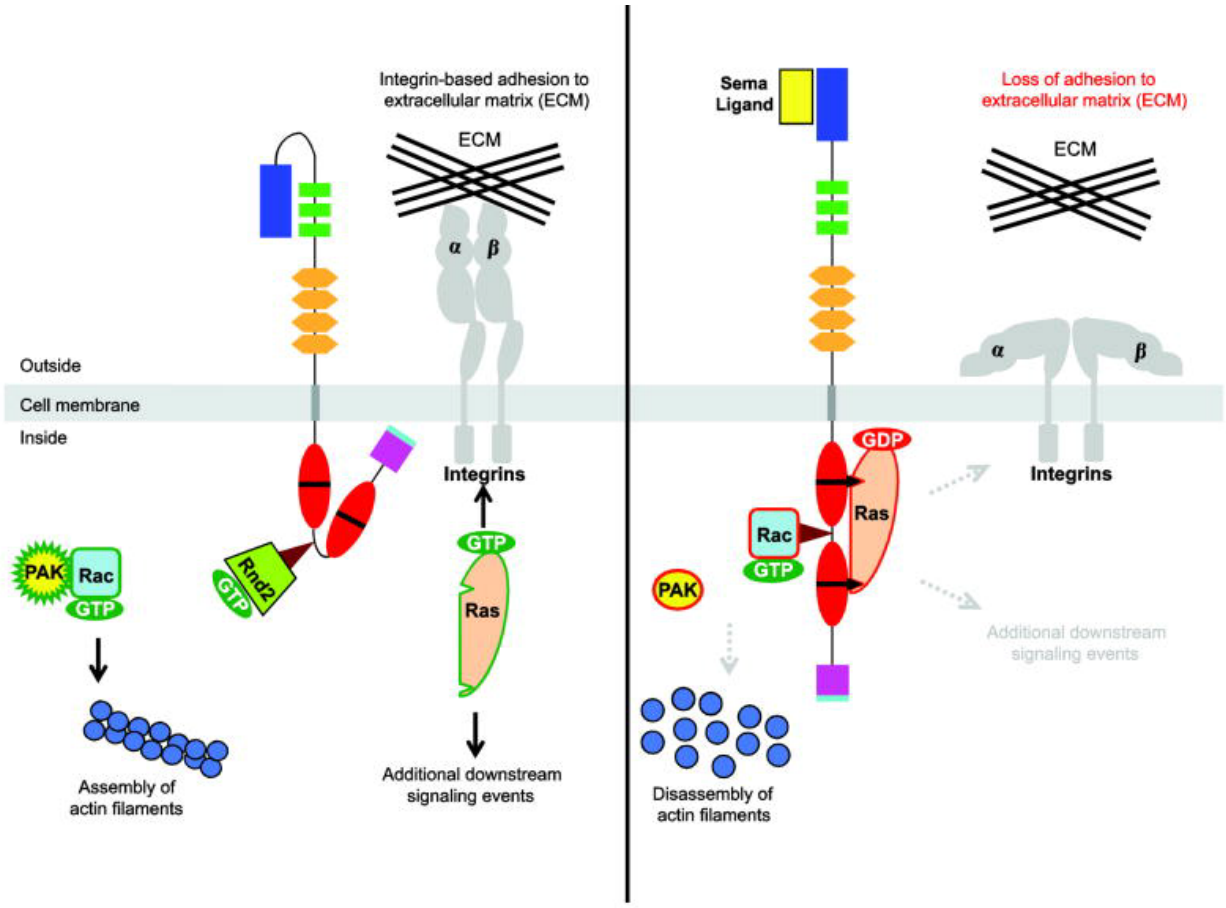

Figure 1-6 ***Cell repulsion mediated by Sema3E-PlexinD1 signaling***
Binding of its Sema3E to PlexinD1 induces a conformational change which makes the intracellular GAP domains of PlexinD1 accessible to the active forms of Rac and R-Ras small GTPases. Then, PAK pathway involved in remodeling of cytoskeletal actin is inactivated downstream of Rac leading to the collapse of the cytoskeleton and cell retraction. In parallel,Sema3E- PlexinD1 signaling inactivates R-Ras leading to the loss of integrin-based adhesion to the ECM and finally cell retraction. There might be other signaling pathways affected by Sema3E-PlexinD1 which has not been clearly addressed, so far.

1.12 Summary points

- Allergic asthma is a heterogeneous chronic inflammatory disease of the airways characterized by bronchial hyperresponsiveness and remodeling.
- Pulmonary dendritic cell subsets play distinct crucial roles in initiation and development of allergic airway inflammatory response.
- Activation and recruitment of neutrophils into the airways upon allergen encounter in response to inflammatory mediators is a key issue in severe refractory asthma.
- Enhanced thickening of ASM mediated primarily by increased ASM cell proliferation and migration is the characteristic feature of airway remodeling in allergic asthma.
- A complex network of mediators such as cytokines and growth factors are implicated in pathogenesis of allergic asthma via affecting the function of airway structural as well as effector inflammatory cells.
- Semaphorins, originally discovered as axon guidance molecules, have been shown to be ubiquitously expressed and control essential cellular functions in several diseases including cancers, autoimmune disorders and congenital defects.
- Emerging evidence suggest that versatile semaphorins could be involved in allergic diseases such as asthma and atopic dermatitis as well as airway disorders including COPD and pulmonary fibrosis.
- Sema3E-PlexinD1 signaling contributes to regulation of inflammatory and structural cell functions in various contexts; though its role in allergic asthma has not been studied.
- Sema3E exerts its regulatory functions via targeting multiple pathways including small GTPase, MAPK and PI3K signaling.

1.13 Thesis overview

1.13.1 Study rationale

Chronic airway inflammation is a pathological hallmark of allergic asthma which is characterized by dendritic cell-mediated presentation of indoor allergens to naïve T cells leading to induction of type 2 response. Airway remodeling is an essential feature of allergic asthma which includes increased smooth muscle mass, mucus overproduction and collagen deposition developed (in)dependent of inflammatory milieu. From a clinical point of view, airway hyperresponsiveness (AHR) is the most important issue in allergic asthma.

Despite tremendous efforts and remarkable progress, the mechanisms underlying regulation of airway inflammation, remodeling and AHR have not been fully addressed. Understanding the expression and function of unknown mediators involved in these processes is an essential step towards developing better therapeutic approaches to treat allergic asthma. For instance, emerging evidence suggest that a neural chemorepellent, semaphorin 3E (Sema3E), plays a crucial role in regulation of cell proliferation, migration, adhesion, differentiation, and angiogenesis in several organs beyond the nervous system. Considering dysregulation of these cellular processes in allergic asthma, Sema3E may be implicated in the regulation of airway inflammation and remodeling as well. However, the role of Sema3E in allergic asthma has not been investigated so far. As a novel approach, we intend to decipher expression and function of Sema3E and its receptor, PlexinD1, in allergic asthma by focusing on its role and mechanism(s) of action in dendritic cells, T and B lymphocytes, neutrophils and airway smooth muscle cells by using various *in vitro* and *in vivo* approaches on both human asthmatic samples and mouse models of the disease.

1.13.2 General Hypothesis

Sema3E, a guidance cue with chemorepellent function, is downregulated in allergic asthma which orchestrates the function of inflammatory (dendritic cells and neutrophils) and structural (airway smooth muscle) cells. Replenishment of Sema3E, which is suppressed under asthmatic conditions, could confer protection against allergic asthma by modulation of cellular functions.

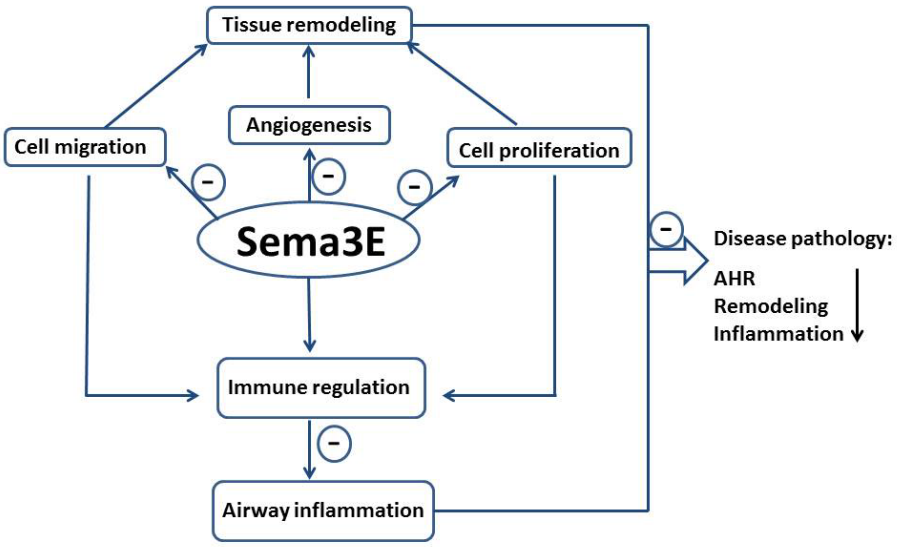

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Figure 1-7 Schematic representation of the rationale underlying our general hypothesis
According to the literature Sema3E is a negative regulator of cellular functions such as migration, proliferation and angiogenesis. It could be considered to minimize these processes which are dysregulated in both inflammatory and structural cells in allergic asthma.

1.13.3 Bridging theme

According to background information presented in *Chapter1*, Sema3E might be involved in regulation of both airway inflammation and remodeling in allergic asthma. Specifically, this thesis aims to address the following issues: Investigate the potential inhibitory role of Sema3E and its high affinity receptor, PlexinD1, in proliferation and migration of human airway smooth muscle cells *(Chapter 2)*; Elucidate expression of Sema3E in allergic asthma *(Chapter 3)* followed by deciphering its role in allergic airway inflammation via modulation of dendritic cell functions *(Chapter 3)* and finally understand the role of Sema3E in neutrophil migration and allergen-induced airway neutrophilia *(Chapter 4)*.

2 CHAPTER 2:

NEURONAL CHEMOREPELLENT SEMAPHORIN 3E INHIBITS HUMAN AIRWAY SMOOTH MUSCLE CELL PROLIFERATION AND MIGRATION

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Authors' contribution: H.M. drafted and produced 100% of the experiments required for the realization of this manuscript. L.S. assisted for migration assay and immunohistochemistry. A.J.H., M.R. and M.T. provided us with human ASM cells. J.C. obtained lung tissue sections used for IHC. A.S.G. designed the experiments and corrected the manuscript.

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2.1 Abstract

Background: Chronic airway diseases including asthma are characterized by increased airway smooth muscle (ASM) mass which is due in part to growth factor-mediated ASM cell proliferation and migration. However, the molecular mechanisms underlying these effects are not completely understood. Semaphorin 3E (Sema3E) has emerged as an essential mediator involved in cell migration, proliferation and angiogenesis though its role in ASM cell function is not investigated.

Objectives: We sought to determine: expression of Sema3E receptor, plexinD1 in human ASM cells (HASMC); effect of Sema3E on basal and platelet-derived growth factor (PDGF)-induced proliferation and migration; and signaling pathways involved in these events.

Methods: The expression of plexinD1 in HASMC was studied using RT-PCR, immunostaining and flow cytometry. The effect of recombinant Sema3E on HASMC proliferation and migration was evaluated by EdU incorporation, cell count and Boyden chamber assay. Sema3E-mediated intracellular signaling was investigated using fluorescence microscopy, flow cytometry, Rac1 activation and Western blot.

Results: Primary HASMC from healthy and asthmatic individuals expressed plexinD1 which was lower in asthmatic HASMC. Recombinant Sema3E inhibited PDGF-mediated HASMC proliferation and migration. Effect of Sema3E on HASMC was associated with F-actin depolymerization, suppression of PDGF-induced Rac1 GTPase activity, Akt and ERK1/2 phosphorylation. Bronchial biopsies from mild asthmatics displayed immunoreactivity of plexinD1, suggesting the potential *in vivo* role of Sema3E-PlexinD1 axis in HASMC function.

Conclusion: This study provides the first evidence that Sema3E receptor is expressed and plays functional roles in HASMC. Our data suggest a regulatory role of Sema3E in PDGF-mediated proliferation and migration leading to downregulation of ASM remodeling.

Clinical Implications: Anti-proliferative and anti-migratory effect of Sema3E on human airway smooth muscle may provide a novel therapeutic approach to minimize airway remodeling in chronic airway diseases.

Capsule summary: Airway smooth muscle (ASM) hyperplasia is a critical pathological event in chronic airway diseases. Sema3E displayed a modulatory role in ASM cell hyperplasia through a mechanism that involves F-actin depolymerization, Rac1, ERK1/2 and Akt inactivation.

Key words: Airway smooth muscle cell, Migration, Platelet-derived growth factor, PlexinD1, Proliferation, Semaphorin 3E

Abbreviations/acronyms:

ASM: Airway smooth muscle

EdU: 5-ethynyl-2´-deoxyuridine

ERK: Extracellular signal-regulated kinase

HASMC: Human airway smooth muscle cells

MAPK: Mitogen-activated protein kinase

PDGF: Platelet-derived growth factor

PI3K: Phosphatidylinositol 3-kinase

Rac1: Ras-related C3 botulinum toxin substrate 1

Sema3E: Semaphorin 3E

2.2 Introduction and rationale

Asthma is a complex and heterogeneous syndrome, which is characterized by reversible airway obstruction, inflammation, hyperresponsiveness and remodeling (1). Human airway smooth muscle cells (HASMC) are a key cell type in asthma owing to their ability to contract in response to inflammatory cell products. Due to their intrinsic plasticity, HASMC also exhibit the capacity for multifunctional behaviour and are actively involved in local inflammation and airway remodeling (2).

Increased airway smooth muscle (ASM) mass, one of the hallmarks of airway remodeling, is commonly observed in patients suffering from chronic airways diseases, including asthma and chronic obstructive pulmonary disease (COPD) (3-5). This pathology is believed to occur via multiple mechanisms such as cell proliferation and migration (6-7). There is a remarkable increase in proliferation rate of HASMC obtained from asthmatic patients compared to non-asthmatic subjects (8). HASMC proliferation is increased in response to allergen challenge, growth factors and inflammatory mediators such as platelet-derived growth factor (PDGF)-BB (9), epidermal growth factor (EGF) (10) and leukotriene B4 (11) suggesting an important role of these factors in HASMC hyperplasia and airway remodeling. In addition, increased accumulation of ASM cells in asthma is not solely because of HASMC proliferation and it might be partly attributable to migration of their progenitors from outside the muscle toward the lumen or immigration of proliferating cells within the muscle bundles (12). Previous studies have demonstrated pro-migratory effects of various growth factors and inflammatory mediators including PDGF, transforming growth factor (TGF) β, interleukin (IL)-8 on HASMC (13-14).

PDGF-BB modulates the contractile phenotype of HASMC to a proliferative phenotype, and stimulates their proliferation and migration *in vitro* (14). Furthermore, Hirota et al. have shown

mitogenic effect of this growth factor *in vivo*, where PDGF-BB over expression in mice airways induced ASM hyperplasia and changed lung mechanics (15). However, the precise regulatory mechanisms underlying PDGF-induced HASMC proliferation and migration have remained elusive.

Semaphorins are a family of conserved secreted and membrane associated proteins originally discovered as axon guidance cues in neuronal development. More recently, their role in processes other than neuronal guidance was documented which encompasses angiogenesis, differentiation, cell proliferation and migration (16). Semaphorin 3E (Sema3E) was previously described as an intrinsic mediator involved in axon path finding (17) and vascular patterning (18). Sema3E interacts with its receptor, plexinD1, with high affinity and regulates migratory functions. Sema3E-PlexinD1 axis has also emerged as a pivotal pathway in cell migration and angiogenesis in immune and endothelial contexts, respectively (19-20). Anti-angiogenic effect of Sema3E is mediated through inhibition of endothelial cell proliferation (20). Therefore, it is a likely candidate to play a significant role in the pathogenesis of airways diseases, where the mechanisms regulating these processes are impaired.

In this study we aimed to investigate the expression of Sema3E receptor on HASMC and whether it affects basal and PDGF-induced HASMC proliferation and migration. We demonstrated that both normal and asthmatic HASMC constitutively express Sema3E high affinity receptor plexinD1, importantly to a lesser extent in asthmatic HASMC compared to normal cells. Sema3E significantly inhibited PDGF-BB induced HASMC proliferation and migration via mechanisms that involve decreased activation of Ras-related C3 botulinum toxin substrate 1 (Rac1) GTPase, Akt and extracellular signal-regulated kinases 1/2 (ERK1/2). PlexinD1 immunoreactivity was detected within ASM bundle in bronchial sections of mild

allergic asthmatics. Our data suggest that Sema3E and its receptor are involved in the regulation of ASM remodeling in chronic airway diseases such as asthma.

2.3 Methods

For details on the methods used in this study, please see the Methods section in the Online Repository at www.jacionline.org

2.4 Results

2.4.1 PlexinD1 is constitutively expressed by HASMC in vitro

It has been previously shown that Sema3E binds plexinD1 with high affinity directly in neuronal and cardiovascular systems (17, 21). *In vitro* expression of Sema3E receptor in HASMC was evaluated. As shown in Fig 2-1A, mRNA for plexinD1 was expressed in primary HASMC from four different donors. Human Universal Reference Total cDNA (Clontech, CA) was used as a positive control in all RT-PCR experiments and no cDNA served as negative control tubes. In parallel, flow cytometry using specific mAb directed against human plexinD1 demonstrated its surface expression on HASMC (Fig 2-1B). We further confirmed expression of plexinD1 by performing immunocytochemistry on HASMC (Fig 2-1C).

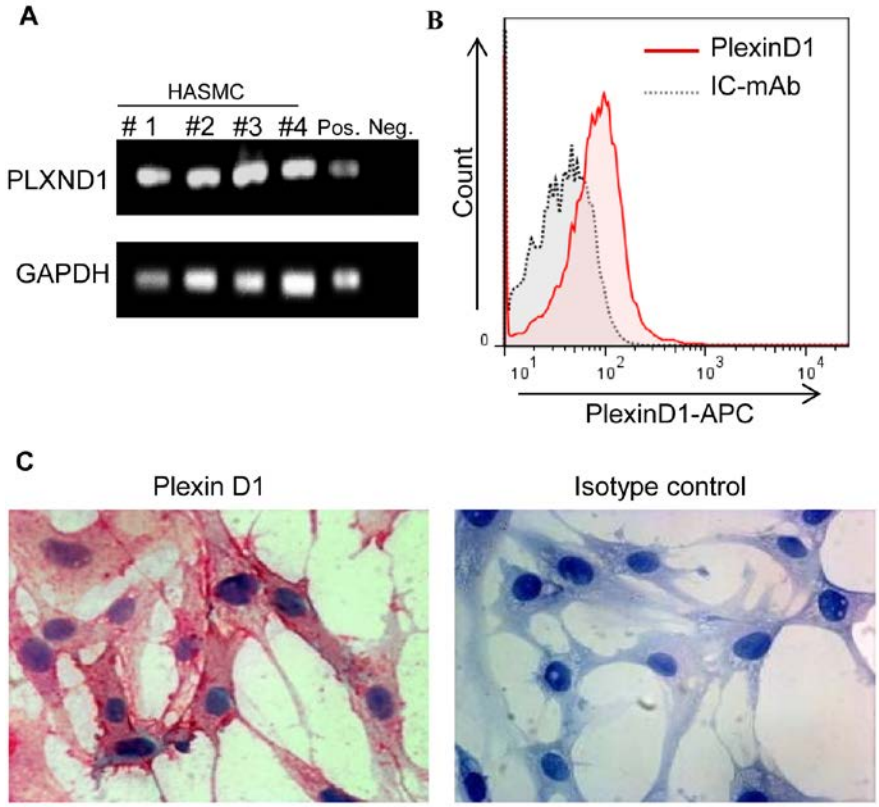

Figure 2-1: Expression of plexinD1 on HASMC.
"Expression of plexinD1 on primary HASMC was examined by RT-PCR using specific primers (A), flow cytometry (B) and immunocytochemistry (C) by specific antibodies. Staining with isotype control antibody showed no immunoreactivity in B and C. RNA and protein expression studies were performed on at least three different HASMC under the same conditions."

2.4.2 PlexinD1 is expressed in allergic asthma with a differential surface expression between normal and asthmatic HASMC

To examine whether plexinD1 is expressed *in vivo*, we performed immunostaining in bronchial tissue sections obtained from allergic asthmatic individuals (n=5). The clinical characteristics of the patients are provided in Table 2-1. Fig 2-2A-C reveals that plexinD1 is expressed in ASM bundles and adjacent endothelium (red staining). Tissue sections stained with isotype antibody showed no immunoreactivity (Fig 2-2D). Immunostaining of bronchial sections from normal non-atopic subjects revealed plexinD1 expression in ASM bundles and airway endothelium (n=5) in a level comparable to asthmatic patients. However, FACS analysis (Fig 2-2E-F) demonstrated a significant decrease in plexinD1 surface expression on asthmatic vs. normal bronchial HASMC which was increased upon stimulation with Sema3E as the ligand (Fig. 2-2 G-H). (n=3, $p<0.05$) (see Table 2-2 in the Online Repository).

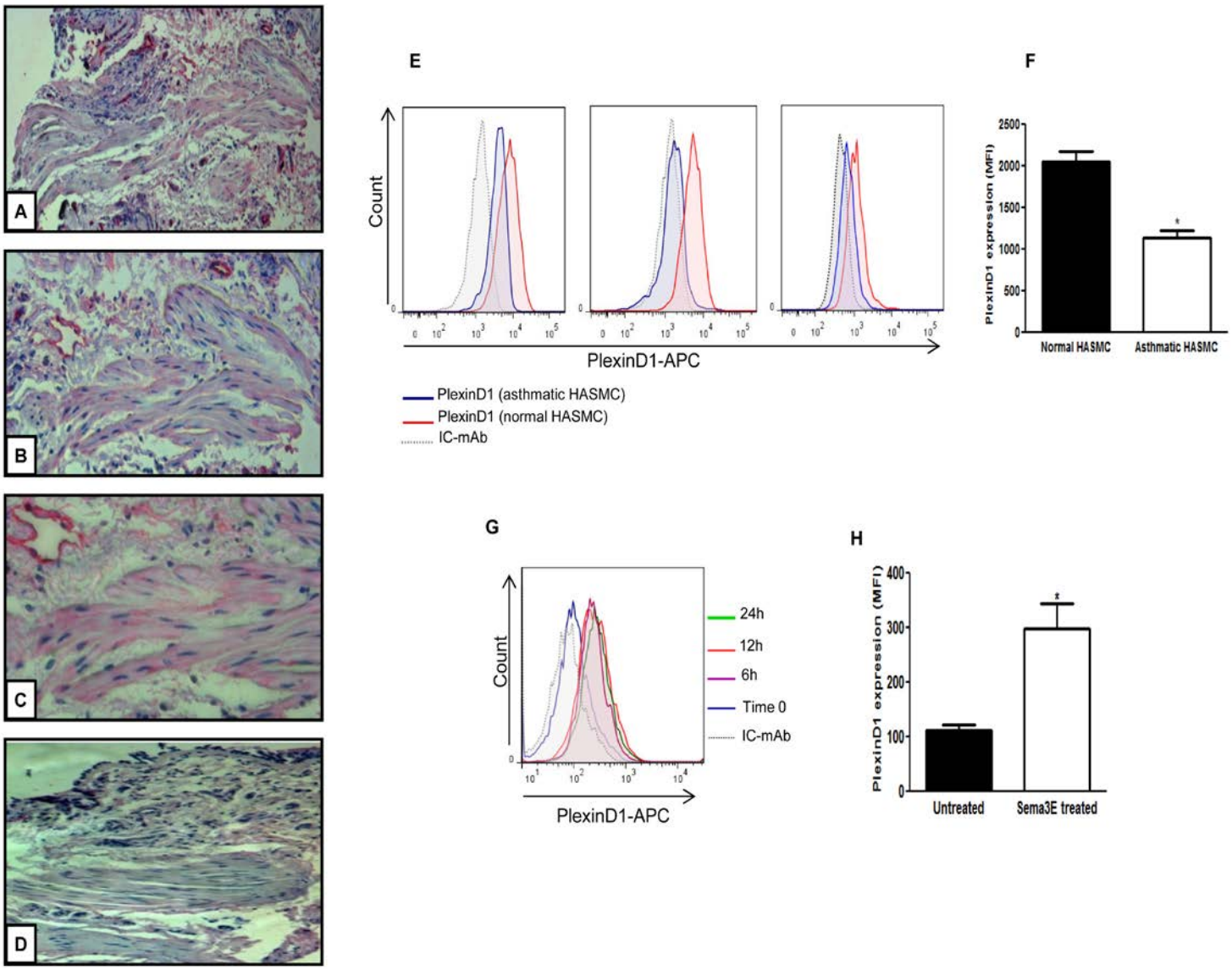

Figure 2-2: Immunohistochemical and FACS analysis of plexinD1 expression.
"Bronchial sections of mild allergic asthmatic subjects were stained using anti-plexinD1 antibody and visualized by 100X (A), 200X (B) and 400X (C) magnifications. No immunoreactivity was observed after isotype antibody staining (D). Surface expression of plexinD1 was compared between asthmatic and normal bronchial HASMC by FACS (E-F). G and H, Asthmatic HASMCs were treated with Sema3E and plexinD1, and surface expression was studied at indicated time points (The statistical significance was determined by performing Student t-test in F and H; n=3, $p<0.05$). APC, Allophycocyanin; IC, isotype control."

2.4.3 Recombinant Sema3E inhibits PDGF induced HASMC proliferation and migration

According to previous studies, anti-angiogenic effect of Sema3E is mediated through inhibition of endothelial cell proliferation (20). However, there is no study indicating inhibitory effect of Sema3E on proliferation of other cell types. In order to study the effects of recombinant Sema3E on HASMC proliferation, serum-deprived HASMC were treated with different concentrations of recombinant Sema3E in presence or absence of PDGF-BB (10ng/ml). Then, incorporation of a fluorescent-labeled thymidine analogue, EdU, into newly synthesized DNA was measured as an indicator of proliferation. PDGF-induced proliferation was inhibited by Sema3E in hTERT immortalized bronchial (Fig 2-3A) and primary tracheal (Fig 2-3C) HASMC ($n=4$, $P<0.01$). Sema3E was also able to inhibit proliferation of primary bronchial HASMC isolated from asthmatic individuals (Fig 2-3E) (see Table 2-2 in Online Repository for clinical characteristics of asthmatic subjects that their HASMC proliferation was studied). As demonstrated in Fig 2-3B, 2-3D and 2-3E, Sema3E inhibited cell proliferation in a dose-dependent manner in immortalized bronchial and primary tracheal HASMC as well as asthmatic bronchial ones, respectively. In a different set of experiments, the anti-proliferative effect of Sema3E on HASMC was further confirmed by performing cell count 4 days after Sema3E±PDGF stimulation of HASMC (Fig E1, $n=4$). In addition, Sema3E+PDGF co-stimulation of HASMC negatively affected cell cycle distribution and led to a decreasing, but not significant, trend of DNA synthesis in the presence of PDGF as a mitogen. However, the results were not statistically significant (see Fig E2 in the Online Repository).

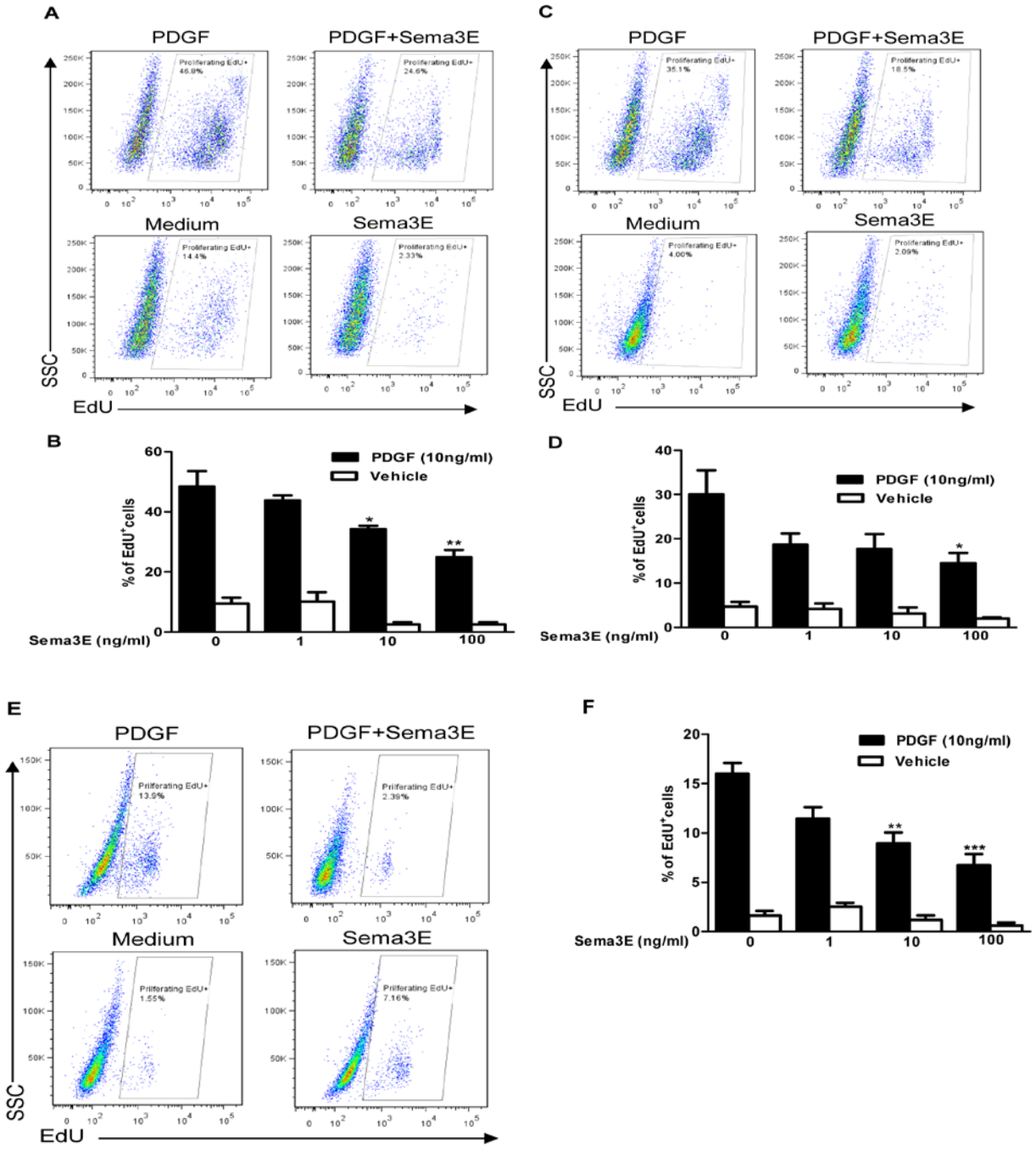

Figure 2-3 ***Inhibition of HASMC proliferation in response to Sema3E.***
"Basal and PDGF-mediated proliferation of immortalized bronchial (A), primary tracheal (C) normal HASMC and also primary bronchial asthmatic HASMC (E) was studied by EdU incorporation assay 48 hours after stimulation. The results were quantified and statistically compared by performing one-way ANOVA and Bonferroni test (B, D and F). The graphs are based on at least three independent proliferation experiments."

To ascertain whether Sema3E effect on HASMC is mediated through plexinD1, cells were treated with exogenous human recombinant plexinD1 and stimulated with PDGF or PDGF combined with Sema3E (20). Human plexinD1 recombinant protein significantly reversed the anti-proliferative effect of Sema3E on HASMC (n=3, P<0.05) as revealed by EdU incorporation assay (Fig 2-4A-B) and manual cell count (Fig 2-4C).

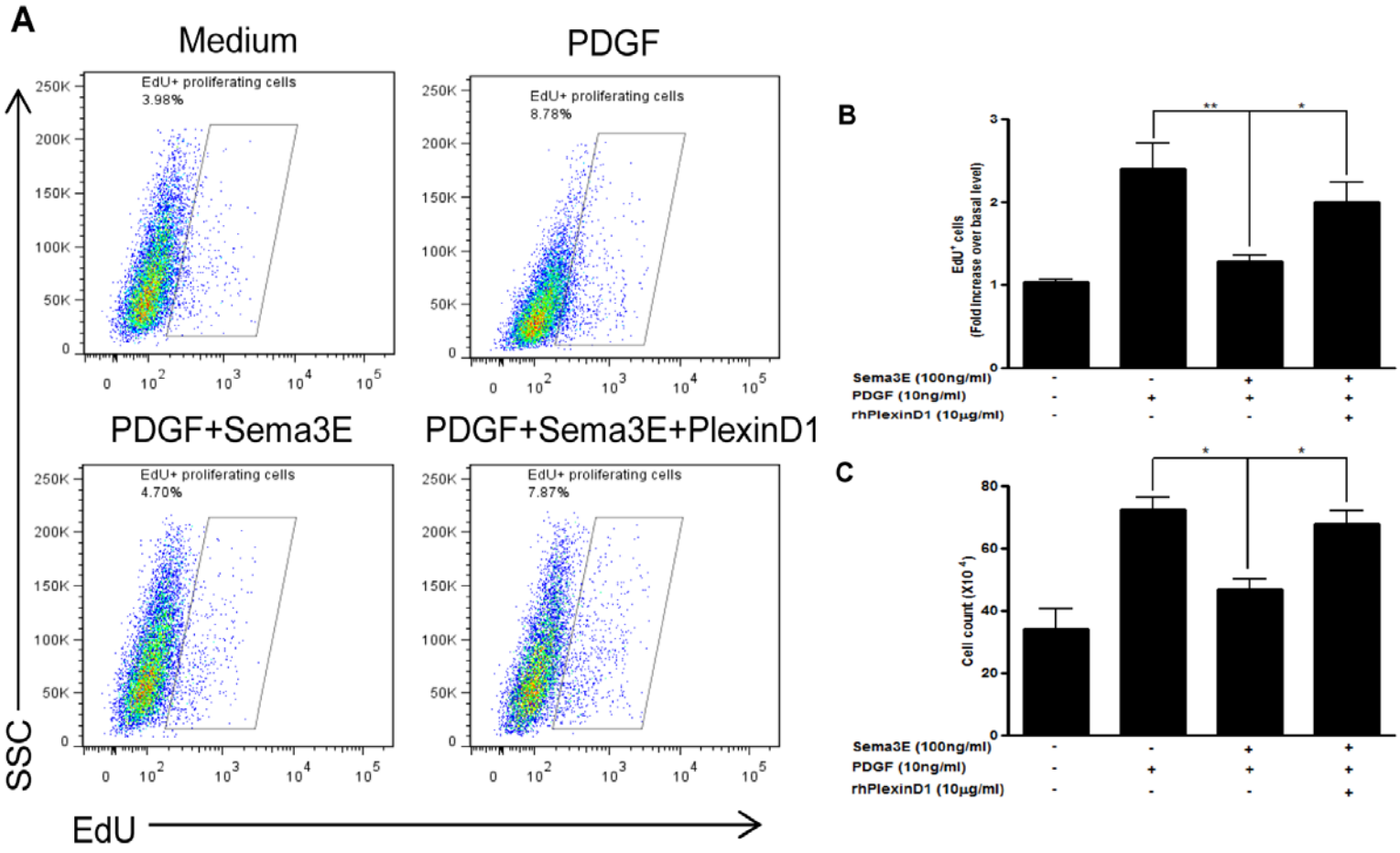

Figure 2-4 PlexinD1-mediated fashion of Sema3E effect on HASMC proliferation.
"HASMC were stimulated with Sema3E and PDGF in the presence or absence of recombinant human plexinD1, followed by EdU incorporation assay (A-B) and cell count (C). Sema3E inhibitory effect on PDGF-induced proliferation was significantly abrogated in presence of exogenous plexinD1 in three independent experiments. The statistical significance was determined by performing one-way ANOVA and Bonferroni test"

Next we evaluated the effect of recombinant Sema3E on HASMC migration using a Boyden chamber assay. Cultures were treated with Sema3E±PDGF and the number of migrated cells was counted after 4 hour. Fig 2-5A-B reveals a significant inhibitory effect of Sema3E on PDGF-induced HASMC migration in a dose dependent fashion ($n=5$, $p<0.001$). Sema3E concentrations used in migration assays are based on previous studies (19). Collectively, our functional studies suggest that Sema3E regulates PDGF-mediated HASMC proliferation and migration.

