

A role of Semaphorin-3E in regulating natural killer-cell migration in natural killer cell– dendritic cell crosstalk

BY

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ABSTRACT

Semaphorin-3E (Sema-3E) is a member of the Semaphorin family that plays important regulatory roles in nervous and immune system. Natural Killer (NK) and dendritic cells (DC) are immune cell types that mediate/shape innate and adaptive immunity. The role of Sema-3E in regulating NK cell biology has not been reported.

I first demonstrated that IL-2 activated NK cells expressed Sema-3E receptors on cell surface. I compared NK migratory responses towards conditioned media of DC (immature, LPS- or Poly I:C stimulated) derived from Sema-3E^{+/+} or Sema-3E^{-/-} mice. Of interest, I observed that activated NK cells exhibited a two-fold increase in the migrations toward immature Sema-3E^{-/-} DC conditioned medium when compared to that of the immature Sema-3E^{+/+} DC. My work revealed a novel role of Sema-3E in limiting NK-cell migrations toward immature DC in NK-DC crosstalk. Future delineations of the impact and signaling of the Sema-3E pathway in NK-DC crosstalk may support novel development of immunotherapy for cancer and infectious diseases.

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DEDICATION

*I dedicate this accomplishment to my wife Abeer Alamri
and my little princess Aleen Alamri*

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
BM	Bone marrow
BMDC	Bone marrow derived dendritic cell
CD	Cluster of differentiation
CD40	Cluster of differentiation 40
CD40L	CD40 ligand
CD40R	CD40 receptor
CD107a	Cluster of differentiation 107a
CLP	Common lymphoid progenitor
CpG	Cytosine phosphate guanine
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte antigen 4
CXCL	CXC chemokine ligand
DAP 10	DNAX-activation protein10
DAP 12	DNAX-activation protein12
DEC 205	The Dendritic Cell Receptor for Endocytosis
DC	Dendritic cell
ECM	Extracellular Matrix
ECP	Eosinophil Cationic Protein
ECs	Endothelial Cells
EGF	Epidermal Growth Factor

ERK	Extracellular signal regulated kinase
ETS	E26 transformation- specific
FACS	Fluorescence-activated cell sorting
Fas-L	Fas-Ligand
Fas-R	Fas-receptor
FBS	Fetal bovine serum
Fc γ -R	Fc-gamma receptor
Fc	Crystallizable fragment
Flt3-L	Fms-related tyrosine kinase 3 ligand
GAP	GTPase Activating Domain
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GM-CSF	Granulocyte monocyte colony stimulating factor
GPI	Glycophosphatidylinositol
GTP	Guanosine Triphosphate
HIV	Human immunodeficiency virus
HSC	Hematopoietic stem cell
ICAM-1	Intercellular adhesion molecule-1
iDC	Immature dendritic cells
IFN- γ	Interferon gamma
IFN- α	Interferon alpha
IFN- β	Interferon beta
Ig	Immunoglobulin

IL	Interleukin
IP-10	Interferon- γ -inducible protein 10
IS	Immunological synapse
iNK	Immature NK cells
ITAM	Immunoreceptor tyrosine based activation motif
ITIM	Immunoreceptor tyrosine based inhibitory motif
JAK / STAT	Janus Kinase / Signal Transducer and Activator of Transcription
KDa	Kilo Dalton
KIR	Killer-cell immunoglobulin-like receptors
KO	Knockout
LC	Langerhans cells
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinases
MDSC	Myeloid derived suppressor cells
mNK	Mature NK cells
mDC	Mature DC
MCMV	Mouse cytomegalovirus virus
MC	Mast cell
MHC	Major Histocompatibility Complex
MICA	MHC class I polypeptide- related sequence A
MICB	MHC class I polypeptide- related sequence B

mRNA	Messenger ribonucleic acid
NK	Natural killer cells
NKP	NK-progenitors
NCR	Natural cytotoxicity receptor
NRP	Neuropilin
PAMPs	Pathogen associated molecular patterns
PI3K	Phosphatidylinositol-3 kinase
pDC	Plasmacytoid DC
PLGF	Placenta growth factor
PG	Prostaglandin
PH Domain	Pleckstrin Homology Domain
PI (4,5) P2	Phosphatidylinositol 4,5-Bisphosphate
PMN	Polymorphonuclear neutrophils
PSC	Progenitor stem cell
PSI	Plexin Semaphorin Integrin
POLY I:C	Polyribonucleosinic polyribocytidylic acid
Real-Time PCR	A real-time polymerase chain reaction
RNA	Ribonucleic acid
RQ	Relative quantitative
SCF	Stem cell factor
SEM	Standard error of mean
SEMA	Semaphorin
SEMA-3E	Semaphorin-3E

SHP-1	Src Homology 2 Domain Phosphatase-1
SHP-2	Src Homology 2 Domain Phosphatase-2
SHIP	SH2-containing Inositol 5'-Phosphatase
SLT	Secondary lymphoid tissue
STAT3	Signal transducer and activator of transcription 3
TCR	T cell receptor
TF	Transcription factor
TGF- β	Transforming growth factor beta
Th1	T helper type 1
Th2	T helper type 2
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAIL	TNF-related apoptosis inducing ligand
Treg	Regulatory T cell
TREM2	Triggering Receptor Expressed On Myeloid Cells 2
ULBP	UL16 binding protein 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WT	Wild Type

1.0 CHAPTER 1

INTRODUCTION

1. Natural killer cells

1.1 Discovery of Natural Killer cell:

Natural killer (NK) cells are granular lymphocytes that initialize innate immune responses to confront abnormal cells such as tumor and pathogen infected cells. Unlike other adaptive immune cells, NK cells mediate cytotoxicity without a need of prior target activation in the recognition and elimination of abnormal cells [1-2]. In addition, NK cells produce numerous cytokines/chemokines (e.g., IFN- γ , TNF, and GM-CSF) that play important roles in immune-cells interactions and adaptive immunity response [3-4].

NK cells were discovered more than 35 years ago as murine lymphoid cells exhibiting cytotoxicity and cytokines production [5-6]. In early 1970s, Herberman and colleagues identified murine lymphoid cells exhibiting cytotoxicity against several syngeneic and allogeneic mouse tumors without any prior stimulation[7-8]. Further characterization by Kiessling and colleagues documented the selective target recognition feature of these lymphoid cells, as the identified cells were demonstrating impaired cytotoxicity against mastocytoma line P815 and few other leukemic cells of non-Moloney origin. Since these cells were found unique in terms of their cytotoxic responses as well as lacking key T and B-lymphocytes receptors at that time, thus formally referred as “Natural” killer cells[9].

1.2 Natural Killer cell maturation subset, distribution and development:

NK cells are heterogeneous population of innate immune system. These cells display differences in their homing, cytotoxic potential, competence for cytokine production as well as activation threshold that classify NK cells into characteristic subpopulations [10-11]. In human, the density of CD56 expression classified NK cells into two subgroups CD56^{bright} and CD56^{dim}. Recent studies indicate both subsets have unique functional attributes and, therefore, distinct roles in the human immune response [12]. CD56^{bright} subset has lower natural cytotoxicity and Fcγ receptor III (CD16) expression comparing to CD56^{dim} subset. CD56^{bright} CD16^{dim/-} is a minor population mostly resides in the secondary lymphoid tissue with mainly expression of L-selection (CD62L), CCR7, and CXCR3. This subset needs continued activation to be able to produce ample IFN-γ, TNF and other immunoregulatory cytokines [13-14]. In contrast, the majority of human NK cells appear as a CD56^{dim} CD16^{high} phenotype. This population has more cytotoxic ability and less amount of NK cytokines production [15].

Mouse mature NK cells of different effector function are defined by the expression of CD11b, CD27, and CD43 surface receptors and acquire effector functions [16]. Mature NK cells have been divided into two different subpopulations based on CD27 surface expression, which differently mediate distinct effector functions [16]. For instance, CD11b^{hi} CD27^{hi} secrete profound amount of IFN-γ and show strong cytotoxicity against abnormal cells compared to CD11b^{hi} CD27^{low} in response to IL-12/IL-18 stimulation. In contrary, CD11b^{hi} CD27^{low} cells mostly reside in the blood, spleen, lung, and liver; acquire an increased expression of inhibitory receptors, which might explain their hyporesponsive effector functions [16-17-18].

In 2006, another subset of NK cell expressing B220⁺CD11c⁺CD49⁺, a hybrid phenotype of NK cell and dendritic cell, was called interferon producing killer dendritic cells (IKDC). This subset is considered as non-conventional NK cells reside in the lymph nodes, spleen, BM and secrete several cytokines [19]. In contrast to bone marrow derived NK cells, another subset of NK cells were reported with CD127 surface expression that originated from thymus and demonstrates higher dependency on GATA3 transcriptional factor. These cells also reside in secondary lymphoid tissues (SLT), able to produce more IFN- γ [20]. The identification of all reported developmental intermediates of human and mouse NK lineage in this tissue rationalize BM as the primary site of NK cell development [21]. However, NK-cell intermediates have also been reported in several other anatomical locations (such as lymph node (LN), liver, spleen, thymus intestine, uterus, and liver) suggest multiple potential sites of NK development in vivo [22-23-24]. Clonal populations of mature classical NK cells can also be defined by their acquisition of specific NK cells activation and inhibitory receptors on a single NK cell surface (see section10).

The development of NK cells occurs predominantly in the bone marrow (BM) [25-26]. These cells are generated from hematopoietic stem cells (HSCs) and require BM environment [27]. Cytokine signaling and transcription factors actions derived from the BM stromal cells regulate NK developmental process that turns HSC into common lymphoid progenitor (CLP). These cells have the potential to develop into bipotent T/NK progenitor (T/NKP) to the committed NK progenitor (NKP) [28-29]. In the mouse fetal liver and thymus, (T/NKP) and (NKP) have been identified [30-31]. Fms-related tyrosine kinase-3 ligand (Flt3L), stem cells factor (SCF) and IL-7 cytokines are involved in the

early development stages, maintain, enhance the extension of HSCs, and induce CD122 (IL-2R β) expression on NKP [32-33].

NKP known as IL-15 responsive intermediate represent a heterogeneous population. IL-15 stimulation supports survival, proliferation and differentiation signals as well as mediating the transition of committed progenitors to an immature stage. Impaired IL-15 signaling associated with JAK3 and STAT 5a/b impairs the generation of effector NK cells [34-35].

In comparison to the mouse, human NK developmental process is still unclear. The most interesting human data generated from mouse models and *in vitro* differentiation system. Some studies indicated potential cellular intermediates of human NK cells but were unable to clarify the developmental NK process.

Human NK cells are defined by CD56⁺ and CD3⁻. Undetectable levels of CD122 in CD34⁺ HSC (a human developmental intermediate) prior to the expression of CD94, poses a challenge for identifying NK cells lineage [36]. It is a general consensus that BM-derived hematopoietic progenitor cells (HPCs) through a common lymphoid progenitor (CLP) has the potential to develop as mature human NK cells [37-38]. CLP represent a heterogeneous population found in BM and shows responsiveness to IL-15 signaling *in vitro*. Similarly, more than two NK cell developmental intermediates have been identified in the human secondary lymphoid organs. These intermediates are able to generate mature NK cells in response to IL-15 stimulation *in vitro* [39]. These cells show the phenotypes of mature human NK cells that express surface receptors such as CD56, NKG2D, and CD161. The expression of killer immunoglobulin-like receptors (KIRs) and CD16 is limited to the late maturation stages [34].

Expressions of high-level of inhibitors of DNA binding proteins (Id1, Id2, Id3, Id4), which bind E proteins to drive NK lineage, differentially regulate NK development. Over-expression of Id3 in CD34⁺ progenitors blocks T-cell development and promotes NK development. However, the frequency of mature NK cells in the secondary lymphoid organs has been linked with Id2 deficiency in mice. Interestingly, this mouse model exhibits relatively normal frequency of NK-precursors and immature NK cells [40-41]. Ikaros and E4BP4 (Nfil3) play important role during early and late phases of NK development. Mutation in Ikaros gene affects the transition of CLP to earliest defined NK progenitors and the maturation of NK cells in periphery [42-43]. Moreover, E4BP4 expression helps the development and maturation of NK cell. E4BP4 deficient mice exhibit aberrant progression of NKP to immature and mature phases [44]. Ets1 (E26 transformation-specific) family proteins are key regulators of T-bet and Id2 expression transcriptional factors needed in the early phases of NK cell development [45]. PU.1, one of the Ets family members mediates lymphopoiesis and its deficiency has been shown to correlate with impaired progression of HSC to NKP [29]. Beyond the above-mentioned TF the role of many other TF in NK cell development is still unclear.

1.3 Target recognition of NK cells:

NK activating and inhibitory surface receptors recognize specific ligands on potential targets to discriminate between self and non-self [71-72]. However, the balance between activating and inhibitory signals decides the functional outcome of an activated NK cell [72-73]. Since NK cells are cytotoxic immune cells, de-regulation in their effector functions could result in deleterious consequences. Therefore, it is believed that NK

activation must be tightly regulated to ensure their protective responses. Several models have been proposed which explicate the mechanisms involved in NK target recognition and self-tolerance. In this regard, Ljunggren and Karre (1986) proposed the first model major histocompatibility complex MHC-I dependent model of education, formally termed as “missing self-hypothesis” [74- 75].

A- Missing self-hypothesis:

TYPE OF MHC CLASS I	Target cells MHC CLASS I MOLECULE	NK CELLS LYSIS
SELF NON SELF	Presence Absence	Inhibitory signal
SELF NON SELF	Absence Absence	Activating signal
SELF NON SELF	Absence Presence	Activating signal

Table1.3 A: NK-cell self-tolerance and the 'missing-self' hypothesis. **SELF:** On interacting with a normal target cell, the target cell expresses the self-MHC class I, NK cell does not lyse the target cell. **MISSING SELF:** If the target cell loses expression of MHC class I molecules, due to viral infection or transformation, NK cell does not receive inhibitory signals and therefore lyses the target cell. **NON-SELF:** NK cells interact with donor target cells that express foreign MHC class I alleles. This event leads to lysis of the allogeneic cells by host NK cells.

According to this hypothesis, target cells lacking or having altered expression of MHC-I, as happened frequently in viral infection or tumorigenesis, become sensitive to NK cell killing. MHC-I molecule, a hallmark of “self” ubiquitously expressed on normal cells, generate dominant protective signals to inhibit NK activation. Identification of Ly49A and Killer cell immunoglobulin-like receptors (KIR) inhibitory receptors in mice and human NK cells respectively and their specificity in subsequent investigations reinforced the credibility of this model [77-78]. NK cells inhibitory receptors demonstrate a positive functional correlation with the expression of MHC-I on target cells. It has been shown previously that NK education and self-tolerance was confined to the bone marrow. However, subsequent studies demonstrated NK effector functions could be modulated and reprogrammed by tuning the MHC-I environment. NK cells become anergic when transferred to MHC-I deficient host whereas NK cells derived from $\beta 2m$ deficient or Transporter associated with Antigen Processing 1 (Tap1) mutated mouse background acquired functional competence when adoptively transferred to MHC-I sufficient environment, suggest how MHC-I expression regulates NK education and target recognition [79-80].

Interestingly, MHC-I dependent recognition (responsiveness) is not an independent event and NK cell requires the involvement of activating receptors to mediate effector functions. It is thought that activating receptors are involved in the education and self-tolerance of NK cells during development [81-82]. Therefore, the role of activating receptors in NK education and self-tolerance cannot be ruled out.

Collectively, NK cell activation (responsiveness) is predominantly under control of inhibitory receptors that specifically recognize and binds with class I MHC molecules. In

addition, different subset of NK cells may acquire one or multiple MHC-I specific inhibitory receptors by a process that is largely random. To anticipate these variations in MHC-I context, following models postulated to define NK education and self-tolerance.

B- NK cell receptors:

NK RECEPTOR	NK RECEPTOR LIGAND	NK RECEPTOR FUNCTION
KIR2DS1	HLA-C	ACTIVATING
KIR2DS4	HLA-A,C	ACTIVATING
KIR2DL4	HLA-G	ACTIVATING
CD94NKG2C	HLA-E	ACTIVATING
KIR3DL2	CpG	ACTIVATING
CD16	IgG	ACTIVATING
NKG2D	MIC, ULBP	ACTIVATING
NCRs	B7-H6	ACTIVATING
KIR2DL 1/2/3	HLA-C	INHIBITORY
KIR3DL1	HLA-B	INHIBITORY
KIR3DL2	HLA-A	INHIBITORY
CD94/NKG2A	HLA-E	INHIBITORY
LIR-1	HLA-A-G	INHIBITORY
KLRG-1	CADHERINS	INHIBITORY
CEACAM1	CEACAM5	INHIBITORY
TIGIT	PVR and PVRL2	INHIBITORY
2B4	CD48	CO-RECEPTOR
NTBA	NTBA	CO-RECEPTOR
CS1	CS1	CO-RECEPTOR
NKP80	AICL	CO-RECEPTOR
TLR	TLRL	CO-RECEPTOR
DNAM1	PVR, NECTIN-2	CO-RECEPTOR
CD96	PVR	CO-RECEPTOR

Table1.3 B: NK cell surface receptors and their ligands. Receptors of NK cells are classified based on their primary function (inhibitory receptors, activating receptors, and activating co-receptors).

1.3.1 NK cell inhibitory receptors:

Twenty-three years ago, Yokoyama and colleagues discovered the first NK cell inhibitory receptor [83-84]. Recently, these receptors specific to MHC-I molecules have been divided to three families including Ly49 in mice, KIR in human and CD94/NKG2A both in mice and human [85-86]. NK cells express combinations of activating and inhibitory receptors during their development to balance NK functions in discrimination between self and non-self MHC-I molecules [87]. Inhibitory receptors contain ITIM (immunoreceptor tyrosine-based inhibitory motif), which get phosphorylated after the engagement of inhibitory receptors with specific ligand. ITIM phosphorylation leads to activate SHP-1 (src-homolgy 2 domain-containing tyrosine phosphatases), SHP-2 and SH2-containingInositol 5'-Phosphatase (SHIP). This activation of phosphatases is important in controlling NK responsiveness inhibitory pathways [88-89-90].

Ly49 receptors:

Ly49 receptors are lectin like type II transmembrane proteins expressed on the surface of NK cells, NKT-cells and $\gamma\delta$ T lymphocytes [89]. The family of Ly49 receptors comprise of both inhibitory and activating receptors. In mouse, inhibitory isoforms like A, G, C and I are well characterized. Certain polymorphic epitopes on MHC-1 molecule are the primary ligand for most of the Ly49 inhibitory receptors, however Ly49H, member of Ly49 family recognize Murine cytomegalovirus m157 on mouse cytomegalovirus virus MCMV infected cells and act as activating receptors. Majority of the Ly49 receptors have unknown ligands other than MHC-I molecules [91].

Killer Immunoglobulin-like receptors (KIR):

KIR receptors expressed by human and showed both activating and inhibitory receptors. KIR receptors are type I integral membrane proteins and part of Ig-superfamily. KIR expresses two extracellular Ig domain (cytoplasmic domain). Long cytoplasmic tail KIR proteins associated with inhibitory signals whereas short tail domain restricted to the activating signals [92-93].

CD94/NKG2A receptor:

CD94/NKG2A is a C-type lectin superfamily expressed on NK cell surface in early developmental stages. In tumor and infections, upregulation of human leukocyte antigen (HLA-E) and Qa1 connected to CD94/NKG2A inhibitory receptors and mediate the destruction of immune responses [94-95].

1.3.2. NK cell activating receptors:

NK cells have shown the expression of several activating receptors in both mice and human. These receptors get activated once they recognize non-self MHC-I molecule. Activating receptors mediated their signaling through immunoreceptor tyrosine-based activation motif (ITAM) present in the cytoplasmic tail. The phosphorylation of ITAM results in the activation of downstream signaling molecules such as phosphatidylinositol 3-kinases (PI3K), phospholipases, and extracellular signal-regulated kinases (ERK). Upon activation, NK cells secrete the pro-inflammatory cytokines and/or cytotoxic granules to activate the immune responses [96-97].

Ly49/KIR receptors:

Some of Ly49 and KIR receptors are activating receptors and associated with DAP12 containing ITAM. For example, Ly49H, Ly49D, Ly49P and Ly49W have different functions including recognition and specificity. In addition, KIR2DS1 and KIR2DS2 are members of KIR receptors mediate activating signaling pathways [98-99].

Natural cytotoxicity receptors (NCR):

NCR contains many activating receptors such as NKp46, NKp44, and NKp30 mediate anti-viral and anti-tumor activity in human. In the literature, not many of these receptors ligand yet clear. Recently, the role of NKp46 (the only NCR with conserved sequence reported in mammals including mice) was proven to control T responses. Moreover NKp30 is ligand to tumor nuclear factors such as BAT3 and B7-H6 [100-101-102].

NKG2D receptor:

NKG2D is a type II transmembrane glycoprotein found on all NK cells. NKG2D is important activating receptor has two different isoforms associated with DAP10 and DAP12 adaptor molecules to mediate signaling cascade. Stress molecules/proteins expressed on infected or transformed cells that share structural homology with MHC-I molecule such as Rae1, H60, Murine ULBP-Like Transcript 1 (MULT1) in mouse and MHC class I polypeptide- related sequence A (MICA), MHC class I polypeptide- related sequence B (MICB), UL16 binding protein 1(ULBP1-4) in human are the primary ligands of this receptor [103].

NKP-P1C (NK1.1) receptor:

This receptor is the most specific and widely distributed surface marker on NK cells in C57BL/6 mice and associated with Fc γ mediated degranulation and cytokine production in antibody cross linking. NKR-P1C transmembrane is associated with Fc γ involved in NK mediated degranulation and cytokine production following antibody cross linking, yet its natural ligand remains unknown [104].

CD16 receptor:

CD16 receptor is a low affinity Fc γ - receptor binds to Fc portion of the immunoglobulin G IgG antibody. NK cells use low affinity receptor to eliminate cellular targets [105].

1.3.3 Dual functions receptors:

2B4 (CD244):

This receptor binds to non-MHC related molecules. CD244 recognizes CD48; a glycoprotein expressed on hematopoietic cells and mediates different functions. The strength of cross-linking is based on the level of 2B4 [106-107].

1.4. Activation of NK cell by Cytokines:

NK cells express surface receptors for several cytokines such as IL-15, IL-2, IL-12, IL-18 and IFN- α . Such cytokines in combination with other cellular factors regulate NK responses. For example, IL-12, IL-18, and IL-2 cytokines are associated with the increase of granzyme-B and perforin in the resting NK cells, which enhanced the effector functions [46]. IL-18, IL-2 and IL-12 promote proliferation and stimulate resting NK to produce cytokines such as IFN γ and TNF- α through JAK/STAT pathways [47]. IL-2 mediates the induction of Fas-L expression on tumor cells through promoting proliferation and stimulation of naïve cells to produce cytokines such as IFN- γ and TNF- α [48]. In addition to the previous cytokines, IL-15 is important for NK cells early development and maintains their proliferation, survival, and cytotoxicity [49-50].

1.5. Effector NK cell Functions (cytotoxicity, cytokines):

Granules exocytosis is considered as the principle cytotoxic pathway that NK cells used to eliminate infected cells [51]. The process of NK cells cytotoxicity begins when NK cells contact target cells and form an immunological synapse (IS), which more triggers the transport of lytic granules containing perforin and granzyme for entry at IS. Perforin is a cytolytic protein that disrupts the membrane at IS, assisting granzymes to enter the cytoplasm of target cell [52]. Alternatively, perforin and granzymes stay in a giant endosome and endocytosed into the cytosol. Perforin makes pores in the endosome to release granzyme into the cytosol [53]. Human and mice NK cells express granzymes (A, B, C, D, E, F, G, D, K, and M) with different cytosolic substrates. However granzyme B, in combination with perforin, has been found critical in inducing apoptosis of target

cell by cleaving proteins such as pro-caspases-3 and 7 [54]. NK cells express Fas-ligand (Fas-L), a molecule that belongs to TNF-receptor family, mediates NK killing of tumor cells. NK cells express also TNF-related apoptosis-inducing-ligand (TRAIL), a type II transmembrane protein, mediates downstream signaling by activating target cells caspase-8 and subsequently caspase-3 to induce cell death. However, cytokine stimulations including IL-2, IL-15 and IFN- α can induce TRAIL expression on NK cells as well [55-56-57].

1.6. Regulatory role (s) of NK cells:

NK cells establish either cell-cell contacts or provide soluble factors to communicate with other immune cells such as T cells, macrophages, neutrophils and dendritic cells (DCs). NK derived IFN- γ in particular has been implicated in a wide variety of immune responses including viral defense, skewing Th1 and promoting cytotoxic T lymphocyte CTL responses as well as in the maturation of DC and macrophages. The secretion of IFN- γ , TNF- α by activated NK cells and cell-cell contact dependent signals induced DC maturation before they gain access to T-cell enriched zones in the lymph nodes, promoting antigen specific T cells responses [58-59]. In addition, NK cells eliminate deregulated immature DCs from the site of infection [60-61]. Moreover, activated macrophages and resting microglia are the targets of NK mediated cell cytotoxicity via NKG2D and NKp46 activating receptors [62- 63]. Such immune regulatory functions preclude inappropriate T cell polarization and avoid immunopathology. Releasing IFN- γ and providing 2B4-CD48 mediated co-stimulation to the T cells, NK cells have been involved in skewing Th1 responses studies have also demonstrated that NK cells

eliminate highly activated and auto-reactive T cells as well. These cells express stress ligands during infection, which greatly increase their susceptibility to NKG2D, and NKp46 mediated NK killing *in vitro*. These studies emphasize the significance of NK mediated negative regulation in the prevention of potential autoimmune disorders [64-65].

