REVIEW OF LITERATURE
In 1971, Cudkowicz et al showed that lethally irradiated F1 progeny were capable of rejecting bone marrow grafts from parental mouse strains. In 1975 a population of cells were found capable of spontaneously lysing ("natural killing") of certain tumor transformed cells in vitro in a MHC unrestricted manner. Hence NK cells were studied for their distinct physiological functions in parallel systems. NK cells were studied in vivo for their bone marrow graft rejection, a phenomenon termed as "hybrid resistance" and in vitro for NK mediated "natural killing" of tumor targets. Subsequently it was found that depletion of NK cells in vivo also resulted in enhanced incidences of tumor formation in various mouse tumor models. Further research identified and characterized the cells, responsible for 'hybrid resistance' and 'natural killing' as large granular lymphocytes that were relatively radio-resistant, did not require prior sensitization and were derived from bone marrow progenitors (Herberman 1981, Ortaldo et al 1984, Trinchieri 1989, Murphy et al 1993). NK cells produce a wide variety of cytokines, especially upon activation. They secrete interferon-γ, tumor necrosis factor-α, interleukin1, granulocyte-macrophage colony stimulating factors (GM-CSF) and transforming growth factor-β (Allavena 1985). NK cells mediate cytotoxicity primarily through perforin and granzyme dependent processes. NK cells also express Fas ligand and TRAIL and kill target cells through apoptotic pathway also.

**Role of NK cells in tumor immunity**

Depletion of NK cells in vivo leads to enhanced tumor formation in several mouse tumor models (Smyth et al 2001, Diefenbach et al 2002). Klas Karre and colleagues (1986) showed that the RMA T cell lymphoma grew progressively in syngeneic mice, but the MHC-I negative variant, RMA-S was rejected by the host NK cells. The fact that NK cells could kill target cells based on the absence of self-MHC class I molecules formed the basis of "missing self hypothesis" (Ljunggren and Karre 1990). Ljunggren and Karre proposed two potential mechanisms to explain the inverse correlation between MHC class I expression on target cells and their susceptibility to NK cells.
1. The target interference model that suggests that the MHC molecule on the target cell may physically interfere with the recognition of target cell ligands by NK cells.

2. Effector inhibition model that proposes the existence of receptors on NK cells that recognize MHC molecules on target cells and send inhibitory signal into NK cells.

Subsequently several inhibitory receptors for MHC I molecules were discovered on humans (KIRs) and rodents (Ly49 receptors) NK cells (Lanier et al 2001). Although an overwhelming array of evidences support the effector inhibition model, there has been some evidence to provide support to the target interference model as well (Haridas et al 1995 a, b, Saxena et al 1996, 1997).

Role of NK cells in protection against viral infection

Cytotoxic T lymphocyte plays a significant role in the elimination of virally infected host cells. However this protection against viral infection is dependent on MHC class I expression on infected cells. Many viruses have evolved mechanisms to escape T-cell surveillance by downregulating MHC I expression of the host cells. Herpes virus evade CTL attack by disrupting MHC I heavy chains and the peptide transporter associated with antigen processing (TAP) which in turn renders the virus infected cell susceptible to NK mediated lysis. This is due to lack of engagement of inhibitory NK receptors for MHC class I (Huard et al 2000, Orange et al 2002)

Human cytomegalovirus, HCMV encodes a glycoprotein UL18 and mouse cytomegalovirus, MCMV encodes a protein m144 both being structurally similar to MHC class I (Fletcher et al 1998). UL18 and m144 inhibit NK cell mediated killing by binding to inhibitory receptors on NK cells. UL18 binds to leukocyte immunoglobulin like receptor (LIR 1) (Cosman 1997) which is expressed at high levels on monocytes and macrophages and at low levels on NK cells. By binding to LIR on monocytes and macrophages, and inhibiting the production of inflammatory cytokines, that subsequently augments NK cell mediated IFNγ production. Leong et al 1998, showed that human fibroblasts infected with HCMV strain lacking UL18
were killed at similar rates to UL 18 containing strains by polyclonal NK cell lines and NK cell clones.

Another HCMV gene product, UL40 binds to HLA-E (Tomasec 2000) which, in turn binds to CD94/NKG2A (CD159a) and blocks NK cell mediated cytotoxicity (Braud et al 1998, Brooks et al 1999). UL16 is an early HCMV gene product, which is not necessary for viral replication in tissue culture (Kaye et al 1992). UL16 binds to two ULBP (UL16 binding protein) family members ULBP1 and ULBP2 and MIC B (MHC class I related B antigen) (Cosman et al 2001, Vales-Gomez et al 2003). ULBP1, ULBP2 and MIC B binding to NKG2D was blocked by UL16 protein (Cosman et al 2001), thus preventing costimulation of CD8+ T cells during HCMV recognition (Groh et al 2001). However, neither the NKG2D mediated NK activation nor the host T cell response has been found to be completely abrogated by UL16. Therefore the role of UL16 in HCMV pathogenesis and host immunity has not been well established yet.

Mice with genetic deficiencies in NK cell function were sensitive to MCMV and the activating receptor Ly49H has been shown to be involved in NK mediated protection against MCMV (Brown et al 2001, Daniels et al 2001, Lee et al 2001, 2003). Ly49H receptor signaling is coupled to adapter molecule DAP 12 (Smith et al 1998). However patients suffering from Nasui-Hakola disease (DAP12 null patients) display chronic pathology in the brain and bones and die of pre-senile dementia but show no susceptibility to herpes virus infections (Paloneva et al 2000, Verloes 1997). Hence Ly49H-DAP12 does not play a pivotal role in immunity against CMV.

NK cells have been shown to participate in defense against other viruses as well. Mandelboim and colleagues (2001) showed that NKP46 immunoglobulin fusion protein binds to haemagglutinin of influenza virus and to haemagglutinin-neuraminidase of parainfluenza virus. Hence activating receptor has been shown to be directly involved in anti-viral immunity.

Direct role of NK cell mediated immunity in AIDS has not been shown but the Nef protein of human immunodeficiency virus 1 selectively downregulates MHC I expression, thus making the infected cells susceptible to NK mediated cytotoxicity.
Human herpesvirus 8 also codes for a protein K5 that downregulates MHC I thus falling prey to NK cells (Coscoy et al 2001, Ishido et al 2000). However the human herpesvirus 8 also reduces the expression of ICAM 1 (CD54), B7 2 (CD86), LFA1 and CD28. NK cells express LFA-1 which is important in NK mediated cytotoxicity.

An activating NK receptor CD244 binds to CD48 adhesion molecule (Brown et 1998, Latchman et al 1998, Kubin et al 1999) and transduces NK activation signal by associating with lymphocyte activation molecule (SLAM) associated protein (SAP or SH2D1A) (Sayos et al 1998). Patients suffering from X-linked lymphoproliferative syndrome, lacking functional SAP when infected with EBV, show fulminating and fatal infectious mononucleosis.

**Role of NK cells in hybrid resistance.**

MHC class I expression level on target cells correlate inversely with their susceptibility to NK cells. Bone marrow grafts from β2 microglobulin knockout mice were universally rejected by NK cells from any strain of mice used as recipient (Ljunggren et al 1988, Raulet et al 1995). The characterization of inhibitory receptors that recognize MHC class I molecules provide support to this observation. However, the mechanism of NK mediated graft rejection has not been fully understood since the target in the bone marrow graft has not been determined. Moreover, NK cells expressing both activating and inhibitory receptors appear to interact in hybrid resistance. Removal of single subsets of inhibitory or activating receptors does not abrogate graft rejection suggesting a synergistic role of receptors in graft rejection (Raziuddin et al 2000).

**NK mediated ADCC and reverse ADCC**

Elucidation of molecular structures responsible for triggering an NK cell ADCC program revealed striking similarities to the TCR and BCR. In NK cells the ADCC receptor complex includes a cell surface Ig G binding molecule, FcγRIIIA (CD 16) in non-covalent association with CD 3ζ and FcγRIy disulfide linked dimers
(Anderson et al 1990, Vivier et al 1991). In humans, the ADCCR comprises a variety of disulfide linked heterodimers and homodimers associated with CD 16, CD 3ζ - CD 3ζ, FcεRIγ-CD 3ζ, FcεRIγ- FcεRIγ. However in murine NK cells, CD 16 only associates with FcεRIγ (Kurosaki et al 1991). The FcεRIγ and CD 3ζ polypeptides express in their intracytoplasmic domain one or three immunoreceptor tyrosine based activation motifs (ITAMs). CD 16 stimulation leads to activation of p56

\[ \text{p56}^{\text{bk}} \]

protein tyrosine kinase, which induces ITAM phosphorylation and recruitment of tandem SH-2 domain of ZAP-70/p72syk PTK. It also leads to tyrosine phosphorylation of phospholipase C-γ1/2 (PLC-γ), phosphatidylinositol 3 kinase, Shc, Grb-2 and pp36-38 and activation of a set of genes encoding for surface activation molecules like CD 69 and cytokines like IFN-γ (Kanakaraj et al 1994, Galadrini et al 1996, Anegon et al 1988).

