MECHANISM OF LEUKEMIC CELL KILLING BY IL-2 ACTIVATED NATURAL KILLER CELLS, ROLE OF CELL ADHESION MOLECULES

by

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Pharm D, University of Paris XI, 1980

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENT FOR THE DEGREE OF
DOCTOR IN PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Microbiology and Immunology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

October 1997

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Date October 10 - 1997

ABSTRACT

Natural killer (NK) cells and lymphokine activated NK (LAK) cells, contribute to the elimination and growth control of malignant and virally infected cells. The binding of killer cells to their targets is a prerequisite for the lysis of malignant cells by NK cells, which utilize cell adhesion molecules (CAMs) to establish initial attachment to target cells. This thesis examined the possibility that defective expression of CAMs on some leukemic cells may be the primary cause of resistance to NK cell-mediated killing. To elucidate the mechanisms by which some leukemic cells are resistant to NK cytotoxicity, a model system was established with the human NK cell line NK-92, and the NK resistant leukemic cell line SR-91 which were established and characterized. SR-91 cells express very low levels of ICAM-1 and they failed to bind to NK-92 cells. NK-92 is highly cytotoxic and kills virtually all leukemic cell lines with the only exception being SR-91. Pre-treatment of SR-91 cells with TNF-α or IFN-γ, two cytokines known to upregulate ICAM-1 expression, increased both ICAM-1 expression on SR-91 cells and binding to NK-92 cells. However, only TNF-α treated SR-91 cells became sensitive to killing by NK-92 cells. The increased binding to NK-92 cells and sensitivity to their killing were abrogated by anti-LFA-1 antibody or a combination of antibodies against ICAM-1, ICAM-2 and ICAM-3, indicating that LFA-1 interaction with the three ICAMs is essential for effector-target cell binding, which is a prerequisite for subsequent target cell lysis. These results underline the importance of ICAM-1 expression on the target cell SR-91 to allow adequate conjugate formation. However, this is, on its own, insufficient to allow target cell lysis by NK-92 cells. TNF-α, but not IFN-γ, also induced the activation of LFA-1, CD44 and β1 integrins on SR-91 cells.

Based on these observations, it was hypothesized that the differential effect of TNF-α and IFN-γ could be due to the TNF-α activation of LFA-1 and CD44 on the surface of SR-91 cells that bind to their counter-receptors and activate NK-92 cells. Preliminary experiments showed that engagement of ICAM-3 and CD44 on NK-92 cells induced tyrosine phosphorylation of several proteins including the tyrosine kinase $p56^{lck}$. Further confirmation of these results would not only suggest a role for these adhesion molecules in signal transduction events in NK-92 cells, but perhaps implicates the protein tyrosine kinase $p56^{lck}$ as an early intermediate in the subsequent lysis of SR-91 cells. These data suggest that NK resistance of leukemic cells can be overcome by some cytokines. Although increased conjugate formation is induced by both TNF-α and IFN-γ, only TNF-α functionally activates LFA-1 and CD44 on target cells that may, upon interaction with counter-receptors on NK-92 cells induce signal transduction events in the latter that lead to target cell lysis. Therefore, treatment of patients with cytokines to overcome NK cell resistance and to eradicate tumor cells may not only activate and stimulate immune effector cells function but may also have direct effects on leukemic cells to make them more susceptible to the lytic effects of NK cells.

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ADCC: antibody dependent cytotoxicity
APC: antigen presenting cell
BFU-E: burst-forming unit-erythrocytes
BSA: bovine serum albumin
BM: bone marrow
BMT: bone marrow transplantation
CAM: cell adhesion molecule
cAMP: cyclic adenosine monophosphate
CFU-E: colony-forming unit-erythrocytes
CFU-GEMM: colony-forming unit-granulocytes, erythrocytes, macrophages, megakaryocytes
CFU-GM: colony-forming unit-granulocytes, macrophages
CHS: Chediak Higashi syndrome
CMC: cell mediated cytotoxicity
CMV: cytomegalovirus
DAG: diacyl glycerol
DMSO: dimethyl sulfoxide
DNA: desoxyribonucleic acid
EBV: Epstein Barr virus
ECM: extracellular matrix
EDTA: ethylene diaminetetraacetic acid
EGTA: ethylene glycol-bis tetraacetic acid
FACS: florescent activated cell sorter
FcR: Fc receptor
FCS: fetal calf serum
FITC: fluorescein isothiocyanate
FN: fibronectin
GM-CSF: granulocyte-macrophage colony stimulating factor
GVL: graft versus leukemia
HA: hyaluronan
HIV: human immunodeficiency virus
HLA: human leukocyte antigens
HPC: hematopoietic precursor cell
HSV: herpes simplex virus
HHSV: human herpes simplex virus
ICAM-1: intercellular adhesion molecule-1
ICAM-2: intercellular adhesion molecule-2
ICAM-3: intercellular adhesion molecule-3
ICAM-1 Rg: soluble ICAM-1 immunoglobulin fusion protein
ICE: interleukin-1 β converting enzyme
IFN: interferon
IL-2: interleukin-2
IL-2 R: IL-2 receptor
IP: immunoprecipitation
IP3: inositol-1,4,5-triphosphate
ITAM: immune receptor-tyrosine based activation motif

JNK: Jun nuclear kinase
kDa: kilo dalton
KIR: killer cell inhibitory receptor
LAD: leukocyte adhesion deficiency
LAK: lymphokine activated killer cell
LFA-1: leukocyte function-associated molecule-1
LFA-3: leukocyte function-associated molecule-3
LN: laminin
LPD: lymphoproliferative disorder
MAP: mitogen-activated protein
MHC: major histocompatibility complex
MW: molecular weight
Na_2CrO_4: sodium chromate
NaF: sodium fluoride
NaPPi: sodium pyrophosphate
NaVO3: sodium orthovanadate
NK: natural killer
NO: nitric oxide
NOS: nitric oxide synthetase
PBMC: peripheral blood mononuclear cells
PBS: phosphate saline buffer
PCD: programmed cell death
PE: phycoerythrin
PF: perforin
PI: phosphoinositide
PI3K: phosphatidylinositol 3-kinase
PKC: protein kinases C
PLC: phospholipase C
PMA: phorbol myristate acetate
PTK: protein tyrosine kinase
Rg: receptor immunoglobulin
SAPK: stress-activated protein kinase
SCID: sever combined immunodeficiency
SDS: sodium dodecylsulfate
sICAM-3: soluble ICAM-3
TBS: tris buffer saline
TBS.T: TBS-Tween
TCL: total cell lysate
TCR: T cell receptor
TIL: tumor infiltrating lymphocytes
TNF: tumor necrosis factor

ACKNOWLEDEGMENTS

I would like to thank Dr. Hans Klingemann and Dr. Fumio Takei, my co-supervisors for their advice and support through these years. Special thanks to Drs. Pauline Johnson, Gerald Krystal, Ling Liu, Jacqueline Damen, Neidi Gong, and Ying Tam for helpful discussion and their support. I would also like to thank Dr. Frank Tufaro and Dr. Graeme Dougherty, members of my advisory committee, for their help and advice.

Chapter 1. INTRODUCTION

1.1.NK cells

1.1.1. Definition, morphology, and cell surface markers

Natural killer (NK) cells are large granular lymphocytes (LGL), defined by their ability to lyse without prior sensitization certain tumor cells, virus and intracellular bacteria infected cells [1]. NK activity may be enhanced by in vivo or in vitro treatment with cytokines such as interleukin-2 (IL-2) and interferons (IFN). These lymphokine activated killer (LAK) cells exhibit higher cytotoxic activity compared to primary NK cells, and acquire a larger spectrum of target cell specificities and the expression of activation antigens such as the IL-2 receptor α chain (CD25), HLA-DR, CD71 (transferrin receptor) and CD69 [1,2].

NK cells do not rearrange nor express surface immunoglobulin or functional T cell receptor (TCR) and CD3 complex. They express a unique pattern of cell surface markers such as CD16 (FcγR III: Fc receptor for IgG) present on the majority of NK cells (80%), CD56 in humans and NK1.1 and asialo GM1 in mice. They also express, associated to CD16, the ζ chain of CD3 complex. Other cell surface molecules expressed on NK cells are IL-2 receptor β chain (p75), LFA-1 (CD11a/CD18), CD2, LFA-3, CD7, while activation antigens such as IL-2 R α chain (CD25), CD71 (transferrin receptor), and CD69 are expressed on activated cells. Most NK cells express the receptor for C3bi (CR3 or CD11b/CD18 or Mac-1), not for (CR1 or CD35) or C3d (CR2 or CD21) [1-3].

1.1.2. Origin, differentiation, and tissue distribution

NK cells are bone marrow derived lymphocytes found at highest frequency in peripheral blood (PB) where they constitute up to 5-15% of mononuclear cells (PBMC), spleen (up to 25% of lymphoid cells), and at lower frequencies in lung, liver, gastrointestinal tract, and peripheral lymphoid organs. Although they are bone marrow (BM) derived, the level of BM-NK activity is low.

A number of studies have shown that human CD3- CD56+ NK cells can be obtained from BM derived CD34+ hematopoietic progenitor cells (HPC) cultured in the presence of IL-2 and an allogeneic feeder cell layer, or IL-2 and other hematopoietic growth factors such as c-kit ligand. The failure to detect IL-2 production by BM stromal cells and the presence of NK cells in IL-2 deficient mice [4], and the observation that mice [5] ,and humans [6] lacking the γ subunit of the IL-2R (IL-2Rγ) lack NK cells, together suggested that cytokines other than IL-2 which use IL-2Rγ may participate in NK cell differentiation from HPCs in vivo [7]. BM stromal cells produce IL-15, a novel cytokine that can activate T and NK cells through components of the IL-2R. In vitro, CD3- CD56+ NK cells can be obtained from 21 days culture of CD34+ HPCs supplemented with IL-15 in the absence of IL-2, stromal cells or other cytokines [7].

NK cell differentiation is thymus independent, and NK activity is normal in patients with the hypoplasic thymus of Di George's syndrome [8]. The thymic independence of NK cells

is also supported by the fact that athymic nude mice and rats have stronger NK cell activity than do their euthymic littermates [8-10].

NK maturation, however, requires an intact bone marrow environment. This is shown by depression of splenic NK cell activity by treatment of mice with ^{89}Sr (a bone-seeking isotype) [11,12]. The role of the bone marrow as a necessary microenvironment for NK cell differentiation is further suggested by the failure of NK cell differentiation in congenital or 17β-estradiol-induced osteoporotic mice and microphtalmic (mi/mi) mice that are congenitally osteoporotic, with reduced marrow and a deficiency in natural killing. Experimentally, osteoporosis and loss of NK cell activity can be observed by treating mice with 17β-estradiol for six weeks [13,14].

Data from irradiated patients and experimental animals suggest that NK cells might be relatively irradiation resistant [15-17]. Animal studies have suggested that mature NK cells are resistant, but that NK progenitors are relatively sensitive to radiotherapy. In humans, there have been some evidence suggesting that T and B cell subsets seem to be equally radiosensitive after in vivo total body irradiation with the exception of the immature thymocyte subset, while CD34+ progenitor cells and NK cells seem to be more radioresistant [18]. While radiotherapy-induced lymphopenia affects T cells markedly, resulting in a continuing depletion up to three years post treatment, the effect of radiotherapy on NK cell number and function depends on the extent of the underlying disease, the dosage delivered, and the area of the tissue irradiated [15].

1.1.3. NK cells and cytokines

NK cells secrete a number of cytokines in response to IL-2 treatment and other stimuli [19,20], which includes all the cytokines that are potent stimuli for monocyte/macrophages such as IFN-γ, GM-CSF and TNF-α. Therefore, IL-2 stimulated NK cells "communicate" with monocytes and together these cells are responsible for so-called "innate" immunity. Upon stimulation with sensitive target cells, CD16 cross linking, or IL-2 treatment, NK cells produce IFN-γ, TNF-α and GM-CSF [19,20]. Production of IL-3 upon nonspecific stimulation (phorbol esters, and calcium ionophores), and of IL-5 upon stimulation with IL-12 have also been reported [21]. Unlike monocytes, NK cells do not produce TNF-α upon endotoxin stimulation. NK cells are also potent producers of macrophage inflammatory protein (MIP-1 alpha). Although fresh NK cells produce negligible amounts of MIP-1 alpha, abundant NK cell production of this cytokine is seen after costimulation with IL-12 and IL-15 [22].

Some cytokines are also known to stimulate NK cells. NK cells proliferate in response to IL-2, IL-4, IL-7, IL-12 [23-26], and IL-15 [27]. IL-2, IL-7, IL-12 and IL-15 also enhance NK cytotoxic functions. IL-12 synergizes with IL-2 in NK activation and induction of LAK activity [23-27]. IL-2, IL-4, IL-7, IL-9 share the same IL-2Rγ chains [5,28-31], while IL-15 utilizes IL-2Rβ and γ chains for transmitting signals [31,32].

1.2. NK activity

1.2 1. Determination of NK activity

NK activity is usually quantified in a four hours chromium release assay in which target cells are labeled with radiolabeled ^{51}Cr ($Na_2\ ^{51}CrO_4$), and mixed with the effector cells at different effector: target ratios. The released radioactivity at the end of incubation time is directly proportional to the extent of target cell lysis by NK cells. Another way of determining NK activity is the use of lytic units (LU), defined as the number of effector cells required to lyse a given proportion (optimally 50%, but often 20 or 30%) of target cells in the assay period. The most widely used target cells are the NK sensitive cell line K562, and the NK resistant LAK sensitive cell lines Daudi and Raji in humans; the NK sensitive cell line Yac-1, and the NK resistant, LAK sensitive cell line P815 in mice [1].

1.2.2. NK recycling and inactivation

Combined use of the single-cell cytotoxic assay in agarose and estimation of the maximum NK cytotoxic potential by ^{51}Cr release to study recycling of effector cells indicated that, NK cells recycle and are capable of killing multiple target cells. After delivering the lethal hit to the target cell, the NK cell detaches from the target cell and recycle, starting other cycles of target binding and lysis. Studies have shown that one NK cell can lyse on average, two to three target cells [33]. It has been reported that NK cells lose their lytic activity, for both

direct cell mediated cytotoxicity (CMC) and antibody dependent cytotoxicity (ADCC), after interaction with sensitive targets (not with resistant targets) and remain refractory for a period of time; that this loss of activity is reversible and can be recovered by incubation with IL-2 (18 hours) [34]. However, recent reports indicated that this NK inactivation involves down regulation of certain cell surface molecules such as CD16 and CD2, and results in the induction of programmed cell death (PCD) [35,36].

1.2.3. Targets of NK cell mediated cytotoxicity

NK cells and CD8+ CTLs represent two major populations of cytotoxic lymphocytes able to kill autologous cells infected with intracellular pathogens, as well as tumor cells and MHC incompatible grafted cells. Prior to the description of NK cells and the natural immunity, nearly two decades ago, T cells had been thought to play the principal role in host immune surveillance against malignancies. CTLs capable of specifically lysing target cells in vitro have been implicated as principal effector cells in allograft rejection [37,38], tumor immunity [39,40], and lysis of virally infected [41,42]. CTLs capable of killing autologous transformed cells in vivo have also been demonstrated in tumor bearing hosts [43]. It has now become clear that NK cells play an important role in host immune surveillance against malignancies and viral infections. There is much evidence to suggest that NK cells play an important role in controlling many tumors and metastasis. In contrast to CTLs that need to be sensitized and recognize target cells by binding to specific antigens on target cell in the context of major histocompatibility complex (MHC), NK cells are capable of mediating this cytotoxic function without the need for prior activation or sensitization, therefore, they are

an important component of innate immunity. Innate immunity, is generally followed by adaptive immunity, which is mediated by clonally selected and expanded antigen-specific B and T lymphocytes.

A. Tumor cells

While most primary malignant solid tumors are NK resistant [44,45], a significant role for NK cells in the in vivo regulation of some tumors, particularly in the control of metastases, has been reported [46]. NK cells are effective against blood-borne tumor cells such as leukemic and metastatic cells of renal or melanoma origin. NK regulation of tumor metastases is conceivable since a major physiologic reservoir of NK activity is the peripheral blood, the common route by which metastases typically disseminate. Recent in vitro studies have shown that leukemic blasts from patients with acute myelogenous leukemia (AML) are susceptible to the lytic action of autologous and non-autologous LAK cells [47]. Ex vivo activated NK cells along with IL-2 have also been shown to be effective in the treatment of metastatic malignant melanoma and renal cell carcinoma and leukemia [48]. In addition, NK cells, along with IL-2 and IL-12, have been shown to be effective on some NK resistant neuroblastoma cell lines [49].

Homing of NK cells to the site of tumors have been thought to be fairly poor, and their presence in tumor infiltrating lymphocytes (TILs) is controversial [50-53]. It has been shown that activation of TILs with IL-2 results in significant antitumor activity [54,55]. However, due to the heterogeneity of the cell population in TILs, controversy exists as to whether or not these cells are of NK cell lineage [56,57]. NK cells have been shown to be present within breast, lung, ovary, colon, and a variety of other tumors. NK activity of TILs has been found to be reduced in patients with lung and colorectal carcinomas, compared to

NK activity of PBLs [58,59], and also TILs in ^{51}Cr release assay have been shown to have reduced recycling capability (ability to kill multiple target cells) [60]. However, single cell assays have shown that the proportion of cells binding to target cells is comparable to the proportion of cells binding to both TILs and PBLs. Therefore, NK cells in TILs appear to have functional impairment, the cause of which is unknown [61].

B. Virus infected cells

Anti viral activity of NK cells is mainly directed against Herpes virus: human herpes simplex virus-1 (HHSV-1)[62,63], mouse herpes virus [64], mouse cytomegalovirus (CMV)[65], and mouse hepatitis virus [66]. CMV and Epstein Barr virus (EBV) are ubiquitous herpes viruses that generally cause only mild symptoms in normal individuals. While EBV related diseases are uncommon following conventional BMT, CMV reactivation remains the most common single cause of death following BMT. Although NK cells of BMT recipients are highly effective against EBV infected target cells they are inactive against CMV infected target cells. This different cytotoxicity seems to be associated with disparate expression of CAM on the target cells [67]. HIV infected patients have also depressed NK activity characterized by a defect at post binding stage of lysis [68,69]. As has been reported for NK resistant target cells, removal of sialic acid residues rendered retrovirus infected target cells sensitive to NK mediated lysis[69] .

C. Parasites and intracellular bacteria

In vitro, NK cells have been shown to be capable of lysing cells infected with intracellular bacteria such as Shighella flexneri [70], Legionella pneumophila [71] or Mycobacterium avium [72]. NK cells have also been reported to be effective in lysis of Mycobacterium leprae infected macrophages and the destruction of the bacteria either by newly migrated

competent monocytes or by the components of the NK cell cytolytic granules [73]. Murine and human NK cells can bind and inhibit growth of fungi such as Cryptococcus neoformans and NK deficient beige mice are less resistant to Cryptococcus neoformans [74,75]. Studies employing beige mice, split-dose irradiation, and adoptive transfer of an NK cell clones have suggested a possible role for NK cells in the clearance of Leishmania from spleen and liver [76,77]. NK cells show little spontaneous cytotoxicity for trypanosomes, but efficient ADCC and appear to be potentially effective against Toxoplasma gonidii [78-80].

D. Hybrid resistance

NK cells have been implicated as the effector cells responsible for the hybrid resistance (HR) phenomenon in which homozygous parental BM grafts are rejected by irradiated F1 progeny [81,82]. HR could be explained by the presence in F1(AxB) mice, of a subset of NK cells which do not express inhibitory MHC class I receptors specific for parent A or B. HR prevents only engraftment of hematopoietic tissues, whereas other parental grafts such as skin are readily tolerated.

E. Normal tissue

Because NK cells can reject incompatible BM cells in healthy irradiated recipients (hybrid resistance), it has been postulated that they also play a role in the regulation of hematopoiesis. Conflicting reports exist regarding the role of NK cells in BM graft rejection and regulation of hematopoiesis. The majority of earlier studies on NK cells using both in vitro and in vivo assays have suggested that they primarily exert a negative influence on hematopoiesis. However, IL-2 activated NK cells have been shown to promote marrow engraftment in mice and support hematopoiesis in vivo [83,84]. Purified human IL-2 activated NK have been shown to be capable of producing the hematopoietic growth factor

GM-CSF, furthermore, these LAK cells have also shown to have little cytotoxic activity against normal BM cells, and no inhibitory effect on hematopoietic progenitor growth in CFU-GM (colony-forming unit-granulocytes, macrophages), CFU-E (colony-forming unit-erythrocytes), BFU-E (burst-forming unit-erythrocytes) or CFU-GEMM (colony-forming unit-granulocyte, erythrocytes, macrophages, megakaryocytes) assays [85]. Subpopulations of NK cells may exist that exert primarily growth promoting versus growth inhibitor functions on hematopoiesis based on the cytokine they produce. Recently, human allospecific NK cell clones have been generated that recognize distinct specificities inherited recessively and controlled by genes linked to MHC. The alloreactive NK cells were proposed to be the human counterparts of the cells mediating hybrid resistance in mice and to play clinically important roles in rejection or in graft versus leukemia (GVL) reactions after allogeneic BMT [86]. Thus, based on cytokine production, presence or absence of alloreactive NK clones, and the state of NK cell activation, NK cells may exert dual regulatory role on hematopoiesis

1.2.4. Evidence for anti-tumor and anti-viral activities of NK cells.

A. Congenital and acquired defects in NK cells

Complete absence of NK cells in humans is extremely rare and has been described in only a few patients [87,88]. All reported cases were characterized by life threatening and relapsing herpes and recurrent viral infections, as young adults, with normal immunological parameters but lack of NK activity against K562 and HSV-1 infected target cells, particularly the case of four siblings with recurrent infections and pneumonia including EBV

infection [87]. Biron et al. have described an adolescent patient with an apparent selective NK deficiency. Patient's B, T cells and macrophage functions appeared normal, but peripheral blood lymphocytes were devoid of CD16+ or CD56+ subsets and were incapable of mediating conjugation to or cytolysis of Daudi, or K562 target cells, this young girl suffered from severe herpes-virus infections including varicella, and CMV infections [88].

The bg/bg mouse, which is functionally deficient in NK activity, exhibits an elevated frequency of spontaneous lymphomas and is more susceptible to the formation of pulmonary metastases of the B16 melanoma than heterozygote bg/+ littermates, increased sensitivity to mouse CMV, hepatitis virus, herpes virus and influenza virus in vivo. In normal young mice, the ability to control the metastatic potential of tumors correlates with the appearance of NK activity at approximately three weeks of age [89].

B. Experimental NK depletion and restoration by adoptive transfer

Experimental NK cell depletion in mice, by in vivo administration of anti asialo-GM1 or anti NK1.1 antibodies results in animals that are NK deficient and express increased frequencies of metastases as well as increased susceptibility to murine CMV and hepatitis. Cyclophosphamide treatment of mice, which ablates NK activity, also results in increased experimental metastases [90]. In contrast to these NK depletion studies, activation of NK cells in vivo with either poly I:C, IFN-γ or IL-2 yields elevated NK activity and reduced pulmonary colonization by metastatic melanoma [91] and adoptive transfer of NK cells to NK deficient recipients confers resistance to metastases and to some virus infections [92].

C. Other deficiencies affecting NK activity

Leukocyte adhesion deficiency (LAD) which is a defective expression of the CD18 molecules (the common β2 chain of β2 integrins) also causes a defective NK activity. These patients suffer from severe bacterial and viral infections including herpes-virus infections, varicella, CMV with complete absence of NK cell activity [93].

Chediak-Higashi syndrome (CHS), with hyporesponsiveness NK cells is a rare autosomal recessive disease characterized by a defect in cytoplasmic granules and degranulation in lymphocytes and granulocytes, associated with defective pigmentation and increased susceptibility to infections [94]. CHS is a state of NK hyporesponsiveness with a normal number of NK cells, where NK cell activity is 10 to 100 times lower than in normal controls [95,96]. Children usually die of pyrogenic infections presumably resulting from their neutrophils abnormality. Survivors generally succumb to a lymphoproliferative disorder that may be malignant [97].

1.3. Mechanism of NK mediated cytotoxicity

Extensive studies to elucidate the molecular mechanism underlying the killing mediated by CTL and NK cells, have proposed a common death pathway that generally involves a three steps mechanism: (i) binding of the target cell (conjugate formation), (ii) activation of the effector cell leading to the delivery of the lethal hit, (iii) target cell disintegration. Binding occurs rapidly within minutes at 37^{0}C and requires Mg^{2+}, not Ca^{2+}, while the following events are temperature dependent (optimum 37^{0}C), Ca^{2+} dependent, sensitive to Ca^{2+} channel

blockers and calmodulin inhibitors. These events are energy and cytoskeleton dependent and could be blocked by various inhibitors of energy production and cytoskeletal function. One of the hallmarks of this killing is the highly selective nature of its lethal effects, as shown by "innocent bystander" experiments in which bystander cells not bearing target antigen were mixed with antigenic target cells and CTLs added, little or no killing of bystander cells was observed, therefore, the lethal hit is highly polarized.

1.3.1. Models of NK mediated cytotoxicity

Two major molecular pathways for target cell lysis, that are not mutually exclusive, have been proposed: (i) a secretory membranolytic mechanism (necrosis) and (ii) a non secretory and receptor mediated triggering of target cell apoptosis.

A) Granule exocytosis and the content of the granules

The major mechanism by which NK cells and CTLs kill their targets is the regulated exocytosis of specialized granules, termed lytic granules [98]. The discovery of perforin or cytolysin in the cytoplasmic granules of CTLs and NK cells [98,99], about 10 years ago, gave the first explanation for the ability of cytotoxic effectors to damage target cell membranes [100-103]. Perforin molecules together with some contribution from serine esterases [104,105] lead to target cell death. This pathway can be triggered through two mechanisms: direct cell mediated cytotoxicity (CMC), and antibody dependent cytotoxicity (ADCC) which is the Fc receptor dependent lysis of antibody coated target cells, mostly against virus infected cells coated with antiviral antibodies. In this necrotic type of cell

death, target cell membranes are disrupted, and this leads to osmotic lysis initiated by the formation of pores in target cell membranes by secreted molecules of lymphocyte origin, such as perforin. The release of cytotoxic granules is vectorial, delivering cytotoxic factors into the contact area between effector and target cells. Morphological analysis of NK cell interaction showed extensive cell-cell adhesion of NK cell with target cell, evidence of activation and degranulation in NK cell, with membrane material of probably granule origin present in the space between the two cells [106]. Upon delivery, perforin monomers form homopolymers in the presence of extracellular calcium and insert into the membrane of the target cell forming pores of 150-170^{0}A diameter. These cause loss of cell integrity and osmotic lysis, and also allows granzymes to access target cell where they can activate the apoptotic pathway of cell death.

Morphological and metabolic inhibitor studies suggest that the essential feature of this model is a polarized and highly regulated secretion of the cytotoxic factors which are presynthesized and stored in secretary granules. Degranulating agents were shown to both deplete granules and inhibit killing [107]. Each granule contains two structural domains: an electron dense core of homogenous appearance surrounded by a thin cortex of vesicles or lamellas. Cytoplasmic granules from effector lymphocytes have been isolated and found to contain mainly cytotoxic factors or "lytic associated proteins" (perforin[103], granzymes [108], proteoglycan [109]), lysosomal proteins, and other granule membrane proteins such as Calreticulin [110]. The soluble, secreted proteins which are killer cell-specific are all concentrated in the dense core domain of the granules, as demonstrated by immunogold labeling of thin frozen electron microscope (EM) sections [111,112]. Conversely, most of the lysosomal proteins are confined to the multivesicular domains.

Perforin (or cytolysin) is a complement like protein which in the presence of Ca^{2+} undergoes a conformational change and forms homopolymers which can insert into the target cell membrane and destroy its integrity. While only activated T cells contain perforin, NK cells are the only resting lymphocytes expressing detectable levels of perforin [113].

Granzymes are serine proteases expressing esterase activity. In mice seven of these granule associated proteases, named granzymes A-G, have been characterized, whereas three granzymes have been identified in human, granzymes A, B and 3 [114,115]. Together, the proteolytic spectrum of the granzymes is very broad including tryptases (granzyme A) and Asp-ase (granzyme B). A role for granzymes in cellular cytotoxicity has been postulated for several years, principally on the basis that cytotoxicity could be completely abrogated by a variety of proteinase inhibitors [116]. Granzymes A and B have been also shown to be involved in triggering apoptosis in target cells [104,115,117,118]. Purified NK granule proteases can induce extensive chromatin condensation, and oligonucleosomal DNA fragmentation [105,119] in the presence of perforin. Therefore, at least a portion of the granzyme family can initiate nuclear damage which is indistinguishable from apoptosis; however, they are inactive on their own and have an absolute requirement for perforin [105,118]. Experiment of degranulation of mast cells expressing granzyme A to cross-linked targets showed that granzymes, whether in simple solution or vectorially delivered at the intercellular site of contact, are not sufficient to initiate apoptosis [118]. This observation virtually eliminates the possibility that proteases activate a membrane receptor such as Fas. However, it does not mean that they have no effect at the membrane. Perforin is able to promote granzyme B or granzyme A induced apoptosis not only at doses that induce membrane damage but also at sublytic levels [105,119]. Another suggested role for granzymes is that they act as auxiliary proteins to perforin [120]. Presumably, they act in

both aspects by proteolytically activating other proteins. Experiments with granzymes A and B knock out mice have recently shown profound alteration in the perforin dependent necrosis pathway suggesting that granzymes may be involved in the processing and/or activation of perforin molecules [121]. The similarity of the enzyme specificity of granzyme B to members of the CED-3/ interleukin-1 β converting enzyme (ICE) family proteases, which are important for cell death has led to the speculation that granzyme B may either mimic the effects of ICE proteases on substrates required for apoptosis, or it may activate one or more members of the ICE family [122]. Several ICE family members have been found to be correctly processed by granzyme B in vitro, including CPP32 [123], and therefore could be candidate in vivo substrates. Recently, experiments in gene-targeted ICE-deficient mice, which have defective granzyme B induced apoptosis, have confirmed the requirement for ICE in granzyme B induced apoptosis. These studies have also shown the involvement of CPP32 protease and $p34^{cdc2}$ kinase in granzyme B-induced apoptosis and suggested that they act upstream and downstream ICE respectively [124].

The third type of lysis associated granule protein is TIA-1 and its relative TIAR. The 15 kDa protein generated after TIA-1 proteolytic cleavage is capable of causing DNA fragmentation [125].

The granules are also rich in highly acidic chondroidin sulfate proteoglycan. At the intragranular pH of 5.5, these proteoglycans are resistant to degradation by the granzymes and can bind to them. The proteoglycan-protease complexes minimize serine esterase-induced auto-degradation and remain intact even after exocytosis [126]. Therefore, the proteoglycans seem to play important structural roles as chaperone, protective, or carrier

molecules. First, they serve as a means of packaging lysis-specific proteins at high concentrations, second, they maintain these proteins in inactive forms until the pH and other environmental conditions change upon exocytosis.

Calreticulin is a highly conserved, ubiquitously expressed intracellular Ca^{2+} binding protein [110] shown to bind to integrins, as well as to regulate gene expression by interacting with the DNA-binding domain of nuclear hormone receptors [127,128]. In addition, considering the essential role of calcium in lytic process, calreticulin could serve as a molecular chaperone by chelating calcium in the lytic granules and thus preventing premature conformational changes in lytic proteins [110,129].

B) The nonsecretory Fas mediated pathway of apoptosis

Several lines of evidence suggested that in addition to perforin-based mechanism, at least another mechanism is involved in lymphocyte mediated cytotoxicity. The existence of an alternative pathway(s) was clear from earlier findings that some target cells can be killed under Ca^{2+} free conditions in which degranulation does not occur, nor can perforin cause membrane damage. Perforin deficient mice obtained by perforin gene knock out have profound defects in the clearance of viral infection, delayed type hypersensitivity and tumor rejection. The perforin knock out mice have normal number of CTL and NK cells but the activity of these cells, tested in vitro, is severely impaired [130-132]. This model strongly supported that perforin is a dominant pathway of lymphocyte mediated cytotoxicity, and showed the existence of a less effective alternative lytic pathway mainly active on certain tumor cells. This second pathway involves direct effector cell-target cell membrane

interactions during which intracellular signals are transduced within the target cell that culminate in target cell's DNA fragmentation and programmed cell death (PCD). Such signals probably occur through the apoptosis-inducing target cell membrane molecule Fas. Fas antigen is a cell surface molecule capable of transducing apoptotic signals, and expressed on a variety of cells including those of lymphoid lineage. In lymphocytes, Fas mediated apoptosis regulates activation induced cell death and participates in cytotoxicity leading to target apoptosis.

Further studies on CTLs showed that CTLs from perforin deficient mice can still exert a Fas based cytotoxicity against Fas expressing target cells. Most, if not all, target cells tested display the Fas receptor at the surface and are lysed by perforin free CTLs and NK cells. Fas based killing accounts in most cases for approximately 25% of the observed lysis in a short term assay. Cells undergoing apoptosis show characteristic morphological changes including plasma and nuclear membrane blebbing, cell shrinkage, chromatin condensation and fragmentation, these changes distinguish apoptosis from cell death by necrosis. Fragmentation of the target cell's DNA into units, consisting of multiples of 180 bp and some smaller fragments, precedes overt damage to the target cell's membrane and release of the affected target cell's cytoplasmic contents (usually monitored by ^{51}Cr release). Fas based cell mediated cytotoxicity is rapid, leading to detectable ^{51}Cr release (a late indicator of cell death), does not require extracellular Ca^{2+} as it is detectable in the presence of EGTA [133].

Fas (also termed APO-1) antigen (CD95) is a novel member of the nerve growth factor receptor/tumor necrosis factor (TNF) receptor family, expressed on immature thymocytes, activated T cells, and nonlymphoid cells in liver, ovary, and heart [134]. Fas expression and

Fas mediated cytotoxicity is greatly increased on target cells by treatment with IFN-γ and TNF-α [134,135]. Triggering of the Fas receptor pathway by its ligand or specific monoclonal antibody results in apoptosis of susceptible normal and malignant cells of lymphoid origin [136-138]. Transfection of Fas into an NK resistant T lymphoma cell line was shown to significantly increase the susceptibility to NK cytotoxicity suggesting that Fas mediated cytotoxicity is involved in NK cell cytotoxicity, while the parental or cells transfected with mutated Fas were still resistant to NK cell killing. These transfectants were shown to be similarly sensitive to cytotoxicity by alloreactive CTLs, suggesting that Fas ligand (Fas L) expression on NK cells is functional and mediates cytotoxicity against specific lymphocyte populations or tumor cells expressing Fas [139,140].

Fas L is a 40 kDa type II transmembrane glycoprotein that belongs to the TNF family [141]. It is expressed on activated T cells or some T cell lines [142] and NK cells [139,140]. Interaction between Fas L on the effector cell and Fas receptor on target cell is capable of inducing apoptosis in target cell and Fas L expression in Cos cells was shown to induce apoptosis in Fas expressing target cells [141].

1.3.2. Other mediators of NK cell mediated cytotoxicity

A. TNF

The availability of perforin and Fas L deficient mice offered an opportunity to investigate roles of perforin and Fas L dependent and independent cytolytic mechanisms in NK and LAK cells. Very recently, perforin and Fas knock out mice experiments have shown the

existence of a TNF-based long term cytotoxicity for LAK cells that is efficient against TNF sensitive targets, while perforin and Fas were required for acute target cell lysis [143]. LAK cells deficient in both perforin and Fas L are essentially noncytolytic in short term cytotoxicity (four hours) assay with the exception of highly TNF sensitive target cells demonstarting that perforin and Fas L are the main cytotoxic molecules used by LAK cells under these conditions. However, significant TNF-mediated cytotoxicity is observed in long term (16-24 hours) assay.

B. Nitric Oxide

Nitric oxide (NO) is a multifunctional molecule that is found in a variety of mammalian cells. NO is generated by both immune and nonimmune cells via the inducible NO synthetase (NOS) by cleavage of terminal guanidino nitrogen from L-arginine. Several lines of evidence first suggested that NO is involved in tumor cell killing by mononuclear phagocytes. NO can cause DNA strand break and mutations and is capable of inducing DNA fragmentation. Triggering through NKR-P1 known to induce NK cell activation and to mediate reverse ADCC, was found to induce arginine metabolism with consequent increase of NO levels. Tumoricidal activity of fresh or IL-2 activated NK cell assessed against Yac-1 and P815 target cells respectively was found to be dependent on the arginine level in the media as it was significantly reduced when cytotoxic assays were performed in arginine free medium or in the presence of L-arginine analog which inhibits NO formation. Moreover, NOS activity was found to gradually increase during the LAK generation and correlated well with the increased capability of these cells to lyse NK resistant targets such as P815 [144,145]. These observations suggested a role for NO as one mediator of murine NK cell mediated DNA fragmentation and cell lysis.

1.4. Cell adhesion molecules

Cell adhesion molecules (CAMs) play a key role in stabilizing and strengthening cell-matrix and cell-cell interactions. Leukocyte CAM provide antigen nonspecific recognition and are involved in a wide range of intercellular interactions including those between helper T cells and antigen presenting cells (APC), CTL, NK cells and their targets [146,147]. Direct effector-target interaction is essential but not sufficient step in this direct cell mediated cytotoxicity. Although specific receptor-ligand structures in NK/LAK recognition of the target cell and subsequent signals involved in this process are still largely elusive, the involvement of certain CAMs in this interaction has now been well established [148].

Recent studies have shown that LFA-1/ICAM-1 and CD2/LFA-3 interactions are two predominant pathways of effector-target interaction for both CTL and NK cells [149-151]. Extracellular matrix (ECM) proteins, specially fibronectin (FN) and laminin (LN) and their receptors of β1 integrin family of adhesion molecules may also play an important role in NK/LAK interaction with leukemic cells [148,152,153]. Since NK/LAK cells express a variety of adhesion receptors, most studies have come to the conclusion that the presence and distribution of adhesion molecules on the target cell may be more important, and that downregulation of adhesion molecules on neoplastic cells may represent a potent escape mechanism of the tumor cell.

