1. ABSTRACT

There are various mechanism by which, tumors are able to escape from the immune attack of NK cells. The various mechanisms are related to the NK cell adhesion or activation interventions, triggered inhibition and NK cell modulation of effector functions through the interplay of huge pool of receptors on NK cell surface. NK cells are blessed with the innate ability to kill target cell. Thus, they have a major role to defend tumors as well as cells infected by viruses. The sensitivity of infected cell to NK cell lysis may open new prospectives for NK cell-based immunotherapy. Natural Killer cells have been known so far to act against many murine tumors used in experimentation, but in humans their antineoplastic attribute is not agreed upon always. A detailed concept of the mechanisms imposed by the tumor microenvironment in the modulation of cytotoxic immune cells is essential before approving their use in cancer therapies. The multifacet recognition pattern of tumor derived proteins by NK activating receptors and every accessible surface involved in this binding event should be explored. The knowledge of affinity of NK cell receptor with tumor ligands will help in understanding the binding patterns required for the activation of NK cell activity. The interactions between these tumor ligands with their activators are potentially addressable by computational approaches and can further help to develop NK based cancer therapeutic strategies. This thesis aims at investigating new factors released from respective NK sensitive cell line, and to further study the NK cell modulation that results from such factors that can be a potential targets of NK cell based therapy as well as also demonstrated the growth kinetics and growth pattern of the NK sensitive cell line. Various proteins factors were isolated from the supernatants, lysates and whole membrane preparation and separated using SDS-PAGE, which further need to be studied through NK receptor profiling.

2. INTRODUCTION

Rolf Kiessling and Ronald Heberman in the 1970s and 1980s investigated and initially characterised natural killer (NK) cells, by freshly isolating certain population of cells from normal hosts that are unimmunized, capable of lysing allogeneic tumor cells that are allogeneic prior any sensitization. With the help of modern techniques like flow cytometry, hybridoma technology, population of cells has been key out as NK cells, denoted as $CD56^+$ $CD3^-$, phenotypically. Currently, it is well known that they acts as killer and immunoregulatory cells releasing cytokines and chemokines. NK cells recognises self-MHC class I, that recently been recognized to be called as "licensing" or "education" for the acquisition of full function. These have large granular lymphocytes like morphology that are $CD34^+$ progenitor cells of the haematopoetic lineage derived.

Killing of the target cell can be directly brought about via antibody-dependent cellular cytotoxicity (ADCC), the perforin- granzyme pathway, and death receptor ligand-induced apoptosis. These ligands includes Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL).

Natural Killer cells of human origin can be divided into two subsets; $CD56_{bright}$ (10%) and $CD56_{dim}$ (90%). The $CD56_{bright}$ has the ability to produce plenty of cytokines, while the $CD56_{dim}$ have higher cytotoxic activity and possesses receptor, FcγRIIIa (CD16) of low affinity specific for the immunoglobulin G constant region.

NK cells are one of the arm of the innate immune system. NK cells are different from phagocytes (granulocytes and macrophages) that relies on conserved pattern-recognition receptors, like the toll-like receptors. Natural killer cells can distinguish between abnormal cells and normal healthy cells with the help of a vast repertoire of surface receptors that control their effector functions, proliferation, and activation. They do not require prior sensitization with an antigen and is capable of dealing with broad range of virus infected cells.

Natural killer cells lies in the subcategory of WBCs. Since 1975, NK cells have been regarded as lymphocytes on the basis of their morphology, presence of many lymphoid markers, and origin in the bone marrow from the lymphoid progenitor cell. They make up to 15% of blood lymphocytes which are found in peripheral tissues, like liver, peritoneal cavity and placenta. Bulk of NK cells are localized in lymph nodes, bone marrow, spleen, and peripheral blood (Ferlazzo, G.*et al*., 2004) but can be made to migrate to the inflammation sites by various chemo attractants (Robertson, M J., 2002). NK cells acts as the early defense against virus infection and in tumor immunosurveillance in humans and mice.

NK cells are involved in tumor immunosurveillance; this is known by their ability to recognize tumors that may have evaded T cell-mediated lysis by aberrant HLA expression. There is a significant correlation between increased survival and elevated levels of intratumoral NK cells that has been shown in different kinds of cancer.

2.1 NK CELL RECEPTORS

NK cells are able to distinguish between normal and transformed cells with the help of the vast repertoire of receptors expressed by NK cells on themselves. The various mechanisms are related to the NK cell adhesion or activation interventions, triggered inhibition and NK cell modulation of effector functions through the interplay of huge pool of receptors on NK cell surface.

There are two types of receptors

1. Activating receptors
2. Inhibitory receptors

The MHC class I receptors belong to the large family of inhibitory receptors that mediate their function by signaling through intracytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs). Thus, NK cells spare healthy cells that express self-MHC class I molecules and low amounts of stress-induced self molecules, whereas they selectively kill target cells “in distress” that down-regulate MHC class I molecules and/or up-regulate stress-induced self molecules such as NKG2D ligands.

Cancer is the result of loss of normal growth control. In normal tissues, cell growth and cell death is balanced. In cancer, this balance is disturbed. This disruption results from uncontrolled cell growth or loss of the ability of cell to undergo a process called "apoptosis" which is an orchestrated series of event by which old or damaged cells normally undergo self-destruction.

The steady rise in the number of multiplying cells gives rise to a mass of tissue called a "tumor" or "neoplasm." If there is no "suicide" signals and rate of cell division is comparatively rapid, the tumor will grow quickly in size. Tumor can be cancerous or non cancerous depending on whether they can spread by invasion and metastasis or not. Also, tumors can be benign or malignant.

2.2 TUMOR CELL LINES AND CELL CULTURE

Cell line is obtained , after the first successful subculture. Cell lines obtained from primary cultures have a finite life span and as they are passaged, cells with good capacity to grow predominate, thus they have phenotypic and genotypic uniformity in the population.

On the grounds of the life span of culture, these are grouped into two types:

1. **Finite cell Lines** - These have a limited life span and possess a restricted no. of generations of cells (usually 20-80 doublings of population) are called as Finite cell lines. These cell lines possess the property of anchorage dependence, density limitation and contact inhibition. They have a slower growth rate and an around 24-96 hours of doubling time.
2. **Continuous Cell Lines** - These Cell lines are transformed under in vitro conditions or laboratory conditions which gives rise to these cell lines. These exhibits the property of

ploidy, lacks contact inhibition and anchorage dependence. Cultured either as suspension or monolayer cultures. They have a rapid growth rate and 12-24 hours of doubling time.

3. **Monolayer cultures** – When there is a continuous layer of cells at the bottom of the culture vessel which usually have a thickness of one cell, they are termed as monolayer cultures. Bulk of continuous cell lines are cultured as monolayers.
4. **Suspension cultures** -Some cells are non-adherent, like those of leukemia or certain cells which can mechanically sustain in suspension, can be grown in suspension.

2.3 CELL CULTURE

Cell culture relates to the isolation of cells from any living organism (human or animal or plant), or they can be taken from a cell line or cell strain and they are subsequently propagated in a artificial, favorable environment & under strict sterile state. The cells are removed from the tissue directly and before cultivation by means of enzymatic or mechanical means, the cells are disaggregated.

2.4 PRIMARY CULTURE

Primary Culture relates to the stage of the culture after their removal from the tissue and their multiplication under the desired conditions until they reach confluency. At this level, the cells are passaged by transferring them to fresh growth medium for continued growth.

YAC-1

It is a murine T-lymphoma cell line susceptible to NK-cells, induced by Moloney sarcoma virus in A/Sn mice. The cells do not express Fc receptors. These are used to study the cytotoxic activities of NK, LAK cells, and other lymphocytes. These are lysed by NK-lysin.

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 posses the effector activity in ADCC. This cell line can be used for tumor-host relationship in in vivo experiments.

3. LITERATURE SURVEY

NK cells are formed from the hematopoietic stem cells (HSC) in the bone marrow (Colucci, F. *et al*., 2003). On the basis of phenotypic markers determined by flow cytometry, three stages of development of NK cell have been theorised: precursors, immature, and mature NK cells (Di Santo, J.P. 2006).
NK cells are classified into tissue-resident NKs (trNKs) and circulating conventional NKs (cNKs) (Sojka *et al.*, 2014). So far, much of the functional studies on NK cells have been focused on cNKs, which are abundantly present in the spleen.

It was found that animals lacking NK cells were more prone to develop spontaneous tumors and had higher growth rates of transplantable tumors in comparison to immunocompetent animals (Ostrand-Rosenberg, 2008). Also, there is a positive correlation between NK cell infiltration and survival in several cancer types (Ishigami, S.*et al*., 2000; Villegas, F.R. *et al*., 2002).

NK cells can be modulated on the basis of the microenvironment in which they live. Such modulations are associated with the growth factors, cytokines, or chemokines present in the microenvironment.

Liu *et al*. (2012) have revealed the significance of activated NK cells during tumor destruction by observing their capacity to root out large solid tumors. Elimination of tumors was dependent on the release of IL-15 by cancer cells, leading to highly proliferative, granular NK cell subset that augments killing in a perforin dependent manner (Liu, Engels *et al*., 2012). However, most studies in non-transgenic tumor models, have shown that NK cells do not eradicate established tumors. The difficulty lies in inducing NK cells in an immunosuppressive milieu where there are few activating cytokines – such as IL-15.

Recently, melanoma cells have been reported to express indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) that are found to down-regulate activating NK receptors that disable T/NK cell function. Clinical trials are on the way that are exploiting small molecules that interferes with IDO and PGE2.

3.1 NK CELL RECEPTOR

NK cell effector functions are regulated by several receptors. Karre's initially observed that NK cells are able to lyse cell that do not possess major histocompatibility complex (MHC),(Karre, K.*et al*., 1986) that led to missing-self hypothesis (Ljunggren, HG., Karre, K., 1990) that indicated the existence of negative regulatory receptors on NK cells that can interact with MHCs and thus spare target cell lysis by NK cells.
There are two types of receptors

1. Inhibitory receptors
2. Activating receptors

Several classes and alleles of inhibitory receptors(IR) for MHC are present in vertebrates [including the killer cell immunoglobulin-like receptor (KIR) family in human, Ly49 receptors in mice, CD94/NKG2A complex in both humans and mice] (Long, EO., 1999; Lanier, LL., 1998; Raulet, DH.*et al*., 2001).

IRs have immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domain which recruit SHP-1 and SHP-2 tyrosine phosphatases, upon interaction with MHC ligands, thereby decreasing activation signals (Colucci, F., 2002; Long, EO., 1999; Lanier, LL., 1998).

INHIBITORY RECEPTORS ON NK CELLS FOR MHC

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

Table-1: Referred James P. Di Santo. (2006); Cerwenka, A. and Lanier, LL. (2001)

IR, alone do not control NK cell regulation, instead work in cooperation with various activating receptors (AR) (Lanier, LL. 2005). In the past recent years, a plethora of AR expressed by NK cells have been identified, which includes the KIR-S family in humans, subset of Ly49 receptors in mice, and the NKG2D receptor and the CD94/NKG2C complex in both humans and mice (Lanier, LL. 2005; Moretta , A.*et al*., 2001; Raulet, DH. 2003; Yokoyama,WM.2003). AR requires the association of DAP10, DAP12, and SAP/Fyn adaptors proteins for signal transduction pathways (reviewed in Latour, S.*et al*. 2004; Lanier, LL. 2005; Moretta, A.*et al*., 2001; Colucci, F.*et al*., 2003; Vivier, E.*et al*.,2004). NK cells also perform antibody-dependent cell cytotoxicity (ADCC) through their cell surface receptor, FcγRIIIA (CD16) that are able to recognise antibody-coated cells. CD16 is associated with the FcRγ and CD3ζ signal transduction polypeptides that possesses immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains.

ACTIVATING RECEPTORS ON NK CELLS AND THEIR LIGANDS

Species	**Receptor**	**ligands**
Humans	NKp30	
Humans	KIR2DS	HLA-C, others?
Humans	NKp44	Influenza haemagglutinin, others?
Humans	CD25(IL-2Rα)	IL-2
Mouse	Ly49D	H-2Dd
Mouse	Ly49H	MCMV-induced(m157gp)
Human ,mouse	CD16(FcγRIII)	IgG

Human ,mouse	NKp46	Influenza haemagglutinin, others?
Human ,mouse	CD94/NKG2C	HLA-E /Qa-1
Human ,mouse	NKG2D	MICA, ULBP(H) RAE-1(H,M), H60(M), B,
Human ,mouse	CD244	CD48

Table-2: Referred James P. Di Santo. (2006); Cerwenka, A. and Lanier, LL. (2001)

3.2 BALANCE BETWEEN ACTIVATING AND INHIBITORY SIGNALS

Cytotoxicity of NK cell is regulated by a balance between inhibitory and activating signals. Initially, NK cells were found to be effector cells with the power to direct nonspecific killing of target cells. Klas Karre and group (1986) first proposed the "missing self" hypothesis. This hypothesis, says that inhibitory receptors (KIRs, killer cell immunoglobulin- like receptor) on NK cells possesses the specificity to recognize self-MHC class I molecules present on normal cells, and this causes the inhibition of their killing activity (Stern-Ginossar,N. Mandelboim,O. 2010).

In case of viral infection or transformation, the self-MHC class I present on normal cells are either altered or absent, then the inhibitory signal will decrease, leading to killing of the target cell. Recent studies have shown that inhibitory receptors also recognizes non-MHC-molecules, although the best discovered inhibitory receptors recognize MHC class I molecules (Stern-Ginossar, N., Mandelboim, O. 2010) .

For example, the NKG2D activating receptor that recognizes MICA/B and ULBP 1-5, which are the stress-induced self-proteins are upregulated in transformed or infected cells. This is called as "induced self". It is now well known that NK cells must exhibit specific activating signals as well as should lack inhibitory signals in order to display killing activity.

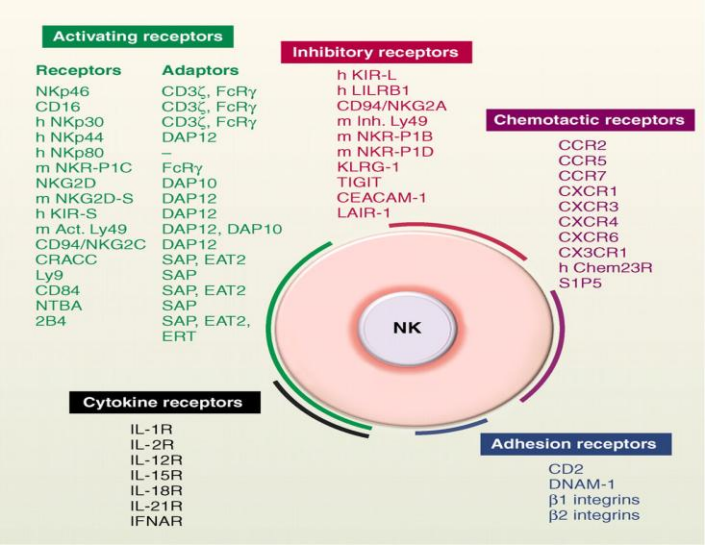

Figure-1: NK cell receptors. (adopted from Vivier, E.*et al*.2011)

3.3 NK CELL ACTIVATION AND CYTOTOXICITY

NK cell cytotoxicity is a highly regulated process. Regulation is brought about by NK cell adhesion to target cells (tumor, virus infected cells) and the interaction between NK cell

activating receptors and their specific ligands expressed on the surface of target cell. This leads to intracellular signaling pathways causing polarization and release of cytotoxic granules responsible for lysis of target cells. Many experimental systems are being used to study signaling resulting from receptor stimulation.