In normal ADCC, antibody binds target cell and interacts with effector cells through Fc-CD16 interaction. Target lysis may also be facilitated if the direction of antibody bridge is reversed (reverse ADCC or redirected lysis). In reverse ADCC antibody reacts with effector NK cells and interacts with target cells by Fc-FcR interaction (Saxena et al 1982).

**Non specific modulators of NK cell activity**

**CD 69**

CD 69 is expressed as a 60 kD disulfide linked dimer present on activated NK and T cells and also on neutrophils, platelets and Langerhans cells. CD 69 is however variable in size since the subunit monomers may be glycosylated differently (Karlhofer et al 1991, Lanier et al 1988). Mitogen and IL-2 promote increased CD 69 expression on NK and T cells. CD 69 gene is NKC linked in both mouse and humans and is transcriptionally regulated by NF-kb/Rel transcription family. Activation of NK cells leads to increased CD 69 expression (Pisegna et al 2002). Recent evidence however shows a novel role of CD69 as a negative regulator of anti-tumor responses and shows the possibility of a novel approach for tumor therapy (Esplugues et al 2003).
IL-2 and its receptor

IL-2 is a soluble cytokine with four α-helices, produced exclusively by activated T cells, predominantly those of the CD4⁺ class. (Powell, J. D. 1998) The IL-2 receptor is expressed by T cells, B cells, NK cells, and cells of the monocyte/macrophage lineage. The receptor complex consists of three distinct subunits, which are designated IL-2Rα, IL-2Rβ and γc. Depending on which IL-2R subunit is expressed by a cell, three different affinities for ligand can be measured. The α subunit alone comprises a low-affinity receptor (Kd ≈ 10nM) that is unable to signal, whereas IL-2Rβ and γc together form an intermediate affinity receptor (Kd ≈ 1nM) that is fully competent to signal if adequate concentrations of ligand are supplied. Finally, expression of all three IL-2R subunits creates a high-affinity receptor (Kd ≈ 10 pM) found on the surface of activated leukocytes (Nelson et al 1998). Intracellular signals from the IL-2R originate from the cytoplasmic domains of the IL-2Rβ and γc subunits, which associate with the tyrosine kinases Jak1 and Jak3, respectively. When IL-2 binds to the extracellular region of the IL-2R complex, it induces heterodimerization of IL-2Rβ and γc, which in turn promotes catalytic activation of Jak1 and Jak3. As a result, cytoplasmic tyrosine residues on IL-2Rβ and γc become phosphorylated. Once phosphorylated, three key tyrosine residues on IL-2Rβ serve as docking and activation sites for downstream molecules. Y338 recruits the adaptor protein Shc, and Y392 and Y510 recruit the transcription factor Stat5. The IL-2R signal appears to bifurcate at this point into distinct Shc- and Stat5-mediated pathways (Nelson et al 1996). Shc serves as an adaptor protein to recruit and activate components of the MAP kinase and PI3 kinase pathways, whereas Stat5 serves as a transcription factor that directly binds to and transactivates a battery of target genes. (Lord et al 1998, 2000).

IL-2 plays an important and complex role in the immune system, serving as a growth factor, a differentiation factor, and a regulator of cell death. It shares many of these functions with other cytokines such as IL-15. Severe combined immunodeficiency diseases (SCID) represent a spectrum of diseases with similar
clinical manifestations (Leonard et al 1996, Buckley et al 1999), which can be subdivided into several categories depending upon the presence or absence of T cells, B cells and NK cells. In 1993 it was shown by Naguchi et al that XSCID results from mutations in the common cytokine receptor γ chain, γc. A major indispensable role of IL-2 signaling, at least in mice, is to limit the number of activated T cells in the periphery after exposure to self or environmental antigens. Quite often, peripheral CD4+ and CD8+ T cells react to self-antigens but under normal circumstances, such autoreactive cells are eliminated by other CD4+ and CD8+ T cells. Somehow this regulatory activity against autoreactive T cells is dependent on IL-2 signaling (Papiernik et al 1998, Brad et al 2003). IL-2 has a boosting effect on NK cells (Misawa et al 2003, Fehniger et al 2003). There is clear evidence of altered cytokine profiles in IL-2 or IL-2R deficient mice. For example, CD4+ T cells from these animals show abnormal expression patterns of Th1 and Th2 cytokines (Van Parijs et al 1997, Suzuki et al 1995, Khoruts et al 1998).

**IL-15 and its receptor**

IL-15 is structurally related to IL-2 and, moreover, uses the IL-2Rβ and γc subunits to generate intracellular signals. The only distinguishing feature of the IL-15R is its α subunit, which is structurally related to IL-2Rα yet binds IL-15 exclusively. To respond to IL-15, a cell must express IL-15Rα, IL-2Rβ and γc (Ma, A. 2000). Immature NK cells express the IL-15Rα, IL-2/15Rβ and γc but do not express the IL-2Rα chain that is necessary for formation of high affinity IL-2R. Therefore NK cell proliferation requires high doses of IL-2 stimulation. IL-15, secreted by bone marrow stromal cells is necessary for differentiation of functional NK cells from progenitor CD 34+ cells (Jaleco et al 1997, Kogure et al 2002, Prlic et al 2003, Koka et al 2003)

**Fc receptors**

Most human and mouse Fc receptors belong to the Ig superfamily. Two main classes of Fc receptors are known, the activation receptors, characterized by
the presence of ITAM motifs and the inhibitory receptors, characterized by the presence of ITIM motifs. Fc receptors present on NK cells are capable of triggering cell activation upon binding to the Fc portion of antibodies. These Fc receptors possess one or several intracytoplasmic activation motifs. These motifs are composed of twice repeated YXXL sequences designated as immunoreceptor tyrosine based activation motifs (ITAM)s (Rath M.G. 1989 and Cambier J.C. 1994).

Fc receptor mediated signaling requires aggregation of Fc receptor on cell surface by antibodies and multivalent antigens (Siraganian et al 1975 and Ishizaka et al 1978). There are high and low affinity Fc receptors. High affinity Fc receptors bind to noncomplex monomeric immunoglobulins while low affinity Fc receptors do not bind monomeric immunoglobulins but bind aggregated antibodies complexed to multivalent antigens with high avidity. By convention high affinity FcR are designated as FcR I and the low affinity receptors are further subdivided into FcR II and FcR III.

Crosslinking of extracellular domain of FcγRIII (CD16) results in tyrosine of ITAM motif by members of src family of kinases like lck. Subsequently SH2 containing signaling molecule ZAP70 binds to the phosphorylated ITAM and transduces an activating signal by activation of PI3 kinase that in turn leads to production of PIP3 and recruitment of PLCγ. PLCγ activation finally leads to generation of IP3 and DAG and a sustained calcium mobilization (Salcedo et al 1993, Cone et al 1993 and Bonilla et al 2000, Galandrini et al 2002). Cellular phenotypes associated with FcR activation includes degranulation, phagocytosis, ADCC, transcription of cytokine genes and release of inflammatory mediators (Andersson et al 1990 and Ravetch et al 1991).

Interferon γ and its receptor

Interferon γ is synthesized exclusively by T lymphocytes and NK cells upon induction by immune and inflammatory stimuli (Farrar et al, 1993, Lieberman et al 2002). Interferon γ is a noncovalent homodimer consisting of two identical 17kD polypeptide chain (Gray et al, 1982,83). During biosynthesis the polypeptide is N-glycosylated giving to a mature form of 50kD (Kelker et al 1984). Interferon γ
induces varied effects on a wide range of target cells. Interferon $\gamma$ can protect cells from viral infections and induces anti proliferated effects on normal and neoplastic cells. Interferon $\gamma$ also upregulates MHC I expression and induces MHC II on a variety of leukocytes and epithelial cells.

**Interferon $\gamma$ receptor**

There are two subunits of Interferon $\gamma$ receptor, $\alpha$ and $\beta$ of which $\alpha$ subunits is constitutively expressed on nearly all cells while $\beta$ subunit expression is regulated by external stimuli.