In infection, it has been demonstrated that NK cells accelerate antiviral and antitumor CD8 T cell responses *in vivo* [66]. NK cells are among early responders in infection, they orchestrate subsequent immune responses in collaboration with other immune cells. In mice model of influenza, NK cells facilitate CD8 T cell recruitment to the peripheral LN “by secreting IFN- γ ”[67]. In turns, NK mediated DC maturation results in IL-12 production, which promote priming and generation of CTLs. [68]. In inflammatory conditions, NK cells are implicated in many physiological situations. NK cells are involved in immunoglobulin Ig isotype switching and regulate B cell differentiation as well as decidual NK cells are found a source of vascular endothelial growth factor VEGF, placenta growth factor PLGF, which further promote angiogenesis during pregnancy [69-70].

2. Dendritic cells (DCs):

Dendritic cells (DCs) are important immune cells were defined by Ralph Steinmann and colleagues in 1974 and formally named them “DC”. DCs are known as professional antigen presenting cells (APCs). Due to their peculiar characteristic shape these cells were initially described as part of the nervous system [108]. DCs are known as master regulator of the immune system because of their ability to modulate innate and adaptive immune systems. However, DCs divided into several subgroups based on anatomical distribution and functions [109].

Strong evidences support that DC belongs to the myeloid lineage and is derived from hematopoietic stem cells [110]. BM myeloid precursors turn into mature DC in the presence of Granulocyte monocyte colony stimulating factor (GM-CSF). More evidence of DC lineage commitment, common myeloid progenitors transplantation into an irradiated mouse yields various subtypes of DCs in the spleen and thymus. However, the acquisition of lymphoid markers by a subtype of DC confined in the secondary lymphoid tissues such as CD8a, CD4 and CD2 suggest an alternate lymphoid lineage [111]. It has become clear now that DC follows various differentiation pathways, as shown their generation by common myeloid progenitors CMP and common lymphoid progenitors CLP both in vitro and in vivo studies [112].

2.1 Functions of DCs:

DCs are professional antigen processing and presenting cells. By producing a broad range of cytokines such as (IL-18, IL-12, IL-15, IFN- α , IFN- β), these cells have been implicated in several immune regulations at the interface of both innate and adaptive

immunities [123- 124]. BM derived immature DCs or their precursors distribute throughout the body predominantly at the potential sites of pathogen entry. Immature DCs express low levels of MHC and other co-stimulatory molecules such as (CD40, CD80, CD86), however they are able to sense and capture different pathogens by expressing a broad range of pattern recognition receptors (PRR) such as Toll-like receptors (TLR), C-type lectin receptors (CLR), RIG-I-like receptors (RLR), NOD-like receptors (NLR), which recognize pathogen associated molecular patterns (PAMPs). Processing antigens from self, virus, or bacteria involves two distinct pathways the endogenous pathway, and the exogenous pathway of antigen processing to present peptides derived from proteins. Capturing and processing of antigen induce higher expression of MHC-I, MHC-II, co-stimulatory and adhesion molecules to enhance immune activation such as CD4 and CD8 T cells. However, such cells have less ability of phagocytosis and endocytosis [125-126]. Moreover, DCs have been shown to play a critical role in mediating immune tolerance to evade injurious effects of foreign or self-antigens. More functions of DCs have been studied such as their appearance to eliminate deregulated T cells from the thymus. However, the antigen loaded immature DCs can enhance peripheral tolerance by activating apoptosis and anergy of T cell and induce T-regulatory cells [127-128]. Many studies have indicated that autoimmune diseases associated DC functions impairment [129-130].

2.2. Types of DC:

DCs represent a heterogeneous class of immune cells. Current classification of DC cells is basically based on their phenotype, location where they reside and develop their

functions [109]. Generally, both mouse and human DCs are classified as conventional and plasmacytoid categories with multiple subtypes. Mouse DCs acquire MHC-II, CD4, CD8a, CD11b and DEC-205 receptors. However all classes of these DCs have a defined expression of CD11c [113].

2.2.1 Conventional DCs:

Conventional DCs (cDC) predominantly reside in the lymphoid tissues such as, spleen, thymus and secondary lymph nodes. These cells are designated as classical DCs, express high levels of MHC-II and CD11c, also express a several pattern recognition receptors[114]. CD8a expression further mark cDC into two subclasses i.e., CD8a⁺CD4⁻CD11b⁻CD205⁺ and CD8a⁻CD4⁺CD11b⁺CD205⁻, which constitute 20% and 40% spleen DC population respectively [115-116]. Likely, CD8a⁺ DCs are found in the LN as well as constitute predominantly in the thymus. Resemble to the CD8a⁻ cDC population, which reside in the marginal zones and induce Th-2 cytokine responses, CD8a⁺ DC predominantly found in the T cell zone, promoting Th-1 biased responses and produce IL-12 profoundly. Interestingly, cDC are able to collect and present antigens from the organ of their residence. Human DCs are found deficient of CD8a expression [116- 117].

2.2.2 Non-lymphoid tissue DCs:

The example of this class is Langerhans cells (LC). These cells reside in the epidermis layer of the skin in steady state. However, during inflammatory insults these DCs uptake the antigens and migrate to the LN for T cell polarization. These cells acquire expression of MHC-II and other co-stimulatory surface molecules and skew Th-1 immune responses

in CD4⁺ T cells. Based on their surface phenotype, skin LC can be defined into two distinct subtypes (CD103⁺CD11b^{lo} Langerin⁺ and CD103⁻CD11b^{hi} Langerin⁻). Importantly, CD103⁺ LC has been implicated in several viral and bacterial infections. Similarly, other DCs populations with CD103 expression have also been shown in the mucosal surfaces, lungs, kidney and liver [109].

2.2.3 Inflammatory DCs:

During infection, human and mice accommodate a subtype of inflammatory DCs. In addition to classical and other migratory DCs. Inflammatory DC cells differentiate from monocytes and acquire characteristic phenotype (MHC-II⁺ CD11b⁺ CD11c⁺ F4/80⁺ Ly6C⁺) and prefer to relocate themselves to the site of infection [118].

2.2.4 Plasmacytoid DCs (pDC):

Plasmacytoid DC cells identified first in human spleen and bone marrow, these cells produce abundant amounts of type I interferon. In infection, pDCs differentiate and acquire the phenotypes and some of the functions of the conventional DCs [119]. In contrast to the classical DCs, pDCs acquire low expression of MHC-II, CD11c as well as co-stimulatory molecules expression [120]. However, this type of cells does not express Toll-like receptor (TLR) 1,2,3,4,5 and 6, however demonstrate higher activation levels through TLR7, and TLR9 and produce IFN- α , IL-12, TNF- α and several chemokines (such as CCL3, CCL5, CXCL10) to promoting NK-cytotoxicity, survival of T cells, macrophages and promoting antibody production by B-cells [121-122].

3.0. Natural Killer Cells- Dendritic Cells Crosstalk:

Recently, an attention and focus were giving to the area of NK-DC interaction (crosstalk) and has been an important immune regulatory mechanism that brings innate and adaptive immunities together to provide a robust mechanism regulating the initiation and amplification of cellular and humoral immune response. NK-DC communication is bidirectional, involving different cytokine signals and/or direct cell - cell contact [131-132]. However, the understanding of this physiological relevance of crosstalk in vivo and the molecular mechanisms underlying the NK-DC crosstalk remain to be under investigation.

3.1 Prospect sites of NK/DC interactions:

The interaction between NK and DC takes place in the lymphoid organs or non-lymphoid tissues [133, 134]. Immature DCs reside in different places such as the skin and intestinal mucosa to counter invading foreign antigens. Uptake and processing of antigen result the upregulation of MHC-II, co-stimulatory molecules (CD40, CD80, CD86) The mature DCs are able to secrete cytokines and chemokines, acquire antigen-presenting ability before they migrate to the LN, a potential meeting point with NK cell [135-136]. Human NK cells express high level of CCR7, L-selectin and CXCR3 resulting in NK cells residing in the LN. By contrast, mouse NK cells express low level of LN homing receptor, therefore excluded from the LN. NK cells constitute about 0.5% of the population in this organ, however in infection the recruitment increased via CXCR3 pathway [133-137]. Other studies indicate that small population of human and mouse NK-like cells expressing NKp44/NKp46 and IL-22 are found in the close proximity of

resident DCs in the gut mucosa [138-139]. Based on these findings, it has been proposed that LN, and the gut mucosa is among the key sites for NK/DC interaction.

3.2 Nature of NK/DC interactions:

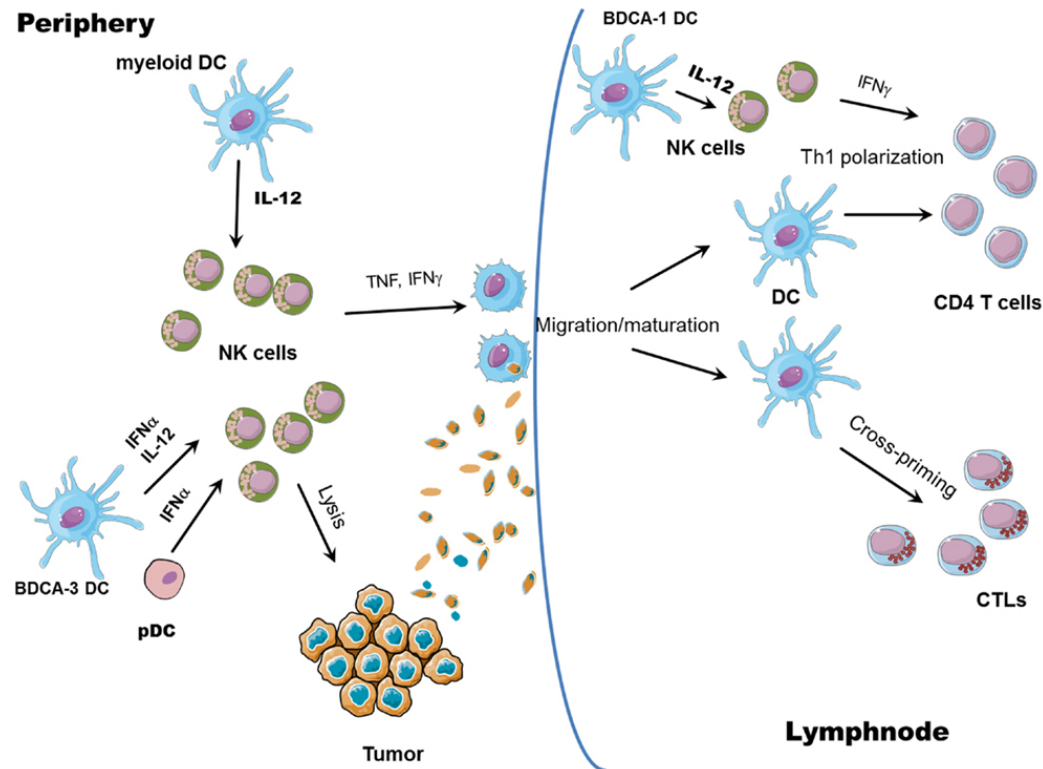


Figure3.2. The interaction between NK cells and DCs, NK-cell and DC interactions result in reciprocal activation: cytokines such as IL-12 and IFN- α released distinct DC subsets reflect different capability to promote NK cells functions may promote NK cell proliferation. Therefore, activated NK cells released IFN- γ that promotes Th1 polarization and, together with TNF, DC maturation, and migration to lymph nodes. Activated NK cells can also lyse tumor cells, leading to the generation of tumor antigenic material that engulfed by DCs and presented to both CD8+ and CD4+ T cells [140].

DCs secrete a range of signals, which regulate NK cell activation, proliferation and stimulate NK-cells to mount effective immune responses [141-142]. DC derived cytokines IL-12, is critical in the generation of IFN- γ producing NK cells. Interestingly, DCs also derive soluble factors such as IL-1 and IL-18 which have implication in the acquisition of IL-12-receptor on NK cells [143]. The roles of IL-15 and TNF alpha proteins produced by DC have promoted survival, proliferation and prime NK cell to secrete pro-inflammatory cytokines and cell cytotoxicity [141]. Mutually, NK cells promote DC maturation and activation by inducing MHC molecules expression and enhancing the ability to secrete IL-18, IL-12, P70 via upregulation of CD86 molecules and activation of Triggering Receptor Expressed On Myeloid Cells 2 (TREM2) and NKp30 signaling [84-144]. Studies have shown that the frequency and the activation of DC in the LN is drastically compromised after NK depletion [145]. Another study indicated immature DCs polarization and protection from lysis is dependent on activated NK cells [146]. Multiple studies focus on interplay between these two cells and suggest that NK-cells might regulate DC homeostasis. However, activated NK cells are capable to eliminate autologous immature DCs (iDCs) with reduced expression of MHC molecules, primarily through NKp30 and TNF-related apoptosis inducing ligand (TRAIL) [147- 148-149]. However, mature DCs become resistant to NK-mediated killing because of substantial MHC expression. Several other NK-receptors including NKG2D, NKp46 and their ligands have also been implicated in killing of deregulated DCs in several infection models [150].

3.3 NK/DC crosstalk in different models:

3.3.1 NK/DC crosstalk in infection:

In infections, maturation of DCs by NK cells regulation has been shown to be important shaping subsequent immune responses. Several human viral infections such as herpes virus, influenza, HIV, poxvirus and papilloma infections are associated with impaired NK cell functions [151-152]. Importantly, recent study indicates the progression and the severity of such infections have association with the disruption of NK/DC crosstalk [153]. For example, early phases of HIV, pro-inflammatory cytokines such as cellular FLICE-like inhibitory protein (c-FLIP) and inhibitor of apoptosis protein (CIAP2) mediated immunosuppression and the acquisition of anti-apoptotic that impair NK mediated infected DC elimination, [154]. Not only NK cells functions affected by HIV but also DCs from HIV patients are poor producer of cytokines (such as IL-12, IL-15, IL-18), which has an effect on NK cells activation and effector functions [155]. In HIV, NK cells were unable to recognize and eliminate immature DCs. The exact mechanism is not known, however the defective function of NKG2D has been implicated in this process [156]. Different studies had showed the impairment of NK/DC crosstalk by the modulation of NK activating receptors such as NKG2D and NCR-1. Such a modulation affects the progression and persistence of herpes viral infection [157]. Interestingly, MCMV infection impaired the ability of infected DC to produce cytokines such as IL-18 and IL-12. As a result, the activation, proliferation and the cytotoxic responses of NK cells were compromised in this model. [142]. NK cells also constitute first line of defense against influenza-A infection. The depletion of NK cells drastically impairs T cells immune responses by blocking the recruitment of DCs to the LN [153].

3.3.2 NK/DC crosstalk in tumor:

The impairment of NK cell functions is associated with the progression of tumors and malignancies [144-158]. In mice, the reduction of self-MHC-1 expression and/or over-expression of NKG2D related stress molecules enhance NK cells sensitivity, which mediate tumor lysis. Tumors have several strategies to evade immune system by mimicking self; preventing recruitment of immune cells, causing anergy or apoptosis of immune cells, inducing immune suppression by activating regulatory T-cells and causing NK exhaustion [157-159]. As infection models, tumor microenvironment disrupted bi-directional cross talk between NK/DC. For example, Myeloid derived suppressor cells (MDSC) in the tumor microenvironment have been shown to promote the development of regulatory T-cells (Treg) which in turn produce IL-10 to induce immune suppression[160]. However, depletion of Treg induces the recruitment of DC in the LN and improves the NK cells activation and proliferation in tumor mouse model [161]. Moreover, NKG2D down regulation abrogates NK cytotoxicity. Additionally, TGF- β derived by myeloid derived suppressor cells (MDSC) is reported in impairing NK/DC communication by blocking NKp30 expression and inhibiting NK cells derived IFN- γ production, thus promote accumulation of immunosuppressive iDC in tumor environment [162]. Most recently, Proteoglycans (PG) promotes tumor progression by disrupting NK/DC communications. The presence of PG in the tumor microenvironment modulates DC functional phenotype, which in turn limit NK activation [163].

4.0. Semaphorins

4.1 Semaphorin classes:

Semaphorins are a large family of proteins that are first discovered as axon guidance molecules in the nervous system. Recently, this family has received an immense attention due to their importance in other systems including immune, respiratory and cardiovascular systems as well as various processes such as angiogenesis, embryogenesis, and tumor formation [164-165-166].

Semaphorins are classified into eight main classes, 1-7 and V. Classes 1 and 2 are found in invertebrate, however, classes 3-7 exist in vertebrate whereas class V is unique to viruses [166]. The differences between these classes are related to their sequence and structure; however, all members have conserved extracellular domain, which consist of 500 amino acids, called Semaphorin (Sema) domain. This domain is a cysteine-rich sequence that is a crucial component for receptor binding specificity and protein function [167]. Semaphorin molecules are also classified based on their localization [168]. Accordingly, Classes 1, 4, 5, and 6 are known as membrane-bound proteins, whereas classes 2 and 3 are characterized as secreted proteins, and class 7 is identified as glycosyl-phosphatidyl-inositol (GPI)-linked proteins [168].

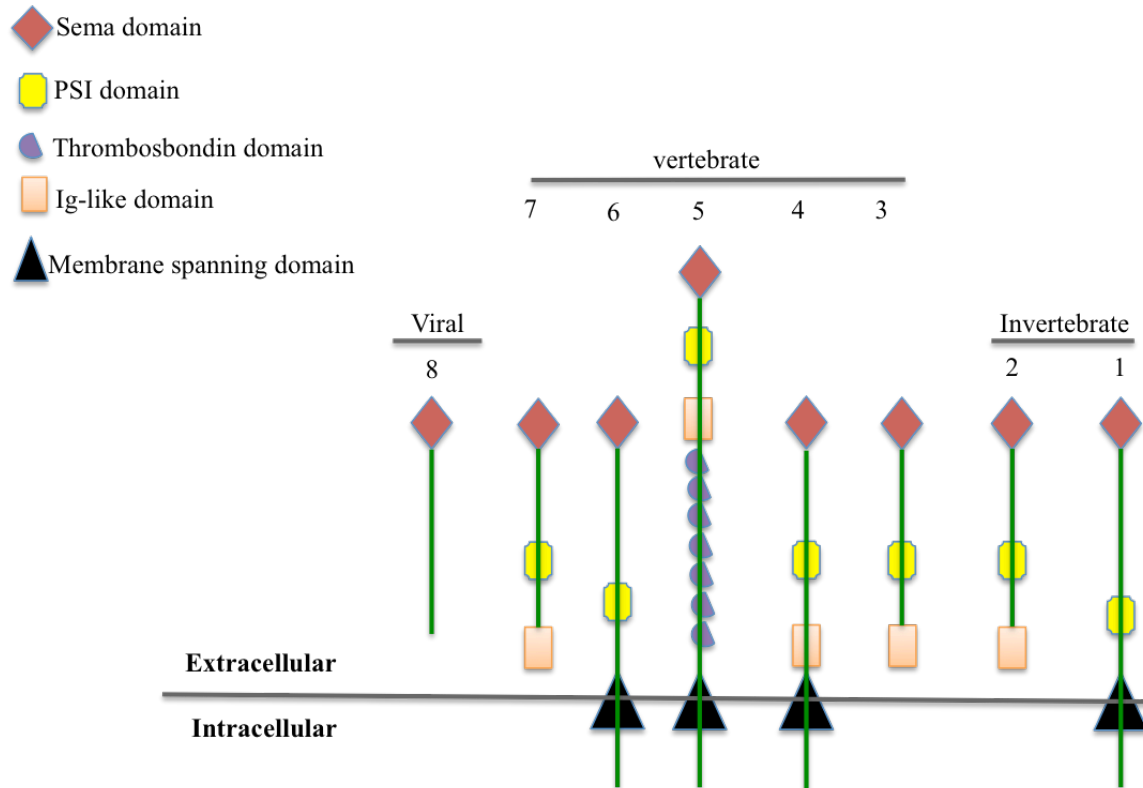


Figure4.1. Semaphorins classes and structure. Semaphorins are represented in their classification into eighth classes. Class 1 and 2 Semaphorins are found in invertebrates. Class 3–7 Semaphorins are found in vertebrates. Sema domains characterize both Semaphorins and Plexins. Additional domains present in Semaphorins and Plexins include PSI domains (Plexin, Semaphorin, and integrin) and immunoglobulin (Ig)-like domains. They can be either secreted or membrane-bound proteins.

4.2. Semaphorins Functions:

Recent studies have showed the involvement of Semaphorins in different body organs and systems such as immune, respiratory, cardiovascular systems, and in pathological disorders including cancer [169-170]. As such, Semaphorins influence cellular processes, such as cell division, differentiation and survival [171-172]. And in directing the tissue morphogenesis by regulating cell migration, proliferation, adhesion and cytoskeletal organization [167].

In neuronal system, Semaphorins- mediated repulsive axon guidance, cell migration, invasive growth and growth cone collapse by several post-translational modifications [173] and oligomerization [174]. Moreover, Semaphorins contribute to cancer progression or suppression by affecting angiogenesis, metastasis, chemotaxis and tumorigenesis [175-176]. In summary, Semaphorins play a crucial role in maintaining physiological homeostasis in many organs and contribute significantly to diseases processes.

4.3. Semaphorin receptors and signaling:

The majority of Semaphorin molecules mediate their effector functions by signaling through Plexins alone. Most Semaphorins bind directly to Plexins, however, depending on cell type; various other membrane-associated proteins can also act as receptors or co-receptors for Semaphorins. This include among others Neuropilins (NRP), vascular endothelial growth factor receptor (VEGFR), CD72, and T-cell immunoglobulin and mucin domain (TIM) [177].

A-Plexins

Plexins are large 200-kDa transmembrane proteins that have been identified in vertebrates (Plexins A1-A4, B1-B3, C1, D1) and two in invertebrates (Plexin A and Plexin B) [178]. The extracellular part of Plexins contains a Sema domain, which is an important portion for binding to Semaphorins [179] followed by three-PSI domains (Plexin-Semaphorin-integrin) and three IPT domains (immunoglobulin, Plexin and transcription factors). PSI domain is a small cystein-rich domain (CDR), which is crucial for protein-protein interactions [180]. IPT domains are required for proper ligand binding to Plexins.

The intracellular domain or cytoplasmic tail of Plexin molecule is highly conserved and plays a crucial role in transmitting the signals following ligand binding. It contains a putative tyrosine phosphorylation sites, but doesn't show any kinase activity. The intracellular domain of Plexin contains glutamine amidotransferases (GTPase)-binding domain and a segmented GTPase-activating protein (GAP) domain, the most important characteristics of that regulates many cellular responses. [181-182-183].

B- Neuropilins:

Two single-pass transmembrane receptors or Neuropilins have been identified in vertebrates: Neuropilin 1 (NRP1) and NRP2 [180]. Most of the Semaphorins signal through Plexins. However, class 3 Semaphorins, except Sema-3E, bind to Neuropilin receptors [176- 177]. These groups of receptors are transmembrane proteins (~900 a.a.) with a short cytoplasmic tail. Due to this characteristic, they lack intrinsic signaling capabilities [184]. The extracellular portion of NRPs contains two repeat complement-binding (CUB) domains (a1 and a2 domains), two coagulation factor-like domains (b1 and b2 domains) and a juxta-membrane meprin/A5/mu-phosphatase (MAM) homology domain

(c domain) [185]. The binding of class 3 Semaphorins to Plexins is facilitated by Neuropilins [183]. Moreover, Neuropilin can act as a core receptor for other Semaphorins with binding domain.

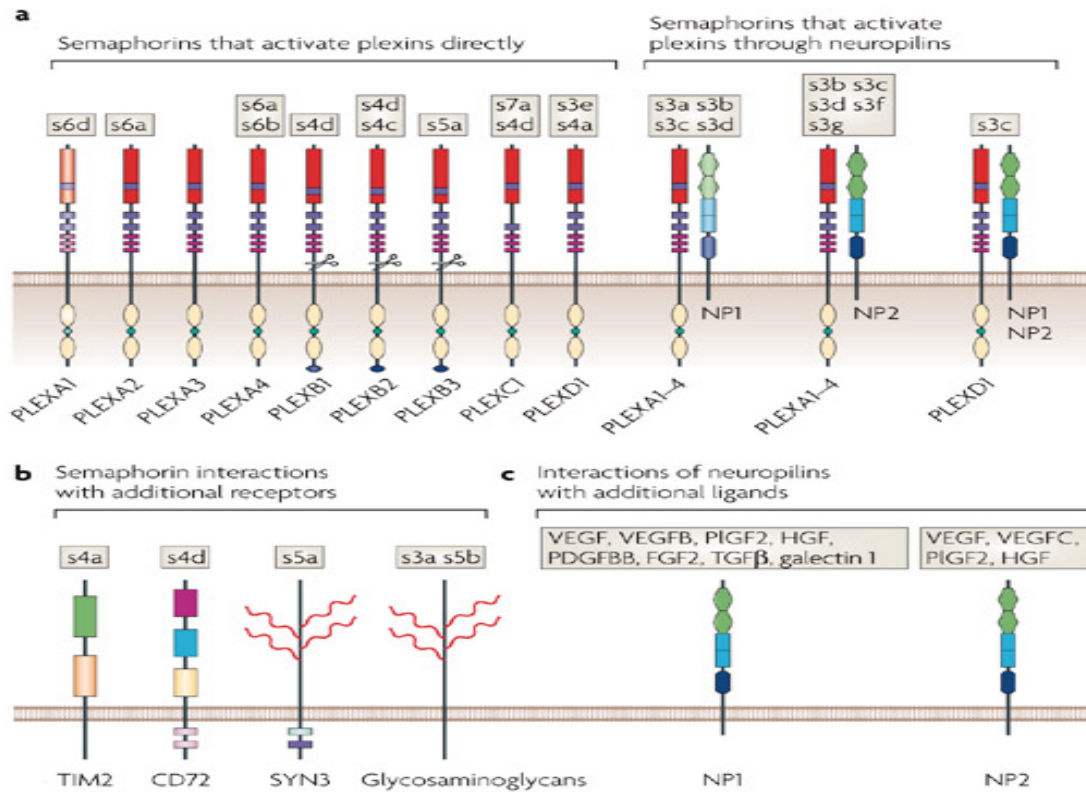


Figure4.3. Semaphorins interact with Plexins and NRP receptors: **a)** The interactions between Semaphorins and Plexins or Neuropilins (NRP) are important to mediate their functions. In the case of the Neuropilins, the Plexins form complexes with these Neuropilins and serve as the signal-transducing elements in response to the binding of the shown Semaphorins are also indicated. **b)** The interactions of Semaphorins with receptors like the T-cell receptor TIM2, the lymphocyte receptor CD72 and proteoglycans. **c)** Neuropilins bind with ligands that do not belong to the Semaphorin family, like (VEGF) gene family are shown [186].

