Diverse Regulation of Natural Killer Cell Functions by Dendritic Cells

BY

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Abstract

Natural killer (NK) cells are innate lymphocytes with inherent ability to eliminate infected cells and produce several cytokines/chemokines. They express surface receptors to sense environment and interact with other immune cells including the Dendritic cells (DC). Classically, DCs are shown to activate NK cells, which is believed to be important for the induction of T-cell immune responses. NK/DC cross-talk is well-documented, yet the molecular interactions and the diverse NK cell activities (such as NK cell recognition, migration and differentiation) regulated by DC remain unclear.

Several target proteins such as MHC-I, Qa-1 mediate NK cell target recognition. One such antigen, Ocil/Clr-b functions as a cognate ligand of inhibitory NK-receptor NKR-P1B/D. I examined whether expression of this protein on DC surface affects mature NK-DC target recognition. I demonstrated that the Ocil ligand was an important immune modulator in NK/DC crosstalk. Its down-regulation enhanced the susceptibility of immature-DC towards NK-mediated lysis. Further analyses revealed that NK cells from Ocil$^{-/-}$ mice were hyporesponsive. My data suggested NKR-P1B/D:Ocil to be another receptor:ligand system, besides Ly49:MHC-I, that regulated NK responsiveness during development.

Src homology region 2-containing protein tyrosine phosphatase-1 (SHP-1) transmits inhibitory signals of the specific NK inhibitory receptors, including NKRP-1B/D, as demonstrated in previous studies. It remained to be determined whether disrupting SHP-1 signalling in mature C57BL/6 NK cells would adversely affect global NK-cell target recognitions. I observed that the SHP-1 silenced NK cells and the control were comparable in their abilities to mediate recognition and direct cell-mediated cytotoxicity against tumor targets. Interestingly, the lentiviral-mediated SHP-1 gene-silenced NK cells demonstrated an unexpected “self-killing” phenotype in vitro. Thus, my current work might implicate SHP-1 phosphatases as an important regulator of other NK-functions.

Although NK cells are bone marrow (BM)-derived lymphocytes, they are distributed in various organs. Several chemical mediators/chemokines secreted by the infected tissues

and/or immune cells are implicated in NK cell migration, however the precise underlying molecular mechanisms need to be investigated. DCs also secrete chemokines that can subsequently stimulate NK cells. Using a novel microfluidic-based approach, I reported a direct involvement of DC-derived soluble factors in the regulation of NK cell migration largely via CXCR3/IP-10 axis. Surprisingly, GM-CSF was found to induce repulsive migration in the activated NK-cells. Together, my study found that DC regulated trafficking of NK cells, potentially into the infected sites in-vivo.

While BM environment provides molecular and cellular signals to NK cell development, the precise nature and origin of these signals are still elusive. DC secretes also soluble factors which could be critical for NK development, thus offering a rationale to examine whether DC could regulate NK cell differentiation. Here, I established a multi-stage in-vitro NK-differentiation model, and identified that immature DC was able to induce NK1.1 and Ly49 receptors on developing NK-cells, in a contact-dependent manner.

In summary, my thesis work highlighted some novel interactions between NK and DC. Such information will be valuable in the future development of DC-based NK immune therapies in cancers and/or infections.

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DEDICATION

I dedicate this accomplishment to my children, Enaaya Mahmood, Insharah Mahmood, Saboor Mahmood and my wife Tahira Jabeen

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LIST OF ABBREVIATIONS

7AAD	7-Aminoactinomycin D
AIRL	antibody induced redirected lysis
APC	antigen presenting cell
BM	bone marrow
BMDC	bone marrow derived dendritic cell
CADM1	cell adhesion molecule 1
CAM	cell adhesion molecule
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD40	cluster of differentiation 40
CD40L	CD40 ligand
CD40R	CD40 receptor
CD107a	cluster of differentiation 107a
Dy	chemokine gradient
CI	chemotactic index
Clr-b	C-type lectin-related family member-b
CMFDA	chloromethylfluorescein Diacetate
CLP	common lymphoid progenitor
CpG	cytosine phosphate guanine
CTL	cytotoxic T lymphocyte
CTLA4	cytotoxic T lymphocyte antigen 4
CXCL	CXC chemokine ligand
DC	dendritic cell
DMEM	dulbecco's Modified Eagles Medium
ELISA	enzyme linked immunosorbant assay
ERK	extracellular signal regulated kinase
E:T	effector:target
Fab	antigen binding fragment
FACS	fluorescence-activated cell sorting

Fas-L	Fas-Ligand
Fas-R	Fas-receptor
FBS	fetal bovine serum
FCS	fetal calf serum
Fcγ-R	Fc-gamma receptor
Fc	crystallizable fragment
Flt3-L	fms-related tyrosine kinase 3 ligand
GFP	green fluorescent protein
GIST	Gleevic-induced lichenoid dermatitis in gastrointestinal stromal tumor
GM-CSF	granulocyte monocyte colony stimulating factor
Grb-2	growth factor receptor-bound protein 2
HEK293T	human embryonic kidney 293 T cell
HIV	human immunodeficiency virus
HBSS	hanks Buffered Salt Solution
MDSC	myeloid derived suppressor cells
HMGB1	high mobility group box 1
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
ICAM-1	intercellular adhesion molecule-1
Id proteins	inhibitors of DNA binding proteins
iDC	immature dendritic cells
IFN-γ	interferon gamma
IFN-α	interferon alpha
IFN- β	interferon beta
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cells
IMDM	Iscove's modified Dulbecco's medium
IONO	ionomycin
IP-10	interferon-γ-inducible protein 10
I/P	intra peritoneal

IS	immunological synapse
iNK	immature NK cells
ITAM	immunoreceptor tyrosine based activation motif
ITIM	immunoreceptor tyrosine based inhibitory motif
KDa	kilo Dalton
KIR	killer-cell immunoglobulin-like receptors
LAMP-1	lysosomal-associated membrane protein-1
Lin^-	lineage negative
LPS	lipopolysaccharide
mNK	mature NK cells
mDC	mature DC
MBP	major basic protein
MCMV	mouse cytomegalovirus virus
MC	mast cell
MCP-1	monocyte chemotactic protein 1
ME	mercaptoethanol
MIP	macrophage Inflammatory Protein
MDC	macrophage derived chemokine
MHC	major histocompatibility complex
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NK	natural killer cells
NKP	NK-progenitors
NCR	natural cytotoxicity receptor
Ocil	osteoclast inhibitory ligand
PAMPs	pathogen associated molecular patterns
PB	polybrene
PBMC	peripheral blood mononuclear cells
PFU	plaque forming units
PI3K	phosphatidylinositol-3 kinase
PKA	protein kinase A

PKB	protein kinase B
pDC	plasmacytoid DC
PLGF	placenta growth factor
PLC-γ	phospholipase C gamma
PG	prostaglandin
PMA	phorbol myristate acetate
PMN	polymorphonuclear neutrophils
PSC	progenitor stem cell
pVav1	phosphorylated Vav1
RANTES	regulated upon activation, normal T cell expressed, and secreted
RNA	ribonucleic acid
RORγt	retinoic acid related-receptors orphan receptor gamma t
RSV	respiratory syncytial virus
SCF	stem cell factor
shEGFP	short hairpin enhanced green florescent protein
SEM	standard error of mean
SH2	src (Sarcoma) homology 2
SHP-1	src Homology 2 Domain Phosphatase-1
shRNA	short hairpin RNA
SLP76	SH2-containing leukocyte protein of 76 kDa
SLT	secondary lymphoid tissue
STAT3	signal transducer and activator of transcription 3
SYK	spleen tyrosine kinase
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
V	velocity (average speed)
VEGF	vascular endothelial growth factor
VSVG	vesicular stomatitis virus G

1.0 CHAPTER 1

INTRODUCTION

1.1. Natural Killer cell:

Natural killer (NK) cells are known granular lymphocytes that are part of the innate immune system. These cells are equipped with competent cytolytic machinery and unlike T cells do not require prior sensitization to recognize and eliminate abnormal cells[1-3]. NK cells also secrete numerous cytokines/chemokines (e.g., IFN-γ, TNF-α, GM-CSF, MIP-1α, MIP-1β, CCL5 and RANTES) that link innate and adaptive immune systems [4-7]. In addition to the spontaneous cytotoxicity, the involvement of these cells in controlling cancer growth, immune surveillance, pregnancy and stress is well documented [8-11]. The knowledge of NK cell biology has been expanding continuously. The emerging role of NK cells in autoimmune diseases as well as the ability of these cells to demonstrate recall responses in infection models, a feature exclusively attributed to T cells and B cells, received a considerable attention with huge implications in clinical settings[12-16].

1.2. Discovery of NK cell:

In early 1970s, Herberman and colleagues identified murine lymphoid cells exhibiting cytotoxicity against several syngeneic and allogeneic mouse tumors without any prior stimulation[17, 18]. 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 they were referred as “Natural” killer cells[19].

1.3. NK subsets and distribution:

NK cells are a 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 [20,

21]. Terminally differentiated mouse NK cells express CD11b (Mac-1), CD27, CD43 surface receptors and acquire effector functions. In 2006, Hayakawa and colleague dissected mature NK cells into two distinct subpopulations based on CD27 surface density[22]. These subtypes demonstrate distinct effector functions, distribution to anatomical locations as well as acquisition of surface receptors. In response to IL12/IL-18 stimulation, $CD11b^{hi}CD27^{hi}$ NK cell population compare with $CD11b^{hi}CD27^{low}$ secrete profound amount of IFN-γ and exhibit strong cytotoxicity against target cells. These cells also acquire higher expression of CXCR3 and are enriched in secondary lymphoid tissues (SLT)[22, 23]. Conversely, $CD11b^{hi}CD27^{low}$ cells predominantly reside in the blood, spleen, lung, and liver; acquire an increased expression of inhibitory receptor repertoire, which may be correlated with their hyporesponsive effector functions [22, 24]. In the same year, Chen and colleagues reported another subset of NK cell expressing B220+CD11c+CD49+ a hybrid phenotype of NK cell and dendritic cell, referred as interferon-producing killer dendritic cells (IKDC) in the literature [25]. These non-conventional NK cells reside in the lymph nodes, spleen, BM and secrete several cytokines including IFN-γ, IFN-α, IFN-β and IL-12. Initially these cells were referred as activated NK cells because of their ability to produce copious amount of IFN-γ and eliminate tumor cells in vivo more efficiently than conventional NK cells. Current work of Sylvie Lesage and colleagues however demonstrated that these NK cells lose B220 and CD11c expression and acquire CD11b+CD27- phenotype of a mature conventional NK cells during adoptive transfer experiment. They describe IKDCs as an immediate precursor to mature NK cell rather than a defined subset[26, 27].

In addition, Di Santo and colleagues reported a subset of NK cells with a characteristic CD127 surface expression. In contrast to bone marrow derived NK cells, this subset originates from thymus and demonstrates higher dependency on GATA3 transcriptional factor. These cells also reside in SLT and produce more IFN-γ and show close resemblance with human $CD56^{bright}CD16^{dim/-}$ NK cells[28].

In contrast to mice, in human the density of CD56 expression classify NK cells into two functionally distinct subgroups. [29]. $CD56^{bright}CD16^{dim/-}$ is a minor population that reside predominantly in the secondary lymphoid tissues with substantial expression of L-selectin (CD62L), CCR7 homing receptor and CXCR3. These cells require prolonged activation

signals and deemed less cytolytic; however they are able to secrete abundant IFN-γ, TNF-α and other immunoregulatory cytokines [7, 30, 31]. On the other hand, majority of the human NK cells bear a $CD56^{dim}CD16^{high}$ phenotype. These cells are recognized as potent cytotoxic lymphocytes, however they produce negligible amounts of NK-signature cytokines following *in vitro* stimulations [32]. In contrast to the $CD56^{bright}$, CXCR1 and CX3CR1 chemokine receptors expression have been well documented on these cells, which regulate trafficking of these cells to the peripheral circulation and tissues with inflammatory insults[32, 33]. It is also interesting that most of the mature NK population reside in various organs and acquire distinct phenotypes. Human uterus also harbors $CD56^{bright}$ NK cells, however these cells acquire higher expression of KIR and CD69 activation marker compare to the $CD56^{bright}$ NK population residing in the peripheral circulation[34, 35]. Similarly, murine liver is enriched with TRAIL+ resident NK cells distinct from other murine NK cells[36, 37]. It is speculated that these phenotypic changes have been implicated with tissue specific activation signals.

1.4. Innate Lymphoid cells:

In recent years, NK cells are also classified as member of a relatively newly described group of cells called innate lymphoid cells (ILC) [38]. In mice, these cells are identified in the gut associated lymphoid tissues expressing a characteristic NKp46+ NK-receptor. In addition, they also acquire the expression of retinoic acid related-receptors orphan receptor gamma t (RORγt) and produce IL-22 in abundance. Moreover, id2 transcription factor and common γ chain signaling have been implicated in the development of these cells[38]. The inability of these cells to secrete IFN-γ and lacking perforin expression distinguished them from classical NK cells of bone marrow origin[39]. A population with similar characteristics of mouse NK-like cells expressing CD127+CD56+CD44+ phenotype is also reported in human mucosa. This subset of NK-like cells secretes IL-22 following IL-23 stimulation and express RORγt [39, 40]. The emerging evidence suggests that gut associated NK-like cells are involved in maintaining a critical association between gut microbiota and the mucosal immune responses.

1.5. Natural Killer cell differentiation:

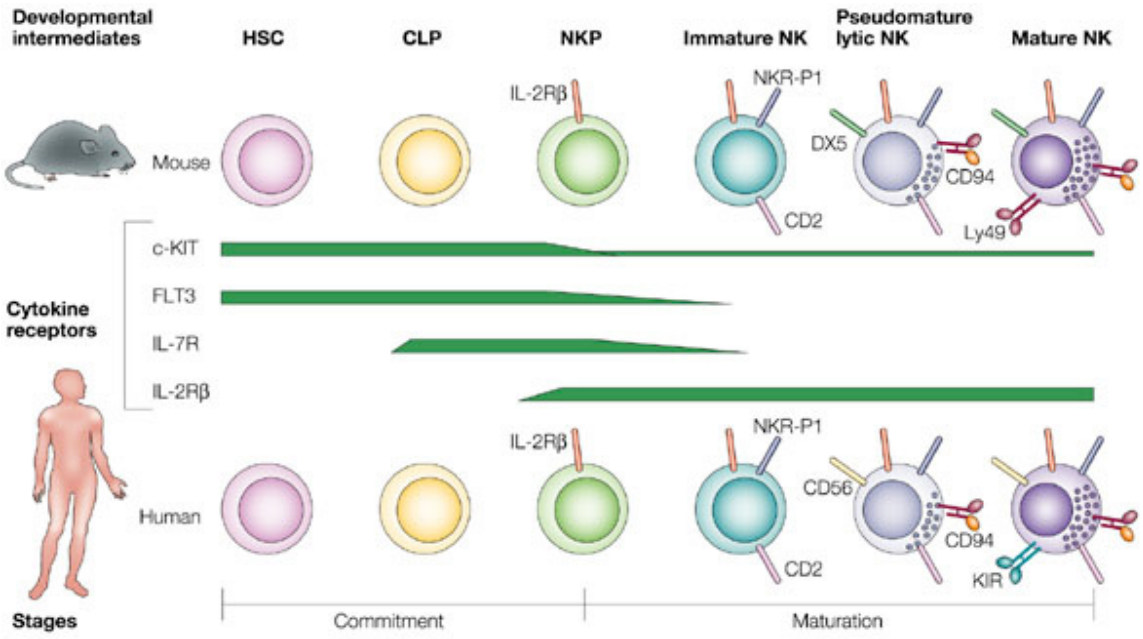

Figure 1.5: Model of NK cell differentiation: NK-cell differentiation occurs as precursors interact with cytokines and stromal cells in the bone marrow. Cell-surface molecules are sequentially expressed by maturing NK cells and can be used as markers of developmental intermediates in mice and humans. Although some markers differ between the two species, many are shared and can be used to delineate common stages of NK-cell differentiation. Stage 1 (commitment) is marked by the acquisition of expression of interleukin-2 receptor- (IL-2R). Stage 2 (maturation) can be subdivided into further steps. Acquisition of expression of NK-cell receptor protein 1 (NKR-P1) molecules (NK1.1 in mice and CD161 in humans) and CD2 identify immature NK cells that are not lytic. DX5 in mice and CD56 in humans are subsequently acquired together with cytolytic potential. Expression of the CD94–NKG2 complex is acquired later, but before the final maturation, which results in the expression of MHC-specific receptors (Ly49 in mice and killer-cell immunoglobulin-like receptors (KIRs) in humans). For clarity, other markers, such as CD38, CD7 and 2B4, are not shown. Cytokine profiles seem to be developmentally regulated in human NK cell, but evidence in mice is lacking [41].

It is believed that the development of NK cells occurs predominantly in the bone marrow (BM) [42-44]. These cells are generated from hematopoietic stem cells (HSCs) and require BM environment[41]. Coordinated actions of cytokine signaling and transcription factors 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) [45, 46]. In mice HSCs defined as Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}Kit^{+}$ population, endure a sequential loss of pluripotency and give rise to CLP with the potential to generate lymphoid cells such as T, B and NK cells [47, 48]. Several early acting cytokines including fms-related tyrosine kinase-3 ligand (Flt3L), stem cell factor (SCF) and IL-7 have been implicated in the regulation of early developmental stages. These three cytokines maintain and enhance the expansion of HSCs as well as induce CD122 (IL-2Rβ) expression on differentiating cells termed as earliest committed NK progenitors [43, 49]. In addition to BM, bipotent T/NK-progenitors as well as committed NK-progenitors have also been identified in mouse fetal liver and thymus [50, 51]. NK-progenitors, also known as IL-15 responsive intermediate, represent a heterogeneous population with a predominant characteristic $Lin^{-}CD122^{+}NK1.1^{-}DX5^{-}$ phenotype. IL-15 stimulation provides survival, proliferation and differentiation signals and mediates 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 [45, 52]. The transition of NK-progenitors to the next stage i.e., immature NK (iNK) cells is accompanied by the sequential expression of NK1.1, NKG2D, CD94/NKG2A/C surface receptors followed by the induction of DX5, ly49 receptor repertoire, CD43 and CD11b expression have been considered events in terminally differentiated NK cells, transforming these cells to mature NK cells [45, 53, 54]. Transition of NKP to immature and the subsequent maturation events in NK cell development defined as IL-15 dependent stages. Mice with IL-15 deficiency demonstrate relatively normal number and genesis of NKP, however the frequency of mature NK cell population and their cytotoxic functions are severely compromised. Many studies have shown that IL-15 signaling alone is insufficient to prime and generate functionally competent NK cells *in vitro*. However, in combination with other factors such as the use of stromal cells lines (OP9, DL1) IL-15 signaling is found critical to induce effector functional phenotype in the terminally differentiated NK cells *in vitro* [42, 45, 55].

Compared to the mouse, the developmental process in human NK cells has been considered more complicated and still unclear. Most of the existing human data regarding the potential developmental stages, molecular and cellular factors involved in the differentiation process has been generated from mouse models and *in vitro* differentiation

system. Several groups independently reported potential cellular intermediates of human NK cells, however these investigations were unable to establish a conclusive linear relationship to explain the developmental process till now. It is important to take into account that despite several commonalities, human NK cells also differ from their mouse counterparts. Human NK cells are defined by $CD3^{-}$ $CD56^{+}$ compared to the mouse NK cells, which are recognized by NK1.1 and DX5 expression [56]. Moreover, identified $CD34^{+}$ HSC, a human developmental intermediate shows undetectable levels of CD122 prior to the expression of CD94, poses a considerable challenge in identifying committed precursors of NK lineage [57]. However, despite these challenges, it is a general consensus that BM-derived $Lin^{-}CD34^{+}CD38^{dim/-}CD45RA^{-}CD10^{-}$ hematopoietic progenitor cells (HPCs) through a common lymphoid progenitor (CLP) have the potential to develop as mature human $CD3^{-}CD56^{+}$ NK cells [58, 59]. Although CLP represents a heterogeneous population, a subset with $CD34^{+}CD45RA^{+}CD117^{+}CD10^{+}$ phenotype primarily found in the BM demonstrates responsiveness to IL-15 signaling *in vitro*. Similarly, two distinct NK cell developmental intermediates with characteristic phenotypes $Lin^{-}CD34^{dim}CD45RA^{+}a_4\beta_7^{bright}CD117^{+}CD161^{+/-}CD94^{-}$ and $Lin^{-}CD34^{-}a4b7^{-}$ $CD117^{+}CD161^{+}CD94^{-}$ are identified in the human secondary lymphoid organs, able to generate mature NK cell in response to IL-15 stimulation *in vitro* [57]. These cells acquire the phenotype of a mature human NK cell by expressing surface receptors such as CD161, CD56, CD94/NKG2A, and NKG2D. The expression of killer immunoglobulin-like receptors (KIRs) and CD16 are confined to the late stages of their maturation [45]. It has become clear now that BM derived stromal cells maintain and regulate NK differentiation, however the precise nature of additional molecular and cellular signaling as well as the involvement of other stromal factors in the process of NK differentiation are yet to be known.

1.6. Transcription Factors in the regulation of NK cell development:

In addition to the common gamma chain and early acting cytokines, the development of NK cells relies on the expression and appropriate function of transcriptional factors (TF). Several TF have been identified regulating early commitment and the development of terminal stages directly or indirectly.

TF such as Ets, PU.1 and zinc-finger family members including Ikaros, and Helios have been shown central in early lymphocyte development [60]. The inhibitors of DNA binding proteins (Id1, id2, id3, id4) differentially regulate NK development. Over expression of id3 in CD34+ progenitors block T-cell development and promote NK-lineage 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 [61-63]. Other transcriptions factors such as Ikaros and E4BP4 (Nfil3) have also been shown critical during early and late phases of NK-development. Mutation in Ikaros gene not only affects the transition of CLP to earliest defined NK progenitors but also influences the maturation of NK cells in the periphery [64, 65]. Similarly, E4BP4 expression positively correlates with the development and maturation of NK cell. Mouse deficient of E4BP4, specifically exhibits aberrant progression of NKP to immature stage and to the subsequent maturation phases in a mouse model without affecting other hematopoietic cell lineages [66]. Ets-1 deficient NK cells exhibit poor cytotoxic responses. In addition to the regulation of NK maturation and acquisition of effector functions by regulating the expression of key NK markers such as NKp46, Ly49H, Ly49D, Ets1 family is also involved in the expression of T-bet and Id2 transcriptional regulators required in the early phases of NK development [67]. PU.1, another key member of Ets family is a known mediator of lymphopoiesis that regulates the development of multipotent lymphoid progenitors. Impaired progression of HSC to NKP has been associated with the deficiency of PU.I, however it is yet not clear that defective transition from lymphoid progenitor to committed NK progenitors is the direct consequence of PU.1 deficiency [45]. GATA-3, the zinc-finger TF, and master regulator of Th2 differentiation also modulate NK development. It has been shown that GATA-3 is dispensable since its deficiency cannot abrogate NK cell generation in the bone marrow in a mouse model. However, due to aberrant Ly49 expression and inability to produce IFN-γ, these cells are described as immature NK cells [68]. Conversely, NK cells of thymus origin demonstrate considerable reliance on GATA-3 expression during development [69]. Differential expression of GATA-3 is a consequence of the environment where they develop; a question remains to be answered. There is a growing list of TF including KLF1, T-bet,

MEF, TOX also linked with the regulation of NK development during maturation stages, however whether these TF have association with a specific developmental stages, work independently or in combination with other TF, remains to be determined.

1.7. Sites of NK cell development:

BM microenvironment has been well documented as a rich source of growth factors and cytokines (e.g., IL-15, IL-7, SCF, Flt3L) essential for the development NK cell *in vitro* [21, 42, 46, 55, 70, 71]. In mice, selective bone marrow ablation negatively impacts the genesis and functions of mature NK cells, indicating the significance of the intact BM for NK specific lineage [72, 73]. Additionally, 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. On the other hand, NK-cell intermediates have also been reported in several other anatomical locations (such as LN, spleen, thymus intestine, uterus, and liver) suggest multiple potential sites of NK development in vivo [36, 68, 74-78]. In 2006, Calliguiri and colleagues identified four distinct stages of human NK lineage within tonsils and LN. Interestingly, each of the isolated intermediate is able to differentiate into a fully mature $CD56^{bright}$ NK cells ex-vivo [79]. Based on these findings, this group proposed secondary lymphoid tissues (SLT) as an additional site of NK development other than the BM. Moreover, the ability of human stromal cells derived from liver, spleen and SLT to secrete stromal factors as well as signature cytokines essential for NK development *in vitro* further reinforces the credibility of the proposed model [80-83].

Mature NK cells acquire a panel of chemokine surface receptors during development and are distributed in lymphoid as well as non-lymphoid tissues. Similarly, the anatomical distribution and/or redistribution of NK precursor or other downstream intermediates to the peripheral locations cannot be ruled out. It is speculated that BM may only be involved in the genesis of committed NK precursors that migrate to SLT to receive secondary signals to turn into tissue specific and functionally distinct NK subsets. Characterization of chemokines receptor repertoire on mature NK cells as well as on their developmental intermediates would indeed be helpful to understand the distribution and development of these cells in the body.

1.8. Cytokine regulation of NK cells:

Since discovery, NK cells have been shown to recognize and eliminate transformed or pathogen infected cellular targets [1-3]. In addition, these cells produce several immunoregulatory cytokines to modulate adaptive immune responses[4, 5]. It is well documented that quality of NK mediated responses largely relies on the microenvironment where they reside. Major histocompatibility complex-1 (MHC-I) molecules intervene in the early education and induction of self-tolerance in developing NK cells. Indeed, NK cells developing in MHC-I deficient background turned hyporesponsive [84]. In mice, resting NK cells have been shown with reduced cytotoxic potential and cytokine production, which can be enhanced with the cytokine stimulation or infection with [85-87]. This reflects that NK cell indeed requires secondary signals for an appropriate activation. NK cells express surface receptors for several cytokines including IL-15, IL-2, IL-12, IL-18 and IFN-α. These cytokines in combination with other cellular factors have been implicated/shown to provide earliest activation signals to regulate the quality and the magnitude of NK-mediated innate immune responses. IL-15, is important not only during early development of NK cell but also maintains their survival, proliferation and cytotoxicity [88, 89]. In addition to IL-15, cytokines like IL-12 IL-18 and IL-2, stimulation has been associated with the dramatic increase of granzyme-B and perforin reservoirs in the resting NK cells, which further correlate with enhanced effector functions [90]. IL-18 synergize with IL-12 to promote proliferation and stimulate naïve cells to produce cytokines such as IFN-γ and TNF-α [91]. IL-2 mediated induction of Fas-L expression on tumor cells is a classical example of cytokine regulation [92]. Apart from cytokines, NK cells also acquire activation signals from biological active secreted proteins known as chemokines, key regulators of NK cell trafficking. The varied expression of CCR2, CCR5, CXCR3 and CX_3CR1 has been associated with the recruitment of NK cells to different tissues during inflammatory conditions, thus regulate NK effector functions [93-96]. In addition, several cellular interactions have also been identified, critical for the effector functions of NK cell. In particular, dendritic cells (DCs) derived soluble factors (IFN-α, IL-18 and IL-12) as well as engagement of cell surface receptors required to activate naive NK cells. Activated DC also trans-present IL-15 to the NK cells [97]. In general, NK cells are the early producer of IFN-γ in the lymph nodes

during infection. It is believed that IL-2 derived from $CD4^+$ T-cell is the primary source that boost and maintain NK cell activation and proliferation during these conditions [98]. The dynamics of NK cell: neutrophil interaction and its impact on NK-biology is fairly a new concept. In a recent study, neutrophils have been presented as a regulator of NK development and functions. NK cells derived from neutrophil deficient mouse as well as from patients with severe neutropenia demonstrated impaired cytotoxicity [99]. Interestingly, several studies have shown that NK cells also produce cytokines like IL-10 during mouse cytomegalovirus virus (MCMV) infection or IL-17 during toxoplasmosis, indicating that the nature of infection and microenvironment can also skew the fate of NK cells; however, the molecular mechanisms involved in these process have yet to be elucidated [100, 101]. Collectively, these observations demonstrate the pleiotropic nature of NK cell to anticipate variety of stimuli. Given the facts, NK cell functional acquisition is a complex phenomenon with multiple layers of regulation and indeed numerous cytokines, chemokines and immune cells are involved to modulate NK cell cytotoxicity and regulatory functions.

1.9. Effector NK cell functions (cytotoxicity, cytokines):

NK cells use several mechanisms to mediate cell cytotoxicity, however granules exocytosis is recognized as the principal and frequently used pathway by the NK cells to eliminate infected cells [102]. The process of cell cytotoxicity begins when NK cells come in contact with the target cells and form an immunological synapse (IS), which further triggers the transport of lytic granules containing perforin and granzyme to aggregate at IS. Perforin is a cytolytic protein, which disrupt the membrane at IS, facilitating granzymes to access target cell cytoplasm [103]. Alternatively perforin and granzymes stay in a giant endosome and endocytosed into the cytosol. Perforin then generates pores in the endosome to release granzyme into the cytosole [104]. Both human and mice NK cells express several granzymes (A, B, C, D, E, F, G, H, K, 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 [105].

In addition, NK cells express Fas-ligand (Fas-L) and TNF-related apoptosis-inducing-ligand (TRAIL), which can interact with target cells bearing cognate death receptors. Fas ligand, a molecule belonging to the TNF-receptor family and expressed on NK cells, can modulate killing by NK cells and tumor cells bearing Fas-receptor exhibit sensitivity to NK cell mediated killing. In general, it has been shown that many tumors exhibit no expression of Fas-receptor, a possible immune evasion mechanism, however NK cell derived IFN-γ stimulation has been implicated to induce Fas-receptor (CD95) and promote their NK-mediated elimination [92, 106]. Upon binding, Fas receptors go through trimerization, which lead to the activation of caspases, subsequently to induce apoptosis [107, 108]. TRAIL, a type II transmembrane protein, constitutively expressed on mouse liver NK cells. However cytokine stimulations including IL-2, IL-15 and IFN-α can induce TRAIL expression on NK cells as well [109-113]. Specific interaction with receptor on tumor cell, TRAIL mediate downstream signaling by activating caspase-8 directly, which in turn activates caspase-3 to induce cell death. Alternately, TRAIL induced stimulation cause DNA fragmentation of target cells by releasing Cytochrome C into the cytosol that mediate processing caspase-9 which in turn facilitate the activation of caspase 3 in collaboration with caspase-8 [114].

1.10. Regulatory role (s) of NK cells:

Besides targeting tumor and infected cells, the role of NK cell in mediating key regulatory functions has also been well defined. 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).

Among other cytokines, NK derived IFN-γ in particular has been implicated in a wide variety of immune responses including viral defense, skewing Th1 and promoting CTL responses as well as in the maturation of DC and macrophages. Activated NK cells secrete IFN-γ, TNF-α and cell-cell contact dependent signals to induce DC maturation before they gain access to T-cell enriched zones in the lymph nodes, promoting antigen specific T cells responses [115-117]. In addition, NK cells also eliminate deregulated immature DCs from the site of infection [118, 119]. Similarly, highly activated macrophages and resting microglia are the targets of NK mediated cell cytotoxicity via

NKG2D and NKp46 activating receptors [120, 121]. These immune regulatory functions preclude inappropriate T cell polarization and avoid immunopathology. Although NK cells have been involved in skewing Th1 responses by releasing IFN-γ and providing 2B4-CD48 mediated co-stimulation to the T cells, 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 [122, 123].

Also in infection, it has been demonstrated that NK cells accelerate antiviral and antitumor CD8 T cell responses in vivo [124]. Since NK cells are among early responders in infection, they orchestrate subsequent immune responses in collaboration with other immune cells. In murine model of influenza, NK cells facilitate CD8 T cell recruitment to the peripheral LN by secreting IFN-γ [125]. Subsequently, NK mediated DC maturation results in IL-12 production, which in turns promote priming and generation of CTLs. [126]. Similarly, NK cell deficiency has also been linked with aberrant induction and/or maintenance of alloantigen-specific CTLs, which yields uncontrolled growth of adoptively transferred tumors in various mouse models [127-129]. Apart from the inflammatory situations, NK cells are also implicated in many physiological conditions. NK cells are involved in Ig isotype switching and regulate B cell differentiation as well as decidual NK cells are found a source of VEGF, PLGF, which further promote angiogenesis during pregnancy [130, 131].

1.11. Target recognition of NK cells:

It is widely accepted that NK activating and inhibitory surface receptors recognize specific ligands on potential targets to discriminate between self and non-self [132-134]. Several investigations confirmed that a fine balance between activating and inhibitory signals eventually decides the functional outcome of an activated mature NK cell [134, 135]. 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 MHC-I dependent model of education, formally termed as "missing self hypothesis" [136, 137].

1.11.1 Missing self hypothesis:

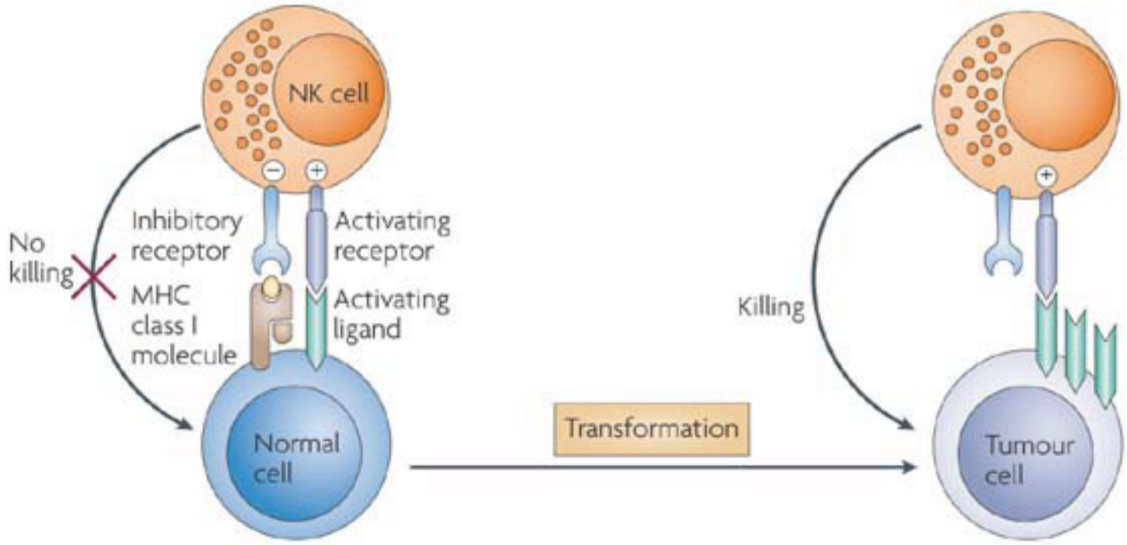

Figure 1.11.1: Missing-self recognition of tumor cells. NK-cell activation is regulated by the balance of signals mediated through activating and inhibitory receptors. Upon cellular transformation, MHC class I ligands for inhibitory receptors are often reduced or lost. In parallel, cellular stress and DNA damage lead to the upregulation of ligands for activating NK-cell receptors on the tumor cell. Together, these events shift the balance towards NK-cell activation and induction of cytolytic effector functions resulting in target-cell killing. During tumor progression, tumor variants may evolve that upregulates ligands for inhibitory receptors and/or lose ligands for activating receptors. These tumors may escape NK-cell-mediated recognition [138].

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. Later on, identification of Ly49A and KIR inhibitory receptors in mice and human NK cells respectively and their specificity in subsequent investigations reinforced the credibility of this model [139, 140]. NK cells bearing these inhibitory receptors demonstrate a positive functional correlation with the expression of MHC-I on the target cells. It has been shown

previously that NK education and self-tolerance is confined to the bone marrow, however the subsequent studies demonstrated that NK effector functions could be modulated and reprogrammed by tuning the MHC-I environment. Normal NK cells become anergic when transferred to MHC-I deficient host whereas NK cells derived from β2m deficient or Tap1 mutated mouse background acquired functional competence when adoptively transferred to MHC-I sufficient environment, suggest how MHC-I expression regulate NK education and target recognition [141, 142].

