HOST TUMOR INTERACTION AND ACTIVATION OF THE EFFECTOR FUNCTION

THESIS

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CERTIFICATE

The research work presented in this thesis has been carried out at the Centre for Cellular and Molecular Biology, Hyderabad. This work is original and has not been submitted in part or full for any other degree or diploma of any other university.

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Abbreviations

β-ME	β-mercaptoethanol
^{51}Cr	radiolabelled chromium
A	absorbance
Ab	antibody
ADCC	antibody dependent cellular cytotoxicity
APC	antigen presenting cell
APS	ammonium persulphate
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
CD	cluster designation
CTL	cytotoxic T lymphocyte
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
E : T	effector : target
EDTA	ethylene diamino tetra acetic acid
EGTA	ethylene-glycol-bis (B-aminoethylether) N,N,N'N' acetic acid
ELIFA	enzyme linked immunofiltration assay
FCS	fetal calf serum
Fig	figure
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage colony stimulating factor
h	hour
HEPES	N-(2-hydroxyethyl) piperazine-n'-(2-ethane sulphonic acid)
i.p	intraperitoneal
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
mAb	monoclonal antibody

MHC	major histocompatibility complex
min	minutes
MOPS	3-(N-morpholino)propanesulfonic acid
NBT	nitro blue tetrazolium
ND	not detectable
NK	natural killer
NKT	natural killer T
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEC	peritoneal exudate cells
PI	propidium iodide
PMSF	phenyl methyl sulfonyl fluoride
R	receptor
RT-PCR	reverse transcriptase – polymerase chain reaction
s.c	subcutaneous
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCR	T-cell receptor
TEMED	N,N,N',N',-tetramethyl ethylene diamine
Th	T helper
TIL	tumor infiltrating lymphocytes
TIM	tumor infiltrating mononuclear
TNF	tumor necrosis factor

Abstract

Natural killer (NK) cells play a significant role in controlling viral and possibly other infections and may play a role in controlling malignancies. They employ cytolysis and cytokine production as major effector mechanisms. While the mechanism used by NK cells to discriminate susceptible from non-susceptible target cells are still incompletely understood, great progress has been made recently in uncovering several families of NK cell receptors that confer specificity for class I MHC molecules. Many of these receptors are inhibitory and consequently prevent NK cells from attacking normal self cells that express high levels of autologous class I MHC molecules. Thus, cells that extinguish expression of class I MHC molecules as a consequence of infection, transformation or mutation are rendered sensitive to cytolysis by NK cells. Some tumor cells expressing high levels of autologous class I molecules are nevertheless sensitive to cytolysis by NK cells, indicating that class I deficiency is not the only mechanism of target cell discrimination by NK cells.

NK cells synthesize and secrete several cytokines including GM-CSF, TNF-α and IFN-γ. TNF-α plays a pivotal role in the activation or inhibition of function of NK cells, depending on the nature of stimulus used. Such dual function of TNF-α is reported by different groups. Among the cytokines produced by NK cells, IFN-γ is thought to be particularly important as it activates macrophages to kill phagocytosed pathogens and to secrete various cytokines. IFN-γ also promotes Th1 immune responses. Therefore, IFN-γ production by NK cells seems to be one of the important links between innate and adaptive immunity.

AK-5 is a highly immunogenic rat histiocytic tumor which regresses spontaneously in syngeneic animals when transplanted subcutaneously. The AK-5 tumor follows a site specific growth pattern when transplanted in syngeneic hosts. Upon s.c. transplantation (5×10^6 cells) the tumor grows upto day 15 (growth phase), after which it starts getting rejected during the regression phase followed by the healing phase when the animal is totally normal but immune to subsequent challenges of AK-5 tumor. Tumor regression of s.c. transplanted animals are mediated by NK cells through antibody dependent cellular cytotoxicity (ADCC) and apoptosis. However, the i.p. transplanted tumors (1×10^7 cells) divide rapidly and form a peritoneal bulge by day 3 and the animals starts dying by day 5. The death of 100% animals in i.p. injected tumors was attributed to the rapid growth in the peritoneum, not giving sufficient time to the immune system to mount a strong immune response and to the immune escape strategies adopted by AK-5 cells.

It is observed that naive NK cells are ineffective in the killing of AK-5 tumor cells in vitro. But NK cells isolated from animals transplanted (s.c.) with AK-5 tumors exhibited cytotoxic activity against the tumor cells, suggesting the requirement of some sort of priming / activation of NK cells before they induce death in the target cells. The purpose of our present study is to find out the specific interaction taking place between NK cells, tumor cells and other accessory cells, which are actually responsible for the activation of NK cell cytotoxic function in vitro.

Recent experiments *in vitro* have implicated antigen dose as an important factor in immune response and a major regulator of effector cell function. Interaction of antigen specific receptor of T-lymphocytes with its antigenic ligand can lead to either cell

activation or a state of profound unresponsiveness (anergy). It has been demonstrated that high dose of antigen can lead to a paradoxical suppression of T cell response. T and NK cells are developmentally related and they also undergo functional anergy and apoptosis. However, a direct correlation between dose of antigen and NK cell cytotoxic function has not been demonstrated. The aim of our present study is to find out whether AK-5 tumor cell can modulate NK cell cytotoxic function in a dose dependent manner both in vivo and in vitro.

The present study deals with the specific interaction taking place between NK cells, AK-5 cells and other accessory cells, which regulate the NK cell cytotoxic function.

Chapter I presents a brief introduction of NK cell development, phenotypic characteristics, activation and effector mechanism and its role in adaptive immunity and antitumor activity.

Chapter II deals with the experimental procedures used in this study.

Chapter III deals with induction of NK cell anergy following treatment with high dose of tumor antigen in vitro. The roles of various soluble and marker proteins in the induction of anergy in NK cells were deciphered. Phenotypic changes in NK cells after induction of anergy were also worked out.

Chapter IV deals with activation of NK cell cytotoxic function following treatment with low dose of tumor antigen in vitro. The role of other accessory cells and interleukins involved in the activation process of NK cells were studied. The expression of different markers involved in the activation of NK cell cytotoxic function were examined.

Chapter V deals with the regulation of NK cell cytotoxic function in vivo by the dose of tumor transplanted in the peritoneum. Comparative study of the expression of different cytokines, interleukins and markers in NK cells following exposure of different doses of tumor transplantation were studied.

CHAPTER 1

INTRODUCTION

1.1 NK cells : An Overview

Natural killer (NK) cells play a significant role in controlling viral and other infections and play a role in controlling malignancies (Trinchieri, 1989). They employ cytolysis and cytokine production as major effector mechanisms. While the mechanisms used by NK cells to discriminate susceptible from non-susceptible target cells are still incompletely understood, great progress has been made recently in uncovering several families of NK cell receptors that confer specificity for class I MHC molecules (Lanier, 1998). Many of these receptors are inhibitory and consequently prevent NK cells from attacking normal self-cells that express high levels of autologous class I MHC molecules. Thus, cells that extinguish expression of class I MHC molecules as a consequence of infection, transformation or mutation are rendered sensitive to cytolysis by NK cells (Ljunggren & Karre, 1990). Some tumor cells expressing high levels of autologous class I molecules are nevertheless sensitive to cytolysis by NK cells, indicating that class-I deficiency is not the only mechanism of target cell discrimination by NK cells.

1.2 Early state of NK cell development

NK cells, like other hematopoietic cells, are derived from pluripotent hematopoietic stem cells. Evidence for a restricted NK/T cell progenitor came initially from analysis of early fetal thymocytes, many of which - like NK cells - express the Fcγ receptor (R) III (Rodewald et al., 1992). This FcγRIII$^+$ fetal thymocyte population gave rise to TCRαβ$^+$ T cells after intrathymic transfer or to NK cells after intravenous transfer but was incapable of giving rise to myeloid cells or B cells.

More recent studies in mice have indicated that the FcγRIII$^+$ fetal thymic population is heterogenous (Carlyle et al., 1997). A fraction of the cells expresses the NK cell markers NK1.1 and DX5 but fails to express CD117 (c-kit) - NK1.1$^+$, DX5$^+$, CD117$^-$ phenotype. The CD117$^-$

subset exhibits *ex vivo* cytolytic activity against YAC-1 target cells, suggesting that it contains functionally mature NK cells. The $CD117^+$ subset was capable of reconstituting αβT cell development in fetal thymic organ culture and NK cell development when cultured with the OP-9 stromal cell line but failed to give rise to B cells or myeloid cells. The $CD117^-$ population failed to give rise to αβT cells (Carlyle et al., 1998). Possibly related is the finding that human precursor thymocytes express NKR-P1A, a human isoform of the murine NK1.1 antigen (Poggi et al., 1996). It was concluded that the $CD117^+$ population represents restricted progenitor cells for the T and NK lineages while the $CD117^-$ population represents mature NK cells. While these studies suggest the existence of a restricted NK/T cell progenitor, it will be important in future experiments to determine whether individual $NK1.1^+$, $DX5^-$, $CD117^+$ cells can give rise to descendants in both the T and NK cell lineages.

