Effect of tumor-derived factors on splenic NK cell receptor modulation

A major project dissertation submitted

in partial fulfilment of the requirement for the degree of

Master of Technology

In

Biomedical Engineering

Submitted by

Richa Mishra

(2K13/BME/05)

Delhi Technological University, Delhi, India

Under the supervision of

Dr. Asmita Das

Department of Biotechnology
Delhi Technological University
(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road,
Delhi-110042, INDIA

CERTIFICATE

This is to certify that the M. Tech. major project dissertation entitled **"Effect of tumor-derived factors on splenic NK cell receptor modulation"**, submitted by **Richa Mishra (2K13/BME/05)** in partial fulfilment of the requirement for the award of the degree of Master of Engineering, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance.

The information and data enclosed in this dissertation is original and has not been submitted elsewhere for honouring of any other degree.

Date:

Dr. Asmita Das
(Supervisor)
Department of Bio-Technology
Delhi Technological University
(Formerly Delhi College of Engineering, University of Delhi)

DECLARATION

I, **Richa Mishra**, hereby declare that the work entitled "**Effect of tumor-derived factors on splenic NK cell receptor modulation"** has been carried out by me under the guidance of Dr. Asmita Das, in Delhi Technological University, Delhi.

This major thesis is part of partial fulfilment for the degree of M.Tech in Biomedical Engineering. This is the original work and has not been submitted for any other degree in any other university.

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Date:

Dr. Asmita Das
(Project Mentor)

Prof. D. Kumar
(Head of the department)

Department of Bio-Technology
Delhi Technological University
(Formerly Delhi College of Engineering, University of Delhi)

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Effect of tumor-derived factors on splenic NK cell receptor modulation

Richa Mishra

Delhi Technological University, Delhi, India

1.Abstract

Natural Killer (NK) cells have natural capacity to kill tumor cell. They do not require prior antigenic sensitization and is capable of dealing with broad range of virus infected cells. Thus, NK cells play important role in host defense against tumor cells as well as virus infected cell. The sensitivity of infected cell to NK cell lysis may open new prospectives for NK cell-based immunotherapy. NK cell activation and function are strongly influenced by the interplay between inhibitory and activating signals. Tumors have evolved various mechanisms by which they can evade NK cell attack. These mechanisms include interference with NK cell activation, inhibition as well as modulation of NK cell function. The modulation may be of recognition and inhibition in NK cell receptor and modulation of co-stimulation, adhesion or susceptibility to apoptosis. Co-culturing of different cell line,one is resistant (P815) and another is sensitive (YAC-1) with the purified NK cell recovered from spleenocytes of C57BL/6 mice strain. Modulation caused in NK cell receptor was analyzed and profiled by the use of flow-cytometric methods. We found that tumor cells inhibited major NK cell receptors expression which are responsible to intiate and stimulate their immune function, including NK 1.1(mouse) inturn activate some inhibitory receptor expression which include Ly49A and Ly49C of NK cell against NK resistant cell lines.

2. Introduction:-

Natural Killer (NK) cells are studied as a class of large, granular cytotoxic lymphocytes of the innate immune system that display lytic activity towards tumor cells and virus infected cells without prior sensitization. NK cell recognize target cells in two different ways i.e. lack of MHC-class 1 expression and absence of expression of appropriate ligands of NK cell receptors renders the target cell susceptible to NK mediated lysis. NK cell receptor utilize several parallel recognition pattern that enables them to distinguish between abnormal cell and healthy cell. NK cell cytotoxicity is regulated by at least two families of receptors that recognize classical MHC class I molecules on the surface of target cells and enable them to discriminate between healthy cells and pathogen infected or tumor cells by monitoring the expression levels of MHC molecules. Functions of NK cells varies differently in different organs due to some organ specific functions and cell-cell interaction. Recognition of NK cells is based on an array of inhibitory and activating receptors and they utilizes these recognition for eliminating and differentiating aberrant cells (Lanier, 2005).

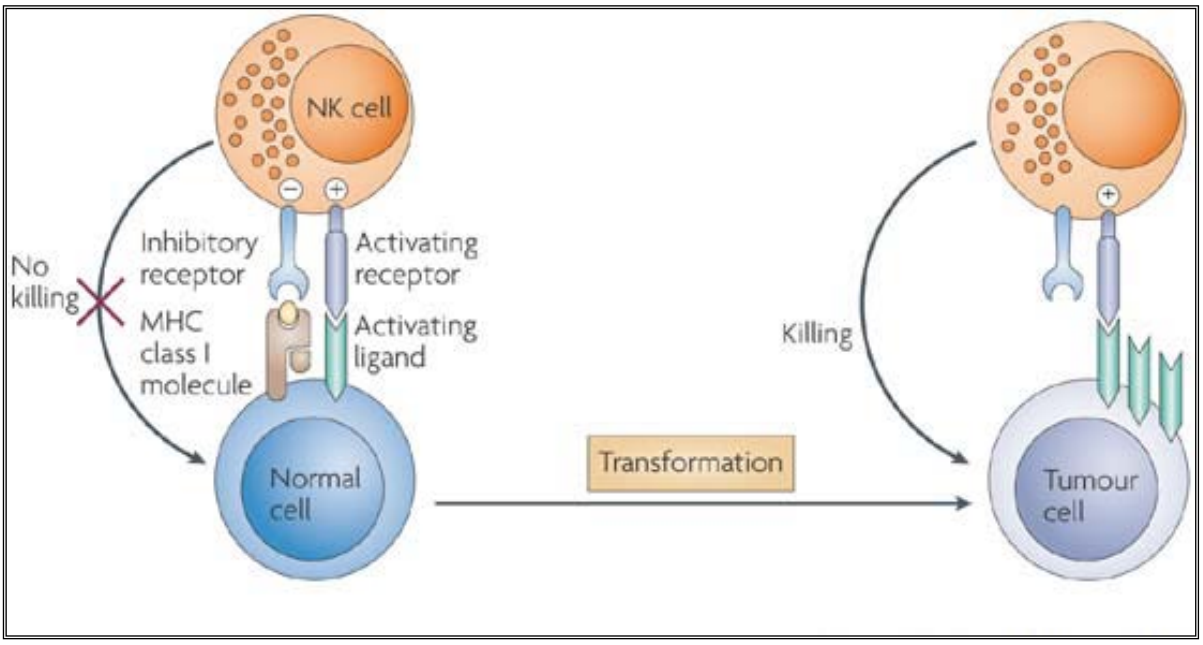


Figure 1:- Transformation of Normal cell to tumor cell: Regulation and Activation of Natural killer (NK)-cell depends on the signal balance between inhibitory and NK 1.1 receptor. (David *et al., 2006)*

MHC class I ligands on target cells associate with the inhibitory receptors present on NK cells and they are often found to get reduced during the cellular transformation of normal cells to tumor cells and this event on tumor cells are followed by the upregulation of ligands for activating receptors present on the tumor cells due to cellular stress and DNA damage. Altogether, these incidents lead to the activation of NK cell and inturn induces cytolytic effector functions which results in killing of tumor cell (Ljunggren et al., 2007)

Tumors are found to escape recognition by NK cell and killing due to the upregulation of ligands which are liable to get associated with inhibitory receptors and in parallel looses or downregulate ligands for activating receptors which mainly occur during tumor progression events.

NK cells respond to various biological agents, for example IFN or IL-2 by enhancing its functions like cytolytic and proliferative action. NK cells are classified as a vital component of antitumor immunity liable for eradicating tumor cells from blood.
Some inhibitory and activating receptors which are expressed on NK cell of human and mouse:-

Table 1: Inhibitory Receptors on NK cell:-

Species	Receptor	Receptor super-family	Ligand
Mouse	Ly49A	C-type lectin	H-2D
Mouse	Ly49C	C-type lectin	H-2Kb
Mouse	CD94/NKG2A	C-type lectin	HLA-E
Human	KIR2DL	Ig	HLA-C
Human	KIR3DL	Ig	HLA-Bw4, HLA-A
Human	CD94/NKG2A	C-type lectin	HLA-E

Table-2: Activating Receptors on NK cell:

Species	Receptor	Receptor super-family	Ligand
Human,Mouse	NKG2D	C-type lectin	CD48
Mouse	Ly49H	C-type lectin	MCMV-induced
Mouse	Ly49D	C-type lectin	H-2D
Mouse	NK1.1	C-type lectin	Clr-g (NKR-P1F)

Adaptive immune system are responsive to highly specific antigen which are recognized by the receptors present on T and B lymphocytes, whereas splenic NK cell induced by IL-2 respond in non-specific manner, as there are variability in target preferences due to the presence of subpopulation of NK cell. It has been seen that different subsets of IL-2 induced NK cell population, which although overlaps are engaged in lysis of YAC-1 tumor cells.

NK 1.1 surface receptor on NK cell is encoded by the NKR- P1C/ NKR-P1B gene, referred as CD161b and also Ly-55. It is usually expressed on NK cells and its subpopulation NK-T cells in C57BL/6. NK1.1 has been observed to participate in NK cell activation and and they are commonly used in experiments as mouse NK cell markers.

Ly49A and Ly49C are inhibitory receptor expressed mainly on NK cell surface of C57BL/6 mice strain. Ly49A is C- lectin type mouse NK cell receptor, which mainly function by interacting with major histocompatibility complex class I molecule. Ly49C is a cell surface molecule expressed on a subpopulation of murine natural killer cells. It transduces a negative signal to NK cells upon recognition of class I antigens.

3.Review of Literature:-

3.1 Role of tumor microenvironment in establishment of NK cell mediated anti-tumor immunity:-

It has been noticed that the tumor microenvironment is not only passive recipient of immune cells, but it also plays an active role in the establishment of immunosuppressive conditions.

Immune cells are capable of adapting to the tumor microenvironment and secrete certain immuno modulatory signals or mediators which affect the functioning of immune cells either through directly mechanism or through stimulating other cells present on the tumor site.

Tumor cells have been reported with the capability of activating their own resistance mechanisms, example autophagy to escape the effective immune response.

Tumor cells synthesis various number of environmental factors which includes growth factors, cytokines , microRNAs, exosomes which impact the immune cell response for target killing or escaping from immune cell mediated killing.

NK cells are activated in the peripheral blood by NK cell stimulatory factors, such as interferon (IFN)-α and –β, interleukin IL- 15, IL-12, or IL-2 (Smyth *et al.*, 2002). NK cell activity is regulated by germline-encoded inhibitory and activating receptors. Recognition of ligands derived and induced by stress, pathogen is funtioned by activating receptors, whereas the inhibitory receptors are found to be associated with binding with self-molecules presented by normal cells. Inhibitory and activating receptors diversity makes NK cells capable of recognizing and killing various forms of tumor (Moretta *et al., 2008*). NK cells play role in the regulation of anti-tumor adaptive immunity and innate immunity both. (Paust *et al., 2010*; Zingoni *et al.,2004*)

3.2 Destruction of NK cell function by recruited immune cells to tumor areas:

Tumor cells are noticed to induce homing of certain cells to tumor areas such as bone marrow-derived CD45+ myeloid cells (Mantovani *et al., 2008*). Tumor cells are functioned to recruit immune cells by production of cytokines such as tumor necrosis factor (TNF)-α and stromal cell-derived factor 1 (SDF-1), in order to create an immunosuppressive microenvironment which activate their resistant to be killed by NK cell.

Major components of the immunosuppressive network capable of impairing NK cell function are Myeloid-derived suppressor cells (MDSCs) (Monu *et al., 2012*), this function is linked with the production of IL-10 which ultimately decreases the IL-12(pro-inflammatory cytokine) production by macrophages, which is involved in NK cell activation (Sinha *et al., 2007*). Researchers have also found that MDSCs expanded through cancer cell presence, induces NK cells' anergy by inhibiting cytotoxicity or NKG2D expression, and membrane-bound transforming growth factor (TGF)-β inhibit IFN-γ production. (Li H *et al., 2009*).

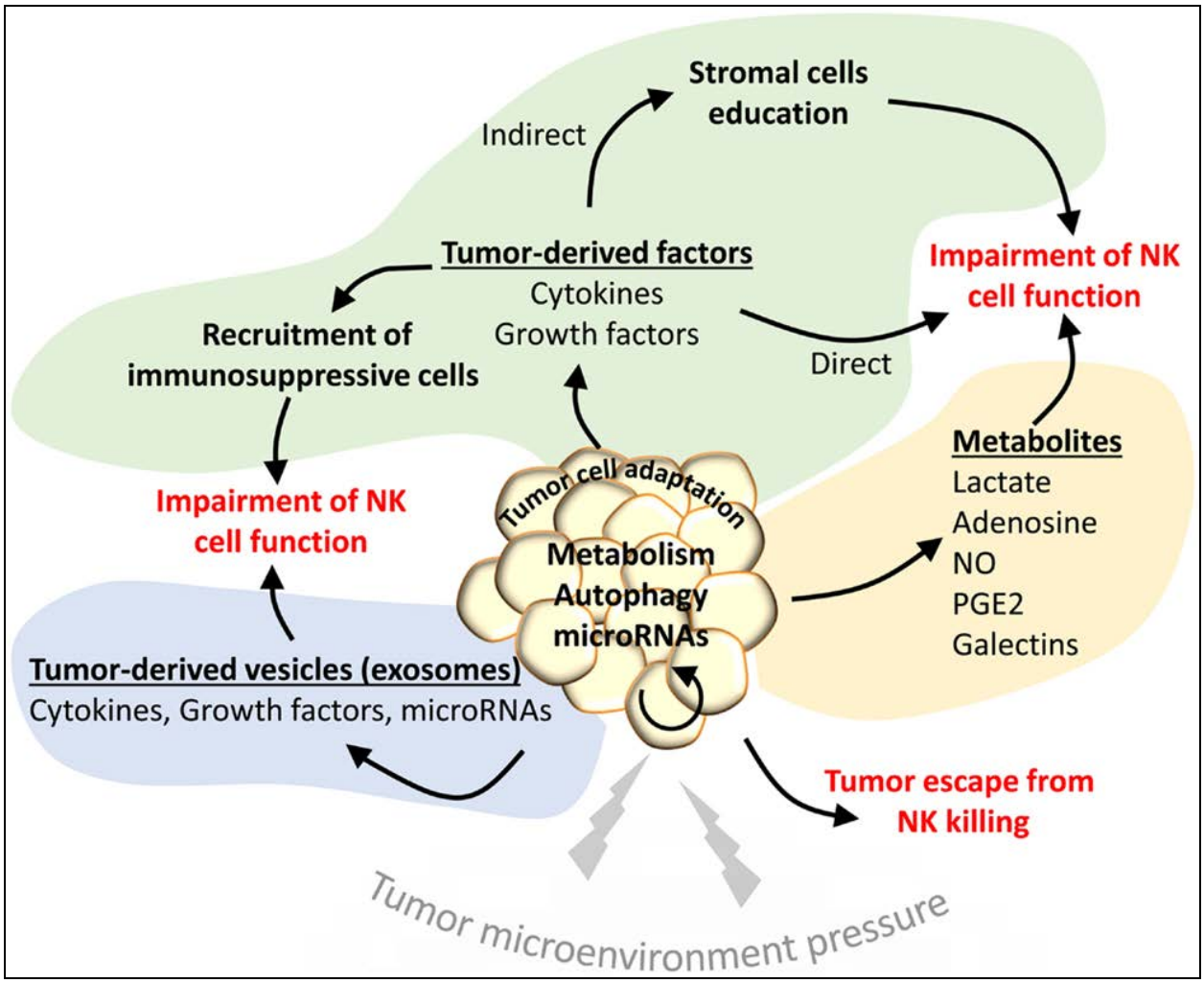


Figure 2: **Different mechanisms involved in impairement the NK-mediated anti-tumor immunity.**(Baginska *et al.*, 2013)

Under tumor microenvironment pressure, tumor cells adapt to stress by activation of intrinsic resistance mechanisms (autophagy). Such regulation results in the secretion of

several metabolites that impair NK cells function in the tumor site .Tumor cells may activate the secretion of tumor-derived vesicles which includes cytokines, growth factors microRNAs to directly affect NK cells functions. These factors are secreted directly in the tumor environment to recruit immunosuppressive cells or to direct stromal cells which are involved in the depletion in the function NK cell functions.

Researchers have demonstrated that hypoxic tumor cells are also involved in upregulation of the matrix metalloproteinase (MMP)-7 protein expression on the tumor associated macrophage surface, which inturn leads to the splitting of Fas ligand from adjoining cancer cells, making them least responsive to NK cells (Burke *et al., 2003*).

Capability of melanoma cells in activating cancer-associated fibroblasts (CAFs) by the release of TGF-β or IL-6 creates the immunosuppressive microenvironment which is seen to interfere with cytokine production by NK cell and also its cytotoxicity function (Lohr *et al., 2001;* Giannoni *et al., 2010*). CAFs are also capable of inhibiting the upregulation of the activating receptors such as DNAX accessory molecule-1 (DNAM-1), NKp30 and NKp44 at the NK cell surface which are induced by IL-2.

Activating receptors expression (NKp44 and NKp30) present on NK cell is notably seen to get modulated by prostaglandin E2 (PGE2) which is secreted from CAFs, while cell-to-cell interaction was necessary for regulation of DNAM-1 regulation. These inhibition results in malfunctioning of NK cell-mediated killing of tumor target cells (Balsamo *et al., 2009*).

3.3 Effect of tumor-derived factors on NK cell inhibition:-

Tumor-derived factors have ultimate effect on NK cell mediated killing. MICA and MICB, the MHC class I chain-related molecules, and UL16-binding proteins (ULBPs) is seen on the carcinomas. The interaction of NKG2D receptors present on NK cell and tumor cell surface MIC molecules plays critical role in activation of mechanism to kill the target cell. Hypoxia has been noticed to interefere in the increment of MICA shedding from the cancer cell surface by nitric oxide(NO) signalling malfunctioning and thus associated with NK cell-mediated target cell killing. Down regulated NKG2D and CXC chemokine receptor(CXCR)1 expression was seen on the NK cell surface and was found to be only associated with soluble MIC molecules shed off from cancer cells.(Barsoum *et al., 2011*). Dimers of the HLA-G (a

non-classical MHC class I molecule) are induced at the surface of melanoma cells by hypoxic stress, thereby escaping tumor cells to be killed by NK cells. Expression of MICA2 ligands (which are recognized by activating receptor, NKG2D) was only observed in NK-sensitive cell lines, whereas its expression was not seen in NK resistant cell lines.

