Novel Roles of Semaphorin-3E in the Regulation of Phenotypes and Functions of Natural Killer Cells and Dendritic Cells

by

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Abstract

Natural killer (NK) cell is an important innate immune cell that mediates direct antiviral and antitumor immunity. Dendritic cells (DCs) are antigen-presenting cells that undergo maturation/activation programs to link innate and adaptive immunity, and to regulate specific adaptive T cell responses. Semaphorin-3E (Sema-3E) is a member of the semaphorin family of proteins that play diverse regulatory functions in various immune responses in vitro and in vivo upon binding to the receptor PlexinD1. Whether Sema-3E and its cognate receptor PlexinD1 were involved in the regulation of NK-cell and DC phenotypes and functions, and thereby NK/DC crosstalk remained to be investigated.

First, I investigated the role of Sema-3E in regulating NK cell development and function. I observed that Sema-3E deficiency did not affect NK cell development, the major NK cell receptors, or cell migration properties. Interestingly, I observed that Sema-3E-deficient NK cells were impaired in cell-mediated cytotoxicity and cytokine release.

Next, I investigated the role of Sema-3E in regulating DC phenotype and function. I found that Sema-3E-deficient mice displayed higher $CD11c^+$ cell number in bone marrow-derived DCs (BMDCs). Moreover, immature and mature splenic DCs from Sema-3E-deficient mice display an altered MHC class II phenotype. Of interest, Sema-3E-deficient immature and LPS-matured BMDCs showed upregulated expression of IL-12/IL-23p40 whereas Sema-3E-deficient immature splenic DCs downregulated IL-12/IL-23p40 compared to wild type (WT) controls.

Sema-3E binds directly to PlexinD1to mediate different effector functions. Interestingly, steady-state PlexinD1-deficient BMDCs displayed higher production levels of proinflammatory cytokines, including IFN-γ, IL-12/IL-23p40, and IL-6. PlexinD1-deficient LPS-matured splenic DCs, but not BMDCs, selectively upregulated IFN-γ and IL-12/IL-23p40.

Lastly, I investigated the role of Sema-3E in regulating NK cell migration in NK/DC crosstalk. I observed that the conditioned medium of immature Sema-3E-deficient BMDCs contained higher MCP-1 and MIP-1α chemokine levels compared to WT controls. Unlike for BMDCs, the conditioned medium of Sema-3E-deficient immature and LPS-matured splenic DCs did not affect NK cell migration. Together, these findings established novel roles of Sema-3E in the regulation of NK-cell and DC functions. The latter could be important in understanding immune dys-regulation in allergic asthma and/or future development of immunotherapy for cancer and infectious diseases.

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Dedication

To my wife, Abeer Alamri,

my princess, Aleen Alamri,

and my little son, Faisal Alamri

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List of Abbreviations

ADCC	Antibody-dependent cell-mediated cell cytotoxicity
ADP	Adenine dinucleotide phosphate
AIDS	Allergy and Infectious Disease
ANOVA	Analysis of variance
APC	Antigen-presenting cells
ASM	Airway smooth muscle
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
CDP	Common myeloid DC progenitors
CLA	Cutaneous lymphocyte antigen
CLP	Common lymphoid progenitors
CTL	Cytotoxic T lymphocytes
CyTOF	Cytometry by time of flight
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DP	Double-positive
ECM.	Extracellular matrix
EpCAM	Epithelial cell adhesion molecule
FACS	Fluorescence-activated cell staining
FAK	Focal adhesion kinase
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanidine exchange factors
GMC	Genetic Models Center
GnRH	Gonadotropin-Releasing Hormone
GTP	Guanosine Triphosphatase

HBSS	Hank’s balanced salt solution
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Histocompatibility leukocyte antigen
HSC	Hematopoietic stem cells
HSV	Herpes simplex virus
HEV	High endothelial venules
ICE	IL1β-converting enzyme
IIR	Immune-inflammatory response
IgG	Immunoglobulin
IKDC	Interferon-producing killer dendritic cells
ILC	Innate lymphoid cells
IPT	Ig-like, plexins, transcription
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	Jun N-terminal kinase
KIR	Killer immunoglobulin-like receptor
KL	Kit ligand
LC	Langerhans cell
LN	Lymph node
LNHEV	Lymph node high endothelial venules
LMPP	Lymphoid-primed multipotential progenitor
LPS	Lipopolysaccharide
M.M	Mouse medium
MAM	Meprin/A5-protein/PTPmu
MAPK	Mitogen-activated protein kinase
MCMV	Molecules in mouse cytomegalovirus virus
MDSC	Myeloid-derived suppressor cell
MET	Mesenchymal-epithelial transition
MFI	Mean fluorescent intensity

MHC	Major histocompatibility complex
MHCII	MHC class II
MIC	Methylisocitric acid
MICAL	Molecule interacting with Cas ligand
MSD	Mesoscale Discovery
MULT1	Murine UL16-binding proteinlike transcript 1
NCR	Natural cytotoxicity receptor
NK	Natural killer cell
NKD	Natural killer deficiency
NKT	Natural killer-T cells
NOD	Non-obese diabetic
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PLGF	Placental growth factor
PFA	Paraformaldehyde
PPD	Protein purified derivative
PSI	Plexin-sema-integrin
PTK	Protein tyrosine kinase
PVR	Poliovirus receptor
RasGAP	Ras GTPase activating protein
RAFTK	Related adhesion focal tyrosine kinase
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RAP	Related proteins
RBD	Rho GTPase binding domain
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RQ	Relative quantitative
SelR	Sulfoxide reductase

SEMAH.	SemaphorinH
SLT	Secondary lymphoid tissues
SP	Sex-plexins
STAT	Signal Transducers and Activators of Transcription
Th1/2	Helper T cell
TF	Transcriptional factors
TLR	Tool like receptors
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand-receptor
Treg	Regulatory T cell
ULBP	UL16-binding proteins
VEGF	Vascular endothelial growth factor
WT	Wild type

Chapter 1: Introduction

1.1 Natural killer cells: History and general overview

Natural killer (NK) cells are part of the innate immune system and were initially defined as effector lymphocytes characterized by their unique ability to kill virus-infected and tumor cells with no priming or expansion of specific clones (*1*).

In the early 1970s, Herberman and colleagues identified NK cells morphologically as large granular lymphocytes (LGL) (*2, 3*). Kiessling and colleagues further characterized the particular target recognition feature of these lymphoid cells, as the identified cells were impaired in cytotoxicity against the mastocytoma line P815 and other leukemic cells of non-Moloney origin (*4*). These cells were referred to as "natural" killer cells since they lacked key T- and B-lymphocyte receptors and were unique in terms of their cytotoxic responses (*4*). Unlike T cells, NK cells are armed with capable cytolytic machinery that does not need prior activation to recognize and eliminate abnormal cells (*5*) Additionally, NK cells secrete numerous cytokines/chemokines (e.g., IFN-γ, TNF-α, GM-CSF, MIP-1α (CCL3), MIP-1β (CCL4), CCL5 and RANTES) that connect the innate and adaptive immune systems (*5*). Recently, the involvement of NK cells in controlling cancer growth, immune surveillance, pregnancy, and stress is well documented and has received considerable attention, with significant implications in clinical settings (*6-9*).

1.2 NK cell development, maturation, and subsets

Early studies suggested NK cells developed exclusively in the bone marrow (BM), and bone marrow microenvironment ablation or disruption can abrogate the development and function of NK cells (*10, 11*). However, in humans and mice, current evidence suggests that secondary lymphoid tissues (SLTs) are the potential locations where NK cells can also develop and mature, including tonsils, spleen, and lymph nodes (LNs) (*12*). In mice, development of NK cells occurs in specialized BM niches; the hematopoietic niche is most often localized in the perivascular regions proximal to sinusoidal vessels (*5*). The integrated cytokine milieu regulates the multipotent self-renewing hematopoietic stem cells (HSCs) that contain transient and long-term self-renewing populations (*13*). HSCs are known to generate leukocytes and red blood cells, a part of which forms the common lymphoid progenitors (CLPs) (*13*). CLPs give rise to pro-B cells, pre-T cells, innate lymphoid cells (ILCs), lymphoid tissue inducers, and $CD122^{+}$ pre-T/early NKP lineages.

The NK cells cellular origin in humans and mice can be linked to oligopotent CLPs (*13*). In murine, the earliest step of CLP transition into the lymphoid lineage is the appearance of interleukin (IL)-7 receptor-alpha (IL-7Rα, CD127) in $Lin^{-}CD244^{+}$ cells. Pre-NK cell precursors (Pre-NKPs), which express the IL-2 receptor β chain (CD122) to become NKPs, are defined as a subset of this early progenitor (*14*). The receptor complex NKG2D/DNAX that activates protein of 10 kDa (DAP10) defines stage A of the immature NK (iNK) population (*15, 16*). Activation of transcription factors such as inhibitor of DNA binding 2 (Id2) and E4-binding protein 4 is required for the maintenance and progression of NKPs to the iNK cell stage (*17-20*). Of interest, different NK cell maturation stages express different receptors. For instance, at the iNK stage, NK cells were found to express NK1.1, NKG2A, DNAM-1 (CD226), NCR1, Leukosialin (CD43) and the cell adhesion molecule L-selectin (CD62L) (*21*). The initial stage of mature NK cells is defined based on the expression of CD49b (DX5, Integrin VLA-2α) and CD51 (Integrin αV). The expression of CD11b (Mac-1) and CD43 (Leukosialin) defines NK cells that are terminally mature. Furthermore, the acquisition of Ly49 receptors defines licensed mature NK cells (*22*).

In 2006, mature NK cells were elucidated by Hayakawa and colleagues and divided into two distinct subpopulations based on CD27 surface density (*23*). In addition to CD27, CD11b can also define functional NK cell maturation, in which NK cells develop through a four-stage progression (*24*): beginning with the double-negative NK population (stage A, in which cells express neither receptor), progressing to $CD27^{+}CD11b^{-}$ (stages B, C, and D), double-positive (DP, stage E), and finally $CD27^{-}CD11b^{+}$ (stage F), which are believed the most mature NK cells (*22, 24*) (**Figure 1.1**).

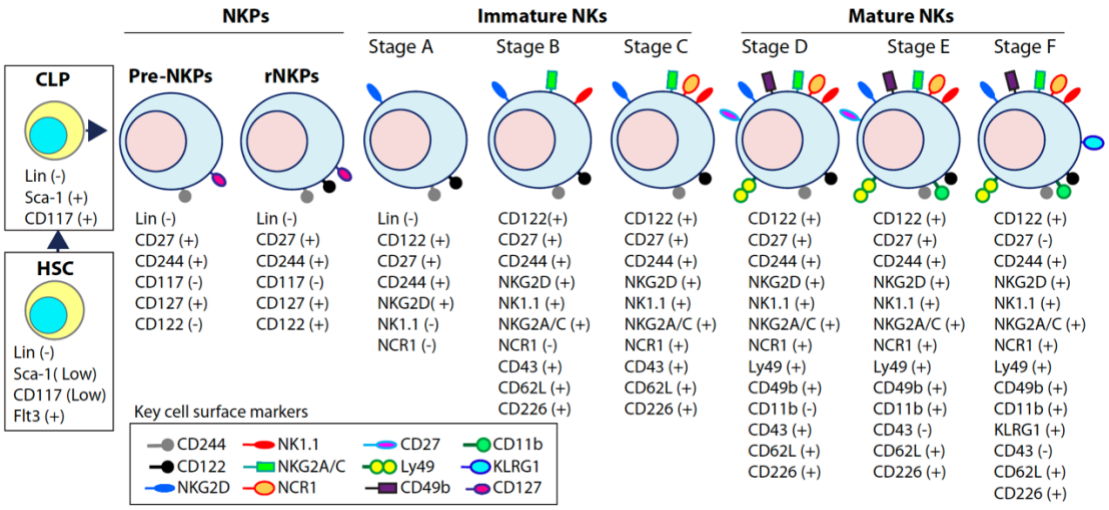


Figure 1.1. The developmental stages of murine NK cell. Lineage negative (Lin−) Sca+ CD117+ hematopoietic stem cells (HSCs) separate into common lymphoid progenitors (CLPs) (Lin−ScaLowCD117LowFlt3+). The CLPs commitment into pre-NK cell precursors (Pre-NKPs) mediated by the expression of IL-7 receptor-alpha (IL-7Rα) (CD127), CD27, and CD244. The expression of IL-2Rβ (CD122) determines the commitment of NKPs transition from Pre-NKPs to refined-NKPs (rNKPs). The expression of NKG2D mediates the transformation of rNKPs into iNK cells. NK cells express NK1.1(iNK stages) and NKG2A/C followed by NCR1 (Stage A through C). Ly49s defined iNK cells and mNK cells and help to identify different subsets (Stage D). CD27 and CD11b (Stage E) expression determine NK cells maturation then followed by Killer cell Lectin-like Receptor G1 (KLRG1) (Stage F). Reproduced with permission from (*5*).

Different NK cell subpopulations demonstrate distinct effector functions, location distribution, and surface receptor acquisition. For instance, the $CD11b^{hi}CD27^{hi}$ NK cell population produces large amounts of IFN-γ and exhibits strong cytotoxicity against target cells in response to IL12/IL-18 stimulation compared with $CD11b^{hi}CD27^{low}$. This same NK cell population expresses higher levels of CXCR3 and is localized to SLTs (*25*, *26*).

Another subset of NK cells, a hybrid phenotype of NK cell and dendritic cell, was reported by Chen and colleagues to express $B220^{+}CD11c^{+}CD49^{+}$ and referred to as interferon-producing killer dendritic cells (IKDC) (*27*). These nonconventional NK cells reside in the spleen, BM, and LNs. IKDCs are a source of several cytokines, including IL-12, IFN-α, IFN-β, and IFN-γ. Because of their ability to produce numerous amounts of IFN-γ (thus eliminating tumor cells in vivo more efficiently than conventional NK cells), IKDC were referred to as activated NK cells (*28*). Pletneva and colleagues demonstrated that IKDC simultaneously produced IL-12p40 and upregulated major

histocompatibility complex (MHC) class II and co-stimulatory molecules in mouse cytomegalovirus virus (MCMV)-infected mice (*29*). An adoptive transfer experiment reported by Blasius and colleagues showed that these NK cells lose B220 and CD11c expression and acquire $CD11b^{+}CD27^{-}$ phenotype of mature conventional NK cells. Based on their observations, they describe IKDCs as a rapid precursor to mature NK cells rather than a defined subset (*30*). Also, Di Santo and colleagues reported a subset of NK cells with a characteristic CD127 surface expression. In contrast to bone-marrow-derived NK cells, this subset originates from the thymus and resides in SLT. This NK subset is unique in that it demonstrates a higher dependency on the transcription factor GATA3, produces more IFN-γ, and shows close similarity to human $CD56^{bright}CD16^{dim/-}$ NK cells (*31*).

In humans, NK cells have been shown to mature in the BM and secondary lymphoid organs such as LNs (*12*). $Lin^{-}CD34^{+}CD133^{+}CD244^{+}$ HSCs distinguish into $CD45RA^{+}$ lymphoid-primed multipotential progenitor (LMPP) in stage 1. The expression of CD7 (Ig family, co-stimulatory molecule) (*32*), CD38 (cyclic adenine dinucleotide phosphate (ADP) ribose hydrolase) (*33*), CD10 (neutral endopeptidase) (*34*), and the cytokine receptor CD127 (IL-7Rα) help the transition of LMPPs into CLPs to make lineage commitments into Pro-B, Pre-T, NKPs, or other ILCs (*35*). The expression of CD122 (IL-2Rβ) markers mediated the irreversible critical decision of CLPs into NK lineage. Therefore, the final transition of iNK into mNK cells is indicated by CD56 (NCAM). It has also been suggested that iNK cells can directly give rise to the $CD56^{dim}$ population (*36*), but this remains to be investigated (**Figure 1.2**).

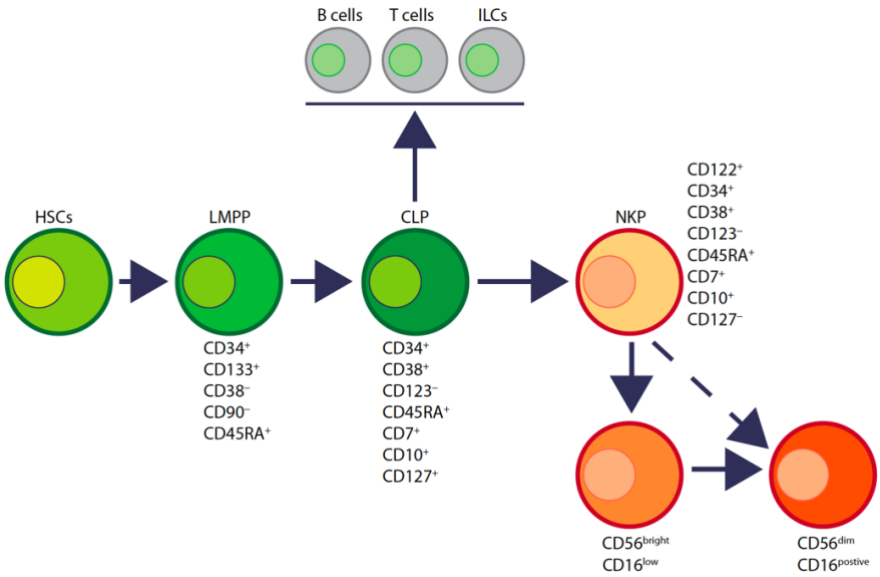


Figure 1.2. **Human NK cells developmental origin.** NK cells can mature from the lymph nodes (LNs). Lin−CD34+ hematopoietic stem cells (HSCs) differentiate into CD45RA+ lymphoid-primed multipotential progenitor (LMPP). The transition of LMPPs into common lymphoid progenitors (CLPs) is mediated by the expression of CD38, CD7, CD10, and IL-7 receptor-alpha. The final transition of immature NK cells (iNK) into mature NK cells showed CD56 expression. iNK cells (CD56bright population) (~5%) convert into major (CD56dim) (>90%) population. It is possible that iNK cells can directly enhance the CD56dim population (dotted arrow). Reproduced with permission from (5).

1.3 Target cell recognition: The missing self and/or induced self hypothesis

According to missing self and/ or induced self hypothesis, NK cells can distinguish "self" and "non-self". In 1986, the first MHC-I-dependent target recognition and self-tolerance was proposed by Ljunggren and Kärre and was called the "missing self-hypothesis" (*37*). The missing self-hypothesis states that target cells lacking or having altered MHC-I expression (similar to what happens typically in viral infection or tumorigenesis) become vulnerable to NK cell action. To inhibit NK activation, the MHC-I molecule, a hallmark of "self" expressed ubiquitously on normal cells, generates a protective signal. Later, this model's credibility was reinforced by identifying Ly49A and KIR inhibitory receptors and their specificity in mouse and human NK cells, respectively (*38, 39*). NK cells and T cells expressed activating receptor NKG2D that interacts with stress-inducible ligands (*40*). Most benign cells in mice do not express NKG2D ligands; however, some virally infected cells upregulated NKG2D ligands, which become sensitive to NK

cell killing. Such observation has initiated the "induced or stressed-self" hypothesis, hypothesizing that normal cells do not express NKG2D ligands but upregulated in response to infection or malignant cell transformation that caused cellular changes (*41*). Together, the missing self-hypothesis and the induced self hypothesis infer that NK cell recognizes non-self molecules, and infection caused many cellular changes, including the self-molecules suppression or induction.

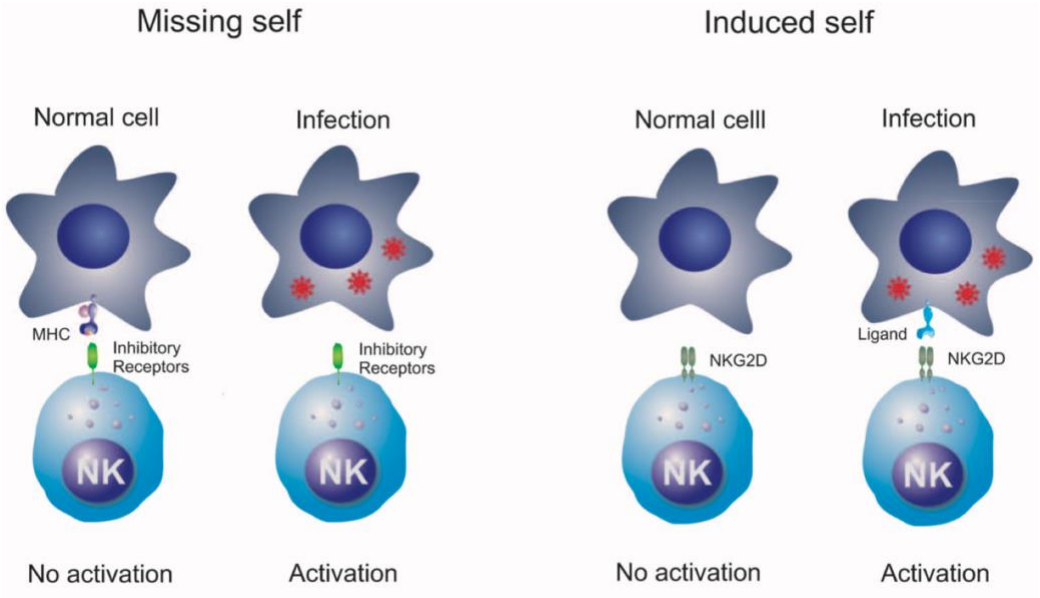


Figure 1.3. Infection caused cellular changes recognized by Innate immunity. NK cells take advantage of inhibitory receptors to discriminate "self" from "missing self". The missing of MHC class I expression (missing self) enhances NK cell activation, thereby targeting cell lysis. In addition, NKG2D (activating receptor) is expressed by NK cells, which may identify ligands that are enhanced in response to the infection (induced self). Reproduced with permission from (*42*).

The stimulation and effector function of NK cells depend on the signals derived from two distinct types of receptors: inhibitory and activating (**Table 1.1**). As a result, MHC class I molecules expressed on normal healthy cells act as ligands for inhibitory receptors to contribute to NK cells' self-tolerance. However, shifts in balance are mediated by the signal received from activating receptors in NK cells, However, shifts in balance are mediated by the signal received from activating receptors in NK cells, resulting in NK cell activation and target cells elimination directly via NK cell-mediated cytotoxicity or indirectly via proinflammatory cytokines secretion (*43*).

Table 1.1. NK cells inhibitory and activating receptors.

NK Receptor Type	NK Receptor	NK Receptor ligand	Species
Inhibitory	KIR2DL 1/2/3	HLA-C1/2	Human
	KIR3DL1	HLA-Bw4	Human
	KIR3DL2	HLA-A3, -A11	Human
	LIR-1	HLA-A-G	Human
	CEACAM1	CEACAM5	Human
	ILT2 (CD85j)	HLA-A, -B, -C, HLA-G1, HCMV UL18	Human
	NKR-P1A	LLTI	Human
	Ly49A	H-2D^{b,d,k,p}, H2M3	Mouse
	Ly49C	H-2D^{b,d,k} , H-K^{b,d,k}m157	Mouse
	Ly49I	H-2D^{b,s,q,v}	Mouse
	Ly49P	H-2D^{d,k}	Mouse
	NKR-P1B	Clr-b	Mouse
	NKR-P1D	Clr-b	Mouse
	CD94-NKG1A	Mouse: Qa1b Human: HLA-E	Mouse/ Human
	CD94/NKG2A	Mouse: Qa1b Human: HLA-E	Mouse/ Human
	KLRG-1	Cadherins -E, -N, and -R	Mouse/ Human
	TIGIT	PVR and PVRL2	Mouse/ Human
	CD244 (2B4)	CD48	Mouse/ Human

Activating	**KIR2DS1**	**HLA-C2**	**Human**
	KIR2DS4	HLA-A11	Human
	KIR2DL4	HLA-G	Human
	KIR3DS1	HAL-Bw4	Human
	KIR3DL2	CpG	Human
	NKP30	B7H6, BAT3, pp65, PfEMP, viral HA	Human
	NKP44	viral HA, HN, PCNA, proteoglycans	Human
	CD16	IgG	Human
	Ly49D	H-2D^{d}	Mouse
	Ly49H	M157	Mouse
	NKR-P1C	Unknown	Mouse
	NKR-P1F	Clr-g, Clr-c	Mouse
	NKR-P1G	Clr-g, Clr-f	Mouse
	NKG2D	Mouse: Rae1a-e, MULT-1,H60 Human: MIC-A/-B, ULBP1-4	Mouse/ Human
	CD94-NKG2C	Mouse: Qa1b Human: HLA-E	Mouse/ Human
	NKP46	Heparin, viral HA and HN	Mouse/ Human
	DNAM-1	CD112, CD155	Mouse/ Human

1.4 NK cell receptors for inhibition and activation

1.4.1 Inhibitory receptors

Inhibitory receptors have been divided into three families based on MHC-I molecule specificity, including Ly49 in rodents, KIR in humans, and CD94/NKG2A in both humans and rodents (*38, 39, 44, 45*). Inhibitory receptors comprise immunoreceptor tyrosine-based inhibitory motifs (ITIM) to mediate their signals. To neutralize the activating signals, ITIMs undergo phosphorylation and recruit phosphatases such as Src homology-containing tyrosine phosphatase 1 (SHP-1), SHP-2, and lipid phosphatase SH2 domain-containing inositol-5-phosphatase (SHIP) (*46*). Immunoreceptor tyrosine-based activation motif (ITAM)-bearing Vav-1 molecules dephosphorylated via SHP-1 and SHP-2 to prevent the downstream signaling (*47*, *48*).

1.4.1.1 Ly49 receptors

Ly49 receptors are expressed on the surface of NK cells, NKT cells, and γδ T lymphocytes and represent unique mouse NK cell inhibitory receptors prominent families (*49*). Ly49 receptors are lectin-like type II transmembrane proteins composed of a carboxy-terminal lectin domain

known as an NK domain (NKD) (*50*). Independently, Ly49 receptors bind to MHC class I molecules through their NKD. The Ly49 receptor family contains inhibitory and activating receptors. In mice, inhibitory isoforms like Ly49A, Ly49C, Ly49I, and Ly49P molecules are well characterized. While Ly49C binds to H-2K^{b} and H-2D^{b} molecules, The prototype member Ly49A binds to H-2D^{d}, H-2D^{k}, and the nonclassical MHC-I molecule H2-M3 (*51*). However, Ly49H, a member of the Ly49 family, recognizes m157 on MCMV-infected cells and activates receptors (*52*).

1.4.1.2 Killer cell immunoglobulin-like receptors (KIR):

The KIR family of proteins are human NK cell receptors that are functionally equivalent to Ly49 (*53*). KIRs express two or three extracellular Immunoglobulin G (IgG)-like domains with a characteristic short or long cytoplasmic tail. KIR proteins with short tails are restricted to activating signals via DAP12 adaptor proteins, whereas those with long cytoplasmic tails exhibit ITIM (I/VxYxxL/V)-associated inhibitory signals (*54, 55*). In contrast to the Ly49 family, most members of the KIR family demonstrate specificity to the HLA class-1 molecules and maintain NK functions (*56*).

1.4.1.3 CD94/NKG2A

CD94/NKG2A is a C-type lectin superfamily of the inhibitory receptor that forms a heterodimer and contains ITIM. This receptor recognizes explicitly nonclassical MHC molecules on target cells and protects the host cell against improper NK cell activation (*57, 58*). Respectively, upregulation of HLA-E and Qa1 in infections and tumors recognized by the NKG2A receptor in humans or mice (*59, 60*). However, some cytokines in the tissue microenvironment can also regulate the expression of NKG2A and therefore modulate NK cell function (*61*).

1.4.1.4 NKR-P1D/NKR-P1B

NKR-P1 receptors are homodimeric type II transmembrane C-type lectin-like molecules. There are five inhibitory or activating receptors in mice (NKR-P1A, NKR-P1B/D, NKR-P1C, NKR-P1F, and NKR-P1G) (*62, 63*). The inhibitory receptor NKR-P1D was found in C57BL/6 mice, whereas inhibitory receptor NKR-P1B homolog was found in the Balb/c genetic background. The cytoplasmic tails of these receptors contain ITIM sequence to facilitate SHP-1 recruitment and induce NK-cell-mediated inhibition. The cognate ligand for NKR-P1D/B is broadly expressed on hematopoietic and non-hematopoietic cell receptors and identified as members of the C-type lectin molecule (Ocil) (*64*).

1.4.2 Activating receptors

Unlike T cells, a wide variety of surface receptors are required to initiate NK cells effector functions (*7*). It has become clear that engagement of NK inhibitory receptors alone is not enough to counter NK activation. A full NK cell activation also requires recognizing stress-induced molecules by NK cell-activating receptors (*7, 65*). Therefore, a delicate balance of signals generated from activating and inhibitory signals defines a mature NK cell (*65*). Activating surface NK receptors in humans and mice leads to the secretion of proinflammatory cytokines and/or cytotoxic molecules to combat inflammatory insults, depending on the specific engagement or activation. Most activating receptors mediate signaling through ITAM sequences present in the cytoplasmic tail (*7*).

1.4.2.1 Ly49/KIR receptors

Although most Ly49 receptors are inhibitory receptors, some Ly49 and KIR members are activating. For example, in C57BL/6 mice, Ly49D and Ly49H show an activation function. These receptors are associated with the DAP12 molecule and transduce the signal through ITAM (*66*). The recognition of the m157 decoy ligand, expressed on MCMV-infected cells, is mediated by Ly49H and imparts resistance to MCMV infection, whereas specificity for self-molecule such as H-2D$_d$ is mediated by Ly49D (*67*). In non-obese diabetic (NOD) mice, other Ly49 activating receptors such as Ly49D, Ly49P, and Ly49W have been reported to bind H-2D$_d$ and H-2D$_k$, respectively (*68-70*). The activating KIR, such as KIR2DS1 or KIR2DS2, binds to the DAP12 adapter molecule for downstream signaling (*71*). It is thought that both receptors, Ly49 and KIR, result from convergent evolution from their respective ancestral inhibitory receptors (*72*).

1.4.2.2 Natural cytotoxicity receptor (NCR)

NCRs are another immunoglobulin superfamily of activating receptors. In humans, NK cells express NCRs, including NKp30, NKp44, and NKp46, which are involved in the antiviral and antitumor activity of NK cells, while in mice, NK cells express only NKp46 (NCR1 or CD335) (*73-75*). It was reported that resting and activated NK cells express NKp46 and NKp30, whereas NKp44 expression is restricted to activated NK cells. To transduce the signal through ITAM, NCRs can bind to adaptor proteins such as FcεRI-γ and CD3-ζ (*76*). NCRs also bind to viral, bacterial, and parasite proteins, as well as to molecules from tumor cells (*76*). Notably, a role for NKp46 in modulating T cell immune responses was reported (*77*). Moreover, the tumor-associated

nuclear factors BAT3 and B7-H6 have also been shown to be a potential ligand of NKp30 (*78, 79*).

1.4.2.3 NKG2D receptor

The NK group 2, member D (NKG2D) receptor is an essential activating receptor. It is a C-type lectin-like type II transmembrane glycoprotein predominantly found on the surface of all murine and human NK cells, NKT cells, $CD8^+$ T cells in mice, $CD8^+$ T cells and a subset of γδ T cells in humans and macrophages (*80*). In mice, two isoforms of NKG2D have been identified in NK cells: a short isoform (NKG2D-S) and a long isoform (NKG2D-L) (*81*). These isoforms employ DAP10 (NKG2D-S) and DAP12 (NKG2D-L) adaptor molecules, respectively, to mediate a downstream signaling cascade (*81*). Unlike their murine counterparts, human NK cells do not present NKG2D-S, and NKG2D-L can only associate with DAP10 (*81*). It was reported that a deficiency of DAP10 and DAP12 impaired both cytotoxicity and cytokine secretion, whereas signaling through DAP10 activates cytotoxicity in both mice and humans (*81-83*). Therefore, cytokines such as IL-12, IL-7, and IL-15 promote NK and $CD8^+$ T cells to express NKG2D on their surface, whereas IFN-γ and transforming growth factor-β (TGF-β) reduce it (*84-86*). In both humans and mice, the NKG2D molecule binds several ligands expressed by infected or transformed cells that share structural homology with MHC-I molecule, including Rae1, H60 (H60a, H60b, H60c), murine UL16-binding protein like transcript 1 (MULT1) in mice and MIC-A, MIC-B, UL16-binding proteins (ULBP)1-4 in humans (*87, 88*). In both mice and humans, NKG2D ligands are expressed at low levels in healthy adult cells, whereas upregulation of these ligands is widely expressed in tumors of diverse tissue origin (*87, 89*). In mice, NKG2D ligands have been identified in lymphoma cells, and cancer cell lines including lung, colon, rectal and prostate (*89, 90*). However, the lack of NKG2D ligands was found in the lymphoma cell lines RMA, RMA-S and the melanoma cell line B16F10 (*91*). The expression of human methylisocitric acid (MIC) and ULBP were found in a wide range of cancers such as neuroblastoma (NB), glioma, leukemia, melanoma, kidney, breast, colon, lung, and prostate (*92-96*). Different factors are known to regulate the expression of NKG2D ligands: the tumor type, stage of tumor progression, the presence of cytokines, toll-like receptors (TLR) signaling, activation of the DNA damage repair pathway, and the presence of ataxia-telangiectasia mutated kinase and Rad3-related kinase (ATR) (*97-99*).

1.4.2.4 CD16 receptor

The CD16 receptor is known to trigger cytolytic activity in NK cells (*100*). The low-affinity Fcγ receptor, which binds to the Fc portion of the IgG antibody, eliminates cellular targets—a phenomenon known as antibody-dependent cell-mediated cell cytotoxicity, where NK cells activated to kill infected or tumor cells (*101*). The use of cross-linking antibodies to NK receptors showed that CD16 could trigger cytokine and chemokine secretion by NK as well as the degranulation of resting human NK cells (*61, 102*).

1.4.2.5 NKR-P1C (NK1.1) receptor:

The NK cell receptor protein 1 (NKR-P1) (CD161) family comprises a series of type II membrane glycoproteins predominantly expressed by NK cells. The association of NKR-P1C with NK1.1 provides an activating signal, where the NK1.1 receptor is the most specific and widely distributed surface marker on murine NK cells (NKT cells in C57BL/6 mice background) (*103-105*). It has been shown that the NKR-P1C (NK1.1) receptor possesses activating functions in NK cell signaling. The NKR-P1C transmembrane domain is associated with Fcγ and involved in NK-cell-mediated cytotoxicity of target cells, calcium ion (Ca^{2+}) flux, phosphatidylinositol turnover, kinase activity, and cytokine production following antibody cross-linking (*106*). The natural ligand of this receptor is complex and remains to be investigated (*107*).

1.4.2.6 DNAM-1 (CD226) receptor

DNAM-1 or CD226 is considered to be a co-stimulatory activating receptor and is a member of the Ig superfamily. The recognition of CD155 (Poliovirus receptor or PVR) and CD112 (Nectin-2) on tumor cells is mediated by DNAM-1 and induces NK cell-mediated lysis (*108*). Additionally, DNAM-1 binds to lymphocyte function-associated antigen 1 to promote adhesion of monocytes. This observation suggested that DNAM-1 is an important player in NK cell migration (*109*).

1.4.3 NK cell dual function receptors: 2B4 (CD244)

The 2B4 receptor (CD244) is present in mouse and human NK cells and binds with non-MHC related molecules (*110*). The 2B4 receptor belongs to a family of membrane receptors involved in lymphocyte activation. Of interest, 2B4 can also act as an inhibitory receptor depending on the adapter protein recruited at the cytoplasmic tail; indeed, in mice 2B4 predominantly acts as an inhibitory receptor. NK cells from 2B4 deficient mice were shown to have enhanced cytotoxicity and cytokine secretion and the rejection of B16F10 melanoma (*111,*

112). CD48, a glycoprotein expressed on hematopoietic cells, can be recognized by 2B4 to mediate multiple functions. Therefore, neutralizing antibodies to human 2B4 blocks the killing of CD48-expressing target cells. It has been shown that human NK cells can kill mouse cell lines transfected with human CD48. Collectively, these observations suggest that 2B4 acts as an activating or inhibitory receptor in human and murine NK cells (*113, 114*). Other inhibitory or activating receptors or molecules involved in the downstream signaling can modulate the activating or inhibitory function of CD244 in humans and mice. The switch in function of 2B4 in human and murine NK cells is based on expression levels of 2B4 and their cross-linking. 2B4 NK inhibitory function is associated with strong cross-linking, whereas 2B4 NK activating function is associated with low 2B4 expression and weak antibody cross-linking (*61, 115*).

1.5 MHC-I and NK-cell licensing

MHC-I expression on the target cells positively and functionally correlated with NK cells that carry these inhibitory receptors. It has been reported that both the "licensing" and self-tolerance education of NK cells is confined to the BM (*44*). However, in mice and humans, many NK cells lacking self-specific MHC-I receptors exist and are consequentially "unlicensed" (*116, 117*). Contrary to the initial school of thought, in which NK cell "license" education occurs during NK cell development in the BM, unlicensed mature NK cells regained their function when transferred from MHC-I-deficient mice into wild-type (WT) mice. Therefore, mature NK cells can also acquire licensing through an inhibitory interaction between their Ly49 receptors and host MHC-I molecules (*44, 118*). It has been suggested that the tuning of the MHC-I environment could modulate and reprogram NK effector functions. As a clue to how MHC-I regulates NK education and target recognition, normal NK cells become anergic when transferred to an MHC-I-deficient host (*44*). However, NK cells derived from β2m-deficient or Tap1-mutated mice acquired functional competence when adoptively transferred to an MHC-I-positive environment (*44, 119*). Of interest, MHC-I-dependent recognition (responsiveness) is not an independent event; therefore, the involvement of activating receptors is required by NK cells to mediate effector responses. It is thought that activating receptors are involved in the education and self-tolerance of NK cells during their development. A limited number of studies report that continuous interaction of differentiating NK cells' self-specific activating receptors (Ly49H, NKG2D) with their cognate ligands induces the generation of hyporesponsive mature NK cells but reduced their ability to proliferate (*120-122*) (**Figure 1.3**). Therefore, the role of activating receptors in NK education and self-tolerance

remains to be investigated. This model collectively suggests that NK cell activation (responsiveness) is predominantly controlled by inhibitory receptors that specifically recognize and bind class I MHC molecules. Although NK cell "licensing" education is vital for NK cell effector functions, unlicensed NK cells respond efficiently toward target cells under specific conditions, such as the high levels of MHC-I expressed by target cells escape NK detection by interacting with the inhibitory Ly49 receptors. These cells' ability to detect MHC-I-expressing tumors and viruses may explain why up to 50% of NK cells are unlicensed (with respect to self-Ly49 expression) and still preserved in immunocompetent mice (*123*, *124*).

1.6 Cytokines, chemokines, and immune cells as regulators of NK cell function

NK cell functions have been shown to include recognizing and eliminating and eliminating transformed or pathogen-infected cellular targets (*125*). These functions are mediated by direct cytotoxic mechanisms and by producing immunoregulatory cytokines to modulate adaptive immune responses (*126*). The microenvironment where NK cells reside determines the quality of the NK-cell-mediated response. As mentioned previously, MHC-I molecules control the early education and induction of self-tolerance in developing NK cells. NK cells that develop in the absence of MHC-I were reported to turn hyporesponsive (*127*). Cytokine stimulation enhanced the cytotoxic potential and cytokine production in resting NK cells in mice (*5*). Indeed, NK cells require secondary signals for an appropriate activation. Cytokines play a critical role in NK cell maturation, activation, and survival, where NK cells express surface receptors for those cytokines (*126*). For instance, interleukin (IL)-2, IL-12, IL-15, IL-18, IL-21, and type I interferons positively regulate NK cell function. Independently or in combination with other cellular factors, these cytokines have been implicated in supporting the earliest activation signals to regulate NK-cell-mediated innate immune responses. In contrast, other cytokines, such as IL-23 and IL-27, may increase or decrease NK cell function depending on the context (*126*). Interestingly, cytokines produced by NK cells, such as IL-10 during MCMV infection or IL-17 during toxoplasmosis, can skew the fate of NK cells based on the nature of infection and the microenvironment (*128*, *129*). IL-15 is an important cytokine in the development, survival, maintenance, proliferation, and cytotoxicity of NK cells (*130*). It was reported that other cytokines, such as IL-12, IL-18 and IL-2, have been associated with a dramatic increase in granzyme B and perforin reservoirs in resting NK cells, and therefore correlate with increased effector functions (*130*). The combination of IL-18 with IL-12 promotes naive cell proliferation and stimulation, resulting in the production of

cytokines such as IFN-γ and TNF-α (*131*). FasL expression on tumor cells is mediated by IL-2, a classic example of cytokine regulation (*5*). Biologically active secreted proteins known as chemokines, which are critical regulators of NK cell trafficking, provide activation signals for NK cells. Under inflammatory conditions, the expression of CCR2, CCR5, CXCR3, and CX3CR1 has been associated with the recruitment of NK cells to different tissues, thus regulating NK effector functions (*132*). Additionally, cellular interactions between NK cells and other immune cells are critical for the effector functions of NK cells. For example, IFN-α, IL-18, and IL-12, derived from dendritic cells (DCs), and cell-surface receptors engagement are required to activate naive NK cells. Another example is the trans-presentation of IL-15 to the NK cells mediated by activated DCs (*133, 134*). In human immunodeficiency virus (HIV) infection, the production of IFN-γ by NK cells was associated with IL-2 derived from $CD4^+$ T-cell boosts and maintained NK cell activation and proliferation (*135*). Finally, interactions between NK cells and neutrophils modulate NK cell development and function. It was reported that NK cells derived from both neutrophil-deficient mice and patients with severe neutropenia demonstrated impaired cytotoxicity (*136*). Collectively, the modulation of NK cell cytotoxicity and regulatory functions is a complex phenomenon with multiple layers of regulation, including numerous cytokines, chemokines and immune cells.

1.7 Effector NK cell functions: cytotoxicity, cytokine release

NK cells mediate their immunomodulatory effects via two important effector functions. First, NK cells are cytotoxic lymphocytes that can directly lyse cells that have become infected with a virus or undergone a malignant transformation (*137*). The primary initiators of this function are degranulation and death receptor ligation. When an NK cell contacts a target cell, it forms an immunological synapse (IS), which further triggers the aggregation of lytic granules containing perforin and granzyme at the IS. The disruption of the membrane at the IS to access target cell cytoplasm is mediated by perforin, a cytolytic protein (*138*). The initiation of the extrinsic apoptotic pathway is mediated by the activation of death receptors presented on the target cell surface (*139*). Death receptors such as TNF-related apoptosis-inducing ligand-receptor (TRAIL-R T) and Fas (CD95) are activated by binding to their respective ligands, TRAIL and Fas ligand (FasL, CD95L), and expressed by NK cells (*140*). In turn, IFN-γ derived from NK cells can induce the expression of death receptors on the surface of target cells, thus initiating pro-apoptotic signaling programs (*141-143*). The utilization of a cytoplasmic death domain characterizes the

death receptor superfamily and activates an apoptotic initiator such as caspase-8 or -10 (*144, 145*). Caspases-8 and -10 promote the IL1β-converting enzyme (ICE) superfamily of proteases, including caspase-3, resulting in the formation of an apoptosome after the induction of mitochondrial damage and cytochrome C release (*146, 147*). Substrate cleavage and DNA fragmentation via caspase-activated DNase are mediated by apoptosome and caspase-3, resulting in apoptosis (*148, 149*). The release of lytic molecules to the target cell is directed by NK cell-mediated cytotoxicity (*150*). These molecules are stored by NK cells in cytolytic granules and delivered through membrane fusion at the IS to the target cell (*138*). Cytoskeletal reorganization is required for this process (*151, 152*). Such reorganization events include actin polymerization at the IS and the polarization of the microtubule organizing center toward the target cell (*153*). Molecules contained in the lytic granules include perforin, granzymes, FasL (CD178), TRAIL (CD253), and granulysin. Among these, granzyme B and perforin are the most critical components (*140, 154, 155*). Once inside the target cell, granzyme B can trigger apoptosis through caspase-dependent apoptosis by directly cleaving the apoptotic initiators caspase-8 and caspase-3 (*156, 157*). In a caspase-independent manner, granzyme B can induce apoptosis through the proteolytic cleavage of the pro-apoptotic protein, Bid (*158*).

Secondly, a variety of inflammatory cytokines are produced by NK cells in response to activation receptor stimulation and inflammatory cytokine-induced activation signaling (*159, 160*). Depending on the inflammatory environment, NK cells are known to produce a wide range of cytokines that primarily produce Th1-type cytokines when responding to intracellular pathogens or tumor ligands (*161-164*). For example, IFN-γ, TNF, and granulocyte/monocyte colony-stimulating factor (GM-CSF) facilitate the activation of T cells and/or other innate immune cells such as dendritic cells (DCs), macrophages, and neutrophils (*165, 166*). NK cells also produce chemokines such as CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CXCL8 (IL-8), which play a role in the recruitment of effector lymphocytes and myeloid cells to the site of inflamed tissues (*167*). Transcription factors, such as T-bet and Signal Transducers and Activators of Transcription (STAT) 4 and 5, regulate the production of cytolytic molecules and inflammatory cytokines (*168, 169*). Collectively, NK cell effector functions (cytotoxicity, cytokines) are the primary mechanisms in which NK cells mediate their protective immune responses.

1.8 Regulatory functions of NK cells

Recent reports suggest NK cells also play a vital role in mediating regulatory functions (*170, 171*), either by cell-cell contact or by soluble factors that communicate with other immune cells such as dendritic cells (DCs), macrophages, and T cells (*172-175*). NK cell effector functions are thought to dictate the threshold and outcome of the immune response during the early phase. Such NK-cell-mediated regulatory functions occur during infections, antitumor immune responses, and autoimmune diseases (*5*). During the initiation of the immune response, IL-10-producing NK cells can control excessive inflammation (*176*). For example, NK cells produced IL-10 that controls liver inflammation in acute murine cytomegalovirus (MCMV) infection (*177, 178*). NK-derived IFN-γ has been implicated in a wide variety of immune responses such as viral defense, skewing Th1 and promoting cytotoxic T lymphocytes (CTL) responses, and maturation of DC and macrophages (*5*). IFN-γ, TNF-α, and cell-cell contact-dependent signals induce DC maturation before they gain access to T-cell-enriched zones in the LNs, enhancing antigen-specific T cells responses (*179, 180*). Moreover, NK cells mediate the elimination of deregulated immature DCs from the site of infection (*181, 182*). Similarly, the activating receptors NKG2D and NKp46 mediated NK cell cytotoxicity targets in highly activated macrophages and resting microglia (*183, 184*). Immune regulation by NK cells also mediates the blocking of an inappropriate T-cell polarization to avoid immunopathology. By releasing IFN-γ and providing 2B4-CD48-mediated co-stimulation to T cells, NK cells have been involved in skewing Th1 responses. However, other studies have shown that the elimination of highly activated and auto-reactive T cells is mediated by NK cells. During infection, highly activated and auto-reactive T cells express stress ligands that significantly increase their susceptibility to NKG2D- and NKp46-mediated NK killing (*185, 186*). Shedding of these activating ligands is a critical negative regulatory function on both T and NK cells (*187-189*). These observations suggest the significance of NK-cell-mediated negative regulation to prevent possible autoimmune disorders (*190, 191*). Additionally, it has been reported that NK cells also accelerate antitumor and antiviral $CD8^+$ T-cell responses in vivo (*192*). In a murine model of influenza, NK-cell-derived IFN-γ facilitates $CD8^+$ T-cell recruitment to the peripheral LNs (*193*). The maturation of DCs mediated by NK cells resulted in IL-12 production and subsequently promoted priming and generation of CTLs (*194*). Similarly, NK cell deficiency has been linked with aberrant induction and/or maintenance of alloantigen-specific CTLs, which results in uncontrolled growth of adoptively transferred tumors in different mouse models (*195,*

196). Besides inflammatory conditions, NK cells have been implicated in many physiological situations. For instance, NK cells were reported to be involved in B-cell differentiation and Ig isotype switching and decidual NK cells. Finally, NK cells are a source of vascular endothelial growth factor (VEGF), and placental growth factor (PLGF), which promote angiogenesis during pregnancy (*197, 198*).

1.9 NK cell migration: adhesion molecules and chemokine receptors

The ability of leukocytes to traffic consistently throughout the body is an essential requirement to maintain immunosurveillance. Spatially and temporally, a plethora of chemokines, chemoattractants, and adhesive molecules belong to the selectin, integrin, and Ig families regulate the migration of NK cells and other leukocytes across the endothelium (*132*). Selectins and integrins, which are adhesion molecules, contribute to the initial tying and rolling of leukocytes along the vessel endothelium. In contrast, integrins mediate strong leukocyte adhesion to the vascular endothelium and, later, diapedesis into the extravascular tissue (*199*). NK cell migration steps are tightly regulated: adhesion receptors must undergo cycles of attachment to and detachment from their endothelial ligands for effective migration (*5*).

Regarding the selection receptors family, L-selectin (CD62L) is expressed by the $CD56^{high}$ subset in peripheral blood, and NK cell activation modulates L-selectin expression (*200*). It was reported that IL-2, IL-15, and TGF-β downregulate L-selectin in the $CD56^{high}$ NK cell subset. On the contrary, IL-12, IL-10, and IFN-α are observed to be increased in both NK cell subsets. The binding of L-selectin ligands expressed by $CD56^{high}$ NK cells to peripheral lymph node high endothelial venules (LNHEVs) with higher efficiency results in a selective advantage of this population in extravasation compared to the $CD56^{low}$ subpopulation (*201*). Additionally, NK cells express PSGL-1 and cutaneous lymphocyte antigen (CLA), a marker for tissue-infiltrating leukocytes. Of interest, PEN5, sulfated polylactosamine carbohydrate epitope, and CLA expression are exclusive to mature NK cells, suggesting that distinct NK cell subsets exhibit different trafficking properties (*202*). Collectively, the differential expression of adhesion molecules and integrins following NK cell activation suggests the specialized recruitment of NK cell subsets in response to inflammation.

It was reported that NK cells express several receptors for C, CC, CXC, and CX3C chemokines (*203*). Resting versus activated NK cells have reported a significant heterogeneity in the chemokine receptor repertoire (*132*). It has been reported that human peripheral blood NK cells

express CXCR1 and CXCR2 as CXCL8 (IL-8) receptors and CX3CR1 as CX3CL1 (fractalkine) receptors (*200, 204*). Campbell and colleagues reported for the first time that distinct (CD56+CD16+) and (CD56+CD16–) peripheral blood NK cell subsets express a unique chemokine receptor repertoire (*205*). $CD16^+$ NK cells consistently express low levels of CXCR2 and CXCR3, and no CXCR5, high levels of CXCR1 and CX3CR1(*205*). Conversely, CD16–NK cells express low levels of CX3CR1, and are negative for CXCR1, CXCR2 and CXCR5, high levels of CXCR3 (*205*). However, both NK cell subsets express high levels of CXCR4, the receptor for CXCL12 (SDF-1α/β). A lack of CCR1-7 and CCR9 expression is reported in most NK cells, and high CCR5 and CCR7 levels are mainly associated with the homing of lymphocytes to secondary lymphoid organs, found only in the $CD16^-$ NK cell subset (*205*). Of interest, CX3CL1 (fractalkine) and CXCL8 (IL-8) preferably recruit the $CD16^+$ NK cell subset, which can respond to the CXCL11 (I-TAC), CXCL10 (IP-10) and CXCR3 ligands. In contrast, $CD16^-$ NK cells respond significantly to CCL19 (ELC/MIP-3β), CCL19 (ELC/MIP-3β), CCL21 (SLC), CXCL11 (I-TAC), CXCL10 (IP-10), CCR7, CXCR3 ligands. $CD16^-$ NK cells respond poorly to CCL2 (MCP-1), CCL4 (MIP-1β), CCR2 ligands, CCR5 ligands, and CCL5 (RANTES). Both $CD16^+$ and $CD16^-$ NK cell subsets strongly migrate in response to CXCR4, CXCL12 (SDF-1α/β) the ligand (*205, 206*).

1.10 Signaling events controlling chemokine-induced integrin-supported NK cell migration

Despite the evidence for the role of chemokines and integrins in leukocyte adhesion and dynamic regulation of migration, the integrin-supported leukocyte migration signaling pathways elicited by chemokines are still under investigation (*207*). The propagation of migratory signals depends on different factors such as complex interact between molecules that regulate actin, myosin, and cytoskeleton components, resulting in protrusive structures at the front of migrating cells and retraction at the rear (*208, 209*). The migration of NK cells and other leukocytes depends on the signaling pathways activated by integrin and chemokine receptors (*132*). Protein tyrosine kinase (PTK) activation, a required event for leukocyte migration, controls integrin adhesiveness and chemotactic response (*210*). The involvement of PTK belongs to the Src and Syk/Zap families in the cell migration and documented mainly in T lymphocytes and myeloid lineage cells. In NK cells, the engagement of LFA-1 results in both Src and Syk kinase activation (*211*). However, these events have been associated with the cytotoxic function of NK cells rather than migratory

ability (*212*). Inhibition of PTK by the specific Lck inhibitor damnacanthal, the general tyrosine kinase inhibitor herbimycin A, or the Syk inhibitor piceatannol, leads to tyrosine phosphorylation and activation of Lck and Src kinase-induced NK cell chemotaxis (*200, 213*).

The p125 focal adhesion kinase (p125Fak) and the proline-rich tyrosine kinase 2 (Pyk-2), recognized as two members of cell adhesion kinase-β (CAK-β) or related adhesion focal tyrosine kinase (RAFTK), were investigated (*214, 215*). Rabinowich H and colleagues reported that NK cells β1 integrin engagement results in p125 focal adhesion kinase (FAK) activation. The p125 FAK is known to be associated with Fyn and Zap-70 PTK (*215*). On the contrary, the expression of Pyk-2 by human peripheral blood NK cells has been reported to be significantly associated with the cytoskeletal protein paxillin but not with p125 FAK(*210*). The tyrosine phosphorylation of both Pyk-2 and paxillin is mediated by the engagement of β1 or β2 integrins on human NK cells (*200, 216*). Moreover, the trigger of MAPK (Mitogen-activated protein kinase) cascades and the development of NK cell natural cytotoxicity are controlled by Pyk-2 when it acts as an upstream mediator of β1 and β2 integrin (*216-218*). It was reported that the binding of NK cells to the endothelium activates both Pyk-2 and the small GTP-binding protein Rac, critical regulator of actin cytoskeleton dynamics (*219*). The functional involvement of Pyk-2 and Rac in chemokine-induced NK cell migration was reported: the use of recombinant vaccinia viruses encoding dominant-negative mutants of Pyk-2 and Rac showed that the Pyk-2/Rac pathway plays a pivotal role in the control of NK cell transendothelial migration (*210, 220*).

Other signaling intermediates such as PI3K and its products play a crucial role in cell migration. In this regard, the involvement of PI3K on chemokine-mediated NK cell chemotaxis was explored. The inhibition of C, CC, and CXC chemokine-induced NK cell chemotaxis was reported in response to wortmannin and anti-PI3K-γ, but not anti-PI3K-α antibodies suggesting that PI3K IB plays a critical role in the activation of NK-cell-induced chemokines (*221, 222*). Of interest, NK cells stimulated by regulated on activation, normal T Cell Expressed and Secreted (RANTES) recruited PI3K-γ into their cell membranes (*223*).

1.11 Dendritic cells (DCs)

Dendritic cells (DC), a distinct class of leukocytes, are specialized antigen-presenting cells (APCs) first defined by Ralph Steinmann and colleagues in 1974 (*224*). DCs are described as the master regulators of the immune system, mediating innate immunity and shaping adaptive immunity. Upon stimulation, DCs undergo a maturation process that allows them to migrate to

secondary lymphoid organs to activate and shape T cell differentiation (*225*). Therefore, DCs play critical roles in mediating the induction of $CD4^+$ and $CD8^+$ T cell responses against different pathogens. Various DC subsets have been identified, and DC expression profiles have also revealed a remarkable heterogeneity (*226*). It is believed that DCs belong to the myeloid lineage and are derived from HSCs (*227*). BM myeloid precursors turn into differentiated DCs in the presence of GM-CSF (*228*). Furthermore, irradiated mice yield various subtypes of DCs in the spleen and thymus when common myeloid progenitors are transplanted into them, suggesting commitment to DC lineage (*229*). However, a subtype of DCs acquires some lymphoid markers such as CD8a, CD4, and CD2, suggesting an alternate lymphoid origin (*230*). Overall, DCs follow various differentiation pathways, as shown by their generation from CMPs and CLPs in vitro and in vivo (*231*).

1.11.1 Types of DCs

Broadly, DCs are classified as conventional (cDCs), plasmacytoid (pDCs), and inflammatory DCs that include monocyte-derived DC (mDCs) and Langerhans cells (LCs). These are the major DC subsets studied in most infection/ inflammatory models (*232*). In mice, DCs acquire MHC class II, CD4, CD8a, CD11b or/and DEC-205 receptors. However, all DCs express CD11c (*229*).

1.11.2 Conventional DCs

Also designated as classical DCs, cDCs predominantly reside in lymphoid tissues such as the thymus, spleen, and secondary LNs. cDCs can collect and present antigens from the organ of their residence. Higher expression levels of MHC class II, CD11c and the expression of several pattern recognition receptors defined cDCs (*233*). In mouse, Based on CD8a expression, cDCs have been divided into cDC1 and cDC2, constituting 20% and 40% of the spleen DC population, respectively (*226, 233*). cDC1 is mostly associated with CD8+ T cells initiation responses, whereas cDC2 has several functions that are usually regulatory (*234*). It has been reported that cDC1 cells are located in the blood, LNs, spleen, BM, skin, tonsils, intestine, liver, and lungs (*232*), and because they are effective APCs, they significantly contribute to viral clearance through the induction of $CD8^+$ T cells (*234*). In contrast, cDC2 cells have been found in the lungs, liver, blood, and intestines (*232*). It was reported that responses of type 1 helper T cells (Th1) to herpes simplex virus (HSV) type 1 are mainly dependent on cDC1 antigen presentation (*235*). However, cDC2 have demonstrated their ability to enhance Th1, Th2, Th17, T follicular helper and $CD8^+$ T cell

responses (*234*, *236*, *237*). While CD141 and Clec9A expression characterized cDC1, CD1c defines cDC2 (*234*). Unlike mouse cDCs, human cDCs do not express CD8. Of interest, MacDonald K and Hart D defined the CD141 (BDCA3)$^+$ DC subsets in human blood that displays mouse CD8+ DC-like features (*238*). The transcriptome meta-analysis of mouse and human DC subsets showed a similar characteristic between mouse CD8+ DCs and human blood BDCA3+DCs (*239*).

1.11.3 Plasmacytoid DCs (pDCs)

Initially, in the human spleen and BM, pDCs were identified and shown to circulate in peripheral blood (*240*). These cells are defined by their ability to produce tremendous amounts of type I interferon (IFN), IL-12, TNF-α, and several chemokines (such as CCL3, CCL5, CXCL10), which enhance NK cell cytotoxicity, T-cell survival, and macrophages in addition to promoting antibody production by B cells to impair the replication of viruses (*241*, *242*). Furthermore, pDCs are characterized by the expression of CD303, CD304, CD123, and CD45RA in humans. In contrast, murine pDCs acquire low CD11c, MHC class II, and the expression of co-stimulatory molecules, which might further compromise their ability to function as efficient APC (*242*, *243*). pDCs derived from the BM and generated from both common myeloid DC progenitors (CDPs) and CLPs but are mainly of "myeloid" origin (*243*). Unlike myeloid DCs, these cells do not express TLR 1, 2, 3, 4, 5, or 6. However, among all DC subsets, pDCs appear to be unique in their expression of TLR9 (*244*). In infection, differentiated pDCs acquire the phenotypes and some of the functions of the conventional DCs, which are called pre-cDCs populations in the literature (*245*). pDCs constitute a minor population in the lymphoid organs. In contrast to classical DCs, the entrance of pDCs into the LN is mediated by HEVs (*246*). pDCs further contribute to the first line of defense against viruses by recruiting cytotoxic NK cells to infection sites (*242*, *247*).

1.11.4 Inflammatory DCs

In addition to classical and other migratory DCs, human and mice harbor a subtype of inflammatory DCs called monocyte-derived dendritic cells (moDCs). These cells differentiate from monocytes after interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation in vitro, tend to acquire the characteristic phenotype (MHCII$^+$ CD11b$^+$ CD11c$^+$ F4/80$^+$ Ly6C$^+$), and thus relocate to the site of infection. Although moDCs in vivo equivalents have been debated, they most closely resemble CD14$^+$ CD1c$^+$ SIRPα$^+$ CD206$^+$ FcεRI$^+$ inflammatory DCs (*226*). In HIV type 1 (HIV-1) infection, Granelli-Piperno et al. reported that

infected moDCs mediated IL-10, an immune evasion strategy, resulting in immunosuppression and the inhibition of moDCs maturation and antigen presentation (*248*).

Langerhans cells (LCs) reside in the skin's epidermis layer in a steady steady-state, share many functional roles with DCs and, oddly, possess macrophage- and DC-specific transcription factors (*249*). They can be distinguished from cDC2 by their higher expression of Langerin, CD1a and epithelial cell adhesion molecule (EpCAM) alongside their lower expression of CD11c, CD11b and CD13 (*232*). LCs can sample antigen in multiple tissues during inflammatory conditions, such as mucosal oral, epidermis, vaginal epithelium, and lungs. They then migrate to draining lymph nodes (dLNs) to act as APCs (*249*, *250*). For example, LCs play a crucial role in preventing ectromelia virus dissemination by coordinating a type Immune-inflammatory response (IIR) cytokine response in dLNs (*251*).

1.12 DC functions

DCs are specialized antigen processing and presenting cells that produce a broad range of cytokines (such as IL-18, IL-12, IL-15, IFN-α, IFN-β) to regulate immune function at the interface between innate and adaptive immunity (*252*). DCs exist in two different functional states, "mature" and "immature," which are distinguished by many features. However, mature DCs have the ability to trigger antigen-specific naive T cells in secondary lymphoid organs (*253-255*). Pathogen-associated molecular pattern (PAMP) or damage-associated molecular pattern (DAMP) recognition detected tissue homeostasis disturbances that triggered DC maturation (*256*). Therefore, metabolic, cellular, and gene transcription programs turned on by maturation allow DCs to migrate from peripheral tissues to T-cell-dependent areas in secondary lymphoid organs to activate T lymphocytes (*226*).

Through maturation, DCs lose adhesive structures and endocytic activity, reorganize their cytoskeleton, and increase their motility (*257*). However, mature DCs increase MHC class II and co-stimulatory molecules (such as CD40, CD80, CD86) expression (*258*). For T cell activation, mature DCs expressed higher levels of the chemokine receptor (CCR7) and cytokines (*259*, *260*). Therefore, antigen-specific immune responses are triggered by the interaction between mature DCs and antigen-specific T cells (*261*). Upon interaction, DC may induce $CD4^+$ T cell differentiation into different helper T cell (Th) subsets such as Th1, Th2, Th17, or other $CD4^+$ T cell subtypes (*262-266*). This differentiation is mediated by different factors such as cytokines in the DCs' tissue of origin, their maturation state, and the cause of tissue imbalance (*267-269*). The

ability to cross-present is a unique characteristic of DCs (*270*). This phenomenon was explained by Bevan in 1976 and defined as the presentation of antigens captured from the extracellular environment (*271*). This phenomenon allows DCs to trigger responses against the intracellular antigens of other cell types, deal with threats that circumvent specialized APCs, and prime $CD8^+$ lymphocytes in the absence of $CD4^+$ T cells (*272*, *273*). Induced tolerance to self-antigens that are not expressed by APC, called cross-tolerance, is also mediated by the cross-presentation process (*274*).

Before DCs receive maturation stimuli, DCs are considered "immature." The hallmark characteristics of immature DC are less induction of naive lymphocyte effector responses, low surface expression of co-stimulatory molecules, low expression of chemokine receptors, and an absence of immunostimulatory cytokines (*226*). However, immature DCs efficiently capture antigens through receptor-mediated endocytosis (lectin or Toll-like receptors) or micropinocytosis (Fc-complement receptors) (*275-277*). Thus, immature DCs act as guards against invading pathogens (*255*). These cells can also act as tissue scavengers, capturing apoptotic and necrotic cells (*278*). This allows immature DCs to play a crucial role in the initiation and maintenance of immune tolerance (*279*). For example, apoptotic cells are internalized by DCs, but this does not induce DC maturation (*280*). Antigens from the internalized cells are then presented to T cells with no delivery of activating co-stimulatory signals, resulting in the apoptosis of T cell, anergy, or regulatory T cells development (*281-284*). These "tolerogenic DCs" are essential to prevent responses against healthy tissues through less expression of co-stimulatory molecules and proinflammatory cytokines, upregulation of inhibitory molecules expression (like PD-L1 and CTLA-4) and the secretion of anti-inflammatory cytokines (such as IL-10) (*283, 285, 286*). However, immature DCs can be harmful to the body when they fail to induce lymphocyte effector responses to fight infections or tumors, resulting in "immune control escape" (*287, 288*). Collectively, the status of DC maturation, mature or immature, determines the cell's function (**Figure 1.4**).

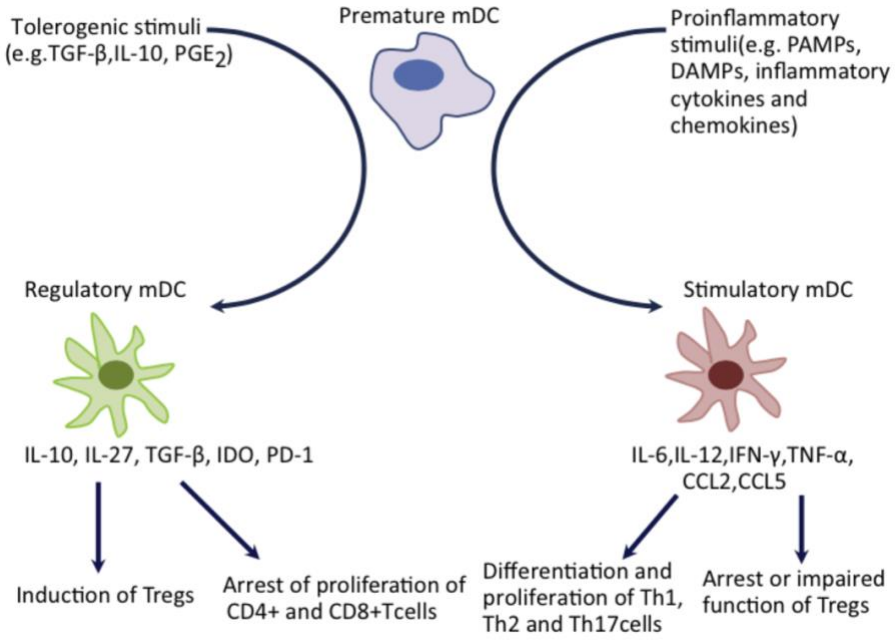


Figure 1.4. Stimulatory and regulatory function of mDCs. Inflammatory cytokines, chemokines, and pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) drive the differentiation of immature mDCs to stimulatory mDCs. Mature stimulatory DCs produce cytokines and chemokines that promote naive T cell differentiation to effector T cells (Th1 and Th17) cells. Stimulatory mDCs can inhibit Tregs immunosuppressive function. On the other hand, anti-inflammatory cytokines such as (TGF)- ß, interleukin-10 (IL-10), and prostaglandin E2(PGE2) activate the regulatory properties of mDCs. Regulatory mDCs produce indoleamine 2,3-dioxygenase (IDO) and programmed cell death protein 1 (PD-1) ligand to suppress effector T cells activation and proliferation. Reproduced with permission from (*289*).