1.4.1. LFA-1/ICAMs interaction

A. LFA-1.

LFA-1 (CD11a/CD18) is an αLβ2 heterodimer, which binds to ICAMs. Like other integrins, LFA-1 is not constitutively avid for its ligands but exhibits a rapid and inducible change in avidity upon activation with stimuli such as phorbol esters. The α chain of LFA-1 is constitutively phosphorylated, whereas β chain becomes phosphorylated in response to stimuli such as phorbol esters. Although protein kinases play a role in the activation of LFA-1, and phorbol ester treatment causes direct phosphorylation of both α and β subunits of β2 integrins, mutational analysis have shown that phosphorylation is not necessary for PMA induced binding to ICAM-1 [154], and the exact mechanism by which the change in its avidity occurs has not been elucidated [155].

LFA-1 is a bifunctional molecule capable of transmitting signals from outside of the cell to inside and from inside cell to the outside [156]. Stimulation with PMA converts LFA-1 to a state of high avidity thereby promoting interaction with its ligands (inside out signaling). On the other hand, LFA-1 itself is capable of signal transducing and second messenger generation upon cross linking or activation with its ligand (outside in signaling) [156]. LFA-1 was shown to be linked to protein tyrosine kinase (PTK) signaling pathway that stimulate tyrosine phosphorylation and activation of phospholipase C γ1 (PLCγ1) [157]. This signaling through LFA-1 involves calcium mobilization, phosphoinositide (PI) hydrolysis, amplification of CD3 dependent IL-2 production, proliferation and enhancing

antigen presentation in B cells [157]. LFA-1-dependent interactions are important in many immune effector mechanisms, they are Mg^{2+}-dependent, temperature-dependent and trypsin-sensitive, and require an intact cytoskeleton [158]. It has been repeatedly described that in the ICAM-1/LFA-1 interaction, LFA-1 plays the most important role in signal transduction. Experiments with ICAM-1 receptor immunoglobulin (Rg) and ICAM-2 Rg fusion proteins coimmobilized with anti TCR mAb have shown that ICAM-1 and ICAM-2, exert a strong costimulatory effect during TCR-mediated activation of T cell [159,160].

Monoclonal antibodies (mAbs) directed against LFA-1 determinants significantly inhibit NK-mediated conjugate formation and cytolysis of susceptible target cell lines. Inhibition is at effector level and target cell pretreatment with either anti CD18 or anti CD11a does not affect NK sensitivity [151,161]. However, the degree of anti LFA-1 inhibition observed in NK assays varies significantly depending on the target cell analyzed [162]. The importance of LFA-1 in NK cytotoxic functions is further supported by the recent observation that NK from CD11a knock out mice, generated by disruption of murine CD11a gene in embryonic stem (ES) cells, have markedly reduced cytotoxic activity [163]. ICAM-1 knock out mice, on the other hand, have been shown to have impaired inflammatory and immune responses. Lymphocytes from these ICAM-1 deficient mice have been shown to have negligible stimulatory effect in mixed lymphocyte reaction (MLR), while proliferating normally as responder cell population, this further supports a role for ICAM-1 as co-stimulatory rather than signal transducing molecule [164-166].

B. ICAMs ; three ligands for LFA-1

ICAM-1, 2 and 3, the three ligands for LFA-1, are members of the immunoglobulin (Ig) superfamily and are variably expressed on the surface of leukocytes. ICAM-1 (CD54), is a widely distributed 90 kDa glycoprotein with five Ig like domains in its extracellular portion [167]. It is expressed on activated leukocytes, epithelial and endothelial cells, and its expression is upregulated by cytokines. ICAM-1 binds also to CD11b/CD18 or Mac-1, CD43, rhinovirus and plasmodium falciparum [168-170]. ICAM-2 (CD105) is a 55kDa glycoprotein constitutively expressed on lymphocytes, monocytes and endothelial cells. It has two Ig domains and binds only to LFA-1 [171]. ICAM-3 (CD50) is a 124kDa glycoprotein with five Ig like domains, mainly expressed on hematopoietic cells and at particularly high levels on resting leukocytes, neutrophils, monocytes, and is absent from endothelial cells [172,173].

ICAMs are highly homologous in their extracellular domains, but their cytoplasmic portion is poorly conserved with no serine or tyrosine residues for ICAM-1 and ICAM-2 [167,171], while ICAM-3 has five serine and two tyrosine residues [172,173]. LFA-1 binds to all ICAMs with different affinities. LFA-1 affinity for ICAM-1 seems to be higher than for ICAM-2 or ICAM-3 [159,171,174]. The extracellular domain of LFA-1 α subunit contains two domains thought to be of functional significance. These include a putative divalent cation binding region also found in other integrins, and a 200 amino acids inserted or I domain which is also present in α chains of αM (Mac-1), αX (p150, 95), α1(VLA-1), α2 (VLA-2), αE (αEβ7) and is involved in ligand binding [155,175]. Three distinct epitopes Ide A, B, and C within the CD11α I domain recognized by antibodies that block binding of

LFA-1 to ICAM-1, ICAM-2 and ICAM-3 have been identified suggesting that different conformation of LFA-1 is required to support adhesion to ICAM-1, ICAM-2, and ICAM-3, and that these ligands may bind on different sites of LFA-1 molecule [176]. Recently, using a series of human/murine CD11α I domain chimeras, a fourth epitope within the I domain, epitope Ide D, was identified that is recognized by anti CD11α antibody that block selectively binding to ICAM-3 [177]. The existence of ICAM-3 receptors other than LFA-1 was first suggested by the observation in human T cell lines Jurkat and JM, that ICAM-3 could regulate both LFA-1/ICAM-1 dependent and independent pathways involved in cell-cell interaction [178]. Recently a cDNA encoding a 4th α chain αd that associates with CD18 was identified. This novel leukointegrin αdβ2 exhibits preferential recognition of ICAM-3 over ICAM-1 [179].

Whereas direct intracellular signaling pathways through LFA-1 have been extensively described, there are few data about signal transducing effects through ICAM-1 and ICAM-2. ICAM-1 cross linking was recently shown to induce an oxidative burst from mononuclear leukocytes [180], to induce transient tyrosine phosphorylation and inactivation of cdc2 kinase in T cells [181]. The majority of the available data however, suggest that the main functional role for ICAM-1 and ICAM-2 is their binding to LFA-1 which triggers signals that can directly, or through other stimulatory surface proteins, enhance cell activation. The important difference in cell distribution, intracytoplasmic domain, and phosphorylation sites observed in ICAM-3 first suggested that ICAM-3 could play a more active role in signal transduction than ICAM-1 and ICAM-2.

C. CD50/ICAM-3

In contrast to ICAM-1 and ICAM-2, ICAM-3 has a longer cytoplasmic domain that contains five serine and two tyrosine (Y) residues that can be phosphorylated upon stimulation with different stimuli, and this phosphorylation is believed to be involved in regulating ICAM-3 functions [182-184]. A sequence motif termed "Ag recognition activation motif" is present in the cytoplasmic domain of CD3γ, δ, ε as well as ζ and plays critical role in signal transduction. This motif is based on a Y and leucine (or isoleucine) residues arranged in the general pattern YXXL. The Y in antigen recognition activation motifs are phosphorylated after receptor stimulation possibly by members of src kinases and this is believed to play a role in receptor function. One of the cytoplasmic tyrosine residues in ICAM-3 is present in a YXXL motif. Recent studies reported that ICAM-3 exert strong stimulatory activity in both resting and activated T cells, showing that certain antibodies to ICAM-3 or ICAM-3 cross linking induce activation of β1 and β2 integrins in T cells [185,186], an increase in intracellular calcium concentrations and tyrosine phosphorylation of a number of intracellular proteins in neutrophils and T cells [178,184,187]. In human T leukemic cell lines JM and Jurkat, ICAM-3 was suggested to play an important role in the regulation of LFA-1/ICAM-1 dependent and independent pathways involved in cell-cell interaction [178,188] also suggesting the existence of other receptors for ICAM-3 that LFA-1, or triggering of intracellular signals through ICAM-3 that would activate other CAMs [178]. More recently, ICAM-3 which has no intrinsic kinase activity has been reported to associate with Src family PTKs *lck* and *fyn* in Jurkat T cell line [187].

1.4.2. CD44

CD44 is broadly distributed integral membrane glycoprotein [189,190] shown to be involved in many adhesion dependent cellular processes including lymphocyte recirculation and homing process [191], hemopoiesis [192], cell interaction with extracellular matrix proteins (FN, hyaluronan, Collagen) [190,191], lymphocyte activation [194-197], NK mediated killing [198-200], and tumor metastasis [190]. Alternative splicing events can generate a large number of differentially expressed CD44 isoforms with molecular weights (MW) ranging between 80 and 250 kDa, that contain additional peptide sequences of varying length inserted into a single site within the extracellular domain [201-204]. Currently 10 alternatively spliced exons encoding sequences present within the extracellular domain and two encoding sequences present within the cytoplasmic domain have been identified. CD44H, 80-85 kDa , is the major CD44 isoform and widely expressed on many cell types. CD44 variants are structurally similar to CD44H isoform except for the presence of 132-338 amino acids inserts in the gag region of the COOH terminal of CD44 extracellular domain [205,206]. CD44E, with 132 amino acids insert, corresponds to exons 13,14,15 (V8,V9, and V10) and is expressed in monocytes, macrophages, epithelial cells and immature myeloid cells [201,205]. CD44R1 which differs from CD44E by a single amino acid, and CD44R2 which corresponds to exon 15 (V10) are expressed on transformed epithelial and certain activated or malignant hemopoietic cell types [203].

CD44 functions as a receptor for glycosaminoglycan hyaluronan (HA), and this binding is dependent on cell activation. There is also increasing evidence that binding to hyaluronan cannot explain all CD44 dependent adhesion events, and homotypic binding between

different isoforms of CD44 has recently been reported. CD44R1 has been shown to bind to CD44R1 and CD44H, while CD44H cannot bind to CD44H [207]. CD44 has also been reported to be implicated in signal transduction events and to trigger cytotoxic functions of CTLs [208-210] and NK cells [196-198], it has been recently reported to associate with $p56^{lck}$ in T cells [211].

1.4.3. CD2-LFA-3

Virtually all blood or splenic NK cells express the 50 kDa integral membrane glycoprotein CD2 which is not an NK specific receptor but serves as an important accessory molecule in both NK and T cells. The CD2 molecule on the effector cell interacts with its ligand LFA-3 (CD58) on the target cell. LFA-3, a 40 to 65 kDa glycoprotein, is expressed on various tissue types including leukocytes and erythrocytes. It can exist in either a GPI-linked form or an integral membrane form. Interaction between CD2 and LFA-3 occurs efficiently at both 4°C and 37°C, and do not require the presence of divalent cations such as Mg^{2+} or Ca^{2+} [157]. Both CD2 and LFA-3 are sensitive to trypsin digestion [212]. Antibody inhibition studies have shown the involvement of CD2 at NK level and of LFA-3 at target level [212-214]. Furthermore, phosphatidyl inositol-specific phospholipase C (PIPLC) treatment of Jurkat or MOLT-4 target cell which removes the GPI-linked form of LFA-3, partially inhibits target cell recognition by NK cells [215-217]. Finally transfection of human LFA-3 into mouse L cells confers sensitivity to human NK cells [218].

1.5. Other cell surface receptors involved in signal transduction

1.5.1. IL-2 receptor (IL-2R)

The IL-2 receptors expressed on the surface of activated T cells and NK cells exhibit a variety of affinity states depending on their subunit composition. The functional IL-2R may consist of 3 subunits α, β and γ chains, none of which contains an intrinsic PTK domain that is the hallmark of other growth factor receptors. The low affinity binding, with rapid association and dissociation, is associated with a 55kDa α chain (CD25 or Tac antigen). The intermediate affinity receptor, with slow association and dissociation binding, associates with the 70-75 kDa β chain, and the high affinity receptor is composed of the α and β chains with rapid association and slow dissociation. A third subunit for IL-2 R termed the γ chain, 64 kDa, is associated with the β subunit and was originally discovered by co-immunoprecipitating with β chains from cells incubated with IL-2 [219,220]. NK cells are unique among all 3 types of lymphocytes in that they constitutively express IL-2Rs and thus they are always IL-2 reactive [221,222]. Only 10% of NK cells express high affinity IL-2R, whereas the remaining 90% lack the 55 kDa α chain and only express the intermediate affinity receptor [220-222]. Expression of p55 is induced upon stimulation with IL-2, regulated by NK-target cell stimulation, or CD16-ligand, which modulate proliferation of NK cells in response to IL-2 (interaction with IL-2Rβ or αβ). Recent studies have demonstrated the critical role of IL-2Rβ and γ cytoplasmic regions in IL-2 signaling and the importance of the cooperation between β and γ cytoplasmic domains in activating downstream signaling pathways [223,224].

1.5.2. Fc receptors

CD16, the FcRγ, is a low affinity receptor that binds IgG in immune complexes with soluble or insoluble antigen (e.g. antibody coated cells) but does not bind monomeric IgG. FcγR is a multimeric receptor that associates with dimers of γ or ζ chains of TCR. None of these subunits possess intrinsic kinase activity, but stimulation of FcγR rapidly activates PTK signaling pathway and tyrosine phosphorylation of substrates critical for cell activation including ζ and PLC, tyrosine phosphorylation and increase in the catalytic activities of both Syk family PTKs, Zap70 and *syk*. Using a vaccina virus expression system, it has been shown that *lck* overexpression of the wild type , but not the kinase deficient mutant, of *lck* in cloned human NK cells increases this phosphorylation and the association of Zap70 with ζ chain of FcγR, suggesting a role for Src family PTK, $p56^{lck}$, in FcγR initiated regulation of tyrosine phosphorylation of Zap70, *syk* and PLCγ [225,226].

FcμR, the Fc R for IgM, initially reported on a small proportion of NK cells, has recently been shown, by the use of more sensitive techniques, to be expressed on the majority of resting NK cells [227], and shown to be involved in ADCC mediated by NK cells against target cells coated with IgM . Very recent studies have shown that like FcγRIII, FcμR also associates physically and functionally with ζ and γ chains which become tyrosine phosphorylated upon cross-linking of the FcμR, and induces the activation of Src and Syk family members PTKs [228].

1.5.3. CD69

CD69, also designated activation inducer molecule (AIM), is a new member of the calcium dependent lectin superfamily of type II transmembrane receptors which includes the human NKG2, the rat and mouse NKR-P1 families of NK specific genes, and shares functional characteristics with most members of this superfamily which act as transmembrane signaling receptor in early phases of cellular activation. CD69 is a phosphorylated disulfide-linked heterodimer of a 28 and a 32 kDa chains. It is not expressed by resting peripheral blood lymphocytes but on activated T, B and NK cells and platelets and seems to be the earliest inducible cell surface glycoprotein. Its expression on IL-2 activated NK cells parallels the acquisition of lytic activity preceding the appearance of HLA-DR, IL-2R (CD25), and the transferrin receptor (CD71). CD69 and phorbol esters are comitogenic for T lymphocytes [2].

1.5.4. CD28

CD28 is a disulfide-linked homodimer expressed by peripheral T lymphocytes, and thymocytes. It binds to CD80 and CD86 (B7-1 and B7-2 respectively), that are expressed on activated B cells, macrophages, and dendritic cells. CD28 on T cells provides one of the most important costimulatory signals for T cell proliferation induced by stimulation via TCR. CD28 mediated signals result in cytokine production, in particular IL-2. Although CD28 is generally absent from PB NK cells [229], its involvement as an important

costimulatory molecule for NK cells have been shown in a number of studies using MHC unrestricted cytotoxicity of the NK cell lines YT2C2 and YT which express CD28. Ligation of CD28 receptor on YT cells induces granule exocytosis, which was also induced by mAb to LFA-1 [230]. The participation of CD28/B7 interaction in MHC-unrestricted cytotoxicity mediated by YT2C2 was demonstrated by correlation of target cell sensitivity with the level of B7 expression, and the inhibition of cytotoxicity by CD28 or anti B7 antibodies and by making both murine and human cell lines susceptible to YT2C2-mediated lysis by genetic transfection with expression vectors containing B7 cDNA. However, CD28/B7 interactions alone were insufficient to initiate cytotoxicity.

1.6. MHC class I and NK receptors

Both CTL and NK cells distinguish self from nonself through target cell MHC class I. NK activity was originally defined as MHC non-restricted as opposed to that of CTLs which recognize foreign antigen in the context of MHC class I. Subsequent studies showed that the pattern of MHC class I expression of the target cell may in fact determine its sensitivity to NK cell killing. Several reports indicated an inverse correlation between MHC class I molecules expression on tumor cells and their susceptibility to NK cells: in some cell lines low expression of MHC class I correlated with high sensitivity to NK-mediated killing and limited in vivo growth potential, whereas high MHC class I variants were resistant to NK cells and highly metastatic. MHC class I molecules seem, at least in some cases, to "protect" tumor cells from NK effector cells by masking some recognition structures on target cells

(target interference model) or by giving a negative signal to NK cell (effector inhibition model). Selected MHC class I molecules have been shown, in some but not all systems, to protect the target cell from NK cell lysis by delivering a predominantly inhibitory signal [231-234]. The protective effect of MHC class I would occur at a post-binding step hence over-riding activating signals induced by target cell. It has been shown that while NK cell binding to both MHC class I deficient and MHC class I transfected target cells induces rapid PTK activation, the more distal phospholipase C (PLC) dependent signaling events (phosphoinositol release and intracellular calcium increase) were only induced by NK cell binding to MHC class I deficient target cells [235]. To date, not a unique NK-receptor responsible for initiating the cytolytic response has been identified. Target cell lysis by NK cells is regulated by a balance between activating and inhibitory receptors.

1.6.1. Activating molecules

A number of activating receptors capable of inducing cytotoxicity and cytokine production, have been identified. They are type II integral membrane proteins with calcium dependent lectin domain, that bind to carbohydrate moieties. Although their ligands are not yet known it can not be excluded that they also recognize MHC class I molecules. These receptors termed NK1.1, NKR-P1 and CD69 in mouse, and NKG2 and CD69 in human, are encoded by a family of related genes, on or near the NK gene cluster on the mouse chromosome 6 and on the human chromosome 12 respectively. NKR-P1 has first been cloned and sequenced in rat [236], and mouse[237] where several isoforms occur simultaneously; and then the human homologous the NKG2 family was cloned [238].

A. Mouse NKR-P1 family

NKR-P1 is a type II integral membrane protein formed of a 60 kDa homodimer and expressed on rat and mouse NK cells. A role for NKR-P1 molecules as activating receptors capable of triggering effector functions of NK cells has been suggested by the observation that antibody to NKR-P1 was able to enhance redirected lysis of FcR+ NK resistant target cells by IL-2 activated NK cells [239]. More recently, a number of oligosaccharides have been shown to bind, with different affinities, to NKR-P1 and such ligands have been further identified on NK susceptible tumor cells. Inhibition studies using soluble forms of NKR-P1 or the ligand oligosaccharide provided evidence that NKR-P1 interaction with oligosaccharide ligands on target cells is crucial and results in activation of NK cells leading to target cell lysis. These studies further postulated that different classes of oligosaccharide constitute ligands for NKR-P1 depending on the nature of target cell [240].

B. Human NKG2 family

NKG2 family of genes preferentially expressed on human NK and some T cells with NKG2-A and B alternatively spliced product of the same gene product, while NKG2-A, C and D are different gene products [234]. A recombinant soluble form of NKG2-C was shown to bind specifically to NK sensitive target cells K562, but not to several other hematopoietic cell lines tested. This binding structure on surface of K562 as well as the susceptibility to killing disappeared when K562 were induced to differentiate with phorbol esters and calcium ionophores. This suggests the presence of specific target molecules for NKG2 on K562 and propose that NKG2-C is functionally important in NK cell killing process [241].

1.6.2. Inhibitory molecules

A. mouse Ly-49 family

Ly-49 is a family of nine closely related genes on mouse chromosome 6, encoding type II integral membrane proteins (members of C type lectins) that are preferentially expressed by NK cells. They may bind to carbohydrate moieties on the target cell [242,243]. LY-49A was the first inhibitory receptor identified on murine NK cells. Originally identified as YE1/48 or A1, a T cell antigen of unknown function [244-246], it was later cloned [247,248] and shown to be a member of the Ly 49 multigene family [249]. It was subsequently observed that Ly-49A+ NK cells were unable to kill H-2D^d and H-2D^k tumor cells, whereas Ly-49A- NK cells efficiently lysed these target cells [250,251]. Among other identified Ly49 members are Ly49C, also termed 5E6, that reacts with H-2^b, H-2^d, H-2^k, and H-2^s [252,253], and Ly49G2, also termed LGL-1, that has been reported to react with H-2D^d, and H-2L^d [254].

B. Human inhibitory receptors

In common with murine NK cells, human NK cells also express receptors for polymorphic MHC class I molecules that inhibit killing of target cells bearing appropriate alleles. Two classes of inhibitory receptors have been identified thus far, namely killer inhibitory receptor (KIR) family which belongs to the Ig-superfamily and CD94 which is a C-type lectin.

KIRs are members of the immunoglobulin (Ig) superfamily encoded by a small family of related genes located on the human chromosome 19, and have no homology to the murine Ly-49 genes. They comprise two groups of molecules: p58 and p70 that interact with HLA-

C and HLA-B alleles respectively. The p58 family with two Ig like domains is characterized by mAb GL183 and EB6 that react with serologically distinct epitopes on molecules termed p58. Expression of p58 defines two groups of NK cells specific for HLA-C. Group 1 recognizes HLA-Cw4, and group 2 recognizes HLA-Cw3 [255,256]. p70 has three Ig like domains and is defined by DX9 monoclonal antibody (previously called NKB1). p70 recognizes HLA-Bw4 [257]. p58 molecule appear to recognize polymorphism at residues 77 and 80 of HLA-C molecule (GL183+ group 1 corresponds to Ser 77 and Asn 80, whereas EB6+ group 2 corresponds to Asn 77 and Lys 80)[258,259], and NKB1 recognizes corresponding region on HLA-B [260]. Recently a new member of the KIR family designated NKAT4, encoding a 70 kDa receptor specific for HLA-A3 has also been described [261]

CD94 (Kp43) is a type II integral membrane protein expressed on NK cells, a subset on γ/δ T cells, and rare α/β CD8+ CD56+ T cell clones, and reported to provide specific recognition for HLA-B (B7, B8, B14)[262,263], but its ability to recognize MHC molecules is controversial [260,263]. Although the general consensus is a post binding inhibitory signal delivered by target MHC class I binding to its receptor on NK cells, there is some discrepancy between the available data with studies indicating that cross linking of some of these same receptors can trigger activating signals and granule exocytosis. An anti p58 antibody, presumably directed against epitopes serologically distinct from that recognized by EB6 or GL183, was reported to directly activate TNF-α, IFN-γ production as well as serine esterase secretion by NK cells [264]. Stimulation through CD94 with an anti Kp43 mAb was shown to enhance the ability of IL-2 stimulated NK cells to synthesize TNF-α, through an LFA-1/ICAM-1 interaction [265]. This was suggested to be due to the great

heterogeneity of different clonal populations in terms of quantitative and qualitative differences in their expression of MHC class I receptors [266]. The functional ambivalence of CD94 was also recently reported with CD94 specific mAbs shown to either trigger or inhibit cytotoxicity in distinct subsets of NK clones [267]. These clonotypic differences have been shown to lead to differential signaling and cellular responses. CD94 ligation in group A clones, expressing low levels of CD94, induced proximal PTK cascade including activation of PTKs *lck* and Zap70, PLC, and PI3K, while in group B clones, expressing high levels of CD94, it did not induce any of these activation events, instead it inhibited FcR induced signaling events such as tyrosine phosphorylation of Zap70, and PLCγ1, formation of phospho-ζ/Zap70 complex, and PI release [268].

The cytoplasmic region of these KIRs includes a motif characterized by two tyrosine-X-X-leucine (YXXL) pairs spaced by 26 amino acids. The integrity of this intracytoplasmic sequence appears to be critical for KIR-mediated inhibition, since a naturally occurring form of the molecule, truncated after the first tyrosine residue, mediates NK cell activation rather than inhibition [269,270]. The configuration of tyrosine residues in KIRs is reminiscent of the immune receptor tyrosine-based activation motif (ITAM), also called antigen receptor activation motif (ARAM), associated with the Fc, B, and T cell receptors [271]. ITAMs transduce activation signals upon receptor cross-linking by serving as substrates for src family tyrosine kinases such as *lck* or *lyn*, and by subsequent association with SH2 domains contained in tyrosine kinases such as ZAP-70 and *syk* [271]. However, the unusual spacer region between the two YXXL, spanning 26 instead of 6-8 amino acids present in ITAM, suggests an independent, and possibly different, function for these two motives. The hematopoietic cell-specific tyrosine phosphatase (HCP) [272] or PTPase PTP1C (also called

SHP, SH-PTP1), expressing two SH2 domains is involved in the termination of many inhibitory signaling pathways, such as erythropoietin and Kit/stem cell factor (SCF) receptor signals [273]. Its association with several receptors has suggested a role for this phosphatase in either modulating or blocking activation signals [274]. Association of HCP/PTP1C with tyrosine phosphorylated p58 has been recently reported providing functional evidence that HCP plays a role in the delivery of a negative signal that prevents target cell lysis by NK cells [275]. Recruitment of HCP/PTP1C by p58 KIR was shown to be mediated by the specific binding of p58 cytoplasmic phosphotyrosine-containing sequences to PTP1C, after phosphorylation induced by either the pharmacological agent phenylarsine oxide or by conjugation with target cells [276]. Tyrosine phosphorylation of the NKB1 KIR consensus motif $YXXL(x)_{26}YXXL$ was also shown to induce an association with PTP1C and to be capable of inhibiting T cell activation in Jurkat cells [277]. CD94 was recently shown to assemble covalently with members of NKG2 family of C-type lectins [278,279]. NKG2A/B possess two ITIM sequences in their cytoplasmic domain, which may be responsible for the inhibitory function of these receptors, whereas other NKG2 proteins lack ITIMs and may potentially transmit positive signals [278]. Association of CD94 with different NKG2 members available on different NK clones could therefore explain clonotypic differences in terms of activatory versus inhibitory signals provided by CD94 engagement.

At present no human homologs to Ly49 genes or murine counterparts to the KIR family have yet been identified. However, despite distinct evolutionary origins, it has recently been reported that both human p58 KIR and $H\text{-}2D^{d/k}$-specific mouse Ly49A receptors recruit the same protein tyrosine phosphatases, PTP1C and PTP1D, upon phosphorylation of critical intracytoplasmic tyrosine residues [280].

1. 7. Signal transduction in NK cells

Two major signal transducing pathways have been extensively studied in NK cells, those following IL-2 binding to IL-2R, and those involved in triggering cytotoxic functions through ADCC or direct binding to target cell.

1.7.1. IL-2 R

IL-2 potently stimulates NK proliferation and cytotoxic functions, with acquisition of higher cytotoxic activity and larger spectrum of target cells. The pleiotropic effects of IL-2 on NK cells are initiated and regulated by a complex and multistep interaction between different PTKs including $p56^{lck}$ and members of the Janus family (JAK) of PTKs that are critical enzymes in signaling pathways via hematopoietic receptors. IL-2R β chain interacts both physically and functionally with $p56^{lck}$ which is activated by IL-2, and also with *Jak1* and *syk* of the Syk/Zap70 family PTKs [223], while IL-2Rγ binds to *Jak3* [281]. Although very small percentage (1%) of total cell $p56^{lck}$ associates with IL-2R β chain, this association has been shown to be critical for the IL-2 induced activation of $p56^{lck}$. IL2 stimulation induces tyrosine phosphorylation of a number of intracellular proteins, a rapid and substantial increase in catalytic activity of $p56^{lck}$ (measured in an in vitro kinases assay). This activation is associated with a pronounced reduction in the electrophoretic mobility of $p56^{lck}$ due to an increase in the phosphorylation of the NH2 terminal region of $p56^{lck}$ containing multiple sites of serine/threonine phosphorylation, as well as in the COOH terminal peptide

containing the regulatory Tyr505 residue of $p56^{lck}$ [282,283]. Quantitative analysis have shown that 10-40% of total $p56^{lck}$ is converted into $p60^{lck}$ presumably due to phosphorylation of $p56^{lck}$ on serine/threonine and/or tyrosine residues. Therefore, $p56^{lck}$ serves as a substrate for both protein serine and tyrosine kinases activated during stimulation with IL-2 [281]. Recent studies reported that *syk* activation precedes $p56^{lck}$ activation which in turn activates PI3K in the cascade of IL-2R stimulation[282].

1.7.2. Cytotoxic functions

Activation of NK cells following conjugate formation with their target cell has been shown to be associated with intracellular calcium mobilization, increased PI turnover, protein kinase C (PKC) activation and tyrosine phosphorylation of a number of NK cell proteins.

CD16 being one of the first NK receptors known to trigger cytotoxic functions and target cell lysis, is the most studied NK cell surface molecule with respect to the induced signaling events. Early studies showed that PKC together with calcium ionophores induce granule exocytosis in CTL and NK cells [284]. PI turnover and increased intracellular calcium concentrations were shown to be induced upon FcR ligation or exposure of NK cells to NK sensitive, but not NK resistant target cells suggesting a possible involvement of PI metabolism in ADCC and CMC. It was subsequently shown that FcR cross linking as well as contact with NK sensitive, but not NK resistant, target rapidly induced tyrosine phosphorylation of a number of distinct proteins. PTK inhibitors, herbimycin A and genestein, inhibit NK cytotoxic functions for both ADCC and CMC, suggesting that

tyrosine phosphorylation provides an early and requisite signal for activation of NK cytotoxic functions [285]. The ζ chain constitutes a substrate for CD16 induced tyrosine phosphorylation, as the activation of NK cells with either anti CD16 Ab or Ab coated target cells but not other activating stimuli (phorbol esters, ionomycin, IL-2, NK sensitive targets) triggers tyrosine phosphorylation of ζ. FcR activation is coupled to phospholipase C (PLC) via a G protein independent pathway, and it induces tyrosine phosphorylation of both PLCγ1 and PLC γ2 isoforms [286,287]. Herbimycin A abrogates this phosphorylation as well as the subsequent PI turnover, suggesting that FcR-initiated PI turnover in human NK cells is regulated by tyrosine phosphorylation of PLCγ. cAMP has been reported to exert a potent inhibitory effect on NK cytotoxicity (ADCC or CMC) probably by uncoupling NK receptor from PLC mediated PI hydrolysis. Activation of PLC cleaves PIs into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) which in turn mediate mobilization of intracellular calcium and activation of PKC respectively. Furthermore, Wortmannin, a selective PI3K inhibitor, inhibits FcR initiated granule exocytosis and ADCC, suggesting that PI3K activation in NK cells is associated with this pathway. Despite some similarities in the signaling events such as early PTK activation, PI turnover and increases in intracellular calcium concentration, ADCC and CMC are differentially regulated by PKC and PI3K: ADCC operating through PI3K dependent and CMC though a PKC dependent pathway [288]. More recently, FcγR was reported to induce tyrosine phosphorylation and increase the catalytic activity of both Syk family PTKs, *syk* and Zap70, this phosphorylation was markedly increased by *lck* overexpression of wild type, but not the inactive mutant, suggesting a regulatory interplay between Src and Syk families PTKs and that early FcγR initiated activation of *lck* results in subsequent regulation of Syk family PTKs [225,226].

1.8. TNF-alpha and IFN-gamma signal transduction pathways

1.8.1. TNF -α signaling pathway

TNF-α is a cytokine with pleiotropic action. It is known to induce DNA fragmentation [289], and expression of a number of cellular genes, including c-fos, c-myc, HLA class I and II, ICAM-1, ELAM-1, VCAM-1, diverse cytokines (IL-1, IL-6, IL-8, TNF-α, CSFs), and their receptors [290-294], There is increasing evidence that induction of many TNF-responsive genes is mediated, at least in part, through activation of nuclear transcription factor system NF-*k*B and its binding to *k*B site which is found in the enhancer element of many genes [295-299]. NF-*k*B was originally described as being present in the cytosol of most cell types as an inactive heterodimer composed of 50 kDa and 65 kDa subunits [300]. This heterodimer is bound to an inhibitor protein or proteins I*k*B. Induction of NK-*k*B binding involves dissociation of NK-*k*B from I*k*B, followed by translocation of the p50-65 heterodimer to the nucleus, where it directly binds to its cognate DNA sequence [301,302]. TNF-α has been identified as one of the few naturally occurring endogenous NF-*k*B inducers. TNF-α, ionizing radiation, or daunorubicin cause the activation of NF-*k*B by its rapid nuclear translocation through degradation of I*k*B. This protects cells from killing, and inhibition of NF-*k*B nuclear translocation enhances apoptotic killing by these reagents, but not by apoptotic stimuli that do not activate NF-*k*B [303,304]. Experiments in NF-*k*B knock out mice, and in cells expressing a dominant negative I*k*B, have recently brought some information on the mechanism of cellular resistance to TNF-α induced PCD and confirmed

an essential role for NF-*k*B in preventing TNF-α induced apoptosis [305,306]. Since cell killing by TNF-α is enhanced by protein synthesis inhibitors [307], it was suggested that a negative feedback mechanism results from TNF-α signaling in which NF-*k*B activation suppress the signals for cell death. Thus the activation of NF-*k*B probably functions to transcriptionally up-regulate a gene or group of genes encoding proteins involved in protection against cell killing [305]. Thus, although it triggers a biochemical pathway leading to PCD, TNF-α activates a key molecule, NF-*k*B, that can block this same pathway and so sets a delicate life-death balance within the cell [305,306]. NF-*k*B is activated in many cell types by PMA, a direct activator of PKC. However, IL-1 and TNF-α have been shown to activate NF-*k*B in PKC depleted cells and in presence of PKC inhibitors [295].

TNF-α signaling is initiated by interaction of TNF-α with two distinct cell surface receptor molecules that have apparent molecular sizes of 55-60 kDa (TNF R-55, or TNFR1), and 75-80 kDa (TNF R-75, or TNFR2) [308]. These receptors share homology in the extracellular ligand binding region; however, the cytoplasmic domains are distinct and lack any inherent enzymatic activity, which suggests that ligand binding and subsequent receptor clustering leads to the association of active signaling molecules with TNFRs [308]. While information about TRF R-75 signaling is scarce, TRF R-55 has been shown to trigger specific signal cascades, including phospholipase A2 (PLA2), phosphatidylcholine (PC)-phospholipase C, protein kinase C (PKC), and sphingomyelinase (SMase)[309]. Two of the most important activities of TNF, apoptosis and NF-*k*B activation, are signaled by TNFR1 following its oligomerization by the trimeric TNF [308]. TNF-α signals via unknown second messengers leading to activation of NF-*k*B [299] and Jun nuclear kinase/stress-activated protein kinase (JNKs/SAPKs), and a weaker stimulation of MAP kinase activity [310,311]. Ceramide has

been suggested to be an important mediator of the effects of TNF-α on growth inhibition, c-myc down-regulation, apoptosis, and the activation of the nuclear factor NF-*k*B [311]. Ceramide has also been shown to be the link between the binding of TNF-α to TNFR1 and activation of a cytoplasmic kinase cascade that results in stimulation of JNK/SAPK activity and c-jun expression [311]. By activating JNK/SAPK, TNF-α and ceramide activate a subset of AP-1 transcription factors, such as c-jun and ATF-2 which in turn will preferentially induce genes with specific non-consensus binding sites, such as the c-jun itself [312]. The weak activation of MAP kinase activity by TNF-α may be the result of ceramide-independent activation of a component (MEK or MAP kinase itself) downstream of Raf-1 and/ or by activation of protein kinase C [313].

1.8.2. IFN-γ signaling pathway

IFN-γ is a potent immunomodulatory cytokine that exerts its pleiotropic effects by interacting with a single high affinity receptor expressed on the surface of nearly all host-derived cells [314]. IFN-γ receptors consist of two subunits: a 90 kDa α chain that is responsible for ligand binding, ligand trafficking through the cells, and signal transduction [315] and a 62 kDa β chain that plays only minor role in ligand binding but which is obligatorily required for function [316,317]. IFN-γ signal transduction is known to require at least three other components in addition to receptor polypeptides. Two of these are the tyrosine kinases JAK-1 and JAK-2 [318], which become rapidly activated following IFN-γ receptor ligation and affect the tyrosine phosphorylation of the third component, a latent cytosolic transcription factor, Stat1, which subsequently forms an activated homodimer,

translocates to the nucleus, and initiates transcription of IFN-γ inducible genes [319,320]. Although it is not yet clear whether JAK kinases phosphorylate STATs directly, they are required for tyrosine phosphorylation and activation of STATs [321,322]. Recent studies clearly demonstrate that the IFN-γ receptor α and β subunits do not constitutively associate with one another but rather become associated upon exposure to ligand. These results also demonstrate that each receptor subunit associates with a specific JAK kinase. JAK-1 associates with the IFN-γ receptor α chain, and JAK-2 with the IFN-γ receptor β chain [323]. Thus ligand-dependent association of the IFN-γ receptor subunits brings into close juxtaposition inactive forms of receptor subunit-associated JAK-1 and JAK-2, which transactivate one another to initiate the IFN-γ signaling responses [324].