NK cell development normally occurs extrathymically, posing the question of whether restricted NK cell progenitors exist in the periphery. Analysis of fetal mouse blood cells revealed a $CD90^+/CD117^{low}$ population that was capable of giving rise to αβT cells after intrathymic transfer. It was concluded that this population represents a restricted αβT cell progenitor (Rodewald et al., 1994). Subsequent studies have shown that most of these cells express NK1.1 and exhibit a similar overall phenotype to the NK/T-cell-restricted progenitors in the fetal thymus (Carlyle et al., 1998). Most significantly, cells in this population can differentiate into either T or NK cells, but not myeloid or B cells. Collectively, these studies suggest that restriction of a progenitor cell to the NK/T cell lineages may occur prethymically. NK cell development - while it may occur briefly in the fetal thymus - occurs primarily extrathymically with critical steps occurring in the bone marrow (Moore et al., 1995).

1.3 Phenotypic and genotypic characteristics of NK cells

1.3.1 Identification of NK cells

Identification of NK cells is based solely on their ability to mediate spontaneous and antibody-dependent cytotoxicity, a function shared with other cell types, such as monocyte/macrophages and activated T cells and which has represented a major limitation in the analysis of NK cells. One of the most significant contributions to the study of NK cells has been their identification as a relatively homogenous cell type on the basis of physical and phenotypic characteristics and their LGL morphology (Saksela et al., 1979). Human NK cells were originally described as non-adherent, non-phagocytic, FcγR-positive cells with lymphoid morphology. Although velocity sedimentation experiments demonstrated that human NK cells were larger than the bulk of T lymphocytes (Trinchieri et al., 1975), it was not until Saksela and collaborators (Saskela et al., 1979) analyzed cytotoxic effector cells adsorbed-eluted from both fibroblast and cell line target cells that NK cells were identified as LGL, i.e., large lymphocytes with high cytoplasmic-nuclear ratio and few discrete azurophilic granules. A separation technique involving a discontinuous percoll gradient has been widely used for the enrichment of LGLs based on their light buoyant density (Timonen & Saskela, 1980). This technique has contributed much to the progress of studies of NK cells, allowing investigations utilizing semi-purified preparation of NK cells. Such preparations have been used for the analysis of surface phenotype and morphology as well as functional characteristics of NK cells (Bloom, 1981).

1.3.2 Surface phenotype of human NK cells

Early studies of human NK cells showed that virtually all of these cells express FcγR and about 50% of them form low affinity rosettes with sheep erythrocytes at 4°C, but, unlike T cells, only a small portion forms high-affinity rosettes at 29°C (Santoli et al., 1978). The use of

monoclonal antibodies has revealed no surface antigen unique to NK cells, but rather a unique combination of antigens, each shared with other cell types, mainly T cells and myelomonocytic cells.

1.3.2.1 FcR (CD16) Antigen

Various types of FcγR have been identified on human hematopoietic cells. Monocytes and macrophages express at least two types of FcγR: a high affinity (Ka-10^8 / M) receptor (p72 or FcRI) able to bind monomeric IgG, and a low-affinity (Ka- 10^6 / M) receptor (gp40, FcRII) also expressed on PMNs and B cells. PMN also express a third type of FcγR (CD16), when activated by IFN-γ. CD16 FcR is also expressed on majority of NK cells and on tissue macrophages as well as on monocyte-derived macrophages. CD16 FcR is a low affinity receptor that binds IgG in immune complexes with soluble and insoluble (e.g., antibody-coated cells) antigen but does not bind monomeric IgG. Several monoclonal antibodies produced against CD16 FcR (Perussia et al., 1983 & Perussia et al., 1984) bind to few different antigenic determinants on the CD16 molecule. During differentiation of PMNs, CD16 antigen appears at a late state of myeloid differentiation in the bone marrow (metamyelocytes or later). In the peripheral blood, CD16 is expressed in virtually all neutrophils, but only on eosinophils with a more mature morphology. Basophils do not express CD16 FcR. Circulating monocytes express little, if any, CD16 FcR, but *in vitro* cultured monocytes express it at high density. Due to the limited information on NK cell differentiation, it remains unknown when CD16 FcR is first expressed on these cells.

1.3.2.2 NKH-1/ Leu-19 antigen (CD56)

A series of antibodies were produced that react with most NK cells and precipitates a molecule of molecular weight 200-220 kD, often referred to as NKH-1 or Leu19 or CD56

antigen. The CD56 antigen has been recently shown to express the natural adhesion protein, N-CAM. The CD56 antigen is expressed at very low density on peripheral blood NK cells, but its density increases significantly following *in vitro* stimulation and growth of NK cells. (Perussia et al., 1987). The subset of PBLs expressing the CD56 antigen (on average, 15% of lymphocytes and 90% of LGL) almost completely overlaps with that expressing the CD16 antigen (Lanier et al., 1986). The $CD16^-$, $CD56^+$ cells representing 2-3% of PBLs, can be subdivided into two subsets based on the expression of CD3 antigens (Lanier et al., 1986). $CD3^-$, $CD56^+$, $CD16^-$ cells are probably NK cells that do not express the CD16 antigen because of differentiation or activation state. $CD3^+$ and $CD56^+$cells are mostly $CD16^-$ cells, which represent a minor subset of T cells with low but significant non-MHC restricted ability (Lanier et al., 1986).

1.3.2.3 HNK-1/Leu-7 antigen (CD57)

The reactivity of antibody HNK-1 (anti-Leu-7), originally described as NK cell specific (Abo et al., 1981), is complex. This IgM antibody precipitates a 110-kD antigen from PBLs and reacts with 30 to 70% of peripheral blood NK cells, with variability among the donors (Lanier et al., 1983). Unlike the observation of CD16 and CD56 antigens, there is no correlation between the percentage of PBLs positive for CD57 antigen and NK cytotoxicity (Perussia et al., 1983). The expression of CD57 is rapidly lost *in vitro*, and neither bulk culture nor clones of NK cells express it (Lanier et al., 1986). Cord blood NK cells, which normally express CD16 antigen and have reduced but significant NK cell activity, do not express CD57 antigen (Lanier et al., 1983).

Four subsets of PBLs have been distinguished on the basis of reactivity of PBLs with HNK-1 and anti CD16 antibody (Lanier et al., 1983): $CD3^-$, $CD16^+$, $CD57^-$ NK cells with highest cytotoxicity; $CD3^-$, $CD16^+$, $CD57^+$ NK cells, with intermediate cytotoxic activity; $CD3^+$, $CD16^-$, $CD57^+$ T cells with low (or null) cytotoxic activity; and $CD3^+$, $CD16^-$, $CD57^-$ small T

cells, with no cytotoxic activity. Interestingly, the T cells that can be induced to become cytotoxic by treatment with IL-2 are mostly induced in the $CD3^+$, $CD57^-$ subset (Phillips and Lanier, 1986). Moreover, $CD3^+$, $CD4^+$, $CD57^+$ PBLs bind to but do not lyse NK cell-sensitive target cells.

1.3.2.4 CD11/CD18 antigens and Myelomonocytic antigens

CD11/CD18 is a family of three molecules composed of a common β subunit (CD18, 95kD) and different α subunits: CD11a or LFA-1, CD11b or CR3, and CD11c or p150 (Springer et al., 1987). All three molecules are expressed on human NK cells (Timonen et al., 1988). CD11a of LFA-1 is expressed on all lymphocytes, whereas CD11b and CD11c tend to be expressed preferentially on NK cells/LGLs (Kay and Horwitz, 1980 & Timonen et al., 1988). CD11b is strongly expressed on PMNs and monocytes (Breard et al., 1980). The reactivity of anti-CD11b antibody OKM-1 with NK cell was first reported as evidence for the myeloid nature of NK cell (Kay and Horwitz, 1980). However, CD11b is present at low intensity in the majority of, but not all, NK cells, is expressed on some T cells, and rapidly disappears from NK cells maintained in culture.

1.4 Activation and effector mechanisms of NK cells

The most striking characteristic of NK cells is that resting circulating NK cells, present at all times in all healthy individuals, are "natural" functionally active cells, i.e., they can be triggered to lyse a target cell within minutes when confronted with the appropriate target structure or with an antibody-coated target cell. Other NK cell functions, such as lymphokine production and the regulation of hematopoietic and adaptive immune cells, are also mediated by resting NK cells. This ability of NK cells to respond to a triggering stimulus without the need for pre-activation enables them to participate in the first line of defense against various pathogens. In

this respect, NK cells resemble other effector cell types of nonadaptive immunity such as granulocytes and monocyte/macrophages. Moreover, the functional activity of NK cells, like that of other nonadaptive effector cells, is rapidly enhanced by cytokines such as IFN-γ and IL-2. This modulation of NK cell functional activity does not require cell division. *In vitro*, however, conditions such as virus infection or a strong antigenic stimulus induce both the activation of NK cells and an increase in NK cell number, due to increased proliferation, probably at the bone marrow level.

