A study on the role of Semaphorin 3E-PlexinD1 axis in dendritic cells for immunity to chlamydial infection

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ABSTRACT

Recent studies have identified semaphorin 3E (Sema3E) as a novel mediator of immune responses. However, its function in immunity to bacterial infection has yet to be investigated. Using a mouse model of chlamydial lung infection, we show that Sema3E plays a significant role in the host immune response to the infection. We found that Sema3E is induced in the lung after chlamydial infection, and Sema3E deficiency has a detrimental impact on disease course, dendritic cell (DC) function, and T cell responses. Specifically, we found that Sema3E knockout (KO) mice exhibited higher bacterial burden, severe body weight loss, and pathological changes after *Chlamydia muridarum* lung infection than wild-type (WT) mice. The severity of disease in Sema3E KO mice was correlated with reduced Th1/Th17 cytokine responses, increased Th2 response, altered Ab response, and a higher number of regulatory CD4 T cells. Moreover, DCs isolated from Sema3E KO mice showed lower surface expression of co-stimulatory molecules and production of IL-12 but higher expression of PD-L1, PD-L2, and IL-10 production. Functional DC-T cell co-culture studies revealed that DCs from infected Sema3E KO mice failed to induce Th1 and Th17 cell responses compared with DCs from infected WT mice. Upon adoptive transfer, mice receiving DCs from Sema3E KO mice, unlike those receiving DCs from WT mice, were not protected against challenge infection.

Moreover, we found that Sema3E signaling on DC is crucial for their enhanced migration after chlamydial infection. Specifically, we observed reduced numbers of $CD103^+$ lung DC and $CD8\alpha^+$ spleen DC in Sema3E KO mice compared to WT mice after *C. muridarum* infection. Migration studies revealed that *C. muridarum* infected Sema3E deficient DCs have impaired ability to respond to CCL19 in vitro and migrate to the lymph node in vivo. Further analysis of downstream

signaling events showed that Sema3E deficiency reduces Rac1GTP, F-actin polymerization, Erk, and Akt phosphorylation in *Cm* infected DC upon CCL19 stimulation.

Our data also demonstrated that dendritic cells express the Sema3E high-affinity receptor, PlexinD1, which makes them responsive to Sema3E. Therefore, we studied whether deficiency of PlexinD1 in dendritic cells increases susceptibility to chlamydial infection. PlexinD1 deficiency on dendritic cells leads to increased severity of *Cm* infection with higher bacterial load, bodyweight loss, and pathological changes in the lung. More importantly, we observed lower IFN γ production by CD4 and CD8 T cells and IL-17 cytokine production in CD11c PLXND1$^{-/-}$ mice (mouse deficient in PlexinD1 on dendritic cells) compared to CD11c PLXND1$^{+/+}$ mice. We also found that PlexinD1 deficiency altered the phenotype and cytokine production pattern of DCs following *Chlamydia muridarum* infection. Adoptive transfer of PLXND1^{+}DCs provided more robust protection with enhanced Th1/Th17 response against *Cm* infection than PLXND1^{-}DCs. To investigate the translational significance of our findings, *Chlamydia muridarum i*nfected mice were treated with exogenous Sema3E. Intranasal Sema3E treatment reduced chlamydial infection in the lung by promoting the Th1/Th17 response and inhibiting Treg response. Sema3E treatment also enhanced the recruitment of Th1 promoting CD103^{+} lung and CD8α^{+} spleen DC subsets. In conclusion, our data evidenced that Sema3E acts as a critical factor for protective immunity against intracellular bacterial infection by modulating DC functions and T cell subsets.

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DEDICATION

I dedicate this thesis to my husband, daughters, and parents

This thesis is also dedicated to mice I sacrificed for the completion of this thesis

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Figure 5: Structure of the Sema receptor PlxnD1. Reprinted with permission from Dev Biol. 2011. Jan 1; 349(1): 1–19 by Elsevier.

LIST OF ABBREVIATIONS

Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen-presenting cell
APC	Allophycocyanin
BAL	Bronchoalveolar lavage
BCR	B-cell antigen receptor
BMDM	Bone marrow derived macrophage
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
CCL19	Chemokine ligand 19
CD	Cluster of differentiation
cDCs	Conventional DCs
CFSE	Carboxyfluorescein succinimidyl ester
cGAS	Cyclic GMP-AMP synthase
Cm	*Chlamydia muridarum*
C. muridarum	*Chlamydia muridarum*
CPAF	Chlamydia protease-like activity factor
CXCL-1	CXC-chemokine ligand
DC	Dendritic cell
EB	Elementary body
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent activated cell sorting
FcR	Fc receptor
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus One
FPR2	Formyl peptide receptor 2
GAP	Guanosine triphosphatase (GTPase)-Activating Proteins
GM-CSF	Granulocyte-macrophage colony stimulating factor

GRO	Growth-related oncogene
HBSS	Hank's balanced salt solution
H&E	Hematoxylin and eosin
HtrA	High temperature requirement A protein
IFNγ	Interferon-γ
IFU	Inclusion-forming unit
Ig	Immunoglobulin
IL	Interleukin
i.n.	Intranasally
IPT	Immunoglobin-like fold shared by Plexins and Transcription factors
i.v.	Intravenous
KO	Knock out
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MLNs	Mesenteric lymph nodes
MOI	Multiplicity of infection
MRS	MET-Related Sequence
NET	Neutrophil extracellular traps
Neto1	Neuropilin tolloid-like 1
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NOD	Nucleotide-binding oligomerization domain
Nrp1	Neuropilin-1
PBS	Phosphate Buffered Saline
pDC	Plasmacytoid dendritic cell
pi	Post infection
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDGFR	Platelet-derived growth factor receptor

PE	Phycoerythrin
PID	Pelvic inflammatory disease
PLXND1	PlexinD1
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptors
PSI	Plexin–semaphorin–integrin
Rac 1	Ras-related C3 botulinum toxin substrate 1
RB	Reticulated body
RBD	Rho GTPase-binding domain
RORγt	Retinoic acid receptor–related orphan nuclear receptor γt
Sema3E	Semaphorin 3E
SP	Sex and Plexins
SPG	Sucrose-phosphate-glutamic acid buffer
STING	Stimulator of interferon genes
TCR	T cell receptor
Tc	Cytotoxic T cell
Th	T helper cell
TLRs	Toll-Like Receptors
TNF	Tumor necrosis factor
T3SS	Type III secretion system
STD	Sexually transmitted diseases
UVEB	Ultraviolet-killed elementary body
VEGFR2	Vascular endothelial growth factor receptor type 2
WHO	World Health Organization
WT	Wild-type

CHAPTER 1

GENERAL INTRODUCTION

1.1. CHLAMYDIAL DISEASES

1.1.1. Etiology and Epidemiology

Chlamydia are the most ancient Gram-negative obligate intracellular bacteria [1]. *Chlamydiae* were identified independently by evinthal, Cole, and Lillie in 1929–1930 [2] and first isolated by Tang in 1955 [3]. Chlamydial-like eye diseases had been known for centuries and described in ancient Chinese and Egyptian writings [4]. The genus *Chlamydia* belong to the family *Chlamydiaceae*, order *Chlamydiales* and phylum *Chlamydiae*[5]. Initially, *Chlamydia* was taxonomically arranged into 4 species namely *Chlamydia (C.) trachomatis, Chlamydia psittaci, Chlamydia pneumoniae* and *Chlamydia pecorum* [4]. Later in 1999, based on ribosomal RNA (rRNA) gene sequences, Everett et al. proposed to divide the family *Chlamydiaceae* into two genera, *Chlamydia* and *Chlamydophila* [6]. This revision incorporated nine species into *Chlamydiacea* namely *Chlamydia trachomatis*, *Chlamydia muridarum*, *Chlamydia suis, Chlamydophila (Cp.) abortus, Cp. caviae, Cp. felis, Cp. pecorum, Cp. pneumoniae, and Cp. Psittaci* [6]. Division of *Chlamydiaceae* into *Chlamydia* and *Chlamydophila* has been discussed controversially. Therefore proposal was made to include all currently recognized species to a single genus genus *Chlamydia* [5]. Currently, 14 species have been described within this genus: *C. abortus*, *C. psittaci*, *C. avium*, *C. gallinacea*, *C. buteonis*, *C. caviae*, *C. felis*, *C. muridarum*, *C. pecorum*, *C. pneumoniae*, *C. poikilothermis*, *C. serpentis*, *C. suis* and *C. trachomatis* [4].

C. psittaci, *C. avium*, *C. gallinacea*, *C. buteoni* cause infections in birds [4]. *C. serpentis* and *C. poikilothermis* cause infection in snakes [7]. *Chlamydia suis* has only been isolated from swine and is associated with conjunctivitis, enteritis and pneumonia in swine [6]. *C. pecorum* was associated with different pathological conditions in ruminants, swine and koalas [8]. *C. abortus* cause infectious abortion in sheep and goats and induces a persistent subclinical infection in nonpregnant sheep and goats [9]. *C. caviae* was first isolated from the conjunctiva of guinea pigs and can also infect the genital tract to cause disease similar to human genital infection [6]. *C. felis* cause chronic conjunctivitis in cats [10]. *Chlamydia muridarum* (MoPn) have been isolated from mice and hamsters [11, 12]. *Chlamydia muridarum* causes lung and genital infections in mice.

C. trachomatis and *C. pneumonia* are the two chlamydial species pathogenic to humans [13]. Halberstaedter and von Prowazek first observed *C. trachomatis* in the conjunctival scrapings of patients with trachoma and they proposed the name Chlamydozoa [14]. Later, Tang et al. isolated *C. trachomatis* from patients with trachoma using chick embryos [14]. *C. trachomatis* currently exists in 19 serovars (A, B/Ba, C, D/Da, E, F, G/Ga, H, I/Ia, J, K, L1, L2, L2a and L3) based on the expression of epitopes of Major Outer Membrane Protein (MOMP) [15]. Serovars A, B/Ba, and C are responsible for trachoma. Serovars D-K causes occulo-genital infections and serovars L1-L3 are associated with lymphogranuloma venereum [15]. The genome of *C. trachomatis* genitotropic strain D/UW-3 is composed of a single chromosome of 1,042,519 base pair, 894 coding sequences and a plasmid of 7,493 base pair [16, 17]. Even though most *C. trachomatis* strains infect mucosal epithelial linings, lymphogranuloma venereum (LGV) strains attack lymph nodes and lymphatics [13]. Trachoma caused by *C. trachomatis* is the common cause of infectious blindness around the world [18]. It is characterized by papillary and/or follicular inflammation of the conjunctiva [18]. This inflammation causes scarring, eyelid distortion and turning of eyelashes

to eyeball (trichiasis), resulting in blindness [18]. Trachoma is primarily seen in young children, and the prevalence of the disease reduces with age. According to World Health Organization (WHO) estimates of 2011, trachoma affects 21 million people worldwide, of which 2.2 million are blind or visually impaired [18].

Apart from trachoma, *C. trachomatis* also causes sexually transmitted diseases (STD). Globally, a total of 131 million new cases of chlamydial STDs were reported [19]. Chlamydial STD is highly prevalent in the United States, with reported cases of 1.7 million in 2017 [20]. In Canada, between 2010 and 2015, chlamydial STD rates increased by 16.7 %, and an estimated 116,499 cases were reported in 2015 [21]. In women, chlamydial infections are mostly asymptomatic, but when left untreated can result in pelvic inflammatory disease (PID) characterized by chronic pelvic pain, ectopic pregnancy and infertility [19, 22]. Infected pregnant women can also transfer the infection to babies at the delivery time, causing pneumonia and neonatal ophthalmia (conjunctivitis) [21, 22]. In men, untreated sexually transmitted chlamydial infection complications involve chronic prostatitis and urethritis [23, 24]. In addition, *C. trachomatis* infection facilitates infection of Human Immunodeficiency Virus and human papilloma virus [25, 26].

C. pneumoniae causes common respiratory infections, including community-acquired pneumonia, sinusitis, pharyngitis and bronchitis [27]. *C. pneumoniae* is prevalent in the majority of individuals with the existence of antibodies against *C. pneumoniae* in 50% of individuals by age 20 and 80% by 60-70 years old [27]. *C. pneumoniae* is morphologically as well as genetically distinct from *C. trachomatis*. *C. pneumonia* exists inside the cell in the form of a pear-shaped elementary body (EB) whereas *C. trachomatis* exist as round-shaped EBs [28]. In addition, *C. pneumonia* has less than 5% DNA homology to *C. trachomatis* [28]. Recent studies also identified the relationship of *C. pneumonia* with several inflammatory diseases, namely, atherosclerosis, arthritis, asthma, and

neurological disorders like Alzheimer's disease, schizophrenia, and multiple sclerosis [27, 29]. Even though broad-spectrum antibiotics such as erythromycin and tetracycline are effective against chlamydial infections, it is challenging to diagnose infection at an early stage due to their asymptomatic behavior [30]. In fact, antimicrobial treatment can hinder the development of natural immunity to chlamydial infection, thereby increasing susceptibility of transmission of chlamydia [30]. This situation is worrisome due to the lack of vaccines for preventing the disease. The main hindrance for effective vaccine development is the poor knowledge of the protective immunity and immunopathology of chlamydial infections. Thus, the investigation of the host immune response to *Chlamydia* is vital for a better understanding the disease.

1.1.2. Developmental cycle of *Chlamydia*

Chlamydia has a biphasic developmental cycle with two distinct forms, elementary body (EB) and reticulate body (RB) (Fig. 1) [31]. EB is a metabolically inactive form, which infects mucosal epithelial cells. Binding of EB to host cells involves a two-step process. First, a reversible interaction occurs between heparin-sulphate proteoglycans and EBs [32]. Second, an irreversible interaction occurs with host receptors namely, mannose receptor, estrogen receptor, epidermal growth factor receptor and platelet-derived growth factor receptor (PDGFR) [31, 32]. After this step, EBs are internalized and reside in a vacuole formed from the endosomal membrane to form an inclusion body [31]. EBs are confined to the inclusion body and interfere with host antimicrobial pathways by the secretion of Type III secretion system (T3SS) effector proteins as well as chlamydial proteins like the chlamydia protease-like activity factor (CPAF) and the high-temperature requirement A protein (HtrA) [31, 33]. Later, EB transforms into a reticulate body (RB), which is metabolically active and divides by binary fission within 24 h post-infection [31]. After 48-72 hours, multiplication ceases, and nucleoid condenses to transform RBs to infectious

EBs. EBs are then released from cells to infect more cells [30, 34]. The time for the completion of the developmental cycle varies based on chlamydial strain and host cell environment [31]. Under stressful conditions (amino acid deprivation), chlamydia enters a persistent state where the bacteria are viable but non-infectious [35]. This persistence allows *Chlamydia* to maintain a long-term infection inside the host cell [35].

The phenotypical changes in these developmental stages of *Chlamydia* correlate with changes in gene transcription [36]. Stage I genes are expressed at early time points of infection. Stage II genes are activated by 18 hours post-infection, and stage III genes are transcriptionally activated by 36 hours post-infection. Gene transcripts expressed at high levels during stage I are oligopeptide binding protein (*oppA*), *groEL*, and inclusion membrane protein (*incC)* [36]. Stage II gene products are involved in RB production and re-organization. Major genes expressed at this stage are polymorphic membrane protein (*pmp*), protease genes (*clpP* and *lon),* integration host factor (*himD*), and an SWI/SNF family helicase (CT708) [36]. These genes are involved in DNA condensation during the transition from RB to EB. Other genes expressed at this stage are outer membrane protein (*ompA* and *ompB*), *glgA* glycogen synthase, disulfide bond isomerase (CT783), and *yscC* (structural component of the type III secretion system) [37]. Stage III gene transcripts are required for nucleoid condensation and EB formation. The genes transcribed at this stage are outer membrane proteins (*omcA* and *omcB*), DNA topoisomerase (CT643), DNA gyrase A paralogue (CT660), histone-like protein 2 (*hctB*) and a regulator of type III secretion (*lcrH*) [36, 37].

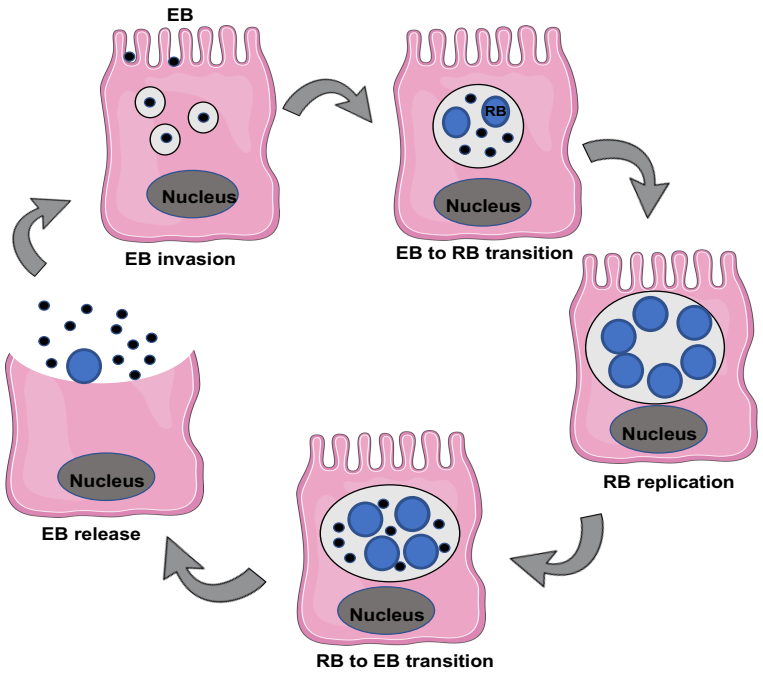

Figure 1: Developmental cycle of *Chlamydia*. Elementary bodies after internalization reside inside the cell within a vacuole called inclusion. EBs then differentiate into metabolically active RBs, which divide by binary fission and increase in numbers. RBs then differentiate back to EBs and subsequently released from host cell by cell lysis.

1.2. ANIMAL MODELS FOR STUDYING CHLAMYDIAL INFECTION

Many animal models from rats, primates, guinea pigs and mice have been used for studying chlamydial infection [34]. Mice are the most commonly used animals for understanding basic immunological mechanisms because of their low cost, widespread availability, ease of handling, and, more importantly, the availability of genetically modified mouse strains [30, 38]. Pathogenicity and immunity of chlamydial respiratory infections are most widely studied using models of respiratory tract infections caused by *C. muridarum* (mouse biovar of *C. trachomatis*) *and C. pneumoniae*. In particular, *C. muridarum* is used often in the study of both respiratory and genital tract infections because it can readily infect the mice through both routes. Moreover, the observed changes in histopathology and immune responses mimic human diseases, especially for acute infection. Pathological alterations in the lung include infiltration of polymorphonuclear leukocyte and mononuclear cells in the early and late stages of infection [39]. Severe inflammation in the lungs are characterized by inflammatory cell infiltration into perivascular, peribronchiolar, alveolar septae, and air space [40]. Infected mice start losing their weight in the initial stages, peaking around 10 days and then gradually recover. The infection with *C. muridarum* is commonly resolved within 2-3 weeks [39].

1.3. CHLAMYDIAL INFECTION AND IMMUNE RESPONSES

Chlamydial infection of host epithelial cells and tissue-resident innate immune cells activates the host's innate and adaptive immune response to release several cytokines and chemokines as well as promote the infiltration of leukocytes, T cells and B cells [30, 41]. The mucosal barrier acts as the first line of defense to prevent chlamydial invasion. Toll-Like Receptors (TLRs) expressed on innate as well as epithelial cells recognize specific chlamydial components. For example, chlamydial lipopolysaccharide (LPS) is recognized by TLR2, chlamydial heat shock protein 60

(cHSP60) by TLRs 2 and 4 and lipoprotein by TLRs1/2 or TLRs2/6 [42]. In addition to TLRs, other pattern recognition receptors (PRRs) such as nucleotide-binding oligomerization domain (NOD), Stimulator of interferon genes (STING), cyclic GMP-AMP synthase (cGAS) are also reported to recognize chlamydial PAMPS [42, 43].

Chlamydial infection of host cell results in the release of proinflammatory cytokines and chemokines like interleukin (IL)-1, IL-8, tumor necrosis factor (TNF), granulocyte-macrophage colony stimulating factor (GM-CSF), growth-related oncogene (GRO)-α, CXC-chemokine ligand (CXCL-1), CXCL-16, IFN-γ, IL-12, chemokine ligand 5 (CCL5), interferon α (IFN-α), IFN-β, tumor-necrosis factor (TNF) and IL-6 [30]. These inflammatory mediators recruit innate cells such as neutrophils, dendritic cells, natural killer (NK) cells and monocytes to the site of infection [42].

1.3.1. Innate immune responses to chlamydial infection

1.3.1.1. NK cells and neutrophils

After chlamydial infection, NK cells and neutrophils are the first cells to reach the site of infection [41]. Cytokines such as IL-8 and IL-17 secreted by *chlamydia*-infected cells attract neutrophils to the mucosal site of infection [41, 44]. Even though higher neutrophil infiltration was observed after chlamydial infection, neutrophils were not efficient for controlling chlamydial growth in the lung [45]. This observation may be related to the recent findings that *Chlamydia trachomatis* evade host immune response by paralyzing neutrophils [46]. In addition, *Chlamydia*-secreted effector protein, chlamydial-protease-like activity factor (CPAF) causes cleavage of formyl peptide receptor 2 (FPR2) expressed on the surface of neutrophils and inhibit neutrophil extracellular traps (NET) activity [46].

Similar to neutrophils, NK cells are recruited to the site of infection as early as 12 h after genital tract infection with *C. muridarum* [47]. It is found that *C. trachomatis* infection of human

epithelial cells and dendritic cells (DC) results in interleukin-18 (IL-18) and IL-12 production, which in turn induced IFN-γ production by NK cells [48]. Moreover, recent studies shows that NK cell depletion leads to phenotypic and functional changes in the DC and promoted chlamydial infection in the lung [49]. In addition, NK cells enhanced IL-12 production by DC and promoted Th1 and Th17 responses [49, 50]. On the other hand, NK cells significantly suppressed regulatory T cells (Treg) response after *C. muridarum* lung infection [51]. Similar to these findings, NK-cell-depleted mice showed an exacerbated *C. muridarum* genital infection with higher Th2 response [47]. Overall, these studies suggest that IFN γ production by NK cells modulates DC to induce strong Th1/Th17 immunity to chlamydial infection.

1.3.1.2. NKT Cells

In response to intranasal *Chlamydia pneumonia*, intravaginal *Chlamydia muridarum*, and intra-articular *Chlamydia trachomatis* infections, stimulation of invariant natural killer T (iNKT) cells using α-GalCer provided protection to infection [52-54]. In contrast, alpha-Galcer treatment after intranasal *Chlamydia muridarum* infection resulted in a pathological response to chlamydial infection [52]. NKT cell response during chlamydial infection was also analyzed using CD1d-KO mice (which lack iNKT cells and type 2 NKT cells). Intra-articular *Chlamydia trachomatis* infection of CD1d-KO mice resulted in resistance to infection compared to WT mice [54]. On the other hand, pulmonary *Chlamydia muridarum* infection of CD1d-KO mice resulted in resistance to infection compared to WT mice [52]. Studies in Jα18-KO mice (which lack iNKT cells) showed that *Chlamydia muridarum* infection resulted in higher lung chlamydia load and pathology compared to WT mice [52]. In contrast to this observation, studies in Jα18-KO mice in *Chlamydia pneumonia* infection showed a protective role of iNKT cells to infection with a predominantly Th1 response [52]. Studies in Yang lab also showed how iNKT impacts DC function to provide an

optimal immune response to chlamydial infection. Transfer of DC from *Chlamydia*-infected Jα18-KO (lack iNKT cells), in contrast to WT, mice promoted chlamydial growth upon challenge with chlamydial infection [55]. Subsequent studies on DC subsets identified that CD8α+ DCs are the major DC subset that interacts with iNKT cells to induce protective Th1 immunity [56].

1.3.1.3. Macrophage

Macrophages play a vital function in the innate immune response to *Chlamydia* infection. Nitric oxide (NO) production by macrophages is required to inhibit chlamydial growth inside the macrophage [57]. However, *Chlamydia* species survive inside the macrophage by subverting phagosome maturation [58]. It is found that in response to *Chlamydia pneumonia* infection, macrophages secrete IFN-γ production to control chlamydial growth [59]. Depletion of macrophage prior to *Cm* infection resulted in increased chlamydial load and morbidity [60]. Moreover, adoptive transfer of macrophages to RAG-$1^{-/-}$/IFN-$\gamma^{-/-}$ mice reduces lung pneumonia burden and dissemination [61]. These studies highlight the significant role played by macrophages in the control of intracellular chlamydial infection.

1.3.1.4. Dendritic cells

DCs are the major antigen-presentation cells (APCs) of the immune system and have a crucial role in both sensing pathogens and tuning the immune responses [62, 63]. DCs reside in an immature form at various portals of pathogen entry. Under steady-state conditions, DCs express low levels of major histocompatibility complex (MHC) and costimulatory molecules [63]. On exposure to pathogens, TLRs and other receptors on the surface of DCs recognize molecular patterns associated with microbes, which initiates DC maturation, upregulation of CCR7 and consequent migration to the local draining lymph nodes where interaction with naive T cell occurs [63]. Mature DCs express high levels of MHC and co-stimulatory molecules, which enable them to activate

naive T cells in T cell areas of secondary lymphoid organs [64]. Priming and modulation of T cells by DCs involves the interaction of CD80 (B7-1)/CD86 (B7.2) and CD40 with CD28/CTLA4 (CD152) and CD40L on T cells, respectively [63, 65]. In addition, activated DCs produce proinflammatory and immunomodulatory cytokines and chemokines, which shape the pattern of immune responses [63, 66].

1.3.1.4.1. Dendritic cell subtypes

Dendritic cells consist of different subtypes and are classified based on their phenotype, location and function [63, 67]. Conventional DCs (cDCs) mainly reside in the lymphoid tissues such as the spleen, thymus and secondary lymph nodes (LNs). These conventional DCs express higher MHC-II and CD11c and can be further divided into $CD8\alpha^+$ and $CD8\alpha^-$ DCs in mice. Compared with $CD8\alpha^+$ DCs, which more often induce Th0 cells to elicit Th1 response, $CD8\alpha^-$ DCs more likely induce Th2 responses [68]. In addition, cDCs in the non-lymphoid tissues such as the intestine and the lung consist of two major subsets; $CD103^+$ and $CD11b^{hi}$ DCs. Interestingly, $CD103^+$DC in the non-lymphoid organs, including lung, gut and skin, form a unified subset, which is developmentally related to the $CD8^+$cDC in lymphoid organs [69]. This correlation is demonstrated by their shared dependence on certain transcriptional factors such as *Batf3* and *Irf8* and functional characteristics of antigen cross-presentation. The linkage between $CD8^+$DC and $CD103^+$DC was further strengthened by the reports showing unique common expression of XCR1, a chemokine receptor, by these DC subsets [70, 71]. Since XCR1 are also expressed in human $BDCA3^+$DC and sheep $CD26^+$DC (the equivalents of mouse $CD8^+$DC), the term "$XCR1^+$DC" could be designated to "$CD8^+$ type DC" in both lymphoid and peripheral tissues across all mammalian species. Plasmacytoid DCs (pDCs) represent a small subset of DCs that enter the lymph nodes through blood circulation. Upon activation through Toll-like receptor (TLR)-7 and

TLR9 stimulations, pDCs secrete profound amounts of IFN-α and several chemokines (CCL3, CCL5, CXCL10) [72].

In humans, DCs express high levels of MHC II and lack markers such as CD3, CD19/20 and CD56. They can be classified as either myeloid or plasmacytoid [73]. Myeloid DCs (mDCs) correspond to mouse cDCs and express myeloid antigens such as CD11c, CD13, CD33 and CD11b. They are divided into $CD1c^+$ and $CD141^+$ DCs, which share homology with mouse $CD11b^+$ DC and $CD8/CD103^+$DC, respectively. $CD14^+$ DCs, described initially as interstitial DCs, are a third subset $CD11c^+$ myeloid DC found in tissues and lymph nodes. Human plasmacytoid DCs lack myeloid antigens and express CD123, CD303 and CD304 [73].

The role of DCs in chlamydial antigen presentation and protective immunity was shown by in vitro and in vivo studies. In response to infection with *Chlamydia muridarum*, DCs were recruited to the site of infection [74]. DCs internalize *Chlamydia* non-specifically by macropinocytosis, and macropinosomes later fuse with lysosomes [74]. In the case of human DC infection *with C. trachomatis*, chlamydial entry to DC occurs in a heparan sulfate-dependent manner, and the attachment is inhibited by the coating of *Chlamydia* with heparin [75]. The interaction of DC with *Chlamydia* leads to DC maturation and chlamydial antigen presentation to T cells [74]. *Chlamydia* infected DC upregulate the expression of CCR7, MHC class II, CD40, CD80 and CD86 molecules [76, 77]. In addition, activated DC secrete profound amounts of cytokines such as interleukin-1beta (IL-1beta), IL-6, IL-8, IL-12p70, IL-18, IL-10 and tumor necrosis factor (TNF) [76, 77]. It is found that live and UV-killed *C. muridarum* exhibits different levels of DC activation; live *C. muridarum* induced higher expression of CD40, CD80, CD86, and higher levels of IL-12 and TNF-alpha compared to UV-killed *C. muridarum* [78]. Cytokine production by DC plays an important role in determining the type of T cell response. It is observed that interleukin 12 (IL-12)

produced by dendritic cells upon infection with *Chlamydia* induce a Th1 cell-dominant response [79]. Similar to lL-12, IL-10 cytokine produced by DCs influences T cell response. IL-10 deficient DCs are strong inducers of Th1 cell response from naive or immune T cells and act as an efficient cellular vaccine against genital chlamydial infection [80]. Furthermore, deficiency of IL-10 resulted in maturation of *Chlamydia* pulsed DC with augmented ability to present chlamydial antigen for rapid T cell response [80]. This is in line with previous studies in IL-10 KO mice, which showed stronger Th1 response and higher IL-12 production after *C. muridarum* infection [81].

Recent studies documented that lung and spleen DC subsets showed differential abilities to induce protective T cell immune response to chlamydial infection. The functional role of $CD8\alpha^+$ and $CD8\alpha^-$ DC spleen subsets were analyzed using an adoptive transfer experiment. After *C. muridarum* infection, mice receiving $CD8\alpha^+$ DC showed lower body weight loss, pathogen load, and pathological changes than those receiving $CD8\alpha^-$ DC [82]. Cytokine analysis also showed an enhanced Th1 response and reduced Th2 response in $CD8\alpha^+$ DC recipients than $CD8\alpha^-$ DC recipients [82]. Recently, our laboratory also studied the immune function of lung $CD103^+$ and $CD11b^{hi}$ DCs. The adaptive transfer of $CD103^+$ DC induced stronger protection from *C. muridarum* challenge infection than from the transfer of $CD11b^{hi}$ DC in the recipient mice [83]. $CD103^+$ DC recipients exhibited stronger Th1 (IFNγ) and Th17 (IL-17) responses than $CD11b^{hi}$ DC recipients [83]. Altogether, these studies highlight the critical role of different DC subsets for protection against chlamydial infection.

1.3.2. Adaptive immune response to chlamydial infection

1.3.2.1.T-cell responses to chlamydial infections

T cells hold an essential role in mediating adaptive immune response to infection. $CD4^+$T cells activate immune cells such as $CD8^+$T cells, B cells, and other immune cells by direct contact and cytokine release [84]. $CD8^+$T cells are involved in cytotoxic and cytokine response.

1.3.2.1.1.Th cell differentiation

Interaction of naive $CD4^+$T cells with antigen-MHC complex activate T cells to differentiate into specific subtypes depending on the cytokine milieu and activation of specific transcription factors [84]. The $CD4^+$ T cells differentiate into subsets such as Th1, Th2 and Th17 cells. Cytokines produced by DC play a major role in inducing T cell differentiation [85]. IL-12 produced by dendritic cells are involved in the differentiation of naïve T cells into Th1 cells. Interleukin 12 (IL12) and interferon γ (IFN γ) initiate the downstream signaling cascade involving signal transducer and activator of transcription 4 (STAT-4), STAT-1, and T box transcription factor T-bet to induce differentiation of Th1 cells [85]. IL-4 and IL-2 are the cytokines favor the Th2 differentiation [85]. IL4-induces STAT-6, leading to the upregulation of GATA3 (GATA-binding protein and Th2 differentiation [86]. Th17 cells require transcription factor, RORγt, and cytokines such as IL-6, IL-23 or IL-21 in combination with transforming growth factor-β (TGF-β) for their differentiation [84].

1.3.2.1.2.Th1/Tc1 responses to chlamydial infection.

IFN-γ production by $CD4^+$ and $CD8^+$ T cells is the critical factor for protection against chlamydial infection [87]. Adoptive transfer of $CD4^+$ T cells from *Chlamydia* infected mice following resolution of a primary infection provided protective immunity to genital tract infection of naïve mice [88]. Even though CD4 and CD8 T cells' depletion resulted in a higher chlamydial burden

in the lungs, CD4 T cells play a dominant role in anti-chlamydial immunity [89]. It is found that IFN-γ production rather than the cytotoxic effect of CD8 T cells provides protective response to chlamydial infection [61]. For example, animals deficient in perforin, Fas or Fas ligand, or of both perforin and Fas, were able to clear *C. muridarum* similar to WT mice [90].

The role of IFN-γ in controlling chlamydial infection is evidenced from in vitro and in vivo studies utilizing mice deficient in IFN-γ, IFN-γ receptor and IFN-γ neutralization [30, 87]. IFN-γ restricts intracellular growth of *Chlamydia* mainly by inducing indoleamine-2, 3-dioxygenase (IDO) enzyme. IDO can degrade tryptophan, an essential amino acid for chlamydial replication [91]. IFN-γ can also induce Nitric Oxide production, which inhibits chlamydial growth and promotes a protective type 1 immune response [92]. Recent studies have also shown that some strains of *Chlamydia* can synthesize tryptophan in the presence of IFN-γ by utilizing indole from local microbial flora. This strategy allows them to evade IFN-γ mediated inhibition and establish chlamydial persistence [93]. This persistence also induces epithelial cells to continuously release proinflammatory cytokines that in turn leads to tissue damage.

1.3.2.1.3.Th2 responses to chlamydial infection

In contrast to the Th1 immune response, which primarily provides protection to chlamydial infection, the activation of Th2 cells is associated with pathology in mouse models of primary chlamydial lung infection [94]. $CD4^+$ T cells producing IL-4 and IL-5 mediate inhibition of protective immune response [94, 95]. Studies showed that increased IL-4 cytokine production was associated with persistent chlamydial infection and pathogenesis in trachoma [96]. Moreover, adoptive transfer of *C. muridarum* specific Th2 cells, in contrast to Th1 cells, was unable to eradicate chlamydia from the infected genital tract [97].

1.3.2.1.4. IL-17/Th17 responses to chlamydial infection

Initial studies by Infante-Duarte *et al.* identified $CD4^+$ T cells producing interleukin-17A (IL-17A) as a T helper cell subset distinct from Th1 cell and Th2 cell subsets [98]. This subset called Th17 cells predominantly produce IL-17A, IL-17F, IL-21, and IL-22 [99]. IL-17A is a homodimeric glycoprotein with 155 amino acids linked by disulfide bonds. It was originally identified as a pro-inflammatory cytokine involved in the generation, migration, and activation of neutrophils [100]. Recent studies identified the relevance of IL-17A in coordinating innate and adaptive immune responses [100, 101]. IL-17F, also produced by Th17 cells, shows 55% similarity with IL-17A, and they form IL-17F homodimers, IL-17A homodimers, or IL-17A-IL-17F heterodimers [102]. IL-17 binds to its receptor (IL-17R), a transmembrane protein highly expressed in the spleen, kidney, liver, and lungs of rats and mice [103]. Th17 cells express CCR6, which induces their migration to the site of inflammation [104].

Once they reach the site of inflammation, IL-17 released by Th17 cells stimulates the expression of proinflammatory cytokines like granulocyte–macrophage colony-stimulating factor, granulocyte-colony stimulating factor, IL-6, and tumor necrosis factor-alpha (TNF) [105]. In addition, IL-17 also promotes the secretion of CXC chemokines like CXCL1 (keratinocyte/growth-regulated oncogene-α), CXCL2 (macrophage inflammatory protein 2), CXCL5, CXCL8 (IL-8), and CXCL10 (IFN-inducible protein 10), which attracts neutrophils *in vivo* [105]. Moreover, IL-17 stimulates the production of antimicrobial peptides such as β-defensin and S100 proteins providing defense against a wide range of microorganisms [106, 107]. Even though most studies on IL-17A producing cells focused on Th17 cells, γδ T cells are also reported as a main source of IL-17A [101]. In contrast to Th17 cells differentiated in response to antigen,

γδT cells are functionally differentiated in the fetal thymus before antigen exposure and therefore act as innate first responders [108].

The critical role of IL-17A and its producers in immunity to chlamydial infection is very well documented in chlamydial lung infection. *C. muridarum* lung infection in mice induces IL-17A production, which in turn modulates dendritic cell function to promote protective type 1 T cell immunity [109]. Further examination identified IL-17A producers at early and later stages of chlamydial lung infection. Previous studies in Yang lab have shown that γδ T cells are the major producers of IL-17A at the initial stage of infection and quickly returned to the background level at day 4 post-infection. Studies on the γδ T cell subsets further identified that $V\gamma4^{+}$T cells are the major IL-17A producing γδ T cell subset at early stages of chlamydial lung infection [110]. IL-17A produced by γδ T cells has a promoting role on Th17 responses but no significant influence on T helper 1 response [111].