In unstimulated cells, the $\alpha$ and $\beta$ subunits are not preassociated. $\alpha$ and $\beta$ subunits are associated with specific Janus family kinases, Jak 1 and Jak 2 respectively. Upon ligand induced assembly, there is a homodimerization of the receptor and the intracellular domains of the two $\alpha$ and two $\beta$ subunits carrying the inactive Jak 1 and Jak 2 are juxtaposed. In this complex, Jak 1 and Jak 2 transactivate and phosphorylate the critical Y 440 site on the $\alpha$ subunit thereby forming the STAT-docking site. Two STAT molecules then associate with the docking site and are phosphorylated. Tyrosine phosphorylated STAT 1 molecules dissociate and form homodimeric complexes. Activated STAT 1 complex is then phosphorylated on a C-terminal serine residue by a MAP kinase like enzyme and this activated STAT 1 then translocates to the nucleus and affects gene transcription of Interferon $\gamma$ inducible genes.

**NK cell development**

NK cells like other hematopoietic cells, are derived from pluripotent hematopoietic stem cells. Initial evidence for a restricted NK/T progenitor came from analysis of fetal thymocytes. A Fc$\gamma$RIII$^+$ fetal thymocyte population was shown to give rise to TCR$\alpha$$\beta^+$ T cells after intrathymic transfer or to NK cells after intravenous transfer but was incapable of giving rise to myeloid cells or B cells (Rodewald et al 1992). The Fc$\gamma$RIII$^+$ fetal thymocyte population was later found to be heterogeneous (Carlyle et al 1998). A fraction of the cells express NK markers, NK1.1 and DX5 but fails to express CD117 (c-kit) also while the other fraction express NK1.1, CD117 but not DX5. The NK1.1$^+$ DX5$^+$ CD117$^-$ subset exhibits ex
vivo cytolytic activity against YAC-1 target cells suggesting that this population contain functionally mature NK cells. The CD117⁺ population was capable of reconstituting αβ T cells in thymic organ cultures and NK cell development when cultured with OP9 stromal cell line but failed to give rise to myeloid or B cells. Similarly, in human NK cells, the NK1.1 isoform, NKR-P1A was found on precursor thymocytes (Poggi et al 1996). NK cell development occurs primarily extrathymically, with critical steps in the bone marrow and only briefly in the thymus (Moore et al 1995). Analysis of fetal mouse blood revealed a NK1.1⁺CD90⁺CD117lo population that has the potential to differentiate into either NK cells or T cells but not myeloid or B cells (Carlyle et al 1998).

NK cell development requires IL-15, the receptor for which employs a unique IL-15Rα but shares the IL-2/-15Rβ chain and the common γ chain with IL2 receptor. IL-15 is secreted by bone marrow stromal cells, which supported NK development from human CD34⁺ progenitor cells (Mrozek et al 1996) and in murine system from Sca2⁺CD117⁺ progenitor population (Williams et al 1997). Preculturing cells in a cocktail of cytokines like IL-6, IL-7, SCF (stem cell factor) and flt-3 ligand is necessary to render the cells responsive to IL-15 (Williams et al 1997).

CD94 is expressed on NK like cells of human fetal liver suggesting that these receptors are expressed early in NK cell ontogeny (Jaleco et al 1997). Mouse fetal liver or spleen of new born mice exhibit class I dependent inhibition of cytotoxicity inspite of the absence of Ly49 receptors, suggesting the possibility of CD94-NKG2 receptors expressed early in NK development (Sivakumar et al 1997, Toomey et al 1998, Kumar et al 2002). CD94-NKG2 receptor family expression is induced by IL-15 (Mingari et al 1997).

Ly49 receptor expression occurs relatively late in ontogeny of NK development, the onset being after few weeks of birth and reaches a plateau after one month (Dorfman et al 1998). The expression of KIR or Ly49 is not dependent on cytokines like IL-15, IL-6, and IL-7, flt3 ligand (Williams et al 1998). Ly49 receptors are expressed predominantly mono allelically, though in some case biallelic expression is also observed (Held et al 1999a). Acquisition of Ly49 expression requires binding of TCF-1, a high mobility group protein that binds to the Ly49A promoter during NK development (Held et al 1999b).
Natural killer cell receptor repertoire

Initial NK cell receptor expression is a stochastic mechanism but the final functional repertoire is shaped by an education process based on MHC class I expressed by the host cells. The education process ensures that the NK cells are self-tolerant and also do not allow transformed tumor cells to escape surveillance. The frequency of NK cells coexpressing a given combination of receptors can be estimated as the product of frequencies of NK cells expressing each receptor (product rule), indicating a stochastic initiation of receptor expression (Raulet et al 1997, Valiante et al 1997). However some combinations of receptor expression and especially some KIR co-expression with CD94/NKG2D often show deviations from the product rule (Smith et al 2000). These deviations are justified since there exists an education process to adjust the repertoire such that each NK clone expresses at least one inhibitory receptor for self-MHC class I molecule. Analysis of Ly49A+ cells in H-2b mice showed Ly49A expression by 20% NK cells, although Ly49A is not the cognate receptor for the H-2b haplotype. However these NK cells were self-tolerant and this inhibition in cytotoxicity was not blocked by blocking Ly49A receptor using monoclonal antibodies. These results corroborate the 'at least one model' as an education process following stochastic repertoire development (Dorfman et al 1996). However it cannot rule out the possibility of existence of anergic cells in the population.

In class I deficient mice, certain hyporesponsive NK cells have been found that are unable to attack class I deficient lymphoblasts and bone marrow cells (Liao et al 1991). Thus, anergy reflects a dampening of the sensitivity of NK cell activating pathways in response to target stimulation, sufficient to prevent lysis of non transformed somatic cells (Hoglund et al 1991).

Cell surface levels of Ly49 receptors are found to be higher in the absence of strongly binding cognate MHC class I ligands. Hence this ‘receptor calibration model’ could be regulating the cell surface levels of Ly49 receptor that modulates the sensitivity of NK cells to self-MHC ligand expressing untransformed cells (Olsson-Alheim et al 1997, Hoelsbrekkan et al 2003). Moreover receptor genes that share commonality in regulation often result in preferential co-expression.
Ly49 genes exhibit sequence polymorphism, hence it is possible to detect two Ly49 alleles at the same locus in heterozygous mice. Ly49 has been found to be expressed in a monoallelic manner (Held et al 1995, 1998).

**Developmentally ordered expression of Ly49 receptor genes and maintenance of receptor gene expression**

Evidences suggest that Ly49 receptor expression is sequential and cumulative during development (Williams et al 2000). It was found that transferred Ly49A+ NK1.1+ cells in vivo or in in-vitro cultures never initiated expression of Ly49A but do initiate Ly49G2, C/I, F on fraction of cells (Dorfman et al 1998). Hence Ly49A expression is presumed to precede NK1.1 expression, similarly Ly49G2+ NK1.1+ cells did give rise to Ly49C/I expression and hence Ly49G2 expression preceded C/I expression (Roth et al 2000).

There are two models to explain this orderliness in Ly49 gene expression. One model suggests a sequential expression of Ly49 genes i.e. Ly49A only->Ly49G2 only ->Ly49C/I etc. The other model suggests that all Ly49 genes are initially available for expression but during development the capacity to activate some genes is lost before others e.g. Ly49A/G2/C/I -> Ly49G2/C/I -> Ly49C/I.

Most evidences suggest that once a NK receptor gene is successfully activated, it is stably maintained over various rounds of proliferation (Moretta et al 1990, Dorfman et al 1998). However, when NK cells were removed from their in vivo environment their target cell specificity might undergo changes. Moreover expression of MHC I ligand downregulates the Ly49A expression on mature cells (Karlhofer et al 1994, Held et al 1996). These evidences suggest that cells retain the capacity for continually modulate their Ly49 receptor expression, depending upon changes in the MHC class I environment, throughout the life span of NK cells.

**Natural Killer Gene Complex (NKC)**

The NKC was first defined by Yokoyama (1990) as a cluster of genes on mouse chromosome 6 encoding type II transmembrane, C-type lectin proteins.
GENOMIC ORGANIZATION OF NK RECEPTOR GENES IN HUMAN AND MOUSE

Figure A.
preferentially expressed on NK cells. Human and rat NKCs are carried on the syntenic human chromosome 12p12-13 and rat chromosome 4 (Brown et al 2000, Renedo et al 2000). The CD94, NKG2 family and the CD69 receptors encode C type lectins and are located in the centromeric end of the human NKC. However the functional equivalent genes of Ly49 family of receptors in humans, encode killer inhibitory receptors (KIR) that belong to the immunoglobulin superfamily. These receptor genes form a cluster called the leukocyte receptor cluster (LRC) on human chromosome 19q13.42. Other members of the IgSF such as the leukocyte Ig like receptors (ILTs), like LIRs/CD85, the leukocyte associated inhibitory receptors (LAIRs), FcαR and the activating receptor NKp46 also reside in this complex (Wilson et al 2000). The equivalent paired Ig-like receptor (PIRs) in mice resides on chromosome 7 (Kubagawa et al 1997).

