TUMOR CELL MEDIATED MODULATION OF NK RECEPTORS AND THEIR ROLE IN CANCER

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By

RICHA SHARMA

DEPARTMENT OF BIOTECHNOLOGY
DELHI TECHNOLOGICAL UNIVERSITY
DELHI-110042 (INDIA)

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Dedicated to My Parents

DECLARATION

I, Richa Sharma, certify that the work embodied in this Ph.D. thesis is my own bonafide work carried out under the supervision of Dr. Asmita Das (Assistant Professor, Department of Biotechnology, Delhi Technological University) for a period of November, 2011 to April, 2018. The matter embodied in this Ph.D thesis has not been submitted for the award of any other degree/diploma.

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(Reg. No: 2K11/PhD/BT/01)

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This is to certify that the Ph.D. thesis entitled **"Tumor Cell Mediated Modulation of NK Receptors and their Role in Cancer"** submitted by **Richa Sharma** (Reg. No: 2K11/PhD/BT/01) to the Delhi Technological University, Delhi for the award of the degree of **Doctor of Philosophy** is based on the original work carried out under our supervision. It is further certified that the work embodied in this thesis has neither partially nor fully submitted to any other University or Institution for the award of any degree or diploma.

Dr. Asmita Das
Asst. Professor
Department of Biotechnology
Delhi Technological University
Shahbad Daulatpur
Bawana, Delhi-110042

Prof. Jai Gopal Sharma
Head of Department
Department of Biotechnology
Delhi Technological University
Shahbad Daulatpur
Bawana, Delhi-110042

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(Richa Sharma)

ABSTRACT

Natural Killer (NK) cells are large granular lymphocytes of the innate immune system with the capacity to kill tumor and virus infected cells without prior sensitization. This is distinct from the mechanism acquired by T-cells to kill target cells, which involves recognition of tumor antigens in a major histocompatibility restricted manner. NK cytotoxicity is regulated by a fine balance between activation and inhibition signals by a multitude of receptors stochastically expressed on each NK cell. NK cells preferentially kill target cells that lack surface expression of major histocompatibility (MHC) class I molecules. Inhibitory NK receptors recognize MHC class I molecules to induce inhibition of NK cell cytotoxicity.

IL-2 is the first cytokine to be tested and successfully used as therapeutic agent in cancer. IL-2 stimulation has been shown to cause an expansion of NK cells and T-cells. In the present study, we explored the effect of IL-2 stimulation on proliferation of NK cell population and modulation of NK cell activity. IL-2 stimulation of NK cells resulted in proliferation of $NK1.1^{+}$ NK population and increase in expression of Ly49A (non-cognate) inhibitory receptor. However neither there was any change in cognate inhibitory receptor, Ly49C nor activating receptor, Ly49D. Hence, IL-2 was found to cause potentiation of NK population but did not result in auto-immunity due to NK activation in absence of tumor.

Despite, potential role of immune system in controlling tumor progression, the tumor cells often evolve strategies to escape immunosurveillance. It has been established that tumor cell mediate modulation in the NK cell receptor profile results in effector function against target cells. In the present study, tumor derived factors from NK resistant tumor were tested for NK receptor expression. In this study we showed that membrane derived factors of P815 interfere with the NK cell function by actively modulating the surface expression of NK cell inhibitory receptors, Ly49A and Ly49C. The inhibitory receptor modulation may be responsible for hypoactivity of NK cells against P815 target tumor cells. Thus, identifying the factors responsible for modulation in NK cell receptor expression and frequency will help to optimize the potential of NK cells in current therapies in combinatorial therapies against tumors.

NK cells express a wide range of inhibitory receptors and it was observed that NK inhibitory receptor and non-classical HLA interaction resulted in better inhibition of NK cell cytotoxicity than with classical HLA molecules. In the present study, the basis for this difference in inhibition potential of NK inhibitory receptors was explored. Since each NK cell expresses a multitude of receptors on a single cell; it was difficult to study binding affinities and residues involved in receptor-ligand interaction through experimental studies. Hence, binding affinity of different inhibitory receptors with classical and non-classical HLA molecules was explored through computational approach. The present study conclusively showed that there was greater affinity of NKG2 receptors for their ligands, non-classical HLA molecules due to engagement of more residues at the receptor-ligand interface.

NK cells have been used as potential immunotherapeutic agents against tumors. Various NK based therapies such as adoptive cell transfer therapy, chimeric antigen receptors, and cytokines based therapies have shown promising results but did not result in complete regression of tumors in most cases. Our findings would help to design strategies to strengthen the potential of NK cells in current NK based regimens for cancer therapy.

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LIST OF ABBREVIATIONS

ACT	:	Adoptive cell transfer
APC	:	Allophycocyanin
BCA	:	Bicinchoninic acid
CD	:	Cluster of Differentiation
DMEM	:	Dulbecco's Modified Eagle's Medium
DTT	:	Dithiothreitol
ECL	:	Electrochemiluminescence
EDTA	:	Ethylenediaminetetraacetic acid
EGTA	:	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	:	Enzyme-linked immunosorbent assay
FITC	:	Fluorescein isothiocyanate
H_2O_2	:	Hydrogen peroxide
H_2SO_4	:	Sulphuric Acid
HLA	:	Human Leukocyte Antigen
HRP	:	Horseradish peroxidase
IFN	:	Interferon
IL	:	Interleukin
KIR	:	Killer Immunoglobulin Receptors
MHC	:	Major Histocompatibility Complex
MIP	:	Macrophage Inflammatory Proteins
NK	:	Natural Killer Cells
PBS	:	Phosphate buffered saline
PDB	:	Protein Database

PE	:	Phycoerythrin
PFA	:	Paraformaldehyde
PVDF	:	Polyvinylidene fluoride
RMSD	:	Root Mean Square Deviation
RT	:	Room Temperature
TEMED	:	Tetramethylethylenediamine
TMB	:	Tetramethylbenzidine

LIST OF PUBLICATIONS

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Chapter 1
Introduction

CHAPTER 1
INTRODUCTION

Natural killer (NK) cells are crucial component of the innate immune system and have the ability to kill tumor and virus infected cells without prior sensitization to tumor antigens (Larsen et al., 2014). This is distinct from the mechanism employed by T-cells to kill target cells, which involves recognition of tumor antigens expressed in the context of major histocompatibility class I or II by a specific T-cell receptor (Marcus et al., 2014). NK cells act as a first line of defense against tumor targets and referred as 'Natural Killer cells'. NK cells produce cytokines through the interaction of a variety of cell surface receptors with their cognate ligands (Larsen et al., 2014). NK cells express multitude of activating and inhibitory receptors that transduce activating or inhibiting signals. Integration of these opposing signals determines the effector functions which involve cytotoxicity and cytokine production. Human NK cells express are characterized by surface marker CD56 and mouse NK cells by surface markers NK1.1 and DX5 (CD94b) (Mandal and Viswanathan, 2015).

Human NK cells can be categorized into two subsets on the basis of intensity of CD56 and CD16 surface expression. Systemic NK cells display two types of subsets, $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ (Amand et al., 2017). Approximately 90 % of peripheral blood NK cells (pNK) are $CD56^{dim}CD16^{+}$, which produced cytokines and lysed the target cells. By contrast, $CD56^{bright}CD16^{-}$ NK cell subsets preferentially expressed in certain lymphoid organs were able to produce large amounts of cytokines, including interferon-γ (IFN-γ), tumor necrosis factor (TNF), and granulocyte–macrophage

colony-stimulating factor, but cytotoxicity was attained only on their prolonged activation (Fauriat et al., 2010). Vossen and his group reported majority of circulating NK cell population, which were $CD56^{dim}$ and also $CD27^{-}$, released high levels of perforin and granzyme B and had strong cytotoxic activity (Vossen et al., 2008). On the other hand, NK cells that were $CD27^{+}$ and $CD56^{bright}$ had markedly low levels of perforin and granzyme B and hence low cytotoxic potential. Also, recruitment of NK cells to different inflamed tissues is controlled via different chemokine receptors repertoire, and there exists a unique representation of NK cell subset in different tissues/organs (Shi et al., 2011). These phenotypically different NK cell subsets showing disparate functions might be activated during different stages of infection and other pathological conditions. Murine NK cells do not express functional homologue of human CD56. However, murine NK cells were categorized on the basis of CD11b (αM integrin) and CD27 (TNFR) expression. $CD11b^{hi}CD27^{+}$and $CD11b^{hi}CD27^{-}$ NK cell subsets in mice displayed distinct expression of activating and inhibitory receptors and cytokine production. $CD11b^{hi}CD27^{+}$ NK cell subset showed enhanced cytotoxicity and the high levels of cytokine production Conversely, $CD11b^{hi}CD27^{low}$ showed low cytolytic potential against tumor targets (Shi et al., 2011).

Natural killer (NK) cells play a key role in innate host defense against tumors, virus as well as bacterial pathogens. Numerous studies in human and mice model had demonstrated the antiviral role of NK cells that included either direct killing of the infected target cell or production of cytokines that mediated effector response (Vidal et al., 2011, Jost and Altfeld, 2013). NK cells were found to mount an effective immune response against Human papilloma virus infection and HINI infection (Zhao et al., 2017, Fox et al., 2012). Tumor cells have a tendency to down-regulate MHC-I

molecules and this feature makes tumor cells susceptible to NK mediated lysis. On target cell interaction, they produce perforin, granzyme and cytokines and mediate anti-tumor immunity (Chester et al., 2015). NK cell frequency in tumors has found to be associated with clinical outcome of various cancers. The density of activated NK cells was correlated with tumor load in lung cancer and it was observed that more number of NK cells infiltrate the tumor site and are able to lyse the tumor cells (Larsen et al., 2014). NK cells also play important role against bacterial pathogens through pattern recognition receptors, by producing cytokines or by up-regulation of activating receptors. It was observed that stimulation of bacterial antigen and IL-22, resulted in activation of duodenal NK cells that showed enhanced IFN-γ production by NK cells in response to *Helicobacter pylori* infection (Yun et al., 2005). It was reported that activated NK cells were involved in direct lysis of *Mycobacterium tuberculosis* infected monocytes by secreting IFN-γ, perforin, granzyme B and granulysin and enhanced production of IL-1β, IL-18, CCL4/MIP-1β by monocytes in response to infection (Paidipally et al., 2018). Furthermore, it has been recently established that activated memory-like NK cells were associated with vaccine-induced protective immunity against *Mycobacterium tuberculosis* (Venkatasubramanian et al., 2017).

NK cells exert strong cytotoxicity against tumor targets. However, the effector function of NK cells is compromised to a variable extent due to various mechanisms opted by tumor cells to evade immune response. These mechanisms may include interference with NK cell activation as well as modulation of NK cell function and frequency by tumor-derived factors (Vitale et al., 2014). Various strategies have been implemented to increase the NK cell number, viability and their cytotoxic function at the tumor microenvironment by the tumor cells. Different approaches include cytokine

based expansion and activation of NK cells, check point inhibitors targeting the NK cell receptors, development of specific antibodies crosslinking tumor specific antigens to CD16 on NK cells that resulted in NK cell activation (Wiernik et al., 2013). Immunotherapy using IL-2 had proved to be one of the main treatment modalities within cancer immunotherapy. IL-2 has been known to cause in NK and T-cells proliferation. Both NK and T-cells are responsive to IL-2 with different affinities, since, they express two different forms of IL-2 receptors (IL-2R), namely IL-2Rαβγ (high affinity) in T-cells and IL-2Rβγ (intermediate affinity) in NK cells. Tumor microenvironment is characterized by the presence of different cell types (such as T-cells, NK cells, dendritic cells, myeloid cell) and high local levels of IL-2 secreted by infiltrating T-lymphocytes, the present study explored the role of IL-2 in modulation of NK activity in the presence or absence of tumor in the tumor microenvironment. Tumors also develop strategies for immune evasion and subversion of NK and T-cell activity. Hence we focused on the role of tumor derived factors on NK receptor modulation and NK activity. Further our study throws light on the relative potential for inhibition through distinct inhibitory receptors engaging classical and non-classical HLA molecules.

Our study therefore aimed to provide insights for developing better immunotherapies especially for tumors resistant to NK and IL-2 driven immunotherapies. Also, combinatorial therapy involving cytokines and multiple immune effector cells necessitates the study of tumor and cytokine mediated modulation of immune function and our study tried to address key issues in this immunomodulation therapy.

Objectives

The present study was focused on the following objectives:

1. To study the response of cytokine mediated proliferation of NK cells.
2. To study the effect of tumor cells on NK activity in presence of IL-2 mediated NK stimulation in the tumor microenvironment.
3. Elucidation of the structural basis for the difference in affinities of different NK cell inhibitory receptor interaction with their cognate ligands.

Chapter 2

Review of Literature

CHAPTER 2
REVIEW OF LITERATURE

2.1 History of NK cells

In early 1970s, a study was intended to detect murine B cells (immunized) producing IgM against Sheep Red Blood Cells (SRBC) in Florida University, researchers observed deflated balloon appearance of the IgM-complement lysed SRBC by Scanning Electron Microscopy (SEM). Formation of SRBC monolayer on the microscope slide surface depicted the immune cellular interactions. Interestingly, unusual plaques were observed that were being formed in these immunized mice in which lysed SRBC were absent. In fact, a central cell was seen in the 'clear' plaque that discharged some lytic agent that completely destroyed the surrounding SRBCs without prior immunisation. Regardless, of the presence of complement components, these central cells lysed SRBCs, creating clear plaques. These cells were called as complement independent plaque forming cells (CIPFC) and were also observed in spleens and lymph nodes of non-immunized mice (Thornthwaite et al., 1974a, Thornthwaite et al., 1974b). Later in 1975, Kiessling and co-workers observed a population of lymphocytes distinct from T-cells were able to kill leukaemia cells *in vitro* without previous sensitization. They designated these cells as 'Killer cells' that showed natural cytotoxicity against mouse Moloney leukemia cells (Kiessling et al, 1975). Further, many groups of researchers had shown their anti-tumor activity (Herberman et al., 1975a, Herberman et al., 1981, Robertson et al., 1990) and Kiessling and co-workers coined the term 'Natural Killer cells' in their publications (Kiessling et al., 1975b). Later, it was observed that anti-tumor activity of NK cells can be significantly augmented on stimulation with cytokines

like IL-2. This activated population was shown to have heightened cytotoxic potential as compared to unstimulated population. This separate active sub-population of NK cells was termed as 'Lymphokine activated Killer' (LAK) cells with ability to target wide spectrum of tumor. Adoptively transferred LAK has been shown to possess a potential anti-tumor efficacy against established metastasis (Davis et al., 2015).

2.2 NK cells as a bridge between innate and adaptive immunity

Natural Killer (NK) cells represent 10–15% of circulating lymphocytes and functional ability to kill tumors and pathogen infected cells without prior sensitization. They are considered as effectors of innate immunity by virtue of their ability to secrete cytokines such as IFN-γ, TNF-α, IL–10, IL-5, IL-13, growth factors such as GM-CSF (granulocyte macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor), and many chemokines, including CCL2 (MCP-1), CCL3 (MIP1-α), CCL4 (MIP1-β), CCL5 (RANTES), XCL1 (lymphotactin), and CXCL8 in response to physiological and pathological conditions (Fauriat et al., 2010, Vivier et al., 2011). NK cells have traditionally been known to be a part of the innate immune system. T-cells being key component of the adaptive response possess major characteristics of adaptive immunity; specificity and memory response (Mitrovic et al., 2012). However, in the recent years there has been growing evidence of memory response being exhibited by NK cells (Peng et al., 2013). In response to viral infections, NK cells secrete IFN-γ that helps in immune recognition of virus infected cells and activate naive CD4+ T cells into Th1-type helper effector cells leading to clearance of infected cells. MCMV infected mice had shown increased IFN-γ levels and mediated protection by interfering with viral replication pathway (Mitrovic et al., 2012). Similarly, NK cells were reported to produce IFN-γ without prior sensitization

and resulted in T-cell independent control of vaccinia virus in respiratory tract (Abboud et al., 2016). Moreover, NK cells stimulated with antibody coated target cell and IL-2/IL-12 had shown to produce a broad array of cytokines such as IFN-γ, TNF-α, GM-CSF and chemokines including MIP-1α, MCP-1, RANTES, IL-8, responsible for recruitment of T-cell at tumor site, therefore induced indirect adaptive response (Roda et al., 2006). Immunological memory and specificity has been considered as a hallmark of adaptive immunity. NK cells have shown the potential to persist for longer duration in the infected organ and have capacity to re-activate and undergo rapid expansion on subsequent exposure to pathogen or antigen. Therefore, NK cell has the ability to retain memory of previously exposed specific antigen, for example in RAG-deficient mice model, which lack T-cells and B-cells, NK cells alone were sufficient to generate contact hypersensitive response and hapten- induced memory (O'Leary et al., 2006). Moreover, it was evident that the NK cell mediated contact hypersensitive response was antigen-specific, because re-stimulation with different haptens did not result in memory-like response (Paust et al., 2010). Adoptive transfer of hepatic NK cells from donor but not splenic NK were capable of transferring memory to a naive recipient. These memory-like NK cells in liver expressed CXCR6, chemokine receptor, suggesting their role in liver homing and memory persistence but not antigen recognition (O'Leary et al., 2006, Paust et al., 2010). Peng et al. 2013 divided hepatic NK cells into two phenotypically distinct subsets, $CD49a^{+}DX5^{-}$ and $CD49a^{-}DX5^{+}$. They reported that $CD49a^{+}DX5^{-}$ NK cells possessed the potential to recall and upon hapten challenge, conferred hapten-specific contact hypersensitive responses (Peng et al., 2013). On the other hand, conventional NK $CD49a^{-}DX5^{+}$ were involved in enhancing T-cell mediated anti-viral response against HBV infection via IFN-γ secretion (Zheng et al., 2016). Interestingly, unlike conventional NK, NK

expressing $CD49a^{+}DX5^{-}$ were known to originate from hepatic hematopoietic progenitor/stem cells (HPCs/HSCs) but not from the bone marrow (Peng et al., 2013). Furthermore, there are evidences of antigen-specific memory-like NK cell response in viral infections. In mice, interaction of $Ly49H^{+}$ NK cells with m157 glycoprotein, specifically expressed by MCMV-infected cells induced expansion, selective activation and production of inflammatory cytokines by the NK cells (Smith et al., 2002, Dokun et al., 2001). On subsequent exposure to MCMV, specific memory NK cells showed a more efficacious response than the naive NK cells (Sun et al., 2009). Similarly, in humans $CD94/NKG2C^{+}$ subset triggered adaptive response to control HCMV infection but cognate ligand for NK receptors is still unknown (Della-Chiesa et al., 2013). As expected from the memory cells, $CD56^{dim}$ $CD16^{bright}$ $NKG2C^{+}$cell subset did not confer specific cytotoxicity against fibroblasts infected with HCMV. Nevertheless, cells displayed an increased ability to expand (Schlums et al., 2015) and produced cytokines like TNF-α upon stimulation by opsonized $HCMV^{+}$ target cells via CD16 receptor (Costa-Garcia et al., 2015, Muntasell et al., 2016). These studies have shown that NK cells can remember prior exposure to antigens resulting in NK cell memory generation. Therefore NK cells interplay between both innate and adaptive arms of immune system that control tumors and pathogenic infections. Further studies on their *in-vitro* expansion, maintenance of the memory NK cell pools and also the understanding of pathways involved in antigen recognition would open new horizons in the development of therapeutics for cancer.

2.3 NK cell receptors

NK cells employ a distinct mechanism to distinguish and eliminate aberrant cells (Lanier, 2005). NK cells express a multitude of receptors that activate or inhibit effector

function. Activating receptors often bind to cognate ligands that are up-regulated on infected or tumor cells. Lack of surface MHC class I molecules and expression of appropriate ligands for NK cell receptor results in NK mediated cytolysis of the target cells. The NK cell receptors may belong to either of receptor superfamily, Ig-superfamily of type I membrane protein or C-type lectin family of type II membrane protein (Warren and Smyth, 1999).

2.3.1 NK cell receptors in mice

2.3.1.1 Ly49 receptors

NK cell receptors that recognize MHC class I molecules are entirely different in mice. Ly49 receptors are type II C-type lectin-like membrane receptors coded by a family of highly polymorphic and polygenic genes and recognize H-2 class-I molecules on target cells. The Ly49 receptors present on the chromosome 6 in mice 'NK gene complex' which includes various related genes that encode both activating and inhibitory receptors, but majority of these receptors are involved in inhibition of NK cell function (Pegram et al., 2011). Ly49A is the prototypic member of klr gene family and contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains. Proteins encoded by different alleles of H-2 bind with different specificities and affinities to Ly49 receptor. NK cell inhibitory receptor Ly49A recognize H-2D^d, D^k and D^p on the potential target cells. Ly49A receptor from C57BL/6 strain has the highest affinity for H-2^d with a K_D ~10 mM (Jonsson et al., 2010). Ly49C binds H-2K^b and H2D^b (Dam et al., 2003). Ly49I is also known to inhibit NK cell cytotoxicity and binds with H-2K^b, D^d, K^d, and K^k. While inhibitory receptor Ly49G2 binds H-2D^d. Ly49D is NK cell activating receptor that stimulates cytotoxicity of murine NK cell (Goerge et al., 1999). Ly49D recognizes H-2D^d antigen. Ly49D is known to be associated with

DAP-12 that contains an immunoreceptor tyrosine-based activation motif and mediates activation of NK cells (Smith et al., 1998). Polygenic and polymorphic nature of Ly49 receptors results in heterogeneity in both the type and level of Ly49 molecules expressed in different mouse strains. Ly49 members are best characterized in C57BL/6 strains. It was reported that NK cells from C57BL/6 expressed activating receptor Ly49H and efficiently recognized m157 viral protein on the surface of MCMV-infected cells, thereby, confer cytotoxicity against MCMV infection. In contrast 129 or BALB/c mouse strains did not express NK cell activating receptor Ly49H and were susceptible to MCMV infection (Lee et al., 2001, Daniels et al., 2001). Other Ly49 activating receptors Ly49P and Ly49W had been reported to interact with H-2D^{d} (Silver et al., 2001, Silver et al., 2000).

2.3.1.2 NKR-P1 receptors

The NK cell receptor protein 1 (NKR-P1) (CD161) molecules belongs to family of type II transmembrane C-type lectin-like receptors. In C57BL/6 mice, NKR-P1 comprises four genes, NKR-P1A, NKR-P1C, NKR-P1D, and NKR-P1F. NK1.1 represent NKR-P1C gene and is the most specific serological marker on murine NK cells (Lanier, 2005). The PK136 mAb (anti-NK1.1) commonly used to detect NK cells reacts with the NKR-P1C gene product in C57BL/6 mice (Ljutic et al., 2005). NK1.1 functions as an activating receptor (Reichlin et al., 1998). NKR-P1D/B are known to have inhibitory function and both contain an ITIM in their cytoplasmic domain (Carlyle et al., 1999, Plougastel et al., 2001) while NKR-P1A receptor signalling has not been fully characterized. Thus, NKR-P1 family possess both activating and inhibitory receptors and recognizes target cell in MHC-independent manner.

2.3.2 NK cell receptors in humans

2.3.2.1 CD16

CD16 is the low-affinity activating receptor that binds with Fc region of IgG (FcγRIII) and mediate antibody-dependent cellular cytotoxicity (ADCC). CD16 is 70kD glycoprotein belong to Ig superfamily and are expressed on the surface of NK cells, some DC, T lymphocytes, monocytes, and macrophages. Upon engagement of CD16, NK cells recognise specific antigen on the target cell and bind to tumor cell coated with antibodies that result in tumor cell death (Lanier, 2005).

2.3.2.2 CD2

CD2 is 50kD glycoprotein and is the member of Ig superfamily. CD2 express on the surface of NK cells and T-cells. CD2 is known to interact with different ligands like CD58, CD48, CD59 and CDL. Interaction between CD2 and CDL resulted in spontaneous cytotoxicity against prototypic cell line K562 (Warren et al., 1996). Moreover, transfection of NK resistant cell line P815 with CD2 ligand, CD58 rendered P815 cells susceptible to NK mediated lysis (Lanier, 1997). Recently, it was investigated that binding of CD2 with CD58 resulted in activation of NK cell and increased production of cytokines by NK cells and generated anti-viral response against CMV infection (Rolle et al, 2016). Further, co-stimulation of NK cells with CD2 showed enhanced antibody dependent response of NK cells in the absence against HCMV infection in NKG2C-deficient pateints (Liu et al., 2016).

2.3.2.3 CD94/NKG2 heterodimer receptors

Another C-type lectin family of receptors is the NKG2 family expressed as disulfide-bonded heterodimer form with CD94. The NKG2 family includes four genes, namely

NKG2A, NKG2C, NKG2E and NKG2D present on the chromosome 12 in human NK gene complex. CD94 lacks cytoplasmic domain and thus do not play role in transducing signal. There is evidence that NKG2 glycoproteins solely unable to express on the cell membrane and they require to bind CD94 glycoprotein. Thus, CD94 may help in transport of NKG2 receptors to the cell surface (Pegram, 2011). Like Ly49 receptors in mice, human NKG2/CD94 recognize MHC class-I molecules. NKG2A/CD94 designated as the first HLA class-I specific inhibitory receptor expressed by NK cells (Mingari et al., 1998). NKG2A/CD94 receptor complex binds to the non- classical MHC molecule (HLA-G/ HLA-E) expressed on the surface of the target cell or healthy cell. Thus, play important role in prevention of inappropriate NK cell activation. It was seen that NK inhibitory receptor and non-classical MHC interaction resulted in better inhibition of NK cell cytotoxicity than with classical MHC molecule. It had been reported that extravillous trophoblasts (EVT) cells lack HLA-A/B expression and expressed an unusual combination of classical MHC class I molecule HLA-C and non-classical class I molecules HLA-E and HLA-G, which protected foetal tissues from maternal immune system (Gonen-Gross et al., 2010). Moreover, non-small-cell lung cancer (NSCLC) tumor showed higher expression of non-classical HLA-E and HLA-G and interaction of NKG2A/CD94 inhibitory receptor with non-classical HLA protected tumors from NK cytotoxicity and resulted into tumor progression (Platanova et al 2011). In mice, NKG2A/CD94 receptor recognizes $Qa1^b$.

NKG2C/CD94 and NKG2E/CD94 heterodimers have been shown to associate with DAP-12, an adaptor molecule that participates in activation of NK cell function. Thus, both NKG2C/CD94 and NKG2E/CD94 are considered as NK cell activating receptors.

2.3.2.4 NKG2D receptor

Natural Killer group 2D (NKG2D), C-type lectin surface activating receptor belongs to NKG2 family. Unlike other members of the family NKG2D does not couple with CD94 and expressed as homodimer. Activation of NK cell via NKG2D is distinct from other activating receptors as NKG2D requires association adaptor protein termed DAP-10. DAP-10 possess a negatively charged residue in the transmembrane portion and a tyrosine (YINM) motif, which is different from ITAM in the cytoplasmic tail, that, upon tyrosine phosphorylation, triggers activation of signalling pathway leads to granule polarization and degranulation (Moretta et al., 2001). It was reported that one NKG2D homodimer associated with four DAP-10 chains (Garrity et al., 2005) and there were two isoforms of the NKG2D molecule in mice strains. The longer isoform (NKG2D-L), which associated with DAP-10, stimulated a strong cytotoxic response and a shorter isoform (NKG2D-S), which recruited either DAP-10 or DAP-12 is results in both cytokine secretion and cytotoxicity (Gilfillan et al., 2002, Lanier, 2008). While Human NK cells only expressed the long isoform of NKG2D, which recruited DAP-10 and mediate both cytotoxic and cytokine-mediated response (Figure 2.3) (Billadeau et al., 2003). NKG2D recognize retinoic acid early transcript-1 (Rae-1) molecules, also murine ULBP-like transcript-1, (MULTI-1) and histocompatibility 60 (H60) molecules expressed by stressed or tumor transformed cells in mice (Cerwenka et al., 2000, Carayannopoulos et al., 2002, Mistry et al., 2007). In humans, ligands for NKG2D include the stress-inducible MHC class I chain-related gene (MIC), MIC-A and MIC-B, and UL-16 binding proteins (ULBP), ULBP1, ULBP2, ULBP3 and ULBP4. MICA and MICB are known to express by the tumors of epithelial tissues including breast, ovary, colon, kidney, and lung carcinomas and interaction between NKG2D and MICA/B leads to NK cell activation (Moretta et al., 2001).

2.3.3 Natural cytotoxicity receptors

Natural cytotoxicity receptors (NCRs) are activating receptors and are the members of the Ig-superfamily in humans. Mainly, NCRs include NKp46, NKp44, NKp80 and NKp30 receptors expressed on the surface of NK cells and are strong mediators of NK cell cytotoxicity on interaction with their cognate ligands. NCRs are reported to bind broad range of ligands including viral hemagglutinin, bacteria, other parasite derived and even self-derived ligands but there is limited information about the cognate ligands for NCRs.

2.3.3.1 NKp46

NKp46 molecules are expressed on activated as well as resting NK cells (Moretta, 2001). The NKp46 gene is located on the human chromosome 19. NKp46 associates with CD3ζ and the γ-chain of FcεRI that activate NK cells via their ITAM motif. NKp46 contains two extracellular domain, a transmembrane domain with positively charged arginine and short cytoplasmic tail. NKp46 are known to have potential role in lysis of tumors. It was observed that $NKp46^{bright}$ clones showed strong cytolytic activity against tumor targets (Sivori et al., 1999). Moreover, Knockout of NCR 1 gene, encoding NKp46 resulted in impairment of NK cell function and progression of lymphoma cells (Halftech et al., 2009). The ligand identified so far for NKp46 is viral hemagglutinins (HA) (Arnon et al., 2004). It was reported that pancreatic β cells expressed unknown ligand for NKp46 responsible for development of type I diabetes in both mice and humans (Gur et al., 2011). In addition, hepatic stellate cells shown to express unknown ligands for NKp46 and lead to activation of NK cells which resulted in inhibition of liver fibrosis (Gur et al., 2011).

2.3.3.2 NKp30

NKp30 is 30 kDa protein encoded by NKp30 gene present on the class III region of the human MHC. NKp30 is expressed by all NK cells including immature NK cells derived in vitro from $CD34^+$ precursors. The NKp30 molecule is composed of one extracellular immunoglobulin domain with positively charged arginine residue in their transmembrane domain (Joyce et al., 2011). Reported cognate ligand for NKp30 is B7-H6 (Li et al., 2011). Although, NKp30 was reported to bind HLA-B-associated transcript 3 (BAT3) (Pogge von Strandmann et al., 2007) but the structural relevance of NKp30 interaction with BAT3 has remain elusive. Initially there were reports on binding of NKp30 with heparan sulphates (HS) but later studies demonstrated that HS were not ligands for NKp30 and there was no influence on the NK cell activation after complete removal of HS (Warren et al., 2005).

2.3.3.3 NKp44

NKp44 is 44kDa glycoprotein and expressed only on activated NK cells. Thus represent as specific marker for activated NK cells. The NKp44 gene is encoded in the class III region of the MHC locus on chromosome 6 in humans. The protein of NKp44 is composed of a single transmembrane domain and a short cytoplasmic tail that contains a sequence (EILYHTVA), corresponds to an immuno-receptor tyrosine-based inhibitory motif (ITIM). The ITIM motif reported to be functional only upon interaction with certain ligand like proliferating cell nuclear antigen (PCNA) on tumor cells and inhibited NK cell mediated lysis of tumors (Rosental et al., 2011). Unlike, NKp30 and NKp46, NKp44 contain amino acid lysine in their transmembrane domain which interacts with ITAM-containing adaptor molecules, DAP-12 and transduce activating signal (Kruse et al., 2014). Like NKp46, NKp44 has been reported to bind HA and

Hemagglutinin neuraminidases (HN) of different viruses (Mandelboim et al., 2001, Chisholm et al., 2006, Jarahian et al., 2009). In line with these studies, NKp44 also known to bind certain unknown bacterial derived ligands but their clinical relevance still need to be addressed (Esin et al., 2008, Esin et al., 2013).

2.3.3.4 DNAM-1 receptor

DNAX accessory molecule-1, DNAM-1 receptor (CD226) is transmembrane glycoprotein and is co-stimulatory activating receptor belong to Ig-superfamily. DNAM-1 binds to CD155, Polio virus receptor (PVR or Necl-5) and CD112 (Nectin-2). Upregulation of DNAM-1 ligand, CD155 induce increased susceptibility of tumor cells to NK cell attack in multiple myeloma (Soriani et al., 2009). It was seen that DNAM-1 was involved in cytolysis of tumors that lack ligand expression for NK activating receptor and therefore broadened the spectrum of tumors to be recognized by the NK cells (Gilfillan et al., 2008). Furthermore, DNAM-1 had found to be associated with an adhesion molecule, lymphocyte function-associated antigen-1 (LFA-1) and had shown crucial role in interaction between target and NK cell; therefore played key role in tumor recognition (Barao et al., 2003). In addition, DNAM-1 had shown to be involved in T-cells proliferation and co-stimulation (Shibuya et al., 2003).

2.3.3.5 PIL receptor

The paired Ig-like 2 receptor (PILβ) is a type 1 glycoprotein molecule. These receptors are comes under category of activating receptors as they are known to be associated with DAP-12. Whereas PILα subunit contains ITIM sequence in its cytoplasmic domain and involved in inhibition of NK cell function (Pegram et al., 2011). The ligand for these receptors is PILR-L (CD99). These receptors recognized carbohydrate chains as opposed to proteins on the surface of target cell (Wang et al., 2008).

2.3.3.6 2B4 receptor

The 2B4 receptor (CD244) is considered as multi-functional receptor binds CD48 molecules expressed on all hematopoietic cells. There are two isoforms of 2B4 receptors, one isoform signal for activation and other isoform for inhibition. In humans, only one isoform is expressed that is involved in activation of NK cell function. In mice strains, both the isoforms are expressed with different cytoplasmic domains that induce either activation or inhibition of NK cell function (Mathew et al., 2009, Vaidya et al., 2005).

2.3.3.7 KLRG1 receptor

Killer cell lectin-like receptor G1 (KLRG1) is an inhibitory receptor that is reported to bind with classical cadherins, (E-, N- and R-cadherins). These cadherin ligands are expressed in healthy, solid tissues and thus play role in protecting healthy tissues from NK mediated lysis. It was found that ligation of KLRG1 with E-cadherin expressing targets resulted in inhibition of NK mediated lysis of the target cell. Thus, spared the cadherins expressing healthy cells and therefore KLRG1 played role in the 'missing self '-mediated activation of NK cells (Ito et al., 2006).

2.3.3.8 KIR family receptors

Killer immunoglobulin receptors (KIR) belong to Ig superfamily and KIR genes encode glycoproteins located on the human chromosome 19. There are two sub-families of KIR based on the number of Ig-like domains in the extracellular regions. The KIR3D sub-family possess three Ig-like domains and KIR2D sub-family contain two Ig-like domains (Lanier, 1998). There exists heterogeneity in the length of the cytoplasmic domains. KIRs with long cytoplasmic domains designated as KIR2DL and KIR3DL,

comprises two ITIM sequences that participate in inhibitory function of these molecules. In contrast, KIRs containing short cytoplasmic domain identified as KIR2DS and KIR3DS molecules that lack ITIM and rather contain a charged amino acid in the transmembrane domain containing ITAM that are responsible for activation of NK cell function. KIRs exists in monomeric form and crystal structures of five members of KIR family are known, KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS2 and KIR2DS4 (Joyce and Sun, 2011). KIRs specifically bind to classical MHC class-I molecules and their cognate ligands are shown in Table 2.1. Inhibitory KIRs are known to bind various HLA-A, HLA-B and HLA-C alleles (Rajagopalan and Long, 2012). However the ligands for most activating KIRs are not known. Killer cell immunoglobulin-like receptors (KIR) regulate natural killer cell response against infection and malignancy. Individual differences in NK-cell interactions and respond to infections are dependent on the combinations of variable killer immunoglobulin-like receptor (KIR) and HLA class-I gene products. Interaction between KIR and HLA- B allele, HLA-B*40 and HLA-B*08 was associated with the risk of breast cancer (Augusto, 2016). Also, unusual receptor-ligand interactions resulted in altered NK-cell-mediated immunity. It was proposed that altered KIR-HLA-C interaction resulted in over-activation of activating genes that contributed to disorders such as recurrent miscarriage (Faridi and Aggarwal, 2010). Studies had been carried out to identify KIR genes distribution and their association with tuberculosis. It was also reported that inhibitory genes KIR3DL1, KIR2DL3 and activating genes KIR2DS1, KIR2DS5 involved in susceptibility towards *Mycobacterium tuberculosis* either individually or in haplotype combinations (Pydi et al 2013).