On the other hand, IL-17A produced by Th17 cells at later stages of chlamydial infection has a significant impact on the development of protective type 1 immunity [111]. These studies collectively suggest that IL-17A mediated protection against chlamydial lung infection depends mainly on Th17 cells rather than γδ T cells [111]. In contrast to the findings in lung infections, either IL-17 receptor signaling or IL-23 dependant induction of IL-22 and IL-17 is indispensable for resolving genital tract infection [44, 112].

1.3.2.1.5. Regulatory T cells

Regulatory T cells (Tregs) are a key member of the family of immunoregulatory cells involved in preserving self-tolerance and fine-tuning the immune response. Treg cells suppress inflammation by cell-cell contact or by releasing cytokines such as IL-10 or TGF-β, and they require the transcription factor FoxP3, for their differentiation [104]. Research in recent years identified two

different Treg cells called natural Treg cells (nTreg) and inducible Treg cells (iTreg) or as recently named, thymic-derived Tregs (tTregs) and peripheral-induced Tregs (pTregs), respectively. nTreg cells develop in the thymus, and when they enter peripheral tissues, they suppress self-reactive T cell activation. Studies in mice and humans found that nTreg cells constitute around 10% of CD4 T cells in the periphery [113]. They express FoxP3 before they are released from the thymus, and expression of TGF-β help in their maintenance after they migrate from the thymus [104]. Upon antigen exposure, iTreg cells develop from naive T cells in the secondary lymphoid organ. Following interaction with TCR, TGF-β induce the FoxP3 expression in $CD4^{+}CD25^{-}$ cells thereby converting them to $FoxP3^{+}$ $CD4^{+}$ $CD25^{+}$ cells [114]. These iTreg cells mediate their inhibitory activities by secretion of IL-10 or TGF-β.

Tregs play a beneficial or detrimental role based on the type and time of infection. There are a few studies on the involvement of Treg cells in chlamydial infections. Treg cells are observed in chlamydial infection sites of both humans and mice [115-117] [118]. In a mouse model of genital infection with *Chlamydia muridarum*, it is found that Tregs is involved in inducing oviduct pathology [115, 116]. Also, in the case of *Chlamydia trachomatis* infection in the genital tract of $ICOS^{(-/-)}$ mice, the reduction of Treg cells is associated with increased Th1 response [117, 118]. Moreover, plasmacytoid dendritic cells (pDC) contribute to severity of genital *C. muridarum* infection by inducing Treg cells [119]. Similarly, our recent studies suggested that NK cells provide protective immunity to chlamydial lung infection by inhibiting Treg expansion [51, 120]. Adoptive transfer of DCs from NK cell-deficient mice induced Treg cells in the recipient mice, which promote pathological response [120]. Altogether, these studies suggest that higher Treg responses contribute to tissue pathology after chlamydial infection.

1.3.2.2. B cells and antibody responses in chlamydial infection

The role of B cells during chlamydial infection has been studied for a long time. The effects of B cells in host defense against infections are likely mediated by antibody effector functions including neutralization, antibody-dependent cell-mediated cytotoxicity (ADCC) and enhanced antigen presentation to T cells [121]. However, in the primary infection of the female genital tract, B cell deficient mice and WT mice exhibit similar levels of protection against *C. trachomatis* infection [122]. But B cells are found to be important in the resolution of secondary genital tract infection [123]. In the genital tract infection, presence of IL-10 producing B cells is associated with reduced Th1 cell responses and impaired bacterial clearance [124]. In lung infection, B cell-deficient mice exhibit a significantly higher mortality rate than wild-type mice and failed to produce IFN-γ after *Chlamydia*-specific in vitro restimulation, suggesting a role of B cell in the development of T cell responses after infection [125]. Also, in the lung infection, the effect of antibody response in chlamydial infection varies depending on the isotype of the antibody. Higher IgG1 response observed in Balb/c mice compared to C57BL/6 mice, was associated with a lower ability to clear *C. muridarum* infection while higher IgG2a response appears to be associated with protection [126]. Moreover, enhanced IgG2a antibody produced after intranasal *C. trachomatis* infection resulted in optimal protective immunity to genital re-infection [127]. The presence of secretory IgA was inversely correlated with the numbers of *C. trachomatis* isolated from the cervix of female patients [128]. Overall, these studies indicate that antibody response mounted after chlamydial infection contributes to protection in secondary infections but the role in primary infection is limited. Summary of the innate and adaptive responses involved after chlamydial infection is shown in Fig. 2.

1.3.3. Chlamydial evasion of host immune response

Chlamydia utilizes several molecular means to evade host immune response. Under unfavorable conditions (presence of antibodies, antimicrobial substances, and proinflammatory cytokines), *Chlamydia* enters into a persistence stage [129]. For example, IFN-*γ* produced by host immune cells induces expression of indoleamine-2,3-dioxygenase (IDO) enzyme, which depletes tryptophan required for *C. trachomatis* growth [130]. To avoid this stressful situation, *C. trachomatis* enters a persistence stage until tryptophan supply is restored [131]. Moreover, *C. trachomatis* release tryptophan synthase (TrpBA) protein [132, 133]. Tryptophan synthase converts indole into tryptophan required for bacterial metabolism [133]. Recent studies also showed a neutrophil evasion strategy used by *Chlamydia*. *Chlamydia* release a protease called chlamydial-protease-like activity factor (CPAF), which inhibits neutrophil activation by degrading neutrophil surface receptor, formyl peptide receptor 2 (FPR2) [46]. *Chlamydia* is also shown to avoid phagocytosis of host cells. Rac1 regulatory subunit of NADPH oxidase is found to be relocated to chlamydial inclusion after infection of HeLa cells [134]. NADPH oxidase is the host enzyme necessary for reactive oxygen species (ROS) production [135]. Low levels of ROS enable *Chlamydia* to survive longer inside host cells.

Chlamydia also inhibits host cell apoptosis. Proapoptotic factors such as BCL-2-like protein 11 (BIM), p53 upregulated modulator of apoptosis (PUMA), and BCL-2-associated death promoter (BAD) transmit death signals to mitochondria to initiate apoptosis [136, 137]. CPAF released by *Chlamydia* degrades these proapoptotic factors and confers resistance to apoptosis [136]. It is also observed that *C. trachomati*s inhibit Class II MHC expression by degradation of the upstream stimulatory factor-1 (USF-1) [138]. Interestingly, CD1d, an MHC-like molecule expressed by epithelial cells, is also downregulated by *C. trachomatis* in human penile urethral epithelial cells

[139]. This process also involves CPAF-mediated degradation of CD1d heavy chain and relocation to chlamydial inclusion. Additionally, *Chlamydia* induces the expression of PD-L1 in host cells to evade the immune response [140]. PD-L1 engagement leads to reduced IFN-γ secretion from T cells and enhanced bacterial survival [83].

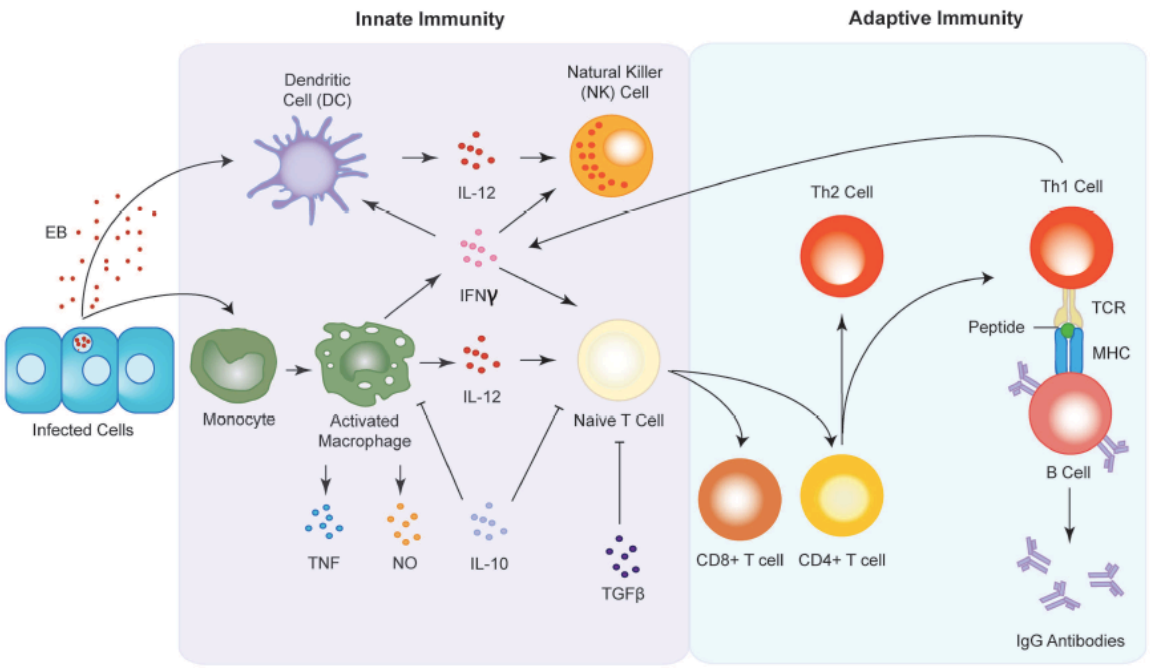

Figure 2: Innate and adaptive immune responses to *Chlamydia* infection. Upon infection, antigen presenting cells (APC) such as macrophages and dendritic cells are sequestered to the site of infection where they begin to release pro-inflammatory cytokines such as IFN γ and IL-12. The chemokines in turn activate natural killer (NK) cells and induce the maturation of T cells into either $CD8^+$ or $CD4^+$ T cells. $CD4^+$ T cells go on to form either T-helper 1 (Th1) or T-helper 2 type (Th2) T cells. Th1 cell interact with B cells via the T cell receptor (TCR) and the major histocompatibility complex (MHC) to produce antibodies against the chlamydial infection [141]. Adapted from Front Immunol. 2014; 5: 534.

1.4. SEMAPHORINS

The first member of semaphorins was discovered as a new axonal glycoprotein in the grasshopper named “fasciclin IV” [142]. Later studies identified another protein called “Collapsin-1” in the chick brain, which shares homology with fasciclin IV [143]. A group of molecules showing similar structural features such as a ”Sema” domain were then identified and categorized into the semaphorin family [144]. Although semaphorins were described initially as guidance molecules that were required to direct neuronal axons to their respective targets, recent studies indicate the relevance of semaphorins outside the nervous system, including angiogenesis, cardiogenesis, tumor progression, osteoclastogenesis, and immune cell homeostasis [145].

Semaphorins exist in secreted and membrane-associated forms and share a conserved ‘Sema’ domain with approximately 500 amino acid residues [144]. They are divided into eight subclasses based on their structural elements and similarities in the amino acid sequences. Invertebrate semaphorins fall in to class I and II, whereas vertebrate semaphorins are grouped into classes III-VII [145-147]. In addition, there are reports of semaphorin molecules encoded within viral genomes and are classified as class VIII semaphorins [145, 148]. Out of these, classes II, III and VIII semaphorins exist in the secreted form [145]. Structurally, semaphorins are characterized by a sema domain at N terminals followed by a cysteine rich plexin semaphorin integrin (PSI) domain [146].

All semaphorins structurally consist of an N-terminal 500 residue sema domain with a seven-blade β-propeller fold conformation [149]. The sema domain is tightly coupled to adjacent cysteine-rich domain named the “PSI domain”(plexin–semaphorin–integrin) [149]. Except for class VI semaphorins, all other semaphorins contain “immunoglobulin (Ig)-like domains” or

"thrombospondin type 1 repeats" [149]. The sema domain of semaphorins undergoes homodimerization and interacts with receptors, such as neuropilins and plexins (Figure 3).

1.4.1. Semaphorin receptors and co-receptors

Plexins and neuropilins are the two high affinity receptors of semaphorins. Plexins (previously referred to as B2) was initially identified in Xenopus as a neuronal cell surface molecule [150]. Later sequence analysis revealed that plexins are single type 1 transmembrane receptors involved in neuronal cell adhesion [146, 151]. They are divided into four subclasses including plexin-A, plexin-B, plexin-C and plexin-D [146]. Plexins consist of a N-terminal Sema domain, a combination of three-PSI domains and six IPT (Ig domain shared by plexins and transcription factors) domains in their extracellular regions [152]. N-terminal Sema-PSI domain of plexin mediates the binding of plexins to semaphorins. Plexins also have an R-Ras and M-Ras GTPase-activating protein (GAP) domain in their cytoplasmic region. Cytoplasmic tail of plexins are involved in signal transmission upon ligand binding [153]. Following binding of semaphorins to plexins, there is an activation of GAP domain resulting in downstream signaling through molecules such as GTPase, protein kinase and associated molecules [154].

In addition to plexins, classes III semaphorins need neuropilin as a co-receptor for their activity. Neuropilins are divided into Nrp-1 and Nrp-2. They are single-pass transmembrane glycoproteins with large extracellular domains, a short transmembrane domain and a cytoplasmic sequence [155]. The extracellular portion of neuropilin contains two a1 and a2 domains, b1 and b2 domains and a MAM (meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu) domain [149]. The a1 and a2 domains bind to class III semaphorins, b1 and b2 domains bind to ligands such as vascular endothelial growth factor [149]. The MAM domain of neuropilins is involved in the dimerization of neuropilins [149] (Fig. 3).

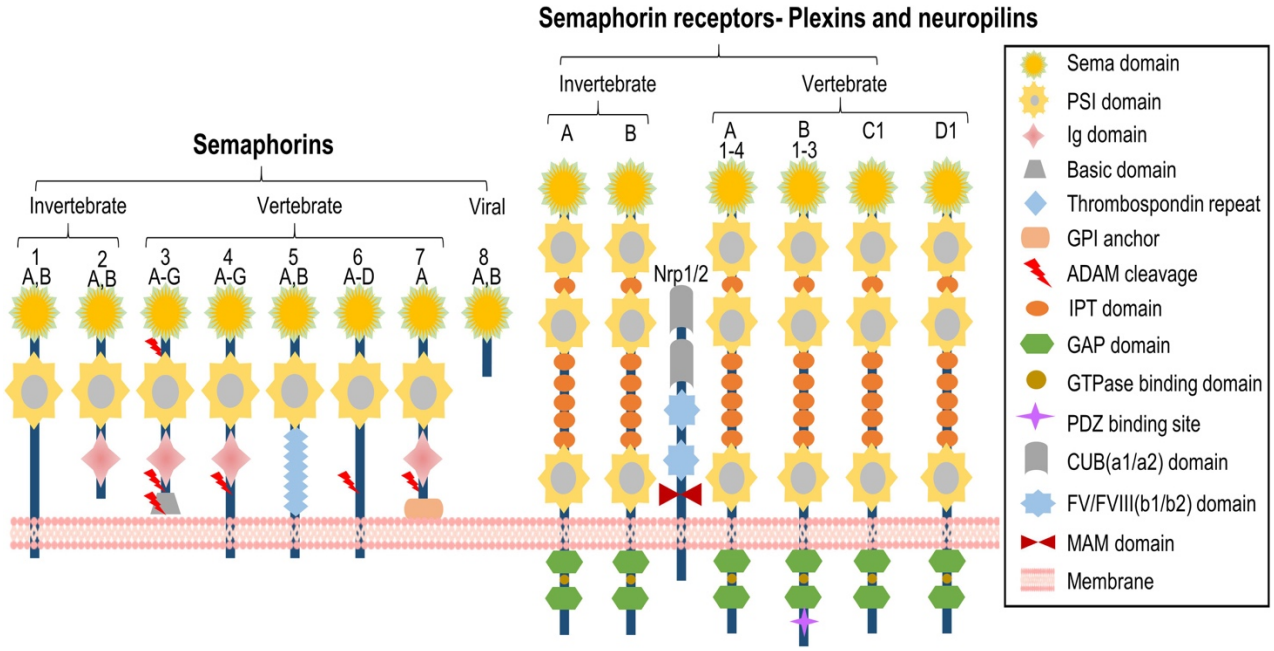

Figure 3: Schematic representation of semaphorins and semaphorin receptors. The semaphorin family can be divided into eight classes. Classes 1 and 2 are found in invertebrates and classes 3–7 belong to vertebrates. Classes 2 and 3 and the viral semaphorin 8 are secreted, whereas classes 4–6 are transmembrane proteins. Class 7 is the only GPI-anchored protein. Each semaphorin consists of a large sema domain, a plexin-sema-integrin domain (PSI), immunoglobulin (Ig)-like domains, and thrombospondin repeats. Semaphorins signal through their receptors, plexins. Plexins A and B are found in invertebrates. Vertebrates have plexins A1–4, plexins B1–3, PlexinC1, and PlexinD1. Plexins contain a sema domain, a PSI domain, and an Ig-like, plexins, transcription factors (IPT) domain. Structurally, the cytoplasmic domain of the plexin contains two GTPase activating protein (GAP) domains, including one GTPase-binding domain and one PDZ domain (postsynaptic protein PSD-95/SAP90, the Drosophila septate junction protein Discs-large, and the tight junction protein ZO-1 domain) (B-type plexins only). In invertebrates, semaphorins 1 and 2 signal through Plexin A and Plexin B. In vertebrates, semaphorins 3, 5, and 6 signal via Plexin A, while semaphorin 3 requires a combination of neuropilins (Nrp1 or Nrp2) for signal transduction. Neuropilin is a transmembrane receptor composed of two complement-like (CUB) domains, two FV/FVIII clotting factor-like domains, one meprin-like MAM domain, and a short cytoplasmic tail [156]. Figure adapted from Front Physiol. 2018; 9: 1236.

1.4.2. Semaphorins and immune system

In recent years, a growing body of evidence indicates the roles of different classes of semaphorins in various phases of immune response, including immune cell trafficking, migration, development and cell-cell interaction [152].

1.4.2.1. Class 3 semaphorins in immune cell function, inflammatory and infectious diseases.

Class 3 semaphorins generally bind to class A plexins and require neuropilins as co-receptor for their function [157]. However, Sema3E can function by binding to plexinD1 independent of neuropilin-1[157]. Recently, Sema3A is found to promote migration of DC to lymphatics by activating myosin II [158]. Sema3A also influences human T cell proliferation and cytokine production [159]. Later studies identified the requirement of Plexin-A4–semaphorin 3A signaling for inflammatory cytokine production induced by sepsis [160]. Furthermore, treatment with neutralizing anti-Sema3A monoclonal antibodies improved the survival of LPS treated mice, possibly through reducing inflammatory cytokine production [161]. On the other hand, intranasal treatment with Sema3A alleviates allergic symptoms by inhibiting Th2/Th17 response and enhancing Th1/Treg response. Exogenous Sema3A inhibits growth factor-induced proliferation of human airway smooth muscle cells, minimizing airway remodeling [162]. Sema3E, a secreted semaphorin, is found to be involved in the regulation of thymocyte development [163]. Sema3E promotes migration of CD69+ double positive (DP) thymocytes to the medulla in response to CCL19/21by inhibiting CCR9 signaling [163]. Cumulative evidence also suggests that Sema3E is involved in several inflammatory diseases (discussed in a later section). Moreover, recent studies established the role of class 3 semaphorins like Sema3A, Sema3C and Sema3F in promoting human DC migration by inducing F-actin organization [164].

1.4.2.2. Class 4 semaphorins in immune cell function, inflammatory and infectious diseases.

Class 4 semaphorins are membrane-bound and bind to class B plexins for their activity [157]. In addition, Sema4A bind to neuropilin-1 and plexinD1[157]. Sema4A is highly expressed in Th1 cells and is involved in their differentiation and T cell priming [165]. Sema4A deficient mice showed reduced Th1 response and higher Th2 response[166]. Sema4A deficiency also exacerbated Th2 like lung inflammation in asthma [167]. Later studies identified that Sema4A is required for Treg stability in tumors [168]. In addition to T cells, Sema4A is also expressed by dendritic cells [166]. Sema4A-deficient DCs, compared to wild-type DCs, showed reduced stimulation of allogenic T cells [166].

Sema4B is another semaphorin expressed by T cells, basophils and B cells. In basophils, recombinant Sema4B inhibited IL-4 and IL-6 production [169]. Sema4B-KO mice had enhanced basophil-mediated IgE production, despite normal lymphocyte and dendritic cell functions [169]. Sema4D (also known as CD100) was the first semaphorin discovered to have immunoregulatory function and is highly expressed in dendritic cells, macrophages, activated B cells and T cells [170]. Several studies suggest that Sema4D promotes B cell activation by shutting off CD72 inhibitory signals [171]. Sema4D promotes the viability of B cells and fine-tune B-cell antigen receptor (BCR) signaling to generate a proper immune response [171, 172].

Moreover, Sema4D and its receptor CD72 is expressed in large amounts in T cells and DC, respectively [173]. Sema4D deficient DC exhibit reduced expression of co-stimulatory molecules and IL-12 cytokine production [173]. In addition to T cells and DC, Sema4D is also expressed in neutrophils. Sema4D-plexinB2 interaction decreased NET formation and act as a negative regulator of neutrophil activation [174]. More recent studies also showed a protective role for

Semaphorin 4D in HIV infection [175]. It is found that $CD8^+$ T cells lacking Semaphorin 4D expression were functionally impaired and present in increased numbers in HIV-1–infected individuals. The function of Sema4D is also examined in the context of endodontic infections. *Enterococcus faecalis*, a bacterial pathogen implicated in endodontic infections, specifically induce the expression of semaphorin 4D (Sema4D) in osteoclast precursor cells to inhibit bone formation [176].

1.4.2.3. Class 5 semaphorins in immune cell function, inflammatory and infectious diseases.

Few studies identified the role of class 5 semaphorins in the functions of the immune system. Initial studies described Sema5A as a factor that increase endothelial cell proliferation to promote angiogenesis [177]. Other studies suggest the association of Sema5A with metastatic ability in gastric and pancreatic cancers [177, 178]. Furthermore, soluble Sema5A stimulates T cell and NK cell proliferation and cytokine production [179].

1.4.2.4. Class 6 semaphorins in immune cell function, inflammatory and infectious diseases.

Class 6 semaphorins are membrane-bound semaphorins and bind to class A plexins. Among the class 6 semaphorins, Sema 6D is expressed by T cells, B cells and NK cells [180]. Sema6D receptor (plexinA1) deficient dendritic cells, compared with wild-type dendritic cells, poorly stimulated allogeneic T-cells [180]. In addition, soluble Sema6D induced IL-12 production and upregulation of MHC class II-expression in dendritic cells [180]. T cells express Sema6D upon TCR stimulation and regulates T cell activation at later stages of immune response [181]. Furthermore, Sema6D receptor, plexin-A1 play a crucial role during entry of DC into lymphatics [182].

1.4.2.5. Class 7 semaphorins in immune cell function, inflammatory and infectious diseases.

Out of the class 7 semaphorins, Sema7A (also known as CD108) reported acting as immune semaphorins. Sema7A is expressed by several immune cells including T cells and dendritic cells. Soluble SEMA7A promote differentiation of $CD4^+$ T cells into Th1 and Th17 subclasses by inducing T-bet and retinoic acid receptor–related orphan nuclear receptor γt (RORγt), respectively [183]. SEMA7A could also be used as a therapeutic agent in rheumatoid arthritis as anti-SEMA7A antibody treatment reduced arthritis scores in mice with collagen-induced arthritis [183]. Fibrocytes also express Sema7A and Sema7A deficiency reduces TGF-β1–induced lung fibrosis as well as alveolar remodeling [184]. Further studies identified association of Sema7A expressing Treg cells with disease progression in people with idiopathic pulmonary fibrosis (IPF) [185]. Moreover, adoptive transfer of these cells induced fibrosis and airway remodeling in the TGF-β1–exposed murine lung [185]. Furthermore, West Nile virus (WNV) infection of Sema7A deficient mice exhibited lower viral load, reduced blood-brain barrier permeability and increased survival [186]. Studies of Sema7A in DC showed that Sema7A is needed for dendrite formation and migration of DC [187]. These studies showed critical functions of semaphorins in various phases of immune response and further investigations are required to understand their role in inflammatory and infectious diseases (Fig. 4).

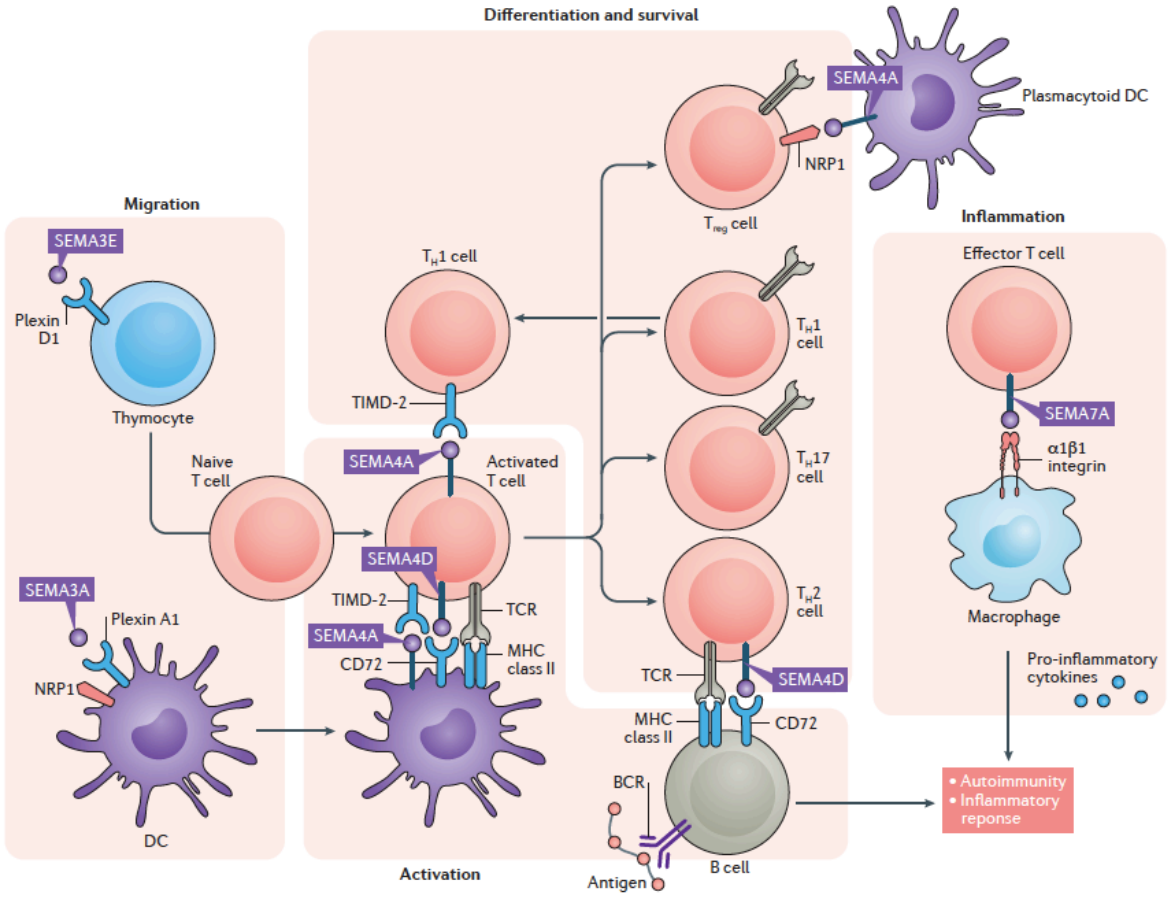

Figure 4: The involvement of immune semaphorins in various aspects of immune responses. During dendritic cell (DC) transmigration, SEMA3A binds to the plexin A1–neuropilin 1 (NRP1) receptor complex expressed on the rear sides of DCs, inducing their transmigration into the lymphatics. SEMA3E binds to plexin D1 expressed on thymocytes and contributes to thymocyte development by regulating thymocyte migration. SEMA4A regulates the differentiation of CD4+ T cells by amplifying T helper 1 (Th1) cell differentiation through the binding of T cell immunoglobulin and mucin domain-containing protein 2 (TIMD-2, also known as TIM-2) on TH1 cells. SEMA4A promotes the survival of regulatory T (Treg) cells by binding to NRP1 expressed by these cells. In the initial phase of T cell immune responses, SEMA4A expressed by DCs promotes the activation of T cells capable of recognizing alloantigens presented on DCs, through binding to TIMD-2 on activated T cells. SEMA4D expressed by T cells positively regulates humoral immune responses by activating B cells via CD72. SEMA4D expressed on T cells interacts with CD72 on DCs and promotes DC activation and maturation. SEMA7A is expressed on activated T cells and stimulates macrophages via integrins to produce pro-inflammatory cytokines. BCR, B cell receptor; TCR, T cell receptor [157]. Adapted from Nat Rev Rheumatol. 2018 Jan;14(1):19-31.

1.4.3. Semaphorin 3E

Semaphorin 3E (Sema3E), originally named as M-SemaH in mice, was discovered as a novel semaphorin family member shown to positively correlate with tumour progression [188]. The Sema3E transcripts encode 775 amino acids with the features of a secreted glycoprotein [188]. Initial studies by Christensen et al. suggest the involvement of the M-SemaH gene in embryonic development. It is found to be expressed in developing lungs, skeletal elements, and neural tube [188]. In humans, Sema3E is located on chromosome 7 and shares 87.4% similarity with mouse Sema3E [189]. Later studies identified the association of Sema3E with genetic disease conditions. Mutations in SEMA3E was proposed as a molecular mechanism by which CHARGE syndrome developed in a subset of patients [190]. A point mutation in SEMA3E was observed in patients with Kallmann syndrome (KS), a condition of inherited deficiency in gonadotropin-releasing hormone (GnRH) [191]. Recombinant wild-type Sema3E protected maturing GnRH neurons from cell death by triggering a plexin D1–mediated activation of PI3K signaling [191].

During embryogenesis, Sema3E is expressed in developing somite and functions as a repulsive cue to regulate blood vessel growth to intersomitic regions [192]. Sema3E is found to directly associate with plexinD1 independently of neuropilins [192]. Later studies identified the role of Sema3E-plexin D1 axis as a critical regulator of cardiovascular disease, tumor growth, and angiogenesis [193-195]. Metastasis of colon cancer, melanoma cancer, ovarian cancer and breast cancer is found to correlate positively with Sema3E expression [196-198]. However, in colorectal cancer and pancreatic cancer, Sema3E expression is inversely related to tumor prognosis [196]. *In vitro* and *in vivo* experiments by Chen *et al.* demonstrated that Sema3E is epigenetically downregulated in gastric cancer and the expression of Sema3E is inversely proportional to tumor progression. Sema3E could suppress gastric cancer cell proliferation by inhibiting cell cycle

progression and promoting apoptosis [196]. Sema3E also inhibits angiogenesis by blocking vascular endothelial growth factor (VEGF) signaling pathway [199].

1.4.3.1 Functions of Sema3E in the immune system

The role of Sema3E in immune system is being elucidated in recent years. Sema3E is highly expressed on DCs, Th2 cells and thymic epithelial cells [163]. Recent studies strongly suggest the importance of Sema3E in directing the migration of maturing thymocytes to the medulla. In activated $CD4^+CD8^+$ double-positive (DP) thymocytes, Sema3E-plexinD1 signaling inhibit CCR9/CCL25 signaling in the cortex [163]. As a result, activated DP thymocytes migrate to the medulla in response to CCL19/21 signaling[163]. Later studies identified that the Sema3E-plexinD1 axis control β1 integrin adhesion to regulate thymocyte migration [200]. Recent studies by Ueda *et al.* identified the relevance of Sema3E in the regulation of thymocyte trafficking. Sema3E directly inhibits Rap1 activation through plexinD1, leading to impaired immunological synapse formation in thymocytes [201].

The role of Sema3E has been started to be evaluated in inflammatory diseases as well. In the mouse model of dietary obesity, the expression of Sema3E and its receptor plexinD1 was shown to be upregulated [202]. In adipose tissue, Sema3E act as a chemoattractant for macrophages, and p53-induced upregulation of Sema3E leads to tissue inflammation [202]. On the other hand, Sema3E is expressed in macrophages of atherosclerotic plaques and inhibit macrophage migration to chemokine by disrupting the re-organization of the actin cytoskeleton [203]. Influence of Sema3E on macrophage function was analyzed during the LPS-induced acute inflammatory response. Both Sema3E-deficient mice and mice with specific deletion of plexinD1in macrophages exhibit better clinical score compared to wild-type controls after LPS-induced sepsis [204]. Reduced early

inflammatory response to LPS in Sema3E KO mice was associated with reduced phosphorylation of ERK1/2, AKT, STAT3, and NF-κB in macrophages [204].

Mouse NK cells also express Sema3E receptor, plexinD1, on their surface [205]. Activated NK cells showed higher migration towards the conditioned medium of immature Sema3E$^{-/-}$ DCs than Sema3E$^{+/+}$ DCs, suggesting a suppressive effect of Sema3E produced by immature DCs in NK-cell migration [205]. Moreover, Sema3E promote susceptibility to *Leishmania major* infection in mice by negatively regulating Th1 immune response [206]. In Sema3E deficient colitic mice, there was an exacerbated disease severity and was ameliorated by recombinant Sema3E treatment[207]. Sema3E modulates the pro-inflammatory activity of CD11c^{+} cells via the NF-κB-dependent pathway to reduce colitis [207]. Furthermore, recombinant Sema3E treatment reduced apoptosis and p53-associated genes in intestinal epithelial cells [208]. Together, these findings suggest Sema3E as a novel inhibitor of intestinal inflammation.

There are numerous studies on the functions of Sema3E in allergic asthma. Human airway smooth muscle cell proliferation and migration, one of the key events in the development of asthma, was inhibited by Sema3E [209]. This effect was mediated by inducing F-actin depolymerization, suppressing Rac1 GTPase activity, and inhibiting phosphorylation of Akt and ERK ½ [209]. The expression of Sema3E was significantly reduced in the airways of severe asthmatic patients and the mice with allergen sensitization and challenge [210, 211]. Consistently, Sema3E deficiency in mice leads to exaggerated allergic airway inflammation, remodeling and airway hyperresponsiveness, while intranasal recombinant Sema3E treatment reduced house dust mite-induced allergic asthma [211, 212]. Sema3E$^{-/-}$ mice showed higher numbers of CD11b^{+} pulmonary DCs than WT controls after sensitization with allergen and adoptive transfer of these cells to WT recipient mice enhanced house dust mite-induced Th2/Th17 inflammation [212]. Intranasal

exogenous Sema3E protects mice from allergic asthma by reducing eosinophilic inflammation, serum IgE, IgG1, and Th2/Th17 cytokines [211]. Also, upon co-culture with T cells, $CD103^+$ cDCs from Sema3E-treated, HDM exposed mice promoted IFN-γ production by T cells [211].

Apart from pulmonary DCs, Sema3E is also involved in regulating pulmonary neutrophil recruitment in a mouse model of allergic asthma. An enhanced accumulation of neutrophils is observed in the lungs of Sema3E KO mice after allergen challenge, whereas exogenous Sema3E treatment reduced allergen-induced neutrophil recruitment [213]. Moreover, human neutrophils exhibited a constitutive expression of Sema3E high-affinity receptor, PlexinD1; and Sema3E inhibited CXCL8/IL-8–induced neutrophil migration via suppression of Ras-related C3 botulinum toxin substrate 1 GTPase activity and F-actin assembly [213]. Recently Sema3E is also reported to influence angiogenesis, an important feature of asthmatic airway remodeling. Sema3E inhibits the formation of new blood vessels in the allergic asthmatic airway by modulating pro-and anti-angiogenic factors such as vascular endothelial growth factor (VEGF), VEGF receptor 2 protein, and soluble VEGF receptor 1 [214].

1.4.3.2 The phenotype of Sema3E KO mice

Previous studies showed that Sema3E deficient mice exhibit developmental, cardiovascular, and neurological defects. In Sema3E deficient mice embryos, intersomitic vessels were disorganized, resulting in loss of normal segmented pattern [192]. This phenotype was also observed in plexin D1 knock out mice [192]. Sema3E deficient mice also exhibit defects in angiogenic patterns leading to abnormal branched aortic plexus [215]. Further studies analyzed whether Sema3E inactivation affected adult behavior. Sema3E KO mice showed reduced anxiety levels and a moderately impaired spatial working memory [216]. In another study examining Sema3E deficiency on adipose tissue inflammation, there was no observed differences in body weight,

visceral fat mass, or food intake between Sema3E KO and WT mice [202]. Sema3E deficiency also affects the organization of the primary lymphoid organ of the immune system. In contrast to their localization inside the medulla in wild-type mice, $CD69^{+}$ thymocytes from $Sema3E^{-/-}$ mice were observed outside the medulla [217]. These studies showed a lack of a clear delineation of the corticomedullary junction in the thymus of Sema3E KO mice. However, immunophenotyping of Sema3E KO mice revealed that the numbers of alveolar macrophages, T, B, NK, and NKT cells of $Sema3E^{-/-}$ mice were similar to that of WT mice although the numbers of eosinophils and $CD11c^{+}$ DC numbers appeared higher in Sema3E KO mice compared to WT mice [212]. Collectively, these studies imply that Sema3E play roles in different systems and the Sema3E KO mice are suitable for investigating its function in immunity to infection.

1.4.3.3 Sema3E isoforms

It is interesting to find that growth repellant activity of Sema3E can be changed to growth attractant by furin-dependent processing of full-size Sema3E [218]. Furin and related proprotein convertases are expressed frequently in tumor cell lines and human cancers of the lung, breast, and head and neck. During experimental lung metastasis, full-size Sema3E is processed by furin to a p61-sema3E isoform. This p61-sema3E isoform promotes lung metastasis, cell migration, invasive growth and extracellular signal–regulated kinase 1/2 activation of endothelial cells [218]. In fact, p61 isoform is the active and predominant form of Sema3E observed in invasive and metastatic cancer cells [219]. Later studies used UnclSema3E, a point-mutated Sema3E isoform resistant to furin-mediated cleavage, to understand the Sema3E signaling cascades in cancer progression. Uncl-Sema3E does not promote metastatic spreading and acts as an anti-angiogenic factor. Moreover, Uncl-Sema3E competes with p61-Sema3E isoform for binding to the receptor and

inhibit the metastatic ability of endogenous p61 [220]. The potential therapeutic efficacy of the systemic delivery of UnclSema3E should be investigated in contexts other than cancer.