Interestingly, MHC-I dependent recognition (responsiveness) is not an independent event and NK cell requires the involvement of activating receptors to mediate effector responses. It is thought that activating receptors are involved in the education and self-tolerance of NK cells during development. Limited but some convincing reports demonstrated that continuous interaction of self-specific activating receptors (Ly49H, NKG2D) on differentiating NK cells with their cognate ligands induce generation of hyporesponsive mature NK cells with reduced ability of proliferation [143-145]. Therefore, the intriguing role of activating receptors in NK education and self-tolerance cannot be ruled out. Although, huge progress have been made in NK biology for the last two decades, the activating receptors and their associated cognate ligands involved in the rejection of MHC-I deficient targets still unclear.

Collectively, this model suggests that NK cell activation (responsiveness) is predominantly under the control of inhibitory receptors that specifically recognize and bind with class I MHC molecules. In addition, only a subset of NK cells demonstrates a given MHC-I specific receptor rather than a homogeneous expression. Moreover, it has been shown that 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 have been postulated to define NK education and self-tolerance.

1.11.2. At Least One Model:

Each developing NK cell acquires and maintains at least one MHC-I molecule specific inhibitory receptor. According to this model, only developing NK cells express inhibitory receptor successfully will be selected with full functional competence. Although, this model is widely accepted and in general support “missing self hypothesis” it does not

explain well the mechanism of self-tolerance for those NK cells derived from MHC-I deficient background. These cells exhibit self-tolerance because of their inability to reject autologous class I deficient BM graft *in vivo*. In addition, a subset of mouse NK cells has been reported, displaying tolerance to self even expressing none of the known inhibitory receptor specific to self MHC-I [141, 146].

1.12. NK cell surface receptor:

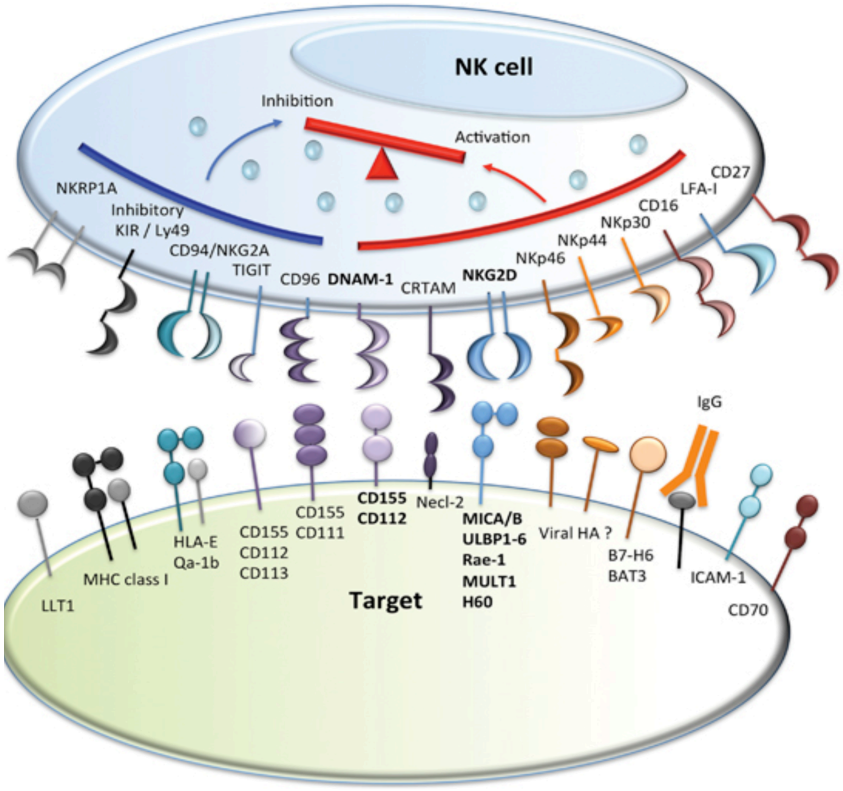

Figure 1.12.1: **NK cell receptors and their cognate ligands.** Major inhibitory and activating receptors on NK cells and their cognate ligands on targets are depicted. BAT3, human leukocyte antigen (HLA)-B-associated transcript 3; CRTAM, class I-restricted T-cell-associated molecule; HA, hemagglutinin; HLA-E, HLA class I histocompatibility antigen, alpha chain E; IgG, immunoglobulin G; LFA-1, leukocyte function-associated antigen-1; LLT1, lectin-like transcript 1; TIGIT, T cell immunoglobulin and ITIM domain [147].

1.12.1. Inhibitory receptors:

Since the discovery of first inhibitory receptor by Yokoyama and colleagues in 1992, three families of inhibitory receptors specific to MHC-I molecules have been described so far: Ly49 in rodents, KIR in humans and CD94/NKG2A both in human and rodents [139, 148]. During development, NK cells acquire different combinations of Inhibitory receptors, and each cell expresses only a few types of inhibitory receptors. However, in comparison to the activating receptor, the frequency of inhibitory receptors on an individual NK cells always dominate [149]. It is generally believed that every NK cell expresses at least one inhibitory receptor to discriminate between self and non-self MHC-I molecules.

1.12.1.1. Ly49 receptors:

Ly49 receptors are lectin like type II transmembrane proteins expressed on the surface of NK cells, NKT-cells and γδ T lymphocytes [150, 151]. 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-I molecule are the primary ligand for most of the Ly49 inhibitory receptors, however Ly49H, member of Ly49 family recognize m157 on MCMV infected cells and act as activating receptors. Majority of the Ly49 receptors have unknown ligands other than MHC-I molecules [151].

1.12.1.2. CD94/NKG2A:

CD94/NKG2A is a C-type lectin superfamily, constituting a heterodimer form expressed on the surface of NK [54, 152, 153]. Upregulation of HLA-E and Qa1 in infections and tumors is implicated to subvert NK mediated immune responses by engaging CD94/NKG2A inhibitory surface receptor. CD94/NKG2A is among first few inhibitory receptors, NK cells acquire in early developmental stages and thought to play a role in self-tolerance mechanisms [154, 155].

1.12.1.3. Killer Immunoglobulin-like receptors (KIR):

Unlike mice and other rodents, humans encode KIR, type I integral membrane proteins and part of Ig-superfamily [156]. Like their Ly49 counterpart KIR show high polymorphism with both activating and inhibitory receptors and each receptor is expressed in a variegated pattern. KIR expresses two or three extracellular Ig domains with a characteristic cytoplasmic domain. KIR proteins with a long cytoplasmic tail

exhibit ITIM (I/VxYxxL/V) associated inhibitory signals whereas short tail associated KIR proteins are restricted to the activating signals via DAP12 adaptor proteins. Majority of the KIR demonstrate specificity to the HLA-class-1 molecules and maintain NK functions [157, 158].

1.12.1.4. NKR-P1D /NKR-P1B Receptors:

Inhibitory receptor NKR-P1D in C57BL/6 and its homolog NKR-P1B in Balb/c background are C-type lectin proteins belonging to the family of NKR-P1 receptors. The cytoplasmic tails of these receptors contain ITIM sequence that facilitate SHP-1 recruitment and induce NK mediated inhibition. The cognate ligand for NKR-P1D/B is a C-type lectin molecule Ocil, broadly expressed on hematopoietic and non- hematopoietic cells [159, 160].

1.13. Inhibitory receptor signaling:

Despite having considerable structural differences, all inhibitory receptors contain ITIM (Immunoreceptor tyrosine-based inhibitory motif) sequences in their cytoplasmic domain [161]. The engagement of inhibitory receptor with its specific ligand induces ITIM phosphorylation that further facilitates the recruitment and activation of SHP-1 (src-homology 2 domain-containing tyrosine phosphatases) [162]. Activated SHP-1 phosphatases have been shown to dephosphorylate Vav, LAT and PLC-γ, as well as target several other intracellular proteins such as SLP-76, ZAP-70, and Syk. SHP-1 signaling downstream of inhibitory receptor generate a dominant inhibitory environment to regulate NK responsiveness [163-166]. In addition to SHP-1, several other phosphatases including SHP-2 and SHIP have been reported to play a role in inhibitory pathways [163].

1.14. Activating receptors:

Unlike T cells, NK cells rely on a wide variety of surface receptors to initiate effector functions. It has become clear that engagement of NK inhibitory receptors alone is not enough to counter NK activation, rather a fine balance of signals generated from activating and inhibitory signals define the fate of a mature NK cell [167]. Both human and mice NK cells carry numerous activating surface receptors, upon specific engagements/activation secrete several pro-inflammatory cytokines and/or cytotoxic

granules to combat inflammatory insults. Most of the activating receptors mediate signaling through ITAM sequences present in the cytoplasmic tail.

1.14.1. Ly49/KIR receptors:

Some Ly49 and KIR members belong to the family of activating receptors. These receptors are associated with DAP12 containing ITAM. Ly49H recognizes m157 decoy ligand expressed on MCMV infected cells, whereas Ly49D shows specificity to self-molecule like H-2D^{d} [168]. Few other Ly49 activating receptors such as Ly49P and Ly49W have been reported in NOD mice that exhibit specificity against H-2D^{d} and H-2D^{k} respectively [169]. KIR2DS1 and KIR2DS2 are the examples of KIR activating receptors [170].

1.14.2. NKG2D receptor:

NKG2D is an important activating receptor. It is a type II transmembrane glycoprotein predominantly found on all NK cells, some T cells and macrophages. Two isoform of NKG2D have been identified in NK cells, which employ differentially DAP10 and DAP12 adaptor molecules to mediate downstream signalling cascade [171]. Stress molecules/proteins expressed on infected or transformed cells that share structural homology with MHC-I molecule such as Rae1, H60, MULT1 in mouse and MICA, MICB, ULBP1-4 in human are the primary ligands of this receptor [172].

1.14.3. Natural cytotoxicity receptor (NCR):

NCR is a group of many activating receptors including NKp30, NKp44, and NKp46 involved in anti-viral and anti-tumor activity of NK cells in human. NKp46 (Ncr1) is the only NCR with conserved sequence so far reported in mammals including mouse [173]. Despite the demonstrated functional significance in infection, the precise nature of cellular ligands of these receptors is not yet clear. However, few binding partners of NRC including haemagglutinin protein of influenza, Newcastle disease and Vaccinia viruses are reported in the literature [174, 175]. Recently, the role of NKp46 is also recognized in tuning T cell responses. Moreover, tumor associated nuclear factor BAT3 and B7-H6 are also shown as potential cellular ligands of NKp30 [176, 177].

1.14.4. CD16 receptor:

Low affinity Fcγ – receptor bind with Fc portion of the IgG antibody. NK cells use these low affinity receptors to eliminate cellular targets, a phenomenon referred as antibody-dependent cell-mediated cell cytotoxicity (ADCC) [178].

1.14.5. NKR-P1C (NK1.1) receptor:

A type II membrane glycoprotein, identified as the first member of the family NKR-P1. It is most specific and widely distributed surface marker on murine NK cells in C57BL/6 mice background. NKR-P1C transmembrane is associated with Fcγ involved in NK mediated degranulation and cytokine production following antibody cross linking, yet its natural ligand remains unknown[179].

1.15. Activating receptor signaling:

Several activation receptors including NKRP1C, Ly49H, Ly49D, initiate protein tyrosine kinase-dependent pathway. These receptors deliver signals through ITAM containing adaptor proteins such as CD3-zeta, FcR-γ or DAP12 [180]. Following stimulation Src family kinases induce phosphorylation of tyrosine sequences within the ITAM, which in turn recruit Syk and ZAP70 protein tyrosine kinases as well as transmembrane adaptor proteins including LAT and LAT2, resulting in the activation of downstream signaling molecules in particular PI3K, phospholipases, Vav and ERK [181, 182]. Importantly, ITAM mediated activation primes NK cells to display cytotoxicity as well as secrete IFN-γ. However, certain other activating receptors such as NKG2D, employed DAP10 adapter proteins containing cytoplasmic YINM motif. Following stimulation, a direct recruitment of PI3K and Grb-2-Vav1 complex has been demonstrated which trigger downstream signaling cascade, sufficient to induce only cytotoxic responses in activated NK cells [182].

1.16. Receptors with dual functions:

1.16.1. 2B4 (CD244):

This receptor demonstrates a conserved sequence both in mice and human and binds with non-MHC related molecules [183]. 2B4 recognizes CD48; a glycoprotein expressed on hematopoietic cells and mediates multiple functions. The relative expression levels of 2B4 and their cross linking potential which further influence recruitment of intracellular

adapter proteins have been considered important regulating the switching functions of human and murine NK cells. A strong cross linking result in NK inhibitory functions compared to the NK activation which is associated with relatively low 2B4 expression and weak antibody stimulation [184, 185].

1.17. Dendritic cells:

Dendritic cells (DC) are well-defined professional antigen presenting cells. Due to their peculiar characteristic shape these cells were initially described as part of the nervous system. However, in 1974, Ralph Steinmann and colleagues defined these cells a distinct class of leukocytes, and formally named them "DC" [186]. Because of their ability to modulate innate as well as the acquired immune systems, DCs are also known as master regulator of the immune system. Based on anatomical distribution and functions, these cells are classified into several subgroups [187].

It is generally believed that DC belongs to the myeloid lineage and derived from hematopoietic stem cells [188]. In the presence of GM-CSF, BM myeloid precursors turn into differentiated DC. Also, transplantation of common myeloid progenitors into an irradiated mouse yields various subtypes of DCs in the spleen and thymus is an evidence of their lineage commitment. 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 [189]. It has become clear now that DC follows various differentiation pathways, as shown their generation by common myeloid progenitors and common lymphoid progenitors both *in vitro* and in vivo studies [21].

1.17.1. Types of DC:

DCs represent a heterogeneous class of immune cells. Current segregation of these cells is primarily based on phenotype, location where they reside and their unique functions [187]. In general both mouse and human DCs are broadly classified as conventional and plasmacytoid categories with multiple subtypes.

Mouse DCs acquire MHC-II, CD4, CD8a, CD11b or/and DEC-205 receptors. However all classes of these DCs have a defined expression of CD11c [190].

1.17.2. 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 higher levels of MHC-II and CD11c, also express several pattern recognition receptors [191]. CD8a expression further mark cDC into two subclasses i.e., CD8α +CD4-CD11b-CD205+ and CD8α -CD4+CD11b+CD205-, which constitute 20% and 40% spleen DC population respectively [192, 193]. Similarly, CD8a+ DC also found in the LN as well as constitute predominantly in the thymus. Compared to the CD8α - cDC population, which reside in the marginal zones and induce Th-2 cytokine responses, CD8α+ 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 CD8α expression [193, 194].

1.17.3. Non-lymphoid tissue DCs:

Langerhans cells are the example for this class. 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 Langerin and MHC-II and other co-stimulatory surface molecules and skew Th-1 immune responses in CD4+ T cells. Skin LC can be defined into two distinct subtypes based on their surface phenotype (CD103+CD11b^{lo} Langerin+ and CD103-CD11b^{hi} Langerin-, however CD103+ LC have been implicated in several viral and bacterial infections. Similarly, there are several DC populations with CD103 expression have also been shown in the mucosal surfaces, lungs, kidney and liver [187].

1.17.4. Inflammatory DCs:

In addition to classical and other migratory DCs, both human and mice harbor a subtype of inflammatory DCs, which appear during infection. These cells differentiate from monocytes, acquire characteristic phenotype (MHC II^{+} CD11b^{+} CD11c^{+} F4/80^{+} Ly6C^{+}) and tend to relocate to the site of infection [195].

1.17.5. Plasmacytoid DCs (pDC):

Originally, these cells were identified in human spleen and bone marrow, produce profound amounts of type I interferon. pDC derived from the BM, however, yet it is not clear that these cells belong to the myeloid or lymphoid developmental pathways [196,

197]. In infection, these cells differentiate and acquire the phenotypes and some of the functions of the conventional DCs, thereby consider pre-cDC population in the literature [198]. pDCs constitute a minor population and confined predominantly to the lymphoid organs, however in contrast to the classical DCs, these cells entered into the LN through high endothelial venules (HEV). Unlike myeloid DCs, pDCs do not express 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 [199-201]. Murine pDC acquire low CD11c expression, a characteristic surface marker of DC, compared to the human counterpart. Interestingly, pDCs demonstrate low expression of MHC-II as well as co-stimulatory molecules expression, which further compromised the ability of these cells to function as efficient APC [202].

1.18. Functions of DCs:

DCs are professional antigen processing and presenting cells. Given the nature of these cells to produce 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 regulatory functions at the interface of both innate and acquired immunities [203, 204].

BM derived immature DCs or their precursors distribute throughout the body predominantly at the potential sites of pathogen entry. These cells express low levels of MHC and other co-stimulatory molecules (such as CD40, CD80, CD86), however able to sense and capture the invading pathogens. DCs express a broad range of pattern recognition receptors (PRR) such as TLR, CLR, RLR, NLR, which recognize pathogen associated molecular patterns (PAMPs). Capturing and processing of antigen induce higher expression of MHC-I and MHC-II molecules to stimulate CD8 and CD4 T cells respectively. Additionally, these cells express higher levels of co-stimulatory molecules, adhesion molecules; however exhibit reduced ability of phagocytosis and endocytosis [205-208]. In addition to the immune activation, DC appears to play a crucial role in mediating central and peripheral immune tolerance to avoid injurious effects of foreign or self-antigens. DC appears to eliminate deregulated T cells from the thymus. However,

The antigen loaded immature DCs can induce peripheral tolerance by inducing apoptosis and anergy in T cell and by activating T-regulatory cells [209, 210]. Several autoimmune disorders have been associated with failed tolerance and impaired DC functions [211-213].

1.19. NK/DC Crosstalk:

In recent years, concept/phenomenon of NK-DC crosstalk has gained a considerable attention being an important immune regulatory mechanism bridging both innate and adaptive immunities and providing a robust mechanism regulating the initiation and amplification of cellular and humoral immune response. Recent publications also suggest that NK-DC communication is bidirectional, involving multiple cytokine signals and direct cell - cell contacts [214, 215]. However, the exact nature of this interaction in vivo is yet to be known.

1.19.1. Potential sites of NK/DC interactions:

The potential interaction between NK and DC takes place in the lymphoid organs or non-lymphoid tissues [216, 217]. Immature DCs predominantly reside in the skin and intestinal mucosa to counter invading pathogens, where they constantly sense the invading pathogens. Uptake and processing of antigen result the upregulation of MHC-II, co-stimulatory molecules (CD40, CD80, CD86) and the maturing DCs able to secrete cytokines and chemokines, acquire antigen-presenting ability before they migrate to the LN, a potential meeting point with NK cell [218, 219]. In human NK cells with high expression of CCR7, L-selectin and CXCR3 reside in the LN in steady state. By contrast, mouse NK cells at large lack LN homing receptor, therefore excluded from the LN. NK cells constitute only 0.5% of the population in this organ, however in infection the recruitment increased many folds via CXCR3 pathway [216, 220]. In patients with Atopic eczema/dermatitis syndrome and Gleevic-induced lichenoid dermatitis in gastrointestinal stromal tumor (GIST), NK cells are reported in close association with DCs [216, 221]. Similarly, a small population of human and mouse NK-like cells expressing NKp44/NKp46 and IL-22 are also found in the close proximity of resident DCs in the gut mucosa [222, 223]. Consistent with these findings, it has been proposed that LN, skin and the gut mucosa are among the key sites for NK/DC interplay.

1.19.2. Nature of NK/DC interactions:

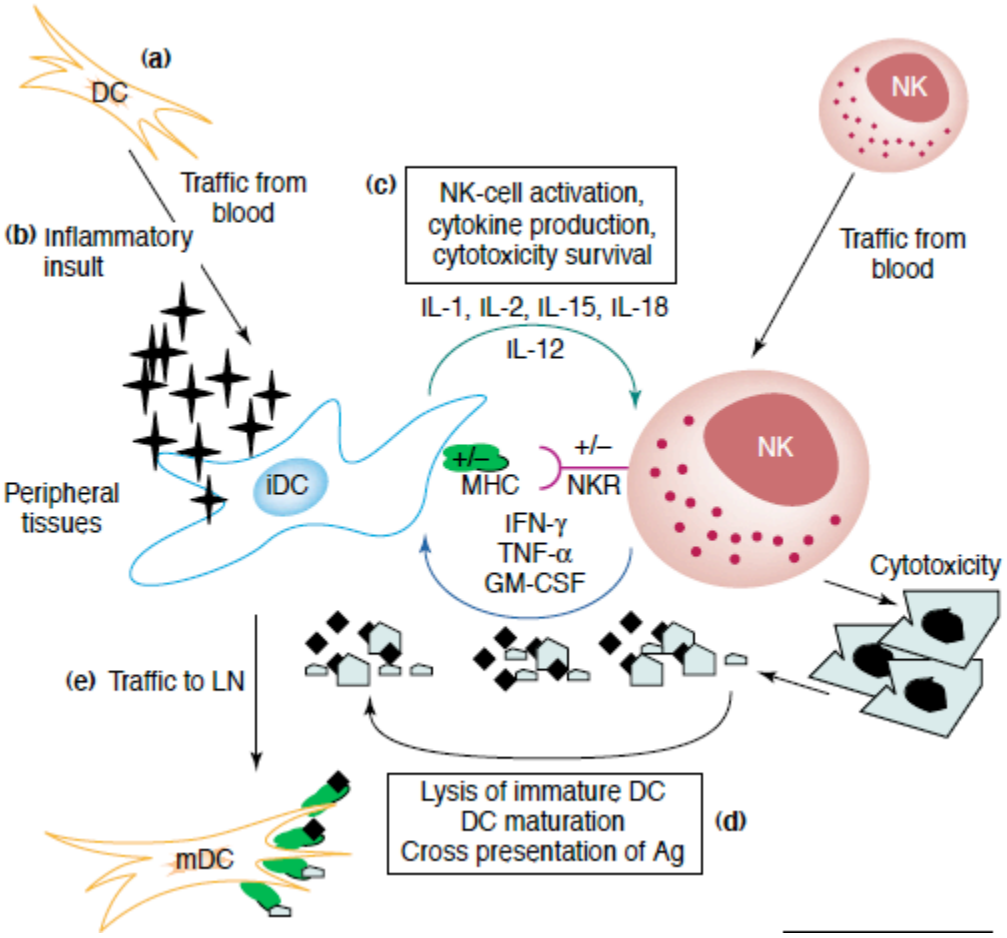

Figure: 1.19.2. NK-cell and DC interactions in the periphery. (a) Blood DCs traffic to sites of inflammation in response to inflammatory signals. (b) At the site of an infectious insult pathogens are taken up by, and activate, immature DCs (iDCs). (c) Maturing DCs release a variety of cytokines that could then act on natural killer (NK) cells recruited from the periphery by inflammatory signals and/or DC-derived chemokines. Through cytokines and cell-contact signals, mature DCs (mDCs) could induce NK-cell survival and proliferation, cytokine production, activation and cytotoxicity. (d) NK cells could then induce DC maturation and activation, including the upregulation of MHC class I molecules. In addition, NK cell-mediated lysis of infected cells could participate in cross-presentation of incoming antigens. NK cells are also capable of lysing iDCs, and might do so to limit the immune response or lyse those DCs that do not respond properly to a particular inflammatory signal. (e) Finally, mDCs will traffic to lymph nodes (LNs). NK–DC interactions probably continue while the maturing DC is acquiring the receptors necessary for trafficking to LNs. Abbreviations: Ag, antigen; GM-CSF, granulocyte–macrophage-colony stimulating factor; IFN-γ, interferon-γ; IL-1, interleukin-1; NKR, NK receptor; TNF-α, tumor necrosis factor-α [224].

DCs, classically described as the sentinels of the immune system, provide/secrete a range of signals, which in turn regulate NK cell activation, proliferation and stimulate NK-cells to mount effective *in vivo* antiviral and antitumor immune responses [225-228]. DC derived IL-12 is critical in the generation of IFN-γ producing NK cells and in combination with type I interferon amplify perforin mediated cell cytotoxicity. Interestingly, DCs derived soluble factors such as IL-1 and IL-18 have also been implicated in the acquisition of IL-12-receptor on NK cells, essential to anticipate downstream signaling [224]. The role of DC-bound IL-15 and TNF alpha proteins have been discussed extensively in the context of NK/DC cross talk. These plasma membrane proteins promote survival, proliferation as well as prime NK cell to secrete pro-inflammatory cytokines and cell cytotoxicity [228, 229]. DCs are known to produce prostaglandin (PG), a lipid mediator that influences DC as well as NK cell functions. PG pulsed DC are unable to stimulate resting NK cells to produce IFN-γ and exhibit cell cytotoxicity that subsequently modulate T cell responses [215, 230]. Reciprocally, NK cells accelerate DC maturation and activation by inducing the expression of MHC molecules and enhancing the ability to secrete IL-18, IL-12p70 via upregulation of CD86 molecules and activation of TREM2 and NKp30 signaling [115, 116, 231]. Studies have shown that the frequency as well as the activation of DC in the LN is drastically compromised following NK depletion [232]. Immature DCs (iDCs) polarization and protection from lysis is also dependent on the production of HMGB1 (high mobility group box 1) by the activated NK cells [233].

In contrast to human NK cells that exhibited partial disruption in the acquisition of their effector functions, murine NK cells failed to acquire cytotoxicity when separated from DCs in a transwell settings, indicating the significance of underlying receptor: ligand contact interactions during maturation [234]. Recently CD30-CD30L interaction appeared critical for the ability of NK cell to secrete IFN-γ and TNF as well as for the maturation of DCs [235]. However, the molecular interactions or other receptor: ligand that could be involved in NK/DC cross talk are still largely unknown.

Apart from the demonstrated regulatory interplay between these two cells, multiple studies suggest that NK-cells might regulate DC homeostasis, which in turn maintain the

balance between immunity and tolerance. Activated NK cells are capable to eliminate autologous immature DCs (iDCs) with reduced expression of MHC molecules, primarily through NKp30 and TRAIL [118, 119, 236]. However, matured DCs become resistant to NK-mediated killing because of substantial MHC expression. Several other NK-receptors including NKG2D, NKp46 and DNAM-1 and their respective ligands have also been implicated in killing of deregulated DCs in several various infection models [227, 237]. Why iDCs are the target of NK cells and the functional relevance of this mechanism remains unknown. However, it is thought that NK mediated DC-editing *in vivo* may have an implication to ensure the quality of the subsequent adaptive immune responses by limiting inappropriate T cell polarization.

1.19.3. NK/DC crosstalk in infection:

NK regulated DC maturation has been shown critical for the induction of early as well as the transition to subsequent acquired immune responses. Several human viral infections including HIV, herpes virus, influenza, poxvirus and papilolloma infections are associated with impaired NK cell functions [238, 239]. Interestingly, current literature also implicates disrupted NK/DC communication associated with the progression as well as the severity of these infections[125]. In early phases of HIV, pro-inflammatory cytokines mediated immunosuppression as well as the acquisition of anti-apoptotic molecules such as c-FLIP and CIAP2 impair NK recognition to eliminate infected DC, thus promoting the persistence of HIV infection [240]. Additionally, DCs from HIV patients are poor producer of key cytokines (like IL-12, IL-15, IL-18), which may further compromise NK activation and effector functions [241]. Moreover, NK cells lose the ability to get activated from DC derived type-1 interferon *in vitro*. In chronic infections, NK cells are also unable to recognize and eliminate immature DC. The exact mechanism is not known, however the defective function of NKp30 has been implicated in this process [242]. Similarly, progression and persistence of herpes viral infection are also linked with impaired NK/DC bidirectional crosstalk. These viruses are also involved in the modulation of NKG2D and NCR-1 NK-activating receptors, which in turn suppress T cell priming as reported in MCMV infection model [159, 237]. Ly49H+ NK cells specifically target MCMV infected cells. However, due to impaired ability of infected DC to produce cytokines such as IL-18 and IL-12, the activation, proliferation and the cytotoxic

responses of NK cells compromised in MCMV infection. Reciprocally, Ly49H+ NK cells are deemed important to maintain CD8a+ DC population in the spleen to anticipate viral infection [226]. 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 [125].

1.19.4. NK/DC crosstalk in tumor:

Well-documented *in vivo* and *in vitro* studies have shown that NK cells are capable of eliminating tumor cells [118, 243]. NK cell deficiencies and/or impaired effector functions are associated, at least in part, with the progression of several solid tumors as well as malignancies of hematological origin [231, 244]. In mice, reduced expression of self-MHC-I and/or over-expression of NKG2D related stress molecules enhance the sensitivity of NK mediated tumor lysis. Tumors, in general, have evolved several strategies including the acquisition of certain proteins 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 to evade NK-mediated immune responses [159, 237]. However, recent evidences also suggest that bi-directional cross talk between NK/DC is also disrupted in tumor microenvironment. 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 generalized immune suppression[245]. Depletion of Treg rapidly induces the recruitment of DC in the LN as well as improves the activation and proliferation of NK cells in a tumor mouse model [246]. Moreover the inability of infiltrated NK cells to produce IFN-γ in B-cell lymphoma might have an association with impaired IL-12 expression by dendritic cells in lymphoma tumor model[247]. The down regulation of NKG2D that abrogates NK cytotoxicity, MDSC derived TGF-β is involved in inhibiting NK cells derived IFN-γ production. Additionally, TGF-β is also reported in impairing NK/DC communication by blocking NKp30 expression, thus promoting accumulation of immunosuppressive iDC in the environment [141]. Similarly, the induction of IFN-γ producing NK cells following the administration of DC in B16F10-OVA tumor bearing mice is another evidence to support the significance of NK/DC crosstalk and its potential role in tumor control [159, 215]. Recent data also suggest that PG promotes tumor

progression by disrupting NK/DC communications. The presence of PG in the tumor microenvironment modulates DC functional phenotype, which in turn compromise NK activation[215]. In head and neck cancer the infiltrated DCs also demonstrate impaired secretion of IFN-α, a key cytokine required for NK cell activation [248]. Interestingly, the induction of NK/DC cross talk which subsequently improves T cell responses in these patients following the administration of therapeutic monoclonal antibody treatment is reported recently in a clinical setting [159].

1.20. Gap of Knowledge and Study Rationale:

The accumulated data documented the importance of NK/DC interactions in the shaping of the innate and adaptive arms of the immune responses. These interactions depend on cell: cell contact and cytokine signals, and occur in lymphoid and non-lymphoid tissues. Dendritic cells are the source of several cytokines (IL-15, IL-12/IL-18, IFN-α/β,) that specifically stimulate NK cells to proliferate, produce cytokines and acquire cytotoxicity. NK cells promote iDC maturation, however de-regulated iDC are also susceptible to activated NK-mediated lysis during "DC editing" process. Such interactions are important to induce high quality T-cell immune responses [231, 249]. The phenomenon of NK/DC cross talk emerged decades ago and studies have identified several important factors that regulate this interaction. However, most studies examined NK activation by its established effector functions such as cytotoxicity activity and/or signature cytokine (IFN-γ, TNF) responses. Recent data showed that cytolytic activity and cytokine responses might be regulated independently via different signaling pathways [232, 250]. The underlying molecular mechanisms that regulated various NK functions i.e. migration, cytotoxicity, chemokine/cytokine in specific microenvironment remain to be defined. A better understanding of the NK/DC interplays involved in the specific regulation of NK cell functions will enable us to better manipulate NK cell mediated responses in anti-tumor and/or anti-viral immunity.

1.21. Global Hypothesis:

Diverse NK cell functions such as migratory properties, cytotoxicity, and cytokines/chemokines production in specific microenvironments can be differentially regulated by dendritic cells.

1.22. Overarching Goals:

The overarching goal of this thesis is to elucidate novel factors/process involved in the NK cells and DC interactions.

1.22.1. Specific Aims and Sub-Hypotheses:

1) To determine the role of the cognate NKR-P1B/D:Ocil receptor:ligand in NK-DC cross talk. I tested the sub-hypothesis that surface expression of Ocil on DC regulates NK cell function.
2) To determine the importance of SHP-1 phosphatases in the regulation of the mature NK cell functions. I tested the sub-hypothesis that gene silencing of SHP-1 in mature NK cells rendered them hyper-responsive. To exclude the possible role of the SHP-1 in NK education during development in the SHP-1 gene deficient mice, I used the lentiviral vector shRNA delivery system established in the Kung laboratory to introduce SHP-1 gene silencing in the mature NK cells of the inbred C57BL/6 mice and functional properties of these cells were studied.

3) To examine the migratory properties of NK cells in the context of NK-DC crosstalk. I tested the sub-hypothesis that NK cell migration was tightly regulated by states of DC activation. I employed microfluidic platform and conventional transwell assays in this aim to elucidate the role of DC-derived soluble factors regulating NK migration.

4) To examine the role of DC in the regulation of NK-differentiation. I tested the sub-hypothesis that DC could regulate the differentiation of mature NK cells. I used established *in vitro* NK differentiation culture conditions to examine the role of DC in promoting differentiation of NK progenitor at specific stages.

2.0 CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Mice:

C57B/6NCr1 (wild type) and BALB/c (wild type) 6-8 weeks old mice were purchased from Genetic Modeling of Disease Centre (GMC), Animal Care Services (CACS), University of Manitoba. Tissue samples of Ocil$^{-/-}$ mice and age-matched wild type C57BL/6 were kindly provided by James Carlyle, Department of Immunology the university of Toronto. 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.

2.2. Cell lines:

293T cells used for virus production and titration were cultured in Iscove's modified Dulbecco's medium (IMDM) (HyClone, Logan, Utah) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin/streptomycin/L-glutamate (PSG) (Gibco, Grand Island, NY). BWZ and BWZ-Ocil cell lines were also maintained in IMDM. OP-9, YAC-1, RMA-s, P815 were cultured in Roswell Park Memorial Institute (RPMI)-1640 (HyClone) supplemented with 10% FBS, 1% PSG, and 1.6 mM 2-ME (Sigma, St. Louis, MO).

2.3. Reagents

2.3.1. Cytokines & Chemokines:

Recombinant murine IL-15, IL-7, and human FMS-related tyrosine kinase 3 ligand (Flt3L) used in the differentiation and other co-culture experiments were purchased from Peprotech. Human IL-2 and GM-CSF were used to stimulate splenic NK cells proliferation and BMDCs development respectively. Chemokines like CCL19, murine and human stromal cell factor (SCF) were also purchased from Peprotech.

2.3.2. Antibodies:

Purified and conjugated monoclonal antibodies against murine NK cell, dendritc cell and their progenitors were used in this study. A summary of all used antibodies, their

clones and source are listed in Table 1.

Serial No	Antibody	Clone	Company
1	NK1.1 (APC, PE, purified)	PK136	eBiosciences
2	Ly49 C/I (PE, FITC)	5E6	BD Biosciences
3	Ly49 C/I/F/H (PE, FITC)	14B11	eBiosciences
4	CD49b (FITC, PE, APC)	DX5	eBiosciences
5	CD27 (FITC)	LG.7F9	eBiosciences
6	NKG2D (PE)	CX5 or A10	eBiosciences
7	NKp46 (APC, PE, FITC)	29A1.4	Biologend
8	CD69 (PE)	H1.2F3	Biolegend
9	CD107a (APC, PE)	1D4B	Biolegend
10	KLRG1 (FITC, APC)	2F1	eBiosciences
11	RAE1 gamma (PE)	CX1	eBiosciences
12	$H2K^b$ (PE)	AF6-885	Biolegend
13	$H2D^b$ (PE)	KH95	Biolegend
14	CXCR4 (PE)	2B11	eBiosciences
15	CXCR3 (PE)	CXCR3-173	eBiosciences
16	CCR5 (PE)	HM-CCR5	eBiosciences
17	CD122 PE	5H4	eBiosciences
18	GM-CSF (APC)	698423	R&D systems
19	Purified GM-CSF	MP1-22E9	eBiosciences
20	CD117 (APC,PE)	2B8	eBiosciences
21	CD132 (PE)	TUGm2	Biolegend
22	CD11b (PE, APC)	M1/70	BD Pharmingen
23	IgG2aκ	A110-2	BD-bioscience
24	Ly-6A/E (Sca-1) (PE)	D7	Biolegend
25	IFN-g (PE, APC, FITC)	XMG1.2	BD Pharmingen
26	TNF alpha (APC, FITC)	MP6-XT22.	eBiosciences
27	MHC-class 11 (PE)	M5/114.15.2	eBiosciences
28	Rabbit anti-SHP-1 antibody	Y476	Abcam
29	CD3 (APC)	17A2	eBiosciences
30	CD40 (APC)	1C10	eBiosciences
31	CD86 (PE)	GL1	eBiosciences
32	CD80 (PE)	16-10A1	eBiosciences
33	CD11c (APC, FITC,	N418	eBiosciences

2.4. Purification and culturing of cells:

2.4.1. Primary mouse NK cells:

Primary NK cells ($CD3^{-}NK1.1^{+}$) were purified from mouse spleen as described previously[251]. Briefly, splenocytes were obtained from homogenized spleen by density gradient centrifugation using Ficoll (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). In degranulation, cytokines analyses and proliferation experiment, purified NK cells were cultured at 37C and 5% CO_2 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 except where indicated otherwise) (obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, National Institutes of Health, contributed by M. Gatley, Hoffman-LaRoche, Nutley, NJ).