The response of NK cells to an external stimulus can be divided into three sequential phases. In the first phase, interaction of NK cells with target cells or with immune complexes induces a rapid response (1-10 minutes) associated with cytotoxicity and the release of granular contents. These interactions and also stimulation by IL-2 induce (10 minutes to 2 hours), independently and synergistically, the second phase, in which genes encoding lymphokines and surface activation antigens, including the p55 chain of the IL-2 receptor (CD25 antigen), are transcribed and expressed. In the presence of IL-2, the NK cells proceed into the third phase (1-3 days) of the response, with blast formation, DNA synthesis, and proliferation. The various stimuli and modulating factors affect these three phases of the NK cell response differently, and the role of each phase in the various *in vivo* and *in vitro* functions of NK cells differs.

1.4.1 Sensitivity of target cells to NK cell-mediated killing

The prototype target cell lines used in each species, the K562 cell line for human NK cells and the YAC-1 cell line for mouse and rat NK cells, are among the most sensitive cell lines in each system. However, almost any cell is sensitive to a certain extent to NK cells, if the concentration of effector cells is sufficiently high or if the NK cells are activated by IFN-γ or IL-2. When evaluating the sensitivity of target cells to lysis, it should be considered that several

different factors play a role in determining cell lysis. The ability of a cell line to bind to NK cells is necessary but not sufficient to render it sensitive to lysis (Timonen et al., 1982). In order to activate the cytotoxic mechanism in the NK cells, a structure on the target cells, possibly distinct from the one responsible for cell binding, must trigger the effector cells (Trinchieri et al., 1981 & Write et al., 1982). This second requirement can be circumvented if the target cells present molecules that can interact directly with functional receptors on the NK cell surface, such as (1) IgG antibodies, binding to CD16 (ADCC); (2) C3, presumably binding to CD11b (C3bi receptor) (Kai et al., 1988); (3) antibodies to CD16 or CD2 antigens on NK cells and binding with the Fc fragment to the FcR on target cells (reverse ADCC) (Griend, 1987); and (4) heterocross-linked antibodies that recognize an NK cell receptor (eg., CD16) and an antigen on the target cells (Titus et al., 1987). When target cells are bound to NK cells and the lytic mechanism is activated, lysis of the target cells still depends on the intrinsic sensitivity of the target cells to the lytic mechanism. Certain types of target cells may activate the cytotoxic ability of NK cells and therefore might appear to be very sensitive to NK cells. For example, NK cell activation is observed with target cells infected with viruses or mycoplasma (Santoli et al., 1978), but may require the participation of accessory cells and IFN (Bandyopadhyay et al., 1986), and is characterized by an increase of the rate of lysis during the cytotoxic assay.

1.4.2 NK cell receptors

NK cell reactivity is controlled by inhibitory and stimulatory receptor interactions. Inhibitory receptors specific for major histocompatibility complex (MHC) class I molecules endow NK cells with the capacity to attach self cells that have down-regulated or extinguished expression of class I molecules (Ljunggren et al., 1990). Three such inhibitory receptor families have been discovered; the killer cell immunoglobulin-like receptors (KIR) in primates, the Ly49

lectin-like receptors in rodents and the CD94-NKG2A lectin-like receptors shared by primates and rodents. Members of these receptor families are typically expressed by overlapping subsets of NK cells. Although inhibitory receptors play a key role in regulating NK cells, stimulatory receptor interactions are believed to be crucial in initially activating them. The balance of inhibitory and stimulatory receptor interactions would finally determine the outcome of an NK cell-target cell interaction. Indeed, each of the aforementioned MHC-specific receptor families includes MHC-specific stimulatory isoforms (Lanier et al., 1998). In addition, NKp44 and NKp46 have been implicated in the recognition of various tumor and normal target cells (Sivori et al., 1999). The identity of the ligands for these receptors is not known. The Lag-3 receptor, which may interact with MHC class II molecules, is implicated in the recognition of a subset of the tumor cell lines that NK cells attack. Other stimulatory or costimulatory receptors, such as NKR-P1A and 2B4, have also been identified but their physiological function remains to be defined.

The lectin-like NKG2D receptor has also been implicated in the activation of NK cells and some T cells. cDNA clones of the gene encoding NKG2D were first characterized by Houchins (Houchins et al., 1990). The NKG2D gene is located next to the NKG2A, NKG2C, NKG2E (and NKG2F in humans only) genes in both the human and mouse NK gene complex. These NKG2 isoforms show a high degree of sequence identity to one another and pair with a unique subunit, CD94, to form receptors specific for the non-classical class I molecules HLA-E (human) or Qa-1 (mouse) (Lance et al., 1999). In contrast, NKG2D is only distantly related to the other NKG2 isoforms and apparently does not pair with CD94 (Lazetic et al., 1996). Recent evidence indicates that human NKG2D forms a homodimer that associates with the signaling subunit DAP10 on the surface of NK cells and $CD8^+$ T cells. Human NKG2D has been shown to

bind to MICA on target cells. The MHC-encoded proteins MICA and the closely related MICB are distantly related to MHC class I proteins (Bauer et al., 1999). MICA and MICB are normally expressed on a subset of intestinal epithelial cells, but their expression is upregulated by cellular stress. MICA and MICB expressions were strongly upregulated on a large number of different human epithelial tumor cell isolates (Groh et al., 1999). These results suggest that human NKG2D represents one of the stimulatory receptors that NK cells employ to attack tumor cells and stressed cells.

1.4.3 Mechanisms of cytotoxicity

Several morphological and metabolic inhibitor studies suggest that lysis is mediated by a vesicular secretory mechanism, involving polarization of the granules to that part of the effector cell in contact with the target, followed by discharge of the granular contents (Carpen et al., 1988). Drugs that block vesicular secretion in other cell types inhibit NK cell killing without affecting the ability of the effector cells to bind the target cells (Carpen et al., 1981). Degranulating agents both deplete the granules from LGLs and inhibit killing (Quan et al., 1982). The programming phase has been shown to involve transfer of a protease-sensitive material from the effector cells to the target cells (Hiserodt et al., 1983).

Wright and Bonavida demonstrated that a soluble lytic factor is secreted by NK cells following lectin stimulation or NK-target cell interaction. This NK cell cytotoxic factor (NKCF) is lytic for NK cell-sensitive target cells, but not for most NK cell-resistant target cells (Farram and Targan, 1983). Detailed studies have shown that successful NK cell-mediated lysis requires that the target cells: (1) be recognized by NK cells, allowing conjugate formation, (2) be able to induce release of NKCF (or other lytic mediators) from NK cells, and (3) be sensitive to the effect of the lytic mediators (Wright and Bonavida, 1983). IFN-γ treated cells form conjugates

with NK cells and are sensitive to NKCF but fail to induce release of the factor (Wright and Bonavida, 1983). These findings might explain the failure of IFN-γ to protect target cells from ADCC mediated by NK cells (Trinchieri et al., 1981): The interaction of the antibodies with FcγR on NK cells may induce release of lytic mediators, circumventing the step blocked by IFN-γ treatment of the target cells.

Work from several laboratories has established that during NK cell-mediated lysis, tubular lesions with an average internal diameter of 150-170 Å are observed on the target cell membrane and that isolated granules are able to mediate the formation of similar lesions (Young and Cohn, 1987). The granular molecule able to form the pores is a 70-kDa protein called pore-forming protein (PFP), or perforin (Young and Cohn, 1987). PFP requires Ca^{2+} for pore formation in membranes and is rapidly aggregated and inactivated in the presence of Ca^{2+} in the medium; thus, PFP cannot represent a lytic factor present in the supernatant fluid, such as NKCF.

1.4.4 Regulation of NK cell cytotoxic activity and proliferation

IFN efficiently enhances the cytotoxic activity of NK cells (Trinchieri et al., 1978). This effect can be readily demonstrated and quantitated by pre-incubating lymphocytes in the presence of IFN and then testing their cytotoxic ability against target cells unable to induce IFN production (Trinchieri and Santoli, 1978). All three known types of IFN, fibroblast (β), and different species of leukocyte type 1 (α), and leukocyte type II or immune (γ) are able to enhance human NK cell cytotoxicity (Perussia et al., 1980). However, IFN-γ is not effective with cells from all donors and always enhances NK cell cytotoxicity at a lower extent and with slower kinetics than does IFN-α or IFN-β (Trienchiri et al., 1984). Human NK cells, as well as other lymphocytes, express high affinity receptors for IFN-α / β and IFN-γ (Faltynek et al., 1986). IFN treatment of NK cells induces 2'-5'-oligodenylate (2',5'A) synthetase and, under appropriate

experimental conditions, 2',5'A augments NK cell cytotoxicity, suggesting that, as in the case of IFN mediated antiviral activity, the pathway of IFN-mediated augmentation of NK cell cytotoxicity may involve 2',5'A (Schmidt et al., 1987). Although most species of recombinant IFN-γ enhance NK cell cytotoxicity, the recombinant IFN-γJ, with potent antiviral and antiproliferative activity, fails to do so (Ortaldo et al., 1984). These results indicate possible differences in the mechanisms of action of IFN in inducing antiviral activity or augmenting NK cell cytotoxicity.