2.4.4 Actin depolymerization is induced following Sema3E treatment in HASMC

Based on the canonical importance of actin dynamics in ASM cell migration (22) and also the effect of Sema3E on actin depolymerization (23), F-actin alterations was studied by phalloidin staining. HASMC were stimulated with Sema3E for 0-5min, stained with-phalloidin-Alexa-fluor-647 and visualized by immunofluorescence microscopy. Fig 2-5C depicts reduced F-actin content, as revealed by decreased immunofluorescence signal, upon sema3E stimulation. Actin depolymerization was also confirmed using flow cytometry quantification and represented as median fluorescence intensity (MFI) values. HASMC showed a substantial decrease in F-actin content in response to Sema3E (Fig 2-5D-E, $n=3$, $p<0.01$).

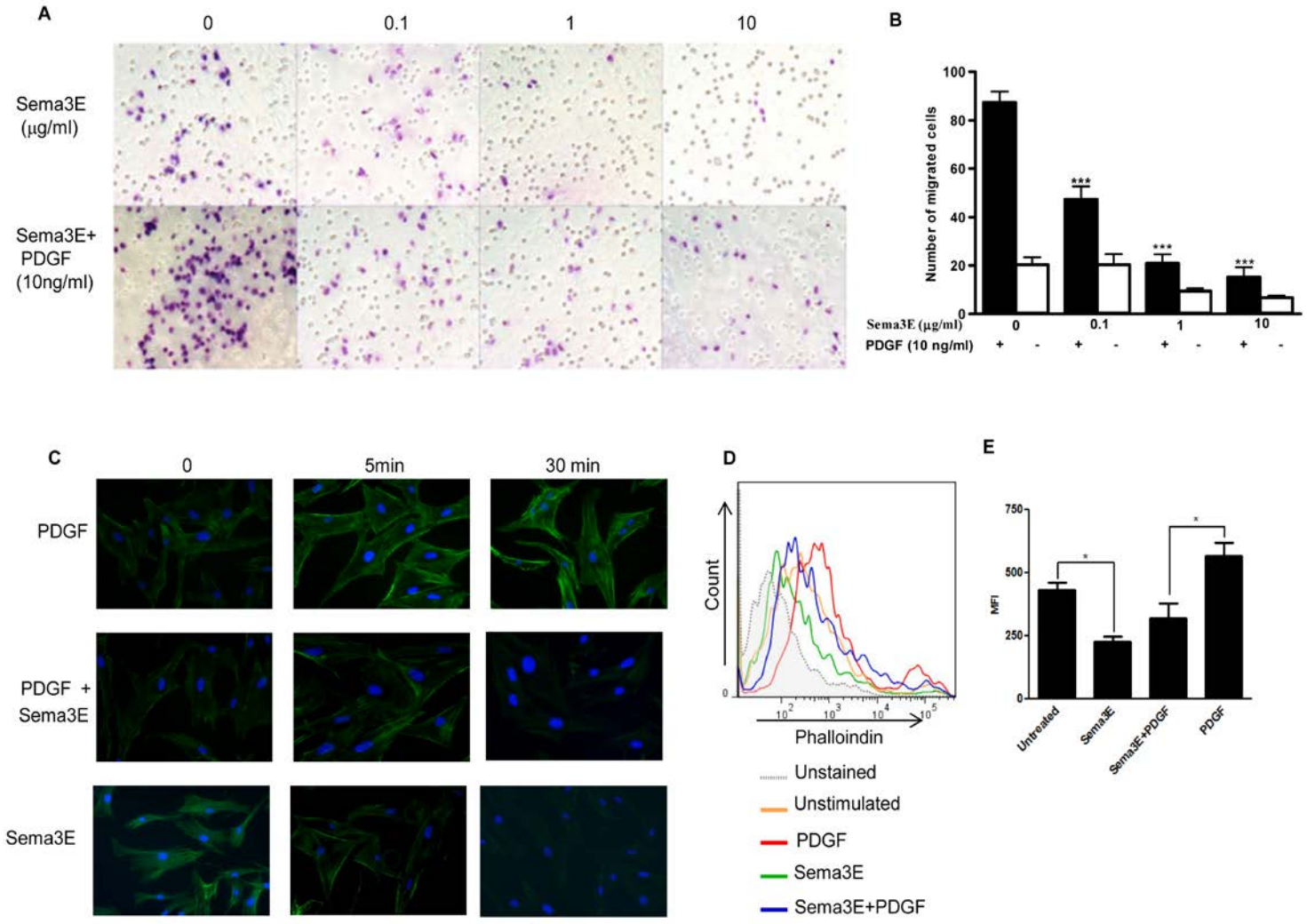

Figure 2-5 Inhibitory effect of Sema3E on HASMC migration.

"HASMC migration towards Sema3E±PDGF was studied in a double blind manner (A-B). Phalloidin staining was performed to detect F-actin content of HASMC following Sema3E treatment (C). F-actin content was quantified using flow cytometry 0-5 min after Sema3E treatment (E-F). The values represent the average number of migrated cells±SEM from triplicate experiments. The statistical significance was determined by performing one-way ANOVA and Bonferroni test in B and E."

2.4.5 Sema3E suppresses PDGF-induced Rac1 GTPase activity in HASMC

Considering the role of Rac1 signaling in PDGF-induced smooth muscle proliferation and migration (9, 24), Rac1 GTPase activity was measured in indicated time points to determine the mechanism by which Sema3E inhibits PDGF-induced HASMC proliferation and migration. As shown in Fig 6, PDGF induced a rapid and robust increase of Rac1 GTPase activity 1 min after stimulation which was reduced to the basal level of activity in the presence of Sema3E (n=5, $P<0.01$). However, stimulation with Sema3E alone at the same time points did not change the activation of Rac1 (Fig 2-6), as Sema3E treatment alone did not significantly affect basal proliferation and migration of HASMC (Fig 2-3 and 2-5).

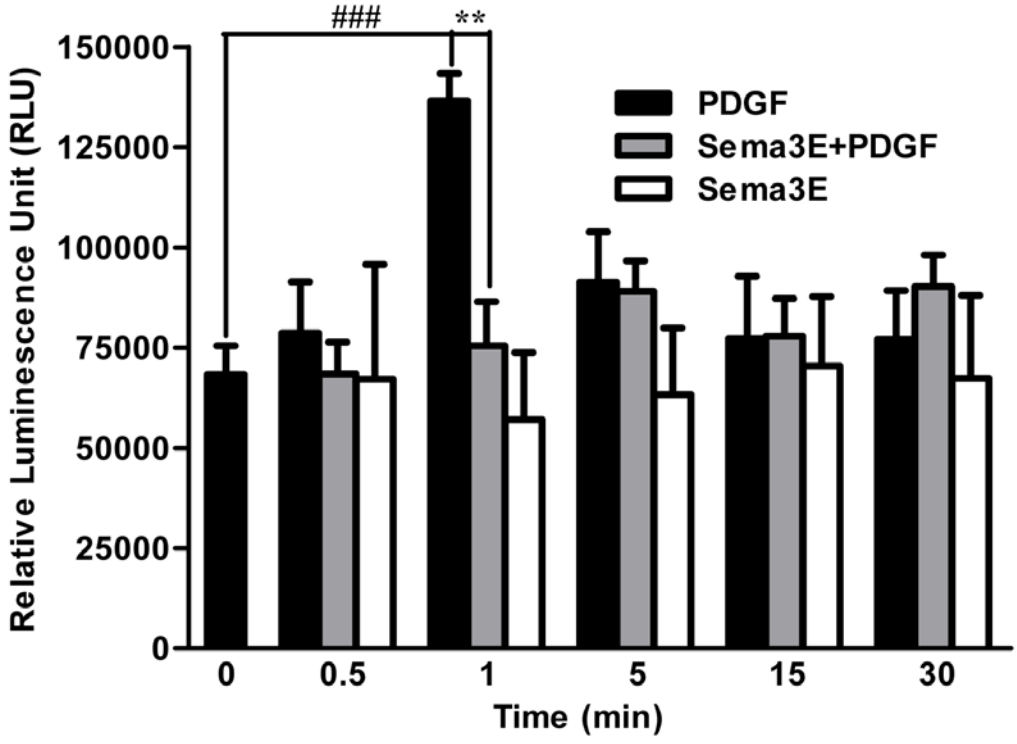

Figure 2-6 Suppression of PDGF-induced Rac1 GTPase activity by Sema3E in HASMC.
"Rac1 GTPase activity was measured after Sema3E±PDGF stimulation by G-LISA. Sema3E suppressed PDGF-induced Rac1 GTPase activity 1 min after co-stimulation without significant effect on the basal activity. Data represent mean±SEM of five independent experiments performed in the same conditions. The statistical significance was determined by performing two-way ANOVA."

2.4.6 Sema3E inhibits PDGF-mediated phosphorylation of PI3K/Akt and MAPK/ERK1/2 in HASMC

To further elucidate the signaling pathways leading to the inhibitory effect of Sema3E on HASMC function, we investigated the role of PI3K/Akt and MAPK/ERK1/2 by Western blot analysis using specific Abs. PDGF induced a dramatic and reproducible increase in Akt and ERK1/2 phosphorylation in HASMC at indicated time points after stimulation. Combination of PDGF with Sema3E treatment reduced PDGF-induced phosphorylation of Akt and ERK1/2 significantly. Sema3E alone did not induce noticeable phosphorylation in HASMC (Fig 2-7A-B). These data suggest that Akt and ERK1/2 are involved in Sema3E inhibition of PDGF-mediated HASMC proliferation and migration.

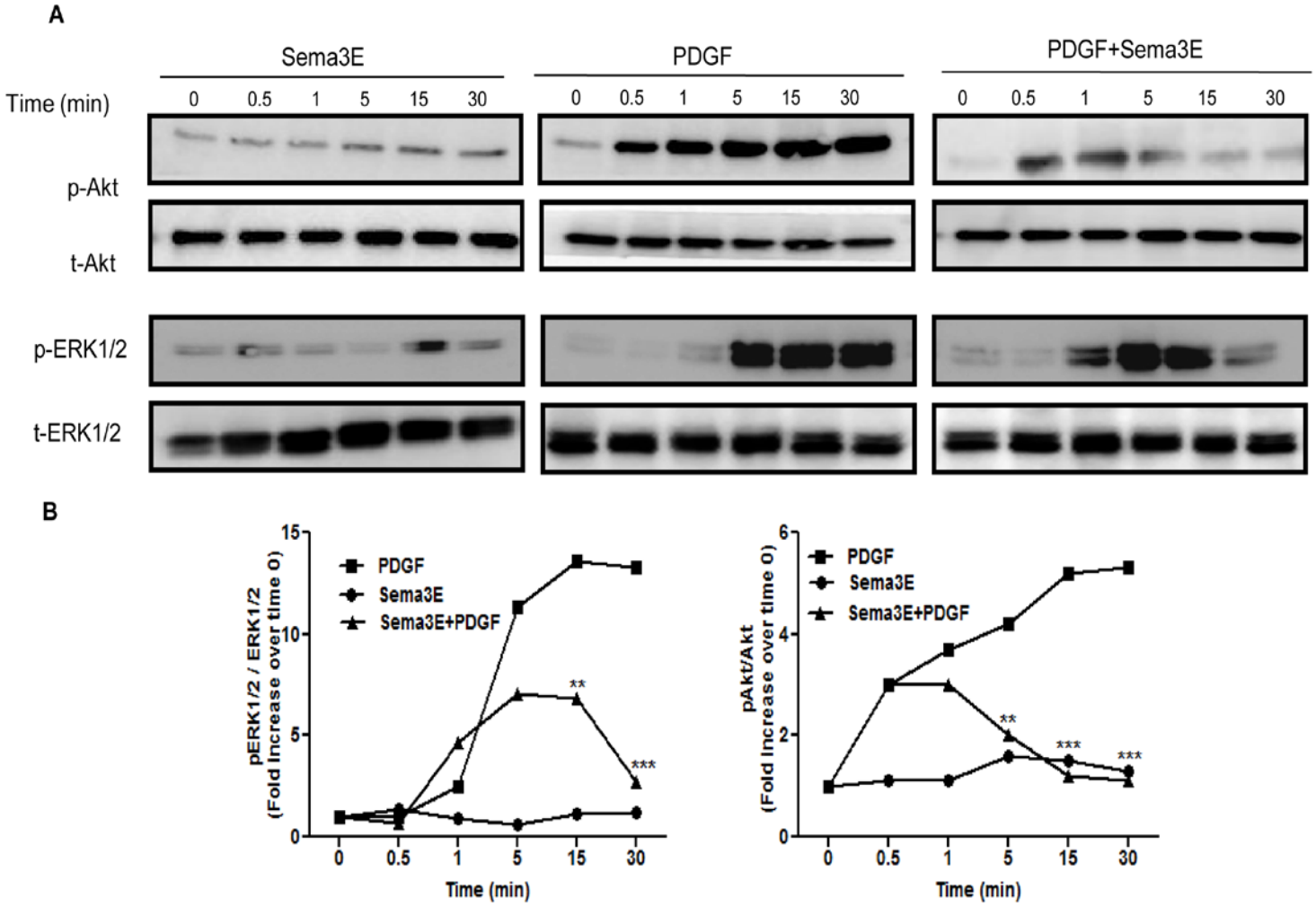

Figure 2-7 Kinetic analysis of signaling pathways downstream of Sema3E stimulation in HASMC.
Sema3E effect on HASMC was associated with a dramatic decrease in Akt and ERK1/2 phosphorylation (A). Densitometric analysis was performed on Akt and ERK1/2 phosphorylation presented as the ratio of phospho over total compared with time zero (B). Data represent mean±SEM of three independent experiments The statistical significance was determined by performing one-way ANOVA and Bonferroni test in B.

2.4.7 Online repository figures

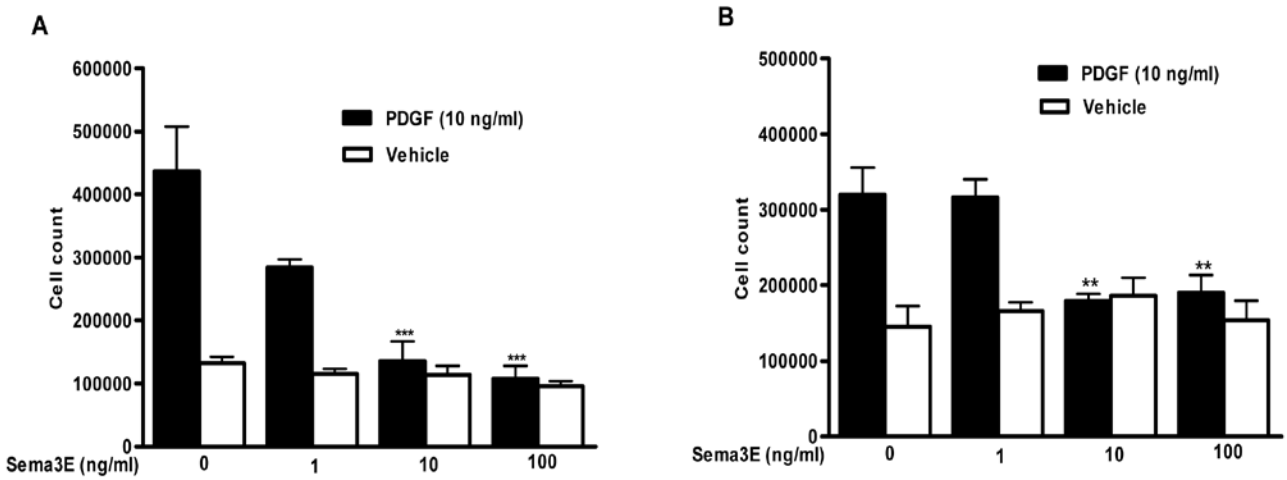

Figure 2-8 Inhibition of HASMC proliferation in response to Sema3E.
"Basal and PDGF-mediated proliferation of HASMC was studied by manual cell count 4 days after stimulation. The graphs are based on four independent proliferation experiments on bronchial (A) and tracheal (B) HASMC. The statistical significance was determined by performing one-way ANOVA and Bonferroni test."

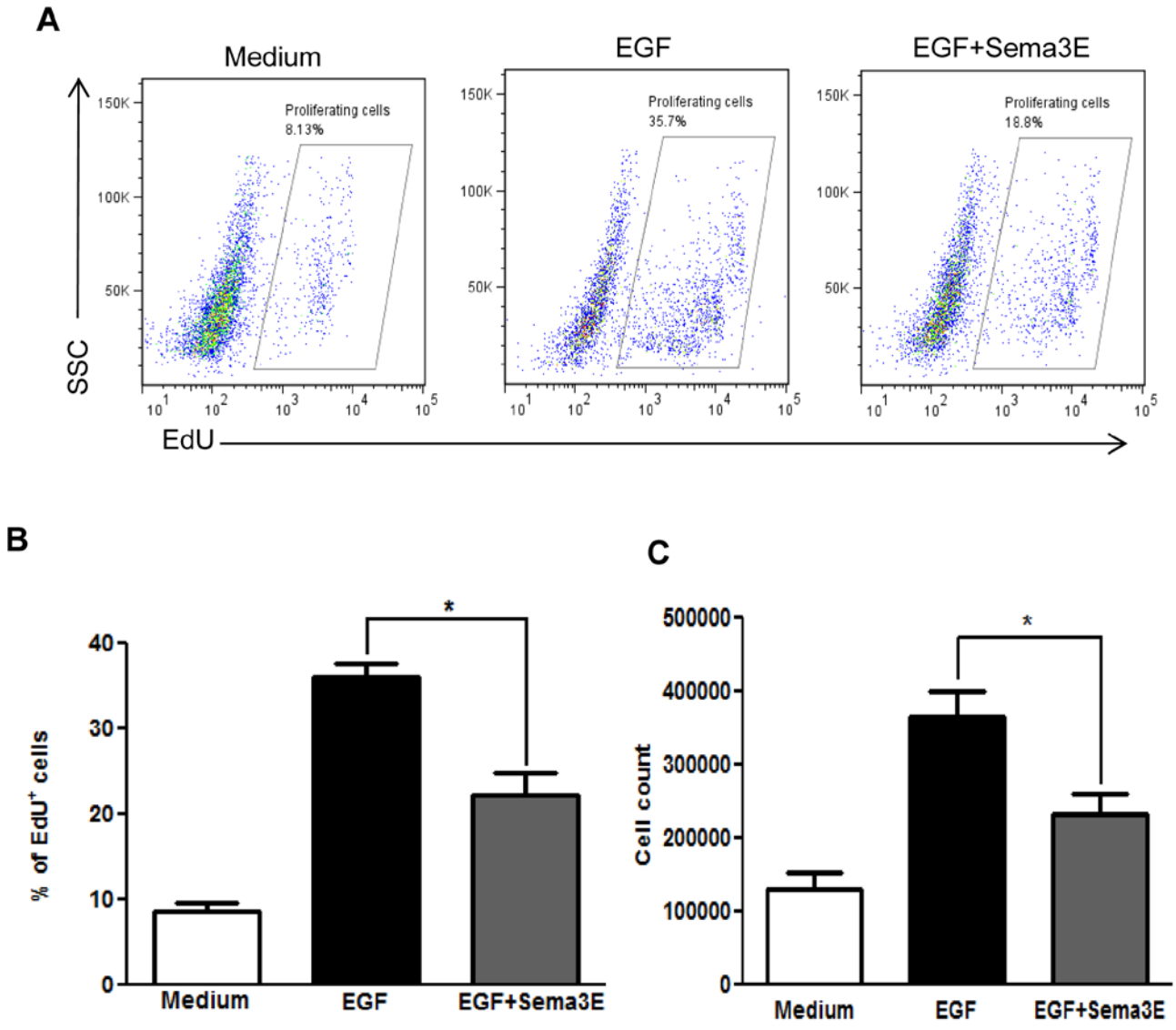

Figure 2-9 Sema3E inhibitory effect on EGF-induced HASMC proliferation.
"Basal and EGF-induced proliferation of ASMC was assessed using EdU incorporation assay. The graphs represent based on three independent proliferation experiments (The statistical significance was determined by performing one-way ANOVA and Bonferroni test; n=3, $p<0.05$)."

	Sema3E	%G0	% S Phase	% G1/G2/M
- PDGF	-	9.22	2.06	88.71
	+	8.43	0.358	90.34
+ PDGF	-	21.3	31.4	47.2
	+	23.7	18.3	58.3

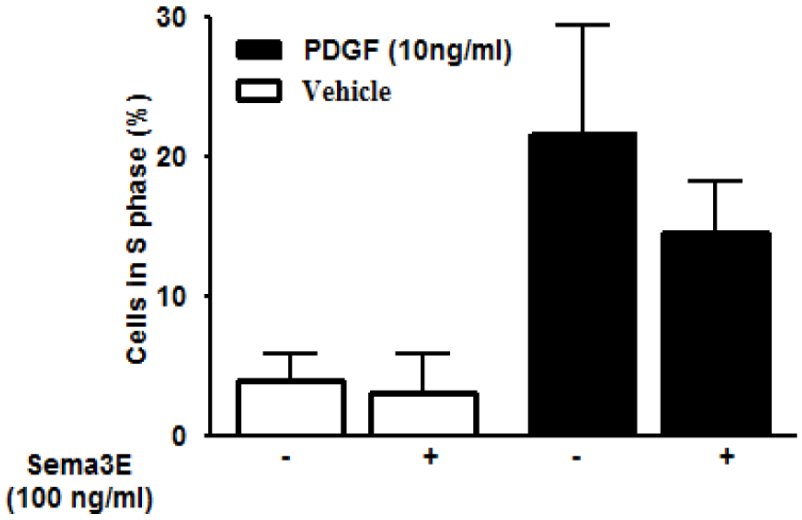

Figure 2-10 Analysis of HASMC cycle after Sema3E stimulation.
"Cell cycle distribution was assessed upon Sema3E with or without PDGF treatment by PI staining upon EdU incorporation assay. The graph (A) and table (B) are the results obtained from three independent experiments."

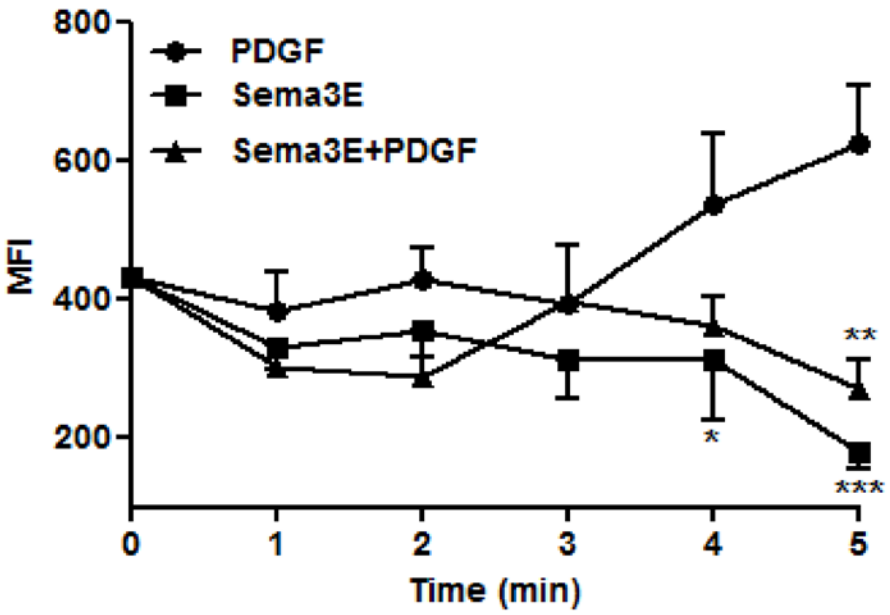

Figure 2-11 Quantification of F-actin content in HASMC.
"F-actin content was measured using flow cytometry 0-5 min after PDGF (A-B) Sema3E (C-D), or Sema3E+PDGF (E-F) stimulation. The values represent the average number of migrated cells±SEM from triplicate experiments. The statistical significance was determined by performing one-way ANOVA and Bonferroni test."

2.5 Discussion

Our study is the first to describe the expression of plexinD1, high affinity receptor for Sema3E, and functional consequences of its ligation with exogenous recombinant Sema3E on HASMC proliferation and migration. We found that Sema3E significantly inhibits PDGF-induced HASMC proliferation and migration. This effect is associated with depolymerisation of F-actin, down regulation of Rac1 GTPase activity as well as decrease in MAPK/ERK1/2 and PI3K/Akt phosphorylation. HASMC from asthmatics displayed less plexinD1 surface expression compared to HASMC from healthy donors.

Understanding the regulatory mechanisms of HASMC proliferation and migration is of potential clinical value and may provide further insight into the new treatment strategies of airway diseases associated with smooth muscle hyperplasia such as asthma and COPD.

Semaphorins are multifaceted proteins involved in pathogenesis of several diseases from different types of cancer to autoimmune and neurological disorders (25-29). It was previously shown that Sema3E contributed to tumor progression (30), ischemic retinopathy (31) and congenital defects (32) through affecting angiogenesis, cell proliferation and migration. However, expression and function of this neuronal guidance molecule in asthmatic and other allergic conditions has remained to be addressed.

We have shown that plexinD1 is expressed by and present on HASMC. Unlike other class 3 semaphorins, Sema3E interacts directly with plexinD1. However, gating of Sema3E-PlexinD1 complex by neuropilin 1 (Nrp1) or vascular endothelial growth factor receptor 2 (VEGFR2) switches axonal repulsion to attraction (33-34). Since Nrp1 and VEGFR2 are abundantly expressed in asthma and chronic rhinosinusitis conditions compared to normal subjects (35-36),

differential interaction of Sema3E with them on asthmatic HASMC and the cell response should be further investigated. Nevertheless, Sema3E repulsive effect on asthmatic HASMC proliferation and also reversion of this effect by recombinant plexinD1 treatment indicate that Sema3E signaling in HASMC may be mediated exclusively through plexinD1.

We confirmed expression of plexinD1 on human ASM bundles in asthmatic and non-asthmatic tissue sections *in vivo*. Decreased surface expression of plexinD1 on primary bronchial HASMC from asthmatic subjects compared to normal ones suggests that Sema3E-plexinD1 inhibitory axis might be abrogated under pathological circumstances such as airway remodeling in which HASMC functions are highly dysregulated. However, isolated asthmatic HASMC responded to exogenous Sema3E through plexinD1 activation; probably by induction of receptor expression upon ligand stimulation as demonstrated for many surface receptors (37-39).

Our functional studies revealed that Sema3E inhibited PDGF-induced HASMC proliferation and migration. Although basal proliferation and migration of HASMC in response to Sema3E showed a decreasing trend compared to unstimulated HASMC, the differences were not statistically significant. Sema3E-mediated inhibition of HASMC migration was associated with F-actin depolymerisation. Therefore, it may be speculated that anti-migratory effect of Sema3E on HASMC is mediated by actin remodeling that is in line with previous findings in endothelial cells (23). It should be noted that semaphorins are more likely to provoke repulsion of target cells than attraction and an individual semaphorin can induce either repulsive or attractive responses in a cell-type-specific manner (40). As a bifunctional semaphorin, Sema3E repulsive effect can be reversed by proteolytic processing (41) and its interaction with coreceptors (33-34). However, in our *in vitro* system, Sema3E negatively regulated HASMC proliferation and migration.

In an effort to understand the mechanism of action of Sema3E in HASMC, considering involvement of Rac1 in smooth muscle proliferation and migration (9, 24) as well as unknown function of Rac1 in Sema3E-PlexinD1 signaling, we investigated the role of Rac1 GTPase in Sema3E-mediated HASMC repulsion. As a novel finding, we revealed here Sema3E induced inactivation of Rac1 GTPase in the presence of PDGF which has been shown to strongly stimulate Rac1 GTPase activity in HASMC. Semaphorin signaling is mainly converged to small GTPases including Ras homology (Rho) proteins such as Rac1. When bound to GTP, small GTPases are activated and attach to effector proteins to promote cell proliferation and migration (42). Along with Sakurai et al. study on human umbilical vein endothelial cells (HUVECs) (23), we found that treatment of HASMC with Sema3E alone did not affect the basal Rac1 GTPase activity in HASMC.

As previously reported in HUVECs, Sema3E decreased growth factor-induced activation of Akt and ERK1/2 (20) which are essential mediators of HASMC proliferation and migration (14, 43-44). Therefore, based on our findings, Rac1, Akt and ERK1/2 seem to play a role in the signaling events which are influenced upon Sema3E+PDGF co-stimulation in HASMC. On the other hand, it seems Sema3E alone does not affect these pathways which may explain non-significant effect of Sema3E on basal HASMC proliferation and migration in agreement with finding in endothelial cells (23).

In summary, our study provides the evidence that semaphorins may contribute to asthma pathology by affecting ASM cell function and airway remodeling. As a novel approach, our results suggest a new mechanism through which Sema3E inhibits HASMC proliferation and migration giving a new clue to control airway remodeling and clinical manifests of allergic asthma.

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2.7 Online Repository Materials

2.7.1 Methods

2.7.1.1 Ethics statement

Bronchial and tracheal human airway smooth muscle cells (HASMC) used in this study were obtained from healthy non-asthmatic and asthmatic individuals in accordance with procedures approved by the Human Research Ethics Board of the University of Manitoba, Winnipeg, Canada, and the Ethics Committee at University Hospital Basel, Switzerland. Written informed consent was obtained from each individual. The clinical characteristics of asthmatic patients are described in Table 2-1.

Table 2-1: Clinical characteristics of mild asthmatic individuals subjected to bronchoscopy

Mild asthmatic	Gender	Age	Smoking status	Allergy	Medication	Dose (mg/day)	PC20 (mg/ml)	FEV1 (%)
Subject #1	F	19	No	Yes	Salbutamol	pr	0.26	71
Subject #2	M	26	No	Yes	Salbutamol	pr	0.43	94
Subject #3	F	28	Ex	Yes	Salbutamol	200	3.7	111
Subject #4	M	21	Ex	Yes	Salbutamol	pr	0.04	100
Subject #5	F	19	No	Yes	Salbutamol	pr	0.41	93

PC20: Provocative concentration of methacholine causing a 20% fall in FEV1
FEV1: Forced Expiratory Volume in 1 second
Ex: Ex-smoker, pr: per need

Bronchoscopy was performed in accordance with procedures approved by the Human Research Ethics Board of Laval University, Quebec, Canada, following the receipt of the subjects' written informed consent. Clinical features of asthmatic patients from whom bronchial biopsies were obtained have been categorized in Table 2-2.

Table 2-2: Clinical profile of asthmatic patients used for bronchial HAMC isolation

Patient	Gender	Age	FEV1%	Medication	MEF25	MEF50	MEF75
Subject #1	M	30	81	Seretide	39	59	59
Subject #2	M	42	80	Symbicort	28	43	85
Subject #3	M	42	78	Symbicort	25	48	79

FEV1: Forced Expiratory Volume in 1 second
MEF: Maximal Expiratory Flow

2.7.1.2 Reagents

Recombinant human Sema3E, plexinD1 and PDGF-BB as well as APC-conjugated mouse anti-human plexin D1 monoclonal antibody and also goat anti-human plexin D1 affinity purified polyclonal antibody were purchased from R&D Systems (Minneapolis, MN). Unlabelled and APC-conjugated isotype control antibodies were obtained from Sigma-Aldrich Canada (Oakville, Ontario, Canada) and eBiosciences (San Diego, CA), respectively. Cell culture media were obtained from Invitrogen (Burlington, Ontario, Canada). FBS and Insulin-Transferrin-Selenium-X (ITS-X) were purchased from HyClone Laboratories (Logan, UT) and Invitrogen, respectively. Alkaline phosphatase-conjugated streptavidin was purchased from Jackson Immuno-Research Laboratories (West Grove, PA). The p42/p44 ERK inhibitor 1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadiene (U-0126) was purchased from Calbiochem

(Mississauga, Ontario, Canada). All other reagents are from Sigma-Aldrich unless otherwise indicated.

2.7.1.3 Isolation and culture of HASMC

HASMC were obtained as described previously (1-2). In brief, primary HASMC were isolated from lower tracheal section of healthy donors from whom recipient cannot be matched. Asthmatic primary bronchial HASMC were isolated from dissected airway muscle bundles of the bronchial biopsies from patients diagnosed with mild to moderate asthma. The patients did not manifest exacerbations, and received no medication 1 week before bronchoscopy (3). Cells retain α smooth muscle-specific actin (filament form), SM22, calponin and/or SMHC protein expression, as well as negative staining for fibronectin and E-cadherin and mobilize intracellular Ca^{2+} in response to acetylcholine (3-4). Primary HASMC were used at passage 2-5 in all experiments. Bronchial HASMC (D9) immortalized by stable ectopic expression of human telomerase reverse transcriptase (hTERT) was used at passages 15-20, and was described in details (5).

HASMC were grown on uncoated plastic dishes in complete DMEM (supplemented with 100µg/ml streptomycin, 100U/ml penicillin, and 10% fetal bovine serum). Unless otherwise mentioned, cells were grown to a subconfluent (~60-70%) condition and serum starved to synchronize for 48 h in Ham's F12 supplemented with 1X ITS (5µg/ml insulin, 5µg/ml transferrin, and 5ng/ml selenium), 100µg/ml streptomycin and 100U/ml penicillin, and before each experiment.

2.7.1.4 RNA extraction and RT-PCR

Total RNA was extracted from HASMC (passages 2-5) using TRIzol™ (Invitrogen). 2 μg of RNA was subjected to MultiScribe™ Reverse Transcriptase to synthesize cDNA according to manufacturer's instructions (Applied Biosystems, ABI). Expression of PLXND1 and GAPDH was analyzed by RT-PCR. PCR products were run on 2% w/v agarose gel electrophoresis and visualized by ethidium bromide staining. PCR conditions and specific primers have been mentioned in Table 2-3.

Table 2-3 Primer sequence, amplicon length and RT-PCR conditions

Primer sequences	Amplicon size (bp)	Annealing temperature (°C)	Cycles (#)
PLXND1: Fwd: 5´-TGGATGTCGCAGCTTACTTG -3´ Rev: 5´-CCCCAACCCACAGTTCTCTA-3´	156	58	30
GAPDH: Fwd:5´-AGCAATGCCTCCTGCACCACCAAC-3´ Rev: 5´-CCGGAGGGGCCATCCACAGTCT-3´	137	60	30

2.7.1.5 Flow cytometric analysis of receptor expression

Primary cultured confluent HASMC were detached using Versene® (0.02% EDTA, Lonza, Walkersville, MD) without trypsinization. The single cell suspensions ($1x10^5$ cells) were separately incubated with APC-conjugated mouse anti-human plexinD1 or isotype control Ab (IgG2b subclass) for 30 min at 4°C. The cells were washed twice with flow buffer, resuspended in PBS containing 0.1% BSA and analyzed on FACScan (BD Biosciences, San Jose, CA). FACS analysis was performed by FlowJo software (Tree Star, Ashland, OR).