2.4.2. Primary mouse bone marrow derived cells:

The bone marrow precursor cells were pulsed with GM-CSF to generate mature dendritic cells [252]. Briefly, precursor cells extracted from the femur and tibia and incubated with ACK buffer for 2-minutes to lyse red blood cells. Approximately 0.5-$1x10^6$ 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 5^{th}, cultures were replenished with more fresh GM-CSF medium while maintaining total volume 1-ml/well. On day-8, lipopolysaccharide (LPS-from Sigma) with 1µg/ml concentration introduced in the culture for 24-hours to acquire matured DC-phenotype. DCs with or without LPS 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.

2.4.3. Purification of progenitor stem cells (PSC):

Bone marrow is a rich source of progenitor stem cells, therefore C57BL/6 mouse bone marrow used to enrich PSC for this purpose. The Lineage Cell Depletion Kit

(Miltenyi Biotec), a magnetic labeling system, was employed for the depletion of NK cells, NKT-cells, T-cells and B-cells and their committed precursors. Extracted bone marrow cells are magnetically labeled with a cocktail of biotinylated antibodies against a panel of so-called lineage antigens (CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4, and Ter-119) and anti-biotin microbeads. This labeling procedure leaves an enrichment of progenitor stem cell population while deplete mature hematopoietic cells.

2.4.5. Culturing of PSC (NK-cell differentiation *in vitro*):

A modified multi-stage model was used to generate mature NK cells *in vitro* [43, 45]. The enriched Lin^- PSCs were cultured in NK differentiation conditioned medium (mouse complete RPMI 1640 medium containing 10% FBS, 1% PSG, 1.6 mmol/l 2-ME and supplemented with early acting cytokines such as murine IL-7 (0.5 ng/ml), of murine stromal cell factor (SCF), 30 µg/ml and human FMS-related tyrosine kinase 3 ligand (Flt3L) 50 ng/ml. On day 6, cells were pelleted, old media was removed, and replaced with 0.5 ml of complete RPMI media containing 30 ng/ml of murine IL-15 (all cytokines from Peprotech). On day 11, differentiating cells transferred to the 24-well plate containing monolayer of OP-9 cells and cultured for an additional 5-days in the presence of IL-15. Immature and mature BMDCs were introduced on day 0, 6 and 11 during all three stages of NK differentiation. The acquisition of corresponding surface markers on differentiating and matured NK cells was studied using flow cytometry analyses.

2.5. NK/DC co-culture:

Dendritic cells are potent stimulator of NK cells activation [253]. To examine proliferation and expression of CD69 activation marker on resting NK cells, a co-culture setting of naïve NK cells and dendritic cells was established. Primary NK cells were purified from spleens of wild type mice using EasySep mouse NK negative selection kit (StemCell Technologies, Vancouver, BC). The BMDCs derived from Ocil KO mice were stimulated with lipopolysaccharide (LPS) (1µg/ml) overnight, then co-cultured with naïve NK cells at NK:DC ratio 1:1 for 36 hours. Cells were harvested and the expression of CD69 was assessed by flow cytometry. For proliferation analysis, viable cells were counted using trypan blue. IL-15 (10ng/ml) concentrations were used to maintain the survival of naïve NK cells without DC culture whereas cultures with 100ng/ml IL-15

concentration was used as positive control in these experiments [91]). In NK differentiation experiments, BMDCs (immature vs matured) were cultured with progenitor stem cells, NK-progenitors and immature NK cells for 5 days in appropriate cytokines depending upon differentiation stage.

2.6. NK cell cytotoxicity and cytokine analysis:

2.6.1. CD107a degranulation assay:

CD107a lysosome-associated membrane protein-1 (LAMP-1) expression was used as a surrogate marker to measure NK-cell cytotoxicity [254, 255]. Briefly, effector NK cells were incubated with or without target cells at an effector:target (E:T) ratio of 1:1 (except where indicated otherwise) for 1-hour in V-shaped 96-well plate to a final volume of 0.2 ml. CD107a antibody (BD, San Diego, CA) and 6 µg/ml Monensin (Sigma) were added to the cultures for an additional 4 hours at 37C. Cells were washed and surface stained with NK1.1 or CD49b before acquisition in flow cytometry.

2.6.2. Intracellular staining and cytokine assay:

To detect the induction of IFN-γ and/or TNF, NK cells were incubated with target cells (DCs, Yac-1 and P815) in the presence of Brefeldin A (10µg/ml – from Sigma) for 4 hours. Cells were washed and subjected to surface and intracellular staining as described previously [251]. Briefly, cells were incubated with Fc-block (ebiosciences) in flow tube for 10 minutes on ice. Cells were stained with NK1.1 monoclonal antibody for 30 minutes on ice. After washing with flow buffer, cells were fixed with 2% para-formaldehyde (PFA) and incubated on ice for 10 minutes, while vortexing after every 3 minutes interval to avoid clumping. Fixed and surface stained cells were permeabilized with 0.1% saponin (Sigma-Aldrich) in flow buffer and then stained with specific fluorochrome-conjugated monoclononal antibodies against cytokine of interest. Samples acquisition was performed on a FACSCanto II (BD Biosciences) using Diva software and data was analyzed using FlowJo software

2.7. Transwell based migration and cytokine/chemokine analysis:

2.7.1. NK cell migration:

In this study NK cell chemotaxis/chemo-repulsion against immature, LPS-

matured conditioned medium or soluble recombinant cytokine/chemokine was performed in the transwell system as described previously [256]. Briefly, $0.2x10^6$ murine IL-2 activated NK cells (in 100 ml) were loaded on the upper chamber (5 mm pore Trans-well insert), whereas 600 ml mouse medium (or conditioned medium) was placed in the lower chamber and incubated at 37C. After 90 minutes, the cells migrated in the lower well were transferred to a polypropylene tube, centrifuged at 300g for 10 minutes and then counted. Migrated cell were also stained with anti-NK1.1 and counted by FACS as percentage of input cells. Alternatively, BMDCs were placed on the upper and immature NK cells on the lower chamber of the transwell system in our differentiation experiments. Cells from the lower chamber were then collected after 5 days and surface stained for Ly49 expression.

2.7.2. CXCR3 and GM-CSF Neutralization assay:

In order to study the significance of CXCR3 chemokine surface receptor in the migration of NK cells, purified murine NK cells (0.2×10^6 per well) were incubated with different concentrations (5µg, 10µg, 20µg and 30µg per ml) of neutralizing anti-CXCR3 antibody at 37C for 30 minutes. Cells were then transferred to the upper chamber while the LPS-stimulated DC-conditioned medium was placed in the lower chamber of the transwell system. Cells migrated into the lower chamber after 90-minutes of incubation at 37C were removed, resuspended in 100 µl PBS and counted. Similarly, to examine the role of GM-CSF in the trafficking of NK cells in the transwell system, residual GM-CSF in the LPS-stimulated DC-conditioned medium was removed using anti-GM-CSF neutralizing antibodies (R&D system). Two different concentrations (5µg, 10µg) of anti-GM-CSF antibody were mixed with conditioned medium for 1-hour at 37C. Treated medium transferred to the lower chamber while the purified murine NK cells (0.2×10^6 per well) were placed in the upper chamber of the transwell. After 90-minutes of incubation, migrated cells in the lower chamber in all wells were collected and resuspended in 100 µl PBS and counted.

2.7.3. Quantitative analysis of chemokines and cytokines:

In order to study the significance of soluble factors released by the BMDC, cells

were cultured in 24 wells plate for 7 days, then stimulated for 24 hours with LPS (1µg/ml). The supernatants were collected, centrifuged at 1200rpm for 6 minutes at 4C to remove cellular debris, and stored at -80C until further use. Interferon-gamma-induced protein (IP-10) and GM-CSF present in the culture supernatants were quantified using sandwich ELISA kits purchased from eBioscience. CCL5 quantification in the conditioned medium was performed with the Bio-Plex system (Bio-Rad) with matched Abs according to basic laboratory protocol provided by the manufacturer. All proteins were quantified in reference to the serial dilutions of standards falling within the linear part of the standard curve for each specific chemokine sample measured.

2.8. Microfluidic device based NK cell migrations assays:

2.8.1. Microfluidic device and gradient generation:

Simple ‘‘Y’’ shape microfluidic device was used for cell migration experiments in this study. The microfluidic device was designed and fabricated as explained earlier. Using SU-8 photo resist (Micro Chem, MA), the design was patterned on a silicon wafer by photolithography. Using this pattern, PDMS replicas were prepared fabricated by molding PDMS (Sylgard 184 silicon elastomer, Dow Corning, MI) against the master. The inlets and outlet are punched as described earlier. 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) Immature and mature BMDC conditioned medium and chemokine solutions (Recombinant Human CCL19/MIP-3 beta and recombinant murine CXCL4 (1µg/ml) from R&D systems of suitable concentrations were prepared in migration medium (RPMI-1640 with 0.4% BSA). FITC-Dextran 10 kD 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 where the gradient yields a smooth profile [257, 258].

2.8.2. Cell migration in the microfluidic platform:

To attach the NK cells, the microfluidic channel was coated with fibronectin (BD Biosciences) for 1 hour at room temperature and blocked with 0.4% BSA in RPMI for another hour before the experiment. For each experiment, $0.4x10^6$cells were loaded into the microfluidic device and allowed to settle in the fibronectin-coated channel for 5 min. The temperature of the device was maintained at 37C. Medium, DC-conditioned medium and chemokine solutions were 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). Cell migration was 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) [257, 258].

2.8.3. 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, average 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 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 diagram showing the distribution of angles grouped in defined intervals, with the radius of each wedge indicating cell number. The parameters between different conditions were compared by the 2-sample t test. Two-three independent experiments were repeated for each condition. The figures in the thesis were generated using one representative experiment for each condition [257, 258].

2.9. Lentiviral vector transduction:

2.9.1. Generation of shRNAs:

For short-hair pin (shRNA) induced gene silencing work, pseudotyped lentiviral vectors expressing specific (shRNA against mouse SHP-1 (TRCN0000028964-68) or

EGFP) were obtained from the RNAi consortium (TRC) Lentiviral shRNA library (Open Biosystems, Thermo Scientifc) maintained at the University of Manitoba. Human embryonic kidney (HEK) 293T cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (HyClone, Logan, UT) (supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin/glutamate (PSG) (Gibco, Grand Island, NY). Lentiviruses were generated by calcium phosphate-mediated transfection of 293T cells and used for transduction of NK cells as described elsewhere [255]. In brief, $14x10^6$ 293T cells were seeded into a T175 flask overnight. They were transfected with 5 μg of pCMV-VSV-G (envelope vector), 12.5 μg of pCMVΔR8.2DVPR (packaging vector) and 12.5 mg of the lentiviral shRNA plasmid vector (SHP-1 or control EGFP). Culture supernatant containing the lentiviruses was collected on day 3 post-transfection, and filtered through a 0.22-μm-pore-size filter. The Lentvirus particles were centrifuged at 17,000rpm for 90 minutes at 4C to concentrate to 1X.

Lentivirus titers were measured by infecting 293T cells. Briefly, 293T cells ($5 x10^4$/well) were cultured in a 24-well plate overnight ($1x10^5$ cells number expected next day) and were infected with pseudotyped lentivirus particles by incubating the cells with 250μl of virus supernatant (undiluted [1X] and 1/10X) in the presence of of polybrene (8μg/ml) in CO2 incubator for 2 hours. The virus supernatant was then replaced with 400ul complete medium. The percentages of GFP expressing cells or puromycin selective cells) were determined on day 3 and 5 post infection respectively. The lentivirus titer in plaque forming units (PFU)/ml was determined as $1x10^5$ 293T cells [the input of cells]) x (%/100 transfected cells [use as decimal]) x (4 [bring to I ml from original 250μl]) x (dilution factor). The multiplicity of infection (MOI) is calculated as: (total number of cells per well) x (desired MOI) = total infectious units (IU). Finally lentivirus titer is multiplied with total IU to calculate the required volume of lentivirus particles for transduction.

2.9.2. Lentiviral vector transduction in primary NK cells:

Throughout in this study, I used "spin protocol" to transduce primary mouse NK cell described earlier [251, 255]. Briefly, purified NK cells (ex vivo isolated or LAK, $1x10^6$) were collected in 1.5 ml eppendorf tube, centrifuged at 4000 rpm for 6 minutes in a micro centrifuge machine The culture supernatant was discarded and the cell pellet was

resuspended in 0.25 ml viral mixture (virus + 8 μg/ml Polybrene + complete RPMI medium (10% FBS, 1% PSG, 1.6 mM 2-mercaptoethanol) at MOI of 10-20. Transfer the contents of the eppendarf tube into a 48-well culture plate and centrifuged at 2000 rpm for 2 hour at room temperature. To avoid NK cell loss, virus-containing supernatant in the culture plate was carefully removed, the cell pellets were resuspended in 1 ml of complete RPMI medium containing 1,000 u/ml IL-2. After 24 hours of incubation at 37C and 5% Co_2, culture plate was subjected to 15 min spin at 2000 rpm and the supernatant was replaced with viral mixture and second round of transduction was performed as stated above. Cells were finally resuspended in 1 ml of complete RPMI medium containing IL-2 (1,000 u/ml) and incubated at 37C and 5% Co_2 for 2 more days. To select transduced NK cells, 4μg/ml puromycin (Sigma Aldrich) in IL-2 containing culture medium was added for 48-hour. Cells were washed and cultured further in mouse medium containing IL-2 for 2-3 days (depending on the experiment and the NK cell number required) before final analysis.

2.9.3. Antibody induced redirected lysis (AIRL) assay:

In the antibody induced redirected lysis (AIRL) assay, mock, shEGFP and SHP-1 shRNA transduced NK cells were treated with purified NK1.1, Ly49 C/I, IgG2a antibodies (5 μg/ml) for 20 minutes at room temperature, as follows: Panel A: No antibody; Panel B: NK1.1+IgG2a; Panel C: NK1.1+Ly49 C/I; Panel D: Ly49 C/I + IgG2a; **Panel E**: IgG2a. The cells were washed with mouse medium to get rid of unbound antibodies and employed in CD107a degranulation assay using P815 target cells. Percentage of $NK1.1^{+}CD107a^{+}$ cells was calculated for final statistical analysis [259].

2.9.4. Live Cell Imaging of NK cell cytotoxicity:

Microscopic approach was employed to visualize the mechanism of spontaneous killing of SHP-1 KD cells. Cell Tracker $^{(TM)}$ Green CMFDA dye (Invitrogen Catalog No. C2925) was mixed in 5 ml serum free RPMI-1640 mouse medium containing 1.0% BSA to a final concentration of 1.0μm. Target cells (Yac-1, P815) were immersed in 5ml labeling medium for 15 minutes at 37C and washed twice with mouse medium to remove

excess dye. The labeled target cells were incubated in pre-warmed RPMI-1640 medium containing 10% fetal calf serum (FCS), for 30 minutes at 37C and 5% CO_2. After another round of washing with complete mouse medium, the labeled target cells were resuspended in Hanks Buffered Salt Solution (HBSS) containing 10% FCS and 50µ/ml of IL-2. Finally, effector cells and labeled target cells were mixed at Effector-Target ratio of 1:1 in a final volume of 500µl containing 5 µl 7AAD in a 24 well plate. The cells were allowed to settle down on the bottom of the wells for 2 minutes and image acquisition was immediately started every 25 seconds for up to 5 hours using 10x magnification objective on a Zeiss Observer 710 station while maintaining the cells at 37C. The images in the figures were excised from live cell imaging movies at different time points and were analyzed using AxioVision software version 4.8.1. Dying cells were characterized by the loss of cytoplasmic dye and subsequent uptake of 7AAD nuclear stain visually appeared as reduction in green and increase in red fluorescence of the dying target cells.

2.10. Statistical analysis:

Data were analyzed statistically using the computer software GraphPad Prism. Results are shown as the mean ± SEM. Two-tailed student's t-test was used in a single, two-group comparison of microfluidic-derived 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.

2.11. Ethics Statement:

The University of Manitoba's review board has approved all the animal work required to complete these studies.

3.0 CHAPTER 3

A CRITICAL ROLE OF OSTEOCLAST INHIBITORY LECTIN IN FINE TUNING NATURAL KILLER CELL RESPONSIVENESS

3.1. SPECIFIC INTRODUCTION AND RATIONALE

NK cells are cytotoxic lymphocytes of the innate immune system, specifically designed to mediate anti-viral and anti-tumor immune responses [233, 234]. Regulation of NK effector functions (such as cytotoxicity, cytokine/chemokine release) is stringently controlled by activating and inhibitory surface receptors [260, 261]. These receptors bind specific ligands on the cell surface and allow NK cell to discriminate self from non-self [132, 262]. How NK cell maintain self-tolerance at cellular and molecular level remains a major interest of current research.

The relevance of MHC class 1 molecules in NK target recognition is well defined [235, 263]. A frequent correlation between elevated NK cell cytotoxicity and insufficient self-MHC-I expression on infected cells indicates that NK cells sense the absence of MHC-I. [264, 265]. It is well documented that these molecules engage Ly49 receptors on NK cell and stimulate the inhibitory pathway, which normally supersedes the stimulatory NK-target interactions [236, 266].

In addition to Ly49 receptor, mouse NK gene complex (NKC) also encodes several other lectin-like receptors including NKR-P1B and NKR-P1D [159]. Interestingly, these inhibitory receptors recognize MHC-I independent ligand known as osteoclast inhibitory lectin (Ocil) or C-type lectin-related family member-b (Clr-b), which is expressed predominantly on the surface of many hematopoietic and non-hematopoietic host cells [237-239], and is frequently down regulated on transformed/malignant cells [159]. Ocil is a type-II membrane bound lectin like molecule, described originally as inhibitor of osteoclast formation, yet recent studies implicated it in several inflammatory immune responses [267, 268]. Recently, Ocil expression has reported to be substantially down regulated in bone marrow derived macrophages with either Vaccinia or Ectromelia infection [241]. Moreover, like class 1-MHC molecules, Ocil expression was previously

shown to be downregulated on rat cytomegalovirus (CMV) infected cells [240]. Stress mediated loss of Ocil expression was also documented on leukemic cells, which further enhance the sensitivity of these cells towards NK cell cytotoxicity [268].

The crosstalk between NK cells and DC is well documented [269]. Activated NK cells regulate DC-maturation and functions including cytokine/chemokine production, T-cell polarization. Reciprocally, NK cells require close association with activated DC to gain innate and effector functions. In addition to the soluble mediators, membrane bound cellular ligands have been shown vital for the bidirectional communication between these two cell types and [214, 215]. Ocil, a well-recognized and relatively well-studied molecule is also expressed on DC surface [159]. Moreover, evidences also suggest that Ocil affects various aspects of the immune system in infection. Apart from the cooperative dialogue between NK cells and DCs, it is well documented that NK cells also target immature DCs, a process required for the induction of appropriate adaptive immune responses [214, 270]. Therefore, the ability of NK cell to recognize Ocil ligands prompted the question of whether this interaction affects NK mediated DC recognition responses during “DC-editing process”.

The main aim of this study is to examine the impact of Ocil deficiency on the effector functions of NK cells in the context of NK/DC crosstalk *in vitro*. I used murine BMDCs of Ocil deficient background in this study and found that DCs in general and immature DCs in particular stimulate NK cell cytotoxicity in CD107a degranulation assay, a surrogate marker for NK cell cytotoxicity [251, 271-273]. Furthermore, I showed that activated NK cells derived from Ocil deficient mice displayed reduced cell cytotoxicity *in vitro*. In the present study, I also established a lentiviral mediated model system to expand the current understanding of Ocil/NKR-P1B/D axis and the potential consequences on NK cell functions.

Collectively, these findings support the direct involvement of Ocil in regulating NK cell effector functions, thus revealing a previously unrecognized role in NK/DC crosstalk.

3.2. RESULTS

3.2.1. Ocil deficient ($Oci^{-/-}$) immature dendritic cells (iDCs) are sensitive targets of NK cell cytotoxicity:

Previously, it was shown that Ocil is expressed on normal hematopoietic cells including DCs, yet frequently reduced on tumor cells and render them sensitive to NK mediated killing [159, 268]. Similarly, macrophages demonstrated marked reduction in Ocil expression following viral infection [241]. DCs manifest variations in Ocil expression in infection and how it affects NK target recognition is yet to be known. To gain better insight into the regulation of Ocil expression on DC, I examined whether Ocil expression influence the sensitivity of DCs towards NK cell mediated responses *in vitro*. To this end, immature and LPS-stimulated mature BMDC derived from Ocil deficient mice were used as potential targets in CD107a degranulation assay. Importantly, I used CD107a as a surrogate marker for NK cell cytotoxicity here as well as in subsequent experiments throughout the thesis [251, 271-273]. In agreement with the published data, I also found immature DCs more susceptible targets of NK cell compared to the mature DCs [214, 270]. Strikingly, (as shown in Figure 3.1), Ocil deficiency enhanced the sensitivity of immature DCs even more. Compared to control group, wild type effector NK cells acquired significant expression of CD107a when incubated with immature DCs. No statistical difference was observed in CD107a degranulation when compared effector cells co-cultured with LPS-stimulated mature DCs of either background. However, in Ocil deficient mature DCs co-culture settings, effector cells consistently maintained elevated levels of degranulation in all experiments (Fig. 3.1). Thus these results suggest that enhanced cytotoxicity mediated by LAK is due to loss of Ocil expression on immature DCs.

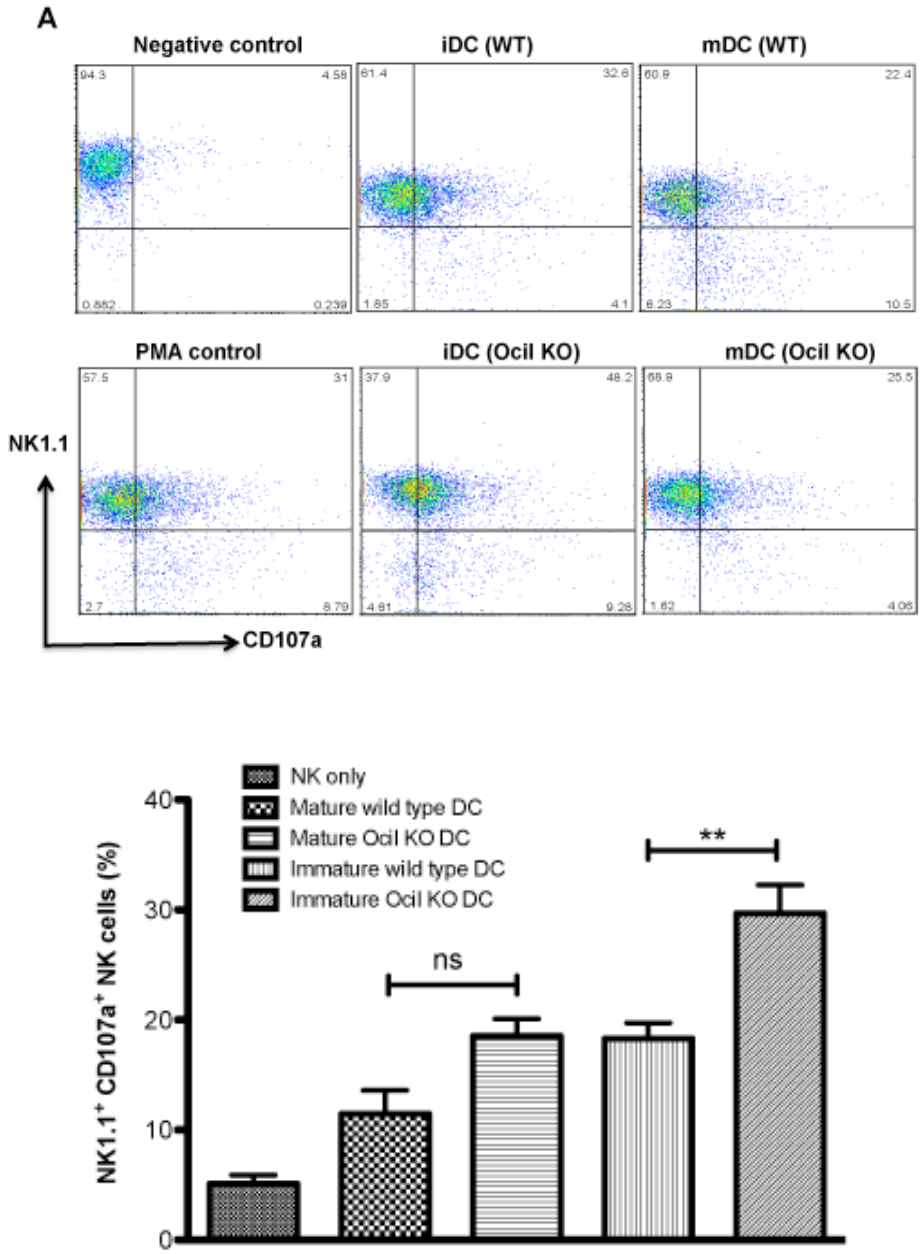

Figure 3.1. A &B: Ocil$^{-/-}$ immature DCs induce higher NK-mediated CD107a responses: LAK cells were used as effector against immature and LPS-stimulated mature BMDCs targets of Ocil deficient backgrounds in CD107a degranulation assay. BMDCs from wild type mouse background used as control in this assay. Briefly, effector and target cells were co-incubated at 1:1 (E:T ratio) in the presence of monensin and CD107a antibody as described in materials & methods section. Cells were washed and surfaced stained with NK1.1 antibody. **A:** Represents one experiment. **B:** Figure is generated with the data of 3 independent experiments. NK cells stimulated with PMA/IONO served as positive control for degranulation. Analysis was performed by flow cytometry. (**$p < 0.01$).

3.2.2. Deficiency of Ocil on DCs does not alter NK mediated cytokine responses:

In addition to the natural cytotoxic immune responses, activated NK have been reported to secrete several pro-inflammatory cytokines when interacting with susceptible targets (such as cells with reduced expression of MHC-I) [243]. Studies have shown previously, that NK cells also secrete cytokines such as IFN-γ when coming in contact with immature DC [118]. I have shown in this study that Ocil deficient immature BMDC are highly sensitive targets of NK-mediated cell cytotoxicity (Fig. 3.1). Therefore, I next investigated whether Ocil deficiency further triggers IFN-γ responses in effector cells. IL-2 stimulated NK cells were incubated with Ocil deficient immature DC. Since LPS-stimulated Ocil deficient mature DCs, compared to the control, also exhibited slightly higher sensitivity towards NK cell cytotoxicity (Fig. 3.1), therefore these cells were also included in this assay. As shown in Figure 3.2, Ocil deficiency did not trigger any such response as it was expected initially. Effector NK cells released amounts of IFN-γ comparable to those of control groups, when exposed to DCs of either background and maturation status. Thus the results of this part indicate that Ocil deficiency has no significant impact on the stimulation of NK cells to produce cytokine like IFN-γ.

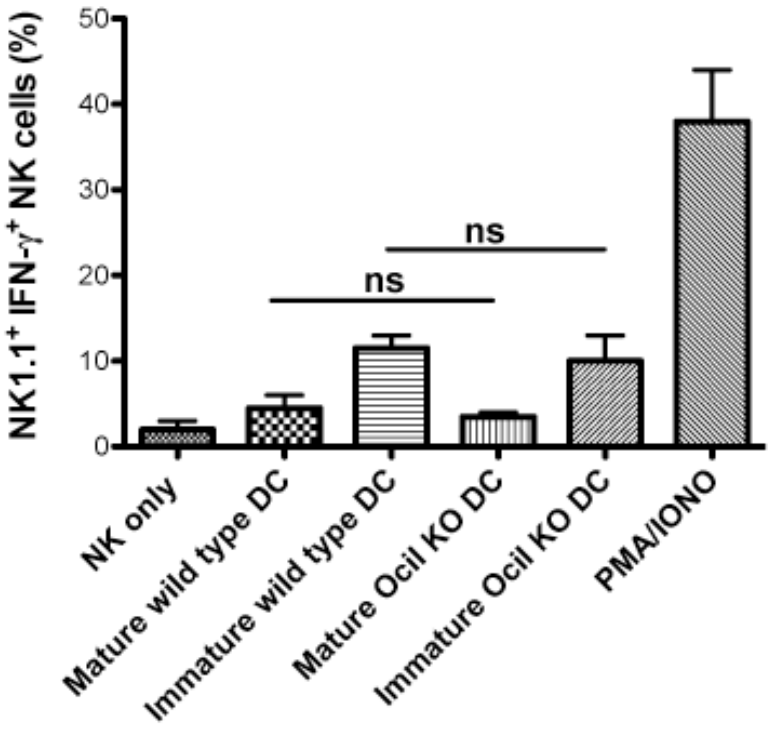

Figure 3.2. Ocil$^{-/-}$ DCs unable to stimulate NK cells to produce more IFN-γ: To detect the induction of IFN-γ, activated NK cells were co-cultured with immature and LPS-stimulated mature DCs of either background at E:T ratio of 1:1 for 4 hours in the presence of brefeldin A. Cells were surface stained with NK1.1 antibody, fixed and permeablized followed by intracellular staining for IFN-γ. LAK stimulated with PMA/IONO served as positive control. Figure is generated with the data of 3 independent experiments. Sample acquisition was performed by flow cytometry.

3.2.3. Dendritic cells with Ocil deficiency donot promote activation and proliferation of resting NK cells:

Several investigations described that matured dendritic cells with higher expressions of co-stimulatory molecules can induce activation and proliferation in resting NK cells [231, 244]. I found that LPS-stimulated Ocil deficient DCs also acquire higher expressions of maturation markers. To assess the ability of these DCs to stimulate naïve NK cells, the latter were purified from the spleen and incubated with LPS-stimulated mature DCs. However, when compared with Ocil deficient DCs with wild type as resting NK cell activators, the outcomes were not strikingly different. Co-cultures with matured Ocil$^{-/-}$

DCs induced $CD69^+$ activation marker on 10% NK cells compared to the 15% acquisition in co-culture settings with DCs of wild type background. Similarly, no considerable difference in the expansion/proliferation of resting NK cells was observed in all NK-DC co-cultures experiments. Thus the data also suggest that Ocil deficiency does not alter or significantly affect the intrinsic capabilities of DCs to activate naive NK cells.

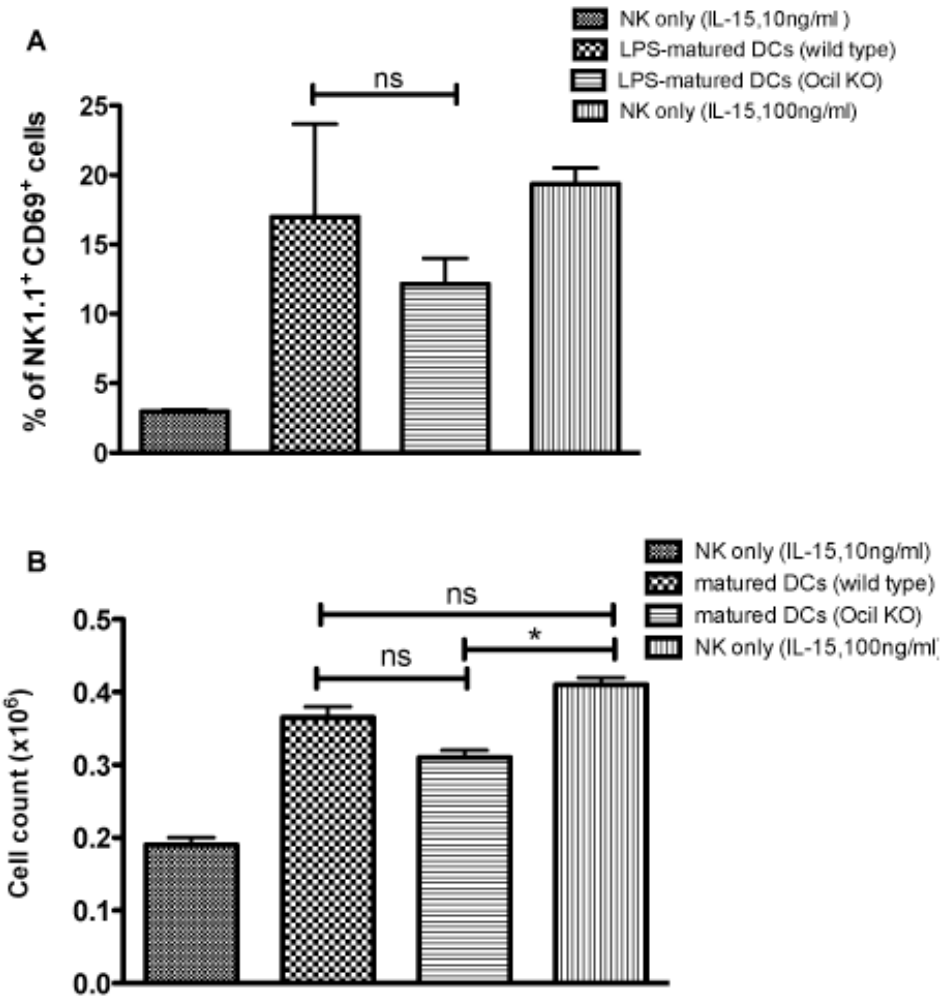

Figure 3.3. Ocil$^{-/-}$ DCs donot affect naïve NK cells activation and proliferation: A: Freshly isolated resting NK cells were co-cultured with LPS-stimulated mature DCs at 1:1 E:T ratio for 36-hours. Cells were surface stained with NK1.1 and CD69 to assess activation status of NK cells. IL-15 with 10ng/ml was used for the survival of NK cells, represented as a negative control, whereas 100ng/ml was used to stimulate resting NK cells in the absence of DCs, served as a positive control. **B:** Viable cells were counted using trypan blue. Similar results were obtained in two independent experiments**.** Figures were generated with the data of 2 independent experiments. Acquisition of cells was performed by flow cytometry. (*$p < 0.05$ and **$p < 0.01$).

3.2.4. Confirming the functional significance of NKR-P1B:Ocil axis using prototypic tumor target cells.

The observed increase in CD107a expression when Ocil KO DC were incubated with activated NK cells suggested that NKR-PIB receptor expressed on NK cells were functionally active in recognizing their cognate Ocil ligands. To confirm the functional significance of identified receptor: ligand axis, and to validate further my findings, I set up a model system utilizing prototypic tumor target cells in standard CD107a assay. Interestingly, BWZ-Ocil expressing targets demonstrated sufficient resistance to NK cell cytotoxicity compared to the parental BWZ cell line, which is devoid of Ocil expression (Fig. 3.4). Yac-1 and P815 tumor target cell lines, as NK cell sensitive and relatively resistant targets respectively, were also used as control in this assay.

