SEMAPHORIN3E IS A NOVEL REGULATOR OF IGE THROUGH REGULATION OF GERMINAL CENTER RESPONSES IN A MOUSE MODEL OF ALLERGIC ASTHMA

By

FATEMEH SEDAGHAT

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Department of Immunology

University of Manitoba

Winnipeg, Manitoba, Canada

THESIS ABSTRACT

Semaphorin3E (Sema3E) is a protein that plays a critical role in immunity to diseases, including asthma, by regulating cell migration, proliferation, and angiogenesis. Little is known about the role of Sema3E in immunoglobulin E (IgE) regulation. Recent data showed that *Sema3e*$^{-/-}$ mice have a significantly higher serum level of total and allergen-specific IgE in allergic asthma. These results strongly indicate a crucial involvement of Sema3E in modulating IgE production. In my project, we hypothesised that Sema3E negatively regulates IgE levels through germinal center (GC) responses.

To investigate the regulatory role of Sema3E in IgE production and GC formation, *Sema3e*$^{-/-}$ and WT mice underwent an acute HDM challenge. Flow cytometry was performed to investigate the number of GC B cells, T follicular helper (Tfh) cells, and inflammatory cells. The antibodies and inflammatory cytokines levels were detected by ELISA and Mesoscale, respectively. Immunofluorescence (IF) staining and H&E staining were performed to investigate GC formation and lung inflammation, respectively. Additionally, to check if Sema3E plays a role through the ICOS/ICOS-L pathway, GC formation was blocked by intraperitoneal administration of anti-ICOS-L or rat IgG as an isotype control.

The results demonstrated that at the steady-state, *Sema3e*$^{-/-}$ mice had a significantly higher baseline number of GC B cells, Tfh cells, plasmablasts, plasma cells, and serum IgE and IgG1 than the WT counterparts. Interestingly, baseline expression of IgE and IgG by B cells is regulated by Sema3E, and *Sema3e*$^{-/-}$ mice demonstrated higher expression of IgE and IgG1 in their B cells. Upon immunization with HDM, *Sema3e*$^{-/-}$ mice exhibited enhanced GC responses, IgE production, and lung inflammation. We demonstrated that the deletion of Sema3E in mice led to an increase in the expression of ICOS on $CD4^+$ T cells and disruption of ICOS/ICOS-L signalling, remarkably reducing the number of inflammatory cells, plasma cells, serum levels of IgE and IgG1, and lung inflammation indicating the importance of ICOS/ICOS-L signalling pathway in Sema3E regulatory effect.

In conclusion, Sema3E plays a critical regulatory role in GC response and IgE production at the steady-state and post-immunization through ICOS/ICOS-L signalling, suggesting the critical negative regulatory role of Sema 3E in allergic inflammation.

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DEDICATIONS

I dedicated this thesis with heartfelt gratitude to my beloved parents, whose unwavering love, support, and encouragement have been my greatest source of strength throughout my life. They have done everything to help me achieve my dreams.

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LIST OF ABBREVIATIONS

AHR: Airway hyperresponsiveness

Akt: protein kinase B

APCs: antigen-presenting cells

AID: activation-induced cytidine deaminase

ASM: airway smooth muscle cells

BAFF: B cell activating factor

BM: Bone marrow

BCR: B cell receptor

CRD: cystine-rich domain

CSR: class switch recombination

DCs: Dendritic cells

DZ: dark zone

ECM: Extracellular matrix

ECP: eosinophil cationic protein

EDN: eosinophil-derived neurotoxin

EPO: eosinophil peroxidase

EBI2: Epstein-Barr virus-induced molecule 2 (EBI2)

FcεRI: high-affinity IgE receptors

FDCs: follicular dendritic cells

FO: Follicular B cells

GAP: segmented GTPase-activating protein

GC: Germinal center

GPI: glycosyl phosphatidyl-inositol

GTP: guanosine triphosphate

GDP: guanosine diphosphate

GM-CSF: granulocyte-macrophage colony-stimulating factor

GATA-3: GATA box 3

HASMCs: human airway smooth muscle cells

ICAM-1: intercellular cell adhesion molecule-1

IF: immunofluorescence

Ig: Immunoglobulin

IgV: immunoglobulin variable region

ILCs: innate lymphoid cells

IPT: immunoglobulin, plexin, and transcription factors

JAK: Janus Kinases

MAPK: Mitogen-activated protein kinases

MBP: major basic protein

MICAL: Molecule Interacting with CasL

MZ: Marginal zone

NE: neutrophil elastase

NK: Natural Killer cells

PAF: platelet-activating factor

PDGF: platelet-derived growth factor

PI3K: Phosphoinositide 3-kinases

PSI: plexin semaphorin integrin

RBD: Ras Binding Domain

Sema3E: semaphorin 3E

SHM: somatic hypermutation

SLO: Secondary lymphoid organs

STAT: signal transducer and activator of transcription

TCR: T cell receptors

Tfh: T follicular helper cells

TIM: T cell Ig and mucin domain

TLR: Toll-like receptor

TSLP: thymic stromal lymphopoietin

UNG: uracil-N-glycosylase

VEGF: vascular endothelial growth factor

VCAM-1: vascular cell adhesion molecule-1

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Chapter 1: Introduction

1. Asthma

1.1.1 General Overview of Asthma

Asthma is a heterogeneous chronic inflammatory disease of the airways that is clinically characterized by coughing, and recurrent airflow obstruction leading to difficult breathing, wheezing, and a sensation of tightness in the chest. From a pathophysiological point of view, asthma can be identified as a persistent inflammatory respiratory disease, marked by airway inflammation, airway hyperresponsiveness (AHR), mucus overproduction, and lung tissue remodelling (1).

Asthma ranks as a prevalent chronic lung disease globally, with its occurrence differing among regions. Despite a significant geographical heterogeneity in asthma prevalence, asthma is more prevalent in developed countries (2). However, the prevalence gap between developed and developing countries is decreasing due to more urbanization, modern living, and allergies in developing regions. Asthma affects over 300 million people globally; by 2025, another 100 million people may be at risk (3). In Canada, it affects about 3 million people; about 20% of them are children under 14. It is estimated that 250 Canadians die annually, from asthma and the direct and indirect cost of the disease is about $2.1 billion per year (4). Asthma is a huge socioeconomic burden on the health system. The treatment is estimated at least $56 billion annually, and according to reports, it ranks 28th in terms of disease burden and the 16th most common cause of living with a disability (5, 6).

In childhood, asthma is more commonly seen in boys until the age of 20, but in adulthood, it tends to be more common in women. This difference is often attributed to hormonal factors, lifestyle differences, and airway size and function variations between males and females (7). Additionally, there's a familial and genetic aspect to asthma. Various studies demonstrated that children of asthmatic parents have a higher susceptibility to asthma (8). Interestingly, studies on monozygotic and dizygotic twins demonstrated a positive correlation in allergic disease traits. Specifically,

monozygotic twins exhibit twice the levels of total serum IgE, heightened methacholine sensitivity in the lungs, and more pronounced skin test reactions compared to dizygotic twins (8, 9).

Genetic factors play a crucial role in the development of allergic diseases and asthma. Specific genes such as those encoding T cell Ig and mucin domain (TIM) family, thymic stromal lymphopoietin (TSLP), Toll-like receptor 4 (TLR4), and other TLRs are involved in allergic disease and asthma. Not only genetic but also environmental factors such as smoking, and obesity significantly contribute to the risk of developing asthma, highlighting the interplay between external factors and the onset of the disease. For instance, obesity is directly linked to increased asthma prevalence, with higher body mass index (BMI) correlating strongly with higher asthma rates, underscoring the impact of body weight on respiratory health (10, 11).

A combination of existing medications, such as an anti-inflammatory inhaled corticosteroid and a broncho-constricting β2-adrenergic agonist, could partially control asthma (12, 13). However, due to the refractory nature of asthma, poor understanding of disease etiology, and more importantly, the mechanisms that govern the disease pathogenesis, satisfactory control of the disease is hampered.

1.1.2 Pathogenesis of Asthma

The clinical characteristics of asthma, such as difficult breathing, wheezing, coughing, mucus production, the tendency for exacerbations, loss of lung function, and severity, indicate various underlying disease mechanisms. These mechanisms involve interactions between structural and immune cells that lead to the disease's pathological features. The extent to which these features contribute can differ among individuals with asthma, resulting in diverse clinical presentations and variations in inflammatory biomarkers (14).

1.1.2.1 Airway inflammation

Asthma is a heterogeneous airway inflammation marked by an increase in neutrophils, eosinophils, mast cells, macrophages, and activated lymphocytes in the airways, as well as Th2/Th17-biased immune response (15). Numerous modulators, including cytokines, chemokines, histamine, immunoglobulins (Ig), growth factors, and lipid mediators, are secreted by these inflammatory cells and cause airway smooth muscle hypertrophy or hyperplasia, hyperreactivity, mucus overproduction, collagen deposition, and ultimately airway remodelling (15) (Figure 1.1.1).

Aeroallergen exposure causes airway inflammation in people with allergic asthma. This reaction results from a dynamic interplay between the innate and adaptive immune systems and respiratory epithelium, which can last for a long time and result in the airways' structural changes. The initial encounter with the allergen triggers an acute early-phase reaction, characterized by type I immediate hypersensitivity, and is often followed by a late-phase reaction. Continuous exposure to the allergen leads to ongoing allergic inflammation and changes in tissue structure (16).

After an environmental allergen exposure, IgE antibodies will be rapidly sensitized and released by plasma cells and bind to its high-affinity IgE receptors (FcεRI), expressed on the cell surface of mast cells and basophils (15). This interaction leads to the release of various mediators. Three different inflammatory mediators that aid in the initial symptoms of the asthma reaction include 1) newly generated mediators such cytokines, chemokines, and growth factors; 2) performed mediators kept in cytoplasmic granules and released upon degranulation; and 3) mediators derived from lipids (17, 18). Late-phase reactions occur 2-6 hours after allergen exposure involving tissue-resident cells and recruited leukocytes. Continuous allergen exposure leads to chronic airway inflammation, sustaining the inflammatory response and causing structural changes in the airways, known as airway remodelling (18, 19).

- **T helper 2 (Th2) cells**

Th2 lymphocytes are crucial in the development of allergic asthma (20, 21). Upon exposure to allergens, naïve $CD4^+$ T cells undergo differentiation into Th2 cells, which release various cytokines, including IL-4, IL-5, IL-9, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF). The bulk of these cytokines are encoded on the long arm of human chromosome 5 (5q31-33), and they are strictly regulated by phosphorylation of a crucial transcription factor termed GATA box 3 (GATA-3) (22, 23). These cytokines facilitate cell communication and maintain inflammation (20, 21). IL-4 is essential for converting Th0 cells to Th2 cells. When IL-4 binds to the type 1 IL-4 receptor (IL-4Rα/γc) on naïve $CD4^+$ T cells, the receptor dimerizes, causing Janus Kinases (JAK) to phosphorylate its tyrosine residues. The cytosolic signal transducer and activator of transcription 6 (STAT6) then attach to this phosphorylated site and is subsequently phosphorylated by JAK. The phosphorylated STAT6 (pSTAT6) dimerizes and moves to the nucleus, where it binds to target DNA to regulate gene expression (24). Notably, STAT6 knockout mice do not exhibit airway eosinophilic inflammation, chemokine production, or goblet cell hyperplasia (25). Furthermore, STAT6 is crucial for regulating GATA3, the key transcription factor for Th2 cell development (26). IL-4 also stimulates endothelial cell expression of vascular cell adhesion molecule-1 (VCAM-1), increasing the recruitment of inflammatory cells to the airways (27), upregulating FcεR2 (CD23), and inducing B cells' IgE class switching (28).

IL-5 and IL-9 play critical roles in the differentiation, activation, maturation, and recruitment of eosinophils and mast cells to the airways. Research on humans indicates that inhibiting IL-5 or its receptor can decrease both local and systemic eosinophilic inflammation. However, this approach does not significantly improve the late airway response triggered by allergens or the baseline AHR in asthma patients (29, 30). It was discovered that, in the absence of allergen exposure, lung-specific over-expression of IL-9 causes AHR and airway inflammation by inducing the production of IL-5 and IL-13 by non-T cells (31). Furthermore, the development of allergen-induced airway eosinophilic inflammation and AHR is inhibited by anti-IL-9 antibody therapy, indicating the key role of this cytokine in lung inflammation (32). In contrast to other type 2 cytokines, IL-9 can directly promote mast cell progenitor migration to the airways (33).

IL-13 shares both structural and functional similarities with IL-4 and binds to a heterodimer consisting of IL-4Rα and IL-13Rα subunits. Elevated levels of IL-13 lead to goblet cell

hyperplasia, airway remodelling, IgE synthesis, increased mucus production, AHR, and eosinophilia (34, 35). Interestingly, it has been shown that inhibiting IL-13 protects mice against allergen-induced AHR. This protective effect may be related to direct impacts on smooth muscle and airway epithelial cells, rather than inflammatory cells (36, 37).

- **T helper 17 (Th17) cells**

Th17 cells are another subset of $CD4^+$ T cells that play a significant role in the pathology of airway diseases and asthma. Biopsies from individuals with asthma show increased levels of IL-17A cytokines, produced by Th17 cells. IL-17A stimulates the release of IL-8 and IL-6 from various cell types including airway smooth muscle cells, lung epithelial cells, and pulmonary vascular endothelial cells. The chemoattractant IL-8 facilitates neutrophil migration to the site of inflammation. Moreover, VCAM-1 and intercellular cell adhesion molecule-1 (ICAM-1) are secreted in response to IL-17A. As a result, IL-17A encourages neutrophil recruitment at the site of inflammation (38, 39). The level of IL-17A in lung diseases shows a strong correlation with neutrophils. It has been shown that insufficient IL-17A leads to weakened neutrophil responses to allergens, decreased AHR, and airway remodelling (40, 41). These findings suggest that the activation of Th17 cells could contribute to the development of neutrophilic asthma. However, IL-17A could also potentially involve recruiting eosinophils to the airway in allergic asthma since it prompts the production of the chemokine eotaxin-1/CCL11 by airway smooth muscle cells (42, 43).

- **Eosinophil**

Eosinophil is a critical player in the pathogenesis of allergic asthma (44). Following allergen exposure, eosinophil release from bone marrow (BM), is stimulated by Th2 cytokines, especially IL-5 (45). Then, eosinophils migrate to the lung by expressing the chemokine receptor CCR3, which binds to its chemoattractant eotaxin-1/CCL11, produced by airway epithelial cells and other inflammatory cells. Once in the lungs, eosinophils accumulate in the airway mucosa and submucosa, where they encounter the allergen. They adhere to the endothelial cells lining the blood vessels and transmigrate into the airway tissue. In contact with the allergen, eosinophils become

activated and release various mediators, including eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), major basic protein (MBP), and eosinophil-derived neurotoxin (EDN) and each of these mediators play a role in the pathogenesis of allergic disease (46). for example, ECP has cytotoxic effects on epithelial cells and can induce cell death by disrupting cell membranes, contributing to tissue damage, and inflammation in the airways, exacerbating asthma symptoms (46). EPO contributes to airway inflammation and tissue damage by generating ROS and promoting oxidative stress. Moreover, MBP contributes to airway inflammation, hyperresponsiveness, and tissue remodelling by causing damage to airway epithelial cells and airway smooth muscle cells (ASM) (44, 47, 48).

Besides causing damage to structural cells, eosinophils can also produce various substances including cytokines (primarily IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-13, and TNFα), chemokines (especially IL-8 and RANTES), lipid mediators (such as leukotrienes and prostaglandins), and oxygen radicals. They also release fibrogenic cytokines like TGFβ, significantly contributing to airway remodelling. Overall, eosinophil activation causes damage to epithelial and endothelial cells, inhibits muscarinic receptors, and induces fibrosis, which in turn causes remodelling of the airways and AHR (27, 49, 50).

- **Neutrophil**

Upon allergen exposure in asthma, neutrophils can also be recruited to the lung, although they are more commonly associated with non-allergic forms of asthma or severe exacerbations. In addition to eosinophils, allergen-induced inflammation can also attract neutrophils to the site of inflammation (51). It is believed that neutrophils are the initial responders to inflammation, arriving at the affected site first. Various chemotactic signals are released in the inflamed airways, attracting neutrophils from the bloodstream to the lung tissue, including interleukin-8 (IL-8), leukotriene B4 (LTB4), and bacterial products such as formyl peptides signalling. Neutrophils adhere to the endothelial cells lining the blood vessels in response to chemotactic signals. They then transmigrate across the endothelium and migrate towards the site of inflammation in the lung tissue. Once in the lung tissue, neutrophils become activated and release various inflammatory

mediators, such as O2, matrix metalloprotease-9 (MMP-9), leukotrienes-4 (LTB-4), neutrophil elastase (NE), and platelet-activating factor (PAF) (51, 52). MMP-9 plays a role in remodelling the airways, while NE prompts the hyperplasia of airway goblet cells, excessive mucous secretion, and proliferation of airway smooth muscle cells (53, 54). Additionally, other substances released by neutrophils, including myeloperoxidase, bFGF, PDGF-BB, VEGF, oxidation products, and serine-neutral proteases, are implicated in causing epithelial damage, fibrosis, and angiogenesis (55-58). As a result, AHR, bronchospasm, lung tissue injury, excessive mucus production, and airway remodelling that results in a permanent change in airway structure are all brought on by activated neutrophils.

- **Dendritic cells and macrophages**

Dendritic cells (DCs) play a crucial role in allergic asthma as key regulators of the immune response to inhaled allergens. DCs are the main antigen-presenting cells (APCs) that capture, process, and present allergens to T lymphocytes in the airway mucosa. Upon encountering inhaled allergens, DCs migrate to the draining lymph nodes, where they present allergen-derived peptides to naïve T cells to polarize to Th2 cells, initiating the adaptive immune response (20). Interestingly, allergens with a low level of endotoxin tend to elicit Th2 responses, whereas those with a high level of endotoxin trigger Th1 responses. Furthermore, besides influencing Th1 and Th2 activation, DCs also play a role in directing T cell differentiation towards the Th17 lineage and promoting IgE class switching (59, 60).

Macrophages are the most abundant cells in the airway, and they are found in various regions of the lung, including the alveoli, interstitium, conducting airways, and distal airspaces. Based on their location, lung macrophages are divided into alveolar macrophages found on the alveolar lumen and interstitial macrophages in the lung interstitium. Generally, lung macrophages play a pivotal role in airway inflammation, remodelling, presenting allergens to T cells, and expressing low-affinity IgE receptors leading to the activation of Th2 cells and the production of inflammatory cytokines. However, each lung macrophage subclass has its unique role. Alveolar macrophages are the first immune cells to encounter inhaled pathogens and particulates and they engulf and digest pathogens and produce inflammatory cytokines that start lung inflammatory responses. Furthermore, they play a role in dampening inflammation by clearing apoptotic cells and secreting

anti-inflammatory mediators. However, interstitial macrophages patrol the interstitial space, providing ongoing surveillance for pathogens and damage. They process and present antigens to T cells, initiating adaptive immune responses and secreting various cytokines, contributing to the amplification and regulation of the immune response. Moreover, the role of interstitial macrophages in tissue repair and remodelling processes by secreting growth factors and extracellular matrix components has been well studied (61).

- **Innate lymphoid cells**

A recently identified subset of immune cells known as innate lymphoid cells (ILCs) mimics the appearance and capabilities of T cells. Following exposure to allergens, group 2 innate lymphoid cells (ILC2) in humans and mice are activated by IL-33, IL-25, and TSLP. This results in the production of huge amounts of IL-5 and IL-13 cytokines, which promote airway eosinophilic inflammation, mucus production, and AHR. Interestingly, compared to other cells, activated ILC2 cells produce a comparatively large amount of IL-4, which serves as a significant promoter of Th2 differentiation and the production of airway allergic inflammation (62-64).

- **Mast cell and basophil**

Mast cells are tissue-resident immune cells found in various tissues, including the airways. Basophils are a type of circulating white blood cell that are closely related to mast cells and share similar functions. Mast cells and basophils both express Fcε receptor I (FcεRI), a high-affinity receptor. When allergens cross-link nearby IgE-FcεRI receptors, these cells get activated and degranulate to release several mediators (27). Mast cells' cytosolic granules are home to a variety of lipid mediators, including prostaglandins, leukotrienes, PAF, and sphingolipids; they also house biogenic amines, such as histamine and serotonin; mast cell-derived proteases, such as tryptase and chymase; serglycin; and proteoglycans. The recruitment of eosinophils, smooth muscle contraction, mucus overproduction, vasodilation, airway remodelling, and airway hyper-reactivity are among the hallmarks of allergic asthma caused by these mediators. By encouraging the recruitment of eosinophils and the overproduction of mucus, basophils control the late stage of allergic reactions (21, 65).

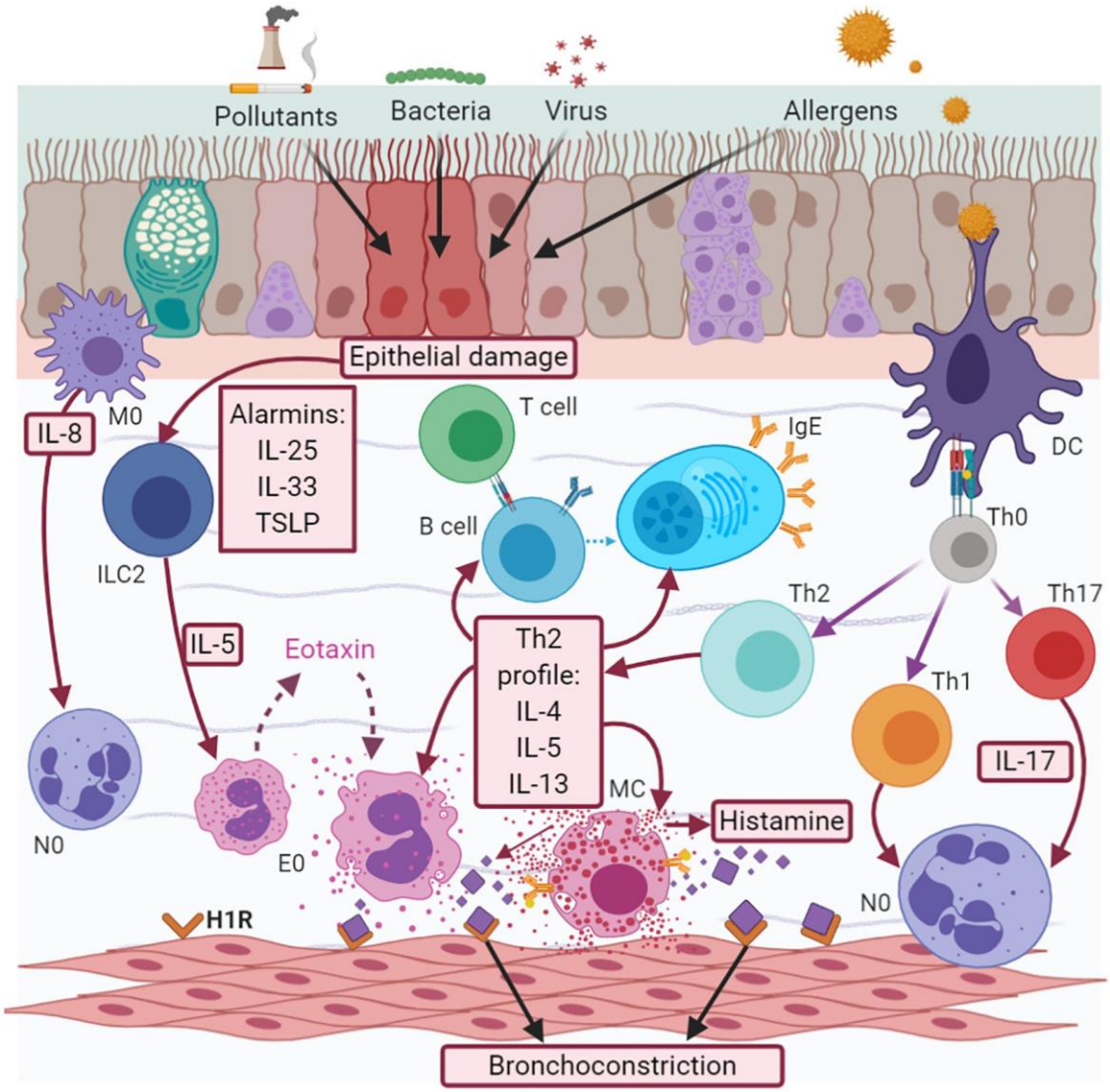


Figure1.1.1 Pathophysiology of lung inflammation in allergic asthma.

Asthma is a complex condition with diverse origins, arising from intricate interplays between genetic elements and environmental triggers such as allergens and viruses. Allergens are captured by dendritic cells in the airway, processed into antigenic components, and presented to naive T helper (Th0) cells. Upon activation, Th0 differentiates to allergen-specific Th2 cells which produce IL-4 and IL-13, and stimulating B cells to generate IgE antibodies. Furthermore, Th2 cells release IL-5, prompting the maturation, migration, and survival of eosinophils. Epithelial cell-derived cytokines (TSLP, IL-33, and IL-25) activate Type 2 innate lymphoid cells (ILC2), which in turn release IL-5 to activate eosinophils and IL-13 to induce mucus production. Mast cells undergo IgE-dependent degranulation, releasing preformed and newly synthesized mediators. Th17 cells, another crucial T lymphocyte subset, contribute to asthma pathology by producing IL-17A, which recruits and expands neutrophils. Moreover, numerous mediators, cytokines, and growth factors secreted by various cell types may impact the functions and proliferation of airway structural cells, including epithelial cells, fibroblasts, smooth muscle cells, and endothelial vascular cells.

1.1.2.2 Airway hyperresponsiveness

Airway hyperresponsiveness (AHR), a key characteristic of asthma, is described as an excessive narrowing of the bronchial airways, resulting in an amplified bronchoconstrictive reaction to various inhaled triggers (66). These triggers, whether chemical or allergenic, induce bronchoconstriction by binding to receptors on the smooth muscle of the airways. There is a correlation between the severity of AHR and asthma. Since higher airflow blockage and increased airway hyperresponsiveness are correlated, airway resistance causes AHR (67). A bronchial challenge test is used to evaluate AHR. In this test, methacholine or other constrictors are used to induce bronchospasm in healthy individuals, but the threshold is lowered in asthmatic patients (68). Based on methacholine challenge results, there are two types of AHR: persistent baseline hyperresponsiveness, typically seen in chronic asthmatics and indicative of structural changes in the airways, and variable episodic hyperresponsiveness, reflecting fluctuations in airway inflammation and the severity of asthma symptoms (69).

Genetic predisposition, chronic inflammation, and airway structural changes are considered the main causes of AHR. Studies have demonstrated the presence of familial clustering in both airway hyperresponsiveness and asthma, indicating a genetic predisposition to asthma alongside environmental influences. Hence, both genetic and environmental factors significantly contribute to the predisposition to airway hyperresponsiveness (70).

The second cause of AHR is chronic inflammation. Research confirms that in asthma, an increased number of various inflammatory cells, especially eosinophils, and neutrophils, release different cytokines and mediators including Th2 cytokines, Tumor Necrosis Factor-alpha (TNF-alpha), and leukotrienes in the airways that promote damage to the epithelium, thickening of the basement membrane, releasing mediators that bind to the ASM receptor and cause contraction of the bronchial smooth muscle, and finally causing plasma exudation, which results in AHR (71). Furthermore, through signalling via muscarinic receptors found on ASM, secretory glands, and various inflammatory cells, parasympathetic nerves play a crucial role in the symptoms and inflammation of allergic disorders (72). Parasympathetic nerves connect to secondary nerves that release a neurotransmitter called acetylcholine, which then binds to specific receptors on ASM and glands, causing airway constriction and increased mucus production respectively (73, 74).

Furthermore, eosinophils produce MBP which triggers the release of acetylcholine, thus intensifying bronchoconstriction in allergic asthma (75). The severity of AHR is linked to changes in the airway structure of the airway (76). Prolonged exposure to allergens results in a permanent structural alteration of the airway, which in turn induces persistent AHR. The structural changes include increased α-SMA in ASM, damage to epithelial cells, edema from plasma exudation, goblet cell hypertrophy and hyperplasia, subepithelial fibrosis, angiogenesis, collagen deposition at the extracellular matrix, and excessive production of mucus (71, 77-79). Additionally, the degree of epithelial structure loss correlates with AHR (80), because a significant quantity of bronchoconstrictor mediators can reach ASM which increases bronchoconstriction in the airway. Moreover, the damaged epithelium reduces the release of substances that dilate the bronchi, further contributing to smooth muscle contraction (71).

In mouse models of allergic asthma, three specific parameters are used to measure AHR (81):

1. "Airway resistance" indicates the resistance in the central or conducting airways, providing a quantitative measure of constriction.

2. "Tissue resistance" reflects the energy dissipation in the alveoli, indicating how easily the lungs can expand.

3. "Tissue elastance" reveals the energy conservation in the alveoli, indicating the elastic rigidity or stiffness of the lungs.

1.1.2.3 Airway remodelling

In general, airway remodelling refers to the structural changes that occur in the lung's airways due to chronic inflammation, most seen in conditions like asthma and chronic obstructive pulmonary disease (COPD). Chronic asthmatic patients experience structural remodelling in the airway wall. This includes increased mucus production by goblet cells, collagen deposition in the lamina reticularis, and development of myofibroblasts. There is also increased angiogenesis and thickening of the muscle bundles, with hypertrophy and hyperplasia of airway smooth muscle (ASM) (Figure 1.1.2). Airway remodelling has been noted across all levels of asthma severity and affects both small and large airways (82, 83).

There are two categories of remodelling: (I) Physiological airway remodelling, which can be repaired, occurs either as a result of inflammation or damage or during normal lung development. (II) Pathological airway remodelling results in a permanent change in the structure of the airway wall and is due to persistent injury or inflammation or disrupted lung development (84).

The airway epithelial barrier is vital in physiological conditions by facilitating the mucociliary clearance of particles, preserving fluid balance, and stimulating innate immune responses. The first step in the pathophysiology of asthma is the direct damage of the airway epithelial barrier by various stimuli (85), which promotes epithelial peeling, goblet cell hyperplasia, ciliated cell loss, increased growth factor release, and increased expression of the epidermal growth factor receptor (EGFR) (86-88). Compared to a healthy individual, the epithelium in asthmatics has a higher turnover rate (loss exceeds proliferation) and a weaker connection to the basement membrane This lack of integrity leads to allergen's effortless entry into the airway (89). Dysregulated epithelium can further stimulate remodelling by releasing various inflammatory cytokines and growth factors, including TNF, IL-1β, IL-6, PGE2, IL-8, MCP-1, eotaxin-11/CCL11, 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 (90). Furthermore, bronchial epithelial cell hyperplasia can contribute to mucosal thickening in asthma. Indeed, airway epithelial cells isolated from asthmatic patients exhibit more Ki-67 and less Bcl-2, indicating higher rates of cell proliferation and lower rates of apoptosis (91, 92). It has been shown that patients with severe asthma have more permeable airway epithelium due to decreased expression of occludins and claudins (93). The over-expression of the epidermal growth factor (EGF) receptor, a hallmark of epithelial damage (94), is indicative of an increased susceptibility to epithelial injury due to the downregulation of adherens junction proteins, such as E-cadherin (95).

One common feature of airway remodelling in asthma is sub-epithelial fibrosis of the conducting airways, which includes increased deposition of extracellular matrix (ECM) components such as collagen I, III, and V, fibronectin, tenascin, lumican, and biglycan secreted by fibroblast, in the submucosal space and thickening of the basement membrane, limited to the lamina reticularis (96, 97). In asthmatics, fibroblasts and myofibroblasts not only express cell surface receptors but also generate cytokines and chemokines which are crucial for cell adhesion and the activation of leukocytes, contributing to the onset of airway inflammation (98).

Goblet cell hyperplasia and excessive mucus production are additional factors that contribute to the thickening and narrowing of the airways in asthmatic patients. Goblet cells, which are found in the epithelium of the airway, secrete mucin glycoproteins into the respiratory tract to function as a defence mechanism. In asthma, there is an increase in the number of goblet cells due to hyperplasia and metaplasia, increasing mucin synthesis, which in turn causes excessive mucus production in the airway, leading to airway constriction and obstruction (99, 100). Patients with asthma exhibit elevated expression of the genes encoding mucin glycoproteins (MUC), particularly MUC5AC. It has been demonstrated that the type 2 cytokine IL-13, through the TGF-β2, forkhead box 2 (FOXA2), and 15-lipoxygenase (LO)1 signalling pathways, indirectly stimulates goblet cell hyperplasia and mucus formation (101, 102). Another significant inflammatory mediator, neutrophil elastase, stimulates the secretion of mucus and may play a role in the pathophysiology of severe asthma, which is characterized by substantial luminal mucus clogging in both large and small airways (103, 104).

One of the key components of airway remodelling is increasing the mass of ASM. The asthmatic's airway has more smooth muscle mass because of hyperplasia, or a rise in number, and hypertrophy, or an increase in size of ASM. ASM hyperplasia is brought on by either higher or lower rates of ASM cell division or apoptosis respectively (105). Different mediators, such as cytokines, matrix metalloprotease-2 (MMP-2), ECM components, mechanical stress, and reactive oxygen species, all contributed to the rise in the number of ASM cells. Notably, chemokines can promote ASM cell migration to the subepithelial region of asthmatic patients' airways (106, 107). This process is crucial in thickening the airways, obstructing general airflow, and causing hyperresponsiveness in the airways. In allergic asthma, prolonged airway inflammation, allergic hyperresponsiveness, and airway obstruction are the final results of ASM cell hypertrophy and hyperplasia-induced airway structural alteration (108).

Another important aspect of airway remodelling in allergic asthma is vascular remodelling, or angiogenesis, in the airways (109). Angiogenesis is the process by which pre-existing vessels grow new ones through several intricate steps. Studies indicate increased levels of vascular endothelial growth factor (VEGF) in the airways of individuals with asthma, correlating with increased angiogenesis (110). Consequently, the outcome of airway wall angiogenesis includes edema, and

the influx of inflammatory and remodelling agents into the airway wall, ultimately leading to airway inflammation and remodelling.

Overall, the airway epithelium is a critical component in both the normal functioning of the respiratory system and in the pathology of asthma. Its role in barrier function, immune defence, and tissue repair is crucial for maintaining airway integrity. In asthma, epithelial dysfunction leads to increased susceptibility to allergens and pathogens, driving inflammation and contributing to airway remodelling, which exacerbates disease severity and progression.

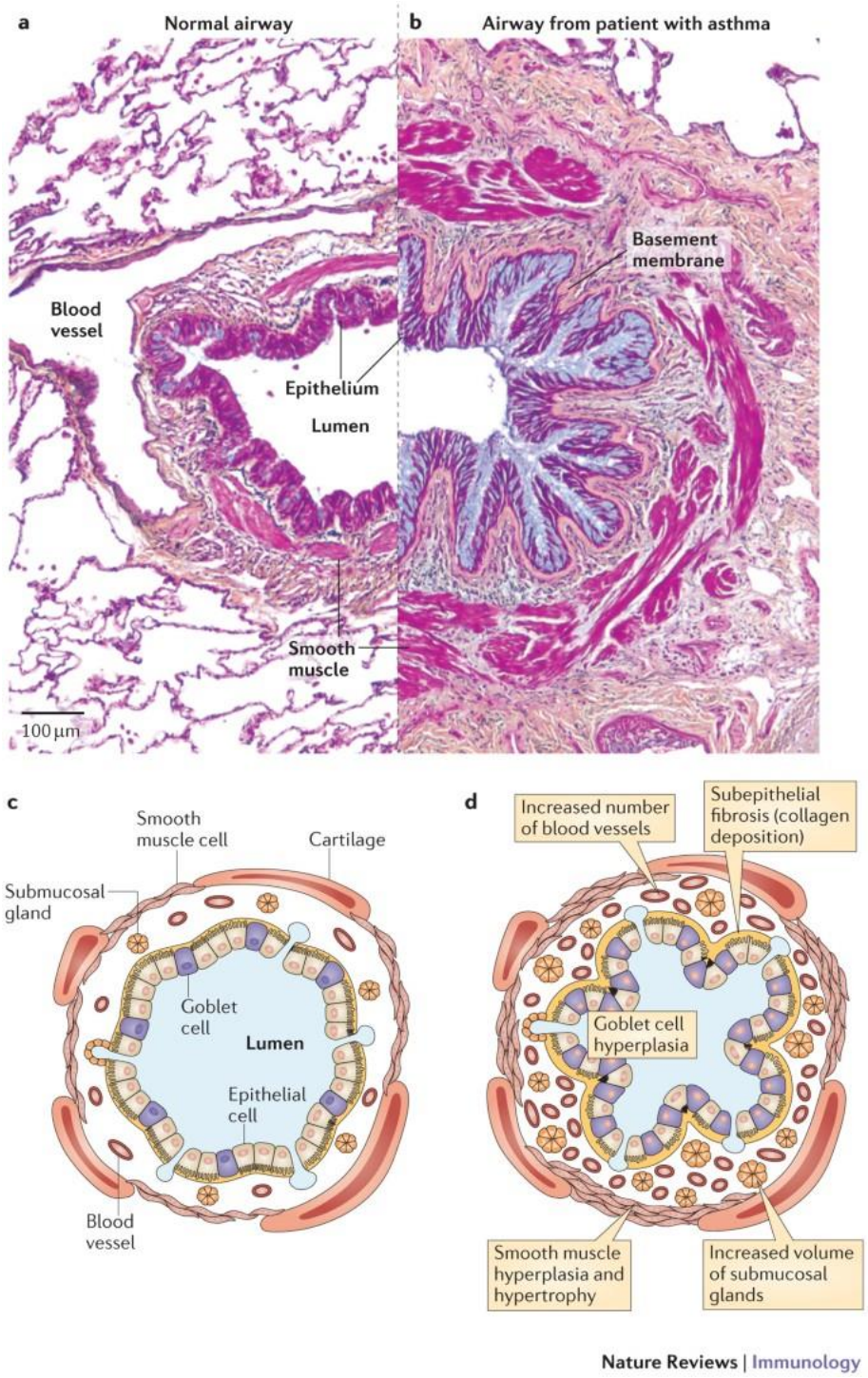


Figure 1.1.2 Airway pathology in asthma. Airway structure in medium-sized normal airways (a and c) compared to those in asthmatic airways (b and d). Asthma-related airways exhibit notable structural changes, such as increased goblet cell multiplication, angiogenesis, sub-epithelial fibrosis, and enlargement of bronchial smooth muscle.

1.1.3 Eosinophilic vs neutrophilic allergic asthma

Over the past decade, extensive studies involving large groups of asthma patients have provided detailed characterizations, including analyses of inflammation in airway secretions and tissues. These studies have led to the understanding that asthma is a heterogeneous disease comprising several distinct pathological phenotypes. Each phenotype follows different pathophysiological pathways, resulting in the varied clinical manifestations of asthma.

The primary marker of airway inflammation in allergic asthma patients is an elevated eosinophil count in both peripheral blood and the airways of most patients, which has been shown in certain studies to correlate with the severity of the disease (111, 112). Nonetheless, it is essential to differentiate between eosinophilic and non-eosinophilic instances of allergic asthma due to their distinct clinical and pathological presentations, which may call for different approaches to therapy (113).

As mentioned earlier, eosinophils are the primary inflammatory cells contributing to asthma's pathobiology. Their development, activation, longevity, and migration to the bronchial wall and airway lumen are crucial pathogenic processes involved in both allergic and nonallergic asthma types. Eosinophils are present in both the large or central airways and the peripheral parts of the lungs in asthmatic patients (114). Asthmatics' eosinophils terminally differentiate both locally in the irritated airways and within the BM (36). A crucial mediator in the biology of eosinophils, IL-5 plays a role in differentiation, maturation, activation, survival, and recruitment (27). MBP and eosinophil peroxidase, two preformed granular cytotoxic mediators that cause epithelium damage in asthmatic patients, are released by eosinophils to carry out their duties. Furthermore, eosinophils exacerbate allergen-induced airway inflammation and remodelling by secreting oxygen radicals, lipid mediators like leukotriene (LT)B4, and various cytokines and chemokines.

Eosinophilic asthma develops through intricate immunologic and proinflammatory processes, predominantly directed by Th2 lymphocytes that secrete interleukins such as IL-5, IL-4, and IL-13. Besides being influenced by adaptive immune responses, Th2-mediated eosinophilia in the airways can also be linked to significant innate immune reactions. These reactions involve interactions between dendritic cells, bronchial epithelial cells, and innate lymphoid cells (115, 116).

Unlike eosinophilic asthma, which is dominated by eosinophils, neutrophilic asthma involves an abundance of neutrophils in the sputum and airways of asthmatic patients. Numerous investigations have shown that severe asthmatics have higher neutrophil counts compared to mild or moderate asthmatics (117, 118). Furthermore, asthma exacerbations and fetal asthma (119) are associated with neutrophilic inflammation and some characteristics in patients include increased sputum levels of IL-8, a positive smoking history, increased airflow obstruction, frequent viral exacerbations, increased numbers of neutrophils in airway secretions during acute exacerbations, and basement-membrane thickening, indicating an important role of neutrophils in asthma pathology (52).

Neutrophilic airway inflammation is initiated by Th1 and particularly Th17 lymphocytes (97, 120). Lung tissue samples from asthmatic patients show increased levels of IL-17A and IL-17F, which are associated with the severity of asthma, especially in those with neutrophilic, steroid-resistant forms of the disease (121). Moreover, severe asthma patients have higher levels of Th1 cells and the Th1-derived cytokines TNF and IFN-γ, which may indicate that Th1 cells are also involved in mediating airway neutrophilic inflammation (122). Inflammatory cells and lung epithelial cells produce key mediators including IL-17A, IL-8, and TNF-α. These factors play a vital role in neutrophil attraction, activation, and survival, leading to their accumulation in the airways. In the lung, neutrophils release granules containing enzymes like elastase, myeloperoxidase, and matrix metalloproteinases (MMPs), and these enzymes degrade ECM components and contribute to tissue damage and airway remodelling. Additionally, by releasing ROS, neutrophils damage airway epithelial cells and further exacerbate inflammation.

Th17 cell-associated neutrophilic asthma frequently exhibits severe clinical manifestations that are incredibly challenging to treat due to recurrent steroid resistance. Unlike their proapoptotic effect on eosinophils, corticosteroids prevent neutrophil apoptosis, extending the lifespan of these inflammatory cells (123). However, it is unclear whether the increased neutrophils in the airways of some asthmatics are due to corticosteroid treatment or an unknown change in the airway microbiome (124). Therefore, the treatment of severe neutrophilic asthma requires the development of innovative antineutrophil medicines.

1.1.4 IgE role in asthma

IgE is the immunoglobulin found in the lowest quantities in vivo, and its levels are closely regulated. In healthy individuals, free serum IgE concentrations range from 50 to 200 ng per ml of blood, whereas other immunoglobulin isotypes have concentrations between 1 and 10 mg per ml. Furthermore, of all the immunoglobulin isotypes, IgE has the lowest serum half-life, lasting between 5 and 12 hours in mice and around 2 days in human (125). However, IgE can exhibit a longer half-life when it is bound to its high-affinity receptor, FcεRI, on the surface of certain immune cells like mast cells and basophils. When IgE binds to FcεRI, it is protected from degradation and can remain in the body for a longer period. It has been shown that mice's serum IgE responses are usually transient, and strong, long-lasting IgE responses are seldom seen (126).

Following allergen entry, it is captured by APCs and presented to naïve T lymphocytes which promote their differentiation into Th2 lymphocytes. Then Th2 secretes many cytokines, such as IL-5, IL-4, and IL-13. The only two cytokines that may induce germline ε transcription and switch recombination to create IgE in B cells are IL-4 and IL-13 (127).

IgE production can occur via two main pathways: the extrafollicular (EF) pathway and the germinal center (GC) pathway. These two routes represent distinct mechanisms by which B cells can generate IgE, each with different implications for immune responses, particularly in allergic diseases. The extrafollicular pathway is a rapid, short-term response that occurs outside of the germinal centers (in the extrafollicular regions of lymphoid tissues such as the spleen and lymph nodes). It leads to the early production of low-affinity IgE because it is not subjected to somatic hypermutation (SHM). These EF antibodies are generated rapidly, providing an early defense against allergens but are less effective in the long term compared to high-affinity IgE (128, 129).

However, The germinal center pathway is a long-term, high-affinity response that occurs within the germinal centers of secondary lymphoid organs. This pathway leads to the production of high-affinity IgE and is associated with memory B cell formation and long-lived plasma cells. Unlike the extrafollicular pathway, B cells in the GC undergo somatic hypermutation in their immunoglobulin (Ig) genes, a process that increases the affinity of the IgE they produce for specific antigens (allergens). These antibodies are more effective at recognizing and binding allergens

compared to IgE produced through the EF pathway and they provide a long-term, potent allergic response (130).

IgE shares a similar molecular structure with other Igs, comprising two identical light (L) chains and two identical heavy (H) chains, each with variable (V) and constant (C) regions formed by Ig domains (Figure 1.1.3 a). About 110 amino acids make up each Ig domain, which is made up of four β-strands in a C-type topology and three β-sheet sandwiches (131). Unlike IgG, IgD, and IgA, which only include three heavy chains, IgE has epsilon (ε) H chains, which, like IgM, contain one variable heavy chain (VH) and four heavy chain constant domains (Cε1-4). The hinge region in IgG is replaced by the extra domain Cε2 in IgE, which is a crucial factor in determining its unique physical characteristics and functions unique to the IgE isotype. Two identical antigen-binding sites are formed when the V regions of the H- and L-chains couple together. The antibody's antigen-binding fragment (Fab) region is composed of these and the CH1 domain pair. The remaining Ig domains form the constant fragment (Fc) region, which interacts with cellular receptors (FcR). IgE binds to its receptors at the Cε3 region. IgE is highly glycosylated, and these glycosylation patterns are crucial for interacting with receptors (132).

IgE attaches to its receptors on immune cells located in tissues or the bloodstream. IgE is recognized by two receptors: the low-affinity Fc receptor (FcεRII; also called CD23) and the high-affinity Fc receptor (FcεRI) (Figure 1.1.3) (17). Although the murine FcεRI is a tetramer composed of one α chain, one β chain, and two identical disulfide-linked γ chains, human FcεRI can be found in both tetrameric and trimeric (αγ2) forms. The Fc binding sites for IgE are in the extracellular domain of the FcεRI-α chain. It is made up of one transmembrane domain containing a conserved aspartic acid residue, two extracellular Ig-related domains, and a brief cytoplasmic tail (133). The cytoplasmic tails of the amino (N) and carboxy (C) termini are separated by four transmembrane domains of the β chain (134). The γ chain is a transmembrane segment and cytoplasmic tail antigen receptor component that belongs to the γ/ζ/η family. The distinct immunoreceptor tyrosine-based activation motifs (ITAM) on both the FcεRI-β and -γ chains are phosphorylated when FcεRI-bound IgE molecules cross-link with antigens. It has been demonstrated that the β chain acts as an amplifier for both the cell-surface production of FcεRI and FcεRI signalling (135). Conversely, the γ chains function as essential FcεRI signalling components. Mast cells and basophils express FcεRI, which when activated, leads to cellular degranulation, eicosanoid synthesis, and cytokine

production. Dendritic cells and macrophages in humans also express FcεRI, and when these cells are activated, IgE-bound antigens are internalized for processing and presentation on the cell surface, and cytokines are produced to support Th2 immune responses (17). Moreover, FcεRI can be expressed by lung structural cells including ASM, epithelial cells, and endothelial cells (136).