1.13 NK/DC crosstalk

Recently, the concept of NK/DC crosstalk concept has attracted much attention as a regulatory mechanism linking both innate and adaptive immunity. This concept provides a mechanistic framework explaining the initiation and amplification of both the cellular and humoral immune responses. NK/DC crosstalk is bidirectional, where NK cells interact with DCs through cell-to-cell contact and/or soluble factors. This interaction results in maturation, activation, and cytokine production by both NK cells and DCs. DCs mediate the activation of NK cells to contribute to innate immunity. In turn, activated NK cells provide signals essential for DC activation and maturation, thus enhancing adaptive immunity responses (*290*) (**Figure 1.5**). *In vivo*, the bidirectional crosstalk between NK cells and DCs arises in the LNs, inflammation site, peripheral tissues, and tumor microenvironments (*291-293*).

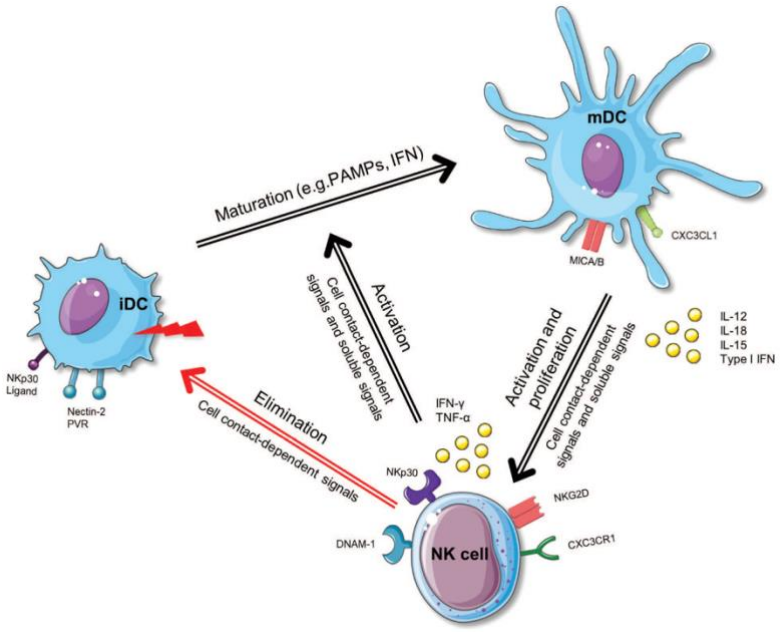


Figure 1.5. **Interplay between DCs and NK cells (NK/DC crosstalk).** The exposure of iDCs to pathogen-associated molecular patterns (PAMPs) and IFN can mature DCs. The mDCs produce IL-12, IL-18, IL-15 and type I IFN, which triggers NK cell proliferation and activation, resulting in further production of IFN-γ. The interaction of NK-DC via NKG2D with MIC-A/B or CXC3CR1 with CXC3CL1 can also result in NK cell activation. Therefore, activated NK cells can enhance DC maturation via IFN-γ and TNF-α and the interaction of the NKp30 receptor on NK cells with its ligand on DCs. On the other hand, activated NK cells eliminate MHC-I low-expressing iDCs via NKp30 receptor with NKp30 ligand and DNAM-1 with Nectin-2 or PVR. Reproduced with permission from (*294*).

1.14 DC-activated NK cells

DCs have a potential role in influencing the function of NK cells. For example, it was reported that CD8α DC-depleted mice showed a reduction of NK-cell-dependent antitumor functions, suggesting that DC triggering of NK cells is dependent on cell-to-cell contact and soluble factors (*293*). Further studies have shown that IL-12 produced by DC is a critical factor for NK cell activation, precisely for NK cell IFN-γ production (*295*). Such interactions mediate the establishment of stimulatory synapses between DCs and NK cells to enhance the polarization of pre-assembled stores of IL-12 secretion by DCs toward NK cells (*296*). Additionally, DCs treated with poly(I:C) and IFN-α promote NK cells to secrete IFN-γ through the binding of the activating NK cell receptor NKG2D to its ligands, like MHC class I-related chains A and B (MIC-A/B) (*297*, *298*). Furthermore, DCs express CXC3CL1, which binds to CXC3CR1 expressed by NK cells, resulting in NK cell cytotoxicity and IFN-γ release (*299*).

DC-derived IL-15 mediates NK cell activation, proliferation, survival and enhances NK cytotoxic functions (*300*). Moreover, NK cell proliferation, CD69 expression, and IFN-γ secretion are enhanced by transmembrane tumor necrosis factor (TNF) and membrane-bound IL-15 in DCs (*301*). Of note, CD40 and B7 molecules expressed by DCs bind to their respective NK cell receptors (CD40L and CD28), enhancing NK cell proliferation (*302*).

IL-18 expressed by DCs has been implicated in NK cell activation, and both IL-18 and IL-12 are considered essential players involved in the upregulation of NK cell cytotoxicity (*303*). However, regulatory DCs produced an insufficient amount of IL-18 to restrain IFN-γ secretion by NK cells, therefore, downregulates NK cell activation (*304*). In mice, the expression of IL-18 and IL-12 by $CD8\alpha^+$ DCs is involved in expanding $Ly49H^+$ NK cells (*305*). In NK/DC crosstalk, secreted type I IFN by plasmacytoid DCs (pDCs) is required for NK cell cytotoxicity in response to virus infection (*306, 307*). In the context of TLR stimulation, the recognition of type I IFN signals and production and trans-presentation of IL-15 by DCs to resting NK cells are vital in priming NK cells (*134*). In vitro and in vivo, the production of IL-2 by activated myeloid DCs is required for NK cell activation and IFN-γ production (*308*).

1.15 DC modulation by activated NK cells

Over the last 20 years, multiple studies have shown that NK cells can interplay with DCs to modulate the DC maturation process, either by direct stimulation or by eliminating DCs that do not acquire a mature phenotype (*309*). Activated NK cells help improve DC maturation and activation, increasing their ability to induce T cell responses. When NK cells are grown in culture with immature DCs in the presence of a maturation stimulus such as lipopolysaccharide (LPS), NK cells support DCs maturation, indicated by the upregulation of DC co-stimulatory molecules such as IL-12 and CD86. IL-2-activated NK cells can also enhance DC maturation and improve their ability to induce allogeneic naive $CD4^+$ T cells. These effects of NK cells on DC maturation are cell-contact-dependent (*310, 311*). Furthermore, DCs activated by NK cells produce higher levels of IL12p70 after the stimulation of CD40 ligands, leading to enhanced induction of T cell activity (*312, 313*). The type of NK cell activation determines the effect on DCs: IL-2-primed "effector" NK cells can kill DCs; conversely, dependent on CD40L stimulation, IL-18-primed NK cells enhance the production of IL-12p70 by DCs (*314*). Indeed, the trigger of immature DCs to produce IL-18 is mediated by NK cells via a Ca^{2+}-dependent and tubulin-mediated recruitment of IL-18-containing secretory lysosomes to the NK cell. Thus, the induction of DC maturation and

the protection of DCs from lysis is facilitated by NK cells activated with IL-18 through the secretion of proinflammatory "danger signal" high-mobility group B1 (HMGB1) (*315*). Also, the exposure of human NK cells to different cytokines promotes the priming of Th1. NK cell activation by IL-12 or IL-2 induces DC maturation capable of priming IFN-γ-production by Th1 cells, whereas NK cells activated with IL-18 stimulate Th1 polarization when co-cultured with both DCs and T cells, which release IL-12 and IL-2, respectively, enhancing IFN-γ production. Thus, the local expansion of such cytokines suggests a possible modulation of NK/DC interaction at the inflammatory sites of inflammation, resulting in different naive T cell polarization outcomes (*316*). In a recent study, IL-23 was proposed to increase the ability of NK cells to stimulate DCs: IL-23 enhanced NK cell activation and displayed a synergistic effect with IL-18 on IFN-γ production. IL-23 also supported CD86 expression and IL-12 secretion by LPS-stimulated DCs upon contact with IL-18-stimulated NK cells (*317*).

The type of NK-cell-activating receptors and the number (density) of MHC class I molecules present on the DC membrane determine the process by which immature DCs are lysed (*318, 319*). In vitro, it has been reported that activated NK cells can recognize and kill DCs via the NKp30 NCR, suggesting that the NKp30 ligand, which is still unknown, is possibly expressed by DCs. However, when MHC-I antibodies block the NK cell inhibitory signal, mature DCs may be lysed by NK cells, confirming that mature DCs are naturally spared due to their high expression of MHC class I molecules (*181*). Furthermore, the DNAM-1 receptor and its ligands, poliovirus receptor (PVR) and Nectin-2, have been reported to play a critical role in NK-cell-mediated lysis of immature DCs. In the NK-cell-mediated elimination of DCs, the DNAM-1 receptor on NK cells interacts with the NKp30 receptor. The degree of contribution of DNAM-1is determined by how it binds its specific ligands PVR and Nectin-2 on the DC surface (*320*). In vivo, NK cells kill injected immature BM-derived DCs via a TRAIL-dependent pathway (*321*). Likewise, NK cells were found to kill immature DCs via the TRAIL–Death Receptor 4 (DR4) pathway in the viral infection context (*322*). Collectively, such observations support the hypothesis that NK cells can kill immature DCs and promote the survival of immunogenic DCs, to facilitate efficient and protective immune responses. For example, autologous NK cells kill immature DCs and thus significantly expand cancer-specific CTLs (*323*).

In contrast, in chronic viral infections, NK cells produced IL-10, resulting in phenotypic changes to DCs. In this context immature DCs show resistance to elimination by NK cells, whereas

mature DCs have increased sensitivity to NKG2D-dependent elimination. This process leads to the accumulation of poorly immunogenic DCs in LNs, resulting in immune dysfunction (*324*). Therefore, the lysis of DCs by NK cells can negatively regulate viral T-cell responses in vivo by limiting T cell exposure to infected APCs, negatively impacting the quality of the T cell response and its ability to limit viral persistence (*325*). In organ transplantation, it was reported that the elimination of allogeneic DCs is mediated by host NK cells via the perforin pathway. As a result, the allogeneic antigen presentation to host lymphocytes will be limited, reducing T cell-mediated graft-versus-host disease (*326*).

1.16 NK/DC interplay in tumors

Cancer immunotherapy aims to shift the balance from cancer cell escape or equilibrium to elimination. Recent studies have therefore been focused on increasing the activation status of both innate and adaptive immunity in a number of ways, including cytokine administration, chimeric antigen receptor (CART) cells, DC- and NK-based vaccines, monoclonal antibodies, and checkpoint inhibitors engineered to target oncogenic signaling pathways (*327, 328*). The development of such approaches was based on the definition of an optimal antitumor immune response. An optimal response depends on a complex network interplay where DCs, NK cells, and $CD8^+$ and CD4+ T cells are involved in mediating the interface between innate and adaptive immunities and the capacity to interact and modulate immune effector functions (*294*). Since the 1990s, DCs have been described as desirable cells with full translational and clinical potential, and DCs have been tested in clinical trials as cellular antitumor vaccines (*329*). As mentioned previously, NK cells can eliminate tumor cells “directly” or “indirectly” to enhance adaptive antitumor immunity by enhancing DC maturation and by killing immature DCs, thus favoring immunogenic DC populations that polarized antigen-specific CTLs (*309, 310*). Clinically, NK cells have been investigated recently in many immunotherapeutic strategies for cancer. Some shreds of evidence have shown that the NK/DC crosstalk is disrupted in the tumor microenvironment (*330*). Myeloid-derived suppressor cells (MDSC) in the tumor microenvironment were reported to show their ability to promote the development of regulatory T cells (Treg), which in turn results in IL-10 production to induce immune suppression (*331*).

In contrast, Treg depletion enhances DC recruitment in the LN and improves NK cell activation and proliferation in a murine tumor model (*332, 333*). In B-cell lymphoma, the ability of infiltrated NK cells to produce IFN-γ may be associated with a reduction in IL-12 expression

by dendritic cells (*334*). MDSC-derived TGF-β is involved in the inhibition of IFN-γ production by NK cells (*335*). Additionally, TGF-β blocks NKp30 expression, impairing NK/DC communication and accumulating immunosuppressive iDC (*336*). Likewise, in B16F10-OVA tumor-bearing mice, the induction of IFN-γ by NK cells following administration of DCs further supports the significance of NK/DC crosstalk in tumor immunity (*337*). Another study has shown that the disruption of NK/DC communication is mediated by proteoglycans (PGs), where PGs modulate DC functional phenotype and compromise NK activation (*338*). Tumor-infiltrated DCs reported the impairment of the IFN-α production that is required for NK cell activation (*339*). Of interest, in the clinical setting, the induction of NK/DC crosstalk subsequently improves the T-cell response following administration of therapeutic monoclonal antibody treatment (*340*).

1.17 NK/DC crosstalk in infection

NK/DC crosstalk plays a vital role in controlling viral infection. A role for NK/DC interaction in shaping the adaptive immune response has been reported in different infection models such as HIV, hepatitis C virus (HCV) and mouse cytomegalovirus (MCMV) infection (*341*).

During HIV-1 infection, Mavilio and colleagues have shown that there was an impairment in the interactions between CD56– NK cells and autologous DCs from HIV-1-infected viremic but not aviremic individuals, resulting in flawed NK/DC bidirectional activation and DC maturation as well as abnormalities in immature DCs apparently caused by NK cells (*342*): an increase in CD56– NK cells with defective NKp30 receptor function was responsible for the immature DC abnormalities. Therefore, HIV-1-infected DCs become resistant to TRAIL-mediated apoptosis induced by NK cells. Such resistance results from HMGB1, which mediates the upregulation of the anti-apoptotic molecules cellular inhibitor of apoptosis 2 (c-IAP2) and cellular-Flice like inhibitory protein (c-FLIP) (*343*). *In vitro*, HIV infection induces IL-10 production by DCs, which supports the resistance of immature DCs to elimination by NK cells (*324*). Also, pDCs from HIV-infected individuals have a deficiency in IFN-α and TNF production (*344, 345*). Similarly, cDCs from HIV-1-infected individuals showed impairment in IL-12, IL-15, and IL-18 secretion, resulting in less NK cell activation; therefore, stimulation of NK cells with these cytokines restored the ability of NK cells to eliminate HIV-1-infected cells (*346, 347*).

In MCMV infection, pDCs recognize viral CpG DNA through the TLR9/MyD88 pathway and produce IFN-α/β and IL-12. Those cytokines promote NK cell cytotoxicity, IFN-γ production,

and cDC functional maturation, which together promote an effective antiviral $CD8^+$ T-cell response (*348*). Activation of NK cells is mediated by $CD11b^+$ myeloid DCs through NKG2D-ligand interactions and through IFN-α/β, IL-18, and IL-12 production (*349*). Such effects require TLR2 and/or TLR3 activation but not TLR9 (*350*). $CD8\alpha^+$ DCs infected with MCMV produce high levels of IL-12 and IL-18, which supports the expansion of $Ly49H^+$ NK cells (*305*). Also, MCMV infection was reported to induce high levels of IFN-α/β in cDCs, promoting their survival and delaying the antiviral response of $CD8^+$ T cells (*351*). Of interest, during the early stages of MCMV infection, $Ly49H^+$ NK cells prevent the release of IFN-α/β to immunosuppressive levels, in turn preventing the loss of splenic $CD8\alpha^+$ DCs normally induced by MCMV infection (*192*). Collectively, creating a balance between the positive and negative effects of IFN-α/β and other cytokines is required for the optimal control of MCMV infection through NK cell and DC crosstalk.

In vitro, NK cells from chronic HCV-infected donors express higher NK inhibitory receptors such as CD94/NKG2A. Hepatic cells express HLA-E that binds to CD94/NKG2A, which results in HCV NK cells producing IL-10 and transforming growth factor-β (TGFβ) (*352*). These cytokines were found to modify DC activation and result in IL-10-producing T cells and $CD25^+CD4^+$ Treg (*351, 353*). However, the blocking of NKG2A on NK cells stimulated DCs to generate Th1-polarized CD4+ T cells. Such observations suggest NKG2A modulates the ability of DCs to induce HCV-specific adaptive immune responses, where HCV-infected DCs lose their production of IL-15 that is normally mediated by type I IFN. Compromised IL-15 production reduces DC expression of MHC class I-related chains A and B (MIC-A/B). NKG2D binds its ligands, MIC-A and MIC-B, to transduce the signals required for NK cell activation, which might explain the reduction in NK cells observed in individuals with chronic HCV infection (*354*, *355*). A recent in vitro culture study using DCs pulsed with HCV peptide showed a maturation defect when co-cultured with HCV NK cells. Alternatively, both activation and inhibitory markers were upregulated in HCV NK cells when co-cultured with healthy DC. Co-culture of DCs and NK cells both from a chronic HCV patient, or healthy NK cells co-cultured with DCs from a chronic HCV patient, resulted in higher apoptosis of both NK cells and DCs, suggesting the impairment of NK/DC crosstalk upon HCV infection (*356*).

1.18 Semaphorins

1.18.1 General overview: History, classification, structure

Semaphorins were first described in 1992–1993 by Alex Kolodkin and colleagues. They initially found a molecule named fasciclin IV, homologous to semaphorin 3A in different species, with repulsive activity in neurons of grasshoppers developing limp insect buds. (*357, 358*). In 1993, Yuling Luo and colleagues at the University of Pennsylvania did another study which led to the isolation of a new protein called "Collapsin-1" from the chicken brain, homologous to human semaphorin 3A (*359*).

The semaphorin/collapsin family is categorized based on identifying these unique sequences and functions (*360*). In addition to the nervous system where semaphorins are noted as axon-guidance molecules, other systems, such as gastrointestinal, endocrine, cardiovascular, musculoskeletal, respiratory, and immune systems, ubiquitously express semaphorins (*357, 359, 361, 362*). Semaphorins are involved in regulating differentiation, morphogenesis, angiogenesis, cell adhesion, proliferation, and cell migration (*363*).

In 1999, a standard nomenclature system was proposed for semaphorins. More than 20 semaphorin family members were identified and phylogenetically classified into eight classes. Classes 1 and 2 are found exclusively in invertebrates, while classes 3, 4, 6, and 7 semaphorins are found in vertebrates. Class 5 semaphorins are reported in vertebrates and invertebrates, and 8 is a class-specific to viruses. Classes 1 and 4–7 are considered membrane-associated proteins, whereas secreted semaphorins include classes 2, 3, and 8 (*360, 364*).

The N-terminal "sema domain" is the structural hallmark of all semaphorins. It comprises nearly 500 amino acids arranged in a seven-blade β-propeller fold. In the extracellular regions, the sema domain is tightly coupled to a cysteine-rich domain named the PSI (plexin-semaphorin-integrin) domain and IPTs (immunoglobulin domains shared by plexins and transcription factors). All mammalian semaphorins except class 6 contain "immunoglobulin (Ig)-like domains" or "thrombospondin type 1 repeats". According to structural and functional studies, homodimer forms are the only form semaphorins exert their roles, and no other shreds of evidence for functional semaphorin monomers or heterodimers were found. The sema domain is responsible for homodimerization and interaction with the receptors (*365, 366*). Some studies on semaphorin structure have recently shown that each sema domain of a semaphorin homodimer binds to a plexin sema domain to enhance plexin dimerization mediating signal transduction (*367, 368*). Plexins are

semaphorin receptors and are associated with other co-receptors in different tissues to endorse pleiotropic functions (*369*) (**Figure 1.6**).

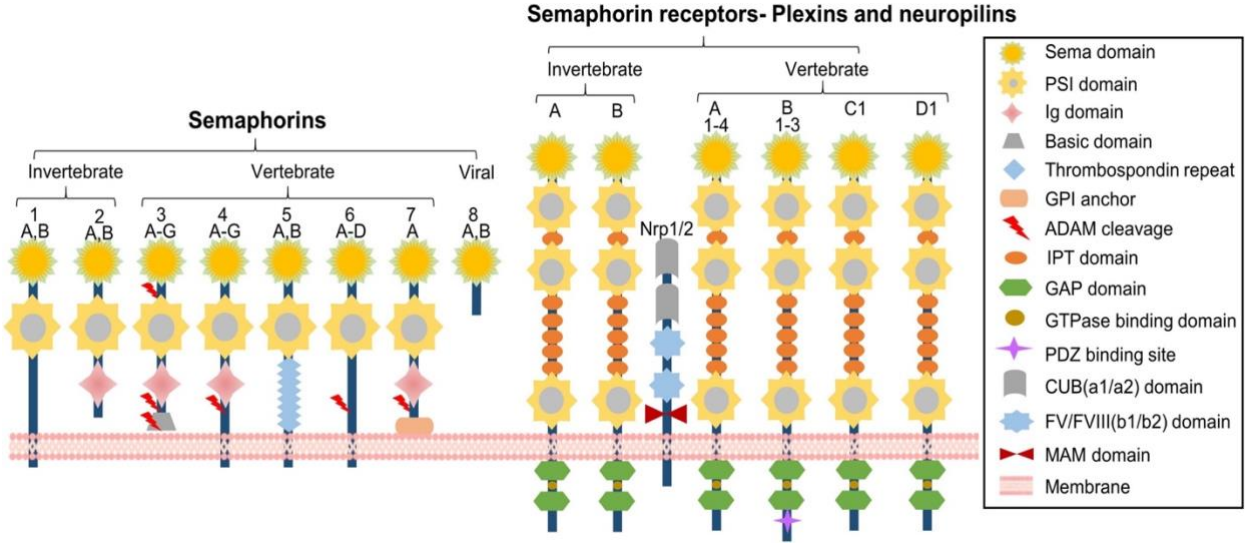


Figure 1.6. Semaphorins and semaphorin receptors. The semaphorin family is divided into eight classes. Classes 1 and 2 belong in invertebrates, whereas classes 3–7 are found in vertebrates. Semaphorins 2 and 3, and 8 are secreted, while semaphorins 4–6 are transmembrane proteins. Semaphorin members composed a large sema domain, a plexin-sema-integrin domain (PSI), immunoglobulin (Ig)-like domains, and thrombospondin repeats. Plexins (semaphorins receptors) found in invertebrates such as plexins A and B and vertebrates such as plexins A1–4, plexins B1–3, PlexinC1, and PlexinD1. Plexins constitute a sema domain, a PSI domain, and an Ig-like, plexins transcription factors (IPT) domain. Neuropilin is a transmembrane co-receptor consisted of complement-like (CUB) domains, FV/FVIII domains, meprin-like MAM domain, and a cytoplasmic tail. Reproduced with permission from (*370*).

1.18.2 Semaphorin receptors and co-receptors

Semaphorins mediate their effector functions through receptors are known as plexins. In mammals, plexins are classified into four groups based on their ectodomains' sequence similarity (*369*). Plexins are highly critical in axon guidance and vascular patterning; therefore, a loss of plexin function has been correlated with several pathologies such as autoimmune diseases, congenital disorders, and cancer (*371*). Of interest, plexins share a similar structure with semaphorins, including an N-terminal sema-PSI binding domain. Unlike the semaphorin sema domain, however, the plexin sema domain does not undergo dimerization (*372*). The transmembrane proteins neuropilin (NRP)-1 and NRP-2 have been reported to act as semaphorin co-receptors with plexin (*373*). The neuropilin extracellular domain contains two CUB (complement C1r/C1s) domains which bind class 3 semaphorins, two coagulation factor V/VIII

homology domains, which bind VEGF, and a MAM (meprin, A-5 protein, and receptor protein tyrosine phosphatase mu) domain involved in neuropilins dimerization (*373*). The interaction of NRP-1 and class A plexins is required for the binding of class 3 semaphorins except for semaphorin 3E (Sema-3E), which directly binds to PlexinD1 and independent of NRP-1 (*374*). Neural cell adhesion molecule L1 (L1CAM), another semaphorin co-receptor, has been reported to interact with NRP-1 (*375*). Moreover, receptor tyrosine kinases erythroblastic leukemia viral oncogene homolog 2 (ERBB2) and mesenchymal-epithelial transition (MET) interact with PlexinD1 and PlexinB (*376*). Sema-7A interacts with plexin-C and β1 integrin receptors in the immune, nervous, and cardiovascular systems (*377*). Sema-4A and Sema-4D, known as the "immune semaphorins," bind to TIM2 (T-cell immunoglobulin and mucin domain-containing protein 2) and CD72, respectively, on the surface of lymphocytes. However, they bind plexin receptors with a much lower affinity than semaphorin classes B and D (*378*). The functional outcome of semaphorin action in target cells appears to be determined by the relative interaction with specific (co)receptors. Therefore, it is critical to address the binding and signaling partners of each semaphorin, considering that semaphorin-holoreceptor interactions are context-specific and might differ from one body system to another.

1.18.3 Semaphorin functions

The repulsive role of semaphorins in guiding migrating axonal growth cones during development was the first finding described for these proteins in the nervous system. However, in 1999, an attractive guidance cue for semaphorins was described for the first time (*379*). Recently, semaphorins have been shown to enhance either attracting or repelling signals based on where their receptors and co-receptors are expressed and the context of the responding cells. Functionally, semaphorins are more likely to support target cell repulsion than attraction. Sema3A and Sema4D, for example, induce axonal collapse in the growth cone region of neuronal cells (*370, 380*). Sema3F has also been proven to inhibit tumor growth by reducing endothelial cell migration and adhesion (*381*).

In contrast, Sema5A enhances angiogenesis by increasing endothelial cell proliferation and migration and suppressing apoptosis (*382*). However, individual semaphorins may exhibit bifunctional properties (i.e., either repulsive or attractive responses). Inhibition vs activation as a semaphorin outcome is determined by the context (i.e., the biological environment) of the repulsive or attractive signal (*383*). For instance, neurons stimulated by cyclic nucleotides convert

the repulsive activity of Sema3A into attraction, suggesting that individual semaphorins exert different and sometimes opposite functions depending on the cell type or biological system (*384*). Such observations highlight the complexity of semaphorins' roles in specific cells, systems, and disease, all of which need to be separately investigated.

1.18.4 Semaphorin downstream signaling

It is not fully understood how the semaphorin-plexin binding mechanism induces plexin-dependent downstream signaling. However, it is believed that upon binding to semaphorin the cytoplasmic domain of inactive plexin undergoes a conformational change to become functionally active (*380*). Cytoskeletal components are affected by semaphorin signaling via actin filaments and reorganization of the microtubule network (*385*). The molecular mechanisms of semaphorin/plexin signaling include actin regulation via a rat sarcoma protein (Ras) homolog (e.g., Rac-1) or integrin-mediated cell adhesion by Ras proteins (e.g. R-Ras) (*380*).

Monomeric p21 GTP-binding proteins called small GTPases regulate cellular functions via GTP hydrolysis and cycling from the guanosine diphosphate GDP to the GTP-bound state with the aid of guanidine exchange factors (GEFs). Small GTPases are activated when they bind to GTP and attach to effector proteins, carrying out event cascades such as cell migration, proliferation, and angiogenesis (*386*). The plexin cytoplasmic domain contains a GTPase activating protein (GAP) domain divided into two segments by a Rho GTPase binding domain (RBD); therefore, they interact with Ras-related proteins (RAP), which play a crucial role in cell migration, proliferation, adhesion, and cell-cell junctions (*387*).

In addition to small GTPases, other pathways such as MAPK, PI3K, and STAT signaling mediate the multifaceted functions of semaphorins (*388, 389*). Furthermore, a family of cytosolic flavoprotein monooxygenases known as "molecule interacting with Cas ligand" (MICAL) is crucial for semaphorin-plexin mediated F-actin reorganization in neurons (*390*). In contrast, specific methionine sulfoxide reductase enzyme (SelR) opposes MICAL redox activity leading to semaphorin-plexin repulsion neutralization (*391*). To develop semaphorin- therapeutic new interventions based, semaphorin functions involved signaling pathways should be further investigated.

1.19 Semaphorin 3E

1.19.1 General overview

Semaphorin 3E (Sema-3E) was previously called by different names, including M-SemaH, coll-5, M-SEMAH, M-SemaK, and SemaH. Later, this gene was officially named semaphorin 3E and designated *SEMA3E* in humans (http://www.ncbi.nlm.nih.gov/gene/9723). Human *SEMA3E* was cloned by Nagase and colleagues from a size-fractionated brain cDNA library and reported to share 87.4% identity with mouse *Sema3E* (*392*). Christensen and colleagues sequenced the *SEMA3E* gene for the first time in tumor cell lines. They were able to clone two cDNA transcripts as spliced variants and estimated a protein comprising 775 amino acids with close similarity to the semaphorin and collapsin families of proteins. Such similarities of structural characteristics, particularly the sema domain and the immunoglobulin-like domain, identify the molecule as a new member of the semaphorin family. Also, it was suggested that Sema-3E could be a glycosylated and secreted molecule based on other features, for example, glycosylation sites, an NH_2-terminal signal sequence, and a positively charged COOH terminus, attached to the cell surface via COOH terminus as suggested for collapsin 1 (*393*). It was shown in humans that the *SEMA3E* gene is located on the long (q) arm of chromosome 7 at position 21.11 (7q21.11), encompassing base pairs 83,363,905–83,649,162 (http://www.ncbi.nlm.nih.gov/gene/9723).

CHARGE syndrome is a sporadic condition characterized by "choanal atresia, coloboma of the eye, dysfunction of cranial nerve, ear abnormalities, cardiac defects, abnormalities of genitourinary, and growth retardation." Screening for *SEMA3E* gene mutations in CHARGE syndrome patients showed a de novo mutation. The translocation breakpoints were plotted by Lalani and colleagues and identified the *SEMA3E* gene within 200 kb of the breakpoint on 7q21.11 (*394*).

In 2015, it was revealed that in Kallmann syndrome, dysfunctional Sema-3E signaling underlies the deficiency of gonadotropin-releasing hormone (GnRH) in neurons. In the same study, the Sema-3E mutation site was identified to cause an inherited deficiency of GnRH. Interestingly, neurons from male GnRH-deficient mice showed impairment in testes growth, a characteristic feature of Kallmann syndrome (*395*). In addition to the role of the Sema-3E/PlexinD1 axis in the nervous and cardiovascular systems, it also negatively affects immune system regulation. It was reported that the migration of $CD4^+CD8^+$ (DP) T cells is modulated by Sema-3E via inhibition of CCL25-CCR9 chemokine signaling (*396*). Of interest, the downregulation of PlexinD1 was

reported in thymocytes, highlighting the critical regulatory role of the Sema-3E/PlexinD1 in clinical settings (*397*).

Sema-3E-deficient mice were recently shown to have dramatically enhanced airway remodeling and Th2/Th17 inflammation (*398*). Therefore, Sema-3E plays a critical role in limiting the hallmarks of chronic airway disease. Sema-3E and its receptors are expressed by human asthmatic and non-asthmatic airway smooth muscle (ASM) and exogenous Sema-3E inhibited platelet-derived growth factor (PDGF)-mediated human ASM cells proliferation and migration. Sema-3E has also downregulated Rac1 guanosine triphosphatase (GTPase) activity and the phosphorylation of AKT and ERK1/2 in ASM (*388*, *389*).

1.19.2 Sema-3E/PlexinD1 binding and signaling

Sema-3E binds directly to PlexinD1, which is expressed in several embryonic tissues and vasculature endothelial cells. The expression of the *Plxnd1* gene has been found in different tissues and cells. These include podocytes, adrenal and mammary glands, lung mesenchyme, smooth muscle of the small intestine, osteoblastic cells and/or bone, and immune cells (*399*).

In cardiovascular development, conventional genetic ablation of PlexinD1 expression in mice resulted in cardiovascular defects (*400*). In contrast, Sema-3E deficiency in mice shows no developmental impact (*401*); therefore, the observed cardiovascular development defects are suggestive of additional PlexinD1 ligand(s). Unlike other class 3 semaphorins, Sema-3E does not bind to other plexins, and thus PlexinD1 is recognized as its unique receptor (*402*). However, PlexinD1 binds several semaphorin family members, including Sema-3A, Sema-4A, and Sema-3C (*374*). Regardless, PlexinD1 serves as the high-affinity receptor for Sema-3E (*402*). The extracellular region of PlexinD1 contains the sema domain that is necessary for Sema-3E binding and signaling (*402*). The ~630 amino acid intracellular domain of PlexinD1 contains the so-called sex-plexins (SP) domain that includes two highly conserved C1 and C2 regions known as RasGAP domains (*399*). Those regions are similar to the GAP sequence and specifically bind the R-Ras family of small GTPases (*387*). Direct interaction with small GTPases is mediated only by plexins (*387*). Downstream from C2 region, the T-segment is a ~40-60 C-terminal stretch that is highly conserved in plexin receptors. Unlike other protein domains, the T-segment is potentially important for providing signaling specificity to each plexin (*399*). A BLAST analysis of PlexinD1 reported 94–100% identity in T-segments from mammals, birds, amphibians, and fish, unlike other plexin family members of the same organism, which showed less similarity (*403*). The PlexinD1-

GAP activity limits integrin-mediated cell adhesion to the extracellular matrix (ECM) and downregulates PI3K and MAPK signaling pathways involved in proliferation, migration, and cell survival (*399*). Therefore, the PlexinD1 ectodomain interacts with Sema-3E, possibly priming the receptor through intracellular conformational changes (**Figure 1.7**). As a result, activated small GTPases (such as Rac1, Cdc42, and Rnd) bind to the RBD, leading to an inhibitory association between the C1 and C2 regions and the disruption of GAP activation (*399*).

Endothelial cells treated with Sema-3E have decreased FAK activity, a key molecule that plays a role in modulating the turning of integrin-containing focal adhesions. Rnd1, 2, and 3 have been reported to interact with the intracellular domain of PlexinD1 through RBD (*399*). In vivo, these GTPases are required for Ras-GAP activation of PlexinD1. Upon Sema-3E/PlexinD1 interaction, Ras GAP might play a role as potential signaling components mediating the early signaling events, regardless of the functional attractive vs repulsive outcome. Another possibility is that PlexinD1 could provoke R-Ras GTPase activity by sequestering these enzymes without catalytic intervention (*399*). The alteration in cytoskeletal and ECM compartments, such as actin polymerization and integrin localization, is the final target of semaphorin signaling and determines the its functional outcome (**Figure 1.7**) (*399*). However, the exact mechanism underlying semaphorin effects has not been completely understood. Upon Sema-3E stimulation and based on the above observations, it has been suggested that PlexinD1 in pre-existing complexes with Rnd2/RLG undergoes an Rnd2/RLG-dependent intracellular conformation change that results in the translation of extracellular Sema-3E cues into an intracellular gradient of PlexinD1 activities (*399*). Of note, the plexin family does not show endogenous kinase activity even though they are phosphorylated at cytosolic tyrosine (*404*). In all plexins, intracellular conserved tyrosine deposits exist and undergo phosphorylation. However, their localization is different between PlexinD1 (SP domain) and others (RBD) (*399*). In a model of lung cancer, the furin cleavage product p61 induces the formation of a PlexinD1/ErbB2 complex. Both phosphorylated on their tyrosine residues, and kinase activity of ErbB2 could induce phosphorylation of PlexinD1 (*405*). Altogether, the specific mechanism in which Sema-3E/PlexinD1 signaling mediates function is not fully understood. It is thought that the final functional outcome is cell- and context-dependent, where the co-receptor ligation, intracellular conformational modifications on receptor domains, and post-translational proteolytic processing should be investigated based on each cell and/or disease model.

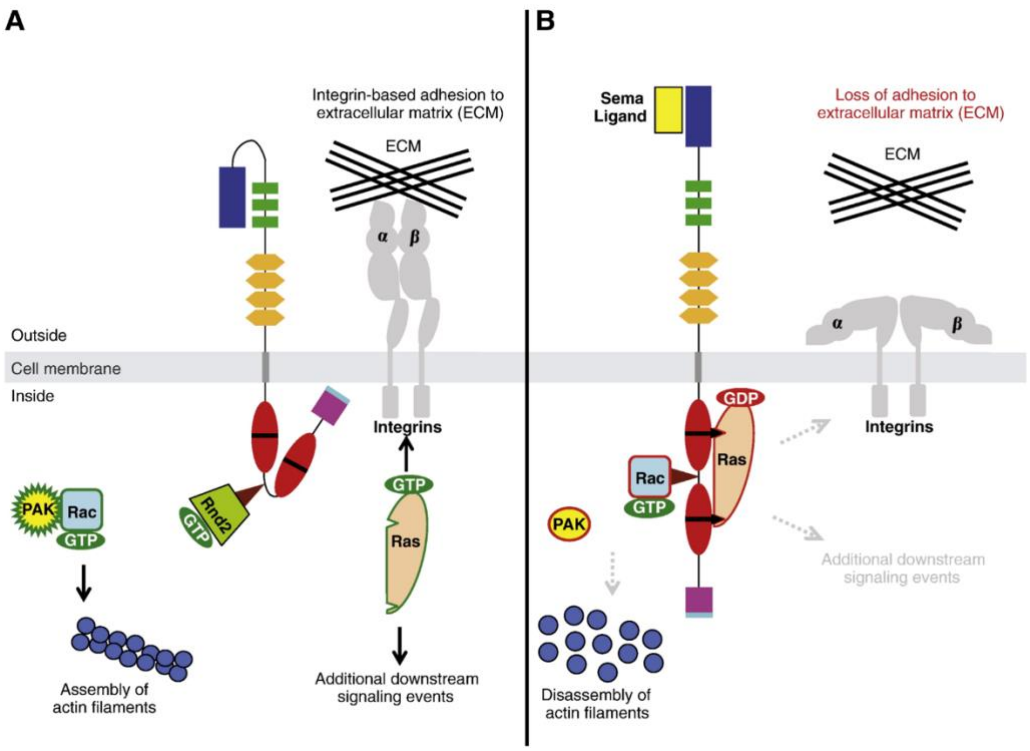


Figure 1.7. Repulsion effect of Sema3/PlexinD1 axis. A, the loss of ligand-mediated plexinD1 enables its link with GTP-bound Rnd2 while blocks its interaction with active, GTP-bound Rac and R-Ras. Therefore, GTP-bound Rac can bind to PAK (p21-activated kinase) and stimulate the assembly of actin filaments to enhance cell migration. In contrast, active GTP-bound Ras activates integrin-promoted adhesion to the extracellular matrix (ECM) and supports downstream signaling pathways. **B,** PlxnD1 undergoes a conformational change and links both Rac and R-Ras GTPases active forms. By preventing Rac, PlexinD1 leads to PAK inactivation and actin-based cytoskeleton failure, leading to repulsive responses. The upregulation of GTP hydrolysis resulted in plexinD1 inactivates R-Ras GTPases and thus the loss of integrin-based adhesion to the ECM. Reproduced with permission from (*399*).

1.19.3 Sema-3E-binding co-receptors and their effects

Neuropilin-1 (NRP-1) and vascular endothelial growth factor receptor 2 (VEGFR2) are the co-receptors that might be associated with PlexinD1 (*406*). Of interest, Sema-3E binds to PlexinD1 independently of NRP-1. Therefore, PlexinD1-mediated endothelial cell repulsion is also independent of NRP-1 (*407*). Upon binding to Sema-3E, NRP-1 and VEGFR2 switch the repulsive effect of Sema-3E/PlexinD1 signaling into an attractive effect (*407*). The pattern of Sema-3E function in neuronal cells is determined by NRP-1 expression: in subiculo-mamillary tract neurons the absence of NRP-1 ensures a repulsive effect, whereas NRP-1 expression by these cells results in attraction (*408*). As another layer of complexity, Sema-3E chemo-attraction has been reported

to be mediated by a PlexinD1/Nrp1/VEGFR2 ternary complex, in which PlexinD1 acts as the ligand-binding partner and VEGFR2 as the signal-transducing factor to mediate axonal growth increase (*409*). However, the co-receptor scenario does not seem to happen in endothelial cells: Sema-3E/PlexinD1 interaction retracts these cells, leading to angiogenesis inhibition (*410*). Very recently, the SARS-CoV-2 virus has been shown to bind to angiotensin-converting enzyme 2 (ACE2) on human cells (*411, 412*); however, it appears that NRP-1 can be involved in the entry step (*413-415*). Of interest, our recent study demonstrated the expression of NRP-1 by immature or mature stimulated DCs generated from BM (*416*). Together, these observations suggest the interaction of NRP-1 with the spike (S) protein of SARS-CoV-2 may increase the functional response of DCs and be a potential antiviral target with which to treat SARS-CoV-2 infection.

1.20 Thesis overview

1.20.1 Knowledge gap and study rationale

Under inflammatory conditions, Sema-3E is known to regulate the trafficking of immune cells (e.g., DCs, neutrophils) (*417*). Recent studies showed that Sema-3E-deficient mice dramatically enhanced airway remodeling and Th2/Th17 inflammation and modulated basal and HDM-induced pulmonary DCs recruitment (*398, 418*). Another study showed Sema-3E regulated DC functions in intestinal inflammation (*419*). Of interest, the expression of Sema-3E and its receptors (PlexinD1 and NRP-1) was tightly regulated in NK cells and DCs, suggesting these cells' ability to sense and respond to the Sema-3E/PlexinD1 axis, therefore modulating their effector functions (*420*). In NK/DC crosstalk, Sema-3E produced by immature DCs suppressed NK cell migration (*420*). Considering the regulation of these cellular processes, Sema-3E may be implicated in NK cell and DCs regulation. However, the role of the Sema-3E in regulating the phenotype and functions of NK cells and DCs has not been reported. Therefore, Understanding the role of Sema-3E in modulating NK and DCs function(s) may provide novel therapeutic targets to control specific NK or DCs effector functions in clinical settings. As a novel approach, I sought to decipher the role of Sema-3E, and its receptor PlexinD1 in modulating the phenotype and functions of NK cells and DCs by using various in vitro and ex vivo approaches on Sema-3E-/- and CD11c-PlexinD1-/- mouse models.

1.20.2 General hypothesis

Semaphorin 3E is a novel factor that regulates NK and DCs functions (Figure 1.8).

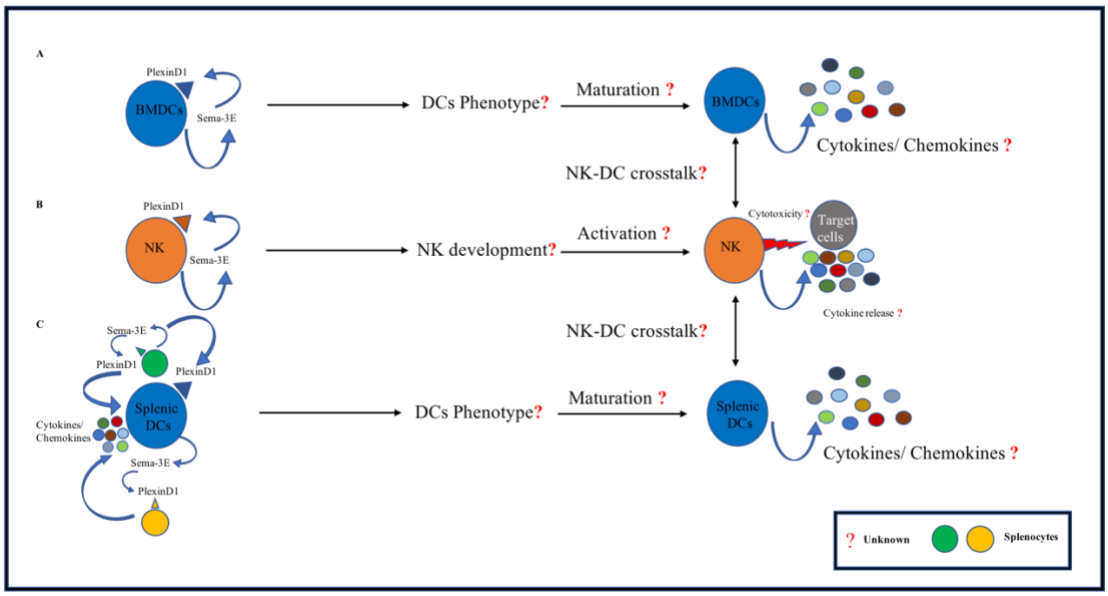


Figure 1.8. Schematic representation of our general hypothesis. According to our previous study, Sema-3E and PlexinD1 are expressed by both NK and DC. **(A)** Bone-marrow-derived DCs (BMDCs) and **(C)** splenic DCs might undergo the effect of the Sema-3E-PlexinD1 axis, which results in DCs maturation and thus functions (cytokines and chemokines expression). **(B)** NK cells might also be affected by the Sema-3E-plexinD1 axis, which leads to their activation to mediated cytotoxicity and/ or cytokines release. Collectively, modulation of NK and DCs phenotype and functions regulates NK-DCs crosstalk.

1.20.3 Specific aims

As presented above in this chapter, the Sema-3E/PlexinD1 axis may be involved in regulating phenotype and functions of both NK cells and DCs. This thesis has the following specific aims:

1. To investigate the potential role of Sema-3E in regulating NK cell function, specifically cytotoxicity and cytokine release (Chapter 3).
2. To elucidate the role of Sema-3E in regulating DC phenotype and function, specifically cytokine and chemokine productions (Chapter 4).
3. To determine whether this effect is mediated via the Sema-3E/PlexinD1 (Chapter 5).
4. To determine the role of Sema-3E/PlexinD1 axis in NK migration resulting from NK/DC crosstalk (Chapter 6).

Chapter 2: General Materials and Methods

2.1 Animals and ethics statement

Parent breeders of *Sema-3E*$^{-/-}$ mice (B6, 129 P2) were originally from Université de la Méditerranée, Marseille, France, and kindly provided by Dr. G. Chengua (Harvard Medical School, Boston, USA). These mice were backcrossed to BALB/c background for 10 generations at the Genetic Models Center (GMC) at the University of Manitoba (U of M), Winnipeg, Canada. *PlexinD1*$^{flox/flox}$ mice (B6, 129-*Plxnd1*$^{tm1.1Tmj/J}$) were kindly provided by Dr. T. M. Jessell (Columbia University/Howard Hughes). All mice were maintained in the U of M Central Animal Care Facility under pathogen-free conditions and used according to the Canadian Council for Animal Care guidelines. The U of M Research Ethics Board approved the current study (protocol number 17-012).

2.2 Sema-3E-knockout phenotype

Sema3E gene ablation in mice was used in most of the in vitro and ex vivo data presented in this thesis. Chenghua Gu from Harvard Medical School, Boston, kindly provided breeding pairs of Sema-3E knockout (Sema-3E-/-) mice (*407*). These mice were generated by disrupting the first exon of the *Sema3E* gene using a homologous recombination strategy in embryonic stem cells (*407*). Mice are viable, fertile and show a normal birth rate, with no visible abnormalities (*407*). Immunologically, Sema-3E-deficient mice show no difference in alveolar macrophages, T cells, Treg, B cells and NKT cell numbers within the spleen or lungs (*418*). However, activated macrophages are significantly higher in total numbers in *Sema3E*-/- mice than WT (*421*). In the steady state, Sema-3E-deficient mice also showed higher total and $CD11b^+$ but lower $CD103^+$ DCs compared to WT mice lung (*418*). BM-derived dendritic cells generated from these mice resulted in higher in vitro expression of CD11c in Sema3E$^{-/-}$ than WT (Dr. Hesam Movassagh, personal communication). Therefore, the phenotype and functions of neither NK cells nor DCs in the absence of Sema-3E have been investigated. A comprehensive study to address the potential role of Sema-3E in modulating the phenotype and functions of both NK cells and DCs between Sema-3E-/- and WT mice is necessary and will provide great insight in determining the potential baseline differences in those specific cell functions between *Sema3E*-/- and WT mice before investigating its role in different disease models.

2.3 CD11c-PlexinD1 conditional knockout phenotype

Mice with genetic deletion of PlexinD1 in CD11c+ lymphocytes were used in the in vitro and ex vivo data presented in this thesis. These mice were kindly provided by Dr. T. M. Jessell, Columbia University/Howard Hughes. These mice, *plexinD1*^{flox}, possess *loxP* sites flanking exon 1 of the PlexinD1 (*Plxnd1*) gene. These mice are viable, fertile, average in size, and do not show any gross physical or behavioral abnormalities. When these mice are bred to mice that express tissue or cell-specific Cre recombinase, the resulting offspring will have exon 1 deleted in the Cre-expressing tissues and/or cells, abolishing gene function. We bred two genetically modified animals in house to generate specific deletion of PlexinD1 in CD11c cells: *plexinD1*$^{flox/flox}$ and *B6.Cg-Tg*$^{(Itgax\text{-}cre)1\text{-}1Reiz/J}$. This breeding results in Cre-mediated recombination and deletion of the floxed sequences in CD11c cells of the offspring (Schedule 13, Genetically Engineered Laboratory Animals, University of Manitoba). There is no known phenotype for these mice. However, we observed no developmental and/or behavioral defects, physical abnormalities, peripartum lethality, or unexpected death incidence. Based on our findings that Sema-3E KO mice modulate the function of NK cells and DCs, we expect that CD11c/PlexinD1-deficient mice will delineate the direct vs indirect impact of the Sema-3E/PlexinD1 axis on DC phenotype and function by modulating NK/DC crosstalk for comparison to WT (Cre-negative or regular C57BL/6) controls.

2.4 Cell lines

A mutant lymphoma cell line (RMA-s) with decreased cell-surface expression of MHC class I molecules was cultured in Roswell Park Memorial Institute (RPMI)-1640 media (HyClone) supplemented with 10% fetal bovine serum (FBS), 1% PSG, and 1.6 mM 2-mercaptoethanol (2-ME; Sigma, St. Louis, MO). A breast cancer cell line (4T1) derived from the mammary gland tissue of a mouse BALB/c strain was cultured RPMI-1640 (HyClone) supplemented with 10% FBS and 1% PSG with no 2-ME. All cell lines were cultured in 10-cm tissue culture plates or T25 or T75 culture flasks and incubated in a tissue culture incubator at 37°C and 5% CO_2 (*422*). Cultured cells were split 2 to 3 times per week at 50% to 80% confluence to maintain cell viability.

2.5 Reagents

2.5.1 Recombinant proteins

Human IL-2 was received from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, National Institutes of

Health, (Nutley, NJ, USA). GM-CSF was purchased from Peprotech (Cranbury, NJ, USA). Mouse recombinant Sema-3E-Fc protein was a generous gift from Dr. Jonathan Duke-Cohan, (Dana Farber Cancer Institute, Harvard Medical School). Recombinant human PlexinD1 was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant mouse CXCL12, CXCL10, and CCL19 were purchased from eBiosciences (San Diego, CA, USA).

2.5.2 Antibodies

Purified and conjugated monoclonal antibodies against murine NK cells, dendritic cells, progenitors, and surface markers were used in this study. A summary of all antibodies used, their clones, and sources are listed in

Table 2.1.

Table 2.1. NK cell and DC antibodies, clones, and sources

Serial No	Antibody	Clone	Company
1	CD49b (DX-5) (APC)	DX5	eBiosciences
2	CD49b (DX-5) (PE)	DX5	eBiosciences
3	CD3 (PE)	17A2	eBiosciences
4	CD11c (APC)	N418	BioLegend
5	CD11c (PE)	N418	BioLegend
6	CD11c (FITC)	N418	invitrogen
7	CD40 (FITC)	1C10	eBiosciences
8	CD86 (Pacific Blue)	GL1	eBiosciences
9	CD80 (PE/Cy7)	16-10A1	eBiosciences
10	MHC class II (APC)	M5/114.15.2	eBiosciences
11	MHC class II (PE)	M5/114.15.2	eBiosciences
12	CD4 (PE/Cy7)	GK1.5	eBiosciences
13	CD8a (Pacific Blue)	53-6.7	eBiosciences
12	CD11b (APC)	M1/70	BD Pharmingen
13	CD27 (PE/Cy7)	LG.3A10	BioLegend
14	Ly49 C/I/FH (FITC)	14B11	BioLegend
15	NKP46 (FITC)	24A1.4	BioLegend
16	NKG2D (FITC)	C7	BioLegend
17	KLRG1 (FITC)	2F1/KLRG1	BioLegend
18	CD107 (PE)	ID4B	BioLegend
19	IFN-γ (PE)	XMG1.2	BioLegend

2.6 NK cell purification

The splenocytes were purified using mechanical and enzyme digestion? method followed by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Sweden) according to the manufacturer's described protocol. Primary NK cells (CD3-DX5+) were enriched using the EasySep mouse NK negative selection kit (StemCell Technologies, Vancouver, BC). NK cells were cultured at 37°C and 5% CO_2 in mouse medium containing 10% FBS (Hyclone), 1% PSG (Invitrogen), 1.6 mmol/L 2-ME and 1000U/mL IL-2 (used in all experiments). The purity of NK cells was checked before the experiments (Figure S.1). The purity of NK cells was routinely > 90 % by CD3-DX5+ in flow cytometry.

2.7 Primary DC preparations

To address the role of Sema-3E and PlexinD1 in DCs phenotype and function, I used BMDCs and splenic DCs. The use of BMDCs illustrated a better controlled in vitro culture system that devoid relatively the influence of other cell types in DC, to study the autocrine effect of Sema-3E on DC phenotype and function. On the other hand, the use of (whole tissue) splenic DCs before isolation (ex vivo) or after isolation (isolated DCs) mimics, to a certain extent, the in vivo system, where DCs were in close contact with other cells such as NK cells, T cells, and macrophages. Such an approach provides us a tool to dissect the relative contribution of the autocrine or paracrine effects of the Sema-3E on DCs.

BM precursor cells were extracted from the femur and tibia and incubated with Ammonium-Chloride-Potassium (ACK) buffer for 2 min to lyse red blood cells. Cells were washed with 1xPBS and centrifuged for 5 minutes (1200 RPMI). BM cells were counted then (0.5–1×10^6 per well) were seeded in a 24-well plate containing RPMI-1640 (Hyclone) medium supplemented with 1% PSG, 10% FGS, 1.6 mmol/L 2-ME and 20 ng/mL GM-CSF (Peprotech). On day 3, non-adherent cells was removed by aspiration and the culture was supplemented with fresh GM-CSF (20ng/mL) containing medium. On day 6, cultures were replenished with new GM-CSF medium, maintaining the total volume at 1 mL/well. On the same day, lipopolysaccharide (LPS; Sigma) at 1 μg/μL was introduced for 24 h to induce DC maturation. DCs with or without LPS treatment and the corresponding conditioned media were tested in different combinations and settings throughout these experiments. The expression of CD40, CD80, CD86 and MHC class II surface markers represented the phenotype of mature DC. All cells were phenotyped for maturity before experimentation (Figure S.2).

For splenic DC isolations, splenocytes were first isolated by homogenization using collagenase type IV (2.5 mL STEMCELL Technologies, Vancouver) for 30 minutes at 35°C followed by density gradient centrifugation (Ruffell lab).The cells were then incubated with ACK buffer for 2 min to lyse red blood cells, and proceed to CD11c+ positive isolation (StemCell Technologies Vancouver, BC). Isolated cells were seeded (1–2x10^6 cells per well) in a 48-well plate containing RPMI 1640 (Hyclone) medium supplemented with 1% PSG, 10% FBS, 1.6 mmol/L 2-ME and 20ng/mL GM-CSF (Peprotech). LPS was added at 1μg/μl to the culture media for 24 h to induce DC maturation. Isolated splenic DCs with or without LPS treatment and the corresponding culture conditioned media were tested in several combinations and settings throughout these experiments.CD40, CD80, CD86 and MHC class II. All cells were phenotyped for maturity markers CD40, CD80, CD86 and MHC class II before experimentation (Figure S.3). The purities of the BMDC and splenic DC are routinely > 85 % and 90 CD11c+ respectively by flow cytometry.

2.8 RNA isolation and cDNA preparation

Total Ribonucleic acid (RNA) was extracted from NK cells or DCs using TRIzol (Invitrogen, Life Technologies, CA, USA) according to the manufacturer's protocol. RNA concentration was measured using a BioPhotometer (Eppendorf AG, Hamburg, Germany). For cDNA synthesis, reverse transcription was perfumed with 2 μg of total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA, Cat #: 4368814) in a total volume of 20 μL following to the manufacturer's protocol. The cDNA samples and sequence-specific primers (10 μM) were combined with Master Mix (Applied Biosystems, USA, Cat #: 4472908), and polymerase chain reaction (PCR) was performed in 0.5 mL tubes using Mastercycler gradient for 25 cycles.

2.9 Quantitative real-time PCR

Real-time PCR was performed in 96-well optical plate with an initial denaturation step for 10 min at 95°C followed by 40 cycles of amplification (95°C for 15 s, 60°C for 35 s, 72°C for 35 s), one final cycle of melting and then cooling (Applied Biosystems 7500 Real-Time PCR system). Average data group and detection of fluorescent products were performed at the end of the 72°C extension period. Product specificity was assessed by melting curve analysis and examination of the amplification curves. Normalizing was performed using the house keeping gene

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and used to calculate the amplification of target genes. The following primers were used in real-time PCR

Table 2.2.

Table 2.2. Real-time PCR primers and amplicon size

Gene	Forward (5′- 3′)	Reverse (5′- 3′)	Size (bp)
Sema-3E	5′- AAAGCATCCCCAACAAACTG-3′	5′- CTGGCTCGAGACCCTTACTG-3′	124
PlexinD1	5′-TGGATGTCGCAGCTTACTTG-3′	5′- CCCCAACCCACAGTTCTCTA-3′	325
NRP1	5′- TATTCCCAGAACTCTGCCC-3′	5′- TGTCATCCACAGCAATCCCA-3′	546
GAPDH	5′-AACTTTGGCATTGTGGAAGG-3′	5′-ACACATTGGGGGTAGGAACA-3′	217
IL-10	5′-CGGGAAGACAATAAC-3′	5′-CGGTTAGCAGTATGTT-3′	300

2.10 NK cell cytotoxicity and cytokine analysis

2.10.1 CD107a degranulation assay

CD107a lysosome-associated membrane protein-1 (LAMP-1) expression was used as a surrogate marker to measure NK cell cytotoxicity (*423*). Briefly, effector NK cells were incubated with or without target cells (RMA-s) at an effector/target (E:T) ratio of 1:1 for 1 h in a V-shaped 96-well plate to a final volume of 0.2 mL. CD107a antibody (BD, San Diego, CA) and 6 μg/mL monensin (Sigma) were added to the cultures for an additional 4 hours at 37°C to retain CD107a in the cytoplasm for detection during the degranulation process. PMA/IONO were used as positive control to stimulate NK cells degranulation. Cells were washed and surface-stained with CD49b (DX-5) and CD107a antibodies before acquisition in flow cytometry.

2.10.2 Intracellular staining and cytokine assay

NK cells were incubated with target cells (RMA-S) in the presence of 10 μg/mL Brefeldin A (from Sigma) for 4 hours to detect the induction of IFN-γ. Cells were washed and subjected to surface and intracellular staining as described previously (*424*). Briefly, cells were incubated with Fc Block (eBiosciences) in a flow tube for 10 min on ice. Cells were stained with CD49b (DX-5) monoclonal antibody for 30 min on ice. After washing with flow buffer, cells were fixed with 2% paraformaldehyde (PFA) and incubated for 10 min on ice to retain IFN-γ in the cytoplasm for detection during intracellular staining (ICS), vortexing every 3 min to avoid clumping. Fixed and surface-stained cells were permeabilized with 0.1% saponin (Sigma-Aldrich) in a flow buffer and then stained with specific fluorochrome-conjugated monoclonal antibodies against cytokine of interest. Samples were analyzed by fluorescence-activated cell sorting (FACS)

using a FACSCanto II (BD Biosciences) and Diva software. Data were analyzed using FlowJo software (FlowJo, BD Life Sciences).

2.10.3 Transwell based migration and cytokine/chemokine analysis

NK cell chemotaxis/chemorepulsion against conditioned medium of immature, and LPS-matured DC was performed in a Transwell system. Murine IL-2-activated NK cells (0.2×10^6 cells in 100 μL) were loaded on the upper chamber (5 μm pore Transwell insert), whereas 600 μL conditioned medium was placed in the lower chamber and incubated at 37°C. Recombinant Sema-3E (6, 12, 25, 50, 100, or 200 ng/mL) was added to conditioned medium in some experiments. Other recombinant proteins such as human PlexinD1, CXCL12 (50 ng/mL), CXCL10 (100 ng/mL), CCL19 (100 ng/mL), and CCL21(50 ng/mL) were also added to conditioned medium. After 90 min, the cells that had migrated to the lower well were transferred to a polypropylene tube, centrifuged at 300*g* for 10 min, and counted. Migrated cells were counted as proportional to the absolute number of input cells (*425*).

2.11 Mesoscale Discovery-based cytokine and chemokine assay

Biomarker molecules (such as cytokines and chemokines) were measured using Mesoscale Discovery (MSD) assays. MSD assays were used to measure up to 20 cytokines and chemokines in the conditioned medium of immature and LPS-matured DCs. A cocktail mix of lyophilized cytokine standards was provided with the MSD multiplex kits at the same stock concentration of 10,000 pg/mL for each cytokine. Negative controls were prepared from the plain mouse medium. 20-plex kits (for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL17A, IL-12/IL23P40, IL-12/IL23p70, IL-13, IFN-γ, TNF-α, MIP-1a, MCP, IP-10, KC/GRO, and VEGF) were purchased from MSD (K110101A-4), and reagents were provided with the MSD kit. The assay's 96-well plate had 10 carbon electrodes in the bottom of each well that were pre-coated with one of the 20 anti-cytokine/chemokines antibodies of interest. The standards were reconstituted in the assay diluent provided. First, 10 μL of assay diluent was added to all wells. The plate was sealed and incubated for 30 s at room temperature on an orbital shaker (600 rpm). Next, 10 μL of samples, standards, or controls were added per well. The plate was sealed again and incubated for 2 h at room temperature on an orbital shaker (600 rpm). At the end of the incubation, the wells were washed three times using 200 μL phosphate-buffered saline (PBS) + 0.05% Tween 20, soaking for 30 s and then discarding after each wash. Detection antibody (10 μL) was then added to each well,

and the plate was sealed and incubated for 1 h at room temperature on an orbital shaker (600 rpm). The plate was washed again three times, as before, at the end of the incubation. MSD Read Buffer (150 µL) was next added to each well, and the MSD plates were measured on the MSD Sector Imager 2400 plate reader. The data was measured as an electrochemiluminescence signal (light) detected by photodetectors and analyzed using Discovery Workbench 3.0 software (MSD). A 4-parameter logistic fit curve was generated for each analyte using the standards and each sample concentration.

2.12 Tumor injection and monitoring

4T1 cells cultured in vitro were harvested and counted. A total of 1×10^5 4T1 cells in a 100-µL volume were loaded into a 1-mL tuberculin syringe using a 27G hypodermic needle. Female Sema-3E deficient mice and WT BALB/c mice (8 weeks old) were injected with 4T1 cells subcutaneously (s.c.) in the mammary gland by gently penetrating the skin. Daily monitoring of mice and tumor growth was performed. The tumor diameters were measured with vernier calipers and tumor volumes were calculated according to the formula $V\,(\mathrm{mm}^3) = L$ (major axis) x W^2 (minor axis)/2 (*426*). Animals were sacrificed when tumor size reached 10 mm or when the mice became moribund according to our animal protocol guidelines.

2.13 Lung metastatic assay

Lungs were collected from both *Sema-3E*$^{-/-}$ and WT mice and washed with Hank's balanced salt solution (HBSS). Then the lungs were digested by collagenase type IV (2.5 mL STEMCELL Technologies, Vancouver) for 75 minutes at 4°C. Next, digested cells filtered through 70-µm cell strainers and washed twice with 1× HBSS. Cells were resuspended in medium containing 60 µM 6-thioguanine (Sigma) and serially diluted in 6-well tissue culture plates. Cultured cells were kept at 37°C and 5% CO_2 in a tissue culture incubator. After 10–14 days, plates were fixed with methanol, and 6-thioguanine-resistant colonies were stained with 0.03% methylene blue and counted. After accounting for dilution factors, data were expressed as total number of metastatic colonies per lung.

2.14 NK depletion

For NK cell depletion, mice were given an intraperitoneal (i.p.) injection of 50 μL anti-asialoGM1 (ASGM1; Wako Pure Chemicals, Osaka, Japan) at four consecutive time points (days 3, 7, 10, and 13). At each time point, blood was collected, and red blood cells were lysed with ACK buffer. The remaining cells were stained with DX-5 and CD3 antibodies to confirm the depletion of NK cells by FACS. Stained cells were analyzed by FACSCanto II (BD Biosciences) using Diva software, and data were analyzed using FlowJo software (Figure S.4).

2.15 Statistical analysis

GraphPad Prism was used to analyze the collected data statistically. Results are shown as mean ± SEM. A two-tailed *t* test was used in a single, two-group comparison data. One-way or two-way analysis of variance (ANOVA) was used for comparing data from more than two groups. A *P* value of <0.05 is considered statistically significant.

Chapter 3: Semaphorin-3E Deficiency Impairs NK Cell Function

3.1 Abstract

Natural killer (NK) cells exert their innate immunity either directly through cell-mediated cytotoxicity and/or cytokines/chemokines production or indirectly through crosstalk with other cell types (such as DC). Unlike classical T cells, NK cells use germ-line encoded receptors to recognize and have the kill virus-infected or tumor cells without the need of prior priming. Recent data supports important functional roles of Semaphorin 3E (Sema-3E) in regulating immune responses. Yet, the importance of Sema-3E in the regulation of NK cell migratory properties and effector functions has not been investigated. Here, I observed that Sema-3E deficiency did not result in a block in NK cell development in the Sema-3E deficient mice despite a small decrease in the absolute number of the mature NK cells isolated from the spleen. Also, activated NK cells from the Sema-3E deficient mice showed comparable migratory responses toward conditional medium from DC, when compared to the WT NK cells. To further address the importance of Sema-3E in regulating NK cell function, I compared the expression of CD107a and IFN-γ in wild type (WT) and Sema-3E-deficient NK cells using the major histocompatibility complex (MHC) class I-negative mutant mouse lymphoma cell line (RMA-s) as target cells in vitro. I observed that Sema-3E-deficient NK cells were impaired in cell-mediated cytotoxicity and cytokine response. Together, these results revealed, for the first time that, Sema-3E is a novel factor that regulating effector functions of NK cells.