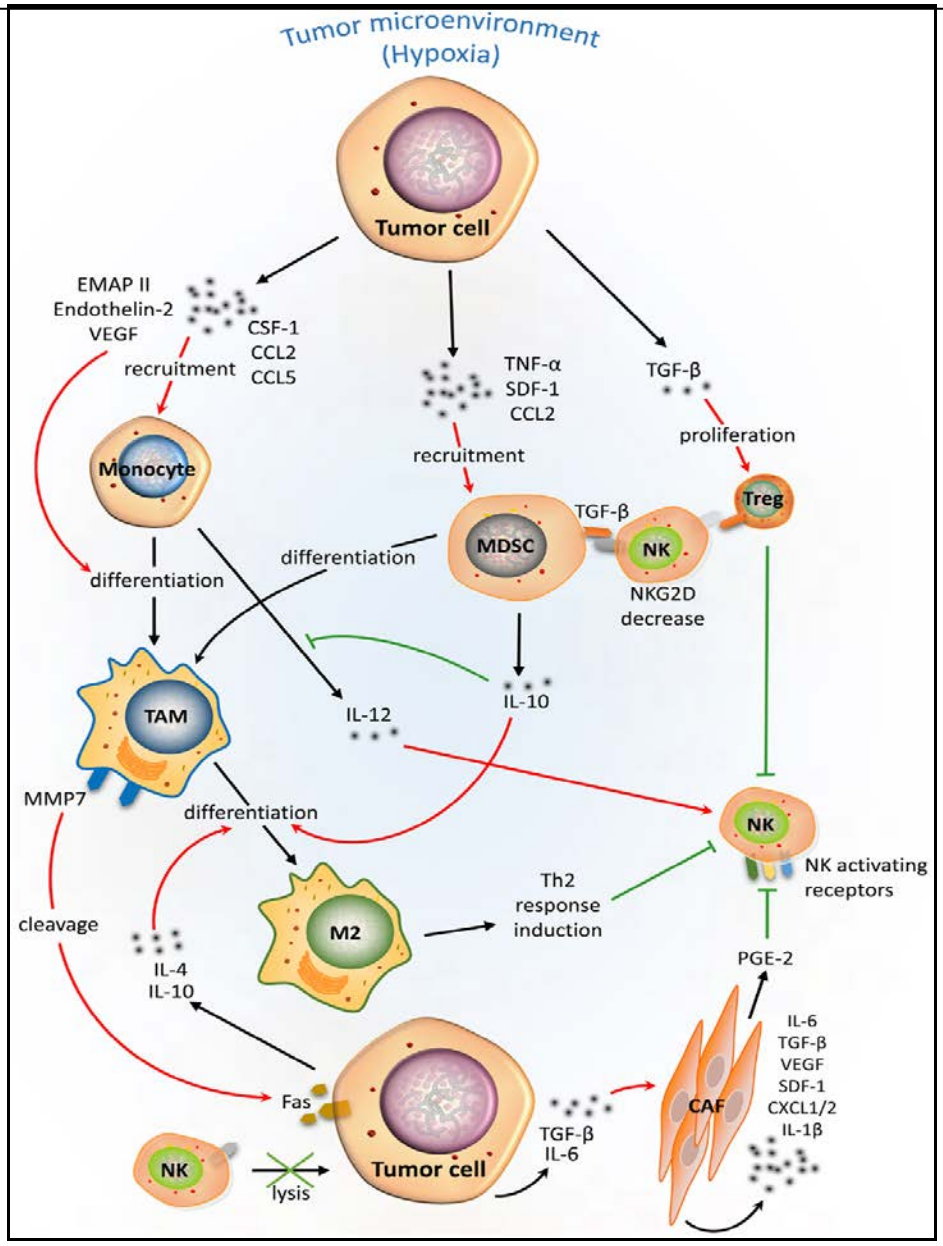


Figure 3:- Cellular crosstalk in the tumor microenvironment inhibits NK-cell mediated killing of target cells.(Baginska *et al.,* 2013)

Soluble factors are secreted in tumor environment which are responsible for educating immune cells This figure illustrate the effects of tumor-derived factors on activation, proliferation, differentiation, and recruitment of tumor-linked cells (red arrows) and their activities associated with immunosuppression (green lines) which interfere in tumor cell lysis by NK cell.

3.4 Role of TME in converting NK cell into Myeloid-derived Suppressor cells (MDSCs):-

At some maturation stage NK cells are noted to get converted into MDSCs. It has also been noticed that phenotype of NK cells were lost. Maturation process of NK cell proceeds in a co-ordinated manner as: $CD11b^{low}CD27^{neg/low}$ –> $CD11b^{low}CD27^{neg/high}$ –> $CD11b^{high}CD27^{neg/high}$ –> $CD11b^{high}CD27^{low}$ (Chiossone *et al.,2009*), and NK cells of $CD11b^{high}CD27^{high}$ types are converted into MDSCs which ultimately result in the decrement of the precursors of mature NK cells of $CD11b^{high}CD27^{low}$ type, which decrease both the populations, and thus led to NK cell reduction in the tumor microenvironment.

3.5 Tumor immunoediting to escape NK mediated killing:-

When tumor cells come in contact with NK cells, they undergo for the altered ligand expression for either activating or inhibitory NK-cell receptors. Resistant towards NK cell meditaed cytotoxicity is observed to occur because of the enhanced expression of both nonclassical and classical HLA-I molecules (Balsamo *et al., 2012*).

Further, in a tumor mouse model, which was induced by methylcholanthrene (MCA), a carcinogen with polycyclic aromatic hydrocarbon, the NKp46, an activating receptor on NK cell was seen to be responsible for editing its ligand expression at the surface of tumor cell. Moreover, when tumors observed in mice-deficient in NKp46 expression, it displayed a higher level of expression of the NKp46 ligand as compared to the wild-type mice (Elboim *et al., 2011*).

Tumor escape mechanism further continue by downregulation of NKG2D ligands on tumor cell surface or upregulation of Proliferating Cell Nuclear Antigen (PCNA), so, classical or non-classical HLA-1 molecules get associated with the activating NKp44 and inturn decreases the chances of NK mediated killing.(Rosental *et al., 2011;* Horton *et al., 2011*)

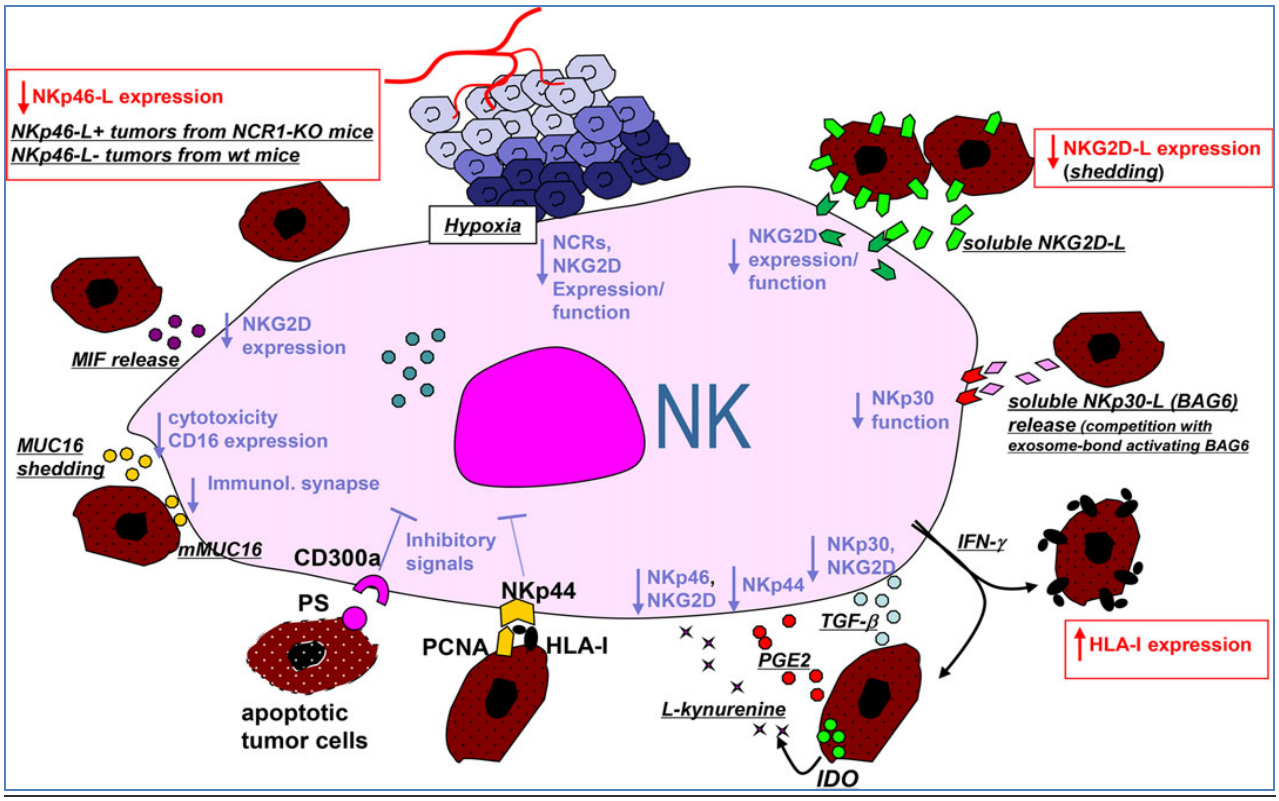


Figure 4:- Various mechanism of tumor evasion from NK cells.(Massimo *et al.,2014)*

3.6 Reasons for tumor resistance towards NK cell killing in most types of cancer:-

There could be so many reasons for the resistance of tumor to NK cell mediated killing, some of them includes:

It can be due to the reduced expression of NKG2D on the tumor cell surface which is facilitated by soluble factors like TGF-β (Lee JC *et al., 2004*; Castriconi R *et al.*,2003) and one of the product of tryptophan degradation, L-kynurenine. It was also observed reducing NKp46 expression on NK cells of human. (Chiesa Della *et al.,2006)*

Pietra G et al. have suggested that the co-culture of tumor cell lines with IL-2 induced NK cells led to the inhibition of the upregulated IL-2-induced NCRs which includes NKG2D ,NKp44 and NKp30, all of which are responsible for killing of tumor cells (Pietra G *et al., 2012).* Lateron, it was reported that this effect resulted from tumor-derived factors like prostaglandin E2 and indoleamine 2,3-dioxygenase secreted by tumor cells. Inhibitory receptors expressions remained unchanged, which indicate that activating receptors are major

targets of tumor-mediated elimination. Evidences support that activating NK cell receptors expression in the tumor environment is regulated at the transcriptional level.

3.7 Some Tumor-derived factors affecting NK cell function:-

Several tumor-derived factors which includes

Lactate

Oxygen depriving condition in tumor cells undergo a significant alteration of glucose uptake metabolism which is a characteristic of high glycolysis. HIF-1α induces expression of multiple genes responsible for catalyzing glucose uptake mechanism (Brahimi *et al.,* 2011) for instance,expression of pyruvate dehydrogenase kinase and monocarboxylate transporter is regulated by HIF-1α, which inturn inhibit reduction of pyruvate to acetyl CoA (Kim *et al.,* 2006). Pyruvate accumulation in cells destruct its metabolism to move to tricarboxylic acid cycle. Pyruvate is converted to lactate and released from the tumor cells. NK-mediated killing is affected by lactate by the upregulated MDSCs, which establishes immunosuppressive environment.

Adenosine

Accumulation of adenosine in the tumor space is reported as mechanism for modulation of immune cells (Sitkovsky *et al.,* 2013).when released in the extracelluar space, it exerts several modulatory effects through binding on receptors of adenosine (like A1, A2A, A2B, and A3) which are expressed on various immune cells like NK cells

Nitric Oxide

Evidences are found that the cells exposure to hypoxic condition are linked to the inhibition of production of NO (McCormick *et al., 2000*). Siemens et al. suggested that disfuntioning of NO signaling in tumor cells led to tumor evasion from NK killing. Shedding up of MIC from tumor cell surface contribute to NO signaling in several types of cancer reported. These shed off can be blocked by the administration of NO mimicking agent(Siemens *et al., 2008*) and this contribution can reactivate NO and could prevent tumor escape.

Prostaglandin E2

Researchers have suggested misregulation of cyclooxygenase (COX)-2 is a major factor in tumor escape from the immune response (Kalinski *et al.,* 2012). Additionally, COX-2 is seen to be over expressed in various types of known tumor through HIF-1α. dependent mechanism. This upregulation is linked to PGE2 production in excess in the tumor space(Pietra *et al., 2012*). Some studies reports that PGE2 downregulates NK cells cytolysis activity (Goto *et al.,* 1983*;* Bankhurst *et al., 1982*) through a process that consequence in IFN-γ production inhibition.

Galectin

Galectins are reported as a proteins, which belong to the lectin family participating in the signals delivery when it binds to the glycolipids and glycoproteins present on target cell surface. Some have evidences that Gal-3 on tumor cells is involved in the modulation of MICA-NKG2D crosstalk which results in severe impairement in the activation of NK cell and thus property of killing target cells. (Tsuboi *et al.,* 2012)

4. Material:-

4.1 Mouse strain

C57BL/6 (WT) mice were issued from INMAS animal facility. Mice were housed under specific pathogen-free condition and used in experiments at 7-19 weeks of age. Experiments were done according to guidelines provided by animal experimental ethics committee.

Figure 5: C57BL/6 mice strain

4.2 Cell lines

Name	Cell Type	Medium
YAC-1	Moloney murine leukemia virus (Mo-MuLV) induced	RPMI
P815	Murine mastocytoma	DMEM

4.3 Cell culture media

Name	Cell line
DMEM	P815
RPMI	YAC-1

Figure 6: RPMI for YAC-1 cell line and DMEM for P815 cell line

4.4 Kits

Name	Company
EasySep™ Human NK Cell Enrichment Kit	Stemcell Technologies

4.5 Antibodies

Name	Company
FITC-labelled anti- NK 1.1	eBioscience
PE- labelled anti- Ly49A	eBioscience
PE- labelled anti-Ly49C	eBioscience
FITC- labelled anti- CD49b	BD Bioscience
APC- labelled anti- CD3	BD Bioscience

4.6 Cytokines

Name	Company
Recombinant Interleukin-2	BD Bioscience

IL-2 Dilution

- Take 20ul of IL-2 from commercial vial in sterilized 15 ml falcon tube inside the hood.
- Add 4980 ul of sterilized PBS in it.
- Transfer 500ul of prepared sample in 10 eppendorf tube.
- Keep it in -20^0 C

4.7 Phosphate Buffer Saline (PBS)

For 1 Litre

Chemicals	**Concentration**
Sodium chloride (NaCl)	8 g
$Na_2HPO_4.H_2O$	1.44 g
Potassium chloride (KCl)	0.2 g
KH_2PO_4	0.2 g
Distilled H_2O	900 ml
pH	**7**.3-7.4

4.8 Medium for NK Cell purification (Recommended media)

For 400ml

Chemicals Required	Concentration
PBS	392 ml
FBS	8 ml
EDTA	1 mM

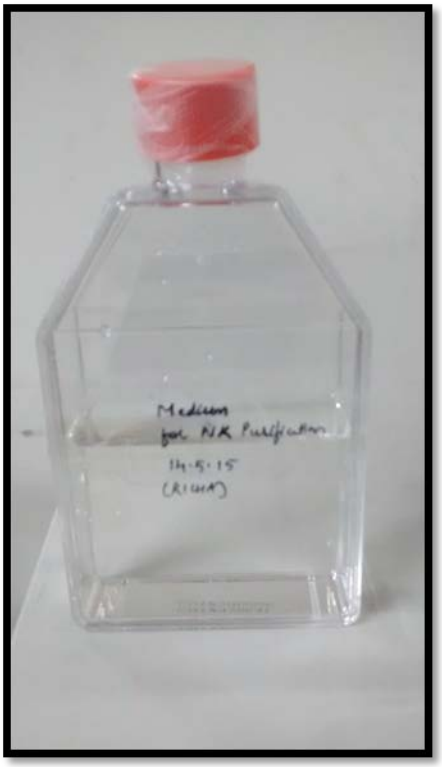


Figure 7 : NK purification media(recommended media)

4.9 **Hank's Balanced Salt Solution (HBSS):-**

For 1 Litre

Chemicals	**Requirement**	**pH**	**Storage**
HBSS(9.76g/L)	9.76 g/L. 1 vial in 1 L	-	Store at below 8^{o} C
HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) (23.83g in 100ml=1M	10mM	6.6-8	Store at room temperature
NaOH (4g in 100ml)	1 N	-	Corrosive. Store at room temperature

Figure 8: **Hank's Balanced Salt Solution (**HBSS)

5.Method:-

5.1 Cell culture methods

5.1a : Thawing cells

1. Take out the cryovials from -80^{0}C.
2. Transfer it into a waterbath maintained at 37^{0}C.
3. Resuspend the thawed culture in 5ml media in falcon tubes.
4. Spin at 1500 rpm for 10 minutes.
5. Discard the supernatant to remove freezing media.
6. Resuspend cells in 5 ml DMEM/RPMI.

5.1 b: Splitting of cells

Cells in suspension reaching an optimal density or confluency were split in ratios ranging from 1:5 up to 1:20 by adding the appropriate volume of medium.