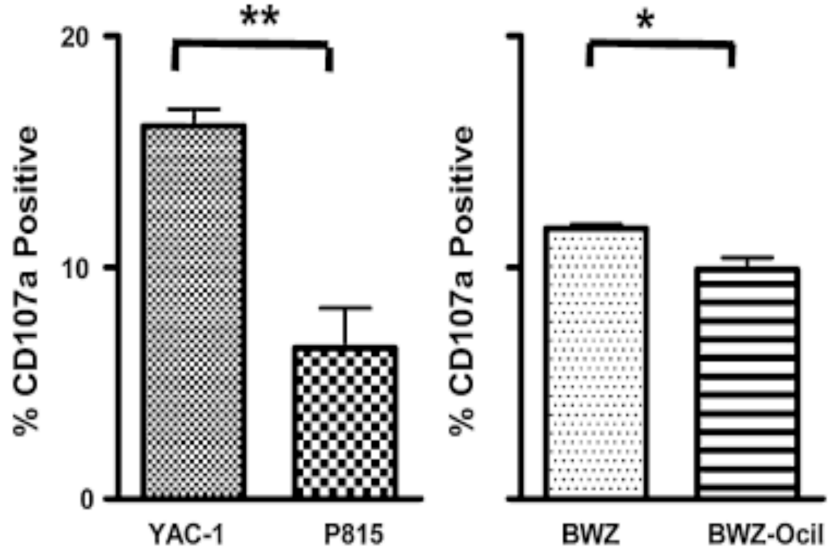

Figure 3.4. Ocil expression on BWZ cells provides resistance to NK mediated CD107a response: BWZ-Ocil expressing cells, parental BWZ, Yac-1 and P815 were used as target cells against day 5 IL-2 activated NK cells in a CD107a degranulation assay. Briefly, NK cells were co-cultured with target cells at a 1:1 E:T ratio, incubated at 37 C for 5 h in the presence of anti-CD107a antibody and monensin. Analysis was performed by flow cytometry. (*$p < 0.05$ and **$p < 0.01$).

3.2.5. Genetically modified NK cells with higher expression of NKR-P1B^{SJL} display reduced CD107a degranulation when interacting with target cells expressing Ocil:

The functional outcome of a mature NK cell primarily relies on the balance of signal strength generated from the activating and inhibitory NK receptors, following their cognate interactions with target cell [245]. Earlier, I confirmed the importance of Ocil expression on target cells and its consequences in maintaining NK cell tolerance (Fig. 3.1 & 3.4). In this part of the study, I utilized a replication-incompetent human immunodeficiency virus type 1-based lentiviral vector to genetically modify primary NK cells to over-express the model NKR-P1B^{SJL} NK inhibitory receptor. I hypothesized that ectopic expression of the NKR-P1B^{SJL} receptor that recognized Ocil ligands; on the NK cells will tip the balance of signals further towards inhibition. To test this, mock or NKR-P1B^{SJL} transduced effector NK cells were used against BWZ-Ocil and BWZ target cells in CD107a assay. Interestingly, the NKR-P1B^{SJL} transduced NK cells developed more "resistance" in responses to the Ocil bearing target cells. In comparison to the untransduced group, a lower percentage of transduced NK cells expressing CD107a were observed when incubated with BWZ-Ocil targets. However, transduced NK cells exhibited no significant difference compared to the control NK cells in the cytotoxicity of parental BWZ targets (Fig. 3.5A). To avoid variations in NK activation status that may differ between independent experiments and individual activated NK culture, data was further analyzed as the folds decrease in CD107a degranulation against BWZ versus the BWZ-Ocil targets within individual NK effector population. Interestingly, NKR-P1B^{SJL+} effector population showed a significant increase in the fold decrease of CD107a degranulation upon interaction with BWZ and BWZ-Ocil targets (~2.5-fold) compared to mock and NKR-P1B^{SJL-} effectors NK cells (~1.2-fold) ($p < 0.001$) (Fig: 3.5B).

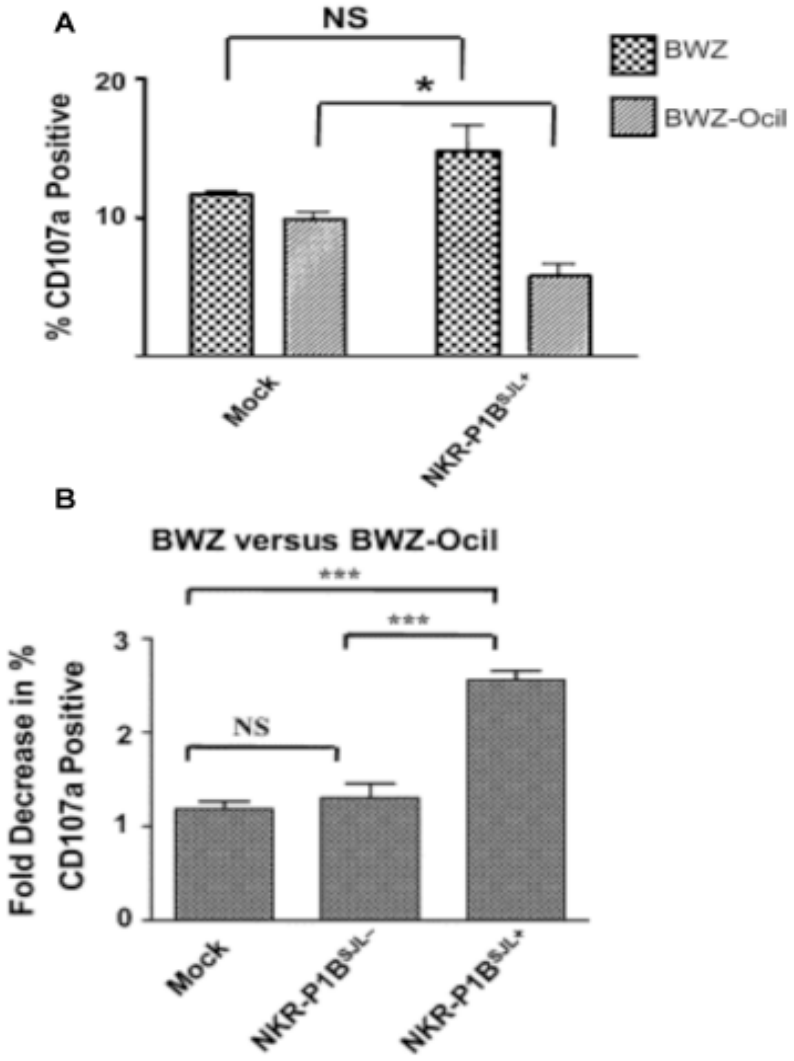

Figure 3.5. NKR-P1B^{SJL} over-expression results in decreased CD107a expression against Ocil-expressing targets. Mock-transduced and lentiviral-transduced IL-2 activated Balb/c NK cells were cultured for 3 more days before they were co-cultured with target cells and examined for CD107a expression. NKR-P1B^{SJL+} transduced cells were detected by surface staining of anti-NK1.1 monoclonal antibodies. BWZ and BWZ-Ocil cells were used as targets in the assay. The percentages of CD107a at E:T ratio 1:1 was determined by flow cytometry. **A:** Direct statistical analysis of the CD107a expression in the mock and the NKR-P1B^{SJL+} gated population upon BWZ, BWZ-Ocil target interactions. **B:** The fold-decrease changes in the CD107a expression (% CD107a in BWZ/% CD107a in BWZ-Ocil) in the mock-transduced NK (mock), NKRP1B^{SJL-} gated population and NKR-P1B^{SJL+} gated population of the transduced cells, using BWZ and BWZ-Ocil targets. Statistical significance was indicated (*$p < 0.05$, and ***$p < 0.001$). Data was a summary of 3 independent analyses.

3.2.6: NK cells developed in Ocil deficient environment show impaired DC-recognition in CD107a assay:

Although, MHC class I ligands for NK inhibitory receptors are not required for generation of normal numbers of NK cells, however these receptor:ligand interactions on the developing NK cells were found essential for the genesis of functionally competent NK cells [118, 274]. Ocil expression significantly alters NK cell target specificities (Fig. 3.1, 3.4 & 3.5), whether the lack of Ocil expression can influence the functional development of NK cell, was the next question addressed in this study. To this end, effector LAK cells of Ocil deficient mice were used against wild type immature and LPS-stimulated mature BMDCs targets in standard CD107a assay. Unlike mature, immature DCs are relatively sensitive NK targets [118], in line with previously published data; Ocil deficient NK cells also displayed similar trends. However, in comparison to the control group these cells demonstrated significantly reduced cell cytotoxicity against immature DCs targets (Fig. 3.6). No considerable differences were observed, when LPS stimulated mature DCs were co-incubated with NK cells of either background (Fig. 3.6). Thus results of this study indicated that presence of Ocil ligand is essential for the optimal functional development of NK cells.

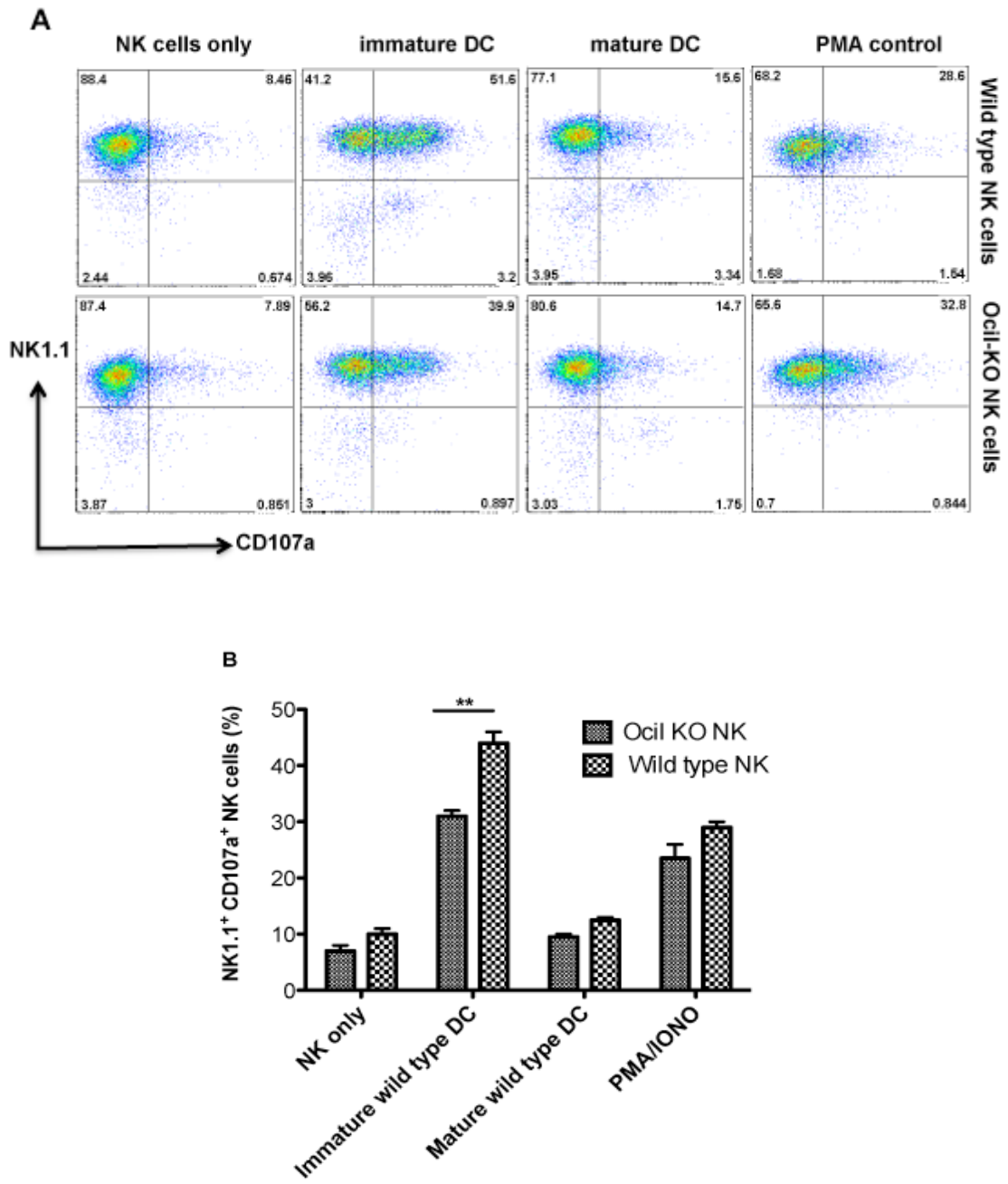

Figure 3.6. NK cells from Ocil deficient background mediate impaired CD107a expression against immature DC targets: Ocil deficient LAK cells were incubated with immature and LPS-stimulated mature DCs of wild type background in a CD107a degranulation assay. Wild type NK cells were used as control effector cells in this assay. Briefly, effector and target cells were mixed at E:T ratio 1:1 and incubated for 5 h in the presence of anti-CD107a antibodies and monensin. The percentages of CD107a positive NK cells were determined by flow cytometry. NK cells stimulated with PMA/IONO were used as positive control in this setting. **A:** representative figure of one experiment. **B**: Data represented three independent analyses. Statistical significance was indicated as **$p < 0.01$.

3.2.7. Ocil deficiency negatively influences NK cell cytotoxic responses towards prototypic tumor target cells:

In the initial analysis, I observed that Ocil deficient NK cells relative to wild type control group exhibited defective degranulation against immature DC targets (Fig. 3.6). To further examine whether the observed hyporesponsiveness is primarily confined to DC-recognition or it prevails as an intrinsic defect in these cells during their development in Ocil deficient environment, I therefore employed established degranulation assay and tested the cytotoxic potential of these cells against P815, RMA-s and Yac-1 standard tumor cell lines. Interestingly, Ocil deficient NK cells demonstrated hyporesponsive cytotoxic responses in all co-culture settings (Fig. 3.7A). As compared to the control group, Ocil deficient NK cells demonstrated a considerable reduction in CD107a expression while interacting with Yac-1. However, no statistical difference in degranulation was observed when NK cells of any background were incubated with P815 and RMA-s tumor cell lines. Unlike P815 tumor cells, which are relatively resistant, Yac-1 and RMA-s are confirmed sensitive NK targets. Strikingly, Ocil deficient as well as control wild type NK cells did not manifest any noticeable difference in the cytotoxic responses against RMAs. Deficiency of Ocil in Yac-1 cells might also be implicated as an additional factor enhancing the susceptibility of these cells in CD107a assay [159]. Since NK cells secrete pro-inflammatory cytokines when come in contact with sensitive targets [243], however as shown in the Figure 3.7B, I did not observe any differential regulation in IFN-γ responses when Ocil deficient NK cells incubated with sensitive Yac-1 target cells. Thus the data suggest that Ocil is involved in regulating NK cell cytotoxicity, which is independent of IFN-γ mediated responses.

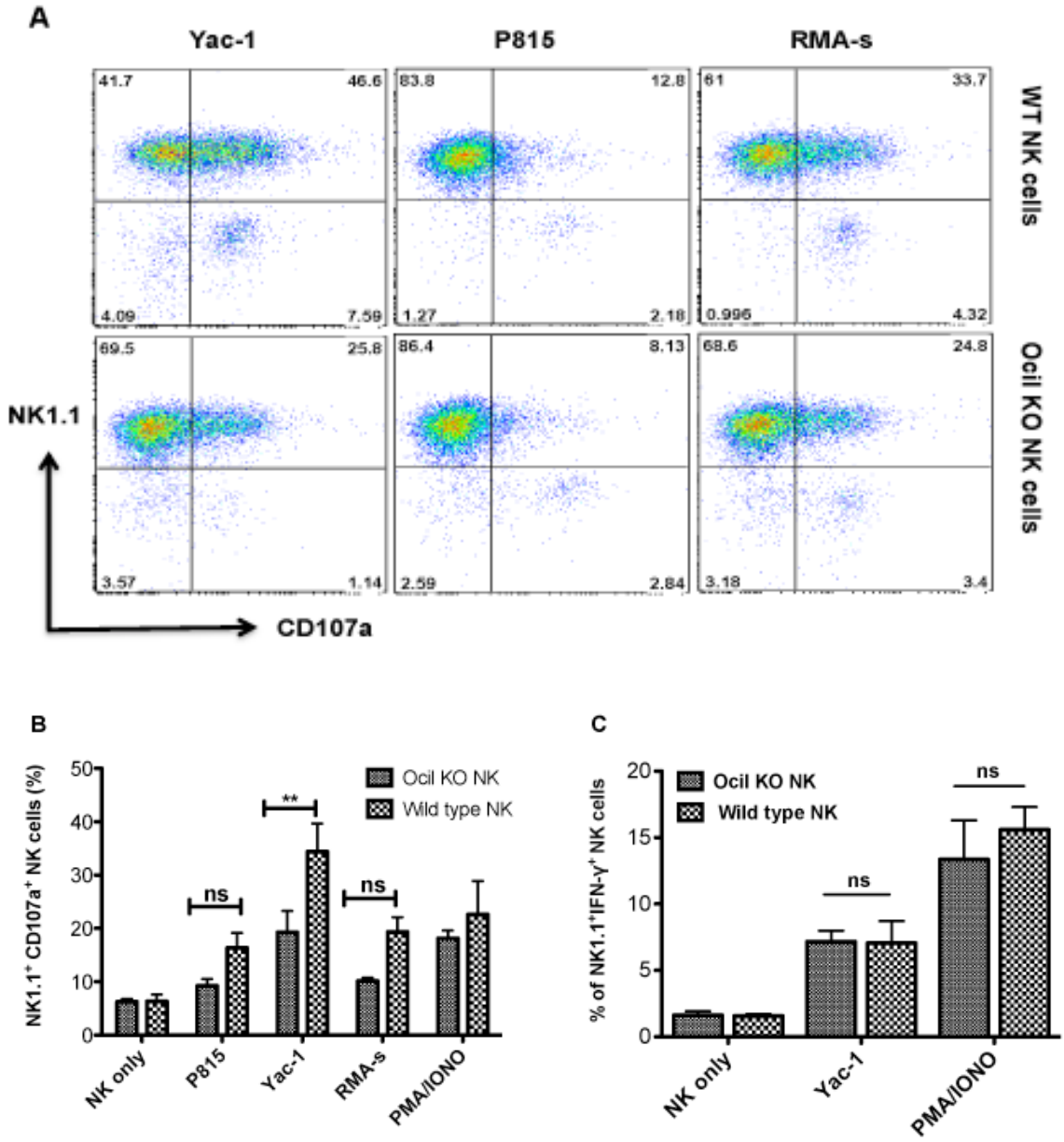

Figure 3.7. Ocil deficiency results in hyporesponsiveness in NK cells but irrelevant to IFN-γ responses: A & B: Activated NK cells from Ocil deficient mice were used as effector cells against P815, RMA-s and Yac-1 target cell lines. NK cells of wild type background were used as control in this setting. Both effector and target cells were mixed at E:T ration 1:1 for 5-hours in standard CD107a assay. Using flow cytometry platform the percentages of effector cells with degranulation marker were calculated. A: Representative experiment showing cell cytotoxicity. B: Statistics was determined using ANOVA and shown as *$p < 0.05$, and ***$p < 0.001$. Data represented three independent analyses. **C:** Activated Ocil deficient NK cells were used as effector cells against sensitive Yac-1 targets at E:T ration 1:1 for 5-hours. Cells were surface stained with NK1.1 first, then detected IFN-γ expression intracellularly. Data represent two independent experiments. PMA/IONO treated cells were used as positive control. (**$p < 0.01$).

3.3. SPECIFIC DISCUSSION

The phenomenon of reciprocal co-activation following NK/DC co-cultures has been well defined. There is accumulating evidence showing that NK cells can target and eliminate iDCs while matured DCs demonstrate sufficient resistance from NK mediated lysis[215, 230]. Whether this phenomenon happens in vivo, remains unclear. In addition to the TRAIL-R as well as the acquisition of NKp30 ligands, the susceptibility of iDC is greatly relied on the varied expression of MHC-I molecules [247]. The involvement of other additional receptor:ligand pairings mediating DC-lysis is still unknown.

In this report, I investigated the role of non-MHC-I molecule. Ocil, in the susceptibility of NK mediated DC-killing. I also demonstrated the significance of Ocil and the cognate NKR-P1B/D receptors interactions modulating NK cell target recognition functions. I observed that Ocil deficiency renders immature DCs highly sensitive to NK mediated cell cytotoxicity (Fig. 3.1). Moreover, LPS-matured DCs also exhibited less resistance yet insignificant to NK-mediated cytotoxicity in the absence of Ocil (Fig. 3.1). However, Ocil deficiency did not alter NK cells IFN-γ production (Fig. 3.2). Interestingly, I did not find the involvement of Ocil in the differential regulation of wild type resting NK cell activation (Fig: 3.3A). I also confirmed these findings by manipulating the balance of receptor:ligand pairing using lentiviral platform and found over expression of NKR-P1B on primary NK cells enhanced inhibition towards Ocil bearing target cells (Fig 3.4 & 3.5 A&B). Further, analyses of NK cells derived from Ocil KO background also demonstrated impaired recognition against DCs and other prototypic target cell lines (Fig 3.6 & 3.7A&B).

NK cells use a number of activating and inhibitory receptors; recognize cellular ligands to discriminate non-self from self [235]. In general, NK mediated resistance to self is linked with effector functions of inhibitory receptors specific to molecules like MHC-I[137, 275]. Results of this study suggest the involvement of additional inhibitory receptors on NK cells like NKR-P1B/D that bind with the non-MHC molecule such as Ocil inducing NK-tolerance. I observed that autologous immature DCs from Ocil deficient background demonstrated higher sensitivity towards NK mediated cell cytotoxicity in CD107a assay (Fig. 3.1). Surprisingly, LPS-matured BMDC, predominantly resistant cells, from the similar background also lost resistance to some extent (Fig. 3.1). Recently, Ocil

downregulation has been linked with the enhanced sensitivity of virally infected macrophages [241], thus these results are providing direct evidence to support the published data and predict the involvement of NKR-P1B/D axis in NK-target recognition. Interestingly, Ocil deficient immature DC did not appear to alter NK cell functions (Fig. 3.2 & 3.3A&B) which supports the association of Ocil with the activation of ITIM regulated NK cytotoxic responses. Indeed, further work is required to examine the Ocil mediated cytokine signaling in future. The available data also show that NK cells target autologous immature DCs *in vitro* [214, 270], the precise adaptive value of this mechanism to the immune system however is unknown. Examining the kinetics of Ocil down regulation in an infection model, at both protein and mRNA levels, might improve our understanding regarding immature DC killing and the functional relevance of this mechanism *in vivo*.

To further delineate the role of Ocil in regulating mature NK cell recognition I used lenti-vector platform to over express NKR-P1B inhibitory receptor on NK cells. Engineered NK cells demonstrated enhanced functional inhibition as well as higher tolerance against Ocil bearing target cells (Fig. 3.4 & 3.5A&B). These observations reinforced the results of this study that NKR-P1B/D:Ocil pair established an independent NK-target recognition different from the existing missing-self model. Of note, I used CD107a degranulation as a surrogate marker of NK cell cytotoxicity in the study. Previously, CD107a was identified as a marker of degranulation (associated with perforin and granzyme release) on human CD8+ cytotoxic T and NK lymphocytes upon antigen stimulation and target interactions [271-273]. Additionally, Kung lab has also established a correlation between target cell ^{51}Cr-release and effector NK cell CD107a expression against NK-sensitive YAC-1 and NK-resistant P815 cell lines [251].

Several investigations have previously confirmed the importance of MHC-I molecules in NK education and self-tolerance [275]. NK cells from MHC-I deficient background exhibit generalized hypo-responsiveness [141]. In this study I also found that Ocil deficiency abrogated the effector functions of NK cells in CD107a assay. NK cells developed in the absence of Ocil demonstrated impaired recognition when co-cultured

with both immature DCs and some other tumor cell lines (Fig. 3.6 & 3.7A). I speculate that Ocil deficiency, similar to MHC-I, may be involved in inducing generalized hyporesponsiveness. Since functional correlation of NK cells with MHC-I have been shown previously [141], thus future function studies involving the adoptive transfer of NK cells from Ocil deficient background to in Ocil sufficient background might explain the extent of impaired cytotoxicity and its association with Ocil expression. The functional analysis of *in vitro* generated mature NK cells in the absence of Ocil ligand would also be useful t precisely sort out the exact role of these non-MHC-I molecules in developing NK cell education and self-tolerance.

4.0 CHAPTER 4

SHP-1 PHOSPHATE IS A CRITICAL REGULATOR IN PREVENTING NATURAL KILLER CELL SELF-KILLING

4.1. SPECIFIC INTRODUCTION AND RATIONALE

NKR-P1B/D receptors like other inhibitory NK-receptors contain ITIM sequences in their cytoplasmic domain, which become charged upon engagements with the cognate cellular ligands [276]. Importantly, the activated ITIM sequences in turn generate inhibitory signals by recruiting Src homology region 2 containing protein tyrosine phosphatase-1 (SHP-1). The importance of SHP-1 in transmitting inhibitory signals of the specific NK inhibitory receptors has been demonstrated [277]. Transient, over-expression of catalytically inactive dominant negative form of SHP-1 (dnSHP-1) in human and murine NK cells resulted in diminished KIR and Ly49-mediated inhibition *in vitro* [278, 279]. The latter is further supported by other studies of the mature NK cells of the transgenic animals or motheaten (*me*) and motheaten viable (*mev*) mice [279]. However, as SHP-1 signaling might be involved in both NK cell development and mature NK cell functions, analyses of mature NK cells of dnSHP-1 transgenic or SHP-1 deficient motheaten mice might represent NK defects associated with the loss of SHP-1 function in NK development and/or in mature NK cell functional regulation [280]. In this part of the study, I utilized lentiviral platform to down-regulate protein expression [255], and functions of the SHP-1 in mature primary NK cell to directly examine the importance of SHP-1 in regulating NK cells effector functions.

4.2. RESULTS

4.2.1. Gene silencing of SHP-1 expression does not affect CD107a expression upon exposure to prototypic tumor target cells:

Recognition of target cells and the induction of cell cytotoxicity are regulated by the integration of the activities of various kinases or phosphatases recruited by the activating and inhibitory receptors in NK cells [281, 282]. As the SHP-1 phosphatase is associated with a majority of mouse inhibitory NK receptors [281]. I examined whether the SHP-1

knockdown NK cells were hyper-responsive in their cytotoxic responses against prototypic tumor targets (YAC-1, P815 and RMA-s). I first confirmed successful gene silencing of the SHP-1 protein expression in primary mouse LAK cells (Fig. 4.1A). To test the functional consequences of SHP-1 inhibition, mock transduced and the shEGFP-transduced LAK cells were used as controls, and the SHP-1-shRNA-transduced LAK cells were used as effectors in a lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) flow cytometry assay as a marker of cell-mediated cytotoxicity [103, 272, 273, 283]. I did not observe any statistical difference in the YAC-1 killing whether the mock transduced, the shEGFP-transduced or the SHP-1-shRNA transduced NK cells were used as effector cells in the assay (Fig.4.1B). Similarly, no statistical differences in their cytotoxicity were observed when other tumor targets, such as P815 (a relatively NK-resistant tumor cell) or RMA-S (a NK-sensitive MHC class I deficient cell) were used in the assay (Fig.4.1B).

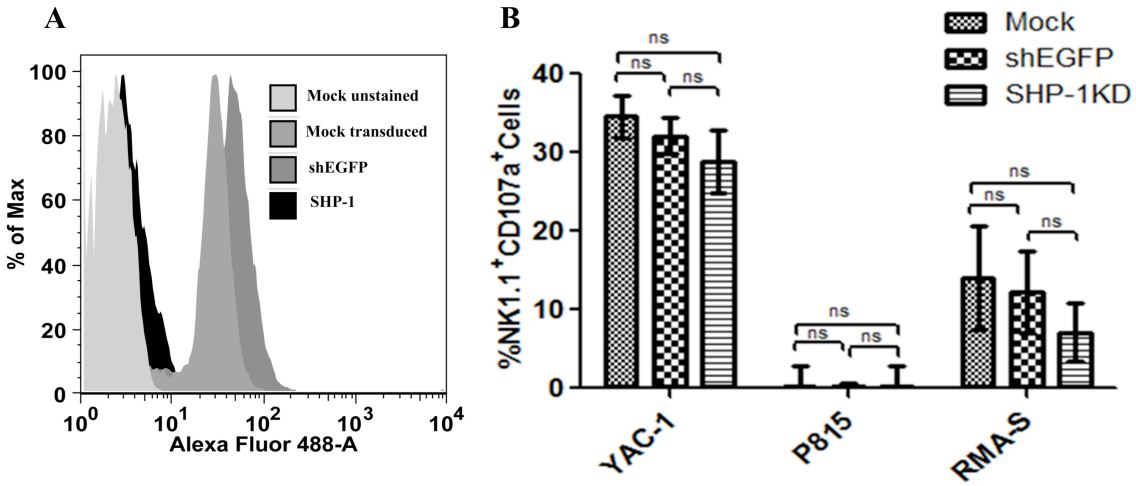

Figure 4.1. A: Efficient SHP-1 gene silencing in primary murine IL-2-activated NK cells. A: Purified C57BL/6 IL-2 activated NK cells were transduced on two consecutive days with TRC lentiviral vectors and incubated for 3 days post-transduction. Transduced cells were puromycin selected for 48 hours followed by 3 days incubation. Cells were assayed for SHP-1 expression by intracellular staining with primary rabbit anti-SHP-1 and secondary anti-rabbit Alexa Fluor 488 antibodies by flow cytometry. Data is representative of 3 experiments. **B: SHP-1 gene knockdown doesnot alter CD107a expression.** IL-2 activated NK cells were transduced with mock, shEGFP and SHP-1 shRNA and incubated for 3 more days. Cells were puromycin selected then used as effectors against Yac-1, P815 and RMA-S targets at E:T ratio 1:1. Data is representative of 4 experiments.

4.2.2. SHP-1 knockdown NK cells showed loss of inhibition in the antibody induced redirected lysis assay:

In an antibody-induced redirected lysis (AIRL) assay, specific NK receptor function/signaling was analyzed using an antibody specific to the NK receptor, and a target cell (e.g. P815, Daudi) that bears Fc-receptors (FcR) on the cell surface. The fragment antigen-binding (Fab) portion of an antibody specific to a NK receptor of interest (activation or inhibitory) binds specifically to the receptor on the surface of the NK cells while its Fc portion binds to the target cell Fc receptor that provides a cross-linking effect [259, 284, 285]. Depending on the activation or inhibitory nature of the NK receptor, such cross-linking triggered the receptor signaling/function to induce or suppress, respectively, the cytotoxic activity of the NK cells against the FcR-bearing P815 target cells in the CD107a assay. Addition of an antibody specific to the activating NK1.1 receptor stimulated C57BL/6NCrl NK cells to kill the relative resistant P815 target cells [259]. Co-engagement of an inhibitory receptor (such as Ly49C/I), however, overrode the stimulatory signals induced by the NK1.1 receptor in a SHP-1 dependent manner [286-288]. Therefore I used this assay system to determine the impact of the SHP-1 gene silencing on the ability of the specific inhibitory NK receptor to exhibit a dominant inhibitory signal over the activation signal triggered by the NK1.1 engagement. Similar to the mock-transduced NK cells, the shEGFP-transduced and the SHP-1-shRNA-transduced NK cells were induced to lyse the P815 target cells in the presence of anti-NK1.1 mAb. Co-engagement of the Ly49C/I inhibitory receptors on the mock and shEGFP-transduced cells inhibited the activation induced by the anti-NK1.1 mAb. In contrast, I observed that the SHP-1 knockdown NK cells lost their ability to inhibit the NK1.1-induced activation upon Ly49C/I receptor engagement (Fig.4.2). Collectively, the data demonstrated that specific SHP-1 gene silencing in primary NK cells exerted a functional impact on NK inhibitory receptor signaling/function.

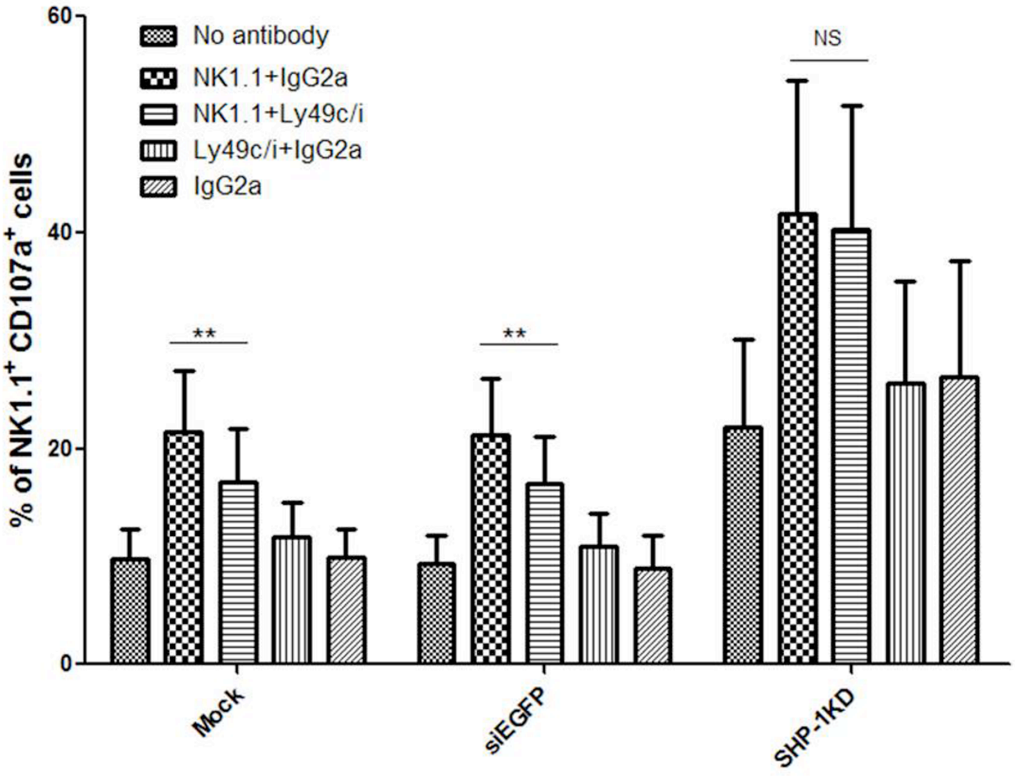

Figure 4.2. SHP-1 knockdown NK cells showed loss of inhibition in the antibody induced redirected lysis assay. Mock, shEGFP and SHP-1 shRNA transduced and puromycin selected LAK cells incubated with purified antibodies for 20-minutes. Effector : target (P815) cells (1:1 ratio) in V-shaped 96-well plate incubated for 5 & 4 hours in the presence of CD107a antibody and monensin respectively. Percentages of $NK1.1^{+}CD107^{+}$ cells were calculated and statistically analyzed using graph pad prism software. A p-value of <0.05 was considered statistically significant. Data is representative of 5 experiments.

***4.2.3. SHP-1 knockdown NK cells show increased "spontaneous degranulation"*:** The balance of signals generated from simultaneous ligand interaction with inhibitory and activating receptors regulates NK-mediated self-non-self discrimination[289]. Normal cells predominantly express ligands for inhibitory receptors. This skews the balance of receptor signaling towards attenuation of NK cell activity leading to self-tolerance [141, 275] . I examined whether gene silencing of SHP-1 promoted their "spontaneous" cytotoxic activity of the SHP-1 knockdown NK cells. The expression of CD107a degranulation in the SHP-1 knockdown NK cells, the mock and shEGFP-transduced control NK cells were measured in the absence of any target cells,

over a period of 7 days *in vitro*. On day 1, 5.9% of the SHP-1 knockdown NK cells showed degranulation as compared to 2.7% and 2.9% degranulation from the mock and the shEGFP-transduced NK cells, respectively (Fig. 5.3). The differences however, were not statistically significant. On day 2, 3.7% of the SHP-1 knockdown NK cells showed the degranulation activity as compared to 1.0% and 1.4% degranulation from the mock and the shEGFP-transduced NK cells, respectively and the differences were significant (p<0.05). On day 3 and day 7, the SHP-1-shRNA-transduced NK cells continued to show a significant increase in CD107a expression as compared to the mock and the shEGFP-transduced NK cells (p<0.0001). I observed that the SHP-1 knockdown NK cells underwent increased "spontaneous" CD107a expression *in vitro*.

