1. ABSTRACT

Natural killer cells which have the cytolytic activities against target cells are the white blood cells of the innate immune system, originally identified in 1975. Their classification as lymphocytes depends on their etiology, expression of lymphoid markers, and their genesis from the lymphoid progenitor cell within the bone marrow. NK cells vary from phagocyte like macrophages and granulocytes which completely depend on conserved pattern recognition sequences present on receptors as toll like receptors. Tumors develop numerous mechanisms through which they elude NK cell attack. The NK cells show great role in host defense against virus infected and tumor cells. The interactions between the known factors such as galectin-3, CD63(AD1 antigen), gp70 and p30 present on the surface membrane of p815 tumor with their inhibitory receptors Ly49A were potentially addressable by computational approaches and which could further help to develop NK based cancer therapeutic strategies.

A set of proteins were obtained upon extraction with tris-EDTA from plasma membrane of p815 mastocytoma which upon fractionation through amicons provided with proteins between 30 KDa-100KDa. These fractions were then analysed and bound to splenic NK cells to examine the modulations. The sensitivity and resistance of various tumor cell lines to NK cells might open the gateways for NK cell-based immunotherapy including intervention with NK cell activation and inhibition induction as well as NK cell function modulation which might be of inhibition and recognition in NK cell receptor and modulation of the co-stimulation, susceptibility to apoptosis or killing.

2. INTRODUCTION

Natural killer cells are important for immune surveillance which distinguish foreign cells from normal healthy host cells, and kill virus infected and tumor cells. NK cell population is not identical and posses different roles. The NK cell functioning is linked to the receptors which are expressed on their surface and relay specific messages to the cell. The two kinds of NK receptors are activating and inhibitory receptors. Activating receptors are needed for NK cells so that they can respond against problems like infection and tumor while the inhibitory ones are required for NK cells so as to maintain self tolerance that is to prevent immune response against healthy cells in the body.

Tumors and virus infections are solemn hazard for human beings. Immune system relies on detecting changes on the surface of infected cells while the effector molecules cannot easily access intracellular pathogens and this is achieved in turn by specialized effector cells which carry the ability to selectively destroy infected host cells via contact dependent cytotoxicity. There are basically two main classes of cytolytic lymphocyte. One is cytotoxic T-cells which recognize and present processed target cells in form of peptides to MHC class I molecules on the other hand NK cells lyse target cells in a MHC non-restricted fashion. Activity of NK cell is linked with the large granular lymphocytes subpopulation (Timonen *et al*., 1981) which is around five percent of the peripheral blood lymphocytes and 1-3% of all mononuclear cells.

There are different mechanisms which are known to engage in the demolition of tumor cells through NK cells:
Cytotoxicity mediated by perforin or granzyme- Cytotoxic granules released are composed of perforin and granzymes which is the fastest medium to kill tumor cells where a synapse is created with the target cell and NK cells will drop at the junction, releasing perforin and granzyme molecules that induces the killing of the target cell (Bryceson YT *et al*., 2006). Perforin deficient mice less are effective (about 10 to100 times) in suppressing syngeneic MHC-I deficient tumor cells, putting forward that this molecule is needed for NK cell cytotoxicity (Kagi D *et al*., 1994). Besides, many studies demonstrate that perforin is essential for the immunosurveillance of various spontaneous malignancies (Smyth MJ *et al*., 2000) while the role of granzymes is less characterized.
Apoptosis mediated by death receptor- Induction of target cell death is mediated by apoptosis through tumor necrosis factor family ligands, like Fas ligand (CD178), TNF, and TRAIL (tumor-necrosis factor-related apoptosis-inducing ligand), which is a substitute to the release of granules. This mechanism needs the presence of the TNF family ligand that is expressed on the NK cells surface. These particular ligands will bind to a receptor Fas on the target cell surface.

NK Cell Tumor Cell

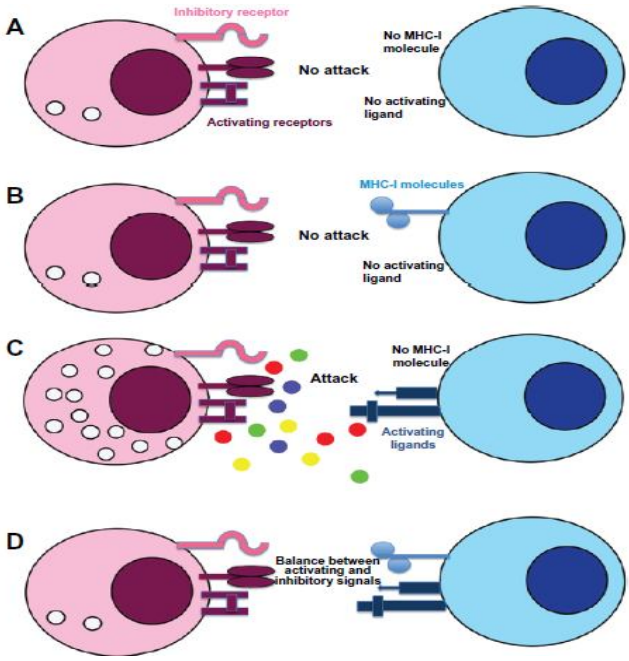


Fig1: Mechanism of recognition of target cells through NK cells.

This pathway is very effective and is controlled by numerous factors such as receptor expression of FasL or TRAIL through the cancer cells which give protection against apoptosis.

Interferon-γ effector functions: On NK cell activation, cytokines such as interferon γ, TNF-α, GM-CSF, IL-10, or IL-13 are secreted and the antitumor activities may be mediated by IFN-γ (Trinchieri G *et al*., 1995). Significantly, IFN-γ produced by NK cells eliminates tumor metastases and sarcoma produced by methylcholanthrene in a murine model (Street SE *et al*., 2001). This cytokine prevents proliferation of tumor cells in vitro and indirectly inhibits the tumor growth in vivo by the induction of antiangiogenic factors, IP-10 (Angiolillo AL *et al*., 1995). IFN-γ also increases NK cell mediated cytotoxicity by the overexpression of adhesion molecules or by elevating the sensitiveness of tumor cells to cytotoxicity which is mediated by granule release or death receptor involvement (Biron CA *et al*., 1995). Murine liver natural killer cell expresses TRAIL that is depends on IFN-γ expression and gives contribution to the natural antimetastatic role of the NK cells (Takeda K *et al*., 2001). IFN-γ induced TRAIL diction is also involved in the IL-12 mediated anti-metastatic effect.

While tumor progression starts, tumor cells evolve various mechanisms to escape from NK-cell recognition and killing which include diminishing expression of adhesion molecules and ligands for activating receptors, MHC class I upregulation, soluble MIC, FasL expression, that secrete immunosuppressive factors such as IL-10, TGF-β and indoleamine 2,3-dioxygense and resists

Fas or perforin mediated apoptosis (Waldhauer I *et al*, 2008). Modulation can be of recognition and inhibition in NK cell receptor and co-stimulation, adhesion or susceptibility to killing. Regis and his co-workers (2002) concluded their analysis on a fact that the NK cells are unable to kill leukemic cells due to decrement in NCR surface expression and down regulation of ligands for NCR-mediated target cell recognition.

Various findings show that tumor-derived factors raise not only MSC recruitment but also their maturation coming closer to an immunosuppressive phenotype. Tumor expression is linked with an increase in the levels of IL-1, IL-6 at the time of later stages of tumor growth (Trikha M *et al*., 2003). Vascular endothelial growth factor depicts a key role while forming blood vessels during embryogenesis, hematopoiesis, and tumor neovascularization. Recently it was found out that the link among MSCs, VEGF and tumor progression can be complex and convey that VEGF is the molecule regulating crosstalk among tumor and the tumor-associated MSCs (Gabrilavich D *et al*., 2004).

In the nucleated cells glycoproteins show complexity while studying murine tumor cell lines**.** For instance, if P815 cell line is taken, there are only eight out of a total 80 separate glycoproteins which are common in other murine cell lines.

Current understanding of NK cell activation molecular mechanism has opened the gateways for the possibility to specifically target cancer cells by NK cells. NK cells are very effective killers of tumor cells and may help to elicit an adaptive immune response against cancer and tumors. A better understanding of the biology of NK cells is a lead to progress strategies for manipulation of NK cells in therapeutics.

3. REVIEW OF LITRATURE

Natural Killer (NK) cells are cytolytic lymphocytes as originally defined, which selectively eliminate tumor cells inspite of having antigen specific receptors. NK are lymphocytes of innate immune system which destroy an array of target cells and produce cytokines that participate in shaping adaptive immune response.

NK cells have capacity to distinguish stressed cells from normal healthy cells through germline encoded recognition receptors. NK cell activity regulation depends on a variety of cell surface activating and inhibitory receptors which govern the dynamic equilibrium. NK cells have short life span and at a time more than two billion cells circulate in an adult and are derived from $CD34^+$ hematopoietic progenitor cells.

NK cells can secrete cytokines and chemokines which influence the host's immune response by killing infected cells through perforin or granzyme or death receptor related pathways. INF-γ is thought to be prototypic NK-cell cytokine whose production shapes the Th-1 immune response by activating APC's to up-regulate MHC class-1 expression, to activate macrophage killing of viral and tumor cells.

NK cells are potent to mediate bone marrow rejection as well as promote engraftment along with their ability to elicit antitumor effects. By understanding NK cell biology from activating and inhibitory receptor functions we can draw out the clinical potential of NK cells.

3.1NK receptors

NK cell cytotoxicity is mediated by two families of receptors which recognize classical MHC class-1 molecules on the surface of target cells and help them to distinguish between healthy pathogen or tumor infected cells by gauging the level of expression of MHC molecules.

Both of these receptors are structurally distinct as belonging to the Ig superfamily such as KIR or as members of the C- type lectin like domain superfamily as CD94NKG2s.

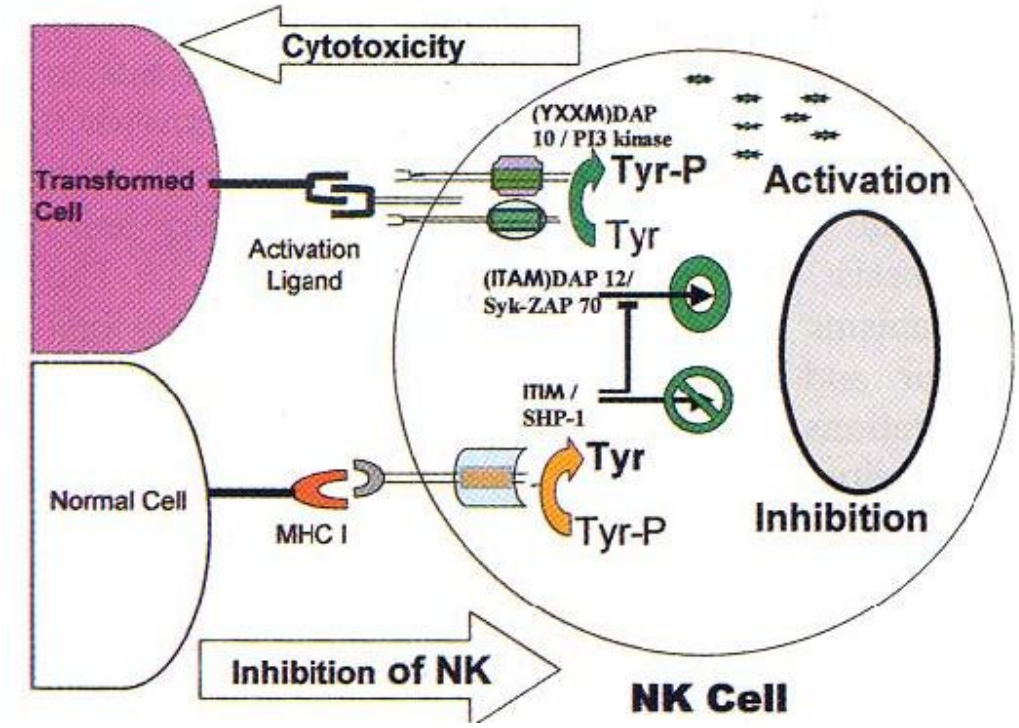


Fig2: Representation of interaction involving different receptors and ligands on NK cell and target cell.