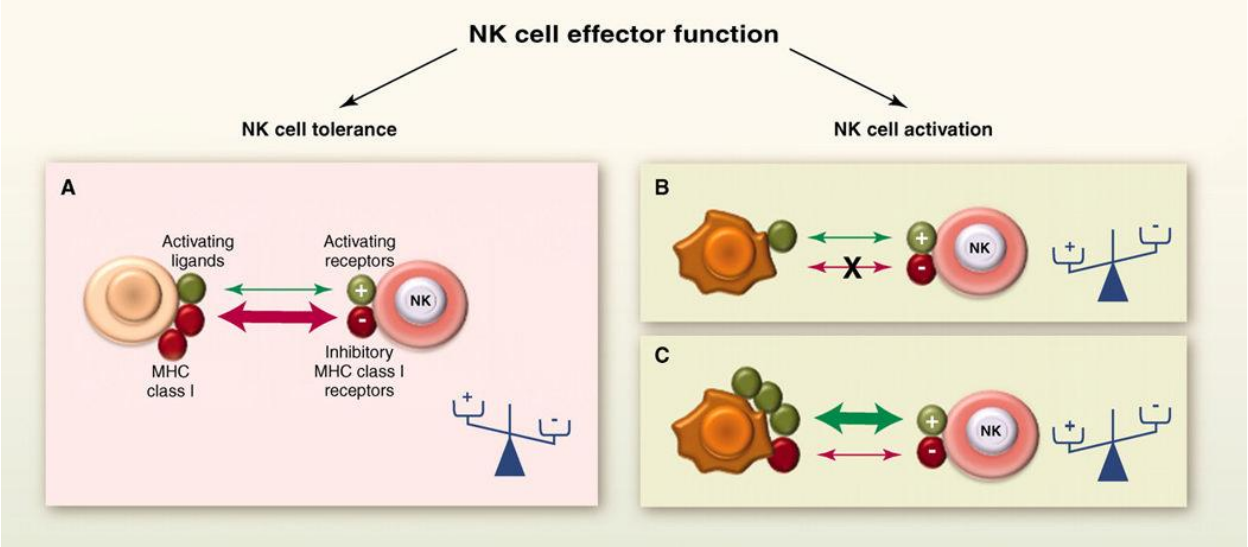

Figure-2: The regulation of NK cell effector function. (adopted from Vivier ,E.*et al*.2011)

3.4 NK CELL ACTIVATING RECEPTOR AND LIGANDS INTERACTION

The interaction between specific ligands on tumor cells and their NK surface receptors greatly contribute to the NK-mediated immune responses. It is quite evident that an activating signal must be produced when NK cells interact with target cells. This activating response can be readily discovered when target cells donot express HLA-class I molecules (Moretta, A. 2001). The NK activating receptors in the process of natural cytotoxicity had remained elusive for many years. Two groups identified three novel surface molecules for the first time, collectively known as natural cytotoxicity receptors (NCR) (Moretta, A.*et al*., 2000; Bottino, C.*et al*., 2004).

Ly49H- It was the first NK cell-activating receptor identified to be vital for viral control in vivo, which is essential to clear MCMV (murine cytomegalovirus) infection (Brown, MG.*et al*., 2001). Ly49H is a C-type lectin-like receptor that specifically recognizes the m157 expressed by MCMV. Two independent groups, identified this ligand using heterologous reporter cells exposed to MCMV-infected cells (Smith, HR.*et al*., 2002; Arase, H.*et al*., 2002). Ly49H activation by m157 is required for NK cell-mediated killing of MCMV-resistant mice. Also, m157 deletion causes viral escape and persist later in the infection. The Ly49 lectin-like receptors are not expressed in humans. The DAP12-associated receptor Ly49H is important for the clearance of MCMV. $Ly49H^{+}$ NK cells control MCMV multiplication by both direct cytotoxic mechanisms and by secretion of IFN-γ. (Loh, J.*et al*., 2005*;* Tay, CH.*et al*., 1997).

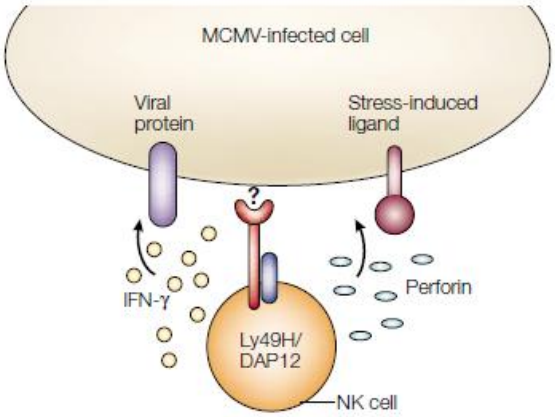

Figure-3: Ly49H and mouse cytomegalovirus (MCMV). (figure adopted from Adelheid Cerwenka and Lewis L. Lanier, 2001)

3.5 NATURAL CYTOTOXICITY RECEPTORS (NCRS) AND THEIR STILL SUBTLE CELLULAR LIGANDS

NKp46 (Sivori, S.*et al*., 1997; Pessino, A.*et al*., 1998) and NKp30 (Pende, D.*et al*., 1999) are present on both resting and activated NK cells, while NKp44 (Vitale, M.*et al*., 1998) is expressed upon NK cell activation. They are most specific NK cell markers as well as the highly important receptors involved in the NK cell-mediated cell lysis. The measure of cytolytic activity of NK cell has been shown to correlate with their surface density. They associate with different transmembrane-anchored polypeptides bearing immune tyrosine-based activating motifs (ITAM)(Bottino, C.*et al*., 2004). Although the ligands expressed by tumor cells are recognized by NCR are still unknown, different experiments have provided evidence about their pattern of expression in several different cell types. So, NCRs play a major role in the lysis of various tumors, including melanomas, myeloid, neuroblastomas, carcinomas, and multiple myeloma ,lymphoblastic leukemias,.

3.6 TUMORS, TUMOUR DERIVED FACTORS AND NK CELL RESPONSE

The evasion of tumors inspite of host immune defenses is by the production of soluble factors that affects the function of host cells involved in immune responses. So, different tumor-derived factors may affect the function of natural killer (NK) cells, macrophages and, lymphocytes or may raise the expansion of cells with down-regulatory activities. (Nelson, D.S.*et al*., 1987; Lopez, D.M., 1991).

Moreover, the release of factors in abnormal quantities by tumor-bearing hosts may change normal cytokine network and cause a harmful imbalance of the immune system (Arteaga, C.L.*et al*., 1993; Huang, M.*et al*., 1995). Reports have shown that the growth of the Ehrlich ascites tumor (EAT) causes the dysfunction of NK and T cell immunosurveillance, and the macrophages and suppressor cells are partly responsible for this suppressive effect (Parhar, R.S. *et al*., 1985; Subiza, J.L. *et al*., 1989; Ruiz de Morales, J.*et al*., 1999).

The interaction between immune cells and tumor cells within tumor microenvironment can be divided into three steps; recruitment, education and response. Tumor cells produce chemokines that enables them to recruit immune cells to the tumor microenvironment. Then, tumor cells polarize immune cells by secreting cytokines that regulate differentiation of immune cells, as well as develop immune tolerance. These differentiated immune cells produce cytokines, growth hormones, growth factors, and chemokines in the tumor microenvironment that promotes tumor growth and immune tolerance (Basith, S. *et al*., 2012).

Sub-populations of tumor cells overexpress various immune markers like immunoglobulin molecules (Ig), Toll-like receptors (TLR) and recombination activating genes 1 and 2 (RAG1 and RAG2), which are normally restricted to the immune system cells.

TLRs play a vital role in tumor progression and carcinogenesis in chronic inflammation that involves the tumor microenvironment. TLRs are expressed on infiltrating immune cells, tumor stromal cells and many types of cancer cells. Such TLRs are activated by PAMPs and DAMPs. DAMPs are derived from necrotic cancer cells and injured normal epithelial cells are present at significant levels in the tumor microenvironment (Sato,Y., 2009).

Tumor cells also secrete assembled Ig protein out of cell membrane. Through the culturing of several cancer cell lines, the existence of Igα heavy chain in the culture supernatant have been reported with the help of ELISA assay. Other evidences supported IgG secretion in the culture supernatants of uterine cervical cancer cell lines (Qiu, X.*et al*., 2003). These secreted IgG promotes growth and survival of these cells.

In 1977, Troy, FA.*et al*., detected and characterized Moloney leukemia virus-induced cell surface antigen, expressed on YAC-1 cells, using sodium dodecyl sulfate gel electrophoresis. MCSA was detected as a protein exposed at the surface which has distinct antigenic determinants and differ in size from the major envelope proteins (gp7l, p30, p15, p15(E), p12, and pl0) and core protein of Moloney leukemia virus and the H-2 antigens.

Many molecular weight entities expressing antigenic determinants for MCSA, H-2a, and gp7l were detected. This experimental approach has resolved the ambiguities regarding the structural relationship between known Moloney leukemia virion proteins membrane and antigens whose synthesis is directed by Moloney leukemia virus. The major peak of a viral envelope glycoprotein, gp71, lies in the region of Mr 68,000-78,000 and a minor crossreacting peak between 40,000 and 48,000 may represent gp45. The p30 (cell surface protein), was detected as a single minor peak in the expected region whereas H-2a antigen consisted of two peaks as previously noted for this particular cell line.

Roder, JC.*et al* (1978) identified Moloney cell surface antigen (MCSA), gp7l, p30, H-2, and NK-TS were localized in different fractions of gels. The reduced MCSA migrated as three molecular species in SDS-PAGE gels with apparent molecular weight values similar to the 50,000-60,000, 90,000,and 180,000-190,000 reported earlier by Troy, F. A.*et al*.

John C. Roder and group (1979), found that natural killer cell target structure (NK-TS), were not present on NK-insensitive targets viz., P815, A9HT, YWA, or EL-4. The NK-TS comprises of some or all of the four characteristic molecular entities, assigned molecular weights of 140kD,

160 kD, 190 kD, and 240 kD in SDS-PAGE gels. On comparing these, NK-TS molecules using cross-inhibition assays, the 240 kD molecule carried the unique NK specificity mostly. NK-TS modulation was also found to occur in a very different manner. YAC cells, when grown in vitro after removing them from the selective pressures of an NK-containing milieu in vivo, an expression of NK-TS were increased concomitantly and exhibited enhanced sensitivity to NK-mediated lysis.

In a study ,the immunomodulating activity of ascophyllan (a sulphated fucan preparation isolated from brown algae A.nodosum) was examined by injecting it intraperitoneally in mice, that enhanced the splenic NK cell activity against YAC-1 cells. It also increased the cytotoxic activity activity of mouse macrophage cell line, RAW264.7 cells (in vitro) towards YAC-1 cells in a concentration dependent manner.It is thought that multiple soluble factors that might be released by stimulated RAW264.7 cells is responsible for the inhibition of the growth of YAC-1 cells in the coculture system with RAW264.7 cells.Thus, it was found that the NO is the cytotoxic factor released from RAW264.7 cells that were a ascophyllan stimulated (Nakano, K.*et al*., 2012).

Unlike CTLs, target cell lysis brought about by NK effector cells is not restricted by MHC class I antigens (Herberman and Ortaldo, 1981).

A Sarin *et al*. (1995) investigated the relationship between the degree of MHC class 1 antigen expressed on tumour cells and their susceptibility to xenogenic and allogenic NK cells. MHC 1 antigen expression were augmented or induced on human K562 tumour cells and murine YAC using human and Mouse NK-RIF (natural killer-resistance inducing factor) preparations. So, the YAC cells became relatively resistant to cytolysis by murine NK but not to rat NK cells. In the same way, induction on K562 cells decreased their susceptibility to human NK but not to monkey NK cells. The outcomes, suggest that NK lysis may not be affected by augmented MHC expression on xenogenic targets. A soluble factor was used for upregulating the expression of class I MHC antigens on tumour cell lines. Such a potent agent was purified from the supernatants of activated mouse spleen cells or human PBMCs. The factor was NK-RIF, a 12 kDa protein, different from IL2 and IFN, which stimulates NK but not CTL resistance in several tumour cell lines (Saxena *et al.,* 1988, 1992).

In an study on F344 rats, it was shown that under various stressful conditions, the release of adrenal catecholamines and activation of β1- and β2-adrenoceptors can inhibit NK activity and enhances sensitivity to experimental metastasis of the MADB106 tumor line (Ben-Eliyahu *et al*., 1999, 2000; Stefanski, 1994).

Pietra and colleagues (2012) show that melanoma cells produce indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2), two natural immunosuppressants that down-regulate activating NK receptors. Interestingly, the activating NK receptors inhibited by IDO and PGE2 are functionally counteracted by NKG2A, an inhibitory receptor utilized by both T and NK cells. Like IDO and PGE2, the NKG2A inhibitory ligand HLA-E is expressed and functional in tumor cells, including melanoma and colorectal carcinoma as reported by Lo Monaco *et al*. (2011).

Despite the existence of different mechanisms by which NK cells may eliminate cancer cells, NK cell-based immunotherapy has resulted in limited clinical benefit (Sutlu *et al*., 2009). For example, transforming TGF- β, IL-10 and prostangladin E2 (PGE2) as well as the activity of the tryptophan metabolising enzyme indoleamine 2, 3-dioxygenase (IDO) may contribute to the establishment of immune tolerance within the tumor microenvironment. To date, limited information is available on the effect of melanoma cells on NK cell function.

According to one hypothesis, Gangliosides (sialic acid-containing glycosphingolipids) are found to shed by certain tumour cells, have shown immunomodulatory effects on the generation of antibody-synthesizing cells in vitro (Miller, HC. and Esselman, WJ., 1975).