1.8. Thesis objective

The overall goal of this thesis is to elucidate the mechanism by which leukemic cells escape NK cell mediated cytotoxicity. Although NK cells have been shown to be effective in eliminating leukemic cells in vitro as well as in vivo, not all leukemic cells are sensitive to their cytotoxic effects. Killing of target cells by NK cells is a multistep process that involves the initial binding of NK cells to their target, activation of NK cells, and eventual lysis of target cells. This thesis focuses on the first two steps of this process and examines whether NK cells fail to kill certain leukemic cells due to their failure to achieve either binding or activation steps. In these studies, the NK resistant leukemic cell line SR-91 and the human NK cell line NK-92 were established. It was found that SR-91 expresses very low level of ICAM-1 which has been demonstrated to play an important role in NK cell cytotoxicity, it was therefore hypothesized that the low level of ICAM-1 expression is responsible for the resistance of SR-91 to NK-mediated cytotoxicity. The second hypothesis is that some adhesion molecules are important not only for the binding of NK cells to target cells, but also for the generation of activation signals in NK cells. To test these hypotheses, the relevance of ICAM-1 expression on the NK resistant leukemic cell line, SR-91, to its sensitivity to NK-mediated killing is examined, and the effect of upregulation of ICAM-1 expression on conjugate formation and lysis by NK cells is studied. The second part of these studies focuses on the cellular mechanism of TNF-α induced sensitivity of SR-91 cells to NK-mediated killing, and two major directions are investigated:

1) the possibility of induction of apoptosis in the target cells upon TNF-α treatment, and 2) activation of CAMs on SR-91 cells by TNF-α.

Finally, the possibility that the cell adhesion molecules ICAM-3 and CD44 may function as activating receptors on NK-92 cells is examined.

Chapter 2. MATERIALS AND METHODS

2.1. Cells and culture medium

K562 (erythroleukemia), HL-60 (promyelocytic), U937 (myelomonocytic), KG1a (variant of the AML cell line KG1), Daudi (Burkitt's lymphoma), Raji (B-cell lymphoma), Jurkat (T-cell lymphoma), NCI H929 (IgA myeloma), U266 (IgE myeloma), and RPMI 8226 (myeloma, light chain secreting) cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). NK-92 cells were maintained in myeloid long-term culture medium (Myelocult™, StemCell Technologies Inc., Vancouver BC) supplemented with 200 units/ml recombinant IL-2 (provided by Amgen Canada, Inc., Mississauga, ON). All other cell lines were maintained in continuous suspension culture in RPMI 1640 supplemented with 10% FCS, penicillin at 10^{-4} units/ml and streptomycin at 10mg/ml. DA3, the murine IL-3 dependent myeloid cell line (provided by Dr G Krystal, Terry Fox Laboratory, Vancouver, BC) was maintained in RPMI 1640+10% FCS, supplemented with 5 ng/ml murine recombinant IL-3 (provided by Recombinant Protein Production Facility, Terry Fox Laboratory, Vancouver, BC).

2.2. Antibodies and reagents

The following murine monoclonal antibodies (mAbs) to human antigens were used: affinity purified antibodies to *CD54* (anti ICAM-1, clone RR1.1, IgG1a, provided by Dr. R. Rothlein, Boehringer Ingelheim Inc. Ridgefield, CT), *CD102* (anti ICAM-2, clone CBR IC2/2, IgG2a, provided by Dr. T. Springer, Boston, MA), *CD50* (anti ICAM-3, clone ICR-2, IgG1, provided by Dr. M. Gallatin, ICOS Corporation, Bothell, WA), *CD11a* (anti LFA-1, clone NB107, IgG1, provided by Dr. F. Takei, Terry Fox Laboratory, Vancouver, BC), *anti CD44* (clones 3C12, 8D8, 7F4, and 2G1, IgG1, provided by Dr. G. Dougherty, Terry Fox Laboratory, Vancouver, BC), anti MHC class I (W6/3, IgG2a, provided by Dr P Landsdorp, Terry Fox Laboratory, Vancouver, CA). Mouse anti Lck mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antisera specific to Lck was provided by Dr. P. Leibson (Mayo Clinic, Rochester, MN), free and Protein A conjugated anti phosphotyrosine mAb, clone 4G10 was from Upstade Biotechnology Inc. (Lake Placid, NY), horse radish peroxidase conjugated second antibody was obtained from Jackson Immunoresearch (West Grove, PA), soluble ICAM-3 (sICAM-3) was provided by Dr. M. Gallatin. Human ICAM-1 cDNA was provided by Dr. T. Springer (Boston MA). Recombinant IL-2 was obtained from Amgen Canada Inc., recombinant TNF-α was provided by Genetech Inc. (San Francisco, CA), IFN-γ was purchased from Collaborative Research Inc. (Bedford, MA), IL-1 from R&D Systems (Mineapolis, MN, USA), IL-4 from DNAX (Palo Alto, CA, USA), IL-6 and IL-7 from Immunex (Seatle, WA, USA), IL-12 from Genetics Institute(Cambridge, MA, USA), IFN-α from Schering Canada Inc. (Pointe

Claire, Quebec, Canada), GM-CSF from Behringwerke (Marburg, Germany), murine recombinant IL-3 was obtained from Recombinant Protein Production Facility at Terry Fox Laboratoy (Vancouver BC), protein grade Nonidet P-40 (NP-40) was purchased from Calbiochem (San Diego, CA), and the enhanced chemoluminescence Western blotting reagents were purchased from Amersham (Arlington Heights, IL). All other reagents were obtained from Sigma (St Louis, MO), unless otherwise indicated.

2.3. Purification of human NK cells

Human peripheral blood mononuclear cells (PBMC) were separated from peripheral blood of healthy volunteers by Ficoll-Hypaque. 2 ml of PBMC ($2x10^8$ cells) were then placed on top of a seven-layer iso-osmolar(290 mOsm, pH 7.4) discontinuous Percoll (Pharmacia, Piscataway, NJ) density gradient (density of 1.052, 1.055, 1.058, 1.061, 1.064, 1.067, and 1.071). After centrifugation at 550xg for 30 mn at room temperature, the NK cell enriched population with a density of 1.055, and 1.058 was collected and cultured in MyeloCult supplemented in the presence of 500 U/ml IL-2. After 4-5 weeks of culture in IL-2, a highly purified IL-2 activated NK cell population (>90% CD56+, CD3- cells by direct immunofluorescence) was obtained that lysed target cells K562 and Daudi.

2.4. Cell surface marker analysis

HLA typing for NK-92 was performed by Dr B. Thiele (laboratory of immunology, Kalserslautern, Germany) using the polymerase chain reaction sequence specific primer (PCR-SSP) based technique (Dynal, Oslo, Norway) [325]. All other cell surface markers were determined by flow cytometry and indirect immunofluorescence with appropriate isotype-matched antibodies used for control experiments. $1x10^5$ cells were washed twice in phosphate buffered saline (PBS) +2% FCS +0.02% sodium azide, and incubated with unconjugated primary antibody, or with FITC or PE conjugated antibodies for 30 min at 4°C in the dark. Staining with unlabeled antibodies was performed by sequential incubation with saturating concentration of test antibodies and FITC conjugated goat $F(ab)'_2$ anti mouse IgG as a secondary antibody. Cells were then washed twice in washing solution and resuspended in PBS+2%FCS+0.2%NaN3, supplemented with propidium iodide at a final concentration of 1 μg/ml. IgG1 and IgG2 isotype controls, secondary antibody alone or normal saline (unstained) controls were included in all experiments. Cells were then analyzed in a flow cytometer FACSort/FACScan (Becton-Dickinson, Mountain View, CA). Propidium iodide was used to set up a live gate on FL-3 to exclude dead cells. For each sample, 5000 events were collected and analyzed using Hewlett Packard HP340 software.

2.5. Cytotoxicity assay

Target cells (K562, Daudi or SR-91) were pelleted and labeled with 30μCi (Du Pont) at 37°C for 1 h, washed and placed into 96-well round-bottom microtiter plates (Nunc) at $1x10^4$ cells/well. NK-92 effector cells were added at different effector: target cell ratios to a final volume of 200 μl. After incubation at 37°C for four h, 100 μl of the supernatant was removed from each well and the released radioactivity counted in a gamma counter (Gamma 5500, Beckman instruments Inc., Palo Alto, CA). The percent cytotoxicity was determined according to the formula :

$$\% \text{ Specific cytotoxicity} = \frac{\text{Experimental release - Spontaneous release}}{\text{Maximum release - Spontaneous release}} X100$$

Spontaneous release (target cells incubated with medium alone) was always less than 15% of maximum release (target cells lysed by medium containing 20% Triton X-100).

Antibody inhibition experiments were performed using antibodies against LFA-1, ICAM-1, ICAM-2 and ICAM-3. Different antibodies were either preincubated with effector or target cells for 30 min at 25°C and the excess antibody was removed by two washes prior to the cytotoxicity assay, or antibodies were added to the reaction mixture and were present during the cytotoxicity assay.

For the cold target cell competition assay, varying numbers of unlabeled and radiolabeled TNF-α and IFN-γ treated SR-91 cells were mixed with effector cells, keeping the total target cell number constant ($1x10^4$ cells/well), and a cytotoxicity assay was performed as described above. For calcium free conditions, the assay was performed in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS) supplemented with 5mM MgCl and 1mM EGTA.

2.6. Proliferation assay

Cells were washed and resuspended at $4x10^5$ cells/ml in RPMI 1640 medium supplemented with 10% FCS or alpha medium for SR-91 and NK-92 cells respectively. 100 µl of cell suspension were seeded in a 96 well flat-bottomed plate (Falcon, Becton Dickinson) and an equal volume of the media alone or media containing different cytokines at various concentrations was added to each well, and incubated at 37°C in a humidified incubator at 5% CO2. Serum free medium was used when testing erythropoietin. SR-91 cells were cultured for 48 h, ^3H thymidine (Amersham, Oakville, ON, Canada) was then added at 0.5 µC/well (in 20 µl). After six hours incubation, cells were harvested and samples collected using a cell harvester (Cambridge Technology, Watertown, MA, USA) and ^3H-thymidine uptake counted in a liquid scintillation counter (Beckman Instruments). For NK-92 cells after 3 days of incubation, alpha medium was replaced by RPMI 1640 medium and plates were further incubated for two hours. This was necessary as alpha medium contains thymidine which can interfere with the ^3H thymidine incorporation. Plates were incubated for further two hours. Cells were then pulsed with ^3H-thymidine and harvested as described above.

2.7. Irradiation of NK-92 cells

NK-92 cells were irradiated in T25 flasks (Falcon, Beckton Dickinson) using a cesium source (Cis-US, Bedfors, MA). A dose range of 200-2000 cGy was tested. After irradiation, the cells were washed twice and resuspended in medium and cultured for 72 hours in IL-2. Cytotoxicity (^{51}Cr release assay) and proliferation (^{3}H-thymidine) assays were performed with these cells as described above.

2.8. Assay for clonogenic hematopoietic progenitor cells

Normal PBMCs were co-cultured with irradiated (1000cG) NK-92 cells for two days. Cells were then plated in replicate 1.1 ml aliquots of methylcellulose-containing media at densities adjusted to give approximately 10-100 large colonies of erythroid cells (from burst-forming units, or BFU-E), granulocytes and macrophages (from colony-forming units-granulocyte/macrophage or CFU-GM), and combinations of all these (from CFU-granulocyte/erythroid macrophage/megakaryocyte, or CFU-GEMM). The medium was supplemented with 30% FCS, 1% de-ionized bovine serum albumin (BSA), 10^{-4} M 2-mercaptoethanol, 3 units/ml human erythropoietin, and 10% human leukocyte conditioned medium. Plates were incubated for 18-21 days in 5% CO2 humidified air at 37^{o}C and the different types of colonies scored in situ using established criteria. Colonies were counted

under an inverted microscope two weeks late. This assay was performed by Stem Cell Assay laboratory at Terry Fox Laboratory.

2.9. Conjugate formation assay

To quantitate the binding between SR-91 and NK-92 cells, a conjugate formation assay was performed as previously described [326,327]. Briefly, effector and target cells were washed separately in serum free PBS, resuspended at two times the desired concentration in 500 µl and mixed with an equal volume of 1-2 µg/ml of fluorescent cell linkers PKH26 (red fluorescence) and PKH2 (green fluorescence) (Sigma), respectively. The cells were incubated in the dark for 2-5 min at 25°C. The staining reaction was stopped by adding an equal volume of FCS and incubating at 25°C for one min. After adding 2 ml RPMI+10%FCS, cells were centrifuged at 400x g for 10 min, the supernatant removed and the pellet resuspended in HBSS +2% FCS, transferred into a new tube and washed three times with HBSS+10%FCS. The cell pellets were then resuspended at the desired concentration in PBS containing 5mM $MgCl_2$ and 1mM EGTA. To allow for conjugate formation, 100 µl aliquots of effector and target cells were mixed, centrifuged at 400x g for three min, incubated for 10 min at 37°C and then analyzed by flow cytometry. To eliminate non-specific conjugate formation, two-color labeled effector/effector and target/target cells were also examined. A total of 5000 events were examined. The percentage of conjugated cells was determined by dividing the number of dual labeled particles by the total number of cells bearing that label.

2.10. Transfection

Human ICAM-1 cDNA was subcloned into the EBV derived expression vectors pREP9 and pCEP4 (Invitrogen. San Diego. CA) which contain neomycin and hygromycin resistance genes respectively. Not I/Hind III sites were used for pREP9, and the Nhe I/Xba I sites for pCEP4. SR-91 cells ($5x10^6$ in 500 µl of media), were transfected with 20 µg of either pREP9:ICAM-1 or pCEP4:ICAM-1 plasmid by electroporation (0.25 kVolt, 500 µFD) using a Gene Pulser™ (Bio-Rad, Hercules, CA). The transfectants were selected in 0.4 mg/ml G418 or 0.5 mg/ml hygromycin respectively.

2.11. Adhesion assay

To determine the activation state of LFA-1, CD44 and the β1 integrins, adhesion assays using fluorescent tagged SR-91 cells were performed essentially as previously described [174]. Briefly, 96 well flat bottom plates (Falcon) were coated with 4µg/ml sICAM-3 in TSM buffer (25mM Tris-HCl, pH 8.2, 150 mM NaCl, 2mM $MgCl_2$) for 2 h at 37°C (to measure the activation state of LFA-1), or with fibronectin (1 mg/ml in PBS) (for the β1 integrins) overnight at 4°C. 24 well plates (Falcon) were coated overnight at 4°C with hyaluronan (Sigma) (5mg/ml in PBS) to assess CD44 activation. As a control, 0.1% bovine serum albumin (BSA) was coated onto the same plates. At the end of the incubation time, wells were washed twice with PBS prewarmed to 37°C, and saturated with PBS containing 0.1% BSA for 30 min at 37°C.

For fluorescent labeling, SR-91 cells were washed three times with serum-free HBSS, resuspended in 1 ml serum-free HBSS and 1-5 μg/ml calcein AM (Molecular Probes Inc., Eugene, OR) added to the cell suspension. After incubation at 37°C for 45 min, cells were washed once in HBSS and once with HBSS+2%FCS and resuspended in HBSS+2%FCS. For binding, 500 μl or 50 μl of the cell suspension ($1x10^6$cells/ml) were added to the 24 or 96 well plates, respectively, and incubated for 30 min at 37°C. Non-adherent cells were gently removed, the plates gently washed twice with prewarmed PBS and the wells overlaid with PBS. The intensity of the fluorescence bound to the wells was determined using a fluorescent plate reader (Bio-Instruments, Highland Park, VT, model EL30q). The percentage of bound cells was determined by referring to a standard curve established using known numbers of fluorescent cells.

2.12. DNA fragmentation assay

Cells were incubated overnight with different doses of TNF-α, $1x10^6$ cells were used for each condition. Cells were washed in PBS, pelleted and DNA obtained by lysing the cell pellet in 20 μl of apoptosis lysis buffer (0.1% Na Citrate, 0.1% Triton X-100, pH 8). DNA samples were then incubated with 1μl proteinase K (at 10 mg/ml), followed by 10 μl RNase (at 1μg/ml), both incubations were for 1 h at 50°C. Samples were further incubated at 70°C for 10 min after the addition of 10 μl of loading buffer, and loaded in 1% agarose gel containing 0.1mg/ml ethidium bromide, and run at 90 volts.

2.13. Flow cytometric cell cycle analysis for the detection of apoptosis

The decreased binding of DNA-binding dyes to apoptotic cells which appears as a distinct peak below the G0/G1 peak, allows the discrimination of apoptotic cells from their healthy counterparts by conventional cell cycle analysis [328]. Cell cycle analysis was performed as previously described [329]. Briefly, $1x10^6$ cells were washed once in PBS and lysed in lysis buffer (0.1% Na Citrate, 0.1% Triton X-100, pH 8) containing 20 μg/ml of propidium iodide, cell lysates were kept at 4°C in the dark for 10 min, and then analyzed by flow cytometry. The IL-3 dependent DA3 cells were deprived of IL-3 and FCS for 24 h. SR-91 cells as well as TNF sensitive cell lines U937 and HL60 were cultured overnight in the presence of different doses of TNF-α.

2.14. Immunoprecipitation and immunoblotting

Immunoprecipitations and Western blot analysis were performed as described previously [330]. Briefly, NK-92 cells were deprived of IL-2 for 4 h, and, after washing and resuspending in PBS, they were incubated with different doses of the indicated antibodies for various times (5 to 45 min) at 25°C. The cells were then washed twice in PBS to eliminate excess antibody and lysed with 1% NP-40 in phosphorylation solubilization buffer (PSB) (50mM HEPES, pH 7.4, 100mM NaF, 10mM NaPPi, 2mM NaVO3, 4mM EDTA,

2mM DMSO) containing 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 2mM PMSF for 1 hour at 4°C. Cell lysates were then centrifuged at 400x g for 10 min to pellet nuclei and any intact cells, and the supernatant centrifuged at 18000x g for 10 min to pellet insoluble material. For immunoprecipitation, the supernatants were incubated with antiphosphotyrosine (4G10) coupled Protein A sepharose beads for four h at 4°C, or sequentially with rabbit anti-Lck antiserum for 1 h at 4°C and Protein A coupled Sepharose beads for three h at 4°C. Immunoreactive proteins were then eluted specifically by 20 mM phenylphosphate (for 4G10) or by boiling in SDS sample buffer. The samples were resolved by SDS-polyacrylamide gel electrophoresis, and the proteins transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Blots were then blocked with 5% BSA in PSB or 5% skim milk in TBS (20mM Tris, pH 7.5, 150mM NaCl) and incubated with 4G10 or mouse monoclonal anti-Lck antibody (at 0.5 μg/ml) for 1 h at 25°C. Membranes were washed five times in TBS, 0.025% Tween (TBST) and then incubated with anti-mouse IgG coupled to horse radish peroxidase (HRP) for 45 min at 25°C. After washing in TBST, the proteins were visualized by incubation with ECL substrate solution and exposed to Kodak Xomat film (Eastman Kodak).

Chapter 3. Establishment of a model system to investigate leukemic cell resistance to NK mediated killing.

The data presented in this chapter has been incorporated into the following manuscripts:

Klingemann HG, Gong J, Maki G, Horsman DE, Dalal BI, Phillips GL. 1994. Establishment and characterization of a human leukemic cell line (SR-91) with features suggestive of early hematopoietic progenitor cell origin. Leuk Lymphoma 12: 463.

Gong J, Maki G , Klingemann HG. 1994. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia 8: 652.

Klingemann HG, Wong E, Maki G. 1996. A cytotoxic NK-cell line (NK-92) for *ex vivo* purging of leukemia from blood. Biol Blood Marrow Transplant 2: 68.

3.1. Rationale

Despite the use of more aggressive regimens of chemo-radiotherapy in the treatment of cancer patients, tumor relapse remains a major problem. In patients with leukemia, even after bone marrow transplantation (BMT), the relapse rate is still about 40-60% for

autologous BMT suggesting that myeloablative doses are not sufficient to eliminate residual disease. The relapse rate is much lower with allogeneic BMT. This is thought to be due to generation of graft versus leukemia (GVL) effect in allogeneic BMT. GVL is an immune reaction involving CTLs, NK cells and cytokines such as IL-2, IFN-γ and TNF-α. One possible mechanism of inadequate GVL in autologous BMT, and tumor resistance in general, can be a lack of expression of MHC and/or accessory molecules on tumor cells.

NK cells and CTLs represent two major cytotoxic lymphocyte populations able to kill malignant cells. They become activated and proliferate in response to IL-2 among other cytokines. Several studies employing IL-2 as an adjuvant immunomodulatory agent have demonstrated that IL-2 also enhances the proliferation and cytotoxicity of T and NK cells in vivo [331-333]. Beside their direct antitumor capacity, NK and T cells are also able to secrete a number of cytokines upon activation, including cytokines with antineoplastic activity such as TNF-α and IFN-γ [334,335]. Thus, activated CTLs and NK cells generated post-BMT contribute to this antineoplastic activity and GVL [336]. Induction of cytokine secretion, in addition to enhancement of the cytotoxic effector functions, is thought to contribute to the antineoplastic effects of IL-2 therapy. Investigations performed with purified T and NK cells from adult BMT patients receiving IL-2 therapy demonstrate that, as a response to IL-2, both cell populations are capable of secreting IFN-γ and TNF-α in vitro [334]. IFN-γ and TNF-α are also known to induce the expression of adhesion molecules on tumor cells [337], rendering the neoplastic cells more sensitive to cytotoxic effector-cells by providing initial cell-cell contact.

In contrast to CTLs that need to be sensitized and recognize the target cell only in the context of MHC class I, NK cells are capable of mediating this cytotoxic function without prior activation or sensitization [1]. Therefore, they are important components of innate immunity functioning as a first line of defense against primary and metastatic tumors. In vitro studies have shown that most acute myeloid leukemia (AML) cells are sensitive to LAK killing, and a number of animal studies as well as clinical trials in humans have demonstrated that combined use of LAK cells and IL-2 can be effective in reducing tumor burden and prolonging survival in patients with renal cancer [338-340], melanoma [341,342], lymphoma [343], and leukemia [344-346]. However, not all leukemic cells are sensitive to cytotoxic effect of LAK cells [162].

The objective of this study was : (i) to delineate why some leukemic cells are resistant to LAK cell killing, and (ii) to define the contribution of cell adhesion molecules expressed on leukemic cells to their susceptibility to LAK cell killing. Information obtained from these studies could be useful as to how resistance of malignant cells to NK/LAK cells could be overcome for example by modulating the expression of these adhesion molecules on leukemic target cells.

The low number of NK cells in peripheral blood (PB) (10-15% of PBMCs), and technical difficulties to obtain pure populations of NK cells free of contaminating T cells constitute a major obstacle to the study of this lymphocyte subset. Enriched NK fractions can be isolated by Percoll density gradient. Further depletion of monocytes and T cells can be achieved by using nylon wool column or plastic adherence, and antibody coated magnetic beads coupled to anti CD3 antibody, respectively. Although combination of these methods can allow to obtain highly enriched NK cell populations, the purity of the NK population obtained is still

compromised by the contaminating T cells. Moreover, the long term maintenance of normal NK cells is difficult. Therefore, the availability of NK cell lines is of great practical advantage. To date, only very few permanent NK cell lines have been established including one rat NK cell line (RNK-16), and four human NK cell lines [347-350] that are listed in table 1. The other alternative option to obtain pure NK cells for biological studies is the generation of NK clones. A number of such NK clones have been isolated and characterized [256,267,351-353]. They have proven to be very useful tool for the studies of MHC class I receptors on NK cells. NK cell clones isolated from single donors display different patterns of cytolytic activity against a panel of allogeneic cells, thus indicating that an NK cell repertoire exists. Each NK clone shows clonal specificity to recognize HLA class I. Expression of given HLA class I alleles protects target cells from lysis by different groups of NK clones [256,267,351-353]. Target specificity of these NK clones is therefore limited by MHC class I molecules expression on the target cells.

To investigate the underlying cause(s) for the resistance of certain leukemic cells to killing by LAK cell, a model system was established with the NK resistant leukemic cell line SR-91, and the human NK cell line NK-92 that I established and characterized. The NK-92 cell line which has identical features as NK/LAK cells proved to be essential for the hypothesis to be tested. The following chapter describes the details as to how the cell line was established including its characterization.

Table 1. Other available human NK cell lines

Cell line	phenotype	Reference
NK3.3	CD3-, CD56+, CD16+	Kornbluth 1982 [347]
YT	CD3-, CD56+, CD16+	Yodoi 1985 [348]
YT2C2	CD3-, CD56+, CD16-	Teshigawara 1987 [349]
NKL	CD3-, CD56+, CD16+	Robertson [350]

3.2. Results

3.2.1. Establishment and Characterization of a human NK cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells.

A. Establishment of NK-92 cell line

The human NK cell line NK-92 was established from blood lymphocytes of a 50-year old male patient with rapidly progressive non-Hodgkin's lymphoma, whose marrow was diffusely infiltrated with large granular lymphocytes (LGL). Immunophenotyping of marrow blasts and peripheral blood lymphocytes showed expression of CD56, CD2, and CD7 but not the T-cell marker CD3. Cytotoxic activity of peripheral blood mononuclear cells against the NK sensitive cell line K562, and the NK resistant LAK sensitive cell line Daudi, at an effector:target ratio of 50:1 was 79% and 48% respectively. To establish the line, PMBCs were cultured in tissue culture flasks, at $1x10^5$ cells/ml in enriched alpha medium supplemented with FCS (12.5%) and horse serum (12.5%) (MyeloCult™, StemCell Technologies, Vancouver, BC) in the presence of 1000U/ml of human recombinant IL-2. Cells were cultured at 37^oC in a humidified atmosphere of 5% CO2. The NK-92 cell line has been continuously proliferating in suspension culture with twice weekly medium change for over 18 weeks. Under these conditions, NK-92 cells grew as non adherent cells forming large loose aggregates. Cell growth and survival was dependent on IL-2 and alpha medium, as the cells died within 72 h when IL-2 was omitted or when the alpha medium was replaced by RPMI 1640 supplemented with either 12.5% human AB serum or 12.5% FCS.

B. Cell surface marker analysis

Flow cytometric analysis showed that NK-92 cells are positive for CD56, CD2, CD25, CD28, CD54 (ICAM-1), CD11a/CD18 (LFA-1); and negative for CD16, CD3, CD4, CD8, CD34 (Fig 1). Expression of the IL-2 R alpha-chain (p55) was found to be dependent on the amount of IL-2 in the culture medium. At higher doses of IL-2 (1000U/ml) 10% of the cells were positive whereas about 50% of NK-92 cells express this receptor at intermediate doses of IL-2 (100U/ml). The IL-2R beta-chain (p75) expression was independent of the concentration of IL-2 in the medium (Fig 1). NK-92 cells also express CD95 (Fas Ag), CD44, VLA-4, but not VLA-5 (Table 2). The MHC phenotype of NK-92 cells is listed in table 3.

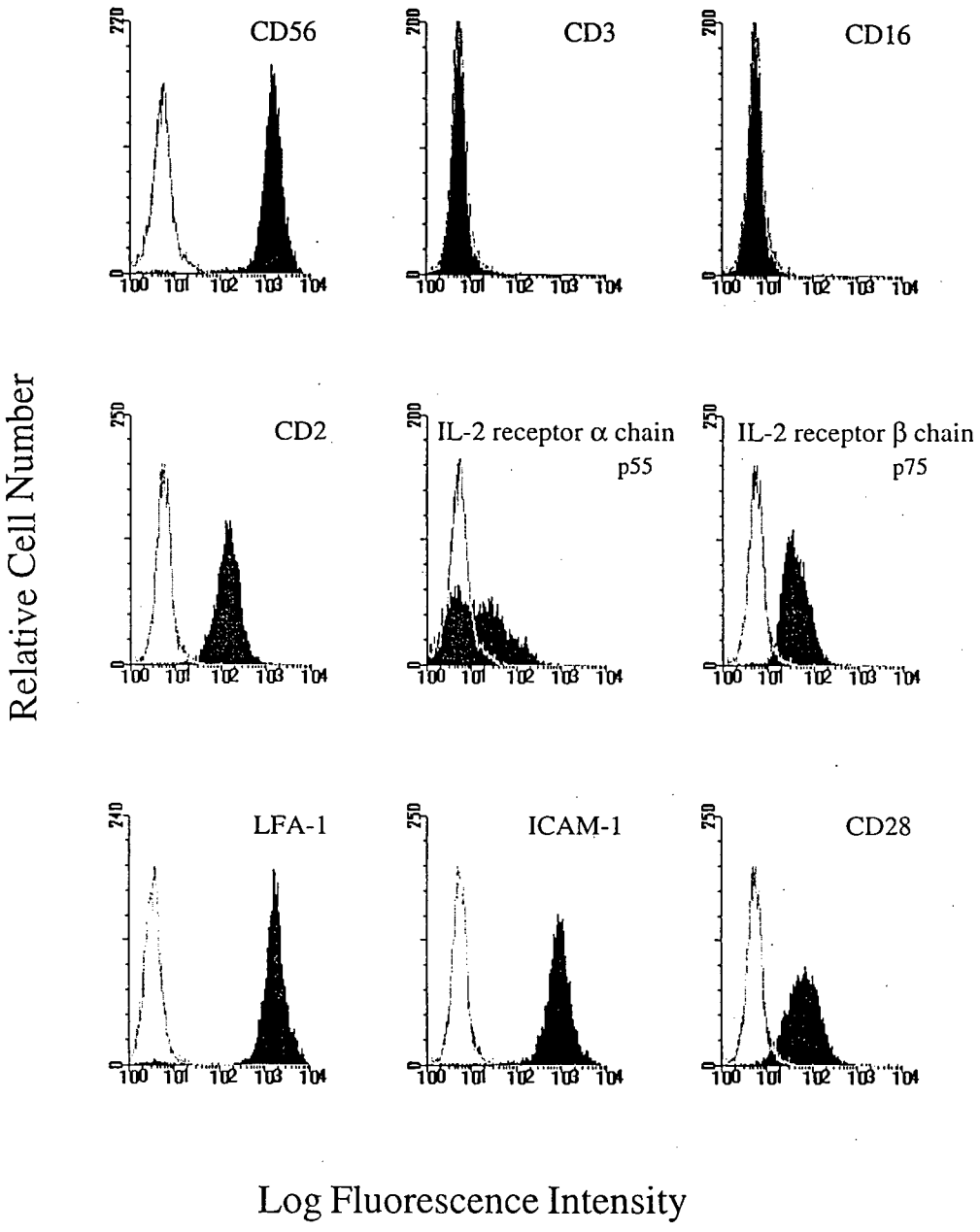


Figure 1. Flow cytometric analysis of cell surface molecules expression on NK-92 cells. NK-92 cells were stained with saturating concentrations of monoclonal antibodies against CD56, CD3, CD16, CD2, IL-2 receptor α and β chains, LFA-1, ICAM-1 and CD28 (filled). Background fluorescence of cells stained with the secondary antibody alone is also shown (open). Expression of the IL-2 R alpha-chain (p55) was found to be dependent on the amount of IL-2 in the culture medium. At higher doses of IL-2 (1000U/ml) 10% of the cells were positive whereas about 50% of NK-92 cells express the receptor at intermediate doses of IL-2 (100U/ml).

Table 2. Flow cytometric analysis of cell surface molecules expression on NK-92 cells.

Cell surface antigen expression level[a]	
CD34	-
CD3	-
CD4	-
CD8	-
CD56	+++
CD16	-
CD2	++
CD54 (ICAM-1)	+++
CD102 (ICAM-2)	++
CD50 (ICAM-3)	+
CD11a (LFA-1 α chain)	+++
CD29 (β1 integrins β chain)	+++
CD49d (VLA-4 α chain)	+++
CD49f (VLA-5 α chain)	-
CD44H	+++
CD44R1[b]	++
CD95 (Fas Ag)	++

[a]Expression of cell surface antigen was determined by indirect immunofluorescence staining with the corresponding mAb and FACS analysis as described in Materials and Methods. Expression levels of each surface antigen is coated as +++(bright), ++ (intermediate). + (low expression), or - (not expressed) above background fluorescence. [b]Based on CD44 staining with mAb 2G1 (recognizing exon V10), NK-92 cells express one or more CD44 isoform(s) containing the alternatively spliced exon V10 (isoforms R1 or R2).

Table 3. HLA phenotype of NK-92 cells

MHC class I [a]	+ for HLA A3, 11 HLA B4, 44
MHC class II [a]	+ for HLA DR7, 15, 51, 53 HLA DQ2, 6

[a] MHC class I and II phenotype was determined by PCR-SSP based technique as described in Materials and Methods.

C. Effects of various cytokines on the growth of NK-92 cells

NK-92 cells were cultured in the presence of different cytokines and ^{3}H-thymidine incorporation measured after 48 h. Different doses of cytokines were tested. No evidence of NK-92 cell proliferation was found after two days in medium alone. IL-1α, IL-4, IL-6, IFN-α and IFN-γ, tested over a range of 50-1000 U/ml, had no effect on the proliferation of NK-92 cells (Fig 2). NK-92 cell growth was IL-2 dose dependent and concentrations as low as 1U/ml induced measurable proliferation with a maximum at 50 U/ml of IL-2 (Fig 3). IL-7 also stimulated NK-92 cell proliferation. However, cells could not be maintained in IL-7 alone for longer than one week. IL-12 neither induced short-term proliferation nor was it able to support long-term growth of NK-92 cells.

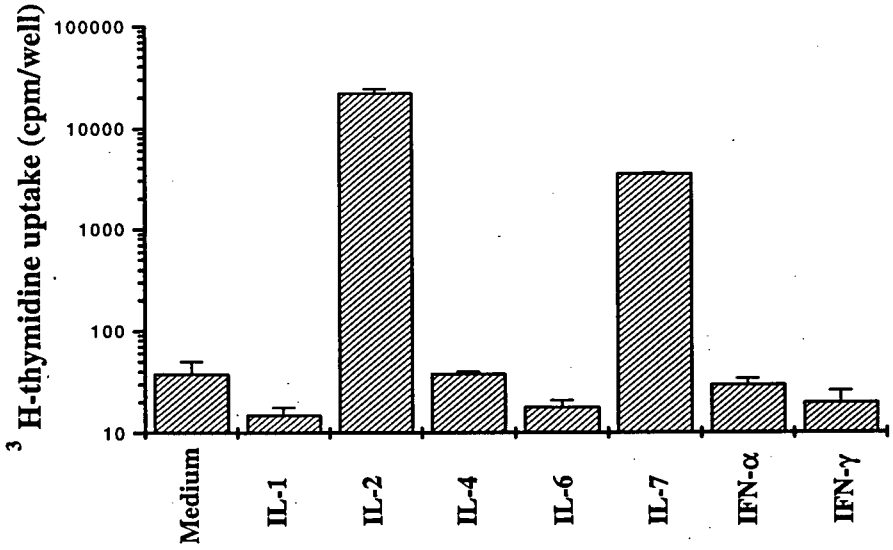


Figure 2. Effects of various cytokines on growth of NK-92 cells.
^{3}H-thymidine incorporation was performed as described in Materials and Methods. The cytokines were used at the following concentrations: IL-1α 1000U/ml, IL-2 50U/ml, IL-4 1000U/ml, IL-6 1000U/ml, IL-7 100U/ml, IFN-α 100U/ml, IFN-γ 1000U/ml. Results represent mean ± SD of triplicates.

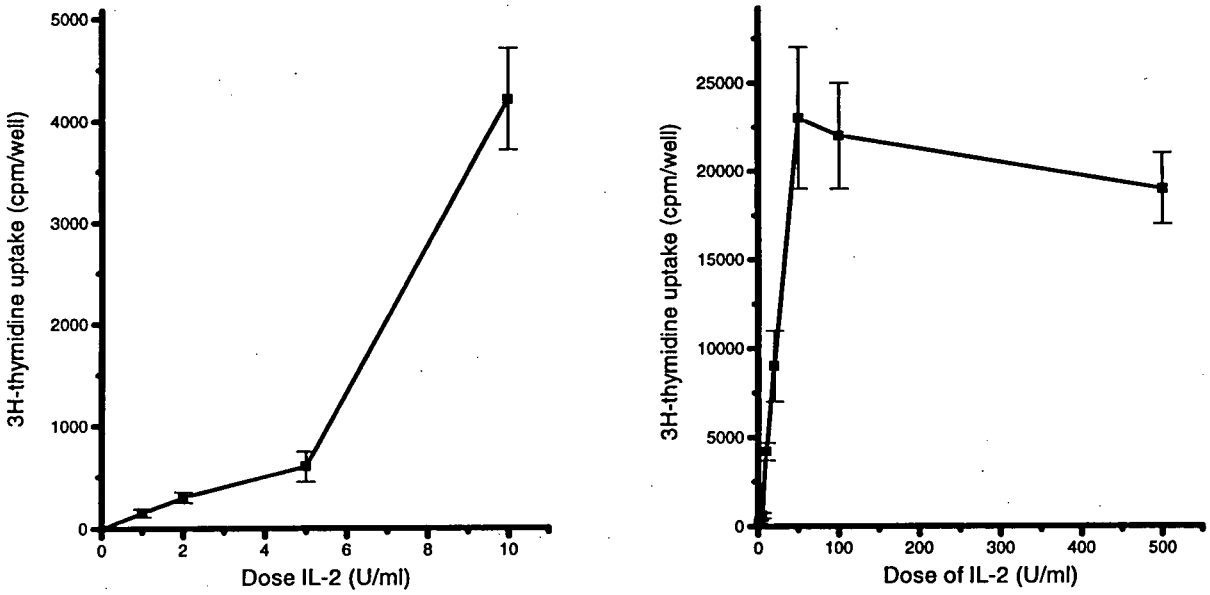


Figure 3. Dose response curve of the effect of IL-2 on proliferation of NK-92 cells.
NK-92 cells were cultured with different doses of IL-2, and ^{3}H-thymidine incorporation assay was performed after 48 h as described in Materials and Methods. Results represent mean ± SD of three different experiments.