(Source: Raghvendra M Srivastava)

3.2Inhibitory receptors

NK cells express a range of inhibitory receptors which regulate their activation, out of which some are specific to MHC class-I whereas others bind to non-MHC ligands. Some inhibitory receptors like KIR (Killer cell Ig like receptors) and LILR's (Leucocyte Ig like receptors) are monomeric type I glycoproteins of the Ig superfamily but some of them as Ly49 and CD94-NKG2A receptors are type-2 glycoproteins with a C-type lectin like scaffold (Ravetch, J. V. et al.,2000).

These receptors share a common signaling motif in cytoplasmic region without having diversity in their extracellular domains. Inhibitory receptors have Tyrosine-based inhibitory motif (ITIM's) immunoreceptor in their cytoplasmic domains which recruit intracellular phosphatases SHP-1 or SHP-2.

KIR and Ly49 demonstrate allelic polymorphism where Ly49A and Ly49C are inhibitory receptors and Ly49H and Ly49D are activating receptors which are recognized on their MHC

class I ligands. CD94/NKG2A receptors bind to ligands that have limited polymorphism while CD85j react with α-3 domain of HLA class I which is highly conserved in the class I molecules.

Species	**Receptor**	**Ligand**
Mouse	Ly49	H-2K, H-2D
Mouse	CD94/NKG2A	Qa-1^b
Human	KIR2DL	HLA-C
Human	KIR3DL	HLA-Bw4, HLA-A
Human	CD94/NKG2A(CD159a)	HLA-E
Human	CD85j, CD85d	HLA class 1

Table: 1 Inhibitory NK receptors for MHC

3.3Activating receptors

When NK cells interact with potential target cells lacking MHC-I molecules, activating signals are generated. They are collectively grouped as natural cytotoxicity receptor. Upon NK cell activation NKp44 is expressed which represent highly specific NK cell markers and are the most important receptors of NK cell-mediated tumor cell lysis. NCR play a great role in NK mediated lysis of tumors, carcinomas, neuroblastomas and transformed B cells which show that NCR ligands are expressed by different histotypic cells when they undergo tumor transformation.

Receptor	Species	Ligand
CD16	Mouse , human	IgG
NKp46	Mouse , human	Influenza haemagglutinin
KIR2DS	Human	HLA-C
CD94/NKG2C	Mouse, human	HLA-E(Qa-1)
Ly49D	Mouse	H-$2D^d$
Ly49H	Mouse	MCMV induced
NKp44	Human	Influenza haemagglutinin
NKG2D	Human , mouse	MIC, ULBP (RAE-1,H60)

Table 2: Activating NK receptors for MHC

3.4 NK cytotoxicity with receptors

It is well known that cytokine secretion leads to NK cell effector functions during innate immune responses particularly through (INF)-γ which is really important to cut down the spread of

infections. NK cells stimulate to secrete tumor necrosis factor (TNF)-a, produced during the anti-tumor immune response. Two main cell populations which are present in peripheral blood human NK,90% are cytotoxic NK cells that are characterized by a $CD56^{dim}CD16$ phenotype, while the rest 10% are not cytotoxic and secrete immunoregulatory cytokines which display a $CD56^{bright}CD16^{dim}$ phenotype (Bielekova B. *et al*.,2006)

The NK cells induce apoptosis of target cells by secreting cytotoxic granules containing granzymes and perforin. Recently, it has been shown that NKp30 triggers NK cell-mediated cytotoxicity while IFN-c which is secreted by human NK cells on recognition of two novel ligands BAT3 and B7-H6 present on tumor cells. From tumor cells BAT3 was released in exosomes and in this manner, this intracellular protein which participates in DNA damage induces NKp30-mediated cytotoxicity (] Brandt, C. S.*et al*., 2009). Apoptosis of target cells can be induced by NK cells through death receptor-mediated cytotoxicity such as the Fas-FasL system and through the TNF related apoptosis-inducing ligand (TRAIL).

The cytokines play a deciding role in regulation of immune responses like lymphoid development, homeostasis, differentiation, tolerance, and memory. IL-2 stimulates NK cells and become lymphokine-activated killer (LAK) cells exhibiting accelerated cytotoxic activity.

Some cytokines such as IL-2, IL-15, IL-18, IL-21, and IFN-a play a critical role for the activation of NK cells against tumor cells and infectious agents. Few novel cytokines which belong to the IL-12 family such as IL-23 and IL-27 may also regulate NK cell mediated responses. Also, NK cell activation is found to negatively regulate cytokines such as transforming growth factor (TGF)-β.

NK cell-mediated cytotoxicity is regulated by IL-2 and IL-15 like LAK cells exhibit cytotoxicity towards tumor cells which are resistant to NK cell-mediated lysis. It has been observed that IL-15 stimulates the NK cell cytotoxicity against tumor cells which over-express the NKG2DL MICA but it is not induced against cells which do not express NKG2DLs without stimulating an upregulated expression of NKG2D.

IL-12 signals are promoted by the activation of Jak2 and STAT3/STAT4 by triggering cytokine secretion and by NK cells cyotoxicity. NK cell proliferation is not triggered by IL-18 or IFN-c production, this cytokine participates in the stimulation of the migratory potential of NK cells while having contribution between NK cells and DCs for activating and polarizing adaptive immune response.

IL-21 regulates the expression of various NK cell receptors like few inhibitory members of the Ly49 family which were stimulated by IL-2 and IL-15 in mouse cells and the activating NKG2D receptor in human cells.

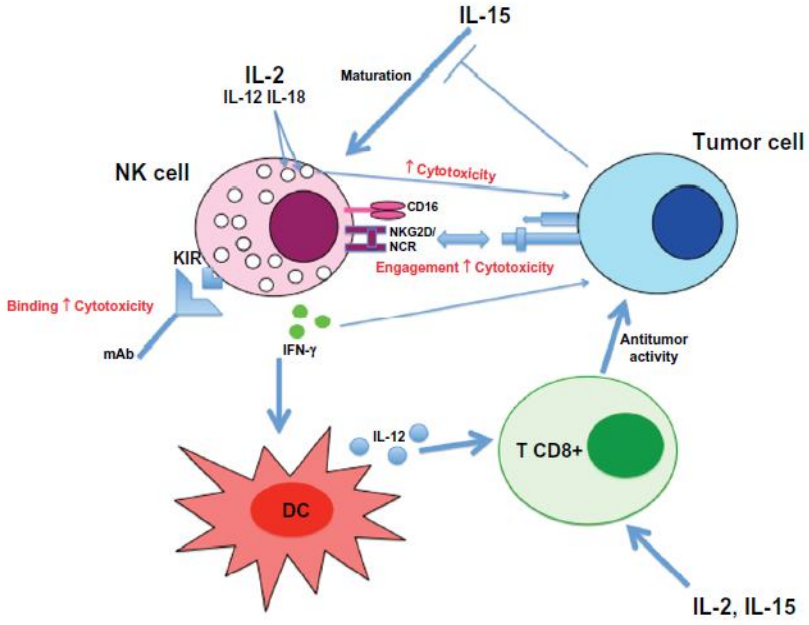


Fig3: Tumor cells showing NK cell responses.

3.5 NK receptor signalling

Signals which are broadcasted by activating and inhibitory receptors regulate NK cell immune responses. In the cytoplasmic domains of inhibitory NK receptors, immunoreceptor tyrosine-based inhibition motifs recruit tyrosine or lipid phosphatases that modulate the activation signals which are transmitted by receptors linked to the Syk and ZAP70 tyrosine kinases and phosphatidylinositol-3 kinases.

NK cells use positive and negative signaling pathways that share many common features with the immune receptors expressing on B and T lymphocytes.

Small transmembrane anchored adaptor proteins which transmit signals possess immunoreceptor tyrosine-based activation motifs in their cytoplasmic domains. NK cells also express the ITAM-bearing CD3z, FceRIg and DAP12 adaptor proteins. Attatchment of any ITAM-bearing receptor complexes gives output by recruiting and activating tyrosine kinases Syk and ZAP70, which are expressed by all natural killer cells (Sjolin H *et al*., 2002)

In NK cells CD16 signalling is initiated by either CD3z or FceRIg is somehow identical to TCR-induced signal transduction in T cells where phosphorylation of the ITAMs is mediated through src family kinase, which facilitate recruitment of Syk and ZAP70 (Colucci F. et al., 2002).

The nuclear factor of activated T cells is activated through CD16 signalling which results in the production of cytokines, involving IFN-g, GM-CSF and various chemokines that causes degranulation of NK cells. Likewise in TCR signaling, activation of CD16 may cause apoptosis

in IL-2-activated NK cells. CD3z or FceRIg, stimulation of NK cells via DAP12 activates Syk, and ZAP70 and stimulates NK cell-mediated cytotoxicity and cytokine production.

Inhibitory KIRs and Ly49 receptors have immunoreceptor tyrosine-based inhibition motifs in their cytoplasmic domains. When MHC class I ligand is involved, these ITIMs are phosphorylated and recruit phosphatases to prevent cellular activation. Among human KIR molecules, KIR2DL4 (CD158d) is schizophrenic, which contain an ITIM in the cytoplasmic domain but also exhibit activating functions (Yusa S *et al*.,2002). Upon introduction into a chimeric receptor, the KIR2DL4 cytoplasmic domain counteracts NK cell activation.

NK cells have the ability to develop in the absence of ITAM-mediated signaling which has been validated by examining in mouse NK cells that lack both Syk and ZAP70. NK cells from the mice which do not posses both Syk and ZAP70 killed NK-sensitive tumors such as Yac-1 and RMA-S at stage comparable to wild-type mice, directing that non-ITAM-based signaling pathways are responsible for this function (Sjolin H. *et al.*, 2002)

The major finding which results from the study of NK cell signal transduction is the outome of inhibitory pathways which control NK cell effector function (Long E O *et al.*, 1999). Cell surface receptors which are expressed on NK are the human killer cell inhibitory receptor–L (KIR-L) and mouse Ly49 transduces inhibitory signals upon involving with the major histocompatibility complex (MHC) class I ligands. On the other hand, oligomeric ITAM-dependent receptors, inhibitory receptors are monomeric and express one or more intracytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIM). On phosphorylation of tyrosine, ITIMs recruit and activate the tandem SH2 protein tyrosine phosphatases SHP-1 and SHP- 2 (Ve´ly F. *et al.*, 1997). ITIM-bearing receptors inhibit the signaling mediated by ITAM-coupled Receptors which is saturable and its involvement is equal to magnitude of engagement of both receptors with their ligands. Equilibrium is established between the potential of activating and inhibitory signals which is being relayed by the extent of tyrosine phosphorylation of critical signaling components that may serve as targets for both Syk-family protein tyrosine kinase and SHP-1 or SHP-2 protein phosphatases.

3.6 NK cells and tumor

While tumor progression starts tumor cells evolve various mechanisms to escape from NK-cell recognition and killing or to induce defective NK cells which include diminishing expression of adhesion molecules, ligands for activating receptors, upregulation of MHC class I, soluble MIC, FasL or NO expression, that secrete immunosuppressive factors such as IL-10, TGF-b and indoleamine 2,3-dioxygense (IDO) and resists Fas- or perforin-mediated apoptosis(Waldhauer I *et al*,2008). Modulation can be of recognition and inhibition in NK cell receptor and co-stimulation modulation, adhesion or susceptibility to apoptosis. Regis and his co-workers (2002) concluded their analysis on this fact that the NK cells are unable to kill leukemic cells due to reduction in NCR surface expression and down-regulation of ligands for NCR-mediated target cell recognition.