4.4 Class 3 semaphorins:

Class 3 Semaphorin members (**Sema3A–G**) are the only secreted vertebrate Semaphorins [184]. Respectively, SEMA3 family signals through a receptor complex composed of Neuropilin (NRP1) or 2 and type A or D Plexin. Secreted SEMA-3 class contains (from the N- to C-terminus) a seven-blade β -propeller Sema domain, a Plexin–Semaphorin–integrin (PSI) domain, an immunoglobulin (Ig) domain and a conserved short basic domain [187].

Class 3 SEMA has been recognized as key players in immune system, cardiovascular, bone metabolism and neurological system [188-189-190]. For example, Sema-3A was first identified to induce the collapse and paralysis of axonal growth cones from sensory neurons in vitro. Furthermore, Sema-3A regulates bone development through its modulation of sensory innervation, works as immunosuppressive molecule by reducing levels of pro-inflammatory cytokines, such as IFN γ and IL-17, and increasing levels of the anti-inflammatory cytokine IL-10 [191-192]. Another example, in obese mice, Sema-3E has been shown to decrease tissue inflammation and improved insulin resistance [193].

4.4.1 Semaphorin-3E receptors, signaling:

Sema-3E is 85- to 90-kDa protein binds with high affinity to PlexinD1. Sema-3E gene is located on chromosome 7 [194]. In vivo, Sema-3E binds to the intersomitic regions of mouse embryos, where PlexinD1 is expressed [195]. Sema-3E is well studied in cancer and it is up-regulated in metastatic cancer cells[185]. Furin dependent cleavage is exclusive to Sema-3E but not others class sema3 members [196]. . High expression of Sema-3E and PlexinD1 was observed in human colon cancer, liver metastasis, and

melanoma progression [197]. Another study showed Sema-3E promoted invasiveness of tumor cells and inhibited tumor growth. This metastatic potential was dependent on plexinD1 expression but was independent of NRP expression [198].

Sema3/PlexinD1 signaling modulates angiogenic pathfinding by restricting the migration of neuronal growth cones. PlexinD1 intracellular tail contains two highly conserved intracellular domains known as the SEX-PLEXIN domain and SEMA/PLEXIN domains [199]. SEMA/PLEXIN domains of PlexinD1 include two C regions RasGAP domain. Each RasGAP domain includes a short motif of (GTPase)-Activating Proteins (GAPs) and monomeric GTPases of the R-Ras subfamily. A monomeric Rho GTPase-Binding Domain (RBD) is sandwiched between the C regions. PlexinD1 acts as a RasGAP to antagonize both integrin-mediated cell extracellular matrix (ECM) adhesion and PI3K a modulator of cell survival, growth and migration signaling. Rho family GTPase 2 (Rnd2) is required for the activation of the RasGAP activity of PlexinD1. P61Sema-3E requires both PlexinD1 and Rnd2 to exert its inhibition of tumor vascularization. Upon Sema-3E-PlexinD1 stimulation, pre-existing PlexinD1-Rnd2/RLG (resistance-nodulation-division/Release Guard signal) complexes undergoes Rnd2/RLG-dependent intracellular conformation that change translates the concentration and distribution of extracellular Sema-3 cues into an intracellular gradient of distinct PlexinD1 activities [200].

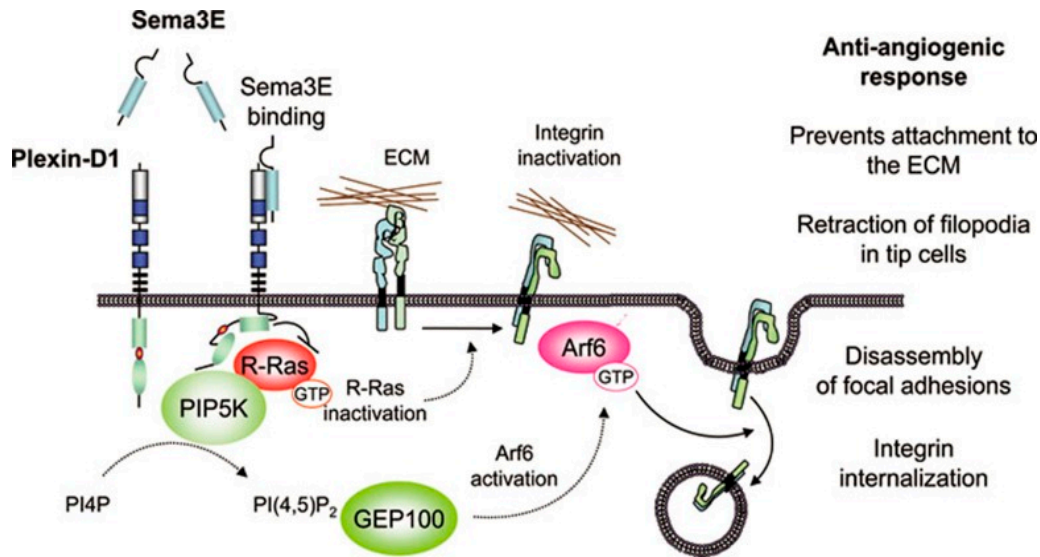


Figure4.4.1. Sema-3E/PlexinD1 signaling: The activation of PlexinD1 by Sema-3E induces the association of the Ras GAP domain of plexinD1 with R-Ras. The activation of Arf6 mediated by the inactivate integrins and enables their subsequent internalization by the PlexinD1. Phosphatidylinositol-4-phosphate-5-kinase β activation leads to promote Arf6 activation, likely by stimulating Arf6 GEFs. This results in the inactivation of integrins and their subsequent internalization, respectively, thus inhibiting endothelial cell adhesion to the ECM by disrupting integrin-mediated adhesive structures and causing filopodial retraction in endothelial tip cells, thereby inhibiting angiogenesis [201].

4.4.2 Immunological Phenotypes of Sema-3E^{-/-} mice:

Breeding pairs of Sema3E knockout mice were kindly provided by Chenghua Gu, Harvard medical School, Boston [202]. Dr. David Ginty at Johns Hopkins University School of Medicine developed these mice by disrupting the first exon of *Sema3E* gene. Immunophenotyping of Sema3E KO mice reveal no major immunological defects. In brief, Sema-3E deficient mice showed no difference in T cells, T reg, B cells and NKT cells numbers within the spleen or lungs (Drs. Hesam Movassagh and Ifeoma Okwor, personal communication). However, total cells numbers as well as expression of CD11c were increased in Sema3E KO bone marrow derived dendritic cells compared to wild type.

5. Gap of Knowledge, Hypothesis and Aims:

5.1. Gap of knowledge, rationale

The accumulated data documented the importance of NK/DC interactions in shaping of the innate and adaptive immunity [143]. These interactions depend on cell: cell contact and cytokine signals, and occur in lymphoid and non-lymphoid tissues [203]. NK cells can either promote iDC maturation or NK-mediated lysis of iDC during a “DC editing” process [150]. On the other hand, dendritic cells produce several cytokines (IL-15, IL-12/IL-18, IFN- α/β ,) that specifically stimulate NK cells to proliferate, produce cytokines and acquire cytotoxicity. A better understanding of factors that regulate NK and/or DC cells in NK/DC crosstalk will enable us to better manipulate NK-cell immune responses in anti-tumor and/or anti-viral immunity.

Semaphorin-3E (Sema-3E) is an axon-guidance secreted protein in neuronal system that has emerged as an essential mediator involved in cell migration and proliferation. However, a role of Sema-3E and its receptors in regulating effector functions (cytotoxicity and cytokine production) and migratory responses of NK cells has not been reported. As migratory properties of NK cells are less studied, and that Sema-3E has been reported to regulate migrations of neural cells [204], I focused on studying the roles of Sema-3E/Plexin D1 in regulating NK-cell migrations in the context of NK- DC crosstalk.

5.2. Global Hypothesis:

Semaphorin3E is a novel factor that regulates NK-cell migration in NK-DC crosstalk.

5.3. Overarching Goals and specific aims:

The overarching goal of this thesis is to elucidate whether Sema-3E exerts a direct or indirect effect on NK-cell migrations in NK-DC crosstalk.

Specific Aims:

- 1- To determine the expression of Sema-3E and its receptors (PlexinD1 and NRP1) on NK and DC cells.
- 2- Examine the migratory properties of activated NK cells in the presence of recombinant Sema-3E alone or in the context of NK-DC crosstalk.

2.0 CHAPTER 2

GENERAL MATERIALS AND METHODS

1. Ethics statement:

Natural Killer and dendritic cells were obtained from Sema-3E^{+/+} or Sema-3E^{-/-} BALB/c mice from GMC animal house at University of Manitoba, Winnipeg, Canada. All mice were maintained in Animal Care facility, the University of Manitoba under pathogen free conditions and used according to the guidelines specified by the Canadian Council for Animal Care. Parent breeders of these animals were gifted by Dr. F. Mann, Université de la Méditerranée, Marseille, France. Research ethics boards of the University of Manitoba, Winnipeg, Canada, approved the current study.

2. Reagents:

Mouse NK cells isolation kit was purchased from StemCell Technologies (Vancouver, BC). Sema-3E, PlexinD1 and NRP1 primers were designed by Integrated DNA Technologies Inc. Mouse recombinant Sema-3E-Fc protein was gifted from Dr. Jonathan Duke-Cohan, Dana Farber Cancer Institute, Harvard Medical School). Monoclonal human anti-Sema-3E, PlexinD1, and NRP1 were purchased from R&D Systems (Minneapolis, MN).

Table2. 1 A summary of used NK and DCs antibodies, their clones and source

Serial No	Antibody	Clone	Company
1	CD49b (APC)	DX5	eBiosciences
2	CD3 (PE)	17A2	eBiosciences
3	CD40 (APC)	1C10	eBiosciences
4	CD86 (PE)	GL1	eBiosciences
5	CD80 (PE)	16-10A1	eBiosciences
6	MHC-class II (PE)	M5/114.15.2	eBiosciences
7	CD11b (APC)	M1/70	BD Pharmingen

3. Natural Killer purification:

Primary NK cells (CD3⁻DX-5⁺) were purified from mouse spleen. The obtaining of splenocytes was from homogenized spleen by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Sweden) following the manufacturer's described protocol. Primary NK cells were enriched using the EasySep mouse NK negative selection kit (StemCell Technologies, Vancouver, BC). NK cells cultured at 37C and 5% CO₂ in mouse medium containing 10% fetal bovine serum (FBS from Hyclone), 1% PSG (invitrogen), 1.6 mmol/l 2-mercaptoethanol (2-ME) and IL-2 (1000U/ml IL-2 concentration was used in all experiments). The purity of NK cells was checked before the experiments (see supplementary figure 7.1).

4. Primary mouse bone marrow derived dendritic cells:

The bone marrow precursor cells were stimulated to generate mature dendritic cells [205]. Precursor cells were extracted from the femur and tibia and incubated with ACK buffer for 2-minutes to lyse red blood cells. $0.5-1 \times 10^6$ BM cells 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 20ng/ml GM-CSF (Peprotech). On day 3, one third of the culture medium was aspirated to remove non-adherent cells and supplemented with fresh GM-CSF containing medium. On day 5th, cultures were replenished with fresh GM-CSF medium while maintaining total volume 1-ml/well. On day-8, lipopolysaccharide (LPS- and Polyinosinic:polycytidylic acid (Poly I:C from Sigma) both at 1 μ g/ μ l were introduced in the culture for 24-hours to acquire matured DC-phenotype. DCs with or without LPS or Poly I:C treatment and the corresponding culture conditioned media were used in various combinations and settings throughout these experiments. The expression of CD40, CD80 and CD86 surface markers represent matured-DC phenotype. Throughout the project all mature and immature culture cells were phenotype before the experiments (see supplementary figure 7.2).

5. Conventional PCR:

Analysis Total RNA was extracted from NK or DCs by using TRIzol (Invitrogen, Life Technologies, Cat #: 15596026, CA, USA) according to manufacturer's protocol. RNA concentration was measured using BioPhotometer (Eppendorf AG, Hamburg, Germany). To synthesize cDNA, reverse transcription was performed with 2 μ g of total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA, Cat #:

4368814) in a total volume of 20 μ L according to the manufacturer's protocol. cDNA of each sample and sequence specific Sema-3E, PlexinD1, NRP1 and GAPDH primers (10 μ M) were added to Master Mix (Applied Biosystems, USA, Cat #: 4472908). PCR was performed in small tubes using Mastercycler gradient for 25 cycles. All samples including 100kb DNA ladder were loaded to 2% agarose gel electrophoresis running into 1%TEA buffer for 25 minutes with 90 voltages. The gel image was taking by Alphaimager EC (Thermo scientific).

6. Quantitative Real-Time PCR:

Real-Time PCR was performed in 96-well optical plate with an initial 1 cycle denaturation step for 10 minutes at 95°C, 40 cycles of PCR (95°C for 15 s, 60 °C for 35 s and 72°C for 35 s), 1 cycle of melting and 1 cooling cycle (Applied Biosystems 7500 Real-Time PCR system). Average data collection and detection of fluorescent products were performed at the end of the 72°C extension period. Performing melting curve analysis and examining the quality of amplification curves assessed products specificity. Normalizing to the amplification of GAPDH and then normalizing to control groups calculated the amplification of target genes. Then the normalized values were expressed as fold increase / decrease of relative quantitative (RQ) over the values calculated with other groups.

Table6. Conventional PCR and Real-Time PCR primers (forward and reverse) and size of the amplification

Gene	Forward (5'- 3')	Reverse (5'- 3')	Size (bp)
Sema-3E	5'- AAAGCATCCCCAACAACTG-3'	5'- CTGGCTCGAGACCCTTACTG-3'	124
PlexinD1	5'-TGGATGTCGCAGCTTACTTG-3'	5'- CCCCACCCACAGTTCTCTA-3'	325
NRP1	5'- TATCCCAGAACTCTGCCC-3'	5'- TGTCATCCACAGCAATCCCA-3'	546
GAPDH	5'-AACTTTGGCATTGTGGAAGG-3'	5'-ACACATTGGGGGTAGGAACA-3'	217

7. Surface and Intracellular staining:

NK and DC cells were incubated with Fc-blocker (ebiosciences) in flow tube for 10 minutes on ice. For surface staining, NK cells were stained with DX5⁺ mAb (10 ug/ml) and/or Sema-3E, PlexinD1 and NRP1 fluorochrome-conjugated monoclonal antibodies all at (10ug/ml) for 30 minutes on ice. However, DCs were stained with CD11c monoclonal antibody and/or Sema-3E, PlexinD1 and NRP1 fluorochrome-conjugated monoclonal antibodies on ice. After washing with flow buffer, cells were fixed with 2% para-formaldehyde (PFA).

For Intracellular staining, fixed and surface stained cells were permeabilized with 0.1% saponin (Sigma-Aldrich) in flow buffer and then stained with Sema-3E, PlexinD1 or NRP1 fluorochrome-conjugated monoclonal antibodies for 30 minutes on ice. Samples acquisition was performed on a FACSCanto II (BD Biosciences) using Diva software and data was analyzed using FlowJo software.

8. Transwell based migration and cytokine/chemokine analysis:

NK cell chemotaxis/chemo-repulsion against mouse medium, immature, LPS or PIC matured conditioned medium was performed in the transwell system. 0.2×10^6 murine IL-2 activated NK cells (in 100 μ l) were loaded on the upper chamber (5 μ m pore Trans-well insert), whereas 600 μ l mouse medium (or conditioned medium) was placed in the lower chamber and incubated at 37C. In some experiments, Recombinant Sema-3E (6, 12, 25, 50, 100, or 200 ng/ml) was added to mouse medium or conditioned medium. After 90 minutes, the migrated cells in the lower well were transferred to a polypropylene tube, centrifuged at 300g for 10 minutes then counted. Migrated cells counted as absolute number of input cells [206].

9. Microfluidic device based NK cell migrations assays:

9.1 Microfluidic device and gradient generation:

Simple “Y” shape microfluidic device was used for cell migration experiments in this study. Using SU-8 photo resist (Micro Chem, MA), the design was patterned on a silicon wafer by photolithography. Using this pattern, PDMS (Polydimethylsiloxane) replicas were fabricated by molding PDMS (Sylgard 184 silicon elastomer, Dow Corning, MI) against the master. The inlets and outlet are punched. The devices were bonded against glass slides and polyethylene tubing (PE-20, Becton Dickinson, MD) was inserted into the inlet holes to connect the microfluidic device to syringe pumps (Model V6, Kloehn, Inc., NV). Mouse medium and (recombinant Sema-3E (50 ng/ml) were diluted in RPMI-1640/0.4% BSA. FITC-Dextran 10 KDa that has similar molecular weight of the chemokine molecule was added to the supernatants /chemokine solution. The migration medium and

supernatants/chemokine solutions were continuously infused into the device by syringe pumps through tubing and the inlets of the device at the total flow rate of 0.2 ml/min. The gradient was confirmed by measuring the fluorescence intensity profile of FITC-Dextran inside the microfluidic channel and the cells were imaged at 3mm downstream of the “Y” junction [207- 208].

9.2 Cell migration in the microfluidic platform:

To attach the NK cells, the microfluidic channel was coated with fibronectin (BD Biosciences) for hour at room temperature and blocked with 0.4% BSA in RPMI for 1 hour before the experiment. 0.4×10^6 cells were loaded into the microfluidic device and allowed to settle in the fibronectin-coated channel for five minutes. The temperature of the device was maintained at 37C. Mouse medium and Sema-3E solution was infused into the device by syringe pumps through tubing and the inlets of the device. The device was placed on a microscope stage (Model No. BX60, Olympus). Cells migrations were recorded by time-lapse microscopy at 6 frames/min for 19 to 44 min using a CCD camera (Model No. 370 KL 1044, Optikon, Canada). The acquisition of the images was controlled by NIH ImageJ program (v.1.34s) [206- 207].

9.3 Microfluidic derived data analysis:

Using NIH ImageJ (v.1.34s), individual cell movement was recorded. Only the cells that migrated within the microscopic field were selected and tracked using the “Manual Tracking” plug-in in NIH ImageJ. The quantitative parameters of cell migration such as chemotaxis percentage, chemotactic index (CI) (the ratio of the displacement of cells

toward the chemokine gradient (dy) to the total migration distance (d) using the equation $CI = dy/d$, and average speed (v) (calculated as d/dt) were quantified. Statistical analysis of migration angles was performed using Origin 8.5 software to examine the directionality of the cell movement. Specifically, migration angles (calculated from x-y coordinates at the beginning and the end of the cell tracks) were summarized in a direction plot, which is a rose diagrams. These diagrams show the distribution of angles grouped in defined intervals with the radius of each wedge indicating cells numbers. Two-three independent experiments were repeated for each condition [206- 207].

10. Statistics:

Data were analyzed statistically using GraphPad Prism (commercial scientific graphing and statistic software published by GraphPad Software, Inc). Results are shown as the mean \pm SEM. Two-tailed student's t-test was used in a single, two-group comparison data. One-way ANOVA was used for comparing data from more than two groups in all trans-well migration assays. A p-value of <0.05 was considered statistically significant.

3.0 CHAPTER 3

This chapter examined the expression of Sema-3E and its receptors (PlexinD1 and NRP1) in NK and DCs at mRNA and/ or protein levels. NK cells were isolated from spleens of Sema-3E^{+/+} or ^{-/-} BALB/c animals (resting, inactivated NK cells). They were used either as “resting” NK cells or further activated in IL-2 for 4 days (activated NK cells) before the experiments. Immature DCs were generated from bone marrow cells under GM-CSF. Mature DCs were prepared by stimulating immature DC with LPS or Poly I:C overnight.

Results:

3.1. Resting and/or IL-2 activated NK cells express Sema-3E protein in vitro

I isolated total RNA from resting NK or IL-2 activated NK cells for cDNA preparations. Sema-3E mRNA expression was analyzed by conventional PCR and qPCR using Sema-3E specific primer. GAPDH was used as an internal control in all PCR analyses. I used 4T1 breast cancer cells and Sema-3E^{-/-} activated NK cells as positive and negative control, respectively. In conventional PCR, the Sema-3E primers were shown to be specific, as I detected no Sema-3E mRNA expression in the Sema-3E^{-/-} NK cells and high level of Sema-3E mRNA expression (124 bp PCR fragment) in 4T1 cell. I observed that resting or IL-2 activated NK cells expressed low level of Sema-3E mRNA transcripts (Figure 3.1).

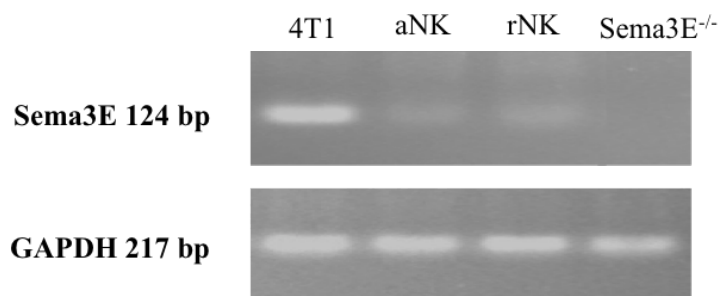


Figure3.1. Low levels of Sema-3E mRNA expressions in resting and IL-2 activated NK cells. Resting NK cells (rNK), the cells were collected right after isolation. Activated NK cells (aNK), the cells were activated in IL-2 for 4 days. Sema-3E^{-/-}, NK cells were isolated from Sema-3E^{-/-} mice and further activated in IL-2 for 4 days (negative control). All previous samples were isolated from BALB/c mice spleen. 4T1 cell was included as a positive control. RNA was extracted by using TRIzol method. Furthermore, cDNA was generated for each sample and mixed with target primers (Sema-3E or GAPDH). The samples were loaded to the gel after PCR performed.

Next, I used real-time PCR (qPCR) to further quantify the mRNA expression of Sema-3E in the resting or IL-2 activated NK cells. Sema-3E^{-/-} activated NK cells were used as a negative control. The activation of NK cells with IL-2 down regulates Sema-3E expression at mRNA (P value = 0.0006) (Figure 3.2). The dissociation curve following RT-qPCR confirmed the amplicon specificity. A single peak in the melting curve analyses for sema-3E primer referred to high specificity (Figure 3.13 A)

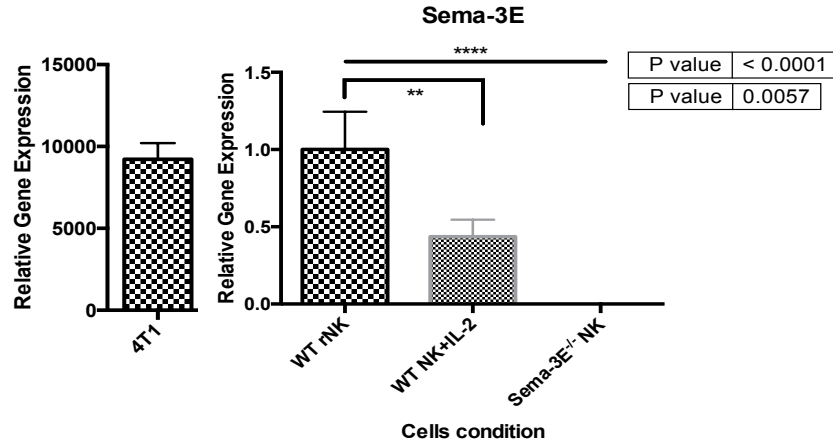


Figure3.2. NK cells express different mRNA levels of Sema-3E. Further confirmation by qPCR showed different level of Sema-3E expression in resting (NK), activated NK cells (NK+IL-2) and activated NK cells obtained from Sema-3E^{-/-} mice (Sema-3E^{-/-}) (negative control). All samples were isolated from BALB/c mice spleen. 4T1 cell lines were used as positive control. RNA was extracted by using TRIzol method. Furthermore, cDNA was generated for each sample and mixed with target primers (Sema-3E). In qPCR, I used $\Delta\Delta CT$ method. All groups were normalized to control group (untreated cells WT rNK). Using GraphPad Prism preformed the statistical analysis for n value=3. Two-tailed student's t-test was used in a single, two-group comparison data (P ** = 0.0057). One-way ANOVA was used for comparing data from than three groups (P **** < 0.0001).