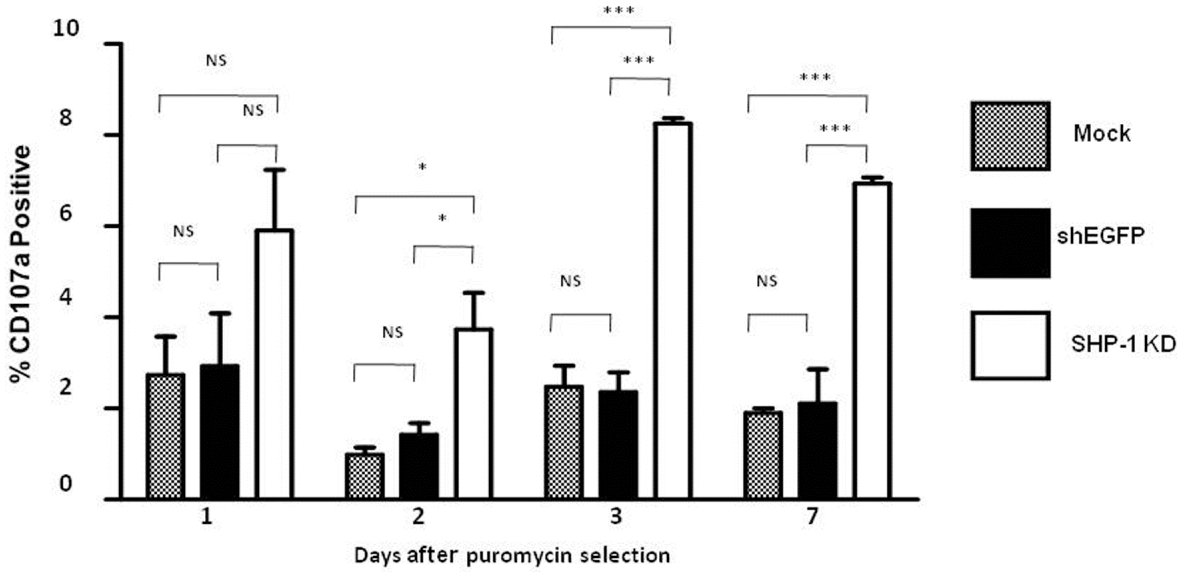

Figure 4.3. SHP-1 gene knockdown NK cells results in increased spontaneous CD107a expression: Mock, shEGFP and SHP-1 shRNA transduced and puromycin selected LAK cells were incubated in IL-2 supplemented media for 1,2,3 or 7 days. Cells were then evaluated for CD107a expression. Briefly, cells were incubated with CD107a antibody and monensin in 5 ml centrifuge tubes for 5 hours at 37$^{\circ C}$ before analysis in flow cytometry. Data is representative of 3 experiments. NS, non-significant; *, $p < 0.05$; ***, $p < 0.0001$.

4.2.4. Development of in vitro imaging platform to analyze NK target interactions, conjugate formation and apoptosis of target cells.

The observed increase in spontaneous degranulation (Fig. 4.3) suggested that SHP-1 inhibits NK cell killing. To directly visualize these events *in vitro*, I therefore established a microscopic based live imaging platform. I used 7AAD to detect dying cells as they take up 7AAD and appear red under microscope. The untransduced primary NK cells of C57BL/6NCrl and the prototypic YAC-1 targets were used first to validate the system. Cell tracker green CMFDA dye was used to label target cells (YAC-1) in the experiments that required discrimination between target andfrom effector cells. The CMFDA-labeled YAC-1 cells were mixed with unlabelled primary NK cells at 1:1 ratio in the presence of 7AAD. Specific interactions and killing of YAC-1 cells events were observed by tracking a green target cell that turned red (upon incorporation of 7AAD) over time (Fig. 4.4C). Unlabelled primary NK cells were found actively engaged with the labeled YAC-1 target cells. YAC-1 cells elimination was subsequently visualized upon reduction in the green fluorescence signal and an increase in red fluorescence due to the uptake of nuclear stain 7AAD (Fig. 4.4C). Unlabelled NK cells were also imaged alone in the presence of 7AAD in this assay to confirm no non-specific killing of the primary NK cells by each other over time (Fig. 4.4A). I also confirmed that the CMFDA dye did not affect the viability of the labeled target cells within the time line used in the imaging procedure because no 7AAD uptake was observed over time (Fig. 4.4B).

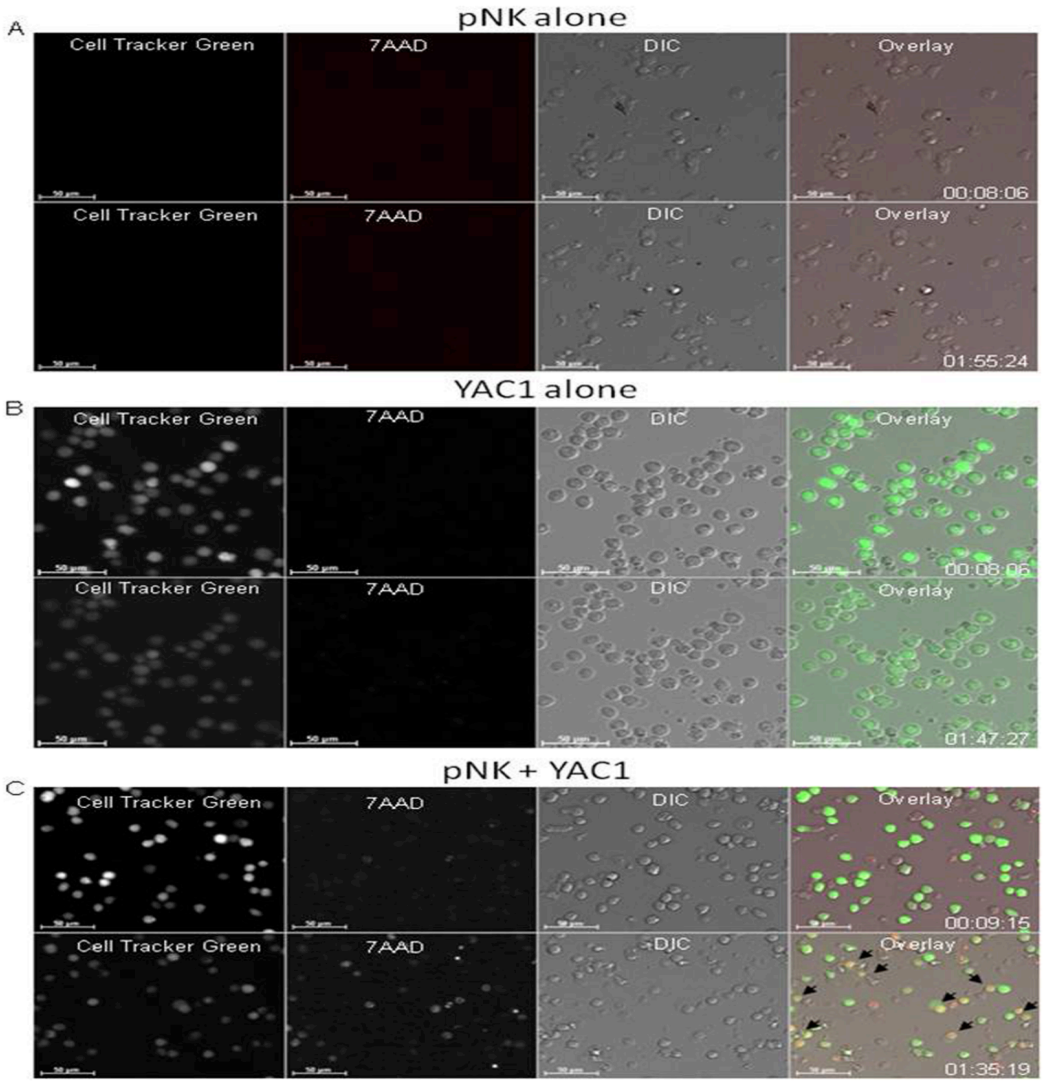

Figure 4.4: Development of *in vitro* imaging platform to examine NK target interactions, conjugate formation and apoptosis of target cells: A live cell in-vitro imaging system developed where events were imaged every 25 seconds using 10X magnification objective on a Zeiss Observer 710 station. Images in the figure were taken from videos. Unlabeled primary NK cells alone (pNK) were cultured in Hanks Buffered Salt Solution (HBSS) with 10% FCS and 50 U/ml IL-2 in the presence of 7AAD. Early and late time points images, showing no evidence of non-specific killing in the culture (A**)**. Similarly, cell tracker green CMFDA dye was used to label target cells (YAC-1) and analyzed in live cell in-vitro imaging system over time in the presence of 7AAD (B). Viability of YAC-1 appeared uncompromised and all cells eventually maintained green florescence throughout acquisition time frames. (C) Real conjugate of NK cells and its prototypic YAC-1 target cells were formed, and subsequent led to apoptosis in YAC-1 cells. The green CMFDA-labelled YAC-1 cells were mixed with unlabelled primary NK cells at 1:1 ratio in HBSS with 10% FCS and 50 U/ml IL-2 containing 7AAD. Images showed stable conjugate formations between NK and YAC-1 cells. Target cells lost the intensity of green fluorescence and picked 7AAD staining, an indication of apoptosis.

4.2.5. Real-time imaging of spontaneous killing of the SHP-1 knockdown NK cells in vitro:

Next, I used this cell-imaging platform to track cytotoxic activities of the mock transduced, shEGFP-transduced and SHP-1-shRNA transduced NK cells *in vitro*. Unlabelled primary NK cells, mock transduced cells and the SHP-1 knockdown cells were imaged in the presence of 7AAD for up to 5 hours using 10x magnification objective on a Zeiss Observer 710 station at the rate of 4 images per minute. No conjugate formation or active killing was observed in mock and shEGFP-transduced controls (Fig: 4.4). In contrast, stable cell contacts and conjugate formation were detected in the SHP-1 knockdown NK cells. Real-time tracking of these same cells at a later time point demonstrated that the SHP-1 knockdown NK cells recognized each other as potential targets, which subsequently led to killing and 7AAD uptake over time.

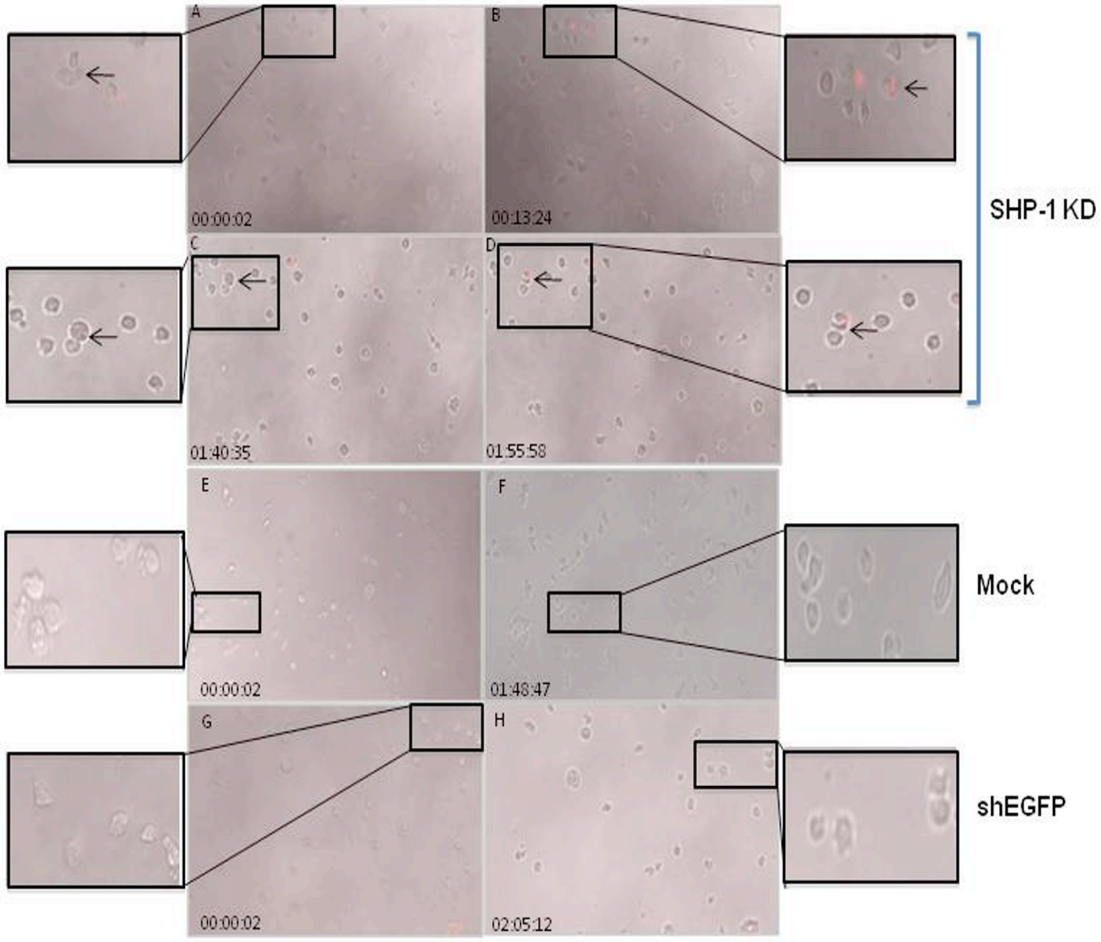

Figure 4.5: Real-time imaging of spontaneous killing of the SHP-1-knockdown NK cells in vitro. Mock, shEGFP transduced and SHP-1 shRNA transduced and puromycin selected purified NK cells resuspended in Hanks Buffered Salt Solution (HBSS) with 10% FCS and 50U/ml IL-2 containing 7AAD. Cells were imaged every 25 seconds for up to 5 hours using 10X magnification objective on a Zeiss Observer 710 station. The images in the figures were excised from live cell imaging movies at different time points. Dying cells appeared red due to 7AAD staining. Frames A-B, C-D: events at different time points of the real-time live cell imaging. Frames B, D represented late time points of the same cells after 10-20 minutes of interactions (as noted in A and C respectively, indicated by the arrows). The inset images showed the zoom-in regions of interest for clarity.

4.2.6. SHP-1 knockdown NK cells specifically target other knockdown cells:

To further confirm the observations of self-killing, I chose to perform a mixing experiment in which unlabeled SHP-1 knockdown NK cells were co-cultured with green CMFDA-labeled normal primary NK cells at 1:1 ratio. As described above, I observed active engagement of the SHP-1 knockdown cells with each other followed by cytolytic activity (Fig. 4.6A). However, I did not observe any stable cell-cell conjugate formation between the SHP-1 knockdown and normal NK cells. Also, labeled green normal NK cells did not turn red in this mixing experiment, demonstrating specific recognition and subsequent killing of the SHP-1 knockdown NK cells as non-self targets by the other SHP-1 knockdown NK cells (Fig. 4.6A & B).

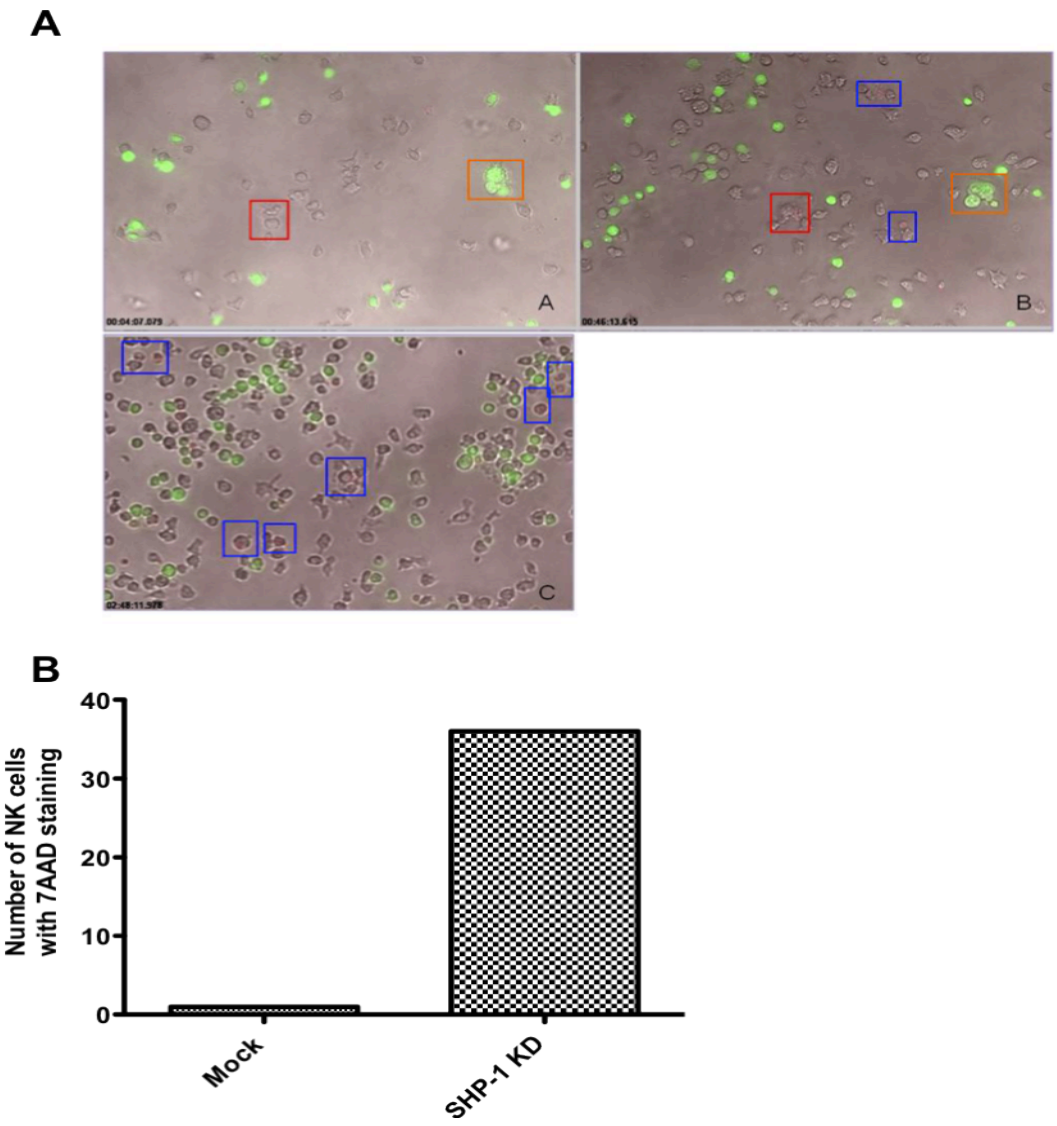

Figure 4.6 A &B: SHP-1 KD NK cells recognize other KD as targets. SHP-1 KD NK cells mixed with green CMFDA-labelled normal C57BL/6 NK cells at 1:1 ratio, resuspended in Hanks Buffered Salt Solution (HBSS) with 10% FCS and 50U/ml IL-2 containing 7AAD. Cells were imaged every 25 seconds for up to 5 hours using 10X magnification objective on a Zeiss Observer 710 station. The images in the figure were taken from the live cell imaging videos at different time points. Red box in insert A indicated real cell conjugate formation between two unlabelled NK cells. These conjugated cells can be tracked 46 minutes later (red box, insert B) to show subsequent cytolysis as the dying cells took up the 7AAD staining. Orange box indicated transient aggregates that disintegrated at later time points, and showed no evidence of cytolysis. At late time-points (such as 150 minutes of imaging), more apoptotic cells (red) were observed in the SHP-1 KD cells (Blue box). **A:** Represents one experiment. **B:** Figure is generated from the data of experiment A indicating 7AAD uptake.

4.2.7. SHP-1 knockdown does not affect MHC-I and Rae-I expression:

Numerous studies have shown that NK cells recognize and eliminate target cells with reduced expression of MHC-I [141]. Alternatively, NK cells also mediate lysis of target cells by recognizing stress surface molecules including H60, retinoic acid early inducible (Rae-1) [290]. There are reports showing that NK cells also acquired Rae-I expression on the surface after interacting with certain tumors [291]. More importantly, these NK cells become sensitive targets of other wild NK cells. In this study, I also observed that SHP-1KD NK cells killing of other KD NK cells. To rule out the possibility of MHC-I down regulation or acquisition of Rae-1 ligands in SHP-1 KD NK cells due to lentiviral transduction, cells were surface stained with anti-H-2D^b /anti-H-2K^b and anti-Rae-1 antibodies. Interestingly, SHP-1 KD NK cells exhibited expression of MHC-I and Rae-1 comparable to the mock transduced and shEGFP transduced control groups (Fig. 4.7A&B). Moreover, SHP-1 KD cells compared to controls, acquired distinct expression of NKG2D, NKp46 and CD69 surface molecules (Fig. 4.7C). Thus this data suggest that some other pathways are involved in mediating SHP-1 KD self-killing.

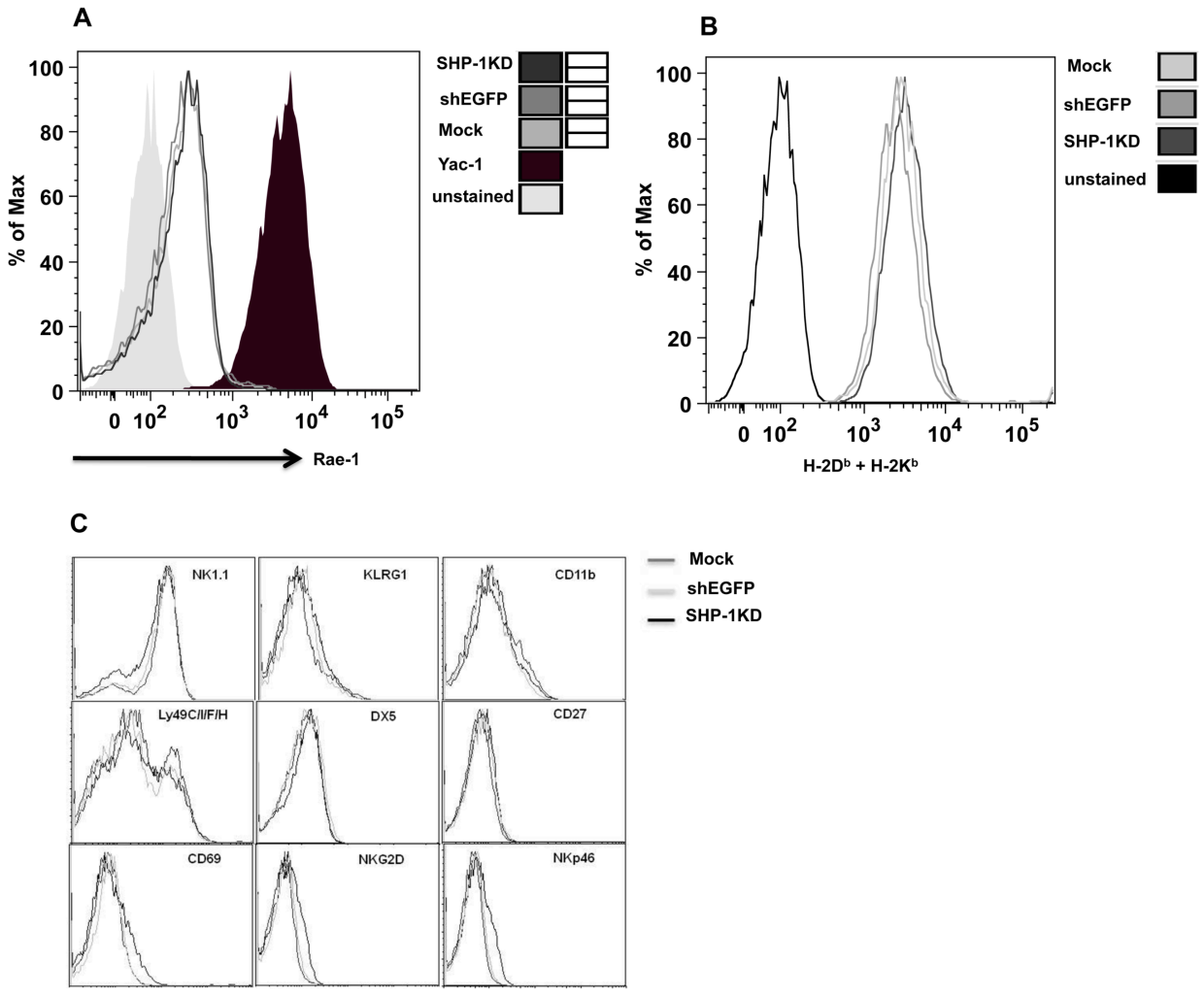

Figure. 4.7. A & B: SHP-1 knockdown NK cells demonstrate sufficient MHC-I and Rae-I expressions. A: Mock, shEGFP and SHP-1 shRNA- transduced and puromycin selected NK cells were surface stained for MHC-I (anti-H-2D^b and anti-H-2K^b) and Rae-1 molecules. YAC-1 cells were used as positive control for Rae-1 staining. Staining was confirmed using flow cytometry analysis. **C: SHP-1 knockdown NK cells acquired higher expression of stimulatory surface proteins**. Mock, shEGFP transduced and SHP-1 shRNA transduced and puromycin selected NK cells were subjected to phenotypic analysis by standard flow cytometry using antibodies against NK1.1, KLRG1, CD11b, Ly49C/I/F/H, DX5, CD27, CD69, NKG2D and NKp46 surface receptors. SHP-1-shRNA transduced NK cells showed enhanced expression level of CD69-activation marker and NK activating receptor molecules like NKG2D and NKp46 as compared to the mock and the shEGFP-transduced controls.

4.3. SPECIFIC DISCUSSION

In the current study, I examined directly the importance of the SHP-1 phosphatases in regulating mature primary NK cell functions. Previously, the Kung laboratory identified a specific shRNA target sequence against mouse SHP-1 gene (unpublished data). I used the same shRNA sequence in this study to silence/knock down SHP-1 in primary mouse NK cells (Fig. 4.1A). I also tested SHP-1 knocked down NK cells in CD107a expression assay using prototypic tumor target cells and found that underthese conditions it was unaffected (Fig. 4.1B). However, the SHP-1 gene silencing in these cells abrogated the ability of the ITIM-containing NK inhibitory receptors to suppress activation signals induced by the NK1.1 activating receptor (Fig. 4.2). For the first time I was able to follow the fates of the stably transduced SHP-1 gene silenced, primary NK cells over a longer period of time in IL-2 containing cultures, I observed an increased in spontaneous CD107a degranulation in the SHP-1 knockdown NK cultures over time (Fig. 4.3). Using a real-time live cell microscopic imaging system, these "de-regulated" NK cells appear to mediate specific self-killing *in vitro* (Fig. 4.5, 4.6 A&B).

The ability to introduce stable gene silencing of the SHP-1 molecules in mature splenic NK cells provided us with a tool to pinpoint the functional importance of the SHP-1 molecules at the mature NK cell level. It is of importance that the SHP-1 silenced NK cells lost the ability to inhibit the NK1.1-induced activation upon Ly49C/I inhibitory receptor engagement (Fig. 4.2). First, it established that tested RNA interference approach could prove useful in studying protein function(s) in primary NK cells; second, the importance of the SHP-1 phosphatases in specific inhibitory receptor signalling could not be compensated by other phosphatases or signalling molecules; third, despite the presence of some "residual" amount of the SHP-1 protein in the SHP-1-shRNA transduced cells, these SHP-1 silenced NK cells had a functional phenotype similar to the NK cells of the SHP-1 dominant negative transgenic or SHP-1 deficient motheaten mice [279, 280, 292] . Current data therefore demonstrated a definitive role of SHP-1 in regulating mature NK cell function(s) that is independent of potential effect of SHP-1 signaling on NK cell development/education.

The apparent lack of undesirable toxicity associated with lentiviral vector transduction

facilitated long-term analyses of the stably modified NK cells in vitro. Strikingly, I observed an increase in spontaneous CD107a degranulation and direct NK cell self-killing in the SHP-1 silenced NK cultures over time (Fig. 4.3).

To directly visualize the cell killing events in vitro, I developed a real-time live cell microscopic imaging system to assay for NK cell cytotoxicity (4.4). This imaging system allowed real-time visual monitoring of cytolytic events that endpoint assays (such as Chromium release cytotoxicity) did not support. When used with a CMFDA cell tracker dye, it captured two confirmatory indicators of cell killing - the loss of cytoplasmic cell tracker dye and subsequent uptake of the 7AAD viability dye in the dying cells. In addition, one can further correlate cytolytic potentials with the other parameters (such as their migration ability, morphology) that have a direct impact on the cytolytic ability.

It is conceivable that the self-killing of the SHP-1-gene silenced NK cells *in vitro* may account for increased cell death. However, it might also be possible that these observations were independent events that revealed other previously unappreciated functional roles of the SHP-1 phosphatase in NK cell regulation. Of note, this data seems to suggest also that the gene silencing of the SHP-1 phosphatase induced NK receptor ligands and/or adhesion molecules that promote specific NK cell target recognition. First, the SHP-1-gene silenced NK cells were not rendered hyper-reactive to all targets, due to a "global" impairment of NK inhibitory receptors signalling and therefore a tipping of the NK receptors signalling balance towards activation. I observed comparable cytotoxic activities in prototypic NK cell tumor targets (YAC-1, P815 and RMA-S) (Fig. 4.1B). Second, in a mixing experiment in which normal NK cells were added in the SHP-1-knockdown NK cell culture as potential targets, knockdown NK cells selectively "recognized" other SHP-1 knockdown NK cells in the real-time live cell microscopic examination (Fig. 4.6 A&B). Third, phenotypic analysis of various receptor molecules including NK1.1, KLRG1, CD11b, Ly49C/I/F/H, DX5, CD27, CD69, NKG2D & NKp46 showed that the SHP-1 knockdown NK cells were selective in up-regulating some of the known activating receptors (Fig. 4.7C). I observed only enhanced expression levels of activation marker (CD69) and NK activating receptor (NKG2D and NKp46) in the SHP-1 knockdown NK cells when compared to the mock, the shEGFP-transduced controls (Fig. 4.7C). It remained to be determined whether a specific subset of NK cells is responsible

for the recognition and subsequent killing of the SHP-1 knockdown NK cells; also which receptor/ligand is involved in such recognition. Analyses of MHC class I (Db and Kb) and Rae-1 expression on the SHP-1 knockdown NK cells and the control NK cells did not reveal any differences in their surface expressions (Fig. 4.7 A&B).

Kung lab previous work established the efficiency and feasibility of using VSV-G pseudotyped lentiviral vectors in genetic engineering of primary NK cells [293]. In a gain-of-function study, I used the NKR-P1B^{SJL} inhibitory receptor as a model receptor to formally demonstrate that over-expression of an NK receptor on primary NK cells was able to manipulate balance of NK receptor signalling, and thus NK-target cell specificity [251]. Here, using SHP-1 phosphatase as a gene-silencing target in a loss-of-function study, I demonstrated that lentiviral vectors are efficient in delivering specific shRNA (RNA interference) in primary NK cells. NK cells that were transduced by the shEGFP irrelevant silencing control vectors did not show any observable differences in functional activities when compared to the mock (untransduced) NK cells. It therefore established that transduction and puromycin selection procedures, de novo production of shRNA inside NK cells, and the lentiviral vector integrations has little/no observable impact on the functional activities of the transduced NK cells.

In summary, the current work established a stable gene-silencing platform that can be applied to study any protein of interest in primary NK cells (mouse or human)[294, 295]. The development of the real-time imaging system to study NK cell cytotoxicity will prove useful in visualizing steps (such as on/off target cell binding and conjugate formation) involved in target cells lysis. Future examinations of the SHP-1 knockdown NK cells will reveal further molecular pathways regulated by the SHP-1 phosphatase in NK cells.

5.0 CHAPTER 5

MICROFLUIDIC-BASED, LIVE-CELL ANALYSIS ALLOWS ASSESSMENT OF NK-CELL MIGRATIONS IN RESPONSE TO CROSS-TALK WITH DENDRITIC CELLS

5.1. SPECIFIC INTRODUCTION AND RATIONALE:

Natural killer (NK) cells are motile bone marrow derived lymphocytes that play a key role in innate immunity against viral, microbial infections and transformed cells [56, 132]. They are capable of killing transformed or infected cells [8, 132] , and/or producing cytokines/chemokines that can profoundly influence the quality and magnitude of the adaptive immune responses[7, 56, 289]. They acquire several chemokine surface receptors during development and maturation [296-299]. Chemokine receptors such as CCR7, CCR5 and CXCR3 are involved in the preferential migration and localization of NK cells into the lymph nodes [220, 256, 300, 301], whereas NK cells residing in blood; liver, spleen and inflammatory sites exhibit higher CXCR1 and CX_3CR1 expression [302-304] . Non-chemokine family proteins such as chemerin and SIP_5 are also involved in the regulation of NK cell trafficking [305, 306]. Collectively, they highlight the complexity of the environmental regulation of NK migrations in physiological and pathological conditions.

NK cell activation and functions are regulated by cytokine/chemokine and/or dendritic cell (DC) in the microenvironments [118, 307]. The NK-DC crosstalk is bidirectional, involving multiple cytokine signals and direct cell - cell contacts [308, 309]. NK cells induce DC maturation and augment IL-12p70 secretions while eliminating deregulated immature DCs from the periphery [215, 310] . Reciprocally, DC, when stimulated by pathogens or Toll-like receptor (TLR) ligands, constitutes a key source of cytokines (such as IL-12, IL-18, IL-15) known to regulate NK cell proliferation, cytotoxicity and cytokine production [215, 309]. DC produces also a number of biologically significant chemokines such as CXCL8/IL-8, CXCL9, CXCL10 and CXCL11 capable of stimulating NK cell

migration [311, 312]. Efficient recruitment of NK cells to peripheral organs or inflamed lymph nodes via direct or indirect interaction with DC is therefore essential in NK cell-mediated immune-surveillance. However, how different DC subsets regulate migratory properties of NK cells (such as chemotaxis, chemokinesis, chemo-repulsion) are not fully defined *in vitro*.

Microfluidic devices can configure well-defined chemical gradients and support single cell-based quantitative cell migration analysis [258, 313]. In this report, I demonstrated how such microfluidic device could be utilized to examine how soluble factors produced by DC regulated NK cell migration *in vitro.*

5.2. RESULTS

5.2.1. A microfluidic platform to perform live cell imaging of NK cell migrations:

I used the established Y shaped microfluidic device to examine chemotactic or chemo-repulsive movements of NK cells at a single cell level in vitro (Fig. 5.1A). In this system, fluidic channels were coated with fibronectin to facilitate cell adhesion. Chemokine or conditioned medium of interest was injected into the channels through the designated inlets using a syringe pump at a total flow rate of 0.4 μl/min. Two channels were used in each experiment - one for the control culture medium and the other one for the chemokines/conditioned medium of interest. Cell migration was recorded by time-lapse microscopy. To visualize the integrity of the gradient of interest, FITC-Dextran 10 KD (molecular weight approximately closer to chemokines) was mixed into these test samples. Its fluorescence intensity was monitored for gradient formation at different time points to confirm the test solution gradient in the microfluidic channel. Migrated cells were tracked using the ‘‘Manual Tracking’’ plug-in in NIH ImageJ. All the tracking data were exported to the Excel and Origin 8.5 for final analyses. Cell movements were quantitatively evaluated by (a) the percentage of cells that migrated toward the chemokine gradient; (b) the Chemotactic Index (C.I.), which is a ratio of the displacement of cells toward the chemokine gradient (dy) to the total migration distance (d) (Fig. 5.1B). In the absence of any chemotactic factor, no specific directional NK cell movement was

observed in angular histogram (Fig. 5.1C) and cell track analyses (Fig. 5.1D). Moreover, the covered distance and the calculated average speed of the selected NK cells in the field were found low.