1. Take the cells from culture flask in falcon.
2. Centrifuge the tube at 2000 rpm for 15.
3. Discard supernatant and suspend the cellular pellet in 1ml DMEM+10% FBS (in case of P815) and 1ml RPMI+10% FBS(in case of YAC-1) in culture flask.

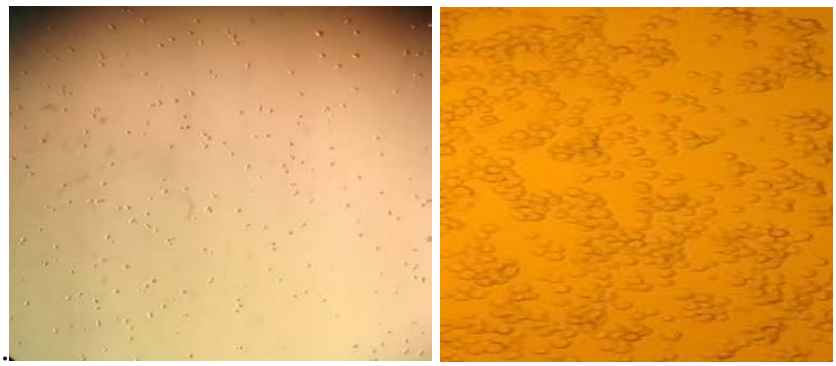


Figure 9: YAC-1 and P815 cells in phase contrast microscope.

4. Take 10µl of culture to count the number of cells.
5. After counting add cells to 5ml media.
6. Observe the cells under microscope.
7. Incubate the cells in CO_2 incubator maintained at 37^0C

Figure 10: P815 cells in T-25 flask

5.1 c: Determination of cell numbers

Cells were counted with a Neubauer counting chamber (0.1 mm depth). The number of live cells per ml cell suspension was calculated as the following:

$$\text{Concentration (cells/ml)} = \frac{\text{Counting of cells}}{4} \times \text{Dilution factor} \times 10^4$$

Total no. of cells = Concentration (cells/ml) X Volume of sample (ml)

5.1 d: Cell Viability Assay

Take the average of live (transparent color, which do not take up the dye) and dead cells (blue color, which take up the dye) seen in the microscope.

$$\text{Count percentage viability (\%)} = \frac{\text{Average of live cells}}{\text{Average of live cells + Average of dead cells}} \times 100$$

$$\text{Cell density (cells/ml)} = \frac{\text{Average live cells X Dilution Factor}}{\text{Volume of squares (ml)}}$$

Total Cells = Cell density (cells/ml) X Volume (ml)

$$\text{Final Volume (ml)} = \frac{\text{Total cells}}{\text{Target density (cells/ml)}}$$

5.1 e: Procedure to count cells

1. Clean the hemocytometer with alcohol.
2. Slide the cover slip over the grid .
3. Take 10µl of the cell suspension into both the sides of grid chamber.
4. View and focus the slide under an inverted phase contrast microscope at 10x magnification.
5. Count the number of cells in all four 16 square grids.

Figure 11: Haemocytometer

5.2 Freezing media (10 ml, kept at -20^{0}c after preparation)

50% serum (5ml)

40% media (4ml)

10% DMSO (1ml)

Media and DMSO are filtered through a filter to remove any contamination before mixing them with serum.

5.2a Freezing of cells

1. For cell freezing, cell suspensions were centrifuged (1200 rpm, 10 min, RT)
2. The pellet was resuspended in freezing medium at a concentration of 5 x 106 cells/ml.
3. 1.8 ml of cell suspension was aliquoted in cryovials and placed in freezing containers.
4. After 24 h at -80°C vials were transferred to liquid nitrogen for long term storage.

Procedure for making HBSS:-

- Transfer 1 vial of HBSS into 1 L bottle.
- Add 950 ml pf 18 MilliQ water.
- Dissolve contents and autoclave
- Add 10ml of 1M sterile NaOH to adjust pH 7.4.
- Make up volume to 1L using autoclaved milliQ water
- Put samples of filtered HBSS for test at 37 degree Centrigrade, if test samples do not show turbidity, change in pH or precipitation;HBSS is safe to use.

5.3 Organ Dissection

Spleen

Animals were sacrificed by dislocation of the neck. The spleen was excised using sterile forceps and kept in ice-cold PBS. Single cell suspensions were obtained by mincing the spleen through a 70 µm cell strainer followed by washing with HBSS and adding recommended media during the crushing and centrifuge at 1500 rpm for 10 min at 4°C). Discard the supernatants and resuspened in 1ml of NK purification media (designated "**MASTER TUBE**"). Counting of cells was performed using haemocytometer in order to ensure the required number of cells to perform experimental setup.

Figure 12:- Crushing of spleen through frozen slides for isolating spleenocytes

5.4 NK cell Purification assay protocol:-

Master tube (as discussed above) exhibit spleenocytes suspended in 1 ml of NK purification media/recommended media.

1.Take 25ul of spleenocytes in another tube for staining and add 75ul of recommended media.Add 1ul anti-CD3 and anti-CD49b and store it at 4^0C overnight and wrap it with aluminium foil.

2.Enrich NK cell by following manual of Stem Cell (as discussed below) followed by counting the cell.

3.Take 50ul of enriched NK cell population and add 50ul of recommended media. Add 1ul anti-CD3 and anti-CD49b and store it at 4^0C overnight and wrap it with aluminium foil.

5.5 Enrichment of NK cell from spleenocytes

This enrichment procedure is used for processing 500 µL - 1.5 mL of sample (upto 1.5×10^8 cells). For efficient performance, each steps must be done at room temperature nearly 15 - 25°C.

1. Single cell suspension is prepared at a concentration of 1×10^6 cells/mL in recommended medium. Place the cells in a 12 x 75 mm polystyrene tube to fit properly into the EasySep® Magnet.

2. Quick spin is recommended to the tube of EasySep® Negative Selection Mouse NK Cell Enrichment Cocktail before use inorder to ensure recovery of entire liquid in a tube. Add Enrichment cocktail at 50 µL/mL of cells (e.g. for 1 mL of cells, add 50 µL of cocktail). Mix it well and consequently incubate it at room temperature at 15 - 25°C for 15 minutes.

3. Add EasySep® Biotin Selection Cocktail at 200 µL/mL cells (for instance, adding 200 µL of selection cocktail for 1 mL of cells,). Mix it well and consequently incubate it at room temperature at 15 - 25°C for 15 minutes.

4. This step is the most important step which is followed by vortexing the EasySep® D Magnetic Particles for 30 seconds. Ensure that magnetic particles are suspended fully with no visible aggregates.

5. Carefully add magnetic particles at 200 µL/mL cells. Mix it well and incubate at room temperature (15 - 25°C) for 10 minutes.

6. Add recommended medium so as to maintain the cell suspension to a total volume of 2.5 mL. Gently pipette up and down the cells in the tube 2-3 times for mixing . Place the polysterane tube without cap into the magnet and set aside for 5 minutes.

7. Hold the Magnet, and invert the magnet in one continuous motion, pour off the desired fraction into a new polystyrene tube. Magnetically labeled unwanted cells is seen to remain bound in the original tube, which is held by the magnetic field of the magnet..

8. Remove the original tube which contains the magnetically labeled unwanted cells from the EasySep® Magnet and kindly get the new tube which contain the desired enriched cells inside the magnet to perform a enrichment for the next time. Set it aside for **5** minute. The enriched cells are ready for use.

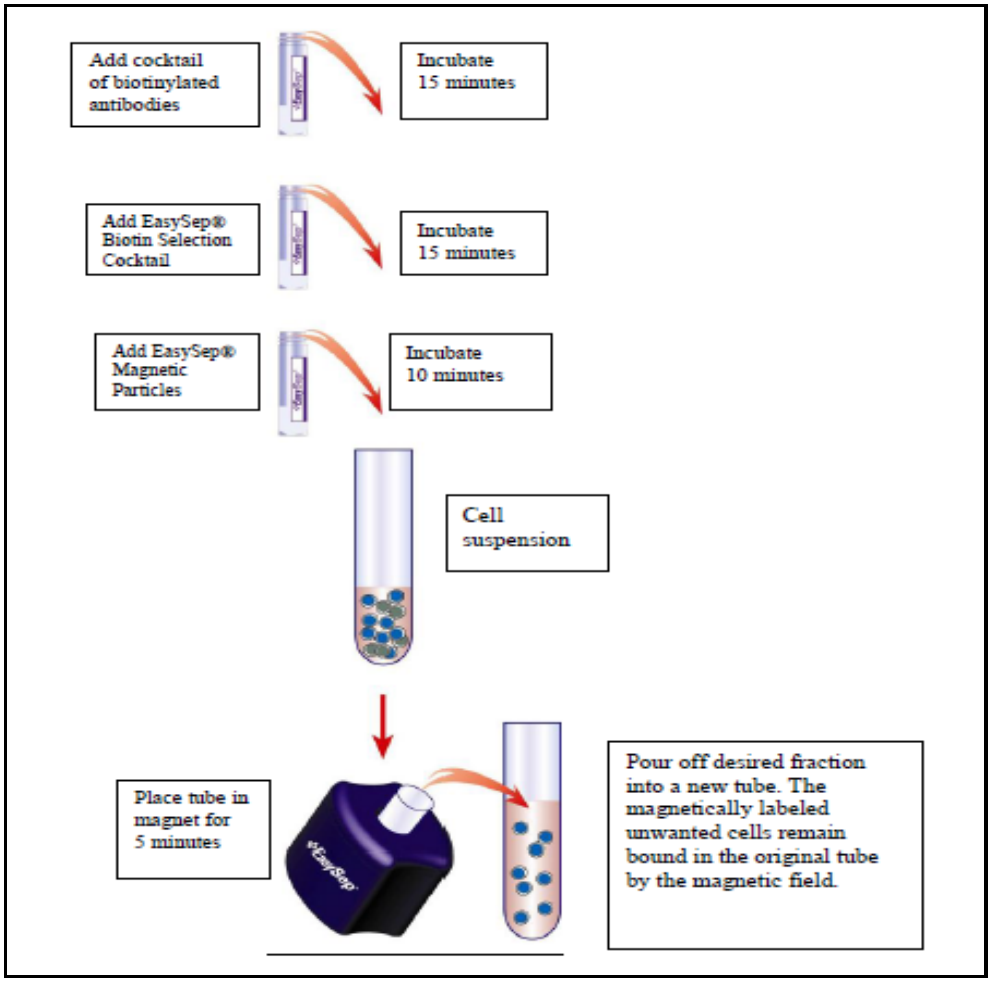


Figure 13:- **NK enrichment steps as mentioned by EasySep™ Mouse NK Cell Enrichment Kit**

5.6 Antibody staining Protocol

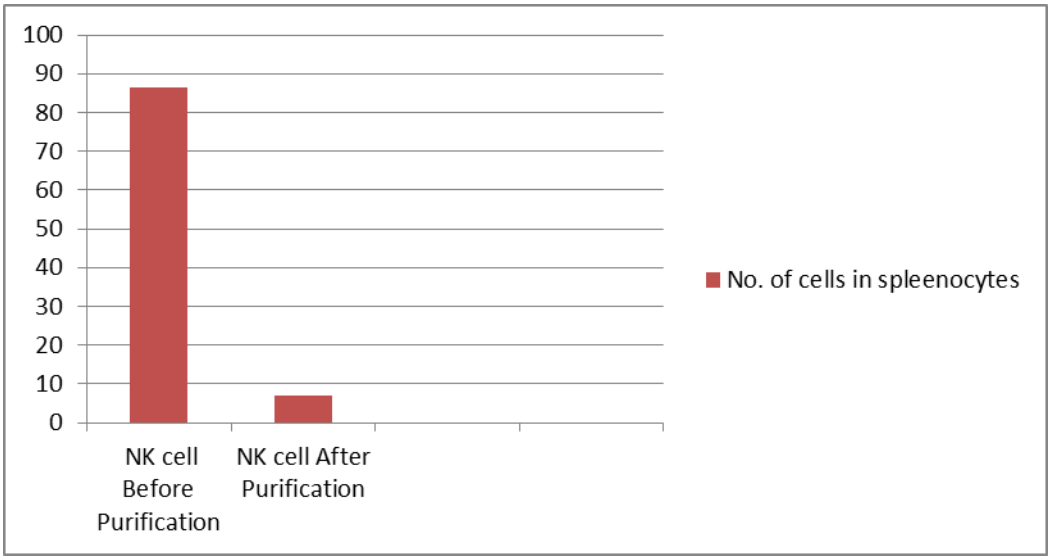
1. Isolate spleenocytes from C57BL/6 mice and subject the cells for RBC lysis. Recover lynphocytes from the cell suspension.
2. Wash purified cells 1X in staining buffer (PBS).
3. Adjust concentration of cell suspension to 0.1 $X10^6$ cells/ml. Cells should be greater than 90% viable as determined by trypan blue exclusion.
4. Determine no. of tubes/eppendorfs necessary for the experiment and add the purified cells (0.1 $X10^6$ cells/ml) from spleen to each Eppendorf.

5. Make the antibody dilutions and add 10µl antibody of desired dilution to the respective tube.
6. Keep the samples at 4^0C for 45min.
7. Now, wash with 500µl PBS at 3500 rpm for 10min.
8. Discard the supernatant and 200 µl PBS to each tube, mix well and transfer the cells to FACS tube.
9. Perform analysis on flow cytometer.

5.7 Flow Cytometry:-

5.7 a NK cell proliferation assay:-

Proliferating and purification assay was done using recombinant IL-2 and EasySep Mouse NK Enrichment kit and staining it with different antibodies against NK cell receptors e.g. FITC-tagged anti-NK 1.1 and PE-tagged anti-Ly49A.



Graph 1:- Spleenocytes cell count before and after enrichment of NK cell by using EasySep Mouse NK Enrichment kit. NK cell before purification was found to be 86 X 10^6 cells/ml and after enrichment they were found to be 7.5 X 10^6 cells/ml.

Stock solutions were prepared and was suspended in respective tubes, which are arranged according to the experimental set-up.

IL-2 containing stock contains 100U of IL-2 for each tube, in addition NK cell was added along with RPMI media,which was purified (as described in section 5.5) and was suspended in respective well plates.

Another stock was prepared without IL-2, so as to compare the proliferation obtained after its addition and in its absence.

Antibodies were stained in following table below:

Table 3: **Stimulation of purified NK cell by IL-2:**

(FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49A)

IL-2	**Unstained NK cell**	**NK 1.1**	**Ly49A**
IL-2 (+) 100U	Sample 1	Sample 2	Sample 3
IL-2 (+) 100U	Sample 4	Sample 5	Sample 6
IL-2 (-)	Sample 7	Sample 8	Sample 9
IL-2 (-)	Sample 10	Sample 11	Sample 12

5.8 Protocol for Membrane protein isolation of P815 and YAC-1 cell lines:-

1. Buffer A:-

   50mMTris-Cl (pH=7.4) + 1mM EDTA

2. Buffer B:-

   50mM Tris-Cl (pH=7.4)

Protocol:-

- To the pellet, add 40ml buffer A, make homogeneous suspension.
- Leave on ice 30 min.
- Centrifuge for 20 min at 35,000g at 4^0C.
- Resuspend the pellet in minimum volume of buffer B. Pass through a fine (26g) needle to ensure homogeneous suspension.
- Estimate the protein in table 1.

- Store at -70^{0}C at concentration of 5ug/ul in Buffer B.

5.8 Protein Estimation by Bicinchoninic Acid:-

- Take 96 well-plate for protein estimation.
- Add BSA and samples according to the table below, then add MilliQ.
- Prepare the BCA (Bicinchoninic Acid) solution by adding $CuSO_4$ and BCA in ratio (1:50).
- Incubate the plate at 37^{0}C for 30 minutes.
- Place the plate in plate reader and set the spectrophotometer at 570nm and take the reading and estimate the protein by using Origin S software.

5.9 Co-culturing of enriched NK cell with NK resistant cells, P815:-

Co-culture of NK cell with P815 cells was performed by preparing stock solutions.

Stock solutions are prepared prior to suspending in each tube, according to the above chart:-

Stock solution 1:-

Required concentrations for each well: 100 U IL-2 + NK cells (0.5 X 10^6 cells/well) suspended in RPMI + P815 cells (0.25 X 10^6 cells/ml)

Stock solution 2:-

Required concentrations for each well: 100 U IL-2 + NK cells (0.5 X 10^6 cells/well) suspended in RPMI

Antibodies were stained according to designed experimental set-up (as described in Table

5.10 Co-culturing of enriched NK cell with whole membrane protein of NK resistant cells, P815:-

Stock solution 1:-

Required concentrations for each well: 100 U IL-2 + NK cells (0.5 X 10^6 cells/well) + 50ug P815 whole membrane protein.

Stock solution 2:-

Required concentrations for each well: 100 U IL-2 + NK cells (0.5 X 10^6 cells/well).

5.11 Co-culturing of enriched NK cell with P815 cells membrane protein, which are more than 100 kDa in size and are obtained by amicons.

Stock solutions are prepared prior to suspending in each tube.

Stock solution 1:-

Required concentrations for each well: 100 U IL-2 + NK cells (0.5 X 10^6 cells/well).

Stock solution 2:-

Required concentrations for each well: 100 U IL-2 + NK cells (0.5 X 10^6 cells/well) + 50ug P815 of more than 100 kDa protein obtained after amicon cut and suspended into each well.

5.12 Antibody Staining:

Below listed antibody staining combinations was followed in each experiment for NK resistant cell line, P815 cells, its whole membrane protein and protein of size more than 100 KDa, which was obtained by performing amicons.

- Unstained
- NK 1.1
- Ly49A
- Ly49C
- NK 1.1 + Ly49A
- NK 1.1 + Ly49C

6. Results:-

6.1. NK Cell Profiling with the expression level of surface inhibitory and activating receptors by flow cytometry :-

Spleenocytes harvested from C57BL/6 mice and further it was stained for isotype control for PE and FITC to match with immunoglobulin isotypes IgG2a of antibodies being used i.e. PE-conjugated anti-Ly49A, PE-conjugated anti- Ly49C, and FITC-conjugated anti- NK 1.1.