To examine Sema-3E at protein level, I used Sema-3E specific antibodies in flow cytometry using standard surface and intracellular staining procedures. Sema-3E^{-/-} activated NK cells (Sema-3E^{-/-}) was used as a negative control and 4T1 cell as a positive control. NK cells were identified by CD3⁻DX5⁺ staining. Samples acquisition was performed on a FACSCanto II and analyzed using FlowJo software (Figure 3.3A). I observed low level of Sema-3E staining in resting NK cells both at surface and intracellularly. The activation of NK cells with IL-2 down regulates Sema-3E expression on surface and intracellular (Figure 3.3B).

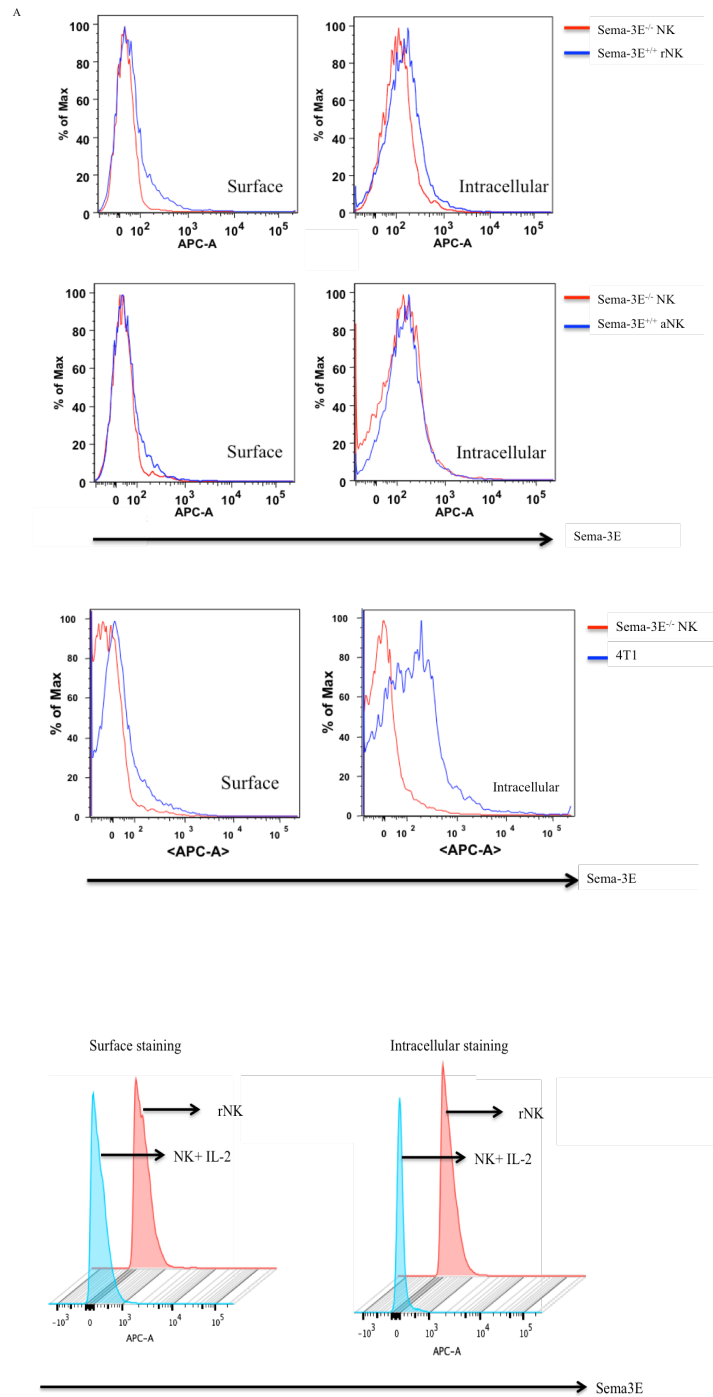


Figure3.3 Sema-3E protein was detected on the surface and intracellularly in the resting (rNK) and IL-2 activated NK cells (aNK) and down-regulated upon IL-2 activation. A: Resting NK cell (rNK), the cells were stained after isolation. Activating NK cells (aNK), the cells were collected after culture with IL-2 for 4 days. 0.2×10^6 NK cells were counted in this experiment. We used APC anti-human Sema-3E mAb (cross-reactive to mouse Sema-3E) to detect Sema-3E protein on the surface and intracellular.

Histogram was used to compare the expression of Sema-3E to Sema-3E^{-/-} activated NK cells. **B:** In this experiment, (rNK) NK cell has not activated. (aNK) NK cell was activated with IL-2 for 4 days. IL-2 (1000u/ml) concentration was used to activate NK cells. I used APC anti-human Sema-3E mAb to detect surface and intracellular Sema-3E protein expression on the surface and intracellular. Histogram was used to compare the expression of Sema-3E by rNK to aNK.

3.2 Resting and/or IL-2 activated NK cells express Sema-3E receptors (PlexinD1 and Neuropilin 1) in vitro

To investigate the expression of PlexinD1 and NRP1 receptors in NK cells, I isolated mRNA of resting NK or IL-2 activated NK cells. cDNA samples were processed by conventional PCR or qPCR using PlexinD1 and NRP1 primers. At protein level, I used FACS staining using mAbs anti-human PlexinD1 or NRP1.

In conventional PCR, the PlexinD1 and NRP1 primers were shown to be specific, as I detected no PlexinD1 or NRP1 mRNA expression in the negative control (RNA's free water) and high level of PlexinD1 and NRP1 mRNA expression in immature dendritic cells (see DCs expression analysis (figure 3.10)). The expression of PlexinD1 (Sema-3E receptor) was observed on resting and activated NK cells at mRNA level (325 bp). NRP1 (Sema-3E co-receptor) was observed on resting but down-regulated in cells activated NK cells at mRNA level (546 bp) (Figure 3.4).

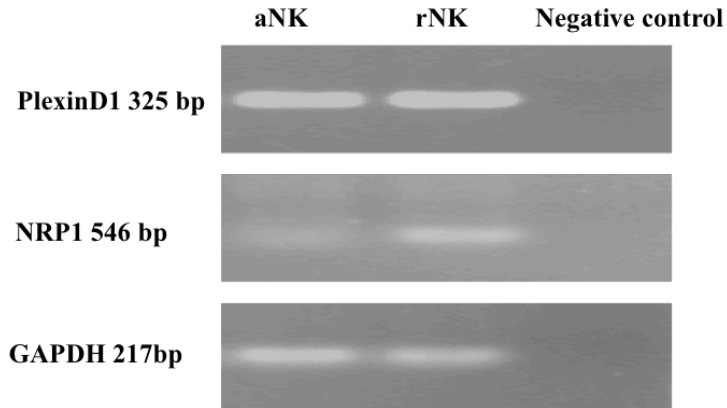


Figure3.4. Resting and IL-2 activating NK cells express Sema-3E receptors (PlexinD1 and NRP1). Conventional PCR showed inactivated NK cells express PlexinD1 and NRP1 at mRNA transcript. However, IL-2 activated NK cells showed the expression of PlexinD1 but not NRP1 transcripts. Resting NK cells (rNK), the cells were collected right after isolation. Activated NK cells (aNK), the cells were activated in IL-2 for 4 days. NK cells were isolated from BALB/c mice spleen.

I used real-time PCR (qPCR) for further quantify the mRNA expression of PlexinD1 and NRP1 in the resting and IL-2 activated NK cells. My data showed the expression of PlexinD1 and NRP1 in the resting NK cells. Sema-3E receptors (PlexinD1 and NRP1) were down-regulated upon IL-2 activation (P value = 0.0119) (P value = 0.0003) (Figure 3.5). The dissociation curve following RT-qPCR confirmed the amplicon specificity. A single peak in the melting curve analyses for PlexinD1 and NRP1 primers referred to high specificity (Figure 3.13 A&B)

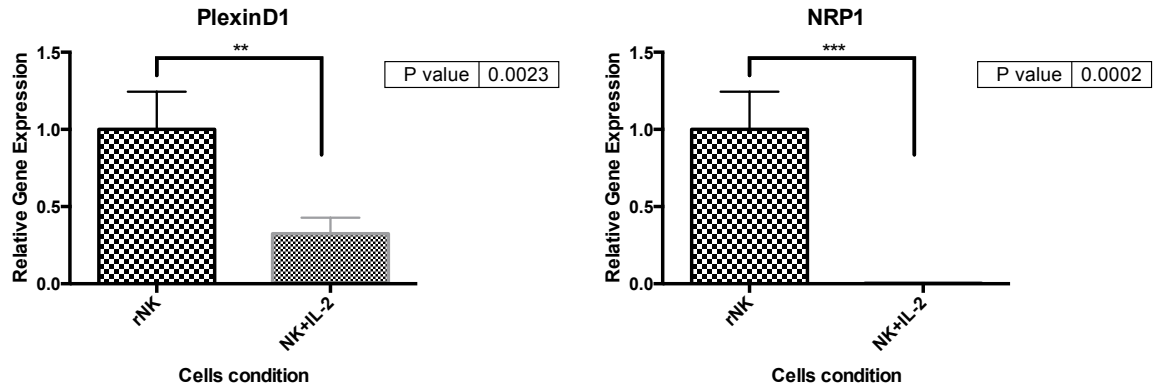
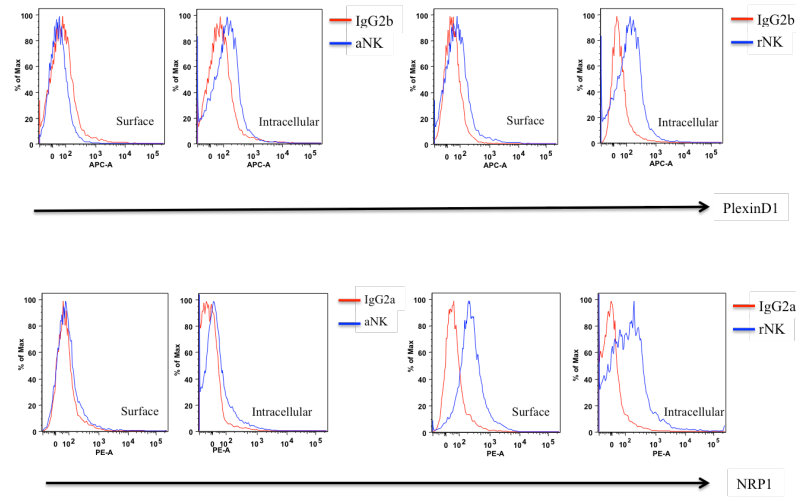


Figure 3.5 Resting NK cells express PlexinD1 and NRP1 but down-regulated upon IL-2 activation.

qPCR showed Sema-3E receptors (PlexinD1 and NRP1) expression in resting (NK) and activated NK cells (NK+IL-2). All samples were isolated from BALB/c mice. RNA was extracted by using TRIzol method. Furthermore, cDNA was generated for each sample and mixed with target primers (PlexinD1 or NRP1). In qPCR, I used $\Delta\Delta CT$ method. All groups were normalized to control group (untreated cells rNK). Using GraphPad Prism preformed the statistical analysis for n -value = 3. Two-tailed student's t-test was used in a single, two-group comparison data (P ** 0.0023) and (P *** = 0.0002).

At protein level, I used mAb anti-human PlexinD1 and NRP1, I was able to detect the both receptors on the cells surface and intracellular of the resting NK cells (rNK) and IL-2 activated NK cells (aNK). I used IgG2b in this setting as PlexinD1 isotype control. However, IgG2a isotype control was used for NRP1 (Figure 3.6A). The activation of NK cells with IL-2 down-regulates PlexinD1 and NRP1 expression at protein levels (Figure 3.6B).

A



B

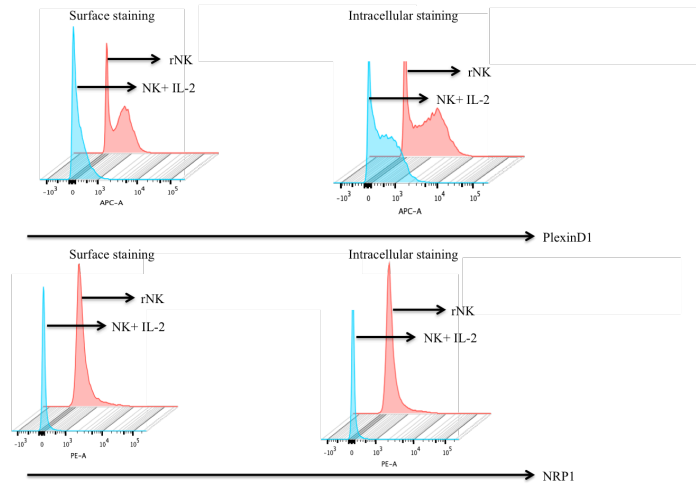


Figure3.6. Sema-3E receptors were detected in the surface and intracellular of both resting (rNK) and IL-2 activated NK cells (aNK) and down-regulated upon IL-2 activation. A: Resting NK cell (rNK), the cells were stained after isolation. Activating NK cells (aNK), the cells were collected after culture with IL-2 for 4 days. 0.2×10^6 NK cells were counted in this experiment. We used APC mAb anti-human Sema-3E to detect PlexinD1 and NRP1 proteins on the surface and intracellular. Histogram was used to compare the expression of PlexinD1 and NRP1 to Isotype controls. **B:** (rNK) NK cell has not activated. (aNK) NK cell was activated with IL-2 for 4 days. IL-2 (1000u/ml) concentration was used to activate NK cells. We used APC mAb anti-human PlexinD1 or NRP1 to detect Sema-3E protein on the surface and intracellular. Histogram was used to compare the expression of Sema-3E by rNK to aNK.

3.3 In vitro, Sema-3E protein is expressed by immature and mature DCs

In our study, NK cells have been shown to express Sema-3E, PlexinD1 and NRP1. However, the interaction between NK cells and DCs has involvement of different factors. Sema-3E one of the factor that I hypothesize has an effect on NK-DC crosstalk. For further investigations about the role of Sema-3E in NK-DC interaction, I asked if dendritic cell does express Sema-3E and its receptors (PlexinD1 and NRP1). mRNA samples were obtained and used for conventional PCR and qPCR using Sema-3E primers. I used FACS staining using mAbs anti-human Sema-3E for studying protein level.

In conventional PCR, Sema-3E was observed in immature and/or mature DCs stimulated with LPS or Poly:IC at mRNA level (124 bp). In the experiment setting, I used generated Sema-3E^{-/-} immature BMDC cells (KO iDC) as a negative control where no signal found and breast cancer cell line (4T1) as a positive control where high signal detected (Figure 3.4.1).

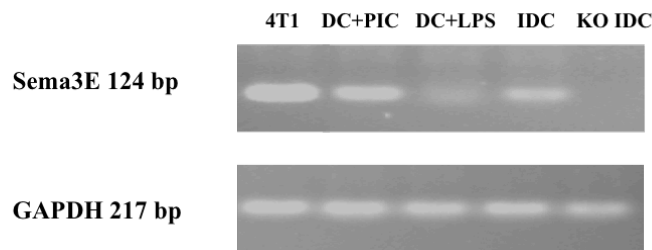


Figure3.7. Unstimulated or stimulated DCs with Ploy I:C express Sema-3E which down-regulated upon LPS stimulation. Conventional PCR showed unstimulated and stimulated DCs are differently express Sema-3E at mRNA level. The experiment setting includes KO IDC (negative control) and 4T1 (positive control). Immature dendritic cells (iDC), the cells were generated from bone marrow and culture with GM-SCF for 7 days. However, stimulated dendritic cells by LPS (1µg/ml) or Poly:IC (1µg/µl) for overnight were collected. All samples were isolated from BALB/c mice bone marrow. RNA was extracted and cDNA was generated for each sample and mixed with target primers (Sema-3E or GAPDH). The samples were loaded into the gel after PCR performed.

Next, I used real-time PCR (qPCR) to further quantify the mRNA expression of Sema-3E in immature or mature dendritic cells. Sema-3E was down-regulated upon LPS (1µg/ml) or PIC (1µg/µl) stimulations (P value = 0.0163). Immature DCs generated from Sema-3E^{-/-} (Sema-3E^{-/-} iDC) were used as a negative control. Moreover, breast cancer cell line (4T1) was included as a positive control (Figure 3.8). The dissociation curve following RT-qPCR confirmed the amplicon specificity. A single peak in the melting curve analyses for sema-3E primer referred to high specificity (Figure 3.13 A)

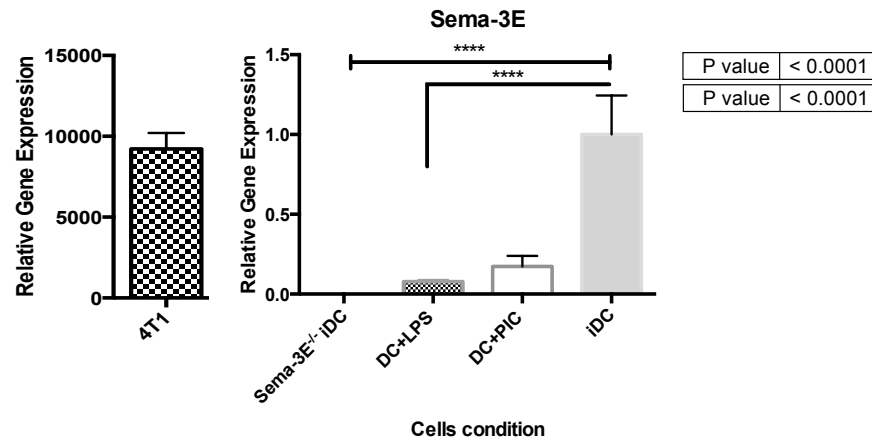
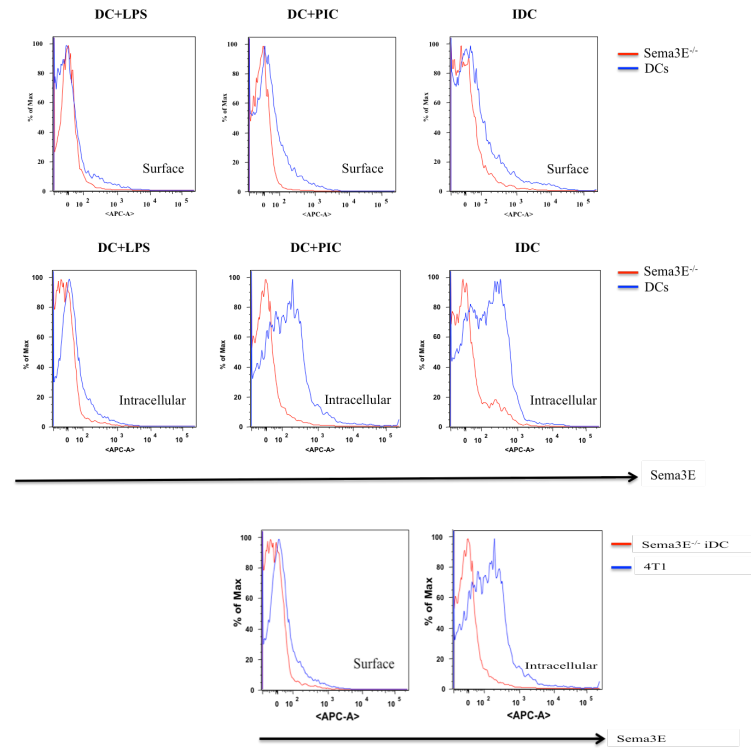


Figure3.8. Unstimulated or stimulated DCs express different quantity of Sema-3E. Significantly, qPCR showed the down regulation of Sema-3E upon the stimulation of DCs by LPs (1µg/ml) or PIC (1µg/µl) (P **** < 0.0001). cDNA was generated for each sample and mixed with target primers (Sema-3E). I used $\Delta\Delta$ CT method. All groups were normalized to control group (untreated cells iDC). The statistic figure obtained from 3 combined experiments. One-way ANOVA was used for comparing data for three groups.

Sema-3E protein using mAbs anti-human Sema-3E was detected on the cell surface and intracellular. I used Sema-3E^{-/-} immature DC cells (Sema-3E^{-/-}) as a negative control and 4T1 as a positive control. Dendritic cells were stained with CD11c⁺ monoclonal antibody and Sema-3E fluorochrome-conjugated monoclonal antibodies (Figure 3.9A). Interestingly, the stimulation of dendritic cells with LPS (1µg/ml) or Poly:IC (1µg/µl) down regulates Sema-3E expression on surface and intracellular (Figure 3.9B).

A



B

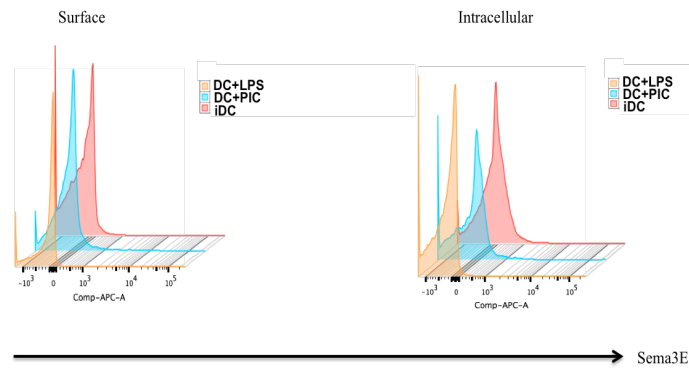


Figure3.9. Sema-3E protein was detected in the surface and intracellular of the immature dendritic cells (iDC) and down-regulated upon TLRs stimulation. A: Sema-3E protein was detected on the surface and intracellular of iDC and/or stimulated DCs by using APC mAb anti-human Sema-3E. Sema-3E protein expression was down -regulated upon LPS stimulation. 0.1×10^6 DC cells were counted and used APC mAb anti-human Sema-3E to detect Sema-3E protein on the surface and intracellular. **B:** On surface and intracellular, immature DCs express higher level of Sema-3E protein compared to mature DCs. In this experiment, (iDC) DC cell has not stimulated. (DC+LPS or DC+PIC) DC was stimulated with LPS (1 μ g/ml) or Poly:IC (1 μ g/ μ l) for overnight. I used APC mAb anti-human Sema-3E to detect Sema-3E protein on the surface and intracellular.

3.4 Immature and mature DC cells express Sema-3E receptors (PlexinD1 and Neuropilin 1)

In conventional PCR, PlexinD1 (Sema-3E receptor) and NRP1 (Sema-3E co-receptor) expression were observed on immature and/or mature DC cells at mRNA level (325 bp). The PlexinD1 and NRP1 primers were shown to be specific, as I detected no PlexinD1 or NRP1 mRNA expression in the negative control (RNA's Free Water) and high level of PlexinD1 and NRP1 mRNA expression in immature dendritic cells (positive control)(Figure 3.10).

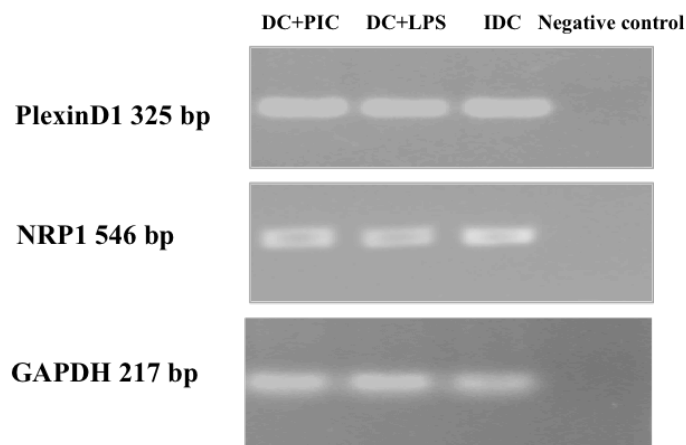


Figure3.10. Dendritic cells express PlexinD1 and NRP1 receptors. Conventional PCR showed unstimulated and stimulated DCs are expressing PlexinD1 and NRP1 at mRNA level. This experiment includes negative control (RNA's free water) and Immature dendritic cells (positive control). All samples were collected for cDNA generation after RNA extraction.

For further quantify the mRNA expression of PlexinD1 and NRP1 in immature or mature dendritic cells, I used real-time PCR (qPCR). PlexinD1 receptor and NRP1 co-receptor were expressed by immature (IDC) and mature DCs. However, upon stimulation with LPS (1 μ g/ml) or Poly:IC (1 μ g/ μ l) the expression level of PlexinD1 receptor and

NRP1 co-receptor were down regulated (P value = 0.0013) (P value = 0.0001). Immature DCs generated from Sema-3E^{-/-} (Sema-3E^{-/-} iDC) and breast cancer cell line (4T1) were included (Figure 3.11). The melting curve following RT-qPCR confirmed the amplicon specificity. A single peak in the melting curve analyses for both PlexinD1 and NRP1 primers referred to high specificity (Figure 3.13 A&B).

