“Study of tumor driven alteration of T and NK cell activity”

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In

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Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled "**Study of tumor driven alteration of T and NK cell activity"** submitted by **Hari Krishnan Balasubramanian (2K15/IBT/04)** in partial fulfilment of the requirement for the award of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by him under my guidance.

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

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LIST OF SYMBOLS, ABBREVIATIONS AND NOMENCLATURE

S. No	Abbreviation	Expansion
1.	ATP	Adenosine tri phosphate
2.	BAT-3	Antigen-B associated transcript - 3
3.	$CD4^{+}$ T cell	Cytotoxic T cells with CD4 surface protein
4.	$CD8^{+}$ T cell	Cytotoxic T cells with CD8 surface protein
5.	CTL	Cytotoxic T lymphocytes
6.	DMEM	Dulbecco's Modified Eagle's Medium
7.	DNAM-1	DNAX accessory molecule 1
8.	EDTA	Ethylene diamine tetra acetic acid
9.	EGTA	Ethylene glycol-bis (2-aminoethylether) tetra acetic acid
10.	HEPES	2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid
11.	IDO	Indole amine 2,3 dioxygenase
12.	IFN-γ	Interferon-γ
13.	IL-2	Interleukin-2
14.	KIR	Killer immunoglobulin like receptors
15.	LILR-B1/ILT2	Leukocyte Ig like receptor B1 - ILT2 isoform
16.	MHC	Major histocompatibility complex
17.	MICA/B	MHC class 1 related chain - A/B
18.	MLV	Moloney leukaemia virus

19.	NK cell	Natural killer cell
20.	NL buffer	Nuclear lysis buffer
21.	NO	Nitrous Oxide
22.	PB	Peripheral blood
23.	PBS	Phosphate buffered saline
24.	PGE2	Prostaglandin E2
25.	PVR	Polio virus receptor
26.	RPMI medium	Roswell Park Memorial Institute medium
27.	SDS-PAGE	Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis
28.	SF buffer	Subcellular fractionation buffer
29.	TEMED	Tetra methyl ethylene diamine
30.	TGF-β	Transforming growth factor – β
31.	TME	Tumor Micro Environment
32.	ULBP	UL16 binding proteins
33.	w/v	Weight by volume
34.	v/v	Volume by volume

1. ABSTRACT

Both NK cells and T cells play an important role in anti-tumor immunity. T-cells are activated through primary signal constituted by recognition of tumor antigen presented upon self MHC and co-stimulation by other co-stimulatory molecules. Hence tumor cells need both primary and co-stimulatory ligands to be susceptible to T cell cytolysis. NK cells on the other hand do not require MHC mediated antigen presentation and can bind directly to the ligands expressed by the tumors. NK cells express inhibitory receptors which upon binding to HLA molecules send a strong inhibitory signal that prevents NK cell mediated tumor cell lysis. Each NK cell expresses a multitude of activating and inhibitory receptors and a delicate balance of the two opposing signals determines the fate of NK cell stimulation. It has been shown that tumors themselves modulate the activity of T cells and NK cells by altering the expression of different ligands, in order to evade the immune system. In the present study we analyzed the expression levels of both activating as well as inhibitory ligands in various tumors for both NK and T cells. The tumors under consideration were themselves clubbed into two groups based on the levels (high/low) of HLA expression. Our studies showed that in case of the tumors that expressed high levels of HLA, the tumor cells try to actively decrease co-stimulatory signals for T cells in order to evade T cell mediated cytotoxicity. However this also results in increased activation signals in NK cells. Multiple activation signals synergize in NK cells and may also overcome the inhibition through KIR-MHC-I ligation, in such tumors with high MHC expression. These findings suggest that in case of tumors with high MHC expression that are resistant to T cell therapies, a new strategy of combinatorial therapy using co-stimulation of NK cells may prove to be more effective.

Key words: NK cell, T cell, HLA, MHC, co-stimulation, cytotoxicity, combinatorial therapy.

2. INTRODUCTION

The immune system evolved as a defence mechanism against the different adverse factors an organism encounters in its lifetime. The immune system is capable of providing defence against a wide range of pathogens, be it a tiny microbe or a long tapeworm. This is possible due to the enormous variety of cells and molecules produced by the immune system, which act in synchronization to identify and eliminate the foreign invaders.

The mode of action of the immune system can be divided into two main functions - recognition and response, i.e., the ability to recognize and differentiate the foreign particles from those of the self and subsequently target them in a selective manner, shielding the self. This is due to the ability of the immune system to recognize molecular patterns that differentiate the self from the non-self. The immune system is also able to recognize the damaged host cells which may lead to the formation of tumors.

The immune system comprises of two components - the innate immune system and the adaptive immune system. The innate immune system is considered to be the first line of defence in an organism and comprises of cells and molecules that are pre-deployed even before the onset of an infection. The innate immune system is capable of eliminating most infections at the very onset or within hours of first onset. However, those infections which escape the innate immune system or persist in spite of it are challenged by the adaptive immune system which comes into action generally a few days after the onset of the infection. Unlike the innate immune system, which can only differentiate the self from the non-self, the adaptive immune system is capable of recognizing the subtle differences in the molecular patterns of the foreign invaders. This leads to a highly specific and targeted attack mounted by the adaptive immune system, which is much more effective than the innate immune response.

The Natural Killer (NK) cells are lymphoid cells derived from the bone marrow. The NK cells can induce apoptosis and/or cell death in tumor cells and cells infected by viruses even in the absence of specific immunization [1]. NK cells are a fundamental part of innate immunity, though recent evidence of memory function in NK cells has also been reported.

The NK cells were identified in 1975 [2, 3] and were classified as lymphocytes due to their morphology, source of origin (lymphoid progenitor cells in the bone marrow) and expression of lymphoid markers. However, due to the lack of antigen-specific cell surface receptors, these cells were recognized as components of the innate immune system.

Various studies have established the involvement of NK cells in defence against viral infections (especially herpes virus infections) [4] as well as in tumor associated immune response [5]. NK cells are present in non-human mammals and the presence of their orthologs has been identified in various other vertebrates as well, adding to the speculation about their importance [6]. The activation, proliferation and effector functions of NK cells are controlled by a sophisticated repertoire of cell surface receptors which also help differentiate between normal healthy cells and abnormal/infected cells. These receptors are coded by normal genes and not generated by somatic recombination, as in the case of adaptive immune system.

T cells originate in the bone marrow and migrate to the thymus for maturation. T cells can recognize antigens only when they are bound to MHC (Major Histocompatibility Complex), a class of proteins expressed in the cell-membrane. T cells are a part of the adaptive immune system.

Even though most of the cancer cells express antigens readily recognizable by the T cells, spontaneous rejection of such cancer cells is extremely rare. In such cases, a potential absence of role of co-stimulatory receptors has been implicated. For example, most cancer cells are lacking in terms of expression of co-stimulatory molecules like CD80 and CD86. In the absence of co-stimulatory signals, T cells are not able to exculpate immunity against the antigens.

3. REVIEW OF LITERATURE

1. NK cells - Mode of action

The killing mechanism of NK cells is similar to that of the $CD8^+$ cytotoxic T lymphocytes (CTL) in that both the mechanisms use perforin and granzymes. NK cells secrete interferon-γ, similar to the CTL and $CD4^+$ T_h1 cells, however; the main difference, which distinguishes NK cells from CTL and $CD4^+$ cells, is the inability to secrete IL-2. It has been found that majority of the NK receptors are also expressed on at least a subset of T lymphocytes, in particular on $\gamma\delta TCR^+$ T cells and on activated $CD8^+$ T cells [7].

The NK cells probably coevolved with the T cells, given the fact that both are focused on recognition of conventional and nonconventional Major Histocompatibility Complex (MHC) molecules. This also distinguishes the NK cells from phagocytes (macrophages and granulocytes) which rely only on the conserved pattern-recognition receptors like the toll-like receptors. Functional MHC molecules have been identified in fish, but not in lower organisms. Similarly, NK cells are also not found in organisms lower than the fish. These factors (lineage relationships, receptor repertoire and effector functions) indicate NK cells to be a transient cell type between the innate and adaptive immune systems [7].

In B cells and T cells, the antigen receptors regulate the differentiation, activation, and effector functions whereas in NK cells, it is much more complicated. There are inhibitory receptors present on the NK cells, which act as a fail-safe to prevent inadvertent stimulation, resulting in its action against normal host cells. NK cell recognition involves the following steps:

1. Initial binding to potential target cells
2. Interactions of the ligands available on the target cell with the activating and inhibitory receptors on NK cells
3. Integration of the signals generated by these interactions
4. Determination of the course of action by NK cells - whether to detach or to stay on and respond.

The main differentiating factor between NK cells and naive T cells is that the NK cells are designed as action-ready effector cells ready for an immediate response, unlike naive T cells which require undergoing transcription, translation and/or cell proliferation. This is possible due to the expression of granzymes and perforin by NK cells, enabling the trigger of lytic response within minutes [7]. Transcripts of IFN-γ are expressed and readily available in

the NK cells for the immediate initiation of cytokine synthesis upon activation. A study of mouse bone marrows has shown the presence IFN-γ transcripts in the NK cell progenitors even during their early developmental stages [8]. Thus, at no stage of development does an NK cell lineage resemble a 'naive' T cell, which has to undergo proliferation, chromatin remodelling of cytokine genes, de novo transcription and translation of granzymes and perforin before it becomes a competent effector cell. Considering this, the phenotype of a resting NK cell is more similar to an effector CD^+ T cell in terms of cell surface receptors expression and presence of effector molecules. The NK cells are highly suited for early defence as they are present in a ready-to-act state, combined with the fact that their receptors are present on a large proportion of cells [7].

2. Reconsidering the 'Missing-self hypothesis'

The initial notion was that the recognition process of NK cells was not restricted by MHC (Major Histocompatibility Complex) molecules as they were able to target and kill both the cells that were lacking MHCs as well as the ones which expressed allogenic MHC molecules. However, this notion was proven wrong as studies by Karre and colleagues demonstrated that NK cells seemed to be actively inhibited when they encountered certain tumor cells expressing MHC class 1 molecules [9]. Further, it was shown that NK cells were able to recognize and eliminate normal host hematopoietic cells that lacked MHC class 1 molecules, by studying the cell-dependent rejection of bone marrow cells from β2-microglobulin deficient syngeneic mice [10, 11]. The 'missing-self' hypothesis [12] states that NK cells monitored those cells that downregulated MHC class 1, as this was a common side-effect of cellular transformation or viral infection.

A common misconception that prevailed was that the NK cells attacked any cell that lacked MHC molecules, as they were not able to engage an inhibitory NK cell receptor for MHC class 1. The missing-self hypothesis can be modified as 'NK cells patrol for abnormal cells that lack MHC class 1 or overexpress ligands for activating NK cell receptors' [7]. It can be said that the inhibitory MHC class 1 receptors on NK cells act as a rheostat that regulates and weakens the signals generated by activating receptors. Experimental data indicate that the role of MHC class 1 inhibitory receptors may be restricted to weakening the NK cell effector function, rather than completely stopping it. The extent to which the effector function is weakened may be proportional to the amount of MHC class 1 on the target cell surface. Studies have proven that NK cells can effectively eliminate the target cells which stimulate multiple activating NK receptors simultaneously [13] or stimulate a sufficiently strong activating NK receptor [14, 15], even if they ligate the MHC class-1 receptors on the NK cells.

As stated by Lanier [7], a corollary of the missing-self hypothesis is that the failure of NK cells to respond to a potential target can be due to either the active inhibition mediated by the inhibitory receptors or the absence of sufficient activation signals to initiate a response.

An example of the latter situation may be represented by encounters between human erythrocytes and NK cells circulating in peripheral blood. Although human red blood cells do not express MHC class I, NK cells do not attack them; therefore, erythrocytes may lack ligands capable of engaging the activating NK cell receptors. An alternative explanation for the inability of NK cells to harm normal tissues possessing low (e.g., neural tissues) or no (e.g., erythrocytes) MHC class 1 is the possibility that this target cell protection is mediated by inhibitory receptors recognizing non-MHC ligands [7].

Figure 1 demonstrates the simultaneous interaction of activating and inhibitory receptors of NK cells in varying degrees and the corresponding outcome (either activation or inhibition of the NK cells).

3. NK cells and tumors

The major inhibitory receptors on NK cells are mainly HLA class I-binding receptors, comprising Killer Immunoglobulin-like Receptors (KIRs), CD94/NKG2A and Leukocyte Ig-like Receptor B1 (LILR-B1/ILT2) [16]. The major activating receptors are NKp46, NKp30, NKp44 [17], NKG2D [7] and DNAX accessory molecule-1 (DNAM-1) [18].