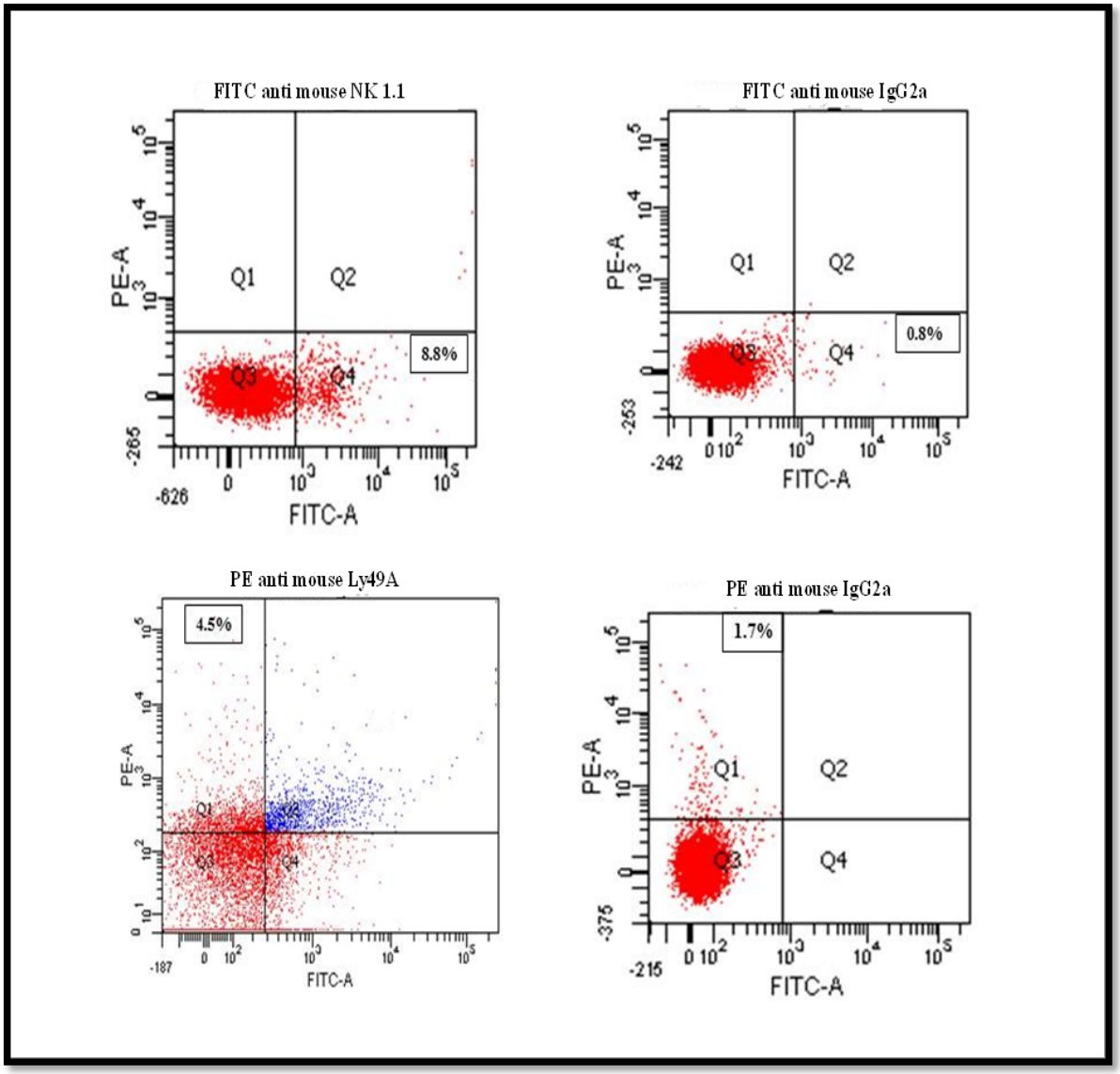


Figure 14:- Spleenocyte staining for NK receptors. FITC-tagged NK 1.1 and PE-tagged Ly49A. The upper left panel shows NK1.1 FITC staining while upper right panel shows matched isotype control for FITC. The lower left panel shows Ly49A PE staining while lower right panel shows matched isotype control for PE.

The above flow cytometric assays indicate that the antibodies being used for NK cell profiling i.e. FITC-conjugated anti- NK 1.1 and PE-conjugated anti-Ly49A are indeed stained efficiently and antibodies dilution are standardized and they work best.

6.2 Enrichment of NK cells from spleenocytes through EasySep™ Mouse NK Cell Enrichment Kit:-

Mature spleen NK cells were enriched through EasySep™ Mouse NK Cell Enrichment Kit (as described in Section 5.5)from C57BL/6 mice strain which is based on negative selection, as recommended by the manufacturer (Stem cell Technologies), with a typical yield of 70% CD49b-positive CD3-negative NK cells.

Unwanted cells were targeted for removal with tetrameric Antibody complexes which recognizes non-NK cell and dextran-coated magnetic particles.

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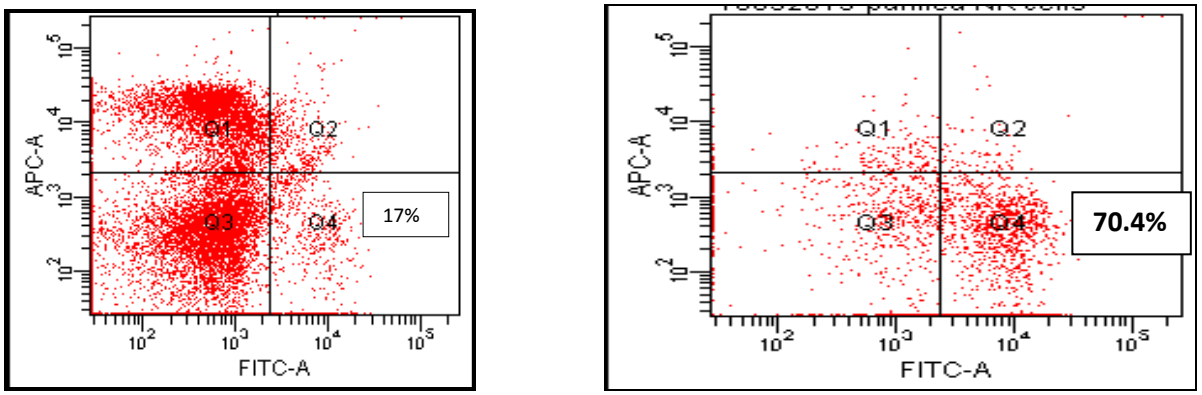

Figure 15:- Comparision of spleenocytes NK cell and enriched NK cells through use of EasySep™ Mouse NK Cell Enrichment Kit: FITC-tagged anti-CD49b and APC-tagged anti-CD3.

Quadrant (say ,Q) are defined as following:

Q1: $CD49b^{-}CD3^{+}$; Q2: $CD49b^{+}CD3^{+}$; Q3: $CD49b^{-}CD3^{-}$; Q4: $CD49b^{+}CD3^{-}$

The left panel and right panel shows the comparision in percentage of CD3 (APC-conjugated) and CD49b (FITC conjugated) population in spleenocytes and purified NK cells respectively

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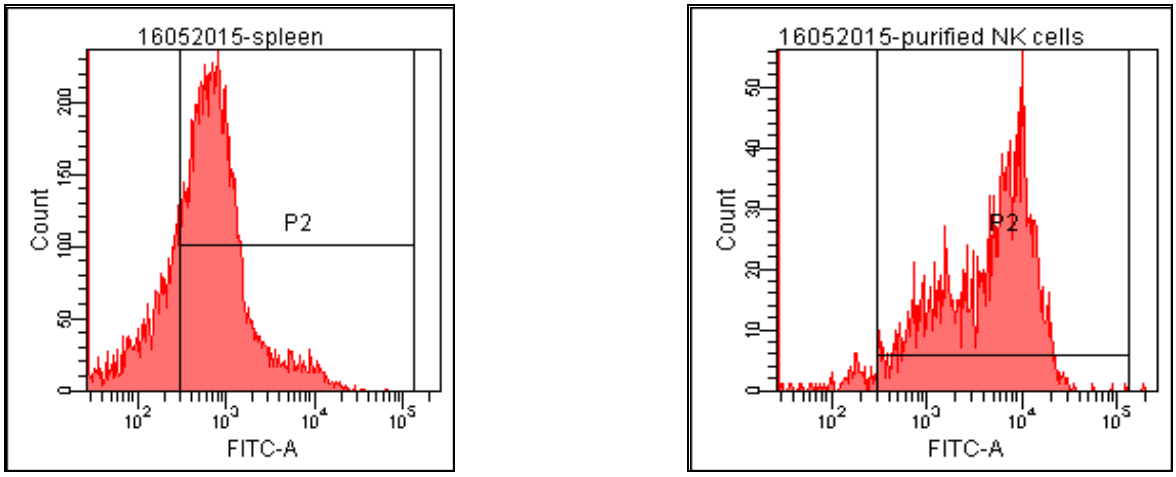

Figure 16:- Comparision of spleenocytes NK cell and enriched NK cells through histogram by EasySep™ Mouse NK Cell Enrichment Kit :

The left panel indicates the $CD49b^+$ spleenocytes (lesser population), while significant shift in peak on right panel indicates purified $CD49b^+$ NK cells.

6.3 Stimulation of NK cells with IL-2 and their effect on NK cell receptor profile:-

Enriched NK cells were stimulated with IL-2 and characterized for NK cells receptors expression to analyse the alteration in NK cell receptor profiling. It was observed that the expression of surface receptor NK 1.1 was enhanced on induction by IL-2, whereas there was decrement in the expression of Ly49A and Ly49C. From this, it may be concluded that IL-2 down-regulates the expression of Ly49A and Ly49C, which are inhibitory recepetors and this might contribute to increase in activity of NK cells against target cells.

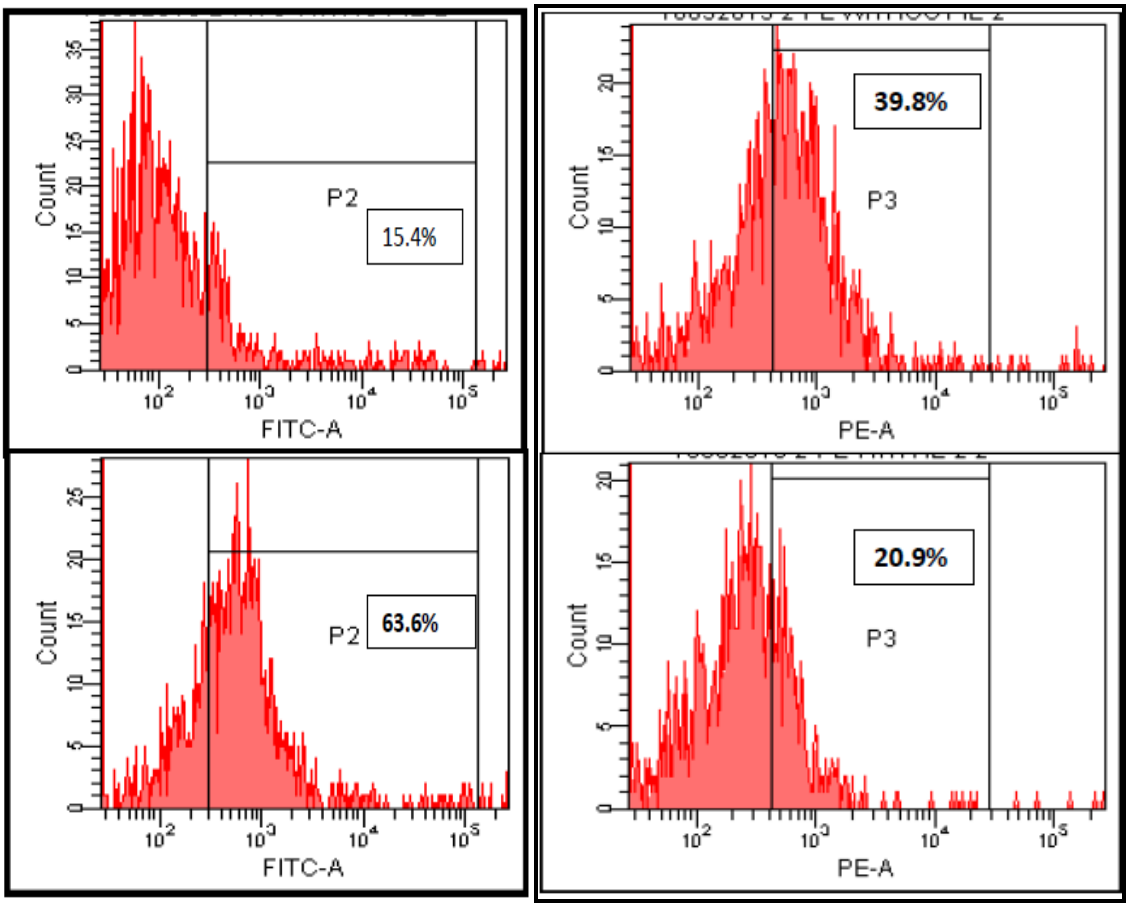

Figure 17:- IL-2 induced NK cells receptor profiling: FITC-tagged anti-NK1.1 (IL-2^-):15.4; FITC-tagged anti-NK1.1(IL-2^+):63.6%; %; PE-tagged anti-Ly49A(IL-2^-):39.8%;PE-tagged anti-Ly49A(IL-2^+):20.9%.

The upper left panel depicts the expression of NK cell surface marker NK 1.1 (FITC conjugated anti-NK 1.1) and upper right panel depicts the expression of NK cell receptor Ly49A (PE conjugated anti-Ly49A) on purified NK cells in the absence of IL-2 (IL-2^-) .

Lower left panel describes the expression of NK cell surface marker NK 1.1 (FITC conjugated anti-NK 1.1) and lower right panel depicts the expression of NK cell receptor Ly49A (PE conjugated anti-Ly49A) on purified NK cells in presence of IL-2(IL-2^+)

6.4 Co-culturing of splenic NK cell and P815 cells

Mature spleen NK cells were isolated from C57BL/6 mice strain in which 90 X 10^6 cells/ml were counted in the spleenocytes further NK cell purification procedure was performed (as described in Section 5.5) after which 7.5 X 10^6 cells/ml was observed.

P815 cell and NK cells were co-cultured at a ratio of 0.25 X 10^6 P815 cells : 0.5 X 10^6 NK cells in the presence of IL-2 and were further stained with FITC-conjugated anti-NK 1.1, PE-conjugated anti-Ly49 A and PE-conjugated anti-Ly49 C (antibody section)

6.4 a Comparison of purified splenic NK cell receptor profile with and without co-culturing with P815 cells by staining it with FITC-conjugated anti-NK 1.1

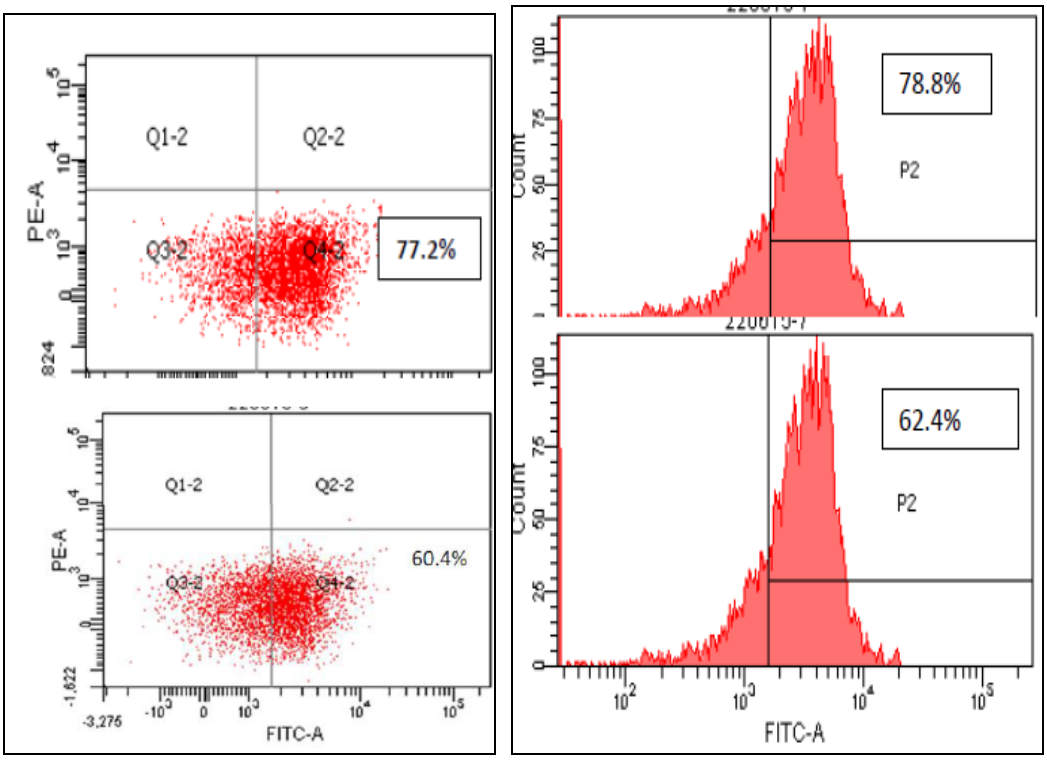

Figure 18: **NK surface receptor (NK 1.1) profiling in the presence and absence of P815 cells**. Left panel is dot diagram for NK when co-cultured with P815 cell (lower left) and without co-culture with P815 cell (upper left). Right panel is histogram for NK when co-cultured with P815 cell (lower right) and without co-culture with P815 cell (upper right), stained with FITC-conjugated anti-NK 1.1.