Low-affinity FcεRII/CD23 belongs to the C-type lectin class and is a type II transmembrane glycoprotein. The membrane-bound form of CD23 features three globular lectin domain 'heads' connected to the membrane by a triple α-helical coiled-coil 'stalk' region (Figure 1.1.3 b) (137). T and B lymphocytes, neutrophils, monocytes, follicular DCs, and intestinal epithelial cells are all known to express CD23 (138).

A variety of soluble fragments (sCD23) are produced when proteases like ADAM10 digest the stalk region of CD23. These sCD23 fragments may all attach to IgE (139). Two splice variants of the N-terminal sequence of CD23 are known to exist: CD23b is IL-4-inducible on inflammatory cells, monocytes, macrophages, B cells, and epithelial cells, whereas CD23a is produced by antigen-activated B cells and eosinophils (140). CD23 activation affects IgE synthesis in activated B cells differently depending on its oligomerization state: monomeric sCD23 inhibits IgE synthesis, while trimeric sCD23 stimulates it. Studies demonstrating that CD23-deficient mice had greater antigen-specific IgE levels while CD23 transgenic animals had lower IgE levels demonstrate that CD23 can control IgE production in vivo (141). Additionally, sCD23 possesses a wide range of cytokine-like properties. For example, it inhibits germinal center B cell apoptosis, promotes the development of germinal center centroblasts towards the plasma cell pool (in conjunction with IL-1α), and keeps active mature B lymphocytes growing (142).

IgE is a crucial mediator in the development of asthma and other allergic conditions, and its release promotes the activation of mast cells and basophils, the release of inflammatory cytokines, and airway inflammation and bronchoconstriction. Numerous studies have shown a link between high serum IgE levels and allergic asthma (143). The level of total and allergen-specific IgE is significantly increased in asthmatic patients and treatment with Omalizumab (an anti-IgE monoclonal antibody), reduces the level of serum IgE and asthma exacerbation in patients with moderate to severe asthma (144). The FcεRI-mediated release of mast cell components triggered by allergen-bound IgE causes a swift rise in mucus production and constriction of airway smooth muscle, setting the stage for inflammatory cell infiltration. These immediate reactions contribute

to increased airflow obstruction and a subsequent decline in lung function (145). Omalizumab dramatically lowers submucosal IgE-positive and FcεRI-positive cells in allergic asthma patients, and this IgE depletion in airway tissue is linked to a large decrease in mucus production and airway eosinophils (146).

ASM cells were long thought to be exclusively engaged in bronchoconstriction during acute asthmatic exacerbations. However, it's important to highlight that ASM cells, which express FcεRIs on their surface, can be activated partly due to the IgE pathway (147). IgE can directly trigger ASMCs to release various substances such as cytokines (IL-4, IL-5, IL-13, TNF-α, TSLP), chemokines (CCL5, CCL11, CXCL8, CXCL10), and traditional mediators, leading to ASMC growth and contraction (148). The hypertrophy and hyperplasia of ASM cells result from their activation, and these changes are correlated with the severity of asthma (149). Furthermore, ECMs, which are essential for airway remodelling, are produced and secreted by ASM cells in response to IgE-driven stimulation. These outcomes explain why anti-IgE monoclonal antibodies prevent airway remodelling, extracellular matrix, and collagen deposition (150).

Overall, understanding the role of IgE in asthma is crucial for the development of targeted therapies aimed at reducing IgE levels or blocking its effects, thereby helping to manage allergic asthma more effectively. Medications such as anti-IgE antibodies (e.g., omalizumab) and anti-inflammatory drugs (e.g., corticosteroids) are commonly used in the treatment of allergic asthma to reduce inflammation and alleviate symptoms.

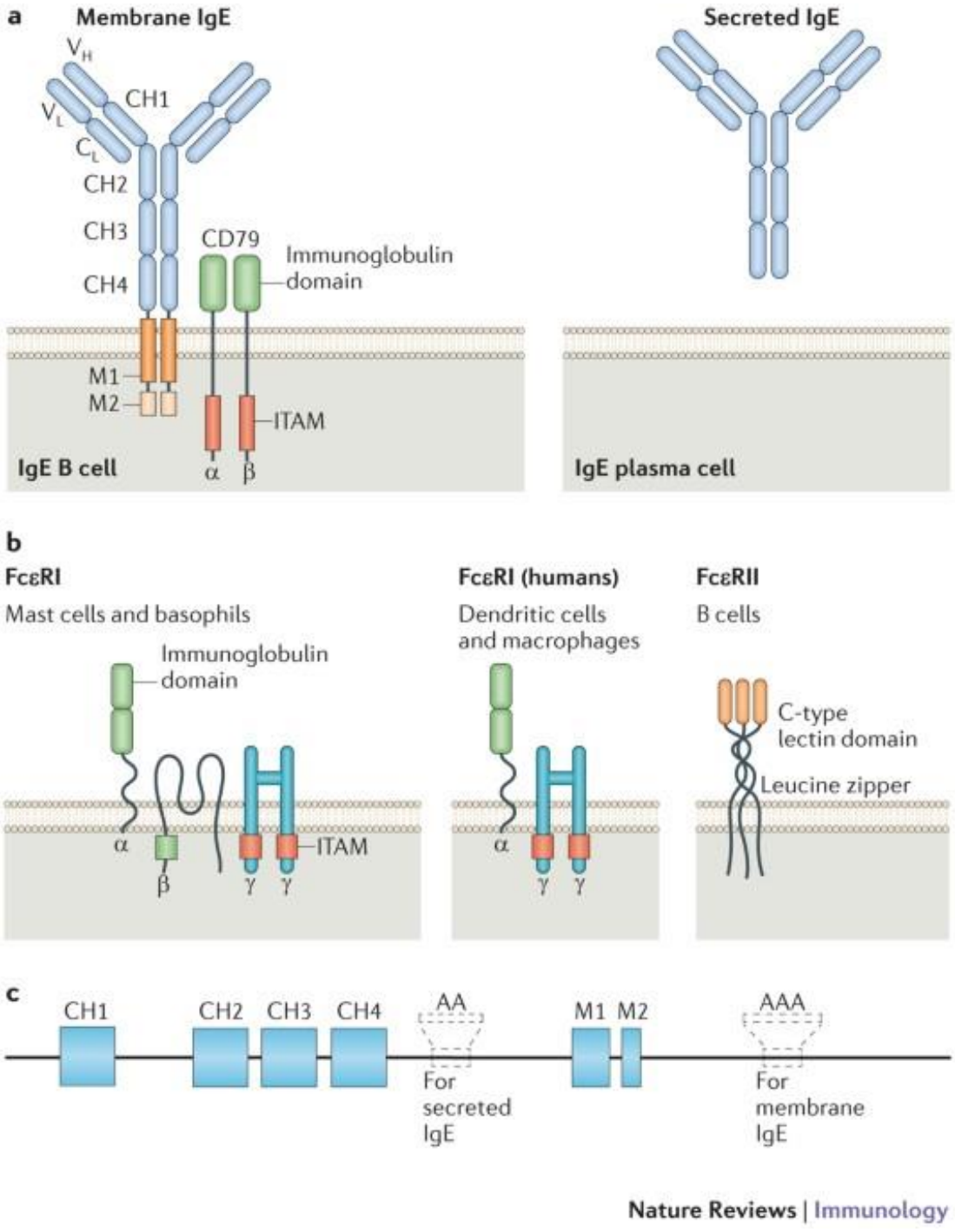


Figure 1.1.3 IgE and its receptors. IgE is produced in two forms: a secreted form made by plasma cells, and a membrane B cell receptor form expressed on the surface of B cells that have switched classes to IgE. The membrane-bound IgE facilitates antigen uptake and presentation by B cells and, along with co-stimulatory signals, induces B cell proliferation and differentiation. Through CD79, which possesses an

immunoreceptor tyrosine-based activation motif (ITAM), membrane IgE can communicate (a). On mast cells and basophils, the high-affinity Fc receptor for IgE (FcεRI) is expressed as a tetrameric complex consisting of one α subunit, one β subunit, and two γ subunits. The γ-subunit, which has an ITAM, serves as the receptor's signalling component. FcεRI is also constitutively expressed as a trimeric complex on dendritic cells, macrophages, and other cell types in humans, but not in mice. This complex consists of one α-subunit and two γ-subunits. The homotrimeric complex of the low-affinity Fc receptor for IgE (FcεRII; sometimes referred to as CD23) is expressed on B cells and other types of immune cells (b). The four heavy-chain constant domains (CH1–CH4), a transmembrane domain (M1), and a cytoplasmic domain (M2) are encoded by exons that make up the IgE genomic locus. Whereas the polyadenylation sites (AA) for secretory IgE are situated between the CH4 and M1 exons, the suboptimal sequences of membrane IgE's polyadenylation sites (AAA) are situated after the M2 exon. The immunoglobulin light chain's CL, or constant region, and VH, or variable region.

1.1.5 Asthma current treatment and limitations

Over the past decade, our growing understanding of asthma's heterogeneity and the underlying mechanisms of airway inflammation has shifted the treatment approach and led to the development of targeted biologic and individualized therapies. Although huge progress was achieved in controlling and treatment of eosinophilic asthma, neutrophilic asthma which is more resistant to standard therapies like corticosteroids and is often associated with more severe forms of the disease needs more attention and a specific clinical approach. The current treatment for asthma, particularly allergic asthma, primarily involves managing symptoms, preventing exacerbations, and reducing inflammation in the airways. The main approaches include medications, lifestyle changes, and in some cases, immunotherapy.

For a long time, it was believed that the primary treatment and control of asthma involved inhaled corticosteroids (ICS). However, this belief has changed with the recognition of different phenotypes of asthma. While corticosteroids are still the cornerstone of treatment in eosinophilic asthma and are effective in controlling symptoms and preventing exacerbations, their effectiveness in neutrophilic asthma remains questionable (151). Unlike their effect on increasing eosinophil apoptosis, corticosteroids extend neutrophil lifespan by inhibiting apoptosis therefore they are not good options for the management and treatment of neutrophilic asthma (152). Furthermore, Corticosteroids target Th2 immune response, which drives eosinophilic asthma. By suppressing Th2 cytokines like IL-4, IL-5, and IL-13, corticosteroids reduce eosinophil recruitment and activation in the airways, leading to reduced inflammation (153). While neutrophilic asthma is

driven by Th1 and Th17 immune responses rather than Th2. Corticosteroids are less effective at targeting cytokines like IL-17 and IL-8, which promote neutrophil recruitment and activation. This difference in the inflammatory pathway makes corticosteroids less impactful in controlling neutrophil-mediated inflammation (154).

The difference in therapeutic approaches and control of eosinophilic and neutrophilic asthma is not solely limited to the use of corticosteroids. Due to significant differences in the underlying mechanisms involved in each phenotype, the treatment strategies for them vary accordingly. Omalizumab is a monoclonal antibody that targets IgE and by binding to the FC portion of secreted IgE, it prevents IgE from interacting with its receptors on mast cells and basophils, reducing allergic inflammation, eosinophil activation, and the subsequent release of inflammatory mediators (155). It has been demonstrated that Omalizumab therapy significantly reduces asthma symptoms, prevents asthma exacerbation, and improves disease outcomes in eosinophilic asthma (156). In addition, the use of antibodies targeting Th2 cytokines such as IL-4, IL-5, IL-13, or their receptors represents an alternative therapeutic option for the treatment of eosinophilic asthma (156).

Neutrophilic asthma represents a lower percentage of patients but with more severe symptoms, yet they are the most difficult to manage and treat. Like ICS, Omalizumab is less effective in patients with neutrophilic asthma because this phenotype is typically non-allergic and not associated with elevated IgE levels. Therefore, Omalizumab is not typically recommended for neutrophilic asthma as these patients do not show the same benefit from anti-IgE therapy as those with allergic, eosinophilic asthma (156). Macrolide therapy may be beneficial for patients with severe asthma who have a non-Th2 phenotype and neutrophilic sputum (157). It has been demonstrated that Azithromycin decreases asthma exacerbations and improves asthma outcomes in non-eosinophilic asthma (158). Furthermore, alternative treatments targeting the underlying Th1/Th17 immune pathways are being researched and developed. Despite significant advances in asthma management, the complete control of this disease remains a challenge. Incomplete response to standard therapies, including corticosteroids and biologics, leaves some patients with uncontrolled symptoms. Moreover, other factors including environmental triggers, poor adherence, and comorbid conditions complicate long-term control. The heterogeneity of asthma phenotypes necessitates personalized approaches, but effective treatments for all phenotypes remain lacking, particularly in severe cases. These challenges in the satisfactory control of asthma underscore the

need for ongoing research and development of new treatments to improve outcomes for patients with allergic asthma.

2. B cells

1.2.1 B cell development

B cells, a type of lymphocyte, primarily defend against invading pathogens by producing diverse antibodies. Apart from generating antibodies, B cells have been found to have significant immunoregulatory functions and influence the behavior of other immune cells either directly or indirectly through antigen presentation and cytokine production, thereby playing a crucial role in the adaptive immune response (159-161)

Research into B cells and their characteristics began in the mid-1960s and early 1970s using experimental models and clinical assessment of individuals with immune-deficiency disorders (162). The discovery of B cells didn't begin with the recognition of a specific cell, but rather with the identification of a protein (Ig or antibody) (163). Recognizing serum gammaglobulin as the origin of antibodies marked the beginning of the eventual discovery of cells that produce antibodies. The “B” cell name comes from the Bursa of Fabricius, a lymphoid organ exclusive to birds that these cells were identified for the first time within it. Studies using transplant models in mice have demonstrated the origin of these antibody-producing cells. In mammals, B cell development takes place in primary lymphoid organs, which are the fetal liver during embryonic development but become the bone marrow (BM) in adults. The development of B cells follows a similar pattern in mice and humans, occurring throughout their lifetime (Figure 1.2.1) (164-167).

The development of B cells in the bone marrow is closely regulated by a mix of extrinsic and intrinsic signalling pathways. The bone's medullary cavity is home to a diverse range of cells that assist the B cells development. For instance, the presence of stromal cells in this microenvironment is crucial because they provide support in the form of extrinsic signals for the primitive B cells

(168). Common lymphoid progenitors that formed in BM during hematopoiesis are the source of B cell precursors (169). Precursor B cells are classified into different stages of early B cell development based on the varying expression of surface markers. Eventually, they are released into the periphery (170, 171).

Early stages of B-cell development in the BM are organized around the functional rearrangement process of the immunoglobulin gene segments (172). The rearrangements involve V (variable), D (diversity), and J (joining) segments of the heavy chain (H-chain), along with VL-JL rearrangements of the light chain (L-chain) gene segments, and known as V(D)J recombination (173). This process is essential for creating a wide variety of B cell antigen receptors (BCRs) that can bind to antigens with varying affinities. The involvement of recombination-activating genes 1/2 (RAG1 and RAG2) is pivotal in facilitating this rearrangement of Ig genes and B cell development (174-176).

Three developmental stages are identified in B cells based on the rearrangement of the H-chain and L-chain gene segments (Figure 1.2.1). The first identifiable stage in B cell development is the pro-B cell stage. At this stage, the HSCs commit to becoming B cells by expressing the precursor B cell receptor (pre-BCR), which is composed of a surrogate light chain (SLC) and a μ heavy chain. Rearrangement of the immunoglobulin heavy chain gene locus (IgH) occurs at this stage. Pro-B cells rearrange the H-chain's D and J segments, and a second rearrangement joins the rearranged DJ segment to an upstream V region. The onset of V(D)J recombination as well as the survival and activation of these early B cells are facilitated by IL-7 produced from stromal cells. Successful rearrangement leads to the expression of a functional μ heavy chain and opens the entry into the next phase. Pre-B cells are characterized by the expression of a functional μ heavy chain paired with the surrogate light chain. In pre-B cells, rearrangement of the immunoglobulin light chain gene locus (IgL) takes place (170, 177). This rearrangement results in the expression of a functional light chain (either kappa κ or lambda λ) (178). Mice lacking RAG gene function halt the development of B cells during the pro-B cell phase because they cannot carry out rearrangement of the Ig heavy chain (175, 176). The assembly of the μ heavy chain with the light chain forms the pre-B cell receptor (BCR) is crucial for cell survival and proliferation at this stage. The primary functions of the pre-BCR involve two main objectives. Initially, it works to halt the operations and manifestation of the enzyme apparatus responsible for rearranging the H-chain gene

segments, a phenomenon known as allelic exclusion (179). This mechanism prevents the cell from expressing two distinct H-chains with different specificities simultaneously. Subsequently, the pre-BCR instigates the rearrangement of the L-chain genes. Once the BCR is successfully assembled and expressed on the cell surface, pre-B cells transition to the last developmental stage, the immature B cells which express membrane-bound IgM (170, 180).

To keep the BM in a constant state, B cell differentiation, apoptosis, and proliferation are all balanced. Apoptosis eliminates B cells exhibiting deficiencies in antigen recognition or Ig gene recombination (180). To guarantee the selection of normal B cells, these cells must navigate several checkpoints during the BM's developmental stages. The first checkpoint is at the pre-B cell stage when B cells will be positively selected for additional development if they show a functional pre-BCR otherwise they will undergo apoptosis (170, 181). The second checkpoint arises during immature B cell differentiation, where cells display mature, functional B cell receptors (BCRs) primarily in the form of IgM on their membranes. However, over half of these generated BCRs may be autoreactive and target self-antigens so they are diminished by negative selection, and the remaining normal immature B cells will exit the BM and migrate to the spleen, where they complete early development by differentiating into various subsets of mature B cells (182).

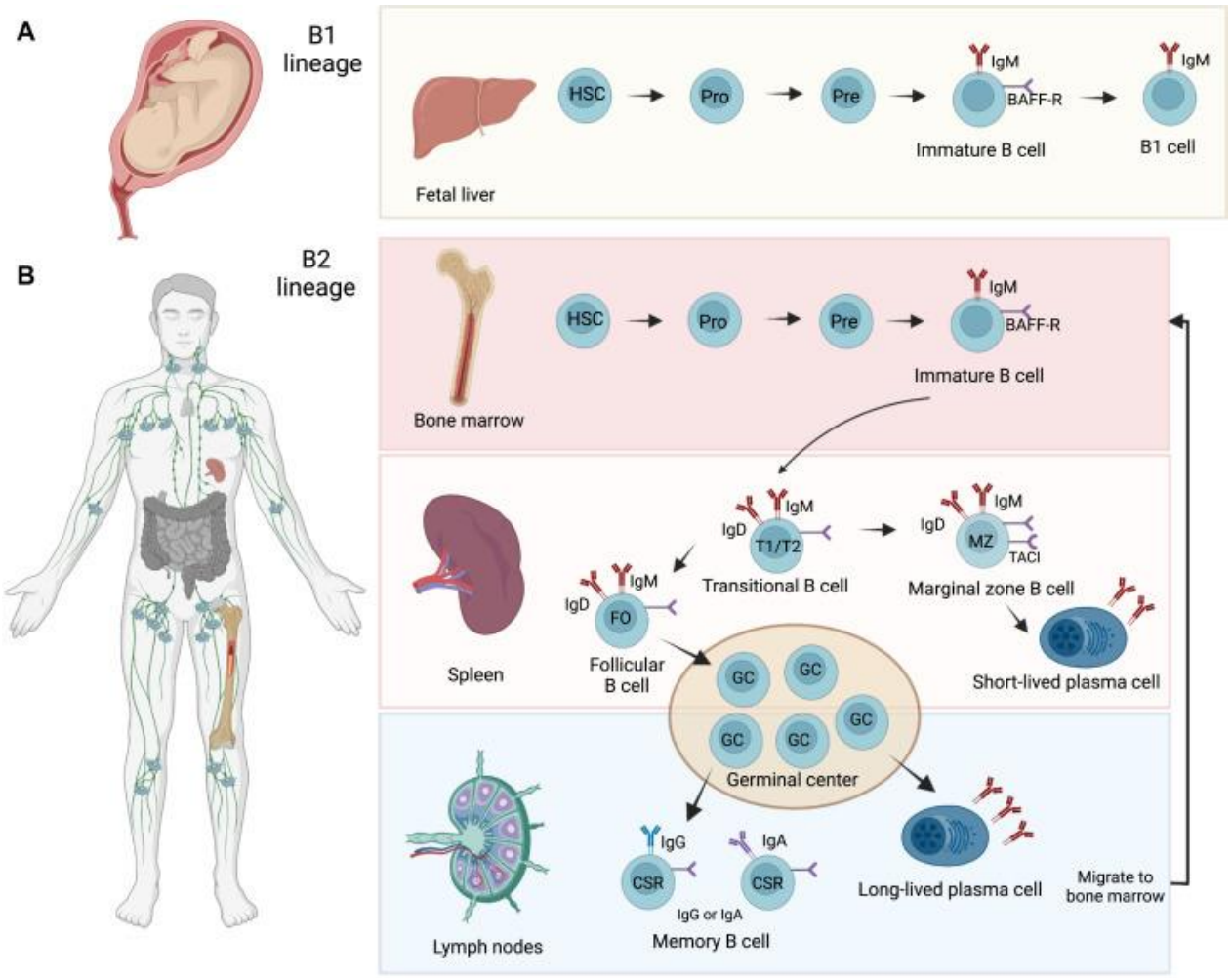


Figure 1.2.1 B cell development. B cells produced from stem cells obtained from the fetal liver differentiate into the B1-cell lineage before to birth (A). After birth, B cells from HSCs in the BM develop into the B2-cell lineage. This process involves progression through stages of pro-B cells, pre-B cells, and immature B cells, sequentially expressing IgM. These immature B cells then migrate to the spleen as transitional B cells, where they receive survival signals via the B-cell-activating factor receptor (BAFF-R). Depending on the specificity of their BCRs, transitional B cells further mature into either follicular B cells or marginal zone (MZ) B cells. Upon encountering antigens, MZ B cells differentiate into short-lived plasma cells, while follicular B cells initiate germinal center (GC) formation. Within GCs, B cells undergo further differentiation into memory B cells (MBCs) or long-lived plasma cells. Somatic hypermutation (SHM) and class switch recombination (CSR) occur in MBCs, modifying BCR affinity and isotype (from IgM to IgG, IgA, or IgE). Both MBCs and long-lived plasma cells migrate to the bone marrow, facilitated by the transmembrane activator and calcium modulator (TACI) and cyclophilin ligand interactor (B).

1.2.2 B cell maturation in secondary lymphoid organs

After being released from BM and entering the bloodstream, naïve immature B cells enter the secondary lymphoid organs (SLOs), including the spleen, lymph nodes, and Peyer patches where they undergo differentiation into transitional and various subsets of B cells to complete their maturation process. B cell maturation in the SLOs especially the spleen is regulated by various factors. Peripheral B cells rely on ongoing basic signalling from their BCRs for survival, even without specific antigen stimulation. Stopping RAG expression and V(D)J recombination after BM departure depends on tonic BCR signalling. These tonic impulses must reach a specific threshold for them to differentiate into transitional B cells. Notably, the process of B cell selection and maturation in bone marrow and SLOs shows remarkable similarities between human and mice (183-185).

1.2.2.1 Transitional B cell

Transitional B cells refer to B cells that have recently exited the bone marrow and are transferred in the bloodstream to SLOs. This population is considered the least mature subset of B cells found in the periphery (186). They represent an intermediate stage between immature B cells and mature, antigen-primed B cells and can be found in the BM, peripheral blood, and spleen. A few percentages of these subsets start to differentiate inside the bone marrow itself, even though the majority of them are observed in the periphery (187-189). It is hypothesized that peripheral checkpoints are applied to human transitional B cells after they leave the bone marrow in order to prevent the development of autoantibodies (182). Transitional B cells that pass through auto-reactivity selection, eventually mature into naive B cells (190). It is generally accepted that the transitional B cell compartment serves as an important negative selection checkpoint for autoreactive B cells since so few immature B cells make it to the mature naive stage (191, 192). It has been shown that transitional B cell deficiency would lead to immunodeficiency and autoimmunity (192).

Compared to their mature counterparts, all transitional B cells have higher levels of heat-stable antigen (HSA, CD24) and express the phenotypic surface markers. AA4 (193). Compared to mature B cells, IgM concentrations are elevated in transitional B cells, and surface expression of CD22 and B220 is reduced (194). While mature B cells are activated and multiply upon BCR stimulation, the same signals cause transitional B cells to halt in an inactive state or undergo cell death (195-197).

B cell has two transitional stages: T1 and T2. T1 is the stage from which the B cell migrates from the bone marrow to the spleen, and T2 is the stage in which the B cell matures within the spleen (198). These two phases are characterized by changes in surface marker expression, functional properties, and sensitivity to survival signals (192). Furthermore, the various stages of transitional B cells exhibit distinct positions within the spleen. The initial transitional B cells seem to be confined to the outer layer surrounding the arteries, known as the periarteriolar lymphoid sheath. In contrast, the T2 B cells demonstrate a propensity to move into the follicles where B cells gather (192, 194, 199). T1 B cells can be identified based on high expression levels of IgM and lack IgD, CD21, and CD23. In contrast, T2 B cells, as more mature transitional B cells, still have high IgM levels but also express IgD, CD21, and CD23 (200). Interestingly, Allman et al. identified a third population, called T3, using the AA4 antibody, which shares similarities with T2 cells except for lower levels of surface IgM. These transitional B cell populations, delineated by surface markers, also display unique functional traits (188). T1 B cells undergo apoptosis as part of the central tolerance mechanisms to eliminate autoreactive B cells. They have limited exposure to antigens and are primarily involved in tolerance induction and selection processes. In contrast, T2 B cells are less sensitive to apoptosis, indicating that they have passed through some degree of negative selection and are less likely to be auto-reactive. They have increased sensitivity to survival signals and are poised to respond to antigens. T2 B cells may undergo further differentiation into mature B cells under the influence of appropriate stimuli, such as antigen engagement and signals from helper T cells (201).

1.2.2.2 B cell peripheral maturation

B cells play a pivotal role in adaptive immune responses by foreign antigen recognition and producing antibodies and cytokines against these antigens. Therefore, their development and maturation process are complex and occur within 2 phases. Although the primary development phase takes place in the BM, B cells need to migrate to SLOs to complete their maturation and turn into potent activated B cells. SLOs are highly organized structures with various areas for B cells, T cells, and other immune cells. SLOs unique structure facilitates interactions between immune cells, antigen-presenting cells, and antigens. The spleen contains two distinct regions known as the red pulp and the white pulp, with the latter serving as the lymphoid compartment. Within the white pulp, the histological arrangement comprises a layer of lymphocytes encircling the splenic arterioles, termed the periarteriolar lymphocyte sheath (PALS). Predominantly, the PALS consists of T cells, while B cells aggregate into spherical structures called primary follicles situated at the periphery of the PALS. These primary follicles mainly harbor naive and recirculating B cells (202, 203). Upon exposure to antigens, these follicles undergo transformation into germinal centers, also known as secondary follicles, responsive to T cell-dependent antigens (204). The interface between the white pulp and the red pulp is designated as the marginal zone, housing specialized subsets of macrophages such as metallophilic and marginal zone macrophages, along with a population of nonrecirculating IgD^- B cells (205).

Self-reactive BCRs are present in a portion of immature B cells in the spleen. Strong BCR signalling upon recognition of self-antigens prompts some of these cells to undergo either anergy-mediated selection or apoptosis-induced cell death. The type of self-antigen determines whether B cells experience tolerance or anergy. These self-reactive B cells are driven to anergy by soluble monovalent antigens and clonal deletion by multivalent antigens (206, 207). Anergy, an essential peripheral tolerance mechanism, necessitates the long-term activation of B cells through persistent antigen receptor binding and signalling, which induces an unresponsive state in the cells (208). Key characteristics that distinguish anergic B cells from their normal counterparts encompass a shortened lifespan and weakened antibody production, thereby constraining their efficacy (209). Although anergic cells may join GCs with assistance from T cells, they typically do not transition into antibody-secreting cells unless alterations within the GC mitigate their self-reactivity (210). In mice subjected to prolonged antigen exposure, B cells undergo a state of anergy, halting their

progression at the transitional stage and impeding further maturation. Despite initially following a typical developmental path, these B cells show a marked decrease in the absolute count of mature follicular cells in peripheral tissues. Moreover, their capacity to generate an antibody response upon immunization is notably diminished (196, 211). The cytokine BAFF (B-cell activating factor), which is secreted by a variety of cells including macrophages, monocytes, and dendritic cells, is a crucial modulator of B cell survival in the periphery. Elevated BAFF levels can have a major impact on B cell populations in the periphery, even though they have little effect on B cell formation in the BM. Without BAFF, mature B cells in the periphery can diminish entirely (199). To differentiate into transitional B cells, immature B cells egress the BM and express BAFF-R (212). On the other hand, an aberrant increase in BAF can reverse anergy in self-reactive B cells located in the periphery. Excessive BAFF production is linked to B cells hyperplasia and the emergence of autoantibodies (213).

1.2.3 B cell subsets

To produce an effective B cell-mediated immune response, B cells need to be situated in a specific microenvironment within secondary lymphoid organs. Based on their function, and location, B cells can be classified into various subsets including B1 B cells, Marginal zone (MZ) B cells, and follicular B cells (214, 215) (Figure 1.2.2) . MZ B cells and follicular B cells are also called B2 B cells or conventional B cells.

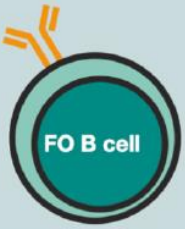
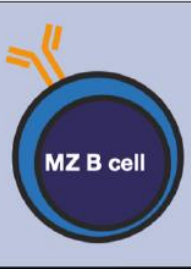
			
Primary localization	Secondary lymphoid organs; mainly in B cell follicles	Secondary lymphoid organs; mainly in the splenic marginal zone	Peritoneal and pleural cavities
Distinguishing surface markers	$B220^{+}$, $CD19^{+}$, $CD1d^{lo}$, $CD21^{+}$, $CD23^{hi}$, $CD43^{-}$, IgM^{lo}, IgD^{hi}	$B220^{lo/+}$, $CD19^{+/hi}$, $CD1d^{hi}$, $CD21^{hi}$, $CD23^{lo}$, $CD43^{-}$, IgM^{hi}, IgD^{lo}	$B220^{-/lo}$, $CD19^{hi}$, $CD1d^{+}$, $CD23^{-}$, $CD43^{+}$, IgM^{hi}, IgD^{lo}
Principal function	Participation in T-dependent immune responses	Housekeeping function (natural IgM), first responders to blood-borne pathogens	Housekeeping function (natural IgM), first responders to mucosal pathogens

Figure 1.2.2 Overview of various B cell subsets characterization.

1.2.3.1 B1 B cell

Different from ordinary B cells (B2 cells), which are created after birth and replenished in the bone marrow, B1 cells are first produced in the fetal liver and the majority of them undergo self-renewal in the periphery (216, 217). Although lacking memory and not part of the adaptive immune system, B1 B cells carry out many functions similar to other B cell subsets, such as producing antibodies against antigens and serving as antigen-presenting cells. they are a type of innate-like B cell that plays a role in an early response to infection or vaccination by secreting IgM. B1 B cells are Typically located in specific peripheral tissues such as the peritoneal and plural cavity and less frequently in the spleen or bloodstream (218).

B1 cells are distinguished from other B cells by the varying levels of CD5, CD86, IgM, and IgD (218). In the mice, B-1 cells are categorized into B-1a and B-1b cells, which originate from different precursors and differ in their expression of CD5, which is not present in the B-1b population. Unlike B1a cells, B1b cells can originate from precursors in the adult bone marrow. Additionally, the precursors for B1a and B1b cells differ in their levels of CD138 expression (219). B1b cells have demonstrated the ability to produce memory responses (220). Additionally, Compared to B1a cells, B1b cells seem to recognize more types of antigens including intracellular antigens. These cells can identify protective antigens within bacteria, which is noteworthy because they target internal components (218).

B-1 cells naturally secrete antibodies even in the absence of antigen exposure. These cells are responsible for producing over 80% of the natural IgM found in the blood under normal conditions. The antibodies they generate are derived from the germline and have broad reactivity, but due to the lack of somatic mutation, they exhibit limited diversity and specificity. B-1 cell-derived antibodies can target a variety of bacterial antigens as well as some self-antigens. Besides producing natural antibodies, B-1 cells can be activated by thymus-independent (TI) stimulations, such as TLR agonists or other microbial products (217, 221). One more protective function of naturally occurring IgMs in the bloodstream is to aid in the removal of apoptotic cells. Because of defective apoptotic processes, B cells are exposed to intracellular antigens, including DNA, RNA, or nuclear proteins (222).

1.2.3.2 Marginal zone B cell

Marginal zone (MZ) B cells are named after their specific location in the spleen's marginal zone, which is a distinct area marking the boundary between the white and red pulp. In this region, arterial blood is filtered, and pathogens are captured by specialized cells such as marginal zone macrophages, marginal metallophilic macrophages, and MZ B cells. These cells are constantly exposed to sphingosine-1-phosphate (S1P) in the bloodstream, and the high levels of sphingosine 1-phosphate receptor 1 (S1P1) and the integrins lymphocyte function-associated antigen-1 (LFA-1) and α4β1 help maintain the localization of MZ B cells in this area (223, 224).

Marginal zone (MZ) B cells make up approximately 5-10% of all splenic B cells and are characterized as $CD19^{+/hi}$ B cells with high levels of IgM expression but lack IgD on their surface. While BCR signalling is essential for MZ B cell survival, other pathways are also known to play a role in determining the destiny of these cells (225, 226). The decision for a B cell to become a marginal zone (MZ) or follicular (FO) B cell is made in the spleen during the transitional B cell stages, specifically the T1 and T2 stages (192, 227, 228). A critical point occurs at the T1 stage, where effective signalling through the BCR triggers the movement of ADAM10 to the cell surface. ADAM10 is an enzyme that cleaves Notch2, which is crucial for activating the transcriptional program leading to MZ B cell development (229-231). A recent study found that when Notch2 is conditionally deleted in B cells, there is a significant reduction in the MZ B cell population (231). Conversely, if an antigen binds too strongly to the BCR, it will activate Bruton's tyrosine kinase (BTK), potentially inhibiting Notch2 signalling. As a result, weak BCR signalling in transitional B cells promotes differentiation into MZ B cells, whereas strong signalling favors the development into FO B cells (232).

Unlike FO B cells that mainly produce mono-reactive BCRs leading to highly specific, high-affinity antibodies, following immunization, innate-like MZ B cells generate poly-reactive BCRs and undergo proliferation and differentiation into short-lived plasma cells and quickly produce low-affinity antibodies with self-reactivity to eliminate pathogens and apoptotic cell debris in a T cell-independent manner. Furthermore, MZ B cells have heightened sensitivity to stimulation via Toll-like receptors (TLRs), a factor that expedites their initial antibody response, specifically against T-independent antigens and blood-borne infections (233, 234).

Furthermore, MZ B cells move between the marginal sinuses and the follicular region. Due to their high expression of the CD21 receptor, MZ B cells can capture antigens trapped by immune complexes and transport them to follicular B cells. This activity of MZ B cells indirectly supports the antibody response within the follicles (235).

1.2.3.3 Follicular B cell

Follicular (FO) B cells primarily reside in the B-cell follicles within secondary or tertiary lymphoid organs such as the spleen and lymph nodes. They make up about 80–90% of all splenic B cells and make up the majority of the B cell population in the periphery. Unlike MZ B cells, FO B cells has the ability to move between the spleen and blood (232).

FO B cells are distinguished from other B cell populations by expressing specific surface markers including high levels of surface IgD and CD23, intermediate to low levels of membrane IgM, and CD21and no expression of CD1 or CD5.

The BCR repertoire within the follicular B cell compartment seems to undergo positive selection during its final maturation in the spleen. This compartment exhibits significantly greater diversity compared to the B1 B and MZ B cell compartments. Crucially, follicular B cells need assistance from T follicular helper (Tfh) cells through the CD40-CD40L interaction to differentiate into plasma cells and facilitate effective primary immune responses, antibody isotype switching, and the establishment of high-affinity B cell memory (236).

1.2.4 Germinal center

One of the key features of the humoral immune response involves a gradual enhancement in the effectiveness of antibodies over time (237). This enhancement occurs through a process called somatic hypermutation (SHM), which is driven by activation-induced cytidine deaminase (AID). SHM targets the variable regions of immunoglobulin (Ig) genes (238, 239). This process generates a variety of mutated B cells. These cells undergo selection based on their affinity, leading to the proliferation and differentiation of high-affinity B cells into antibody-secreting plasma cells and memory B cells. This selection process takes place within specialized microanatomical structures called germinal centers (GCs) (240, 241). GC is a distinct anatomical structure found within secondary lymphoid organs such as the spleen, lymph nodes, and mucosa-associated lymphoid tissues (MALTs) (242). In 1884, Walther Flemming first identified regions with significant mitotic

activity in lymph nodes and other lymphoid tissues. He termed these regions with intense cell division as GCs, believing they were the primary source of lymphocytes (242-244). Despite this initial theory being later disproved, GCs continue to be a vital source of effector B-cell populations and by producing high-affinity antibodies and memory cells, play an essential role in defending against various infectious agents and the development of humoral immunity and effectiveness of nearly all vaccines (245, 246).

The dynamic nature of the GC reaction is one of its essential features. GC B cells continuously move between different microanatomical compartments at the cellular level. During this process, they repeatedly undergo SHM and selection, seeking survival signals from other cell populations within the GC.

1.2.4.1 Initiation of GC

GCs develop within the central regions of B cell follicles in SLOs, where they are interspersed among stromal cells called follicular dendritic cells (FDCs) (247). FDCs have an organizing function in primary follicles (follicles without GCs), assisting B cells in clumping together into compact, distinct follicles (248). In secondary follicles containing GCs, FDCs are situated within the GC itself, performing two essential functions. The primary and most studied function is the long-term retention of intact antigens within complement-coated immune complexes, facilitating the affinity-dependent testing of somatically hypermutated BCRs during GC selection (247). According to a recent study, antigen recycles between non-degradative endosomal compartments and the FDC surface, indicating a mechanism by which antigen can be kept on these cells for the long times necessary for effective affinity maturation (249). Additionally, FDCs support the survival of GC B cells and enhance the overall GC response. The observation that blocking FDC activation through TLR4 causes smaller GCs and lower antibody titers in response to immunization lends credence to this (250).

The first step in the development of GCs is when resting B cells and T cells capture antigens (251, 252). B cells can directly bind to soluble antigens or antigens presented on the surfaces of FDCs, macrophages, or dendritic cells (253-255). Under hemostatic conditions, naive T and B

lymphocytes are separated into distinct niches that facilitate their survival and movement between the bloodstream and lymphoid organs (256). T cells and the majority of DCs are found in the T-cell zone, which is situated in the paracortical region of the lymph nodes, while B cells are found in follicles within the lymph node cortex (257). In the spleen, B cells are also found in follicles within the white pulp, and T-cell-rich areas encircle the central arteriole (258). The chemotactic signals that direct the various cell types to their specific niches within the lymph node or spleen control this cell placement (256). The G-protein coupled receptor (GPCR) CXCR5, which is expressed by naive B cells, attracts them to the lymph node's follicles where the receptor's ligand, CXCL13, is expressed (259, 260). Naive T cells express CCR7 and are located in the T-cell zone where its ligands, CCL21 and CCL19, are produced by stromal cells (261). Following an antigen contact, B cells become activated and increase the expression of the chemokine receptor CCR7 and downregulate CXCR5. These changes enable B cells to migrate to the T cells zone toward the CCL19 and CCL21 ligands following a chemokine gradient (262). Besides CCR7 expression in B cells, the Epstein-Barr virus-induced molecule 2 (EBI2) is also upregulated when B cells are activated (263, 264). BCR-mediated EBI2 induction is essential for the efficient placement of B cells within the B-cell follicle upon antigen stimulation, but not for the first CCR7-dependent localization events of B cells following antigen contact (263, 264).

Simultaneously, naive CD 4^{+}T cells within the T zone recognize and bind to the same specific antigen presented by DCs through their T cell receptors (TCRs) and differentiate into multiple types of effector cells with specific functions, including Tfh cells (265-267). Tfh cells are a unique subset of $CD4^{+}$ T cells that assist B cells during the GC responses and they are identified by high expression of surface markers including CXCR5, PD1, Bcl-6, and ICOS (265, 268). The formation of Tfh cells involves several stages. Initially, DCs prime naïve CD4+ T cells. T cells need two signals to be activated during these interactions: the first is the TCR binding to the peptide MHC, and the second is a co-stimulatory signal that is produced when the receptor CD28 is ligated by its ligands, CD80/86, which are expressed on the surface of DCs. T cells integrate signals from several cytokines during this T: DC interaction, which skews their development toward a Tfh cell destiny. Here, pre-Tfh cells, or Tfh cell progenitors, upregulate the master transcription factor Bcl-6 and the chemokine receptor CXCR5 and downregulate CCR7. Bcl-6 represses genes involved in alternative T cell fates and promotes the expression of genes necessary for Tfh cell function and CXCR5 upregulation allows them to migrate to the boundary between the T and B cell zones (265,

269). At the T: B border, B cells display peptide antigen fragments on MHC-Class II molecules to Tfh cells. This interaction provides B cells with essential survival and co-stimulatory signals (262, 270). The last stage in the formation of GCs involves activated Tfh cells and B cells migrating from the T:B border into the follicle, where they become GC B cells and fully differentiated Tfh cells. Both Tfh cells and B cells decrease the expression of CCR7 and EBI2 while maintaining stable levels of CXCR5. This allows them to move away from the chemotactic signals of the T cell zone and outer follicle towards the center of the follicle (263, 264, 271, 272). Once at the center of the follicle, signals exchanged between T and B cells provide the final instructions for Tfh cell differentiation and stimulate GC B cell proliferation to initiate the formation of the GC (268).

Following antigen recognition outside of the follicle, B cells can have another fate. They can develop into memory B cells or short-lived extrafollicular plasma cells (273-275). The first wave of antibodies is produced by extrafollicular plasma cells, which thereafter undergo apoptosis within a few days. This initial burst of antibodies is crucial for the early suppression of infection while the GC response is being developed (276).

1.2.4.2 GC structure

Based on various histological characteristics, Mature GC consists of two distinct zones, dark zone (DZ) and light zone (LZ) which are surrounded by naive follicular mantle B cells. The DZ, located near T-cell regions, is densely packed with large, rapidly proliferating B cells known as centroblasts, which have reduced surface immunoglobulin expression. In contrast, the LZ is situated at the opposite end and contains fewer B cells due to a network of FDCs and Tfh cells that are FDCs critical for the positive selection of centrocytes.

FDCs are a unique population of cells that do not express class II MHC molecules and are not produced from hematopoietic progenitors. Nonetheless, unprocessed antigens are captured and held by FDCs via complement and Fc receptors, forming immune complexes (277, 278). As a result, they act as long-term antigen reservoirs and are thought to play a crucial role in selecting high-affinity GC B-cell clones and in the formation and maintenance of immunologic memory. After activation, FDCs start to express more CXCL13 and BAFF, which help the GC develop and

keep the LZ intact (279). By producing cytokines like IL-6 and IL-15, FDCs facilitate SHM and B cells proliferation respectively (280, 281). Additionally, FDCs are a major source of the CXCL13 in the LZ of GCs, where it accumulates in their cellular processes (277, 282). FDCs are crucial for the GC response since studies on mice have demonstrated that their ablation causes GC termination because of decreased GC B cell survival and localization (248).

Tfh cells, which make up just 5% to 20% of GC cells, are a second population of accessory cells found in the LZ of GCs. recent findings indicate that these cells play a vital role in initiating GC responses by delivering essential signals necessary for the survival of GC B cells (272, 283). Moreover, LZ is populated by centrocytes, small non-dividing B cells that express surface immunoglobulins (242, 284). The organization of GC B cells into dark and light zones is regulated by the differing concentrations of chemokines CXCL12 and CXCL13 in these zones, as well as the controlled expression of CXCR4 on centrocytes and centroblasts (285). Centroblasts exhibit higher levels of CXCR4 compared to centrocytes, and the ligand for this receptor, CXCL12, is more prevalent in the DZ of GC thus this chemokine upregulation facilitates their migration to the DZ of GC. In contrast, the accumulation of centrocytes, which have lower CXCR4 levels, in the LZ is driven by the upregulation of CXCR5 and the abundant presence of its ligand, CXCL13, in these regions of GC (285).

Centrocytes need to migrate to the LZ to acquire antigens and present them to Tfh cells, which is crucial for the survival of high-affinity B-cell clones (286). Signals received in the LZ enable a subset of centrocytes to re-express the CXCR4 receptor and migrate back to the DZ via CXCL12-mediated migration. This process allows them to undergo additional rounds of proliferation and SHM (287). Consequently, this bidirectional trafficking between the LZ and DZ facilitates multiple cycles of proliferation and selection, refining the affinity of the GC B cells (287).

1.2.4.3 Affinity maturation

Several decades ago, researchers noted that following immunization with a foreign antigen, the average affinity of serum antibodies for that antigen gradually increased over time (288). This phenomenon, later named affinity maturation, was found to result from somatic hypermutations

(SHM) in the variable region of antibodies (289) (Figure 1.2.3). GCs are considered the main location where antigen-experienced B cells undergo diversification and affinity maturation. Further research revealed that compared to extrafollicular short-live plasma cells, GC B cells had a high frequency of somatic mutations, indicating that an SHM process is triggered in GC (240, 241).

Through the process of SHM, B cells multiply in GCs and acquire high rates of mutations in their immunoglobulin variable region (IgV) genes (on the order of 10^{-3} to 10^{-4} per base pair each generation (290). This process involves DNA strand breaks and primarily introduces single nucleotide substitutions in the targeted IgV genes. SHM relies on the activity of the enzyme AID, which is mainly expressed in the GC dark zone (291). AID demethylates the deoxycytidine bases in the heavy and light chain IgV genes, which are then eliminated by uracil-N-glycosylase (UNG) which is an enzyme normally involved in error-free base excision repair. This removal of uracil bases by UNG initiates a DNA repair process that can result in the introduction of mutations, particularly single nucleotide substitutions. Mutations occur as the resulting gaps in the DNA sequence are filled in by an error-prone polymerase, leading to random alterations in B-cell receptor affinity and specificity (292). GC B cells that express variations that have higher binding capacity to the immunizing antigen expand preferentially, while alterations that diminish antigen binding trigger apoptosis in GC B cells.