2.7.1.6 Immunocytochemistry

HASMC were seeded onto sterile glass coverslips and grown to 70% confluency. Cells were then fixed in 4% paraformaldehyde (PFA) for 20 min; permeabilized and non-specific Ab binding was blocked. Immunolabeling was performed using goat anti-human plexinD1 Ab, as well as appropriate control goat IgG at 4°C overnight. Cells were incubated with biotin labelled rabbit anti-goat IgG as secondary antibody, washed and followed by streptavidin-alkaline phosphatase-conjugated secondary Abs for 1 hour at room temperature. The signals were detected by addition of Fast-red and then cells were counterstained with modified Mayer's haematoxylin (Fisher Scientific, Fair Lawn, NJ). Coverslips were mounted using crystal mount and visualized by AxioVision software (Carl Zeiss, Inc.).

2.7.1.7 Immunohistochemistry

Subjects. Bronchial biopsies were obtained from mild asthmatic patients fulfilling the American Thoracic Society selection criteria (6). Mild asthmatics used no inhaled corticosteroids, had symptoms < 2 days per week, and rare exacerbations. These atopic nonsmokers had not received long acting β_2-agonists or inhaled corticosteroids in the last 3 months (see Table 2-3 in the Online Repository).

Paraffin sections were stained as described previously (2). Briefly, formalin-fixed tissues were paraffin embedded, and 5-µm-thick sections were prepared, deparaffinized in xylene, and rehydrated through graded concentrations of alcohol to water and then boiled with microwave for 10 min in sodium citrate buffer (pH, 6.0). Sections were washed and then incubated with blocking solution (1% BSA, 0.1% cold fish skin gelatin in TBS) for 60 min at room temperature. Goat anti-human plexin D1 Ab or control goat IgG (both at 10 µg/ml) were added, and sections

were incubated overnight at 4°C. Slides were then washed twice with TBS followed by incubation for 1 h at room temperature with biotin-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). Slides were then washed extensively with TBS and incubated with streptavidin alkaline phosphatase for 30 min at room temperature. After washing with TBS, the slides were developed using Fast Red and counterstained with modified Mayer's hematoxylin (Fisher Scientific, Fair Lawn, NJ).

2.7.1.8 EdU cell proliferation assay and cell cycle analysis

Serum deprived HASMC were stimulated with aforementioned concentrations of Sema3E with or without PDGF. Click-iT™ EdU flow cytometry assay kit (Invitrogen,) was used to further investigate Sema3E effect on HASMC proliferation. Briefly, 5-ethynyl-2´-deoxyuridine reagent was added at a 10 μM concentration 40 h after stimulation and cells were harvested 8 h later. Cells were collected, fixed and then incubated with saponin-based permeabilization buffer. Click-iT reaction cocktail containing copper sulphate and fluorescent dye azide (Alexa Fluor® 488) was freshly prepared and added to the samples. Finally, cell cycle was analyzed using propidium iodide (PI) staining. EdU incorporation into DNA and cell cycle distribution were assessed using flow cytometry.

2.7.1.9 Cell count

HASMC were seeded at 5×10^4 cells/well in triplicates in 6-well plates and maintained in DMEM to become 50% to 70% confluent. After serum starvation in F12 medium containing ITS-X and sodium pyruvate for 48 h, cells were treated with Sema3E (0, 1, 10 and 100 ng/ml) with or without PDGF (10 ng/ml). Two days later, cells were collected and counted using a

hemocytometer and cell viability was determined by trypan blue exclusion. Cell count experiments were performed double-blind and twice by two independent individuals.

2.7.1.10 Cell migration assay

A Boyden chamber (Neuro Probe Inc. Gaithersburg, MD) assay developed specifically to study smooth muscle cell migration was employed as described previously (7). Sema3E (0, 0.1, 1 and 10 μg/ml) with or without PDGF (10 ng/ml) was added to the wells of the bottom chamber in triplicate. Then, collagen-coated filter membrane was placed on the bottom chamber and serum-deprived HASMC (5X105cell/ml) were added to the upper chamber wells. After 4 h of incubation in 37°C, non-migrated cells that did not pass through the membrane pores were wiped out from the top side of the insert membrane and then migrated cells in each well were stained with Protocol Hema 3 stain (Biochemical Sciences Inc. Swedesburg, NJ) and counted in random fields.

2.7.1.11 Phalloidin staining and immunofluorescence

The cells cultured on sterile coverslips were serum-starved and treated with Sema3E (100 ng/ml) for 0-5 min, fixed with 4% PFA in PBS for 20 min, and permeabilized with 0.05% Triton X-100. Filamentous (F) actin was visualized using Alexa Fluor® 647 Phalloidin (Invitrogen). Samples were mounted in gold anti-fade mounting medium (Invitrogen) and visualized under fluorescence microscope.

2.7.1.12 Quantification of F-actin content by flow cytometry

HASMC were seeded in triplicate in 12-well plates, serum starved and stimulated with Sema3E (100 ng/ml) for 0-5 min. Cells were fixed, permeabilized and stained with Phalloidin as described earlier. Then, intracellular fluorescence was determined using flow cytometry. After appropriate gating to remove cell debris from analysis, histograms of cell number versus log fluorescence intensity and forward angle light scatter were recorded. Relative F-actin content following Sema3E treatment was expressed as the mean fluorescence intensity (MFI) and was compared to that of non-stimulated HASMC.

2.7.1.13 Measurement of Rac1 GTPase activity

Rac1 GTPase activity was measured in snap-frozen cell lysates harvested from unstimulated or Sema3E±PDGF stimulated HASMC using a luminometric-based G-LISA Rac1 Activation Assay Kit (Cytoskeleton, Inc. Denver, CO). Briefly, cell lysates were subjected to Rho binding domain of Rac1 in a Rac-GTP affinity 96-well plate. Then, Rac1-GTP was detected using specific primary Ab followed by HRP-conjugated secondary Ab incubation and development with a chemiluminescent reagent. A constitutively active Rac1 provided in the kit was used as positive control in all experiments.

2.7.1.14 Assessment of MAPK/ERK1/2 and PI3K/Akt activation

Protein lysates obtained from G-LISA experiments were investigated for downstream signaling using Western blotting. Protein lysates (10 µg) were loaded on 10% SDS-PAGE, transferred to PVDF membranes, blocked and then incubated overnight at 4°C with Abs specific for phosphorylated Akt (S473), ERK1/2 (T202/Y204), p38 (T180/Y182), and STAT3 (Y705).

After washing, the blots were incubated with HRP-conjugated secondary Abs and bands were revealed with ECL reagents. Total anti-Akt, ERK1/2, p38, and STAT3, were used as loading control. Densitometric analysis was performed by using AlphaEase FC software (Alpha Innotech, San Leandro, Calif) and integrated density value was presented as the fold-increase in phosphorylated over total compared to time zero.

2.7.1.15 Statistical analysis

GraphPad Prism 5.0 software was used for statistical analysis and values were presented as the mean±SEM of at least three independent experiments. Depending on the number of groups and treatments, data were analyzed by unpaired *t* test, one-way or two-way ANOVA, followed by the Bonferroni's multiple comparison post-hoc test. Differences were considered to be statistically significant at $*p<0.05$, $**p\leq0.01$ and $***p\leq0.001$.

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3 CHAPTER 3:

SEMAPHORIN 3E EXPRESSION IS SUPPRESSED IN SEVERE ASTHMA AND ALLEVIATES ALLERGIC AIRWAY DISEASE BY REGULATING DENDRITIC CELL FUNCTIONS

This collaborative work is under revision with *PLOS BIOLOGY* journal.

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H.M. designed, performed and analyzed the experiments, and prepared the manuscript. L.S. contributed in conducting both human and mouse experiments. J.C. participated in processing human lung tissue sections and RNA/protein isolation from human bronchial epithelial cells and also revision of the manuscript. J.S.D-C. cloned, expressed and purified mouse Sema3E-Fc and revised the manuscript. J.F.M. conducted BALF preparation and processing from human asthmatics and healthy individuals. A.J.H. contributed in mouse lung mechanics studies and establishing HDM model. A.S.G. designed experiments, analyzed data, and prepared the manuscript. All authors revise d and approved the final version of the manuscript.

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3.1 Abstract

Allergic asthma is characterized by airway inflammation primarily mediated by presentation of aeroallergens via dendritic cells (DC) which leads to bronchial hyper-responsiveness and remodeling. The precise protective mechanisms that govern DC-mediated airway inflammation have not been fully understood. Herein, we report that expression a neural chemorepellent, semaphorin Sema3E (Sema3E), is reduced in bronchial biopsies of asthmatic patients compared with healthy controls and the level of Sema3E secretion in the airways inversely correlates with disease severity. Mice lacking the gene that encodes this mediator (*Sema3e*$^{-/-}$), develop exaggerated airway hyperresponsiveness, mucus production, collagen deposition, IgE synthesis and Th2/Th17 inflammation upon allergen exposure. Furthermore, *Sema3e*$^{-/-}$ mice had increased CD11b^{+} pulmonary DC inducing enhanced Th2/Th17 inflammatory response. In addition, DC from *Sema3e*$^{-/-}$ mice had higher migration and allergen uptake capacity along with increased CCR7 expression, Rac1 GTPase activity and F-actin polymerization. Adoptive transfer of CD11b^{+} pulmonary DC from *Sema3e*$^{-/-}$ mice into wild type littermates exacerbated allergic airway inflammation. Recombinant Sema3E protected mice from allergic asthma through decreased CD11b^{+} and increased CD103^{+} pulmonary DC, reduction of Th2/Th17 and increased Th1 response. Together, these findings show that Sema3E expression is reduced in allergic asthma and modulates pathological features of the disease by altering DC functions. Sema3E thereby appears as a novel regulatory mediator, upstream of pro-allergic events, suggestive of a new approach to attenuate asthma deficits.

Key words: Allergic asthma, Dendritic Cells, House Dust Mite, PlexinD1, Semaphorin 3E

3.2 Introduction and rationale

Asthma is a heterogeneous chronic disorder of the airways in which the inflammation is associated with airway hyperresponsiveness (AHR) leading to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing. It is among the commonest chronic conditions in Western countries affecting approximately 14% children and 8% adults (1, 2). Pathophysiological features of asthma include granulocyte-rich inflammatory cell infiltrates, mucus hypersecretion, and airway wall remodeling (3).

In allergic asthma, the most common form of the disease, inhalation and subsequent presentation of allergens by DC induce airway inflammation and recruitment of granulocytes into the lungs which lead to tissue damage and bronchial hyperreactivity in atopic individuals (4). Allergen specific type 2 response is characterized by production of interleukin (IL)-4, IL-5 and IL-13 which play critical roles in IgE synthesis, eosinophil recruitment, mast cell growth and AHR. However, the containment of type 2 inflammation by activation of Th1, Th17, and T_{reg} cells is important in pathogenesis of allergic asthma (5). Considering the essential role of DC in induction of these responses (6), interference with their pro-allergic functions is considered as an efficient therapeutic strategy in allergic asthma (7, 8).

Semaphorins, initially discovered as axon guidance molecules, are ubiquitously expressed beyond the nervous system and control immune regulation (9-11). Among them, semaphorin 3E (Sema3E) and its receptor plexinD1 have emerged as an essential axis involved in cell migration, proliferation and angiogenesis (12-15), which are key features associated with inflammation and tissue remodeling. We have previously demonstrated that Sema3E inhibits human airway smooth muscle (ASM) cells proliferation and migration via modulation of MAPK, PI3K and Ras-related

C3 botulinum toxin substrate 1 (Rac1) GTPase pathways suggesting a potential role of this pathway in airway remodeling (16). However, the *in vivo* function of Sema3E in allergic asthma has not been investigated.

Our objective in the present study was to determine the role of Sema3E in development and maintenance of allergic asthma. We demonstrated that Sema3E immunoreactivity was reduced significantly in bronchial biopsies of human severe asthmatics compared to healthy donors and Sema3E level in human bronchoalveolar lavage fluid (BALF) was inversely correlated with forced expiratory volume in 1 second (FEV1). Sema3E deficient mice developed an exacerbated form of allergic airway disease characterized by increased Th2/Th17 responses, goblet cell hyperplasia, collagen deposition and AHR. These events were associated with augmented total and specific serum IgE and airway eosinophilia. Intranasal (i.n.) administration of exogenous recombinant Sema3E prevented lung pathology in allergen-induced airway disease. Interestingly, we demonstrated that Sema3E mediates its role in allergic asthma at least in part through regulation of DC migration, allergen uptake and naïve T cell proliferation and differentiation.

3.3 Results

3.3.1 Sema3E expression negatively correlates with allergic asthma

Sema3E was previously reported as a potent inhibitor of postnatal angiogenesis (13). However, the role and expression of Sema3E in allergic asthma is not clear. We investigated expression of Sema3E in bronchial biopsies of allergic asthmatics with different severity and healthy control group (Table 3-1). Sema3E was highly expressed in bronchial biopsies of healthy donors and its expression was significantly decreased in severe asthmatics (Fig. 3-1A). Mild and moderate allergic asthmatics showed also a reduced tendency according to pathological score but it was not significant. It is noteworthy that bronchial epithelial and infiltrating inflammatory cells within the submucosa displayed Sema3E immunoreactivity (Fig. 3-1A).

Since the functional form of Sema3E is a secreted protein, we compared extracellular Sema3E in human BALF obtained from normal and severe asthmatics (Table 3-2). There was a significant decrease of Sema3E in BALF isolated from severe asthmatics compared to healthy donors (Fig. 3-1B) which was negatively correlated with FEV1 (Fig. 3-1C). Decrease in Sema3E expression in allergic asthma was further confirmed at protein and mRNA levels in bronchial epithelial cells isolated from severe allergic asthmatics (Figure 3-1D-E) compared to those of healthy individuals.

To ascertain the reduced expression of Sema3E in allergic asthma, a mouse model of allergic asthma using intranasal (i.n.) instillation of house dust mite (HDM) extract was performed as shown in Fig. 3-2 (17). Sema3E immunoreactivity was significantly decreased in mice exposed to HDM compared to saline-treated mice (Fig. 3-9). Taken together, our data indicate that Sema3E expression is decreased in allergic asthmatic conditions.

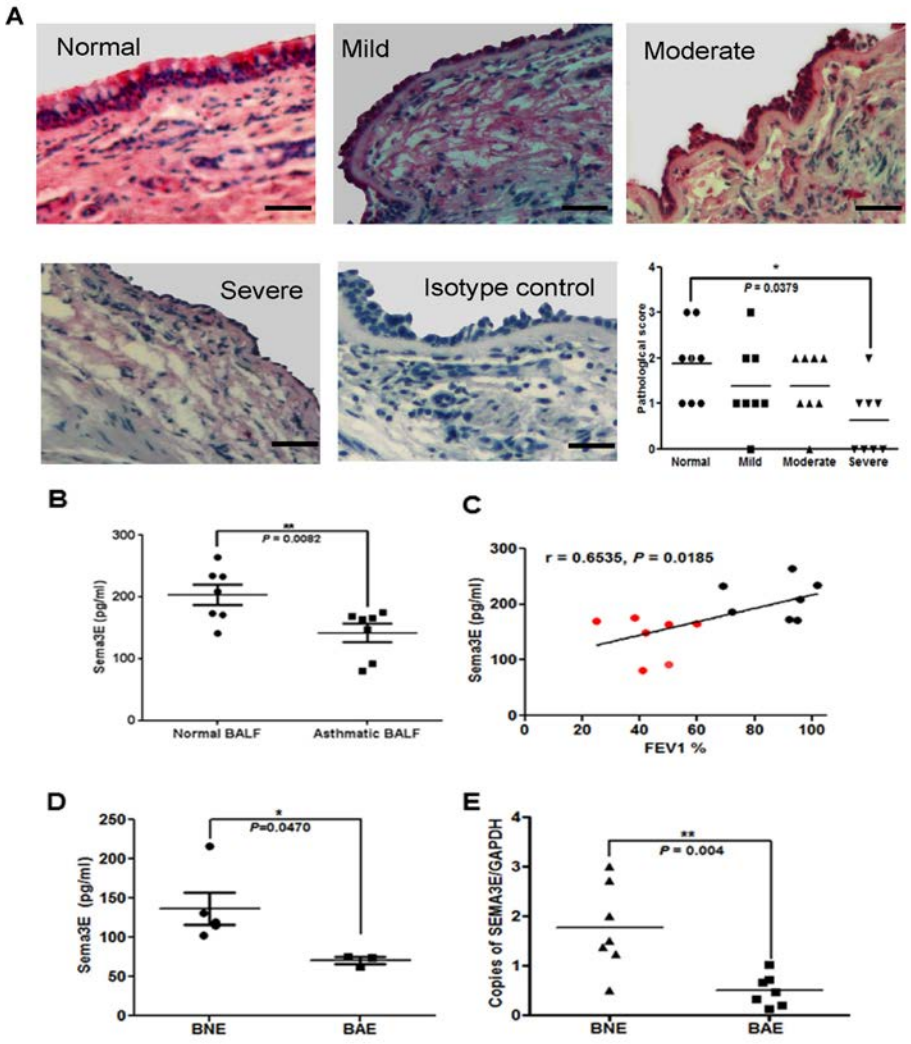

Figure 3-1 Expression of Sema3E is reduced in asthmatic conditions.
(A) Expression of Sema3E was compared between healthy and asthmatic subjects by performing IHC on airway bronchial sections (n = 8 per group), Scale bars: 50 μm. (B) Sema3E level on BALF from severe asthmatic and healthy subjects was measured by ELISA. (C) Correlation of Sema3E level with FEV1 was assessed by Pearson's R test. Red and black dots represent severe asthmatic and healthy subjects, respectively (n = 7 per group). (D) Ex vivo Sema3E level was measured in supernatant of cultured human airway epithelial cells from normal (n = 5) and severe asthmatic (n = 3) individuals. (E) mRNA expression of SEMA3E in airway epithelial cells from severe asthmatic and healthy individuals was determined by qPCR (n = 7 per group). BNE: Bronchial normal epithelial cells, BAE: Bronchial asthmatic epithelial cells. The data are expressed as mean ± SEM. P values have been shown for each graph; Scale bars: 50μm. (n = 4 mice per group). The data are expressed as mean ± SEM. P values have been shown for each graph. The statistical significance was determined by performing Student t-test in A, B, D and E.

3.3.2 Sema3E deficient mice demonstrate exaggerated allergic asthma

Repeated inhalational exposure to HDM induces AHR and inflammation (17-19). We then compared development of allergic asthma in the presence or absence of Sema3E in an HDM acute murine model as depicted in Fig. 3-2A (19). After exposure to nebulized methacholine, HDM-challenged *Sema3e*$^{-/-}$ mice showed a significant increase in airway resistance (Fig. 3-2B), tissue resistance (Fig. 3-10A) and tissue elastance (Fig. 3-10B) compared to WT littermates. HDM-challenged *Sema3e*$^{-/-}$ mice had increased total inflammatory cell numbers in BALF compared to WT mice (Fig. 3-2C) characterized by a significant increase in granulocytes and decrease in mononuclear cells upon HDM challenge (Fig. 3-2D). Increased recruitment of inflammatory cells to the airways was further confirmed by performing FACS analysis on BALF cells. Concomitantly, lung histology studies revealed exacerbated peribronchial inflammation (Fig. 3-2E), goblet cell hyperplasia (Fig. 3-2F and Fig. 3-10C) and collagen deposition (Fig. 3-2G) in HDM-challenged *Sema3e*$^{-/-}$ compared to WT mice.

Corresponding to their having an exaggerated allergic response, HDM-challenged *Sema3e*$^{-/-}$ mice showed a statistically significant increase in the levels of BALF IL-4, IL-5, IL-13 and IL-17A compared to WT controls. However, IFN-γ and IL-9 levels did not change significantly (Fig. 3-3A). Intracellular staining of IFN-γ , IL-4 and IL-17A on lung draining mediastinal lymph node (MLN) cells further confirmed the cytokine response (Fig. 3-10D-F). In HDM-challenged *Sema3e*$^{-/-}$ mice, total and HDM-specific IgE and IgG1 levels were significantly increased whereas IgG2a remained unchanged (Fig. 3-3B). These data are collectively suggestive of an enhanced Th2/Th17-biased inflammation in *Sema3e*$^{-/-}$ mice upon HDM exposure.

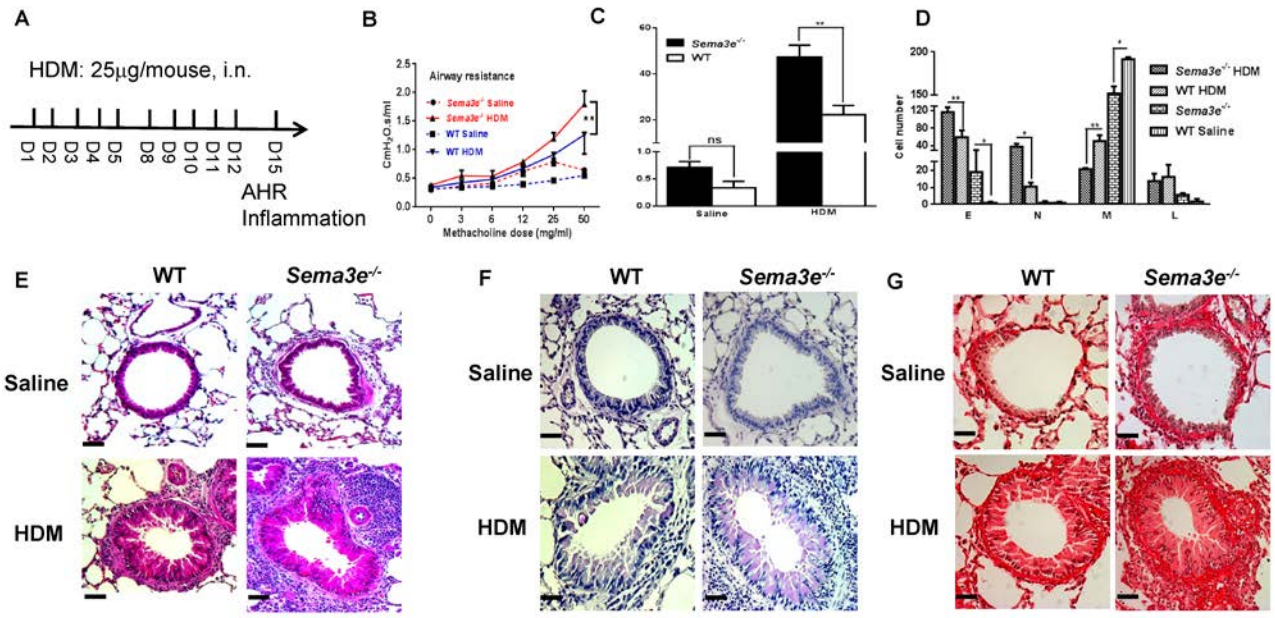

Figure 3-2 Sema3E deficient mice have elevated AHR, airway inflammation and remodeling.
*Sema3e$^{-/-}$ or WT mice underwent tracheotomy accompanied by methacholine challenge to measure airway resistance (A). Total (B) and differential (C) cell count was performed on BALF. E: Eosinophil, N: Neutrophil, M: Macrophages, L: Lymphocytes. Airway inflammation, mucus secretion and collagen deposition was studied by performing H&E (D-I), PAS (D-II) and Sirius Red (D-III) staining, respectively. Upper and lower parts of DI-III panels represent histological data from saline and HDM-exposed mice, respectively. Scale bars: 100μm. The statistical significance was determined by performing one-way ANOVA. n = 5-6 per group, *P<0.05 and **P<0.01.*

3.3.3 Sema3E is involved in regulation of pulmonary DC recruitment and function

Mononuclear cells, mostly alveolar macrophages are the dominant resident cell types in healthy airways (20, 21). However, aeroallergen exposure induces migration of DC, another subset of mononuclear cells, to the airways to sample and present the antigens to naïve T cells (22). We then studied recruitment of DC into the airways of *Sema3e*$^{-/-}$ and WT mice upon HDM encounter (23). Our flow cytometric analysis on BALF cells revealed that continuous HDM exposure for 2 weeks induces higher accumulation of total DC in the airways of *Sema3e*$^{-/-}$ mice compared to WT littermates (Fig. 3-3C-E).

Pulmonary DC have been shown to sample aeroallergens from the airways, process and present them to naïve T cells in lymph nodes after migration (24). CD103^{+} and CD11b^{+} DC are two major subsets of lung conventional DC (cDC) which exert distinct functions (25). CD103^{+} pulmonary cDC subset is involved in regulation of Th1/Th17 responses (25) and also induction of tolerance to inhaled allergens (26); while CD11b^{+} cDC are potent promoters of Th2 (25, 27) or Th17 (28) responses upon allergen encounter. Considering differential effect of these subsets on allergen-induced T cell differentiation (27), we compared their frequency between *Sema3e*$^{-/-}$ and WT littermates 2d after i.n. sensitization with a single high dose (100 μg) of HDM. CD11b^{+} pulmonary DC were increased, whereas CD103^{+} DC decreased in HDM exposed *Sema3e*$^{-/-}$ mice compared to WT littermates (Fig. 3-4A-C). Similarly, pulmonary DC from naïve *Sema3e*$^{-/-}$ mice showed higher CD11b^{+} and lower CD103^{+} subsets at the baseline compared to WT mice (Fig. 3-11) suggesting that Sema3E play an important role in DC type recruitment to the airways.

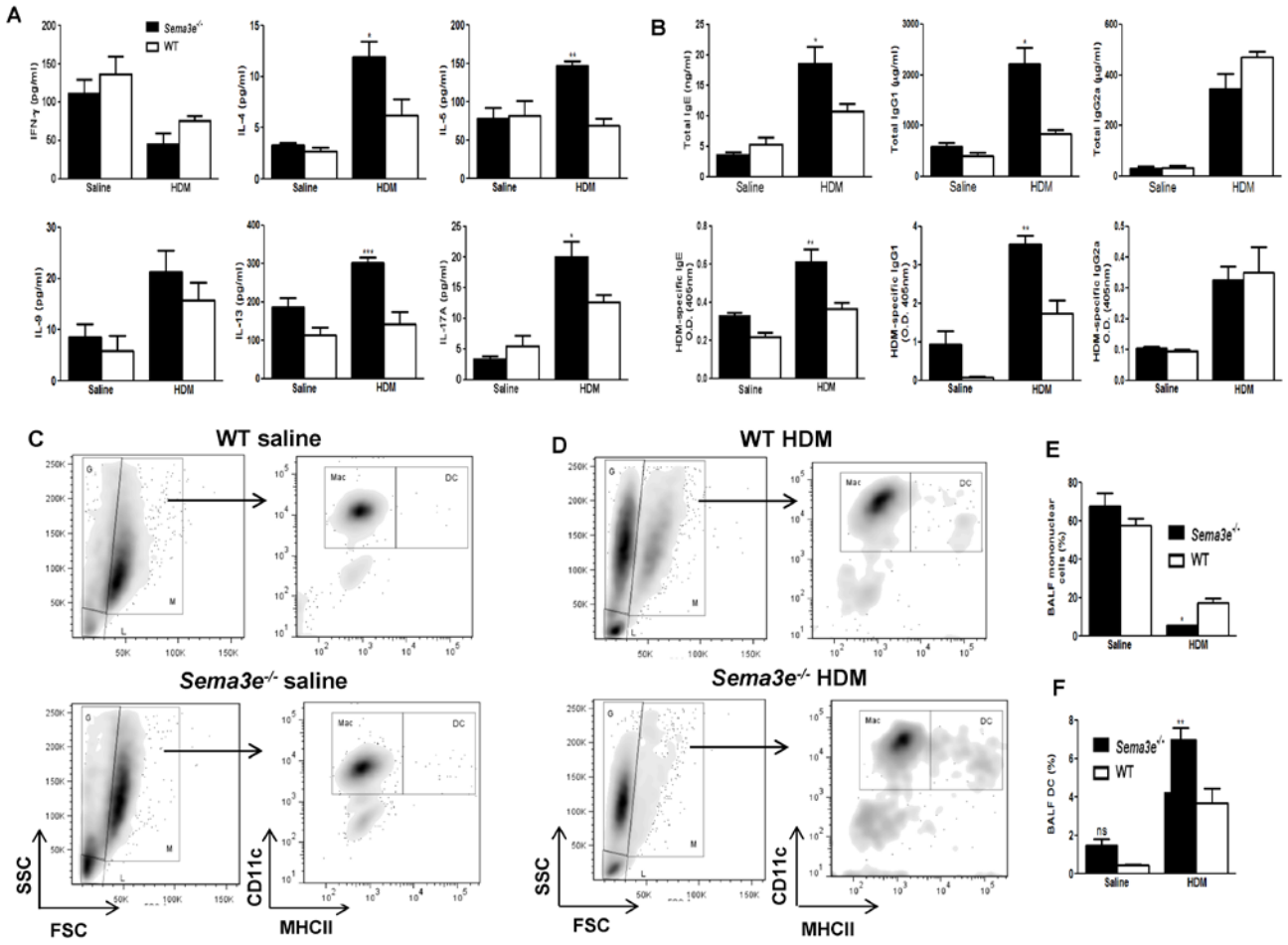

Figure 3-3 Sema3E deficient mice demonstrate a hyper-inflammatory phenotype in the airways.
*Airway levels of IL-4, IL-5, IL-9, IL-13, IL-17A and IFN-γ were measured upon either HDM or saline exposure by ELISA in BALF supernatants obtained from Sema3e$^{-/-}$ or WT mice after intranasal exposure to saline or HDM (A). Serum levels of total and HDM-specific antibodies IgE, IgG1, and IgG2a were measured by ELISA (B). BALF inflammatory cells underwent flow cytometry to determine major populations including granulocytes, lymphocytes and mononuclear cells based on their size and granularity. Extracellular staining for CD11c and MHCII further characterized mononuclear cell subpopulations including macrophages and DC after saline (C) or HDM (D) exposure. The basal and HDM-induced recruitment of mononuclear cells (E) and DC (F) was compared between Sema3e$^{-/-}$ and WT mice as absolute percentage. G: Granulocyte, L: Lymphocyte, M: Mononuclear cells, Mac: Macrophage and DC: Dendritic cells. The statistical significance was determined by performing one-way ANOVA and Bonferroni test. All data are representative of 5 mice per group. *P<0.05, **P<0.01 and ***P<0.001.*

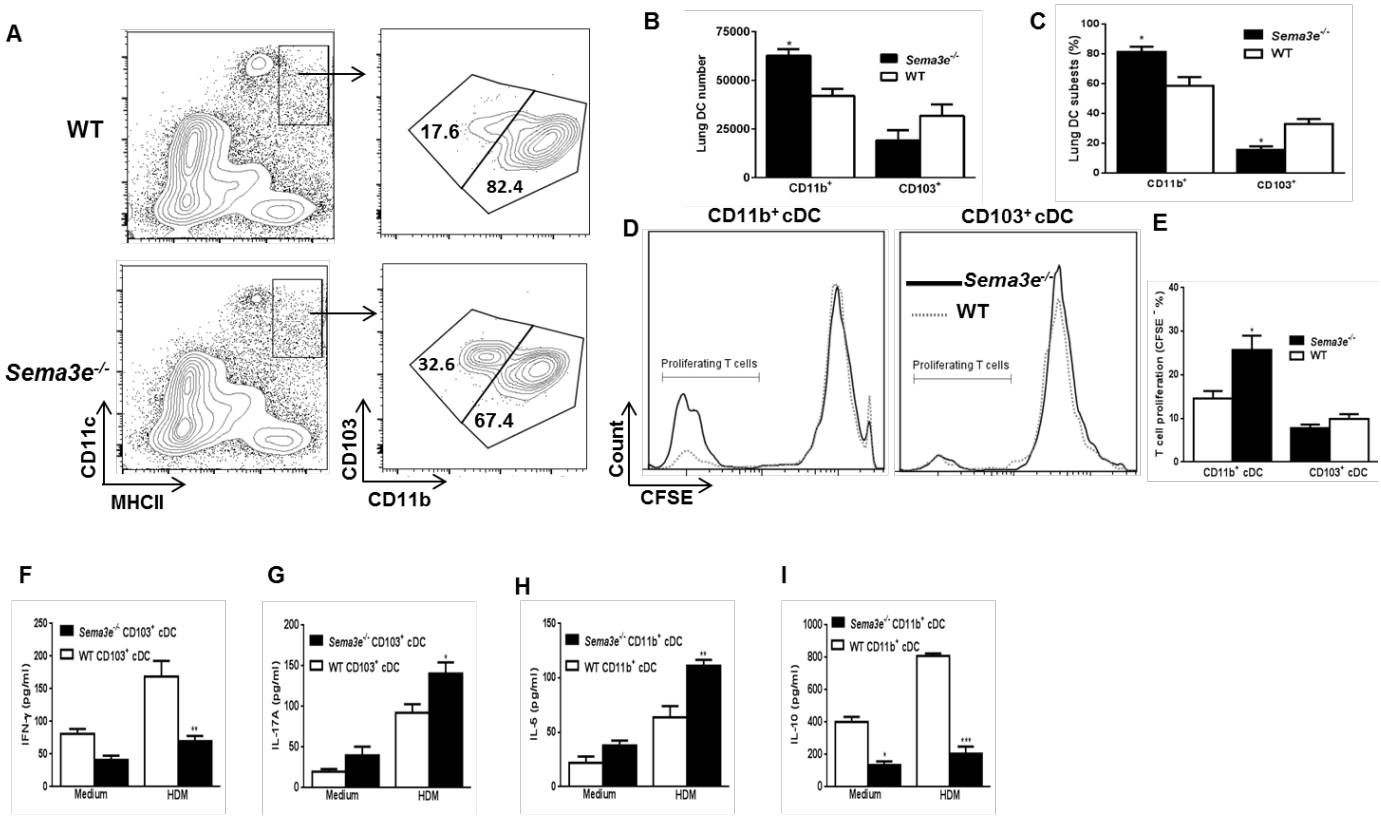

Figure 3-4 Sema3E is involved in allergen-induced pulmonary DC functions.
*Frequency of CD11b$^+$ and CD103$^+$ lung DC subsets was studied 2 days after exposure with a single high dose of HDM (A- C) (n = 9 per group). Proliferation of CFSE-labeled T cells co-cultured for 5d with lung DC subsets sorted from HDM-exposed Sema3e$^{-/-}$ or WT mice was studied by flow cytometry (D-E). Cytokine secretion was measured in supernatants of naïve T cells: pulmonary DC co-cultures after ex vivo re-stimulation with either HDM or vehicle (F-I) (n=4 per group). Pulmonary DC data represent 3 independent experiments generated from 3 pooled whole lung samples in each group per experiment. The statistical significance was determined by performing one-way ANOVA and Bonferroni test. *P<0.05, **P<0.01 and ***P<0.001.*

To detect the role of Sema3E in induction of primary immune response to HDM, we purified above mentioned pulmonary cDC subsets from *Sema3e*$^{-/-}$ and WT mice after *in vivo* exposure to HDM for 2d. Both subsets of DC were then co-cultured with CFSE-labeled T cells from WT littermates for 5d *in vitro* followed by measurement of T cell proliferation (27). CD11b^{+} pulmonary DC from *Sema3e*$^{-/-}$ mice induced significant proliferation of naïve T cells compared to CD11b^{+} from WT controls. However, effect of CD103^{+} pulmonary DC on induction of T cell proliferation was independent of Sema3E (Fig. 3-4D-E). We then measured the levels of IFN-γ, IL-5, IL-10 and IL-17A in the supernatants of the same co-cultures as the respective hallmarks of Th1, Th2, T_{reg} and Th17 responses (27). CD103^{+} pulmonary DC from *Sema3e*$^{-/-}$ mice induced secretion of significantly lower IFN-γ (Fig. 3-4F) and higher IL-17A (Fig. 3-4G) levels compared to CD103^{+} DC from WT littermates. On the other hand, CD11b^{+} pulmonary DC from *Sema3e*$^{-/-}$ mice induced higher IL-5 (Fig. 3-4H) and lower IL-10 (Fig. 3-4I) secretion.