D. Effect of NK-92 cell on normal and malignant hematopoietic cells

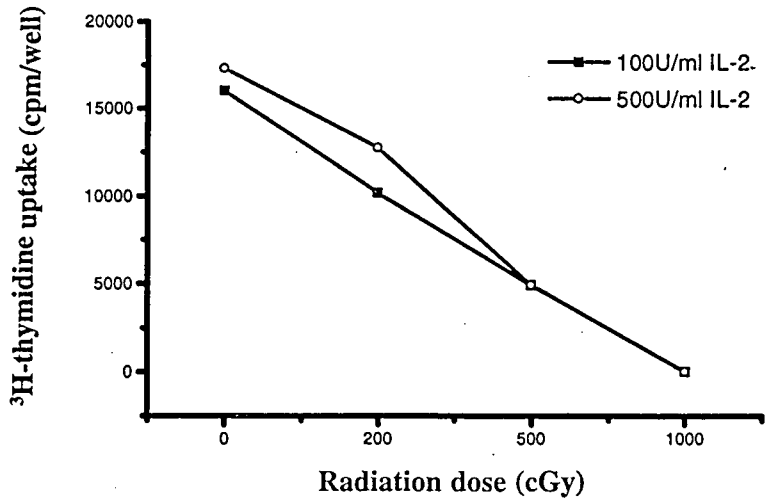
NK-92 cells spontaneously kill the NK sensitive cell line K562 and the NK resistant, LAK sensitive Daudi cells with high efficiency. Even at E:T ratio of 1:1, 83% of K562 and 76% of Daudi cells were killed by NK-92 cells. Moreover, it has a very potent cytotoxic activity against a broad spectrum of tumor target cells including a number of leukemia (K562, KG1a, U937, HL-60), lymphoma (Daudi, Raji, Jurkat, DHL-10), and myeloma (NCI-H929, RPMI 8226, U266) cell lines (Table 4). To test whether proliferation of NK-92 could be suppressed while maintaining their cytotoxic activity, NK-92 cells were irradiated with a cesium source at different doses and tested in proliferation and cytotoxicity assay. It was found that at a dose of 1000cGy, their proliferation, as measured by ^{3}H-thymidine was almost completely inhibited (Fig 4) without significant alteration of their cytotoxic activity (Fig 5).

Table 4. Comparison of cytotoxic activity of NK-92 cells and IL-2 activated PBMCs against various leukemia, lymphoma, and myeloma cell lines

			Percentage of cell lysis [a]				
			Effector: target ratio				
			50:1	20:1	10:1	5:1	1:1
Leukemia	**K562**	NK-92	68	68	64	59	50
		PBMC+IL-2	63	73	67	51	19
	HL-60	NK-92	97	90	77	46	40
		PBMC+IL-2	31	26	17	2	0
	KG1a	NK-92	90	91	80	67	39
		PBMC+IL-2	15	11	12	6	0
	U937	NK-92	99	98	96	91	85
		PBMC+IL-2	57	43	23	13	2
Lymphoma	**Daudi**	NK-92	94	87	71	48	39
		PBMC+IL-2	65	57	29	16	6
	Raji	NK-92	81	75	74	70	54
		PBMC+IL-2	72	67	57	35	13
	Jurkat	NK-92	100	100	98	93	80
		PBMC+IL-2	67	50	36	27	4
Myeloma	**NCIH929**	NK-92	94	89	89	86	51
		PBMC+IL-2	75	58	39	24	5
	RPMI 8226	NK-92	82	72	70	72	41
		PBMC+IL-2	95	83	81	67	25
	U266	NK-92	84	77	85	81	53
		PBMC+IL-2	84	74	73	56	21

[a] Cell lysis was determined in a four h ^{51}Cr release assay as described in Materials and Methods, using various E:T ratios. PBMCs were cultured in the presence of 1000U/ml IL-2 for four days and then tested in cytotoxicity assay. Data are representative of one out of three independent experiments.

A



B

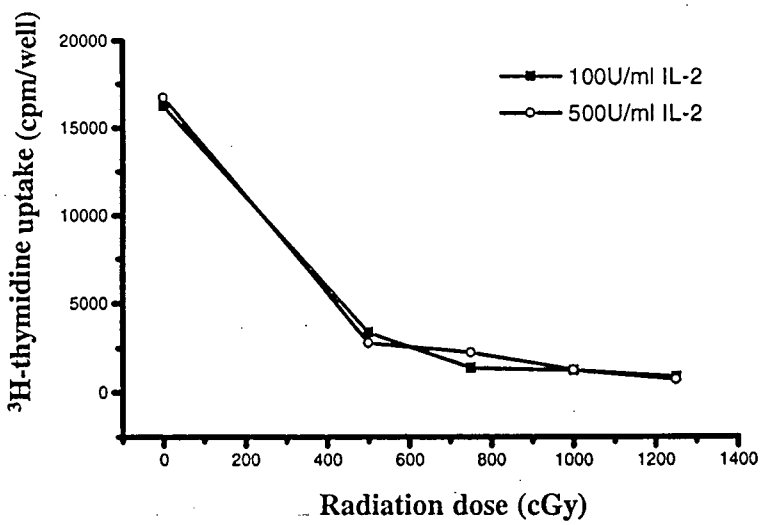


Figure 4. Effects of γ irradiation on NK-92 cells proliferation.
NK-92 cells were irradiated with a cesium source at the indicated doses and cultured for 48 h at 37°C in the presence of 100 or 500 U/ml IL-2. Two h prior to the addition of ^{3}H-thymidine (0.5μCi/well), the culture medium was replaced with thymidine free RPMI. ^{3}H-thymidine uptake was measured in a liquid scintillation counter four h later. The counts per minute (cpm) from two independent experiments are presented in panels **A** and **B**.

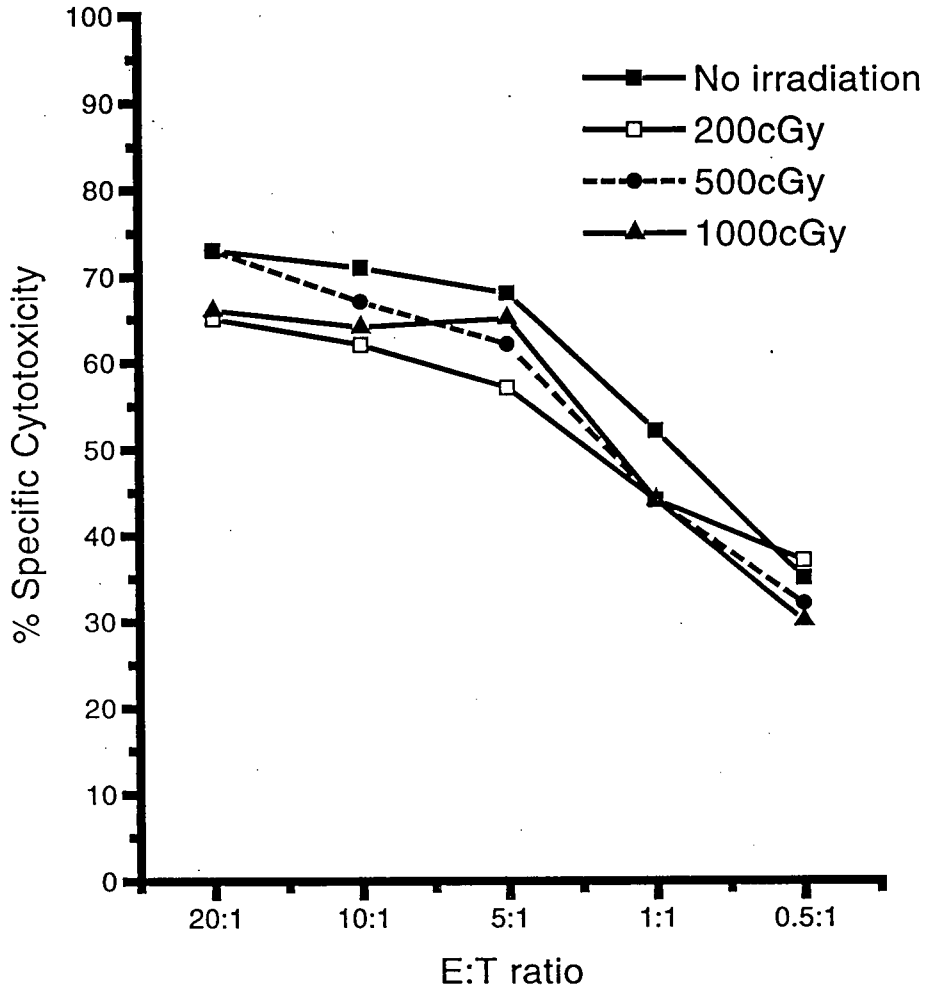


Figure 5. Effects of various doses of radiation on NK-92 cytotoxic activity.
NK-92 cells were irradiated with a Cesium source at doses ranging between 200 and 1000cGy. After 24 h in IL-2 containing medium to allow cell recovery, cells were then tested in a four h Cr release assay against K562 target cells. Mean values of two independent experiments are presented. Error bars are not shown, they were always less than 7% the mean values.

To determine whether NK-92 cells have any cytotoxic or inhibitory effect on normal hematopoietic progenitor cells, normal PBMCs were co-cultured with irradiated NK-92 cells (1000cGy) for two days and hematopoietic progenitor cell assay was performed as described in Materials and Methods. No growth inhibitory effect on hematopoietic progenitors by NK -92 cells was noted (Table 5). Furthermore, NK-92 cell conditioned supernatants obtained after maintaining the cells for 2 days in either intermediate (100U/ml) or high (1000U/ml) concentrations of IL-2 had neither stimulatory nor inhibitory effects on myeloid colony formation when tested in standard BFU-E, CFU-GM, and CFU-GEMM containing non-adherent cell suspensions prepared from human marrow.

Table 5. Effect of NK-92 cells on colony formation of normal hematopoietic progenitor cells.

Experiment number	CFU-GEMM[a]	BFU-E[a]	CFU-C[a]
1	100	46	94
2	200	98	64
3	33	104	103

[a]NK-92 cells were irradiated with 1000cGy (cesium source) and co-cultured with normal PBMCs at a 1:1 ratio. The cells were then plated in methylcellulose and counted two weeks later as described in Materials and Methods. Results obtained from different donors are presented as percentage of normal controls.

E. IL-2 deprivation maintains NK-92 activity for 48 hours

To test how long NK-92 cells would maintain their cytotoxic activity in the absence of exogenous IL-2 in the culture medium, NK-92 cells were deprived of IL-2 and ^{51}Cr-release was measured in 24-hour intervals. The results obtained suggested that the cells maintain full cytotoxic activity for at least 48 hours. Thereafter, the activity dropped rapidly to negligible levels (Fig 6).

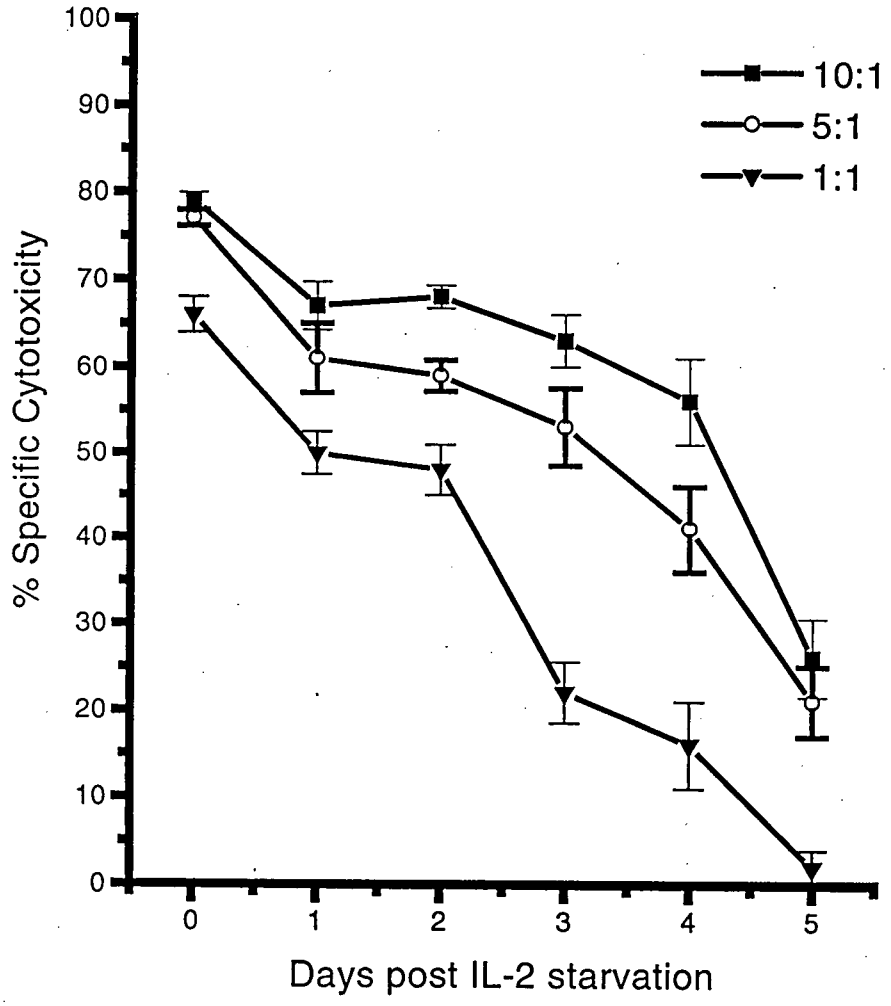


Figure 6. Effects of IL-2 starvation on NK-92 cytotoxic activity.
NK-92 cells were cultured in medium without IL-2. ^{51}Cr release assay was performed against K562 target cell line every 24 h. Results represent mean values of two independent experiments ± SD.

3.2.2. Establishment of NK resistant human leukemic cell line SR-91

A. Establishment of SR-91 cell line

SR-91, a leukemic cell line, was derived from the peripheral blood of a 22-years old male patient with a diagnosis of undifferentiated acute leukemia with a peripheral blast cell count of $25x10^{10}$/L. His bone marrow at diagnosis showed >95% infiltration with blast cells which were uniform in size and 10-20 μ in diameter. To establish the cell line, the thawed peripheral blood obtained at diagnosis were cultured in RPMI 1640/10% FCS and 10% 5637 conditioned medium which was produced by collecting the supernatant from a cell line that had originated from a patient with bladder carcinoma. The cells were maintained at 37°C in a humidified incubator with 5% CO2, with medium changed twice weekly. After four weeks, cells became independent of the presence of 5637 medium and were maintained in RPMI 1640/10% FCS.

B. Characterization of SR-91

Flow cytometric analysis showed that SR-91 cells are positive for CD33, CD34, CD45, CD56, and negative for T cell and B cell markers. SR-91 cells were found to express low levels of ICAM-1 and high levels of MHC class I (Fig 7). The MHC class I phenotyping of SR-91 cells showed that they were positive for HLA-A2, B44, B57, C5. To study the effect of various cytokines on the growth behavior of SR-91 cells, ^{3}H-thymidine uptake assay was performed. An increase in thymidine incorporation was observed in a dose-dependent manner when cells were cultured in the presence of GM-CSF. IL-3 and IL-6 only slightly promoted cell growth. No increase or inhibition of proliferation was seen with IL-1, IL-2,

IL-4, erythropoietin, TNF-α, IFN-α or IFN-γ (Fig 8). The sensitivity of SR-91 cells to NK mediated cytotoxicity was assessed in a standard four h ^{51}Cr release assay. They were resistant to IL-2 activated NK cell killing by both peripheral blood mononuclear cell (PBMC)-derived NK cells and NK-92 cell line (Fig 9)

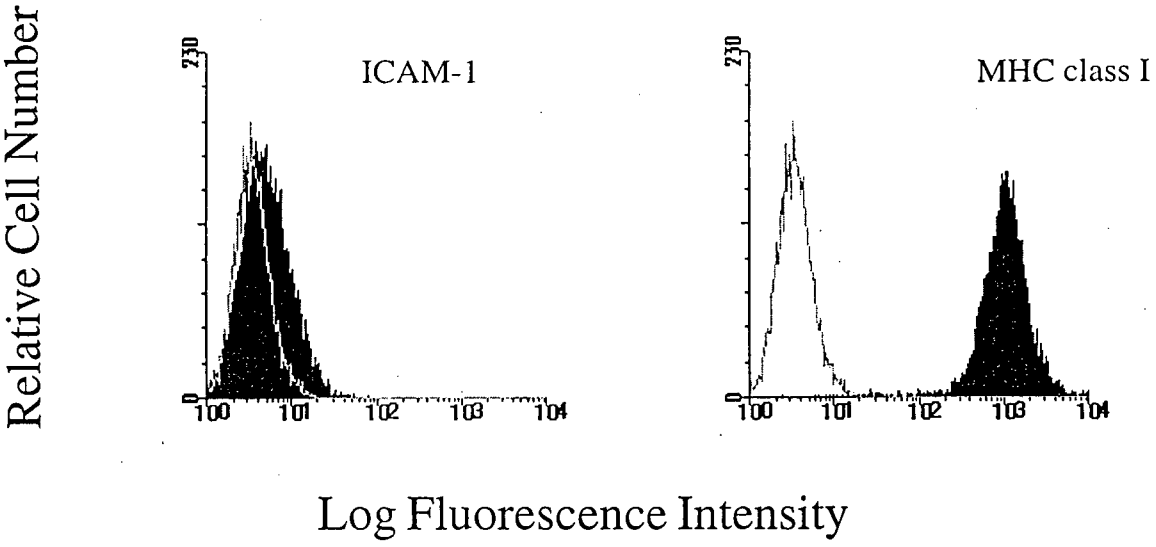


Figure 7. Expression of ICAM-1 and MHC class I on SR-91 cells.
SR-91 cells were stained with saturating concentrations of mAbs against ICAM-1, and MHC class I (W6/32 mAb) (filled). Background fluorescence of cells stained with the secondary antibody alone is also shown (open).

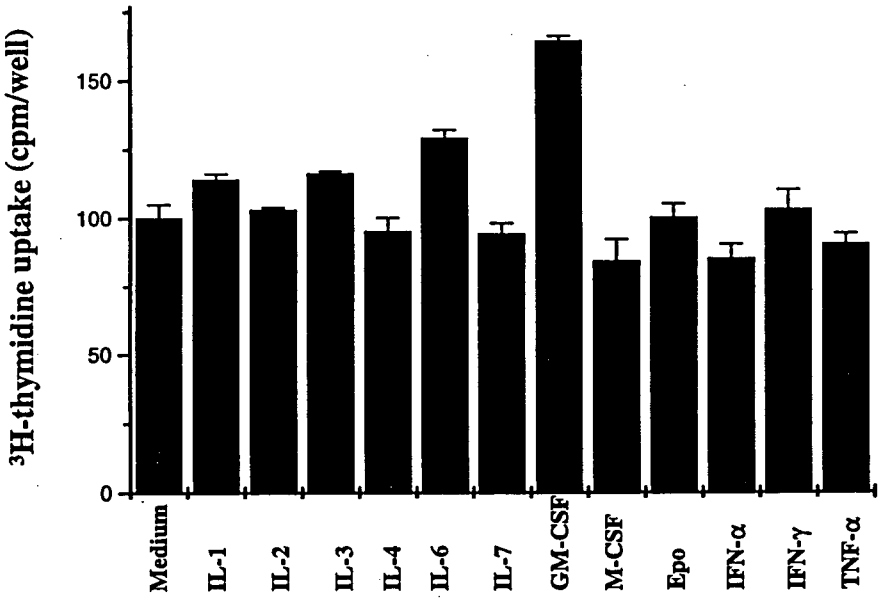


Figure 8. Effect of various cytokines on growth of SR-91 cells.
SR-91 cells were cultured at an initial concentration of $2x10^4$ in 200 µl per well in the presence of recombinant cytokines as indicated (IL-1α 1000U/ml, IL-2 1000U/ml, IL-3 100ng/ml, IL-4 1000U/ml, IL-6 1000U/ml, IL-7 1000U/ml, M-CSF 360 ng/ml, GM-CSF 100ng/ml, IFN-γ 1000U/ml, TNF-α 1000U/ml, erythropoietin 10U/ml). After 48 h of incubation, ^{3}H-thymidine uptake (over six hours) was measured. Results represent mean ± SD of triplicates.

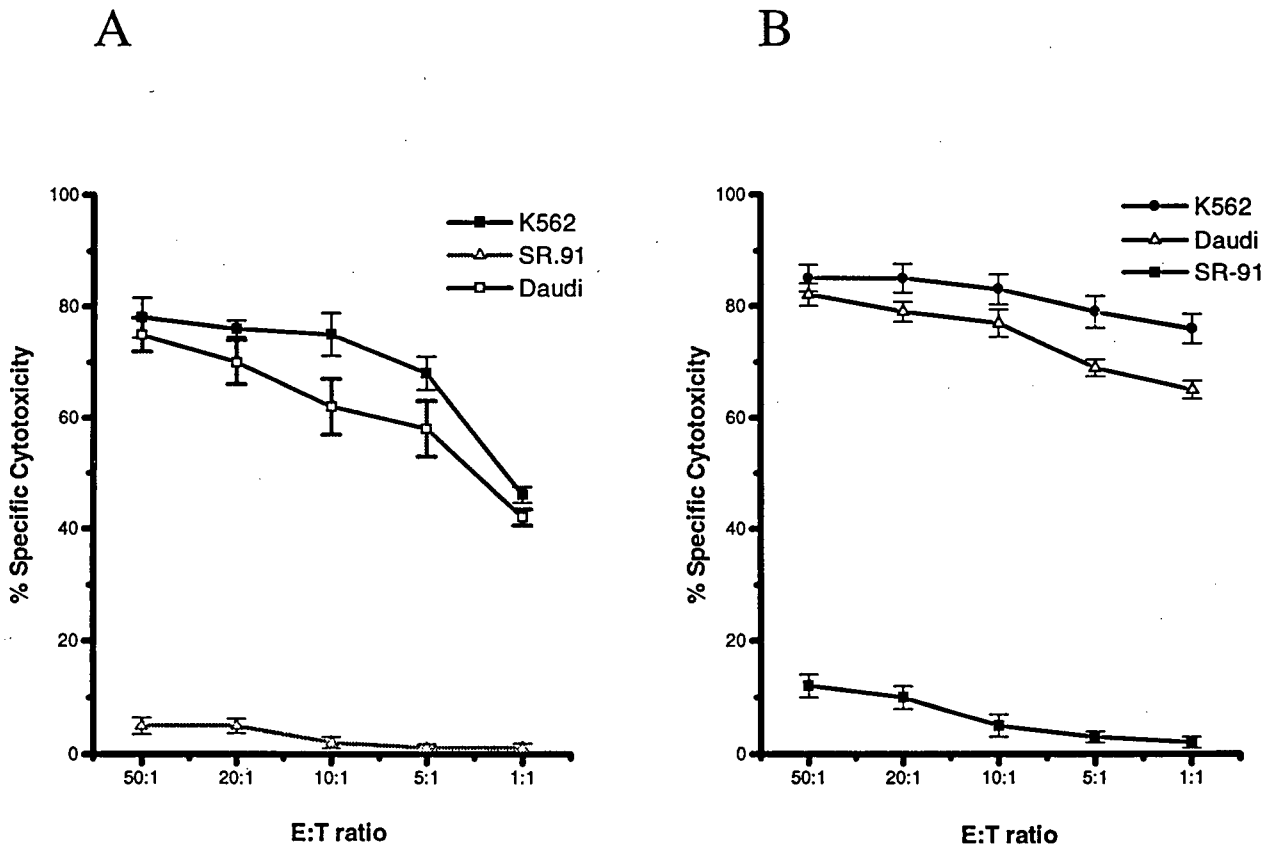


Figure 9. Sensitivity of SR-91 cells to NK mediated cytotoxicity.
Cytotoxic activity of (A) peripheral blood NK cells and (B) NK-92 cells against K562, Daudi and SR-91 cells measured in a four h ^{51}Cr release assay using various E:T ratios. Results are expressed as mean values of three independent experiments ± SD.

3.3. Discussion

In this chapter, establishment, cell surface markers, growth and functional characteristics of the human NK cell line NK-92 and the NK-resistant leukemia line SR-91 are described.

NK-92 is an IL-2 dependent human NK cell line with characteristics of IL-2 activated NK cells and able to lyse very efficiently a broad spectrum of tumor target cells, including a number of leukemia, lymphoma and myeloma cell lines. Unlike NK clones, NK-92 has a broad target specificity and is not inhibited by specific MHC class I on target cells. Extensive studies undertaken by other investigators on the relevance of MHC class I molecules on target cells which could react with specific NK receptors on NK-92 cells have not shown protection from NK-92 cell mediated lysis (E Long personal communication), but it has recently been reported that the infection of NK-92 cells, with recombinant vaccina virus encoding the two p58 receptors specific for HLA-Cw3 and HLA-Cw4 conferred to NK-92 cells the ability to receive an inhibitory signal and reduced significantly their lytic activity against Cw3 and Cw4 expressing target cells [275].

NK-92 cell line is unique among other available human NK cell lines because it has exceptionally high cytotoxic activity against a broad spectrum of target cells. Indeed NK-92 has proven to be a useful cell line to study the mechanism by which NK cell cytotoxicity is regulated [354-357]. The lack of expression of CD16 represents an advantage of this cell

line, allowing it to be used to selectively analyze direct cell-mediated killing mechanisms without the involvement of ADCC.

SR-91 is a leukemia cell line resistant to killing by both PBMC derived NK cells and NK-92 cells. SR-91 expresses low levels of ICAM-1. Although the cytotoxic activity of NK-92 is considerably higher than that of IL-2 activated peripheral blood NK cells, the leukemic cell line SR-91 cells was still resistant to its killing. The hallmark of SR-91 is that it expresses low levels of ICAM-1 and high levels of MHC class I, and thus provides a model with which to investigate the role of two potentially important factors in determining NK resistance : 1) lack of adequate expression of an important cell adhesion molecules, ICAM-1; 2) high expression of a "protective" molecule, MHC class I.

Chapter 4. ROLE OF ICAMs IN LEUKEMIC TARGET CELL INTERACTION WITH EFFECTOR NK-92 CELLS

4.1. Rationale

Target cell killing by NK cells involves three steps: 1) binding of NK cells to the target cell and conjugate formation, 2) activation of the effector cell leading to the delivery of lethal hit, and 3) target cell lysis. A number of cell adhesion molecules (CAMs) have been shown to be critical in this process, including members of integrin family (LFA-1, VLA-4, VLA-5) and members of the immunoglobulin superfamily (ICAM-1, CD2, LFA-3)[148]. Recent studies have shown that LFA-1/ICAM-1 and CD2/LFA-3 interactions are two predominant pathways of effector-target interaction for both CTL and NK cells [149-151]. Since NK/LAK cells express a variety of adhesion receptors, most studies have come to the conclusion that the presence and distribution of adhesion molecules on the target cell may be very important in determining its sensitivity to effector cytotoxic cells, and that downregulation of adhesion molecules on neoplastic cells may be a potent escape mechanism of the tumor cell from the host immune system.

Considering the importance of LFA-1/ICAM-1 pathway in tumor target interaction with effector killer cell, potential contribution of a defective expression of ICAM-1 on target cell to its resistance to NK-mediated lysis was investigated in this chapter. For these studies, the model system using the NK resistant leukemic cell line, SR-91 and the human NK cell line

NK-92 described in the previous chapter was used. SR-91 cells were found to express low levels of ICAM-1 and were shown to be resistant to highly enriched CD56+/CD3- NK cells obtained from peripheral blood as well as to NK-92. The hypothesis to be tested was that resistance of SR-91 to NK mediated lysis is due to lack of conjugate formation, the consequences of inadequate expression level of ICAM-1. To test this hypothesis, two approaches were taken to upregulate ICAM-1 expression. First, the ICAM-1 cDNA was transfected into SR-91. Second, inflammatory cytokines known to induce ICAM-1 upregulation were used to treat SR-91, and their effects on conjugate formation and sensitivity to NK-mediated killing were investigated.

4.2. Results

4.2.1. ICAM-1 transfection

To determine whether the low levels of ICAM-1 expression on SR-91 is responsible for its resistance to NK cells, ICAM-1 cDNA was transfected into SR-91 using the two EBV derived mammalian expression vectors pREP9 and pCEP4. ICAM-1 expression of pREP9:ICAM-1 transfectants, followed by repeated rounds of fluorescent cell sorting of transfected cells after selection by G418 resistance allowed us to isolate populations with about a fivefold increase in expression of ICAM-1 over the background expression of non transfected SR-91 cells (Fig 10A). pCEP4:ICAM-1 transfectants (selected by hygromycin resistance) showed higher levels of ICAM-1 expression (five to sixflod) (Fig 10B), and were tested in a cytotoxicity assay (Table 6). However, the sensitivity of these higher ICAM-1 expressing transfectants to NK-92 mediated lysis was not different from that of the cells transfected with the vector alone (Table 6).

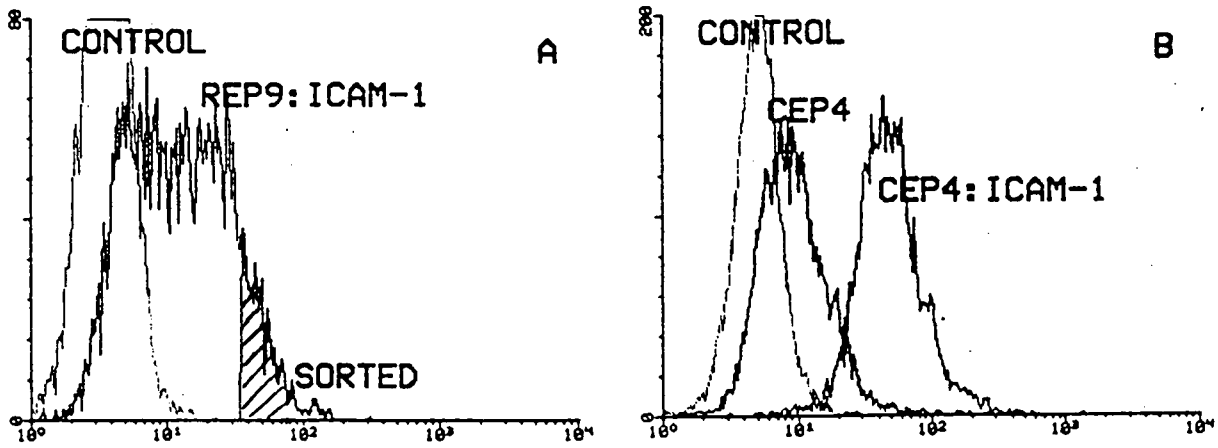


Figure 10. ICAM-1 expression on SR-91 transfectants.
ICAM-1 expression of transfectants obtained by electroporation of SR-91 with human ICAM-1 cDNA subcloned into two EBV derived expression vectors pREP9 and pCEP4, was tested: **(A)** with pREP-9:ICAM-1 after selection in G418, the hatched area shows higher expressing transfectant population isolated by fluorescent activated cell sorting, **(B)** with pCEP4: ICAM-1, after selection in hygromycin B. The expression level of non transfected SR-91 cells is also shown as control.

Table 6. Sensitivity of SR-91 transfectants to NK-92-mediated cytotoxicity.

	Percentage of cell lysis [a]			
	Effector:target ratio			
	10:1	5:1	1:1	0.5:1
SR-91	8±3	7±2	6±3	3±3
SR-91. Cep4	18±5	15±4	10±4	8±5
SR-91. Cep4:ICAM-1	19±2	15±4	11±2	9±2
K562	83±2	80±2	72±1	63±4
Daudi	80±2	70±2	65±2	51±3

[a] SR-91 transfectants (pCep4: ICAM-1) selected by hygromycin resistance and showing higher levels of ICAM-1 expression were tested in a cytotoxicity assay. Hygromycin resistant selected SR-91 transfected with vector alone (pCep4) as well as the sensitive cell lines K562 and Daudi were also included in the assay. Results represent mean ± SD of triplicates.

4.2.2. Upregulation of surface expression of ICAM-1 by TNF-α and IFN-γ

In an attempt to achieve higher levels of ICAM-1 expression, pro-inflammatory cytokines TNF-α and IFN-γ, known to up-regulate ICAM-1 expression on a number of cells, were used to investigate whether ICAM-1 up-regulation on NK resistant SR-91 allows conjugate formation and lysis by NK cells. Both cytokines were tested at various concentrations (100-2000U/ml) for induction of ICAM-1 expression. The dose of 500 U/ml for both was found to be optimum and used throughout the experiments. SR-91 cells were treated with 500U/ml of either TNF-α or IFN-γ for 16 h at 37^{0}C and tested for the expression of ICAMs by flow cytometry. Both TNF-α and IFN-γ treatment of SR-91 substantially up-regulated ICAM-1 expression by about 15-fold (Fig 11). In contrast to ICAM-1, the two other members of the ICAM family, ICAM-2 and ICAM-3 have not been shown to be induced by these cytokines. The expression levels of ICAM-2 and ICAM-3 were not significantly affected by TNF-α or IFN-γ treatment (Fig 11).

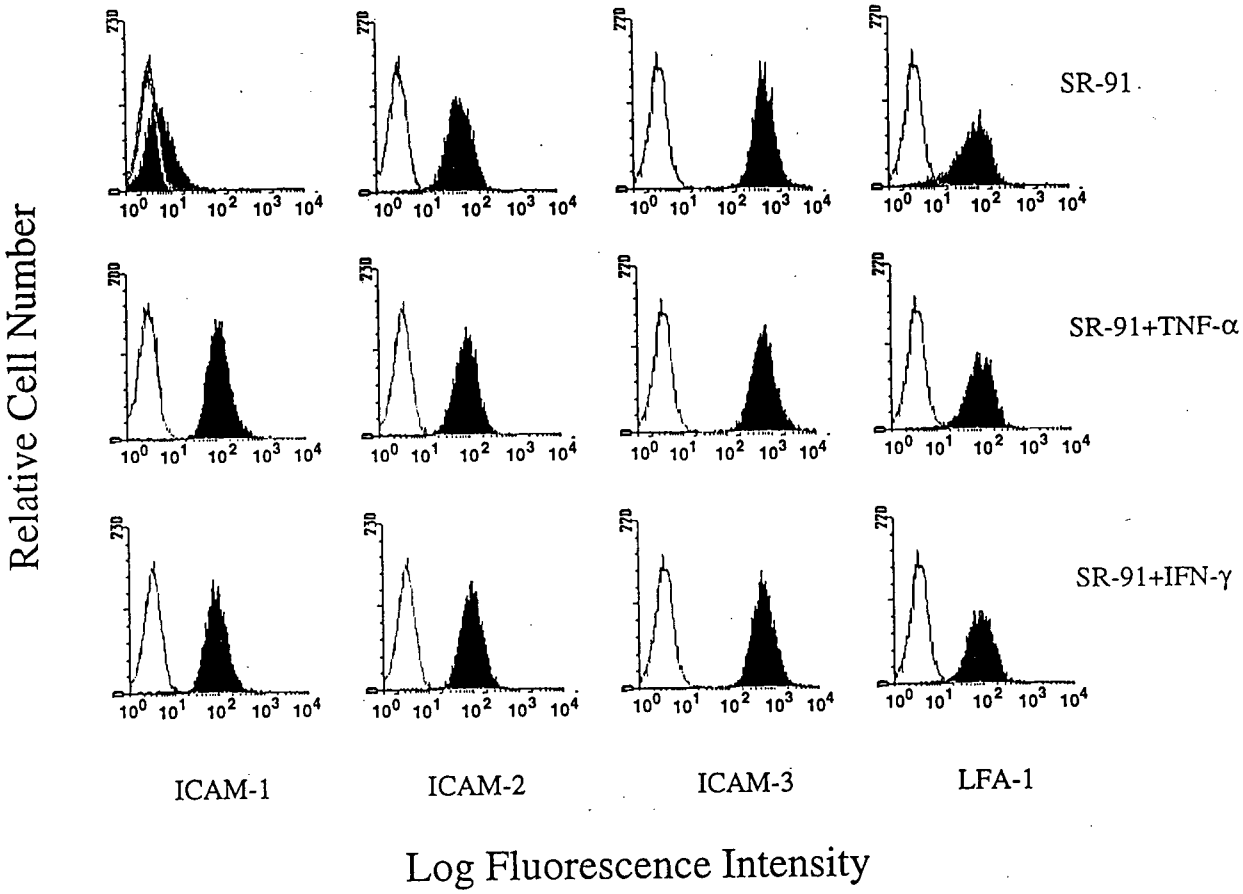


Figure 11. Flow cytometric analysis of ICAM-1, ICAM-2, ICAM-3, and LFA-1 on SR-91 cells and the effect of cytokine treatment.

SR-91 cells (untreated and after 16 h treatment with TNF-α or IFN-γ at 500U/ml) were stained with saturating concentrations of mAbs against ICAM-1, ICAM-2, ICAM-3, and LFA-1 (filled). Background fluorescence of cells stained with the secondary antibody alone is also shown (open).

4.2.3. Effects of TNF-α and IFN-γ treatment of SR-91 on sensitivity to NK cytotoxicity

A. Cytotoxicity and Cold target competition assay

SR-91 cells were treated with TNF-α or IFN-γ overnight, and the effects of the treatment on sensitivity of SR-91 to NK-92 cytotoxicity was examined. Interestingly, although the level of ICAM-1 on IFN-γ treated SR-91 cells was comparable to that on the TNF-α treated cells, only TNF-α treated cells became sensitive to NK-92 mediated lysis (Fig 12). Thus it can be concluded from this that upregulation of ICAM-1 per se is insufficient to enhance susceptibility to NK-mediated killing. To determine if upregulation of ICAM-1 is required to enhance conjugate formation and to compare the ability of NK binding induced by the two cytokines, a cold target competition assay between TNF-α and IFN-γ treated SR-91 was performed. Varying numbers of unlabeled and radiolabeled TNF-α and IFN-γ treated SR-91 cells were mixed and used as target while keeping the total target cell number constant. The results of this assay showed that IFN-γ treated SR-91 cells were able to compete with TNF-α treated cells as targets of NK-92 cells. Therefore, IFN-γ and TNF-α seem to induce the same level of binding of SR-91 to NK-92 cells (Table 7). These results suggest that increased ICAM-1 expression on the target cell does induce a higher level of binding to NK cells, but that this only allowed increased killing by NK cells of the TNF-α treated SR-91 cells. Untreated SR-91 cells showed low binding to NK cells in conjugate formation assay, consistent with the hypothesis that increased ICAM-1 expression does lead to increased conjugate formation. However, this alone seems to be insufficient to cause lysis and that multiple factors induced by TNF-α, but not IFN-γ, were required.