Together, according to the cyclic re-entry model, centroblasts first undergo cell division and SHM in the DZ. After exiting the cell cycle, they re-express surface immunoglobulin and move to the LZ, where they become centrocytes. Within the LZ, GC clones with mutations leading to higher affinity for antigens are favored through their interaction with antigens held by FDCs and signals from Tfh cells. This increased affinity provides advantages such as better competition for limited antigen resources, enhanced recruitment of T-cell assistance, and potentially positive signals from FDCs. Selected centrocytes may then return to the DZ for further rounds of proliferation, selection, and differentiation into memory B cells or long-lived plasma cells which exit the GC and form humoral immunity. However apoptotic cells are cleared by specialized macrophages within the GCs called tingible body macrophages (204, 293).

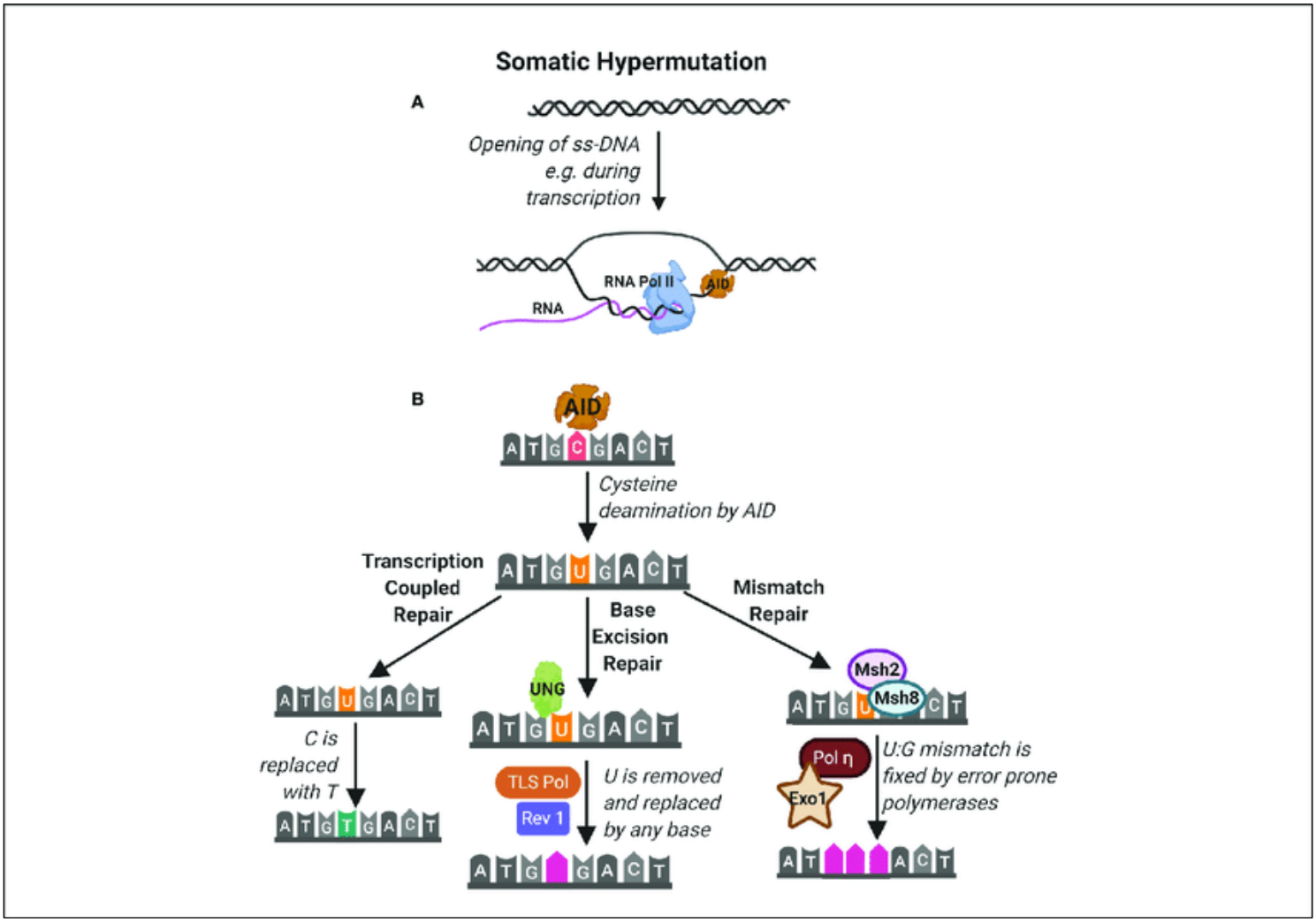


Figure 1.2.3 Somatic hypermutation process. (A) SHM involves the process facilitated by activation-induced cytidine deaminase (AID) on the immunoglobulin heavy chain. AID acts on single-strand DNA (ss-DNA) exposed during RNA transcription, typically by RNA polymerase II (RNA pol II). (B) Specifically, AID converts cytidine (C) nucleotides into uracil (U), which is considered a form of DNA damage. This damage can be rectified through several pathways: (1) transcription-coupled repair, where uracil is transcribed as thymidine (T); (2) base excision repair (BER), where Uracil-DNA glycosylase (UNG) identifies and removes U, followed by replacement by any base via specialized polymerases such as TLS polymerase and Rev1; and (3) mismatch repair (MMR), where Msh2 and Msh8 proteins recognize the U and initiate repair using error-prone DNA polymerases and exonucleases like Pol and Exo1.

1.2.4.4 Class-switch recombination

The key feature of adaptive immunity is the way antibodies (Abs) diversify from the primary IgM and IgD subclasses, which are co-expressed on immature B cells, into the more specialized subclasses including IgG, IgA, and IgE. This transformation process is called class-switch recombination (CSR), or Immunoglobulin class-switching.

Both naïve mature B cells and mature B cells that have been activated by antigen produce both IgM and IgD, the first two heavy chain segments in the immunoglobulin locus. Ag interaction leads to GC B cells proliferation and activation of the CD40 receptor on B cells by CD154 (CD40L) or the Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) and delivers essential signals for CSR. Moreover, AID expression is induced in GC following immunization and these processes result in antibody class switching to produce IgG, IgA, or IgE (239). During class switching, the Ig-heavy chain constant (C) region changes but the variable (V) regions remain intact, so the antigenic specificity remains unchanged. This enables different daughter cells from the same activated B cell to produce antibodies of varying isotypes or subtypes (e.g., IgG1, IgG2, etc.) (294). During CSR, segments of the antibody-heavy chain locus are excised from the chromosome (Figure 1.2.4). The remaining gene segments are then reconnected to maintain a functional antibody gene that encodes an antibody of a different isotype.

In general, targeting, cleavage, and DNA repair are the three simple stages that make up the CSR process (295). To start CSR, one must first target the highly repetitive sequences known as switch (S) regions, which are located upstream of gene segments encoding the C regions of antibody-heavy chains. These S regions are present next to all heavy chain constant region genes except for the δ-chain. Sterile germline transcripts (GLTs), which are activated by cytokines, control the accessibility of the S region (295, 296). Subsequently, DNA cleavage occurs and DNA breaks at two specific S-regions as a result of the introduction of lesions into the targeted S regions by activation of some enzymes such as uracil DNA glycosylase, apyrimidinic/apurinic (AP)-endonucleases, and AID (297, 298). AID initiates the class-switching process by deaminating (removing an amino group from) the cytosines in the S regions. This transforms the original C bases into deoxyuridine and permits the base to be excised by uracil glycosylase. As a result, the freshly created abasic site can be cut by AP-endonucleases, resulting in the first single-strand

breaks (SSBs) that subsequently spontaneously produce double-strand breaks (DSBs) (299). A γ, α, or ε constant region gene segment can be substituted when the intervening DNA between the S-regions is removed from the chromosome, eliminating undesirable μ or δ heavy chain constant region exons. In the last step, DNA repair machinery rejoins the severed ends, looping out a circular DNA-containing stretch between Sμ and the desired S region. Non-homologous end joining (NHEJ) is the technique used to rejoin the free ends of the DNA to connect the variable domain exon of the antibody heavy chain to the desired downstream constant domain exon (300). The specific antibody isotype produced is influenced by the microenvironment, where B cells receive signals from cytokines and receptor interactions, leading to the creation of various antibody isotypes.

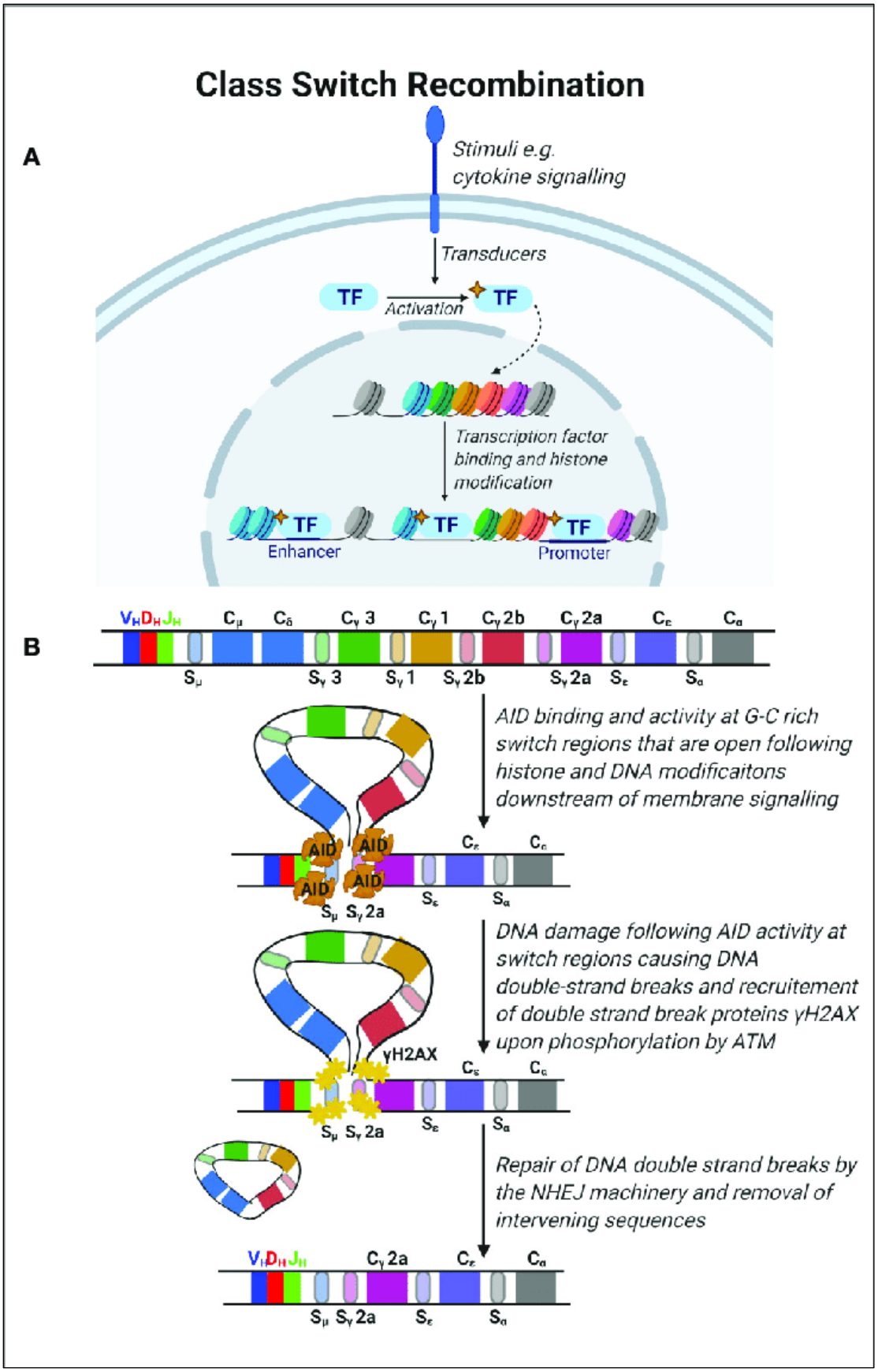

Class Switch Recombination
A
Stimuli e.g.
cytokine signalling
Transducers
TF
Activation
TF
Transcription factor
binding and histone
modification
TF
TF
TF
Enhancer
Promoter
B
VHDHJH
Cμ
Cδ
Cγ 3
Cγ 1
Cγ 2b
Cγ 2a
Cε
Cα
Sμ
Sγ 3
Sγ 1
Sγ 2b
Sγ 2a
Sε
Sα
AID binding and activity at G-C rich
switch regions that are open following
histone and DNA modificaitons
downstream of membrane signalling
AID
AID
AID
AID
DNA damage following AID activity at
switch regions causing DNA
double-strand breaks and recruitement
of double strand break proteins γH2AX
upon phosphorylation by ATM
γH2AX
Repair of DNA double strand breaks by
the NHEJ machinery and removal of
intervening sequences

Figure 1.2.4 Ig class switch recombination. (A) Cytokine signalling from the microenvironment sends signals to B cells, activating specific transcription factors that move into the nucleus. These factors then facilitate the unwrapping of tightly packed DNA at particular sites, revealing the switch regions located at the 5' end of the immunoglobulin constant (C) segments. (B) The immunoglobulin gene's constant region is made up of the variable region (VDJ) followed by the constant regions for various antibodies including IgM (Cμ), IgD (Cδ), IgG3 (Cγ3), IgG1 (Cγ1), IgE (Cε) and IgA1 (Cα). Switch areas rich in G-C surround constant regions. DNA damage at these switch areas is caused by the deaminase activity of activation-induced cytidine deaminase (AID), which in turn causes ABM to initiate the double-strand break repair response by phosphorylating H2AX and enlisting the non-homologous end joining (NHEJ) machinery. As a result, intervening sequences are eliminated, and DNA double-strand breaks are repaired.

1.2.4.5 IgE CSR

Signals originating from T cells, such as those from CD40 and IL-4Rα, stimulate the initiation of transcription in the germline of the Cε gene, responsible for encoding the heavy chain of the IgE isoform. This process results in the production of initial transcripts (εGLTs) that do not encode for functional IgE. However, these εGLTs play a crucial role in triggering IgE CSR. Additionally, T cell signals prompt the expression of the AID enzyme, necessary not only for general CSR but specifically for IgE CSR as well.

CSR to IgE is regulated by various cytokines and transcription factors. Activation of signalling by IL-4 and IL-13 upregulated IgE CSR while IFNγ, TGFβ, and IL-2 play an inhibitory role in this process (301-304). STAT6 is phosphorylated and undergoes nuclear translocation in response to signalling via the IL-4R. For the Iε promoter to be activated, phosphorylated STAT6 (pSTAT6) must bind to it. Additionally, pSTAT6 stimulates the transcription factor NFIL3, which attaches itself to the Iε promoter and enhances transcription (305, 306). pSTAT6 works in concert with other transcription factors, such as E2A, NF-κB, PU.1, Pax5, AP-1, and C/EBP, to activate germline Iε transcription. On the other hand, GC B cell-expressed BCL6, a transcriptional inhibitor involved in IgE production, blocks CSR to IgE (307). The transcription factor Id2, which is induced downstream of TGFβ signalling, also negatively regulates the Iε promoter (308). Transcription factors such as STAT6, NFκB, PAX5, and E47 have binding sites in the 5' upstream region of the AID gene. Likewise, the induction of AID is similarly subject to the synergy between STAT6 and

NFκB (309). Overall, CSR to IgE is driven by the general mechanisms of B cell activation and signalling via the IL-4/IL-13 receptor. The regulation of CSR to IgE by cytokines secreted by CD4 T cells makes IgE responses highly sensitive to the cytokine environment created by T cells.

Class switching to IgE can happen either through direct recombination between the switch (S) regions of the Cμ and Cε genes (known as Sμ and Sε, respectively) or through a sequential process where B cells that have already recombined to produce IgG subsequently switch to IgE (310). DNA recombination is used to achieve both at the DNA level, first creating a hybrid between Sε and Sμ or Sμ/γ. Then, VDJ is left next to the Cε sequence after the DNA sequence from Sμ or Sμ/γ to Sε is removed. Ultimately, the full-length, functional IgE heavy chain is produced by the ligation of the remaining Sμ and Sε (311). Sequential switching is crucial for producing high-affinity IgE, which is believed to significantly contribute to the development of IgE-mediated allergic reactions like anaphylaxis (311). To summarize, sequential switching produces IgE plasma cells and is necessary for the development of high-affinity IgE, whereas direct class switching to IgE is involved in the generation of GC IgE cells and is associated with the production of low-affinity IgE.

1.2.4.6 Outcome of GC

In order to establish productive humoral immunity, GC responses are required. This results in the significant generation of high-affinity antigen-specific antibodies of switched isotypes, which can then be induced more quickly and abundantly upon a secondary challenge of the same antigen. Because GC B cells stimulate the enzymes necessary for immunoglobulin hypermutation and class switch recombination, these cells can differ in their binding affinities to the antigen and BCR isotypes (239, 312). Since GC B cells must compete for the few antigens trapped on FDCs as well as for the opportunity to engage with homologous GC T cells in order to get survival signals, particularly CD40 ligands, antibody affinity maturation occurs within GCs (313). Low-affinity BCR-expressing GC B cells are less able to compete for antigen binding and will eventually undergo apoptosis and are removed by macrophages phagocytose (314). Class-switched plasmablasts and memory B cells must differentiate within GCs, once they have done so, they will leave GCs to become plasmablasts and memory B cells (315).

Plasmablasts migrate to the bone marrow, where they develop into long-lived, antibody-producing plasma cells. In contrast, memory B cells in the bloodstream tend to migrate back to the follicles of secondary lymphoid organs, where they are primed for quick responses if the antigen is encountered again (315, 316).

1.2.5 Role of B cell in asthma

B cells play a complex role in the pathogenesis of asthma, contributing to both the immune response and the chronic inflammation characteristic of the disease. Clinical studies demonstrated that in asthmatic patients B cells migration to the lung is increased and during an asthma attack, there is an elevated presence of B cells at various stages of activation and development in both the bloodstream and the bronchial mucosa of affected patients (317).

The primary established role of B cells in allergies involves generating IgE antibodies targeted at allergen components. Upon activation by antigens, B cells differentiate into plasma cells, which produce immunoglobulins, including IgE. In allergic asthma, IgE bind to the FcεRI receptor, found abundantly on mast cells and basophils. These cells are preloaded with IgE, so when allergen-derived antigens bind to specific IgE, it triggers FcεRI cross-linking and signal transmission. This process can lead to rapid degranulation, releasing pre-stored granules containing mediators like histamine, which underlie the characteristic symptoms of early phase of allergic asthma (318). It's crucial to remember that additional B cell-derived antibody isotypes are also significant in allergy. IgG1 and IgA are two other antibody isotypes that may be able to neutralize allergens and lessen the chance that they may bind to IgE. IgG linked to allergens can generate immune complexes that ligate inhibitory FcγRIIb receptors on B cells, basophils, and some subsets of mast cells, thereby suppressing these cells' responses (319). Thus, it is crucial to keep in mind that B cells may be essential for both triggering allergic reactions through IgE and inhibiting them through IgG and potentially IgA.

B cells not only produce antibodies but also have the ability to control immune responses by presenting antigens and producing immunosuppressive cytokines. B cells can act as potent APCs,

capturing allergens or other antigens and presenting them to T cells. It has been shown that B cells capture allergens directly, regardless of their B-cell receptor's specificity. Once sensitized, these B cells then display the captured allergens to CD4+ T cells using MHC-II molecules. Consequently, they transform into effector Th2 cells. In addition, B cells can prime Th1 and Th17 responses, and promote the recruitment of eosinophils and neutrophils to the lung, and all these interactions are crucial for initiating and amplifying the immune response in asthma (320). However, Dullaers et al showed that B cells are not crucial initially for the development of allergen-responsive T cells, but they become essential for the complete expansion of sensitized T cells upon secondary allergen exposure. They observed that the significance of B cells in HDM-induced asthma became evident when the allergen dosage was lowered, resulting in diminished asthma symptoms in mice lacking B cells. This research highlights that B cells play a significant role in the adaptive immune response to inhaled HDM allergens, especially when the allergen exposure is minimal, by promoting the proliferation of allergen-specific T cells (321). Interestingly, another study demonstrated that B cells can trigger asthma symptoms independently of T cells and with limited reliance on IgE involvement (322). B cells are also capable of secreting various cytokines that modulate the immune response. For example, B cells can produce IL-10, which has anti-inflammatory properties and can suppress immune responses. Conversely, B cells can also produce pro-inflammatory cytokines such as IL-6 and TNF-α, which contribute to airway inflammation in asthma.

Given their multifaceted roles in asthma pathogenesis, B cells represent a potential therapeutic target. Biologic therapies targeting B cell-related pathways, such as anti-IgE antibodies (e.g., omalizumab) or B cell-depleting agents (e.g., rituximab), have shown promise in certain subsets of asthma patients, particularly those with severe, refractory disease.

1.2.6 ICOS/ICOS-L signalling

ICOS is a member of the CD28 family that can be quickly induced on T cells upon T cell activation through signals from the TCR and CD28. ICOS is a 199 amino acid protein that is encoded by a gene that consists of five exons and spans 2,620 nucleotides. ICOS was initially discovered in humans and later found in mice where it is significantly similar to the immunological inhibitor CTLA-4 and the costimulatory protein CD28 (323). Inducible costimulatory factor (ICOS) is a word that fits well because it is not often produced in naïve T cells. ICOS is quickly increased on activated CD4 and CD8 T cells following TCR cross-linking and/or co-stimulation with CD28, indicating that ICOS may control adaptive T cell responses. Among various T-cell subsets, Tfh specifically relies on ICOS/ICOS-L signalling during an immunological response and following DC priming to preserve their phenotype and interact with GC B cells (324).

ICOS ligand is a cell surface protein called ICOSL (also known as B7RP-1), which is constitutively expressed on specialized APCs like B cells (where its regulation involves BAFFR and non-canonical NFκB signalling), macrophages, and DCs. However, certain endothelial cells and lung epithelium also express ICOSL (325). ICOS-L is categorized as a member of the B7 family and exhibits significant homology with CD80/CD86 (326). ICOSL is expressed in hematopoietic cells and non-hematopoietic cells following inflammation. Immunohistochemical analysis indicates high ICOS expression around tonsil germinal centers, fetal medulla, and human juvenile thymus, implying its crucial role in T/B cell maturation alongside B cells and early T cell development (326).

By binding to its ligand, ICOS activates some downstream signalling pathways. It is proven that the ICOS/ICOS-L pathway leads to the activation of several other pathways including Phosphoinositide 3-kinase (PI3K), Nuclear Factor-kappa B (NF-κB), and JAK-STAT pathway which in turn these pathways promote T-cell survival, proliferation, differentiation, and metabolism. It also regulates cytokine production and the effector function of T-cells (327, 328). The ICOS/ICOSL signalling specifically supports Th2 cell differentiation and a positive correlation was found between ICOS expression and the production of IL4 and IL5, which was accompanied by the upregulation of GATA3, a crucial factor for Th2 differentiation. To further investigate ICOS's role in Th2 differentiation, naïve CD4+ T cells isolated from BALB/c mice

were treated with anti-ICOS mAb, resulting in a significant increase in the Th2-associated cytokines IL4 and IL5 upon stimulation (329). In the absence of ICOS/ICOS-L signalling T cells, especially Tfh accumulation, maintenance, and function are impaired and GC formation and Ig production are disturbed. T cells lacking ICOS showed a reduced ability to proliferate and differentiate in vitro compared to WT CD4+ T cells. Interestingly, after immunization, lymph nodes from *ICOS*$^{-/-}$ mice were smaller than those from wild-type mice, indicating a similar proliferation defect in vivo (330, 331).

The molecular mechanisms through which ICOS influences Th2 cells and impacts allergic diseases are not well understood. Studies have shown that the enhancement of IL-4 receptor-induced Stat6 phosphorylation and primary GATA-3, both of which are necessary for Th2-mediated differentiation of Ag-stimulated initial CD4 + T cells, is how the ICOS/ICOS-L co-stimulatory pathway-mediated effects on Th2 differentiation are accomplished. Additionally, the NFAT1 and MAPK pathways are implicated in the expression of ICOS genes downstream of TCR signalling, with NFAT1 co-expressing ICOS genes and GATA-3 in Th2 cells (332, 333).

Furthermore, ICOS/ICOS-L signalling affects B cell activation and functions. When ICOS binds to ICOSL, it promotes the expression of CD40L on activated CD4+ T cells from intracellular vesicles. This interaction enhances the CD40-CD40L connection, providing a co-stimulatory signal for B cell activation. Consequently, ICOS-ICOSL and CD40-CD40L form a positive feedback loop that coordinates B cell terminal differentiation, including germinal center formation, isotype class switching, and memory B cell development and the generation of IgE-producing plasma cells by increasing the production of IL-4 and IL-10 (323). it has been shown that *ICOS*$^{-/-}$ mice demonstrated very small GC follicles and a remarkable reduction in Tfh cells (334). In the absence of ICOS/ICOS-L, primary GC formation was delayed and less frequent compared to normal situations. However, after a recall challenge, *ICOS*$^{-/-}$ mice were unable to form GC. Moreover, Ig production was impaired. *ICOS*$^{-/-}$ mice demonstrated early deficiency in Ag-specific Ab, CSR, and produced Ab lacked affinity maturation, clearly indicating the importance of ICOS/ICOS-L pathway in GC responses and Ab production (335, 336).

1.2.6.1 ICOS/ICOS-L role in asthma

Recently, it has been noted that ICOS can be expressed in a few of the immune cells that have been linked to the development of allergic airway disease and asthma, such as Tregs, iNKT cells, Tfh cells, and ILC2s (324, 337-339). A series of cellular and animal studies have been conducted to investigate allergic airway inflammation by focusing on the ICOS costimulatory pathway. However, these studies have produced conflicting outcomes: some show notable improvement in the disease and others reveal a strong increase in lung inflammation.

ICOS/ICOSL-mediated signalling is crucial for effector Th2 cells in managing allergic inflammation. In several allergic airway disease models, inhibiting ICOS/ICOSL signalling notably decreased airway inflammation and suppressed effector Th2 cytokine production such as IL-4, and IL-5. OVA-sensitized ICOS-L deficient mice showed a significant reduction in Th2 cells with decreased Th2 cytokines such as IL4 in comparison to wild-type mice. It makes sense that IL4 is necessary for the synthesis of particular IgE, which lessens IgE crosslinking with mast cells and eosinophils and may eventually stop OVA-induced AHR. Additionally, in an animal model of asthma, the function of ICOS in the polarization toward Th2 responses was noted. OVA-induced *ICOS*$^{-/-}$ mice's spleens contained CD4 + T cells that produced more Th1 cytokines (IFN-γ) and less Th2 cytokines than the wild-type control (340).

It has been revealed that the introduction of ICOS-enriched T cells, followed by an allergen airway challenge, caused recipient T and B cells to infiltrate, and intrapulmonary plasma cells to produce allergen-specific IgE locally. Transferring the T-cell fraction depleted of ICOS led to the recruitment of a notably smaller number of B cells that did not produce IgE locally. According to these findings, ICOS expression characterizes a subset of T effector cells necessary for local IgE synthesis and B cell infiltration in lung tissue (341). Another study observed that upon the onset of allergic airway disease (AAD), blocking the ICOS/ICOS-L pathway leads to a depletion of Tfh cells and a significant reduction in germinal centers. This blockage diminishes the germinal center response, allergen-specific IgE production, inflammatory response, intrapulmonary IL-13 production, and AHR (342). Additionally, the inhibitory effect of treatment with ICOSL antibodies on airway resistance and elasticity as well as increased lung compliance is well established (342). Interestingly, ICOS/ICOS-L interaction mediates mucosal tolerance, as studies have indicated that

pulmonary DCs in the bronchial lymph nodes of mice exposed to respiratory allergen-induced costimulation of T reg cells through the ICOS/ICOS-L pathway (343).

Overall, understanding the importance of ICOS/ICOS-L in allergic asthma sheds light on potential therapeutic strategies aimed at modulating immune responses to alleviate symptoms and improve outcomes for individuals with this condition.

3. Semaphorins

1.3.1 General overview

Semaphorins are a family of membrane-bound and secreted proteins that can act in the paracrine or autocrine manner and play a critical regulatory role in cell migration, proliferation, differentiation, trafficking, and angiogenesis (344-347). Following the identification of the first semaphorin in 1992-1993, the semaphorin family was discovered through the homology of an extracellular semaphoring (Sema) domain (348, 349). At first, these proteins were identified as repulsive axon guidance molecules that guided neuronal axons to the proper targets (350). However, emerging evidence suggested that semaphorins can be ubiquitously expressed beyond the nervous system by the endocrine, musculoskeletal, cardiovascular, gastrointestinal, immune, and respiratory systems (351). Thus far, more than 20 types of semaphorin proteins have been identified (352) and they play a critical role in physiological conditions including angiogenesis (353), vasculogenesis (354), cardiogenesis (355), osteoclastogenesis (356), and immune regulation (357, 358). Moreover, semaphorins are linked to the development of several pathological conditions, such as immunological disorders (359), neurological diseases (360), tumorigenesis/metastasis (361, 362), and sudden death (363).

Based on sequence similarities and unique structural characteristics, semaphorins are divided into 8 various classes (352, 364). Class 1 and 2 are expressed in invertebrates, class 3-7 are found in

vertebrates, and class 8 of semaphorin is restricted to some DNA viruses (345, 352, 365). In another system based on their localization site, semaphorins are classified into membrane-bound proteins including semaphorin 1, 4, 5, and 6, secreted proteins such as semaphorins 2, 3, and 8, and glycosyl phosphatidyl- inositol (GPI)-linked protein (Sema 7) (365, 366).

1.3.2 Semaphorins structure

The common feature of all semaphorin proteins is an extracellular N-terminal seven-blade beta-propeller fold structure called "Sema domain" which consists of ~500 amino acids and is responsible for homodimerization and protein-protein interaction with receptor(Figure 1.3.1) (350, 367).

In the extracellular region of semaphorin proteins, the Sema domain is tightly followed by a cysteine-rich domain called plexin semaphorin integrin (PSI). Depending on the presence of other domains like immunoglobulin (Ig-like domain) (in class 2, 3, 4, and 7 semaphorins), thrombospondin (class 5 semaphorin), or GPI linking sites (class 7 semaphorin), semaphorin proteins can range in size from 400 to 1000 amino acids (368-370).

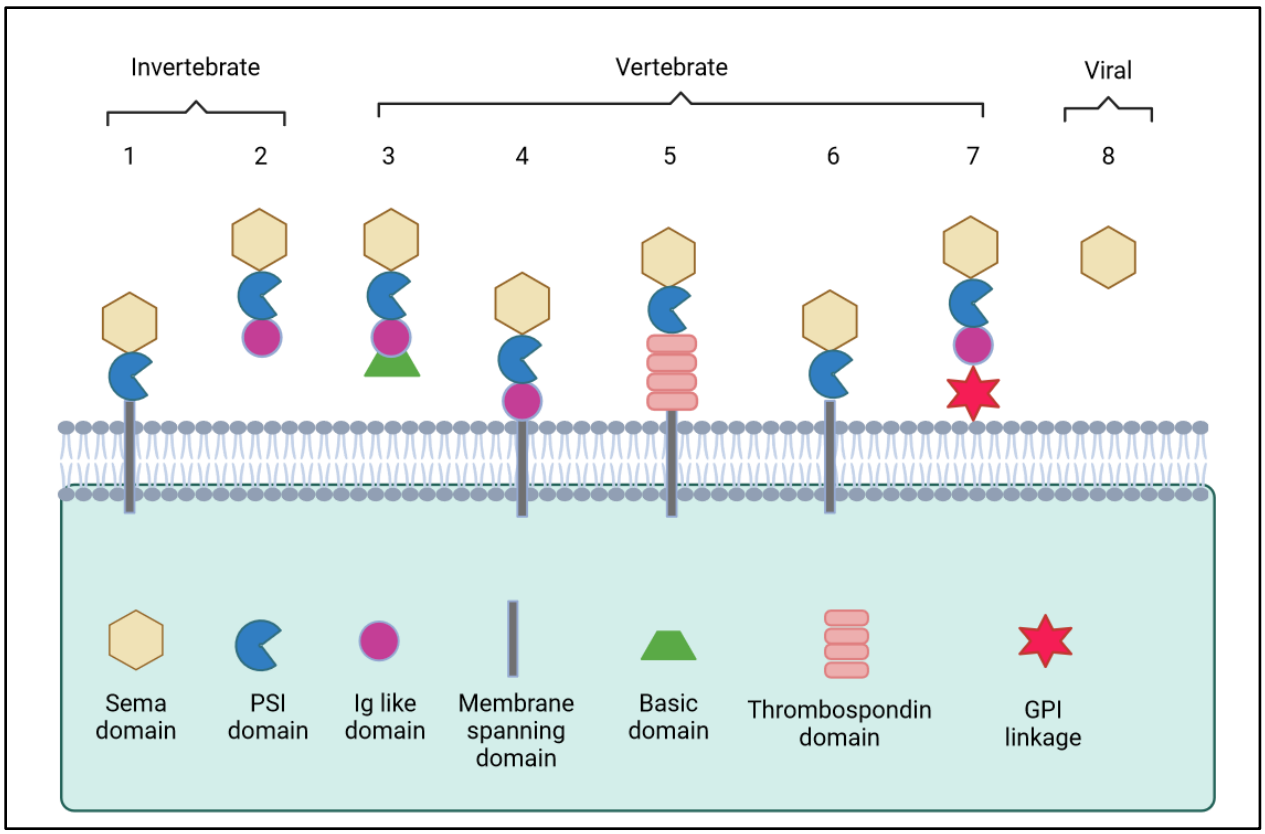


Figure 1.3.1 The structure of the Semaphorin proteins. Class 1 and 2 of semaphorins are found in invertebrates, and classes 3-7 are found in vertebrates however, class 8 of semaphoring is only limited to some viruses. All semaphorins share the same Sema domain and PSI domain (except viral semaphorin). Some semaphorins have Ig-like domains and semaphorins 3, 5, and 7 are distinguished from others due to additional domains (basic domain, thrombospondin domain, and GPI linkage respectively).

1.3.3 Semaphorin receptor

To play their regulatory roles, semaphorins bind to two main receptors; plexins and neuropilins (Figure 1.3.2) (371, 372). The majority of semaphorins are directly coupled with plexin protein as their receptor. However, class 3 semaphorin (except Sema3E) requires neuropilin coreceptors to active cellular signalling pathways (373-375). On the other hand, recent data indicate that semaphorin receptor binding may be more complex and other proteins are involved. For example, Sema7A functions in the immune and neurological systems via integrin receptor (376, 377). Furthermore, CD7229 and T-cell immunoglobulin and mucin domain-containing protein 2 (TIM-

2), two molecules unrelated to plexins and neuropilins, functionally interact with Sema4D and Sema4A, respectively, in the immune system (378, 379). Additionally, it merits attention that some other proteins including tyrosine kinase, GTPase, G-coupled protein, and other adhesion molecules are involved in semaphorin signalling activity (351) and these multiple protein interactions can lead to numerous signal transductions.

1.3.3.1 Plexins

Plexins are a family of large proteins (~200 kDa) that are the main canonical receptor for the majority of semaphorins (374, 375). Similar to semaphorin, plexin proteins have two forms; membrane-bound and soluble (373) and based on structural similarity, they are divided into 4 different classes: A, B, C, and D (380). There are two plexins in the invertebrate: plexin-A and plexin-B and four classes of plexins are found in the vertebrate: class B (Plexin-B1, B2, B3), class C (C1), class D (D1), and class A (Plexin-A1, A2, A3, A4).

Plexins exhibit structural similarities to tyrosine kinases receptor, MET, and RON, which are significant in development, tissue regeneration, and cancer (381).

Plexins are structurally homologous to semaphorins, and they consist of extracellular and cytoplasmic (intracellular) portions (Figure 1.3.2). A Sema domain, three plexin-semaphorin-integrin (PSI) domains, and three immunoglobulin, plexin, and transcription factors (IPT) domains form the extracellular part of plexin molecules (375, 382, 383).

Each extracellular domain of plexins plays an important role. The N-terminal sema domain is responsible for ligand binding and activating signalling pathways. Moreover, the sema domain functions as an autoinhibitory factor that limits the activation of plexin molecules (381, 382, 384). However, the sema domain found in plexins does not engage in dimerization, in contrast to the sema domain of semaphorins (385).

The PSI domain which is also known as a small cystine-rich domain (CRD) or MET-related sequence, plays a crucial role in facilitating precise protein-protein interactions (386). The IPT domains, also known as Ig domains or transcription factor Ig domains (TIG), play a crucial role in ligand binding (387). Research involving the blockade of the IPT domain in the β2 integrin has

indicated its importance in facilitating the dimerization of the αβ subunits (388). Additionally, investigations into MET proteins lacking the sema domain have demonstrated that the IPT domains alone are capable of facilitating ligand binding (389).

Despite lacking kinase activity, the cytoplasmic region of plexin molecules is crucial for signal transduction following ligand binding (390). A segmented GTPase-activating protein (GAP) domain is featured in cytoplasmic part of plexins and they play a crucial regulatory role upon activation (380). R-Ras/M-Ras GAP domain of plexins is separated into two segments by a Ras Binding Domain (RBD) (Figure 1.2) (391, 392). Across all plexin classes, the intracellular domain is strongly conserved (393). Furthermore, aside from the domains that present in all plexin molecules, plexin-B1 and B2 additionally feature sites susceptible to convertase cleavage (375, 394).

Semaphorins interact with plexins in a specific manner. Different plexins have specificity in binding to various semaphorins. For example, Plexin A receptors are activated by class 5 and class 6 semaphorins. Plexin B is activated by class 4 and class 5 semaphorins, while class 7A semaphorins binds to plexin C1. Some semaphorins from class 3 semaphorins bind to plexinD1 in a neuropilin-dependent manner, whereas semaphorin3E and semaphorin4A can bind to plexinD1 independently of neuropilins (369, 395).

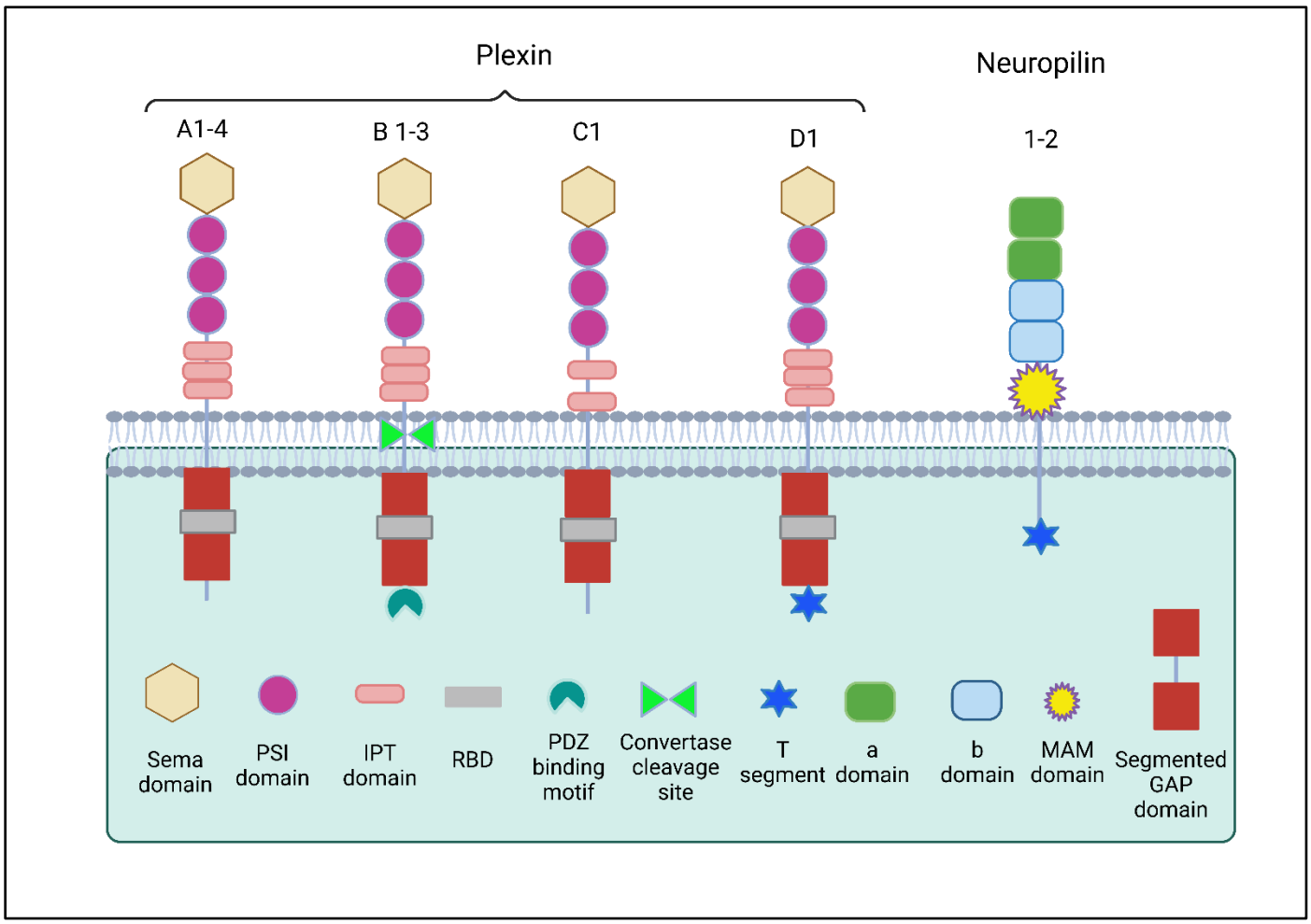


Figure 1.3.2 The structure of the Plexins and Neuropilin. Plexins are characterized by an N-terminal Sema domain, along with a mixture of PSI domains and IPTs in their extracellular sections, and based on their origin and structural similarity, they are categorized into four distinct groups, A–D. All members of the plexin family are membrane-bound, however, plexins in Group B, have the capability to be secreted. Neuropilins are coreceptors that class 3 semaphorin (except Sema3E) need them to become fully activated. Neuropilins are characterized by a, b, and MAM domains in their extracellular domain.

PSI, plexins, semaphorins and integrins; IPT, Ig domains shared by plexins and transcription factors; RBD, Rho GTPase-binding domain; MAM, Meprin, A5, and Mu-phosphatase.

1.3.3.2 Neuropilins

Some semaphorins require a coreceptor beside plexins to fully activate signalling pathways. This coreceptor is a 900 amino acids transmembrane protein called Neuropilin (Figure 1.3.2) (396). There are two main types of neuropilins: neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2) and contain a large extracellular and a short intracellular domain. Two CUB (complement C1r/C1s) a1 and a2 domains, two coagulation factor V/VIII homology b1 and b2 domain, and a MAM (meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu) domain, also know as c domain, are found in the extracellular portion of neuropilins. The a and b domains are responsible for binding to semaphorin class 3 and vascular endothelial growth factor (VEGF) respectively and the MAM domain is involved in the dimerization of neuropilins (369, 396, 397). Due to their short intracellular domains, neuropilins lack inherent signalling abilities. Instead, they team up with other cell surface molecules to facilitate signal transmission as co-receptor complexes (380). Notably, except for Sema3E, class 3 semaphorins rely on neuropilin co-receptors for proper functioning (354, 398).

1.3.4 Semaphorins downstream signalling

The ability of semaphorin and plexin to function as repulsive or attractive cues is more complex than initially presumed and it depends on the receptor-ligand combination, the organism's developmental stage, cell type, and/or the cellular context (399-401). Modulating cytoskeletal structure via reorganization of microtubule network and actin filaments is a key function of semaphorin-plexin signalling, which is partly accomplished by controlling the activity of small GTPases from the Rho-family and RAS (362).

The exact mechanism by which semaphorin triggers downstream signalling through binding to plexin is not clear. Nonetheless, it is believed that semaphorin-plexin binding leads to a structural alteration in the cytoplasmic segments of the inactive plexin and shifts it to a functional state (372). The plexin GAP domain assumes a closed conformation and is unable to accept R-Ras or M-Ras

at its substrate-binding site when semaphorin ligation is not present (402). The presence and binding of semaphorin facilitate the recruitment and activation of Guanine Nucleotide Exchange Factors (GEFs) proteins that are involved in promoting the exchange of inactive guanosine diphosphate (GDP) for an active guanosine triphosphate (GTP) on small GTPases. This activation triggers a cascade of downstream signalling pathways involved in cell migration, angiogenesis, and proliferation processes (362, 372, 403). An RBD divides the GAP domains found in the cytoplasmic portion of plexins into two segments. Ras-related proteins (RAP), which have been demonstrated to be essential for cell division, migration, adhesion, and cell-cell junctions, are directly interacting with the plexin GAP domains (404, 405).

The pleiotropic function of semaphorins is not limited to the GTPase signalling pathway and semaphorin-plexin binding leads to the activation of another downstream signalling such as PI3K, MAPK, and STAT signalling (372, 406). Moreover, phosphorylation of tyrosine residues within the plexin cytoplasmic domain after ligand-receptor interaction led semaphorins to encompass kinase activity (407). Emerging evidence revealed that the cytoplasmic segment of plexin also can connect with the Molecule Interacting with CasL (MICAL) family of cytosolic flavoprotein monooxygenases enzymes, recognized for their involvement in reduction-oxidation (redox) enzymatic processes (408). A recent discovery indicates that MICAL controls actin filament (F-actin) dynamics precisely by directly interacting with semaphorin signalling. Through its redox activity, the MICAL protein directly binds F-actin and disassembles actin filaments that are bundled together as well as individually (409).

Together, these findings suggest that signals from semaphorin–plexin binding play a role in controlling cell morphology through the breakdown of F-actin, actomyosin contraction, microtubule instability, and integrin-mediated adhesion.

1.3.5 Semaphorins functions in the immune system

The initial discoveries suggested semaphorins as a repulsive axonal guidance protein in the nervous system. Further studies completely changed previous understandings by demonstrating semaphorins as an attractive guidance cue. Nowadays semaphorins are recognized as bidirectional proteins that, depending on the expression of their receptors and co-receptors and the type of responding cells, can generate either an attracting or repulsive signal in a context-dependent way and they are involved in regulating immune hemostasis and pathological conditions (359, 410). Several semaphorins have been found to play a role in immune cell trafficking in the primary and secondary lymphoid structures, cell growth, apoptosis, and cytokine production (378, 411-413). For example, it has been shown that Sema3E is involved in thymocyte development through the plexinD1 signalling pathway (346).

Sema4D, also referred to as CD100, was the first member of the semaphorin family identified to play an immunoregulatory roles (414, 415). Sema4D is expressed by mature DCs, T cells, and activated B cells and stimulates DC and B cell activation leading to the generation of antibodies and antigen-specific T cell responses (378, 411). The absence of Sema4D influences the production of Th2 cytokines and the activation of T cells, resulting in reduced allergic inflammation in a mouse model of asthma (416). Following antigen immunization, $CD4^+$ T cells derived from the draining lymph nodes of *Sema4D*$^{-/-}$ mice exhibit significantly diminished proliferative responses and cytokine secretion upon antigen restimulation clearly showing the importance of this protein in the regulation of immune responses (411, 417). Sema4A is constitutively expressed in DCs and T cells and plays a crucial role in antigen-specific T cell priming and promoting T helper cell differentiation towards the Th1 phenotype (418).