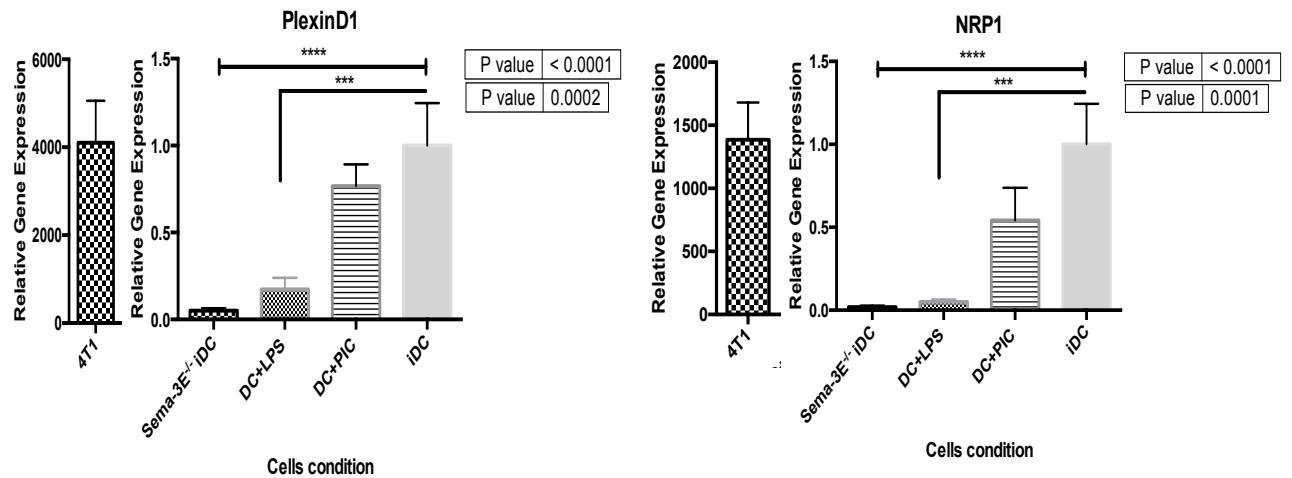
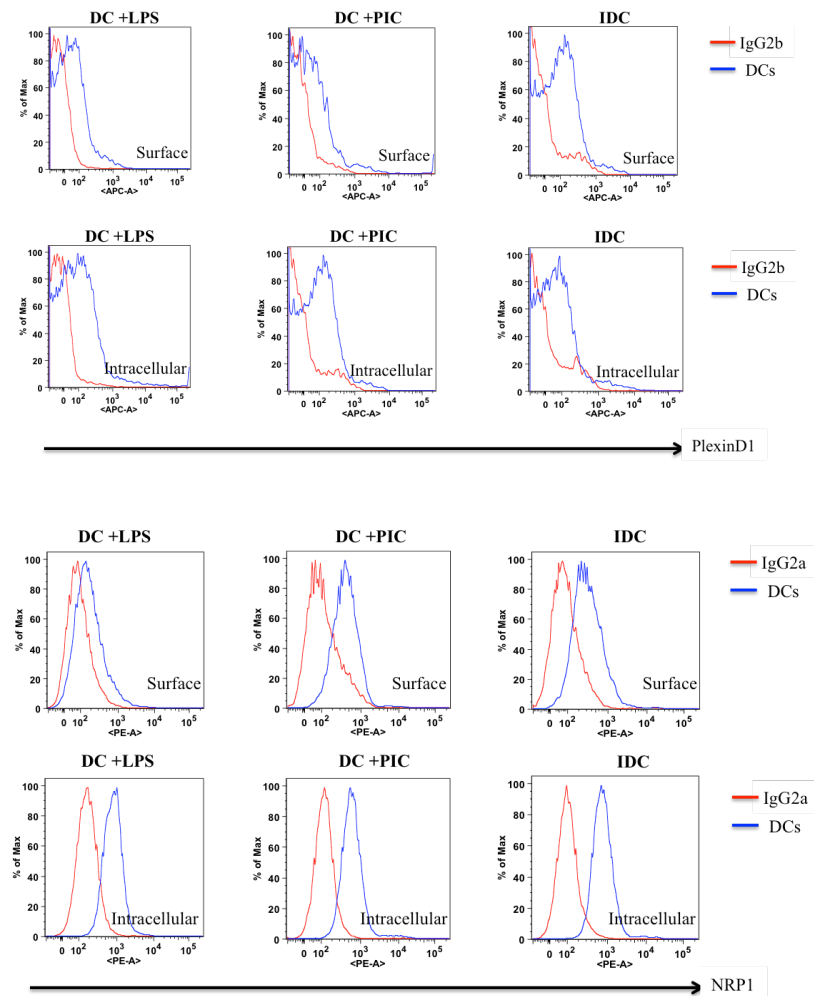


Figure.3.11. Dendritic cells are differently express PlexinD1 and NRP1. qPCR showed the confirmation of PlexinD1 and NRP1 receptors expression in immature and mature DCs. All samples were isolated from BALB/c mice bone marrow. RNA was extracted by using TRIzol method. cDNA was generated for each sample and mixed with target primers (PlexinD1 or NRP1). $\Delta\Delta CT$ method was used for all groups that normalized first to GAPDH then to control group (untreated cells iDC). Using GraphPad Prism preformed the statistical analysis. One-way ANOVA was used for comparing data from more than two groups (P **** <0.0001), (P *** = 0.0002) and (P *** = 0.0001). N value=3.

At protein level, I used mAb anti-human PlexinD1 or NRP1 and I was able to detect the both receptors on the cells surface and intracellular of immature DC cells (iDC) and mature DC cells (DC+LPS or DC+PIC). I used IgG2b in this setting as PlexinD1 isotype control. However, IgG2a was used for NRP1 (Figure 3.12 A). Dendritic cells stimulation with LPS (1 μ g/ml) or Poly:IC (1 μ g/ μ l) down-regulates PlexinD1 and NRP1 expression (Figure 3.12 B).

A



B

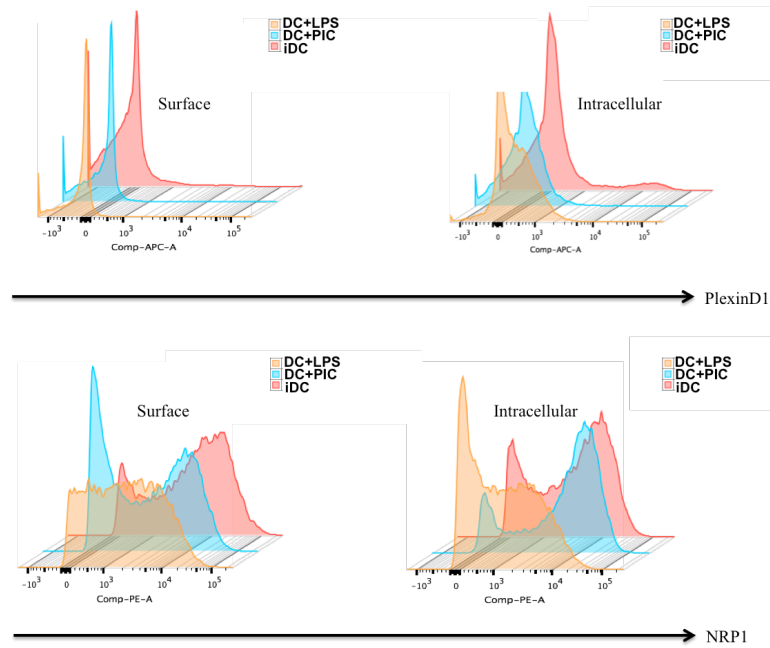
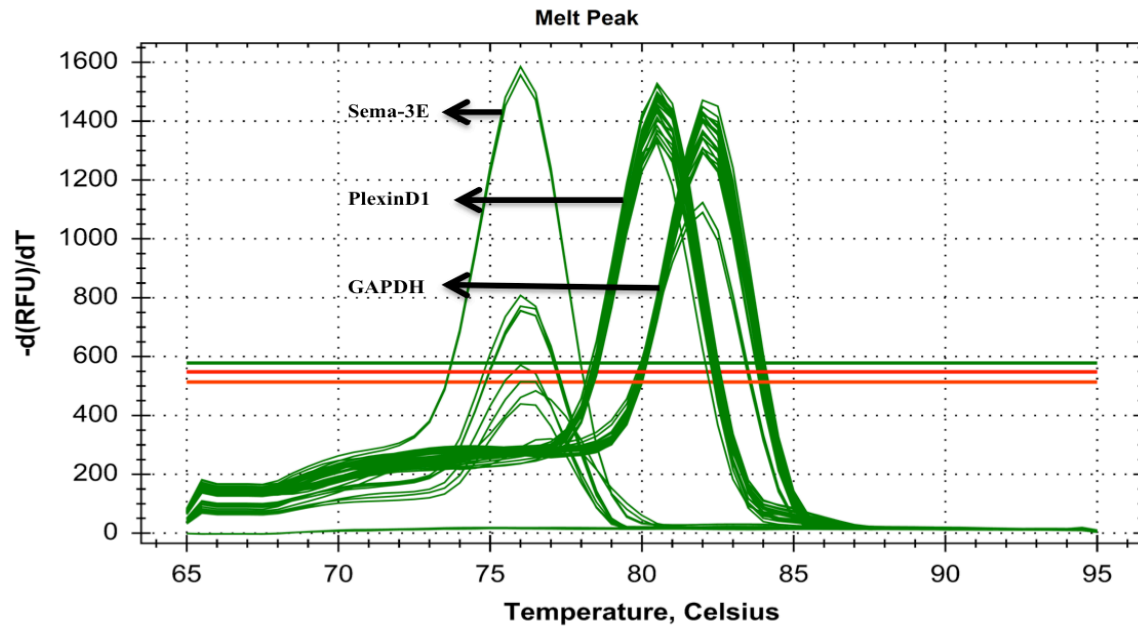


Figure3.12. Sema-3E receptors (PlexinD1 and NRP1) were detected in the surface and intracellular of the immature and mature dendritic cells but down-regulated upon TLRs stimulation. A: Immature DC cells were cultured with GM-CSF and stained without further stimulation. DC cells stimulated with LPS or PIC were collected after culture with GMCSF for 7 days and stimulated for overnight. In this experiment, 0.1×10^6 DC cells were counted and used APC mAb anti-human APC mAb anti-human PlexinD1 or PE NRP1 to detect protein expression on the surface and intracellular. **B:** On surface and intracellular, immature DCs express higher level of Sema-3E protein In comparison to mature DCs. In this experiment, (iDC) DC cell has not stimulated. (DC+LPS or DC+PIC) DC cell was stimulated with LPS ($1 \mu\text{g/ml}$) or Poly I:C ($1 \mu\text{g}/\mu\text{l}$) for overnight. I used APC mAb anti-human PlexinD1 or PE NRP1 to detect receptor protein level.

A



B

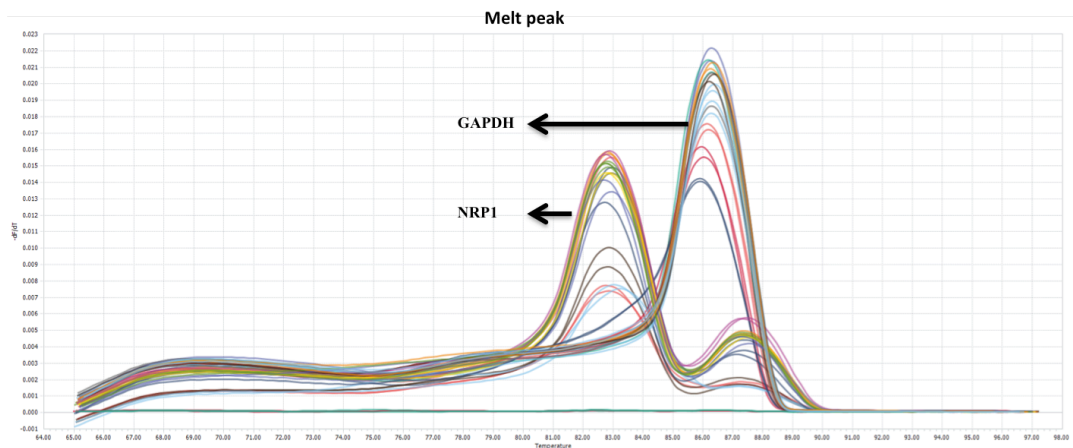


Figure3.13. Melt Curves from qPCR of Sema-3E, PlexinD1, and NRP1. Performing melting curve analysis and examining the quality of amplification curves assessed products specificity. Normalizing to the amplification of GAPDH and then normalizing to control groups calculated the amplification of target genes. **A:** Shows the melt curves for 2 different amplicons (Sema-3E and PlexinD1). **B:** An amplicon from NRP1 produces 2 peaks, often interpreted as representing multiple amplicons, when in this case, there is only one amplicon generated. In qPCR, I used delta CT method that quantifies the gene expression. All genes were normalized against the reference gen (GAPDH).

4.0 CHAPTER 4

This chapter examined the role of Sema-3E in regulating NK-cell migrations in context of NK-DC crosstalk. NK cells were isolated from spleens of Sema-3E ^{+/+} animals. They were activated in IL-2 for 4 days before the experiments. Immature DCs were generated cells under GM-CSF from bone marrow of Sema-3E ^{+/+} or Sema-3E ^{-/-} animals. Mature DCs were prepared by stimulating immature DC with LPS or PolyI:C overnight. Activated NK-cell migrations were examined in the Trans-well and microfluidic assays using mouse medium or DCs conditioned medium.

Results

4.1 Recombinant Sema-3E does not affect the migration of activated NK cells

Because activated NK cells expressed receptors for Sema-3E, we first examined whether Sema-3E alone could regulate activated NK-cells migration in vitro. 0.2×10^6 murine IL-2 activated NK cells (in 100 μ l) were loaded on the upper chamber whereas 600 μ l mouse medium was placed in the lower chamber and incubated at 37C. Different Recombinant Sema-3E concentrations (from 6 ng/ml to 200 ng/ml) were added to mouse medium. After 90 minutes, the cells migrated in the lower well were counted as absolute number of input cells. Plain mouse medium (negative control) and LPS (1 μ g/1 μ l) added to dendritic cells condition medium (positive control) were used in experiment setting ($P < 0.0001$) I observed that recombinant Sema-3E alone had no chemo-attractive effect on NK cell migration (P value = 0.4208) (Figure 4.1).

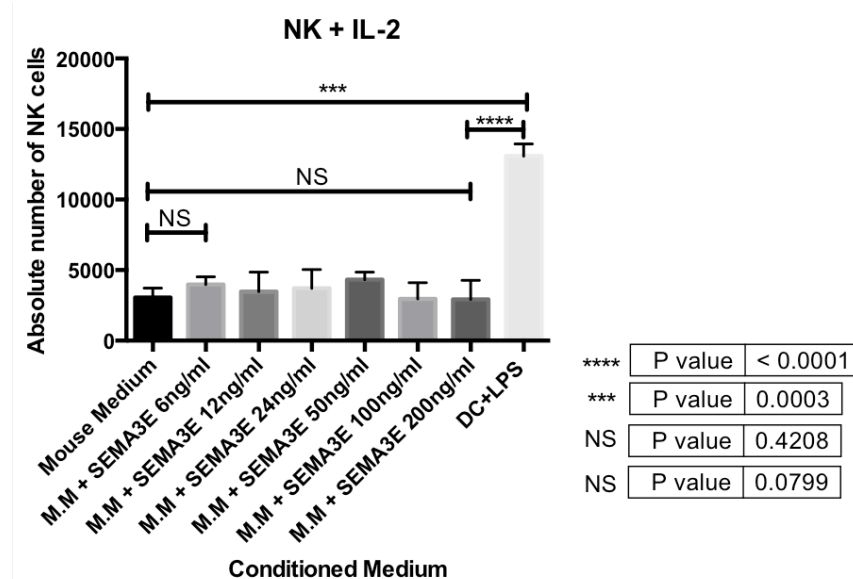


Figure4.1. Recombinant Sema-3E has no effect on activated NK cells migration: NK cells were purified and activated with IL-2 for 4 days. Sema-3E was added to mouse medium using different concentrations (6-200 ng/ml). However, recombinant Sema-3E showed no statistical significance in recruiting activated NK toward mouse medium ($P=0.4208$). Plain mouse medium (negative control) ($P=0.0799$) or LPS ($1\mu\text{g/ml}$) added to dendritic cells conditioned medium (positive control) were used in this experiment. One-way ANOVA was used for comparing data from more than two groups ($P^{****}<0.0001$). The statistic analysis was obtained from $n\text{-value}=3$.

Microfluidic devices support single cell-based, quantitative cell migration analysis in well-defined chemical gradients. It allows us to examine chemotactic and even chemo-repulsive movements of NK cells at a single cell level. I therefore examined the effect of recombinant Sema-3E (50 ng/ml) alone on NK-cell migrations in the established microfluidic system in vitro. I observed that NK cells exhibited random migratory movements similar to the control experiment in the mouse medium alone control groups. Cell tracks in black are cells migrated towards the gradient (chemotactic). Cell tracks in light gray are cells migrated away from the gradient (chemo-repulsive). Additionally, chemotactic Index (C.I.) showed random migration and no significance level for both conditions (Figure 4.2. A and B).

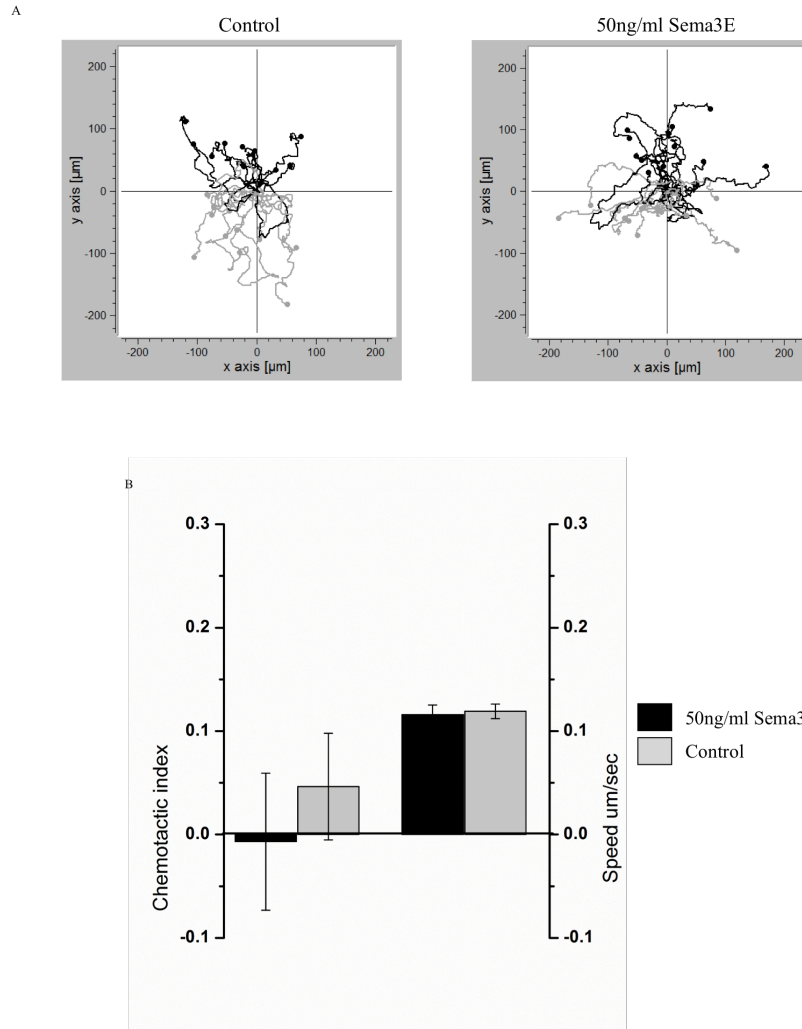


Figure4.2. Recombinant Sema-3E does not affect NK cell chemotaxis: mouse medium was used to establish the gradient in the microfluidic device. Migratory responses of IL-2 activated NK cells were recorded and analyzed. **A:** Cell tracks in black are cells migrated towards the gradient; however, cell tracks in light gray are cells migrated away from the gradient. The percentage of chemotactic cells was determined by the number of cell migrated towards the gradient x 100/Total migrating cells tracked). **B:** Chemotactic Index (C.I.) and speed of NK cells in a gradient of mouse medium plus recombinant Sema-3E and its comparison with the medium control group. Based on one set of experiments with triplicated samples, the C.I. shows random migration and no significance level for both conditions. The significance level for each comparison from 2-sample *t* test is shown.

4.2 Sema-3E^{+/+} Conditioned medium of the LPS or Poly I:C-stimulated mature DC promoted activated NK cell migration

Our laboratory has previously reported that conditioned medium from immature and mature DC supported chemotaxis of activated NK cells [209]. I showed in Chapter 3 that immature and mature DCs stimulated with LPS or Poly I: C produced different levels of Sema-3E mRNA and proteins (Figure 3.4.2 and Figure 3.4.3). I therefore investigated Sema-3E regulate NK-cell migration in the context of NK-DC crosstalk i.e. whether Sema-3E modulates migratory responses of NK cells induced by chemotactic factors produced by DC. My data showed activated NK cells migrated respectively to Sema-3E^{+/+} stimulated and unstimulated DC conditioned medium. LPS or PolyI: C stimulated mature DC promotes activated NK cells migration. (P value < 0.001) (Figure 4.3) Therefore I examine activated NK-cell migratory responses towards conditioned medium of immature or mature DC preparations in the absence of DC-derived Sema-3E. Trans-well assay in vitro was used. Interestingly, immature Sema-3E^{-/-} DCs conditioned medium promotes strong activated NK cells migration than mature Sema-3E^{-/-} DCs conditioned medium (P value = 0.0232) (Figure 4.4).

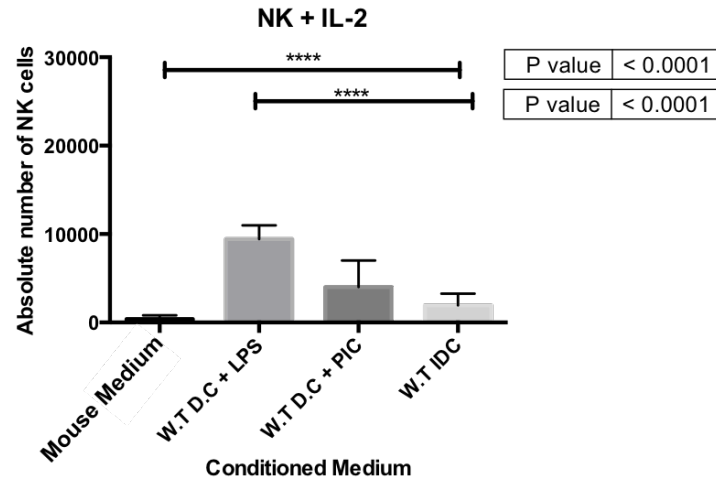


Figure4.3. Sema-3E^{+/+} Mature DC conditioned medium enhance activated NK cells migration. NK cells were purified and activated with IL-2 for 4 days. DCs conditioned medium were collected at day 8 of DCs culture. DCs conditioned medium undergo different conditions including immature DCs (iDC), DC+LPS (1μg/ml), or DC+PIC (1μg/μl) condition medium. Activated NK cells migration toward DCs condition medium was studied by migration assay. Activated NK cells migrate respectively to stimulated and unstimulated DC conditioned medium. One-way ANOVA was used for comparing data from more than two groups (P **** <0.001). N -value=3.

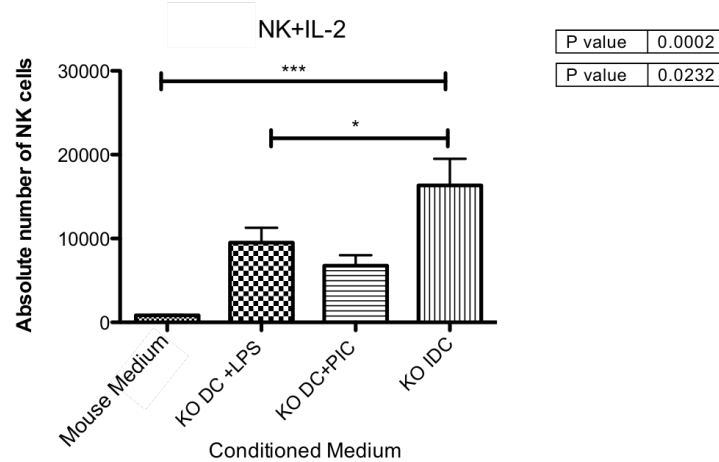


Figure4.4. Sema-3E^{-/-} conditioned medium of the immature DC promoted activated NK cells migration. To further examine how activated NK cells migrate to Sema-3E^{-/-} DCs condition medium, NK cells were purified and activated with IL-2 for 4 days. DCs condition medium were collected at day 8 of DCs culture. Different DCs conditioned medium (iDC, DC+LPS (1μg/ml), and DC+PIC (1μg/μl) were used in this experiments. NK cells migration toward DCs conditioned medium was studied by migration assay. One-way ANOVA was used for n=3 to compare data from more than two groups (P***<0.0002) (P*= 0.0232).

4.3 In vitro, Sema-3E expression level modulates activated NK cells migration

Previously, I showed that immature and mature DCs stimulated with LPS or Poly I: C express different levels of Sema-3E mRNA and proteins (Figure 3.4.2 and Figure 3.4.3). I therefore investigated the effect of different TLRs such as LPS or Poly I: C on Sema-3E expression level. Therefore, does Sema-3E level modulates migratory responses of NK cells toward DCs conditioned medium? I further compared Sema-3E^{+/+} to Sema-3E^{-/-} DCs conditioned medium affecting activated NK cells migration. In migration assay, no significant differences were observed in activated NK cells migrate to Sema-3E^{+/+} compared to Sema-3E^{-/-} conditioned medium upon LPS stimulation (P value = 0.1849) (Figure 4.5 A). However, Sema-3E^{-/-} DCs conditioned medium stimulated with poly I:C showed significant recruitment of activated NK cells compared to Sema-3E^{+/+} DCs conditioned medium (P value = 0.0010) (Figure 4.5 B). Moreover, unstimulated Sema-3E^{-/-} DCs conditioned medium enhanced activated NK cells migration (P value = 0.0002) (Figure 4.5 C).