6.4 b Comparison of purified splenic NK cell receptor profile with and without co-culturing with P815 cells by staining it with PE-conjugated anti-Ly49 A

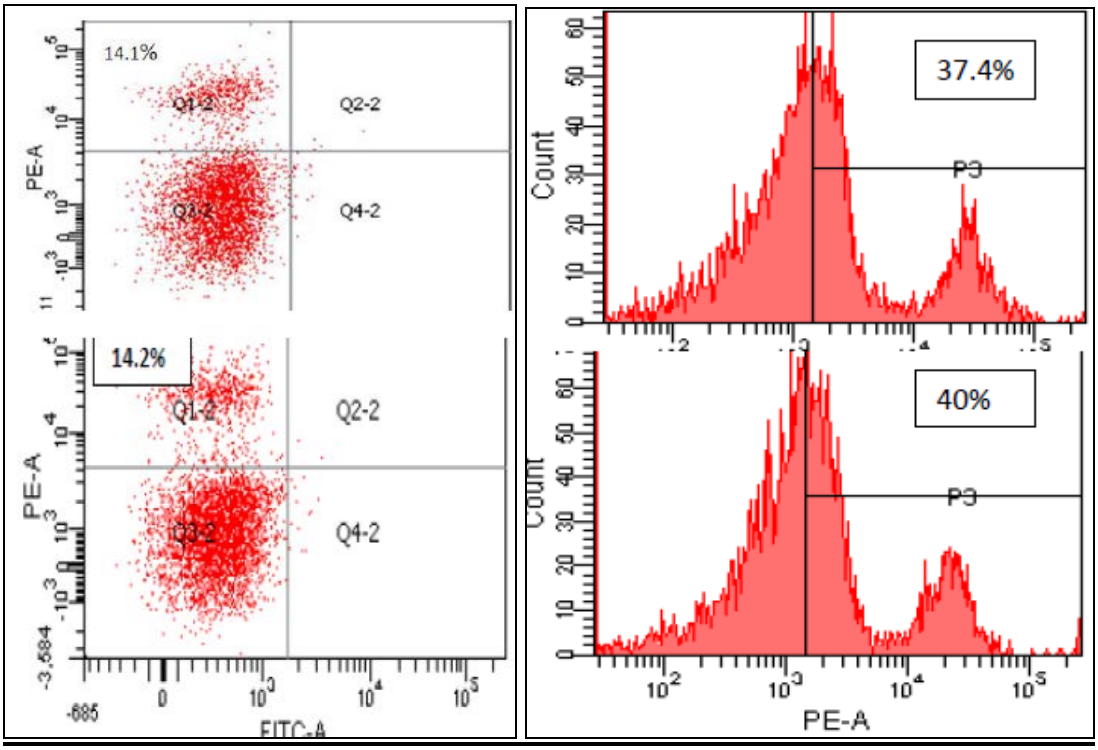

Figure 19: NK inhibitory receptor (Ly49A) profiling in the presence and absence of P815 cells. Left panel is dot diagram for NK when co-cultured with P815 cell (lower left) and without co-culture with P815 cell (upper left). Right panel is histogram for NK when co-cultured with P815 cell (lower right) and without co-culture with P815 cell (upper right), stained with PE-conjugated anti-Ly49 A.

6.4 c. Comparison of purified splenic NK cell receptor profile with and without co-culturing with P815 cells by staining it with FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 C

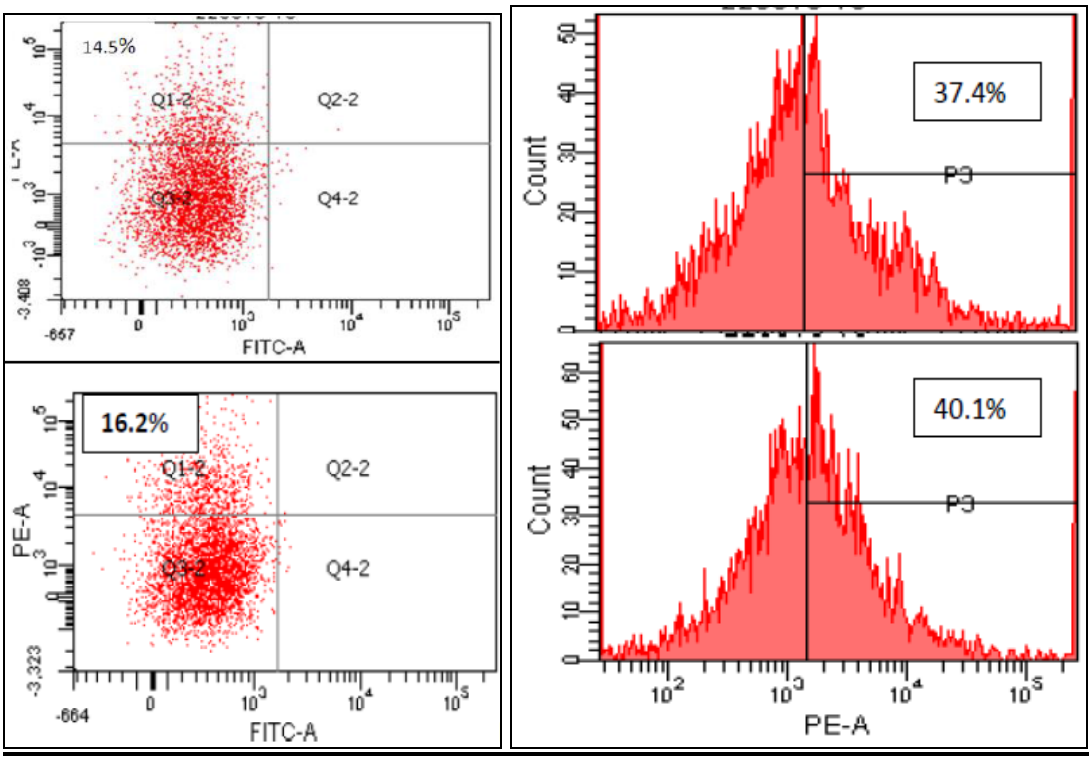

Figure 20: NK inhibitory receptor (Ly49C) profiling in the presence and absence of P815 cells. Left panel is dot diagram for NK when co-cultured with P815 cell (lower left) and without co-culture with P815 cell (upper left). Right panel is histogram for NK when co-cultured with P815 cell (lower right) and without co-culture with P815 cell (upper right), stained with PE-conjugated anti-Ly49 C.

6.4 d. Comparison of purified splenic NK cell receptor profile with and without co-culturing with P815 cells by staining it with FITC-conjugated anti-NK 1.1 PE-conjugated anti-Ly49 C

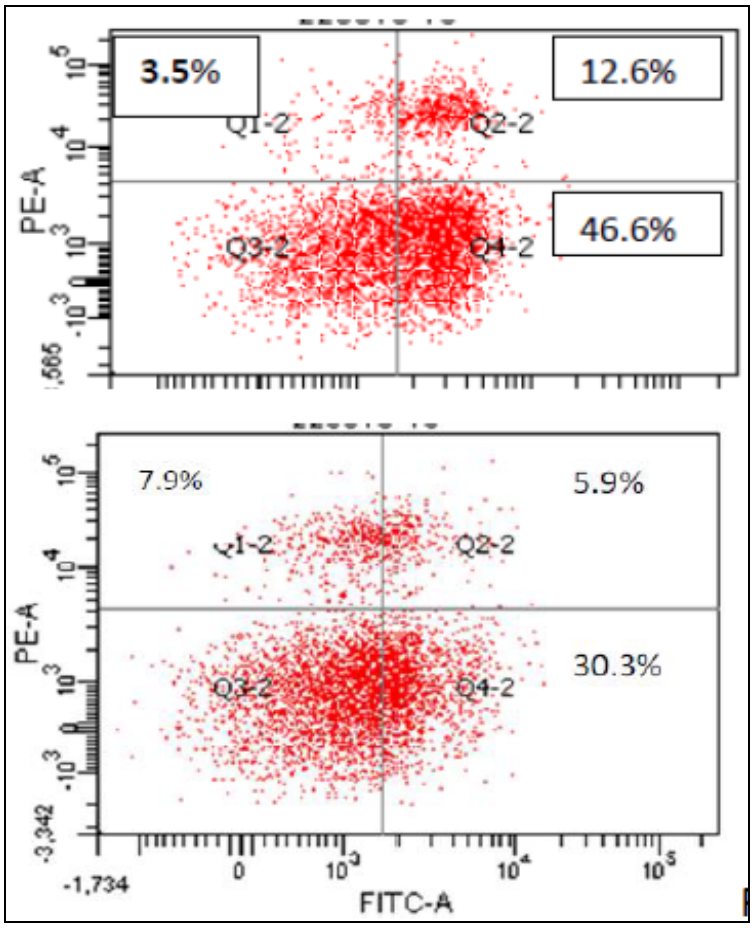

Figure 21: Dot plot depicting the NK cell stained with FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 A in presence and absence of P815 cells.

Q1-2: $NK1.1^{-}Ly49A^{+}$; Q2-2: $NK1.1^{+}Ly49A^{+}$; Q3-2: $NK1.1^{-}Ly49A^{-}$; Q4-2: $NK1.1^{+}Ly49A^{-}$.

Upper panel shows the dot diagram of NK cell surface receptor (NK 1.1 and Ly49A)without co-culturing it with P815 cells. Lower panel shows above stated receptor on NK cell co-culture with P815.

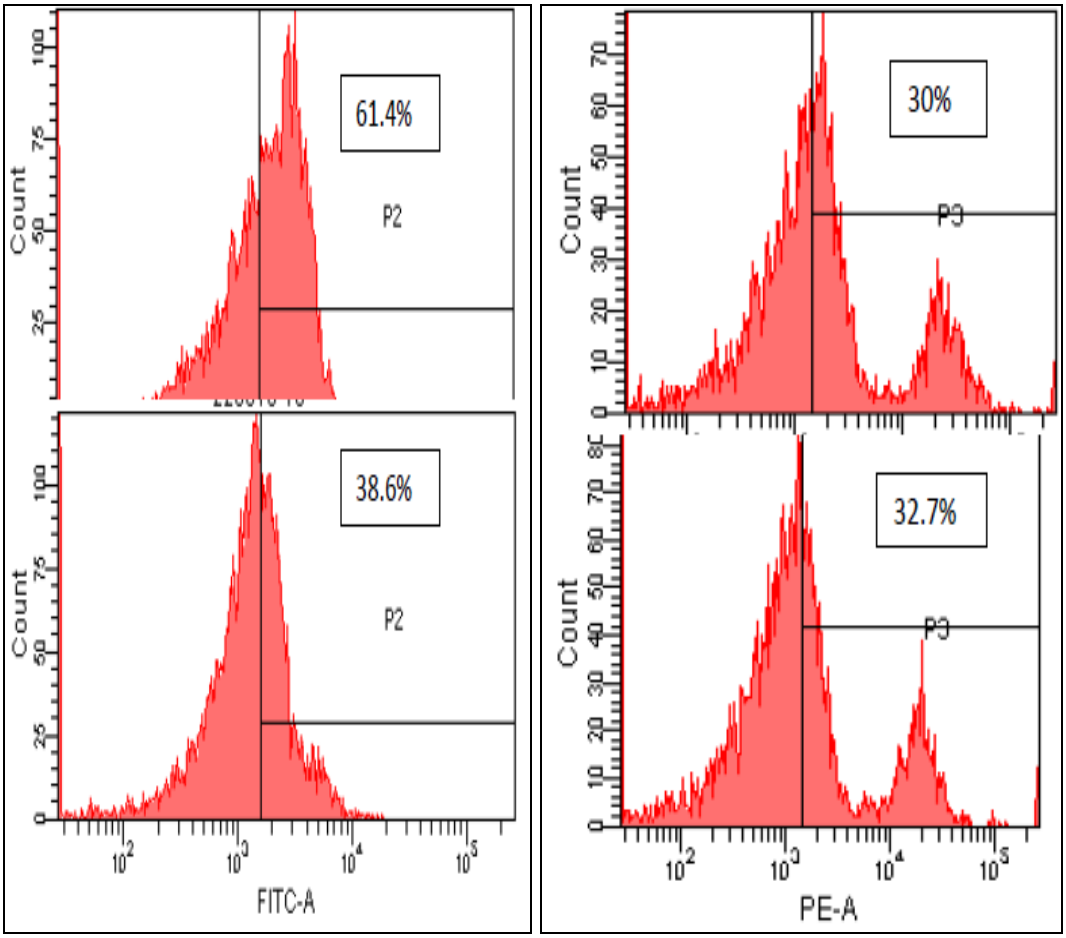

Figure 22 : **NK cell receptor (NK 1.1) and NK inhibitory receptor (Ly49A) profiling in the presence and absence of P815 cells**. Upper left and right panel shows histogram for NK cell receptor profiling without co-culture with P815 cells for FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 A respectively.

Lower left and right panel shows histogram for NK cell receptor profiling with P815 cells co-culture stained with FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 A respectively.

6.4 e Comparison of purified splenic NK cell receptor profile with and without co-culturing with P815 cells by staining it with FITC-conjugated anti-NK 1.1 PE-conjugated anti-Ly49 C.

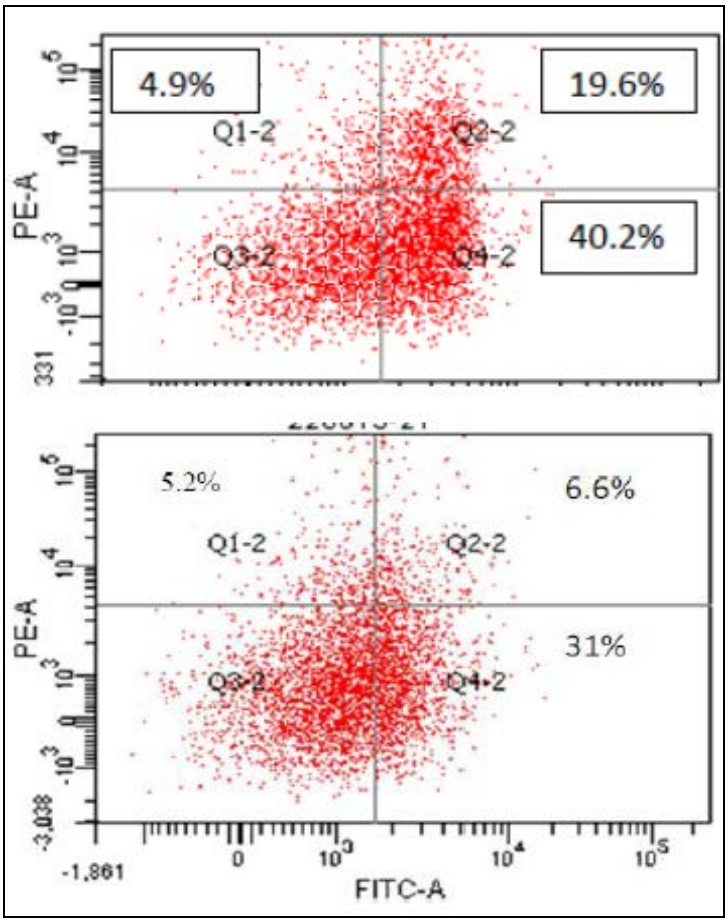

Figure 23: Dot plot depicting the NK cell stained with FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 C in presence and absence of P815 cells.

Q1-2: $NK1.1^{-}Ly49C^{+}$; Q2-2: $NK1.1^{+}Ly49C^{+}$; Q3-2: $NK1.1^{-}Ly49C^{-}$; Q4-2: $NK1.1^{+}Ly49C^{-}$.

Upper panel shows the dot diagram of NK cell surface receptor (NK 1.1 and Ly49C)without co-culturing it with P815 cells. Lower panel shows above stated receptor on NK cell co-culture with P815.

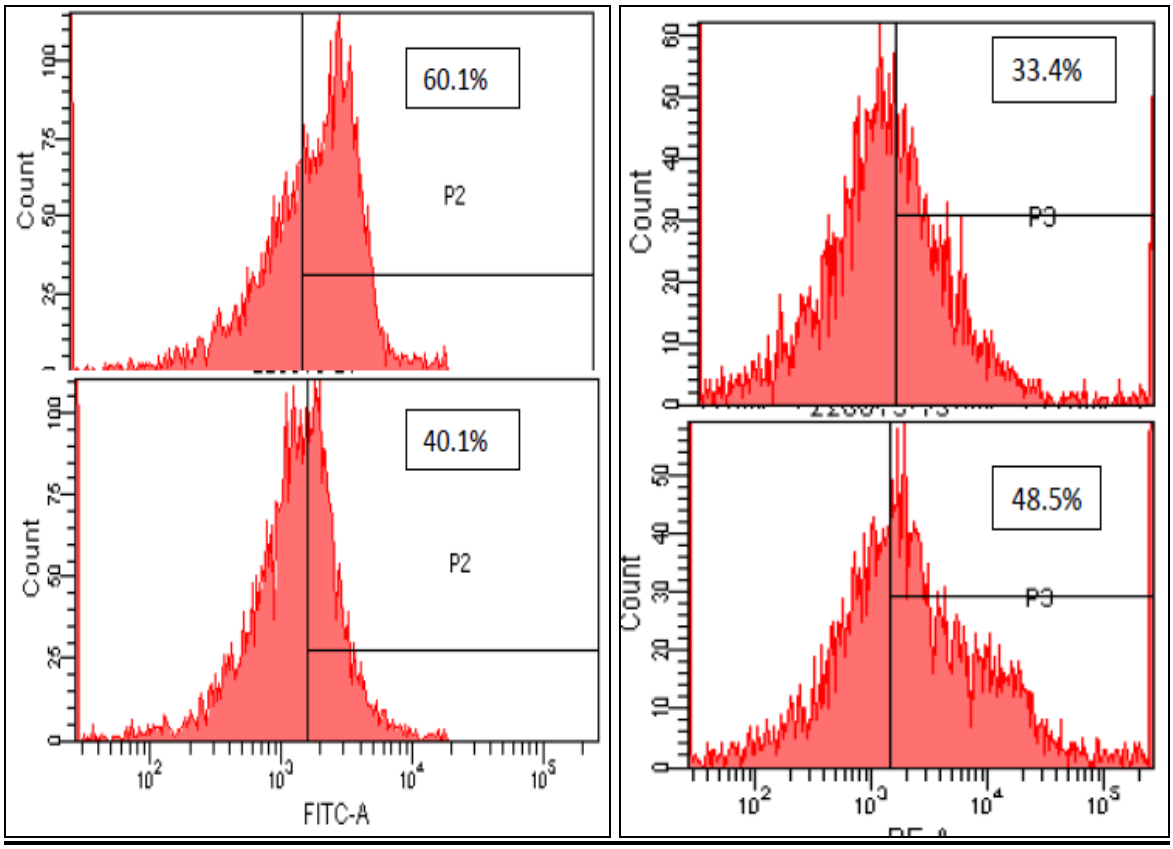

Figure 24 : **NK cell receptor (NK 1.1) and NK inhibitory receptor (Ly49C) profiling in the presence and absence of P815 cells**. Upper left and right panel shows histogram for NK cell receptor profiling without co-culture with P815 cells stained with FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 C respectively.