The ligands for activating receptors are stress inducible molecules including major histocompatibility complex (MHC) class 1 - related chain A/B (MICA/B) and UL16 binding proteins (ULBPs) recognized by NKG2D [19, 20]. Other ligands include the poliovirus receptor (PVR, CD155) and Nectin-2 (CD112) that bind to DNAM-1 [21], the human leukocyte antigen-b associated transcript 3 (BAT-3) and the recently discovered B7-H6 molecule both recognized by NKp30 [22, 23]. Generally, these ligands are not found on normal cells, whereas they become highly expressed in tumor cells. A series of costimulatory receptors and adhesion molecules such as 2B4, NTBA, NKp80 and LFA-1 are also involved in the regulation of NK cell activation [24]. NK cells can recognize tumors that successfully evade T-cells due to loss or down-regulation of MHC class 1 antigens, thus playing a complementary role in anti-tumor activity.

NK cells are promising candidates in adoptive immunotherapy of cancer [25]. A high degree of natural cytolytic activity of peripheral blood (PB) lymphocytes has been associated with a reduced cancer risk in a 11-year follow-up study [26]. In addition, NK cell infiltration in solid tumors has been found to be associated with a better prognosis [27]. Notably, recent

evidences also suggest that NK cells, besides their direct cytolytic effect on the tumor cells, may also be involved in shaping the subsequent adaptive immune response towards a T_H1 profile, thought to favour antitumor responses [28, 29].

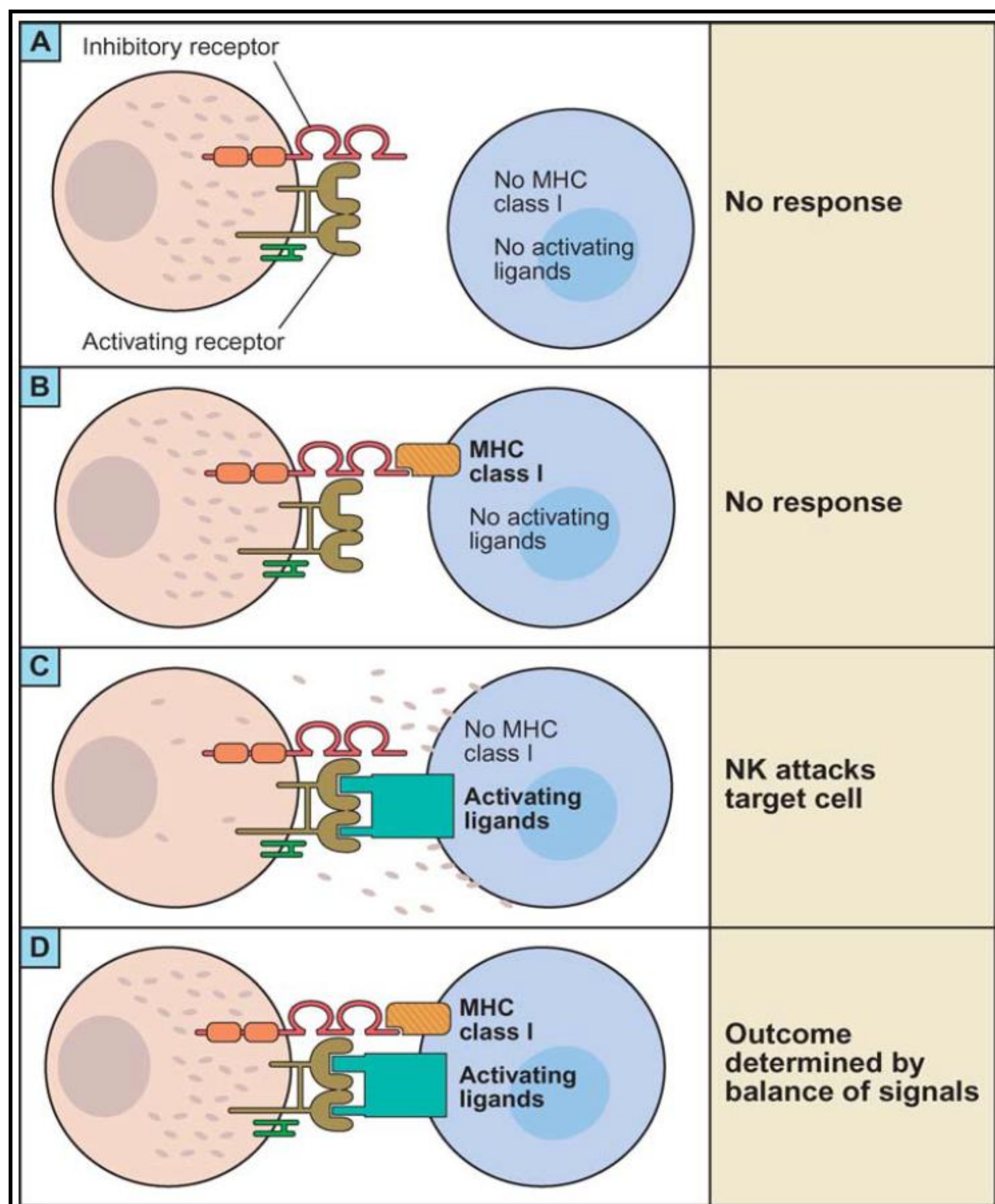


Figure 1: Graphic depiction of encounters between NK cells and potential targets and possible outcomes. In some circumstances, inhibitory receptors recognizing ligands other than MHC class I proteins may suppress NK cell responses. When interacting with target cells expressing ligands for both inhibitory and activating receptors, the outcome is determined by the summation of the strength of signals. The amount of activating and inhibitory receptors on the NK cells and the amount of ligands on the target cell, as well as the qualitative differences in the signals transduced, determine the extent of the NK cell response. (Taken from: Lewis L. Lanier, 2005).

Despite the existence of different mechanisms by which NK cells may eliminate cancer cells, NK cell-based immunotherapy has resulted in limited clinical success [30]. This may be due to the poor capacity of adoptively-transferred NK cells to home in on tumor sites. In addition, tumor cells may have developed various escape mechanisms to avoid NK mediated response. In this context, a number of cytokines, growth factors and enzymes synthesized by tumors and/or stromal cells have been reported to exert suppressive effects on cells involved in immune response [31, 32]. For example, transforming TGF- β, IL-10 and prostaglandin E2 (PGE2) as well as the activity of the tryptophan metabolising enzyme indole-amine 2,3-dioxygenase (IDO) may contribute to the establishment of immune tolerance within the tumor microenvironment. Limited information is available on the effect of melanoma cells on NK cell function. Melanoma cells co-cultured with PB NK cells sharply downregulate the expression of major activating NK receptors including NKp30, NKp44 and NKG2D, thus markedly affecting the NK-mediated cytotoxicity against melanoma cells [16].

4. Tumor microenvironment and NK cell mediated immune response

NK cells possess a diverse range of activating and inhibitory receptors which enables them to recognize and kill a wide array of tumor cells [33]. Apart from their role in the inhibition of early stages of tumor formation, NK cells are also capable of eradicating large solid tumors. This is dependent on the massive infiltration of proliferating NK cells as a result of the release of IL-15 by tumor cells in the tumor microenvironment (TME) [34].

3.4.1. Impairment of NK cell function by cells from the tumor microenvironment

The tumor microenvironment is made immunosuppressive through the activation of resistance mechanisms. Hypoxic tumor cells induce the homing of bone marrow derived CD45+ myeloid cells to tumor areas by secreting cytokines such as tumor necrosis factor (TNF)- α and stromal cell-derived factor 1 (SDF-1) [35]. This results in a highly immunosuppressive environment for NK cells [36]. Myeloid-derived suppressor cells (MDSCs) are major components of the immunosuppressive network that compromises the NK cell- and T cell-dependent anti-cancer activity [37]. The MDSCs produce IL-10 which decreases the production of IL-12, a pro-inflammatory cytokine involved in the activation of NK cells, by the macrophages [38].

Hypoxia in TME induces the secretion of the immunosuppressive cytokine TGF-β from gastric tumor cells, which results in the proliferation and accumulation of regulatory T (T_{reg}) cells [39]. Human T_{reg} cells have the ability to induce anergy of NK cells through

membrane-bound TGF-β and subsequently downregulate NKG2D, an activating receptor on the surface of NK cells.

Cancer cells have the ability to activate cancer associated fibroblasts (CAFs) via the secretion of TGF-β or IL-6 [40, 41]. The IL-2 induced upregulation of the activating receptors NKp44, NKp30 and DNAX accessory molecule-1 (DNAM-1) on the NK cell surface is inhibited by the CAFs [42].

3.4.2. Impairment of NK cell function by tumor cell derived factors

The MHC class 1 chain-related (MIC) molecules MICA and MICB, along with the UL16-binding proteins, are known to be expressed on the surface of majority of the carcinomas and few hematopoietic malignancies. Hypoxia increases the shedding of MICA from the surface of tumor cells through the impairment of nitrous oxide (NO) signalling. The interaction of cell surface MIC molecules with NKG2D is vital for the activation of target cell killing. Presence of soluble MIC in the TME results in a downregulated expression of NKG2D and CXC chemokine receptor (CXCR) 1 on the surface of NK cells [43].

Hypoxic stress induces the formation of HLA-G (a non-classical MHC class I molecule) dimers on the surface of melanomas, which protects from NK cell mediated killing through the secretion of INF-β and INF-γ [44]. Under hypoxic stress, NK cells lose the ability to up-regulate expression of NKp46, NKp30, NKp44 and NKG2D receptors in response to activating cytokines.

5. Ligand(s) distribution profile: NK susceptible vs. NK resistant cell lines

In a recent study by Richa et. al. (in press), several ligands (p30CA, p12, p15MA and p15E) present on the surface of a NK susceptible cell line (YAC1) were found to exhibit significant binding towards activating receptors (Ly49H) on the NK cell surface. Similarly, several ligands (galactin-3, CD-63, gp70 and p30) present on the surface of a NK resistant cell line (P815) were found to exhibit significant binding towards inhibiting receptors (Ly49A) on the NK cell surface. This study indicates a possible involvement of ligands in the engagement of activating and/or inhibiting receptors, which might have a role to play in determining whether the NK cells mount an attack.

6. Natural Killer Cell Receptors

NK cells originate from the $CD34^+$ hematopoietic progenitor cells and their maturation requires the presence of cytokines found in the bone marrow. There are at least a couple of known families of receptors involved in the recognition of classical MHC class I

molecules expressed on the target cells' surface and differentiating them as healthy or diseased (virus infected as well as tumor) based on the MHC expression levels. Almost all of the NK cell surface receptors fall into either one of the two categories: Immunoglobulin (Ig) superfamily (eg: KIRs) and C-type lectin-like domain (CTLD) superfamily (eg: CD94/NKG2s) [45]. The probable reason behind such nomenclature is that the extracellular part of CTLD receptors resembles the carbohydrate recognition domain from C-type lectin whereas in case of KIR receptors, the extracellular part comprises of immunoglobulin-like domains. Both inhibitory as well as activating receptors are found in either of the superfamilies. It it to be noted here that unlike B and T cell receptors, which form as a result of rearrangement; NK cell receptors are preformed and their variability is attributed to the genetically defined subset of genes that get modulated during NK cell development into a variety of complex combinatorial expression patterns [46,47]. This preformed receptor pool enables the NK cells to regulate/act upon pathogen attacks or cellular transformation events even before the time period required for the clonally expanded set of antigen-specific B and T cells to take action [48]. The members of CTLD family in humans are NKG2A, NKG2B, NKG2C, NKG2D, NKG2E and NKG2F.

3.6.1. Killer Immunoglobulin-like Receptors (KIR)

KIRs are a group of polymorphic cell surface molecules found on NK cells as well as a small population (8%) of Natural Killer T (NKT) cells. By virtue of recognition of HLA class I molecules, they provide an alternative mode of modulation of immune response to foreign/damaged cells [49]. KIR proteins comprise of either two or three Ig-like domains, as denoted in their nomenclature as 2D or 3D. In addition, an S on an L is used to indicate whether the cytoplasmic tail present is short or long. When contacted with MHC class I ligands present on the target cells' surface, KIRs regulate the NK cell response by passing-on activating or inhibitory signals [50]. HLA-C allotypes are recognized by the two-domain KIRs, whereas HLA-B allotypes are recognized by the three-domain KIRs.