Sema4B expression is observed in both T cells and B cells, where it exerts a negative regulatory effect on basophil function through the facilitation of T cell-basophil interactions (410). A previous study demonstrated that even though lymphocyte and DC activities are normal, serum IgE concentrations are significantly higher in *Sema4B* $^{-/-}$ mice. Sema4B is produced from T cells and decreases basophil-mediated Th2 skewing in addition to inhibiting basophils' IL-4 production. Moreover, basophil-mediated memory IgE production is increased in *Sema4B*$^{-/-}$ mice (410). Taken

together, humoral memory responses and basophil-mediated Th2 are negatively regulated by Sema4B.

In the immune system, activated T cells express Sema 7A and this protein triggers macrophages to produce proinflammatory cytokines through the α1β1 integrin (376) indicating a vital role for Sema 7A in the effector phase of the inflammatory immune responses. It has been shown that When an asthmatic patient is exposed to allergens, eosinophils in bronchoalveolar lavage specimens express more Sema7A (419). Recombinant Sema7A stimulates human bronchial fibroblasts to produce alpha-smooth muscle actin in vitro, indicating that Sema7A may regulate eosinophil profibrotic activities in asthmatic airway remodelling (419). Sema7A has the potential to be a therapeutic target for severe allergic asthma with eosinophilic and neutrophilic airway inflammation. An anti-Sema7A antibody decreased AHR and neutrophilic inflammation in a murine model (420).

1.3.6 Semaphorins' role in B cell and GC responses

The regulation of GC formation and IgE production involves a complex interplay of various factors within the immune system and several key elements contribute to their regulation (421). While the role of some factors has been widely studied, others are less known. Accumulating evidence suggests that several semaphorins are involved in B cell -T cell interaction, GC formation, and antibody production.

The functional role of Sema4D in B cells has been validated in both human and mouse models. Initial investigations into human Sema4D revealed its capacity to stimulate B-cell differentiation and aggregation (422). Studies using *Sema4D*$^{-/-}$ mice have further illustrated the significance of Sema4D in B-cell biology. Subsequent research in mice confirmed human findings and demonstrated that although B cells have low basal expression of Sema4D, over-expression of this protein following CD40 or Lipopolysaccharides (LPS) induction augmented B cell proliferation and antibody production (423). It was demonstrated that, by reducing CD72 inhibitory signals and enabling subsequent BCR signalling, sema4D controls B cell immunological responses (378, 424).

Sema4D expression has been observed on germinal center B cells (422). Interestingly, studies have indicated that Sema4D deficient mice, when immunized with T cell-dependent antigens, exhibit impaired antibody affinity maturation and reduced generation of antigen-specific germinal center B cells (411). Hence, interactions between Sema4D and CD72 could potentially promote the proliferation of germinal center B cells through B-cell interactions and facilitate the survival signals necessary for the selection of high-affinity B cells by enhancing signals from helper T cells (357). In addition, the absence of Sema 4D changed B cell hemostasis and led to lower B-1 B cell populations at younger ages but higher marginal zone B cell counts at older ages (423). Furthermore, *Sema4D*$^{-/-}$ mice also exhibited impaired antibody production and antigen-specific T-cell priming (411, 417).

Sema 4C is another member of the semaphorins family that plays a crucial role in the maturation of B cell follicles in secondary lymphoid structures (425). Both human and murine B cells express Sema 4C. Sema 4C exhibited one of the highest expression levels after B-cell activation, standing out by its notably elevated expression compared to other Semaphorin family members. *Sema4C*$^{-/-}$ mice showed reduced B-cell follicle maturation in the spleen, resulting in reduced populations of both follicular and marginal zone B-cells, alongside compromised production of IgG and IgA antibodies. Moreover, Sema 4C plays a role in B cell polarization because in *Sema 4C*$^{-/-}$ mice B cell polarization was impaired (425). Sema 4C is essential for plasma cells (CD138^{+} B cells) to produce optimal regulatory cytokines. Intriguingly, CD19^{+}CD138 cells lacking Sema4C show a clear divergence from regulatory cytokine-producing cells to proinflammatory cytokine-producing cells (426, 427).

Yan H, et al observed that Sema4C expression can be elevated on Tfh cells, and its receptor, plexin B2 (PlxnB2) is abundantly expressed by GC B cells (428). They demonstrated that in order to facilitate GC access, Sema4C acts as a receptor on TFH cells, detecting the presence of PlxnB2 cues on GC cells, thereby guiding TFH migration inward toward the GC periphery. The absence of either PlxnB2 in the GC or Sema4C on Tfh cells leads to Tfh accumulation along the GC boundary, disrupting T-B cell interactions within the GC, and resulting in compromised plasma cell generation and affinity maturation. Consequently, Sema4C and PlxnB2 play pivotal roles in regulating both TFH recruitment and function within the GC, thereby optimizing antibody responses (428). Thus, although PlxnB2 expression on B cells is dispensable for GC formation, it

is crucial for normal plasma cell development and the refinement of antibody affinity. PlxnB2's role is indispensable in ensuring proper TFH recruitment into the GC.

Another study indicated that plexin-D1, a novel molecule belonging to the plexin family and the receptor for Sema3E, regulates the GC reaction and is necessary for secondary humoral immune responses (429). They observed that Plexin-D1 was not necessary for B cell maturation, B1/B2 development, dark and light zone formation, marginal zone precursor development, or the initial extrafollicular response. However, upon B cell activation, Plexin-D1 expression was upregulated, and during T-dependent immunological activation, *PlxnD1*$^{-/-}$ mice displayed impaired GC responses. Moreover, *PlxnD1*$^{-/-}$ B cells exhibited impaired migration towards GC and they showed reduced levels of long-lived bone marrow plasmacytes and recall humoral memory responses, accompanied by diminished production of IgG1 and IgG2b serum antibodies (429). These findings unveil a novel role for immune plexins in regulating the GC responses and immunologic memory generation.

4. Semaphorin 3E

1.4.1 General overview

Sema3E, a semaphorin secreted by vertebrates, is produced by various cell types including structural cells such as epithelial cells, endothelial cells, fibroblasts, and diverse immune cells like DCs, and macrophages (430).

The human *sema3e* gene first was sequenced by Christensen et al in 1998 in the tumor cell line (431). The *sema3e* gene spans base pairs 83,363,905 to 83,649,162 in humans and is located at 21.11 (7q21.11) position on the long (q) arm of chromosome 7 (431). Interestingly, Nagase and his team showed that the human *sema3e* variant exhibits an 87.4% similarity to the mouse *Sema3e* (432). Based on sequence analysis, the *sema3e* gene encodes a 775 amino acids protein (89.2 kDa) recognized as Sema3E protein with significant structural similarity to other semaphorin and

collapsin protein families (431). In general, the Sema3E protein consists of four main segments: a Sema domain, a PSI domain, an Ig-like C2 type domain, and a positively charged C terminus (basic domain) (Figure 1.4.1) (433). The Sema domain is an N-terminal domain characterized by its abundance of cysteine and its ability to create disulfide bonds within the same subunit. These bonds play a crucial role in enhancing the stability of the Sema3E structure. This domain, which is conserved across all semaphorins proteins, is recognized as the receptor-binding domain and by binding to its specific receptor, plexinD1, the sema domain activates downstream signalling pathways and serves as an essential element for the functioning of Sema3E (433). The PSI domain, located between the Sema domain and the Ig-like C2 type domain acts as a connector bridging the Sema domain and the Ig-like C2 type domain, ensuring proper alignment of the ligand-binding site with the receptor (434). Additionally, the Ig-like C2 type domain and the small basic domain are rich in positively charged amino acids, which play a crucial role in enhancing the effects of the Sema domain (433).

Sema3E has two different forms: isoform-1, known as p87-Sema3E, represents the standard form, while the proteolytic fragment p61-Sema3E, weighing 61 kDa, is considered the shortened version (435, 436). The canonical isoform, p87-Sema3E, is susceptible to a proteolytic process mediated by a furin enzyme known as PACE (paired basic amino acid cleaving enzyme), which belongs to the subtilisin-like pro-protein convertase family (435). . This process leads to the generation of truncated isoforms, namely p61-Sema3E and p25-Sema3E (25 kDa) (436). However, the proper function and the inhibitory effects on growth exhibited by class 3 semaphorins rely on the presence and activity of complete dimeric forms (437, 438). Indeed, extracellular cleavage of full-length Sema3E to a shortened isoform (p61-Sema3E) converts Sema3E role from a repelling cue into an invasive pro-metastatic factor, as demonstrated by research on human cancer cell lines and experimental lung metastasis assay (439).

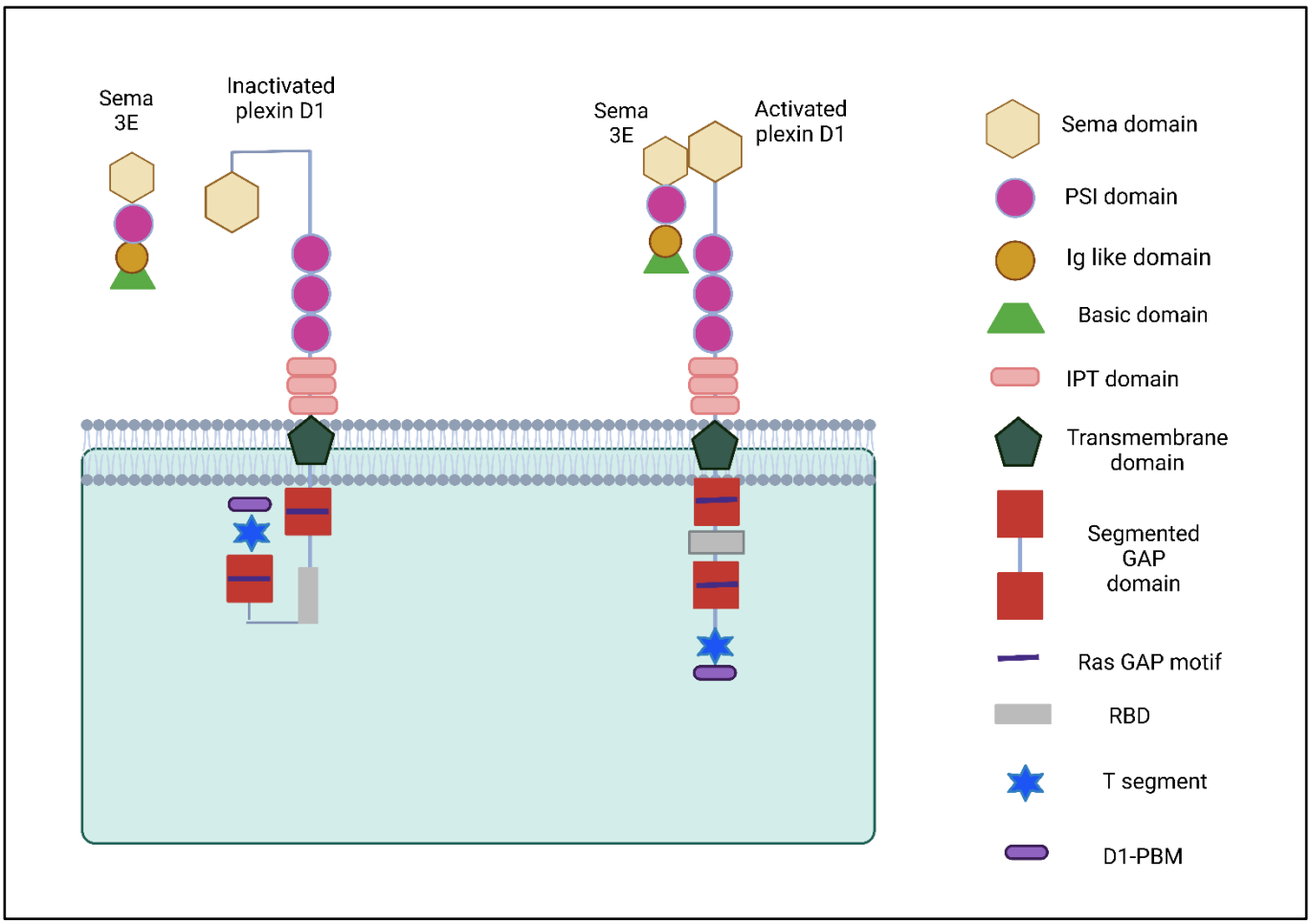


Figure 1.4.1 The structure of the Sema3E and PlexinD1. A full-length Sema3E contains 4 domains: sema domain, PSI domain, Ig-like domain, and basic domain. Sema3E receptor, PlexinD1 structure is different in inactive vs active state. In general, plexinD1 contains a Sema domain, three PSI domains, and three IPT domains in the extracellular portion. The transmembrane domain acts as a connector between the extracellular and intracellular parts of plexinD1 and the intracellular part consists of the segmented GAP domain, RBD, Ras GAP motif, T segment, and D1-PBM.

PSI, plexins, semaphorins, and integrins; IPT, Ig domains shared by plexins and transcription factors; RBD, Rho GTPase-binding domain.

1.4.2 Semaphorin 3E receptor

Unlike other semaphorin class 3 proteins, Sema3E exclusively binds to plexinD1 protein (354, 440). Other Semaphorin 3 proteins are dependent on the plexin-neuropilin complex while the binding between Sema3E and plexinD1 occurs independent of neuropilin (Nrp1), leading to plexinD1-mediated repulsion of endothelial cells (354).

Among the four classes of plexins, plexinD1 is thought to have the most structural diversity. At first, plexin-D1 was recognized for its crucial involvement in the development of blood vessels (441). It is expressed by a wide range of embryonic tissues as well as different tissues and cells after development, such as the cardiovascular system, lung mesenchyme, neurons, osteoblastic cells and bone tissues, endothelium, muscle of the small intestine, fat cells, airway smooth muscle cells, activated B cells, dendritic cells, neutrophils, and macrophages (369, 436, 442-445).

PlexinD1 is a transmembrane protein that consists of extracellular and intracellular (cytoplasmic) portions. The mature form of plexinD1 comprises 1879 amino acids, weighing 208 kDa (446). Its extracellular segment contains a sema domain which is essential for binding with Sema3E, initiating a cascade of signalling processes within the target cell (354, 447). Although Sema3E binds exclusively to the sema domain of plexinD1, the receptor lacks specificity and can also be activated by Sema3A or Sema3C, potentially in conjunction with or without Nrp1 association and varying between cell types (441, 448, 449). The sema domain is followed by three PSI repeats, four IPT domains, and a transmembrane (TM) domain (436).

The intracellular section of PlexinD1 comprises around 630 amino acids and includes a domain known as "Sex and Plexins" SP domain, comprising two closely preserved regions named C1 and C2 regions, collectively referred to as the RasGAP domain due to its resemblance to GAPs and its specificity for the R-Ras family of small GTPases.

The cytoplasmic segment of plexinD1, known as the Sex and Plexins (SP) domain with approximately 630 amino acids, features a GAP domain. This GAP domain comprises two closely preserved sections, termed C1 and C2 which are separated by an RBD domain. The C1 and C2 regions, respectively, contain Ras GAP motif 1 (RasGAP1) and Ras GAP motif 2 (RasGAP2) which encompass conserved arginine residues crucial for inhibiting R-Ras protein activity.

Collectively, this part of Plexin is referred as the RasGAP domain due to its resemblance to GAPs and its specificity for the R-Ras family of small GTPases (373, 450). Remarkably, Plexins stand out as the sole transmembrane receptors that directly interact with small GTPases (451, 452). Following the GAP domain, the C-terminal of plexinD1 harbors a terminal segment (T-segment) connected to a brief PDZ binding motif (D1-PBM) that exhibits high conservation among members of the same plexins subfamily (Figure 1.4.1) (436). This section is thought to be a viable candidate region that could provide each plexin its signalling specialization because it has no resemblance to recognized protein domains. In PlexinD1, comparative analysis using BLAST reveals that T-segments across mammals, birds, amphibians, and fish exhibit "94–100% identity," yet they display minimal similarity with T-segments of other plexin family members within the same organism (436, 453).

1.4.3 Semaphorin 3E/PlexinD1 signalling

There are two states for plexinD1: inactive and active. When plexinD1 is not bound by a ligand, it remains in an inactive state where the intracellular C1 and C2 portions of the GAP domain are wrapped around each other, and the sema domain is folded to the remainder of the extracellular domain. Sema3E, a known ligand of PlexinD1, triggers a conformational change in PlexinD1 upon binding to its sema domain, leading to the activation of the GAP domain (Figure 1.4.2) (436).

In general, Sema3E/plexinD1 signalling alters actin filaments and the microtubule structure, thus impacting cytoskeleton compartments (362). Sema3E/plexinD1 signalling is regulated by molecular mechanisms such as the regulation of actin by Ras homologs (e.g., Rac-1), or the modulation of integrin-mediated cell adhesion by Rat sarcoma (Ras) proteins (e.g., RRas). When plexinD1 is not in its active state, Rnd2, which is bound to GTP, interacts with RBD, blocking the active GTP-bound Ras and Rac from binding. Consequently, the active GTP-bound Rac can stimulate PAK (p21-activated kinase) to promote the formation of F-actin filaments, while the active GTP-bound Ras triggers integrin-mediated adhesion to the extracellular matrix (ECM), facilitating cell migration (Figure 1.4.2).

The interaction between Sema3E and plexinD1 triggers a structural alteration causing the GAP domain to bind to an activated form of R-Ras, and the RBD to bind to an active form of Rac GTPase which breaks the inhibitory link between the C1 and C2 areas and activates the GAP. Additionally, this interaction leads to the sequestration of Rac and Ras, resulting in the deactivation of integrin-dependent cell adhesion to the ECM, the conversion of GTP to GDP, and the downregulation of MAPK and PI3K signalling pathways which are critical pathways for cell survival, proliferation, and migration (Figure 1.4.2) (347, 436, 454).

It is crucial to note that, even while the plexin family is phosphorylated at cytosolic tyrosine residues in vivo, which is a necessary process controlling multiple cellular processes, they do not exhibit endogenous kinase activity (373). All plexins that undergo phosphorylation have conserved tyrosine residues inside the cell. Nonetheless, PlexinD1 (SP domain) and others (RBD) have distinct localizations for them. Although there is no experimental support for this theory, VEGFR2, a tyrosine kinase receptor, has been proposed to phosphorylate PlexinD1 in order to alter its activity (436). In a lung cancer model, the furin cleavage product p61 causes the formation of a PlexinD1-ErbB2 complex in which both proteins are phosphorylated on their tyrosine residues, and PlexinD1 may also get phosphorylated due to EebB2's kinase activity (361). Overall, the exact mechanism of Sema3E-PlexinD1 signalling remains unclear, and the final functional result is likely context- and cell-dependent. Therefore, in each cell/disease model, a thorough investigation of co-receptor ligation, intracellular conformational modifications on receptor domains, and post-translational proteolytic processing is necessary.

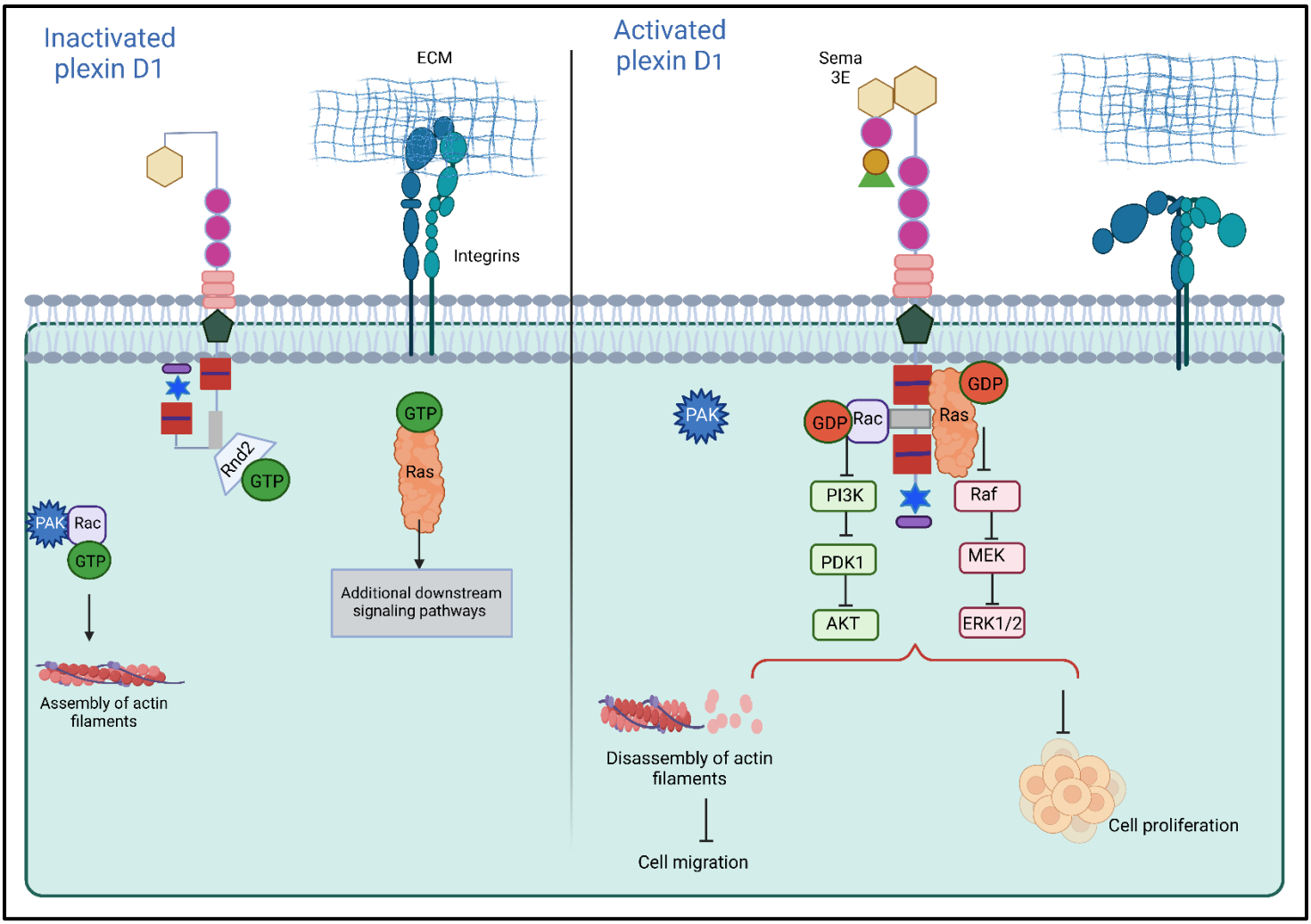


Figure 1.4.2 The Sema3E-plexinD1 signalling pathway. In the absence of Sema3E, the extracellular and cytoplasmic portions of plexinD1 are folded and Rac and Ras are blocked to bind to the plexinD1. Following Sema3E binding conformational changes occur in the plexinD1 structure allowing the active forms of Rac and R-Ras small GTPases to access PlexinD1's intracellular GAP domains and deactivating the PAK, AKT, and ERK1/2 signalling pathways. As a result, F-actin disintegration and restricted cell movement are seen. Conversely, the deactivation of AKT and ERK1/2 may control cell cycle signalling and prevent mitosis and cell division.

1.4.4 Semaphorin 3E/PlexinD1 functions

The Sema3E/plexinD1 axis is significantly involved in various aspects such as nervous system formation, embryo development, tumor suppression, angiogenesis and vasculature, thymocyte maturation, immunological pathology, and cancer metastasis (346, 354, 361, 436, 455, 456).

Sema3E and its receptor plexinD1 are crucial molecules involved in organizing neuronal circuits by guiding axons during the nervous system's development in embryos. The Sema3E/plexinD1 axis can play a dual role as chemorepellent and chemoattractant during nervous system development. Specifically, when the dimeric form of Sema3E binds with plexinD1 without neuropilin, it stops axon growth cone migration. However, when the monomeric form of Sema3E binds with plexinD1 in the presence of neuropilin, it attracts axon growth cones, promoting migration. Research indicates that plexinD1's repulsion activity prevents synapse formation necessary for sensory-motor connectivity. Furthermore, Sema3E/plexinD1 is crucial for hippocampal development at various stages from embryonic to adult, and the absence of plexinD1/Sema3E leads to abnormal hippocampal formation (436, 455).

Through controlling endothelial cell positioning and cardio-vasculature patterning, Sema3E/plexinD1 functions as a crucial regulator of angiogenesis (354, 457). Its absence promotes the formation of new blood vessels because the Sema3E/plexinD1 signalling pathway suppresses VEGF-induced angiogenesis by triggering the decoy receptor VEGFR1, which competes with VEGFR2 for VEGF binding. Consequently, VEGF is sequestered by VEGFR-1, preventing its interaction with VEGFR2 and thus inhibiting endothelial growth mediated by VEGF (458). Furthermore, the Sema3E/plexinD1 axis impedes endothelial cell migration and proliferation by disrupting actin filament assembly and affecting integrin binding to the ECM. Moreover, the Sema3E/plexinD1 axis impedes endothelial cell migration and proliferation by disrupting actin filament assembly and affecting integrin binding to the extracellular matrix. This disruption ultimately inhibits endothelial cell migration and proliferation, thereby suppressing angiogenesis (459). Additionally, the Sema3E/plexinD1 axis inhibits the development of extraretinal vessels by suppressing VEGF-induced blood vessel growth (457).

PlexinD1's role in tumor development and cancer progression appears to be like a two-edged sword, with reports indicating both tumor-promoting and tumor-suppressing effects depending on

the type of cancer, its location, and the specific interaction between plexinD1 and Sema3E isoforms. While the short proteolytic fragment Sema3E (P61-Sema3E) is pro-metastatic, the full-length Sema3E (P87-Sema3E) is not. Tumour metastatic progression is favorably linked with plexinD1 expression (456). In human colon cancer, both plexinD1 and Sema3E levels escalate in metastatic cancer cells compared to tumor cells, and the expression of Sema3E, along with its signalling through plexinD1, correlates positively with metastatic advancement (361). Nevertheless, the metastatic effect of P61-Sema3E is negated when plexinD1 is deleted from tumor cells (361). Additionally, Sema3E prompts the proliferation of intestinal, gastric, and pancreatic cancer cells, with heightened Sema3E expression correlating with gastric cancer metastasis in the intestine and poorer survival rates in pancreatic cancer patients (460, 461). The interaction between plexinD1 and Sema3E appears to inhibit tumor apoptosis, promoting breast cancer metastasis. Conversely, blocking plexinD1 signalling induces apoptosis in breast cancer cells (462). On another note, a mutated furin-resistant Sema3E isoform, when in its full-length form, inhibits tumor angiogenesis and the spread of metastasis (463).

The importance of Sema3E and its receptor plexinD1 in immunological physiology and pathology is well identified. Through controlling T cells, B cells, eosinophils, neutrophils, macrophages, DC, and natural killer (NK) cells, the Sema3E/plexinD1 axis exhibits modulatory involvement in immune-mediated pathology (429, 464-466). Sema3E functions as a chemoattractant (as opposed to its natural repellent role) for macrophages in adipose tissue by binding to the plexinD1/Nrp1/VEGFR2 ternary complex (445). This recruitment of plexinD1-expressing macrophages by Sema3E results in TNF production and subsequent inflammation within adipose tissues (445). Interestingly, in atherosclerotic plaques, Sema3E interferes with the actin cytoskeleton's reorganization and prevents macrophage migration (444). Furthermore, Sema3E can affect macrophage polarization toward a more anti-inflammatory phenotype. The regulatory effects of Sema3E are mediated through reduced phosphorylation of STAT3, NF-κB, ERK1/2, and AKT in the macrophages, which results in decreasing phagocytosis, antigen-presenting ability, and inflammation and leads to better disease outcome (465).

T cells are another subset of immune cells that are affected by the Sema3E/PlexinD1 signalling pathway and recent research indicates the role of Sema3E in controlling the migration of developing thymocytes to the thymus medulla throughout inactivating CCR9/CCL25 signalling

(346). Moreover, Subsequent research revealed that the Sema3E/plexinD1 axis regulates thymocyte migration by controlling β1 integrin adhesion (467). Recent findings by Ueda et al. highlight the significance of Sema3E in thymocyte trafficking regulation. Thymocyte immunological synapse development is hampered by Sema3E's direct inhibition of Rap1 activation via plexinD1(468). Treg can also be affected by Sema3E signalling however, Sema3E plays a complex role in regulating Tregs, influencing their function, migration, and contribution to immune homeostasis.

Sema3E is also involved in regulating neutrophils, DCs, and NK cell migration and activation. PlexinD1 receptor expression in human neutrophils raises the possibility that Sema3E may target these cells. Sema3E inhibited the migration of human neutrophils mediated by CXCL8/IL-8 through a decrease in Rac1 GTPase activity and actin filament depolymerization. Furthermore, it dramatically lowered neutrophils' IL-8-induced chemotactic index (443). Additionally, dendritic cells derived from *Sema3e*$^{-/-}$ progenitors in the bone marrow exhibit enhanced migration both at baseline and in response to CCL21. This increased migratory capacity is associated with overexpression of the chemokine receptor CCR7, increased Rac1 GTPase activity, and F-actin polymerization (469). Moreover, the Sema3E receptor, plexinD1, is expressed on the surface of mouse NK cells. Activated NK cells migrated more toward the conditioned media of immature *Sema3e*$^{-/-}$ DCs than WT DCs, indicating that Sema3E generated by immature DCs has a suppressive effect on NK-cell migration (464).

Sema3E is crucial for maintaining the balance and function of the immune system and plays a pivotal role in responses to infections. Its regulatory influence on immune cell migration and tissue inflammation directly impacts the effectiveness of the immune response during pathogen encounters, highlighting its dual importance in both immune system homeostasis and infection management. Sema3E play a pathogenic role in early LPS-induced inflammation in a mouse model of sepsis by controlling proinflammatory cytokine production and temperature. Following exposure to LPS, macrophages lacking Sema3E demonstrated a decreased inflammatory response, as seen by notable decreases in TNF, IL-6, and inducible NO synthase production (465).

Sema3E deficiency has been linked to more severe lung infections and pathology in a mouse model of Chlamydia infection. This has been linked to greater expression of IL-4 and IL-10 and lower levels of IFN-γ and IL-17. This suggests that Sema3E plays a part in the immune response against

Chlamydia, particularly in the production of pathogen-specific IgG2a antibodies associated with IFN-γ production (466). In a recent investigation on host immunity against Leishmania major infection, it was found that mice lacking the Sema3E gene were more resilient to the infection. Specifically, dendritic cells expressing CD11c+ from infected Sema3E knockout mice exhibited heightened levels of co-stimulatory molecules and IL-12p40, a cytokine known to promote Th1 immune responses, in comparison to WT mice. This finding suggests that Sema3E typically suppresses the expression of co-stimulatory molecules (470). Additionally, reduced proinflammatory cytokine production in CD11c + cells was seen in infected Sema3E KO mice, which inhibited CD4+ cell activation and differentiation into CD4+ Th1 cells (470). Taken together, these findings underscore the intricate role of Sema3E in immune-related disorders.

1.4.5 Semaphorin 3E/PlexinD1 in allergic asthma

Interestingly, a genome-wide association study (GWAS) in African-American children with asthma found that a specific genetic variation called rs17446324 in the Sema3E gene might have a direct impact on asthma (471). Over the past ten years, studies conducted in our lab have revealed that the Sema3E-plexinD1 pathway reduces airway inflammation in asthma by regulating various inflammatory cells and the substances they release (430).

Studies on asthmatic patients revealed that due to a faster rate of proliferation of human airway smooth muscle cells (HASMCs) in asthma patients compared to healthy individuals, airway smooth muscle (ASM) mass is increased (472). It's noted that both healthy individuals and those with asthma display consistent expression of plexinD1 in HASMCs; however, its expression is reduced in asthma patients (472). The presence of Sema3E hampers platelet-derived growth factor (PDGF)-induced proliferation and movement of HASMCs, resulting in the reduction of ASM mass. Thus, by preventing ASM cell growth, plexinD1/Sema3E appears to downregulate airway remodelling (472).

It has been demonstrated that collagen deposition in the airways is influenced by the Sema3E/plexinD1 axis. Upon acute or chronic HDM challenges, mice lacking Sema3E showed increased collagen deposition in the submucosa in comparison to WT mice (473, 474) and

treatment with Sema3E-Fc decreased the collagen deposition and related fibrosis in *Sema3e*$^{-/-}$ mice (474).

Sema3E shows significant expression levels in the lungs according to RNA-Seq examination of healthy human tissues, with airway epithelial cells (AECs) being identified as one of the main producers of Sema3E. It has been shown that the level of Sema3E expression significantly decreases in both bronchial biopsy samples and bronchoalveolar lavage (BAL) fluid from patients with severe asthma (475). Similarly, downregulation of Sema3E expression was observed in both in vitro bronchial epithelial cells (BECs) from severe asthmatics and in vivo BECs within lung sections of a chronic HDM mouse model (473). Moreover, a significant inflammatory response was linked to the downregulation of Sema3E in lung epithelial cells upon HDM exposure and forced expiratory volume in one second (FEV1). Sema3E expression has a negative connection to the number of inflammatory cells suggesting that Sema3E may inhibit the recruitment of effector inflammatory cells to the airway by decreasing the release of inflammatory mediators from epithelial cells (475).

Mouse studies have confirmed the result of human studies on the importance of Sema3E and plexinD1 in the pathology of allergic asthma. Previous studies in our lab demonstrated that the absence of Sema3E worsens the symptoms of acute and chronic asthma through increasing airway inflammation and hyperresponsiveness following HDM-induced allergic airway disease. Increased Th2 and Th17 cytokine responses, eosinophils, neutrophils, mucus overproduction, collagen deposition, and total and HDM-specific IgE production were the consequences of Sema3E deficiency (473, 476). However, administering exogenous Sema3E-Fc recombinant protein mitigates these asthma manifestations (474). Additionally, exposure to allergens exacerbated the neutrophilia in the airway of *Sema3e*$^{-/-}$ mice and recombinant Sema3E therapy inhibits allergen-induced neutrophil infiltration to the airway (443). In a recent study, *Sema3e*$^{-/-}$ mice who underwent acute HDM challenge, exhibited increased angiogenesis in the airway compared to WT control. Yet, administration of Sema3E-Fc recombinant protein decreased the levels of the pro-angiogenic factors VEGF and VEGFR2, while boosting the presence of the anti-angiogenic factor VEGFR1 (477). Overall, these findings suggest that Sema3E/plexinD1 has a significant role in mitigating asthma by suppressing airway hyperresponsiveness, inflammation, and remodelling.

Chapter 2: Rationales, hypothesis, and aims

2.1 Rationale:

Asthma is a chronic inflammatory disease, that predominantly affects the airways, characterized by lung inflammation, airway remodelling, airway hyperresponsiveness, mucus overproduction, and increased numbers of eosinophils, neutrophils, mast cells, and IgE.

Semaphorin3E (Sema3E) is a protein that plays a critical role in immunity to diseases, including asthma, by regulating cell migration, proliferation, and angiogenesis (346, 347). However, the mechanisms that govern Sema3E regulatory effects in asthma are poorly investigated. We have previously reported that Sema3E expression is significantly reduced in the airways of human asthmatics and mouse models of allergic asthma (473, 475). Furthermore, mice deficient in Sema3E showed disease exacerbation (increased airway inflammation, hyperresponsiveness, and remodelling) during HDM-induced allergic inflammation (442, 476). Treatment of WT mice with Sema3E Fc-Ig was able to attenuate HDM-induced asthma-associated disease features (476), clearly showing the importance of Sema3E in asthma control. Interestingly, more recent data from our lab showed that *Sema3e*$^{-/-}$ mice have a significantly higher level of total and HDM-specific IgE) in their sera (476), a key immunoglobulin that is known as a critical mediator and driver of asthma.

2.2 The gap in the knowledge

While the regulatory roles of many mediators on GC responses and IgE production have been well studied, the role of Sema3E in these processes is still unknown.

2.3 Global hypothesis

Sema3E is a novel regulator of IgE levels through germinal center (GC) formation in vivo.

2.4 Aims

2.4.1 To investigate if Sema3E deficiency can affect steady-state GC responses.

2.4.2 To investigate the role of Sema3E in HDM-induced GC responses and IgE production in allergic asthma.

2.4.3 To determine whether the dissociation of Tfh and GC reaction during HDM-induced allergic inflammation will abolish the excessive GC responses and production of IgE in *Sema3e*$^{-/-}$ mice.

2.5 Significance of the study

The overarching goal of this project is to understand how Sema3E regulates IgE production. The significance of my study lies in its exploration of the previously uninvestigated role of Sema3E in regulating key immune processes, precisely GC response. By showing that *Sema3e*$^{-/-}$ mice have significantly higher baseline GC formation and IgE production than WT mice, both under steady-state conditions and following HDM challenge, my research reveals a novel regulatory function of Sema3E in immune responses. My results suggest that Sema3E may be a key modulator of GC formation and IgE responses, potentially influencing allergic asthma and other IgE-mediated diseases. By uncovering this regulatory pathway, my study opens new avenues for understanding the molecular mechanisms underlying allergic diseases. It offers potential targets for therapeutic interventions in conditions involving excessive IgE production, such as asthma and allergies.

Chapter 3: Materials and Methods

3.1 Animal model

Six to eight-week-old male and female C57BL/6 *Sema3e* $^{-/-}$ mice (with129P2 Bl/6 background were kindly gifted by Dr. Fanny Mann, Developmental Biology Institute of Marseille Luminy, Université de la Méditerranée, Marseille, France) and sex-age matched C57BL/6 wild type (WT) mice were used (mice were backcrossed for 4 generations). All the mice were kept pathogen-free conditions in the animal center facility at the University of Manitoba, Winnipeg (MB)-Canada, and utilized following the Canadian Council for Animal Care guidelines.

3.2 Ethics statement

All the animal procedures and experiments were approved by the University of Manitoba Animal Care and Use Committee (protocol # 19035).

3.3 HDM-induced airway inflammation model

HDM-induced allergic asthma is a well-established and widely used model. House dust mites (HDM), especially Dermatophagoides pteronyssinus (D. pteronyssinus) and Dermatophagoides farinae (D. farinae) species, are the most important indoor allergen responsible for human allergic asthma (478, 479). Therefore, in animal studies, HDM-induced allergic Asthma more closely mimics the heterogeneous character of human allergic asthma.

In our study, to induce acute allergic airway inflammation, HDM extract (Dermatophagoides pteronyssinus, Greer Laboratories, Lenoir, NC, LOT: 259585, protein concentration: 5.34 mg protein/vial) was reconstituted in sterile saline (2.5mg/ml) as a stock. Then 25µg of HDM stock was freshly mixed in 35 µl sterile saline (for each mouse) and administered intranasally (I.N.) to *Sema3e*$^{-/-}$ and WT mice under anesthesia with isoflurane for 5 consecutive days per week for two weeks (480) (Figure 3.1). Forty-eight hours after the last challenge, mice were anesthetized with isoflurane gas, and after direct cardiac blood collection, mice were sacrificed by cervical

dislocation and lung, mediastinal lymph nodes (MLNs), spleen, and Bronchoalveolar lavage fluid (BALF) were collected to measure the outcomes.

It is noteworthy that before lung collection, we did lung perfusion by directly injecting 5 ml of 1X PBS into the right ventricle of mice slowly till the lung became completely white. We did this to ensure all the inflammatory cells analyzed within the lung are tissue-resident cells, not cells, not cells brought there by circulation.

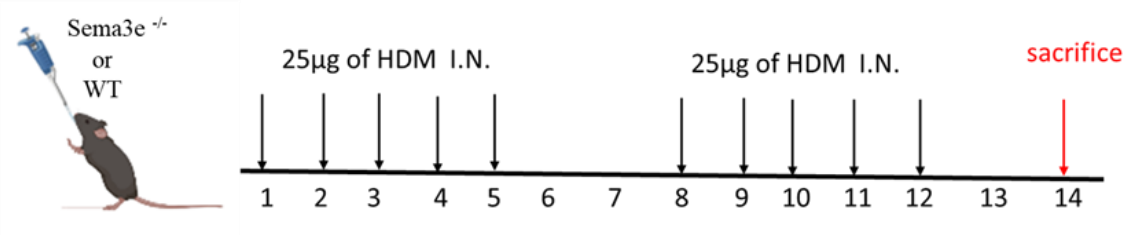


Figure 3.1. Acute model of allergic asthma. To induce allergic asthma, 6-8 weeks *Sema3e*$^{-/-}$ and WT mice received intranasal (I.N.) HDM for two weeks.

3.4 ICOS/ICOS-L blocking strategy in vivo

The ICOS/ICOS-L pathway was blocked in *Sema3e*$^{-/-}$ and WT mice by co-administration of 150 µg per mouse anti-ICOS-L (Clone: HK5.3, BioXCell, NH, USA) in 200 µL sterile PBS intraperitoneally, three times per week for two weeks as depicted in figure 4A. The control group received rat IgG as isotype control (Clone: 2A3, BioXCell, NH, USA) (342).

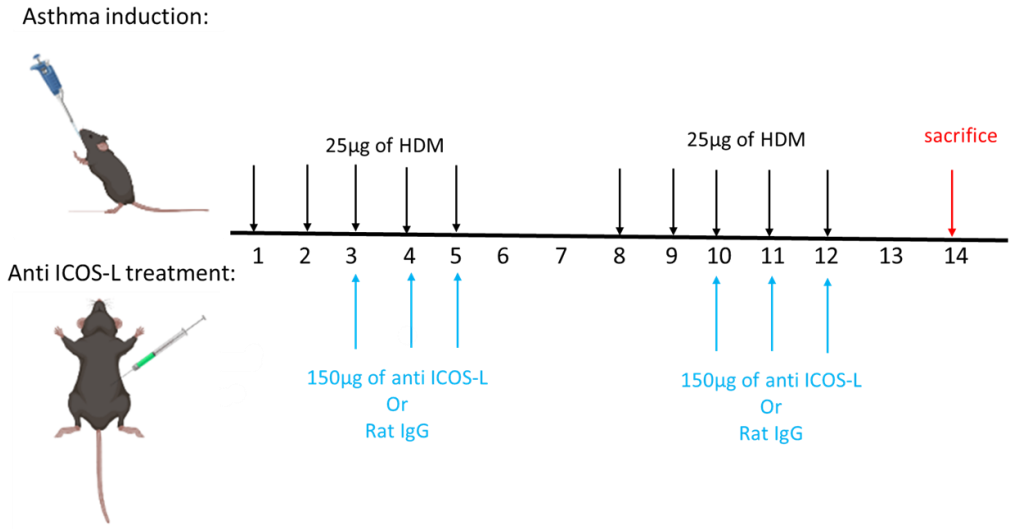


Figure 3.2. ICOS/ICOS-L blocking method. To disrupt ICOS/ICOS-L signalling pathway, 6-8 weeks male and female *Sema3e*$^{-/-}$ and WT mice underwent HDM challenge then, the ICOS-L or rat IgG as isotype control was intraperitoneally co-administrated for 3 consecutive days per week for two weeks.

3.5 Bronchoalveolar Lavage Fluid (BALF) collection and processing

Bronchoalveolar Lavage (BAL) is an experimental procedure that is used to evaluate the level of inflammation in the airways and lungs of asthmatic animals. By analyzing the cellular composition of BALF, we can gain insights into the extent and type of inflammation in the lungs.

In our experiments, for Bronchoalveolar lavage fluid (BALF) collection, first we prepared a sterile 1X PBS buffer containing 0.5M ethylenediaminetetraacetic acid (EDTA) (1µl of 0.5M EDTA/ 1ml of 1X PBS). Following mice euthanasia, a small incision was made in the trachea by a sterile scissor, and a catheter was inserted. Then a 1 ml syringe loaded with PBS buffer containing EDTA was instilled slowly into the trachea and bronchioles throughout the catheter. After that, the solution was aspirated gently, and we repeated this step 3-4 times. Finally, the syringe was removed from the catheter and the recovered lavage fluid was transferred into a two separate 1.5 ml Eppendorf tube. BALF was centrifuged at 1200 rpm for 5 min at 4°C and supernatants were aliquoted and stored at -80ºC to measure cytokines by mesoscale. In the next step, total cells in

BALF were counted using trypan blue by hemocytometer. Cytospin slides of BALF were prepared ($1x10^5$ cells/slide) Thermo Shandon CytoSpin 3 Cytocentrifuge, UK). Then cytospin slides were fixed and stained by quick dipping in the the HEMA 3 solution I and II (Fisher Scientific, Cat: 123-869). Differential cell counts were evaluated by counting at least 200 various cells including eosinophils, neutrophils, macrophages, and lymphocytes, using a light microscope (Primo Star Digital Microscope and INFINITY camera coupled to infinity capture software) under X40 magnification. Cell counts were performed in a blinded manner by two different individuals.

3.6 Single-cell preparation

Spleen, lung-draining mediastinal lymph nodes (MLNs), and Peyer's patch were collected from *Sema3e*$^{-/-}$ and WT groups. Peyer's patches are a group of well-organized lymphoid follicles that located in the gastrointestinal tract. Peyer's patch can be a rich reservoir of B cells, Tfh cells, and GC responses, so in the steady state that we didn't have MLNs in naïve mice, we collected Peyer's patch instead and studied GC responses in these follicles. Then, the single-cell suspension was prepared by grinding these tissues in a 40µl cell strainer. After using Ammonium-Chloride-Potassium (ACK) lysing buffer to lyse RBC, cells were resuspended in RPMI 1640 (Gibco, Thermo Fisher Scientific, US) supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin (complete RPMI or CRPMI buffer).

Lungs were minced by scissor and then enzymatically digested using 1 mg/ml collagenase IV (Worthington Biochemical, Lakewood, NJ) and 0.5 mg/ml DNase from bovine pancreas (Promega, Madison, WI USA) in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, US). In the next step, lung cells were ground in a 70 µl cell strainer. After lysing RBC cells with ACK buffer, cells were resuspended in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin.

After single-cell suspension preparation, total live cells were counted by using a hemocytometer. Briefly, the single-cell suspension was diluted with CRPMI (1:10 dilution) in a 1 ml Eppendorf tube and then mixed with trypan blue (1:2 dilution). Live cells were counted by a microscope (Leica, DMIL-090-135-001, Canada) at 40X magnification.

3.7 Extracellular staining of the cell marker

4×10^6 cells from the spleen, lung, and MLNs were washed with flow buffer (containing distilled water, 10X PBS, and FBS) and incubated with Fc blocker for 10 minutes. After washing with flow buffer, cells were incubated and stained with anti-mouse antibodies including CD4, PD-1, ICOS, B220, GL7, Fas (CD95), CD138, IgE, IgD, CD-80, CD-86, MHC II, CD-40, Gr-1, CD11C, SinglecF, and CXCR5 for 20 min (all details are mentioned in table 3.1). In the next step, cells were washed with 1X PBS and incubated with Zombie viability (Table 1) dye for 15 min. Then, cells were fixed using 4% paraformaldehyde (PAF) for 30 min. After washing, the supernatant was discarded, and cells were resuspended in 400µl flow buffer ready to acquire by Cyto FLEX LX machine (Beckman Coulter Life Sciences, USA).