Lower left and right panel shows histogram for NK cell receptor profiling with P815 cells co-culture when stained with FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 C respectively.

6.5 Co-culturing of P815 cells whole membrane protein with splenic NK cell :-

Mature spleen NK cells were isolated from C57BL/6 mice strain in which 100 X 10^6 cells/ml were counted in the spleenocytes further NK cell purification procedure was performed (as described in Section 5.5) after which 8 X 10^6 cells/ml was observed.

P815 membrane protein was isolated from whole cells of P815 by the membrane protein isolation protocol (as described in section 5.7) and NK cells were co-cultured by taking 50ug protein for each well prepared for examination of NK cell receptor modulation and 0.5 X 10^6 NK cells was suspended in each well in the presence of IL-2 and were further stained with several antibodies like FITC-conjugated anti-NK 1.1, PE-conjugated anti-Ly49 A and PE-conjugated anti-Ly49 C.

6.5 a Comparison of purified splenic NK cell receptor profile with and without co-culturing it with P815 whole membrane protein by staining it with FITC-conjugated anti-NK 1.1

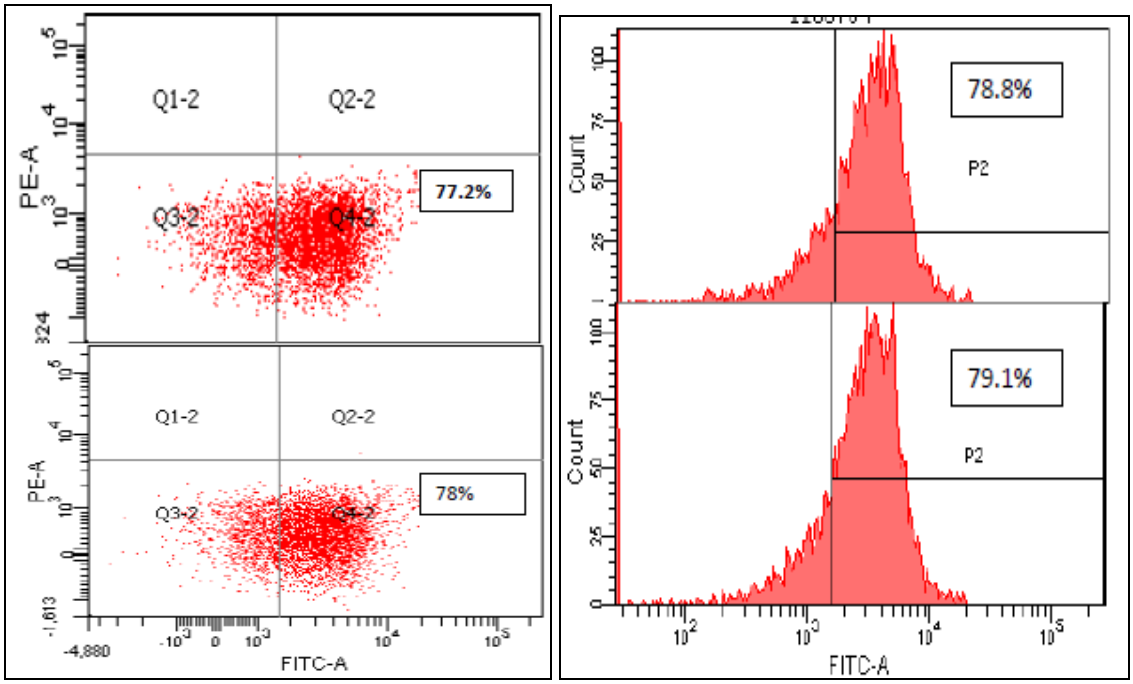

Figure 25: Dot plot and histogram of whole membrane protein:Upper left panel and Lower left panel depict the dot plot for NK cell in the absence of its co-culture with whole membrane protein and in presence of P815 cells whole membrane protein respectively, which are stained with FITC-conjugated anti-NK 1.1 Upper right panel and lower left panel shows the histogram which shows little peak shift in absence and presence of P815 cells whole membrane protein respectively.

6.5 b.Comparison of purified splenic NK cell receptor profile with and without co-culturing with P815 whole membrane protein by staining it with PE-conjugated anti-Ly49A.

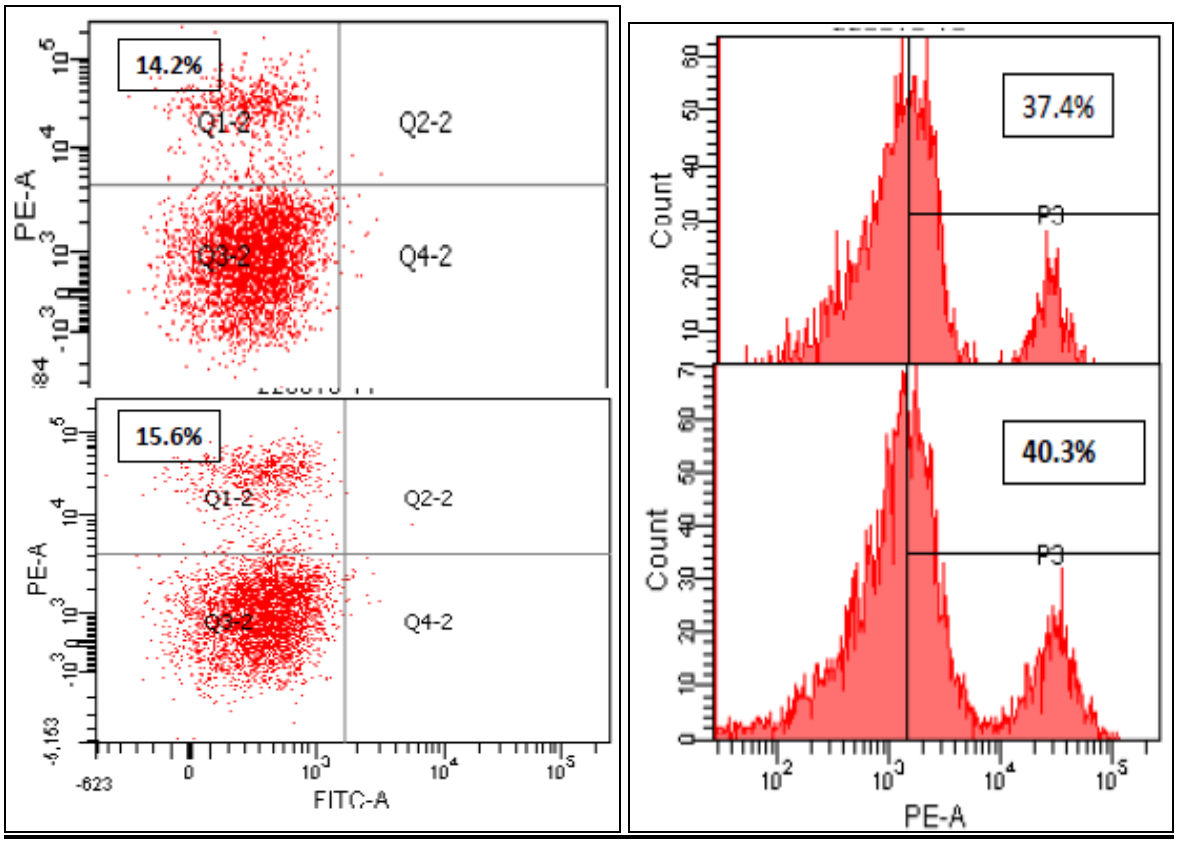

Figure 26: **: Dot and histogram analysis of P815 cells whole membrane protein** Upper left panel and Lower left panel depict the dot plot for NK cell in the absence of its co-culture with whole membrane protein and in presence of P815 cells whole membrane protein respectively, which are stained with PE-conjugated anti-Ly49A .Upper right panel and lower left panel shows the histogram which shows little peak shift in absence and presence of P815 cells whole membrane protein respectively.

6.5 c. Comparison of purified splenic NK cell receptor profile with and without co-culturing with P815 whole membrane protein by staining it with PE-conjugated anti-Ly49C.

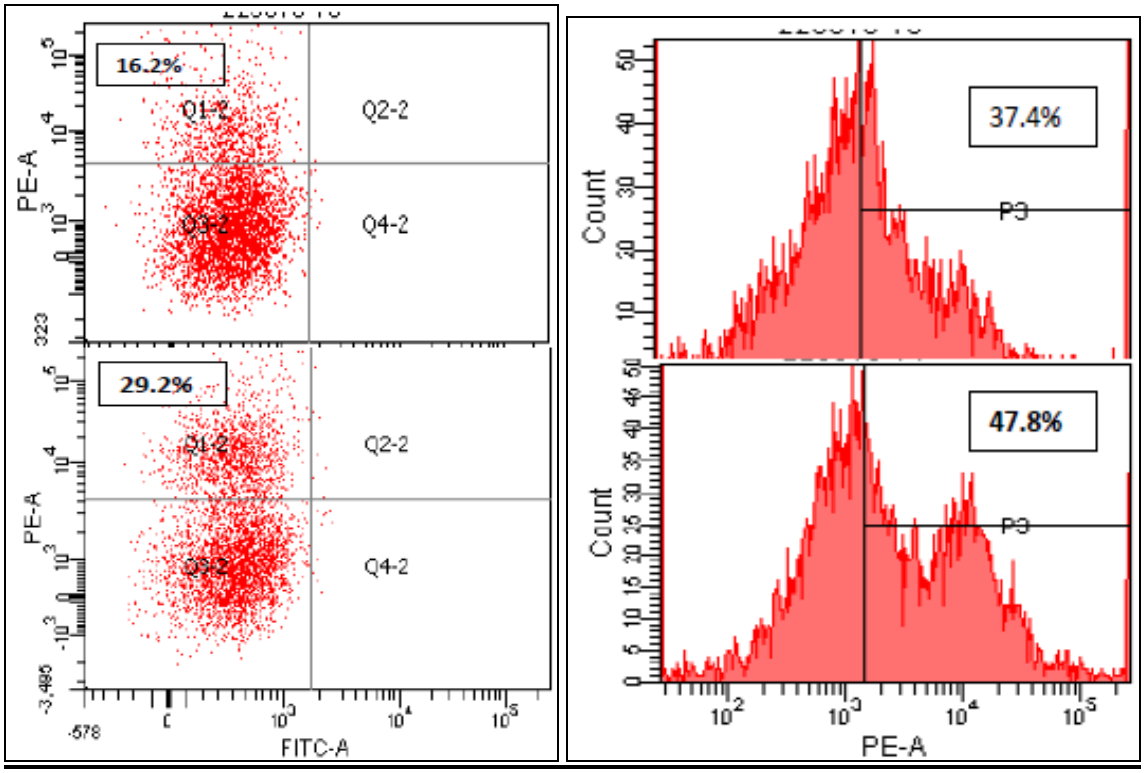

Figure 27: Dot and histogram analysis of P815 cells whole membrane protein. Upper left panel and Lower left panel depict the dot plot for NK cell in the absence of its co-culture with whole membrane protein and in presence of P815 cells whole membrane protein respectively, which are stained with PE-conjugated anti-Ly49C.

Upper right panel and lower left panel shows the histogram which shows significant peak shift in absence and presence of P815 cells whole membrane protein respectively.

6.5 d Comparison of purified splenic NK cell receptor profile with and without co-culturing with P815 whole membrane protein by staining it with FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49A.

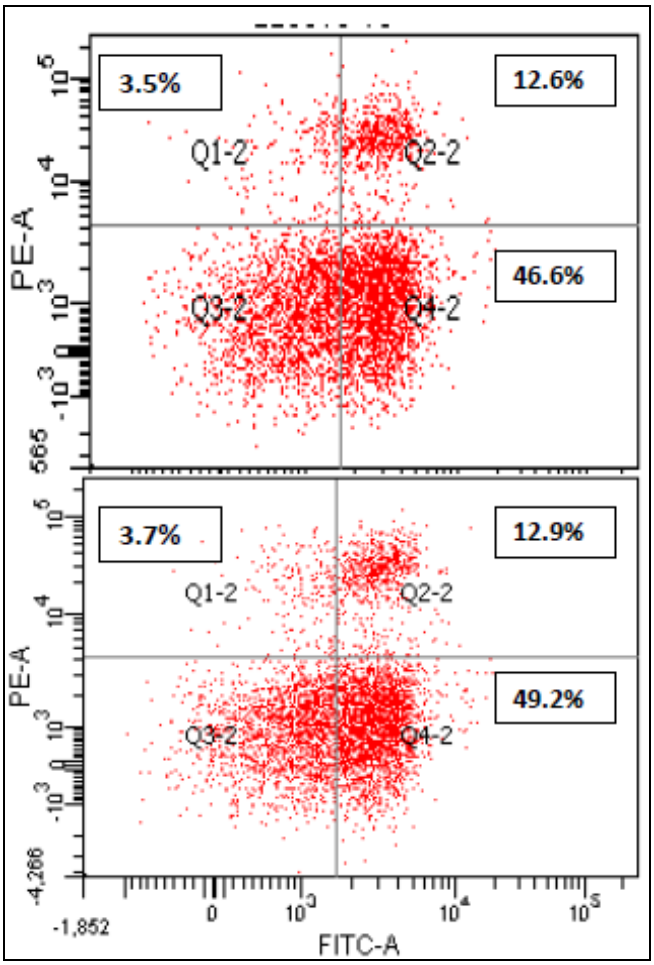

Figure 28: Dot plot of P815 cells whole membrane protein: Upper panel and Lower panel depict the dot plot for NK cell in the absence of its co-culture with whole membrane protein and in presence of P815 cells whole membrane protein respectively, which are stained with with FITC-conjugated anti NK 1.1 and PE-conjugated anti-Ly49A.

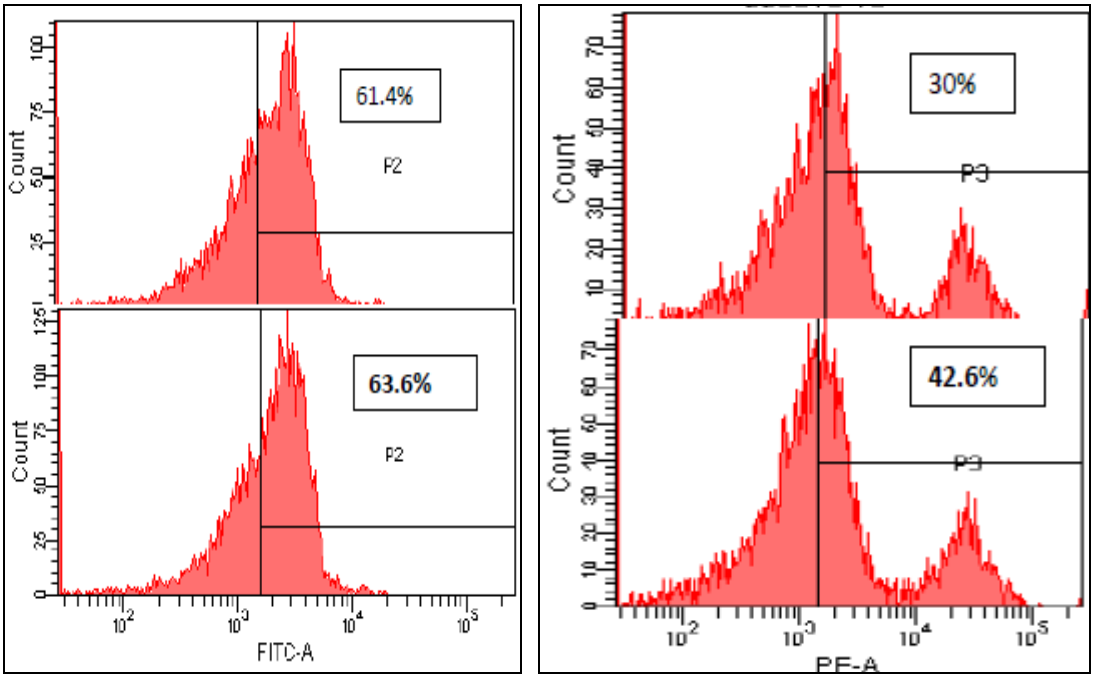

Figure 29: **NK receptor (NK 1.1) and NK inhibitory receptor (Ly49A) profiling in the presence and absence of P815 cells whole membrane protein**. Upper left and right panel shows histogram for NK cell receptor profiling without co-culture with P815 cells whole membrane protein for FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 A respectively.

Lower left and lower right panel shows histogram for NK cell receptor profiling when it was co-cultured with P815 cells whole membrane protein for FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 A respectively. It shows significant peak shift in absence and presence of P815 cells whole membrane protein respectively.

6.6 e Comparison of purified splenic NK cell receptor profile with and without co-culturing with P815 whole membrane protein by staining it with FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49C.