3.2 Introduction and rationale

NK cells are part of the innate immune system, which mediates antiviral and antitumor immune responses (*1*). The interaction of NK cells with self-MHC class I determines the functional outcomes for these cells (*427*). To discriminate between self and non-self MHC class I, activating and inhibitory surface receptors on NK cells recognize specific ligands (KIR in humans and Ly49 in mice) on potential target cells, thus regulating NK cell functions such as cytotoxicity and cytokine/chemokine release (*427-429*). Of interest, activating and inhibitory NK cell receptors are expressed differently based on the cells' maturation stage (*22, 24*). Therefore, different NK cell subpopulations demonstrate distinct effector functions (*25, 26*).

Semaphorins are ubiquitously expressed beyond the nervous system and control immune regulation (*362*). Semaphorin 3E (Sema-3E) and its receptor PlexinD1 have emerged as an

essential axis involved in cell migration, proliferation and angiogenesis (*396*). I have previously demonstrated that Sema-3E and its receptors (PlexinD1 and NRP-1) are expressed by resting and IL-2-activated NK cells in vitro (*420*). Furthermore, IL-2-activated NK cell migration was reported to be suppressed by Sema-3E produced by immature DCs (*420*). However, the role of Sema-3E in shaping NK cell development and functions is not known. Here, I made use of the Sema-3E knockout mice available in the laboratory to examine whether Sema-3E deficiency impact the ability of NK cells to develop and exert their functions (effector functions and chemotactic responses).

3.3 Results

3.3.1 Sema-3E-deficient mice developed fewer NK cells

Previously, it was demonstrated that a lack of NK cells in mice fails $CD4^+$ T cell commitment initiation, cytokine production, and Ab responses (*430, 431*). Furthermore, Sema-3E-deficient mice showed no difference in alveolar macrophages, T cell, Treg, B cell and NKT cell numbers within the spleen or lungs (*418*). Of interest, LPS treatment induced a shift in macrophage numbers in Sema-3E-deficient mice compared to WT (*421*). In the steady state, Sema-3E-deficient mice also showed higher total and $CD11b^+$ DC counts, but fewer $CD103^+$ DCs compared to WT mouse lung (*418*). Here, I sought to investigate whether Sema-3E deficiency affects NK cell percentage and absolute number in the BM and spleen in vivo.

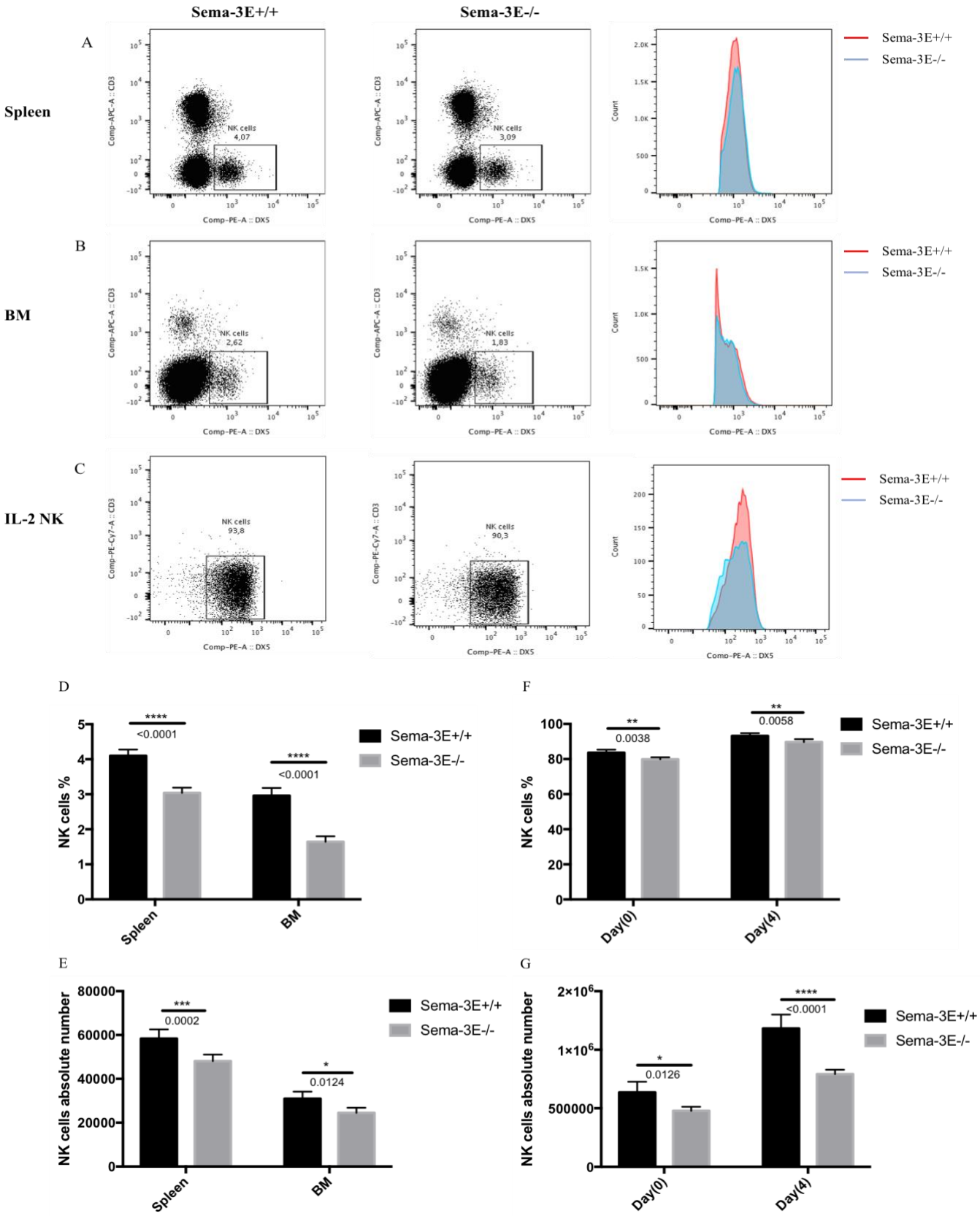

Sema-3E+/+
Sema-3E-/-
A
Spleen
NK cells
4,07
NK cells
3,09
B
BM
NK cells
2,62
NK cells
1,83
C
IL-2 NK
NK cells
93,8
NK cells
90,3
Comp-APC-A :: CD3
Comp-PE-Cy7-A :: CD3
Comp-PE-A :: DX5
Count
D
NK cells %
****
<0.0001
****
<0.0001
Spleen
BM
F
NK cells %
**
0.0038
**
0.0058
Day(0)
Day(4)
E
NK cells absolute number
***
0.0002
*
0.0124
G
NK cells absolute number
*
0.0126
****
<0.0001

Figure 3.1. Sema-3E-deficient mice developed fewer NK cells. (A–C) Flow cytometry assay of spleen (A), bone marrow (BM; B), and IL-2-activated NK cells (C) harvested from WT (Sema-3E+/+, left column) and Sema-3E-deficient (Sema-3E-/-, middle column) BALB/C mice. Right-hand column shows plots corresponding to the images in the first two columns. Cells were surface-stained with monoclonal antibodies against CD49b (DX5) PE and CD3 PE-Cy-7. **(D, E)** The percentage (D) and absolute number (E) of NK cells in harvested spleen and BM are plotted from all collected data. **(F, G)** NK cells were isolated from the spleen of WT (Sema-3E+/+) or Sema-3E-deficient (Sema-3E-/-) BALB/C at Day 0 and cultured in IL-2 (1000 U/mL) for 4 days (Day 4). Data are shown as mean ± SEM, n = 15. *P* values are from two-way Analysis of variance (ANOVA) used for comparing two groups: **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

Interestingly, Sema-3E-deficient mice showed a significantly lower percentage and absolute number of NK cells in the spleen (**Figure 3.1**A, D, E) and BM (**Figure 3.1**B, D, E) compared to WT littermate controls at the baseline. To confirm the low number of NK cells in spleen, I isolated NK cells. Similarly, the percentage and absolute number of freshly isolated NK cells from the spleen of Sema-3E-deficient mice were less than that of cells from WT littermates (**Figure 3.1**F, G). I then sought to determine if stimulation of NK cells with IL-2 in vitro would recover the reduced NK cell number in Sema-3E deficient mice. To address this question, an equal number (0.5×10^6) of freshly isolated NK cells from the spleen of Sema-3E-deficient and WT mice were cultured with IL-2 for 4 days. IL-2-stimulated NK cells isolated from Sema-3E-deficient mice persisted in showing fewer NK cells than WT controls (**Figure 3.2**C, F, G). Sema-3E deficiency therefore diminished both NK cell percentage and absolute number, and stimulation with IL-2 was insufficient for recovery of NK cell number. Therefore, NK cell function may be impacted.

3.3.2 Sema-3E deficiency had no impact on NK development and maturation

Early studies suggested NK cells develop exclusively in the BM, and ablation or disruption of the BM microenvironment abrogated the development and function of NK cells (*10, 11*). However, in humans and mice, current evidence suggests that NK cells can also develop and mature in SLTs, including tonsils, spleen, and LNs (*12*). Based on CD27 and CD11b surface density, maturation of functional NK cells occurs in three stages: beginning with a double-negative (DN) NK population ($CD27^-CD11b-$) cells progress to $CD27^+CD11b^-$, DP, and finally $CD27^-CD11b^+$ NK cells, which are considered the most mature NK cells (*22, 24*). Different NK cell subpopulations demonstrate distinct effector functions, location distribution, and surface

receptors acquisition (*25, 26*). Therefore, I wanted to explore if Sema-3E deficiency modulated the early stages of NK cell development and maturation, explaining the impairment in NK cell count described above. I observed that Sema-3E deficiency did not impact NK cell development or maturation stage in NK cells from either spleen (**Figure 3.2**A, C) or BM (**Figure 3.2**B, D), as evidenced by expression of CD27 and CD11b. This observation suggests that the impairment of NK cell number displayed in Sema-3E-deficient mice is not correlated with the dysregulation of NK cell development or maturation stage.

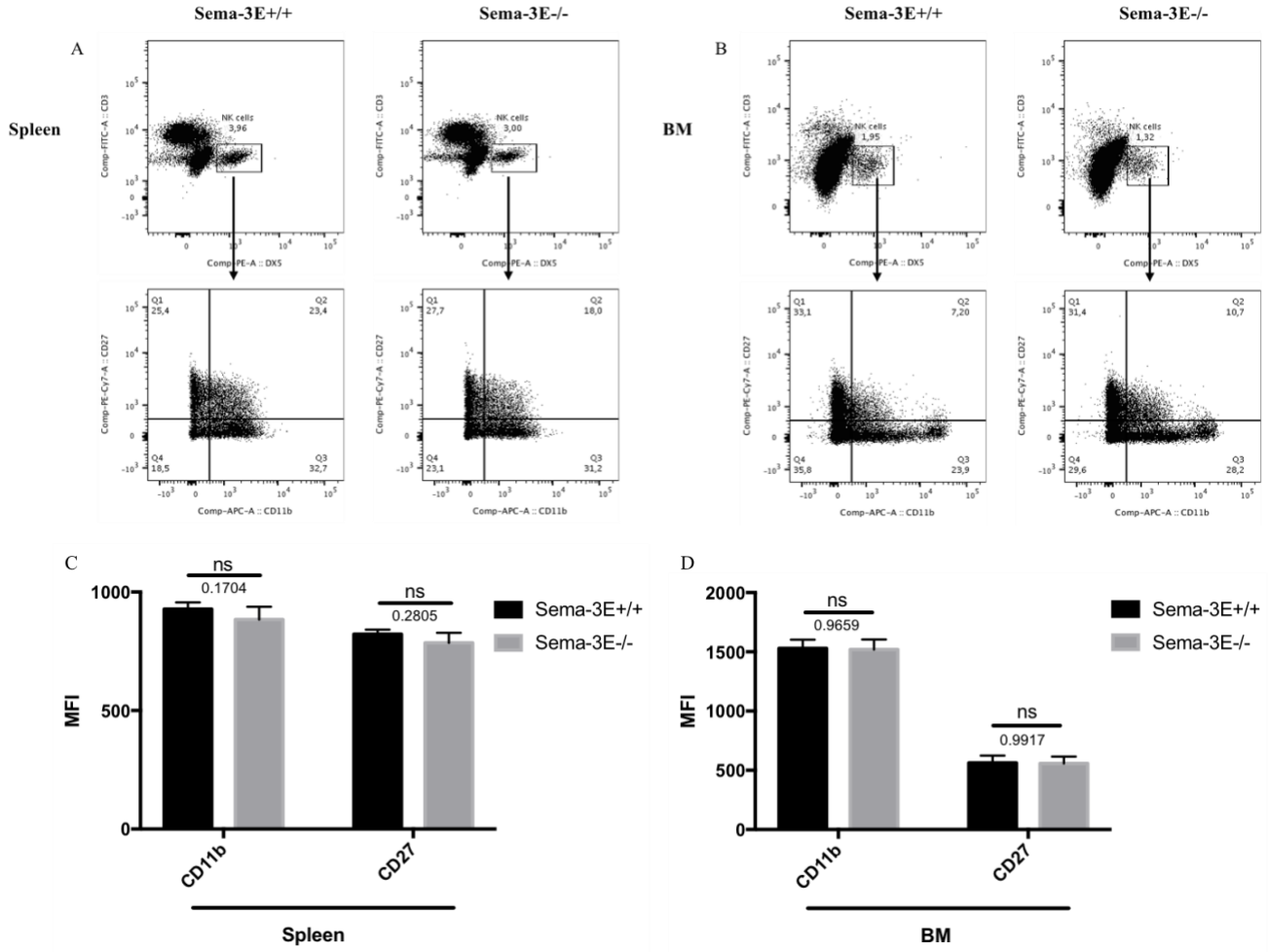


Figure 3.2. Sema-3E deficiency did not affect NK cell maturation. (A, B) Flow cytometry assay of spleen (A), bone marrow (BM; B). Cells were surface-stained with monoclonal antibodies against CD49b (DX5) PE, CD3 FITC, CD11b APC, and CD27 PE-Cy-7. **(C, D)** The mean fluorescent intensity (MFI) of CD11b and CD27 from the spleen (C) and BM (D) of NK cells in harvested spleen and BM are plotted from all collected data. Data are shown as mean ± SEM, n = 6. *P* values are from two-way ANOVA used for comparing two groups: ns *P*>0.05.

3.3.3 Sema-3E deficiency did not alter surface NK cells major receptor expressions

The stimulation of NK cell and effector function depend on the signals derived from two distinct types of receptors: inhibitory and activating (*64*). To discriminate between self and nonself MHC class I, activating and inhibitory NK cell receptors recognize specific ligands on potential target cells (*428*, *429*). The balance between activating and inhibitory signals decides the functional outcome of an activated mature NK cell (*429*). However, the role of Sema-3E in modulating inhibitory and activating NK cell receptors remains to be investigated. Here, I examined inhibitory and activating receptors such as Ly49 C/I/F/H, NKP46, NKG2D, and KLRG1 using flow cytometry (**Figure 3.3**).

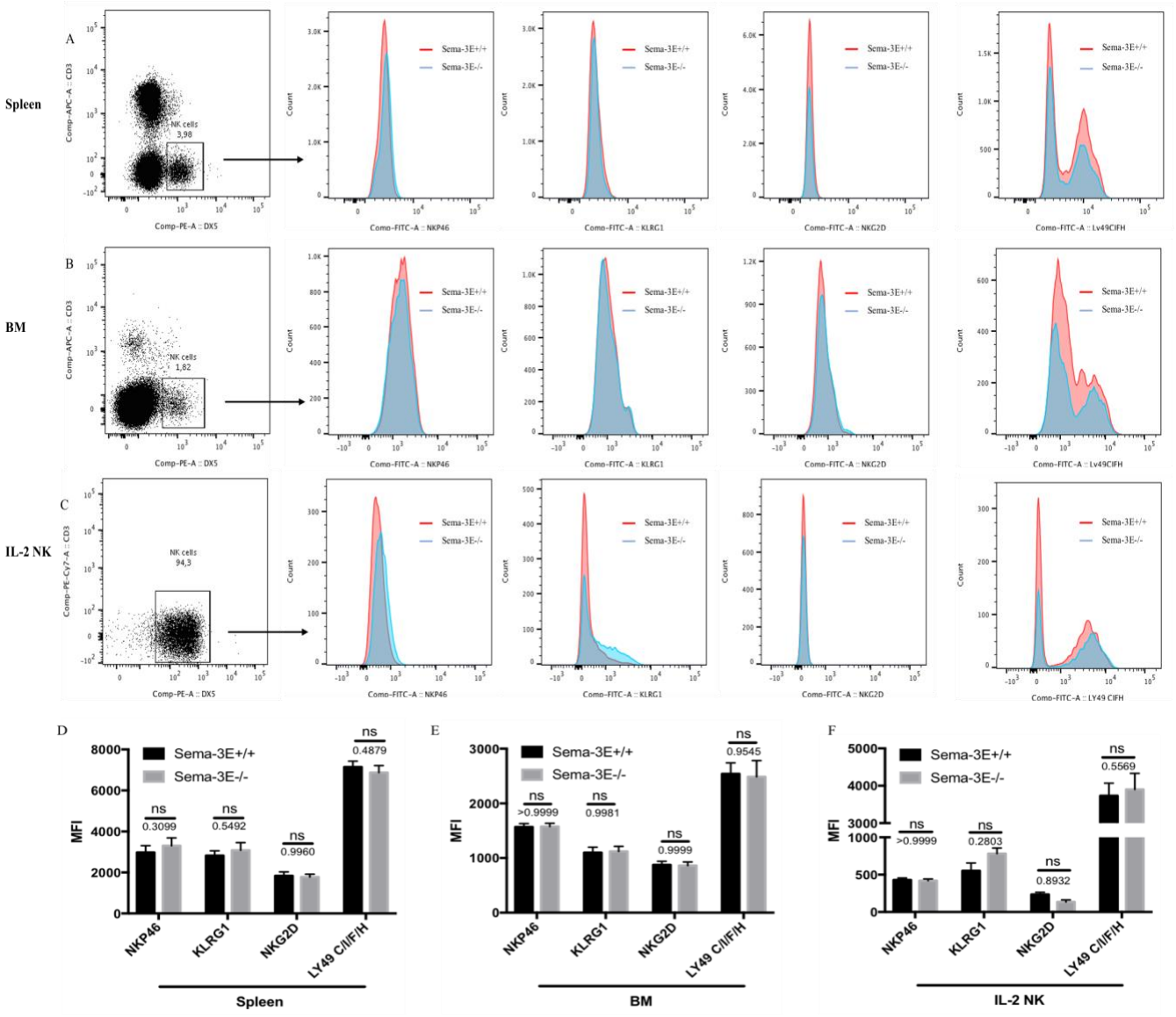


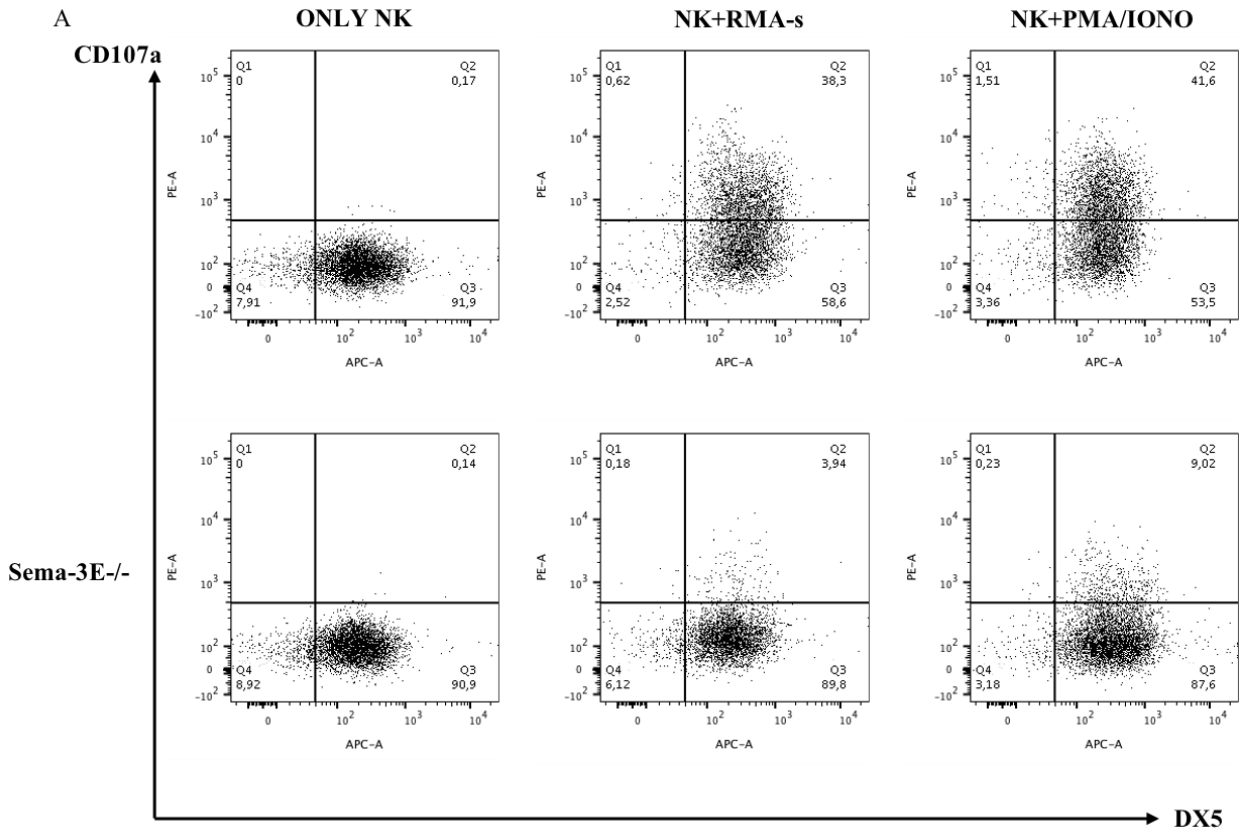
Figure 3.3. Sema-3E deficiency did not affect surface expression of major NK cell receptors. **(A–C)** Flow cytometry assay of spleen (A), bone marrow (BM; B) and IL-2-activated NK cells (C). Right-hand four columns show plots corresponding to WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-). Cells were surface-stained with monoclonal antibodies against CD49b (DX5) PE, CD3 (APR, PE-Cy-7), NKP46, KLRG1, NKG2D, and Ly49 C/I/F/H. The mean fluorescent intensity (MFI) of NKP46, KLRG1, NKG2D, and Ly49 C/I/F/H receptors from the spleen (D) and BM (E) and IL-2 NK cells (F) are plotted from all collected data. Data are shown as mean ± SEM, n = 6. *P* values are from two-way ANOVA used for comparing two groups: ns $P>0.05$.

No significant differences were observed in NK cells isolated from the spleen (**Figure 3.3**, A and D) or the BM (**Figure 3.3**, B and E) of Sema-3E-deficient mice compared to WT littermate controls. Similar results were obtained in IL-2-activated NK cells from Sema-3E-/- mice and WT littermate controls (**Figure 3.3**, C and F). Together, our data suggest that Sema-3E has no direct effect on the NK cell receptors NKP46, KLRG1, NKG2D, or Ly49 C/I/F/H.

3.3.4 NK cells developed in Sema-3E-deficient background demonstrated less cytotoxicity

The recognition of target cells and the induction of cell cytotoxicity are regulated by inhibitory and activating NK cell receptors (*428, 429*). I showed that Sema-3E-deficient mice developed fewer NK cells (**Figure 3.1**). However, the NK maturation stage (**Figure 3.2**) and major receptors (**Figure 3.3**) were not affected by the absence of Sema-3E. The lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) is considered a marker of NK cells degranulation and cytotoxicity function (*432*). To better understand the correlation between Sema-3E deficiency and NK cell cytotoxicity function, I examined the surface expression of CD107a on NK cells after degranulation toward RMA-s target cells *in vitro*. To this end, isolated splenic NK cells from Sema-3E-deficient and WT mice were stimulated with IL-2 for 4 days and used in CD107a degranulation assay. Strikingly (as shown in **Figure 3.4**, A, B), NK cells isolated from Sema-3E-deficient mice showed less toxicity to RMA-s cells compared to those from WT littermate controls. NK cells in the absence of RMA-s target cells were used as negative control, and phorbol myristate acetate (PMA)/ionomycin-stimulated NK cells served as a positive control. Thus, these results suggest that the absence of Sema-3E signaling decreases NK cell cytotoxicity.

A


ONLY NK
NK+RMA-s
NK+PMA/IONO
CD107a
Sema-3E-/-
DX5
PE-A
APC-A
Q1 0
Q2 0,17
Q4 7,91
Q3 91,9
Q1 0,62
Q2 38,3
Q4 2,52
Q3 58,6
Q1 1,51
Q2 41,6
Q4 3,36
Q3 53,5
Q1 0
Q2 0,14
Q4 8,92
Q3 90,9
Q1 0,18
Q2 3,94
Q4 6,12
Q3 89,8
Q1 0,23
Q2 9,02
Q4 3,18
Q3 87,6

B

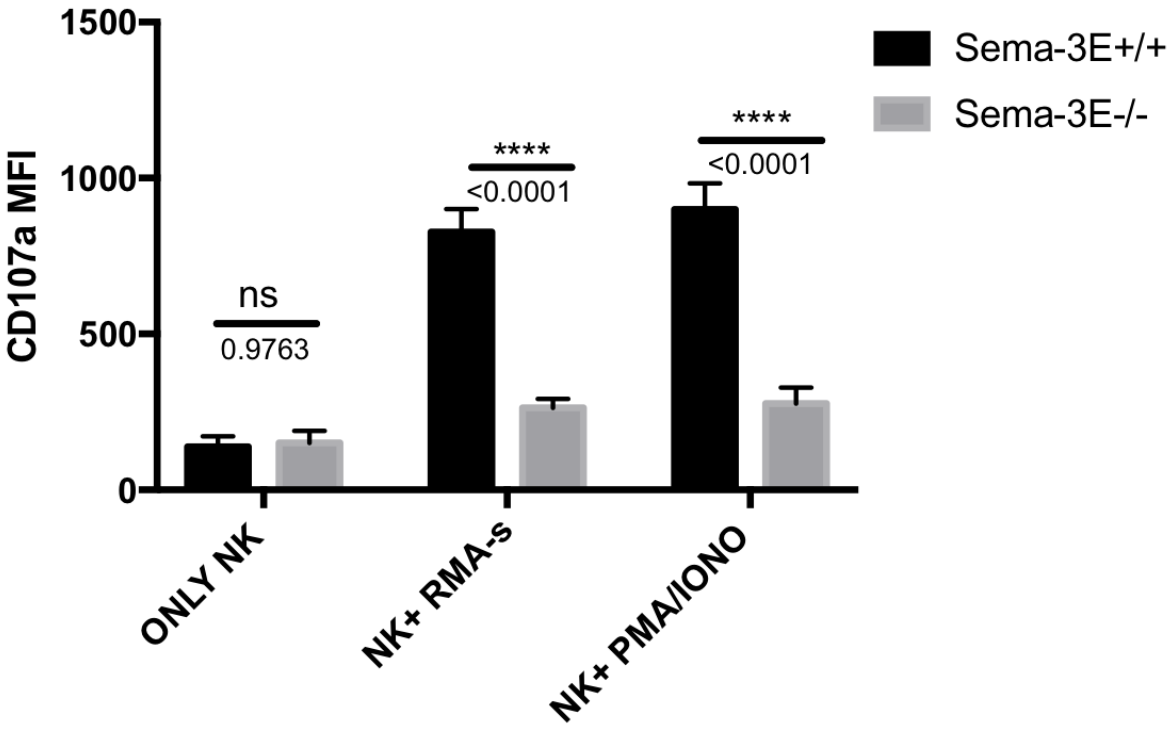
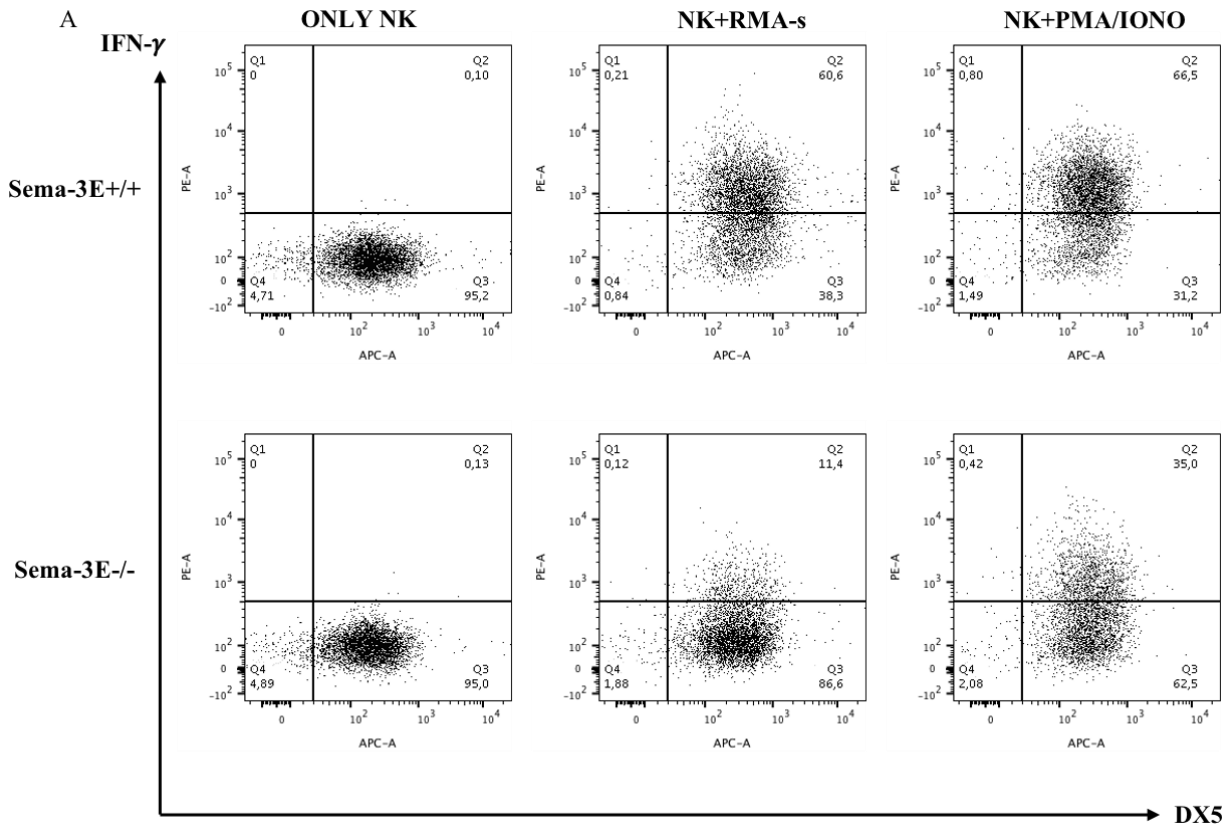

CD107a MFI
1500
1000
500
0
Sema-3E+/+
Sema-3E-/-
ns
0.9763
****
<0.0001
****
<0.0001
ONLY NK
NK+ RMA-s
NK+ PMA/IONO

Figure 3.4. Sema-3E deficiency reduces NK-mediated CD107a response. NK cells were isolated from the spleen of WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) BALB/C mice and cultured in IL-2 (1000 U/ml) for 4 days. **(A)** Flow cytometry assay of IL-2 NK cells. Cells were surface-stained with monoclonal antibodies against CD49b (DX5) APC and CD107a PE. Lift column represented, negative control, IL-2 NK cells alone (ONLY NK). Middle column represented IL-2 NK cells cultured with target cells (RMA-s) incubated at **1:1 (E:T ratio)**. Right column represented positive control, IL-2 NK cells stimulated with PMA/IONO. **(B)** MFI of CD107a is plotted from all collected data in mentioned conditions. Data are shown as mean ± SEM, n = 4. *P* values are from two-way ANOVA used for comparing two groups: ns $P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

3.3.5 Sema-3E deficiency impaired the NK cell-derived cytokine response

In addition to the natural cytotoxic immune responses, activated NK cells secrete several proinflammatory cytokines when interacting with susceptible targets (such as cells with reduced expression of MHC class I) (*5*). These include IFN-γ, TNF-α, GM-CSF, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 and RANTES NK cell-derived IFN-γ has been implicated in a wide variety of immune responses such as viral defense, skewing Th1, promoting CTL responses and maturation of DCs and macrophages (*5*). Since NK cells developed in Sema-3E-deficient mice displayed less CD107a expression compared to WT controls (**Figure 3.4**), I sought to investigate the ability of NK cells to produce IFN-γ in vitro. Isolated splenic NK cells from Sema-3E-deficient and WT mice were stimulated with IL-2 for 4 days and subsequently used in an IFN-γ cytokine release assay. Similar to what was observed in the CD107a assay, NK cells developed in Sema-3E-deficient mice exhibited significantly lower NK cell IFN-γ production levels in the presence of RMA-s compared to NK cells isolated from WT controls (**Figure 3.5**A, B). NK cells alone were used as negative control while PMA/ionomycin-stimulated NK cells were positive control. The level of IFN-γ was also significantly reduced in PMA/ionomycin-stimulated NK cells from Sema3E-deficient mice compared to those isolated from WT littermates. Collectively, **Figure 3.4** and **Figure 3.5** suggest that Sema-3E deficiency diminishes NK cell cytotoxicity and cytokine release to kill target cells.

A


ONLY NK
NK+RMA-s
NK+PMA/IONO
IFN-γ
Sema-3E+/+
Sema-3E-/-
DX5
PE-A
APC-A
Q1 0
Q2 0,10
Q4 4,71
Q3 95,2
Q1 0,21
Q2 60,6
Q4 0,84
Q3 38,3
Q1 0,80
Q2 66,5
Q4 1,49
Q3 31,2
Q1 0
Q2 0,13
Q4 4,89
Q3 95,0
Q1 0,12
Q2 11,4
Q4 1,88
Q3 86,6
Q1 0,42
Q2 35,0
Q4 2,08
Q3 62,5

B

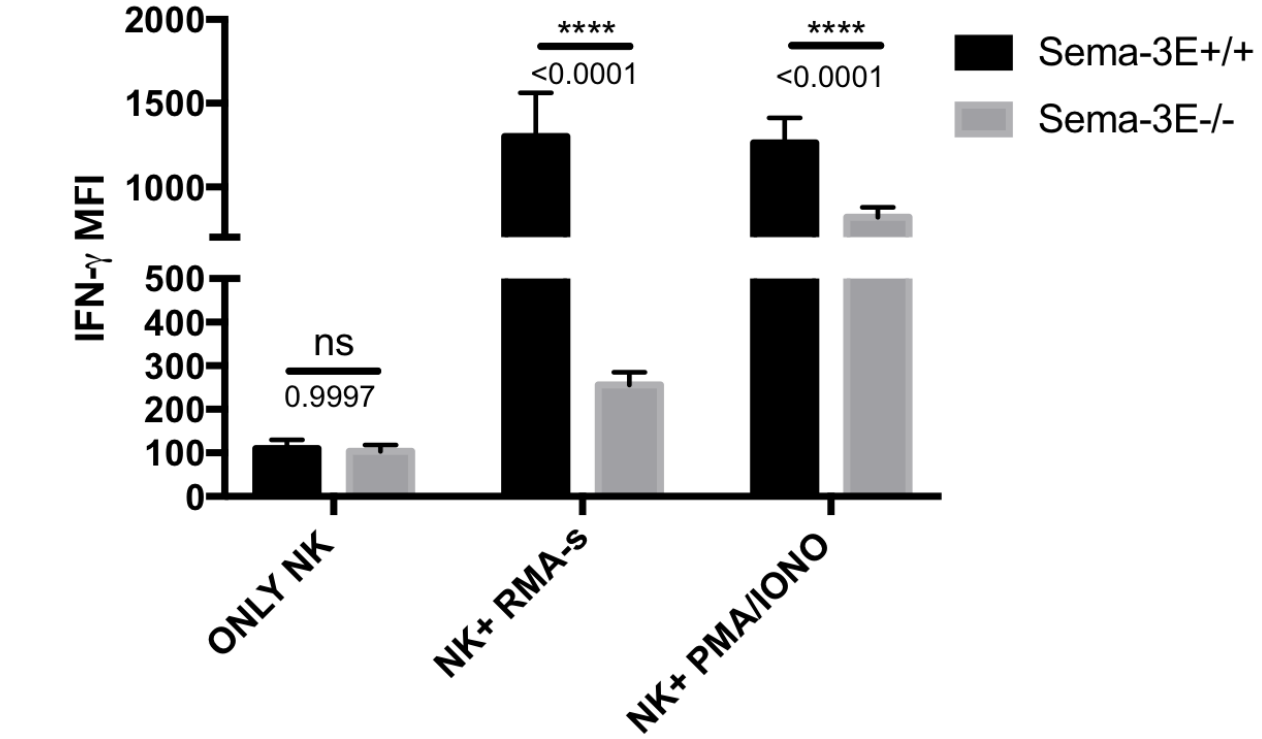

IFN-γ MFI
2000
1500
1000
500
400
300
200
100
0
****
<0.0001
****
<0.0001
ns
0.9997
Sema-3E+/+
Sema-3E-/-
ONLY NK
NK+ RMA-s
NK+ PMA/IONO

Figure 3.5. Sema-3E deficiency reduced NK-derived IFN-γ production. NK cells were isolated from the spleen of WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) BALB/C mice and cultured in IL-2 (1000 U/ml) for 4 days. **(A)** Flow cytometry assay of IL-2 NK cells. Cells were surface-stained with monoclonal antibodies against CD49b (DX5) APC and intracellular-stained with IFN-γ PE. Left column represented negative control, IL-2 NK cells alone (ONLY NK). The Middle column represented IL-2 NK cells cultured with target cells (RMA-s) incubated at **1:1 (E:T ratio)**. The right column represented positive control; IL-2 NK cells stimulated with PMA/IONO. **(B)** MFI of IFN-γ is plotted from all collected data in mentioned conditions. Data are shown as mean ± SEM, n = 3. *P* values are from two-way ANOVA used for comparing two groups: ns$P$>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

3.3.6 Sema-3E did not affect IL-2-activated NK cell migration

In NK-DC crosstalk, I observed that conditioned medium from Sema-3E-deficient immature BMDCs enhanced IL-2-activated NK cell migration in NK (*420*). With this data suggesting that Sema-3E produced by immature BMDCs suppresses NK cell migration, I next wanted to confirm whether Sema-3E deficiency affects NK cell migration properties in vitro. First, I isolated splenic NK cells from Sema-3E-deficient and WT mice and stimulated them with IL-2 for 4 days. Second, BMDCs were generated from WT BALB/c mice. BMDCs were cultured in the presence of GM-CSF for 7 days. Cells were stimulated with LPS (1 μg/μL) for 24 h to acquire the mature DC phenotype. The conditioned medium of these DCs were collected and used with the IL-2-activated NK cells in a transwell migration assay. Plain mouse medium was used as a negative control, and the conditioned medium of LPS-stimulated BMDCs was used as a positive control. Interestingly, I observed no significant differences in IL-2-activated NK cell migration of Sema-3E-deficient mice toward BMDCs conditioned media compared to WT controls (**Figure 3.6**). My observation suggested Sema-3E produced by NK cells has not directly impacted NK cell migration.

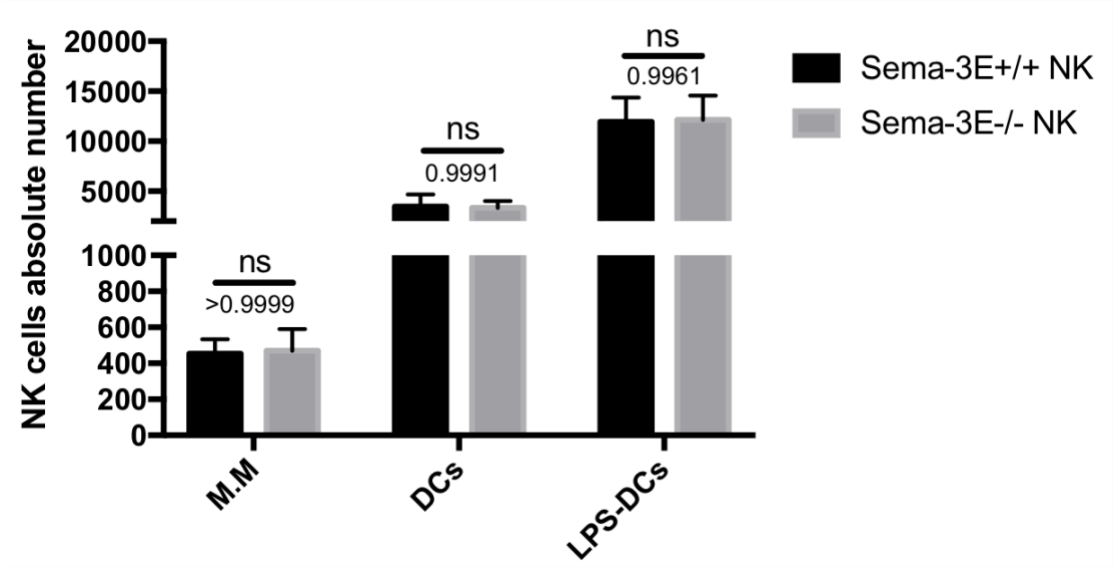


Figure 3.6. Migration of IL-2-activated NK cells is not affected by Sema-3E deficiency. Transwell migration assay of IL-2-activated NK cells toward the conditioned medium of immature (DCs) and LPS-stimulated (LPS-DCs) BMDCs generated from BALB/c mice. NK cells were isolated from Sema-3E deficient (Sema-3E-/- NK) and WT mice (Sema-3E+/+ NK). Plain mouse medium (M.M) was used as a negative control, and the conditioned medium of LPS-stimulated BMDCs (LPS-DCs) served as a positive control for migration. Data are shown as mean ± SEM, n = 4 independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups. ns, $P > 0.05$.

3.4 Discussion

NK cells are part of the innate immune system that can kill virus-infected and tumor cells mediated by the cytolytic machinery or cytokine release (*5*). In this chapter, I examined for the first time the importance of Sema-3E on NK cell biology. I reported that Sema-3E-deficient mice developed fewer NK cells than the WT controls (**Figure 3.1)**. Next, I investigated the effect of Sema-3E on NK cell development. I observed that deficiency of Sema-3E did not affect the maturation stage of NK cells, based on the CD27/CD11b phenotype **(Figure 3.2)**. Expression of the major NK cell receptors (Ly49 C/I/F/H, KLRG1, NKP46, and NKG2D) was also not affected by the absence of Sema-3E (**Figure 3.3)**. Moreover, the migration of IL-2-stimulated NK cells was not affected by the absence of Sema-3E **(Figure 3.6)**. Interestingly, diminished NK cell cytotoxicity and cytokine release were observed in NK cells isolated from Sema-3E-deficient mice compared to WT littermates (**Figure 3.4, Figure 3.5)**. Understanding the role of Sema-3E in NK cell function regulation may provide further insight into new treatment strategies targeting infected and/or tumor cells.

It was reported that the differentiation of NK cells is a gradual step that is driven by the transcriptional factors (TFs) regulation and cytokine signals coordination from HSCs (*433, 434*). For example, deficiency of flt3 and c-kit (receptors of fms-like tyrosine kinase 3 ligand (FL) and kit ligand (KL), respectively), resulted in NKPs reduction, suggesting these cytokines are essential for early stages of NK cell differentiation (*435*). IL-3 is another cytokine known to maintain lymphoid progenitor development and promote NK cell differentiation (*436, 437*). In addition, the deficiency of CXCR4 signaling resulted in NK cell number reduction (*438*). In my study, I observed that Sema-3E-deficient mice developed fewer NK cells in the BM and spleen than WT controls (**Figure 3.1**). However, Sema-3E did not impact the maturation stage of NK cells represented by the expression of CD27 and CD11b (**Figure 3.2**). Sema-3E is known to exert its effects on immune response, including proliferation (*388*) and cytokine release (*439*). Therefore, my data suggested that Sema-3E might regulate NK proliferation and differentiation via the downregulation of key cytokines driver NK proliferation independent of NK cell maturation. The cytokine IL-2 is known to activate STAT1, STAT3, and STAT5 resulting in NK cell proliferation and response *in vivo* and *in vitro (440, 441)*. However, I found that IL-2-stimulated NK cells isolated from Sema-3E-deficient mice persisted in showing fewer NK cells than WT controls (**Figure 3.1**). This observation suggests the impairment in NK cell number may occur at the early

stage of NK cell proliferation and differentiation and independent of IL-2 stimulation and activation of signaling pathways. It is possible that IL-2 stimulation is specific to NK cell functions independent of their proliferation. The activation of the IL-2 receptor (IL-12R*a*) increases phosphorylation of JAK1/3-STAT5 and downstream signaling, including MAPK, JAK-STAT, and PI3K/AKT-mTOR (*442*). Of interest, It was reported that STAT5 deficient mice displayed mature NK cell number reduction (*443*). However, the role of Sema-3E in IL-2 NK proliferation and survival remains to be investigated in vitro. It is possible that Sema-3E deficiency might suppress IL-2R expression, and STAT5 signaling on NK cell results in NK reduction. The impairment of IL-2R on NK may also modulate the crosstalk of NK with other immune cells (such as DCs) that produced critical cytokines for NK cell proliferation.

NK cell migration is an important step that allows NK cells to mediate their effector functions (*444*). NK cell migration is tightly regulated: adhesion receptors must undergo cycles of attachment to and detachment from their endothelial ligands for effective migration (*5*). Sema-3E is known to regulate the trafficking of immune cells (DCs, neutrophils, and NK cells) (*417, 420*). Previously, I reported that Sema-3E produced by immature DCs suppressed NK cell migration in NK/DC crosstalk (*420*). In neutrophil migration, Sema-3E suppressed CXCL8/IL-8–induced neutrophil migration via impairment of F-actin polymerization and Rac1 GTPase activity (*445*). NK cells express CXCR1 and CXCR2 as CXCL8 (IL-8) receptors (*200, 204*). CXCR3 was expressed by NK cells and played an essential role in driving NK cell migration (*446*). Of interest, I reported IL-2-activated NK cells of Sema-3E-deficient mice displayed similar migration toward BMDC conditioned medium compared to WT controls **(Figure 3.6)**. This result suggests that Sema-3E does not impact NK cell chemotaxis toward BMDCs rich chemokines conditioned media. However, chemokines receptors expressed by NK cells remain to be investigated.

The natural cytotoxic immune response and cytokine release are critical mechanisms by which NK cells kill target cells (*5*). NK cell function is regulated by the signals received from activating or inhibitory receptors (*447*). I, therefore, examined whether Sema-3E regulates the cytotoxic and cytokine release function of NK cells. In vitro, I showed that Sema-3E deficiency in IL-2- stimulated splenic NK cells impaired NK cell cytotoxicity represented by the surface expression of CD107a (**Figure 3.4**) and cytokine release represented by IFN-γ (**Figure 3.5**). However, I observed no differences in the expression of NKP46, KLRG1, NKG2D, or Ly49

C/I/F/H in Sema-3E-deficient spleen, BM, or IL-2-stimulated splenic NK cells (**Figure 3.3**). NK cell cytotoxicity is known to be regulated through three main processes: recognition of target cell, a target cell contact and IS formation, and NK cell-induced target cell death (*5*). In addition, NK cells responded to tumor ligands and intracellular pathogens by producing cytokines, including IFN-γ (*164*). Recently, it was reported that Sema-3A inhibited T cell IS assembly, thereby impairing the killing of tumor cells (*448*). Moreover, Sema-3E was found to prevent mature thymocyte (IS) formation on lipid bilayers presenting agonist pMHCs and intercellular adhesion molecules1 (ICAM-1) (*397*). In contrast, my results may suggest a different role Sema-3E in promoting IS formation specific to RMA-s. It is also possible that Sema-3E enhanced the process of degranulation and protein transport, independent of activation/inhibitory receptor activation. Therefore, the absence of Sema-3E in NK cells resulted in degranulation delay or inhibition which might explain the reduction in cytotoxicity and cytokine release observed when NK cells are stimulated with PMA/ionomycin reagent. NK cells have the unique ability to kill tumor cells without any existing priming and expansion of specific clones (*1*). In vivo, to better understand the relevance of Sema-3E in modulating antitumor immunity, 4T1 cells were injected into Sema-3E-deficient mice and WT controls. Interestingly, I observed that Sema-3E-deficient mice injected with 4T1 cells developed significantly larger tumors than their WT controls (**supplementary Figure S.5).** NK cells have the unique ability to kill tumor cells without any existing priming and expansion of specific clones (*1*). Despite there being other immune cells (such as T, macrophages, and DCs) that may mediate antitumor immunity in the 4T1 tumor model (*449-451*), I observed that NK cell depletion in WT mice led to a significant acceleration of tumor growth compared to the nondepleted group (**supplementary Figure S.9)**. Our results suggested a critical role for Sema-3E in modulating 4T1 tumor growth in vivo, possibly due to the lack of NK cell effector function. Similarly, NK cell depletion in Sema-3E-deficient mice led to a significant acceleration of tumor growth compared to the nondepleted group **(supplementary Figure S.10)**. This suggests that Sema-3E-deficient NK cells remain able to kill tumor cells but with lower efficacy than WT NK cells.

In conclusion, our study provides evidence for the first time of a novel role for Sema-3E in regulating NK cell function. I demonstrated that Sema-3E-deficient mice developed fewer NK cells and presented evidence for regulatory effects of Sema-3E on NK cell cytotoxicity and cytokine production (**Figure 3.7**). Future work will establish if and how Sema-3E impacts the

ability of NK cells to target RMA-s cells in vivo and will examine the expression of NK cell exhaustion markers (such as NKG2A, PD-1, and TIM-3) both in the steady state and in a tumor model. Understanding Sema-3E's role in modulating NK cell effector function(s) may provide novel therapeutic targets to manipulate specific NK cell functions in clinical settings.

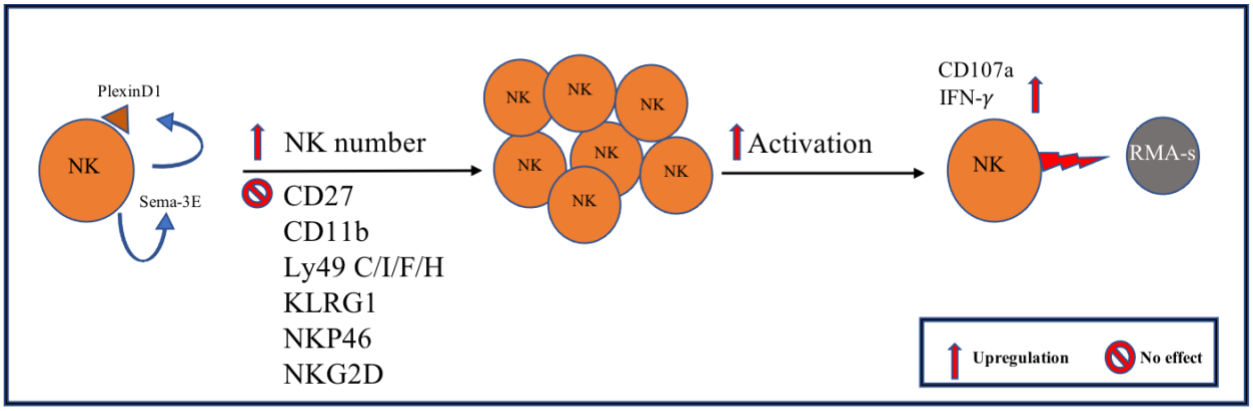


Figure 3.7. Sema-3E modulated NK cell function. The Sema-3E was examined in NK cells. Through modulation of the phenotype and function of NK cells, the Sema-3E modulates NK cell absolute cell number, CD107a, and IFN-γ to target RMA-s cells.

Chapter 4: Semaphorin 3E Deficiency Altered Dendritic Cell Phenotype and Function

4.1 Abstract

Dendritic cells (DCs), a distinct class of leukocytes, are specialized antigen-presenting cells described as the master regulators of the immune system, mediating innate immunity and shaping adaptive immunity. Maturation of DCs upregulated co-stimulatory molecules, promoted DCs migration thus T-cell activation and generation while steady-state DCs reside in immature or semi-mature states to maintain self-tolerance. Semaphorin 3E (Sema-3E) is a secreted axon-guidance protein that has emerged as a novel mediator of DC function in various disease models. However, the role of Sema-3E in regulating DC phenotype and function in the steady-state has not been investigated. Here I report that a deficiency in Sema-3E resulted in higher proportion and absolute cells number in bone marrow DCs. However, splenic DCs from Sema-3E-deficient mice and WT littermates showed no apparent difference in CD11c expression. Splenic DCs expressed both Sema-3E and PlexinD1 mRNA, and their expression were tightly regulated by LPS. Sema-3E-deficient splenic DCs showed an altered MHC class II phenotype and favored the $CD4^{+}CD8^{-}$ subset that promotes T cell immune responses and innate lymphoid cells (ILC) 2 and 3 activation. In terms of DC function, Sema-3E-deficient immature and LPS-matured bone marrow-derived DCs showed upregulated expression of IL-12/IL-23p40. However, Sema-3E-deficient immature splenic DCs downregulated IL-12/IL-23p40 while LPS-stimulated splenic DCs showed a similar expression level compared to WT controls. Our results revealed a critical role of Sema-3E in modulating DC phenotype and function presumably through autocrine or paracrine pathways.

4.2 Introduction and rationale

DCs belong to the myeloid lineage and are derived from HSCs (*227*). BM myeloid precursors turn into differentiated DCs in the presence of GM-CSF and IL-4 (*228*).Pre-cDCs migrate from the BM to different tissues, including the spleen where they differentiate further into cDC1s and cDC2s (*233*). DCs are found in two distinct functional states, mature and immature, distinguished by many features. In mice, DCs acquire MHC class II, CD4, CD8a, CD11b or/and DEC-205 receptors. However, all classes of DCs have a defined expression of CD11c (*229*). Upon stimulation, DCs underwent maturation and upregulated the expression levels of CD40, CD80, and CD86 co-stimulatory molecules compared to immature DCs (*254*). Mature DCs also produce

a broad range of cytokines (including IL-2, IL-6, IL-10, IL-18, and IFN-γ) that allow them to activate immune cells such as T and NK cells (*225*, *252*).

Semaphorin 3E (Sema-3E) and its receptor PlexinD1 have emerged as an essential axis involved in immune regulation (*396*). In vitro, I have previously demonstrated that Sema-3E and its receptor (PlexinD1) are expressed by immature and LPS-stimulated BMDCs. Furthermore, Sema-3E produced by immature BMDCs suppressed NK cell migration (*420*). It was reported that Sema-3E-deficient mice modulated basal and HDM-induced pulmonary DC recruitment (*398*, *418*). Sema-3E also regulated DCs functions in intestinal inflammation (*419*). Following *Leishmania major* infection, $CD11c^+$ cells from Sema-3E deficient mice showed increased expression of co-stimulatory molecules and IL-12p40 production (*452*). In chlamydial infection, Sema-3E was induced in the lung and impacted DC function. Interestingly, DCs from Sema-3E-deficient mice showed impairment of both the surface expression of co-stimulatory molecules and IL-12 production. However, DCs displayed a higher expression of PD-L1, PD-L2, and IL-10. In DC-T cell co-culture system, DCs from Sema-3E-deficient mice failed to induce Th1 and Th17 cell responses compared to DCs from WT mice (*453*). An adoptive transfer experiment demonstrated that mice receiving DCs from Sema-3E-deficient mice were not protected against chlamydial infection were those receiving DCs from WT mice (*453*). All these published works show the association of Sema-3 expression and DC function. However, there is no formal study to examine the impact of Sema-3E in modulating steady-state or mature DCs phenotype and function.

This study focused on the ability of Sema-3E to regulate DC phenotype and function using murine BM and splenic DCs from Sema-3E deficient mice. The use of BMDCs illustrated a better controlled in vitro culture system that devoid relatively the influence of other cell types on DC, to support analyses of possible autocrine effect of Sema-3E on DC phenotype and function. On the other hand, the use of (whole tissue) splenic DCs before isolation (ex vivo) or after isolation (isolated DCs) mimics, to a certain extent, the in vivo system, where DCs were in close contact with other cells such as NK cells, T cells, and macrophages. Such an approach provides us a tool to dissect the relative contribution of the autocrine or paracrine effects of the Sema-3E on DCs.

I hypothesized that Sema-3E modulated DCs phenotype and function. My aim in this study was to determine the role of Sema-3E in modulating DC phenotype and cytokine production in the steady-state or following stimulation by LPS.

4.3 Results

4.3.1 Sema-3E deficiency in immature BMDCs resulted in higher CD11c+ cells number and IL-12/IL-23p40 cytokine production.

All classes of DCs are defined by the expression of CD11c (*229*). Here, I sought to investigate whether Sema-3E modulates immature BMDC CD11c expression. In vitro, I observed that BMDCs resulted in a higher proportion and the absolute number of CD11c cells (percentage and absolute cell number) in Sema-3E-deficient mice compared to WT (**Figure 4.1**). Furthermore, I wanted to explore whether Sema-3E modulates BMDC maturation phenotype. I found that Sema-3E-deficient and WT cells exhibited similar expression levels of co-stimulatory molecules (CD40, CD80, CD86), and maturation phenotype (MHC class II) in flow cytometry (**Figure 4.2**).

Next, to investigate if Sema-3E would affect immature BMDC cytokine production, conditioned media of both Sema-3E-deficient and WT BMDCs was collected and analyzed for cytokine productions by MSD assay. Interestingly, I observed that immature BMDCs of Sema-3E-deficient mice displayed higher level of IL-12/IL23p40 compared to WT mice (**Figure 4.3**). However, other cytokines such as IFN- γ, IL-6, and IL-10 were below the detection level in both Sema-3E-deficient, and WT conditioned media (data not shown). Together, these results suggest a direct impact of Sema-3E on BMDC function ($CD11c^+$ cell number and cytokine production), independent of their phenotype (maturation status).

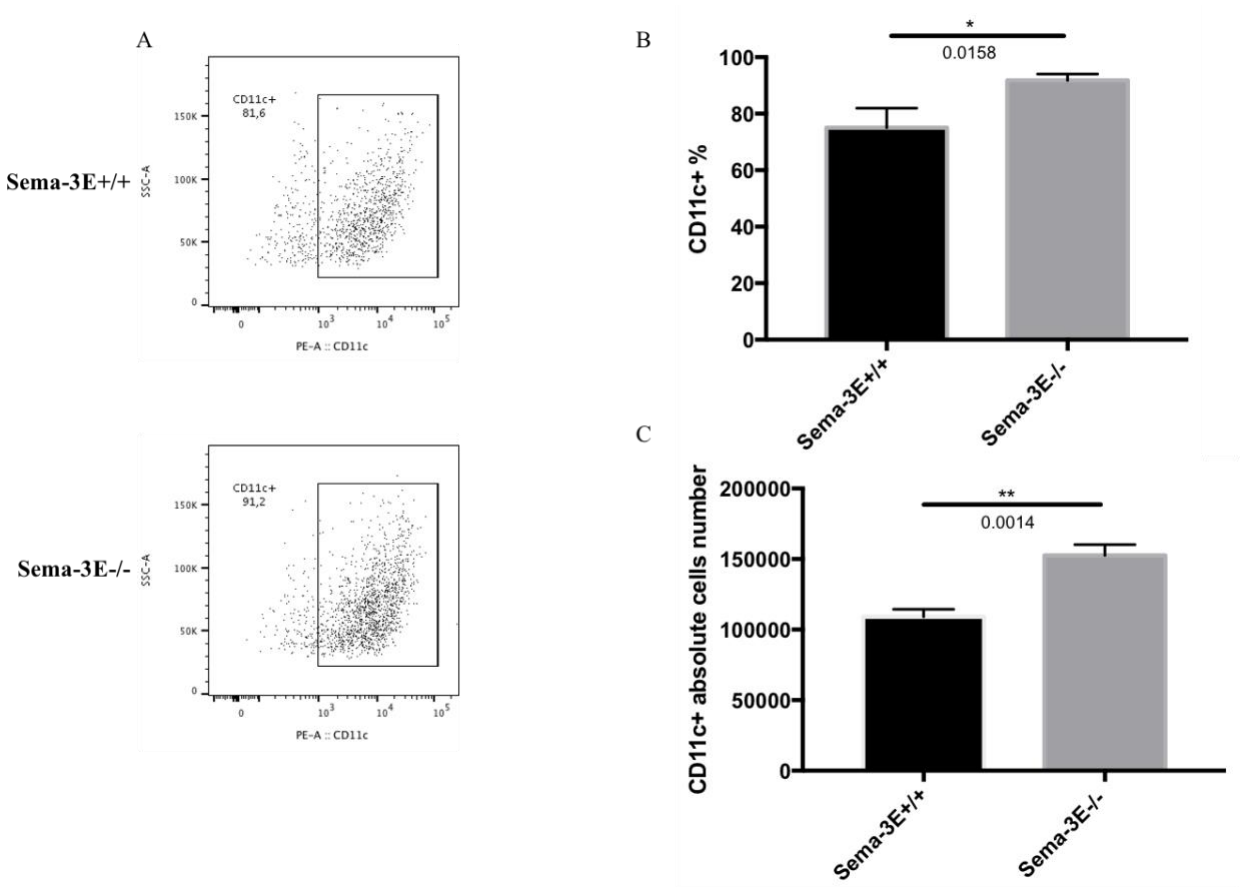
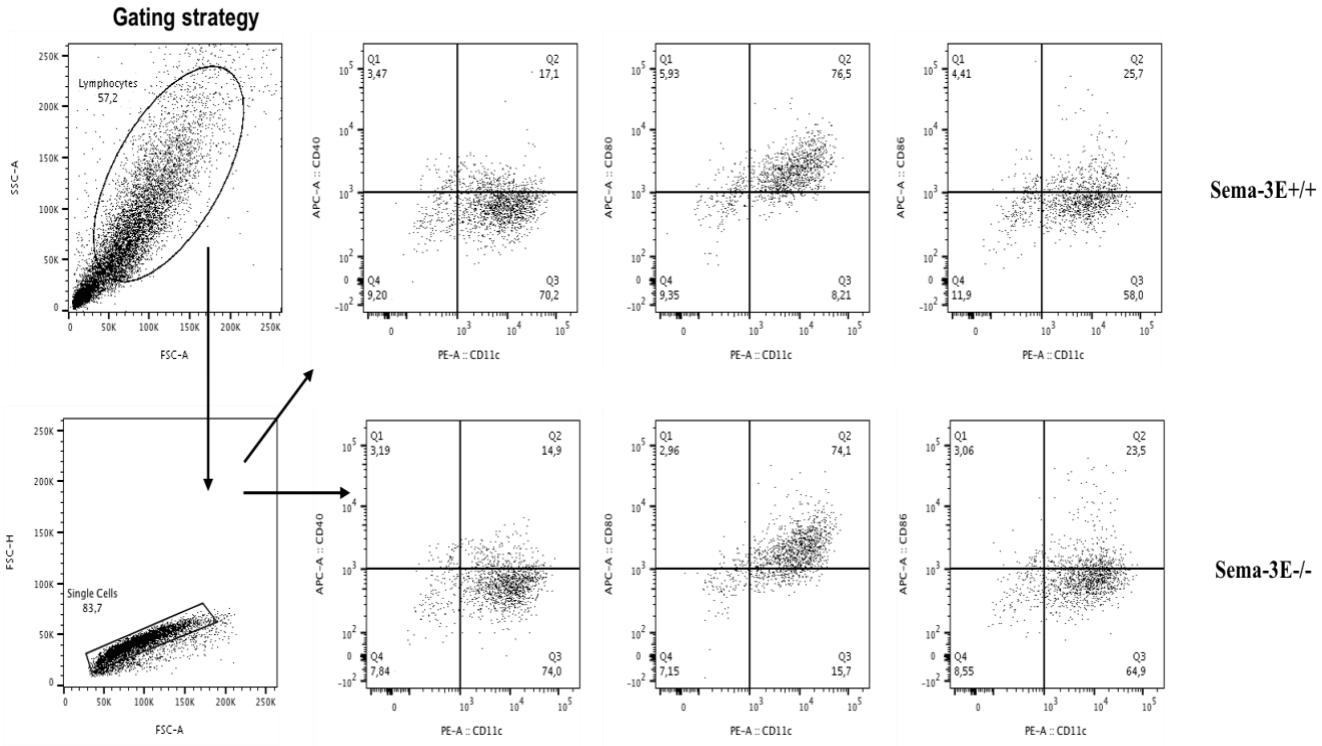


Figure 4.1. Sema-3E deficiency resulted in an increase in and the cell number of immature BMDCs at steady-state. BMDCs were cultured in GM-CSF medium, and the expression of CD11c was detected by surface staining. **(A)** Representative flow cytometry; **(B)** $CD11c^+$ percentage; **(C)** $CD11c^+$ absolute cell number in BMDCs from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) mice. Data are shown as mean ± SEM, $N = 5$. A two-tailed *t* test was used to compare two groups as indicated. $*P < 0.05$; $**P < 0.01$.