To compare the capacity of *Sema3e*$^{-/-}$ vs. WT CD11b^{+} DC to induce Th2/Th17 immunity, we sorted either WT or *Sema3e*$^{-/-}$ pulmonary CD11b^{+} DC from HDM-sensitized mice after 2d and then adoptively transferred them to WT recipients via i.n. route followed by HDM exposure (27) (Fig. 3-5A). Mice receiving CD11b^{+} pulmonary DC from *Sema3e*$^{-/-}$ mice, displayed a significant increase in BALF cells particularly eosinophils and to a lesser extent neutrophils (Fig. 3-5B-C) concomitantly with an increased frequency of MHCII^{+}CD11c^{+} CD11b^{+} DC in the airways (Fig. 3-12A). These events were accompanied by a significant production of IL-4 and IL-17A (Fig. 3-5D), but not IFN-γ (Fig. 3-12B), by CD4^{+} T cells, in the MLN of *Sema3e*$^{-/-}$ mice. Similarly, peribronchial cell infiltrate was more pronounced in mice receiving CD11b^{+} pulmonary DC from *Sema3e*$^{-/-}$ mice compared to those of WT littermates (Fig. 3-5E). Thus, Sema3E deficiency may

increase pulmonary CD11b^{+} DC recruitment and function which affects Th cell polarization upon HDM challenge.

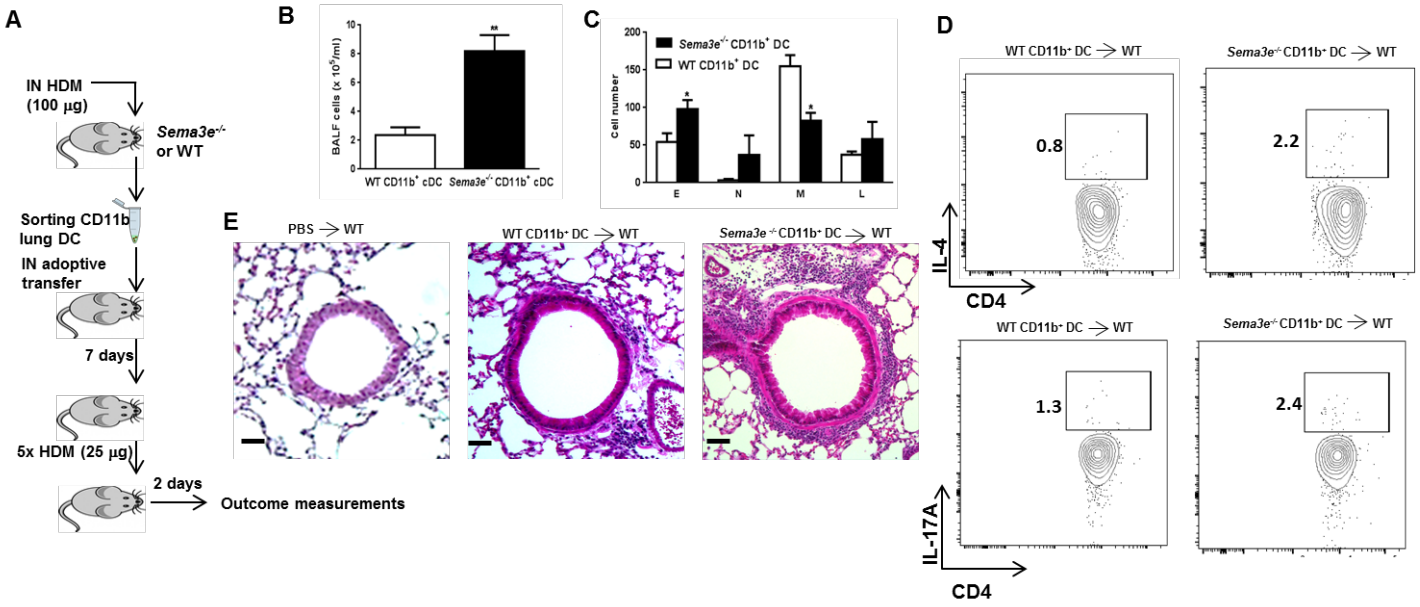

Figure 3-5 Adoptive transfer of CD11b^{+} pulmonary DC from Sema3e deficient mice exacerbates allergic airway inflammation.

*Cartoon depicting allergic airway induction by adoptively transferred CD11b^{+} cDC from HDM-sensitized Sema3e$^{-/-}$ or WT mice into naïve WT recipients (A). Total (B) and differential (C) counts of inflammatory cells recovered in the BALF after HDM exposure. E: Eosinophil, N: Neutrophil, M: Mononuclear cells, L: Lymphocytes. IL-4 and IL-17A production in MLN cells (D). Histological examination of lung sections of recipient mice stained with H&E (E). Scale bars: 100µm. Data are representative of four independent experiments. The statistical significance was determined by performing Student t-test in B and one-way ANOVA and Bonferroni test in C. n= 5-7 per group; *P < 0.05 and **P<0.01.*

3.3.4 Sema3E-regulated DC function is mediated by CCR7 expression, Rac1 GTPase activity and F-actin polymerization

It has been shown that pulmonary DC migrate to the MLN upon allergen encounter which highly express CCR7 as an essential mediator of DC migration (27). To delineate the potential impaired regulatory mechanism underlying hyperinflammatory phenotype in the absence of Sema3E, we studied surface expression of CCR7 in MLN DC. Three days exposure with a single high dose of HDM (100 μg) (27) induced higher CCR7 expression in $CD11b^+$ MLN DC from *Sema3e*$^{-/-}$ compared to those of WT mice (Fig. 3-6A) which was not significantly different earlier (36h). Therefore, enhanced accumulation of $CD11b^+$ DC in the absence of Sema3E could be partly mediated by HDM-induced overexpression of CCR7, earlier (36h) at the lung and later (72h) at the MLN.

Although pulmonary DC are essential for controlling effector T cell response and also AHR upon allergen exposure, they have been originally recruited from bone marrow into the airways (29). Bone marrow-derived dendritic cells (BMDC) from *Sema3e*$^{-/-}$ mice had higher level of CCR7 surface expression at the baseline and also upon stimulation with either CCL21 (Fig. 3-6B) or HDM (Fig. 3-13A). Concurrently, *Sema3e*$^{-/-}$ BMDC displayed a higher basal and CCL21-induced migration capacity compared to their WT counterpart (Fig. 3-6C). Therefore, Sema3E may exert its inhibitory effect on allergic asthma partly through reduction of DC migration via CCR7 pathway.

In addition, we studied allergen uptake ability by stimulating mature BMDC from *Sema3e*$^{-/-}$ and WT mice with a fluorescent OVA. Allergen uptake was higher in BMDC from *Sema3e*$^{-/-}$ than those of WT littermates. Sema3E treatment of BMDC from *Sema3e*$^{-/-}$ mice decreased

allergen uptake; though it was not observed in BMDC from WT mice probably via presence of some unknown compensatory mechanisms (Fig. 3-13B).

Activation of small GTPases such as Rac1 is indispensable for acquisition of the migratory cell phenotype and allergen uptake by DC (30). Hence, we compared the level of Rac1 GTPase activity between BMDC from *Sema3e*$^{-/-}$ and WT mice which was higher in the absence of Sema3E (Fig. 3-6D). F-actin polymerization is an essential component of cell migration (31) which was higher in BMDC from *Sema3e*$^{-/-}$ compared to WT mice (Fig. 3-6E). *In vitro* differentiation of bone marrow progenitors to mature BMDC resulted in higher expression of CD11c in *Sema3e*$^{-/-}$ than WT BMDC (Fig. 3-13C). Therefore, our data suggest that crucial DC functions such as migration and allergen uptake and also their downstream signaling pathways are dysregulated in the absence of Sema3E which may account for the higher inflammation in *Sema3e*$^{-/-}$ vs. WT mice which was heightened upon HDM exposure.

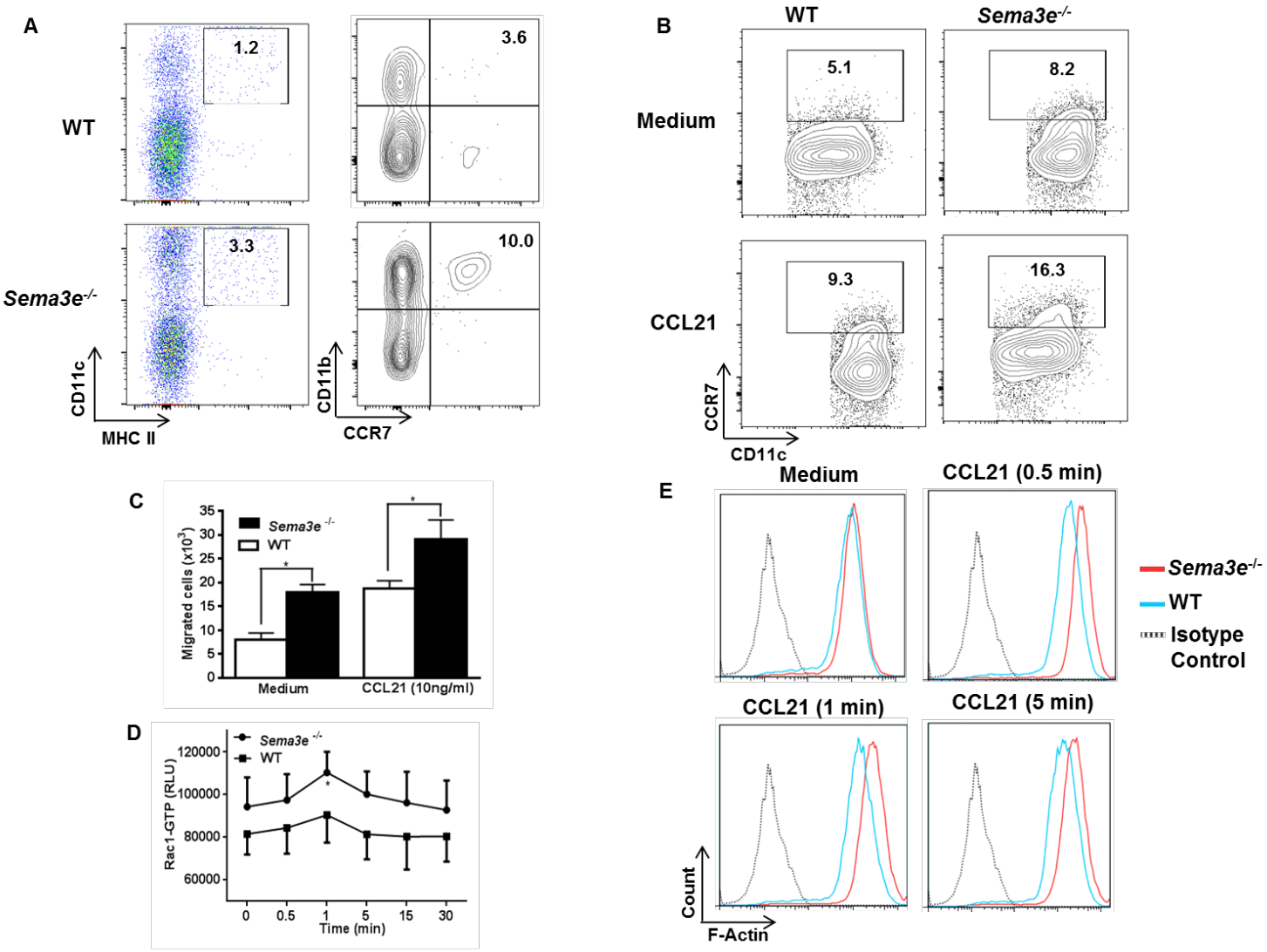

Figure 3-6 Sema3E is involved in regulation of DC migration.

*HDM-induced CCR7 surface expression was studied in pulmonary CD11b^{+} DC from Sema3e$^{-/-}$ or WT mice (A). CCL21 stimulation induced higher levels of CCR7 surface expression in BMDC from Sem3e$^{-/-}$ mice than those of WT littermates as determined by flow cytometry (B). Both basal and chemokine-induced migration was compared between BMDC obtained from Sema3e$^{-/-}$ and WT mice (C). Rac1 GTPase activity was compared between BMDC from Sem3e$^{-/-}$ and WT mice before and after CCL21 stimulation by performing G-LISA (D). Kinetic study of actin polymerization by Phalloidin staining at the baseline and upon stimulation with CCL21 revealed higher F-actin content in the absence of Sema3E over time (E). Data represent at least three independent experiments. The statistical significance was determined by performing one-way ANOVA and Bonferroni test (n=5 per group), *P<0.05.*

3.3.5 Sema3E treatment prevents allergic airway disease

To address the therapeutic potential of Sema3E in allergic asthma, we first exposed Balb/c WT mice with either Sema3E-Fc or saline-Fc control followed by i.n HDM or saline exposure 1h later for two consecutive weeks (Fig. 3-14A). Mice were mechanically ventilated and lung function parameters were measured by exposure to a gradient of methacholine concentration. We observed a significant decrease in airway resistance (Fig. 3-7A), tissue resistance (Fig. 3-14B) and elastance (Fig. 3-14C) in mice receiving Sema3E-Fc before HDM exposure compared to HDM exposed Fc alone treated group. Mice treated with Sema3E-Fc exhibited decreased numbers of total inflammatory cells (Fig. 3-7B), eosinophils and neutrophils (Fig. 3-7C) in BALF compared to HDM-Fc treated mice. Lung leukocytic infiltration (Fig. 3-7D-I), mucus secretion (Fig. 3-7D-II), and collagen deposition (Fig. 3-7D-III) were also decreased upon Sema3E-Fc treatment.

To understand whether Sema3E inhibitory effect on airway inflammation is related to alteration in cytokines associated with asthma, the levels of IL-4, IL-5, IL-9, IFN-γ, IL-12, IL-17A, TNF and IL-1β was measured in BALF obtained from HDM±Sema3E-Fc and vehicle treated mice. The level of IL-4, IL-5, IL-9 and IL-17A was significantly decreased in mice treated with Sema3E-Fc compared to HDM challenged mice. In contrast, IFN-γ was increased in BALF upon Sema3E-Fc treatment and IL-12 level was not significantly changed (Fig. 3-7E). In addition, HDM-induced secretion of pro-inflammatory mediators TNF and IL-1β were significantly decreased in Sema3E-Fc treated mice (Fig. 3-14D-E).

Considering the importance of IgE and IgG1 in initiation and persistence of Th2 inflammation in allergic asthma (32), total and HDM-specific forms of these antibodies were measured in mouse sera from HDM-challenged mice with or without Sema3E-Fc treatment. Sema3E

significantly suppressed total and HDM-specific IgE levels (Fig. 3-7F) which was associated with significant decreased HDM-specific but not total IgG1 (Fig. 3-7G). Collectively these data suggest a potential therapeutic implication of Sema3E treatment in allergic asthma by regulation of inflammatory responses and airway remodeling features.

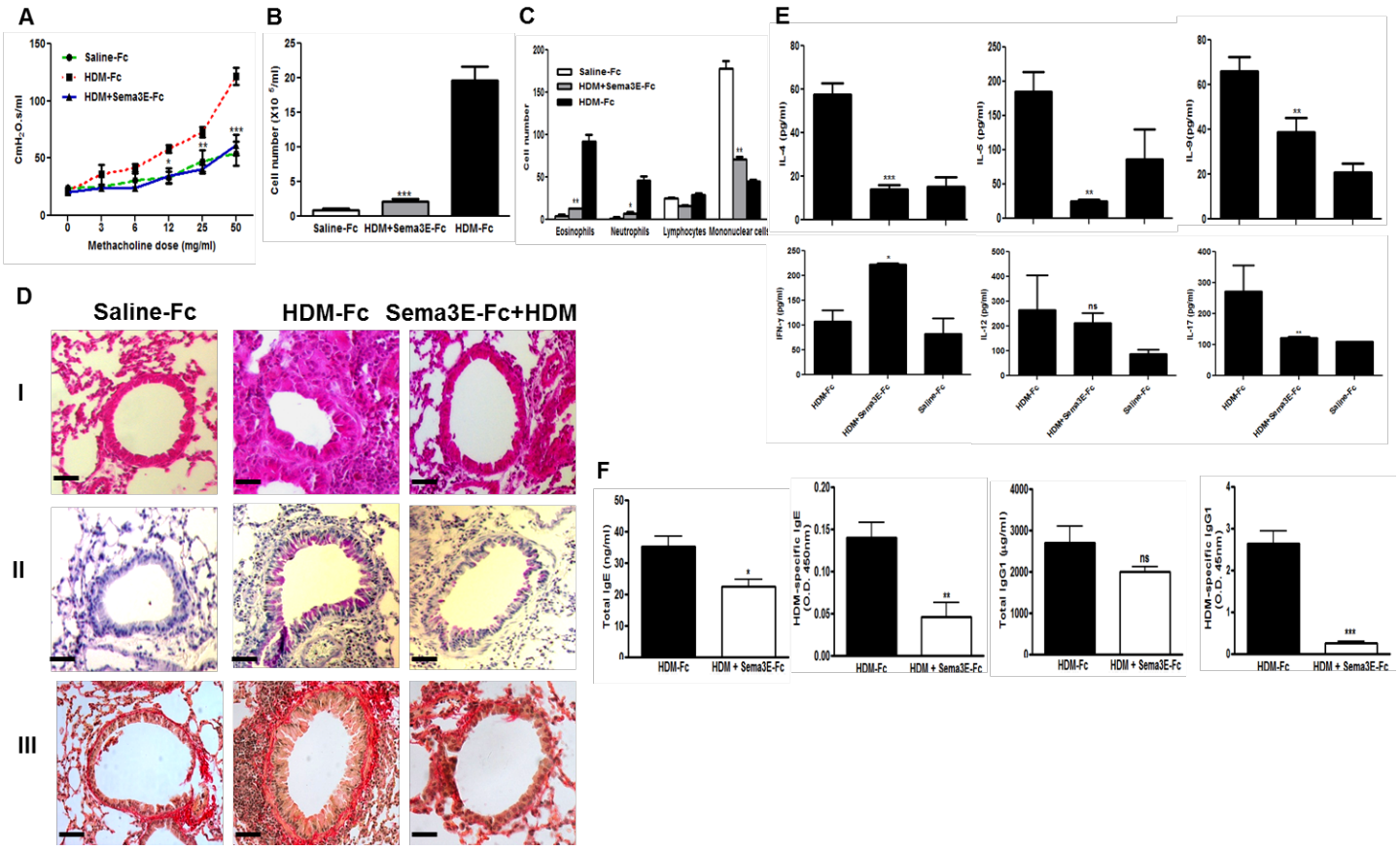

Figure 3-7 ***Sema3E prevents allergen-induced experimental asthma.***
*Intranasal administration of Sema3E hampers HDM-induced AHR (A) and airway inflammation characterized by decreased BALF total inflammatory cells (B) and granulocytes but increased macrophages (C). Sema3E treatment also decreased HDM-induced lung inflammation (D-I), mucus overproduction (D-II) and collagen deposition (D-III) in a magnitude comparable to naïve saline-treated mice. Sema3E treatment decreased HDM-induced IL-4, IL-5, IL-9, IL-17A, TNF and IL-1β in BALF while IFN-γ secretion was increased (E). Total and HDM-specific IgE (F) as well as HDM-specific but not total IgG1 IgG1 (G) levels were decreased after Sema3E treatment in serum. All data represent two independent experiments. The statistical significance was determined by performing one-way ANOVA and Bonferroni test in A, B, C and E and Student t-test in F. (n=6), *P<0.05, **P<0.01 and ***P<0.001.*

3.3.6 Sema3E protective effect is mediated by pulmonary DC subsets

In order to address the mechanism of Sema3E inhibitory effect on HDM-induced airway inflammation, major pulmonary DC subsets were compared between mice exposed to HDM±Sema3E. *In vivo* Sema3E-Fc treatment significantly reduced the frequency of Th2-inducing $CD11b^+$ pulmonary DC compared to HDM-challenged mice (Fig. 3-8A). Furthermore, $CD103^+$ pulmonary DC sorted from Sema3E-Fc treated mice, when co-cultured with naïve T cells, induced higher proliferation of naïve T cells compared to those isolated from Fc alone treated HDM-exposed mice. Along with CFSE staining, CD44 production was also studied to evaluate the role of Sema3E in DC-mediated effector T cell generation. Our data revealed that Sema3E treatment decreases HDM-induced T cell proliferation and effector generation in co-culture with $CD11b^+$ pulmonary DC. Inversely, Sema3E treatment increased T cell proliferation and effector generation in $CD103^+$ pulmonary DC:T cell co-culture (Fig. 3-8B) (27). These events were accompanied with a significant increase of IFN-γ, decrease of IL-17A and no significant change in IL-5 levels. In contrast, $CD11b^+$ pulmonary DC from Sema3E-Fc treated mice decreased naïve T cell proliferation and both Th2 and Th17 responses *in vitro* (Fig. 3-8C).

We further examined Sema3E effects on HDM-induced cytokine pattern by intracellular cytokine staining of single cell suspensions prepared from lung draining MLN. *In vivo* treatment with Sema3E resulted in decreased IL-4 in MLN (Fig. 3-15A). IFN-γ production was unchanged in MLN obtained from Sema3E-Fc treated mice (Fig. 3-15B). Finally, expression of Sema3E high affinity receptor, plexinD1, was detected on the surface of both $CD11b^+$ and $CD103^+$ pulmonary DC subsets (Fig. 3-16) further supports DC responsiveness to Sema3E treatment. Therefore, our studies suggest that Sema3E protective effect on allergic asthma is mediated through modulating the function of $CD11b^+$ and $CD103^+$ pulmonary DC subsets which leads to balancing T cell responses.

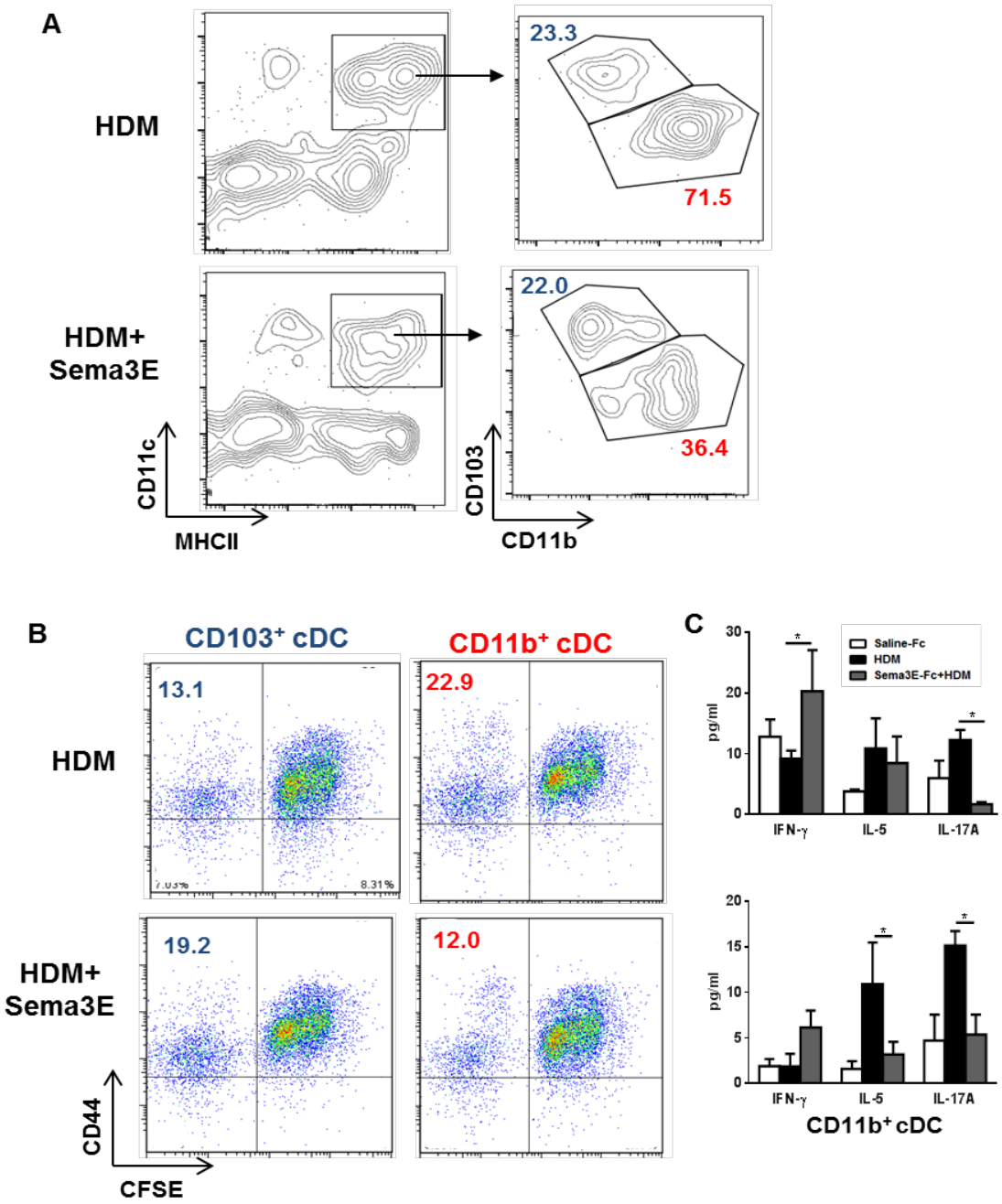

Figure 3-8 Sema3E treatment regulates pulmonary DC functions.
*Intranasal administration of Sema3E decreased the number of CD11b$^+$ pulmonary DC as determined by flow cytometry (A). Proliferation and effector generation of T cells, induced by CD11b$^+$ pulmonary DC in co-culture, were also decreased upon Sema3E treatment as shown by CFSE labeling and CD44 staining, respectively (B). Effect of Sema3E treatment on DC-mediated cytokine response in T cells was assessed by performing ELISA on supernatants from co-culture of naïve T cells and CD11b$^+$ or CD103$^+$ pulmonary DC subsets (C). The statistical significance was determined by performing one-way ANOVA and Bonferroni test (n = 4), *P<0.05.*

3.3.7 Supporting figures

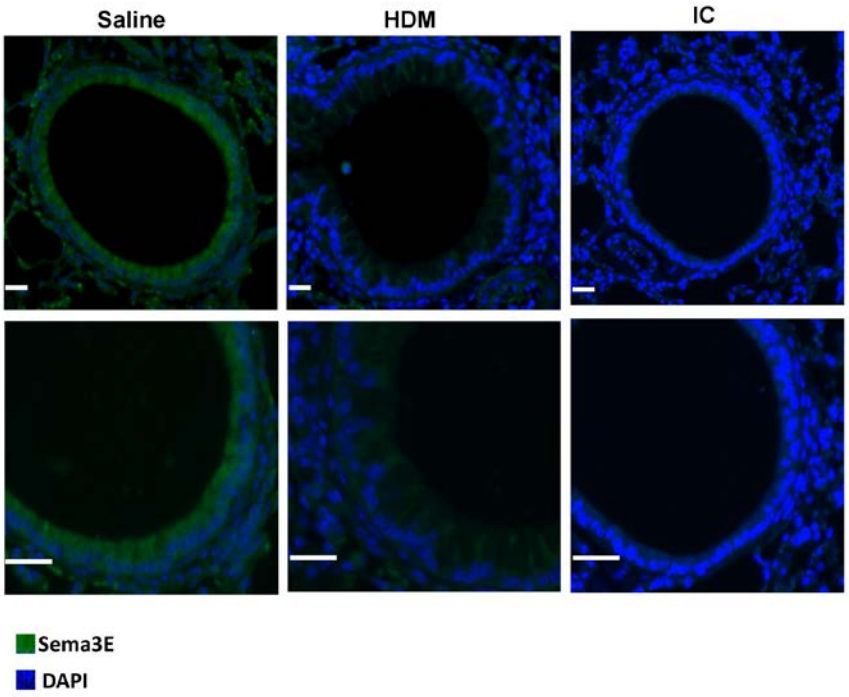

Figure 3-9 Sema3E expression is reduced in the airways of HDM-exposed mice.
Mouse Sema3E expression was investigated by performing immunofluorescence staining on airway sections obtained from mice exposed to either HDM or saline for two weeks through intranasal route as explained in Fig. 2A. Sema3E expression was visualized after incubation of tissue sections with FITC-labeled anti-Sema3E specific antibody and counter staining of nuclei with DAPI. IC: Isotype control, Scale bars: 50 μm. (n=4 mice per group).

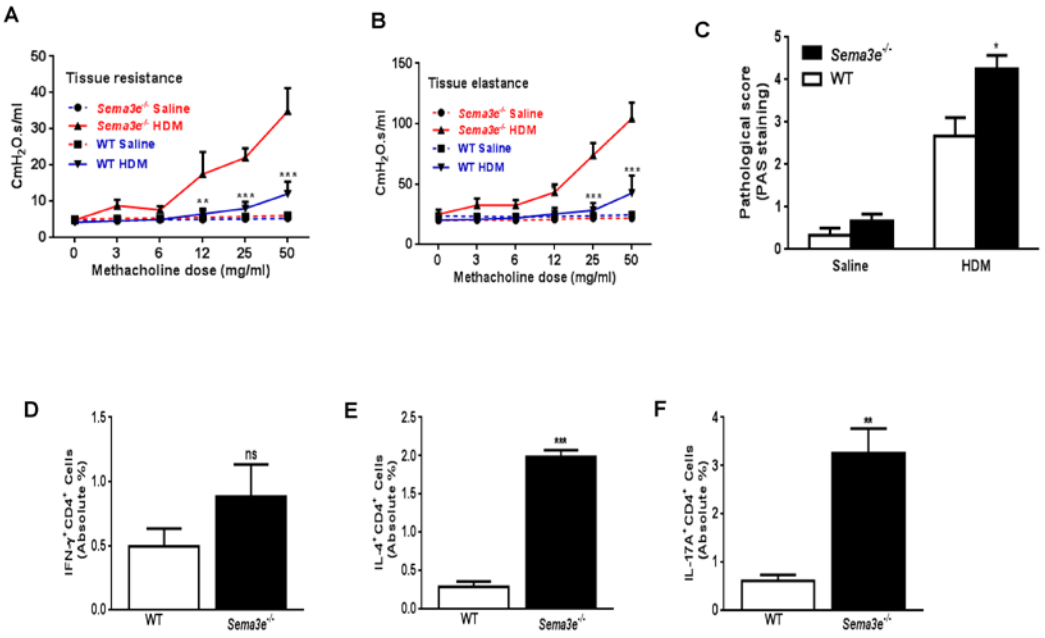

Figure 3-10 HDM-induced allergic responses are higher in Sema3e$^{-/-}$ compared to WT mice.
*Lung tissue resistance (A) and tissue elastance (B) were assessed in response to an increasing concentration of metacholine after 2 weeks of IN HDM or saline intranasal exposure. Lung tissue sections were stained with PAS as explained previously (Fig. 2F) and pathological scoring was performed in blind manner (C). IL-4 (D), IFN-γ (E) and IL-17A (F) production in CD4^{+} T cells was compared in single cell suspension of MLN obtained from HDM exposed Sema3e$^{-/-}$ and WT mice by flow cytometry. The statistical significance was determined by performing one-way ANOVA and Bonferroni test in A-C and Student t-test in D-F (n = 5-6), **P<0.01 and ***P<0.001.*

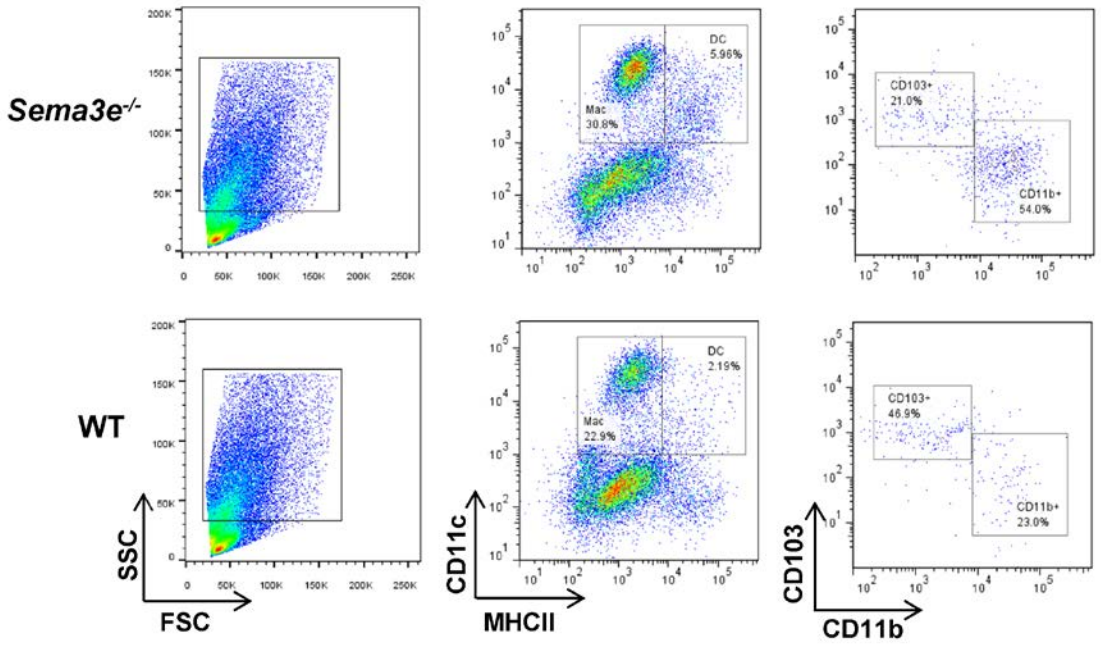

Figure 3-11 Comparison of pulmonary DC subsets between Sema3e$^{-/-}$ and WT mice at homeostatic conditions. *The frequency of CD11b^{+} and CD103^{+} pulmonary DC subsets was determined by performing FACS analysis on non-autofluorescent MHCII^{+}CD11c^{+} pre-gated cells undergone lung digestion as described in Methods. CD11b^{+} and CD103^{+} pulmonary DC subsets were respectively higher and lower in absence of Sema3E at the baseline. (n = 4 per group).*

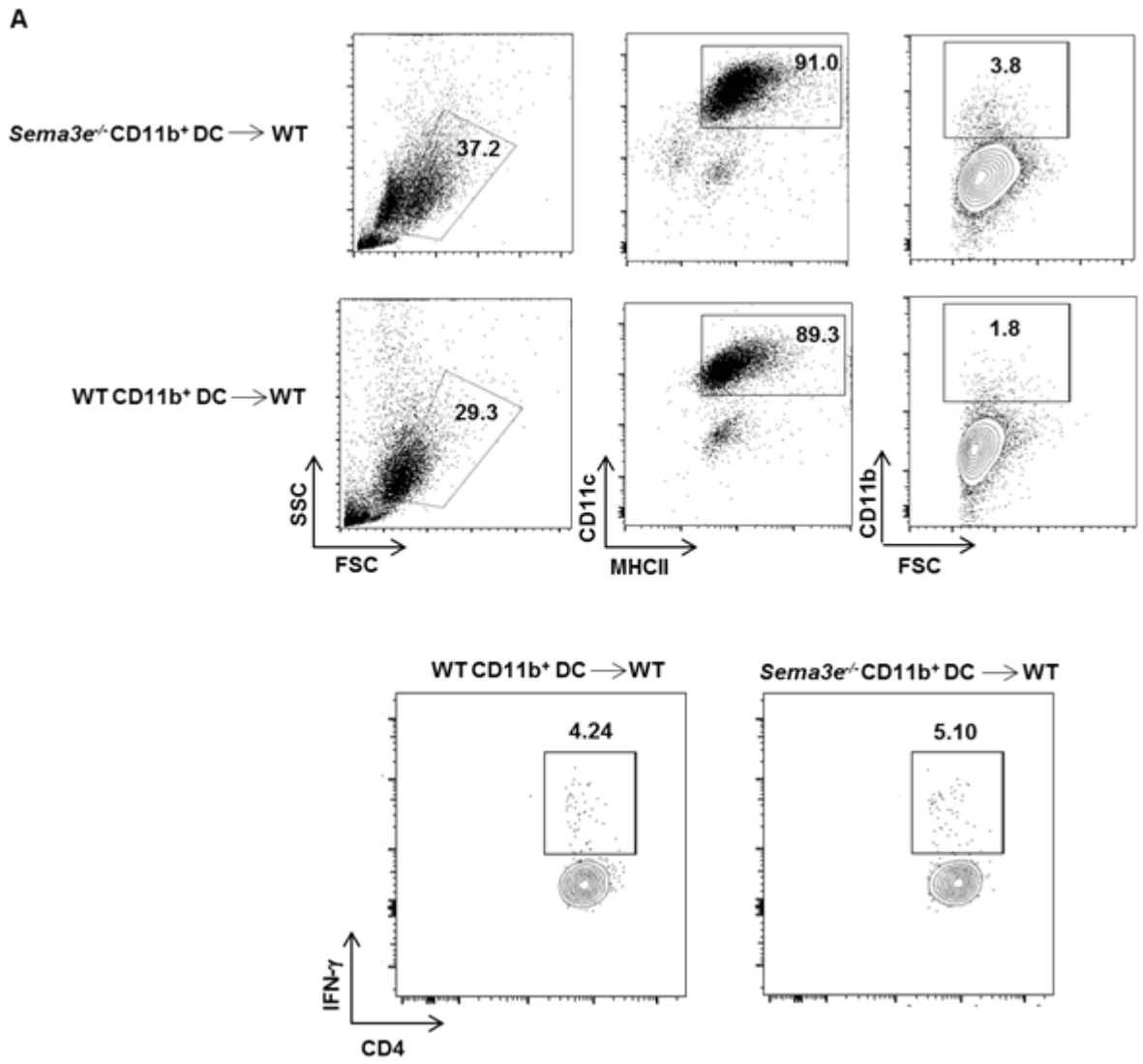

Figure 3-12 Absence of Sema3E increased CD11b$^+$ DC recruitment into the airways.
BALF (A) and MLN (B) cells were stained with antibodies against DC markers including MHCII, CD11c and CD11b after adoptive transfer and HDM challenge as explained in Fig. 5A. Upon HDM exposure, the frequency of CD11b$^+$ DCs was higher in the airways of those WT mice that received Sema3E deficient cells compared to their WT littermates. (n = 5 per group).