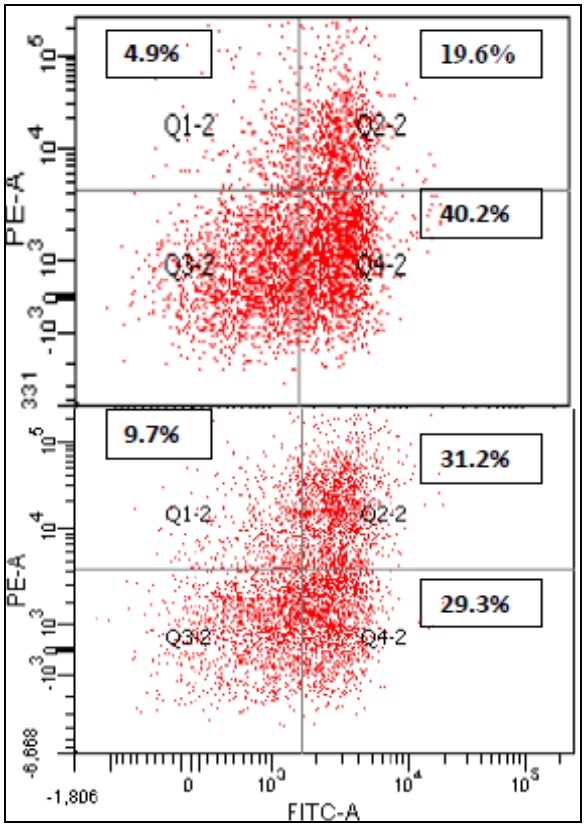

Figure 30: Dot plot of P815 cells whole membrane protein. Upper panel and Lower panel depict the dot plot for NK cell in the absence of its co-culture with whole membrane protein and in presence of P815 cells whole membrane protein respectively, which are stained with with FITC-conjugated anti NK 1.1 and PE-conjugated anti-Ly49 C.

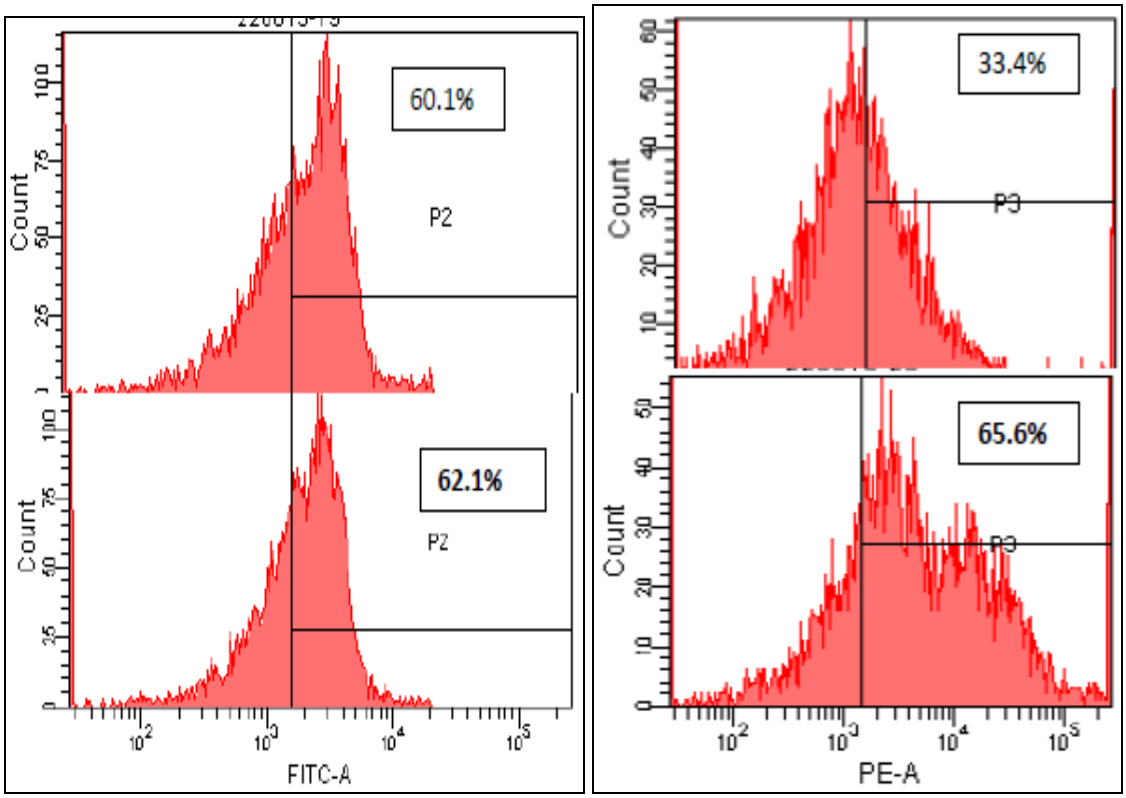

Figure 31: NK cell receptor (NK 1.1) and NK inhibitory receptor (Ly49C) profiling in the presence and absence of P815 cells whole membrane protein.. Upper left and right panel shows histogram for NK cell receptor profiling without co-culture with P815 cells for FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 C respectively.

Lower left and right panel shows histogram for NK cell receptor profiling when it was co-cultured with P815 cells whole membrane protein for FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 C respectively.

6.6 Co-culturing of P815 cells membrane protein, which are more than 100 KiloDalton (>100KDa) (obtained through amicons of 100 KDa) with splenic NK cell

Mature spleen NK cells were isolated from C57BL/6 mice strain in which 110 X 10^6 cells/ml were counted in the spleenocytes further NK cell purification procedure was performed (as described in Section 5.5) after which 8.5 X 10^6 cells/ml was observed.

P815 membrane protein was isolated from whole cells of P815 by the membrane protein isolation protocol (as described in section 5.7) and where further fractioned by using amicons and protein was estimated by BCA method . Furthermore, NK cells were co-cultured by taking 50ug protein of more than 100KDa for each well prepared for examination of NK cell receptor modulation and 0.5 X 10^6 NK cells was suspended in each well in the presence of IL-2 and were further stained with several antibodies like FITC-conjugated anti-NK 1.1, PE-conjugated anti-Ly49 A and PE-conjugated anti-Ly49 C.

6.7 a Comparison of purified splenic NK cell receptor profile with and without co-culturing it with more than 100KDa (>100KDa) membrane protein of P815 cell by staining it with FITC-conjugated anti-NK 1.1.

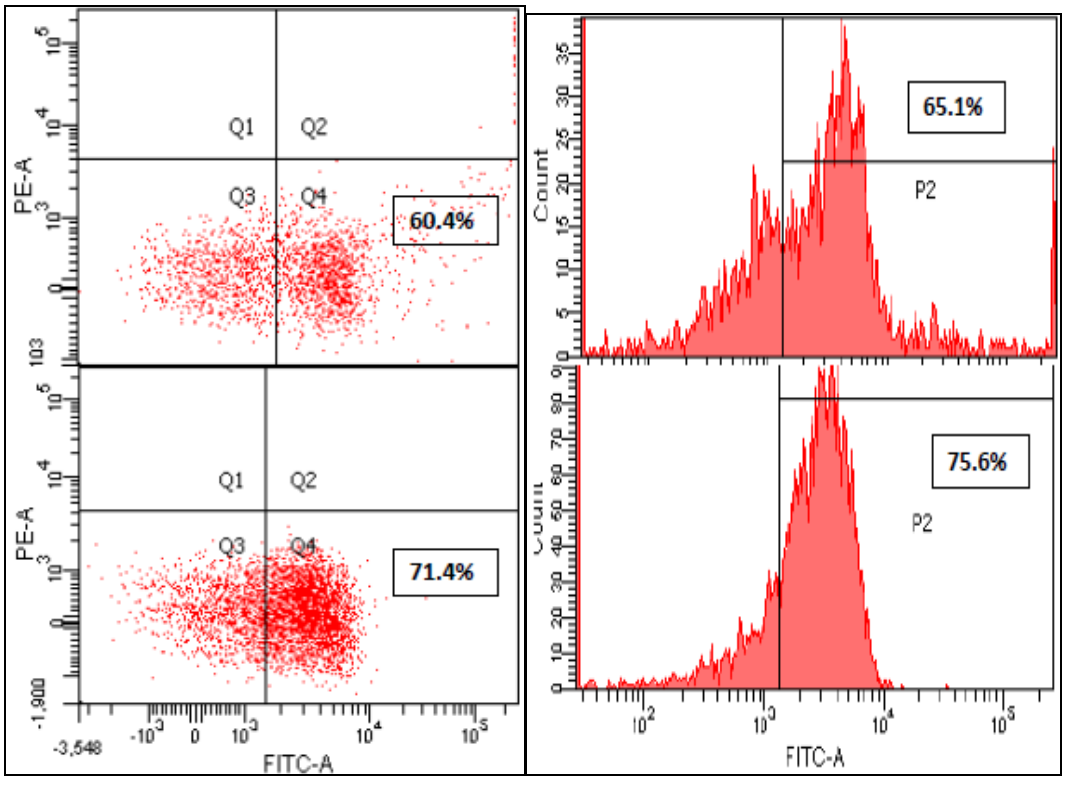

Figure 32: Dot plot and histogram analysis of more than 100KDa (>100KDa) membrane protein of P815 cell .Upper left panel and Lower left panel depict the dot plot for NK cell in the absence and presence of its co-culture with more than 100KDa (>100KDa) membrane protein of P815 cell respectively, which are stained with FITC-conjugated anti-NK 1.1 Upper right panel and lower right panel shows the histogram which shows significant peak shift in absence and presence of more than 100KDa (>100KDa) membrane protein respectively.

6.6 b Comparison of purified splenic NK cell receptor profile with and without co-culturing with more than 100KDa (>100KDa) membrane protein of P815 cell by staining it with PE-conjugated anti-Ly49A

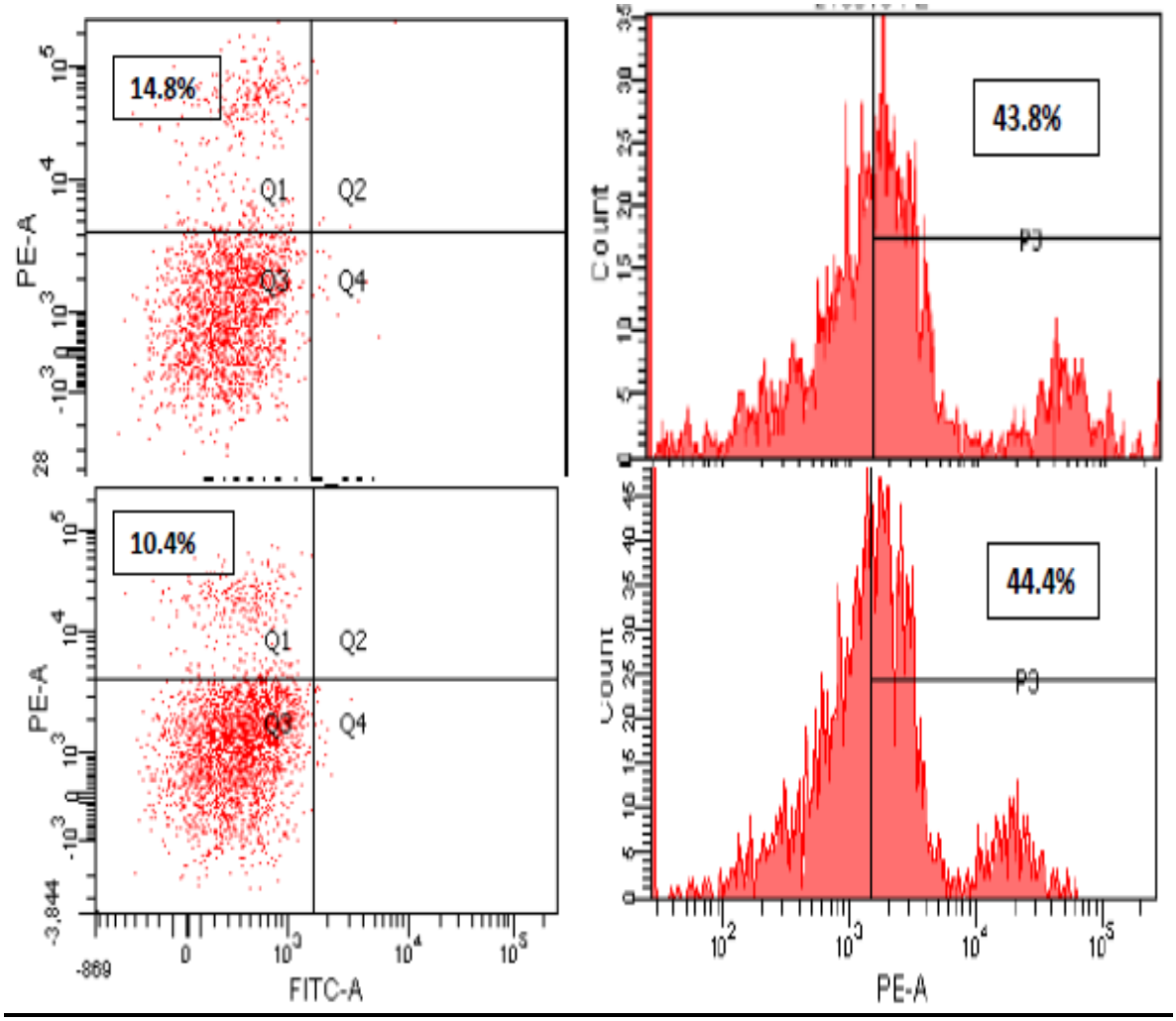

Figure 33: **Dot plot and histogram analysis of more than 100KDa (>100KDa) membrane protein of P815**
Upper left panel and Lower left panel depict the dot plot for NK cell in the absence of its co-culture with more than 100KDa (>100KDa) membrane protein of P815 cell respectively, which are stained with PE-conjugated anti-Ly49A.Upper right panel and lower right panel shows the histogram which shows little peak shift in absence and presence of more than 100KDa (>100KDa) membrane protein of P815 cell respectively.

6.6 c Comparison of purified splenic NK cell receptor profile with and without co-culturing with more than 100KDa (>100KDa) membrane protein of P815 cell by staining it with PE-conjugated anti-Ly49C

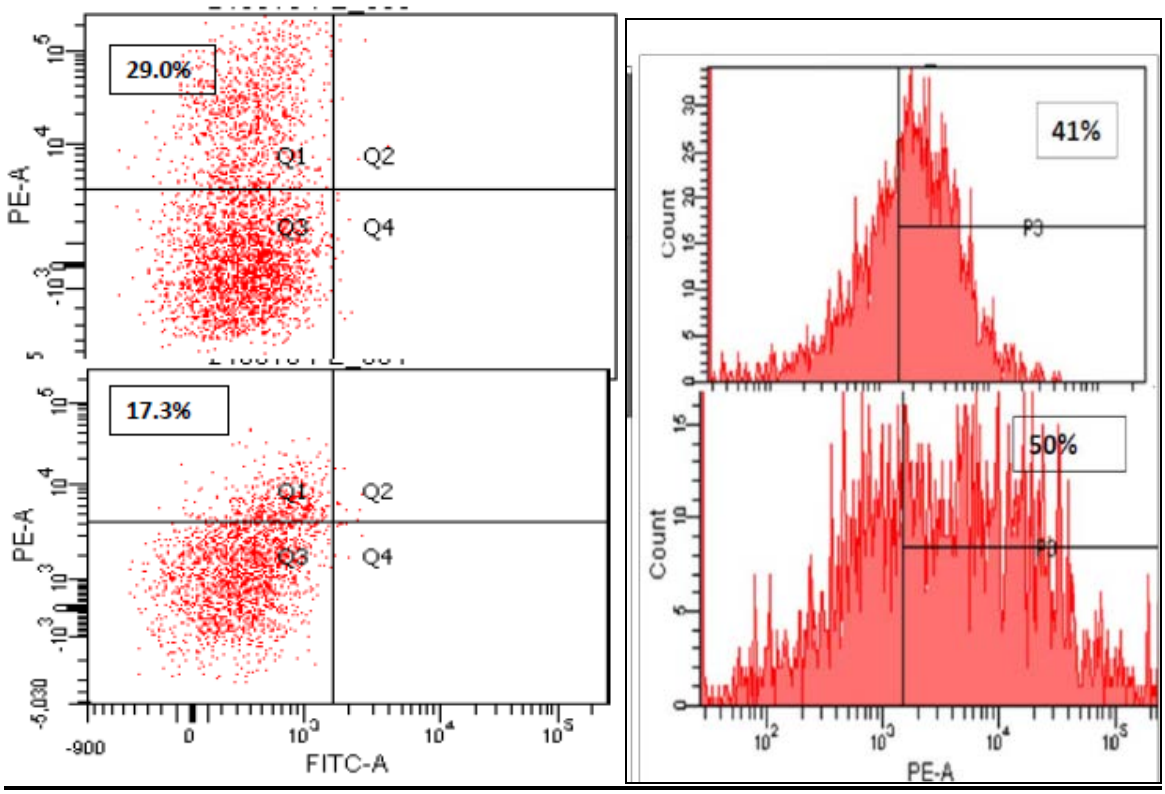

Figure 34: Dot plot and histogram analysis of more than 100KDa (>100KDa) membrane protein of P815 cell .Upper left panel and Lower left panel depict the dot plot for NK cell in the presence and absence of its co-culture with more than 100KDa (>100KDa) membrane protein of P815 cell respectively, which are stained with PE-conjugated anti-Ly49C

Upper right panel and lower right panel shows the histogram which shows significant peak shift in absence and presence of more than 100KDa (>100KDa) membrane protein of P815 cell respectively.

6.6 d Comparison of purified splenic NK cell receptor profile with and without co-culturing with more than 100KDa (>100KDa) membrane protein of P815 cell by staining it with FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49A.