A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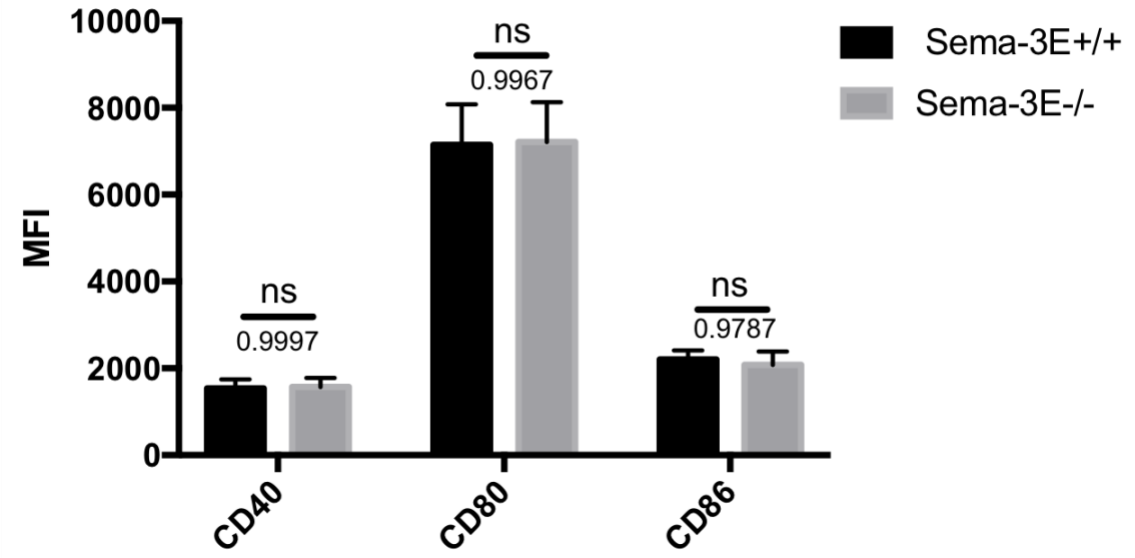
57,2
SSC-A
FSC-A
Single Cells
83,7
FSC-H
APC-A :: CD40
APC-A :: CD80
APC-A :: CD86
PE-A :: CD11c
Q1 3,47
Q2 17,1
Q4 9,20
Q3 70,2
Q1 5,93
Q2 76,5
Q4 9,35
Q3 8,21
Q1 4,41
Q2 25,7
Q4 11,9
Q3 58,0
Q1 3,19
Q2 14,9
Q4 7,84
Q3 74,0
Q1 2,96
Q2 74,1
Q4 7,15
Q3 15,7
Q1 3,06
Q2 23,5
Q4 8,55
Q3 64,9
Sema-3E+/+
Sema-3E-/-

B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6000
4000
2000
0
ns
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ns
0.9967
ns
0.9787
CD40
CD80
CD86
Sema-3E+/+
Sema-3E-/-

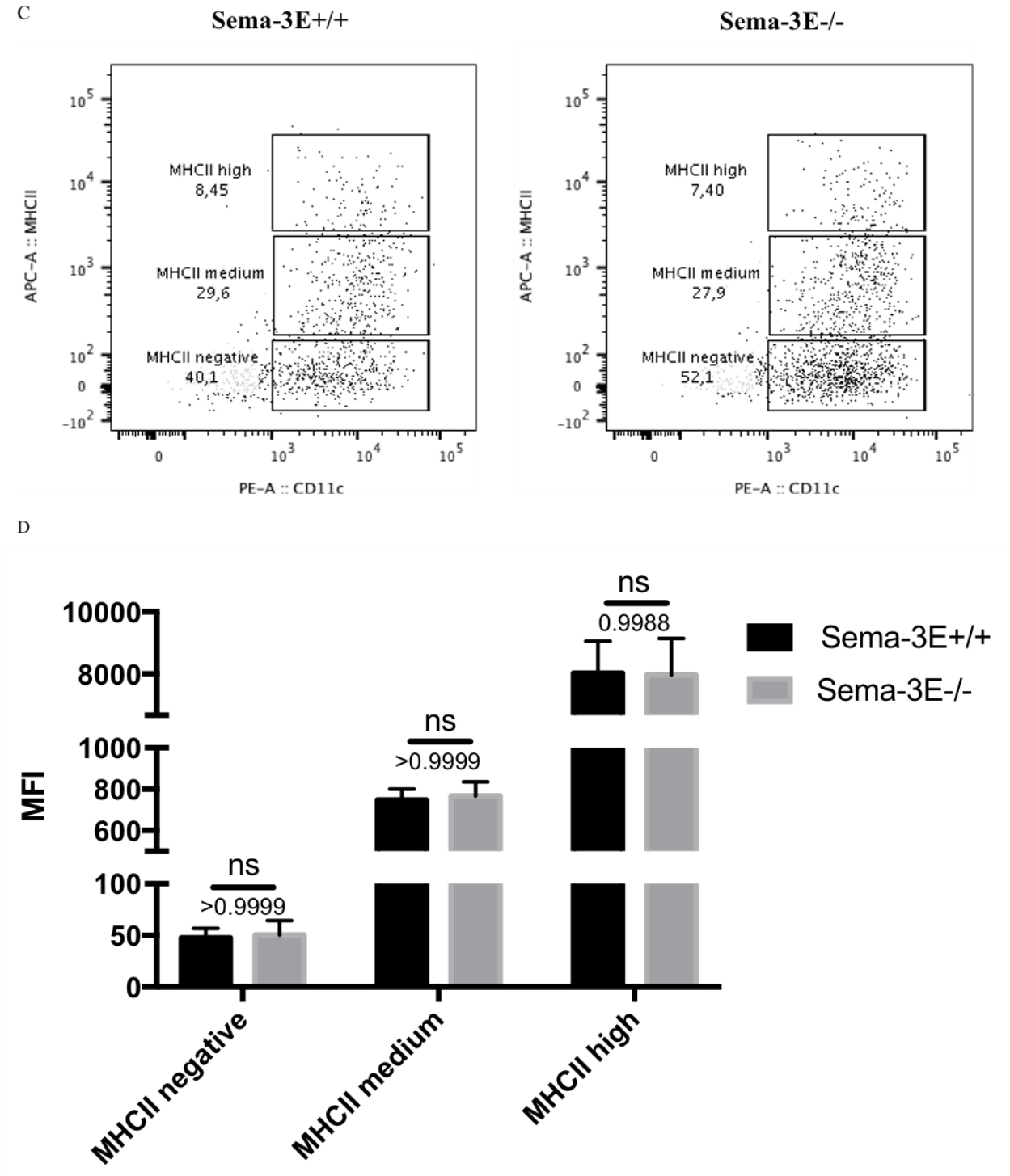


Figure 4.2. Sema-3E deficiency did not affect BMDC maturation phenotype. BMDCs from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) mice were cultured in GM-CSF medium. **(A, B)** Expression of CD40, CD80, CD86, and **(C, D)** MHC class II (MHCII) was detected by surface staining. **(A, C)** Representative flow cytometry; **(B, D)** Mean Fluorescent Intensity (MFI) for collective data (N = 5) P values are shown for two-way ANOVA to compare data from two groups as indicated. ns, not significant.

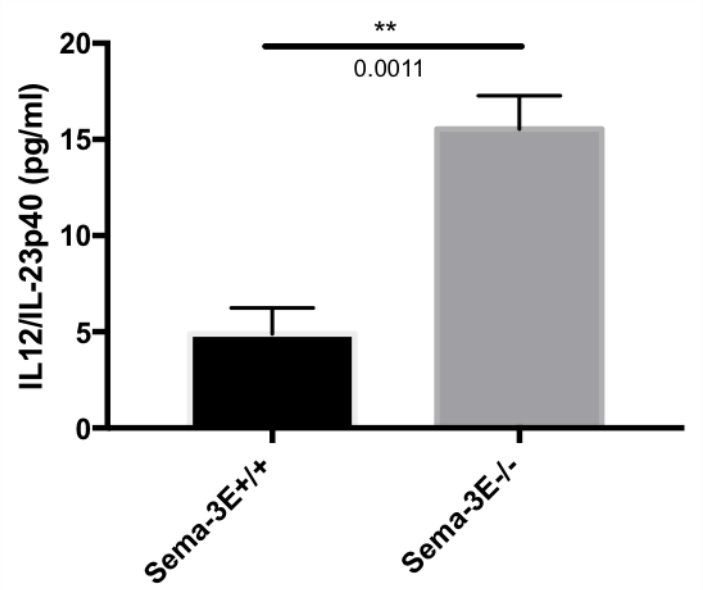


Figure 4.3. Sema-3E deficiency resulted in the upregulation of IL-12/IL-23p40 production in BMDC. BMDCs from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) mice were cultured in GM-CSF medium. On day-6, conditioned mediums were collected and used in MSD assay. **(A)** The concentration of IL-12/IL23p40 was measured. Mean calculated concentration of the samples (in pg/mL)—concentrations adjusted for the dilution factor. Data are shown as mean ± SEM, $N = 3$. A two-tailed t test was used to compare two groups as indicated. $^{**}P < 0.01$.

4.3.2 Splenic DCs expressed Sema-3E and PlexinD1.

In the steady-state, non-migratory conventional DCs (cDCs) represent the majority of the splenic DC population (*454*). Similar to BMDCs, steady-state splenic DCs are immature. However, they can process and present foreign antigens, resulting in a T cell response (*454, 455*). Previously, I have reported that Sema-3E and PlexinD1 are expressed by immature BMDCs (*420*). However, the expression of Sema-3E and PlexinD1 in splenic DCs remains to be investigated. To study the role of Sema-3E in regulating splenic DC phenotype and function, I examined Sema-3E and PlexinD1 mRNA expression in isolated splenic DCs from both WT and Sema-3E-deficient mice. Splenic DCs were isolated using a CD11c PE positive selection kit (described in section 2.6 (chapter 2)) and checked for purity using CD11c PE antibody in flow cytometry (purity >90%) (supplemental Figure S.3). Isolated cells were processed for RNA isolation using the Trizol method then cDNA was synthesized (described in section 2.7(chapter 2)). Sema-3E and PlexinD1

mRNA expression was analyzed using primers specific to Sema-3E and PlexinD1 in quantitative (real-time) PCR (qPCR) assays (described in section 2.8 (chapter 2)) (**Figure 4.4**). GAPDH was used as an internal control in all qPCR analyses. For Sema-3E expression, I used immature BMDCs from WT and Sema-3E-deficient mice as a positive and negative control, respectively. Similarly, for PlexinD1 expression, I used immature BMDCs of WT mice as positive control and CD11c-PlexinD1 conditional knockout (described in section 1.18.5 (chapter 1)) BMDCs as a negative control. Immature splenic DCs expressed Sema-3E (**Figure 4.4**A) and PlexinD1 (**Figure 4.4**B) at the mRNA level. These results suggest that Sema-3E may affect splenic DCs through the PlexinD1 receptor and regulate their phenotypes and functions.

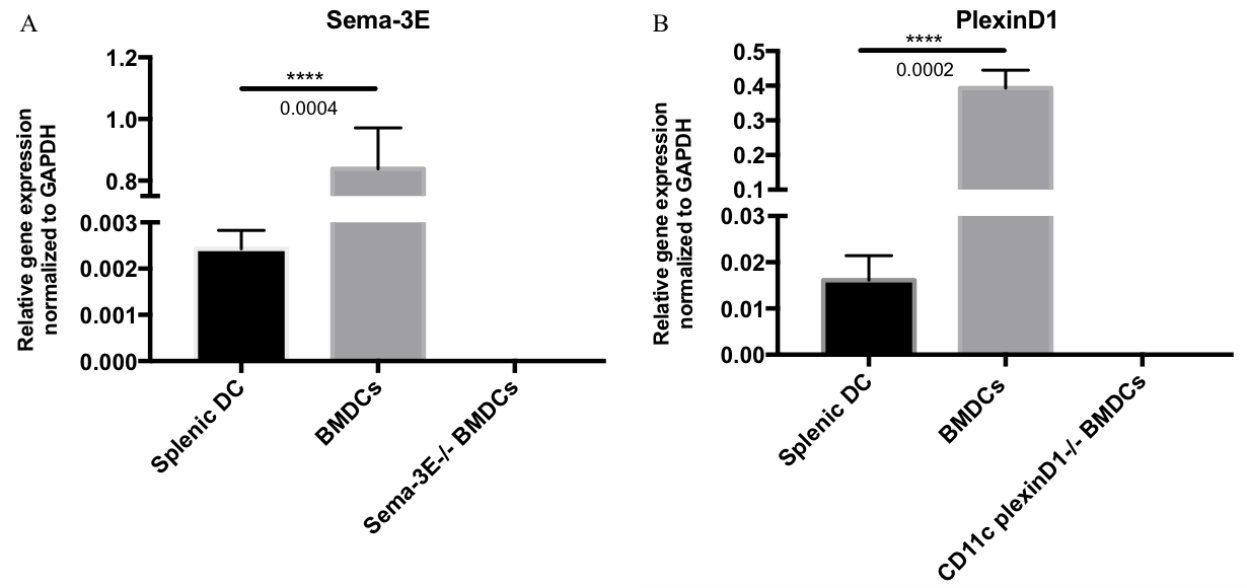


Figure 4.4. Splenic DCs expressed lower level of Sema-3E and PlexinD1 when compared to BMDCs. Splenic DCs were isolated from WT (Sema-3E+/+) BALB/c mice using a CD11c PE positive selection kit. RNA from these cells was used in a qPCR assay to measure Sema-3E (**A**) and PlexinD1 (**B**) expression, respectively. BMDCs from Sema-3E-deficient mice (**A**, Sema-3E-/-) or CD11c-PlexinD1 conditional knockout mice (**B,** CD11c-PlexinD1-/-) BMDCs were used as respective negative controls. BMDCs were used as a positive control in both cases. Data are shown as mean ± SEM of 4 independent experiments. *P* values from two-tailed *t* tests are shown for comparison of two groups as indicated. ****$P < 0.0001$.

4.3.3 Sema-3E-deficient splenic DCs exhibited altered MHC class II phenotype and CD4$^+$ subsets.

As shown previously, Sema-3E-deficient BMDCs displayed high CD11c proportion and number (**Figure 4.1**) and upregulated IL-12/IL23p40 expression (**Figure 4.3**). However, the co-stimulatory molecules CD40, CD80, CD86, and MHCII remained unaffected (**Figure 4.2**). Moreover, splenic DCs expressed both Sema-3E and PlexinD1 at the mRNA level (**Figure 4.4**). Next, I investigated whether Sema-3E would modulate the phenotype of splenic DCs. To address this question, splenic DCs were collected from both WT and Sema-3E-deficient BALB/c mice. Unlike BMDCs (in **Figure 4.1**), splenic DCs showed no change in CD11c$^+$ percentage and absolute cell numbers in Sema-3E-deficient mice than WT controls (**Figure 4.5**). Cells were stained with CD11c, CD40, CD80, CD86, and MHC class II in flow cytometry (**Figure 4.6**). No apparent differences were reported in the co-stimulatory molecules CD40, CD80, and CD86; however, Sema-3E-deficient splenic DCs displayed higher levels of MHC class II molecules than WT controls (**Figure 4.6**).

In mice, cDCs are categorized based on CD4 and CD8α expression into CD4$^-$CD8$^+$, CD4$^+$CD8$^-$, and CD4$^-$CD8$^-$ (*456*). Here, I investigated whether Sema-3E would modulate splenic DC subpopulations (**Figure 4.7**). Splenic DCs were collected from both WT and Sema-3E-deficient mice. Cells were stained for CD11c, CD4, and CD80α in flow cytometry. Our data showed that Sema-3E deficiency resulted in a larger CD4$^+$CD8$^-$ DC population than WT controls. However, both Sema-3E-deficient and WT splenic DCs displayed similar CD4$^-$CD8$^+$ populations (**Figure 4.7**). Our observations suggest that Sema-3E plays a critical role in modulating splenic DC phenotypes and populations; therefore, I hypothesized it would also modulate DC effector functions.

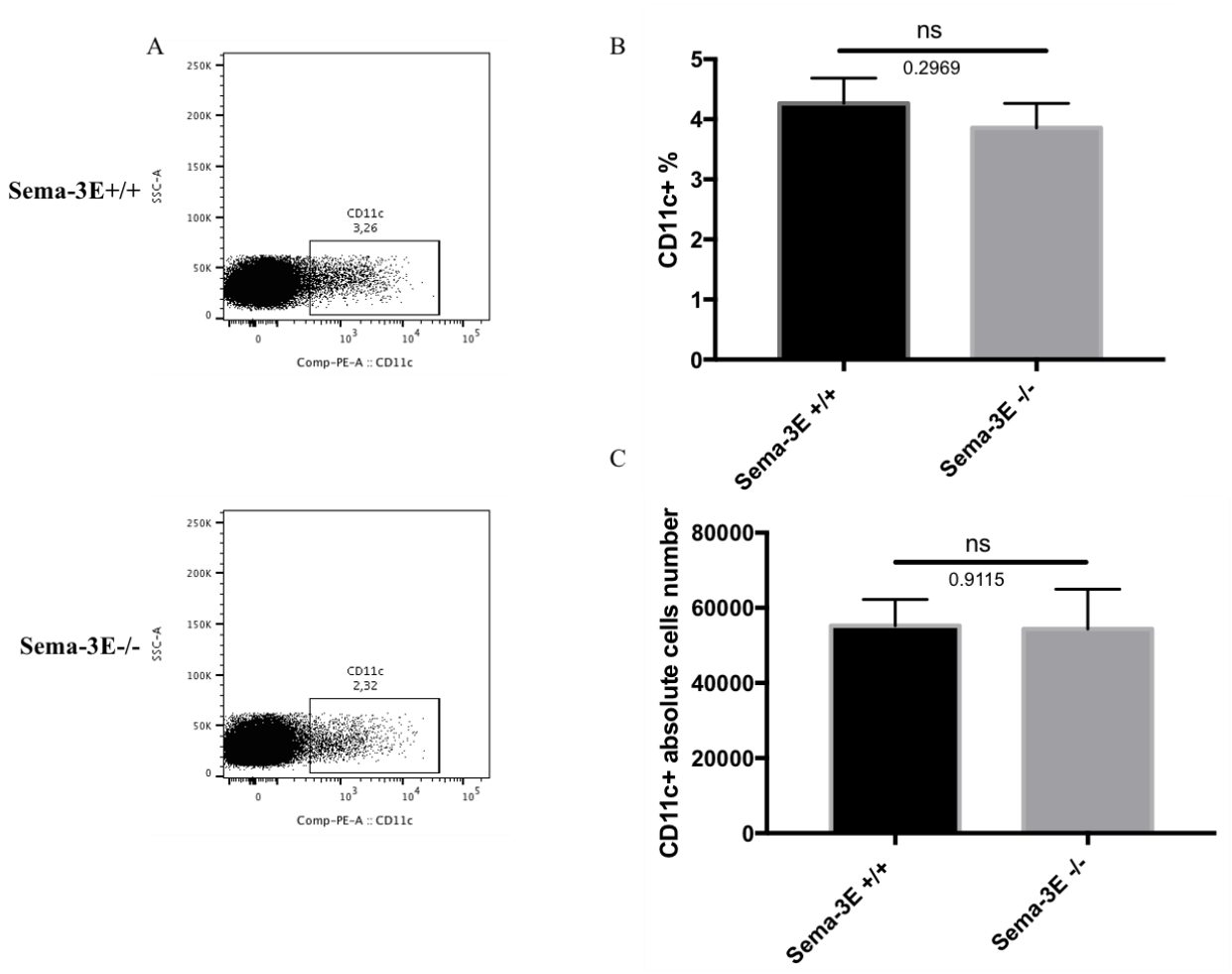
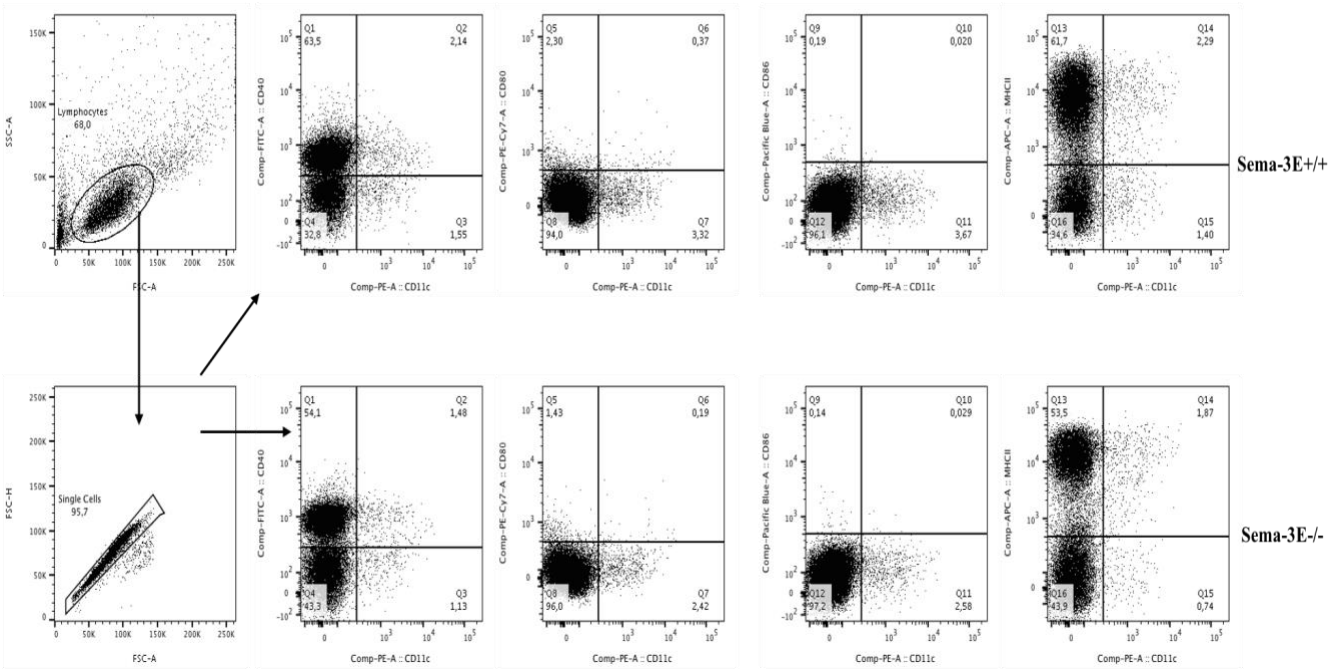


Figure 4.5. Sema-3E deficiency did not affect percentage and absolute cell number in splenic DCs. Spleens were collected from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) BALB/c mice and the cells were surface-stained to detect the expression of CD11c. **(A)** Representative flow cytometry; **(B)** $CD11c^+$ percentage; **(C)** $CD11c^+$ absolute cell number. Data shown as mean ± SEM, $N = 5$. A two-tailed *t* test was used to compare two groups as indicated. ns, not significant.

A


Lymphocytes
68,0
SSC-A
FSC-A
Comp-FITC-A :: CD40
Comp-PE-A :: CD11c
Comp-PE-Cy7-A :: CD80
Comp-Pacific Blue-A :: CD86
Comp-APC-A :: MHCII
Q1 63,5
Q2 2,14
Q4 32,8
Q3 1,55
Q5 2,30
Q6 0,37
Q8 94,0
Q7 3,32
Q9 0,19
Q10 0,020
Q12 96,1
Q11 3,67
Q13 61,7
Q14 2,29
Q16 34,6
Q15 1,40
Sema-3E+/+
Single Cells
95,7
FSC-H
Q1 54,1
Q2 1,48
Q4 43,3
Q3 1,13
Q5 1,43
Q6 0,19
Q8 96,0
Q7 2,42
Q9 0,14
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Q14 1,87
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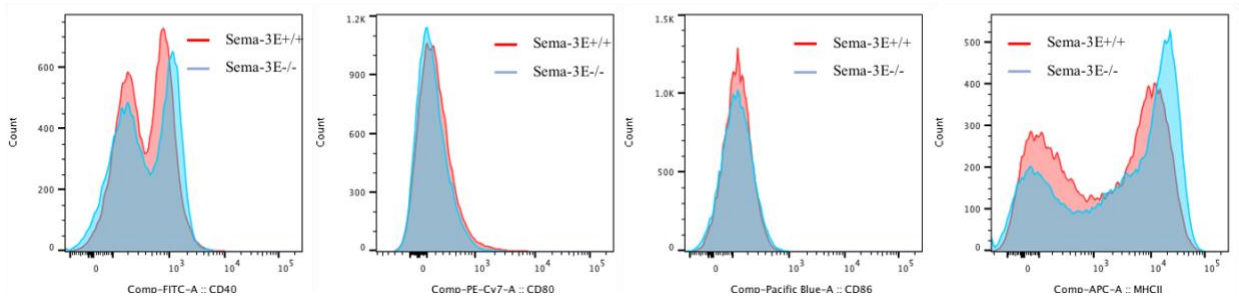
Sema-3E-/-

B


Sema-3E+/+
Sema-3E-/-
Count
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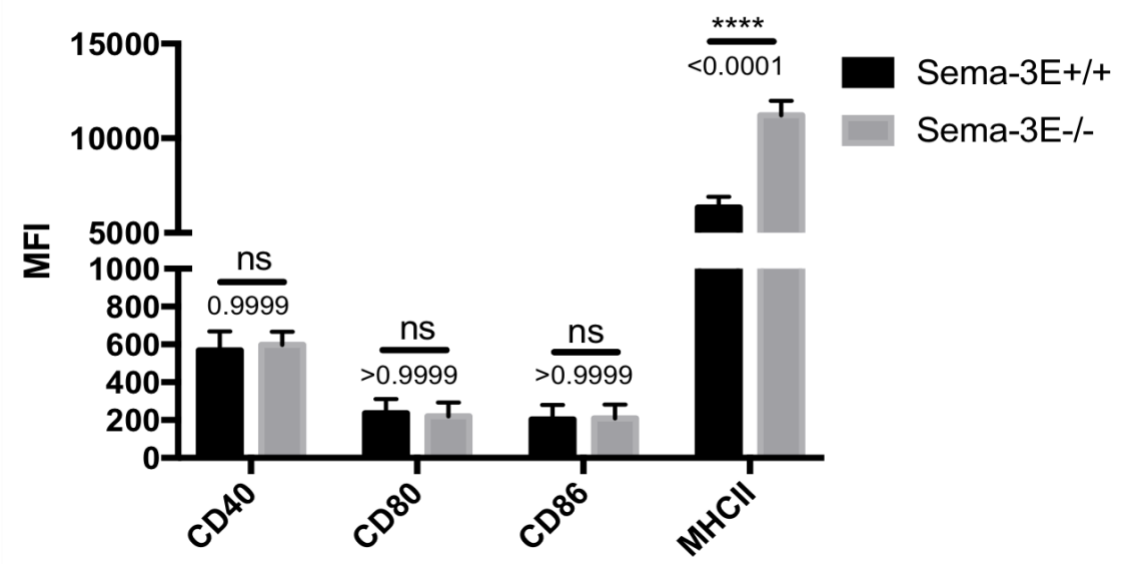
Comp-PE-Cy7-A :: CD80
Comp-Pacific Blue-A :: CD86
Comp-APC-A :: MHCII

C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<0.0001
Sema-3E+/+
Sema-3E-/-
CD40
CD80
CD86
MHCII

Figure 4.6. Sema-3E deficiency resulted in an upregulation of MHC class II expression in in splenic DCs. Spleens were collected from Sema-3E-deficient (Sema-3E-/-) and WT (Sema-3E+/+) BALB/c mice. Expression of CD40, CD80, CD86, and MHC class II (MHCII) was detected by surface staining. **(A, B)** Representative flow cytometry; **(C)** Mean Fluorescent Intensity MFI for collective data ($N = 5$). P values are shown for two-way ANOVA to compare data from two groups as indicated. ns, not significant; ****$P < 0.0001$.

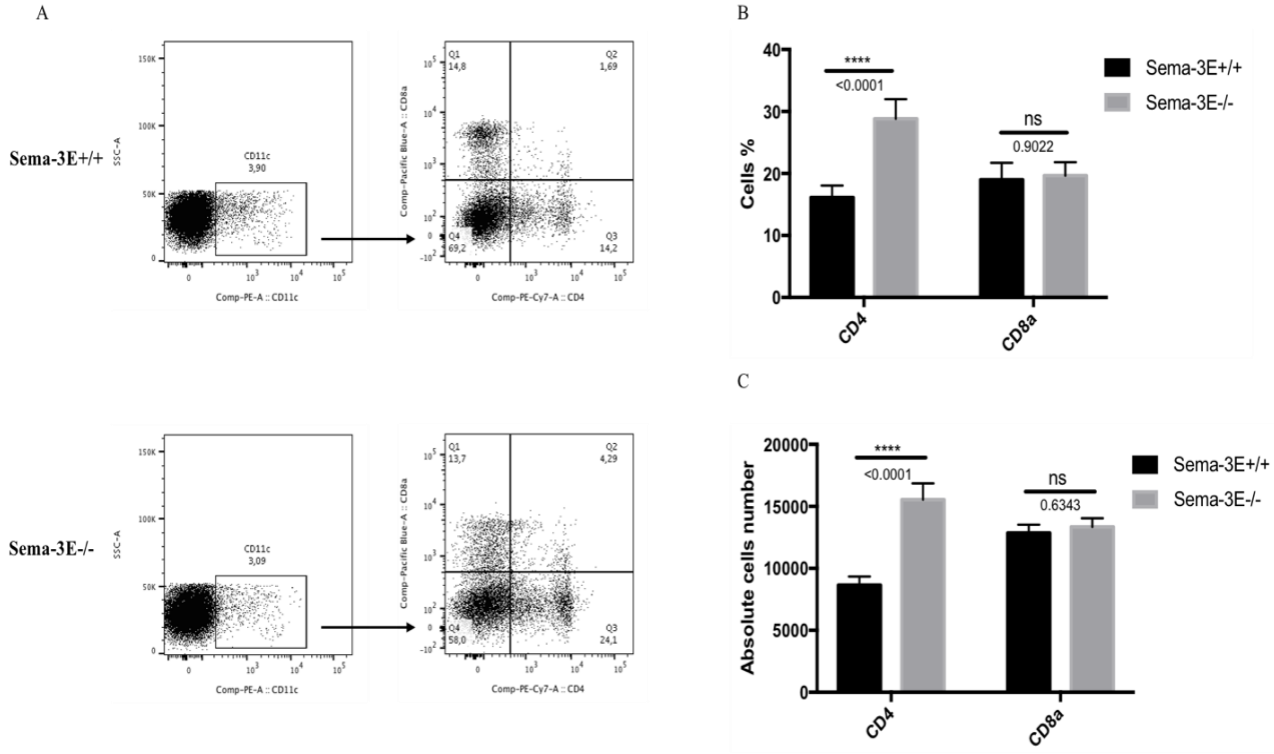


Figure 4.7. Sema-3E deficiency resulted in an increased in the CD4+ DC subsets in the spleen. Spleens were collected from WT (Sema-3E+/+) and Sema-3E-/- BALB/c mice. Expression of CD11c, CD4, and CD8a was detected by surface staining. **(A)** Representative flow cytometry, **(B)** $CD4^+$ and $CD8a^+$ percentage; **(C)** $CD4^+$ and $CD8a^+$ absolute cell number in splenic DCs from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) mice. Data are shown as mean ± SEM, $N = 5$. A two-tailed t test was used to compare two groups as indicated. ns, not significant; ****$P < 0.0001$.

4.3.4 Sema-3E deficiency in splenic DCs upregulated IL-10 production in vitro.

It has been reported that different cDC subsets such as $CD4^+CD8^-$ and $CD4^-CD8^-$ differ in their profile of chemokine production but otherwise seem to be functionally similar (*457*). However, other cDC subsets such as $CD8^-$ and $CD8^+$ differ markedly in cytokine production,

antigen processing, and Toll-like receptor expression (*458*). As shown in **Figure 4.7**, the absence of Sema-3E increases the $CD4^{+}CD8^{-}$ subset. However, the role of Sema-3E in modulating DC function remains unexplored. To investigate if Sema-3E would impact the production of key cytokines (such as IFN- γ, IL-12/IL-23p40, IL-6 and IL-10) by immature splenic DCs, DCs were isolated from the spleen of Sema-3E-deficient and WT mice and then cultured in GM-CSF medium for 24 h. Conditioned media from both Sema-3E-deficient and WT control cells was collected and analyzed for IFN-γ, IL-12/IL-23p40, IL-6, and IL-10 production by MSD assay. Interestingly, Sema-3E-deficient immature splenic DCs displayed a lower level of the proinflammatory cytokines IFN-γ, IL-12/IL-23p40, and IL-6 compared to WT controls (**Figure 4.8**). However, the anti-inflammatory cytokine IL-10 is upregulated in Sema-3E-deficient splenic DCs compared to WT control. These results suggest that the absence of Sema-3E in steady-state splenic DCs might result in an anti-inflammatory (tolerogenic) DC function.

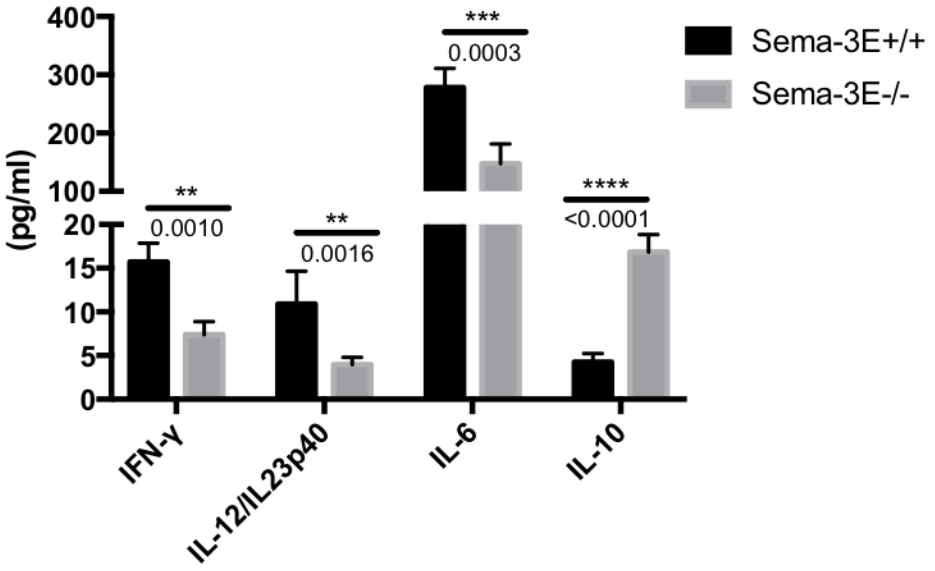


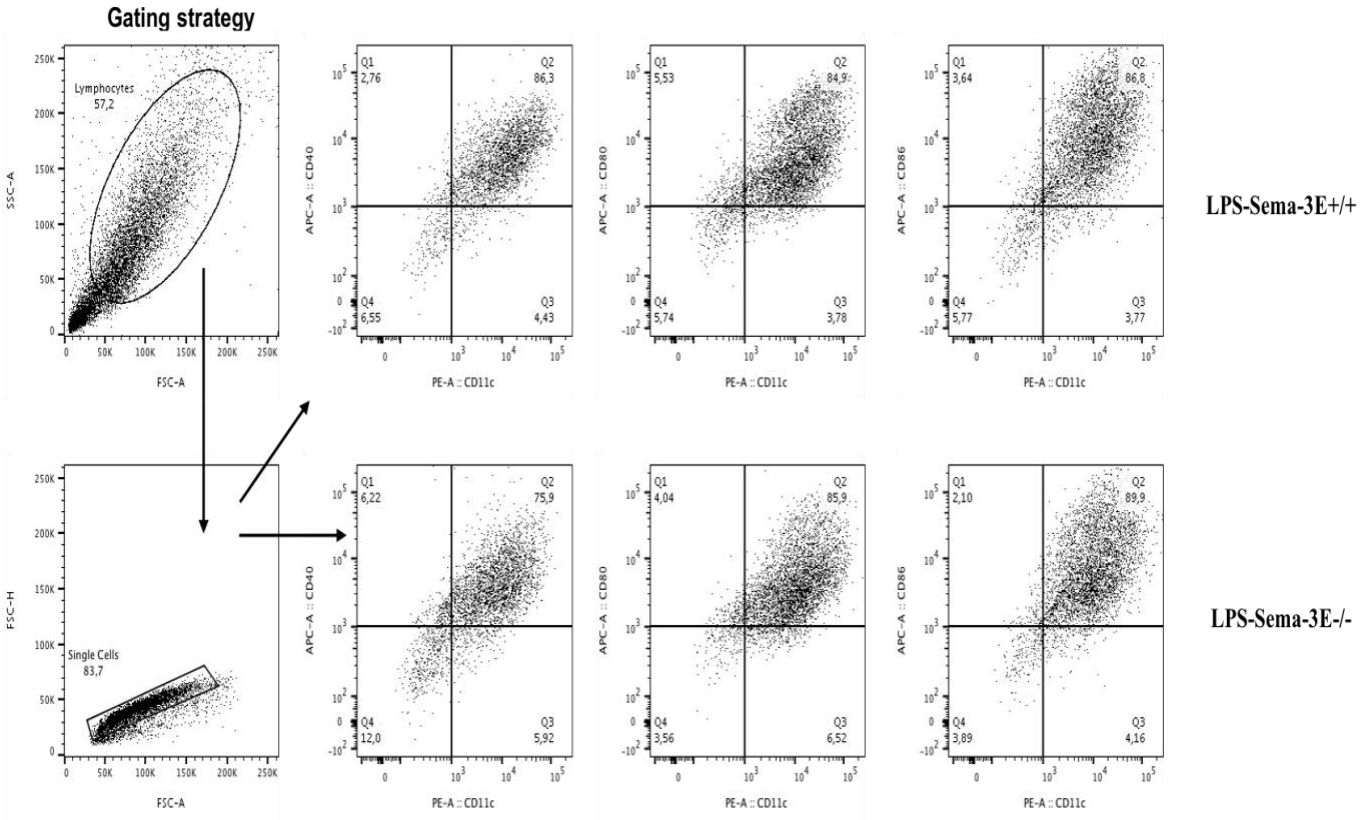
Figure 4.8. Sema-3E deficiency in splenic DCs resulted in IL-10 cytokine upregulation. Splenic DCs were isolated from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) BALB/c mice and cultured in GM-CSF medium for 24 h. Conditioned medium was collected and used to measure the proinflammatory markers IFN-γ, IL12/IL23p40, and IL-6 and the anti-inflammatory marker IL-10 by MSD assay. Data are shown as mean ± SEM of the calculated concentrations in pg/mL, adjusted for the dilution factor, for 3 independent experiments. *P* values from two-tailed *t*-tests used to compare two groups as indicated are shown. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

4.3.5 LPS-stimulated Sema-3E-deficient BMDCs upregulated IL-12/IL23p40 and IL-10 production in vitro.

The maturation of DCs - upregulates the expression of the co-stimulatory molecules CD40, CD80, and CD86 (*254*). I have reported a high level of Sema-3E and PlexinD1 mRNA expression in immature WT BMDCs; however, maturation of these cells by LPS stimulation downregulated Sema-3E and PlexinD1 mRNA expression (*420*). To investigate the role of Sema-3E in modulating mature DC phenotype and function, BMDCs were cultured in GM-CSF medium and stimulated with LPS (1 μg/μL) for 24 h to acquire the mature phenotype. The expression of CD40, CD80, CD86 and MHC class II surface markers (representing the mature DC phenotype) was assessed by flow cytometry, using immature BMDCs as the negative control.

Both WT and Sema-3E-deficient LPS-stimulated BMDCs showed upregulation of these co-stimulatory molecules (CD40, CD80, CD86), and maturation phenotype (MHC class II) compared to immature DCs (supplementary Figure S.2). However, I observed no difference between WT and Sema-3E-deficient LPS-stimulated BMDCs in the expression of these molecules **(Figure 4.9**). Next, I wanted to determine if Sema-3E deficiency would modulate cytokine production in LPS-stimulated mature BMDCs. After LPS stimulation, conditioned media of Sema-3E-deficient cells and WT controls were collected and examined by MSD assay. I observed an upregulation of both IL-12/IL23p40 and IL-10 with Sema-3E deficiency compared to WT control cells; however, there was no change in IFN-γ, IL-2, or IL-6 (**Figure 4.10**). These results suggest Sema-3E modulates the function of LPS-stimulated mature BMDCs to mediate dual pro- and anti-inflammatory functions.

A


Gating strategy
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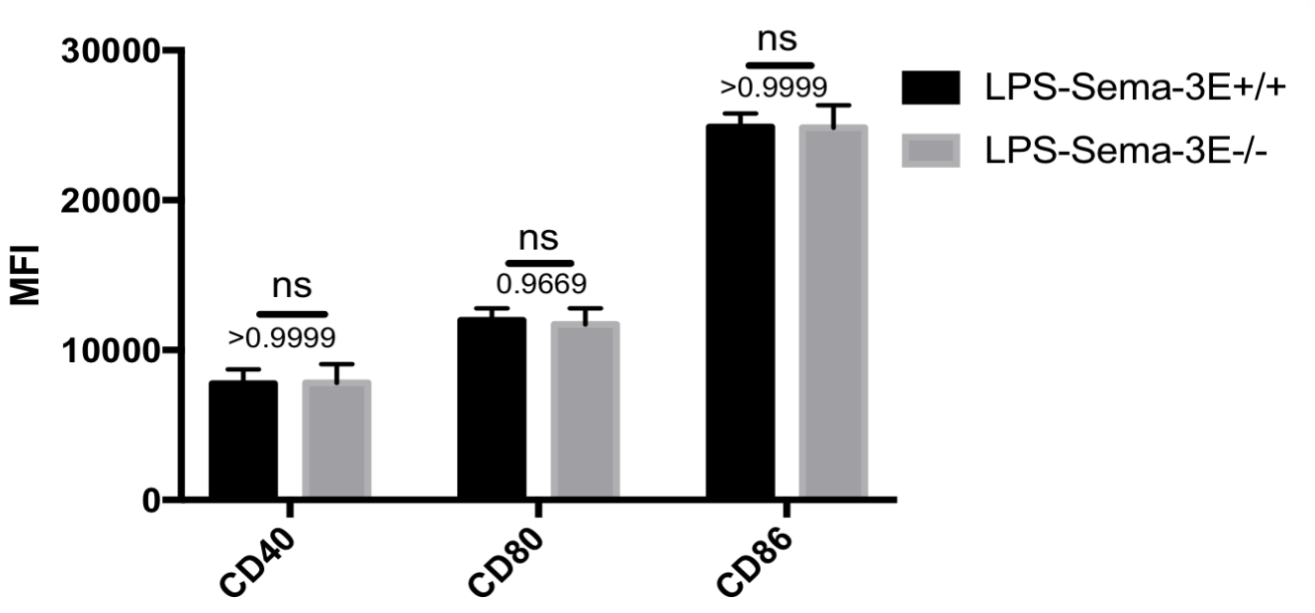
57,2
SSC-A
FSC-A
Q1
2,76
Q2
86,3
Q4
6,55
Q3
4,43
APC-A :: CD40
PE-A :: CD11c
Q1
5,53
Q2
84,9
Q4
5,74
Q3
3,78
APC-A :: CD80
PE-A :: CD11c
Q1
3,64
Q2
86,8
Q4
5,77
Q3
3,77
APC-A :: CD86
PE-A :: CD11c
LPS-Sema-3E+/+
FSC-H
FSC-A
Single Cells
83,7
Q1
6,22
Q2
75,9
Q4
12,0
Q3
5,92
APC-A :: CD40
PE-A :: CD11c
Q1
4,04
Q2
85,9
Q4
3,56
Q3
6,52
APC-A :: CD80
PE-A :: CD11c
Q1
2,10
Q2
89,9
Q4
3,89
Q3
4,16
APC-A :: CD86
PE-A :: CD11c
LPS-Sema-3E-/-

B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CD40
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LPS-Sema-3E+/+
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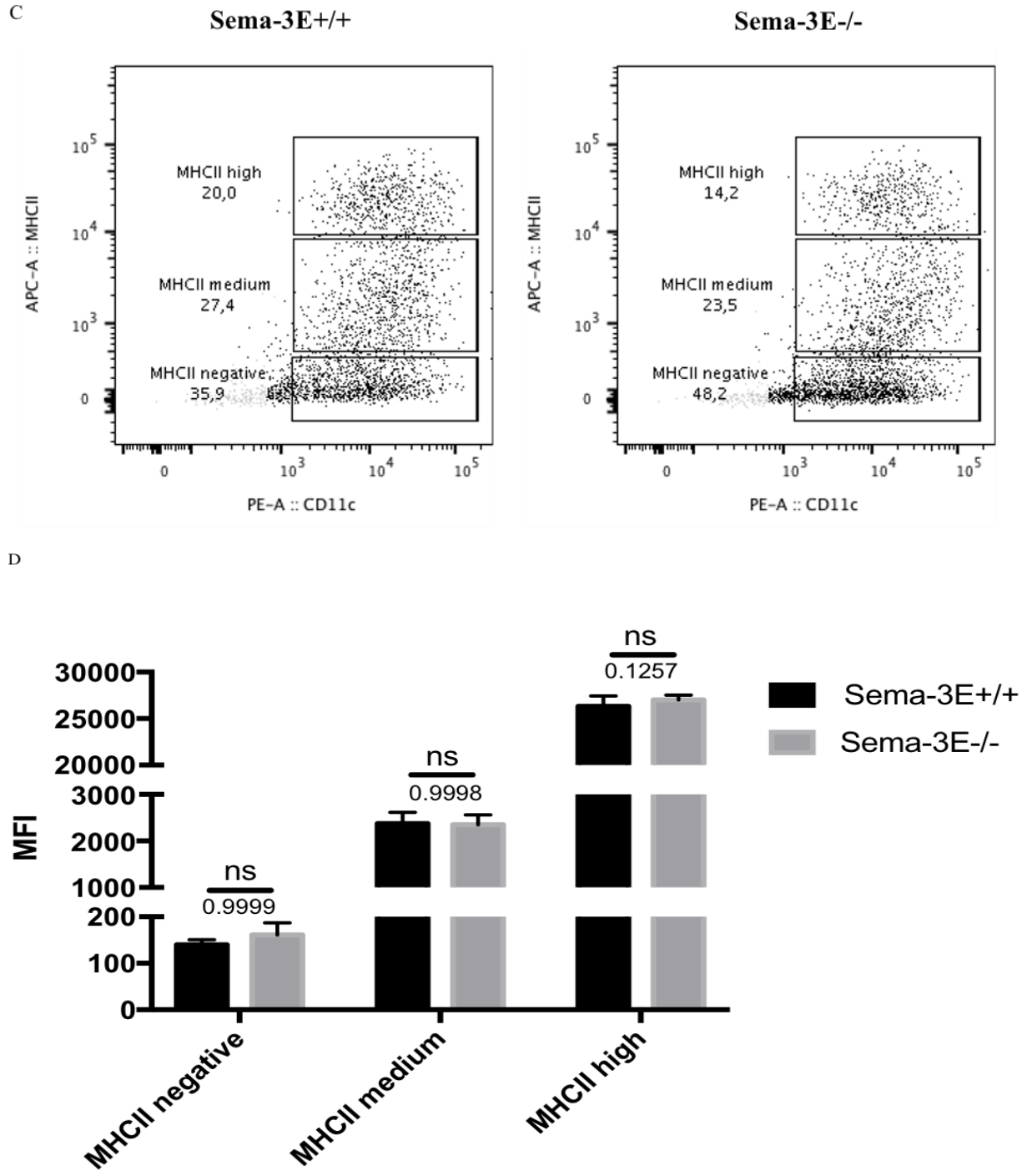


Figure 4.9. Sema-3E deficiency did not affect the maturation phenotype of the LPS-stimulated BMDCs. BMDCs from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) mice were cultured in GM-CSF medium. On day 6, LPS (1 μg/μL) for 24 h to acquire matured DC phenotype. **(A, B)** Expression of CD40, CD80, CD86, and **(C, D)** MHC class II (MHCII) was detected by surface staining. **(A, C)** Representative flow cytometry; **(B, D)** Mean Fluorescent Intensity (MFI) for collective data ($N = 5$) *P* values are shown for two-way ANOVA to compare data from two groups as indicated. ns, not significant.

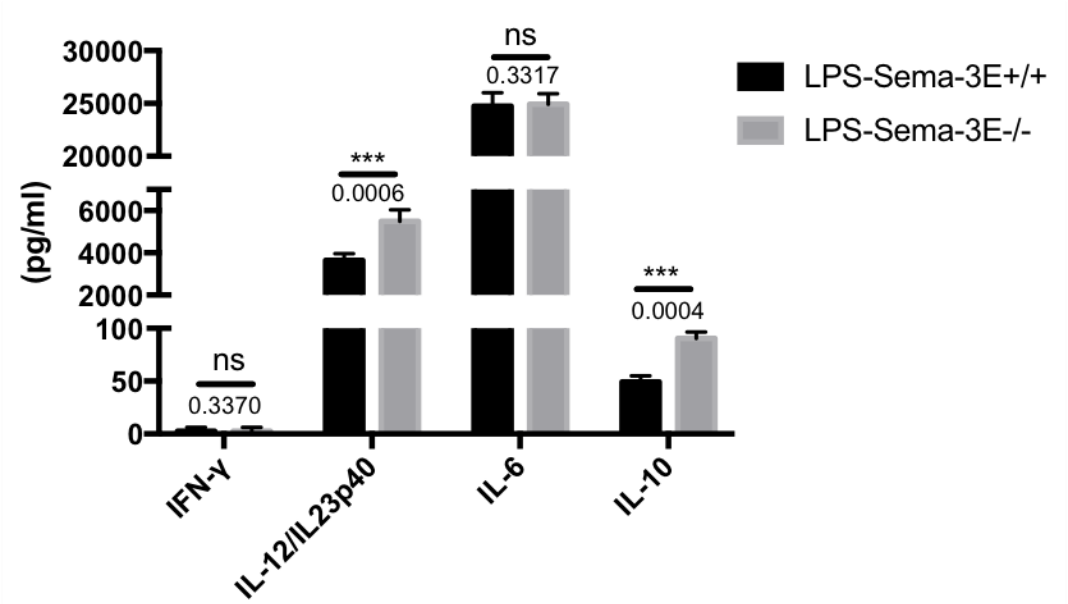


Figure 4.10. LPS-stimulated BMDCs from Sema-3E-deficient mice upregulated IL-12/IL23p40 and IL-10 production. BMDCs were generated from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) BALB/c mice and cultured in GM-CSF medium for seven days. On day 6, LPS (1μg/μl) for 24-hours to acquire matured DC. Conditioned medium was collected and used to measure the proinflammatory markers IFN-γ, IL12/IL23p40, and IL-6 and the anti-inflammatory marker IL-10 by MSD assay. Data are shown as mean ± SEM of the calculated concentrations in pg/mL, adjusted for the dilution factor, for 3 independent experiments. *P* values from two-tailed *t* tests used to compare two groups as indicated are shown. ns, not significant; ***$P < 0.001$.

4.3.6 LPS stimulation of isolated splenic DCs differentially modulated Sema-3E and PlexinD1 expression.

Previously, I have reported that immature BMDCs express Sema-3E and PlexinD1; however, stimulation of immature WT BMDCs with LPS induced downregulation of Sema-3E and PlexinD1 mRNA expression (*420*). Here I have demonstrated that immature splenic DCs expressed Sema-3E and PlexinD1 mRNA (**Figure 4.4**). The regulation of Sema-3E and PlexinD1 expression upon LPS stimulation remains to be investigated. To study the critical role of Sema-3E in regulating LPS-stimulated splenic DC phenotype and function, I examined Sema-3E, and PlexinD1 mRNA expression in isolated splenic DCs stimulated with LPS. Splenic DCs were

isolated from both WT and Sema-3E-deficient mice using a CD11c PE positive selection kit and checked for purity using CD11c PE antibody in flow cytometry (purity >90%; see supplementary Figure S.3). Isolated cells were stimulated with LPS (1 μg/μL) for 24 h. Next, the cells were processed for RNA isolation using the Trizol method then cDNA was synthesized. Sema-3E and PlexinD1 mRNA expression was analyzed by Sema-3E and PlexinD1-specific primers in qPCR assays. GAPDH was used as an internal control in all qPCR analyses. For Sema-3E expression, I used immature splenic DCs from WT mice as a positive control and immature splenic DCs from Sema-3E-deficient mice as a negative control. For PlexinD1 expression, I used immature splenic DCs from WT mice as positive control and splenic DCs from CD11c-PlexinD1 conditional knockout mice as a negative control. I demonstrated that LPS stimulation downregulated the expression of Sema-3E. In contrast, PlexinD1 receptor expression was upregulated in splenic DCs compared to immature splenic DCs at the mRNA level (**Figure 4.11**). This result suggests that LPS stimulation differently modulates the expressions of Sema-3E and PlexinD1, which may thus exert different effects on splenic DC phenotype and function.

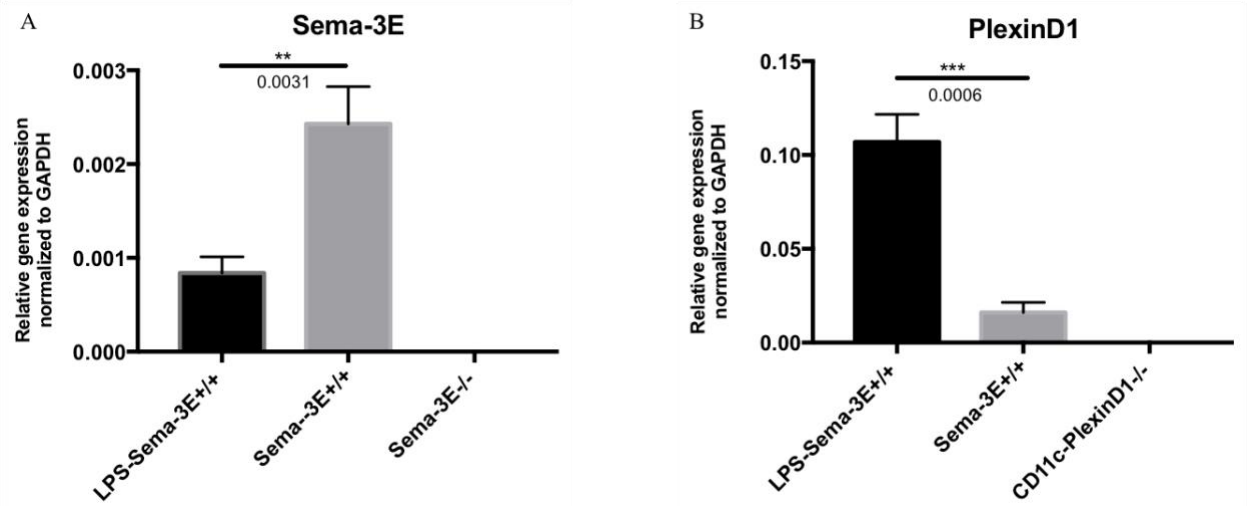


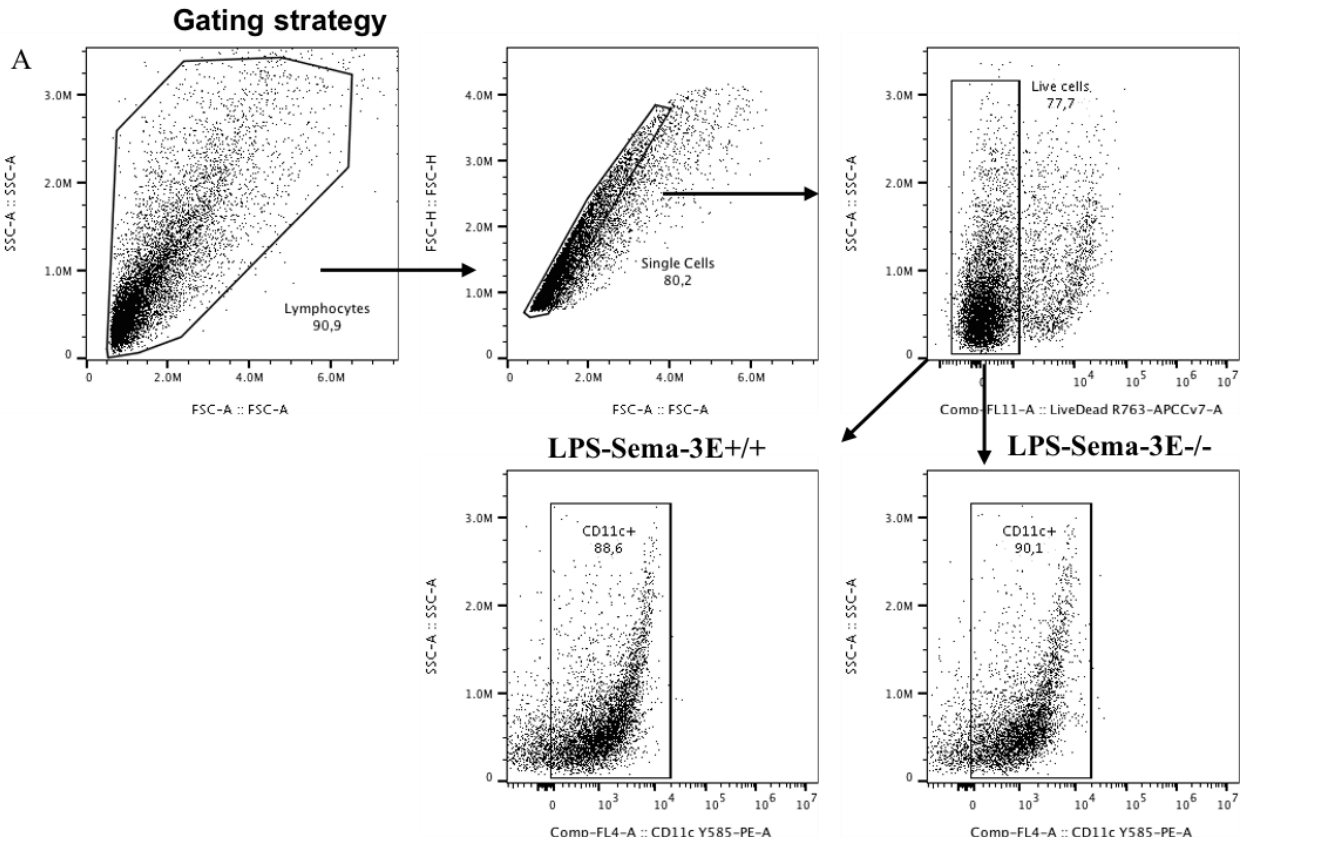
Figure 4.11. Splenic DCs downregulated Sema-3E and upregulated PlexinD1 expression upon LPS stimulation. Splenic DCs were isolated from WT (Sema-3E+/+) BALB/c mice. Isolated DCs were cultured in GM-CSF medium overnight, then LPS (1 μg/μL) was added to the culture medium for a further 24 h to induce DC maturation. RNA from these cells was used in a qPCR assay to measure Sema-3E (**A**) and PlexinD1 (**B**) expression. BMDCs from Sema-3E-deficient mice (**A**, Sema-3E-/-) or CD11c-PlexinD1 conditional knockout mice (**B,** CD11c-PlexinD1-/-) BMDCs were used as respective negative controls. Immature isolated splenic DCs from WT (Sema-3E+/+) were used

as a positive control in both cases. Data are shown as mean ± SEM of 4 independent experiments. *P* values from two-tailed *t*-tests are shown for comparison of two groups as indicated. ***P* <0.01; ****P* < 0.001.

4.3.7 LPS-stimulated splenic DCs from Sema-3E deficient mice exhibited higher MHC class II phenotype and IFN-γ production.

Next, similar to the approach taken with immature splenic DCs, I wanted to explore the role of Sema-3E in modulating mature (LPS-stimulated) splenic DC phenotype and function. Splenic DCs were isolated from WT and Sema-3E-deficient BALB/c mice. After isolation, cells were cultured in the presence of GM-CSF and stimulated with LPS (1 μg/μL) for 24 h to acquire the mature DC phenotype. The expression of the maturation cell-surface markers CD40, CD80, CD86, and MHC class II was measured by flow cytometry. To confirm maturation, I used isolated immature splenic DCs as a negative control. Maturation was therefore confirmed in these cells (supplementary Figure S.3).

Of interest, while there were no apparent differences in the expression of the co-stimulatory molecules CD40, CD80, and CD86 between WT and Sema-3E-deficient mature splenic DCs, I did observe an upregulation in MHC class II expression in Sema-3E-deficient cells relative to WT controls (**Figure 4.12**). Next, I wanted to determine if Sema-3E would modulate cytokine production in these cells. Conditioned medium of WT and Sema-3E-deficient LPS-stimulated splenic DCs were collected and examined by MSD assay. I observed an increase in IFN-γ expression in Sema-3E-deficient cells compared to WT controls (**Figure 4.13**), with no change in any of the other pro- (IL-12/IL23p40, IL-6) or anti-inflammatory (IL-10) cytokines I assayed. These results suggest that Sema-3E, selectively modulates proinflammatory functions upon LPS stimulation in splenic DCs.


Gating strategy
A
Lymphocytes
90,9
Single Cells
80,2
Live cells
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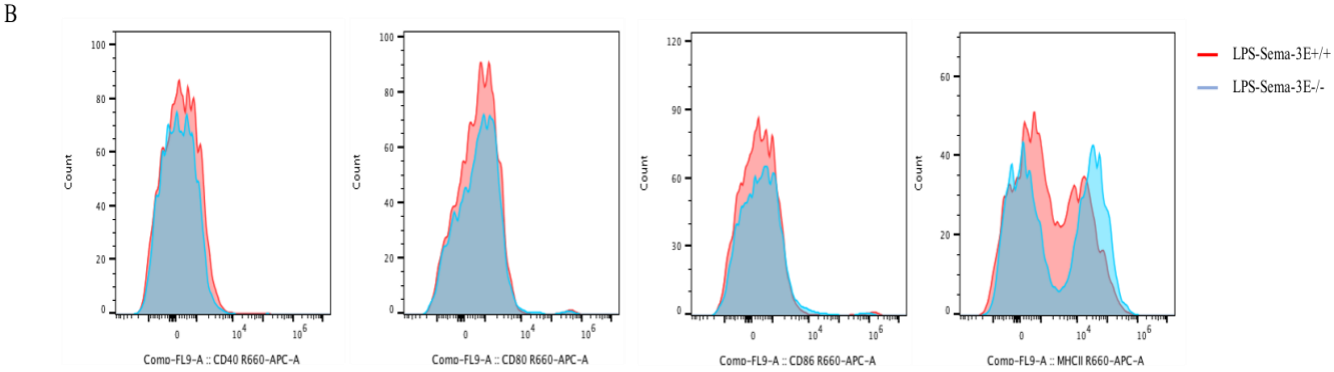
LPS-Sema-3E-/-
CD11c+
88,6
CD11c+
90,1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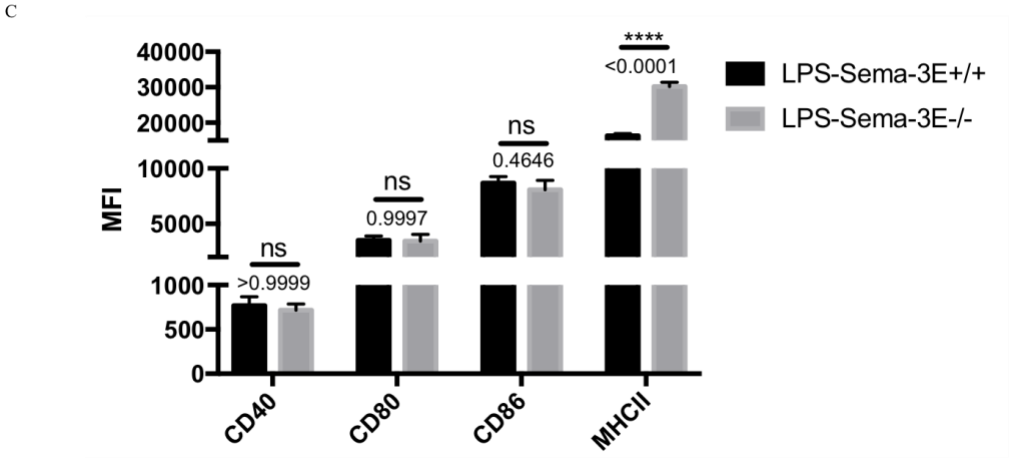
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CD40
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CD86
MHCII
LPS-Sema-3E+/+
LPS-Sema-3E-/-

Figure 4.12. Sema-3E deficiency altered LPS mature splenic DCs ability to upregulate MHC class II phenotype. Spleens were collected from Sema-3E-deficient (Sema-3E-/-) and WT (Sema-3E+/+) BALB/c mice. CD11c+ cells were isolated and cultured in GM-CSF medium overnight. LPS (1μg/μl) was introduced to the culture medium for 24-hours to acquire matured DC phenotype. Expression of CD40, CD80, CD86, and MHC class II (MHCII) was detected by surface staining. (A, B) Representative flow cytometry; (C) Mean Fluorescent Intensity MFI for collective data (*N* = 5). *P* values are shown for two-way ANOVA to compare data from two groups as indicated. ns, not significant; ****$P < 0.0001$.

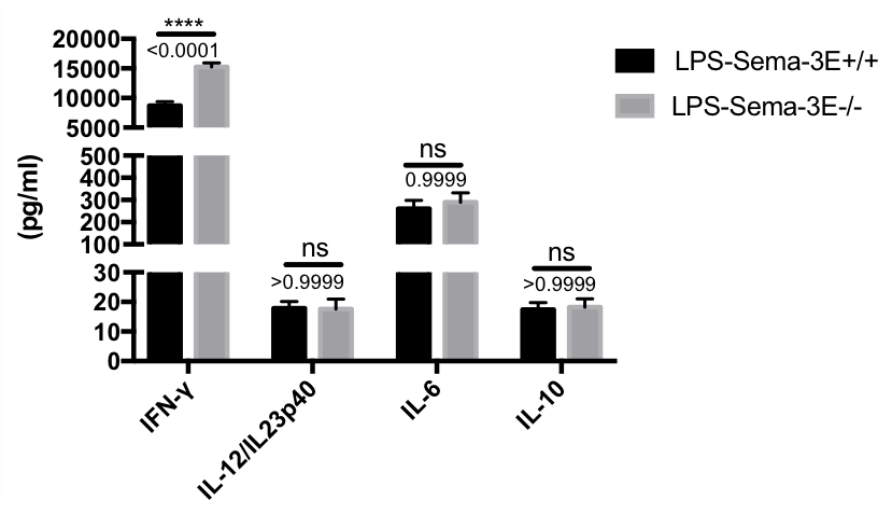


Figure 4.13. Sema-3E deficiency upregulated IFN- γ production in LPS mature splenic DCs. Splenic DCs were isolated from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) BALB/c mice. CD11c+ cells were isolated and stimulated with LPS (1μg/μl) for 24-hours to acquire matured DC phenotype. Conditioned medium was collected and used to measure the proinflammatory markers IFN-γ, IL12/IL23p40, and IL-6 and the anti-inflammatory marker IL-10 by MSD assay. Data are shown as mean ± SEM of the calculated concentrations in pg/mL, adjusted for the dilution factor, for 3 independent experiments. *P* values from two-tailed *t*-tests used to compare two groups as indicated are shown. ns, not significant; ****$P < 0.0001$.