7. Synergy of coactivation receptors leads to activation of NK cells

NKG2D and the NCRs (NKp46, NKp44 and NKp30) are among the receptors on NK cells considered to be activating [51]. The expression of ligands that bind to NKG2D on cells which are resistant to NK cytotoxicity can lead to the lysis of target cells [52]. This observation led many to infer that NKG2D was sufficient in induce natural cytotoxicity and that it is resistant to MHC-I mediated inhibition. Antibodies targeted towards any one or more NCRs resulted in the blocking of tumor cells' lysis [53]. This led to the inference that NCRs are necessary for the activation of cytotoxicity. Through cross-linking experiments on NK

receptors with antibodies, CD16 was identified to be the only receptor that was sufficient for the activation of degranulation in NK cells [54]. Other receptors like NKG2D, NKp46, NKp44, DNAM-1 etc needed a combination with one or more of the other receptors to stimulate degranulation. Certain but not all combinations of receptors in pairs led to the induction of granulation, giving proof of synergistic activation in NK cells [51].

8. T cell co-stimulation

The activation of T cells requires a combination of primary stimulation as well as co-stimulation. The primary stimulation is in the form of antigens displayed by APCs where they are bound to MHC molecules. This step helps identify the antigen and enables the elucidation of antigen specific response. An additional stimulatory signal, termed as co-stimulatory signal due to its action in conjunction with an antigen-bound APC, is essential and provided by the binding of certain molecules on the APCs with corresponding co-stimulatory receptors [55]. If the stimulatory signal is not accompanied by a co-stimulatory signal, T cells either die or enter anergy (state of unresponsiveness) [56]. Several co-stimulatory ligands and their corresponding co-stimulatory receptors have been identified.

3.8.1. B7.1-CD28 and B7.2-CD28 pathways of co-stimulation

B7.1 (CD80) and B7.2 (CD86) are known to bind with CD28 in T cells and are perhaps the strongest co-stimulatory signal system discovered so far [57]. Binding of B7.1/B7.2 with CD28 can induce one or more of the following responses:

1. Increased cytokine expression [58]
2. Cytokine mRNA stabilization [59]
3. Enhancement in the uptake and utilization of glucose [60]
4. Up-regulation of T cell survival [61]
5. Progression of T cell response in case of subsequent exposure to same antigen [62]

3.8.2. OX-40 and OX-40L

OX40 (CD134, TNFSF4) is a co-stimulatory molecule of the TNF receptor superfamily and its ligand OX40L (CD252, TNFSF4) is present on T cells as well as endothelial cells [63, 64]. Binding of OX40 with its corresponding receptor on the T cells induces proliferation, enhancement in T cell survival and cytokine secretion. The survival of

T cells is enhanced in part due to the increase in the expression of Bcl-2 family anti-apoptotic molecules [65].

3.8.3. HVEM and LIGHT

HVEM (Herpes Virus Entry Mediator) is a member of TNF receptor superfamily, also known as CD270 and TNFRSF14. Both resting as well as activated T cells have shown the expression of this receptor. LIGHT (CD258, TNFSF14) binds to HVEM and acts as a co-stimulatory signal for T cells. LIGHT can induce the production of chemokines, resulting in the recruitment of T cells.

3.8.4. ICOS and ICOS-L

Inducible co-stimulator or ICOS (CD278), belonging to the B7 family, is found mainly in $CD4^+$ and $CD8^+$ T cells. Its ligand ICOSL (CD275, B7-H2) has been found to be expressed mainly on APCs (macrophages, B cells and dendritic cells).

3.8.5. GITR and GITRL

Glucocorticoid-induced TNF receptor or GITR (TNFRSF18) is found mainly in $CD4^+$ and $CD8^+$ T cells. Its ligand GITRL (TNFSF18) has been found to be expressed mainly on APCs (macrophages, B cells and dendritic cells).

3.8.6. CD40 and CD40L

CD40 is a member of the TNF receptor superfamily and plays an important role in the functioning of B cells. Its ligand CD40L (CD154) in found to be expressed on activated T_h cells and also on NK cells and platelets. The ligation of CD40L with CD40 in APCs results in the secretion of IL-12, which in turn drives the effector CTL mediated immune response.

3.8.7. CTLA-4

CTLA-4 (CD152) is a strong negative regulator of T cell activation [66]. CTLA-4 has the ability to bind to both B7.1 and B7.2 with an estimated 10-20 times higher affinity than that of CD28.

3.8.8. PD1

Programmed cell death-1 or PD1 (CD279) is a strong inhibiting co-stimulatory receptor, belonging to the Ig (Immunoglobulin) superfamily. There are two known ligands with affinity towards the PD1 receptor, namely PD-L1 (B7-H1) and PD-L2 (CD273, B7-DC).

PD-L1 expression is found in many tumors like Glioblastoma, melanoma etc. and in cancers of the head, neck, colon, lung, breast, ovary, kidney and stomach [67, 68].

4. AIM

“Study of tumor driven alteration of T and NK cell activity”

5. OBJECTIVES

1. Identification of tumors with high/low expression of HLA.
2. Identification of activating/inhibitory receptors on NK and their corresponding ligands.
3. Identification of activating/inhibitory co-receptors on T cells and their corresponding ligands.
4. Determination of the level of expression of ligands by the tumors for both activating/inhibitory receptors and co-receptors on NK cells and T cells respectively.
5. Analysis and interpretation of the data for useful inferences w.r.t the working hypothesis.
6. Identify an NK susceptible and an NK resistant cell line for in-vitro testing of the hypothesis.
7. Study of growth kinetics for the aforementioned cell lines.
8. Determination and standardization of a suitable method for the extraction and fractionation of membrane protein fractions from the aforementioned cell lines.

6. WORKING HYPOTHESIS

In case of tumors with high levels of HLA expression, antigens are presented to T cells in conjunction with self MHC and through co-stimulation by other co-stimulatory ligands like CD80, OX40L, and ICOS-L etc. The expression of HLA by tumors results in a strong inhibitory signal for NK cells through the KIR-MHC-I ligation. However, it is also well known that there is a multitude of activating receptors which work in synergy.

Hence we explored the role of tumor cells in terms of their ability to alter the T cell or NK cell activity in tumor microenvironment.

7. METHOD

7.1. Identification of tumors with high/low expression of HLA

1. The HLA expression levels were determined for a wide range of cancers through literature survey.
2. Based on the HLA expression data, the tumors were categorized as high/low HLA expressing tumors.

7.2. Identification of activating/inhibitory receptors on NK cells and their corresponding ligands

1. An exhaustive list of previously reported receptors on the NK cells was prepared and characterized as activating/inhibitory.
2. The ligands with affinity towards the corresponding receptors identified above were also identified through literature survey.

7.3. Identification of activating/inhibitory co-receptors on T cells and their corresponding ligands

1. An exhaustive list of previously reported co-stimulatory receptors on the T cells was prepared and characterized as activating/inhibitory.
2. The ligands with affinity towards the corresponding co-stimulatory receptors identified above were also identified through literature survey.

7.4. Determination of the level of expression of ligands

1. The expression levels of the ligands identified above with affinity towards the corresponding activating/inhibitory receptors on the NK cells were determined from Human Protein Atlas.
2. The expression levels of the ligands identified above with affinity towards the corresponding activating/inhibitory co-stimulatory receptors on the T cells were determined from Human Protein Atlas.

7.5. Analysis and interpretation of the data

1. The ligands' expression level data was analyzed for determining the validity of the working hypothesis.

7.6. Study of growth kinetics for NK susceptible and NK resistant cell lines

1. YAC-1 was used as a prototypic NK susceptible cell line whereas P815 was used as a prototypic NK resistant cell line.
2. **Cell culture of NK resistant cell line (P815):**

P815 is cultured in high glucose DMEM medium (formulation given in Appendix A).

The additives added are:

1. Antibiotics (Streptomycin, Penicillin, Nystatin) to prevent contamination.
2. 10% fetal bovine serum as protein supplement.
3. Sodium bicarbonate for maintaining pH in the range of 7.0 - 7.5.
4. Glutamine as amino acid source.
5. HEPES buffer (formulation given in Appendix B).
6. Sodium pyruvate to aid production of ATP.

3. **Cell culture of NK susceptible cell line (YAC-1):**

YAC-1 is cultured in RPMI-1640 medium (formulation given in Appendix C).

The additives added are:

1. Antibiotics (Streptomycin, Penicillin, Nystatin) to prevent contamination.
2. 10% fetal bovine serum as protein supplement.
3. Sodium bicarbonate for maintaining pH in the range of 7.0 - 7.5.
4. Glutamine as amino acid source.
5. HEPES buffer (formulation given in Appendix B).
6. Sodium pyruvate to aid production of ATP.

4. Cells were seeded at 1 x 10^5 per ml.
5. Cell count was performed by extracting 100 µl sample each time at time intervals of 24 hours up to 96 hours.

7.7. Determination and standardization of a suitable method for the extraction and fractionation of membrane

1. Isolation of cell membrane:

1. Sub-cellular Fractionation of Cultured Human Cell Lines

Note: The sample has to be kept at 4 °C on ice at all times! All buffers must be ice-cold when used. All centrifugations are done in the Eppendorf Micro-centrifuge unless stated otherwise.

1. The cell culture is centrifuged to obtain the cells as pellet.

2. The pellet is washed twice with ice-cold PBS (formulation given in Appendix D), 500 µl of SF buffer (formulation given in Appendix E) is added immediately and put on ice.

3. Cell scraper is used to collect the lysate and transferred to a 1.5 ml Eppendorf tube.

4. The lysate is agitated at 4 °C for 30 min at around 30-50 rpm on a tube roller.

5. The lysate is centrifuged at 720x g at 4 °C for 5 min. The supernatant is carefully transferred to a new 1.5 ml tube.

6. The supernatant is centrifuged at 10,000x g at 4 °C for 10 min.

7. The supernatant is transferred to a new 1.5 ml tube. This is the cytosolic and membrane fraction.

8. The cytosolic and membrane fraction is centrifuged in an ultracentrifuge at 100,000x g at 4 °C for 1 h.

9. The pellet is washed with 500 µl of SF buffer and re-suspended by pipetting.

10. The pellet is centrifuged at 100,000x g at 4 °C for 1 h.

11. The supernatant is removed and the pellet is re-suspended in NL buffer (formulation given in Appendix F). This is the membrane fraction.

2. Preparation of Soluble and Membrane Protein Fractions

Note: The sample has to be kept at 4 °C on ice at all times! All buffers must be ice-cold when used. All centrifugations are done in the Eppendorf Micro-centrifuge unless stated otherwise.

1. The cells are centrifuged to obtain a pellet.

2. The pellet is washed twice with ice-cold PBS.

3. 1 ml of cold homogenization buffer (composition given in Appendix J) is added to the pellet in the bottom of a micro-centrifuge tube.

4. The pellet is homogenized using a Teflon pestle with 20 manual up and down strokes.

5. The sample is sonicated using two 5-seconds pulses separated by 30 seconds in between.

6. Centrifugation is done to remove the intact cells, nuclei and cell debris. Pellet is discarded.

7. The supernatant is centrifuged at 100000 g for 1 hour at 4ºC.

8. The supernatant is removed, which comprises of soluble proteins.

9. The pellet is washed with homogenization buffer.

10. The sample is centrifuged at 100000 g for 1 hour at 4ºC.

11. The supernatant is discarded. The pellet contains the membrane fraction.

2. Fractionation by SDS PAGE:

1. Preparing the separating gel:

The casting frames were set by clamping two glass plates in the casting frames on the casting stand.

The gel solution was prepared in a small beaker as per the concentration needed as given in the following table:

Acrylamide percentage	6%	8%	10%	12%	15%
H_2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
1.5 M Tris (pH=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% (w/v) SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) Ammonium persulfate (AP)	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

Table 1: Concentration of different ingredients for separating gel preparation.

The solution was swirled gently but thoroughly.

Appropriate amount of the separating gel solution, as listed in the table below, was pipetted into the gap between the glass plates.

Thickness of the gel	Vol. of stacking gel	Vol. of separating gel
0.75mm	2ml	4ml
1.0mm	3ml	6ml
1.5mm	4ml	8ml

Table 2: Volume of gel solutions to be added for obtaining the necessary thickness.

In order to obtain a smooth horizontal top surface in the gel, isopropanol is added into the gap till it overflows.

The gel is left undisturbed for 20-30 min for proper setting of the gel.