1.4.4 PlexinD1

Sema3E binds to the receptor PlexinD1(PlxnD1), which is highly expressed in embryonic tissues, osteoblasts, lung mesenchyme, adrenal gland, mammary gland, small intestine and immune cells [221]. Unlike other class 3 semaphorins, Sema3E-plexinD1 binding is a Neuropilin-independent process. However, Neuropilin-1(Nrp1) and vascular endothelial growth factor receptor type 2 (VEGFR2) could be associated with plexinD1. Binding of these co-receptors switches Sema3E-PlexinD1 repulsive signaling to attraction [222, 223]. Moreover, Sema3E-induced axonal attraction also involves formation of a PlxnD1/Nrp1/VEGFR2 ternary complex in which VEGFR2 as the signal transducing subunit and PlxnD1 functions as the ligand binding subunit [221]. Sema3E deficient mice are viable but PlxnD1 deficiency induces perinatal lethality [193, 221]. This suggests the existence of additional PlxnD1 ligands. Sema3C, Sema3F and Sema4A also act as PlxnD1 ligands. Sema3C binds directly to both Npn-1 and Npn-2 but its binding is enhanced by PlxnD1 [193]. Sema4A-PlxnD1 binding is Neuropilin-independent and Sema3F specifically binds to PlxnD1/Neuropilin tolloid-like 1(Neto1) co-receptor complexes [221, 224].

PlxnD1 is a type I transmembrane protein with an extracellular N-terminal Sema domain consists of three MRS (MET-Related Sequence) repeats, four IPT (Immunoglobin-like fold shared by Plexins and Transcription factors) domains, transmembrane domain and cytosolic domain [221]. Sema domain consists of 500 amino acids and structurally resemble to integrins with a seven-bladed beta-propeller topology [225]. Sema domain is likely involved in ligand binding, maintaining the receptor in an inactive state and mediating Plxn-Plxn associations [225]. MRS

repeats or PSI (Plexins, Semas and Integrins) domains are 50 amino acids long composed of three cysteine-rich motifs [221]. Each IPT domains consists of glycine and proline rich repeats [221]. The cytosolic tail of PlxnD1 contains the Sex and Plexins (SP) domain with two highly conserved C regions (C1 and C2). C regions are collectively called RasGAP domain because of sequence similarity to Guanosine triphosphatase (GTPase)-Activating Proteins (GAPs). Ras GAP motif have conserved arginine residues. A Rho GTPase-binding domain (RBD) is located between the C1 and C2 regions [221]. Lastly, a short conserved C-terminal region follows the GAP domain. C-terminal is designated as T-segment and consists of 40–60 amino acids that includes the COOH terminus. In the absence of Sema3E, PlexinD1 is conformationally inactive folded state, in which the Sema domain contacts the rest of the extracellular portion and the GAP domain is non-functional (Fig. 5). Upon Sema3E binding, PlexinD1 undergoes conformational changes that activate its GAP domain and thereby promoting downstream signaling cascade [221]. Activated small GTPases such as Rac1, Cdc42 and Rnd, bind to the RBD which leads to disruption of the inhibitory association between the C1 and C2 regions and activation of the GAP [221]. Collectively, the exact mechanism of Sema3E-PlexinD1 signaling is not completely understood and extensive studies are required to understand their function in a specific cell/ disease conditions.

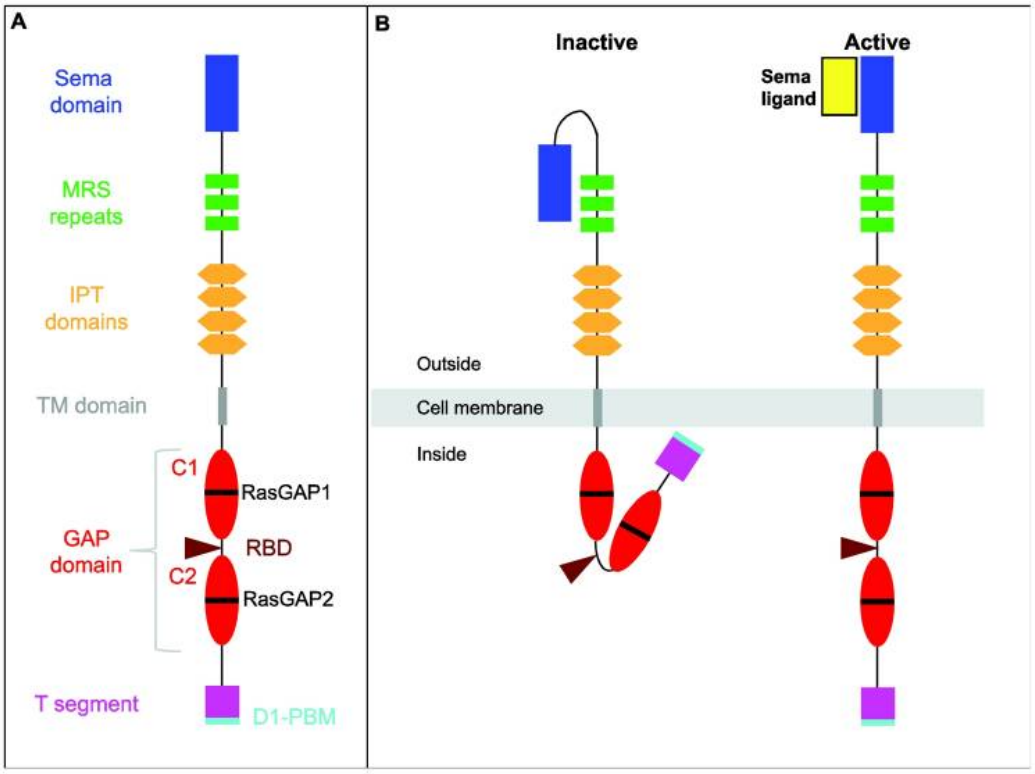

Figure 5: Structure of the Sema receptor PlxnD1. A, PlxnD1 is a type I transmembrane protein. The extracellular N-terminal portion contains a Sema domain (blue), likely involved in mediating ligand binding. Following the Sema domain there are three MRS (MET-Related Sequence) repeats (green), four IPT (Immunoglobin-like fold shared by Plexins and Transcription factors) domains (orange) and the transmembrane domain (brown). The cytosolic tail of PlxnD1 is also known as the Sex and Plexins (SP) domain. It contains a split GAP (GTPase Activating Protein) domain with two highly conserved C regions (C1 and C2; red). Each C region contains a Ras GAP motif (RasGAP1 and RasGAP2; black), each of which includes conserved arginine residues required to inhibit the activity of R-Ras proteins. A Rho GTPase-binding domain (RBD, beige) is located between the C1 and C2 regions. Finally, the GAP domain is followed by a short C-terminal region that lacks any resemblance to known protein domains and which is highly conserved between members of the same Plxn subfamily. Here we designate this region as the terminal (T) segment (pink). The T segment of PlxnD1 ends in a short PDZ-binding motif (D1-PBM; aqua) that physically associates with GIPC1. B, Activation model of PlxnD1. In the absence of its Sema ligands, PlxnD1 is in a conformationally inactive folded state, in which the Sema domain contacts the rest of the extracellular portion and the GAP domain is non-functional. Upon Sema binding PlexinD1 undergoes conformational changes that activate its GAP domain and likely enable additional protein-protein interactions [226]. Adapted from Dev Biol. 2011 Jan 1; 349(1): 1–19.

1.5. SUMMARY OF LITERATURE

- *Chlamydia* is an obligate intracellular bacterium causing wide range of diseases in humans called as chlamydial diseases or chlamydiosis.
- *C. trachomatis* and *C. pneumonia* are the two chlamydial species pathogenic to humans. *C. trachomatis* causes trachoma and sexually transmitted diseases (STD). *C. pneumoniae* causes common respiratory infections, including community-acquired pneumonia, sinusitis, pharyngitis and bronchitis
- Currently, no vaccines are available for chlamydial diseases. The main hindrance for effective vaccine development is the inadequate knowledge of the mechanisms underlying protective immunity and immunopathology in chlamydial infections.
- *C. muridarum* lung infection mice is one of the most commonly used mouse model in studying host defense mechanisms against chlamydial infection
- IFN-γ production by $CD4^+$ and $CD8^+$ T cells is critically important for protection against chlamydial infection
- Th2 type immune response is associated with pathology in animal models of chlamydial infection
- IL-17A produced by Th17 cells has a significant promoting impact on the development of protective type 1 immunity in *Chlamydia* lung infection and Tregs contribute to tissue pathology after chlamydial infection.
- Interaction of DC with *Chlamydia* leads to DC maturation and chlamydial antigen presentation to T cells.
- *Chlamydia* infected DC upregulate the expression of CCR7, MHC class II, CD40, CD80 and CD86 molecules. In addition, activated DC secrete profound amounts of cytokines

such as interleukin-1beta (IL-1beta), IL-6, IL-8, IL-12p70, IL-18, IL-10 and tumor necrosis factor alpha.

- Cytokine production by DC plays an important role in determining the type of T cell response.
- In the lung and spleen, cDC1 and cDC2 subsets showed differential abilities to induce protective T cell immune responses to chlamydial infection.
- Semaphorins were described initially as guidance molecules that were required to direct neuronal axons to their respective targets.
- Recent studies indicate the relevance of semaphorins outside the nervous system, including angiogenesis, cardiogenesis, tumor progression, osteoclastogenesis, and immune cell regulation.
- The function of Sema3E-PlexinD1 signaling in inflammation and host defense has started to be reported but its role in immunity to bacterial infection has not been studied.

CHAPTER 2

RATIONALE, HYPOTHESIS AND RESEARCH AIMS

2.1. RATIONALE

Chlamydiae, an obligate intracellular pathogen, is the causative agent of various human and animal diseases. *Chlamydia pneumonia* and *Chlamydia trachomatis* are the two major species of *Chlamydiae* responsible for human diseases, causing pneumonia [28], trachoma [18], and sexually transmitted diseases [227]. In addition, *Chlamydia pneumonia* has been found to be associated with chronic inflammatory diseases like asthma [228], multiple sclerosis [228], and Alzheimer's disease [229]. In mouse models for studying human genital tract and respiratory infections, *Chlamydia muridarum (Cm),* a natural mouse strain, has been commonly used [230]. Although great effort has been made, a vaccine is currently unavailable for human chlamydial diseases. The factor impeding rational vaccine development is the incomplete understanding of protective immune response and immunopathology associated with the infections. Studies by Yang lab and others have shown that CD4 and CD8 T cell responses characterized by IFN-γ production are required for protective immunity to chlamydial infections [126, 231]. Additionally, IL-17 and Th17 cells work synergistically with Th1 cells to inhibit chlamydial growth, at least in lung infection models [109]. In contrast, Th2 response and the development of regulatory T cells (Tregs) is associated with slower clearance of the bacteria and increased immunopathology in chlamydial infections [81, 120]. Moreover, studies also show an inconsistent role of antibody responses in protective and pathological responses in chlamydial infections [65, 81, 126]. In particular, it is reported that IgG2a antibody response is associated with Th1 cytokine production, whereas IgG1 antibody response is associated with Th2 cytokine response and pathology in the lung infection [81, 126].

Dendritic cells (DCs) are the major antigen-presenting cells (APC) with the incredible ability to activate and direct the development of T cell responses [65]. Upon sensing inflammatory or infectious stimuli, DC matures by upregulating co-stimulatory molecules and migrate to the draining lymph node to drive T cell response [65]. DC phenotype and cytokine production pattern profoundly determines the development of different types of adaptive T cells. It is found that IL-12 produced by DCs is essential for the induction of Th1 response [79], whereas IL-10 produced by DC is related to inhibition of Th1 response and enhancement of regulatory T cells [232]. In addition, IL-6 production by DC is found to promote Th17 responses [233]. The expression of immunoregulatory molecules, such as programmed cell death ligand-1 (PD-L1) by DCs, is critical for Tregs' initiation and maintenance [234].

Semaphorins were first described as axon guidance molecules essential to guide neuronal axons to their respective targets [235]. Semaphorin 3E (Sema3E), a subclass of semaphorin, is a secreted protein that signals through receptor plexin D1 with high affinity [236]. Sema3E-plexin D1 axis has emerged as a critical regulator of cardiovascular disease, tumor growth, and angiogenesis[193, 195, 237]. In recent years, a growing body of evidence reveals a role for Semaphorin 3E in various phases of the immune responses, including cell proliferation, migration, and function [202, 209, 217]. Sema3E is produced by multiple cell types, including DCs [238], macrophages [239], tumor cells [237], hepatocytes [240], adipocytes [202], osteoblasts [241], and thymic epithelial cells [217]. In addition, Plexin D1, the Sema3E receptor, is reported to be required to regulate IL-12/IL-23p40 production by DCs [238]. Some studies also showed that genetic deletion of Sema3E in mice resulted in increased severity of colitis [208] and asthma [209, 212], suggesting an inhibitory role of Sema3E for pathological inflammatory responses. However, the role of Sema3E in protective immunity to infections is not investigated. Considering that the major unanswered

question in chlamydial immunobiology is the mechanisms of protective and pathological immune responses, a study on the role of Sema3E in chlamydial infection may provide new insights into the molecular basis of immune regulation in chlamydial infection.

2.2. HYPOTHESIS

We hypothesize that Sema3E may play a significant role in the immune regulation of host defense against chlamydial infections. Specifically, based on the previous reports showing the inhibitory role of Sema3E for Th2 mediated allergic inflammation and the results of DCs from Sema3E treated mice promoting Th1 cells, we predict that Sema3E may promote the protective Th1 response but inhibit the Th2 response related to pathology in chlamydial infection. In addition, based on the nature of Sema3E production by DCs and its role in DC migration, we hypothesize that Sema3E may have a significant impact on DC phenotype, migration, cytokine production, and function in chlamydial infection. The goal of this thesis was to use a mouse lung infection model to test the hypothesis.

2.3. RESEARCH AIMS

The overall goal of our study was to assess the role of Sema3E in host defense against chlamydial lung infection using a mouse model. The specific aims of the study were:

1) Investigate the effects of Sema3E on protective immunity and pathological changes in *C. muridarum* lung infection;
2) The relationship of these effects of Sema3E with its influence on dendritic cell function and migration.
3) Understand the importance of Sema3E receptor, Plexin D1, in dendritic cell function for protection to chlamydial infection.

4) To analyze the effect of exogenous Sema3E treatment on host defense against chlamydial lung infection.

CHAPTER 3
MATERIALS AND METHODS

3.1. Animals

Sema3E$^{-/-}$ BALB/c mice were obtained from GMC animal house at University of Manitoba, Winnipeg, Canada. Sema3E$^{-/-}$ mice in B6 background were gifted by Dr. F. Mann, Université de la Méditerranée, Marseille, France. These mice were back crossed for 10 generations to obtain Sema3E$^{-/-}$ mice in BALB/c background. All mice were maintained in the Animal Care facility of the University of Manitoba. The immunophenotypic analysis of Sema3E$^{-/-}$ mice has been shown previously [212]. All mice used in the study were males of 6-8 weeks of age.

PlexinD1^{flox} (The Jackson Laboratory, Stock No: 018319) were bred to Cd11c-Cre mice (The Jackson Laboratory, Stock No: 008068) to generate offspring with plexinD1 deficiency on dendritic cells. Cd11c-Cre mice express Cre recombinase under the control of the CD11c promoter or enhancer regions within the BAC transgene. The use of all mice in this study was in adherence to the ethical standards prescribed by the Canadian Council on Animal Care (CCAC) and The University of Manitoba Animal Ethics Committee (Protocol # 19-029).

3.2. Organism

Chlamydia muridarum (*Cm*) used in this study was propagated and cultured, as described previously [82]. Shortly, HeLa 229 cell monolayers in Eagle's MEM (composed of 10% FBS and 2 mM L-glutamine) were infected with *Cm* for 48 h. Using sterile glass beads, infected cells were harvested, and *Cm* elementary bodies (EBs) were isolated by discontinuous density gradient centrifugation. The purified *Cm* elementary bodies were stored in the sucrose-phosphate-glutamic acid buffer (SPG) at −80°C.

3.3. Infection of mice and quantification of chlamydial *in vivo* growth

Mice were anesthetized and intranasally infected with 1×10^3 inclusion-forming units (IFU) of *Cm* in 40 µl of SPG buffer. Mice were sacrificed at specified days post-infection. The lung chlamydial load was determined as described previously [82]. Briefly, lung tissue suspensions aseptically isolated from mice were homogenized using a cell grinder in SPG buffer. Homogenized tissue was centrifuged at 1900 × g for 30 min at 4°C, and the supernatant was collected and kept at −80°C. For *Cm* quantitation, HeLa 229 cells were grown to confluence in 96-well flat-bottom microtiter plates. The monolayers were then washed in 100 µl of Hank's Balanced Salt Solution (HBSS) and inoculated in triplicates with 100 µl of serially diluted samples and incubated at 37°C for 2 hours. After washing plates, 200 µl of MEM containing cycloheximide (1.5 µg/ml) and gentamicin (10 µg/ml) was added. The plates were incubated at 37°C in 5% CO2. After 48 hours, the culture medium was removed, and the cells were fixed with absolute methanol. Fixed cells were washed, incubated with *Chlamydia* genus-specific murine mAb at 37°C for 70 minutes. The cells were washed, stained with HRP conjugated goat anti-mouse IgG, and developed with a substrate containing 4-chloro-1-naphthol (Sigma-Aldrich). The number of inclusions was counted under a microscope. Five fields per well were counted, and the chlamydial load was analyzed based on dilution titers of the original inoculum.

3.4. Semaphorin 3E treatment of mice

Recombinant mouse Semaphorin 3E Fc protein and control IgG Fc (0.3µg/mice) were purchased from R&D SYSTEMS and used according to the manufacturer's instructions. WT BALB/c mice and Sema3E KO BALB/c mice were treated intranasally with either Sema3E-Fc or saline-Fc, two hours before *Cm* infection and day 1 to day 6 consecutively after *Cm* infection. Mice were sacrificed on day 7 post-infection and analyzed for bacterial load and cytokine response.

3.5. Isolation of lung, spleen, local draining lymph node (LN) cells, and collection of bronchoalveolar lavage (BAL) fluids.

For obtaining single lung cell suspensions, lung tissues isolated from mice at designated time periods were digested in 2 mg/ml collagenase XI (Sigma-Aldrich, Oakville, Ontario, Canada) dissolved in RPMI 1640 medium at 37°C for 60 min. In the last 5 min of incubation, EDTA (2 mM, pH 7.2) was also added to the medium. After filtering cells through 70 μm cell strainers, red blood cells (RBC) were lysed by ACK lysis buffer (composed of 150 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA). Spleen single-cells were made by digesting spleens with 2 mg/mL collagenase D (Roche Diagnostics, Meylan, France) in RPMI 1640 for 30 min at 37°C. The RBCs were then lyzed by using ACK lysis buffer. Draining Lymph nodes (LN) were homogenized in RPMI 1640, and RBCs were removed by ACK lysis buffer. The isolated cells were washed and resuspended in complete RPMI-1640 medium (RPMI-1640 supplemented with 10% FBS, 1% L-glutamine, 25 mg/mL gentamicin) for further analysis. For collection of BAL fluid (BALF), the lungs of WT and Sema3E KO mice were washed twice with 1 ml of PBS by tracheal cannulation. BALF was prepared as mentioned previously [242]. Briefly, BALF was centrifuged at 1000 rpm for 10 min at 4°C. Supernatants were collected for cytokine analysis by ELISA. BAL cell pellets were resuspended in 200ul of ACK lysis buffer and incubate for 2 minutes at room temperature. 1 mL of cold PBS was added to dilute the ACK lysis buffer. Cells were centrifuged, and the supernatant was discarded. Cells were then resuspended in PBS, counted, and $1x10^5$ cells were spun onto slides [243]. Cytospins from BALF cells were stained by Fisher HealthCare™ PROTOCOL™ Hema 3™ Manual Staining System and Stat Pack according to the manufacturer's instructions.

3.6. Quantification of Sema3E protein, cytokines, and antibodies

Single-cell suspensions were cultured with UVEB (1×10^5 IFU/ml) at a concentration of 7.5×10^6 (for spleen) and 5×10^6 (for lung and LN) cells/well. The supernatants were collected after 72 hours and assayed for the production of cytokines IFN-γ, IL-17, IL-12p40, IL-10, IL-4, and IL-6 (eBioscience or BD Biosciences) by enzyme-linked immunosorbent assay (ELISA). Supernatants obtained after centrifugation of BALF samples were used for cytokine analysis. For quantification of Sema3E protein levels, lung tissue isolated from mice was homogenized, centrifuged, and the supernatant was collected. Sema3E protein levels in the supernatant at different time points were analyzed using a customized Sema3E assay kit (Mesoscale Discovery) according to the manufacturer's instructions. Serum was obtained from WT and Sema3E KO mice on day 7 and day 14 post-infection.

ELISA kits for measuring total and *cm*-specific IgG1 and IgG2a levels were purchased from Southern Biotech (Birmingham, AL). An alkaline phosphatase-based ELISA measured total and *Cm*-specific IgG1 and IgG2a. For measuring *Cm*-specific antibodies, 96-well microtiter plates were coated with *Cm* elementary bodies [126]. After overnight incubation, plates were blocked, washed and incubated with serially diluted serum samples for 3h at 37^0c. After washing, biotinylated goat anti-mouse antibody was added. After overnight incubation, alkaline phosphatase-conjugated streptavidin was added for 45 min at 37^0c. After washing, substrate p-nitrophenyl phosphate (Sigma-Aldrich) was added, and the microplate reader read plates at 405nm [244].

3.7. DC purification and culture

Purification of DC was performed, as described previously [245]. Briefly, splenic and lung single-cell suspensions were incubated with CD11c microbeads (Miltenyi Biotec) for 15 minutes at 4^0C

and passed through magnetic columns for positive selection. The purity of DC was found to be >95%. The cytokine production by DC was detected by culturing freshly isolated DC in the presence of UVEBs (1×10^5 IFUs/ml) in complete RPMI 1640 medium for 72 h at 5×10^5cells/well. Cytokines such as IL-12, IL-6 and IL-10 were measured in the culture supernatants by ELISA [55].

3.8. Flow cytometry

Surface marker expression on DC was analyzed by staining freshly isolated lung and splenic DC with anti-CD11c-APC, anti-MHC class II PE-Cy7, anti-CD40 FITC, anti-CD80 FITC, or anti-CD86 FITC, anti-PD-L1-PE, anti-PD-L2-PE, anti-F4/80-PE, anti-plexinD1 PE (eBioscience, San Diego, CA), or isotype controls in a flow staining buffer (composed of Dulbecco's PBS mixed with 2% FBS as well as 0.09% NaN3). After surface staining, cells were fixed using 2% paraformaldehyde for 30 minutes, washed twice, and resuspended in staining buffer. Fluorescence minus one controls were also used in addition to isotype controls. For intracellular cytokine staining of DC, cells were cultured (10^6 cells) in the presence of UV-EB (10^4 IFUs) in complete RPMI 1640 medium for 6 h at 37°C. To accumulate the cytokine intracellularly, monensin (eBioscience) was supplemented to the culture medium during the last 4 h of incubation. Cultured cells were isolated and incubated with Fc receptors blocking Abs (anti-CD16/CD32 antibody; eBioscience) for 15 min at 4°C to prevent non-specific staining. Following this step, cell surface markers were stained, fixed, and permeabilized with Cytofix/Cytoperm (BD Pharmingen) for intracellular staining. Intracellular staining was performed using anti–IL-12 (p40/70)–APC, anti-IL-10-APC or isotype control antibodies (eBioscience) in permeabilization buffer (BD Pharmingen). After staining in permeabilization buffer, the cells were washed and resuspended in staining buffer for flow cytometric analysis. $CD11c^{hi}MHC\text{-}II^{hi}$ cells were analyzed as spleen DCs

(Appendix 1A). $CD11c^{hi}MHC\text{-}II^{hi}F4/80^{-}$ cells were analyzed as lung DCs by flow cytometry (Appendix 1B).

For T cell intracellular cytokine analysis, spleen and lung single-cell suspensions were cultured at 7.5×10^6 cells/well in the presence of phorbol 12-myristate 13-acetate (50 n g/ml; Sigma-Aldrich, St Louis, MO, USA) and ionomycin (1 μg/ml; Sigma-Aldrich) in complete RPMI 1640 medium for 6 h at 37°C. After this step, brefeldin A (5 μg/ml; eBioscience) was added at the last 3 hours of incubation to accumulate cytokines intracellularly. Cultured cells were then washed twice, incubated with Fc receptors blocking Abs (anti-CD16/CD32 antibody; eBioscience) for 15 min on ice to prevent non-specific staining. Following this step, surface marker staining was done on cells using fluorescent-labeled anti-CD3 PE-Cy7, anti-CD4 FITC, anti-CD25 APC, or anti-CD8a PE mAbs (eBioscience). The surface stained cells were then fixed and permeabilized using Cytofix/Cytoperm (BD Pharmingen). Later, cells were intracellularly stained with anti-IFN-γ-APC, anti-IL-17-APC, anti-IL-4-APC or isotype control antibodies (eBioscience). After staining, cells were then washed, resuspended in staining buffer, and data collected by BD FACSCanto™ II (BD Biosciences) and analyzed using FlowJo. FoxP3 staining was done using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions and stained using anti-Foxp3-PE or isotype control antibodies (eBioscience). For CD4 and CD8 T cells, the analysis was performed on gated CD3+ cells (Appendix 2). $CD4^+$ $FoxP3^+CD25^+$ cells gated on CD4 T cells were analyzed as Tregs (Appendix 2).

3.9. DC-T cell co-culture

To determine the function of DC to modulate *Cm*-specific T cell responses, $CD4^+$ T cells (1×10^6 cells/well) were isolated from inactivated *Cm*-immunized mice and co-cultured with the DC (5×10^5 cells/well) from different groups of mice in the presence of UVEB (1×10^5 IFUs/ml) for 72 h

in 200 µl of complete RPMI medium. Cells were isolated after culture for analyzing T cell IFN-γ, Th17 cells, and Treg cells. *Chlamydia*-specific T cells were isolated from inactivated *Cm*-immunized mice as described previously [109]. Briefly, inactivated *Cm* (1×10^6 IFU) was injected i.p., and boosted with the same infection dose after 2 weeks. A week after administering the booster dose, spleen $CD4^+$ T cells were isolated using EasySep™ Mouse $CD4^+$ T Cell Isolation Kit (STEMCELL Technologies Canada Inc) according to manufacturer's instructions.

3.10. Generation of bone marrow-derived DC (BMDC) and infection of BMDC with *Cm*

BMDC were isolated from naive CD11c $PLXND1^{-/-}$ mice and CD11c $PLXND1^{+/+}$ mice, as described previously [246]. Briefly, murine femurs were isolated and bone marrow cells were flushed with RPMI-1640 medium using a 1-ml insulin syringe. After this step, cells were washed, RBCs were lyzed by ACK, and cultured (2×10^6 cells) in complete medium (RPMI-1640 + 10% FBS + 20 mM penicillin/streptomycin) in the presence of GM-CSF (20 ng/ml) in a 100-mm cell culture dish for 8 days. On days 3 and 6, fresh complete medium with GM-CSF was added and nonadherent cells were harvested at day 8. $CD11c^+$ DC was purified using CD11c microbeads and 1×10^6 cells were cultured in a final volume of 1 ml. DCs were infected with *Cm* at a 2:1 multiplicity of infection [246]. The cells were then cultured for 36 h at 37°C in 5% CO_2. DCs were then harvested and used for adoptive transfer experiments.

3.11. Adoptive transfer of DC and challenge infection

Splenic $CD11c^+$DC (5×10^5 DC/mouse) isolated from several mice groups were injected intravenously to syngeneic naive recipient mice. Two hours after adoptive transfer, the recipient mice were then challenged intranasally with *Cm* (1×10^3 IFUs). Body weights of the recipient mice were recorded daily, and mice were sacrificed on day 7 post-infection to investigate chlamydial loads in the lung and immune responses.

For adoptive transfer of PLXND1^{+} DC, BMDC (5×10^5 DC/mouse) isolated from several mice groups were injected intravenously to syngeneic naive recipient mice. Two hours following adoptive transfer, the recipient mice were then challenged intranasally with *Cm* (1×10^3 IFUs) . Body weights of the recipient mice were documented daily, and mice were sacrificed at day 7 post-infection for investigation of chlamydial loads in the lung and immune responses.

3.12. Histopathological analysis

The lung tissues aseptically obtained from different mice groups at indicated time points were fixed in 10% formalin. Haematoxylin and Eosin (H&E) staining was done on tissue sections, and histopathological changes were observed under light microscopy, as described [109]. The degree of lung inflammation was analyzed using a semi-quantitative grading system [40]; grading scale: 0, normal; 1, mild inflammation, granuloma formation, cellular infiltration of less than 25% of the area, no prominent infiltration into adjacent alveolar septae or air space; 2, mild interstitial pneumonitis, diffused cellular infiltration in some area (25%–50%), septal congestion, interstitial edema; 3, inflammatory cell infiltration into perivascular, peribronchiolar, alveolar septae, and air space (50%–75% of the area); 4, over 75% of the area of the lung filled with infiltrating cells.

3.13. In vitro migration assay

Migration of naïve and *C. muridarum* infected BMDC to CCL19 was analysed by using Transwell plate (Corning; Sigma-Aldrich). Naïve and *C. muridarum* infected BMDC were seeded in the upper chamber of the Transwell, and CCL19 (20ng/ml, PeproTech, Rocky Hill, NJ) was added to the lower compartment. Cells were incubated at 37°C in 5% CO2 for 2 h, and cells migrated to lower chamber were counted [247].

3.14. In vivo migration assay

To analyze the effect of Sema3E on migration of DCs to the draining lymph nodes (LNs) *in vivo*, *C. muridarum* infected BMDCs from Sema3E KO, and WT mice were labeled with CFSE (Sigma-Aldrich). The labeled BMDCs ($1x10^6$) were given intranasally to WT recipient mice. After 2 hours of BMDC transfer, mice were infected with *C. muridarum* ($1x10^3$ IFU). The lymph node was collected at 48 hours post-infection, processed into single-cell suspensions, and analyzed by flow cytometry. The percentage of $CFSE^+CD11c^+$ BMDCs that migrated to the lymph nodes was identified and calculated by flow cytometry [182, 247].

3.15. Western blotting of BMDC

BMDCs were lysed using lysis buffer containing M-PER (Thermo Fisher Scientific) as well as protease inhibitors (Roche) [204] . Cell lysates were then centrifuged at 10,000 rpm for 10 min at 4°C and the supernatants were collected. The supernatants were stored at −80°C for further analysis. Protein concentration was estimated using BCA assay (Thermo Fisher Scientific). Protein lysates prepared (10 μg) were loaded onto 10% SDS-PAGE, transferred to PVDF membranes, blocked, and then incubated overnight at 4°C with Abs for phosphorylated ERK1/2 (Thr202/Tyr204) and AKT (Ser473). The blots were then washed and incubated with HRP-conjugated secondary Abs, and bands were developed using ECL reagents. Total anti-ERK1/2 and Akt were used as loading controls [204].

3.16. Rac1 GTPase activity

BMDCs from *C. muridarum* Sema3E KO and WT mice were first stimulated with CCL19 (20 ng/ml) for 0, 1, 2, 5 min. Then, Rac1-GTP was measured in snap-frozen BMDC by G-LISA assay based on manufacturer's instructions (Cytoskeleton, Denver, CO).

3.17. Actin polymerization

BMDCs from *C. muridarum* Sema3E KO and WT mice were first stimulated with CCL19 (20 ng/ml) for 0, 1, 2, 5 min and immediately fixed with 4% paraformaldehyde. Then, cells were washed and permeabilized for 30 min. Permeabilized cells were then stained with Alexa488 conjugated Phalloidin (Life Technologies) for 30 min. Finally, the $CD11c^+$ $Phalloidin^+$ BMDCs were analyzed by flow cytometry to detect F-actin.

3.18. Statistical analysis

Unpaired Student's t-test (GraphPad Prism software v4, GraphPad, San Diego, USA) was used to assay the statistical significance for the comparison of two different groups. For comparing several groups of mice, one-way analysis of variance (ANOVA) was used. A p-value of less than 0.05 was considered significant.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Semaphorin 3E deficiency leads to susceptibility to *Chlamydia muridarum* infection in mice and impacts dendritic cell and T cell functions.

This part of the results is published in the Journal of Immunology

Thomas R, Wang S, Shekhar S, Peng Y, Qiao S, Zhang C, Shan L, Movassagh H, Gounni AS, Yang J, Yang X. Semaphorin 3E protects against chlamydial infection by modulating dendritic cell functions J Immunol. 2021 Mar 15; 206 (6):1251-1265. PMID: 33504621

4.1.1. *Chlamydia muridarum* lung infection induces production of Sema3E, which is involved in protection against *Cm* lung infection

Firstly, we examined the kinetics of Sema3E production after *Cm* lung infection by measuring Sema3E protein levels in the lung. As shown in Fig. 6A, Sema3E protein levels in the lung increased very quickly with peak expression on day 3 and decreased on day 7 post-infection.

To test the involvement of Sema3E in immunity to *Cm* lung infection, Sema3E KO mice and WT mice were infected intranasally with a sublethal dose (1x 10^3) of *Cm* and observed for bodyweight change, pathological changes, and chlamydial loads in the lungs. As shown in Fig. 6B, Sema3E KO mice showed much more serious bodyweight loss than WT mice. The histological analysis also showed severe tissue inflammation and pathology in Sema3E KO mice at early (day 7) and late (day 14) stages of infection (Fig. 6C & 6D). To understand the nature of infiltrating cells, BALF cells of WT and Sema3E KO mice were analyzed (Fig.6E). At day 7 as well as day 14 post-infection, Sema3E KO mice showed significantly higher infiltration of neutrophils compared to WT mice. On day 14 post-infection, WT mice showed only mild inflammation with higher numbers of lymphocytes and macrophages. Consistently, the Sema3E KO mice showed

significantly higher bacterial loads on day 7 as well as day 14 post-infection (Fig. 6F). Taken together, this data suggests that Sema3E plays a crucial role in protection against chlamydial infection.

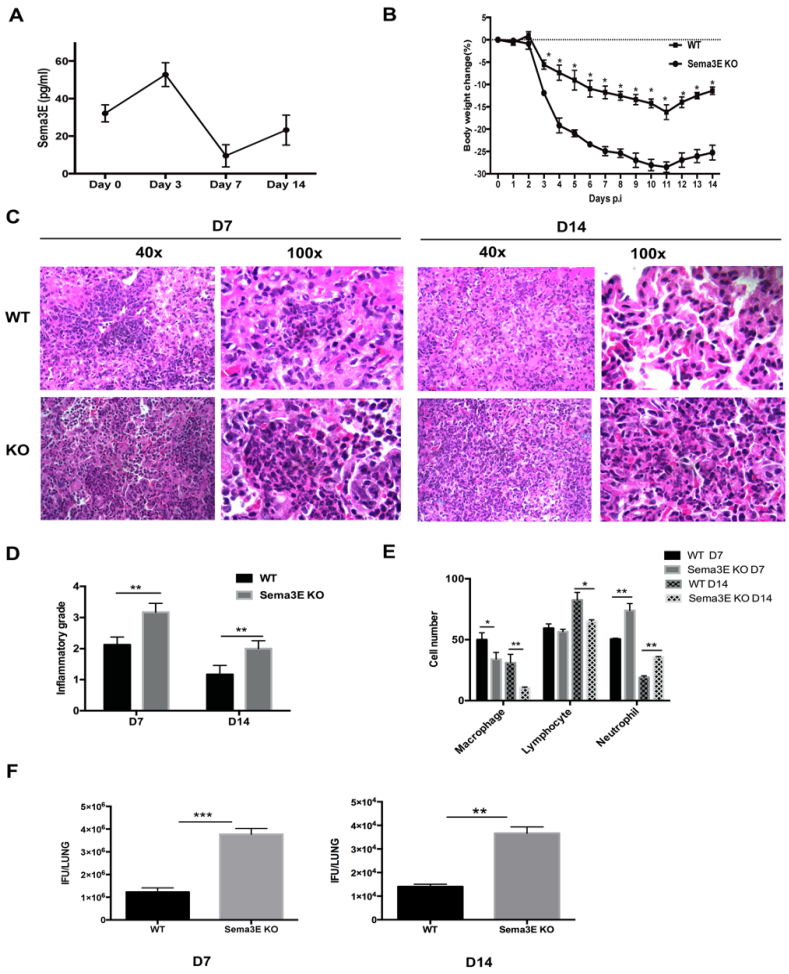

Figure 6: Chlamydial lung infection induces Semaphorin 3E protein production in the lung, and Sema3E deficiency leads to severe disease than WT mice following infection. A) Murine Semaphorin 3E protein levels in lung tissue homogenates of Balb/c mice at different time points post-infection. Sema3E KO and WT mice were intranasally infected with $1x10^3$ IFUs of *Cm*, sacrificed on day 7 or 14 p.i B) Severe bodyweight loss in Sema3E-KO mice compared with WT mice following *Cm* lung infection. Mice were monitored daily for bodyweight changes. The initial body weights of the two groups of mice were similar. C) Pathological changes in the lungs of Sema3E KO mice compared with WT mice following *Cm* lung infection. Lung sections were stained by H&E for histological analysis under light microscopy at day 7 (D7) and day 14 (D14) p.i. D) Lung inflammation was analyzed semi-quantitatively by a blinded pathologist as detailed in Materials and Methods. E) BAL fluids were isolated from Sema3E KO and WT mice at day 7 and day 14 post-infection. The number of macrophages, lymphocytes and neutrophils per 200 cells was calculated based on their morphological and staining properties. F) lungs from Sema3E KO and WT mice were collected and quantified for chlamydial loads at day 7 and day 14 p.i. Data are shown as mean ± SD (n = 3) and represent one of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.1.2. Sema3E is involved in the regulation of T cell response and antibody response after *Cm* infection.