Table 2.1: NK cell receptors in human and mice model

Receptor Family	Ligands	Species
Ly49 family		
Activating receptors		
Ly49D	H-2Dd	Mice
Ly49H	m157	
Inhibitory receptors		
Ly49A	$H\text{-}2D^{d,k,p}$	Mice
Ly49C	$H\text{-}2D^{b,d,k}$ $H\text{-}2K^{b,d}$	
Ly49I	$H\text{-}2K/D^{b,d,s,q,v}$	
KIR family		
Activating receptors		
KIR2DS1	HLA-C2	Human
KIR2DS2	HLA-C1	
KIR2DS3	unknown	
KIR2DS4	unknown	
KIR2DS5	unknown	
KIR3DS1	HLA-Bw4	
KIR2DL4	HLA-G	
Inhibitory receptors		
KIR2DL1	HLA-Cw2, HLA-Cw4, HLA-Cw5	Human
KIR2DL2	HLA-Cw1, HLA-Cw3, HLA-Cw7	
KIR3DL1	HLA-Bw4	
KIR3DL2	HLA-A3, -A11	
NKG2/CD94 family		
Activating receptors		
NKG2C/CD94	Human- HLA-E/G	Mice/ Human
NKG2E/CD94	Mice- Qa1b	
Inhibitory receptor		
NKG2A/CD94	Human:MIC-A/-B, ULBP1/2/3/4	
NKG2D	Mouse: RAE-1, H60, MULTI-1	
NCR family		
Activating receptors		
NKp30	BAT-3, B7-H6	Mice/ Human
NKp44	Viral HA	
NKp46	Viral HA, HSPG	
Others		
Activating receptors		
2B4	CD48	Human/Mice
NKR-P1		Mice
DNAM-1	PVR, CD122	Human/Mice
PILR	CD99	Mice
Inhibitory receptors		
KLRG1	Cadherins	Mice

2.4 NK cell signalling pathway

NK cell inhibitory and activating receptors utilize opposing signalling motifs to stimulate or inhibit activation. NK cell activation involves the interaction of activating receptors with their ligands on the target cell surface, which initiates the intracellular signalling pathways. This results in polarization and release of cytotoxic granules towards the target.

2.4.1 NK activation

NK cell activation is mediated by conserved sequences present in the cytoplasmic domain of NK receptors or their associated adaptor proteins. NK cells activating receptors are associated with adaptor proteins which express three immunoreceptor tyrosine-based activation motif (ITAM) containing adaptor proteins FcεRIγ, CD3ζ and DAP12 also called KARAP. ITAM consists of prototype sequence, Asp/Glu-x-x-Tyr-x-x-Leu/Ile x6−8 Tyr xx Leu/Ile, where x is any amino acid. On interaction of NK cell activating receptor with their ligand on target cell, tyrosine residue in ITAM sequence gets phosphorylated leading to recruitment of the Src homology 2 (SH2) domain containing kinases (Syk or ZAP70) and the transmembrane adaptor proteins LAT and LAT2 that in turn activate downstream effector molecules, including phosphoinositide 3-kinase (PI3K), the phospholipases PLCγ1 and PLCγ2, the guanosine triphosphate-guanosine diphosphate exchange (Grb) factors Vav2 and Vav3 and the MAP kinase extracellular-regulated kinase (ERK). These events cause a Ca^{2+} influx, degranulation, and transcription of cytokine and chemokine genes (Lanier, 2005, Long et al., 2013). Activating receptors CD16 (FcγRIII), is associated with the ITAM-containing FcεRI γ chain and T-cell receptor (TCR) ζ chain. NKp46 and NKp30 are associated with the

TCR ζ chain and NKp44, KIR2DS, and CD94/NKG2C signal through ITAM-containing DAP-12 molecule (Bryceson et al., 2006). While NKG2D adopt an alternate signalling mechanism, NKG2D associates with DAP-12 and DAP-10 in mice whereas in human NKG2D signal via DAP-10 adapter protein, which is not associated with ITAM. DAP-10 possesses a cytoplasmic YINM motif and involves direct recruitment of PI3K and the Grb2-Vav1 complex (Figure 2.1) (Long et al., 2013). This DAP10-dependent pathway alone triggered NK cell activation and cytotoxicity against target cell but was incapable to stimulate cytokine production (Lanier, 2009).

Other receptors like 2B4 tranduce signal through their own cytoplasmic tail that contains an immunoreceptor tyrosine-based switch motif, which recruited SH2 domain containing adapter proteins SAP, resulted in activation of NK cell (Bryceson et al., 2009).

2.4.2 NK inhibition

Human NK cell inhibitory receptors mainly include KIRs with long cytoplasmic domains (KIR2DL1, KIR2DL2, KIR2DL3) and NKG2A/CD94 molecules which are specific for different HLA molecules (Beldi-Ferchiou and Caillot-Zucman, 2017).

2.4.2.1 KIRs associated NK inhibition

KIRs bind specifically to classical MHC class molecules, HLA-C and HLA-B and possess two immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domains (Rajalingam, 2016). ITIM consists of consensus sequence Ile/Val/Leu/Ser-x-Tyr-x-x-Leu/Val, where x is any amino acid. After phosphorylation of tyrosine residue in ITIM sequence, Src homology 2 domain containing phosphatases (SHP-1 and SHP-2) are recruited via ITIM. SHP convert phosphatidylinositol-3,4,5-

trisphosphate (PI-3,4,5-P3) to phosphatidylinositol-3, 4-bisphosphate (PI-3,4-P2) and dephosphorylate downstream effector molecules vav1 and vav 2 (Figure 2.1). These events inhibit activation of Rac-1 and result in phosphorylation of the adapter Crk by Abl, leading to disassembly of the Cbl–Crk–C3G complex. This process inhibits activation of events responsible for NK cell activation and cytotoxicity (Watzl and Long, 2010). It was observed that engagement of inhibitory KIRs with classical HLA resulted in unsuccessful phosphorylation of ZAP-70 and LAT adaptor protein, thereby blocking NK cell cytotoxicity (Held, 2013).

2.4.2.2 NKG2A/CD94 associated NK inhibition

NKG2A exist in heterodimeric form with CD94 and recognizes non-classical MHC class I molecules HLA-E and HLA-G (Gaynor and Colucci, 2017). Like KIRs, NKG2A contain two conserved ITIMs in their cytoplasmic domains, CD94 molecule does not contain cytoplasmic tail and thus lacks signal transduction capacity. Binding of HLA-E/G to NKG2A/CD94 complex results in activation of Src family kinases, leading to phosphorylation of tyrosine residues and enable recruitment of the tyrosine phosphatases SHP-1 and SHP-2 that causes de-phosphorylation of protein substrates of tyrosine kinase linked to activating NK cell receptors (Long et al., 2013).

It is believed that ITIM mediated signalling interfere with activation signal by de-phosphorylation. This de-phosphorylation turn NK cell "off" and block cascade initiated by activation receptor (Held, 2013). It was reported that β-arrestin 2 mediated recruitment of the tyrosine phosphatases SHP-1 and SHP-2 to inhibitory receptor and facilitated inhibitory signalling events (Watzl and Long, 2010). Further, was found that

interaction of inhibitory receptor with their ligand resulted in phosphorylation of tyrosine adaptor, crk accompanied by de-phosphorylation of vav-1 and prevented NK cytotoxicity (Liu et al., 2012).

Although both KIRs and NKG2A/CD94 belong to different families and have structural differences (Figure 2.2) but all of these inhibitory receptors utilize common strategy to inhibit NK cell activation (Long et al., 2013).

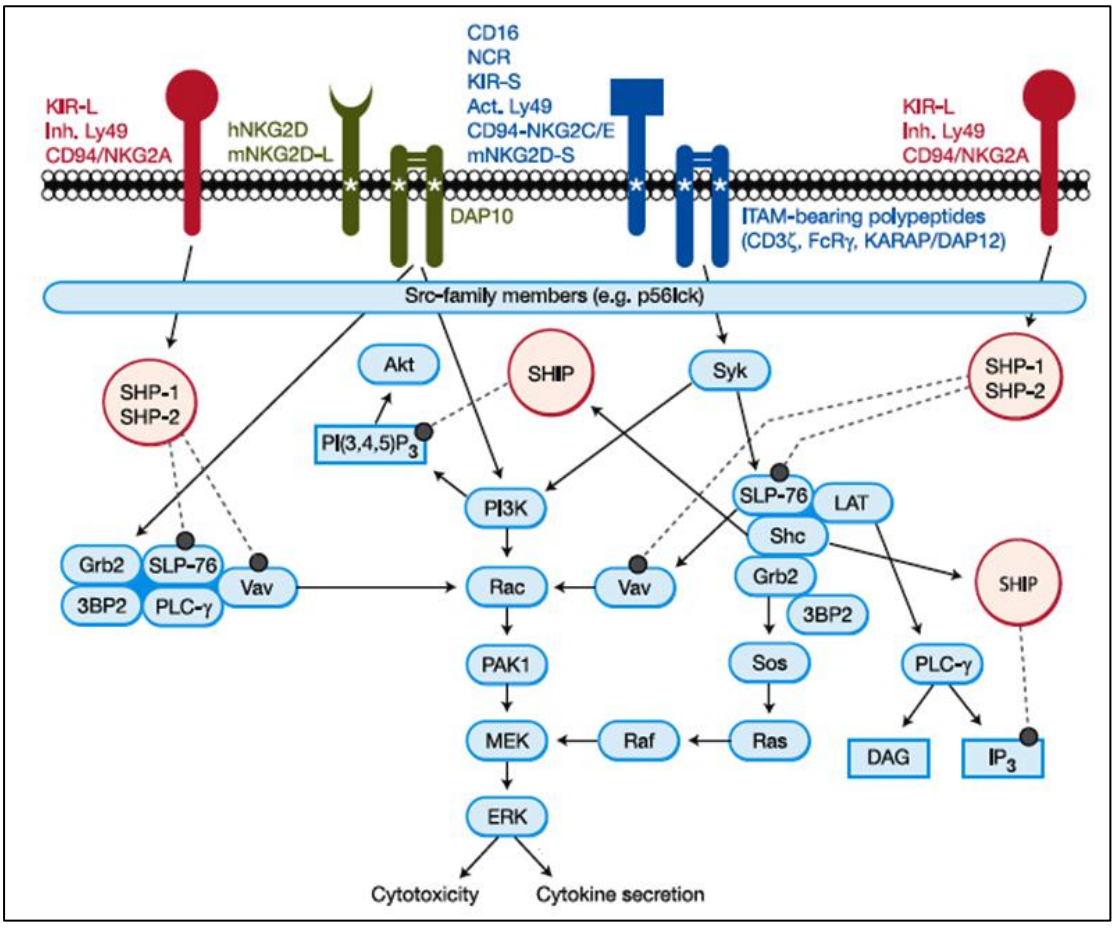

Figure 2.1: NK cell Signalling (Vivier et al., 2004)

Activating receptors, associated with ITAM-bearing molecules are represented in blue and with DAP10 in green, inhibitory MHC class I–specific receptors are shown in red. 'h' stands for human while 'm' represent mice. ***** indicate the presence of a transmembrane charged amino acid. KIR-S are activating killer cell Ig-like receptors with a short intracytoplasmic domain and no intrinsic signalling properties, whereas KIR-L are inhibitory receptors with an intracytoplasmic ITIM.

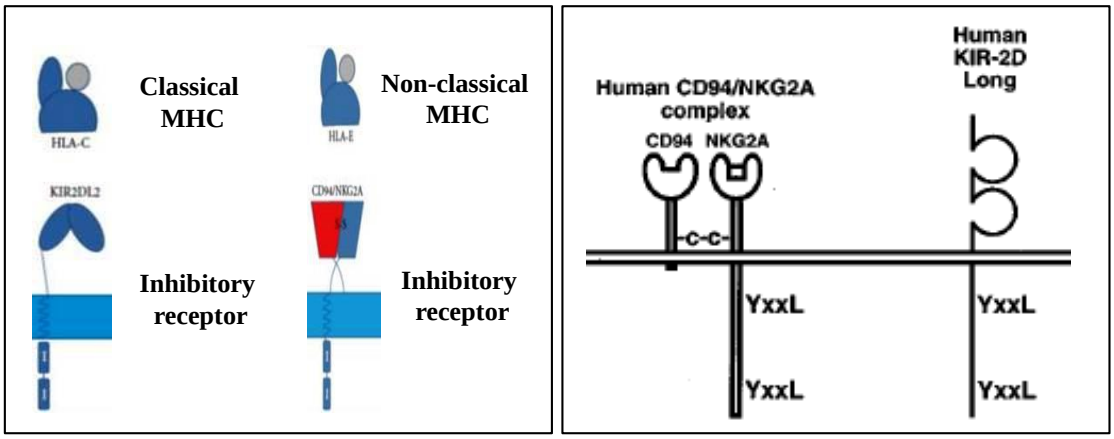

Figure 2.2: Structures of NKG2A/CD94 and KIR inhibitory receptors. KIRs bind to classical MHC (HLA-C) and NKG2A/CD94 bind to non-classical MHC (HLA-E) NKG2A receptors expressed as disulphide-bonded heterodimer form with CD94, and inhibitory KIR containing long cytoplasmic tail. Both receptors contain two ITIM sequences in their cytoplasmic domain while CD94 lack cytoplasmic tail (Adapted from Joyce and Sun, 2011, Lanier, 1998)

2.5 NK cell response

2.5.1 NK cell recognition

NK cell response is regulated by the balance between the expression of activating and inhibitory receptors. The outcome of immune response is determined by the extent of strength of activating/inhibitory signals. Different NK cell inhibitory and activating receptors are shown in Figure 2.3. The NK cells preferentially kill target cells that lack surface expression of MHC class I molecules and upon engagement of ligand for activating receptor by the infected or tumor transformed cell. For example, expression of MHC class I chain-related gene MICA/MICB, ligand for activation receptor NKG2D by the virus infected cell lead to activation of NK cell function (Ghadially et al., 2017). On the other hand, inhibitory NK receptors recognize major histocompatibility (MHC) class I molecules to modulate the immune response by inhibiting the NK cell cytotoxicity and spare the healthy cell. Thus, presence of MHC class-I molecule on the

cell prevents NK cell activation and provide the basis of "missing-self" whereas cell transformation lead to down-modulation of MHC-I molecules resulting in NK cell recognition and tumor cell lysis (Figure 2.4) (Langers et al., 2012). Normal self-cells usually expressed MHC-I molecules and were protected from NK cell mediated cytotoxicity. For example, although decidual NK cells (dNK) cells were in close contact with fetal-derived trophoblasts at the fetal-maternal interface yet they were not cytotoxic against them (Wallace et al., 2013). It was observed that activated peripheral blood (pNK) cells lysed trophoblast cell lines JAR and JEG3 but decidual NK (dNK cells) did not show cytotoxicity against trophoblast cells (Vacca et al., 2008).

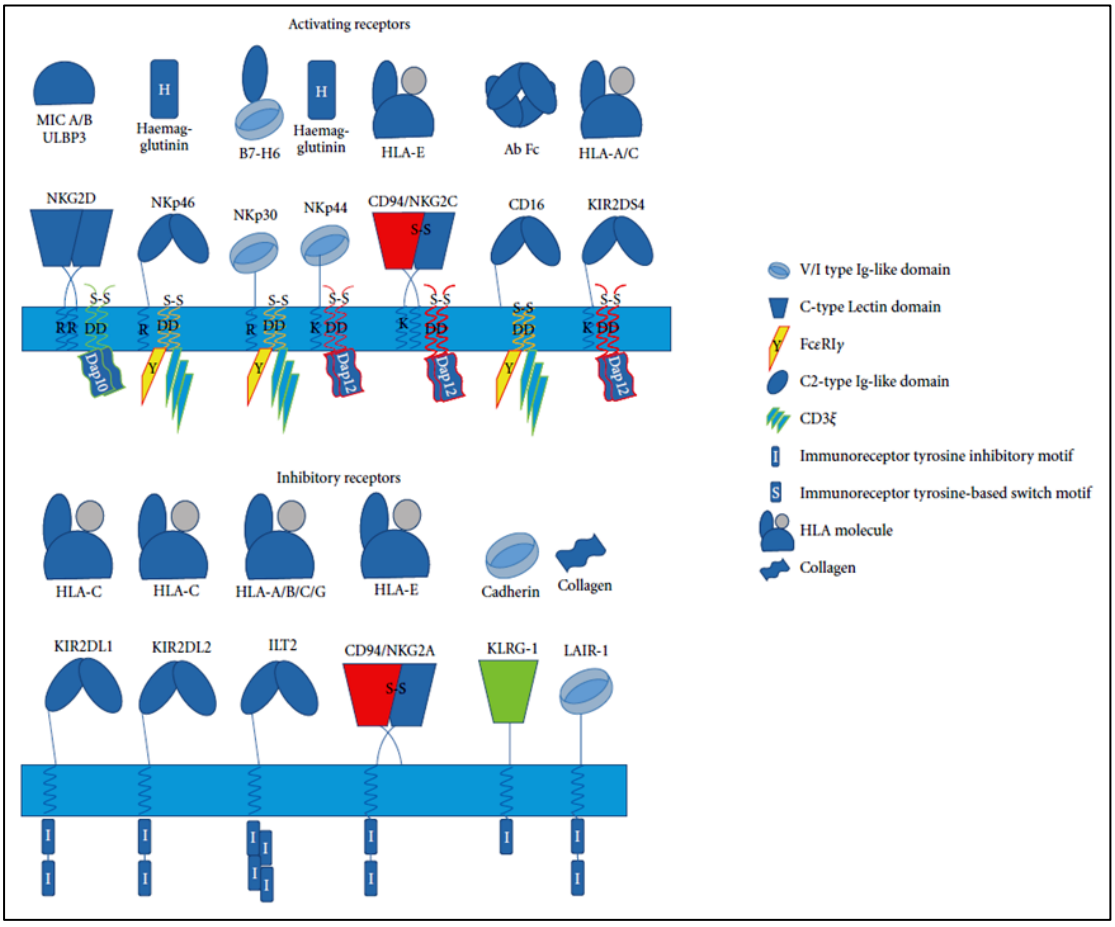

Figure 2.3: NK cell activating and inhibitory receptors (Sun and Joyce, 2011)

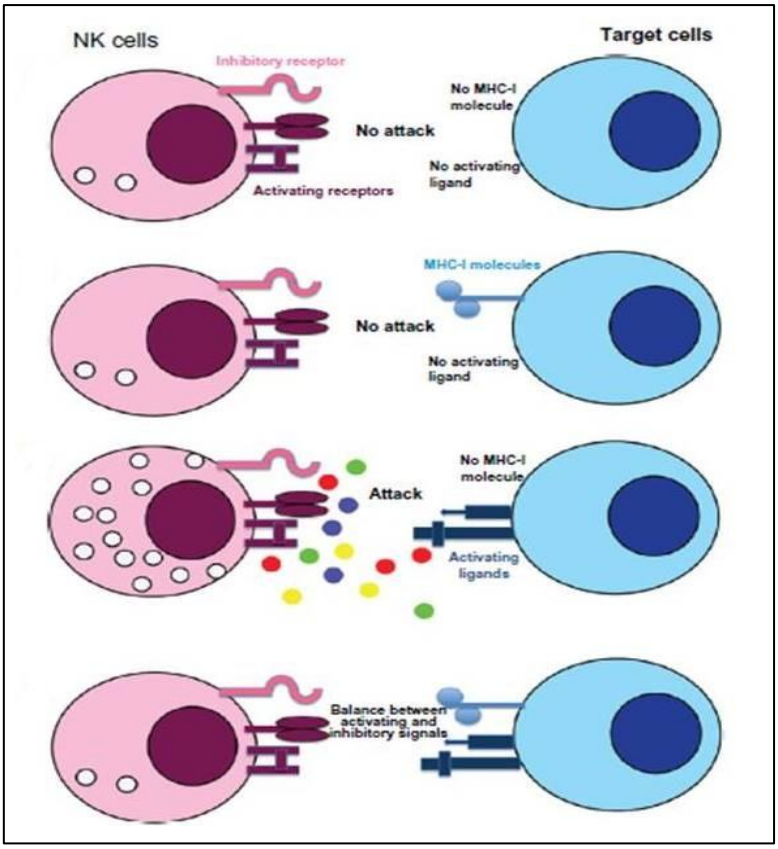

Figure 2.4: 'Missing-self' mechanism of NK cells (Langers et al., 2012)

2.5.2 NK cell mediated cytolysis

NK cells may utilize two basic mechanisms for target cell lysis:

1. Secretory pathway or Cytolytic granule-mediated cytotoxicity- Upon target cell interaction NK cells release pore forming protein, perforin and granzymes (including granzyme B, GrB), a family of serine proteinases, into the extracellular space causing the lysis of target cell. The process involves granule polarization and exocytosis of granules containing perforin and granzymes B. Exocytosis of granules causes homo-polymerisation of perforin in the target cell membrane in a calcium-dependent manner that facilitate the release of granzyme into the target cell cytosol, which in-turn triggers the

caspases dependent cell death cascade. Eventually, causes proteolysis of key cytotoxic and nuclear substrates, thereby results in death of the target cell (Carotta, 2016).

2. Non-secretory pathway or TNF superfamily-mediated cell death- Studies on perforin and granzyme deficient animal models revealed that the existence of another Ca^{2+} independent pathway to induce target cell death (Zamai et al., 1998). It involved the interaction of CD95L molecule (FasL/TNF) on effector cell with CD95, apoptotsis-inducer (Fas/TNFR) expressed by the target cell. Fas belong to tumor necrosis factor (TNF) superfamily that incorporates a conserved intracytoplasmic 'death-domain' that participates in transducing apoptotic signal. Presently, five different death receptors are known, TNF receptor-1, CD95 (Fas/APO-1), TNF-receptor-related apoptosis-mediated protein (TRAMP) and TNF-related apoptosis-inducing ligand (TRAIL) receptor-1 and -2. Crosslinking of receptor ligand complex results in the recruitment of an adaptor protein, Fas-associated death domain (FADD) to the death domain, which interact with FADD-like interleukin-1 b-converting enzyme (FLICE). This interaction leads to the assembly of the receptor associated death-inducing signalling complex (DISC). The DISC-bound FLICE induces cytoplasmic and nuclear condensation and DNA fragmentation leading to apoptotic cell death (Chester et al., 2015, Zhu et al., 2016).

It was observed that unlike T cells, freshly isolated NK cells expressed FasL mRNA and exhibited cytotoxicity against transfectants expressing Fas antigens. It was also observed that NK cells failed to lyse the targets with mutant Fas antigens (Arase et

al., 1995). These findings suggested that FasL/ Fas interaction mediate NK cell cytotoxicity against their susceptible targets.

2.5.2.1 Cell death by cytokine secretion

NK cells has capability to mediate target cell death indirectly by secreting pro-inflammatory cytokines mainly type II interferon, IFN-γ that play critical role to eliminate microbial infection and tumors (Green et al., 2017). In tumor microenvironment, IFN-γ secreted by NK cells stimulated CD4+ T cells to deviate toward Th1 subset and initiated the activation of macrophages and CD8+ T cells that targeted tumor cells and induced tumor cytolysis (Melero et al., 2014). Other multifunctional cytokine is TNF-α that involve necrosis of target by B cell expansion and also differentiation of monocyte and macrophage (Balkwill, 2009, Arango-Duque and Descoteaus, 2014). Together, TNF-α and IFN-γ triggers innate as well as adaptive immune response against the target (Wang et al., 2012).

2.5.2.2 Antibody-dependent cell-mediated cytotoxicity

Antibody-dependent cell-mediated cytotoxicity (ADCC) is the process of killing of an antibody-coated target cell by NK cell by the release of the cytotoxic granules. NK cells express Fc receptors on their surface, FcγRIIIa and/or FcγRIIc (CD16). Binding of Fc receptor to the Fc portion of antibodies transmitted the activating signals to the NK cells, leading to target lysis without priming and secreted cytokines such as IFN-γ. IFN-γ accelerated maturation of antigen presenting dendrtric cells and other adaptive immune cells, thus resulted in producing memory response for the target cell (Wang et al., 2015). The efficiency of ADCC was thought to be dependent on the FcγRIII polymorphism displayed by the NK cell and it was suggested that FcγRIIIa binds with

high affinity to IgG1. Cetuximab treatment to patients with FcγRIIIa polymorphism had shown enhanced clinical benefits (Rodríguez et al., 2012). Similarly, FcγRIIIa polymorphism was shown to associated with increased clinical efficacy of Cetuximab drug in cancer patients (Sclafani et al., 2014).

2.6 Tumor microenvironment

Malignant tumors are aberrantly proliferating cells due to mutation in genes associated with important biological pathways such as proliferation, apoptosis, and angiogenesis (Sever and Brugge, 2015). In comparison to healthy cells, tumor transformed cells over-expressed stress-induced molecules, tumor specific antigens or may downregulated expression of MHC-I molecules (Fang et al., 2017). Interaction between tumors and associated cellular and molecular components creates a tumor microenvironment. The tumor microenvironment consists of malignant cells, stromal tissues (cancer associated fibroblasts, blood), immune cells and myeloid population that include dendritic cells, macrophages and myeloid derived suppressor cells (Cui and Guo, 2016). Immune cells present in tumor microenvironment include both arms of immune system i.e adaptive and innate (Gajewski et al., 2014). As lymphocytes represent the substantial component of immune infiltrates, they are usually called as Tumor infiltrating lymphocytes (TILs). TILs mainly consist of T cells, B cells and NK cells (Whiteside, 2008). Presence of tumor infiltrating lymphocytes within tumor microenvironment has been considered as symbolic of ongoing immune response against developing tumors. Each cell in the tumor microenvironment has the ability to acclimatize in the hostile microenvironment and produce a number of immunomodulatory factors such as cytokines, chemokines, and growth factors (Bussard et al., 2016). Thus, successive changes occur on

interaction of immune cells with tumor cells at the tumor site that decides the fate of the developing tumor.

2.6.1 Tumor microenvironment in immune evasion

Primarily, immune system has three major roles in prevention of tumor growth. First, elimination and suppression of virus induced tumors. Second, inhibit formation of inflammatory environment conducive to tumorigenesis by prompt elimination of pathogens. Third, identification and elimination of tumor specific antigens expressed by tumors and cellular stress induced molecules before the onset of their harmful effect, referred to as immunosurveillance. (Beatty and Gladney, 2015). Despite, potential role of immune system in controlling tumor progression, the tumor has evolved to escape immunosurveillance, thus, resulted in tumor escape from host immune system (Wang et al., 2015). Therefore, process of tumor progression includes immune-editing that divides into three phases, elimination, equilibrium and escape. Elimination involves the identification and elimination of tumor cells that has developed as a consequence of failed intrinsic tumor suppression mechanism. In case of partial elimination of tumor, a temporary state of equilibrium develops in which tumor cells either remain dormant or continue to evolve and induce modulation of tumor-specific or stress-induced antigens. In this phase tumor usually continue to remain in dormant state until microenvironment become favourable for its growth. These modulations create an environment where immune system fails to control tumor progression and results in selection of tumor cells that are able to resist or suppress the cytotoxicity of immune cells, leading to escape phase (Chew et al., 2012b).

2.6.1.1 Cells in tumor microenvironment

Cellular components of immune system comprised of TILs and tumor associated myeloid populations of dendritic cells, macrophages and myeloid-derived suppressor cells (MDSC) (Figure 2.5) (Bussard et al., 2016). Tumor-infiltrating lymphocytes (TILs) were associated with the improved survival in different types of cancers, suggesting their role in anti-tumor immunity. Many studies had documented that immune system played a critical role in immunosurveillance and immunodeficient mice with defective T-lymphocytes, NK cells and impaired IFN-γ, perforin activity develop tumor spontaneously (Smyth et al., 2000, Street et al., 2001, Van Den Broek et al., 1996). Traditionally, T-lymphocytes had been considered the major component of TILs (Hadrup et al., 2013).

2.6.1.1.1 T-lymphocytes

Cytotoxic T lymphocytes are the major effector cells that recognize tumor specific antigens associated with MHC-I molecules and produce perforin and cytokines that lead to killing of target tumor cells (Harimoto et al., 2013). Numerous studies had documented the role of infiltrating T-lymphocytes in good prognosis of several types of cancer (de Reuter et al., 2017, Hannani et al., 2012). High numbers of T cells infiltrated tumor microenvironment and $CD8^+$ memory T cells and $CD4^+$ helper T-cells (Th1) secreted IL-2 and IFN-γ within tumor stroma, thus favoured tumor regression (Fridman et al., 2012). T-cell based immunotherapy had demonstrated most efficacious results in clinical trial (Wang et al., 2014). Although various T-cell based therapies utilizing genetically engineered T cells, and immune checkpoint inhibitor antibodies, adoptive cell transfer (ACT) of tumor-infiltrating T lymphocytes are the promising approach for cancer treatment and have yielded clinical benefits but all the modalities are in their

infancy (Houot et al., 2015). These modalities require further optimization with respect to extent of possible antitumor effects and toxicities for better clinical outcomes (Wang et al., 2016).

2.6.1.1.2 B-lymphocytes

B cells, on antigen specific activation produce antibody producing plasma cells and mediate humoral anti-tumor activity. Nielsen et al., 2012 found that tumor-infiltrating $CD20^+$ B cells in conjunction with T cells were associated with better prognosis of ovarian cancer than T cells alone. Also, tumor infiltrating B cells played important role in control of tumor growth in Hepatocellular carcinoma (HCC) patients via activation of T cells that resulted in enhanced expression of IFN-γ and granzyme B (Garnelo et al., 2017). Similar findings of other groups on cutaneous melanoma (Ladanyi et al., 2011), colorectal cancer (Maletzki et al., 2012), breast cancer (Yamaguchi et al., 2012) had shown the potential role of high density tumor infiltrating B cells in anti-tumor response. Thus, cancer therapy using TILs had provided consistently favourable results in anti-tumor response.

2.6.1.1.3 NK cells

NK cells are more valuable and more versatile alternative to T-cell mediated cytotoxicity against tumors. Unlike T cells, NK cells does not require prior sensitization and they have ability to kill target cell that lack MHC-I expression. Tumor cells have tendency to down-regulate MHC-I molecules and this feature makes tumor cells susceptible to NK mediated lysis. On target cell interaction, they produce perforin, granzyme and cytokines and mediate anti-tumor immunity (Chester et al., 2015). NK cell frequency in tumors has found to be associated with clinical outcome of various

cancers. The density of activated NK cells was correlated with tumor load in lung cancer and it was observed that more number of NK cells infiltrate the tumor site and able to lyse the tumor cells (Larsen et al., 2014). Increased population of NK cells had also been associated with better prognosis of leukaemia (Krzywinska et al., 2015). Moreover, it had been seen that chemokines expressed in tumor microenvironment induce NK cells infiltration and leading to enhanced tumor death in HCC (Chew et al., 2012a). It had been reported that less infiltration density of NK cells in tumor lesion provokes tumor escape from NK cytotoxicity in HCC (Sun et al., 2015a). There was an evidence for low expression of protein responsible for recruitment of NK cells in tumor tissue may subject to poor prognosis of HCC (Lin et al., 2011). Also, reduced NK population and lower expression of activating receptors promotes incidence of HCC and Non-small cell lung carcinoma (NSCLC) (Al Omer et al., 2011, Jiang et al., 2012, Sun et al., 2015b). Thus, the above findings suggested that the defects in NK cells function and population was associated with advanced cancer stage.

2.6.1.1.4 Natural Killer T- lymphocytes

Another lymphocyte subset distinct from NK cells with respect to their origin, phenotype, antigen recognition, and certain effector functions are NKT cells. NKT cells share characteristics with NK and T cells; they represent both NK (CD16, CD57, CD161 or NK1.1) and T cell (CD3) specific markers on their surface (Gapin, 2010). NKT recognize glycolipid antigens presented by CD1d, non-classical MHC class I-like molecule and secreted large quantities of cytokines and chemokines. NKT population is divided into two subsets, Type I NKT or invariant NKT (iNKT) and Type II NKT. Type I NKT express single invariant TCRα chain (Vα14-Jα18 in mice, Vα24-Jα18 in human) paired with certain TCRβ chains (using Vβ8.2, 7 or Vβ2 in mice, Vβ11 in

human) (Gapin, 2010). Both murine and human Type I NKT cells recognize the α-galactosylceramide (α-GalCer), Type I NKT cell ligand. Type II NKT are more diverse, similar to conventional T cells and bind to lipid antigens such as Sulfatides, lysosulfatide, lysophospholipids (Liao et al., 2013). Tumor infiltrated NKT cells had resulted in favourable outcome in neuroblastoma through activation of tumor associated macrophages (Metelitsa et al., 2009, Song et al., 2009). Moreover, NKT targeted immunotherapy had shown safe, feasible and better clinical outcome in NSCLC, head and neck tumors (Fujii et al., 2013). Although, NKT cells had shown important role in the immunosurveillance of different cancers but NKT cell defects both in frequency and function being observed in a variety of solid tumor and hematologic cancer (Nair et al., 2017). However, inconsistent characterization of NKT-cell subsets and heterogeneity of patient populations studied make a generalized conclusion of the NKT cell role in human cancer challenging (Favreau et al., 2016). A deeper understanding of the factors determining the induction of NKT-cell subsets during tumor development is an important goal of further investigation.

2.6.1.1.5 Tumor associated macrophages (TAM)

TAM express CD206 marker on their surface and are present in abundant in tumor microenvironment. TAMs are known to originate from circulating monocyte and their recruitment to the tumor milieu is primarily mediated by tumor-derived chemotactic factor, CCL2. Initially, TAMs undergo polarization toward M1 response and induce anti-tumor response. However, as tumor progresses the interaction with tumor derived cytokines and skewed regulatory T cells, dendritic cells orient them to polarized type II/ M2 response. M2-polarized macrophages produce immunosuppressive factors such as IL-10 and TGF-β, colony stimulating factors contributing towards tumor progression

(Quatromoni and Eruslanov, 2012, Owen and Mohamadzadeh, 2013). Role of TAM and other tumor-associated myeloid cells, including DCs, and MDSCs in immune evasion was further explained by the expression of Adenosine A2A receptors (A2ARs), which inhibited anti-tumor T-lymphocytes and NK activity. Myeloid-selective A2AR deletion resulted in significantly increased population and activation of M1 macrophages, T-lymphocytes and NK in Lung infiltrates (Cekic et al., 2014).

2.6.1.1.6 Myeloid-derived suppressor cells (MDSCs)

MDSC ($CD34^{+}CD33^{+}CD13^{+}CD11b^{+}CD15^{-}$) in the tumor microenvironment are known to involve in supressing anti-tumor activity by down regulating the cytotoxic function of T lymphocytes and has been co-related with poor prognosis in cancer patients (Barnie et al., 2017, Chaudhary and Elkord, 2016). In tumor microenvironment, MDSC showed increased expression of iNOS and arginase which were responsible for enhanced production of RNS, ROS and depletion of extracellular L-Arg leading to supressed T-cell function (Monu and Frey, 2012). In addition, MDSCs were involved in impairing NK cell cytotoxicity by down-regulating expression of activating receptor NKG2D and inhibition of perforin and STAT activation of NK cells (Hasmim et al., 2015, Shvedova et al., 2015).

2.6.1.1.7 Regulatory T cells (Tregs)

Tregs are the subsets of immunosuppressive cells that participate in maintaining homeostasis and self-tolerance. They display Forkhead Box P3 (FoxP3) transcription factor and the IL-2 receptor α chain (CD25) ($CD4^{+}CD25^{bright}$ $Foxp3^{+}$ T cells) on their surface (Chaudhary and Elkord, 2016). Chemokines such as CCL17/22-CCR4, CCL5-CCR5, CCL28-CCR10 and CXCL9/10/11-CXCR3 secreted by tumor cells and immune

cells facilitate their infiltration in tumor microenvironment (Chaudhary and Elkord, 2016). Tregs played a critical role in tumor progression and producing tumorigenic microenvironment. It was reported that Tregs isolated from tumors from colorectal cancer (CRC) patients inhibited the T- cell mediated toxicity by secreting TGF-β and IL-10 and contributed to cancer progression (Scurr et al., 2014). In similar studies, in patients with HCC, pancreatic cancer and gastric cancer , Tregs were reported to limit T-cell anti-tumor effector function via secretion of TGF-β and IL-10 and PGE2 (Amedei et al., 2013, Yi et al., 2013, Yuan et al., 2010). Also, Tregs were found to involve in strong inhibition of NK cell function mediated by TGF-β in gastrointestinal tumor (Vitale et al., 2014). In an *In vitro* study, Tregs were involved in direct inhibition of NK cell Cytotoxicity and expression of the CD69 activation marker. Moreover, depletion of Tregs resulted in NK cell proliferation (Hasmim et al., 2015).

2.6.1.1.8 Dendritic cells (DC)

Dendritic cells (DC) are major antigen presenting cells and have ability to acquire, process and present tumor antigens to T lymphocytes. DCs were involved differentiation of naïve T cells into activated tumor-specific lymphocytes (Hanke et al., 2013). They played a key role in mounting immune response by interacting with myeloid cells and other cells of the immune system (Yang et al., 2013). Infiltration of DC in tumor microenvironment had shown controlled tumor growth and overall survival of patients with lung carcinoma, gastric cancer, head and neck cancer, oral squamous cell carcinoma (Yang et al., 2013). However, several factors secreted by tumor associated fibroblast cells and TAM such as IL-10, IDO, TGF-β, VEGF and chemokines resulted in skewed DC differentiation towards tolerogenic DC (Tran-Janco et al., 2015). These tolerogenic tumor infiltrating DC expressed high levels of

Programmed cell death receptor (PD-1) and T cell immunoglobulin and mucin domain 3 (TIM-3) which prevent antigen presentation to T-cells and inhibit tumor cytolysis (Karyampudi et al., 2014, Du et al., 2017). Furthermore, it had been investigated that sustained STAT3 phosphorylation in DCs is associated with enhanced TGF-β secretion, which prevented NK cell activation, and blocking of TGF-β restored NK cell functions (Sarhan et al., 2015). Also, IL-6 and IL-10 secretion by DC was resulted in inhibition of cytokine production and cytotoxicity of NK cells leading to poor survival and higher risk of leukaemia relapse (Perez-Martinez et al., 2011).

Therefore, cellular components of tumor microenvironment played an important role to calibrate the immune response to malignant cells and promoted events such as tumor invasion, angiogenesis, proliferation and metastasis (Hasmim et al., 2015).