Divergent and convergent evolution of NK cell receptors

Comparisons of the LRC and the NKC in humans and mice show that these two regions are orthologous in the two species. The evolution of the PIRs and ILTs probably took place independently in rodents and primate lineages by repeated duplication of a different locus in each species that originated from a single common ancestral sequence (Nei et al 1997). The evolution of genes encoding Ly49 and KIR proteins has taken a parallel route, both duplicating several times, both expressed in a clonal fashion and both employing similar signaling pathways upon recognition of different parts of the intact MHC I molecules. However there exist some drastically different evolution patterns in the evolution of Ly49 and KIRs. Gumperz and Perham (1995) showed that humans evolved from an ancestral species with Ly49 genes, only a single pseudogene is now left as evidence to this heritage. Conversely KIR loci never evolved in rodents or were lost early in evolution since there is no evidence of any related genes on mouse chromosome 7.

NK cells were found to lack T cell receptor or B cell receptor and were capable of lysing tumor transformed or virally infected cells in a MHC unrestricted manner. This posed the question as to the type of receptors on NK cells for detection
Figure B. Schematic representation of various forms of receptors on NK cells.
of target cells. NK cells are a unique class of lymphocytes that co-express receptors that transduce negative signal (inhibitory receptors) and those that transduce positive signal (activating receptors) for cytotoxicity of NK cells.

**Inhibitory NK receptors**

NK cells are known to spare normal cells expressing adequate amounts of MHC class I molecules while they kill transformed cells that have lowered levels of MHC I or have lost the expression of MHC I molecules. This "missing self hypothesis" proposed by Ljunggren and Karre has been supported by identification of multigene families of inhibitory receptors on mice and humans that send an inhibitory signal upon encountering self MHC I on target cells (Lanier 1998).

**Human Inhibitory Receptors**

NK cells are triggered into action by target cells that downregulate their cell surface MHC class I expression. This trigger is a common consequence of intracellular infection and tumor transformation since they evolve mechanisms of subverting the CD8+ T cell mediated surveillance by virtue of down regulating their MHC I expression (Algarra et al 2000).

NK cells detect this down regulation of MHC I by means of specific inhibitory receptors. Two main categories of inhibitory receptors have been detected in humans:


In addition some members of ILT/LIR/MIR family, related evolutionarily to KIR are also expressed by NK cells and recognize HLA- class I molecules (Borges et al 1997, Colonna et al 1997).
Killer Inhibitory Receptors

The KIR family of receptors (CD 158) are type I (extracellular amino terminus) membrane protein that contain two or three extracellular immunoglobulin-like domains (Wagtman et al 1995, Colonna et al 1995) and hence are designated as KIR2D or KIR3D receptors respectively. The cytoplasmic domains of KIR can be either long (L) or short (S) functioning as inhibitory or activatory receptors respectively. Inhibitory KIRs contain one or two ITIM sequences (V/IXYXXL/V) in their cytoplasmic domains.

Allelic Polymorphism-

Most KIR genes exhibit allelic polymorphism but the extent varies in different KIR genes. Most individuals are heterozygous at one or more KIR genes and this diversity is generated by point mutation and homologous recombinations (Gardiner et al 2001, Shilling et al 1998, Rajalingam et al 2001). The gene and allele diversity in human KIR haplotypes suggest that they have evolved rapidly in comparison to most other human genes (Valiante et al 1997).

Structural basis of HLA-KIR recognition-

Direct interaction between recombinant KIR and HLA molecules by native gel electrophoresis revealed a 1:1 stoichiometry (Fan et al 1996). The kinetics and thermodynamics of this binding measured by surface plasmon resonance (SPR), has revealed that the affinity of KIR-HLA C interaction is in the range of affinity of TCR for specific peptide bound MHC I, though the binding/detachment rate of KIR and MHC I is much higher (Maenaka et al 1999, Boyington et al 2000). This fast on and off rates allow for more efficient NK cell surveillance of potential target cells. Alleles of all three MHC I molecules, HLA-A,B and C can confer protection from lysis by NK clones (Wagtman et al 1995). In general, KIR3D receptors recognize HLA-A and B alleles whereas KIR2D receptors recognize HLA-C alleles (Litwin et al 1993). KIR2D receptors can be subdivided into two groups, one that recognize
HLA-C with Lys80 (e.g.: HLA-Cw2, 4,5,6) and those that recognize HLA-C with Asn80 (e.g.: HLA-Cw1, 3,7,8) in the MHC α helix (Colonna et al 1993, 2003).

Site directed mutagenesis has identified the KIR binding site on MHC I to be the region around position 80 of the HLA C α helix and its following loop (Luque et al 1996). Unlike TCR-peptide bound MHC interaction, where the interaction is largely dependent on the specific peptide fitting into the groove on MHC molecule, no significant changes in the peptide contributes to the MHC-KIR interaction (Fan et al 2001, Fan et al 1999).

KIR diversity-

In humans, KIR is encoded by a compact family of genes occupying 150kb of the leucocyte receptor cluster (LRC) on chromosome 19q13.4 (Martin et al 2000, Bartin et al 2001, Wende et al 1999). There are three criteria that have been used to classify the encoded KIR protein into 13 groups-

a. The number of extracellular Ig-like domains
b. Length of cytoplasmic tail
c. Sequence similarity

In the standard nomenclature, the number of Ig like domains is given by 2D for two domains and 3D for three domains and the length of cytoplasmic tail is given by L for long and S for short. Different KIRs with similar overall organization but sequence divergence of >2% are generally numbered in series. The prototypical KIR is long tailed and contains 3 extracellular Ig-like domains, KIR3DL1 and KIR3DL2 (Martin et al 2000). The majority of human KIRs have 2 extracellular Ig-like domains. These are of two types: Type 1 KIR2D having domains homologous to D1 and D2 of KIR3DL and type 2 KIR2D having domains homologous to D0 and D2 of KIR3D (Vilches et al 2000).

Signal transduction:

Engagement of various members of inhibitory Ig-like receptor family with cognate MHC I molecules leads to the early termination of NK cell activation cascades (Watzl et al.2003). In humans, inhibitory receptors for MHC I belonging to immunoglobulin superfamily like KIR, ILT also called LIR or the C type lectin
superfamily like CD94/NKG2A heterodimers, despite their structural heterogeneity, use common inhibitory mechanisms (Burshtyn et al 1997, Vivier et al 1997, Binstadt et al 1997). These receptors contain one or two immunoreceptor tyrosine based inhibition motifs (ITIMs) characterized by the presence of I/V/L/SxYxxL/V in their intracytoplasmic portion (Long 1999, Bolland et al 1999, Daeron et al 1999). Upon receptor engagement, there is activation of src family of protein tyrosine kinases like lck and phosphorylation of the tyrosine residues in ITIM motifs. As a consequence there is recruitment of the tandem SH2 domains of the intracytoplasmic protein tyrosine phosphatases SHP-1 and/or SHP-2, which are responsible for the inhibition of the NK cell activity (Leibson 1997, Ono et al 1997). This dephosphorylation induces the dissociation of LAT (linker for activation in T cells) and PLCγ in NK cells (Valiante et al 1996). SHP-1 can also dephosphorylate ZAP-70 and Syk, which induce early activation signals (Plas et al 1996, Dustin et al 1999). Hence, SHP-1 is capable of terminating the activating signals by dephosphorylating key Src and Syk kinases and adapter molecules responsible for downstream signaling by activating receptors. The role of recruitment of SHP-2 on ITIMs is not well understood as SHP-2 is preferentially involved in activating signaling cascades (Huyer et al 1999).

**CD94-NKG2A**

These two types of inhibitory MHC I receptors on NK cells employ different strategies for MHC class I recognition. CD94-NKG2A receptors are non-polymorphic and recognize a sequence element conserved in the signal peptide of most HLA class I that are presented by HLA-E which has little polymorphism. HLA-E signal peptide complexes are the ligands for CD94-NKG2A receptor and hence HLA class I diversity does not play a role in this recognition (Lee et al 1998). On the other hand, the KIR family includes highly polymorphic family of receptors that recognize the diversity in the HLA class I molecules (Valiante et al 1997).