1.4.5 Production of Lymphokines by NK cells

NK cells are powerful producers of IFN-γ when stimulated with IL-2 (Young and Ortaldo, 1987). The IFN-γ induced in total PBL preparations by IL-2 treatment is produced predominantly by NK cells and in part by T cells (Trinchiri et al., 1984). The production of IFN-γ by resting NK cells, as well as by resting T cells, however, requires the participation of HLA-DR^+ accessory cells, with a mechanism which is still unclear (Wilson et al., 1988). Because the majority of NK cells are rapidly induced by IL-2 to produce IFN-γ, it is likely that *in vivo*, during an immune response, the few antigen-specific T cells that may respond to antigen with production of IL-2, recruit NK cells as the major producers of IFN-γ.

NK cells have been shown to produce B cell growth factors (Rambaldi et al., 1985) and various types of colony-stimulating factors. During the studies on the effect of human NK cells on bone marrow colony formation, it was found that NK cells, when cultured with bone marrow cells or NK-sensitive target cells, release low levels of TNF (Degliantoni et al., 1985). This result was surprising because TNF was considered a macrophage product, but production of TNF by both NK and T lymphocytes was subsequently confirmed at the protein (Yamamoto et al., 1986) as well as RNA (Anegon et al., 1988) levels.

Stimulation of purified NK cells with CD16 ligand and IL-2 induce high levels of mRNA accumulation and release of IFN-γ, TNF-α, GM-CSF, and CSF-1 (Anegon et al., 1988). Nonspecific stimulation with phorbol diesters and calcium ionophore also induce IFN-γ, TNF, GM-CSF, and IL-3 release (Cuturi et al., 1988). In neither case was accumulation of transcripts for GM-CSF, IL-1α or IL-1β observed (Cuturi et al., 1988). The lack of detection of IL-1α or β mRNA was surprising, because previous studies have shown that NK cells are powerful producers of IL-1 in response to endotoxin (Rambaldi et al., 1985). However, NK cells, unlike monocyte/macrophages, are not stimulated to produce TNF by endotoxin (Cuturi et al., 1988), and it is possible that the IL-1 production in the NK cell preparation previously reported was due to contamination with a small number of monocytes, activated by NK cells as shown for the CL response, or that the IL-1 activity reported was due to a cytokine different from IL-1α or IL-1β.

1.5 NK cells and adaptive immunity

1.5.1 Immunoregulatory role of NK cells on B cell response

Moretta et al. (1977) originally showed that E-rosetting $Fc\gamma R^+$ lymphocytes, after interaction with immune complexes, suppress the polyclonal B cell differentiation induced by pokeweed mitogen (PWM). E-rosetting $Fc\gamma\text{-}R^+$ cells are now known to be almost exclusively $CD2^+$, $CD16^+$ NK cells. Lobo (1981) showed that non-E-rosetting $Fc\gamma R^+$ cells, probably corresponding to the $CD2^-$ subset of NK cells, spontaneously enhanced PWM-induced B cell differentiation but suppressed it after interaction with immune complexes, providing the first experimental evidence that NK cells might have both enhancing and suppressive effects on B cell response. The effect of NK cells on PWM-induced B cell differentiation was attributed to an indirect effect of NK cells on helper T cells rather than to a direct effect on B cells. A murine NK cell clone was shown to inhibit B cell response both *in vivo* and *in vitro*. Although some studies

have shown that B cells at different stages of activation are sensitive to the lytic effect of NK cells, this sensitivity has not always been confirmed (Froelich et al., 1987) and major evidences from different experimental systems suggest that B cell lysis by NK cells during an immune response is not a predominant mechanism by which NK cells modulate B cell response.

Evidence for an enhancing effect of NK cells on the B cell response was provided by studies showing that NK cells, in the absence of T cells, support the *in vitro* antigen-specific murine B cell response in T cell-replacing factor-dependent systems or upon *in vitro* stimulation with T cell-independent antigens (Mond and Brunswick, 1987). In these systems, the enhancing effect of NK cells was mediated by the production of IFN-γ (Mond and Brunswick, 1987).

Human NK cell clones can produce B cell differentiation factors that induce Ig production from B cell lines and can induce Ig synthesis from purified B cells only when the NK cell clones are cocultured with B cells (Vyakarnam et al., 1985). TNF and IFN-γ are among the factors produced by NK cell clones and by non-MHC-restricted CTL clones that enhance *in vitro* antibody formation.

1.5.2 Immunoregulatory role of NK cells on T cell response

Treatment of mice with anti-asialo-GM_1 serum prevents the induction of alloantigen-specific CTLs *in vitro* by immunization with allogeneic spleen cells (Suzuki et al., 1995). The same requirement for asialo-GM_1^+ cells in the generation of *in vitro* alloantigen-specific CTLs was shown in one study (Suzuki et al., 1995), whereas many other studies (Varkila et al., 1987) have shown that asialo-GM_1^+ murine NK cells or $CD16^+$ human NK cells suppress *in vitro* T lymphocyte proliferation or generation of CTLs and that this suppressive effect is enhanced by IFN-γ.

NK cells suppress CTL generation and T cell proliferation in allogeneic or autologous mixed-leukocyte culture by suppressing or eliminating dendritic cells that have interacted with antigen (Gilbertson et al., 1986). In secondary mixed-leukocyte cultures, which are efficiently stimulated by either dendritic cells or macrophages, NK cells suppress only the stimulation by dendritic cells (Shah et al., 1987). On the other hand, studies using Percoll-purified LGL preparations have suggested that subsets of human LGLs provide accessory cell functions for T cell proliferation in autologous and allogeneic mixed-leukocyte cultures (Scala et al., 1985) and for *in vitro* generation of virus-specific CTLs (Burlinton et al., 1984). However, these studies did not exclude contamination of the LGL preparation with accessory cells such as dendritic cells or the HLA-DR^+ IFN-α-producing cells that copurify with LGLs in Percoll gradient. Purified human NK cells are unable to function as antigen-processing cells, although they can present alloantigens after *in vitro* activation with phytohemagglutinin and IL-2 (Brook and Moore, 1986). Purified $CD16^+$ human NK cells are also unable to function as accessory cells in various types of T cell-proliferative responses (Silvennoinen, 1988), although they did support phytohemagglutinin-induced T cell proliferation to a very low extent (Silvennoinen, 1988) and, in the presence of a source of accessory cells, enhanced a mixed-leukocyte reaction (Weissler et al., 1988).

1.6 Anti-tumor activity of NK cells

1.6.1 Studies using experimental animals

In order for NK cells to play a role in the control of tumor growth, they require the ability to interact with and destroy syngeneic tumor cells or to indirectly activate other adaptive and nonadaptive mechanisms of antitumor resistance. The ability of NK cells to lyse syngeneic cells was proven using transformed cell lines as the target (Santoli et al., 1976), but fresh tumor cells

are almost insensitive to NK cell lysis. Studies in which NK cells were enriched and/or activated with IFN or IL-2 showed that allogeneic and autologous fresh tumor cells are sensitive to NK cell mediated cytotoxicity (Grimm et al., 1982). However, NK cells are not specifically cytotoxic to tumor or transformed cells, and normal cells, like fibroblasts, may be as sensitive or more sensitive to NK cell lysis than tumor cells (Santoli et al., 1976). The *in vivo* existence of cytotoxic NK cells with a possible function in the surveillance against tumor suggests the importance of *in vivo* regulatory mechanisms to recruit and activate NK cells locally, in analogy with other nonadaptive mechanisms of defense in the organism (Trienchieri and Santoli, 1978).

In experimental animals the *in vivo* effect of NK cells against tumors was investigated by evaluating long-term growth of tumors (Kiessling et al., 1976), metastasis formation (Hanna et al., 1981), and short-term elimination of radio labelled tumor cells from the whole animal or from certain organs (like lungs) (Gorelik et al., 1979). The experimental protocols used involved analysis of the correlation of NK cell activity and tumor resistance (Kaminsky et al., 1985), the use of NK cell-deficient mice (eg., beige mice) (Talmadge et al., 1980), or the use of experimental procedures able to enhance (eg., treatment with IFN or IFN-inducing substances) (Barlozzari et al., 1985) or suppress NK cell activity. The latter was achieved by the use of ^{89}Sr, split-dose irradiation, anti-asialo-GM_1 antiserum, anti-NK cell alloantisera, anti-NK-1.1 monoclonal antibodies, and anti-IFN antisera. Altogether, these experiments have clearly shown that NK cells are effective *in vivo* and are able to destroy tumor cells. Transplanted NK cell-sensitive tumors and experimental tumor metastasis can be inhibited by NK cells. The direct role of NK cells in the prevention of metastasis formation was confirmed by reconstitution experiments in which formation of metastasis in NK cell-depleted animals was prevented by adoptive transfer of purified NK cells (Barlozzari et al., 1985) or cloned cell lines with NK

activity (Strong et al., 1981). However, the evidence for an effective role of NK cells in resistance to spontaneously arising neoplastic cells is much less compelling (Loutit et al., 1980).