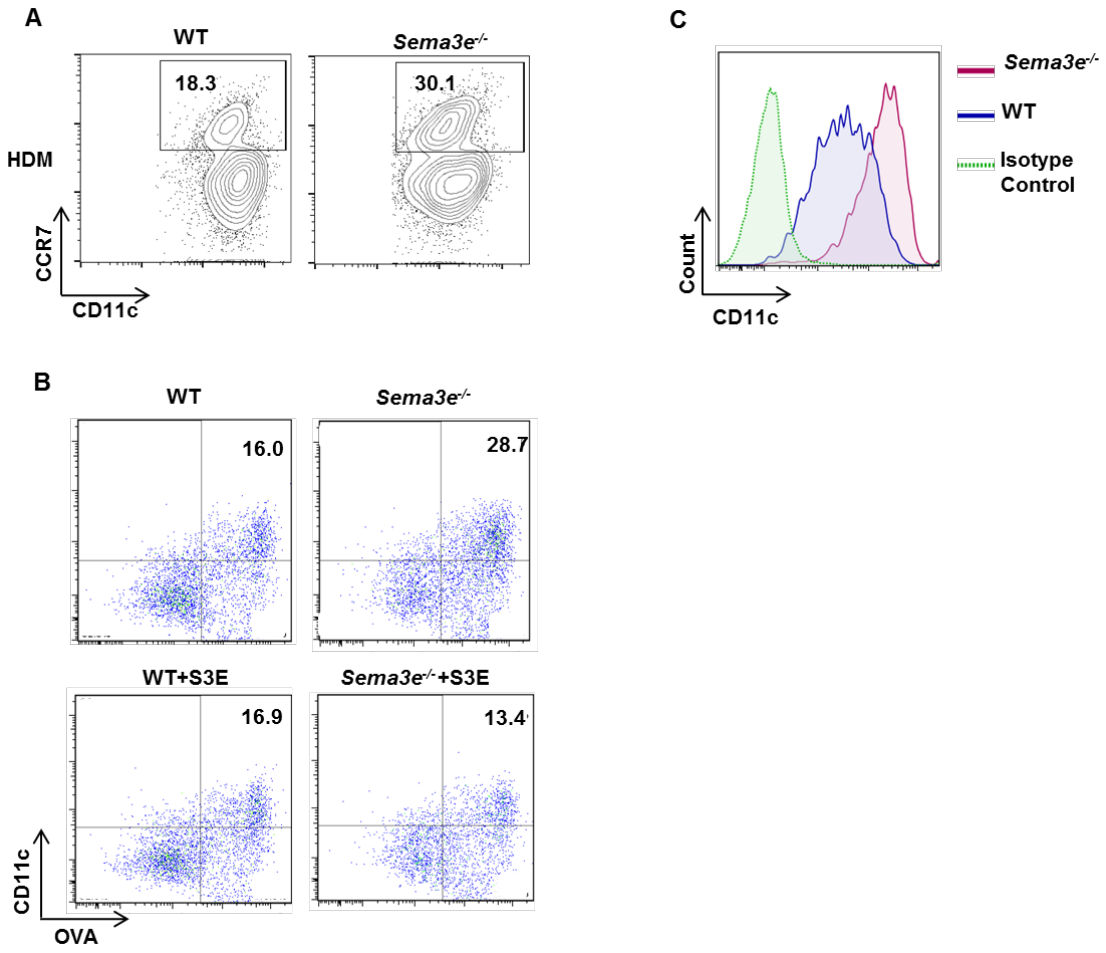

Figure 3-13 Sema3e deficient BMDC have enhanced CCR7 and CD11c expression as well as allergen uptake. *In vitro HDM stimulation induced higher levels of CCR7 surface expression in the Sema3e$^{-/-}$ than WT BMDC (A). Allergen uptake ability was higher in Sema3e$^{-/-}$ than WT BMDC which was replenished upon exogenous Sema3E treatment in Sema3e$^{-/-}$ but not WT BMDC in vitro; S3E: Sema3E (B). Stimulation of bone marrow progenitor cells isolated from Sema3e$^{-/-}$ mice with GM-CSF followed by LPS maturation induced higher surface expression of CD11c compared to WT mice (C). Data represent five independent experiments. (n = 5-7 per group), *P<0.05.*

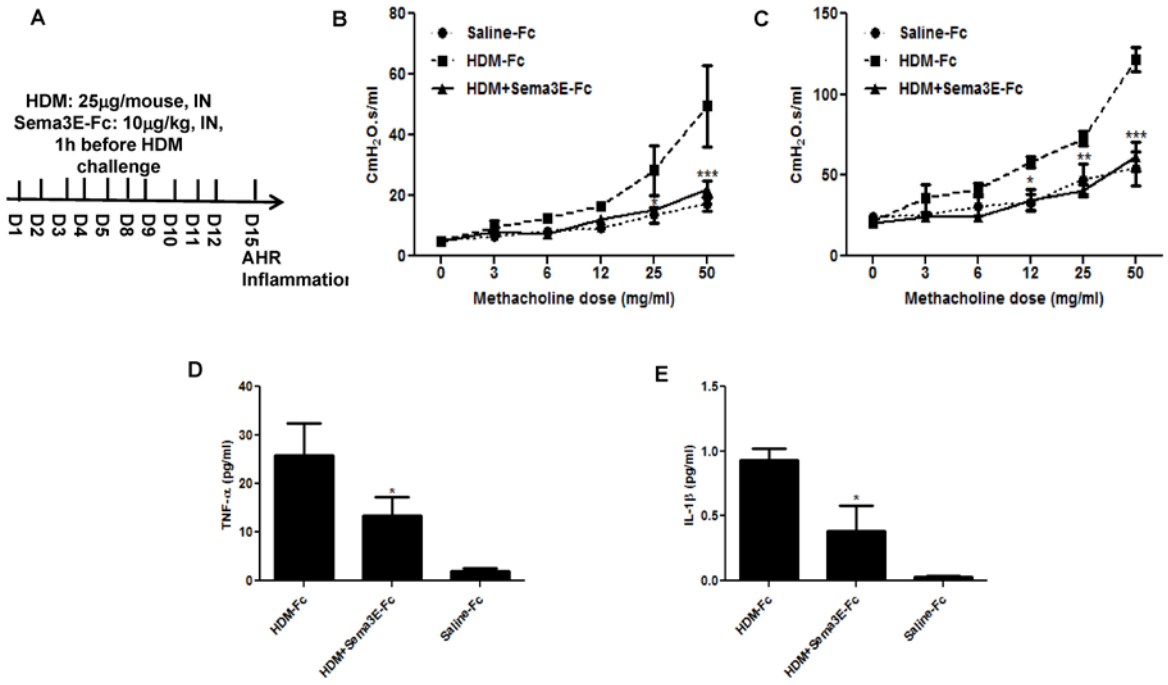

Figure 3-14 Sema3E treatment decreases AHR, BALF TNF and IL-1β expression.
*Exogenous recombinant Sema3E-Fc was administered i.n. 1h prior to each HDM-Fc exposure for 2 weeks (A). Tissue resistance (B) and tissue elastance (C) were measured after metacholine nebolization in HDM-exposed mice treated with either Sema3E-Fc or saline-Fc. The levels of TNF and IL-1β were measured by multiplex MesoScale system. The statistical significance was determined by performing one-way ANOVA and Bonferroni test. (n = 6-8), *P<0.05, **P<0.01 and ***P<0.001. Data are from two independent experiments.*

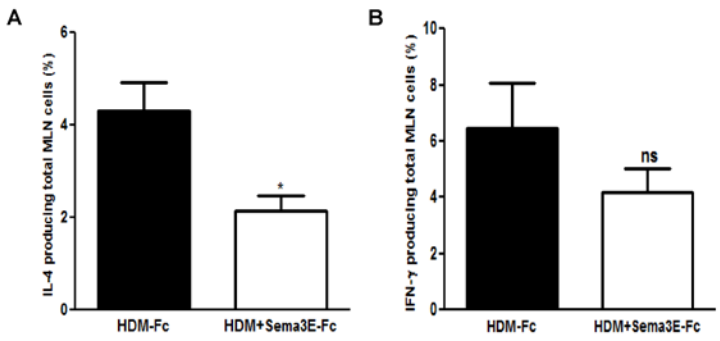

Figure 3-15 Sema3E treatment reduces IL-4 but not IFNγ production in MLN.
*Intracellular cytokine staining on MLN single cell suspensions was performed by flow cytometry. In vivo Sema3E treatment decreased HDM-induced IL-4 production (A); but did not affect IFN-γ production (B) in MLN cells. The statistical significance was determined by performing Student t-test (n = 4), *P<0.05 and **P<0.01.*

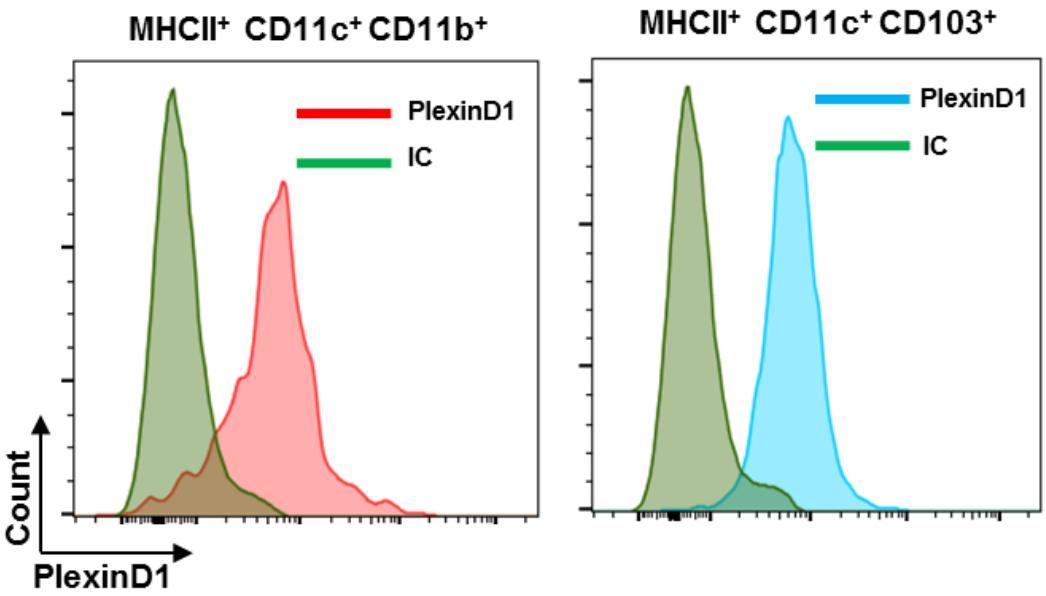

Figure 3-16 PlexinD1 is expressed by pulmonary DC subsets.
Surface expression of PlexinD1 was studied by performing FACS analysis. Enzymatically digested mouse lung single cells were stained by specific antibodies against DC markers. MHCII$^+$ pre-gated cells were subdivided into CD11c$^+$CD11b$^+$ or CD11c$^+$CD103$^+$ groups prior to PlexinD1$^+$ population. Isotype control antibody shows no cross reactivity. IC: Isotype control. (n = 4).

3.4 Discussion

Semaphorins are ubiquitously expressed and play diverse roles in many biological processes including immune regulation (9, 33). Recently, we have shown that Sema3E inhibits proliferation and migration of human ASM cells, as a characteristic feature of airway remodeling (16), but our understanding of the role of Sema3E in allergic asthma has been surprisingly limited.

In the present study, we demonstrate that Sema3E expression plays a critical role in controlling allergic asthma in a well described HDM model of the disease. Sema3e$^{-/-}$ mice showed increased AHR, goblet cell hyperplasia, collagen deposition, and Th2/Th17 inflammation upon HDM challenge in comparison to WT littermates. Mechanistically, *Sema3e*$^{-/-}$ mice present a higher frequency of CD11b+ pulmonary DC than WT controls that may account for enhanced induction of T cell proliferation and Th2 response after HDM exposure. Furthermore, adoptive transfer of CD11b+ pulmonary DC from *Sema3e*$^{-/-}$ mice into WT littermates increased HDM-induced airway inflammation. BMDC from *Sema3e*$^{-/-}$ mice had higher migration and allergen uptake capacity alongside with an enhanced CCR7 expression, Rac1 GTPase activity and F-actin polymerization. From a translational point of view, i.n. administration of Sema3E protected mice from HDM induced allergic asthma features through decreased CD11b+ and increased CD103+ pulmonary DCs, downregulation of Th2/Th17 and upregulation of Th1 response. Equally importantly, we have found that Sema3E is reduced in bronchial biopsies of allergic asthmatics particularly in the severe form which negatively correlates with disease outcome (FEV1) suggestive of a novel biomarker for asthma. Collectively, our findings suggest that Sema3E attenuates allergic asthma deficits, partially via

modulation of DC functions, and reveal an unrecognized role of this protein as a novel therapeutic in this disease.

The significance of Sema3E expression in the airways was addressed using HDM model of allergic asthma wherein *Sema3e*$^{-/-}$ mice displayed exaggerated airway inflammation compared to WT littermates. Increased levels of IL-5 (34, 35) and IL-17A (36) might be part of mechanism underlying higher HDM-induced eosinophil and neutrophil accumulation in the airways of *Sema3e*$^{-/-}$ mice, respectively. Furthermore, increased goblet cell hyperplasia and AHR in *Sema3e*$^{-/-}$ mice could be in part due to increased IL-13 secretion by Th2 cells (37, 38). Higher levels of both total and HDM-specific IgE and IgG1 in the sera of Sema3e-/- mice and reduction of these antibodies upon exogenous Sema3E treatment suggest a negative regulatory role for Sema3E in Ig class-switching as a cardinal feature of B cell development and humoral responses (39, 40) which should be further investigated. These findings could be explained by elevated levels of IL-4 in Sema3e$^{-/-}$ mice upon HDM exposure, and its down regulation by Sema3E treatment, which is considered as a key player for immunoglobulin class-switching to IgE (39, 40). In the future, it will be crucial to further study the mechanism underlying Sema3E mediated down regulation of Th2 and Th17 cytokines.

Sema3e$^{-/-}$ mice revealed higher DC recruitment into the airways associated with higher DC-mediated Th2 and Th17 responses. In particular, the frequency of pulmonary CD11b+ and CD103+ cDC was respectively higher and lower in Sema3e-/- than WT mice. This finding is in agreement with recent studies where CD11b+ DC were shown to be a major source of inducing Th2 response upon HDM exposure (27); whereas CD103+ DC were revealed to induce pulmonary tolerance to inhaled allergens (26) and also Th1 deviation (25). Along with more Th2/Th17-biased response in the airways of Sema3e-/- mice, we found that the higher frequency

of CD11b+ DC was also accompanied by privileged in vitro secretion of Th2/Th17 cytokines in co-culture with naïve T cells and also in our adoptive transfer model in vivo. Conversely, treatment of HDM-challenged mice with exogenous Sema3E reduced CD11b+ pulmonary DC subset and Th2/Th17 response while increased CD103+ tolerogenic pulmonary DC (26) and Th1 responses. This finding implies that Sema3E may play a role in regulating tolerogenic versus pathogenic response in the airway which needs further investigation.

AHR, the most characteristic clinical facet of asthma, is triggered mainly by chronic airway inflammation (41) which is initiated by activated pulmonary DC (42). Allergen challenge provokes substantial recruitment of DC into the airways (43) and specific elimination of cDC can prevent allergic airway inflammation (7). Therefore, regulated recruitment of DC subsets or modulation of their functions by Sema3E, as shown in this study, may be linked to decreased AHR observed in our model and consequently be considered as a novel treatment option. Importantly, the Sema3E receptor, plexinD1, has been previously demonstrated to be highly expressed on BMDC (44) as we also showed its surface expression on pulmonary DC subsets which suggests them as responder cells to Sema3E treatment. However, it is important to highlight that AHR can be driven directly by structural cells (45). As we previously reported an inhibitory effect of Sema3E on proliferation of human ASM cells (16), it is plausible that the absence of Sema3E in allergic asthma setting may drive ASM cell proliferation and or migration leading to exaggerated AHR *in vivo*. This adds another layer of complexity to Sema3E's role in allergic asthma.

The functional role of Sema3E in allergic asthma was further confirmed in the therapeutic model in which i.n. administration of Sema3E reduced the levels of Th2/Th17 mediators leading to improve AHR, inflammation and remodeling features. We have also demonstrated anti-

proliferative role of Sema3E in T cell proliferation upon co-culture with $CD11b^+$ DC. Our *in vitro* polarization experiments confirmed that naïve T cell differentiation to Th2 cells is higher in the presence of $CD11b^+$ pulmonary DC from HDM-exposed *Sema3e*$^{-/-}$ than WT mice. In addition, Sema3E treatment reduced T cell proliferation which may account for the decreased secretion of Th2 cytokines observed *in vivo*. The beneficial effect of Sema3E on modulation of DC functions was further supported by decrease in HDM-induced expression of co-stimulatory molecules on DC in BALF (unpublished data). Higher airway inflammation upon adoptive transfer of $CD11b^+$ pulmonary DC from HDM-sensitized *Sema3e*$^{-/-}$ compared to those of WT mice in WT recipients provides the proof of concept for the essential role of Sema3E in allergic asthma which could be mediated at least in part by regulation of this specific cDC subset. Increased recruitment of DC into the airways of *Sema3e*$^{-/-}$ mice, higher migration ability and CCR7 expression in DC from *Sema3e*$^{-/-}$ mice suggest that Sema3E acts as a guidance cue for DC, which is analogous to the original function of semaphorins in axon guidance (46, 47). This finding is in line with the previous studies on repulsive effect of Sema3E on human ASM cell (16) and atherosclerotic plaque macrophage (48) migration. However, Sema3E seems to act as a macrophage chemoattractent to adipose tissue leading to inflammation in obesity mode (49). In that study, the mechanism of Sema3E chemoattraction is mediated through co-ligation of plexinD1 with co-receptors neuropilin 1 (Nrp1) and vascular endothelial growth factor receptor 2 (VEGFR2) which revert the repulsive activity to chemoattraction (49). However, VEGFR2 surface expression was not detected in spleen, blood, bone marrow and pulmonary cDC (50). In our experiments neither Nrp1 nor VEGFR2 were expressed on the surface of pulmonary cDC from HDM-exposed mice (unpublished data) suggesting that the co-ligation is less likely to occur in our system.

It has been shown that small GTPases such as RhoA, cell division control protein 42 homolog (Cdc42) and Rac tightly regulate DC migration, antigen uptake and T cell priming (51-53). Furthermore, Sema3E inhibitory effect on macrophages (48) and ASM cells (16) is mediated through Rac1 signaling pathway. Tata et al. have recently reported Sema3E-induced hypercollapse of endothelial cells after silencing *Rac1* gene expression which was rescued upon treatment with constitutively active Rac1 (54). Elevated Rac1 GTPase activity in mature BMDC from *Sema3e*$^{-/-}$ mice may explain the mechanism underlying higher DC migration, allergen uptake and T cell priming in the absence of Sema3E. Increased F-actin content and higher surface expression of CCR7 in BMDC from *Sema3e*$^{-/-}$ mice may further provide potential mechanistic evidence behind their higher migration ability compared to those of WT littermates which was further supported by higher level of CCR7 induction in CD11b^{+} pulmonary DC upon HDM exposure *in vivo*. Moreover, Rac1 is an essential component downstream of CCR7 signaling and controlling ERK pathway activation in DC (55). Thus, higher GTPase activity could connect CCR7 over-expression to consequent increased F-actin polymerization in *Sema3e*$^{-/-}$ BMDC upon CCL21 stimulation. Because of the low frequency of highly pure CD11b^{+} pulmonary DC, performing Rac1 GTPase activity and F-actin polymerization assays were not feasible on these cells. It should be mentioned that there might be other potential signaling targets involved in regulation of Sema3E-mediated effects in DC which have not been investigated so far.

Sema3E immunoreactivity was clearly observed in bronchial biopsies of healthy donors and decreased gradually with disease progression particularly in the severe form. Similarly, we previously showed a reduced expression of plexinD1 on human ASM cells from allergic asthmatic subjects (16) suggesting a dysregulated expression of both ligand and receptor in

allergic asthma. We also found that the major cells displaying Sema3E immunoreactivity in bronchial biopsies include epithelial cells which may account for the presence of Sema3E protein in the BALF. Moreover, plexinD1 was shown to be expressed on DC (44) and ASM cells *in vitro* as well as smooth muscle bundle in bronchial biopsies of allergic asthmatics *in vivo* (16). We previously revealed that Sema3E modulates growth factor induced ASM cell proliferation and migration (16). Taken together, it is tempting to speculate that *in vivo* Sema3E released from epithelial cells modulates airway inflammation by regulating DC function; AHR and tissue remodeling through inhibiting the migration and proliferation of ASM cells (16). This speculation is in accordance with decreased Sema3E protein expression and enhanced inflammation, AHR and remodeling observed in the airways of *Sema3e*$^{-/-}$ mice compared to WT littermates upon HDM exposure.

In summary, we report that Sema3E plays an essential immunoregulatory role in experimental allergic asthma. The absence of Sema3E led to higher airway inflammation in allergic asthmatic mice, which was associated with exacerbated features of AHR and remodeling. Mechanistically, Sema3E deficiency resulted in uncontrollable recruitment and function of DC into the airways upon allergen encounter. Given that Sema3E expression was abrogated in clinical asthma and its administration reduced the pathological features of experimental disease, re-introducing Sema3E to the airways might prove efficacious in the treatment of clinical asthma. This previously unknown role for Sema3E in allergic asthma may lead to the develop therapeutic strategies in other diseases involving AHR and airway inflammation.

3.5 Materials and Methods

All methods are described in detail in *SI Materials and Methods*.

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3.7 Supporting Information

3.7.1 Materials and Methods

3.7.1.1 Human subjects and study approval

Bronchial biopsies were obtained from the tissue bank of the Respiratory Health Network of the FRQS (Québec, Canada). Asthmatic subjects fulfilling the American Thoracic Society criteria for asthma (3-1) were recruited from the Asthma Clinic at the Institut Universitaire de Pneumologie et de Cardiologie de Québec. Subject characteristics are summarized in Table 1. Mild asthmatic subjects were atopic with at least one positive response to common allergens on allergy skin prick tests. They were non-smokers and used only an inhaled β_2-agonist on demand and did not use inhaled or systemic corticosteroids (CS). In the month preceding the study, none of the subjects reported a respiratory infection or an increase in asthma symptoms. Severe asthmatic subjects have severe asthma according to the ATS refractory asthma definition (2). Subjects were on continuous treatment of high doses of inhaled CS (1000 mg of fluticasone proprionate or the equivalent in combination with a long-acting β_2-agonist). They were non-smokers and their asthma was stable with no exacerbation for the last 4 months. Healthy subjects were non-atopic and non-smokers with no history of asthma or atopy or any respiratory diseases. The study was approved by the Institut Universitaire de Cardiologie et de Pneumologie (Québec, Canada) ethics committee and all subjects signed an informed consent form. Human BALF was obtained from severe asthmatic and healthy subjects (Table 3-2) according to procedures approved by the Human Research Ethics Board of Department of Medicine, University of Chicago following the receipt of the subjects' written informed consent.

3.7.1.2 Animals

The 129 P2 *Sema3e*$^{-/-}$ mouse was a kind gift from Dr. F. Mann (Developmental Biology Institute of Marseille Luminy, Université de la Méditerranée, Marseille, France) which was described previously (3) and 129P2 WT littermates were used as control groups. Female 6-8 week old Balb/c mice purchased from the Central Animal Care Services (CACS) University of Manitoba were used in Sema3E treatment experiments. All experiments using mice were approved by Center for Animal Care Services, the University of Manitoba, Winnipeg (MB)-Canada. All of the mice were maintained at the CACS facility under specific pathogen-free conditions and used according to guidelines stipulated by the Canadian Council for Animal Care.

3.7.1.3 Immunohistochemistry

Formalin-fixed tissues were paraffin embedded, and 5-µm-thick sections were prepared, dewaxed in xylene, and rehydrated through graded concentrations of alcohol to water and then boiled for 10 min in sodium citrate buffer. Then, sections were incubated with blocking buffer for 1h at room temperature. Anti-human Sema3E ab R&D Systems (Minneapolis, MN) or isotype control IgG Jackson ImmunoResearch Laboratories (West Grove, Pa) were added, and sections were incubated overnight at 4°C. Slides were then washed twice with TBS followed by incubation for 1h at room temperature with biotin-conjugated secondary abs. Slides were washed extensively with TBS and incubated with streptavidin alkaline phosphatase for 30 min at room temperature afterwards. At the end, slides were developed using Fast Red (Sigma-Aldrich Canada Ltd., Oakville, ON) and counterstained with modified Mayer's hematoxylin (Fisher Scientific, Fair Lawn, NJ). Expression of Sema3E in mouse lung sections was studied using FITC-conjugated anti-mouse Sema3E ab and immunofluorescence microscopy. Mounted slides

were visualized by Axioskop 2 mot plus microscope using AxioVision software (Carl Zeiss, Inc, Thornwood, NY).

3.7.1.4 House dust mite exposure model

Lyophilized HDM protein extract was obtained from Greer Laboratories (Lenoir, NC) which was reconstituted in sterile saline as 2.5 mg/ml stock concentration before treatment. Working concentration (25 μg per mouse in 35 μL of saline) was freshly prepared and the acute model of the disease was established via i.n. administration under gaseous anaesthesia for 5 d per week during two consecutive weeks in *Sema3e*$^{-/-}$ and WT mice (4). In therapeutic model, recombinant mouse Sema3E-Fc (10 μg/kg in sterile PBS) was i.n. administered 1h prior to HDM exposure (25 μg per mouse) for two weeks in Balb/c mice. Control group received sterile saline-Fc at the same time points. All experiments were performed 48h after the last HDM exposure, unless otherwise indicated. Murine Sema3E-Fc recombinant protein was produced as fusion protein N-terminal to a functional mouse γ2c Fc domain. As we described previously, the complete cDNA was amplified from total RNA of mouse brain, ligated into pFUSE-mfc1 vector and electroporated into CHO cells. Finally, secreted Sema3E-Fc protein was purified from the conditioned media by protein A-affinity chromatography (5).

3.7.1.5 Airway responsiveness

48h after the last HDM challenge, mice were anesthetized and canulated via the trachea. Increasing concentrations of methacholine were administered intratracheally, and AHR parameters including airway resistance, tissue resistance and tissue elastance were measured by using FlexiVent small animal ventilator system (SCIREQ, Montreal, QC, Canada).

3.7.1.6 BALF cytology and flow cytometry

BALF was performed with 2 instillations of 1 ml of sterile saline containing 0.1 mM EDTA. Red blood cells were lysed using ACK buffer. Total BALF cells were spun down at 1500 rpm/4°C for 5 min and supernatants were separated and stored at -80°C to measure cytokines. Cytospins from BALF cells were prepared, stained and different cells types were characterized morphologically and counted by two individuals in a blind manner. After Fc blocking, flow cytometry was performed on the rest of BALF cells as described previously (6). Cells were stained with the following abs: MHCII-FITC (Clone: M5/114.15.2), CD11c-APC (Clone: N418), CD3 eFluor® 450 (Clone: 17A2) all from eBiosciences (San Diego, CA) and CCR3-PE (Clone: 83101) from R&D systems (Minneapolis, MN). Samples were acquired on a FACSCanto II (BD Biosciences, San Jose, CA) and major leukocyte populations in the BALF were analyzed by FlowJo software (Treestar, Costa Mesa, CA). DCs were identified as non-autofluorescent CD3-, MHCIIhi and CD11chi. Alveolar macrophages were distinguished from DCs as large autofluorescent MHCIIint CD11cint cells (6). Airway granulocytes were characterized as small (FSClo) highly granular (SSChi) MHCII- cells expressing CCR3 in eosinophils but not in neutrophils.

3.7.1.7 Lung histology

Dissected left lobes of mouse lungs were inflated, fixed in formalin, and embedded in paraffin. Then, sections were stained with H&E, periodic acid Schiff (PAS) and Sirius Red for assessing the presence of airway inflammation, mucus overproduction and collagen deposition, respectively.

3.7.1.8 Quantification of Sema3E, cytokines and immunoglobulins

ELISA of mouse BALF for IL-4, IL-5, IL-9, IL-10, IL-12, -13, IL-17A, and IFN-γ was performed according to manufacturer's instructions. ELISA plates were read with SpectraMax plate reader and analyzed with SoftMax Pro software (Molecular Devices). All cytokine ELISA kits were from BioLegend (San Diego, CA) except IL-13 which was from eBioscience (San Diego, CA). The level of TNF and IL-1β was measured by MesoScale Discovery system. (Meso Scale Diagnostics, LLC). Similar procedure was performed on supernatants obtained from co-culture of lung DCs and naïve T cells. Sema3E level was determined in human BALF from asthmatic and healthy donors by using an ELISA kit according to the manufacturer's instructions (R&D systems, Minneapolis, MN). Considering the low amount of secreted Sema3E in human BALF, samples were concentrated 10 fold by centrifugation prior to performing ELISA.

Serum was obtained from saline- and HDM-treated WT and *Sema3e*$^{-/-}$ mice 48h after the last allergen challenge. In a different set of experiments, serum was obtained from female Balb/c mice exposed intranasally with saline-Fc or HDM-Fc with or without Sema3E-Fc. Total and HDM-specific IgE, IgG1 and IgG2a levels were quantified using commercial ELISA kits according to manufacturer's instructions as we described previously (7). ELISA kits for measuring total and HDM-specific IgE, IgG1 and IgG2a levels in serum samples were purchased from Southern Biotech (Birmingham, Al).

3.7.1.9 Lung dendritic cell isolation

Major pulmonary conventional DC subsets were purified from Sema3e-/- or WT mice 3d after i.n. exposure with a single high dose of HDM (100 μg/mouse) (8). Briefly, lungs were removed from mice and enzymatically digested using 1 mg/ml collagenase IV (Worthington Biochemical

Corporation, Lakewood, NJ) and 0.5 mg/ml DNase from bovine pancreas in RPMI 1640 medium. After Fc blocking, DCs were stained and sorted by anti-mouse CD11c-APC (Clone: 418, eBioscience), MHCII eFluor® 450 (Clone: M5/114.15.2, eBioscience), CD11b-PE-Cy7 (Clone: M1/70, BioLegend), and CD103-PerCP-Cy5.5 (Clone: 2E7, BioLegend) antibodies using a BD FACSAria-III digital cell sorter. The purity of both CD11b+ and CD103+ subsets sorted from MHCII+ CD11c+ gated cells was higher than 95%. The same strategy was applied to sort pulmonary DC subsets from Balb/c mice to investigative mechanism of Sema3E therapeutic effect.

3.7.1.10 T cell-DC co-culture experiments

$CD4^+$ $CD25^-$ T cells were sorted from single cell suspension of naive mouse spleen using FACSAriaIII digital cell sorter (purity>%98). T cells were labeled with CFSE (eBioscience) and co-cultured with $CD103^+$ or $CD11b^+$ lung DCs (10 T cells: 1 DC). To study T cell proliferation, cells were first stained with combination of CD3 e-Fluor® 450 (Clone: 17A2), CD4-PE (Clone: RM4-5) from eBioscience and CD44-APC-Cy7 (Clone: IM7) from BD Pharmingen. Then, CFSE dilution was assessed 5d after co-culture. Cytokines were measured in supernatant harvested from co-cultures with commercially available ELISA kits as mentioned above.

3.7.1.11 In vivo adoptive transfer of pulmonary DCs

To evaluate the differential ability of $CD11b^+$ pulmonary DCs from *Sema3e*$^{-/-}$ and WT mice to drive Th2/Th17 cell polarization, mice were first challenged with a single high dose of i.n. HDM (100 μg) and then lungs were harvested and enzymatically digested after 2 days. $CD11b^+$ pulmonary DCs were sorted from the population pre-gated on $MHCII^+$ and $CD11c^+$ cells. 15,000

$CD11b^+$ pulmonary DCs from either *Sema3e*$^{-/-}$ or WT mice were administered into 129P2 WT littermates through the nasal route. 1 week later, mice were challenged with 10 μg of i.n. HDM for five consecutive days. After 2d, BALF cellularity, lung tissue inflammation, MLN DC frequency and cytokine production were evaluated as described above.

