PLEXIND1 DEFICIENCY IN MACROPHAGE EXACERBATES HOUSE DUST MITE-INDUCED ALLERGIC ASTHMA

By

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ABSTRACT

Semaphorin3E (Sema3E) and its receptor plexinD1 are involved in cell migration, proliferation, and angiogenesis considered key features of asthma. The absence of Sema3E exacerbates asthma features, and treatment with recombinant Sema3E reduces inflammation and airway hyperresponsiveness (AHR). However, whether Sema3E-plexinD1 axis regulates airway macrophages function in allergic asthma has not been studied. Therefore, we investigated the role of plexinD1 deficient macrophage in allergic asthma. Genetic ablation of plexinD1 receptor in interstitial macrophages was performed *in vivo* by crossing CX3CR1Cre/ERT with *Plxnd1* floxed mice followed by tamoxifen treatment. AHR, airway inflammation and remodeling were measured by flexivent, flow cytometry, histochemistry, ELISA, and RT-PCR techniques respectively. We found that the absence of *Plxnd1* in lung interstitial macrophages increased AHR, airway leukocytes number, allergen-specific IgE, goblet cell hyperplasia, and Th2/Th17 cytokines response in the acute house dust mite model of allergic asthma. Also, the expression of *Muc5ac, Muc5b* and *α-SMA* genes were increased in mice with *Plxnd1* deficient interstitial macrophage compared to WT mice.

Our data suggest that lung interstitial macrophage via Sema3E/plexinD1 axis negatively regulates airway inflammation, AHR, and airway remodeling in a murine model of allergic asthma.

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DEDICATIONS

This dissertation is dedicated to:

My parents and my siblings.

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LIST OF ABBREVIATIONS

AHR: Airway Hyper Responsiveness

ACK: Ammonium-Chloride-Potassium

AM: Alveolar macrophage

ANOVA: Analysis of variance

ASM: Airway Smooth Muscle

ASMC: Airway Smooth Muscle Cell

BALF: Broncho-Alveolar Lavage Fluid

bFGF: basic Fibroblast Growth Factor

BM: Bone marrow

BMDM: Bone marrow-derived macrophage

BSA: Bovine Serum Albumin

CACS: Centre of Animal Care

CCL: Chemokine ligand

CCR: Chemokine receptor

CD: Cluster of Differentiation

cDNA: Complementary Deoxyribonucleic acid

CUB: Complement C1r/C1s, Uegf, Bmp1

CXCL: Chemokine Ligand

CXCR: Chemokine Receptor

DC: Dendritic cell

ddH2O: Double-distilled water

DNA: Deoxyribonucleic acid

DP: Double-positive

ECM: Extracellular Matrix

ECP: Eosinophil Cationic Protein

EC: Endothelial Cell

EDN: Eosinophil-derived neurotoxin

EDTA: Ethylenediaminetetraacetic acid

EEF2: Eukaryotic Elongation Factor

EGF: Epidermal Growth Factor

EGFL: Epidermal Growth Factor Like Protein

EGFR: Epidermal Growth Factor Receptor

ELISA: Enzyme-Linked Immunosorbent Assay

EMR: EGF-like module-containing mucin-like hormone receptor-like

EMT: Epithelial-to-mesenchymal transition

EPO: Eosinophil Peroxidase

FACS: Fluorescence-Activated Cell Sorting

FBS: Fetal Bovine Serum

Fc: Fraction crystallizable

FcγR: Fc γ receptor

FEV1: Forced expiratory volume in one second

FGF: Fibroblast Growth Factor

FMO: Fluorescence minus one

FOT: Forced Oscillation Technique

GAP: GTPase Activating Domain

GDP: Guanosine Diphosphate

GMC: Genetic Models Center

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

GPCR: G-protein-Coupled Receptor

GPI: Glycophosphatidylinositol

GTP: Guanosine Triphosphate

H & E: Hematoxylin and Eosin

H2O2: Hydrogen peroxide

HASMC: Human Airway Smooth Muscle Cell

HDM: House Dust Mite

HIF: Hypoxia Inducible Factor

HRP: Horseradish peroxidase

HSPG: Heparan sulfate proteoglycan

i.n: Intranasal

ICAM: Intercellular cell adhesion molecule

IFNγ: Interferon-γ

Ig: Immunoglobulin

IGF-1: Insulin-like Growth Factor 1

IL: Interleukin

ILC: Innate lymphoid cell

IM: Interstitial macrophage

IPT: Ig-like Plexins and Transcription Factors

KO: Knock out

LPS: Lipopolysaccharides

LTB: Leukotrienes

Lyve: Lymphatic vessel endothelial hyaluronic acid receptor

mAb: Monoclonal Antibody

MAM: Meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu

MBP: Major Basic Protein

MCh: Methacholine

MCP-1: Monocyte Chemoattractant Protein-1

MerTK: MER proto-oncogene tyrosine kinase

MHC: Major histocompatibility complex

MLN: Mediastinal lymph node

MMP: Matrix Metallopeptidase

MPO: Myeloperoxidase

MRS: MET-Related Sequence

MyD88: Myeloid differentiation primary response 88

МΦ: Macrophage

NE: Neutrophils elastase

NK cell: Natural Killer cell

NRP: Neuropilin

OD: Optical Density

PAF: Platelet-activating factor

PAK: p21-activated kinase

PAS: Periodic acid-Schiff

PBM: PDZ-binding motif

PBS: Phosphate Buffered Saline

PCR: Polymerase chain reaction

PDGF: Platelet Derived Growth Factor

PDGFR: Platelet Derived Growth Factor Receptor

PGE2: Prostaglandin E2

PMA: Phorbol 12-myristate-13-acetate

PSI: Plexin Semaphorin Integrin

Q-PCR: Quantitative polymerase chain reaction

RBC: Red blood cell

RBD: Rho-GTPase-binding domain

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

RNA: Ribonucleic acid

ROI: Reactive oxygen intermediates

ROS: Reactive oxygen species

RPMI: Roswell Park Memorial Institute medium

RQ: Relative quantitative

RT: Room temperature

SEM: Standard error of the mean

Sema: Semaphorin

SMA: Smooth Muscle Actin

SP: Sex and Plexins

SP: Single-positive

SP: Surfactant protein

SR: Sirus red

TGF: Transforming Growth Factor

Th cell: T helper cell

TIM: T-cell Immunoglobulin and Mucin Domain

TLR: Toll-like receptor

TM: Transmembrane

TNF: Tumor Necrosis Factor

Treg: T regulatory

TSLP: Thymic Stromal Lymphopoietin

VCAM: Vascular Cell Adhesion Molecule

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

VSMC: Vascular Smooth Muscle Cell

WHO: World Health Organization

WT: Wild Type

1 INTRODUCTION

1.1 Chapter 1: Asthma

1.1.1 Asthma

Asthma is a chronic relapsing disease of the lung. The clinical symptoms of asthma may include whistling sound during exhale because of repeated episodes of wheezing, shortness of breath, chest tightness, and nighttime or early morning coughing[1, 2]. However, according to the pathological view, asthma is defined as a chronic inflammatory disease of the lung, which is characterized by airway hyperresponsiveness, airway inflammation, and tissue remodelling [3-6].

1.1.2 Prevalence of asthma

Asthma is one of the most common chronic lung diseases in the world [7, 8]. The prevalence of asthma varies among different countries across the globe. Although asthma rates were higher in developed countries, gaps are closing day by day because of the rising prevalence in low and middle-income countries.

The increasing incidence of asthma is associated with a modern and urban lifestyle and allergy. It is estimated that around 300 million people have asthma with 250,000 annual death worldwide. Mortality rates of asthma are the highest in some countries where access to appropriate medication is lacking. The prevalence of asthma is increasing in both adults and children day by day; thus, it has become one of the major public health problems. According to the World Health Organization (WHO, 2007 report), the number of people with asthma will increase by 100 million by 2025[9].

1.1.3 Pathogenesis of asthma

The pathogenesis of asthma is quite complex. Different types of pathological changes happen during asthma. Immune inflammatory cells and structural cells play a vital role in the pathogenesis of asthma. The pathological change involves airway inflammation, airway remodelling, airflow obstructions, and airway hyperresponsiveness.

1.1.3.1 Airway inflammation

Asthma is defined as an inflammatory disease of the airways, which is characterized by Th2/Th17-biased responses with an increased number of neutrophils, eosinophils, mast cells, macrophages, and activated lymphocytes in the airways. These inflammatory cells secrete different types of modulators, such as cytokines, chemokines, histamine, immunoglobulins, growth factors, and lipid mediators. These modulators induce hyper-reactivity, mucus overproduction, collagen deposition, airway smooth muscle hypertrophy or hyperplasia, and thus airway remodelling [10]. The role of these inflammatory cells in the progression of asthma is explained below:

Eosinophils: Eosinophils play a significant role in the pathogenesis of allergic asthma [11]. In the presence of airway antigen, Th2 cytokines, including IL-5 and eotaxin-1/CCL11 promote the release of eosinophils from bone marrow. Chemokine receptor (CCR3) present on eosinophils binds to chemoattractant (eotaxin) migrate to the lung where they release granule proteins, including eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil-derived neurotoxin (EDN) ECP. EDN proteins are cytotoxic and have ribonuclease activity whereas EPO catalyzes the reaction of hydrogen peroxide with halogen to produce hypo halides [12, 13]. Like MBP, EPO promotes the cytotoxic process leading to

epithelial cell's damage. They can also promote the degranulation of mast cells [11]. MBP has also the ability to bind to the muscarinic M2 receptor that causes constriction of the airway.

In addition to the role of eosinophils on structural cells damage, they can synthesize and release cytokines (mainly IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-13, and TNFα); chemokines (mainly IL-8 and RANTES), lipid mediators (leukotrienes and prostaglandins); and oxygen radicals. Eosinophils also release fibrogenic cytokines TGFβ and is the main source of TGFβ during asthma; thus it plays a vital role in airway remodelling. Collectively, eosinophil activation results in epithelial and endothelial cells damage, inhibition of muscarinic receptor, induction of fibrosis leading to airway remodelling and airway hyperresponsiveness [10, 14, 15].

Neutrophils: Although it has been commonly considered that Th2 immune response and eosinophilia are the main cause of allergic asthma, some asthma patients show neutrophils-dominating disease with low or even absent Th2 responses suggesting a key role of these cells in the development of certain types of asthma [16-18].

It is thought that neutrophils come first at the site of inflammation [16]. At the inflammatory site neutrophils release O2, matrix metalloprotease-9(MMP-9), leukotrienes-4(LTB-4), neutrophils elastase (NE), and platelet-activating factor (PAF)[17]. MMP-9 are involved in airway remodelling [19], NE induces airway goblet cells hyperplasia, mucous secretion [20]; NE and exosome regulate the proliferation of airway smooth muscle cells[21]; and α-defensin damage the airway epithelial cells[22]. Other mediators secreted from neutrophils including myeloperoxidase, bFGF, PDGF-BB, VEGF, oxidation product, and serine neutral proteases are involved in epithelial damage, fibrosis, and angiogenesis [23-28]. Thus, activated neutrophils cause AHR, bronchospasm, lung tissue damages, mucus overproduction, and airway remodelling leading to irreversible alteration of airway structure.

Th2 cells: Allergic asthma is mainly regulated by type 2 immunity. Th2 cells are the main orchestrators of type2 inflammation. Allergen exposure promotes the differentiation of naïve CD4+ T cells to Th2 cells, which secrete different types of cytokines, particularly IL-4, IL-5, IL-9, and IL-13 [29-31]. IL-4 is critical for the differentiation of Th0 to Th2 cells and for inducing isotype switching to IgE production. IL-5 and IL-9 are independently responsible for differentiation, activation, and recruitment of eosinophils and mast cells to the airway. On the other hand, IL-13 is accountable for goblet cell hyperplasia, mucin and mucus production, and AHR. T_{FH} cells are also able to promote IgE production by interacting with B-cells [32, 33].

Th17 cells: Th17 cells are activated by airway sensitization with the allergen. IL-17A cytokines, secreted by Th17 has an elevated level in the biopsy of asthmatics. IL-17A promotes the production of IL-8 and IL-6 by airway smooth muscle cell[34-37], lung epithelial cells [38, 39], and in the pulmonary vascular endothelial cells [40-42]. IL-8 (CXCL8) acts as a chemoattractant to promote the migration of neutrophils to the site of inflammation. IL-17A also induce secretion of vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1). Thus IL-17A promotes the recruitment of neutrophils at the site of inflammation [43, 44]. A strong correlation was found between the level of IL-17A and neutrophils in lung diseases. Deficiency of IL-17A causes impaired neutrophilic responses to allergen, reduced AHR and airway remodelling [42, 45]. All this information says the activation of Th17 cells might play role in the predisposition of neutrophilic asthma [46]. However, IL-17A also induce chemokine eotaxin-1/CCL11 production by airway smooth muscle cells [47], which indicates IL-17A may play role in the recruitment of eosinophils to the airway in the allergic asthma by inducing eotaxin-1/CCL11 production.

B-lymphocytes: B cells directly uptake allergen independently of B-cell receptor specificity. Sensitized B cells present uptaking allergen to CD4+ T cells through MHC-II and in turn, convert to effector Th2 cells [48]. Besides, B-cells activate Th2 cells to produce different cytokines including IL-4 and IL-9 that activate B cells to class switching to release IgE antibody. These IgE antibodies bind to high-affinity IgE receptor FcεRI present on mast cells, eosinophils, and basophils, therefore sensitize these cells to the allergen. Cross-linking of adjacent FcεRI-IgE complex by allergen exposure activates these cells to degranulate. As a result, they release various types of mediators that lead to smooth muscle contraction, vasodilation, mucous secretion, airway remodelling, and ultimately AHR[10].

Mast cells and Basophils: Both mast cells and basophils express high-affinity Fcε receptor I (FcεRI). Cross-linking of adjacent IgE- FcεRI receptor by allergen activate these cells and release different types of mediators through degranulation [10]. Cytosolic granules of mast cells contain cytokines (IL-4, IL-5, IL-6, and IL-13), different lipid mediators (prostaglandins, leukotrienes, platelet-activating factor, and sphingolipids), biogenic amines (histamine and serotonin), mast cell-derived proteases (chymase and tryptase), serglycin, and proteoglycans. These mediators' effects lead to allergic asthma features including recruitment of eosinophils, smooth muscle contraction, mucus overproduction, vasodilation, airway remodelling, and airway hyper-reactivity. Basophils regulate the late phase of allergic responses by inducing eosinophils recruitment and mucus overproduction [30, 49].

Innate lymphoid cells: Innate lymphoid cells (ILCs) are a newly discovered member of the immune cells group that mirror the phenotypes and function of T cells. Both human and mouse group 2 innate lymphoid cells (ILC2) are activated by IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) upon allergen exposure and produce a vast amount of IL-5 and IL-13

cytokines, which promote airway inflammation and hyperresponsiveness[50-55]. Activated ILC2 cells produce relatively high levels of IL-4 compared to other cells thus act as a major promoter of Th2 differentiation leading to induction of airway allergic inflammation[50, 51, 55]. ILC3 are also able to produce IL-17 during asthma[46].

Dendritic cells and macrophages: Dendritic cells (DCs) are the main antigen-presenting cells that promote cytokines production. In the presence of an allergen, dendritic cells acquire the allergen and migrate to the secondary draining lymph node where it presented it to naïve T cells to polarize to Th2 cells [29]. Allergen with low level of endotoxin induce Th2 responses, however, high level of endotoxin induce Th1 responses[56, 57]. In addition to activation of Th1 and Th2, DC also regulates T-cells lineage to the Th17[58, 59]. Moreover, macrophages, are the most frequent cells in the airway, express low-affinity IgE receptor. They release inflammatory mediators and cytokines once activated by allergens[60].

All this above information indicates that leukocytes play an important role in airway inflammation, which plays a crucial role in the pathogenesis of asthma. Airway inflammation is the initial stage of asthma that trigger and promote other physiological change in allergic asthma such as airway hyperresponsiveness and airway remodelling.

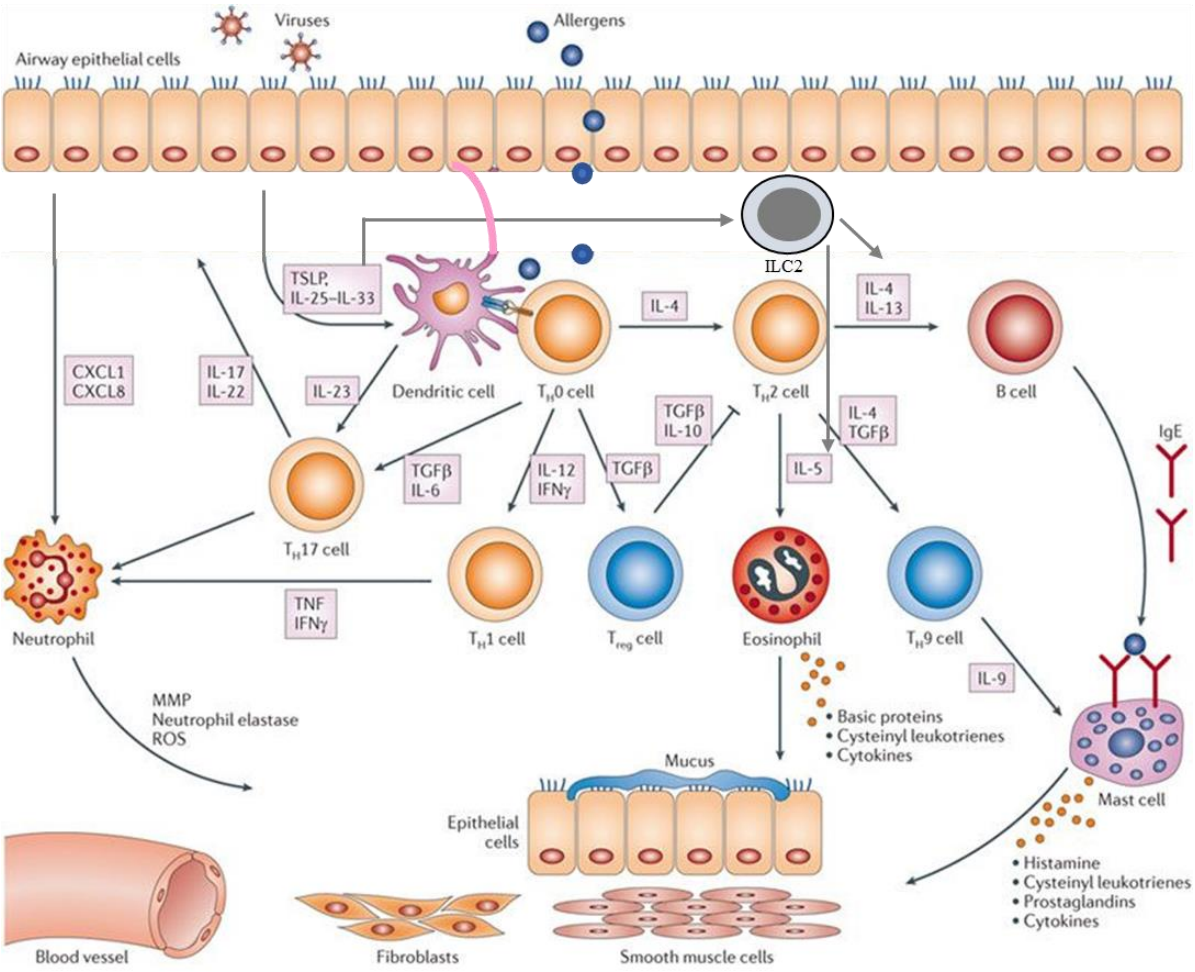


Figure 1. 1: Pathophysiology of airway inflammation in asthma. Asthma is a heterogenious diseases, which originates from complex interactions between genetic factors and environmental agents including allergen and viruses. Allergens can be taken up by dendritic cells in the airway lumen which is processed to antigenic molecules and present them to naive T helper (Th0) cells. The activated allergen-specific Th2 cells produce IL-4 and IL-13, which promote B cell to synthesize IgE antibodies. In addition, Th2 cells secrete IL-5, which induces eosinophil maturation, migration, and survival. All these events are promoted due to functional defect in IL-10- and TGFβ-producing TReg cells, which normally regulate Th2 cell-mediated responses by an immunosuppressive function. Type 2 innate lymphoid cells (ILC2) are activated by epithelial

cells derived cytokines (TSLP, IL-33, and IL-25). ILC2 release IL-5 to activate eosinophils and IL-13 to induce mucus production. In addition to Th2 and ILC2 cells, activated Th9 cells secrete IL-9, which promote the growth and recruitment of mast cells. IgE-dependent degranulation of mast cells secrete both preformed and newly synthesized mediators. Another important T lymphocytes are Th17 cells, which contribute to asthma pathology by producing IL-17A and IL-17F. These cytokines induce neutrophil recruitment and expansion. Moreover, IL-12-dependent Th1 cells secrete IFNγ due to airway infections. Finally, many mediators, cytokines and growth factors secreted by various cells may also affect the functions and proliferation of airway structural-type cells, including epithelial cells, fibroblasts, smooth muscle cells, and endothelial vascular cells. IL-4, interleukin-4; IgE, immunoglobulin E; TGFβ, transforming growth factor-β; TReg, T regulatory; IFNγ, interferon-γ; TSLP, thymic stromal lymphopoietin; TNFα, tumor necrosis factor-α; CXCL1, chemokine CXC motif ligand 1; MMP, matrix metalloproteinase; ROS, reactive oxygen species [61].

Adapted and modified from: The potential of biologics for the treatment of asthma. *Nature Reviews Drug Discovery,* 2012.

1.1.3.2 Airway hyperresponsiveness

Airway hyperresponsiveness is a characteristic feature of asthma and is defined as an increased sensitivity of the airways to any stimuli (allergen or chemical) that leads to bronchoconstriction and the predisposition of the airways to excessively narrow. All the stimuli (chemical or allergen) causes bronchoconstriction by binding to a receptor present on airway smooth muscle (ASM). AHR has been considered a hallmark of asthma [62, 63]. The severity of asthma correlates with the severity of AHR [64]. AHR is the result of airway resistance as the increased airflow obstruction correlates with the increased airway hyperresponsiveness [65].

Causes of airway hyperresponsiveness:

There are three causes of airway hyperresponsiveness that happen sequentially or simultanously:

1). Genetic Predisposition

2). Airway inflammation

3). Structural change of airway

Genetic predisposition

It has been shown that familial clustering exists for airway hyperresponsiveness and asthma [66, 67]suggesting that genetics predispose to asthma in combination to environmental factors. Therefore, a combination of genetics and environment play a significant role in the predisposition of airway hyperresponsiveness.

Airway inflammation

Exposure to an allergen, a virus or any other components induces the recruitment of leukocytes to the airway that leads to the transient airway hyperresponsiveness. It is now confirmed that an increased number of different leukocytes, mainly eosinophils and neutrophils release various mediators and cytokines at the airway. These mediators and cytokines cause changes in the airway, including epithelial damages, thickening of the basement, release mediators that bind to the receptor of ASM to cause bronchial smooth muscle contraction and plasma exudation that ultimately causes the airway hyperresponsiveness (Figure 1.2) [62]. Moreover, Parasympathetic nerves play an essential role in the symptom and inflammation of allergic diseases mainly by signaling through muscarinic receptor present on ASM, secretory gland and different inflammatory cells [68]. Parasympathetic nerves travel to postganlionic nerves that release neurotransmitter (acetylecholine), which bind to the M3 muscarinic receptor on ASM and mucus gland lead to airway bronchoconstriction and mucus secretion respectively [69, 70]. In addition, major basic protein produced by eosinophils induce acetylecholine release that causes bronchoconstriction in allergic asthma [71]

Structural change of airway

The degree of airway hyperresponsiveness correlates with the structural change of the airway[72-75]. Over exposure to the allergen causes the permanent structural change of the airway that leads to persistent airway hyperresponsiveness. The structural changes include mucus overproduction, goblet cell hypertrophy and hyperplasia, subepithelial fibrosis, angiogenesis, collagen deposition at the extracellular matrix, airway smooth muscle hypertrophy or hyperplasia, increased α-SMA in ASM, epithelial cell damage, and edema due to plasma exudation [62, 76-79].