Metastasis often advances by hematogenous spread; the presence in the blood of NK cells with cytotoxic activity that can be up regulated to lyse tumor cells present in the circulation before these cells colonize to form metastasis. The experiments of *in vivo* clearance of intravenously injected tumor cells, especially when clearance from the lung is measured, mostly measure intravasal destruction of tumor cells, because NK cell-mediated effects are observed before appreciable extravasation of the tumor cells occurs starting at 4 hours (Fidler et al., 1978). The demonstration that NK cells can eliminate tumor cells in circulation does not exclude, however, the possibility that prevention of metastasis takes place also at the tissue level. An extravasal anti-metastatic effect of NK cells in the lung and the liver was demonstrated using mice treated sequentially with MVE-2 and anti-asialo-GM1 antiserum, which have increased NK cell activity in both the lung and the liver but suppressed circulating NK cells. In these mice metastasis formation was suppressed, suggesting that organ-associated extravasal NK cell activity is a possible mechanism for the antimetastatic therapeutic effects of *in vivo* treatment with NK cell-activating substances (Wiltrout et al., 1985).

IL-2 activated lymphocytes (i.e., LAK cells) suppress metastasis formation. The role of NK cells in this activity was determined (Habu et al., 1981) by comparing the effect of unfractionated rat LAK cells with that of enriched IL-2-stimulated NK cells obtained by the plastic adherence method (Vujanovic et al., 1988). The enriched NK cell preparation in combination with IL-2, compared to unfractionated LAK cells, demonstrated a dramatic and superior antimetastatic effect both at the liver and the lung levels and significantly prolonged survival of the host after treatment (Vujanovic et al., 1988).

1.6.2 Studies of cancer patients

In patients with advanced cancer, NK cell cytotoxic activity is usually suppressed (Pandolfi et al., 1982) which appears to be secondary to tumor invasion and due either to interaction of NK cells with tumor cells or to the presence of suppressor cells (Allavena et al., 1981). Pross and Baines (Pross et al., 1986) reported the analysis of data from the first 307 patients in a study of a total of 1600 randomly chosen cancer patients. Randomly chosen control healthy donors, patients with no evidence of disease, and patients with local disease had comparable cytotoxic activity; patients with metastatic disease and more so, patients with advanced metastases, displayed significantly lowered NK cell cytotoxic activity (Pross et al., 1988).

The suppression of NK cell activity in cancer patients is probably due to several different mechanisms, reflecting the complexity of NK cell regulation *in vivo*. Competition or inactivation by tumor cells, reduced number of NK cells, reduced responsiveness to IFN or IL-2, inability to produce IFN or IL-2, presence of suppressor cells (including monocyte/macrophages acting through release of prostaglandins), presence of inhibitory substances such as glycoproteins and glycolipids, and other mechanisms have been described as responsible for NK cell suppression in cancer patients (Pross et al., 1988).

Most of the studies on NK cell cytotoxic activity in cancer patients have been performed using cells from peripheral blood. It is therefore possible that the decrease in NK cell function or number is in part due to altered circulation of the cells or their sequestration at tumor sites or in draining lymph nodes. However, virtually no NK cell activity is found in malignant effusions or among tumor-infiltrating lymphocytes (Eremin et al., 1981). The lack of NK cell activity at tumor sites could be due in part to an *in situ* inhibition of NK cell activity, because in some

studies (Uchida et al., 1983) functional cytotoxic NK cells have been enriched from ascitic fluid and tumor-infiltrating lymphocytes using Percoll gradient fractionation. Highly cytotoxic $CD2^+$, $CD3^-$, $CD16^+$ cells have been grown in IL-2-containing medium from the ascitic fluid or pleural effusions of patients with advanced ovarian or metastatic breast cancer (Blanchard et al., 1988). NK cell activity was demonstrated in breast tumor draining lymph nodes, whereas it was almost absent in normal lymph nodes (Cunningham et al., 1982); however, NK cell activity was suppressed in the lymph nodes more proximal to the tumor and/or with tumor infiltration (Cunningham et al., 1982), indicating that both alteration of NK cell localization and *in situ* suppression takes place in cancer patients.

The regulation of NK cell activity in patients with hematopoietic tumors is somewhat different from that observed in patients with solid tumors. Patients with preleukemia or myelodysplastic syndrome have generally reduced NK cell activity. The number of phenotypically identifiable NK cells is, however, normal in most patients and defects in the ability of patients' cells to produce IFN-α or to respond to IFN-α have been reported. The alteration in the bone marrow environment in these patients is probably responsible for deficient production / differentiation of NK cells, analogous to the situation in 17β-estradiol-treated mice in which noncytotoxic NK-1.1^+ NK precursor cells are found (Hacekett et al., 1986). Reduction in NK cell activity is also observed in patients with acute or chronic leukemia; B cell and myeloid chronic leukemia patients often present a significant proportion of cells with the $CD3^+$, $CD16^+$ phenotype and non-MHC-restricted cytotoxicity (Fijimiya et al., 1987). Cells with this phenotype are rare or absent in healthy donors (Lanier et al., 1985). In patients with pure RBC aplasia associated with B cell chronic lymphocytic leukemia, $CD3^+$, $CD16^+$ cells have been

shown to suppress RBC colony formation *in vitro* and have been proposed to be responsible for the *in vivo* erythropoietic defect (Trienchiri et al., 1987).

If NK cells play a role in surveillance against malignancies, low NK cell activity should have a prognostic value in determining the risk of developing tumors. Patients with genetic diseases such as CHS or X-LPD, with a primary and secondary suppression of NK cell activity respectively, have a high probability of developing an LPD. In these cases, the etiology of the disorder is probably viral and the role of NK cells may reflect their antiviral, rather than their antitumor, activity (Pross and Herberman, 1989). In familial melanoma, relatives of the patients, who have an increased risk of developing the tumors, also showed a suppressed NK cell cytotoxic activity, suggesting a possible role of NK cells resistance to tumor growth (Hersey et al., 1979). Unlike patients with other solid tumors, those with primary noninvasive melanoma have low NK cell activity (Hersey et al., 1979). Strayer et al (1984) reported NK cell cytotoxicity lower than controls in family members of patients with a higher incidence of tumors and observed that NK cell activity varied inversely with the number of family members with cancer. However, in another study of 155 women at high relative risk for breast cancer (Pross et al., 1984), no difference in NK cell activity was found compared to normal controls, with the exception of women with benign breast syndrome, who had slightly elevated NK cell cytotoxicity, possibly because of systemic hormonal changes. Because NK cell activity in healthy donors is variable and the disease itself affects NK cell activity, it is still unknown whether NK cells really have any role in tumor surveillance despite many years of investigation for a possible relationship between low versus high NK cell activity and the probability of developing primary tumors (Pross et al., 1986).

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Animals and tumors

Wistar rats from the inbred colony of this laboratory were used in this study. The AK-5 tumor was maintained as ascites in 6- to 8-week old rats by injecting 5 x 10^6 cells intraperitoneally (i.p). Solid AK-5 tumor was developed by injecting 5 x 10^6 cells subcutaneously (s.c.) into 5-6 week old rats.

2.1.2 Reagents

Recombinant rat IFN-γ was obtained from Genzyme, recombinant murine IL-12 was kindly provided by Dr U. Gübler,Hoffman-la-Roche, recombinant rat IL-2 was obtained from R&D systems and recombinant TNF-α was obtained from Genzyme.