3.7.1.12 Flow cytometry analysis for intracellular cytokines

Briefly, MLN were collected from HDM-exposed mice and their single cell suspension was prepared by using a cell strainer. The cells were resuspended at a concentration of $4x10^6$ cells/ml in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5x10−5 M 2-ME, plated in 24-well tissue culture plates. Then, MLN cells were incubated with a freshly prepared cocktail containing 50 ng/ml PMA, 500 ng/ml ionomycin and 10 μg/ml brefeldin A (BFA), all from Sigma-Aldrich (Oakville, Ontario, Canada) for 4h at 37°C and 5% CO2. Extracellular staining was performed by using anti-mouse CD3 e-Fluor® 450 (Clone: 17A2) and CD4-FITC both from eBioscience. Fixed and surface-stained MLN cells were permeabilized with 0.1% saponin in flow cytometry buffer and then stained with specific fluorochrome-conjugated mAbs including anti-mouse IFN-γ-PE (Clone: XMG1.2), IL-4-APC (Clone: 11B11) and IL-17A-PE (Clone: eBio17B7) all from eBioscence. Samples were acquired on a FACSCanto II and analyzed using FlowJo software.

3.7.1.13 Differentiation of BMDCs

Naive 129P2 WT and *Sema3e*$^{-/-}$ mice were euthanized, their femurs and tibias were dissected, and the BM was flushed out by injecting complete DMEM through the marrow cavities and cells were cultured for 7d in DMEM containing mouse recombinant GM-CSF

(PeproTech, Rocky Hill, NJ) followed by LPS-induced DC maturation (9). The preparations were stained with CD11c antibody followed by FACS analysis to assess their purity (9). In some experiments, surface expression of CCR7 was studied on fully mature BMDC, as well as MLN DC, at the baseline or upon stimulation with CCL19 or HDM.

3.7.1.14 BMDC migration assay

BMDCs from *Sema3e*$^{-/-}$ or WT mice were seeded (15×10^4/well in 0.1 ml) in the upper compartment of a transwell chamber. 20ng/ml of mouse recombinant CCL21 (PeproTech, Rocky Hill, NJ) or PBS as vehicle was added to the lower compartment as a chemoattractant. After 90 minutes incubation at 37°C, migrated cells towards the lower chamber were counted and compared with control groups.

3.7.1.15 Antigen uptake assay

2×10^5 BMDC were incubated for 10 min at 37°C with 1 μg/ml of LPS or PBS. Cells were then stimulated by OVA-Alexa647 (0.2 mg/ml) with or without rmSema3E (100ng/ml) for 10 min. Uptake was terminated by washing the cells 3x with ice-cold PBS containing 2% FBS, Experiments were also conducted on ice to inhibit intracellular uptake as a control for surface binding of the Ag. Cells were resuspended in FACS buffer and stained with FITC-conjugated anti-CD11c (Biolegend). After staining for CD11c, AF647 fluorescence of the CD11c positive cells was measured using a flow cytometer.

3.7.1.16 Rac1 GTPase activity

BMDCs from *Sema3e*$^{-/-}$ and WT mice were first stimulated with CCL21 (20 ng/ml) for 0, 0.5, 1, 5, 15 and 30 min. Then, GTPase activity of Rac1 was measured in snap-frozen BMDC extracts by G-LISA activation assay according to the manufacturer's instructions (Cytoskeleton, Denver, CO).

3.7.1.17 Actin polymerization

BMDCs were stimulated with CCL21 (20 ng/ml) for 0, 0.5, 1, and 5min and immediately fixed with 4% paraformaldehyde. Then, cells were washed and permeabilized with %0.05 Triton-X100 in for 30 min before F-actin staining with Alexa488 conjugated Phalloidin (Life Technologies) for 30 min. Finally, the CD11c^{+} pre-gated BMDCs were analyzed by flow cytometry to detect F-actin content.

3.7.1.18 Statistics

GraphPad Prism 5.0 software was used for statistical analysis and values were presented as the mean±SEM of at least three independent experiments. Depends on the number of groups and treatments, data were analyzed by unpaired t test, one-way or two-way ANOVA, followed by the Bonferroni's multiple comparison post-hoc test. Differences were considered to be statistically significant at *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$. Statistical correlation between Sema3E expression and FEV1% was examined by performing Pearson's R test.

3.7.2 Supporting Tables

Table 3-1 Demographic and clinical characteristics of asthmatic and healthy individuals subjected to bronchoscopy

Asthma severity	Gender	Age	Smoking status	Allergy	Medication	Dose (mg/day)	PC20 (mg/ml)	FEV1 (%)
Mild #1	F	19	No	Yes	Terbutaline	prn	0.41	93
Mild #2	F	24	No	Yes	Salbutamol, Antihistamine	prn, prn	2.93	104
Mild #3	M	29	No	Yes	Salbutamol	prn	0.04	88
Mild #4	M	33	Ex	Yes	Salbutamol	200	1	88
Mild #5	F	28	Ex	Yes	Salbutamol	prn	0.71	116
Mild #6	F	19	No	Yes	Salbutamol	prn	3.70	111
Mild #7	F	55	No	Yes	Salbutamol	prn	0.26	71
Moderate #1	F	21	No	Yes	Salbutamol	prn	0.06	118
Moderate #2	F	20	Ex	Yes	Formoterol, Salbutamol, Fluticasone	24, Prn, 250	0.16	104
Moderate #3	F	21	No	Yes	Terbutaline	prn	0.16	83
Moderate #4	M	32	No	Yes	Terbutaline, Beclomethasone	Prn, 100	3.27	58
Moderate #5	F	26	No	Yes	Salbutamol	prn	2.08	100
Moderate #6	F	25	No	Yes	Fluticasone	1000	0.85	101
Moderate #7	F	25	No	Yes	Formoterol, Terbutaline, Fluticasone	prn, 500, 625	0.44	93
Moderate #8	F	55	No	Yes	Salmeterol-Fluticasone, Salbutamol	250, prn	0.06	64
Severe #1	M	20	Ex	Yes	Montelukast, Salbutamol, Salmétérol-Fluticasone	10, prn, 500, Two puffs per day	0.16	65
Severe #2	F	21	Ex	Yes	Terbutaline, Budesonide	prn, 1200	2.7	103
Severe #3	F	65	Ex	Yes	Salmeterol, Salbutamol, Beclomethasone	50, prn, 600	0.11	77
Severe #4	F	23	No	Yes	Fluticasone	1000	0.17	98
Severe #5	F	38	No	Yes	Salmeterol, Salbutamol, Fluticasone	100, prn, 1000	0.81	93
Severe #6	F	45	No	Yes	Salbutamol, Terbutaline, Budesonide	prn, prn, 1600	0.58	105
Severe #7	F	21	Ex	Yes	Terbutaline, Budesonide	prn, 1200	0.16	103

Severe #8	F	52	No	Yes	Salbutamol, Fluticasone	400, 1000	2.7	54
Healthy #1	M	23	Ex	No			50.88	91
Healthy #2	M	20	No	No			53.31	97
Healthy #3	F	31	No	No			169.6	107
Healthy #4	M	19	Ex	No			>128	106
Healthy #5	F	23	No	No			219.45	111
Healthy #6	M	27	No	No			>64	100
Healthy #7	F	27	No	No			>128	105
Healthy #8	M	31	No	No			>128	108

FEV1: Forced Expiratory Volume in 1 second
PC20: Provocative concentration of methacholine causing a 20% fall in FEV1
Ex: Ex-smoker,
prn: per need

Table 3-2 Demographic and clinical profile of asthmatic and healthy subjects used in human BALF studies

Subjects	Gender	FEV1 % pred	Steroid1	Steroid2	Steroid3	Other asthma medications	Asthma duration (yr)	Total IgE	Allergens (serum)
Severe #1	F	50	Advair 500/50	OCS		Singulair, Albuterol	24	1131	Cat, Dog, Aspergillus, Candida, *A. alternata*, *S. rostrata*, Ragweed
Severe #2	F	38	Advair 500/50	OCS	Budesonide	Combivent	57	368	Cat, Aspergillus, HDM (med+)
Severe #3	M	42	Advair 500/50			Singulair, Albuterol	46	66.9	Cat, Dog
Severe #4	F	25	Advair 250/50			Singulair, Spiriva, Albuterol	72	299	*S. herbarum*, *P. betae*, *E. purpurascens*, *T. rubrum*, *B. cinerea*, *S. rostrata*, *F. proliferatum*
Severe #5	F	50	Advair 250/50			Albuterol	7	881	Negative
Severe #6	F	59	Symbicort 160/4.5			Singulair, Albuterol	70	25.7	Negative
Severe #7	F	41	Fluticasone 220			Salmeterol 50, Maxair	14	87	Negative
Healthy# 1	M	95							
Healthy# 2	F	102							
Healthy# 3	F	96							
Healthy# 4	M	69							
Healthy# 5	F	92							
Healthy# 6	F	93							
Healthy# 7	M	72							

FEV1: Forced Expiratory Volume in 1 second
OCS: Prednisone (oral corticosteroids)

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4 CHAPTER 4:

NEUROIMMUNE SEMAPHORIN 3E MODULATES CHEMOKINE-INDUCED NEUTROPHIL MIGRATION AND CONTRIBUTES TO ALLERGIC AIRWAY NEUTROPHILIA

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This manuscript is has been finalized to be submitted to the journal of *Mucosal Immunology*.

H.M. designed, performed and analyzed all *in vivo* experiments in mice and also *in vitro* signaling experiments in human neutrophils, and prepared the manuscript. A.S. performed expression and migration assays on human neutrophils. S.N. performed the microfluidic migration experiments. L.S. contributed in isolation of human neutrophils and transwell migration assays. F.L. analyzed the microfluidic data and revised the manuscript. A.S.G. designed experiments, analyzed data, and revised the manuscript.

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4.1 Abstract

Neutrophil migration is an essential step in leukocyte trafficking during inflammatory responses. Semaphorins, originally discovered as axon guidance cues in neural development, have been shown to regulate cell migration beyond the nervous system. However, the potential contribution of semaphorins in regulation of neutrophil migration has not been completely understood. This study examines the possible role of a secreted chemorepellent, Semaphorin 3E (Sema3E), in neutrophil migration. We found that human neutrophils constitutively express Sema3E high affinity receptor, PlexinD1. Additionally, Sema3E displayed a potent ability to inhibit CXCL8/IL-8-induced neutrophil migration as determined by an *in vitro* transwell system and microfluidic device coupled to real-time microscopy. Anti-migratory effect of Sema3E on human neutrophil migration was associated with suppression of chemokine-mediated Rac1 GTPase activity and actin polymerization in a timely manner. We further addressed the role of Sema3E in regulation of neutrophil migration by using *in vivo* models in which both allergen and LPS exposure induced higher neutrophil recruitment into the lung of *Sema3e*$^{-/-}$ mice compared to WT controls which was replenished upon Sema3E treatment. Our data suggest that Sema3E could be considered as an essential regulatory mediator involved in modulation of neutrophil migration throughout the course of neutrophilic inflammation.

Key words: House dust mite, Migration, Neutrophil, PlexinD1, Semaphorin 3E

4.2 Introduction

Neutrophils are the most abundant circulating leukocyte in humans and the first cells to be recruited towards the site of inflammation. These cells are extremely important in several inflammatory and infectious diseases and play a critical role in maintenance of homeostasis (1). Since imbalanced neutrophil recruitment and also its deregulated activity contribute to human disease, neutrophil functions should be tightly regulated (2). Neutrophil chemotaxis towards inflammatory sites requires spatiotemporal control of signaling events triggered by a chemokine milieu that promote neutrophil migration (3). However, the precise regulatory mechanism underlying chemokine-mediated neutrophil migration has not been fully understood.

Emerging evidence suggest that axon guidance proteins could be involved in regulation of neutrophil migration (4, 5). Guidance cues have been primarily identified as essential mediators of development which direct the cells to reach their targets via exerting attracting or repulsive signals (6-8). Semaphorins are a versatile family of axon guidance molecules ubiquitously expressed in diverse tissues (9), though their post-developmental functions have remained to be investigated.

Class 3 semaphorins comprise all vertebrate secreted semaphorins which play a key role in cell migration on a context-dependent manner (10-12). Apart from their classical chemorepulsive function in axon guidance, class 3 semaphorins signaling has been shown to be indispensable for the immune regulation (13). We have previously reported that human airway smooth muscle (ASM) cells express semaphorin 3E (Sema3E) high affinity receptor, PlexinD1, and their migration is mitigated upon ligation with Sema3E as a crucial component of airway remodeling (14). In addition, other studies controversially suggest either repulsive or attractive role of Sema3E in macrophages in atherosclerotic plaque (15) or obesity (16) models, respectively.

However, whether Sema3E regulates neutrophil migration during homeostatic and inflammatory conditions is unknown. Sema3E-PlexinD1 signaling is mediated primarily by two GTPase-activating proteins (GAPs) located on the intracellular domain of PlexinD1. In response to Sema3E treatment, they interact with small Rho GTPases such as Ras-related C3 botulinum toxin substrate 1 (Rac1) which critically regulates actin cytoskeleton during the process of directional cell migration (17). In addition, Rac1 has been previously shown as a key signaling component involved in regulation of neutrophil chemotaxis (3).

The current study aimed to determine the role of Sema3E in neutrophil migration by using *in vitro* and *in vivo* approaches. Our results indicate that Sema3E treatment inhibits chemokine-induced human neutrophil migration via suppression of Rac1 GTPase activity and F-actin polymerization. We observed that Sema3E is able to suppress chemotactic movements of primary human neutrophils induced by CXCL8/IL-8 which was further confirmed in a standard transwell migration assay. Genetic ablation of Sema3E led to enhanced airway neutrophilic infiltration in mice which was sustained upon house dust mite (HDM) allergen challenge encounter. Furthermore, *in vivo* Sema3E treatment reduced HDM-induced airway neutrophilia. Taken together, these data suggest that Sema3E could be considered as a novel mediator involved in modulation of neutrophil extravasations with a potential therapeutic implication in neutrophilic inflammatory diseases.

4.3 Results

4.3.1 Human neutrophils constitutively express PlexinD1

The expression of PlexinD1 has been identified in a wide variety of cells including T lymphocytes (18), endothelial (19, 20) and airway smooth muscle (ASM) (14) cells. However, there is no report addressing the expression of PlexinD1 on human neutrophils. Here, in order to verify the functional response of neutrophils to Sema3E, we first investigated the expression of PlexinD1, the high affinity receptor of Sema3E, on freshly isolated highly pure peripheral blood human neutrophils (Fig.4-8). As shown in Fig. 4-1A, human neutrophils from four healthy donors expressed PlexinD1 at the mRNA level as compared to peripheral blood mononuclear cells (PBMC) used as a positive control.

Surface expression of PlexinD1 was then studied by FACS on peripheral blood human neutrophils and also in PBMC as a positive control using goat anti-human PlexinD1 PE-conjugated antibody. As shown in Fig. 1B, PlexinD1 is highly expressed on human neutrophils from healthy donors (n = 12) similar to PBMC (Fig.4-1C). Then, we sought to confirm PlexinD1 expression in primary human neutrophils utilizing immunocytochemistry. PlexinD1 immunoreactivity was detected in 99% of human neutrophils and no cross-reactivity was observed in cells stained with isotype control Ab (Fig. 4-1D). Taken together, this data indicate that human neutrophils express PlexinD1 receptor and suggest that these cells are a potential target of Sema3E.

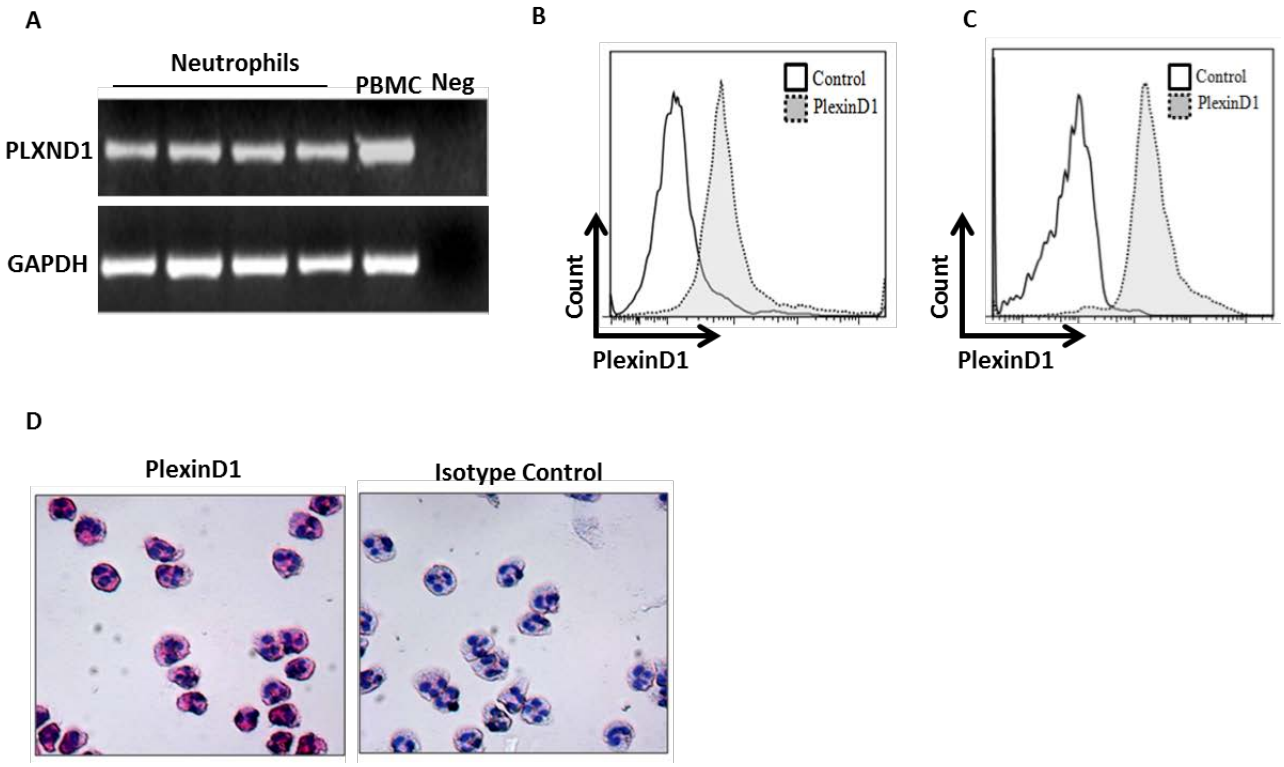

Figure 4-1 Human neutrophils constitutively express PlexinD1.
RT-PCR analysis was performed using specific primers of PLXND1 and PCR products were run on 2% agarose gel. GAPDH is served as a housekeeping gene, and N is referring to the negative control (A). FACS analysis was performed to examine the surface expression of PlexinD1 protein on human neutrophils by using either specific antibody or isotype-matched goat IgG as negative control (B). Representative experiment of 12 human samples is shown. The MFI was calculated by subtracting the isotype control. Utilizing ICC on neutrophil cytospins, PlexinD1 protein expression was further confirmed and our isotype control stained with goat IgG revealed no immunoreactivity.

4.3.2 Sema3E reduces chemokine-induced directional migration of human neutrophils

It was previously reported that Sema3E inhibits chemokine-mediated migration of double positive ($CD4^+$ $CD8^+$) T cells from the cortex into the medulla of thymus during maturation (18). Whether Sema3E may have an effect on chemokine-induced neutrophil migration is not yet understood. Considering the importance of CXCL-8 (IL-8) in neutrophil migration during inflammatory processes and unknown role of Sema3E in these events, we investigated whether Sema3E influences CXCL-8-induced migration of human neutrophils. Utilizing a microfluidic device that is optimized mainly to test cell chemotaxis in response to chemokines gradient (21-23), we found that Sema3E reduced CXCL-8-mediated human neutrophil migration. As shown in Fig. 4-2A and Supplemental video 1, neutrophils exhibited chemotactic activity toward IL-8 gradient alone. In a Sema3E gradient, the percentage of migrated cells is reduced; interestingly, among the migrated cells we tracked, more cells moved to the opposite direction of Sema3E gradient (Fig. 4-2B and Supplemental video 2). However, this movement was not significantly different than the baseline migration (Fig. 4-2C and 4-2F and Supplemental video3).

We then assessed neutrophil chemotaxis in response to co-existing gradients of Sema3E and IL-8 using microfluidic device. Competing gradients were created in which 10 ng/ml of IL-8 was configured in one side and 100 ng/ml of Sema3E along in the opposite directions (Fig.4-2D). Neutrophil migration toward IL-8 gradient was significantly reduced in the presence of Sema3E gradient (Fig. 4-2D and Supplemental video 4) compared to IL-8 gradient alone (Fig 4-2A). In addition, when we configured IL-8 and Sema3E gradients competing in the same side of microfluidic channel, neutrophils randomly moved within the microchannel and negatively responded to IL-8/Sema3E gradients compared to IL-8 alone experiment (Fig. 4-2E and

Supplemental video 5). In both configurations, neutrophil motility was significantly inhibited as shown by the very low percentage of migrated cells and the much less migration path of the migrated cells comparing to it in the IL-8 gradient control (Fig. 4-2C-D and Supplemental video 3-4). More interestingly, Sema3E significantly decreased IL-8-induced-neutrophil chemotactic index (CI) (Fig. 4-2F) which was associated with random cell migration in different directions (Supplemental videos 4 and 5). Analysis of neutrophil speed showed a maximal response with IL-8 gradient reflecting cell migration (Fig. 4-2G). However, in Sema3E and IL-8 competing or the same side gradients, speed, along with CI, was significantly decreased compared to IL-8 alone. Altogether, these experiments revealed that neutrophils undergo random migration in response to competing gradients of IL-8 and Sema3E and suggest that Sema3E has a potential inhibitory role in IL-8-induced directional neutrophil migration**.**

Using a traditional standard transwell migration assay, we further confirmed the inhibitory effect of Sema3E on human neutrophil chemotaxis. In fact, administration of Sema3E alone into upper chamber of the transwell insert slightly reduced basal neutrophil migration which was not significantly different compared to the negative control (Fig. 4-3) and as observed with the microfluidic device experiment (Fig. 4-2B and 4-2F). Recombinant human Sema3E was able to significantly inhibit IL-8-induced neutrophil migration in all doses tested (1, 10, 50, and 100 ng/ml). ($p < 0.01$, Fig. 4-3A). We then investigated neutrophils migration when Sema3E was added to the lower chamber mixed with 10 ng/ml of IL-8 (Fig. 4-3B). Sema3E had a dose-dependent inhibitory effect on human neutrophil migration when introduced in the lower chamber with IL-8 (n=5, Fig. 4-3B) suggesting a negative correlation between the number of migrated cells and Sema3E concentration. Furthermore, Sema3E alone did not significantly affect baseline neutrophil migration (Fig. 4-3B) in this experiment as well. Altogether, Sema3E impairs IL-8-induced human peripheral blood neutrophils migration without affecting their homeostatic migration.

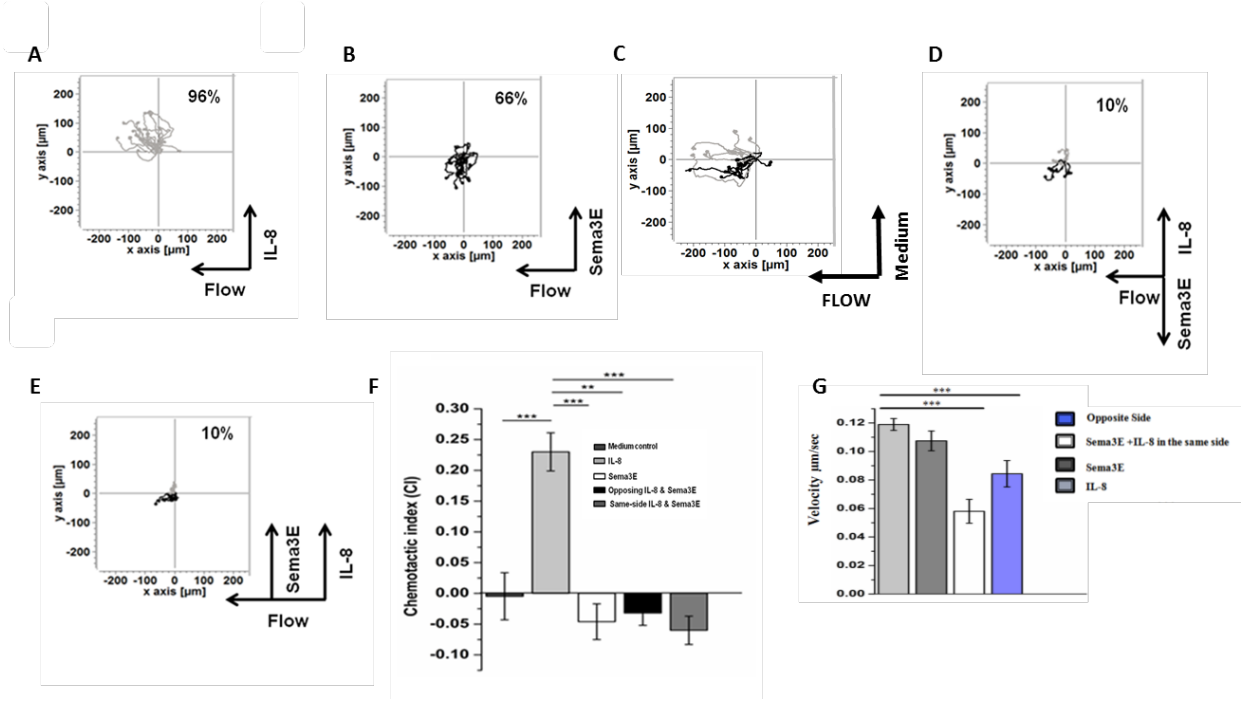

Figure 4-2 Sema3E negatively regulates migration and chemotaxis of human neutrophils in a microfluidic device.

*(A) Cell tracks in an IL-8 gradient; (B) Cell tracks in a Sema3E gradient; (C) Cell tracks in a medium; (D) Cell tracks in co-existing gradients of IL-8 and Sema3E along the opposite direction (i.e. opposing gradients); (E) Cell tracks in co-existing gradients of IL-8 and Sema3E along the same direction (same-side gradients); Black tracks are cells moving downward; grey tracks are cells moving upward; The direction of gradients and flow are indicated by arrows for each condition. The percentage of significantly moving cells for each condition is indicated; (F) Comparison of Chemotactic Index (CI) for the experiments in A-D; The error bar represents Standard Error of the Mean (S.E.M). Positive CI indicates upward cell migration; Negative CI indicates downward cell migration. The significance level of statistical comparison for each specific paired conditions (indicated by the line) from t-test is indicated (** for p<0.01; *** for p<0.001). The figure used data from one representative experiment for each condition. (G) Cell speed of neutrophils in the presence of IL-8 alone control, Sema3E alone or their combination was determined by calculating the distance travelled toward the gradient in unit time (per second) using chemotaxis and migration tools. (A and B) Data are shown as mean ± SEM of one sample representative of three independent experiments. The statistical significance was determined by performing one-way ANOVA and Bonferroni test. ***p < 0.001 (two-sample t-test).*

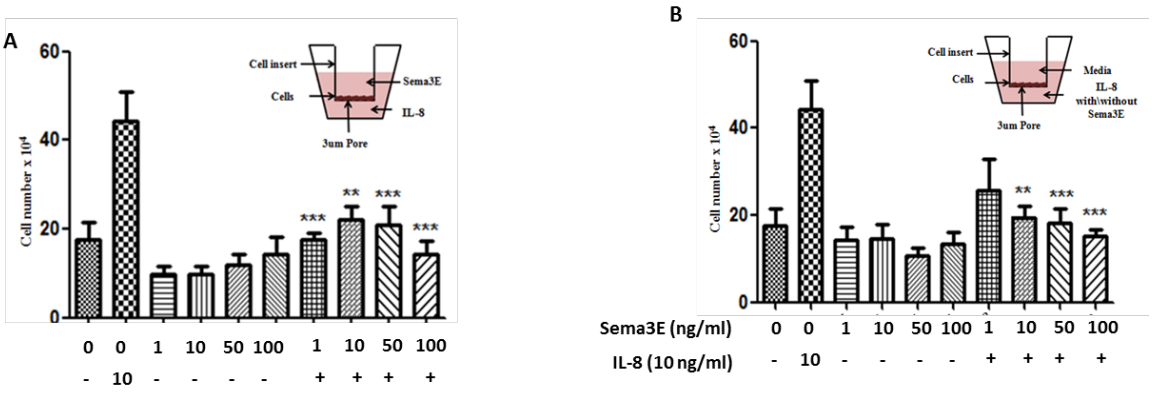

Figure 4-3 Sema3E inhibits IL-8 induced human neutrophils migration in a transwell system.
In a transwell system, human recombinant Sema3E with or without CXCL8/IL-8 was added to the top insert with human neutrophils. After 1h incubation, migrated cells to the bottom chamber were collected and subjected to cell count. (A). Isolated fresh human neutrophils were added to the insert, whereas Sema3E was added to the bottom well with or without CXCL8/IL-8 (B). After 1h incubation, migrated cells were collected and counted using haemocytometer. The data represents five independent experiments repeated in duplicate. The statistical significance was determined by performing one-way ANOVA and Bonferroni test.

4.3.3 Sema3E treatment decreases IL-8-mediated Rac1 GTPase activity and F-Actin polymerization

It has been demonstrated that IL-8 promotes neutrophil migration via activation of a small GTPase called Rac1 (24-26). We and others have previously reported that repulsive effect of Sema3E on cell migration is partly mediated by suppression of Rac1 GTPase activity as an early signaling component affected upon Sema3E treatment (14, 15). Therefore, the potential role of Sema3E in abrogation of IL-8-induced Rac1 GTPase activity was assessed by GLISA in primary human neutrophils. As shown in Fig. 4-4A, Sema3E significantly decreased Rac1 GTPase activity promoted by IL-8 without any significant effect at the baseline.

Induction of F-actin polymerization has been shown as an essential cytoskeletal target to promote neutrophil migration (24, 27, 28). However, the role of Sema3E in regulation of F-actin dynamics has not been addressed in neutrophils, so far. We stimulated human peripheral blood neutrophils with Sema3E (100 ng/ml) in the presence or absence of CXCL-8 (100 ng/ml) and then stained them with a fluorescent conjugated phalloidin to specifically quantify F-actin polymerization. As depicted in Fig. 4-4B, stimulation of human neutrophils with CXCL-8 alone robustly increased F-actin polymerization which was significantly reduced upon co-stimulation with Sema3E and CXCL-8 and. F-actin content of human neutrophils after treatment with Sema3E alone was comparable to un-stimulated neutrophils (Fig. 4-4B); along with its non-significant effect on neutrophil migration and Rac1 GTPase activity. Therefore, our data suggest that repulsive effect of Sema3E on CXCL8-induced migration of human neutrophils is associated with reduction of Rac1 GTPase activity and depolymerization of actin filaments.

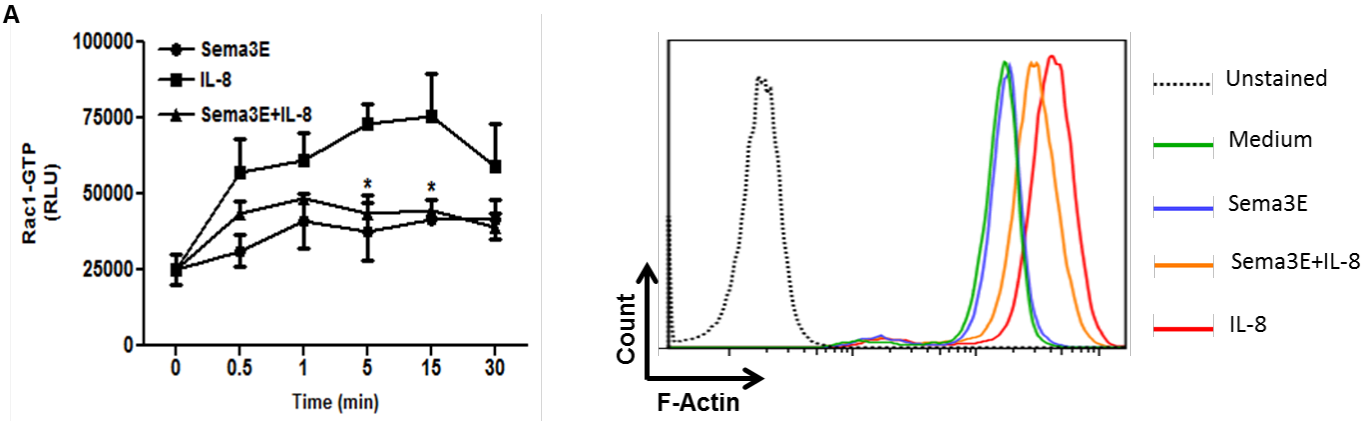

Figure 4-4 ***Sema3E inhibits IL-8-induced Rac1 GTPase activity and actin polymerization in human neutrophils.*** *Rac1 GTPase activity was measured in snap-frozen human neutrophils cell lysates harvested from either unstimulated or Sema3E±IL-8 stimulated cells at the indicated time points by performing G-LISA. Constitutively active Rac1 was used as a positive control, P, and the negative control, N, refers to blank well in all experiments (A). Phalloidin staining was performed to detect F-actin content of human neutrophils upon Sema3E±IL-8 treatment (B). * $p < .05$ and *** $p < .001$. The data represent 4 independent experiments.*

4.3.4 Pulmonary neutrophilic inflammation is exacerbated the absence of Sema3E *in vivo*

Neutrophil influx into the lung is a key feature of airway inflammatory diseases in which sustained accumulation of these cells, mainly due to dysregulated migration, leads to perpetuate pathological manifestations (29). Therefore, understanding mechanisms regulating homeostatic neutrophil migration which are impaired during inflammatory processes are of great importance. In order to evaluate the functional relevance of our findings *in vivo*, we investigated the potential role of Sema3E in neutrophilic airway inflammation in homeostatic conditions and in mouse model of allergic asthma. As a conventional gating strategy, $CD11b^{bright}$ $Gr\text{-}1^{bright}$ subset from live singlet FSC^{lo} SSC^{hi} granulocyte population was considered as neutrophils in the following mouse flow cytometry experiments (Fig. 4-9) (30). First, we observed that genetic deletion of Sema3E led to a higher neutrophilia in the lungs of naïve mice (Fig. 4-5) which was further confirmed in the airways by performing the same experiment on mouse bronchoalveloar lavage fluid (BALF) (Fig. 4-10A). We also investigated whether mouse genotype has any effect on these events and observed that in both 129P2 B6 (Fig. 4-10A) and Balb/c (Fig. 4-10B) strains the frequency of airway neutrophil was higher in *Sema3e*$^{-/-}$ mice than those of corresponding WT littermates (n = 4 per group).

We induced allergic airway neutrophilia by sensitization of *Sema3e*$^{-/-}$ and WT mice with a clinically relevant allergen, house dust mite (HDM), via intranasal route as depicted in Fig. 4-6A. Along with exacerbation of other asthma deficits such as IgE synthesis and mucus hypersecretion in *Sema3e*$^{-/-}$ mice (Chapter 3), HDM exposure induced higher recruitment of $CD11b^{bright}$ $Gr1^{bright}$ neutrophils into the airways of *Sema3e*$^{-/-}$ than those of WT controls (Fig. 4-6B, n=4). In addition, our FACS analysis on blood samples from the same mice revealed higher

frequency of CD11b^{bright} Gr1^{bright} circulating neutrophils in *Sema3e*$^{-/-}$ mice compared to WT littermates two weeks after HDM exposure (Fig. 4-11). Enhanced pulmonary neutrophilia induced by HDM was also evident in *Sema3e*$^{-/-}$ mice with Balb/c background (data not shown). Taken together, it could be implied that Sema3E deficiency is associated with a hyper-neutrophilic phenotype which may lead to higher susceptibility during the course of inflammation.