Ladisch *et al*. (1983) directly tested the above hypothesis using a murine tumor system, the YAC-1 lymphoma. According to the findings, in vitro cultured YAC-1 cells synthesize gangliosides which inhibit mitogen- and antigen-induced lymphoproliferative responses; in vivo propagated YAC-1 cells and in the ascites fluid, similar gangliosides are also found. These results supported the above hypothesis that synthesis of gangliosides may serve to protect tumor cells from host immune destruction. In a comprehensive study of shedding to identify the natural forms of shed gangliosides by Kong, Y., Ladisch, S.(1983) it was confirmed that gangliosides are shedded by the tumour cells exist in their three natural forms: membrane vesicles, micelles, and monomers.

Chiba, S.*et al* (2014) showed that the NK-mediated lysis of tumor cells that express N-glycan structures [tumor-associated molecular patterns (TAMPs)] at high levels is critically brought about by innate immune receptor Dectin-1 expressed on dendritic cells and macrophages.

For this study, the lung carcinoma 3LL and YAC-1 cells with strong sDectin-1 binding and colon carcinoma SL4 cells with weak binding, were selected respectively and subjected them to the in vitro killing assay.

These results support the notion that Dectin-1 ligands expressed on tumor cells at high levels are more sensitive to NK cell lysis, in a manner dependent on Dectin-1 signaling. On the basis of the chemical cross-linking data and MS, for the Dectin-1 recognition, the N-glycan structures need to be bound to proteins.

Yogeeswaran, G. *et al*. (1981) quantitated glycosphingolipids and sialoglycoconjugates in a number of variants derived from the YAC-1 lymphoma, sensitive to natural killer (NK) cell lysis. A strong positive correlation between chromatographic migration of asialo-GM2 with the total cell neutral glycolipid and susceptibility to endogenous or activated NK-cell-mediated lysis were came to know on analysis of glycosphingolipids among the variants. Significant correlations were not found with any other neutral glycolipids. The ratio of asialo-GM2 to GM2 had a highly significant positive correlation with sensitivity. These results suggest that the above findings could be involved in lytic events or binding NK cell:target cell interactions, and moreover high degree of sialic acid and sialylation on the surface might be responsible for such interactions. Such studies with these YAC variants could be useful for examining the biochemical bases of target cell-NK cell interactions.

Research shows that microvesicles carry a vast array of molecular information such as a variety of biologically active proteins, oncogenic receptors and pieces of RNA material (Al-Nedawi *et al*., 2009).

3.7 OTHER TUMOR DERIVED FACTORS

Tumor cells forms a complex "society" and possess genetic heterogeneity termed as "tumor microenvironment". The tumor microenvironment, contains various non-transformed cells such as endothelial cells, fibroblasts, and inflammatory cells along with extracellular matrix components that are densely packed and in communication each other and with tumor cells.

A consistent supply of myelomonocytic cells is required by the tumor cells, for the process of the stroma remodeling and angiogenesis required for their growth, which is mediated by tumor-derived factors (TDFs), which is needed for sustained myelopoiesis as well as the recruitment of myelomonocytic cells, most of which are macrophages, at the tumor site.

Tumors develops ways to escape immune surveillance by an action called immune editing, which gives a selective edge in the TME that leads to malignant progression. Numerous tumor-derived factors causes the occurrence of complex regional and local immunosuppressive networks, which includes interleukin-10, prostaglandin E2, vascular endothelial growth factor, soluble phosphatidylserine , and transforming growth factor-B, soluble Fas, soluble Fas ligand. These secreted factors could pass the immunosuppressive effects into the spleen and local lymph nodes, promoting metastasis and invasion.

Using clinical samples and mouse models, the molecular mechanisms through which cancer cells escape the host immune system have been investigated. Many important mechanisms have been reported, such as loss of tumor antigen (Jager, D. *et al*. 2001), alteration of HLA class I antigen (Algarra, I. *et al.*, 2004), defective death receptor signaling (French, LE.*et al*., 2002), lack of costimulation (Byrne, SN.*et al*., 2003), immunosuppressive cytokines (Toutirais, O.*et al*., 2003), and immunosuppressive T cells (Somasundaram, R.*et al*., 2002).

Transforming growth factor-β (TGF-β) and interleukin (IL)-10, are the immunosuppressive cytokines that are secreted from tumor cells and supresses the maturation of dendritic cells and function of T-cell through regulatory T cells (Berger, CL.*et al*., 2005). Apart from tumor-derived cytokines, prostaglandin E2 (PGE2) hampers T cell function as well as that of IL-10 and TGF-β (He,X., Stuart,JM., 1999).

Vascular endothelial growth factor (VEGF) is one of the important tumor-derived soluble factor (TDSF), which produces iMC (immature myeloid cells) from the bone marrow, by acting as a chemoattractant, then through the peripheral circulation they are recruited at the primary tumor site in a mouse model (Gabrilovich, D.*et al*., 1998).

Other TDSFs, like soluble MHC class I–related chain A gene (sMICA) products and soluble Fas ligand (sFasL), also play critical roles in immune escape, which inhibit NKG2D- and Fas-mediated killing of immune cells (Webb, SD.*et al*., 2002; Doubrovina, ES.*et al*., 2003).

Soluble phosphatidylserine (sPS), is another TDSF that are capable of inducing an anti-inflammatory behavior of TAMs, that results in the release of anti-inflammatory molecules, like TGF-β, IL-10, and PGE-2, that inhibits DCs and T cells immune response(Kim, R.*et al*., 2005).

During the anti-inflammatory response, the activation of NK cells and CTLs are inhibited as the TGF-β secreted from TAMs prevents antigen-presenting DCs from maturing (Bondanza, A.*et al*., 2004).

sFas derived from tumour cells interact with present on CTLs, through which Fas-mediated apoptosis is prevented. Also the altered FasL expressed by tumor cells is able to interact with CTLs, which causes their apoptosis (Igney, FH.*et al*., 2005). This mechanism induces activation of FasL-mediated -cell death of CTLs (Li, JH. *et al*., 2002).Thus, the soluble factor, sFas and the altered expressions of FasL, and sFasL, so the tumor cells escapes from killing by CTLs that leads to their immune evasion.

NK cells, through NKG2D-mediated apoptosis play a vital role in antitumor immunity (Smyth, MJ.*et al*., 2005). Regulation of this pathway occurs through the interaction between ligand, MICA expressed on tumor cells and the NKG2D receptor on NK and CD8+ T cells. Tumor cells derived soluble form of MICA (sMICA), intervene with the NKG2D/MICA interaction between NK and tumor cells and inhibit NKG2D-mediated apoptosis (Holdenrieder, S.*et al*. 2006). This internalizes with the NKG2D receptor, leading to the down-regulation of the NKG2D expression, thus hampering the NK-mediated killing of MICA+ tumor cells (Raffaghello, L.*et al*., 2004).

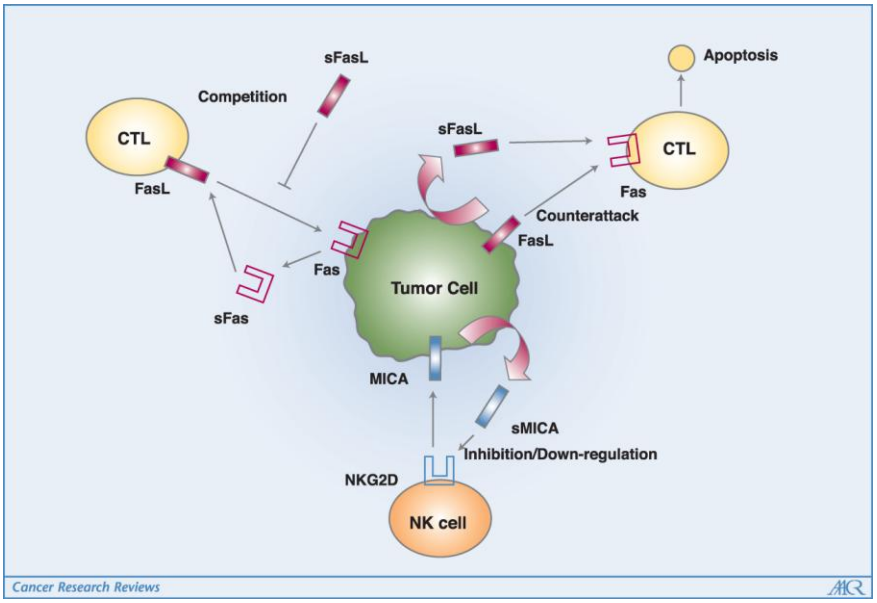

Figure-4: Possible roles of sFas, sFasL, and sMICA in promoting immune escape.
(adopted from Kim, R.*et al*., 2006)

Various findings have shown that tumor-derived factors (TDFs) boosts myeloid suppressor cells (MSCs) recruitment and maturation causing immunosuppressive effects. Gabrilovich, DI.*et al* in 1996 have shown that conditioned media obtained from tumor cell lines can inhibit the in vitro differentiation of DC. For over 10 years, efforts are being done to identify the TDFs. A large amount of chemokines, cytokines or other molecules that, induce MSCs recruitment causing their maturation into suppressive cells.

The release of CSF-1, also known as colony stimulating factor, is being described in various cancers. CSF-1 recruits immunosuppressive macrophages and changes the normal maturation of DCs (Lin, EY.*et al*., 2002).

During the later stages in the growth of tumor, its expression is linked with an increase in the amounts of acute-phase proteins, IL-6, and IL-1 (Trikha, M.*et al*., 2003). IL-6 produces both anti-inflammatory and pro-inflammatory effects in the immune system. Also IL-6 affects the differentiation of macrophages and DCs, both in vivo and in vitro (Park, SJ.*et al*., 2004) by activating the transcription factor STAT3.

GM-CSF is also released by several murine cells lines like squamous cells carcinoma(Smith, CW.*et al*., 1998), mammary and colon adenocarcinoma (Bronte,V.*et al*., 1999) and plasmacytoma (Merchav, S.*et al*., 1987). Out of 75 tested human tumor cells lines, almost 31% secreted this cytokine (Bronte,V., 1999). Tumor-derived GM-CSF suppresses CD8+ T cells that are antigen-specific and recruits MSCs in the secondary lymphoid organs (Bronte,V.*et al*., 1999)..

Arginase (ARG) can be expressed or released either by tumor cells or tumor-associated myeloid cells, also putative MSCs (Bronte, V.*et al*., 2003; Rodriguez, PC.*et al*., 2004). ARG activity is seen in the serum and TMI of patients with colorectal carcinoma , breast , skin , and prostate cancer. ARG convert L-Arg into L-ornithine, an important molecule for the formation of polyamines, substance essential for tumor proliferation and cell transformation (Medina, MA.*et al*., 1999).

Nitric oxide synthase (NOS) activity has been found in tumor cells of different origins and has been linked with the proliferation index and tumor grade (Xu, W.*et al*., 2002), involved in promoting or inhibiting cancer.

Galectins are secreted by tumours and, can modulate many of the inflammatory responses (Rabinovich, G. A.*et al*., 2004; Liu, F.*et al*., 2000). Galectins belongs to a family of animal lectins that binds to various glycoproteins and glycolipids or β-galactosides in extracellular matrices or on the cell surface. Galectins amplifies the inflammatory response, stops immune effector functions (Rabinovich, G. A. *et al.*, 2002*)*. Recent data have shown that some galectins secreted by the tumor might cause the tumor evasion from the immune surveillance. Galectin inhibits T-cell activation (Chung, C. D.*et al*., 2000) induces apoptosis of activated T cells (Blaser, C. *et al.* 1998; He, J.*et al*., 2004), and inhibits the release of proinflammatory cytokines (Rabinovich, G.A. *et al., 1999*).

4. MATERIALS AND METHODS

4.1 RECEPTOR MODELING :

Ly49H: A 265 amino acid sequence of Ly49H of mouse origin was retrieved from NCBI (accession no. = AAR03586.1). Since the crystal structure of Ly49H was not available, the protein was modeled using a hybrid modeling server named Phyre[2] (Soding, J.2005). The platform incorporates ab initio folding simulation called Poing[2] to model regions of proteins with no detectable homology to the known structure (Jefferys *et al.,* 2010).

Other activating receptor of mouse origin, Ly49D donot have a sequence available on NCBI. So, the receptor of interest chosen is Ly49H.

4.2 LIGAND PREPARATION :

We have taken ligands which are derived from YAC-1 cell line, viz., p30CA, p12, p15MA, p15E.

The following information about p30CA (Accession no.= NP_955585.1, 263 amino acids, of Moloney murine leukemia virus origin), p12 (Accession no.= 0804277A, 84 amino acids, of

Moloney murine leukemia virus origin), p15MA (Accession no.= NP_955583.1, 130 amino acids, of Moloney murine leukemia virus origin), p15E (Accession no.= NP_955589.1, 196 amino acids, of Moloney murine leukemia virus origin) were retrieved from NCBI. The crystallized structure of above mentioned ligands were not available in RCSB Protein Data Bank (Berman *et al.*,2000), so the structure of these ligands were modeled using a hybrid modeling server named Phyre^2 (Soding , J.2005). The platform incorporates ab initio folding simulation called Poing^2 to model regions of proteins with no detectable homology to the known structure (Jefferys *et al.*,2010).

4.3 MOLECULAR DOCKING USING PATCHDOCK (AN AUTOMATIC SERVER FOR MOLECULAR DOCKING):

PatchDock is an algorithm used for molecular docking. Here, the input is two molecules of any type eg.,proteins, DNA, drugs. The output obtained is a list of potential complexes that are sorted by the 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 divided into patches depending upon the surface shape. These patches corresponds to the patterns that distinguish between the puzzle pieces. Once these patches are identified, then they can be superimposed using the shape matching algorithms.

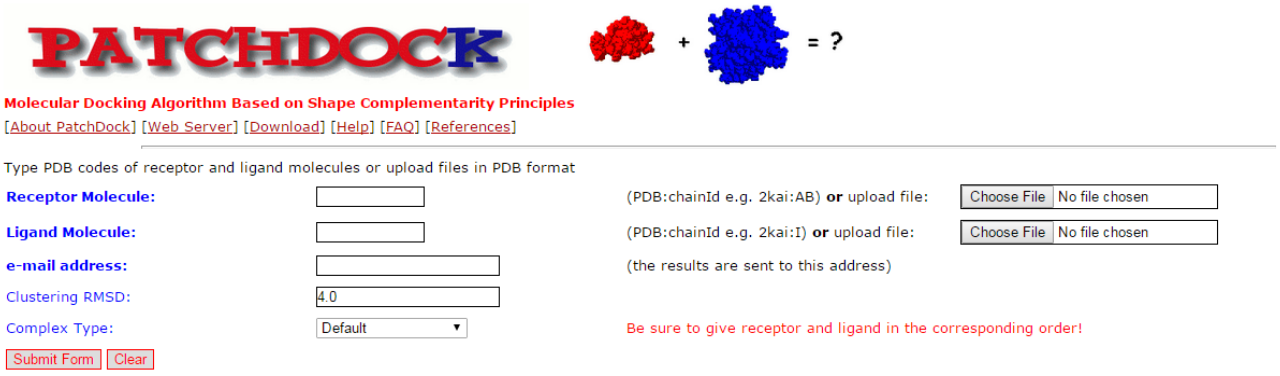

Figure- 5 : Screenshot of the PATCHDOCK server

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.