2. Preparing the stacking gel:

The separating gel is obtained by discarding the excess water.

Stacking gel solution is pipetted until it overflows.

The comb for forming wells in the gel is carefully inserted, avoiding the formation of air bubbles.

The gel is left undisturbed for 20-30 min for proper setting of the gel.

3. Setting up the apparatus:

After the gel gets set properly, the comb is gently removed. The glass plates are removed and set in the cell buffer dam. The running buffer or electrophoresis buffer (formulation given in Appendix G) is poured until it overflows and reaches sufficient level in the outer chamber as well.

4. Preparation of the samples:

The samples are mixed with the loading buffer (formulation given in Appendix H) and heated in boiling water for 5-10 min.

5. Running the samples:

The samples are carefully poured into the wells and it has to be ensured that no overflow takes place. Protein marker is added into the first lane. The top was covered and the electrodes were connected.

An appropriate voltage is set and electrophoresis is run.

The SDS PAGE is stopped when the lowermost band of the protein marker reaches near the bottom of the glass plate.

8. RESULTS

HLA or ‘Human Leukocyte Antigens’ are a group of cell surface molecules which are involved in the presentation of antigenic peptides to TCRs (T-cell receptors) present on the surface of T cells.

HLA class-I comprises of HLA-A, HLA-B and HLA-C along with some non-classical HLAs like HLA-E and HLA-G. HLA class-II comprises of the HLA-DP, HLA-DQ and HLA-DR groups in humans.

In case of tumors which exhibit a high level of HLA expression, both the NK cells as well as the T cells would play an important role in the immune reaction. However, it is expected that the role of T cells would be more significant due to the enhanced possibility of MHC mediated antigen presentation as a virtue of high HLA expression in these tumors.

In context of such tumors, by virtue of possessing a high HLA concentration, these tumors would present antigens to T cells in cognition with self MHCs. Each clone of T cells has a receptor specific towards a unique yet different antigen; hence, it would not be viable to down-regulate the T cell mediated cytotoxicity through the down-regulation of primary antigens. There would always be a possibility that one or the other clonal set of T cells may be stimulated. As an alternate strategy, these tumors have till date been found to suppress the levels of MHC-I expression. In our study, we are showing another mechanism by which the tumor cells evade T cell immunity by down-regulating the expression of co-stimulatory ligands, which would be universally effective against polyclonal T cells.

The activation of NK cells requires a synergy of multiple activating signals, whereas the inhibition is more robust, potent enough to inhibit the effect of activation that happens through the synergy of multiple activating signals. Due to the presence of MHC-I, these tumors would be expected to send a strong inhibitory signal through the inhibitory receptors on NK cells.

This led us to explore the role of activating ligands for T cells in context of tumors with high HLA expression while also looking at the role of NK activating ligands acting in synergy for activation of NK cells.

1. Categorization of cancers based on level of HLA expression

Based on the level of expression of HLA type-1 and HLA type-2 in 167 cancer cells lines grouped according to their type/tissue of origin, the following tumors were identified

and categorized as exhibiting either high or low levels of expression of HLA (as reported by Boegel. S. et. al.) [92]:

Tumors with high HLA expression	Tumors with low HLA expression
Breast Cancer	Melanoma
Skin Cancer	Ovarian Cancer
Glioblastoma	Lymphoma
Colorectal Cancer	Prostate Cancer
Gastric Cancer	Lung Cancer

Table 3: Tumors segregated based on their level of HLA expression.

2. **Identification of T cell co-receptors and their corresponding ligands**

From literature review, the following list of co-receptors on the T cells and their corresponding ligands were identified:

Receptor	Ligand	Effect	Reference
CD28	CD80 (B7.1)	Activating	69
CD28	CD86 (B7.2)	Activating	69
OX40 (CD134, TNFRSF4)	OX40L (CD252, TNFSF4)	Activating	70,71
HVEM (CD270, TNFRSF14)	LIGHT (CD258, TNFSF14)	Activating	70
ICOS (CD278)	ICOS-L (CD275, B7-H2)	Activating	72,73
GITR (TNFRSF18)	GITRL (TNFSF18)	Activating	70
CTLA-4 (CD152)	CD80 (B7.1)	Inhibitory	74
CTLA-4 (CD152)	CD86 (B7.2)	Inhibitory	74
PD-1 (CD279)	PD-L2 (CD273, B7-DC)	Inhibitory	75,76
LFA-1 (CD11a/CD18)	CD54 (ICAM1)	Activating	77,78
LFA-1 (CD11a/CD18)	CD50 (ICAM3)	Activating	77,78
BTLA (CD272)	HVEM (CD270, TNFRSF14)	Inhibitory	79
CD154	CD40	Activating	80
CD28	CD80	Inhibitory	69
CD226 (DNAM1)	CD112	Activating	83
CD226 (DNAM1)	CD155	Activating	83
TIGIT (VSIG9,VSTM3)	CD112	Inhibitory	83
TIGIT (VSIG9,VSTM3)	CD155	Inhibitory	83
TIGIT (VSIG9,VSTM3)	CD113	Inhibitory	83
TIM2 (TIMD2)	SEMA4A	Inhibitory	83
CD2 (LFA2,OX34)	CD48	Activating	83
CD160 (BY55,NK28)	HVEM (CD270, TNFRSF14)	Inhibitory	81,82

Table 4: T cell co-receptors and their corresponding ligands.

3. Identification of NK cell receptors and their corresponding ligands

From literature review, the following list of cell receptors on the NK cells and their corresponding ligands were identified:

Receptor		Ligand	Effect	Reference
CTLDs				
NKG2A	In complex with CD94	HLA-E	Activating/ Inhibitory	86
NKG2B				86
NKG2C				86
NKG2E				86
NKG2F				86
NKG2D		MIC-A	Activating	84
		MIC-B	Activating	84
		ULBP-1	Activating	84
		ULBP-2	Activating	84
		ULBP-3	Activating	84
		ULBP-4/ RAET1E	Activating	84
		ULBP-5/ RAET1G	Activating	84
KLRG1		Cadherins	Inhibitory	87
DNAM-1		PVR	Activating	87
		CD122	Activating	87
NCRs				
Nkp46/NCR1		HSPG	Activating	87
Nkp44/NCR2		Viral HA	Activating	85
		PCNA	Inhibitory	85
Nkp30/NCR3		BAT-3/BAG6	Activating	87
		HSPG	Activating	87
		B7-H6	Activating	87
Nkp80		AICL	Activating	87
KIRs				
KIR2DL1		HLA-C2	Inhibitory	87
KIR2DL2		HLA-C1	Inhibitory	87
KIR2DL3		HLA-C1	Inhibitory	87
KIR2DS1		HLA-C2	Activating	87
KIR2DS2		HLA-C1	Activating	87
KIR2DS3		Unknown	Activating	87
KIR2DS4		Unknown	Activating	87
KIR2DS5		Unknown	Activating	87
KIR2DL4		HLA-G	Inhibitory	87
KIR2DL5		Unknown	Inhibitory	87
KIR3DL1		HLA-Bw4	Inhibitory	87
KIR3DL2		HLA-A3	Inhibitory	87
		HLA-A11	Inhibitory	87
KIR3DS1		HLA-Bw4	Activating	87

Table 5: NK cell receptors and their corresponding ligands.

4. Level of expression of ligands for NK cell receptors in tumors

The ‘Human Protein Atlas’, a database that contains the expression profiles of proteins corresponding to 44 normal tissue types and 20 cancer tissues, was used to determine the levels of expression of different ligands having affinity to the aforementioned receptors on NK cells as well as to the co-receptors on T cells.

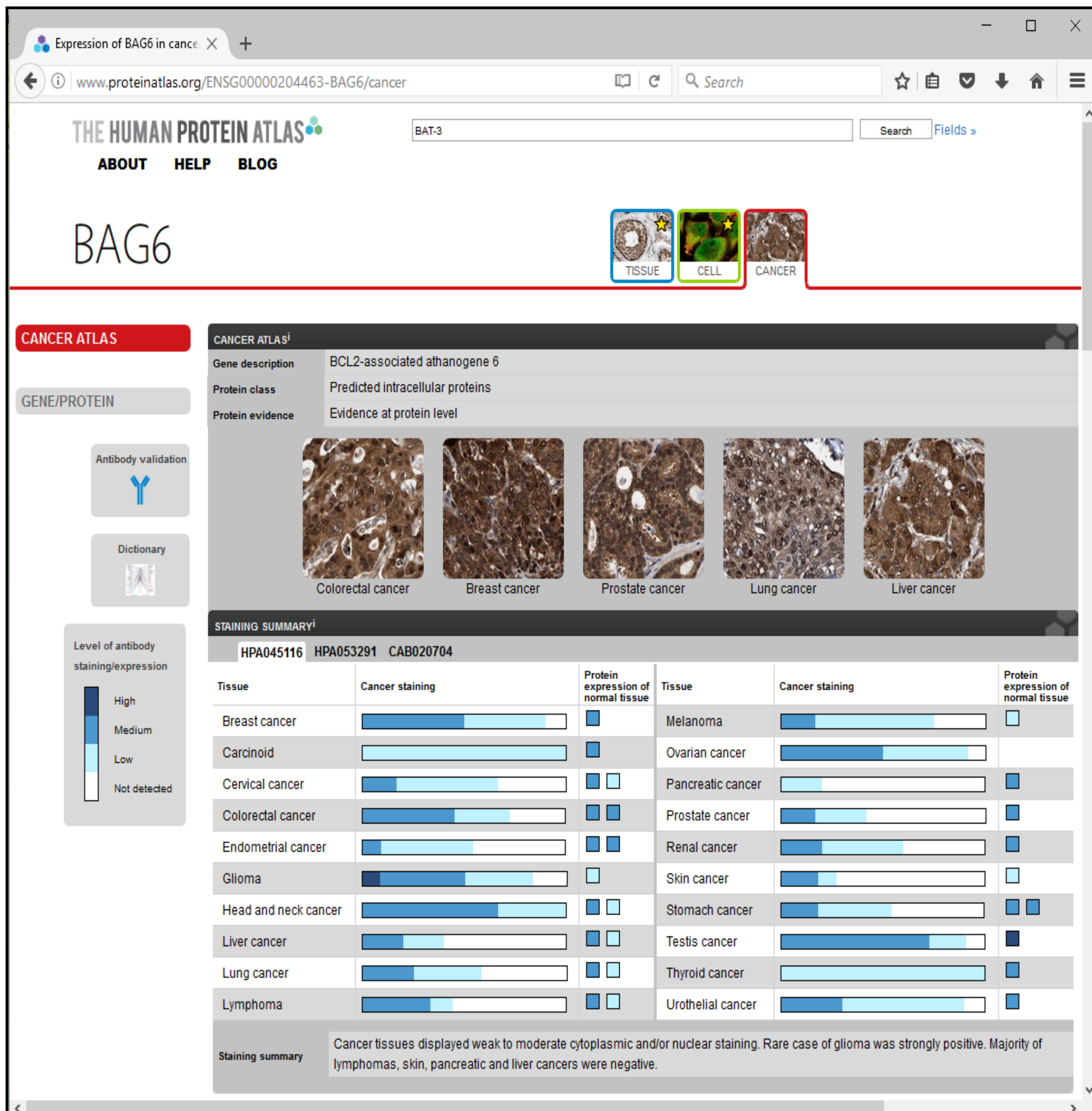


Figure 2: A screenshot of the working window in Human Protein Atlas.

From Human Protein Atlas, the following data regarding the level of expression of different NK ligands in different cancers was obtained:

Ligand	Receptor	Effect	Breast Cancer	Skin Cancer	Colorectal Cancer	Gastric Cancer
HLA-E	NKG2A	-	0.318	0.316	0.545	0.474
HLA-E	NKG2C, NKG2E	+	0.318	0.316	0.545	0.474
ULBP-1	NKG2D	+	0.091	0	0	0.083
ULBP-2	NKG2D	+	0	0	0	0
ULBP-3	NKG2D	+	0.091	0	0	0
PVR	DNAM-1	+	0	0.091	0.364	0.3
CD122	DNAM-1	+	0	0	0	0
HSPG	Nkp46/NCR1	+	0	0.091	0	0.091
HSPG	Nkp30/NCR3	+	0	0.091	0	0.091
PCNA	Nkp44/NCR2	-	1	0.936	1	0.932
BAT-3/ BAG-6	Nkp30/NCR3	+	0.548	0.366	0.485	0.364
B7-H6	Nkp30/NCR3	+	0.455	0.091	0.583	0
AICL	Nkp80	+	0.2	0	0.083	0
HLA-G	KIR2DL4	+	0	0	0	0

Table 6: NK cell receptor ligands and their levels of expression in tumors with high HLA.