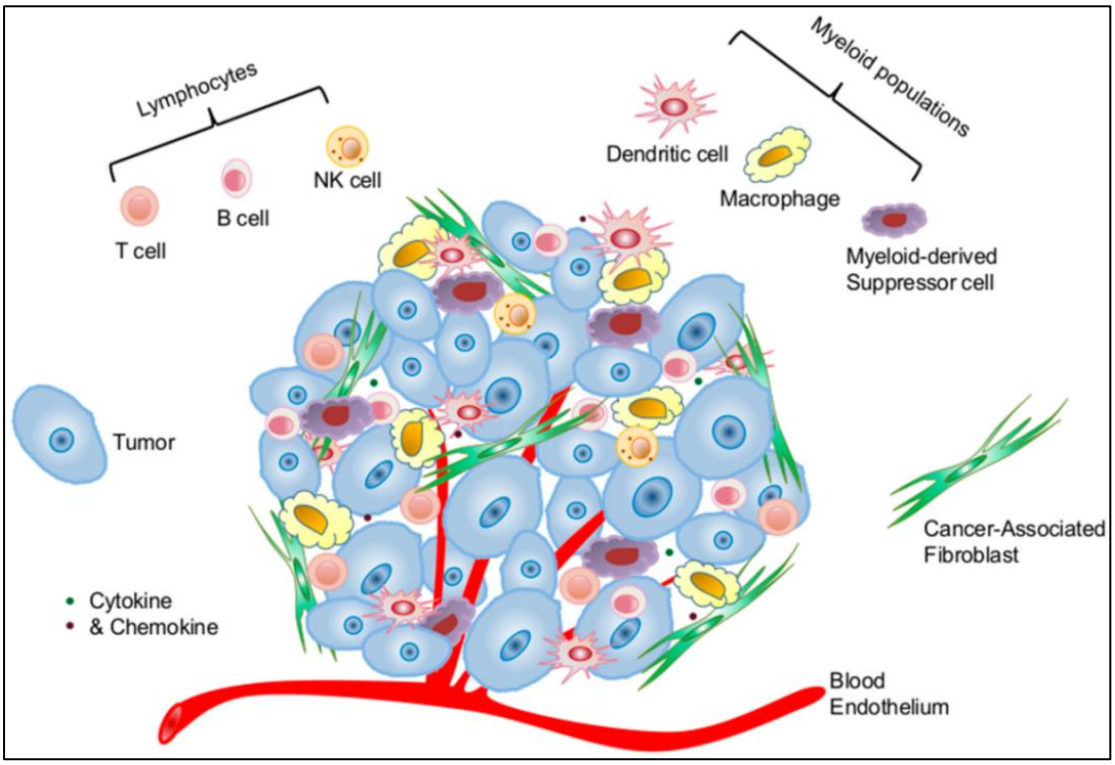

Figure 2.5: Cells in tumor microenvironment (Cui and Guo, 2016)

2.6.2 Inflammation as tumor inducer

Inflammation is the physiological response of immune cells that helps to heal and restore the injured or damaged tissue affected by pathogen, physical or chemical agents. However, if inflammation is unregulated, it results in onset of cellular events such as apoptosis, increased proliferation rate, and angiogenesis that promote malignant transformation of cells and carcinogenesis (Landskron et al., 2014). Many studies had investigated that inflammation is associated with increased risk of cancer (Long et al., 2017, He and Lin., 2017, Conway et al., 2016, Xiao et al., 2016). In early phase of tumor development, a state of hypoxic stress is established by activation of hypoxia responsive genes in tumor cells that favour migration of macrophages, granulocytes, and neutrophils (Semenza, 2016). Activation of these cells triggered abundance of Reactive Oxygen and Nitrogen species (ROS and RNS) production, leading to increased angiogenesis, cellular oxidative stress, damage of lipids, proteins, and DNA damage (Li et al., 2013). Further, recruitment of immune cells to the affected tissue resulting in secretion of pro-inflammatory cytokines such as TNF-α and sustained activation of signalling pathways such as NF-κB and STAT3 that favoured tumor progression (Laura D'Ignazio et al., 2017, Fan et al., 2013). Therefore, in a host reaction to the tumor, infiltrating immune cells together with tumor associated tissues contributed to establish an inflammatory milieu that nourished and promoted invasion and metastasis of cancer (He and Lin., 2017).

2.6.3 Immunosuppressive cytokines

Cytokines secreted by the cells in the tumor microenvironment has potential to modulate anti-tumor response and induce cell transformation and malignancy.

2.6.3.1 TNF-α

TNF-α is the member of TNF superfamily and is recognized by their receptors: TNF-α receptor-1 (TNF-αR-1) and TNF-αR-2. TNF-α play important role in regulation of hematopoiesis, cell proliferation, angiogenesis, differentiation and immune cell activation. TNF-α is a prototypical proinflammatory cytokine and its role in cancer has been controversial (Landskron et al., 2014). TNF-α had found to play dual role either in tumor inhibition or in progression (Ham et al., 2016). In clinical trials, local administration of TNF-α-expressing adenovirus (TNFerade) gene therapy combined with chemotherapy found safe and effective in patients with advanced pancreatic and oesophageal cancer (Herman et al., 2013, Cheng et al., 2013). In contrast, it was analysed that treatment of tumor-bearing mice strain B16F10 with rhTNF-α resulted in a significant increase in tumor burden and metastatic foci. It was observed that rhTNF-α activated TNF-αR-2 on pulmonary Tregs, lead to expansion of immunosuppressive Tregs and enhanced lung metastasis (Chopra et al., 2013). Moreover, it was found that TNF-α signalling via TNF-αR-2 promotes accumulation of immunosuppressive MDSCs in tumor milieu helps tumor cells evade the immune system (Zhao et al., 2012). Suganuma and co-workers (2012) investigated that TNF-α-inducing protein secreted by *Helicobacter pylori* was responsible for increased TNF-α levels in preneoplastic lesions and associated with increased risk of gastric cancer (Suganuma et al., 2012).

2.6.3.2 IL-6

Binding of IL-6 to IL-6 receptor, IL-6Rα and co-receptor gp130 results in activation of the JAK/STAT signalling pathway that promote proliferation and reduced apoptosis of tumor cells. In a mouse model, IL-6 was shown to be secreted by stromal fibroblasts in microenvironment with gastric cancer (Kinoshita et al., 2013). IL-6 was known to

involve in tumor progression by promoting conversion of non-cancer cells into tumor stem cells. It was observed that non-cancer stem cells cultured in low-attachment plates secreted IL-6 that induced activation of IL-6R/JAK/STAT3 signalling pathway and upregulated tumor stem cell associated *OCT4* gene expression (Kim et al., 2013). Interleukin-6 (IL-6) was one of the major cytokines which was found at high concentrations in various cancers as compared to healthy controls. Elevated serum IL-6 levels in cancer patients were reported to be associated with tumor progression by regulation of all cancer hallmarks such as apoptosis, survival, proliferation, angiogenesis, invasiveness and metastasis (Landskron et al., 2014). Moreover, IL-6 promoted the tumor proliferation by protecting from oxidative stress, apoptosis and therapy-induced DNA damage by induction of countersignalling pathways involving antioxidant and anti-apoptotic/pro-survival (Kumari et al., 2016).

2.6.3.3 TGF-β

TGF- is a pleiotropic cytokine, with immune-suppressing and anti-inflammatory properties and play important role in embryogenesis, cell proliferation, differentiation, apoptosis, adhesion and invasion. TGF-β considered as most potent immunosuppressive cytokines which was directly involve in inhibition of clonal expansion of NK cells and CTLs and impairing cytotoxicity of NK cells, macrophages, and CTLs (Burkholder et al., 2014). It was observed that TGF-β mediate upregulation of the transcription factor Forkhead box protein P1 (Foxp1) that induce suppression of anti-tumor T cells proliferation and activation (Stephen et al., 2014). Deletion of TGFβ receptor gene *Tgfbr2* specifically in myeloid cells from advanced stage cancer patients significantly inhibited tumor metastasis, suggested that myeloid-specific TGFβ signalling was a vital component of the tumor metastasis (Pang et al., 2013). Additionally, TGFβ reported to

interfere with the activation of antigen-presenting dendritic cells and maturation of NK cells, thus inhibited effective anti-tumor response (Bellomo et al., 2016). In the tumor microenvironment, primary sources of TGF-β were tumor and stromal cells, including fibroblasts and immune cells (Landskron et al., 2014). TGF-β targeted anti-tumor therapies in advanced cancer patients had shown safe and promising results in preclinical and clinical studies (Connolly et al., 2012). In a clinical trial, TβRI inhibitor, LY2157299 monohydrate was tested in patients with advanced cancer and glioma showed strong beneficial effects with no cardiotoxic side effects suggesting the promising anti-TGFβ therapy (Rodon et al., 2015).

2.6.3.4 IL-10

IL-10 is an anti-inflammatory cytokine released by various tumor cells and also produced by TILs, tumor-associated macrophages and almost all immune cells. It appeared to modulate the differentiation and antigen-presenting function of mature myeloid cells such as macrophages and dendritic cells. In tumor microenvironment, tumor-induced IL-10 was found to be specifically responsible for DC dysfunction and thus dampen antigen presentation capacity of DC allowing tumor to evade immune response (Hargadon, 2013). It was observed that DC secreted IL-10 was associated with supressing NK cytotoxicity and inhibition of cytokine production by NK cells in hematopoietic stem cell transplantation patients (Perez-Martinez et al., 2011). In contrast to IL-6, which led to transient activation of STAT3, IL-10 induced a sustained STAT3 activation that resulted in enhanced metastatic potential of by Lung cancer cells by promoting angiogenesis and resistance to apoptosis (Braun et al., 2013, Landskron et al., 2014). Similarly, elevated IL-10 were associated with poor prognosis of breast

cancer, Liver cancer, Oral squamous cell carcinoma (Esquivel-Velázquez et al., 2015, El-Emshaty et al., 2015, Aziz et al., 2015).

2.6.4 Tumor-derived factors

One of the most important mechanisms of immune evasion is the tumor cell derived growth factors which encourages tumor outgrowth. A variety of tumor derived soluble factors including vascular endothelial growth factor (VEGF), IL-10, TGF-β, prostaglandins, soluble phosphatidylserine are known to contribute to immunosuppression (Wang et al., 2017). VEGF is 34-42 kD protein secreted by almost all tumor cells and was primarily known as a proangiogenic factor. VEGF induce dysfunction of antigen-presenting dendritic cells by increasing expression of immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) and ineffective antigen presentation results in tumor evasion from immune system (Marti et al., 2013). Moreover, IDO shown to produce immunosuppressive tryptophan catabolites down regulated the expression of NK cell activating receptors and therefore inhibited NK function against tumors (Vitale et al., 2014). Recent studies had reported that like DCs, macrophages express VEGF receptor that caused infiltration of macrophages at the tumor site which promote neovascularization for continued tumor growth (Li et al., 2016). Soluble phosphatidylserine derived from cancer cells interacted with macrophages and released immunosuppressive factors such as IL-10, TGF and PGE2 contributed to anti-inflammatory response to macrophages, leading to anti-tumor response (Birge et al., 2016). Fibroblasts were associated with maintaining the structure and function of healthy tissues through remodeling and transient tissue repair during wound healing. However, fibroblasts were the major constituent of tumor stroma, especially in breast, prostate, and pancreatic cancers and involved in tumorigenesis (Ohlund et al., 2014). Tumor associated fibroblasts (TAFs) promoted tumor growth by

recruiting macrophages and secreting inflammatory cytokines and neoangiogenesis (Bussard et al., 2016). TAFs cells secreted certain immunosuppressive mediaters such as IL-6,TGF-β, VEGF,SDF-1, and CXCL1/2 at the tumor site that interfered with NK cell cytotoxicity (Hasmim et al., 2015). It was reported that CAFs derived factors like PGE2, matrix-metalloproteinases (MMP) is responsible for down-regulation of ligands for NK cell activating receptors and resulted in impaired activity of NK cells against melanoma targets (Vitale et al., 2014, Inoue et al., 2016, Ziani et al., 2017). Prostaglandins are the immnomodulators secreted mainly by antigen presenting macrophages and DC. Mainly prostaglandin E2 (PGE2) had been implicated in the tumor-associated alteration of immune function (Figure 2.6) (Wang and DuBois, 2016). Cyclooxygenases (COX) commonly overexpressed in various cancer tissues and responsible for biosynthesis of arachidonic acid to prostaglandins (PGs) and thromboxane. Interaction of PGE2 to its receptor triggered activation signal that led to tumor cell proliferation, invasion, angiogenesis, and immunosuppression (Zelenay and Sousa, 2016).

2.6.5 Alteration in NK cell receptor profile

In particular, NK cells are recognized as potent anti-tumor component of immune system. However, tumor microenvironment associated cell subsets, tumor derived factors, and metabolites with immunosuppressive roles mediate alteration in NK cells receptor expressions. This alteration of receptor expression led to impairment in cytotoxic activity and also cytokine production by NK cells (Baginska et al., 2013). The expression of activating receptors NKp30, NKp46 and NKG2D were found to be down-regulated in breast cancer patients (Nieto-Velázquez et al., 2016). It was observed that colorectal carcinoma derived fibroblasts released PGE2 on co-culturing with NK cells and resulted in downregulating activating receptor expression level and NK activity

against tumor (Li et al., 2013). Moreover, Neuroblastoma-derived TGF-β supressed the NK cytotoxicity by decreasing the NKp30 expression level (Castriconi et al., 2013) and upregulating the surface expression of inhibitory receptor, NKG2A in lung cancer (Jin et al., 2014). Tumor cells had reported to escape NK mediated recognition by down-regulating the expression of ligands for activating receptors and over-expressing the ligands for inhibitory receptors, thus reduced NK cytotoxicity (Cremer et al., 2012, El-Gazzar et al., 2013, Mathew et al., 2016). Thus, tumor microenvironment play a pivotal role in constituting immunosuppressive networks that remain an important obstacle to cells involve in immune system and contribute to the tumor progression and metastasis.

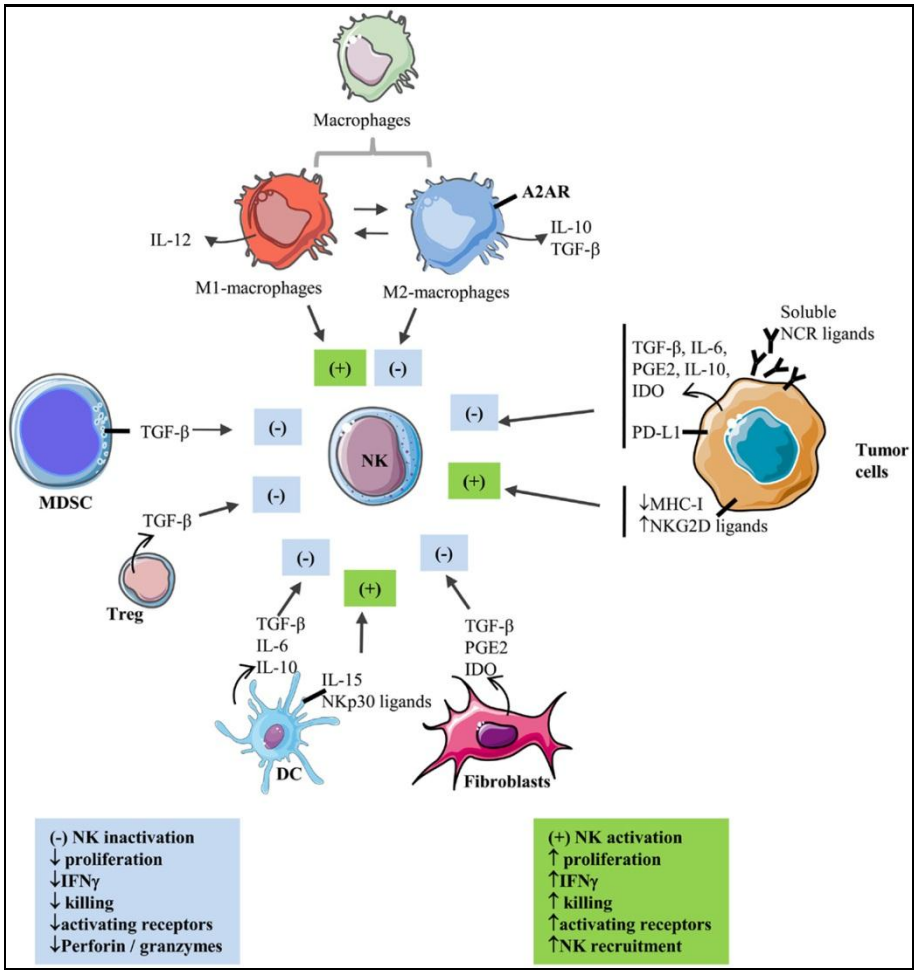

Figure 2.6: Interaction between NK and stromal cells in tumor microenvironment (Hasmim et al., 2015)

2.7 NK and virus infection

Natural killer (NK) cells play a key role in innate host defense against viral infections. Numerous studies in human and mice model had demonstrated the antiviral role of NK cells that included either direct killing of the infected target cell or production of cytokines that mediated effector response (Vidal et al., 2011, Jost and Altfeld, 2013). Recent studies had shown that activated NK cells accumulated in the liver at the onset of hepatitis B Virus (HBV) infection and secrete antiviral cytokine IFN-γ and contribute to HBV clearance (Tong et al., 2017). NK cells had found to mount an effective immune response against Human papilloma virus infection (Zhao et al., 2017). Moreover, severity of H1N1 infection had been correlated with NK cells deficiency; it was observed during a clinical study that percentage of NK cells reduced gradually with symptoms progression but counts returned to normal following the recovery of patients (Fox et al., 2012). Despite the loss of MHC class I on infected cell, viruses had some mechanisms to evade NK cell recognition. Iwasaki and Pillai, 2014 reported that influenza virus evaded the NK cell innate immune defence by directly infecting NK cells and induced cell apoptosis. In another finding, Yoon et al., 2011 suggested that direct interaction of NK cells with HCV-infected cells negatively modulated functional capacity of NK cells which was associated with down-regulation of NK-activating receptors such as NKG2D and NKp30 on NK cell surfaces. The ability of NK cells to secrete IFN-γ also diminished after exposure to HCV-infected cells. Therefore, contributed to the establishment of chronic infection. Patients with HHV8 viral infection had shown down-modulated expression of NKp30, NKp46 and CD161 receptors associated with impaired NK-cell cytotoxicity against target cells (Dupuy et al., 2012).

2.8 NK cell in fetal implantation

Human decidua possesses almost 70% of NK cells among total lymphocytes during pregnancy (Feyaerts et al., 2017). They suggested that the higher percentage of NK cells during pregnancy play crucial role in implantation and vascular modification in the decidua, hence are vital for normal pregnancy. Decidual NK cells expressed CMKLR1 (for a chemoattractant, chemerin); CMKLR1/chemerin interaction led to NK cells recruitment to deciduas from periphery and formation of capillary- like tube, therefore mediated vascular remodelling during early pregnancy (Carlino et al., 2012). Extravillous trophoblast (EVT) cells lack HLA-A/B (human leukocyte antigen) expression and expressed non-classical MHC class I molecules HLA-G, which may protected fetal tissues from maternal immune system (Tilburgs et al., 2015b). Interaction between the non-classical class I MHC molecule HLA-G and KIR2DL4 initiated the production of proinflammatory cytokines and proangiogenic factors (Ang1, Ang2, VEGF-A, VEGF-C, IFN-γ, and TGFβ1) that promoted vascular remodeling as well as induced surface co-expression of HLA-E on trophoblast cells and thus led to a strong inhibitory signals in decidual NK cells through CD94/NKG2A (Tilburgs et al., 2015a). Moreover, uterine NK cells, but not peripheral NK cells, secreted VEGF-C that activated TAP-1 expression on trophoblast cells and thus imparted protection from uterine NK cell-mediated cytotoxicity (Tilburgs et al., 2015b, Ferreira et al., 2017). In line with these studies, Yokota et al., 2013 established that NKp46 expression on uterine NK cells up-regulated the production of cytokines that facilitated normal pregnancy. Hence, during pregnancy there was increased migration of immature NK cells from periphery which underwent significant change in their receptor profile upon interaction with decidual components. Thus, NK cells play a crucial role in spiral artery

remodelling in fetal trophoblast and loss of decidual NK cells may lead to pathological conditions like preeclampsia and recurrent spontaneous abortion (Wallace et al., 2012).

2.9 NK cell in autoimmunity

Auto-reactivity and autoimmunity reactions are considered as aggravated immune response to self-cells due to altered function of immune cells. In healthy conditions, normal cell express HLA-I molecules that are recognize by the NK inhibitory receptors and inhibitory signal outweighs the activation thereby avoiding the self-reactivity of NK cells. Various studies had documented the dual role of NK cells in autoimmune diseases as disease-promoting and disease-controlling. Autoimmune disorders were generally associated with impaired function and frequency of NK cells. Reduced NKG2D expressing NK population and impaired function of NK cell resulted in progression of Type I diabetes (Zhang et al., 2017). Moreover, it was observed that expression of NK activating receptor NKG2D was upregulated and there was significant decrease in population of NK cells expressing inhibitory receptor KIR3DL1 in LADA patients as compared to healthy controls (Akesson et al., 2010). NK cells expressed various activating and inhibitory KIRs on their surface that influences the NK cell licensing and cytotoxicity against the targets. Interaction of KIRs with HLA had seen to be involved in NK mediated protection against various infections. However, same KIR/HLA combination had inclined individuals to certain autoimmune disorders including systemic lupus erythematosus (SLE), multiple sclerosis (MS), scleroderma and Type-I diabetes.(Pellett et al., 2007, Fusco et al., 2010, Van der Slik et al., 2007). However, interaction of KIR3DL1 with its ligand HLA-Cw4 marked a strong protective response against MS in African American patients (Hollenbach et al., 2016).

Surprisingly, activating KIR/HLA combination exhibited protective response against an autoimmune blistering skin disease, Pemphigus Foliaceus in urban population of western Brazil (Augusto et al., 2012). Therefore, evidences from studies suggested that NK cells can affect the development of autoimmune disorders as well as provide protection against them and further studies are needed to clarify potential mechanism behind the role of NK cells in disease aggravation and protection from disease.

2.10 IL-2 responsiveness of NK cells

It is well known that administration of Interleukin-2 (IL-2) lead to expansion of TILs and results in complete or apparently curative regression of tumor in cancer patients (Rosenberg et al., 2011, Stevanovic et al., 2015). IL-2 is the one of the key cytokine with multiple effects on immune cells like differentiation of $CD4^+$ T cells into a variety of subsets, NK cell cytotoxic function, and modulates T-cell function in response to antigen (Jiang et al., 2016). IL-2 is a small cytokine with molecular mass 15.5-kDa. IL-2 is predominantly produced by activated T-cells, NK cells and activated dendritic cells. IL-2 receptor consists of the three subunits IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132). IL-2 binds to its receptors with different affinities. IL-2Rα binds IL-2 with low-affinity (K_d-10^{-8} M) and does not transduces intracellular signals and the heterodimeric IL-2Rβγ binds IL-2 with intermediate-affinity (K_d-10^{-9} M) and results in signal transduction. Combination of all three IL-2 receptor subunits form an IL-2Rαβγ trimeric complex and have highest binding affinity towards IL-2. Small number of resting NK cells expressed IL-2Rα on cell surface and IL-2Rβ, IL-2Rγ subunits are expressed by activated NK cells and T cells. The optimal doses of IL-2 that are able to stimulate NK mediated cytotoxicity are higher than those required for T-cell activation.

This is because IL-2 binds with intermediate affinity to NK cells and high affinity to T-cells (Hodge et al., 2000). Binding of IL-2 to the IL-2Rβγ or IL-2Rαβγ complex initiate activation of signalling pathway cascade which mediate the recruitment of Janus family tyrosine kinases (JAK1 and JAK3) to the cytoplasmic domains of IL-2Rβγ or IL-2Rαβγ. The activation of JAK kinases bring about recruitment and phosphorylation of signal transducer and activator of transcription 1 (STAT1) (Figure 2.7). Eventually, lead to activation of major downstream signalling pathways phosphoinositide 3-kinase (PI3K-AKT), and the mitogen-activated protein kinase (MAPK) signalling pathways. Activation of these pathways mediates the survival, proliferation, differentiation, activation, cytokine production in different types of immune cells.

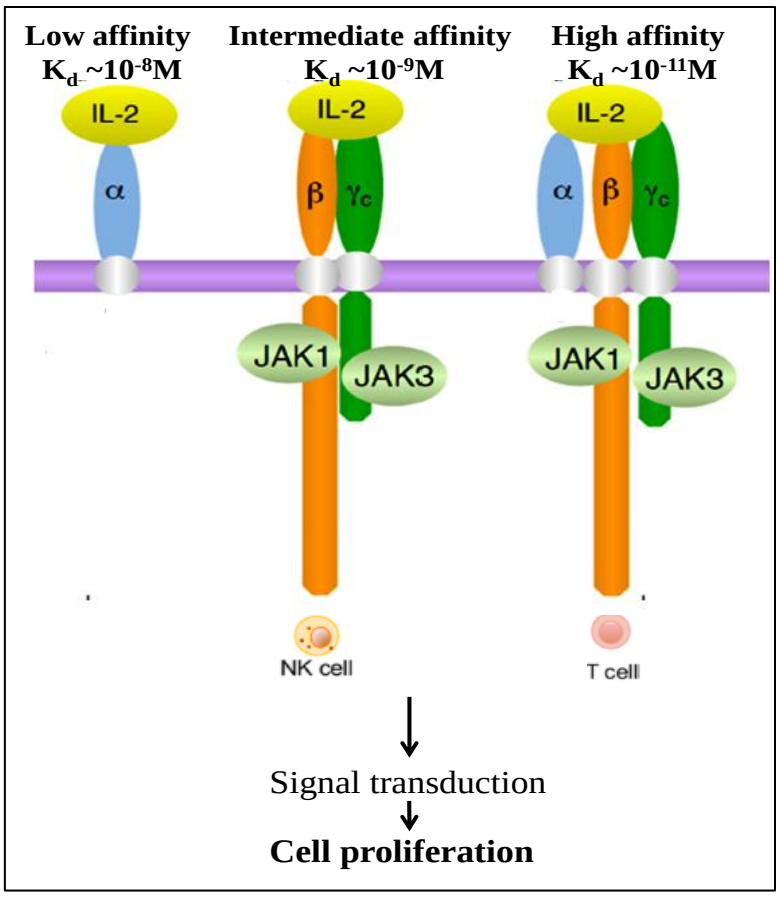

Figure 2.7: Structure of IL-2 receptor (adapted from Liao et al., 2013)

2.11 NK cell based immunotherapies

Several experimental and clinical evidences have demonstrated the potential role of NK cells in the recognition and eradication of tumors. However, tumors had developed various immunosuppressive mechanisms that eluded NK cell cytotoxicity thereby facilitated tumor progression. Therefore, new therapeutic strategies were focussed at to reverse tumor mediated NK cell suppression, retain their activation potential and accelerate their anti-tumor response. The promising anti-tumor response of NK cell in clinical studies necessitates development of NK cell-based therapy against tumor. The following primary approaches are used for NK cell-based cancer therapy aimed at improving the clinical outcome.

2.11.1 Cytokines

Cytokines are known to be involved in NK cell proliferation and activation. IL-2 is one of the most promising cytokine for boosting NK cell efficacy in cancer treatments. IL-2 has diverse biological actions including ability to proliferate and activate anti-tumor NK cells and T cells. This feature makes it a potent therapeutic agent in cancer treatment. Adoptive Cell therapy (ACT) using IL-2 had proved to be one of the main treatment modalities within cancer immunotherapy, in which tumor infiltrating lymphocytes mostly, T cells were removed from tumor samples and propagated *in-vitro* on IL-2 stimulation (Qian et al., 2014). The expanded and activated lymphocytes were infused into cancer patients with the goal of recognizing, targeting, and destroying tumor cells. Immunotherapy using tumor-specific T-cells was first established by Steven Rosenberg in 1980's and eventually human trials of *ex vivo* expanded TILs had shown promising results when combined with systemic high-dose interleukin-2 (IL-2) in

patients with metastatic melanoma (Rosenberg et al., 2011). Also, infusion of HPV-TILs from Human papilloma virus (HPV) positive patients into cervical cancer patients resulted in cancer regression (Stevanovic et al., 2015). Schleypen and co-workers (2006) separated tumors in two groups based on frequency of infiltrating NK cells in tissues with low NK-TIL and high NK-TIL isolated from renal cell carcinomas and observed that high NK-TIL showed more cytolytic potential against K562 target cells on low dose IL-2 stimulation (Schleypen et al., 2006). Also, It was reported that on high doses of IL-2 administration at regular short intervals, significantly more activated NK (A-NK) cells were accumulated in the tumors and were able to eliminate well established lung metastasis in C57BL/6 mice (Yang et al., 2003). Sarkar and co-workers (2013) had shown that activation of NK cells with IL-2 led to restoration of NK cells cytotoxicity even under hypoxic conditions in multiple myeloma (Sarkar et al., 2013). Modified form of IL-2 called 'super-2' with high affinity towards IL-2Rβ demonstrated an enhanced capacity to expand NK cell population in mice models (Levin et al., 2012). Ghasemi et al., 2016 developed a recombinant fusion protein comprised of combination of mutated IL-2 and cowpox virus encoded NKG2D ligand (OMCP- mutIL-2). Systemic administration of the fusion protein in different mice models reported to promoted the NK cell proliferation and enhanced cytotoxicity of $NKG2D^{+}$ NK cells against YAC-1 targets (Ghasemi et al., 2016). Additionally, IL-2 had been used a potential candidate to expand NK cells for adoptive cell transfer therapies (Romee et al., 2016, Yang et al., 2016, Shaffer et al., 2016). Another cytokine involved in NK cell proliferation and activation is IL-15. IL-2 and IL-15 share common receptor β chain and γc chain (Miller et al., 2015). In a clinical study, bolus intravenous infusion of recombinant IL-15 resulted in redistribution, proliferation and activation of

NK cells in patients with metastatic malignancy (Colnol et al., 2015). Approaches in devising good manufacturing practice (GMP)-compliant used combination of IL-2 and IL-15 that resulted in reduced tumor cell load in NOD/SCID mice transplanted with human AML (Siegler et al., 2010). Cytokines like IL-12 and IL-21 also had shown immunomodulatory effects in clinical trials (Lasek et al., 2014, Croce et al., 2015) but repeated dose regimen had demonstrated some side effects in cancer patients (Floros and Tahini, 2015) . Further studies are required to focus on dose and frequency optimization.

2.11.2 Other factors

Factors like serum supplementation dosage, culture media and expansion platform play important role in enhancing NK yield, activity and efficacy of NK cells. It was observed that NK cells cultivated in large scale bioreactor under feeder-free conditions resulted in significantly higher expansion and demonstrated promising anti-tumor response and in clinical trials of adoptive immunotherapy (Sutlu et al., 2010). In addition, cGMP quality cell culture media was another important factor for NK cell expansion. Usually, media are supplemented with human AB serum or fetal bovine serum such as stem cell growth medium (SCGM; CellGenix, Freiburg, Germany), X-VIVO serum-free media (BioWhittaker, Verviers, Belgium), or AIM V (Life Technologies, Grand Island, NY, USA) (Klingemann et al., 2004, Mckenna et al., 2007, Parkhurst et al., 2011).

2.11.3 Adoptive cell transfer therapy

Clinical trials using autologous adoptive NK cells had proved to be safe and efficient therapy against different tumor targets such as non-small cell lung cancer, breast cancer, glioma renal cell carcinoma, lymphoma, and adenocarcinoma (Lim et al., 2015). ACT

therapies utilized patient-specific autologous or HLA-matched allogeneic lymphocytes which were activated *in vitro* with stimulation of cytokines; growth factors including IL-2, IL-12, GM-CSF (Qian et al., 2014). Administration of Hsp70 peptide+IL-2 activated autologous NK cells displayed higher cytotoxicity against Hsp70 membrane-positive colon carcinoma cells and NSCLC (Specht et al., 2015). However, adoptive transfer of autologous NK did not showed remarkable regression in tumor growth such as relapsed lymphoma metastatic, melanoma and renal cell carcinoma (Rezvani and Rouce, 2015). Later, in a clinical trial, NK cells were expanded *ex vivo* with IL-2 and OK-432 stimulation. Infusion of expanded NK cells in patients diagnosed with advanced digestive cancer resulted in better clinical outcome with no adverse effects (Sakamoto et al., 2015). Using allogeneic NK cells has the risk of developing graft-versus-host disease (GvHD), so it is strictly necessary to adopt measures to avoid such complications like depletion of $CD3^+$ T cells (Simonetta et al., 2017). However, patients infused with *ex vivo* expanded HLA-mismatched NK have shown better survival with no side effects (Martin-Antonia et al., 2017). In comparison to autologous or allogeneic NK cells, the NK cell lines expanded under GMP conditions had found to be better and safe candidates for clinical adoptive therapy. NK-92 cells were the only ones among all NK cell line to be approved by FDA for investigational treatment of advanced malignant melanoma and renal cell carcinoma patients. However, KHYG-1 cells and NKL cells had shown more cytotoxicity against tumors than NK-92 cells (Qian et al., 2014) making them potential candidates holding a therapeutic promise.

2.11.4 Genetically modified NK cells

Genetic modification is the most promising strategy to design more efficacious NK therapy products. Since, NK cells are resistant to retroviruses, the limitation of gene

delivery was overcome by targeting intracellular viral defense mechanism and increasing transduction efficiency by using inhibitors molecule (Brandstadter et al., 2011, Sutlu et al., 2012). Therefore, genetic modification of NK cells would help in designing cytokine transgene, increased specificity, over-expressed activating receptors and silenced inhibitory receptors to contribute towards enhanced anti-tumor activity.

2.11.5 Monoclonal antibodies (mAbs)

Another promising tool to manipulate anti-cancer immune response is to devise tumor specific mAbs targeting the tumor cells that bind to the FcγRIIIa receptor (CD16a) on the NK cell, results in ADCC response. Various mAbs such as anti-CD20 (Retuximab), anti-Her2 (Trastuzumab), anti-CD52 (Alemtuzumab), anti-EGFR (Certuximab), and anti-CD38 (Daratumumab) have been developed for targeting specific tumor antigens (Mentlik-James et al., 2013). Daratumumab treatment showed mild infusion-related reactivity, overall good response rate with reduced bone marrow plasma cell levels of patients with relapsed myeloma (Lokhorst et al., 2015). In clinical trials, second generation humanized anti-CD20 antibody, Veltuzumab considered safe at all tested doses with no adverse effects in patients with non-Hodgkin's lymphoma and chronic lymphocytic leukemia (CLL) (Morschhauser et al., 2009, Kalaycio et al., 2016). Third-generation anti-CD20 mAbs, obinutuzumab, ocaratuzumab and ublituximab had designed to show increased binding affinity to CD16a, thus aims at more efficient NK mediated cytolysis of tumors (Le Garff-Tavernier et al., 2011, Kern et al., 2013, Cheney et al., 2014). Treatment of obinutuzumab in patients with CLL and Non-Hodgkin Lymphoma had considered as safe, effective monotherapy and overall response rate was higher than rituximab (Wierda et al., 2011, Sehn et al., 2015).

2.11.6 Bi-specific and tri-specific antibodies

In this method, Fab fragment of the antibody targeting the tumor cell antigen is fused in combination with another Fab region recognizing CD16 on NK cell, results in activation of NK cell. It was observed that fully humanized bi-specific killer engagers (BiKE) CD16 × CD33 were able to produce cytokines and triggered effective NK cytotoxicity against acute myeloid leukaemia targets (Wiernik et al., 2013). Similarly, BiKE CD16 × CD33 reported to stimulate NK activation and significant increase in secretion of IFN-γ and TNFα against Myelodysplastic syndromes CD33+ targets (Gleason et la., 2014). In addition, tri-specific killer engagers (TriKE) had been constructed by incorporating modified IL-15 as cross-linker between single chain Fv against CD16 and CD33 (designated as 161533) to increase NK cell specificity, viability and expansion. TriKE directed enhanced cytotoxicity and cytokine stimulation against CD33 targets than BiKE, rendered it more promising anti-cancer tool (Miller et al., 2015, Schmohl et al., 2016).

2.11.7 Chimeric antigen receptors (CARs)

CARs were developed by fusing tumor antigen antibodies with intracellular lymphocyte stimulatory molecules (CD3ξ, CD28, 4-1BB) that led to high- affinity specific recognition of tumor antigens and tumors. Although CARs considered as promising approach but only few studies using NK-CARs were under clinical trial had been approved (Suck et al., 2016). Therefore, there is need to standardize the construct design, specifically intracellular stimulatory adapter molecules in order to elicit more efficient anti-tumor response by NK cells.

2.11.8 Immunomodulatory drugs (iMiDs)

IMiDs like thalidomide, lenalidomide, and pomalidomide are known to involve in both NK cells and T cells stimulation, leading to better targeting tumors (Shortt et al., 2013). Lenalidomide enhance expression of TRAIL molecules on NK cells and thus cytotoxicity (Jungkunz-Stier et al., 2014). Combination of anti-inhibitory KIR antibody (IPH2101) and lenalidomide resulted in increased NK proliferation and activation of NK cells against targets (Bensen et al., 2015).

2.11.9 Combination treatment

Immunosuppressive effects of tumors can be combated by the increasing the efficacy of immunotherapy by combining the NK therapy with the other products. Combination of NK cells with drugs or immunomodulatory drugs had been extensively studied in different tumors and had shown promising anti-tumor effect (Veluchamy et al., 2016). Another way to boost NK cytotoxicity was to combine drug therapy with NK cell stimulating cytokines such as IL-2, IL-12, IL-15, and IL-21 (Romee et al., 2014). It was observed that combination of high dose fludarabin and cyclophosphamide and administration of IL-2 resulted in *in vivo* expansion NK cells led to complete remission of malignancy in patients with AML (Miller et al., 2005).