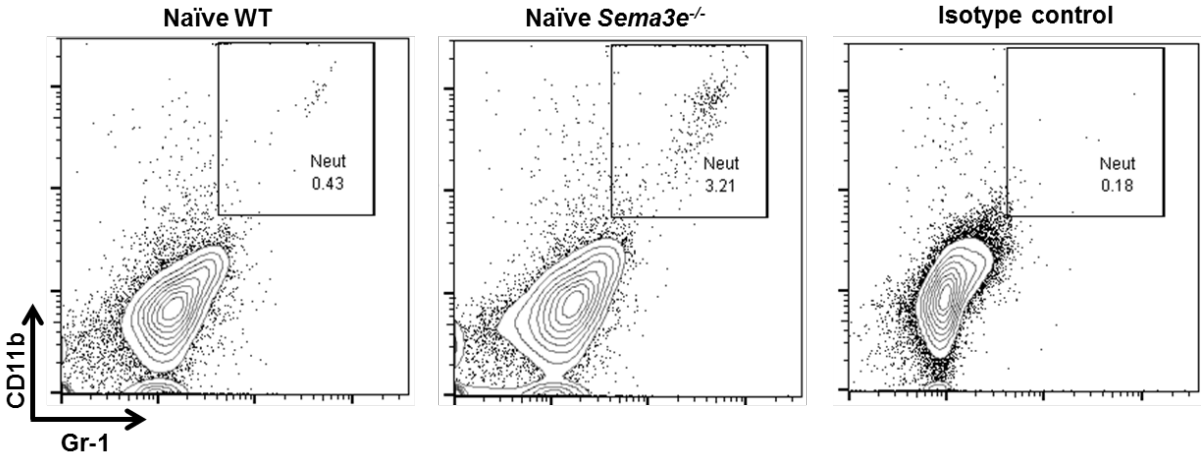

Figure 4-5 Pulmonary neutrophil accumulation is enhanced in Sema3e$^{-/-}$ mice at homeostatic conditions.
BALF samples from Sema3e$^{-/-}$ and WT mice were processed and stained with specific antibodies to detect neutrophil surface markers. Flow cytometry determined the frequency of CD11b ^{bright} Gr1^{bright} neutrophil population in the airways of those mice at the baseline. Staining with isotype control antibody revealed no immunoreactiviry. (N = 4 per group).

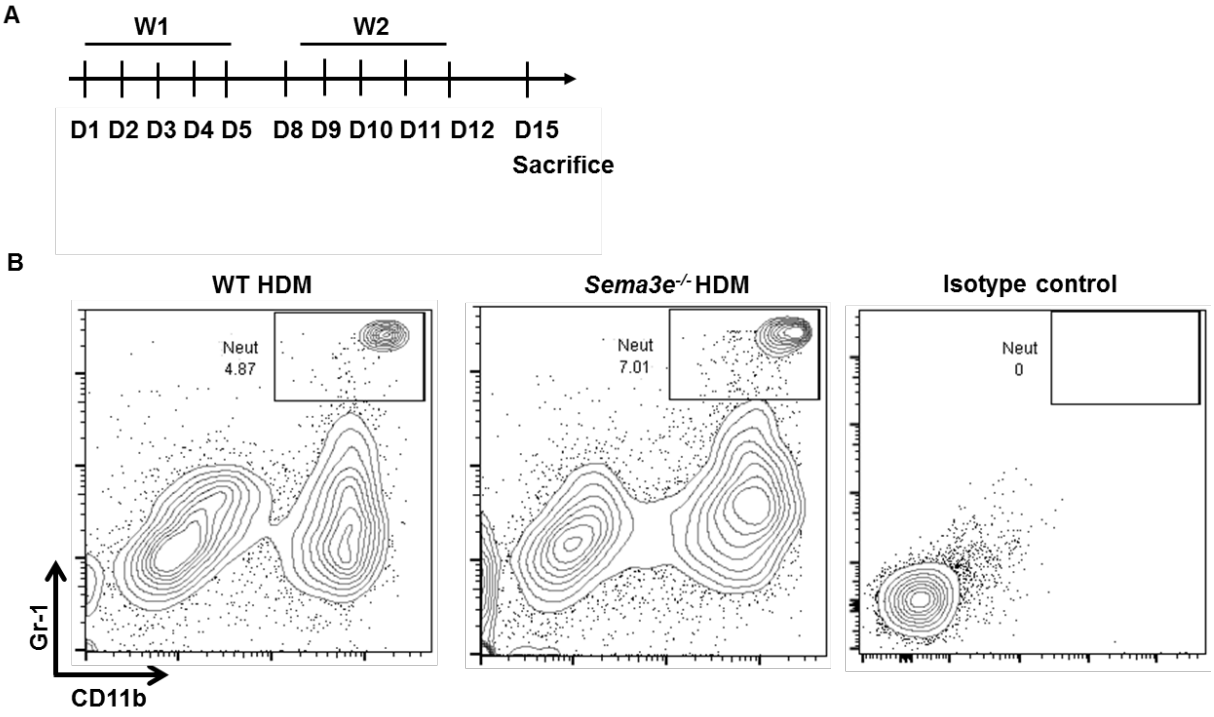

Figure 4-6 HDM-induced neutrophil recruitment into the lung is exacerbated in Sema3e$^{-/-}$ mice.
Age and sex-matched Sema3e$^{-/-}$ and WT mice were exposed to HDM for two consecutive weeks via intranasal route (A). Then, the mice were anesthetized and BALF collection was performed. Recruitment of neutrophils into the airways was assessed by flow cytometry (B). Staining with isotype control antibody revealed no immunoreactiviry. (N = 4 per group).

4.3.5 Exogenous Sema3E treatment protects mice from allergen-induced neutrophilic airway inflammation

To address the potential therapeutic relevance of this study, we treated HDM-challenged WT mice with exogenous recombinant Sema3E-FC intranasally and then re-exposed them with HDM as demonstrated in Fig. 4-7A. Sema3E-treated mice demonstrated lower $CD11b^{bright}$ $Gr1^{bright}$ neutrophil influx compared to the control group (Fig. 4-7B,). These results were further confirmed in the lungs of the same mice (Fig. 4-12, n = 4 per group). In addition, related to our human neutrophil data, Sema3E treatment decreased HDM-induced levels of murine keratinocyte chemoattractant (CXCL1/KC) (Fig. 4-7C); which is orthologous to human CXCL-8 and involved in neutrophil migration and prompting neutrophilic inflammation (31-34). Finally, surface expression of PlexinD1 was also detected on mouse blood, airway neutrophils after HDM exposure suggesting that Sema3E treatment may affect their function *in vivo* (Fig. 4-7D). These findings collectively suggest a regulatory role of Sema3E in neutrophilic allergic airway inflammation and modulation of neutrophil recruitment to the lung most likely via reducing neutrophil migration.

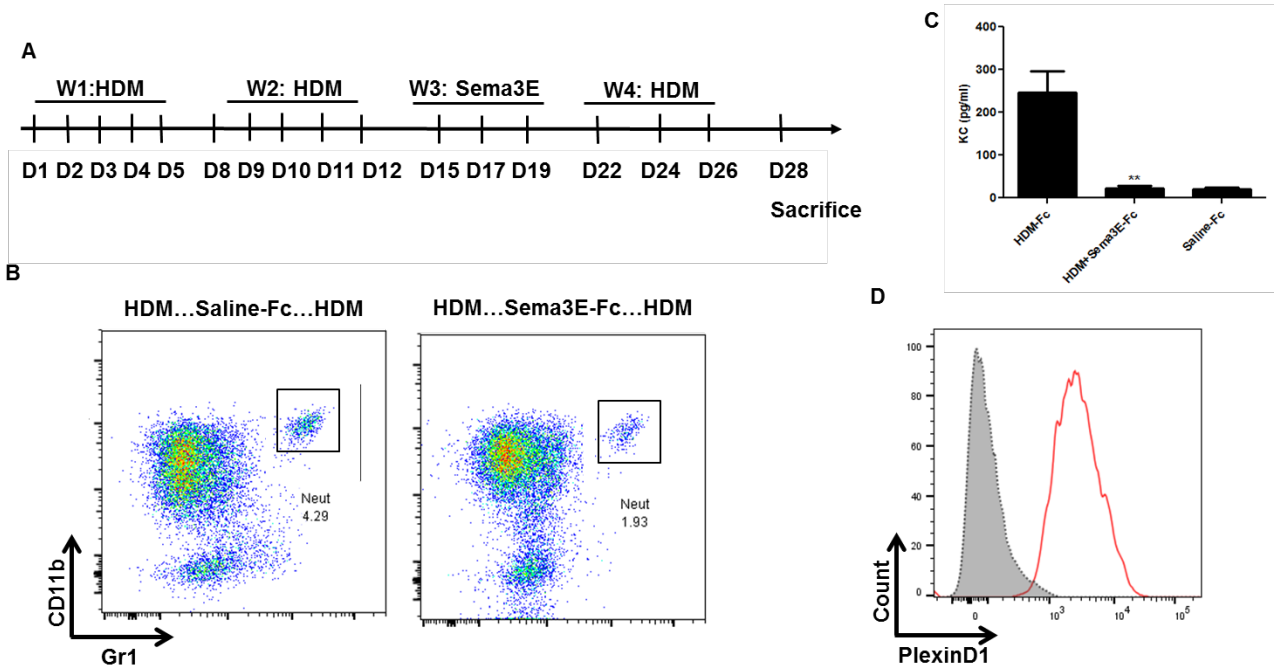

Figure 4-7 Exogenous Sema3E treatment decreases HDM-induced pulmonary neutrophilia and KC secretion into the airways.

*The timeline of HDM challenge and Sema3E treatment model has been depicted (A). Intranasal treatment with recombinant murine Sema3E after inducing experimental allergic asthma by HDM challenge, decreased airway neutrophilia upon HDM re-exposure (B) which was associated with reduction of HDM-induced mKC secretion in the airways (C). Surface expression of PlexinD1 on mouse neutrophils was examined by flow cytometry. The statistical significance was determined by performing one-way ANOVA and Bonferroni test in C; (N = 4, ** $p<0.01$).*

4.4 Supplemental figures

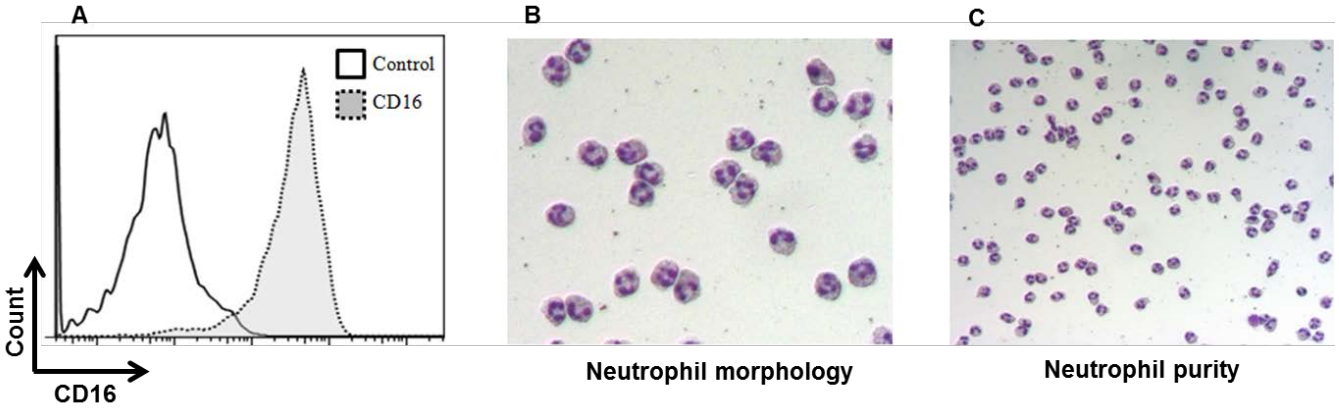

Figure 4-8 CD16 expression on the surface membrane of freshly isolated neutrophils.
CD16 expression in isolated human neutrophils was examined utilizing FACS analysis. $5x10^5$ of neutrophil suspension resuspended in 100 µl of PBS were blocked with Fc blocking buffer for 30 min. After two times washing, cells were incubated with anti-human CD16-Alexa 488 conjugated for 30 min on ice. As a negative control, cells were labeled with isotype-matched goat IgG (A). Neutrophil morphology direct after isolation, indicating healthy non-stimulated cells (B). Neutrophil purity was always above 96% (C).

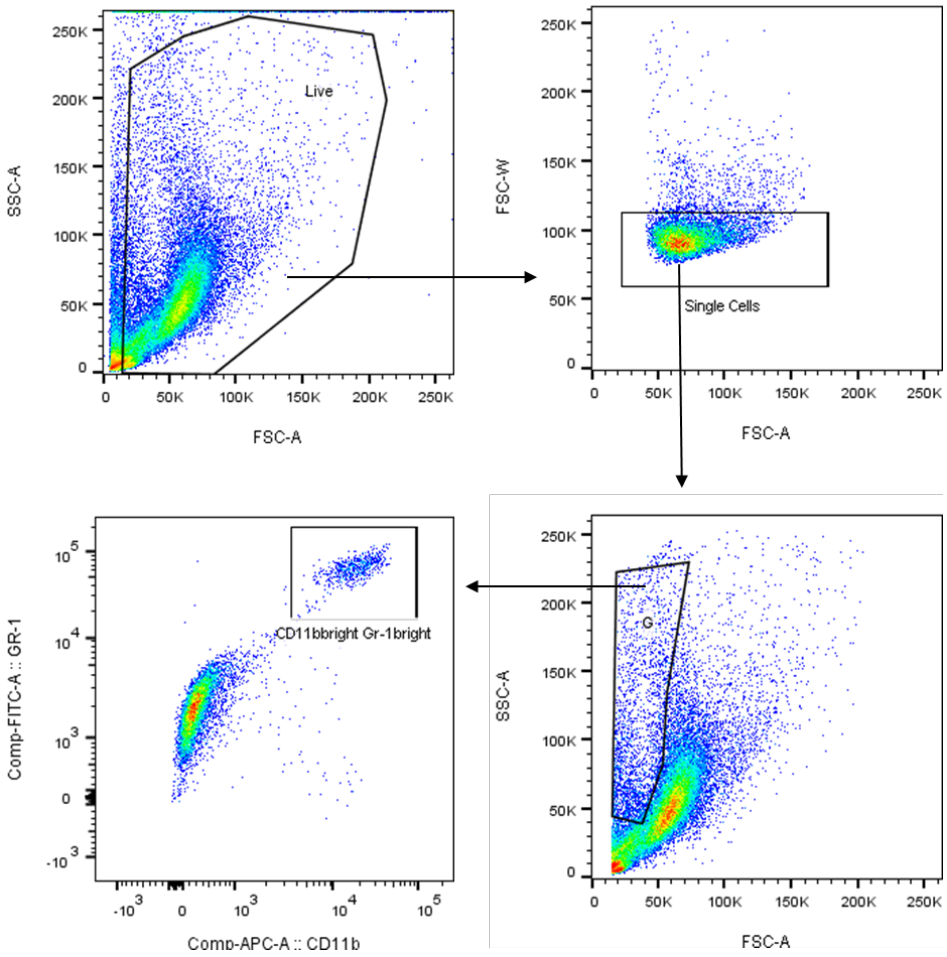

Figure 4-9 Gating strategy to characterize mouse pulmonary neutrophils.
Single cell population was first selected on live cells followed by gating on total granulocytes as $FSC^{lo}SSC^{hi}$ cells. Then, neutrophils were traditionally characterized by high expression of both CD11b and Gr-1 surface markers on either BALF or lung samples.

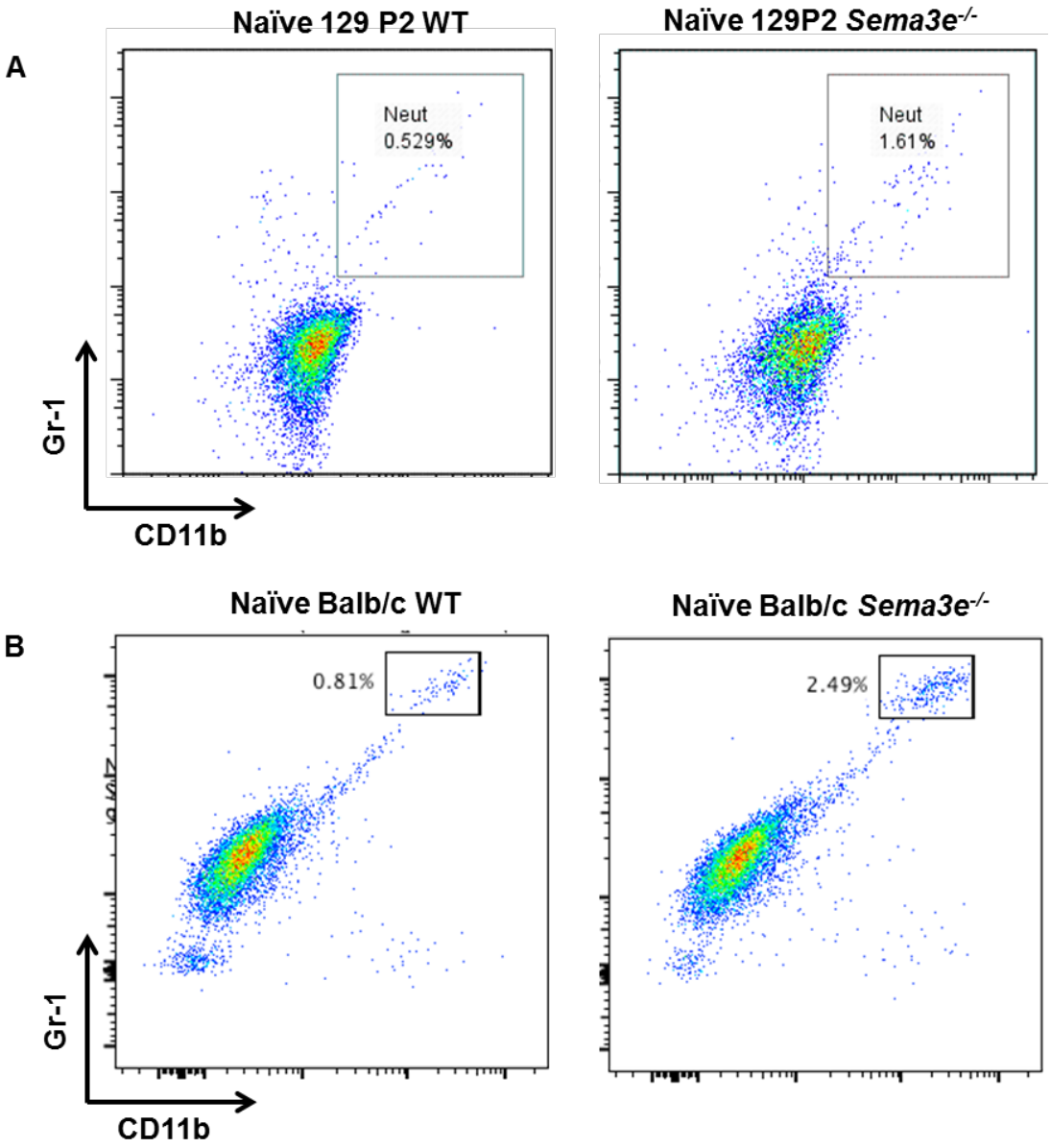

Figure 4-10 Neutrophil accumulation is enhanced in the airways of Sema3e$^{-/-}$ mice at the baseline.
Basal accumulation of CD11b^{hi} Gr-1^{hi} neutrophils in the airways was compared between Sema3e$^{-/-}$ and WT littermates in naïve 129 P2 (A) and Balb/c (B) mice without any encounter with inflammatory stimuli (n= 4 mice per group).

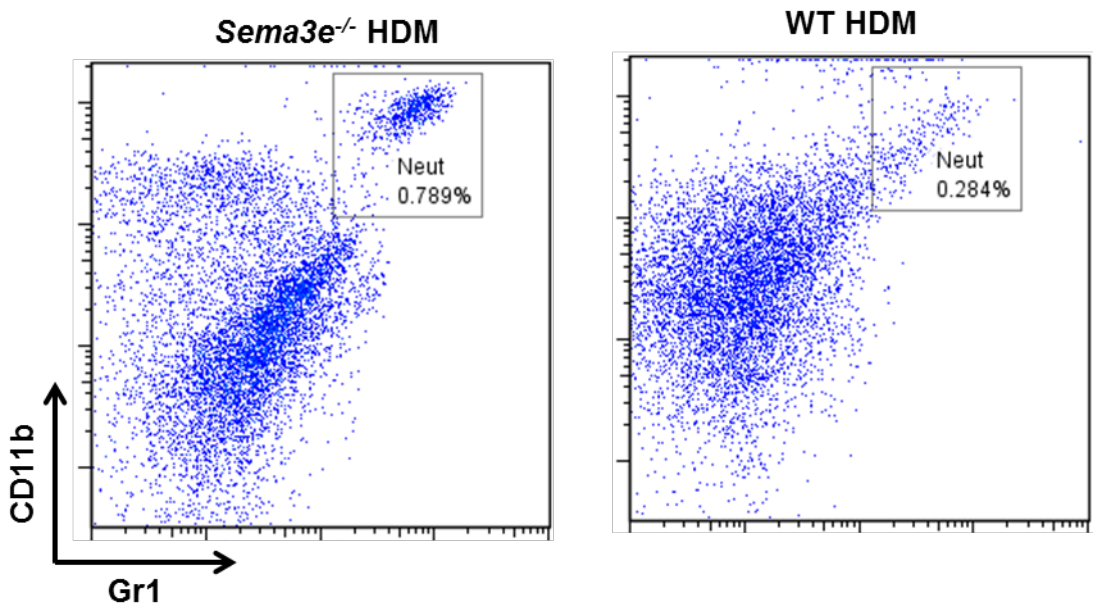

Figure 4-11 Blood neutrophils are higher in Sema3e$^{-/-}$ mice than WT controls upon HDM challenge.
Blood collection was performed at the end of HDM challenge protocol from Sema3e$^{-/-}$ and WT mice followed by RBC lysis. Extracellular staining was performed to determine surface expression of CD11b and Gr1 in the circulating neutrophils by using previously mentioned specific fluorescent labeled-antibodies. (N = 4 mice per group).

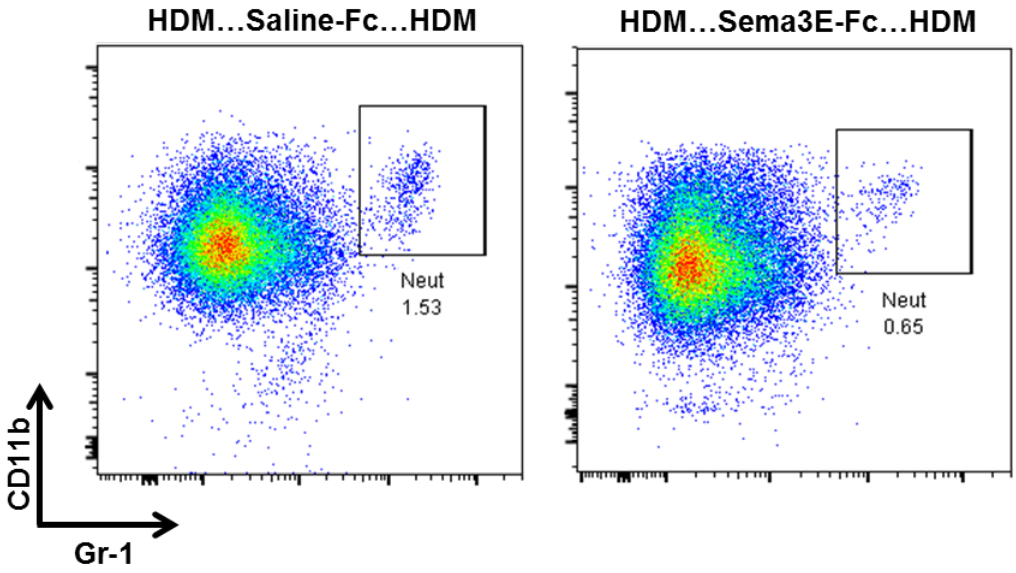

Figure 4-12 Exogenous Sema3E treatment decreases HDM-induced neutrophil influx in the lung.
Intranasal Sema3E treatment was performed as explained in Fig. 4-7A. Lung neutrophilia upon HDM re-exposure was assessed by performing extracellular staining of CD11b and Gr-1on enzymatically digested lung cells upon HDM re-exposure. (N = 4 per group).

4.5 Discussion

Neutrophil influx to the site of inflammation is a double-edged sword: In one hand, it is absolutely required for the inflammatory response to occur. On the other hand, considering destructive capacity of neutrophil-derived mediators, it should be tightly regulated to avoid tissue damage caused by an excessive inflammatory reaction. Confining the neutrophil extravasation once they are adequate for inflammogen containment and protective response is a complex and largely unknown process which is essential for resolution of inflammation (35). Understanding novel mediators and pathways implicated in this process is an essential step towards designing better strategies to control neutrophilic inflammatory disorders.

Originally discovered as neural chemorepellents, the role of semaphorins in the immune regulation has emerged during the recent past years (13). In the present study, we investigated the role of Sema3E in regulating neutrophil migration. Our *in vitro* studies on primary human neutrophils and also our *in vivo* model of allergic airway inflammation show a repulsive role of Sema3E in neutrophil migration and its potential to diminish airway neutrophil influx.

A growing body of evidence during the last decade illustrates that semaphorins and their receptors are implicated in regulation of neutrophil functions. For instance, a viral semaphorin, A39R, was previously shown to inhibit phagocytosis by neutrophils (36). More recent studies reveal a crucial pathological role for Sema7A-PlexinC1 axis in promoting neutrophil migration in acute lung injury (37) and hypoxia (38) models. On the other hand, Sema3C therapeutic effect on lung injury is mediated by decreased lung neutrophil influx and silencing its expression leads to a significant increase in neutrophil activity (4). However, to our knowledge, the role of Sema3E in neutrophil migration has not been previously investigated.

We have shown the Sema3E high affinity receptor, plexinD1, is constitutively expressed on neutrophils obtained from healthy individuals. It should be mentioned that all secreted mammalian semaphorins bind neuropilins except Sema3E which interacts directly with plexinD1 as the binding receptor (9, 12). According to previous studies on neuronal cells, the repulsive functional outcome of Sema3E-plexinD1 signaling could be reversed via interaction with neuropilin 1 (Nrp1) (39) or vascular endothelial growth factor receptor 2 (VEGFR2) (40). However, the anti-migratory effect of Sema3E on human neutrophil suggests it may be mediated through plexinD1 independent of gating by Nrp1 or VEGFR2. In fact, our unpublished data revealed that Nrp1 is not expressed in human neutrophils signifying the anti-migratory is mainly mediated via plexinD1.

In this study, we employed a microfluidic-based system to assess directional neutrophil migration at a single-cell level in defined stable gradient of IL-8, Sema3E and their combinations. The results further confirmed the inhibitory effect of Sema3E on IL-8-induced neutrophil migration as we demonstrated by a traditional transwell assay. In both methods, Sema3E inhibitory effect was evident when neutrophils were stimulated with Sema3E and IL-8 as either opposite or same side gradients. The microfluidics-based results not only confirmed the Sema3E inhibitory effect by the significantly reduced number of migrated cells as seen in the transwell assay, but also further showed the inhibitory effect of Sema3E on the directionality of cell migration by the significantly reduced chemotactic index.

Monitoring neutrophil functions such as chemotaxis by microfluidics devices has been recently considered as a novel approach for sensitive asthma diagnosis (41, 42). Here, we provide evidence that Sema3E affects neutrophil cell migration by reducing cell motility and

directionality. In addition, this technical approach could be applicable in mechanistic and translational studies in which unknown repulsive potency of a therapeutic candidate on neutrophil migration is aimed to be assessed.

From a mechanistic point of view, Sema3E inhibited neutrophil migration at least in part via reduction of CXCL-8-mediated F-actin polymerization and Rac1 GTPase activity. These findings are in line with our previous study in which decreased ASM cell migration and proliferation was associated with reduced F-actin polymerization and Rac1 GTPase activity (14). Similar results on macrophages (15) and endothelial cells (19) further support involvement of these key elements underlying Sema3E repulsive roles. Indeed, other signaling pathways activated by IL-8 stimulation could undergo negative regulation upon Sema3E treatment. As we previously reported, both MAPK and PI3K signaling could be targeted by Sema3E which might lead to repulsion of other neutrophil functions such as phagocytosis and survival (14). Treatment with Sema3E alone did not desirably affect basal Rac1 GTPase activity or F-actin rearrangement in neutrophils. It may explain the non-significant effect of Sema3E on basal neutrophil migration in agreement with findings in ASM cells and endothelial cells (14).

Neutrophil influx into the lungs is a hallmark of many airway inflammatory diseases such as allergic asthma. In fact, severe asthmatic patients who are refractory to glucocorticoid therapies are characterized by a massive recruitment of neutrophils to their lungs where they accumulate due to higher migration or survival compared to normal conditions (43-45). In our HDM-induced model of allergic asthma, lung neutrophils were significantly higher in *Sema3e*$^{-/-}$ mice than those of WT littermates suggestive of a regulatory role for Sema3E in pulmonary neutrophil extravasation. In addition, higher amount of neutrophils in naïve *Sema3e*$^{-/-}$ mice indicates an

intrinsic defect at homeostatic conditions which could potentially make the animal more prone to airway neutrophilia upon allergen exposure. To further support this notion, our previous studies on severe asthmatic patients and HDM mouse model demonstrated a significant suppression of Sema3E expression suggestive of an impaired Sema3E signaling in asthmatic conditions underlying a potential hyper-inflammatory phenotype in the airways characterized by enhanced neutrophilia. It was recently reported that deterious role of Sema7A receptor, PlexinC1, in acute lung injury model is mediated by induction of pulmonary neutrophil migration and its genetic or pharmaceutical inhibition could be a new therapeutic option (37). Endothelial-derived Sema7A has been reported to propagate the extravasation of neutrophils from the vascular space during hypoxia (38). However, to our knowledge, this is the first study reporting the role of a semaphorin family member, Sema3E, in allergen-induced airway neutrophilia. Furthermore, to address the translational relevance, Sema3E intranasal treatment reduced HDM-induced recruitment of neutrophils into the lungs. It further suggests Sema3E as a potential treatment option for refractory severe asthma which deserves more mechanistic studies.

HDM model was chosen primarily because it is known as a clinically relevant common indoor aeroallergen (46). Besides the mite-derived allergens, pattern recognition receptor (PRR) ligands derived from microbial components, especially lipopolysaccharide (LPS), are highly present in HDM extracts (47, 48). It has been shown that HDM induces a TLR4/MyD88-mediated response which are able to sense bacterial LPS on airway structural (49, 50) and myeloid cells (51). Phipps et al reported that both TLR4 and MyD88 knockout mice are protected from the airway inflammation and hyperreactivity. More relevant to our study, they have shown that HDM-induced airway neutrophilia and IL-17 production are attenuated in MyD88 but not TLR4 deficient mice (52). HDM also releases IL-8 from airway epithelium as a neutrophil

chemoattractant and it may also contribute to the airway neutrophilic inflammation in acute asthma exacerbations or severe asthma (53, 54). It further highlights the importance of Sema3E endogenous defect as a predisposing factor in various contexts regarding pulmonary neutrophilia especially where bacterial endotoxin contributed to the inflammation.

All in all, our data provides novel insights into the potential contribution of Sema3E in regulation of neutrophilic inflammatory responses. This study provides evidence to understand a previously unknown mechanism of modulating neutrophil migration which could be considered as a therapeutic approach in neutrophil-dominated inflammatory disorders.

4.6 Materials and Methods

4.6.1 Subjects

This study was approved by the Ethics Committee of the Faculty of Health Sciences, College of Medicine, University of Manitoba. Adult non-smoker non-allergic healthy subjects volunteered to donate blood. 30-80 ml of venous blood was taken from each donor, between 9:00-11:00 am on weekdays only. EDTA coated tubes from RD biosciences were used to store the blood, which was immediately transferred to a cell culture hood for further processing.

4.6.2 Human peripheral blood neutrophil isolation and purification

Blood was collected from the peripheral vein of healthy donors using sterile syringes. Every 30 ml of blood was then mixed with 30 ml of normal saline. 10 ml of the mixture was added to 3.5 ml of Ficoll (Amersham) rotated for 30 min in a centrifuge at room temperature at 1000 RPM. The upper layer of plasma, Ficoll and peripheral blood mononuclear cells (PBMC) were disposed, while the bottom layer of the granulocyte-erythrocyte was kept. Then, 6 ml of dextran-saline was added to the bottom layer to sediment red blood cells. The granulocytes on clear layer on top were further purified by using hypotonic lysis and then normal saline 0.85%. The hypotonic shock procedure was repeated if necessary; otherwise the pellet was re-suspended in RPMI 1640 (HyClone Laboratories) medium. This step was followed by repeated centrifugation and re-suspension to ensure a clean population of cells. Samples were then taken for manual counting and viability assessment using trypan blue exclusion. Furthermore, cytological examination of stained neutrophils by the Wright-Giemsa method was carried out on a sample of 100,000 neutrophils spread on a glass slide by a cytospin, routinely yielding neutrophil that were between 95-98% pure.

4.6.3 RNA isolation and RT-PCR analysis

RNA was purified from primary human neutrophils using *TRIzol* (Invitrogen) followed by measuring RNA concentration and integrity. Reverse transcription was performed with 2 μg of total RNA using a high capacity reverse transcription kit (Applied Biosystems) to synthesize cDNA. The annealing temperature and size of the amplified fragment for PLXND1 were 58ºC and 156 bp, respectively and the following specific primers were used: FWD 5'-CCCCAACCCACAGTTCTCTA-3' and REV 5'-CAAGTAAGCTGCGACATCCA-3'. In parallel, human PBMCs, used as a positive control, were cultured in RPMI 1640, and reverse transcription was performed as mentioned above. Primers for housekeeping gene GAPDH and standard controls were developed in our laboratory. Product specificity was determined by a melting curve analysis and by visualization of the PCR products on agarose gel.

4.6.4 Immunocytochemistry

Immediately, after neutrophil isolation, a sample of 100,000 neutrophils was spread on a glass slide by a cytospin and fixed with 4% paraformaldehyde. Then, slides were washed twice with l% peroxidise blocking solution for 10 min and left to dry over night at room temperature. Slides were blocked with normal serum blocking solution (5% donkey or 5% rabbit and 5% human sera) for 1 hour at room temperature. Anti-PlexinD1 (R&D Systems) was diluted in 1%PBS (1 μg/ml) and added to slides (Overnight, 4ºC). Then, slides were washed extensively three times for 10 min each with Cyto-TBS. Immediately the slides were incubated with the biotinylated secondary antibody (rabbit–anti-Goat IgG, 1:200 dilution in PBS) for 1 hour at room temperature. After extensive washing with TBS cyto, the slides were then incubated with streptavidin-alkaline phosphatase for 30 min at room temperature. The slides were developed

with Fast Red dissolved in the alkaline phosphatase substrate followed by counterstaining with Mayer's hematoxylin. Isotype-matched control mAb was used as negative control.