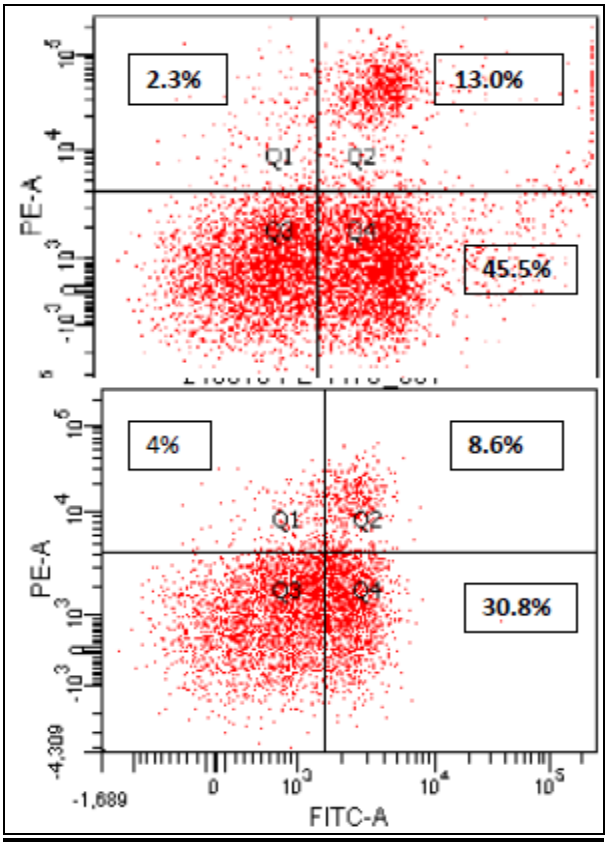

Figure 35: Dot plot for more than 100KDa (>100KDa) membrane protein of P815 cell. Upper panel and Lower panel depict the dot plot for NK cell in the absence and presence of its co-culture with more than 100KDa (>100KDa) membrane protein of P815 cell respectively, which are stained with with FITC-conjugated anti NK 1.1 and PE-conjugated anti-Ly49A

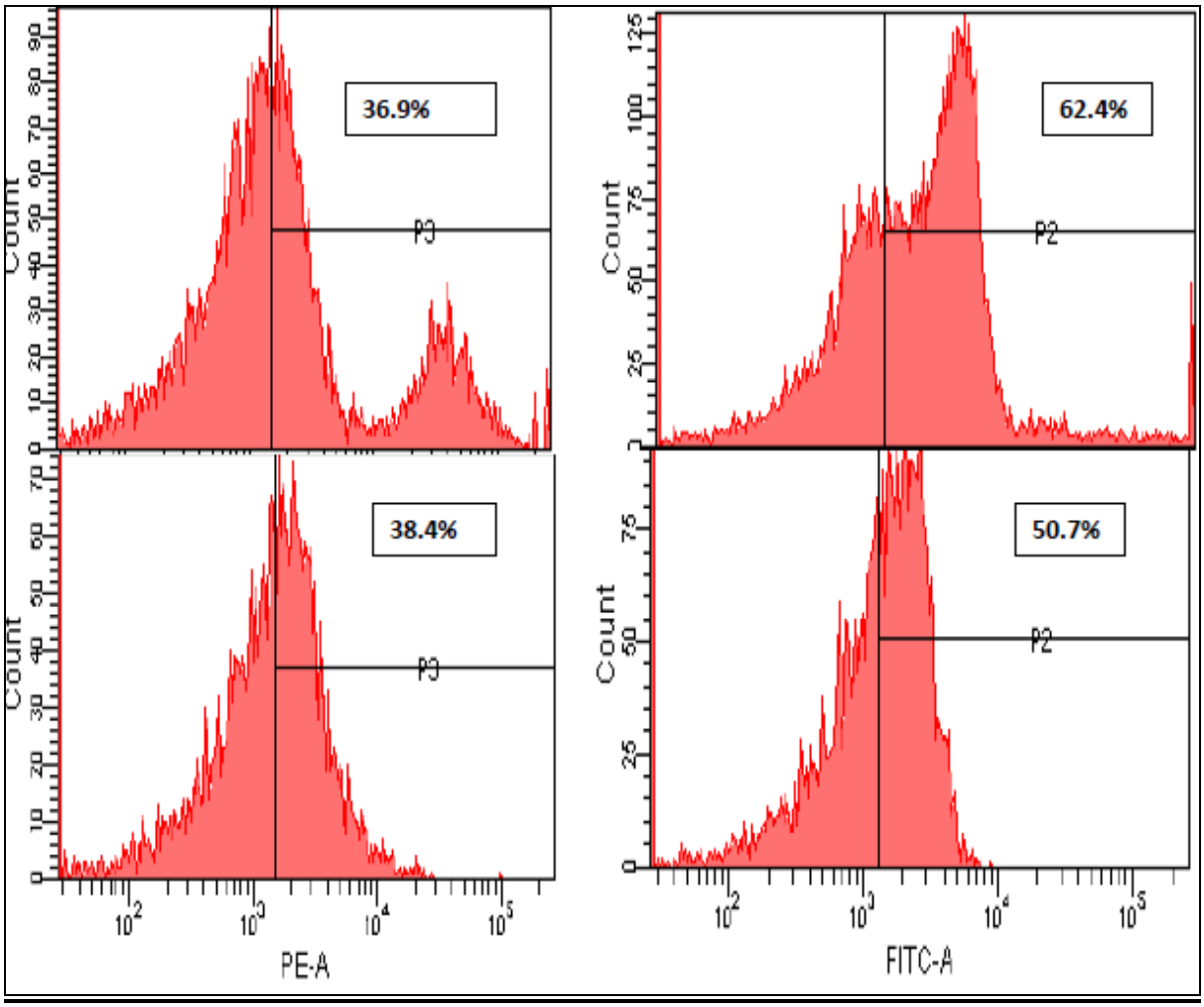

Figure 36:NK cell receptor (NK 1.1) and NK inhibitory receptor (Ly49A) profiling in the presence and absence of more than 100KDa (>100KDa) membrane protein of P815 cell.Upper left and upper right panel shows histogram for NK cell receptor profiling without co-culture with more than 100KDa (>100KDa) membrane protein of P815 cell for FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 A respectively.

Lower left and lower right panel shows histogram for NK cell receptor profiling when it was co-cultured with more than 100KDa (>100KDa) membrane protein of P815 cell for FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 A respectively

6.6 e Comparison of purified splenic NK cell receptor profile with and without co-culturing with more than 100KDa (>100KDa) membrane protein of P815 cell by staining it with FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49C

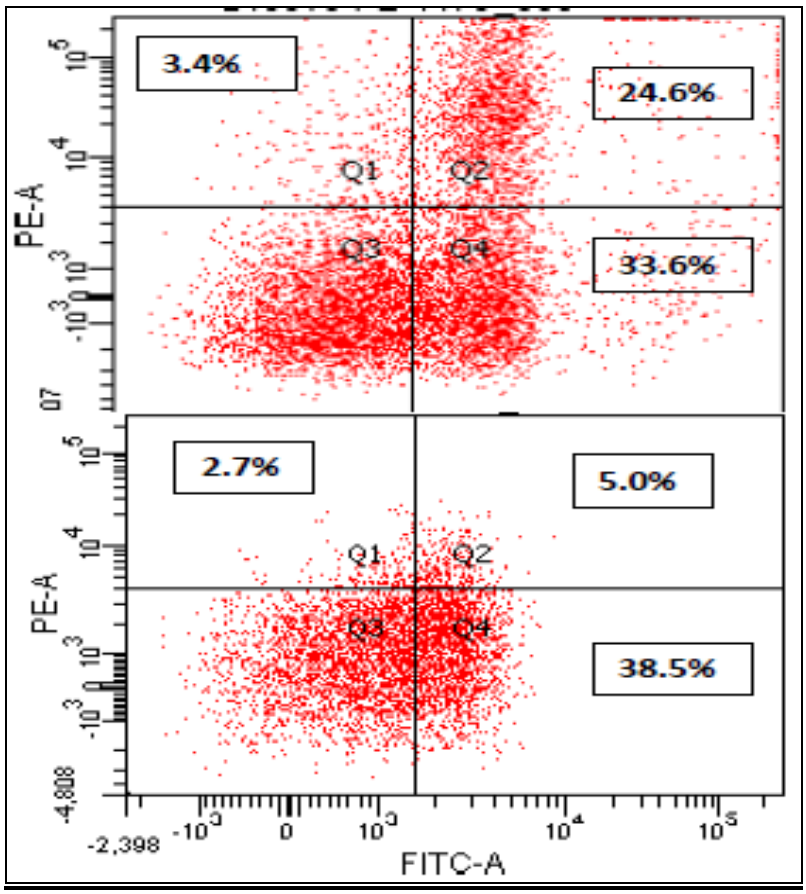

Figure 37: Dot plot for with more than 100KDa (>100KDa) membrane protein of P815 cell Upper panel and Lower panel depict the dot plot for NK cell in the absence and presence of its co-culture with more than 100KDa (>100KDa) membrane protein of P815 cell respectively, which are stained with with FITC-conjugated anti NK 1.1 and PE-conjugated anti-Ly49C

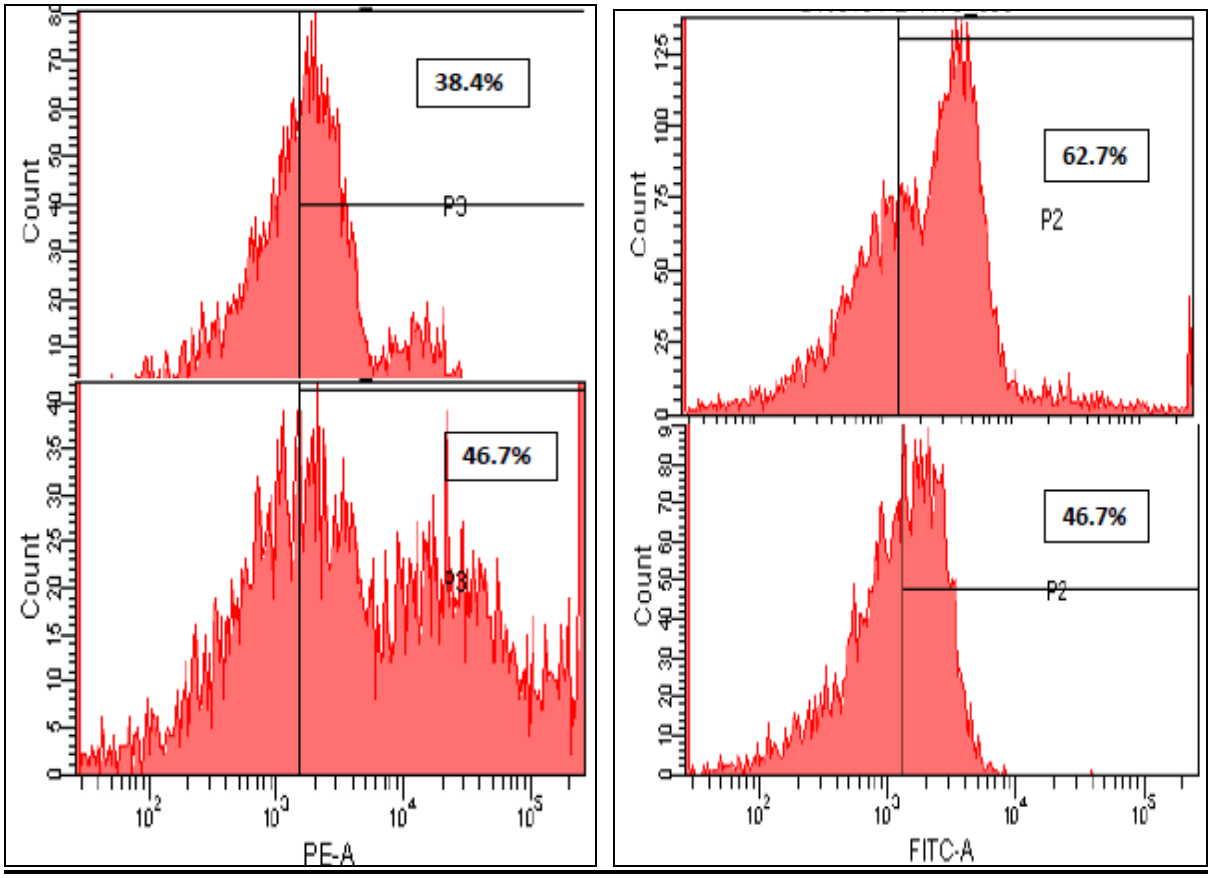

Figure 38: NK cell receptor (NK 1.1) and NK inhibitory receptor (Ly49C) profiling in the presence and absence of more than 100KDa (>100KDa) membrane protein of P815 cell. Upper left and right panel shows histogram for NK cell receptor profiling without co-culture with P815 cells for FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 C respectively.

Lower left and right panel shows histogram for NK cell receptor profiling when it was co-cultured more than 100KDa (>100KDa) membrane protein of P815 cell for FITC-conjugated anti-NK 1.1 and PE-conjugated anti-Ly49 C respectively

7. Conclusion:

In the presence of tumor resistant cell line (P815) or tumor sensitive cell lines (YAC-1), NK cell surface receptors are found to modulate in both aspects either their up-regulation or down-regulation, which play vital role in the interplay of activation and inhibition signals of NK cell towards tumor lysis.

NK cell surface receptor modulation were analysed by flow cytometric method in the presence of P815 whole cells, P815 whole membrane protein and P815 membrane protein fraction of more than 100 KDa. No significant receptor modulation was observed in NK cell when they were co-cultured with P815 whole cells, but when whole membrane protein of P815 cells was isolated (as discussed in Section 5.8), receptor profiling was observed in NK cell which describes several significant shifts towards tumor resistance to undergo NK-mediated killing.

In this regard, upregulation of inhibitory receptors (Ly49A and Ly49C) was observed, which is previously reported by many researchers to be involved in tumor evasion from NK mediated killing and down-regulation of NK receptor (NK 1.1) was also reported, which resist NK cell activation to perform tumor lysis.. These regulations altogether resist tumor from NK attack and evade immune system.

As from the Figure 31 figure 34 and figure 38, it is evident from the histogram that there is not only increase in expression of Ly49C but there is also an increase in population of cells expressing Ly49C or greater proliferation response induced by the tumor cells.

In the above cases of P815 cells co-culture, there is no any significant increase in NK 1.1 receptor that may change in NK cell receptor proliferation is specifically on NK cell population on per cell basis.

Increase in frequency of NK receptors may be due to an increase in the percentage of inhibitory receptor on per cell basis or it may be contributed by an induced proliferation of Skewed population of cells that inherently express higher frequency of inhibitory receptors.

When P815 membrane protein fraction of more than 100 KDa was co-cultured with NK cell, down-regulation of inhibitory receptors (Ly49A and Ly49C) and up-regulation of NK receptor (NK 1.1) was observed in several cases (Section 6.6) although same profiling was seen as discussed in case of P815 cells and P815 whole membrane protein.

8. Discussion and Future Perspective:-

Efficient recognition of tumor cells by NK cells is instrumental for NK cell activation. Thus, up-regulation of activating ligands and/or down-regulation of inhibitory ligands on tumor cells would increase NK cell activation against tumors. Expression of definite patterns of surface markers on NK cells may be associated to the inactivated and activated state of cells.Sum total of inhibiting and activating receptors results in outcome of NK cell killing activity.

NK cells were recovered from spleenocytes of C57BL/6 mice strain, and was purified from NK cell enrichment kit (as described in section 5.5), before purification spleenocytes count was too high (say, 90 X 10^6cells/ml) and after enrichment it was reported to be equal to nearly 5-7% of total spleenocytes (say, 7.5 X 10^6 cells/ml) which minimized the error-prone results which we were getting earlier in our results.

To evaluate, if NK cells in activated and resting state express comparatively different surface markers, flow cytometric methods was used to study receptor profiling of NK cell. NK cells were incubated with and without IL-2 and it was reported that there was increase in total number of cells which includes NK, NKT and T cells, some researchers have also suggested that interleukin 2 (IL-2) induce activation of unstimulated resting NK cells.It was observed that in the presence of IL-2, NK cell receptor, NK 1.1 was up-regulated and inhibitory receptors Ly49A and Ly49C show a significant down-fall and thus potentiates growth function of NK cells.

In this study, we also infer that tumor cell lines have a great impact on NK cell functionality by regulating Nk 1.1 and inhibitory receptors present on NK cell surface by performing several experiments using NK resistant cell lines (P815).

Co-culture of splenic NK cell with tumor-resistant cells (P815) was performed and we demonstrated that NK cell receptor (NK 1.1) was down-regulated and inhibitory receptors (Ly49A and Ly49C) were upregulated in the presence of cells, but they were not so significantly marked.

For obtaining significant receptor profiling for NK cells, we isolated whole membrane protein of P815 cells (as described in section 5.7) and we observed significant results,in which we observed significant potentiation of inhibitory receptor, Ly49C in comparison to another inhibitory receptor, Ly49A and downregulation in NK 1.1, which is predominant receptor, which tip the balance towards inhibition of killing mechanics of NK cells towards P815 cells and hence P815 cells were spared which ultimately contribute to its resistance towards NK mediated killing.

For the identification of active components secreted by NK resistant cell (P815), amicons were performed (unpublished work of Ms. Ruchi Verma) for more than 100 KDa protein, expecting our interested component or tumor-derived factor to be present in this kilodalton of protein, which can be responsible for the modulation of NK cell surface receptors towards tumor lysis.

Several implications of this study can be done in future, which includes:

Identification of active compounds secreted by tumors, which has capability for enhancing NK 1.1 receptors and down-regulating inhibitory receptors on NK cell, which can efficiently target tumor cell for killing.

Preparation of monoclonal antibody which can block inhibitory receptors and can potentiate killing of tumor by NK cell.

Preparation of bi-specific antibodies that can promote NK cell targeting of tumor cells.

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