It has also been shown that the degree of airway hyperresponsiveness correlates with the loss of epithelial structure [80]. This is because the partial loss of epithelial barrier allows a considerable amount of bronchoconstrictor mediators to reach ASM or other cells, thus enhance bronchoconstriction in the airway. On the other hand, the release of bronchodilation mediators is decreased from epithelial barrier due to damage, that enhances bronchial smooth muscle contraction [62].

Lung functions use to measure AHR

Airway hyperresponsiveness is determined by measuring lung airway resistance (Rrs), airway conductance (Grs), and airway elastance (Xrs) by forced oscillation technique (FOT). The severity of AHR correlates with lung high airway resistance, low airway conductance, and high airway elastance[81].

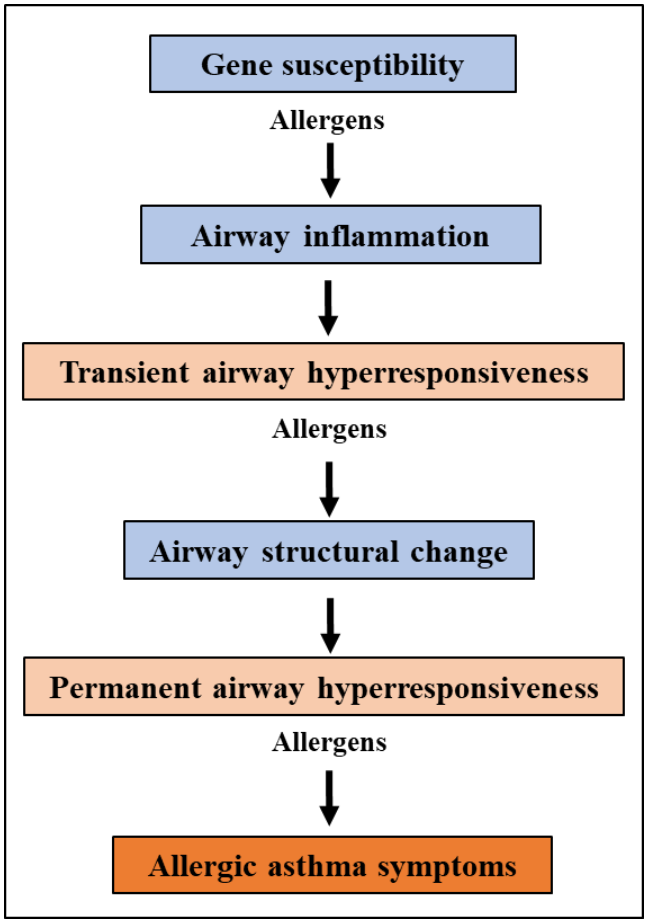


Figure 1. 2: Causes of allergic asthma symptoms. The interaction of the susceptible genes and the environmental factors including allergens cause airway inflammation and transient airway hyperresponsiveness in asthma patients. Over exposure to the allergen causes structural change leading to permanent airway hyperresponsiveness [62].

1.1.3.3 Airway remodelling

Airway remodelling is defined as any change in composition, distribution, mass or volume, thickness, and the number of the structural components in the airway in patients compared to healthy subjects. There are two types of remodelling: (I) Physiological airway remodelling that happens during normal lung development or due to injury or inflammation, which can be restored.

(II) Pathological airway remodelling that occurs due to disturbed lung development or chronic injury/inflammation, which leads to permanent alteration of airway wall structure [82].

Airway remodelling has been observed in all types of asthma severities, and both small and large airways[83, 84]. Structural changes that happen during airway remodelling include epithelial damage[85], subepithelial fibrosis[86], thickening of basement membrane[87], goblet cell hyperplasia and mucus overproduction[88], submucosal gland enlargement[89], smooth muscle cell hyperplasia and hypertrophy[88], increased bronchial vasculature including barrier dysfunction and airway angiogenesis [90, 91], and decreased cartilage integrity[92]. (Figure 1.3)

Epithelial change: Airway epithelial barrier plays an essential role in maintaining healthy lung by particle clearance, fluid balance, and innate immune responses. Direct injury of the airway epithelial barrier by different triggers is the initial step of the asthma pathogenesis [93-95]. Epithelial change in asthma includes epithelial peeling, goblet cell hyperplasia, loss of ciliated cells, increase growth factor release, and increased expression of epidermal growth factor receptor (EGFR)[85, 88, 96-98]. The asthmatic epithelium has a higher turnover (loss is higher than proliferation) than a healthy person, and it has a weak attachment to the basement membrane. The loss of epithelial integrity allows the allergen to enter the airway easily that ultimately leads to asthma. Epithelial damage correlates with airway hyperresponsiveness [80]. Besides, dysregulated epithelium produce and release different types cytokines and growth factors such as TNF, IL-1β, IL-6, PGE2, IL-8, MCP-1, RANTES, eotaxin-11/CCL11, oncostatin-M, IL-11, IL- 10, IL-16, IL-18, GM-CSF, b-FGF, TGF-β1 and TGF-β2, IGF-1, PDGF-BB, IL-5 and IL-13. These cytokines and growth factors induce remodelling of the underlying mesenchyme of epithelium. Thus, epithelium-mesenchymal interaction has a significant role in the airway wall thickening[99].

Subepithelial fibrosis: Subepithelial fibrosis is a second important feature of airway remodelling in asthma[86, 100]. Subepithelial fibrosis starts with increased number of myofibroblasts and fibroblasts under the bronchial epithelial basement membrane [101]. Subepithelial fibrosis occurs in the lamina reticularis layer, which causes thickening of the basement membrane just under the epithelium.

In asthmatics, in addition to the expression of cell surface receptors, fibroblast and myofibroblast produce cytokines and chemokines, which play an essential role in the cell adhesion and leukocytes activation leading to the development of airway inflammation [102]. Fibrosis occurs due to increased deposition of extracellular matrix proteins, including collagens I, III, and V, fibronectin, tenascin, lumican, and biglycan secreted by fibroblast [87, 103-106]. Thus, subepithelial fibrosis causes airway wall thickening and has been associated with the severity of asthma.

Goblet cells hyperplasia and mucus overproduction: Goblet cells hyperplasia and mucus overproduction is another cause of airway wall thickening and narrowing. Both goblet cells and submucosal hyperplasia have been documented in the airway of patients with asthma, especially in fatal asthma[88, 89, 107]. Goblet cells are in the epithelium of the airway and act as a defensive system by secreting mucin glycoproteins into the respiratory tract. The number of goblet cells increased in the asthmatics airway due to the increase of goblet cells hyperplasia and metaplasia, which is the conversion of non-granulated cells to goblet cells. As a result, the production of mucin increased and thus, the accumulation of the high amount of mucus in the airway leads to the airway thickening and narrowing ultimately causes airway obstruction [108, 109]. Of note, mucus overproduction has been confirmed by showing a higher expression of MUC5AC in the lung of patients with asthma compared to healthy control [110].

Although the mechanism of goblet cell hyperplasia is not completely understood, Th2 cytokines (IL-4 & IL-13) play an essential role in the induction of goblet cell hyperplasia, mucin gene expression, and mucus overproduction [111, 112]. Moreover, epidermal growth factor receptor (EGFR) present on airway epithelium plays an important role in the goblet cell metaplasia [113]. The activation of EGFR signalling by EGF, oxidative stress or oxygen-free radical secreted by activated neutrophils causes overproduction of mucus in the airway [113-116]. Therefore, goblet cells hyperplasia and mucus overproduction contributes to airway thickening and narrowing which causes airway hyperresponsiveness.

Airway smooth muscle mass: Increasing the mass of airway smooth muscle (ASM) is an essential feature of airway remodelling. Airway smooth muscle mass is increased in the asthmatic's airway due to hyperplasia (increase in number) and hypertrophy (increase in size). Importantly, ASM cells can migrate to the subepithelial area of the airway of patients with asthma [88, 117, 118]. Chemokines can induce the migration and contractility of ASM cell which may play an essential role in the airway thickening and thus obstruction of the overall airflow and airway hyperresponsiveness.

ASM cells also play a vital role in the inflammation and remodelling process by releasing proinflammatory cytokines, chemokines, and ECM proteins[119-121]. Increased rates of division or decreased rates of apoptosis of ASM cells causes ASM hyperplasia[122]. The number of ASM cells increased due to the influence of different mediators including cytokines, matrix metalloprotease-2 (MMP-2), components of extracellular matrix (ECM), mechanical stress, and reactive oxygen species.

Moreover, different mediators and cytokines that stimulates ASM cells hyperplasia including growth factors PDGF-BB, EGF, FGF-2 and insulin-like growth factor that activate

tyrosine kinase activity), contractile agonist (α-thrombin, serotonin, thromboxane, endothelin-1, leukotriene that work through G-protein coupled receptors), and pro-inflammatory cytokines (IL-1β, TNF, IL-6 that works through cytokine receptors) [122-126]. It has been shown that abnormality in smooth muscle contractility, leads to hypercontractile phenotype, has a role in the ASM cell hypertrophy. ASM cell hypertrophy and hyperplasia lead to exacerbation of airway structural change, which ultimately causes persistent airway inflammation, AHR and airway obstruction in allergic asthma [123].

Vascular remodelling and angiogenesis: Vascular remodelling or angiogenesis in the airway is another critical characteristic of airway remodelling in allergic asthma [127]. Angiogenesis is multiple complex processes in which pre-existing vessels generate a new one[78]. It has been found that higher expression of vascular endothelial growth factor (VEGF) in the airway of asthmatics that are associated with airway vascularity [128]. The subsequent result of airway wall angiogenesis is airway wall edema, delivery of inflammatory and remodelling mediators into the airway wall that ultimately causes airway inflammation and remodelling.

Cartilage integrity loss: Airway wall stiffness and integrity is maintained by cartilage. Increased cartilage proteoglycan degradation and reduced cartilage volume have been found in the airway of patients with asthma [90]. Cartilage degradation leads to the chronic airway obstruction and strong bronchoconstriction in the ASM bundles[129].

All these structural changes cause airway remodelling in the airway of patients with asthma. Thus, the severity of asthma correlates with airway remodelling [83] (Figure 1.4).

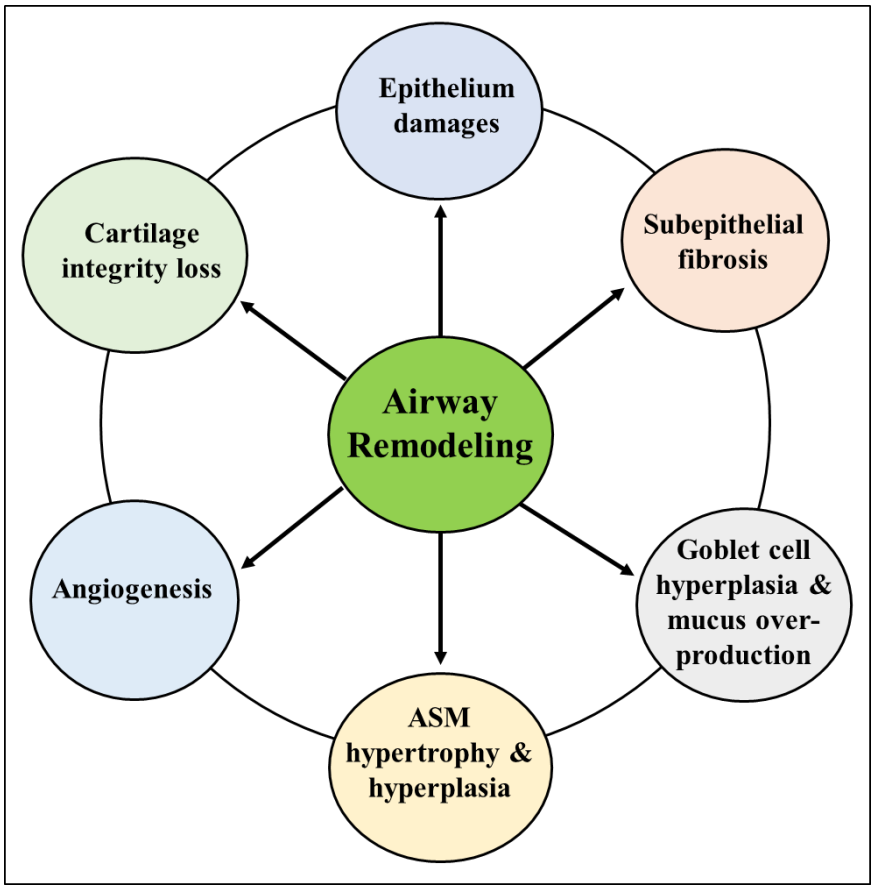


Figure 1. 3: Airway remodelling in asthmatics. Airway remodelling is defined as the structural changes in the airways of patients with asthma. These changes include epithelium damage, subepithelial fibrosis, goblet cell hyperplasia and mucus overproduction, airway smooth muscle (ASM) cell hyperplasia and hypertrophy, angiogenesis, and loss of cartilage integrity [83].

1.2 Chapter 2: Macrophages in asthma

1.2.1 Macrophages in asthma

Macrophages are one of the inflammatory cells that are found in different spaces of the lung, such as alveoli, interstitium, conducting airways, and distal airspaces. The number of macrophages in the lung is more significant compared to other leukocytes, [130-132] suggesting that they have a vital role in the protection of the host from environmental insults. Although it is well established that type 2 inflammation (cells and cytokines) play a central role in the predisposition to allergic asthma, different studies have shown a role of lung macrophages in airway inflammation and airway remodelling [32, 133, 134].

Types of lung macrophages

lung macrophages have mainly been classified into two types:

- Alveolar macrophages (AMs)
- Interstitial macrophages (IMs)

AMs are residing in the alveolar lumen when IMs reside in the interstitium of the lung (figure1.4). AMs act in a nonspecific innate defence mechanism, whereas, IMs interact with other lymphocytes leading to their activatation [135-137]. Although the origin of IMs is not currently well defined, a study on developmental origin of lung macrophages has shown that 'primitive' IMs originate from yolk sac when 'definitive' IMs originate bone marrow. Primitive IMs develop from yolk sac during embryogenesis and populated the lung interstium (peripheral and perivascular zone) during first week of post natal period where they maintain themselves by self-renewing, whereas

definitive IMs develop from bone marrow during first week of birth and maintain their number from blood monocytes throughout the life (Figure 1.4) [138].

On the other hand, early studies have shown that AMs originate from bone marrow-derived blood monocytes [139, 140]. Whereas, later it has been shown that AMs originated from fetal monocytes and populate in the empty alveolar space immediately after birth, where they maintain their population by self-proliferation without recruiting blood monocyte [138, 141]. However, AMs can also originate from blood monocyte where IMs act as an intermediate state between AMs and monocytes [141-143]. Although AMs pool is maintained by local proliferation during type 2 inflammation[144, 145], it has also been shown that AM can arise from blood monocyte after allergen exposure [146].

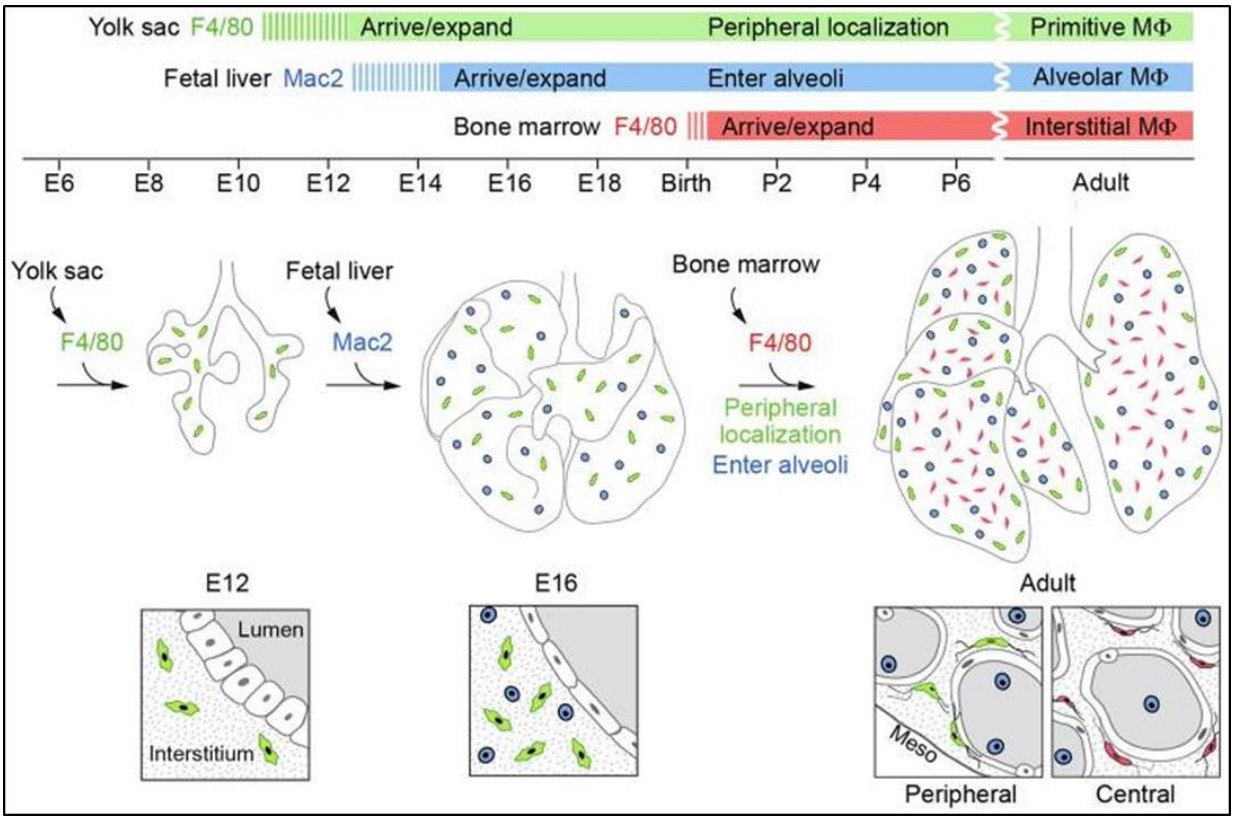


Figure 1. 4: Model of developmental origin of lung macrophage diversity. Timeline (top) and schematics (middle) of lungs at E12, E16, and in the adult illustrating the major developmental events and global localization of the three lung macrophage lineages shown in green, blue and red. Schematics at the bottom are close-ups showing interstitial and lumenal localization of the macrophage lineages, with airway epithelial cells indicated in white, the airway lumen in gray and the interstitium stippled. F4/80 embryonic macrophages (green) arrive from yolk sac in the earliest wave beginning at E10.5. Two days later, Mac2 embryonic macrophages (blue) begin arriving, most likely from the fetal liver [141]. During the first week of postnatal life, Mac2 macrophages enter the lumen to become alveolar macrophages, which self-renew. F4/80 embryonic macrophages persist at specific locations (peripheral and perivascular) to become the 'primitive' interstitial macrophages, whereas those in the parenchyma are replaced by a new population of 'definitive' interstitial macrophages (red) from the circulation, presumably of bone marrow origin. Both F4/80 lineages turn off F4/80 and turn on MHCII during the first 3 weeks of postnatal life (not shown). Meso, mesothelium; MΦ, macrophage.

Adapted from: Developmental origin of lung macrophage diversity by Serena Y.S Tan et al, *Development.* 2016 Apr 15; 143(8): 1318–1327 2016. doi**:** **10.1242/dev.129122**

1.2.2 Alveolar macrophages in asthma

AMs play both as an anti-inflammatory and pro-inflammatory, depending on the origin of AMs proliferation during allergic asthma. Depletion of blood monocytes caused less eosinophilic inflammation in allergen-challenge mice [146]. On the other hand, depletion of resident AMs by the administration of clodronate through the intratracheal route results in increased eosinophilic inflammation[145]. All these data suggest that resident AMs serve as an anti-inflammatory cell to maintain lung homeostatic when recruited monocytes to lung induce allergic inflammation[145, 146].

Anti-inflammatory role of AMs: Anatomic location of AMs suggests that they are the first line of defence to protect the lung from different pathogens, through activating immunological responses, and from excessive tissue damage by inducing an anti-inflammatory response.

Allergen-induced airway inflammation and hyperresponsiveness were ameliorated upon transferred of AMs from unsensitized mice to macrophage -depleted sensitized mice [147]. AMs which are not primed with allergen has higher regenerative capacity than that of AMs from sensitized mice, thereby it provides protection against allergic asthma symptoms [148, 149]. Moreover, unsensitized AMs can suppress the super-proinflammatory function of the dying cells in the lung through appropriate phagocytosis and inhibiting other antigen-presenting cells to activate inflammatory cells[150, 151]. AMs actively suppress the maturation of lung DC, thus inhibit the antigen-presenting function of DC in the lung[151].

AMs can also attenuate Th2 cells' behaviour in asthma through secreting IL-10. Because IL-10 production by AMs is higher in asthmatics, and thus these cells are involved in the downregulation of inflammation[152]. AMs play their anti-inflammatory role in the maintaining of the lung homeostatic by secreting IL-10 [153-155]. Adoptive transfer of AMs treated with prostaglandin

E2 (PGE2) *in vitro* into lung ameliorate HDM- induced allergic asthma. Here, PGE2 boost macrophages to produce a large amount of IL-10[156]. These IL-10 inhibit the proliferation of type2 cells and DC maturation during allergen exposure[157, 158]. Moreover, AMs can reduce inflammation in lung epithelium by providing suppressor of cytokine signalling (SOCS) proteins into the extracellular vesicle [159]. PGE2 or IL-10 contributes to packaging SOCS proteins within secreted vesicles of AMs. Moreover, AMs produces nitric oxide, which increases in the asthmatic state. Increased nitric oxide inhibits the production of inflammatory cytokines by AMs, and thus AMs acts as an immunosuppressive [160].

Pro-inflammatory role of AMs: Although so far, AMs have been showed to play an anti-inflammatory role in allergic asthma; different studies have indicated a role in the development of asthma and driving Th2/Th17 inflammation. AMs from allergic asthma mice are capable of inducing Th2 cytokines secretion, eosinophilic inflammation, and airway hyperresponsiveness upon transfer to unsensitized mice followed by allergen challenge[134]. Furthermore, AMs rather than Th17 is the main producer of IL-17 in ova-induced asthma, and their number increased after allergen challenge[161]. Histamine and serotonin released from mast cells activate AMs to produce IL-17, which recruits neutrophil to the airway[162].

Moreover, AMs derived from recruited monocytes are pathogenic, and blocking their recruitment reduces allergic asthma [145, 146]. Blocking CCR2 of monocytes results in the decreased number of eosinophils, lower number of AMs, lower airway hyperresponsiveness in bronchoalveolar lavage [163]. In humans, AMs from allergic asthma patients produce more pro-inflammatory cytokines upon activation of the IgE receptor and thus promote airway inflammation[164].

Therefore, all of the information conclude that AMs play a dual role, either anti-inflammatory (embryonic macrophages) or pro-inflammatory (monocyte-derived), during allergic asthma.

1.2.3 Interstitial macrophages

Lung macrophages mainly studied based on the well defined and characterized AMs. There is another type of macrophage that remains in the lung tissue interstitium so; thus, it is called "interstitial macrophages" [165].