*Mouse inhibitory receptors*
Mouse inhibitory receptors belong to the C type lectin family of receptors and recognize MHC class I molecules on target cell surface as ligands. Similar to the humans, inhibitory receptors in mice also belong to two groups of receptors:

1. The Ly49 receptor family
2. CD94-NKG2 A receptors

Ly49 receptor family in mice

The Ly49 genes compose a large multigene family, 20 or more Ly49 genes exist within the mouse genome, of which products of at least 14 genes have been identified (Smith et al 1994). The prototypical mouse Ly49 A receptor is an inhibitory receptor for H-2\textsuperscript{d} and H-2\textsuperscript{k} MHC I molecules (Karlhofer et al 1992).

The inhibitory Ly49 receptors possess cytoplasmic tail bearing immune receptor tyrosine based inhibition motifs (ITIMs) through which they functionally associate with the SHP-1 phosphatase and inhibit NK cell effector functions (Nakamura et al 1997). Detailed analysis of the signaling through inhibitory Ly49 receptors show that the inhibition of cytotoxicity by NK cells is mediated by interruption of early signaling events and not by delivering a global inhibitory signal per se. Cold target inhibition assays demonstrated that Ly49 A can prevent the lysis of an H-2\textsuperscript{d} protected target while allowing simultaneous killing of unprotected targets (Nakamura et al 1997).

Structural basis of MHC I ligand binding to Ly49 inhibitory receptors

Extensive studies showed that the $\alpha_1/\alpha_2$ domains of D\textsuperscript{d} was involved in the interaction of Ly49 A with H-2\textsuperscript{d}. Both functional and binding interactions could be blocked by monoclonal antibodies to $\alpha_1/\alpha_2$ domains of D\textsuperscript{d} and not to the $\alpha_3$ domain (Orihuela et al 1996). Studies of Ly49A interaction with D\textsuperscript{d}/ D\textsuperscript{b} chimeric MHC molecules suggested that the allelic specificity of Ly49A is restricted by the $\alpha_2$ domain of D\textsuperscript{d} (Sundback et al). It was found that the residues 53-65 in the $\alpha_1$ domain and residues 90-107 in the N-terminal $\alpha_2$ domain of H-2D\textsuperscript{d} are important for Ly49 A specificity (Matsumoto).
Interestingly however, co-crystal structure of Ly49 A and H-2D\textsuperscript{d} (Tormo et al 1999) revealed two sites of interaction between ligand and receptor. Site 1 involves the N-terminal end of the \(\alpha_1\) domain and the C-terminal end of the \(\alpha_2\) domain. At this site 8 residues are involved in hydrogen bond interactions with Ly49 A including polymorphic residues that differ between MHC class I alleles. Site 2 involves a cavity beneath the H-2D\textsuperscript{d} peptide binding platform that partially includes the CD 8 binding site and that spans the \(\alpha_1,\alpha_2,\alpha_3\), and \(\beta_2\) microglobulin domains of the molecule (Tormo et al 1999). Although the interaction of activating receptor like Ly49 D with H-2D\textsuperscript{d} is essentially similar to inhibitory receptor, Ly49A interaction with H-2D\textsuperscript{d}, in contrast to Ly49A/ D\textsuperscript{d}, Ly49D/ D\textsuperscript{d} interaction was easily disrupted by single amino acid changes in H-2D\textsuperscript{d} (Nakamura et al 2000). These included mutations in the exposed \(\alpha\) helices at position 73,80,83,104,155,156 and 169. These differences in the distinct interaction sites for activating and inhibitory receptors for MHC I ligands may be explained by the fact that the avidity of activating receptor Ly49 D may be much lower than that of Ly49 A and therefore the interaction of activating receptor with the MHC ligand can be easily broken by small conformational changes in the molecule. Similar observations have been reported for activating and inhibitory human NK receptors (Vales-Gomez et al 1998).

Ly49 diversity:

Members of the Ly49 family are type II glycoproteins belonging to the C type lectin superfamilly. The Ly49 gene products are expressed as disulphide linked homodimers on the NK cell surface. The Ly49 receptor gene family is located on chromosome 6 in the NK gene cluster (NKC) (Yokoyama et al 19993). There are at least 11 active genes (a-j and q) and 5 pseudogenes (k-n and v) in the Ly49 family (Mc Queen et al 1999 a, b, Depatie et al 2000).

Signal transduction:

Ly49 A, the prototype inhibitory receptor contains an immunoreceptor tyrosine based inhibition motif (ITIM) in the intracytoplasmic region. Upon receptor engagement the tyrosine residues in the ITIM motif gets phosphorylated by members of src family protein tyrosine kinase. The phosphorylated ITIM then
serves as a docking site for the intracellular tyrosine phosphatase SHP-1 (Olcese et al 1996) and activation of SHP-1 is a key regulator through which activation signals by NK activating receptors are blocked. SHP-1 dephosphorylates tyrosines in the active site of other kinases that are essential for transducing an activating signal.

**CD94-NKG2A**

Mouse CD94-NKG2A transduce inhibitory signal on perceiving MHC class Ia molecules indirectly by specifically recognizing peptides processed from the leader sequences of class Ia molecules bound into the groove of a non-classical class Ib molecule, Qa-1 in mice (Vance et al 1998).

**Activating NK receptors**

Upon ligation of activating receptors NK cells undergo blastogenesis, cytokine production, cytotoxicity and migration. The effector function of NK cells is regulated by a fine balance between the activating and inhibitory signals transduced by receptors on NK cell surface. However coligation of activating and inhibitory receptors leads to a dominant inhibitory effect that downregulates activating signals initiated by activating receptors.

**Human Activating NK cell receptors**

Surface molecules expressed by NK cells that can trigger a stimulatory signal have been known for a long time, including CD2 (Bolhuis et al 1986), CD16 (Lanier et al 1988) and CD69 (Moretta et al 1991). However none of these molecules are involved directly in triggering natural cytotoxicity, although a role of CD2 as coreceptor has been shown (Lanier et al 1997).

**MHC specific activating receptors**
There are two main classes of HLA specific activating receptor on human NK cells.

1. The HLA-C specific p50 activating receptor
2. CD94-NKG2C complex that recognizes HLA-E molecules

**p50 receptors**

Molecular cloning revealed that the activating isoforms of p58 receptors were shorter (thereby termed p50) and lacked ITIM sequences in their cytoplasmic domain. The short intracytoplasmic domain associates with other signal transducing adapter molecules to transduce an activating signal (Bottino et al 1996). There is a charged amino acid lysine in the transmembrane domain that is essential for the association of the adapter molecule (Biassoni et al 1996).

The p50 receptors bind HLA-C alleles with lower affinity than the corresponding p58 inhibitory receptor. Moreover this binding can be influenced by single amino acid substitutions in the positions that are critical for the interaction of p50 to the HLA-C molecule (Biassoni et al 1997).

**Signal transduction**

The p50 activating receptor displays a transmembrane sequence containing a positively charged amino acid lysine, which engages in electrostatic interactions with the negatively charged aspartic acid in the transmembrane region of the adapter molecule (Moretta et al 1996, Lanier et al 1998). KIR2DS (p50) assemble with disulphide linked dimers of DAP 12, a 13 Kd ITAM bearing protein that shows similarity to the ITAM bearing ζ chain of the TCR (Lanier et al 1998). The DAP 12 molecule was found to be structurally related to a previously isolated molecule called “killer activating receptor associated protein” (KARAP) (Olcese et al 1997). KARAP has been cloned and now referred to as DAP 12 (Lanier et al 1998). Phosphorylated human DAP 12 recruits ZAP 70 and syk tyrosine kinases and thus triggering the activating signal.
CD94-NKG2C

The human NKG2 family of NK cell genes was identified by Houchins et al 1991 by subtractive hybridization. The function of these gene products remained elusive until in 1996 Phillips et al discovered that NKG2 chains form heterodimers with CD 94 NK cell receptors (Phillips et al 1996). CD 94-NKG2 A/B complexes transduce inhibitory signals through ITIM residues in the cytoplasmic tail of NKG2 A/B. However, NKG2C and NKG2E lack cytoplasmic ITIM residues and possess transmembrane charged residues for docking DAP 12 molecules.

The human CD 94/NKG2 receptors recognize the non-classical MHC I molecule HLA-E. HLA-E transcripts are broadly expressed in human cells. HLA-E protein expression on cell surface however requires occupancy of the MHC peptide-binding groove (Braud et al 1998). HLA-E binds peptides derived from selected leader sequences of various classical MHC I molecules. Thus, targets expressing specific HLA molecules can contribute leader peptides to HLA-E stabilizing its expression on the surface of targets. Thus, HLA-E binding to CD 94-NKG2 allows to monitor indirectly a broad array of MHC I molecules.