Previous studies have shown that type 1 T cell response and Th17 response are involved in protection against chlamydial lung infection [109, 126, 231]. On the other hand, Th2 response is associated with pathology [231] .To explore the cellular basis for the protective role of Sema3E, we analyzed the Th1, Th17 and Th2 responses in the lungs of Sema3E KO mice and WT mice after *Cm* infection. Cell-specific cytokine analysis of lung cells showed a significant reduction of IFN-γ producing CD4 (Fig. 7A) and CD8 T cells (Fig. 7B) at day 7 and day 14 p.i. in comparison with WT mice. Moreover, IL-17 production by CD4 T cells (Fig. 7C) was also significantly lower in Sema3E KO mice compared to WT mice. In contrast, IL-4 production by T cells was found to be higher in Sema3E KO mice compared to WT mice (Fig. 7D).

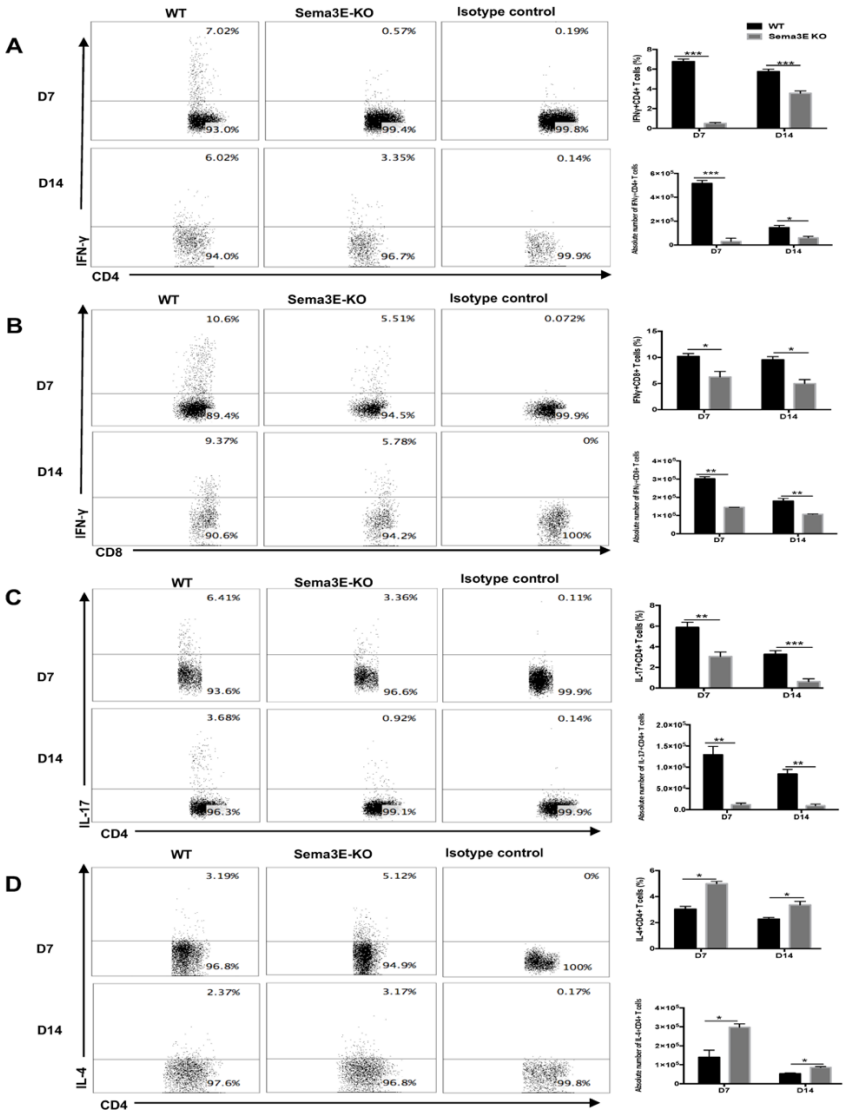

Figure 7: Reduced Th1/Th17 response and increased Th2 response of lung tissues of Sema3E-KO mice at day 7and day 14 post-infection. Mice were intranasally inoculated with $1x10^3$ IFUs of *Cm*. Lung cells were collected at day 7 (D7) and day 14 (D14) post-infection. The cytokine production of lung CD4 and CD8 T cells was analyzed by flow cytometry. A) Representative flow cytometric images (left) and summary of flow cytometric analysis to show the percentage and absolute number (right) of IFN-γ producing CD4 T cells. B) Representative flow cytometric images (left) and summary of flow cytometric analysis to show the percentage and absolute number (right) of IFN-γ producing CD8 T cells. C) Representative flow cytometric images (left) and summary of flow cytometric analysis to show the percentage and absolute number (right) of IL-17 producing CD4 T cells D) Representative flow cytometric images (left) and summary of flow cytometric analysis to show the percentage and absolute number (right) of IL-4 producing CD4 T cells. Data are shown as mean ± SD (n = 3) and represent one of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Consistently, the analysis of lung homogenates (Fig. 8A) and BALF (Fig. 8B) showed that Sema3E KO mice produced lower levels of IFN-γ and IL-17, but higher levels of IL-4 and IL-10 at day 7 and day 14 p.i, than WT mice. Further analysis of cytokine responses in spleen and draining LNs of Sema3E KO mice also showed a significant reduction of IFN-γ and IL-17 production than WT mice following *Cm* infection (Fig. 8C). In contrast, IL-10, as well as IL-4 production, was higher in the spleen and draining LNs of Sema3E-KO mice compared to WT mice (Fig. 8C). Thus, these findings suggest that Sema3E enhances Th1 and Th17 responses but inhibits Th2 responses for protection to *Cm* lung infection. Moreover, analysis of antibody response showed that Sema3E KO mice exhibited higher total and as well as *Cm*-specific IgG1 (Fig. 9A and B) but lower *Cm*-specific IgG2a (Fig.9D). These results indicated that Sema3E is involved in regulating antibody response by inducing IgG2a, and suppressing IgG1, associated with pathological response [248].

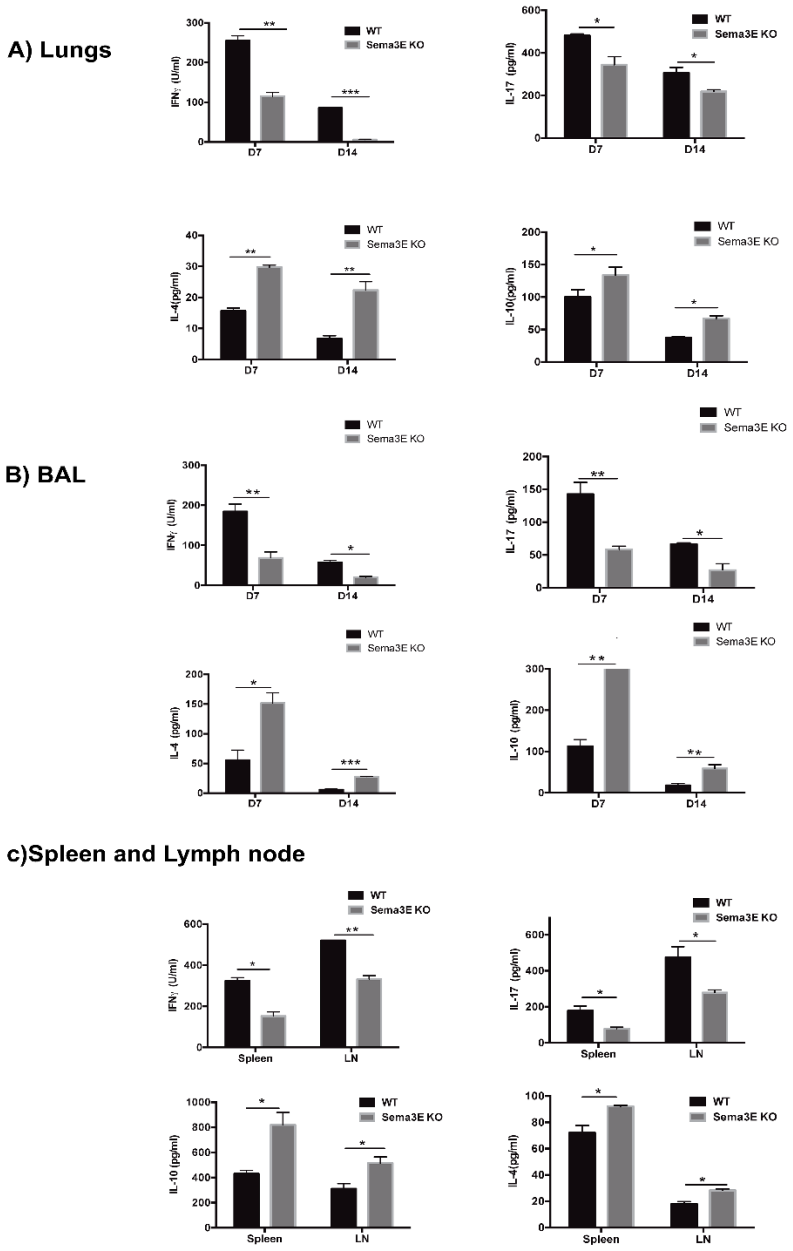

Figure 8: Altered cytokine production in Sema3E KO mice after chlamydial infection. Sema3E KO and WT mice were infected intranasally with *Cm* and sacrificed at day 7 and day 14 p.i. The lung, spleen, and draining lymph node (LN) cells were cultured with UV-killed elementary bodies (UVEB). IFN γ, IL-17, IL-10 and IL-4 levels in 72-h culture supernatants were determined by ELISA. BALF isolated from WT and Sema3E KO mice were centrifuged and supernatants collected for cytokine analysis A) IFN γ, IL-17, IL-10 and IL-4 cytokine production in lung cells at day 7 and day 14 p.i. B) IFN γ, IL-17, IL-10 and IL-4 cytokine production in BALF at day 7 and day 14 p.i. C) IFN γ , IL-17, IL-10 and IL-4 production in spleen and lymph node cells at day 7 p.i. Data are shown as mean ± SD (n = 3) and represent one of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

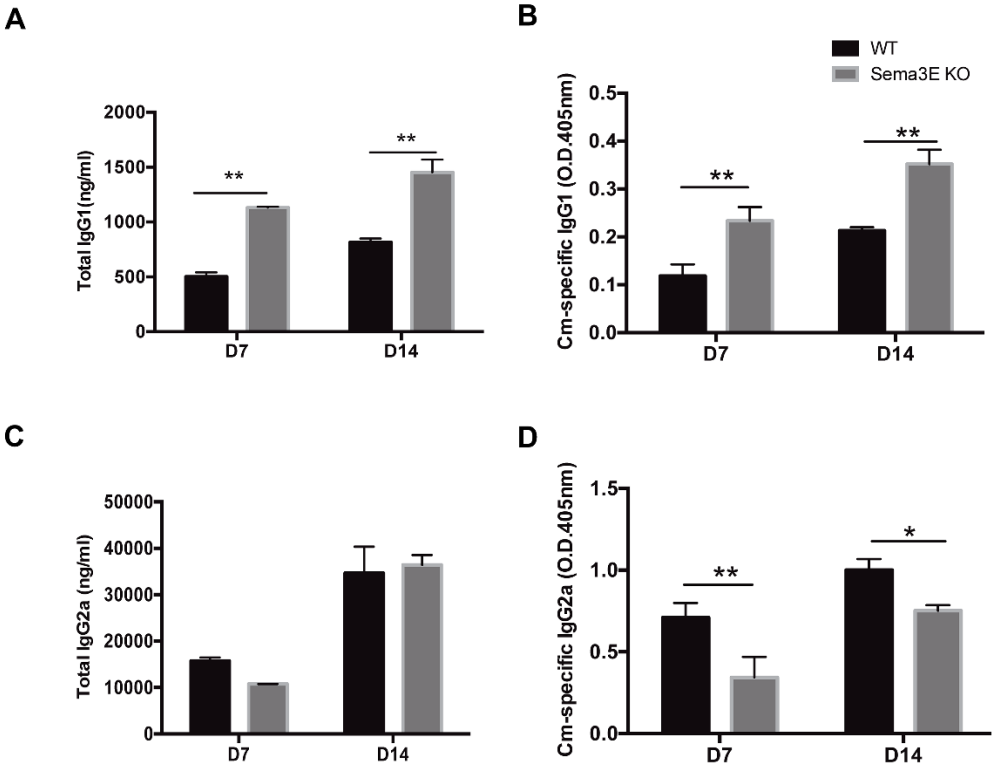

Figure 9: Sema3E is involved in the regulation of antibody response after chlamydial infection. Sema3E KO and WT mice were infected with $1x10^3$ IFUs of *Cm*, and serum samples were collected at day 7 and day 14 p.i. The levels of total and *Cm*-specific IgG1(A and B) and IgG2a (C and D) were measured by ELISA. O.D, Optical Density. The data represent one of two similar experiments (n=3). *$p < 0.05$, **$p < 0.01$.

4.1.3. Sema3E deficiency leads to increased $CD4^+CD25^+FoxP3^+$ regulatory T cell responses

Given that we observed reduced Th1 and Th17 responses in Sema3E KO mice and increased production of IL-10, which suppresses T cell immune responses [118], we next examined the number of $CD4^+CD25^+FoxP3^+$ regulatory T cells in WT and Sema3E KO mice after *Cm* infection. We found the proportion (Fig. 10A, B) and numbers (Fig. 10C) of $CD4^+CD25^+FoxP3^+$ regulatory T cells were significantly higher in Sema3E KO mice than WT mice in the lung and spleen at day 7 p.i.. The results show an inhibitory role of Sema3E on Treg cells after *Cm* infection.

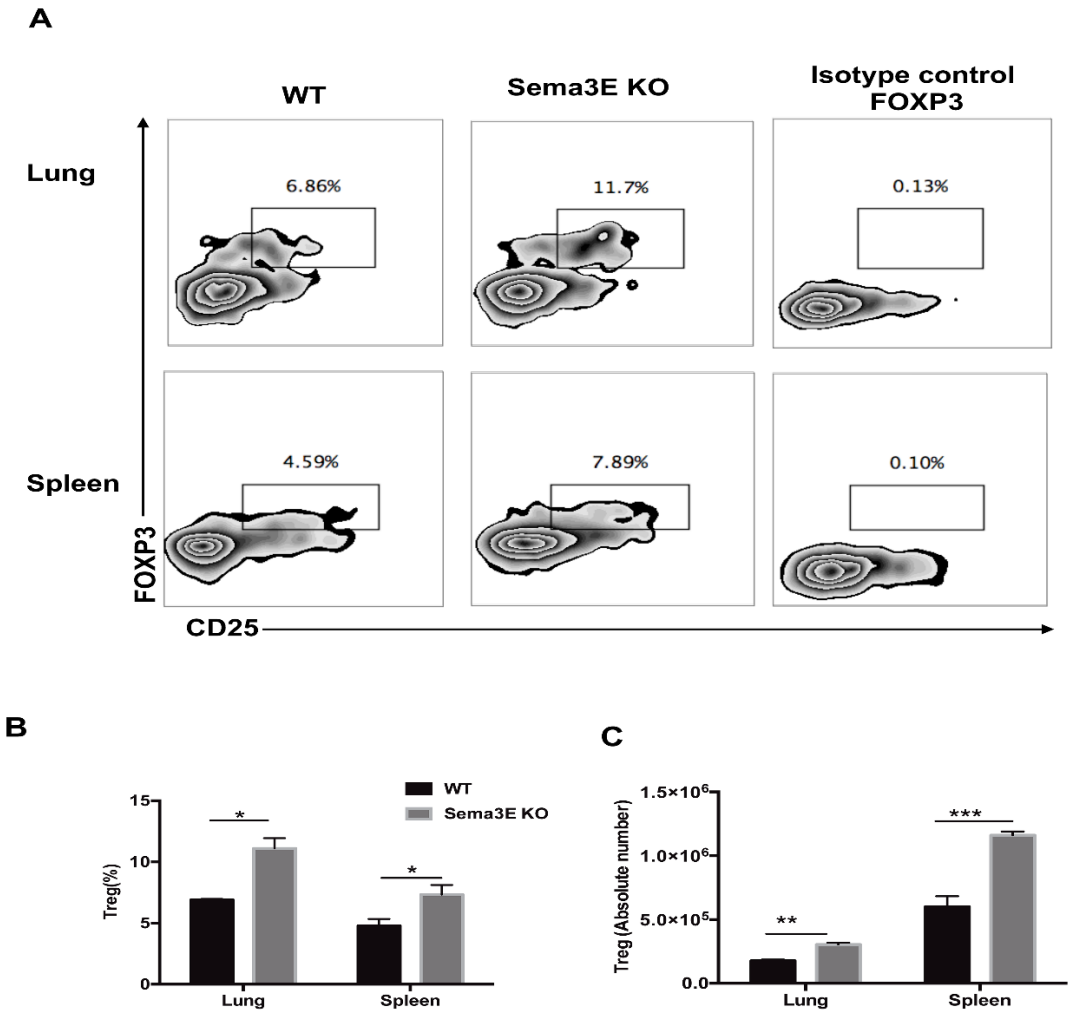

Figure 10: Higher Treg cells in the lung and spleen of Sema3E KO mice following *Cm* infection. Mice were intranasally inoculated with 1x10^3 IFUs of *Cm*. Lung and spleen cells were collected at day 7 post-infection and stained for expression of CD3, CD4, and CD25. FoxP3 intranuclear staining was done on T cells for analysis of Treg cells as described in Materials and Methods. A) Representative flow cytometric images of Treg cells in the lungs and spleen. The percentages (B) and number (C) of Treg cells in lung and spleen. Data are shown as mean ± SD (n = 3) and represent one of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.1.4. Sema3E deficiency leads to alteration of DC phenotype and cytokine production

It is established that DCs play a major role in promoting type 1 T cell response against chlamydial infection [79]. Since Th1 response is significantly reduced in Sema3E KO mice after *Cm* infection, we further analyzed the impact of Sema3E on DC phenotype and function. DCs were isolated from the spleen and lungs of infected Sema3E KO and WT mice and analyzed for DC maturation status, expression of co-stimulatory and inhibitory molecules. We observed lower surface expression of CD40, CD80, CD86 on lung DCs of Sema3E KO than WT mice (Fig. 11). MHC II expression was also lower in the lung and spleen DC from Sema3E KO mice compared to WT mice (Fig. 11). In contrast, *Cm* infected Sema3E-KO mice showed significantly higher expression of inhibitory molecules such as PD-L1 and PD-L2 on the surface of lung and spleen DCs (Fig. 11).

Cytokine production by DCs has a significant impact on the type of T cell immune response. For example, IL-12 production by DC is critical for the development of Th1 cell response [50, 79], whereas IL-10 production by DC impedes type 1 T cell response [232]. In addition, IL-6 production by DC is found to promote Th17 responses [233]. Therefore, we further analyzed the DC cytokine production pattern in Sema3E KO and WT mice following *Cm* lung infection. Intracellular cytokine analysis showed higher percentages of IL-10 producing DC and lower percentages of IL-12 producing DC in the spleen and lung of Sema3E KO mice after chlamydial infection (Fig. 12A, C). Similarly, cytokine analysis of culture supernatants of spleen DCs showed that DCs from Sema3E KO mice exhibited lower IL-12 and IL-6 production but higher IL-10 cytokine production than that of WT mice (Fig. 12B). Altogether, this data suggests that Sema3E significantly modulates both the phenotype and cytokine production of DC after *Cm* infection.

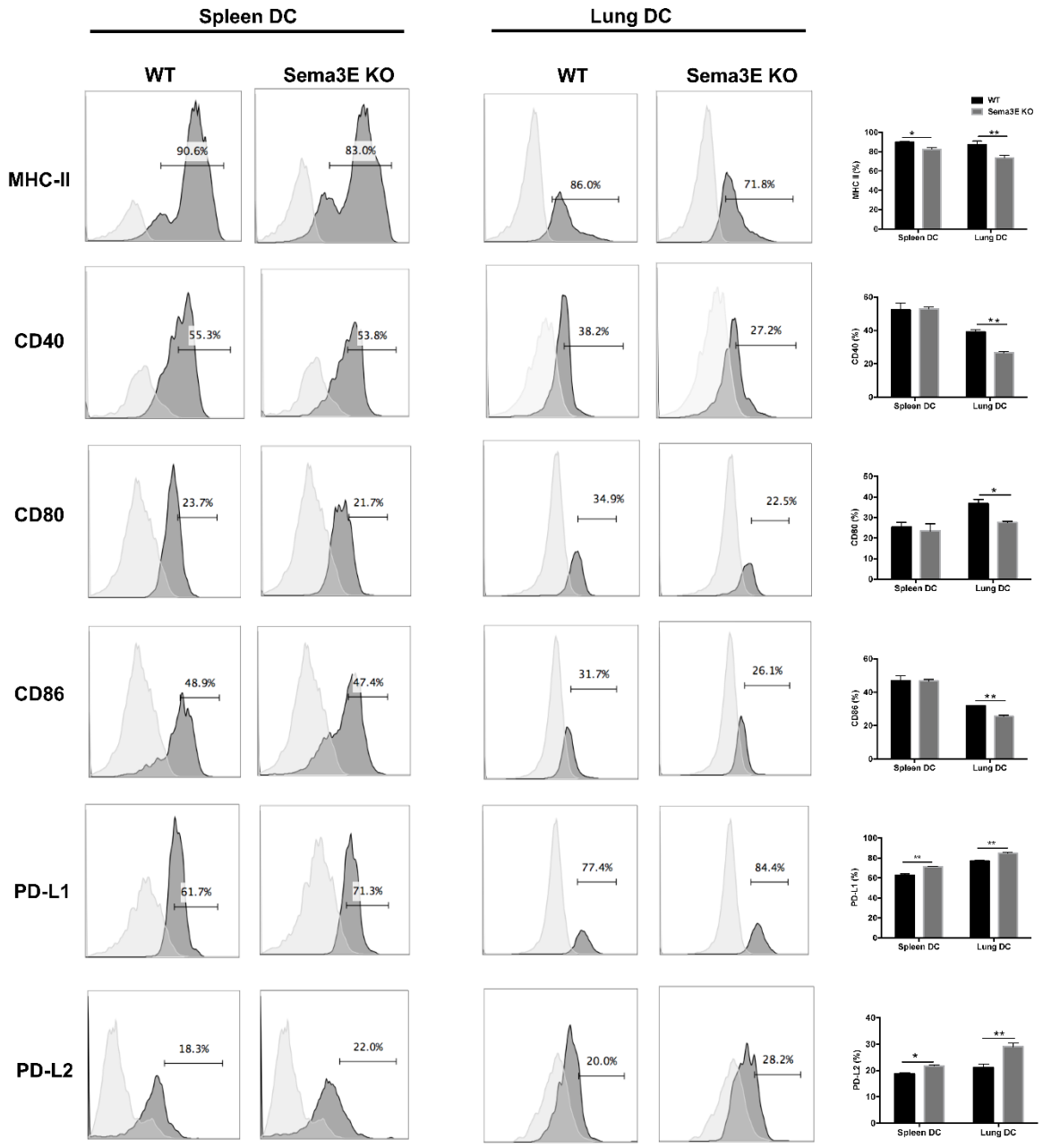

Figure 11: Sema3E KO mice showed altered surface phenotype of DCs following *Cm* infection. Sema3E KO and WT mice were sacrificed at 3 days after intranasal infection with *Cm*. DCs were isolated from the lungs and spleen using CD11c microbeads, and MACS columns and cells were stained for surface markers and analyzed using flow cytometry. Expression of PDL-1, PD-L2, CD40, CD80, and CD86 on $CD11c^+$ $MHCII^+$ cells (dark shaded histograms) and isotype control (light shaded histograms) were shown. MHCII expression on $CD11c^+$ cells and isotype control were recorded. The percentages of positive cells were indicated. One of the three independent experiments with similar results is shown (n = 3). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

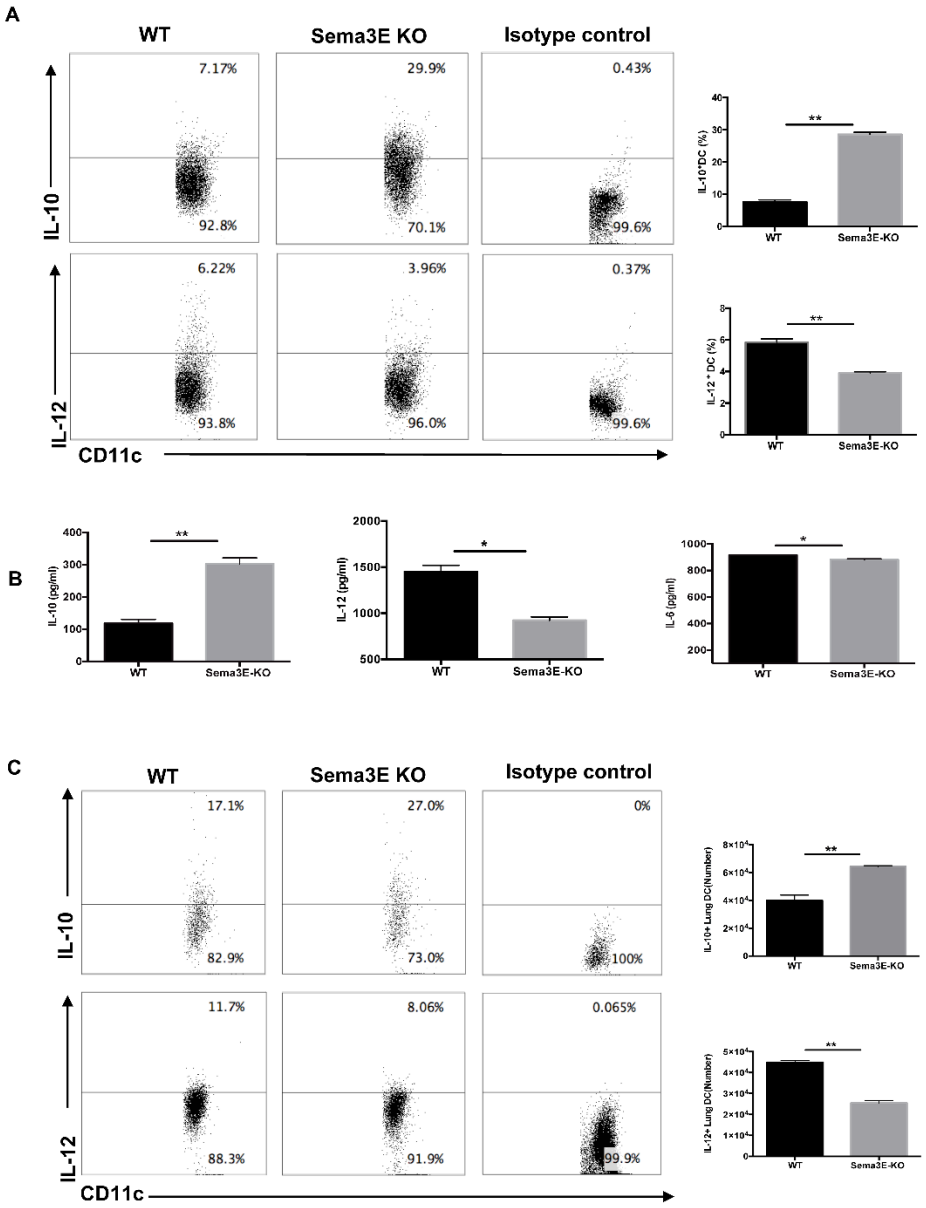

Figure 12: Cytokine production pattern of the spleen and lung DC from Sema3E KO and WT mice. Spleen and lungs were harvested from Sema3E KO and WT mice at day 3 p.i., processed into single-cell suspensions, and DCs were isolated using CD11c microbeads and MACS columns. Flow cytometric analysis was performed on gated $CD11c^{+}MHCII^{+}$ cells. A) The percentages of IL-12 and IL-10 producing spleen DC from Sema3E KO and WT mice were calculated and graphically summarized. B) Cytokine production by DC. Spleen DC was cultured (5×10^5 cells in 200 µl culture medium) in 96 well plates for 72 h. Concentrations of IL-12, IL-10, and IL-6 in supernatants were determined by ELISA. C) IL-12 and IL-10 producing lung DC from Sema3E KO and WT mice were calculated and graphically summarized. The results are shown as mean ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001. One of three similar experiments is shown.

4.1.5. Sema3E impact the ability of DC to direct T cell responses

To study whether Sema3E modulates the functional ability of DC to promote *Cm*-specific T cell responses, we co-cultured T cells from *Cm*-immunized mice with DCs from infected WT and Sema3E KO mice in the presence of killed *Cm* (UVEB). IFN-γ and IL-17 cytokine production by CD4 T cells were examined in the co-culture. As compared with WT DC, Sema3E KO DC showed a dramatically reduced ability to promote $CD4^+$ T cells to produce IFN-γ (Fig. 13A) and IL-17 (Fig. 13B). Similarly, cytokine analysis using ELISA showed lower IFN-γ and IL-17, but higher IL-10 production in Sema3E KO DC-T cell co-culture supernatants compared to WTDC-T cell supernatants (Fig. 13D). Moreover, we observed that Sema3E KO DC induce the production of more Treg cells than WT DC (Fig. 13C). Collectively, this data shows that Sema3E promotes DC's ability to polarize T cells towards type 1 response but inhibits its ability to induce Treg responses after *Cm* infection.

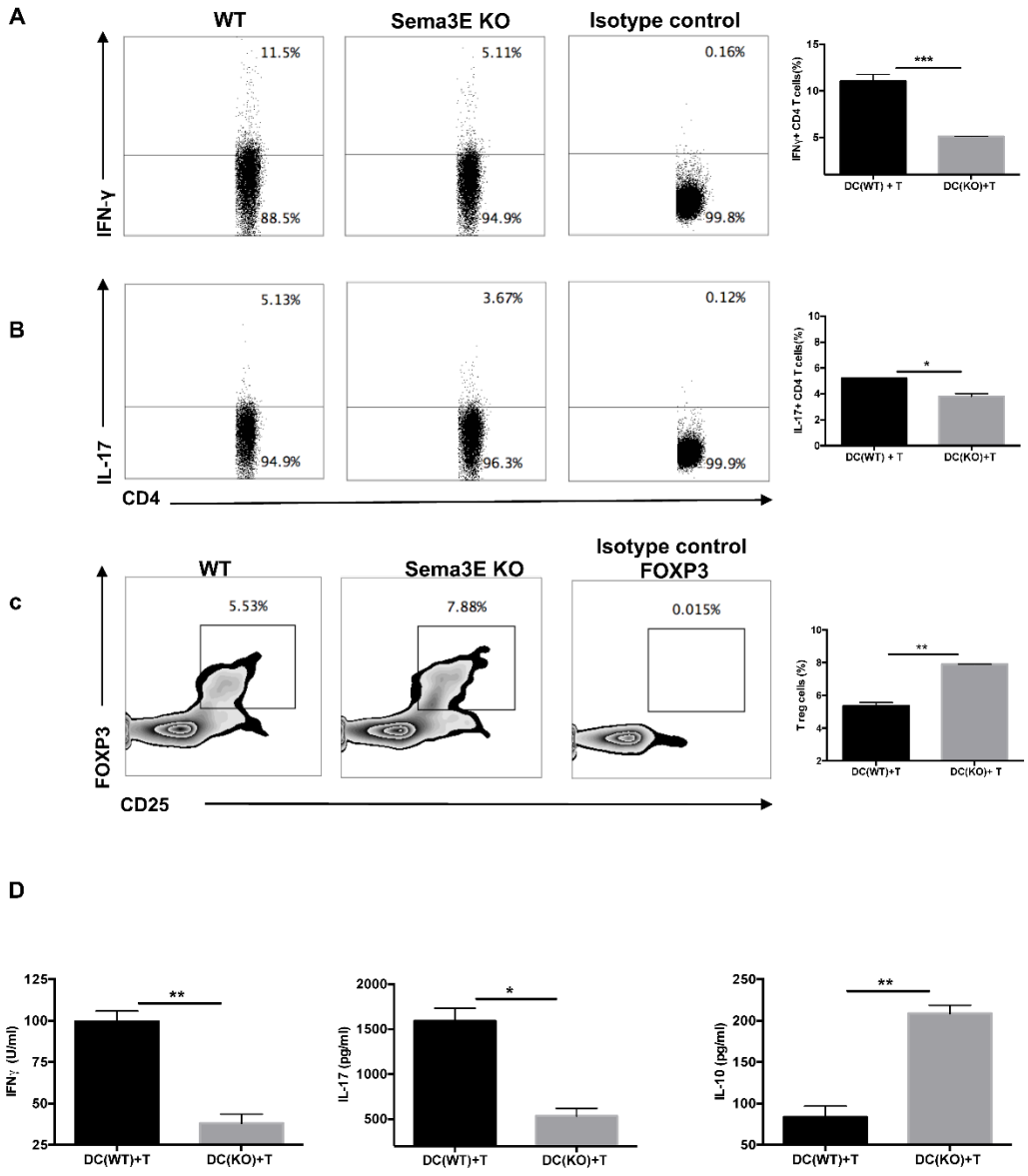

Figure 13: Sema3E is critical for DCs to induce Th1 and Th17 response and suppress Treg cells. Spleen DCs were isolated from Sema3E KO and WT mice at day 3 post-*Cm* infection, and co-cultured with $CD4^+$T cells isolated from *Cm*-immunized mice. After 48-h, cells were analyzed for surface CD3, CD25 and CD4, and intracellular IL-17 and IFN γ. FoxP3 intranuclear staining was done on T cells for analysis of Treg cells as described in Materials and Methods. Cells were gated on $CD3^+CD4^+$ cells. The percentages of (A) IFNγ^+CD4^+ T cells (B) IL-17^+CD4^+ T cells (C) $CD25^+FoxP3^+$ cells in total $CD4^+$ T cells ($CD3^+CD4^+$) are indicated. The culture supernatants were analyzed for the production of IFN-γ , IL-17 and IL-10 by ELISA (D). Data are shown as mean ± SD (n = 3). *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$. One representative data of three independent experiments are shown.

4.1.6. Adoptive transfer of DCs isolated from *Cm*-infected WT mice provides better protective immunity against chlamydial challenge infection than the transfer of those isolated from infected Sema3E KO mice.

To further confirm the functional difference of DC from WT and Sema3E KO mice *in vivo,* we adoptively transferred DCs from naive and infected WT and Sema3E KO mice to syngeneic recipient mice and analyzed their ability to induce immunity to chlamydial challenge infection. DCs were isolated from the spleen of infected Sema3E KO and WT mice (Cm^+DC) as well as naïve mice (naïve DC) and then intravenously transferred to naïve syngeneic recipients. The recipients of different types of DCs were subsequently challenged with *Cm* and sacrificed at day 7 post-challenge. Mice group that received PBS with the same challenge infection were used as controls. We found that mice receiving DCs from infected mice (WT Cm^+DC) were well protected from challenge infection with minimal bodyweight loss, unlike the PBS controls, which lost about 20% body weight on day 7 p.i. (Figure 14A). However, the recipients of DCs from infected Sema3E KO mice (KO Cm^+DC) failed to be protected to the challenge infection, showing similar body weight loss to PBS controls. Similarly, the transfer of DCs from naïve WT (WT naïve DC) or KO (KO naïve DC) mice did not show significant protection by the measurement of body weight change (Figure 14A). The observed differences in body weight change among groups were consistent with the levels of chlamydial load in the lung, exhibiting dramatically lower IFUs in WT Cm^+DC recipients compared to other groups, including in the KO Cm^+DC recipients (Figure 14B). Moreover, the recipients of infected Wild-type-DC (WT Cm^+DC) recipients exhibited mild inflammatory and pathological changes in the lungs compared to infected Sema3E KO-DC (KO Cm^+DC) recipients and the control groups (Fig. 14C& D). The *in vivo* data confirmed the role of Sema3E in influencing the function of DCs to mediate protection against challenge chlamydial infection.