Chapter 3

Materials and Methods

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

Table 3.1: List of chemicals and reagents

Biochemicals, Chemicals and Reagents	Brand/ Manufacturer
2-mercaptoethanol	Sigma-Aldrich, USA
Acrylamide	Himedia, India
Ammonium per sulphate	Sigma-Aldrich, USA
Anti-alpha-tubulin	GeneTex, USA
Anti-cadherin	GeneTex, USA
Bicinchoninic Acid	Sigma-Aldrich, USA
Bovine Serum Albumin (BSA)	Merk, India
Copper (II) Sulphate pentahydrate	Sigma-Aldrich, USA
Dithiothreitol	Himedia, India
Dulbecco′s Modified - Eagle′s Medium (DMEM) with high glucose	Sigma-Aldrich, USA
Ethyleneglycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)	Sigma-Aldrich, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, USA
Fetal bovine Serum (FBS)	Gibco, Life Technologies, USA
FITC anti-mouse NK1.1	BD biosciences, USA
Glycerol	Thermo Fisher scientific, India
Glycine	Himedia, India
HEPES	Sigma-Aldrich, USA
HRP Goat anti-mouse Ig	BD biosciences, USA
HRP Rabbit anti-mouse IgG	BD biosciences, USA
Magnesium Chloride ($MgCl_2$)	Himedia, India
Membrane filter	Millipore, Merk
Methanol	Thermo Fisher scientific, India

Biochemicals, Chemicals and Reagents	Brand/ Manufacturer
Mouse IFN-γ Duoset Elisa Kit	R&D systems, USA
Mouse MIP-1α Duoset Elisa Kit	R&D systems, USA
Mouse NK cell enrichment Kit	Stem cell technologies, Canada
NK purification Magnet	Stem cell technologies, Canada
Nystatin	MP Biomedicals, France
PE anti-mouse Ly49A	BD biosciences, USA
PE anti-mouse Ly49C	BD biosciences, USA
PE anti-mouse Ly49D	BD biosciences, USA
PE anti-mouse NK1.1	BD biosciences, USA
Penicillin	MP Biomedicals, France
Ponceau S	Sigma-Aldrich, USA
Potassium Chloride	Himedia, India
Potassium dihydrogen Phosphate	Himedia, India
Pre-stained protein Ladder	BIOCHEM, Life Sciences
Protease Inhibitor	Sigma-Aldrich, USA
Purified Mouse Anti-β-Catenin	BD biosciences, USA
PVDF membrane	MDI, India
Recombinant Human IL-2	Biolegend, USA
RPMI-1640	Sigma-Aldrich, USA
Sodium Chloride	Himedia, India
Sodium deoxycholate	Sigma-Aldrich, USA
Sodium dihydrogen Phosphate	Himedia, India
Sodium dodecyl sulphate (SDS)	Himedia, India
Sodium Pyruvate	Sigma-Aldrich, USA
Streptomycin Sulfate	MP Biomedicals, France
Sulphuric Acid	Thermo Fisher scientific, India
Tetramethylbenzidine	R&D system, USA
Tetramethylethylenediamine (TEMED)	Thermo Fisher Scientific, India
Transwell Plates	Corning, USA
Tris HCl	Himedia, India
Trypan Blue	Gibco, Life Technologies
Tween 20	Sigma-Aldrich, USA

All other chemicals were of analytical grade.

Table 3.2: List of buffers/solutions

Reagents/Buffers	Composition
4 % PFA (fixative)	4 % paraformaldehyde (v/v) in 1X PBS
Blocking Buffer	5 % (w/v) BSA in 1X PBST
Blocking buffer	1% BSA in PBS, pH 7.2-7.4
De-staining solution	30 % (v/v) methanol and 10 % (v/v) acetic acid
Laemmili buffer (2 X)	2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol , 10% (v/v) glycerol and 0.004% (w/v) bromophenol blue, 0.5M EDTA
Nuclear Lysis (NL) buffer	50mM Tris HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS. 10% glycerol and 1X PI cocktail were added to 10ml of NL buffer at the time of use
Phosphate buffered saline	NaCl 138 mM, KCl 2.7 mM, Na_2HPO_4 4.3 mM, KH_2PO_4, 1.5 mM (pH 7.4)
Ponceau S staining solution	2% Ponceau S in 30 % trichloroacetic acid
SDS-Running Buffer (1X)	Tris glycine Buffer which contains 25mM Tris base, 250 mM glycine (electrophoresis grade) (pH 8.3), 0.1% SDS
Staining solution	0.25 % (w/v) coommasie R 250 in 50 % (v/v) methanol and 10 % (v/v) Glacial acetic acid
Sub-cellular fractionation (SF) buffer	Sucrose, Tris, 1mM EDTA, 1mM EGTA, 10mM KCl, 20mM HEPES, 1.5mM $MgCl_2$. 1mM Dithiothreitol (DTT) and 1X protease inhibitor (PI) cocktail were added to 10ml of SF buffer at the time of use
Transfer buffer	Tris 25Mm, Glycine 192mM, methanol 20%; SDS 0.1%
Wash buffer	0.05% Tween 20 in PBS, pH 7.2-7.4

Table 3.3: Resolving gel composition (for 10 mL)

Components	For 10% gel (ml)	For 12% gel (ml)
Water	4.0	3.3
30% Acrylamide solution	3.3	4.0
1.5 M Tris (pH 8.8)	2.5	2.5
10 % Ammonium per sulphate	0.1	0.1
10 % SDS	0.1	0.1
TEMED	0.004	0.004

Table 3.4: Stacking gel composition

Components	5 (ml)	10 (ml)
Water	3.4	6.8
30 % Acrylamide solution	0.83	1.7
1.0 M Tris (pH 6.8)	0.63	1.25
10 % Ammonium per sulphate	0.05	0.1
10 % SDS	0.05	0.1
TEMED	0.005	0.01

3.1.2 Equipments and instruments

Table 3.5: List of equipments and instruments

Equipment / Instrument	Manufacturer
Centrifuge	REMI
CO_2 Incubator	Thermo Fisher Scientific, USA
Electronic Balance	Mettler Toledo, India
ELISA reader	Bio-Tek, Winooski, USA
Flow cytometer	BD biosciences, USA
Gel documentation system	MiniBIS-Pro Bio Imaging system
Inverted microscope	Olympus CKX41, Japan
Light Microscope	Olympus BX 60
pH meter	Hanna
Refrigerated centrifuge	Galaxy Lab Instruments, India
SDS-PAGE assembly	BIO-RAD, USA
Ultracentrifuge	Beckman coulter
Western Blot apparatus	BIO-RAD, USA

3.2 Methodology

3.2.1 Preparation of cells culture medium

Dulbecco's modified Eagle's medium with 2mM L-glutamine and glucose 4.5 g/l supplied as dry powder was dissolved in 900ml Milli-Q water and supplemented with 25mM $NaHCO_3$. Thereafter, 10mM HEPES 1mM Sodium pyruvate and antibiotics *viz.* penicillin, streptomycin sulfate and antimycotic *viz.* nystatin was added at a

concentration of 50Units/ml, 50µg/ml and 2µg/ml respectively. Final pH was adjusted to 7.4. The medium was filtered using membrane filter assembly (0.22 µm) syringe. The sterility was routinely checked by incubating the medium at 37ºC for 72h. The medium was thereafter stored at 4ºC. Growth medium was finally prepared by addition of 10% FBS (heat inactivated) to the filter sterilized medium.

RPMI medium with 2mM L-glutamine supplied as dry powder was dissolved in 900ml Milli-Q water and supplemented with 25mM $NaHCO_3$. Thereafter, 10mM HEPES, 1mM Sodium pyruvate and antibiotics *viz.* penicillin, streptomycin sulfate and antimycotic *viz.* nystatin was added at a concentration of 50Units/ml, 50µg/ml and 2µg/ml respectively. Final pH was adjusted to 7.4. The medium was filtered using membrane filter assembly (0.22 µm) syringe. The sterility was routinely checked by incubating the medium at 37ºC for 72h. The medium was thereafter stored at 4ºC. Growth medium was finally prepared by addition of 10% FBS (heat inactivated) to the filter sterilized medium.

3.2.2 Maintenance of cell culture

P815 is mastocytoma cell line was obtained from School of Life Sciences, Jawaharlal University (JNU), Delhi, India. The cells were routinely cultured and maintained in DMEM with high glucose at 37°C temperature under 5% CO_2 atmosphere and 95% humidity supplemented with 10% heat-inactivated FBS. Cells were passaged 2-3 times per week for maintaining them in healthy log phase. Cell growth was estimated by counting cells with the help of hemocytometer at different time intervals.

YAC-1 cell line was procured from NCCS, Pune, India. Stock cells were routinely cultured and maintained as a monolayer culture in RPMI at 37°C temperature under 5% CO_2 atmosphere and 95% humidity supplemented with 10% heat-inactivated FBS. Cells were passaged 2-3 times per week for maintaining them in healthy log phase.

3.2.3 Experimental animal model

Animals used in the experiments were 6-8 weeks old, male C57BL/6 mice weighing 25.0±2.0 gm. Animals were issued by the Institutional Animal Facility (IAF), Institute of Nuclear Medicine and Allied Sciences (INMAS), Delhi under MoU with Delhi Technological University. All animals were maintained under controlled conditions of temperature (28.0 ±5.0ºC), humidity (55%) and light 12:12h of light and dark period. The animals had free access to sterile water and food (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd., Pune, India) *ad libitum*. Animals were housed in polypropylene cages containing sterile paddy husk (procured locally) as bedding throughout the experiments. Animals were kept under observation and weighed daily for a week before experiments initiation. The animals not appearing healthy or inactive, sluggish or observed to lose their weight were not included in the experiments. All experiments were performed in accordance with the recommendations of the Institutional Animal Ethics Committee (IAEC).

3.2.4 NK cell preparation

Spleens were removed aseptically from C57BL/6 mice and disrupted in recommended medium (PBS+2% FBS + 1mM EDTA). The cell suspension was

then passed through a 70µm nylon mesh filter to remove unwanted debris and clumps to prepare single-cell suspension. The cell suspension was then washed with recommended media and spleenocytes were re-suspended in fresh recommended media. Erythrocytes were lysed by using RBC lysis buffer and NK cells were isolated from these spleenocytes through EasySep™ Mouse NK Cell Enrichment Kit through negative selection kit. In brief, 50µl of NK cell enrichment cocktail was added to spleenocytes cell suspension with concentration of $1x10^8$cells/ml. After, mixing, the cells were incubated at room temperature (RT) (18-25°C) for 15min. Further, 200µl of biotin selection cocktail was added, mixed and incubated for 15min at RT. In the next step, 200µl of magnetic particles were added, mixed, incubated for 10min at RT and the cell suspension was brought to 2.5ml volume by adding recommended media. The cells were then mixed gently by pipetting and tube (without cap) was placed into the magnet for 5min. The desired fraction was poured in one continuous motion into the new tube and magnetically labelled unwanted cells were remained inside the original tube. The purity of NK cells was measured by staining cells with antibodies against NK cells marker (FITC anti-mouse NK1.1) and T-cell marker (APC anti-mouse CD3).

3.2.5 Evaluation of cell viability

The viability of NK cells and tumor cell lines was evaluated by trypan blue exclusion method (Strober, 2001). In which, 0.4% trypan blue dye was added to the cell suspension and number of dead and live cells were counted using haemocytometer. The live cells with intact membrane excluded the dye while dead cells did not, so appeared blue. The percentage of viable cells was counted by dividing the number of viable cells by the number of total cells and multiplying by 100.

3.2.6 Staining and flow cytometry

IL-2 stimulated and unstimulated NK cells were adjusted to the concentration of 0.5 $X10^6$ cells/ml. The antibodies anti-Ly49A and anti-Ly49C conjugated with PE, anti-Ly49D and anti-NK1.1 conjugated with FITC were diluted to 1:100 in PBS. After mAb staining for 30 min at 4^oC, cells were washed with and re-suspended in ice-cold PBS buffer. All flow cytometry data were acquired using FACS LSR flow cytometer (BD Biosciences).

3.2.7 Isolation of Membrane fraction from cell lines

Membrane fractions from P815 cell line were isolated by using the modified protocol utilized in publication by Huang et al., 2012. The cells were cultured in T-25 tissue culture flask until 80% confluent at 37^oC with 5% CO_2. Cells were then washed with ice-cold PBS and suspended in SF buffer. The cell lysate were then agitated at 30-50rpm on tube roller and then centrifuged at 720xg at 4 °C for 5 min. The supernatant was collected and centrifuged at 10,000xg at 4 °C for 10 min. The supernatant was collected and ultracentrifuged at 100,000x g at 4 °C for 1 h. The pellet was re-suspended in SF buffer and ultracentrifuged at 100,000x g at 4 °C for 1 h. The supernatant was removed and the pellet was re-suspended in NL buffer, which contain membrane fraction. The total protein concentration of the isolated membrane fractions were quantified by BCA method (Smith et al., 1985). Absorbance values were determined at 562 nm on spectrophotometer. Purity of isolated membrane fractions were analysed by western blot technique using beta-catenin, cadherin, alpha-tubulin as internal controls. In this technique, the membrane and cytosolic fractions were separated on the SDS-PAGE on the basis of molecular weight. In the next step, the

separated proteins were transferred out of the gel to PVDF membrane (0.45µm). The transferred proteins were then verified by staining with Ponceau S staining solution for 5min incubation. After appearance of clear bands on PVDF membrane, the membranes were de-stained using PBST buffer. After electro-transfer of protein to a membrane, the blots were blocked to prevent non-specific binding with blocking buffer for 45min at 20-30rpm. On completion of incubation, the blocking buffer was removed by washing with PBST. Thereafter, diluted primary antibody (1:1000) specific either for cadherin (anti-mouse cadherin), alpha-tubulin (anti-mouse alpha tubulin), beta-catenin (anti-mouse beta-catenin) were added to each blot with cytosolic and membrane fractions and kept for overnight incubation at 4^0C. Thereafter, blots were washed with PBST two times for 5min. After washing, diluted HRP conjugated secondary antibody (1:2000) specific for primary antibody was added to the blots and incubated for 1.5h at 20-30rpm at RT. Blots were then washed three times for 10min. Then, the blots were detected using Electrochemiluminescence (ECL) reagents, A:B (1:1). These reagents identify the HRP enzyme conjugated to the secondary antibody and catalyze the ECL reaction and produce light. The proteins bands were then detected on transilluminator (DNR MiniBis-Pro).

3.2.8 Estimation of IFN-γ, and MIP-1α cytokines secretion using ELISA

Quantitative estimation of cytokines was carried out according to the manufacturer's instructions using ELISA (RandD Systems). In brief, the plates were coated by adding 100µl/well of capture antibody to the 96-well plates. The plates were sealed and incubated overnight at room temperature (RT). Upon completion of incubation period, the plates were aspirated and washed three times with wash buffer by firmly tapping

the plates upside down on absorbent paper. The plates were then blocked by adding 300µl/well blocking buffer and incubated at RT for 1h. Thereafter, plates were aspirated and washed three times. In the next step, 100µl/well of standards and test samples were added to the coated plates. The plates were sealed and incubated for 2h at RT. Upon completion of incubation period, samples were aspirated and plates washed three times with wash buffer. 100µl of diluted detection antibody specific either to IFN-γ or MIP-1α) was added to each well sealed and incubated for 2h at RT. Thereafter, plates were washed again four times with wash buffer. Then 100µl of diluted Streptavidin-HRP solution (1:40) was added to each well, sealed and incubated for 20 minutes at RT. Plates were washed again 4 times with wash buffer. Then, 100µl of substrate solution (1:1 H_2O_2: Tetramethylbenzidine (TMB) was added and incubated in the dark for 20 minutes. Positive wells should turn bluish in colour upon incubation with this step. Chromogenic reaction was stopped by adding 50µl of stop solution (2N H_2SO_4) to each well. Positive wells should turn from blue to yellow. Final absorbance was read at 450nm within 20 minutes of stopping reaction. Quantitative estimation of the expression of individual cytokine (IFN-γ, MIP-1α) was carried out by extrapolation in terms of pg/ml of protein obtained from a standard curve prepared in parallel using standard of individual cytokines supplied along with the individual kit.

3.2.9 Prediction of 3D structures of NK receptors and ligands

The structures of NK receptors and NK receptor complex and HLA ligand were retrieved from RCSB Protein Data Bank (Table 3.6) while structure of NK receptor KIR2DL1, classical HLA molecules were not available so they were predicted through

de novo method using Iterative Threading ASSEmbly Refinement (I-TASSER). I-TASSER is web-based protein 3D structure prediction tool based on threading approach. It simulates the generated structure and defines its *C*-score, TM-score, and active sites. *C*-score refers to a confidence level of predicted structure of a given protein sequences where a high *C*-score (range from −5 to 2) indicates an absolute precise quality of the predicted structure. TM-score is structural assessment parameter in which, a smaller distance between the structures is weighted high. TM-score defines the topology of the structure, and it shows that a score more than 0.5 ensures a model of absolute topology (Yang et al., 2015).

Generated 3D structures were further refined by using 3Drefine, a webserver that involves optimization of hydrogen bonding network combined with atomic-level energy minimization of optimized model (Bhattacharya et al., 2016) and GalaxyRefine server which rebuilds all side-chain conformations and repeatedly relaxes the structure by short molecular dynamics simulations (Lim et al., 2013). Structures with lowest RMSD value were selected for protein-protein interactions.

Table 3.6: List of NK receptors, ligand and complex structures available on PDB

	Resolution (Å)	PDB entry	Reference
Receptors			
KIR2DL2	2.9	2DL2	Snyder et al., 1999
NKG2A/CD94	2.5	3BDW	Sullivan et al., 2007
Ligands			
HLA-G	1.9	1YDP	Clements et al., 2005
NK receptors-HLA complex			
NKG2A/CD94-HLA-E	2.5	3CDG	Petrie et al., 2008
KIR2DL2/HLA-Cw3	3.0	1EFX	Boyington et al., 2000
KIR2DL1/HLA-Cw4	2.8	1IM9	Fan et al., 2001

3.2.10 Molecular docking of NK receptor–ligand complex

Interaction analysis of NK receptors and their putative ligand were predicted by ClusPro protein–protein molecular docking program. ClusPro server is a rigid docking program based on fast Fourier trans-formation, which clusters the interaction complexes with low energy and identifies the stability of the interaction clusters using the medium-range optimization algorithm. Resulting top docking score NK receptor-ligand were selected, and its docked complex were analysed to determine the molecular interactions.

3.2.11 Receptor-Ligand interaction analysis

NK receptor-ligand interactions in terms of Hydrogen bonds, non-hydrophobic interactions, Sulphide bonds, salt-bridges were analysed by using PDBsum server. Further, interface residues involved in interactions were determined by PDBsum. NK receptor-ligand complexes were evaluated for its stereo-chemical properties through Ramachandran plot using PDBsum (Procheck). Ramachandran plot determines the dihedral angles [phi (Φ) and psi (ψ)], and the number of residues lying in favoured, allowed, and outlier regions of the protein structure (LaskowskI et al., 2001).

3.3 Statistical Analysis

All experiments were repeated at least three times and data obtained were expressed as mean±standard deviation. The variation between the control and test groups was estimated. Significant variation between these groups were determined by one-way ANOVA (one way analysis of variance) followed by bon ferroni test. $p<0.05$ was considered significant across the entire experimental groups.

Chapter 4

Results

CHAPTER 4

RESULTS

4.1 NK cell isolation

NK cells were isolated from C57BL/6 mice spleenocytes through EasySep™ Mouse NK Cell Enrichment Kit. The EasySep™ Mouse NK Cell Enrichment Kit was designed to isolate NK cells from single cell suspensions of spleenocytes by negative selection upon depletion of non-NK cells, using antibody cocktail for non-NK cells (T-cells, B-cells, DCs and other non-NK). The non-NK cells were specifically labelled with dextran-coated magnetic particles using biotinylated monoclonal antibodies cocktail against cell surface antigens expressed on the unwanted cells, and bispecific tetrameric antibody complexes (TAC). The TAC binds to both dextran and biotin, small sized magnetic dextran allow efficient binding to the TAC-labelled cells. Magnetically labelled non-NK cells were then separated from unlabelled target NK cells by magnetic separation. Purity of NK cells was quantified by flow cytometry after staining with fluorochrome-conjugated antibodies against NK marker NK1.1 and T-cell marker CD3 (FITC anti-CD49b and APC anti- CD3 respectively). NK1.1 represent NKR-P1C gene and is the most characteristic serological marker expressed on murine NK cells. The PK136 mAb (anti-NK1.1) that reacts with the NKR-P1Cgene productwas used to detect NK cells before and after purification upon staining with anti-mouse NK1.1and anti-mouse CD3(Figure 4.1a and 4.1b). Purified NK population comprised of CD3 depleted and NK1.1 expressing cells ($NK1.1^{+}CD3^{-}$). The purity of NK cells was 92.7%.

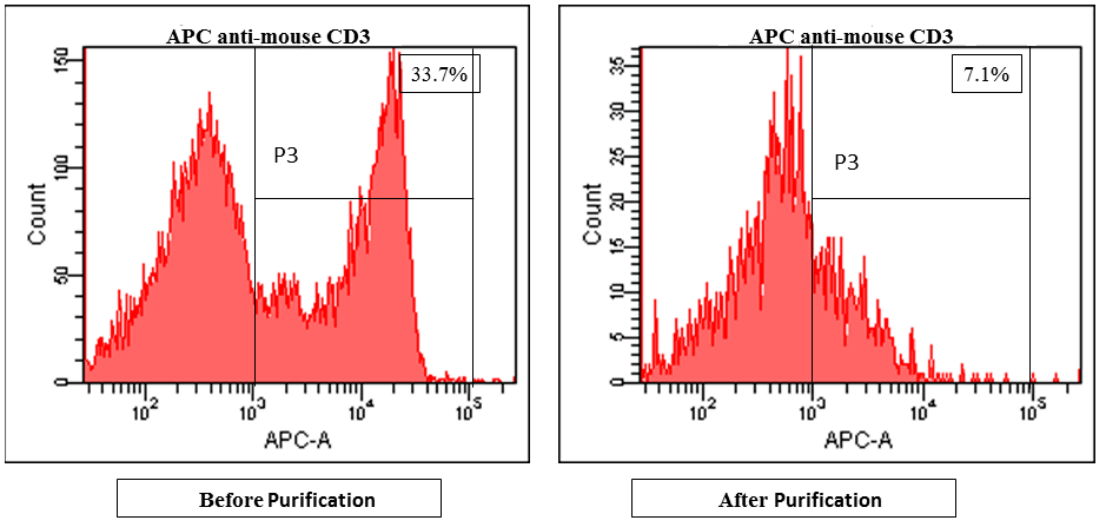

Figure 4.1a: Purification of NK cells. Spleenocytes subjected to purification (negative selection) were stained for $CD3^+$cells (APC anti-mouse CD3). Left panel shows population before purification and right panel show population after purification.

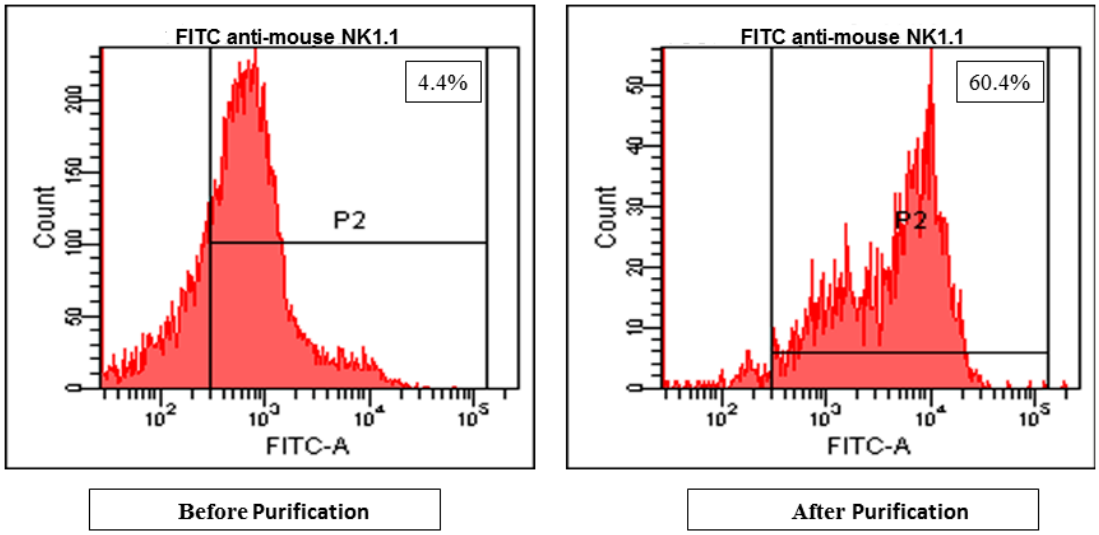

Figure 4.1b: Purification of NK cells. Spleenocytes subjected to purification (negative selection) were stained for $NK1.1^+$cells (FITC anti-mouse NK1.1). Left panel shows population before purification and right panel show population after purification.

4.2 Expansion of NK cells upon IL-2 stimulation

IL-2 is one of the key cytokine with multiple effects on immune cells like proliferation and differentiation of $CD4^+$ T cells and NK cells into a variety of subsets, modulation of

T-cell function in response to antigen and NK cell cytotoxic function. NK cells have IL-2Rβγ receptor and hence IL-2 binds with intermediate affinity to NK cells and optimal doses of IL-2 were able to stimulate NK cell expansion (mentioned in section 2.10). Therefore, in our study, purified NK cells were stimulated with IL-2 (100U) and NK cell proliferation and viability was evaluated by trypan blue exclusion method. The cells were counted by haemocytometer on overnight incubation at 37^0C, 5% CO_2. A definitive increase in NK cell population was observed upon IL-2 stimulation in comparison to un-induced controls ($p<0.05$) (Figure 4.2).

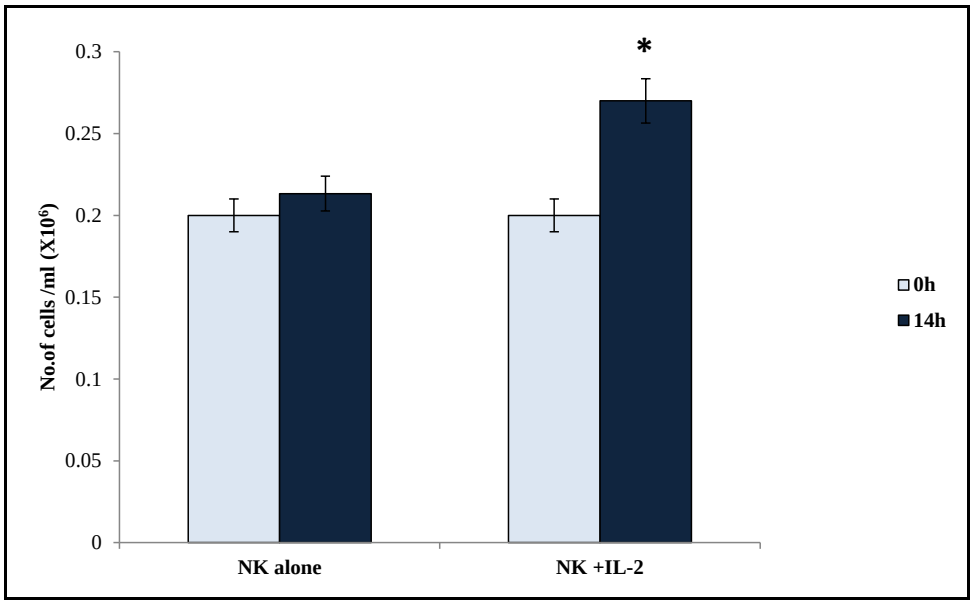

Figure 4.2: Viable cell count upon IL-2 stimulation of NK cells. Viable NK cell count at 0h and 14h incubation in absence (NK alone) and presence (NK+IL-2) of IL-2 (*$p<0.05$). Values represent mean ± SD of 3 independent experiments.

4.3 Effect of IL-2 stimulation of NK cells on NK cell inhibitory receptor profile

NK cells are known to be responsive to IL-2 and in tumor microenvironment *in situ*, tumor infiltrating lymphocyte stimulation leads to IL-2 secretion by T cells and hence

we wanted to study the effect of IL-2 stimulation upon NK receptor expression profile. Since, IL-2 stimulation resulted in proliferation of population of NK cells (Figure 4.2), we wanted to explore the effect of IL-2 stimulation on the expression level of inhibitory receptors. Effect of IL-2 stimulation was studied on inhibitory NK cell receptors, Ly49A and Ly49C, (Figure 4.3).

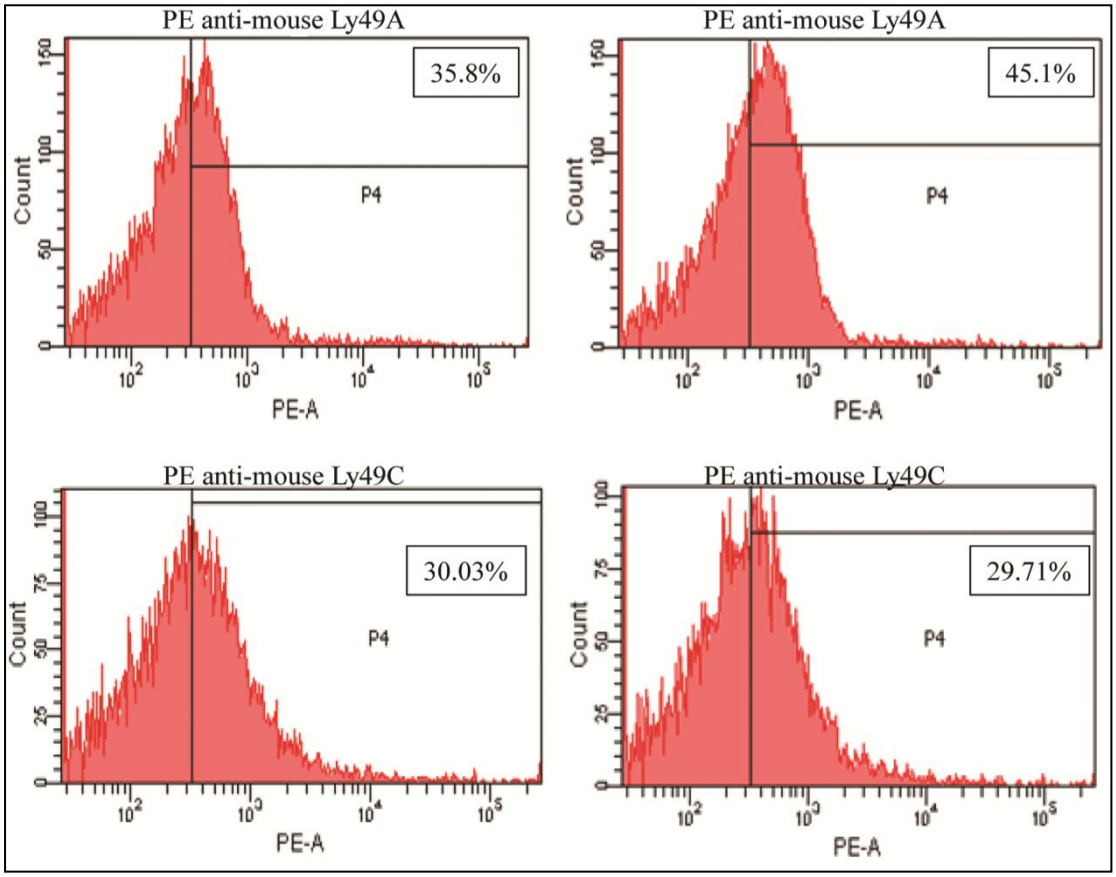

Figure 4.3: Effect of IL-2 stimulation on NK cell inhibitory receptors. The upper panels describe the expression of Ly49A (PE anti-mouse Ly49A), while lower panels depict the expression of NK cell inhibitory receptor Ly49C (PE anti-mouse Ly49C) on purified NK cells in absence (left panels) and presence of IL-2 (right panels).

It was observed that the expression of inhibitory receptor Ly49A increased upon stimulation with IL-2 ($p<0.05$) while there was no significant alteration in inhibitory receptor Ly49C expression (Table 4.1). It may be noted that C57BL/6 mice used in this

study had MHC haplotype H2b. Ly49A is the cognate inhibitory receptor for H2k and H2d. While Ly49C is the cognate receptor for H2b. The NK harvested from C57BL/6 mice had H2b background and hence our results showed that IL-2 induced a more pronounced stimulation of inhibitory receptor expression on non-cognate inhibitory receptor, as compared to the cognate receptor.

Table 4.1: Percentage of activating and inhibitory receptors expressing NK cells in absence (NK alone) and presence (NK+IL-2) of IL-2

NK receptor	NK alone*	NK+IL-2*
Ly49A	35.8	45.1
Ly49C	30.03	29.71
Ly49D	32	32.6
NK1.1	10.9	30.2

*Values represent mean ± SD of 3 independent experiments.

4.4 Effect of IL-2 stimulation of NK cells on NK cell activating receptor profile

IL-2 is known to induce NK cell proliferation therefore, we wanted to explore if IL-2 stimulation also resulted in increased NK activating receptor expression and thus modulated NK cytolytic potential. So, role of IL-2 induced NK cell proliferation was studied in the context of expression level of activating receptors on NK cells. We noticed a significant increase in expression level of a pan-NK marker, NK1.1 (Figure 4.4). However, there was no significant increase in the expression of Ly49D upon IL-2 stimulation of NK cells ($p>0.05$). Thus, IL-2 stimulation resulted in NK cell expansion but did not cause upregulation of activation receptor, Ly49D expression. Figure 4.4 shows that there was no significant change in Ly49D expression on per cell basis upon

IL-2 stimulation. Further, a drop in peak was observed, which also signified a decrease in per cell basis of Ly49D expression despite proliferation of NK cells. We hypothesize that this was a significant strategy employed by the immune system to ensure that proliferative stimulation of NK cells did not result in hyper-responsiveness of NK cells. In the context of any infection and stimulation of T cells, there would be a local increase in the levels of IL-2, however although this would resulted in significant expansion of functional NK cells, such NK cells were not hyperactive or potentially deleterious to self-cells.

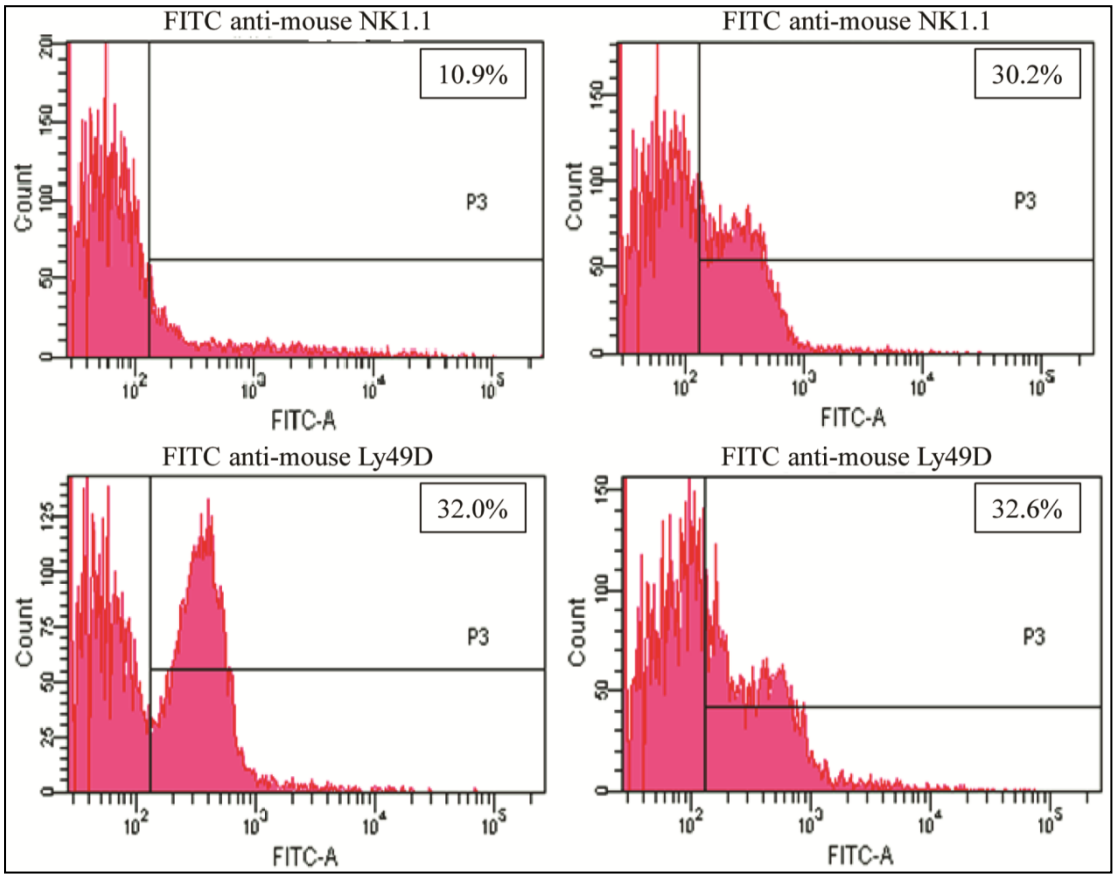

Figure 4.4: Effect of IL-2stimulation on NK cell activating receptors. The upper panels describe the expression of NK cell activating receptor NK1.1 (FITC anti-mouse NK1.1) and lower panels describe the expression of Ly49D (FITC anti-mouse Ly49D) on purified NK cells in absence (left panels) and presence of IL-2 (right panels).