4.4 Discussion

DCs are specialized APCs described as the master regulators of the immune system, mediating innate immunity and shaping adaptive immunity. In this study, I examined the effect of Sema-3E on DC phenotype and function. I reported that Sema-3E-deficient mice altered the CD11c (**Figure 4.1)** and MHC class II (**Figure 4.6)** phenotype of these cells. However, other co-stimulatory molecules, including CD40, CD80, and CD86, remained unaffected in BM (**Figure 4.2)** and spleen (**Figure 4.6).** The role of Sema-3E in modulating DC phenotype establishes the relevance of studying Sema-3E in DC function. I observed that Sema-3E-deficient immature BMDCs displayed higher levels of IL-12/IL23p40 compared to control cells (**Figure 4.3)**. However, Sema-3E-deficient immature splenic DCs showed downregulated IFN-γ, IL-12/IL-23p40, and IL-6 but upregulated IL-10 production (**Figure 4.8).** Upon LPS stimulation, Sema-3E-deficient BMDCs displayed the upregulation of both IL-12/IL-23/p40 and IL-10 **(Figure 4.10),** whereas Sema-3E-deficient splenic DCs upregulated IFN-γ production (**Figure 4.13).** My findings collectively support the role of Sema-3E in modulating DC phenotype and function (measured by cytokine production) via autocrine (BMDCs) or paracrine (splenic DCs) mechanisms.

DCs are defined by different markers, including CD11c and MHC class II. CD11c is the most widely used to define DCs (*459*). CD11c couples with the integrin beta chain-2 (*ITGB2)* to form an integrin heterodimer that in turn pairs with the complement fragment iC3b and mediates in vitro phagocytosis. It is also referred to as complement receptor 4 (*460, 461*). Activator protein 1 (AP-1) bound to JunD and Fos-related antigen 2 (Fra2) plays a critical role in enhancing the CD11c transcription level gene in DCs and thus in their differentiation and proliferation (*462*). In intestinal inflammation, lack of Sema-3E increased the expression of CD11c marker and resulted in a greater inflammation response (*463*). However, the role of Sema-3E in modulating CD11c in the steady-state remains to be investigated. Interestingly, I have found that Sema-3E deficiency resulted in a higher $CD11c^+$ percentage and absolute number in isolated immature BMDCs relative to WT controls (**Figure 4.1)**. However, Sema-3E deficiency in these cells did not affect other co-stimulatory molecules, including CD40, CD80, CD86, and maturation phenotype MHC class II (**Figure 4.2**). This suggests that the autocrine effect of Sema-3E in DCs might limit DC proliferation via modulating AP-1, reducing the inflammatory response. In chlamydial infection, DCs from Sema-3E-deficient mice failed to induce Th1 and Th17 cell responses and displayed a higher expression of PD-L1, PD-L2, and IL-10 compared to DCs from WT mice (*453*). We can

speculate that if Sema-3E failed to mediate proinflammatory function, $CD11c^+$ DCs might undergo the exhaustion or apoptotic process mediated by cytokines such as IL-1 or IL-10. The observed increase in $CD11c^+$ numbers in Sema-3E-deficient isolated BMDCs may not support DC development in vivo, but it indicates that Sema-3E autocrine function may affect BMDC generation in vitro.

MHC class II is an antigen-presenting molecule highly expressed in epithelial cells and DCs (*464*). On encounter with microbial antigens, DCs process the antigens and load the peptides onto MHC class II molecules. Concurrently, MHC class II loaded with antigens will be presented to antigen-specific naive $CD4^+$ T cells that differentiate to effector T cells capable of producing inflammatory cytokines and combating infection (*465*). It was reported that the post-translational ubiquitination of MHC class II plays a critical role in controlling its intracellular transport (*466*). Functionally, the ubiquitination of MHC class II mediates the suppression of MHC class II expression by DCs and reduces antigen presentation in response to IL-10 and regulatory T cells (Treg) (*467*). For example, DCs from Sema-3E-deficient mice infected with chlamydia showed impairment of the surface expression of co-stimulatory molecules. However, DCs displayed a higher expression of PD-L1, PD-L2, and IL-10. (*453*) Interestingly, I observed that Sema-3E-deficient immature (**Figure 4.6**) and mature (**Figure 4.12**) splenic DCs had altered (increased) MHC class II expression compared to WT controls, suggesting that a paracrine effect of Sema-3E on DCs might downregulate ubiquitination, resulting in more MHC class II expression and thus displaying proinflammatory features. It is known that different cDCs subsets such as $CD4^+CD8^-$, $CD4^-CD8^-$, $CD8^-$, and $CD8^+$ differ in their profile of chemokines, cytokines, antigen processing, and Toll-like receptor expression (*457, 458*). Along with the altered MHC class II that I observed in steady-state splenic DCs, I observed that Sema-3E-deficient splenic DCs displayed a higher $CD4^+CD8^-$subpopulation compared to WT controls (**Figure 4.7**). It was previously reported that cDC2 expressed MHC class II and CD4 and associated with innate lymphoid cells 2 (ILC2) and Th2 activation and ILC3 and Th17 immune responses induction (*468*). This, therefore, establishes the relevance of studying the role of Sema-3E in DC function. Future studies should include more DC-specific markers such as Ly-6C, CLEC9A, or SIRPα to exclude any cells other than DCs that might express CD11c and/or MHC class II. Collectively, our observations suggest that Sema-3E deficiency in DCs selectively upregulates CD11c and MHC class II independent of other co-stimulatory molecules including CD40, CD80, and CD86. This necessitates the reconsideration of

current thinking which defines maturation based on upregulation of CD40, C80, CD86, and MHC class II.

DCs are considered a primary source of IL-12 and IL-23, and these are critical factors for immune defenses against microorganisms. Both cytokines share a common p40 subunit that functions in conjunction with either p35 (to form IL-12) or p19 (to form IL-23) to support T and NK cell proliferation and cytolytic activity (*469*). The IL-12/IL-23 common pathway (STAT4) has been found to play a role in the induction of inflammation in adaptive immune responses (*470*). Notably, IL-23 promotes naive T helper cell differentiation into Th17, whereas IL-12 induces Th1 polarization (*470*). Sema-3E-deficient mice were recently shown to have dramatically enhanced airway remodeling and Th2/Th17 inflammation (*418*). Upon HDM challenge, GATA3 and RORγt, critical transcriptional factor in T cell differentiation, were found to be higher in mesenteric lymph node (MLN) $CD4^+$ T cells of Sema-3E-deficient mice compared to WT controls (*418*). Sema-3E-deficient splenocytes and splenic CD11c+ cells in a colitis model produced more IL-12/23 compared to WT controls, and recombinant Sema-3E reduced their release (*463*). It was recently reported that following *Leishmania major* infection, CD11c+ cells from Sema-3E-deficient mice has increased expression of IL-12p40 (*452*). Collectively, those observations suggest a critical role for Sema-3E in regulating IL-12/IL23p40 production in DCs. However, the role of Sema-3E in modulating IL-12/IL23p40 production in either steady-state or mature DCs has not been investigated. Interestingly, I observed that immature (**Figure 4.3**) and LPS-matured BMDCs (**Figure 4.10**) from Sema-3E-deficient mice produced more IL-12/IL-23p40 than WT controls. These results might suggest that the Sema-3E autocrine signal associated negatively with IL-12/IL-23p40 production in steady-state and mature DCs to preserve DCs homeostasis. In chlamydial infection, Sema-3E was induced in the lung and impacted DC function (*453*). Sema-3E-deficient DCs showed an impairment in IL-12 production. Interestingly, I observed immature Sema-3E-deficient splenic DCs showed the downregulation of IL-12/IL-23p40 production (**Figure 4.8**) whereas LPS mature splenic DCs showed no change in IL-12/IL-23p40 production (**Figure 4.13**) compared to WT controls. My observations suggested Sema-3E paracrine signal regulates IL-12/IL-23p40 production at steady-state and mature DCs. Collectively, my data suggest that DC-derived IL-12/IL23p40 production is modulated differently via Sema-3E autocrine signal (downregulation) or paracrine signal (upregulation). Therefore, my study reveals

the importance of Sema-3E autocrine and/or paracrine modes in differential modulation of inflammatory responses in steady-state and LPS-stimulated DCs.

IFN-γ is known to modulate the Th1/Th2 balance, enhance macrophage activation, promote antigen presentation, and control cellular proliferation and apoptosis (*471*). It was reported that IL-12 induced IFN-γ production in macrophages and DCs, governed by STAT4 (*472*). Therefore, I investigated whether Sema-3E would affect IFN-γ production in DCs. I observed that Sema-3E-deficient immature splenic DCs produced less IFN-γ compared to WT controls (**Figure 4.8**), thus establishing the correlation between IL-12/IL-23p40 and IFN-γ production under the influence of Sema-3E. However, this correlation seems to be disturbed in mature DCs (i.e., upon LPS stimulation): LPS-matured BMDCs displayed similar IFN-γ production in the presence or absence of Sema-3E (**Figure 4.10**), whereas LPS-matured splenic DCs retained a higher level of IFN-γ production in the absence of Sema-3E compared to WT controls (**Figure 4.13**). It was previously reported that in intestinal inflammation, splenocytes and splenic $CD11c^+$ cells of Sema-3E-deficient mice produced more IFN-γ than WT control and that this might be associated with upregulated IL-12/23 production (*463*). Similarly, following *Leishmania major* infection, Sema-3E-deficient mice enhanced resistance was linked with significantly increased IFN-γ production by $CD4^+$ T cells (*452*). Collectively, my results suggest a critical role of Sema-3E in regulating IFN-γ production in steady-state DCs that is possibly associated with IL-12/IL-23p40 production to promote proinflammatory responses.

LPS mediates DC maturation via TLR4 that activates signaling proteins such as MAPK, STATs, AKT, and NF-kappa-B (NF-κB) (*473*), and results in different DCs effector functions, including migration and antigen uptake (*474*). It was found that deficiency of Sema-3E in BM-derived macrophages (BMDM) impaired the phosphorylation of LPS-related signaling protein ERK1/2, AKT, STAT3, and NF-kB (p65) (*421*). We can speculate that the paracrine effect of Sema-3E on DC-derived IFN-γ production is disturbed upon LPS stimulation via ERK1/2, AKT, STAT3, and NF-kB regulation. It is also possible that the observed positive correlation between IFN-γ and IL-12/IL23p40 in Sema-3E-deficient steady-state DCs in different models (such as colitis or *Leishmania major* infection) may not carry over to mature DCs; therefore, the effect of maturation in the absence of Sema-3E remains to be explored in these disease models.

IL-6 is produced by different cells (such as macrophages, dendritic cells, and T and B cells) in response to tissue damage and infections (*475*). It has been reported that DC-derived IL-

6 acted back in an autocrine manner to promote their ability to prime pathogenic Th17 cells (*476*). It was shown that IFN-γ regulates the secretion of IL-6, where a defect in IL-6 expression accompanies a deficiency in IFN-γ. The reconstitution of IFN-γ restored IL-6 production, thus identifying a direct role for IFN-γ in controlling local IL-6 generation (*477*). Here, I wanted to investigate if Sema-3E would modulate IL-6 production in DCs. I observed that Sema-3E-deficient immature splenic DCs produced less IL-6 compared to WT controls (**Figure 4.8**). Of interest, Sema-3E upregulated IL-6 production in adipose tissue macrophages, resulting in insulin resistance by blocking AKT phosphorylation and signaling (*478*). Our results suggested Sema-3E paracrine effect upregulated DCs proinflammatory response. Therefore, the deficiency of Sema-3E in the steady-state maintains DC homeostasis. However, LPS stimulation of both BMDCs (**Figure 4.10**) and splenic DCs (**Figure 4.13**) demonstrated similar IL-6 production levels in the presence or absence of Sema-3E. In LPS-induced systemic inflammation, the level of IL-6 in the serum was significantly lower in the Sema-3E-deficient mice compared to WT controls, suggesting the deficiency of Sema3E protective role in the early systemic inflammatory response (*421*). We can speculate that LPS stimulation disturbs the autocrine or paracrine effects of Sema-3E on WT DCs, resulting in similar IL-6 production in Sema-3E-deficient DCs. To further confirm this, future studies should investigate IL-6 signaling pathways such as Jak-STAT, or MAPK.

Interleukin 10 (IL-10) is a critical regulatory cytokine that limits excessive T-cell responses to microbial pathogens, preventing adverse effects such as chronic inflammation, tissue damage, autoimmune diseases, transplant rejection, or allergic reactions (*479*). It was recently reported that in chlamydial infection, Sema-3E-deficient DCs showed a higher expression of IL-10 production, resulting in failed induction of Th1 and Th17 cell responses (*453*). However, the role of Sema-3E in modulating IL-10 cytokine production at steady-state DCs remains to be investigated. I found that Sema-3E-deficient immature splenic DCs produced a higher amount of IL-10 than WT control cells (**Figure 4.8**). My observation may suggest the absence of Sema-3E in steady-state DCs supports tolerogenic DC functions; however, this may translate either to a protective role in inflammatory response or to increased susceptibility to disease. Interestingly, I also observed that LPS stimulation modulated IL-10 production in BMDCs: LPS-stimulated Sema3E-deficient BMDCs showed a higher IL-10 production level than WT counterparts (**Figure 4.10**). However, LPS stimulation did not affect IL-10 production levels in either WT or Sema-3E-deficient splenic DCs (**Figure 4.13**). Of interest, I observed that LPS-stimulated Sema3E-deficient BMDCs

upregulated both IL-12/IL-23p40 and IL-10 cytokine production (**Figure 4.10**). Depending on the DC phenotype and type of secreted cytokines, DCs may exhibit either proinflammatory or tolerogenic function (*480*). The β-catenin, a major component in the Wingless and Int-1 (Wnt) signaling pathway, is a critical factor in thus dual function (*481*). β-catenin was detectable in immature monocyte-derived DCs (moDCs) and further increased in LPS-stimulated (mature) moDCs (*480*). Furthermore, it was reported that the activation of β-catenin by 6-bromoindirubin-3′-oxime (6-BIO) in LPS-stimulated moDCs decreased IL-12p70 and increased IL-10 production (*5)*. On the other hand, the inhibition of β-catenin by ICG-001 enhanced the proinflammatory signature cytokine IL-12p70 and downregulated the anti-inflammatory signature molecule IL-10 (*480*). We can speculate that LPS stimulation and the Sema-3E autocrine effect regulate, respectively, the dual functions of LPS-stimulated BMDCs: Sema-3E deficiency in immature BMDCs produced higher IL-12/IL-23p40 level than WT controls (**Figure 4.3**), and LPS stimulation presumably increased the expression of β-catenin that resulted in the observed increase in IL-10 production (**Figure 4.10**). It is also possible that the (WT) Sema-3E autocrine signal inhibits β-catenin after LPS stimulation, resulting in increased IL-12/IL-23p40 production. The expression of the BMDC inhibitory markers PD-L1 and PDL-2 before and after LPS stimulation remains to be investigated in BMDCs.

In conclusion, our results provide evidence, for the first time, of a novel role of Sema-3E in the regulation of DC phenotype and function. I demonstrated that Sema-3E-deficient mice show altered MHC class II phenotype and DC subsets. This suggests a regulatory effect of Sema-3E on DC function in the form of cytokine production (**Figure 4.14**). Future work will characterize how Sema-3E modulates downstream signaling pathways such as STAT3 and STAT4 in the steady state and following LPS stimulation. Understanding the role of Sema-3E in modulating DC phenotype and effector function may provide unique therapeutic targets to manipulate DCs functions in clinical settings.

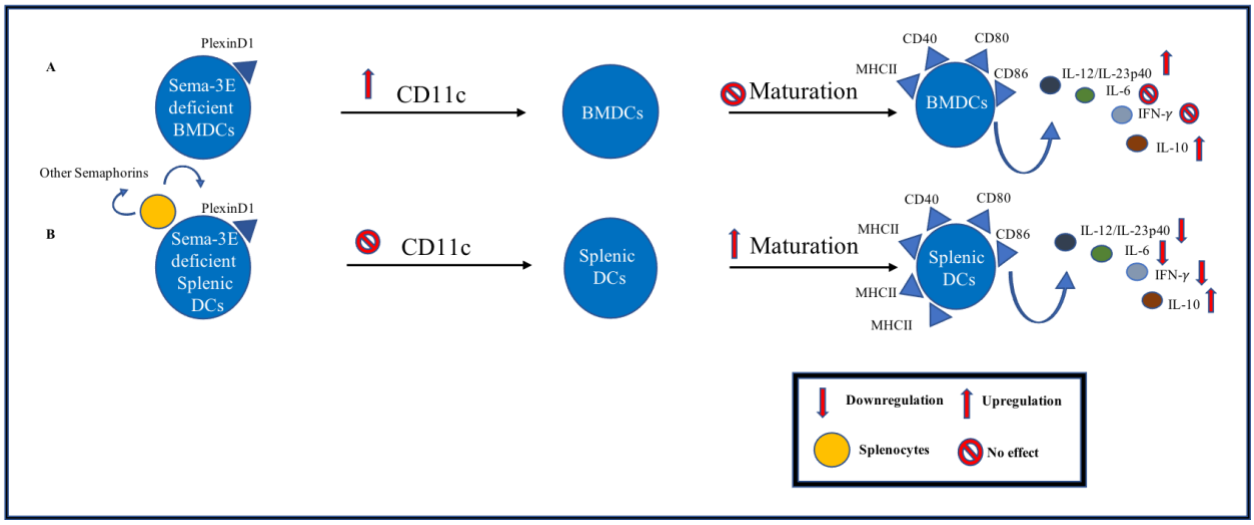


Figure 4.14. The Sema-3E deficiency modulated DC phenotype and function. The Sema-3E was examined in **(A)** BMDCs, and **(B)** splenic DCs. Through modulation of the phenotype and function of DCs, the Sema-3E modulates DC absolute cell number, maturation and cytokine production.

Chapter 5: PlexinD1 Deficiency Upregulated DC-derived Proinflammatory Cytokines

5.1 Abstract

PlexinD1 (Semaphorin-3E receptor) is expressed in several organ systems, including the immune system. PlexinD1 is expressed in various immune cells (such as DCs) and has been involved in cell migration and cell-cell interaction during the immune response. In this study, the role of PlexinD1 expressed by DCs in regulating DCs phenotypes and functions was investigated. Unlike the deficiency of Sema-3E, the deficiency of PlexinD1on DCs did not affect BMDCs and splenic DCs phenotype. However, steady-state PlexinD1 deficient DCs displayed higher production levels of proinflammatory cytokines such as IFN-γ, IL-12/IL-23p40, IL-6. Upon LPS stimulation, PlexinD1 deficient splenic DCs selectively upregulated IFN-γ and IL-12/IL-23p40 while BMDCs remain unaffected. Also, PlexinD1 deficiency in LPS-stimulated DCs has been correlated positively with Sema-3E expression. My results revealed the critical role of PlexinD1expressed by DCs in modulating DCs phenotype and proinflammatory cytokine productions, thus possibly impact immune responses.

5.2 Introduction and rationale

Sema-3E binds directly to PlexinD1 and the expression of the *Plxnd1* gene has been found in different tissues and/or cells such as podocytes, adrenal and mammary glands, lung mesenchyme, the smooth muscle of the small intestine, osteoblastic cells and/or bone, and immune cells (*399*). The extracellular region of PlexinD1 contains RasGAP domains bind the R-Ras family of small GTPases and PlexinD1-GAP activity limits integrin-mediated cell adhesion to the extracellular matrix (ECM) and downregulates PI3K and MAPK signaling pathways involved in proliferation, migration, and cell survival (*399*). Therefore, the PlexinD1 ectodomain interacts with Sema-3E, possibly priming the receptor through intracellular conformational changes. In mice, conventional genetic ablation of PlexinD1 expression resulted in cardiovascular defects (*400*). In contrast, Sema-3E deficiency shows no developmental impact (*401*); therefore, the observed cardiovascular development defects suggest there are additional PlexinD1 ligand(s).

In 2012, Holl et al. (*482*) reported that PlexinB2 and PlexinD1 are expressed in myeloid and plasmacytoid DC populations and are modulated upon TLR ligand activation, TNFα, and anti-CD40. Interestingly, they also found that PlexinB2 and PlexinD1-deficient DCs produced

selectively higher levels of IL-12/IL-23p40, a critical cytokine involved in cytotoxic activity and IFN-γ production in NK cells.

In my previous report (*420*) and Chapter 4 of this thesis, *I found that both BM and splenic DCs expressed PlexinD1 and that this expression is tightly regulated upon LPS stimulation. Of interest,* I have observed that PlexinD1 receptor expression levels were downregulated significantly in the immature BMDCs of Sema-3E−/− mice when compared to immature BMDCs of WT controls (*420*). In addition, Sema-3E/PlexinD1 axis modulated DCs phenotype and functions. For example, I reported the deficiency of Sema-3E upregulated CD11c (BMDCs) and MHC class II (splenic DCs) phenotype but not CD40, CD80, or CD86. Moreover, Sema-3E upregulated proinflammatory cytokine such as IL-12/IL23p40 in BMDCs but not in splenic DCs (chapter 4). The expression of PlexinD1 on DCs suggests a functional relevance. However, the role of DC-derived PlexinD1 in regulating DCs phenotype and cytokine productions at steady-state and LPS stimulation remain unexplored.

To address this question, CD11c-specific PlexinD1 conditional knockout (cKO) mice were generated by crossing *Plxnd1*$^{flox/flox}$ mice to inducible CD11c-Cre mice (B6. Cg-Tg$^{(Itgax\text{-}cre)1\text{-}1Reiz/J}$).

5.3 Results

5.3.1 PlexinD1 deficiency in immature BMDCs altered proinflammatory cytokine production

It was reported that the deficiency in PlexinB2 and PlexinD1 did not affect BMDCs maturation markers including CD40, CD80, CD86, and MHC class II (*482*). However, the role of DCs-derived PlexinD1in regulating DCs phenotype at steady-state and LPS stimulation remain to be investigated. Here, I investigate whether PlexinD1 expressed by BMDCs would modulate $CD11c^+$ numbers and expression of co-stimulatory molecules in the presence of Sema-3E but the absence of PlexinD1.

In vitro, I observed steady-state BMDCs generated from CD11c-PlexinD1 cKO mice displayed similar numbers of $CD11c^+$ cells compared to WT controls (**Figure 5.1**). I also observed similar expression levels of the co-stimulatory molecules (CD40, CD80, CD86), and maturation phenotype (MHC class II) in the presence and absence of PlexinD1 (**Figure 5.2**). As shown in Chapter 4, Sema-3E deficiency in immature BMDC upregulated IL-12/IL-23p40 production (Figure 4.3). Therefore, I wished to investigate Sema-3E action on immature BMDCs cytokine expression is dependent on the PlexinD1. Conditioned media from cultured CD11c-PlexinD1 cKO and WT BMDCs were collected and analyzed for cytokine production by MSD assay (**Figure 5.3**).

Interestingly, as shown for Sema 3E deficient BMDCs, IL-12/IL-23p40, IL-6, and IFN-γ were upregulated, while IL-10 expression level was diminished, in CD11c-PlexinD1 cKO cells when compared to WT controls. My data suggest the absence of PlexinD1 alters DCs cytokine response, and thus Sema-3E/PlexinD1 axis maintains DC homeostasis that.

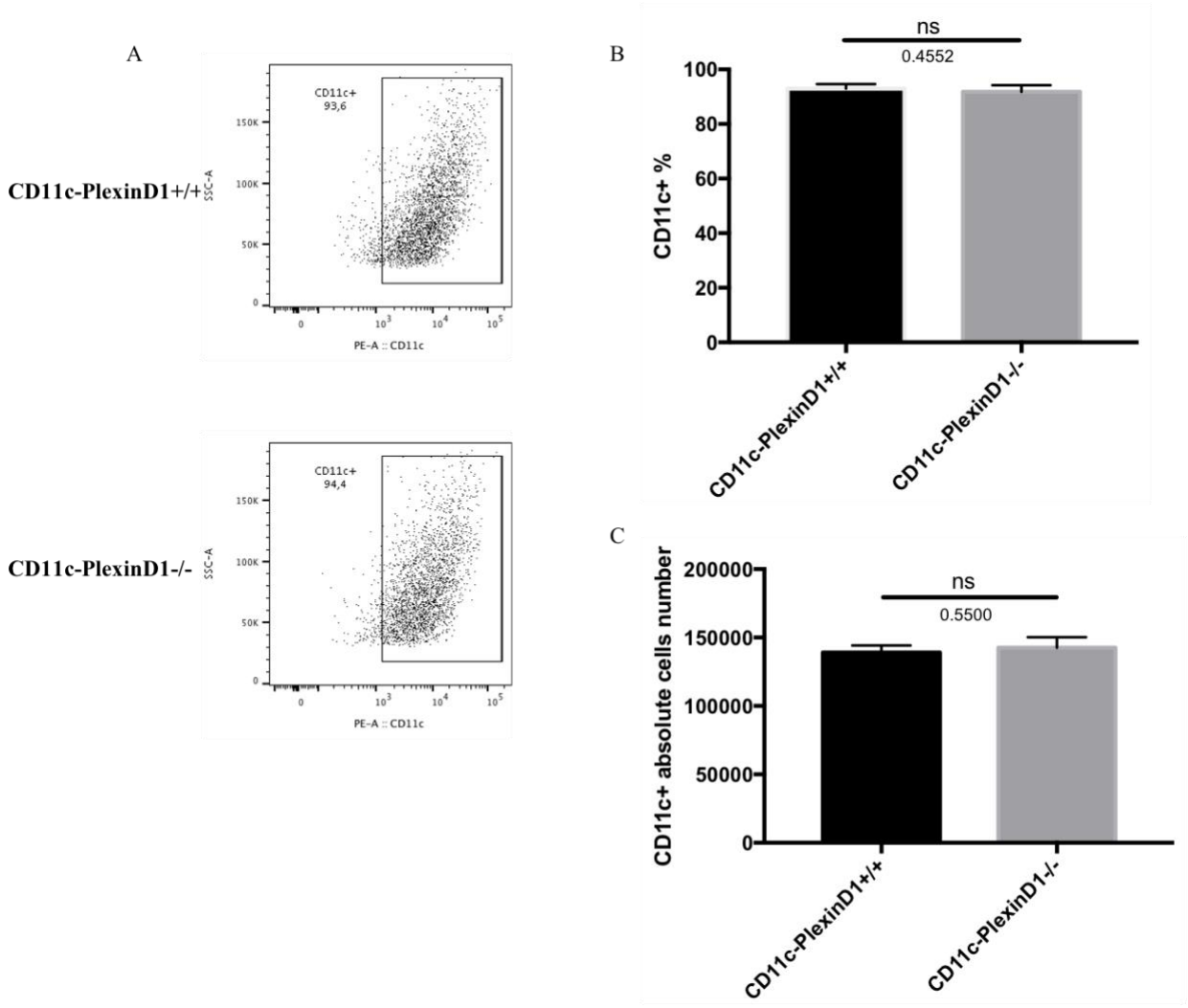
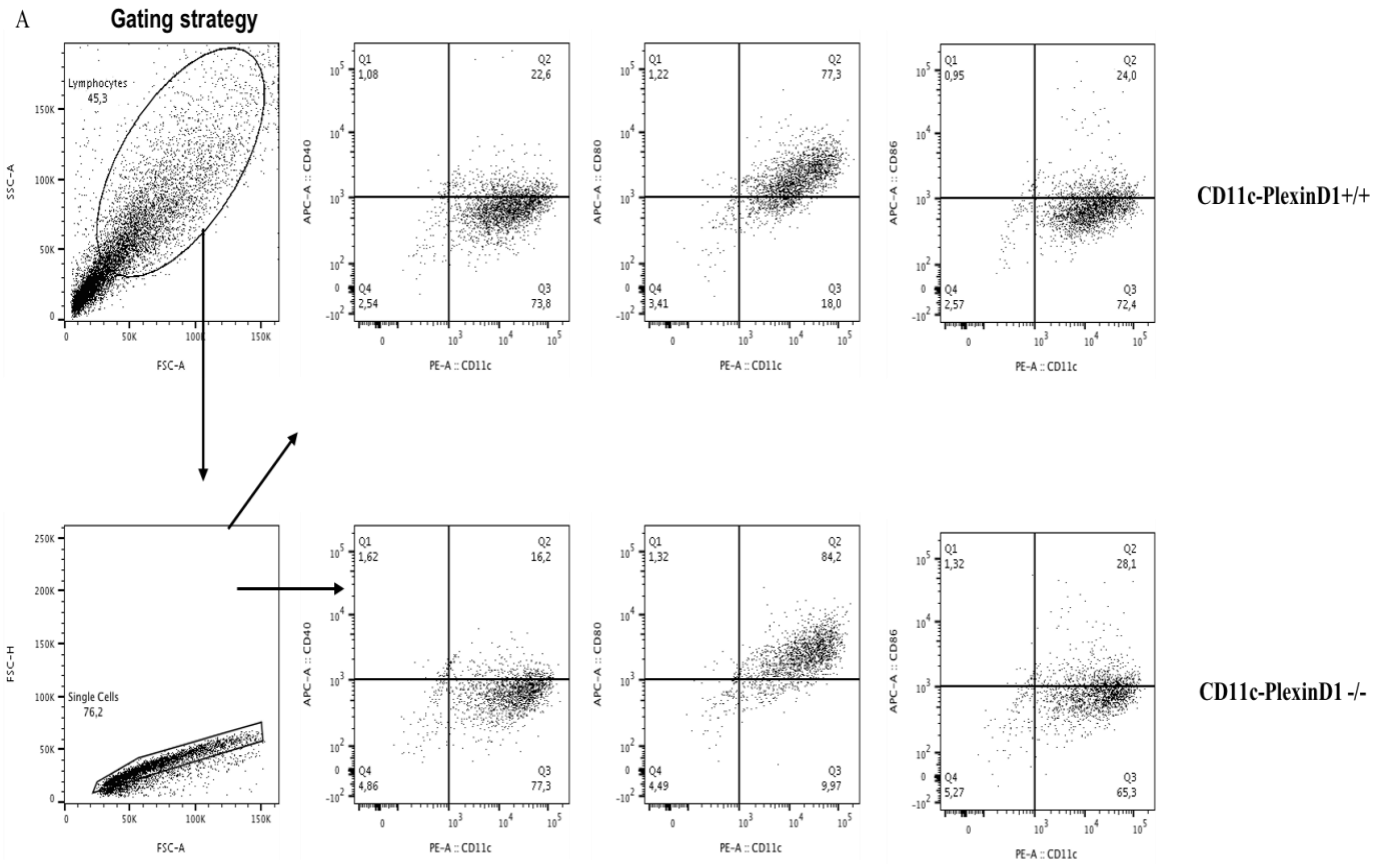
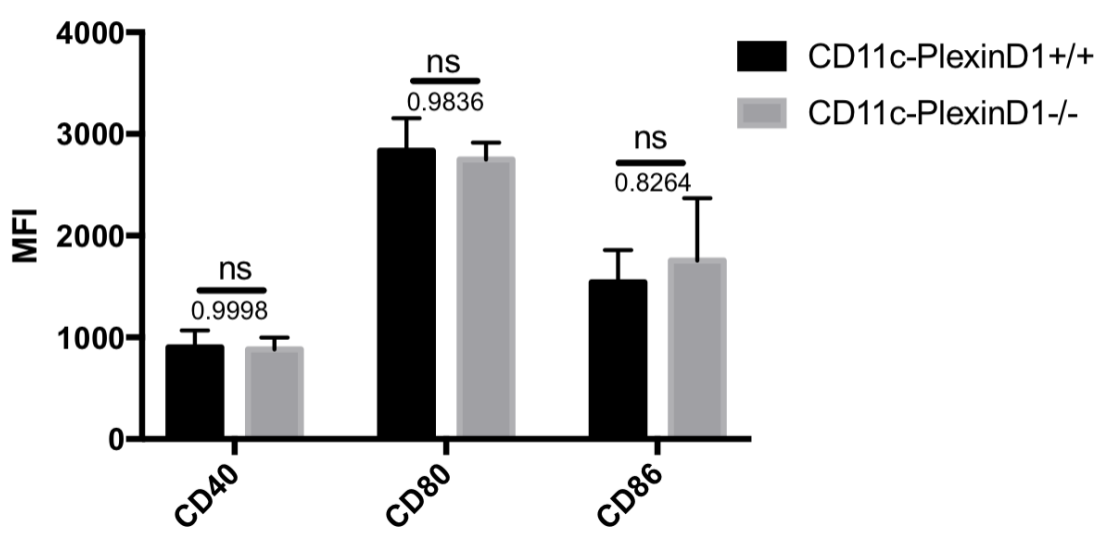


Figure 5.1. PlexinD1 deficiency did not affect CD11c$^+$ BMDCs cell numbers. (A) Flow cytometry assay of BMDCs generated from WT (CD11c-PlexinD1+/+) and CD11c-PlexinD1conditional knockout (CD11c-PlexinD1-/-) mice. Cells were cultured in GM-CSF medium for 7 days, then surface-stained with a monoclonal antibody against CD11c PE. **(B, C)** The percentage (B) and absolute number (C) of CD11c$^+$ BMDCs are plotted from all collected data. Data are shown as mean ± SEM, n = 5. *P* values are from a two-tailed *t* test used for comparing two groups. ns, $P > 0.05$.


A
Gating strategy
Lymphocytes
45,3
SSC-A
FSC-A
APC-A :: CD40
APC-A :: CD80
APC-A :: CD86
PE-A :: CD11c
Q1 1,08
Q2 22,6
Q4 2,54
Q3 73,8
Q1 1,22
Q2 77,3
Q4 3,41
Q3 18,0
Q1 0,95
Q2 24,0
Q4 2,57
Q3 72,4
CD11c-PlexinD1+/+
FSC-H
Single Cells
76,2
Q1 1,62
Q2 16,2
Q4 4,86
Q3 77,3
Q1 1,32
Q2 84,2
Q4 4,49
Q3 9,97
Q1 1,32
Q2 28,1
Q4 5,27
Q3 65,3
CD11c-PlexinD1 -/-

B


MFI
4000
3000
2000
1000
0
ns
0.9998
ns
0.9836
ns
0.8264
CD40
CD80
CD86
CD11c-PlexinD1+/+
CD11c-PlexinD1-/-

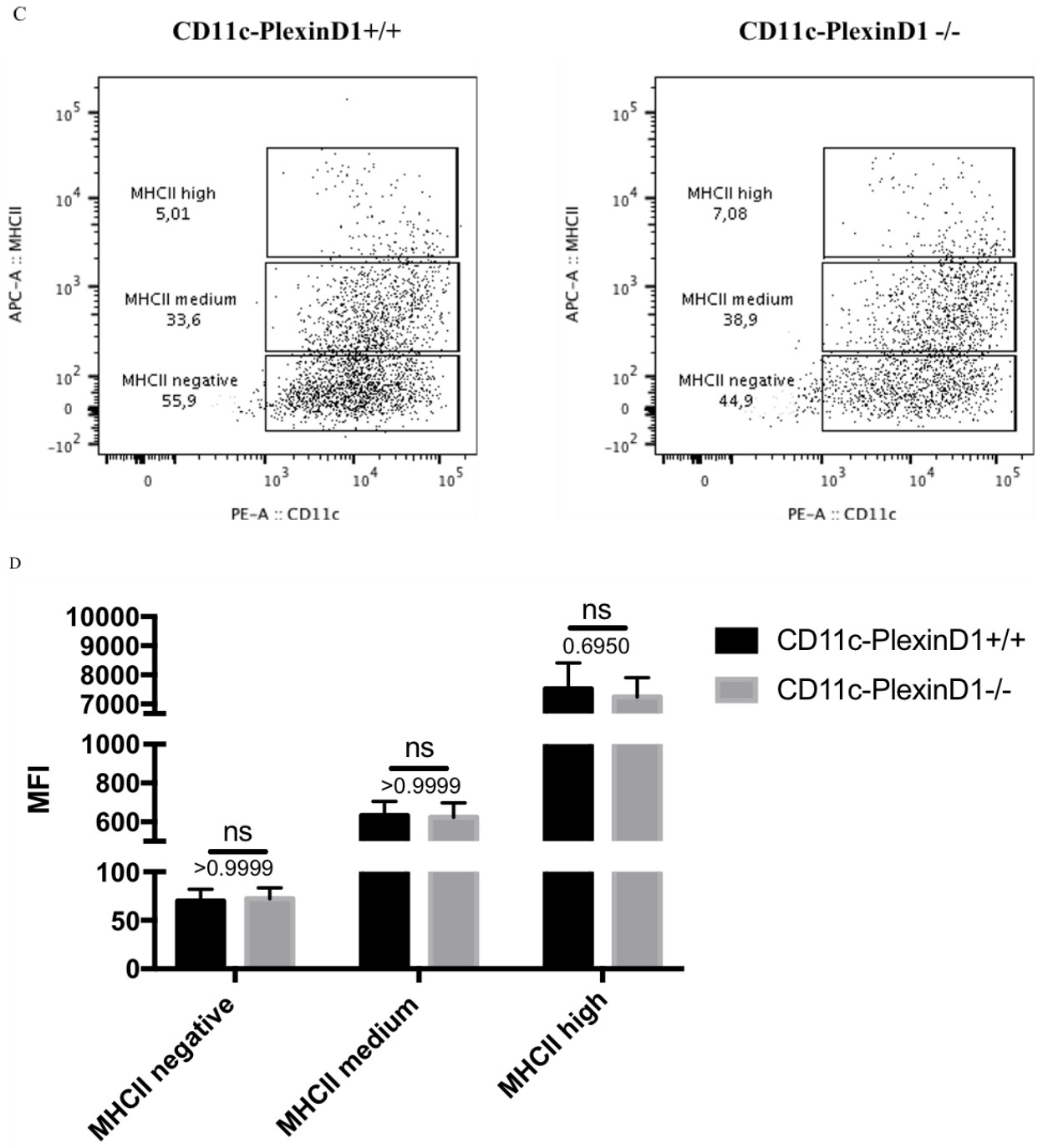


Figure 5.2. PlexinD1 deficiency did not affect maturation phenotype of BMDC. (A, C) Flow cytometry assay of BMDCs generated from WT (CD11c-PlexinD1+/+) and CD11c-plexinD1 conditional knockout (CD11c-PlexinD1-/) mice. BMDCs were cultured in GM-CSF medium for 7 days, then surface-stained with monoclonal antibodies against CD11c PE, CD40 APC, CD80 APC, CD86 APC, and MHC class II APC. **(B, D)** MFI of $CD11c^{+}$ BMDCs plotted from all collected data. Data are shown as mean ± SEM, n = 5. *P* values are from two-way ANOVA used for comparing data from two groups. ns, $P > 0.05$.

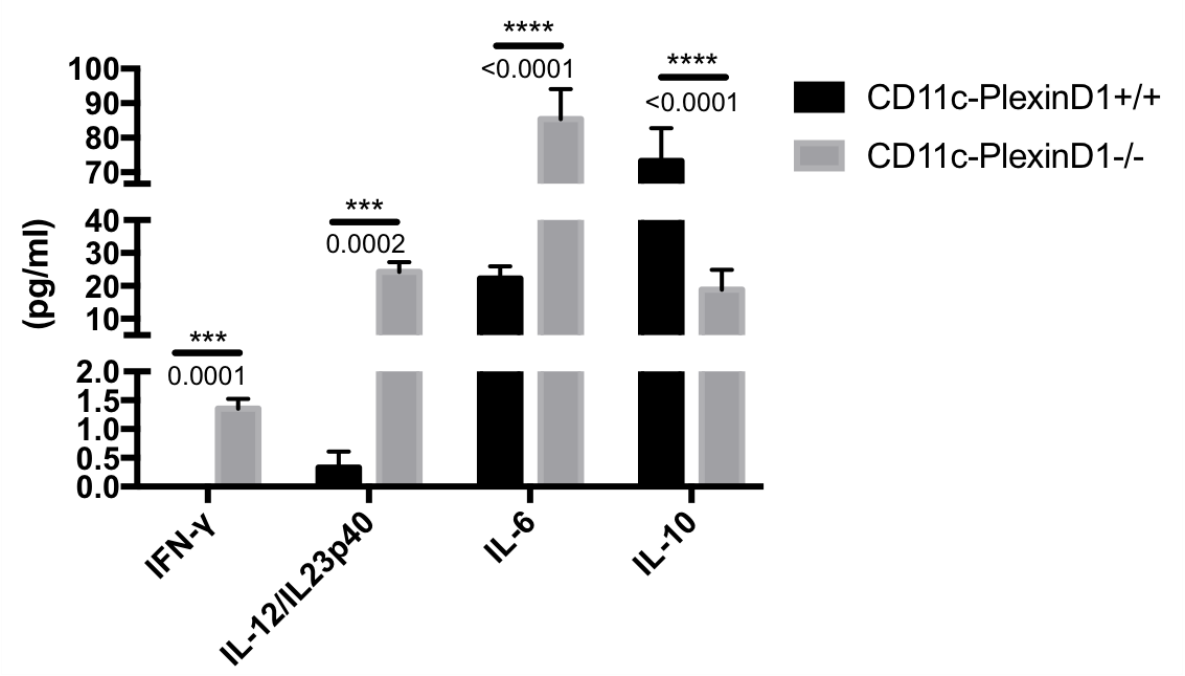


Figure 5.3. PlexinD1 deficiency promoted BMDCs to upregulate proinflammatory cytokine production. Analyses of cytokine profiles of the BMDCs generated from WT (CD11c-PlexinD1+/+) and CD11c-PlexinD1 conditional knockout (CD11c-PlexinD1-/-) mice. BMDCs were cultured in GM-CSF medium. On day 6, conditioned medium of immature DCs was collected to measure IFN-γ, IL-12/IL-23p40, IL-6 and IL-10. Mean calculated concentrations (pg/mL) are adjusted for the dilution factor. Data are shown as mean ± SEM, n = 3. *P* values are from two-way ANOVA used for comparing two groups. ****P* < 0.001, *****P* < 0.0001.

5.3.2 PlexinD1 deficiency in splenic DCs did not affect DC phenotype

In Chapter 4, I observed ex vivo (isolated and uncultured) splenic DCs displayed similar $CD11c^+$ cell numbers in both Sema-3E-deficient and WT mice **(Figure 4.5)** and that Sema-3E-deficient mice expressed higher levels of MHC class II compared to WT control **(Figure 4.6)**.

I next wished to determine whether this modulation of splenic DC phenotype by Sema-3E is dependent on PlexinD1 expressed by splenic DCs themselves (i.e., autocrine action). Ex vivo splenic DCs were collected from both CD11c-PlexinD1 cKO and WT mice and stained with CD11c and MHC class II using flow cytometry. No differences were found in CD11c expression level in PlexinD1 deficient DCs and WT controls (**Figure 5.4**). Moreover, ex vivo splenic DCs exhibited similar MHCII expression levels in splenic DCs of CD11c-PlexinD1 cKO and WT mice

controls **(Figure 5.5)**. My observations suggested that DCs phenotype is independent of PlexinD1-expression.

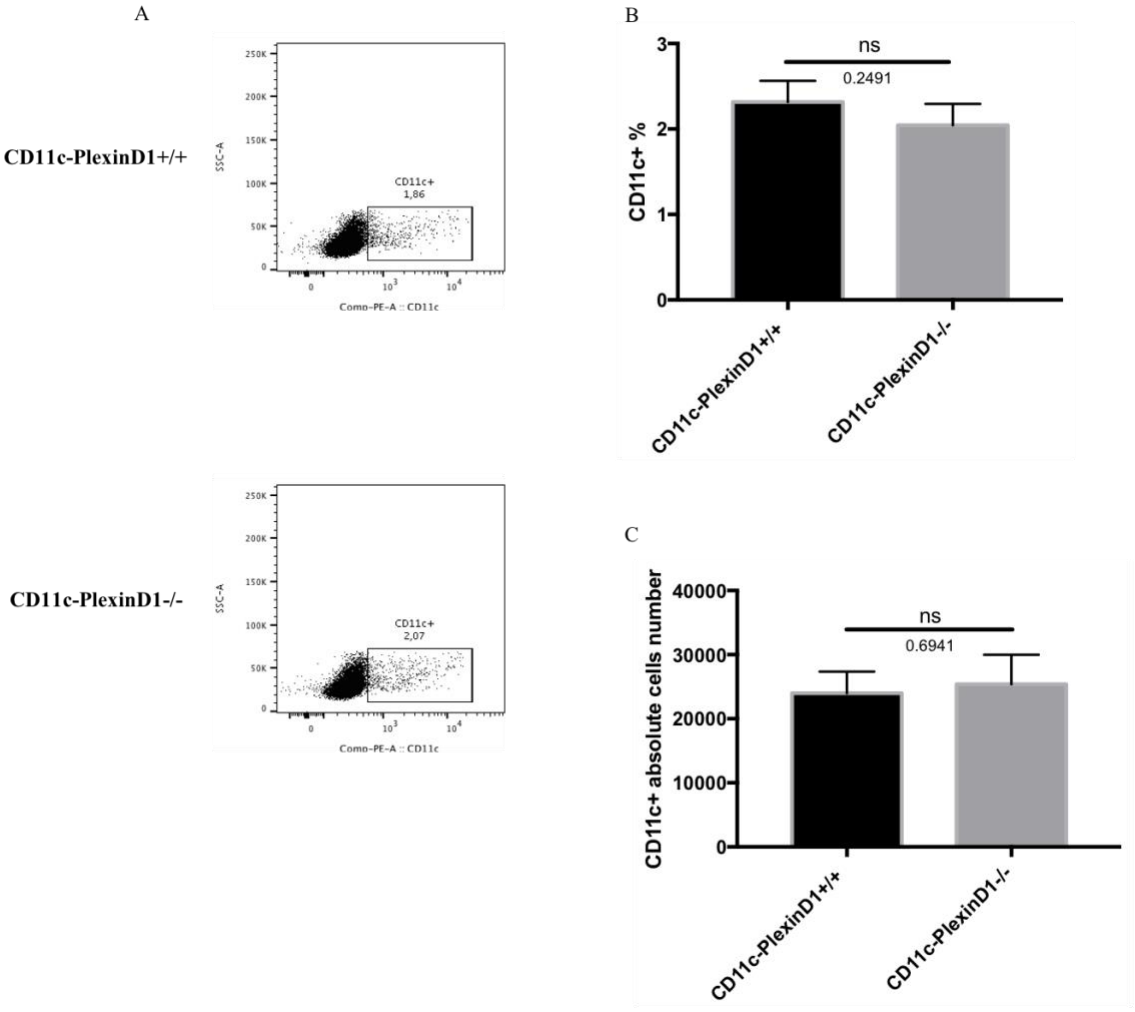


Figure 5.4 PlexinD1 deficiency did not affect CD11c expression in ex vivo splenic DCs. (A) Flow cytometry assay of spleen from WT (CD11c-PlexinD1+/+) and CD11c-PlexinD1 cKO (CD11c-PlexinD1-/-) mice. Cells were surface-stained with a monoclonal antibody against CD11c PE. **(B, C)** Percentage (B) and absolute number (C) of CD11c+ splenic DCs from all collected data. Data are shown as mean ± SEM, n = 5. *P* values are from a two-tailed *t* test used for comparing two groups. ns, $P > 0.05$.

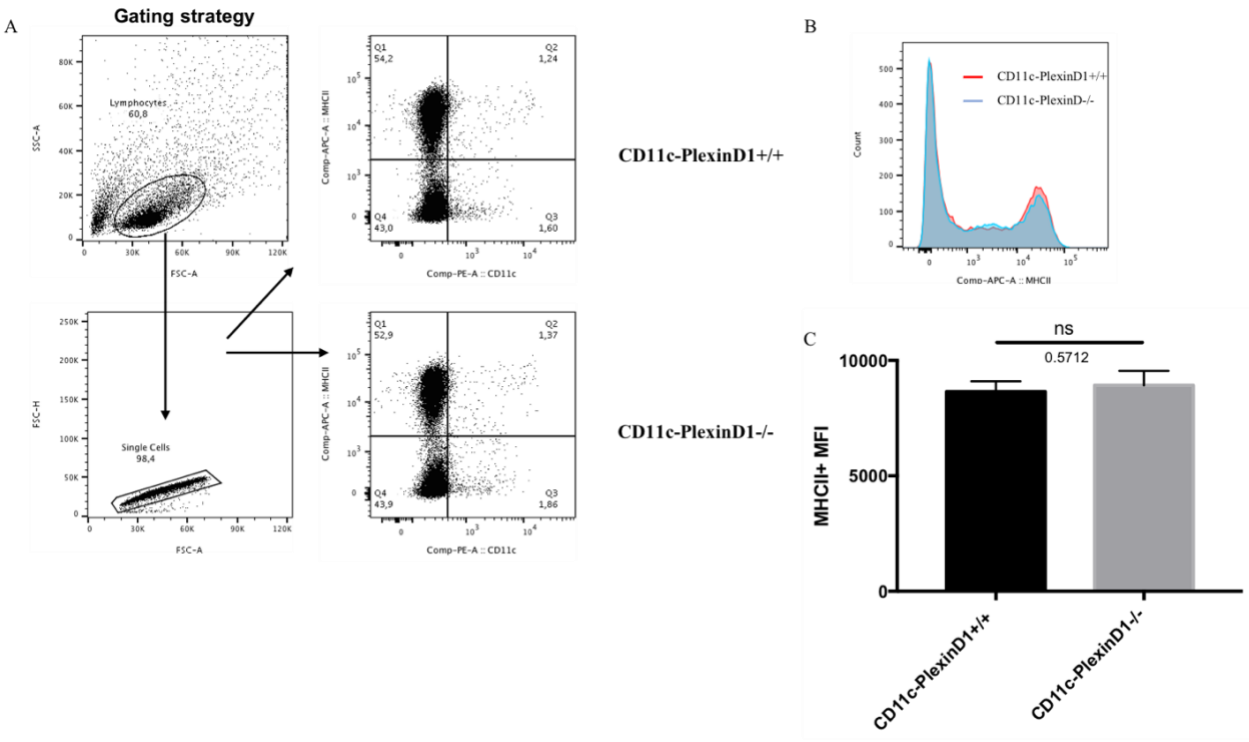


Figure 5.5. PlexinD1 deficiency did not affect MHC class II phenotype in ex vivo splenic DCs. (A) Flow cytometry assay of spleen from WT (CD11c-PlexinD1+/+) and CD11c-PlexinD1 conditional knockout (CD11c-PlexinD1-/-) mice. Cells were surface-stained with a monoclonal antibody against MHC class II APC. **(B, C)** the histogram is plotted from CD11c+ cells (B), and MFI (C) of $MHCII^{+}$ splenic DCs are plotted from all collected data. Data are shown as mean ± SEM, n = 5. *P* values are from a two-tailed *t* test used for comparing two group. ns, $P > 0.05$.

5.3.3 PlexinD1 deficiency in splenic DCs upregulated IL-12/IL-23p40 production

it was reported that PlexinB2- and PlexinD1-deficient BMDCs and isolated splenic DCs produced selectively higher levels of IL-12/IL-23p40 (*482*). In contrast, I showed that Sema-3E deficiency in splenic DCs upregulated IL-10 production while other cytokines (IFN- γ, IL-12/IL-23p40, and IL-6) were downregulated **(Figure 4.8)**. I therefore wished to determine the role of PlexinD1 in modulating DC function in ex vivo steady-state splenic DCs. I isolated splenic DCs from both CD11c-PlexinD1 cKO and WT mice using CD11c PE positive selection kit then checked for cell purity using CD11c PE antibody in flow cytometry (purity >90%) (supplement figure S3). Isolated splenic DCs were cultured in GM-CSF medium for 12 h, then conditioned mediums were collected, and cytokine production was measured by MSD assay. I observed that immature splenic DCs of CD11c-PlexinD1 cKO mice had increased IL-12/IL-23p40 production

compared to WT splenic DCs. However, PlexinD1 deficiency in immature splenic DCs resulted in no change in the secretion levels of IFN-γ, IL-6, and IL-10 compared to WT controls (**Figure 5.6**). My results suggested that Sema-3E/PlexinD1 axis modulated DCs functions independent of PlexinD1expressed by DCs. However, PlexinD1 negatively regulate IL-12/IL-23p40 production in splenic DCs.

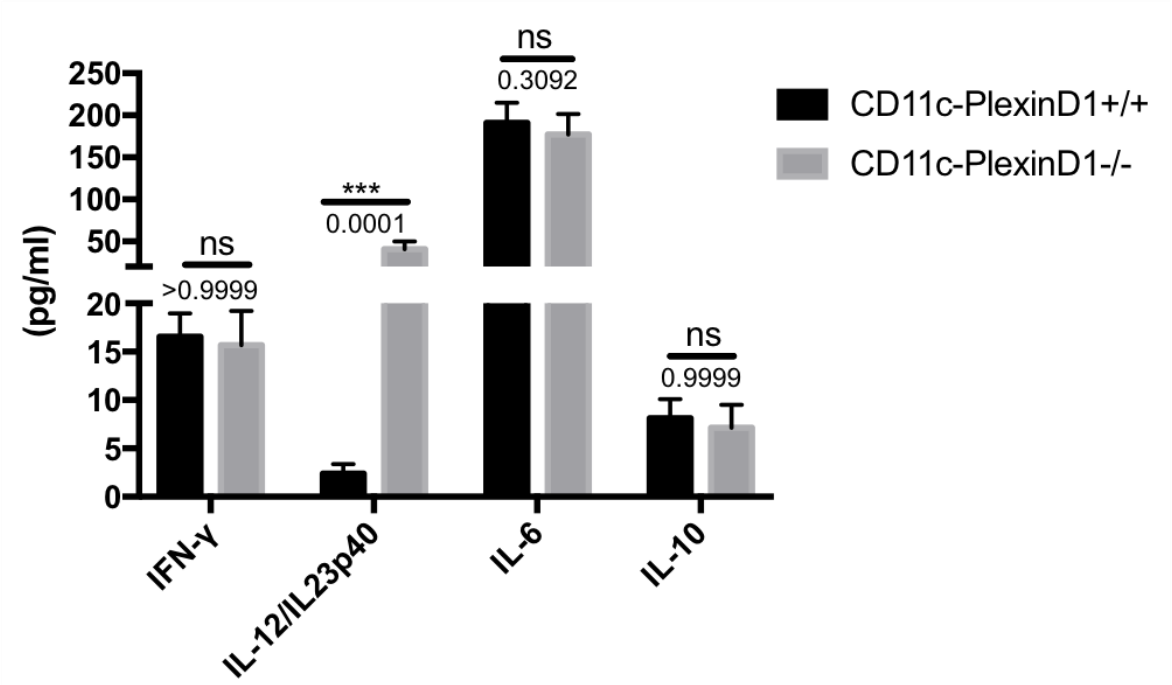


Figure 5.6. PlexinD1 deficiency promoted splenic DCs to upregulate IL-12/IL-23p40 production. (A) MSD assay of splenic DCs isolated from WT (CD11c-PlexinD1+/+) and CD11c-PlexinD1 conditional knockout (CD11c-PlexinD1-/-) mice. Isolated splenic DCs were cultured in GM-CSF medium for 24 h. The next day, conditioned mediums were collected to measure IFN-γ, IL-12/IL-23p40, IL-6 and IL-10. Mean calculated concentrations (pg/mL) are adjusted for the dilution factor. Data are shown as mean ± SEM, n = 3. *P* values are from two-way ANOVA used for comparing two groups. ns, $P > 0.05$; ***$P < 0.001$.

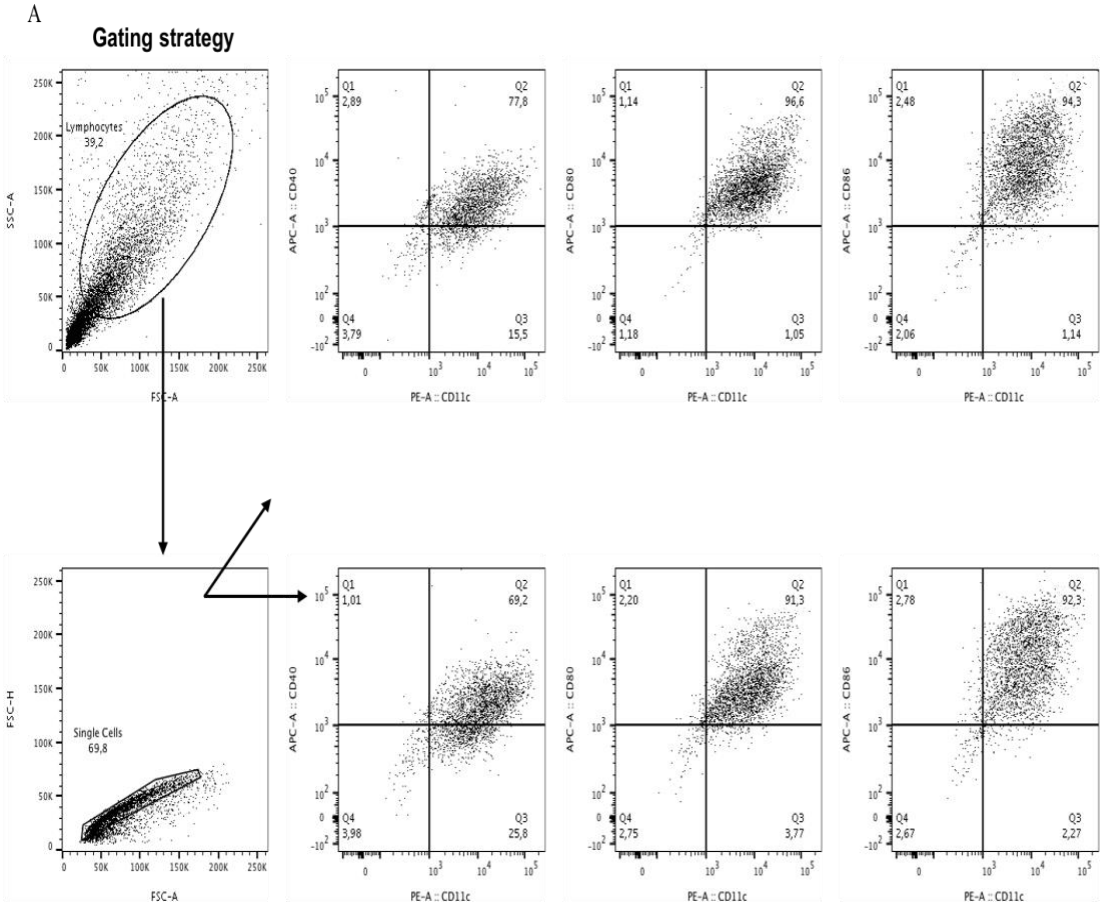
5.3.4 PlexinD1 deficiency did not affect LPS-stimulated BMDC maturation phenotype or function

I have previously reported that immature WT BMDCs express high levels of PlexinD1 mRNA and that stimulation of these cells with LPS reduced this expression (*420*). It was observed that LPS-stimulated BMDCs showed no change in the expression of the co-stimulatory molecules CD40, CD80, CD86, and I-Ab in the absence of either PlexinB2 or PlexinD1 compared to WT controls (*482*).

In Chapter 4, I report that Sema-3E deficiency does not affect the phenotype of LPS-stimulated BMDCs (Figure 4.9). To investigate the role of PlexinD1 expressed by DCs in modulating the phenotype and function of LPS-stimulated (mature) BMDCs, I generated BMDCs from CD11-PlexinD1 cKO mice and WT littermates. BMDCs were stimulated with LPS (1 μg/μL) for 24 h to induce maturation. The expression of CD40, CD80, CD86, and MHC class II surface markers were taken to validate the mature phenotype as assessed by flow cytometry, and immature BMDCs were used as negative controls (*483*). LPS-stimulated BMDCs from both Sema-3E-deficient and WT showed upregulated expression of co-stimulatory molecules compared to immature DCs (supplementary figure S.2). LPS-stimulated BMDCs of CD11c-PlexinD1 cKO and WT controls showed no differences in expression of the co-stimulatory molecules (CD40, CD80, CD86), and maturation phenotype (MHC class II) (**Figure 5.7**).

LPS-stimulated BMDCs of Sema-3E-deficient mice had upregulated IL-12/IL-23p40 and IL-10 production (Figure 4.10). I therefore investigate whether the upregulated IL-12/IL-23p40 depends on the expression of PlexinD1 by the mature DCs. After LPS stimulation, conditioned medium of BMDCs from CD11c-PlexinD1cKO mice and WT controls was collected and examined by MSD assay. LPS-stimulated BMDCs produced similar levels of IFN-γ, IL-12/IL-23p40, IL-6, and IL10 with or without PlexinD1 **(Figure 5.8**). As shown before, CD11c-PlexinD1 cKO immature BMDCs upregulated IL-12/IL-23p40, IL-6, and IFN-γ, while IL-10 expression level was diminished compared to WT controls (**Figure 5.3**). My results suggest that LPS treatment limited the direct impact of PlexinD1 expressed by BMDCs on the modulation of either their phenotype or function.

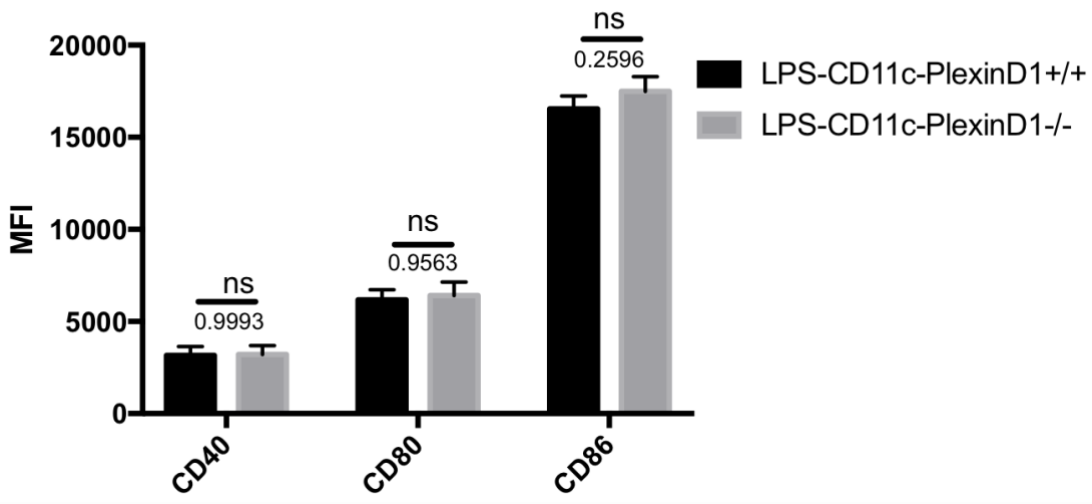
A



LPS-CD11c-PlexinD1+/+

LPS-CD11c-PlexinD1 -/-

B



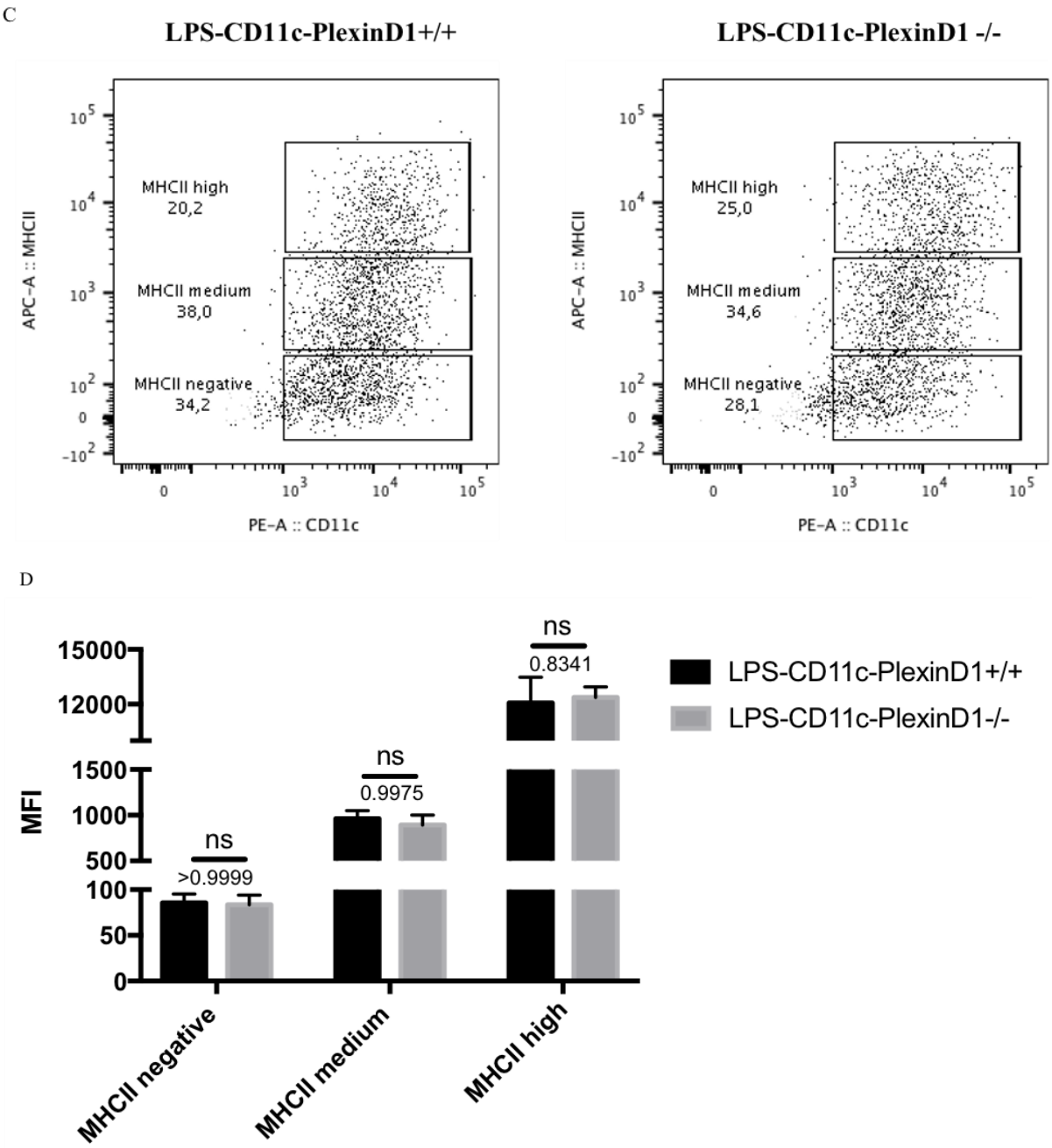


Figure 5.7. PlexinD1 deficiency did not affect the maturation phenotype of LPS-stimulated BMDCs. (A, C) Flow cytometry assay of BMDCs generated from WT (LPS-CD11c-PlexinD1+/+) and CD11c-PlexinD1 conditional knockout (LPS-CD11c-PlexinD1-/-) mice. BMDCs were cultured in GM-CSF medium for 7 days. On day 6, LPS (1μg/μL) for 24-hours to acquire matured DC phenotype then surface-stained with monoclonal antibodies against CD11cPE, CD40 APC, CD80 APC, CD86 APC, and MHC class II (MHCII) APC. **(B, D)** The MFI of CD11c+ BMDCs are plotted from all collected data. Data are shown as mean ± SEM, n = 5. *P* values are from two-way ANOVA used to compare data from two groups. ns, $P > 0.05$.