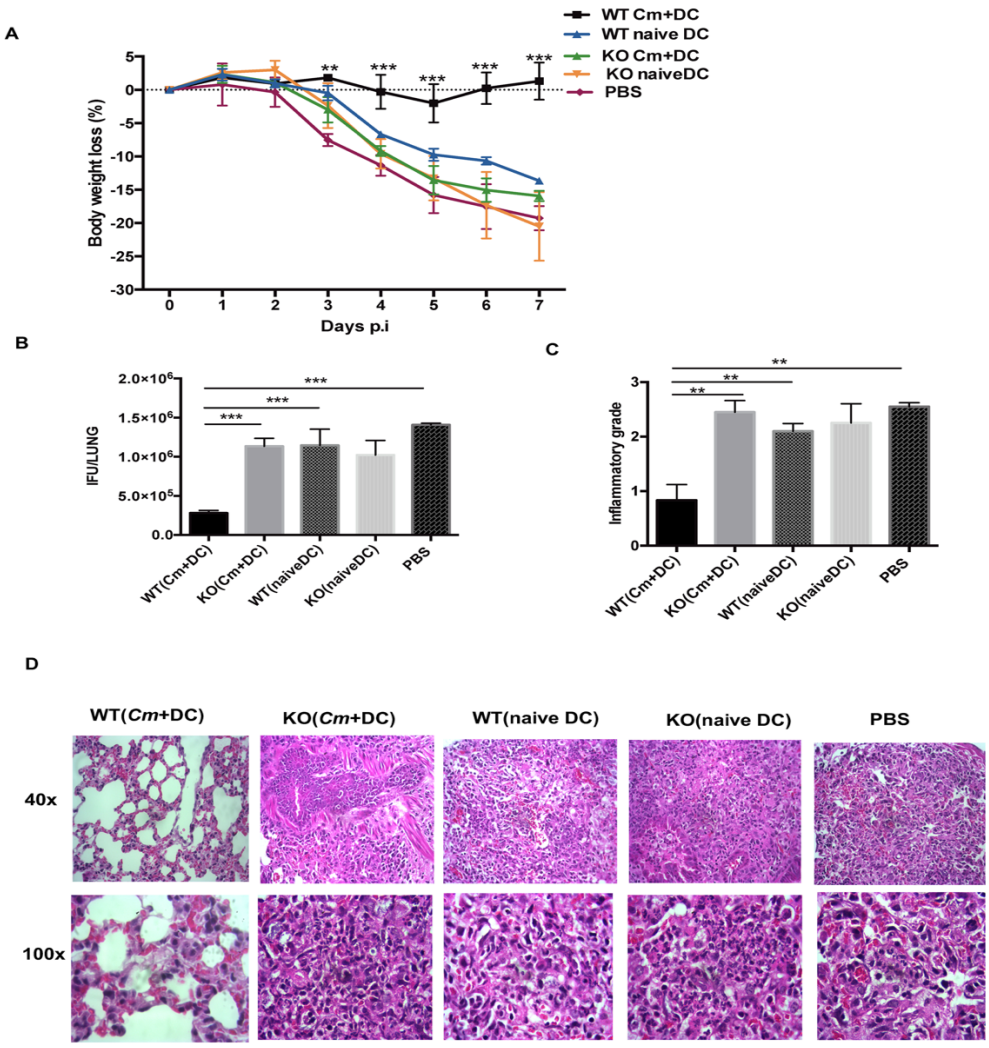

Figure 14: Adoptive transfer of DCs from WT mice, but not from Sema3E KO mice provide protection against chlamydial infection *in vivo*. DCs were isolated from spleens of WT (WT *Cm*+DC) and Sema3E KO mice (KO *Cm*+DC) day 3 post infection ($1x10^3$ IFU of *Cm*) using CD11c magnetic beads and adoptively transferred to recipient WT mice ($5x10^5$ DC/mouse intravenously). Later after 2 hours, recipient mice were challenged intranasally with $1x10^3$ IFU of *Cm*. DCs were also isolated from spleens of naïve WT (WT naïve DC) and Sema3E KO mice (KO naïve DC) and transferred to recipient WT mice. Mice that received PBS alone (PBS) with the same challenge infection used as controls. The body weight changes p.i. were monitored daily. The mice were sacrificed at day 7 p.i., and analyzed for bacterial loads and pathological changes in the lungs. A) Bodyweight loss in recipient mice after transfer of DCs. Mice were observed daily for body weight changes. The initial body weights of the two groups of mice were similar. B) Chlamydial growth in the lung. C) Inflammatory grade. Lung inflammation in different mice groups was analyzed semi-quantitatively by a blinded pathologist as detailed in Materials and Methods. D) Lung histopathological analysis. The slides were stained for H&E and analyzed under light microscopy (magnification ×40 and ×100). Data are expressed as mean ± SD. One representative of the two independent experiments is shown (n = 3). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4.1.7. Adoptive transfer of Sema3E KO DC failed to induce protective Th1/Th17 responses following challenge chlamydial infection

To understand the mechanism by which adoptively transferred Sema3E KO-DC and WT-DC exhibit distinct effects in protection, we evaluated the cytokine production pattern of the corresponding recipient mice following *Cm* challenge infection. We found that the mice that received infected KO-DCs showed a lower number of IFN-γ^+ CD4 T cells compared to mice that received infected WTDC at day 7 post-infection (Fig. 15A). In addition, Sema3E KO DC-recipient mice had lower levels of IFN-γ and IL-17 in their lungs than the infected WT DC- recipients and naïve DC- recipients (Fig.15B). Together, this data shows that Sema3E KO-DC, in contrast to WT-DC, failed to induce type 1 and IL-17 immune responses.

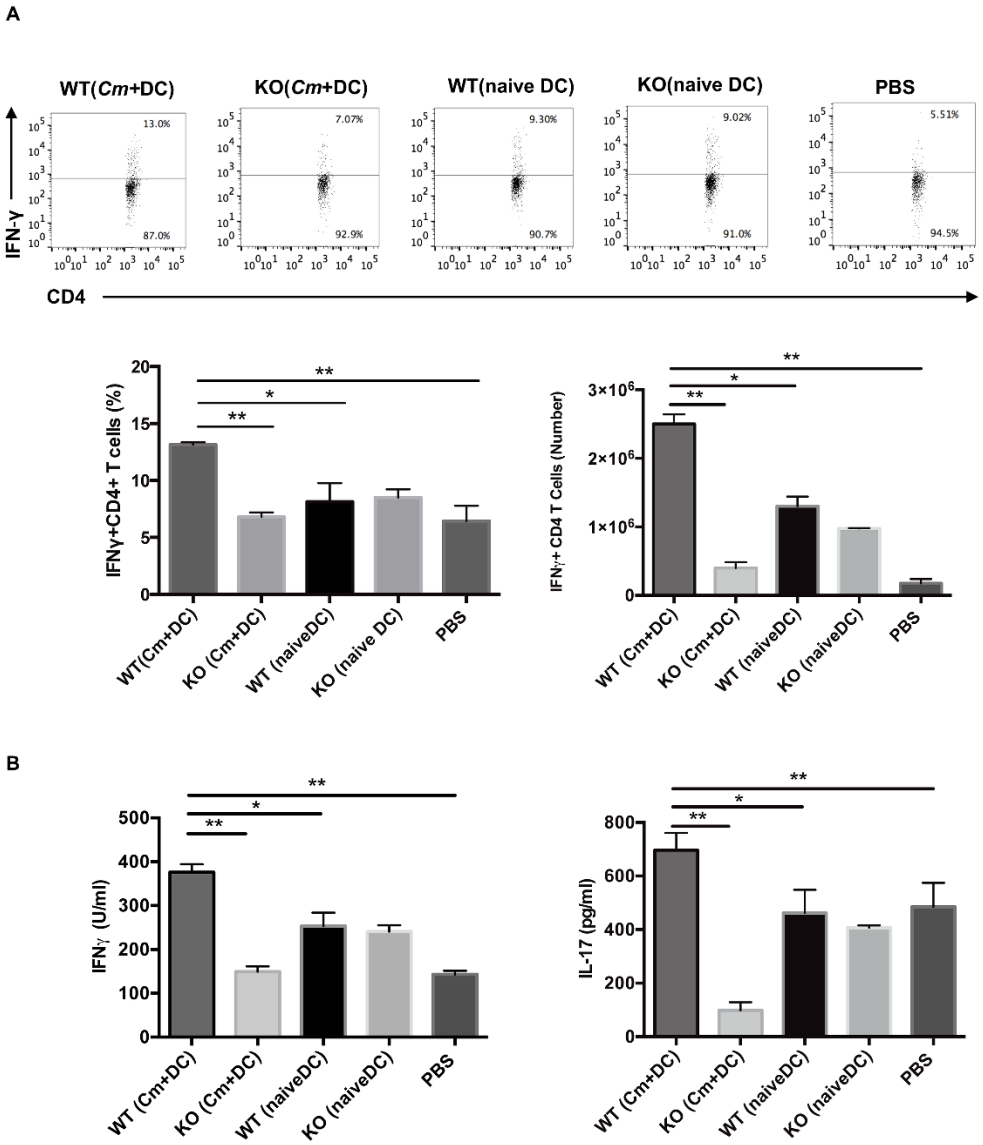

Figure 15: Adoptive transfer of DC from WT mice, but not from Sema3E KO mice enhances Th1 and Th17 cytokines. DCs were isolated from spleens of WT (WT *Cm*+DC) and Sema3E KO mice (KO *Cm*+DC) day 3 post-infection (1x10^3 IFU of *Cm*) using CD11c magnetic beads and adoptively transferred to recipient WT mice (5x10^5 DC/mouse intravenously). Later after 2 hours, recipient mice were challenged intranasally with 1x10^3 IFU of *Cm*. DCs were also isolated from spleens of naïve WT (WT naïve DC) and Sema3E KO mice (KO naïve DC) and transferred to recipient WT mice. Mice that received PBS alone (PBS) with the same challenge infection served as controls. The cytokine production pattern of lung CD4 T cells was analyzed by flow cytometry. A) The percentages and numbers of $IFN\gamma^+CD4^+$ T cells in the lungs of recipient mice were summarized. B) IFN-γ and IL-17 production in the lungs of recipient mice were examined by ELISA. The data represent one of two similar experiments (n = 3). ∗ $p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.1.8. Discussion

In this study, we evaluated the role of Sema3E in host immunity to *Chlamydia muridarum* lung infection. Our results showed that Semaphorin 3E is produced in the local infected tissue after intranasal infection, while deficiency of Sema3E leads to severe *Cm* lung infection characterized by higher lung chlamydial loads, more severe pathology, and body weight loss. Cellular analysis of the BALFs in the Sema3E KO mice showed more neutrophils, but less lymphocytes and macrophages, similar with IFN-alpha/beta receptor KO mice ($IFNAR^{-/-}$) and IL-17 neutralized mice, supporting the involvement of neutrophils in pathology and the importance of lymphocytes and macrophages in protection [60, 81, 109, 231]. Cytokine analysis of local tissues, BALF, draining LN, and spleen also showed lower levels of IFN-γ, IL-17, and higher levels of IL-10 in Sema3E KO mice compared to WT mice. We also observed higher expression of Th2 cytokine IL-4 in Sema3E KO mice after chlamydial infection.

Further T cell cytokine analysis revealed that Th1/Th17 response is reduced and Th2 response is enhanced in Sema3E KO mice compared to WT mice. IFN-γ production is found to be associated with the production of IgG2a antibodies [249]. We found that Sema3E deficiency reduced *Cm*-specific IgG2a antibody response, highlighting the role of Sema3E in Th1-associated antibody response to *Chlamydia.* This influence of Sema3E in regulating T cell response and antibody response leads to the clearance of *Chlamydia* in the lung. Moreover, we found changes in DCs for their expression of surface co-stimulatory molecules, production of immune-modulatory cytokines, and, more importantly, *in vitro* and *in vivo* function. To our knowledge, this is the first report showing the immune-modulatory role of Sema3E in an intracellular bacterial infection.

The influence of Sema3E on cytokine patterns of chlamydial infection showed some similarities and some discrepancies with reports in other models. On the one hand, previous reports on allergic

asthma have shown that exogenous Sema3E supplement treatment increased IFN-γ production by spleen CD4 T cells [211], while Sema3E KO mice exhibited a significant increase in Th2 cytokines compared with WT mice [212]. This data concurs with our current study and support our observation of Sema3E as an enhancer of Th1 responses and inhibitor of Th2 responses. On the other hand, higher IL-17A levels were observed in Sema3E KO mice after allergen exposure, while our chlamydial infection model showed low levels of IL-17A in Sema3E KO mice. The discrepancy could be due to the difference in dominant immune responses in corresponding models, meaning dominant Th2 responses in the allergy model but dominant Th1 responses in chlamydial infection [250]. Moreover, house dust mite (HDM) induced the recruitment of $CD11b^{+}$cDCs in the Sema3E KO mice, which promote Th17 response [212], while the numbers of $CD11b^{+}$cDC were similar in WT and Sema3E KO mice after chlamydial infection (data not shown here).

An important finding in this study is the influence of Sema3E in the functional development of DC for inducing Th1 and Th17 responses. Our previous studies have shown the crucial role of DCs in inducing Th1 and Th17 response to *Cm* lung infection [82, 83, 109]. The present study demonstrates that Sema3E deficiency induced phenotypic and functional changes in DC following chlamydial lung infection. DCs from Sema3E KO mice showed reduced expression of maturation marker MHC II and co-stimulatory molecules, but higher expression of inhibitory molecules, PD-L1 and PD-L2. Our recent studies have shown that blockade of PD1/PD-L1 signaling in DC induces Th1/Th17 responses after chlamydial infection [83]. Conforming to this, it is evident that higher PD-L1 and PD-L2 expression on DCs of Sema3E-KO mice suppress Th1/Th17 response in this mouse model. Moreover, compared to DCs of WT mice, DCs of NK cell-depleted mice exhibited higher expression of PD-L1, lower IL-12 but higher IL-10 cytokines after chlamydial infection [120]. Similarly, Sema3E KO DC produced significantly lower levels of Th1 promoting

cytokine IL-12, as well as Th17 inducing cytokine IL-6. On the other hand, Sema3E KO DC produced higher levels of IL-10, which is detrimental for host defense against chlamydial infection [251]. Our study provides new data on the impact of Sema3E on the suppression of IL-10 production by DCs. Further studies are required to understand how Sema3E signaling in DC impacts specific cytokine production after chlamydial infection.

Another significant finding in this study is the higher numbers of Treg cells in Sema3E KO mice after *Cm* infection. Tregs play a significant role in regulating the effector immune response. In a model of genital infection with *Cm*, it is found that the depletion of Tregs resulted in a reduction of oviduct pathology [115]. Also, in the case of *Chlamydia trachomatis* infection in the genital tract of $ICOS^{(-/-)}$ mice, the reduction of Treg cells is associated with augmented Th1 response [118]. Similarly, our recent studies suggested that NK cells provide protective immunity to chlamydial lung infection by inhibiting Treg expansion [51, 120]. Tregs release suppressive cytokines such as IL-10 to modulate T cell activation [252]. In line with this, IL-10 cytokine production was higher in the lungs, spleen, and LNs of Sema3E KO mice compared to WT mice. This indicates that Sema3E deficiency leads to higher numbers of Tregs, which release IL-10 to suppress Th1 and Th17 effector response to *Cm* infection. Upon chlamydial infection, DCs have an incredible ability to suppress the excessive expansion of regulatory T cells [120]. DCs from Sema3E KO mice induced Treg cells upon co-culture with *Chlamydia*-specific $CD4^+$ T cells, suggesting that Sema3E influence DC to suppress Tregs after chlamydial infection. Higher IL-10 cytokine produced by Sema3E KO DCs contributes to the induction of Tregs after chlamydial infection. To our knowledge, our findings are the first to show the impact of Sema3E on suppressing regulatory T cells.

Importantly, our data demonstrate that changes in DC phenotype and cytokine production occurred in the absence of Sema3E resulted in the impairment of DC to generate Th1 and Th17 response and enhanced Treg response *in vivo*. In addition, we observed a marked decrease in the proportion of chlamydia-specific Th1 and Th17 cells upon co-culture with DC from Sema3E KO mice compared to WT mice. More importantly, we found that, in contrast to the dramatic reduction of lung chlamydial loads, body weight loss, and pathology in the recipient mice upon adoptive transfer of DC from *Cm* infected WT mice, the transfer of DC from *Cm* infected Sema3E KO mice failed to generate protection against chlamydial challenge infection. The severity of infection in the recipients of DCs from infected Sema3E KO mice was correlated with the reduction of Th1 responses. These results suggest that Sema3E signaling after *Cm* infection functionally activates DC to induce a protective adaptive immune response to infection. Notably, the involvement of Sema3E on DC function *in vivo* has been studied in experimental allergic asthma. It is found that Sema3E deficiency promoted the recruitment of Th2 skewing $CD11b^+$ cDCs to the airways [212]. Upon adoptive transfer, Sema3E deficient $CD11b^+$ cDCs promoted the secretion of Th2 cytokines and exacerbated allergic asthma [212].Taken together, it is tempting to speculate that Sema3E is critical for DC function to promote Th1 response and inhibit Th2 response.

What cells produce Sema3E and how Sema3E influences DC during chlamydial infection remains a question. It has been reported that Sema3E is highly expressed in bronchial epithelial cells of the lung [253], and we found its production is induced after *Cm* infection in the lung (Fig.6A). Since Sema3E can be produced by multiple cell types [202, 209, 217] [187]and previous studies on *Cm* have shown the capacity of the chlamydial strain to activate different cells, it is likely to be produced by multiple cell types. This question needs to be answered in the future study.

4.2. Semaphorin 3E deficiency leads to defective dendritic cell migration in response to chlamydial infection

4.2.1. Sema3E deficiency alters the number of lung and spleen DC after *C. muridarum* infection.

In order to understand the effect of Sema3E on DC migration, we analyzed the number of lung DCs in Sema3E KO and WT mice after *C. muridarum* infection. Lung DCs (LDCs) are identified by CD11c expression and exclusion of autofluorescent cells. Autofluorescence was measured by keeping the FL-1/FITC channel empty, i.e., without using any fluorochrome-conjugated antibodies for this channel in the sample [251]. $CD11c^{hi}$ non-auto fluorescent cells were characterized as LDCs [251]. We found a reduced absolute numbers and percentages of the lung (Fig. 16 A & B) in Sema3E KO mice in comparison with WT mice at day 3 p.i. However, in the naïve mice, no difference was found in the percentages of lung DCs between WT and Sema3E KO mice. We next analyzed whether Sema3E preferentially modulate DC subsets in the lung and spleen DCs. $CD11c^{hi}$MHC II^{+} cells were identified as spleen DCs. $CD103^{+}$ LDC subset plays a predominant role in providing Th1/Th17 response to chlamydial infection [83] and the splenic $CD8\alpha^{+}$ DC subset is shown to induce Th1 cytokine production and inhibit Th2 cytokines [82]. We found that at day 3 after *Cm* infection, the percentages and absolute numbers of splenic $CD8\alpha^{+}$ DC (Fig. 17A) and $CD103^{+}$ lung DC (Fig. 17B) were reduced in Sema3E KO mice compared to WT mice.

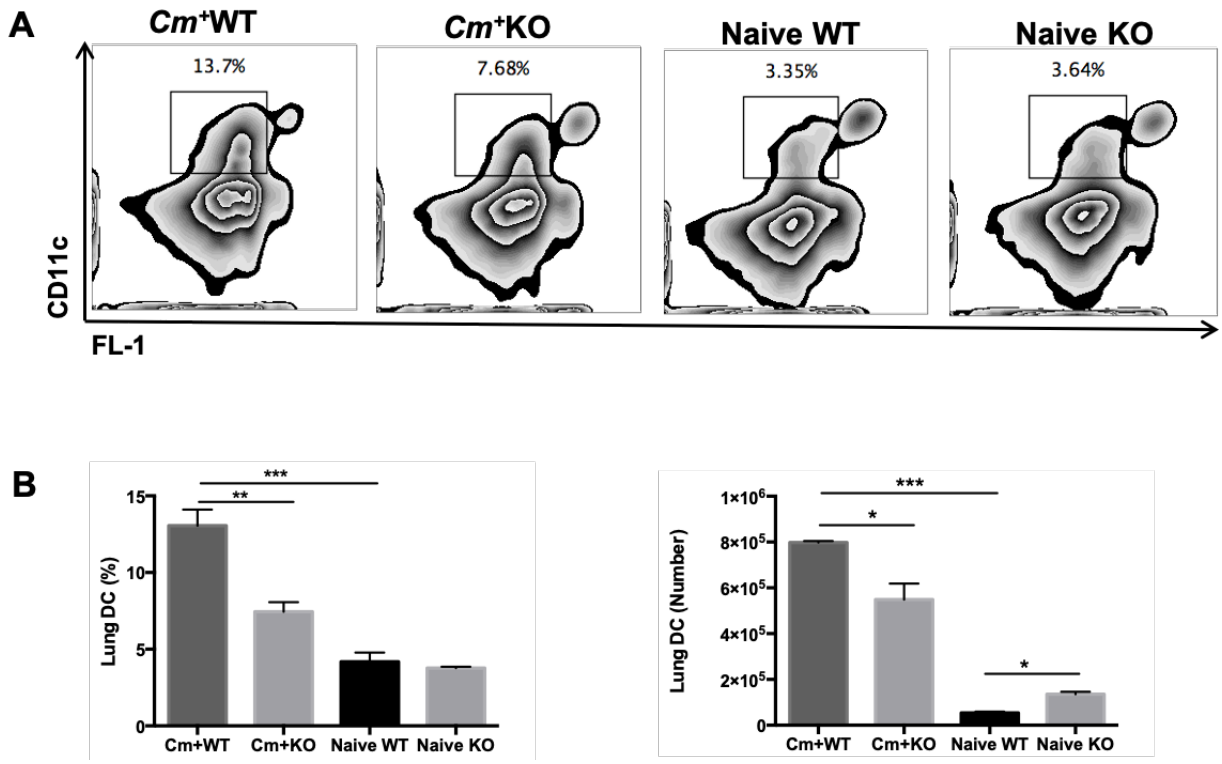

Figure 16: Reduced numbers of lung DCs after *C. muridarum* infection in Sema3E KO mice. Mice were intranasally inoculated with $1x10^3$ IFUs of *C. muridarum*. Lung cells were collected from Sema3E KO mice and WT mice, stained for surface markers, and analyzed using flow cytometry. A) Representative flow cytometric images of *C. muridarum* infected and naïve lung DCs in Sema3E KO and WT mice. B) The percentages and number of lung DC. $*p < 0.05$, $**p < 0.01$.

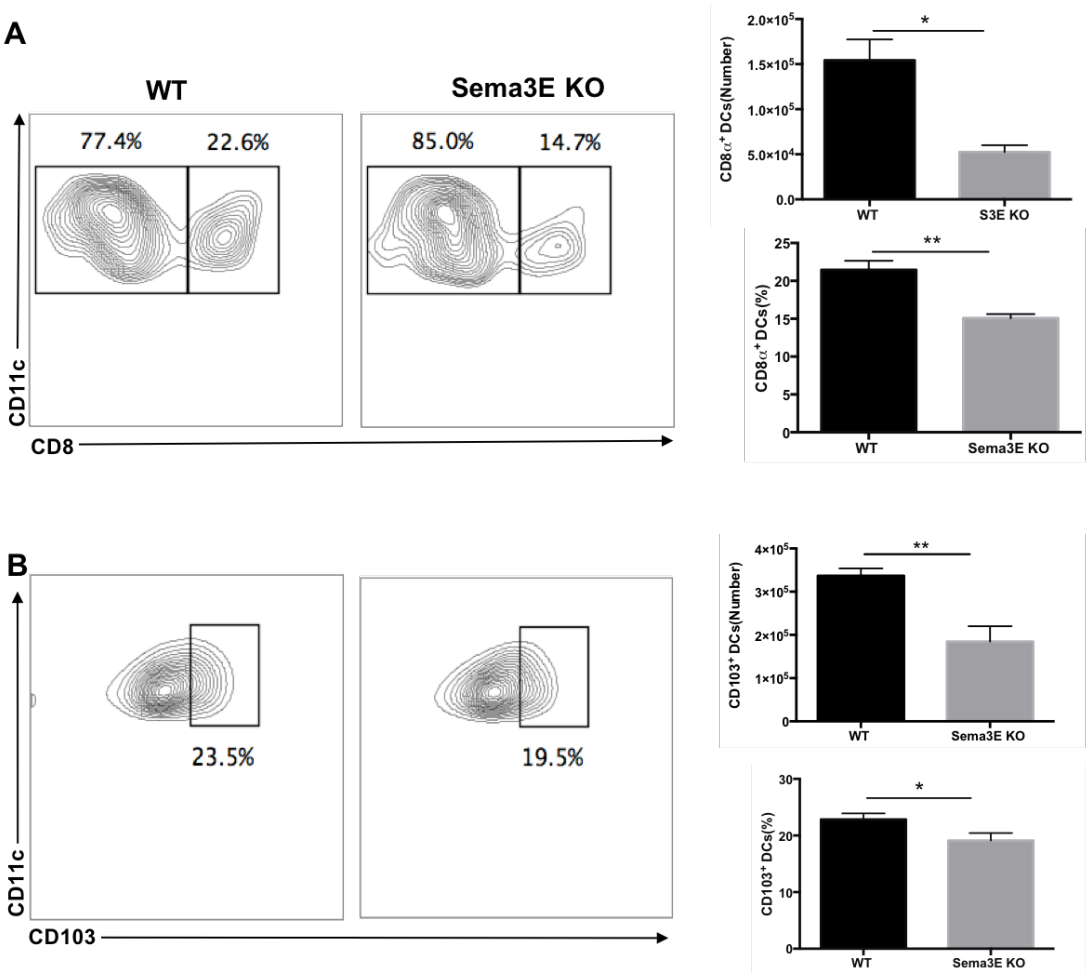

Figure 17: Lower CD8α$^+$ spleen DC subset and CD103$^+$ lung DC subset in Sema3E KO mice compared WT mice after *C. muridarum* infection. Mice were intranasally inoculated with 1x10^3 IFUs of ***C. muridarum***. Lung and spleen cells were collected from mice at day 3 post-infection, stained for surface markers and analyzed using flow cytometry. A) Representative flow cytometric images (left) and numbers CD8α$^+$ spleen DC subset (right). B) Representative flow cytometric images (left) and numbers CD103$^+$ lung DC subset (right). Data are shown as mean ± SD (n = 3) and represent one of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$.

4.2.2. Sema3E deficiency affect the migration of dendritic cells after *C. muridarum* infection

To study the effect of Sema3E in migration of DC, we first did an in vitro trans-well migration assay to examine the response of DCs to a chemokine, CCL19. BMDCs were isolated from naïve WT and Sema3E KO mice. The migration of the in vitro infected and uninfected DCs to CCL19 was analyzed. We observed that *Cm* infected (Cm^+) Sema3E KO DC showed a reduced migration to CCL19 compared to infected WT DC (Fig. 18A). No significant differences in the migration were observed between naïve WT and Sema3E KO DCs. Since CCL19 chemokines are needed for the migration of infected DCs to lymph node, we next analyzed the migration of WT and Sema3E KO DCs to the lymph nodes (LNs) *in vivo*. *Cm* infected BMDCs from WT and Sema3E KO mice was labeled with CFSE and transferred intranasally to WT recipient mice after *Cm* infection. The percentage and absolute number of $CFSE^+$DCs in the lymph node were analyzed as described [182]. The results showed a reduced percentage and number of $CFSE^+$Sema3E KO DC in the lymph nodes of recipient mice compared to the $CFSE^+$WTDC (Fig. 18B).

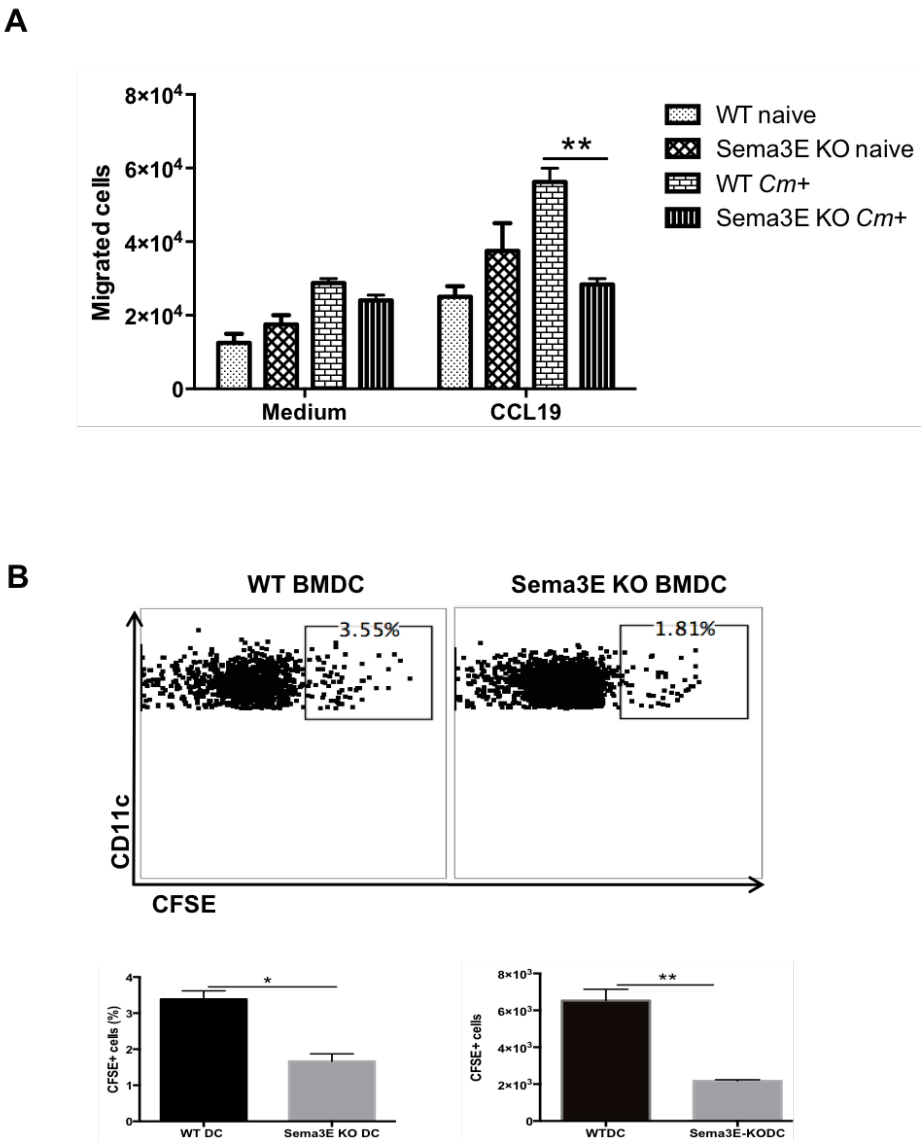

Figure 18: Defective migration of Sema3E KO dendritic cells after *C. muridarum* infection. BMDC isolated from Sema3E KO mice and WT mice and infected with *C. muridarum*. A) Migration of naïve and *C. muridarum* infected Sema3E KO and WT BMDC to CCL19 assessed using transwell migration assay. B) *C. muridarum* infected BMDC from WT, and Sema3E KO mice were labeled with CFSE and transferred intranasally to recipient mice after *C. muridarum* infection. The percentage of $CFSE^{+}$DCs in the lymph node was analyzed.

4.2.3. CCR7 chemokine receptor expression is reduced in the DCs of Sema3E KO mice compared to WT mice

The chemokine receptor, CCR7, is induced on DCs after chlamydial stimulation and is required for their migration to draining lymph nodes to initiate T cell responses [76, 254]. Since we observed reduced migration of *Cm* infected Sema3E KO DC to lymph node, we tested whether this is due to alteration in the expression of the CCR7 receptor on DCs. The data showed a marked decrease of the percentage of $CCR7^+$ lung DCs in Sema3E KO mice compared to WT mice in the early stage of in vivo infection (day 3 p.i.) (Fig. 19). This data suggest that the reduction of CCR7 expression by DCs of Sema3E KO mice is likely an important reason for the reduced migration of these cells to draining lymph nodes.

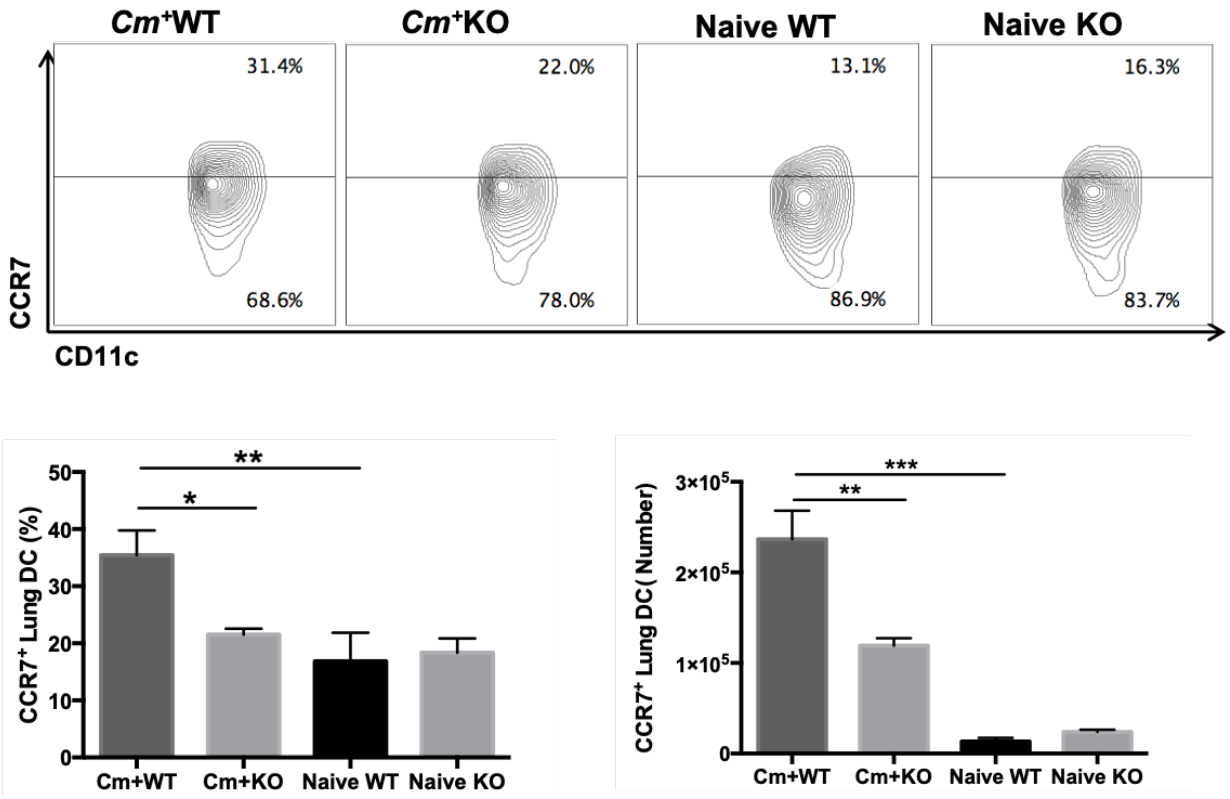

Figure 19: Sema3E deficiency leads to reduced numbers of CCR7$^+$ DC in the lungs after*C. muridarum* infection. Mice were intranasally inoculated with 1x10^3 IFUs of *C. muridarum*. Lung cells were collected from mice at day 3 post-infection and stained for surface markers. CCR7$^+$lung DC number and percentages were analyzed by flow cytometry.

4.2.4. Sema3E deficiency leads to reduced Rac1 GTPase activity, Erk and Akt activation, and F-actin polymerization in *Cm*-infected DCs in response to CCL19

Next, we compared the downstream signaling molecules which are activated in response to CCL19 by *Cm*-infected Sema3E KO and WT DCs. Since the activation of Rac1 is required for the motility of mature DCs [255], we measured Rac-1 GTP by G-LISA in *Cm* infected Sema3E KO and WT DCs upon stimulation with CCL19. We observed that Rac-1 GTP levels were reduced in *Cm*-infected Sema3E KO DC compared to WT DCs in response to CCL19 (Fig 20A). It is reported that Rac-1 activation leads to F-actin polymerization resulting in enhanced migration of dendritic cells [256]. Therefore, we next analyzed the F-actin content of *Cm*-infected Sema3E KO and WT DCs upon stimulation with CCL19. As shown in Fig. 20B, F-actin polymerization was reduced in *Cm*-infected Sema3E KO DC compared to WT DC after CCL19 stimulation. Further analysis of downstream signaling pathways involved in CCL19-CCR7 signaling in DCs showed reduced phosphorylations of Akt and Erk in *Cm*-infected Sema3E KO DC compared to WT DCs. (Fig. 20C) Altogether, our data suggest that Sema3E is critical for the activation of downstream signaling pathways of the DCs in response to chemokines, such as CCL19, following chlamydial infection.

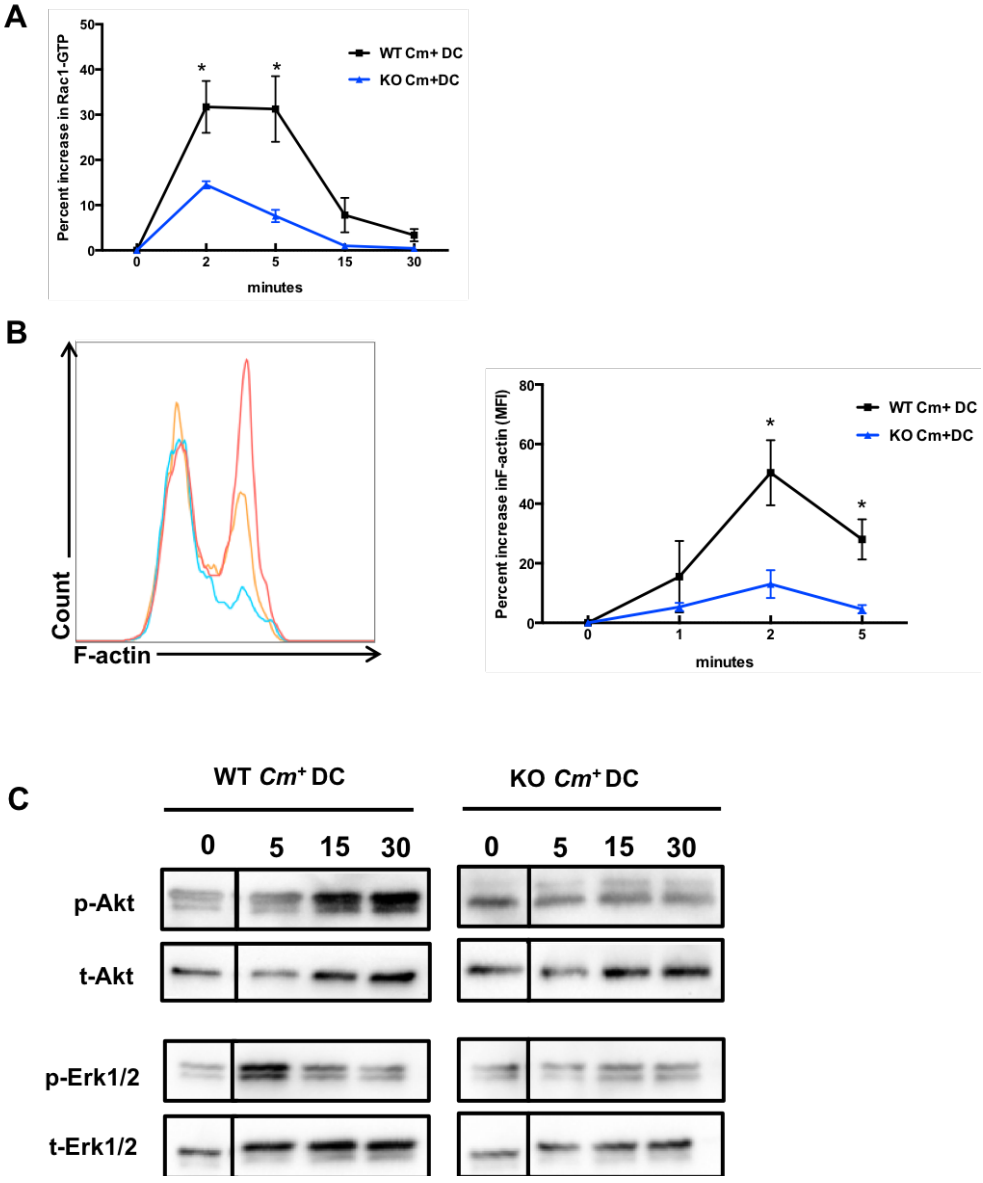

Figure 20: Sema3E deficiency affects downstream signaling molecules activated in response to CCL19. A) Sema3E deficiency leads to reduced Rac1 GTP in BMDC in response to CCL19 as analyzed by G-LISA. B) Reduced F-actin polymerization in *C. muridarum* infected Sema3E KO mice in response to CCL19. Representative histogram representation (left) of actin polymerization at baseline (blue line) and 2 minutes after CCL19 stimulation of *C. muridarum* infected Sema3E KO DC (yellow line) and WTDC (red line). Kinetics (right) of increase in actin polymerisation in *C. muridarum* infected Sema3E KO DC and WTDC after CCL19 stimulation. C) Reduced Erk and Akt activation in *C. muridarum* infected Sema3E KO BMDC compared to WTDC after CCL19 stimulation.