4.5 Effect of IL-2 on NK mediated cytokine secretion

NK cells have the capacity to directly mount an immune response to aberrant cell by virtue of their ability to secrete cytokines and chemokines. IFN-γ and MIP-1α are among most prominent cytokines produced by NK cells against their targets. We further showed that although IL-2 stimulation resulted in significant increase in NK population and increase in expression of NK activation marker NK1.1 as shown in Figure 4.1 and 4.4, this did not result in hyperactivity of the NK cells. Figure 4.5 and 4.6 showed that there was no appreciable increase in MIP-1α and IFN-γ production by the NK cells upon IL-2 stimulation ($p>0.05$). Incubation of NK cells with IL-2 resulted in only 0.2-fold increase in secretion of MIP-1α and IFN-γ (Figure 4.5 and 4.6) as compared with unstimulated NK cells. Thus, IL-2 alone caused proliferation of NK cells but did not result in hyperactivity of NK cells. However, these IL-2 expanded NK cells were not inactivated and in the presence of susceptible tumor cells (YAC-1) potentially mounted a very robust response in MIP-1α and IFN-γ production as represented in Figure 4.5 and 4.6. NK cells co-cultured with tumor cells in absence of IL-2 showed significantly lower activity.

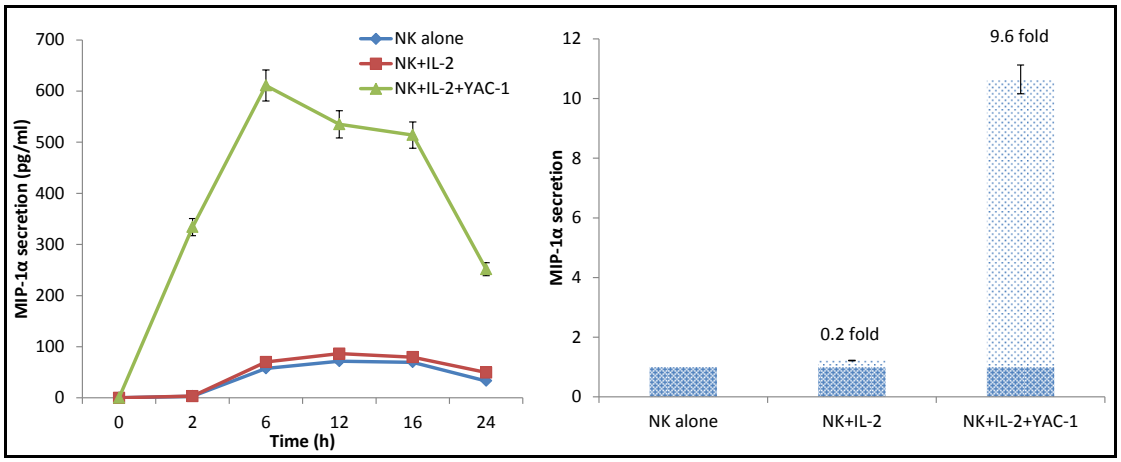

Figure 4.5: Kinetics and profile of MIP-1α secretion on IL-2 stimulation. NK cells were incubated alone or with IL-2 or with YAC-1 cells as positive control at 37^0C. Supernatants were harvested at different time points and as indicated MIP-1α secretion was determined by using ELISA kit. Values represent mean ± SD of 3 independent experiments.

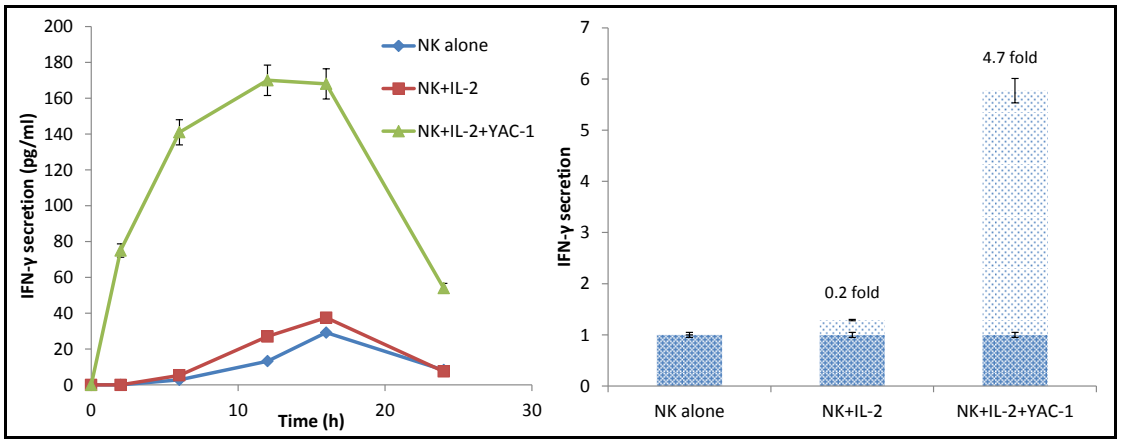

Figure 4.6: Kinetics and profile of IFN-γ secretion on IL-2 stimulation. NK cells were incubated alone or with IL-2 or with YAC-1 cells as positive control at 37^0C. Supernatants were harvested at different time points and as indicated IFN-γ secretion was determined by using ELISA kit. Values represent mean ± SD of 3 independent experiments.

Hence in the presence of tumor, we observed a specific increase in activity of NK cells. However, in absence of tumor the hypo-responsiveness of the IL-2 induced expansion of NK population was a measure of extensive proliferation of potentially active NK cells but refrained from hyperactivity. Such IL-2 proliferated cells were potentiated and hyperactive upon encountering target cells like tumor cells and can immediately cause significant increase in NK activity.

4.6 Effect of culturing NK cells with NK resistant cell line P815 on NK cell receptor profile

In order to investigate whether P815 cells could affect the expression of NK cells receptors relevant for tumor cell killing, NK cells were co-cultured overnight with P815 cells at 37°C in 5% CO_2. Before, co-culturing of NK cells with P815 cells, generation of a growth curve and viability count was performed to evaluate the growth characteristics and assess the viability of P815 targets. The generation time observed was approx. 19 hours in DMEM with high glucose and 10% FBS. NK cells were then

cultured with P815 cells (2:1 ratio). Trypan blue exclusion assay was used to assess the viability of NK cells and P815 cells on overnight incubation of cells at 37°C, 5% CO_2 (Figure 4.7). There was also no difference in the peak pattern of Ly49D suggesting no significant change in Ly49D on a per cell basis too.

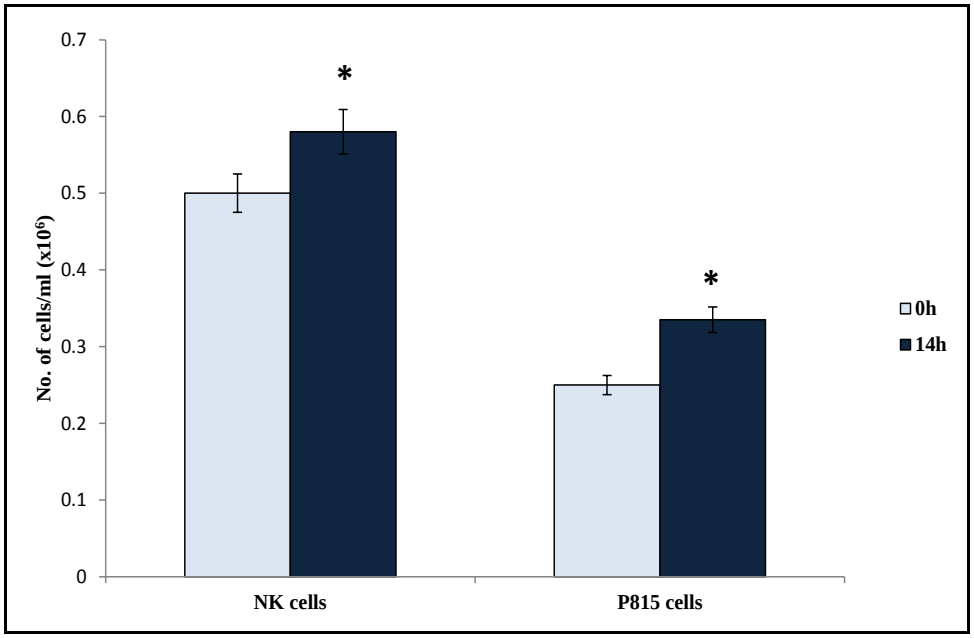

Figure 4.7: Viable cell count upon co-culture of NK cells with tumor cells. Viability of NK cells and P815 cells at 0h and 14h incubation on co-culture assayed by trypan blue exclusion method (*$p<0.05$). Values represent mean ± SD of 3 independent experiments.

After 14h in culture, the viability of cells was more than 90%. The expression level of NK inhibitory receptors Ly49A and Ly49C as well as surface marker NK1.1 and activating receptor Ly49D were examined through flow cytometry (Figure 4.8 and 4.9). It was observed that co-culture of NK cells with P815 cells showed increase in expression of Ly49A and a significant increase in the inhibitory receptor Ly49C ($p<0.05$), which is cognate receptor for the MHC haplotype of the mouse model (C57BL/6).

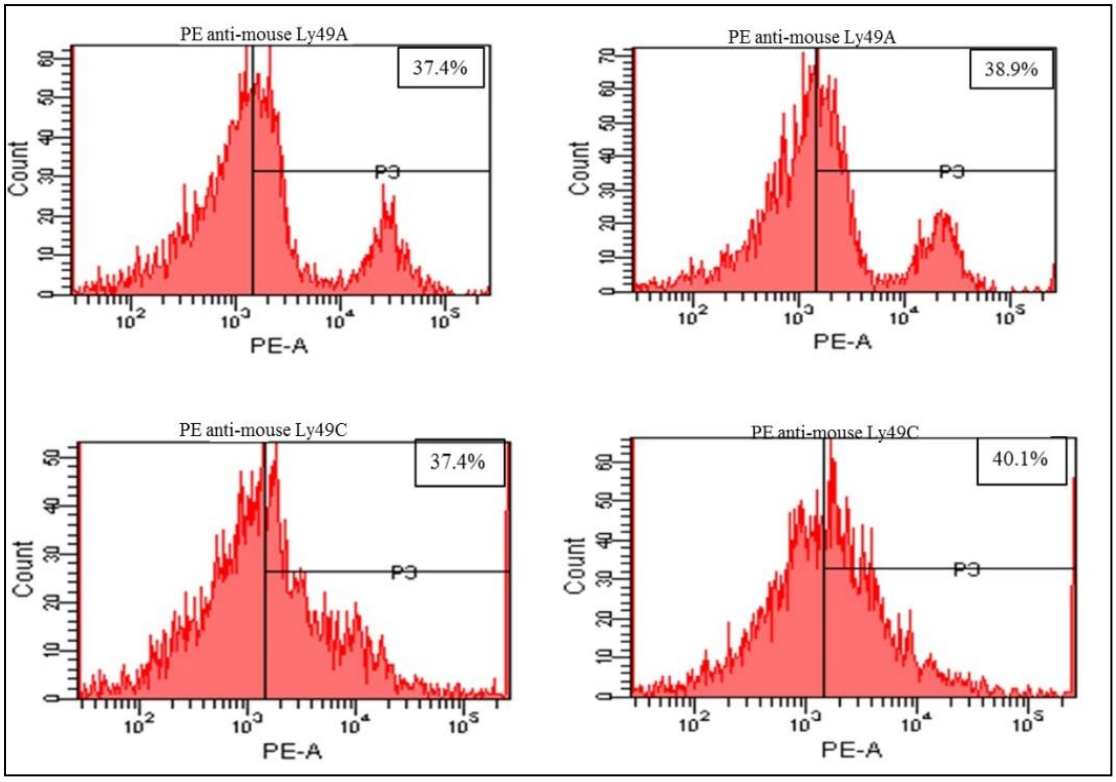

Figure 4.8: Effect of co-culture of NK cells with P815 cells (2:1) on inhibitory receptors expression profile. The upper panel depict the expression of NK cell inhibitory receptor Ly49A (PE anti-mouse Ly49A), while lower panel describe the expression of NK cell inhibitory receptor Ly49C (PE anti-mouse Ly49C) on IL-2 stimulated NK cells in absence of P815 tumor cells (left panels) and NK cells with P815 cells (right panels).

NK cell proliferation and activation was determined by the expression of surface marker NK1.1. We found a significant increase in the expression of NK1.1 upon overnight incubation of NK cells with P815 cells ($p<0.05$). Ly49D is known to be associated with DAP-10 and DAP-12 that contains an immunoreceptor tyrosine-based activation motif and mediates activation of NK cells (as mentioned in section 2.3.1). Hence, the expression of activating receptor Ly49D was also studied on co-culturing NK cells with P815 cells. We found that there was no increase in expression of Ly49D (Figure 4.9). There was also no difference in the peak pattern of Ly49D suggesting no significant change in Ly49D on a per cell basis too.

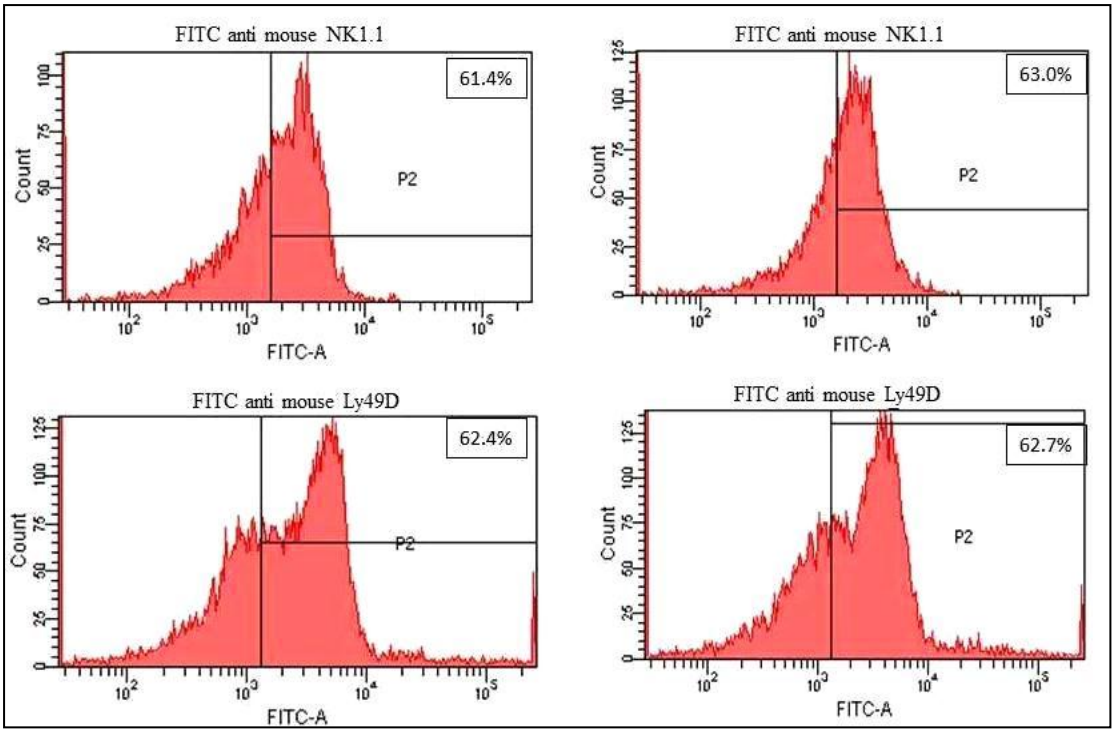

Figure 4.9: Effect of co-culture of NK cells with P815 cells (2:1) on activating receptors expression profile. The upper panel describe the expression of NK cell surface marker NK1.1 (FITC anti-mouse NK1.1) lower panel depict the expression of NK cell activating receptor Ly49D (FITC anti-mouse Ly49D) on IL-2 stimulated NK cells in absence of tumor (left panels) and NK cells with P815 cells (right panels).

4.7 Effect of culture of NK cells with P815 supernatant on NK receptor profile

The above findings concluded that NK-target interaction caused receptor modulation. To determine the factors responsible for the NK receptor modulation, NK cells were incubated with supernatant harvested from P815 cells. Prior to the experiment, P815 cells were adapted to grow in serum-free medium to eliminate the serum constituents that might interfered with P815-derived factors or may itself be involved in alteration of NK cell receptor profile. However it raised the question whether such serum starved cells are metabolically viable comparable to the tumor cells grown in the presence of serum. Viability of P815 cells adapted to serum-free medium was also checked and were observed to be more than 90% viable (Figure 4.10).

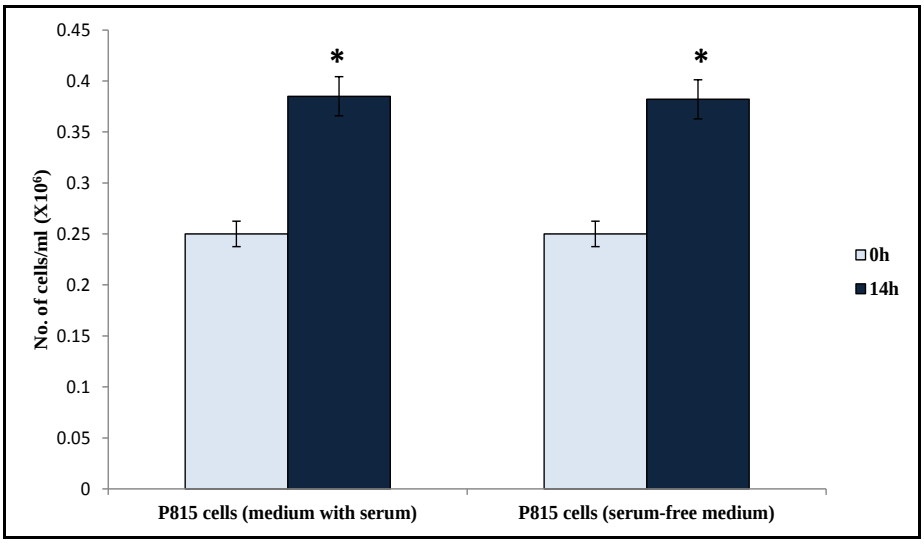

Figure 4.10: Viable cell count upon adaptation of P815 cells in serum-free medium. Viability of P815 cells at 0h and 14h incubation on culturing in medium with serum and serum-free medium assayed by trypan blue exclusion method (*$p<0.05$). Values represent mean ± SD of 3 independent experiments.

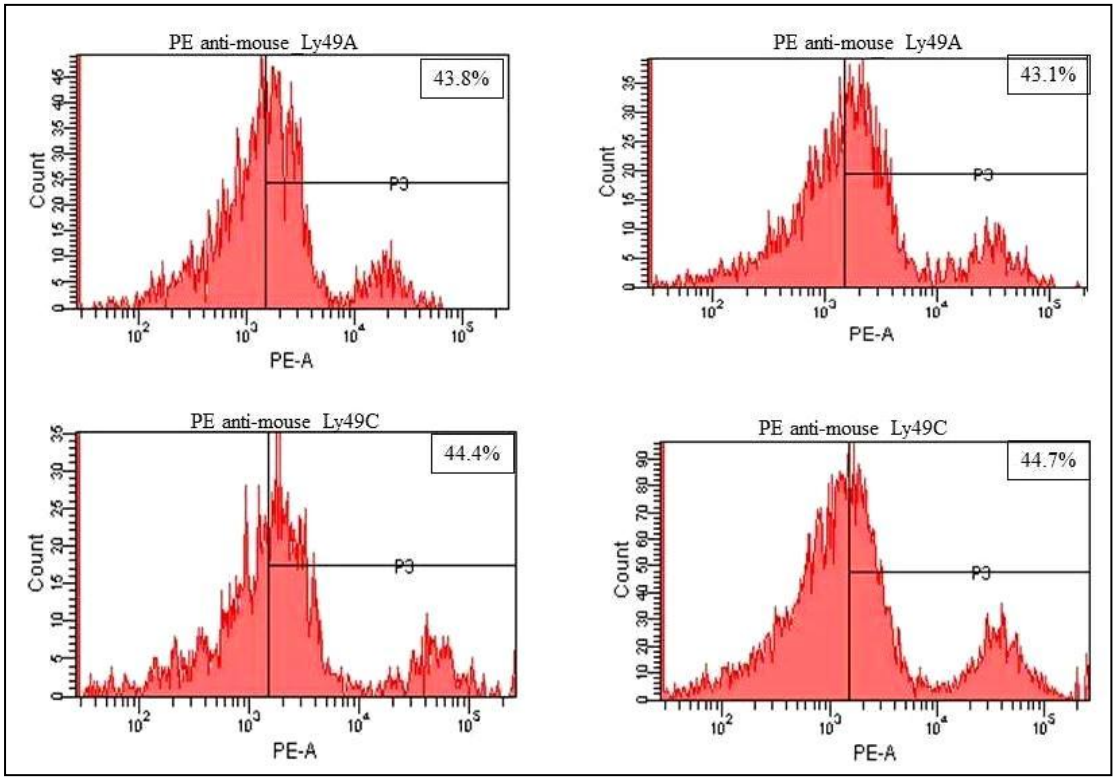

Figure 4.11: Effect of co-culture of NK cells with P815 cell supernatant on inhibitory receptors expression profile. The upper panel depict the expression of NK cell inhibitory receptor Ly49A (PE anti-mouse Ly49A), while lower panel describe expression of NK cell inhibitory receptor Ly49C (PE anti-mouse Ly49C) on IL-2 stimulated NK cells alone (left panels) and NK cells with P815 supernatant (right panels).

NK cells were incubated with P815 cell supernatant at 5% CO_2, 37^0C. The receptor profile of NK cells inhibitory receptors Ly49A and Ly49C as well as surface receptor NK1.1 and activating receptor Ly49D were analysed. There was no alteration in NK receptor expressions as compared to the IL-2 stimulated NK cells in the absence of tumor ($p>0.05$) (Figure 4.11 and 4.12). This suggested that the NK cell receptor alteration seen upon co-culture of P815 cells with NK cells was not due to soluble factors produced by NK resistant tumor, P815 cells.

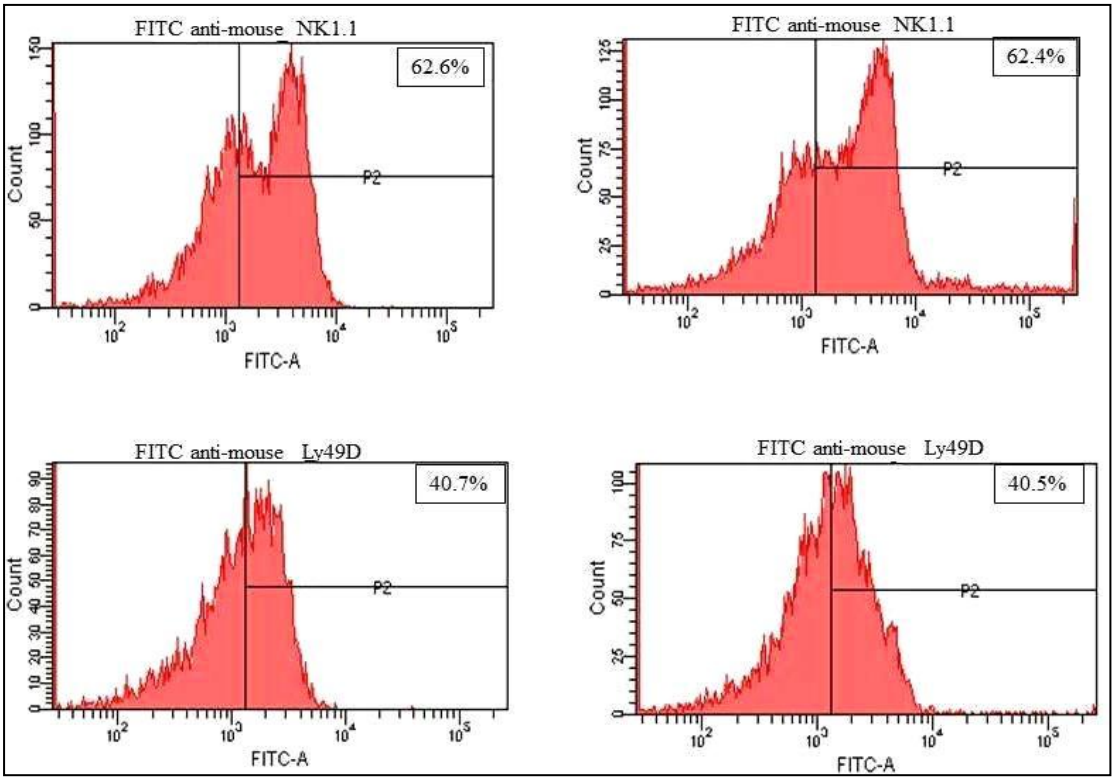

Figure 4.12: Effect of co-culture of NK cells with P815 cell supernatant on activating receptors expression profile. The upper panel describe the expression of NK cell surface marker NK1.1 (FITC anti-mouse NK1.1). Lower panel depict the expression of NK cell activating receptor Ly49D (FITC anti-mouse Ly49D) on IL-2 stimulated NK cells without tumor supernatant (left panels) and NK cells with supernatant (right panels).

4.8 Effect of co-culture of NK cells with P815 cells using transwell assay on NK cells receptor profile

Since there was significant alteration in inhibitory receptor profile upon co-culture of P815 cells with NK cells and there was no alteration in Ly49A and C expression on NK cell upon co-culture with P815 cell supernatant, it raised the possibility that direct contact between the target and effector cells was required for this modulation. In an attempt to investigate this mechanism, NK cells were cultured in serum-free medium in microtiter well plates with P815 cells (NK/P815 ratio: 4/1) and in the transwell separated by semi permeable membrane (NK cells in lower transwell and P815 cells in upper transwell). Upon overnight incubation, NK cells and P815 cells were harvested, counted, and analysed for the expression of activating receptors NK1.1, Ly49D and inhibitory receptors Ly49A and C (Figure 4.13 and 4.14).

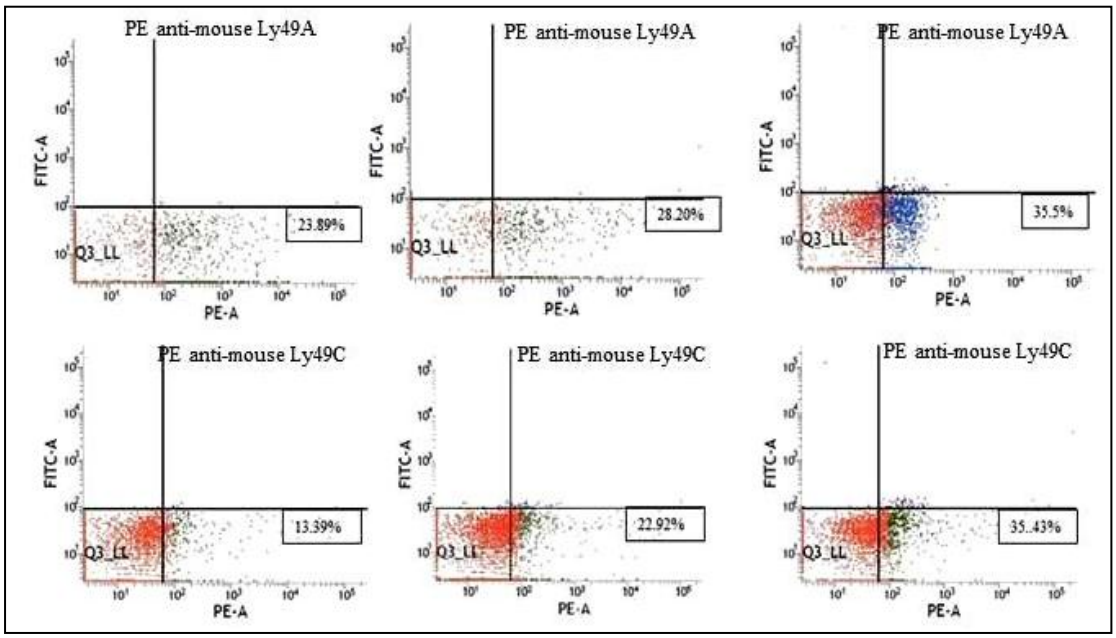

Figure 4.13: Effect of NK cells inhibitory receptors on co-culture of NK cells with P815 cells in transwells. The upper panel describe the expression of NK cell inhibitory receptor Ly49A (PE anti-mouse Ly49A), while lower panel describe the expression of NK cell inhibitory receptor Ly49C (PE anti-mouse Ly49C) on IL-2 stimulated NK cells in absence of tumor (left panels), NK cells with P815 cells in trans-wells (middle panels) and NK cells with P815 cells in same wells (right panels).

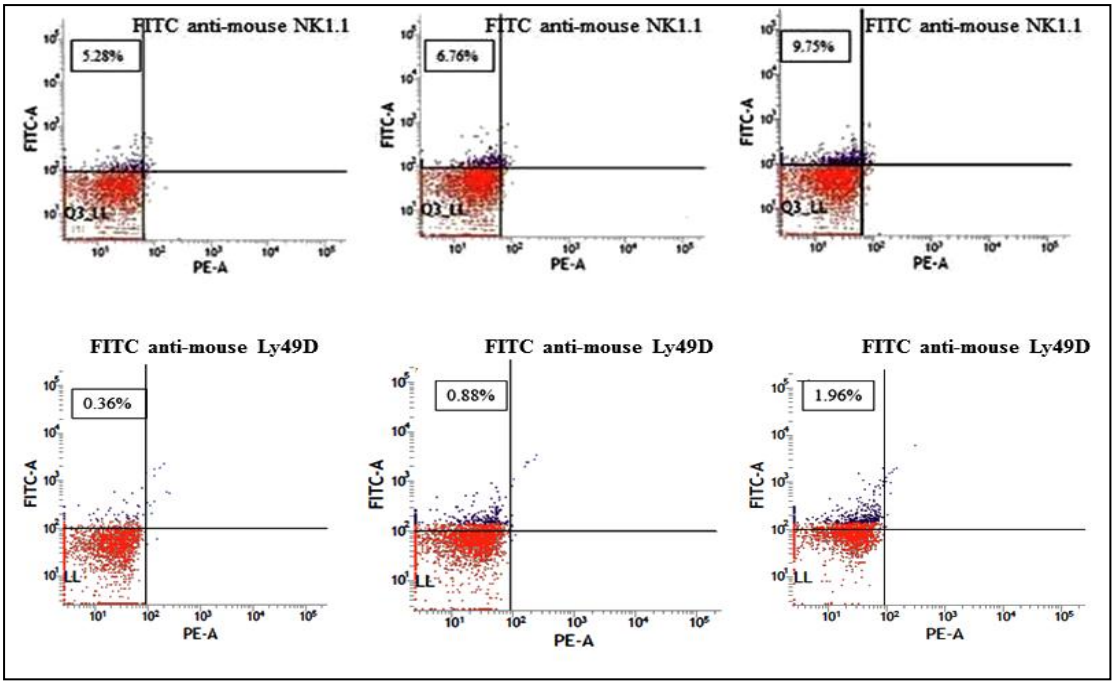

Figure 4.14: Effect of NK cells activating receptors on co-culture of NK cells with P815 cells in transwells. The upper panel describe the expression of NK cell receptor NK1.1 (FITC anti-mouse NK1.1), while lower panel describe the expression of NK cell activating receptor Ly49D FITC anti-mouse Ly49D) on IL-2 stimulated NK cells in absence tumor (left panels), NK cells with P815 cells in trans-wells (middle panels) and NK cells with P815 cells in same wells (right panels).

Population of both cells increased on 14h incubation and more than 90% cells were viable as seen by trypan blue exclusion assay. There was a significant increase in the expression of NK cell inhibitory receptors on co-cultured cells in same well compared to cells grown in transwell plates, where direct contact between target and effector cell was prevented by the presence of the semipermeable membrane. However the semipermeable membrane did allow supernatant factors to pass through.

From these findings, it may be concluded that direct cell to cell interaction between target and effector was required for alteration in NK cell receptor profile. Moreover, the target cells harvested from the upper well in transwell plate were also analysed for the expression of NK cells receptors (data not shown). But the target cells did not express any of these NK specific receptors being studied. Thus, conclusively showed that there was no

penetration of NK cells through the semipermeable membrane and no cross contamination was ruled out. Thus, NK receptor modulation strategy employed by tumor cell expressed ligands was indeed mediated by NK-target surface interaction.

4.9 Effect of culturing NK cells with P815 membrane derived peptides on NK cells receptor profile

In the above findings, we observed that neither supernatant harvested from P815 cells nor transwell culture of NK cells and target cells caused any modulation in NK cell receptor profile. Therefore tumor derived soluble factors were not responsible for alteration of NK receptor profile. We therefore isolated cell membrane of P815 cells to investigate whether tumor derived membrane components were capable of modulation of NK cell receptor profile.

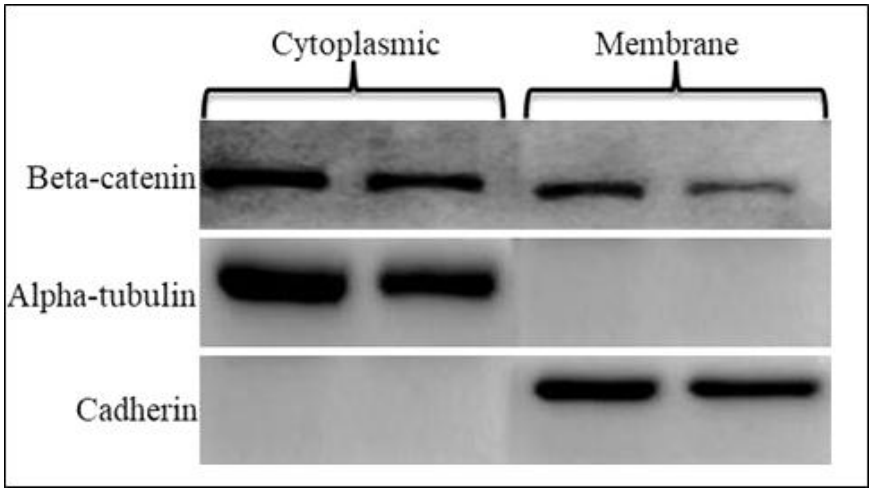

Figure 4.15: Western blot of P815 cells membrane derived protein. The P815 membrane derived components expressed beta-catenin which is expressed by all cytoplasmic and membrane bound proteins and also cadherin which is specific marker for membrane bound proteins. The P815 membrane derived components did not express alpha-tubulin (internal control, expressed by cytoplasmic proteins).

The tumor membrane isolated from P815 cells were analysed for purity by western blot (Figure 4.15). The P815 membrane derived components expressed beta-

catenin which is expressed by all cytoplasmic and membrane bound proteins and also cadherin which is a specific marker for membrane bound proteins. The P815 membrane derived components did not express alpha-tubulin which is expressed by cytoplasmic proteins. Thus, P815 membrane fraction was conclusively identified as membrane derived fraction, not contaminated with soluble factors derived from the tumor. P815 membrane peptide was co-cultured with IL-2 stimulated NK cells. These were then analysed for alteration in expression of activating NK cell receptors NK1.1 and Ly49D as well as inhibitory receptors Ly49 A and Ly49C. It was observed that there was slight increase in expression of Ly49A but a very significant increase in expression of inhibitory receptor Ly49C ($p<0.05$) (Figure 4.16).

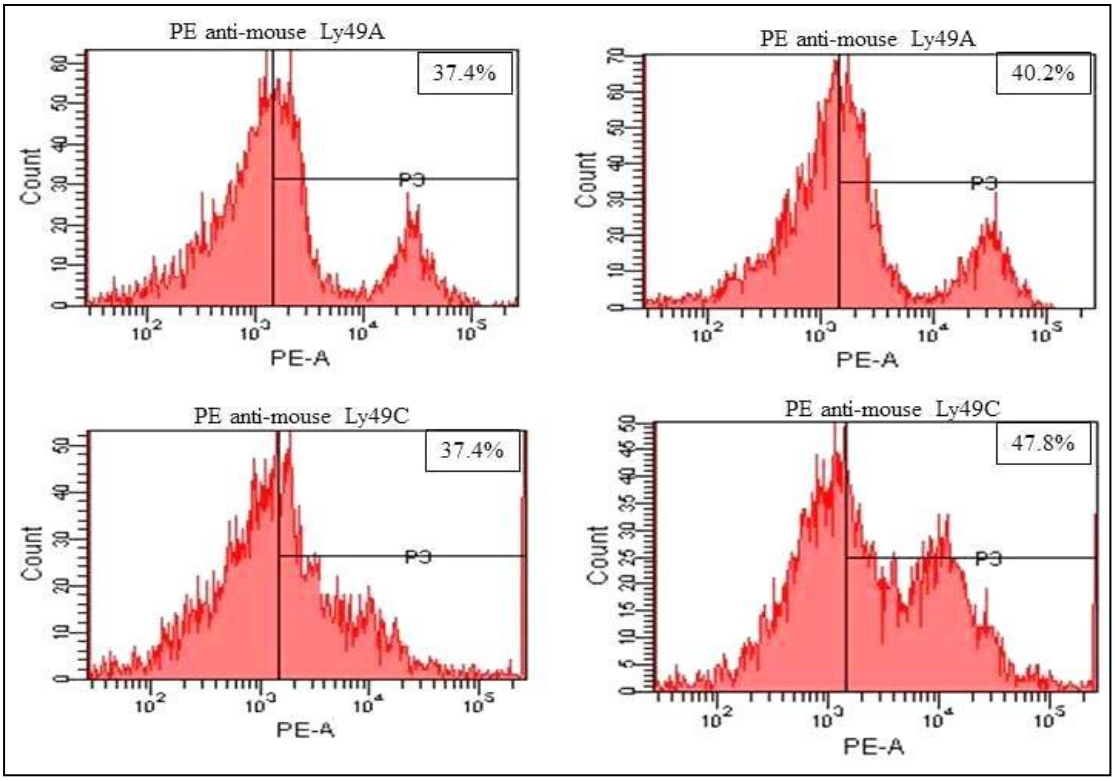

Figure 4.16: Effect of culturing NK cells with P815 membrane derived peptide on inhibitory receptors expression profile. The upper panel depict the expression of NK cell inhibitory receptor Ly49A (PE anti-mouse Ly49A), while lower panel describe the expression of NK cell inhibitory receptor Ly49C (PE anti-mouse Ly49C) on IL-2 stimulated NK cells in absence of tumor (left panels) and NK cells with P815 membrane derived peptide (right panels).