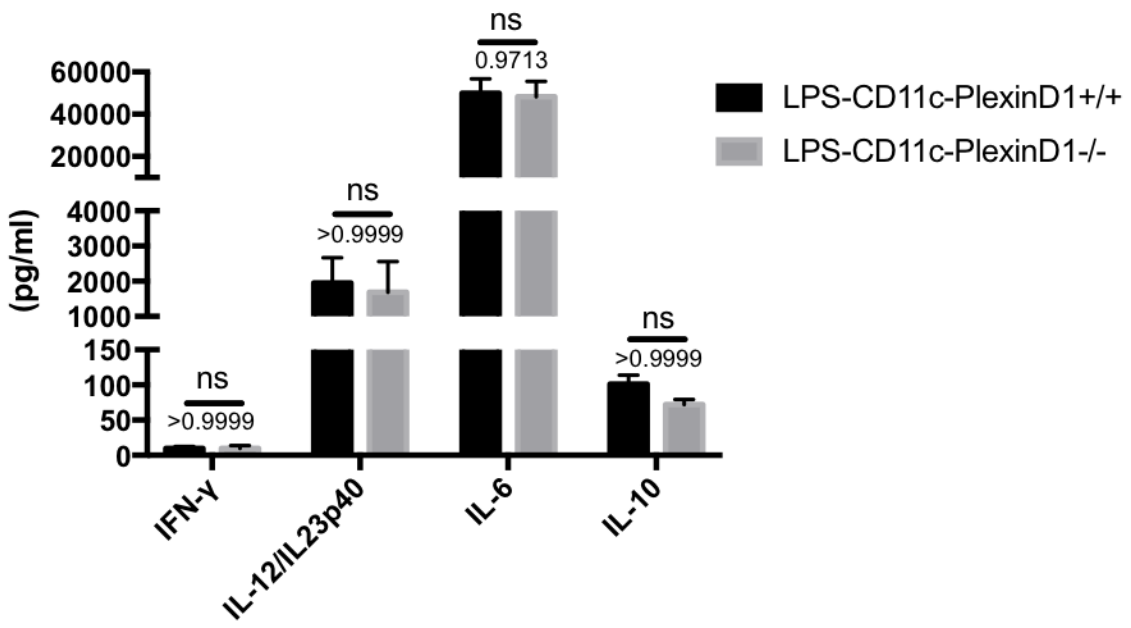


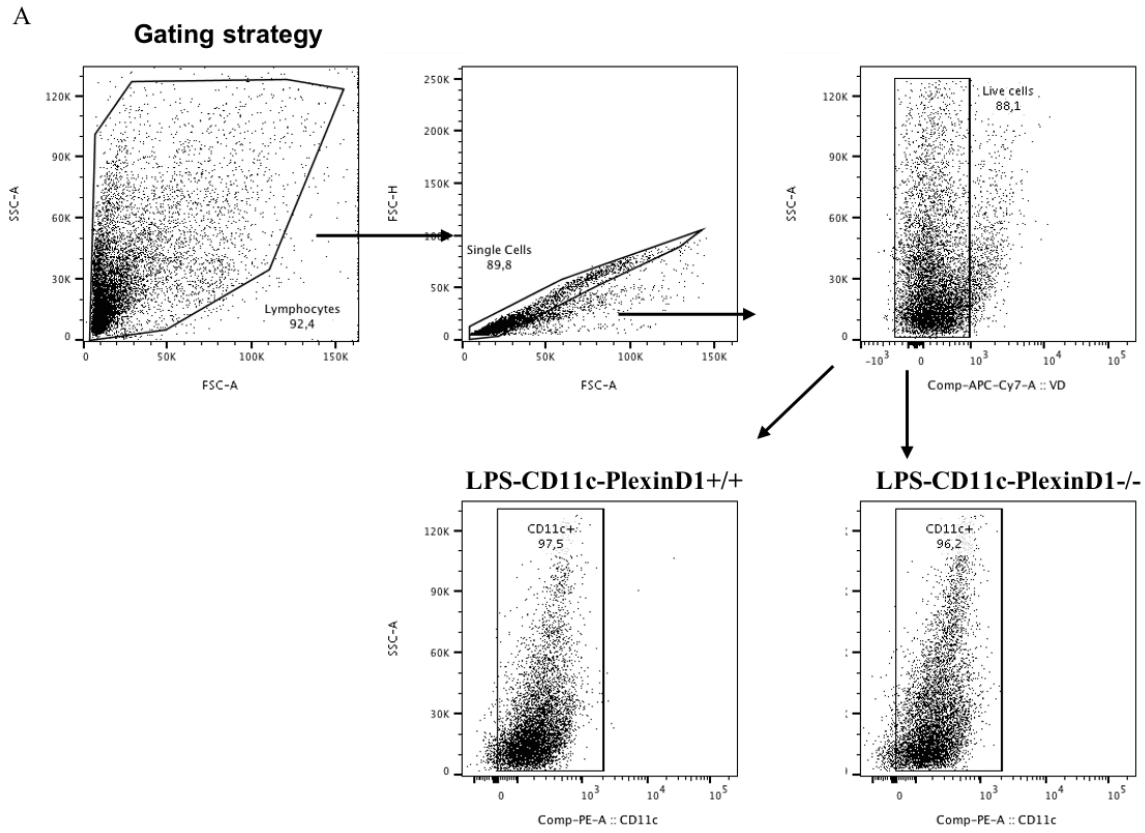
Figure 5.8. PlexinD1 deficiency had no effect on cytokine release by LPS-stimulated BMDCs. MSD assay of BMDCs generated from WT (LPS-CD11c-PlexinD1+/+) and CD11c-PlexinD1 conditional knockout (LPS-CD11c-PlexinD1-/-) mice. BMDCs were cultured in a GM-CSF medium. On day 6, LPS (1μg/μL) for 24-hours to acquire matured DC phenotype then conditioned medium of LPS BMDCs were collected to measure IFN-γ, IL-12/IL-23p40, IL-6 and IL-10. Mean calculated concentrations (pg/mL) are adjusted for the dilution factor. Data are shown as mean ± SEM, n = 3. *P* values are from two-way ANOVA used for comparing two groups. ns, $P > 0.05$.

5.3.5 PlexinD1 deficiency resulted in an upregulation of IFN-γ and IL-12/IL-23p40 in LPS-stimulated splenic DCs

In Chapter 4, I reported that LPS stimulation downregulated Sema-3E but upregulated PlexinD1 mRNA expression in splenic DCs (Figure 4.11). Moreover, the Sema-3E deficiency in these cells altered the MHC class II phenotype (Figure 4.12) and increased IFN-γ production (Figure 4.13). To assess the specific role of self-expressed PlexinD1 in modulating LPS-stimulated (mature) splenic DC phenotype and function, I repeated these experiments using CD11c-PlexinD1 cKO mice. Splenic DCs from CD11c-PlexinD1 cKO mice and WT controls were isolated using a CD11c PE positive selection isolation kit, cultured in GM-CSF medium, and stimulated with LPS (1 μg/μL) for 24 h to acquire the mature phenotype. As before, the expression of CD40, CD80, CD86, and MHC class II surface markers were taken to represent the mature DC phenotype by

flow cytometry. Immature isolated immature splenic DCs served as a negative control, and I confirmed upregulation of these co-stimulatory molecules after LPS stimulation in the presence or absence of PlexinD1 (supplementary Figure S.3). I observed that LPS-stimulated splenic DCs from CD11c PlexinD1 cKO mice showed no differences in expression of the co-stimulatory molecules CD40, CD80, CD86, or MHC class II compared to WT controls (**Figure 5.9**).

In Chapter 4, I showed that LPS-stimulated splenic DCs of Sema-3E-deficient mice had increased IFN-γ production (Figure 4.13). In addition, it was reported that PlexinB2 and PlexinD1-deficient BMDCs and isolated splenic DCs produced selectively higher levels of IL-12/IL-23p40 (*482*). I therefore wished to determine the role of self-expressed PlexinD1 the modulation of mature splenic DC function. To address this, splenic DCs were isolated from CD11c-PlexinD1 cKO and WT mice and stimulated with LPS. Conditioned medium from these cells was then examined by MSD assay. LPS-stimulated splenic DCs from CD11c-PlexinD1 cKO mice released more IFN-γ and IL-12/IL-23p40; however, IL-6 and IL-10 remained unaffected (**Figure 5.10**). These observations suggest that the action of the Sema-3E/PlexinD1 axis is dependent on PlexinD1 self-expression by DCs.



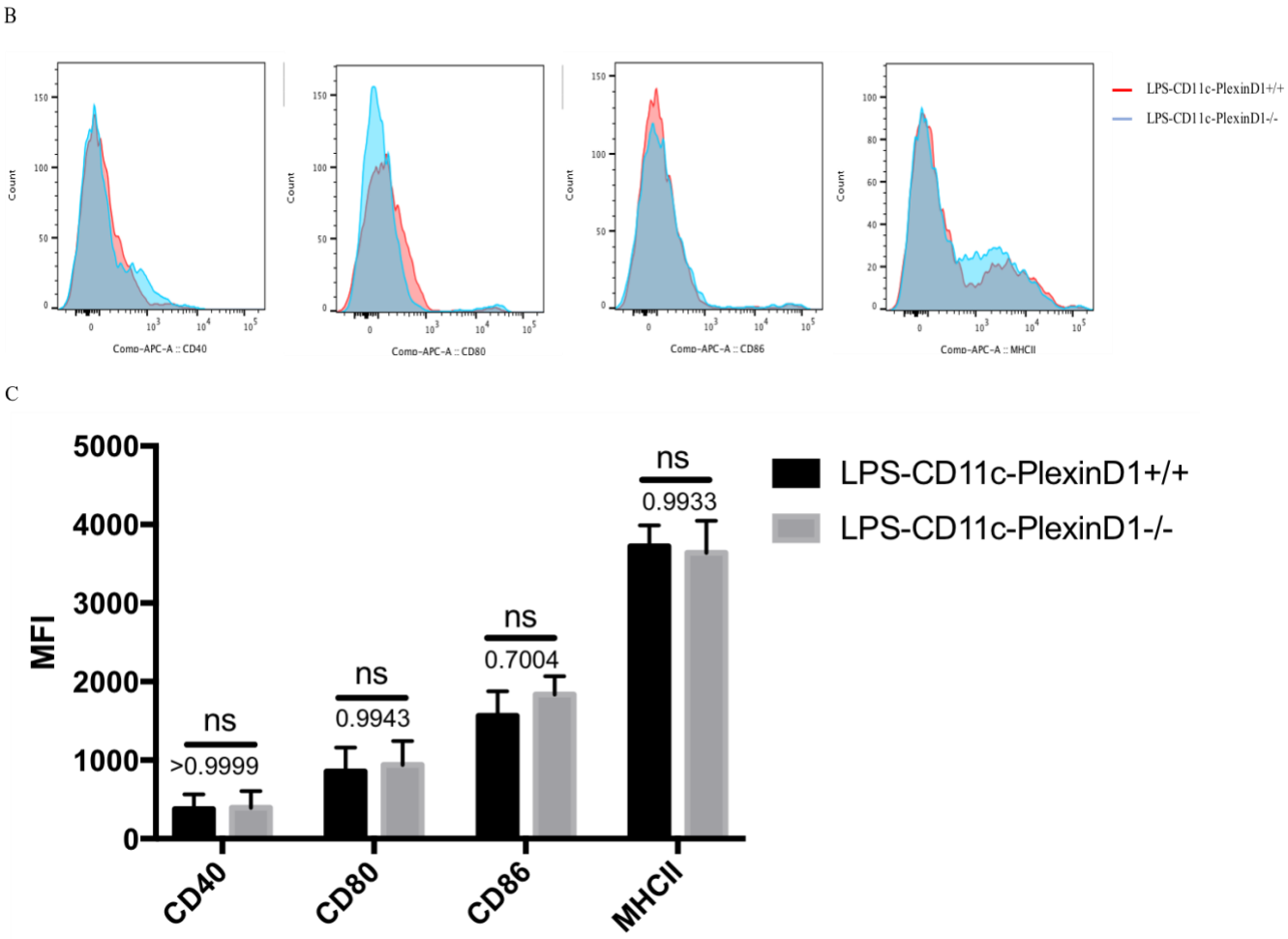


Figure 5.9. PlexinD1 deficiency did not affect the maturation phenotype of LPS-stimulated splenic DCs. (A) Flow cytometry assay of splenic DCs isolated from WT (LPS-CD11c-PlexinD1+/+) and CD11c-PlexinD1 conditional knockout (LPS-CD11c-PlexinD1-/-) mice. Isolated DCs were cultured in GM-CSF medium for 24 hours after LPS (1μg/μL) stimulation to acquire matured DC phenotype. cells were surface-stained with monoclonal antibodies against viability dye (APC-Cy-7), CD11cPE, CD40 APC, CD80 APC, CD86 APC, and MHCII APC. **(B)** The representative histograms were plotted from CD11c+ cells. **(C)** The MFI represented all collected data. Data are shown as mean ± SEM, n = 5. *P* values are from two-way ANOVA used for comparing data from two groups. ns, $P > 0.05$.

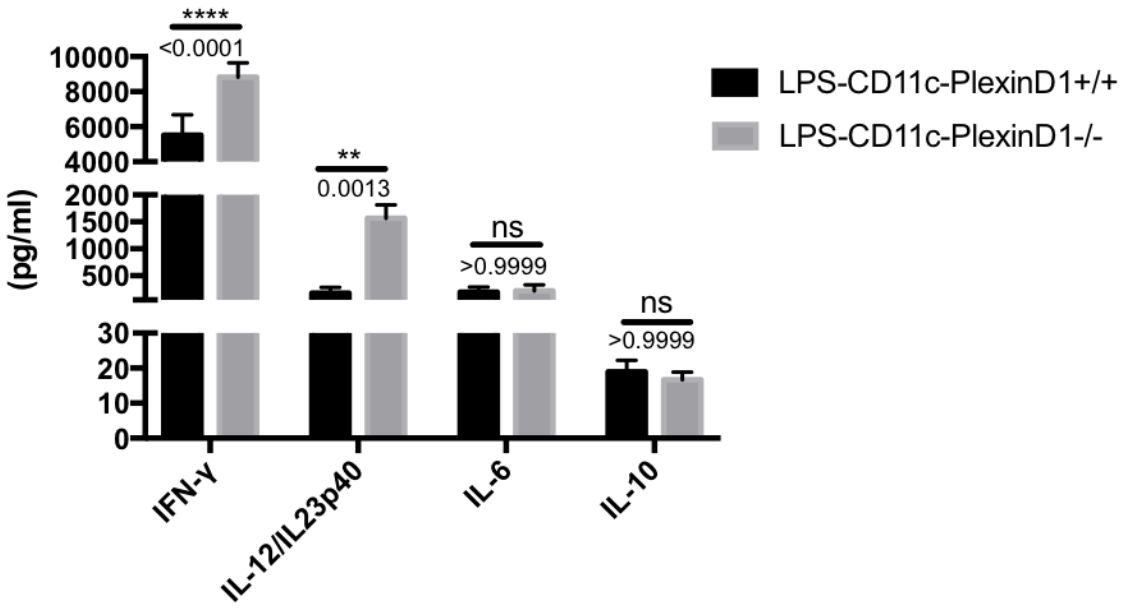


Figure 5.10. LPS treatment upregulated IFN-γ and IL-12/IL-23p40 production in PlexinD1-deficient splenic DCs. (A) MSD assay of splenic DCs isolated from WT (LPS-CD11c-PlexinD1+/+) and CD11c-PlexinD1 conditional knockout (LPS-CD11c-PlexinD1-/-) mice. Isolated splenic DCs were cultured in GM-CSF medium and stimulated with LPS (1μg/μL) for 24 hours, and then conditioned mediums were collected to measure IFN-γ, IL-12/IL-23p40, IL-6 and IL-10. Mean calculated concentrations (pg/mL) are adjusted for the dilution factor. Data are shown as mean ± SEM, n = 3. *P* values are from two-way ANOVA used for comparing two groups. ns, $P > 0.05$; ***$P < 0.001$; ****$P < 0.0001$.

5.3.6 PlexinD1 deficiency in LPS-stimulated splenic DCs correlated with an increase in Sema-3E expression.

Sema-3E binds PlexinD1 as its main receptor and NRP-1 as a co-receptor to mediate its effector functions (*406*). Previously, I have reported that BMDCs expressed PlexinD1 and NRP-1 at both the mRNA and protein levels (*420*). Interestingly, I reported that LPS stimulation downregulated the expression of PlexinD1 in BM (*420*) but upregulated in *splenic DCs* (**Figure 4.11**). Here, I investigated the correlation between PlexinD1 and Sema-3E or NRP-1 expression at the mRNA level in both BM (**Figure 5.11**) and splenic (**Figure 5.12**) DCs. First, BMDCs from CD11c-PlexinD1 cKO mice and WT controls were stimulated with LPS (1 μg/μL) for 24 h to induce maturation. Both immature and LPS-matured BMDCs were processed for RNA isolation using the Trizol method, and cDNA was synthesized. Sema-3E and NRP-1 mRNA expression was

analyzed by Sema-3E- and NRP-1-specific primers in qPCR assays (Chapter 2, table 2.2). GAPDH was used as an internal control in all qPCR analyses. Immature BMDCs from both CD11c-PlexinD1 cKO and WT controls displayed similar expression levels of Sema-3E and NRP-1 (**Figure 5.11**). LPS-stimulated BMDCs also showed no difference in Sema-3E and NRP-1 expression levels with or without PlexinD1 (**Figure 5.11**).

The same experiment was repeated in splenic DCs isolated from CD11c-PlexinD1 cKO and WT control mice. Isolated cells were stimulated with LPS (1 μg/μL) for 24 h to induce maturation. Immature splenic DCs of CD11c-PlexinD1 cKO and WT mice expressed similar Sema-3E and NRP-1 expression (**Figure 5.12**). Interestingly, LPS-stimulated splenic DCs of CD11c-PlexinD1 cKO expressed a higher level of Sema-3E when compared to WT control; however, NRP-1 expression remained unaffected (**Figure 5.12**). These results suggest that Sema-3E expression is independent of, or negatively correlated with, the expression of PlexinD1 by DCs, suggesting Sema-3E might use an alternative mechanism to modulate DCs functions.

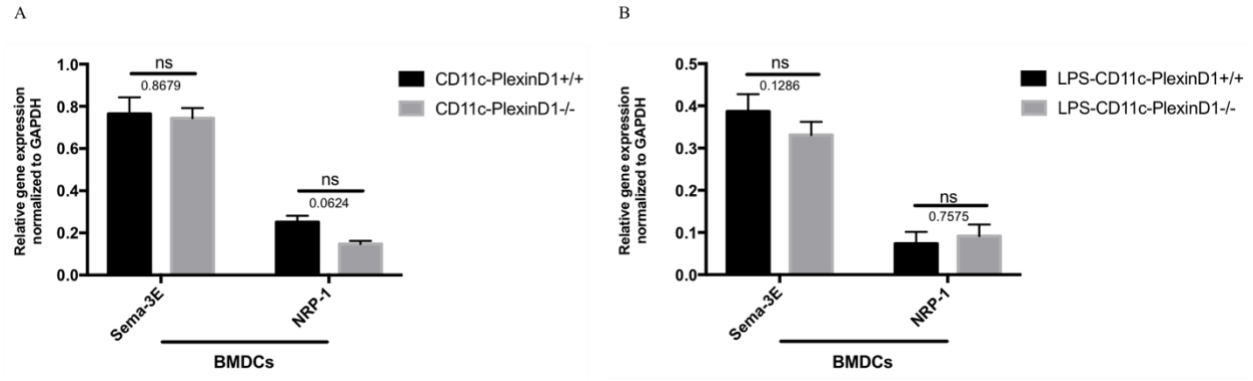


Figure 5.11. PlexinD1 deficiency had no effect on Sema-3E and NRP-1 expression in BMDCs. (A-B) Real-time PCR of BMDCs generated from WT (CD11c-PlexinD1+/+) and CD11c-PlexinD1 conditional knockout (CD11c-PlexinD1-/-) mice. Isolated cells were cultured in GM-CSF medium for 7 days. One day 6, LPS (1 μg/μl) was introduced to the culture medium for 24 h to induce maturation. Cells were harvested and used in qPCR assay to measure Sema-3E and NRP-1 expression in **(A)** immature BMDCs or **(B)** LPS-stimulated BMDCs. Data are shown as mean ± SEM, n = 4. *P* values are from two-way ANOVA used for comparing two groups. ns, $P > 0.05$.

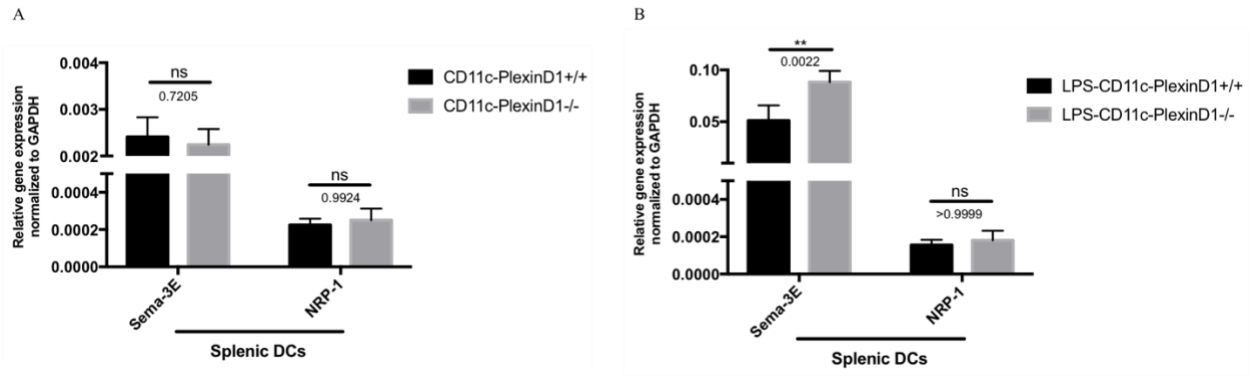


Figure 5.12. PlexinD1 deficiency promoted mature splenic DCs to enhance Sema-3E expression. (A-B) Real-time PCR of isolated splenic DCs from WT (CD11c-PlexinD1+/+) and CD11c-PlexinD1 conditional knockout (CD11c-PlexinD1-/-) mice. Isolated cells were cultured in GM-CSF medium overnight. LPS (1 μg/μL) was added to the culture medium for 24 h to acquire matured DC phenotype. Cells were harvested and used in qPCR assay to measure Sema-3E and NRP-1 expression in **(A)** immature or **(B)** LPS-stimulated splenic DCs. Data are shown as mean ± SEM, n = 4. *P* values are from two-way ANOVA used for comparing two groups. ns, $P > 0.05$; $^{**}P < 0.01$.

5.4 Discussion

PlexinD1 is a member of the plexin family of receptors, whose members are expressed in many organ systems implicated in cell trafficking and cell-cell interaction in immune response (*484*). In this study, I first examined the role of DC-expressed PlexinD1 on DC phenotype. I reported that PlexinD1-deficient at steady-state BMDCs (**Figure 5.1** and **Figure 5.2**) and splenic DCs (**Figure 5.4 and Figure 5.5)** displayed a similar $CD11c^+$ absolute cell number and MHC class II expression levels compared to WT controls. Moreover, I reported no significant differences in the expression of the co-stimulatory molecules CD40, CD80, or CD86 in immature (**Figure 5.2**) and LPS-matured (**Figure 5.7**) CD11c-PlexinD1 cKO BMDCs when compared to WT controls. Secondly, I investigated the effect of DC-expressed PlexinD1 in regulating steady-state BMDCs or splenic DC cytokine production in vitro. I observed that PlexinD1-deficient immature BMDCs upregulated IFN-γ, IL-12/IL-23p40, and IL-6 cytokine production but released less IL-10 (**Figure 5.3**). In contrast, PlexinD1-deficient immature splenic DCs produced more IL12/IL-23p40, whereas the other cytokines (IFN- γ, IL-6, and IL-10) remained unaffected (**Figure 5.6**). Upon LPS stimulation, I observed no change in IFN- γ, IL-6, IL-12/IL-23p40, or IL-10 production between CD11c-PlexinD1 cKO mature BMDCs and WT controls **(Figure 5.8**). Interestingly, PlexinD1-deficient mature splenic DCs showed upregulated IFN-γ and IL-12/IL-23p40 production with no change in IL-6 or IL-10 compared to WT controls (**Figure 5.10**). The absence of PlexinD1 in either immature or LPS-matured BMDCs did not affect Sema-3E and NRP-1 expression compared to WT control cells (**Figure 5.11**). The same was also true for immature splenic DCs (**Figure 5.12**). In contrast, LPS-matured splenic DCs showed increased expression of Sema-3E in the absence of PlexinD1 compared to WT; however, the expression level of NRP-1 remained unaffected (**Figure 5.12**). These findings collectively support that DC-expressed PlexinD1 is a critical factor in the Sema-3E/PlexinD1 signaling that upregulates DC-derived proinflammatory cytokines.

In Chapter 4, I reported that Sema-3E-deficient BMDCs contained a higher $CD11c^+$ percentage and absolute cell number compared to WT **(Figure 4.1)**. However, Sema-3E-deficient and WT BMDCs exhibited similar expression levels of the co-stimulatory molecules CD40, CD80, CD86, and MHC class II **(Figure 4.2).** Unlike other class 3 semaphorins, Sema-3E does not bind to other plexins, and thus PlexinD1 is recognized as its sole high-affinity receptor (*402*). Here, I focused on whether the ability of the Sema/PlexinD1 axis ability to regulate DC phenotype is

dependent (autocrine) or independent (paracrine) on DC-expressed PlexinD1. It was reported that integrin CD11c was crucial in the binding and uptake of missing-self CD47 cells (*459*). It was further reported that increased expression of co-stimulatory molecules including CD40, CD80, CD86 and maturation phenotype (MHC class II) specifically represents mature DCs (*258*). Therefore, DCs can activate antigen-specific naive T cells in secondary lymphoid organs (*253-255*). Of interest, deficiency in PlexinB2 and PlexinD1 did not affect the BMDC maturation markers CD40, CD80, CD86, or MHC class II or the cells' ability to take up antigen and thus stimulate T cells in vitro (*482*). In vitro, I observed that in the steady-state, BMDCs generated from CD11c-PlexinD1 cKO mice demonstrated a similar level of CD11c expression compared to cells from WT littermates (**Figure 5.1**). In the spleen, ex vivo (uncultured) steady-state DCs showed no apparent differences in $CD11c^+$ numbers in the absence of PlexinD1 (**Figure 5.4**). Moreover, immature (**Figure 5.2**) or LPS-matured (**Figure 5.7**) BMDCs showed similar expression levels of CD40, CD80, CD86, and MHC class II. Similarly, steady-state (**Figure 5.5**) or LPS-matured (**Figure 5.9**) splenic DCs showed similar expression levels of the co-stimulatory molecules CD40, CD80, CD86, and MHC class II. Collectively, my observations suggested the Sema-3E/PlexinD1 signaling axis modulated DC phenotype in Sema-3E deficient mice in vivo and in vitro specifically through Sema-3E expression, independently of DC-expressed PlexinD1. It is possible that Sema-3E may exert its effect on $CD11c^+$ cell proliferation in vitro via PlexinD1 expressed by the other cell types (5-10%) present in the BMDC-rich culture. In the spleen, my observations may also suggest that Sema-3E binds to PlexinD1 expressed by other cell types (such as T, NK, and B cells), limiting the maturation of DCs as evidenced by MHC class II upregulation.

It was shown that PlexinB2 and PlexinD1 deficiency in DCs resulted in selectively higher levels of IL-12/IL-23p40 (*482*). It was reported that neutralizing PlexinD1 in Sema-3A/PlexinD1 autocrine axis enhanced Th1 differentiation but reduced Th2 and Th17 skewing (*485*). Such observations were associated with the upregulation of T-bet in Th1 and downregulation of GATA3 and RORγt in Th2 cells and Th17 cells (*485*). In Chapter 4, I observed immature (**Figure 4.3**) and LPS mature BMDCs (**Figure 4.10**) from Sema-3E-deficient mice produced more IL-12/IL-23p40 than WT controls. I also observed immature Sema-3E-deficient splenic DCs showed the downregulation of IL-12/IL-23p40 production (**Figure 4.8**), whereas LPS-matured splenic DCs showed no change in IL-12/IL-23p40 production (**Figure 4.13**) compared to WT controls. Collectively, those observations suggest a critical role for self-expressed PlexinD1 in modulating

DCs function via a PlexinD1-dependent signaling axis. However, it remains unclear whether this axis regulates DC production of IL-12/IL23p40 in a manner dependent on (autocrine) or independent of (paracrine) DC-expressed PlexinD1. I observed that steady-state BMDCs (**Figure 5.3**) and splenic DCs (**Figure 5.6**) from CD11c-PlexinD1 cKO mice upregulated the expression level of IL-12/IL-23p40 compared to WT controls. In contrast, this effect persisted in mature (LPS-stimulated) splenic DCs (**Figure 5.10**) but not in mature BMDCs (**Figure 5.8**). Together, my results suggested that self-expressed PlexinD1 negatively regulates IL-12/IL-23p40 production by DCs. Furthermore, IL-12/IL-23p40 production modulated by the Sema-3E/PlexinD1 axis appears to be dependent on self-expressed PlexinD1 in steady-state DCs. However, my results with LPS stimulation suggest this dependence disappears with DCs maturation. It is also possible that LPS may activate alternative pathways to reduced IL-12/IL-23p40 production in CD11c-PlexinD1 cKO or upregulated IL-12/IL-23p40 in WT DCs. To explore this possibility, the STAT4 pathway, often associated with IL-12/IL-23 activity, is a strong candidate for investigation.

Previously, it was reported that IL-12 induced IFN-γ production in macrophages and DCs (*472*). It was reported that neutralizing PlexinD1 in Sema-3A/PlexinD1 autocrine axis in CD4T cells significantly enhanced the secretion of IFN- γ (*485*). In the previous chapter, I observed that the Sema-3E-deficient immature splenic DCs produced less IFN-γ and correlated positively with IL-12/IL23p40 production compared to WT controls (**Figure 4.8**), thus establishing the role of DC-expressed PlexinD1 in modulating IFN-γ production in DCs. However, the role of DC-expressed PlexinD1 in regulating DC-derived IFN-γ remains to be explored in steady-state or LPS-matured DCs. I was therefore interested in whether self-expressed PlexinD1 would affect IFN-γ production by these cells. Immature BMDCs from CD11c-PlexinD1 cKO mice produced more IFN-γ than WT control (**Figure 5.3**), while immature splenic DCs showed no significant difference in IFN-γ with or without PlexinD1 (**Figure 5.6**). With LPS-stimulated DC maturation, the observed effect is reversed by cell source: mature splenic DCs produced more IFN-γ than WT controls (**Figure 5.10**), with no observed change IFN-γ production by mature BMDCs with or without PlexinD1 (**Figure 5.8**). It was reported that IFN-γ in BMDCs correlated with CD40, CD80, CD86, MHC class II upregulation and induced IL-12 and TNF-*a* production (*486*). My results together suggested that IFN-γ correlates positively with IL-12/IL-23p40 production by DCs mediated by PlexinD1 signaling that is autocrine (dependent on DC-derived PlexinD1) in the steady state and paracrine (independent of DC-derived PlexinD1) upon LPS-stimulated

maturation. It was reported that the defect in IL-12-induced IFN-γ production resulted in poor Th1 cell differentiation (*487, 488*). Therefore, whether the DC-expressed PlexinD1 signaling axis is a critical factor in maintaining T cell differentiation in the steady state and following LPS stimulation can be delineated in future studies in a DC/T cell co-culture system.

Previous reports indicated that DC-derived IL-6 acts back on DCs in an autocrine manner to boost their ability to prime pathogenic Th17 cells (*476*). That same study showed that IFN-γ regulates the secretion of IL-6, in that a defect in IL-6 expression resulted from a deficiency in IFN-γ. Furthermore, the reconstitution of IFN-γ restored IL-6 production, thus identifying a direct role for IFN-γ in controlling local IL-6 generation (*477*). It was reported that deficiency of PlexinB2 and PlexinD1 in DCs had not affected the levels of IL-6 cytokine production compared to WT DCs (*482*). I reported that DC-expressed PlexinD1 deficiency resulted in higher IFN-γ production correlated positively with IL-12/IL-23p40 (**Figure 5.3**). Such observations suggest that DC-expressed PlexinD1 might modulate IL-6 production in DCs. PlexinD1-deficient immature BMDCs produced more IL-6 (correlating positively with IFN-γ) than WT control cells (**Figure 5.3**), whereas this change was not observed in PlexinD1-deficient immature splenic DCs (**Figure 5.6**). IFN-γ and IL-6 are known to signal through the JAK/STAT pathway (*489*). The Sema-3E/PlexinD1 axis might regulate the IL-6 and IFN-γ shared pathway (JAK/STAT), resulting in the positive correlation between IFN-γ and IL-6. Upon LPS stimulation, mature DCs from neither BM (**Figure 5.8**) nor spleen (**Figure 5.10**) displayed any changes in IL-6 production with or without PlexinD1. Together, my results suggest that IL-6 production by DCs correlates positively with IFN-γ, and this is mediated by self-expressed PlexinD1 in a dependent (autocrine) manner in the steady state. The upregulation of IL-12/IL23p40, IFN-γ, and IL-6 in steady-state DCs strongly suggests that the DC-expressed PlexinD1 dependent (autocrine) axis is a master regulator of DC function, independent of their maturation.

IL-10 is a critical immunosuppressive cytokine. A lack of IL-10 or IL-10 receptor resulted in severe chronic inflammation such as inflammatory bowel disease (IBD) (*490*). Recently it was reported that in chlamydial infection, Sema-3E-deficient DCs showed a higher expression of IL-10 production, which resulted in failed induction of Th1 and Th17 cell responses (*453*). In Chapter 4, I also showed that Sema-3E-deficient immature splenic DCs produced a higher amount of IL-10 than WT controls (**Figure 4.8**). Moreover, LPS-stimulated, Sema-3E-deficient BMDCs showed a higher IL-10 production level than WT controls (**Figure 4.10**). The role of self-expressed

PlexinD1 in modulating IL-10 cytokine production in steady-state and mature DCs remained to be explored. In this chapter, I observed that PlexinD1-deficient immature BMDCs produced less IL-10 compared to WT control cells (**Figure 5.3**). Of interest, no such difference was found in immature splenic DCs (**Figure 5.6**). LPS-stimulated BM (**Figure 5.8**) and splenic (**Figure 5.10**) DCs also displayed similar expression levels of IL-10 in CD11c-PlexinD1 and WT control. My results suggest that PlexinD1 expressed by BMDCs is a crucial factor in an autocrine axis that supports tolerogenic DC functions. Therefore, a lack of autocrine Sema-3E/PlexinD1 signaling may result in more susceptibility to autoimmune disease. Interestingly, IL-10 was found to prevent DC-mediated $CD8^+$ tumor-infiltrating lymphocyte apoptosis via the regulation of IFN-γ production (*491*). The gene silencing of Plexin D1 in human metastatic carcinoma cells limited their metastatic potential (*492*). These observations suggest that the deficiency of DC-expressed PlexinD1 may limit tumor metastasis via T-cell antitumor immunity. The blocking of the DC-expressed PlexinD1-mediated axis might be a potential target for limiting tumor metastasis.

I previously reported that Sema-3E, PlexinD1, and NRP-1 were expressed by immature DCs and downregulated upon LPS stimulation (*420*). Of interest, PlexinD1 and NRP-1 mRNA expression was downregulated significantly in the Sema-3E-deficient immature BMDCs compared with WT controls (*420*). In turn, I wished to investigate whether PlexinD1 deficiency would modulate the expression levels of Sema-3E and NRP-1therfore modulate DCs functions. I observed that immature or LPS-matured BMDCs from CD11c-PlexinD1cKO and WT mice showed no differences in Sema-3E and NRP-1 expression (**Figure 5.11**). A similar finding was observed in immature splenic DCs (**Figure 5.12**), but interestingly, LPS-stimulated, PlexinD1-deficient splenic DCs expressed higher Sema-3E mRNA levels compared to WT controls, while NRP-1 remained unaffected in these cells (**Figure 5.12**). My results suggest that PlexinD1 limits Sema-3E expression, and PlexinD1 deficiency in LPS-stimulated splenic DCs might activate alternative compensatory mechanisms that allow overexpressed Sema-3E to mediate its effector functions on DCs independent of self-expressed PlexinD1.

In conclusion, my results provide evidence of DC-expressed PlexinD1 as a novel factor to regulate DC function via Sema/PlexinD1 dependent (autocrine) or independent (paracrine) manner. Furthermore, I demonstrated that PlexinD1-deficient DCs had altered proinflammatory cytokine productions, suggesting a regulatory effect of PlexinD1 on DC function (**Figure 5.13**). Future work will establish how self-produced PlexinD1 modulates DC function through the Sema-

3E/PlexinD1 axis. Understanding the mechanism by which the Sema-3E/PlexinD1 axis modulates steady-state and LPS-stimulated DC effector function(s) may present novel therapeutic targets to control specific DC functions in clinical settings.

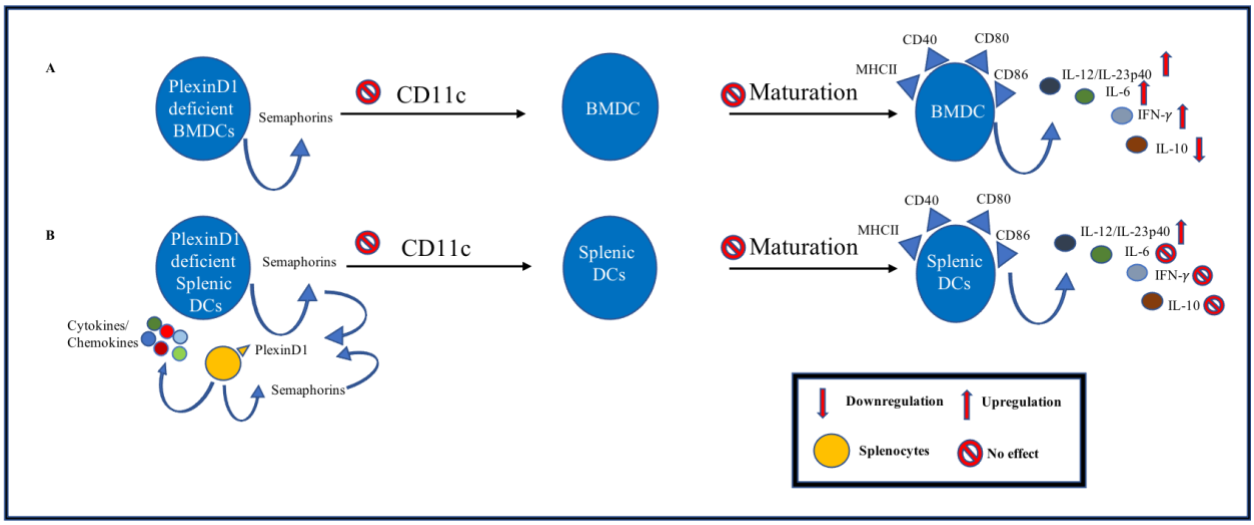


Figure 5.13. The PlexinD1 deficiency modulates DC function. The role of PlexinD1 was examined in **(A)** BMDCs, and **(B)** splenic DCs. Through modulation of the phenotype and function of DCs, the PlexinD1 modulates DC cytokine production independent of absolute cell number and maturation.

Chapter 6: Semaphorin-3E Modulated IL-2-activated NK Cell Migration in NK/DC Crosstalk

6.1 Abstract

NK cells and DCs are part of the innate immune system and critical in regulating both innate and adaptive immunity. NK cell or DC function and migratory response are thought to be modulated by NK/DC crosstalk, and this may include direct cell-cell contact and/or multiple cytokine signals. However, a possible role for the Sema-3E in the regulation of NK cell migration has not been studied. To formally address the importance of the DC-derived Sema-3E in regulating NK cell migration, I compared the in vitro migratory responses of IL-2-activated NK cells toward the conditioned medium of DCs (immature or lipopolysaccharide-stimulated) derived from the BM or spleens of Sema-3E-deficient or CD11c-PlexinD1 conditional knockout mice. I observed that the conditioned medium of immature Sema-3E-deficient BMDCs contained an upregulated MCP-1 and MIP-1α chemokine levels compared wild type (WT) controls. These conditioned medium (i.e from the immature Sema-3E-deficient BMDCs) induced an increase in IL-2-activated NK cell migration compared wild type (WT) controls. Blocking of Sema-3E in the conditioned medium of WT immature BMDCs enhanced NK cell migration. However, the addition of exogenous recombinant Sema-3E to the conditioned medium of the Sema-3E-deficient mature BMDCs had no effect on NK cell migration. To study the paracrine effect of Sema-3E on DCs function that modulated NK migration, I used splenic DCs conditioned medium. Unlike BMDCs, conditioned medium of Sema-3E-deficient immature and LPS mature splenic DCs did not affect NK migration. This work reveals a novel role for the Sema-3E in modulating NK cell migration, and therefore NK/DC crosstalk, specifically in splenic DCs.

6.2 Introduction and rationale

NK/DC crosstalk is bidirectional, where NK cells interact with DCs through cell-to-cell contact and/or soluble factors (*290*). This interaction is critical in cell maturation, activation, and cytokine production. DCs mediate the activation of NK cells to contribute to innate immunity. In turn, activated NK cells provide signals essential for DC activation and maturation, thus enhancing adaptive immunity responses (*290*).

NK cell migration is a crucial mechanism for maintaining immunosurveillance (*132*). During inflammation, viral infections, and tumor growth and invasion, NK cells are recruited to

the injured organs where they are activated and release cytokines and chemokines, kill target cells, and participate in the recruitment and activation of other leukocytes (*200, 493*).

Under inflammatory conditions, Sema-3E is known to regulate the trafficking of immune cells (e.g., DCs,T cells neutrophils) (*397, 418, 445*). Of interest, I previously reported that the expression of Sema-3E and its receptors (PlexinD1 and NRP-1) is tightly regulated in NK cells and DCs, suggesting these cells' ability to sense and respond to the Sema-3E/PlexinD1 axis, therefore modulating their effector functions (*420*). Furthermore, in NK/DC crosstalk, Sema-3E produced by immature DCs suppresses NK cell migration (*420*). Sema-3E plays an essential role in modulating NK cell function (Chapter 3) and DC phenotype and function (Chapters 4). However, the role of the Sema-3E role in regulating NK cell migration in NK/DC crosstalk is not fully understood.

6.3 Results

6.3.1 Blocking Sema-3E produced by immature BMDCs enhanced IL-2-activated NK cell migration

Previously I have reported NK and BMDCs expressed Sema-3E and PlexinD1 at both the mRNA and protein levels. However, stimulation of the immature WT BMDCs with LPS induced downregulation of Sema-3E and PlexinD1 mRNA expression (*420*). In addition, I observed that conditioned medium from Sema-3E-deficient immature BMDCs enhanced IL-2-activated NK cell migration (*420*). Adding back recombinant murine Sema-3E to the conditioned medium of these cells reversed this effect (*420*). With this data suggesting that Sema-3E produced by immature BMDCs suppresses NK cell migration, I next wanted to confirm whether the upregulation in NK cell migration I observed is Sema-3E-specific. First, I used recombinant PlexinD1 receptor protein in solution as a decoy to remove the secreted Sema-3E present in the conditioned medium of immature WT BMDCs (*410*). Bovine serum albumin (BSA; 1% = 100 ng/mL) and PBS 1% = 100 ng/mL) added to the conditioned medium were used as negative controls. The conditioned medium of Sema-3E-deficient immature BMDCs was used as a positive control. Recombinant PlexinD1, BSA, or PBS were added to the conditioned medium of WT immature BMDCs at the beginning of the transwell migration assay. I found no significant difference in the migratory response of IL-2-activated NK cells to conditioned medium containing BSA or PBS; however, I did observe an effect after blocking of Sema-3E using recombinant Plexin D1 protein (**Figure 6.1**). I reported Sema-3E deficiency enhanced NK cell migration (*420*). However, conditioned medium of the

Sema-3E-/- immature DC treated with recombinant Sema-3E downregulated NK cell migration (*420*). Interestingly, the addition or blocking of Sema-3E in the conditioned medium of either Sema-3E-deficient or WT LPS-stimulated BMDCs had no effect on IL-2-activated NK cell migration. The conditioned medium of WT immature BMDCs were used as negative controls, and the conditioned medium of LPS-stimulated WT (LPS-Sema-3E+/+) was used as a positive control (**Figure 6.2**).

The chemokines CXCL10, CXCL12, CCL19, and CCL21 play a critical role in regulating immune cell recruitment (*494, 495*). I therefore wished to investigate whether Sema-3E down-regulation of NK cell migration is mediated through blocking the effect of these chemokines. To address this question, I used plain mouse medium (RPMI alone) in a transwell assay, in which recombinant CXCL10 (100 ng/mL), CXCL12 (50 ng/mL), CCL19 (100 ng/mL), or CCL21 (50 ng/mL) was added plus or minus recombinant murine Sema-3E protein (50 ng/mL). I observed no Sema-3E effects on NK migration induced by these single chemokines (**Figure 6.3**). My results suggest that the Sema-3E alone did not affect NK cell migration independent of immature BMDCs supplement factors, a crucial modulator for NK cell migration. The level of Sema-3E in the conditioned medium of immature BMDCs remain to be evaluated.

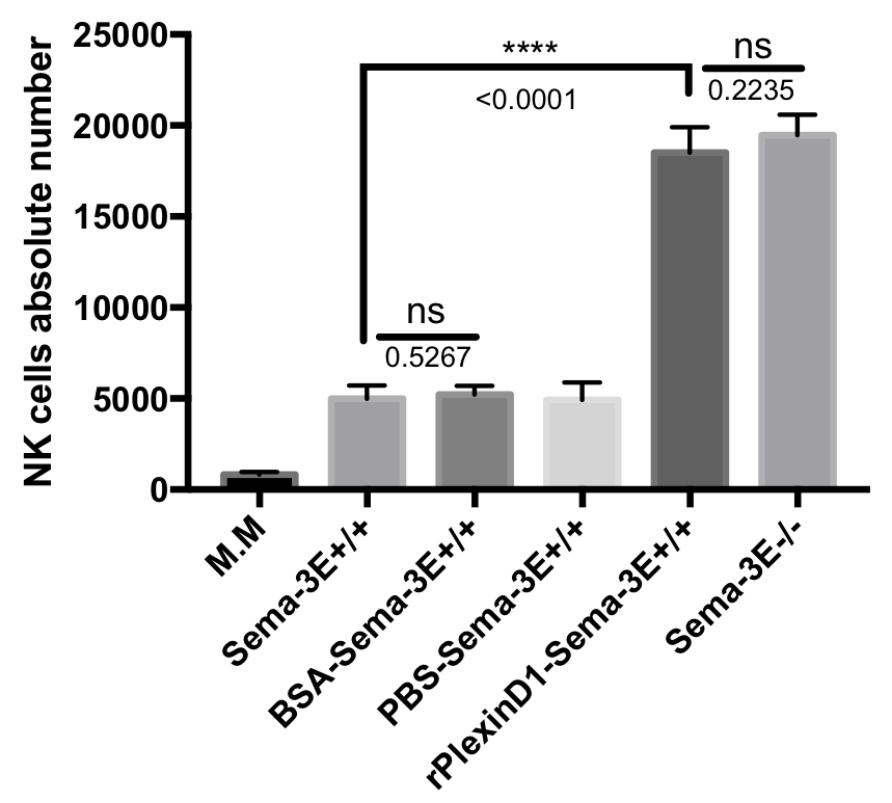


Figure 6.1. Blocking of Sema-3E produced by immature BMDCs enhanced activated NK cell migration. Migration of IL-2-activated NK cells toward the conditioned medium of immature BMDCs generated from Sema-3E-/- and WT (Sema-3E+/+) mice was assessed in a transwell assay. Recombinant Plexin D1 (rPlexinD1; 100 ng/mL) was added to the WT (Sema-3E+/+) conditioned medium at the time of running the migration assay. Mouse medium (M.M), 1% BSA and 1% PBS (100 ng/mL) were used as negative controls. Conditioned medium of cells from Smea-3E-deficient mice (Sema-3E-/-) was used as a positive control. Data are shown as mean ± SEM, n = 3. independent experiments. *P* values are from two-tailed *t* tests used for comparing two groups. ns, $P > 0.05$, ****$P < 0.0001$.

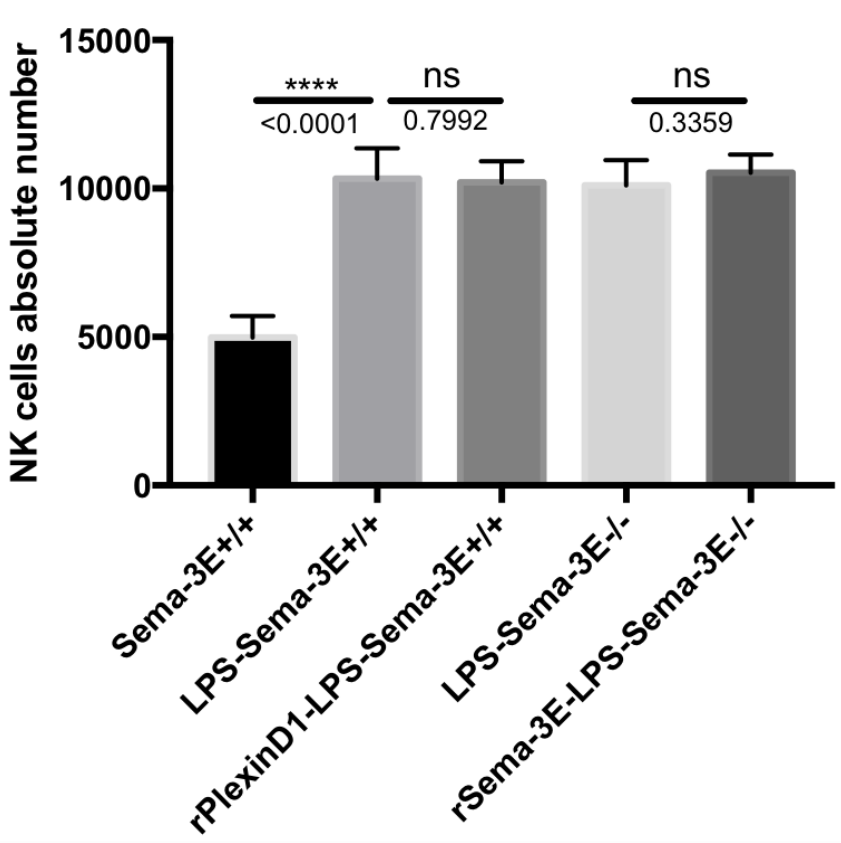


Figure 6.2. Sema-3E in conditioned medium of LPS-stimulated BMDCs did not affect activated NK cell migration. Migration of IL-2-activated NK cells toward the conditioned medium of LPS-stimulated BMDCs generated from Sema-3E-/- and WT (Sema-3E+/+) mice was assessed in a transwell assay. Recombinant Plexin D1 (rPlexinD1; 100 ng/mL) was added to the LPS-stimulated WT (LPS-Sema-3E+/+) conditioned medium at the time of running the migration assay. Recombinant Sema-3E (rSema-3E; 50 ng/mL) was added to the LPS-stimulated SEMA-3E-deficient (LPS-Sema-3E-/-) conditioned medium at the time of running the migration assay. Conditioned medium of the WT (Sema-3E+/+) was used as a negative control, while the conditioned medium of LPS-stimulated WT cells (LPS-Sema-3E+/+) was used as a positive control. Data are shown as mean ± SEM, n = 3 independent experiments. *P* values are from two-tailed *t* tests used for comparing two groups. ns, $P > 0.05$, ****$P < 0.0001$.

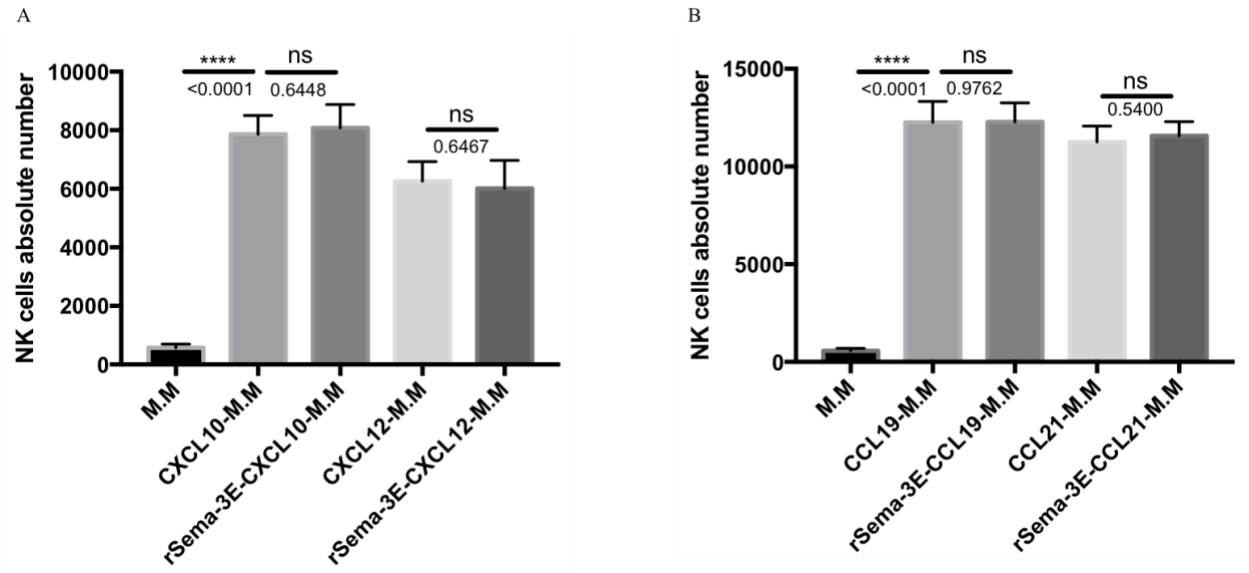


Figure 6.3. Recombinant Sema-3E did not suppress activated NK cell migration induced by CXCL10 and CCL21. NK cells were purified and activated with IL-2 for 4 days, and cell migration was assessed in a transwell migration assay. **(A)** The chemokines CXCL10 (100 ng/mL) and CXCL12 (50 ng/mL) were added to mouse medium (M.M) plus or minus recombinant Sema-3E (rSema-3E). Mouse medium (M.M) was used as a negative control, and CXCL10 or CXCL12 alone served as positive controls. **(B)** The chemokines CCL19 (100 ng/mL) and CCL21 (50 ng/mL) were added to mouse medium (M.M) plus or minus recombinant Sema-3E (rSema-3E). Mouse medium (M.M) was used as a negative control, where only CCL19 or CCL21 was used as a positive control. Data are shown as mean ± SEM, n = 3 independent experiments. *P* values are from two-tailed *t* tests used for comparing two groups. ns, $P > 0.05$, ****$P < 0.0001$.

6.3.2 Deficiency of Sema-3E in immature BMDCs upregulated MCP-1 and MIP-1α chemokine production.

Other chemokines that are also critical regulators of NK cell migration include MCP-1 (CCL2), MIP-1α (CCL3), IP-10 (CXCL10), and KC/GRO (*493*). As I have reported previously, immature BMDCs conditioned medium suppressed IL-2-activated NK cell migration compared to Sema-3E deficient conditioned medium (*420*). As expected, blocking of Sema-3E restored IL-2-activated NK cell migration (**Figure 6.1**). In Chapter 4 I showed that Sema-3E modulated BMDC cytokine production. However, the role of Sema-3E in modulating BMDC-derived key chemokines that modulate NK cell migration remains to be investigated. To investigate the effect of Sema-3E on BMDC chemokines production, conditioned medium of both Sema-3E-deficient

and WT BMDCs was collected and analyzed by MSD assay for the chemokines MCP-1, MIP-1α, IP-10, and KC/GRO. Interestingly, I observed that compared to WT cells, Sema3E-deficient immature BMDCs had increased production of MCP-1 and MIP-1α while IP-10 and KC/GRO remained unaffected (**Figure 4.10)** for MIP-1α and MCP-1, respectively). Of interest, LPS-stimulated Sema-3E-deficient BMDCs displayed similar levels of the chemokines MCP-1, MIP-1α, IP-10 and KC/GRO compared to WT controls (**Figure 6.5**). My results may suggest that Sema-3E produced by immature BMDCs may mediate its suppressive effects on NK cell migration through the chemokines MCP-1 (CCL2) and MIP-1α (CCL3) that can be confirmed by blocking of these chemokines in future studies.

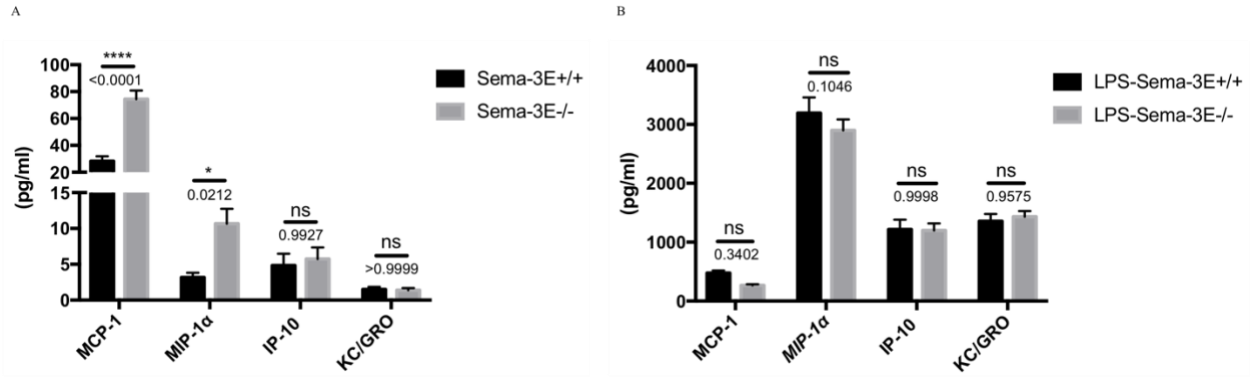


Figure 6.4. Immature BMDCs from Sema-3E-deficient mice upregulated MCP-1 and MIP-1α chemokine production. BMDCs were generated from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) BALB/c mice and cultured in GM-CSF medium for 7 days. On day 6, LPS (1 μg/μL) was added for 24 h to acquire the mature DC phenotype. Conditioned medium was collected and used to measure the chemokines MCP-1, MIP-1α, IP-10, and KC/GRO by MSD assay in **(A)** immature BMDCs and **(B)** LPS-stimulated BMDCs. Data shown are mean ± SEM of the calculated concentrations in pg/mL, adjusted for the dilution factor, for three independent experiments. *P* values from two-way ANOVA used for comparing data from two groups. ns, not significant; **P* < 0.1; *****P* < 0.0001.

6.3.3 Conditioned medium of splenic DCs from Sema-3E-deficient mice did not enhance IL-2-activated NK cell migration.

In Chapter 4, I found that immature splenic DCs express Sema-3E and PlexinD1 at mRNA levels **(Figure 4.4);** however, LPS stimulation induced Sema-3E downregulation but PlexinD1 upregulation at the mRNA level **(Figure 4.11)**. In addition, I observed that Sema-3E deficiency in immature **(Figure 4.8)** or LPS-stimulated **(Figure 4.13)** splenic DCs regulated their function (measured by cytokine production).

I next wished to investigate the role of Sema-3E produced by splenic DCs in modulating IL-2-activated NK cell migration. First, I isolated splenic NK cells from WT BALB/c mice and stimulated them with IL-2 for 4 days. Second, splenic DCs were isolated from WT and Sema-3E-deficient BALB/c mice using a CD11c PE positive selection isolation kit. After isolation, cells were cultured in the presence of GM-CSF and stimulated with LPS (1 μg/μL) for 24 h to acquire the mature DC phenotype.

The conditioned medium of these DCs was collected and used with the IL-2-activated NK cells in a transwell migration assay. Plain mouse medium was used as a negative control, and the conditioned medium of LPS-stimulated BMDCs was used as a positive control. The results are shown in **(Figure 6.5)**. Interestingly, I observed no significant differences in IL-2-activated NK cell migration toward conditioned media from Sema-3E-deficient immature splenic DCs compared to WT controls. Moreover, no differences in NK cell migration were observed with or without Sema-3E using conditioned media from LPS-stimulated splenic compared to WT controls ($n = 4$, $P > 0.05$).

Next, I wanted to investigate if Sema-3E produced by splenic DCs would affect their chemokine production and thus modulate NK cell migration. Conditioned medium of immature and LPS-stimulated splenic DCs isolated from both Sema-3E-deficient and WT mice were collected and analyzed for the chemokines MCP-1, MIP-1α, IP-10, and KC/GRO by MSD assay. I observed no changes in chemokine production between WT and Sema-3E-deficient immature splenic DCs (**Figure 6.6)**. Similarly, no changes were observed in LPS-stimulated splenic DCs (**Figure 6.6**). These results suggest Sema-3E produced by splenic DCs has no effect on DC chemokine production.

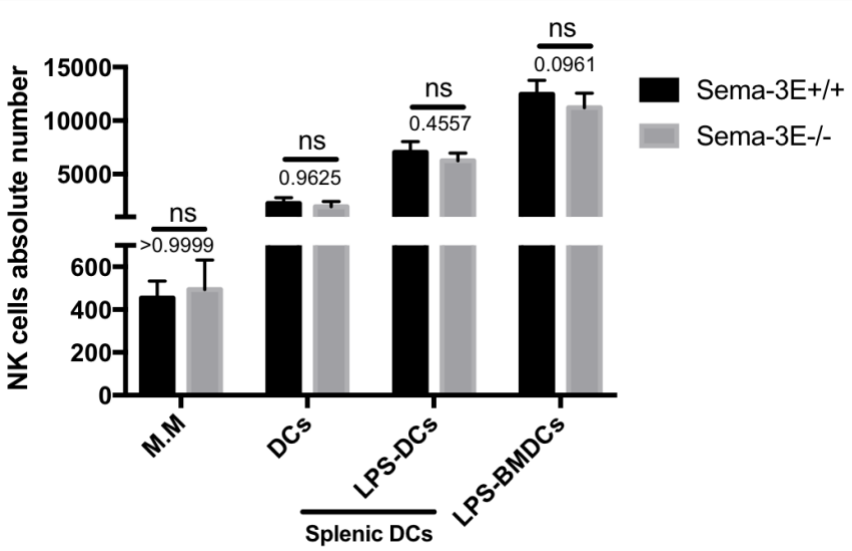


Figure 6.5. Conditioned medium of splenic DCs from Sema-3E-deficient mice did not enhance IL-2-activated NK-cell migration. IL-2-activated NK cells migrations toward the conditioned medium of the immature and LPS-stimulated splenic DCs isolated from Sema-3E-/- and WT (Sema-3E+/+) were assayed. Plain mouse medium (M.M) was used as negative controls while the conditioned medium of LPS-stimulated BMDCs (LPS-BMDCs) was used as a positive control. Data are shown as mean ± SEM, n = 4 independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups. ns $P > 0.05$.

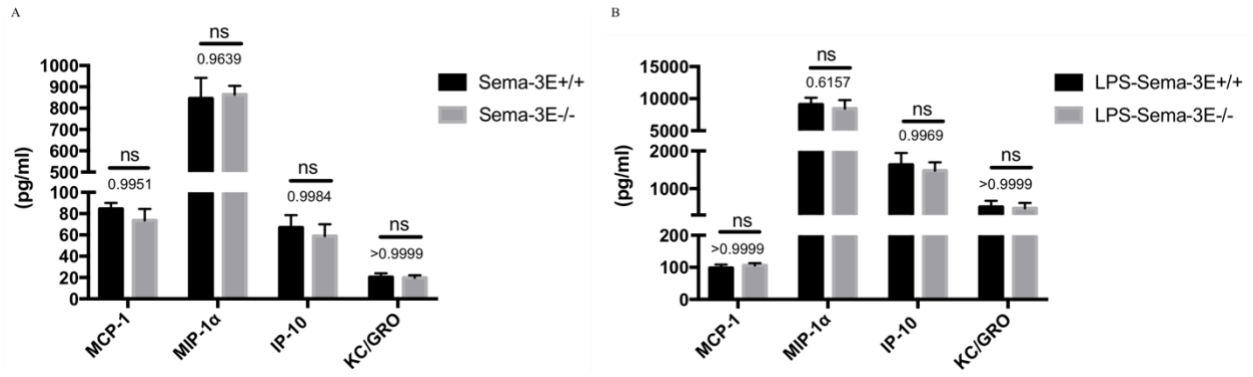


Figure 6.6. Sema-3E deficiency in immature or LPS-stimulated splenic DCs did s not affect their chemokine production. (A, B) Splenic DCs were isolated from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) BALB/c mice and cultured in GM-CSF medium for 24 h. After isolation, LPS (1 μg/μL) was introduced to the culture for 24 h to acquire matured DCs. Conditioned medium was collected and used to measure the chemokines MCP-1, MIP-1α, IP-10, and KC/GRO by MSD assay in **(A)** immature splenic DCs and **(B)** LPS-stimulated splenic DCs. Data are shown as mean ± SEM of the calculated concentrations in pg/mL, adjusted for the dilution factor, for three independent experiments. *P* values from two-way ANOVA used for comparing data from two groups. ns, not significant.