Molecular Docking Algorithm Based on Shape Complementarity Principles

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

Receptor	Ligand	Complex Type	Clustering RMSD	User e-mail	Receptor Site	Ligand Site	Distance Constraints
ly49h.pdb	p15E.pdb	Default	4.0	aqua_sds@yahoo.com	-	-	-

Solution No	Score	Area	ACE	Transformation	PDB file of the complex
1	14584	1805.30	23.28	1.10 -0.19 2.91 -15.93 24.82 30.83	result.1.pdb
2	13428	1888.20	268.53	-1.45 1.13 -0.75 -10.85 39.97 17.60	result.2.pdb
3	13386	1682.20	-87.59	1.30 -0.28 2.17 17.05 14.09 47.47	result.3.pdb
4	13288	1675.50	-235.71	-2.84 0.25 -2.49 -27.91 -11.96 8.74	result.4.pdb
5	13094	1535.10	378.01	1.60 0.65 1.13 -25.55 -17.85 33.95	result.5.pdb
6	13076	1646.50	124.98	1.02 0.02 2.99 -14.64 16.49 26.78	result.6.pdb
7	13020	1616.00	-116.89	1.78 0.04 1.25 -7.27 -18.35 34.65	result.7.pdb
8	13004	1881.30	-205.26	0.97 -0.18 2.64 -13.28 26.38 33.55	result.8.pdb
9	12956	1633.40	-79.23	1.06 -0.01 2.78 -15.84 21.71 29.61	result.9.pdb
10	12906	2309.30	348.80	-2.94 0.42 -3.03 -8.15 8.96 7.12	result.10.pdb
11	12834	1680.20	-126.12	2.01 -0.44 0.34 -16.73 5.56 22.00	result.11.pdb
12	12816	1653.00	78.65	1.42 -0.95 -1.13 -43.68 2.54 27.41	result.12.pdb
13	12774	2231.00	-363.11	1.10 0.15 2.17 -14.03 20.85 30.48	result.13.pdb
14	12730	1913.20	-560.11	-2.17 -0.53 -1.93 -12.95 0.34 29.20	result.14.pdb
15	12728	1791.20	-111.53	1.73 -0.58 -0.02 -30.46 7.41 27.18	result.15.pdb
16	12554	1893.60	152.14	1.68 -1.24 1.30 0.07 -13.65 23.35	result.16.pdb
17	12496	1773.80	480.52	-0.31 0.77 -2.33 13.25 16.79 38.00	result.17.pdb
18	12480	2180.30	3.31	0.91 0.14 2.95 14.67 27.77 33.08	result.18.pdb
19	12458	1733.90	35.27	1.16 0.33 2.01 -15.21 22.44 30.07	result.19.pdb
20	12442	1855.90	-367.55	-2.70 -0.05 3.00 -27.87 -3.98 28.85	result.20.pdb

show next 20 >>

Figure-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.

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 along with 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	TransFile
ly49h.pdb	p15E.pdb	fd_trans.txt

Rank	Solution Number	Global Energy ↓	Attractive VdW	Repulsive VdW	ACE	HB	Structure show/hide
1	8	-11.92	-21.68	9.82	2.29	-2.16	☑
2	4	-8.47	-34.17	58.68	-1.56	-2.92	☐
3	7	-6.06	-7.68	5.44	-4.66	-0.57	☐
4	9	7.62	-2.54	1.53	2.06	-0.89	☐
5	3	11.36	-18.24	6.35	0.77	-1.75	☐
6	2	17.94	-10.01	5.33	8.27	0.00	☐
7	6	19.74	-10.58	5.15	4.07	-0.25	☐
8	1	23.62	-35.11	42.45	3.07	-1.89	☐
9	5	24.89	-30.24	16.98	14.02	-2.18	☐
10	10	8224.13	-64.07	10356.00	7.72	-12.47	☐

Figure-7: Screenshot of results obtained with FireDock

This results into the solutions with hydrogen bonds ,the D COMPLEX predicts the binding affinity of the protein complex , determining the energy in kcal/mol.

4.5 TUMOR CELL LINES - YAC-1 (NK-SENSITIVE TUMOR CELL LINE) CELL LINE FEATURES

Organism	*Mus musculus*, mouse
Cell Type	Moloney murine leukemia virus (Mo-MuLV) induced
Product Format	Frozen
Morphology	Lymphoblast
Culture Properties	Suspension
Disease	Lymphoma
Strain	A/Sn
Applications	This cell line can be used to assay of NK cell cytotoxicity.
Storage Conditions	liquid nitrogen vapor phase
Complete Growth Medium	The base medium for this cell line is ATCC-formulated RPMI-1640 Medium. To make the complete growth medium, add fetal bovine serum to a final concentration of 10%.
Subculturing	Cultures can be maintained by addition or replacement of fresh medium. Start cultures at 3 X 10^5 cells/mL and maintain between

	2 X 10^5 and 2 X 10^6 cells/mL. Medium Renewal: Every 2 to 3 days
Cryopreservation	Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO Storage temperature: liquid nitrogen vapor phase
Culture Conditions	Atmosphere: air, 95%; carbon dioxide (CO_2), 5% Temperature: 37^0C.

Table-3: Showing YAC-1 cell line features.

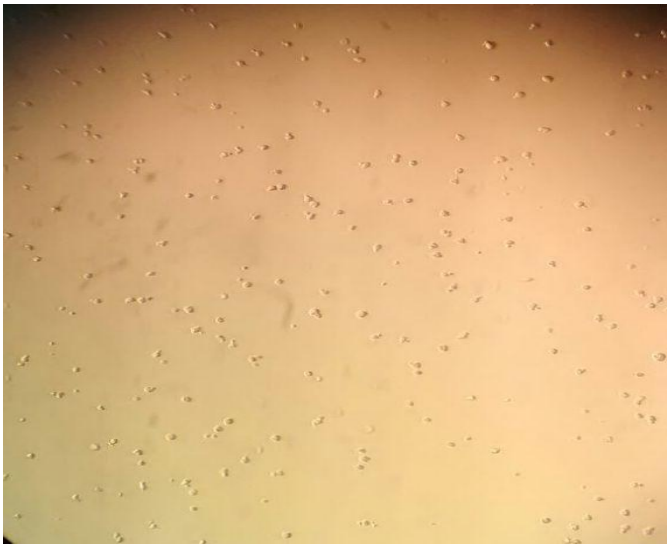

Figure- 8: YAC-1 cells observed under phase contrast microscope.

4.6 CULTURE MEDIA

a) RPMI – 1640

L-glutamine	2 Mm
HEPES	10 mM
sodium pyruvate	1 mM
Glucose	4500 mg/L
sodium bicarbonate	1500 mg/L

Table-4: Showing details of culture media

Figure- 9: RPMI media used for culturing of YAC-1 cells.

RPMI (Roswell Park Memorial Institute Medium) 1640 medium (Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal calf serum (FCS), was used throughout the study.

b) 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.

4.7 SDS-PAGE ELECTROPHORESIS (LAEMMLI'S METHOD, 1970) BUFFERS

- 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

- Known markers

4.7.1 STOCK SOLUTIONS

4.7.1.1 Solution A- 30% Acrylamide solution

29.2g acrylamide
0.8g bis-acrylamide
Add water to makeup volume 100ml.

4.7.1.2 Solution B- 1.5 M Tris buffer (pH-8)

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

4.7.1.3 Solution C- 0.5 M Tris buffer(pH-6.8)

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

4.7.1.4 Solution D- 10% APS

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

4.7.2 COMPOSITION OF GEL SOLUTION FOR A 1MM THICK GEL

	Separating gel (12.5%) in ml	Separating gel (10%) in ml	Concentrating gel (4.5%)in ml
Solution A	7.5	7	0.9
Solution B	4.5	5	-
Solution C	-	-	1.5
Solution D	0.07	0.100	0.018
TEMED	0.01	0.015	0.01
Water	6	8	3.6

Table- 5: Composition of gel solution for a 1mm thick gel

4.7.3 Stock solution-10% SDS solution

10g of SDS in 100 ml water

4.7.4 Sample solubilizing buffer (SSB) (1X)

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

Table-6: Showing composition of sample solubilising buffer

For 10 ml

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

Table-7: Showing composition for 10ml solution

Tris – Cl (pH6.8)

Stock (0.1M) = 100ml

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

4.7.5 Electrophoretic buffer

Tris	0.025M
Glycine	0.192M
SDS	0.1%

Table-8: Showing 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.

4.7.6 Staining solution

CBB	2.5g
Methanol	500ml
Acetic acid	100ml

Table-9: Showing composition of staining solution

Make volume upto 1000 ml

4.7.7 Destaining solution

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

4.7.8 Silver staining reagents (for 300ml)

Solution ι (fixer)

Methanol	120 ml
Acetic acid	36 ml
Formalin(37%)	150 ml
Water	144 ml

Table-10. Showing composition of solution I for silver staining.

Solution ιι (50% ethanol) (wash)

Ethanol	150 ml
Water	150 ml

Table-11. Showing composition of solution II for silver staining.

Solution ιιι (sensitising)

Sodium thiosulphate	0.6 g

Table-12. Showing composition of solution III for silver staining.

Solution iv (silver nitrate solution)

Silver nitrate	0.6 g
Formalin	225 ul

Table-13. Showing composition of solution IV for silver staining.

Solution v (developer solution)

Sodium carbonate	18 g
Sodium thiosulphate	24 ul
Formalin	100 ul

Table-14. Showing composition of solution V for silver staining.

Solution vi (stopper solution)

Methanol	120 ml
Acetic acid	30 ml
Water	150 ml

Table-15. Showing composition of solution VI for silver staining.

4.8 PHOSPHATE SALINE BUFFER COMPOSITION (pH: 7.3 -7.4)

Sodium chloride	8 g
Sodium orthophosphate	1.44 g
Potassium chloride	0.2 g
Potassium orthophosphate	0.2 g
Water	Make up the final volume to 1000 ml

Table-16. Showing composition of phosphate saline buffer composition .

4.9 WHOLE CELL LYSIS BUFFER (For 30 ml)-

150 mM NaCl- 262.98 mg
1% triton-X- 300 ul
0.5 M sodium deoxycholate- 150 mg
0.1%SDS- 150 mg
50 mM tris (pH -8.0)- 1.5 ml of 1M
Make up the volume with water**.**

4.10 MEMBRANE FRACTIONATION BUFFERS

Buffer A- Tris-Cl (pH – 7.4) + 1mM EDTA
Buffer B- 50mM Tris-Cl (pH – 7.4)
Protease inhibitor(PI)

4.11 METHODS IN ANIMAL CELL CULTURE (YAC-1 CELL LINE)

Suspension cell lines ,Yac-1(Non-adherent) were cultivated in filter-capped T-25 flasks (BD falcon) as well as in 24 well plates in 5 ml of complete RPMI medium. Cell cultures were grown in a CO2 incubator maintained at 37°C and 5% CO2. Cells were seeded at the density of 2 $x10^6$ to 5 $x10^6$ cells/ml.

Figure-10: YAC-1 suspension cultured in T-25 flasks.

a) 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 RPMI media and mix it well.
4. Take 10µl of culture to count the number of cells.
5. After counting add cells to 5ml media.

b) 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

c) THAWING OF CELLS

1. Take out the cryovials from -80^0C.

2. Transfer it into a water bath 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 RPMI.

4.12 CELL NUMBER DETERMINATION USING NEUBAUER CHAMBER

4.12.1 Counting through hemocytometer (Thermo Fischer scientific)

Priniple:

It is a type of glass slide that has a rectangular indentation that has an 'H' shaped chamber at the centre. A laser-etched grid consisting of perpendicular lines is engraved on the chamber. There are two counting regions with ruled grates that are partitioned by the horizontal groove of the 'H'. it makes use of a flat,thin cover slip. 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 remains constant, so the precise result of the volume of fluid at each square of the grid can be known.

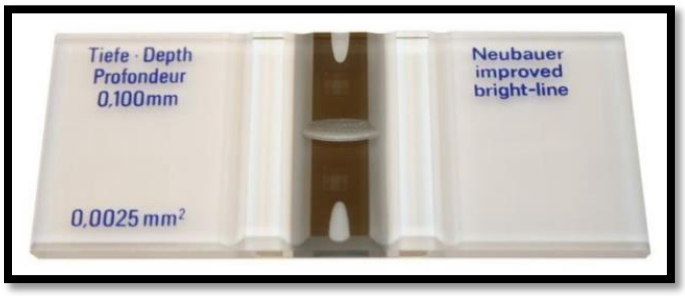

Figure- 11: Haemocytometer

4.12.2 PROCEDURE TO COUNT CELLS

1. Clean the hemocytometer with alcohol.
2. Slide the cover slip over the grid .
3. When required cells were diluted in trypan blue solution to distinguish dead cells.
4. Take 10µl of the cell suspension into both the sides of grid chamber.View and focus the slide under an inverted phase contrast microscope at 10x magnification.
5. Count the number of cells in all four major quadrants.

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)

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

Cells viability as calculated by trypan blue exclusion, was found to vary between 78-96%.

4.13 COLLECTION OF YAC-1 SUPERNATANT

YAC-1 cells were cultured in T-25 flask at concentration of $2 * 10^6$ cells/ml. Supernatents were collected by centrifugation at 1500 rpm for 10 minutes, after different time intervals of incubation in 10% FBS containing media as well as in starved media. YAC-1 supernatant under the same conditions were stored at -20^0C. Protein concentration was estimated using BCA method.

4.14 GENERATION OF GROWTH CURVE OF NK CELL SUSCEPTIBLE CELL LINE –YAC-1

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.

Procedure:

1. Add the freshly grown culture (YAC-1) to the falcon and centrifuge the cells at 1500rpm for 10min.
2. Resuspend the pellet in 5mL 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 0.1×10^6 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 METHOD FOR CELL LYSATE PREPARATION-

1. To the cell pellet, add lysis buffer (RIPA +PI in the ratio of 90 ul: 10 ul)
2. Keep in ice for 45 min.
3. Vortex at every 10 min for 5-7 sec.
4. Spin at 14000 rpm for 15 min.
5. Collect the supernatant in fresh eppenddorf
6. Store at -20^{0}C.