4.2.5. Discussion

DCs are the major antigen-presentation cells (APCs) of the immune system and have a crucial role in both sensing pathogens and tuning the immune responses [63]. On exposure to pathogens, TLRs and other pattern recognition receptors on the surface of DCs recognize molecular patterns associated with microbes, leading to DC maturation, upregulation of CCR7, and consequent migration to the local draining lymph nodes. The migrating DCs meet and interact with naive T cells in the lymph nodes, leading to T cell activation and differentiation [63]. In this part of the study, we focused on the influence of and mechanisms by, Sema3E expressed on DCs on DC migration following chlamydial infection. The data showed that Sema3E signaling on DCs is crucial for the upregulation of CCR7 and the migration of these cells to the draining lymph nodes. Specifically, we found reduced numbers of lung and spleen DCs in Sema3E KO mice compared to WT mice following *C*m infection. More interestingly, we found that the reduction of DCs in Sema3E KO mice following *Cm* infection was preferentially in the $CD103^+$ lung DC and $CD8\alpha^+$ spleen DC subsets which are critically important for the induction of protective T cell response to chlamydial lung infection [82, 83].

The migration experiments in the present studies revealed that the ability of DCs of *Cm*- infected Sema3E deficient mice to respond to a chemokine, CCL19, in vitro is impaired, and the DCs could not migrate to the draining lymph nodes in vivo as WT DCs (Fig. 18). In contrast, we observed that Sema3E deficiency did not significantly impact the migration of naïve DC in vitro in response to CCL19, which fits the equal low level CCR7 expression on the naïve DCs of WT and Sema3E KO mice (Fig. 19). The observation is also in line with a previously reported study where PlexinD1(Sema3E receptor) deficient naïve DCs migrate similar to WT naïve DC [238]. The data suggests that Sema3E mediated activation of DCs following chlamydial infections can enhances

their ability to respond to chemokines. More importantly, we confirmed that Sema3E can impacts the ability of DC to migrate to the draining lymph node in vivo following *Cm* infection. The reduced numbers of Sema3E KO DCs in draining lymph nodes is line with the observation of lower numbers of CCR7 expressing DCs in Sema3E KO mice following *Cm* lung infection.

The critical role of Sema3E in the response of DCs to chemokine for migration following chlamydial infection is also confirmed by the further analysis of downstream signaling events of CCL19 stimulation. We found that Sema3E deficiency reduces Rac 1 GTP, F-actin polymerization, Erk and Akt phosphorylation of infected DCs upon CCL19 stimulation. Rac 1 activation and associated actin rearrangements were the early events occurring in DCs in response to CCL19 [256, 257]. We observed reduced Rac1 activation and F-actin polymerization in *Cm*-infected Sema3E KO DC compared to WTDC after CCL19 stimulation. It was reported that CCL19-CCR7 signaling on DCs particularly leads to activation of Erk and Akt downstream signaling molecules leading to chemotaxis and migration [258]. Our observation of reduced phosphorylation of these key signaling molecules in *Cm*-infected Sema3E KO DCs further confirm the involvement of Sema3E in specific CCL19-CCR7 interaction following chlamydial infection. The finding is in line with a recent study on macrophages which showed reduced phosphorylation of Erk and Akt in the bone marrow-derived macrophages of Sema3E KO mice upon in vitro LPS stimulation [204]. Our study by examining the DCs isolated from infected WT and KO mice strengthened the previous findings and expanded the finding to DCs. These studies further suggest the critical importance of Sema3E in the activation of signaling molecules involved in chemotaxis following infections. Altogether, our studies suggest that Sema3E mediated signaling on DCs following chlamydial infection promote their migration in response to chemokines.

4.3. Plexin D1 deficiency in dendritic cells exacerbates chlamydial infection in mice.

Rationale: We showed that Sema3E deficiency impacts the ability of dendritic cells to influence effective T cell response against *Cm* infection, suggesting that Sema3E can modulate the function of dendritic cells. To further test if the effect of Sema3E is mediated through the pathway of ligand-receptor interaction in dendritic cells, we analyzed the susceptibility of mice that have deficiency of Sema3E high-affinity receptor, plexin D1 on their dendritic cells, to chlamydial infection. We also examined the function of dendritic cells from the mice deficient of plexinD1 receptor on their dendritic cells to confirm the role of Sema3E-plexin D1 axis in dendritic cells for the induction of protective immune responses to *Cm* infection.

4.3.1. Deficiency of plexinD1 on dendritic cells leads to enhanced susceptibility to chlamydial infection in mice.

Since Sema3E deficiency impacts dendritic cell function and Sema3E high-affinity receptor, plexin D1 (PLXND1) is reportedly expressed on DCs; we examined this receptor expression on DCs after chlamydial lung infection. We found that plexin D1 is indeed expressed on spleen DCs (Fig. 21A) after chlamydial infection. To test the functional importance of this receptor, we analyzed the impact of deficiency of plexin D1 on DCs in immunity to chlamydial infection. Mice deficient in PLXND1 on DC (CD11c PLXND1$^{-/-}$) and WT mice (CD11c PLXND1$^{+/+}$) were infected with *Chlamydia muridarum* ($1x10^3$) and analyzed for bacterial load, body weight loss, and lung pathology. As shown in Fig. 21B, CD11c PLXND1$^{-/-}$ mice showed significantly higher bacterial loads on day 7 post-infection. Following intranasal *Cm* infection, CD11c PLXND1$^{-/-}$ mice showed much more severe bodyweight loss than CD11c PLXND1$^{+/+}$ mice (Fig. 21C). Lung histological analysis showed that CD11c PLXND1$^{-/-}$ mice exhibit severe pathology with higher cellular infiltration than CD11c PLXND1$^{+/+}$ mice (Fig. 21D & E). These observations demonstrate that PLXND1 expression on DC is critical for clearance of chlamydial lung infection and control of the diseases.

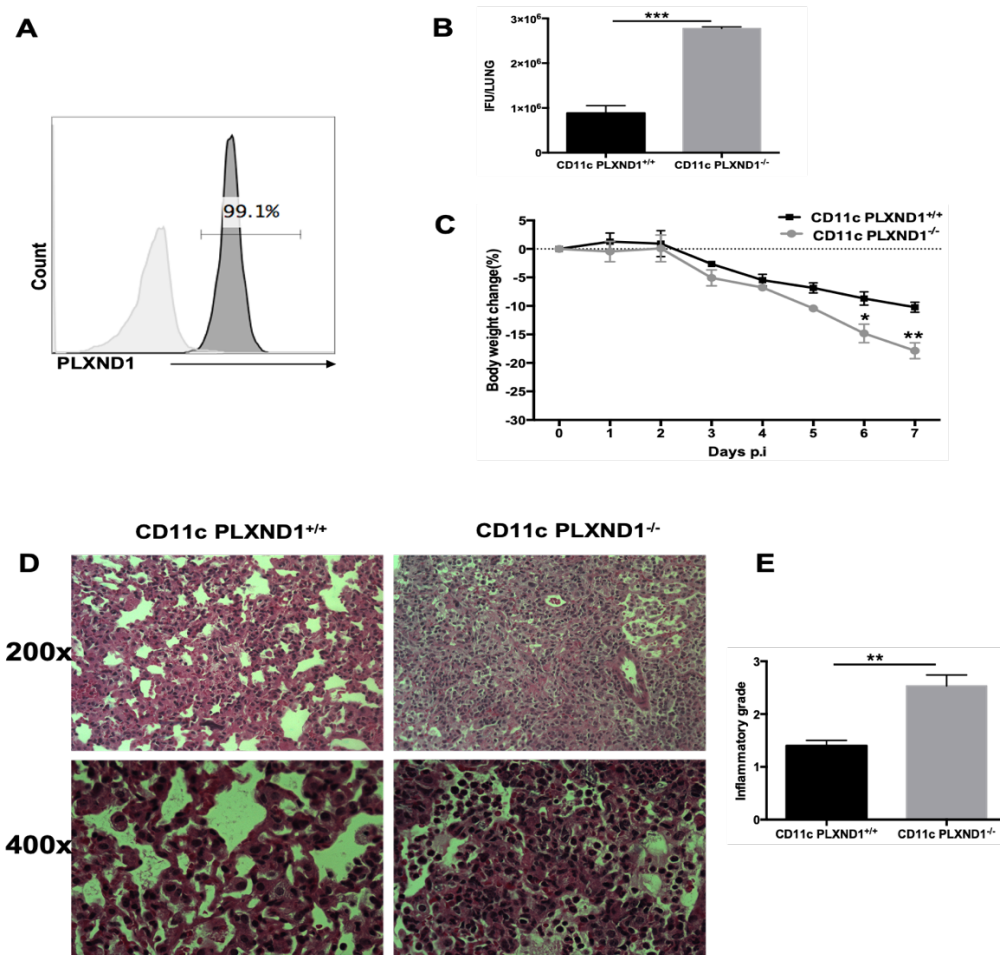

Figure 21: Deficiency of plexinD1 on dendritic cells leads to more severe disease and higher bacterial growth following *C. muridarum* lung infection. CD11c PLXND1$^{-/-}$and CD11c PLXND1$^{+/+}$ were infected with *Chlamydia muridarum* (1x10^3), sacrificed on day 7 post-infection, and analyzed for chlamydial load, bodyweight loss, and lung histopathological changes. Bodyweight changes were monitored daily after chlamydial infection. Each point represents the mean ± SD of three mice. A) expression of PLXND1 receptor on the surface of spleen DCs of WT mice after *Cm* infection. B) Higher bacterial load *in vivo* after *C. muridarum* infection in CD11c PLXND1$^{-/-}$ mice. Lungs isolated from mice were homogenized and analyzed for bacterial loads as described in Materials and Methods. C)The percentage of body weight changes in the two groups of mice. D)The lung tissue sections from both groups of mice were stained with H&E and examined with light microscopy. E) Lung pathology was analyzed semi-quantitatively by a blinded pathologist as detailed in Materials and Methods.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.3.2. Deficiency of plexinD1 on dendritic cells leads to lower Th1/Tc1 cytokine and IL-17 responses to *Cm* infection

IFN-γ producing T cells and Th17 cytokine response play a critical role in resistance and resolution of *Cm* lung infection [109, 126, 231]. To determine whether deficiency of plexinD1 on dendritic cells influences adaptive T cell immune responses, we analyzed cytokine response in mice after *Cm* infection. Lung cells isolated from both groups of mice were stained for intracellular IFN-γ production by CD4 and CD8 T cells. As shown in Fig. 22A-C, the frequency and the absolute number of IFN-γ producing CD4 and CD8 T cells were significantly lower in CD11c PLXND1$^{-/-}$ mice compared to CD11c PLXND1$^{+/+}$ mice following the infection. Moreover, lung cytokine analysis showed that the levels of IFN-γ and IL-17 was significantly lower in CD11c PLXND1$^{-/-}$ mice compared to that in CD11c PLXND1$^{+/+}$ mice (Fig. 22D). The data suggest that PLXND1 expression on dendritic cells has dramatic impact on T cell cytokine responses to *Cm* infection.

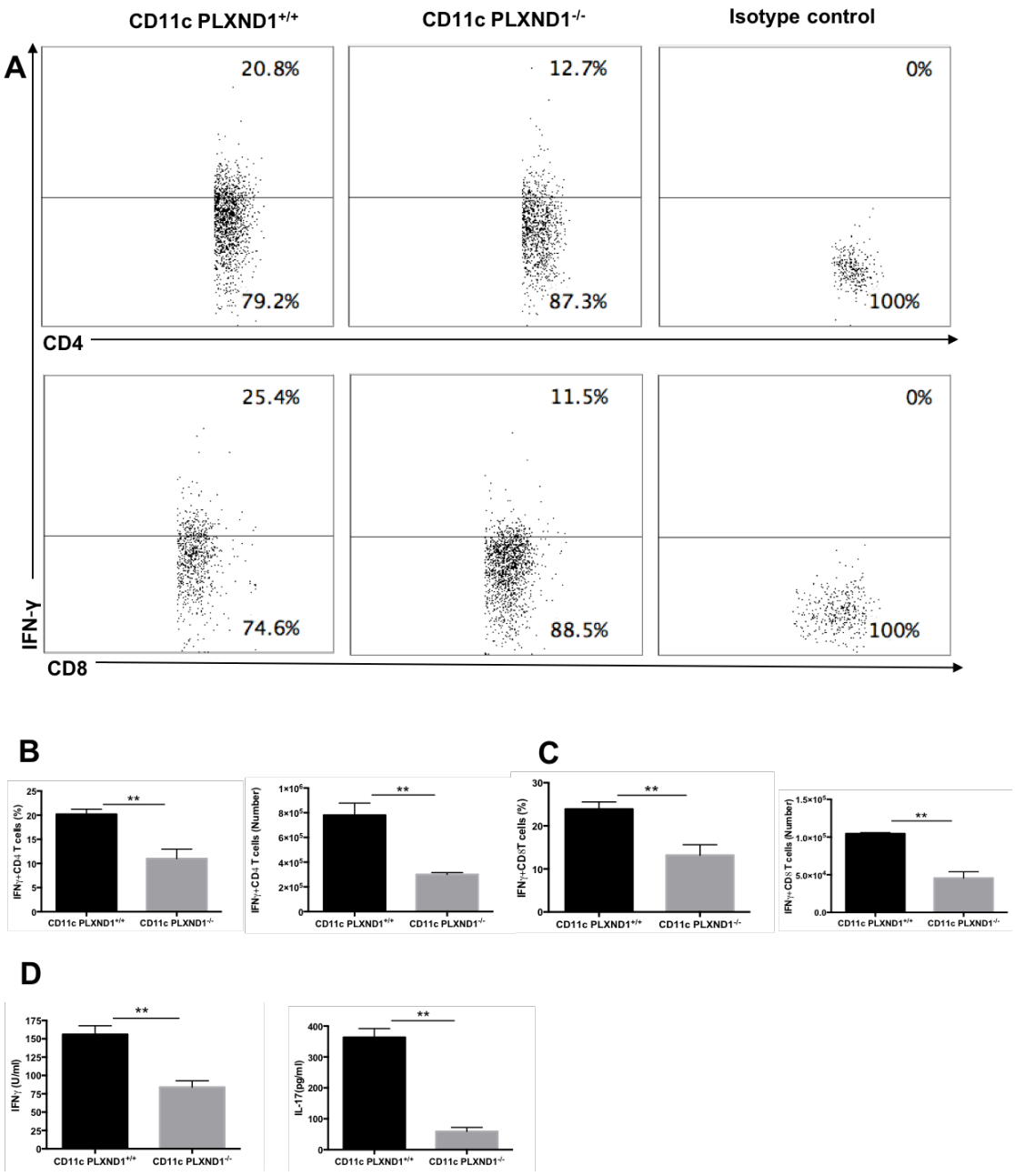

Figure 22: Reduction of Th1/Tc1 and IL-17 responses in CD11c PLXND1$^{-/-}$ mice following *Cm* lung infection. The lung cells isolated from *Cm* infected mice at day 7 post-infection were analyzed for cytokine production by intracellular cytokine staining, as described in Materials and Methods. Representative flow cytometric images (A) and the percentage and absolute number of IFN-γ producing CD4 T cells (B) and CD8 T cells (C). D) IFN-γ and IL-17 cytokine production in the lungs of *Cm* infected mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.3.3. PLXND1 deficiency on dendritic cells alters DC phenotype and cytokine production following *Cm* infection.

Since the surface marker expression and cytokine production are important for DC function, we next evaluated whether the deficiency of PLXND1 receptor on dendritic cells affects the surface expression of costimulatory and inhibitory molecules of these cells and their cytokine production. Compared to CD11c PLXND1$^{+/+}$ mice, CD11c PLXND1$^{-/-}$ mice showed lower percentages of co-stimulatory CD40, CD80, and CD86 molecules on the surface of DCs following the infection (Fig. 23). On the other hand, a higher percentage of PD-L1, an inhibitory surface molecule, expressing DCs were found in CD11c PLXND1$^{-/-}$ mice compared to CD11c PLXND1$^{+/+}$ mice (Fig. 23). No changes were observed in the percentages of MHC-II and PD-L2 expressing DC between the two groups. The cytokine profile of DCs plays a crucial role in skewing T-cell responses. IL-12 secretion by DCs promotes Th1 responses (IFN-γ), whereas IL-10 production by DCs impedes Th1 response. The intracellular cytokine analysis of lung DCs showed that IL-12 production by CD11c PLXND1$^{-/-}$ DCs was significantly lower than CD11c PLXND1$^{+/+}$ DCs (Fig. 24A & C). In contrast, the IL-10 production by CD11c PLXND1$^{-/-}$ DCs was higher than the PLXND1$^{+/+}$ DCs (Fig. 24A & B). Overall, these data support that PLXND1 mediated signaling on DCs can significantly modulate the phenotype and cytokine production of DCs, preferentially promoting IL-12 production but regulating IL-10 cytokine production.

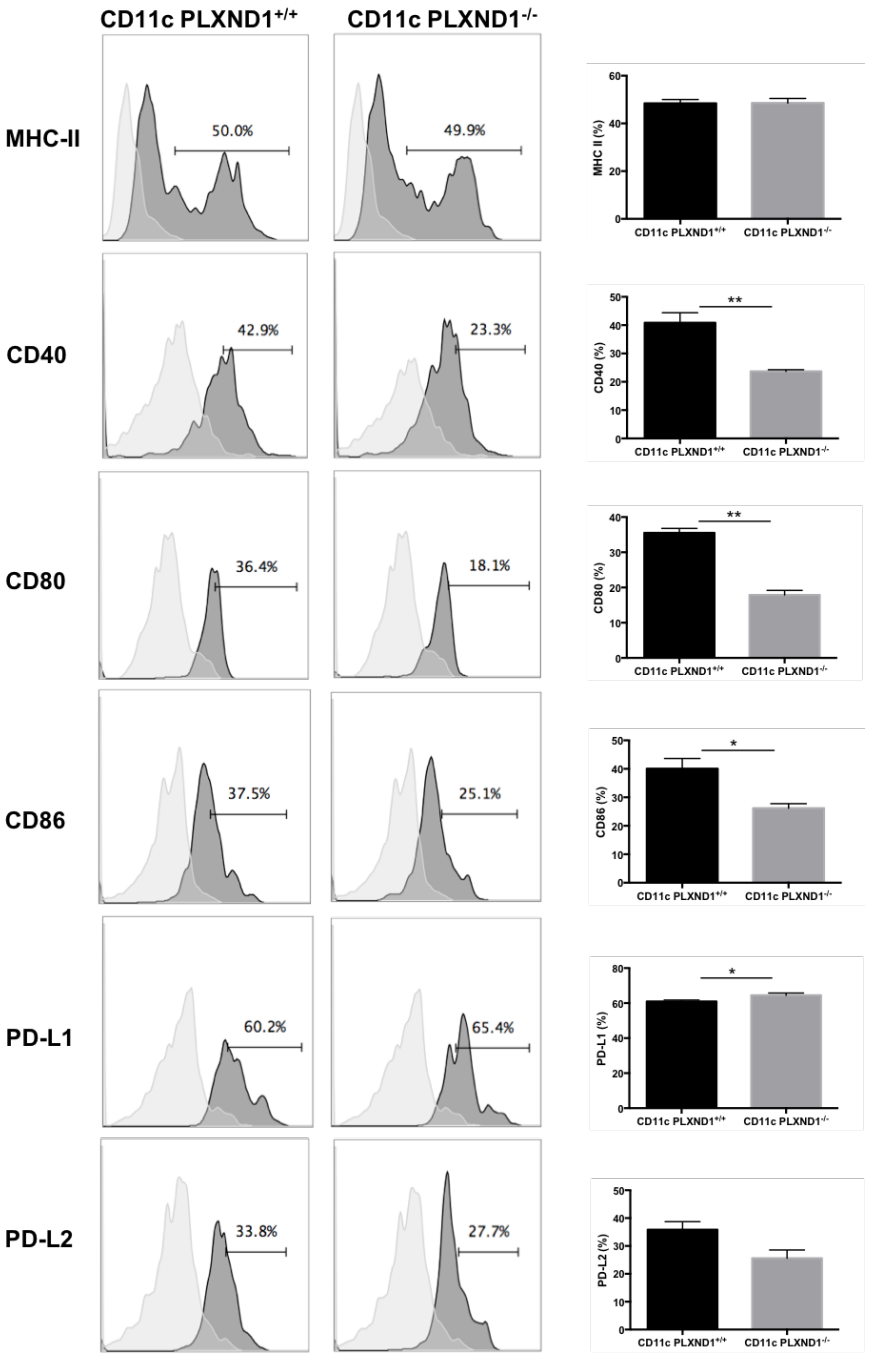

Figure 23: Altered surface phenotype of DCs of CD11c PLXND1$^{-/-}$ mice following *Cm* infection. CD11c PLXND1$^{+/+}$ and CD11c PLXND1$^{-/-}$ mice were infected with *chlamydia* and lung DCs were stained for surface markers and analyzed using flow cytometry. Expression of CD40, CD80,CD86, PD-L1 and PD-L2 on CD11c^{+} MHCII^{+} cells (dark shaded histograms) and isotype control (light shaded histograms) were shown. MHCII expression on CD11c^{+} cells and isotype control were recorded. The percentages of positive cells were indicated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

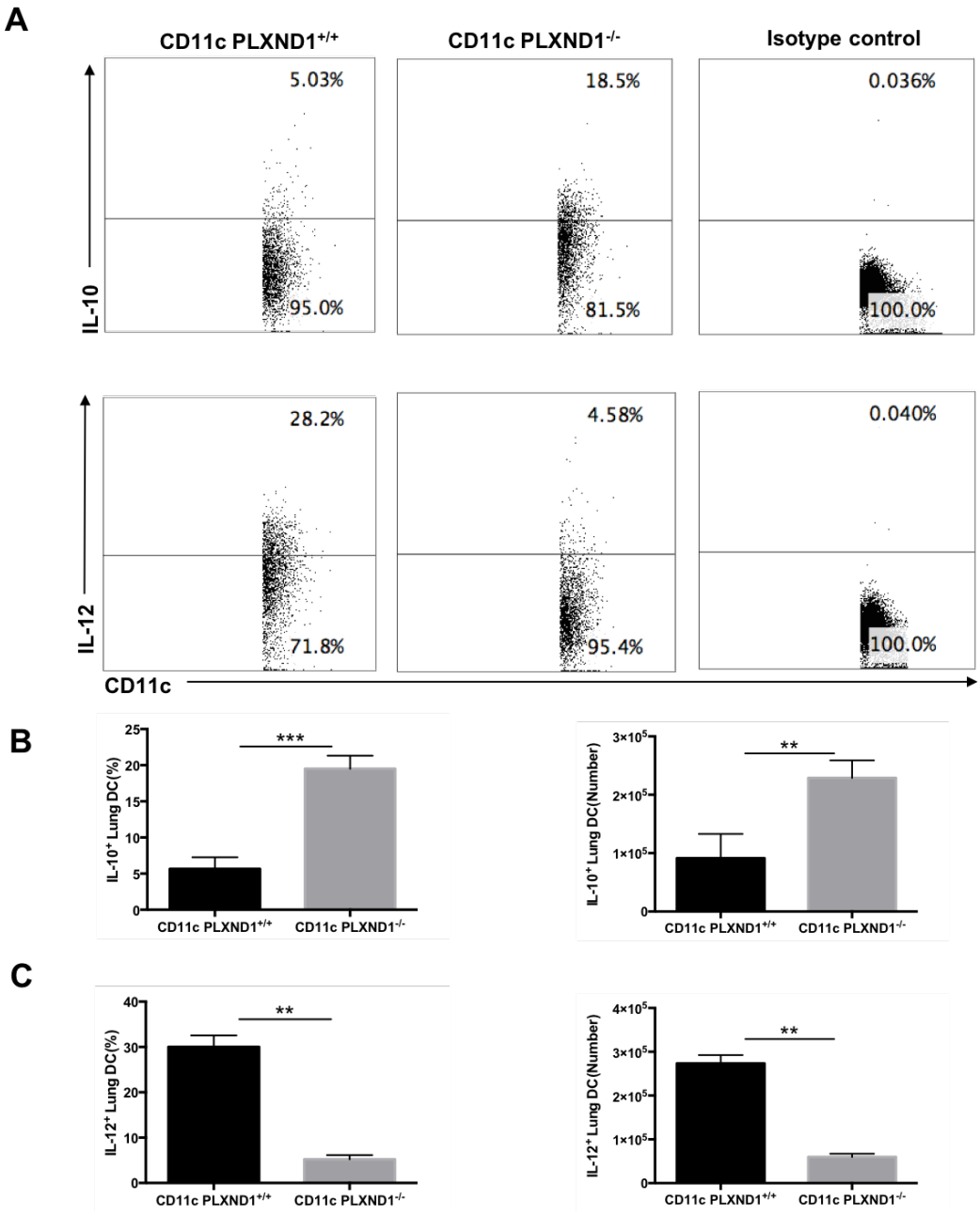

Figure 24. Analysis of cytokine profile of CD11c PLXND1$^{+/+}$ and CD11c PLXND1$^{-/-}$ DCs following *Cm* infection. Lungs harvested from both groups of mice after *Cm* infection were processed into single-cell suspensions and flow cytometric analysis was performed on gated CD11c^{+} F4/80^{-}MHC-II^{+} lung DCs. A) Representative dot plot showing percentages of IL-12 and IL-10 producing LDC from CD11c PLXND1$^{+/+}$ and CD11c PLXND1$^{-/-}$ mice. B)Percentages and numbers of IL-10 producing lung DC. C)Percentages and numbers of IL-12 producing lung DC mean ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.

4.3.4. PLXND1 deficiency on DC impacts the recruitment of Th1 promoting CD103^{+} lung DC subset.

Previous studies showed that *Cm* infection induces lung DC subsets with different abilities to enhance protective immunity. CD103^{+} lung DC subset induces stronger Th1/Th17 response to *Cm* infection than CD11b^{+} lung DC subset . We therefore analyzed the effect of DC plexin D1 expression on the DC subsets in the lung following *Cm* infection. We observed that the proportion and absolute number of CD103^{+} lung DC subset is reduced in CD11c PLXND1$^{-/-}$ mice compared to CD11c PLXND1$^{+/+}$ mice (Fig. 25A & B), while the proportion and absolute numbers of lung CD11b^{+} DC subset did not change significantly between CD11c PLXND1$^{-/-}$ mice and CD11c PLXND1$^{+/+}$ mice (Fig. 25A & C). Altogether, the data suggest that plexin D1 expression on DC has a critical impact on the expansion/recruitment Th1/Th17 promoting CD103+ DC subsets in the lung following chlamydial infection.

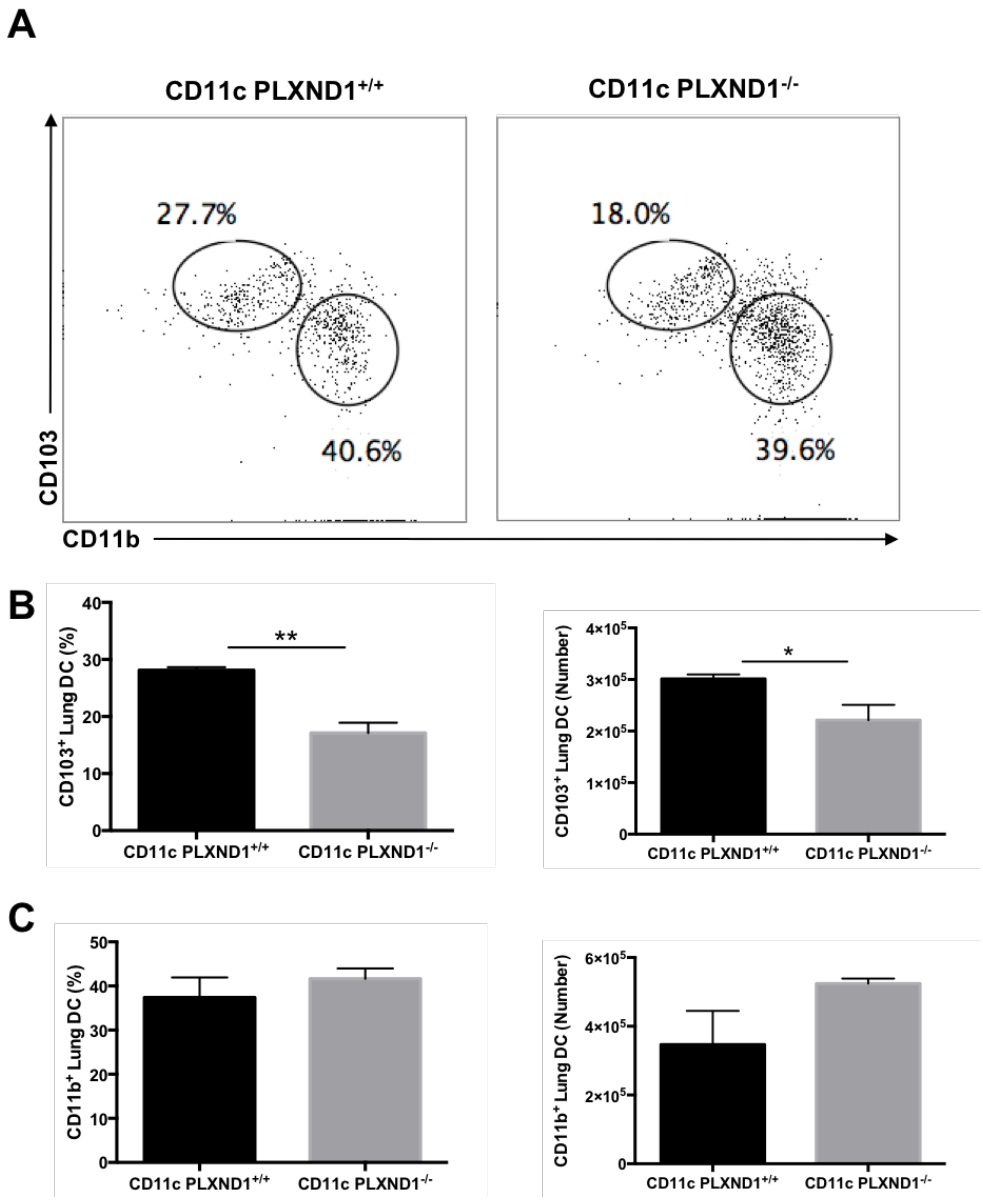

Figure 25: PLXND1deficiency on DC impact recruitment of CD103$^+$ lung DC subset after *Cm* infection. Mice were intranasally inoculated with 1x10^3 IFUs of *Cm*. Lung cells were collected from CD11c PLXND1$^{-/-}$ mice and CD11c PLXND1$^{+/+}$ mice, stained for surface markers and analyzed using flow cytometry. A) Representative flow cytometric images of CD103$^+$ and CD11b$^+$lung DC subset. B) The percentages and number of CD103$^+$ lung DC subset. C) The percentages and number of CD11b$^+$lung DC subset. *$p < 0.05$, **$p < 0.01$.

4.3.5. Adoptive transfer of DCs from CD11c PLXND1$^{+/+}$ mice provided better protection against chlamydial infection than DCs from CD11c PLXND1$^{-/-}$mice.

To analyze the function of plexin D1 in modulating DC function *in vivo*, we conducted adoptive transfer experiments with DCs from *C. muridarum*-infected CD11c PLXND1$^{+/+}$ mice (PLXND1^{+}DC) and CD11c PLXND1$^{-/-}$mice (PLXND1^{-}DC). Bone marrow-derived dendritic cells (BMDCs) isolated from both groups of mice were infected with *Cm* and then intravenously transferred to naïve syngeneic recipients. The recipient mice were subsequently challenged with *Cm* and sacrificed at day 7 post-challenge. Mice group that received PBS with the same challenge infection were used as controls. We found that the transfer of PLXND1^{+}DC enhanced protection to *Cm* infection with faster recovery of body weight loss (Fig. 26A) and lower chlamydial loads (Fig. 26B) in the lung of the recipients than the PBS treated mice. However, the mice receiving PLXND1^{-}DC did not show enhanced protection because their body weight loss and chlamydial loads were similar to PBS treated mice. Moreover, the histopathologic analysis showed mild pathologic changes in the lungs of PLXND1^{+}DC received mice compared with the other groups (Fig. 26C). These results more directly confirm the critical protective role of plexin D1 expression on DCs in host defense against *Cm* infection *in vivo*.

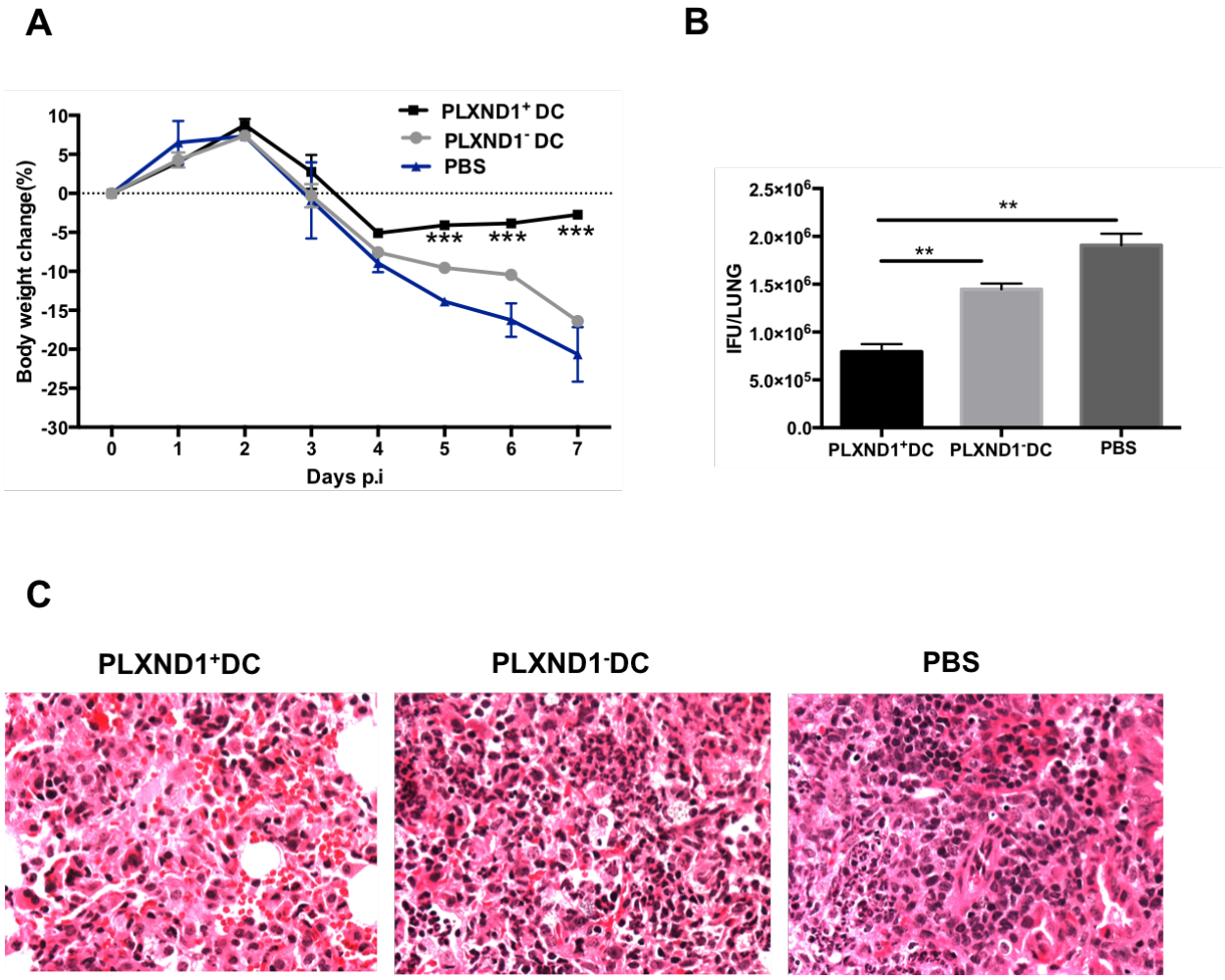

Figure 26. Adoptive transfer of PlexinD1^{+}DC and PlexinD1^{-}DC to evaluate their function *in vivo*. PlexinD1^{+}DC and PlexinD1^{-}DC were transferred intravenously to naïve recipient mice . The mice were challenged with *Cm* and sacrificed at day 7 to analyze bacterial load, histopathological changes and T cell cytokine pattern in the lung. A) Bodyweight loss B) Chlamydial growth in the lung. C) Lung histopathology. The data represent one of two similar experiments. ∗ $p < 0.05$, **$p < 0.01$(one-way Anova).