Ontogeny and tissue location: Study on developmental origin of lung macrophages has shown that 'primitive' IMs originate from yolk sac when 'definitive' IMs originate bone marrow. Primitive IMs develop from yolk sac during embryogenesis and populated to the lung submesothelial and perivascular zones during first week of post natal period where they maintain themselves by self-renewing throughout the life. On the other hand, definitive IMs develop from bone marrow during first week of birth and difusley populate in the lung interstium, even replacing parenchymal embryonic lineage population and maintain their number from blood monocytes through out the life (Figure 1.4 [138]. During homeostasis, IMs maintain their number by self-renewal of embryonically derived tissue-resident macrophages with minimal contribution of bone-marrow-derived monocytes [138, 166].

At steady-state conditions, there are three subsets of IMs found in the different locations of the lungs. All these three subsets express canonical macrophages marker CD64 and MerTK; however, different in the expression of CD11c, CD206, CCR2, lymphatic vessel endothelial hyaluronic acid receptor 1 (Lyve-1), and MHCII. Based on the degree of this marker expression IMs subsets are IM1 ($MHCII^{lo}CD206^{high}$), IM2 ($MHCII^{+}CD206^{high}$), IM3 ($MHCII^{high}CD206^{lo}CCR2^{lo}$) [167].

IMs subsets remain in different locations of the lung. Immunostaining against F4/80 and CD11c showed that IMs ($F4/80^{+}CD11c^{-}$ cells) remain in the parenchyma of the lung [168]. On the other hand, another study showed that IMs remains in the bronchial interstitium near to the lymphatic vessel, but not in the parenchyma while they found IMs ($MertK^{+}Cx3cr1^{+}$cells) in the interstitium

after staining (figure 1.4) [167]. Although at a glance, IMs show a lower turnover rate than AMs, IM3 shows a higher turn over rate compared to the other two types IM1 and IM2, and IM3 readily replaced by the bone-marrow-derived monocytes [167].

Cell-surface marker expressed by IMs: All three IMs subsets have strong transcriptional macrophages signature including the expression of MER proto-oncogene tyrosine kinase (MerTK), cluster of differentiation 64 (CD64) also known as Fc γ receptor Ia (FcγRI), EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1 also known as F4/80), and integrin alpha M (CD11b) also known as complement receptor 3. Although all three subsets differentially express MHCII, CD206, Lyve-1, and CDllc, however, they all expressed high levels of CD14 and CX3CR1[167].

CX3CL1 chemokine receptor 1 (CX3CR1) is also expressed in various immune cells, including macrophages, monocytes, dendritic cells, T-cells, and natural killer cells. However, CX3CR1 expression is highly cell type-specific depending on tissue and organ [169]. CX3CR1 is mostly expressed on monocyte in the blood, macrophages in the gut, microglia in the brain, macrophages, CD8+ T cells, and NK cells in the liver [170-175]. However, in the lung tissue, CX3CR1 is mainly expressed by lung interstitial macrophages at a steady-state. It is also expressed by a fraction of $CD4^+$ T cells in the asthma condition [169, 176, 177].

1.2.3.1 Regulatory role of IMs in asthma

Both human and mice IMs have been documented to secrete anti-inflammatory cytokine IL-10 in the steady-state [165, 168, 178-180]. Production of IL-10 by IMs is lung microbiota independent at steady-state. In the lung, IMs may extend their dendrites into the lung lumen to take the antigen

and thus, it recognizes surfactant protein SP-A resulting in continuous production of IL-10 through the activation of TLR4/MyD88 pathway [178]. Thus, IMs maintain lung homeostasis in the naïve condition. In the mice lung, IL-10 producing IMs are more prevalent than the persistently IL-10 producing $Foxp3^{+}T_{reg}$ cells. Moreover, IL-10 production by IM, but not $Foxp3^{+}T_{reg}$, increased in the mice upon HDM-challenge [178] via aTLR4/MyD88-dependent hypoxia-inducible factor-1α (HIF1α) expression, which robust the expression of cytokine IL-10 by IMs [180]. As a result, IM through IL-10 production reduced neutrophilic inflammation by negatively regulate Th2 and Th17 responses in HDM-induced allergic asthma. Transplantation of wild-type IM to IL-10 KO mice reduced goblet cell mucous production, neutrophilic inflammation, and expression of IL-13, IL-17, TNFα, and GM-CSF[178].

IMs play an essential role in maintaining lung homeostasis, even in the presence of environmental LPS [168]. Low concentration of airborne LPS promotes the induction of DC-driven Th2 responses to harmless inhaled allergen result in allergic asthma. However, a large number of people do not develop lung DC-driven Th2 responses upon exposure to environmental LPS due to the prevention of Th2 responses by IMs IL-10. Here, low dose LPS induce IMs to produce IL-10, which inhibits maturation and migration of DCs loaded with airborne harmless antigen, thereby prevent Th2 immune responses. Therefore, IMs play an important role in lung homeostasis [168].

According to the hygiene hypothesis, exposure to environmental and commensal microbes or their products reduces the risk of development of allergic asthma[181, 182]. In addition to bacterial LPS, bacterial DNA, which is present in high amount in the dust, play an important role in the reduction of development of allergic asthma[183]. DNA containing non-methylated CpG motifs (CpG-DNA) can strongly expand the lung IMs populations, and it is the most potent stimulator of

IL-10 expression in mouse IMs. Therefore, CpG-induced IMs expansion and production of IL-10, confer protection against allergic asthma even in the microbe-rich environment [179].

All these above data suggest that IMs play an important role in the downregulation of allergic asthma through IL-10 production, while, surfactant protein (SP-A) or environmental microbial products (LPS or CpG-DNA) or HDM promote the stimulation of IL-10 production by IMs.

Very little has been documented about the pro-inflammatory activities of IMs. It has been shown that IMs play an important role in the inflammatory response of the lung to acute endotoxemia. Chemotaxis, phagocytosis, and production of reactive oxygen intermediates (ROI) by lung IMs is increased after acute endotoxemia[184].

1.2.4 Difference between interstitial macrophages and alveolar macrophages

Table 1. 1 Difference between IM and AM:

Characteristics	**Interstitial Macrophages**	**Alveolar Macrophages**	**Reference**
Location	Lung vicinity of bronchi, in the bronchial interstitium.	Alveolar lumen.	[167, 168, 179]
The number at steady-state of the	8×10^6 cells, or ≅2% of the total lung cell population.	1.3×10^7 cells, or ≅3% of the total lung cell population.	[165]

total lung population			
Size	Smaller than AM, size and morphology closer to monocyte.	Larger than IM.	[135, 137, 167, 184-186]
Cellular structure	High nuclear/cytoplasm ratio, cytosol contains vacuoles. It has smoother surface with more irregular nucleolus.	It has more prominent pseudopodia.	[137, 184, 186]
Phagocytosis	Phagocytes bacteria and particles.	Phagocytes bacteria and particles.	[135, 137, 167, 168, 179]
Defence	The second line of defence. The microorganism that has evade phagocytosis by luminal AM.	The first line of defence.	[184]
Antigen presentation	IM express high level of MHC-II. So, it has the capacity to present antigen and activate T cells.	AM are more capable of phagocytosis than antigen presentation.	[137, 167, 179]
Antigen presentation to DC	IM are capable of processing and presenting Ag to DCs.	No report has been documented regarding presentation of Ag to DCs.	[178]

Originate	IM originate from blood monocyte of embryonic yolk-sac and blood. They maintain through local self-proliferation and circulating monocytes.	Originate from embryonic fetal liver and can self-maintain by local proliferation with minimal contribution from circulating monocytes in the steady-state	[138, 141, 166, 167, 179, 187-193]
Subtypes	Three subtypes of IMs including IM1, IM2, and IM3.	No subtypes of AMs. Although the different studies have shown resident AMs & recruited AMs. In sddition, AMs are M1 & M2 based on polarization.	[167, 194]
Change in number upon inflammation	The number of IM increases upon microbes and allergens	The number of AM decreases upon allergen.	[179]
Intermediate cell	IMs act as the intermediate of the monocytes and AMs during inflammation. IMs infiltrate to airway lumen in response to microbes (LPS and CpG-DNA).	At inflammatory condition regulatory AMs convert to pathogenic AMs.	[145, 146, 179]
Autofluorescence	Autofluorescence	Non- autofluorescence	[167, 179]

Cell surface marker	IMs are SiglecF^{-} CD11c$^{+/-}$ CD11b^{+} CCR2$^{+/-}$ CX3CR1^{+} cells.	AMs are SiglecF^{+} CD11c^{+} CD11b^{-} CCR2^{-} CX3CR1^{-} cells.	[167, 179]
Common marker	IMs are Mertk^{+}CD64^{+}F4/80^{+}	AMs are Mertk^{+}CD64^{+}F4/80^{+}	[167]
Regulatory role	IMs play a regulatory role by expressing immunosuppressive cytokines IL-10 at the steady-state,upon microbes and allergen exposure. IMs produce more IL-10 than AMs.	AMs act as both anti-inflammatory and pro-inflammatory.	[145, 146, 168, 178, 179]

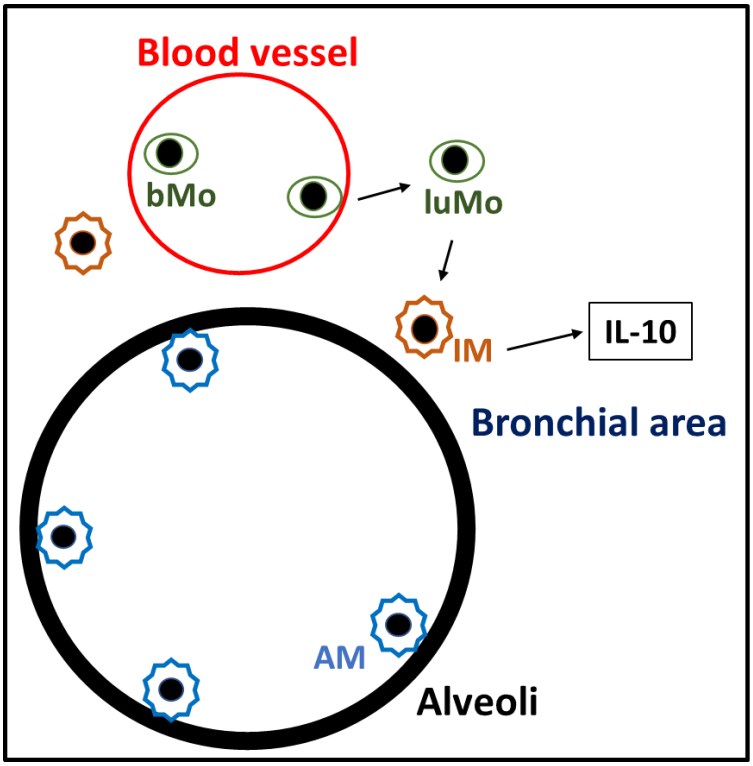


Figure 1. 5: The location of IM and AM in the lung. At the steady-state, lung interstitial macrophages (IM) reside in the lung interstitium, and alveolar macrophage (AM) reside in the lumen of the alveoli. At steady-state, interstitial macrophages produce IL-10 and play role in the suppressive regulation of the lung immune system. IM is originated from blood monocytes (bMo) through intermediate state lung monocyte (luMo), and when some portion is maintained through self-proliferation of local IMs [178, 179, 195].

1.3 Chapter 3: Semaphorins and plexins

1.3.1 Semaphorins

Semaphorins are cell-membrane bound or secretory proteins that regulate cell migration, differentiation, proliferation, and cell morphology. Semaphorins are guidance cue that has either repulsive or attractive effect on growth cones and thus determine their direction toward or away from a target place. Moreover, they act as either chemorepellent or attractive molecules in other different systems[196]. Semaphorins initially discovered as an axon guidance molecule that is essential in the nervous system development[197, 198]. However, growing evidence shows that they have an important role in the other systems, including the immune system, cardiovascular, and respiratory system, as well as they are involved in the process of angiogenesis, organogenesis and progression of cancers[198, 199].

The first semaphorin was discovered in 1993 that was initially in the collapsin family. The semaphorin family was later expanded due to the discovery of new proteins that have similarity or homology in their amino acid sequence and structure[200, 201]. There are more than 20 semaphorins have been identified, and they have been divided into 8 classes based on their amino-acids component and structural similarity[202, 203]. Classes 1 and 2 are found in invertebrate, Classes 3-7 are found in vertebrate and class V (sema-8) is found in virus only[199]. According to their localization, they have been divided into other categories. Such as, semaphorins class 1,4,5 and 6 are membrane-bound proteins, class 2, 3, and 8 are secreted proteins, and class 7 is glycosyl-phosphatidyl-inositol (GPI)-linked proteins[204] (Figure 1.5).

Structure of semaphorins: Semaphorins are made up of different domains. The first extracellular domain is called 'Sema domain' that consists of around 500 amino acids, which is the conserved domain among all semaphorin proteins[205, 206]. Semaphorin activities are mainly regulated by the 'sema domain'. The next domain, which is tightly coupled with sema domain, is the cysteine-rich domain called PSI (plexin, semaphorin, and integrins) domain. Ecto-domain of class 1 & 6 consists of sema domain and PSI domain. Some of them have another domain called Immunoglobulin (Ig)-like domain or thrombospondin domain. Ig-like domain present in semaphorins class 2, 3,4,5,7 whereas thrombospondin only present in semaphorin class 5 [198]. Some semaphorins (class 3) undergo proteolytic cleavage to produce an active form [207-209] (figure 1.5).

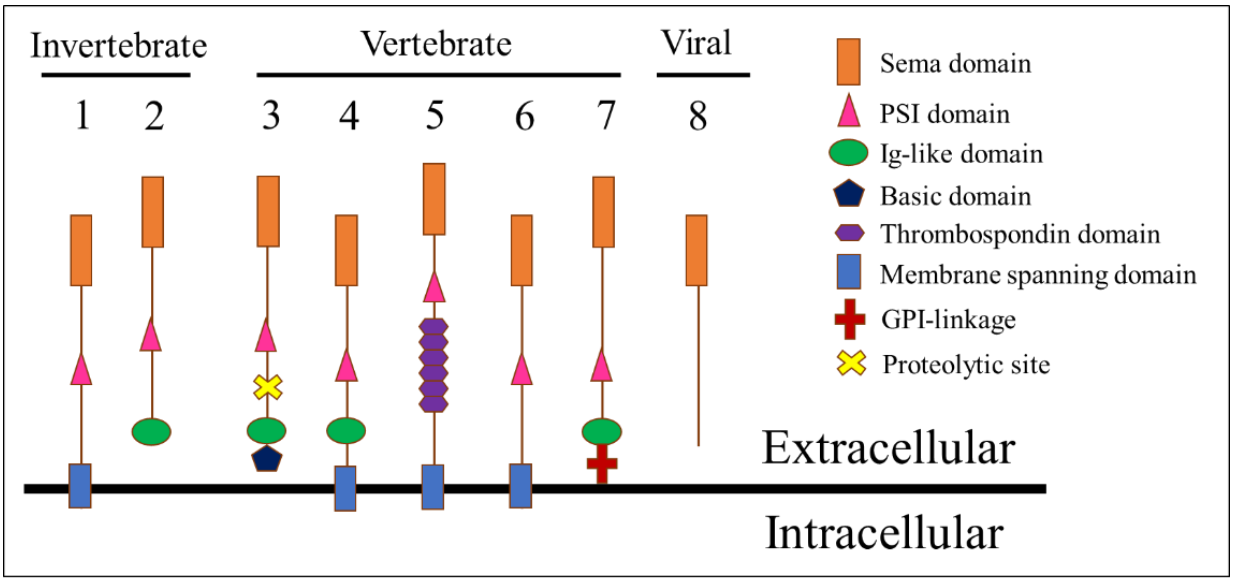


Figure 1. 6: Semaphorins family. Semaphorins are categorized into eight classes. Class 1 and 2 semaphorins are identified in invertebrates. Class 3–7 semaphorins are found in vertebrates. Class V (sema-8) is found in the virus. All semaphorins share a conserve region named sema domain. Semaphorins 2,3, & 8 are secretory proteins, whereas class 1, 4,5,6, & 7 are membrane-bound

proteins. Some semaphorins (class 2,3,4 & 7) have Immunoglobulin (Ig)-like domain when class 5 has thrombospondin domain. Class 7 semaphorin is linked membrane-associated GPI-linkage at its carboxyl terminus[203, 210].

Informations from: Unified nomenclature for the semaphorins/collapsins. *Cell*, 1999 and Semaphorins and Their Receptors: From Axonal Guidance to Atherosclerosis. *Front Physiol.*, 2018.

1.3.2 Semaphorin receptors

Semaphorins transmit their signals into the cells through two main receptors: plexins and neuropilins [211, 212]. Most of the semaphorins molecule mediates their signal through plexins alone. However, semaphorins class 3, except semaphorin3E, mediate their signal through the combination of plexins and neuropilins. They need co-receptor neuropilins with plexins to transmit the signal properly [213, 214]. Moreover, semaphorins molecules can transmit their signals through other receptors. These are CD72, T-cell immunoglobulin and mucin domain-containing protein-2 (TIM-2), heparan sulfate proteoglycan (*HSPG*) and chondroitin sulfate proteoglycans[199, 212]. Different proteins including G-coupled proteins, tyrosine kinase, GTPase, and other adhesion molecules are involved in their signal process [198].

1.3.2.1 Neuropilins

In addition to plexins, several other transmembrane receptors act as co-receptor for semaphorins or are engaged in the signalling of semaphorins molecules[207]. The well-described co-receptor

of plexins is neuropilins. There are two types of neuropilins so far documented including neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2). The extracellular part of neuropilins contains two CUB domains named a1 and a2 domain, two coagulation factor V/VIII homology domains called b1 and b2 domain, and a MAM domain called c domain[215]. Vascular endothelial growth factor (VEGF) binds to this b1 and b2 domains, whereas semaphorins class 3, except Sema3E, bind to a1 and a2 domains, thereby activate vascular endothelial growth factor receptor (VEGFR) and plexins [207]. The MAM domain is responsible for the dimerization of the neuropilins. These transmembrane receptors have a short cytoplasmic tail. As a result, they can not transmit signals into the cells alone and therefore act as a co-receptor of another receptor. For instance, semaphorins class 3, except semaphorin3E, use neuropilins to activate plexin class A for axon guidance (axon repulsion or attraction), and VEGF uses the NRP-1 as a co-receptor to activate VEGFR-2 on endothelial cells [212, 216].

1.3.2.2 Plexins

Most of the semaphorins mediate their function through plexin receptors. Plexins are, single-pass, a group of nine transmembrane proteins [217, 218]. They are categorized into 4 classes including A, B, C, and D. All four classes of plexins play a vital role in the transmitting signal into cells. These plexins includes class A (Plexin-A1, A2, A3, A4), class B (Plexin-B1, B2, B3), class C (PlexinC1), and class D (PlexinD1), whereas only Plexin-A and Plexin-B play a role in invertebrate [218, 219].

Structure of plexins: The overall structure of all plexins is quite similar to each other. The extracellular portion of plexins contains around 500 amino acid sema domain followed by cysteine-rich PSI (plexin-semaphorin-integrin) domain and three IPT (immunoglobulin-plexin-

transcription factors) domain [205, 220]. The difference of plexin's sema domain from the one of semaphorins is that the former does not dimerize [207]. Sema domain act as a ligand-binding domain, while the PSI domain is important for protein-protein interaction, and the IPT domain is essential for perfect ligand binding [221-223]. Sema domain act as an autoinhibitory domain, thereby constraint the activation of plexins in the absence of ligand [221].

The cytoplasmic domains play a critical role in the transduction of the signal after ligand binding[224] .Cytoplasmic domain, itself does not have kinase activity, however, can be tyrosine phosphorylated by receptor or non-receptor tyrosine kinase, which indicates plexins transduce signal into cytoplasm by associating a tyrosine kinase [218]. Highly conserved regions of the cytoplasmic tail are GTPase-binding domain and GTPase-activating protein (GAP) domain. These domains play a critical role and regulate many responses upon activation[219].

Semaphorins-plexins interaction: Binding of semaphorins with plexins has specificity. Different plexins bind to various semaphorins with specificity. For instance, membrane-bound class 5 and class 6 semaphorins directly activate class A plexins, whereas secreted class 3 semaphorins, except semaphorin3E, require neuropilins as co-receptors to stablize the semaphorin–plexin interaction. [207, 216]. Semaphorins class 4 and class 5 activate class B plexins, and class 7A semaphorins activate plexin C1. Several proteins of class 3 semaphorins bind to plexinD1 in a neuropilin-dependent manner, whereas semaphorin3E and semaphorin4A can bind to plexinD1 independently of neuropilins [207, 218].

1.3.2.3 PlexinD1

PlexinD1 is considered the most structurally diverse protein among the 4 classes of plexins. PlexinD1 is expressed by different cells including neuron cells, endothelium, airway smooth muscle cell, fat cell, thymocytes, activated B cells, dendritic cells, neutrophils, and macrophages [207, 225-228].

Mature plexinD1 consists of 1879 amino acids with 208KDa molecular mass[229]. Extracellular part N-terminal contains sema domain, three MRS (MET-Related Sequence) or PSI repeats, four IPT domains, and transmembrane (TM) domain. The cytoplasmic portion of the plexinD1 name as Sex and Plexins (SP) domain. It has a GTPase activating protein (GAP) domain. This GAP domain consists of two highly conserved regions C1 and C2. A Rho-GTPase-binding domain (RBD) is present between C1 and C2 regions. Ras GAP motif 1 (RasGAP1) and Ras GAP motif 2 (RasGAP2) is in the C1 and C2 region, respectively. Each of the motifs contains conserved arginine residues required for inhibiting the activity of the R-Ras protein. After the GAP domain, C-terminal of plexinD1 contains a terminal segment (T-segment), which is linked to a short PDZ-binding motif (D1-PBM). T-segment is highly conserved among members of same plexins subfamily (figure 1.6) [230].

Activation of PlexinD1: PlexinD1 has two states: inactive and an active state. In the absence of ligand, plexinD1 remains in an inactive form in which sema domain folded to the rest of the extracellular domain, and intracellular C1 and C2 region of GAP domain wrapped to each other (figure 1.6). Sema3E is a canonical ligand of plexinD1. Upon binding of Sema3E to sema domain of plexinD1, a conformational change of plexinD1 occurs that activates GAP domain [230]. Activation of the cytoplasmic tail of plexinD1 is involved in a different cellular process including integrin-mediated cell adhesion, cell proliferation, cell-cell junction, and establishment of cell

polarity [231]. For instance, in inactive form GTP bound Rnd2 bind to RBD that prevents binding of active GTP-bound Ras and Rac. As a result, GTP-bound Rac can activate PAK (p21-activated kinase) to induce the assembly of F-actin filaments, and active GTP-bound Ras induce integrin-mediated adhesion to the extracellular matrix (ECM) leads to cells migration. Binding of Sema3E with plexinD1 leads to a conformational change in which GAP domain and RBD binds to an active form of R-Ras and Rac GTPase respectively. By sequestering Rac and Ras, plexinD1 inactivate PAK and hydrolyze GTP to GDP. As a result, disassembly of actin filaments and inhibition of integrin binding to ECM leading to cell retraction. Binding of Sema3E with plexinD1 inhibits Ras-mediated other downstream signalling events [230].