4.16 METHOD FOR WHOLE MEMBRANE EXTRACTION

1. Cultured cells from the flask were transferred to the 15ml falcon, and centrifuged at 1500 rpm for 10 minutes.
2. Supernatant containing the media were discarded and the pellet is dissolved in PBS solution and stored at -20^{0}C.
3. Further the whole procedure was carried out in ice incubation.
4. Culture is thawed in ice and centrifuged at 1500 rpm for 10 minutes.
5. Removed the supernatant containing the PBS.
6. To the pellet, 200 ul of buffer A and 20 ul of PI was added.
7. Incubated in ice for 30 minutes.
8. Then, it was centrifuged at 21000 rcf at 4^{0}C for 20 minutes.
9. Discard supernatant, resuspend the pellet in 500 ul of buffer B.
10. Pass the sample through 26g syringe to ensure homogeneous suspension.
11. Estimated the Protein concentration using BCA method.
12. Stored at -20^{0}C.

4.17 CHARACTERISATION OF PROTEIN BY SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)-

Principle:

Electrophoresis is the analytical technique for the separation of charged molecules in solution by the application of an electric field. As these molecules in an electric field migrate with a speed depending on their shape, size and charge. Here, in SDS-PAGE proteins are separated on the basis of their molecular weights.

It is used to determine the relative molecular mass of the protein present in the sample as well as the presence of desired protein molecule. A support medium used is in the form of a polymerized gel like matrix. The movement of molecules under the electric field is on the basis of three parameters, 1) applied electric field, 2) pore size of the gel matrix and 3) the shape, size, and charge of the molecules to be separated.

a) 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 1.00 mm 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 stacking gel.
9. Insert the comb to make wells and allow it to polymerize.

b) SAMPLE PREPARATION

1. Protein concentration of each sample is calculated using BCA method of protein concentration determination.
2. Prepare the samples in the appropriate volume of 1x sample solubilising buffer (laemmli buffer) and lysis buffer.
3. Heat the sample in boiling water bath/ dry bath for 10 minutes (keep the sample at $4^{O}C$ for 1to 2 min and centrifuge at 10,000 rpm for 30sec, take the supernatant in sample).

c) SAMPLE LOADING

1. 1x reservoir buffer was poured into the electrophoretic unit.
2. Load the protein samples into the corresponding well along with molecular weight marker protein in respective wells.
3. Electrophoretic unit were run at 30mA until the tracking dye were about to leave the separating gel. (run the gel at 70V, when dye front enters the separating gel, increase the voltage to 100-120V).

1. After that, gel were removed and placed in the staining tray and flooded with staining solution, incubated for overnight.
2. After that staining solution was discarded and gel were 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).

d) SILVER STAINING PROTOCOL

1. 20 minutes wash in solution I(fixative).
2. 10 minutes wash in solution II.
3. Exactly 1 minute in solution III.
4. Water washes twice for 20 seconds.
5. 10 minutes wash in solution IV ($AgNO_3$).

6. Water washes twice for 20 seconds.
7. Incubation in solution V (developer step) for 5 minutes.
8. Incubation in solution VI (stopper solution).
9. Store the gel in 1% acetic acid at 4^0C.

5. RESULTS

5.1 3D structure of NK inhibitory receptor Ly49H-

The 3D structure of Ly49H is not available at PDB, so it was predicted using hybrid modeling server named Phyre[2] (Soding, J.2005).

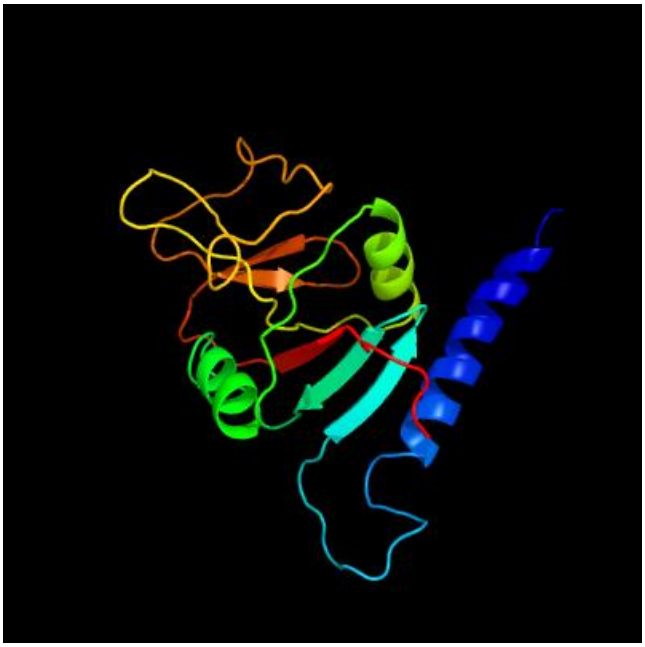

Figure-12. Modeled Crystal structure of murine Ly49H

5.2 Predicted structure of YAC-1 surface ligands:

For all the YAC-1 surface ligands including, p30CA, p12, p15MA, p15E, the structures were not present at PDB. So the structures were also predicted with Phyre[2].

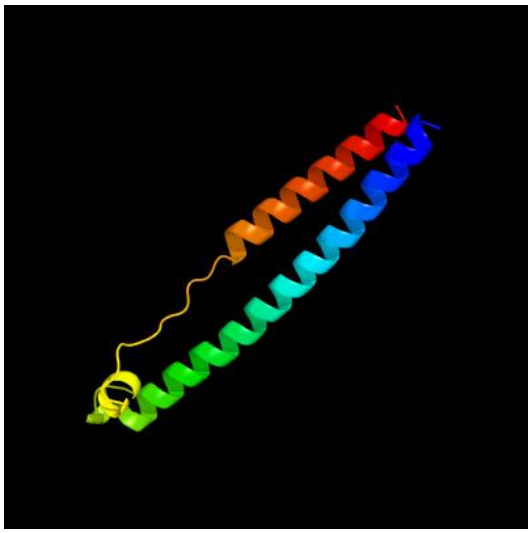

Figure-13.Modeled crystal structure of p15E

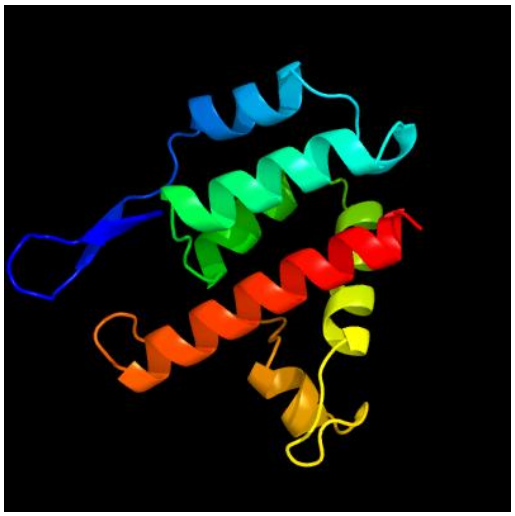

Figure-14.Modeled crystal structure of p30

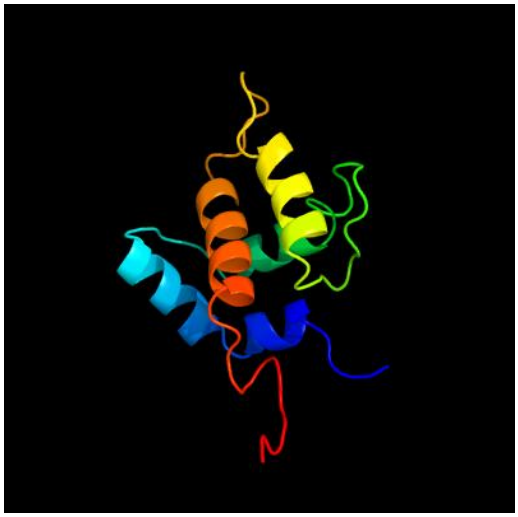

Figure-15.Modeled crystal structure of p15MA

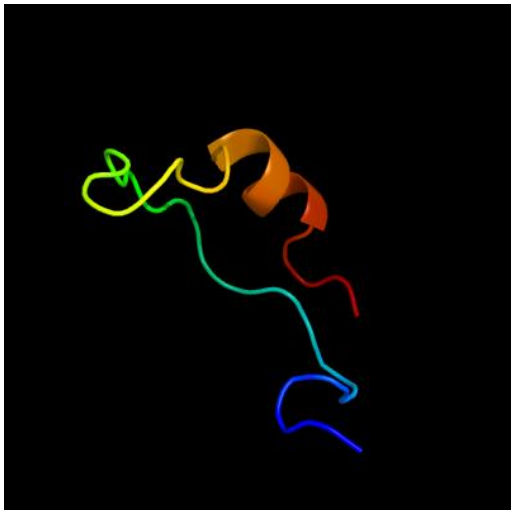

Figure-16.Modeled crystal structure of p12

5.3 Murine NK activating receptor interaction with YAC-1 surface ligands :

PATCHDOCK was used to perform molecular docking Ly49H with the YAC-1 ligands; p15E, p30CA, p15MA, p12. PATCHDOCK determines top 10 complexes and for each of the complexes, the D-complex energy was calculated.

5.3.1 Ly49H-p15E

The results obtained after the docking of receptor Ly49H with p15E were observed. For each complex obtained, following data was observed. Here, the complex with minimum energy is the second one with D-complex energy -12.518432 Kcal/mol and the complex representing minimum energy is shown below.

Rank	Solution no.	Score	Area	D-complex (kcal/mol)
1	8	13004	1881.30	-7.958298
2	4	13288	1675.50	**-12.518432**
3	7	13020	1616.00	-6.078592
4	9	12956	1633.40	-6.998742
5	3	13386	1682.20	-3.906250
6	2	13428	1888.20	-6.315068
7	6	13076	1646.50	-6.355111
8	1	14584	1805.30	-11.026176
9	5	13094	1535.10	8.240053
10	10	12906	2309.30	-6.856760

Table-17. Representing minimum energy for Ly49H-p15E.

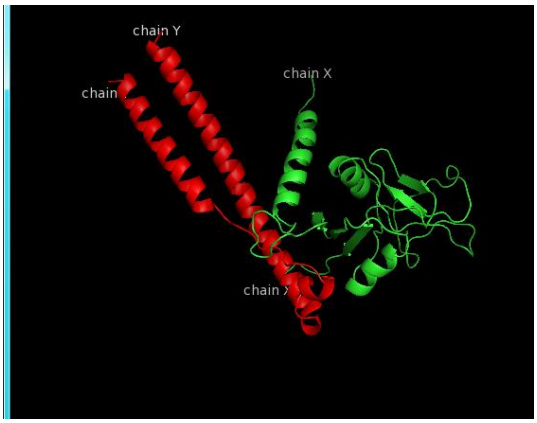

Figure-17. Complex representing minimum energy for Ly49H-p15E

5.3.2 Ly49H-p30CA-

Here , the complex with minimum energy is is the first one with D-complex energy - 11.061380 Kcal/mol and the complex representing minimum energy is shown below.

Rank	Solution no.	Score	Area	D-complex (kcal/mol)
1	10	12816	1782.60	**-11.061380**
2	1	15552	2125.10	-11.043210
3	3	13640	2610.20	3.583099
4	9	12842	1828.80	-6.233670
5	8	12864	1538.10	-10.211391
6	2	14556	1712.60	-10.105183
7	7	12866	1910.70	-6.869576
8	6	13456	2312.60	-4.854557
9	5	13596	1979.00	-8.113094
10	4	13596	2295.70	-4.700000

Table-18. Representing minimum energy Ly49H-p30CA

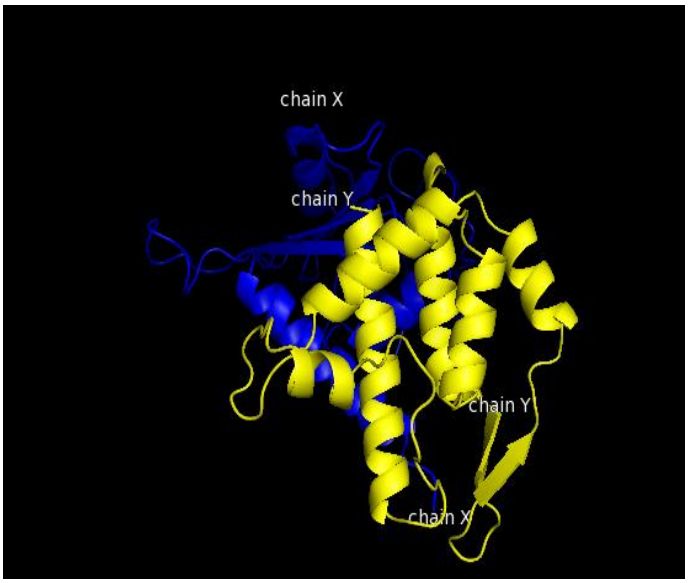

Figure-18. Complex representing minimum energy Ly49H-p30CA

5.3.3 Ly49H-p15MA-

Here , the complex with minimum energy is is the first one with D-complex energy - 12.205013 Kcal/mol and the complex representing minimum energy is shown below.

Rank	Solution no.	Score	Area	D-complex (kcal/mol)
1	3	13172	1629.80	**-12.205013**
2	8	12380	1610.70	-7.548349
3	9	12376	1735.30	-9.758832
4	10	12344	2049.90	-3.523870
5	4	12922	2142.90	-10.070923
6	1	13532	2317.30	-3.179320
7	6	12582	2069.70	-4.670235
8	5	12630	1824.10	-4.355385
9	2	13396	1937.70	10.206377
10	7	12444	1942.00	-4.854555

Table-19. Representing minimum energy Ly49H-p30CA

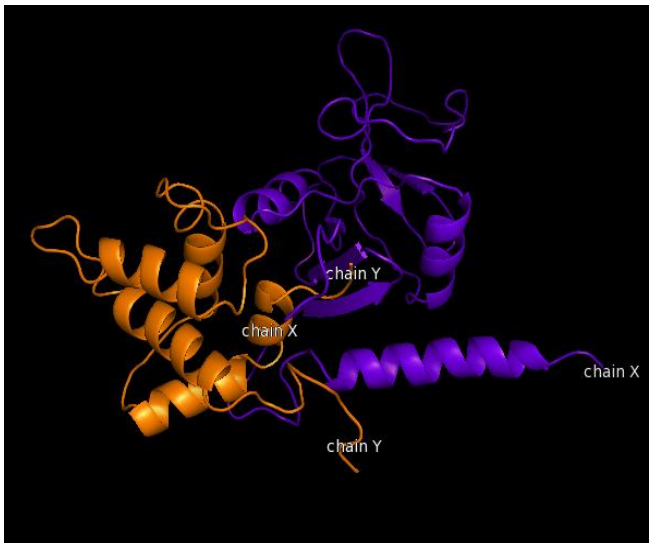

Figure-19. **C**omplex representing minimum energy for Ly49H-p15MA

5.3.4 Ly49H-p12-

Here , the complex with minimum energy is is the tenth one with D-complex energy - 8.455273 Kcal/mol and the complex representing minimum energy is shown below.