Table 3.1: Anti-mouse surface marker antibody details

Surface marker	Fluorescence label	Company	Clone	Lot #
CD4	APC-Cy7	BD Pharmingen	GK 1.5	B381129
PD1	PerCP-eFluor 710	eBioscience	RMPI 1-30	E15816-103
ICOS	APC	Invitrogen	7E.17G9	-
ICOS-L Biotin antimouse	-	Biolegend	HK5.3	B315300
Streptavidin	APC	BD Biosceinces	-	8354846
B220	FITC	eBioscience	RA3-6B2	B343459
B220	PerCP-Cy5. 5	Invitrogen	RA3-6B2	1933255
B220	APC	BD Pharmingen	RA3-6B2	7153546
GL7	FITC	BD Pharmingen	GL7	7180722

Fas (CD95)	PE	BD Pharmingen	JO2	7067991
CD138	PerCP-Cy5. 5	Biolegend	281-2	B379422
IgE	PE	eBioscience	23G3	E02005-1633
IgG	PE-Cy7	Biolegend	RMG1-1	B357926
IgD	efluor 450	Invitrogen	11-26c	1929630
Sca1	FITC	Biolegend	D7	B245339
CD80	PE	BD Pharmingen	16-10A1	6323505
CD86	APC-Cy7	eBioscience	GL1	B248850
MHCII	PB	Biolegend	M5/114.152	B360614
CD40	PE-Cy7	Biolegend	3/23	B344284
Gr-1	FITC	eBioscience	RB6-8C5	4322602
CD11c	eFluor 450	eBioscience	N418	4300055
SinglecF	PE	Biolegend	S17007L	B357623
CXCR5	PE	eBioscience	SPRCL5	4339666
CD3e	PE-Cy7	Biolegend	17A2	B356288
Zombie Aqua ™ Fixable Viability	Amcyan	Biolegend	-	B386903
CD45	APC-Cy7	Biolegend	13/2.3	B362428
CD11b	PE-Cy7	Biolegend	M1/70	B390649
CD64	PerCP-Cy5.5	Biolegend	X54-5/7.1	B348169
MertK	APC	Invitrogen	DS5MMER	2504628

3.8 Intracellular staining

Intracellular staining complements extracellular staining in flow cytometry by providing a more complete picture of cytokines or antibodies synthesis and production. Indeed intracellular staining is crucial for detecting IgE and IgG synthesis and the full antibody production pathway in B cells. We did intracellular staining for IgE and IgG on $B220^{+}$ cells, and this would help us to detect

antibodies bound to the surface of B cells, typically through Fc receptors, as well as antibodies that are produced but still inside the B cell, providing insight into IgE and IgG synthesis.

Following the extracellular staining and fixing step, cells were permeabilized by incubation with 1ml Saponin buffer (0.1% saponin in flow buffer) for 30 min. Then, cells were separated by centrifugation and incubated with anti-mouse IgE and IgG labelled antibodies (diluted in saponin buffer) (antibody details are mentioned in table 3.1) for 30 min. Next, cells were washed with saponin buffer and flow buffer, respectively. Finally, the supernatant was removed, and cells were resuspended in a 400µl flow buffer.

3.9 Flow cytometry analysis

After extracellular and intracellular staining of cells, data were acquired using BD CytoFLEX LX (Beckman Coulter Life Sciences, USA) following the standard instrumental protocol. At least 100,000 events of stained cells were acquired and the gating strategies for the inflammatory cells and markers are indicated in the corresponding figures. We used fluorescence minus one (FMO) control of flow antibody to get accurate gating for cells. In the end, acquired flow data were analyzed by FlowJo software version 10.81.

3.10 Histologic examination

Paraffin-embedded tissues

left lobe of the lung was collected and fixed in formalin for 48 hours, followed by embedding in paraffin. Then tissue sections were cut into 5 µm thick sections by microtome (Leica Microsystem Inc, Ontario, Canada) and slides were prepared. After dewaxing with Xylene and rehydrating in various concentrations of ethanol (100%, 95%, and 75%), hematoxylin and eosin staining (H&E), Sirius red, and periodic acid-Schiff (PAS) staining were done to evaluate baseline and HDM-induced airway inflammation, collagen deposition, and mucus production respectively (476).

Using a Carl Zeiss AxioCam MRc 5 camera and AxioVision Rel 4.8 software, images were captured at x200 using a digital Zeiss Axioskop 2 mot plus microscope.

Fresh frozen tissue

Freshly harvested spleen was fixed in 2ml PAF4% for 6 hours at 4ºc (481). After rinsing with 1X PBS, the spleen was left immersed in 30% sucrose for 12 hours at 4 ºc till the tissue sank. Next, tissue was washed with 1X PBS and transferred to a cryomold embedded in OCT and snap-frozen in liquid nitrogen. The OCT frozen tissues were stored at -80 ºc before cutting. Finally, the frozen blocks were cut into 5µm thick cryosections by cryostat microtome (Epredia HM525 NX, USA) attached to frost-free slides, and stored in -80 ºc in a sealed box for immunofluorescence (IF) staining.

3.11 Immunofluorescence (IF) staining

Paraffin-embedded lung slides were first deparaffinized in xylene and rehydrated through graded ethanol concentrations to water (100%, 95%, 75% ethanol). The antigen retrieval step was done by boiling tissue slides in sodium citrate buffer (pH 6.0) in a microwave for 10 min. After cooling down at room temperature, slides were washed with washing buffer (containing 1X PBS and 0.5% Tween 20). Following washing slides, tissues were blocked by incubation with blocking buffer (5% BSA and 5% goat serum in 1X PBS) for 1 hour at room temperature in a humid dark box. Next, tissues were incubated with permeabilization buffer (2.5% Triton 100X in 1X PBS/gelatin 0.2% w/v) for 20 min at room temperature to allow antibodies to access intracellular epitopes. Fluorescent-conjugated antibodies were diluted in dilution buffer (1% BSA in 1X PBS) (details are mentioned in table 3.2) added to tissues and incubated in a humid dark box at 4ºc overnight. The next day, tissues were washed with a washing buffer and DAPI was added to each tissue for 3 minutes. Finally, slides were washed and mounted with a diamond anti-fade mountant (Invitrogen, USA, Lot# 2406613).

For OCT tissue, after cutting and preparing slides, tissues were fixed with 4% PAF for 30 min. Then, we rehydrated our slides with a washing buffer 3times× 5 min. In the next step, slides were incubated with a blocking buffer for 1 hour. The rest of the steps until the end were like paraffine-embedded tissue.

Images were taken by CY10 microscope (BioTek, Agilent, USA) at 100X, 200X , and 1000X magnification and analyzed by ImageJ software.

Table 3.2 Immunofluorescence (IF) staining antibodies

Antibody	Fluorescence	Dilution	Isotype control	Company and Cat #
Rat anti-mouse IgD	Alexa Fluor 647	1:300	Rat IgG2a	Biolegend Lot: B350163
Hamster anti-mouse CD3e	FITC	1:300	Armenian hamster IgG	Biolegend Lot: B347489
Rabbit anti-mouse KI-67 (First antibody)	-	1:100	Rabbit IgG	Abcam Lot: ab16667
Rat IgG2a	APC	1:300	-	eBioscience Lot: E07263-1631
Armenian Hamster IgG	FITC	1:300	-	eBioscience

				Lot: E00617-1319
Goat anti-rabbit Secondary Ab	Alexa Fluor 568	1:300	-	Invitrogen Lot: 2273773
Rabbit monoclonal [SP6] to Ki67 IgG (first antibody)	-	1:100	-	Abcam

3.12 Enzyme-Linked Immunosorbent Assay (ELISA) for serum immunoglobulin assessment

Total and HDM-specific IgE and IgG1 were measured in the serum sample of *Sema3e*$^{-/-}$ and WT mice using commercial ELISA kits, according to the manufacturer's instructions (482). Briefly, the blood was collected from the mouse's heart then serum was separated by centrifuge at 10000 rcf /4ºC for 5 min. 96 well ELISA kits were purchased from Life Sciences (Corning® 96-well EIA/RIA, product no.:3369, US) and coated with a 50 µl/well coating buffer (containing 2µg coating Ab/ 1ml coating buffer) (antibody details are mentioned in table 3.3). For HDM-specific antibody detection, ELISA plates were coated with HDM in PBS (5µg HDM/1ml PBS). Plates were firmly wrapped and stored in a humidified container, to avoid evaporation and variance in wells, at 4ºc overnight. On the second day, plates were washed and dried by hitting them several times on paper towels. To avoid non-specific bindings, plates were incubated with 80µl/well blocking buffer for 2 hours at 37ºc. After washing, standard (20ng/ml) and samples were added and serially diluted with dilution buffer within the plates (with the final volume of 50µl/well). Then, plates were tightly wrapped and incubated in a humid box at 4ºc overnight for maximal sensitivity. On the third day, first we washed and dried the plates. Next, biotinylated goat anti mouse IgE and IgG1 detection antibodies buffer (1µg detection Ab/ml dilution buffer) were added, and plates were sealed in a moisture box for 2 hours at 37ºc. Following washing and drying plates,

50 µl of Alkaline phosphatase (ALP) buffer was added to wells for 45 min at 37ºc. Alkaline phosphatase (ALP) is one of the most used enzyme labels that catalyze a colorimetric reaction. After the final washing step, 50 µl of ELISA phosphate substrate buffer (1 ELISA phosphate substrate tablet (Sigma Diagnostic, USA) in 6 ml substrate buffer) was added to the plates, and the plates were covered with aluminum foil in the dark. Progress of the assay was observed visually until the yellow color appeared. Finally, following the proper color development, plates were read at 450 nm with Spectra Max 190 ELISA reader (Molecular Devices, CA, USA), and data were analyzed using SoftMax Pro software version 5.4.1. The data obtained from the serial dilution of the standard was used to create a standard curve. The resulting four-parameter curve was created by calculating the OD on the Y-axis and the concentration of IgE and IgG1 in the samples on the X-axis (483).

Table 3.3 ELISA antibodies

Antibody	Type	Company	Lot #
Goat anti-mouse IgE-UNLB	Coating	Southern Biotech (Birmingham, AL, USA)	J3210-Z751Z
AffiniPure sheep anti-mouse IgG (H+L)	Coating	Jackson ImmunoResearch USA	162151
Goat anti-mouse IgE-BIOT	Detection	Southern Biotech (Birmingham, AL, USA)	K5313-MF28
Goat F(ab')2 anti-mouse IgG1-BIOT	detection	Southern Biotech (Birmingham, AL, USA)	I2611-MI12

3.13 BALF cytokines measurement

The levels of the inflammatory cytokines including IL-4, IL-5, IL-13, and IL-17A were measured in BALF by the MesoScale Discovery system (MSD U-PLEX, US. Cat#K15069M-1) according to manufacturer's guidelines. Briefly, BALF was centrifuged at 1200 rpm at 4ºc for 5 min and supernatant was collected. 25µl of diluent 41 (Cat#R50AH-2) was added to each plate well. Then, 25 µl of calibrator standards with different concentrations and diluted BALF samples were added to each well and incubated at room temperature for 1 hour while shaking. Following washing the plate with wash buffer (0.05% Tween 20 in PBS) 3 times, detection antibodies master mix solution was added, and the plate was sealed and incubated for another 1 hour at room temperature while shaking. After final washing steps (3 times each time for 5 min), 150 µl of 2X read buffer (Cat# R92TC-3) was added to each well. the plate was read by an MSD reader machine (1300 MESO QuickPlex SQ120, USA) and data were analyzed by GraphPad Prism 9.0 software.

3.14 Statical analysis

The statistical analysis was performed using GraphPad Prism 9.0 software, and data were shown as the mean ± SEM. Based on the group size and treatments multiple statistical tests including unpaired t-test, one-way ANOVA, or two-way ANOVA, followed by the Bonferroni's multiple comparisons post hoc test were performed. Differences at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered to be statistically significant.

Chapter 4: Results

4.1 Sema3E regulates the steady-state germinal center responses.

The key role of Sema3E in the regulation of various immune cells is well-established (346, 347) however, the role of this protein in regulating GC responses and IgE production has not been studied. To determine whether Sema3E plays a role in optimal GC responses at the steady state, I measured the total cell numbers of lung, spleen, and Peyer's patches as well as the frequency and absolute numbers of GC B cells and Tfh cells in unimmunized *Sema3e*$^{-/-}$ and WT littermate controls. The regulatory role of Sema3E in cell migration is well-studied (443, 467, 469) therefore, it was not unexpected that the absence of Sema3E would affect the total number of cells in steady state condition, leading to a significant increase in cells in the lung, spleen, and Peyer's patches (Figure 4.1 A-C). Intriguingly, I observed that the GC B cells and Tfh may be regulated by Sema3E. The number of GC B cells (gated as B220^{+}GL7^{+}Fas^{+}) (484) is significantly higher in the lung, spleen, and Peyer's patch (Figure 4.1 D-F respectively) in the absence of Sema3E. Consistent with GC B cells, baseline Tfh cells population (CD3^{+}CD4^{+}CXCR5^{+}PD-1^{+}ICOS^{+} cells) (485) demonstrated an increase in naïve *Sema3e*$^{-/-}$ mice compared to the WT control group in the lung and spleen (Figure 4.1 G& H) indicating a substantial negative regulatory role for Sema3E in the baseline GC responses. Although it did not reach a statistically significant difference in Peyer's patches, it seems that the *Sema3e*$^{-/-}$ mice have more Tfh in their Peyer's patches compared to the WT group (Figure 4.1 I).

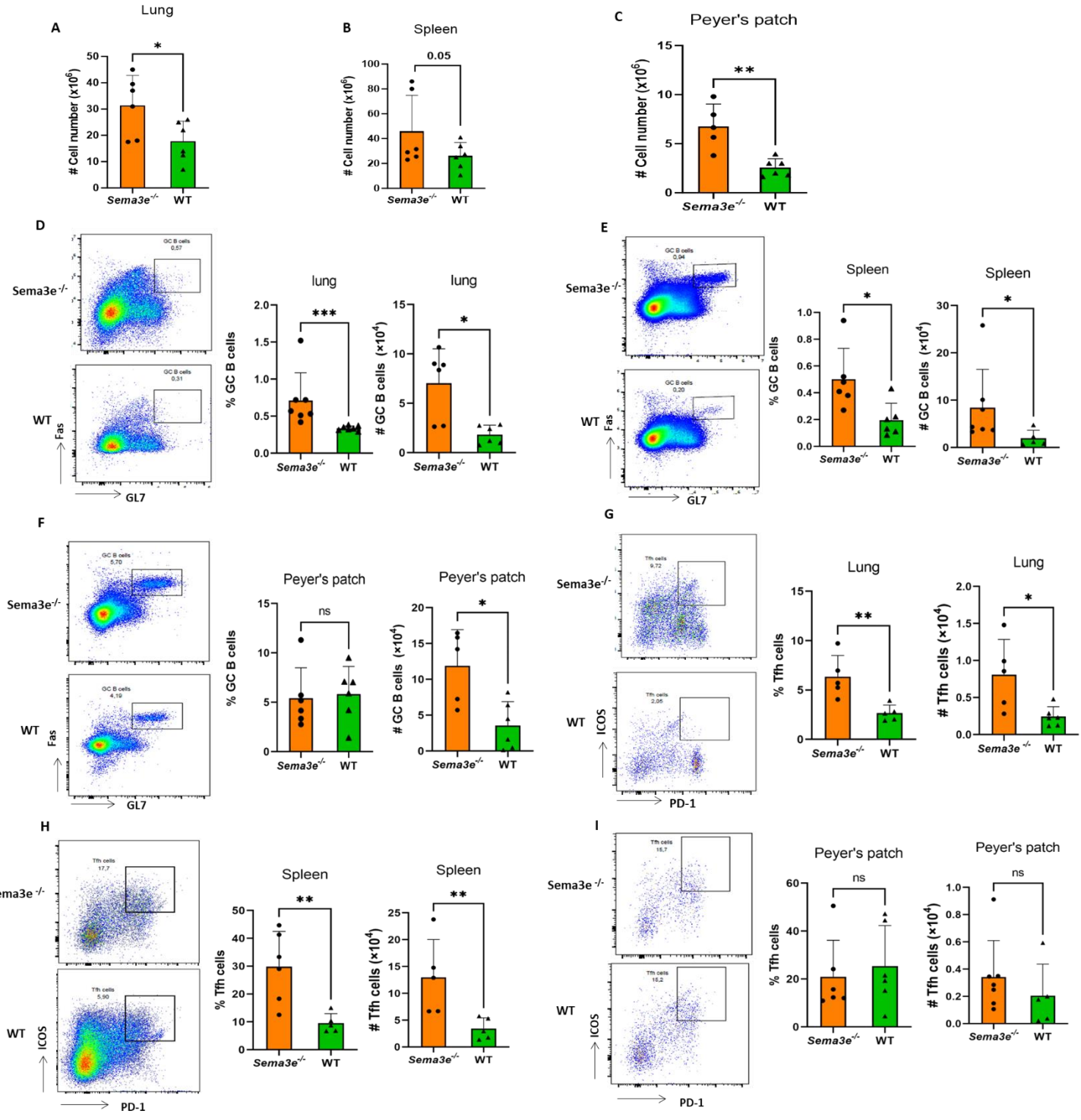

A
Lung
Cell number (x10^6)
*
Sema3e-/-
WT
B
Spleen
0.05
C
Peyer's patch
**
D
Sema3e-/-
WT
Fas
GL7
lung
% GC B cells
***
GC B cells (x10^4)
E
Spleen
F
Peyer's patch
ns
G
Lung
% Tfh cells
# Tfh cells (x10^4)
ICOS
PD-1
H
I

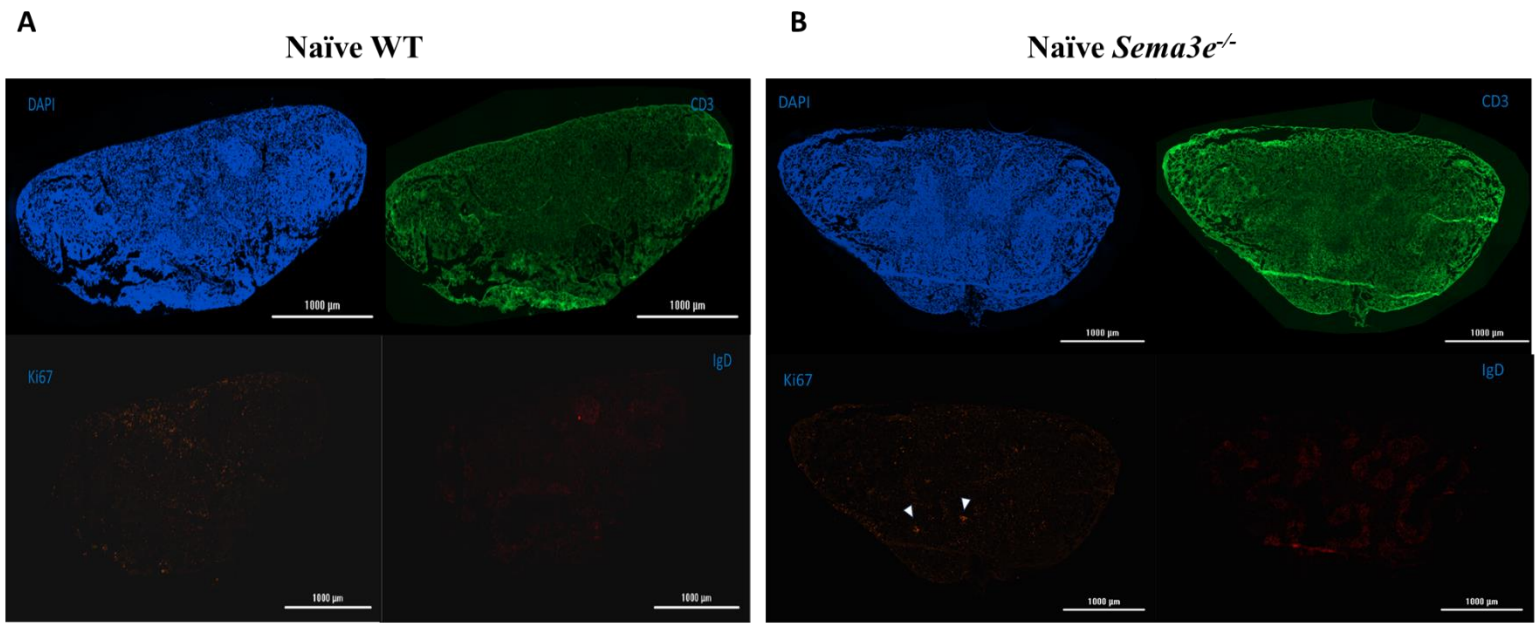
Figure 4.1 Sema3E regulates steady-state germinal center responses.

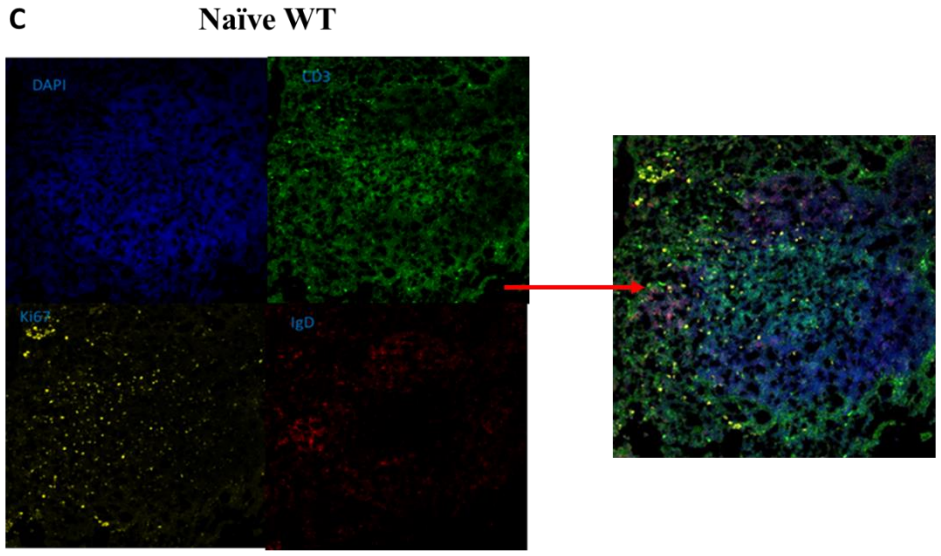
The total cell numbers, percentage and absolute number of GC B cells and Tfh cells were characterized by flow cytometry analysis in 6-8 weeks naive *Sema3e*$^{-/-}$ and WT mice. Total cell numbers in the lung (A), spleen (B), and Peyer's patches (C) were measured and GC B cells were gated as B220^{+}Fas^{+}GL7^{+} in the lung (D), spleen (E), and Peyer's patch (F). Tfh cells population was identified as CD3^{+}CD4^{+}CXCR5^{+}PD1^{+}ICOS^{+} and were measured in the lung (G), spleen (H), and Peyer's patch (I). Flow data were generated from at least two independent experiments. Bars show mean +/-SEM; *, $p < 0.05$; **, $p<0.01$; ***, $p < 0.001$, ns; not significant; N=5-7 mice in each group.

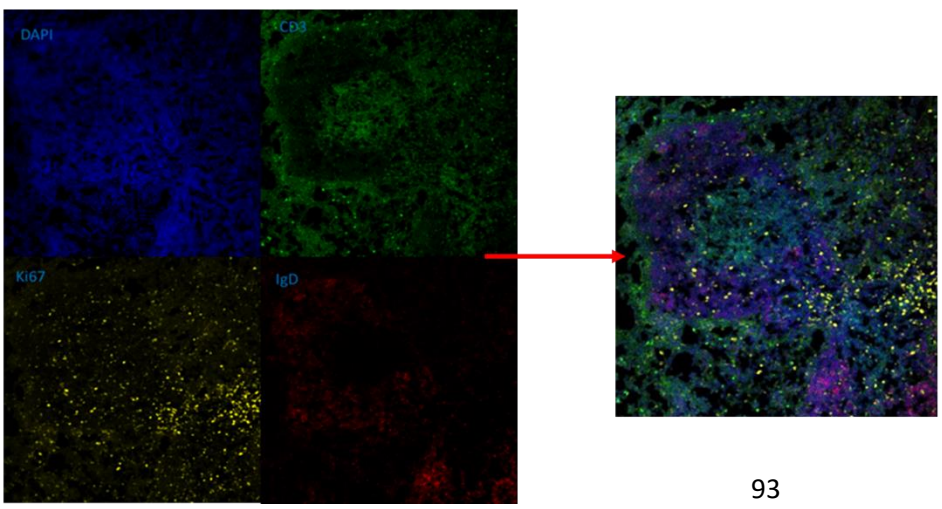
4.2 Sema3E regulates the baseline germinal center formation.

GCs typically form in response to an antigenic stimulus, such as infection, vaccination, or inflammation, which triggers the activation of B cells. Under naive conditions, without infection or inflammation, GC formation is generally not observed in WT mice because it requires the activation of B cells by antigen presentation, Tfh cell interaction, and cytokine signaling. However, the IF staining in the spleen tissue was done to investigate whether the absence of Sema3E protein could affect steady-state GC formation within lymphoid tissue (spleen).

To evaluate GC formation, I used CD3 marker for the T cell zone, Ki67 for proliferating GC B cells, IgD for the B cell follicle (481). I counted every Ki67^{+}IgD^{+} center as one active GC. My IF staining results demonstrated that, as we expected, there was no GC formation in the naïve WT mice (Figure 4.2 A), while in the absence of Sema3E, GCs were formed in the spleen (Figure 4.2 B). Regarding the shape of GC, it doesn't seem the absence of Sema3E could affect the normal shape of GC compared to WT mice (Figure 4.2 C &D). Statical analysis showed a significant difference between baseline GC formation in *Sema3e*$^{-/-}$ and WT indicating a negative regulatory role for Sema3E in steady-state GC responses.


A
Naïve WT
DAPI
CD3
1000 µm
Ki67
IgD
B
Naïve Sema3e-/-
DAPI
CD3
Ki67
IgD


C
Naïve WT
DAPI
CD3
Ki67
IgD


D
Naïve Sema3e-/-
DAPI
CD3
Ki67
IgD

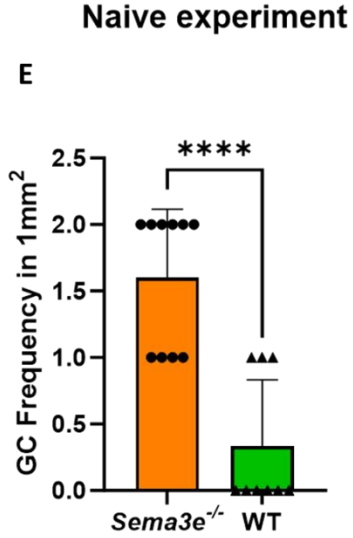

Naive experiment
E
****
GC Frequency in 1mm2
2.5
2.0
1.5
1.0
0.5
0.0
Sema3e-/-
WT

Figure 4.2 Sema3E negatively regulates Baseline germinal center formation.

GC formation was measured by histology (IF staining). Spleen sections (5 µm) of unimmunized *Sema3e*$^{-/-}$ and WT mice were labeled with CD3-FITC (green), Ki-67-AF568 (yellow), IgD-AF647 (red), and DAPI (blue). The image of the whole spleen was captured in WT and *Sema3e*$^{-/-}$ mice and the frequency of Ki67^{+}IgD^{+} area was counted as GC (A & B respectively). The scale bar represents 1000 µm (A & B). The structure of GC is shown in detail in naïve WT (C) and *Sema3e*$^{-/-}$ (D) and the scale bar represents 200 µm. The number of GC was counted in each section and each symbol represents one GC (Ki67^{+}IgD^{+} area) (I). Statistical analysis was done to evaluate the difference between GC frequency in *Sema3e*$^{-/-}$ and WT mice. Bars show mean +/-SEM; ****, $p < 0.0001$.

4.3 The absence of Sema3E increases baseline numbers of plasma cells and plasmablasts.

Following GC formation, GC B cells undergo clonal expansion and differentiation to produce more specialized cells, including plasmablasts and plasma cells leading to high-affinity antibody production (486). In this context, I evaluated the frequency of plasma cells (gated as IgD^{-} Sca1^{+}B220^{-}CD138^{+}) and plasmablasts (IgD^{-}Sca1^{+}B220^{+}CD138^{+}) in naïve *Sema3e*$^{-/-}$ and WT mice to evaluate the effect of Sema3E in these populations. The flow cytometry data demonstrated that in the absence of Sema3E, the percentage and total number of plasma cells and plasmablasts in the lung (Figure 4.3 A&B) was significantly elevated. Consistent with the lung, in secondary lymphoid tissues including the spleen (Figure 4.3 C&D), and Peyer's patch (Figure4.3 E&F) plasma cells and plasmablasts substantially increased suggesting a negative regulatory key role of Sema3E in the regulation of these inflammatory cells.

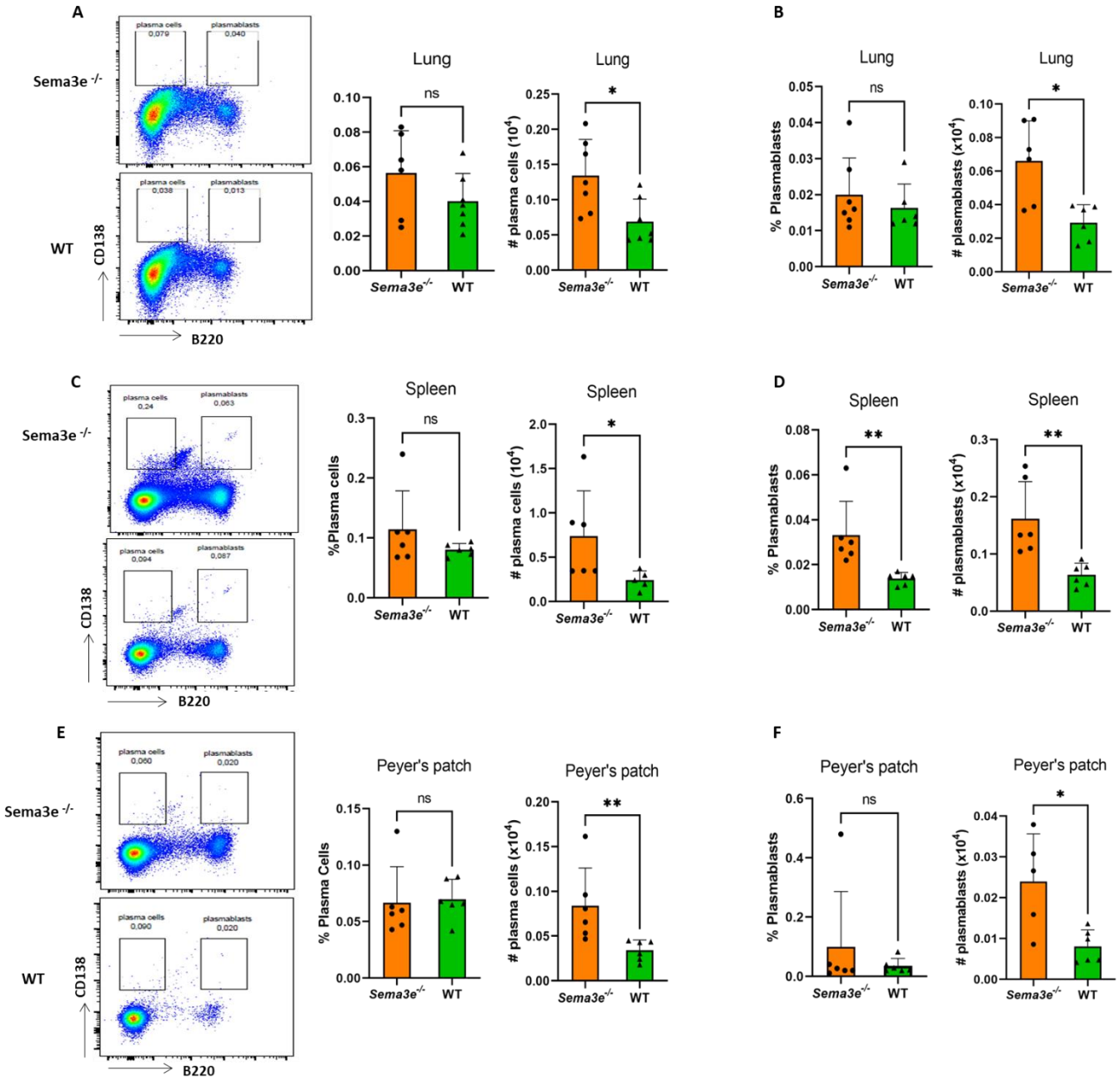


Figure4.3 Naïve *Sema3e*$^{-/-}$ mice had higher plasma cells and plasmablasts.

The percentage and absolute number of plasma cells ($IgD^{-}Sca1^{+}B220^{-}CD138^{+}$) were analyzed by flow cytometry in the lung (A), spleen (C), and Peyer's patches (E). In addition, plasmablasts were identified as $IgD^{-}Sca1^{+}B220^{+}CD138^{+}$ in the lung (B), spleen (D), and Peyer's patch (F) of *Sema3e*$^{-/-}$ and WT control mice. The data are representative of two different experiments with a similar outcome. Bars show mean +/- SEM; *, $p < 0.05$; **, $p<0.01$; ns; not significant; N=5-7 mice in each group.

4.4 Sema3E does not regulate the baseline expression of costimulatory molecules on B cells

Next, I investigated if Sema3E can regulate B cell stimulation or the number of active B cells at the steady-state. For this aim, the baseline expression of co-stimulatory molecules such as CD40, CD80, CD86, MHCII, and ICOS-L were evaluated by flow cytometry on $B220^+$ cells. The number of B cells expressing these markers was analyzed based on total cell number and flow cytometry percentage. Moreover, the expression of the co-stimulatory molecules was evaluated using Mean Fluorescence Intensity (MFI). MFI measures the average fluorescence signal emitted by a population of cells labeled with a fluorescent marker and it provides information about the level of expression of a specific protein or molecule on the surface or inside the cells. In this experiment, MFI demonstrates how much the B cell population expresses the costimulatory molecules on their surface which can suggest the level of B cell activation.

Interestingly I observed that baseline CD80 expression by B cells in the spleen is partially controlled by Sema3E. Although the number of $CD80^+$ B cells and the expression of CD80 was comparable in the lung (Figure 4.4 A), the absence of Sema3E in the spleen led to a decrease in the expression of this costimulatory molecule on B cells compared to the WT control group indicating a possible positive regulatory role for Sema3E (Figure 4.4 B). However, in Peyer's patch, Sema3E deficiency did not affect the expression of CD80 but led to higher numbers of $CD80^+$B cells (Figure 4.4 C), probably because of its effect on cell migration. There were no significant differences in the baseline expression or number of $CD86^+$B cells (Figure 4.4 D-F) and CD40 ($CD40^+B220^+$) (Figure 4.4 G-I) between unimmunized *Sema3e*$^{-/-}$ and WT control group in the lung, spleen, and Peyer's patch. MHCII is another key molecule that plays a significant role in B cell stimulation and B cell- T cell interaction. I observed that baseline MHCII expression by lung and spleen B cells ($MHCII^+B220^+$) (Figure 4.4 J&K) were not affected by the absence of Sema3E however, similar to CD80, the number of Peyer's patch B cells expressing MHCII was significantly higher in *sema3e*$^{-/-}$ mice (Figure 4.4 L). Finally, the ICOS-L expression ($ICOS\text{-}L^+B220^+$) was comparable between *Sema3e*$^{-/-}$ and WT mice (Figure 4.4 M-O). Overall, my results demonstrated that at the steady-state, when B cells are not active, Sema3E only can affect the baseline expression of CD80 on splenic B cells and its absence caused lower CD80 expression.

Moreover, due to its regulatory role in cell migration, Sema3E deficiency increased the number of B cells expressing CD80 and MHCII in Peyer's patches.

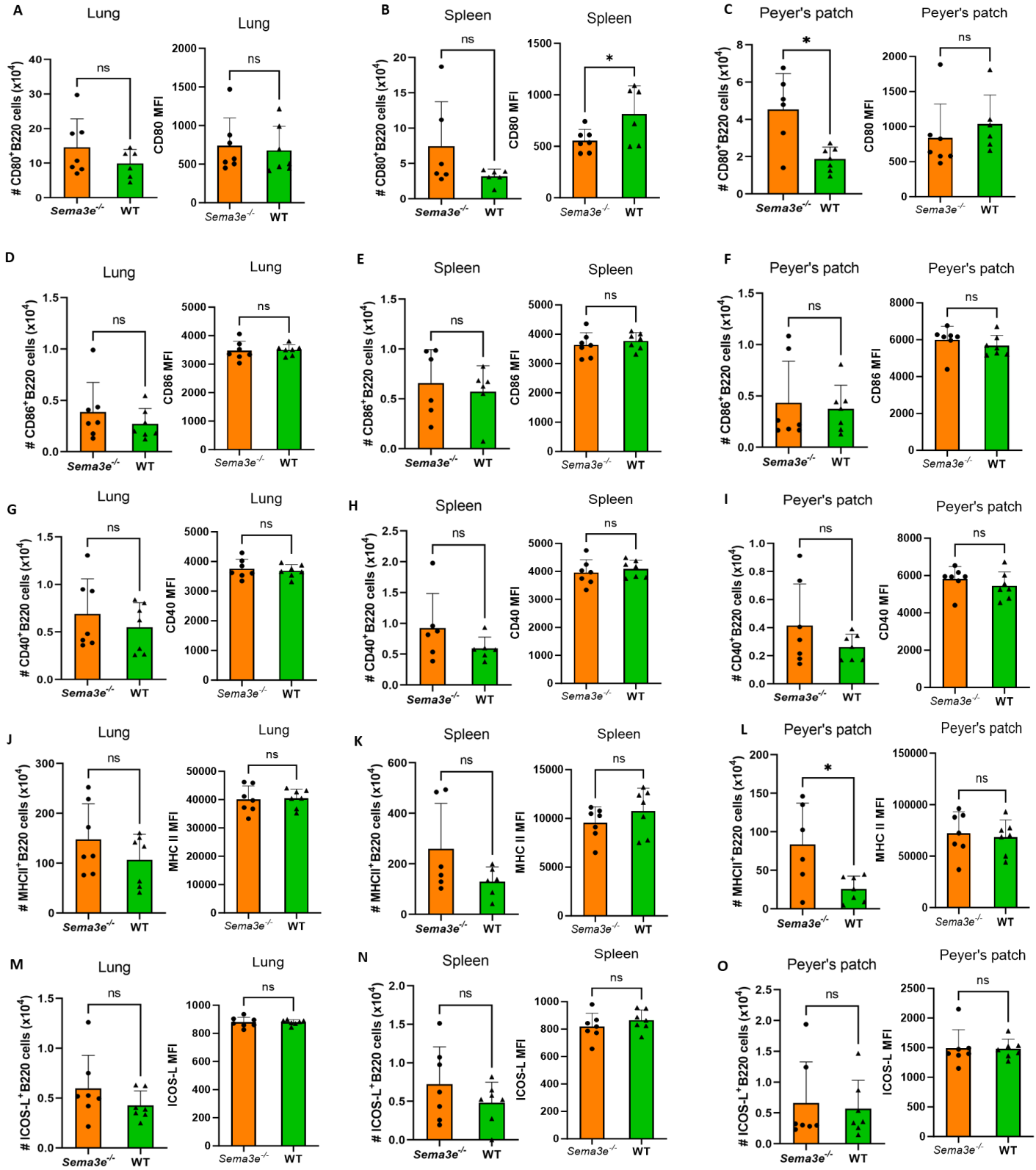


Figure 4.4 Sema3E deficiency does not affect baseline B cell stimulation.

The total cell number and expression of B cells co-stimulatory molecules including CD80 (A-C), CD86 (D-F), CD40 (G-I), MHCII (J-L), and ICOS-L (M-O) were evaluated by flow cytometry in the lung, spleen, and Peyer's patch of naïve *Sema3e*$^{-/-}$ and WT control mice. The data are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$; ns; not significant; N=5-7 mice in each group.

4.5 naïve *Sema3e*$^{-/-}$ mice had a remarkable number of IgE^{+}B cell and IgG1^{+}B cells and serum antibodies.

IgE^{+} B cells and IgG^{+} B cells refer to B cells that express IgE and IgG immunoglobulin on their surface. In a naive condition, before encountering specific antigens, these cells are present in the body but are not actively producing IgE or IgG antibodies. These B cells are part of the diverse repertoire of B cells that are involved in surveillance for antigens. They express membrane-bound IgE or IgG receptors that can bind to antigens directly. However, they are primarily resting until they encounter an antigen that matches their specific receptor. I studied these two populations within the lung, spleen, and Peyer's patch of unimmunized *Sema3e*$^{-/-}$ and WT control mice to observe if they are affected by the absence of Sema3E. The flow cytometry results demonstrated that the baseline number of IgE^{+}B cells in the lung and Peyer's patches were significantly higher in naïve *Sema3e*$^{-/-}$ mice compared to WT control (Figure 4.5 A & C, respectively). However, no statistically significant difference was observed in the number of this population in the spleen in the absence of Sema3E (Figure 4.5 B). Interestingly, the MFI analysis demonstrated that Sema3E regulated baseline expression of IgE on B cells, especially in lymphoid tissue like the spleen and Peyer's patch since its deletion led to higher expression of IgE on splenic and Peyer's patch *Sema3e*$^{-/-}$ B cells compare to WT B cells. Furthermore, the IgG^{+} B cell population and expression of IgG on the B cell surface were affected by Sema3E absence. The number of B cells expressing IgG was remarkably higher in the lung and Peyer's patch in the absence of Sema 3E while no difference was observed in the spleen of Sema*3e*$^{-/-}$ and WT mice (Figure 4.5 D-F). Furthermore, expression of IgG on the B cell surface significantly increased in the spleen and Peyer's patch following Sema 3E deficiency (Figure 4.5 D-F). Concomitantly, ELISA data showed that serum

levels of total IgE and IgG1 are remarkably increased in unimmunized *Sema3e*$^{-/-}$ mice (Figure 4.5 G &H), indicating a crucial role for Sema3E in the regulation of baseline IgE and IgG1 production.

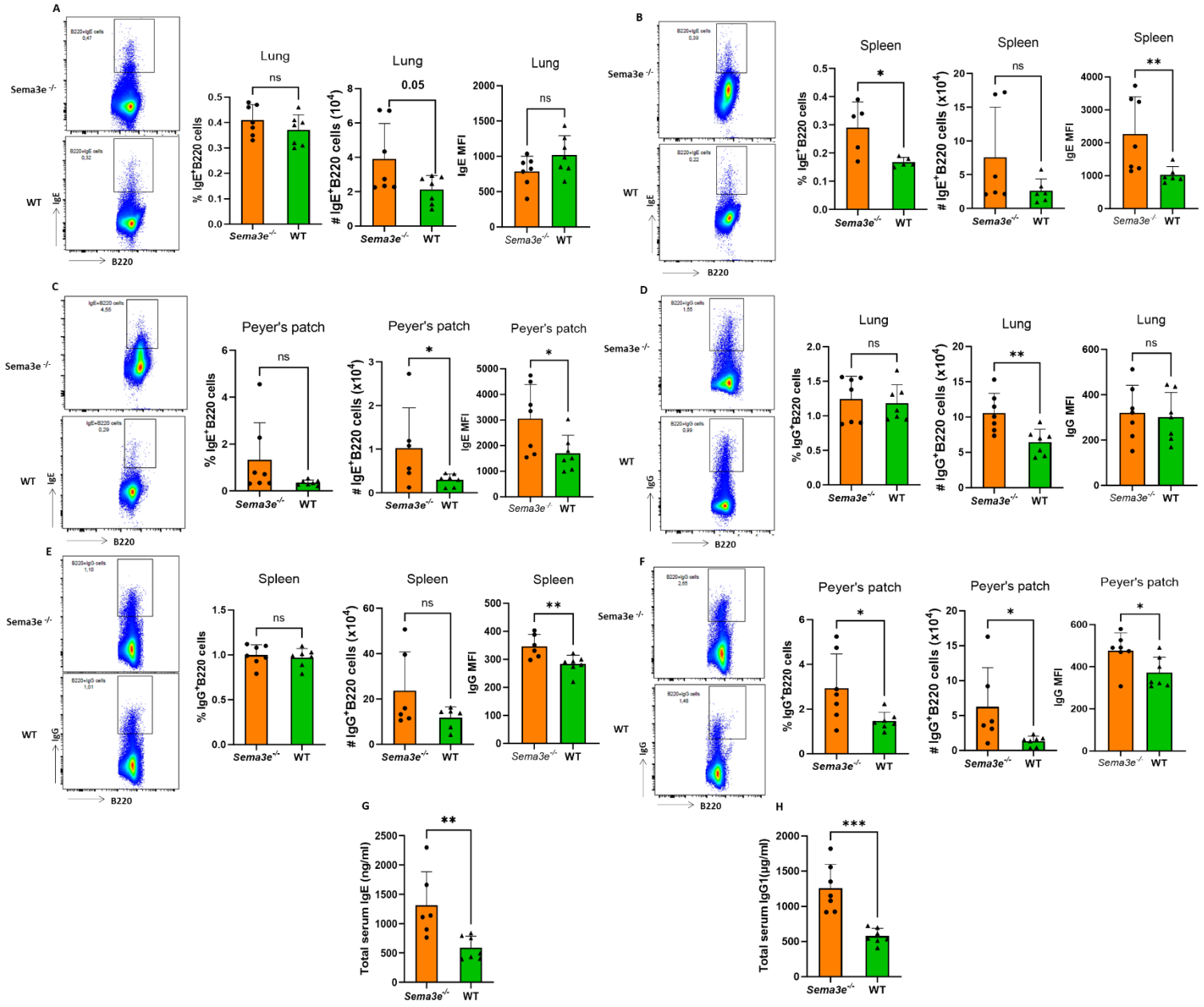
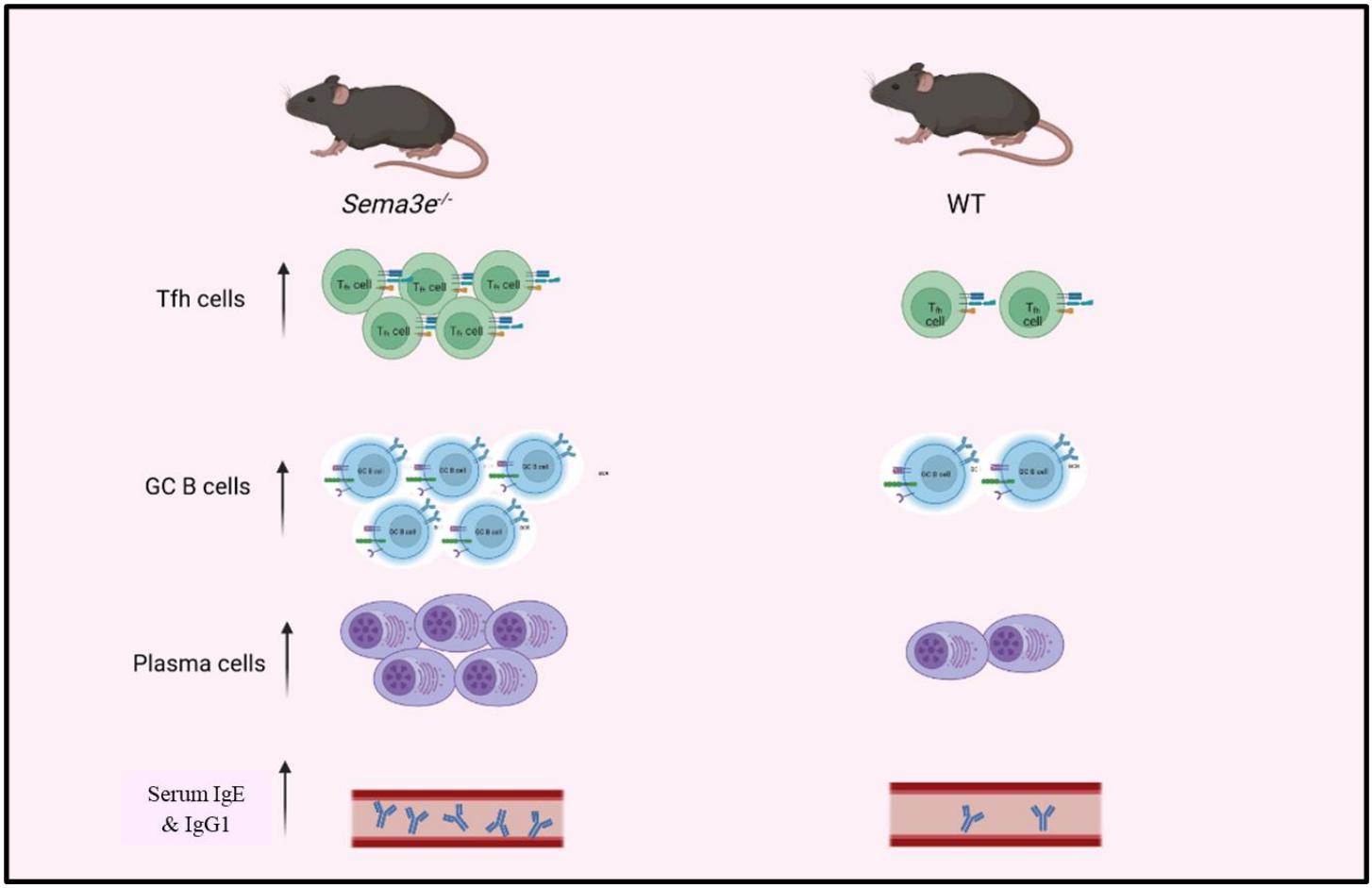


Figure 4.5 The absence of Sema3E led to higher baseline IgE$^+$ and IgG$^+$ B cells and serum immunoglobulin.