2.1.3 Fine Chemicals

Low molecular weight protein markers (Bangalore Genei)

Dyanal immunomagenetic beads (Dyanal Chantilly, V A)

Detach-a- bead (Dyanal Chantilly, V A)

Trizol (Gibco Laboratories, UK)

PPO (2.5 Diphenyeoxazole) (Loba)

POPOP [2,2'-p-phenylene-bis (4 methyl-5-phenyloxazole)] (Loba)

Crystal violet (Loba)

All restriction enzymes (New England Biolab Inc., USA)

Percoll (Pharmacia)

Sephadex G-50 (Pharmacia biotech)

Propidium Iodide (Sigma)

Actinomycin-D (Sigma)

MTT (3-4,5-dimethylthiozol-2-YL 2,5 diphenyl tetrazolium Bromide) (Sigma)

Ficoll Hypaque (Sigma)

NBT (Nitro blue tetrazolium) (Sigma)

BCIP (5 Bromo- 4- chloro 3-indol phosphate) (Sigma)

TEMED (N,N,N',N'-tetra methyl ethylene diamine) (Sigma)

2.1.4 Antibodies (primary)

Mouse anti-rat CD161 mAb (3.2.3 mAb, Endogen Inc.), anti mouse CD3 (145.2C11) mAb were used for isolating pure NK, T and NKT cells. Anti-murine IL-2 (S4B6), anti-murine IFN-γ (XMG 1.2), anti-murine IL-4 (11B11), anti murine IL-10 (SXC1), anti murine IL-12 p70 (C17.15) and anti mouse TNF-α (polyclonal) antibodies from Genzyme were used in this study. Antibody against CD95 was procured from Santa Cruz biotechnology, anti-CD16 was from Serotec (UK), Rabbit anti mouse CD95L was from Santa-Cruz-biotechnology, USA, mouse and anti rat perforin was kindly provided by Dr K Okumura, rabbit anti mouse phosphotyrosine antibody from Promega and murine anti phosphotyrosine (hybridoma). Anti murine CD28 (37N) (kindly provided by Dr J.P. Allison), anti murine B7.1 (IG10), anti murine B7.2 (2D10) (kindly provided by Dr Vijay Kuchroo), anti LFA-1 (WT1) and anti ICAM-1 (IA29) kindly provided by Prof M Miyasaka, were used in our study. Anti mouse iNOS antibody was from Upstate Biotechnology, anti rat MHC- I (OX-18) antibody and anti rat MHC-II (OX-8) antibody were procured from Serotec, UK.

2.1.5 Antibodies (secondary)

FITC conjugated anti rabbit IgG and anti mouse IgG were obtained from Amersham International, UK. FITC conjugated anti rat IgG was purchased from Sigma Chemical Co. USA. HRPO conjugated goat anti-rat IgG was purchased from Amersham International UK. Alkaline

phosphatase conjugated anti rabbit IgG, anti rat IgG and anti mouse IgG were purchased from Boehringer Mannheim.

2.1.6 Cell lines

BC-8, a single cell clone of AK-5; YAC-1, murine lymphoma cell line; L-929, mouse fibroblastoid cell line; Rat-2, rat embryonic cell line and RNK-16, rat lymphoma cell line were used in this study.

2.1.7 Culture Medium

Rosewell Park Memorial Institute-1640 (RPMI), Dulbecco's modified eagle's medium (DMEM) and Iscove's modified Dulbecco's medium (IMDM) were purchased from Sigma Chemical Company, USA. The culture medium was supplemented with 100 μ/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml kanamycin and 10% FBS separately.

2.1.8 Serum

Fetal bovine serum (FBS) was purchased from Sigma Chemical Company, USA. It was heat inactivated at 56°C for 30 min and stored in sterile condition at –20°C.

2.1.9 Antibiotics

Penicillin, streptomycin and kanamycin were purchased from Sigma Chemical Company, USA. The antibiotics were made as 100X stocks and stored at 4°C and added to the culture media just before use.

2.1.10 Radioactive Isotopes

Radioactive sodium chromate ($Na_2{}^{51}CrO_4$), tritiated thymidine (^{3}[H] TdR) and all radio labeled nucleotides were purchased from BRIT, Mumbai. $Na_2{}^{51}CrO_4$ (half life 28 days) ranged between 5-10 milli Ci/ml and was stored at –20°C. The specific activity of ^{3}[H]-TdR was 18,000

μCi/m mole (half life 12 years) and was stored at –20°C. $Na_2{}^{51}CrO_4$ was used for labeling various target cells while ^{3}[H] was used to evaluate proliferating efficiency of lymphocytes.

2.1.11 Lectins

Phytohemagglutin (PHA) purified from Kidney beans, Concanavalin A (Con A) purified from Jack beans were purchased from Sigma Chemical Co., USA. Stock solutions of 2 μg/ml of the lectins were prepared and stored at –20°C.

2.2 METHODS

2.2.1 Purification of anti AK-5 antibody

Serum collected from rats that had rejected AK-5 tumor earlier and was positive in complement fixation assay was used as anti-AK-5 antiserum. The serum was partially purified by precipitation with 18% and 12% sodium sulphate followed by dialysis against PBS. Anti AK-5 antibody free of cytokines like IL-2, IFN-γ and IL-12 as checked by ELIFA was pooled, filter sterilised, aliquoted and stored at –20°C with 0.1% NaN_3.

2.2.2 Preparation of splenocytes and their fractionation

Spleens from one-month-old rats were removed aseptically, teased in RPMI-1640 medium and passed through a 20-gauge needle to obtain single cell suspensions. Mononuclear cells were obtained after fractionation on Ficoll-Hypaque density gradient. The cells were washed twice with the medium and used in different experiments. Macrophages were separated from the splenocytes by allowing them to adhere to polystyrene plates for 1 hour. The floating cells were passed through a nylon wool column and the non-adherent cell fraction comprised of T and NK cells. T and NK cells were separated with immune magnetic beads. Anti- CD161 and anti-CD3 mAb coated magnetic beads were prepared by incubating immunomagnetic beads M-450 (Dynal Chantilly, VA) with each mAb as described in the Dynal manual. For the isolation of

NK and T cells, non-adherent cell fraction from nylon wool column was mixed with anti-CD161 and anti-CD3 mAb coated beads at a ratio of five beads per cell and incubated for 45 min. The cells bound to magnetic beads were collected with a magnet. After three washings, the cells were separated from beads by using DETACH-a- BEAD from Dynal and used as isolated NK and T cells. For the isolation of NKT cells these $CD161^+$ cells were further selected with anti-CD3 mAb coated beads and used as NKT cells. Purity of the cell preparations was checked by flow cytometry.

2.2.3 Fixation of AK-5 cells

AK-5 tumor cells were fixed by suspending the PBS washed cells in 3% para formaldehyde, 3 % para formaldehyde, periodate-lysine- para formaldehyde, 70% methanol, 70% ethanol and methanol acetic acid (2:1) for 30 min at room temperature. Fixed tumor cells were washed several times with PBS, counted and used in co-culture experiments.

2.2.4 Pretreatment of effector cells with fixed AK-5 cells

Normal and immune (AK-5 tumor rejected animals) effector cells were cocultured with fixed AK-5 cells at different ratios for 20-24 h. The NK cells were then separated using mAb 3.2.3 coated dynal beads, detached from the beads, washed and used in different experiments.

2.2.5.1 ^{51}Cr-release assay

Cytotoxicity of the NK cells was measured by 4 h ^{51}Cr release assay. The AK-5 and YAC-1 cells, which are sensitive to NK-mediated cytotoxicity, were used as target cells. Target cells suspended in CM (10^6 cells/200 µl) were incubated with occasional shaking for 45 min with 250 µCi $Na_2{}^{51}CrO_4$ at 37°C. The target cells were then washed thrice with PBS to remove the free radioactivity. The viability of the cells was determined and the cells were suspended at a concentration of 5 x 10^5 cells/ml in CM.

The NK and radio labeled target cells were mixed at an effector: target ratio (E: T) of 50:1 and plated in 96 well microtitre plates in a total volume of 200 µl/well. The plates were centrifuged at 500 rpm for 3 min to facilitate contact between effectors and target followed by incubation at 37°C for 4 h. At the end of the incubation, the assay was terminated by centrifugation at 1000 rpm for 5 min. 100 µl of the supernatants from each well were carefully transferred to bio-gamma tubes and the released radioactivity was measured in a γ-counter (Packard gamma counter). All the assays were done in triplicate. The spontaneous release of ^{51}Cr from the equivalent number of target cells was evaluated by treating the same number of ^{51}Cr labeled target cells with 10 µl of Triton-X 100. The results were expressed as percent of specific lysis.

2.2.5.2 Cytotoxicity assays

Cytotoxicity assay was performed by a 4 h ^{51}Cr-release assay. Purified NK cells were separated from fixed AK-5 cells and incubated either with ^{51}Cr-labelled AK-5 cells in the presence of anti-AK-5 antibody or with YAC-1 cells for 4h. ^{51}Cr released in the medium was counted in a Packard gamma counter, and the percentage cytotoxicity was calculated.

$$\% \text{ Cytotoxicity} = 100 \times (cpm_{exp} - cpm_{spont}) / (cpm_{max} - cpm_{spont}).$$

The augmentation index (AI) represents the fold change in lytic activity of effector cells incubated with fixed cells compared with effector cells incubated without fixed cells, and was calculated by using the following formula:

$$AI = \% \text{ Cytotoxicity}_{\text{fixed cell}} / \%\text{Cytotoxicity}_{\text{media}}.$$

Percent inhibition of NK cell activation by neutralizing antibodies was calculated by the standardized decrease of augmentation (SDA):

$$SDA = 100 \text{ X } (\%Cyt_{\text{fixed cell}} - \%Cyt_{\text{fixed cell+ Ab}}) / (\%Cyt_{\text{fixed cell}} - \%Cyt_{\text{media}}).$$

2.2.6 Enzyme-linked immunofiltration assay (ELIFA)

The cytokine levels in the coculture supernatants were quantified by ELIFA (Pierce Chem. Co., USA). The ligand solution was filtered through a nitrocellulose membrane allowing it to bind to the membrane. The membrane was blocked with PBS-BSA solution and then treated with the primary antibody. The membrane was washed and treated with horse radish peroxidase – conjugated secondary antibody. The membrane were washed and the signal bound to the membrane were developed for peroxidase.