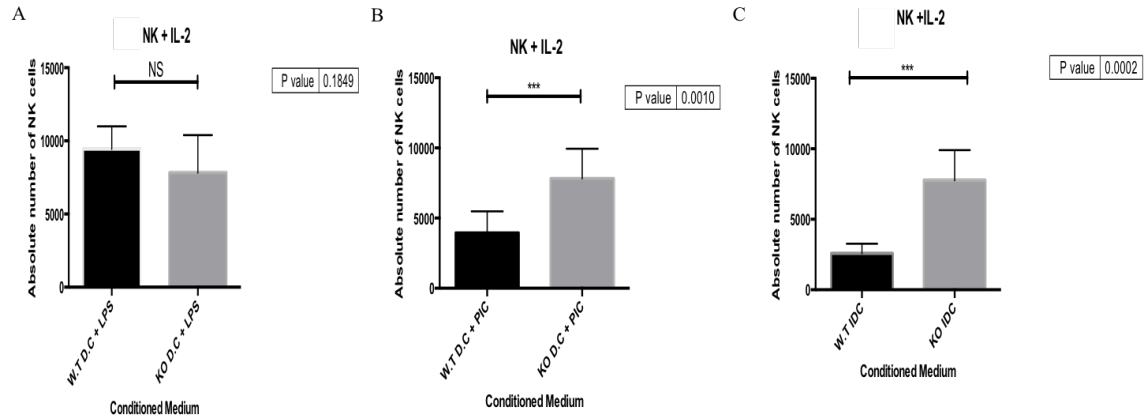


Figure 4.5. The comparison between Sema-3E^{+/+} and Sema-3E^{-/-} mature or immature DCs conditioned medium in recruitment of activated NK cells. NK cells were purified and activated with IL-2 for 4 days. DCs conditioned medium were collected at day 8 of DCs culture. DCs conditioned medium undergo different conditions including iDC, or stimulated DCs with LPS (1 μ g/ml), and DCs stimulated by PIC (1 μ g/ μ l) conditioned medium **A:** activated NK cells migrate equally to WT and/or KO Sema-3E DCs conditioned medium upon LPS stimulation (P value = 0.1849). **B:** Sema-3E^{-/-} DCs conditioned medium stimulated with PIC showed better recruitment of activated NK cells than Sema-3E^{+/+} DCs conditioned medium (P*** = 0.0010). **C:** However, unstimulated DCs conditioned medium from Sema-3E^{+/+} has lower ability to attract activated NK cells migration (P*** = 0.0002). Using GraphPad Prism preformed the statistic analysis for n-value=3. Two-tailed student's t-test was used in a single, two-group comparison data.

4.4 Introduction of recombinant Sema-3E back to the unstimulated Sema-3E^{-/-} DC condition medium inhibited NK-cells migration

As we showed before, the absence of Sema-3E attracted more of activated NK cells toward immature DCs conditioned medium. I further confirmed the role of Sema-3E affecting activating NK cells migration in context of NK-DC crosstalk. I added back mouse recombinant Sema-3E protein (50 ng/ml) to unstimulated Sema-3E^{-/-} DC conditioned medium (KO iDC) at the time migration. Significantly, I observed recombinant Sema-3E (50 ng/ml) suppressing the migration of activated NK cells toward Sema-3E^{-/-} immature DCs conditioned medium (P value = 0.0006) as showed in (Figure 4.6).

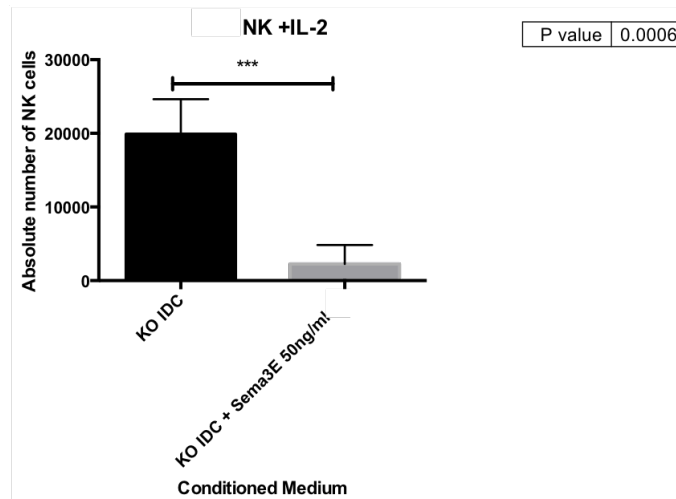


Figure4.6. Recombinant Sema-3E suppresses activated NK cells migration toward unstimulated Sema-3E^{-/-} DC conditioned medium. NK cells were purified and activated with IL-2 for 4 days. DCs condition medium were collected at day 8 of DCs culture. Immature DCs conditioned medium was used in migration assay. Recombinant Sema-3E (50 ng/ml) added to the condition medium at the time of running migration assay. However, recombinant Sema-3E significantly suppresses the migration of activated NK cells toward Sema-3E^{-/-} immature DC condition medium (P*** = 0.0006). The significance level for each comparison from 2-sample *t* test is shown and obtained from combined 3 independent experiments.

4.5 Ex -vivo splenic DC from Sema-3E KO mice exhibited altered DC maturation phenotypes

DCs are professional antigen processing and presenting cells. By producing a broad range of cytokines such as (IL-18, IL-12, IL-15, IFN- α , IFN- β), these cells have been implicated in several immune regulations at the interface of both innate and adaptive immunities [123- 124]. Here I sought if Sema-3E is important for DCs maturation and therefore affect NK-DC crosstalk. To address this hypothesis I collected the spleen from both Sema-3E^{+/+} and Sema-3E^{-/-} Balb/c mice. Equal numbers of splenocytes were used for surface staining using monoclonal antibody CD45 Pacific-Blue, monoclonal CD11c APC and monoclonal antibody MHCII PE. I used gating strategies, which first specify the CD45⁺ cells. The gating on single cell was performed to target CD11c⁺, MHCII⁺ cells. Interestingly, the percentage of DCs in Sema-3E^{-/-} was higher (12.5%) compared to Sema-3E^{+/+} (6.35%) (Figure 4.7A). Interestingly, I found Sema-3E^{-/-} mouse splenic DCs showed higher MHCII expression compared to Sema-3E^{+/+} mouse splenic DCs. (Figure 4.7 B).

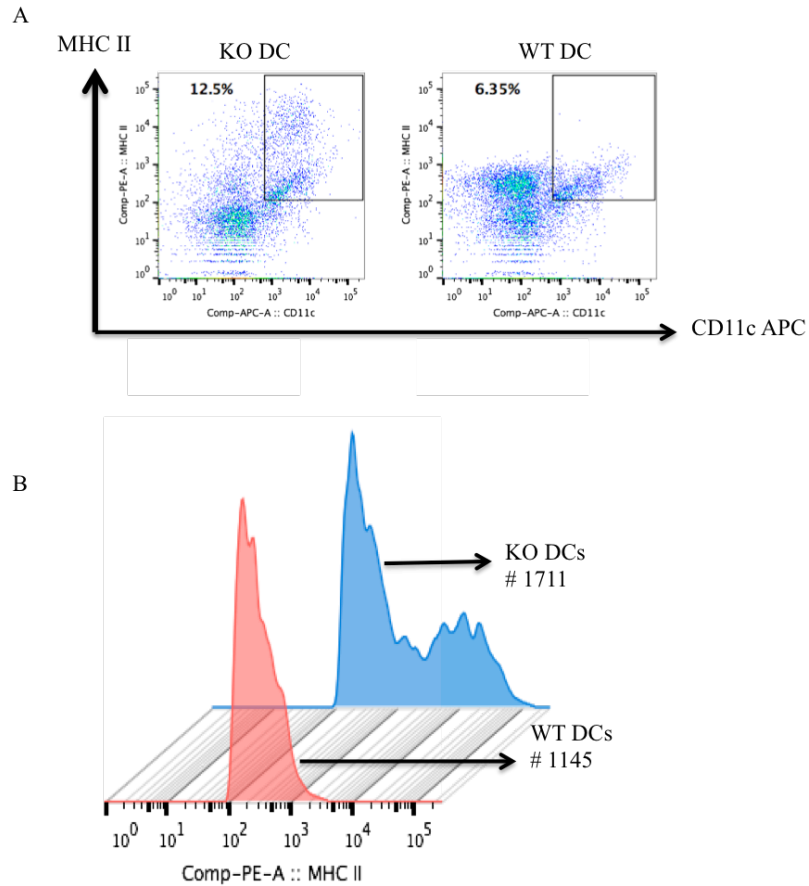


Figure4.7. Sema-3E affects dendritic cells maturation. Splenocytes were collected from Sema-3E^{-/-} or Sema-3E^{+/+} Balb/c mice. 0.2×10^6 splenocytes were used for surface staining using monoclonal antibody CD45 Pacific-Blue (specific for leukocytes), monoclonal CD11c APC and monoclonal antibody MHCII PE (specific for DCs). **A:** KO spleen has more percentage of DCs (12.5%) compared to WT spleen that showed less percentage (6.35%). **B:** MHCII molecule was expressed more in KO (DCs number = 1711) versus WT mouse splenic DCs (DCs number = 1145). Sema3E^{-/-} mice have more mature DCs than Sema-3E^{+/+}.

5.0 CHAPTER 5

General discussion and future directions

In this study, I first examined RNA and protein expressions of Sema-3E, PlexinD1 and NRP1 in primary NK and DC. I observed that resting or IL-2 activated NK cells expressed low level of Sema-3E transcripts and protein [Figures 3.1 and 3.3A]. Unstimulated or DCs stimulated with Poly I: C expressed Sema-3E transcript and protein [Figures 3.7 and 3.9A]. Down-regulation of Sema-3E transcript and protein was observed in DCs stimulated with LPS more significantly than DCs stimulated with Poly I:C [Figures 3.8 and 3.9B]. PlexinD1 receptor was expressed on NK cells and DC cells at both mRNA and protein levels [Figures 3.4, 3.6A, 3.10 and 3.12A]. NRP1 co-receptor was expressed on resting NK cells, LPS, poly I:C-stimulated DC and immature DCs at mRNA and protein level [Figures 3.4, 3.6A, 3.10 and 3.12A] but not IL-2 activated NK cells [Figures 3.4 and 3.6 B]. Second, I investigated the effect of Sema-3E in NK cell migrations. In the Trans-well assays and microfluidic assay, I observed that recombinant Sema-3E does not affect NK cells migration [Figures 4.1 and 4.2]. However, I reported that conditioned medium of the mature Sema-3E^{+/+} DC promoted a stronger chemotaxis of IL-2 activated NK cells than immature DC conditioned medium in trans-well assay [Figure 4.3]. In contrast, activated NK cells exhibited increase in the chemotactic migrations toward the conditioned medium of the immature Sema-3E^{-/-} DC [Figure 4.4]. The suppressive effect of Sema-3E was restored when Sema-3E (50ng/ml) added back to the conditioned medium of the immature Sema-3E^{-/-} DC [Figure 4.6].

NK cells constitute the first line of defense and possess substantial cytotoxic and immune regulatory potential, have also been employed as therapeutic agent in pre-clinical and clinical settings for a long time [210- 211- 212]. However, a growing consensus is that NK cells alone as immune therapeutic agent may not be highly efficient to counter tumor as well as other infectious diseases. Similarly, DC based immunotherapeutic interventions, which are designed primarily to manipulate T cell responses directly, also yielded limited success in the clinical setting [213]. Abilities to augment NK/DC interactions may prove to be important in the effective induction of the innate and adaptive immunities in anti-tumor settings.

Both NK and DCs have the ability to secrete a wide range of cytokines and chemokines, thus widely implicated in regulating different immune functions [214-215]. In addition to the independent role of these two cell types in the regulation of immune system, another relevant feature attributed to NK cells and DCs is their mutual communications [216]. It has been shown that these cells require cell: cell contact and soluble factors to acquire full spectrum of functional phenotype. Previously our lab targeted several proteins involved in the regulation of the NK or DC cells functions such as MHC-I, Qa-I, SHP1, IP-10, CXCR3, and GM-CSF.

Semaphorin-3E (Sema-3E) is an axon-guidance secreted protein in neuronal system that has emerged as an essential mediator involved in cell migration and proliferation [204]. I conducted several studies to cover the role of Sema-3E as a novel factors involved in the regulation of NK cell migration in interplay between NK cell and DC.

To achieve my hypothesis, I sought to explore whether activated NK cells response to Sema-3E or not. As known, Sema-3E protein mediates its functions by binding with high affinity to PlexinD1 and neuropilin1 (NRP1) receptors [194]. I examine PlexinD1 and NRP1 expressions by activated NK cells. My data showed activated NK cells express Sema-3E receptor (PlexinD1) but not NRP1 [Figure 3.4]. Unlike class 3 semaphorins, Sema-3E binds its receptor plexin-D1 directly and independently of the neuropilin1 [217]. Collectively, expression of plexin-D1 on activated NK cells suggested a possible interaction that results in direct effect of Sema-3E on NK cells function(s). Choi YI and her colleagues have reported Sema-3E regulates migrations of neural cells [204]. I desire to explore the direct effect of Sema-3E on activated NK cells migration. My data showed that recombinant Sema-3E alone has no inducer or repulsive effect on activated NK cells migration [Figures 4.1 and 4.2].

NK-DC communication is bidirectional, involving many cytokine signals and/or direct cell-to-cell contacts [130- 131]. Immature and LPS-activated mature DCs released soluble factors, which induced a high level of chemotactic movement of IL-2-activated NK cells in vitro [209]. I showed Sema-3E expressed by immature and mature DCs stimulated with LPS or Poly:IC (Chapter3) [Figures 3.7]. I therefore investigated the potential role of Sema-3E expressed by DCs affect NK migration in context of NK-DC crosstalk. My data showed Sema-3E^{+/+} stimulated DC conditioned medium promoted activated NK cells migration [Figures 4.3]. In contrast, Sema-3E^{-/-} immature DCs conditioned medium promotes strong activated NK cells migration compared to mature Sema-3E^{-/-} DCs conditioned medium [Figures 4.4]. This finding suggests that Sema-3E might affect DCs induce chemotactic factors signaling and therefore activated NK cells migration properties

might affected. Migration of activated NK cells mediated by Chemokines expression in DC for example, CXCR3 chemokine receptor produced by DCs plays a role in the regulation of leukocyte migration [209]. Sema-3E might intersect the signaling pathway downstream of chemokine receptor signaling. Recent Studies in cultured cells had shown that plexin-D1 itself displays R-Ras GAP activity to inhibit migration, and these actions specifically require the small GTPase Rnd2. In addition, Rnd2 was found to bind to Plexin-D1 in cortical neurons, and Sema-3E-Plexin-D1-induced inhibition of axon outgrowth of cortical neurons required Rnd2 and downregulation of R-Ras activity [218].

Based on the findings of chapter 3, immature and mature DCs expressed different level of Sema-3E [Figure 3.8]. I therefore studied the effect of Sema-3E expression level modulating migratory responses of activated NK cells toward DCs conditioned medium. Immature DCs of Sema-3E^{+/+} DCs conditioned medium showed less activated NK cells recruitment compared to Sema-3E^{-/-} immature DCs conditioned medium [Figure 4.5C]. Significantly, the migration of activated NK cells was suppressed when recombinant Sema-3E (50 ng/ml) introduced to Sema-3E^{-/-} immature DCs conditioned medium [Figure 4.6]. This finding suggests that Sema-3E is a novel factor modulates NK cells migration in NK-DC crosstalk. In the context of mature DC conditioned medium and upon LPS stimulation, Sema-3E expression was down regulated. Interestingly, I found no significant differences between Sema-3E^{+/+} DCs conditioned medium compared to Sema-3E^{-/-} DCs conditioned medium in activated NK cells recruitment [Figure 4.5A]. This data suggested LPS stimulation downregulates Sema-3E expression close to the level of Sema-3E^{-/-} DCs conditioned medium. On the other hand, Poly I: C stimulation showed less recruitment of activated NK cells toward Sema-3E^{+/+} DCs conditioned medium compared to Sema-3E^{-/-}

DCs conditioned medium [Figure 4.5B]. Previously, I observed Poly I:C stimulation down regulates Sema-3E expression level [Figure 3.8]. Collectively, these findings suggested that Poly I:C stimulation downregulates Sema-3E expression level but not strong as LPS stimulation. LPS bind to TLR4 and associated with NF-kappaB and IRF3 pathways activation. In contrary, Poly I: C binds to TLR4 and activates NF-kappaB, MAPK, and IRF-3 [219]. It can be speculated that TLRs stimulations downregulate Sema-3E expression levels through different signalling pathways and therefore enhance activated NK cells migration. Moreover, the in vitro data of Sema-3E down regulation in relation to bacterial or viral infection and the migration of NK cells in mouse model could further explain other possible regulatory functions of Sema-3E in the context of NK/DC interactions. In addition, the down regulation of Sema-3E on DCs could be a protective mechanism to alert NK cells to ensure the regulation of T cell immune responses in “DC-editing process”.

To understand the relevance of these results it will be of interest to monitor the down regulation patterns of Sema-3E in the DC maturation. In NK-DC interactions, activated NK cells released IFN- γ and TNF that promote DC maturation, and migration to draining lymph nodes [140]. The mature DCs are able to uptake and processing of antigen result in the upregulation of MHC-II and co-stimulatory molecules (CD40, CD80, CD86)[135-136]. Here I investigated the role of Sema-3E regulating DCs maturation in NK-DC crosstalk. Ex -vivo splenic DC from Sema-3E^{-/-} mice exhibited altered DC maturation phenotypes [Figures 4.7A and B]. My data revealed that Sema-3E is a novel factor enhances DCs maturation.

Mature dendritic cells produce several cytokines (IL-15, IL-12/IL-18, IFN- α/β ,) that specifically stimulate NK cells to proliferate, migrate, produce cytokines and acquire cytotoxicity.[123-124]. As observed in this study, DCs maturation has affected by Sema-3E, which is in correlation with migratory properties of activated NK cells. Future studies in this regard might also delineate some important chemokines mediate the migration of NK cells.

Activated NK cells tightly regulate Sema-3E and PlexinD1 expression. I found the activation of NK cells with IL-2 downregulates the expression level of Sema-3E and its receptors (PlexinD1 and NRP1) compared to resting NK cells (Figs 3.3B and 3.6B). Different studies have showed IL-2 activated several different pathways such as Janus Kinases (JAK1 and JAK3) and STAT5 [219]. This observations press further steps to study downstream signaling pathways that might affect Sema-3E and PlexinD1 expression. Moreover, activated NK cells with IL-15 and IL-18 deserve to explore their characteristics in term of development and functions. I will continue to explore the role of Sema-3E in regulating other NK cells functions such as cytotoxicity, cytokines productions.

In conclusion, the role of Sema-3E in regulating NK cells functions in context of NK-DC crosstalk is fairly a new concept, which needs to be investigated further. Identification of Sema-3E regulatory function(s) might help to manipulate the existing methodologies to improve genesis as well as function of developing NK cells. Understanding the role of Sema-3E regulating NK cells function(s), indeed improve our current understanding of the immune systems, but may also equip us to manipulate NK cell effector functions and their utility as immune therapeutic agent in clinical settings.

References

- 1 **Kerry S, Campbell. and Jun, Hasegawa.,** NK cell biology: An update and future direction. *J Allergy Clin Immunol.* 2013. **132**:536-544.
- 2 **Narni-Mancinelli, E., Vivier, E. and Kerdiles, Y.) M.,** The 'T-cell-ness' of NK cells: unexpected similarities between NK cells and T cells. *Int Immunol* 2011. **23**:427-431.
- 3 **Wang, R., Jaw, J. J., Stutzman, N. C., Zou, Z. and Sun, P. D.,** Natural killer cell-produced IFN-gamma and TNF-alpha induce target cell cytolysis through up-regulation of ICAMY1. *J'Leukoc'Biol* 2012.**91**:299-309.
- 4 **Cooper, M. A., Fehniger, T. A., Turner, S. C., Chen, K. S., Ghaheri, B. A., Ghayur, T., Carson, W. E. and Caligiuri, M. A.,** Human natural killer cells: a unique innate immune regulatory role for the CD56 (bright) subset. *Blood* 2001.**97**:3146-3151.
- 5 **Caligiuri MA.** Human natural killer cells. *Blood* 2008.**112(3)**: 461-469.
- 6 **Herberman, R. B., Nunn, M. E. and Lavrin, D. H.,** Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int'J'Cancer* 1975. **16**:216-229.
- 7 **Herberman, R. B., Nunn, M. E. and Lavrin, D. H.,** Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. *Int J Cancer* 1975. **16**: 216-229.
- 8 **Herberman, R. B., Nunn, M. E., Holden, H. T. and Lavrin, D. H.,** Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int J Cancer* 1975. **16**: 230-239.
- 9 **Kiessling, R., Klein, E. and Wigzell, H.,** "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* 1975. **5**: 112-117.
- 10 **Koepsell, S. A., Miller, J. S. and McKenna, D. H., Jr.,** Natural killer cells: a review of manufacturing and clinical utility. *Transfusion* 2013. **53**: 404-410.
- 11 **Huntington, N. D., Vosschenrich, C. A. and Di Santo, J. P.,** Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat Rev Immunol* 2007. **7**: 703-714.

- 12 **Romee, R., Foley, B., Lenvik, T., Wang, Y., Zhang, B., Ankarlo, D., Luo, X., Cooley, S., Verneris, M., Walcheck, B. and Miller, J.,** NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). *Blood* 2013.**121**:3599-3608.
- 13 **Cooper, M. A., Fehniger, T. A. and Caligiuri, M. A.,** The biology of human Natural killer-cell subsets.*Trends Immunol*2001.**22**:633-640.
- 14 **Poli, A., Michel, T., Theresine, M., Andres, E., Hentges, F. and Zimmer, J.,** CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology*2009.**126**:458-465.
- 15 **Carbone, T., Nasorri, F., Pennino, D., Eyerich, K., Foerster, S., Cifaldi, L., Traidl-Hoffman, C., Behrendt, H. and Cavani, A.,** CD56highCD16-CD62L- NK cells accumulate in allergic contact dermatitis and contribute to the Expression of allergic responses. *J Immunol*2010.**184**:1102-1110.
- 16 **Hayakawa, Y. and Smyth, M. J.,** CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J Immunol* 2006.**176**:1517-1524.
- 17 **Vivier, E., Tomasello, E., Baratin, M., Walzer, T. and Ugolini, S.,** Functions of natural killer cells. *Nat Immunol*2008.**9**:503-510.
- 18 **Hayakawa, Y., Watt, S. V., Takeda, K. and Smyth, M. J.,** Distinct receptor repertoire formation in mouse NK cell subsets regulated by MHC class I expression. *J Leukoc Biol*2008.**83**:106-111.
- 19 **Chan, C. W., Crafton, E., Fan, H. N., Flook, J., Yoshimura, K., Skarica, M., Brockstedt, D., Dubensky, T. W., Stins, M. F., Lanier, L. L., Pardoll, D. M. and Housseau, F.,** Interferon-producing killer dendritic cells provide a link between innate and adaptive immunity. *Nat Med*2006.**12**:207-213.
- 20 **Vosshenrich, C. A., Garcia ojeda, M. E., Samson Villeger, S. I., Pasqualetto, V., Enault, L., Richard Le Goff, O., Corcuff, E., Guy Grand, D., Rocha, B., Cumano, A., Rogge, L., Ezine, S. and Di Santo, J. P.,** A thymic pathway of mouse natural killer cell development characterized by expression of GATAY3 and CD127. *Nat Immunol*2006.**7**:1217-1224.
- 21 **Freud, A. G. and Caligiuri, M. A.,** Human natural killer cell development. *Immunol Rev* 2006.**214**:56-72.
- 22 **Scott, G. B., Bowles, P. A., Wilson, E. B., Meade, J. L., Low, B. C., Davison, A., Blair, G. E. and Cook, G. P.,** Identification of the BCL2/adenovirus E1BY19K protein-interacting protein 2 (BNIP-2) as a granzyme B target during human natural killer cell-mediated killing. *Biochem J* 2010.**431**:423-431.

- 23 **Semino, C., Angelini, G., Poggi, A. and Rubartelli, A.,** NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1. *Blood* 2005. **106**: 609-616.
- 24 **Nedvetzki, S., Sowinski, S., Eagle, R. A., Harris, J., Vely, F., Pende, D., Trowsdale, J., Vivier, E., Gordon, S. and Davis, D. M.,** Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. *Blood* 2007. **109**: 3776-3785.
- 25 **Rosmaraki, E. E., Douagi, I., Roth, C., Colucci, F., Cumano, A. and Di Santo, J. P.,** Identification of committed NK cell progenitors in adult murine bone marrow. *Eur J Immunol* 2001. **31**: 1900-1909.
- 26 **Williams, N. S., Kubota, A., Bennett, M., Kumar, V. and Takei, F.,** Clonal analysis of NK cell development from bone marrow progenitors in vitro: orderly acquisition of receptor gene expression. *Eur J Immunol* 2000. **30**: 2074-2082.
- 27 **Colucci, F., Caligiuri, M. A. and Di Santo, J. P.,** What does it take to make a natural killer? *Nat Rev Immunol* 2003. **3**: 413-425.
- 28 **Fathman, J. W., Bhattacharya, D., Inlay, M. A., Seita, J., Karsunky, H. and Weissman, I. L.,** Identification of the earliest natural killer cell-committed progenitor in murine bone marrow. *Blood* 2011. **118**: 5439-5447.
- 29 **Di Santo, J. P.,** Natural killer cell developmental pathways: a question of balance. *Annu'Rev'Immunol* 2006. **24**: 257-286.
- 30 **Kawamoto, H., Ikawa, T., Ohmura, K., Fujimoto, S. and Katsura, Y.,** T cell progenitors emerge earlier than B cell progenitors in the murine fetal liver. *Immunity* 2000. **12**: 441-450.
- 31 **Rodewald, H. R., Moingeon, P., Lucich, J. L., Dosiou, C., Lopez, P. and Reinherz, E. L.,** A population of early fetal thymocytes expressing Fc gamma RII/III contains precursors of T lymphocytes and natural killer cells. *Cell* 1992. **69**: 139-150.
- 32 **Williams, N. S., Klem, J., Puzanov, I. J., Sivakumar, P. V., Bennett, M. and Kumar, V.,** Differentiation of NK1.1+, Ly49+ NK cells from flt3+ multipotent marrow progenitor cells. *J'Immunol* 1999. **163**: 2648-2656.
- 33 **Ohno, S., Sato, T., Kohu, K., Takeda, K., Okumura, K., Satake, M. and Habu, S.,** Runx proteins are involved in regulation of CD122, Ly49 family and IFN γ expression during NK cell differentiation. *Int' Immunol* 2008. **20**: 71-79.