Rank	Solution no.	Score	Area	D-complex (kcal/mol)
1	4	11670	1548.10	-7.701501
2	7	11378	1659.40	-8.169250
3	10	11220	1631.30	-4.092175
4	2	12534	1759.70	-8.218939
5	9	11258	1641.80	-6.653870
6	5	11626	1527.90	-5.149787
7	3	11872	1649.40	-3.852422
8	1	12808	1783.50	-4.707519
9	8	11366	1673.60	-6.188818
10	6	11378	1796.90	**-8.455273**

Table-20. Representing minimum energy Ly49H-p12

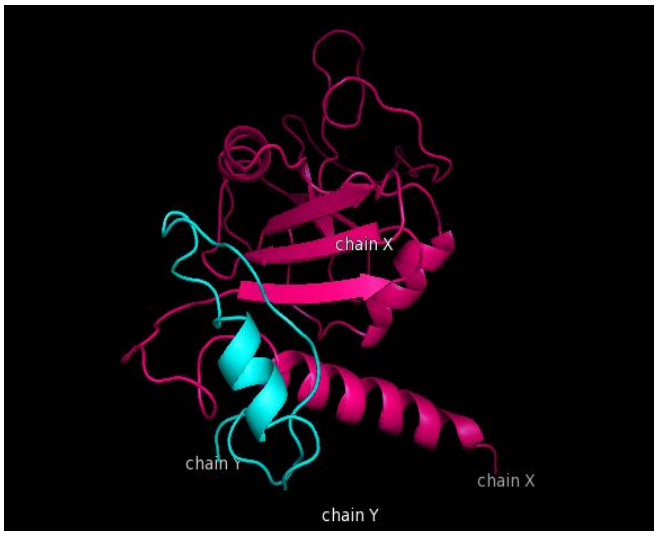

Figure-20. Complex representing minimum energy Ly49H-p12

To understand the interaction between Ly49H, activating receptor (chain-X) and the ligands (chain-Y) present (p15E, p30CA, p15MA, p12) on the surface of YAC-1 cell line, docking was performed and the D-complex energies for each receptor–ligand pair was studied. The results has

shown significant interaction between them. So based upon these results, further experimental studies were carried out.

5.4 CHARACTERIZATION OF TUMOR CELL LINE (YAC-1) TO STUDY THE TUMOR DERIVED FACTORS THAT MAY INDUCE NK CELL MODULATION

To determine the components secreted by YAC-1, YAC-1 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 (RPMI + 10% FBS). So, supernatants were subjected to SDS-PAGE in serum free media (figure 2B). It was observed that YAC-1 could secrete some components in serum free media while no bands were seen in cell free and serum free media (RPMI-FBS control) (figure 2B).

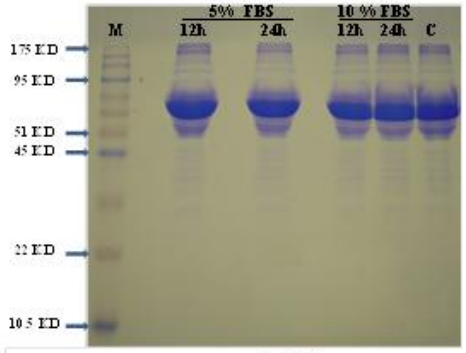

Figure Fractionation of YAC-1 culture on 10% SDS-PAGE.
Lane M- Protein ladder (pre-stained Pink plus,BR Biochem), 12h stands for culture supernatant at 12hour incubation,24h stands for culture supernatant at 24hour incubation with 5% and 10% FBS concentration respectively. Lane C- RPMI media with 10% FBS concentration.

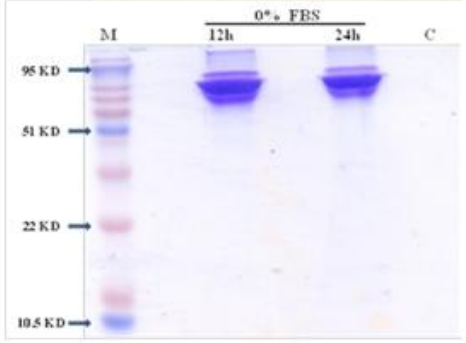

Figure Figure Separation of YAC-1 culture on 10% SDS-PAGE.
Lane M- Protein ladder (pre-stained Pink plus,BR Biochem), 12h stands for culture supernatant at 12hour incubation,24h stands for culture supernatant at 24hour incubation with 0% FBS concentration (serum free RPMI media respectively. Lane C- RPMI medium without serum (0% FBS).

Figure- 21. Fractionation of YAC-1 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 12h, 24h. Marker: BR biochem, Pink plus prestained protein ladder with MW 175KD to 10.5 KD.

5.5 GENERATION OF GROWTH CURVE OF NK CELL SUSCEPTIBLE CELL LINE,YAC-1

Growth curve of YAC-1 was analyzed in 5% and 10% fetal bovine serum and the generation time observed was 20 h in 10% serum concentration and 22 h in 5% serum concentration (Figure and).

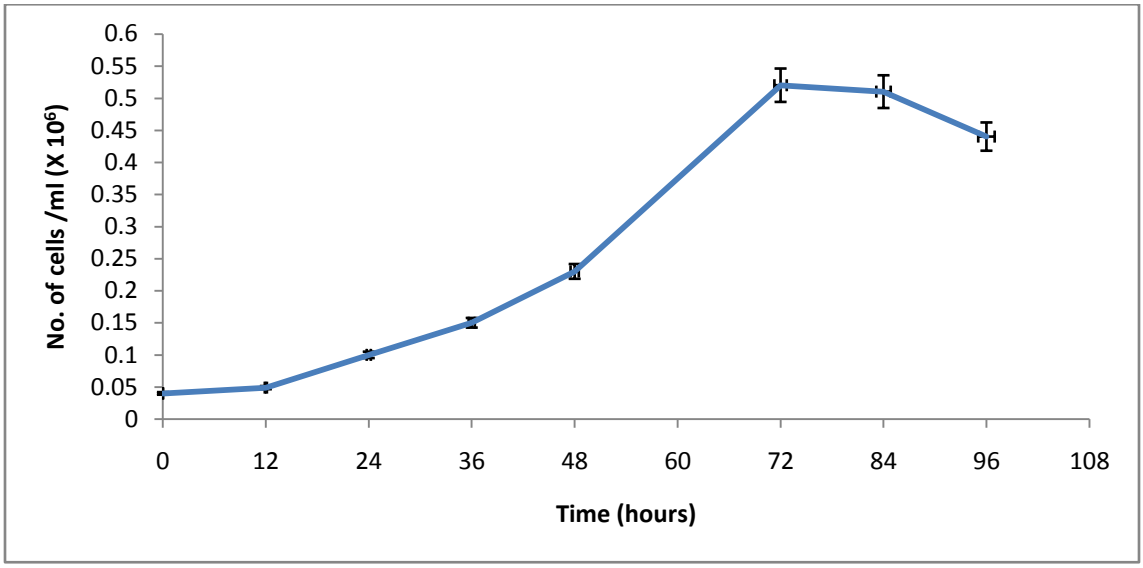

Figure-22. Growth Curve - YAC-1(10% RPMI)

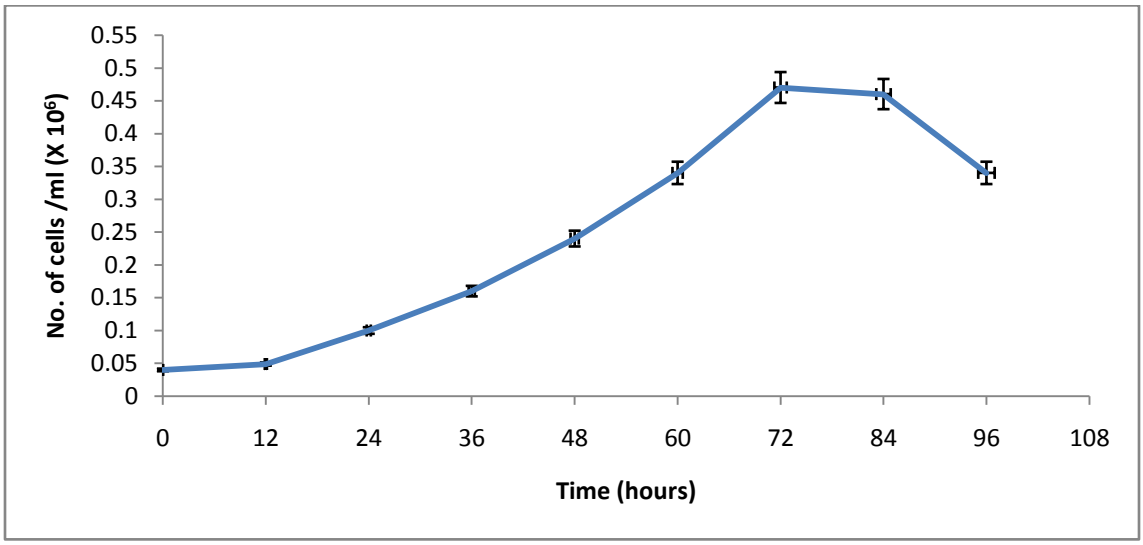

Figure-23. Growth Curve - YAC-1(5% RPMI)

5.6 CHARACTERIZATION OF TUMOR CELL LINE (YAC-1) TO STUDY THE TUMOR LYSATE THAT MAY INDUCE NK CELL MODULATION

YAC-1 cell lysate was obtained by harvesting the cells in their log phase in order to identify the different cytoplasmic or intracellular factors, which may be involved for the modulation of NK cell responses. Various intracellular proteins were characterized on the basis of molecular weight using 12 % gel in SDS-PAGE.

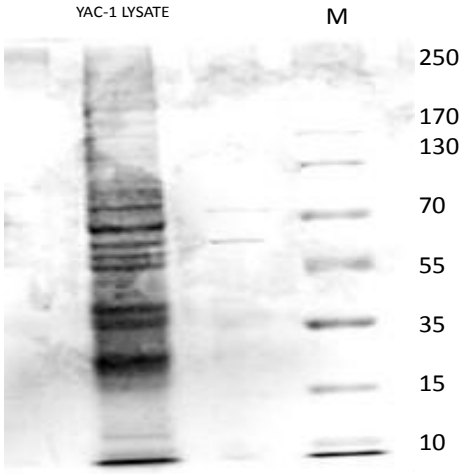

Figure-24. SDS-PAGE profile of YAC lysate containing intracellular protein pool and the gel was stained with coomassie blue followed by silver staining. Marker: BR biochem, Pink plus prestained protein ladder with MW 10 kD to 250 kD.

The YAC-1 cell lysate contains a pool of both intracellular and membrane bound proteins. Membrane bound proteins are the one responsible for inducing NK cell modulation. So, we are interested in isolating whole membrane protein. Therefore, we prepared whole membrane proteins as per the protocol given in materials and methods.

5.7 CHARACTERIZATION OF TUMOR CELL LINE (YAC-1) TO STUDY THE TUMOR LYSATE AND WHOLE MEMBRANE PROTEIN THAT MAY INDUCE NK CELL MODULATION

Whole membrane protein or membrane based factors/ligands were extracted, when cells are in their log phase, which might be involved in their interaction with NK activating receptors, to mount an immune response that leads to lysis of tumor cells. The various components are separated using 10 % gel in SDS-PAGE.

The gel picture also shows the comparative analysis of the different molecular weight components extracted from the YAC-1 lysate and whole membrane protein.

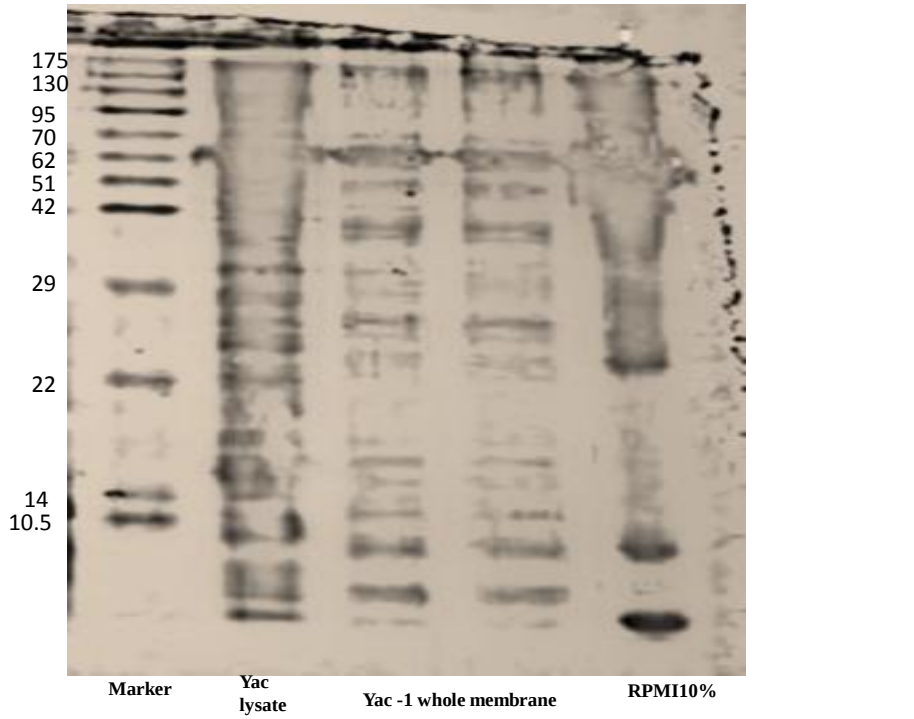

Figure-25. SDS-PAGE profile of YAC lysate and whole membrane protein isolate and the gel was stained with coomassie blue followed by silver staining**.** Marker: BR biochem, Pink plus prestained protein ladder with MW 10.5KD to 175KD.

6. DISCUSSION AND FUTURE PERSPECTIVE

The evasion of tumors inspite of host immune defenses is by the production of soluble factors that affects the function of host cells involved in immune responses. So, different tumor-derived factors may affect the function of natural killer (NK) cells that may raise the expansion of cells with down-regulatory activities. (Nelson, D.S.et al., 1987; Lopez, D.M., 1991).

The present study aims at identifying the multiple factors derived from NK sensitive cell line (YAC-1) that might be involved to bring about changes in the NK receptor profile. Different classes of activating receptors are present in murine NK cell. Here, the sequence of one of the murine NK activating receptor, Ly49H was obtained from NCBI and their structures were modeled with Phyre^2, as it is not available in the PDB. Similarly, through literature study various ligands/surface proteins were chosen that are known to be found on YAC-1 cell surface, viz., p15E, p30CA, p15MA, p12 (Troy, FA.et al.1977). The sequences for the above surface proteins were obtained from NCBI and their structures were modeled with Phyre^2, as it is not available in the PDB.