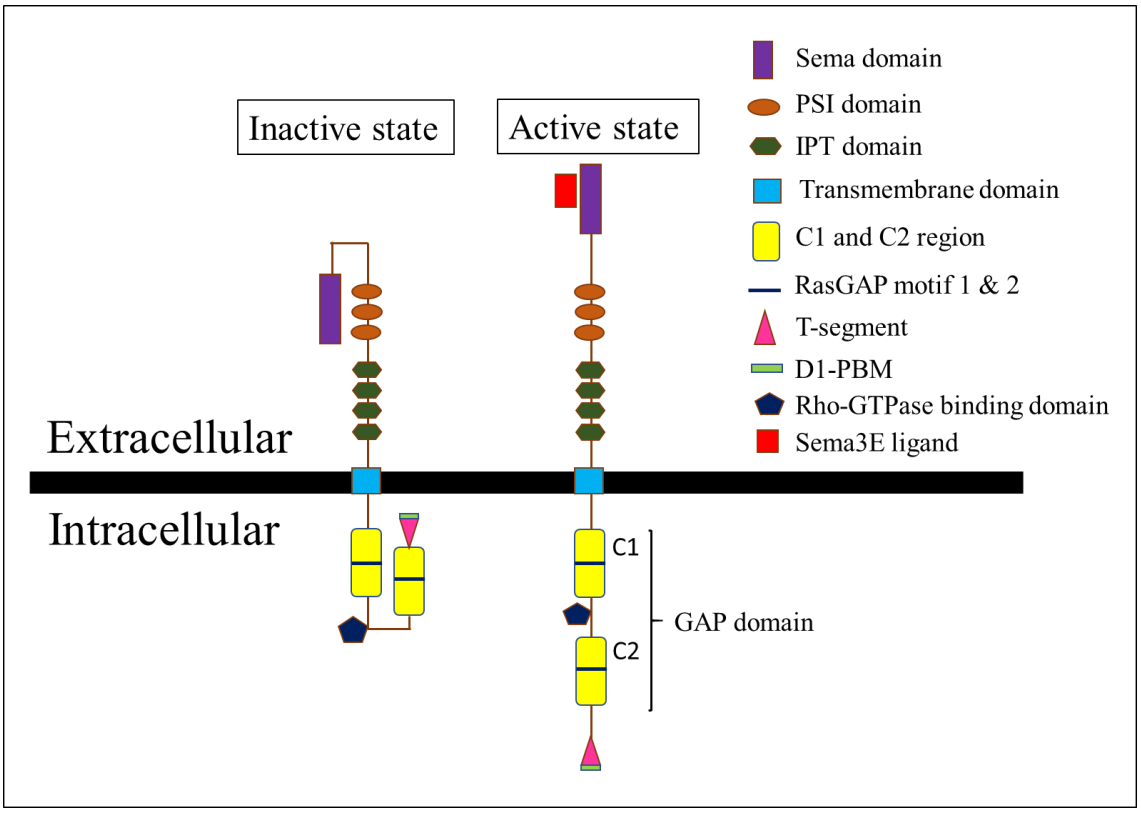


Figure 1. 7: Structure of plexinD1 including active and inactive state. The extracellular N-terminal part contains a sema domain, where Sema3E binds. After the sema domain, three MRS (MET-Related Sequence) also called PSI (plexin, semaphorin, and integrins) repeats, four IPT

(Immunoglobin-like fold shared by plexins and Transcription factors) domains and the transmembrane domain (TM). The cytosolic portion of plexinD1 contains a GTPase Activating Protein (GAP) domain with two highly conserved C1 and C2 regions. C1 region contains a Ras GAP motif-1 (RasGAP1) and C1 region contains RasGAP2. A Rho-GTPase-binding domain (RBD) is present between the C1 and C2 regions. The GAP domain is followed by a short C-terminal region named the terminal (T) segment. PDZ-binding motif (D1-PBM) is the end of plexinD1 connected to T-segment. In the absence of its sema ligands, plexinD1 remains in a conformationally inactive folded state and non-functional. Upon Sema3E binding with sema domain of plexinD1, it goes to conformational changes that activate its GAP domain and downstream signals [230].

Informations from: Diverse functions for the semaphorin receptor PlexinD1 in development and disease. *Developmental Biology*, 2011.

1.3.3 Functions of Sema3E/plexinD1 axis

Sema3E/plexinD1 axis plays a critical role in the development of the nervous system, embryogenesis, vasculature and angiogenesis, tumor inhibition, cancer metastasis, thymocyte development, and allergic asthma.

Nervous system: Sema3E and its receptor plexinD1 are axon guidance molecules that play a vital role in the development of the nervous system during embryogenesis. Sema3E and plexinD1 have been shown to play a role in the organization of neuronal circuits. Sema3E/plexinD1 act as both chemorepellent and chemoattractant during nervous system development. For example, binding of

the dimeric form of Sema3E with plexinD1, in the absence of neuropilin, inhibits axon growth cone migration. However, binding of the monomeric form of Sema3E with plexinD1 acts as a chemoattractant leading to axon growth cone migration in the presence of neuropilin [230]. Studies have shown that the repulsion activity of plexinD1 blocks the synapse formation that leads to sensory-motor connectivity[230]. Again, Sema3E/plexinD1 play an essential role in the development of hippocampus during embryonic, perinatal, postnatal, and adult stage, while the absence of plexinD1/Sema3E leads to abnormal hippocampal formation [232].

Tumor and cancer: PlexinD1 has been reported to act as both pro and anti-tumor based on the cancer type and location and interaction of plexinD1 with an isoform of Sema3E. Full-length Sema3E (P^{87}-Sema3E) has no prometastatic activity when small proteolytic fragment Sema3E (P^{61}-Sema3E) are prometastatic. PlexinD1 expression is positively co-rrelate with the progression of tumor metastatic[233]. In human colon cancer, level of plexinD1 and Sema3E increased in the metastasis cancer cells compared to tumor cells, and Sema3E expression and signaling through plexinD1 positively correlate with metastatic progression [234]. However, deletion of plexinD1 from tumor cells abrogate metastatic effect of P^{61}-Sema3E [234]. Moreover, Sema3E induced intestinal gastric and pancreatic cancer cell growth. Enhanced expression level of Sema3E correlated with the metastasis of gastric cancer in the intestine and poor pancreatic patient survival [235, 236]. Furthermore, Sema3E of ovarian tumor induce epithelial-to-mesenchymal transition (EMT) and cell migration and malignant progression via plexinD1[237]. PlexinD1/Sema3E inhibit tumor apoptosis and induce breast cancer metastasis and inhibition of plexinD1 signaling induced apoptosis of breast cancer cells [238]. On the other hand, mutated furin-resistant Sema3E isoform, full length, inhibit tumor angiogenesis and metastatic spreading [239].

T-cells development: PlexinD1 plays a role in the direction of migration of thymocytes during T-cells maturation. Sema3E/plexinD1 axis regulates the thymocyte movement from cortex to medulla during T-cells development. Here, double-positive (DP) thymocytes ($CD4^+CD8^+$) express PlexinD1, and activation of plexinD1 through Sema3E suppresses CCR9/CCL25 signaling in thymocytes that result in DP thymocytes move from cortex to medulla that leads to maturation of thymocytes to single positive T-cells. Absence of plexinD1, DP thymocytes remain in the cortex and mature to single-positive (SP) thymocytes that form ectopic SP cluster in the cortex result in disruption of the corticomedullary junction in the thymus[240].

Vasculature and angiogenesis: PlexinD1 play a critical role in the angiogenesis where Sema3E/plexinD1 axis regulates the positioning of endothelial cells and cardio-vasculature patterning [214, 241]. Moreover, the absence of plexinD1 induced new blood vessels formation because Sema3E/plexinD1 signalling inhibits VEGF-induced angiogenesis by inducing the decoy receptor VEGFR1 of VEGFR2 receptor. As a result, VEGF is trapped by VEGFR-1 and can't bind with VEGFR2 leading to VEGF mediated endothelial growth inhibition[242]. Sema3E/plexinD1 inhibits endothelial cells (EC) migration and proliferation by disassembly of actin filament and dysregulating integrin to bind to extracellular matrix results in inhibition of EC migration and proliferation lead to inhibition of angiogenesis [243]. Sema3E/plexinD1 axis also inhibits extra-retinal vessel development by inhibiting VEGF-induce blood vessel growth [241].

Although plexinD1 is the canonical receptor of Sema3E, plexinD1 can also bind Sema4A independently of neuropilins. However, major receptor for Sema4A in the immune system is TIM2 receptor [244] though it can functionally interact with TIM2 and CD72 in different immune cells [245]. Sema4A present on DC promote T-cell differentiation to Th1 cells through TIM2 receptor and thus suppress Th2-mediated responses leading to reduce allergic airway inflammation [246,

247]. On the other hand, Sema4A inhibit angiogenesis by suppressing VEGF-mediated migration and proliferation of endothelial cells through binding with plexinD1. Therefore, It indicates that Sema4A exert organ-specific activities through different receptor-mediated signaling such as through plexinD1 in endothelial cells and TIM2 in T-cells [248]. However, macrophages migrate to the ischemic/tumor tissue through Sema4A/plexinD1-mediated interaction and induce angiogenic responses needed for tissue healing and remodeling [249]. In addition, Sema4A/plexinD1-mediated signalling induce IL-17 expression by $CD4^+$ T cells and profibrotic gene expression in dermal fibroblast of healthy and systemic sclerosis (SSc) subjects [250]. Therefore, plexinD1 exert its cytoplasmic function through binding to different ligands e.g; Sema3E or Sema4A.

1.3.4 Sema3E/plexinD1 in macrophages

Macrophages express plexinD1. Binding of Sema3E to plexinD1 on macrophages present in adipose tissue and atherosclerosis plaque induce inflammation that leads to different diseases [227, 228].

Different subsets of macrophages contribute to adipose tissue inflammation[251-253]. In the obese condition, M2 macrophages shift to M1 macrophages, and these M1 macrophages produce proinflammatory cytokines result in promoting insulin resistance in obese persons [228, 253]. Sema3E expression by adipose tissue, and plexinD1 expression by macrophages, is increased in obese persons. Increasing of Sema3E in the adipose tissue attract macrophages to tissue area through Sema3E/plexinD1 axis. As a result, the infiltration of macrophages is increased in the adipose tissue of obese persons. Binding of Sema3E with plexinD1 influences the intracellular signalling pathway of recruited macrophages, and leads to the production of different pro-

inflammatory molecules including TNF, IL-6, MCP-1. Thus, inflammation in the adipose tissue causes different metabolical problems, including dietary obesity and insulin resistance[228].

In addition to recruitment, the presence of plexinD1 on macrophages promotes the retention of macrophages in atherosclerosis plaque that induces inflammation in atherosclerosis. In atherosclerosis plaque, the number of M1 macrophages and Sema3E expression by M1 are increased. Autocrine action of Sema3E on plexinD1 inhibits the migration of macrophage from plaque by disrupting Rho-GTPase signalling and disassembly of the actin polymer. As a result, macrophage remains in the plaque and secret different pro-inflammatory cytokines. Therefore, the retention of M1 macrophages regulates the persistence of inflammation in the advance atherosclerosis plaque [227].

1.3.5 Sema3E/plexinD1 in asthma

Sema3E is the canonical receptor for plexinD1 and can directly bind to plexinD1 with high affinity [214]. Different studies have shown that the PlexinD1/Sema3E axis regulates allergic asthma, and Sema3E deficiency exacerbates allergen-induced airway hyperresponsiveness, airway inflammation, and airway remodelling [254].

PlexinD1/Sema3E axis in human asthma: Airway smooth muscle (ASM) mass is increased in patients with asthma due to higher proliferation rate of human airway smooth muscle cells (HASMCs) compared to healthy subjects [255]. Both healthy and asthmatics HASMCs constitutively express plexinD1; however, expression was decreased in patients with asthma than healthy subjects [225]. Sema3E inhibits platelet-derived growth factor (PDGF)-mediated HASMC proliferation and migration leading to the downregulation of ASM mass [225]. Thereby, this study

suggests that plexinD1/Sema3E downregulates airway remodelling by inhibiting ASM cell proliferation.

Moreover, Sema3E is mainly expressed by airway epithelial cells [256] and the expression of Sema3E robustly decreased in bronchial biopsy as well as bronchoalveolar lavage (BAL) of patients with severe asthma. Furthermore, there is a strong negative correlation between Sema3E expression and forced expiratory volume in one second (FEV1)[256]. These suggest that plexinD1 ligand Sema3E might downregulate asthma through the reducing release of inflammatory mediators from epithelial cells and thus decrease the recruitment of effector inflammatory cells to the airway.

Sema3E inhibits chemokine-induced neutrophils migration *in vitro* and *in vivo*. Human neutrophils constitutively express plexinD1. PlexinD1/Sema3E interaction present on neutrophils suppresses CXCL8/IL-8-induced primary human neutrophils migration by repression of Rac1 GTPase activity and F-actin polymerization. For instance, Sema3E/plexinD1 reduced LPS-induced blood neutrophilia [226].

PlexinD1/Sema3E in asthma model: Sema3E- deficiency exacerbates HDM-induced acute and chronic asthma features, including airway hyperresponsiveness, airway inflammation. This was manifested by an increased number of CD11b+cDC, eosinophils, neutrophils, Th2 & Th17 cytokine responses, and mucus overproduction, and collagen deposition [254, 257]. However, recovery of Sema3E/plexinD1 axis by the treatment with exogenous Sema3E-Fc recombinant protein reduced these asthma features [258]. Furthermore, Sema3E deficiency induced neutrophilia in the airway which was increased upon allergen exposure. However, recovery of this axis by treatment with recombinant Sema3E reduces allergen-induced neutrophils infiltration to the airway [226]. More recently, Sema3E deficient mice subjected to acute HDM challenge

showed an increased angiogenesis in the airway compared to their wild type counterpart. However, treatment with Sema3E-Fc recombinant protein reduced pro-angiogenic factor VEGF and VEGFR2 and promoted anti-angiogenic factor VEGFR1[259].

Therefore, Sema3E/plexinD1 plays an important role in the downregulation of asthma by inhibiting airway hyperresponsiveness, airway inflammation, and airway remodelling.

2 RATIONALE, HYPOTHESIS AND AIMS

2.1 RATIONALE

Asthma is a chronic relapsing inflammatory disease of the airways characterized by an increased number of eosinophils, neutrophils, mast cells, activated lymphocytes, and macrophages associated with increased airway hyperresponsiveness and airway remodelling. Semaphorin3E is one of the neuronal guidance cues that regulate allergic asthma. The absence of Sema3E exacerbates airway hyperresponsiveness, airway inflammation, Th2/Th17 cytokine responses, and airway remodelling upon allergen exposure. These asthma features can be ameliorated upon treatment with Sema3E recombinant protein. However, whether these effects are mediated exclusively through the binding to plexinD1 of inflammatory or structural cells is not known.

Gap in knowledge: PlexinD1 is expressed constitutively by macrophages and Sema3E induced macrophages recruitment and retention in the adipose tissue[228]. Also, binding of Sema3E to plexinD1 promotes retention of macrophages in the advanced atherosclerosis plaques leading to inflammation. However, whether PlexinD1/Sema3E axis on macrophages have an impact on critical features of allergic asthma is not yet determined. Therefore, given the importance of Sema3E/PlexinD1 axis present on macrophages, we aim to investigate the effect of PlexinD1-deficient lung interstitial macrophages (IMs) in allergen-induced asthma.

2.2 HYPOTHESIS

PlexinD1 deficiency in lung interstitial macrophages exacerbates house dust mite-induced allergic asthma.

2.3 AIMS

2.3.1 To investigate the effect of *Pxnd1*-deficient interstitial macrophages on airway hyperresponsiveness and inflammation.

2.3.2 To study the expression of tissue remodelling genes after deletion of *Plxnd1* from interstitial macrophages in an allergic asthma model.

2.3.3 To investigate the change of cytokines and immunoglobulins level after deletion of *Plxnd1* from interstitial macrophages in an allergic asthma model.

3 METHODS AND MATERIALS

3.1 Animal models

CX3CR1-ERT2, B6.129P2(C)-*Cx3cr1*$^{tm2.1(cre/ERT2)Jung}$/J (Jackson Lab, stock number 020940) and Floxed PlexinD1, B6.129-*Plxnd1*$^{tm1.1Tmj}$/J (Jackson Lab, stock number 018319) mice were obtained from the Jackson Laboratory. *Cx3cr1*^{creERT2} mice were hemizygous, while *Plxnd1*$^{fl/fl}$ mice were homozygous. Mice were then crossed in Genetic Models Center (GMC), the University of Manitoba, to get offspring B6.129-*Plxnd1*$^{tm1.1Tmj}$ / *Cx3cr1*$^{tm2.1(cre/ERT2)Jung}$ (*Cx3cr1*^{creERT2}*Plxnd1*$^{fl/fl}$) which carry both *Cx3cr1*^{creERT2} and *Plxnd1*$^{fl/fl}$ genes. All mice are viable and fertile. All the mice were maintained in the Central Animal Care within the pathogen-free facility at the University of Manitoba and used according to ethical guidelines provided by the Canadian Council for Animal Care (Protocol Number 15802).

3.2 *Plxnd1* deletion from CX3CR1 cells

Tamoxifen (Cat T5648; Sigma) was dissolved in corn oil at a concentration of 8mg/100µl. Six to eight weeks old female *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ mouse was treated with 100µl tamoxifen by oral gavage as tamoxifen facilitate deletion of *Plxnd1* from CX3CR1 cells according to figure 3.1. They were treated every other day for a week to get Cx3cr1 cells specific *Plxnd1* knock out (*Cx3cr1*^{creERT2}-*Plxnd1* KO) mice. Non treated mice were used as s a wild-type (WT) mice and these are *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ mice. The deletion of *Plxnd1* from CX3CR1 cells was checked by flow cytometry and quantitative real-time PCR of lung CX3CR1 cells.

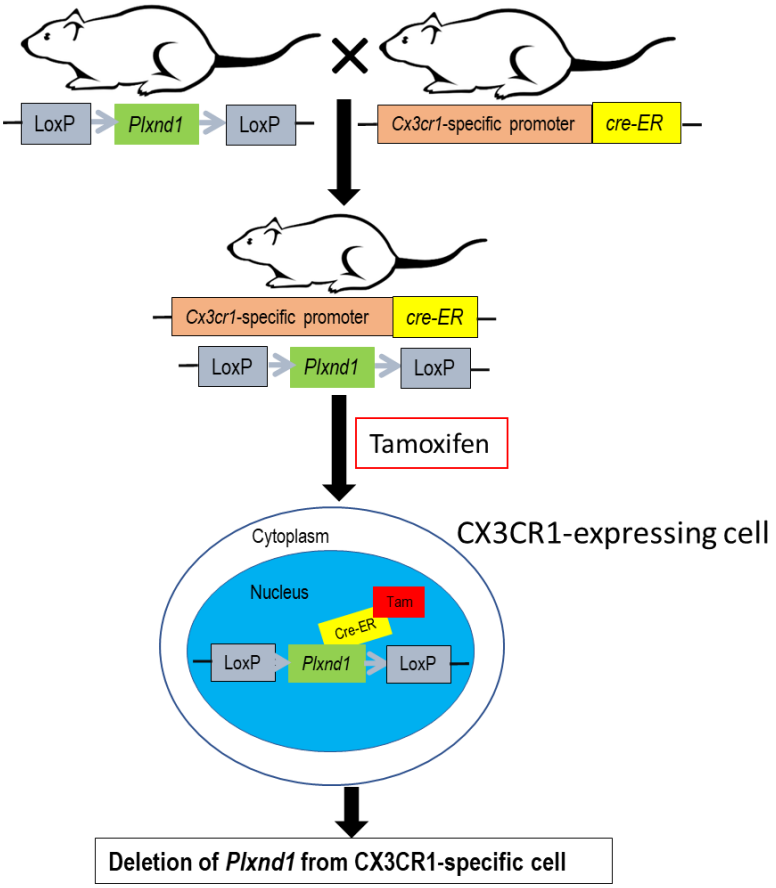


Figure 3. 1: CX3CR1 cell-specific *Plxnd1* KO mice were generated by crossing *Plxnd1*$^{fl/fl}$ mice with inducible CX3CR1-CreERT2 mice followed by tamoxifen treatment. Cre recombinase enzyme is expressed under the CX3CR1 promoter in the CX3CR1-CreERT2 mouse. Tamoxifen treatment facilitates Cre recombinase translocation from cytoplasm to nucleus and Cre recombinase targets the loxP site of *Plxnd1* leading to its deletion. Consequently, this generates CX3CR1 cell-specific *Plxnd1* KO mouse (*Cx3cr1*^{creERT2}-*Plxnd1* KO) and non treated mice are used as WT control (*Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$).

3.3 HDM-induced airway inflammation model

Traditionally, ovalbumin (OVA) has been used to induce allergic inflammation in mouse. However, OVA is not a clinically relevant allergen and in-vivo sensitization with adjuvant is extremely different from human sensitization by allergen only. On the other hand, HDM is one of the common aroallergens worldwide and up to 85% of asthmatics are usually HDM allergic [260]. Therefore, HDM extract was used to develop a mouse asthma model that closely similar to human allergic asthma. Both *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice were challenged with 25 μg HDM extract (*Dermatophagoides pteronyssinus*, Lot 259585; LPS, 615 EU/vial; protein, 5.34 mg/vial;; Greer Laboratories, Lenoir, NC) in 35 μl saline intranasally (i.n) for 5days/week of two consecutive weeks under gaseous anesthesia as we described previously [254, 261]. *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO control mice were challenged with 35 μl sterile saline. The mice were sacrificed 2 days after the last challenge with either HDM or saline to measure the outcomes (Fig 3.2)

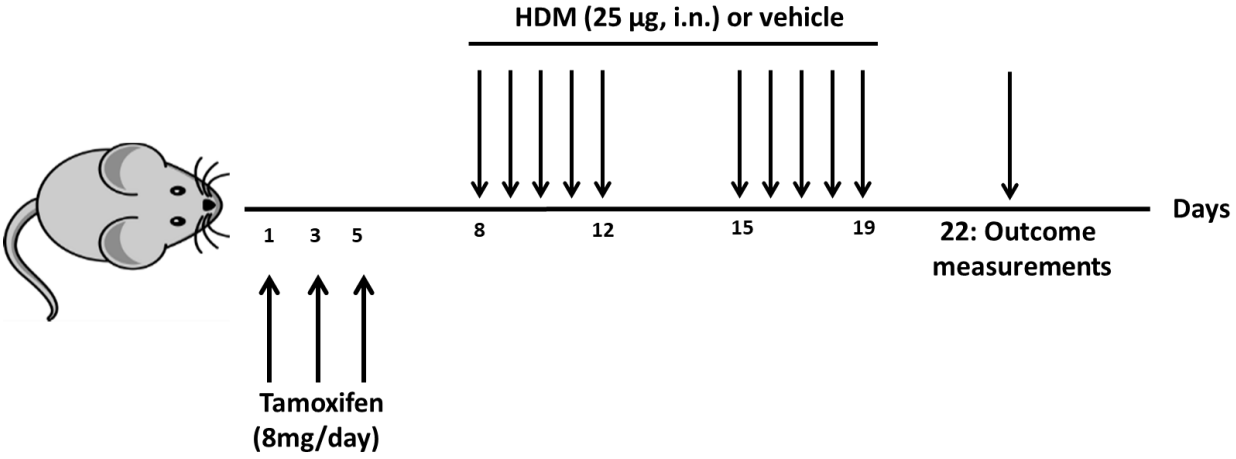


Figure 3. 2: Generation of acute allergic asthma model after tamoxifen treatment. Eight-ten weeks old female transgenic mice were sensitized and challenged intranasally with 25μg of house

dust mite (HDM) extract in 35µl saline for two weeks 5 consecutive days per week with a two-day rest period) to induce asthma.

3.4 Methacholine challenge test

To investigate lung mechanics, *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice were anesthetized with pentobarbital sodium (90 mg/kg, ip injection) after 2 days of last challenge with HDM or saline. Mice were then tracheotomized with a 20-gauge polyethylene catheter that was connected to the FlexiVent small animal ventilator system (SCIREQ, Montreal, QC, Canada). Mice were ventilated with a tidal volume of 10 ml/kg body wt for 150 times/min. Lung mechanics were measured by nebulizing the increasing gradient of methacholine (MCh) dose (0, 3, 6, 12, 25, and 50 mg/ml) intratracheally at 5-mins interval between each dose using flexiVent ventilator [254, 262]. To measure the effects of different concentrations of MCh on lung mechanics, a low-frequency forced oscillation technique was used. Here, the mechanical ventilation was interrupted during low-frequency forced oscillation and then a volume perturbation signal was applied. After these processes, flexiVent software calculated Newtonian resistance (Rn), peripheral tissue damping (G), and tissue elastance (H) and finally, all mentioned parameters were normalized to animal's body weight. Values of each parameter were calculated as the mean of all 12 perturbation cycles conducted after each MCh challenge.