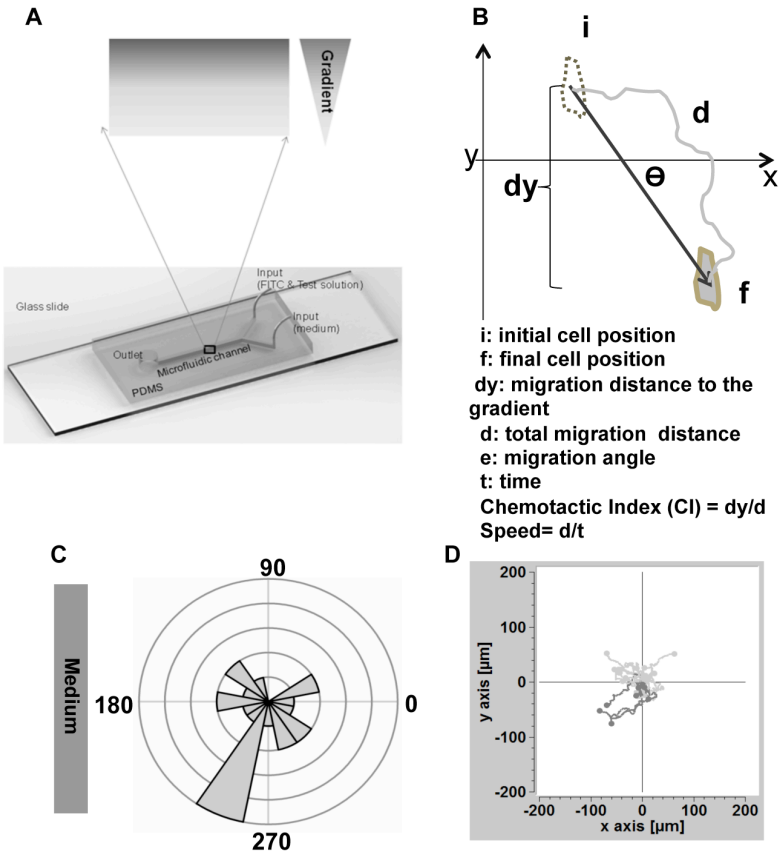

Figure 5.1: Microfluidic platform for NK cell migration analysis. (A). The experimental platform was constituted of a multi-channel fluidic device connected with a time-lapse microscope. Chemokines or conditioned medium was injected into the channels and the cell migration was recorded by time-lapse microscopy. Migrated cells in the selected field were tracked using the ‘‘Manual Tracking’’ plug-in in NIH ImageJ. Movements of cells were quantitatively evaluated by the angle of migration (angular histogram) and the percentage of chemotactic cells (number of cell migrated towards the gradient x 100/Total migrating cells tracked). **(B)**. Additional quantitative parameters of cell migration (chemotactic index, C.I. and cell speed, velocity) were calculated using the following equations: C.I. = Dy/d, a ratio of the displacement of cells toward the chemokine gradient (Dy) to the total migration distance (d). Velocity = d/t, t is the time of travel. i: initial cell position. f: final cell position. dy: migration distance to the gradient. d: total migration distance. e. migration angle. **(C &D):** Angular histogram and cell track analyses of NK cell migration in the absence of any chemotactic gradient. Cell tracks in light gray and dark gray were the cells that migrated towards and away from the gradient respectively.

5.2.2. Conditioned medium of the LPS-stimulated mature BMDC promotes NK cell chemotaxis:

Activated DC produce an array of chemokines, such as CXCL8 (IL-8), CXCL10, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), that have the potential to induce NK chemotaxis [311, 312]. I utilized the microfluidic platform to formally examine the migratory responses of NK cells in the presence of the soluble factors produced in the conditioned medium of the LPS-stimulated matured DC. Angular and cell track based analyses of live cell imaging data (Fig. 5.2A & 5.2B) demonstrated a higher percentage (72%) of NK cells migrated towards the gradient with considerable higher CI value i.e., 0.101 and the average speed was 0.096 μm/sec (Fig. 5.2C). To rule out a possible role of LPS in regulating NK cell trafficking in this system, I examined NK migration in the presence of LPS alone. NK cells exhibited random migratory movements similar to the control experiment in the LPS alone control groups. The data derived from this experiment therefore demonstrated that LPS-stimulated mature DC produced chemotactic factors that induced NK migration in the microfluidic device.

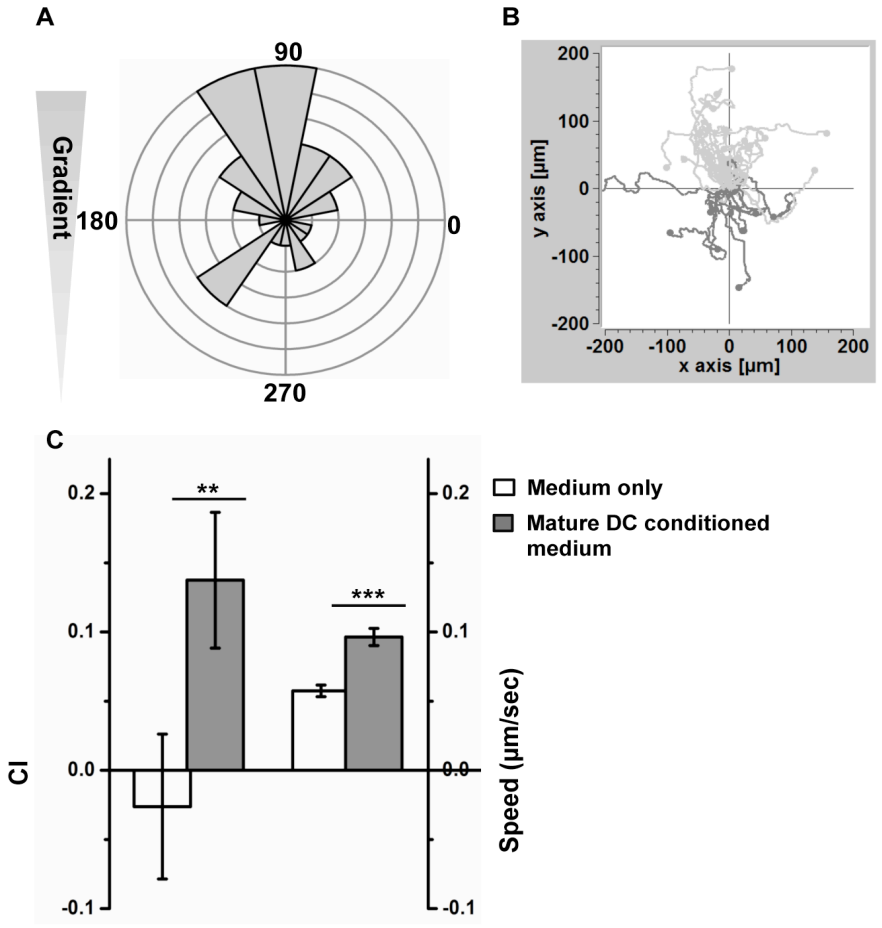

Figure 5.2: Conditioned medium of the LPS-stimulated mature DC promotes NK cell chemotaxis: LPS-stimulated DC-conditioned medium was used to establish the gradient in the microfluidic device. Migratory responses of IL-2 activated NK cells were recorded and analyzed. (**A):** Angular histogram of NK cell migration angles in a gradient of the conditioned medium of the LPS-stimulated mature DC from a representative experiment. (**B):** Cell tracks from the same representative experiment; cell tracks in light gray are cells migrated towards the gradient; cell tracks in dark 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). Data represented three independent experiments. (**C):** Chemotactic Index (C.I.) and speed of NK cells in a gradient of matured DC supernatant and its comparison with the medium control group. The error bars represent the standard error of the mean (SEM). Data represents three independent experiments. The significance level for each comparison from 2-sample *t* test is shown.

5.2.3. Conditioned medium of the immature DC promotes NK cell chemotaxis:

Immature DC primarily reside in the periphery, and require physical interaction with NK cells for their maturation and the induction of Th1 responses [309, 314]. The ability and physiological relevance of immature DC in regulating NK cell migrations have not been fully elucidated. I utilized the microfluidic platform described above to examine whether soluble factors produced by immature DC were capable of directing NK migration *in vitro*. Results of this experiment, demonstrated chemotaxis of activated NK cells towards the conditioned medium gradient of immature DC origin. The percentage of chemotactic NK cells was 66% with an average C.I of 0.105, which was significantly higher than NK cells analyzed under the culture medium alone control (Fig. 5.3A & 5.3B). Moreover, the moving cells exhibited higher average C.I (0.105a) and average speed (0.0751 μm/sec) when compared with control group (Fig. 5.3C). These results demonstrated that the immature DC produced also chemo-attractant factors that induced NK cell migration.

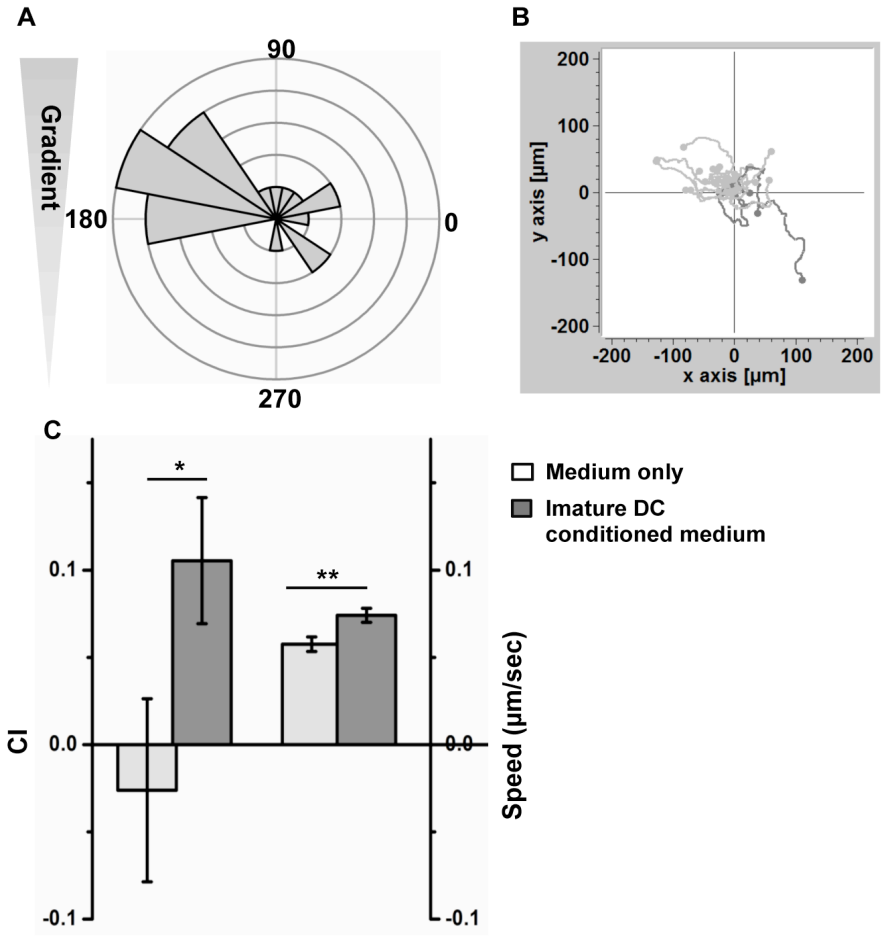

Figure 5.3: Conditioned medium of the immature DC promotes NK cell chemotaxis: Conditioned medium of the immature DC was used to establish the gradient in the microfluidic device. Migratory responses of IL-2 activated NK cells were recorded and analyzed. (**A):** Angular histogram of NK cell migration angles in a gradient of the conditioned medium of the immature DC. (**B):** Cell tracks from the same representative experiment; cell tracks in light gray were cells migrated towards the gradient; cell tracks in dark gray were 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). Data represented three independent experiments. (**C):** Chemotactic Index (C.I.) and speed of NK cells in a gradient of immature DC supernatant and its comparison with the medium control group. The error bars represent the SEM. Data shown represents three independent experiments. The significance level for each comparison from 2-sample *t* test is shown.

5.2.4. Granulocyte macrophage colony-stimulating factor (GM-CSF) induces repulsive migration in NK cells:

Recombinant GM-CSF is a key cytokine routinely used in the generation of bone marrow derived DC in cultures [252]. It was therefore present, along with other soluble factors produced by the DCs, in the conditioned medium I used in the migration studies. To rule out a potential role of GM-CSF in contributing to the NK chemotaxis observed in the conditioned medium of the mature and immature DC (Fig. 5.2 & 5.3), I set up another gradient in the microfluidic device using medium-containing recombinant GM-CSF only. Surprisingly, I observed that NK cells migrated robustly away from the GM-CSF gradient. Both angular histogram as well as cell track analyses of recorded imaging data showed that more than 81% cells migrated away from the GM-CSF gradient (repulsive migration) (Fig. 5.4A & 5.4B). Further analysis to calculate the speed of moving cells and the respective C.I values, I found that in response to the GM-CSF, cells exhibited a distinct negative average C.I (-0.21) and the average speed of 0.076 μM/sec (Fig. 5.4C). Thus result of this part revealed a novel feature of GM-CSF in regulating NK cell migration.

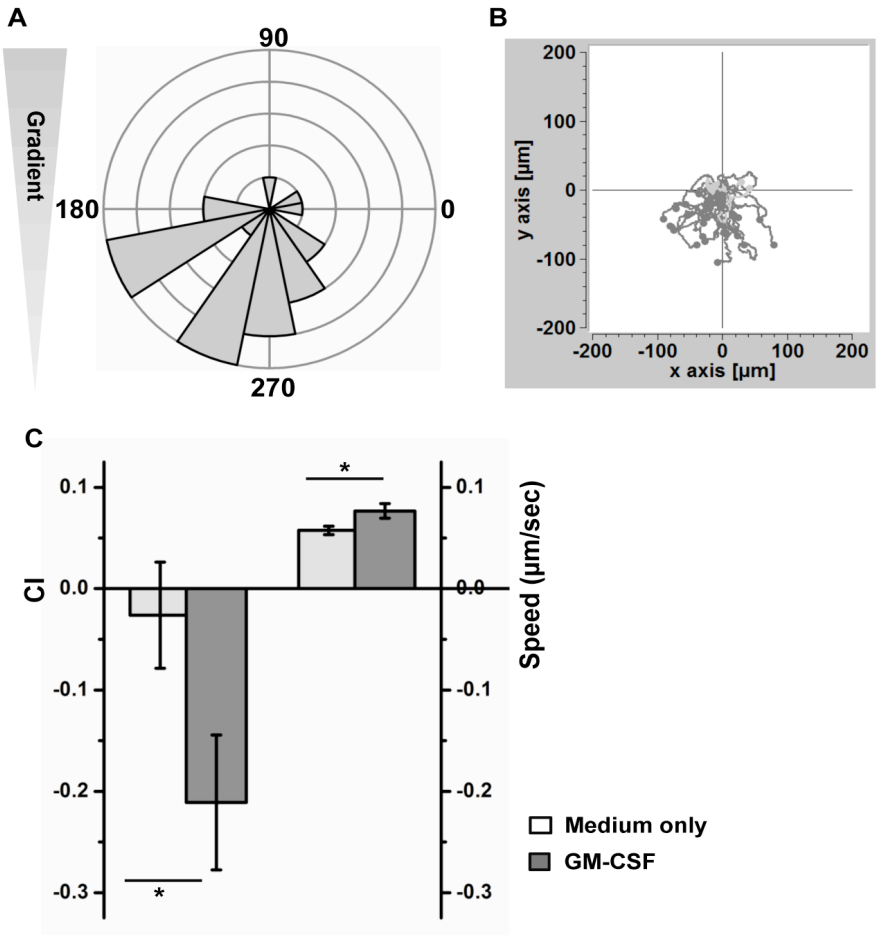

Figure 5.4: GM-CSF induces repulsive migration of NK cells: Culture medium containing recombinant GM-CSF (20 ng/ml) was used to establish the gradient in the microfluidic device. Migratory responses of IL-2 activated NK cells were recorded and analyzed. (**A):** Angular histogram of NK cell migration angles in a gradient of recombinant GM-CSF. (**B):** Cell tracks from the same representative experiment; cell tracks in light gray are cells migrated towards the gradient; cell tracks in dark 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). (**C):** Chemotactic Index (C.I.) and speed of NK cells in a GM-CSF gradient and its comparison with the medium control group. A high negative C.I. confirms the repulsive cell migration. The error bars represent the SEM. Data from one experiment, is representative of three independent experiments. The significance level for each comparison from 2-sample *t* test is shown.

5.2.5. NK cell chemotaxis in a conventional trans-well system:

Trans-well migration assay is a commonly used technique to study the chemotactic responses of immune cells *in vitro* [315]. I therefore, used the conditioned medium of immature and LPS-stimulated mature DC in the lower chamber of trans-well to examine the migration of the IL-2 activated NK cells that were placed in the upper chamber of the trans-well. CXCL12, a chemokine previously studied in the trans-well assay [316], was used as a positive control. In consistent with the results observed in the microfluidic system, I found that both of the conditioned media of the LPS-stimulated mature and immature DC induced a statistically significant (*$p < 0.05$) recruitment of NK cells into the lower chamber of the trans-well (Fig. 5.5A). However, in comparison to the immature DC conditioned medium, LPS-matured DC exerted a stronger NK chemotaxis.

Thus the microfluidic and trans-well data showed the soluble factors released by the DCs were chemotactic (Fig. 5.2, 5.3, & 5.5A). It has been shown previously that DCs produce a broad range of proinflammatory cytokines and chemokines such as IL-8, CXCL10, MIP-1α, MIP-1β, RANTES [218, 317]. These chemokines have the potential to activate chemokine receptors including CCR5, CCR7 CXCR1 and CXCR3 expressed on circulating NK cells [318]. Among them, CXCR3 has been studied more precisely in the regulation of NK cell trafficking in vivo [220]. I therefore examined directly whether CXCR3 was involved in the observed NK cell recruitment. Neutralizing CXCR3 activities in the LPS-stimulated mature DC conditioned medium reduced the NK recruitment significantly (65-75%), Figure 5.5B). It therefore indicated that the observed NK chemotaxis in the conditioned medium of the LPS-stimulated mature DC was partially dependent on the CXCR3 signalling in the IL-2 activated NK cells. Indeed, higher concentrations of interferon gamma inducible proteins (IP-10) were also detected [95, 319] in the conditioned medium of the LPS-stimulated mature DC (Fig. 5.5C) thus corroborating further with the significance of the CXCR3:IP-10 axis in the regulation of NK cell migration in the NK-DC crosstalk.

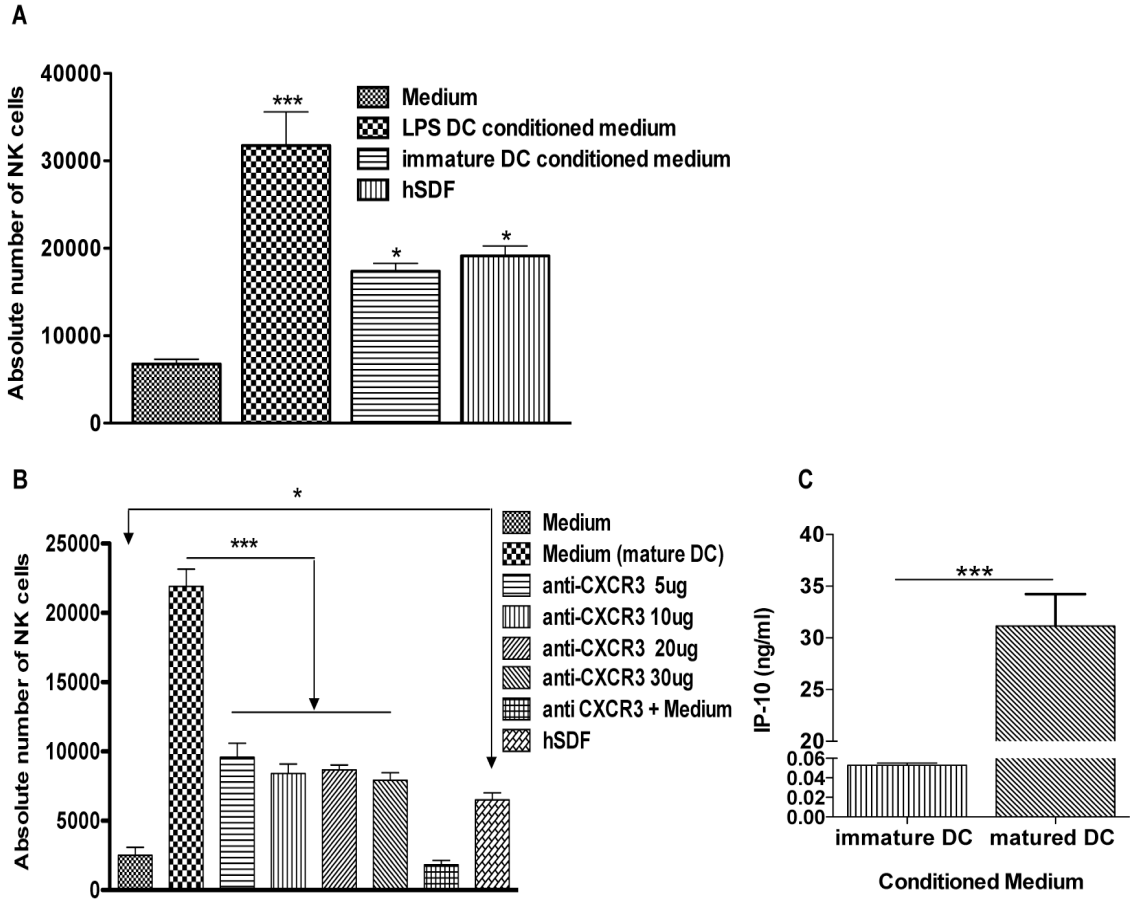

Figure 5.5: Conditioned media of DC induces migrations of NK cells in the conventional trans-well system. (A): Human SDF (hSDF or CXCL12), the conditioned medium of the LPS-stimulated mature or immature DC was added into the lower chamber to test chemotaxis of the IL-2 activated mouse NK cells in a conventional trans-well migration assay. (**B)** CXCR3 partially regulated NK migration in the presence of the conditioned medium of the LPS-stimulated mature DC. IL-2 activated NK cells were treated with three different concentrations (5 μg/ml, 10 μg/ml, and 30 μg/ml) of purified anti-CXCR3 antibodies. Treated NK cells were subjected to test the chemotaxis against the conditioned medium of LPS-stimulated mature DC in the trans-well system. After 90-minute incubation, migrated NK cells into the lower chamber of all wells (**A & B** settings), were collected and counted. Data were shown as mean ± SEM of n=3 samples per group and were representative of one out of three independent experiments, p values <0.05 (*), 0.01 (**) and 0.001 (***); one way ANOVA were considered significant. **(C):** Determination of IP-10 in BMDC derived conditioned media using sandwich ELISA kit.

5.2.6. Confirming GM-CSF mediated NK repulsion:

Live cell imaging of IL-2 activated NK cell migrations under microfluidic platform revealed that GM-CSF induced a high level of repulsive migration *in vitro* (Fig. 5.4). To validate this unanticipated finding further, I examined the effect of GM-CSF on NK migration in the trans-well system. As chemo-repulsion cannot be measured directly in this assay, I examined whether chemotaxis of NK cells towards the conditioned medium of LPS-stimulated DC could be further augmented by the removal of any GM-CSF activities present in the conditioned medium (Fig. 5.5C). Indeed I observed that neutralizing GM-CSF activities in the conditioned medium of the LPS-stimulated mature DC resulted in a stronger chemotactic response of NK cells, when compared to the controls. (Fig. 5.6 A&B). Previously Kung lab observed that LPS-stimulated BMDC conditioned medium induced NK cells recruitment in the peritoneal cavity following intraperitoneal (I/P) injection in mice (unpublished data). To further elucidate the inhibitory function of GM-CSF in the migration of NK cells in-vivo, mice were injected intraperitoneally with diluted LPS-stimulated mature DC conditioned medium containing GM-CSF (1:1) or pure LPS-stimulated mature DC conditioned medium as control. Interestingly, after 36-hours, count of CD3-NK1.1+ cells in the peritoneal cavity of GM-CSF treated mice showed significant reduction in the recruitment of NK cells compare to the control mice group (Fig. 5.6 C&D). Thus data from this part confirming the inhibitory role of GM-CSF in NK cell migration.

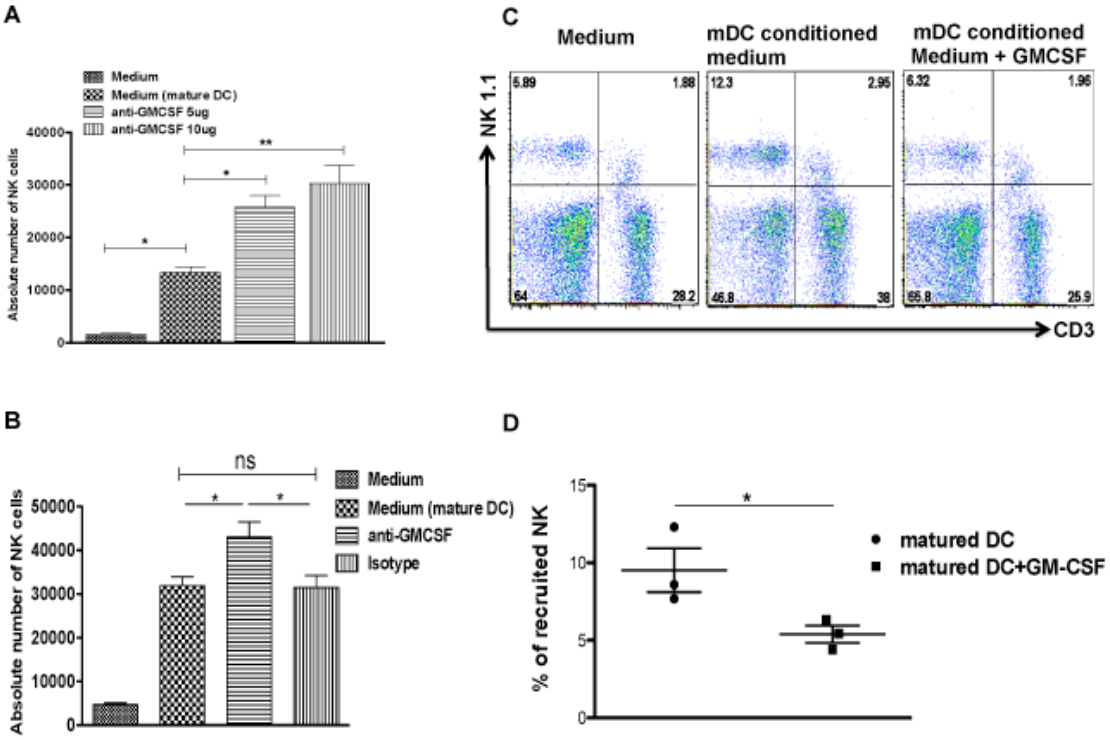

Figure 5.6: A &B. Neutralizing GM-CSF in the conditioned medium of the LPS-stimulated mature DC enhances NK cell migration. (A): GM-CSF activity in the conditioned medium of the LPS-stimulated mature DC was neutralized using anti-GM-CSF antibodies (at 5 mg/ml or 10 mg/ml). Treated conditioned medium was added in the lower chamber of the trans-well to measure the migratory responses of IL-2 activated NK cells placed in the upper chamber. After 90-minutes of incubation, NK cells in the lower chamber were collected and counted. Results were shown from two independent experiments, with three samples in each experiment. **(B):** Neutralizing effect of anti-GM-CSF antibodies was specific. Isotype control antibody did not enhance NK migrations. The data are shown of three independent experiments, each performed with three samples. A & B results are expressed as mean ± SEM of three independent experiments, each performed with three samples. p values <0.05 (*), 0.01 (**) and 0.001 (***) were considered significant. **(C &D): GM-CSF inhibits NK cell recruitment in the peritoneal cavity in vivo:** Mice were injected intraperitoneally with the LPS-stimulated DC conditioned medium (300ul/each mice) or the mature DC conditioned medium supplemented with GM-CSF (20ng/ml) (1:1). Peritoneal washes were collected at 36 hours, centrifuged and washed twice with PBS. The collected cells were analyzed for NK cells ($CD3^-$ $NK1.1^+$) by flow cytometry. (C) Dot plot analyses of one representative experiment. (D) The percentages of the $CD3^-NK1.1^+$ NK cells in all experimental animals were calculated in both groups, n=3/group. The significance of difference (*p < 0.05) was determined using Students t-test.

5.3. SPECIFIC DISCUSSION

Here, I employed a microfluidic platform, which facilitated a real-time microscopic examination of NK cell migration in a stable chemical gradient, at a single cell level. To my best knowledge, this is the first microfluidic-based NK cell chemotaxis study reported to date. I observed that conditioned media of the immature and LPS-stimulated mature DC induced high level of chemotactic movements of IL-2 activated NK cells *in vitro* (Fig 5.2, 5.3 & 5.5A). I confirmed these findings in a standard trans-well migration assay, and identified that CXCR3 was a key receptor on NK cells that mediated the migration (Fig. 5.5B). Interestingly, this study also revealed a novel function of GM-CSF in repulsing NK cell migration for the first time.

Bi-directional interactions of NK and DC led to mutual activation of both cell types. Both soluble factors and direct cell contacts were involved during this process [222]. Such reciprocal crosstalk between NK and DC can occur in lymphoid and non-lymphoid tissues, which in turn leads to induction of subsequent innate and adaptive immune responses. Pathogen exposed mature DC migrated to the tissues like peripheral blood, bone marrow and spleen, where resting NK cells could potentially be activated [118, 220, 320-322]. I observed that the conditioned medium of the LPS-matured DC induced a strong chemotactic migration in NK cells (Fig. 5.2 & 5.5A), thus providing direct evidence to support an important role of DC-secreted soluble factors in the recruitment of NK cells. A number of reports demonstrated that CXCR3, other than CCR2, CCR5, and CX3CR1 receptors, was involved in the migration of cytotoxic NK cells during inflammation and their infiltration into the tumors [95, 200, 220, 318, 323]. In agreement with the published data, we found that neutralizing antibodies against CXCR3 abrogated part of the migratory response induced by the conditioned medium of the LPS-stimulated mature DCs in the trans-well system (Fig. 5.5B). In addition to the CCL5 chemokine, I also verified the concentrations of CXCR3 stimulating chemokine i.e., IP-10 in the LPS-activated DC-conditioned medium (Fig. 5.5C) Although DC provide chemical mediators as well as cell:cell signals to enhance NK cell functions, the ability of these cells to produce chemokines such as IP-10 and the existence of functional receptor like CXCR3 on NK cells further highlight the significance of NK/DC cross-talk in regulating NK

migration. Since DC secreted a diverse array of biological signals [317], it is possible that other chemokine receptors on NK cells were involved in the process. Further investigations of the DC conditioned medium may reveal these factors and their novel associations with NK migration and/or cytotoxic potential [324].

Immature DCs are located primarily in the periphery to counter invading pathogens (infections). In a process called "NK mediated DC editing", NK cells eliminate de-regulated immature DCs from the periphery to maintain the quality of the subsequent adaptive immune responses [325, 326]. The results of my study provided direct evidence that immature DC produced soluble factors that were chemotactic to NK cells (Fig. 5.3 & 5.5A). It remained to be determined whether the factors involved, as well as the nature of the NK cell functions, would be the same as that induced by the mature DC at the site of infection. Several NK cells subsets are equipped with different sets of chemokine receptors, which mediate their distribution across lymphoid and non-lymphoid tissues during physiological and pathological conditions [327]. It will be interesting to use this platform to examine further how different DC subsets or mature DC activated by different TLR ligands regulate migratory properties of NK cells in future.

I found significant recruitment of activated NK cells in response to DC–conditioned medium (immature vs LPS mature) in both of the microfluidic device and trans-well assay (Fig. 5.2, 5.3, & 5.5A). In the conventional trans-well system, the recruitment of NK cells was weaker in the immature DC-conditioned medium (*$p<0.05$) when compared to that of the LPS-matured DC conditioned medium (***$p<0.001$) (Fig. 5.5A). In the microfluidic device, however, I observed comparable NK cells chemotaxis in the immature and LPS-matured DC conditioned medium with higher chemotactic indices (C.I of 0.101 and 0.105 respectively) than the control experiment (Fig. 5.2C & 5.3C). In addition, I also observed that CXCL12 promoted a weak but still significant recruitment of NK cells in the trans-well assay (*$p<0.05$)(Fig. 5.5A). However, using CXCL12 gradient in the microfluidic platform induced chemotaxis in only 52% of NK cells. Collectively, these data highlighted the features and differences of the two migratory assays: In the microfluidic device, cell migration parameters such as speed and chemotactic index were analyzed in real-time at the single cell level on a 2-D plane under a stable chemical gradient. In

contrast, chemotaxis was measured as a net unidirectional downward movement of a population of the migratory cells in the trans-well assay

The established microfluidic platform used in this study was unique also in its ability to assay chemo-repulsive response, which was not possible in the conventional trans-well assay. Strikingly, when I examined recombinant GM-CSF in our microfluidic system, I observed 81% NK cells displayed migration away from the GM-CSF gradient with a strong negative C.I (Fig. 5.4 A,B &C). Interestingly, the chemotactic ability of LPS-stimulated DC conditioned medium amplified following the removal of residual GM-CSF (Fig. 5.6). Whereas in mice NK recruitment into the peritoneal cavity was abrogated when GM-CSF was administered in combination with LPS-stimulated mature DC conditioned medium (Fig. 5.6 C&D). GM-CSF is produced by a number of cell types (such as T cells, NK cells, alveolar epithelial cells and tumor cells), and has been well documented to be an important hematopoietic growth factor and immune modulator [328-330] [330-332]. Its role in regulating cell migration, to our knowledge, has not been reported. The current work however revealed a novel role of GM-CSF in repulsing NK cell migration. It will be of great interest to elucidate further this previously unappreciated property of GM-CSF in different tumor models as well in physiological settings.

Emerging microfluidic device based approach has been developed to provide better control in stable gradient generation and to mimic complex microenvironment for the quantitative analyses of chemotaxis, chemokinesis and chemo-repulsion at a single cell level. Indeed, this “Y” shape microfluidic device I utilized here has been previously used to analyze T cell migration in co-existing chemokine fields (such as CCL19, CCL21) that mimic the lymph node microenvironments in vitro [257]. Understanding NK trafficking mechanism, particularly in inflamed peripheral sites such as skin and lymphoid tissues (e.g., lymph nodes, liver and thymus) will provide new insights into the development of NK-based therapeutic approaches against tumor and infections.

6.0 CHAPTER 6

IMMATURE DENDRITIC CELLS SUPPORT NATURAL KILLER CELL DIFFERENTIATION

6.1 SPECIFIC INTRODUCTION AND RATIONALE

According to the current literature, NK cells development occurs predominantly in the bone marrow[42, 45, 55]. However, the emergence of NK precursors and other intermediate stages in the secondary lymphoid organs such as lymph nodes diversifies the developmental sites[333, 334]. Among various other cellular and molecular interactions identified in the BM microenvironment, several cytokines including c-kit-L, Flt3L, and gamma-chain-dependent cytokines have also been shown/found critical in differentiating HSC into the CLP and NK committed precursors (NKP)[43, 45, 335]. Further differentiation of the committed NK precursors into immature and functionally matured NK cells resulted by the expression of key surface receptors such as Ly49 in mice specific for MHC class I molecules, acquisition of effector functions (cytokine production, cytotoxicity) and self-tolerance "education" [21]. Among the members of gamma-chain-dependent cytokine family, IL-15 emerged as an important cytokine that provides survival signals as well as regulates the activation of NKP, immature and mature NK cells by interacting with IL-15Rβ (CD122) [45, 336, 337]. The precise function of this cytokine specifically in the terminal maturation phases of NK cell development, however, is not completely understood. [338, 339]. Members of the TNF superfamily that constitute the ligands/receptor of the lymphotoxin/LIGHT pathway are also reported to be involved in NK differentiation. An impaired NK cell development and maturation have been reported in $LT\alpha^{-/-}$ and $LT\alpha R^{-/-}$ mice [340, 341]. The current model suggests that lymphotoxin- α bind to the lymphotoxin-βR and activates the stromal cells which in turn facilitates the expression of IL-15 receptors on committed NK precursors. Interestingly, IL-15 signaling has also been shown sufficient for NK-precursors to differentiate into immature NK cells independent of BM stromal cells [45]. However, further differentiation as marked by the upregulation of Ly49 receptors and functional maturation of immature NK cells involves stromal cells via a yet-to-be identified mechanism[45, 55, 342].