Ligand	Receptor	Effect	Melanoma	Ovarian Cancer	Lymphoma	Prostate Cancer	Lung Cancer
HLA-E	NKG2A	-	0.55	0.5	0.917	0.191	0.238
HLA-E	NKG2C, NKG2E	+	0.55	0.5	0.917	0.191	0.238
ULBP-1	NKG2D	+	0	0	0	0	0.083
ULBP-2	NKG2D	+	0	0	0	0	0
ULBP-3	NKG2D	+	0	0	0	0	0
PVR	DNAM-1	+	0.182	0	0	0.091	0.25
CD122	DNAM-1	+	0	0	0	0	0
HSPG	Nkp46/NCR1	+	0.636	0.083	0	0	0
HSPG	Nkp30/NCR3	+	0.636	0.083	0	0	0
PCNA	Nkp44/NCR2	-	1	0.766	0.733	0.717	0.87
BAT-3/ BAG-6	Nkp30/NCR3	+	0.528	0.486	0.452	0.371	0.4
B7-H6	Nkp30/NCR3	+	0.083	0.083	0	0.7	0.091
AICL	Nkp80	+	0.166	0	0.417	0	0.083
HLA-G	KIR2DL4	+	0	0	0	0	0

Table 7: NK cell receptors ligands and their levels of expression in tumors with low HLA.

5. Level of expression of ligands for T cell co-receptors in tumors

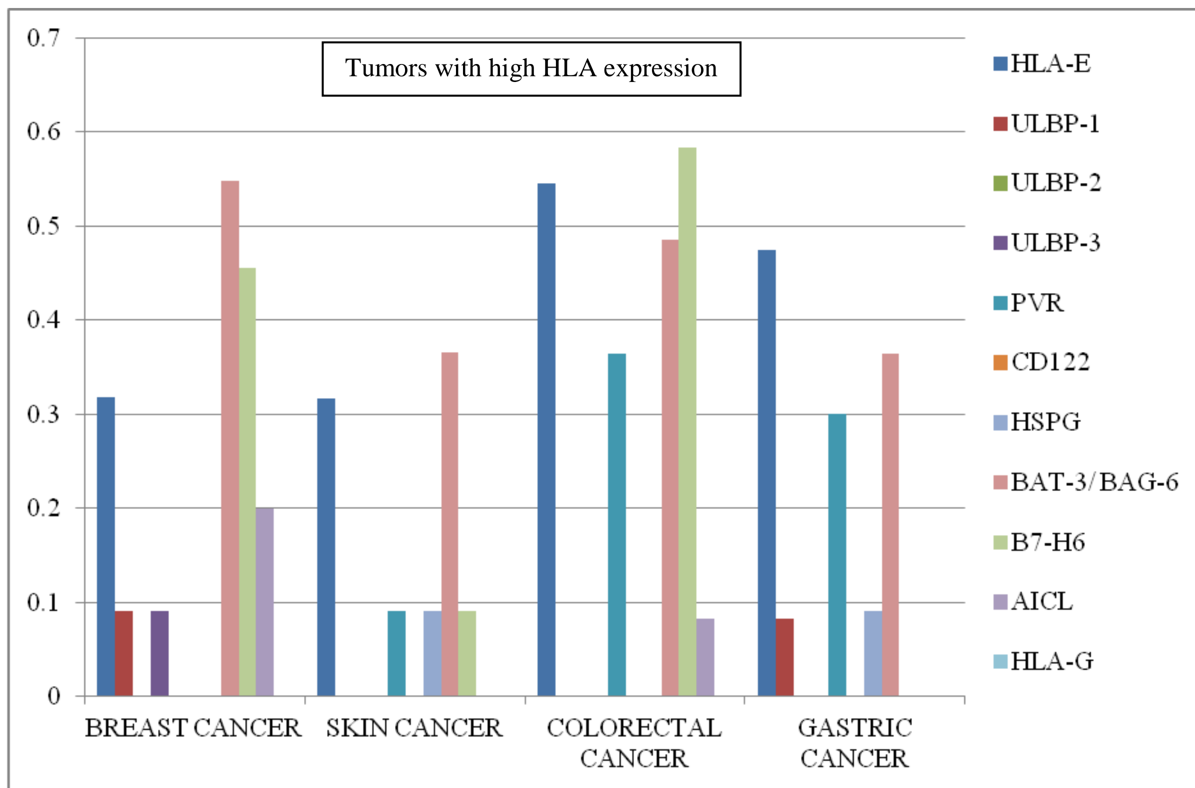
From Human Protein Atlas, the following data regarding the level of expression of different NK ligands in different cancers was obtained:

Receptor	Ligand	Effect	Breast Cancer	Skin Cancer	Colorectal Cancer	Gastric Cancer
CD28	CD80 (B7.1)	+	0	0	0	0
CD28	CD86 (B7.2)	+	0.667	0.417	1	1
OX40 (CD134, TNFRSF4)	OX40L (CD252, TNFSF4)	+	0.1	0.083	0.2	0.25
HVEM (CD270, TNFRSF14)	LIGHT (CD258, TNFSF14)	+	0.667	0	0.75	0.727
ICOS (CD278)	ICOS-L (CD275, B7-H2)	+	0.917	0.5	1	0.364
GITR (TNFRSF18)	GITRL (TNFSF18)	+	0.917	0.583	1	1
CTLA-4 (CD152)	CD80 (B7.1)	-	0	0	0	0
CTLA-4 (CD152)	CD86 (B7.2)	-	0.667	0.417	1	1
PD-1 (CD279)	PD-L2 (CD273, B7-DC)	-	0.727	0.182	0.909	0.917
LFA-1 (CD11a/CD18)	CD54 (ICAM1)	+	0.242	0.172	0.088	0.294
LFA-1 (CD11a/CD18)	CD50 (ICAM3)	+	0	0	0	0
BTLA (CD272)	HVEM (CD270, TNFRSF14)	-	0.125	0	0.303	0.118
CD154	CD40	+	0	0.043	0.042	0.022
CD28	CD80	-	0	0	0	0
CD226 (DNAM1)	CD112	+	0.545	0	0.542	0.389
CD226 (DNAM1)	CD155	+	0	0.091	0.364	0.3
TIGIT (VSIG9, VSTM3)	CD112	-	0.545	0	0.542	0.389
TIGIT (VSIG9, VSTM3)	CD155	-	0	0.091	0.364	0.3
TIGIT (VSIG9, VSTM3)	CD113	-	0.417	0.364	0.708	0.696
TIM2 (TIMD2)	SEMA4A		0	0	0	0
CD2 (LFA2, OX34)	CD48	+	0	0	0	0
CD160 (BY55, NK28)	HVEM (CD270, TNFRSF14)	-	0.125	0	0.303	0.118

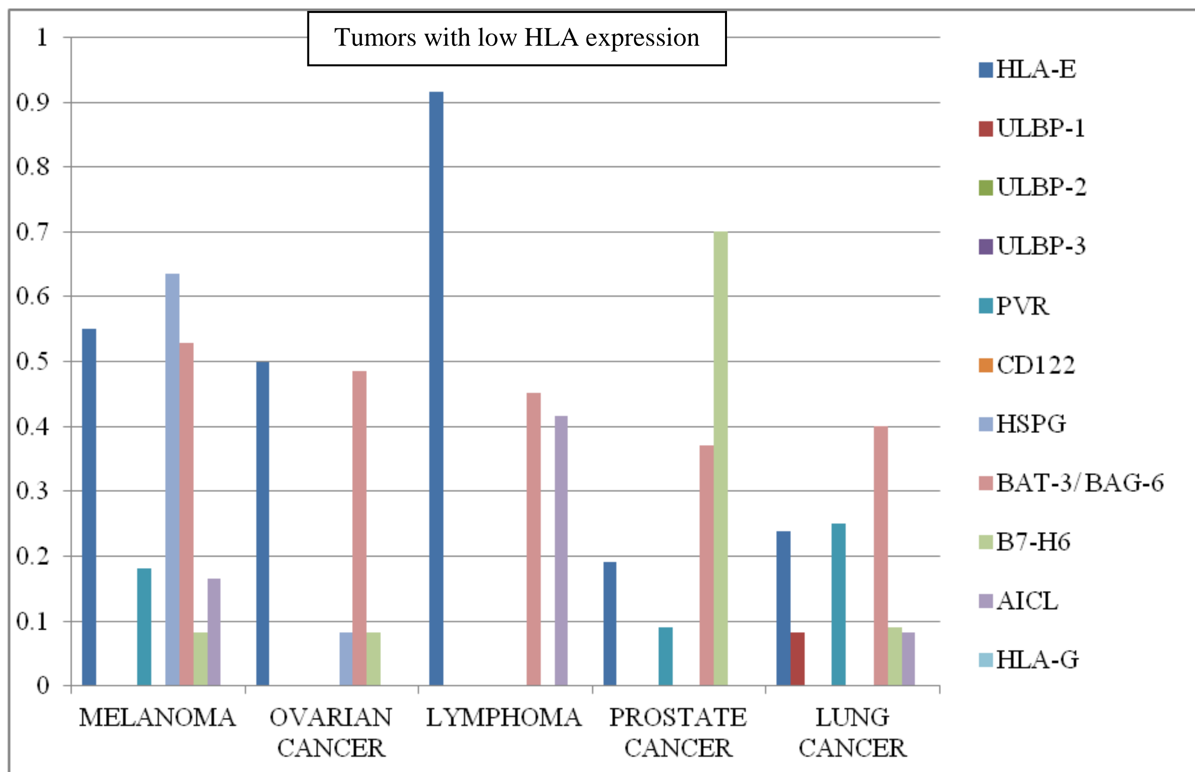
Table 8: T cell co-receptors ligands and their levels of expression in tumors with high HLA.

Receptor	Ligand	Effect	Melanoma	Ovarian Cancer	Lymphoma	Prostate Cancer	Lung Cancer
CD28	CD80 (B7.1)	+	0.044	0	0.044	0.0417	0.05
CD28	CD86 (B7.2)	+	0.75	0.417	0.75	0.333	0.727
OX40 (CD134, TNFRSF4)	OX40L (CD252, TNFSF4)	+	0.222	0.182	0	0.727	0.273
HVEM (CD270, TNFRSF14)	LIGHT (CD258, TNFSF14)	+	0	0.583	0	0.917	0.3333
ICOS (CD278)	ICOS-L (CD275, B7-H2)	+	1	0.667	0.727	1	0.818
GITR (TNFRSF18)	GITRL (TNFSF18)	+	1	1	1	1	0.917
CTLA-4 (CD152)	CD80 (B7.1)	-	0.044	0	0.044	0.0417	0.05
CTLA-4 (CD152)	CD86 (B7.2)	-	0.75	0.417	0.75	0.333	0.727
PD-1 (CD279)	PD-L2 (CD273, B7-DC)	-	0.7	0.75	0.167	1	0.583
LFA-1 (CD11a/CD18)	CD54 (ICAM1)	+	0.618	0.441	0.667	0.345	0.444
LFA-1 (CD11a/CD18)	CD50 (ICAM3)	+	0	0	1	0	0
BTLA (CD272)	HVEM (CD270, TNFRSF14)	-	0.424	0.088	0.294	0.281	0.088
CD154	CD40	+	0	0.128	0.267	0	0.083
CD28	CD80	-	0.044	0	0.044	0.0417	0.05
CD226 (DNAM1)	CD112	+	0.087	0.478	0.087	1	0.044
CD226 (DNAM1)	CD155	+	0.182	0	0	0.091	0.25
TIGIT (VSIG9, VSTM3)	CD112	-	0.087	0.478	0.087	1	0.044
TIGIT (VSIG9, VSTM3)	CD155	-	0.182	0	0	0.091	0.25
TIGIT (VSIG9, VSTM3)	CD113	-	0.542	0.667	0	0.792	0.455
TIM2 (TIMD2)	SEMA4A		0	0	0.25	0.1	0
CD2 (LFA2, OX34)	CD48	+	0	0	0.083	0	0
CD160 (BY55, NK28)	HVEM (CD270, TNFRSF14)	-	0.424	0.088	0.294	0.281	0.088