Various elegant studies demonstrate that NK cells effectively control tumor growth in mice which is intervened through release of perforin and cytokines (Smyth *et al.,* 1998). Anti-tumor immunity is further illustrated by the elevated incidence of leukemia in patients having dysfunctional NK cells (Smith *et al*, 1985) This has been observed that NK cells can efficiently respond to tumor cells possesing defective MHC class I which has promising effectors for immunotherapeutic strategies that target tumor escape variants (Karre *et al*,1986).Tumor cells develop mechanisms to prevent NK cell function, that involve the expression of ligands which interact with NK cell inhibitory receptors (Lanier *et al*,1998).

An important correlation between high intratumoral levels of NK cells and enhanced survival has been demonstrated in various kinds of cancer (SenovillaL *et al*., 2012). Immensely, high levels of NK-infiltrating tumors associate with a significant improvement of clinical results with head and neck squamous carcinoma (HNSCC) patients. It was reported by van Herpen *et al*. that CD56C NK cells in lymph nodes produced IFN- γ which leads to tumor regression in IL-12-treated HNSCC patients (TurksevenMR *et al*., 2010). The density of CD57CNK cells and a good prognosis show a direct positive interaction that has been reported for oral squamous carcinoma and gastric carcinoma (GC) tumors. In addition to it NK cell infiltration found to correlate with the extent of invasion, the clinical stage, and the venous invasion. Hence, the 5-year survival rate of GC patients having high rate of NK infiltration was significant more than that of patients with a low level of NK infiltration (OygurT *et al*., 2010).

Tumors escape NK cell attack by triggering ligands so that the activation threshold for NK cell granule exocytosis is not fulfilled.

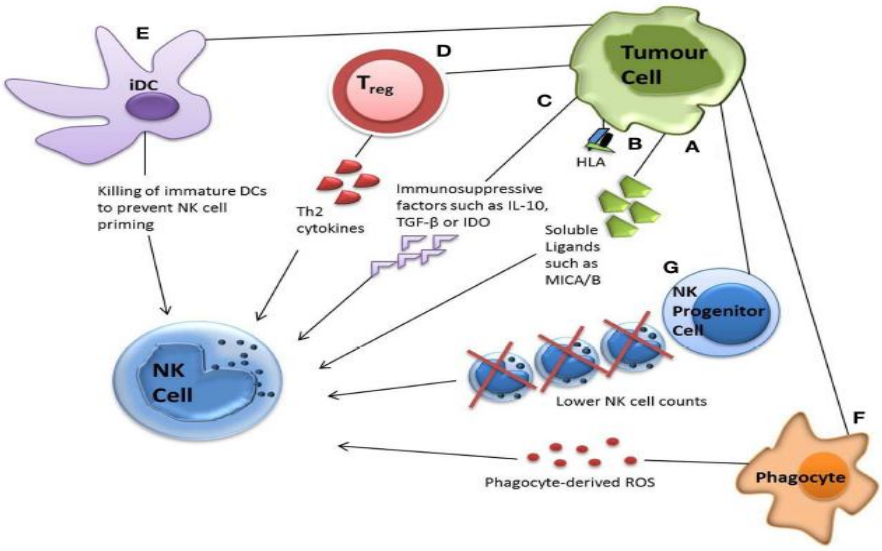


Figure 4: tumor cell evasion via direct mechanisms include(a)Shedding soluble ligands for NK cell activating receptors.(b)Upregulation of HLA molecules(c)Release of inhibitory cytokines and indirect mechanism include (d)Activation of inhibitory regulatory T cells(e)Dendritic cell killing(f)Phagocyte-derived inhibitory cytokines. It is demonstrated that tumor cells decrease the number of NK progenitor cells (g) therefore lowering NK cell counts.

3.7 Tumor derived factors

Various findings show that tumor-derived factors (TDFs) raise not only MSC recruitment but also their maturation getting closer to an immunosuppressive phenotype. Infact, the conditioned media isolated from tumor cells lines may inhibit in vitro differentiation of dendritic cells from their precursors (Gabrilovich *et al*., 1996). Almost from 10 years efforts are being made to recognize these TDFs (Pegoraro L *et al*., 1989). Tumors secrete cytokines, chemokines, and other diffusible molecules that can induce MSCs recruitment and enhance their maturation into suppressive cells. A number of candidate proteins have been identified till date which is discussed below.

Colony stimulating factor 1 (CSF-1) whose secretion is also called Macrophage-colony stimulating factor and is described in many cancers including acute myeloblastic leukemia (Rambaldi A *et al*.,1988), renal cell carcinoma, bladder carcinoma and about 70% of human breast cancers. Furthermore, through a mechanism of starvation, mouse and human monocytes which are exposed to CSF-1 gain ability to suppress antigen and mitogen driven T-cell proliferation.

Tumor expression is linked with an increase in the levels of IL-1, IL-6 at the time of last stages of tumor growth (Trikha M *et al*., 2003). The activity of IL-6 is complex, which produce both pro-inflammatory and anti-inflammatory effects in the immune system. Adding to it , IL-6 affects the differentiation of myeloid lineages, macrophages along with dendritic cells, both in vitro and in vivo by the activation of the transcription factor STAT3, that exerts a negative immune regulatory function while tumor development. An increased IL-10 concentration is found in patients having solid tumors and haematological malignancies (Pawelec G *et al*., 2004) which are being used as a marker of tumor progression. IL-10 production is co-ordinated with the induction of T-cell together with TGF-β which is known to be one of the important immunosuppressive factors released by tumors (Chen ML *et al*., 2001). Vascular endothelial growth factor (VEGF) depict a key role while formation of blood vessels during embryogenesis, hematopoiesis, and tumor neovascularization. Recently it has been found out that the link among MSCs, VEGF and tumor progression can be complex and covey that VEGF is the molecule that regulates crosstalk between tumor and the tumor-associated MSCs (Gabrilavich D *et al*., 2004).

Galectins regulate the inflammatory and immune responses. Various inflammatory cells are activated by the induction of galectin-3 which can sometimes function as the chemokine that could attract monocytes and macrophages. Galectin-3 downregulates IL5 production and blocks the B lymphocyte differentiation into plasma cells. C-terminal domain of galectin-3 suppresses tumor growth and metastasis is inhibited in mouse models of human breast cancer.

P815 which is murine mastocytoma induces CTL responses in opposition to least four distinct Ags (AB, C, D, and E).It has been demonstrated by recent studies that the major component of the CTL response in resistance to the P815 tumor is directed against Ags P815AB and P815E.

A methylcholanthrene-induced tumor P815 where mastocytoma cells were investigated for the presence of type C viral proteins on their surface (Al-Rammahy *et al*., 1980). Two different

glycoproteins (gp70 and p30) with molecular weights 90,000 are expressed on membrane of p815 cells.

In the nucleated cells glycoproteins show complexity while studying murine tumor cell lines**.** For instance, if P815 cell line is taken, there are only eight out of a total 80 separate glycoproteins which are common in other murine cell lines.

CD63 antigen expressesd on the surface showed degranulation in 2RBL-2H3 cells. The experiments with transfected IgE p815 mastocytoma was done where expression in normal p815 was observed but not in p815 variant cells which missed a C-terminal cytoplasmic domain of β or γ.

In a study it was demonstrated that a new GM-CSF on the surface of the P815 mastocytoma cell line could elicit an antitumor immune response in syngeneic DBA/2 hosts. It was shown here that the rejection of P815 cells was quick which transfected to express CD40 L were and this rejection was due to remarkable contribution of NK cells (Nakajima *et al*., 1901).

A set of proteins which remain insoluble upon extraction with the tritonX-100 is found in the plasma membrane of P815 mastocytoma forming a matrix. When this matrix is treated with EGTA, it is found that approximately 25% of protein is released with molecular masses 70, 69, 38, and 36 KD. These proteins are associated with detergent insoluble structures which are named as Agorin1, a 20 KD protein and Agorin2 which is the 40 KD protein.

3.8 Effect of tumour derived factors on NK cells

Hypoxic tumor cells have the capability to activate resistance mechanisms which create an immunosuppressive microenvironment. Certainly, via their ability to produce cytokines such as tumor necrosis factor and stromal cell-derived factor1, tumor cells generate the homing of bone marrow-derived CD45C myeloid cells in tumor areas. Myeloid cells invasion in the TME is shown to be a highly immune suppressive factor for NK cells. One of the main components of the immune- suppressive network are MDSCs which are the cause for the deterioration of NK cells and T cell-dependent anti-cancer immunity (Frey AB *et al*., 2012), its immune supressive function is due to the fact that they produce IL-10 which lowers the production by macrophages of IL-12 where a pro-inflammatory cytokine is involved in the activation of NK cells (Ostrand-Rosenberg S *et al*., 2007).

Macrophages represent another major myeloid component of the infiltrated tumors and constitute up to 80% of the cell mass in breast carcinoma. Tumors which are hypoxic secrete chemoat - tractants (colony-stimulating factor, chemokine ligands), which upshot into the recruitment of monocytes from the blood to the tumor site.

Melanoma cells produce indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2), both are natural immunosuppressants that down-regulate activating NK receptors (Mingari MC *et al*.,2012) . IDO and PGE2 are known to inhibit CD8+ T cells and enhance suppressive, regulatory T cell responses (Fridman WH *et al*., 2012). Exceptionally, the combined inhibitory

effect of IDO on T and NK cells is demonstrated in a seminal paper on the activating NK receptors that are inhibited by IDO and PGE2, functions of which are counteracted by NKG2A, an inhibitory receptor used by both T and NK cells.

Functions of NK cells are expanding exponentially, further understanding the use of NK cells as an immunotherapy of solid tumor metastases play important role. Higher gratitude to the equilibrium among the activating and inhibitory receptors has led to the successful utilization of the anti-leukemic potential of NK cells via KIR ligand mismatches in a haplotype mismatched stem cell transplantation. Current understanding of NK cell activation molecular mechanism has opened the gateways for the possibility to specifically target cancer cells by NK cells. NK cells are very effective killers of tumor cells and may help to elicit an adaptive immune response against cancer and tumors. A better understanding of the biology of NK cells is a lead to progress strategies for manipulation of NK cells in therapeutics. NK based immunotherapy in future, should have focus on how blocking of the inhibitory signals occurs as well as intensifying of the activating signals which are received from tumors to achieve better results in cancer therapy.

4. MATERIALS AND METHODS

4.1Receptor modeling:

Ly49A: A 262 amino acid sequence of Ly49A of mouse origin was retrieved from NCBI (accession no.= AAF99547.1). Since the crystal structure of Ly49A was not available, the protein was modelled using a hybrid modeling server named Phyre[2] (Soding, J.2005). The platform incorporates ab initio folding simulation called Poing[2] which models regions of proteins with no detectable homology to the known structure (Jefferys *et al.*, 2010).

4.2 Ligand preparation:

We have taken ligands which are derived from tumor resistant p815 cell line namely, Galectin-3, CD63 (AD1 Antigen), gp70 and p30.
The following information about Galectin-3 (Accession no. NP_034835.1), 264 amino acids of Mus Musculus origin), CD63 (Accession no. NP_001036045.1), 238 amino acids of Mus Musculus origin), gp70 (Accession no.CAA41992.1), 644 amino acids of Murine Leukemia Virus, p30 (Accession no. AAA46522.1), 160 amino acids of Murine Leukemia Virus were retrived from NCBI and since their structures were not available at PDB so these were modeled using Phyre[2].