3.5 Collection of Bronchoalveolar Lavage Fluid

After the measurement of lung mechanics, bronchoalveolar lavage fluid (BALF) was collected from *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice using 1ml sterile PBS containing

0.05M EDTA twice. BALF was collected into two separate Eppendorfs for two instillations of 1ml PBS. After centrifugation, BALF supernatant of first instillations of 1ml PBS was stored at -80^{o}C to measure airway cytokines response. Total BAL cells were counted using trypan blue by hemocytometer. Then cells cytospins were prepared (Thermo Shandon CytoSpin 3 Cytocentrifuge, UK), fixed by HEMA3 fixative, and stained (cytoplasm and nucleus) by quick dipping in the HEMA 3 solution I and II (Fisher Scientific, Cat: 122-929). Differential cell counts were measured by counting at least total 200 of cells of neutrophils, eosinophils, lymphocytes, and macrophages based on standard morphologic criteria[254, 263]. Cells were counted using a light microscope (Primo Star Digital Microscope and INFINITY camera by using infinity capture software) at x40 magnification. All counts were performed in a blinded manner of different study groups.

3.6 Surface staining of BALF inflammatory cells by fluorochrome-labeled antibodies

A portion of BAL cells was used for the immunophenotyping of inflammatory cells by flow cytometer. After Fc blocking (200µl/tube) for 10mins, cells were washed with flow buffer (2% FBS-PBS) and stained with a mixture (0.5µl each antibody/20µl flow buffer/tube) containing the following anti-mouse Abs: Fixable viability dye-efluor 780 (eBioscience), Siglec F-PE (clone E50-2440; BD Biosciences), CD11b-PE/Cy7 (clone M1/70), CD11c-PerCP/Cy5.5 (clone N418), Ly6G-APC (clone 1A8), F4/80-FITC (clone BM8; all four from BioLegend), CD3- eFluor 450 (clone 145-2C11; eBioscience), and B220- eFluor 450 (clone RA3-6B2; BD Biosciences). After 30mins incubation with antibodies mixture, cells were washed with flow buffer and fixed with 2% paraformaldehyde (500µl/tube) for 5mins. Then cells were washed by flow buffer again, blotted out the excess liquid by paper towel and resuspended in 200µl flow buffer. Then the samples were acquired using a BD FACSCanto-II flow cytometer.

3.7 Surface staining of lung inflammatory cells by fluorochrome-labeled antibodies

Lung was collected from Saline or HDM challenged *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice. The whole lung was minced, enzymatically digested in 1mg/ml collagenase IV (Worthington Biochemical, Lakewood, NJ) containing RPMI medium at 37^{o}C for 1hr. After RBC lysis with 5ml ACK buffer for 4mins, cells were washed with flow buffer and counted by hemocytometer using trypan blue. After blocking with Fc blocker for 10mins, cells were washed with flow buffer and stained with the following anti-mouse Abs mixture (0.5µl each antibody/20µl flow buffer/tube): Fixable viability dye-efluor780 (eBioscience), CD45- eFluor 450 (clone 30F11), MerTK-APC (clone DS5MMER), CD4-PerCP-eFluor710 (clone X54-5/7.1), Ly6G-FITC (clone RB6-8C5; all four from eBioscience), Siglec F-PE (clone E50-2440; BD Biosciences), CD11b-PE/Cy7 (clone M1/70; BioLegend). After 30mins incubation with antibodies mixture, cells were washed, fixed and resuspended in 200µl flow buffer. Then the samples were acquired using a BD FACSCanto-II flow cytometer.

3.8 Intracellular staining of lymph node and spleen cells for cytokines

Lung-draining mediastinal lymph nodes (MLN) and spleen were collected from saline or HDM challenged *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice. A Single-cell suspension of MLN and spleen were prepared using a 70µm cell strainer. After RBC lysis with 5ml ACK buffer for 5mins, cells were washed with RPMI and counted by hemocytometer using trypan blue. Intercellular staining was performed as previously described [254]. In brief, 4×10^6 cells/ml of MLN or spleen were incubated for 4hrs with a cell stimulation cocktail (2µl/ml), which contain phorbol 12-myristate-13-acetate (PMA), ionomycin, and protein transport inhibitor Brefeldin A (Invitrogen, USA), at 37°C with 5% CO2. Cells were then collected, washed with flow buffer and

extracellular staining was performed by using anti-mouse CD3 e-PE/Cy7 (clone145-2C11) and CD4-APC (clone G1.5), both from eBioscience. For intracellular cytokines staining, fixed and surface-stained MLN or spleen cells were permeabilized with 0.1% saponin in flow buffer (1ml/tube) for 15mins. After washing with 0.1% saponin-flow buffer, cells were stained (0.5µl each antibody/20µl 0.1% saponin-flow buffer/tube) with specific anti-mouse IFN-γ PerCPCy5.5 (clone XMG1.2; eBioscience), IL-4 PE (clone 11B11; eBioscience), and IL-17A (clone TC11-18H10.1; BioLegend). After 30mins incubation with Ab mixture, cells were washed with saponin buffer first and then flow buffer. After removing supernatant, cells were dissolved in 200µl flow buffer. Samples were acquired on a FACSCanto II.

3.9 Flow cytometry analysis

Stained BAL, lung, MNL and spleen cells were acquired by using BD FACSCanto II (BD Biosciences, USA) by adopting the standard instrumental protocol. At least 50,000 events of stained cells were acquired and the gating strategies for the inflammatory cells and markers are indicated in respective figures. We used fluorescence minus one (FMO) control of flow antibody conjugated with respective fluorochrome to get accurate gating for cells. Acquired flow data were analyzed by FlowJo software.

3.10 Measurement of cytokines in BALF, lymph node and spleen cells culture

A portion of MNL or spleen cells (4×10^6 cells/ml) were resuspended in RPMI medium (Life technology, cat: 11875-093, USA) supplemented with 10% FBS (Flow laboratories, cat: 2916154, USA), 100 U/ml penicillin, 100 U/ml streptomycin (Life technology, cat: 15140-122), and 50µM

2-mercaptoethanol (Invitrogen, cat:21985-023), and cultured with HDM or without HDM at 37^{o}C with 5% CO2 for 72hrs.

ELISA of IL-4, IL-5, IL-17A, IFNγ, and IL-13 was done in BALF and 72hrs culture supernatant of MLN cells according to the manufacturer's instructions. Briefly, ELISA plates (Immunol VWR, Mississauga, ON) were coated with primary antibody (50µL/well) and incubated at 4^{o}C overnight. The next day, plates were blocked by adding 100µL /well diluent buffer to avoid non-specific bindings. After 2 hours of blocking, plates were washed 4 times with washing buffer (0.05% Tween-20 in 1XPBS). Standards and samples were diluted in assay diluent according to their respective concentration range, and they are added to each well in 50µL volume. Plates were incubated at room temperature with shaking (200rpm) for 2 hours and then washed 4 times with washing buffer. After adding the detection antibody (50 µL/well), plates were incubated at room temperature with shaking for 1 hour. Plates were then washed 4 times with washing buffer and added 50 µL of diluted Avidin-HRP solution to each well. After incubation at RT for 30 minutes with shaking, plates were washed 4 times with washing buffer. Mixture (A+B) of TMB Substrate Solution (50 µL/well) was added and kept in dark for 15-20 minutes. Then the reaction was stopped by adding stop solution (50µl 1M H_2S0_4 /well). Plates were read at 450 nm by Spectra Max 190 ELISA reader (Molecular Devices, CA, USA) after the appropriate color development and data were analyzed using SoftMax Pro software (Molecular Devices). All cytokines ELISA kits were purchased, except IL-13 (from eBioscience), from BioLegend (San Diego, CA). The concentration of the sample was calculated after subtracting blank OD from sample OD.

3.11 Measurement of Immunoglobulins

Serum was obtained from saline- and HDM-challenged *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}-*Plxnd1* KO mice. Total and HDM-specific IgE and IgG1 levels were measured using ELISA, according to the manufacturer's instructions (2, 5). Briefly, ELISA plates (Immulon VWR, Mississauga, ON) were coated with primary antibody (50µL/well) and incubated at 4ºC overnight. The next day, plates were blocked by adding 100µL /well diluent buffer to avoid non-specific bindings. After 2 hours of blocking at 37ºC, plates were washed 4 times with washing buffer (0.05% Tween-20 in 1XPBS). Standards and samples were diluted in assay diluent according to their respective concentration range, and they are added to each well in 50µL volume. Plates were incubated at room temperature with shaking (200rpm) for 2 hours and then washed 4 times with washing buffer. After adding the detection antibody (50 µL/well), plates were incubated at room temperature with shaking for 1 hour. Plates were then washed 4 times with washing buffer and added 50 µL of diluted Avidin-HRP solution to each well. After incubation at RT for 30 minutes with shaking, plates were washed 4 times with washing buffer. Mixture (A+B) of TMB Substrate Solution (50 µL/well) was added and kept in dark for 15-20 minutes. Then the reaction was stopped by adding stop solution (50µl 1N H_2S0_4 /well). Plates were read at 450 nm (Spectra Max) after the appropriate color development and data were analyzed with SoftMax Pro software (Molecular Devices). ELISA antibodies for measuring total and HDM-specific Immunoglobulins in serum were purchased from Southern Biotech (Birmingham, AL, USA).

3.12 Immunohistochemistry

Dissected lower left lobe of the lung was fixed in formalin for overnight and then embedded in paraffin. 5-µm thick tissue section was cut and mounted on slides. Saline and HDM-induced airway inflammation, mucus production, and collagen deposition in lung tissue sections were measured by performing H&E, periodic acid-Schiff (PAS), and Sirius red (SR), respectively. Images were taken by a digital Zeiss Axioskop 2 mot plus microscope at x200 using a Carl Zeiss AxioCam MRc 5 camera and AxioVision Rel 4.8 software. The mucus production and collagen deposition was determined by histological scoring of PAS, and SR-stained slides respectively. 3 individuals preformed the scoring in a blind manner (scoring scale: 1=mild, 2=moderate, and 3=severe) as previously described [254, 262] .

3.13 Generation of bone marrow derived macrophages (BMDMs) and culture with tamoxifen and HDM

Bone marrow (BM) cells were collected from *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice using a 1ml syringe and 25G needle. BM cells were mixed and filter by cells strainer to get a single-cell suspension. Cells were centrifuged at 1250rpm for 5 mins at 4^0C. After RBC lysis with ACK buffer, cells were centrifuged and dissolved in RPMI media. The cell suspension was plated on a petri dish (100 x 20mm) in the concentration of $5X10^5$ cells/10ml/plate in BMDM medium (30% of L929 conditioned medium +20% fetal bovine serum +1%penicillin-streptomycin +50µM 2-mercaptoethanol in RPMI 1640 medium). Cells were incubated at 37°C with 5% CO2 for 6 days. BMDM medium was changed every other the day over 6-day period. On day 6, some portion of BMDMs cells were treated with 5-OH tamoxifen (20µg/ml) at a 4ml/plate and incubate for 24hrs to remove *Plxnd1* from floxed cells. Cells were then washed twice with sterile 1X PBS and

detached by gentle scraping using the sterile scraper. A portion of cells (tamoxifen-treated or without tamoxifen-treated) were cultured with HDM (10µg/ml) for 24hrs. Cells are then detached and centrifuged at 1200rpm for 5 min at 4^{o}C and the cell pellet was stored at -70^{o}C to extract RNA.

3.14 RNA extraction and RT-PCR

Total RNA was extracted from the middle lobe of the lung and BMDM by using TRIzol (Invitrogen, Life Technologies, Cat: 15596018, USA) according to the manufacturer's protocol. The RNA concentration was measured by Nanodrop Lite spectrophotometer (Thermo scientific). MultiScribe Reverse Transcriptase was used for 1µg RNA to synthesize cDNA according to the manufacturer's instructions (Applied Biosystems, Cat: 4319983, Lithuania). Expression of the lung collagen (*Col3)*, mucin (*Muc5AC & MUC5B)*, α-Smooth Muscle Actin *(αSMA), fibronectin* and BMDM IL-10 genes was analyzed by Q-PCR. Eukaryotic Elongation Factor (*EEF2)* was used as a housekeeping gene. cDNA of each sample and *col3, Muc5AC, αSMA, fibronectin* genes specific primers (10 µM of each forward and reverse primer) were added to Power SYBR Green PCR Master Mix (Applied Biosystems, UK, Cat #: 4367659). Q-PCR was done in 96-well optical plate with an initial 1 cycle denaturation step at 95°C for 10 min, 40 cycles of PCR (95°C for 15 s, 60 °C for 30 s and 72°C for 30 s), 1 cycle of melting and 1 cycle of cooling (BIO-RAD CFX96^{TM}Real-Time PCR system). Product specificity was assessed by performing a melting curve analysis and examining the quality of amplification curves. The amplification of target genes was calculated by normalizing to the amplification of *eef2* and then normalizing to control groups.

Table 3. 1 Real time PCR primers (forward and reverse) and amplicon size used in analysis of gene expression.

Genes	Forward (5′-3′)	Reverse (5′-3′)
Muc5ac	GCATGTTGGTACCCCACTCA	GTTGCAGAGACCAGGGAAG T
Muc5b	GAAACTGGAGCTGGGCTCTG	CAGGTGTAAGGCGCTCATGC
αSMA	CCCAGACATCAGGGAGTAATGGT	TCTATCGGATACTTCAGCGT CA
Col3	GCAGGACCCAGAGGAGTAG	TTCCATCATTGCCTGGTC
Fibronectine	GGGGAGACATGGCAAACCTGT	TGGTAGGAGAGTAGTTGGTG G
IL-10	TGCACTACCAAAGCCACAAAGCAG	TGGCCTTGTAGACACCTTGG TCTT
Eef2	5′-TGTCAGTCATCGCCCATGTG-3′	5′-CATCCTTGCGAGTGTCAGTG A-3′

3.15 Statistical analyses

GraphPad Prism 7.0 software was used for statistical analysis. Depending on the number of groups and treatments, data were analyzed by unpaired t-test, one-way ANOVA, or two-way ANOVA, followed by Tukey's test. Differences were statistically significant at *p <0.05, **p < 0.01, and ***p <0.001.

4 RESULTS

4.1 *Plxnd1* deletion from CX3CR1-expressing IMs

Plxnd1 deletion was determined in BMDMs and lung IMs of *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ mice. *Plxnd1* was deleted from BMDMs by treating BMDMs of *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ mice with tamoxifen for 24hrs. More than 80% deletion was found after tamoxifen treatment (Figure 4.1B). Deletion was checked in the lung IMs after sorting from *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}-*Plxnd1* KO mice. Naïve *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ mice were treated with tamoxifen for every other day for a week and after two days of last treatment lung IMs were sorted. Around 60% deletion was found in lung IMs in mice treated with tamoxifen (Figure 4.1D). A small percentage of deletion was also found in lung T-cells.

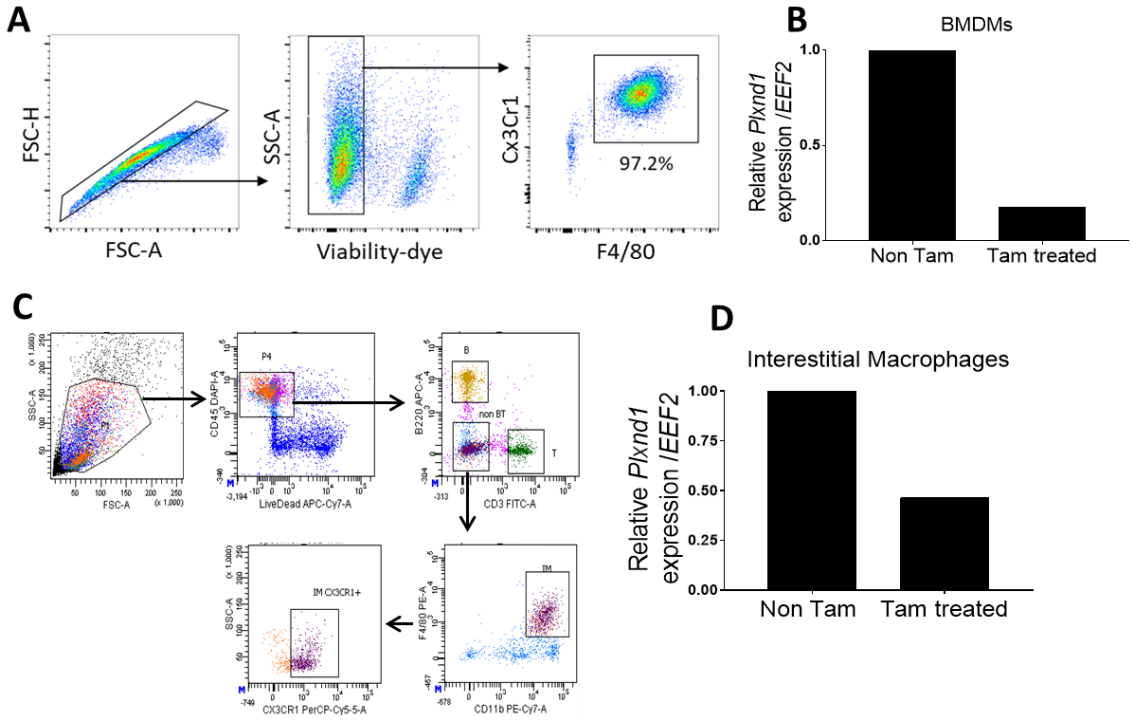


Figure 4. 1: Deletion of *Plxnd1* from CX3CR1 cell. Deletion of Plxnd1 was checked in BMDMs and lung IMs by q-PCR. Representative data of deletion has been shown in B & D. Bone marrow-derived macrophages was generated from bone marrow of *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ mice. 97.2% cells are CX3CR1-expressing macrophages (A). A portion of *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ BMDMs were treated with tamoxifen (tam) and *Plxnd1* deletion was determined (B). FACS analysis was done to sort interstitial macrophages (IMs) from lung of tam and non tam treated mice (C) and *Plxnd1* deletion was checked by q-PCR (D).

4.2 Mice with *Plxnd1* deletion in CX3CR1 cells have no effect on immune cells composition in lung compared to WT at steady state

To understand whether the absence of *Plxnd1* in IMs cell has any effect on immune cells composition in the lung, spleen, lymph node, and blood, immunophenotyping of *Cx3cr1*^{creERT2}-*Plxnd1* KO, *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ as well as wild type (WT) mice were performed at the baseline using FACS analysis [167] (Figure 4.2A)

To get *Plxnd1* deletion in the CX3CR1 cells (then defined as *Cx3cr1*^{creERT2}-*Plxnd1* KO mice) *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ mice were treated with tamoxifen at every other day of a week. After two days of last tamoxifen treatment, lung from WT, *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$, and *Cx3cr1*^{creERT2}-*Plxnd1* KO were collected to analyze immune cells. At homeostatic conditions, comparable absolute number and percentage of eosinophils, neutrophils, alveolar and interstitial macrophages, B and T-cells were observed in the lung of *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$, *Cx3cr1*^{creERT2}-*Plxnd1* KO, and WT mice (Figure 4.2B & 4.2C). These Immunophenotyping data suggest that deletion of *Plxnd1* from CX3CR1 cells has no effect on immune cells compositions at the steady-state.

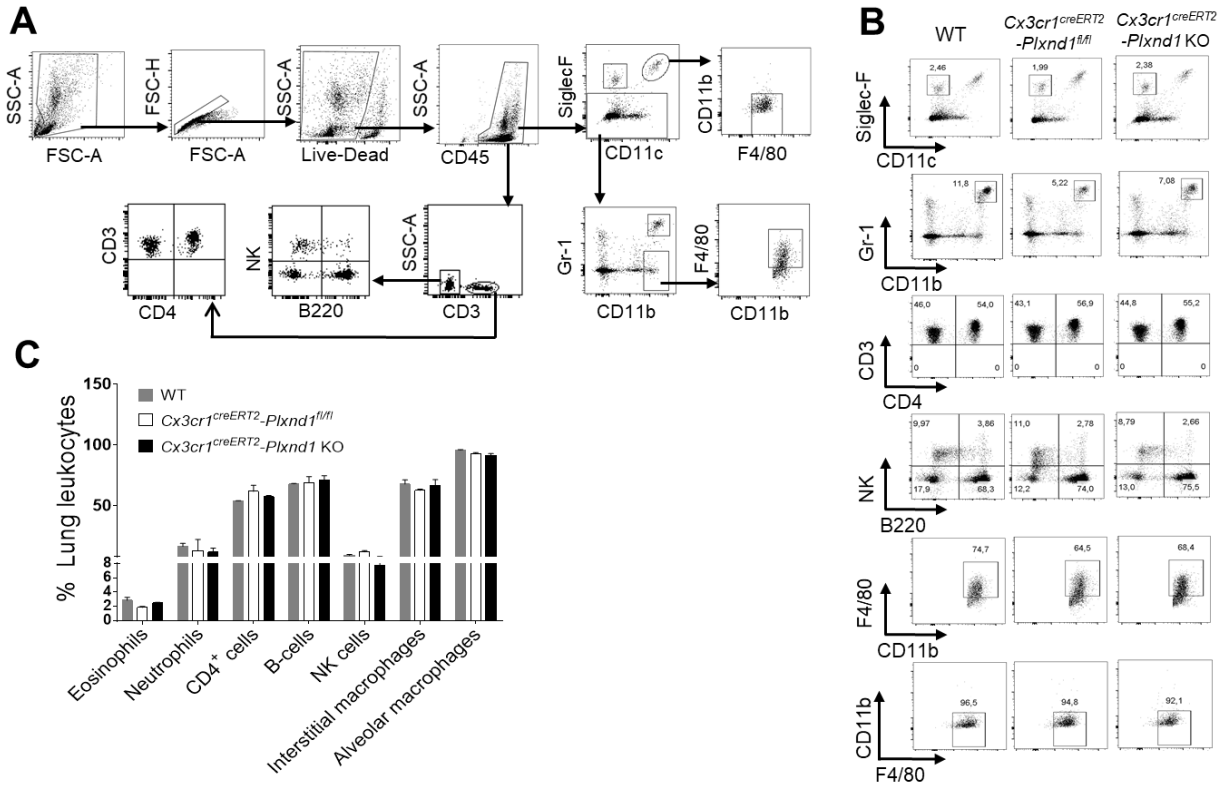


Figure 4. 2: Immunophenotyping of inflammatory cells in lung in *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$* and *Cx3cr1^{creERT2}-Plxnd1* KO mice. FACS analysis was done to characterize inflammatory cells populations in the lung. General gating strategy includes exclusion of debris and doublet, and the inclusion of live cells (A). Single-cells suspension of the lung were stained by target specific surface Abs. Eosinophils were characterized by expression of Siglec-F and CD11c negative. Alveolar macrophages are CD11c, CD11b and F4/80 positive. Neutrophils are characterized by the expression of Gr-1 and CD11b pre-gated on Siglec-F negative cells. Interstitial macrophages are CD11b and F4/80 positive pre gated on neutrophils negative cells. $CD4^+$ T cells were characterized by CD3 and CD4 positive cells. B and NK cells were characterized by B220, and NK respectively pre gated on CD3 negative cells (A). Percentage of each cell type was compared between WT, *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$*, and *Cx3cr1^{creERT2}-Plxnd1* KO mice of lung (C). Data are

expressed as mean with SEM. n=03 data per group (Figure courtesy of Lianyu Shan and Ifeoma Okwor).

4.3 Mice with *Plxnd1* deletion in CX3CR1 cells have no effect on immune cells composition in lymph node, spleen, and blood compared to WT at steady state.