2.2.7 Analysis of NK cell markers by flow cytometry

The paraformaldehyde fixed NK cells were washed twice with PBS containing 1% BSA. Predetermined optimal concentrations of specific mAbs were added to 5 x 10^4 cells in 50 µl of cold PBS-BSA and the cells were kept at 4°C for 60 min. The cells were washed twice and treated with FITC-conjugated secondary antibody (1:100) for 30 min. The cells were washed and analysed by flow cytometry using FACStar Plus.

2.2.8 Flow cytometric analysis of apoptosis

NK cells isolated following treatment with fixed AK-5 cells were washed with PBS and fixed in 80% methanol for 15 min. They were then washed twice with PBS and stained with the DNA-intercalating fluorochrome, propidium iodide (50 µg/ml; Calbiochem) for 5 min. at room temperature and observed under a fluorescensce microscope (Nikon Optiphot). Nuclei showing chromatin condensation were evaluated.

2.2.9 MTT Colorimetric Assay

Cells were suspended in CM, plated in 96 well microtitre plates (2 x 10^4 cell/well) and incubated in presence of different stimulators at 37°C in CO_2 incubator for 24 h to 72 h depending on the experiments. Cells cultured without any stimulator served as control. At the

end of the incubation the cells were washed twice with CM and 20 µl of the stock MTT solution (5mg/ml) was added in each well. The cells were then allowed to incubate further for 4 h at 37°C. The plates were centrifuged at 1500 rpm for 5 min and the supernatants were discarded. To each well 100 µl of DMSO-Methanol (1:1) was added to dissolve the dark blue crystals formed in the cells and allowed to react for a few minutes at room temperature. The absorbance was measured at a wavelength of 540 nm by using ELISA reader (V max, Molecular Devices). Each assay was done in triplicate.

2.2.10 TNF-α Bioassay

The TNF bioactivity was determined by measuring culture supernatant-induced death of TNF sensitive L929 cells by MTT colorimetric assay. The L929 cells in CM were plated in 96 well flat-bottomed culture plates (2 x 10^4 cells/well). Actinomycin D, a transcription blocker was added at a concentration of 1 µg/ml to the cell suspension. Different dilutions of TNF containing culture supernatants were added to the wells in triplicate and the control wells contained. The cells were then incubated at 37°C for 18 h. The cells were washed twice with CM and 20 µl of stock MTT (5 mg/ml) solution was added in each well. The plate was further incubated for 4 h at 37°C. From each well 150 µl of the supernatants were removed and 100 µl of DMSO was added. After thorough mixing for a few minutes, the absorbance was read using a wavelength of 540 nm in an ELISA reader (V max, Molecular Devices).

$$\% \text{ Cytotoxicity} = \frac{\text{OD in experimental well}}{\text{OD in control well}} \times 100$$

2.2.11 ^{3}[H] Thymidine Incorporation Assay

Lymphocytes were suspended in CM were plated in 96 well tissue culture plates (2 x 10^6 cells/well) and cultured in presence or absence of various stimulators at 37°C different length of

time. The cells were pulsed with 1 μCi of ^{3}[H]-thymidine per well for the last 18 h of culture. At the end of the incubation, the cells were harvested using PHD cell harvester (Cambridge, MA) onto glass fiber filters, washed and dried filter discs taken in a scintillation fluid were counted in β-scintillation counter (1500 Tri-carb, Packard). All these assays were done in triplicate and the results were expressed as counts per minute (CPM).

2.2.12 Cell adhesion assay

Adhesion of NK cells to the tumor cells was analysed using ^{51}Cr labeled NK cells. AK-5 cells were fixed with methanol: acetic acid (2:1) and plated in flat bottomed 96 well plates (2 x 10^4 cells/well). The cells were allowed to attach to the wells for 18 h and washed with PBS. NK cells from tumor bearing animals were incubated with [^{51}Cr] sodium chromate for 1 h at 37°C. After washing, the labeled NK cells were plated (10^6 cells/well) in the wells coated with AK-5 cells and incubated with anti LFA-1, anti ICAM-1, anti AK-5 and isotype matched antibodies for different periods of time. After gentle washing with PBS, the adherent NK cells were lysed by the addition of 100 μl of 1% SDS per well and the radioactivity released was measured using a Packard-γ counter. The percentage of adherent NK cells was calculated as:

A / B X 100

'A' represents counts per min (CPM) of the test wells and B represent CPM of total NK cells.

2.2.13 Estimation of proteins

Protein estimation was done by using Bio-Rad protein assay kit-concentrated dye was diluted with four volume of distilled water. 25 μl of sample was added to 100 μl of diluted dye reagent. Reaction mixture was mixed thoroughly and absorbance was measured at 590nM. Different concentration of BSA was used (as a standard) to make a calibration curve.

2.2.14 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The method used was as originally reported by Laemmli (1970) and described by Sambrook et al, (1989)

Solutions required:

(i) Acrylamide stock: 29% (w/v) acrylamide and 1% N, N'-methylene bisacrylamide.
(ii) Stacking gel buffer: 1.0 M Tris.Cl pH 6.8
(iii) Resolving gel buffer: 1.5 M Tris.Cl pH 8.8
(iv) SDS stock: 10% (w/v) solution
(v) Ammonium persulphate (APS) stock: 10% (w/v) solution made fresh
(vi) Gel running buffer (1X): 25 mM Tris (pH 8.3)
250 mM Glycine
0.1% SDS

(vii) Sample loading buffer (4X): 200 mM Tris.Cl (pH 6.8)
8% SDS
40% Glycerol
400 mM β-mercaptoethanol (β-ME)
0.4% Bromophenol blue dye

Gels of 1.5 mm thickness were cast in a mighty small gel apparatus. Resolving gel of 12% (10 ml) was made by mixing 4.2 ml 30% acrylamide, 3.1 ml water, 2.5 ml of 1.5M Tris, pH 8.8 and 0.1 ml of 10% SDS. Stacking gel (2 ml) was made by mixing 0.33 ml of 30% acrylamide, 1.4 ml of water, 0.25 ml of 1M Tris.Cl, pH 6.8 and 0.02 ml of 10% SDS. Gels were polymerized by addition of TEMED and APS ($1/100^{th}$ volume of gel mix). Sample dye (4X) was added to protein samples and were boiled for 3 min, cooled by plunging into ice and loaded into the wells. The gel was run at constant current of 20 mA. When the dye front crossed the stacking gel the current was increased to 40 mA.

2.2.15 Western blotting

The protein samples separated on SDS-PAGE were transferred to nitrocellulose membrane electrophoretically using a Nova Blot apparatus (Pharmacia). The gel and the membrane were wetted with transfer buffer (39 mM glycine, 48 mM Tris base, 0.0375% SDS and 20% methanol). The gel was placed in contact with the membrane and sandwiched between 6 pieces of buffer soaked Whatman No.1 paper on each side. The sandwich was then placed between graphite plate electrodes, with the membrane facing the anode. The transfer was done for 90 min using a current of 0.65 mA/cm^2.

After the transfer, membranes were blocked with 3% BSA in PBS with 0.1% Tween 20, overnight at 4°C or 1 h at room temperature. The blot was incubated with primary antibody for 1 h in binding buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20), washed for 15 min with 3 changes using wash buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% Tween 20) and incubated with alkaline phosphatase or peroxidase conjugated secondary antibody for 1 h and was washed for 15 min with three changes. The proteins detected by the alkaline phosphatase conjugated secondary antibody were visualized by using AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-Cl, pH 9.5) containing 0.005% BCIP and 0.01% NBT. The proteins detected by peroxidase-conjugated antibody were visualized by using ECL kit (Amersham, UK). The reagents A and B were mixed in the ratio of 100:1 and the blot was treated with this solution at a concentration of 0.1 ml/cm^2 for one min in dark and exposed to X-ray film for 1-10 min after which the film was developed.

2.2.16 Isolation of plasmid DNA

Small-scale preparations of high purity plasmid DNA were obtained using Qiagen columns. Bacterial pellet from a 5 ml overnight culture was resuspended in 0.25 ml of P1 solution (50 mM glucose; 25 mM Tris. HCl, pH 8.0; 10 mM EDTA pH 8.0), 100 $\mu g\ ml^{-1}$ Dnase-

free RNase A, followed by the addition of P2 (0.2 N NaOH, 1% SDS) and the tube was gently inverted 5 times. To this was added P3 (5 M potassium acetate) and mixed by gentle inversion. The contents were centrifuged at 12,000 rpm for 10 min at 4^0C. The supernatant was layered on a Qiagen miniprep column. The column was centrifuged at 12,000 rpm for 1 min at 4^0C. The column was loaded with 0.75 ml of PE (70% ethanol) and centrifuged twice to remove last traces of alcohol. TE (50µl) or water was added to the column and kept for 1 min. The DNA bound to the column was eluted by centrifugation as above.