FACS analysis was done to measure the expression and numbers of IgE$^+$B cells (IgE$^+$B220$^+$) (A-C) and IgG$^+$B cells (IgG$^+$B220$^+$) (D-F) in the lung, spleen, and Peyer's patches of naïve *Sema3e*$^{-/-}$ and WT mice. ELISA was performed and baseline serum total IgE (G) and IgG1(H) were evaluated in the absence of Sema3E. The data presented, are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$; **, $p<0.01$; ***, $p < 0.001$; ns; not significant; N=5-7 mice in each group.

Graphical Abstract 1:

Aim 1: To investigate if Sema3E deficiency can affect steady-state GC responses.



To summarize aim 1 results, I observed that the absence of Sema3E led to an increased baseline number of T follicular helper cells, germinal center B cells, plasma cells, plasmablasts, IgE^+ B cells, and IgG^+ B cells in naïve mice. The baseline serum levels of IgE and IgG1 were significantly higher in unimmunized *Sema3e*$^{-/-}$ mice compared to the WT control, indicating a fundamental role for Sema3E in the regulation of steady-state GC formation and antibody production.

4.6 The absence of Sema3E promotes GC responses following HDM immunization.

Next, I aimed to determine whether Sema3E affects GC responses following immunization with HDM in an acute model of allergic asthma. I induced acute allergic asthma by intranasal administration of HDM to *Sema3e*$^{-/-}$ and WT control mice for two weeks (Figure 4.6 A) (476). Flow cytometry data demonstrated following HDM challenge, Sema3E absence led to an increased number of total cells in the lung, spleen, MLN, and BAL (Figure 4.6 B-E). A substantial enhancement was observed in the number of GC B cells (B220^{+} GL7^{+} Fas^{+}) in the lung, spleen, and MLN of *Sema3e* $^{-/-}$ mice compared to the WT mice after HDM exposure (Figure 4.6 F-H respectively).

Tfh cells are essential in GC responses and antibody production upon allergen exposure. In this regard, the number of Tfh cells (CD3^{+}CD4^{+}CXCR5^{+}PD-1^{+}ICOS^{+}) was measured in the *Sema3e*$^{-/-}$ and WT mice after the HDM challenge. Flow cytometry data showed that the absence of Sema3E led to a significant increase in Tfh cells in the lung, spleen, and MLNs (Figure 4.6 I-K, respectively) upon the HDM challenge.

To evaluate whether Sema 3E plays a regulatory role in GC responses following HDM or if the difference observed was a reflection of the baseline difference between *Sema3*$^{-/-}$ mice and WT, I calculated the fold changes for GC B cell and Tfh cell populations in the lung and spleen (Table 4.1). It seems that following immunization with HDM, Sema 3E may play a negative regulatory role on the GC B cell population in the lung and spleen since its absence led to a slightly higher expansion of this population compared to WT group (lung: 7.26 vs 6.62 and spleen: 10.761 vs 9.249) (Table 4.1). This suggests that Sema3E normally may act as a regulatory molecule that controls the extent of GC B cell formation during immune responses. Its loss leads to enhanced GC B cell numbers, indicating that Sema3E has an important role in maintaining the balance of the immune response.

Interestingly, the effect of Sema 3E on regulating Tfh cell population following immunization differs from that of GC B cells. Upon HDM challenge, The fold change of Tfh cells in the lung and spleen were almost similar between *Sema3e*$^{-/-}$ and WT mice (Table 4.1), suggesting that the expansion of Tfh cells following HDM exposure is impaired compared to WT mice. Indeed the

significant difference observed between *Sema3e*$^{-/-}$ and WT Tfh cells in HDM-challenged mice is due to its baseline differences not because of Sema 3E regulatory role following HDM. This indicates that Sema3E may play a role in the optimal expansion of Tfh cells in response to allergic challenges. The absence of Sema3E appears to restrict or limit the magnitude of the Tfh cell response to HDM, resulting in a less pronounced increase in their numbers compared to WT mice. However, in the spleen, the mean number of Tfh cells decreased following the HDM challenge in both WT and *Sema3e*$^{-/-}$ mice (Table 4.1), indicating that upon immunization with HDM, Tfh cells may migrate to the lung as the site of inflammation so their number would be lower following HDM challenge compare to the steady state.

Overall, I showed that upon HDM immunization, the number of GC B cells significantly increased in the absence of Sema3E, indicating a key negative regulatory role for this protein in GC B cells following HDM immunization in the mouse model of allergic asthma. However, the number of Tfh cells slightly increased, especially in the lung but Sema 3E doesn't have a prominent regulatory role on HDM-induced Tfh cells, and the difference between *Sema3e*$^{-/-}$ and WT in the HDM model is the reflection of their baseline difference.

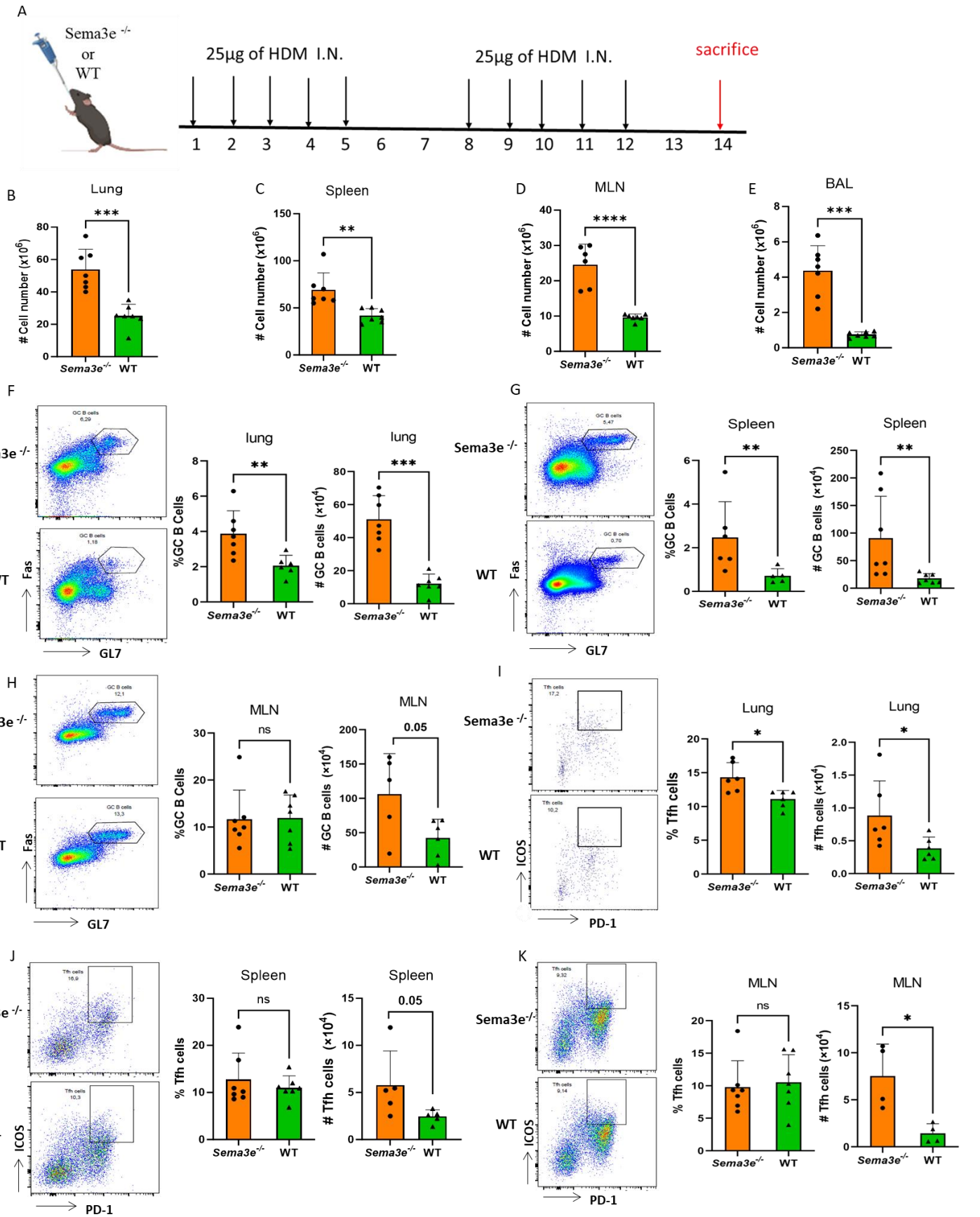

A
Sema3e -/-
or
WT
25µg of HDM I.N.
25µg of HDM I.N.
sacrifice
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
Lung
Cell number (x10^6)
Sema3e-/- WT
C
Spleen
Cell number (x10^6)
Sema3e-/- WT
D
MLN
Cell number (x10^6)
Sema3e-/- WT
E
BAL
Cell number (x10^6)
Sema3e-/- WT
F
Sema3e -/-
WT
Fas
GL7
lung
%GC B Cells
lung
GC B cells (x10^4)
G
Sema3e -/-
WT
Fas
GL7
Spleen
%GC B Cells
Spleen
GC B cells (x10^4)
H
Sema3e -/-
WT
Fas
GL7
MLN
ns
%GC B Cells
MLN
0.05
GC B cells (x10^4)
I
Sema3e -/-
WT
ICOS
PD-1
Lung
% Tfh cells
Lung
Tfh cells (x10^4)
J
Sema3e -/-
WT
ICOS
PD-1
Spleen
ns
% Tfh cells
Spleen
0.05
Tfh cells (x10^4)
K
Sema3e-/-
WT
ICOS
PD-1
MLN
ns
% Tfh cells
MLN
Tfh cells (x10^4)

Figure 4.6 The lack of Sema3E led to increased GC responses following the HDM challenge.

Flow cytometry analysis was performed to measure the total cell numbers in the lung (B), spleen (C), MLNs (D), and BAL (E). The numbers of GC B cells ($B220^+GL7^+Fas^+$) was counted in the lung (F), spleen (G), and MLNs (H) of *Sema3e*$^{-/-}$ and WT mice upon HDM immunization. Tfh cells ($CD3^+CD4^+CXCR5^+PD\text{-}1^+ICOS^+$) were analyzed within the lung (I), spleen (J), and MLNs (K) in the absence of Sema3E. The data presented, are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$; **, $p<0.01$; ***, $p < 0.001$; ns; not significant; N=5-7 mice in each group.

Table 4.1 The fold changes in GC B cells and Tfh cells population in *Sema3e*$^{-/-}$ and WT mice following the HDM challenge.

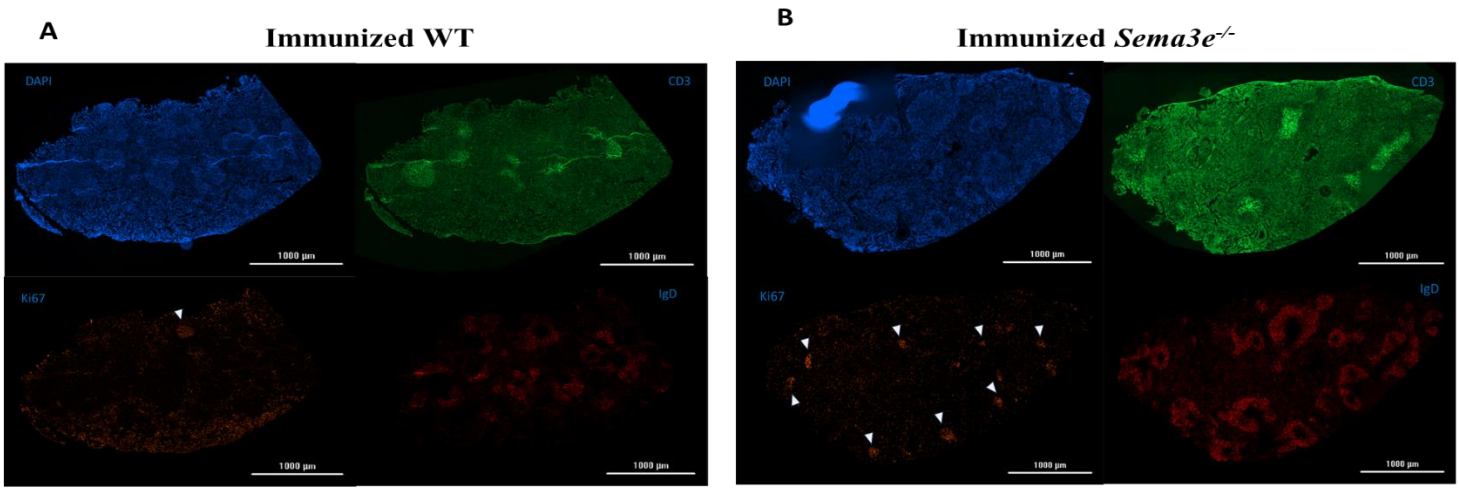
Lung GC B cells			
	Naïve (Mean)	**HDM-challenged (Mean)**	**Fold changes**
Sema3e-/-	7.04	51.386	7.26
WT	1.83425	12.1429	6.62
Splenic GC B cells			
Sema3e-/-	8.44357	90.865	10.761
WT	1.9569	18.1	9.249
Lung Tfh cells			
Sema3e-/-	0.8074	0.8865	1.09
WT	0.243963	0.385292	1.579
Splenic Tfh cells			
Sema3e-/-	12.961	5.7874	0.446
WT	3.4396	2.4416	0.709

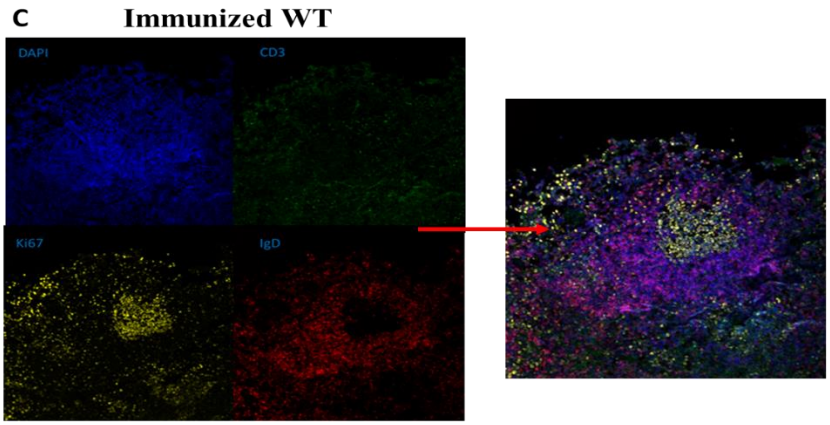
4.7 Sema3E deficiency led to remarkable GCs formation upon HDM challenge.

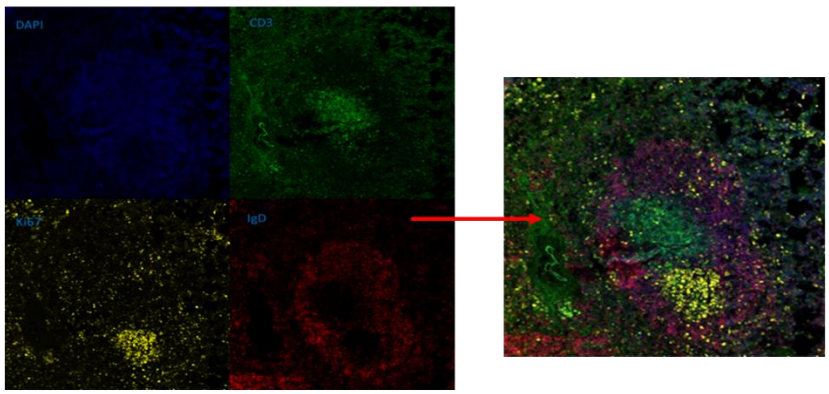
In the next step, I investigated the impact of Sema3E absence on GCs formation in spleen tissue following HDM immunization. IF staining demonstrated that Sema3E absence significantly increased GC formation in the spleen following exposure to HDM (Figure 4.7). Spleen tissue from *Sema3e*$^{-/-}$ mice exhibited a remarkably higher number of GCs ($Ki67^{+}IgD^{+}$) compared to WT controls after the HDM challenge (Figure 4.7 A &B). This observation suggests that Sema3E plays a critical role in modulating GC formation during allergic responses and it supports the hypothesis that Sema3E may act as a negative regulator of GC formation, limiting the magnitude of the immune response during allergen exposure.

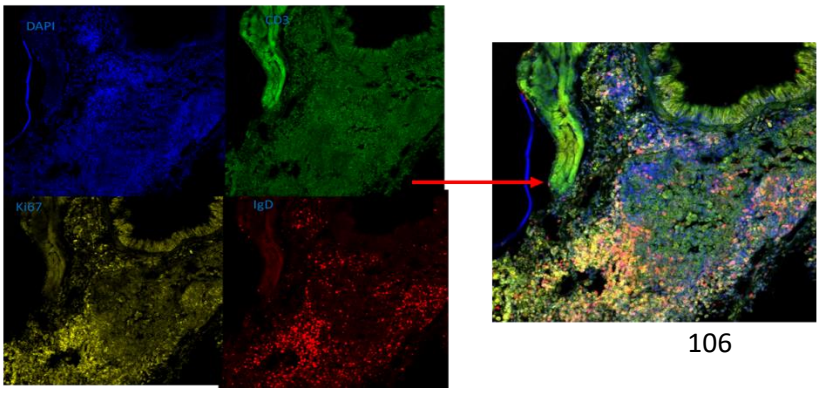
The shape of GC is shown in detail in *Sema3e*$^{-/-}$ and WT mice (Figure 4.7 C & D). It doesn't seem that the absence of Sema3E affects GC shape or structure.

I was also interested in investigating if the absence of Sema3E could lead to the formation of GC-like structures in the lung following HDM immunization. However, Sema3E deficiency did not lead to typical GC formation in the lung following the 2-week acute HDM challenge (Figure 4.7 E). I observed B cells, proliferating cells, and T cells in the lung although, they were not organized in one specific structure like in the spleen. This is likely because the lung is not a primary lymphoid tissue, where GC reactions typically occur. Additionally, the 2-week acute HDM challenge may not have provided a sufficient duration or the chronic antigenic stimulation needed to induce ectopic GC formation in the lung. While chronic inflammatory conditions or prolonged antigen exposure can sometimes result in the formation of tertiary lymphoid structures in non-lymphoid tissues like the lung.


A
Immunized WT
B
Immunized Sema3e-/-
DAPI
CD3
Ki67
IgD
1000 µm


C
Immunized WT
DAPI
CD3
Ki67
IgD


D
Immunized Sema3e-/-
DAPI
CD3
Ki67
IgD


E
Lung of Immunized Sema3e-/-
DAPI
CD3
Ki67
IgD

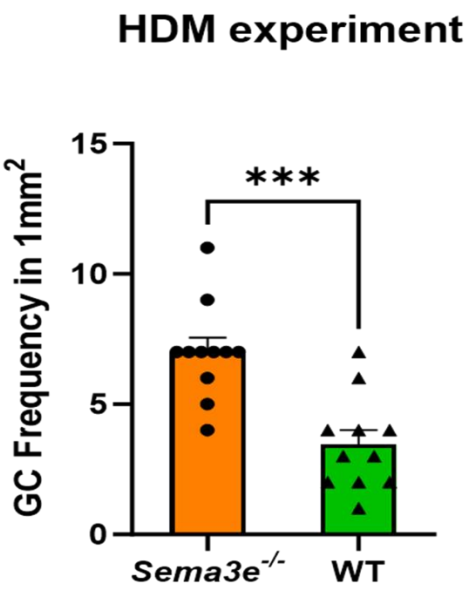

HDM experiment
GC Frequency in 1mm2
15
10
5
0
***
Sema3e-/-
WT

Figure 4.7 Sema3E regulates germinal center formation following HDM immunization.

GC formation was measured by histology (IF staining). Spleen sections (5 μm) of HDM-challenged *Sema3e*$^{-/-}$ and WT mice were labeled with CD3-FITC (green), Ki-67-AF568 (yellow), IgD-AF647 (red), and DAPI (blue). The image of the whole spleen was captured in WT and *Sema3e*$^{-/-}$ mice and the frequency of Ki67^{+}IgD^{+} area was counted as GC (A & B respectively). The scale bar represents 1000 μm (A & B). The structure of GC is shown in detail in the spleen of immunized WT (C) and *Sema3e*$^{-/-}$ (D) and lung of immunized *Sema3e*$^{-/-}$ mice (E). The scale bar represents 200 μm. The number of GC was counted in each section, and each symbol represented one GC (Ki67^{+}IgD^{+} area) (F). Statistical analysis was done to evaluate the difference between GC frequency in *Sema3e*$^{-/-}$ and WT mice. Bars show mean +/-SEM; ***, $p < 0.001$.

4.8 The number of plasma cells and plasmablasts increased upon HDM immunization in *Sema3e*$^{-/-}$ mice.

To investigate whether the absence of Sema3E affects immune cells producing antibodies following immunization with HDM, I evaluated the plasma cells and plasmablasts population in *Sema3e*$^{-/-}$ and WT mice upon 2 weeks of HDM challenge. Consistent with our baseline steady-state data, flow cytometry analysis indicated that the number of plasma cells (IgD^{-}Sca1^{+}B220^{-}CD138^{+}) and plasmablasts (IgD^{-}Sca1^{+}B220^{+}CD138^{+}) dramatically increased in the lung (Figure 4.8 A) and spleen (Figure 4.8 B) of *Sema3e*$^{-/-}$ mice compared to WT control. Even though MLN plasma cells and plasmablasts (Figure 4.8 C) are slightly higher in immunized *Sema3e*$^{-/-}$ mice, it didn't reach statistical significance.

In the lung, the absence of Sema3E led to a massive expansion of plasma cells and plasmablasts following the HDM challenge compared to WT mice (Table 4.2). The fold change for plasma cells (30.942) in *Sema3e*$^{-/-}$ mice is much higher than in WT mice (7.69), and plasmablasts also showed a higher expansion in *Sema3e*$^{-/-}$ mice (21.349) compared to WT (15.314) (Table 4.2). This suggests that Sema3E plays an important regulatory role in controlling the expansion of plasma cells and plasmablasts during allergic lung inflammation. Without Sema3E, the immune response is amplified, resulting in an excessive production of these antibody-secreting cells, which could contribute to increased antibody production (such as IgE and IgG1), exacerbating allergic responses. In the spleen, *Sema3e*$^{-/-}$ mice showed a similar fold change in plasma cells as WT mice (6.28 vs. 6.29) (Table 4.2), indicating that Sema3E deficiency does not significantly affect plasma

cell expansion in the spleen after the HDM challenge. However, the plasmablasts in the spleen exhibited a much larger fold increase in *Sema3e*$^{-/-}$ mice (40.394) compared to WT mice (22.26) (Table 4.2). This indicates that Sema3E selectively controls plasmablast expansion in the spleen, similarly to how it regulates these cells in the lung, although the effect is more pronounced in the spleen.

To summarize, I observed that the absence of Sema3E results in an exaggerated expansion of plasma cells and plasmablasts, particularly in the lung following the HDM challenge, suggesting that Sema3E normally acts to regulate or limit the differentiation and expansion of these antibody-producing cells.

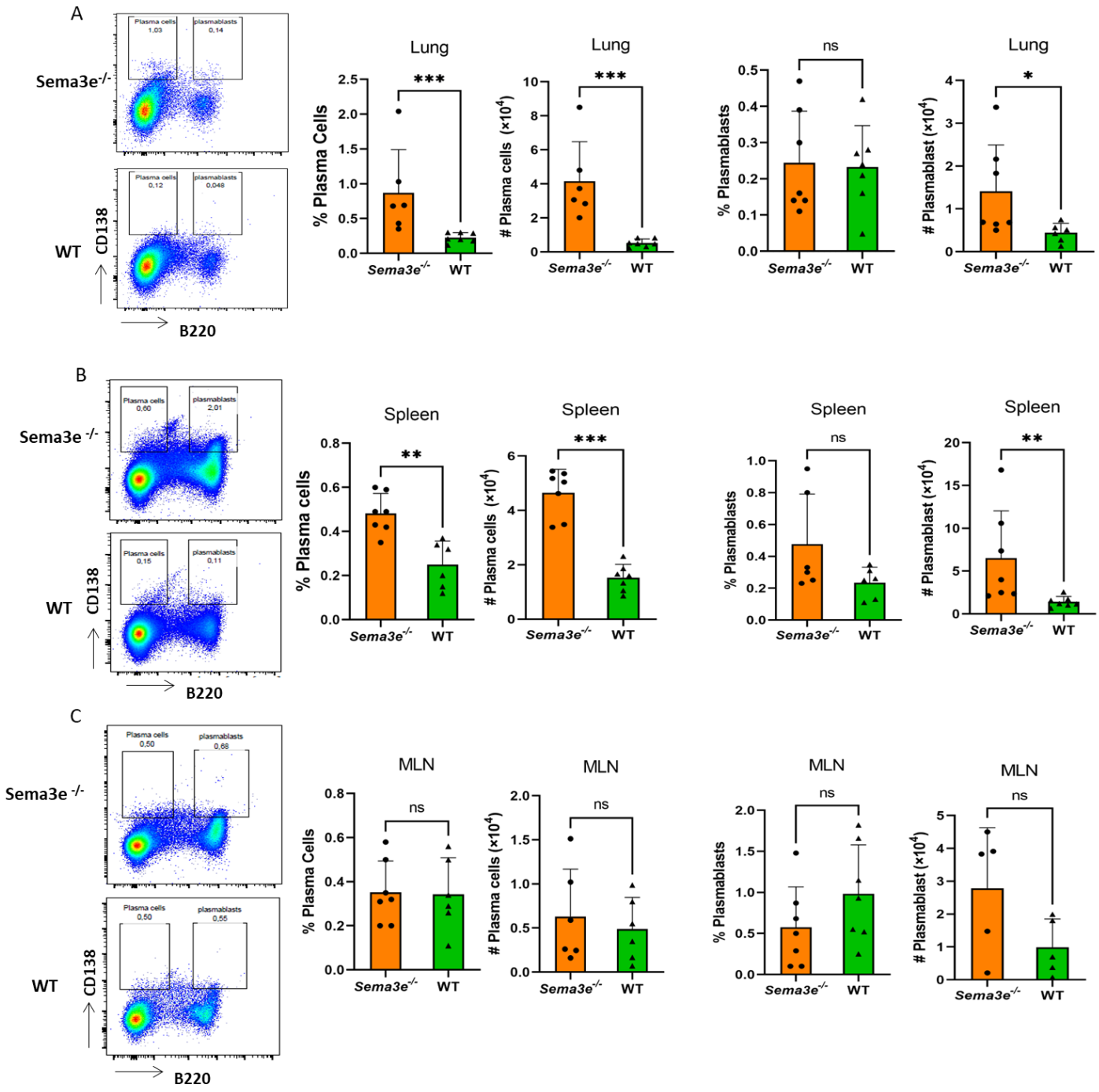


Figure 4.8 Sema3E absence led to increased plasma cells and plasmablasts.

FACS analysis was performed to measure the numbers of plasma cells ($IgD^{-}Sca1^{+}B220^{-}CD138^{+}$) and plasmablasts ($IgD^{-}Sca1^{+}B220^{+}CD138^{+}$) in the lung (A), spleen (B), and MLNs (C) of *Sema3e*$^{-/-}$ and WT mice following HDM immunization. The data presented, are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$; **, $p<0.01$; ***, $p < 0.001$; ns; not significant; N=5-7 mice in each group.

Table 4.2 The fold changes in plasma cells and plasmablasts population in *Sema3e*$^{-/-}$ and WT mice following the HDM challenge.

Lung plasma cells			
	Naïve (Mean)	**HDM-challenged (Mean)**	**Fold changes**
Sema3e-/-	0.134334	4.15667	30.942
WT	0.0688357	0.53	7.699
Splenic plasma cells			
Sema3e-/-	0.738417	4.64179	6.286
WT	0.242257	1.52507	6.295
Lung plasmablasts			
Sema3e-/-	0.0660925	1.41107	21.349
WT	0.02927	0.442983	15.134
Splenic plasmablasts			
Sema3e-/-	0.161581	6.52693	40.394
WT	0.0637317	1.41921	22.26

4.9 Sema3E regulated some B cells' co-stimulatory molecule expression and number of active B cells following allergen exposure.

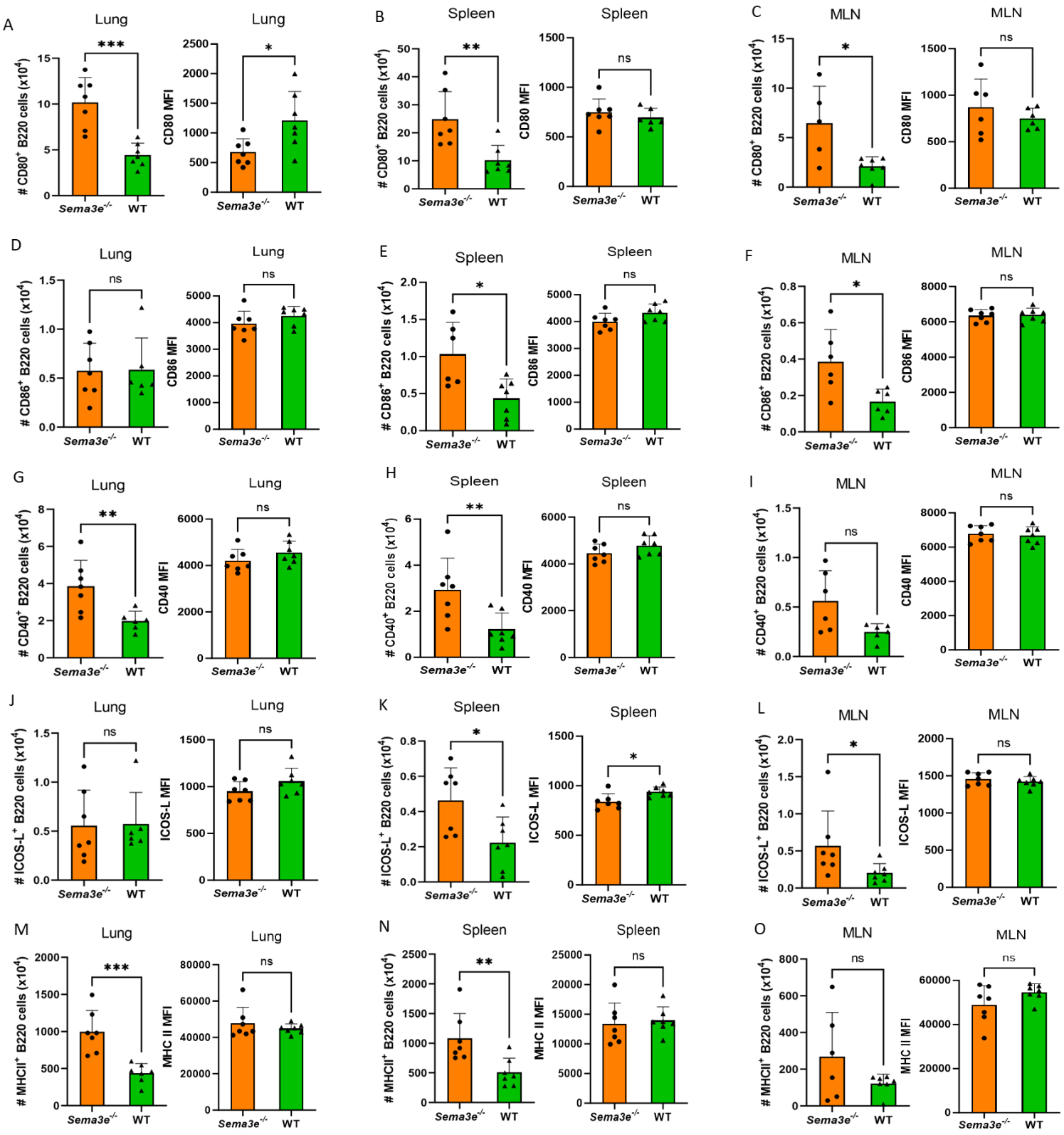
Following antigen exposure, B cells interact with T cells through their receptors, and co-stimulatory molecules obtain the critical additional signalling for T cells and B cells to become fully activated (487). Indeed, without co-stimulatory signals, B cells can not proliferate, and T cell functions are impaired and these unfunctional T cells undergo anergy or apoptosis (488-490). In

this regard, I aimed to investigate if Sema3E deficiency affects co-stimulatory molecule expression on activated B cells following HDM immunization. To address this question, I evaluated the number and the expression of c-stimulatory molecules including CD80 (gated as $CD80^{+}B220$), CD86 (gated as $CD86^{+}B220$), CD40 (gated as $CD40^{+}B220$), ICOS-L (gated as $ICOS\text{-}L^{+}B220$), and MHCII (gated as $MHCII^{+}B220$) on B cells in *Sema3e*$^{-/-}$ and WT mice upon HDM challenge. Flow cytometry data demonstrated that upon immunization and in the absence of Sema3E, the number of B cells that expressed CD80 was significantly higher in the lung, spleen, and MLNs of the *Sema3e*$^{-/-}$ compared to the WT control group (Figure 4.9 A-C respectively). Interestingly, Sema3E deficiency led to a remarkable decrease in the expression of CD80 by lung B cells while there was no difference in the expression of CD80 on B cells from SLO including spleen and MLNs (Figure 4.9 A-C respectively). It seems that CD80 expression on B cells is somehow positively regulated by Sema3E since its deficiency caused a significant reduction in the baseline expression of CD80 by naïve splenic B cells as well as a reduction in CD80 expression by lung residents B cells following HDM immunization.

The number of $CD86^{+}$ B cells was also affected, and *Sema3*$^{-/-}$ mice showed a higher number of B cells expressing CD86 in the lung, spleen, and MLNs (Figure 4.9 D-F respectively) although the absence of Sema3E didn't affect the expression level of CD86. Another co-stimulatory molecule CD40 which plays a critical role in B cell-T cell interaction, was impacted by the lack of Sema3E and *Sema3e*$^{-/-}$ mice significantly had a higher number of $CD40^{+}$ B cells, especially in the lung and spleen (Figure 4.9 G &H respectively) following HDM challenge.

Furthermore, flow cytometry results showed that immunized *Sema3e*$^{-/-}$ mice had a remarkable number of $ICOS\text{-}L^{+}$ B cells compared to WT mice (Figure 4.9 J-L). Additionally, ICOS-L expression can be partially affected by Sema3E after B cell activation upon HDM immunization since Sema3E's absence led to a substantial reduction in the level of ICOS-L expression on the splenic B cells (Figure 4.9 K).

Consistent with the higher number of B cells expressing co-stimulatory molecules, $MHCII^{+}$ B cells were also affected by Sema3E in the acute model of allergic asthma. *Sema3e*$^{-/-}$ mice had notable amounts of B cells expressing MHCII in the lung and spleen in comparison to WT mice (Figure 4.9 M-O). In conclusion, these data indicated the key role of Sema3E in the regulation of active B cell numbers and expression of CD80 and ICOS-L following immunization.


A
Lung
***
CD80+ B220 cells (x10^4)
Lung
*
CD80 MFI
Sema3e-/-
WT
B
Spleen
**
CD80+ B220 cells (x10^4)
Spleen
ns
CD80 MFI
C
MLN
*
CD80+ B220 cells (x10^4)
MLN
ns
CD80 MFI
D
Lung
ns
CD86+ B220 cells (x10^4)
Lung
ns
CD86 MFI
E
Spleen
*
CD86+ B220 cells (x10^4)
Spleen
ns
CD86 MFI
F
MLN
*
CD86+ B220 cells (x10^4)
MLN
ns
CD86 MFI
G
Lung
**
CD40+ B220 cells (x10^4)
Lung
ns
CD40 MFI
H
Spleen
**
CD40+ B220 cells (x10^4)
Spleen
ns
CD40 MFI
I
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ns
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MLN
ns
CD40 MFI
J
Lung
ns
ICOS-L+ B220 cells (x10^4)
Lung
ns
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Spleen
*
ICOS-L+ B220 cells (x10^4)
Spleen
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ICOS-L MFI
L
MLN
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ICOS-L+ B220 cells (x10^4)
MLN
ns
ICOS-L MFI
M
Lung
***
MHCII+ B220 cells (x10^4)
Lung
ns
MHC II MFI
N
Spleen
**
MHCII+ B220 cells (x10^4)
Spleen
ns
MHC II MFI
O
MLN
ns
MHCII+ B220 cells (x10^4)
MLN
ns
MHC II MFI

Figure 4.9 *Sema3e*$^{-/-}$ mice showed a remarkable number of B cells expressing remarkable co-stimulatory molecules.

Following immunization with HDM in the acute model of allergic asthma, FACS analysis was done to measure the total number and expression of CD80 (CD80^{+}B220) in the lung (A), spleen (B), and MLNs (C) of *Sema3e*$^{-/-}$ and WT mice. CD86 (CD86^{+}B220) and CD40 (CD40^{+}B220) expression were measured in the lung, spleen, and MLNs in the absence of Sema3E (D-I respectively) and WT mice. Moreover, ICOS-L expression (ICOS-L^{+}B220) was analyzed in the lung (J), spleen (K), and MLNs (L) of *Sema3e*$^{-/-}$ and WT mice. MHCII (MHCII^{+}B220) expression was also measured (M-O) in immunized *Sema3e*$^{-/-}$ and WT mice. The data are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$; **, $p<0.01$; ***, $p < 0.001$; ns; not significant; N=5-7 mice in each group.

4.10 Sema3E negatively regulated IgE^{+} and IgG^{+} B cell population and serum immunoglobulin production.

Following immunization, GC is formed, and B cells undergo Ig class switching. Some produced IgE or IgG, binds to the B cell membrane (IgE^{+}B220 and IgG^{+} B220 cells respectively) and play a critical assisting role in antigen processing and signal transduction (491). In this regard, I studied IgE^{+} B cells (IgE^{+} B220^{+} cells) and IgG^{+} B cells (IgG^{+} B220^{+} cells) in the lung, spleen, and MLNs of *Sema3e*$^{-/-}$ and WT mice upon the HDM challenge to see if post-inflammation results reaffirm my baseline results indicating a regulatory effect of Sema3E. As shown in Figure 4.10 A & B, the number of IgE^{+} B cells substantially expanded not only in the lung, as the main organ affected by asthma (Figure 4.10 A), but also in the spleen (Figure 4.10 B) of *Sema3e*$^{-/-}$ mice compared to WT group following immunization with HDM, although no significant difference was observed in MLNs (Figure 4.10 C). Consistent with IgE^{+} B cells, the total number of IgG^{+} B cells remarkably increased in the absence of Sema3E (Figure 4.10 D-F).

The fold changes analysis (Table 4.3) demonstrated that Sema3E deficiency results in more expansion of IgE^{+} B cells and IgG^{+} B cells, especially in the lung following the HDM challenge

compared to WT mice (Table 4.3). IgE^+ B cells increased by 4.085-fold in the lung of *Sema3e*$^{-/-}$ mice following HDM, while the WT mice showed a 2.377-fold increase (Table 4.3). Furthermore, IgG^+ B cells increased by 2.976-fold in the spleen in the absence of Sema3E, compared to 1.528-fold in WT mice although the splenic IgE^+B cell population was not remarkably affected by the absence of Sema3E (Table 4.3). These results suggest that Sema3E normally may play a regulatory role, possibly limiting the proliferation or activation of these B cells during allergic inflammation.

Unlike the naïve condition, the expression of IgE and IgG on B cells in the secondary lymphoid tissue did not change following the HDM challenge in *Sema3e*$^{-/-}$ immunized mice (Figure 4.8). It is possible that the differing effects of Sema3E deficiency on IgE and IgG expression in naive steady-state conditions versus following HDM immunization could be attributed to the regulatory roles of Sema3E in steady-state B cell homeostasis versus its interaction with inflammatory pathways activated during allergic responses.

ELISA was performed to evaluate the serum levels of total and HDM-specific immunoglobulin production in the *Sema3e*$^{-/-}$ and WT mice upon HDM immunization. The results indicated that asthmatic *Sema3e*$^{-/-}$ mice have substantially higher serum levels of total and HDM-specific IgE (Figure 4.9 G & H respectively) and IgG1 (Figure 4.9 I & J respectively) compared to WT control. Following HDM immunization, *Sema3e*$^{-/-}$ mice showed a 5.995 and a 1.441-fold increase in serum IgE and IgG1 levels respectively (Table 4.3). However, WT mice showed a slightly higher increase in serum IgE (6.645-fold increase) and a slight decrease (0.89-fold change) upon HDM. Serum IgE levels were slightly lower in *Sema3e*$^{-/-}$ mice compared to WT mice following HDM exposure, even though both groups showed a significant increase from baseline. This indicates that, while Sema3E deficiency still allows for strong IgE production, it seems to restrain the upper limit of IgE secretion compared to WT mice. In the absence of Sema3E, serum IgG1 levels increased by

1.441-fold following HDM, while WT mice showed a slight decrease (0.89-fold) in IgG1 levels. This may suggest that Sema3E absence promotes IgG1 production, while in WT mice, the IgG1 response is more tightly controlled. My data demonstrated that Sema3E plays a nuanced role in regulating IgE and IgG1 antibody production following an allergic challenge.

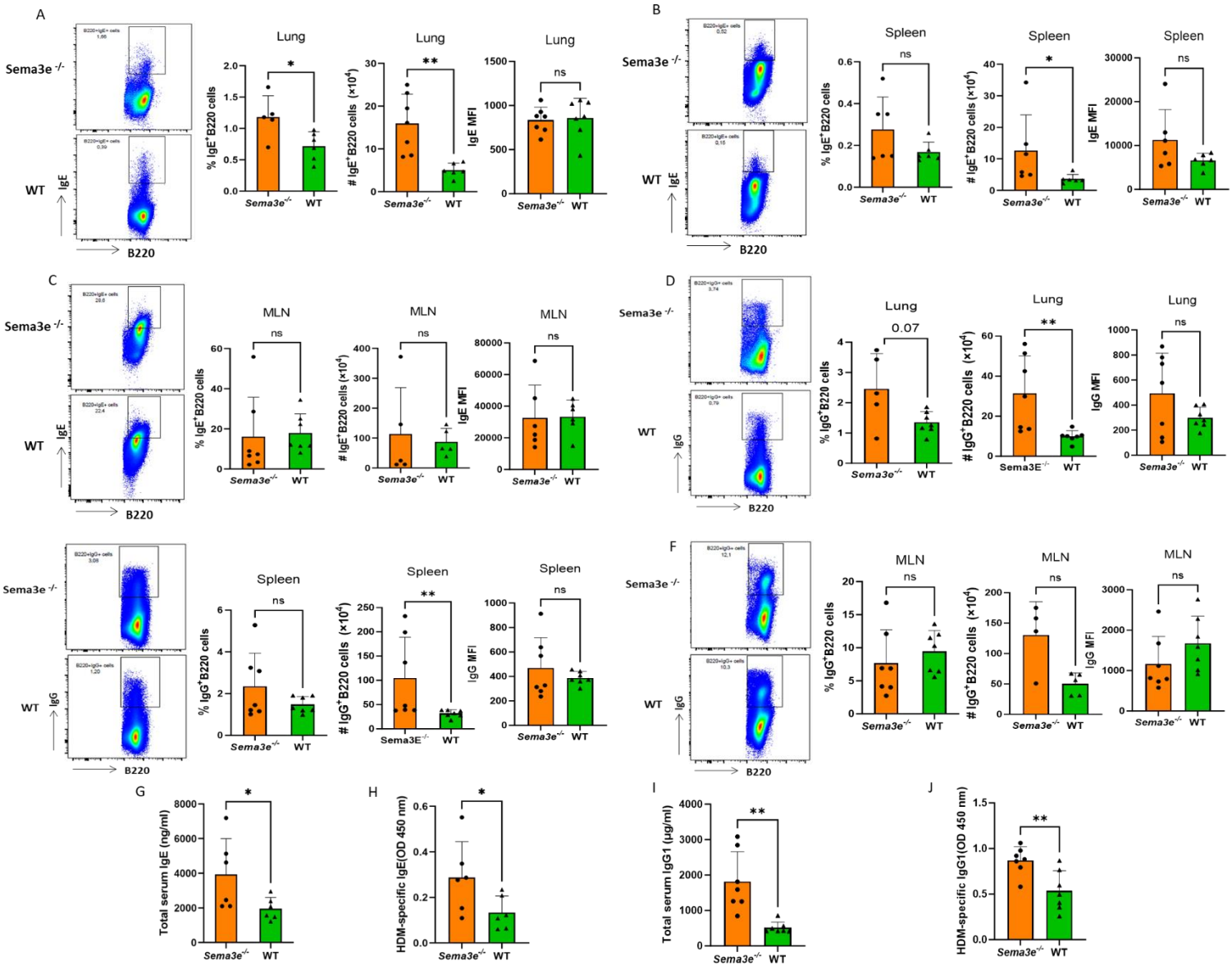


Figure 4.10 Sema3E deficiency led to a higher number of IgE$^+$ and IgG$^+$ B cells and serum antibody production following HDM immunization.