We observed in our studies that IL-2 alone caused a more significant stimulation of Ly49A expression as compared to Ly49C (Sharma and Das, 2018). However in the presence of P815 membrane, the induced boosting of inhibitory receptor expression was greater in Ly49C. In C57BL/6 mice which has H2b background, Ly49C is the cognate inhibitory receptor and upon co-culture of P815 derived membrane fractions with NK cells, there was a more significant change in the cognate inhibitory receptor expression as compared to the non-cognate inhibitory receptor. P815 has high expression of MHC I and significant boosting of Ly49C expression by P815 membrane components indicate greater engagement of cognate inhibitory receptor by P815 expressed ligands thus resulted in tumor mediated specific boost in inhibition of NK mediated cytolysis.

We observed no significant change in expression of activating receptor Ly49D ($p>0.05$) but there was a significant change in expression of surface marker NK1.1 ($p<0.05$) (Figure 4.17). This implies that in response to IL-2 stimulation, NK cells proliferated but in the presence of P815 membrane fraction, there was no significant alteration of activating receptor, Ly49D expression. Thus tumor derived factors were unable to significantly modulate NK activating receptors. Hence P815 membrane resulted in increase in expression of inhibitory receptors for MHC I, thus increasing the overall inhibition signal by the NK cells, helping the tumor cells to evade NK mediated cytolysis.

NK cells have been reported to secrete various cytokines and chemokines, most prominently IFN-γ and MIP-1α upon target cell interaction, which is indicative of NK cell activation potential. So, we determined the secretion profile of IL-2 stimulated NK cells upon co-culture with target P815 derived membrane peptides. We observed no significant increase in IFN-γ (0.1 fold) and MIP-1α (0.07 fold) secretion respectively compared to IL-2 stimulated NK cells in absence of tumor (Figure 4.18). Thus, the

alteration of NK receptor profile resulted in significant increase in inhibitory receptors expression of inhibition of decrease in NK activity.

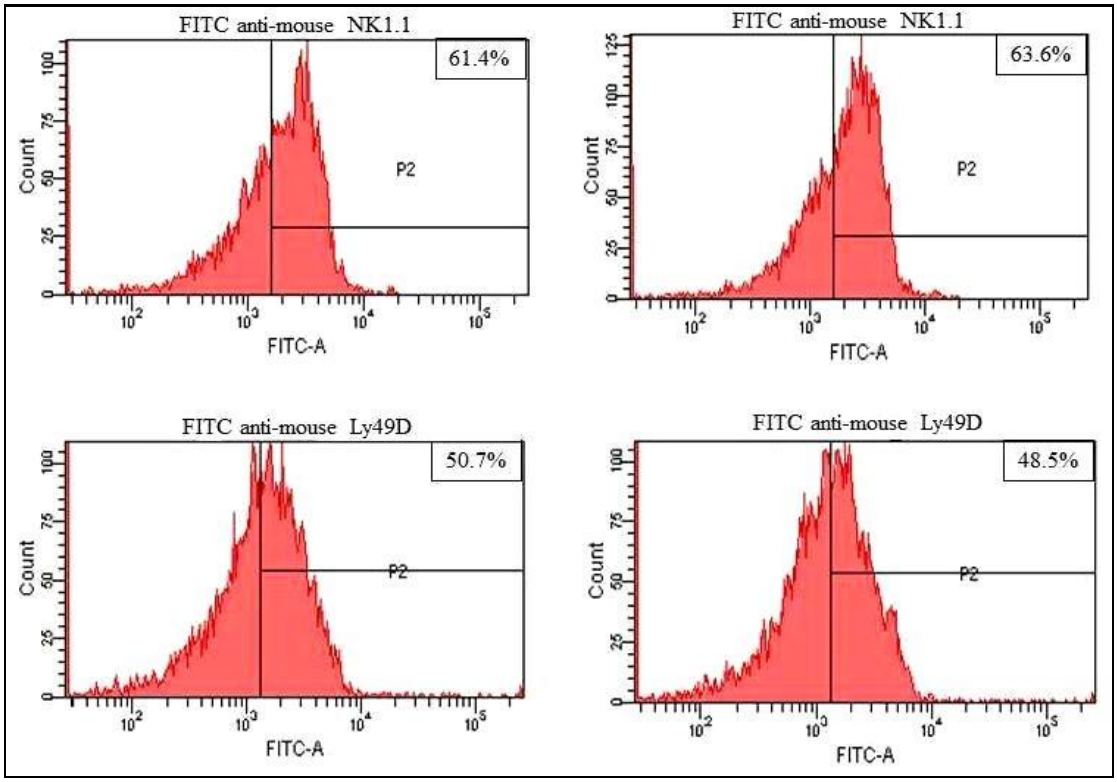

Figure 4.17: Effect of culturing NK cells with P815 membrane derived peptide on activating receptors expression profile. The upper panel describe the expression of NK cell surface marker NK1.1 (FITC anti-mouse NK1.1) and lower panel describe the expression of NK cell activating receptor Ly49D (FITC anti-mouse Ly49D) on IL-2 stimulated NK cells in absence of tumor (left panels) and NK cells with P815 membrane derived peptide (right panels).

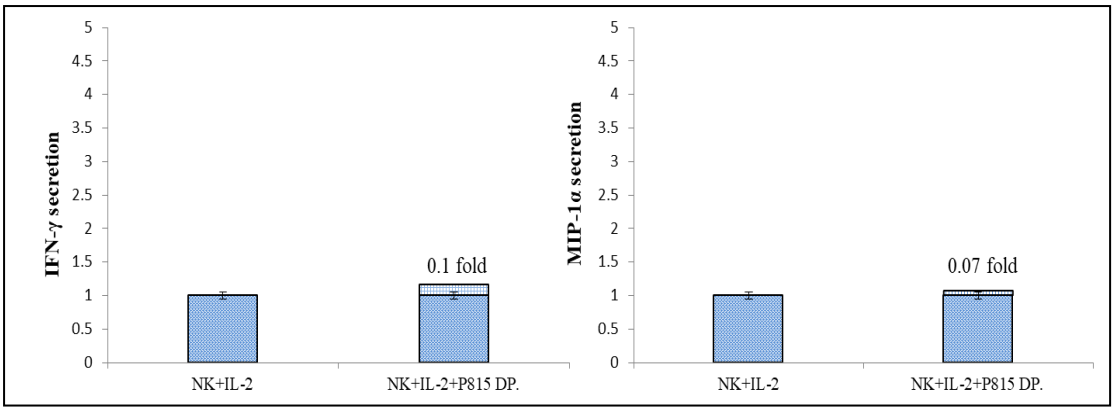

Figure 4.18: Secretion profile of IFN-γ and MIP-1α. Secretion profile of IFN-γ and MIP-1α (pg/ml) on interaction with P815 cells derived protein (P815 DP.) Values represent mean ± SD of 3 independent experiments.

4.10 Analysis of interaction of NK inhibitory receptors with classical HLA molecule

Inhibitory Killer Immunoglobulin Receptors (KIR) belongs to Ig superfamily and contain ITIM sequences in their cytoplasmic domain which initiate an inhibition signal upon engagement of these receptors by corresponding ligand. KIRs specifically bind to classical HLA class-I molecules and transduce inhibition or activation signal depending upon ITIM or ITAM driven signalling processes. Among KIRs, mainly KIR2DL1 and KIR2DL2 expressions have been reported to be associated with cancer progression and inhibitory KIR-ligand combination frequency had been correlated with tumor recurrence (Benson and Caligiuri**,** 2014, Beksac et al., 2015). Therefore, the present study was focussed on interaction studies of KIR2DL1 and KIR2DL2 with their cognate ligands (Table 4.2).

Table 4.2: Cognate ligands for different NK cell inhibitory receptors

Receptors	Ligands
KIR2DL1	HLA-Cw2, HLA-Cw4, HLA-Cw5
KIR2DL2	HLA-Cw1, HLA-Cw3, HLA-Cw7
NKG2A/CD94	HLA-G, HLA-E

The 3D structures of KIR2DL1, HLA-Cw1, HLA-Cw2, HLA-Cw5 and HLA-Cw7 were not available, so the structures were predicted using I-TASSER, which predicted five models for each receptor/ligand molecule. Among these models, the models with high *C*-score, TM-score were subjected to refine for energy minimization, stabilization and flexibility of structures (Table 4.3). It was observed that the RMSD scores obtained

from 3Drefine server were lowest among RMSD scores obtained from other structures refining servers (Table 4.4).

Table 4.3: Lowest scores of modelled 3D structures obtained from I-TASSER

	HLA-Cw1	HLA-Cw2	HLA-Cw5	HLA-Cw7	KIR2DL1
C-score	0.22	-1.15	0.30	-1.45	-2.19
TM-score	0.74±0.11	0.57±0.15	0.75±0.10	0.54±0.15	0.46±0.15
RMSD	4.9±3.2Å	9.2±4.6Å	5.5±3.5Å	10.0±4.6Å	11.6±4.5Å

Table 4.4: RMSD scores (Å) after refinement of structures with different refinement server

	HLA-Cw1	HLA-Cw2	HLA-Cw5	HLA-Cw7	KIR2DL1
ProTsav score	0.540	0.513	0.477	0.587	0.560
#3Drefine Score	0.173	0.209	0.171	0.209	0.225
Galaxyrefine Score	0.380	0.509	0.402	0.450	0.540

#RMSD scores (in red) obtained from 3Drefine server were lowest among all 3 structures refining server

The structures with lowest RMSD scores that correspond to the more stable structure were selected for molecular docking. The receptor-ligand complexes were then docked using ClusPro and H-bond interactions, non-bonded contacts and area involved in interactions were obtained through PDBsum server (summarized in Table 4.5). The interface amino acids residues involved in the interactions at the receptor- ligand interface have been shown in Figure 4.19a to 4.19d. It was observed that more number of chains were involved in interaction of KIR2DL1 with HLA-Cw4 (5 chains) and KIR2DL2 with HLA-Cw3 (4 chains) as compared to interactions of KIRs with their other cognate ligands (2 chains). The presence of multiple amino acid chains in KIR2DL1-HLA-Cw4

and KIR2DL2-HLA-Cw3 complexes signified that more number of amino acid residues was involved in these interactions as compared to other ligand interactions with these receptors. Moreover, multiple hydrogen bonds were involved in interactions of different chains in KIR2DL1-HLA-Cw4 (2-14 hydrogen bonds) and KIR2DL2-HLA-Cw3 (1-13 hydrogen bonds) complexes respectively. On the other hand, the number of hydrogen bonds was comparatively lesser 10, 9, 9 and 8 in KIR2DL1-HLA-Cw1, KIR2DL1-HLA-Cw5, KIR2DL2-HLA-Cw1 and KIR2DL2-HLA-Cw7 complexes. Also, KIR2DL1-HLA-Cw4 complex observed to have lowest energy (-1235.3 Kcal/mol) compared to other KIRs-cognate ligand complexes (Table 4.5).

Table 4.5: Molecular interactions between NK inhibitory receptors with classical HLA molecules

Receptor ligand complex	Energy (Kcal/mol)	No. of interface residues chain:chain	Interface area (A^2)	No. of salt bridges	No. of sulphide bonds	No. of hydrogen bonds	No. of non-bonded contacts
KIR2DL1-HLA-Cw2	-1042.5	29:28 A:B	1630:1768	1	-	10	175
KIR2DL1-HLA-Cw4	-1235.3	32:23 A:B	1234:1384	2	-	14	166
		30:23 E:F	1230:1360	3	-	14	165
		12:15 A:D	699:652	4	-	8	77
		3:3 A:E	183:193		-	2	8
		4:3 B:E	151:156	1	-		14
KIR2DL1-HLA-Cw5	-1094.0	25:27 A:B	1409:1334	2	-	9	149
KIR2DL2-HLA-Cw1	-799.9	14:16 A:B	903:932	3	-	9	82
KIR2DL2-HLA-Cw3	-877.7	31:21 A:B	1220:1330	5	-	13	168
		12:14 A:D	752:666	5	-	8	85
		5:7 D:E	380:322	-	-	1	32
KIR2DL2-HLA-Cw7	-972.6	19:25 A:B	1159:1082	-	-	8	141

Furthermore, NK receptor-ligand complexes were evaluated for its stereo-chemical properties depicting the number of residues lying in favoured, allowed, and outlier regions of the receptor-ligand complex (Figure 4.20) and are summarized in Table 4.6. It was observed that 67.5%, 73.3%, 70.9%, 67.8%, 67.4% and 68.3% residues lied in favoured regions and 28.7%, 23.5%, 26%, 29.5% and 29.9% residues were present in allowed regions of KIR2DL1-HLA-Cw2, KIR2DL1-HLA-Cw4, KIR2DL1-HLA-Cw5, KIR2DL2-HLA-Cw1, KIR2DL2-HLA-Cw3 and KIR2DL2-HLA-Cw7 complexes respectively. Therefore, among KIRs-classical HLA complexes, KIR2DL1-HLA-Cw4 complex was found to be most stable structure in terms of energy minimization, presence of number of hydrogen bonds, non-bonded contacts and its stereochemical properties.

Table 4.6: Ramachandran plot scores of NK receptors interactions with classical HLA molecules

	KIR2DL1-HLA-Cw2	KIR2DL1-HLA-Cw4	KIR2DL1-HLA-Cw5	KIR2DL2-HLA-Cw1	KIR2DL2-HLA-Cw3	KIR2DL2-HLA-Cw7
Residues in favoured region (%)	67.5	73.3	70.9	67.8	67.4	68.3
Residues in allowed region (%)	28.7	23.5	26	29.5	29.7	29.9
Residues in outlier region (%)	3.8	3.2	3.1	2.6	2.9	1.9

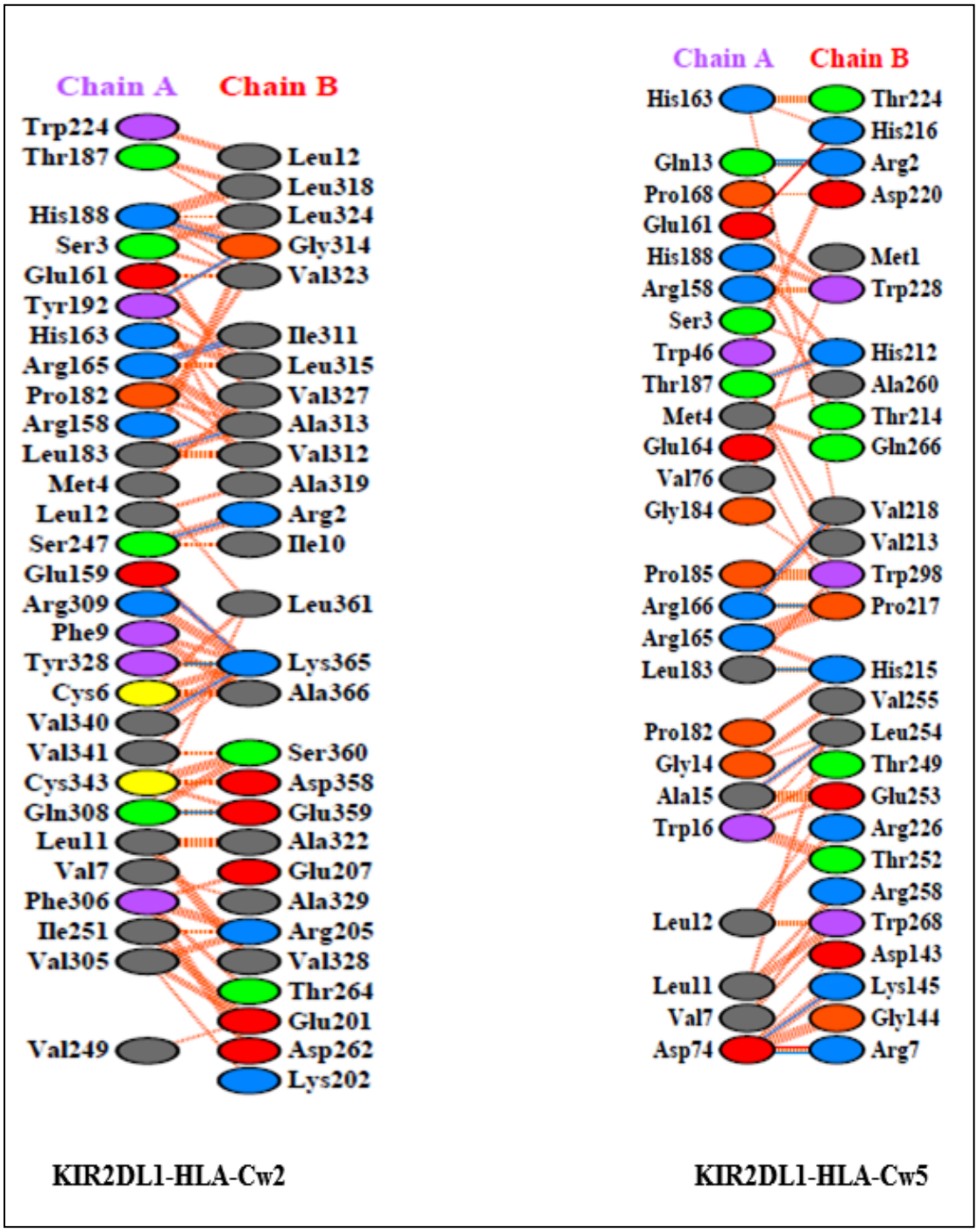

Figure 4.19a: Residues involved in interactions between NK inhibitory receptor and classical HLA molecule. Amino acid residues involved in interactions in KIR2DL1- HLA-Cw2 (left) and KIR2DL1- HLA-Cw5 complex (right).

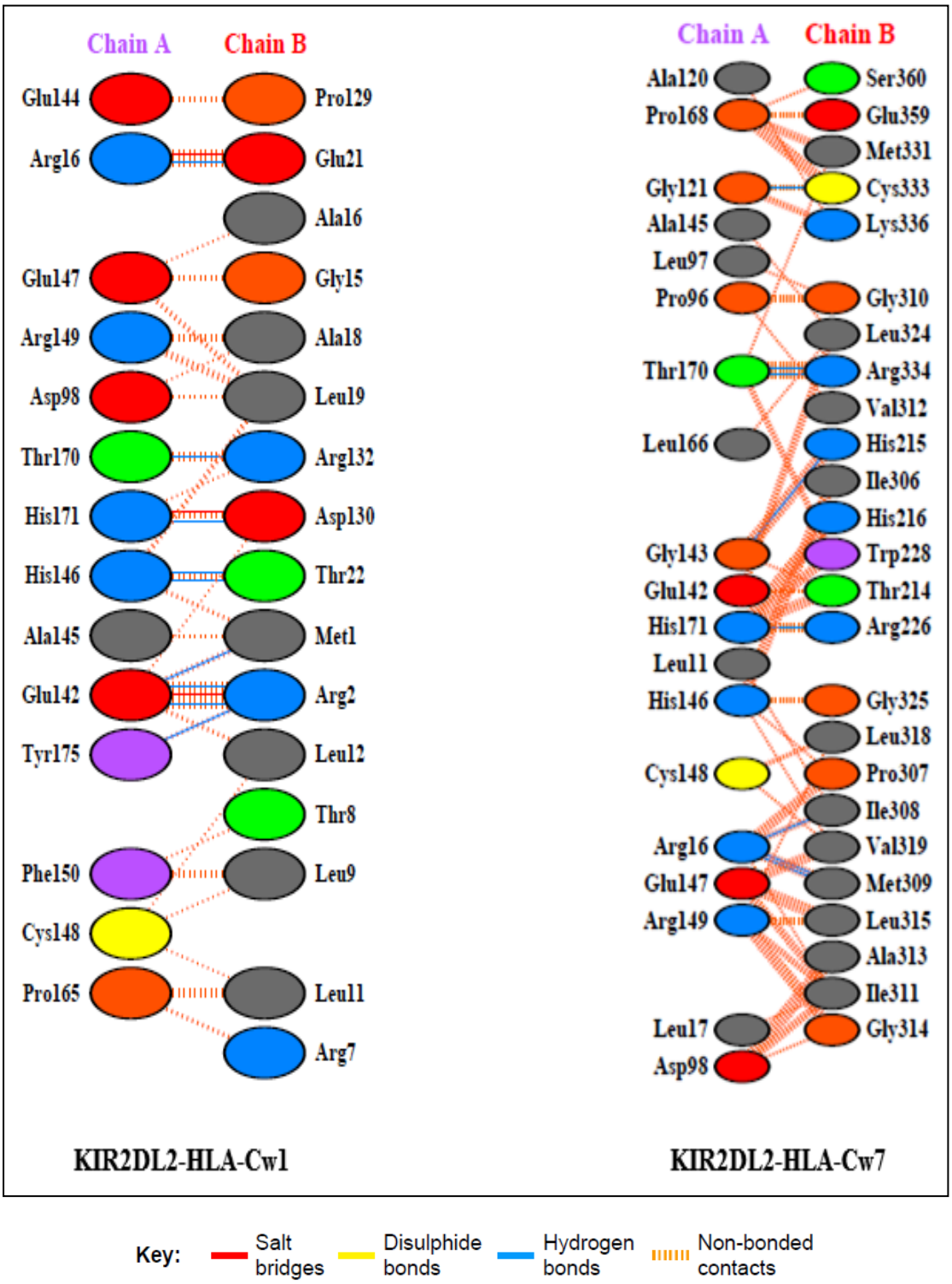

Figure 4.19b: Residues involved in interactions between NK inhibitory receptor and classical HLA molecule. Amino acid residues involved in interaction of KIR2DL2- HLA-Cw1 (left) and KIR2DL2- HLA-Cw7 complexes (right).

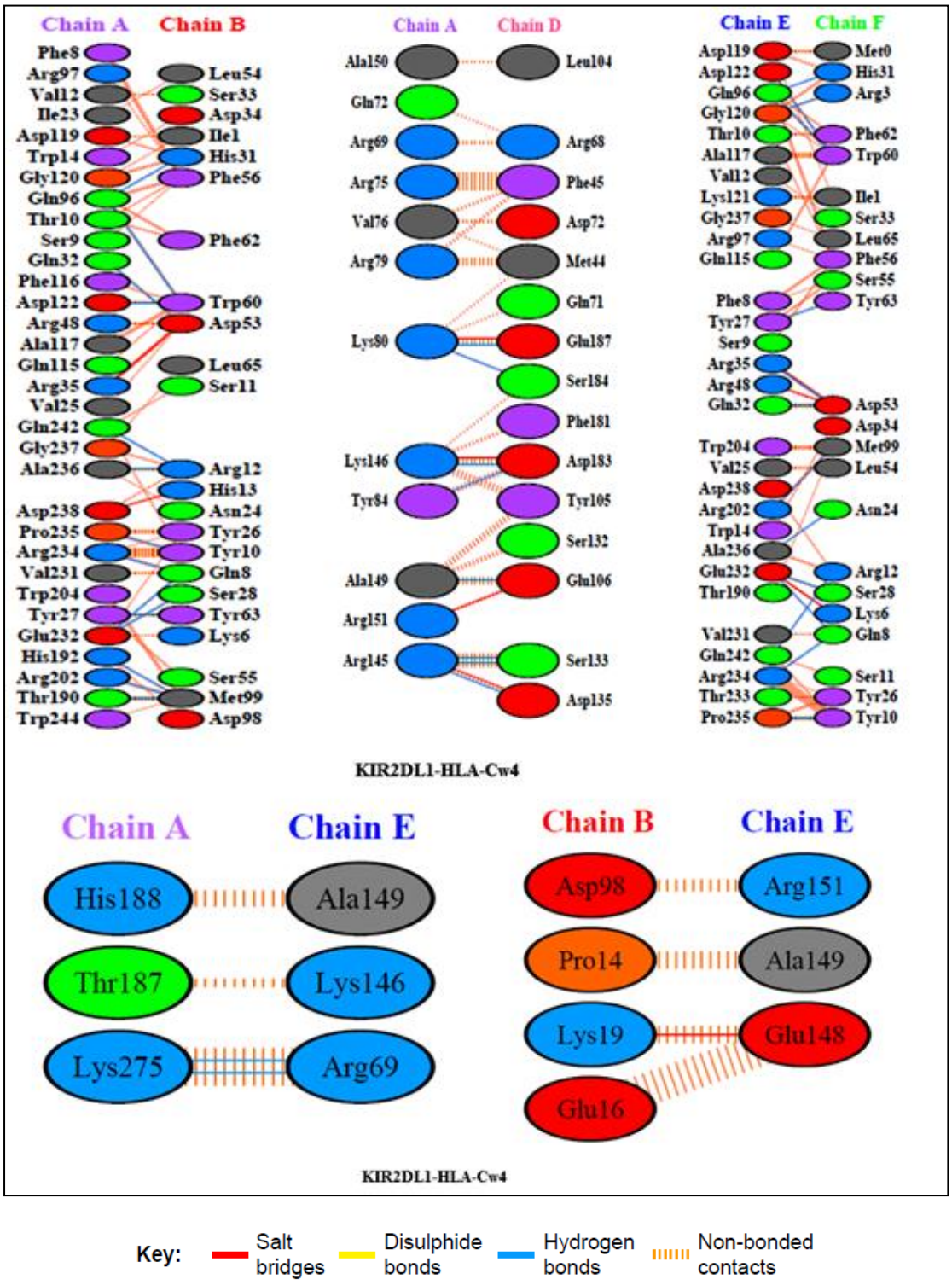

Figure 4.19c: Residues involved in interactions between NK inhibitory receptor and classical HLA molecule. Amino acid residues participated at KIR2DL2- HLA-Cw4 interface.

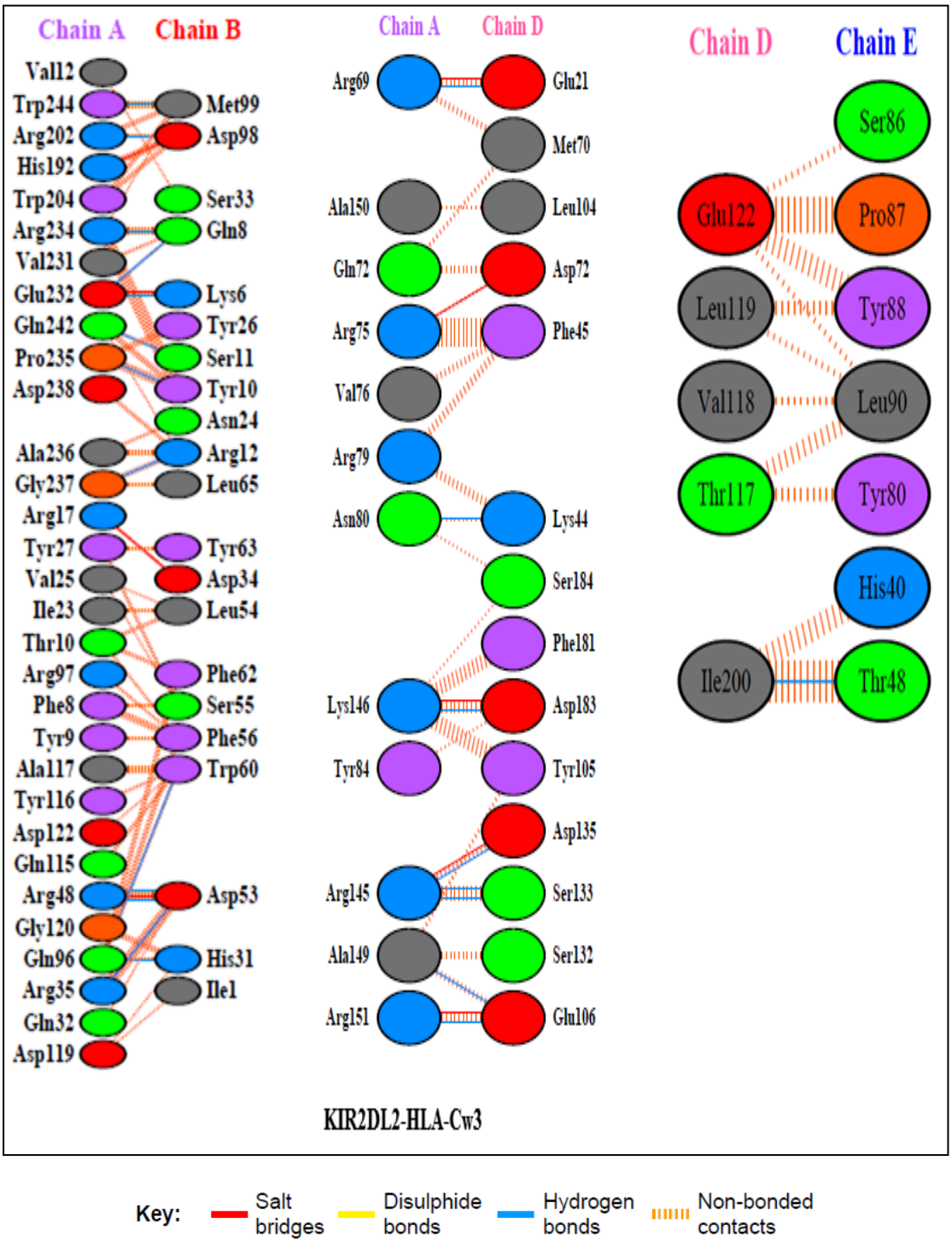

Figure 4.19d: Residues involved in interactions between NK receptor and classical HLA molecule. Amino acid residues participated at KIR2DL2- HLA-Cw3 interface.

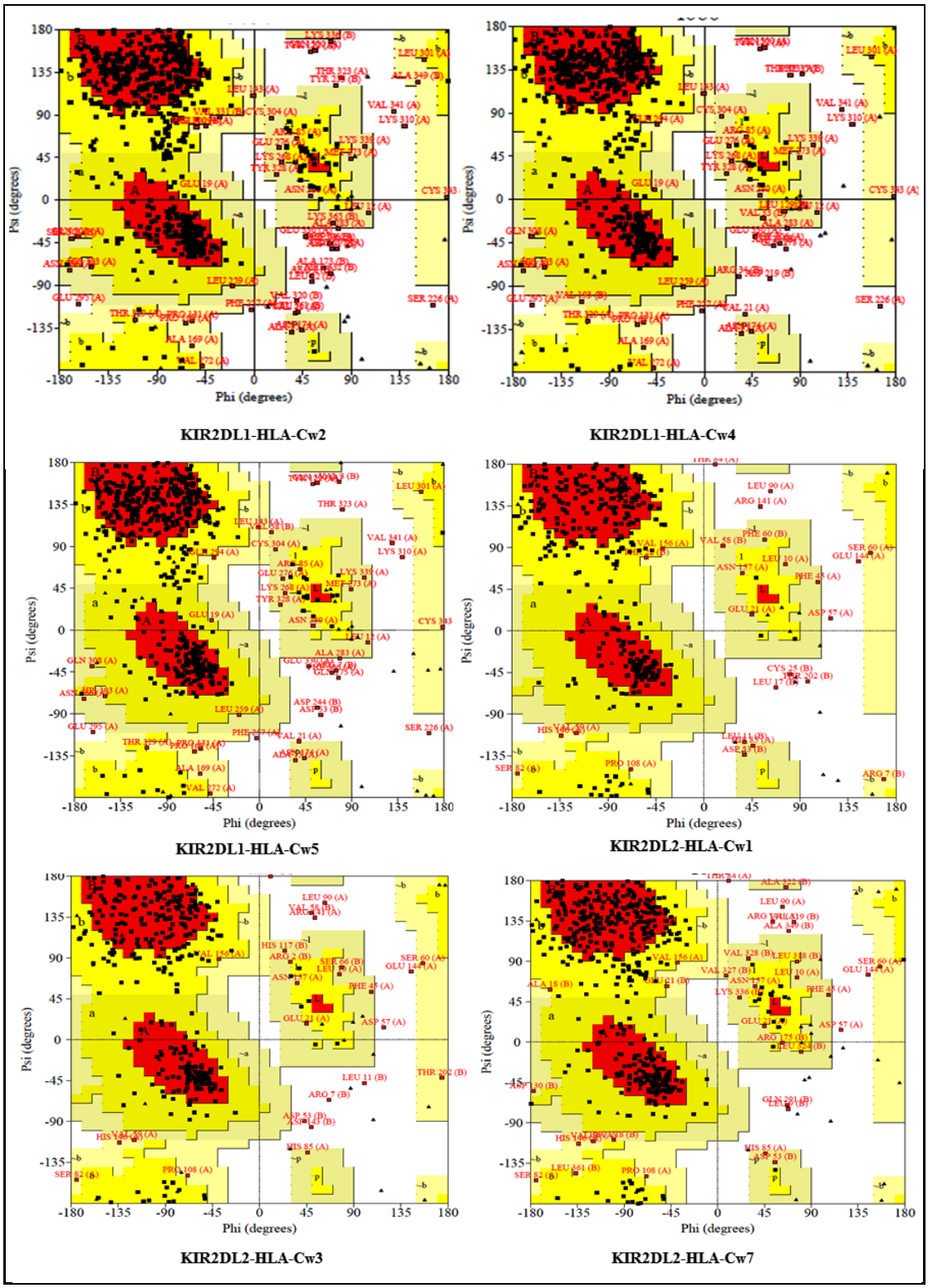

Figure 4.20: Ramachandran plot of NK receptors interactions with classical HLA molecules. Ramachandran Plot were generated from human NK cell inhibitory receptors KIRs and their cognate ligands, classical HLA molecule interaction that represented both α-helix and β-sheet. The red, brown and yellow regions represent the favored, allowed and generously allowed regions as defined by Procheck (PDBsum server).

4.11 Analysis of interaction of NK inhibitory receptors with non-classical HLA molecule

NKG2A/CD94 receptor complex belong to C-type lectin family of receptors and binds to the non- classical HLA molecule (HLA-G/ HLA-E) expressed on the surface of the targets or healthy cell.The docking studies between NKG2A/CD94 and HLA-G as well NKG2A/CD94 and HLA-E complexes were analysed using ClusPro. The binding studies such as hydrogen bond interactions, non-bonded contacts and area involved in interactions were obtained through PDBsum server (summarized in Table 4.7). It was observed that 1-15 hydrogen bonds were present between different chains of NKG2A/CD94-HLA-E complex and 11 hydrogen bonds between chain A and B; Chain C and D each were present in NKG2A/CD94-HLA-G complex. The number of salt-bridges were more in NKG2A/CD94-HLA-E (4,3,2,2,2,1,2,2,1 and 1 in A:B, A:D, A:J, C:E, A:K, B:C, C:F, E:F and J:K respectively) as compared to NKG2A/CD94-HLA-G (2 in A:B and C:D each) complex. Also, the more number of amino acids chains were present in NKG2A/CD94-HLA-E (7 Chains) as compared with NKG2A/CD94-HLA-G (4 chains). The amino acids residues involved in the interactions at the receptor- ligand interface are shown in Figure 4.21a and 4.21b. The stereo-chemical properties of the complexes were then analysed by generating the Ramachandran plots (Figure 4.22). It showed 77.9% residues in most favoured region, 21.3% in allowed region and 0.8% in outlier region of NKG2A/CD94-HLA-E complex. It was observed that 85.6% residues lying in most favoured region, 14.4% in allowed region and no residues in outlier region of NKG2A/CD94-HLA-G (Table 4.8).

4.12 Comparative analysis of KIRs with classical HLA and NKG2A/CD94 with non-classical HLA

It was observed that the receptor interactions with classical HLA as well as non-classical HLA complexes were found to have stable conformation with a low energy score. However, numbers of residues involved in receptor ligand interactions, hydrogen bonds (H-bonds), number of salt bridges in contact**,** number of non-bonded contacts were significantly more in receptor interaction with non-classical HLA complex (NKG2A-HLA-E). Also, we observed sulphide bonds (2) interaction in case of NK receptor with non-classical HLA molecules (Table 4.7) whereas no such interactions were seen in case of KIR-classical HLA complex.