NKG2D

NKG2D is a type II lectin like receptor first identified by Houchins in 1991 by the ability of its cDNA to cross-hybridize with NKG2A on Southern blot analysis. Although this receptor is characterized as an NKG2 family member it shares only 25% amino acid homology to the other members of NKG2 family. The NKG2D receptor does not heterodimerize with CD 94, instead is expressed as a homodimer on NK cells, on many αβ+ and γδ+ T cells and on liposaccharide stimulated macrophages (Uhrberg et al 1997, Bauer et al 1999). NKG2D lacks cytoplasmic ITIM motifs and possess charged residues in its transmembrane region that associates with an activating adapter protein DAP10 (Wu et al 1999).

Human DAP10 and DAP 12 genes lie adjacent on chromosome 19q13.1, in opposite transcriptional orientation and separated by only 130bp. DAP 10 has a short but conserved cytoplasmic tail which contains a YxxM signaling motif, a
potential src-homology 2 (SH2) domain binding site for the p85 regulatory subunit of the phosphatidylinositol 3-kinase (Songyang et al 1993). DAP 10 has an acidic transmembrane segment that electrostatically interacts with a basic residue in the transmembrane region of other receptors like NKG2D.

Human NKG2D have been shown to recognize stress inducible MHC I chain related transmembrane proteins, MIC A and MIC B (Cerwenka et al 2000, Jinushi et al 2003). The expression of these MHC I like molecules do not require association with the β2 microglobulin or occupancy of the peptide binding groove (Groh et al 1998). MIC A and MIC B are found to be under the control of heat shock promoter elements and their expression can be induced on primary and neoplastic targets by cellular stress and viral infection (Groh et al 2001). Additional polymorphic ligand for NKG2D has been identified by Lanier et al and Raulet et al 2000. They identified surface glycosylphosphatidylinositol-linked NKG2D ligands, the retinoic acid early inducible genes (RAE-I) and weakly homologous minor histocompatibility antigen H60 (Diefenbach et al 2000). Hence, NKG2D and its inducible ligands comprise a potent mechanism for the recognition and lysis of stressed, virally infected or neoplastic targets (Hayakawa et al 2002, Borrego et al 2002).

**Non MHC specific activating receptors**

Masking the HLA class I molecules using monoclonal antibodies (Moretta et al 1996) can induce NK cell mediated lysis of HLA class I+ target cells. These observations imply the existence of NK-cell receptors that are responsible for the induction of NK-cell triggering in an HLA-independent fashion. This novel emerging group of receptors are called ‘natural cytotoxicity receptors’ (NCRs).

**NKp46, the prototype NCR**

NKp46 is a 46 Kd glycoprotein characterized by a protein backbone of 33kD, expressed by all NK cells (both resting and activated) but is absent on all other cell types (Sivori et al 1997). Molecular cloning revealed a type I
transmembrane glycoprotein belonging to immunoglobulin superfamily characterized by two C2-type Ig-like domains in the extracellular portion followed by a stretch of amino acids, forming a stem which connects the ectodomain to the transmembrane region. The transmembrane region contains positively charged amino acid residue, Arginine, that is involved in stabilizing the interaction with associated molecules. The cytoplasmic portion is very short comprising of only 30 amino acid residues and therefore does not contain any sequence motifs typically involved in activation signals. Biochemical analysis revealed that NKp46 molecules are coupled to ITAM containing CD 3ζ and/or FceRIγ adaptor proteins (Moretta et al 1999).

It plays an important role in the lysis of tumor-transformed cells of autologous, allogenic and xenogenic origin (Sivori et al 1999, Vankayalapati et al 2002). NKp46 masking by specific monoclonal antibodies inhibited lysis of majority of human tumors belonging to different histotypes including lung, liver and breast carcinomas, melanomas, and Epstein Barr virus transformed cell lines (Pessino et al 1998). NKp46 is the only triggering receptor expressed by human NK cells that is capable of recognizing a ligand on the surface of murine cells (Pessino et al 1998). A NKp46 homologue has been cloned in mouse as well (Biassoni et al 1999).

NKp44

It is known that culturing with IL2 increases the ability of NK cells to mediate non-MHC restricted tumor cell lysis (Trinchieri et al 1989). This phenomenon may reflect the de novo expression of novel NCRs that allow activated NK cells to kill additional targets.

A novel 44 kD surface molecule (NKp44) was identified, (Vitale et al 1998) which is absent on freshly isolated peripheral blood lymphocytes but is progressively expressed by all NK cells in vitro upon culturing with IL2. NKp44 is present on all NK clones analysed so far and absent on activated T cells and T cell clones. Thus NKp44 is a marker specific for activated NK cells (Vitale et al 1998, Cantoni et al 2002).
Masking of NKp44 by monoclonal antibodies partially inhibited cytolytic activity against certain HLA- tumor target cells and this degree of inhibition was greatly increased by simultaneous masking of NKp46. This suggests that NKp44 functions in cooperation with other NCRs in non MHC restricted lysis.

Molecular cloning revealed that NKp46 also belongs to the Ig superfamily. It is characterized by a single extracellular V type domain and by a membrane proximal, hinge-like portion containing a high proportion of Pro, Ser and Thr. The transmembrane region contains a charged amino acid Lys, involved in association with the ITAM bearing signal transducing KARAP/DAP 12 molecule (Vitale et al 1998, Cantoni et al 1999).

NKp30

The failure to completely abrogate cytotoxicity of HLA- targets by simultaneous masking of NKp44 and NKp46, suggested the existence of other NCRs as well. A 30 Kd receptor was identified and cloned and termed NKp30 (Pende et al 1999). NKp30 has been found to be a major receptor involved in the killing of certain tumors that are independent of killing by NKp46 and NKp44. NKp30 has also been found to cooperate with NKp44 and NKp46 in the cytotoxicity against certain tumors. Both resting and activated NK cells express NKp30. Human dendritic cells activate resting NK cells via the NKp30 receptor (Ferlazzo et al 2002).

Molecular cloning of NKp30 revealed a member of Ig superfamily associated with CD3ζ chain and characterized by a single V-type domain in the extracellular domain and a charged residue in the transmembrane portion (Pende et al 1999).

Mouse Activating NK cell receptors

Activating receptors on mouse NK cells constitute of
1. ITAM containing members of the Ly49 family
2. CD94-NKG2C
3. NKR-P1

Activating members of Ly49 receptor family

Ly49 receptors are expressed at the cell surface as transmembrane disulphide bonded homodimers with each subunit displaying a membrane proximal stalk region and a carboxy-terminal carbohydrate recognition domain. The activating receptors lack an ITIM containing cytoplasmic tail and when engaged, activate NK cells to lyse target cells or produce cytokines (Mason et al 1996, Smith et al 2000). Activating Ly49 receptors associate with a small (12kD) disulphide bonded transmembrane homodimeric signaling adaptor protein DAP 12 through an intramembrane noncovalent charge interaction (Smith et al 1998).

Ly49 D

The Ly49 D molecule was first identified in experiments designed to clone molecules highly related to the originally identified Ly49 A receptor (Smith et al 1994). Mason and Ortaldo (1996) produced a monoclonal antibody specific for Ly49 D and showed that Ly49 D is an activating receptor. Smith et al dissected a genetic locus termed Chok, which was responsible for regulating the killing of Chinese hamster ovary (CHO) targets (Idris et al 1998). The chok gene was found to be related to the NKC on mouse chromosome 6. Monoclonal antibody against chok gene product immunoprecipitated a disulphide linked dimeric molecule (Idris et al 1999). Moreover, BALB/c IL2 activated NK cells were infected with a recombinant Ly49 D-vaccinia virus construct and this resulted in the aqisition of the ability of these cells to kill CHO cells while control vector infected NK cell did not. This CHO killing was blocked by monoclonal antibody against chok gene product. Hence, it was proved that the chok gene locus codes for Ly49 D that serves as an activating receptor on NK cells.

Ly49 H
The Ly49 H cDNA was first reported by the Takei group (Brennan et al 1994). Like Ly49 D, Ly49 H significantly differed in the cytoplasmic domain of other Ly49 receptors and contains a charged amino acid residue in the transmembrane segment (Smith et al 2000). DAP 12 was found to be essential for Ly49 H signaling (Smith et al 1998). Although Ly49 H shows significant similarities with Ly49 D, Ly49 H is not involved in killing of CHO cells and Ly49 H showed greater dependence on DAP 12 than Ly49D. Ly49 D and Ly49 H are expressed only on NK1.1+ CD3− NK cells and are absent in NKT or T cells.