In fully mature NK cells, state of responsiveness is controlled by a fine balance of signals generated through simultaneous engagement of activating and inhibitory surface receptors. Similarly, the inhibitory receptor-ligand interaction and signaling have also been suggested to play a role in NK cell development and acquisition of self-tolerance[343, 344]. Since multiple receptors are expressed on both the mature NK cells and its precursors, it is imperative to formally examine how changes in the balance of NK receptor signals regulate NK cell differentiation, acquisition of self-tolerance and NK-target interactions at different NK developmental stages.

The emerging scientific data explicitly place NK cells within the lymphoid lineage. These studies further emphasize that developmental process occurs largely in the bone marrow as well as in other secondary lymphoid tissues[42, 57] However, the developmental events as well as underlying molecular interactions and cellular factors that drive multipotent progenitors, common lymphoid progenitors or committed NK-progenitors to become a mature NK cell remain unclear. Dendritic cells have been shown to activate NK cells, enhance IFN-γ secretion, cytotoxicity and stimulate NK cells in controlling *in vivo* viral/pathogen infections and tumor growth [225-228]. Recent evidence also suggests that DCs are equipped with certain important "stromal" factors including SDF-1**,** IL-15 and members of the TNF superfamily that may potentially regulate NK differentiation[340, 341]. Interestingly, like NK cells, DCs also develop within BM. Additionally; bone marrow derives DCs also found in the secondary lymphoid tissues, potential NK developmental sites. Whether these DCs interact with NK-progenitors or other potential developmental stages is not known. Therefore, I hypothesize that apart from other cytokines and receptors signaling, DCs can be a cellular component that regulate NK differentiation in vivo. In this study I established an in vitro NK cell culture system and examined the possible role of BMDCs and DC-derived soluble factors in regulating NK-development at various stages.

6.2. RESULTS

6.2.1. Development of in vitro co-culture NK-differentiation assay

To examine the role of BMDC in NK differentiation, I first established a culture system, comprising of stage I, II and III to generate mature NK cells in vitro (Fig. 6.1). A lymphoid lineage restricted cell population $Lin^{-}c\text{-}kit^{+}Sca2^{+}$ (day 0) was sorted out from the bone marrow of C57BL/6 mouse. The enriched population cultured in the cocktail of early acting cytokines (IL-7, SCF, Flt3L) for 5-day (defined as stage I). This expanded cellular population expressing CD122 showed responsiveness to IL-15 signaling (defined as committed NK precursors (NKP) and was kept in IL-15 for an additional 5-days (stage II) to generate immature NK cells. In the last stage, differentiating cells ($NK1.1^{+}$) were cultured on a monolayer of stromal cells (OP9) for an additional 5-days (stage III). Primary DC cells (immature versus LPS-matured) were added at different stages of the NK differentiation culture to examine whether the presence of DC affects NK differentiation. Cells were collected after each stage used for down stream analyses.

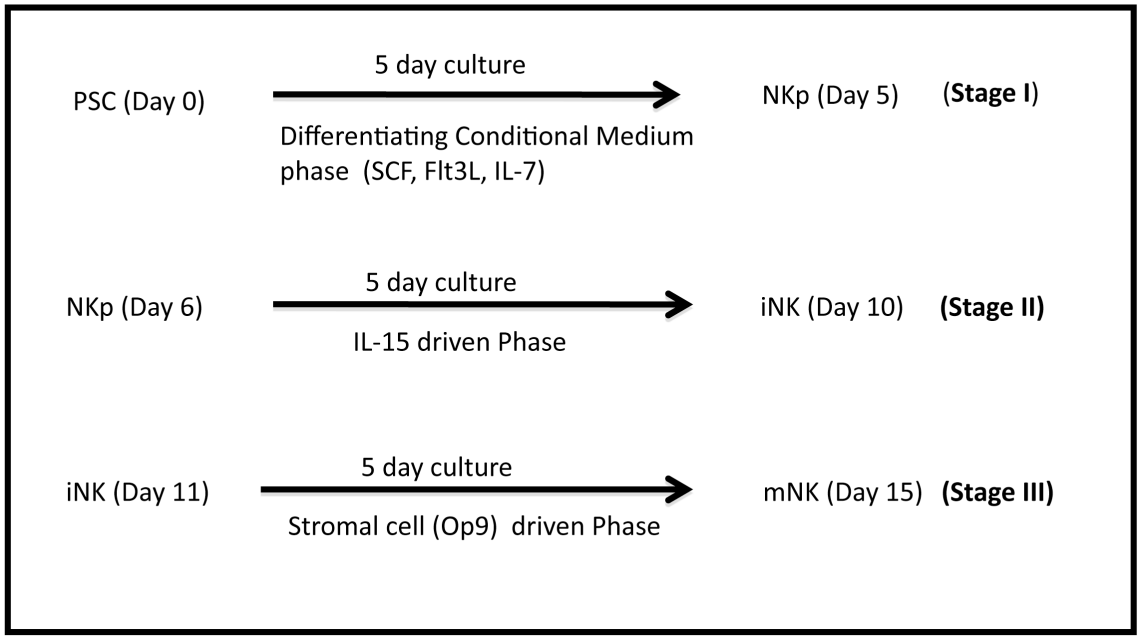

Figure 6.1. In vitro NK differentiation model: A schematic diagram showing the layout of the *in vitro* model used to generate NK cells.

6.2.2. Immature dendritic cells promote the acquisition of Ly49 receptors on stage III developing NK cells:

Continuous IL-15 stimulation drives NKP to transform into immature NK cells. These cells maintain CD122 and acquire NK1.1 expression during this phase. However these cells are lacking Ly49 and other mature NK cell surface receptors, also unable to demonstrate cytotoxicity against sensitive targets [60, 110, 256]. In addition to the IL-15 signalling, several reports have shown that induction of Ly49 surface receptors on immature NK cells indeed dependent on physical interactions with the stromal cells [44, 342]. I used the established differentiation model, and co-cultured immature NK cells (NKP at the end of stage II) with BMDCs (immature vs LPS-matured NK cells) at 1:1 ratio to examine whether BMDCs facilitate the induction of Ly49 on the immature NK (iNK) cells similar to the OP9 cultures, which I used as positive control. Interestingly, immature DCs but not the LPS-stimulated mature DCs induced the expression of Ly49 C/I/F/H on developing NK cells (Fig. 6.2 A&B). The cultures of immature NK cells without BMDCs and OP9 maintained the expression of NK1.1 even at the end of stage III, however failed to acquire Ly49 receptor expression.

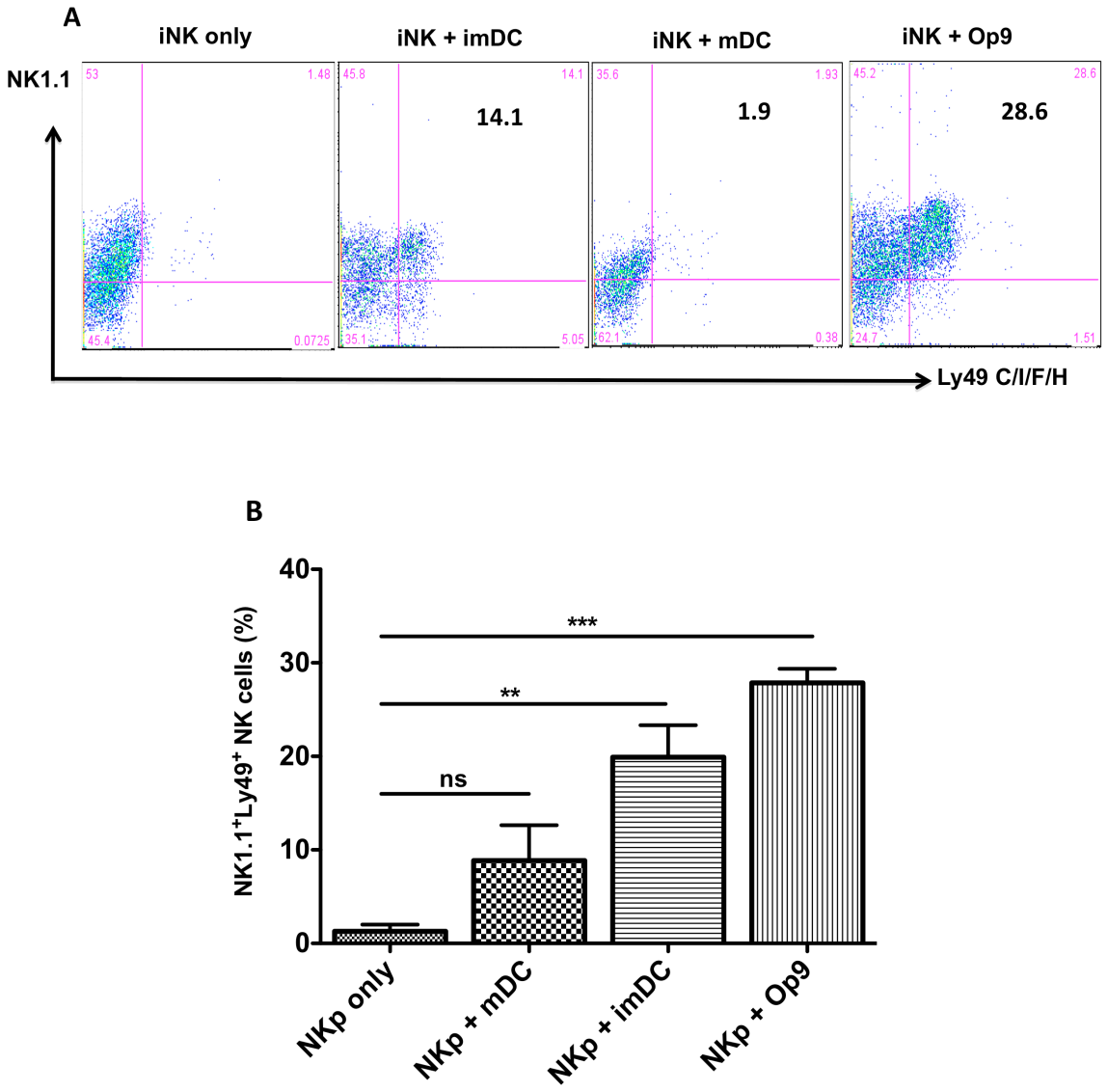

Figure 6.2 A & B: Immature BMDCs support Ly49 (C/I/F/H) acquisition at phase III developing NK cells: iNK of stage II co-cultured with immature and LPS stimulated mature DC (at E:T ratio 1:1) for 5 days. Stage II iNK cells alone were cultured as a negative control whereas Op9 cultures were maintained as positive control in this experimental setting. All culture conditions were maintained in IL-15. After 5 days, cells were harvested, washed and surface stained and analyzed for CD3-NK1.1+ population expressing Ly49 (C/I/H/F) surface receptors. **A**: Representative experiment showing imDC mediated induction of Ly49 surface receptors. **B**: Statistics was determined using ONE WAY ANOVA and shown as **p < 0.01, and ***p < 0.001. Data represented three independent analyses.

6.2.3. Induction of Ly49 (C/I/F/H) on immature NK cells requires cell-cell contact with BMDCs:

In the context of mature NK/DC cross talk, the acquisition of NK-effector functions depend on cell:cell contact and several receptor:ligand pairs are documented in this regard [345, 346]. Similarly, such molecular engagements have also been implicated critical in the development of NK cells. The interaction of BM-stroma bound ligands such as Gas6 and protein S with Tyro3 receptors expressed on NKP have been shown essential for the differentiation and acquisition of several surface receptors (inhibitory and activating) [347]. Therefore, to examine the potential role of DC-based molecular interactions in the induction of Ly49 receptors on stage II differentiating NK cells, I used conventional transwell settings to separate BMDC from the iNK cells. BMDCs (immature vs matured) were seeded on the upper chamber and iNK cells in the lower chamber of the transwell for 5 days. Surface staining analysis of iNK cells showed interesting results, as the iNK cells with matured BMDCs or seeded alone in those settings maintained NK1.1 expression, however immature DCs lost the ability to induce Ly49 expression on iNK cells (Fig.6.3). Thus these findings highlight the significance of cell:cell interaction during terminal maturation phases of NK cell differentiation. I used OP9 co-culture in those settings as a control.

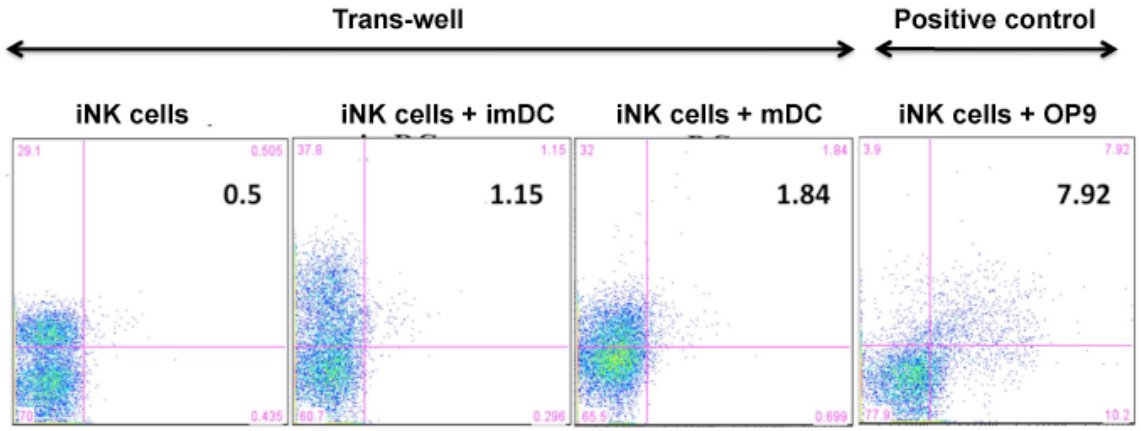

Figure 6.3. Cell-cell contact requires for the induction of Ly49 receptors on immature NK cells: Boyden chamber (Transwell system) was used to separate stage III differentiating iNK cell culture from BMDC ((imDCs vs LPS-stimulated mature DCs). iNK cells were seeded on the lower chamber while DCs were placed in the upper chamber of the transwell system. In one well, only iNK cells were cultured without BMDC as negative control. At the end of 5th day, cells from the lower chamber were collected and surface stained for CD3^{-}NK1.1^{+} population expressing Ly49 (C/I/H/F) surface receptors. OP9 cells were cultured under similar conditions separately in 24-well plate as positive control. Figure represents two independent experiments.

6.2.4. Immature DCs support NK maturation/differentiation at stage-II:

In mice, committed NKP are defined as $Lin^{-}CD122^{+}NK1.1^{+}$ population identified in various organs including BM and thymus. The induction of CD122 expression allows the committed NK precursors to respond to IL-15 signaling which in turn induces the expression of NK1.1 surface receptor on developing NK cells. Many studies highlighted the importance of IL-15 signaling in the generation of mature NK cells. It has also been reported previously that IL-15 stimulation itself is unable to generate $Ly49^{+}$ NK cells in vitro and indeed requires additional stromal factors for the terminal differentiation of NK cells [348, 349]. In order to investigate the potential role of BMDC in this IL-15 driven stage and examined their ability to secrete stromal factors, I generated $CD122^{+}$ NKP in this model and co-cultured them with and without DCs (immature vs matured) for 5 days in the presence of IL-15. Surface staining analyses showed that NKP cultures stimulated with IL-15 alone, unregulated NK1.1 expression as expected. Similarly, cultures in matured DC settings showed no difference in terms of NK maturation and acquired only NK1.1 expression, an attribute of IL-15 driven phase. Interestingly, in immature DC co-culture settings, differentiating NK cells acquired $NK1.1^{+}ly49^{+}$ phenotype. Although the observed difference was insignificant, however these results indicated the potential role of DC in this stage (Fig. 6.4 A&B). I also found considerable impact of OP9 driving NK maturation in terms of Ly49 expression however the frequency of $NK1.1^{+}Ly49^{+}$ cells was considerably reduced compared to the stage III. I also cultured NKP with immature or matured BMDC without exogenous IL-15 stimulation, interestingly these cultures failed to survive at the end of day 5, which also indicates the inability of the DCs to provide enough survival signals to the differentiating cells.

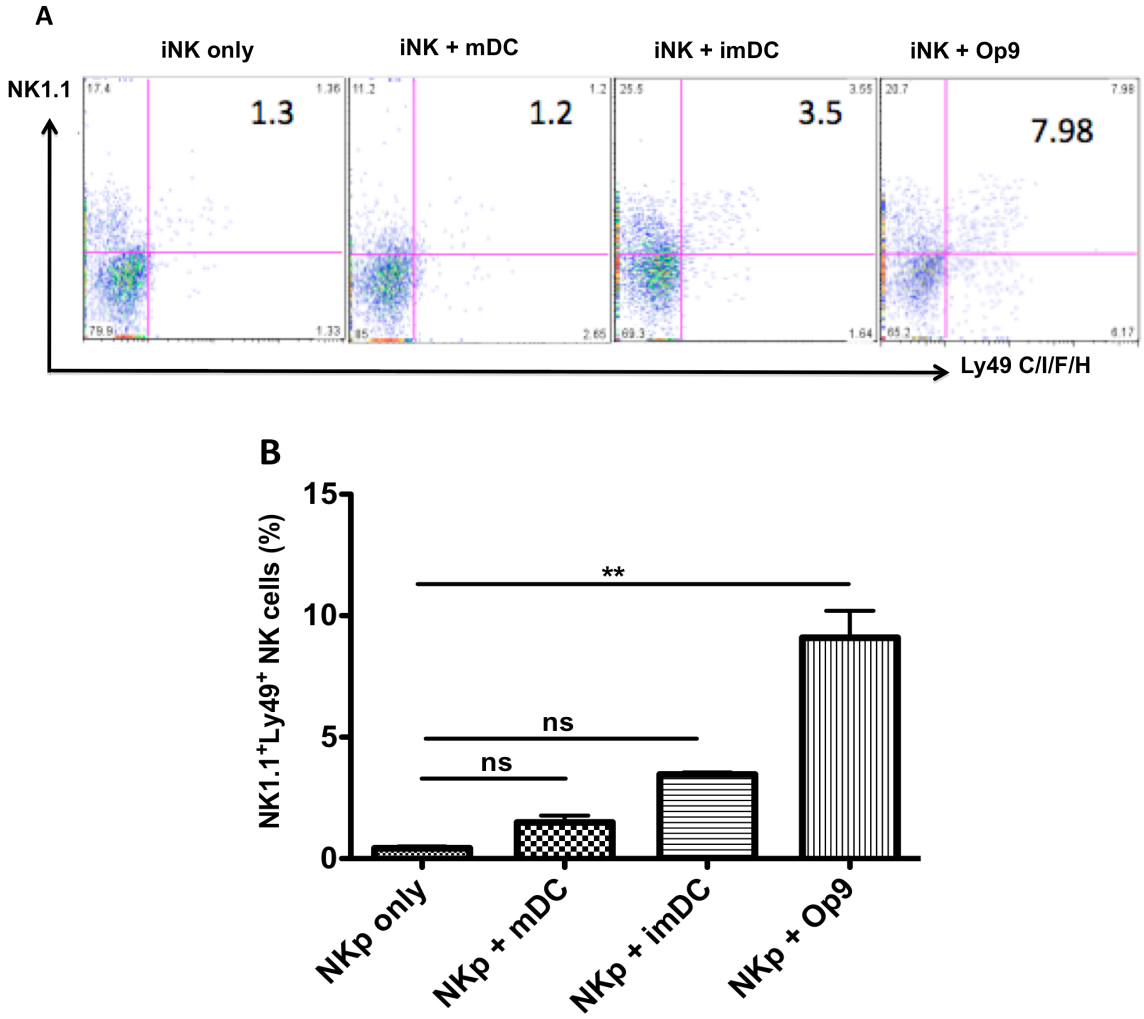

Figure 6.4 A & B: Immature BMDCs influence NK differentiation at stage II: NK progenitors (stage II differentiating cells) with surface expression of CD122 were co-cultured with immature and LPS stimulated mature DC (at E:T ration 1:1) for 5 days. As per the requirement of NK progenitors, all cultures were stimulated with exogenous IL-15. Cells were harvested, washed, surface stained and analyzed for CD3-NK1.1+ population expressing Ly49 (C/I/H/F) surface receptors. **A**: Representative experiment showing Ly49 surface receptors acquisition. **B**: Statistics was determined using ONE WAY ANOVA and shown as **p < 0.01. Figure represents two independent experiments.

6.2.5. Immature DCs regulate NK differentiation at stage-I:

The *in vitro* differentiation models as well as studies with transgenic mice showed the involvement of several cytokines and transcription factors in the early regulation of hematopoietic stem cells (HSC)[60]. However, the mechanisms involved in the early regulation and transition from HSC to NKP is thought to be more complicated and still elusive. It is generally believed that HSC requires stromal cell interactions *in vivo*, as the recovery of mature NK cells yield greatly compromised in liquid culture conditions compared to those cultured on stromal cell lines [43, 71, 350, 351].

To study any possible DCs intervention at the early developmental stages, the isolated HSC were cultured with immature and matured BMDC for 5 days in the presence of early acting cytokine cocktail comprising of IL-7, Flt3L, and SCF. In any culture condition, DCs were unable to induce the expression of NK1.1 on differentiating cells (Fig, 6.5 A&B). As it is clearly documented that common gamma chain cytokines regulate the development and homeostasis of mature NK cells, these cytokines are reported equally essential in the differentiation of NK-precursors as well [43, 97, 352]. However, these cytokines were never tested with HSC previously. Therefore I decided to set up co-cultures of freshly isolated HSC with immature and matured BMDC in the presence of IL-15 or IL-2 for 5 days. The analyses displayed quite interesting results, as immature DCs induced the expression of NK1.1 on differentiating cells under the influence of common gamma chain cytokines compared to the matured DC and cultures with early actiting cytokines only (Fig.6.5 A&B). Thus my data indicate the potential regulatory role of immature DCs in combination with IL-15 or IL-2 in the early development phase.

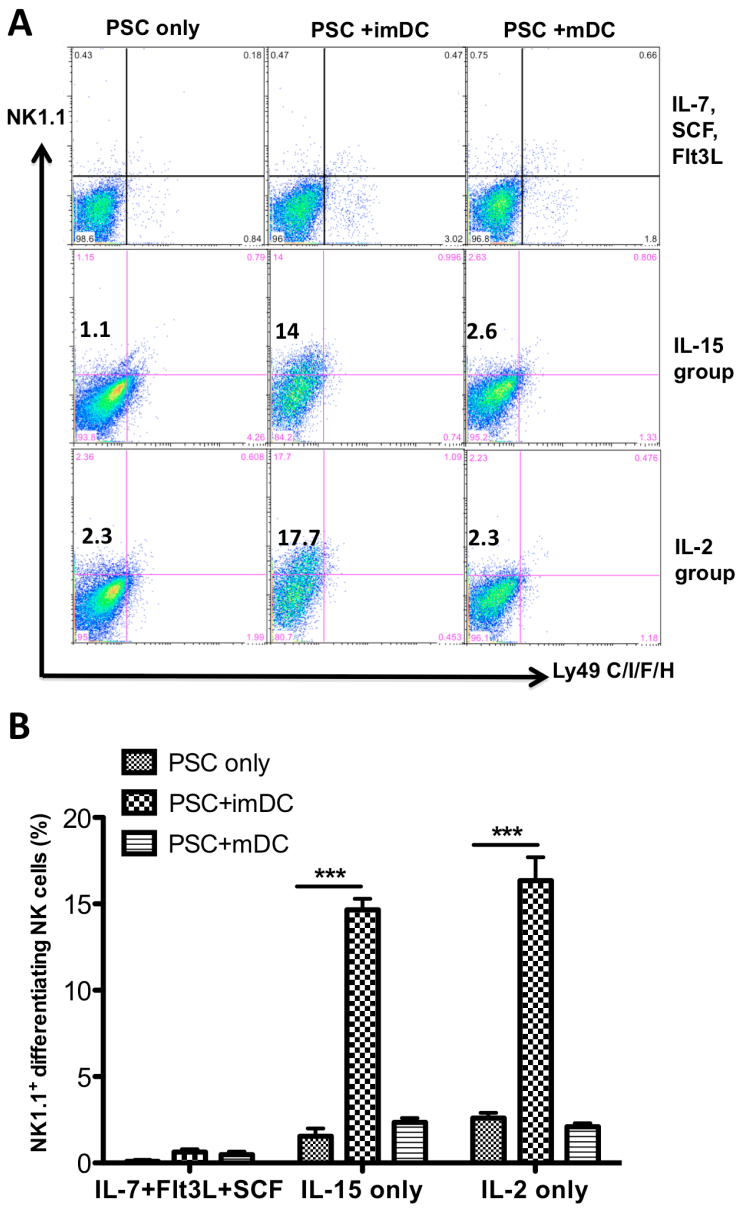

Figure 6.5 A & B. Immature BMDCs regulate the development of progenitor stem cells: Purified, progenitor stem cells (PSC) were isolated from the bone marrow of C57BL/6J mice and incubated with immature vs LPS-stimulated mature BMDC at E:T ratio 1:1, Three different cultures conditions were established, one with early acting cytokines (IL-7, Flt3L, SCF), second and third with IL-15 and IL-2 respectively. After 5 days, cells were harvested, washed and surface stained and analyzed for CD-NK1.1+ population expressing Ly49 (C/I/H/F) surface receptors. **A**: Representative experiment showing NK1.1 receptors acquisition. **B**: Statistics was determined using TWO WAY ANOVA and shown as ***$p < 0.001$. Figure represents two independent experiments.

6.2.6. Stage III differentiated NK cells demonstrate degranulation in CD107a assay:

I evaluated CD107a expression as a putative measure of degranulation to study the cytotoxic potential of stage III differentiated NK cells [273]. The sensitive target cell line Yac-1 was incubated with or without effector cells derived from immature DC culture settings. I also co-cultured target cells with OP9-derived differentiated NK cells. The surface expression of CD107a was used as a read out of degranulation activity. Compared to the control group that was without target cells showed no spontaneous degranulation, immature DC generated differentiated NK cells (stage III) demonstrated CD107a expression (Fig. 6.6). However, the percentages of degranulated NK cells were much lower than those derived from the OP9 cultures (Fig 6.6 A&B). These findings suggest that DC derived differentiation may also equip NK cells with natural cytotoxicity.

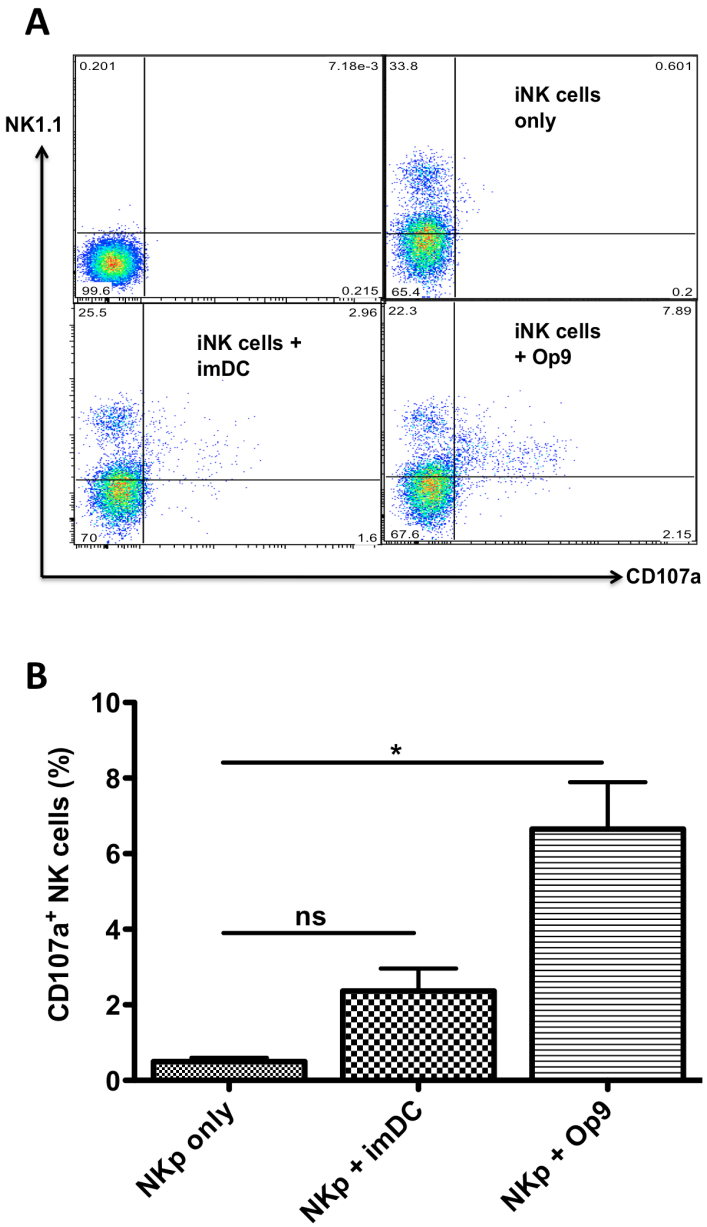

Figure 6.6 A & B: Immature DC-mediated stimulation results the generation of CD107a expressing NK cells: In vitro differentiated NK cells of stage III were generated as shown in Fig. 6.1. Three different co-culture settings were established in this assay. In the first well, effector cells were generated in IL-15 cultures, where as second and third wells contained differentiated NK cells generated under the influence of immature and OP9 cells respectively. All differentiated cells were incubated with and without Yac-1 target cells in an effector:target ration 1:1. After 5-hours cells were harvested and stained for CD3, NK1.1 and CD107a to determine the percentages of NK cells demonstrated degranulation. **A**: Representative experiment showing CD107a expression by differentiating NK cells. **B**: Statistics was determined using ONE WAY ANOVA and shown as *$p < 0.05$. Figure represents two independent experiments.

SPECIFIC DISCUSSION

The interplay between NK and DC has been reported in many studies in the context of innate and adaptive immune responses [216, 217]. However, the significance and mechanistic details of this interaction in physiological environment remain unclear. In this study, I established an *in vitro* culture system (Fig. 6.1 schematic diagram) to generate mature NK cells from multipotent progenitor stem cells. This system/model comprises of three distinct stages. In the first stage, a cocktail of three cytokines composed of flt-3L, SCF ad IL-7 was used to induce the expression of CD122 (IL-15Rβ^{+}) on freshly isolated Lin^{-}c-kit+Sca2+ population. These cells demonstrated commitment towards NK- lineage by responding to IL-15 signaling. The second stage, also known as IL-15 driven phase, promotes NKP to become Ly49^{-}NK1.1^{+} immature NK cells. While the third stage, which involves the use of stromal cells, as the induction of Ly49 surface receptors on the differentiating NK cells is dependent on the presence of stromal cells and associated soluble factors. I introduced immature and LPS-matured BMDC in this culture model to investigate the potential role of these DC in regulating all three stages of NK cell differentiation. In this report I provided evidences that immature DCs are able to induce the expression of Ly49 on immature NK cells (Fig. 6.2 A&B and 6.4 A&B). Importantly, the expression of Ly49 on terminally differentiating NK cells merely depends on cell: cell contacts (Fig. 6.3). Moreover, immature NK cells are found to regulate stage II, and I thus influence the development of NKP and multipotent stem cells respectively (Fig. 6.4 A&B and 6.5 A&B). The primary advantage of *in vitro* differentiation model is that it allows dissection of underlying molecular events/mechanisms, which are otherwise challenging in *in–vivo* studies. Collectively, in this study, I found that imDCs support NK cell differentiation at stages I & III (Fig. 6.5 A&B, 6.2 A&B). At stage II, my conclusion that imDCs can have a positive, yet insignificant, impact on NK differentiation is based on consistently observed trends (Fig. 6.4 A&B). This could be due to variations in individual experiments, a possible limitation of my in vitro differentiation model. Future experiment including the sorting of stage specific differentiating NK cells and their long-term cultures with DC could improve our understanding and apprehend the existing limitations of my in vitro model.

The process of NK cell development involves the transition through several steps reported in the bone marrow [45]. The BM-microenvironment harbors all the potential developmental intermediates yet it is not clear that BM stroma itself provides sufficient molecular signals to generate mature NK cells. It has become evident that several TF such as TF, PU.1, id2, Ikaros Ets-1 GATA3, T-bet and cytokine signaling including C-kit-L, flt3-L and common gamma chain cytokines required for early and late NK cell differentiation are derived from the bone marrow[45]. However, previous studies do not exclude the possibilities of releasing these factors from other cells as well in addition to the BM stroma. Importantly, the information may not be conclusive, as the additional cytokines and growth factors have been emerged which may be involved in the process of NK differentiation. Dendritic cells are professional antigen presenting cells, also developed in the bone marrow. Co-existence of DC-precursors with various NK developmental intermediates in the BM raises the possibility of a novel NK/DC interaction at this site [59]. DCs activate resting NK cells and maintain their survival by releasing type I interferon, IL-12 and IL-15 [353]. Many *in vitro* and *in vivo* studies have also demonstrated the importance of IL-15 signaling in NK development as well [43, 44, 354]. Importantly, BM stromal cells and hematopoietic cells are believed to be the primary sources of IL-15, which regulate lymphocyte development including NK cells in the bone marrow[355, 356]. Interestingly, my findings also show that immature DCs regulate NK differentiation only in combination with exogenous IL-15. Additionally, this *in vitro* culture system also mimics (in part) the bone marrow environment to facilitate cell:cell interactions, as results of this study also confirm that transwell dependent separation completely abrogated DC-mediated Ly49 induction on stage III NK-cells (Fig. 6.3). I also observed stage II (NKP) and III (immature) NK cell developmental progressions completely halted and cells failed to survive in the absence of IL-15 signaling which is in agreement with the published data[45]. Furthermore, IL-15 responses alone were not sufficient for the induction of Ly49 receptors in this culture system (Fig. 6.2), suggesting the importance of underlying molecular interactions. Following a stable contact, iDC also release IL-18 at the immunological synapse to activate naïve NK cell as shown previously [115], this data also reinforce my findings (Fig: 6.3) and assuming that iDC might be establishing molecular interaction with NK-

intermediates to support their development in the BM-microenvironment, however formal studies are warranted to elucidate such underlying molecular interactions. The significance of cell:cell contact in mature NK/DC context is well documented, however such interactions are largely elusive at progenitor level. Therefore, future in vivo studies involving selective ablation of $CD11c^+$ DC may further confirm and elaborate the physiological role of DC in mediating NK development [357].

Collectively, results of this study (Fig. 6.2A&B, 6.3, 6.4A&B) support BM as the site of NK differentiation. However, the identification of committed precursors and other developmental intermediates in secondary lymphoid tissues such as LN, also suggest that BM may not be the only site for NK cell developmental niches [57]. In the lymph nodes, NK cells have been shown in the close proximity of DC and are considered early source of IFN-γ. Moreover, the frequency of these cells amplified many folds in the LN, also correlate with a better outcome to anticipate infection [220, 358]. In this model, I found that matured in contrast to immature DCs turned irrelevant/ineffective in driving NK differentiation. I observed that matured DCs failed to induce Ly49 expression on stage III, II and NK1.1 on stage I population respectively (Fig: 6.2A&B, 6.4A&B and 6.5 A&B). Unlike mature NK cells, differentiating NK cells lacking several surface molecules [115, 359]. Therefore I speculate that matured DC may come in close contact with NK precursors and/or other developmental intermediates in the LN, might be unable to establish stable molecular interactions required for the development of NK cells. Also I did not find any such report showing interactions of NK developmental intermediates with LN resident DC-population. Therefore, further co-culture experiments of LN derived mature DCs displaying higher expressions of CD40, MHC-II, DEC-205 [360] with NK-developmental intermediates ex-vivo might help to dissect the role of matured DC in regulating NK development in SLT.