4.3 Molecular Docking using PATCHDOCK (automatic server for molecular docking):

PatchDock is an algorithm which is used for molecular docking. The input given here is two molecules of any type viz proteins, DNA, drugs etc. and the output obtained is a list of potential complexes which are sorted on the basis of shape complementarity criteria. The PatchDock algorithm has been inspired by image segmentation and object recognition techniques that are used in Computer Vision. Docking could be compared to assembling a jigsaw puzzle as when solving the puzzle we first try to match two pieces, by picking one of the piece and searching for the other complementary one. We basically concentrate on the patterns which are unique for puzzle element and then look for the matching patterns in rest of the pieces. PatchDock server employs a similar technique. If two molecules are given, then their surfaces are then divided into patches depending upon the surface shape. These patches correspond to the patterns that distinguish between the puzzle pieces. Once these patches are identified, then they can be superimposed using the shape matching algorithms. The algorithm has three main stages:

- Molecular Shape Representation
- Surface Patch Matching
- Filtering and Scoring

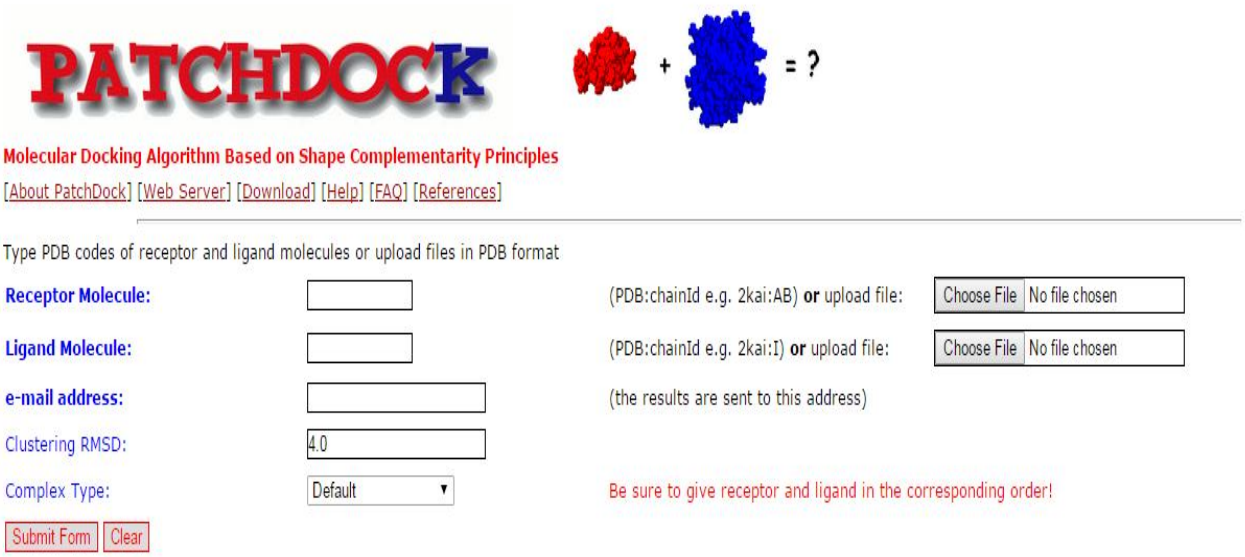
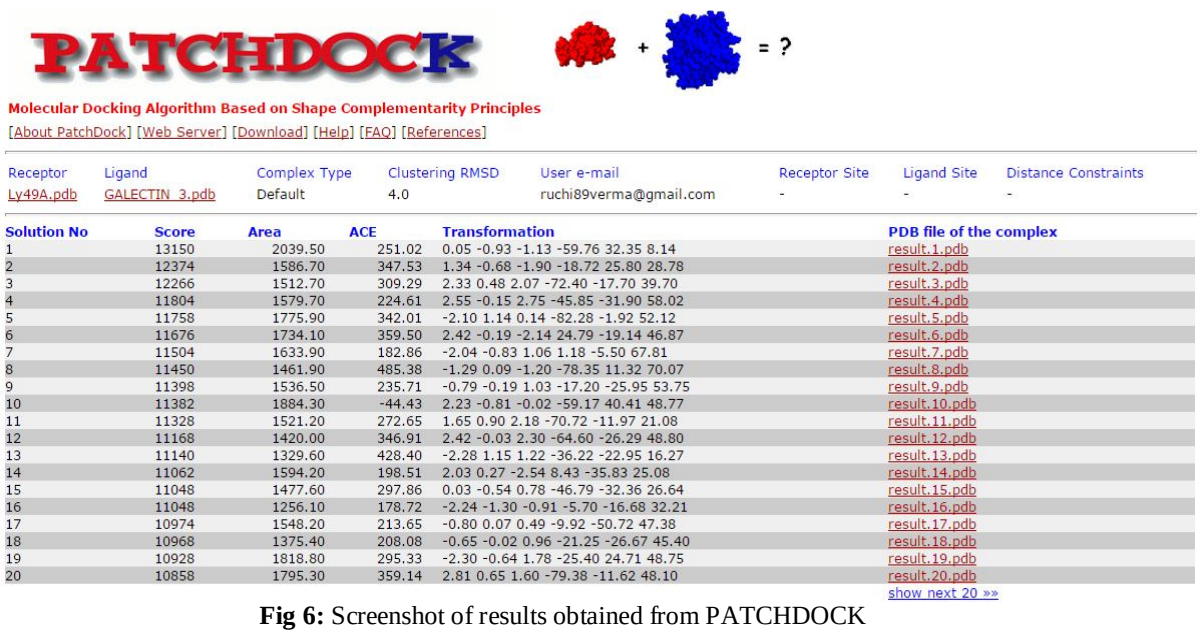


Fig 5: Screenshot of the PATCHDOCK server

The receptor molecule along with its ligand molecule was uploaded in the PATCHDOCK server, the respective e-mail id was entered and then the server gives the results.



Receptor	Ligand	Complex Type	Clustering RMSD	User e-mail	Receptor Site	Ligand Site	Distance Constraints
Ly49A.pdb	GALECTIN_3.pdb	Default	4.0	ruchi89verma@gmail.com	-	-	-

Solution No	Score	Area	ACE	Transformation	PDB file of the complex
1	13150	2039.50	251.02	0.05 -0.93 -1.13 -59.76 32.35 8.14	result.1.pdb
2	12374	1586.70	347.53	1.34 -0.68 -1.90 -18.72 25.80 28.78	result.2.pdb
3	12266	1512.70	309.29	2.33 0.48 2.07 -72.40 -17.70 39.70	result.3.pdb
4	11804	1579.70	224.61	2.55 -0.15 2.75 -45.85 -31.90 58.02	result.4.pdb
5	11758	1775.90	342.01	-2.10 1.14 0.14 -82.28 -1.92 52.12	result.5.pdb
6	11676	1734.10	359.50	2.42 -0.19 -2.14 24.79 -19.14 46.87	result.6.pdb
7	11504	1633.90	182.86	-2.04 -0.83 1.06 1.18 -5.50 67.81	result.7.pdb
8	11450	1461.90	485.38	-1.29 0.09 -1.20 -78.35 11.32 70.07	result.8.pdb
9	11398	1536.50	235.71	-0.79 -0.19 1.03 -17.20 -25.95 53.75	result.9.pdb
10	11382	1884.30	-44.43	2.23 -0.81 -0.02 -59.17 40.41 48.77	result.10.pdb
11	11328	1521.20	272.65	1.65 0.90 2.18 -70.72 -11.97 21.08	result.11.pdb
12	11168	1420.00	346.91	2.42 -0.03 2.30 -64.60 -26.29 48.80	result.12.pdb
13	11140	1329.60	428.40	-2.28 1.15 1.22 -36.22 -22.95 16.27	result.13.pdb
14	11062	1594.20	198.51	2.03 0.27 -2.54 8.43 -35.83 25.08	result.14.pdb
15	11048	1477.60	297.86	0.03 -0.54 0.78 -46.79 -32.36 26.64	result.15.pdb
16	11048	1256.10	178.72	-2.24 -1.30 -0.91 -5.70 -16.68 32.21	result.16.pdb
17	10974	1548.20	213.65	-0.80 0.07 0.49 -9.92 -50.72 47.38	result.17.pdb
18	10968	1375.40	208.08	-0.65 -0.02 0.96 -21.25 -26.67 45.40	result.18.pdb
19	10928	1818.80	295.33	-2.30 -0.64 1.78 -25.40 24.71 48.75	result.19.pdb
20	10858	1795.30	359.14	2.81 0.65 1.60 -79.38 -11.62 48.10	result.20.pdb

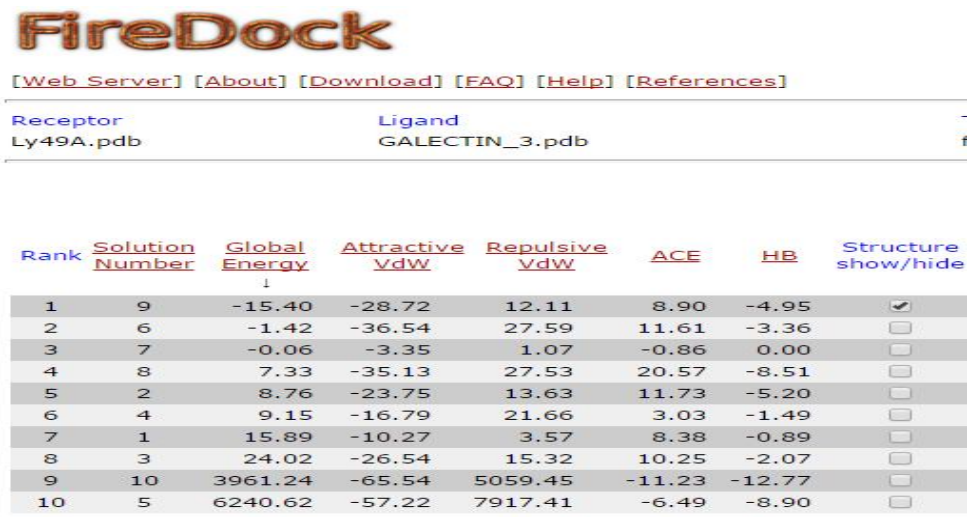
show next 20 »»

Fig 6: Screenshot of results obtained from PATCHDOCK

PATCHDOCK then returns the result in the form of docked files i.e. the PDB files of the complex which is the predicted complex structure, a number of complexes were obtained along with the score, area that is the approximate interface area of the complex and the ACE (atomic contact energy).

4.4 Refining models by FIREDOCK :

After running PATCHDOCK, the top 10 results were refined by using FIREDOCK. The FireDock server then addresses refinement problem of the protein-protein docking solutions. This method simultaneously targets problem of flexibility alongwith the scoring of solutions that are produced by fast rigid-body docking algorithms. Given upto a set of 1000 potential docking candidates, it can refine and score them based on an energy function. This is the first webserver that allows scoring of docking solutions and performing large-scale flexible refinement online.


FireDock

[Web Server] [About] [Download] [FAQ] [Help] [References]

Receptor	Ligand
Ly49A.pdb	GALECTIN_3.pdb

Rank	Solution Number	Global Energy ↓	Attractive VdW	Repulsive VdW	ACE	HB	Structure show/hide
1	9	-15.40	-28.72	12.11	8.90	-4.95	☑
2	6	-1.42	-36.54	27.59	11.61	-3.36	☐
3	7	-0.06	-3.35	1.07	-0.86	0.00	☐
4	8	7.33	-35.13	27.53	20.57	-8.51	☐
5	2	8.76	-23.75	13.63	11.73	-5.20	☐
6	4	9.15	-16.79	21.66	3.03	-1.49	☐
7	1	15.89	-10.27	3.57	8.38	-0.89	☐
8	3	24.02	-26.54	15.32	10.25	-2.07	☐
9	10	3961.24	-65.54	5059.45	-11.23	-12.77	☐
10	5	6240.62	-57.22	7917.41	-6.49	-8.90	☐

Fig7: Screenshot of results obtained with FireDock

FireDock then results the best structures in the form of zip folder along with the Global energy which is the binding energy of the solution , the attractive and repulsiveVdW forces , the contribution of atomic contact energy to global binding energy and the contribution of hydrogen bonds to global binding energy.

Finally the DCOMPLEX calculated the binding affinities of the protein complex which determined the energy in kcal/mol.

4.5 CELL LINE

It is defined as the clones of animal cells which are derived and developed from a primary culture as a first subculture. Cell lines that are obtained from primary cell culture posses a definite or limited life span and with every passaging, cells having greatest growth capacity predominate which outcomes as a measure of uniformity in the population.