4.3.6. PLXND1^{+}DC induced stronger Th1/Th17 immunity compared with PLXND1^{-}DC

To understand the mechanisms by which adoptively transferred PLXND1^{+}DC enhances protection, we analyzed the cytokine responses in the lungs of the recipient mice following *Cm* challenge infection. The intracellular cytokine analysis showed higher numbers of IFN-γ (Fig. 27A & B) and IL-17 (Fig. 28A & B) producing CD4^{+} T cells in the lungs of PLXND1^{+}DC recipients than that of PLXND1^{-}DC. The transfer of PLXND1^{-}DC also showed increase of IFN-γ (Fig. 27A & B) and IL-17 (Fig. 28A & B) producing cells but the increase was significantly lower than the transfer of PLXND1^{+}DC. Consistently, local cytokine production in the lungs of PLXND1^{+}DC recipients also revealed a stronger bias toward Th1/Th17 cytokine pattern with higher levels of IFN-γ and IL-17 (Fig. 27C & 28C) compared to that of PLXND1^{-}DC recipients and PBS controls. Together, these observations from the adoptive transfer experiments confirmed the critical role of PLXND1 mediated signaling on DCs to enhance the Th1/Th17 responses against chlamydial infection.

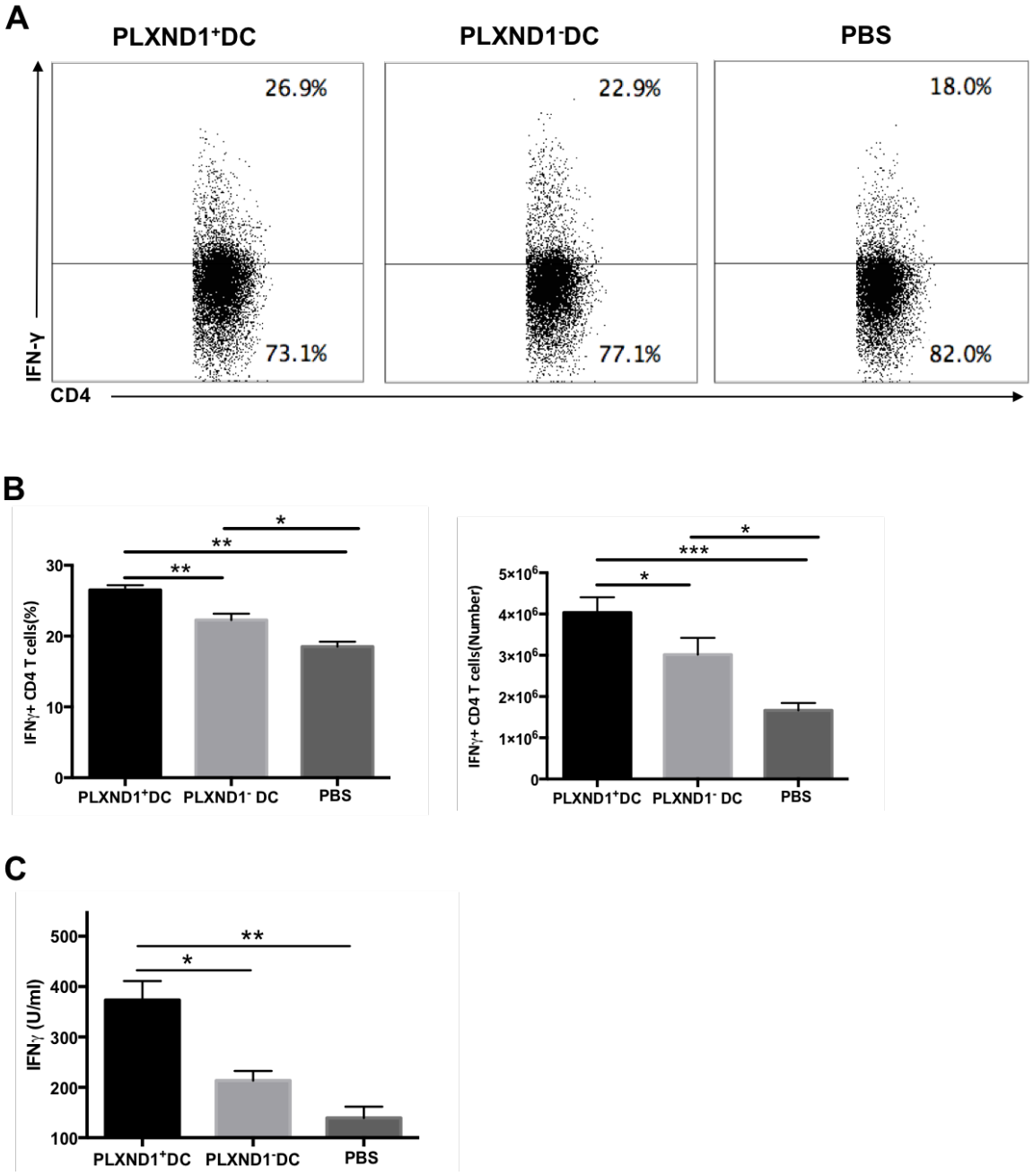

Figure 27. Impact of transfer of PLXND1^{+}DC and **PLXND1^{-}DC on Th1 response in the lungs of recipient mice.** PlexinD1^{+}DC and PlexinD1^{-}DC were transferred intravenously to naïve recipient mice and sacrificed at day 7 p.i. The cytokine production pattern in the T cells was analyzed by intracellular cytokine staining and flow cytometry. A) Representative flow cytometric images showing the percentages of IFNγ $^{+}$CD4 T cells. B) Graphical summary of the percentages and absolute numbers of IFNγ $^{+}$CD4 T cells. C) IFN γ cytokine production in the lungs of recipient mice.

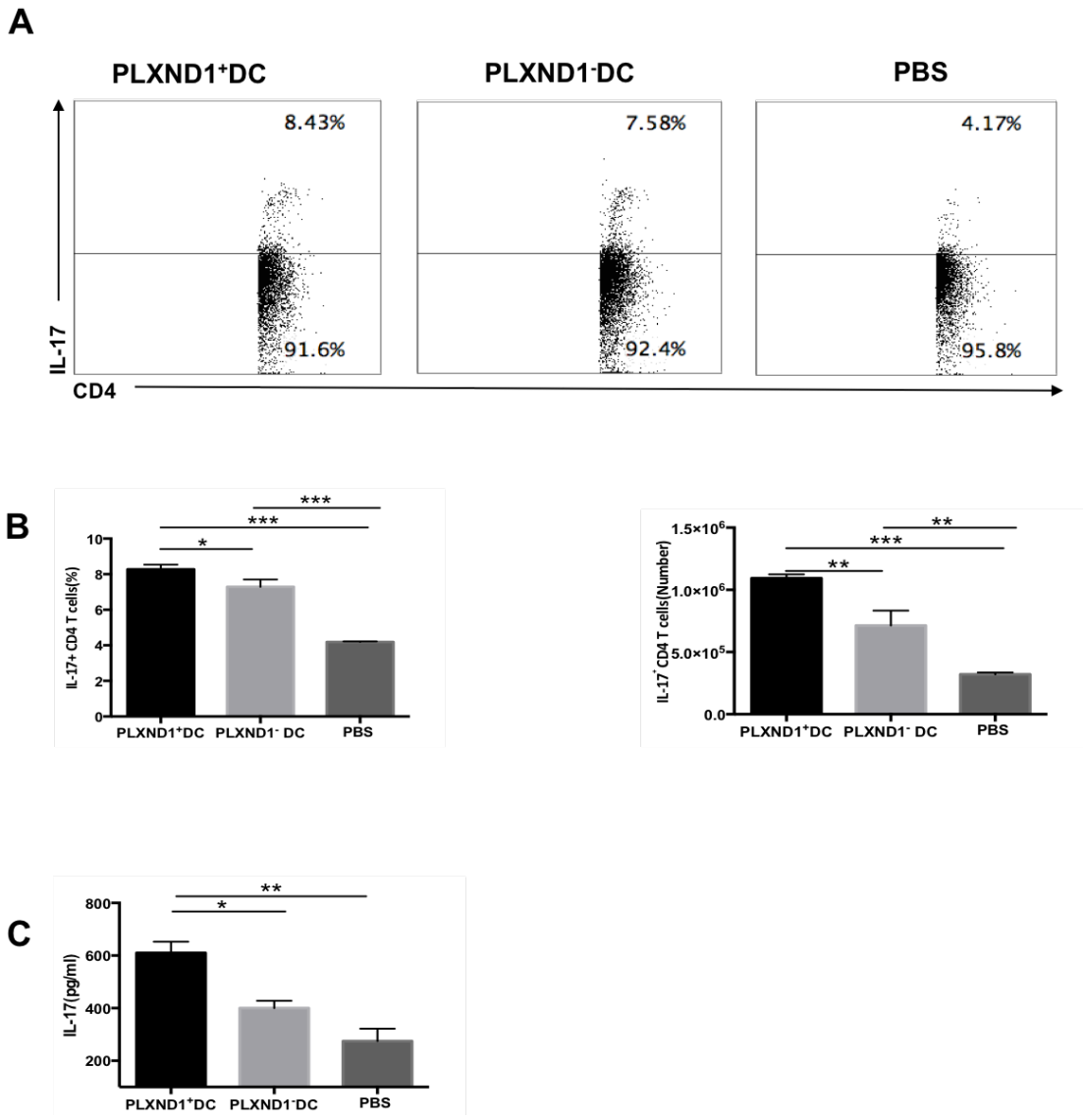

Figure 28. Impact of transfer of PLXND1^{+}DC and PLXND1^{-}DC on Th17 response in the lungs of recipient mice. PlexinD1^{+}DC and PlexinD1^{-}DC were transferred intravenously to naïve recipient mice and sacrificed at day 7 p.i. The cytokine production pattern in the T cells was analyzed by intracellular cytokine staining and flow cytometry. A) Representative flow cytometric images showing the percentages of IL-17^{+}CD4 T cells. B) Graphical summary of the percentages and absolute numbers of IL-17^{+}CD4 T cells. C) IL-17 cytokine production in the lungs of recipient mice.

4.3.7. Discussion

A large body of evidence suggests that Sema3E-PlexinD1 signaling pathway plays a critical role in different phases of the immune response [217, 259, 260]. However, the involvement of Sema3E-PlexinD1 signaling in immunity to infection is not investigated yet. Our studies described in the above sections have shown that Sema3E is required for the protective immune response to chlamydial infection. In particular, the above study has been demonstrated that Sema3E can modulate dendritic cell function to enhance Th1/Th17 response to chlamydial infection. In this study, we further investigated the impact of Sema3E receptor, PLXND1 deficiency on dendritic cells function for immunity to chlamydial infection to confirm the importance of Sema3E-PlexinD1 signaling in DC during this process. Our results revealed that PLXND1 deficiency on dendritic cells led to increased susceptibility/severity to *Cm* infection with higher bacterial load, greater body weight loss, and more severe lung pathology. Moreover, we observed lower IFN γ production by CD4 and CD8 T cells and lower IL-17 cytokine production by CD4 T cells in CD11c PLXND1$^{-/-}$ mice compared to CD11c PLXND1$^{+/+}$ mice. More importantly, we confirmed the functional ability of PLXND1^{+}DC to promote protective immunity to chlamydial infection *in vivo.* Adoptive transfer of PLXND1^{+}DCs provided more robust protection with enhanced Th1/Th17 response against *Cm* infection than PLXND1^{-}DCs. Altogether, our study illustrates the critical role of PLXND1 on DCS for a protective immune response to lung *Cm* infection by modulating the function of DC *in vivo.*

A major finding in this study is the changes in the surface molecule expression and cytokine production of DCs in the absence of PLXND1 receptor and their association with T cell cytokine responses after *Cm* infection. DCs from CD11c PLXND1$^{-/-}$ mice showed lower expression of co-stimulatory molecules but higher expression of inhibitory molecule, PD-L1. Upregulation of co-

stimulatory molecules such as CD40, CD80 and CD86 is required for the development of effective T cell response to chlamydial infection [261]. A critical role of Th1/Th17 response in protective immunity to chlamydial infection is evidenced in previous studies [109, 126, 231]. Reduction in IFN γ and IL-17 cytokine production is also observed in Sema3E KO mice after *Cm* infection, suggesting the essential function of the Sema3E-PLXND1 axis in protective immunity to *Chlamydia.* Recent studies in Yang lab also evidenced the involvement of PD1/PD-L1 signaling in DC for the induction of Th1/Th17 responses after chlamydial infection [83]. Lower co-stimulatory molecules and higher PD-LI expression on DCs of CD11c PLXND1$^{-/-}$ mice suggest reduced Th1/Th17 response observed in this mouse model.

Also, DCs from CD11c PLXND1$^{-/-}$ mice produced significantly lower levels of Th1 promoting cytokine IL-12 but higher levels of IL-10, which is detrimental for immunity to chlamydial infection. Cytokine production by PLXND1 deficient DCs has been reported under steady-state and upon LPS stimulation *in vitro* [238]. Notably, in contrast to our observation, PLXND1deficient BMDCs produced higher levels of IL-12/IL-23p40 than wild-type cells under steady-state and upon LPS stimulation. The reason for the observed difference remains unclear, but in general, the dynamic nature of DCs is better mimicked *in vivo* in a real pathogen than *in vitro* conditions. Moreover, PLXND1 ligands that bind to PLXND1 receptors during *in vitro* and *in vivo* conditions under different infectious/ inflammatory stimuli may differ. Therefore, our data provide new insight into the relationship between SEMA-PLXND1 signaling and DC cytokine responses. Our studies showed that Sema3E is released during chlamydial infection in the lung. Sema3E can act as a ligand to the PLXND1 receptor expressed on DC and contribute to higher IL-12 cytokine production by DCs from *Cm* infected CD11c PLXND1$^{+/+}$ mice. Lower IL-12 cytokine production by *Cm* infected Sema3E KO DC further confirms this observation. Moreover, we found that

PLXND1on DC is required for the inhibition of IL-10 cytokine production. To our knowledge, the data are the first to show the critical function of the PLXND1 receptor on DC for modulating DC cytokine response for protective responses to infection.

Another significant finding is the role of SEMA-PLXND1 signaling in modulating lung DC subsets. The data showed that SEMA-PLXND1 signaling has promoting effect on $CD103^+$ lung DC expansion in the infection site. We observed that the frequency and numbers of $CD103^+$ pulmonary cDCs was reduced in the CD11c $PLXND1^{-/-}$ mice compared to CD11c $PLXND1^{+/+}$ mice. However, the numbers of lung $CD11b^+$DC subset remain unchanged between the two groups. $CD103^+$ pulmonary cDCs were reported as potent inducers of Th1/Th17 response to *Cm* infection [83]. Therefore, lower Th1/IL-17 responses observed in CD11c $PLXND1^{-/-}$ mice after *Cm* infection can also be due to lower numbers of $CD103^+$ lung DC subsets. Similarly, we found lower numbers of $CD103^+$ pulmonary cDCs in Sema3E KO mice than WT mice after chlamydial infection. Together, these findings suggest that Sema3E-PLXND1 signaling on DC preferentially modulates the recruitment of the Th1/Th17 inducing lung DC subset to induce protective immunity to chlamydial infection.

Few studies identified the relevance of plexin D1 in other immune cells. PlexinD1 expression on B cells is required for germinal center reaction during T-dependent immune activation and secondary humoral immune response [262]. Migration studies revealed that activated B cell migration to chemokine CCL19 was reduced in PLXND1 deficient B cells compared to WT B cells [262]. PlexinD1 is also expressed in double-positive thymocytes, endothelial cells, NK cells, and macrophages [193, 204, 205, 217]. PlexinD1 expression on thymocytes is a critical factor needed for the directional migration of thymocytes to the medulla [217]. Moreover, PlexinD1 signaling on macrophages is involved in regulating the inflammatory response to LPS [204]. These

studies show that PlexinD1 exhibit distinct functions in various immune cells based on cell development or immune activation. Future studies are required to understand the function of plexinD1 in other immune cells for immunity to chlamydial/bacterial infections.

4.4 Exogenous Semaphorin 3E treatment protect against chlamydial lung infection in mice

Rationale: Since the above-described study suggests a protective role of Sema3E in chlamydial infection based on data from Sema3E or its receptor-deficient mice, we further planned to test its function using a supplementary approach. On the one hand, this is to confirm the finding from deficient mice, and on the other hand, to examine whether Sema3E has a therapeutic function to chlamydial infection *in vivo.* Previous studies by us and others have suggested that IFN-γ production by T cells plays a critical function in resolving chlamydial infection [109, 126, 231]. In addition, IL-17 production by T cells is also found to provide protection to chlamydial infection in the lungs [40, 109, 263].

4.4.1. Semaphorin 3E treatment provides protection to chlamydial lung infection

To answer the question if the treatment of Sema3E KO mice with exogenous recombinant Sema3E would correct the deficiency of the mice in defense against chlamydial infection; and if the supplement of Sema3E to WT would enhance their capacity to fight against the infection, we treated WT mice and Sema3E KO mice intranasally with either Sema3E-Fc or saline-Fc, two hours before *Cm* infection and day 1 to day 6 consecutively after infection (Fig. 29A). The results showed that Sema3E-Fc treatment significantly reduced the severity of disease of Sema3E KO mice in that the weight loss and bacterial loads of these mice were comparable to the WT mice following *Cm* lung infection. Moreover, the supplement of Sema3E-Fc to WT mice further enhanced the protection in WT mice. In both WT and Sema3E KO mice, we observed a significant decrease in chlamydial load in the mice that received Sema3E-Fc compared to those that received saline-Fc alone (Fig. 29B & C). In addition, lung histological analysis showed that mice treated with Sema3E-Fc exhibited a reduced cellular infiltration compared to saline-Fc–treated controls (Fig. 29D & E). Similarly, it is found that Sema3E-Fc treatment reduced body weight loss in both WT

Balb/c mice as well as Sema3E KO mice compared to saline-Fc treated mice after *Cm* infection (Fig. 29F & G). These observations suggest that Sema3E treatment can correct the deficiency of the Sema3E KO mice in defense against chlamydial infection, and the supplement of Sema3E to WT mice can provide further help for host defense against the infection.

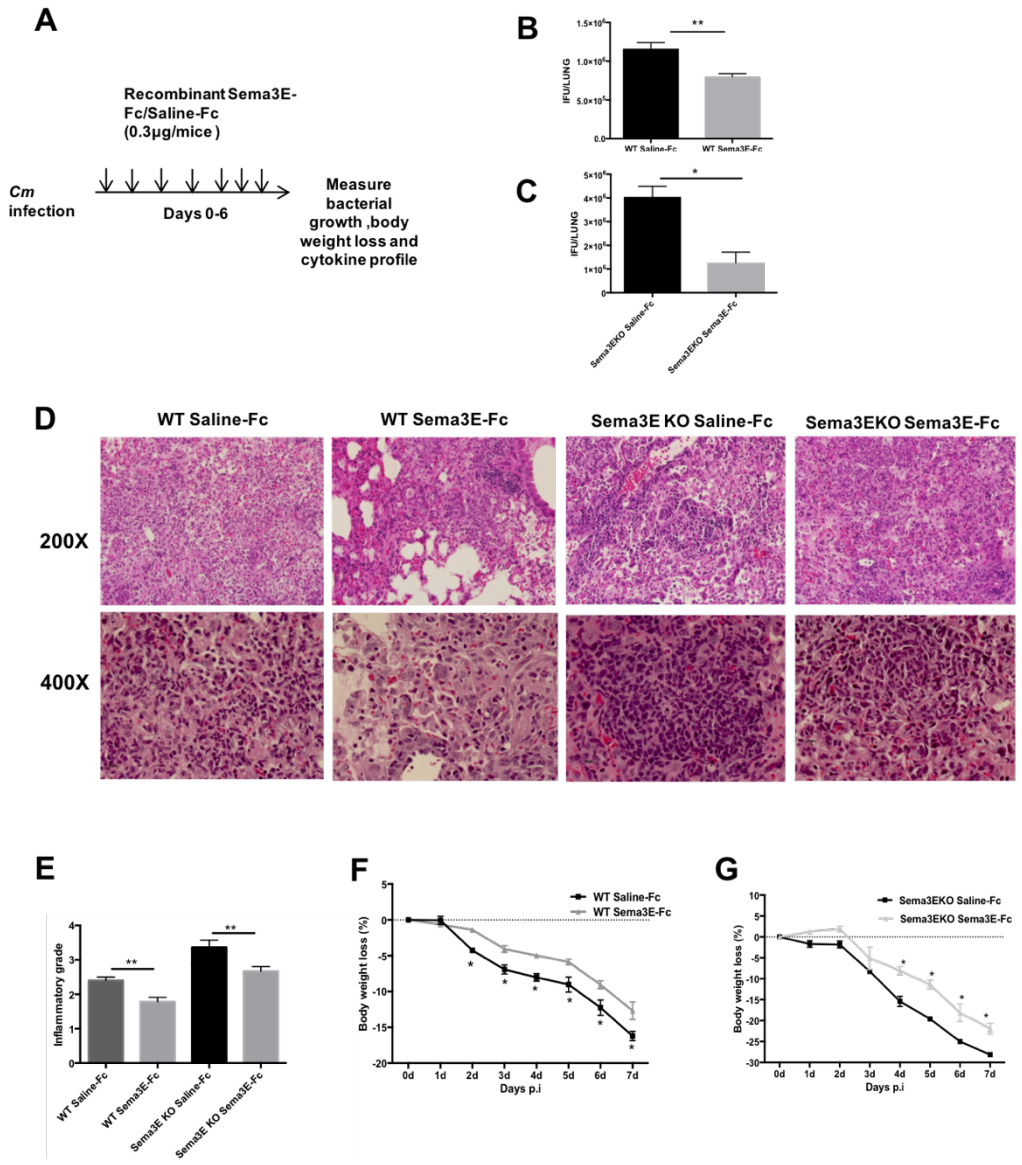

Figure 29: Semaphorin 3E treatment enhances the ability to control *C. muridarum* lung infection. A) Exogenous recombinant Sema3E-Fc was administered intranasally 2 hours before *Cm* infection and day 1 to day 6 after infection. B) Chlamydial growth *in vivo* after Sema3E-Fc or saline-Fc treatment of WT Balb/c mice. C) Chlamydial growth *in vivo* after Sema3E-Fc or saline-Fc treatment of Sema3E KO mice. D) The pulmonary pathology. Lungs from Sema3E-Fc or saline-treated mice were sectioned, and the lung pathology was examined by H&E staining and analyzed in (×200 and ×400x) magnification under light microscopy. E) Lung inflammation was analyzed semi-quantitatively by a blinded pathologist as detailed in Materials and Methods. Mice were monitored daily for body weight changes. Lower body weight loss after chlamydial infection in Sema3E-Fc treated WT mice (F) and Sema3E KO mice (G). Each point represents the mean ± SD of three mice. One representative experiment of three independent experiments with similar results is shown.

4.4.2. Semaphorin 3E treatment promotes Th1/Th17 responses while reduces IL-4/IL-10 responses after chlamydial infection

Next, we investigated the effect of Sema3E-Fc treatment on cytokine response of WT mice after *Cm* lung infection. We first examined cytokine response in the local lung tissues. Sema3E-Fc–treated mice showed a significant increase in IFN-γ and IL-17 cytokines in the lungs compared to the mice given only saline-Fc (Fig. 30A). On the other hand, IL-4 and IL-10 cytokines were reduced in the lungs of Sema3E-Fc–treated mice (Fig. 30A). To further understand the effect of Sema3E treatment on T cell immune response, we studied the antigen-driven cytokine response by ex vivo splenocytes isolated from *Cm* infected mice. Sema3E-Fc–treatment increased antigen-driven IFN γ and IL-17 cytokines, whereas IL-4 and IL-10 cytokines were reduced in the splenocytes culture compared to saline-Fc treated mice (Fig. 30B). These results suggest that the Sema3E modulates Th1/Th17 and Th2 cytokine responses differently by preferentially promoting Th1/Th17 while reducing Th2/IL-10 responses after chlamydial infection.

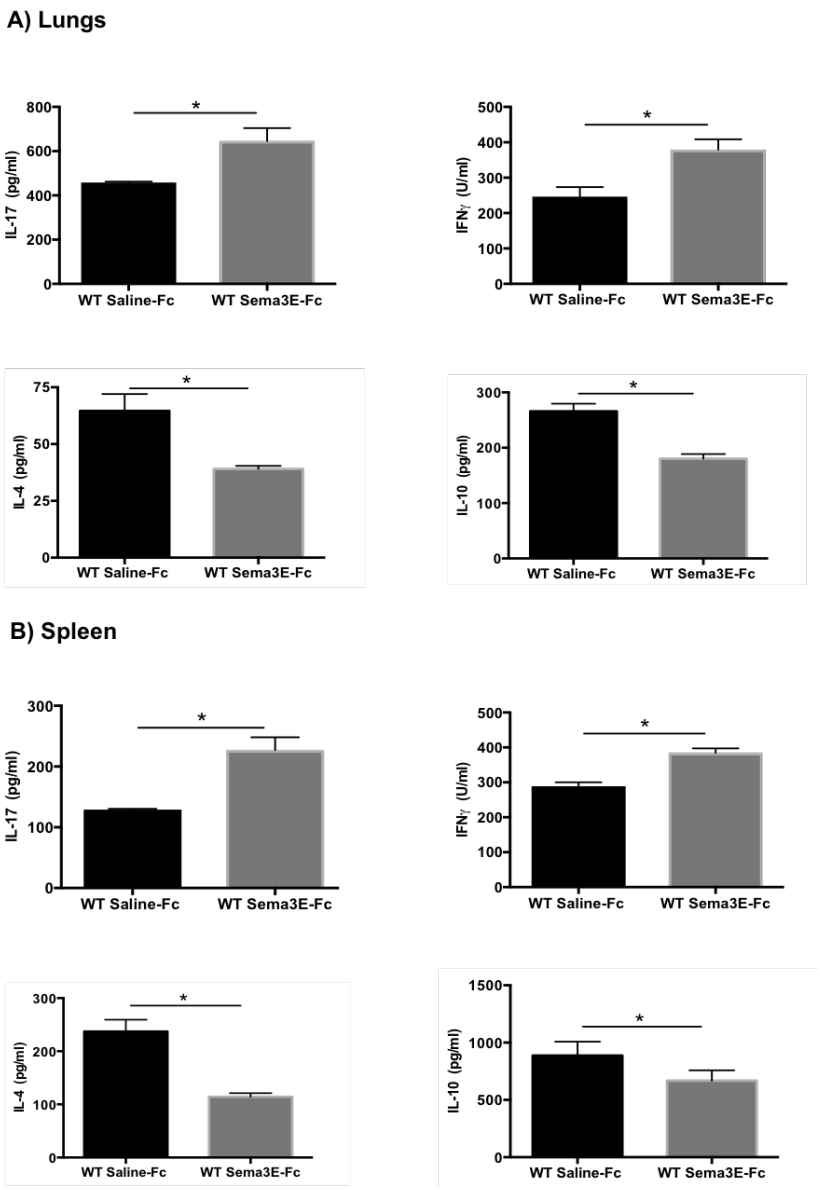

Figure 30: Semaphorin 3E treatment modulates antigen-driven cytokine responses after chlamydial infection. Exogenous recombinant Sema3E-Fc was administered intranasally 2 hours before *Cm* infection ($1x10^3$ IFUs of *Cm*) and day 1 to day 6 after infection. Mice were sacrificed at day 7 post-infection. The spleen cells were cultured with UV-killed elementary bodies (UVEB). IFN-γ IL-17, IL-10, and IL-4 levels in 72-h culture supernatants were determined by ELISA. A) IFN-γ, IL-17, IL-10, and IL-4 cytokine production in lung cells at day 7 p.i. B) IFN-γ, IL-17, IL-10, and IL-4 cytokine production in the spleen at day 7 p.i. Data are shown as mean ± SD (n = 3) and represent one of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.4.3. Sema3E treatment enhances Th1/Tc1 and Th17 cytokine response in the lung after chlamydial infection

To further analyze specific T cell cytokine responses, intracellular cytokine analysis of lung CD4+ and CD8+ T cells was performed by flow cytometry. The intracellular cytokine analysis showed a higher number of IFN-γ producing $CD4^+$ T cells, and $CD8^+$ T cells in the lung of Sema3E-Fc treated mice compared to saline-Fc treated mice (Fig. 31A&B). Moreover, a more significant number of IL-17^+ T cells were found in the lungs of Sema3E-Fc treated mice than saline-Fc treated mice after *Cm* infection (Fig. 32 A&B). Together, these data confirm that Sema3E can promote Th1/Th17 response during *Cm* infection *in vivo.*

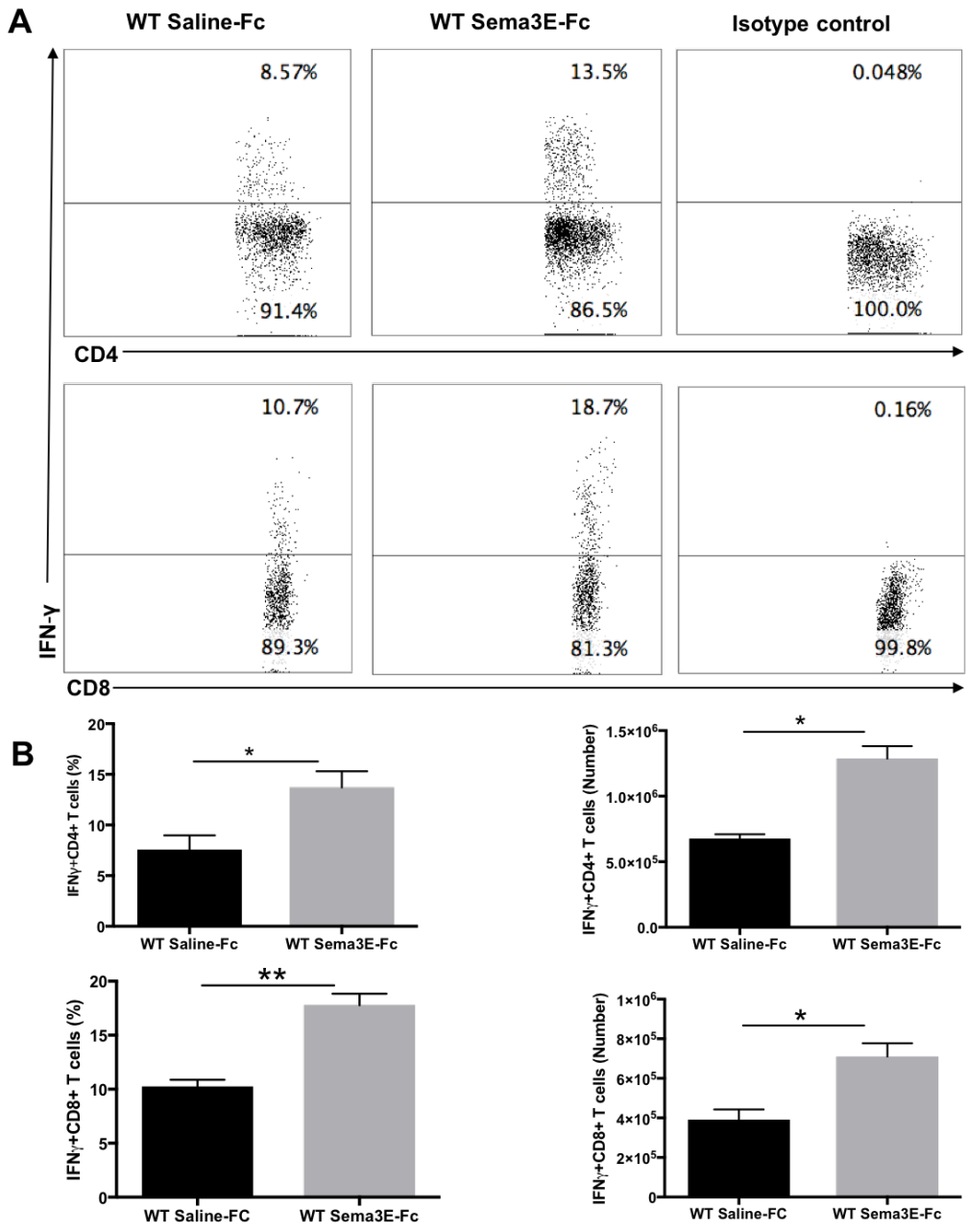

Figure 31: Higher IFN-γ production by CD4 and CD8 T cells after Sema3E-Fc treatment of *Cm* infected WT mice. $CD4^+$ and $CD8^+$ T cells isolated from the lungs of *Cm* infected mice at day 7 post-infection were stained intracellularly for IFN-γ. A) Representative flow cytometric images (top) and summary of flow cytometric analysis (B) to show the percentage and absolute number (bottom) of IFN-γ producing CD4 and CD8 T cells. Data are shown as mean ± SD (n = 3) and represent one of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

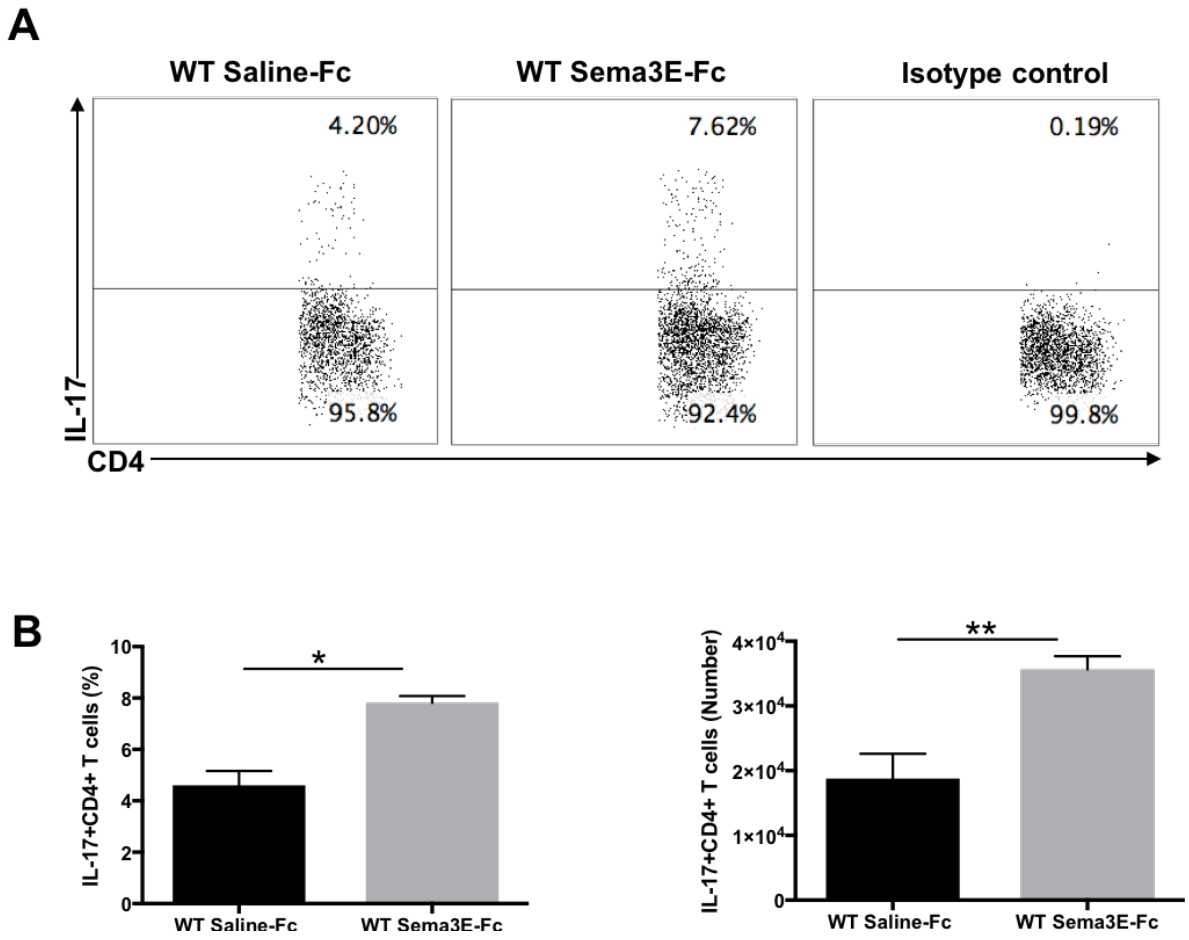

Figure 32: Higher IL-17 production by CD4 T cells after Sema3E-Fc treatment of *Cm* infected WT mice. $CD4^+$ T cells isolated from the lungs of *Cm* infected mice at day 7 post-infection were stained intracellularly for IL-17. A) Representative flow cytometric images (top) and summary of flow cytometric analysis (B) to show the percentage and absolute number (bottom) of IL-17 producing $CD4^+$T cells. Data are shown as mean ± SD (n = 3) and represent one of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.4.4. Sema3E treatment reduced $CD4^+CD25^+FoxP3^+$ regulatory T cell in the lung after *Cm* infection

Since we observed lower IL-10 levels in the spleen and lung of Sema3E-Fc treated mice and Treg is one of the main sources of this cytokine, we next examined $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in the lungs of WT mice after Sema3E-Fc treatment. The proportion and number of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells was significantly lower in Sema3E-Fc treated mice compared to saline-Fc treated mice (Fig. 33A&B). The results show an inhibitory role of Sema3E on Treg responses after *Cm* infection.