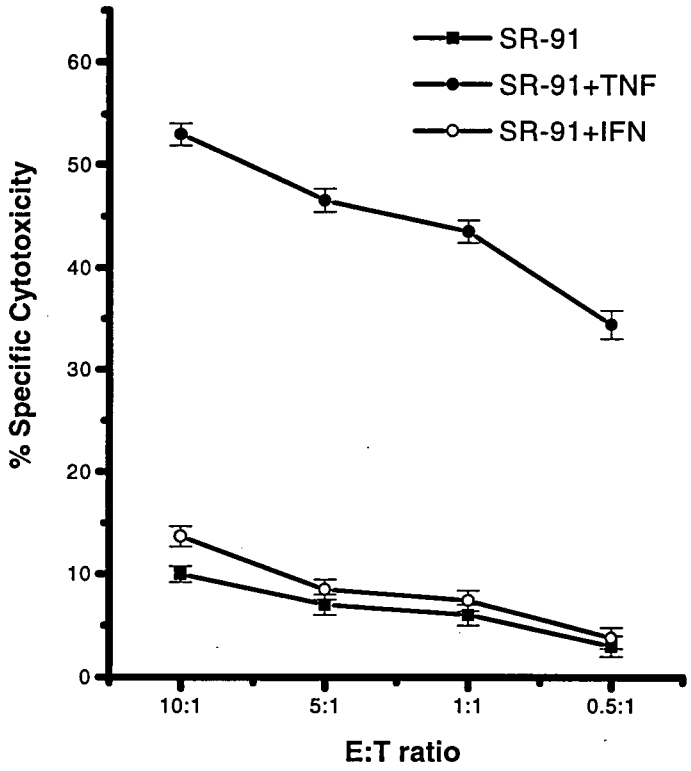


Figure 12. Effect of treatment with TNF-α and IFN-γ on sensitivity of SR-91 cells to NK-92 mediated lysis.

Target cells were incubated with TNF-α or IFN-γ at 500 U/ml for 16 h, and then tested in a four h ^{51}Cr release assay as described in Materials and Methods. Results are expressed as mean values of three independent experiments ± SD.

Table 7. Cold target competition assay

$2x10^4$ target cells per well[a]	Number of lysed cells* $x10^3$ Experiment 1 :	Experiment 2 :
100% TNF*	12.0	11.0
75% TNF* + 25% TNF	8.2	8.1
25% TNF* + 75% TNF	2.8	2.8
75% TNF* + 25% IFN	8.2	7.9
25% TNF* + 75% IFN	3.3	2.4
100% IFN*	0	0

[a] Varying numbers of non labeled (cold) and radiolabeled TNF-α and IFN-γ treated SR-91 cells were mixed with effector cells, keeping constant the total target cell number ($2x10^4$ cells per well). A cytotoxicity assay was performed as described in Materials and Methods. Results of two different experiments are presented. * indicates ^{51}Cr labeled target cells.

B. Potential inhibitory effect of IFN-γ

Both TNF-α and IFN-γ induced ICAM-1 expression (Fig 11) to the same extent and seem to induce similar levels of binding to NK-92 cells (Table 7), but only TNF-α treated cells were NK sensitive. To test the possibility that IFN-γ treatment might have induced some inhibitory mechanisms that over-ride the effect of ICAM-1 up-regulation and inhibits the induction of NK sensitivity, SR-91 cells were treated with both cytokines simultaneously, and chromium release assay performed. SR-91 cells treated with both cytokines were still NK sensitive, ruling out the possibility that IFN-γ has an inhibitory effect on cytotoxicity (Table 8).

Table 8. Effect of simultaneous treatment of SR-91 cells with TNF-α and IFN-γ on sensitivity to NK-92 cell-mediated lysis

	Percentage of cell lysis [a]			
	Effector:target ratio			
Treatment of SR-91 cells	20:1	10:1	5:1	1:1
No treatment	10±3	9±2	7±3	4±3
TNF-α	57±5	55±4	48±4	38±5
IFN-γ	16±2	12±4	9±2	4±2
TNF-α+IFN-γ	59±2	53±6	49±5	37±3

[a] SR-91 cells were incubated with either or both cytokines, and tested in a cytotoxicity assay as described in Materials and Methods. Results are expressed as mean values of three independent experiments ± SD.

C. Effect of antibodies on NK-92 mediated cytotoxicity

To investigate whether or not ICAM-1 up-regulation was required for the lysis of TNF-α treated SR-91, the effect of anti LFA-1 and anti ICAM-1 mAbs on this process was studied. NK-92 mediated cytotoxicity against TNF-α treated SR-91 cells was partially blocked with anti-ICAM-1 antibody, while LFA-1 completely abrogated the cytotoxicity. A combination of antibodies against ICAM-1, -2, and -3 also completely inhibited it, whereas combination of ICAM-1 and ICAM-2 or ICAM-1 and ICAM-3 only partially inhibited the cytotoxicity (Fig 13). Anti ICAM-2 or ICAM-3 alone or in combination had almost no inhibitory effect. These results indicate that LFA-1 is crucial for cytotoxicity of TNF-α treated SR-91 cells by NK-92. In this system LFA-1 primarily interacts with ICAM-1 but ICAM-2 and ICAM-3 are also involved. Thus the upregulation of ICAM-1 appears to enhance the efficiency of killing, but as suggested in the previous assay it is not sufficient to account for the induced cytotoxicity observed by TNF-α.

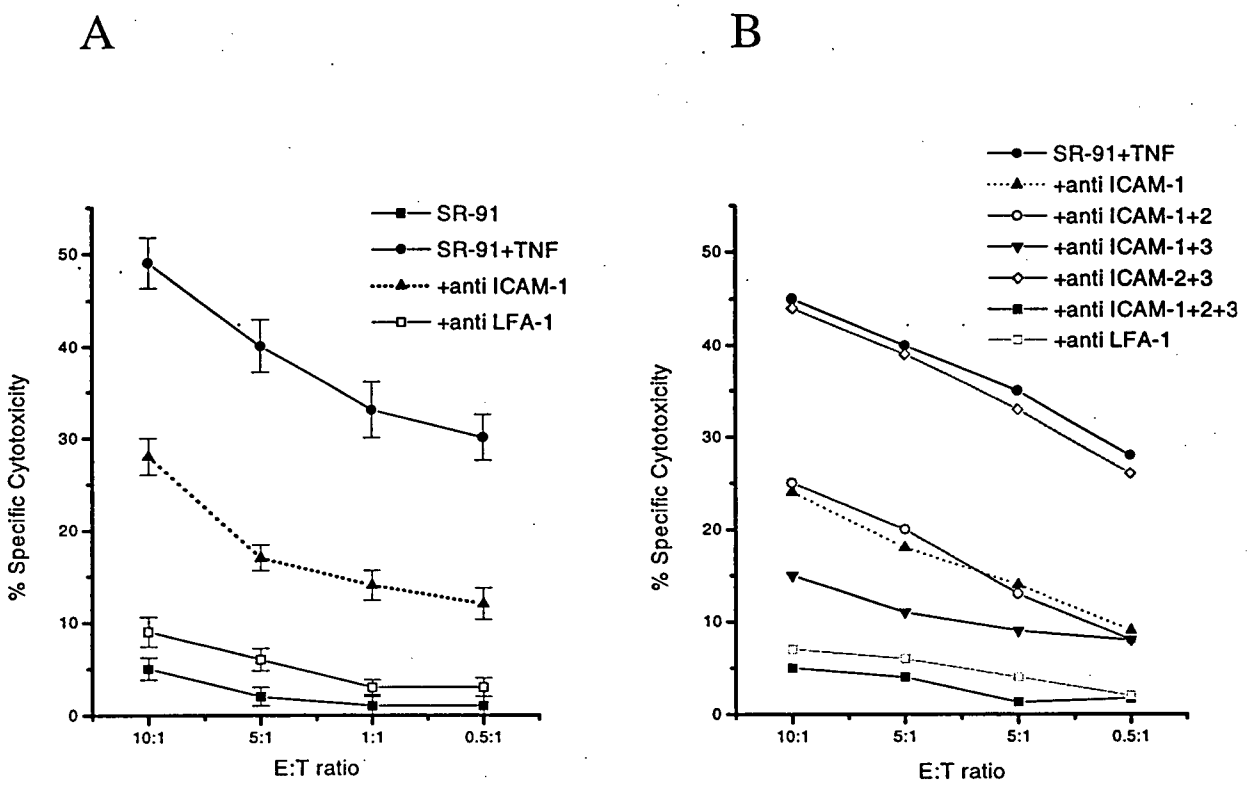


Figure 13. Effect of different antibodies on the cytotoxicity of NK-92 against TNF-α treated SR-91.

Target cells were incubated with TNF-α at 500U/ml for 16 h, and a ^{51}Cr release assay performed after antibodies against different adhesion molecules were added to the cells. Anti LFA-1 mAb was used at 1:20 dilution of hybridoma supernatant, affinity purified mAbs anti ICAM-1, anti ICAM-2 and anti ICAM-3 were used at 5 to 10 μg/ml. Panel A shows the mean of five independent experiments ± SD, one representative experiment (out of three independent experiments) is shown in panel B. Anti CD44 hybridoma supernatant (clone 2G1, IgG1), and the affinity purified mAb OKT9 (CD71, IgG1) and W6/32 (against MHC class I invariant portion, IgG2a) were used as control antibodies, and had no effect on killing of TNF-α treated SR-91 cells.

4.2.4. Effect of cytokine treatment on conjugate formation

A. Dual color flow cytometric analysis

To further establish upregulation of ICAM-1 on SR-91 by pretreatment with TNF-α and IFN-γ enhanced the binding of SR-91 to NK-92 cells, effector and target cells were labeled with two distinct fluorescent dyes and a conjugate formation assay performed. Effector-target cell conjugates were visualized by flow cytometry as double color events. Only 8-16% of untreated SR-91 cells formed conjugates with NK-92 cells. Treatment of SR-91 cells with TNF-α enhanced the frequency of conjugates to 32-44% (Fig 14). IFN-γ pretreatment also enhanced the number of conjugates to the same extent (26-40%). These results indicate that TNF-α and IFN-γ are equally effective at enhancing conjugate formation between SR-91 and NK-92 and is consistent with the increased ICAM-1 expression by both cytokines. Since TNF-α, but not IFN-γ, treatment enhanced the susceptibility of SR-91 to NK-92 cytotoxicity, TNF-α is likely to have additional effects on SR-91 cells.

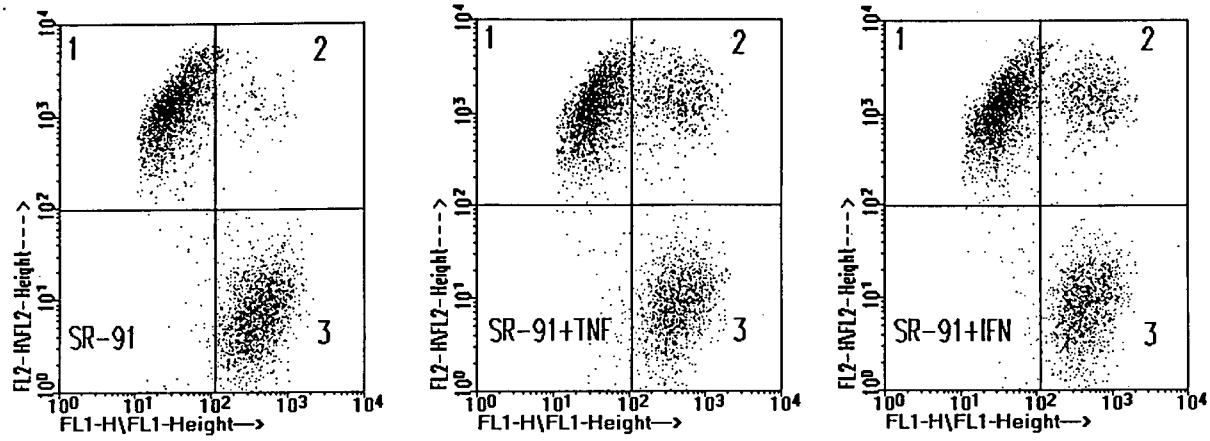


Figure 14. Quantification of effector-target conjugate formation by dual color flow cytometry.

After incubating SR-91 cells with TNF-α or IFN-γ at 500U/ml for 16 h, effector and target cells were labeled with two different fluorescent dyes (red and green fluorescence respectively), and a conjugate formation assay was performed as described in Materials and Methods. Effector-target conjugates were visualized by flow cytometry as double color events, quadrant 1: effector cells, quadrant 2: effector-target conjugates, quadrant 3: target cells. The number of conjugates was obtained by dividing the number of dual labeled particles (quadrant 2) by the number of target cells (quadrant 2+3) multiplied by 100. Results of one representative experiment (out of five independent experiments) are shown.

B. Effect of antibodies on conjugate formation

To examine the contribution of CAMs in conjugate formation in this system, and to investigate whether ICAM-1 up-regulation was responsible for this increased binding to NK cells of TNF-α treated SR-91, the effect of anti LFA-1 and anti ICAM-1 mAbs on this process was studied. Conjugate formation between cytokine treated SR-91 and NK-92 cells was reduced to baseline when anti-LFA-1 antibody or a combination of antibodies against ICAM-1, ICAM-2 and ICAM-3 were used (9% and 7% respectively). Antibody against ICAM-1 alone had not a significant inhibitory effect on conjugate formation (30% versus 33% in the absence of antibodies) and anti ICAM-2 or anti ICAM-3 when used alone, had no effect (Fig 15). This data is consistent with the data using cytotoxicity as a readout (Fig 13) and underlines that ICAM-1 expression on SR-91 cells is crucial for conjugate formation and lysis by NK cells.

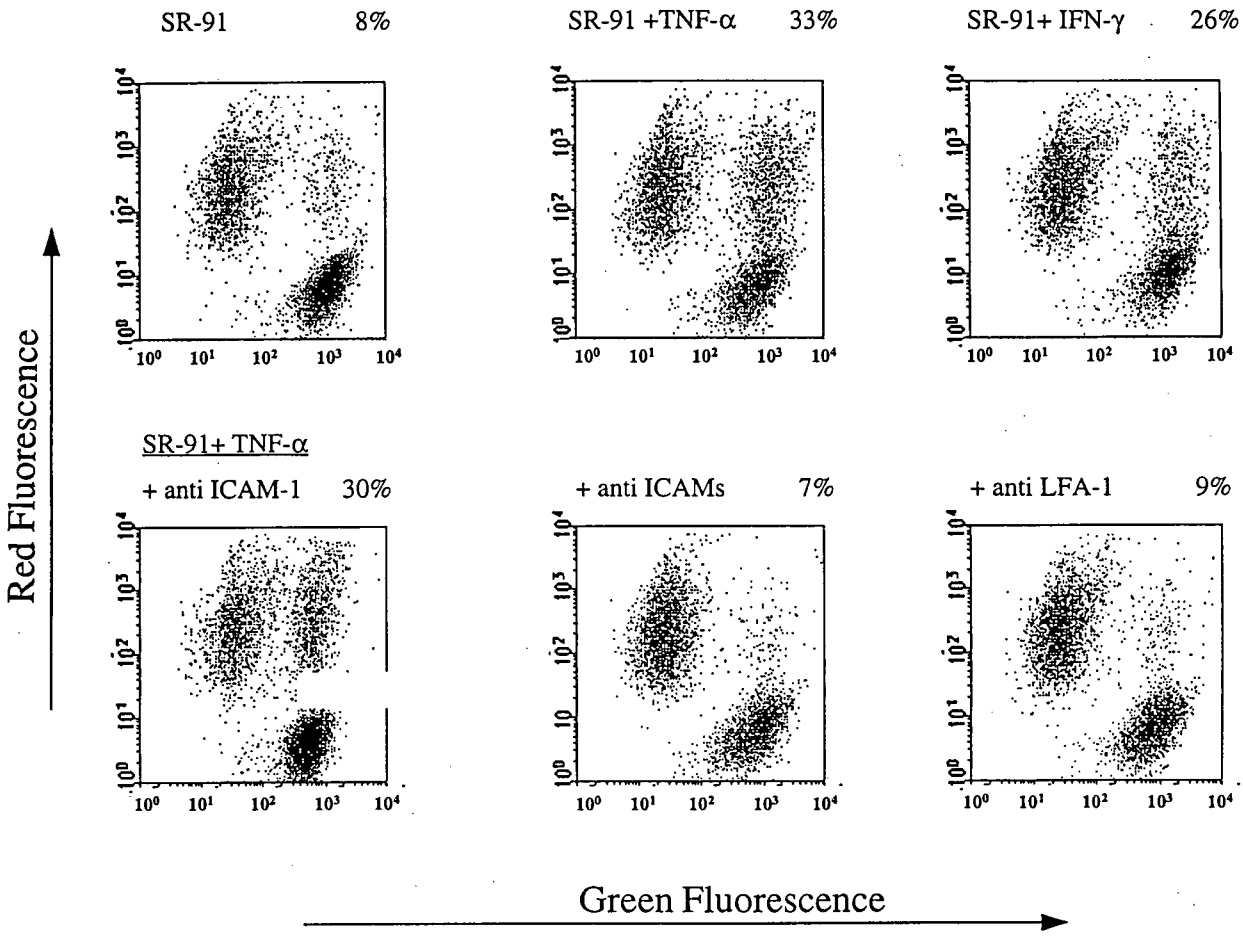


Figure 15. Inhibitory effect of antibodies on conjugate formation.
SR-91 cells were incubated with TNF-α or IFN-γ at 500U/ml for 16 h and conjugate formation determined (as described in the legend to Figure 14) in the presence or absence of antibodies to LFA-1 and the different ICAMs (as described in the legend to Figure 13). The percentage of effector-target conjugates is stated in the heading of each plot. Control antibodies were used as mentioned in the legend to figure 13.

4.3. Discussion

ICAM-1 transfection into SR-91 was not conclusive in that although it allowed about fivefold increase in ICAM-1 expression, these transfectants when tested in cytotoxicity assay showed similar sensitivity as the cells transfected with vector alone. The second approach however, SR-91 treatment with TNF-α, was more effective and allowed higher expression of ICAM-1 (about 15-fold), and increased binding and sensitivity to NK-92 cells.

The results in this chapter demonstrated that: 1) TNF-α and IFN-γ treatment of SR-91 induced the same level of ICAM-1 expression and binding to NK cells, but only TNF-α treated cells became sensitive to NK mediated killing. 2) The increased binding and susceptibility to NK-92 cells was completely abrogated by anti LFA-1 antibody or a combination of antibodies against all three ICAMs, while anti ICAM-1 alone had only a partial inhibitory effect and antibodies against ICAM-2 or ICAM-3 had almost no effect. These results indicate that ICAM-1 expression on SR-91 cells is required for binding to NK cells but, on its own, is not sufficient to induce sensitivity to NK cell mediated killing.

MHC class I expression of SR-91 cells is high and does not change upon treatment with either cytokines. Therefor, it is unlikely that MHC class I is responsible for the resistance of SR-91 cells to NK-92 mediated killing. Moreover, NK-92 cells do not express any known member of the KIR family whether p58 or p70, but express high levels of CD94 (E Long personal communication).

The conjugate formation assay showed inefficient binding between SR-91 and effector NK cells. Considering the crucial role of ICAM-1/LFA-1 in target cell interaction with effector killer cells, and the fact that NK-92 cells express a wide spectrum of CAMs, it was hypothesized that NK resistance of SR-91 cells is due to the lack of adequate binding to NK cells, which in turn is a consequence of low expression of ICAM-1 on SR-91.

The results in this chapter confirm the importance of LFA-1/ICAMs interaction for initial effector-target cell binding and subsequent target lysis by NK cells. They also suggest that ICAM-2 and ICAM-3 participate in conjugate formation, but require the presence of ICAM-1 at a certain level to allow adequate binding. This further underlines that ICAM-1 expression on target cell is essential to allow binding to the effector cell. However, ICAM-1 on its own is not sufficient to induce sensitivity to NK killing. In a number of systems, antibody inhibition studies have previously shown the importance of ICAM-1 at the target level and of LFA-1 at effector level, since preincubation of effector cells with anti LFA-1 and target cells with anti ICAM-1, but not anti LFA-1, have been shown to significantly inhibit NK-mediated conjugate formation and cytolysis of susceptible target cell lines [151,161]. It should be noted that the degree of anti LFA-1 inhibition observed in NK assays varies significantly and is apparently dependent on the given target cell analyzed [162]. In this study, antibodies had to be present in the reaction mixture during the period of the assay to be effectively inhibitory. Preincubation of either effector and/or target cells with antibodies against LFA-1, ICAM-1, ICAM-2 or ICAM-3 alone or in combination followed by removal of unbound antibody did not result in any significant inhibitory effect. This might be due to low affinity of the antibodies used. Therefore, these studies did not allow the role of LFA-1 and ICAM-1(or ICAMs) on effector versus target cells to be delineated.

The fact that both cytokines used in these studies induced the same level of ICAM-1 expression and binding to effector cells, while only TNF-α treated target cells became susceptible to NK-92 mediated lysis argues against an upregulation of ICAM-1 on SR-91 as the sole factor responsible for this observed NK sensitivity. This is further supported by the results of antibody inhibition studies. The importance of LFA-1/ICAMs interaction in conjugate formation and susceptibility to NK lysis was demonstrated by profound inhibitory effects of antibodies to LFA-1 and ICAMs. Among CAMs examined in this study, only ICAM-1 was upregulated by the two cytokines. These results suggest that ICAM-1 upregulation is sufficient for binding of SR-91 to NK-92. However, the differential effects of TNF-α and IFN-γ on the sensitivity of SR-91 to NK cytotoxicity suggest that other molecules or pathways may be involved in subsequent post binding events necessary for the induction of costimulatory signals to the effector cell and granule exocytosis.

Chapter 5. INVESTIGATION INTO THE ROLE OF OTHER CAMs IN THE LYSIS OF TNF TREATED SR-91 BY NK-92 CELLS

5.1. Rationale

Based on the well established role of ICAM-1/LFA-1 pathway in effector-target interaction, the original hypothesis of this project was that the lack of adequate expression of ICAM-1 on target cell is responsible for its resistance to NK cell mediated killing. However, results from the previous chapter showed that induction of ICAM-1 expression by IFN-γ results in increased binding of SR-91 to NK-92 cells, but not enhanced sensitivity to NK-92 cell mediated killing. In contrast, TNF-α treatment induces not only increased binding but also enhanced sensitivity of SR-91 to NK-92. Therefore, it was postulated that TNF-α, but not IFN-γ, may activate molecules on SR-91 cells that, upon interaction with their counter receptors on NK-92 cells, trigger the release of cytotoxic granules and target cell lysis. TNF-α is a cytokine with pleiotropic action. It is known to induce apoptosis and DNA fragmentation [289]. It has also been identified as one of the few naturally occurring endogenous NF-*k*B inducers. Expression of a number of cellular genes, including c-fos, c-myc, HLA class I and II, and cytokine genes are induced by TNF-α [290-292]. Therefore, TNF-α may have many effects at various levels of effector-target interaction.

A number of cell adhesion molecules, including ICAM-1, ELAM-1, and VCAM-1, have also been shown to be induced by TNF-α. In addition to inducing expression of these CAMs, TNF-α may also functionally activate CAMs. It was hypothesized that TNF-α, but not IFN-γ, may activate molecules on SR-91 cells that upon interaction with their counter-receptors on NK-92 cells trigger the release of cytotoxic granules and target cell lysis.

Among several CAMs known to be expressed on NK cells, ICAM-3 and CD44 are of particular interest. CD44 has recently been reported to trigger cytotoxic functions of CTL [208-210] and NK cells [196-198]. Anti CD44 antibodies or the CD44 ligand, hyaluronan, have been shown to be costimulatory for T cells and to trigger cytotoxic functions and tyrosine phosphorylation. Ligation of CD44 on NK cells by selected antibodies, have also been shown to induce NK cytotoxic functions and increased killing of sensitive target cells [198,200]. Bispecific antibodies linking CD44 on NK cells to target molecules on the target cell, have also been shown to induce target cell lysis [199]. Finally CD44 has been recently reported to associate with $p56^{lck}$ in T cells [211]. Moreover, homotypic and heterotypic binding between different isoforms of CD44 has recently been reported [207]. Thus it is possible that a ligand of TNF-α treated SR-91 cells may bind to CD44 on NK cells and trigger cytotoxicity. Another possible counter-receptor on NK cells is ICAM-3. ICAM-3 has been reported to transduce signals and to associate with the Src family members of tyrosine kinases *fyn* and *lck* in T cells [187]. However, the involvement of ICAM-3 in triggering activation signals in NK cells that lead to granule exocytosis has not been reported. Therefore, the hypothesis was that TNF-α induced counter-receptors on SR-91 cells that may interact with CD44 and ICAM-3 on NK-92 cells to trigger the cytotoxic function.

5.2. Results

5.2.1. TNF-α and apoptosis

The results in the previous chapter showed that TNF-α treated SR-91 cells become sensitive to NK mediated cytotoxicity. TNF-α is known to induce DNA damage either directly [289] or indirectly through the induction of other molecular pathways such as Fas-Fas L [135]. To determine whether this is due to induction of apoptosis of SR-91 by TNF-α treatment, DNA was isolated from SR-91 cells treated overnight with different doses of TNF-α and analyzed by a 1% agarose gel electrophoresis. HL-60 and U937 cells were used as positive control for TNF-α induced apoptosis [289]. TNF-α did not induce DNA fragmentation of SR-91 cells, whereas apoptosis of HL-60 and U937 was readily detected by this assay (Fig 16). Apoptosis of SR-91 was also examined by flow cytometric analysis of cell cycle as described in Materials and Methods. The results confirmed that TNF-α treatment does not induce apoptosis of SR-91 cells. As positive control, IL-3 starvation of the IL-3 dependent cell line DA3 induced a 20% increase of the proportion of apoptotic cells, while the TNF sensitive cell line, U937, showed about 8% increase of the apoptotic rate upon TNF-α treatment (Fig 17).

SR-91 and NK-92 cells both express Fas antigen, but the expression of Fas ligand (CD95) on NK-92 cells had not been documented. In contrast to granule exocytosis and perforin mediated killing, Fas pathway of apoptosis is Ca^{2+} independent [133]. To determine whether TNF-α treatment induces Fas-mediated apoptosis of SR-91, a cytotoxicity assay was

performed in the presence and absence of calcium (Fig 18). The results clearly demonstrated that the lysis of TNF-α treated SR-91 by NK-92 is totally dependent on Ca^{2+}. Therefore, Fas-Fas L pathway, which is Ca^{2+} independent, is not involved in this cytotoxicity.

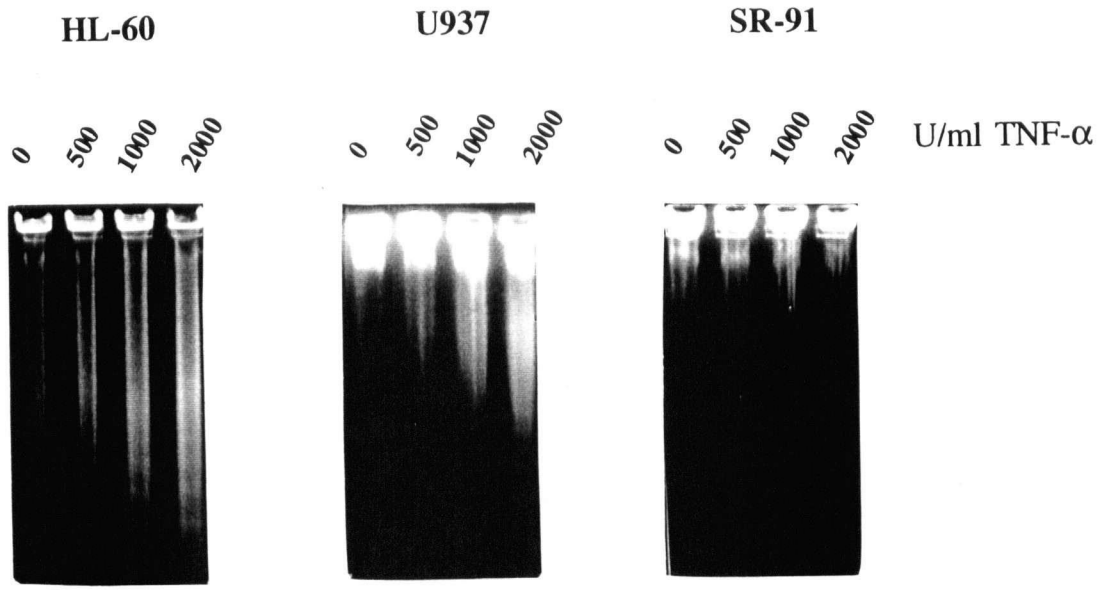


Figure 16. DNA fragmentation assay.
SR-91 and the TNF-α sensitive cell lines HL-60, and U937 cells were exposed to various doses of TNF-α (0, 500, 1000, and 2000 U/ml), for 16 h. DNA was extracted and resolved by agarose gel electrophoresis as described in Material and Methods. $1x10^6$ cells were analyzed in each experiment.

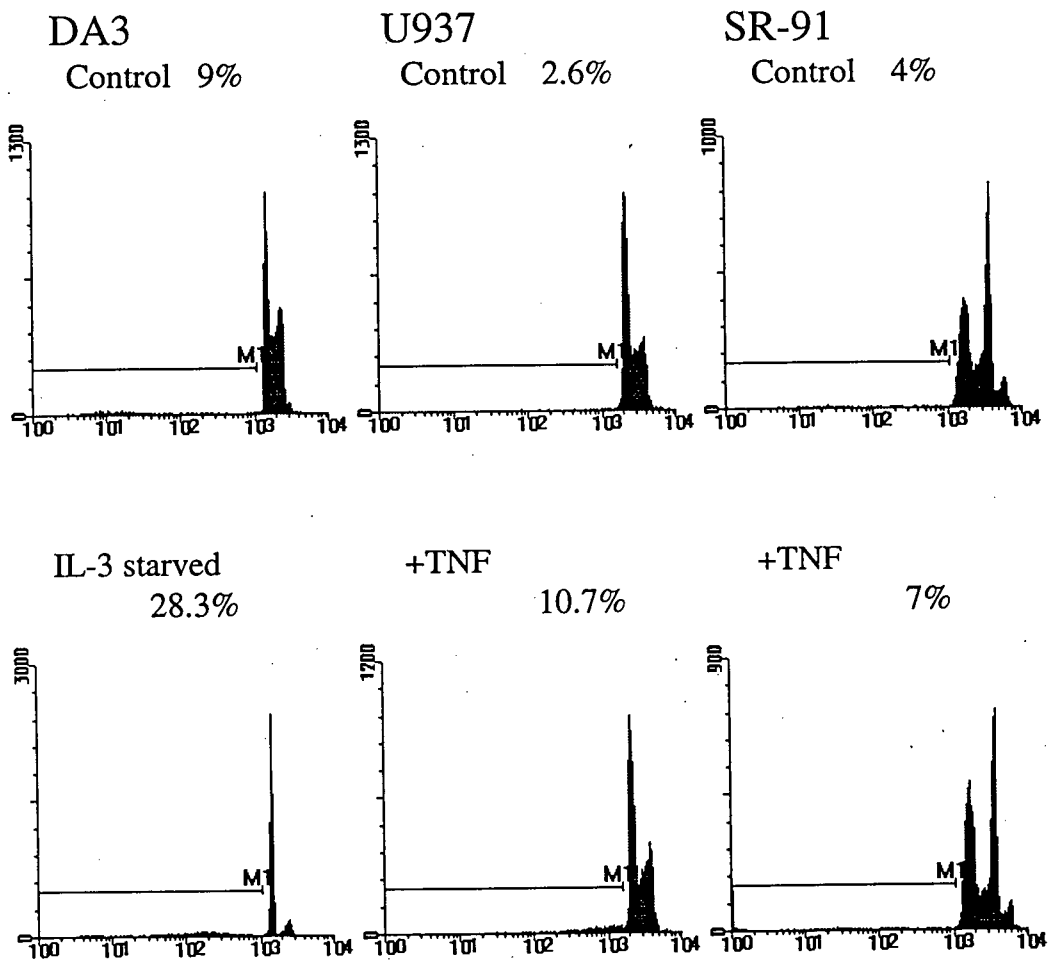


Figure 17. Detection of apoptosis by flow cytometric analysis of cell cycle.
SR-91 cells and the TNF sensitive cell line U937 were exposed overnight to TNF-α. $1x10^6$ cells were lysed and analyzed by flow cytometry as described in Material and Methods. The IL-3 dependent cell line DA3 was also included as a positive control of apoptosis induced upon IL-3 starvation.

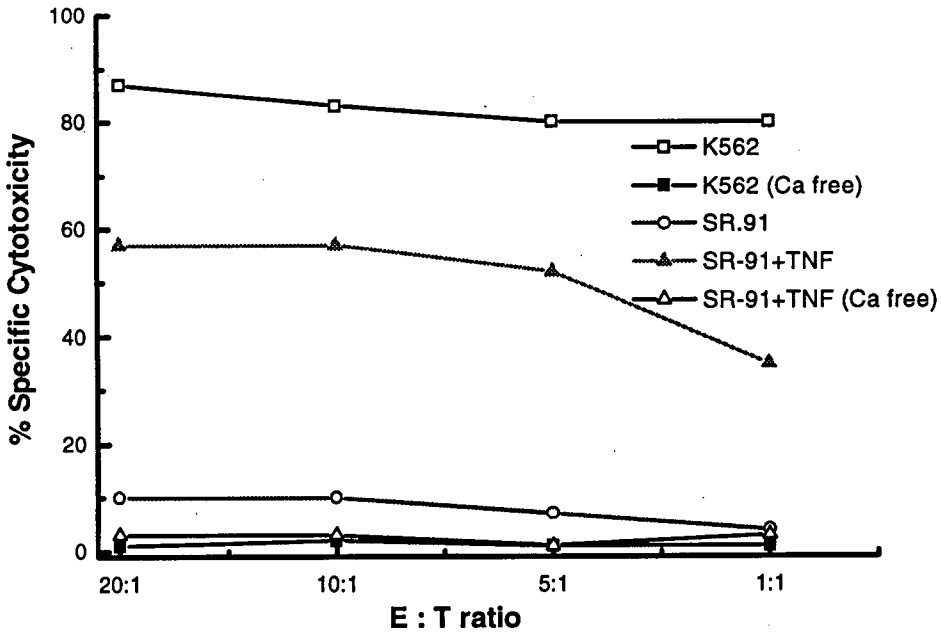


Figure 18. Calcium requirement for NK-92 lysis of TNF-α treated SR-91.
To test the effect of calcium depletion on NK-92 mediated lysis of TNF-α treated SR-91, a cytotoxicity assay, in calcium free conditions, was performed in Ca^{2+} and Mg^{2+} free HBSS supplemented with 5mM $MgCl_2$ and 1mM EGTA as described in Materials and Methods.

5.2.2. TNF-α but not IFN-γ activates the adhesion receptors LFA-1, and β1 integrins on SR-91 cells

To investigate whether CAMs other than ICAM-1 were involved in sending an activating signal to NK-92 cells, cell surface expression and the functional activation state of a number of cell surface molecules on SR-91 and the effects of TNF-α and IFN-γ were examined. Activation of LFA-1 by TNF-α was determined by binding of SR-91 cells to plastic wells coated with sICAM-3. LFA-1 and ICAM-3 were found to be constitutively expressed on both NK-92 and SR-91(Fig 1&11). LFA-1 on NK-92 is in active state, as assessed by binding to sICAM-3 coated wells, presumably because of IL-2 stimulation. On the other hand, SR-91 cells without cytokine treatment did not bind to sICAM-3-coated wells, indicating that it is in inactive form. Upon treatment with TNF-α, but not IFN-γ, SR-91 cells avidly bound to sICAM-3, indicating that LFA-1 on SR-91 is functionally activated by TNF-α, but not IFN-γ (Fig 19). This is consistent with the hypothesis that LFA-1 on SR-91 may bind to ICAM-3 on NK-92 cells and send a signal to kill.

β1 integrins VLA-4 and VLA-5 bind to the ECM protein, FN [358]. VLA-4 also binds the vascular endothelial adhesion molecule (VCAM-1) which is normally expressed on vascular endothelial cells, and its expression is inducible by TNF-α [359,360]. Although usually both VLA-4 and VLA-5 are expressed on NK cells, only VLA-4 is expressed on NK-92 cells. VCAM-1 is not expressed on NK-92 cells nor on SR-91 or cytokine treated SR-91 cells. Therefore,VCAM-1/VLA-4 interaction is not involved in this process. Although SR-91 cells

constitutively express VLA-4 and VLA-5, they do not bind to fibronectin unless they are activated by PMA (Fig 19). However, TNF-α treatment enhanced the adhesion of SR-91 to fibronectin, although not to the same extent as seen with PMA, whereas IFN-γ had no effect.