Depending on the life span of culture, cell lines are divided into two forms:
a) Finite cell line - cell lines having a limited life span undergoing the confined number of generations are called as Finite cell lines. These have the quality of contact inhibition, are anchorage dependent and posses density distribution. Their doubling time is approximately 24 to 96 hours as the growth rate is quite slow.

b) Continuous cell line - Cell lines which are altered under in vitro culture conditions produces continuous cell lines. These exhibit the property of ploidy, are anchorage dependent and donot exhibit contact inhibition property. Their growth is in either monolayer or suspension form. They show rapid growth rate and 12-24 hours is their doubling time.

There are basically two types of cultures when we deal with cell lines:

a) Monolayer cultures – When a continuous layer of cells is formed and covers the complete bottom of culture flasks generally one cell in thickness, this condition is then known to have monolayer cultures. Most of the continuous cell lines produce as monolayers.

b) Suspension cultures – Few cells that are non-adhesive for example leukemia cells are grown or their propagation carried out in suspension. For instance YAC-1 and p815 tumor cell lines are propagated in suspension cultures.

4.5.1 YAC-1

Originated from mouse T-lymphoma cells and are induced through Moloney sarcoma virus. These are sensitive to natural killer cells and in NK assays these are often used as target cells. These do not posses Fc receptor expression.

4.5.2 p815

These are originated from murine mastocytoma (DBA/2) upon treatment with methylcholanthrene. Used in cytotoxic T-cell assays as target cells. These do not possess the effector activity in ADCC. This cell line can be used for tumor-host relationship in vivo experiments.

Cell line- p815

Origin	Mouse strain DBA/2
Cell type	Mast cells
Properties of culture	Suspension culture with few adherent cells
Disease caused	Mastocytoma
Growth medium	Dulbecco's Modified Eagle's Medium formulated by ATCC with catalog no. 30-2002. For complete growth medium 10% fetal bovine serum is added to base medium.
Culture conditions	Humidified incubator with 5% CO_2 at 37^0C temperature
Cryopreservation	95% culture medium and 5% DMSO
Medium renewal	Every 2 to 3 days

Table 3: Description of p815 cell line.

4.6 Culture medium-Dulbecco's Modified Eagle's Medium

DMEM is required by p815 as the growth medium which is lesser complex than enriched media that is used for more specialized cell types. Original DMEM contains 1000mg/L of glucose and Mouse Embryonic Cells MEC's were first cultured in this medium. It includes 10 Essential Amino Acids, and for Mammalian cells Cystine, Glutamine and Tyrosine are added in the culture medium. This basal medium contains glucose, pH indicator, amino acids, vitamins and salts. It has no antimicrobials, growth enhancing agents and proteins. DMEM high glucose consists of high glucose level (4500mg/l) and about four times more concentration of amino acids and vitamins which are added for optimal cultivation of other cell type.

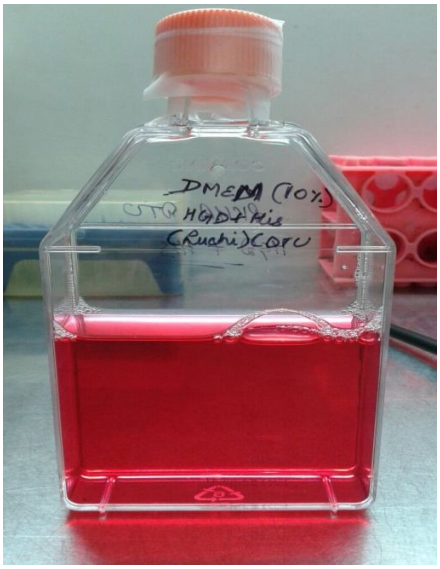


Fig 8: DMEM media for culturing p815 cells.

4.7 Culturing of p815 cell lines

1. 15 ml falcon is sterilized to take culture in it.
2. Centrifuged at 2000 rpm for 15 minutes.
3. Supernatant is discarded and the cellular pellet is suspended in 1ml DMEM+10% FBS in culture flask.
4. The cells are observed under microscope.
5. Incubate the cells in co_2 incubator.

Fig 9: p815 cultures in T-25 flasks.

4.8 Freezing of cells

1. With the help of 10ml sterile pipette pool the culture into falcon tube directly from culture flask.
2. Centrifuge it at 1500 rpm for 15 minutes.
3. Discard the supernatant and add 2ml freezing media.
4. Keep the cryovials at 0^0C for 2 minutes.
5. Now the vials are stored at -80^0C.

4.9 Thawing of cells

1. Take out the cryovials from -80^0C.
2. Transfer it into a waterbath maintained at 37^0C.
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.

4.10 Freezing media (10 ml, kept at -20^0C 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.

4.11 Splitting of cells

1. Take the cells from culture flask in falcon.
2. Centrifuge the tube at 2000 rpm for 15.
3. Discard the supernatant and to the pellet add 1ml DMEM media and mix it well.
4. Take 10µl of culture to count the number of cells.
5. After counting add cells to 5ml media.

4.12 Counting of cells

4.12.1 Counting through hemocytometer

Hemocytometer has a rectangular cavity which forms the 'H' shaped chamber in the centre. A laser-etched grid of perpendicular lines is carved on this chamber. There are two counting regions with ruled grates that are partitioned by the horizontal groove of the 'H'. A cover slip of glass is placed at 0.1 mm above on the surface of the counting regions through ground glass ridges which are present on both the sides of the vertical groove on the H shape. The height is constant and the volume of cell suspension on each square of the grid is known accurately.

4.12.2 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)

4.12.3 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)

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

4.12.4 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.

Fig 10: Haemocytometer

4.13 SDS-PAGE ELECTROPHORESIS

4.13.1 Principle:

Separation of protein in SDS-PAGE is based on molecular masses and is used to determine relative molecular mass, relative presence of major proteins in a sample is estimated, as well as the distribution of protein fractions is detemined .

Macromolecules are separated under the effect of electric field known as electrophoresis which is a common method for protein separation where a discontinuous polyacrylamide gel is used which acts as a support medium and a detergent sodium dodecyl sulfate is utilized to denature the proteins so the is known as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

4.13.2 Molecular characterization of supernatants (p815) on the basis of SDS-PAGE Electrophoresis (Laemmli's method, 1970)

- Reagents
- Acrylamide
- N,N'- Methylene bis acrylamide
- Ammonium persulfate
- N,N,N',N'- Tetramethylethylenediamine (TEMED)
- Tris- hydroxymethylaminomethane
- SDS
- HCl
- Glycine

- Glycerine
- Acetic acid
- Methanol
- Commassie Brilliant Blue G
- Bromophenol Blue

4.13.3 Stock solutions

4.13.3.1Solution A- 30% Acrylamide solution

29.2g acrylamide

0.8g bis-acrylamide

Add water to makeup volume 100ml.

4.13.3.2 Solution B- 1.5 M Tris buffer (pH8)

Dissolve 18.17g of Tris and 0.4g of SDS in water, adjust for pH with HCl and make upto 100 ml.

4.13.3.3 Solution C- 0.5 M Tris buffer (pH6.8)

Dissolve 6.06g of Tris and 0.4g of SDS in water, adjust for pH with HCl and make upto 100 ml.

4.13.3.4 Solution D- 10% APS

Add 1ml of water to 0.1g of APS (PREPARE JUST PRIOR TO USE)

4.13.4 Composition of gel solution for a 1mm thick gel

	Separating gel (12.5%) in ml	Concentrating gel (4.5%)in ml
Solution A	7.5	0.9
Solution B	4.5	-
Solution C	-	1.5
Solution D	0.07	0.018
TEMED	0.01	0.01
Water	6	3.6

Table 4: Composition of gel solution for a 1mm thick gel

4.13.4.1 Stock solution-10% SDS solution

10g of SDS in 100 ml water

4.13.5 Sample solubilizing buffer (SSB) (1X)

SDS	10%
Tris-HCl buffer(pH6.8)	10mM
Glycerine	20%
Bromophenol blue	0.05%

Table 5: Sample solubilizing buffer (SSB) (1X)

4.13.5.1 For 10 ml

Tris-HCl (0.1M)	1ml
Glycerol	2ml
SDS	1g
BPB	0.05g
Water	Volume makeup to 10ml

Table 6: Sample solubilizing buffer (SSB) For 10 ml

Tris – Cl (pH6.8)

Stock (0.1M) = 100ml

Weigh 1.21g and dissolve it in water, volume makeup to 100ml

4.13.6 Electrophoretic buffer

Tris	0.025M
Glycine	0.192M
SDS	0.1%

Table 7: Composition of electrophoretic buffer

Add 10ml of 10% SDS solution to 3g of Tris and 14.4g of glycine and makeup to 1000ml with water.

4.13.7 Staining solution

CBB	2.5g
Methanol	500ml
Acetic acid	100ml

Make volume upto 1000 ml

Table 8: Composition of staining solution.

4.13.8 Destaining solution

Add water to 250ml methanol, 70ml of acetic acid, to make 1000ml.

4.13.9 Gel preparation

1. Clean glass plates and spacers with mild detergent, wipe with 70% ethanol and dry up with tissue.
2. Clamp two plates on casting stand with the help of spacers.
3. Examine the setup for any leakage.
4. Now pour separating gel with the help of micropipette.
5. Immediately pour water or methanol over it to level the gel.
6. Let it polymerize.
7. Drain water.
8. Then after polymerization of separating gel immediately pour separating gel.
9. Insert the comb to make wells and allow it to polymerize.

4.13.10 Sample preparation

1. Take 15µl of protein samples in 2ml eppendorf tubes.
2. 10µl of SSB solution were added in protein sample.
   Heat the sample in boiling water bath for 15minutes (keep the sample at $4^{O}C$ for 1to 2 min and centrifuge at 10,000 rpm for 30sec, take the supernatant in sample).
3. Load the protein samples into the corresponding well along with molecular weight marker protein in respective wells.
4. Electrophoretic unit were run at 30mA until the tracking dye were about to leave the separating gel. (run the gel at 80V, when dye front enters the separating gel, increase the voltage to 100-120V).
5. After that, gel were removed and placed in the staining tray and flooded with staining solution, incubated for overnight.
6. After that staining solution was discarded and gel was kept in destaining solution until bands were clearly visible (for destaining, after adding solution, keep the tray in incubator ($37^{O}C$). (not in microwave or blower, patches will form).

4.14 Generation of Growth Curve of NK cell resistant cell line – p815

Generation of a growth curve can be useful in evaluating the growth characteristics of a cell line. From a growth curve, the lag time, population doubling time, and saturation density can be determined.

4.14.1 Procedure:

1. Add the freshly grown culture (p815) to the falcon and centrifuge the cells at 1500rpm for 10min.
2. Resuspend the pellet in 1mL of medium and count the cells.

3. Dilute the cells suspension in order to have an appropriate amount of medium and cells to achieve a seeding density of 0.5 X 10^6 cells/ml.
4. Mix well and seed appropriate amount of diluted cell suspension to culture plate.
5. Put the plates in CO_2 incubator. Count the duplicate plates at interval of 12 hours till cells density started to reduce.
6. Plot the results on a log-linear scale. The population-doubling time can be determined by identifying a cell number along the exponential phase of the curve, tracing the curve until that number has doubled, and calculating the time between the two.

4.15 Preparation of p815 cell lysate

1. To the cell pellet, add lysis buffer (RIPA+PI) in the ratio of 10:1.
2. Keep on ice for 45 min, vortex in every 10 min for 5-7 second.
3. Spin at 14,000 rpm for 15 min.
4. Collect the supernatant in fresh eppendorf.
5. Store at -20ºC.

4.15.1 Lysis buffer composition (for 30ml)

150ml NaCl	262.98 mg
1% Triton-X	300µl
0.5 M Sodium deoxycholate	150mg
0.1% SDS	30mg
50Mm Tris pH 8.0	1.5ml of 1M

Make up volume with H_2O.

Table 9: Lysis buffer composition (for 30ml)

4.16 Preparation of p815 whole membrane extract

Buffer A	Tris-Cl (pH7.4) + 1Mm EDTA
Buffer B	50Mm Tris-Cl (pH7.4)

Table 10: Preparation of p815 whole membrane extract

4.16.1 Protocol

1. To the pellet of p815 cells add 200 µl buffer A and 20 µl of protease inhibitor (PI), make homogenous suspension.
2. Leave the suspension on ice for 30 min for incubation.
3. Centrifuge for 20 min at 21,000 rcf at 4ºC temperature.
4. Resuspend the pellet in 500 µl of buffer B.