6.3.4 Splenic DCs expressed a lower level of Sema-3E compared to BMDCs.

BMDCs and splenic DCs appear to share many of the DCs functions such as T-cell stimulatory ability (*496*). However, BMDCs showed better antigen processing and presenting than splenic DCs (*496*). Therefore, DCs cell-mediated and soluble factors that are differentially presented in the culture and in vivo determine DCs functions. Therefore, the expression level of Sema-3E in BMDCs and splenic DCs that might modulate NK/DC crosstalk remains to be investigated. I reported that immature BMDCs expressed Sema-3E and its receptor PlexinD1. However, LPS stimulation downregulated expression of both (*420*). In Chapter 4, I showed that immature splenic DCs express Sema-3E and PlexinD1 (Figure 4.4); however, stimulation of immature WT splenic DCs with LPS induced downregulation of Sema-3E but PlexinD1 upregulation at the mRNA level (Figure 4.11). In NK/DC crosstalk, I showed Sema-3E produced by immature BMDCs suppressed IL-2-activated NK cell migration (*420*). Furthermore, Sema-3E-deficient immature BMDCs upregulated the production level of MCP-1 and MIP-1α (**Figure 4.10**). Unlike BMDCs, conditioned medium from Sema3E-deficient immature or LPS-stimulated splenic DCs had no effect on NK cell migration compared to WT controls (**Figure 6.5**). Moreover, I observed that Sema-3E-deficient immature and LPS-stimulated splenic DCs had similar production levels of the chemokines MCP-1, MIP-1α, IP-10, and KC/GRO compared to their WT counterparts (**Figure 6.6**). I next wished to compare the expression level of Sema-3E in BMDCs vs splenic DCs, to possibly explain the different effects on NK cell migration. Interestingly, I observed using qPCR that immature splenic DCs expressed a lower level of Sema-3E than immature BMDCs (**Figure 4.4)**. Although LPS stimulation downregulated the expression level of Sema-3E in BM (*420*) and splenic DCs compared to immature DCs (**Figure 4.4)**, I observed that LPS-stimulated splenic DCs retained a lower level of Sema-3E expression compared to BMDCs (**Figure 4.4**). My results suggest that this difference in Sema-3E expression level leads to difference in the modulation DCs functions, and thus NK-DC crosstalk, between BM and splenic DCs.

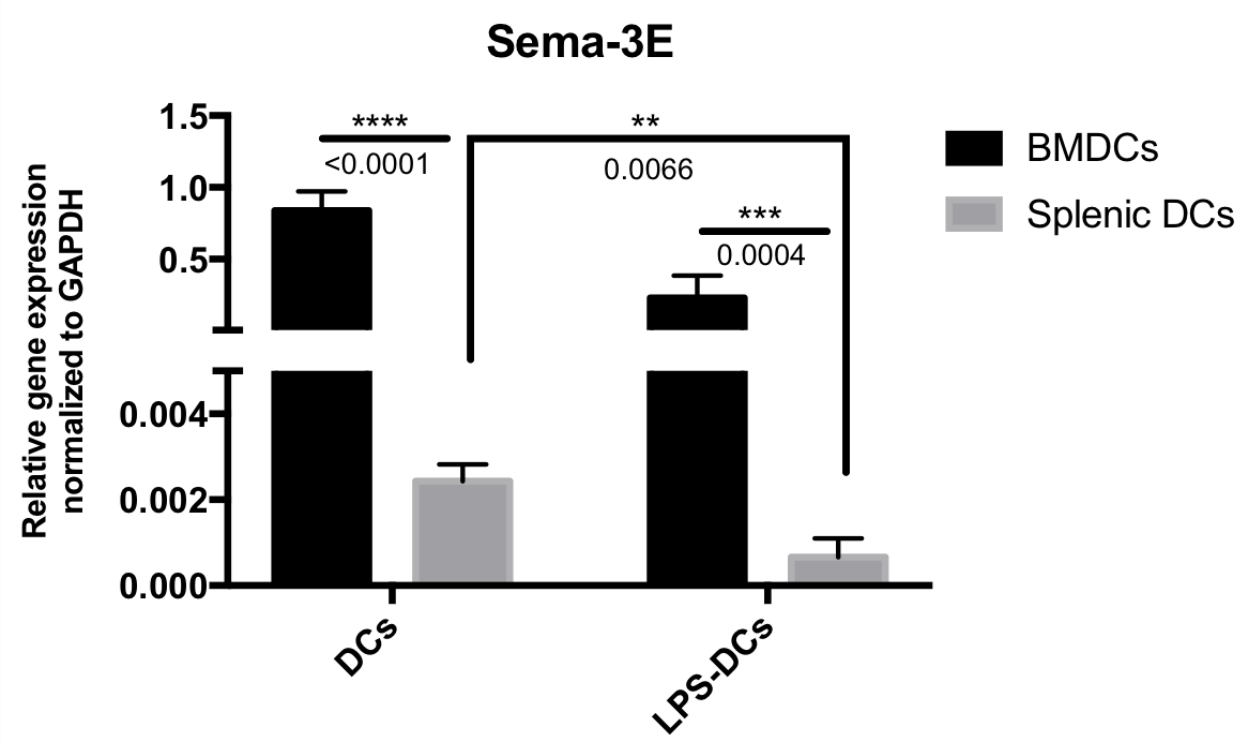


Figure 6.7. Splenic DCs expressed less Sema-3E than BMDCs. Splenic DCs were isolated from WT (Sema-3E+/+) BALB/c mice. BMDCs were generated from WT (Sema-3E+/+) BALB/c mice. RNA from these cells was used in a qPCR assay to measure Sema-3E expression in immature DCs (DCs), and LPS-stimulated DCs (LPS-DCs). Data are shown as mean ± SEM of four independent experiments. *P* values from two-way ANOVA used for comparing data from two groups. ***P* < 0.001; ****P* < 0.001; *****P* < 0.0001.

6.3.5 Recombinant Sema-3E enhanced NK cell migratory property under prolonged incubation with IL-2.

Previously I have reported that resting NK cells expressed Sema-3E and PlexinD1 at both the mRNA and protein levels. However, stimulation of NK cells with IL-2 induced downregulation of Sema-3E and PlexinD1 mRNA expression (*420*). Of interest, recombinant Sema-3E alone did not affect IL-2-activated NK cell migration when added to plain mouse medium in the transwell migration assay (*420*). Here I investigated whether prolonged exposure of NK cells to Sema-3E in culture would affect NK cell migration. To address this question, I isolated NK cells from the spleen of WT BALB/c mice. Isolated cells were cultured with IL-2 plus or minus recombinant Sema-3E (50 ng/mL) for 4 days. The migration of IL-2-activated NK cells toward the conditioned

medium of immature WT BMDCs was examined by transwell migration assay. Plain mouse medium was used as a negative control, and the conditioned medium of LPS-stimulated WT DCs was used as a positive control. Interestingly, IL-2-activated NK cells cultured with recombinant Sema-3E for 4 days showed a higher migration ability compared to IL-2-activated NK cells alone (**Figure 6.8)**. Next, to confirm that recombinant Sema-3E has not caused IL-2-activated NK cells apoptosis, resulting cells detach and cross the membrane, making the correlation essentially an artifact. After culture, cells were stained with 7-aminoactinomycin D and Annexin V (**Figure 6.9**). IL-2-activated NK cells alone were used as negative control while NK cells cultured in plain mouse medium were used as a positive control. Recombinant Sema-3E did not affect the viability of IL-2-activated NK cells. Our data therefore suggest that Sema-3E promoted NK cell migration through prolonged contact.

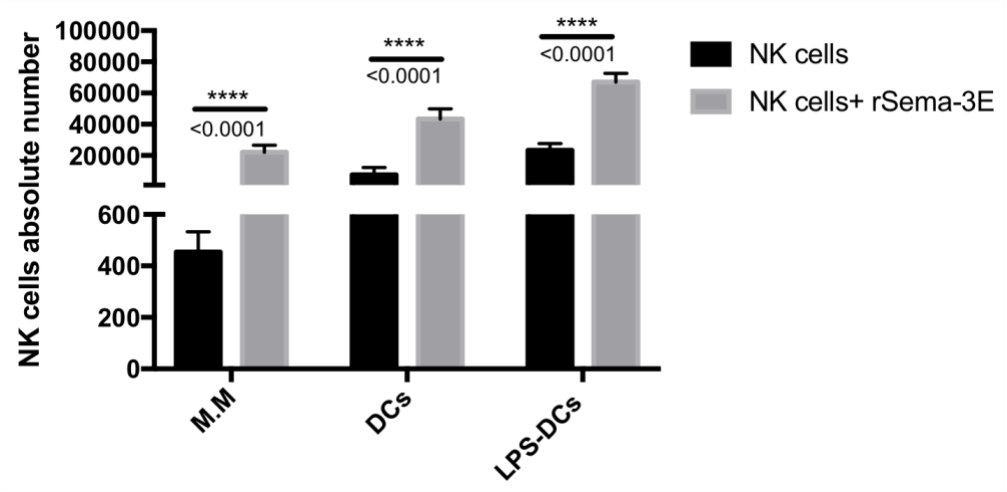


Figure 6.8. Recombinant Sema-3E promoted activated NK cell migration towards conditioned medium of DC. Transwell migration assay of IL-2-activated NK cells alone (NK cells) and with recombinant Sema-3E for 4 days (NK cells+ rSema-3E). Conditioned medium of immature (DCs) and LPS-stimulated BMDCs (LPS-DCs) generated from WT BALB/c mice was used as an attractant in the assay. Plain mouse medium (M.M) served as the negative control. Data are shown as mean ± SEM, n = 4 independent experiments. *P* values shown are from two-way ANOVA used for comparing data from two groups. ****$P < 0.0001$.

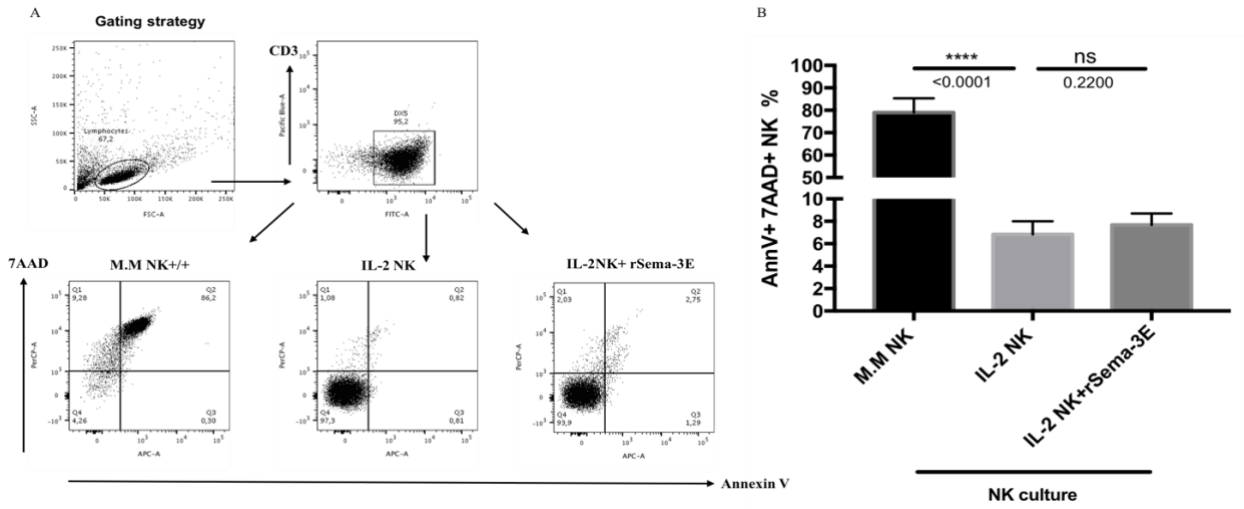


Figure 6.9. Recombinant Sema-3E did not affect activated NK cell survival. (A) The viability of IL-2-activated NK cells alone (IL-2 NK) cultured with recombinant Sema-3E for 4 days (IL-2 NK + rSema-3E) was assayed by flow cytometry. Cells were surface-stained with monoclonal antibodies against Annv (APC) and 7AAD (PerCP). NK cells cultured in plain mouse medium (M.M NK) were used as negative controls. **(B)** Cell double-positive population percentage was represented from all collected data. Data are shown as mean ± SEM, n = 3 independent experiments. *P* values shown are from two-tailed *t* test used for comparing two groups. ns, not significant; ****$P < 0.0001$.

6.3.6 PlexinD1 deficiency in BMDCs did not affect NK-cell migration towards DC CM.

Previously, I have reported that immature BMDCs express PlexinD1 (*420*); however, stimulation of immature WT BMDCs with LPS induced the downregulation of PlexinD1 (*420*). In addition, I observed that deficiency of PlexinD1 in immature (Figure 5.3) or LPS-stimulated BMDCs (Figure 5.8) regulated their function (evidenced by cytokine production). Therefore, I wanted to investigate the role of PlexinD1 produced by BMDCs in modulating IL-2-activated NK cell migration. To address this question, I isolated NK cells from the spleen of WT mice, then stimulated the cells in culture with IL-2 for 4 days. Next, I generated BMDCs from CD11-PlexinD1 cKO and WT mice. BMDCs were stimulated with LPS (1 μg/μL) for 24 h to acquire mature BMDCs. Conditioned medium of both immature and LPS-stimulated PlexinD1-deficient and WT BMDCs was collected for use in a transwell migration assay to measure the migration of IL-2-activated NK cells (**Figure 6.10**). Plain mouse medium was used as a negative control, while the conditioned medium from LPS-stimulated BMDCs served as a positive control. I observed no significant differences in IL-2-activated NK cell migration toward conditioned medium from

PlexinD1-deficient immature or LPS-stimulated splenic DCs compared to that of WT controls. (**Figure 6.10)**.

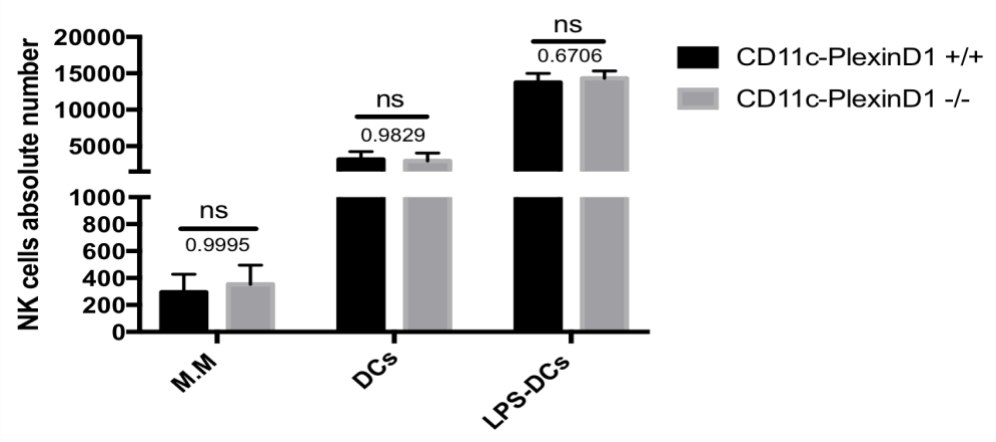


Figure 6.10. PlexinD1 deficiency in BMDCs did not affect NK cell migration. Transwell migration assay of IL-2-activated NK cell migration toward the conditioned medium of the immature and LPS-stimulated BMDCs generated from CD11c-PlexinD1 cKO (CD11c-PlexinD1-/-) and WT mice (CD11c-PlexinD1+/+). Plain mouse medium (M.M) was used as negative controls while the conditioned medium of LPS-stimulated BMDCs (LPS-DCs) served as a positive control. Data are shown as mean ± SEM, n = 4 independent experiments. *P* values shown are from two-way ANOVA used for comparing data from two groups. ns, $P > 0.05$.

Next, I wanted to investigate if PlexinD1 produced by BMDCs would affect DC chemokine production, thus modulating NK cell migration. Therefore, the conditioned medium of immature and LPS-stimulated BMDCs generated from both CD11c-PlexinD1 cKO and WT mice was collected and analyzed for the chemokines MCP-1, MIP-1α, IP-10, and KC/GRO) by MSD assay. I observed that Plexin-D1-deficient immature splenic DCs displayed similar chemokine levels to that of WT controls (**Figure 6.11)**. Similarly, LPS-stimulated splenic DCs also displayed similar production of chemokines with or without PlexinD1 expression (**Figure 6.11)**. These results suggest that the deficiency of PlexinD1 in BMDCs did not impact DC chemokine production function, thus modulated NK cell migration.

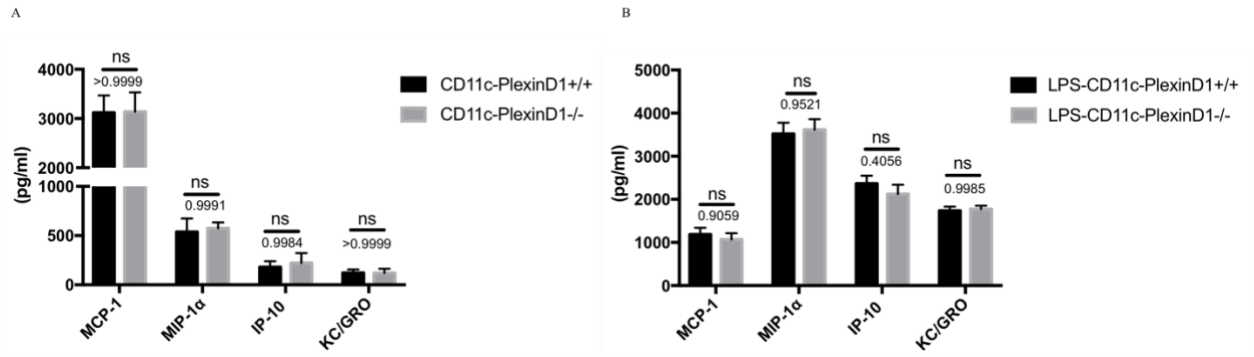


Figure 6.11. PlexinD1-deficient BMDCs express similar chemokine levels. BMDCs were generated from WT (CD11c-PlexinD1+/+) and CD11c-PlexinD1 cKO (CD11c-PlexinD1-/-) mice and cultured in GM-CSF medium for 7 days. On day 6, LPS (1 μg/μL) was introduced to the culture for 24 h to acquire matured DC. Conditioned medium was collected and used to measure the chemokines MCP-1, MIP-1α, IP-10, and KC/GRO by MSD assay in **(A)** immature BMDCs and **(B)** LPS-stimulated BMDCs. Data are shown as mean ± SEM of the calculated concentrations in pg/mL, adjusted for the dilution factor, for three independent experiments. *P* values from two-way ANOVA used for comparing data from two groups. ns, not significant.

6.3.7 PlexinD1 deficiency in LPS-stimulated splenic DCs diminished the ability to promote NK cell migration.

In Chapter 4, I found that immature WT splenic DCs express PlexinD1 **(Figure 4.4)**; however in Chapter 5, stimulation of immature WT splenic DCs with LPS induced upregulation of PlexinD1 mRNA **(Figure 5.6)**. In addition, I observed that deficiency of PlexinD1 in immature (**Figure 5.6**) or LPS-stimulated **(Figure 5.10)** splenic DCs upregulated IL-12/IL23p40 production that critical in NK cell proliferation and cytolytic activity (*469*). Such observations suggested a critical role of DC-expressed Plexind1 in modulating DCs functions. Therefore, I wanted to investigate the role of PlexinD1 expressed by splenic DCs in modulating IL-2-activated NK cell migration. Conditioned medium of WT and PlexinD1-deficient immature and LPS-stimulated splenic DCs were collected. The migration of IL-2-activated NK cells toward the conditioned medium of splenic DCs was examined in a transwell migration assay **(Figure 6.12)**. Plain mouse medium was used as a negative control, while the conditioned medium of LPS-stimulated BMDCs served as a positive control. I observed no significant differences in IL-2-activated NK cell migration toward conditioned medium from immature splenic DCs with or without PlexinD1.

However, conditioned medium from mature splenic DCs PlexinD1 cKO mice induced less NK cell recruitment compared to WT controls (n = 4, $P < 0.0001$).

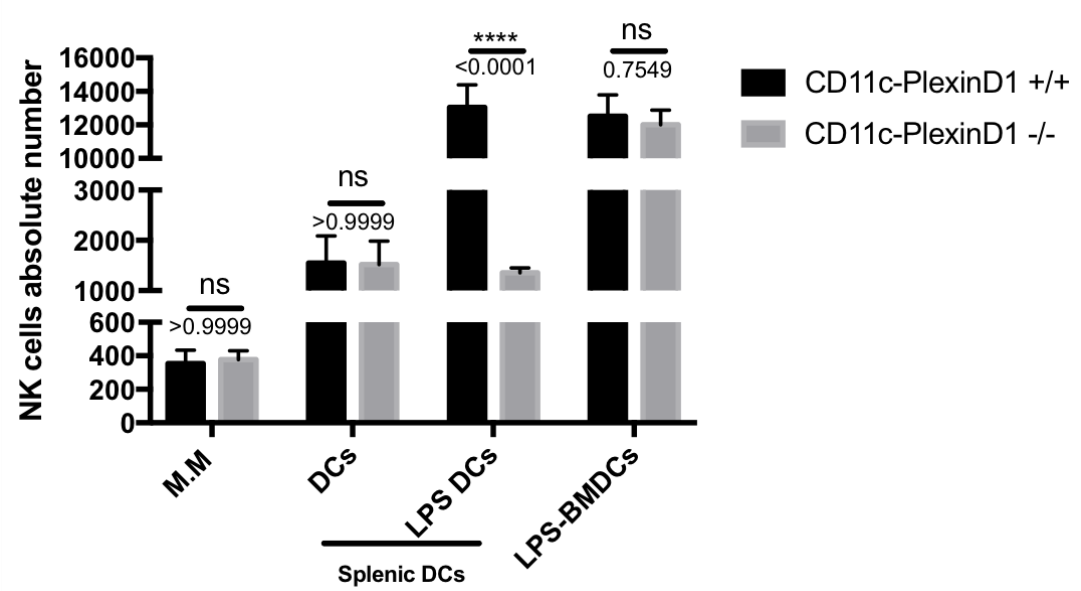


Figure 6.12. PlexinD1 expression promoted LPS-stimulated splenic DCs to enhance NK cell migration. Transwell migration assay of IL-2-activated NK cells toward the conditioned medium of immature and LPS-stimulated splenic DCs isolated from CD11c-PlexinD1 cKO (CD11c-PlexinD1-/-) and WT mice (CD11c-PlexinD1+/+). Plain mouse medium (M.M) was used as a negative control, and the conditioned medium of LPS-stimulated BMDCs (LPS-BMDCs) served as a positive control for migration. Data are shown as mean ± SEM, n = 4 independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups. ns, $P > 0.05$; ****$P < 0.0001$.

Next, I wanted to correlate these observed changes in NK cell migration with changes in DC chemokine production. Therefore, I use an MSD assay to analyze the conditioned medium of immature and LPS-stimulated splenic DCs isolated from both CD11c-PlexinD1 cKO and WT mice for the chemokines MCP-1, MIP-1α, IP-10, and KC/GRO (**Figure 6.13**). I observed no change in chemokine production in CM of immature splenic DCs from WT or KO lexinD1 mice (**Figure 6.13)** However, LPS-stimulated splenic DCs from PLexinD1 cKO released less MCP-1, MIP-1α, and IP-10, compared to WT controls (**Figure 6.13).** Considering that the CM from LPS-stimulated splenic DCs from PLexinD1 cKO induced less migration of NK cells compared to WT, it is tempting to speculate that these chemokines expression is mediated through Sema 3E action on its receptor, PlexinD1 on DCs culminating in NK cells migration. My results confirm that the

observed changes in NK cell migration correlate with changes to DC chemokine production, suggesting a mechanism of action.

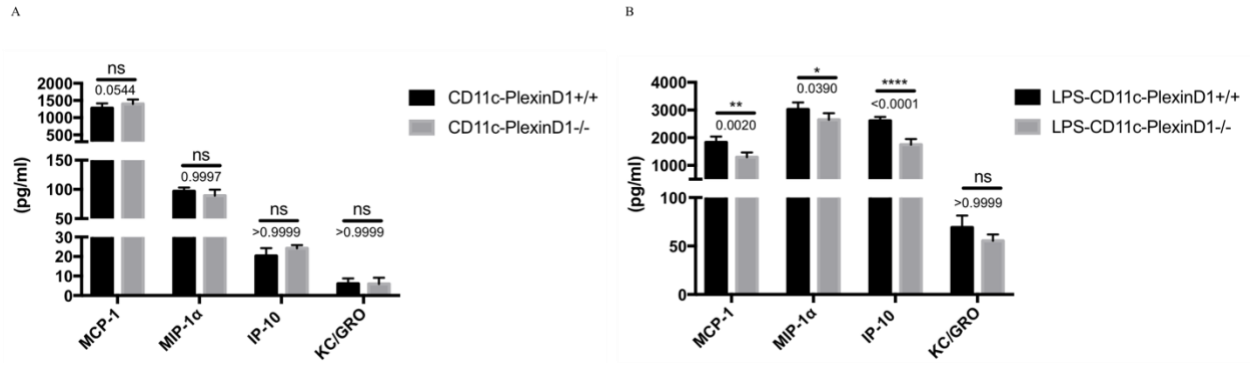


Figure 6.13. PlexinD1 deficiency in LPS-stimulated splenic DCs diminished MCP-1, MIP-1α, and IP-10 chemokine levels. Splenic DCs were isolated from WT (CD11c-PlexinD1+/+) and CD11c-PlexinD1cKO mice (CD11c-PlexinD1-/-) and cultured in GM-CSF medium for 24 h. After isolation, LPS (1 μg/μL) was introduced to the culture for 24 h to acquire matured DC. Conditioned medium was collected and the chemokines MCP-1, MIP-1α, IP-10, and KC/GRO were measured by MSD assay in **(A)** immature splenic DCs and **(B)** LPS-stimulated splenic DCs. Data are shown as mean ± SEM of the calculated concentrations in pg/mL, adjusted for the dilution factor, for three independent experiments. *P* values from two-way ANOVA used for comparing data from two groups. ns, not significant; **P* < 0.1; ***P* < 0.01; *****P* < 0.0001.

6.3.8 Splenic DCs expressed a lower level of PlexinD1 compare to BMDCs.

I have reported that immature BMDCs expressed the Sema-3E receptor PlexinD1 and that LPS stimulation *downregulated* PlexinD1 expression (*420*). In Chapter 4, I showed that immature splenic DCs expressed PlexinD1 (Figure 4.4) and that LPS stimulation of WT splenic DCs *upregulated* PlexinD1 mRNA expression (Figure 4.11). With respect to NK/DC crosstalk, PlexinD1-deficient BMDCs (immature or mature) showed no differences in NK cell migration (**Figure 6.10**) or chemokine production (**Figure 6.11**) compared to WT controls. In contrast, PlexinD1-deficient mature splenic DCs induced less NK cell migration (**Figure 6.12**), and this was associated with a reduction in the chemokines MCP-1, MIP-1α, and IP-10 (**Figure 6.13**). Here, I sought to compare the expression level of PlexinD1 in BM and splenic DCs, to possibly explain the observed different effect on NK cell migration. Interestingly, immature splenic DCs expressed

a significantly lower level of PlexinD1 compared to BMDCs as measured by qPCR (**Figure 6.14)**. Strikingly, LPS mature splenic DCs upregulated the expression level of PlexinD1 compared to immature splenic DCs (**Figure 6.14)**. However, LPS mature splenic DCs retained a relatively a lower level of PlexinD1 expression compared to BMDCs (**Figure 6.14)**. My results therefore suggest that the observed differences in NK/DC crosstalk between BM and splenic DCs can be attributed to differential expression levels of PlexinD1 in DCs of different tissue origins.

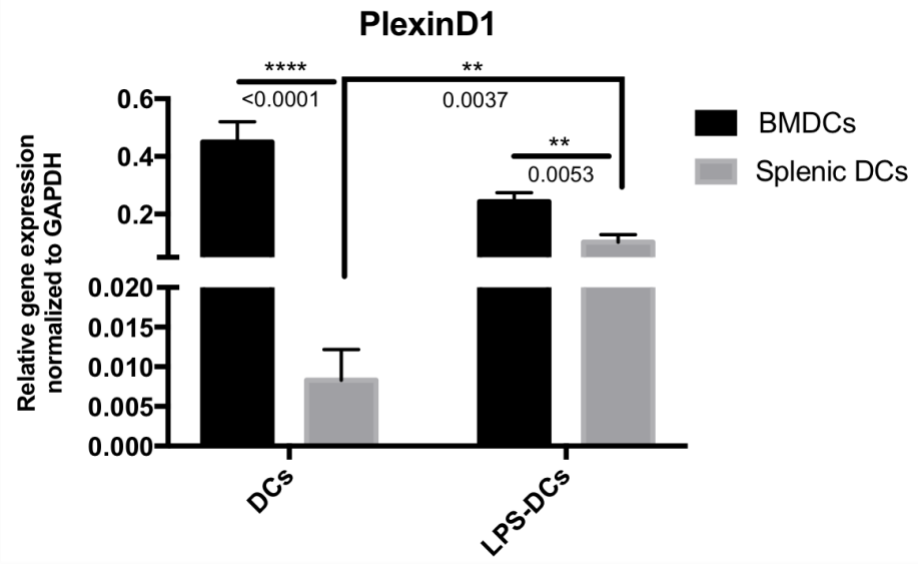


Figure 6.14. Splenic DCs expressed less PlexinD1 mRNA than BMDCs. BM and splenic DCs were isolated from WT C57BL/6 mice. RNA from these cells was used to measure PlexinD1 expression in immature (DCs), and LPS-stimulated DCs (LPS-DCs) by qPCR. Data are shown as mean ± SEM of four independent experiments. *P* values from two-way ANOVA used for comparing data from two groups. $^{**}P < 0.01$; $^{****}P < 0.0001$.

6.4 Discussion

In this study, I observed that blocking Sema-3E in the conditioned medium of immature WT BMDCs enhanced NK cell migration (**Figure 6.1**). Sema-3E-deficient immature but not LPS-matured BMDCs produced more MCP-1 and MIP-1α than WT controls (**Figure 4.10)**. Splenic DCs expressed a lower level of both Sema-3E (**Figure 4.4)** and PlexinD1 mRNA (**Figure 6.14**) than did BMDCs. Sema-3E-deficient immature or LPS-stimulated splenic DCs did not affect IL-2-activated NK cell migration (**Figure 6.5**) and produced similar amounts of MCP-1, MIP-1α, IP-10, and KC/GRO compared to WT splenic DCs (**Figure 6.6**). I observed that conditioned medium from PlexinD1-deficient immature BM (**Figure 6.10**) and splenic (**Figure 6.12)** DCs had no significant effect on NK cell recruitment or chemokine production (**Figure 6.13)**. However, conditioned medium from PlexinD1-deficient LPS-stimulated splenic DCs displayed lower IL-2-activated NK cell migration compared to WT controls (**Figure 6.12)** and associated with MCP-1, MIP-1α, and IP-10 downregulation (**Figure 6.13)**.

Previously, I had reported that immature BMDCs expressed Sema-3E and its receptor (PlexinD1 and NRP-1) mRNA, and that LPS stimulation downregulated their expression (*420*). Similarly, in Chapter 4 I showed splenic DCs expressed Sema-3E and PlexinD1 mRNA (**Figure 4.4**). Of interest, LPS stimulation downregulated Sema-3E in splenic DCs but upregulated PlexinD1 mRNA expression (**Figure 4.11**). However, the role of LPS in modulating Sema-3E and PlexinD1 expression in NK/DC crosstalk remains to be investigated. Of interest, LPS is known to activate macrophages to promote an M1-activated inflammatory phenotype (*497*) and downregulated NRP-1 and PlexinA1 expression in macrophages (*498*). NK cells interact with DCs through cell-cell contact and/or soluble factors. This interaction results in maturation, activation, and cytokine production by both NK cells and DCs. DCs mediate the activation of NK cells to contribute to innate immunity (*290*). In turn, activated NK cells provide signals essential for DC activation and maturation, thus enhancing the adaptive immune response (*290*). PlexinD1 displayed R-Ras GAP activity that required small GTPase Rnd2 to inhibit migration (*499*). Rnd2 binds PlexinD1, and inhibition of Sema-3E/PlexinD1-induced axon outgrowth required Rnd2 and R-Ras activity downregulation (*500*). It is possible that under inflammatory conditions, inflammatory signals such as LPS downregulated Sema-3E and PlexinD1 to allow NK cells and DCs to migrate to the target site, thus engaging in crosstalk and mediating the activation and/or maturation needed for proper immune responses. In turn, in the steady state, we can speculate that

the crosstalk between NK and DCs is controlled by the presence of Sema-3E and PlexinD1 to mediate immune homeostasis. On the other hand, activated NK cells can induce lysis of immature DCs and promote the survival of the most immunogenic DCs to empower immune responses (*319*). When the NK cell inhibitory signal is blocked by MHC class I, mature DCs are also susceptible to elimination (*181*). In colitis, the use of recombinant Sema-3E prevents apoptosis of the intestinal epithelium (*501*). Studies in the tumor setting showed that the Sema-3E/PlexinD1 axis is essential for cell viability, and the interruption of this axis resulted in cell apoptosis (*502*). These observations may also suggest that LPS downregulates Sema-3E and PlexinD1 to allow NK cells to migrate toward DCs and, further, to eliminate ineffective DCs, especially after infection (*503*). LPS is known to enhance DC maturation and thus upregulate antigen uptake ability and T-cell stimulatory function (*504*). Interestingly, I observed that splenic DCs expressed a lower level of Sema-3E (**Figure 4.4)** and PlexinD1(**Figure 6.14**) mRNA than did BMDCs. Such observations might suggest that DCs in the spleen are more mature than BMDCs in the steady state or after LPS stimulation and that this is the reason they expression lower levels of Sema-3E and PlexinD1 mRNA in response to their maturation status.

The guidance of leukocyte recruitment and their positioning in healthy or diseased tissues is mediated by the interaction of chemokines with their transmembrane-domain receptors, resulting in complex signaling events that govern leukocyte migration and elicit a chemotactic response with dynamic regulation (*505*). It was previously reported that recombinant Sema-3E suppressed neutrophil migration in vitro and in vivo by inhibiting CXCL8/IL-8 (*445*). Endothelial cells treated with Sema-3E have decreased FAK activity, a critical molecule that plays a role in modulating integrin-containing focal adhesions (*399*). Upon Sema-3E/PlexinD1 interaction, Ras GAP might play a role as potential signaling components mediating the early signaling events, regardless of the functional attractive vs repulsive outcome (*399*). However, the role of Sema-3E in regulating NK cell migration remains unclear. I observed that IL-2-activated NK cells cultured with recombinant Sema-3E displayed higher migration toward BMDC conditioned medium in a transwell migration assay (**Figure 6.8**). NK cells express several receptors for C, CC, CXC, and CX3C chemokines (*203*), suggesting that Sema-3E-NK cell long exposure upregulates chemokine receptors, thus increasing NK cell sensitivity to respond to specific chemokines. Sema-3E may also activate PTK required for NK cell migration, to control integrin adhesiveness, and for

chemotactic response (*210*). Future studies should investigate chemokine receptors expressed by Sema-3E-deficient NK cells and WT controls.

In vitro, it was previously reported that immature and LPS-stimulated BMDCs released soluble factors that induced different levels of chemotactic movement of IL-2-activated NK cells (*506*). However, the role of Sema-3E in modulating these factors remains to be investigated. Previously, I have reported that Sema-3E produced by immature but not LPS-stimulated BMDCs suppressed IL-2-activated NK cell migration (*420*). In Chapter 4, I also found that immature BMDCs significantly produced less MCP-1 and MIP-1α chemokines than Sema-3E deficient BMDCs (**Figure 4.10**). Unlike in BMDCs, Sema-3E produced by immature splenic DCs did not affect IL-2-activated NK cell migration (**Figure 6.5**) and displayed unchanged MCP-1, MIP-1α, IP-10 and KC/GRO chemokine production (**Figure 6.6**). The overall differences in my results between BM and splenic DCs suggest different roles in this context for Sema-3E derived from diverse DC environments, and this may relate to two possible factors: First, there may be tight regulation of Sema-3E in BMDCs and splenic DCs that result in different effects. For example, BMDCs and splenic DCs produce proteases such as cathepsins B, C, D/E, H, J, and L, which play a critical role in antigen digestion (*507*). This suggests that Sema-3E produced by splenic DCs has been modified (via intrinsic DC proteases) to different isoforms that are not effective in modulating NK cell migration (e.g., p87, p26). Second, Sema-3E may suppress the production of key chemokines that modulate NK cell migration. In turn, once Sema-3E is downregulated upon LPS stimulation, DCs possibly upregulate similar levels of key chemokines including MCP-1, MIP-1α, IP-10, and KC/GRO, resulting in the observed similar NK cell migration toward DC conditioned medium. The similar migration and chemokine production observed in splenic DCs may also suggest that steady-state and LPS-stimulated DCs in fact have the same maturation level with no associated changes to NK/DC crosstalk. Future studies should involve the blocking of MCP-1, MIP-1α, IP-10, and KC/GRO in the conditioned medium of immature Sema-3E-deficient BMDCs to examine this role in NK migration. Of interest, the blocking of Sema-3E produced by immature BMDCs increased IL-2-activated NK cell migration (**Figure 6.1).** Others have previously reported that the Sema3E/PlexinD1 axis modulated small GTPase activity in several cell types, thus mediating the disassembly of actin filaments disassembly through the activation of p21-activated kinase (PAK) (*399*). My observation suggests that blocking Sema-3E may alter the assembled actin filaments remodeling, therefore enhancing NK cell migration. Future studies

should determine the regulation of actin filaments in NK cells upon exposure to Sema-3E for different periods of time.

Recent studies have shown that inhibition of migration is mediated by PlexinD1 that displayed R-Ras GAP activity, which required the small GTPase Rnd2 (*508*). Next, I used PlexinD1-deficient CD11c cKO mice to examine the role of DC-expressed PlexinD1 in modulating NK migration in NK/DC crosstalk. Interestingly, I reported that PlexinD1 expression was upregulated in splenic DCs upon LPS stimulation (but not immature DCs) (**Figure 6.14)** and resulted in the suppression of IL-2-activated NK cell migration (**Figure 6.12**). I observed that the lower levels of NK cell migration prompted by the conditioned medium of PlexinD1-deficient LPS-stimulated splenic DCs were associated with less production of the chemokines MCP-1, MIP-1α, and IP-10 (**Figure 6.13**). Interestingly, I observed LPS-stimulated splenic DCs of CD11c-PlexinD1 cKO expressed a higher level of Sema-3E when compared to WT control; however, NRP-1 expression remained unaffected (**Figure 5.12**). This suggests that LPS stimulation may upregulate Sema-3E only under specific conditions (e.g., deficiency of PlexinD1 in DCs). Therefore, upregulated Sema-3E may bind to PlexinD1 on other cells (paracrine mode) and result in the inhibition of NK cell migration. It is possible that the Sema-3E/PlexinD1 paracrine axis may impact the production of DC-derived chemokines via modulation of R-Ras GAP activity. However, neither NK cell migration nor chemokine production was affected by the absence of Plexin D1 in conditioned media of immature splenic DCs (**Figure 6.12**). I observed that PlexinD1 deficiency in immature BMDCs (**Figure 5.11**) or splenic DCs (**Figure 5.12**) expressed a comparable level of Sema-3E expression compared to WT controls. This could explain why there was no difference in the ability of the conditioned medium of PlexinD1-deficient immature BMDCs (**Figure 6.10**) or splenic DCs (**Figure 6.12**) in the chemotactic responses of IL-2-activated NK. This suggests that the expression of Sema-3E is independent of PlexinD1 expression. Alternatively, Sema-3E may bind either to NRP-1 co-receptor expressed by DCs or to PlexinD1 expressed by other cells to mediate its effector functions.

In conclusion, my results demonstrate a novel role for the Sema-3E/PlexinD1 axis in the regulation of IL-2-activated NK cell migration. I have shown that Sema-3E and PlexinD1 differentially modulate IL-2-activated NK cell migration. The expression of Sema-3E and PlexinD1 is tightly regulated in both BM and splenic DCs both in the steady-state and upon LPS stimulation. This regulation modulates DC chemokine production, which is associated with the

regulation of IL-2-activated NK cell migration (**Figure 6.15**). Future work should establish the regulation of NK/DC crosstalk via Sema-3E and PlexinD1 that could affect DCs maturation and homeostasis in steady-state and/or disease models in vivo. Studying the role of the Sema-3E/PlexinD1axis in regulating NK cell migration may provide a new way to manipulate NK cell effector functions and/or migration in clinical settings.

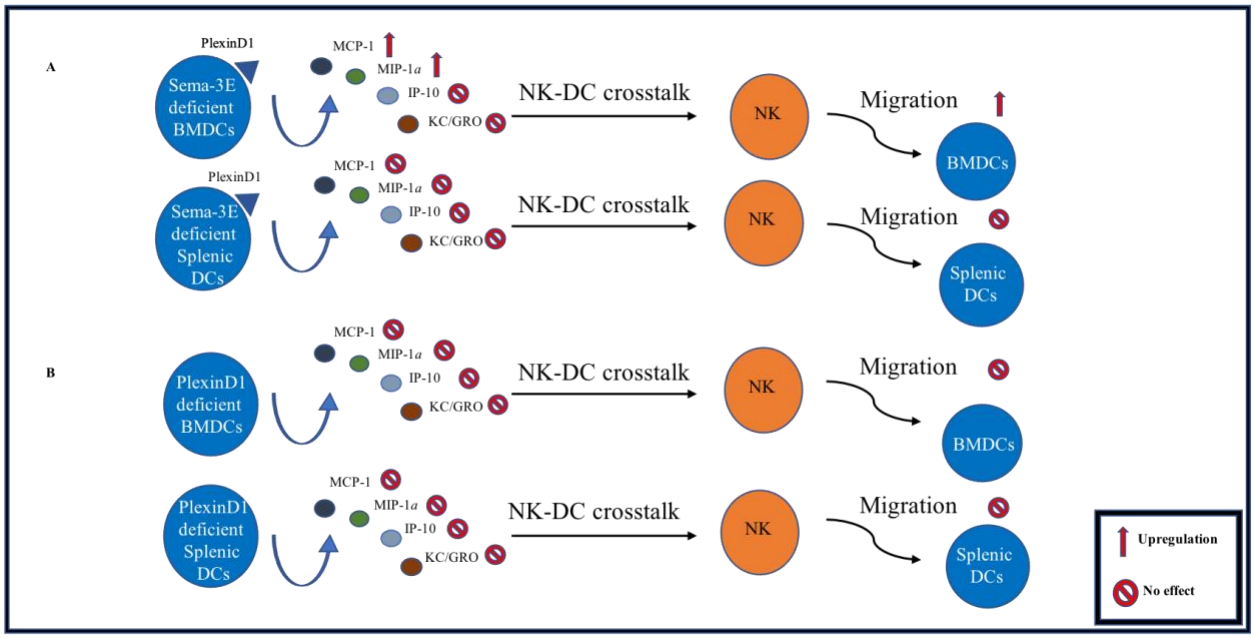


Figure 6.15. Sema-3E deficiency upregulated NK migration. The role of Sema-3E **(A)** and PlexinD1**(B)** were examined in NK migration in NK-DC crosstalk. Through modulation of the chemokines expressed by DCs, the Sema-3E modulates NK cell migration.

Chapter 7: General Discussion and Future Directions

7.1 General discussion

My current thesis revealed novel role of Sema-3E in regulating NK (Chapter3), DCs (Chapters 4,5) and NK migration in NK-DC crosstalk (Chapter 6). I reported that Sema-3E regulated NK cell number (**()** and cytolytic machinery (**()** and (**Error! Reference source not found.**). The regulation of Sema-3E and PlexinD1 expression in DCs and other cell types could impact the DCs activation states. For example, I reported that Sema-3E-deficiency altered the CD11c (**Figure 4.1**) and MHC class II (**Figure 4.6**) DC phenotype. The IL-12/IL23p40 production was higher in Sema-3E-deficient immature (**Figure 4.3**) and LPS-matured BMDCs (**Figure 4.10**). In contrast, Sema-3E-deficient immature splenic DCs had downregulated IFN-γ, IL-12/IL-23p40, and IL-6 but upregulated IL-10 production (**Figure 4.8**). However, in Sema-3E-deficient LPS-stimulated splenic DCs, only IFN-γ production was upregulated (**Figure 4.13**)**.** The use of DC-specific PlexinD1 revealed further that splenic DCs phenotype **(Figure 5.5),** and IL12/IL-23p40 (**Figure 5.6**) or IFN-γ (**Figure 5.10**) productions are independent of PlexinD1 while BMDCs phenotype (**Figure 5.2**)**,** and IFN-γ, IL-12/IL-23p40, IL-6, or IL-10 productions (**Figure 5.3**) **(Figure 5.8**) are dependent on PlexinD1. In NK/DC crosstalk, I have reported the blocking of Sema-3E produced by WT immature BMDCs enhanced NK cell migration (**Figure 6.1**). Of interest, Sema-3E-deficient immature BMDCs produced more MCP-1 and MIP-1α than WT controls (**Figure 4.10**), which was associated with higher NK migration. However, PlexinD1-deficient LPS-stimulated splenic DCs displayed lower IL-2-activated NK cell migration (**Figure 6.12**) associated with MCP-1, MIP-1α, and IP-10 downregulation (**Figure 6.13**).

Sema-3E regulates different systems, including the nervous system, cardiovascular development, cancers, and the immune system (*509*). Many evidences have supported a role of Sema-3E in regulating the immune system under different various diseases and inflammatory conditions (*396, 398, 421, 452, 453, 463*). However, I considered the importance of studying the role of Sema-3E in regulating NK cell and DC phenotype and function in the steady state or after stimulation to provide a better understanding of the role of Sema-3E in early immune responses and the implications of this in different disease models. I found there were fewer NK cells in the spleen and BM in Sema-3E deficient mice in the steady state and after IL-2 stimulation. Moreover, NK cells isolated from Sema-3E-deficient mice showed less toxicity and cytokine release targeting RMA-s cells in vitro. Such interesting observations enhance our knowledge and set up a baseline

for NK cell number and function upon which to study the effect of Sema-3E. These differences observed in NK cell number and function should be considered carefully when studying the role of NK cells in cancers or infectious diseases using Sema-3E-deficient mice. Consequently, conclusions from cancer or infectious disease studies should distinguish the direct effect of Sema-3E on NK cells vs the indirect effect of Sema-3E on NK cells mediated by the disease. For example, it was shown that deleting either Sema-3E or PlexinD1 in human metastatic carcinoma cells inhibited human carcinoma metastatic potential when injected into mice (*492*). In contrast, Sema-3E overexpression in cancer cells enhanced tumor invasiveness, trans endothelial migration, and metastatic spreading, even though tumor vessel formation was inhibited, and tumor growth reduced in mice (*492*). Such studies showed the importance of Sema-3E in modulating carcinoma metastasis and tumor invasiveness. Of interest, Sema-3E can be a double-edged sword that promotes tumor survival (*502*) and tumor immunity (*439*). It will be important to distinguish the different mechanism that Sema-3E use to favour tumor survival versus tumor immunity. However, the effects of Sema-3E on immune cells in general, and NK cells in particular, have not been investigated in tumor models, making Sema-3E a potential target to restore NK cell cytotoxicity and cytokine release (downregulated at the baseline) in the tumor setting. Treating tumor with an exogenous source of Sema-3E that is specifically engineered to target NK cells in tumor microenvironment may be a possible way to boost NK antitumor immunity. Alternatively, the adoptive transfer of Sema-3E WT NK cells may restore NK cell cytotoxicity in tumor microenvironment.

Next, I discovered the role of Sema-3E in modulating DC phenotype and function in two different modes: autocrine (BMDCs) and paracrine (splenic DCs) signaling. I found that Sema-3E-deficient mice altered the CD11c phenotype in BMDCs and the MHC class II phenotype in splenic DCs. Interestingly, IL-12/IL23p40 production was upregulated in immature and LPS-matured BMDCs of Sema-3E-deficient mice. However, immature splenic DCs downregulated IL-12/IL-23p40 but upregulated IL-10 production. Such observations indicate the Sema-3E outcomes are determined by the context (i.e., the biological environment), maturation status, and manner of signaling (autocrine or paracrine). Based on these results, I am speculating that Sema-3E deficiency in autocrine signaling exhibited inflammatory DCs whereas Sema-3E deficiency in paracrine signaling showed tolerogenic DCs. For example, Sema3E was found to protect the mice

from allergic asthma via inhibiting eosinophilic inflammation, serum IgE levels, and the Th2/Th17 cytokine response (*398*).

On the other hand, Sema-3E enhanced protective immunity by modulating DC function and T cell subsets in chlamydial infection (*453*). Therefore, the use of Sema-3E-deficient mice to study DCs in different disease models should consider the different modes of Sema-3E signaling that might result in different DC outcomes (inflammatory or tolerogenic). Therefore, Sema-3E is a potential approach to target DCs to mediate either inflammatory responses or tolerogenic functions depending on the disease conditions.

NK/DC interaction contributes to both innate and adaptive immunity (*290*). Generally, semaphorins have been shown to enhance either attracting or repelling signals based on where their receptors and co-receptors are expressed and the context of the responding cells. Functionally, semaphorins are more likely to support target cell repulsion than attraction (*370, 380*). In NK/DC crosstalk (in the context of NK cell migration), I found that Sema-3E produced by immature BMDCs suppressed NK cell migration (*420*) and is associated with the upregulation of MCP-1 and MIP-1α. However, PlexinD1-deficient LPS-stimulated splenic DCs displayed lower NK cell migration, and this was associated with MCP-1, MIP-1α, and IP-10 downregulation. NK/DC interactions lead to NK activation, DC maturation, or apoptosis (*290*). Cancer immunotherapy aims to shift the balance from cancer cell escape or equilibrium to elimination (*327, 328*). Therefore, the ideal antitumor immune response depends on a complex network interplay where DCs, NK cells, and $CD8^+$ and $CD4^+$ T cells mediate the interface between innate and adaptive immunities and the capacity to connect and regulate immune effector functions (*294*). In infection, NK/DC crosstalk plays a critical role in controlling different viral infections such as HIV, HCV, and MCMV infection (*341*). For example, HIV infection induces IL-10 production by DCs, which supports the resistance of immature DCs to elimination by NK cells (*324*). Of interest, during the early stages of MCMV infection, $Ly49H^+$ NK cells prevent the release of IFN-α/β to immunosuppressive levels, in turn preventing the loss of splenic $CD8\alpha^+$ DCs that is commonly induced by MCMV infection (*192*).

A malfunction in NK/DC crosstalk results in defects in either (or both) NK cells or DCs (*341*). Upon LPS stimulation, NK cells and DCs produce IL-15. This IL-15 is trans-presented by DCs, or NK cells self-present it, to mediate NK cell activation (*510, 511*). Thus, NK cells enhance the secretion of IFN-γ that required IL-15 to be presented in trans to NK cells through IL-15Rα

(*512, 513*). It is possible that Sema-3E plays a critical role in IL-15 regulation, thus modulating NK/DC crosstalk. This will have direct translational implications for NK cells in DC-based immunotherapies activity.

Understanding the role of Sema-3E in NK/DC crosstalk could be used as a new tool to control NK/DC interaction, thus modulating NK cell activation or DC maturation or elimination in different disease models. Collectively, my observations of Sema-3E regulation in either NK cells, DCs, or NK/DC crosstalk provide a physiological basis for the Seme-3E/PlexinD1 axis to maintain immune homeostasis condition in the steady state.

In summary, our observations suggest different scenarios for the modulation of NK cell and/or DC phenotype and function by the Sema-3E/PlexinD1 axis (**Figure 7.1**). First, NK cells express both Sema-3E and PlexinD1. The possible autocrine axis enhances NK cell proliferation and upregulates NK cell cytotoxicity and cytokine release. Second, DCs express Sema-3E and PlexinD1. The autocrine axis upregulates DCs phenotype (CD11c) and modulates DC cytokine and chemokine production independent of their maturation phenotype. Third, the paracrine axis modulates DC maturation phenotype and, therefore, cytokine and chemokine production. These observations suggested that the Sema-3E/PlexinD1 axis regulates DCs maturation at steady-state, and the loss of this axis enhances DCs maturation, therefore modulates DCs cytokine and chemokine productions. Both second and third scenarios might suggest that Sema-3E regulated DCs phenotype and function at different stages that are independent of one another. For example, Sema-3E deficiency did not affect BMDCs phenotype (**Figure 4.2**), yet BMDCs upregulated IL-12/IL23p40 production (**Figure 4.3**). On the other hand, Sema-3E deficient splenic DCs exhibited alter MHC class II (**Figure 4.6**), whereas IFN-γ, IL12/IL23p40, and IL-6 were downregulated (**Figure 4.8**). Finally, the modulation of DCs phenotype and function, through autocrine or paracrine effects of the Sema-3E/PlexinD1 axis, regulates NK cell migration in NK/DC crosstalk.

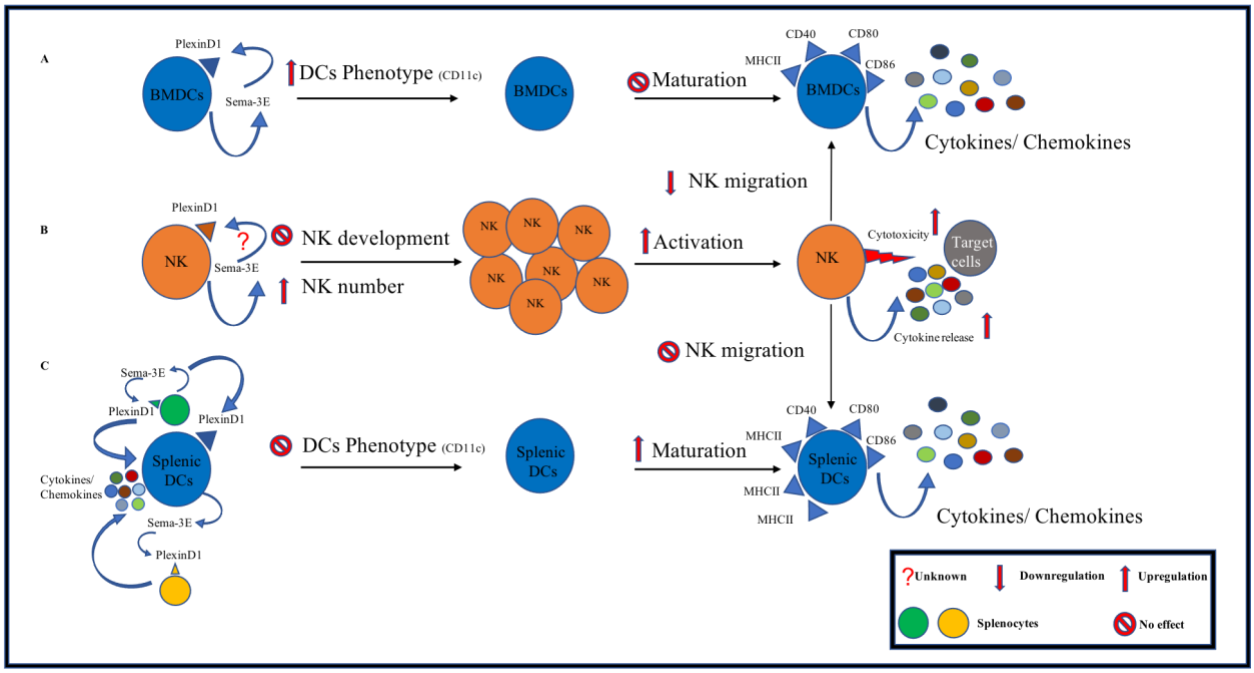


Figure 7.1. The Sema-3E/PlexinD1 axis modulates NK cell and DC phenotype and function. The Sema-3E/PlexinD1 axis was examined in **(A)** BMDCs, **(B)** NK cells, and **(C)** splenic DCs. Through modulation of the phenotype and function of each individual cell type, the Sema-3E/PlexinD1 axis modulates NK cell migration in NK/DC crosstalk.

7.2 Limitations and future directions

The data presented in this thesis support a crucial role of the Sema-3E/PlexinD1 axis in NK cell and DC phenotype and function. However, some limitations should be considered to interpret the results and for future studies.

1. **NK cell number:** In the spleen, the NK cell number is very limited (2-5%); therefore, studying nonactivated (resting) NK cell phenotype and function is challenging and requires many mice to achieve sufficient statistical power. Alternatively, using an NK cell line (such as NK101) (*514*) could be an attractive NK source to detail NK cell functions in vivo and in vitro. Next, I aimed to study the role of Sema-3E in NK cell function in vitro including Genzyme B and TNF-*a* expression. Furthermore, the addition of recombinant Sema-3E to IL-2 NK culture will be examined in vitro to determine the ability of Sema-3E to restore NK cytokine and cytotoxicity responses. Future studies should also focus on studying role of Sema-3E in NK cell survival, and proliferation in vitro. Such studies will provide a better understanding of how Sema-3E modulates NK cell number and function. NK cells key chemokines receptors such as CXCR2, CXCR3, CXCR4, and CXCR5 remain to be investigated to determine the role of Sema-3E in modulating NK migration via chemokines receptors.
2. **Cytokine and chemokine changes:** I observed changes in cytokines and chemokines attributed to the Sema-3E/PlexinD1 axis. Confirmation of these observations using qPCR should be considered in future studies to confirm the cytokine and chemokine expression at mRNA level. Furthermore, measuring other cytokine release in NK function such as TNF-*a*, IL-10, IL-13, and IL-15 should be investigated as well to determine the role of Sema-3E in modulating NK expressed key cytokines. The blocking the critical chemokines produced by DCs and possibly modulating NK cell migration should be investigated in future studies to delineate their role in NK migration.
3. **Mechanistic studies:** Our studies focus mainly on characterizing NK cell and DC phenotype and function in vitro after disturbing/disrupting the Sema-3E/PlexinD1 axis. However, the mechanisms underlying the effect remain to be investigated. Therefore, these key observations will be made more convincing by including some mechanistic studies (such as the activation of crucial signaling pathways or RNAseq assays) to support these observations.

4. **New KO models:** Future studies should consider the use of different KO mice models. NK-PlexinD1 conditional KO, NK-Sema-3E/PlexinD1 conditional KO, or DC-Sema-3E conditional KO will be a great tool to study NK and DCs function defect and whether they are regulating each other' functions or not.

Supplementary Material

S1. The role of the Sema-3E/PlexinD1 axis in regulating breast cancer tumor growth and antitumor immunity

Despite the observation that autocrine Sema-3E/Plexin D1 signaling was kept intact in transplanted 4T1 cells, 4T1 tumors grew significantly larger in Sema-3E-deficient BALB/c mice compared to WT controls **(supplementary Figure S.5)**. The larger tumors were associated with a bigger volume and weight **(supplementary Figure S.6).** However, lung metastasis appeared to be not affected **(supplementary Figure S.5),** suggesting that Sema-3E has minimal or no role in the spread of 4T1 tumor cells from their initial growth place toward other organs. Of interest, others have reported that Sema-3E and PlexinD1 are highly expressed in metastatic cancer cells and correlate with human tumor metastatic progression (*502*). Deleting either Sema-3E or PlexinD1 in human metastatic carcinoma cells inhibited human carcinoma metastatic potential when injected into mice (*492*). In contrast, Sema-3E overexpression in cancer cells enhanced tumor invasiveness, transendothelial migration, and metastatic spreading, even though tumor vessel formation was inhibited, and tumor growth reduced in mice (*492*). I therefore wanted to examine the expression of Sema-3E, PlexinD1, and NRP-1 in the 4T1 tumor microenvironment in Sema-3E-deficient and WT hosts to have a better understanding of why no lung metastases were detected. Our data showed no significant differences in Sema-3E expression in 4T1 tumors in Sema-3E-deficient hosts compared to WT. In contrast, the expression of PlexinD1 and NRP-1 (Sema-3E receptors) were downregulated in the 4T1 tumors of Sema-3E-deficient hosts compared to those of WT hosts **(supplementary Figure S.7).** All the above observations might suggest the following: (i) the equal expression level of Sema-3E detected in both tumors was expressed initially by 4T1 cells; (ii) the lower levels of the Sema-3E receptors PlexinD1 and NRP-1 resulted in less metastatic potential; and (iii) Sema-3E and PlexinD1 expression levels are not sufficient in either Sema-3E-deficient or WT hosts to mediate cell migration and thus lung metastasis.

Interestingly, IL-10 mRNA expression levels were upregulated in the 4T1 tumors of Sema-3E-deficient mice compared to WT controls **(supplementary Figure S.8).** IL-10 is known to promote tumor cell proliferation and metastasis through immunosuppression effects (*515*). IL-10 suppresses ILs-1α, -1β, -6, -8, -12, and -18, TNF-α, and GM-CSF in T cells and macrophages as well as IFN-γ in activated Th cells and peripheral blood mononuclear cells (PBMCs) (*516*). The deficiency of IL-10 is linked with an increase in IL-1 production, promoting tumor growth in mice

(*517*). Others have previously reported that Sema-3E deficiency in DCs resulted in higher expression of PD-L1, PD-L2, and IL-10 (*453*). In Chapter 4, I have observed that Sema-3E deficiency in splenic DCs upregulated IL-10 production (**Figure 4.8**). This suggests that systemic deficiency of Sema-3E upregulates IL-10 production in tumor microenvironment and therefore promotes larger tumor growth in Sema-3E-deficient mice.

NK cells have the unique ability to kill tumor cells without any existing priming and expansion of specific clones (*1*). To better understand the role of NK cells in the 4T1 tumor microenvironment in Sema-3E-deficient and WT host mice, I depleted NK cells in WT and Sema-3E-deficient mice. I observed that NK cell depletion in WT mice led to a significant acceleration of tumor growth than the nondepleted group (**supplementary Figure S.9**). Similarly, NK cell depletion in Sema-3E deficient mice led to a significant acceleration of tumor growth compared to the nondepleted group **(supplementary Figure S.10)**. Our results suggested a critical role for Sema-3E in modulating 4T1 tumor growth in vivo, possibly due to the lack of NK cell effector functions.

To conduct a parallel experiment in PlexinD1-deficient hosts, I used syngeneic E0771 tumors cells transplanted into CD11c-PlexinD1 cKO mice and observed no significant differences in tumor growth between CD11c-PlexinD1 cKO and WT mice **(supplementary Figure S.11).** This observation suggests that PlexinD1 expressed by DCs is not involved in the Sema-3E/PlexinD1 signaling required to modulate tumor growth.

Next, I analyzed the tumor microenvironment for primary immune cells including NK, T, and NKT cells in flow cytometry. I observed that the Sema-3E-deficient 4T1 tumor microenvironment contained fewer $CD3^+$ T cells than the WT environment, whereas NK and NKT cells remained unaffected **(supplementary Figure S.12).** This observation suggests that Sema-3E has the potential to modulate antitumor immunity. In contrast to the flow cytometry results, preliminary data from cytometry by time of flight (CyTOF) showed that the 4T1 tumors in a Sema-3E-deficient host displayed more NK cells, $CD4^+$ or $CD8a^+$ T cells, or Treg compared to WT controls **(supplementary Figure S.13).** The different readouts from flow cytometry and in CyTOF may be due to different means of tumor size, processing the samples or the antibodies used. These results remain to be confirmed by repeat of the experiment using the same antibody clones in flow cytometry or CyTOF.

The interaction of Sema-3E and PlexinD1 is known to inhibit angiogenesis (*410*); therefore, the nonimmune component of the Sema-3E/PlexinD1 axis is worth investigating. Our preliminary data showed no differences in blood vessel formation in 4T1 tumors grown in Sema-3E-deficient or WT hosts **(supplementary Figure S.14).** Overall, our preliminary results point to a role of the Sema-3E/PlexinD1 axis in regulating breast cancer antitumor immunity.

S2. Mouse genetic strains and biology differences

It is known that different genetic backgrounds display different readouts (the Jackson lab). Throughout my studies, BALB/c mice were used to investigate the role of Sema-3E in modulating NK cell and DC phenotype and function, whereas C57BL/6 mice were used to investigate the role of PlexinD1-deficient CD11c cells in similar experiments. It is possible that this difference in genetic background may account for some differences I observed. I therefore compared the BALB/c and C57BL/6 genetic backgrounds in both WT and Sema-3E deficient genotypes. First, I determined the baseline (WT) expression levels of Sema-3E, PlexinD1, and NRP-1 mRNA in steady-state BMDCs of BALB/c versus C57BL/6 mice and observed no differences **(supplementary Figure S.15).** However, I observed that steady-state BMDCs of WT BALB/c mice displayed higher CD40, CD80, CD86, and MHC class II co-stimulatory molecules MFI compared to those of WT C57BL/6 mice **(supplementary Figure S.16).** This difference carried over into the Sema-3E knockout genotype **(supplementary Figure S.17).** This trend was retained after LPS-stimulated BMDC maturation in both WT **(supplementary Figure S.18)** and Sema-3E deficient mice **(supplementary Figure S.19).**

Similar to BMDCs, I observed that steady-state splenic DCs of BALB/c and C57BL/6 mice expressed similar levels of Sema-3E, PlexinD1, and NRP-1 **(supplementary Figure S.20).** Splenic DCs also showed the same MFI trends in the production of the co-stimulatory molecules CD40, CD80, CD86, and MHC class II with BALB/c levels higher than C57BL/6 levels in WT immature splenic DCs **(supplementary Figure S.12)**, in Sema-3E-deficient immature splenic DCs **(supplementary Figure S.22)**, in WT mature splenic DCs **(supplementary Figure S.23)** and in LPS-stimulated mature splenic DCs **(supplementary Figure S.24)**. Collectively, our results indicate a significant variation in DC co-stimulatory molecules that might impact DC biological function.

Finally, as a supplement to the 4T1 tumor data in Sema-3E-deficient mice (**supplementary Figures S.5–S.10**), which were carried out in the BALB/c background, I transplanted E0771 cells into WT and Sema-3E-deficient mice in a C57BL/6 background. Interestingly, I observed no E0771 tumor growth in Sema-3E deficient mice while WT controls did **(supplementary Figure S.25).** This observation may be related to previous reports that E0771 cells result in a poorly metastatic tumor compared to the 4T1 cells (*518*). Future studied should test different cell lines in this model, such as RMA-s, to confirm that the C57BL/6 genetic background is not the reason for the observed lack of tumor growth in Sema-3E-deficient mice.

Supplementary Figures

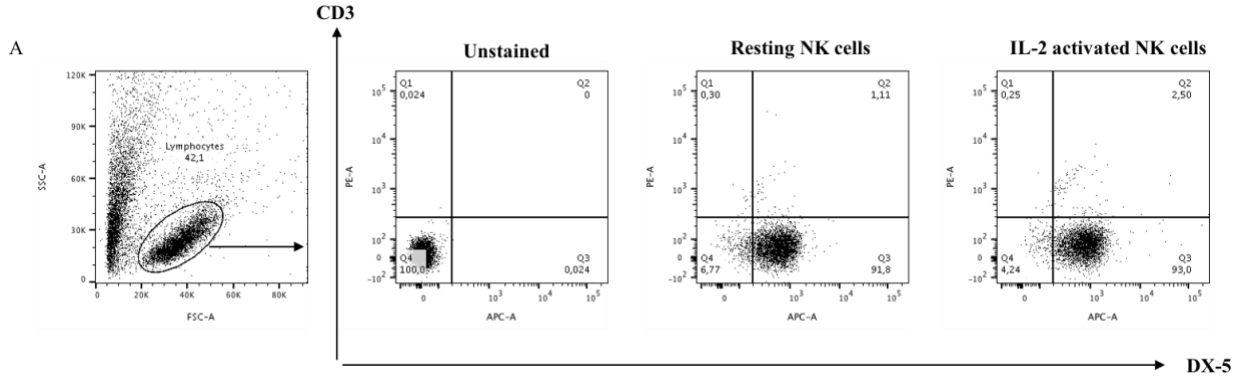


Figure S.7.1. Purity of NK cells sorted by magnetic beads. (A) Flow cytometry assay of splenic NK cells. Cells were isolated from spleen and stained with monoclonal antibodies against surface DX-5 APC and CD3 PE monoclonal Abs. Resting NK cells were used immediately after isolation while activated NK cells were cultured with IL-2 (1000 U/mL) for 4 days.