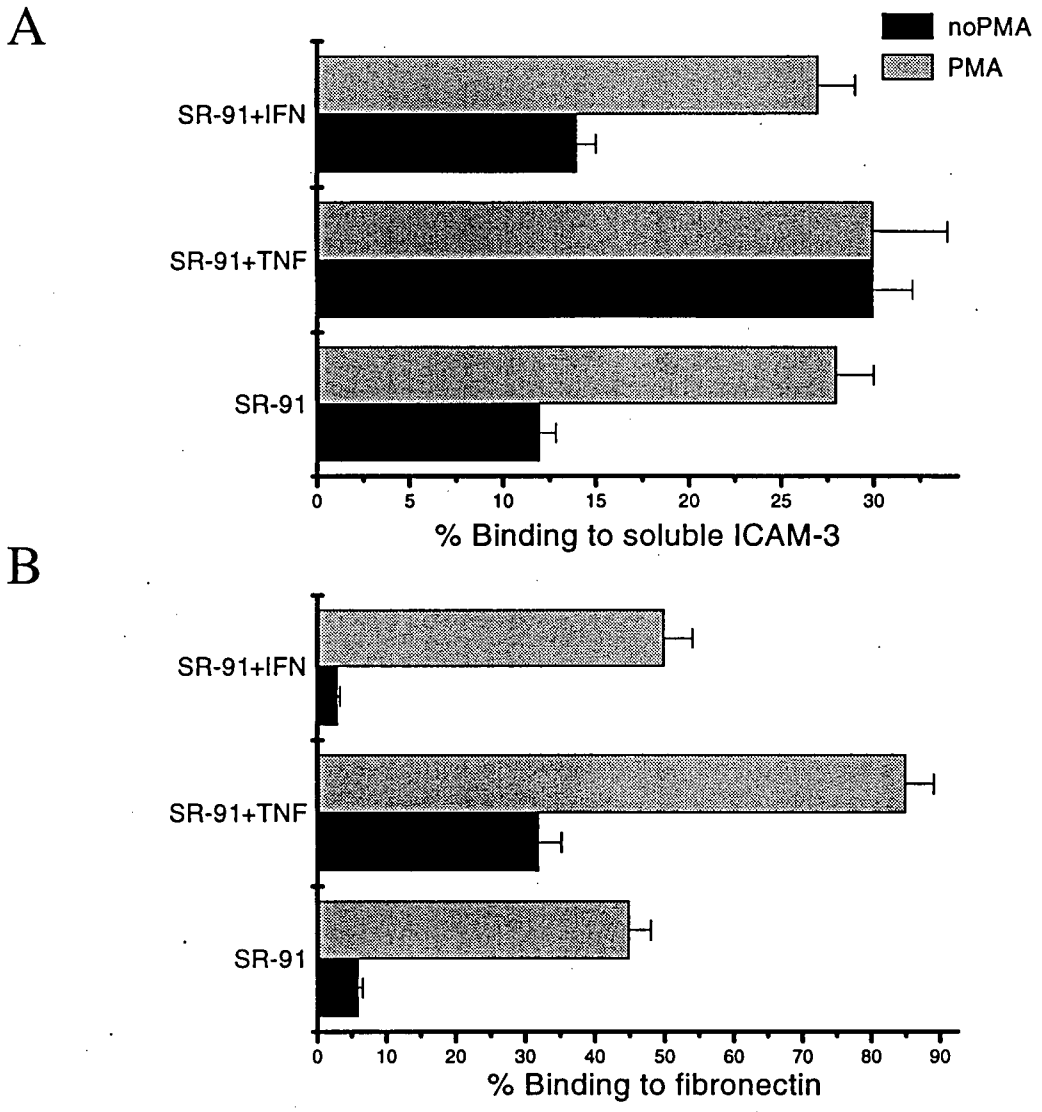


Figure 19. Activation of LFA-1 and β1 integrins on SR-91.
SR-91 cells were incubated for 16 h with TNF-α or IFN-γ and tested for their binding to plastic wells coated with sICAM-3 and fibronectin, respectively, either in the absence or presence of PMA (added at 100 ng/ml during the adhesion assay). The percentage of bound cells was determined by referring to a standard curve established using a series of known number of fluorescent cells. Results are expressed as mean values of two independent experiments ± SD.

5.2.3. Expression and function of CD44 isoforms

CD44 binds hyaluronan (component of ECM). Homotypic adhesion of CD44, mediated by interaction between CD44R1 and CD44R1 or CD44R1 and CD44H, has also been reported [207]. Flow cytometric analysis showed that NK-92 expresses CD44H and CD44R1, as well as the activation epitope defined by the antibody 7F4 (Table 2, Fig 20). SR-91 expresses CD44H, and TNF-α treatment up-regulates CD44R1 expression and slightly enhances the expression of 8D8 epitope (Fig 21). It also induces CD44 activation, as assessed by binding of SR-91 to hyaluronan coated plastic wells and the expression of the activation epitope 7F4 (Fig 22). 7F4 mAb reacts with a CD44 determinant shown to be expressed on peripheral blood T cells following PHA stimulation which parallels the acquisition of the capacity to bind hyaluronan. Therefore, it appears to define an activation epitope associated with functional activation of CD44 molecules [361]. IFN-γ did not induce activation of CD44 (Fig 21& 22).

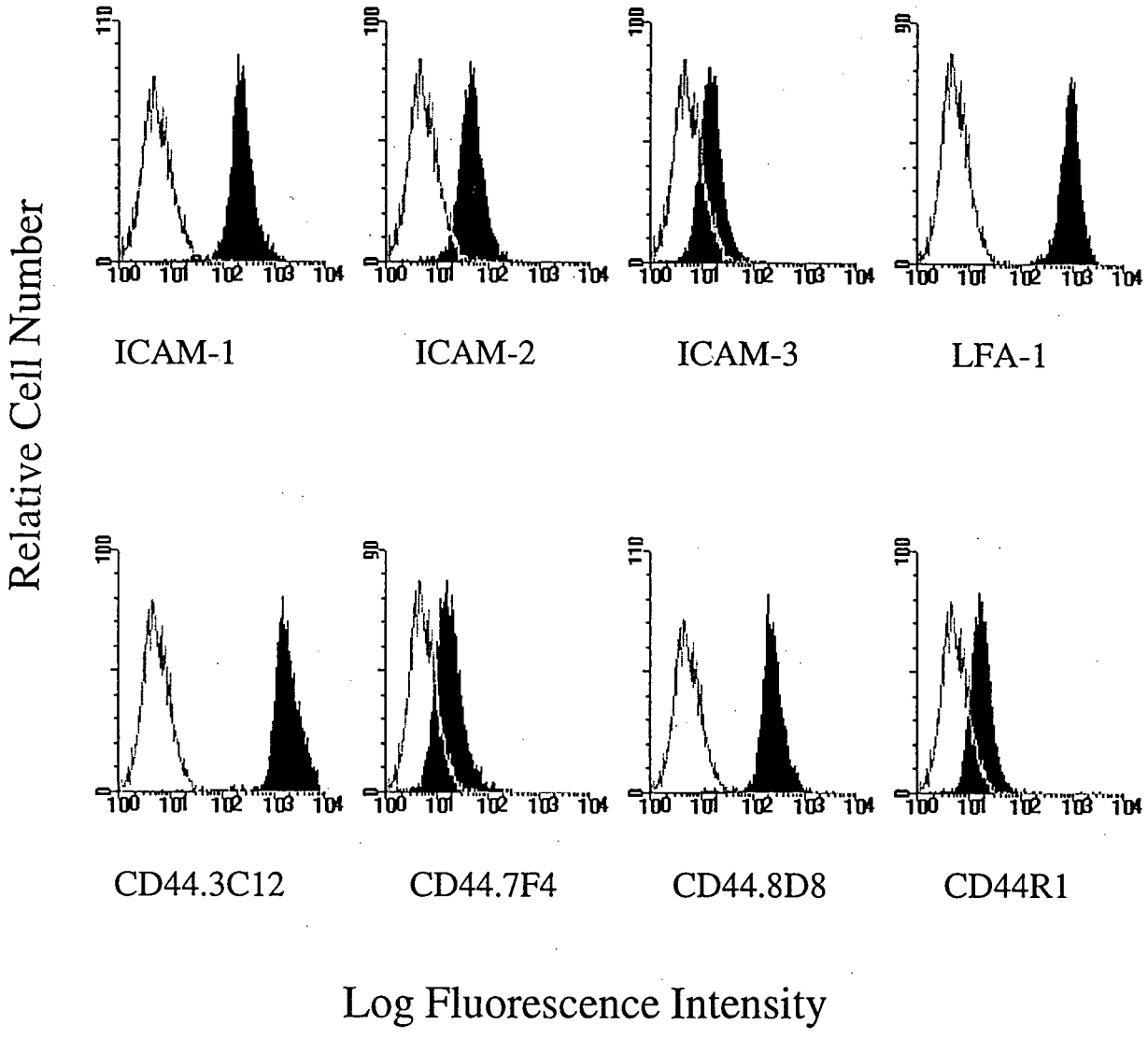


Figure 20. Flow cytometric analysis of ICAM-1, ICAM-2, ICAM-3, LFA-1, and different epitopes of CD44 on NK-92 cells.
NK-92 were stained with saturating concentrations of monoclonal antibodies against ICAM-1, ICAM-2, ICAM-3, LFA-1, and CD44 (filled). Background fluorescence of cells stained with the secondary antibody alone is also shown (open).

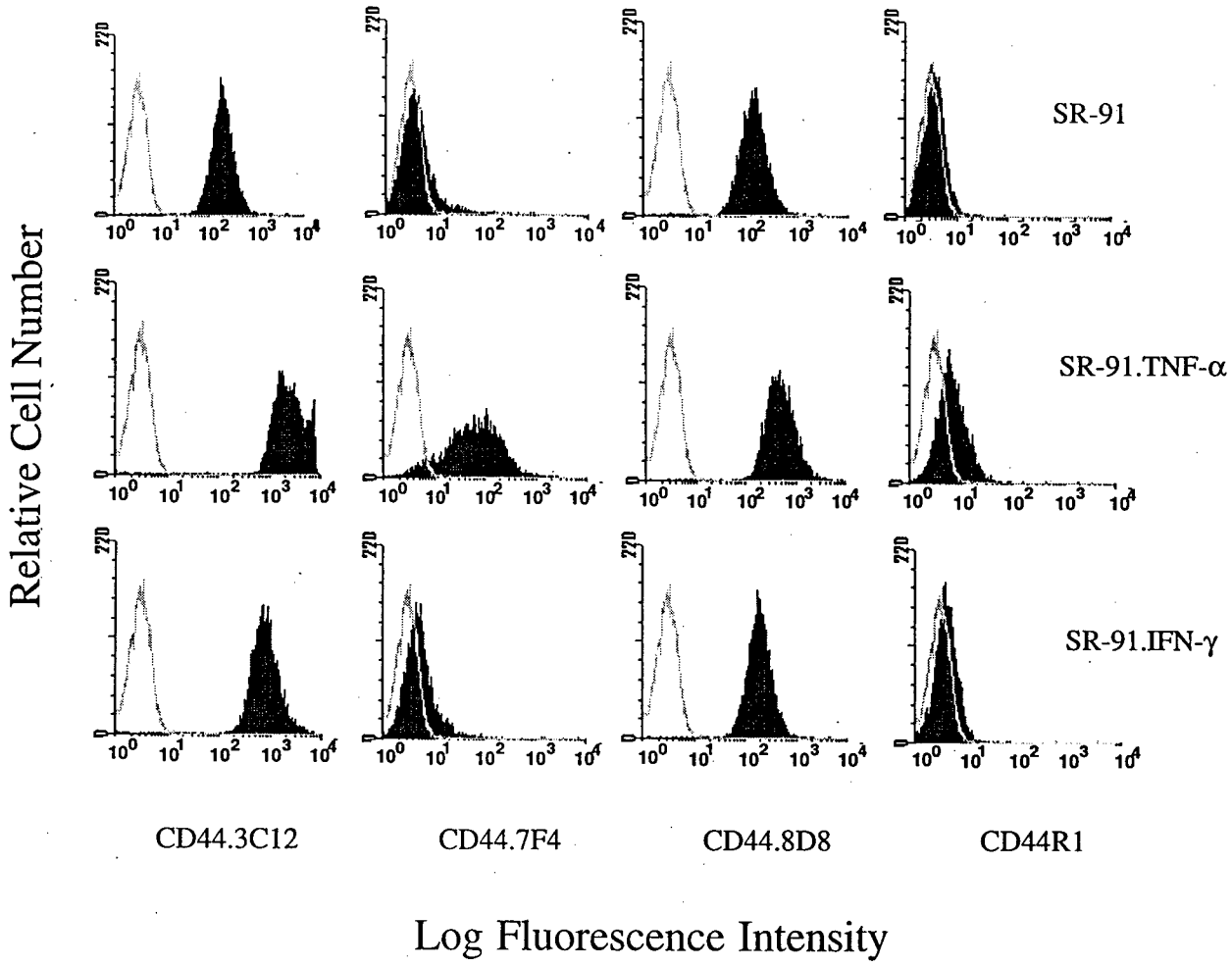
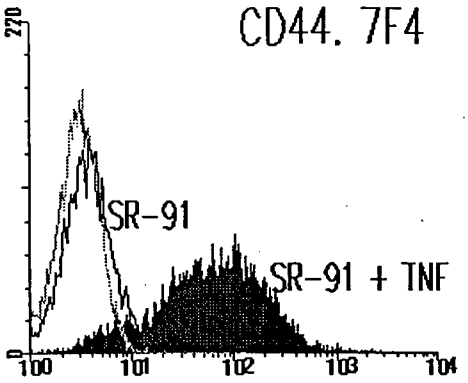


Figure 21. Flow cytometric analysis of different epitopes of CD44 on SR-91 cells and the effect of cytokine treatment.

SR-91 cells (untreated and after 16 h treatment with TNF-α or IFN-γ at 500U/ml) were stained with saturating concentrations of monoclonal antibodies against CD44H (3C12), CD44R1 (2G1) and different epitopes of CD44 (8D8 and 7F4) (filled). Background fluorescence of cells stained with the secondary antibody alone is also shown (open).

A



B

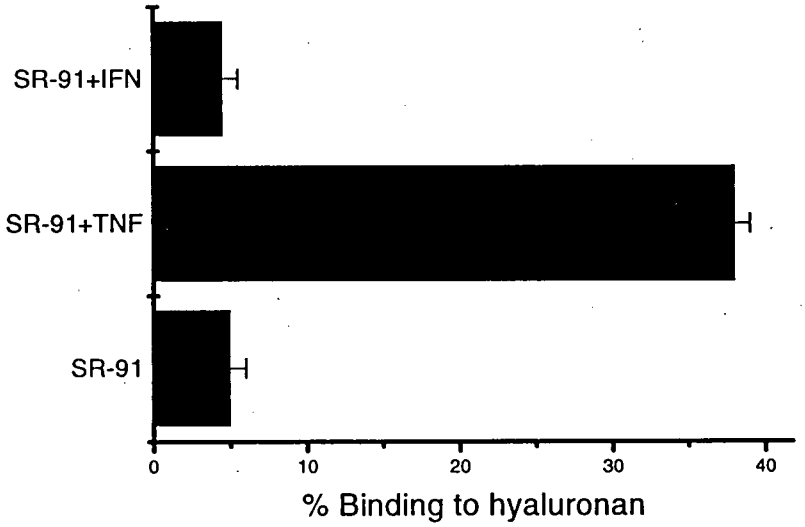


Figure 22. Activation of CD44 on SR-91 cells and TNF-α treated cells.
Untreated and cytokine treated SR-91 cells were analyzed (**A**) by flow cytometry for the expression of the activation epitope of CD44, 7F4 and (**B**) in adhesion assay with hyaluronan coated wells (as described in the legend to Figure 19). Results are expressed as mean values of two independent experiments ± SD.

5.3. Discussion

In this chapter, I investigated the mechanisms by which TNF-α-induces sensitivity of SR-91 cells to NK-92-mediated killing. This effect of TNF-α is not due to direct induction of apoptosis of SR-91 cells and is independent of Fas-Fas L pathway. Functional analysis of CAMs on SR-91 cells demonstrated that TNF-α, but not IFN-γ, functionally activates LFA-1, β1 integrins (VLA-4, and VLA-5), and CD44. These adhesion molecules have been shown to be important for the interaction between NK cells and target. NK-92 expresses ICAMs and CD44 that can function as counter-receptors for LFA-1 and CD44, respectively. Thus it is possible that these CAMs may bind to their counter-receptors on NK-92 and induce activation signals in these NK cells.

A large number of studies have already established that LFA-1/ICAM-1 pathway is critically involved in effector-target cell interaction, suggesting the importance of ICAM-1 as a major adhesion molecule, and of LFA-1 as a dominant adhesion molecule able to transmit both inside-out and outside-in signals [155]. LFA-1, like other integrins, needs to be activated in order to bind its ligands. The original focus of this study was the relevance of ICAM-1 expression on target cell as adhesion molecule necessary to provide adequate conjugate formation, and of LFA-1 on effector cell as signal transducing molecule. Although the importance of LFA-1 on NK cells has been well documented not only as adhesion molecule involved in effector:target conjugate formation, but also as signal transducing molecule [151,161], functional role of LFA-1 on target cells has not been investigated.

ICAM-3 has recently been shown to be a signal transducing molecule mainly in both resting and activated T cells. A sequence motif termed "Ag recognition activation motif" (ITAM) which is present in the cytoplasmic domain of CD3γ, δ, ε as well as ζ and plays critical role in signal transduction, is also found in ICAM-3. This motif is based on a tyrosine and leucine (or isoleucine) residues arranged in the general pattern $(YXXL)_2$. The tyrosines in ITAM motifs are phosphorylated after receptor stimulation possibly by members of the Src family of kinases, and this is believed to play a role in receptor function. ICAM-3 cross linking has been reported to induce activation of β1 and β2 integrins in T cells [185,186], an increase in intracellular calcium concentrations and tyrosine phosphorylation of a number of intracellular proteins in neutrophils and T cells [178,184,187]. In the human T leukemic cell lines JM and Jurkat, ICAM-3 was shown to play an important role in the regulation of LFA-1/ICAM-1 dependent and independent pathways involved in cell-cell interaction [178,188] suggesting the existence of novel receptors for ICAM-3 other than LFA-1. Alternatively ICAM-3 may trigger signals that would activate other CAMs [178]. More recently, ICAM-3 which has no intrinsic kinase activity has been reported to associate with Src family PTKs *lck* and *fyn* in Jurkat T cell line [187].

CD44 has been implicated in triggering cytotoxic functions of CTL and NK cells. CD44 binding to its ligand requires its activation. Homotypic and heterotypic adhesion have recently been reported for CD44R1 which is able to bind CD44R1 as well as CD44H isoforms. NK-92 cells express both isoforms as well as the activation epitope, presumably as a consequence of activation by IL-2, while SR-91 cells express the activation epitope only after TNF-α treatment. It is conceivable that CD44 on SR-91, upon activation with

TNF-α treatment, becomes able to bind its counterparts (CD44H or R1) on NK-92 cells, providing stronger binding and/or post binding signaling events. Therefore, the functional activation of their counter-receptors LFA-1 and CD44, respectively, on SR-91 by TNF-α may be responsible for the susceptibility of TNF-α treated SR-91 cells to NK-92 mediated cytotoxicity.

Thus, susceptibility of leukemic cells to NK-mediated killing seems to depend on the expression and activation state of several CAMs. In this study ICAM-1, LFA-1, CD44, and β1 integrins were shown to be upregulated or activated upon TNF-α treatment. Only ICAM-1 was upregulated by IFN-γ which did not induce sensitivity to NK-mediated cytotoxicity, although ICAM-1 was required for conjugate formation. It is therefore possible that other upregulated or activated CAMs may trigger NK cells to kill. Initial evidence is consistent with this hypothesis and suggest a role for ICAM-3 and CD44 on NK cells in triggering cytotoxicity. Further elucidation of the role of these CAMs in triggering NK cell cytotoxic functions will provide new insight into possible pathways of target induced activation of NK killing mechanisms.

Chapter 6. DISCUSSION

Although high doses of chemotherapy supported by rescue with bone marrow stem cells can cure a certain percentage of patients with leukemia and other hematopoietic malignancies, data from allogeneic transplants suggest that immune-mediated events significantly contribute to elimination of tumor in the recipient. In addition to T-lymphocytes, NK cells are critically involved in such a graft-versus-tumor effect. IL-2 activated NK cells are a major effector population and potent mediators of lysis of autologous and allogeneic leukemic cells in vitro. Adoptive immunotherapy with ex vivo activated NK cells (LAK) cells and IL-2 has been shown to induce tumor regression in some melanoma and renal cell carcinoma patients [362,363]. Such an approach has also shown promising results in patients after high dose chemotherapy and bone marrow transplantation. Although most leukemic cells are effectively killed by IL-2 activated NK cells in vitro, a certain proportion are resistant to LAK cell-mediated cytotoxicity [364]. The objective of this thesis was to investigate why some leukemic cells were resistance to NK cell killing.

Cell adhesion molecules (CAMs) are critical in effector-target conjugate formation which is a prerequisite for target cell lysis by effector CTL and NK cells, and the LFA-1/ICAM-1 pathway has been shown to be one of the most relevant adhesion pathways involved in effector-target interaction [146-148]. Therefore, this study focused on the contribution of CAMs in effector cell-target cell conjugate formation and their potential involvement in a post-binding step as signal transducing molecules that might be able to provide stimulatory

signals for NK cytotoxic functions. As LFA-1/ICAM-1 pathway is considered the most important for killer cell functions and interactions, the main objective of this study was to characterize the role of ICAM-1 in conjugate formation and of ICAM-3 and CD44 as candidates to deliver activating signals to NK cells.

An NK resistant leukemic cell line, SR-91, was isolated and found to express ICAM-1 at very low density, raising the possibility that its resistance to NK mediated killing may be due to lack of expression of ICAM-1. To examine this hypothesis, a human NK cell line, NK-92, was established. NK-92 was found to be an IL-2 dependent human NK cell line with characteristics of IL-2 activated NK cells. NK-92-mediated cytotoxicity is exceptionally potent against a broad spectrum of target cells tested so far, with the exception of SR-91. The lack of expression of CD16 is also an advantage of NK-92 cells, since it allows to analyze direct cell mediated killing mechanisms without the involvement of ADCC.

SR-91 is a leukemia cell line resistant to killing by both peripheral blood derived NK cells obtained by activation with IL-2, and NK-92 cells. Despite the potent cytotoxic activity of NK-92, which is considerably higher than that of IL-2 activated peripheral blood NK cells, the leukemic cell line SR-91 cells was still resistant to its killing. Because of these characteristics, these cell lines provided an ideal model to investigate how some leukemic cells escape NK cytotoxicity.

The hypothesis to be tested was that resistance of SR-91 to NK mediated lysis is due to lack of conjugate formation, the consequences of inadequate expression level of ICAM-1. To test this hypothesis two approaches were taken. First, human ICAM-1 cDNA was transfected into SR-91 cells. These transfection studies, although they allowed stable

increase of ICAM-1 expression (about fivefold) were not conclusive in that the susceptibility of ICAM-1 transfectants to NK-92 mediated lysis was found to be slightly increased (19% at 10:1 ratio against 8% for untransfected cells) but the same percentage of killing was also observed for SR-91 cells transfected with vector alone.

The second approach was SR-91 treatment with inflammatory cytokines TNF-α and IFN-γ known to induce ICAM-1 expression. It was found that the resistance of the leukemic SR-91 cells to NK-92 cell killing could be overcome by exposure of SR-91 cells to TNF-α but not to IFN-γ. Although both cytokines increased conjugate formation between NK-92 cells and SR-91 cells to similar levels and upregulated the expression of ICAM-1, only TNF-α was able to render target cells sensitive to NK cell killing. This suggests that ICAM-1 upregulation may be required to induce adequate conjugate formation, but does not allow sensitivity to NK mediated killing, which may require additional activating signals. Interestingly, the increased binding induced by both cytokines and the TNF-α induced sensitivity of SR-91 cells to NK-92 cell killing were abrogated by anti-LFA-1 mAb as well as a combination of antibodies against the three ligands of LFA-1, ICAM-1, ICAM-2 and ICAM-3, while anti-ICAM-1 on its own had only a partial effect. These observations suggest that binding of ICAMs to LFA-1 is involved in the initial binding of NK cells to target cells. In this study, antibodies had to be present in the reaction mixture during the period of the assay to be effectively inhibitory. Preincubation of either effector and/or target cells with antibodies against LFA-1, ICAM-1, ICAM-2 or ICAM-3 alone or in combination followed by removal of unbound antibody did not result in any significant inhibitory effect. This might be due to low affinity of the antibodies used, or may underline the importance and involvement of LFA-1 on both effector and target side.

Since enhanced conjugate formation on its own was insufficient to overcome resistance to NK cell killing in these studies, it was hypothesized that TNF-α (but not IFN-γ) may cause the activation of other molecules on SR-91 cells that are important for post binding activation of NK-92. Subsequent studies showed that TNF-α, but not IFN-γ treatment of SR-91 induced functional activation of LFA-1, CD44 and β1 integrins. Therefore, ICAM-3 and CD44 are potentially activating receptors on NK-92. It is possible that activation of SR-91 with TNF-α leads to binding of LFA-1 and CD44 on SR-91 to ICAM-3 and CD44, respectively, on NK-92 which may in turn activate NK-92 to kill SR-91. Although the importance of LFA-1 on NK cells has been well documented not only as adhesion molecule involved in effector:target conjugate formation, but also as signal transducing molecule [151,161], functional role of LFA-1 on target cells has not been investigated. The results of this thesis underline the potential importance of LFA-1 on the target cell where by binding to its counter-receptor on NK cell can lead to the activation of NK cell which may particularly involve ICAM-3. Therefore, the activation state of LFA-1 on the target cell may also be important for the activation of NK cell.

The differential effect of the two cytokines reflects differences in signaling pathways and intracellular targets, and activation of distinct transcription factors. IFN-γ is a potent immunomodulatory cytokine that exerts its pleiotropic effects through JAK/STATs signaling pathway [318-320, 323,324]. Two major signaling pathways known for TNF-α are 1) ceramide and sphingomyelinase, and 2) MAPK and JNK/SAPK pathways [310,311]. TNF-α is known to induce apoptosis in some cells, and the expression of a number of cellular genes, mainly through activation of NF-*k*B. However, TNF-α has not been shown to

induce sensitivity to NK mediated lysis. The possibility of apoptosis induced by TNF-α was ruled out, but TNF-α may have induced not only cell surface molecules, but also expression of other cytokines or cytokine receptors. It was not the objective of this study to analyze those events

It has been proposed that NK-mediated cytotoxicity may not be due to a unique, dominant receptor, but may result from an interaction between an appropriate array of "adhesion" molecules with their ligands, and be triggered by engagement of certain "accessory" or "costimulatory" molecules with their counter-receptors. Interference with individual receptor/ligand pairs could partially or totally inhibit the cytolytic response. However, none of the receptors alone would be sufficient for the initial "recognition" event that results in triggering cytotoxicity. LFA-1 may be an important adhesion and costimulatory molecule not only on NK cells, but also may play an important role on the target cell by binding to its counter-receptor ICAM-3 on the NK cell where it could trigger activating signals to the NK cell.

ICAM-3 has been shown to be a signal transducing molecule, increasing cell adhesion via β1 and β2 integrins, calcium mobilization and tyrosine phosphorylation through $p56^{lck}$ and $p59^{fyn}$ in T cell lines [185,187]. In contrast to the other ligands for LFA-1, ICAM-1 and ICAM-2 which have no serine in their cytoplasmic domains, the cytoplasmic domain of ICAM-3 has five serine and two tyrosine residues that can be phosphorylated upon stimulation with different stimuli. These cytoplasmic tyrosine residues are present in a $(YXXL)_2$ motif, a sequence motif termed "Ag recognition activation motif" (ITAM) which is present in the cytoplasmic domains of CD3γ, δ, ε as well as ζ and plays critical role in

signal transduction. These observations first suggested that ICAM-3 may play a more dominant role in signal transduction than ICAM-1 or ICAM-2 [172,173,183,184]. ICAM-3 has been shown to be costimulatory for both resting and activated T lymphocytes [186] and has been implicated in homotypic cell aggregation through the regulation of both LFA-1/ICAM-1 dependent and LFA-1/ICAM-1 independent cell-cell interactions of leukemic T cells. This suggested the existence of other ligands for ICAM-3, or triggering of intracellular signals through ICAM-3 that would activate other CAMs [178,187]. A fourth chain that associates with CD18 has recently been cloned allowing the identification of an alternative counter-receptor for ICAM-3, a novel leukointegrin, αdβ2 that was found to be expressed at moderate levels on myelomonocytic cell lines [179]. Whether this new ICAM-3 counter-receptor is present on SR-91 or on NK cells is not known, but if it is expressed on SR-91 cell, it would be of interest to investigate its interaction with ICAM-3 on NK-92 cells.

CD44 has also been implicated in signal transduction events and the triggering of cytotoxic functions of CTL [208-210] and NK cells [198-200]. CD44 has recently been reported to associate with $p56^{lck}$ in T cells [211]. Different isoforms of CD44 with molecular weights ranging from 80 to 250 kDa can result from alternative splicing of 10 exons. Although CD44 can function as a receptor for the glycosaminoglycan hyaluronan, there is evidence that binding to hyaluronan cannot explain all CD44 dependent adhesion events, and homotypic binding between different isoforms of CD44 has recently been reported showing that CD44R1 is able to bind to CD44R1 and CD44H while CD44H cannot bind to CD44H [207]. NK-92 cells express activated forms of both the CD44H and CD44R1 isoforms. It is conceivable that activation of CD44 (H and R1) on SR-91 cells that occurs upon treatment with TNF-α (but not IFN-γ), could allow interaction with CD44 on NK-92 cells, providing

costimulatory signals to the NK cells. Hence, overexpression or activation of CD44 on the target cell could contribute to an increased target sensitivity to NK cell mediated lysis.

Although NK cells have been considered as MHC non-restricted with regards to reactivity with target cells, the pattern of MHC class I expression of the target cell may determine its sensitivity to NK cell-mediated killing. Selected MHC class I molecules have been shown, in some but not all systems, to protect target cell from NK effector cells by delivering a predominantly inhibitory signal [231-234]. The protective effect of MHC class I would occur at post binding step, hence over-riding other target induced activating signals. A number of inhibitory receptors have been identified on human NK cells that belong mainly to two structural families of molecules. One is a family of immunoglobulin related molecules termed killer inhibitory receptors (KIRs) with p58 and p70 that interact with HLA-A and HLA-B alleles respectively [255-261]. Another is a type II integral membrane protein Kp43 (CD94) that provides specific recognition for HLA-B molecules [260,262,263]. SR-91 cells express high levels of MHC class I molecules, and NK-92 cells do not express any known member of the KIR family whether p58 or p70, but express high levels of CD94 (E Long personal communication). The study of the relevance of MHC class I molecules on target cells which could react with specific NK receptors on NK-92 cells is currently under investigation by other groups. Extensive studies against a large panel of transfectants expressing single HLA class I alleles have not shown protection from NK-92 cell mediated lysis (E Long personal communication), but it has recently been reported that the infection of NK-92 cells, with recombinant vaccina virus encoding the two p58 receptors specific for HLA-Cw3 and HLA-Cw4 conferred NK-92 cells with the ability to receive an inhibitory signal and reduced significantly their lytic activity against Cw3 and Cw4 expressing target cells [275]. It is not likely that MHC class I is responsible for the

resistance of SR-91 cells to NK-92 mediated killing, because NK-92 cells do not seem to express inhibitory receptor for MHC class I expressed on SR-91(HLA-A2, B44, B57, C5). Furthermore, the fact that TNF-α treated SR-91 cells are sensitive to NK-92-mediated lysis argues against a protective effect of MHC class I molecules in this system, since the MHC class I expression on SR-91 is not altered by TNF-α treatment. TNF-α and IFN-γ are in fact able to induce MHC class I expression in a number of cells [291,314,317,337,365], however, MHC class I expression of SR-91 cells is high and does not change upon treatment with these cytokines.

The study of NK cells and cytotoxic mechanisms is hindered by the difficulty of obtaining pure NK populations exempt of contaminating T lymphocytes. Therefore, the availability of NK cell lines represents a great practical advantage. A number of NK cell clones have been isolated and characterized. They have proven to be important tool in the study of the NK cell repertoire and MHC class I receptors. However, these NK clones have, by definition, limited cytotoxic activity and are only effective against target cells missing the MHC class I molecules recognized by inhibitory receptors expressed on these clones [256,267,351-353]. On the other hand, only a few NK cell lines are available to date [347-350]. The establishment of NK-92 cell line is of particular interest mainly because of (i) its very potent cytotoxic activity against a broad spectrum of target cells and (ii) its lack of expression of CD16 which makes it more suitable for the study of direct cell mediated cytotoxicity without the involvement of ADCC. NK-92 cell line has thus far proven to be a very useful tool to investigate different aspects of NK-mediated cytotoxicity by various groups [275,354-357]. It has been used to determine the specificity of p70 KIR for HLA-B allotypes [354], and to show the association of NKG2A with CD94 [355]. The cell line has

also been used to investigate cytokine induced apoptosis of NK cells [356], and the contribution of NK cells to endothelial cell activation and xenograft rejection [357]. Furthermore, the data presented in the first results chapter of this thesis, show its potential application for *ex-vivo* purging of malignant cells.

The data presented herein demonstrate that ICAM-1 expression on the target cell SR-91 is a prerequisite for conjugate formation between cytotoxic effector and target cells. However, this on its own is insufficient to allow target cell lysis by NK-92 cells. ICAM-2 and ICAM-3 on SR-91 cells also participate in this effector-target binding but their effect is minor relative to that of ICAM-1. Furthermore, preliminary experiments suggested that binding of anti ICAM-3 and CD44 to NK-92 may induce tyrosine phosphorylation of a number of proteins including $p56^{lck}$ in NK-92 cells. Although not completely conclusive, these observations are consistent with the hypothesis that TNF-α activated LFA-1 and CD44 on SR-91 cells may upon interaction with ICAM-3 and CD44, their respective counter-receptors on NK-92 cells, trigger activating signals. ICAM-3 and CD44 may contribute to signal transduction events in NK-92 cells which seem to involve the Src family member of protein tyrosine kinases $p56^{lck}$. However, whether ICAM-3 or CD44 play a major role in NK-92 lysis of TNF-α treated SR-91 cells still remains to be established. Their potential involvement in triggering cytotoxic activity of NK-92 cells could be further examined by testing (i) the ability of antibodies to ICAM-3 or CD44 to increase NK-92 cytotoxicity against other target cells, (ii) in a redirected ADCC against FcR bearing target cells, (iii) by using tetrameric antibodies against ICAM-3 or CD44 and an antigen present only on the target cells [199]. If ICAM-3 and CD44 are signal transducing molecules on NK-92 cells, such bispecific antibodies should be able to induce the killing of IFN-γ treated SR-91 cells,

which bind to NK-92 cells but are still resistant. Finally, the full scope of signal transducing events in NK-92 cells remains to be resolved and is beyond the intention of this thesis.

These results in this thesis demonstrated that the NK-resistant leukemic line SR-91 can become NK-sensitive by TNF-α treatment. Although the precise mechanisms responsible for this effect are still unclear, some were addressed in this thesis. This effect of TNF-α which has not been described before potentially has important implications with respect to the treatment of leukemia. NK/LAK cells have proven to be very effective in the treatment of hematological malignancies and particularly the eradication of residual leukemic disease after myeloablative chemo-radiotherapy supported by BMT [344-346]. One of the problems associated with the use of NK/LAK cells for the treatment of leukemia is that some leukemic cells are resistant to their cytotoxicity. This research was undertaken to investigate underlying cause(s) of this resistance, and if an improvement of this therapeutic modality could be achieved by modulating the expression of CAMs. My results suggest that at least some NK/LAK-resistant leukemic cells can become sensitive by appropriate treatment. It will be of great interest if this observation could be confirmed for other leukemic cells and particularly if it could be extended to fresh leukemic cells. This could improve the outcome of approaches using NK-cells in the treatment of leukemia. Although systemic administration of cytokines such as TNF-α may not be clinically desirable given their side effects, one possible approach to this may be the use of antibody based targeting of cytokines allowing that cytokines like as TNF-α to selectively interact with malignant cells without causing systemic side effects [366-368]. Since NK cells are themselves producers of TNF-α upon activation, bispecific antibodies to a receptor on NK cells able to deliver activating signal upon cross-linking and coupled to a molecule on the surface of leukemic

cell could allow target cytokine to bind directly to leukemic cell and also improve target cell lysis. Moreover, results in the first chapter show a potential application using NK-92 cell line *ex vivo* to eliminate leukemic cells. In conclusion, the observations presented in this thesis could potentially benefit patients with leukemia for whom in addition to chemotherapy, immunotherapy with NK cells is considered.

REFERENCES

1. Trinchieri G. 1985. Biology of natural killer cells. Adv Immunol 47: 187.

2. Lopez-Cabrera M, Santis AG, Fernandez-Ruiz E, Blacher R, Esch F, Sanchez-Mateos P, Sanchez-Madrid F. 1993. Molecular cloning, expression, and chromosomal localization of the human earliest lymphocyte activation antigen AIM/CD69, a new member of the C-type animal lectin superfamily of signal transmitting receptors. J Exp Med 178: 537.

3. Spits H, Lanier LL, Phillips JH. 1995. Development of human T and natural killer cells. Blood 85: 2654.

4. Kundig TM, Schorle H, Bachmann MF, Hengartner H, Zinkernagel RM, Horak I. 1993. Immune responses in interleukin-2-deficient mice. Science 262: 1059.

5. Di Santo JP, Muller W, Guy-Grand D, Fischer A, Rajewsky K. 1995. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. Proc Natl Acad Sci USA 92: 377.

6. Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, Mc Bride OW, Leonard WJ. 1993. Interleukin-2 receptor γ chain mutation results in X-linked severe combined immunodeficiency in humans. Cell 73: 147.