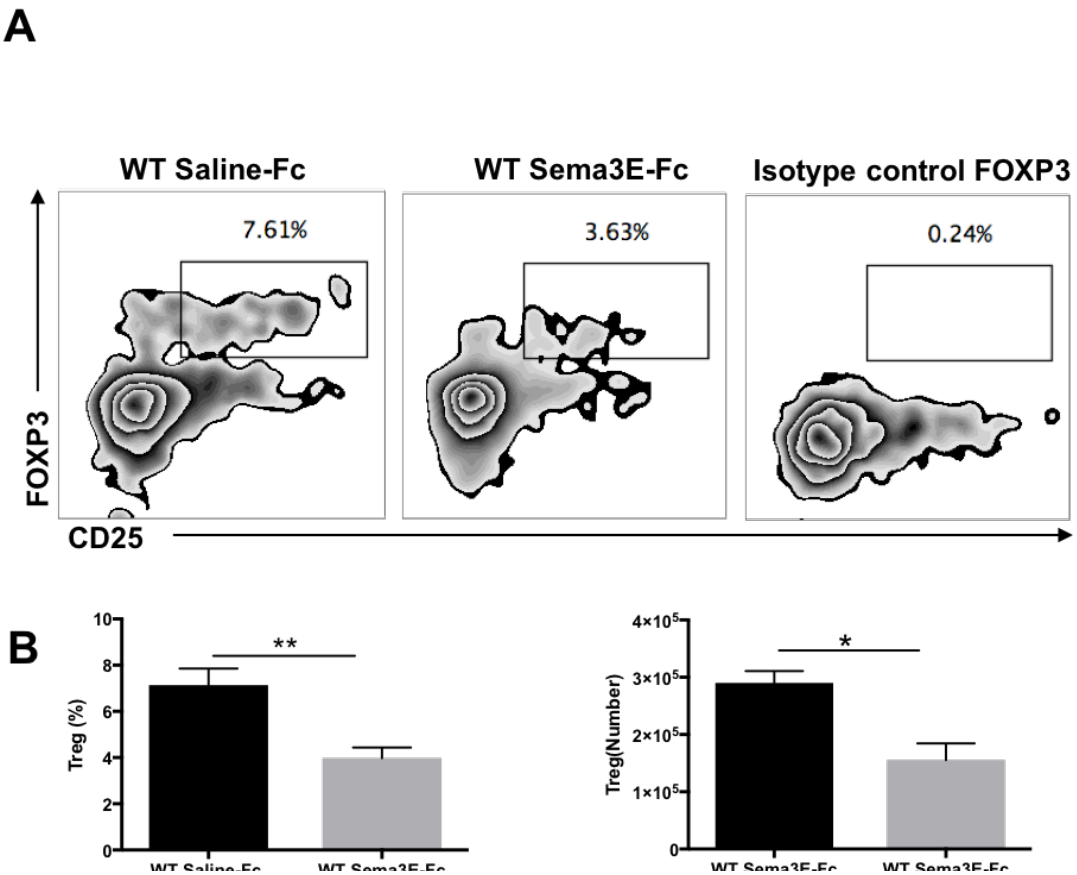

Figure 33: Lower Treg cells in the lungs of Sema3E-Fc treated WT mice following *Cm* infection. Mice were intranasally inoculated with $1x10^3$ IFUs of *Cm*. Lung cells were collected from Sema3E-Fc, or saline-Fc treated mice at day 7 post-infection and stained for expression of CD3, CD4, and CD25. FoxP3 intranuclear staining was done on T cells to analyze Treg cells as described in Materials and Methods. A) Representative flow cytometric images of Treg cells in the lungs. B) The percentages and number of Treg cells in the lung. Data are shown as mean ± SD (n = 3) and represent one of three independent experiments with similar results. *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$.

4.4.5. Sema3E treatment enhanced recruitment of Th1 promoting CD103$^+$ lung and CD8α$^+$ spleen DC subsets.

Previous studies showed that different DC subsets exhibit variable capacity in inducing a protective immune response to *Cm* infection. Notably, CD8α$^+$ spleen DC subset and CD103$^+$ lung DC subset are reportedly more potent in inducing Th1 response to *Cm* infection than CD8α$^-$ spleen DC subset and CD11b$^+$ lung DC subset [82, 83]. We, therefore, analyzed these DC subsets and found that Sema3E treatment increased the proportion and number of CD8α$^+$ DC subset and CD103$^+$ DC subset in the spleen and lung, respectively (Fig. 34 A, B & D). Lung CD11b$^+$ DC subset did not significantly change after Sema3E treatment of *Cm* infected mice (Fig. 34A & C). The percentages of CD8α$^-$ spleen DC subset was reduced after Sema3E treatment (Fig. 34A & F). These results collectively suggest that Sema3E plays a critical role in recruiting and development of specific DC subsets after chlamydial infection.

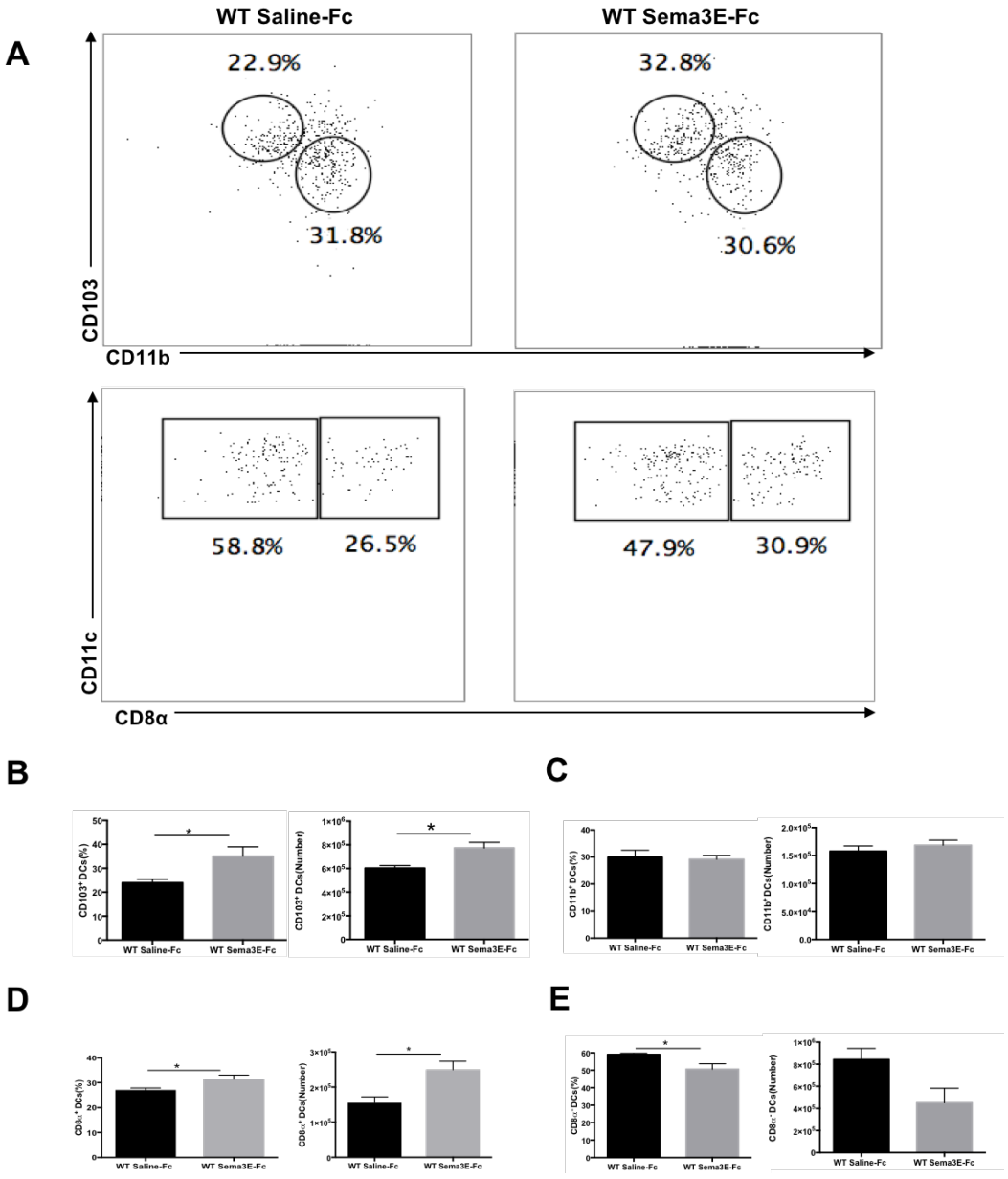

Figure 34: Higher CD8α$^+$ spleen DC subset and CD103$^+$ lung DC subset in Sema3E-Fc treated mice compared to saline-Fc treated mice after *Cm* infection. Mice were intranasally inoculated with 1x10^3 IFUs of *Cm*. Lung cells were collected from Sema3E-Fc, or saline-Fc treated mice at day 3 post-infection, stained for surface markers and analyzed using flow cytometry. A) Representative flow cytometric images of CD103$^+$ lung DC, CD11b$^+$lung DC, CD8α$^+$ and CD8α$^-$ spleen DC subset. B) The percentages and number of CD103$^+$ lung DC subset. C) The percentages and number of CD11b$^+$lung DC subset. D) The percentages and number of CD8α$^+$ spleen DC subset. E) The percentages and number of CD8α$^-$ spleen DC subset. Data are shown as mean ± SD (n = 3) and represent one of three independent experiments with similar results. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

4.4.6. Discussion

A large body of evidence shows that Semaphorin 3E is involved in the regulation of immune response [202, 209, 212, 217]. However, the contribution of Sema3E in host defense against bacterial infection is not examined. On the basis of showing the deficiency of Sema3E KO mice in host defense against chlamydial infection, in the present study, we found that exogenous Semaphorin 3E treatment reduced chlamydial infection in the lung characterized by lower chlamydial growth, less severe pathology, and reduced body weight loss. More interestingly, we found that the administration of exogenous Sema3E to either Sema3E or WT mice can enhance the capacity of mice to fight against the infection. Analysis of cytokine response shed light on the mechanism by which Sema3E treatment inhibits chlamydial infection. We have observed that Sema3E treatment enhances IFN-γ, IL-17 production but reduces IL-10 and IL-4 cytokine response in the lungs and spleen after chlamydial infection. Similar to our observation, studies in allergic asthma also shown that Sema3E treatment enhanced the secretion of IFN-γ and reduced IL-4 in the airways upon House Dust Mite (HDM) challenge [212]. These studies further confirm the role of Sema3E as a promotor of protective immune responses to infection/inflammatory stimuli.

Our laboratory and others have evidenced a critical function of IFN-γ production by CD4 T cells for clearance of chlamydial infection [109, 126, 231]. In the present study, our results showed that Sema3E treatment enhanced IFN-γ production by $CD4^+$ T cells and $CD8^+$ T cells. Also, Th17 response is found to be higher in Sema3E-Fc treated mice compared to saline-Fc treated mice after *Cm* infection. It is established that Th17 response modulates DC function to generate protective type 1 CD4 and CD8 T cell responses to chlamydial lung infection *in vivo* [40, 109, 263].

Therefore, the observed protection in Sema3E treated mice can be the combined effect of enhanced Th1 and Th17 response.

Another significant observation in our study is the inhibition of Treg response after Sema3E treatment of chlamydia infected mice. Treg plays a significant role in regulating immune response, which can be both favorable and detrimental in host defense to infection. In *Chlamydia* studies, Treg is more associated with the inhibition of protective immunity. Several recent studies have shown the detrimental role of Treg cells in immunity to chlamydial infection. Depletion of Tregs reduced genital *Cm* infection by enhancing Th1 response [115]. Inhibition of Treg response is one of the mechanisms by which NK cells protect against chlamydial lung infection [51, 120]. In agreement with reduced Treg response, we observed lower IL-10 cytokine in the lungs and spleen of Sema3E treated mice.

Another significant observation in this study is the influence of Sema3E in regulating DC recruitment. We observed that Sema3E treatment enhanced the frequency and numbers of $CD103^+$ pulmonary cDCs and $CD8\alpha^+$ spleen DC subset. However, the numbers of lung $CD11b^+$ DC subset and $CD8\alpha^-$ spleen DC subset remain unchanged after Sema3E treatment. The preferential protective role of $CD103^+$ pulmonary cDCs and $CD8\alpha^+$ spleen DC subset has been evidenced in chlamydial lung infection. Previous studies in Yang lab showed that adoptive transfer of $CD103^+$ pulmonary cDCs induce more robust Th1 and Th17 responses in the recipient mice than the transfer of $CD11b^+$ DC, resulting in greater protection to *Cm* challenge [83]. Similarly, adoptive transfer of $CD8\alpha^+$ DC induced significantly higher IFN-γ production but lower IL-4 production in the recipient mice [82]. Our current data concurs these studies, showing association of increased recruitment of $CD103^+$ pulmonary cDCs and $CD8\alpha^+$ spleen DC subset with enhanced Th1/Th17 response and reduced IL-4 production in the Sema3E treated mice. The modulating function of

Sema3E in lung DC subsets has been shown in the allergic asthma model. Upon co-culture of T cells with lung DCs, $CD11b^+$ DC from HDM given Sema3E treated mice induce lower IL-5, IL-17 levels where $CD103^+$ DCs from HDM given Sema3E treated mice induce higher IFN-γ production compared to DCs from saline-Fc treated mice [212]. Future studies are needed to delineate the role of Sema3E in specific DC subset function especially its relationship with the type of T cell responses in *Cm* infection.

In summary, using a model of administration of exogenous Sema3E, we report that Sema3E treatment can correct the deficiency of Sema3E KO mice in host defense against chlamydial infection and further supplement of Sema3E can enhance the capacity of protective immunity in WT mice. The study confirms the essential role of Sema3E in immunity to chlamydial infection and suggests the potential of exogenous Sema3E in the prevention and treatment of chlamydial and likely other bacterial diseases. The immunomodulatory function of Sema3E may be considered in the development of preventive/therapeutic strategies in infectious/inflammatory diseases.

CHAPTER 5

GENERAL DISCUSSION, SIGNIFICANCE, LIMITATIONS AND FUTURE DIRECTIONS

5.1. GENERAL DISCUSSION

5.1.1. Sema3E acts as a critical factor for protective immunity against chlamydial infection

Semaphorins are a large family of proteins initially discovered as axon guidance factors in the nervous system. However, recent studies identified their function outside the nervous system, such as cancers, inflammatory and infectious diseases. Sema3E emerged as a critical regulator of the immune response. However, to our knowledge, there is no previously published study investigating this protein's functional role in bacterial infections. The study in this thesis shows that Sema3E production is induced in the lung after chlamydial lung infection of mice and plays a critical role in host defense against the infection. The conclusion is based on the studies using Sema3E KO mice (chapter 4.1), and the mice supplemented with recombinant Sema3E (chapter 4.4).

In chapter 4.1, we found that Sema3E deficiency leads to severe *C. muridarum* lung infection characterized by higher lung chlamydial loads, more severe pathology, and body weight loss. Further T cell cytokine analysis showed that Th1/Th17 response is reduced and Th2 response is enhanced in Sema3E KO mice compared to WT mice. Numerous studies have shown that Th1 immune responses are more effective for fighting against chlamydial infection, but Th2 responses promote pathological responses. Also, we found that Sema3E deficiency reduced *Cm*-specific IgG2a antibody response. These observations highlight the role of Sema3E in regulating T cell response and antibody response for protection against chlamydial lung infection. This point was

supported by an earlier finding in allergic asthma that reported that exogenous Sema3E treatment increased IFN-γ production by spleen CD4 T cells [211], while Sema3E KO mice exhibited a significant increase in Th2 cytokines compared with WT mice [212]. Moreover, our study suggests the involvement of Sema3E for suppressing Treg cells after chlamydial infection. The exacerbated inflammatory lung response in Sema3E KO mice was accompanied by the significantly higher Treg cells in spleens and lungs. Higher numbers of Tregs in Sema3E KO mice were also associated with increased levels of IL-10 cytokine in the spleen and lungs. Since DCs act as a key player in inducing protective T cell response, further analysis was performed on Sema3E KO and WTDC after chlamydial infection.

In chapter 4.4, we found that exogenous Semaphorin 3E treatment reduced chlamydial infection in the lung characterized by lower chlamydial growth, less severe pathology, and reduced body weight loss. More interestingly, we found that the administration of exogenous Sema3E to either Sema3E or WT mice can enhance the capacity of mice to fight against the infection. Moreover, We found that Sema3E treatment enhances IFN-γ, IL-17 production but reduces IL-10 and IL-4 cytokine response in the lungs and spleen after chlamydial infection. The combination of deficiency and supplementary approaches further confirm the protective role of Sema3E in infection.

5.1.2. Impact of Sema3E on DC function after chlamydial infection

The most interesting finding in this study is the modulating role of Sema3E on the phenotype, cytokine production, and, function of DC in a real bacterial infection model, and how it impacts protective T cell responses. The study from multiple aspects examined the impact of Sema3E on the phenotype and functions of DC. First, DC isolated from Sema3E KO mice exhibited lower co-stimulatory molecules and IL-12 production. However, higher inhibitory molecules and IL-10

production were observed in Sema3E KO DC than WTDC after *C. muridarum* infection. Second, coculture of the DC isolated from Sema3E KO mice with T cells showed reduced IFN-γ and IL-17 levels compared to DC from WT mice. To study the function of DC in vivo, an adoptive transfer approach has been employed. We found that the transfer of DC from *C. muridarum* infected Sema3E KO mice failed to generate protection against chlamydial challenge infection. The disease severity in the recipients of DCs from infected Sema3E KO mice was correlated with the lower Th1 responses. These results suggest that Sema3E signaling after *Cm* infection functionally activates DC to induce a protective adaptive immune response to chlamydial infection.

How Sema3E influences DC during chlamydial infection? Since Sema3E high-affinity receptor, plexinD1[236], is expressed on DCs after chlamydial infection, DCs can respond to Sema3E released in the lungs. Direct involvement of Sema3E on DC was identified using mice deficient in Sema3E receptor (PLXND1) on DC. Interestingly, PLXND1 deficiency on dendritic cells promote susceptibility to *Cm* infection leading to higher pathogen load, body weight loss, and pathological changes in lungs. More importantly, we observed lower Th1/Th17 response in CD11c PLXND1$^{-/-}$ mice compared to CD11c PLXND1$^{+/+}$ mice suggesting the requirement of Sema3E-PLXND1 signaling on DC for protective immunity to *Chlamydia*. Similar to Sema3E KO DC, PLXND1$^{-/-}$ DC showed lower IL-12 but higher levels of IL-10. Functionally PLXND1$^{-/-}$DCs, compared to PLXND1$^{+/+}$DCs, were unable to provide enhanced protection upon adoptive transfer to recipient mice. These studies highlight the critical role played by the Sema3E-PLXND1 axis on DC for a protective immune response to chlamydial infection.

5.1.3. Sema3E is required for enhanced migration of dendritic cells after chlamydial infection.

Reduced recruitment of $CD103^+$ DC to the lung is observed in Sema3E KO mice and CD11c $PLXND1^{-/-}$ mice after *Cm* infection. $CD103^+$ pulmonary cDCs were reported as potent inducers of Th1/Th17 response to *Cm* infection [83]. Therefore, lower Th1/IL-17 responses observed in CD11c $PLXND1^{-/-}$ mice and Sema3E KO mice after *Cm* infection can also be due to lower numbers of $CD103^+$ lung DC subsets. DC migration to secondary lymphoid organs is indispensable for the orchestration of adaptive immune responses. Upon exposure to a pathogen, DCs migrate to draining lymph nodes and initiate T cell activation. Our study identified Sema3E as a key component for chemokine responsiveness of DC after chlamydial infection. Further studies identified ability of Sema3E to upregulate chemokine receptor CCR7 and activation of downstream signaling pathways involved in DC chemotaxis. Other than DCs, Sema3E was identified as a critical factor for regulating thymocyte migration [163]. Previous studies suggest that Sema3A promote RhoA dependant myosin II activation during DC transmigration [158]. Role of Sema3E in other phases of DC transmigration such as adhesion disassembly need to be investigated in future. Since plexinD1 is expressed in endothelial cells, it is possible for the Sema3E-plexinD1 axis to be also involved in leukocyte extravasation [193].

5.1.4. Sema3E protein as a therapeutic approach for chlamydial infection

The study in chapter 4.4 showed a therapeutic effect of Sema3E in *Cm* lung infection. On the one hand, the study confirms the role of the molecule in host defense against chlamydial infection, which was first found using Sema3E deficient mice, and on the other hand, suggests the potential of molecule in treating chlamydial diseases. Chlamydial infections are routinely effectively treated with antibiotics such as macrolides or tetracyclines. Considering that the antibiotic treatments are associated with concerns such as side effects of treatment, recurrent infections, antibiotic

resistance, and the safety in pregnancy [264-268], the development of non-antibiotic-based therapy is needed.

Our studies showed that exogenous Sema3E treatment could be considered as a novel therapeutic approach to treat the chlamydial infection. Sema3E treatment reduced chlamydial infection in the lung characterized by lower chlamydial growth, less severe pathology, and reduced body weight loss. Sema3E treatment promotes DC recruitment to the lungs, enhances Th1/Th17 response, and inhibits Treg response. The data suggest that although it is unlikely to become a major treatment method in chlamydial disease control, it might be considered as an alternative therapeutic approach to treat the chlamydial infections in some special scenario.

More than the potential use of Sema3E in chlamydial diseases, accumulating evidence suggests the relevance of semaphorins as novel targets in cancer, autoimmune and allergic disorders. For example, the therapeutic ability of the uncleavable variant of Sema3E (Uncl-Sema3E) was demonstrated in multiple tumor models where it acts as a novel inhibitor of tumor growth, metastasis, and angiogenesis [219]. Also, in vivo studies on mice indicate that Sema3E treatment reduces allergic asthma by reducing eosinophilic inflammation, serum IgE level, Th2 cytokine response and proposed as a novel treatment option for allergic asthma [211]. However, the efficacy of semaphorins has remained to be estimated in clinical settings. More study in this area is needed.

5.2. SIGNIFICANCE

Despite being known for centuries, chlamydial infection remains a highly prevalent bacterial infection in both industrialized and developing countries. Until now, a vaccine is unavailable for chlamydial diseases. Chlamydial vaccine development was delayed by an incomplete understanding of the protective and pathological immune responses. DCs are quintessential APC which play a crucial role in bridging innate and adaptive immunity and directing the type of immune responses. Understanding the mechanisms by which DC functions are modulated and T cell response are influenced remains a critical research priority for successful development of an effective and safe vaccine against human chlamydial infections. Our studies identified that an endogenous guidance cue, Sema3E, is produced after chlamydial infection. Deficiency of Sema3E leads to the severity of chlamydial infection with lower Th1 response and higher Th2 and Treg response, while the supplement of exogenous Sema3E shows opposite effect.

Further studies identified that Sema3E modulates DC function to enhance protective T cell response and inhibit the pathological response. This finding on the role of Sema3E in protective immunity provides new insights into the immune modulation mechanisms in chlamydial infection, thus having implications for developing vaccines against *Chlamydia* and other infections. It has been reported that adoptive transfer of DC pulsed ex vivo with non-viable *Chlamydia* induces Th1 immunity against chlamydial genital challenge [269]. Our finding of Sema3E modulates DC function to enhance protective T cell response may suggest that Sema3E treated *Chlamydia* pulsed DC have implications for developing DC-based vaccines. Moreover, the therapeutic ability of Sema3E can be exploited for the treatment of infectious diseases. In a broader sense, our study reveals a previously unidentified role of Sema3E in immunity to chlamydial infection, which can be explored further to understand immunity to other infectious diseases. This study is the first

report on the influence of Sema3E-PlexinD1 axis in promoting the functional competence of DC for a protective immune response to an infection. Summary of our findings were demonstrated in Figure 35 as an integrative schematic model.

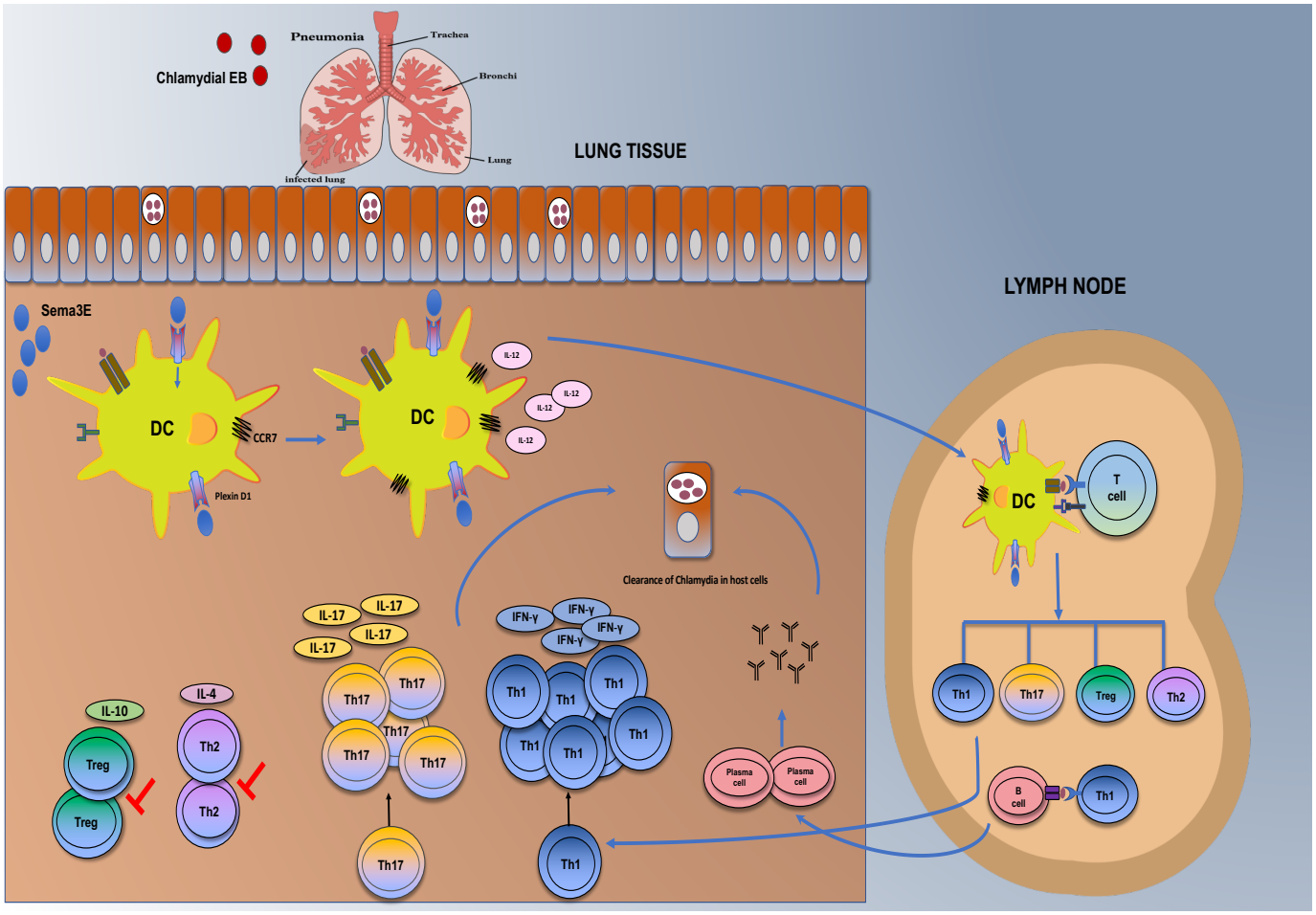

Figure 35: Schematic representation showing the mechanism by which Sema3E protects against chlamydial infection. Chlamydial infection of the lung results in the production of Sema3E. Sema3E binds to plexinD1 expressed on DCs and upregulate co-stimulatory molecules, chemokine receptor (CCR7), and IL-12 cytokine production. Activated DC migrate to draining lymph node to initiate the cellular and humoral immune response. Sema3E modulated DC promotes Th1/Th17 response and inhibits Th2 and Treg response. These responses result in the clearance of *Chlamydia* inside host cells.

5.3. LIMITATIONS

Although our data presented in this thesis elucidate the previously unknown role of Sema3E in immunity to a pulmonary bacterial infection, some limitations need to be considered to interpreting the study results and implications.

- The first and most important limitation is that this study is based on an experimental mouse model. For Sema3E treatment strategies for *Chlamydia* in clinical settings, it is necessary to determine the expression of Sema3E and PlexinD1 in *Chlamydia* infected human tissues. Also, it is important to explore the function of Sema3E in other models of chlamydial infections, such as genital tract infection and infections with other chlamydial strains/species.
- In this study, we observed that Sema3E protein is secreted into the lungs of *Chlamydia* infected mice. However, the exact cellular source of Sema3E in this model was not identified. Therefore Sema3E producing cells after chlamydial infection should be further investigated. Considering it was reported to be produced by bronchial epithelial cells and various immune cells in other models, the tissues/cells should be examined as a priority.
- In exogenous Sema3E treatment studies, we observed an effect of Sema3E after chlamydial infection. However, the impact of Sema3E treatment before chlamydial infection as a preventative strategy needs to be studied in the future.
- There are more and more surface markers and cytokines that can be analyzed by flow cytometry now. The numbers of these molecules examined in the study is limited, an expanded analysis would provide more information on the phenotype of DCs which are potentially influenced by Sema3E.

- Also, to examine the ability of DC to influence *Chlamydia*-specific T-cell responses, we used T cells isolated from mice immunized with *Chlamydia* due to the lack of *Chlamydia*-specific TCR transgenic mouse. This is in some way mimicking a recall response. It would be better if this type of mice is available to us because we can then directly test the effect of DC in primary T cell responses.

5.4. FUTURE STUDIES

- Previous studies showed that the function of Sema3E differs based on Sema3E isoforms expressed. The p61-sema3E isoform obtained by furin-mediated processing of full-length Sema3E promotes lung metastasis, cell migration, invasive growth, and extracellular signal–regulated kinase 1/2 activation of endothelial cells [218]. In fact, p61 isoform is the active and predominant form of Sema3E observed in invasive and metastatic cancer cells and reverses the repulsive effects of full-length Sema3E [219]. But, we haven't determined the expression levels of different Sema3E isoforms in this study. This can be addressed in future studies by western blotting analysis.
- Our studies showed that Sema3E KO mice, compared to WT mice, exhibited reduced numbers of $CD103^+$ lung DC and $CD8\alpha^+$ spleen DC subset after Chlamydial infection. Therefore, it will be interesting to extend our investigation on the modulating effect of Sema3E on DC to the level of DC subsets. Functional studies will be conducted on lung and spleen DC subsets of WT and Sema3E KO mice after chlamydial infection. In addition, the gene transcription factors in DC and T cell subset development could be studied.
- Apart from their involvement in DCs, Sema3E impacts macrophage functions in various disease conditions. Our studies also showed reduced numbers of macrophages in Sema3E

KO mice after chlamydial infection. Macrophages isolated from KO mice and WT mice after chlamydial infection will be analyzed for their phenotype and function.

- To address the mechanism by which Sema3E promotes DC migration, we focused on few signaling pathways involved in Sema3E function and DC chemotaxis, such as Rac1 GTPase activity and F-actin polymerization. But the involvement of other signaling pathways are possible. In future, transcriptomic studies will be conducted on WT, Sema3E KO, Sema3E treated, and untreated DC infected with *Chlamydia.* These studies will help to determine factors downregulated or upregulated in DC as a result of Sema3E signaling.

CHAPTER 6

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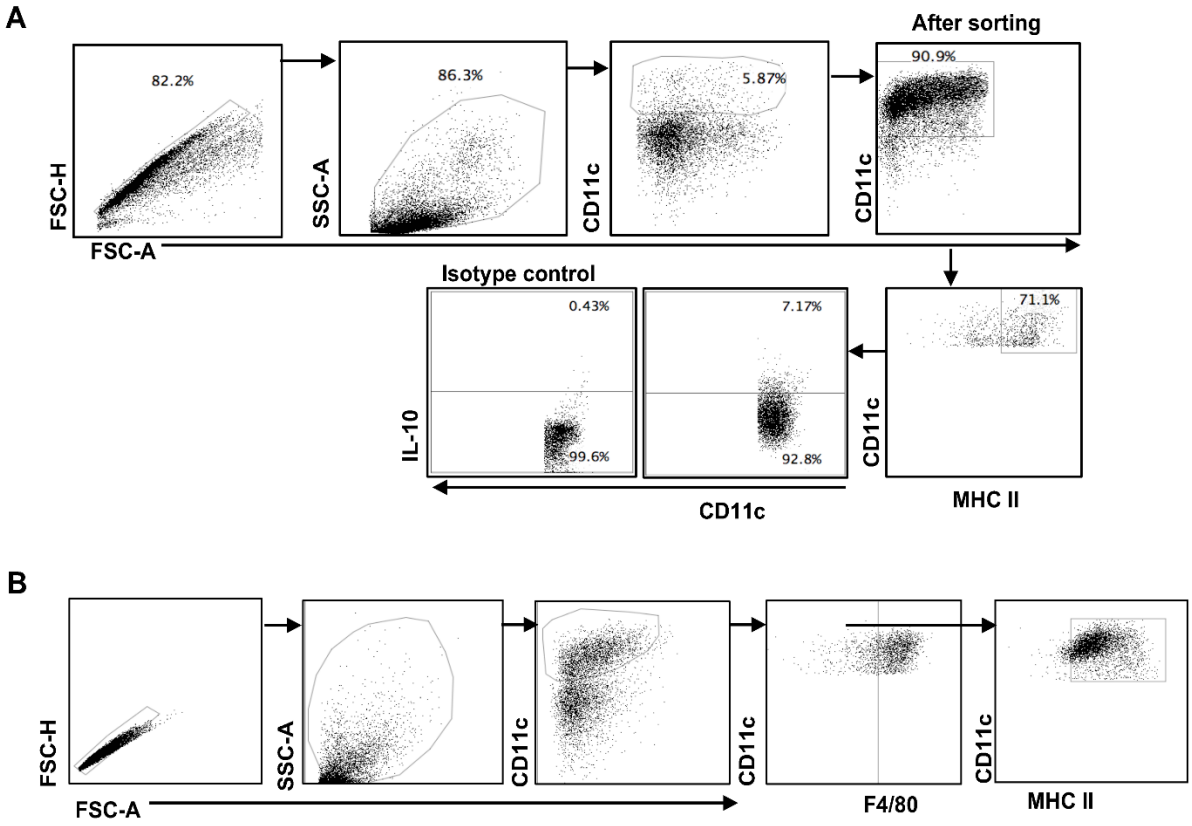
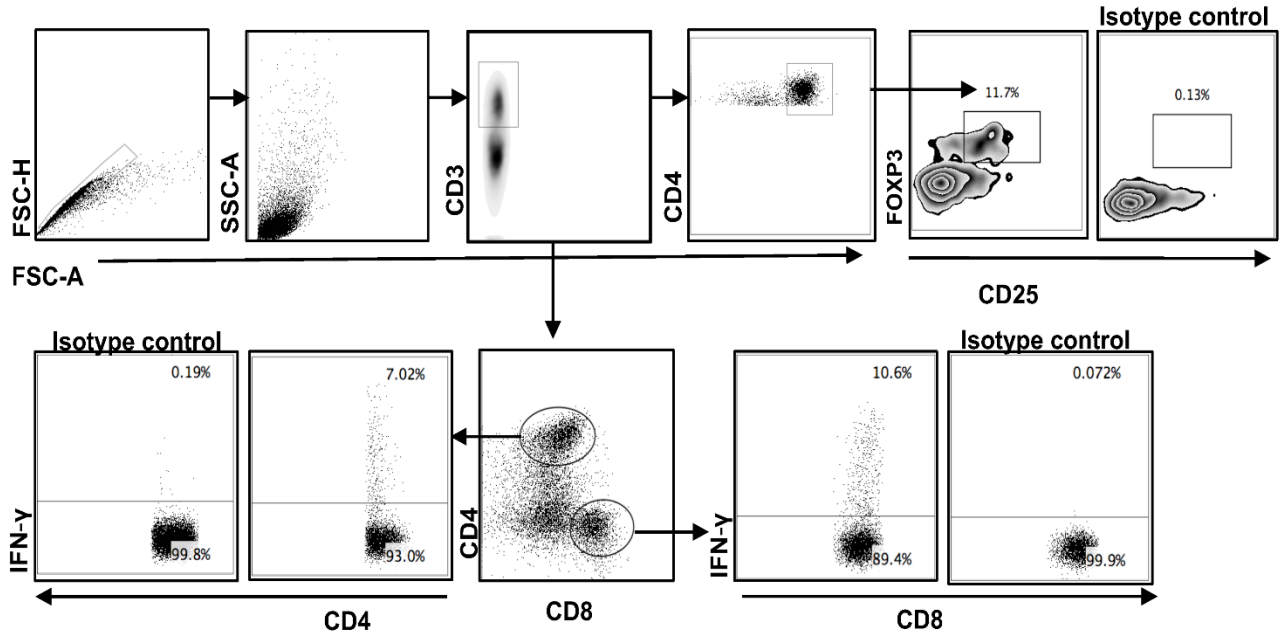
CHAPTER 7

APPENDICES



Appendix 1: Gating strategy of DC. A) Gating strategy of spleen DCs and purity of sorted spleen DCs. CD11c^{hi}MHC-II^{hi} cells were analyzed as spleen DCs by flow cytometry. B) Gating strategy of lung DC. CD11c^{hi}MHC-II^{hi}F4/80^{-} cells were analyzed as lung DCs by flow cytometry.



Appendix 2: Gating strategy of lung T cells. For CD4 and CD8 T cells, analysis was performed on gated $CD3^+$ cells.