Table 4.7: Molecular interactions between NK inhibitory receptors with non-classical HLA molecules

Receptor ligand complex	Energy Kcal/mol	No. of interface residues chain:chain	Interface area (A^2)	No. of salt bridges	No. of sulphide bonds	No. of hydrogen bonds	No. of non-bonded contacts
NKG2A/CD94-HLA-E	-896.9	30:23 A:B	1237:1366	4	-	15	185
		30:22 C: D	1245:1347	3	-	15	182
		15:16 A:C	890:893	-	-	7	82
		2:2 A: D	77:86	2	-		3
		1:1 A:E	63:79	-	-		5
		1:1 C:J	38:51	-	-		5
		9:12 A:J	586:580	2	-	3	44
		10:13 C:E	588:541	2	-	4	53
		6:10 A:K	422:374	1	-		29
		2:2 B:C	85:75	2	-		2
		5:5 B: D	239:241	-	-	5	34
		6:6 C:F	315:295	2	-	1	19
		14:16 E:F	704:714	1	1	6	55
		15:12 J:K	683:694	1	1	4	43
NKG2A/CD94-HLA-G	-718.4	18 :19 A:B	766:746	2	1	11	97
		18:18 C: D	757:741	2	1	11	89
		2: 2 B: D	168:168	-	-	-	4

Table 4.8: Ramachandran plot scores of NK receptors interactions with non-classical HLA molecules

	NKG2A/CD94-HLA-E	NKG2A/CD94-HLA-G
Residues in favoured region (%)	77.9	85.6
Residues in allowed region (%)	21.3	14.4
Residues in outlier region (%)	0.8	0.0

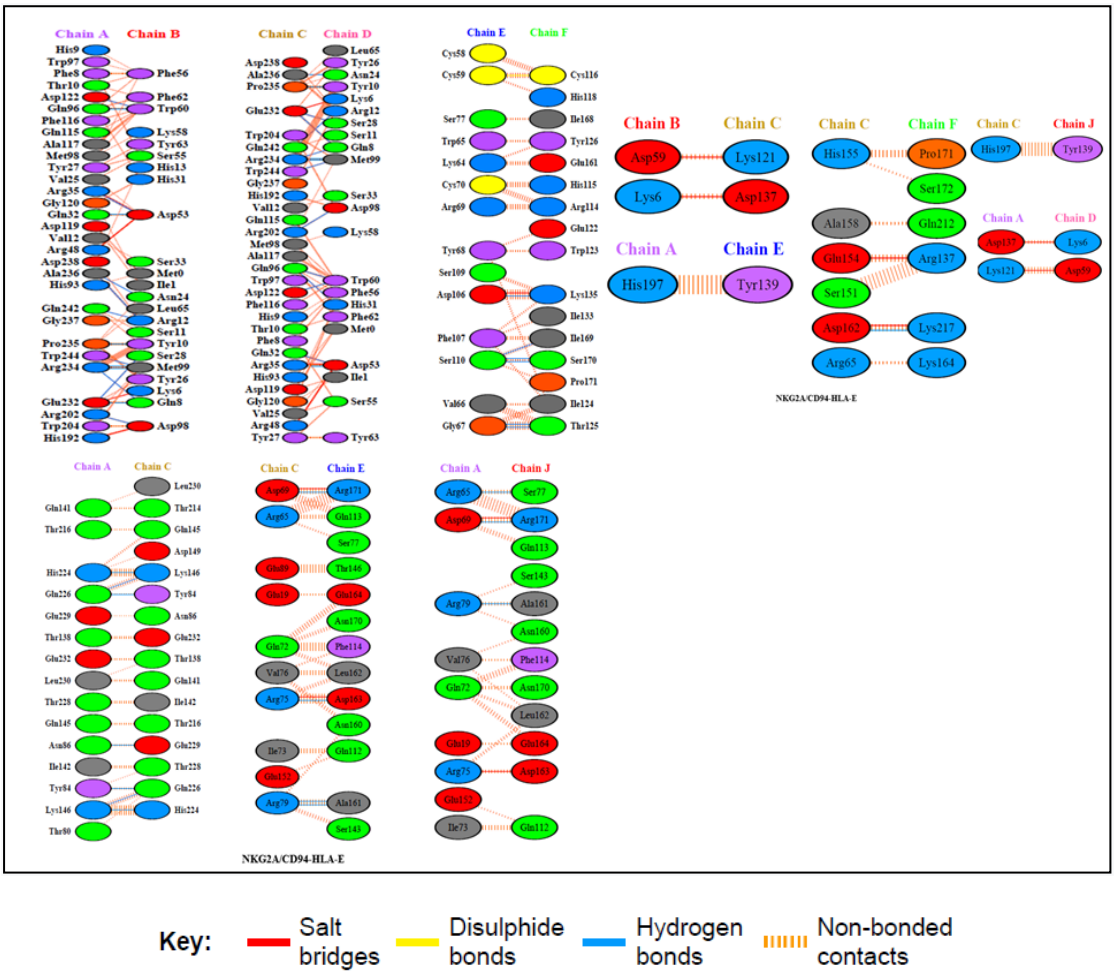

Figure 4.21a: Residues involved in interactions between NK inhibitory receptor and non-classical HLA molecule. Amino acid residues involved in interaction of NKG2A/CD94-HLA-E interaction.

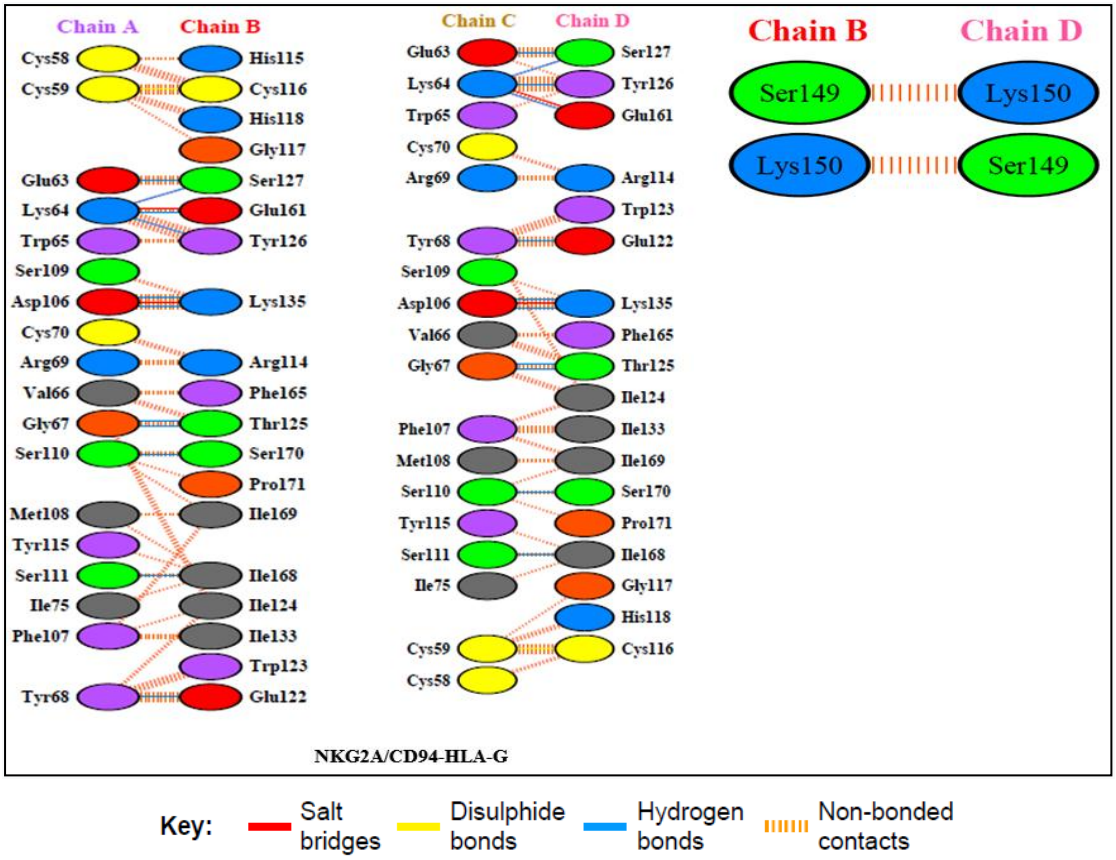

Figure 4.21b: Residues involved in interactions between NK inhibitory receptor and non-classical HLA molecule. Amino acid residues involved in interaction of NKG2A/CD94-HLA-G interaction.

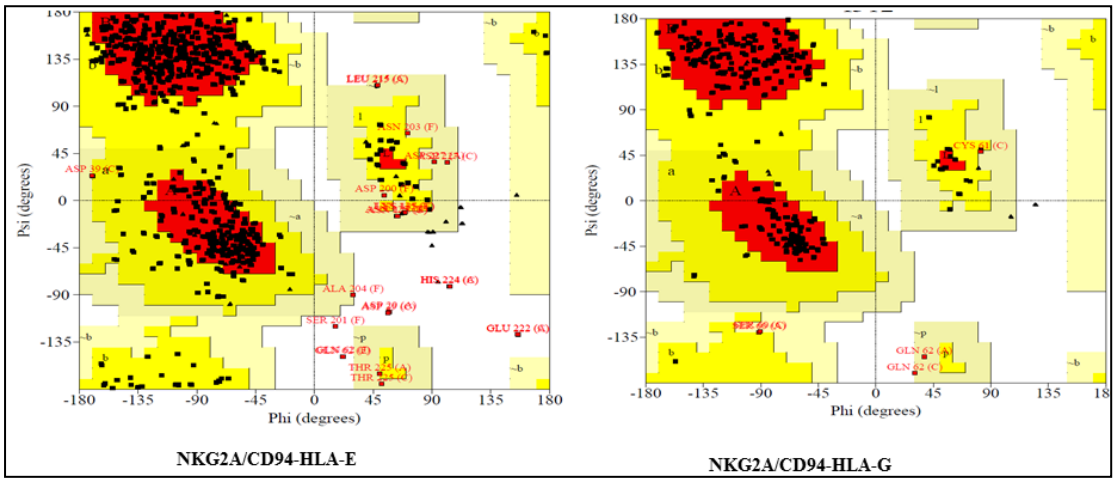

Figure 4.22: Ramachandran plot of NK receptors interactions with non-classical HLA molecules. Ramachandran Plot were generated from human NK cell inhibitory receptors NKG2A/CD94-HLA-E (left) and NKG2A/CD94-HLA-G (right) interaction that represents both α-helix and β-sheet. The red, brown and yellow regions represent the favored, allowed and generously allowed regions as defined by Procheck (PDBsum server). Almost all residues were positioned under favoured and allowed regions.

A significantly higher number of H-bonds contributing to stability of interaction were observed in case of inhibitory receptor interaction with non-classical MHC. It was observed that more number of chains were involved in NKG2A/CD94 receptor-ligand interactions, thus a stronger binding affinity of NKG2A/CD94 NK receptor with non-classical HLA molecule was predicted (Table 4.5 and 4.7). The stereochemical properties showed that 77.9% and 85.6% residues were present in most favoured region; 21.3% and 14.4% residues lied in allowed region; 0.8% and 0.0% in outlier region of NKG2A/CD94-HLA-E and NKG2A/CD94-HLA-Gcomplex respectively. On the other hand, 67-73% of residues lied in favoured regions and 23-30% amino acid residues were present in allowed region and 1.9-3.8% residues were positioned in outlier region of KIRs-classical HLA complexes (Figure 4.20 and 4.22). This showed more stability and stronger affinity of NK receptor and non-classical HLA molecules as compared with NK receptor and classical HLA molecules.

Both classical and non-classical HLA interaction with KIRs or NKG2A/CD94 receptors trigger similar downstream signalling pathways hence structural interaction of KIRs with classical HLA and NKG2A/CD94 with non-classical HLA were evaluated to explore difference in inhibition potential of both receptors. Upon comparing the affinities of NK cell receptor, KIRs with classical HLA and NKG2A/CD94 with non-classical HLA ligands, we found that there was a higher diversity of bonding in case of non-classical HLA binding to inhibitory receptors. Also, number of interface residues involved in interactions and presence of different chains were numerous in case of non-classical HLA molecules. A significantly higher number of H-bonds were observed in case of non-classical HLA interaction with inhibitory receptors.These binding pattern of NK receptors with their cognate ligand may be responsible for stronger affinity of NK receptors towards non-classical HLA molecules.

Chapter 5

Discussion

CHAPTER 5
DISCUSSION

IL-2 is a key cytokine that is well known for its role in immune cell proliferation and activation in tumor microenvironment. NK cells have IL-2Rβγ and had shown to be responsive to IL-2 with intermediate affinity (as discussed in section 2.10). In tumor milieu, there is localized increase in IL-2 concentration by antigen stimulated T cells. Tumor microenvironment consists of variety of cells such as T-cells, NK cells, dendritic cells, myeloid cells (as discussed in section 2.6.1.1). Like T-cells, NK cells expand in high IL-2 milieu and play significant role in immunosurveillance. Therefore, we wanted to study the effect of IL-2 stimulation on NK receptor expression. We explored IL-2 mediated response by using NK1.1 as pan-NK marker to check for NK specific proliferation response. Ly49 receptors are type II C-type lectin-like membrane receptors coded by a family of highly polymorphic and polygenic genes. Ly49 receptors recognize H-2 class-I molecules on target cells. The inhibitory Ly49 receptors are involved in NK cell education, a process in which NK cells acquire function and tolerance toward cells that express "self-MHC-I." C57BL/6 mice model used in the study, NK cells have H2b background in which therefore Ly49A is non-cognate MHC whereas Ly49C is bound by cognate MHC. In a milieu of high IL-2 concentration, we explored the modulation of inhibitory receptors Ly49A and Ly49C upon IL-2 stimulation. We observed significant increase in expression of inhibitory receptor Ly49A upon IL-2 stimulation ($p<0.05$) but no such significant increase in Ly49C expression ($p>0.05$). This implies that IL-2 caused a preferential skewed proliferation of population of NK cells expressing higher levels of Ly49A, which did not bind to

cognate MHC-I. However, Ly49C which binds to its cognate receptor, there was no significant increase in expression upon IL-2 stimulation ($p>0.05$). Probably this was to ensure that there was no significant non-specific activation in the high IL-2 milieu, thus, prevented autoimmune reaction.

IL-2 stimulation also resulted in restoration of degranulation and cytokine producing ability of NK cells against tumor in Wiscott Aldrich syndrome patients (Kritikou et al., 2016). We therefore explored the possibility that IL-2 stimulation of NK cells might resulted in potentiation of NK activity. We determined the expression of activating receptor Ly49D on NK cells upon IL-2 stimulation. There was no significant increase in expression of Ly49D on IL-2 stimulated NK cells as compared to NK cells alone. Also, we detected a significant decrease in expression of Ly49D on per-cell basis despite expansion of NK cell population as seen in Figure 4.4, where there was a decrease in the population peak upon IL-2 stimulation. Thus, IL-2 caused expansion in NK cell population but did not upregulate activation receptor expression. This implies that in high IL-2 concentration the NK cells that underwent proliferation did not however overexpress their activation receptors, thus prevented hyperactivity. Further, we showed that upon IL-2 stimulation there was no significant increase in MIP-1α and IFN-γ production. This raised the question that in the presence of IL-2 in the tumor microenvironment, did IL-2 result in inactivation of NK cells. Therefore, we cultured IL-2 activated NK cells with YAC-1 (NK sensitive) target and explored NK activation potential. We observed a significant and robust increase in MIP-1α and IFN-γ secretion upon co-culture with NK sensitive, YAC-1 target ($p<0.05$). Hence we conclude that in tumor microenvironment where there was presence of tumor cells in context of high levels of IL-2, there was specific increase in activity of NK cells.

However in the absence of tumor cells an inherently hyperactive NK population stimulated by IL-2 would have resulted in non-contextual autoimmunity. Hence the hypo-responsiveness of the IL-2 induced expansion of NK population in a measure of extensive proliferation of potentially active NK cells but refrained from hyperactivity. Although such IL-2 expanded NK cells were hypoactive but upon encountering target cells like sensitive tumor cells, were capable of significantly robust NK activity.

Despite the potential role of immune system in controlling tumor progression, tumor cells adopt various strategies for evading NK cell response including modulation in expression levels of NK cell receptors. The present study involved the investigation of tumor induced modulation of NK receptor expression and activity. P815 cells are known to express high levels of MHC-I, thus being resistant to NK cell mediated cytolysis by engaging inhibitory receptors. We further wanted to explore whether NK resistant tumor cells could actively modulate NK receptor profile resulting in their ability to evade NK mediated cytolysis. Therefore, in our study we cultured IL-2 stimulated NK cells with P815 tumor cells. We observed that co-culturing of NK cells with P815 cells showed increase in expression of Ly49A and a significant higher increase in the inhibitory receptor Ly49C ($p<0.05$), which is the cognate receptor for the MHC haplotype of the mouse model (C57BL/6) used in our study. However no significant change in the expression of activating receptor Ly49D($p>0.05$) was observed. This phenomenon was shown to be caused by P815 membrane derived components not by peptides secreted in the cell supernatant. Our experiments with transwell plates also suggested that direct cell to cell contact was essential for this alteration in NK receptor expression pattern by tumor derived factors. Thus, alteration of inhibitory receptor expressions compounded by over expression of MHC-I,

outweighed the activation signal, thus, ensured better escape of tumor cells by inhibition of NK cell activity. Though we observed increase in expression of NK cell surface marker NK1.1, it implies that presence of tumor membrane derived factors caused skewed proliferation of NK cells with higher expression of inhibitory receptors thus ensured resistance to NK cell cytotoxicity.

Thus, it was assumed that NK cell resistant P815 membrane derived peptides may interfere with NK cell activation potential by up-regulating the expression of cognate inhibitory Ly49 receptors, which significantly increased the inhibition signal and thus provided evidence for an active mechanism of inhibition of NK activity not by overexpression of MHC-I molecules. However the fact that inhibition of NK mediated P815 lysis was observed even if MHC-I molecules were blocked it has been suggested that other mechanisms were also actively employed by such P815 cells. Also, P815 cells showed some basal level of NK cytolytic activity compared to S2 *Drosophila* insect cell system thus further confirming active mechanism of engagement of receptor profile modulation by P815 cells (March et al., 2010). Co-culture of P815 derived peptide with NK cells also showed inhibition of NK cell cytokine release even in IL-2 induced expanded NK population. Such tumor specific NK proliferation of a skewed population and modulation of NK activation potential may be responsible for failure of certain NK mediated immunotherapy and these findings provide insights in devising strategies to interrupt inhibition of potentially NK resistant tumor cells.

NK cell receptors may belong to either Ig-superfamily of type I membrane receptor superfamily or C-type lectin family of type II membrane protein (as mentioned in section 1.3.2). Inhibitory KIRs are known to bind various HLA-A, HLA-B and HLA-

C alleles and CD94/NKG2A inhibitory receptors recognize non-classical HLA-E and HLA-G. It had been established that Extravillous trophoblast (EVT) cells lack classical MHC, HLA-A/B expression and expressed non-classical MHC class I molecules HLA-G, which may protected fetal tissues from maternal immune system (Tilburgs et al., 2015a) and NK inhibitory receptor and non-classical HLA interaction resulted in better inhibition of NK cell cytotoxicity than with classical HLA molecule that protected developing foetus from uterine NK cell cytotoxicity. Previously, it was shown that non-classical HLA molecule interaction with NKG2A as opposed to classical MHC binding to KIRs resulted in greater inhibition (Das and Long 2010). Surface expression of HLA-E was enough to protect target cells from lysis by CD94/NKG2A+ NK-cell clones. It has also been established that inhibition of degranulation confered resistance to NK-cell-mediated lysis (Das and Long 2010).

Despite the fact that both KIRs and NKG2A/CD94 belong to different families and have structural differences, both of these inhibitory receptors pursue similar downstream signalling events to inhibit NK cell activation. Therefore, the difference in inhibition potential is probably at the receptor-ligand interaction level. So, we explored the binding affinities of NK cell receptor with classical HLA vs non-classical HLA ligands to understand the reason for better inhibition potential of non-classical HLA interaction with cognate inhibitory receptor (NKG2A/CD94). Our results showed that there was a high diversity of bonding in NKG2A/CD94 inhibitory receptor and non-classical HLA. Also, number of interface residues involved in interactions and presence of different chains were numerous in case of non-classical HLA molecules and NKG2A/CD94 complex. A significantly higher number of H-bonds were observed in case of non-classical HLA interaction with inhibitory receptors. This binding pattern of

NK receptors with their cognate ligand may be responsible for stronger affinity of NK receptor NKG2A/CD94 towards its ligand non-classical HLA molecules. Also, Ramachandran plot showed that almost all amino acid residues of NKG2A/CD94-HLAE and NKG2A/CD94-HLAG complexes were positioned in the favoured and allowed region compared to KIRs-classical HLAs complexes. This implies better stability and stronger affinity of NK receptor and non-classical HLA molecules as compared to NK receptor and classical HLA molecules, thus providing supportive evidence for difference in inhibition potential upon engagement of the two different types of inhibitory receptors.

In case of some tumor cells there is a increased expression of non-classical HLA molecules and in such tumor microenvironment NK cell receptor interaction with non-classical MHC molecules results in impairment of NK cell activity and thereby promotes cancer progression. Thus, our study provides insights in understanding the difference in inhibition potential of individual NK receptors with their cognate ligands. These findings can be correlated with their impact on cancer susceptibility and will help to design novel therapeutic strategies for cancer.

Chapter 6
Conclusion

CHAPTER 6
CONCLUSION

Natural Killer (NK) cells play a fundamental role against viral infections and tumors. These are known to exert cytotoxicity against tumor cells and secreting cytokines to inhibit tumor progression. Therefore, Natural Killer cells are known to play a very significant role in immunity against tumor cells. NK cell triggering is dependent upon specific combination of NK receptor and ligand on tumor cells. Weak expression of MHC class I molecule on target cell leads to NK cell activation. In contrast, interaction between normal levels of self MHC class I molecule bearing tumor cells and NK cells, induce inhibitory signal. This is the phenomenon of NK activation through 'missing self' hypothesis by which inhibition receptors are engaged by MHC class-I molecules, thus preventing autoimmunity. Tumor cells adopt various strategies for evading NK cell response including modulation in expression levels of NK cell receptors. IL-2 is known to induce durable NK cell expansion and modulation of their cytotoxic function. IL-2 has proved to be one of the main treatment modalities in complete or apparently curative regression of different tumors. It has been established that activation of NK cells with IL-2 lead to enhanced NK cells cytotoxicity against tumor. So, we wanted to explore the effect of IL-2 on NK cell receptor profile. Murine NK cells are best characterized by the expression of surface marker NK1.1. Therefore, in our study, purified NK cells were stimulated with IL-2 (100U) and we observed a definitive increase in NK cell population upon IL-2 stimulation upon overnight incubation as compared with un-induced controls ($p<0.05$). We explored the possibility that IL-2

stimulation of NK cells itself might result in potentiation of NK activity. The inhibitory receptor Ly49A binds to H-2D^d and H-2D^k antigens bearing target cells and Ly49C recognizes H-2b antigens. NK harvested from C57BL/6 mice has H2b background and thus Ly49C is the cognate inhibitory receptor. It was observed that the expression of inhibitory receptor Ly49A increased upon stimulation with IL-2 ($p<0.05$) while there was no alteration in inhibitory receptor Ly49C expression. Hence, our result showed that IL-2 induced stimulation of NK inhibitory receptor expression was more on non-cognate inhibitory receptor, Ly49A. We further determined the expression of activating receptor Ly49D on NK cells upon IL-2 stimulation. There was no significant increase in expression of Ly49D on IL-2 stimulated NK cells as compared to unstimulated NK cells. Thus, IL-2 stimulation resulted in increase in NK cell population but did not induce upregulation of activation receptor expression. Further, we noticed that there was no appreciable increase in MIP-1α and IFN-γ production by the NK cells upon IL-2 stimulation as compared with un-stimulated NK cells.

As we did not notice any stimulation of NK activity as observed by the absence of upregulation of activating receptor expression, Ly49D or significant downregulation of cognate inhibitory receptor, Ly49C, we suspected that IL-2 stimulation of NK cells resulted in hypoactivity. To analyse this possibility, we incubated IL-2 stimulated and un-induced NK cells with NK sensitive YAC-1 tumor cells. NK cells potentially mounted a very robust response in MIP-1α and IFN-γ production in the presence of susceptible tumor cells (YAC-1). This implies that in high IL-2 concentration NK cells undergo proliferation and are hypoactive under in the absence of tumor cells to circumvent non-contextual autoimmunity. However, in the presence of sensitive tumor, IL-2 stimulated NK showed enhanced activity against tumor targets.

Despite various mechanisms followed by immune cells of the body, most significantly T-cells and NK cells, tumor cells in the tumor microenvironment often develop strategies for immune evasion. In the present study, we investigated the tumor induced modulation of NK cell receptors by culturing IL-2 stimulated NK cells with NK resistant, P815 tumor targets. We explored the mechanism of tumor induced immune evasion by co-culturing tumor with IL-2 activated NK cells. Our results conclusively showed that P815 membrane derived factors were responsible for a specific upregulation of inhibitory receptor, Ly49C, which is the cognate receptor for MHC-I expressed in mice model studied (C57BL/6). There was also an increase in the non-cognate inhibitory receptor expression mediated by tumor factors. However, the specific upregulation of cognate receptor mediated by tumor upon engagement of its ligand is clearly a strategy that the tumor cells employ in order to inhibit NK activity. Further, tumor cells did not show any significant modulation of activation receptors on NK cells.

In humans, inhibitory receptor KIRs bind to classical HLA molecules and NKG2A/CD94 bind to non-classical HLA molecules; HLA-G/HLA-E (mentioned in Section 3.10). It has been established that NK inhibitory receptor and non-classical HLA interaction results in better inhibition of NK cell cytotoxicity as compared with interaction of NK inhibitory receptor with classical HLA. Since the inhibitory signal transduction events downstream of KIRs and NKG2 receptors are essentially similar, the difference in their inhibition potential was explored in the context of their affinities for their respective cognate ligands. Our results showed that there was high diversity of bonding (more number of Hydrogen bonds, non-hydrophobic interactions Sulphide bonds, salt bridges) between non-classical HLA binding and NKG2A/CD94 inhibitory receptor complex compared with KIRs and classical HLA complex. Also, more number

of interface residues and different amino acids were involved in interaction of NKG2A/CD94 with non-classical HLA. This binding pattern of NKG2A/CD94 NK receptors with their cognate ligand may be responsible for stronger affinity of NK receptor NKG2A/CD94 with its ligand non-classical HLA molecules.

Altogether, our study conclude that in tumor microenvironment where there is presence of tumor cells in context of high levels of IL-2, there is specific increase in activity of NK cells. However in the absence of tumor cells an inherently hyperactive NK population stimulated by IL-2 would have resulted in non-contextual autoimmunity. Thus, IL-2 plays an important role in proliferative expression and potentiating NK cell activity such that IL-2 stimulated NK cells are able to kill tumor cells more efficiently. Our findings are particularly significant in designing strategies for immunotherapeutics in conjunction with IL-2 combinatorial therapies.

P815 membrane derived peptide interfered with the NK cell function by actively modulating the surface expression of NK cell inhibitory receptors and responsible for downregulation of NK activity against P815 tumor cells. In view of this immunosuppressive effect, a new approach might be developed to interrupt inhibition of potentially NK resistant tumor cells, thus enabling better immunotherapy.

Our study provides further explanation for the fact that NK cells in extravillous trophoblast cells specifically upregulate their non-classical HLA but classical HLA and therefore, spared by maternal NK cell cytotoxicity. Moreover, understanding of difference in inhibition potential of individual NK receptors with their cognate ligands can be correlated with their influence on cancer and will help to design novel therapeutic strategies for cancer.

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ORIGINAL ARTICLE

IL-2 mediates NK cell proliferation but not hyperactivity

Richa Sharma[1] · Asmita Das[1]

Abstract
Natural killer cells play a major role in innate immunity against tumor and virus-infected cells. NK cells express activating and inhibitory receptors to regulate their function. It has been established that modulation in the NK cell receptor profile results in altered function of NK cell against target cells. Here, we study the effect of IL-2 stimulation on NK cell inhibitory receptors Ly49A, Ly49C, and activating receptor Ly49D in C57BL/6 mice. It was observed that there was significant increase in expression of Ly49A but no change in expression of Ly49C and Ly49D on IL-2 stimulation. We further noticed that although IL-2 stimulation increased the NK cell population and expression of activation marker NK1.1 but IL-2 stimulation does not cause hyper-responsiveness in NK cells, as there was no increase in MIP-1α and IFN-γ production in IL-2 stimulated NK cells as compared to unstimulated controls. These findings provide a framework to understand the effect of IL-2 stimulation on cognate and non-cognate receptor ligand interactions and suggest stratagies for immunotherapies in conjunction with IL-2 combinatorial therapies.

Keywords NK cells · IL-2 · NK cell receptors · Ly49A · Ly49C · Ly49D · NK1.1

Introduction

Natural killer (NK) cells are lymphocytes of the innate immune system. They are cytokine producing and have cytotoxic ability to kill both virus-infected cells and tumor cells. It has been established that NK cells have the ability to recognize and kill tumor cells without any prior antigen exposure. So, NK cells are considered as promising agents for cell-based cancer therapies. However, NK cells represent only a minor fraction of the human lymphocyte population. Their skewed phenotype and altered functionality during tumor progression necessitate the expansion and activation of sufficient numbers of functional NK cells to develop possible therapies against tumor [1]. It is now well known that IL-2 affects many types of cells in the immune system including T cells, B cells, and NK cells. IL-2 has been reported to be the first cytokine to be injected to patients diagnosed with metastatic melanoma [2]. IL-2 is known to be crucial for antigen specific T cell response as well as non-specific T cell activation [3] and has been used as monotherapy for several different cancer types [4].

The presence of tumor-infiltrating lymphocytes (TILs) within the tumor microenvironment has been considered as symbolic of an ongoing immune response toward the tumor. Many studies have shown that intense infiltration of lymphocytes can be correlated with tumor remission [5, 6]. Adoptive T cell therapy (ACT) using tumor-infiltrating lymphocytes has proved to be one of the main treatment modalities within cancer immunotherapy, in which lymphocytes mostly; T cells are removed from tumor samples and propagated in vitro on IL-2 stimulation. Then the lymphocytes are infused into cancer patients with the goal of recognizing, targeting, and destroying tumor cells. Immunotherapy using tumor-specific T cells was first established by Steven Rosenberg in 1980s, and eventually human trials of ex vivo expanded TILs have shown promising results when combined with systemic high-dose interleukin-2 (IL-2) in patients with metastatic melanoma [7]. Hwang et al. observed during the meta-analysis study among ovarian cancer patients that the absence of intraepithelial TILs in patients lead to poor survival [8]. Also, infusion of HPV-TILs from human papilloma virus (HPV) positive patients into cervical cancer patients resulted in cancer regression [9]. Moreover, like T cells, infiltrating NK cells have capacity to cope with the tumor outgrowth. Schleypen and colleagues separated

✉ Asmita Das
asmita1710@gmail.com; asmitadas1710@dce.ac.in

[1] Department of Biotechnology, Delhi Technological University, Main Bawana Road, Shahbad Daulatpur, New Delhi, Delhi 110042, India

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tumors in two groups based on frequency of infiltrating NK cells in tissues with low NK-TIL and high NK-TIL isolated from renal cell carcinomas and observed that high NK-TIL showed more cytolytic potential against K562 target cells on low-dose IL-2 stimulation [10]. Also, It was reported that on high doses of IL-2 administration at regular short intervals, significantly more activated NK (A-NK) cells were accumulated in the tumors and were able to eliminate well-established lung metastases in C57BL/6 mice [11, 12]. Sarkar and his co-workers have shown that activation of NK cells with IL-2 lead to restoration of NK cells cytotoxicity even under hypoxic conditions in multiple myeloma [13]. Although various T cell and NK cell based immunotherapies have shown impressive clinical success in the treatment of different cancers but have certain limitations due to several obstacles including reduced effector cell function following infiltration into tumor [14, 15].

It has been suggested that an imbalance between the number and activation status of tumor-infiltrating effector cells may be responsible for resistance of solid tumors to adoptive T cell therapy [16]. TILs hypofunction has been co-related with upregulation of surface inhibitory receptors [14]. Tahtinen et al. analyzed that IL-2 administration could modulate the tumor microenvironment and reduce the tumor resistance to cytotoxic CD8+ T cells within the tumor by down-regulating the expression of CTLA-4 and PD-1 markers on $CD8^+$ T cells [17]. Similarly, infiltrating NK cells isolated directly from renal cell carcinomas showed over-expression of inhibitory receptor NKG2A/CD94 compared to autologous peripheral blood mononuclear cells [18]. Therefore, tumor microenvironment seems to be responsible for altered expression of receptors and MHC represented by tumor cells may act as ligands for inhibitory receptors.

Like T lymphocytes, NK cells should not recognize autologous cells as self cell is equipped either with strong expression of HLA-I antigens able to deliver inhibiting signals to NK cells or weak expression of surface ligands required for triggering NK cell activation. It is becoming evident that NK cells can recognize self cells which display ligands for activating receptors. Several evidences have been reported on the upregulation of the NKG2D ligand MHC class I-related chain A (MICA) in response to infection. In addition, the poliovirus receptor (PVR) or nectin-2, expressed on HIV-infected $CD4^+$ T cells resulted in NK cell recognition through the DNAM1-activating receptor favoring the NK-mediated self aggression [19]. Recently, many studies have documented the upregulation of NK-activating receptors in patients with rheumatoid arthritis, multiple sclerosis (MS), systemic, psoriasis lupus erythematosus (SLE), and latent autoimmune diabetes in adults (LADA) [20–23].

Several reports have also shown an association between NK cell deficits and autoimmune disorders. Similarly, a significantly low percentage of NK cells was observed in LADA patients and Type I diabetes as compared to healthy controls [23, 24]. The above studies suggest that the correlation of decreased NK cell numbers and/or function with autoimmune diseases raises the possibility that autoimmunity may arise from NK cell deficiencies.

One way to overcome the tumor resistance is adequate expansion of cytotoxic effector cells with immunomodulatory cytokine such as IL-2 to ensure efficient lysis of tumor targets. Inappropriate killing of self cells reflect a loss of NK cell tolerance in particular tissue due to downregulation of inhibitory receptors. Thus, identifying the modifications of NK cells with respect to NK cell density and receptor expression will help to optimize the efficiency of NK cells in current therapies. In the current study, we investigated the effect of IL-2 stimulation on NK cell population and NK cell receptors, NK1.1, Ly49D-activating receptors, and Ly49A and Ly49C expressions in mice model.

Materials and methods

Reagents

RPMI 1640, RBC lysis buffer (0.15 M Ammonium chloride, 1 mM Potassium bicarbonate, 0.1 mM disodium EDTA), penicillin, streptomycin, and nystatin were obtained from sigma Aldrich; Fetal bovine serum (FBS) was purchased from Gibco, Thermo Fisher Scientific. Phosphate-buffer saline (PBS) was procured from Himedia Laboratory Pvt. Ltd., Mumbai, India.

NK cell preparation

Spleens were removed aseptically from C57BL/6 mice, and a single-cell suspension was prepared in RPMI1640 supplemented with heat inactivated 10% FBS, penicillin, streptomycin, and nystatin. Erythrocytes were lysed by using RBC lysis buffer, and NK cells were isolated from these splenocytes through EasySep™ Mouse NK Cell Enrichment Kit through negative selection. Cells were stimulated with 100 U of human recombinant IL-2 and incubated overnight at at 37 °C in an atmosphere of 5% CO_2, unstimulated cells were kept as control. NK cell viability was evaluated by trypan blue exclusion method and was counted with hemocytometer after incubation.

Staining and flow cytometry

IL-2 stimulated and unstimulated NK cells were adjusted to the concentration of 0.5 $X10^6$ cells per milliliter. The antibodies anti-Ly49A and anti-Ly49C conjugated with PE and anti-Ly49D and anti-NK1.1 conjugated with FITC were purchased from BD pharmingen. After mAb staining for 30 min, cells were washed with and re-suspended in ice-cold PBS buffer.

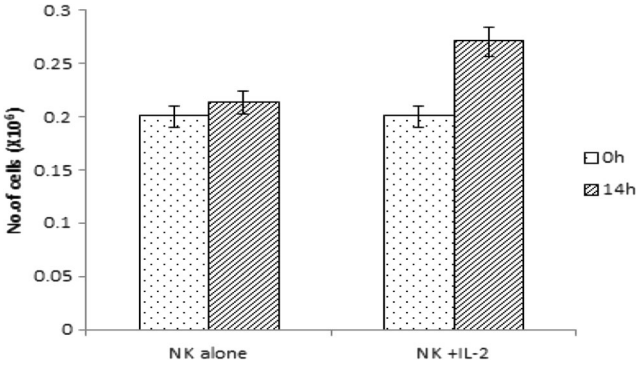

Fig. 1 Viable NK cell count at 0 and 14 h incubation in absence (NK alone) and presence (NK + IL2) of IL-2

All flow-cytometry data were acquired using FACS LSR flow cytometer (BD Biosciences).

Cytokine secretion measurement

Purified NK cells (0.5 X10^6) were washed and incubated for the indicated time at 37 °C in 5% CO_2 in absence and presence of IL-2. Also, NK cells were mixed with NK cell targets YAC-1 (4:1 ratio) as positive control. Thereafter, supernatants were collected. The concentrations of cytokines were quantified using Duoset ELISA kit. To address the kinetics of secretion of cytokines, MIP-1α and IFN-γ were mixed according to the manufacturer's instructions (R&D Systems).

Statistical analysis

Statistical analysis of comparisons between NK alone and NK + IL-2 was performed with using 2-way analysis of variance followed by bon ferroni test. *P* value $p < 0.05$ was considered significant vs respective controls. All experiments were repeated at least three times, and data obtained were expressed as mean ± SD.

Results

Expansion of NK cells with IL-2 stimulation

Purified NK cells were stimulated with IL2 (100 U), and NK cell viability was evaluated by trypan blue exclusion method, and cells were counted by hemocytometer on overnight incubation at 37 °C, 5% CO_2. A definitive increase in NK cell population was observed upon IL2 stimulation in comparison to uninduced controls (Fig. 1).