**CD94-NKG2C**

Similar to the human counterparts CD94-NKG2C lacks cytoplasmic ITIM motifs and act as activating receptors. However in contrast to the human homologue, the mouse NKG2C protein does not contain transmembrane charged residues for docking to DAP 12. Rather, there is a charged residue in the transmembrane domain of CD 94 that recruits the activating adapters in rodent CD94-NKG2C receptors (Vance et al 1997). The mouse non-classical MHC molecule Qa-1b binds to mouse CD 94-NKG2 molecules (Vance et al 1998). Qa-1b binds to peptides derived from mouse MHC I leader peptides.

**NKR-P1 family**

NKR-P1 was the first family of NKC encoded receptor to be discovered. The first member of NKR-P1 family to be discovered was the rat NKR-P1A (Chambers et al 1989). The structure of the rat NKR-P1A revealed a lectin like type II integral membrane homodimer. In both mice and rats various homologous genes designated as NKR-P1 A, B, C and D have been identified (Giorda et al 1991). Although the prototype rat NKR-P1A was found to be an activating receptor, subsequently NKR-P1 family with divergent functions has been identified in mouse as well as human NKR-P1 repertoire. Mouse NKR-P1C encoded NK1.1 and rat NKR-P1A activate cytotoxicity while a homologous human NKR-P designated as NKR-P1A serves as an inhibitory receptor.
Signal transduction:

The ligand for NKR-P1 binding has not yet been identified, carbohydrate binding by the lectin domain or rat NKR-P1A has been demonstrated and glycoprotein ligands on the surface of tumors or immature cells have been speculated as possible targets for NKR-P1 recognition (Bezouska et al 1994 a, b). The cytoplasmic domains of the rat and mouse NKR-P1 can be separated into 3 regions; an amino terminal tyrosine based motif, a proline rich motif and a putative p56^ck tyrosine kinase-binding motif characterized by the sequence CxCP. In human NKR-P1A there is no evidence of either a transmembrane basic residue for association with known adapter molecules, or a cytoplasmic ITIM and yet sends an inhibitory signal through as yet unknown mechanisms (Lanier et al 1994). The proline rich region binds to the src homology 3 domain (SH3) of molecules involved in signal transduction (Yu et al 1994). The activation signal leads to phosphoinositide turnover, mobilization of intracellular calcium, phosphorylation of protein tyrosines, degranulation and release of cytokines.

Co-receptors

Apart from the true triggering receptors, there are some molecules that act as co-receptors and their triggering function is dependent on the simultaneous engagement of other main triggering receptors (Sivori et al 1999).

2B4 (CD 244)

2B4 is expressed as a 70 Kd surface molecule on NK cells as well as CD 8^+ T-cell subset, monocytes and granulocytes (Sivori et al 2000). 2B4 is a member of the CD2 subset of the immunoglobulin superfamily (Valiante et al 1993). 2B4 was initially identified on mouse lymphocytes as a surface receptor capable of mediating non-MHC restricted NK cell cytotoxicity (Garni-Wagner et al 1993). Crosslinking of 2B4 with monoclonal antibodies leads to cytokine production and increased target cell lysis. It has been found that NK cells lacking NKp46 expression are unresponsive to 2B4 stimulation. This suggests that NKp46 interaction with its
ligand provides the first signal for subsequent 2B4 signaling (Sivori et al 1999). This
coreceptor role of 2B4 is not restricted to NKp46 but is shown with CD16 and
NKp44 as well.

Both murine and human 2B4 molecules recognize the GPI-anchored surface
molecule, CD 48. The 2B4 molecule is characterized by one membrane distal Ig V-
type domain and one membrane proximal Ig C2 type domain. The transmembrane
does not contain any charged amino acid residues. There is a long cytoplasmic tail
that contains four tyrosine based motifs (Txyx/L/V) which undergoes
phosphorylation and is able to recruit src homology 2 (SH 2) containing phosphatase
SHP-2 and also binds to the intracellular adapter molecule SLAM associated protein
also called SAP (Tangye et al 1999). Therefore SH2D1A/SAP competes with SHP
for association with 2B4 thus preventing the inhibitory signal mediated by SHP
association. It has also been shown that 2B4 is constitutively associated with the
linker for activation of T cells (LAT). This occurs through the interaction between a
CxC sequence surrounded by positively charged residues (CxxC/+), present in the
2B4-transmembrane or cytoplasmic portion and a CxxC/- motif in the LAT-
transmembrane or cytoplasmic portion (Bosselut et al 1999). Cell triggering upon
2B4 monoclonal antibody mediated crosslinking results in tyrosine phosphorylation
of both 2B4 and the associated LAT and recruitment of intracytoplasmic signaling
molecules including phospholipase C and Grb2 (Bottino et al 2000).

In patients with Epstein-Barr virus induced lymphomas, 2B4 molecule upon
interaction with CD48 on target cells, fail to activate and rather inhibit NK
cytotoxicity. In these XLP NK cells, SH2D1A/SAP is truncated or missing and
hence the 2B4 associated with SHP molecule and sends an inhibitory signal

Co-engagement of 2B4 and inhibitory receptors like KIR and CD94/NKG2A
prevents 2B4 phosphorylation (Watzl et al 2000). It has been shown recently that
LFA1-ICAM1 interaction leads to vav1 phosphorylation and actin cytoskeleton
reorganization, which results in 2B4 recruitment to the lipid membrane rafts. This
recruitment of 2B4 to rafts is essential for 2B4 phosphorylation and transduction of
an activating signal (Watzl et al 2003, Barber et al 2003). KIR engagement can
inhibit clustering of rafts and retention of 2B4 in these rafts. Moreover ITIM
phosphorylation in KIRs also recruits SHP-1, that binds to vav 1 at the catalytic site and leads to vav1 dephosphorylation, thus blocking actin polymerisation and 2B4 phosphorylation.

**NKp80**

A novel-triggering molecule, termed NKp80 was identified that preferentially activates NCR+ NK cells. NKp80 co-receptor expression is confined to NK cells and a small subset of T cells. NKp80 is expressed as a 80 Kd type II transmembrane protein belonging to the C-type lectin superfamily (Vitale et al 2001). The transmembrane region is characterized by non-polar amino acids and there is no classical ITAM motif. The signal transduction by NKp80 is yet to be elucidated.

**Distinct families of Ig like receptors that modulate functions of lymphoid and myeloid cells**

A family of receptors called immunoglobulin like transcripts (ILTs) or leukocyte Ig like receptor (LIRs) or monocyte/macrophage Ig like receptor (MIRs) have been discovered on lymphoid and myeloid cell subsets. They are characterized by the presence of either 2 or 4 homologous extracellular C-2 type Ig like domains and are classified by differing transmembrane and cytoplasmic domains (Samaridis et al 1997). There are both activating and inhibitory receptors in this family. One subset of these receptors (ILT2, ILT3, ILT4, ILT5 and ILT8) displays long cytoplasmic tails containing ITIM motifs. These receptors can inhibit cell activation by recruiting protein tyrosine phosphatases SHP-1 (Fanger et al 1998). Another subset of receptors (ILT1, ILT1-like protein, ILT7, ILT8 and LIR6) contains short cytoplasmic domains that lack signaling motifs. They contain single basic arginine residues within the hydrophobic transmembrane domain and associate with the gamma chain of Fc receptor (FcRγ) which transduces stimulatory signal through cytoplasmic ITAM motifs (Borges et al 1997, Nakajima et al 1999).
Genes encoding ILT/LIR/MIR receptors are located on human chromosome 19q13.4. Their murine counterparts are called paired Ig like receptors (PIRs) and their genetic location is on chromosome 7 (Kubagawa et al 1997). PIRs are characterized by the presence of 4 C2 like and 2 V like extracellular domains but differ in their transmembrane and cytoplasmic domains. Only one PIR isotype (PIR-B) displays cytoplasmic ITIM and mediates inhibition (Blery et al 1998) while 8 known PIR isotypes mediate activation by associating with FcRy via an arginine residue within the hydrophobic transmembrane domain (Kubagawa et al 1999). All ILTs are expressed on peripheral blood monocytes, macrophages and dendritic cells. ILT1, ILT2 and ILT5 are expressed on granulocytes, NK and T cell subsets (Banham et al 1999). The homology between ILTs and KIRs suggest that ILTs may be receptors for MHC I molecules though this interaction has not been clearly understood. MHC class I related molecules like CD1, MR1 and MIC has been suggested as possible ligands for ILTs (Porcelli et al 1995 and Bahram et al 1996).