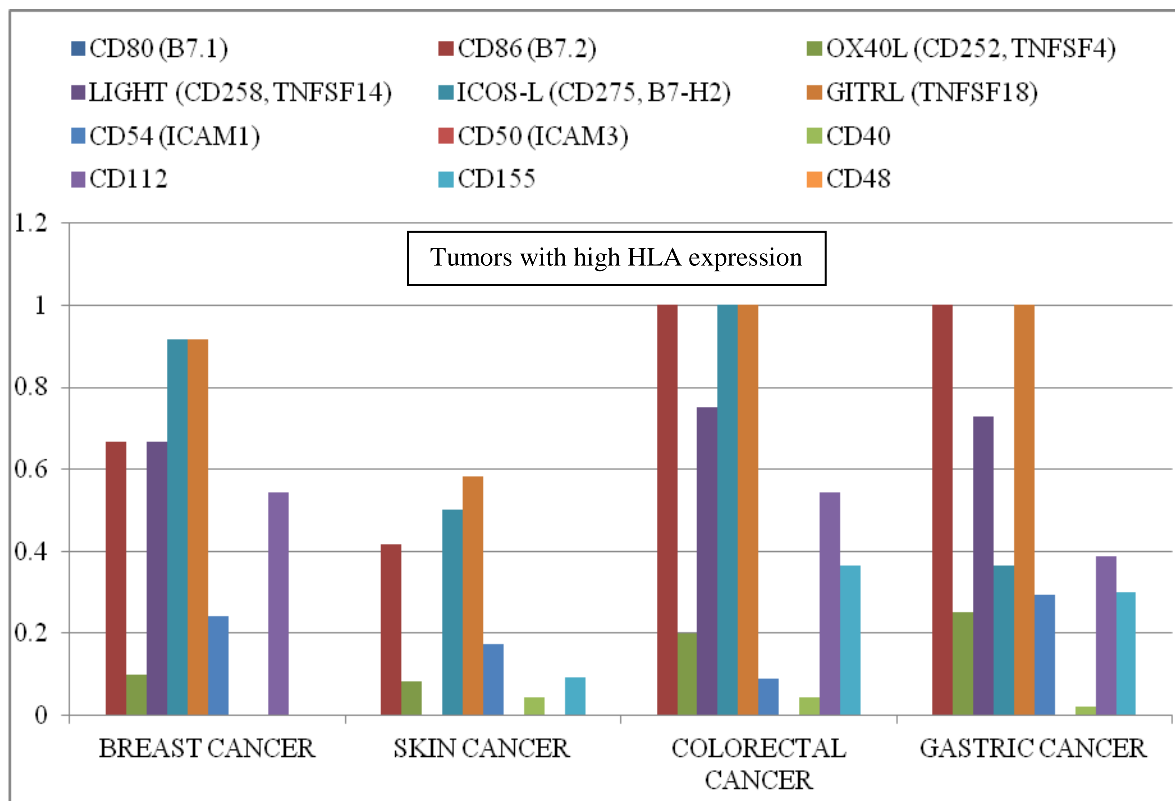
Table 9: T cell co-receptors ligands and their levels of expression in tumors with low HLA.



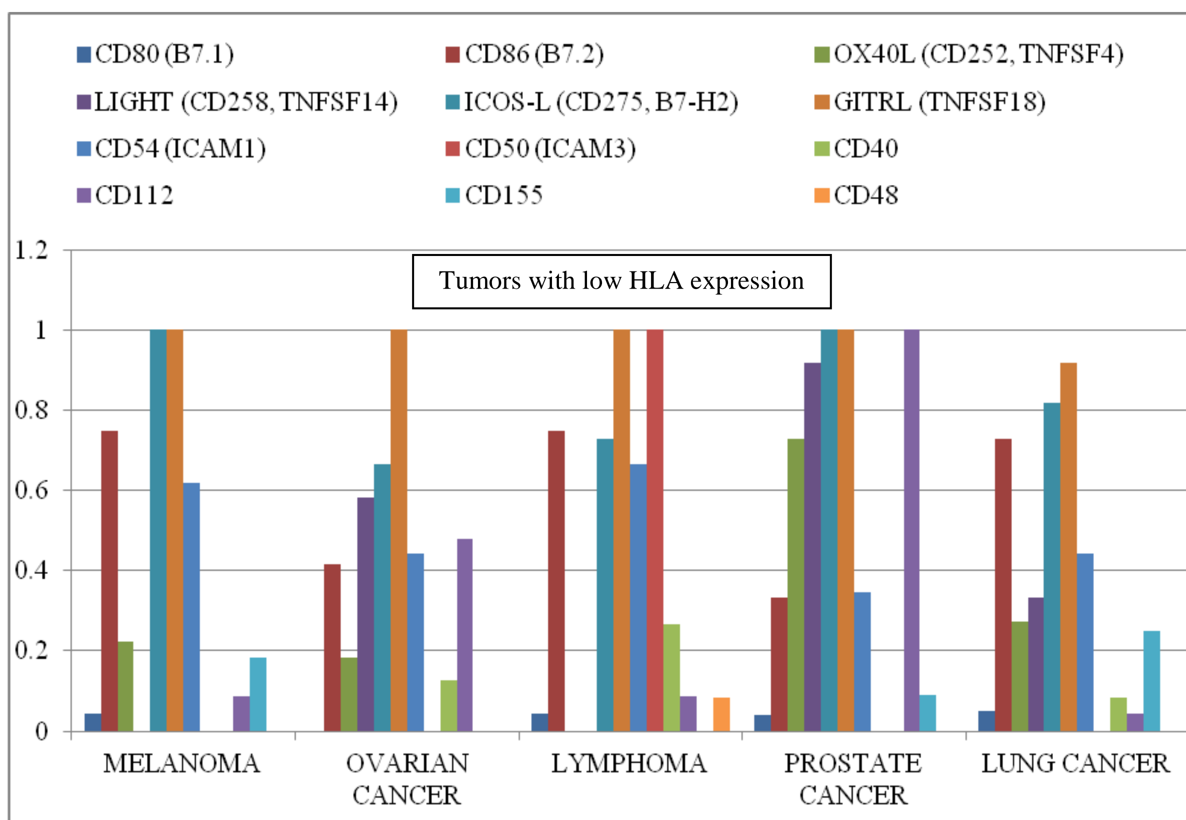
Graph 1: Expression levels of activating ligands for NK receptors in tumors with high HLA expression.



Graph 2: Expression levels of activating ligands for NK receptors in tumors with low HLA expression.



Graph 3: Expression levels of activating ligands for T cell co-receptors in tumors with high HLA expression.



Graph 4: Expression levels of activating ligands for T cell co-receptors in tumors with low HLA expression.

In tumors with high levels of HLA expression, it is expected that the co-stimulatory ligands with affinity towards the T cell co-receptors would cause a much higher potentiation of TCR (T cell receptors) mediated immune response.

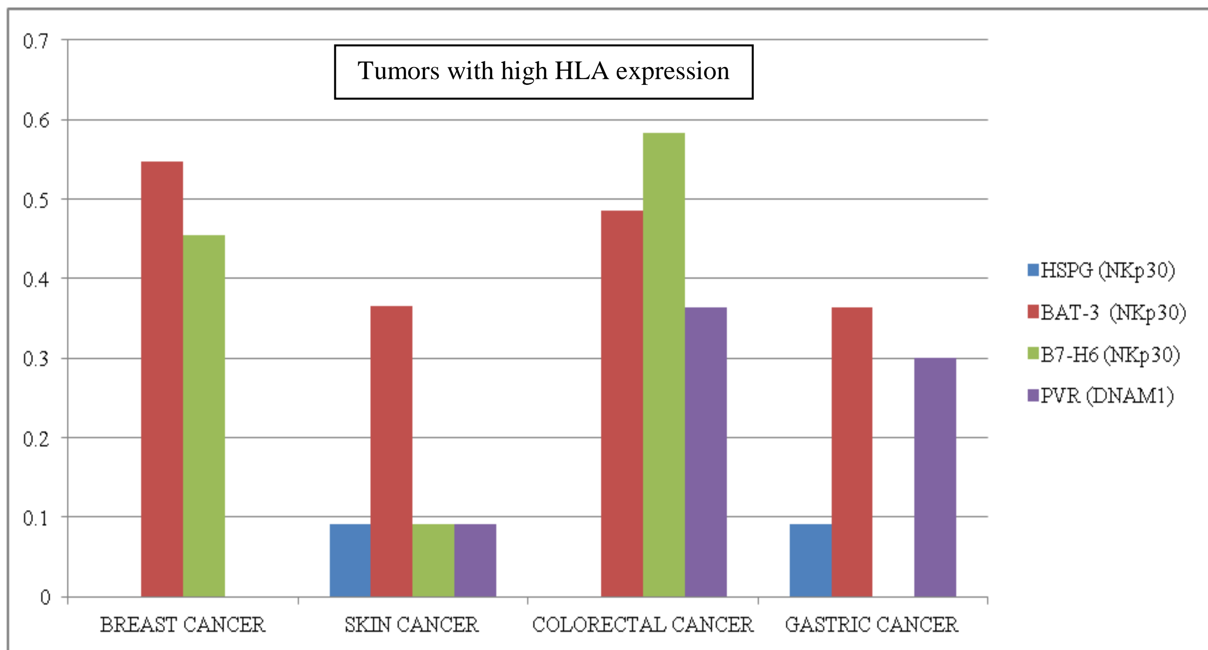
ICAM1, which is a ligand for LFA-1, has been shown to be an essential component involved in the CTL (Cytotoxic T Lymphocyte) recognition [88]. It is a co-stimulatory ligand for T cells and can also work in synergy with activating receptors on the NK cells. LFA-1/ICAM1 interactions have been reported to exhibit remote co-stimulation resulting in the induction of NK mediated cytotoxic activity [89].

CD112 interacts with DNAM1 on the T cells and acts as a co-stimulatory signal for the T cells. It may be noted here that CD40 is an important co-stimulatory ligand that causes T cell activation as well as T cell mediated B cell activation, thus potentiating the entire immune system involving APCs, macrophages and dendritic cells. Studies have shown the ability of CD40 triggered APCs to induce NK mediated cytotoxicity through IL-15, which is secreted as a result of CD40/APC interaction [90, 91]. CD40An interesting observation we made in this context was that the expression of CD40 and ICAMs was restricted in the tumors with high levels of HLA expression as compared to those with low levels of HLA expression. This is probably a reactionary modification adopted by the tumors for reducing the activation of NK cells as well as T cells.

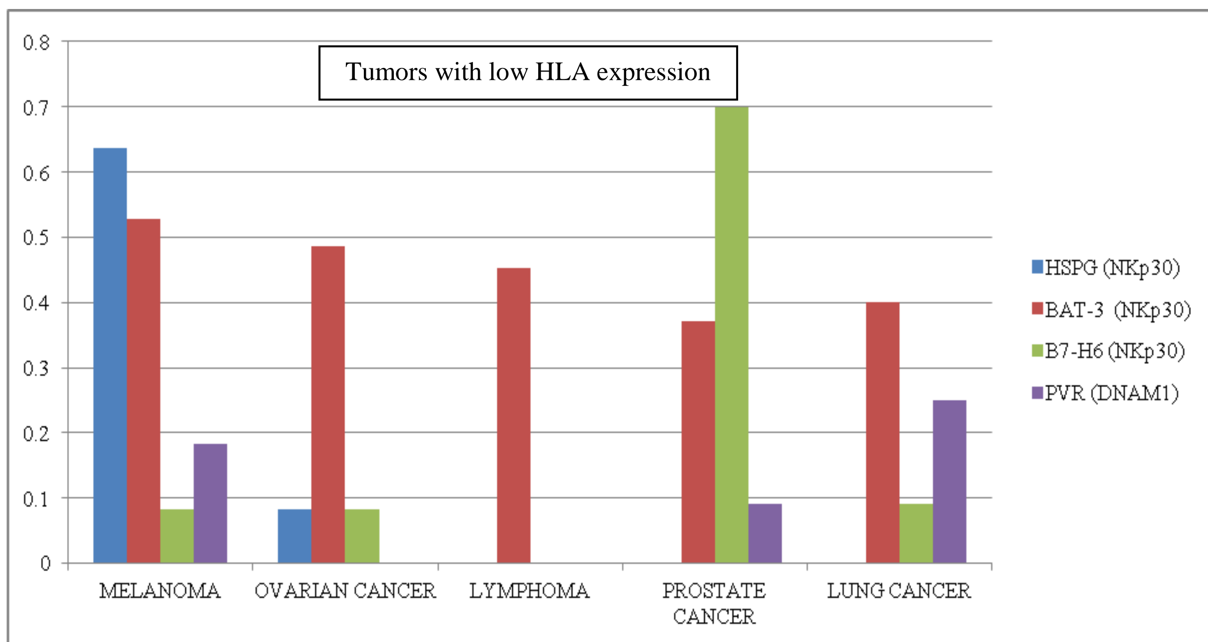
Here, it is prudent of the tumor cells to cause active down-regulation of such activating ligands. However, we found that CD112 is consistently expressed in higher levels in tumors with high HLA expression as compared to those with low HLA expression.