To understand whether the absence of *Plxnd1* in IMs cell has any effect on immune cells composition in the spleen, lymph node, and blood, immunophenotyping of *Cx3cr1*^{creERT2}*-Plxnd1* KO, *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ as well as wild type (WT) mice were performed at the baseline using FACS analysis (Figure 4.3A, 4.3B, and 4.3C)

Cx3cr1^{creERT2}*-Plxnd1*$^{fl/fl}$ mice were treated with tamoxifen for three days at every other day of a week. After two days of last tamoxifen treatment, we collected mediastinal lymph node (MLN), spleen, and blood from WT, *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$, and *Cx3cr1*^{creERT2}*-Plxnd1* KO to analyze immune cells. At homeostatic conditions, comparable immune cells number was found in the spleen, lymph node, and blood (Figure 4.3D & 4.3E, & 4.3F) of *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$, *Cx3cr1*^{creERT2}*-Plxnd1* KO, and WT mice at the baseline. These Immunophenotyping data suggest that deletion of *Plxnd1* from CX3CR1 cells has no effect on immune cells compositions at different organs at the steady state.

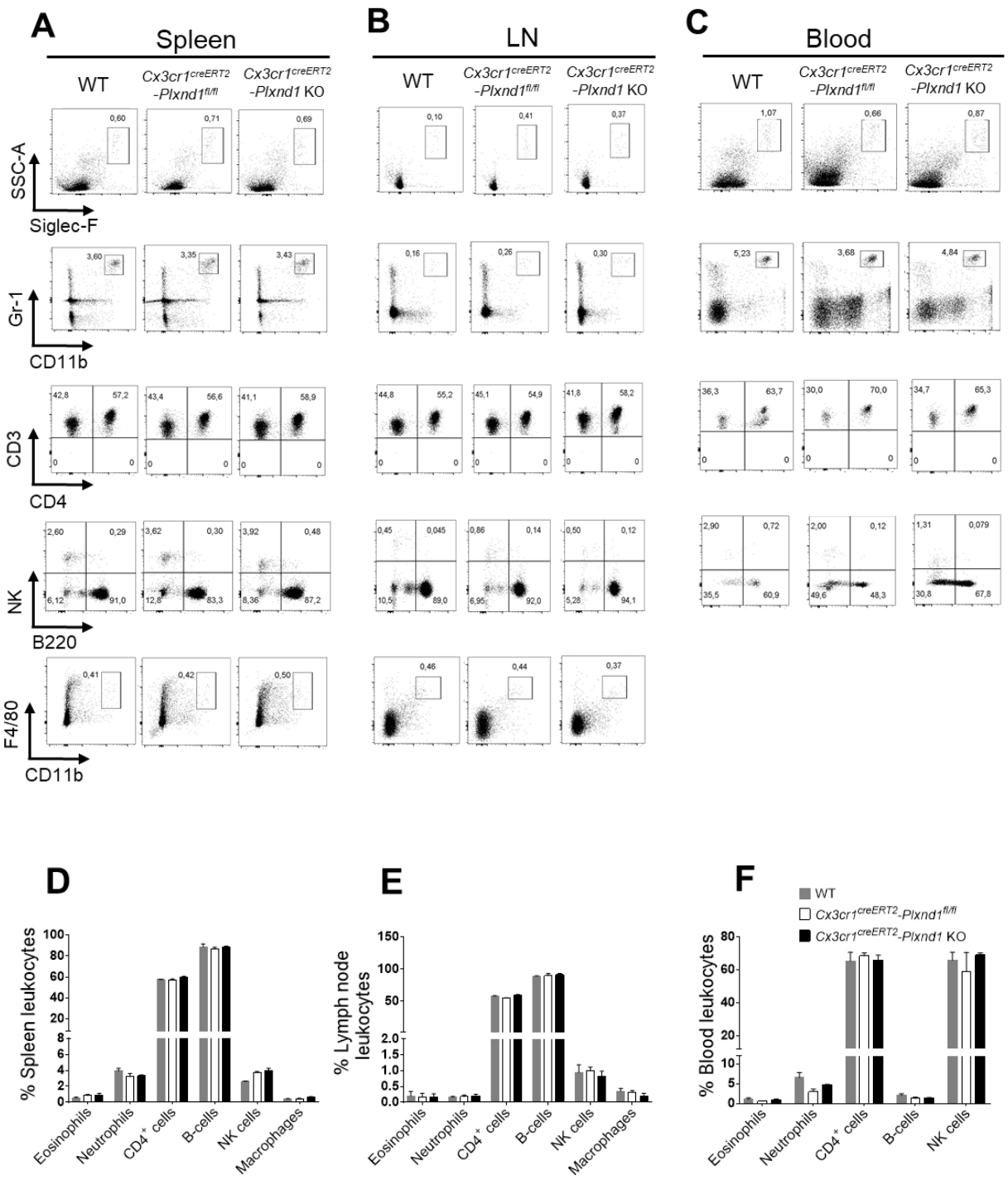


Figure 4. 3: Immunophenotyping of inflammatory cells in spleen, LN, and blood in *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$* and *Cx3cr1^{creERT2}-Plxnd1* KO mice. FACS analysis was done to characterize inflammatory cells populations in the spleen, mediastinal lymph node (MLN), and

blood. General gating strategy includes exclusion of debris and doublet, and the inclusion of live cells. Single-cells suspension of the lung, and spleen, MLN, and blood were stained by target specific surface Abs. Eosinophils were characterized by expression of Siglec-F and CD11c negative. Alveolar macrophages are CD11c, CD11b and F4/80 positive. Neutrophils are characterized by the expression of Gr-1 and CD11b pre-gated on Siglec-F negative cells. Interstitial macrophages are CD11b and F4/80 positive pre gated on neutrophils negative cells. $CD4^+$ T cells were characterized by CD3 and CD4 positive cells. B and NK cells were characterized by B220, and NK respectively pre gated on CD3 negative cells (A, B, C). Percentage of each cell type was compared between WT, *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$, and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice of spleen, MLN, and blood cells (D, E, & F). Data are expressed as mean with SEM. n=03 data per group.

4.4 Absence of *Plxnd1* in CX3CR1 cells induced exaggerated AHR.

I investigated whether a lack of *Plxnd1* in CX3CR1 cells exacerbate allergic asthma features. To do this, we exposed *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice to house dust mite (HDM) allergen for two consecutive weeks (5 days/week) (Figure 4.4A), as repeated exposure to allergen induces airway inflammation, AHR, and airway remodeling [261, 264, 265]. Since airway resistance (Rn) is a key measure of lung function, we measured Rn after 2 days of the last HDM challenge. I observed a significant increased in HDM-induced airway resistance (Figure 4.4B) in *Cx3cr1*^{creERT2} *-Plxnd1* KO mice compared to *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice (****P<0.0001). I also measured tissue dumping and tissue elastance in both groups of mice. However, we did not observe any significant difference in HDM-induced tissue dumping and tissue elastance between *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice and *Cx3cr1*^{creERT2} *-Plxnd1* KO mice (Figure 4.4C & 4.4D). These

results suggest that the deletion of *Plxnd1* from CX3CR1 cells exacerbate HDM-induced airway resistance.

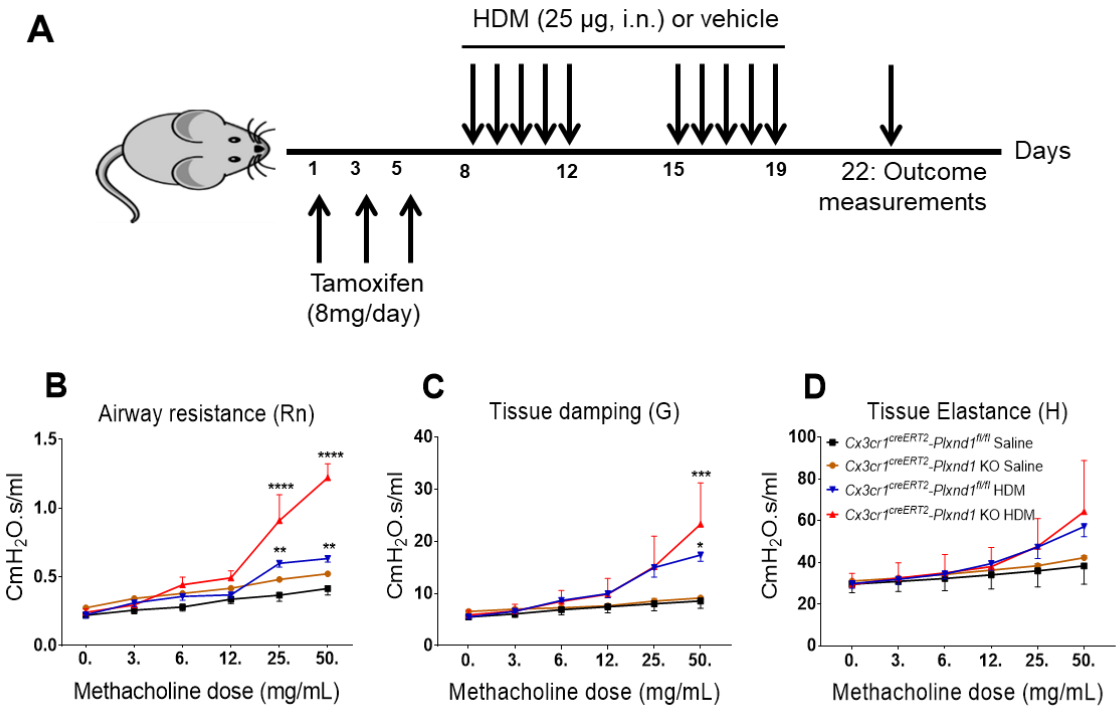
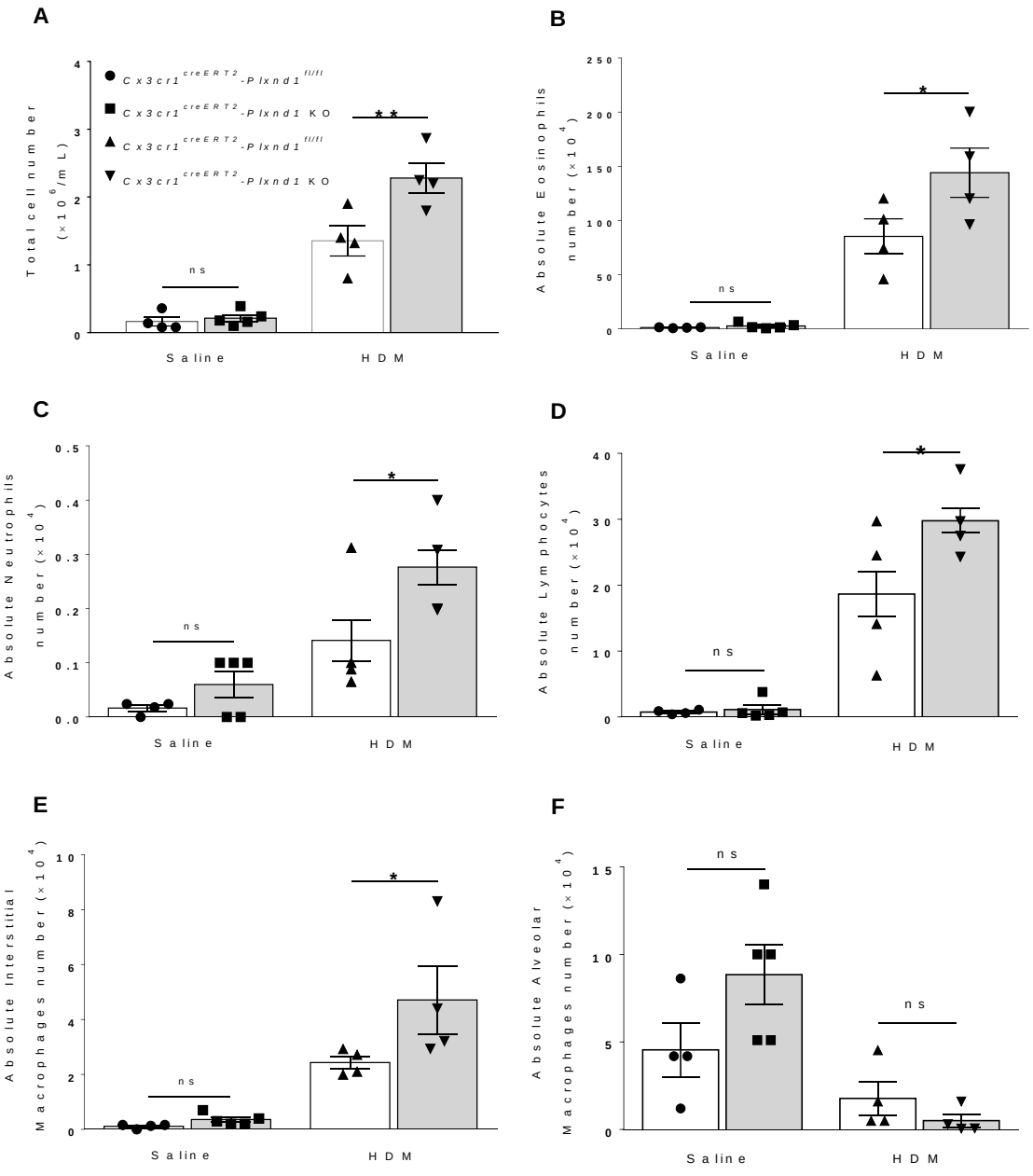


Figure 4. 4: Airway hyperresponsiveness is elevated in HDM-challenged CX3CR1-*Plxnd1*-deficient mice. CX3CR1 cell-specific *Plxnd1*-deficient mouse was established by oral gavage of tamoxifen for 3 alternative days and then induced airway allergic asthma by i.n exposure of HDM for 2 weeks, control mice received saline (A). HDM or saline-exposed *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ or *Cx3cr1*^{creERT2}-*Plxnd1* KO mice underwent tracheotomy accompanied by methacholine challenge to measure airway resistance (Rn) (B), tissue damping (G) (C), and tissue elastance (H) (D). Data are expressed as mean with SEM. n = 03-05 mice per group. *p<0.05 **p<0.01, ***p<0.001, and ****p<0.0001.

4.5 Absence of *Plxnd1* in CX3CR1 cells induced exaggerated airway inflammation

As airway resistance correlates with the airway inflammation in asthma [257], I then measured total and differential inflammatory cells in the bronchoalveolar lavage fluid (BALF). I found a significant increased in total BALF cells (**p<0.01) in *Cx3cr1*^{creERT2} *-Plxnd1* KO mice compared to that of *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice after HDM challenge (Figure 4.5A).

As I got higher total BALF cells in *Cx3cr1*^{creERT2}*-Plxnd1* KO mice, it encouraged me to measure different inflammatory cells in the BALF of both groups. BALF inflammatory cells were analyzed by flow cytometric and cytologic examination on cytospin slides by differential quick staining. The number of HDM-induced eosinophils, neutrophils, lymphocytes including T and B cells, and IMs increased in the airway after deletion of *Plxnd1* from CX3CR1 cells (*p<0.05) (Figure 4.5B, 4.5C, 4.5D, and 4.5E). I got similar results after staining the BAL cells for nucleus and cytoplasm where *Cx3cr1*^{creERT2}*-Plxnd1* KO mice have higher inflammatory cells compared to *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice (*p<0.05 **p<0.01, and ****p<0.0001) after HDM challenge (Figure 4.5G & 4.5H). Inflammatory cells in the lung tissue of *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice were confirmed by hematoxylin and eosin staining of lung tissue section and flow cytometric analysis (Figure 4.5I and 4.5J). Collectively, all data demonstrate that the recruitment of granulocytes and lymphocytes increased in the airway of CX3CR1 cells specific-*Plxnd1* KO mice after HDM challenge.


A
Total cell number (×10^6/mL)
Cx3cr1^creERT2-Plxnd1^fl/fl
Cx3cr1^creERT2-Plxnd1 KO
Cx3cr1^creERT2-Plxnd1^fl/fl
Cx3cr1^creERT2-Plxnd1 KO
ns
**
Saline
HDM
B
Absolute Eosinophils number (×10^4)
ns
*
Saline
HDM
C
Absolute Neutrophils number (×10^4)
ns
*
Saline
HDM
D
Absolute Lymphocytes number (×10^4)
ns
*
Saline
HDM
E
Absolute Interstitial Macrophages number (×10^4)
ns
*
Saline
HDM
F
Absolute Alveolar Macrophages number (×10^4)
ns
ns
Saline
HDM

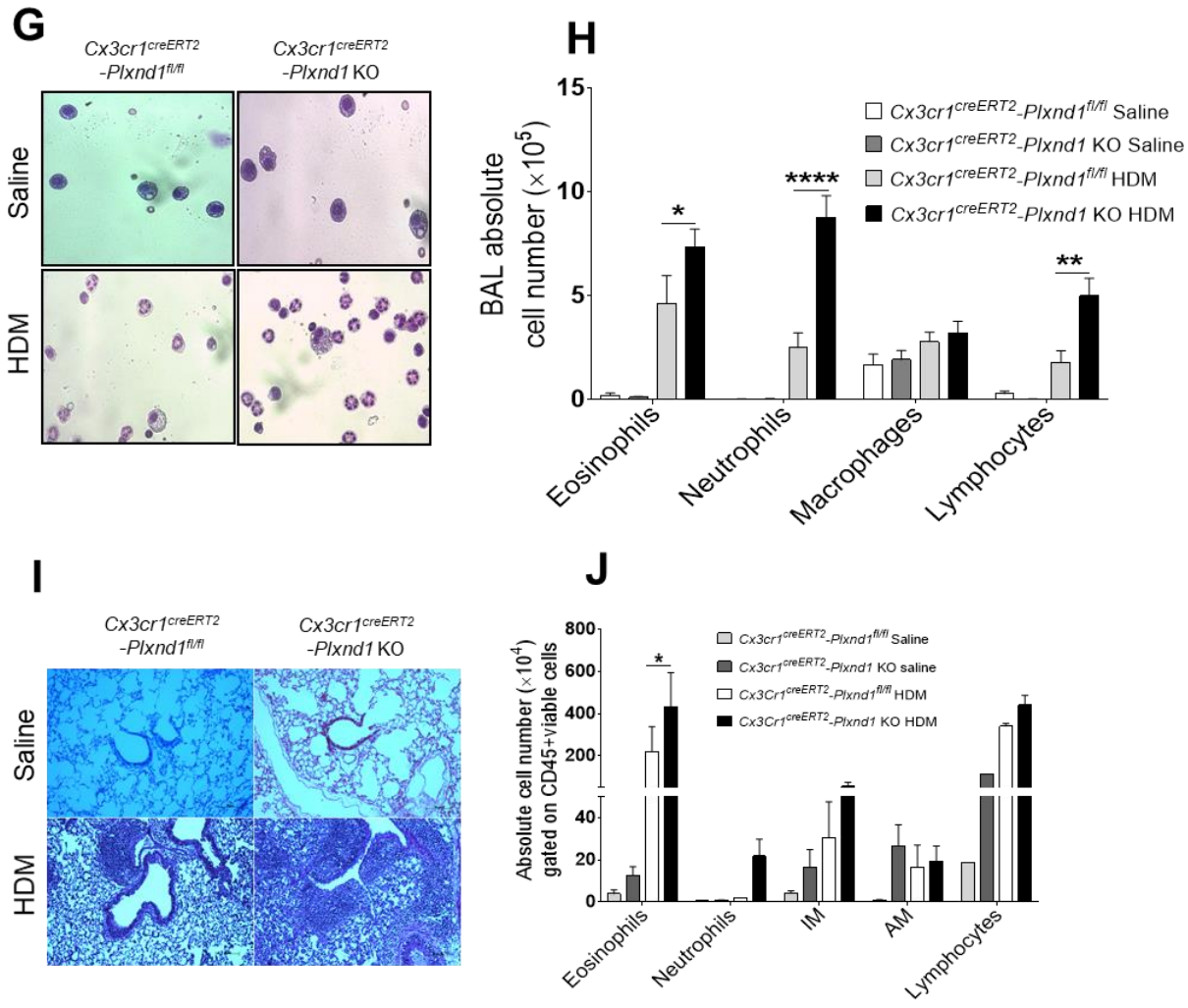


Figure 4. 5: Inflammatory cells increased in the airway of CX3CR1-*Plxnd1*-deficient mice. Total bronchoalveolar lavage fluid (BALF) inflammatory cells of saline or HDM treated *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}-*Plxnd1* KO mice were counted on a hemocytometer using trypan blue (A). FACS analysis has been done using Abs against surface marker. General gating strategy includes single cells and excludes debris and doublet. The absolute number of eosinophils (B), neutrophils (C), lymphocytes (D), interstitial macrophages (E), and alveolar macrophages (F) were measured. A portion of BALF cells was fixed on the cytospin slide and stained for nucleus and cytoplasm (G). BALF inflammatory cells were counted in a blind manner

(H). H & E staining of lung tissue (I). Lung inflammatory cells analyzed by flow cytometry (J). Data are expressed as mean with SEM. n= 03-05 mice per group. *p<0.05 **p<0.01, and ****p<0.0001.

4.6 HDM specific IgE is enhanced in *Cx3cr1^{creERT2}-Plxnd1* KO mice

Allergic asthma is mediated by the triggering of IgE bound mast cells by repetitive exposure of allergens, and the prevalence of asthma is closely related to serum IgE in humans [29, 266, 267]. So, I investigated total and HDM-specific immunoglobulins responses in the serum obtained from *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$* and *Cx3cr1^{creERT2}-Plxnd1* KO mice. I observed that the HDM-specific IgE level significantly increased in *Cx3cr1^{creERT2}-Plxnd1* KO mice compared to *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$* mice after HDM challenge (*P < 0.05) (Figure 4.6B). On the other hand, the HDM-specific IgG1 level decreased in the serum of *Cx3cr1^{creERT2}-Plxnd1* KO mice (*P < 0.05) (Figure 4.6D). However, there is no significant difference in total IgE and IgG1 responses between *Cx3cr1^{creERT2}-Plxnd1* KO mice and *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$* mice (Figure 4.6A & 4.6C). Therefore, it suggests that the absence of *Plxnd1* in CX3CR1 cells could mediate allergic asthma in part through the up-regulation of HDM-induced IgE in the serum.