**NK cell mediated killing**

Studies with perforin deficient mice firmly established perforin as the key element in NK cell mediated cytotoxicity. Results show that in contrast to wild type mice, mice deficient for both granzymes, gzm A and gzm B exhibit an uncontrolled tumor growth with a time kinetic similar to that of perforin-deficient mice. This indicates that a concerted action of perforin and the two granzymes is mandatory for optimal NK cell-mediated tumor control in vivo (Pardo et al 2002). Although the granule exocytosis pathway seems to be the most potent cytolytic mechanism of NK cell-mediated rejection, alternative perforin-independent mechanisms, such as death receptor-induced apoptosis, also exist. By preventing both perforin- and Fas-mediated interactions concurrently, mice were impaired in mediating MHC class I-deficient BMC rejection, killing of intracellular pathogens and tumor rejections (Taylor et al 2002). Inhibitors of apoptosis can block the rejection of tumors mediated by NK cells, by introducing the long form of Fas-associated death domain-like IL-1beta-converting enzyme-associated inhibitory protein (FLIP(L)) and poxvirus cytokine response modifier A (CrmA) into the MHC class I-deficient T
lymphoma cell line RMA-S. RMA-S cells do not normally express Fas in vitro, and it was previously postulated that the rejection of these tumors by NK cells is strictly perforin dependent. However, perforin-deficient NK cells have been shown to directly mediate Fas up-regulation on RMA-S cells and thereafter kill the cells in a Fas-dependent manner, and that RMA-S FLIP(L) and RMA-S CrmA are protected from such killing (Screpanti et al 2001). Addition of antibodies to major histocompatibility complex class I (MHC class I) and F(c) gamma Rill (CD16) antigens resulted in the synergistic augmentation of natural killer (NK) cell death, and the loss of NK cell cytotoxic function. NK cell death was associated with an increase in tumor necrosis factor-alpha (TNF-alpha) secretion, and concomitant inhibition of nuclear factor kappa B (NF kappaB) activation and the induction of c-jun N-terminal kinase (JNK) activity in NK cells (Jewett et al 2001). The levels of death promoting gene products such as the Fas receptor and the Fas ligand were also upregulated in NK cells in the presence of anti-class I and anti-CD16 antibodies (Jewett et al 2000).

**Granzyme, Perforin mediated cytolysis**

Contact between the effector and the target cell induces a calcium dependent release of specialized lytic granules containing various cytotoxins. One of these cytolytic proteins is perforin, which is a 65 Kd glycoprotein that polymerizes to form a pore in the target membrane (Berke et al 1995). Upon release from the granule perforin polymerizes into a cylindrical structure that is lipophilic on the outside and has a hydrophilic hollow center with an inner diameter of 16 nm. This structure can insert into the lipid bilayers, forming a pore that allows water and salts to enter rapidly into the cell, destroying the integrity of the cell membrane and thus results in osmolysis of the target cell.

Another class of cytolytic proteins comprises of the granzymes, which belong to the family of serine proteases and once released into the cytoplasm, activate apoptosis in the target cell. Granzymes are proteases, so they cannot directly fragment DNA in the target cell, rather they activate a cascade of enzymes in the
target cell that leads to apoptosis. Granzyme B can cleave the ubiquitous cellular enzyme CPP-32, which plays a key role in apoptosis.

**Fas signaling pathway**

Clustering of Fas upon binding to Fas ligand recruits the Fas associated 'death domain' (FADD) which is a bipartite molecule with a 'death effector domain' (DED) at the amino terminus and a DD at the carboxy terminus. FADD binds to Fas via a DD-DD interaction and recruits the DED containing caspase 8 and caspase 10 to the receptor via a DED-DED interaction (Vincenz et al 1997) thus forming the death inducing signaling complex. Further this leads to sequential induction of cascade of caspases that ultimately results in the apoptosis of target cells (Spaggiari et al 2002).

**Immunological Synapse**

NK cells have large granular lymphocyte morphology and form part of both innate and adaptive immune responses. NK cells express low affinity Fcγ receptor CD16 that recognizes Ig G coated target cells that can be killed by antibody dependent cell cytotoxicity (ADCC). NK cells also express two types of MHC I recognition receptors: activating receptors containing immunotyrosine based activating (ITAM) motifs in their cytoplasmic tails and inhibitory receptors containing immunotyrosine based inhibitory (ITIM) motifs in their cytoplasmic tails. The activating receptors signal through a kinase cascade involving lck and Syk or Zap-70 kinases. This activating signal is counteracted by the inhibitory receptor that recruits SHP-1 following phosphorylation of the ITIMs by src family kinases. Thus SHP-1 extinguishes signaling through neighbouring ITAMs suggesting that the activating and inhibitory receptors are thoroughly intermixed in the immunological synapse, IS (Binstadt et al 1996). Many adhesion and costimulatory molecules like LFA-1, CD2, 2B4 contribute to NK killing. Antibodies to these molecules inhibit killing of target cells by NK cells (Lanier 1998). The distance spanned by KIR or KAR/ MHC I is similar to that of TCR/MHC I interactions i.e. <15 nm (Boyington...
et al 2000). Davis et al (1999) described an inhibitory immunological synapse between an NK cell line expressing KIR2D and a class I transfected B cell line. In the NK cell immunological synapse a ring of KIR/MHC interaction formed around the central LFA-1/ICAM-1 cluster (Ardouin et al 2003). It was demonstrated that MHC I clustering by KIR2DL may require zinc molecules to stabilize the clusters. It is likely that the KIR/MHC ring of inhibitory NK IS also contains engaged activating receptors surrounded by inhibitory receptors that recruit SHP-1 (Trambas et al 2003, Blanchard et al 2002).

**NKT cells**

NKT cells are a population of T cells that share some of the characteristics of NK cells like NK.1.1 expression. NKT cells are characterized by a heavily biased TCR gene usage, CD1d restriction and high levels of IL4 and IFNγ production (Bendelac et al 1995, 1997, Macdonald et al 1995). Since their discovery in 1987, two distinct populations of NKT cells have been found. They are: thymic αβ TCR⁺ CD4⁺CD8⁻NK1.1⁺ and CD4⁺NK1.1⁺ populations. Both these populations express TCR Vα14Jα281 and secreted high levels of IL4 and IFNγ. Both these populations have been found to belong to the same lineage and their development is dependent on MHC I like β2microglobulin associated molecule, CD1d. Similar populations of NKT cells have been found in humans as well (Exley et al 1997, Dellabona et al 1994, Davodeau et al 1997).

NKT cells are most predominant in the liver (30-50% of mature T cells), 20-30% in bone marrow and 10-20% of mature HSA⁻ T cells in the thymus.

**NKT cell development**

NKT cells have been found to develop in fetal thymic organ cultures (Bendelac 1994) and are found in recent thymic emigrants in both spleen and liver (Hammond et al 1999) and fail to develop in nude or neonatally thymectomised mice (Coles et al 2000, Hammond et al 1998). Such evidences strongly suggest a thymic origin of most NKT cells, however some recent evidences suggest an extrathymic origin as well, especially CD8⁺NK1.1⁺ T cells that are found in normal
concentration in the periphery of neonatally thymectomised mice (Hammond et al 1998, 1999).

Development of all subsets of NKT cells has been found to require interaction with membrane lymphotoxin expressing cells. NKT cell development is also dependent on pre-Tg signaling and on granulocyte-macrophage colony stimulating factor (GM-CSF) signaling. Studies on NKT cells from γ chain deficient mice revealed two stages in NKT cell development, since in such mice DN IL4 producing Vα14Jα281+ cells were present but lacked NK1.1 and Ly49 expression and are not exported to the periphery. Therefore intrathymic selection and IL4 production seem to precede acquisition of NK surface markers and emigration to periphery which are γ chain dependent events.

NKT cell ligands


Physiological significance of NKT cells

The most significant physiological role of NKT cells is to prevent an autoimmune inflammatory response. Vα14 TCR transgenic mice, which have tenfold increased NKT cell populations, show elevated serum IgE and IL4 levels and hence promote Th2 response (Kitamura et al 2000). NKT cells have also been found to suppress Th1 response by production of IL4, IL10, TGFβ. NKT cells play an important role in controlling anterior chamber associated deviation (ACAID) that prevents eye damage by inflammatory immune response (Sonoda et al 1999).

NKT cells have been found to predominate in the granulomatous reactions to M. tuberculosis. NKT cells respond to mycobacterial infection by decreasing IL4 and increasing IFNγ and causes clearing of the pathogen. NKT cells participate in
the immune response to various infectious agents like mycobacteria (Behar et al 1999), plasmodium(Schofield et al 1999), Listeria (Flesch et al 1997), etc.

NKT cells also mediate cytotoxicity against tumor cell lines in vitro (Smyth et al 2000, Chan et al 2003). A major function of NKT cell mediated tumor restriction in vivo is accomplished by activating other effector cells like NK cells (Carnaud et al 1999, Eberl et al 2000).