Further, PATCHDOCK was used to perform molecular docking Ly49H with the YAC-1 ligands; p15E, p30CA, p15MA, p12. PATCHDOCK determines top 10 complexes and for each of the complexes, the D-complex energy was calculated. The results have shown significant interaction between them. So based upon these results, further experimental studies were carried out. This knowledge of affinity of NK cell receptor with tumor ligands will help us in understanding the binding patterns required for the activation of NK cell activity.

In the same way, another important surface proteins can be taken in future studies like Moloney leukemia virus-determined cell surface antigen (MCSA) (Troy, FA.et al.1977), NKTS (Roder, JC.et al.,1979), p71(Roder, JC.et al.,1979; Troy, FA.et al.1977), H-2a , etc., once their sequences are deduced. So, similar understanding of their binding energies can be calculated with other activating receptors also in mouse.

Further, we wanted to study whether TDFs can modulate frequency of expression of activating receptors on NK cells. Our docking studies have shown engagement of activating receptors by multiple YAC tumor derived factors. Further changes in receptor profile by modulation stimulated by YAC membrane components needs to be studied in detail.

Cells were cultured in 5%, 10% and serum free media and supernatants were collected at different time intervals of 12 hrs and 24 hrs. No significant variation was observed in given particular interval of time and in comparison to cell free media (RPMI + 10% FBS). So, supernatants were subjected to SDS-PAGE in serum free media (shown in figure-21). It was observed that YAC-1 could secrete some components in serum free media while no bands were seen in cell free and serum free media (RPMI-FBS control).

Growth curve of YAC-1 was analyzed in 5% and 10% fetal bovine serum and the generation time observed was 20 h in 10% serum concentration and 22 h in 5% serum concentration (shown in figure-22 and 23). It has a characteristic metabolite secretion in a tumor microenvironment. There should be some difference between the NK sensitive and NK resistant supernatant factors. This is to extrapolate the obtained growth curve with the ATCC's growth curve. So by

comparing, growth characteristic is studied to ensure that the supernatant we are collecting are, whether they are in their lag phase or log phase. It is an immortal cell line, so do not follow sigmoidal growth kinetics which can be inferred by its extended log phase along with shorter lag phase.

YAC-1 cell lysate was obtained by harvesting the cells in their log phase in order to identify the different cytoplasmic or intracellular factors and extracellular factors, which may be involved for the modulation of NK cell responses. Various intracellular proteins were characterized on the basis of molecular weight using 12 % gel in SDS-PAGE (shown in figure-24). Multiple number of bands were obtained which overlaps, so for further expansion of bands, lysate proteins were characterized using 10 % gel in SDS-PAGE (shown in figure-25).

For further specification, complete membrane proteins were extracted, when cells are in their log phase, which might be involved in their interaction with NK activating receptors, to mount an immune response that leads to lysis of tumor cells. The various components are separated using 12% gel in SDS-PAGE (shown in figure-25). The gel picture also shows the comparative analysis of the different molecular weight components extracted from the YAC-1 lysate and whole membrane protein.

The whole membrane protein has to be further fractionated using amicon cuts of 30, 50, and 100 kD, respectively. Then, the membrane fractions has to be further characterized using 12 % in SDS-PAGE.

Earlier profiling techniques were not so much developed, so information about the changes in NK receptor profile resulting from NK sensitive tumor targets are known to a lesser extent. In order to study the NK modulation activity, we propose to co- culture YAC-1 membrane protein, membrane fractions obtained from amicon cuts with NK cells, to evaluate the modulation of NK of each fraction.

Molecular basis of YAC target structures with relevant NK receptor has not been known. And also, it is well known that YAC is the prototypic target for NK cells. As single binding is not so informative, so we are looking for multiple ligands on YAC that are responsible for binding to activating receptor. The target for different activating receptors can be identified using a modeled structure of YAC ligands.

This piece of information can be similarly used for finding such targets in other types of cancers, and can be used for diagnostic studies initially and later on for therapeutic success.

Such factors or targets can also be transfected (can be used as adjuvants) into other tumors (that are resistant to killing by NK cell), to evoke immune response against them, that will automatically convert the resistant tumor into a sensitive target. This idea can be applied to construct therapeutic cancer vaccines.

In future, similar type of studies can be performed taking human tumor targets into consideration which can provide new insights for better and more comprehensive understanding of the identity of the tumor derived factors for the development of more promising and novel therapies.

7. CONCLUSION-

The knowledge of affinity of NK cell activating receptor with tumor ligands have helped in understanding the binding patterns required for the activation of NK cell activity. PATCHDOCK determines top 10 complexes and for each of the complexes, the D-complex energy was calculated. The results have shown significant interaction between them as well as good binding affinities. So, it can be said that the presence of such molecules (tumor ligands) and the absence of MHC class I expression on the tumor cells makes them vunerable to NK cell attack.

Various cellular fractions (supernatants, cell lysates, whole membrane protein) are isolated and subsequently separated on SDS-PAGE. The whole membrane proteins are needed to be further fractionated using amicons, each fraction obtained would need to be validated for their effect on NK cell receptor frequency modulation using flowcytometry (unpublished work of Ms. Richa Mishra).

Since, a number of TDFs cause NK activation through multiple NK activating receptor engagement. We propose that NK susceptible tumor cell simultaneously engage multiple activating receptors and culmination of multiple activating signals are responsible for high cytotoxicity of the tumor cell.

8. REFERENCES-

- Algarra I; Garcia-Lora A; Cabrera T; Ruiz-Cabello F; Garrido F. (2004). The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother. *53:904–10.*
- Anderson;TW; Wright; C; Brooks; WS. (2010). The E3 Ubiquitin Ligase NARF Promotes Colony Formation in vitro and Exhibits Enhanced Expression Levels in Glioblastoma Multiforme in vivo. American Journal of Undergraduate Research. ***9***; 2-3.
- Arteaga, C.L; Hurd, S.D; Winnier, A.R; Johnson, M.D; Fendly, B.M; Forbes, J.T.(1993). Anti-transforming growth factor (TGF)-b antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-b interactions in human breast cancer progression.J. Clin.INVEST. *92:2569.*
- Augustinack; JC; Sanders; JL; Tsai; LH. and Hyman; BT (2002). Colocalization and fluorescence resonance energy transfer between cdk5 and AT8 suggests a close association in pre-neurofibrillary tangles and neurofibrillary tangles. J. Neuropathol. Exp. Neurol. ***61***; 557–564.
- Balsamo,M; Scordamaglia,F; Pietra,G; Manzini,C; Cantoni,C; Boitano,M et al.(2009). Melanoma-associated fibroblasts modulate NK cell phenotype and antitumor cytotoxicity. Proc Natl Acad Sci U S A. *106:20847-52*.
- Basith,S; Manavalan,B; Yoo,T.H; Kim,S.G; Choi,S. (2012). Roles of toll-like receptors in cancer: a double-edged sword for defense and offense. Arch Pharm Res. *35(8): 1297-1316.*
- Ben-Eliyahu, S; Shakhar, G; Page, G.G; Stefanski, V; Shakhar, K. (2000). Suppression of NK cell activity and of resistance to metastasis by stress: a role for adrenal catecholamines and betaadrenoceptors. Neuroimmunomodulation. *8 (3); 154–164.*
- Ben-Eliyahu, S; Shakhar, G; Rosenne, E; Levinson, Y; Beilin, B. (1999). Hypothermia in barbiturate-anesthetized rats suppresses natural killer cell activity and compromises resistance to tumor metastasis: a role for adrenergic mechanisms. Anesthesiology. *91(3), 732–740.*
- Bennett, I. M; O. Zatsepina; L. Zamai; L. Azzoni; T. Mikheeva; B. Perussia.(1996). Definition of a natural killer NKR-P1A_/CD56_/CD16_ functionally immature humanNKcell subset that differentiates in vitro in the presence of interleukin 12. J. Exp. Med. *184: 1845–1856.*
- Berger, C.L; Tigelaar R; Cohen J; et al. (2005). Cutaneous T-cell lymphoma: malignant proliferation of T-regulatory cells. Blood. *105:1640–7.*
- Blagosklonny; MV; Toretsky; J; Bohen; S; Neckers; L (1996). Mutant conformation of p53 translated in vitro or in vivo requires functional HSP90. Proc. Natl. Acad. Sci. USA. *93*:8379-83.

- Blaser, C. et al. (1998). β-galactoside binding protein secreted by activated T cells inhibits antigen-induced proliferation of T cells. Eur. J. Immunol. ***28;*** *2311–2319.*
- Bondanza, A; Zimmermann, V.S; Rovere-Querini, P; et al. (2004). Inhibition of phosphatidylserine recognition heightens the immunogenicity of irradiated lymphoma cells in vivo . J Exp Med . *200:1157–65.*
- Bottino, C; L. Moretta; D. Pende; M. Vitale; A. Moretta. (2004). Learning how to discriminate between friends and enemies; a lesson from natural killer cells. Mol. Immunol. *41: 569–575.*
- Brady, J; Y. Hayakawa; M. J. Smyth; S. L. Nutt. (2004). IL-21 induces the functional maturation of murine NK cells. J. Immunol. *172: 2048–2058*.
- Braud. V. M; D. S. Allan; C. A. O'Callaghan; K. Soderstrom; A. D'Andrea; G. S. Ogg; S. Lazetic; N. T. Young; J. I. Bell; J. H. Phillips et al. (1998). HLA-E binds to natural killer cell receptors CD94/NKG2A; B and C. Nature *391: 795–799.*
- Bronte, V; Chappell, D.B; Apolloni, E; Cabrelle, A; Wang, M; Hwu, P; et al.(1999). Unopposed production of granulocyte-macrophage colony stimulating factor by tumors inhibits CD8+ T cell responses by dysregulating antigen-presenting cell maturation. J Immunol. *162(10):5728–37.*
- Bronte, V; Serafini, P; Mazzoni, A; Segal, D.M; Zanovello, P. (2003). Larginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol. *24(6):302–6.*
- Byrne, S.N; Halliday, G.M.(2003). High levels of Fas ligand and MHC class II in the absence of CD80 or CD86 expression and a decreased CD4+ T cell Infiltration; enables murine skin tumours to progress. Cancer Immunol Immunother . *52:396–402.*
- Carson; W. E; J. G. Giri; M. J. Lindemann; M. L. Linett; M. Ahdieh; R. Paxton; D. Anderson; J. Eisenmann; K. Grabstein and M. A. Caligiuri (1994). Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. J. Exp. Med. *180: 1395–1403.*
- Cerwenka, A; L. L. Lanier. (2003). NKG2D ligands: unconventional MHC class I-like molecules exploited by viruses and cancer. Tissue Antigens. *61: 335–343.*
- Cerwenka, A; Lewis L. Lanier. (2001). Natural killer cells; viruses and cancer. NATURE REVIEWS | IMMUNOLOGY. VOLUME 1
- Chiba,S; Ikushima,H; Ueki,H; Yanai,H; Kimura,Y; S, Hangai; Nishio,J; Negishi,H; Tamura,T; Saijo,S; Iwakura,Y; Taniguchi,T. (2014). Recognition of tumor cells by Dectin-1 orchestrates innate immune cells for anti-tumor responses. eLife. *3:e0417.*
- Chung, C.D; Patel, V.P; Moran, M; Lewis, L.A.; Miceli, M. C. (2000). Galectin-1 induces partial TCR ζ-chain phosphorylation and antagonizes processive TCR signal transduction. J. Immunol. ***165****; 3722–3729*.
- Colucci, F; Di Santo, J.P; Leibson, P.J.(2002). Natural killer cell activation in mice and men:different triggers for similar weapons? Nat. Immunol. *3:807–13.*

- Colucci,F; M. A. Caligiuri; et al. (2003). What does it take to make a natural killer?. Nat Rev Immunol. ***3****(5):413-425.*
- Cooper, M. A; T. A. Fehniger; A. Fuchs; M. Colonna; M. A. Caligiuri. (2004).NK cell and DC interactions. Trends Immunol. *25: 47–52.*
- Di Santo, J. P. (2006). Natural killer cell developmental pathways: A question of balance. Annual Review of Immunology. ***24****: 257-286.*
- Doubrovina, E.S; Doubrovin, M.M; Vider, E; et al. (2003). Evasion from NK cell immunity by MHC class I chainrelated molecules expressing colon adenocarcinoma. J Immunol. *171:6891–9.*
- Ferlazzo, G; C. Mu¨nz. (2004). NK cell compartments and their activation by dendritic cells. J. Immunol. *172: 1333–1339.*
- Ferlazzo, G; D. Thomas; S. L. Lin; K. Goodman; B. Morandi; W. A. Muller; A. Moretta; C. Mun. (2004). The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. J. Immunol. *172: 1455–1462*.
- French, L.E; Tschopp, J. (2002). Defective death receptor signaling as a cause of tumor immune escape. Semin Cancer Biol. *12:51–5.*
- Fruci, D.; Monaco, EL.; Cifaldi, F.; Locatelli, F.; Tremante, E.; Giacomini, MB. (2013). T and NK cells: two sides of tumor immunoevasion. Journal of Translational Medicine. *11:30*
- Gabriella Pietra; Claudia Manzini; Silvia Rivara; et al. Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. Cancer Res.
- Gabrilovich D; Ishida T; Oyama T; et al. (1998). Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo . Blood. *92:4150–66.*
- Gabrilovich, D.I; Chen, H.L; Girgis, K.R; Cunningham, H.T; Meny, G.M; Nadaf, S; et al. (1996). Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat Med . *2:1096–103.*
- Glas. R; L. Franksson; C. Une; M. L. Eloranta; C. Ohlen; A. Orn; K. Karre. (2000). Recruitment and activation of natural killer (NK) cells in vivo determined by the target cell phenotype: an adaptive component of NK cell-mediated responses. J. Exp. Med. *191: 129–138*.
- Godin-Ethier J; Hanafi, L.A; Piccirillo, C.A; Lapointe, R. (2011): Indoleamine 2;3-dioxygenase expression in human cancers: clinical and immunologic perspectives. Clin Cancer Res. *17:6985–6991*.
- Golab, J. (2000). Interleukin 18-interferon_ inducing factor—a novel player in tumour immunotherapy. Cytokine. *12: 332–338.*
- Hamerman, J. A, K. Ogasawara; L. L. Lanier. (2005). NK cells in innate immunity. Curr. Opin. Immunol. *17: 29–35.*