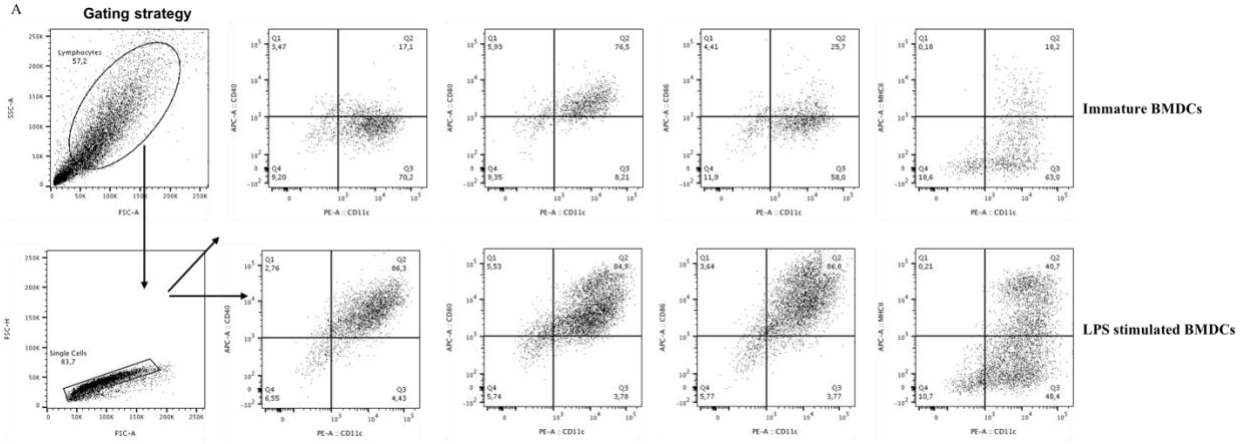


Figure S.7. 2. Phenotype of BM-derived DC preparations. (A) Flow cytometry assay of BMDCs. Cells were stained with monoclonal antibodies against CD40, CD80, CD86, and MHCII surface markers after cultured in GM-CSF medium. On day-8, lipopolysaccharide (LPS; 1 μg/μL) was introduced in the culture for 12-hours to acquire matured DC phenotype.

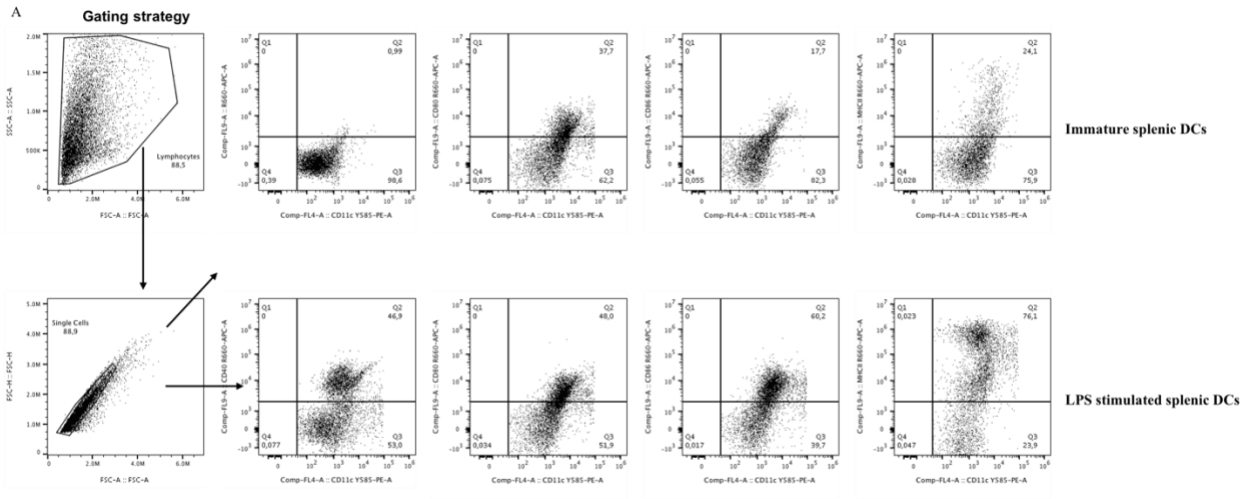


Figure S.7.3. Phenotype of isolated splenic DC preparations. (A) Flow cytometry assay of splenic DCs. Cells were isolated from spleen and stained with monoclonal antibodies against CD40, CD80, CD86, and MHCII markers after cultured in GM-CSF medium for 12 hours. Lipopolysaccharide (LPS; 1 µg/µL) was introduced in the culture for 12-hours to acquire matured DC phenotype.

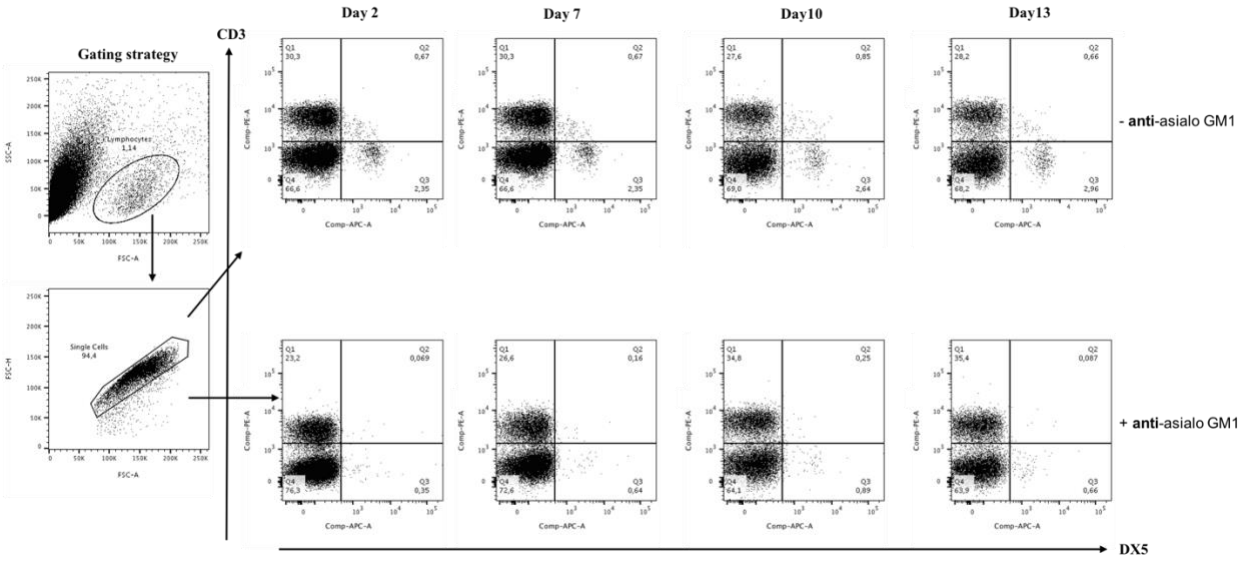


Figure S.7.4. Depletion of NK cells by anti-asialo GM1 antibody. (A) Flow cytometry assay of blood NK cells. WT **(**Sema-3E+/+) BALB/C mice were given an i.p. injection of anti-ASGM1 (50 µL) for four consecutive time points (days 3, 7, 10, and 13). On each mentioned day, mice blood was collected and Stained with monoclonal antibodies against DX-5 (APC) and CD3 (PE) antibodies to confirm the depletion of NK cells. Stained cells were analyzed by FACSCanto II (BD Biosciences) using Diva software, and data were analyzed using FlowJo software.

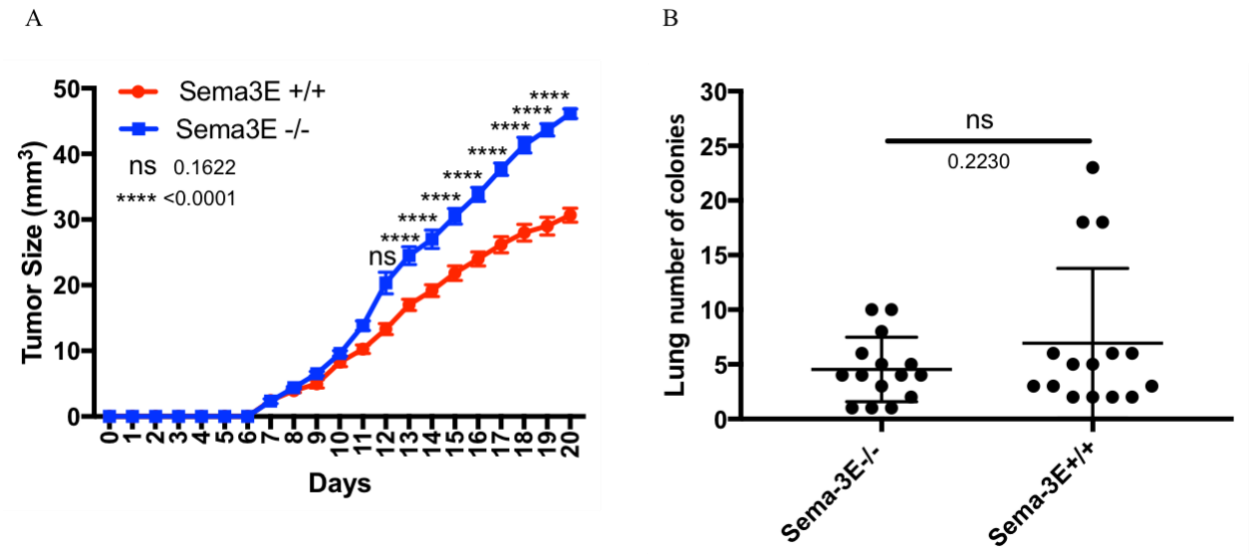


Figure S.7.5. 4T1 breast cancer cells grown larger tumors in Sema-3E-deficient mice. (A) $0.01x10^6$ 4T1 cells were injected into the breast fat pad of WT (Sema-3E+/+) (red) and Sema-3E-deficient (Sema-3E-/-) mice (blue). Tumor size was monitored daily. 4T1 tumor grew bigger in the Sema-3E-/- mice when compared to the Sema-3E+/+ mice. **(B)** Lungs of WT (Sema-3E+/+) and the Sema-3E-deficient (Sema-3E-/-) were collected and processed for lung metastatic assay. Data are shown as mean ± SEM, n = 8. *P* values are from two-way ANOVA used for comparing two groups: ns, $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

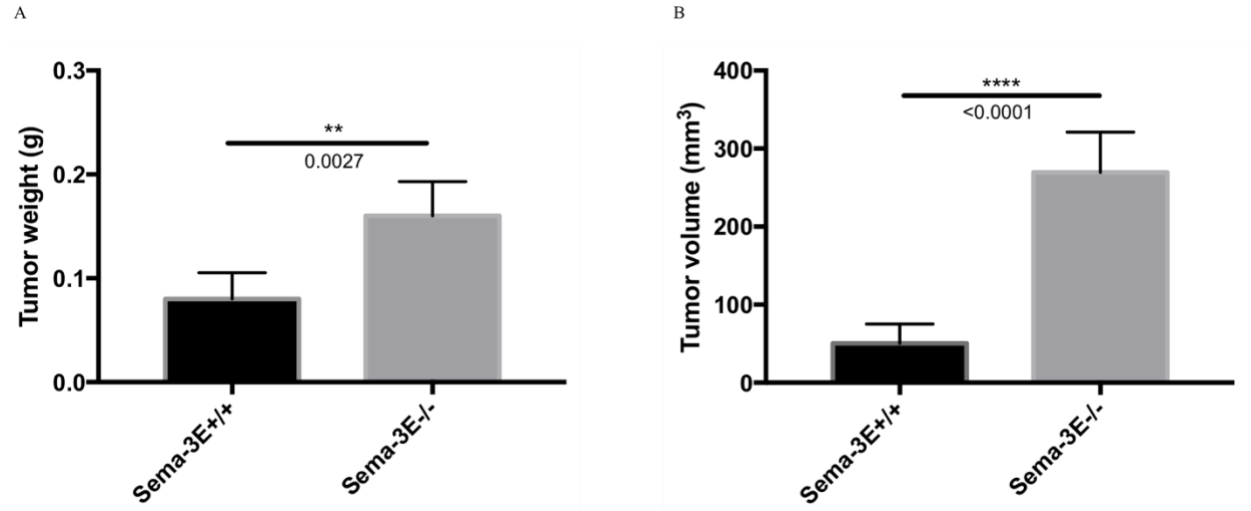


Figure S.7.6. 4T1 breast cancer cells transplanted to Sema-3E-deficient mice resulted in higher tumor weight and volume. (A) 4T1 cells were injected ($0.01x10^6$ cells per injection) into the mammary fat pad of WT (Sema-3E+/+) or Sema-3E-deficient (Sema-3E-/-) mice. Tumor weight was measured at the time of tumor collection. **(B)** Tumor diameter was measured with vernier calipers and volume was calculated from this. Data are shown as mean ± SEM, n = 8 independent experiments (5 mice/ group). *P* values are from two-tailed *t* tests used for comparing two groups: ** $P < 0.01$, **** $P < 0.0001$.

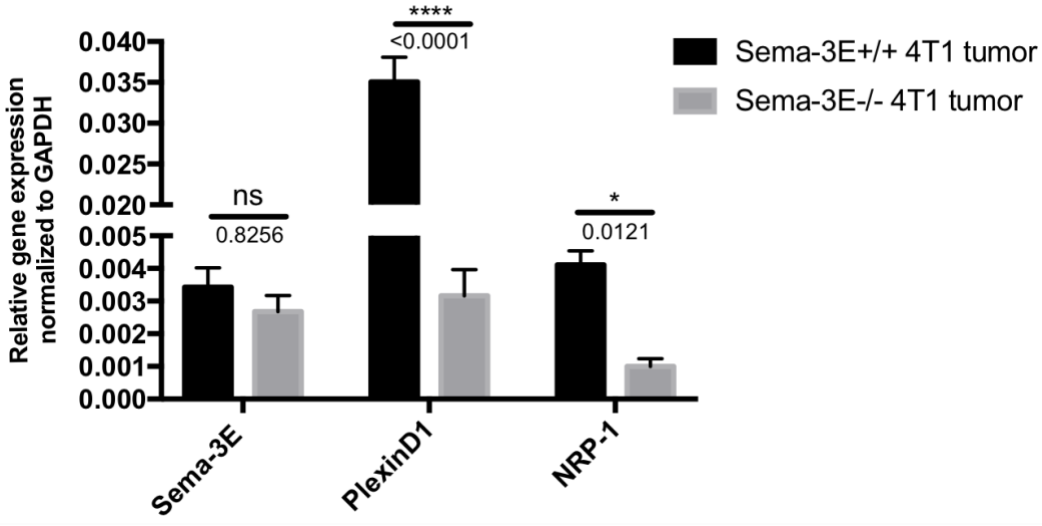


Figure S.7.7. 4T1 breast cancer cells transplanted to Sema-3E-deficient mice expressed less PlexinD1 and NRP-1 mRNA. Tumors were harvested and digested using collagenase IV. RNA from digested cells was used to measure Sema-3E, PlexinD1 and NRP-1 expression in tumors grown in WT (Sema-3E+/+ 4T1 tumor) and Sema-3E-deficient

(Sema-3E-/- 4T1 tumor) mice using qPCR. Data are shown as mean ± SEM, n = 2 independent experiments (4 mice/ group). *P* values are from two-way ANOVA used for comparing two groups: * $P < 0.1$, **** $P < 0.0001$.

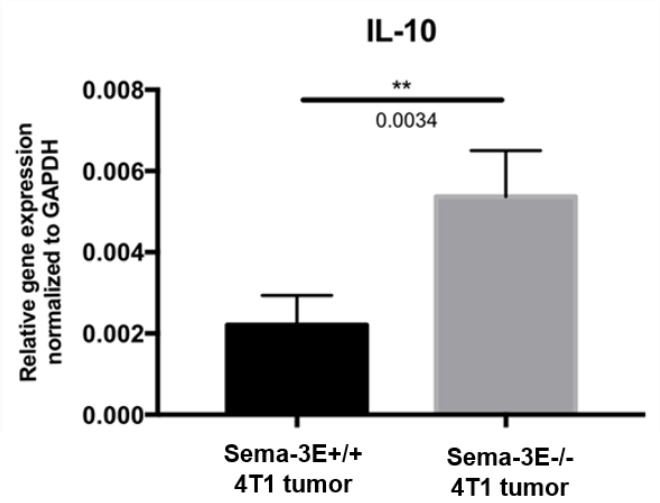


Figure S.7.8. 4T1 breast cancer cells transplanted to Sema-3E-deficient mice expressed higher IL-10. RNA from digested cells was used to measure IL-10 expression by qPCR in tumors grown in WT (Sema-3E+/+ 4T1 tumor) and Sema-3E-deficient (Sema-3E-/- 4T1 tumor) mice. Data are shown as mean ± SEM, n = 2 independent experiments (4 mice/ group). *P* values are from two-way ANOVA used for comparing two groups: ** $P < 0.01$.

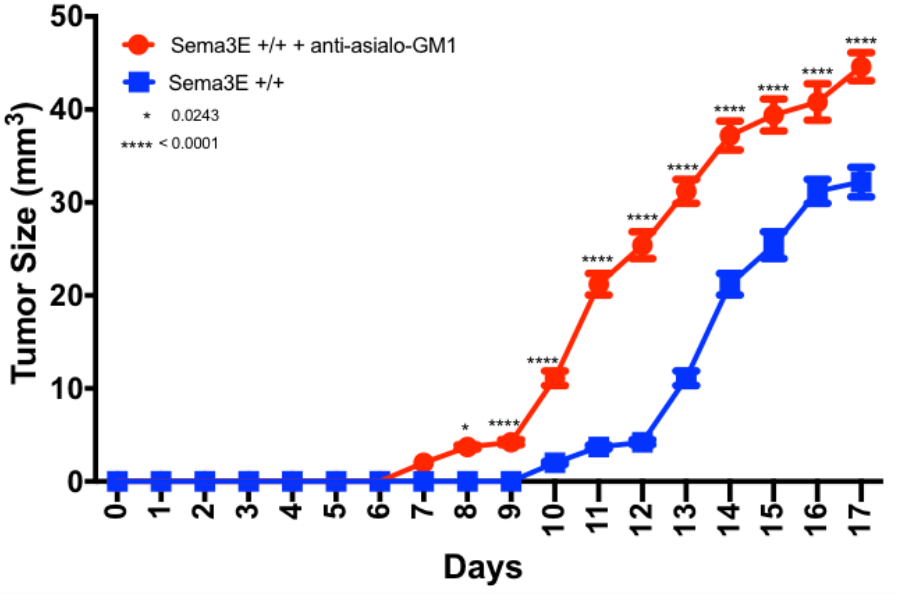


Figure S.7.9. 4T1 breast cancer cells grown faster in WT NK depleted mice. $0.01x10^6$ 4T1 cells were injected into the breast fat pad of the WT NK depleted mice (Sema-3E+/+ + anti-asialo-GM1) (red) and the WT nondepleted mice (Sema-3E+/+) (blue). Tumor size was monitored daily. 4T1 tumor grew bigger in the WT mice + anti-asialo-GM1 when compared to the WT mice. WT (Sema-3E+/+) BALB/c mice were injected with anti-ASGM1 (50 µl) for three consecutive time points (days 3, 7, 10, and 13) after 4T1 cells injection to deplete NK cells and tumor size was monitored daily. 4T1 tumor grew faster in the WT (Sema-3E+/+) + anti-ASGM1 when compared to WT (Sema-3E+/+)**.** Data are shown as mean ± SEM, n = 8. *P* values are from two-way ANOVA used for comparing two groups: ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

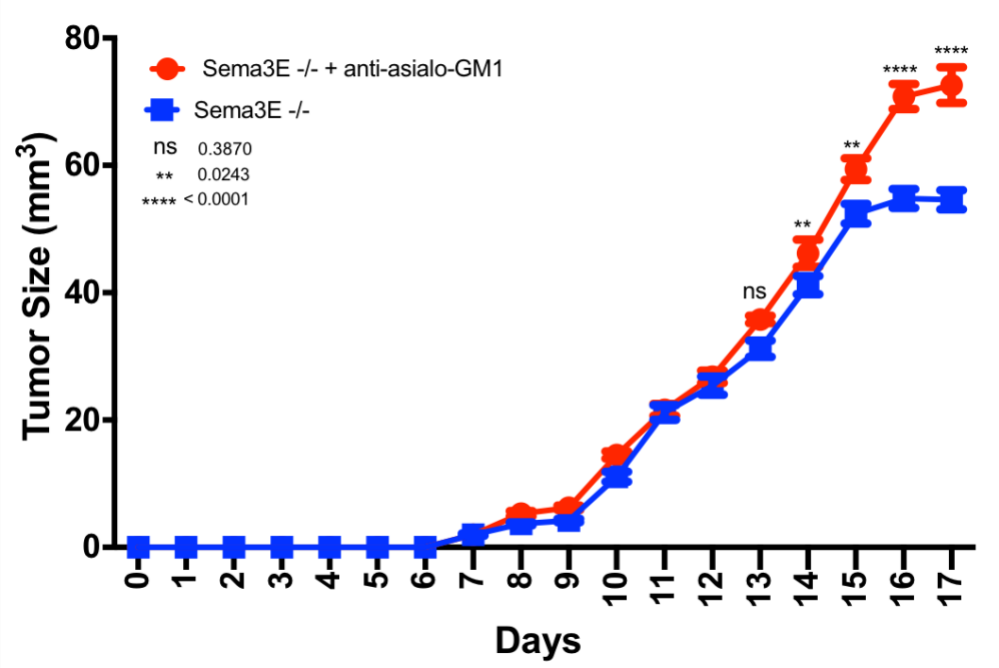


Figure S.7.10. Increased growth of 4T1 breast cancer cells after NK cell depletion in Sema-3E-deficient mice. $0.01x10^6$ 4T1 cells were injected into the breast fat pad of the Sema-3E deficient NK depleted mice (Sema-3E-/- + anti-asialo-GM1) (red) and the Sema-3E deficient nondepleted mice (Sema-3E-/-) (blue). Tumor size was monitored daily. 4T1 tumor grew bigger in the Sema-3E deficient + anti-asialo-GM1 when compared to the Sema-3E deficient mice. Sema-3E deficient (Sema-3E-/-) BALB/c mice were injected with anti-ASGM1 (50 µl) for three consecutive time points (days 3, 7, 10, and 13) after 4T1 cells injection to deplete NK cells and tumor size was monitored daily. 4T1 tumor grew faster in the WT (Sema-3E+/+) + anti-ASGM1 when compared to WT (Sema-3E+/+)**.** Data are shown as mean ± SEM, n = 8. *P* values are from two-way ANOVA used for comparing two groups: ns $P > 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

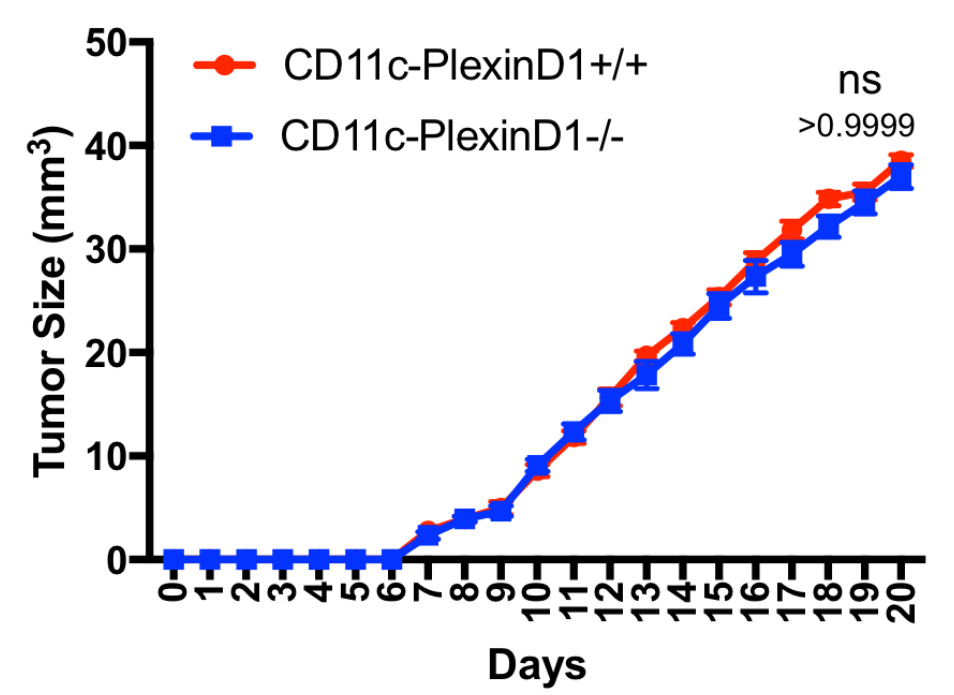


Figure S.7.11. E0771 breast cancer cells transplanted to CD11c-PlexinD1 cKO mice showed similar growth curve to WT hosts. (A) E0771 cells were injected ($0.1x10^6$ cell per injection) into the mammary fat pad of WT (CD11c-PlexinD1+/+; red) and CD11c-PlexinD1 cKO (CD11c-PlexinD1-/-; blue) mice. Tumor size was monitored daily. Data are shown as mean ± SEM, n = 1 from a single experiment (4 mice/ group). *P* values are from two-way ANOVA used for comparing two groups: ns $P > 0.05$.

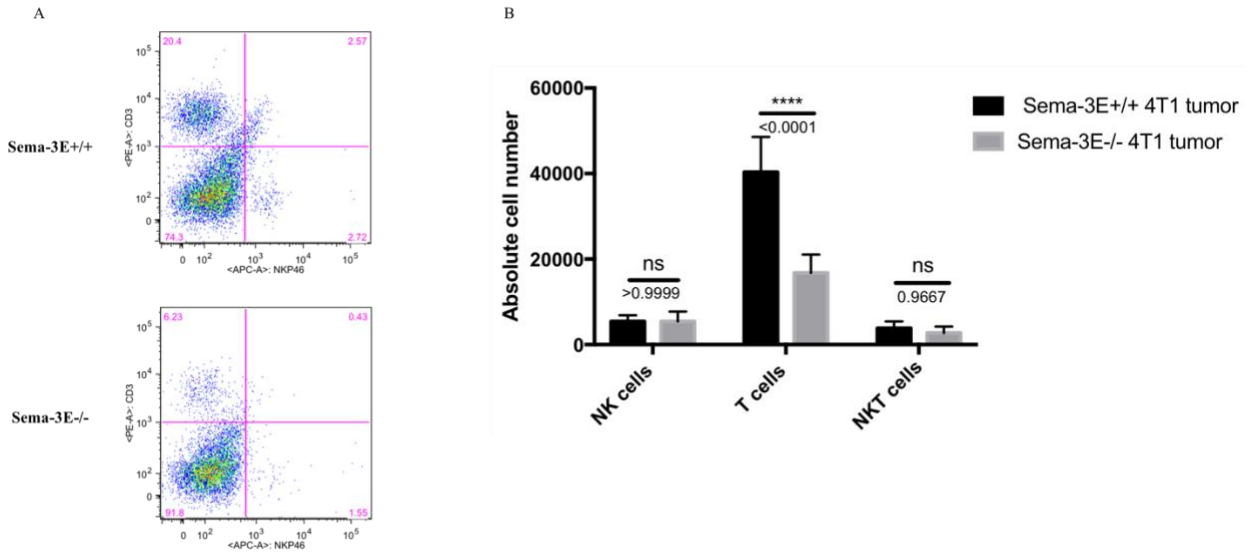


Figure S.7.12. 4T1 cells transplanted to Sema-3E-deficient mice grown tumors with fewer T cells. Tumors were harvested and digested using collagenase IV. Digested cells were analyzed by flow cytometry **(A).** Cells were surface-stained with monoclonal antibodies against NKP46 APC and CD3 PE. (B) absolute cell number of T, NK and NKT cells in digested 4T1 tumor are plotted from all collected data. Data are shown as mean ± SEM, n = 3 independent experiments (1 mouse/group). *P* values are from two-way ANOVA used for comparing two groups. ns $P > 0.05$; ****$P < 0.0001$.

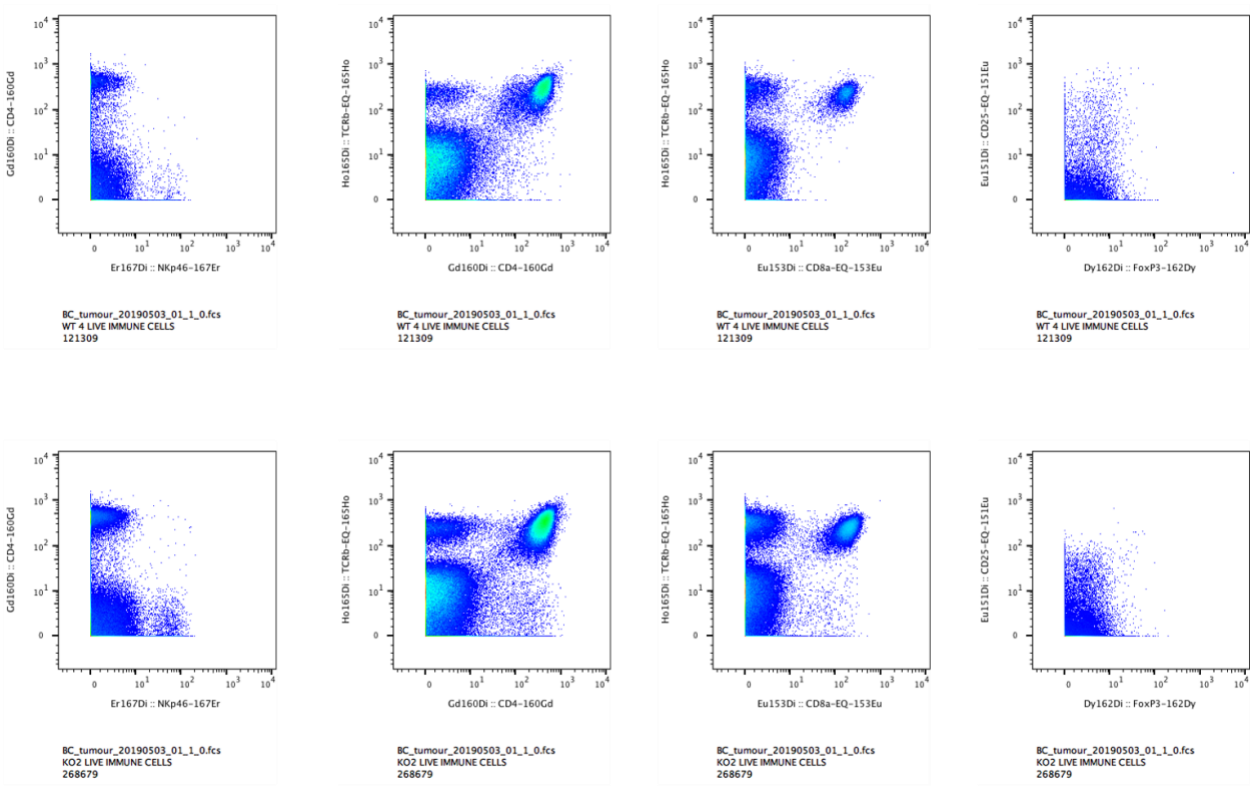


Figure S.7.13. 4T1 cells transplanted to Sema-3E-deficient mice tumors showed higher NK, T, and Treg cell numbers. Tumors were harvested and digested using collagenase IV enzyme. Digested cells were analyzed in CyTOF using monoclonal antibodies against NKP46 (NK cells), CD4 and CD8a (T cells) and FoxP3 (Treg). n = 1 independent experiment (4 mice/ group).

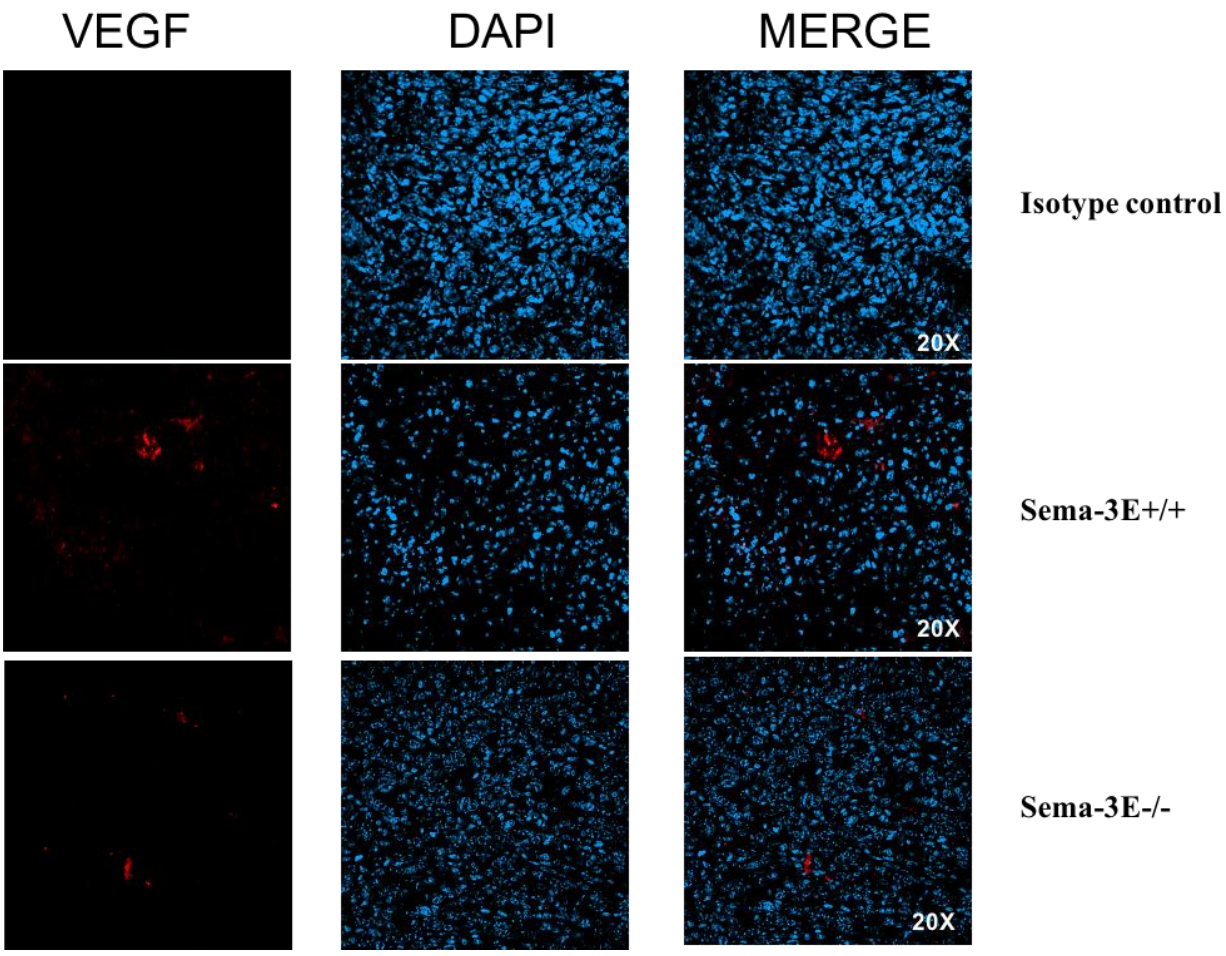


Figure S.7.14. Sema-3E did not affect VEGF expression in 4T1 tumors. Vascular endothelial growth factor (VEGF) was detected by immunofluorescence staining on tumor sections obtained from WT (Sema-3E+/+) and Sema-3E-deficient (Sema-3E-/-) mice. VEGF expression was visualized after incubation of tissue sections with FITC-labeled anti-VEGF specific antibody and counter staining of nuclei with DAPI. IC: Isotype control, Scale bars: 20 μm. (n=1 independent experiment, 1 mouse per group).

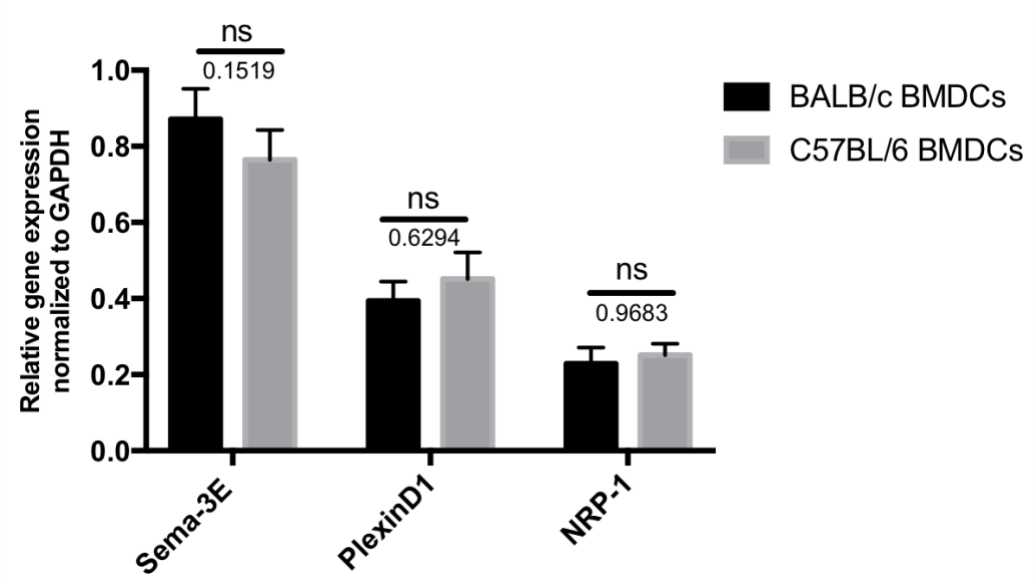


Figure S.7.15. BALB/c and C57BL/6 mice expressed similar levels of Sema-3E, PlexinD1, and NRP-1 mRNA. Immature BMDCs were generated from BALB/c and C57BL/6 mice. RNA from these cells was used in a qPCR assay to measure Sema-3E, PlexinD1, and NRP-1 expression. Data are shown as mean ± SEM of four independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups. ns, $P > 0.05$.

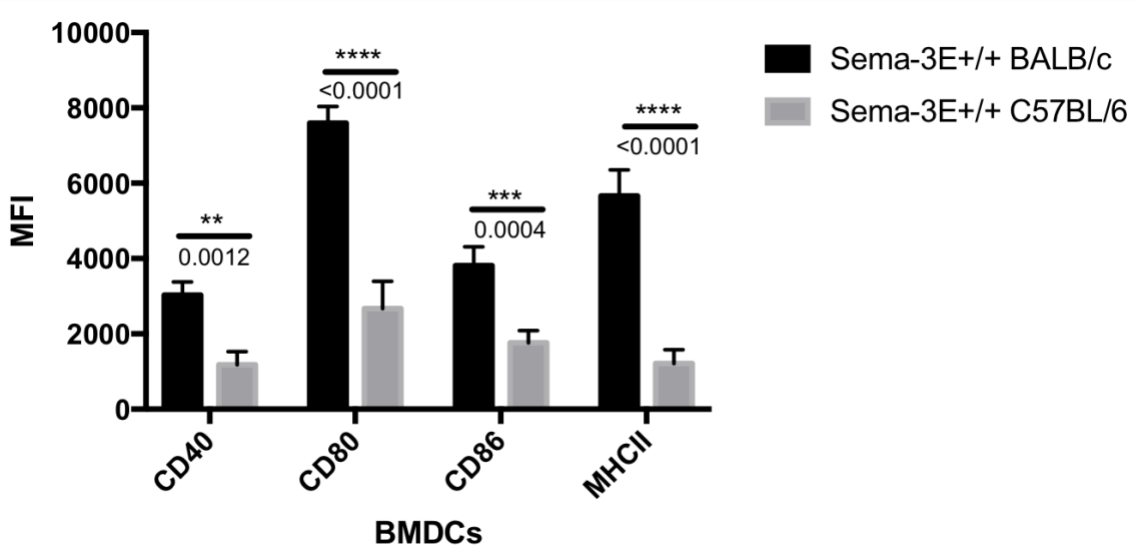


Figure S.7.16. BALB/c BMDCs expressed higher levels of co-stimulatory molecules than C57BL/6 BMDCs. BMDCs were generated from WT BALB/c (Sema-3E+/+ BALB/c) and WT C57BL/6 (Sema-3E+/+ C57BL/6) mice. Cells were collected and surface-stained with monoclonal antibodies against CD40 APC, CD80 APC, CD86 APC, and MHC class II APC. MFI of CD40 APC, CD80 APC, CD86 APC, and MHC class II were plotted from $CD11c^+$

cell data. Data are shown as mean ± SEM, n = 4 independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups. ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

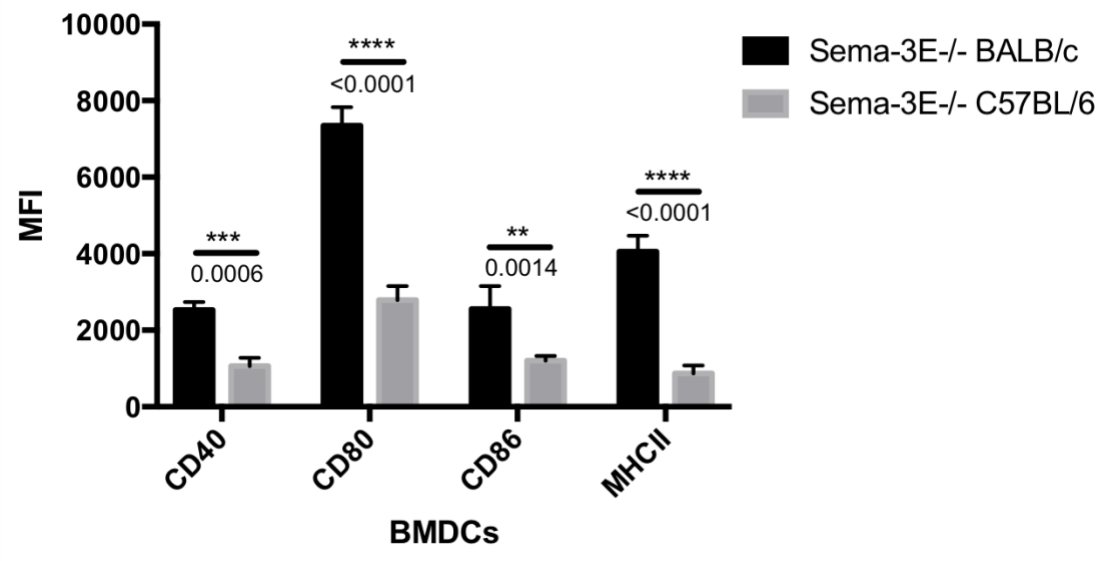


Figure S.7.17. BMDCs from Sema-3E-deficient BALB/c mice expressed higher levels of co-stimulatory molecules than Sema-3E-deficient C57BL/6 BMDCs. BMDCs were generated from Sema-3E-deficient BALB/c (Sema-3E-/- BALB/c) and Sema-3E-deficient C57BL/6 (Sema-3E-/- C57BL/6) mice. Cells were collected and surface-stained with monoclonal antibodies against CD40 APC, CD80 APC, CD86 APC, and MHC class II APC. MFI of CD40 APC, CD80 APC, CD86 APC, and MHC class II were plotted from $CD11c^+$ cell data. Data are shown as mean ± SEM, n = 4 independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups. ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

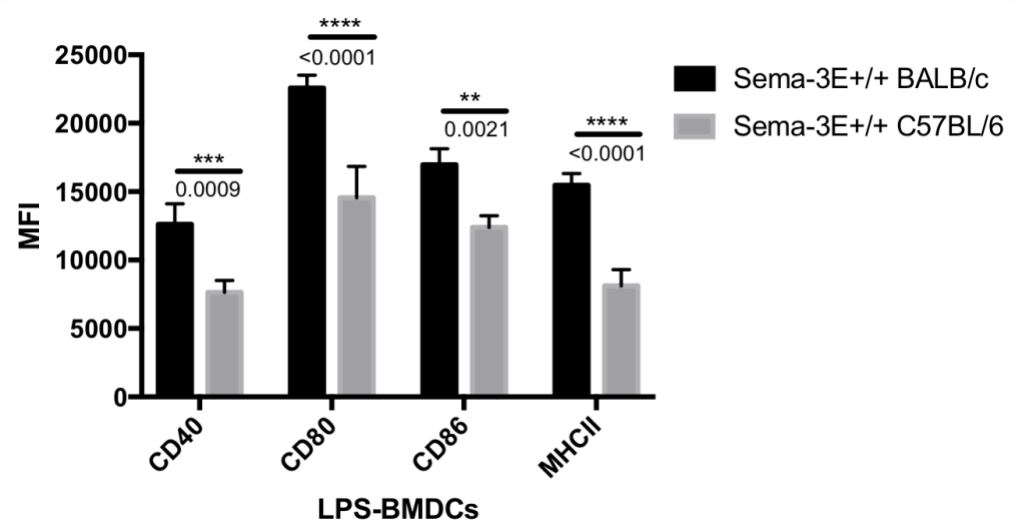


Figure S.7.18. LPS-stimulated BMDCs from BALB/c mice expressed higher levels of co-stimulatory molecules than C57BL/6 BMDCs. BMDCs were generated from WT BALB/c (Sema-3E+/+ BALB/c) and WT C57BL/6 (Sema-3E+/+ C57BL/6) mice. BMDCs were cultured in GM-CSF medium for 7 days. On day 6, LPS (1 μg/μL) was used for 24-hours to acquire matured BMDC phenotype. Cells were collected and surface-stained with monoclonal antibodies against CD40 APC, CD80 APC, CD86 APC, and MHC class II APC. MFI of CD40 APC, CD80 APC, CD86 APC, and MHC class II were plotted from $CD11c^+$ cell data. Data are shown as mean ± SEM, n = 4 independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups: $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

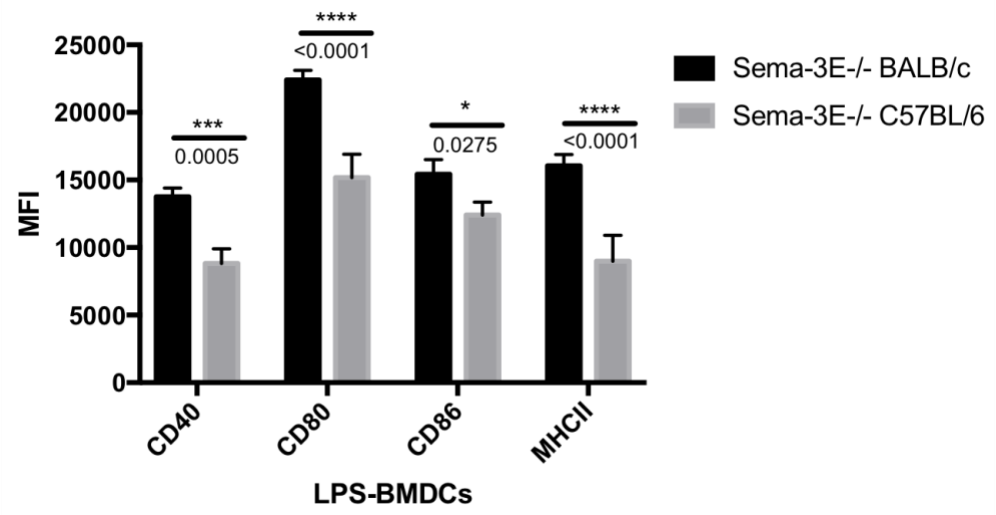


Figure S.7.19. LPS-stimulated BMDCs of Sema-3E-deficient BALB/c mice expressed higher levels of co-stimulatory molecules than C57BL/6 BMDCs. BMDCs were generated from Sema-3E deficient BALB/c (Sema-3E-/- BALB/c) and Sema-3E deficient C57BL/6 (Sema-3E-/- C57BL/6) mice. BMDCs were cultured in GM-CSF

medium for 7 days. On day 6, LPS (1μg/μL) was used for 24-hours to acquire matured BMDC phenotype. Cells were collected and surface-stained with monoclonal antibodies against CD40 APC, CD80 APC, CD86 APC, and MHC class II APC. MFI of CD40 APC, CD80 APC, CD86 APC, and MHC class II were plotted from CD11c+cells data. Data are shown as mean ± SEM, n = 4 independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups: **P* < 0. 1; ****P* < 0.001; *****P* < 0.0001.

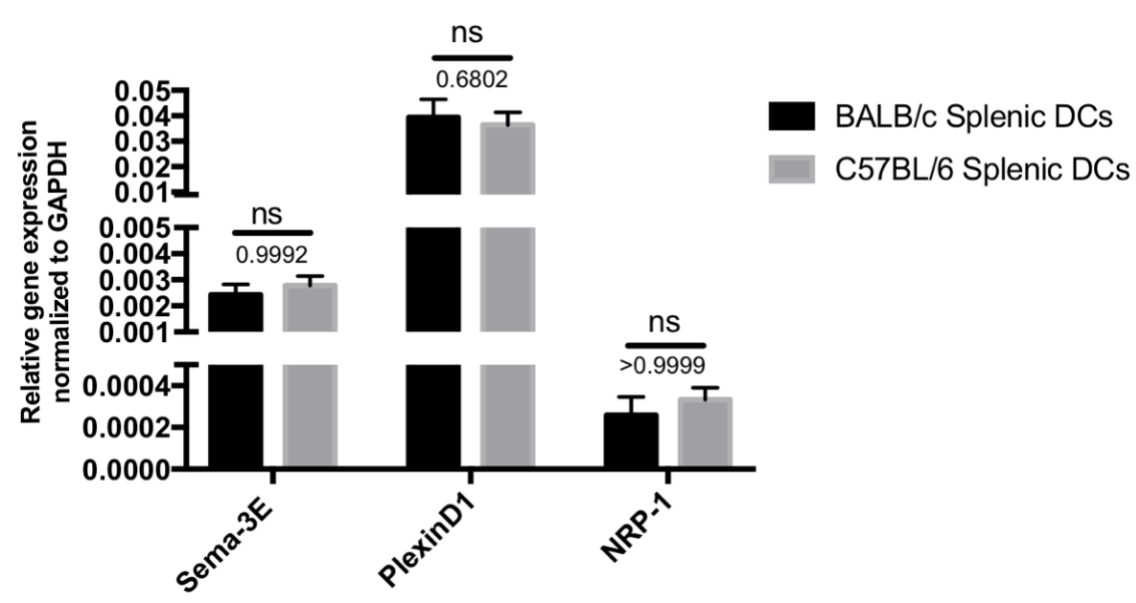


Figure S.7.20. Splenic DCs of BALB/c and C57BL/6 mice expressed similar levels of Sema-3E, PlexinD1, and NRP-1. Splenic DCs were isolated from WT BALB/c (BALB/c Splenic DCs) and WT C57BL/6 (C57BL/6 Splenic DCs) RNA from collected cells was used to measure Sema-3E, PlexinD1, and NRP-1 expression by qPCR. Data are shown as mean ± SEM, n = 3 independent experiments. *P* values are from two-way ANOVA used for comparing two groups. ns, *P* > 0.05.

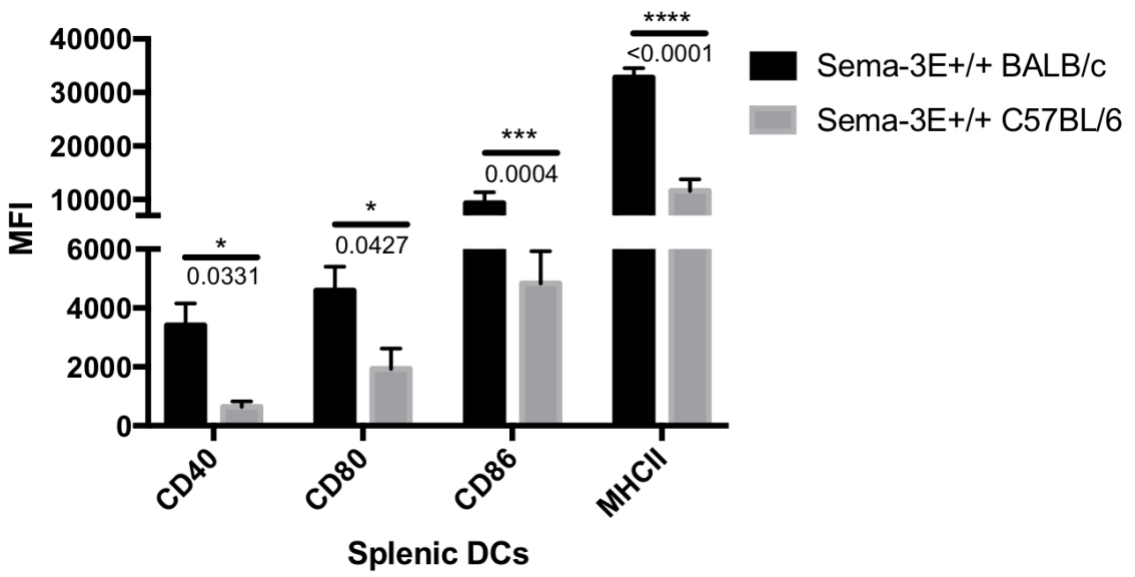


Figure S.7.21. Splenic DCs from BALB/c mice expressed higher levels of co-stimulatory molecules than C57BL/6 splenic DCs. Splenic DCs were isolated from WT BALB/c (Sema-3E+/+ BALB/c) and WT C57BL/6 (Sema-3E+/+ C57BL/6) mice. Cells were collected and surface-stained with monoclonal antibodies against CD40 APC, CD80 APC, CD86 APC, and MHC class II APC. MFI of CD40 APC, CD80 APC, CD86 APC, and MHC class II were plotted from CD11c+cells data. Data are shown as mean ± SEM, n = 3 independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups. **P* < 0.1; ****P* < 0.001; *****P* < 0.0001.

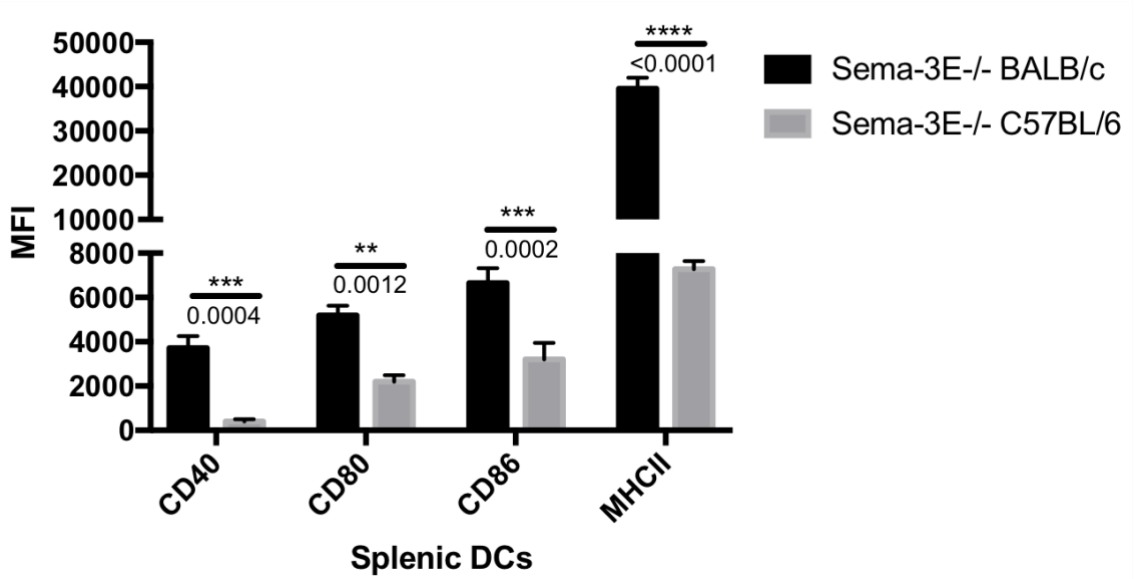


Figure S.7.22. Splenic DCs of Sema-3E-deficient BALB/c mice expressed higher levels of co-stimulatory molecules than C57BL/6 splenic DCs. Splenic DCs were isolated from Sema-3E deficient BALB/c (Sema-3E-/- BALB/c) and Sema-3E deficient C57BL/6 (Sema-3E-/- C57BL/6) mice. Cells were collected and surface-stained with monoclonal antibodies against CD40 APC, CD80 APC, CD86 APC, and MHC class II APC. MFI of CD40 APC, CD80 APC, CD86 APC, and MHC class II were plotted from CD11c+cells data. Data are shown as mean ± SEM, n = 3 independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups. ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

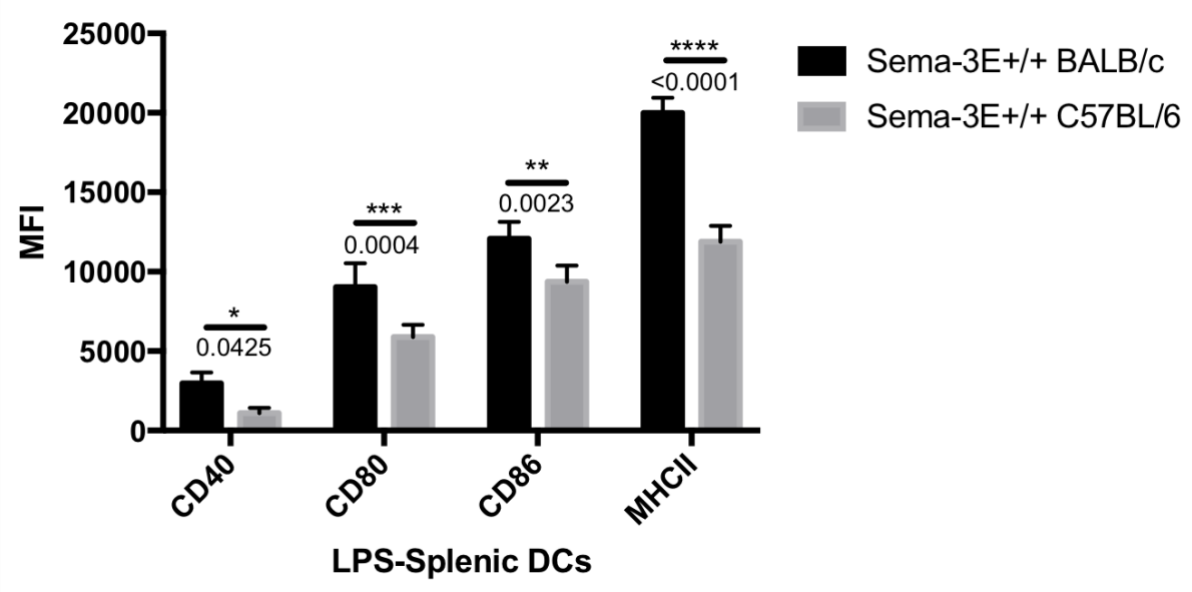


Figure S.7.23. LPS-stimulated splenic DCs of BALB/c mice expressed higher levels of co-stimulatory molecules than C57BL/6 splenic DCs. Splenic DCs were isolated from WT BALB/c (Sema-3E+/+ BALB/c) and WT C57BL/6 (Sema-3E+/+ C57BL/6) mice. LPS (1 μg/μL) was used for 24 h to acquire the mature BMDC phenotype. Cells were collected and surface-stained with monoclonal antibodies against CD40 APC, CD80 APC, CD86 APC, and MHC class II APC. MFI of CD40 APC, CD80 APC, CD86 APC, and MHC class II were plotted from CD11c+cells data. Data are shown as mean ± SEM, n = 3 independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups. **P* < 0.1; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

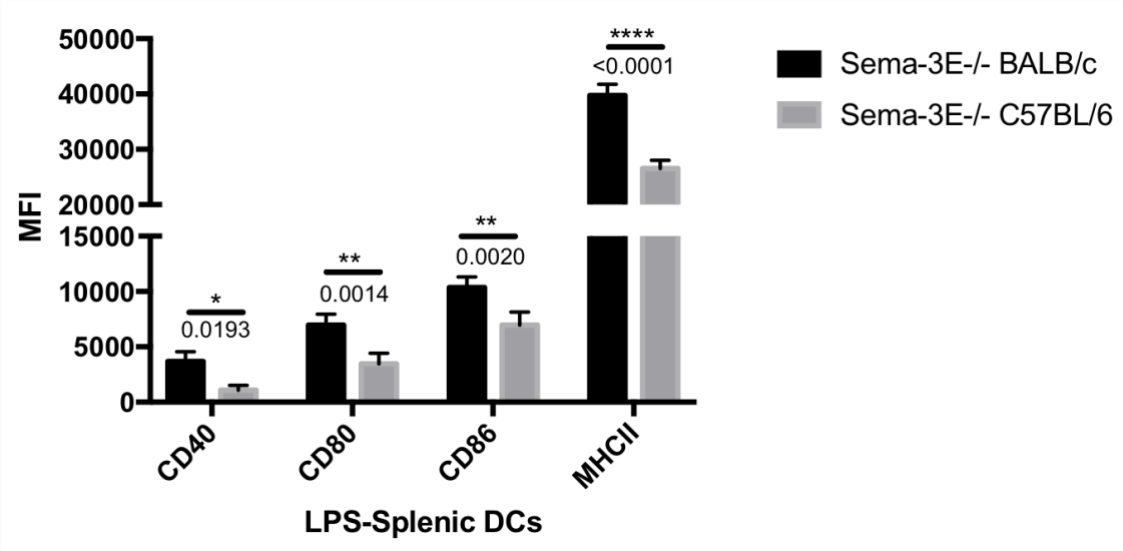


Figure S.7.24. LPS-stimulated splenic DCs of Sema-3E deficient BALB/c mice expressed higher co-stimulatory molecules than C57BL/6 splenic DCs. Splenic DCs were isolated from Sema-3E-deficient BALB/c (Sema-3E+/+ BALB/c) and Sema-3E-deficient C57BL/6 (Sema-3E+/+ C57BL/6) mice. LPS (1µg/µL) was used for 24 h to acquire the mature BMDC phenotype. Cells were collected and surface-stained with monoclonal antibodies against CD40 APC, CD80 APC, CD86 APC, and MHC class II APC. MFI of CD40 APC, CD80 APC, CD86 APC, and MHC class II were plotted from CD11c+cells data. Data are shown as mean ± SEM, n = 3 independent experiments. *P* values are from two-way ANOVA used for comparing data from two groups. **P* < 0.1; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

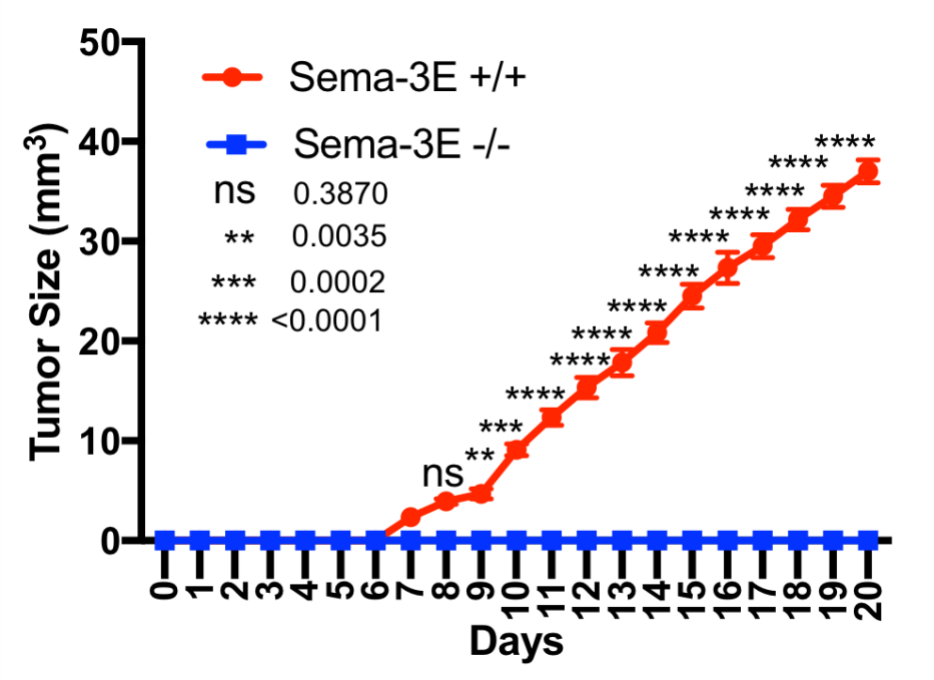


Figure S.7.25. E0771 breast cancer cells grown in C57BL/6 WT but not in Sema-3E-deficient mice. (A) E0771 cells were injected ($0.1x10^6$ cells per injection) into the mammary fat pad of WT (Sema-3E+/+; red) and Sema-3E-deficient (Sema-3E-/-; blue) C57BL/6 mice. Tumor size was monitored daily. Data are shown as mean ± SEM, n = 2 independent experiments (4 mice/ group). *P* values are from two-way ANOVA used for comparing two groups. ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

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Appendix: List of Publications

Published Manuscripts

1. Mahmood, S, Upreti D, Sow I, Alamri A, Nandagopal S, Kung SK. Bidirectional Interactions of NK cells and dendritic cells in immunotherapy: current and future perspective. Immunotherapy, 2015; 7(3):301-8. PMID 25804481
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5. Alamri A. Fisk D, Upreti D, Kung SK. A missing link: Engagement of dendritic cells in the pathogenesis of SARS-CoV-2 infections. International Journal of Molecular Sciences, 2021 Jan 22(3), 1118. PMID: 33498725
5.

Manuscripts in Preparation

1. Alamri, A and Kung, S. Sema-3E deficiency downregulated natural killer cells effector function
2. Alamri, A and Kung, S. Sema-3E/PlexinD1 axis modulated dendritic cells phenotype and function
3. Upreti D, Alamri A. Fisk D, Kung SK. Adoptive transfer of natural killer cells in therapeutic treatment of COVID-19 patients