Effect of IL-2 stimulation of NK cells on NK cell inhibitory receptor profile

NK cells are known to be responsive to IL-2 and in tumor microenvironment in situ; tumor infiltrating lymphocyte stimulation would lead to IL-2 secretion by T cells, and hence, we wanted to study the effect of IL-2 stimulation on NK receptor expression. Since, IL-2 stimulation resulted in proliferation of population of NK cells (Fig. 1), we wanted to explore the

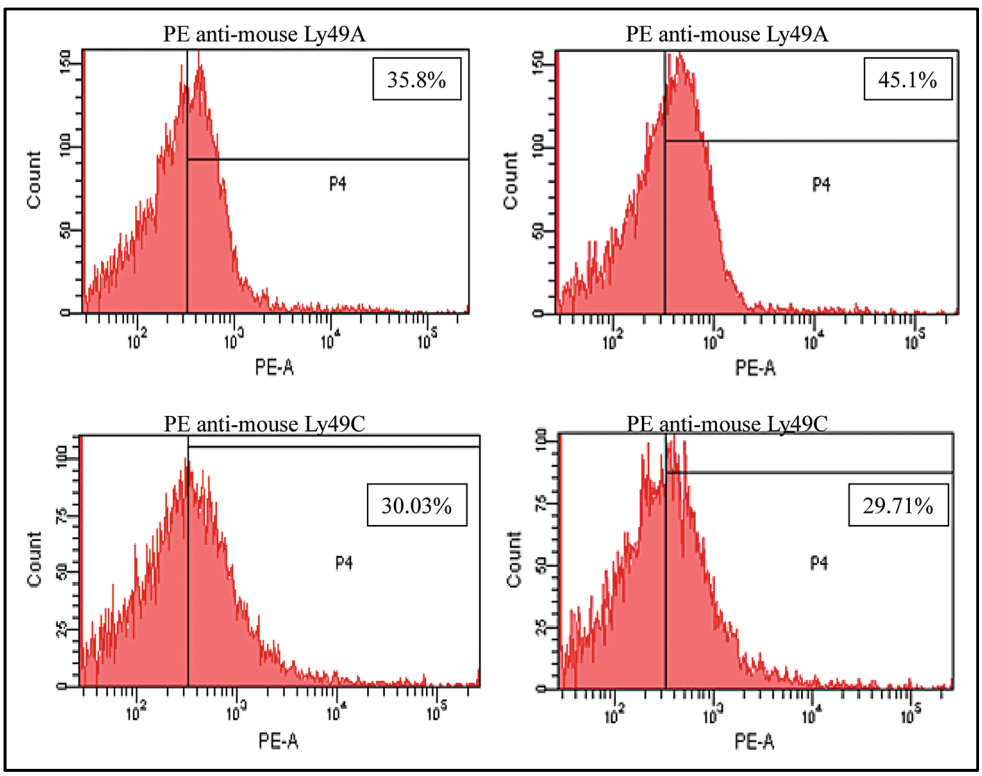

Fig. 2 Charaterization of NK cells inhibitory receptors on stimulation with IL-2. The upper panels describe the expression of Ly49A (PE anti-mouse Ly49A), (lower panels) depicts the expression of NK cell inhibitory receptor Ly49C (PE anti-mouse Ly49C) on purified NK cells in absence (left panels) and presence of IL-2 (right panels)

Table 1 Percentage of activating and inhibitory receptors expressing NK cells in absence (NK alone) and presence (NK + IL-2) of IL-2

NK receptor	NK alone	NK + IL-2
Ly49A	35.8	45.1
Ly49C	30.03	29.71
Ly49D	32	32.6
NK1.1	10.9	30.2

*Values represent mean ± SD of three independent experiments

effect of IL-2 stimulation on the expression level of inhibitory as well as activating receptors. Effect of IL-2 stimulation was studied on inhibitory NK cell receptors, Ly49A and Ly49C, (Fig. 2). It was observed that the expression of inhibitory receptor Ly49A increased upon stimulation with IL-2 (P value < 0.05) while there was no alteration in inhibitory receptor Ly49C expression (Table 1). It may be noted that C57BL/6 mice used in this study have MHC haplotype H2b. Ly49A is the cognate inhibitory receptor for H2k and H2d. While Ly49C is the cognate receptor for H2b, the NK harvested from C57BL/6 mice has H2b background, and hence, our results show that IL-2-induced stimulation of inhibitory receptor expression is more on non-cognate inhibitory receptor.

Effect of IL-2 stimulation of NK cells on NK cell-activating receptor profile

Role of IL-2-induced NK cell proliferation was studied in the context of expression level of activating receptors on NK cells. We noticed the significant increase in expression level of NK1.1 (Fig. 3). Ly49D, which acts as an activating receptor due to the DAP-10 and DAP-12-mediated activation, has shown to be effective in enhancing cytolytic effector function and memory differentiation of NK cells in context of CMV infection [25]. Hence, expression level of Ly49D was studied upon IL-2 stimulation. Figure 3 shows that there is no significant change in Ly49D expression on per cell basis upon IL-2 stimulation. We hypothesize that this is a significant strategy employed by the immune system to ensure that proliferative stimulation of NK cells does not result in hyper-responsiveness of NK cells. In the context of any infection and stimulation of T cells, there would be a local increase in levels of IL-2; however, although this would result in significant expansion in the number of functional NK, such NK cells are not hyperactive or potentially deleterious to self cells.

We further showed that although IL-2 stimulation resulted in significant increase in NK population and increase in expression of NK activation marker NK1.1 as shown in Figs. 1 and 3, this did not result in hyperactivity of the NK cells. Figures 4 and 5 show that there is no appreciable increase in MIP-1α and IFN-γ production by the NK cells upon IL-2 stimulation. Incubation of NK cells with IL-2 resulted in only 0.2-fold increase in secretion of MIP-1α and IFN-γ (Figs. 4 and 5) in comparison to NK cells alone. Thus, NK cells expressing Ly49D in C57BL/6 mice do not mount effective response and provide tolerance in normal conditions. However, these IL-2 expanded NK cells are not inactivated and in the presence of susceptible tumor cells (YAC-1) can

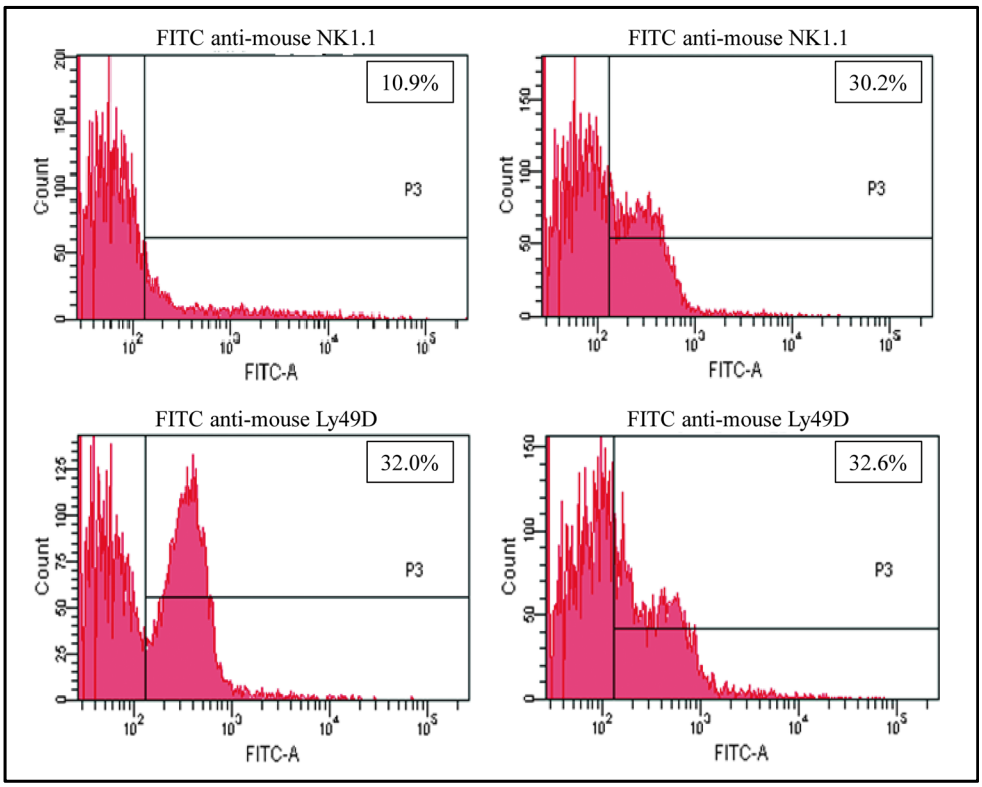

Fig. 3 Charaterization of NK cells activating receptors on stimulation with IL-2. The upper panels describe the expression of NK cell-activating receptor NK1.1 (FITC anti-mouse NK1.1) and lower panels describe the expression of Ly49D (FITC anti-mouse Ly49D) on purified NK cells in absence (left panels) and presence of IL-2 (right panels)

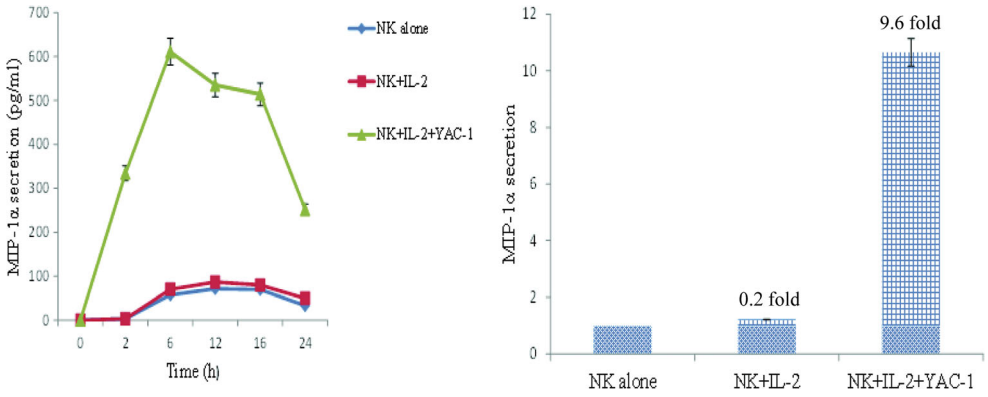

Fig. 4 Kinetics and profile of MIP-1α secretion on IL-2 stimulation. NK cells were incubated alone or with IL-2 or with YAC-1 cells as positive control at 37 °C. Supernatants were harvested at different time points and as indicated, MIP-1α secretion was determined by using ELISA kit. Values represent mean ± SD of three independent experiments

potentially mount a very robust response in MIP-1α and IFN-γ production in Figs. 4 and 5.

Discussion

Murine NK cells are best determined by the expression of surface marker NK1.1 and considered a hallmark of NK cell function. In our present study, we noticed that the stimulation of IL-2 resulted in enhanced proliferation of NK cells and expression of NK1.1 activation marker on overnight incubation. This provides insights toward increasing cytotoxic potential of NK cells on IL-2 stimulation. Previously, studies have shown that NK cells stimulated with anti-NK1.1 and IL-2 had increased cytolytic activity against NK sensitive tumor target, YAC-1 [26]. IL-2 stimulation also resulted in restoration of degranulation and cytokine producing ability of NK cells against tumor in Wiscott Aldrich syndrome patients [27]. Hence, we explored the possibility that IL-2 stimulation of NK cells itself might result in potentiation of NK activity. We therefore determined the expression of activating receptor Ly49D on NK cells upon IL-2 stimulation. There was no significant increase in expression of Ly49D on IL-2-stimulated NK cells as compared to NK cells alone. Thus, IL-2 causes increase in NK cell population but does not cause upregulation of activation receptor expression.

Ly49 receptors are type II C-type lectin-like membrane receptors coded by a family of highly polymorphic and polygenic genes. The inhibitory Ly49 receptors are involved in NK cell education; a process in which NK cells acquire function and tolerance toward cells that express "self-MHC-I." Ly49A inhibitory receptor binds to H-2D^{d} and H-2D^{k} antigens bearing target cells and Ly49C recognizes H-2^{b} H-2k^{d}, H-2k^{b}, and H-2D^{d} antigens. Thus, Ly-49A and Ly-49C differ in both their pattern of expression by NK cell subsets, as well as their specificities for class I molecules and for C57BL/6 mice which have H2b background, Ly49C is the cognate-inhibitory receptor. In our study, we did not observe any significant increase on Ly49C expression on IL-2 stimulation on per cell basis. This raises the possibility to eliminate hyporesponsiveness by effector cells. We observed significant increase in expression of non-cognate ligand Ly49A on IL-2 stimulation. Probably this is to ensure further no cross-reactive activation in the high IL-2 milieu of tumor microenvironment. Thus, preventing autoimmune reaction and may predict the way of decreasing the NK cytotoxic potential through non-cognate cross-reactive receptor. There are also reports of downregulation of Ly49C and Ly49A expression on T cell and NK cell on interaction with their cognate ligands in Ly49C and Ly49A transgenic mice led to autoimmune inflammatory disorders [28, 29].

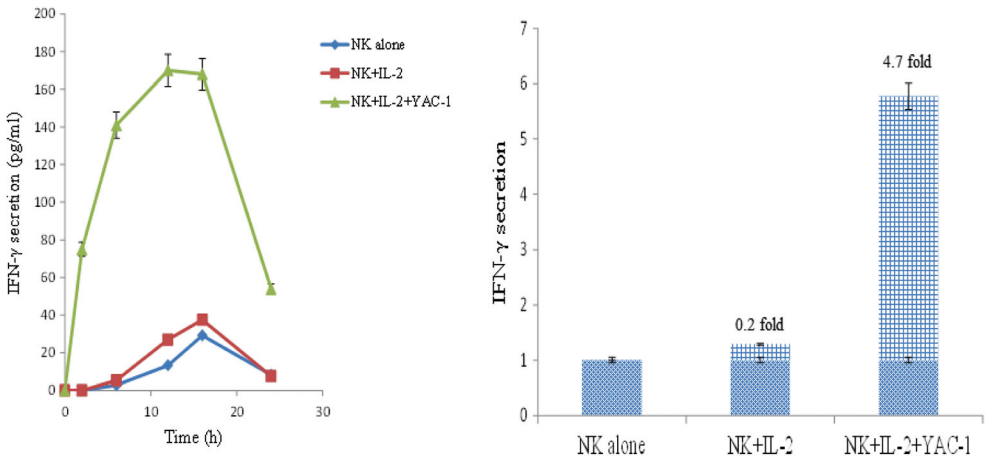

Fig. 5 Kinetics and profile of IFN-γ secretion on IL-2 stimulation. NK cells were incubated alone or with IL-2 or with YAC-1 cells as positive control at 37 °C. Supernatants were harvested at different time points and as indicated IFN-γ secretion was determined by using ELISA kit. Values represent mean ± SD of three independent experiments

Further, we showed that upon IL-2 stimulation there is no significant increase in MIP-1α and IFN-γ production. This increased level upon T cell activation will not result in concomitant increased hyperactivity of NK cells. However upon co-culturing of NK cells stimulated with IL-2 and tumor cells, there is a very significant and robust increase in NK activity (data not shown). Hence, we conclude that in tumor microenvironment where there is presence of tumor cells in context of high levels of IL-2, there is specific increase in activity of NK cells. However, in the absence of tumor cells, an inherently hyperactive NK population stimulated by IL-2 would have resulted in non-contextual autoimmunity. Hence, the hyporesponsiveness of the IL-2 induced expansion of NK population in a measure of extensive proliferation of potentially active NK cells but refrain from hyperactivity. Although such cells are expanded but are hypoactive upon encountering target cells like tumor cells can immediately cause significant increase in activity.

Our findings are particularly significant in designing strategies for designing strategies for immunotherapeutics in conjunction with IL-2 combinatorial therapies.

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All experiments involving animals were performed in Institute of Nuclear Medicine and Allied Sciences. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Organ-specific phenotypic and functional features of NK cells in humans

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Organ-specific phenotypic and functional features of NK cells in humans

Richa Sharma · Asmita Das

Abstract Natural killer (NK) cells kill virus-infected and tumor target cells without prior sensitization. Each NK cell expresses a multitude of activating and inhibitory receptors, and the interplay of signals determines the outcome of NK cell activity. NK cell-mediated cytolysis of target cell involves polarized degranulation at effector–target interface. Peripheral blood NK cell constitutes about 10 % of lymphocytes, and approximately 90 % of peripheral blood NK cells are $CD56^{dim}CD16^{+}$; however, there is a distinct subset of NK cells, $CD56^{bright}CD16^{-}$, expressed by certain lymphoid organs which are able to produce large amounts of cytokines including interferon-γ, tumor necrosis factor, and granulocyte–macrophage colony-stimulating factor, but the cytotoxicity is attained only on their prolonged activation. In this review, we discuss the accumulated data on distinct phenotypes of NK cells in human uterus, liver, intestine, skin, and lung and also attempt to correlate their phenotype with corresponding activity and functions, with significant stress on the role of NK cells in pathology in the specific organs. Our detailed understanding of altered NK cell activity in different organs and their inherent cytotoxic activity against tumor target cells will help us design better immunotherapeutic strategies in NK cell-mediated cancer therapies.

Keywords HCC · Inflammatory bowel disease · Melanoma · NK cells · NSCLC · Pre-eclampsia · Psoriasis

R. Sharma · A. Das (✉)
Department of Biotechnology, Delhi Technological University, Bawana Road, New Delhi 110042, Delhi, India
e-mail: asmitadas1710@dce.ac.in; asmita1710@gmail.com

Introduction

Natural killer (NK) cells are large granular lymphocytes that kill virus-infected and tumor cells without prior sensitization. Each NK cell expresses a multitude of activating and inhibitory receptors, and the cytotoxicity of these cells is determined by a fine balance between activation and inhibition signals [1]. These receptors may belong to either type I Ig-superfamily receptor or C-type lectin family of type II membrane proteins [2]. The activating receptors possess a short cytoplasmic tail and an adaptor molecule like DAP-12 that contain ITAMs (immune receptor tyrosine-based activation motifs, $Yxxl/Ix_{6-8}$, YxxL/I, x is any amino acid residue), leading to NK cell activation and cytokine production. Additionally, there are other cell surface receptors like NKG2D that are not directly coupled to ITAMs. They are non-covalently associated with DAP-10, which contains YxxM motif (x is any amino acid residue). These adaptor molecules, DAP-10 and DAP-12, contain negatively charged aspartic acid residues in the transmembrane domain that are involved in association with killer-activating receptors or NKG2D receptors. Inhibitory NK cell receptors recognize major histocompatibility (MHC) class I molecules and are characterized by immunoreceptor tyrosine-based inhibitory motifs in their long cytoplasmic tail, which recruit intracellular phosphatases, such as SHP1, SHP2, and SHIP that block the cascade initiated by activation receptor, thus causing inhibition of cytotoxicity [3].

Cytolysis of target cell may be achieved by a variety of effector mechanisms that include release of granules containing perforin and granzyme as well as TNF superfamily members (FasL/TNF) and their corresponding ligands (Fas/TNFR). FAS-independent NK cell cytotoxicity has been studied extensively and is mediated by cytolytic granule

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polarization and degranulation. Das and Long (2010) established that granule polarization is the preferred target for inhibitory signal thus preventing NK cell cytotoxicity [4].

Human NK cells can be categorized into two subsets on the basis of intensity of CD56 and CD16 surface expression. Systemic NK cells display two types of subsets, $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$. Approximately 90 % of peripheral blood NK cells (pNK) are $CD56^{dim}CD16^{+}$, which produce cytokines and lyse the target cells. By contrast, $CD56^{bright}CD16^{-}$ NK cell subsets preferentially expressed in certain lymphoid organs are able to produce large amounts of cytokines, including interferon-γ (INF-γ), tumor necrosis factor (TNF), and granulocyte–macrophage colony-stimulating factor, but cytotoxicity is attained only on their prolonged activation [5]. Vossen et al. [6] reported majority of circulating NK cell population, which are $CD56^{dim}$ and also $CD27^{-}$, release high levels of perforin and granzyme B and had strong cytotoxic activity. On the other hand, NK cells that were $CD27^{+}$ and $CD56^{bright}$ had markedly low levels of perforin and granzyme B and hence low cytotoxic potential. Also, recruitment of NK cells to different inflamed tissues is controlled via different chemokine receptors repertoire, and there exists a unique representation of NK cell subset in different tissues/organs [7]. These phenotypically different NK cell subsets showing disparate functions might be involved during different stages of infection and other pathological conditions. In this review, we have summarized the data on the distinct phenotype of NK cells in uterus, liver, intestine, skin, and lung and attempted to relate with their corresponding activity and functions in these different organs.

NK cells in uterus

Human deciduas possess almost 70 % of NK cells among total lymphocytes during pregnancy [8]. The main NK cell subset found in human uterine mucosa is $CD56^{bright}$ $CD16^{-}$, and the level of expression of CD56 on these NK cells is even higher than that of pNK cells [9]. Vacca et al. [10] analyzed hematopoietic precursors in deciduas and showed that CD34 cells differentiated into $CD56^{bright}$ $CD16^{-}$ NK cells on interaction with the components of the deciduas. On successful implantation, there was dramatic recruitment of immature NK cells that attained maturity in uterine microenvironment [11]. Manaster et al. [12] suggested that the higher percentage of NK cells during pregnancy play crucial role in implantation and vascular modification in the decidua, hence are vital for normal pregnancy. Recently, it has been reported that decidual NK cells express CMKLR1 (for a chemoattractant, chemerin); CMKLR1/chemerin interaction leads to NK cells recruitment to deciduas from periphery and formation of capillary-like tube, therefore mediating vascular remodeling during early pregnancy [13]. Though the role of chemerin in successful pregnancy has been established, its physiological role in disease outcome like pre-eclampsia is not yet clear.

Natural killer cells in the uterus remain non-toxic to the fetal-derived trophoblasts at the fetal–maternal interface. Extravillous trophoblast (EVT) cells lack HLA-A/B (human leukocyte antigen) expression and express an unusual combination of classical MHC class I molecule HLA-C as well as non-classical class I molecules HLA-G, which may protect fetal tissues from maternal immune system [14]. Vacca et al. [15] observed that dNK cells did not lyse trophoblast cell lines JAR and JEG3, while pNK cells could easily kill them. Interaction between the non-classical class I MHC molecule HLA-G and KIR2DL4 initiates the production of proinflammatory cytokines and proangiogenic factors (Ang1, Ang2, VEGF-A, VEGF-C, IFN-γ, and TGFβ1) that promote vascular remodeling as well as induce surface co-expression of HLA-E on trophoblast cells and thus lead to a strong inhibitory signals in dNK cells through CD94/NKG2A [16–18]. Moreover, uNK cells, but not pNK cells, secreted VEGF-C that activated TAP-1 expression on trophoblast cells and thus impart protection from uNK cell-mediated cytotoxicity [19]. Fu et al. [20] analyzed that $CD56^{bright}$ $CD27^{+}$ dNK cells maintain fetal–maternal tolerance by suppressing T_H17-mediated inflammatory response, and loss of this regulatory response leads to recurrent spontaneous abortion. Recently, it has been observed that combined factors like TGF-β, hypoxia, and demethylating agent are responsible for VEGF-A secretion, reduced cytotoxicity, and invasion of trophoblast cells by dNK cells [21]. In line with these studies, Yoksta et al. [22] established that NKp46 expression on uNK cells up-regulates the production of cytokines that facilitate normal pregnancy.

Impaired combination of maternal KIR receptors on dNK cells and fetal HLA on EVT cells could increase the risk of pregnancy-associated disorders [23]. Faridi et al. [24] analyzed KIR genotypes in women experiencing recurrent miscarriages and concluded that over activation of activating receptors may contribute to loss of pregnancy.

Pre-eclampsia is characterized by a poor placental perfusion due to failure of transformation of the maternal spiral arteries, leading to hypertension and proteinuria. Goldman-wohl et al. [25] described that reduced expression of HLA-G on EVT cells rendered trophoblasts susceptible to lysis by dNK cells, which in turn resulted in impaired trophoblast invasion and constriction of placental spiral arteries, resulting in restricted blood supply to the placenta. Furthermore, there was increased level of VEGF

soluble receptors, soluble fms-like tyrosine kinase, that act as anti-angiogenic factors, which in turn promotes maternal syndrome. The process of spiral artery remodeling involves apoptosis of vascular smooth muscle cells (VSMCs) and temporary loss of endothelial layer; these events were controlled by cells at maternal–fetal interface. Recently, Doppler ultrasound method was used to categorize NK cells into normal Resistance Index (RI) group and high RI group. dNK cells with normal-RI had shown more extensive vessel remodeling and activated pro-apoptotic factor, FasL, that induced vascular cell apoptosis, whereas dNK cells isolated from pregnancies with high RI failed to induce VSMCs apoptosis and led to impaired trophoblast invasion and impaired artery remodeling and therefore promoted pregnancy complications like pre-eclampsia [26].

Hence, during pregnancy there is increased migration of immature NK cells from periphery which undergo significant change in their receptor profile upon interaction with decidual components. Thus, NK cells play crucial role in spiral artery remodeling in fetal trophoblast and loss of dNK cells leads to pathological conditions like pre-eclampsia and recurrent spontaneous abortion [27].

NK cells in liver

Human liver contains distinct populations of NK cells, T cells, and NKT cells, defined by their expression of $CD3^{-}$ $CD56^{+}$, $CD3^{+}$ $CD56^{-}$, and $CD3^{+}$ $CD56^{+}$, respectively [28]. Majority of liver NK cells are $CD56^{bright}$ $CD16^{-}$ that showed a higher expression of NKG2A/CD94 and lower expression of KIRs resembling $CD56^{bright}$ pNK cells. This subset expresses the activation marker CD69 and showed elevated levels of adhesion molecule CD11a compared to $CD56^{bright}$ NK cells in blood [29]. Interestingly, the cytotoxic potential of $CD56^{bright}$ $CD16^{-}$ NK cells in liver is debatable. Burt et al. showed that liver NK cells were less cytotoxic than pNK cells in hepatic malignancy. They reported that $CD16^{-}$ NK cell subset expressed high level of NKp44, HLA-DR, and CD69 but had poor ability to kill K562- and MHC-I-deficient LCL721.221 targets. Further they showed that the small proportion of $CD56^{dim}$ $CD16^{+}$ subset in liver had lower perforin expression compared to their blood counterparts. Moreover, they observed lower expression of self-$KIR^{+}NKG2A^{-}$ and self-$KIR^{+}NKG2A^{+}$ by $CD16^{+}$ subset in liver as compared to pNK. The self-KIR expression levels on $CD16^{-}$ population in liver and peripheral blood were comparable. Hence, the $CD16^{+}$ population in liver, which contributed more significantly to liver NK cell-mediated cytotoxicity, comprised of a high percentage of unlicensed NK cells [30]. Thus, $CD56^{bright}$ subsets in liver were phenotypically and functionally distinct from same subset in periphery. In direct contrast to the above findings, Moroso et al. reported that $CD56^{bright}$ $CD16^{-}$ $CD\ 69^{+}$ population showed a higher proportion of perforin, granzyme A and B and exert twofold higher cytotoxicity by hepatic NK cells against K562 target cells without 1L-2 stimulation as compared to the same subset in blood. This unique subset of NK cells is transferred from donor to recipient after liver transplantation [29].

Ishiyama et al. [31] observed that upon IL-2 stimulation, the expression of TNF-related apoptosis-inducing ligand (TRAIL) significantly increased and up-regulated killing capacity of NK cells against HepG2, a hepatocellular carcinoma (HCC) cell line compared to pNK cells of the same donor.

Chuang et al. [32] reported that abundant NK cells occupied small bile ducts of liver in patients with primary biliary cirrhosis (PBC) and they recruited from periphery to liver during PBC and exerted higher cytotoxic effect, resulting in aggravated hepatic damage. Upon ligation of TLR4 in the presence of IFN-α, there is activation of NK cells and TRAIL expression in PBC patients [33]. Recently, it has been seen that $CD\ 16^{-}$ NK cell subsets accumulate in liver and contribute to chronic liver disease severity [34].

It has been reported that low density of NK cells in intratumoral cells of HCC patients promoted tumor survival [35]. Lin et al. [36] suggested that reduced NK cell population in HCC tumor is because of poor expression of chemerin protein. Also, lower expression of activating receptors NKG2D, NKP30, and NKP44 and increased expression of inhibitory receptors were observed in advanced cancer stage of HCC patients [37]. Li et al. [38] demonstrated that HCC-derived fibroblast factors induced impaired cytokine production and decreased cytotoxicity of NK cells.

Although studies have been carried out on the involvement of pNK cells in PBC [39] and especially in animal models [40], role of liver-specific NK cells in pathogenesis needs further exploration. Also, hepatic NK cells have been demonstrated to be hypoactive in cytotoxic potential due to weaker expression of activating receptors and poor chemerin expression preventing pNK recruitment to HCC, thus favoring tumor survival. In HCC, reduced NK cell population and hypoactivity of the hepatic NK cells favor tumor growth. Hence, immunotherapeutics using NK cells in HCC will require further understanding of HCC-mediated NK cell receptor modulation.

NK cells in intestine

Natural killer cells can be found throughout the human digestive tract and constitute a diverse phenotype. Most of

the NK cells and NK-like cell population among intraepithelial lymphocytes (IELs) had been shown to express CD56, CD161, CD122, CD69 and minor fraction expressed CD16 but lacked CD57 expression [41]. Leon et al. [42] identified a unique subset of NK-like cells among small intestinal IELs that were $CD3^-$ $CD16^-$ $CD7^+$ and resembled NK cells in other mucoid tissues. They showed a strong lytic activity with increased perforin content on stimulation with IL-2 as compared to pNK cells.

Lindgren et al. [43] demonstrated that $CD8^-CD16^-$ $CD56^{bright}$ NK cell subsets predominate gastrointestinal mucosa. Stimulation of mucosal NK cells with the combination of *Helicobacter pylori* antigen and IL-12 leads to NK cell activation and production of IFN-γ, thus playing important role in bacterial control. Lindgren et al. [44] proposed that TLR2 involved in recognition of *H. pylori* by NK cells in the gastrointestinal mucosa was capable of combating *H. pylori* infection by production of IFN-γ.

Zhang et al. [45] observed increased expression of HLA-Cw*07 gene in patients with ulcerative colitis (UC) and HLA-Cw*12 gene phenotype in Crohn's disease (CD) patients. This HLA overexpression reduced the activity of NK cells toward such target and thus increased IBD susceptibility. Also, IL-23 expression in inflamed mucosa of IBD promoted activation of NK cells and induced pro-inflammatory cytokine secretion such as IFN-γ and TNF that lead to the severity of IBD [46]. However, precise role of IL-23 in NK cell activation in mucosal inflammation needs further investigation.

The frequency of NK cells expressing CD16 increased in lamina propria of patients with CD or UC compared to healthy controls [47]. Takamaya et al. showed two distinct populations of NK cells in intestinal mucosa, $NKp44^-$ $NKp46^+$ $CD122^+$ $CD127^-$ and $NKp44^+$ $NKp46^-$ $CD122^-$ $CD127^+$. $NKp44^-$ $NKp46^+$ NK cells produced large amount of IFN-γ and their population was significantly raised in the intestinal mucosa of individuals with CD. On the other hand, $NKp44^+$ $NKp46^-$ cells were scarce in individuals with CD. Thus, altered balance between $NKp44^+$ $NKp46^-$ and $NKp44^-$ $NKp46^+$ cells may be involved in the pathogenesis of IBD [48].

Reduced NK cell infiltration colorectal carcinoma (CRC) tissue has been reported [49]. NK cell and CRC interaction induced alteration of phenotype on NK cells. The tumor cell-associated NK cells down-regulated the expression of activating receptors NKG2D, NKp44, DNAM-1 and had reduced capacity to produce IFN-γ [50]. Likewise, NK cells derived from colon carcinoma showed low expression of DNAM-1 and NKG2D [51]. Furthermore, NK cells in gastric cancer tissue expressed low levels of NKG2D that might be responsible for impaired NK cell function and cancer growth [52]. Combined blocking of NCRs (NKp30, NKp44, and NKp46) leads to the inhibition of NK cell cytotoxicity against H7 29, SW 480 colon cancer cell line [53]. Thus, modulation of NK cell receptors and function involved in disease progression has been consistently observed but the mechanism for receptors modulation on intestinal NK cells is still unclear.

NK cells in skin

$CD56^{bright}$ $CD16^-$ NK cells predominate in skin, express chemokine receptor CCR8, and lack CCR7 expression in contrast to pNK cells [54].

Skin-derived NK cells have potential to lyse melanoma cells. $CD56^+$ $CD3^-$ NK cells exerted cytotoxicity against SK-Mel2 melanoma cell line and produced perforin [55]. Carrega et al. [55] compared NK cell-mediated lysis of 10 different autologous melanoma cell lines and suggested that either deletion or low expression level of HLA class I allele specific for KIR was responsible for NK cell-mediated autologous melanoma cell lysis.

NK cells are actively engaged in psoriasis, a chronic inflammatory condition of skin, characterized by red, raised, scaly plaques typically on elbows, knees, and scalp. $CD56^{bright}$ $CD3^-$ NK cell population has been shown to infiltrate psoriatic skin in response to CXCL10 and CCL5 ligands for chemokine receptors (CXCR3 and CCR5 resp.) expressed on skin NK cells. This NK cell subset also expressed activation marker CD69 and produces high levels of IFN-γ in vitro on IL-2 stimulation [56]. Batista et al. [57] isolated NK cells from psoriatic and healthy skin and observed significant increase in percentage of $CD57^-$ $CD56^+$ $CD16^+$ population with increased expression of NKG2A on NK cells in lesional skin. However, this $CD57^-$ $CD56^+$ $CD16^+$ population showed higher IFN-γ production on IL-2 stimulation. Thus, it can be concluded that psoriatic skin harbors less differentiated phenotype, and future studies are needed to determine the significance of this phenotype on cytolytic activity of NK cells in psoriasis.

Carbone et al. [58] identified a distinct population of NK cells that are $CD56^{bright}$ $CD16^-$ $CD62L^-$ in skin during allergic contact dermatitis. They expressed chemokine receptor CXCR3, CCR6, CCR5, C-type lectin NKG2A, NKp44, NKp46, NKG2D, and perforin that contributed to the activation of IFN-γ and TNF-α, thus associated with allergic responses.

Balsamo et al. [59] demonstrated that melanoma cell lines displayed increased expression of classical and non-classical HLA-I molecules upon co-culture. They also demonstrated that NK–melanoma interaction resulted in the release of IFN-γ and down-regulation of activating receptors NKG2D, NKp44, and DNAM-1, which render

the melanoma cells resistant to NK cell-mediated lysis. Moreover, NK–melanoma interaction also resulted in lower expression of G-protein-coupled receptor, GPR 56, which was involved in tumor progression and metastasis [60].

Morettas group demonstrated that NK–melanoma interaction on co-culture favored the release of indoleamine 2,3 dioxygenase (IDO) and prostaglandin E2 (PGE2), which trigger down-regulation of activating receptors NKp44, NKp30, and NKG2D. This resulted in restricted NK cell-mediated cytolytic activity against melanoma cell and thus aided in tumor progression [61].

NK cells in lung

The lungs and the upper respiratory tract are constantly prone to infection by various bacterial and viral infections, and innate immune system is known to play a crucial role in preventing disease progression.

The study of NK cell subsets in broncho-alveolar lavage fluid (BALF) as well as blood of healthy individuals and sarcoidosis patients revealed that frequencies of $CD56^{bright}$ (2.1 %) and $CD56^{dim}$ (98 %) NK cells in BALF were similar to $CD56^{bright}$ (2.9 %) and $CD56^{dim}$ (96.5 %) subset in peripheral blood NK cells. They also reported NK cells representing $CD94^{high}$ KIR^{low} subset which were recruited from periphery to lungs during respiratory tract inflammation [62]. Similarly, Pokkali et al. [63] reported that NK cells migrated to lungs during TB infection from peripheral blood in response to IL-8, IP-10, and MCP-1. These $CD56^{bright}$ NK cell subsets also showed up-regulated chemokine receptor expression (CCR1, CCR2, and CCR7), activation marker CD69, and TLRs.

Carrega et al. isolated NK cells from non-small-cell lung cancer (NSCLC) specimen; they displayed $CD56^{bright}$ $CD16^{-}$ subset with higher frequency. NSCLC associated NK cells showed higher expression of activation marker NKp44, HLA-DR, CD-69, failed to express CD107a and thus exerted lower cytotoxicity against NK cell susceptible K562 cell line but retained ability to produce cytokines comparable to pNK cells [64]. Likewise, Platonova et al. reported that NK cells infiltrated NSCLC and were restricted to tumor stroma. Intratumoral NK cells showed increased expression of activating receptors NKp44, CD69, and inhibitory receptors CD161, CD94, NKG2A and reduced expression of NKp30, NKp80, DNAM-1, and CD16. Moreover, NSCLC showed higher expression of non-classical HLA-E and HLA-G, protecting them from NK cell cytotoxicity [65]. In line with these studies, Cremer et al. also described tumor-induced modulation of NK cell receptors NKp30, NKp80, DNAM-1, and NKG2D expression and reduced activity of NK cells against NSCLC tumors [66]. It has been reported that $CD11b^{-}$ $CD27^{-}$ NK cell population infiltered NSCLC tumor tissue, and the frequency of this altered population increased as tumor progressed. Thus, $CD11b^{-}$ $CD27^{-}$ NK cells are associated with progression lung cancer [67]. Thus, NK cells have the capacity to migrate to inflammatory sites with varying expression levels of chemokine receptors, and these NK cell distinct subsets are either detrimental or productive for the defense against infection or anti-tumor response that might open prospective for manipulating NK cells during immunotherapeutics strategies.

Conclusion

Cytotoxicity by NK cells is based on interplay of activating and inhibitory receptors. Migration of NK cells from periphery to different organs and their receptors modulation may be regulated by cytokines and organ-specific microenvironment. Cytotoxic potential of infiltrating NK cells has been greatly exploited for elimination of tumor cells. Adoptive transfer of in vitro activated allogeneic and autologous NK cells has exhibited clinical efficacy toward control of NSCLC and HCC [68, 69]. However, poor clinical responses were observed with adoptively transferred NK cells in melanoma patients [70]. Thus, cytotoxic potential of NK cells against tumors localized in different organs against tumors needs to be explored further to establish promising and safe NK cell-based immunotherapy. Better understanding of the mechanisms behind receptor modulation, activity, and proliferation of organ-specific NK cell- and tumor-mediated modulation of NK cell activity will give insights into devising good manufacturing practice conditions for adoptive transfer of organ-specific NK cells against various tumors and to answer many of our unresolved lacunae in the development of NK cell-based cancer therapeutic strategies.

Conflict of interest None.

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