- 34 **Shuai, K. and Liu, B.,** Regulation of JAK-STAT signalling in the immune system. *Nat'Rev'Immunol*2003.**3**:900-911.
- 35 **Shi, F. D., Ljunggren, H. G., La Cava, A. and Van Kaer, L.,** Organ-specific features of natural killer cells. *Nat'Rev'Immunol* 2011.**11**:658-671.
- 36 **Freud, A. G. and Caligiuri, M. A.,** Human natural killer cell development. *Immunol'Rev* 2006.**214**:56-72.
- 37 **Galy, A., Travis, M., Cen, D. and Chen, B.,** Human! T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity* 1995.**3**:459-473.
- 38 **Mrozek, E., Anderson, P. and Caligiuri, M. A.,** Role of Interleukin-15 in the development of human CD56+ natural killer cells from CD34+hematopoietic progenitor cells. *Blood* 1996.**87**:2632-2640.
- 39 **Freud, A. G. and Caligiuri, M. A.,** Human natural killer cell development. *Immunol'Rev* 2006.**214**:56-72
- 40 **Heemskerk, M. H., Blom, B., Nolan, G., Stegmann, A. P., Bakker, A. Q., Weijer, K., Res, P. C. and Spits, H.,** Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J Exp Med* 1997. **186**: 1597-1602.
- 41 **Boos, M. D., Yokota, Y., Eberl, G. and Kee, B. L.,** Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. *J Exp Med* 2007. **204**: 1119-1130.
- 42 **Georgopoulos, K., Bigby, M., Wang, J. H., Molnar, A., Wu, P., Winandy, S. and Sharpe, A.,** The Ikaros gene is required for the development of all lymphoid lineages. *Cell* 1994.**79**:143-156.
- 43 **Georgopoulos, K., Winandy, S. and Avitahl, N.,** The role of the Ikaros gene in lymphocyte development and homeostasis. *Annu' Rev' Immunol* 1997. **15**: 155-176.
- 44 **Kamizono, S., Duncan, G. S., Seidel, M. G., Morimoto, A., Hamada, K., Grosveld, G., Akashi, K., Lind, E. F., Haight, J. P., Ohashi, P. S., Look, A.= T. and Mak, T. W.,** Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. *J'Exp'Med*2009.**206**:2977-2986.
- 45 **Ramirez, K., Chandler, K. J., Spaulding, C., Zandi, S., Sigvardsson, M., Graves, B. J. and Kee, B. L.,** Gene deregulation and chronic activation in natural killer cells deficient in the transcription factor ETS1. *Immunity* 2012. **36**:921-932.

- 46 Fehniger, T. A., Cai, S. F., Cao, X., Bredemeyer, A. J., Presti, R. M., French, A. R. and Ley, T. J., Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. *Immunity* 2007.**26**:798-811.
- 47 Cooper, M. A., Elliott, J. M., Keyel, P. A., Yang, L., Carrero, J. A. and Yokoyama, W. M., Cytokine-induced memory-like natural killer cells. *Proc' Nat'Acad'Sci'U'S'A* 2009.**106**:1915-1919.
- 48 Bradley, M., Zeytun, A., Rafi- anajreh, A., Nagarkatti, P. S. and Nagarkatti, M., Role of spontaneous and interleukin-2-induced natural killer cell activity in the cytotoxicity and rejection of Fas+ and Fas-tumor cells. *Blood* 1998.**92**: 4248-4255.
- 49 Zhao, Y. M. and French, A. R., Mechanistic model of natural killer cell proliferative response to IL-15 receptor stimulation. *PLoS' Comput' Biol* 2013. **9**:e1003222.
- 50 Tang, F., Sally, B., Ciszewski, C., Abadie, V., Curran, S. A., Groh, V., Fitzgerald, O., Winchester, R. J. and Jabri, B., Interleukin 15 primes natural killer cells to kill via NKG2D and cPLA2 and this pathway is active in psoriatic arthritis. *PLoS'One* 2013.**8**:e76292.
- 51 Grossman, W. J., Revell, P. A., Lu, Z. H., Johnson, H., Bredemeyer, A. J. and Ley, T. J., The orphan granzymes of humans and mice. *Curr' Opin' Immunol* 2003.**15**:544-552.
- 52 Krzewski, K., Gil?Krzewska, A., Nguyen, V., Peruzzi, G. and Coligan, J. E., LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood* 2013.**121**:4672-4683.
- 53 Thiery, J., Keefe, D., Boulant, S., Boucrot, E., Walch, M., Martinvalet, D., Goping, I. S., Bleackley, R. C., Kirchhausen, T. and Lieberman, J., Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B!into the cytosol of target cells. *Nat'Immunol* 2011.**12**:770-777.
- 54 Scott, G. B., Bowles, P. A., Wilson, E. B., Meade, J. L., Low, B. C., Davison, A., Blair, G. E. and Cook, G. P., Identification of the BCL2/adenovirus E1BY19K protein-interacting protein 2 (BNIP-2) as a granzyme B target during human natural killer cell-mediated killing. *Biochem'J* 2010.**431**:423-431.
- 55 Smyth, M. J., Cretney, E., Takeda, K., Wiltrot, R. H., Sedger, L. M., Kayagaki, N., Yagita, H. and Okumura, K., Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma- dependent natural killer cell protection from tumor metastasis. *J' Exp' Med* 2001. **193**: 661-670.

- 56 **Kayagaki, N., Yamaguchi, N., Nakayama, M., Takeda, K., Akiba, H., Tsutsui, H., Okamura, H., Nakanishi, K., Okumura, K. and Yagita, H.,** Expression and function of TNFY related apoptosis-inducing ligand on murine activated NK cells. *J Immunol* 1999. **163**:1906-1913.
- 57 **Boatright, K. M., Renatus, M., Scott, F. L., Sperandio, S., Shin, H., Pedersen, I. M., Ricci, J. E., Edris, W. A., Sutherlin, D. P., Green, D. R. and Salvesen, G. S.,** A unified model for apical caspase activation. *Mol' Cell* 2003. **11**: 529-541.
- 58 **Semino, C., Angelini, G., Poggi, A. and Rubartelli, A.,** NK/iDC interaction results in IL-18 secretion by DCs at the synaptic cleft followed by NK cell activation and release of the DC maturation factor HMGB1. *Blood* 2005. **106**: 609-616.
- 59 **Batalla, E. I., Pino Martinez, A. M., Poncini, C. V., Duffy, T., Schijman, A. G., Gonzalez Cappa, S. M. and Alba Soto, C. D.,** Impairment in natural killer cells editing of immature dendritic cells by infection with a virulent Trypanosoma cruzi population. *J Innate Immun* 2013. **5**: 494-504.
- 60 **Ferlazzo, G., Tsang, M. L., Moretta, L., Melioli, G., Steinman, R. M. and Munz, C.,** Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med* 2002. **195**: 343-351.
- 61 **Spaggiari, G. M., Carosio, R., Pende, D., Marcenaro, S., Rivera, P., Zocchi, M. R., Moretta, L. and Poggi, A.,** NK cell-mediated lysis of autologous antigen-presenting cells is triggered by the engagement of the phosphatidylinositol 3-kinase upon ligation of the natural cytotoxicity receptors NKp30 and NKp46. *Eur J Immunol* 2001. **31**: 1656-1665.
- 62 **Nedvetzki, S., Sowinski, S., Eagle, R. A., Harris, J., Vely, F., Pende, D., Trowsdale, J., Vivier, E., Gordon, S. and Davis, D. M.,** Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. *Blood* 2007. **109**: 3776-3785.
- 63 **Lunemann, A., Lunemann, J. D., Roberts, S., Messmer, B., Barreira da Silva, R., Raine, C. S. and Munz, C.,** Human NK cells kill resting but not activated microglia via NKG2D- and NKp46-mediated recognition. *J Immunol* 2008. **181**: 6170-6177.
- 64 **Olson, J. A., Leveson-Gower, D. B., Gill, S., Baker, J., Beilhack, A. and Negrin, R. S.,** NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood* 2010. **115**: 4293-4301.
- 65 **Roy, S., Barnes, P. F., Garg, A., Wu, S., Cosman, D. and Vankayalapati, R.,** NK cells lyse T regulatory cells that expand in response to an intracellular pathogen. *J Immunol* 2008. **180**: 1729-1736.

- 66 **Robbins, S. H., Bessou, G., Cornillon, A., Zucchini, N., Rupp, B., Ruzsics, Z., Sacher, T., Tomasello, E., Vivier, E., Koszinowski, U. H. and Dalod, M.,** Natural killer cells promote early CD8 T cell responses against cytomegalovirus. *PLoS Pathog* 2007. **3**: e123.
- 67 **Ge, M. Q., Ho, A. W., Tang, Y., Wong, K. H., Chua, B. Y., Gasser, S. and Kemeny, D. M.,** NK cells regulate CD8+ T cell priming and dendritic cell migration during influenza A infection by IFN-gamma and perforin-dependent mechanisms. *J Immunol* 2012. **189**: 2099-2109.
- 68 **Kos, F. J. and Engleman, E. G.,** Requirement for natural killer cells in the induction of cytotoxic T cells. *J Immunol* 1995. **155**: 578-584.
- 69 **Snapper, C. M., Yamaguchi, H., Moorman, M. A., Sneed, R., Smoot, D. and Mond, J. J.,** Natural killer cells induce activated murine B cells to secrete Ig. *J Immunol* 1993. **151**: 5251-5260.
- 70 **Hanna, J., Goldman-Wohl, D., Hamani, Y., Avraham, I., Greenfield, C., Natanson-Yaron, S., Prus, D., Cohen-Daniel, L., Arnon, T. I., Manaster, I., Gazit, R., Yutkin, V., Benharroch, D., Porgador, A., Keshet, E., Yagel, S. and Mandelboim, O.,** Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med* 2006. **12**: 1065-1074.
- 71 **Orr, M. T. and Lanier, L. L.,** Natural killer cell education and tolerance. *Cell* 2010. **142**: 847-856.
- 72 **Pegram, H. J., Andrews, D. M., Smyth, M. J., Darcy, P. K. and Kershaw, M. H.,** Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol* 2011. **89**: 216-224.
- 73 **O'Connor, G. M., Hart, O. M. and Gardiner, C. M.,** Putting the natural killer cell in its place. *Immunology* 2006. **117**: 1-10.
- 74 **Karre, K., Ljunggren, H. G., Piontek, G. and Kiessling, R.,** Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 1986. **319**: 675-678.
- 75 **Ljunggren, H. G. and Karre, K.,** In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* 1990. **11**: 237-244.
- 76 **Vinay, Kumar and Megan E. McNerney.,** A new self: MHC-class-I-independent Natural-killer-cell self-tolerance. *Nature Reviews Immunology* 2005. **5**:363-374.
- 77 **Long, E. O., Colonna, M. and Lanier, L. L.,** Inhibitory MHC class I receptors on NK and T cells: a standard nomenclature. *Immunol Today* 1996. **17**: 100.

- 78 **Karlhofer, F. M., Ribaldo, R. K. and Yokoyama, W. M.,** MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature* 1992. **358**: 66-70.
- 79 **Elliott, J. M., Wahle, J. A. and Yokoyama, W. M.,** MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *J Exp Med* 2010. **207**: 2073-2079.
- 80 **Liao, N. S., Bix, M., Zijlstra, M., Jaenisch, R. and Raulet, D.,** MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science* 1991. **253**: 199-202.
- 81 **Oppenheim, D. E., Roberts, S. J., Clarke, S. L., Filler, R., Lewis, J. M., Tigelaar, R. E., Girardi, M. and Hayday, A. C.,** Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nat Immunol* 2005. **6**: 928-937.
- 82 **Sun, J. C. and Lanier, L. L.,** Tolerance of NK cells encountering their viral ligand during development. *J Exp Med* 2008. **205**: 1819-1828.
- 83 **Leung, W.,** Use of NK cell activity in cure by transplant. *British journal of hematology* 2011. **155**:14-29.
- 84 **Karlhofer, F. M., Ribaldo, R. K. and Yokoyama, W. M.,** MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature* 1992.**358**:66-70.
- 85 **Tripathy, S. K., Keyel, P. A., Yang, L., Pingel, J. T., Cheng, T. P., Schneeberger, A. and Yokoyama, W. M.,** Continuous engagement of a self-specific activation receptor induces NK cell tolerance. *J' Exp' Med* 2008. **205**: 1829-1841.
- 86 **Sun, J. C. and Lanier, L. L.,** Tolerance of NK cells encountering their viral ligand during development. *J'Exp'Med* 2008.**205**:1819-1828.
- 87 **Ryan, J. C., Naper, C., Hayashi, S. and Daws, M. R.,** Physiologic functions of activating natural killer (NK) complex-encoded receptors on NK cells. *Immunol'Rev* 2001.**181**:126-137.
- 88 **Binstadt, B. A., Billadeau, D. D., Jevremovic, D., Williams, B. L., Fang N., Yi, T., Koretzky, G. A., Abraham, R. T. and Leibson, P. J.,** SLP-76 is a direct substrate of SHP-1 recruited to killer cell inhibitory receptors. *J' Biol' Chem* 1998.**273**:27518-27523.

- 89 **Mason, L. H., Gosselin, P., Anderson, S. K., Fogler, W. E., Ortaldo, J. R. and McVicar, D. W.,** Differential tyrosine phosphorylation of inhibitory versus activating Ly-49 receptor proteins and their recruitment of SHP-1 phosphatase. *J'Immunol* 1997.**159**:4187-4196.
- 90 **Stebbins, C. C., Watzl, C., Billadeau, D. D., Leibson, P. J., Burshtyn, D. N. and Long,E.O.,** Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. *Mol' Cell' Biol* 2003. **23**: 6291-6299.
- 91 **Kane, K. P., Lavender, K. J. and Ma, B. J.,** LyY49 receptors and their functions. *Crit'Rev'Immunol* 2004.**24**:321-348.
- 92 **Cavanaugh, V. J., Raulet, D. H. and Campbell, A. E.,** Upregulation of CD94/NKG2A receptors and Qa-1b ligand during murine cytomegalovirus infection of salivary glands. *J'Gen'Virol* 2007. **88**:1440-1445.
- 93 **Boyington, J. C., Brooks, A. G. and Sun, P. D.,** Structure of killer cell immunoglobulin-like receptors and their recognition of the class I MHC molecules. *Immunol'Rev* 2001.**181**:66-78.
- 94 **Selvakumar, A., Steffens, U. and Dupont, B.,** NK cell receptor gene of the KIR family with two IG domains but highest homology to KIR receptors with three IG domains. *Tissue'Antigens* 1996.**48**:285-294.
- 95 **Orr, M. T., Wu, J., Fang, M., Sigal, L. J., Spee, P., Egebjerg, T., Dissen, E., Fossum, S., Phillips, J. H. and Lanier, L. L.,** Development and function of CD94-deficient natural killer cells. *PLoS'One* 2010. **5**: e15184.
- 96 **Vance,)R. E. Kraft, J. R., Altman, J. D., Jensen, P. E. and Raulet, D.H.,**Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b). *J' Exp' Med* 1998. **188**:1841-1848.
- 97 **Lanier, L. L.,** Natural killer cell receptor signaling. *Curr' Opin' Immunol* 2003. **15**:308-314.
- 98 **Tassi, I., Cella, M., Presti, R., Colucci, A., Gilfillan, S., Littman, D. R. and Colonna, M.,** NK cell-activating receptors require! PKC-theta for sustained signaling, transcriptional activation, and IFN-gamma secretion. *Blood* 2008. **112**:4109-4116.
- 99 **Ortaldo, J. R. and Young, H. A.,** Expression of IFN-gamma upon triggering of activating Ly49D NK receptors in vitro and in vivo: costimulation with IL-12 or IL-18 overrides inhibitory receptors. *J'Immunol* 2003.**170**:1763-1769.

- 100 **Marcenaro, E., Carlomagno, S., Pesce, S., Della Chiesa, M., Moretta, A. and Sivori, S.,** Role of alloreactive KIR2DS1(+) NK cells in haploidentical hematopoietic stem cell transplantation. *J'Leukoc'Biol* 2011.**90**:661-667.
- 101 **Biassoni, R., Pessino, A., Bottino, C., Pende, D., Moretta, L. and Moretta, A.,** The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. *Eur' J' Immunol* 1999. **29**: 1014-1020.
- 102 **Jarahian, M., Watzl, C., Fournier, P., Arnold, A., Djandji, D., Zahedi, S., Cerwenka, A., Paschen, A., Schirmacher, V. and Momburg, F.,** Activation of natural killer cells by newcastle disease virus hemagglutinin- neuraminidase. *J'Virol* 2009.**83**:8108-8121.
- 103 **Pogge von Strandmann, E., Simhadri, V. R., von Tresckow, B., Sasse, S., Reiners, K. S., Hansen, H. P., Rothe, A., Boll, B., Simhadri, V. L., Borchmann, P., McKinnon, P. J., Hallek, M. and Engert, A.,** Human leukocyte antigen-B-associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells. *Immunity* 2007. **27**: 965- 974.
- 104 **Gilfillan, S., Ho, E. L., Cella, M., Yokoyama, W. M. and Colonna, M.,** NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nat'Immunol*2002. **3**:1150-1155.
- 105 **Rozbesky, D., Kavan, D., Chmelik, J., Novak, P., Vanek, O. and Bezouska, K.,**High-level expression of soluble form of mouse natural killer cell receptor NKR-P1C(B6) in Escherichia coli. *Protein'Expr'Purif* 2011.**77**:178-184.
- 106 **Clemenceau, B., Vivien, R., Pellat, C., Foss, M., Thibault, G. and Vie, H.,**The human natural killer cytotoxic cell line NK-92, once armed with a murine CD16 receptor, represents a convenient cellular tool for the screening of mouse mAbs according!to their ADCC potential. *MAbs*2013.**5**: 587-594.
- 107 **Chlewicki, L. K., Velikovsky, C. A., Balakrishnan, V., Mariuzza, R. A. and Kumar, V.,** Molecular basis of the dual functions of 2B4 (CD244). *J' Immunol* 2008.**180**: 8159-8167.
- 108 **Garni-Wagner, B. A., Purohit, A., Mathew, P. A., Bennett, M. and Kumar, V.,** A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J' Immunol* 1993.**151**: 60-70.
- 109 **Steinman, R. M., Lustig, D. S. and Cohn, Z. A.,** Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. *J Exp Med* 1974. **139**: 1431-1445.
- 110 **Liu, K. and Nussenzweig, M. C.,** Origin and development of dendritic cells. *Immunol Rev* 2010. **234**: 45-54.

- 111 Schulz, C., Gomez Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S. E., Pollard, J. W., Frampton, J., Liu, K. J. and Geissmann, F., A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* 2012. **336**: 86-90.
- 112 Wang, Y., Zhang, Y., Yoneyama, H., Onai, N., Sato, T. and Matsushima, K., Identification of CD8alpha+CD11c- lineage phenotype-negative cells in the spleen as committed precursor of CD8alpha+ dendritic cells. *Blood* 2002. **100**: 569-577.
- 113 Huntington, N. D., Vosschenrich, C. A. and Di Santo, J. P., Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat Rev Immunol* 2007. **7**: 703-714.
- 114 Shortman, K. and Caux, C., Dendritic cell development: multiple pathways to nature's adjuvants. *Stem Cells* 1997. **15**: 409-419.
- 115 Belz, G. T. and Nutt, S. L., Transcriptional programming of the dendritic cell network. *Nat Rev Immunol* 2012. **12**: 101-113.
- 116 Fukaya, T., Murakami, R., Takagi, H., Sato, K., Sato, Y., Otsuka, H., Ohno, M., Hijikata, A., Ohara, O., Hikida, M., Malissen, B. and Sato, K., Conditional ablation of CD205+ conventional dendritic cells impacts the regulation of T-cell immunity and homeostasis in vivo. *Proc Natl Acad Sci U S A* 2012. **109**: 11288-11293.
- 117 Price, J. D., Beauchamp, N. M., Rahir, G., Zhao, Y., Rieger, C. C., Lau-Kilby, A. W. and Tarbell, K. V., CD8+ dendritic cell-mediated tolerance of autoreactive CD4+ T cells is deficient in NOD mice and can be corrected by blocking CD40L. *J Leukoc Biol* 2014. **95**: 325-336.
- 118 Villadangos, J. A. and Shortman, K., Found in translation: the human equivalent of mouse CD8+ dendritic cells. *J Exp Med* 2010. **207**: 1131-1134.
- 119 Segura, E. and Amigorena, S., Inflammatory dendritic cells in mice and humans. *Trends Immunol* 2013. **34**: 440-445.
- 120 Naik, S. H., Sathe, P., Park, H. Y., Metcalf, D., Proietto, A. I., Dakic, A., Carotta, S., O'Keeffe, M., Bahlo, M., Papenfuss, A., Kwak, J. Y., Wu, L. and Shortman, K., Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat Immunol* 2007. **8**: 1217-1226.

- 121 **O'Keeffe, M., Hochrein, H., Vremec, D., Caminschi, I., Miller, J. L., Anders, E. M., Wu, L., Lahoud, M. H., Henri, S., Scott, B., Hertzog, P., Tatarczuch, L. and Shortman, K.,** Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8(+) dendritic cells only after microbial stimulus. *J Exp Med* 2002. **196**: 1307-1319.
- 122 **Asselin-Paturel, C., Brizard, G., Pin, J. J., Briere, F. and Trinchieri, G.,** Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody. *J Immunol* 2003. **171**: 6466-6477.
- 123 **Gibson, S. J., Lindh, J. M., Riter, T. R., Gleason, R. M., Rogers, L. M., Fuller, A. E., Oesterich, J. L., Gorden, K. B., Qiu, X., McKane, S. W., Noelle, R. J., Miller, R. L., Kedl, R. M., Fitzgerald-Bocarsly, P., Tomai, M. A. and Vasilakos, J. P.,** Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod. *Cell Immunol* 2002. **218**: 74-86.
- 124 **Pashenkov, M., Kouwenhoven, M. C., Ozenci, V. and Huang, Y. M.,** Phenotypes and cytokine profiles of enriched blood dendritic cells in healthy individuals. *Eur Cytokine Netw* 2000. **11**: 456-463.
- 125 **Savina, A. and Amigorena, S.,** Phagocytosis and antigen presentation in dendritic cells. *Immunol Rev* 2007. **219**: 143-156.
- 126 **Villadangos, J. A., Schnorrer, P. and Wilson, N. S.,** Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. *Immunol Rev* 2005. **207**: 191-205.
- 127 **Bordon, Y.,** T cell responses: a dendritic cell designed for two. *Nat Rev Immunol* 2013. **13**: 844-845.
- 128 **Dzhagalov, I. L., Chen, K. G., Herzmark, P. and Robey, E. A.,** Elimination of self-reactive T cells in the thymus: a timeline for negative selection. *PLoS Biol* 2013. **11**: e1001566.
- 129 **Kim, R., Emi, M. and Tanabe, K.,** Functional roles of immature dendritic cells in impaired immunity of solid tumour and their targeted strategies for provoking tumour immunity. *Clin Exp Immunol* 2006. **146**: 189-196.
- 130 **Niino, M. and Miyazaki, Y.,** [Interferons and autoimmune diseases]. *Brain Nerve* 2013. **65**: 1381-1388.
- 131 **Wu, G. F. and Laufer, T. M.,** The role of dendritic cells in multiple sclerosis. *Curr Neurol Neurosci Rep* 2007. **7**: 245-252.