Upon HDM immunization, FACS analysis was done to evaluate the number of IgE$^+$B cells (IgE$^+$B220$^+$) (A-C) and IgG$^+$B cells (IgG$^+$B220$^+$) (D-F) in the lung, spleen, and MLNs of *Sema3e*$^{-/-}$ and WT mice. ELISA was performed to measure serum levels of total IgE (G), HDM-specific IgE (H), and IgG1 (I & J respectively) in immunized *Sema3e*$^{-/-}$ and WT mice. The data are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$; **, $p<0.01$; ***, $p < 0.001$; ns; not significant; N=5-7 mice in each group.

Table 4.3 The fold changes in IgE^+ B cells, IgG^+ B cells, and serum antibody production in *Sema3e*$^{-/-}$ and WT mice following the HDM challenge.

Lung IgE^+B220^+ cells			
	Naïve (Mean)	**HDM-challenged (Mean)**	**Fold changes**
Sema3e-/-	3.91543	15.9979	4.085
WT	2.12843	5.06	2.377
Splenic IgE^+B220^+ cells			
Sema3e-/-	7.57467	12.6466	1.669
WT	2.61233	3.68008	1.408
Lung IgG^+B220^+ cells			
Sema3e-/-	10.5829	31.4993	2.976
WT	6.45571	9.86857	1.528
Splenic IgG^+B220^+ cells			
Sema3e-/-	21.6	104.556	4.840
WT	15.4971	31.7993	2.051
Serum total IgE			
Sema3e-/-	1312.72	7869.89	5.995
WT	588.552	3911.25	6.645
Serum Total IgG1			
Sema3e-/-	1257.7	1813.51	1.441
WT	576.425	514.305	0.89

4.11 *Sema3e*$^{-/-}$ mice showed a significant increase in the lung inflammatory cells following the HDM challenge.

Our lab previously reported that mice deficient in Sema3E showed asthma exacerbation (increased airway inflammation, hyperresponsiveness, and remodelling) during HDM-induced allergic inflammation (476), showing the importance of Sema3E in asthma control. To investigate the compatibility of my results with the previous data, I evaluated lung inflammatory cells in the *Sema3e*$^{-/-}$ and WT mice following the challenge with HDM in the acute model of allergic asthma. Flow cytometry analysis was done to investigate the numbers of eosinophils (MertK^{-}CD64^{-} CD11c^{+}SinglecF^{+}), neutrophils (MertK^{-}CD64^{-}Gr-1^{+} CD11b^{+}), alveolar macrophages (MertK^{+}CD64^{+}CD11b^{+}SinglecF^{low}) and interstitial macrophages (MertK^{+}CD64^{+}CD11b^{+}SinglecF^{high}) in the lung of *Sema3e*$^{-/-}$ and WT mice.

By flow cytometry analysis, MertK^{-}CD64^{-} cells were selected from live CD45^{+} cells as non-macrophage population. Then eosinophil and neutrophil were characterized as CD11c^{+}SinglecF^{+} and Gr-1^{+} CD11b^{+} cells respectively. My data demonstrated that the number of eosinophils and neutrophils increased in the lung of *Sema3e*$^{-/-}$ mice compared to the WT control group (Figure 4.11 A & B respectively) following the HDM challenge. In addition, I measured lung macrophages including alveolar and interstitial macrophages to investigate if the absence of Sema3E had an impact on these cells. Flow cytometry analysis implicated that the number of alveolar macrophages is comparable between *Sema3e*$^{-/-}$ and WT control mice (Figure 4.11 C). However, *Sema3e*$^{-/-}$ mice had an increased interstitial macrophage population compared to the WT mice (Figure 4.11 D). Sema3E deficiency disrupted the balance between macrophages and tipped the scales in favour of the interstitial macrophage population (Figure 4.11 E & F).

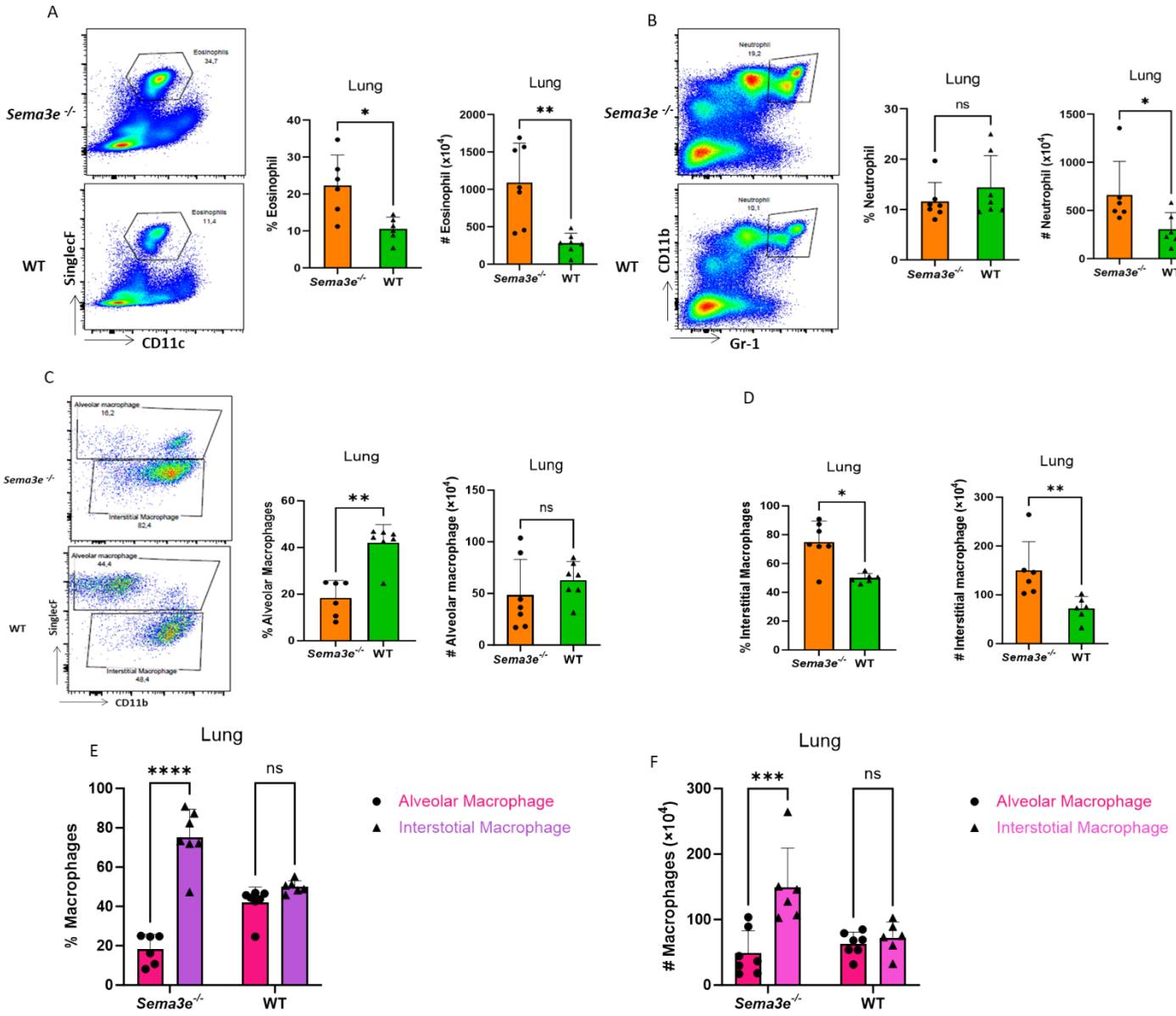


Figure 4.11 The absence of Sema3E increased lung inflammatory cells.

Flow cytometry analysis was performed to evaluate the number of eosinophils (MertK^{-}CD64^{-} CD11c^{+}SinglecF^{+}) (A), neutrophils (MertK^{-}CD64^{-}Gr-1^{+} CD11b^{+}) (B), alveolar macrophages (MertK^{+}CD64^{+}CD11b^{+}SinglecF^{low}) (C) and interstitial macrophages (MertK^{+}CD64^{+}CD11b^{+}SinglecF^{high}) (D) in the lung of *Sema3e*$^{-/-}$ and WT control group upon HDM challenge. The lung macrophage changes were analyzed in the absence of Sema3E (E & F). The data are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$; **, $p<0.01$; ***, $p < 0.001$; ns; not significant; N=5-7 mice in each group.

4.12 Lack of Sema3E increased inflammatory cells in the BALF and induced cytokine production.

BALF is a fluid obtained from bronchoalveolar lavage. It is a crucial diagnostic tool that provides details on the inflammatory, infectious, and immunological processes occurring in the pulmonary alveoli (492). BALF is enriched with various cellular and noncellular content including epithelial cells, macrophages, cytokines, extracellular vesicles, and many more (493, 494). BALF makes the perfect "lung liquid biopsy" since it can be used to extract both cellular and noncellular material from lung alveoli and distal airways. It is currently being thoroughly studied as a potential source of biomarkers for various lung disorders (495). Therefore, I collected BALF from *Sema3e*$^{-/-}$ and WT mice after HDM immunization in the acute model of allergic asthma. To measure the numbers of eosinophils (MertK^{-}CD64^{-}CD11c^{+}SinglecF^{+}), neutrophils (MertK^{-}CD64^{-}Gr-1^{+} CD11b^{+}), alveolar macrophages (MertK^{+}CD64^{+}CD11b^{+}SinglecF^{low}) and interstitial macrophages (MertK^{+}CD64^{+}CD11b^{+}SinglecF^{high}) within the BALF, flow cytometry analysis was performed. Consistent with lung inflammatory cells results, the number of inflammatory cells including eosinophil and neutrophil were remarkably higher in *Sema3e*$^{-/-}$ mice compared to WT littermate control (Figure 4.12 A & B respectively). Although the number of BALF alveolar macrophages didn't show any difference between *Sema3e*$^{-/-}$ and WT mice (Figure 4.12 C), interstitial macrophages were affected by lack of Sema3E and demonstrated a significant increase (Figure 4.12 D) in immunized *Sema3e*$^{-/-}$ mice. No difference was observed between the alveolar and interstitial macrophages ratio in *Sema3e*$^{-/-}$ and WT mice (Figure 4.12 E & F).

Th2/Th17 cytokines levels play an essential role in the exacerbation of allergic asthma. In this regard using Mesoscale analysis, I evaluated the level of Th2 and Th17 cytokines in the BALF of *Sema3e*$^{-/-}$ and WT mice upon HDM challenge. My data indicated that Sema3E deficiency significantly induced levels of inflammatory cytokines including IL-17A, IL-4, IL-5, and IL-13 in the BALF (Figure 4.12 G-J respectively). Collectively, I demonstrated that Sema3E plays a key regulatory role in lung inflammation and inflammatory cytokines production in the acute model of allergic asthma.

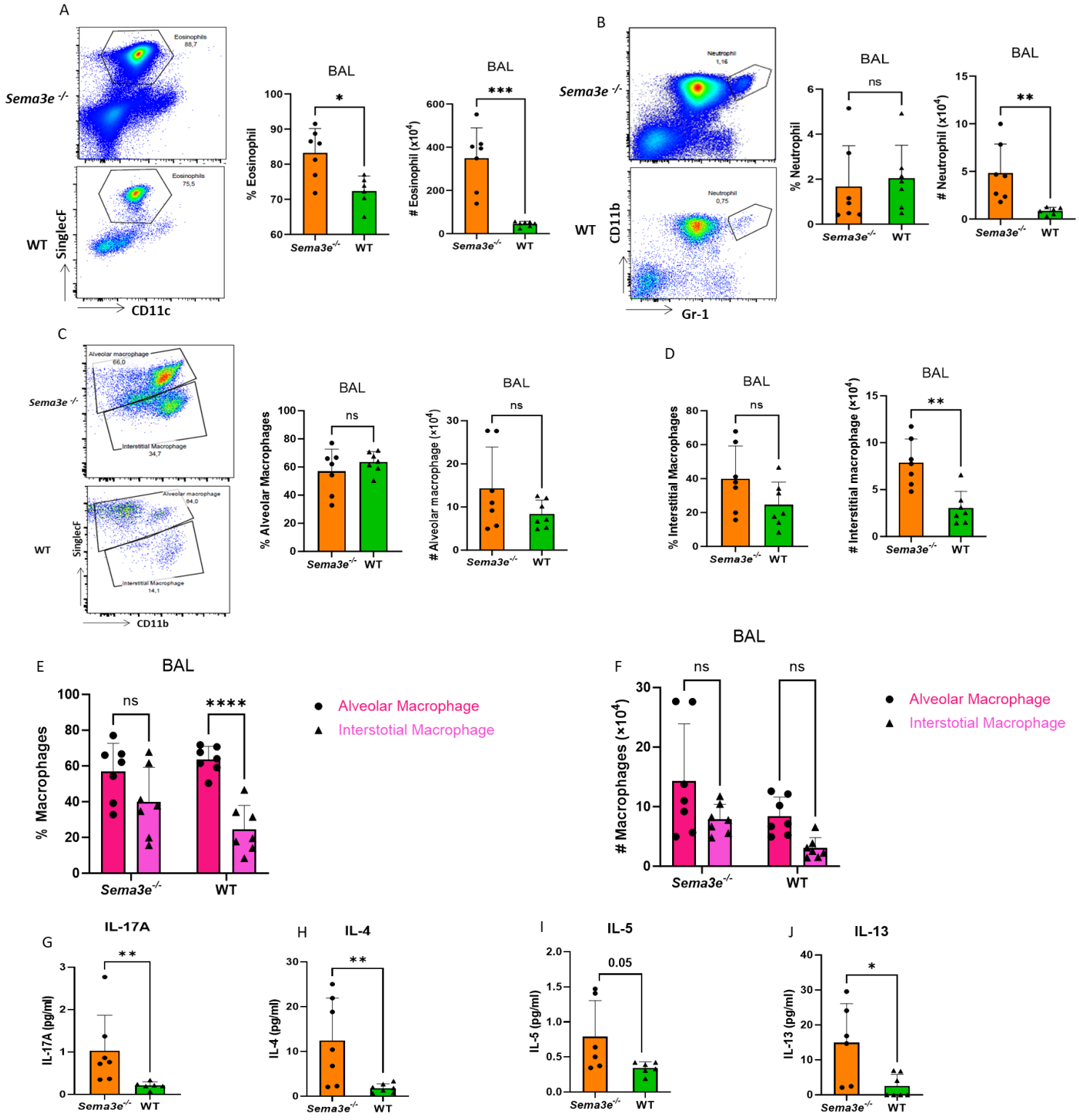

A
Sema3e -/-
WT
SinglecF
CD11c
Eosinophils
88,7
Eosinophils
75,5
BAL
% Eosinophil
# Eosinophil (x10⁴)
Sema3e-/-
WT
B
Neutrophil
1,16
Neutrophil
0,75
CD11b
Gr-1
% Neutrophil
# Neutrophil (x10⁴)
ns
C
Alveolar macrophage
66,0
Interstitial Macrophage
34,7
Alveolar macrophage
64,0
Interstitial Macrophage
14,1
% Alveolar Macrophages
# Alveolar macrophage (x10⁴)
D
% Interstitial Macrophages
# Interstitial macrophage (x10⁴)
E
% Macrophages
Alveolar Macrophage
Interstotial Macrophage
F
Macrophages (x10⁴)
G
IL-17A
IL-17A (pg/ml)
H
IL-4
IL-4 (pg/ml)
I
IL-5
IL-5 (pg/ml)
0.05
J
IL-13
IL-13 (pg/ml)

Figure 4.12 *Sema3*$^{-/-}$ mice had higher BALF inflammatory cells and cytokines compared to WT mice.

FACS analysis was performed to investigate the number of eosinophils (MertK^{-}CD64^{-}CD11c^{+}SinglecF^{+}) (A), neutrophils (MertK^{-}CD64^{-}Gr-1^{+} CD11b^{+}) (B), alveolar macrophages (MertK^{+}CD64^{+}CD11b^{+}SinglecF^{low}) (C) and interstitial macrophages (MertK^{+}CD64^{+}CD11b^{+}SinglecF^{high}) (D) in the *Sema3e*$^{-/-}$ and WT control group. The BALF macrophage changes were analyzed in the absence of Sema3E (E & F). Mesoscale was performed to evaluate the level of IL-17A (G), IL-4 (H), IL-5 (I), and IL-13 (J) in the BALF. The data are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$; **, $p<0.01$; ***, $p < 0.001$; ns; not significant; N=5-7 mice in each group.

4.13 *Sema3e*$^{-/-}$ T cells demonstrated higher stimulation potential.

In GC, Tfh cells are specifically dependent on continuous ICOS signalling after T cell priming to maintain their phenotype and interact with GC B cells, form GC, and produce antibodies (496, 497). various studies showed that ICOS-deficient mice exhibited profound defects in T cell activation, effector function, and Ab responses (498-500). Therefore, in the next step, I investigated whether the Sema3E protein regulates ICOS expression on CD4^{+} T cells. flow cytometry was done to measure the number of T cells expressing ICOS (CD3^{+}CD4^{+}ICOS^{+}) and the level of ICOS expression by T helper cells in *Sema3e*$^{-/-}$ and WT mice in the steady-state and following HDM exposure in an acute model of allergic asthma. Interestingly, naïve *Sema3e*$^{-/-}$ mice not only have a remarkable number of ICOS^{+}T cells but also have increased levels of ICOS expression in the lung, spleen, and Peyer's patches (Figure 4.13 A-C respectively). Consistent with the steady-state data, upon HDM immunization, *Sema3e*$^{-/-}$ mice had a significantly higher number of ICOS^{+}T cells and level of ICOS expression on CD4^{+} T cells in the lung, spleen, and MLNs compared to WT mice (Figure 4.13 D-F, respectively). Although following HDM challenge both *Sema3e*$^{-/-}$ and WT mice demonstrated expansion in ICOS^{+} T cells, the difference between WT and *Sema3e*$^{-/-}$ is more

reflection of their baseline difference cause the fold changes were almost similar in both groups (Table 4.4).

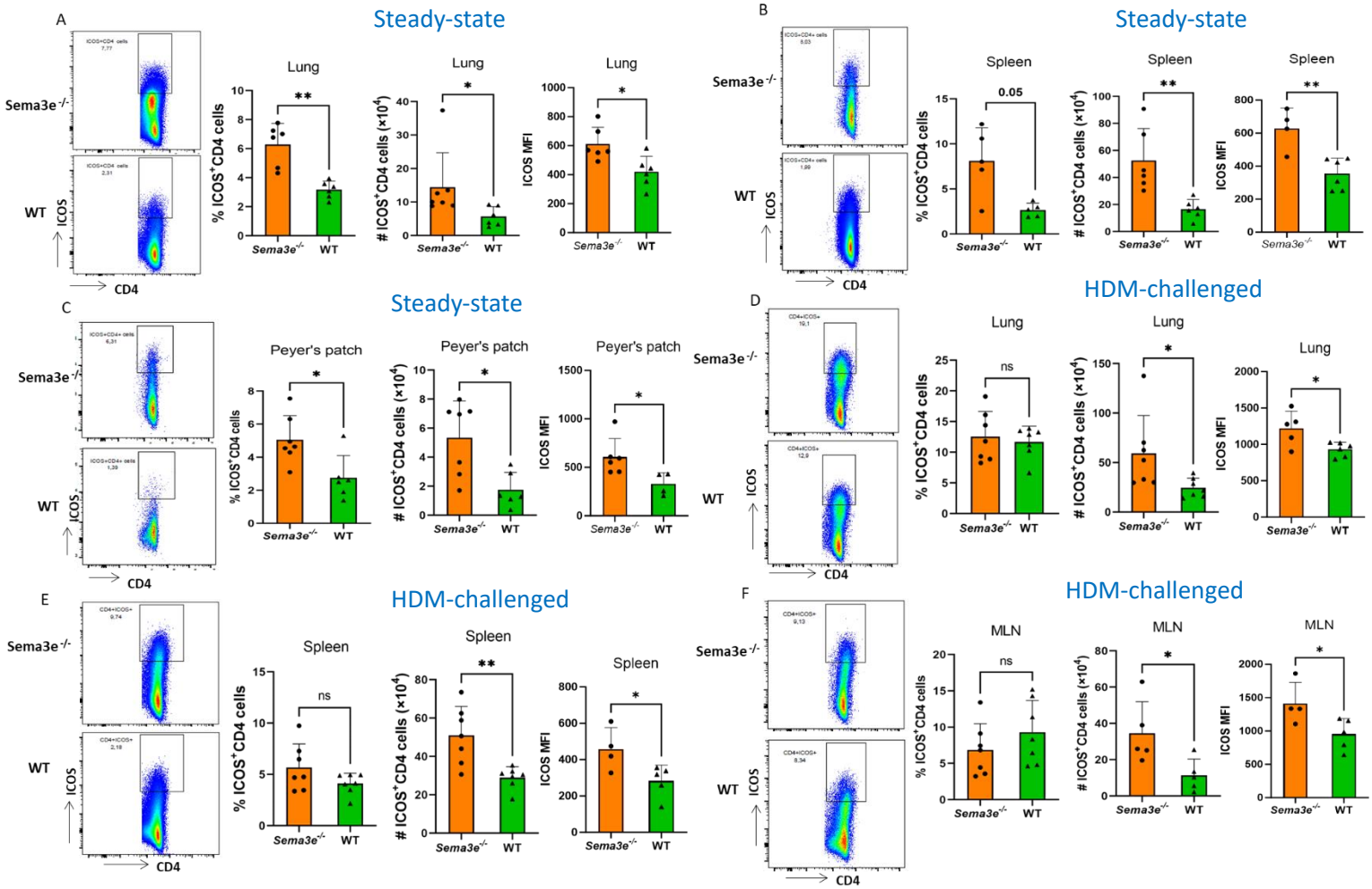


Figure 4.13 The absence of Sema3E led to increased ICOS expression on CD4+ T cells in both steady-state and HDM immunization.

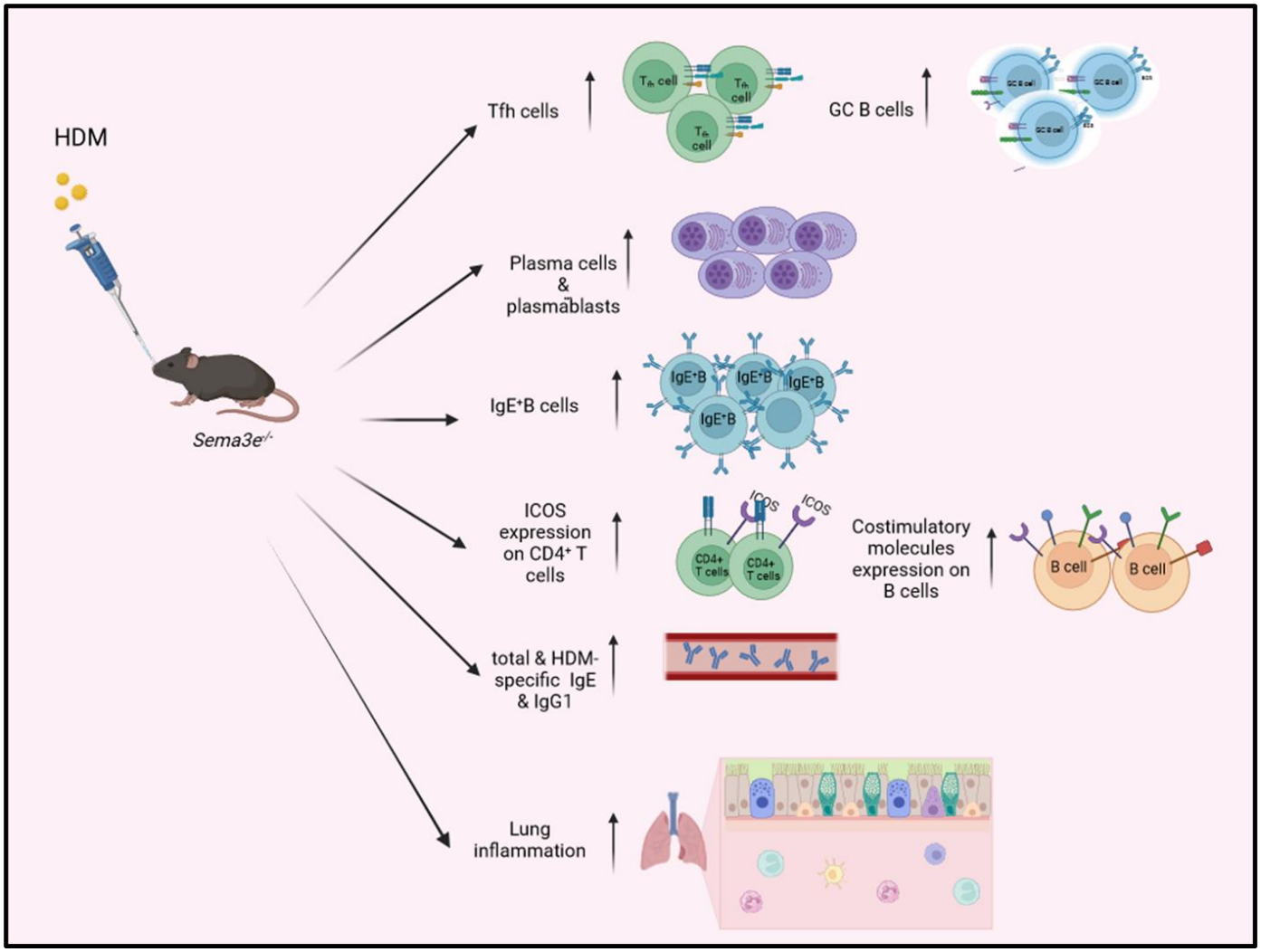
The baseline number of ICOS-expressing T cells ($CD3^+$ $CD4^+ICOS^+$) in the naïve *Sema3e*$^{-/-}$ and WT mice was evaluated by flow cytometry in the lung (A), spleen (B), and Peyer's patches (C). Following immunization with HDM in an acute model of allergic asthma, FACS analysis was performed in *Sema3e*$^{-/-}$ and WT mice to measure the cell number of $ICOS^+CD4$ T cells in the lung (D) and spleen (E) and MLNs (F). The data are representative of two different experiments with a similar outcome. Bars show mean +/- SEM; *, $p < 0.05$; **, $p<0.01$; ns, not significant; N=5-7 mice in each group.

Table 4.4 The fold changes in ICOS$^+$ T cells in *Sema3e*$^{-/-}$ and WT mice following the HDM challenge.

Lung ICOS$^+$ T cells			
	Naïve (Mean)	**HDM-challenged (Mean)**	**Fold changes**
Sema3e-/-	14.4579	59.3343	4.103
WT	5.73083	24.5686	4.287
Splenic ICOS$^+$ T cells			
Sema3e-/-	52.5775	50.9421	0.9688
WT	16.5842	28.9971	1.748

Graphical Abstract 2:

Aim 2: To investigate the role of Sema3E in HDM-induced GC responses and IgE production in allergic asthma.



Collectively, my data demonstrated that following immunization with HDM challenge, Sema3E absence led to exacerbated GC responses, and the number of Tfh, GC B cells, plasma cells, plasmablasts, IgE^+ B cells, IgG^+ B cells, and lung inflammatory cells and cytokines remarkably increased in *Sema3e*$^{-/-}$ mice. Moreover, *Sema3e*$^{-/-}$ $CD4^+$ T cells and B cells expressed higher levels of ICOS and costimulatory molecules respectively. The lack of Sema3E significantly induces serum total and HDM-specific immunoglobulins.

4.14 Sema3E may affect GC responses through the ICOS/ICOS-L signalling pathway.

Higher expression of ICOS on *Sema3e*$^{-/-}$ T cells compared to WT controls led us to consider the hypothesis that Sema3E may affect GC responses and Ig production through the ICOS/ICOS-L signalling pathway. Although the effect of blocking the ICOS/ICOS-L pathway on dampening inflammation is well studied, I aimed to disrupt GC formation in *Sema3e*$^{-/-}$ and WT mice by blocking the ICOS/ICOS-L pathway following the HDM challenge to reaffirm the regulatory role of Sema3E in ICOS expression, and to investigate if this intervention would lead to reduced GC responses and improve allergic asthma outcomes in *Sema3e*$^{-/-}$ mice. For this aim, I induced an acute model of allergic asthma by intranasal administration of HDM five days per week for two weeks. The ICOS/ICOS-L pathway was blocked by intraperitoneal co-administration of anti-ICOS-L or rat IgG as isotype control, three days per week for two weeks (Figure 4.14 A).

I observed that disrupting ICOS/ICOS-L effectively reduced the increased total cell numbers that had occurred due to the absence of Sema3E (Figure 4.14 B-D). As shown in Figure 4.14 B-D, the total cell number of the lung, spleen, and MLN in *Sema3e*$^{-/-}$ not only significantly decreased upon anti-ICOSL treatment compared to the *Sema3e*$^{-/-}$ control treated with rat IgG, but also compared to control WT mice who received rat IgG and this may further suggest a key role of Sema3E in ICOS regulation.

Furthermore, blocking ICOS/ICOS-L interaction caused a remarkable decrease in the Tfh population in the lung, spleen, and MLNs (Figure 1.14 E-G, respectively). Consistent with reduced Tfh cells, I observed a substantial reduction in the number of GC B cells in the lung, spleen, and MLNs of *Sema3e*$^{-/-}$ and WT mice upon anti-ICOS-L treatment (Figure 4.14 H-J respectively).

A noteworthy point is that rat IgG injection affects the regulatory role of Sema3E since the significant difference between the WT and *Sema3*$^{-/-}$ has disappeared following rat IgG injection.

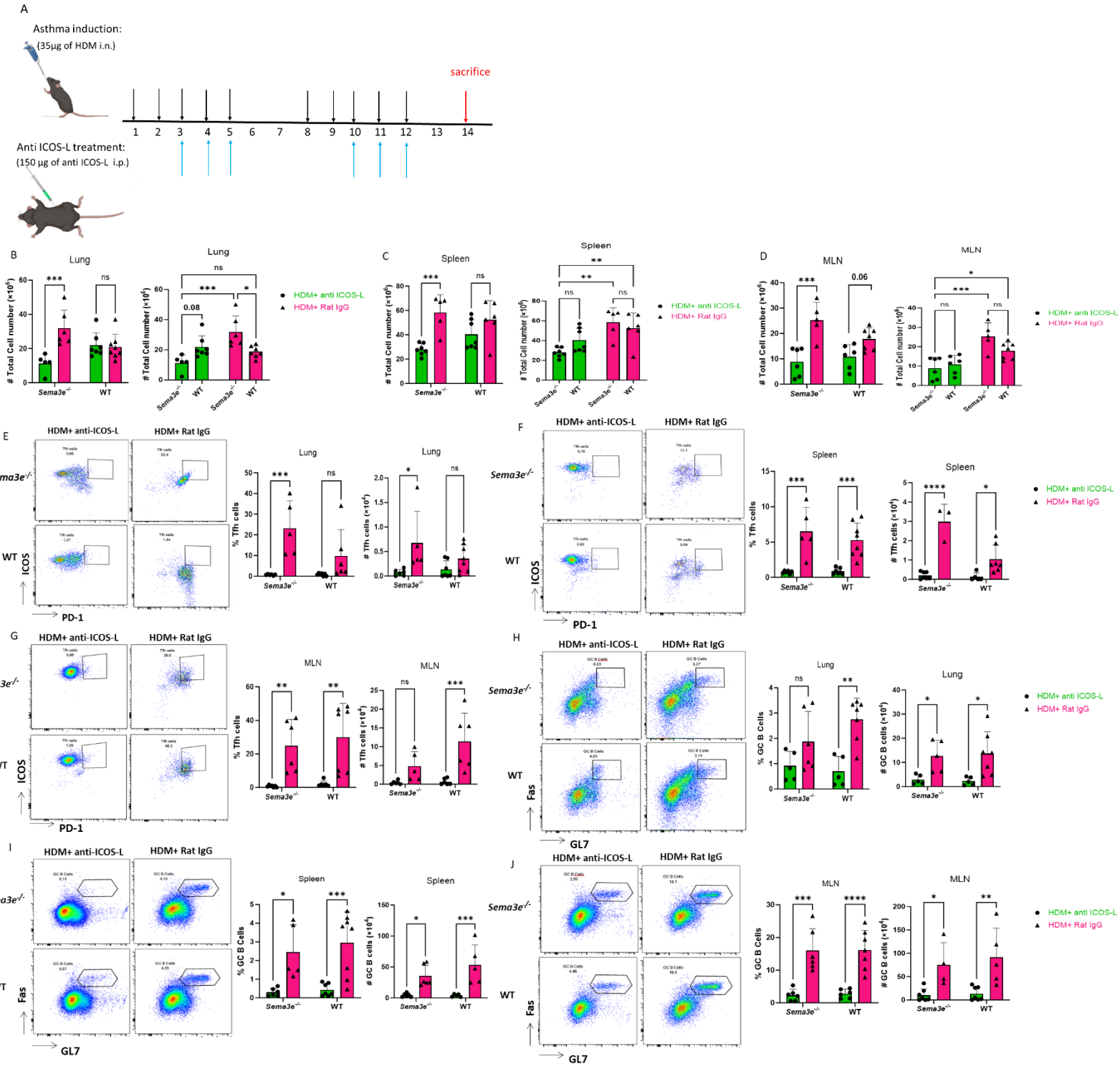


Figure 4.14 ICOS/ICOS-L disruption reduced total cell numbers and GC responses in the absence of Sema3E.

During the HDM challenge, to block the ICOS/ICOS-L pathway, *Sema3e*$^{-/-}$ and WT mice received intraperitoneal injection of anti-ICOS-L or rat IgG (A). The total cell numbers (B-D) and the number of Tfh cells ($CD3^+CD4^+CXCR5^+PD1^+ICOS^+$) were evaluated in the lung (E), spleen (F), and MLN (G). GC B cells ($B220^+GL7^+Fas^+$) were measured by FACS analysis in the lung (H), spleen (I), and MLN (J) of *Sema3e*$^{-/-}$ and WT mice. The data are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$, **, $p<0.01$; ***, $p < 0.001$; ns, not significant; N=4-7 mice in each group.

4.15 ICOS/ICOS-L disruption reduced the high number of plasmablasts, and plasma cells in *Sema3e*$^{-/-}$ mice.

Following GC formation, GC B cells undergo clonal expansion and differentiation to produce plasmablasts and plasma cells leading to high-affinity antibody production (486). Thus, disrupting GC formation causes a significant decrease in plasmablast and plasma cell population, followed by a reduction in antibody production (501). Therefore, I investigated whether blocking the ICOS/ICOS-L pathway in the absence of Sema3E can modify the expanded number of plasma cells and plasmablast populations. Following treatment with anti-ICOS-L, as expected, I observed a remarkable decrease in cell number of plasma cells ($IgD^-Sca1^+B220^-CD138^+$) and plasmablasts ($IgD^-Sca1^+B220^+CD138^+$) in the lung (Figure 4.15 A), spleen (Figure 4.15 B), and MLNs (Figure 4.15 C) of *Sema3e*$^{-/-}$ and WT mice compared to control group who received rat IgG.

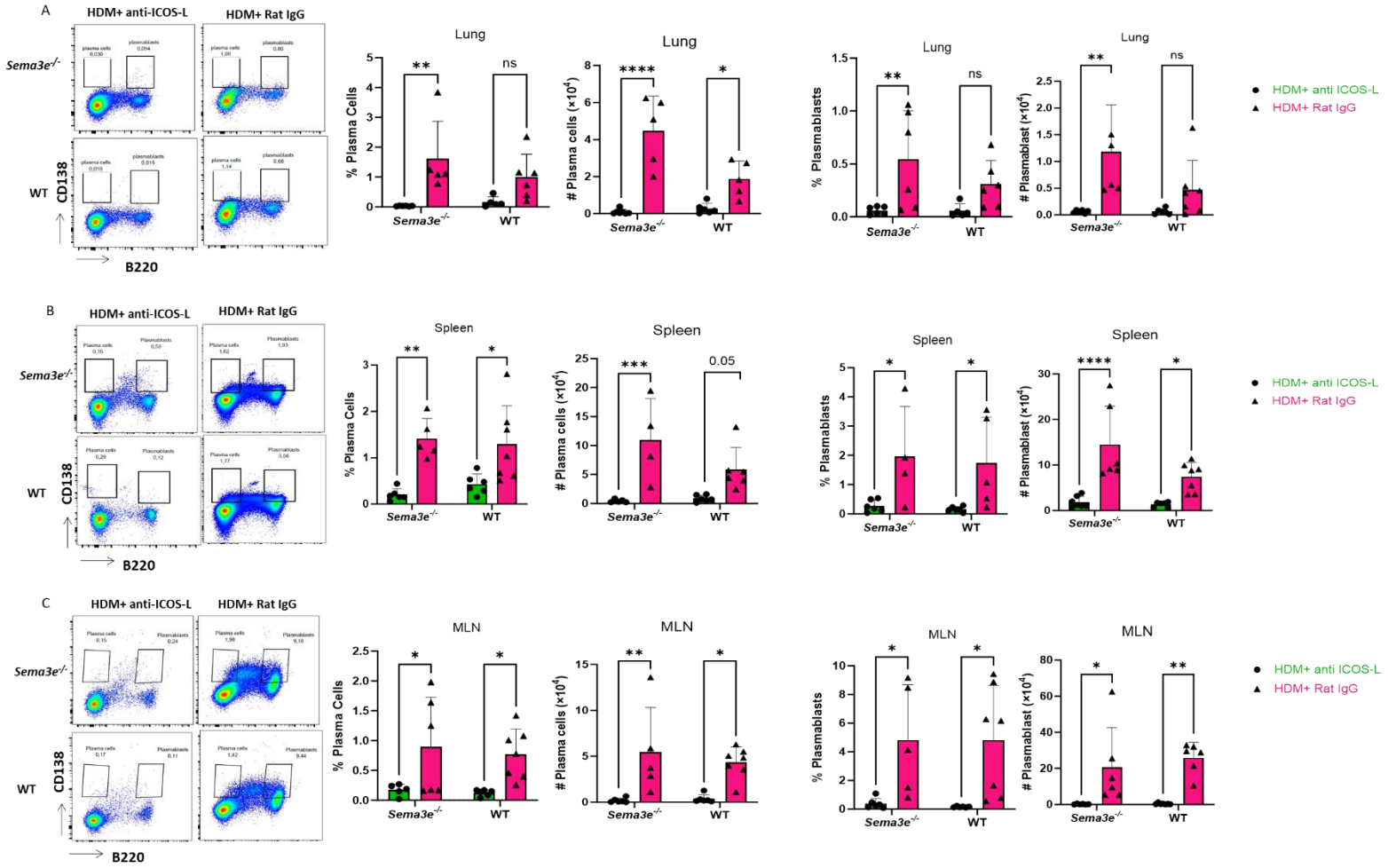


Figure 4.15 The number of plasma cells and plasmablasts in *Sema3e*$^{-/-}$ mice significantly decreased after blocking the ICOS/ICOS-L.

Flow cytometry was performed in *Sema3e*$^{-/-}$ and WT mice upon anti-ICOS-L or rat IgG treatment. Cell number of plasma cells ($IgD^{-}Sca1^{+}B220^{-}CD138^{+}$) and plasmablasts ($IgD^{-}Sca1^{+}B220^{+}CD138^{+}$) were studied within the lung (A), spleen (B), and MLNs (C). The data are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$, **, $p<0.01$, ***, $p < 0.001$; ns, not significant; N=4-7 mice in each group.

4.16 IgE^+B cells and serum immunoglobulin were diminished in the absence of Sema3E following anti-ICOS-L treatment.

As described previously, the absence of Sema3E led to a higher baseline and HDM-induced number IgE^+B cells and serum IgE and IgG1 production. Therefore, I aimed to measure the number of IgE^+B cells (IgE^+B220) and serum antibody production after blocking the ICOS/ICOS-L pathway to investigate if blocking this pathway can successfully dampen the high number of IgE^+B cells and serum immunoglobulins in *Sema3e*$^{-/-}$ mice. My data showed that the number of IgE^+B cells significantly reduced in the lung, spleen, and MLNs of *Sema3*$^{-/-}$ and WT mice who received anti-ICOS-L (Figure 4.16 A-C, respectively) compared to the control group who received rat IgG. Concomitantly, blockade of the ICOS/ICOS-L pathway, remarkably decreased serum levels of total and HDM-specific IgE and IgG1 (Figure 4.16 D-G respectively) in *Sema3e*$^{-/-}$ mice. However, as mentioned before, the baseline differences between *Sema3e*$^{-/-}$ and WT group disappeared after treatment with rat IgG.

Overall, my data confirmed that Sema3E may play its role in GC responses by negative regulation of the ICOS/ICOS-L pathway since exacerbated GC responses and IgE production in *Sema3e*$^{-/-}$ mice can be successfully depleted by blocking the ICOS/ICOS-L pathway and this is associated with reduced GC B cell, Tfh cells, plasma cells, and serum IgE and IgG1 production.

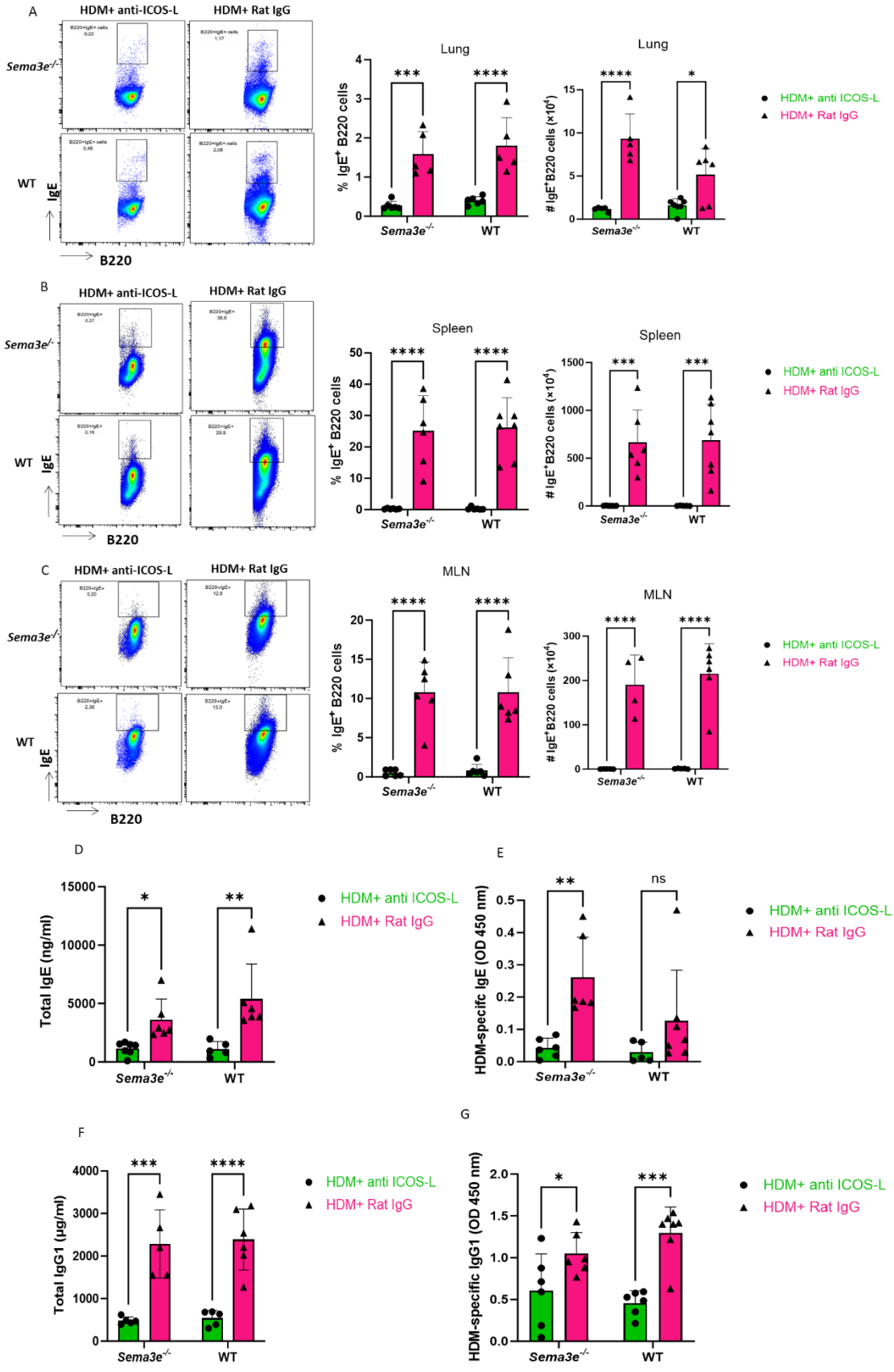

A
HDM+ anti-ICOS-L
HDM+ Rat IgG
Sema3e-/-
WT
IgE
B220
Lung
% IgE+ B220 cells
# IgE+B220 cells (×10⁴)
HDM+ anti ICOS-L
HDM+ Rat IgG
B
Spleen
C
MLN
D
Total IgE (ng/ml)
E
HDM-specifc IgE (OD 450 nm)
ns
F
Total IgG1 (µg/ml)
G
HDM-specific IgG1 (OD 450 nm)

Figure 4.16 IgE$^+$B cells and serum immunoglobulin were diminished in *Sema3e*$^{-/-}$ mice following ICOS/ICOS-L blocking.

FACS analysis was performed to evaluate IgE-producing B cell (IgE$^+$B220) number in the lung (A), spleen (B), and MLNs (C) of *Sema3e*$^{-/-}$ and WT mice after anti-ICOS-L or rat IgG treatment. Serum levels of total and HDM-specific IgE (D & E respectively) and IgG1 (F & G, respectively) were measured by ELISA. The data are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$, **, $p<0.01$, ***, $p < 0.001$; ns, not significant; N=2-4 mice in each group.

4.17 Disrupting the ICOS/ICOS-L pathway noticeably dampened lung inflammation in *Sema3e*$^{-/-}$ mice.

Lung inflammation enhancement is a key indicator of asthma progress. Among all inflammatory cells, eosinophils contribute the most to the exacerbation of allergic asthma and disease severity (502). My results and our lab's previous data demonstrated that *Sema3e*$^{-/-}$ mice had a higher lung inflammation at the baseline and upon HDM challenge (476).