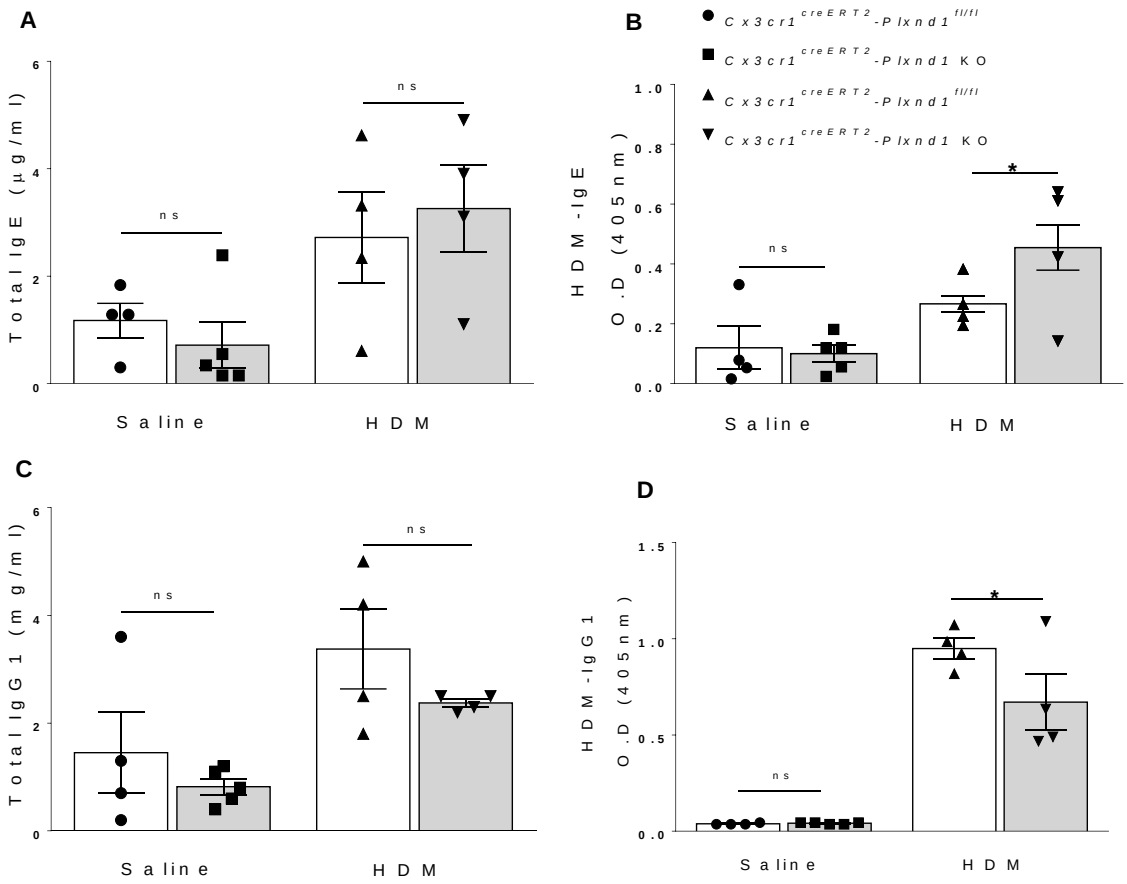


Figure 4. 6: HDM-specific serum IgE increased in *Cx3cr1*^{creERT2}*-Plxnd1* KO mice upon HDM challenge. Serum was collected from *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice exposed to either saline or HDM. The levels of Total IgE (A), HDM-specific IgE (B), Total IgG1 (C), and HDM-specific IgG1 (D) were measured by ELISA. HDM-specific IgE increased in *Cx3cr1*^{creERT2}*-Plxnd1* KO mice compared to *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice after HDM exposure, whereas HDM-IgG1 decreased in *Cx3cr1*^{creERT2}*-Plxnd1* KO mice. Data are expressed as mean with SEM. n=03-05 mice per group. *P < 0.05.

4.7 Th2/Th17 cytokines increased in bronchoalveolar lavage in the absence of *Plxnd1* in CX3CR1 cells during allergic asthma.

Th2/Th17 cytokines levels play an essential role in the exacerbation of allergic asthma. So, I measured Th2/Th17 cytokines level in the BALF of both *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice. HDM-induced IL-4, IL-17A, and IL-13 level significantly increased in the BALF of *Cx3cr1*^{creERT2}*-Plxnd1* KO mice (*P < 0.05) (Figure 4.7A, 4.7B & 4.7C) compared to that mice which had *Plxnd1* in CX3CR1 cells. Increased levels of IL-4 and IL-17A might induce the production of serum HDM-specific IgE [268, 269] and recruitment of airway neutrophils [270] respectively. Increased level of IL-13 in BALF induce goblet cell hyperplasia and mucin production [271]. All these data suggest that the deletion of *Plxnd1* from CX3CR1 cells induced the production of Th2/Th17 cytokines in BALF that correlates with the allergic asthma exacerbation in *Cx3cr1*^{creERT2}*-Plxnd1* KO mice.

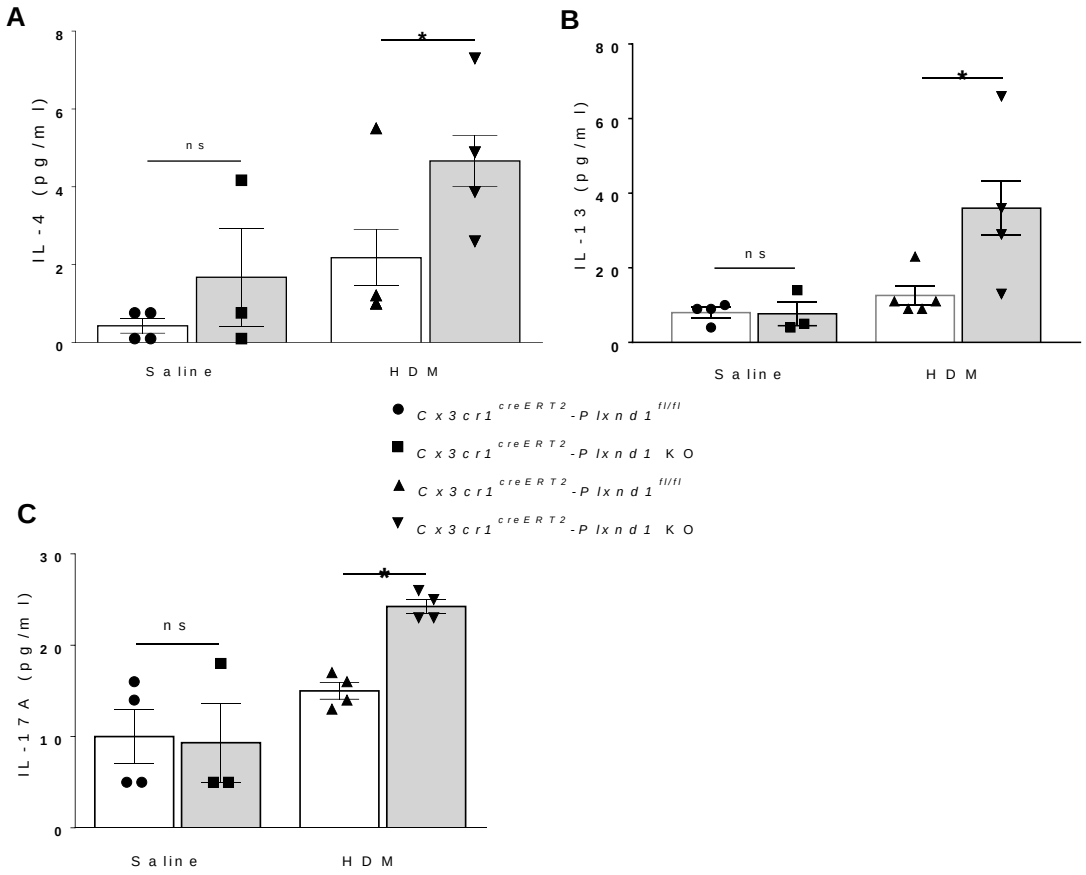


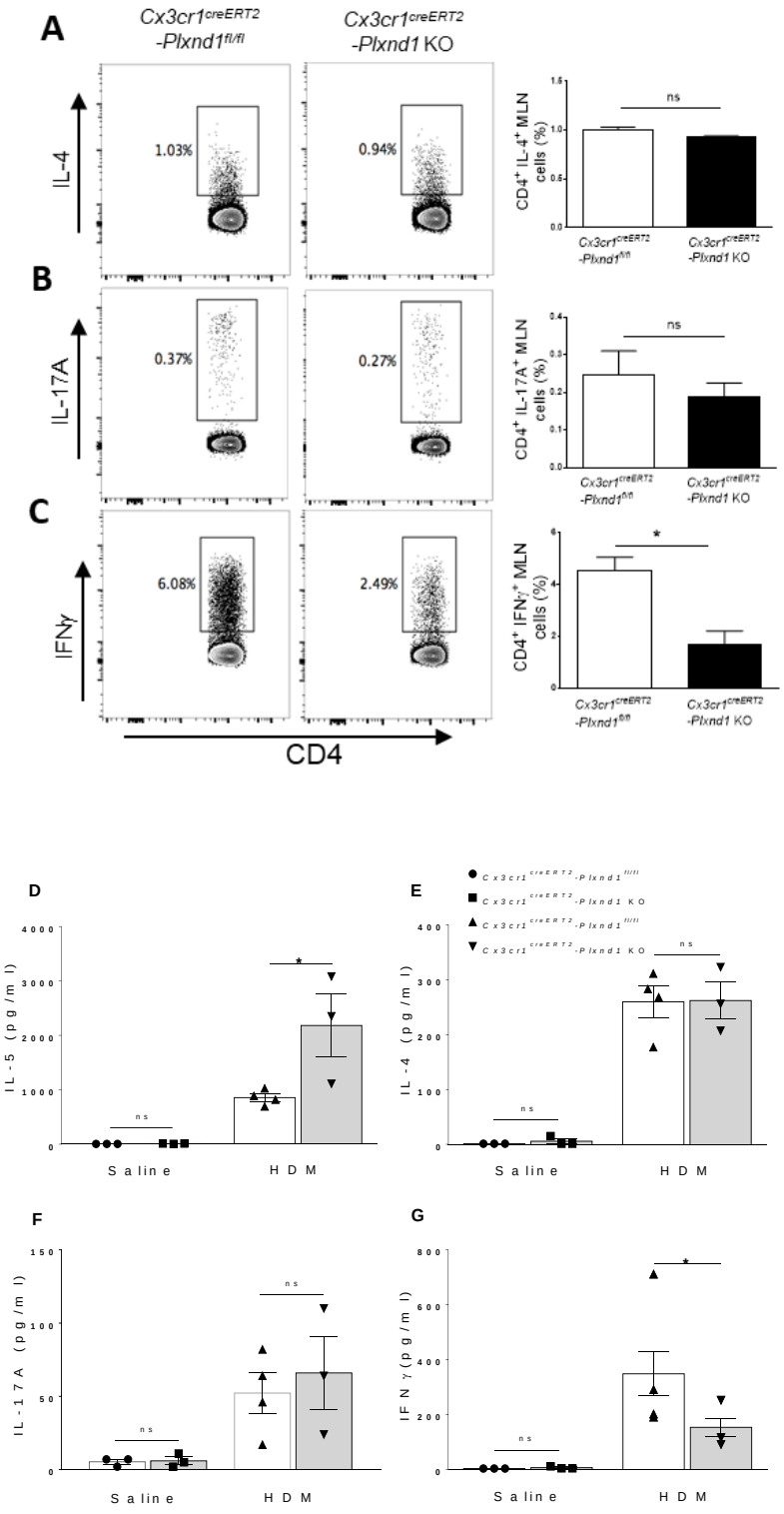
Figure 4. 7: Th2 and Th17 cytokines level of bronchoalveolar lavage fluid (BALF) elevated in *Cx3cr1*^{creERT2}*-Plxnd1* KO mice. BALF was collected from *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice exposed to either saline or HDM. The levels of IL-4 (A), IL-17A (B), and IL-13 (C) were measured by ELISA. IL-4, IL-17A and IL-13 level increased in *Cx3cr1*^{creERT2}*-Plxnd1* KO mice compared to *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice after HDM exposure. Data are expressed as mean with SEM. n=03-04 mice per group. *P < 0.05.

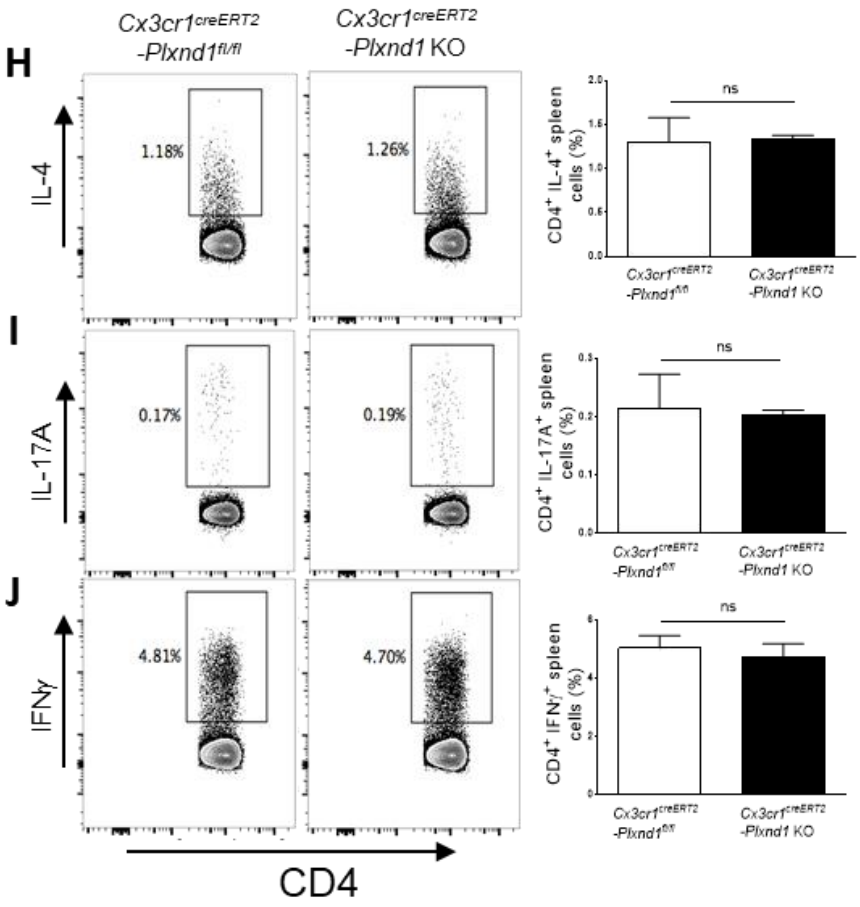
4.8 IL-5 increased and IFNγ reduced in the lymph node and spleen in the absence of *Plxnd1* in CX3CR1 cells during allergic asthma.

As I got higher cytokines responses in BALF of *Cx3cr1*^{creERT2}*-Plxnd1* KO mice, it motivated us to check whether this response is present in the lymph node and spleen. To do this, mediastinal lymph node (MLN) and spleen single cells were isolated from *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice. A single-cell suspension of MLN and spleen were stimulated with phorbol 12-myristate-13-acetate (PMA), ionomycin, and Brefeldin A in *vitro* for 4hrs and then checked IL-4, IL-17A, and IFNγ responses by flowcytometric analysis. IFNγ producing MLN CD4+ cells significantly decreased in *Cx3cr1*^{creERT2}*-Plxnd1* KO mice compared to *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice (*P < 0.05) (Figure 4.8C). I did not find any difference between *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-PLXND1* KO for MLN IL-4 and IL-17A (Figure 4.8A and 4.8B). Again, I did not get the difference for spleen IL-4, IL-17A, and IFNγ (Figure 4.8 H, 4.8I, and 4.8J), which indicates the deletion of *Plxnd1* from CX3CR1-cells does not affect on the systemic system.

Then I investigated whether these cytokines responses are sustained even after HDM recall. To do this a portion of single-cell suspension was re-stimulated with HDM or vehicle *in vitro* for 72hrs to determine the cytokines responses in the culture supernatant. The level of IL-5 increased in the MLN and spleen culture supernatant of *Cx3cr1*^{creERT2}*-Plxnd1* KO mice compared to *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice (*P < 0.05) (Figure 4.8D and 4.8K). On the other hand, the level of IFNγ decreased in the MLN culture supernatant of *Cx3cr1*^{creERT2}*-Plxnd1* KO mice (*P < 0.05) (Figure 4.8G). Although no difference for IL-4 in MLN culture supernatant (Figure 4.8E), *Cx3cr1*^{creERT2}*-Plxnd1* KO mice had higher IL-4 (Figure 4.8L) in the spleen culture supernatant (*P < 0.05).

These results suggest lack of *Plxnd1* in CX3CR1 cells induce Th2 (IL-5) and reduce Th1 (IFNγ) cytokines production result in allergic asthma exacerbation in *Cx3cr1*^{creERT2}*-Plxnd1* KO mice.




H
I
J
Cx3cr1creERT2 -Plxnd1fl/fl
Cx3cr1creERT2 -Plxnd1 KO
IL-4
IL-17A
IFNγ
CD4
1.18%
1.26%
0.17%
0.19%
4.81%
4.70%
ns
CD4+ IL-4+ spleen cells (%)
CD4+ IL-17A+ spleen cells (%)
CD4+ IFNγ+ spleen cells (%)

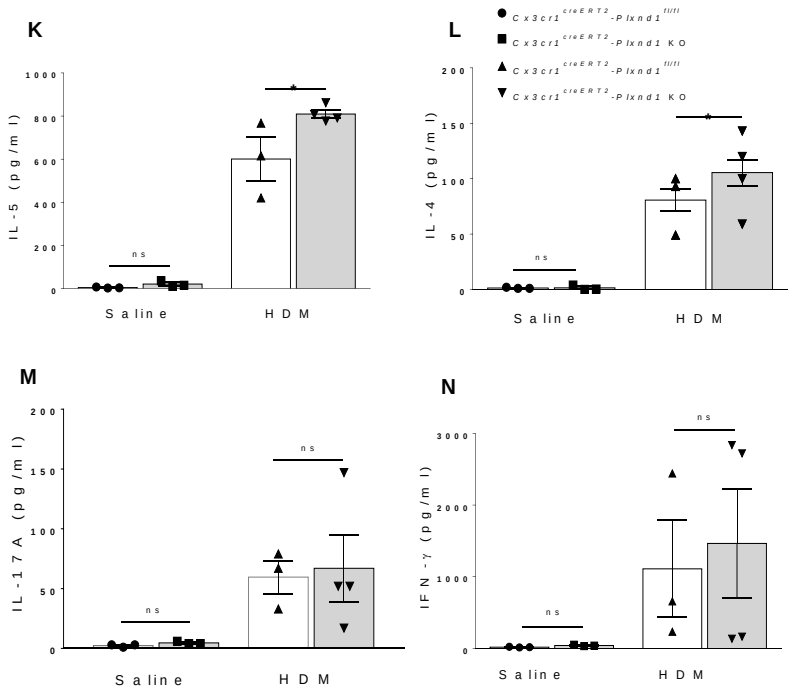

K
L
M
N
IL-5 (pg/ml)
IL-4 (pg/ml)
IL-17A (pg/ml)
IFN-γ (pg/ml)
Saline
HDM
ns
Cx3cr1creERT2-Plxnd1fl/fl
Cx3cr1creERT2-Plxnd1 KO

Figure 4. 8: Mediastinal lymph node (MLN) and spleen Th2 cytokines level increased in *Cx3cr1^{creERT2}-Plxnd1* KO mice. Single-cell suspension of mediastinal lymph node (MLN) and spleen from *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$* and *Cx3cr1^{creERT2}-Plxnd1* KO mice exposed to saline or HDM was prepared and stimulated with phorbol 12-myristate-13-acetate (PMA), ionomycin, and Brefeldin A in vitro for 4hrs. IL-4 (A & H), IL-17A (B & I), and IFNγ (C & J) producing MLN & spleen $CD4^+$ T-cells number were compared in HDM-challenged *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$* and *Cx3cr1^{creERT2}-Plxnd1* KO mice by FACS. A portion of cells were stimulated with medium or HDM in vitro. The levels of IL-5 (D & K), IL-4 (E & L), IL-17A (F & M), interferon (IFNγ) (G & N) were measured in the supernatant of 72 hours cultured cells by ELISA. Data are expressed as means SEM. n=03-04 mice per group *P < 0.05.

4.9 Lack of *Plxnd1* in CX3CR1 cells induced expression of airway mucin proteins

Mucus overproduction is one of pathological causes of allergic asthma, where it plays role in the airway remodeling. The two major secreted airway mucin proteins are MUC5AC and MUC5B. So, I determined the expression level of these proteins in the airway.

I investigated *MUC5AC and MUC5B* genes expression using gene specific primers. I found that *Cx3cr1^{creERT2}-Plxnd1* KO mice had significantly higher *MUC5AC* and *MUC5B* genes expression compared to *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$* mice after HDM challenge (*p<0.05) (Figure 4.9A & and 4.9B). The expression of these proteins was confirmed by staining of lung tissue for mucin. These data indicate absence of *Plxnd1* in CX3CR1-cells induce mucus production in allergic asthma result in airway remodeling.

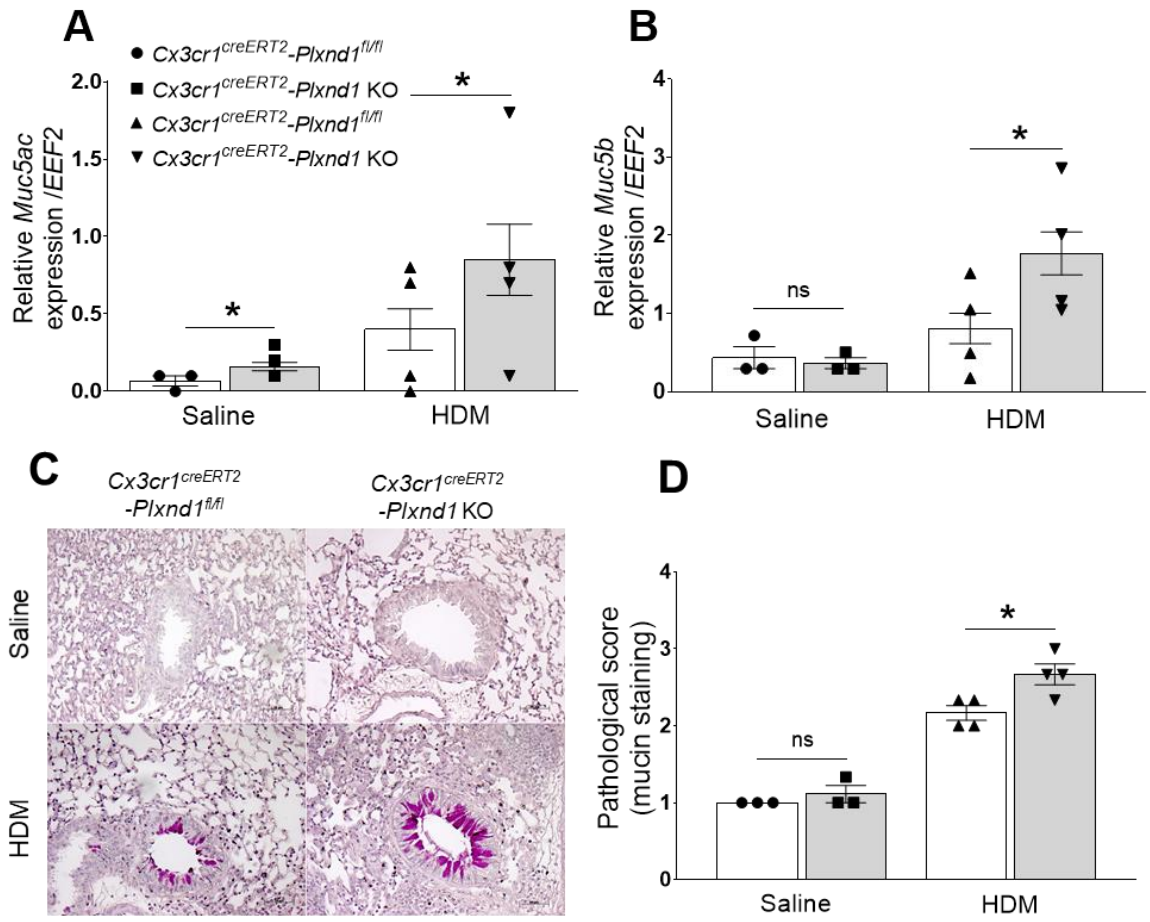


Figure 4. 9: Production of mucin increased in *Cx3cr1^{creERT2}-Plxnd1* KO mice. Expression of lung MUC5AC and MUC5B genes in *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$* and *Cx3cr1^{creERT2}-Plxnd1* KO mice was determined by Q-PCR using specific primers (A & B). Lung tissue section from *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$* and *Cx3cr1^{creERT2}-Plxnd1* KO mice were stained with Periodic acid-Schiff (PAS) to determine mucus production (C). Scoring data of lung mucus staining was done by three peoples in a blind manner (D). Data are expressed as mean with SEM. n= 03-05 mice per group, *p<0.05.