In addition to the Ly49 induction, I also examined the lytic potential of NK cells generated from immature DC cultures in this study (Fig. 6.6 A&B). Yac-1 sensitive targets were used in CD107a assay and found that DC-derived differentiated NK cells were able to degranulate suggesting that these DC-derived NK cells are functional. However, the frequency of $NK1.1^+CD107a^+$ NK cells in DC-derived cultures was much lower as compared to the OP9 cultures. In general, recovery of matured NK cells in all of

my assays was lower in all settings. It remained to be determined further in future whether there were differences between the stromal cell derived and DC-derived NK cells or it may simply be the limitation of short term culture period my system supported *in vitro*. Most of the reported *in vitro* NK differentiation assay allows co-culturing differentiating NK cells with OP9 and IL-15 much longer for better yield. However, co-culture experiments of stage III NK cells with DCs in my hand also do not allow us to extend culture conditions longer than what I attempted in this study. I assume that adequate DC exposure might provide additional signals to improve frequency of matured NK cells in our culture system.

Thus, the data of this study elucidate a novel cross talk mechanism between NK/DC at progenitor level. This study also provides interesting information into the induction of the DC mediated Ly49 surface receptors on the immature as well as terminally differentiating NK cells, an event previously associated with BM stromal cells. Additionally, my study explained the role of immature DCs into the acquisition of NK1.1 receptor on multipotent stem cells. Since the acquisition of Ly49 repertoire has been associated with NK-licensing, my results further suggest that DC may also be involved in early education and self-tolerance.

7.0. CHAPTER 7

GENERAL DISCUSSION, FUTURE DIRECTIONS AND IMPLICATIONS

Based on advanced understanding of the immune system, several therapeutic approaches have been designed to curb/anticipate infections and malignancies. 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 [361-363]. The adoptive transfer of ex-vivo activated NK cells or the administration of cytokines, antibodies, and drugs are some of the key therapeutic approaches/strategies to modulate/prime endogenous NK cell to counter tumor mediated immune suppression [363, 364]. However, a growing consensus is that NK cells alone as immune therapeutic agent may not be sufficient 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 [365].

Following the expansion of NK/DC cross talk mechanism and its potential significance in the field of immunotherapy, the vaccine trends have recently been shifted/focused to exploit NK immune therapeutic potential in conjunction with DCs. Using DC as a natural adjuvant in NK mediated interventions provide some significant advantages, first- release of soluble factors by DC such as IL-18, IL-12 and type I interferon which boost the cytotoxicity as well as immune regulatory functions of NK cells, second, activation of NK cells results in the production of IFN-γ required for DC-maturation and functions which in turn prime/regulate T cell responses and third, NK mediated killing of tumors, pathogen infected cell and bystander immature DC provide additional antigens (apoptotic bodies) to DCs for cross presentation, a mechanism thought to regulate subsequent T cell polarization effectively [366-368]. Additionally, the data also suggest that NK/DC interplay that is induced by pathogens/tumors not only amplifies the innate immune responses to eliminate invading threats but also initiates and develops an appropriate

down stream adaptive immune responses involving T cells [369, 370]. Besides reviewing other biological technicalities involving in current DC-based NK immunotherapies, an in-depth understanding of molecular interactions (receptors: ligands) and cellular pathways regulating communication between these two cell types are also highly desirable.

In an attempt to anticipate some of the above challenges and contribute meaningfully, I conducted several studies to cover various aspects of the interplay between NK cell and DC and identified several DC-derived novel factors involved in the regulation of NK cell functions and development.

7.1. Ocil Mediated NK Cell Target Recognition

Extensive studies have shown the significance of reciprocal communications between NK cells and DCs [215, 370]. A successful stable NK/DC interaction results in the activation and maturation of both cells. In addition to this, NK cells also regulate the elimination of deregulated immature DCs [215]. Interestingly, soluble factors as well as several surface molecules have been shown critical in regulating NK/DC cross talk. Recently, CD30/CD30L receptor/ligand axis and the associated regulatory functions in the context of NK/DC cross talk are reported [346]. The emergence of CD30/CD30L also emphasizes a missing link and the need to explore other unknown molecular interactions involved in the regulation of NK/DC cross talk, which subsequently improves our understanding for the application of these cells as immunotherapeutic agents.

In general NK cell functions, including the discrimination of self from non-self, are tightly regulated by both activating and inhibitory surface receptors [84]. However, the significance of inhibitory receptors such as Ly49 and their interactions with self-MHC-I are well documented to explain NK target recognition. A large number of reports confirm that tumor and pathogen infected cells lose the normal expression of self-MHC-I, which further makes them highly sensitive to NK cell mediated killing. Apart from classical MHC-I, a non-classical MHC-I molecule Qa-1 also reported to inhibit NK cytotoxicity [371]. Recently, Ocil, a cognate ligand of mouse NKR-P1B/D inhibitory receptor, was identified on DCs. The regulation and physiological relevance of this molecule is yet to be determined in the context of NK/DC cross talk [159].

Chapter 3 of this study was aimed at investigating the role of Ocil in mediating DCs

protection from IL-2 stimulated NK cells *in vitro*. In this study, I examined the differential role of Ocil in the regulation of a mature NK cell effector functions. Like other sensitive cellular targets, iDC but not TLR-stimulated mature DCs are the prime target of NK mediated lysis due to the low expression of MHC molecules [372]. Moreover, the increased survival of adoptively transferred iDCs in NK deficient mice indicates the pivotal role of NK cell mediated DC-homeostasis [373]. Since DCs also express Ocil, so the question is: "Do Ocil modulates NK functions?" Interestingly, my in vitro studies demonstrate that Ocil deficient DCs alter NK cell functions. NK cells and GM-CSF pulsed BMDCs from Ocil deficient mouse were used in this study to dissect the significance of Ocil in NK/DC context. I found that Ocil interaction with the cognate inhibitory NK-receptors generates sufficient signals to mediate resistance and modulates NK-cell DC-recognition, as the Ocil deficient immature DCs become highly sensitive to NK killing (Figure: 3.1).

Previously, it was shown that several tumor cell lines exhibit higher susceptibility towards NK cell mediated lysis due to the reduced expression of Ocil molecules [159]. Notably, Ocil was recently found critical molecule in several infection mouse models. Following viral infections bone marrow derived macrophages displayed a drastic down-regulation of Ocil. Interestingly, reduction of Ocil also enhances the susceptibility of these macrophages towards NK-mediated cell cytotoxicity, implicating Ocil as a molecule with inhibitory functions [241]. Indeed, results of my study also reflect a positive correlation between Ocil expression and the level of NK cell cytotoxicity specifically in the immature state of DCs, which is in agreement with the published data. This phenomenon could be related with the "NK mediated DC-editing process" in the periphery. The modulation in Ocil expression in hyperactive de-regulated iDC could also be another "indication" for NK cells in addition to the TRAIL-R and few other surface molecules as reported previously [373]. Several other immunological assays were also employed in this study to examine the impact of Ocil bearing DC in affecting the regulatory functions of NK cells. No considerable role of Ocil deficiency in regulating NK cells was observed, which further suggest the selective association of Ocil with the NK target cell recognition (Fig: 3.2, & 3.3A&B). In my hands, LPS-matured DC compared to the iDC, despite Ocil deficiency, displayed relatively more resistance to NK killing, which is contrary to

hypothesis initially formulated (Fig. 3.1). Therefore, based on my data, if I speculate Ocil as a strong protective molecule, then LPS-matured DCs should also demonstrate reduced resistance towards NK cell killing. As it is well documented that mature DCs express co-stimulatory molecules as well as maintain sufficient MHC expression to acquire protection against NK killing [374]. Additionally, NK cells employ a variety of inhibitory receptors to interact with these endogenous ligands. So in the context of mature DC regulation, it can be speculated that the level of protection mediated by the generation of signals from other ligands to activate other NK-inhibitory receptors overcome the deficiency of Ocil. Moreover, the role of additional unknown underlying molecular interactions in relation to the inability of Ocil-deficient LPS-matured DCs to exhibit differential regulatory functions (such as inducing proliferation in resting NK cells, cytokine production etc) cannot be ruled out. Hence, further understanding and identification of specific receptor (s) involved in this alliance and their functional associations with Ocil molecule could extend the current understanding to devise strategies to manipulate NK cell mediated anti-viral and anti-tumor effector functions.

Based on the findings of chapter 3, I come up with two possibilities, first that Ocil might constitute an additional layer of protection thus facilitating stable inhibitory responses to regulate NK cell responsiveness. Since viruses employ several immune evasion strategies, so it is quite possible that they also aim Ocil as a prime target thus promoting NK cell cytotoxicity. Alternatively, the down regulation of Ocil 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 further understand the relevance of these results it will be of interest to monitor the down regulation patterns of Ocil in the DC subsets. The in vivo kinetic data of Ocil downregulation in relation to the intensity of viral infection and the activation status of NK cells in mouse model could further explain other possible regulatory functions of Ocil in the context of NK/DC interactions.

In addition to the target recognition the significance of inhibitory receptors in the early education of NK cells has already been explained extensively [84]. NK cells education of self-tolerance and the acquisition of functional competence, a process believed to be initiated/happened in the bone marrow after the engagement of self MHC-I molecules with their cognate inhibitory receptors on the developing NK cells [342]. NK cells

deficient of self-MHC-I surface receptors demonstrate intrinsic hypo responsiveness [375]. Similarly, NK cells developed in MHC-I deficient environment are also unable to reject their sensitive targets [142]. Collectively these observations suggest that MHC-I expression regulates a mechanism that supports NK education and effector functions.

Since Ocil like other inhibitory receptor ligands such as MHC-I has been reported to interact with its cognate NKR-P1B/D NK cell inhibitory receptors [159]. In agreement with the MHC-I related published fact, NK cells derived from Ocil deficient background exhibited severe hypo responsiveness towards the sensitive targets in this study (Fig: 3.6 & 3.7A). These cells demonstrate inability to reject sensitive tumor or infected cells *in vivo* yet to be known. In comparison to MHC-I, Ocil is recognized fairly a new and undefined molecule. Despite notable differences in the structure and functions of Ocil vs MHC-I molecules, few similarities were also observed in these molecules, specifically their roles in target cell recognition. NK cells obtained from Ocil deficient mice consistently exhibiting reduced expression of CD107a degranulation when exposed to either DCs or other prototypic tumor targets (Fig. 3.6 & 3.7A). Although, these evidences are still not enough and more work is required to define the role of Ocil in NK cell education and tolerance. However I believe that these data indicate a potential role of these molecules in the early education of NK cells. If Ocil molecules like MHC-I regulate NK cell effector functions, then it can be examined by adoptively transferring Ocil deficient NK cells into a wild type background. Regaining effector phenotype will be a strong rationale to hypothesize the involvement of Ocil in NK education. Moreover, generating NK cells in Ocil deficient environment can draw a definite answer. I have established an *in vitro* NK differentiation model (Chapter 6), which can be used to differentiate NK committed precursors in Ocil deficient stromal cell line. Kung laboratory has already established a lentiviral platform; therefore I do not see any potential problem to knock down Ocil in OP-9 and achieve this goal in future.

NK cells mediated DC-killing has been associated with impaired T cell immunity resulting an uncontrolled tumor mass in mouse models [376]. The data presented in this study suggest that Ocil expression on DC generate inhibitory signals by interacting through its cognate NKR-P1B/D NK inhibitory receptors. Data of this study strongly co-relate the expression of Ocil with the survival of DC as well as the intrinsic cytotoxic

potential of NK cells. These conclusions might have a broader implication in improving DC-based NK immune therapy. In agreement with the published data, I am also predicting that viruses may target Ocil to subvert the immune responses, thus maintaining surface expression of Ocil on DC could be a possible counter strategy to avoid NK cell cytotoxicity in DC-based immunotherapy in animal studies.

7.2. SHP-1 Induced Mature NK Cell Functional Regulation

In chapter 3, I used lentiviral vector platform to manipulate the expression of NKR-P1B inhibitory receptor on primary murine NK cells. Genetically manipulated NK cells with higher expression of inhibitory receptor exhibited significant resistance towards BWZ-Ocil target cells (Fig. 3.4 & 3.5 A&B). This approach successfully validated the functional viability of NKR-P1B: Ocil axis in NK/DC context.

Upon specific stimulation, NKR-P1B like other inhibitory receptor also recruits SHP-1protein to counter activation signalling pathway [377]. For my second study (chapter 4), I examined the effect of down regulating SHP-1 expression in IL-2 activated mature NK cells. Based on previous investigations I hypothesized that disrupting SHP-1 protein expression will abrogate NK cell inhibitory receptor signalling. To directly examine the importance of SHP-1 in regulating activities and cell fate of mature NK cells, Kung lab established lentiviral-based engineering protocol was used in this study to knock down the SHP-1 protein expression in primary NK cells [255]. Contrary to the initial thoughts, the cytotoxic responses of SHP-1 (knock down) KD NK cells remained unchanged (Fig: 4.1B). Gene silencing of the SHP-1 however abrogated the ability of ITIM-containing NK inhibitory receptors to suppress the activation signals induced by NK1.1 activating receptors (Fig. 4.2). More interestingly, knock down NK cells mediated specific self-killing in a real-time live cell microscopic imaging system I developed to study NK cell cytotoxicity *in vitro* (Fig. 4.5, 4.6 A&B).

Previously the role of SHP-1 was studied both in human and mouse NK cells. The NK cells with catalytically inactive dominant negative form of SHP-1 or those derived from SHP-1 knock out mice demonstrated a distinct reduction in their cytotoxic potential when co-incubated with MHC-I deficient targets [278, 279]. Results of my study clearly contradict with the published functions of SHP-1. However, it is important to consider

that motheaten and motheaten viable mice have a short life span, which could be the consequence of the immunological disorders as reported in these mice [378]. Additionally, macrophages and other myeloid cell populations in these mice demonstrate aggressive activation levels, suggesting an inappropriate immune status [292]. It is clear now that NK cells like other immune cells do not function in isolation. These cells frequently interact with DCs, macrophages, T cells and neutrophils to shape up both innate and adaptive immunities [99, 379]. Therefore, one may speculate that NK cells in these mice could also be affected, which could subsequently alter their development and function. Therefore, it remains to be confirmed that cumulative effect of SHP-1 loss in these transgenic mouse represents a real NK cell function as well as the potential implications of this loss on the development and acquisition of effector functions of these cells.

It is well documented that NK cells use inhibitory surface receptors efficiently to spare self-MHC-I expressing cell, thus induce tolerance [380]. Therefore, disrupting the inhibitory pathway by silencing SHP-1would render these cells auto reactive. Although higher expression of CD107a was found in SHP-1 silenced NK cells (Fig. 4.3) surprisingly, these cells maintained normal cytotoxicity responses against prototypic target cells (Fig. 4.1B). In-fact an unexpected finding was noticed that SHP-1 shut down NK cells were recognizing other KD cells as potential targets (Fig. 4.6 A). These findings were validated by live cell imaging analysis and confirmed this unique phenotype is SHP-1 KD specific, as mock-transduced cells remained unaffected (Figure: 4.5, 4.6A&B). Due to the differential acquisition of NKG2D on the KD NK cells, it was speculated that SHP-1 silencing might induce the up-regulation of stress ligands on NK cells surface which enhance NK cell self cytotoxicity via engaging NKD2D receptors [8, 281]. Part of this investigation ruled out the role of Rae-1, a ligand of NKG2D in this phenomenon (Fig. 4.7). However complete profiling of other stress ligands such as H60, MULT is highly desirable to elucidate potential interactions of these molecules with NKG2D receptor [381]. Alternatively, investigating the cytotoxicity of SHP-1 KD NK cells after neutralizing NKG2D receptor will be a useful approach to identify the possible role of this receptor in this setting. Results of these proposed experiments would also reveal the significance of SHP-1 phosphatases in inducing stress responses in NK cells.

It is a well-known fact that phosphorylated Vav1 (pVav1) and its downstream signalling molecule Rac1 mediate F-actin polymerization, which subsequently facilitates the release of perforin granules from a cytotoxic NK cell [165]. In mature NK cell, binding of inhibitory receptor to its cognate ligand on the target cells induces SHP-1 phosphatase recruitment, which subsequently mediates Vav1 dephosphorylation [287, 382]. Signals for cytotoxicity that depend on actin mediated cytoskeleton rearrangement are consequently blocked due to the inactivation (dephosphorylation) of Vav1 and its down stream signalling molecules, resulting a hyporesponsive NK cell [383]. Since SHP-1 KD NK cells mediate self-killing, I, also speculate hyper-activated signalling molecules in these cells. Formal future experiments on these line might reveal the significance of this pathway in NK cell mediated cytotoxicity, otherwise these experiments might propose alternate molecular signaling potentially involved in cytotoxicity mechanism.

The importance of inhibitory receptors signaling is well recognized in NK education and self-tolerance [384]. Since this study highlights the significance of SHP-1 in the inhibitory signalling pathway of NK cells, thereby I further anticipate the involvement of this molecule in the early education and self-tolerance. Indeed, targeted future experiments, disrupting the expression of SHP-1 in NK-progenitors and their differentiation *in vitro* could improve our understanding and provide us definite answers in this regard.

Collectively, this study highlights the significance of SHP-1 in NK cell mediated cytotoxicity. As the primary NK cells are considered relatively resistant immune cells for genetic manipulation. In this report I was successful to establish a lentiviral platform to transduce resting NK cells. This study also proves that shRNA approach is a valuable tool to study the function of protein (s) and can be employed to study other candidate molecules in future as well.

7.3. DC-Regulated NK Cell Migration

NK cells and DCs both constitute the innate part of the immune system, however their role in regulating both innate and adaptive immunities has been demonstrated extensively [24, 222]. NK cells harbor sufficient potential to mediate spontaneous cytotoxic responses against abnormal cellular targets [385]. Whereas, DCs are formally known as professional antigen processing and presenting cells able to regulate adaptive immune

functions effectively [386]. Both NK and DCs have the ability to secrete a wide range of cytokines and chemokines, thus widely implicated in regulating different immune functions [7, 387].

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 communication [345]. It has been shown that these cells require cell:cell contact and soluble factors to acquire full spectrum of functional phenotype. Mice lacking such reciprocal interactions display impaired T-cell mediated immune responses [215]. However, the induction of NK/DC cross talk requires that both cell types either reside in the same location or can migrate from other organs to the site of infection. Available evidence supported the union of these two cells in the skin and LN in infection [220, 314]. DCs cells are predominantly reside in the epidermis and LN whereas NK cells poorly represent these tissues in steady state and even live in different compartments, away from DCs. Indeed the recruitment/infiltrations of NK cells into these tissues have been reported in infection [216, 220, 314]. Since migration and localization of natural killer (NK) cells in the periphery are tightly regulated in normal and pathological conditions. So the question is: "What are the biological mechanisms including any potential intervention of DCs in regulating the union of these two cells?"

Consistent with the published data, the results of this study also demonstrated the ability of DCs to secrete a wide range of chemical mediators including IP-10 (Fig: 5.5C) [201, 311]. NK cells express a number of chemokines receptors [311, 312], which enable them to respond to DC-derived factors suggesting the potential role of DC in mediating NK-recruitment *in vivo.*

In an effort to delineate the underlying mechanism, a novel microfluidic platform as well as conventional trans-well migration assays were employed to formally understand the role of DCs in NK migration in this study (chapter 5). Interestingly, both immature and LPS-mature DC derived soluble factors were found important to regulate NK migration *in vitro*. In contrast to iDC, however matured DC factors demonstrated strong chemotaxis (Fig. 5.2 & 5.3). These differential migratory responses can be explained based on the quantitative analysis of the conditioned media collected from the cultures of immature and mature DCs (Fig. 5.5C). Higher concentrations of IP-10 and CCL5 in matured DC

conditioned media can be associated with the compelling role of mature DC in NK cell recruitment. During infection, heavy recruitment of NK cells in the LN, a site primarily occupied by the matured infected DC compared to the skin which harbors immature DC, might also be a reasonable interpretation of my results, confirming mature DCs as strong inducer of NK-chemotaxis [220, 314, 388]. Similarly, heavy NK cell infiltration in the peritoneal cavity following the I/P administration of matured conditioned medium further validates my in vitro study results (Fig.5.6 C&D). Growing evidences suggest that NK cells mainly stay in the circulation, however they respond to specific signals and migrate to other organs and several chemokine receptor and their ligands have also been implicated in the induction of NK cell migration [94]. In particularly, several *in vivo* mouse models highlighted the role of CXCR3 in the regulation of NK migration in infection [200]. Notably, in human a recent study demonstrated CXCR3/IP-10 and CXCR4/SDF-1 axis, associated with NK migration into the infected TB sites [389]. In response to infection such as HIV, DCs are shown to be the main producer of IP-10 [390]. From the perceptive of my current investigation, CXCR3 also signify a main surface chemokines receptor on NK cells demonstrated specific signalling response to DC-derived conditioned medium. In agreement with this published data [220], I also documented the involvement of CXCR3 in regulating NK migration (Fig. 5.5B). Blocking CXCR3 however was not sufficient to inhibit complete NK migration in response to the LPS-stimulated DC-derived conditioned medium, which strongly suggested the involvement of other chemokines receptors. Characterization of DC-derived factors and their potential interactions with NK chemokine receptors is the focus of future studies. Understanding the precise nature of molecular pathways involved in these interactions will improve our ability to manipulate NK mediated effector functions in various pathological and physiological conditions.

Apart from other potential factors, results of this study however draw an inference that DCs are greatly implicated in recruiting NK cells at the site of infection. Additionally, several tumors have been linked with disrupted NK/DC cross talk, more precisely the inability of DC to improve NK effector functions [391]. Since, DC-maturation has a direct correlation with migratory properties of NK cells, as observed in this report, therefore,

results of my study also stress to determine the ability of tumor infiltrated-DC to produce soluble factors (chemokines) other than IL-15, IL-12 and type I interferon (key cytokines) involved in this cross talk [215, 309]. Future studies in this regard might also delineate some important unknown cellular and/or molecular factors involved in the tumor microenvironment. Kung lab has initiated an investigation to elucidate the potential impact of 4T1-derived condition medium (breast cancer cell line) on the functional phenotype of BMDCs. Conditioned medium pulsed DC demonstrating differential migratory responses as observed in the preliminary experiments (Kung lab unpublished data). The use of genetically engineered DC (to study chemokines) and/or modified NK cells (to study chemokines receptors) both in vitro and in animal studies will be warranted to understand specific receptor/ligand axis involved in the migration of NK cells. Examining the involvement of other NK receptors and their cognate chemokines in future investigations will also help us to comprehend the precise nature of NK/DC cross talk as well as to refine future therapeutic interventions to treat tumors.

While characterizing the potential role of DC-derived soluble factors regulating/mediating NK migration, I also reported an unexpected function of GM-CSF in this study. I demonstrated that exogenously administered recombinant GM-CSF is involved in repelling activated NK cells (chemo-repulsion) in the microfluidic device (Fig. 5.4). Further experiments using conventional trans-well migration platform also confirmed the inhibitory/negative role of GM-CSF. The ability of LPS-matured conditioned medium to attract NK cells enhanced many folds following the depletion of residual GM-CSF (Fig: 5.6 A&B). The inhibitory function of GM-CSF was also validated *in vivo*, as the ability of LPS-conditioned medium to recruit NK cells into the peritoneal cavity was greatly abolished when diluted with recombinant GM-CSF (Fig. 5.6 C&D). However, the exact mechanism regulating GM-CSF mediated NK cell chemo-repulsion is still unknown.

GM-CSF is a known pro-inflammatory cytokine, involved in the regulation of various immune functions [392]. GM-CSF mediated immunogenicity has been associated with the improved development and functions of tumor infiltrated antigen presenting cells as well as mediating Th-1 immune responses, thus widely used in the supportive care of several tumors [393, 394].

In addition to the immune cells including T cells, macrophages and fibroblasts, several tumors were also shown to produce GM-CSF [329, 395, 396]. In particularly, the examination of breast cancer demonstrated higher transcript of GM-CSF [330]. Preliminary work with 4T1-conditioned medium, a breast cancer cell line, confirmed the ability of these cells to secrete abundant GM-CSF. Interestingly, the depletion of GM-CSF from 4T1-conditioned medium amplified the chemotactic nature of the tested conditioned medium (Kung lab unpublished data). Apart from confirming the negative role of GM-CSF, the results of my study also suggest a possible NK-mediated immune evasion mechanism adapted by these tumor cells. Since breast cancer like other malignant tissues contain lower absolute NK cell numbers [397]. The exact mechanism behind low infiltration of NK cells in tumor mass is not yet clear [398], however based on this study I speculate that GM-CSF produced by breast cancer cells inhibits NK cell migration into the tumor microenvironment. GM-CSF is broadly recognized as a potent immunogenic cytokine, therefore arguing its immune suppressive functions seem quite challenging. The ability of GM-CSF to induce the generation of regulatory T-cells that in turn suppress the immune system and facilitate tumor progression also indicate the negative functions of this cytokine, which in turn support my hypothesis [399].

In addition to validating the role of GM-CSF in the microfluidic platform with other tumor cell lines, future experiments involving the determination of GM-CSF transcripts level in other tumor cells as well as elucidating the role of GM-CSF in cancer growth, metastasis and NK cell migration in a mouse model of breast cancer are highly desirable. Results of these studies will establish whether GM-CSF and/or NK cells is a novel prognostic biomarker of disease progression/metastasis, and/or novel target to enhance anti-tumor immunity in patients. Finally, if tumor cells are the continuous source of abundant GM-CSF, a potent stimulator of DCs development, then what other mechanism and factors are involved polarizing DC maturation and impaired functions in tumor microenvironment. Alternatively, if the chemotactic functions of tumor infiltrated DCs are unaffected, then I speculate that strength of chemo-repulsive signals mediated by GM-CSF could be higher than the cumulative chemotactic signals generated by the DCs. I believe these are some of the interesting avenues to unravel the mystery of NK cells in cancer in future studies.

Apart from tumor (s) the expression of GM-CSF has also been reported in several infection. Epstein-Barr virus (EBV) infected nasopharyngeal carcinoma epithelial cells were reported to secrete several immunomodulators including the GM-CSF [400]. Similarly, the induction of GM-CSF by the Respiratory Syncytial Virus (RSV) -infected respiratory epithelial cells was also demonstrated in previous studies [401, 402]. Various investigations demonstrated that viral induced GM-CSF causes eosinophil degranulation and the release of superoxide in addition to the IL-10 production to exacerbate immune responses [400]. However, a direct role of GM-CSF manipulating NK immune responses is never reported. Since viruses employ a variety of defensive mechanisms to impair CD8 T cell and NK cell recognition, based on current investigation I also hypothesize that viruses might use GM-CSF as an additional layer of immune evasion. It is highly likely that GM-CSF is involved impeding NK cell infiltrations, thereby sparing infected cells from NK-mediated killing. Therefore, future experiments are required to evaluate the contributing effect of GM-CSF in relation to the functional inactivity of NK cells in such infections.

Finally, in this study, I also standardized a novel microfluidic platform (Fig: 5.1 A&B). Among some of the notable features, microfluidic platform generate live imaging data at single cell level. Moreover, it can measure chemotactic as well as chemo-repulsive migration patterns, so provide a valuable tool and in combination with conventional trans-well migration assay to understand migration mechanisms. I am confident that the utility of these standardized platforms will also be beneficial for the future experiments to elucidate the role of chemical mediator including GM-CSF or some other DC-derived chemokines in proposed tumor and infection models. It is yet to be determined whether a specific subtype of NK cells responds to the DC-derived soluble factors. In addition, the characterizing of DC-conditioned medium will also help to delineate unknown receptor:ligand interaction (s) involved in this cross talk.

7.4. DC-Induced NK Cell Differentiation

Mounting evidences suggest that NK/DC interactions establish a strong relationship between innate and acquired immunities [365]. However, the mechanisms involved in

calibrating these associations *in vivo* have not been well defined. Phenomenon of NK/DC cross talk emerged more than two decades, until now the exact nature of this dynamic interplay is not clear especially in cancer and infection models. Therefore, additional information is still needed to unfold the significance of this interaction *in vivo*. How and where these cells interact in vivo, what other novel receptor:ligand interactions require to orchestrate immune responses, what other DC release factors modulate NK functions, are the questions yet to be answered. My current study (chapter 6) has examined the physiological significance of NK/DC crosstalk at progenitor level. I first established a culture system, to generate mature NK cells from a subset of purified multipotent stem cells (Fig. 6.1). I demonstrated that iDCs but not the matured DCs, have potential to regulate NK cell differentiation *in vitro* (Fig. 6.4A&B, 6.2 A&B). In addition to the DC-derived soluble factors those play an important role for the induction of Ly49 receptor repertoire on differentiating NK cells, I also confirmed that physical interaction/engagement between DC and developing NK cells is deemed important during this process (Fig. 6.3).

The acquisition/expression of Ly49 surface receptors, a characteristic phenotype of mature NK cell, is considered an important step to generate self-tolerant NK cells [343, 344]. A number of studies demonstrated the significance of several molecules including class-I MHC, the members of lymphotoxin family as well as TF such as TCF-1, PU.1 and GATA3 in regulating the expression of Ly49 receptors [403]. However, the precise nature of the signals and the molecular mechanism involved in the induction of Ly49 are yet to be determined.

BM tissue is constituted of a number of cells, soluble factors and stromal factors with the ability to drive hematopoiesis of many lineages [404]. Previous studies demonstrated overwhelming functions of BM-environment in the development of NK cells [405]. (Indeed results of my study also support BM as the primary site of NK cell development.

The fact is that DCs like NK cell also originate from the bone marrow [406]. Moreover, these cells are capable to produce IL-15, IL-7 and SCF, some of the known factors required for NK development both *in vitro* and *in vivo* settings [407]. Therefore the stimulating effect of these cells driving NK differentiation is quite expected and according to my hypothesis. However, contrary to the previously published work, where mature DC

found key players in NK/DC cross talk and function like a master regulator of this process [215], in this study, iDC emerged as the primary regulator in driving the development process.

Unlike mature DCs, which are characterized as potent stimulator of NK effector functions and producer of several cytokines, iDCs are presented as the prime target of NK cells in the literature [118, 308]. Also these cells require NK mediated stimulation to acquire maturation [247]. No such evidence showing the role of iDC in the development of NK cells or any other cell of different lineage has been reported previously. In the context of mature NK/DC cross talk where cell:cells interactions deemed crucial, I also observed that physical disruption between these cells impaired the acquisition of Ly49 receptors on immature NK cells (Fig. 6.3). These results suggest that differentiating NK cells might be establishing an immunological synapse with iDC, but what are those receptor and ligands involved in this interaction currently not known. Although, there is evidence in the literature supporting that iDC can make a physical contact with naïve NK cells, however the molecular nature of this interaction in this immunological synapse is also elusive. [115]. Therefore, a formal examination of surface receptor/ligand repertoire of differentiating NK cells as well as of the iDC is required in future investigations to uncover the differential signalling events between these two distinct cell populations. These studies would allow us to comprehend the precise nature of underlying recptor:ligand pairing involved in NK/DC communication and their potential implications in the regulations of NK cell development.

IL-15 has been associated previously with NK cell survival and proliferation. Mice deficient of either IL-15 or CD122 expression demonstrates impaired NK cells development suggesting the physiological significance of IL-15 signalling [337, 408-410]. In consistent with published data, my study also suggest that IL-15 might function as a compensatory cytokine, since iDC failed to up regulate the expression of Ly49 on iNK cells (stage II & III) in the absence of exogenous IL-15 stimulation (Fig. 6.2A&B, 6.4A&B). Similarly, the ability of iDC to drive the expression of NK1.1 on stage I differentiating cells is also abrogated in the cultures in the absence of IL-15 and/or IL-2 cytokines (Fig. 6.5 A&B). The reason for these unexpected results is not clear, however these results indicate that acquisition of NK1.1 and Ly49 surface receptors on

differentiating NK cells are independent events and the signals promoting development come from iDC. Moreover, the common gamma chain cytokines tested in this setting may be required to provide survival signals to the differentiating NK cells at various stages. The inability of common gamma chain cytokines to support differentiation process in the absence of iDC not only reinforces the significance of underlying molecular interactions between these two cell populations, but also validates my logical thinking. However, the inference I draw here may not be supported in part by the majority of the published work. As the stage I cells, in comparison to the stage II and III differentiating NK-cells, do not express CD122 expression eventually unable to respond to IL-15 signalling [55]. Interestingly, in human a developmental intermediate lacking CD122 expression yet responds to IL-15 signaling, may also explain the peculiar responses of IL-15 in conjunction with iDC to promote HSC differentiation in this model [79]. There is a possibility that the exogenous IL-15 stimulation may also skew the functional phenotype of iDC in co-cultures, a question need to be addressed in future studies. One potential weakness I realize in this model is the limited exposure of DC to our stage I, II & III developing NK cell population resulted poor yield. Modifying the existing model to prolong the exposure of developing NK cells with iDC (by replenishing with a fresh lot of culturing DC) might be valuable to improve recovery as well as to dissect the underlying mechanism involved in this process.

Collectively, the data presented in this study provide new evidences that DCs are also contributing effectively in promoting NK cell development *in vitro*. Previous studies established the critical involvement of several TF, cytokines and bone marrow derived stromal factors in this process, and now I have demonstrated that these developing NK cells may also need/require additional signals from other accessory cells such as iDC, to acquire maturation. Bone marrow is a complex tissue harboring a diverse cell population. Therefore, in addition to the DC, I cannot exclude the possibility of other cell populations as a prime candidate in NK-cell development *in vivo*. Thus targeted future studies are necessary to reveal the significance of physiological mechanisms as well as the involvement of other cellular components in this process.

NK cell based adoptive immune therapies have shown reasonable promises against various cancer treatments in clinical settings [138, 411, 412]. Such therapeutic

interventions however, require adequate number of clinical relevant, *ex-vivo* generated NK cells, a technical challenge that most protocols anticipate. So far several models of NK development have been proposed using different combinations of cytokines with or without stromal cells, claiming varied percentages of success. In addition to this, recent publications demonstrated that distinct phenotypic differences also exist between *ex-vivo* generated NK cells and of those freshly isolated NK cells, suggesting additional factors are involved in this process [21, 45]. The emergence of DC and DC-derived soluble factors as an important contributing factor in this process in my study further strengthens the involvement of other factors in NK development. Compare to the mature NK cell and DC interactions; the interplay of DC with NK cells at progenitor level is fairly a new concept, which needs to be investigated further. Identification of soluble factors and/or key receptor:ligand involved in this process might help to manipulate the existing methodologies to improve genesis as well as function of developing NK cells. In human, secondary lymphoid tissues are also proposed as potential sites for NK cell developmental [79]. Understanding the interplay between developmental intermediates of NK cells and DC in the extramedullary sites, 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.

8.0 CHAPTER 8

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Appendix

1. List of My Publications

Manuscripts Published

1. **Mahmood S,** S. Nandagopal, F. Lin and S.K.P. Kung. Microfluidic-based, live-cell analysis allows assessment of NK-cell migration in response to cross-talk with dendritic cells. Eur J Immunol. 2014. doi: 10.1002/eji.201344244

2. **Mahmood S,** Kanwar N, Tran J, Zhang ML, Kung SK. SHP-1 phosphatase is a critical regulator in preventing natural killer cell self-killing. ***PLoS One***. 2012; 7 (8).

3. Narni-Mancinelli E1, Jaeger BN, Bernat C, Fenis A, Kung S, De Gassart A, **Mahmood S,** Gut M, Heath SC, Estellé J, Bertosio E, Vely F, Gastinel LN, Beutler B, Malissen B, Malissen M, Gut IG, Vivier E, Ugolini S. Tuning of Natural Killer Cell Reactivity by NKp46 and Helios Calibrates T Cell Responses. ***Science.*** 2012 Jan 20;335 (6066):344-8.

4. Tran J, **Mahmood S**, Carlyle JR, and Kung SK. Altering the specificity of NK: target cell interactions by genetic manipulation of NK receptor expression on primary mouse NK cells. ***Vaccine***. 2010 May 14;28 (22):3767-72.

Papers in Preparation

1. A critical role of Osteoclast inhibitory lectin in fine-tuning Natural Killer cell responsiveness. **Sajid Mahmood** and Sam Kung.

2. Immature dendritic cells support natural killer cell differentiation. **Sajid Mahmood** and Sam Kung.