- He X; Stuart, J.M.(1999). Prostaglandin E2 selectively inhibits human CD4+ T cells secreting low amounts of both IL-2an d IL-4. J Immunol. *163:6173–9.*
- He, J.; Baum, L. G.(2004) Presentation of galectin-1 by extracellular matrix triggers T cell death. J. Biol. Chem. ***279****; 4705–4712.*
- Herberman, R.B; Ortaldo,J,R. (1981). Natural killer cells, their role in defense against disease. Science. ***214*** *24–30.*
- Holdenrieder, S; Stieber, P; Peterfi, A; Nagel, D; Steinle, A; Salih, H.R. (2006). Soluble MICA in malignant diseases. Int J Cancer. *118:684–7.*
- Huang, M; Sharma, S; Mao, J.T; Dubinett, S.M.(1995). Non-small cell lung cancer-derived soluble mediators and prostaglandin E2 enhanced peripheral blood lymphocyte IL-10 transcription and protein production. J. Immunol. *157: 5512.*
- Igney, F.H; Behrens, C.K; Krammer, P.H. (2005). CD95L mediates tumor counterattack in vitro but induces neutrophil-independent tumor rejection in vivo . Int J Cancer. *113:78–87.*
- Ishigami, S; S. Natsugoe; et al. (2000). Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer. ***88****(3): 577-583.*
- Jager, D; Jager, E; Knuth, A. (2001). Immune responses to tumour antigens: implications for antigen specific immunotherapy of cancer. J Clin Pathol. *54: 669–74.*
- Karre, K; Ljunggren, H.G; Piontek, G; Kiessling, R. (1986). Selective rejection of H-2-efficient lymphoma variants suggests alternative immune defence strategy. Nature *319:675–78*
- Kiessling, R; Klein, E; Wigzell. (1975). Natural killer cells in the mouse. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur.J.Immunol. *5(2):112-117.*
- Kim, R; Emi, M; Tanabe, K.(2005). Cancer cell immune escape and tumor progression by exploitation of anti-inflammatory and pro-inflammatory responses. Cancer Biol Ther. *4:924–33.*
- Ladisch,S; Gillard,B; Wong,C; Ulsh,L. (1983). Shedding and Immunoregulatory Activity of YAC-1 Lymphoma Cell Gangliosides.CANCER RESEARCH. *43; 3808-3813.*
- Lanier, L.L. (1998). NK cell receptors. Annu. Rev. Immunol. *16:359–93*
- Lanier, L.L. (2005). NK cell recognition. Annu. Rev. Immunol. *23:225–74.*
- Latour, S; Veillette, A. (2004). The SAP family of adaptors in immune regulation. Semin.Immunol. *16:409–19.*
- Lauwerys, B. R; N. Garot; J. C. Renauld; F. A. Houssiau. (2000). Cytokine production and killer activity of NK/T-NK cells derived with IL-2; IL-15; or the combination of IL-12 and IL-18. J. Immunol. *165: 1847–1853.*
- Li, J.H; Rosen, D; Sondel, P; Berke, G. (2002). Immune privilege and FasL: two ways to inactivate effector cytotoxic T lymphocytes by FasL-expressing cells. Immunology. *105:267–77.*

- Lin, E.Y; Gouon-Evans, V; Nguyen, A.V; Pollard, J.W. (2002). The macrophage growth factor CSF-1 in mammary gland development and tumor progression. J Mammary Gland Biol Neoplasia. *7(2):147–62.*
- Liu, F.-T. (2000). Galectins: a new family of regulators of inflammation. Clin. Immunol. ***97****; 79–88).*
- Liu, R.B; B.Engels; et al. (2012). Densely Granulated Murine NK Cells Eradicate Large Solid Tumors. Cancer Research. ***72****(8): 1964-1974.*
- Ljunggren, H.G; Karre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. Immunol. Today. *11:237–44*
- Lo Monaco, E; Tremante, E; Cerboni, C; Melucci, E; Sibilio, L; Zingoni, A; Nicotra, MR; Natali, PG; Giacomini, P. (2011): Human Leukocyte Antigen E contributes to protect tumor cells from lysis by natural killer cells. Neoplasia. *13:822–830.*
- Long, E.O. (1999). Regulation of immune responses through inhibitory receptors. Annu. Rev. Immunol. *17:875–904*
- Lopez,D.M; Lopez-Cepero,M; Watson,G.A; Ganju,A; Sotomayor, E.M; Fu, Y-X. (2010). Modulation of the immune system by mammary tumor-derived factors. CancerINVEST. *9:643.*
- Medina, M.A; Quesada, A.R; Nunez de Castro, I; Sanchez- Jimenez, F. (1999). Histamine; polyamines; and cancer. Biochem Pharmacol. *57(12):1341–4.*
- Merchav, S; Apte, R.N; Tatarsky, I; Ber, R.(1987). Effect of plasmacytoma cells on the production of granulocyte-macrophage colonystimulating activity (GM-CSA) in the spleen of tumor-bearing mice. Exp Hematol. *15(9):995–1000.*
- Miller, H. C; Esselman, W. J. (1975). Modulation of the immune response by antigen reactive lymphocytes after cultivation with gangliosides. J. Immunol. *775:839-843.*
- Moretta, A; Bottino, C; Vitale, M; Pende, D; Cantoni, C; et al.(2001). Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu. Rev.Immunol. *19:197–223*
- Moretta, L; C. Bottino; D. Pende; M. Vitale; M. C. Mingari; A. Moretta. (2004). Different checkpoints in human NK-cell activation. Trends Immunol. *25: 670–676.*
- Moretta, L; G. Ferlazzo; C. Bottino; M. Vitale; D. Pende; M. C. Zingari; A. Moretta. (2006). Effector and regulatory events during natural killer-dendritic cell interactions. Immunol. Rev. *214: 219–228.*
- Nelson,D.S; Nelson, M. (1987). Evasion of host defenses by tumor. Immunol. Cell Biol. *65: 287.*
- Ostrand-Rosenberg, S. (2008). Immune surveillance: a balance between protumor and antitumor immunity. Curr Opin Genet Dev. ***18****(1): 11-18.*
- Parhar, R.S; Lala, P.K.(1985). Changes in the host natural killer cell population in mice during tumor development. 2. The mechanism of suppression of NK activity. Cell. Immunol. *93: 265.*

- Park, S.J; Nakagawa, T; Kitamura, H; Atsumi, T; Kamon, H; Sawa, S; et al. (2004). IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation. J Immunol. *173(6):3844–54*.
- Parrish-Novak, J; S. R. Dillon; A. Nelson; A. Hammond; C. Sprecher; J. A. Gross; J. Johnston; K. Madden; W. Xu; J. West et al. (2000). Interleukin 21 and its receptor are involved inNKcell expansion and regulation of lymphocyte function. Nature. *408:57–63.*
- Pende, D; C. Bottino; R. Castriconi; C. Cantoni; S. Marcenaro; P. Rivera; G. M. paggiari; A. Pondero; B. Carnemolla; N. Reymond et al. (2005). PVR (CD155) and Nectin-2 (CD112) as ligands of the human DNAM-1 (CD226) activating receptor: involvement in tumor cell lysis. Mol. Immunol. *42: 463–469.*
- Pende, D; P. Rivera; S. Marcenaro; C. C. Chang; R. Biassoni; R. Conte; M. Kubin; D. Cosman; S. Ferrone; L. Moretta; A. Moretta. (2002). Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. Cancer Res. *62: 6178–6186.*
- Pietra, G; Manzini, C; Rivara, S; Vitale, M; Cantoni, C; Petretto, A; Balsamo, M; Conte, R; Benelli, R; Minghelli, S; Solari, N; Gualco, M; Queirolo, P; Moretta, L; Mingari, M.C. (2012): Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. Cancer Res. *72:1407–1415.*
- Qiu,X; Zhu,X; Zhang,L; Mao,Y; Zhang,J; et al. (2003). Human epithelial cancers secrete immunoglobulin g with unidentified specificity to promote growth and survival of tumor cells. Cancer Res. *63(19): 6488- 6495.*
- Rabinovich, G. A; Toscano, M. A; Ilarregui, J. M.; Rubinstein, N. (2004). Shedding light on the immunomodulatory properties of galectins: novel regulators of innate and adaptive immune responses. Glycoconj. *J. **19**; 565–573.*
- Rabinovich, G.A. *et al.* (2002). Galectins and their ligands: amplifiers; silencers or tuners of the inflammatory response? Trends Immunol. ***23**; 313–320.*
- Rabinovich, G.A; Gabrilovich, D; Sotomayor, E.M. (2007) Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol. *25:267-96.*
- Raffaghello, L; Prigione, I; Airoldi, I; et al.(2004). Downregulation and/or release of NKG2D ligands as immune evasion strategy of human neuroblastoma. Neoplasia. *6:558–68.*
- Raulet, D.H. (2003). Roles of the NKG2D immunoreceptor and its ligands. Nat. Rev.Immunol. *3:781–90.*
- Raulet, D.H; Vance, R.E; McMahon, C.W. (2001). Regulation of the natural killer cell receptor repertoire. Annu. Rev. Immunol. *19:291–330.*
- Robertson, M. J. (2002). Role of chemokines in the biology of natural killer cells. J. Leukocyte Biol. *71: 173–183.*
- Rodriguez, P.C; Quiceno, D.G; Zabaleta, J; Ortiz, B; Zea, A.H; Piazuelo, M.B; et al. (2004). Arginase I production in the tumor microenvironment by mature myeloid cells

inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res. *64(16):5839–49.*

- Rosenberg, S. A; M. T. Lotze; J. C. Yang; S. L. Topalian; A. E. Chang; D. J. Schwartzentruber; P. Aebersold; S. Leitman; W. M. Linehan; C. A. Seipp; et al. (1993). Prospective randomized trial of high-dose interleukin-2 alone or in conjunction with lymphokine-activated killer cells for the treatment of patients with advanced cancer. J. Natl. Cancer Inst. *85: 622–632*.
- Ruiz de Morales, J; Vélez, D; Subiza, J.L.(1999). Ehrlich tumor stimulates extramedullar hematopoiesis in mice without secreting identifiable colony-stimulating factors and without engagement of host T cells. Exp. Hematol. *27: 1757.*
- Sarin, A; Saxena, QB.; Herberman, RB. and Saxena, RK.(1995). Enhanced MHC I antigen expression on tumour target cells is inversely correlated to lysis by allogenic but not by xenogenic NK cells. J. Biosci. *20;515–523.*
- Sato,Y; Goto,Y; Narita,N; Hoon,DS. (2009). Cancer cells expressing tolllike receptors and the tumor microenvironment. Cancer Microenviron 2(Suppl 1). *205-214.*
- Saxena,R.K; Saxena,Q.B; Adler,W.H. (1988). Properties and characterization of a rat spleen cell derived factor which induces resistance to NK cell lysis in YAC lymphoma cells. J. Immunol. ***141*** *1782–1787.*
- Saxena,R.K; Saxena,Q.B; Sarin,A; Herberman,R.B. (1992). Natural Killer—Resistance Inducing Factor(NK-RIF): A novel immunomodulatory cytokine; in *NK cell mediated cytotoxicity: Receptor signaling and mechanisms* (eds) E Lotzova and R B Herberman (Ann Arbor: CRC Press) pp *381–392*
- Shanker, A; Marincola, FM. (2011). Cooperativity of adaptive and innate immunity: implications for cancer therapy. Cancer Immunol Immunother. *60:1061–1074.*
- Shanker, A; Verdeil, G; Buferne, M; Inderberg-Suso, EM; Puthier, D; Joly, F; Nguyen, C; Leserman, L; Auphan-Anezin, N; Schmitt-Verhulst, AM. (2007). CD8 T cell help for innate antitumor immunity. J Immunol. *179:6651–6662.*
- Sivori, S; S. Carlomagno; L. Moretta; A. Moretta. (2006). Comparison of different CpG oligodeoxynucleotide classes for their capability to stimulate human NK cells. Eur. J. Immunol. *36: 961–967.*
- Smith, C.W; Chen, Z; Dong, G; Loukinova, E; Pegram, M.Y; Nicholas- Figueroa, L; et al. (1998). The host environment promotes the development of primary and metastatic squamous cell carcinomas that constitutively express proinflammatory cytokines IL-1alpha; IL-6; GMCSF; and KC. Clin Exp Metastasis. *16(7):655–64.*
- Smyth, M.J; Swann, J; Cretney, E; Zerafa, N; Yokoyama, W.M; Hayakawa, Y. (2005). NKG2D function protects the host from tumor initiation. J Exp Med. *202:583–8.*
- Sojka, DK; Plougastel-Douglas, B; Yang, L; Pak-Wittel, MA; Artyomov, MN; Ivanova,Y; Zhong,C; Chase,JM; Rothman,PB; Yu,J; Riley,JK; Zhu,J; Tian,Z; Yokoyama,WM. (2014). Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. eLife. *3:e01659*

- Somasundaram R; Jacob L; Swoboda R; et al. (2002). Inhibition of cytolytic T lymphocyte proliferation by autologous CD4+/CD25+ regulatory T cells in a colorectal carcinoma patient is mediated by transforming growth factor-h. Cancer Res. *62:5267–72.*
- Stern-Ginossar, N; Mandelboim, O.(2010). Receptors on NK cells. In: Lotze, MT; Thomson, AW; eds. Natural killer cells: basic science and clinical application. 1st ed. London; UK: Academic Press.*:155-68.*
- Subiza, J.L; Viñuela, J.E; Rodriguez, R; Gil, J; Figueredo, M.A; De la Concha, E.G.(1989). Development of splenic natural suppressor (NS) cells in Ehrlich tumor-bearing mice. Int. J. Cancer. *44: 307.*
- Sutlu, T; Alici, E. (2009) Natural killer cell-based immunotherapy in cancer. current insights and future prospects. *J Intern Med. 266:154-81*.
- Terabe, M; et al. (2000). NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat. Immunol. ***1****:515– 520.*
- Terabe, M; et al. (2003). Transforming growth factor-{beta} production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. J. Exp. Med. ***198****:1741–1752.*
- Toutirais O; Chartier P; Dubois D; et al. (2003). Constitutive expression of TGF-h1; interleukin-6 and interleukin-8 by tumor cells as a major component of immune escape in human ovarian carcinoma. Eur Cytokine Netw . *14: 246–55.*
- Trikha, M; Corringham, R; Klein, B; Rossi, J.F. (2003). Targeted antiinterleukin- 6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence. Clin Cancer Res. *9(13):4653–65.*
- Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat. Rev. Immunol. *3: 133–146*.
- Villegas, F. R; S. Coca; et al. (2002). Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer. ***35****(1):23-28*.
- Vivier, E; Nunes, J.A; Vely, F. (2004). Natural killer cell signaling pathways. Science3. *06:1517–19.*
- Webb, S.D; Sherratt, J.A; Fish, R.G. (2002). Cells behaving badly: a theoretical model for the Fas/FasL system in tumour immunology. Math Biosci. *179:113–29.*
- Xu, W; Liu, L.Z; Loizidou, M; Ahmed, M; Charles, I.G. (2002). The role of nitric oxide in cancer. Cell Res. *12(5/6):311–20.*
- Yogeeswaran,G; Gronberg,A; Hasson,M; Dalianis,T; Kiessling,R; Welsh, R.M. (1981). Correlation of glycosphingolipids and sialic acid in YAC-1 lymphoma variants with their sensitivity to natural killer cell mediated lysis. Int. J Cancer. *28:517-526.*