4.10 Lack of *Plxnd1* in CX3CR1 cells induced expression of airway α-smooth muscle actin

Collagen and fibronectin deposition, and smooth muscle cells proliferation is the most critical factor for the airway remodeling in the allergic asthma. So, we determined expression levels of these proteins in the airway.

We investigated *col3, fibronectin, and α-SMA* (smooth muscle actin) genes expression using gene specific primers in the lung tissue RNA of *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice. Expression of *α-SMA* gene was significantly higher in Cx3cr1^{creERT2}*-Plxnd1* KO mice compared to *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice after HDM challenge (*$p<0.05$) (Figure 4.10C). We did not find difference of *col3* and *fibronectin* gene expression between *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice (Figure 4.10A and 4.10B). We did not see any difference of collagen deposition after staining the lung tissue for collagen (Figure 4.10D). These data suggest that absence of *Plxnd1* in CX3CR1 cells induce airway remodeling by inducing *α-SMA* expression in allergic asthma.

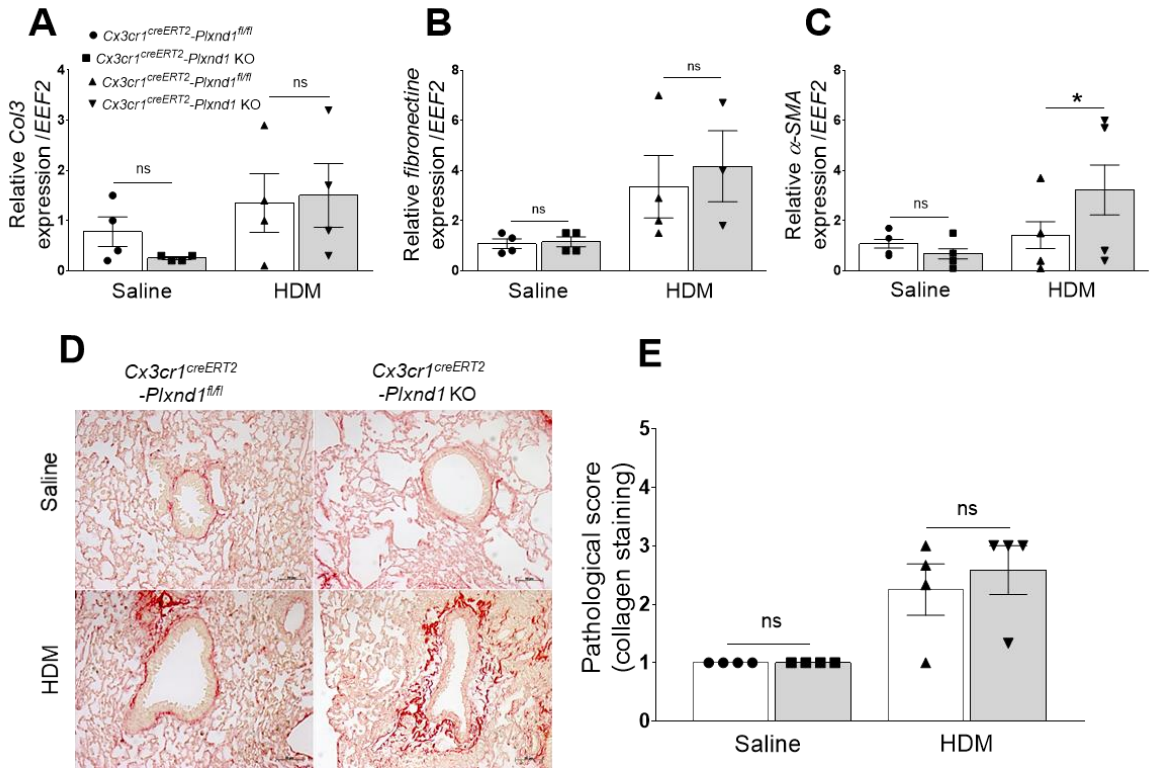


Figure 4. 10: Lung α-smooth muscle actin production increased in *Cx3cr1^{creERT2}-Plxnd1* KO mice. Expression of airway remodeling genes col3 (A), fibronectin (B), α-SMA (C) in *Cx3cr1^{creERT2} Plxnd1$^{fl/fl}$* and *Cx3cr1^{creERT2}-Plxnd1* KO mice was determined by Q-PCR using specific primers. Lung tissue section from *Cx3cr1^{creERT2}-Plxnd1$^{fl/fl}$* and *Cx3cr1^{creERT2}-Plxnd1* KO mice were stained with sirius red to determine collagen deposition (D). Scoring data of collagen staining (F). Data are expressed as mean with SEM. n= 03-05 mice per group, *p<0.05.

4.11 IL-10 expression reduced in *Cx3cr1^{creERT2}-Plxnd1* KO bone marrow-derived macrophages (BMDMs).

Our data shows that absence of *Plxnd1* in the CX3CR1-positive lung interstitial macrophages exacerbate airway allergic asthma. It has been shown that lung interstitial macrophage ameliorates

allergic asthma through secretion of IL-10 that negatively regulate DC function in the airway and thus reduce Th2 and Th17-mediated inflammation[168, 178]. Therefore, we generated BMDM by 6-day culture of bone marrow collecting from *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice and characterize the percentage of CX3CR1-positive macrophages. We found that around 95% cells are CX3CR1-positive macrophages (Figure 4.11A).

BMDMs were treated with tamoxifen to get *Cx3cr1*^{creERT2}*-Plxnd1* KO BMDM and later culture them with HDM. RNA was extracted from *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO BMDM and from HDM treated BMDM. The expression of *IL-10* was determined by Q-PCR using gene specific primer. We found that *IL-10* expression decreased by *Cx3cr1*^{creERT2}*-Plxnd1* KO BMDM compared to *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ BMDM at naïve (**$p<0.01$) as well as after culture with HDM (***$p<0.001$) (Figure 4.11B & 4.11C). This result explains that deletion of *Plxnd1* reduce IL-10 expression by interstitial macrophages that induce allergic asthma exacerbation.

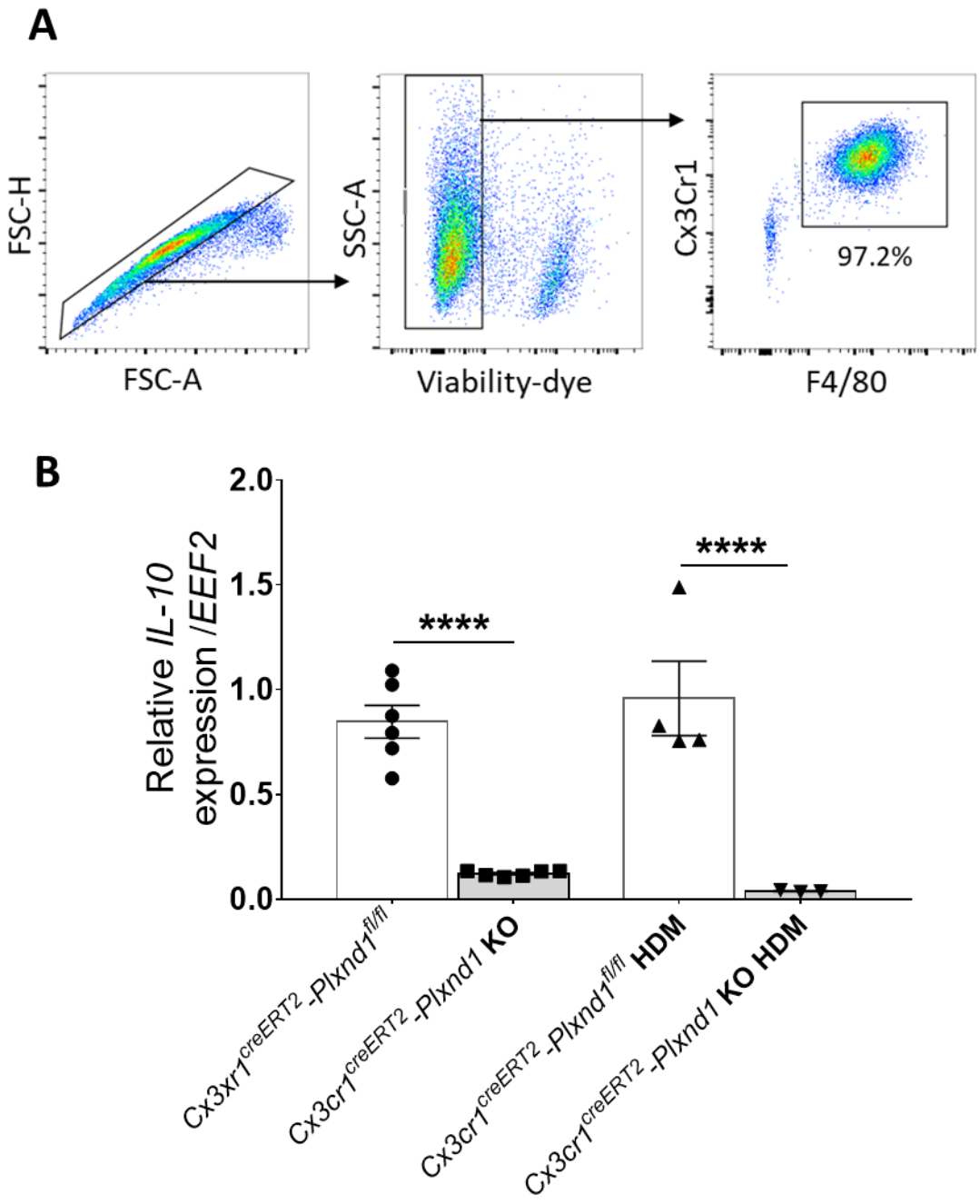


Figure 4. 11: Deletion of *Plxnd1* in CX3CR1 cells negatively regulate IL-10 expression in BMDM. Expression of anti-inflammatory cytokine IL-10 was measured in *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}-*Plxnd1* KO bone marrow derived macrophages (BMDM) by Q-PCR using specific primers. Bone marrow was collected from *Cx3cr1*^{creERT2}-*Plxnd1*$^{fl/fl}$ mice and culture

with L929 media for 6 days to differentiate into macrophages. 97.2% cells are CX3CR1-expressing macrophages (A). A portion of *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ BMDMs were treated with tamoxifen (20µg/ml) to get *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ KO BMDM. RNA was collected from *Cx3cr1*^{creERT2} *Plxnd1*$^{fl/fl}$ BMDMs and Cx3cr1^{creERT2} *Plxnd1*$^{fl/fl}$ KO BMDMs and converted to cDNA. IL-10 expression is decreased in *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ KO BMDMs of both in naïve and after culture with HDM (B). Data are expressed as mean with SEM. n= 03-06 data per group, **p<0.01, and ***p<0.01.

5 DISCUSSION

Plexins are transmembrane receptors of semaphorins protein. In addition to different types of plexins, plexinD1 plays an important role in asthma [225, 226], and semaphorin3E can directly bind to the plexinD1 with high affinity[214]. Previously, we have shown that Sema3E global deficiency exacerbates allergen-induced airway hyperresponsiveness, airway inflammation, and airway remodelling [254]. However, the role of plexinD1 present on lung interstitial macrophages in allergic asthma has not been studied. Therefore, I studied the role of plexinD1 deficient lung interstitial macrophages in allergic asthma. I found that the deletion of plexinD1 from CX3CR1-expressing lung interstitial macrophages exacerbate allergen-induced airway resistance, airway inflammation, Th2/Th17 cytokines, IgE level, mucus production, and α-smooth muscle actin expression.

After the deletion of *Plxnd1* gene from lung CX3CR1 cells, we got a CX3CR1-cells-specific *Plxnd1* KO mouse (named as *Cx3cr1*^{creERT2}*-Plxnd1* KO). No difference was observed in the baseline immune cells at different organs, including lung, lymph node, spleen, and blood, between *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ and *Cx3cr1*^{creERT2}*-Plxnd1* KO mice. Then I challenged these two groups of mice with HDM to induce allergic asthma. Airway resistance, a part of airway hyperresponsiveness, elevated in *Cx3cr1*^{creERT2}*-Plxnd1* KO mice compared to *Cx3cr1*^{creERT2}*-Plxnd1*$^{fl/fl}$ mice. It has previously been shown that the absence of Sema3E in mouse allergic model exacerbates airway hyperresponsiveness, and recovery of Sema3E/plexinD1 axis by treatment with recombinant Sema3E reduced airway hyperresponsiveness, tissue remodelling including angiogenesis [254, 257]. My current study suggests the absence of Sema3E/plexinD1 in CX3CR1-expresing IMs induced allergen-induced airway resistance. Further studies are needed to study at the mechanistic level how plexinD1 in the IMs regulate airway resistance.

Airway hyperresponsiveness (AHR) is a critical characteristic of symptomatic asthma. Various factors have been reported to be involved in causing airway hyperresponsiveness in asthma patients. Among them, the recruitment of inflammatory cells to the airway contributes to AHR. Different studies have reported a strong correlation between the increasing number of eosinophils and the severity of AHR after allergen exposure [272, 273]. Similarly, I observed an elevated number of eosinophils in the airway after allergen challenge, which significantly increased in the mice with *Plxnd1*-deficiency in IMs, which suggest that IMs-plexinD1 regulates eosinophils recruitment.

IL-5 is the most important Th2 cytokines that regulate eosinophils growth, differentiation, maturation, survival, and activation[10], most importantly act as an eosinophil chemoattractant [274]. I measured the higher level of IL-5 in the supernatant of the recall response of MLN of *Cx3cr1*^{creERT2}*-Plxnd1* KO mice. This suggests that the absence of *Plxnd1* in IMs induce, by a mechanism to be determined, IL-5 secretion that promotes eosinophils recruitment to the airway leading to the exacerbation of allergic asthma.

Although it has long been suggested that allergic asthma is associated with elevated eosinophils in the airways, however, recent research found that some asthmatics have an increased level of neutrophils with or without elevated eosinophils [275]. Moreover, neutrophils were associated with increased bronchoconstriction leading to airway closure. In my study, in addition to elevated IL-17A, we found a higher number of neutrophils in the airway of mice with *Plxnd1* deficiency in IMs after allergen exposure. IL-17A induces neutrophils recruitment at the site of inflammation through the activation of CXCL-8/IL-8 during asthma [270], which suggests that the absence of *Plxnd1* in CX3CR1 cells induce neutrophilia at the site of the airway through stimulation of IL-17A production.

In addition to eosinophils and neutrophils, the number of macrophages and lymphocytes increased in the airway of allergic asthma [10]. In my current study, I found that a higher number of interstitial macrophages and lymphocytes in the airway of *Cx3cr1*^{creERT2}*-Plxnd1* KO mice after allergen exposure. Therefore, the deletion of *Plxnd1* from IMs exacerbates allergic asthma by inducing the recruitment of lymphocytes and monocytes-derived IMs. These results are similar to the previous data where *Mellado M et al* showed blocking of monocytes recruitment ameliorate allergic asthma [163]. However, I need to further study how these monocyte-derived IMs exacerbate allergic asthma.

Our lab has shown that the absence of plexinD1/Sema3E interaction makes the macrophage more hyporesponsive to LPS (data not published yet). As a result, macrophages produce less pro-inflammatory cytokines such as IL-6 & TNF. Similarly, another study showed that Sema3E attracts monocytes/macrophages to adipose tissue and these recruited cells cause adipose tissue inflammation [228]. However, in our study, I found a higher number of interstitial macrophages (IM) in the airway after inhibiting the Sema3E/plexinD1 interactions, and the absence of plexinD1 on IMs exacerbate airway allergic asthma. IMs might be originated from recruited blood monocytes through the CCR2 dependent axis upon allergen exposure [163]. Moreover, it has been shown that lung IMs constitutively produce anti-inflammatory cytokines IL-10 through the TLR4/MyD88 pathway that is independent of lung microbiota. Although FOXP3^{+} T_{reg} cells produce IL-10, IL-10 producing IMs are more prevalent than FOXP3^{+} T_{reg} cells in the lung. Therefore, IL-10 producing IMs inhibit HDM-induced allergic asthma exacerbations in mice [178]. In addition, another study has shown that IL-10 producing IM, not alveolar macrophages (AM), reduces airway allergic diseases by inhibiting allergen-loaded dendritic cells maturation and migration that activate Th2 responses [168]. Therefore, it suggests that the absence of

plexinD1/Sema3E interaction might regulate the signaling pathway of IL-10 cytokines production in the interstitial macrophages. To investigate this, I deleted *Plxnd1* from bone marrow-derived macrophages (BMDM) and measured IL-10 expression at both naïve and after culture with HDM. I found that the deletion of *Plxnd1* from BMDMs reduces the expression of IL-10 even after culture with the allergen (HDM). However, I need to study the downstream effect of plexinD1/Sema3E interaction on IMs that might be linked to the IL-10 expression.

Airway remodeling is another important feature of asthma that includes collagen and fibronectin deposition, goblet cell hyperplasia and mucus overproduction, subepithelial fibrosis, angiogenesis, and ASM hypertrophy or hyperplasia [78, 79, 105]. Airway remodeling causes the airway narrowing that leads to airway resistance as airway resistance is inversely correlated to airway radius. Excessive mucin secretion by goblet cells reduces the radius of the airway that inhibits airflow through an airway leading to airway resistance. Previously, it has been shown that the global absence of Sema3E/plexinD1 axis induces mucus overproduction and treatment with recombinant Sema3E reduces mucus hypersecretion [254, 257]. In our study, I observed a higher expression of two major lung secretory mucins proteins *MUC5AC* and *MUC5B* in the *Cx3cr1*^{creERT2}*-Plxnd1* KO mice with goblet cell hyperplasia without affecting collagen and fibronectin deposition. Moreover, the previous data showed that IL-10 producing lung IMs reduced goblet cell mucous production in allergic asthma [178]. Therefore, current results explain that the deletion of *Plxnd1* from lung IMs negatively regulates IL-10 production by IMs that ultimately lead to the induction of expression of *MUC5AC* and *MUC5B* genes. Moreover, IL-13 induces mucin production by working on airway epithelial cells [271]. As I got higher IL-13 in the BAL of *Cx3cr1*^{creERT2}*-Plxnd1* KO mice, it explains the deletion of *Plxnd1* in IMs might exacerbate mucus production by inducing IL-13 production.

Bronchoconstriction, another feature of asthma, is due to constriction of the airway smooth muscle (ASM) surrounding the airway. Increased expression of contractile proteins α-smooth muscle actin (α-SMA) in ASM correlates with the severity of asthma [276]. Furthermore, increased ASM mass, because of hypertrophy or hyperplasia, exaggerate airway narrowing [277, 278]. Previously, our lab has shown that ASM cell express plexinD1 and Sema3E/plexinD1 interaction on ASM cells reduces ASM cell proliferation and migration [225]. Here, I observed that the deletion of *Plxnd1* in the lung IMs induced the expression of *α-SMA* in the asthmatic lung. The deletion of *Plxnd1* from IMs might upregulate the high affinity-IgE receptor (FcεRI) on IMs that lead to the degranulation of various mediators upon allergen exposure result in the induction of *α-SMA* expression [279].

Immunoglobulin IgE plays a critical role in the predisposition of allergic asthma and serum IgE level positively correlated with the severity of asthma [280]. Global deficiency of Sema3E enhanced IgE production [254], which suggests that plexinD1/Sema3E might regulate IgE class switching in the B-cells. In the current study, I found HDM-specific serum IgE level elevated in the absence of *Plxnd1* in IMs while the HDM-specific IgG1 level decreased. IgG is not a risk factor for asthma even some people are tolerant of the high dose of allergens due to the production of IgG instead of IgE [281].

Th1 cytokine IFNγ inhibits allergic asthma through the inhibition of isotype switch recombination to IgE in B cells, and expression of IgE receptors on inflammatory cells [282, 283]. It explains the deletion of *Plxnd1* from IMs might exacerbate airway allergic asthma in another way by suppressing IFNγ production. Furthermore, IL-4 cytokine is very important to regulate the growth, differentiation, and activation of B-cells [268], and it helps B cells to produce IgE by class switching recombination [269]. It promotes the IgE-mediated responses by enhancing the IgE

receptors on the inflammatory cells in the airway [284]. Here, we found that *Cx3cr1*^{creERT2}*-Plxnd1* KO mice had elevated IL-4 in BAL. A high level of IL-4 might induce B-cells to produce more IgE. Therefore, the deletion of *Plxnd1* in IMs exacerbate allergic asthma might be by IgE-mediated responses where lack of IL-10 expression in *Plxnd1*-deficient IMs induce Th2 cell activation through promoting DC maturation and migration.

Limitation of the study is that CX3CL1 chemokine receptor 1 (CX3CR1) is expressed in different immune cells including monocytes, macrophages, dendritic cells, T-cells, and natural killer cells. However, CX3CR1 expression is highly cell type-specific depending on each tissue and organ [169]. CX3CR1 is mostly expressed on monocyte in the blood, macrophages in the gut, microglia in the brain, and macrophages. Other studies have showed that CX3CR1 can be also expressed in subpopulation of CD8+T cells, NK cells in the liver [170-175, 285]. However, in the lung tissue, CX3CR1 is mainly expressed by lung interstitial macrophages [169, 286]. It is also expressed by a fraction of CD4(+) T cells in the asthma condition [169, 176, 177]. As a results, *Plxnd1* deletion was found in the lung T-cells in this model though percentage of deletion was lower in T-cells than IMs. Therefore, different effect of allergic asthma might be in part due to deletion of *Plxnd1* from T-cells.

Although I need to study at the mechanistic level to determine the way plexinD1 in the IMs regulates allergic asthma, for the first time, my study showed that plexinD1 in the CX3CR1-expressing interstitial macrophages play an important role in allergic asthma through regulating airway resistance, airway inflammation, and remodeling.

6 FUTURE DIRECTIONS

- Study of IL-10 expression in lung interstitial macrophages of wild type and CX3CR1 cells-specific *Plxnd1* deficient mice. In my study, I observed that deletion of *Plxnd1* from bone marrow-derived macrophages (BMDMs) reduced IL-10 expression compared to wild type BMDMs. As lung IMs ameliorate allergic asthma through secreting IL-10 [168, 178], deletion of *Plxnd1* in IMs might affect the reduction of IL-10 expression that lead to exacerbation of allergic asthma. IL-10 expression level will be measured in sorted lung IMs from wild type and *Plxnd1* deficient mice.
- To study whether Sema3E/plexinD1 axis play a role in the IL-10 expression in IMs. Sema3E bind with its canonical receptor plexinD1with high affinity; therefore, the effect of Sema3E in the IL-10 expression will be measured by sorting lung IMs from *Sema3e* KO and WT mice as well as in the BMDMs. The role of Sema3E/plexinD1 axis in the IL-10 production will be determined through retrieval of this axis by treatment with recombinant sema3E.
- Investigation of the role of Sema3E/plexinD1 axis in the induction of IL-10 expression in IMs. Lung IMs constitutively produce IL-10 through TLR4/MyD88 activation [178]. Moreover, during helminth infection macrophages produce IL-10 through activation of TLR2/4/MyD88 pathway. Here, MyD88 is recruited to the activated receptor in a PI3K-dependent manner. This event induces phosphorylation of P^{38} and NF-kB proteins (P^{65} and P^{105}) that lead to release of TPL2, which activates MEK/ERK/RSK cascade pathway. Phosphorylation of P38 and RSK induce phosphorylation of CREB, which is recruited to the *Il-10* promoter leading to expression of IL-10 [287]. Again, Sema3E and plexinD1 interaction activate intracellular Ras GAP domain of plexinD1, which induces activation

of PI3K pathway in the cell [243]. Therefore, activation of PI3K might play role in the phosphorylation of CREB in the cells, which ultimately promote IL-10 expression. Activation level of MEK, ERK, RSK and CREB will be determined in the BMDM of wild type and after deletion of *Plxnd1*. Activation of these proteins will also be checked in vivo model.

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