7. Mrozek E, Anderson P, Caligiuri MA. 1996. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. Blood 87: 2632.

8. Lipinski M, Virelizier JL, Tursz T, Griscelli C. 1980. Natural killer and killer activities in patients with primary immunodeficiencies or defects in immune interferon production. Eur J Immunol 10: 246.

9. Herberman RB, Nunn ME, Lavrin DH. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogenic tumors. I. Distribution of reactivity and specificity. Int J of Cancer 16: 216.

10. Lotzova E, Savary CA, Gray KN, Raulston GL, Jardine JH. 1984. Natural killer cell profile of two random-bred strains of athymic rats. Exp Hematol 12: 633.

11. Haller O, Wigzell H. 1977. Suppression of natural killer cell activity with radioactive strontium: effector cells are marrow dependent. J Immunol 118: 1503.

12. Kumar V, Ben Ezra J, Bennett M, Sonnenfeld G. 1979. Natural killer cells in mice treated with ^{89}Strontium: normal target binding cell number but inability to kill even after interferon administration. J Immunol 123: 1832.

13. Heckett J, Tutt M, Lipscomb M, Bennett M, Koo G, Kumar V. 1986. Origin and differentiation of natural killer cells. II. Functional and morphologic studies on purified NK1.1+ cells. J Immunol 136: 3124.

14. Seaman WE, Gindhart TD, Greenspan JS, Blackman MA, Tala N. 1979. Natural killer cells, bone, and the bone marrow: studies in estrogen-treated mice and in congenitally osteoporotic (mi/mi) mice. J Immunol 122: 2541.

15. Blomgren H, Baral E, Edsmyr F, Strender LE, Petrini B, Wasserman J. 1980. Natural killer activity in peripheral blood lymphocyte population following local radiation therapy. Acta Radiol: Oncol radiat Phys Biol 19: 2236.

16. Brovall C, Schacter B. 1981. Radiation sensitivity of human natural killer cell activity: control by X-linked genes. J Immunol 126: 139.

17. Gorelik E, Herberman RB. 1982. Depression of natural antitumor resistance of C57BL/6 mice by leukemogenic doses of radiation and restoration of resistance by transfer of bone marrow or spleen cells from normal, but not beige syngenic mice. J Natl Cancer Inst 69: 89.

18. Clave E, Socie G, Cosset JM, Chaillet MP, Tartour E, Girinski T, Carosella E, Fridman H, Gluckman E, Mathiot C. 1995. Multicolor flow cytometry analysis of blood cell subsets in patients given total body irradiation before bone marrow transplantation. Int J Rad Oncology Biol Phys 33: 881.

19. Cuturi MC, Anegon I, Sherman F, Loudon R, Clark SC, Perussia B, Trinchieri G. 1989. Production of hematopoietic colony-stimulating factors by human natural killer cells. J Exp Med 169: 569.

20. Anegon I, Cuturi MC, Trinchieri G, Perussia B. 1988. Interaction of Fc receptor (CD16) with ligands induces transcription of IL-2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells. J Exp Med 167: 452.

21. Warren HS, Kinnear BF, Phillips JH, Lanier LL. 1995. Production of IL-5 by human NK cells and regulation of IL-5 secretion by IL-4, IL-10, and IL-12. J Immunol 154: 5144.

22. Bluman EM, Bartynski KJ, Avalos BR, Caligiuri MA. 1996. Human natural killer cells produce abundant macrophage inflammatory protein-1 alpha in response to monocyte-derived cytokines. J Clin Invest 97: 2722.

23. Robertson MJ, Manley TJ, Donahue C, Levine H, Ritz J. 1993. Costimulatory signals are required for optimal proliferation of human natural killer cells. J Immunol 150: 1705.

24. Naume B, Gately M, Espevik T. 1992. A comparative study of IL-12 (cytotoxic lymphocyte maturation factor), IL-2, and IL-7-induced effects on immunomagnetically purified CD56+ NK cells. J Immunol 148: 2429.

25. Silva MR, Hoffman R, Srour EF, Ascensao JL. 1994. Generation of human natural killer cells from progenitors does not require marrow stromal cells. Blood 84: 841.

26. Trinchieri G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Ann Rev Immunol 13: 251.

27. Warren HS, Kinnear BF, Kastelein RL, Lanier LL. 1996. Analysis of the costimulatory role of IL-2 and IL-15 in initiating proliferation of resting (CD56 ^{dim}) human NK cells. J Immunol 156: 3254.

28. Kondo M, Takeshita T, Ishii N, Nakamura M, Watanabe S, Arai K, Sugamura K. 1993. Sharing of the interleukin-2 receptor γ chain between receptors for IL-2 and IL-4. Science 262: 1874.

29. Kondo M, Takeshita T, Higuchi M, Nakamura M, Sudo T, Nishikawa S, Sugamura K. 1994. Functional participation of the IL-2 receptor γ chain in IL-7 receptor complexes. Science 263: 1453.

30. Kimura Y, Takeshita T, Kondo M, Ishii N, Nakamura M, Van Snick J, Sugamura K. 1995. Sharing of the IL-2 receptor γ chain with the functional IL-9 receptor complex. Int Immunol 7: 115.

31. Johnston JA, Wang LM, Hanson EP, Sun XJ, White MF, Oakes SA, Pierce JH, O'Shea JJ. 1995. Interleukins 2, 4, 7, and 15 stimulate tyrosine phosphorylation of insulin receptor substrates 1 and 2 in T cells. Potential role of Jak kinases. J Biol Chem 270: 28527.

32. Giri JG, Ahdieh M, Eisenman J, Shanebeck K, Grabstein K, Kumaki S, Namen A, Park LS, Cosman D, Anderson D. 1994. Utilization of the β and γ chains of the IL-2 receptor by the novel cytokine IL-15. EMBO J 13: 2822.

33. Ullberg M, Jondal M. 1981. Recycling and target binding capacity of human natural killer cells. J Exp Med 153: 615.

34. Abrams SI, Brahimi Z. 1988. Target cell directed NK inactivation: concomitant loss of NK and antibody-dependent cellular cytotoxic activities. J Immunol 140: 2090.

35. Jewett A, Bonavida B. 1996. Target-induced inactivation and cell death by apoptosis in a subset of human NK cells. J Immunol 156: 907.

36. Yamauchi A, Taga K, Mostowski HS, Bloom ET. 1996. Target cell-induced apoptosis of interleukin-2-activated human natural killer cells: roles of cell surface molecules and intracellular events. Blood 87: 5127.

37. Cerottini JC, Brunner KT. 1974. Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. Adv Immunol 18: 67.

38. Berke G. 1980. Interaction of cytotoxic T lymphocytes and target cells. Prog Allergy 27:69.

39. Germain RN, Dorf ME, Benacerraf B. 1975. Inhibition of T-lymphocyte-mediated tumor-specific lysis by alloantisera directed against the H-2 serological specifities of the tumor. J Exp Med 142: 1023.

40. Schrader JW, Edelman GM. 1976. Participation of the H-2 antigens of tumor cells in their lysis by syngenic T cells. J Exp Med 143: 601.

41. Zinkernagel RM, Doherty PC. 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngenic or semiallogenic system. Nature 248: 701.

42. Glaser M, Law LW. 1978. Adjuvant-induced thymus-derived suppressor cells of cell-mediated tumour immunity. Nature 273: 385.

43. Herberman RB. 1974. Cell-mediated immunity to tumor cells. Adv Cancer Res 19: 207.

44. Ortaldo JR, Herberman RB. 1984. Heterogeneity of natural killer cells. Annu Rev Immunol 2: 359.

45. Moore M. 1985. Natural immunity to tumors: theoritical predictions and biological observations. Br J Cancer 52: 147.

46. Hanna N, Fidler IJ. 1980. Role of natural killer cells in the destruction of circulating tumor emboli. J Natl Cancer Inst 65: 801.

47. Cortelezzi A, Sarina B, Cattaneo C, Pomati M, Silvestris I, Soligo D, Calori R, Di Stefano M, Hu C, Monza M. 1996. In vitro effects of IL-2 on NK-activity, clonogenic potential, blast cell proliferation and cytokine release of MDS bone marrow patients. Leukemia 10: 1181.

48. Fao R, Meloni G, Guarini A, Vignetti M, Marchis D, Tosti S Gillio Tos A, Vischia F, Mandelli F, Gavosto F. 1992. Interleukin-2 in the management of acute myeloid leukemia: clinical and biological findings. Leukemia 6 (suppl 3): 115.

49. Rossi AR, Pericle F, Rashleigh S, Janiec J, Djeu JY. 1994. Lysis of neuroblastoma cell lines by human natural killer cells activated by interleukin-2 and interleukin-12. Blood 83: 1323.

50. Eremin O, Coombes RR, Ashby J. 1981. Lymphocytes infiltrating human breast cancers lack K-cell activity and show low levels of NK cell activity. Br J Cancer 44: 166.

51. Golub SH, Niitsuma M, Kawate N, Cochran AJ, Holmes EC. 1982. NK activity of tumor infiltrating and lymph node lymphocytes in human pulmonary tumors. In NK cells and other natural effector cells (RB Herberman, ed) p 1113, Academic Press, New York.

52. Mantovani A, Allavena P, Sessa C, Bolis G, Mangioni C. 1980. Natural killer activity of lymphoid cells isolated from human ascitic ovarian tumors. Int J Cancer 25: 573.

53. Vose BM, Vanky F, Argov S, Klein E. 1977. Natural cytotoxicity in man: activity of lymph node and tumor-infiltrating lymphocytes. Eur J Immunol 7: 753.

54. Blanchard DK, Kavanagh JJ, Sinkovics JG, Cavanagh D, Hewitt SM, Djeu JY. 1988. Infiltration of interleukin-2-inducible killer cells in ascitic fluid and pleural effusions of advanced cancer patients. Cancer Res 48: 6321.

55. Rosenberg SA, Spies P, Lafreniere RA. 1986. A new approach to the adoptive immunotherapy of cancer with tumor infiltrating lymphocytes. Science 233: 205.

56. Faure F, Triebel F, Hecend T. 1990. MHC-unrestricted cytotoxicity. Immunol Today 11: 108.

57. Parmiani G. 1990. An explanation of the variable clinical response to interleukin 2 and LAK cells. Immunol Today 11: 113.

58. Pisani RJ, Kco CJ, Wold LE, McKean DJ. 1989. Lymphokine-activated killer (LAK) cell activity in tumor-infiltrating lymphocyte from non-small cell lung cancer. Am J Clinic Pathol 92: 435.

59. Staren ED, Economou SG, Harris JE, Braun DP. 1989. Lymphokine-activated killer (LAK) cell induction in tumor infiltrating leukocytes from colon cancer patients. Cancer 64: 2238.

60. Moy PM, Holmes EC, Golub SH. 1985. Depression of natural killer cytotoxic activity in lymphocytes infiltrating human pulmonary tumors. Cancer Res 45: 57.

61. Brittenden J, Heys SD, Ross J, Eremin O. 1996. Natural killer cells and cancer. Cancer 77: 1226.

62. Fitzgerald PA, Evans R, Kirkpatrick D, Lopez C. 1983. Heterogeneity of human NK cells: comparison of effectors that lyse HSV-1-infected fibroblasts and K562 erythroleukemic targets. J Immunol 130: 1663.

63. Biron CA, Byron KS, Sullivan JL. 1989. Severe herpes virus infection in an adolescent without natural killer cells. New Engl J Med 320: 1731.

64. Bukowski JF, Warner JF, Dennert G, Welsh RM. 1985. Adoptive transfer studies demonstrating the antiviral effect of natural killer cells in vivo. J Exp Med 161: 40.

65. Bancroft GJ, Shellam GR, Chalmer JE. 1981. Genetic influences on the augmentation of natural killer (NK) cells during murine cytomegalovirus infection: correlarion with patterns of resistance. J Immunol 126: 988.

66. Bukowski JF, Woda BA, Habu S, Okumura K, Welsh RM. 1983. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. J Immunol 131: 1531.

67. Ducombe AS, Grundy JE, Oblakowski P, Prentice G, Gottlieb DJ, Roy DM, Reittie JE, Bello-Fernandez C, Hoffbrand AV, Brenner MK. 1992. Bone marrow transplant recipients have defective MHC-unrestricted cytotoxic responses against cytomegalovirus in comparaison with Epstein-Barr virus: the importance of target cell expression of LFA-1. Blood 79: 3059.

68. Chehimi J, Starr SE, Frank I, Rengaraju M, Jackson SJ, Llanes C, Kobayashi M, Perussia B, Young D, Nickbarg E, Wolf SF, Trinchieri G. 1992. Natural killer cell stimulatory factor increases the cytotoxic activity of NK cells from both healthy donors and human immunodeficiency virus-infected patients. J Exp Med 175: 789.

69. Zheng ZY, Zucker-Franklin D. 1992. Apparent ineffectiveness of natural killer cells vis-a-vis retrovirus-infected targets. J Immunol 148: 3679.

70. Klimpel GR, Niesel DW, Klimpel KD. 1986. Natural cytotoxic effector cell activity against Shigella flexneri-infected HeLa cells. J Immunol 136: 1081.

71. Blanchard DK, Stewart WE, Klein II, Friedman H, Djeu JY. 1987. Cytolytic activity of human peripheral blood leukocytes against Legionella pneumophila-infected monocytes: Characterization of the effector cell and augmentation by interleukin-2. J Immunol 139: 551.

72. Blanchard DK, Bia Michelini-Norris M, Friedman H, Djeu JY. 1989. Lysis of mycobacteria-infected monocytes by IL-2 activated killer cells: Role of LFA-1. Cell Immunol 119: 402.

73. Denis M. 1991. Activated murine natural killer cells control growth of Mycobacterium lepaemurium in mouse macrophages; in vitro and in vivo evidence. Int J Immunopharmacology 13: 881.

74. Nabavi N, Murphy JW. 1985. In vitro binding of natural killer cells to Cryptococcus neoformans targets. Infec Immunol 50: 50.

75. Levitz SM, Dupont MP, Smail EH. 1994. Direct activity of human T lymphocytes and natural killer cells against cryptococcus neoformans. Inf Immunity 62: 194.

76. Kirkpatrick CE, Farrel JP, Warner JF, Dennert G. 1985. Participation of natural killer cells in the recovery of mice from visceral leishmaniasis. Cell Immunol 92: 163.

77. Laskay T, Rollinghoff M, Solbach W. 1993. Natural killer cells participate in the early defence against Leishmania major infection in mice. Eur J Immunol 23: 2237.

78. Hauser WE, Tsai V. 1986. Acute toxoplasma infection of mice induces spleen NK cells that are cytotoxic for T. gondii in vitro. J Immunol 136: 313.

79. Hunter CA, Candolfi E, Subauste C, Van Cleave V, Remington JS. 1995. Studies on the role of interleukin-12 in acute murine toxoplasmosis. Immunol 84: 16.

80. Gazzinelli RT, Hieny S, Wynn TA, Wolf S, Sher A. 1993. Interleukin-12 is required for the T-lymphocyte-independent induction of interferon gamm by an intracellular parasite and induces resistance in T-cell-deficient hosts. Proc Natl Acad Sci USA 90: 6115.

81. Cudkowicz G, Stimpfling JH. 1992. Deficient growth of C57BL6 marrow cells transplanted into F1 hybrid mice. Association with the histocompatibility-2 locus. Immunology 7: 291.

82. Yu YYL, Kumar v, Bennett M. 1992. Murine natural killer cells and marrow allograft rejection. Annu Rev Immunol 10: 189.

83. Murphy WJ, Keller JR, Harrison CL, Young HA, Longo DL. 1992. Interleukin-2-activated natural killer cells can support hematopoiesis in vitro and promote marrow engraftment in vivo. Blood 80: 670.

84. Murphy WJ, Bennet M, Kumar V, Longo DL. 1992. Donor-type activated natural killer cells promote marrow engraftment and B cell development during allogenic bone marrow transplantation. J Immunol 148: 2953.

85. van den Brink MRM, Voogt PJ, Marijt WAF, van Luxambourg-Heys SAP, van Rood JJ, Brand A. 1989. Lymphokine-activated killer cells selectively kill tumor cells in bone marrow without compromising bone marrow stem cell function in vitro. Blood 74: 354.

86. Bellone G, Valiante NM, Viale O, Ciccone E, Moretta L, Trinchieri G. 1993. Regulation of hematopoiesis in vitro by alloreactive natural killer cell clones. J Exp Med 177: 1117.

87. Fleisher G, Koven N, Kamiya H, Henle W. 1982. A non-X-linked syndrome with susceptibility to sever Epstein-Barr virus infections. J Pediatr 100: 727.

88. Biron CA, Byron KS, Sullivan JL. 1988. Susceptibility to viral infections in an individual with complete lack of natural killer cells. Nat Immun Cell Growth Regul 7: 47.

89. Roder JC, Lohmann-Matthes M, Domzig W, Wigzell H. 1979. The beige mutation in the mouse. II. Selectivity of the natural killer (NK) cell defect. J Immunol 123: 2174.

90. Hanna N, Burton RC. 1981. Definitive evidence that natural killer (NK) cells inhibit experimental tumor metastasis *in vivo*. J Immunol 127: 1754.

91. Ando K, Hunter N, Peters LJ. 1980. Inhibition of artificial lung metastases in mice by pre-irradiation of abdomen. Br J Cancer 41: 250.

92. Mazumder A, Rosenberg SA. 1984. Successful immunotherapy of natural killer resistant established pulmonary melanoma metastases by the intravenous adoptive transfer of syngeneic lymphocytes activated *in vitro* by interleukin 2. J Exp Med 159: 495.

93. Ross GD, Thompson RA, Walport MJ, Springer TA, Watson JV, Ward TH, Lida J, Newman SL, Harrison RA, Lachman PJ. 1985. Characterization of patients with an increased susceptibility to bacterial infections and a genetic deficiency of leukocyte membrane complement receptor type 3 and the related membarne antigen LFA-1. Blood 66: 882.

94. White JG, Clawson CC. 1980. The Chediak-Higashi syndrome: the nature of the giant neutrophil granules and their interactions with cytoplasm and foreign paticulates. Am J Pathol 98: 151.

95. Haliotis T, Roder J, Klein M, Ortaldo J, Fauci AS, Herbermann RB. 1980. Chediak-Higashi gene in humans. I. Impairment of natural killer function. J Exp Med 151: 1039.

96. Klein M, Roder J, Haliotis T, Korec S, Jett JR, Herbermann RB, Katz P, Fauci AS. 1980. Chediak-Higashi gene in humans. II. The selectivity of the defect in natural killer and antibody-dependent cell-mediated cytotoxicity function. J Exp Med 151: 1049.

97. Dent PB, Fish LA, White JF, Good RA. 1966. Chediak-Higashi syndrome. Observation of the nature of the associated malignancy. Lab Invest 15: 1634.

98. Henkart PA. 1985. Mechanism of lymphocyte-mediated cytotoxicity. Annu Rev Immunol 3: 31.

99. Podack ER. 1985. Molecular mechanism of lymphocyte-mediated tumor cell lysis. Immunol Today 6: 21.

100. Henkart PA, Millard PJ, Reynold CW, Henkart MP. 1984. Cytotic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. J Exp Med 160: 75.

101. Masson D, Tschopp J. 1985. Isolation of a lytic, pore-forming protein (perforin) from cytolytic T lymphocytes J Biol Chem 260: 9069.

102. Podack ER, Young JE, Cohen ZA. 1985. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. Proc Natl Acad Sci USA 82: 8629.

103. Dennert G, Podack ER. 1983. Cytolysis by H-2 specific T killer cells. Assembly of tubular complexes on target membranes. J Exp Med 157: 1483.

104. Shiver JW, Su L, Henkart PA. 1992. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolysin and granzyme A. Cell 71: 315.

105. Shi L, Kraut RP, Aebersold R, Greenberg AH. 1992. A natural killer cell granule protein that induces DNA fragmentation and apoptosis. J Exp Med 175: 553.

106. Frey T, Petty HR, McConnell HM. 1982. Electron microscopic study of natural killer-tumor cell conjugates. Proc Natl Acad Sci USA 79: 5317.

107. Quan PC, Ishizaka T, Bloom BR. 1982. Studies on the mechanism of NK cell lysis. J Immunol 128: 1786.

108. Jenne D, Rey C, Masson D, Stanley KK, Herz J, Plaetnick G, Tschopp J. 1988. cDNA cloning of granzyme C, a granule-associated serine protease of cytolytic T lymphocytes. J Immunol 140: 318.

109. MacDermott RP, Schmidt RE, Caulfield JP, Hein A, Bartley GT, Ritz J, Schlossman SF, Austen KF, Stevens RL. 1985. Proteoglycans in cell-mediated cytotoxicity. Identification, localization, and exocytosis of a chondoidin sulfate proteoglycan from human cloned natural killer cells during target cell lysis. J Exp Med 162: 1771.

110. Dupuis M, Shaerer E, Krause KH, Tschopp J. 1993. The calcium-binding protein calreticulin is a major constituent of lytic granules of cytolytic T lymphocytes. J Exp Med 177: 1.

111. Burkhardt JK, Hester S, Lapham CK, Argon Y. 1990. The lytic granules of natural killer cells are dual function organelles combining secretory and prelysosomal compartments. J Cell Biol 111: 2327.

112. Peters PJ, Geuze HJ, Van der Donk HA, Slot JW, Griffith JM, Stam NJ, Clevers HC, Borst J. 1989. Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes. Eur J Immunol 19: 1469.

113. Liu CC, Steffen M, King F, Young JD. 1986. Identification and characterization of a pore-forming protein of human peipheral blood NK cells. J Exp Med 164: 2061.

114. Hameed A, Lowrey DM, Lichtenheld M, Podack E. 1988. Characterization of three serine esterases isolated from human IL-2 activated killer cells. J Immunol 141: 3142.

115. Trapani JA, Smyth MJ. 1993. Killing by cytotoxic T cells and natural killer cells: multiple granule serine proteases as initiators of DNA fragmentation. Immunol Cell Biol 71: 201.

116. Hudig D, Allison VJ, Pickett TN, Winkler V, Kam C, Powers J. 1991. The function of lymphocyte proteases. Inhibition and restoration of granule mediated lysis with isocoumarin serine protease inhibitors. J Immunol 147: 1360.

117. Smyth MJ, Trapani JA. 1995. Granzymes: exogenous proteinases that induce target cell apoptosis. Immunol Today 16: 202.

118. Shiver JW, Su L, Henkart PA. 1992. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolysin and granzyme A. Cell 71: 315.

119. Shi L, Kam CM, Powers JC, Aebersold R, Greenberg AH. 1992. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. J Exp Med 176: 1521.

120. Hudig D, Ewoldt GR, Woodward SL. 1993. Proteases and lymphocyte cytotoxic killing mechanisms. Curr Opin Immunol 5: 90.

121. Shresta S, Thomas D, Russel J, Hausmann M, Simon MM, Ley TJ. 1996. Cytotoxic lymphocytes derived from mice deficient for granzyme A and B have an unexpected defect in cytotoxicity. Blood 88 Suppl 1: 1831.

122. Henkart PA. 1996. ICE family proteases: mediators of all apoptotic cell death? Immunity 4:195.

123. Darmon AJ, Nicholson DW, Bleackley RC. 1995. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. Nature 377: 446.

124. Shi L, Chen G, MacDonald G, Bergeron L, Li H, Miura M, Rotello RJ, Miller DK, Li P, Seshadri T, Yuan J. 1996. Activation of an interleukin 1 converting enzyme-dependent apoptosis pathway by granzyme B. Proc Natl Acad Sci USA 93: 11002.

125. Tian Q, Streuli M, Saito H, Schlossman SF, Anderson P. 1991. A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. Cell 67: 629.

126. Masson D, Peters PJ, Geuze HJ, Borst J, Tschopp J. 1990. Interaction of chondroidin sulfate with perforin and granzymes of cytolytic T-cells is dependent on pH. Biochemistry 29: 11229.

127. Burns K, Duggan B, Atkinson EA, Famulski KS, Nemer M, Bleackley RC, Michalak M. 1994. Modulation of gene expression by calreticulin binding to the glucocorticoide receptor. Nature 367: 467.

128. Dedhar S, Rennie PS, Shago M, Leung Hagesteijn CY, Yang H, Filmus J, Hawley RG, Bruchovsky N, Cheng H, Matusik RJ, Giguere V. 1994. Inhibition of nuclear hormone receptor activity by calreticulin. Nature 367: 480.

129. Burns K, Helgason CD, Bleackley RC, Michalak M. 1992. Calreticulin in T-lymphocytes. Identification of calreticulin in T-lymphocyte and demonstration that activation of T cells correlates with increased levels of calreticulin mRNA and protein. J Biol Chem 267: 19039.

130. Kagi D, Ledermann B, Burki K, Seller P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM, Hengartner H.1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 369: 31.

131. Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S, Hengartner H, Golstein P. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science 265: 528.

132. Henkart PA. 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. Immunity 1: 343.

133. Rouvier E, Luciani MF, Golstein P. 1993. Fas involvement in Ca^{2+}-independent T cell-mediated cytotoxicity. I Exp Med 177: 195.

134. Watanabe-Fukunaga R, Brannan CI, Itoh N, Yonehara S, Copeland NG, Jenkins NA, Nagata S. 1992. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J Immunol 148: 1274.

135. Macienjewski J, Selleri C, Anderson S, Young NS. 1995. Fas antigen expression on CD34+ human marrow cells is induced by interferon γ and tumor necrosis factor α and potentiates cytokine-mediated hematopoietic suppression in vitro. Blood 85: 3183.

136. Yonehara S, Ishii A, Yonehara M. 1989. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med 169: 1747.

137. Rouvier E, Luciani MF, Golstein P. 1993. Fas involvement in Ca^{2+} independent T cell-mediated cytotoxicity. J Exp Med 177: 195.

138. Hanabuchi S, Koyanagi M, Kawasaki A, Shinohara N, Matsuzawa A, Nishimura Y, Kobayashi Y, Yonehara Y, Yagita H, Okumura K. 1994. Fas and its ligand in a general mechanism of T-cell-mediated cytotoxicity. Proc Natl Acad sci USA 91: 4930.

139. Arase H, Arase N, Saito T. 1994. Fas-mediated cytotoxicity by freshly isolated natural killer cells. J Exp Med 181:1235.

140. Oshimi Y, Oda S, Honda Y, Nagata S, Miyazaki S. 1996. Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells. J Immunol 157: 2909.

141. Suda T, Takahashi T, Goldstein P, Nagata S. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 75: 1169.

142. Suda T, Nagata S. 1994. Purification and characterization of the Fas-ligand that induces apoptosis. J Exp Med 179: 873.

143. Lee RK, Spielman J, Zhao DY, Olsen KJ, Podack ER. 1996. Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine-activated killer cells. J Immunol 157:1919.

144. Filep JG, Baron C, Lachance S, Perreault, Chan JSD. 1996. Involvement of nitric oxide in target-cell lysis and DNA fragmentation induced by murine natural killer cells. Blood 87: 5136.

145. Cifone MG, Festuccia C, Cironi L, Cavallo G, Chessa MA, Pensa V, Tubaro E, Santoni A. 1994. Induction of nitric oxide-synthetizing pathway in fresh and interleukin-2-cultured rat natural killer cells.Cell Immunol 157: 181.

146. Springer TA. 1990. Adhesion receptors of the immune system. Nature 346: 425.

147. Patarroyo M, Prieto J. Rincon J, Timonen T, Lundberg C, Lindbom I, Asjo B, Gahmberg CG. 1990. Leukocyte-cell adhesion: a molecular process fundamental in leukocyte physiology. Immunol Rev 114: 67.

148. Storkus WJ, Dawson JR. 1991. Target structures involved in natural killing (NK): characteristics, distribution, and candidate molecules. Crit Rev Immunol 10:393.

149. Shaw S, Ginther Luce GE, Quinones R, Gress RE, Springer TA, Sanders MA. 1986. Two antigen-independent adhesion pathways used by human cytotoxic T-cell clones. Nature 323: 262.

150. Boyer MW, Orchard PJ, Gorden KB, Anderson PM, McIvor RS, Blazar BR. 1995. Dependency on intercellular adhesion molecule recognition and local interleukin-2 provision in generation of an in vivo CD8+ T-cell immune response to murine myeloid leukemia. Blood 85: 2498.

151. Schmidt RE, Bartley G, Levine H, Schlossman SF, Ritz J. 1985. Functional characterization of LFA-1 antigen in the interaction of human NK clones and target cells. J Immunol 135: 1020.

152. Palmieri G, Serra A, De Maria R, Gismodi A, Milella M, Piccoli M, Frati L, Santoni A. 1995. Cross-linking of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ fibronectin receptors enhances natural killer cell cytotoxic activity. J Immunol 155: 5314.

153. Gismodi A, Mainiero F, Morrone S, Palmieri G, Piccoli M, Frati L. 1992. Triggering through CD16 or phorbol esters enhances adhesion of NK cells to laminin via very late antigen 6. J Exp Med 176: 1251.

154. Hibbs ML, Jakes S, Stacker SA, Wallace RW, Springer TA. 1991. The cytoplasmic domain of the integrin lymphocyte function-associated antigen1 β subunit: sites required for binding to intercellular adhesion molecule-1 and the phorbol ester-stimulated phosphorylation site. J Exp Med 174: 1227.

155. Stewart MP, Cabanas C, Hogg N. 1996. T cell adhesion to intercellular adhesion molecule-1 (ICAM-1) is controlled by cell spreading and the activation of integrin LFA-1. J Immunol 156: 1810.

156. Hynes RO. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11.

157. Kanner SB, Grosmaire LS, Ledbetter JA, Damle NK. 1993. β2-integrin LFA-1 signaling through phospholipase C-γ1 activation. Proc Natl Acad Sci USA 90: 7099.

158. Bierer BE, Burakoff SJ. 1988. T cell adhesion molecules. Faseb J 2: 2584.

159. Damle NK, Klussman K, Linsley PS, Aruffo A. 1992. Differential costimulatory effects of adhesion molecules B7, ICAM-1, LFA-3, and VCAM-1 on resting and antigen-primed CD4+ T lymphocytes. J Immunol 148: 1985.

160. Damle NK, Klussman K, Aruffo A. 1992. Intercellular adhesion molecule-2, a second counter-receptor for CD11a/CD18 (leukocyte function-associated antigen-1) provides a costimulatory signal for T-cell receptor-initiated activation of human T cells. J Immunol 148: 665.

161. Timonen T, Patarroyo M, Gahmberg CG. 1988. CD11a-c/CD18 and GP84 (LB-2) adhesion molecules on human large granular lymphocytes and their participation in natural killing. J Immunol 141: 1041.

162. Ritz J, Schmidt RE, Michon J, Hercend T, Schlossman SF. 1988. Characterization of functional surface structures on human natural killer cells. Adv Immunol 42: 181.

163. Schmits R, Kundig TM, Baker DM, Shumaker G, Simard JJL, Duncan G, Wakeham A, Shahinian A, van der Heiden A, Bachmann MF, Ohashi PS, Mak TW, Hickstein DD. 1996. LFA-1 deficient mice show normal CTL responses to virus but fail to reject immunogenic tumor. J Exp Med 183: 1415.

164. Sligh JE Jr, Ballantyne CM, Rich SS, Hawkins HK, Smith CW, Bradley A, Beaudet AL. 1993. Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule-1. Proc Natl Acad Sci USA 90: 8529.

165. Connolly ES, Winfree CJ, Springer TA, Naka Y, Liao H, Yan SD, Stern DM, Solomon RA, Gutierrez-Ramos JC, Pinsky DJ. 1996. Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. J Clin Invest 97: 209.

166. Soriano SG, Lipton SA, Wang YF, Xiao M, Springer TA, Gutierrez-Ramos JC, Hickey PR. 1996. Intercellular adhesion molecule-1-deficient mice are less susceptible to cerebral ischemia-reperfusion injury. Annals of Neurol 39: 618-24.

167. Staunton DE, Marlin SD, Stratowa C, Dustin ML, Springer TA. 1988. Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell 52: 925.

168. Staunton DE, Dustin ML, Erickson HP, Springer TA. 1990. The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. Cell 61: 243.

169. Ockenhouse CF, Betageri R, Springer TA, Staunton DE. 1992. Plasmodium falciparum-infected erythrocytes bind ICAM-1 at a site distinct from LFA-1, Mac-1, and human rhinovirus. Cell 68: 63.

170. Rosenstein Y, Park JK, Hahn WC, Rosen FS, Bierer BE, Burakoff SJ. 1991. CD43, a molecule defective in Wiskott-Aldrich syndrome, binds ICAM-1. Nature 354: 233.

171. Staunton DE, Dustin ML, Springer TA. 1989. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature 339: 61.

172. Fawcett J, Holness CLL, Needham LA, Turley H, Gatter KC, Mason DY, Simmons DL. 1992. Molecular cloning of ICAM-3 a third ligand for LFA-1, constitutively expressed on resting leukocytes. Nature 360: 481.

173. Vazeux R, Hoffman PA, Tomita JK, Dickinson ES, Jasman RL, St John T, Gallatin WM. 1992. Cloning and characterization of a new intercellular adhesion molecule ICAM-R. Nature 360: 485.

174. De Fourgerolles AR, Stacker SA, Schwarting R, Springer TA. 1991. Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. J Exp Med 174: 253.

175. Huang C, Springer TA. 1995. A binding interface on the I domain of lymphocyte function antigen-1 (LFA-1) required for specific interaction with intercellular adhesion molecule 1(ICAM-1). J Biol Chem 270: 19008

176. Binnerts ME, van Kooyk Y, Simmons DL, Fidgor CG. 1994. Distinct binding of T lymphocytes to ICAM-1, -2, or -3 upon activation of LFA-1. Eur J Immunol 24: 2155.

177. Binnerts ME, van Kooyk Y, Edwards CP, Champe M, Presta L, Bodary SC, Fidgor CG, Berman PW. 1996. Antibodies that selectively inhibit leukocyte function-associated antigen 1 binding to intercellular adhesion molecule-3 recognize a unique epitope within the CD11a I domain. J Biol Chem 271: 9962.

178. Arroyo AG, Campanero MR, Sanchez-Mateos P, Zapata JM, Ursa MA, del Pozo MA, Sanchez-Madrid F. 1994. Induction of tyrosine phosphorylation during ICAM-3 and LFA-1 mediated intercellular adhesion, and its regulation by CD45 tyrosine phosphatase. J Cell Biol 126: 1277.

179. Van der Vieren M, Le Trong H, Wood CL, Moore PF, St John T, Staunton DE, Gallatin WM. 1995. A novel leukointegrin, αd β2, binds preferentially to ICAM-3. Immunity 3: 683.

180. Rothlein R, Kishimoto TK, Mainolfi E. 1994. Cross-linking of ICAM-1 induces co-signaling of an oxidative burst from mononuclear leukocytes. J Immunol 152: 2488.

181. Chirathaworn C, Tibbetts SA, Chan MA, Benedict SH. 1995. Cross-linking of ICAM-1 on T cells induces transient tyrosine phosphorylation and inactivation of cdc2 kinase. J Immunol 155: 5479.

182. De Fourgerolle AR, Klickstein LB, Springer TA. 1993. Cloning and expression of ICAM-3 reveals strong homology to their other immunoglobulin family counter-receptors for LFA-1. J Exp Med 177: 1187.

183. Lozano F, Alberola-Ila J, Places L, Vives J. 1992. Effect of protein kinase C activators on the phosphorylation and surface expression of CDw50. Eur J Biochem. 203: 321.

184. Skubitz KM, Ahmed K, Campbell KD, Skubitz APN. 1995. CD50 (ICAM-3) is phosphorylated on tyrosine and is associated with tyrosine kinase activity in human neutrophils. J Immunol 154: 2888.

185. Cid MC, Esparza J, Juan M, Miralles A, Ordi J, Vilella R, Urbano-Marquez A, Gaya A, Vives J, Yague J. 1994. Signaling through CD50 (ICAM-3) stimulates T lymphocyte binding to human umbilical vein endothelial cells and extracellular matrix proteins via an increase in β1 and β2 function. Eur J Immunol 24: 1377.

186. Hernandez-Caselles T, Rubio G, Campanero MR, del Pozo MA, Muro M., Sanchez-Madrid F, Aparicio P. 1993. ICAM-3, the third LFA-1 counter-receptor, is a co-stimulatory molecule for both resting and activated T lymphocytes. Eur J Immunol 23: 2799.

187. Juan M, Vinas O, Pino-Otin MR, Ordi J, Places L, Martinez-Cacerec E, Barcelo JJ, Miralles A, Vilella R, de la Fuente MA, Vives J, Yague J, Gaya A. 1994. CD50 (ICAM-3) stimulation induces calcium mobilization and tyrosine phosphrylation through $p59^{fyn}$ and $p56^{lck}$ in Jurkat T cell line. J Exp Med 179: 1747.

188. Campanero MR, del Pozo MA, Arroyo AG, Sanchez-Mateos P, Hernandez-Caselles T, Craig A, Pulido R, Sanchez-Madrid F. 1993. ICAM-3 interacts with LFA-1 and regulates the LFA-1/ICAM-1 cell adhesion pathwy. J Cell Biol 123: 1007.

189. Pals ST, Hogervorst F, Keizer GD, Thepen T, Horst E, Fidgor CC. 1989. Identification of a widely distributed 90-kDa glycoprotein that is homologous to the Hermes-1 human lymphocyte homing receptor. J Immunol 143: 851.

190. Lesley J, Hayman R, Kincade PW. 1993. CD44 and its interaction with the extracellular matrix. Adv Immunol 54: 271.

191. Idzerda RL, Carter WG, Nottenburg C, Wayner EA, Gallatin WM, St John T. 1989. Isolation and DNA sequence of a cDNA clone encoding a lymphocyte adhesion receptor for high endothelium. Proc Natl Acad Sci USA 86: 4659.

192. Miyake K, Medina KL, Hayashi SI, Ono S, Hamaoka T, Kincade PW. 1990. Monoclonal antibodies to Pgp-1/CD44 block lymphocyte-hemopoiesis in long-term bone marrow cultures. J Exp Med 171: 477.

193. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. 1990. CD44 is the principal cell surface receptor for hyaluronan. Cell 61: 1303.

194. Haynes BF, Telen MJ, Hale LP, Denning SM. 1992. CD44 a molecule involved in leukocyte adherence and T cell activation. Immunol Today 10: 423.

195. Shimizu Y, Van Sevnter GA, Siraganian R, Wahl L, Shaw S. 1989. Dual role for the CD44 molecule in T cell adhesion and activation. J Immunol 143: 2457.

196. Huet S, Groux H, Caillou B, Valentin H, Prieur AM, Bernard A. 1989. CD44 contributes to T cell activation. J Immunol 143: 798.

197. Denning SM, Le PT, Singer KH, Haynes BF. 1990. Antibodies against CD44 p80, lymphocyte homing receptor molecule augment human peripheral blood T cell activation. J Immunol 144: 7.

198. Galandrini R, De Maria R, Piccoli M, Frati L, Santoni A. 1994. CD44 triggering enhances human NK cell cytotoxic functions. J Immunol 153: 4399.

199. Sconocchia G, Titus JA, Segal DM. 1994. CD44 is a cytotoxic triggering molecule in human peripheral blood NK cells. J Immunol 153: 5473.

200. Tan PHS, Santos EB, Rossbach HC, Sandmaier BM. 1993. Enhancement of natural killer activity by an antibody to CD44. J Immunol 150: 812.

201. Stamenkovic I, Aruffo A, Amiot M, Seed B. 1991. The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. EMBO J 10: 343.

202. Gunthert U, Hofmann M, Rudy W, Reber S, Zoller M, Hausmann I, Matzku S, Wenzel A, Ponta H, Herrlich P. 1991. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 65: 13.

203. Dougherty GJ, Lansdorp PM, Cooper DL, Humphries RK: 1991. Molecular cloning of CD44R1 and CD44R2, two novel isoforms of the human CD44 lymphocyte "homing" receptor expressed by hemopoietic cells. J Exp Med 174: 1.

204. Jackson DG, Buckley J, Bell JI. 1992. Multiple variants of the human lymphocyte homing receptor CD44 generated by insertion at a single site in the extracellular domain. J Biol Chem 267: 4732.

205. Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI. 1992. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc Natl Acad Sci USA 89: 12160.

206. Tolg C, Hofmann M, Herrlish P, Ponta H. 1993. Splicing choice from ten variant exons establishes CD44 variability. Nucleic Acids Research 21: 1225.

207. Droll A, Dougherty ST, Chiu RK, Dirks JF, McBride WH, Cooper DL, Dougherty GJ. 1995. Adhesive interactions between alternatively spliced CD44 isoforms. J Biol Chem 270: 11567.

208. Galandrini R, Albi N, Tripodi G, Zarcone D, Terenzi A, Moretta A, Grossi CE, Velardi A. 1993. Antibodies to CD44 trigger effector functions of human T cell clones. J Immunol 150: 4225.

209. Galandrini R, Galluzzo E, Albi N, Grossi CE, Velardi A. 1994. Hyaluronate is costimulatory for human T cell effector functions and binds to CD44 on activated T cells. J Immunol 153: 21.

210. Bourguignon L, Lokeshwar VB, Chen X, Kerrick WGL. 1993. Hyaluronic acid induced lymphocyte signal transduction and HA receptor (GP85/CD44) cytoskeleton interaction. J Immunol 151: 6634.

211. Taher TEI, Smit L, Griffioen AW, Schilder-Tol EJM, Borst J, Pals ST. 1996. Signaling through CD44 is mediated by tyrosine kinases: association with $p56^{lck}$ in T lymphocytes. J Biol Chem 271: 2863.

212. Springer TA, Dustin ML, Kishimoto TK, Marlin SD. 1989. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. Ann Rev Immunol 10: 370.

213. Petranyi GG, Pocsik E, Kutian B, Gorog G, Benczur M. 1986. Regulatory fuction of cell surface molecules CD2, LFA-1, and β2-microglobulin in natural killer cell activity. Mol Immunol 23: 1275.

214. Bolhuis RLH, Roozemond RC, van de Griend RJ. 1986. Induction and blocking of cytolysis in CD2+, CD3- NK and CD2+, CD3+ cytotoxic T lymphocytes via CD2 50 kD sheep erythrocyte receptor. J Immunol 136: 3939.

215. Seed B. 1987. An LFA-3 cDNA encodes a phosphlipid-linked membrane protein homologous to its receptor CD2. Nature 329: 840.

216. Dustin ML, Selvaraj P, Mattaliano RJ, Springer TA. 1987. Anchoring mechanisms for LFA-3 cell adhesion glycoprotein at membrane surface. Nature 329: 846.

217. Wallner BP, Frey AZ, Tizard R, Mattaliano RJ, Hession C, Sanders ME, Dustin ML, Springer TA. 1987. Primary structure of lymphocyte function-associated antigen 3 (LFA-3) the ligand of the T lymphocyte CD2 glycoprotein. J Exp Med 166: 923.

218. Anasetti C, Martin PJ, June CH, Hellstrom KE, Ledbetter JA, Rabinovitch PS, Morishita Y, Hellstrom I, Hansen JA. 1987. Induction of calcium flux and enhancement of cytolytic activity in natural killer cells by cross-linking of the sheep erythrocyte binding protein (CD2) and the Fc-receptor (CD16). J Immunol 139: 1772.

219. Saito Y, Tada H, Sabe H, Honjo T. 1991. Biochemical evidence for a third chain of interleukin-2 receptor. J Biol Chem 266: 22186.

220. Voss SD, Sondel PM, Robb RJ. 1992. Characterization of interleukin-2 receptor (IL-2R) expressed on human natural killer cells activated in vivo by IL-2: association of the p64 IL-2R γ chain with the IL-2R β chain in functional intermediate-affinity IL-2R. J Exp Med 176: 531.

221. Smith KA. 1993. Lowest dose interleukin-2 immunotherapy. Blood 81:1414.

222. Caligiuri MA, Zmuidzinzs A, Manley TJ, Levine H, Smith KA, Ritz J. 1990. Functional consequences if interleukin 2 receptor expression on resting human lymphocytes. Identification of a novel natural killer cell subset with high affinity receptors. J Exp Med 171: 1509.

223. Minami Y, Nakagawa Y, Kawahara A, Miyazaki T, Sada K, Yamamura H, Taniguchi T. 1995. Protein tyrosine kinase Syk is associated with and activated by the IL-2 receptor: possible link with the c-myc induction pathway. Immunity 2: 89.

224. Miyazaki T, Liu ZJ, Minami Y, Yamada K, Tsujimoto Y, Barsoumian EL, Perlmutter RM, Taniguchi T. 1995. Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation. Cell 81: 223.

225. Vivier E, da Silva AJ, Ackerly M, Levine H, Rudd CE, Anderson P. 1993. Association of a 70 kDa tyrosine phosphoprotein with the CD16: ζ: γ complex expressed in human natural killer cells. Eur J Immunol 23: 1872.

226. Ting AT, Dick CJ, Schoon RA, Karnitz LM, Abraham RT, Leibson PJ. 1995. Interaction between Lck and Syk family tyrosine kinases in Fcγ receptor-initiated activation of natural killer cells. J Biol Chem 270: 16415.

227. Pricop L, Galatiuc C, Manciules M, DeLeo A, Sulica A, Herberman RB, Whiteside TL. 1991. Expression of Fc mu receptors on human natural killer cells. Clin Immunol Immunopathol 59: 355.

228. Rabinowich H, Manciulea M, Metes D, Sulica A, Herberman RB, Corey SJ, Whiteside TL. 1996. Physical and functional association of Fcμ receptor on human natural killer cells with the ζ and FcRI γ chians and with *src* family protein tyrosine kinases. J Immunol 157: 1485.

229. Azuma M, Cayabyab M, Buck D, Phillips JH, Lanier LL. 1992. Involvement of CD28 in MHC-unrestricted cytotoxicity mediated by human natural killer leukemia cell line. J Immunol 149: 1115.

230. Teng JMC, Liu XR, Mills GB, Dupont B. 1996. CD28-mediated cytotoxicity by human leukemic cell line YT involves tyrosine phosphorylation, activation of phosphatidylinositol 3-kinase, and protein kinase C. J Immunol 156: 3222.

231. Trinchieri G. 1994. Recognition of major histocompatibility complex class I antigens by natural killer cells. J Exp Med 180: 417.

232. Yokoyama WM. 1995. Natural killer cell receptors specific for MHC class I molecules. Proc Natl Acad Sci USA 92: 3081.

233. Raulet DH, Held W. 1995. Natural killer cell receptors: the offs and ons of NK cell recognition. Cell 82: 697.

234. Lanier LL, Phillips JH. 1996. Inhibitory MHC class I receptors on NK cells and T cells. Immunol Today 17: 86.

235. Kaufman DS, Schoon RA, Robertson MJ, Leibson PJ. 1995. Inhibition of selective signaling events in natural killer cells recognizing major histocompatibility complex class I. Proc Natl Acad Sci USA 92: 6484.

236. Giorda R, Rudert WA, Vavassori C, Chambers WH, Hiserodt JC, Trucco M. 1990. NKR-P1, a signal transduction molecule on natural killer cells. Science 249: 1298.

237. Giorda R, Trucco M. 1991. Mouse NKR-P1 a family of genes selectively coexpressed in adherent lymphokine-activated killer cells. J Immunol 147: 1701.

238. Houchins JP, Yabe T, McSherry C, Bach FH. 1991. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. J Exp Med 173: 1017.

239. Ryan JC, Niemi EC, Goldfien RD, Hiserodt JC, Seeman WE. 1991. NKR-P1, an activating molecule on rat natural killer cells, stimulates phosphoinositide turnover and a rise in intracellular calcium. J Immunol 147: 3244.

240. Bezouska K, Yuen CT, O'Brien J, Childs RA, Chai W, Lawson AM, Drbal K, Fiserova A, Popisil M, Feizi T. 1996. Oligosaccharide ligands for NKR-P1 protein activate NK cells and Cytotoxicity. Nature 372:150.

241. Duchler M, Offerdinger M, Holzmuller H, Chu CT, Aschauer B, Bach FH, Hofer E. 1995. NKG2-C is a receptor on human natural killer cells that recognizes structures on K562 target cells. Eur J Immunol 25: 2923.

242. Yokoyama WM, Seaman WE. 1993. The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cell: The NK gene complex. Annu Rev Immunol 11: 613.

243. Takei F, Brennan J, Mager DL. 1996. The Ly-49 family: Genes, proteins, and recognition of class I MHC. Immunol Rev 155: 67.

244. Chan PY, Takei F. 1986. Expression of a T cell receptor-like moecule on normal and malignant murine T cells detected by rat monoclonal antibodies to nonclonotypic determinants. J Immunol 136: 1346.

245. Chan PY, Takei F. 1988. Characterization of a murine T cell surface disulfide-linked dimer of 45-kDa glycoprotein (YE1/48 antigen). Comparison with T cell receptor, purification, and partial amino acid sequences. J Immunol 140: 161.

246. Nagasawa R, Gross J, Kanagawa O, Townsend K, Lanier LL, Chiller J, Allison JP. 1987. Identification of a novel T cell surface disulfide-bonded dimer distinct from the alpha/beta antigen receptor. J Immunol 138: 815.

247. Chan PY, Takei F. 1989. Molecular cloning and characterization of a novel murine T cell surface antigen, YE1/48. J Immunol 142: 1727.

248. Yokoyama WM, Jacobs LB, Kanagawa O, Shevach EM, Cohen DI. 1989. A murine T lymphocyte antigen belongs to a supergene family of type II integral membrane protines. J Immunol 143: 1379.

249. Yokoyama WM, Kehn PJ, Cohen DI, Shevach EM. 1990. Chromosomal location of the Ly-49 (A1, YE1/48) multigene family. Genetic association with the NK1.1 antigen. J Immunol 145: 2353.

250. Karlhofer FM, Ribaudo RK, Yokoyama WM. 1992. MHC class I alloantigen specificity of Ly49+ IL-2 activated natural killer cells. Nature 358: 66.

251. Daniels B, Karlhofer FM, Seaman WE, Yokoyama WM. 1994. A natural killer cell receptor specific for a major histocompatibility complex class I molecule. J Exp Med 180: 687.

252. Bennett M, Yu LL, Stoneman E, Rembecki RM, Mttehew PA, Lindahl KF, Kumar V. 1995. Hybrid resistance: 'negative' and 'posotive' signaling of murine natural killer cell. Sem Immunol 7: 121.

253. Brennan J, Mager D, Jefferies W, Takei F. 1994. Expression of different members of the Ly-49 gene family defines distinct natural killer cell subsets and cell adhesion properties. J Exp Med 180: 2287.

254. Masson LH, Ortaldo JR, Young HA, Kumar V, Bennett M, Anderson SK. 1995. Cloning and functional characteristics of murine large granular lymphocyte-1: a member of the Ly-49 gene family (Ly-49G2). J Exp Med 182: 293.

255. Moretta L, Ciccone E, Mingari MC, Biassoni R, Moretta A. 1994. Human natural killer cells: origin, clonality, specificity, and receptors. Adv Immunol 55: 341.

256. Moretta A, Viale M, Bottino C, Orengo AM, Morelli L, Augugliaro R, Barbaresi M, Ciccone E, Moretta L. 1993. p58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specifities. J Exp Med 178: 597.

257. Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. 1994. NKB1: a natural killer cell receptor involved in the recognition of HLA-B. J Exp Med 180: 537.

258. Colonna M, Borsellino G, Falco M, Ferrara GB, Strominger JL. 1993. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK-1 and NK-2 specific natural killer cells. Proc Natl Acad Sci USA 90: 1200.

259. Colonna M, Brooks EG, Falco M, Ferrara GB, Strominger JL. 1993. Generation of allospecific natural killer cells by stimulation across polymorphism of HLA-C. Science 260: 1121.

260. Yokoyama W. 1995. Natural killer cell receptors specific for major histocompatibility complex class I molecules. Proc Natl acad Sci USA 92: 3081.

261. Dohring C, Scheidegger D, Samaridis J, Cella M, Colonna M. 1996. A human killer inhibitory receptor specific for HLA-A. J Immunol 156: 3098.

262. Aramburu J, Balboa MA, Izquierdo M, Lopez-Botet M. 1991. A novel functional cell surface dimer (Kp43) expressed by natural killer cells and γ/δ TCR+ T lymphocytes. II. Modulation of natural killer cytotoxicity by anti Kp43 monoclonal antibody. J Immunol 147: 714.

263. Moreatta A, Vitale M, Sivori S, Bottino C, Morelli L, Augugliaro R, Barbaresi M, Pende D, Ciccone E, Lopez-Botet M, Moretta L. 1994. Human natural killer cell receptors for HLA-class I molecules. Evidence that the Kp43 (CD94) molecule functions as receptor for HLA-B alleles. J Exp Med 180: 545.

264. Molero I, Salmeron A, Balboa MA, Aramburu J, Lopez-Botet M. 1994. Tyrosine kinase-dependent activation of human NK cell functions upon stimulation through a 58-kDa surface antigen selectively expressed on discrete subsets of NK cells and T lymphocytes. J Immunol 152: 1662.

265. Aramburu J, Balboa MA, Rodriguez A, Melero I, Alonso M, Alonso JL, Lopez-Botet M. 1993. Stimulation of IL-2-activated natural killer cells through the Kp43 surface antigen up-regulates TNF-α production involving the LFA-1 integrin. J Immunol 151: 3420.

266. Leibson PJ. 1995. MHC-recongnizing receptors: they're not just for T cells anymore. Immunity 3: 5.

267. Perez-Villar JJ, Melero I, Rodriguez A, Carretero M, Aramburu J, Sivori S, Orengo AM, Moretta A, Lopez-Botet M. 1995. Functional ambivalence of the Kp43 (CD94) NK cell-associated surface antigen. J Immunol 154: 5779.

268. Brumbaugh KM, Perez-Villar JJ, Dick CJ, Schoon RA, Lopez-Botet M, Leibson PJ. 1996. Clonotypic differences in signaling from CD94 (Kp43) on NK cells lead to divergent cellular responses. J Immunol 157: 2804.

269. Moretta A, Sivori M, Vitale D, Pende L, Morelli R, Augugliaro C, Bottino C, Moretta L 1995. Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. J Exp Med 182: 875.

270. Biassoni R, Cantoni C, Falco M, Verdiani S, Bottino C, Vitale M, Conte R, Poggi A, Moretta A, Moretta L.1996. The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. J Exp Med 183: 645.

271. Chan AC, Desai DM, Weiss A. 1994. The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. Annu Rev Immunol 12: 555.

272. Yi TL, Cleveland JL, Ihle JN. 1992. Protein tyrosine phosphotase containing SH2 domains: characterization, preferential expression in hematopoietic cells, and localization to human chromosome 12p12-p13. Mol Cell Biol 12: 836.

273. Stone RL, Dixon JE. 1994. Protein-tyrosine phosphatases. J Biol Chem 269: 31323.

274. Thomas ML. 1995. Of ITAMs and ITAMs: turning on and off the B cell antigen receptor. J Exp Med 181: 1953.

275. Burshtyn DN, Scharenberg AM, Wagtmann N, Rajagopalan S, Berrada K, Yi T, Kinet JP, Long EO. 1996. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitory receptor. Immunity 4: 77.

276. Campbell KS, Dessing M, Lopez-Botet M, Cella M, Colonna M. 1996. Tyrosine phosphorylation of a human killer inhibitory receptor recruits tyrosine phosphatase 1C. J Exp Med 184: 93.

277. Fry AM, Lanier LL, Weiss A. 1996. Phosphotyrosines in the killer cell inhibitory receptor motif of NKB1 are required for negative signaling and for association with protein tyrosine phosphatase 1C. J Exp Med 184: 295.

278. Lazetic S, Chang C, Houchins JP, Lanier LL, Phillips JH. 1996. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. J Immunol 157: 4741.

279. Brooks AG, Posch PE, Scorzelli CJ, Borrego F, Coligan JE. 1997. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. J Exp Med 185: 795.

280. Olcese L, Lang P, Vely F, Cambiaggi A, Marguet D, Blery M, Hippen KL, Biassoni R, Moretta A, Moretta L, Cambier JC, Vivier E. 1996. Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. J Immunol 156: 4531.

281. Minami Y, Kono T, Yamada K, Kobayashi N, Kawahara A, Perlmutter R, Tanigushi T. 1993. Association of $p56^{lck}$ with IL-2 receptor β chain is critical for the IL-2-induced activation of $p56^{lck}$. EMBO 12: 759.

282. Hatakeyama M, Kono T, Kobayashi N, Kawahara A, Levin SD, Perlmutter RM, Tanigushi T. 1991. Interaction of the IL-2 receptor with the Src-family kinase $p56^{lck}$: identification of novel intermolecular association. Science 252: 1523.

283. Einspahr KJ, Abraham RT, Dick CJ, Leibson PJ. 1990. Protein tyrosine phosphorylation and $p56^{lck}$ modification in IL-2 or phorbol ester-activated human natural killer cells. J Immunol 145: 1490.

284. Windebank KP, Abraham RT, Powis G, Olsen RA, Barna TJ, Leibson PJ. 1988. Signal transduction during human natural killer cell activation: inositol phosphate generation and regulation by cAMP. J Immunol 141: 3951.

285. Einspahr KJ, Abraham RT, Binstadt BA, Uehara Y, Leibson PJ. 1991. Tyrosine phosphorylation provides an early and requisite signal for the activation of natural killer cell cytotoxic function. Proc Natl Acad Sci USA 88: 6279.

286. Ting AT, Einspahr KJ, Abraham RT, Leibson PJ. 1991. Fcγ receptor signal transduction in natural killer cells. Coupling to phospholipase C via a G protein- independent, but tyrosine kinase dependent pathway. J Immunol 147: 3122.

287. Ting AT, Karnitz LM, Schoon RA, Abraham RT, Leibson PJ. 1992. Fcγ receptor activation induces the tyrosine phosphorylation of both phospholipase C (PLC)-γ1 and PLC-γ2 in natural killer cells. J Exp Med 176: 1751.

288. Bonnema JD, Karnitz LM, Schoon RA, Abraham RT, Leibson PJ. 1994. Fc receptor stimulation of phosphatidylinositol 3-kinase in natural killer cells is associated with protein kinase-C dependent granule release and cell-mediated cytotoxicity. J Exp Med 180: 1427.

289. Greenblatt MS, Elias L. 1992. The type B receptor for tumor necrosis factor-α mediates DNA fragmentation in HL-60 and U937 cells and differentiation in HL-60 cells. Blood 80: 1339.

290. Pfizenmaier K, Himmler A, schutze s, scheurich P, Kronke M. 1992. In Beutler B (ed). Tumor necrosis factors: The molecules and their emerging role in medicine. Raven Press, New York, NY, 439.

291. Fiers W. 1991. Tumor necrosis factor: characterization at the molecular, cellular and in vivo level. FEBS Lett 285: 199.

292. Heller RA, Kronke M. 1994. Tumor necrosis factor receptor-mediated signaling pathways. J Cell Biol 126: 5.

293. Kronke M, Schutze S, Scheurich P, Pfizenmaier K.1992. TNF signal transduction and TNF responsive genes. In Tumor Necrosis Factor, Structure, Function, and Mechanism of Action, B B Aggarwal and Vilcek, Eds(New York: Marcel Dekker)pp 189-216.

294. Tracey KJ, Cerami A. 1993. Tumor necrosis factor, other cytokines and disease. Annu Rev Cell Biol 9: 317.

295. Meichle A, Schutze S, Hensel G, Brunsing D, Kronke M. 1990. Protein kinase C-independent activation of nuclear factor *k*B by tumor necrosis factor. J Biol Chem 265: 8339.

296. Schutze S, Potthoff K, Machleidt T, Berkovic D, Wiegmann K, Kronke M. 1992. TNF activates NF-*k*B by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. Cell 71: 765.

297. Shimizu H, Mitomo K, Watanabe T, Okamoto S, Yamamoto KY. 1990. Involvement of a NF-*k*B-like transcription factor in the activation of the interleukin-6 gene by inflammatory lymphokines. Mol Cell Biol 10: 561.

298. Lowenthal JW, Ballard DW, Boehnlein E, Greene WC. 1989. Tumor necrosis factor alpha induces proteins that bind specifically to kappa B-like enhancer elements and regulate interleukin-2 receptor a chain gene expression in primary human T lymphocytes. Proc Natl Acad Sci USA 86: 2331.

299. Osborn L, Kunkel S, Nabel GJ. 1989. Tumor necrosis factor a and interleukin1 stimulate the human immunodeficiency virus enhancer by activation of nuclear factor kB. Proc Natl Acad Sci USA 86: 2336.

300. Baeuerle PA, Henkel T. 1994. Function and activation of NF-*k*B in the immune system. Annu Rev Immunol 12: 141.

301. Sen R, Baltimore D. 1986. Inducibility of *k* immunoglobulin enhancer-binding protein NK-*k*B by a posttranslational mechanism. Cell 47: 921.

302. Baeuerle PA, Baltimore D. 1988. IkB: a specific inhibitor of the NK-kB transcription factor. Science 242: 540.

303. Baeuerle PA, Henkel T. 1994. Function and activation of NF-*k*B in the immune system. Annu Rev Immunol 12: 141.

304. Baldwin Jr AS. 1996.The NF-*k*B and I*k*B proteins: new discoveries and insights. Annu Rev Immunol 14: 649.

305. Wang CY, Mayo MW, Baldwin Jr AS. 1996. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-*k*B. Science 274: 784.

306. Beg A, Baltimore D. 1996. An essential role for NF-*k*B in preventing TNF-α induced cell death. Science 274: 782.

307. Wallach D, 1984. Preparations of lymphotoxin induce resistance to their own cytotoxic effects. J Immunol 132: 2464.

308. Tartaglia LA, Goeddel DV. 1992. Two TNF receptors. Immunul Today 13: 151.

309. Wiegmann K, Schutze S, Kampen E, Himmler A, Machleidt T, Kronke M. 1992. Human 55kD receptor for tumor necrosis factor coupled to signal transduction cascades. J Biol Chem 267: 17997.

310. Kyriakis JM, Banerjee, Nikolakaki E, Dai T, Rule EA, Ahmad MF, Avruch J, Woodgett JR. 1994. The stress-activated protein kinase subfamily of c-Jun kinases. Nature 369: 156.

311. Westwick JK, Bielawaska AE, Dbaibo G, Hannun YA, Brenner DA. 1995. Ceramide activates the stress-activated protein kinases. J Biol Chem 270: 22689.

312. Westwick JK, Weitzel C, Minden A, Karin M, Brenner DA. 1994. Tumor necrosis factor α stimultes AP-1 activity through prolonged activation of the c-Jun kinase. J Biol Chem 269: 26396.

313. Brenner DA, O'Hara M, Angel P, Chojkier M, Karin M. 1989. Prolonged activation of jun and collagenase genes by tumor necrosis factor-α. Nature 337: 661.

314. Farrar MA, Schreiber RD. 1993. The molecular cell biology of interferon-γ and its receptor. Annu Rev Immunol 11: 571.

315. Aguet M, Dembic Z, Merlin G. 1988. Molecular cloning and expression of the human interferon-γ receptor. Cell 55: 273.

316. Hemmi S, Bohni R, Stark G, Di Marco F, Aguet M. 1994. A novel member of the interferon receptor family complements functionality of murine interferonnnn γ receptor in human cells. Cell 76: 803.

317. Soh J, Donnelly R, Kotenko S, Mariano TM, Cook JR, Wang N, Emanuel S, Schwartz B, Miki T, Pestka S. 1994. Identification and sequence of an accessory factor required for activation of the human interferon γ receptor. Cell 76: 793.

318. Watling D, Guschin D, Muller M, Silvennoinen O, Witthuhn BA, Quelle FW, Rogers NC, Schindler C, Stark GR, Ihle JN, Kerr IM. 1993. Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in th einterferon-γ signal transduction pathway. Nature 366: 166.

319. Fu XY. 1992. A transcription factor with SH2 and SH3 domains is directly activated by interferon α-induced cytoplasmic protein tyrosine kinase(s). Cell 70: 323.

320. Schindler C, Shuai K, Prezioso VR, Darnell Jr JE. 1992. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. Science 257: 809.

321. Greenlund AC, Farrar MA, Viviano BL, Schreiber RD. 1994. Ligand-induced IFN-γ receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). EMBO 13: 1591.

322. Darnell JE, Kerr IM, Stark GR. 1994. Jak-STAT Pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264: 1415.

323. Kotenko SV, Izotova LS, Pollack BP, Mariano TM, Donnelly RJ, Muthukumaran G, Cook JR, Garotta G, Silvennoinen O, Ihle JN. 1995. Interaction between the components of the interferon γ receptor complex. J Biol Chem 270: 20915.

324. Kotenko SV, Izotova LS, Pollack BP, Muthukumaran G, Paukku K, Silvennoinen O, Ihle JN, Pesteka S. 1996. Other kinases can substitute for Jak2 in signal transduction by interferon-γ. J Biol Chem 271: 17174.

325. Olerup O, Zetterquist H. 1992. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantations. Tissue Antigens 39: 225.

326. Radcliff G, Waite R, LeFevre J, Poulik MD, Callewaert DM. 1991. Quantification of effector/target conjugation involving natural killer or lymphokine activated killer cells by two-color flow cytometry. J Immunol Meth 139: 281.

327. Callewaert DM, Radcliff G, Waite R, Lefevre J, Poulik MD. 1991. Characterization of effector-target conjugates for cloned human natural killer and human lymphokine activated killer cells by flow cytometry. Cytometry 12: 666.

328. Darzynkiewicz Z, Bruno S, Del Bino G, Gorczca W, Hotz MA, Lassota P, Traganos F. 1992. Features of apoptotic cells measured by flow cytometry. Cytometry 13: 795.

329. Mc Gahon AJ, Martin SJ, Bissonnette RP, Mahboubi A, Shi Y, Mogli RJ, Nishioko WK, Green DR. 1995. The end of the (cell) line: methods for the study of apoptosis in vitro. Methods Cell Biol 46: 153.

330. Damen J, Liu L, Cutler RL, Krystal G. 1993. Erythropoietin stimulates the tyrosine phosphorylation of Shc and its association with Grb2 and a 145 kDa tyrosine phosphorylated protein. Blood 82: 2296.

331. Blaise DO, Stoppa AM, Viens P, Pourreau C, Lopez M, Attal M, Jasmin C, Monges G, Mawas C, Mannoni P, Palmer P. Franks C, Philip T, Maraninchi D. 1990. Hematologic and immunologic effects of the systemic administration of recombinant interleukin-2 after autologous bone marrow transplantation. Blood 76:1092.

332. Gottlieb DJ, Prentice HG, Heslop HE, Bello-Fernandez C, Bianchi AC, Galazka AR, Brenner MK. 1989. Effects of recombinant interleukin-2 administration on cytotoxic function following high-dose chemo-radiotherapy for hematological malignancy. Blood 74: 2335.

333. Soiffer RJ, Murray C, Cochran K, Cameron C, Wang E, Schow PW, Daley JF, Ritz J. 1990. Clinical and immunological effects of prolonged infusion of low-dose recombinant interleukin-2 after autologous and T-cell-depleted allogenic bone marrow transplantation. Blood 79: 517.

334. Heslpo HE, Gottlieb DJ, Bianchi AC, Meager A, Prentice HG, Mehta AB, Hoffbrand AV, Brenner MK. 1989. In vivo induction of gamma interferon and tumor necrosis factor by interleukin-2 infusion following chemotherapy or autologous bone marrow transplantation. Blood 74: 1374.

335. Heslop HE, Gottlieb DJ, Reittie JE, Bello-Fernandez C, Meager A, Prentice HG, Brenner MK. 1989. Spontaneous and interleukin-2 induced secretion of tumor necrosis factor and gamma interferon following autologous bone marrow transplantation or chemotherapy. Br J Heamatol 72: 122.

336. Reittie JE, Gottlieb D, Heslop HE, Leger O, Drexler HG, Hazlehurst G, Hoffbrand AV, Prentice HG, Brenner MK.1989. Endogenously generated activated killer cells circulate after autologous and allogenic bone marrow transplantation but not after chemotherapy. Blood 73: 1351.

337. Van Valen F, Hanenberg H, Fried C, Bott U, Burdach S, Winkelmann W, Jurgens H, Gobel U. 1991. Interferon γ and tumor necrosis factor α induce adhesion molecule ICAM-1 and HLA class I antigen expression in Ewing sarcoma cells. 1991. Med Pediatr Oncol 19: 342.

338. Kolitz JE, Mertelsman R. 1993. The immunotherapy of human cancer with interleukin-2: Present status and future directions. Cancer invest 9: 529.

339. Jones M, Philip T, Palmer P, Von der Masse H, Vinke J, Elson P. 1993. The impact of interleukin-2 on survival in renal cancer: a multivariant analysis. Cancer Biother 8: 275.

340. Dillman RO. 1994. The clinical experience with interleukin-2 in cancer therapy. Cancer Biother 9: 183.

341. Heys SD, Franks CR, Eremin O. 1993. Interleukin-2 therapy: current role in surgical oncological practice. Br J Surg 80: 155.

342. Rosenberg SA, Lotze MT, Yang JC, Topalian SL, Chang AE, Schwartzentruber DJ, Aebersold P, Leitman S, Linehan WM, Seipp CA, White DE, Steinberg SM. 1993. Prospective randomized trial of high dose interleukin-2 alone or in conjunction with lymphokine-activated killer cells for the treatment of patients with advanced cancer. J Natl Cancer Inst 2: 622.

343. Lauria F, Raspadori D, Ventura MA, Rondelli D, Zinzani PL, Gherlinzoni F, Miggiano MC, Fiacchini M, Rosti G, Rizzi S, Tura S. 1996. Immunologic and clinical modifications following low-dose subcutaneous administration of rIL-2 in non-Hodgkin's lymphoma patients after autologous bone marrow transplantation. Bone Marrow Transpl 18: 79.

344. Hauch M, Gazzola MV, Small T, Bordignon C, Barnett L, Cunningham I, Castro Malaspinia H, O'Reilly RJ, Keever CA. 1990. Anti-leukemia potential of interleukin-2 activated natural killer cells after bone marrow transplantation for chronic myelogenous leukemia. Blood 75; 2250.

345. Soiffer RJ, Murray C, Gonin R, Ritz J. 1994. Effect of low-dose interleukin-2 on disease relapse after T-cell depleted allogenic bone marrow transplantation. Blood 84; 964.

346. Attal M, Blaise D, Marit G, Payen C, Michallet M, Vernant JP, Sauvage C, Troussard X, Nedellec G, Pico J, Huguet F, Stoppa AM, Broustet A, Sotto JJ, Pris J, Maraninchi D, Reiffers J. 1995. Consolidation treatment of adult acute lymphoblastic leukemia: a perspective randomized trial comparing allogenic versus autologous bone marrow transplantation and testing the impact of recombinant interleukin-2 after autologous bone marrow transplantation. Blood 86: 1619.

347. Kornbluth J, Flomenberg N, Dupont B. 1982. Cell surface phenotype of a clone of human natural killer cells. J Immunol 129: 2831.

348. Yodoi J, Teshigawara K, Nikaido T, Fukui K, Noma T, Honjo T, Takigawa M, Sasaki M, Minato N, Tsudo M, Uchiyama T, Maeda M. 1985. TCGF (IL-2) receptor inducing factor(s): I.Regulation of IL-2 receptor on a natural killer-like cell line (YT cells). J Immunol 134: 1623.

349. Teshigawara K, Wang HM, Kato K, Smith KA.1987. Interleukin 2 high affinity receptor expression requires two distinct binding proteins. J Exp Med 165: 223.

350. Robertson MJ, Cochran KJ, Cameron C, Le JM, Tantravahi R, Ritz J. 1996. Characterization of a cell line, NKL, derived from an aggressive human natural killer cell leukemia. Exp Hematol 24: 406.

351. Carbone E, Terrazzano G, Colonna M, Tuosto L, Piccolella E, Franksson G, Palazzolo G, Perez-Villar JJ, Fontana S, Karre K, Zappacosta S. 1996. Natural killer clones recognize specific soluble HLA class I molecules. Europ J Immunol 26: 683.

352. Vitale M, Sivori S, Pende D, Augugliaro R, Di Donato C, Amoroso A, Malnati M, Bottino C, Moretta L, Moretta A. 1996. Physical and functional independency of p70 and p58 natural killer (NK) cell receptors for HLA class I: their role in the definition of different groups of alloreactive NK cell clones. Proc Natl Aca Sci USA 93: 1453.

353. Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. 1993. Specificity of HLA class I antigen recognition by human NK clones: evidence for clonal heterogeneity, protection by self and non-self alleles, and influence of target cell rype. J Exp Med 178: 1321.

354. Peruzzi M, Wagtmann N, Long EO. 1996. A p70 killer cell inhibitory receptor specific for several HLA-B allotypes discriminates among peptides bound to HLA-B* 2705. J Exp Med 184: 1585.

355. Brooks AG, Posch PE, Scorzelli CJ, Borrego F, Coligan JE. 1997. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. J Exp Med 185: 795.

356. Ross ME, Caligiuri MA. 1997. Cytokine-induced apoptosis of human natural killer cells identifies a novel mechanism to regulate the innate immune response. Blood 89: 910.

357. Goodman DJ, Von Albertini MA, McShea A, Wrighton CJ, Bach FH. 1996. Adenovirus-mediated overexpression of IκBα in endothelial cells inhibits natural killer cell-mediated endothelial cell activation. Transplantation 62: 967.

358. Gismodi A, Morrone S, Humphries MJ, Piccoli M, Frati L, Santoni A. 1991. Human natural killer cells express VLA-4 and VLA-5, which mediate their adhesion to fibronectin. J Immunol 146: 384.

359. Bevilacqua MP. 1993. Endothelial-leukocyte adhesion molecules. Annu Rev Immunol 11: 767.

360. Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyi S, Hemler ME, Lobb RR. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell 60: 577.

361. Dohgherty GJ, Dougherty ST, Dirks JF, Chui RK, Peters CE, Droll A. 1995. Regulation of the functional activity and ligand binding specificity of the adhesion protein CD44. Trends in Glycosci Glycotech 7: 45.

362. Beran M, Hansson M, Kiessling R. 1983. Human natural killer cells can inhibit clonogenic growth of fresh leukemic cells. Blood 61: 596.

363. Fao R, Fierro MT, Cesano A, Guarini A, Bonferroni M, Raspadori D, Miniero R, Lauria F, Gavosto F. 1991. Defective lymphokine-activated killer cell generation and activity in acute leukemia patients with active disease. Blood 78: 1041.

364. Panayotides P, Porwit A, Sydgren AM, Wasserman J, Reizenstein P. 1988. Resistance of some leukemic blasts to lysis by lymphokine activated (LAK) cells. Eur J Haematol 40: 362.

365. Ritchie AJ, Johnson DR, Ewenstein BM, Pober JS. 1991. Tumor necrosis factor induction of endothelial cell surface antigens is independent of protein kinase C activation or inactivation. Studies with phorbol myristate acetate and staurosporine. J Immunol 146: 3056.

366. Klingemann HG, Dougherty GJ. 1996. Site-specific delivery of cytokines in cancer. Mol Med Today 2: 154.

367. Gillies SD, Reilly EB, Lo KM, Reisfeld RA. 1992. Antibody-targeted interleukin-2 stimulates T-cell killing of autologous tumor cells. Proc Natl Acad Sci USA 89: 1428.

368. Sabzevari H, Gillies SD, Mueller BM, Pancook JD, Reisfeld RA. 1994. A recombinant antibody-interleukin 2 fusion protein suppresses growth of hepatic human neuroblastoma metastases in severe combined immunodeficiency mice. Proc Natl Aca Sci USA 91: 9626.