The fact that ICAM1 is expressed at a much higher level in tumors with low HLA expression shows that in case of such tumors, where inhibitory signals through the KIRs is missing, activation by synergy with ICAMs is expected to be significant. It is also to be observed that CD40 expression is higher in tumors with low HLA expression. Hence, synergy of activating signals through the binding of ICAMs and CD40 with their corresponding receptors on the NK would be expected to potentiate the NK mediated anti-tumor immune response in case of such tumors.

It has to be noted here that CD112 expression is much lower in tumors with low HLA expression as compared to those with high HLA expression, probably because T cells play an insignificant role in tumors with low HLA expression, as it has been shown in various reports that in the absence of primary stimulation, co-stimulation is inconsequential.



Graph 5: Expression levels of activating ligands for NK cell receptors which are expressed at a higher level in tumors with high HLA expression.



Graph 6: Expression levels of activating ligands for NK cell receptors which are expressed at a higher level in tumors with high HLA expression.

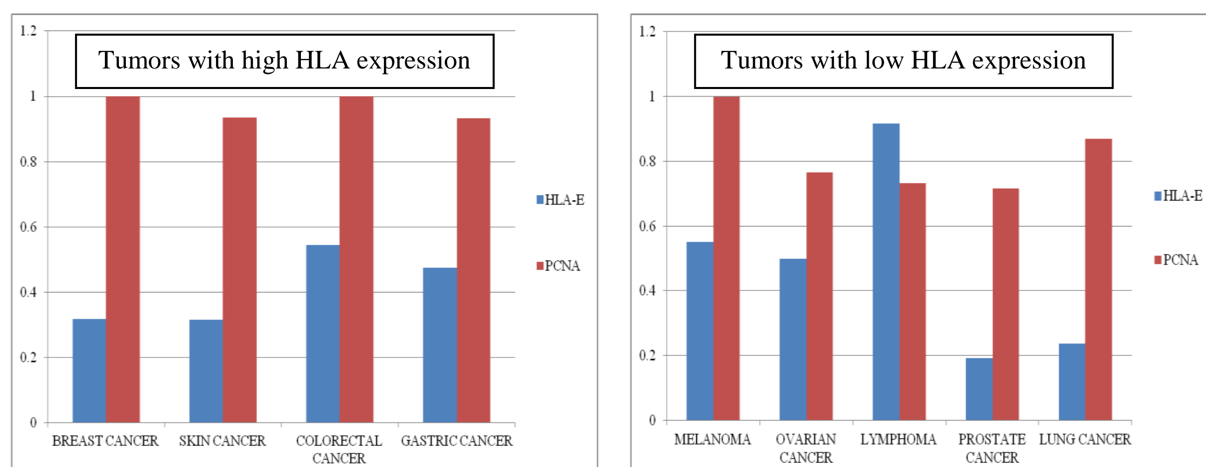
Another interesting finding from the data is that there is a significantly higher expression of activating ligands that bind to the NKp30 receptor on NK cells in the case of tumors with high HLA expression as compared to their counterpart. This indicates a possible role of NK cells in tumor immunity even against those tumors that express higher levels of HLA.

In case of tumors with high HLA expression levels, it may be noted here that due to the presence of MHC-I, there is a generation of strong inhibitory signals through interaction with KIRs. Therefore, NK cells are expected to play a less significant role in immunity against tumors with high HLA expression. However, in our study, we show a significant upregulation of NKp30 ligands on tumors with high HLA expression. Since NKp30 is a receptor present on all the NK cells, it is reasonable to state that the role of upregulation of activating ligands with affinity towards NKp30 signifies an important role played by NK cells in immunity against such tumors as well.

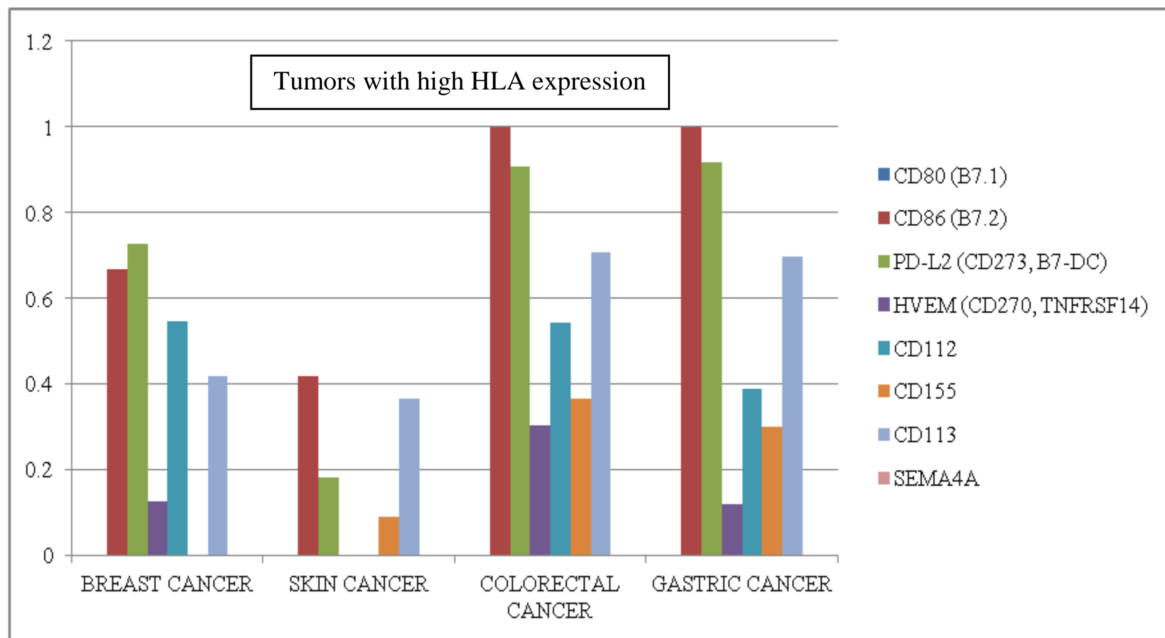
PVR and CD122, both of which are activating ligands that associate with DNAM1 on the NK cells, are also found to be up-regulated in tumors with high HLA expression. Thus, DNAM1 mediated activation signals in NK is up-regulated in the context of tumors with high HLA expression levels. This further corroborates our hypothesis that despite inhibition through KIR-MHC-I ligation, the activation signals may synergize to overcome this inhibition and potentiate the cytolytic activity in NK cells.

The tumor mediated response of down-regulation of activating signals for T cell co-stimulation, in the context of tumors with high HLA expression levels, is therefore in our hypothesis being taken care of by NK mediated activation of immune response.

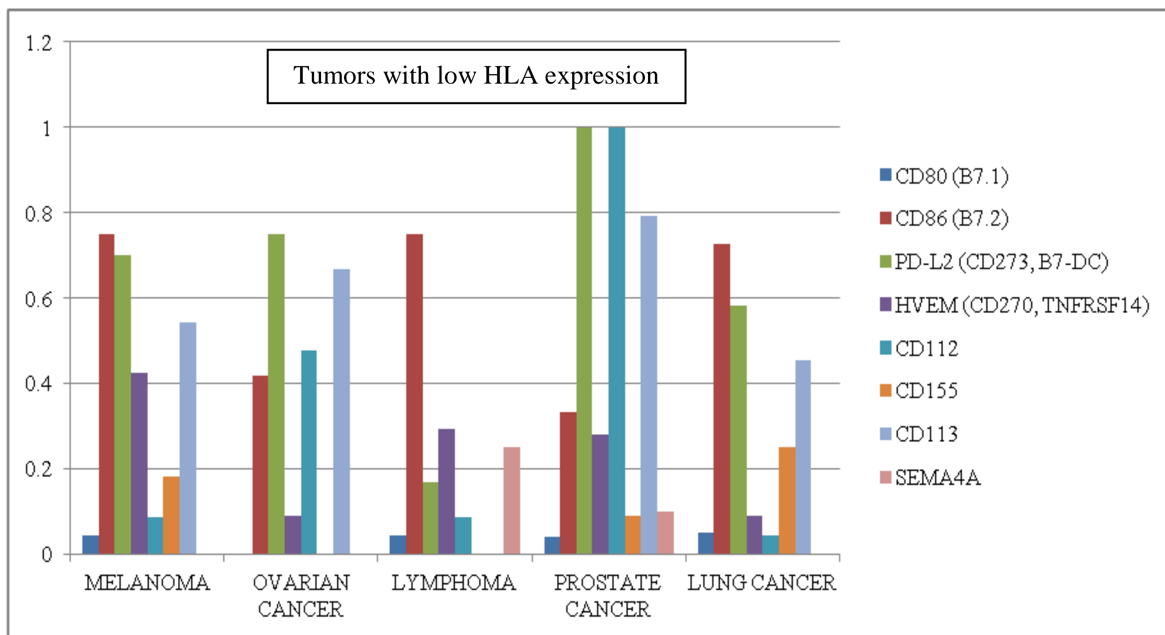
Our previous study has shown that tumors do express ligands that act as activating and inhibitory signals for NK cells and that the expression of these ligands is actively modulated by the tumor cells. A study of surface expressed molecules present on P815 (NK resistant cell line) and YAC-1 (NK susceptible cell line) demonstrated significant binding of the surface molecules present on YAC-1 with NK activating receptor Ly49H whereas those present on the surface of P815 showed a strong affinity towards the NK inhibitory receptor Ly49A.



Graph 7: Expression levels of inhibitory ligands for NK receptors in tumors with high HLA expression and low HLA expression.



Graph 8: Expression levels of inhibitory ligands for T cell co-receptors in tumors with high HLA expression.



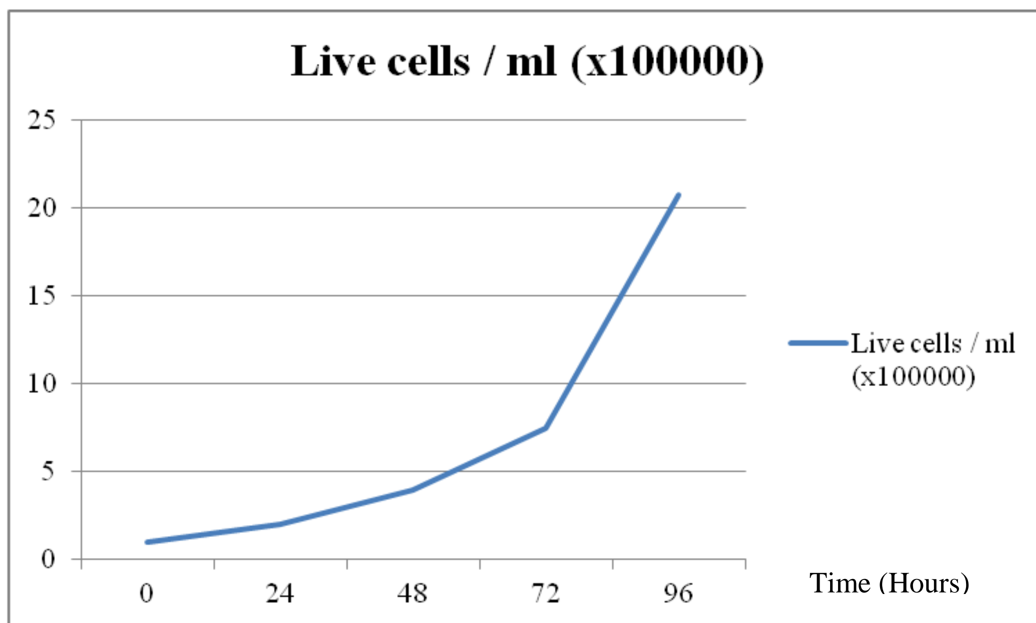
Graph 9: Expression levels of inhibitory ligands for T cell co-receptors in tumors with low HLA expression.