5. Estimate the protein
6. Store at -20ºC.

5. RESULTS

5.1 Structures of NK inhibitory receptor Ly49A:

The crystal structures of Ly49A was not available on PDB, so the protein was modelled using a hybrid modeling server named Phyre[2]

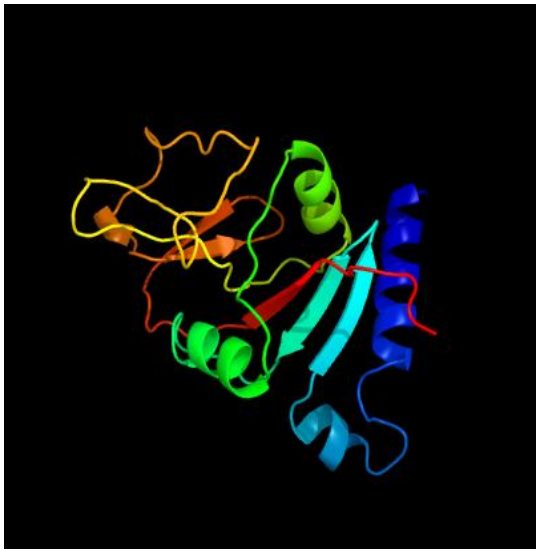


Fig 11: predicted structure of Ly49A.

5.2Predicted structures of ligands: CD63, galectin-3, p30, gp70 these are the p815 surface expressed molecules whose structure was not available on PDB so, the protein structures were modelled using a hybrid modeling server named Phyre[2]

Ligand name	Origin	Structure availability
CD63	Mus Musculus origin	Modelled using Phyre[2]
Galectin-3	Mus Musculus origin	Modelled using Phyre[2]
p30	Murine Leukemia Virus	Modelled using Phyre[2]
gp70	Murine Leukemia Virus	Modelled using Phyre[2]

Table11: p815 surface expressed ligands

Fig 12: Predicted structure of CD63

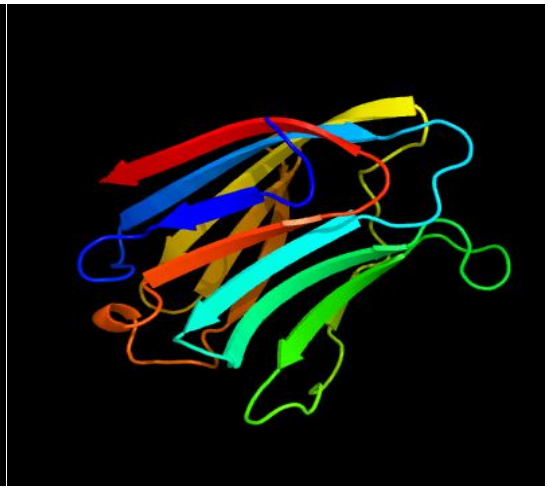


Fig 13: Predicted structure of Galectin-3

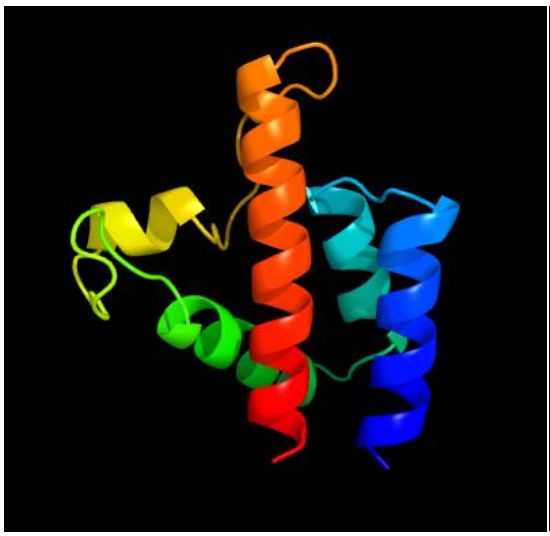


Fig 14: Predicted structure of p30

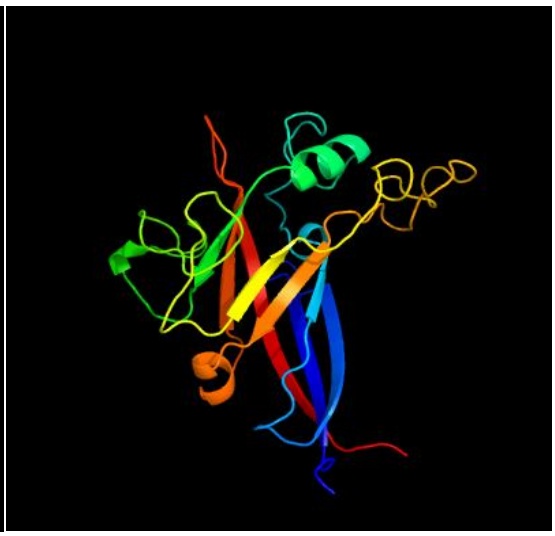


Fig 15: Predicted structure of gp70

5.3 Murine NK Inhibitory receptor interaction studied with surface ligands of p815

PATCHDOCK was used to perform molecular docking of murine inhibitory surface receptor Ly49A with the surface ligands of p815 (CD63, Galectin-3, p30, gp70). PATCHDOCK determines top 10 complexes and for each of the complex, the d-complex energy was calculated.

5.3.1Ly49A- CD63

The results obtained after the docking of receptor Ly49A with CD63 were observed. For each complex obtained, following data was observed.

Rank	Solution no.	Score	Area	D-complex (kcal/mol)
1	1	14340	2093.20	**-6.579559**
2	2	14216	2164.10	-4.585131
3	9	12984	2185.50	-4.631744
4	3	13956	1819.70	-5.695937
5	7	13122	2285.50	-4.700000
6	4	13836	1727.90	-5.003655
7	8	13106	2010.20	-5.607669
8	10	12824	1805.30	-3.485970
9	6	13400	2101.20	-1.997861
10	5	13614	2330.20	-4.003188

Table 12: D-complex energies for Ly49A-CD63

Here , the complex with minimum energy is is the first one with D-complex energy **-6.579559** Kcal/mol and the complex representing minimum enery is shown below

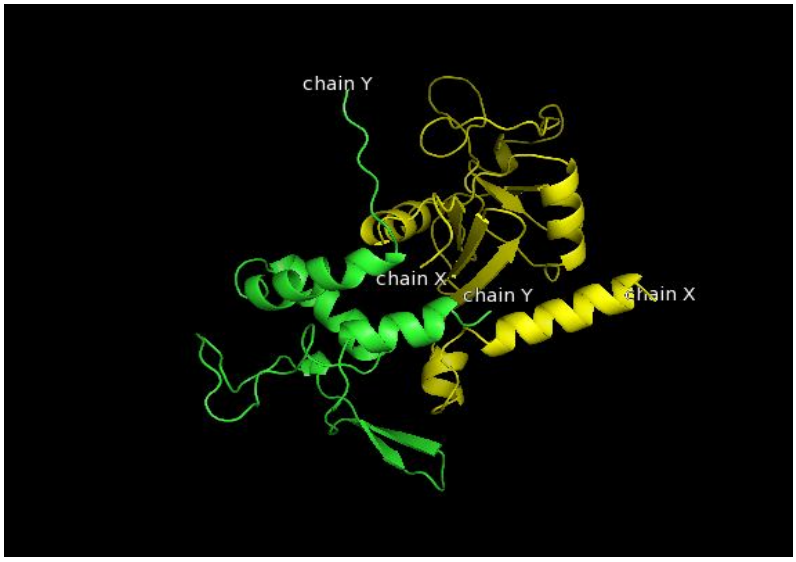


Fig16: Complex representing minimum energy for Ly49A-CD63

5.3.2 Ly49A-Galectin-3:

Rank	Solution no.	Score	Area	D-complex (kcal/mol)
1	9	11398	1536.50	-9.467794
2	6	11676	1734.10	-6.930770
3	7	11504	1633.90	111.539484
4	8	11450	1461.90	-8.500221
5	2	12374	1586.70	-5.118127
6	4	11804	1579.70	-6.278455
7	1	13150	2039.50	-7.149738
8	3	12266	1512.70	**-10.283447**
9	10	11382	1884.30	-4.452348
10	5	11758	1775.90	-4.745992

Table 13: D-complex energies for Ly49A-galectin-3

The results obtained after the docking of receptor Ly49A with Galectin-3 were observed. For each complex obtained, following data was observed.
Here, the complex with minimum energy is is the eigth one with D-complex energy-**10.283447**Kcal/mol and the complex representing minimum enery is shown below.

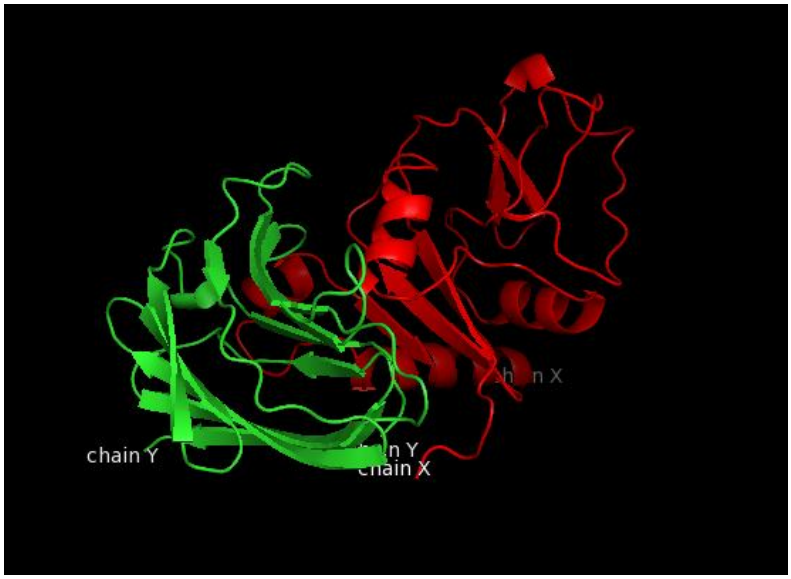


Fig17: Complex representing minimum energy for Ly49A-Galectin-3

5.3.3Ly49A-p30:

The results obtained after the docking of receptor Ly49A with p30 were observed. For each complex obtained, following data was observed.
Here, the complex with minimum energy is is the second one with D-complex energy-**11.074407** Kcal/mol and the complex representing minimum enery is shown below.

Rank	Solution no.	Score	Area	D-complex (kcal/mol)
1	7	11666	1424.20	-7.158205
2	2	12416	1508.90	**-11.074407**
3	8	11572	1458.00	-6.778593
4	6	11796	1786.60	24.503307
5	3	12182	2217.50	-2.055967
6	1	12634	2205.30	-4.072277
7	4	11902	1621.60	-4.700000
8	5	11842	1753.60	-4.519311
9	9	11508	1378.70	-5.943563
10	10	11504	1664.90	-4.636067

Table 14: D-complex energies for Ly49A-p30

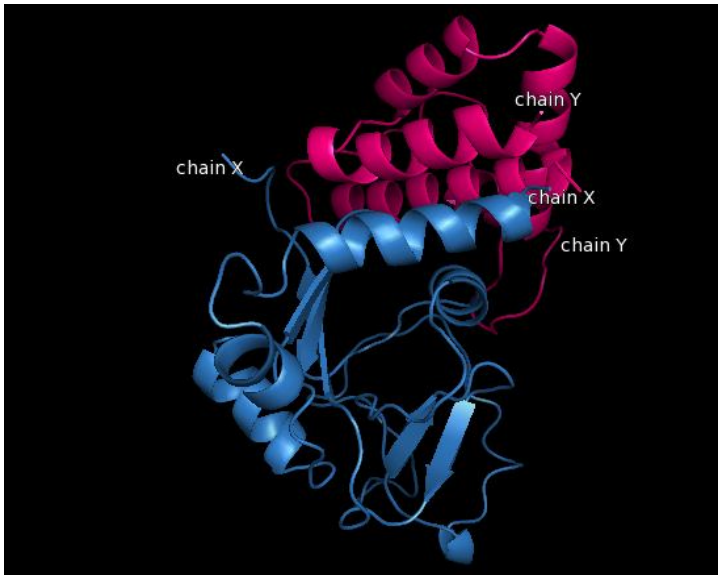


Fig 18: Complex representing minimum energy for Ly49A-p30

5.3.4Ly49A-gp70:

The results obtained after the docking of receptor Ly49A with gp70 were observed. For each complex obtained, following data was observed.