- 132 **Pende, D., Castriconi, R., Romagnani, P., Spaggiari, G. M., Marcenaro, S., Dondero, A., Lazzeri, E., Lasagni, L., Martini, S., Rivera, P., Capobianco, A., Moretta, L., Moretta, A. and Bottino, C.,** Expression of the DNAM-1 ligands, Nectin-2 (CD112) and poliovirus receptor (CD155), on dendritic cells: relevance for natural killer-dendritic cell interaction. *Blood* 2006. **107**: 2030-2036.
- 133 **Harizi, H.,** Reciprocal crosstalk between dendritic cells and natural killer cells under the effects of PGE2 in immunity and immunopathology. *Cell Mol Immunol* 2013. **10**: 213-221.
- 134 **Buentke, E., Heffler, L. C., Wilson, J. L., Wallin, R. P., Lofman, C., Chambers, B. J., Ljunggren, H. G. and Scheynius, A.,** Natural killer and dendritic cell contact in lesional atopic dermatitis skin--Malassezia-influenced cell interaction. *J Invest Dermatol* 2002. **119**: 850-857.
- 135 **Ferlazzo, G., Pack, M., Thomas, D., Paludan, C., Schmid, D., Strowig, T., Bougras, G., Muller, W. A., Moretta, L. and Munz, C.,** Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proc Natl Acad Sci U S A* 2004. **101**: 16606-16611.
- 136 **Marcenaro, E., Carlomagno, S., Pesce, S., Moretta, A. and Sivori, S.,** NK/DC crosstalk in anti-viral response. *Adv Exp Med Biol* 2012. **946**: 295-308.
- 137 **Piqueras, B., Connolly, J., Freitas, H., Palucka, A. K. and Banchereau, J.,** Upon viral exposure, myeloid and plasmacytoid dendritic cells produce 3 waves of distinct chemokines to recruit immune effectors. *Blood* 2006. **107**: 2613-2618.
- 138 **Martin-Fontecha, A., Thomsen, L. L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A. and Sallusto, F.,** Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat Immunol* 2004. **5**: 1260-1265.
- 139 **Kirchberger, S., Royston, D. J., Boulard, O., Thornton, E., Franchini, F., Szabady, R. L., Harrison, O. and Powrie, F.,** Innate lymphoid cells sustain colon cancer through production of interleukin-22 in a mouse model. *J Exp Med* 2013. **210**: 917-931.
- 140 **Rizzello, V., Bonaccorsi, I., Dongarra, M. L., Fink, L. N. and Ferlazzo, G.,** Role of natural killer and dendritic cell crosstalk in immunomodulation by commensal bacteria probiotics. *J Biomed Biotechnol* 2011. **2011**: 473097.
- 141 **Ferlazzo G and Morandi B** Cross-talks between natural killer cells and distinct subsets of dendritic cells. *Front. Immunol* 2014 5:159. doi: 10.3389/fimmu.2014.00159

- 142 **Vujanovic, L., Szymkowski, D. E., Alber, S., Watkins, S. C., Vujanovic, N. L. and Butterfield, L. H.,** Virally infected and matured human dendritic cells activate natural killer cells via cooperative activity of plasma membrane-bound TNF and IL-15. *Blood* 2010. **116**: 575-583.
- 143 **Andrews, D. M., Scalzo, A. A., Yokoyama, W. M., Smyth, M. J. and Degli-Esposti, M. A.,** Functional interactions between dendritic cells and NK cells during viral infection. *Nat Immunol* 2003. **4**: 175-181.
- 144 **Cooper, M. A., Fehniger, T. A., Fuchs, A., Colonna, M. and Caligiuri, M. A.,** NK cell and DC interactions. *Trends Immunol* 2004. **25**: 47-52.
- 145 **Zitvogel, L., Terme, M., Borg, C. and Trinchieri, G.,** Dendritic cell-NK cell cross-talk: regulation and physiopathology. *Curr Top Microbiol Immunol* 2006. **298**: 157-174.
- 146 **Portevin, D. and Young, D.,** Natural killer cell cytokine response to M. bovis BCG Is associated with inhibited proliferation, increased apoptosis and ultimate depletion of NKp44(+)CD56(bright) cells. *PLoS One* 2013. **8**: e68864.
- 147 **Hamerman, J. A., Ogasawara, K. and Lanier, L. L.,** NK cells in innate immunity. *Curr Opin Immunol* 2005. **17**: 29-35.
- 148 **Christensen, M. D. and Geisler, C.,** Recruitment of SHP-1 protein tyrosine phosphatase and signalling by a chimeric T-cell receptor-killer inhibitory receptor. *Scand J Immunol* 2000. **51**: 557-564.
- 149 **Ferlazzo, G., Tsang, M. L., Moretta, L., Melioli, G., Steinman, R. M. and Munz, C.,** Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med* 2002. **195**: 343-351.
- 150 **Spaggiari, G. M., Carosio, R., Pende, D., Marcenaro, S., Rivera, P., Zocchi, M. R., Moretta, L. and Poggi, A.,** NK cell-mediated lysis of autologous antigen-presenting cells is triggered by the engagement of the phosphatidylinositol 3-kinase upon ligation of the natural cytotoxicity receptors NKp30 and NKp46. *Eur J Immunol* 2001. **31**: 1656-1665.
- 151 **Draghi, M., Pashine, A., Sanjanwala, B., Gendzekhadze, K., Cantoni, C., Cosman, D., Moretta, A., Valiante, N. M. and Parham, P.,** NKp46 and NKG2D recognition of infected dendritic cells is necessary for NK cell activation in the human response to influenza infection. *J Immunol* 2007. **178**: 2688-2698.

- 152 **Aust, J. G., Gays, F., Mickiewicz, K. M., Buchanan, E. and Brooks, C. G.,** The expression and function of the NKRP1 receptor family in C57BL/6 mice. *J Immunol* 2009. **183**: 106-116.
- 153 **Plougastel, B. F. and Yokoyama, W. M.,** Extending missing-self? Functional interactions between lectin-like NKrp1 receptors on NK cells with lectin-like ligands. *Curr Top Microbiol Immunol* 2006. **298**: 77-89.
- 154 **Ge, M. Q., Ho, A. W., Tang, Y., Wong, K. H., Chua, B. Y., Gasser, S. and Kemeny, D. M.,** NK cells regulate CD8+ T cell priming and dendritic cell migration during influenza A infection by IFN-gamma and perforin-dependent mechanisms. *J Immunol* 2012. **189**: 2099-2109.
- 155 **Voigt, S., Mesci, A., Ettinger, J., Fine, J. H., Chen, P., Chou, W. and Carlyle, J. R.,** Cytomegalovirus evasion of innate immunity by subversion of the NKR-P1B:Clr-b missing-self axis. *Immunity* 2007. **26**: 617-627.
- 156 **Williams, K. J., Wilson, E., Davidson, C. L., Aguilar, O. A., Fu, L., Carlyle, J. R. and Burshtyn, D. N.,** Poxvirus infection-associated downregulation of C-type lectin-related-b prevents NK cell inhibition by NK receptor protein-1B. *J Immunol* 2012. **188**: 4980-4991.
- 157 **Chung, J. W., Yoon, S. R. and Choi, I.,** The regulation of NK cell function and development. *Front Biosci* 2008. **13**: 6432-6442.
- 158 **Das, S., Varalakshmi, C., Kumari, A. L., Patel, M. and Khar, A.,** Target cell induced activation of NK cells in vitro: cytokine production and enhancement of cytotoxic function. *Cancer Immunol Immunother* 2001. **50**: 428-436.
- 159 **Reschner, A., Hubert, P., Delvenne, P., Boniver, J. and Jacobs, N.,** Innate lymphocyte and dendritic cell cross-talk: a key factor in the regulation of the immune response. *Clin Exp Immunol* 2008. **152**: 219-226.
- 160 **Carlyle, J. R., Jamieson, A. M., Gasser, S., Clingan, C. S., Arase, H. and Raulet, D. H.,** Missing self-recognition of Ocil/Clr-b by inhibitory NKR-P1 natural killer cell receptors. *Proc Natl Acad Sci U S A* 2004. **101**: 3527-3532.
- 161 **Cheent, K. and Khakoo, S. I.,** Natural killer cells: integrating diversity with function. *Immunology* 2009. **126**: 449-457.
- 162 **Abeyweera, T. P., Kaissar, M. and Huse, M.,** Inhibitory receptor signaling destabilizes immunological synapse formation in primary NK cells. *Front Immunol* 2013. **4**: 410.

- 163 **Elliott, J. M., Wahle, J. A. and Yokoyama, W. M.,** MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *J Exp Med* 2010. **207**: 2073-2079.
- 164 **Koncina E, Roth L, Gonthier B and Bagnard D.,** Role of semaphorins during axon growth and guidance. *Adv Exp Med Biol* 2007: **621**: 50-64.
- 165 **Wong, J.T., W.T. Yu, and T.P. O'Connor.,** Transmembrane grasshopper Semaphorin I promotes axon outgrowth in vivo. *Development*1997. **124**: 3597-607.
- 166 **Roney, K., E. Holl, and J. Ting.,** Immune plexins and semaphorins: old proteins, new immune functions. *Protein Cell* 2013. **4**:17-26.
- 167 **Yazdani, U. and J.R. Terman.,** The semaphorins. *Genome Biol* 2006. **7**: 211.
- 168 **Siebold, C. and E.Y. Jones.,** Structural insights into semaphorins and their receptors. *Semin Cell Dev Biol* 2013. **24**:139-45.
- 169 **Takamatsu H and Kumanogoh A.,** Diverse roles for semaphorin-plexin signaling in the immune system. *Trends Immunol* 2012 **33**: 127-35.
- 170 **Capparuccia L and Tamagnone L.,** Semaphorin signaling in cancer cells and in cells of the tumor microenvironment--two sides of a coin. *J Cell Sci* 2009 **122**: 1723-36.
- 171 **Ran TS, Kolodkin AL and Bharadwaj R.,** Semaphorin regulation of cellular morphology. *Annu Rev Cell Dev Biol* 2007 **23**: 263-92.
- 172 **Vanderhaeghen P and Cheng HJ.,**Guidance molecules in axon pruning and cell death. *Cold Spring Harb Perspect Biol* 2010. **2**: a001859.
- 173 **Adams, R.H, Lohrum. M, Klostermann. A, Betz. H, and Püschel. A.W.,** The chemorepulsive activity of secreted semaphorins is regulated by furin-dependent proteolytic processing. *EMBO J* 1997. **16**: 6077-86.
- 174 **Klostermann. A, Lohrum. M, Adams, R.H, and Püschel. A.W.,** The chemorepulsive activity of the axonal guidance signal semaphorin D requires dimerization. *J Biol Chem*1998. **273**:7326-31.
- 175 **Neufeld. G, Shraga.N, Lange. T Guttman. N, Herzog. Y, and Kessler.O.,** Semaphorins in cancer. *Front Biosci* 2005. **10**: 751-60.
- 176 **Autiero, M, Smet. F, Claes. F, and Carmeliet. P.,** Role of neural guidance signals in blood vessel navigation. *Cardiovasc Res* 2005. **65**: 629-38.

- 177 **Kumanogoh, A. and Kikutani, H.**, Immunological functions of the neuropilins and plexins as receptors for semaphorins. *Nat Rev Immunol* 2013. **13**: 802-14.
- 178 **Roney, K, Holl, E, and Ting J.**, Immune plexins and semaphorins: old proteins, new immune functions. *Protein Cell* 2013 **4**: 17-26.
- 179 **Worzfeld, T, and Offermanns, S.**, Semaphorins and plexins as therapeutic targets. *Nat Rev Drug Discov* 2014 **13**: 603-21.
- 180 **Kumanogoh, A, and Kikutani, H.**, Immunological functions of the neuropilins and plexins as receptors for semaphorins. *Nat Rev Immunol* 2013 **13**: 802-14.
- 181 **Gelfand, MV, Hong, S, and Gu, C.**, Guidance from above: common cues direct distinct signaling outcomes in vascular and neural patterning. *Trends Cell Biol* 2009 **19**: 99-110.
- 182 **Mann, F, and Rougon, G.**, Mechanisms of axon guidance: membrane dynamics and axonal transport in semaphorin signalling. *J Neurochem* 2007 **102**: 316-23.
- 183 **Nasarre, P, Gemmill, RM, and Drabkin, HA.**, The emerging role of class-3 semaphorins and their neuropilin receptors in oncology. *Onco Targets Ther* 2014 **7**: 1663-87.
- 184 **Masuda T and Taniguchi M.**, Congenital diseases and semaphorin signaling: Overview to date of the evidence linking them. *Congenit Anom (Kyoto)* 2015 **55**: 26-30.
- 185 **Nakamura, F, and Goshima, Y.**, Structural and functional relation of neuropilins. *Adv Exp Med Biol* 2002 **515**: 55-69.
- 186 **Neufeld, G, and Kessler, O.**, The semaphorins: versatile regulators of tumor progression and tumor angiogenesis *Nature Reviews Cancer* 2008 **8**: 632-645
- 187 **Capparuccia L and Tamagnone L.**, Semaphorin signaling in cancer cells and in cells of the tumor microenvironment-two sides of a coin. *J Cell Sci* 2009 **122**: 1723-36.
- 188 **Feiner L, Webber AL, Brown CB, Lu MM, Jia L, Feinstein P, Mombaerts P, Epstein JA and Raper JA.**, Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption. *Development* 2001 **128**: 3061-70.
- 189 **Hayashi M, Nakashima T, Taniguchi M, Kodama T, Kumanogoh A and Takayanagi H.**, Osteoprotection by semaphorin 3A. *Nature* 2012 **485**: 69-74.

- 190 **Takamatsu H and Kumanogoh A.**, Diverse roles for semaphorin-plexin signaling in the immune system. *Trends Immunol* 2012 **33**: 127-35.
- 191 **Dontchev VD and Letourneau PC.**, Growth cones integrate signaling from multiple guidance cues. *J Histochem Cytochem* 2003 **51**: 435-44.
- 192 **Catalano A.**, The neuroimmune semaphorin-3A reduces inflammation and progression of experimental autoimmune arthritis. *J Immunol* 2010 **185**: 6373-83.
- 193 **Schmidt AM and Moore KJ.**, The Semaphorin 3E/PlexinD1 axis regulates macrophage inflammation in obesity. *Cell Metab* 2013 **18**: 461-2.
- 194 **Klagsbrun M and Shimizu A.**, Semaphorin 3E, an exception to the rule. *J Clin Invest* 2010 **120**: 2658-60.
- 195 **Oh WJ and Gu C.**, The role and mechanism-of-action of Sema-3E and Plexin-D1 in vascular and neural development. *Semin Cell Dev Biol* 2013 **24**: 156-62.
- 196 **Casazza A, Finisguerra V, Capparuccia L, Camperi A, Swiercz JM, Rizzolio S, Rolny C, Christensen C, Bertotti A, Sarotto I, Risio M, Trusolino L, Weitz J, Schneider M, Mazzone M, Comoglio PM and Tamagnone L.**, Sema-3E-Plexin D1 signaling drives human cancer cell invasiveness and metastatic spreading in mice. *J Clin Invest* 2010 **120**: 2684-98.
- 197 **Luchino J, Hocine M, Amoureux MC, Gibert B, Bernet A, Royet A, Treilleux I, Lecine P, Borg JP, Mehlen P, Chauvet S and Mann F.**, Semaphorin 3E suppresses tumor cell death triggered by the plexin D1 dependence receptor in metastatic breast cancers. *Cancer Cell* 2013 **24**: 673-85.
- 198 **Casazza A, Kigel B, Maione F, Capparuccia L, Kessler O, Giraudo E, Mazzone M, Neufeld G and Tamagnone L.**, Tumour growth inhibition and anti-metastatic activity of a mutated furin-resistant Semaphorin 3E isoform. *EMBO Mol Med* 2012 **4**: 234-50.
- 199 **Tamagnone L, Artigiani S, Chen H, He Z, Ming GL, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M and Comoglio PM.**, Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell* 1999 **99**: 71-80.
- 200 **Gay CM, Zygmunt T and Torres-Vazquez J.**, Diverse functions for the semaphorin receptor PlexinD1 in development and disease. *Dev Biol* 2011 **349**: 1-19.

- 201 **Sakurai A, Doci C, Gutkind JS.,** Semaphorin signaling in angiogenesis, lymphangiogenesis and cancer. *Cell Research* 2012 **22**:23-32.
- 202 **Chenghua Gu, Yutaka Yoshida, Jean Livet, Dorothy V. Reimert, Fanny Mann, Janna Merte, Christopher E. Henderson, Thomas M. Jessell, Alex L. Kolodkin, David D. Ginty.,** Semaphorin 3E and Plexin-D1 Control Vascular Pattern Independently of Neuropilins. *Science* 2005 **307**: 265-268
- 203 **Mailliard, R. B., Son, Y. I., Redlinger, R., Coates, P. T., Giermasz, A., Morel, P. A., Storkus, W. J. and Kalinski, P.,** Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. *J Immunol* 2003. **171**: 2366-2373.
- 204 **Choi YI, Duke-Cohan JS, Ahmed WB, Handley MA, Mann F, Epstein JA, Clayton LK, Reinherz EL.,** PlexinD1 glycoprotein controls migration of positively selected thymocytes into the medulla. *Immunity* 2008 **29**:888-98.
- 205 **Zhang, L., Procuik, M., Fang, T. and Kung, S. K.,** Functional analysis of the quantitative expression of a costimulatory molecule on dendritic cells using lentiviral vector-mediated RNA interference. *J Immunol Methods* 2009. **344**: 87-97.
- 206 **Gustafsson, K., Ingelsten, M., Bergqvist, L., Nystrom, J., Andersson, B. and Karlsson-Parra, A.,** Recruitment and activation of natural killer cells in vitro by a human dendritic cell vaccine. *Cancer Res* 2008. **68**: 5965-5971.
- 207 **Lin, F. and Butcher, E. C.,** T cell chemotaxis in a simple microfluidic device. *Lab Chip* 2006. **6**: 1462-1469.
- 208 **Nandagopal, S., Wu, D. and Lin, F.,** Combinatorial guidance by CCR7 ligands for T lymphocytes migration in co-existing chemokine fields. *PLoS One* 2011. **6**: e18183.
- 209 **Sajid Mahmood, Saravanan Nandagopal, Ibrahim Sow, Francis Lin and Sam K. P. Kung.,** Microfluidic-based, live-cell analysis allows assessment of NK-cell migration in response to crosstalk with dendritic cells. *European Journal of Immunology* 2014. **44**:2737-48.
- 210 **Cheng, M., Chen, Y., Xiao, W., Sun, R. and Tian, Z.,** NK cell-based immunotherapy for malignant diseases. *Cell Mol Immunol* 2013. **10**: 230-252.
- 211 **Zecher, D., Li, Q., Oberbarnscheidt, M. H., Demetris, A. J., Shlomchik, W. D., Rothstein, D. M. and Lakkis, F. G.,** NK cells delay allograft rejection in lymphopenic hosts by downregulating the homeostatic proliferation of CD8+ T cells. *J Immunol* 2010. **184**: 6649-6657.

- 212 **Palmer, J. M., Rajasekaran, K., Thakar, M. S. and Malarkannan, S.,** Clinical relevance of natural killer cells following hematopoietic stem cell transplantation. *J Cancer* 2013. **4**: 25-35.
- 213 **Gallois, A. and Bhardwaj, N.,** Dendritic Cell-Targeted Approaches to Modulate Immune Dysfunction in the Tumor Microenvironment. *Front Immunol* 2013. **4**: 436.
- 214 **Fauriat, C., Long, E. O., Ljunggren, H. G. and Bryceson, Y. T.,** Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* 2010. **115**: 2167-2176.
- 215 **Cravens, P. D. and Lipsky, P. E.,** Dendritic cells, chemokine receptors and autoimmune inflammatory diseases. *Immunol Cell Biol* 2002. **80**: 497-505.
- 216 **Fernandez, N. C., Lozier, A., Flament, C., Ricciardi-Castagnoli, P., Bellet, D., Suter, M., Perricaudet, M., Tursz, T., Maraskovsky, E. and Zitvogel, L.,** Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat Med* 1999. **5**: 405-411.
- 217 **Gu C, Yoshida Y, Livet J, Reimert DV, Mann F, Merte J, Henderson CE, Jessell TM, Kolodkin AL, Ginty DD.,** Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins. *Science*. 2005 **307**:265-8.
- 218 **Uesugi K, Oinuma I, Katoh H, Negishi M.,** Different requirement for Rnd GTPases of R-Ras GAP activity of Plexin-C1 and Plexin-D1. *J Biol Chem*. 2009 **284**:6743-5.
- 219 **Thornik Reimer, Marija Brcic, Matthias Schweizer, and Thomas W. Jungi.,** Receptors, Signal Transduction, and Genes poly(I:C) and LPS induce distinct IRF3 and NF- κ B signaling during type-I IFN and TNF responses in human macrophages. *J Leukoc Biol* 2008 **83**:1249-1257.

Supplementary data 1

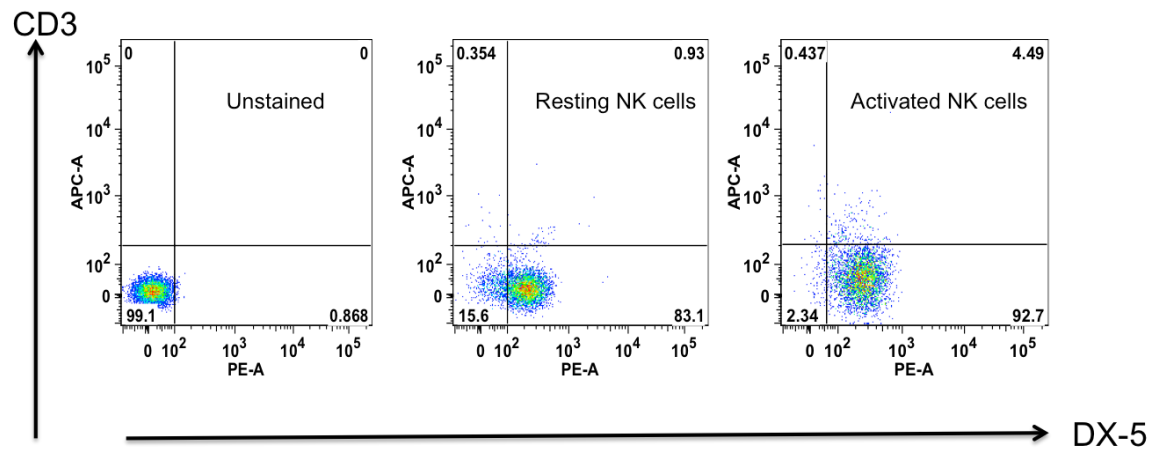


Figure7.1 Representative natural killer cells purity analysis. NK cells were isolated from spleen. Resting NK cells were collected immediately after isolation. However, activated NK cells were cultured with IL-2 (1000U/ml IL-2 concentration was used) for 4 days. Surface staining was used to measure the purity of NK cells using DX-5 and CD3 monoclonal Abs.

Supplementary data 2

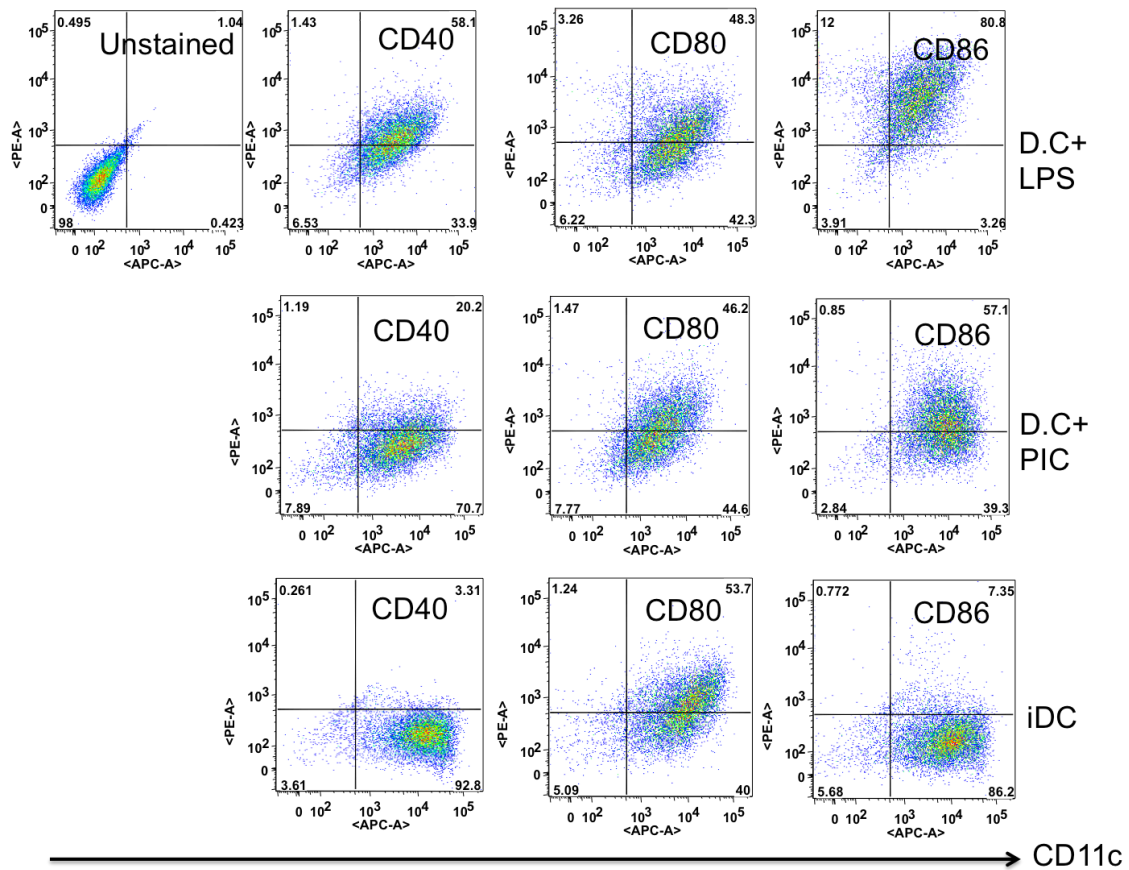


Figure7.2 Representative Dendritic cells maturation analysis. BMDCs were cultured GM-CSF medium. On day-8, lipopolysaccharide (LPS- and Polyinosinic:polycytidylic acid (Poly I:C both at 1µg/µl were introduced in the culture for 24-hours to acquire matured DC-phenotype. Surface staining was used to detect the expression of CD40, CD80 and CD86 surface markers, which represent matured-DC phenotype. In contrary to immature DCs (iDC), mature DCs with LPS up-regulated mature –DC marker.