In the present study, I evaluated the effect of ICOS/ICOS-L on lung inflammation in absence of Sema3E. To evaluate lung inflammatory cells, CD45$^+$ cells were categorized into non-macrophages including eosinophils and neutrophils, and macrophages based on the expression of CD64 and MertK. Flow cytometry data demonstrated that disrupting the ICOS/ICOS-L interactions diminished eosinophils (MertK$^-$CD64$^-$CD11c$^+$SinglecF$^+$) (Figure 4.17 A) and neutrophil (MertK$^-$CD64$^-$Gr-1$^+$ CD11b$^+$) (Figure 4.17 B) cell numbers in *Sema3e*$^{-/-}$ mice compared to the control group who received rat IgG. Furthermore, I noticed that upon disrupting the ICOS/ICOS-L pathway, the number of alveolars (MertK$^+$CD64$^+$CD11b$^+$SinglecF^{low}) and interstitial (MertK$^+$CD64$^+$CD11b$^+$SinglecF^{high}) macrophages significantly reduced in *Sema3e*$^{-/-}$ mice (Figure 4.17 C & D, respectively), indicating the importance of ICOS/ICOS-L signalling in Sema3E regulatory effect on the lung following HDM challenge. However, no significant changes were observed between WT mice receiving anti-ICOS-L treatment and control WT mice receiving rat IgG. It's possible that because of the higher expression of ICOS on T cells in *Sema3e*$^{-/-}$ mice, ICOS/ICOS-L pathway plays a more prominent role in these mice and the absence of Sema3E may amplify the importance of the ICOS/ICOS-L pathway in driving lung inflammation. If WT mice

have lower levels of ICOS/ICOS-L interaction in the lungs, blocking this pathway might have a limited effect on their inflammatory response and other regulatory pathways could maintain control, making ICOS-L blockade less effective.

The effectiveness of the ICOS/ICOS-L disruption in the lung inflammation of asthmatic *Sema3e*$^{-/-}$ mice was confirmed by hematoxylin and eosin (H & E) staining in the lung tissue sections from *Sema3*$^{-/-}$ and WT mice following ant-ICOS-L or rat IgG administration. H&E staining determined that anti-ICOS-L treatment alleviated lung tissue inflammation in *Sema3e*$^{-/-}$ mice compared to the control group who received rat IgG (Figure 4.17 E). Periodic acid–Schiff (PAS) staining was done to measure if blocking the ICOS/ICOS-L pathway could reduce mucus overproduction in asthmatic *Sema3e*$^{-/-}$ mice (Figure 4.17 F). No significant changes were observed in asthmatic *Sema3*$^{-/-}$ mice following ICOS/ICOS-L disruption compared to the rat IgG group. Moreover, to investigate tissue remodelling in the lungs of *Sema3e*$^{-/-}$ and WT mice who underwent anti-ICOS-L treatment, Sirus Red staining was performed (Figure 4.17 G). However, high collagen deposition in the lung of *Sema3*$^{-/-}$ mice was not affected by blocking ICOS/ICOS-L treatment. Since mucus overproduction and collagen deposition in the lung following HDM exposure takes time, maybe anti-ICOS-L treatment needs more time to efficiently reduce mucus or collagen deposition.

Collectively, my data suggested that exacerbated lung inflammation in the absence of Sema 3E could be noticeably alleviated by blocking the ICOS/ICOS-L pathway again indicating the importance of this pathway in the regulatory role of Sema3E.

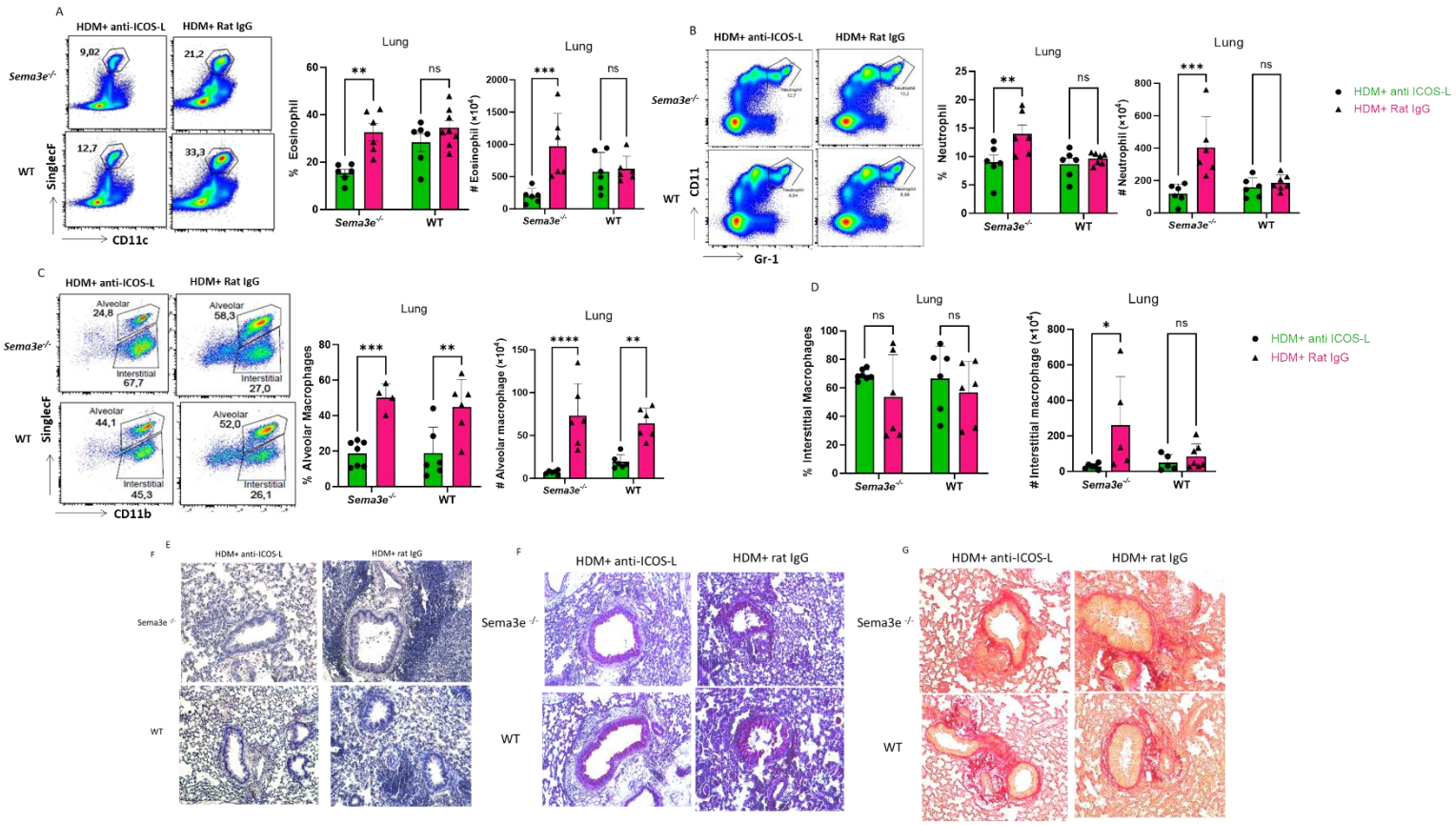


Figure 4.17 anti-ICOS-L treatment efficiently decreased the high number of lung inflammatory cells in *Sema3e*$^{-/-}$ mice.

Flow cytometry was done to measure lung inflammatory cells. Eosinophils (MertK^{-}CD64^{-}CD11c^{+}SiglecF^{+}) (A) and neutrophils (MertK^{-}CD64^{-}Gr-1^{+} CD11b^{+}) (B) were evaluated within the lung of *Sema3e*$^{-/-}$ and WT mice. Moreover, the number of lung alveolar (MertK^{+}CD64^{+}CD11b^{+}SiglecF^{low}) (C) and interstitial (MertK^{+}CD64^{+}CD11b^{+}SiglecF^{high}) (D) macrophages were analyzed in *Sema3e*$^{-/-}$ and WT littermate after anti-ICOS-L or rat IgG treatment. Lung inflammation, mucus overproduction, and tissue remolding were measured by H&E staining (E), PAS (F), and Sirus red staining (G) respectively in *Sema3e*$^{-/-}$ and WT control mice following the blocking of the ICOS/ICOS-L signalling. Scale bars, 50um; The data are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$; **, $p < 0.01$; ns, not significant; N=4-7 mice per each group.

4.18 Anti-ICOS-L treatment of *Sema3*$^{-/-}$ mice significantly reduced inflammatory cells in the BALF.

In the next step, I investigated the effect of blocking the ICOS/ICOS-L pathway in BALF total cell number and inflammatory cells of *Sema3e*$^{-/-}$ mice and WT group. The control *Sema3e*$^{-/-}$ and WT mice received i.p rat IgG. As expected, the high BALF cell number in the absence of Sema3E was significantly decreased following anti-ICOS-L treatment (Figure 4.18 A). There was a significant reduction in the number of eosinophils ($MertK^{-}CD64^{-}CD11c^{+}SinglecF^{+}$) (Figure 4.18 B) and neutrophils ($MertK^{-}CD64^{-}Gr\text{-}1^{+}CD11b^{+}$) (Figure 4.18 C) in BALF of *Sema3*$^{-/-}$ and WT mice who underwent anti-ICOS-L treatment. Moreover, the population of alveolar ($MertK^{+}CD64^{+}CD11b^{+}SinglecF^{low}$) and interstitial ($MertK^{+}CD64^{+}CD11b^{+}SinglecF^{high}$) macrophages significantly reduced in both *Sema3e*$^{-/-}$ and WT mice (Figure 4.18 D & E respectively) after disrupting ICOS/ICOS-L pathway.

Finally, I showed that the levels of Th17 and Th2 cytokines including IL-17A, IL-4, IL-5, and IL-13 were decreased in BAL following anti-ICOS-L treatment (Figure 4.18 F-H respectively).

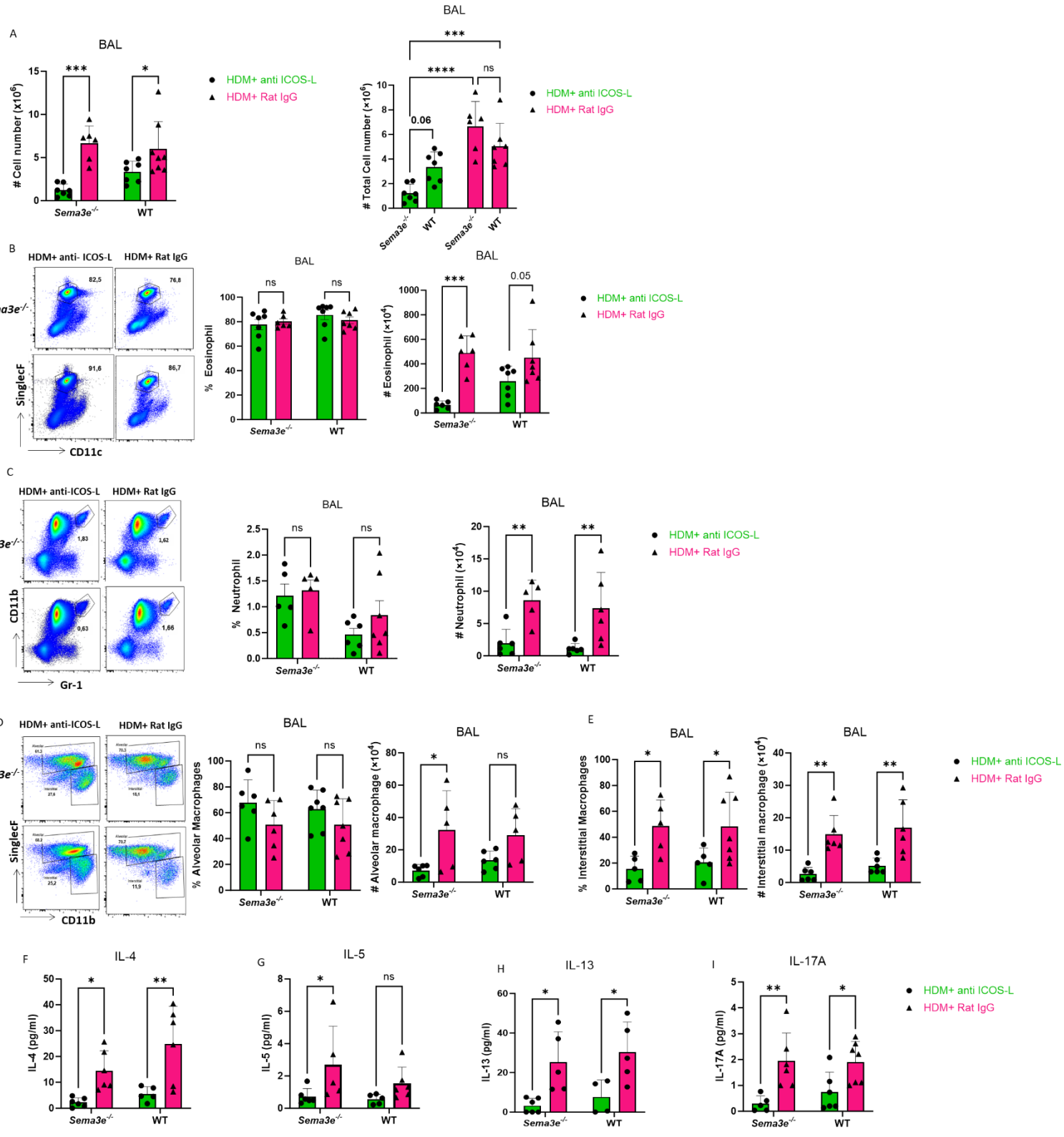
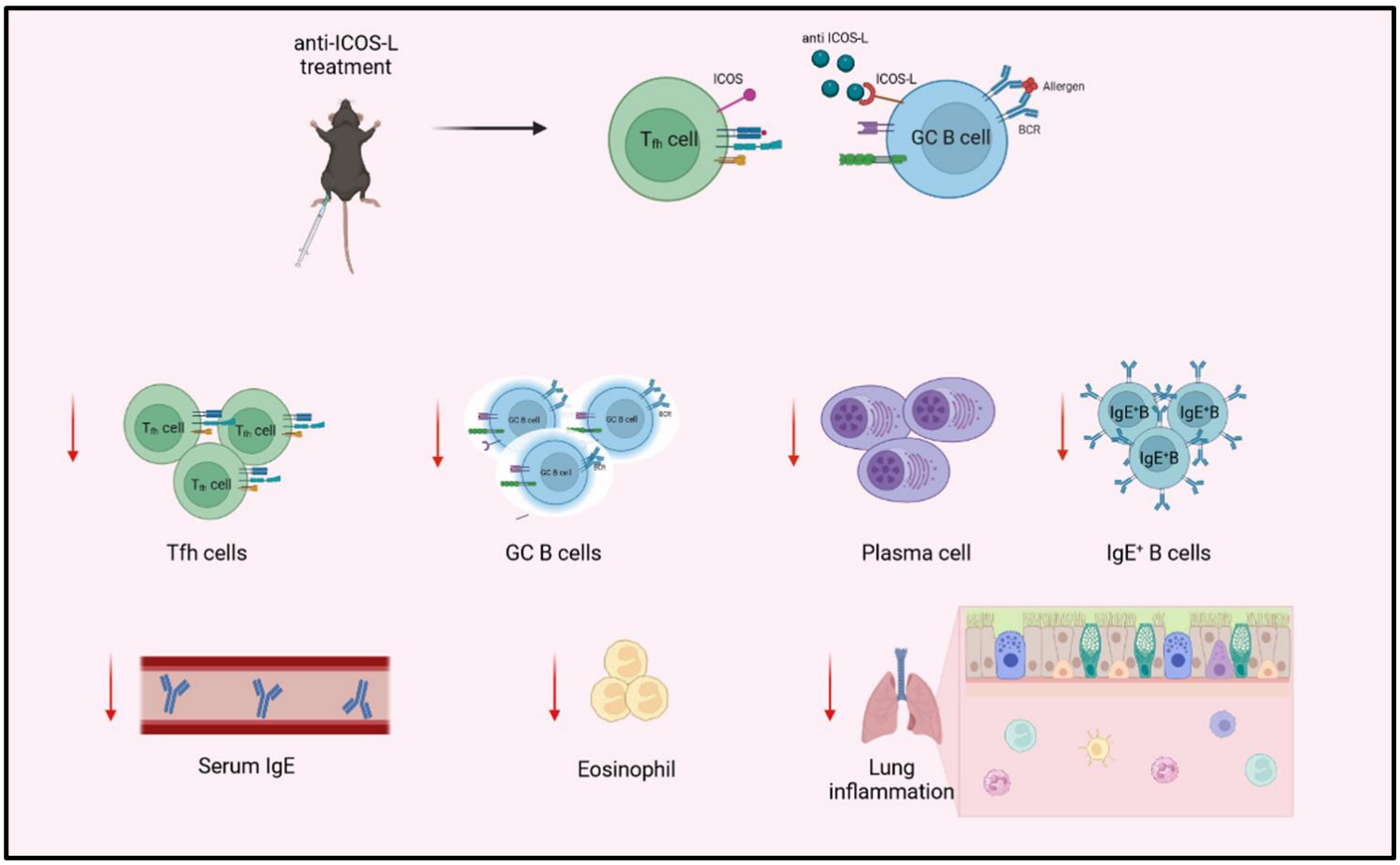

A
BAL
Cell number (x10^6)
HDM+ anti ICOS-L
HDM+ Rat IgG
Sema3e-/-
WT
BAL
Total Cell number (x10^6)
0.06
B
HDM+ anti- ICOS-L
HDM+ Rat IgG
SinglecF
CD11c
% Eosinophil
# Eosinophil (x10^4)
C
HDM+ anti-ICOS-L
CD11b
Gr-1
% Neutrophil
# Neutrophil (x10^4)
D
% Alveolar Macrophages
# Alveolar macrophage (x10^4)
E
% Interstitial Macrophages
# Interstitial macrophage (x10^4)
F
IL-4
IL-4 (pg/ml)
G
IL-5
IL-5 (pg/ml)
H
IL-13
IL-13 (pg/ml)
I
IL-17A
IL-17A (pg/ml)

Figure 4.18 BALF inflammatory cells significantly dampened following anti-ICOS-L treatment in *Sema3e*$^{-/-}$ mice.

FACS analysis was done in the BALF to measure the total number of BAL cells (A) and inflammatory cells including eosinophils (MertK^{-}CD64^{-}CD11c^{+}SinglecF^{+}) (B), neutrophils (MertK^{-}CD64 Gr-1^{+} CD11b^{+}) (C), alveolar (MertK^{+}CD64^{+}CD11b^{+}SinglecF^{low}) (D) and interstitial (MertK^{+}CD64^{+}CD11b^{+}SinglecF^{high}) (E) macrophages in *Sema3e*$^{-/-}$ and WT control after anti-ICOS-L or rat IgG treatment. Mesoscale was done to evaluate the levels of IL-4 (F), IL-5 (G), IL-13 (H), and IL-17-A (I) cytokines in the BAL of mice. The data are representative of two different experiments with a similar outcome. Bars show mean +/-SEM; *, $p < 0.05$; **, $p < 0.01$; ns, not significant; N=4-7 mice per each group.

Graphical Abstract 3:

Aim 3: To determine whether the dissociation of Tfh and GC reaction during HDM-induced allergic inflammation will abolish the excessive GC responses and production of IgE in *Sema3e*$^{-/-}$ mice.



Overall, our data showed that during HDM exposure in acute allergic asthma, excessive GC responses in the absence of Sema3E can be successfully depleted using α-ICOS-L treatment. The ICOS/ICOS-L disruption in *Sema3e*$^{-/-}$ mice is associated with reduced Tfh cells, GC B cells, plasma cells, plasmablasts, IgE^{+} B cells, and lung inflammation. Furthermore, high levels of total and allergen-specific IgE and IgG1 in *Sema3e*$^{-/-}$ mice are diminished following ICOS/ICOS-L blockade.

Chapter 5: Discussion

A bias toward Th2 inflammation characterizes allergic asthma, and IgE plays a prominent role in this inflammatory process (1, 503). In my project using a well-defined acute allergic asthma model, I examined the importance of Sema3E protein in regulating GC responses and IgE production. I observed that Sema3E deficiency led to exaggerated GC responses. Unimmunized *Sema3e*$^{-/-}$ mice had remarkably higher baseline numbers of GC B cells, Tfh cells, plasma cells, and plasmablasts. Moreover, *Sema3e*$^{-/-}$ mice produced considerably higher serum IgE and IgG1 levels than the WT group. Immunization with HDM further emphasized the regulatory role of Sema3E in GC formation and IgE production. Following the HDM challenge, *Sema3e*$^{-/-}$ mice showed disease exacerbation features and the number of immune cells that play a role in GC responses and IgE secretion noticeably increased. Interestingly, the absence of Sema3E affected ICOS expression by T cells as *Sema 3e*$^{-/-}$ mice expressed higher baseline and inflammatory levels of ICOS on CD4^{+} cells. Furthermore, ICOS/ICOS-L pathway intervention in HDM-challenged *Sema3e*$^{-/-}$ mice interrupted GC responses, decreased serum IgE, and airway inflammation. My study identified a novel role for Sema3E protein in GC responses and IgE production, and ICOS upregulation may account for this exaggeration response. Indeed, my data signify that Sema3E is one of the critical regulators of optimal GC responses and IgE production.

Semaphorins were first discovered as axon guidance molecules in the nervous system. However, it is now understood that their expression and roles extend beyond the nervous system, being widely present and functionally important in development, health, and disease. *Sema3e* and its receptor *plexind1* genes are expressed by multiple immune cells including macrophages, DCs, T cells, neutrophils, ILC-2, NK cells, and to a lesser extent by B cells and regulate these cells' proliferation, migration, and function.

Various proteins and molecules interact together to form optimal GC responses. While some of these proteins have been widely investigated, the role of semaphorins has been less noticed. It has been shown that semaphorin 4C is upregulated by Tfh cells, and by interacting with Plexin B2 (PlxnB2) receptor, highly expressed by GC B cells, plays an important role in Tfh cell recruitment

and guidance to GC, Ab affinity maturation, and plasma cells formation (428). My data signify the pivotal regulatory role of Sema3E in the GC responses. Sema3E deficiency substantially increased the baseline of GC responses, plasma cells, plasmablasts, and serum IgG1 and IgE in the steady-state, which intensified after immunization with HDM. Sema3E and its receptor, PlexinD1 are known to function as a guidance cue during immune cell migration and interactions (346). PlexinD1 is expressed by naïve B cells and upregulated post-immunization and plays a key role in B cell function and GC formation (429). The absence of Sema3E likely disrupts the normal Sema3E/PlexinD1 signalling pathways and immune cell movement, leading to increased total inflammatory cell numbers, GC B cells, Tfh cells, and GC responses. Sema3E deficiency may have direct effects on the baseline activation of B cells or could influence other immune cells, such as DCs and T cells, which in turn may indirectly alter B cell activation. This suggests a potential mechanism in which the absence of Sema3E enhances the immune system's basal activation state, either through B cells themselves or via intermediary effects on DCs and T cells, both of which are critical for shaping GC responses. Additionally, it is important to consider that Sema3E deficiency might affect other tissues, including the brain, gut, and metabolic tissues such as the liver, pancreas and fat tissues. These tissues are integral to maintaining immune homeostasis, and dysfunction in these areas could promote chronic systemic inflammation. Such persistent inflammation might indirectly drive altered B cell function and immune dysregulation, highlighting the possibility of broad, systemic effects in the absence of Sema3E.

Consistent with the increased GC responses, the number of plasma cells and plasmablasts were significantly higher in *Sema3e*$^{-/-}$ mice than in WT control in the steady state and following HDM. It is possible that the increased number of GC B cells and Tfh cells in the absence of Sema3E leads

to naturally more GC responses and subsequently to a higher conversion of GC B cells into plasmablasts and plasma cells.

The possible regulatory role of Sema3E in B cell costimulation induction was also investigated in this study. In naïve conditions, the number of B cells expressing costimulatory molecules ($CD80^+$ B cell, $CD86^+$ B cell, $CD40^+$ B cell, ICOS-L^+ B cell and $MHCII^+$ B cell) or the expression of almost all costimulation markers, except CD80, were not regulated by Sema3E protein. However, Sema3E may positively regulate baseline expression of CD80 by B cells since its deletion led to a remarkable reduction in CD80 expression in splenic B cells. Following the HDM challenge in an acute model of allergic asthma, the number of active B cells expressing costimulatory markers significantly increased in *Sema3e*$^{-/-}$ mice compared to WT mice and it may be due to the regulatory effect of Sema3E on cell migration. However, regarding costimulatory molecules expression levels on B cells, my results demonstrated that Sema3E could only positively regulate CD80 and ICOS-L expression on B cells upon immunization since its deficiency led to lower expression of these markers. The role of semaphorins in the stimulation and activation of B cells has received less attention. However, Sema4D has been implicated in the regulation of costimulatory markers like CD40, CD80, and CD86 on B cells and DCs, thereby influencing immune responses (504). B cells lacking Sema4D exhibit reduced responsiveness to BCR stimulation compared to WT B cells (411), and Sema4D knockout DCs show impaired expression of CD80 and CD40. Sema4D boosts B cell activation by inhibiting the CD72 signals (505), and soluble Sema4D increases the surface expression of CD80, CD86, and MHC class II on DCs (504). However, it's important to note that the exact mechanisms and signalling pathways through which Sema3E influences B cell responses would require further research and investigation.

The IgE response can be influenced by various mechanisms beyond GC formation (128). One key pathway involves direct B cell activation through T cell-dependent mechanisms in extrafollicular areas where B cells can produce IgE without needing the GC environment, especially in response to certain pathogens or allergens (128). Cytokines, such as IL-4 and IL-13, secreted primarily by Th2 cells, are potent inducers of class-switch recombination to IgE, enabling B cells to produce IgE outside of GCs (306). Additionally, regulatory T cells (Tregs) and innate lymphoid cells (ILCs) may modulate IgE responses by either promoting or suppressing B cell activation. The local microenvironment, such as inflamed tissues or mucosal surfaces, can also provide cues that affect IgE production through interactions with dendritic cells, macrophages, and epithelial cells (506). Furthermore, chronic inflammation and the presence of IgE-specific receptors on mast cells and basophils may enhance IgE production in a feedback loop, intensifying allergic responses independently of the GC pathway. Therefore, Sema 3E may regulate IgE responses through non-GC pathways, such as extrafollicular foci of antibody production, tissue-resident immune cells, or the modulation of cytokine environments, which can skew the immune response toward an IgE-promoting Th2 phenotype

In my project, I demonstrated that Sema3E may regulate IgE^+ and IgG^+ B cell populations and serum immunoglobulin production. The number of class-switched B cells to IgE^+B cells and IgG^+B cells and IgE and IgG expression on B cells was significantly higher in *Sema3e*$^{-/-}$ mice compared to WT littermate in the steady-state. Moreover, *Sema3e*$^{-/-}$ mice had remarkably increased total and HDM-specific IgE and IgG1 levels in their serum. The novel finding of my project is that Sema3E negatively regulates baseline IgE and IgG production and expression on B cells in secondary lymphoid organs in the naïve condition since its deficiency causes increased baseline levels of IgE and IgG in serum and IgE and IgG expression on B cells.

IgE^+ B cell and IgG^+B cell are B cells that have undergone class switch recombination to produce IgE and IgG, respectively. IgG and IgE can be produced as a plasma membrane-bound isoform or as a secreted antibody molecule. Transmembrane Ig binds to the Igα/β heterodimer (CD79) to create a functional B-cell antigen receptor (BCR) (507, 508) and define the antigen-specificity of the B-cell response, with various Ig subclasses having unique but overlapping signalling characteristics (509). Both IgE and IgG class switching and production are tightly regulated processes influenced by multiple cytokines such as IL-4, IL-13, IL-21, IFN-γ IL-10, etc, transcription factors including STAT, NFκB, Bcl-6, etc, cell interactions, and environmental factors (311). Semaphorins are important but less-known proteins regulating Ig production and class switching by modulating B cell activation, costimulatory molecule expression, and the cytokine environment. These effects can directly or indirectly shape the production of different antibody classes, including IgE and IgG (510). My findings provide important insights into the role of Sema3E in regulating Ig production. My data show that the baseline levels of IgE and IgG1 production and expression by B cells are significantly higher in *Sema3e*$^{-/-}$ mice compared to the WT control. However, following immunization with HDM, the difference between WT and *Sema3e*$^{-/-}$ mice is more a reflection of their differences in the steady-state. In steady state IgE and IgG expression on B cells is negatively regulated by Sema3E (since its absence led to higher expression of these molecules on B cells) while following HDM immunization no difference was observed between *Sema3e*$^{-/-}$ and WT mice regarding IgE and IgG expression on B cell and several factors may be involved. First, after HDM immunization, strong inflammatory and immunological signals may dominate the regulatory role of Sema3E on B cells. The potent stimulation provided by HDM could activate signaling pathways that bypass or overwhelm Sema3E's influence on IgE and IgG expression, leading to similar levels in both KO and WT mice. Moreover, upon HDM

challenge, compensatory pathways might be upregulated in the absence of Sema3E. Other regulatory molecules or pathways (such as cytokines or costimulatory molecules) might take over the role of controlling IgE and IgG expression on B cells, thereby masking the effect of Sema3E deficiency. Additionally, the immune microenvironment in the steady state is different from the environment following HDM immunization, with different cytokines, chemokines, and cellular interactions. This shift in the local immune milieu could affect how B cells respond to the absence of Sema3E, leading to no observable difference in IgE and IgG expression after immunization.

It is noteworthy that Sema3E may have a tissue-specific regulatory role. I noticed that unlike spleen and Peyer's patch, the expression of IgE and IgG on lung B cells is comparable between *Sema3e*$^{-/-}$ and WT. The absence of Sema 3E has a stronger regulatory effect on IgE and IgG expression in B cells of the spleen and Peyer's patch, likely due to the nature of these tissues as key sites for germinal center reactions, class switching, and systemic or mucosal immunity. In contrast, lung B cells may rely on different immune regulatory pathways, which could explain the comparable expression of membrane-bound IgE and IgG in both *Sema3e*$^{-/-}$ and WT mice in the lung. This suggests that Sema 3E regulation is context- and tissue-specific depending on the local immune needs and the role of B cells in each tissue,

Indeed the possible regulatory effect of Sema3E on immunoglobulin production and expression by B cells is a complex process and some factors affect the precise evaluation of this regulatory role. In my naïve experiment, I observed that the expression of IgE on B cells (MFI value) is high in the absence of Sema3E although the number of IgE^{+} B cells is low. IgE can bind to B cells through high-affinity (FcεRI) or low-affinity (FcεRII or CD23) receptors, which can cause non-specific surface staining and lead to inflated MFI values, despite a low actual percentage of IgE-expressing B cells. This phenomenon often complicates flow cytometry analysis, as the IgE

detected might not represent newly synthesized or membrane-bound IgE but instead reflect IgE that has passively adhered to the cell surface from the serum. To address this, most staining protocols incorporate a low pH buffer wash to strip passively bound IgE from cells. This wash disrupts the receptor-ligand interactions without affecting the intrinsic IgE production or membrane-bound forms, thus providing a more accurate representation of IgE^+ B cell populations and reducing the likelihood of artefacts.

Aligned with my results, other similar projects demonstrated that *Sema3*$^{-/-}$ mice have increased total and HDM-specific IgE and IgG1 levels in allergic asthma, further indicating the regulatory role of Sema3E in antibody production (476). Interestingly, the Sema3E receptor, plexinD1, also plays a regulatory role in GC responses, Ig production, and antibody affinity maturation. Holl et al showed in peripheral lymphoid organs, plexin-D1 is constitutively expressed by MZ B cells and is upregulated in FO B cells upon activation. They observed that *plexind1*$^{-/-}$ mice had significantly reduced basal levels of IgG1 and IgG2b compared to the WT control. Following immunization, although the initial antibody-forming cells and primary humoral responses are not affected by the absence of plexinD1, as time passes, humoral responses and antibody affinity maturation are impaired in *plexind1*$^{-/-}$ mice (429). Another semaphorin, Sema4D (CD100), plays a significant regulatory role in antibody production and affinity maturation. In contrast to Sema3E, the absence of Sema4D leads to impaired Ig production and affinity maturation in response to CD40 stimulation in vitro (411). These results indicate that different semaphorin proteins, such as Sema3E and Sema4D, contribute distinct regulatory mechanisms to Ig production and affinity maturation. While Sema4D is crucial for proper B cell activation and antibody maturation via CD40 interactions, Sema3E appears to modulate systemic levels of specific antibodies, with its

absence leading to exacerbated responses in allergic contexts. However, further research is needed to fully elucidate the specific roles and mechanisms by which Sema3E governs these processes.

The key finding of my study is that *Sema3e*$^{-/-}$ T cells expressed remarkable levels of ICOS not only post-immunization with HDM but also at a steady state. Intriguingly, the baseline level of ICOS expression was significantly raised in the absence of Sema3E. This finding is in accordance with emerging research showing that semaphorins, including Sema3E, can play important roles in T cell responses. For example, Sema4A and its receptor neuropilin-1 (NP-1) interaction inhibits Tcell proliferation, hence downregulating T cells function (511). Moreover, Sema3E and plexinD1 are shown to be involved in T cell development by regulating migration in the thymus (346). Our result demonstrated a novel role for Sema 3E in T cell activation. Sema 3E affects T cell activation by regulating ICOS expression therefore in the absence of Sema3E there may be reduced inhibitory signalling that usually keeps ICOS expression in check leading to higher ICOS expression in T cells. This phenomenon, in turn, can affect T cell activation, differentiation, and the immune response.

Following immunization, ICOS expression is induced through T-cell antigen receptor (TCR) and CD28 signalling and regulates Th2 cytokines production which is crucial for the lung mucosal inflammatory responses (325, 512, 513). Various studies demonstrated that ICOS/ICOSL signalling plays a significant role in controlling allergic inflammation by regulating effector T cells and blocking this pathway culminating in reduced Th2 cytokine secretion, IgE production, and airway inflammation (514, 515). ICOS upregulation activates PI3K and Akt signalling, essential to T cell survival, proliferation, and differentiation (325, 516, 517). The upregulation of ICOS coincides with the upregulation of GATA 3, a process essential for Th2 differentiation and this led to the production of IL4 and IL5 (329). Additionally, T cells lacking ICOS showed a defect in

proliferation, Th2 differentiation, and cytokines production compared to normal CD4+ T cells (518, 519). Interestingly, the transfer of ICOS-enriched T cells beforehand of the allergen exposure can lead to infiltration of recipient T and B cells and production of allergen-specific IgE by lung resident plasma cells (341). The crucial role of ICOS on Tfh cell differentiation, function, and GC formation is well-studied (520, 521).

However, based on my results, it seems Sema3E plays an exciting role in B cell-T cell interaction. It may play a positive regulatory role in B cell activation by maintaining levels of CD86 in B cells. Sema3E absence could impair B cell activation or its ability to provide costimulatory signals to T cells, leading to reduced CD86 expression. This reduced CD86 could weaken the activation of T cells, specifically those that rely on B cell-mediated antigen presentation and costimulation (522, 523). On the other hand, Sema3E might normally act as a negative regulator of ICOS expression on Tfh cells. Without Sema3E, Tfh cells could become more activated and overexpress ICOS, potentially driving enhanced Tfh-B cell interactions. This increased ICOS expression could lead to stronger signals to B cells for GC formation, antibody production, and possibly class switching (e.g., IgE or IgG1). This matches my observation of higher IgE and GC B cells in Sema 3E-deficient mice. The opposing effects on B cells and T cells suggest that Sema 3E may play a dual role in modulating B-Tfh interactions: In B cells, Sema 3E may promote costimulatory readiness (higher CD86) to ensure efficient T cell activation while in Tfh cells, Sema 3E may limit ICOS expression to prevent overactivation of Tfh cells and excessive B cell stimulation in the germinal center. Without Sema3E, this balance could be disrupted contributing to the hyperimmune responses.

Tfh are reliant on ICOS expression to maintain their phenotype and play their role in GC. In the absence of ICOS/ICOS-L signalling, Tfh accumulation, maintenance, and function are impaired

(498, 524, 525). In this regard, we hypothesized that Sema3E may contribute to GC responses, IgE production, and asthma exacerbation through the ICOS/ICOS-L signalling pathway. Blocking a critical signalling pathway such as ICOS/ICOS-L which leads to reduced GC responses, antibody production, and inflammatory cytokines and consequently diminishes overall inflammation, is not a novel finding. Numerous studies have thoroughly established this. However, in my project, to test the hypothesis that Sema3E might exert its effects on enhancing GC responses and IgE production via this pathway, I was motivated to block this signalling pathway and investigated whether this blocking results in the reduction of excessive GC responses, decreased antibody production, inflammation, and overall, an improvement in allergic asthma. I observed that blocking the ICOS/ICOS-L pathway led to diminished GC B cells, Tfh cells, plasma cells, serum IgE and IgG1, lung inflammatory cells, and proinflammatory cytokines in *Sema3e*$^{-/-}$ and alleviated lung inflammation. Interestingly, the total inflammatory cell numbers in the absence of Sema3E significantly reduced and reached the WT level following the blocking of this pathway. This indicates that the exacerbated features of inflammation in *Sema3e* $^{-/-}$ mice are, at least in part, driven by increased ICOS signalling. In fact, ICOS can intensify the functions of CD28 during an immune response and induce additional T-effector cell functions (526). Furthermore, by inducing IL-4, and IL-10 production, ICOS promotes B cell and IgE^{+} plasma cell differentiation (527). Several studies demonstrated that blocking the ICOS/ICOS-L pathway upon inflammation significantly reduced GC responses, IgE production, and inflammatory outcomes (342, 518, 528). These findings suggest that ICOS expression specifies a subset of T effector cells necessary for local IgE production and B cell infiltration in lung tissue. However, the exact mechanisms by which Sema3E deficiency affects ICOS expression and GC responses in different contexts require further investigation to fully understand the molecular pathways involved.

It should be noted that, following intraperitoneal administration of rat IgG, the significant difference between *Sema3e*$^{-/-}$ and WT mice disappeared and this may suggest rat IgG treatment has an immunomodulatory effect that impacts both genotypes similarly, thereby masking the previously observed phenotypic differences. Several potential reasons could explain this phenomenon. First, rat IgG could engage Fc receptors on immune cells, such as macrophages, dendritic cells, or B cells, leading to altered signalling pathways. This interaction might suppress the exaggerated GC response, inflammation, and antibody production seen in *Sema3e*$^{-/-}$ mice, effectively levelling the immune response to be more comparable with WT mice. It has been shown that intravenous immunoglobulin (IVIG) treatment can affect B cells and other components of the immune system. IVIG can modulate signaling pathways in B cells, affecting their ability to differentiate into plasma cells and reducing antibody production. Moreover, by binding to inhibitory receptors like FcγRIIB on B cells, IVIG can reduce B activation and proliferation (529, 530). T cells also can be affected by IVIG and it reduces the activation and proliferation of effector T cells (531).Rat IgG may act as a general immunosuppressive agent by engaging with Fcγ receptors, leading to changes in cytokine production, antigen presentation, or cell activation. Moreover, the administration of rat IgG may induce a general anti-inflammatory or immune-regulatory response, potentially through the activation of regulatory pathways involving FcγRIIb, an inhibitory Fc receptor. This receptor can dampen immune responses by reducing the activation of B cells and other immune cells, which may be particularly impactful in the context of hyperactive immune responses in the absence of Sema3E (532). Furthermore, in *Sema3e*$^{-/-}$ mice, the absence of Sema3E likely leads to enhanced ICOS/ICOSL interactions, driving stronger Tfh, GC, and antibody responses. Also, the rat IgG could influence this pathway directly or indirectly, possibly by altering ICOSL or other co-stimulatory molecule interactions. This could lead to

reduced Tfh and GC responses, compensating for the hyperactive state induced by Sema3E deficiency and masking the difference with WT. Further investigation is needed to address this problem.

Chapter 6: Limitations of study

Several limitations to this study should be considered when interpreting the results. A primary limitation is that my experiments cannot definitively determine whether Sema3E directly affects any specific cell types involved in GC responses, such as GC B cells, Tfh cells, or plasma cells. While Sema3E is implicated in immune regulation, its direct influence on these cell populations remains unclear. Moreover, the effect of Sema3E on follicular dendritic cells (FDCs), another key player in the germinal center microenvironment, was not examined. FDCs are critical for GC maintenance and antibody affinity maturation, and the potential impact of Sema3E on these cells should be explored in future studies.

Additionally, the role of Sema3E deficiency in the light zone, where B cells undergo selection, and the dark zone, where B cells proliferate and mutate—was not specifically addressed. Antibody affinity maturation, a crucial process for producing high-affinity antibodies, could also be influenced by Sema3E deficiency, and this aspect warrants further investigation.

Another important limitation relates to the use of anti-ICOS-L treatment. Since ICOS expression is a defining marker for Tfh cells, blocking the ICOS/ICOS-L pathway may alter ICOS expression on these cells, complicating the gating strategy used in flow cytometry. It would be beneficial to use an alternative marker for Tfh cells in future experiments to improve accuracy.

The administration of rat IgG in control groups appeared to mask the differences between *Sema3e*$^{-/-}$ and WT mice. Rat IgG may have exerted non-specific immunomodulatory effects that altered the immune response, potentially confounding the experimental outcomes.

Lastly, a key limitation of this study is that the mice used were global Sema3E knockout mice, meaning that Sema3E was absent in all cell types. This raises the question of which specific cell

populations are driving the observed phenotypic changes. Future studies utilizing conditional knockout models, where Sema3E is selectively deleted in particular cell types, would help pinpoint which cells are responsible for the altered GC responses and immune activation seen in the absence of Sema3E.

Chapter 7: Future Direction

- To address whether Sema3E directly affects specific cells such as GC B cells and Tfh cells. One possible experiment involves isolating GC B cells and Tfh cells from WT and *Sema3e*$^{-/-}$ mice and culturing them under conditions promoting GC and Tfh interactions (533). By adding recombinant Sema3E protein to these cultures, it would be possible to directly observe its effects on B cell proliferation, differentiation into plasma cells, and antibody production, as well as on Tfh cell activation and cytokine secretion. In parallel, co-culture experiments using Sema3E-deficient cells and WT cells could help determine whether the absence of Sema3E impacts cell-cell communication or costimulatory signalling. Furthermore, using conditional knockout models where Sema3E is deleted only in specific cell types, such as B cells or T cells, could provide additional clarity on which cell types are directly influenced by Sema3E.
- To investigate the downstream signalling events following ICOS/ICOS-L engagement in the context of Sema3E deficiency. My study demonstrated that Sema3E deficiency led to higher ICOS expression in T cells, and may affect IgE production via the ICOS/ICOS-L signalling pathway, but the exact mechanism should be investigated. This could involve examining specific transcription factors and signalling molecules activated in T and B cells. Western blotting using phospho-specific antibodies can be performed to detect the activation of key signalling proteins downstream of ICOS/ICOS-L engagement, such as PI3K, Akt, and mTOR in the absence of Sema3E and WT control (328, 333, 516). Moreover, the nuclear translocation and activation of transcription factors (e.g., NF-κB, AP-1, Foxo1) that are downstream of the ICOS/ICOS-L pathway should be examined in the absence and the presence of Sema3E protein (333, 534). Some of these transcription

factors and proteins also can be regulated by Sema3E. For example, *Sema3e*$^{-/-}$ mice have higher levels of both GATA3 and RORγt which play key roles in T cell differentiation (476).

- To investigate how Sema 3E interacts with other costimulatory or inhibitory pathways, such as PD-1/PD-L1 or CTLA-4, in modulating GC responses and antibody production. The ICOS/ICOS-L pathway is not the only signalling pathway that affects GC formation and antibody production. To fully understand through which signalling pathways Sema3E could affect GC responses and IgE production, it would be noteworthy to check the possibility of its effect on other stimulatory or inhibitory pathways.

- To investigate if the Sema3E has an autocrine effect on B cells and if its defect in B cells will affect IgE production. Sema3E is expressed by various cells, specifically alveolar macrophages, T cells, DCs, and ILC-2 (400, 535, 536). B cells also express Sema3E but at a lower level compared to other immune cells. However, B cells do express PlexinD1, which is a specific receptor of Sema3E. It would be helpful if the expression and secretion of Sema3E by B cells would be evaluated in naïve conditions and following B cell activation. Then, to check the autocrine effect of Sema3E on B cells, an adoptive transfer model in which *Sema3e*$^{-/-}$ B cells are transferred in μMT mice that lack B cells can be done. For this objective, naive splenic B cells from either WT and *Sema3e*$^{-/-}$ mice (both are CD45.2^{+}) will be isolated and mixed with WT CD45.1 B cells at a 1:1 ratio, and then the mixture will adoptively be transferred to μMT mice that lack B cells via tail vein (537). Then, the mice will be challenged with HDM, and the effect of Sema3E deficiency on B cells in GC center formation, IgE production, and inflammation will be assessed. We expect the adoptive transfer of *Sema3e*$^{-/-}$ B cells to mice lacking B cells should lead to increased GC responses, IgE production, and overall disease exacerbation following the HDM challenge.

- To investigate if the Sema3E expressed by other immune cells has a paracrine effect on B cells. B cells have a rich connection with other immune cells, which can be affected by

Sema3E, including DCs and T cells. Hence, Sema3E does not directly impact B cells but can also indirectly affect B cells. This objective will investigate the hypothesis that Sema3E, expressed by other immunological cells, can indirectly affect B cells, IgE production, and GC center formation. For this aim, the plexinD1 receptor on B cells should be knocked out so the Sema3E secretion by other cells cannot impact B cells. In this experiment, the B cell-specific Cre (CD19 Cre) mice will backcross with floxed plexinD1 mice to generate plexinD1 receptor B cells KO. These mice will be challenged with HDM for two weeks, and the IgE production and GC formation levels will be assessed. In this experiment, if Sema3E has a paracrine effect, on B cells we will see results are like the KO model, and if it doesn't have the paracrine effect the results will be more like the WT group.

- To investigate lung function in *Sema3e*$^{-/-}$ and WT mice, particularly before and after ICOS-L blockade. This approach would provide insights into whether the heightened immune responses observed in the absence of Sema3E, particularly GC responses, translate into altered lung function or airway hyperresponsiveness (AHR). Pulmonary function tests could be performed to assess baseline lung function, followed by repeated measurements after ICOS-L treatment. This would allow for a detailed understanding of how ICOS-L inhibition impacts respiratory parameters in Sema3E deficiency, potentially linking immune dysregulation to lung pathology.

  In addition to functional assays, it will be important to evaluate structural changes in the lungs, particularly regarding airway remodeling, which is a hallmark of chronic inflammation in diseases like asthma. Proposed measures of airway remodelling could include histological analysis of lung tissue to assess parameters such as airway smooth muscle mass, epithelial thickening, and collagen deposition. Staining for α-smooth muscle actin (α-SMA), which marks smooth muscle cells, should be incorporated to quantify airway smooth muscle remodelling. The inclusion of other markers for fibrosis and extracellular matrix deposition could further refine the understanding of how Sema3E deficiency, combined with altered ICOS-L signalling, affects lung structure. This multifaceted approach could help identify whether targeting ICOS-L has therapeutic potential in preventing airway remodelling and preserving lung function in inflammatory diseases.

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