Rank	Solution no.	Score	Area	D-complex (kcal/mol)
1	4	13626	2245.00	**-11.879547**
2	1	14438	1899.30	-3.268851
3	8	13022	1804.90	-8.566955
4	2	13964	1872.50	-5.879165
5	7	13092	1835.20	-5.508979
6	6	13238	1989.30	-8.161250
7	5	13384	1838.60	-3.141820
8	9	12904	1721.20	-4.220493
9	3	13920	2097.80	-5.034545
10	10	12840	2833.60	9.995013

Table 15: D-complex energies for Ly49A-gp70

Here, the complex with minimum energy is is the first one with D-complex energy**-11.879547** Kcal/mol and the complex representing minimum enery is shown below.

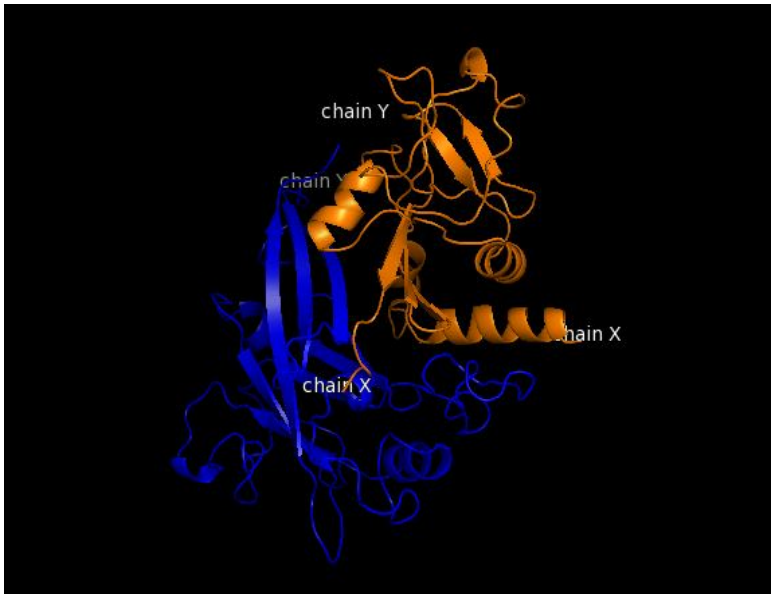


Fig 19: Complex representing minimum energy for Ly49A-gp70

To understand the interaction between Ly49A and the ligands(p30. Gp70, galectin-3, CD-63) present on the surface of p815 cell line, docking was performed and the D- complex energies for each receptor-ligand pair were studied. The result has shown significant interactions between them, so based upon these further experiments studies were carried out.

5.4Culturing of p815 cell line:

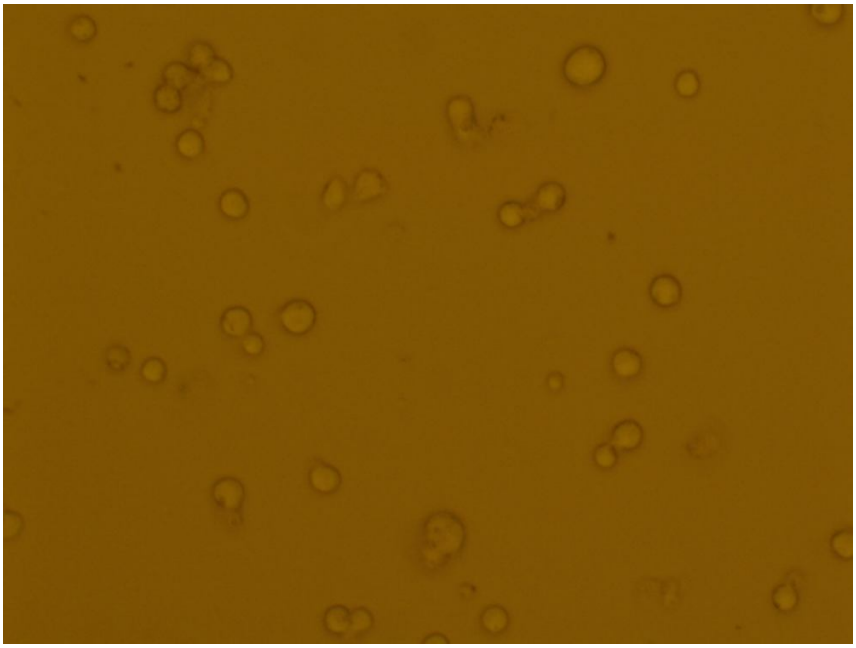


Fig 20: Microscopic view of p815 cells.

p815 cells were cultured in DMEM -10%FBS which were subcultured after every 2 days and cultures were maintained between 3-5×10^5 cells/ml.

5.5Generation of Growth Curve of NK cell resistant cell line –p815

Time (h)	No. of cells/ml **(× 10^6)** DMEM+10% FBS	No. of cells/ml **(× 10^6)** Starved (DMEM+**no** FBS)	Standard deviation (From three independent exp.)
12 h	0.25	0.97	0.50
24 h	0.14	5.40	3.71

Table16: Calculation of no. of cells at different time points

Graphical representation of growth of p815 was analyzed in 10% fetal bovine serum.

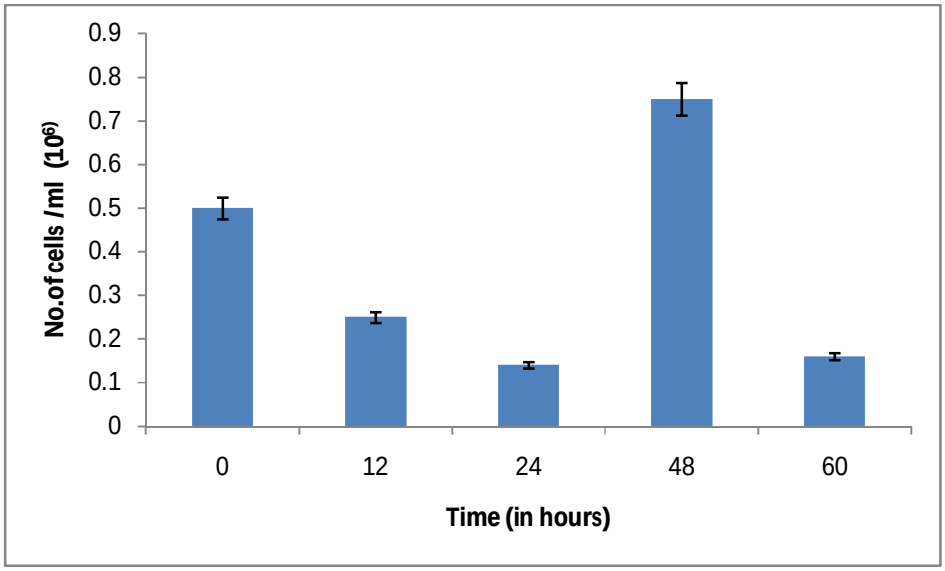


Figure 21: Bar graph of growth - p815(10% DMEM)

Growth curve was performed to show the optimum doubling time of the mastocytoma p815 cells.

5.6Characterization of tumor cell line (p815) to study the tumor derived factors that may induce NK cell modulation:

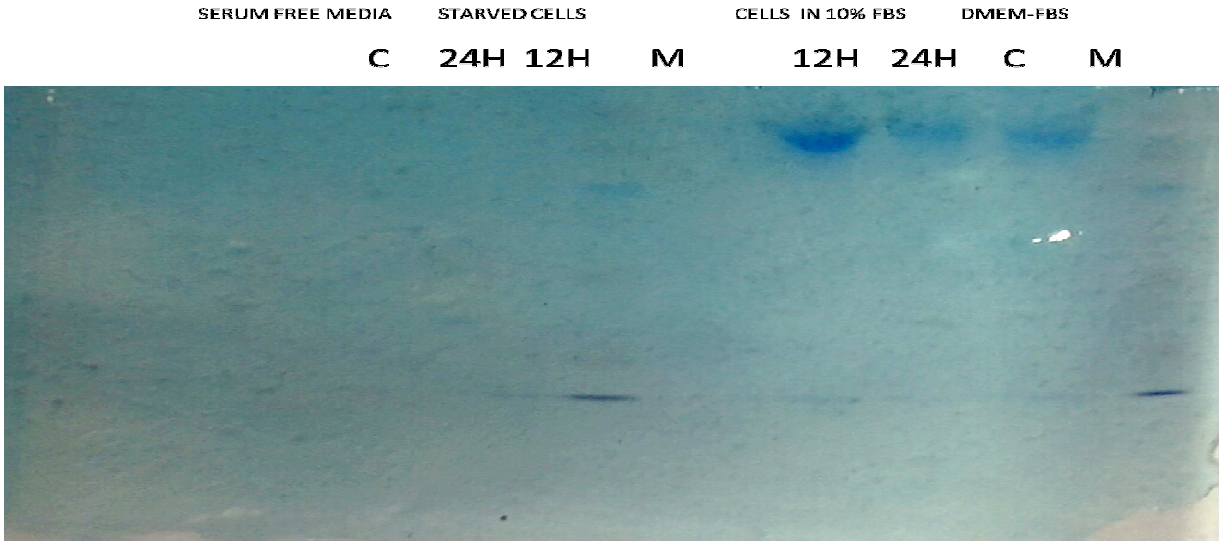


Fig 22: Fractionation of p815 supernatant on SDS-PAGE

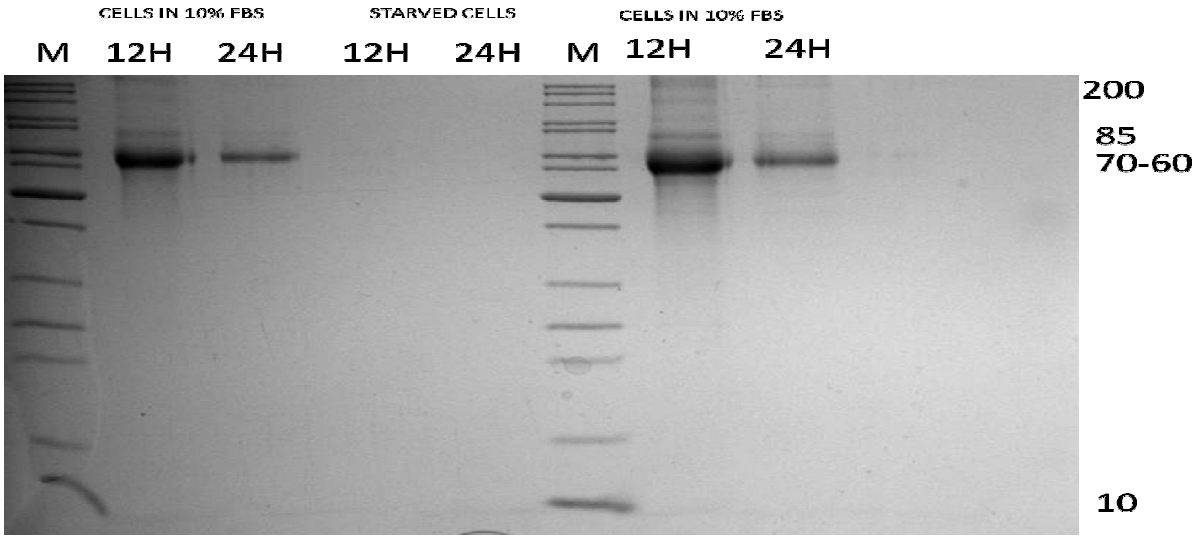


Fig 23: Fractionation of p815 supernatant on SDS-PAGE. A: Fractionation of supernatant in presence of fetal bovine serum (10%) at time interval of 12h, 24h. B: Fractionation of supernatant in absence of fetal bovine serum at time interval of 24h. Marker: BR biochem, Pink plus prestained protein ladder with MW 200KD to 10KD.