The expression of inhibitory ligands on tumor cells shows no significant variation in tumors with both high and low levels of HLA expression. Hence there is no active inhibition of T cells in either of the group, but the active modulation of T cell activity by tumor cells is exhibited by down-regulation of activating ligands through T cell co-receptors. This active tumor driven regulation of T cell co-stimulatory signals helps the tumor cells to dodge a polyclonal subset of T cells and thus effectively evade T cell mediated cytotoxicity.

It is worth noting here that in both the case of tumors with high and low levels of HLA expression, the expression levels of NK inhibitory ligands is more or less similar. Therefore, it becomes all the more significant, that by our hypothesis, we have proposed a role of active involvement of NK cells in immunity against tumors with high HLA expression due to a dynamic tumor cell mediated down-regulation of activation receptor signals in the context of T cells.

Growth kinetics of YAC-1:

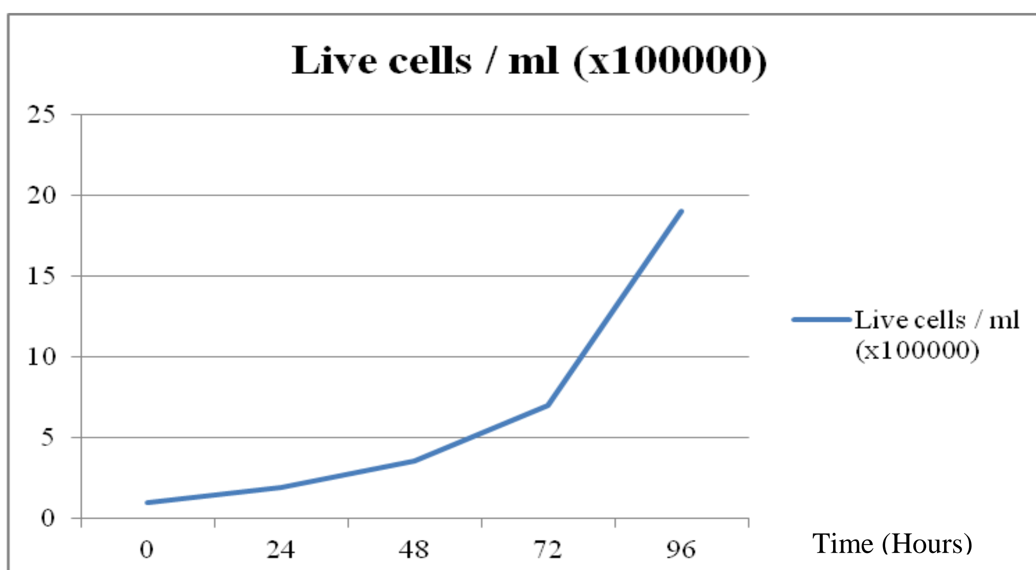
Hours	Live cells/ml
0	1×10^5
24	2.029×10^5
48	3.972×10^5
72	7.468×10^5
96	20.764×10^5



Graph 10: Growth kinetics of YAC-1.

Growth kinetics of P815:

Hours	Live cells/ml
0	1×10^5
24	1.907×10^5
48	3.592×10^5
72	6.983×10^5
96	19.051×10^5



Graph 11: Growth kinetics of P815.

Both NK susceptible cell line (YAC1) as well as NK resistant cell line (P815) were cultured and the cells from multiple passages were pooled to obtain 20 million cells per cycle. Subsequently, membrane fraction was isolated and the isolates from multiple cycles were pooled for future use. Protein estimation was done by BCA method and absorbance was recorded at 562 nm. Cell lysate was prepared using RIPA buffer (composition given in appendix I).

The protein concentrations of various fractions obtained are as follows:

Fraction	**Concentration (µg/µl)**
Cell lysate (YAC1)	7.45
Cell lysate (P815)	8.82
Nuclear fraction (YAC1)	3.23
Nuclear fraction (P815)	7.70
Membrane fraction (YAC1)	2.57
Membrane fraction (P815)	3.15

Total membrane proteins obtained from YAC1 in 1 cycle: 1285 µg

Total membrane proteins obtained from P815 in 1 cycle: 1575 µg

9. CONCLUSION

Tumors often down-regulate their MHC-I expression in order to evade T cell mediated response. However, these tumors are particularly susceptible to NK cell attack since due to absence of MHC-I, inhibitory signal in NK cells through KIRs is missing. Therefore, in our study we were surprised to see a significant down-regulation of co-stimulatory signals in T cells in the context of tumors with high level of MHC expression as well. As co-stimulation is essential for any T cell to exude its activity, lack of co-stimulatory signals can universally down-regulate the cytotoxic activity of all T cell clones. The tumor mediated response of down-regulation of activation signals for T cell co-stimulation also results in upregulation of multiple NK activating signals. Our study has shown a significant up-regulation of activating signals in NK cells which may synergize to overcome inhibition through MHC-I-KIR interaction and thus prove to be effective in cancers with high HLA expression that are resistant to T cell therapy.

Adoptive transfer therapy mediated by T cells and NKT cells has proven to be ineffective in some tumors with high HLA expression which are found to be refractile to T cell mediated therapy. In such cases, combinatorial therapy involving NK cells stimulation through activating receptors may be more effective.

10. APPENDIX

1. Appendix A

DMEM medium composition

Inorganic Salts (g/l)		Vitamins (g/l)	
$CaCl_2$ (anhydrous)	0.11665	D-Biotin	0.00000365
$CuSO_4$ (anhydrous)	0.0000008	Choline Chloride	0.00898
$Fe(NO_3)_3.9H_2O$	0.00005	Folic Acid	0.00265
$FeSO_4.7H_2O$	0.000417	myo-Inositol	0.01261
$MgSO_4$ (anhydrous)	0.08495	Niacinamide	0.00202
KCl	0.3118	D-Pantothenic Acid (hemicalcium)	0.00224
$NaHCO_3$	1.20000	Pyridoxine.HCl	0.00203
NaCl	7.00000	Riboflavin	0.00022
Na_2HPO_4 (anhydrous)	0.07100	Thiamine.HCl	0.00217
$NaH_2PO_4.H_2O$	0.06250	Vitamin B-12	0.00068
$ZnSO_4.7H_2O$	0.000432		

Amino Acids (g/l)		Others (g/l)	
L-Alanine	0.00445	D-Glucose	3.15100
L-Arginine.HCl	0.14750	HEPES	3.57480
L-Asparagine.H_2O	0.00750	Hypoxanthine	0.00239
L-Aspartic Acid	0.00665	Linoleic Acid	0.000044
L-Cysteine.HCl.H_2O	0.01756	Phenol Red, Sodium Salt	0.00810
L-Cystine.2HCl	0.03129	Putrescine.2HCl	0.00008
L-Glutamic Acid	0.00735	Pyruvic Acid.Na	0.05500
L-Glutamine	0.36510	DL-Thioctic Acid	0.000105
Glycine	0.01875	Thymidine	0.000365
L-Histidine.HCl.H_2O	0.03148	Thymidine	0.000365
L-Isoleucine	0.05437		
L-Leucine	0.05895		
L-Lysine.HCl	0.09135		
L-Methionine	0.01724		
L-Phenylalanine	0.03548		
L-Proline	0.01725		
L-Serine	0.02625		
L-Threonine	0.05355		
L-Tryptophan	0.00902		
L-Tyrosine.2Na.$2H_2O$	0.05582		
L-Valine	0.05285		

2. Appendix B

HEPES buffer composition

Reagent	Amount to add (for 1 L)	Final concentration
NaCl	6.72 g	115 mM
$CaCl_2$	133 mg	1.2 mM
$MgCl_2$	114 mg	1.2 mM
K_2HPO_4	418 mg	2.4 mM
HEPES	4.77 g	20 mM
H_2O	to 1 L	

Adjust the pH to 7.4 with HCl or NaOH. HEPES buffer can be stored refrigerated for several weeks.

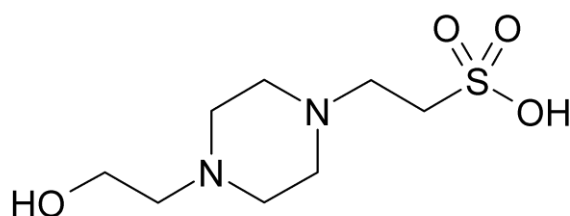


Figure 3: Structure of HEPES.

3. Appendix C

RPMI-1640 medium composition

Inorganic Salts (g/l)

$Ca(NO_3)_2.4H_2O$	0.10000
$MgSO_4$ (anhydrous)	0.04884
KCl	0.40000
$NaHCO_3$	1.50000
NaCl	6.00000
Na_2HPO_4 (anhydrous)	0.80000

Amino Acids (g/l)

L-Arginine (free base)	0.20000
L-Asparagine.H_2O	0.05682
L-Aspartic Acid	0.02000
L-Cystine.2HCl	0.06520
L-Glutamic Acid	0.02000
L-Glutamine	0.30000
Glycine	0.01000
L-Histidine (free base)	0.01500
Hydroxy-L-Proline	0.02000
L-Isoleucine	0.05000
L-Leucine	0.05000
L-Lysine.HCl	0.04000
L-Methionine	0.01500
L-Phenylalanine	0.01500
L-Proline	0.02000
L-Serine	0.03000
L-Threonine	0.02000
L-Tryptophan	0.00500
L-Tyrosine.2Na.$2H_2O$	0.02883
L-Valine	0.02000

Vitamins (g/l)

D-Biotin	0.00020
Choline Chloride	0.00300
Folic Acid	0.00100
myo-Inositol	0.03500
Nicotinamide	0.00100
p-Amino Benzoic Acid	0.00100
D-Pantothenic Acid (hemicalcium)	0.00025
Pyridoxine.HCl	0.00100
Riboflavin	0.00020
Thiamine.HCl	0.00100
Vitamin B-12	0.000005

Other (g/l)

D-Glucose	4.50000
Glutathione (reduced)	0.00100
HEPES	2.38300
Phenol Red, Sodium Salt	0.00500
Sodium Pyruvate	0.11000

4. Appendix D

PBS composition

Reagent	Amount to add (for 1X solution)	Final concentration (1X)	Amount to add (for 10X stock)	Final concentration (10X)
NaCl	8 g	137 mm	80 g	1.37 m
KCl	0.2 g	2.7 mm	2 g	27 mm
Na_2HPO_4	1.44 g	10 mm	14.4 g	100 mm
KH_2PO_4	0.24 g	1.8 mm	2.4 g	18 mm

If necessary, PBS may be supplemented with the following:

$CaCl_2.2H_2O$	0.133 g	1 mm	1.33 g	10 mm
$MgCl_2.6H_2O$	0.10 g	0.5 mm	1.0 g	5 mm

5. Appendix E

SF buffer composition

	Stocks	50 ml 1x solution
250 mM Sucrose	-	4.28 g
20 mM HEPES (pH 7.4)	1 M	1 ml
10 mM KCl	-	0.0373 g
1.5 mM $MgCl_2$	1 M	75 µl
1 mM EDTA	0.5 M	100 µl
1 mM EGTA	0.5 M	100 µl

At time of use, add the following into 10 ml of SF buffer

	Stocks	10 ml 1x solution
1 mM DTT	1 M	10 µl
PI cocktail	40x (dissolve 1 tablet in 2 ml double distilled H_2O)	250 µl

6. Appendix F

NL buffer composition

	Stocks	50 ml 1x solution
50 mM Tris HCl (pH 8)	1 M	2.5 ml
150 mM NaCl	1 M	7.5 ml
1% NP-40	20%	2.5 ml
0.5% sodium deoxycholate	10%	2.5 ml
0.1% SDS	10%	0.5 ml

At time of use, add the following into 10 ml of NL buffer

	Stocks	10 ml 1x solution
PI cocktail	40x	250 µl
10% glycerol	-	1 ml

7. Appendix G

Electrolysis buffer composition

25 mM	Tris-HCl
200 mM	Glycine
0.1% (w/v)	SDS

8. Appendix H

Loading buffer composition

2% w/v	SDS
2 mM	Dithiothreitol or beta-mercapto-ethanol
4 % v/v	Glycerol
0.04 M	Tris-HCl, pH 6.8
0.01% w/v	Bromophenolblue

9. Appendix I

RIPA buffer composition

150 mM	NaCl
1%	Triton-X
0.5%	Sodium deoxycholate
0.1%	SDS
50 mM	Tris HCl, pH 8

10. Appendix J

Homogenization buffer composition

250 mM	Sucrose
1 mM	EDTA
10 mM	Tris HCl, pH 7.2
1x	Protease inhibitor cocktail

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