4.6.5 Flow cytometry (extracellular staining)

Approximately, $1x10^5$ cells of isolated human neutrophils were transferred to FACS tubes and washed twice with 2 ml of ice cold 1X PBS. After centrifugation, the supernatant was gently discarded followed by primary or isotype control antibody incubation (Polyclonal anti-PlexinD1, Sheep IgG) on the shaker for 1 hour on ice. Cells were then washed and incubated with a secondary antibody Alexa Fluor 488 conjugated donkey anti-goat IgG (1:100) (R&D Systems) or Alexa Fluor conjugated donkey anti-sheep in the dark for 1 hour on ice. Cells were then washed once with 1X PBS, and fixed with 200 μl of 2% paraformaldehyde for 2 min, and analyzed on FACS CantoII (BD Biosciences, San Jose, Calif). The results were presented as specific mean fluorescence intensity (MFI) using FlowJo software (Tree Star, Ashland, Ore).

4.6.6 Microfluidic cell migration experiments and data analysis

The microfluidic channel was coated with fibronectin (BD Biosciences) for 1h at room temperature and blocked with 0.4% BSA in RPMI for another hour before the experiment. 0.4×10^6 neutrophils were loaded in the fibronectin-coated channel for 5 min. Medium, recombinant human Sema3E (100 ng/ml) ± IL-8 (10 ng/ml) (both from R&D Systems) were infused into the device by syringe pumps through the inlets. The device was placed on a microscope stage (BX60, Olympus). The temperature in the device was maintained at 37^oC and cell migration was recorded by time-lapse microscopy at six frames/min for about 35–40 min using a CCD camera

(Optikon, Canada). Individual cell movement from the time-lapse images was tracked using the NIH ImageJ v.1.34s.

At least 20 or more migrating cells were selected for each condition in each experiment. Only cells that remained from the beginning to end of the experiment were tracked and analyzed. Chemotaxis percentage (number of cell migrated toward the gradient × 100/total migrating cells tracked), CI (the ratio of the displacement of cells toward the chemokine gradient (*dy*), to the total migration distance (*d*) using the equation $CI=dy/d$), and cell speed ($v=d/t$) were quantified. Three independent experiments were repeated for each condition. The representative figures of microfluidic data were generated from an independent experiment for each condition.

4.6.7 Measurement of Rac1 GTPase activity

Rac1 GTPase activity was measured in snap-frozen cell lysates harvested from unstimulated or Sema3E (100 ng/ml) ± IL-8 (10 ng/ml) stimulated human neutrophils ($5x10^6$ cells/ml) at indicated time points using a luminometric-based G-LISA Rac1 Activation Assay Kit (Cytoskeleton, Inc. Denver, CO). Briefly, cell lysates were subjected to Rho binding domain of Rac1 in a Rac-GTP affinity 96-well plate. Prior to this process, wells were incubated for 30 min with an antigen presenting buffer on a cold orbital microplate shaker for 30 min. Then, antigen presenting buffer was added for 2 min. Rac1-GTP was detected using anti-Rac1 primary antibody after 45 min of incubation at room temperature. Subsequently, HRP-conjugated secondary antibody was added to the wells and incubated (45 min, 400 rpm, room temperature). After washing and vigorous drying, wells were incubated and developed with a chemiluminescent reagent. A constitutively active Rac1 provided in the kit was used as positive control in all experiments and a blank well was also used as a negative control.

4.6.8 Phalloidin staining and F-actin measurements

Primary human neutrophils were stimulated with Sema3E (100 ng/ml) with or without IL-8 for 0, 0.5, 1 and 5 min. Cells were then fixed, permeabilized, and stained with Alexa Fluor 488 Phalloidin (Life Technologies). Then, intracellular fluorescence was determined with flow cytometry as we described previously. Effect of Sema3E±IL-8 treatment on F-actin content was quantified as MFI and compared in each time point by using FlowJo software.

4.6.9 Allergen-induced airway inflammation model

6-8 week old female *Sema3e*$^{-/-}$ and WT mice (129P2 strain) were anesthetized and 25μg of HDM extract (Greer Inc.) per mouse was administered intranasally 5 times a week during two consecutive weeks. Naïve control group did not receive HDM. 48 hours after the last HDM challenge BALF, lung and blood collection was performed as described previously. In some experiments recombinant Sema3E was produced and administered to Balb/c mice 3 times during a week starting 48 hours after the last HDM challenge and then mice were re-exposed with HDM as depicted in Fig. 7A. Neutrophil surface markers were stained by using specific monoclonal antibodies including CD11b-APC and Gr1-PE. Samples were acquired by FACS Canto II and analyzed by FlowJo software.

4.6.10 Statistics

Data were analyzed by one-way ANOVA first to determine if any significant differences may generally exist among various experimental groups. Tukey's test was then performed to detect statistically significant differences to compares all possible pairs of means. GraphPad Prism 5.0 (GraphPad Inc, San Diego, Calif) was the software of choice and $p<0.05$ was considered

statistically significant. Microfluidic data were analyzed using Student's two-sample (two-tailed) t-test to assess the significance of the difference between the parameters relative to the critical p value ($p < 0.05$).

4.7 References

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5 CHAPTER 5: GENERAL DISCUSSION

5.1 Sema3E as a new player in allergic asthma

Semaphorins were originally identified as axon guidance cues in neuronal cells. However, today we know that their expression and function is not restricted to the nervous system and they are ubiquitously expressed and play functional roles in development, health and disease. In the case of Sema3E, to our knowledge, there is no previously published study investigating the potential role of this mediator in allergic asthma or other similar airway disorders, e.g. COPD and cystic fibrosis, in which both inflammatory and structural components of the airways are involved. Furthermore, differential expression of Sema3E between asthmatic and healthy individuals has not been surprisingly reported in genome-wide association studies (GWAS). Previous findings in our lab revealed that SEMA3E gene is differentially expressed in human ASM cells isolated from mild asthmatic versus healthy subjects (Dr. Stephan Dragon PhD thesis, 2010). This encouraged us to further investigate expression and function of Sema3E in allergic asthma.

In general, our data indicate that Sema3E is not only involved in inflammatory aspects of allergic asthma, but also plays a critical role in regulation of airway remodeling and hyperreactivity. Considering the multifunctional nature of semaphorins, it could be implied that Sema3E mainly produced by epithelial cells controls various inflammatory processes underlying airway remodeling and AHR. In parallel, it could also directly regulate the functions of airway structural cells contributing to bronchial remodeling and lung caliber. Therefore, the results obtained in this project suggest that Sema3E could be considered as a novel essential regulatory mediator in the airways which is abrogated under inflammatory/allergic conditions.

5.2 Expression of Sema3E in allergic asthma

In this project we addressed high expression of Sema3E in lung tissue biopsies of normal non-asthmatic individuals as well as naïve mice which was significantly lower in asthmatic conditions in both humans and murine studies. The remarkable suppression of Sema3E, which was confirmed by *in vitro* approaches at mRNA and protein levels, suggests a protective regulatory role of this mediator in allergic asthma. In addition, the negative correlation between the level of secreted Sema3E in the human airways (BALF) and FEV1, as the clinical measurement of asthma severity, suggest that this protein could be considered as a novel biomarker. However, it requires further investigations by increasing sample size and type, especially obtained by using non-invasive methods such as sputum collection from asthmatic patients and relevant healthy controls with different severities.

Considering the importance of proteolytic processing in determining Sema3E functional outcome, it would be an interesting approach to compare expression of different Sema3E isoforms, p61 truncated vs. full length, between healthy and asthmatic individuals or mouse model. Since Sema3E treatment was associated with beneficial protective effects in both our *in vitro* and *in vivo* studies, we might speculate that the furin cleavage is less likely to occur though it should be experimentally addressed by performing Western blot analysis. As previously mentioned and observed in tumor metastasis models (276, 346), expression of p61 isoform reverses repulsive effects of Sema3E which could be detrimental *in vivo*. Creation of a recombinant mutated version of Sema3E which is resistant to be targeted by furin enzymes has been introduced to overcome this potential problem (347).

Similar to Sema3E, we observed a significant reduction in expression of PlexinD1 in human ASM cells obtained from asthmatic patients compared to those of healthy control group. It

provides another evidence to assure that Sema3E-PlexinD1 regulatory axis has been abrogated in allergic asthma. However, as explained in Chapter 2, treatment with Sema3E, as the ligand, induces surface expression of PlexinD1 on human ASM cells which makes them functionally responsive in terms of inhibiting growth factor-mediated proliferation and migration to a level comparable to healthy cells highly expressing PlexinD1 at the baseline. In a mouse study performed at 2012, Holl and colleagues reported higher gene expression of both Sema3E and PlexinD1 in Th2 cells as well as BMDC (363) which is suggestive of a potential contribution in type 2 inflammatory circumstance such as allergic asthma. We also observed surface expression of PlexinD1 in two major pulmonary $CD11b^{+}$ and $CD103^{+}$ DC subsets indicating their responsiveness to exogenous Sema3E treatment in allergic asthma model.

5.3 Functional outcome of Sema3E in ASM cells

In this project, we have shown that Sema3E inhibits proliferation and migration of human ASM cells which is extremely important in airway remodeling. From a technical point of view, proliferation of ASM cells is classically measured by radioactive-based methods such as incorporation of ^{3}H thymidine (364) into DNA or fluorescent-based methods such as BrdU flow cytometry (365). To our knowledge, for the first time we optimized EdU incorporation assay to study ASM cell proliferation. This method is based on Click-iT reaction between copper and an azide dye which is more sensitive and reproducible compared to the other ones (366, 367). Boyden chamber assay utilized to evaluate the anti-migratory effect of Sema3E was specifically developed to investigate the migration of ASM and vascular smooth muscle cells (368).

Although we confirmed the repulsive effect of Sema3E on human ASM cells by using two different growth factors, PDGF and EGF, it is not clear whether Sema3E could exert the same

function against other mitogens *in vitro* or under allergic inflammatory conditions *in vivo*. Since Sema3E effect is mediated by targeting multiple signaling pathways (276, 317, 347, 351, 352, 362), it might be assumed that the inhibitory effect would be the case for other growth factors signaling via different pathways as well.

Sema3E could also affect the other aspects of ASM functions such as apoptosis which has not been studied in these cells but shown to induce Caspase3 expression in endothelial cells (347). This is of great importance for our study because reduced apoptosis of ASM cells for example in response to *in vitro* stimulation with Th17 cytokines (369), IL-8, eotaxin and macrophage inflammatory protein 1 alpha (MIP-1α) (230) has been proposed as a potential mechanism of increased ASM mass in allergic asthma. In addition, adoptive transfer of $CD4^+$ T cells from OVA-sensitized mice increases ASM mass which is associated with inhibition of apoptosis and induction of proliferation in ASM cells (370). Besides the role of Sema3E in the proliferative phenotype of ASM cells, the potential contribution of this chemorepellent in ASM synthetic phenotype, as a crucial facet of remodeling (371, 372), should be further investigated. In fact, Sema3E might regulate autocrine mechanisms of ASM dysfunction by modulating expression of pro-inflammatory/remodeling mediators in these cells. Reduced expression of Sema3E in asthmatic conditions (Chapter 3) further supports its regulatory role in ASM remodeling.

5.4 Mechanism of Sema3E function in ASM cells

As mentioned earlier, semaphorins are typically multifunctional proteins which mediate their effects via targeting several signaling pathways. To our knowledge, this is the first study investigating the mechanism underlying the function of a semaphorin in ASM cells.

We demonstrated that the small GTPase Rac1 is an early signaling component in which its PDGF-induced activity is reduced upon Sema3E treatment. Rac1 has been previously reported as an essential mediator involved in ASM cell proliferation and migration. In fact, silencing *Rac1* gene expression reduces PDGF-induced phosphorylation of STAT3 which is associated with a remarkable decrease in proliferation of ASM cells in response to either PDGF or serum (225). Therefore, targeting Rac1 by replenishing an endogenous mediator such as Sema3E could be a new strategy to minimize ASM remodeling.

Reduction of PDGF-induced MAPK/ERK and PI3K/Akt phosphorylation was another crucial signaling event occurred upon Sema3E treatment in human ASM cells. Pharmacological inhibition of ERK and PI3K pathways has been associated with modulation of ASM cell function and induction of cell senescence (373-375) suggesting them as fascinating targets for therapeutic interventions. However, more comprehensive mechanistic studies including other signaling pathways such as Rho GTPases, STATs and cyclins will determine the precise mode of Sema3E action in ASM cells. Furthermore, Sema3E treatment could partly function through reducing intracellular phosphorylation of growth factor receptors (RTKs) as it was previously shown for VEGFR2 in endothelial cells that results in anti-angiogenic effect of Sema3E (362).

Sema3E reduced PDGF-mediated polymerization of F-actin which is a key cytoskeletal event in ASM cell migration. There is a complex network of signaling pathways which controls actin dynamics in cytoskeleton and it is not still clear how these pathways are influenced by Sema3E treatment in ASM cells. Surprisingly, stimulation by Sema3E alone did not significantly change F-actin content along with no remarkable alteration of Rac1, ERK1/2 and Akt activity suggestive of no effect on basal function of ASM cells. It further provides evidence to explain why Sema3E does not modulate human ASM cell proliferation and migration at the baseline in the absence of

PDGF. Thus, Sema3E might exert its repulsive role specifically under abnormal conditions wherein growth factors are pathologically dysregulated.

5.5 Versatile role of Sema3E in allergen-induced airway inflammation

In this project, we demonstrated that genetic deletion of Sema3E in mice leads to enhanced allergic airway inflammation in terms of higher granulocyte recruitment, Th2/Th17 response and IgE synthesis compared to WT controls. The allergen-induced hyper-inflammatory phenotype observed in the absence of Sema3E has not been previously reported and is of great importance because expression of Sema3E is tremendously suppressed under allergic asthmatic conditions in both humans and mouse models somewhat similar to *Sema3e*$^{-/-}$ model. In contrast, intranasal administration of recombinant Sema3E protected mice from airway inflammation induced by HDM which further suggests the *in vivo* regulatory role of Sema3E in allergic asthma.

Our findings indicate that Sema3E is involved in several aspects of allergic airway inflammation and could affect different inflammatory cell types. However, in order to understand the precise mechanism of Sema3E function in allergic asthma, we first established the disease model, then compared the phenotypic outcomes and finally focused on DC, Th cells and neutrophils. Indeed, other immune cells such as macrophages, B cells, ILC and eosinophils should be extensively investigated in this context to determine whether their functions are influenced by Sema3E or not.

5.6 The role of Sema3E under homeostatic non-inflammatory conditions

Genetic deletion of Sema3E could lead to immune deficits at non-inflammatory conditions. It might be a part of mechanism underlying heightened airway inflammation upon allergen

exposure. To address this issue, some immune responses were compared between naïve *Sema3e*$^{-/-}$ and WT mice at the baseline. We unexpectedly observed that recruitment of both CD11b^{+} DC and neutrophils into the lungs is higher in the absence of Sema3E than those of WT controls. Considering the developmental role of Sema3E in other contexts, it could be indirectly attributed to a novel effect of this chemorepulsive mediator on differentiation of myeloid lineages which has not been previously demonstrated. It could be speculated that lacking Sema3E, as an immune modulator, might provide an abnormal environment wherein DC and neutrophils are undesirably primed to mount an unnecessary response.

It has been shown by other groups that BMDC intrinsically produce Sema3E which might negatively regulate DC functions in an autocrine manner; though it could be also ubiquitously expressed by other cellular sources. In our conventional knockout model, Sema3E expression has been globally abrogated in all inflammatory and structural cells. Absence of Sema3E leads to higher baseline BMDC migration, Rac1 GTPase activity and allergen uptake which is heightened upon HDM stimulation *in vitro*. It is not completely understood how Sema3E deficiency results in such an inflammatory-prone phenotype at the baseline either in DC or neutrophils. As a future direction, the potential contribution of Sema3E in development of these cells should be considered. In addition, Sema3E deficiency might not be crucial only in allergic asthma and it could make the model disposed to other inflammatory, e.g. infectious, autoimmune, etc., insults which should be extensively investigated.

5.7 Sema3E implication in dendritic cell biology

Sema3E deficiency resulted in enhanced accumulation of pulmonary $CD11b^+$ DC that may account for increased type 2 response after HDM exposure in mice. We observed that surface expression of CCR7 is increased in pulmonary $CD11b^+$ DC from *Sema3e*$^{-/-}$ mice upon HDM sensitization which could be the reason of their enhanced accumulation through higher migration capacity compared to WT mice. As a proof of concept, adoptive transfer of this subset from *Sema3e*$^{-/-}$ into WT mice augmented allergic airway inflammation. In addition, migration and allergen uptake capacity alongside with CCR7 expression, Rac1 GTPase activity and F-actin polymerization were enhanced in BMDC from *Sema3e*$^{-/-}$ mice. Therapeutic effect of Sema3E was mediated in part through decreased $CD11b^+$ and increased $CD103^+$ pulmonary DCs which led to balance T cell response and improvement of lung function and airway remodeling.

An accumulating body of evidence suggest that targeting DC is an appropriate approach for development of novel asthma therapeutics (376). It has been shown that DCs are involved not only in shaping the immune response against allergens, but also in regulation of AHR features of the disease (377-379). However, it is important to mention that DC are a heterogeneous population including distinct functional subsets (380). Therefore, considering the “division of labor” concept (76), determining the role of Sema3E in each specific DC subset is crucial for development of a novel of asthma treatment option based on this guidance cue.

The role of pulmonary $CD103^+$ cDC subset in allergic asthma is controversial. Nakano and colleagues at 2012 demonstrated that pulmonary $CD103^+$ cDC prime Th2 response to inhaled allergens and deletion of this subset in mice is associated with reduced Th2 priming to various inhaled allergens (381). In contrast, Khare and colleagues reported that $CD103^+$ cDC are involved in pulmonary tolerance via promoting Foxp3 expression in T cells (85). More recently,

Bernatchez and colleagues revealed that allergic airway inflammation, induced with either OVA or HDM, is exacerbated in *Cd103*$^{-/-}$ mice which was associated with deficiency in the resolution phase of inflammation suggestive of a regulatory role for CD103 in the control asthma (382). This discrepancy could be attributed to different type/dose of allergen or different routes and time of sensitization/challenge (84). In our model, pulmonary CD103^{+} cDC were lower in the absence of Sema3E at the baseline or upon HDM challenge. In addition, co-culture of this subset from *Sema3e*$^{-/-}$ mice with naïve T cells induced lower Th1 cytokine response compared to those of WT mice which was restored in our therapeutic model upon Sema3E treatment.

The crucial role of Sema3E in regulation of Th2-inducing pulmonary CD11b^{+} cDC was addressed in this project and discussed in details in Chapter 3 which was surprisingly associated with increased Th17 differentiation in *Sema3e*$^{-/-}$ mice. Furthermore, it has been shown that plasmacytoid DC (pDC), play protective roles in allergic asthma by providing tolerogenic signals and induction of T_{reg} response (383-385). The potential contribution of Sem3E in regulation of pDC function has not been addressed in this project and would be an interesting direction for future studies.

All these data are based on studies on mouse models of allergic asthma and generally there might be some differences between mouse and human settings affecting the functional outcome of DC-based therapeutic approaches. Human DC play a major role in development of inflammatory response in allergic asthma. However, our knowledge about the precise function of human DC in allergic asthma is limited. It has been previously shown that Der p, a typical allergic component existing in HDM, binds human DC via DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). In fact of DC-SIGN is downregulated in Der p-sensitized human DC from asthmatic patients which is associated with increased endocytosis and

impaired differentiation of DC progenitors (386). Moreover, the efficacy of some asthma therapeutic strategies such as neutralization of IgE by omalizumab (387) or antagonism of LT receptor by montelukast (388, 389) has been shown to be mediated via modulation of DC functions. There is no previous study addressing the role of Sema3E or any other semaphorin in human DC in asthma context. Extending our findings from mouse to human DC will provide more insights into the potential clinical application of Sema3E as a novel therapeutic option for allergic asthma.

5.8 Pulmonary neutrophils as a target cells for Sema3E

Neutrophilic infiltration is a hallmark of refractory severe asthma and understanding the mechanisms underlying enhanced influx of neutrophils in the airway is essential to develop efficient therapeutic strategies. In this project (Chapter 4), we revealed that Sema3E deficiency leads to increased accumulation of neutrophils in the airways at homeostatic conditions which is heightened upon HDM challenge. Intranasal administration of recombinant Sema3E inhibited HDM-induced accumulation of neutrophils in the airways which was sustained upon HDM re-exposure. Our *in vitro* signaling studies on human neutrophils revealed that the inhibitory effect of Sema3E on neutrophil migration is mediated by reduced level of Rac1 GTPase activity and F-actin polymerization.

Expression of Sema3E high affinity receptor, PlexinD1, on both mouse and human neutrophils suggests that these cells could be responsive to treatment with exogenous Sema3E. However, PlexinD1 could also bind other ligands such as Sema4A (301, 390) or Sema3C (355) especially in asthmatic circumstance wherein Sema3E expression has been downregulated (Chapter 3). As

another layer of complexity, it is not clear whether Sema3E-PlexinD1 axis in neutrophils could be gated by co-receptors including Nrp1 and VEGFR2.

Indeed, enhanced neutrophil migration is not the only reason explaining the neutrophilic phenotype in severe asthmatic airways. Increased survival and decreased apoptosis are the crucial features of neutrophils in severe asthma. Our preliminary data indicate that neutrophils isolated from *Sema3e*$^{-/-}$ mice survive significantly longer than those of WT littermates which needs to be further investigated. In addition, the frequency of granulocyte progenitors in the bone marrow of *Sema3e*$^{-/-}$ mice was surprisingly higher than the WT littermates. Understanding the precise role of Sema3E in neutrophils will enable us develop novel strategies to treat severe refractory asthma.

It should be noted that Sema3E-induced signaling might not be necessarily mediated by PlexinD1 in an exclusive fashion. As mentioned previously, Sema3E-PlexinD1 has been shown to be gated by Nrp1 or VEGFR2 which completely reverses the functional outcome. The potential cross talk between PlexinD1 and these (co)receptors depends on the cell type in a context dependent manner as Sema3E-PlexinD1 signaling in endothelial cells is not influenced by Nrp1 but is totally reversed in neuronal cells. In addition, there might be some unknown receptors to cross talk with Sema3E-PlexinD1 complex in allergic asthma model which have not been reported previously. For instance, according to our data from human ASM cells (Chapter 2), Sema3E-PlexinD1 interaction may reduce the activation of PDGF receptor upon Sema3E+PDGF co-stimulation compared to when ASM cells are stimulated with PDGF alone which leads to inhibition of cell proliferation and migration. This is similar to what is shown for Sema3E-inhibitory effect on endothelial cell proliferation/angiogenesis via decreasing VEGF-induced phosphorylation of VEGFR2. The potential cross talk of PlexinD1 with other receptors is a fascinating issue which should be further studied in inflammatory cells in allergic asthma model.

5.9 *In vivo* implication of Sema3E in airway remodeling and AHR

We have shown that genetic deletion of Sema3E leads to increased HDM-induced goblet cell hyperplasia and collagen deposition as the characteristic structural changes in asthmatic airways. Lung function parameters including airway resistance, tissue resistance and tissue elastance were also significantly augmented in the absence of Sema3E upon HDM exposure for two consecutive weeks. However, considering the chronic nature of allergic asthma, it would be very important to assess airway remodeling and AHR at longer time points for example 5-7 weeks after repeated HDM exposure. Therapeutic efficacy of intranasal Sema3E administration on airway remodeling and AHR should be also evaluated at later time points to ascertain the effect is long lasting in a chronic manner.

Besides confirming the role of Sema3E in remodeling events by morphometric analysis on lung tissue sections, expression of the genes involved in mucus overproduction (*Muc5ac*) and collagen deposition (*Col3*) should be compared between *Sema3e*$^{-/-}$ and WT mice in a chronic model of HDM exposure. In addition, our *in vitro* data on the inhibitory effect of Sema3E on human ASM cell functions could be further investigated as another facet of airway remodeling in chronic HDM model. ASM contraction plays a key role in pathogenesis of asthma and the effect of Sema3E on this process needs to be addressed. Along with the non-redundant role of Sema3E in allergic airway inflammation, remodeling and AHR, demonstrating the potential regulation of ASM contraction by Sema3E would provide a more comprehensive insight into the translational value of Sema3E as a novel therapeutic option for allergic asthma.

5.10 Sema3E protein as a therapeutic approach for allergic asthma

Our results collectively suggest that administration of exogenous Sema3E could be considered as a novel therapeutic approach to treat allergic asthma via regulating various aspects of the disease pathology including AHR and airway remodeling as well as type 2 and neutrophilic inflammation. Remarkable reduction of Sema3E level in asthmatic conditions, both in humans and mouse model, further supports the notion of replenishing this new regulator to minimize asthma deficits. All our findings and the proposed therapeutic approach has been presented in Figure as an integrative schematic model. However, similar to other studies, the pharmacokinetic issues such as the optimal dose and time of Sema3E administration and also the appropriate route of delivery should be further determined.

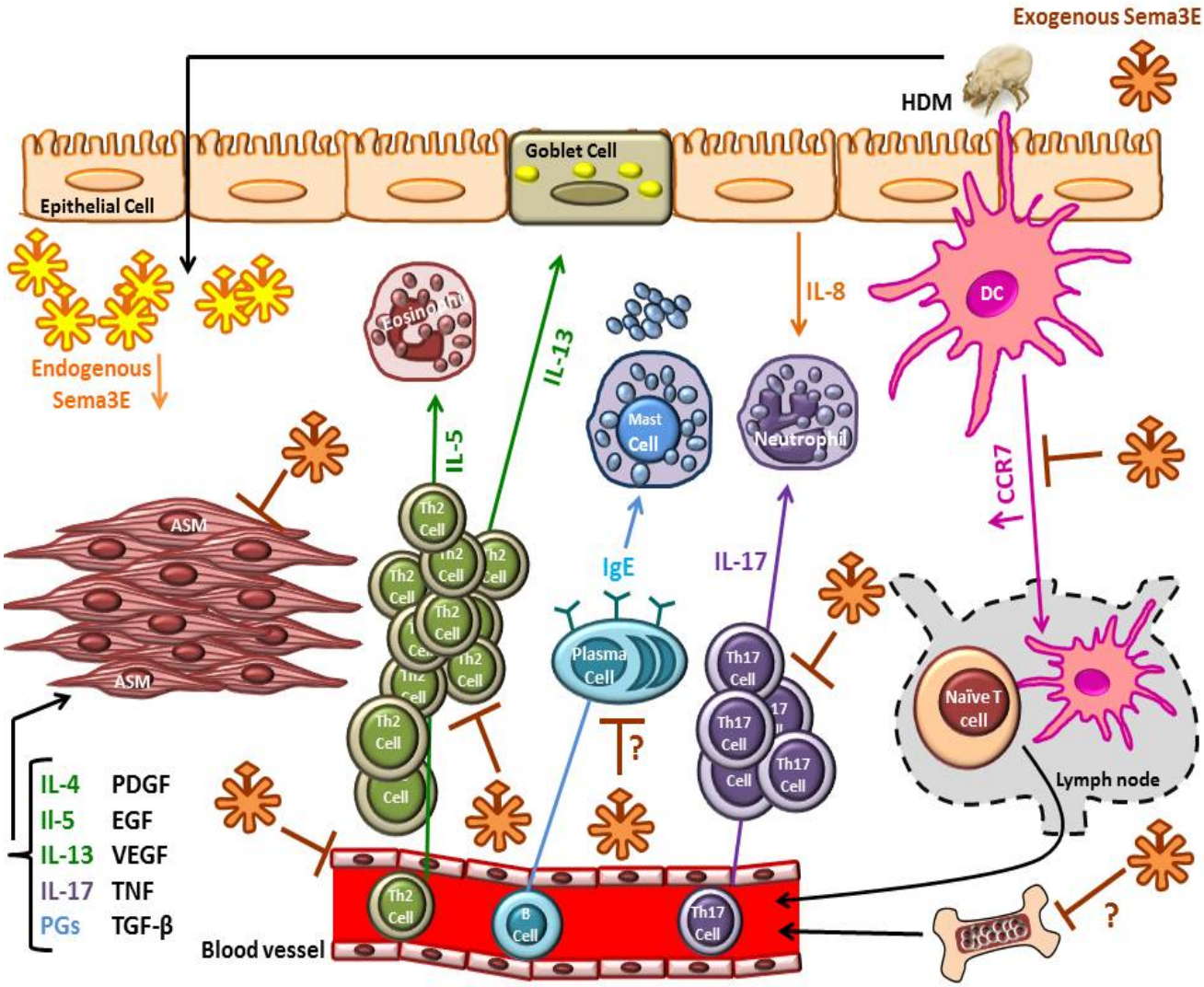

Figure 5-1: An integrative model depicting the potential therapeutic targets for Sema3E administration in asthmatic airways.

Replenishment of Sema3E could lead to minimize DC migration and allergen presentation, Th2/Th17 cytokine response, IgE synthesis by B cells and ASM hyperplasia and recruitment of inflammatory cell progenitors from the bone marrow.

Immunological interventions aiming to treat immune or even non-immune disorders have been extensively examined in preclinical studies or clinical settings. For example, anti-TNF mAb is currently approved to treat inflammatory bowel disease as a successful immunomodulating approach. Blocking cytokines or their receptors by using mAbs or gene silencing techniques are the major approaches in which the target cytokine(s) is pathologically up-regulated or plays adverse roles impairing appropriate immune response. For instance, type 2 mediators such as IgE, IL-4, IL-5, IL-13 and TSLP have been targeted in different clinical trials to treat allergic asthma with some improvements on controlling asthma exacerbation, lung function and inflammation (146). However, the results of these studies indicate that targeting a single mediator/pathway cannot completely overcome various asthma features which could be attributed to the heterogeneity of asthma phenotypes and multiple pathological mechanisms. Conversely, there might be some immune mediators which are down-regulated or do not function properly in a specific disease and their re-introduction could modulate the disease. Nowadays, administration of recombinant IFN is considered as a treatment option for patients suffering from multiple sclerosis.

The potential application of semaphorins as therapeutic approaches for different types of cancer, autoimmune (multiple sclerosis) and allergic (asthma and atopic dermatitis) disorders has been suggested based on experimental models. However, the efficacy of semaphorins has remained to be addressed in clinical settings. The non-redundant role of semaphorins in immune modulation has been previously shown in different mouse models. For example, *in vivo* studies on mice indicate that immune semaphorin Sema4A is involved in induction of Th1 response and proposed as a novel treatment option for allergic asthma. Moreover, Sema7A, besides its pro-fibrotic functions, has been shown to be expressed by airway eosinophils which is increased by

IL-5 family suggestive of a potential contribution in allergic asthma. In this study, we demonstrated an unknown differential expression pattern and the functional role of Sema3E in allergic asthma which could be considered as a novel immunotherapy for this condition. It requires further mechanistic studies in both humans and mice.

6 CHAPTER 6: General conclusions and final comments

6.1 Clinical significance

Asthma is a highly prevalent chronic disorder worldwide and development of effective asthma therapeutics is an unmet health priority. In this project, for the first time, we have found that the level an endogenous guidance cue, Sema3E, is remarkably reduced in asthmatic patients which negatively correlates with the disease severity (FEV1). Allergic deficits are more pronounced in the absence of Sema3E and its intranasal administration alleviates airway inflammation, remodeling and AHR in a mouse model of the disease. Our data introduces a novel biomarker which might be a useful clinical measure to better stratify and endotype the asthma. In addition, replenishment of Sema3E in asthmatic patients, wherein it is surprisingly suppressed, could provide a safe and effective treatment option especially for severe cases which are refractory to currently available treatments.

6.2 Knowledge translation

A dualistic role, repulsion vs. attraction, has been previously described for Sema3E in a context-dependent manner. Here, we provided basic mechanistic evidence to support a regulatory role of Sema3E which is abrogated in asthmatic conditions. It would be of great importance to bridge our basic research findings to a pharmacological feasible approach in which Sema3E could be considered as an applicable therapeutic option in clinical settings for allergic asthma. Furthermore, downregulation of Sema3E in asthma could be translated into a diagnostic approach.

6.3 Limitations and future directions

The data presented in this thesis indicate a previously unknown crucial role of Sema3E-PlexinD1 axis in allergic asthma. However, there are some limitations that should be considered to interpret the results and for future studies. First of all, in our Sema3E expression studies in human samples we are not sure whether previous treatment of the asthmatic patient might have affected Sema3E expression. According to the clinical profiles of the patients, they have not received any medication to treat asthma 2 month before sampling; though it does not rule out any long lasting effect of previous therapies. BALF and lung tissue sections obtained from two different sources and repeating the expression studies using more samples, preferably obtained by non-invasive approaches for example sputum, from diverse human populations would enable us to ascertain the significant down-regulation of Sema3E as a novel biomarker of allergic asthma. Although epithelial cells have been identified as a primary source of Sema3E expression as we found in bronchial epithelial cells, our immunostaining indicates that Sema3E might be expressed by other sources such as inflammatory cells that should be further investigated.

In human ASM cell *in vitro* studies, we used both primary and immortalized cell lines which have been clinically characterized and widely used in similar studies. Although these findings provide some mechanistic insights, they do not necessarily reflect the similar *in vivo* outcome in terms of ASM cell proliferation and migration or underlying signaling events in response to Sema3E treatment. Focusing on the role of Sema3E in ASM cell functions by using a chronic mouse model of allergic asthma would be an appropriate *in vivo* alternative approach.

In mouse studies, we aimed to utilize a comprehensive approach to cover different aspects of asthma pathology such as various aspects of airway inflammation, remodeling and AHR. In future studies, kinetic studies will determine the optimal time and dose of Sema3E effect in each

cellular process. For instance, it is not completely clear that Sema3E role in pulmonary DC subsets is restricted to sensitization phase or it involves the effector phase as well. In addition, different doses of recombinant Sema3E should be examined and compared with current medications in the therapeutic model.

To address the mechanism of Sema3E function, we focused on specific signaling components based on previously published literature considering both semaphorin and asthma points of view. For instance, Rac1 GTPase activity or F-actin rearrangement are involved in Sema3E signaling and also asthma pathology. Although our mechanistic hypotheses were addressed in ASM cells, DC and neutrophils, but there are for sure other unknown pathways and mediators contributing to Sema3E functions in allergic asthma. Therefore, a holistic transcriptomic approach will be extremely helpful to determine which components undergo down/up-regulation upon allergen exposure in the cells of interest from *Sema3e*$^{-/-}$ vs WT mice or from Sema3E treated vs untreated mice. Considering the chronic nature of asthma, the role of Sema3E should be further investigated in a chronic model of allergic asthma wherein HDM exposure would be extended to 7 weeks and then asthma deficits with focus on airway remodeling features studied.

Collectively, according to abovementioned issues, we would further hypothesize for future studies that Sema3E could be considered as a novel biomarker and also a therapeutic option for asthma. The specific aims would include: 1) To measure Sema3E protein level in asthmatic and healthy individuals by a non-invasive approach using sputum instead of invasive methods to obtain human BALF or lung tissue sections; 2) To perform immunophenotyping on inflammatory cells (DCs, neutrophils and lymphocytes) after chronic HDM exposure; 3) To study the involvement of Sem3E in induction of tolerance via pDCs; 4) To investigate the potential role of Sema3E in airway smooth muscle contraction and hyperplasia.

7 CHAPTER 7: REFERENCES

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