To determine the components secreted by p815, p815 cell line supernatant was fractionated on SDS-PAGE after incubation at different time interval in 10% serum concentration. No significant variation was observed in given particular interval of time and in comparison to cell free media (DMEM+ 10% FBS). So, supernatants were subjected to SDS-PAGE in serum free media. It was observed that no bands of p815 secreted protein components were seen in serum free media due to low protein concentration in supernatant as well as no bands were observed in cell free and serum free media (DMEM-FBS control) so, to resolve the bands p815 cell lysate which included all the secreted, membrane bound and intracellular proteins was subjected to SDS-PAGE.

5.7Characterization of p815 cell lysate on SDS-PAGE:

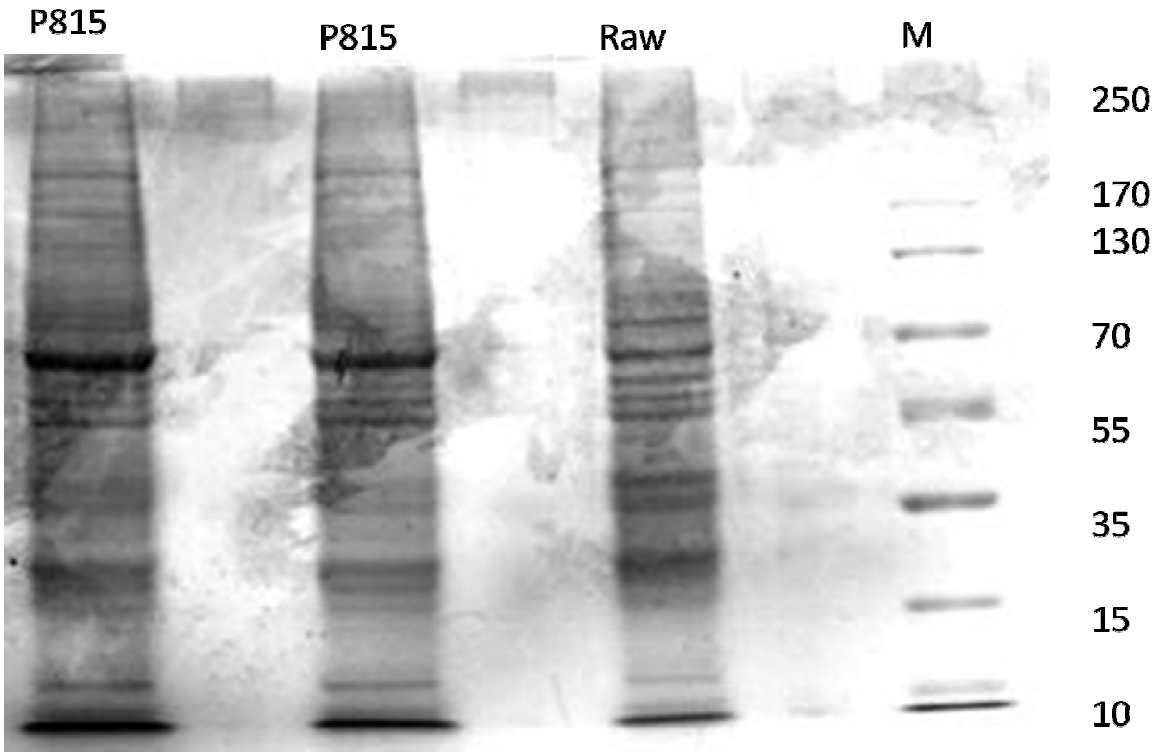


Fig 24: Characterization of p815 cell lysate

Further membrane bound proteins were studied using whole membrane proteins of p815 which were fractionated on SDS-PAGE to determine the intracellular and extracellular cell secreted proteins. Gel depicts multiple overlapping of bands which is further fractionated to see NK modulation.

5.8Characterization of p815 whole membrane protein on SDS-PAGE:

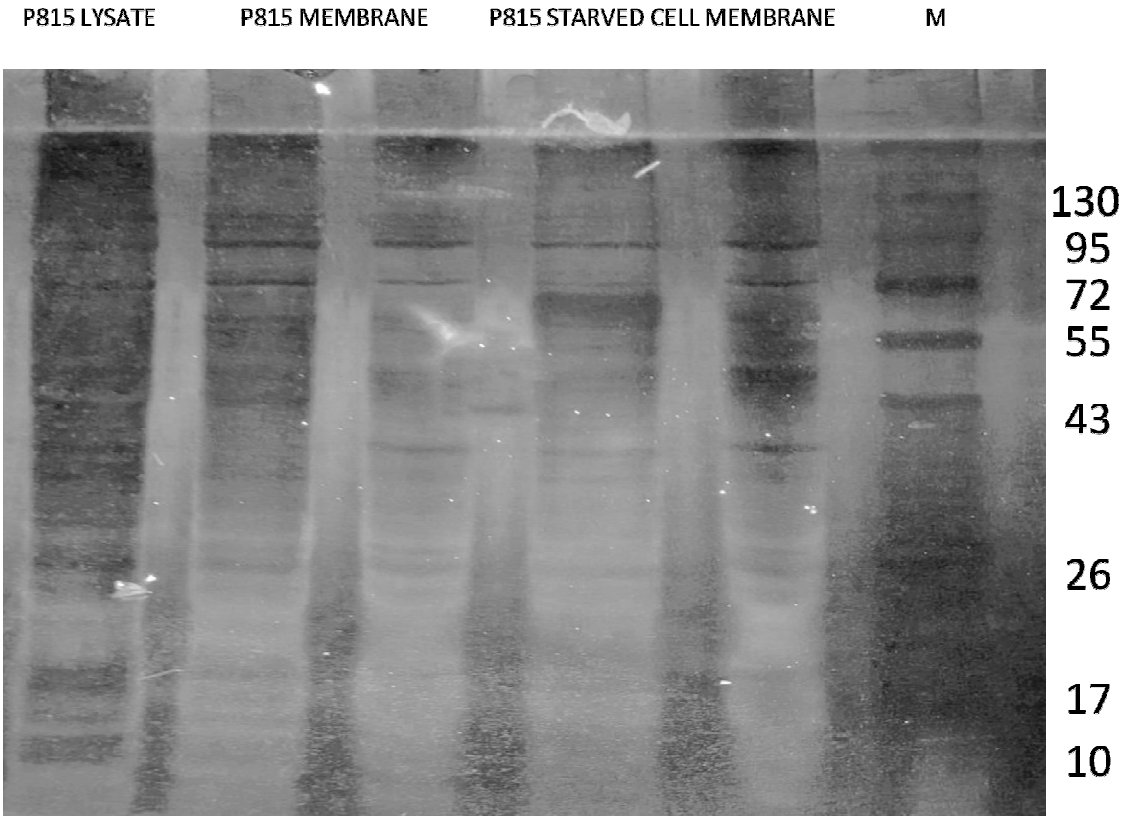


Fig 25: Characterization of p815 whole membrane protein on SDS-PAGE

Further p815 whole membrane was fractionated on SDS-PAGE to analyze different molecular weight cell surface proteins. And to study NK modulation activity, p815 cells were co-cultured with NK. NK receptor profiling has to be studied by co-culturing which has been performed in the lab(unpublished work of Richa Mishra). As per her results, there is a definite induction of inhibitory receptor expression in the presence of IL-2 stimulation upon co-culturing with p815 cells or p815 whole membrane.

6.CONCLUSION

Inhibitory receptor present on NK cell recognise MHC molecule and upon engagement with these receptors, block the ability of NK cells to attack the target cells, if the inhibitory ligand is not present in the target cell , NK cell will cause lysis of the target cell. In the present study, other surface expressed molecules present on p815 (CD63, Glectin-3, p30, gp70), other than MHC were also studied for binding with inhibitory receptors. The results obtained show that the binding affinities obtained with docking of murine inhibitory receptor Ly49A and the surface ligands of mastocytoma p815 were quite significant(CD63 with -6.579559 and galectin-3 having -10.283447, p30 with -11.074407 and gp70 having -11.879547 binding affinities) where this tumor is itself known to modulate NK by changing the receptor profile of it and hence, further increasing the inhibition. Multiple inhibitory receptors are being engaged by p815 cells and further studies need to be carried out whether these ligands of tumor resistant cell line cause inhibitory receptor profiling. This work can further be studied with other inhibitory receptors. Targets for inhibitory receptors which could be responsible for NK cell modulation can be determined by characterizing the cell membrane of tumor resistant cell line p815 after giving amicon cuts (30kDa, 50kDa, 100kDa) on SDS-PAGE to further evaluate the molecular masses of the active components that might be responsible for this enhanced inhibition, which would affect therapeutic vaccines. Similar work can be done with human targets also.

7.DISCUSSION AND FUTURE PROSPECTS

High levels of MHC class-I molecules are expressed by the p815 tumor resistant cell lines which fail to express stress- inducible ligands (Rae-1 and H60) for NK cell activating receptor NKG2D. On tumor rejection in mice NK cells express a transgene, the TCRP1A receptor which is specific for mouse tumor antigen P1A shown to be the major rejection antigen of p815. In our docking studies of mastocytoma p815 surface ligands (CD63, p30, gp70, galectin-3) and murine NK inhibitory receptor (Ly49A), D- complex energies for each receptor-ligand pair were studied where significant interaction was shown indicating the upregulation of inhibitory action of NK cells. Further, we tried to identify the active components present in p815 tumor resistant cells through determination of the components secreted by p815, p815 cell line supernatant was fractionated on SDS-PAGE (12% gel) after incubation at different time interval in 10% serum concentration. No significant variation was observed in given particular interval of time and in comparison to cell free media (DMEM+10% FBS). So, supernatants were subjected to SDS-PAGE in serum free media. It was observed that no bands of p815 secreted protein components were seen in serum free media due to low protein concentration in supernatant as well as no bands were observed in cell free and serum free media (DMEM-FBS control) so, to resolve the bands further we proceeded on to different method for the identification of different components using p815 cell lysate which was fractionated on SDS-PAGE with same gel concentration to determine the intracellular and extracellular cell secreted proteins. Gel depicts multiple overlapping of bands with molecular weights between 30-100KDa, where more overlapping was observed in regions of 70-85 KDa, which is further fractionated to see NK modulation. Further p815 whole membrane was fractionated on SDS-PAGE to analyze different molecular weight cell surface proteins. And to study NK modulation activity, p815 cells were co-cultured with NK cells. NK cell receptor profiling has to be studied by co-culturing which has been performed in the lab(unpublished work of Richa Mishra). As per her results, there is a definite induction of inhibitory receptor expression in the presence of IL-2 stimulation upon co-culturing with p815 cells or p815 whole membrane. Present work shows for the first time inhibitory receptor engagement by multiple ligands, thus contributing to a high intensity of inhibition by p815 cells which are known to be prototypic cells per resistance to NK lysis.

Targets for inhibitory receptor in modelled structure for resistant cell lines would affect therapeutic vaccines. Upregulation of activating receptors can be done using the mimiced ligands found in tumor susceptible cell lines as during tumor evasion from immune system, downregulation of activating receptors is observed which deteriorate NK cell cytotoxicity. Ligands or the components which are found responsible for the resistivity of tumors can be blocked using Mab's so that they no more interact with the inhibitory receptors on NK cells which can lead to tumor cell lysis efficiently. Also, the inhibitory receptors present on NK cells which are responsible for inhibiting NK activation towards tumor can be downregulated to generate negative signalling for tumor immunosurveillance.

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