2.2.17 Estimation of DNA and RNA

Nucleic acids were estimated by measuring the absorbance at 260 nm (Sambrook et al, 1989). An absorbance of 1 corresponded approximately to 50 µg ml^{-1} of double stranded DNA, 40 µg ml^{-1} of RNA and 33 µg ml^{-1} of single stranded oligomers. Purity of the DNA was ascertained by taking the A_{260}/ A_{280} ratio. A ratio of 1.8 corresponds to a pure preparation of nucleic acids

2.2.18 Elution of DNA fragments from agarose gels by Gene Clean

The gel trays were thoroughly cleaned with ethanol before casting the gel. After the gel run, the gel fragments were cut out with a sterile blade using an UV torch to visualize the ethidium bromide stained DNA fragments. The gel piece was cut into smaller pieces and weighed. NaI solution (2.5 times the weight) was added and incubated at 50^0C until the gel pieces dissolved completely. Glassmilk (1 µl/µg of DNA) was added and the contents were mixed by gently inverting the tube. The contents were incubated for 15 mins at room temperature with 2-3 mixing in-between. The glassmilk was then spun down by centrifugation at 8000 rpm at 4^0C for 5 sec. The supernatant was carefully removed and the pellet was given three washes with 0.6 ml of new wash. After the last wash the pellet was allowed to air dry for 1 min and resuspended in 10 µl sterile water. It was incubated at 55^0C for 3 mins and centrifuged at 12,000 rpm for 30 sec. The supernatant was carefully removed and kept at 4^0C and 1 µl was loaded on the gel to confirm that the fragment was of the expected size. The concentration was estimated

by loading a standard DNA marker alongside. The fragments were immediately used in ligation reactions.

2.2.19 Agarose gel electrophoresis

DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to the DNA sample. Based on the size of the DNA sample, either 0.8% or 1% agarose gel was cast in 1 X TAE (40 mM Tris acetate, 1 mM EDTA) containing 0.5 μg/ml ethidium bromide. The gel was electrophoresed in 1 X TAE buffer at a constant voltage of 5 V/cm. Lambda Hind III digested standard DNA marker was loaded alongside for estimating the sizes and concentration of DNA fragments. The DNA stained with ethidium bromide was visualized in a UV transilluminator and documented in a Syngene gel doc system.

2.2.20 Northern analysis

2.22.20.1 Isolation of total RNA

Total cellular RNA from cells was extracted by acid-guanidinium-thiocyanate-phenol-chloroform (AGPC) method described by Chomczynski and Sacchi (1987). BC-8 cells were washed twice with sterile cold PBS. To the cell pellet was added 1 ml of guanidium thiocyanate solution (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), mixed thoroughly and sonicated briefly on ice. The mixture was split into two 1.5 ml microfuge tubes (0.5 ml each). To each tube sequentially 50 μl of 2 M sodium acetate, pH 4.0, 0.5 ml of water-saturated phenol and 100 μl of chloroform were added with thorough mixing after addition of each reagent. The final suspension was vigorously shaken for 10 sec, incubated on ice for 15 min, and centrifuged at 12,000 rpm for 20 min at 4°C. The aqueous phase containing RNA was transferred to a fresh tube and the RNA was precipitated by addition of 1 volume of isopropanol and kept at –20°C for 1 h. RNA was pelleted by centrifugation at 12,000

rpm for 20 min at 4 °C. The pellet was dissolved in 0.3 volumes of AGPC solution (of the initial AGPC volume) and RNA was reprecipitated with isopropanol at –20°C, for 1 h. The total RNA pellet obtained after final centrifugation was washed with 70% ethanol, dried briefly under vacuum and dissolved in sterile water. The RNA preparation was checked on 1% agarose gel containing 0.5 μg/ml ethidium bromides.

2.2.20.2 Northern transfer and hybridization

RNA samples were electrophoresed on 1% agarose/2.2 M formaldehyde gel made in formaldehyde gel-running buffer (20 mM MOPS pH 7.0, 8 mM sodium acetate, 30% formaldehyde and 1 mM EDTA). The samples were prepared by mixing together 10 μg RNA (in 5 μl), 1 μl of 10X formaldehyde-gel running buffer, 3.5 μl of formaldehyde and 10 μl of formamide in a sterile microfuge tube. The samples were heated at 56°C for 15 min and chilled on ice. 2 μl of sterile formaldehyde gel-loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol) was added. Finally 1 μl of a 1 mg/ml solution of ethidium bromide was mixed with the sample and loaded onto the gel. The gel run was performed in a formaldehyde gel-running buffer. After electrophoresis the gels were photographed. The gel was washed in sterile water with 3-4 changes and the RNA was vacuum transferred to Hybond N^+ nylon membrane using 50 mM NaOH for 1-2 h. The filter was washed once in 2 x SSPE and pre-hybridized for 2 h at 65°C and then hybridized to a labeled probe, the specific activity of which was approximately 2-5 x 10^8 cpm/μg. Hybridizations were carried out at 65°C overnight. The blots were washed to a final stringency of 0.1 x SSPE and subjected to autoradiography on a Fuji Bas 1800 Bioimage analyzer.

2.2.20.3 Random priming

The radiolabeled probe was synthesized by random priming of the gene-cleaned insert, by using random priming kit (BRIT). 0.5 μg of DNA was denatured for 5 min by heating and immediately cooling. To the DNA solution were added 4 μl of all the dNTP's except the labeled dNTP, 5 μl of the random priming buffer, 5 μl of random primer, 50 μCi of [α-^{32}P]dATP, 1 μl of Klenow enzyme in a total volume of 50 μl. After mixing the reaction was carried out for 30-45 min at 37°C. The unincorporated nucleotides were separated by spun column chromatography, using sephadex G-50 swollen in TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0) and packed in a 2 ml syringe plugged with glass wool. The labeled mixture was diluted to 100 μl and applied onto the column and spun at 3000 rpm for 10 min. 2 μl of the probe was spotted on filter disc and radioactivity was measured by Cerenkov counting. The specific activity of the labeled probe was roughly 2-5 x 10^8 cpm/μg.

2.2.21 Reverse-transcription (RT) and PCR

RNA was prepared using Trizol Reagent and reverse transcription was carried out with random hexamers using the kit from Life Technologies as per the manufacturer's instruction. RT was carried out in a final concentration of 5 mM magnesium chloride; 1 mM each of dNTPs; 2.5 μM random hexamers; and 1-2 μg total RNA. A master mix was prepared (20 μl per reaction) and final volume was made up with DEPC treated water. Random hexamers were allowed to hybridize RNA at room temperature for 15 min. RT was done in a step cycle comprising extension at 42°C for 15 min (or 1 h for superscript II), denaturation and inactivation at 94°C for 5 min (or at 70°C for 15 min, for superscript II), followed by incubation at 4°C for 5 min.

1 μl of the RT product was used as template in subsequent PCR with forward and reverse primers. The following conditions were used for PCR-denaturation 94°C for 30 sec annealing

64°C for 30 sec and extension 72°C for 60 sec. After 35 cycles, a final extension was done at 72°C for 7 min. The amplified product (10 μl) was run on 1.5% agarose gel with pBR322 HinfI markers at 5 v/cm.

In order to determine the relative mRNA levels of RT16, these mRNAs were coamplified with the mRNA for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as control for the desired number of cycles using 1 μl of RT product derived from 100 ng of total RNA. The cycling conditions were 94°C for 30 sec, 60°C for 1 min, 72°C for 1 min and a final step of extension for 7 min at 72°C. GAPDH was amplified for 23 cycles by adding primer in the PCR reactions.

2.2.22 Primers used in this study

Primer	Sequence	Gene amplified	Amplicon size
CD16 FOR	GCA ATT TTT CAA TCC CCA AA	Rat CD16	293 bp
CD16 REV	ATT TGT CCT GTG GAG CCT TG		
CD25 FOR	CCT GCT ATC AGC ATC TGC AA	Rat CD25	292 bp
CD25 REV	GTG AAC CCA CTC AGG AGG AG		
VAV1 FOR	ACT GCG TGG AGA ATG AGG AG	Mouse VAV1	294 bp
VAV1 REV	CAG ACA GGG CAT CCT TCA GT		
FASL 5' end	GGG GCT GGT TGT TGC AA	Murine Fas L	300 bp
FASL 3' end	CCA CCG CCA TCA CAA CC		
RT16 FOR	GGA CTA TTG GGA GAG GGA GACA	Rat RT16	1183 bp
RT16 REV	AA– CCA AAC AAA GCA AAA CCCC		
NKRP2 FOR	CAT ATT CTT CAA ATG GCA GGC AGT C	Rat NKRP2	450 bp
NKRP2 REV	CTC TGG TTC CAG GCT TTG TTC TCA TTA		
G3PDH FOR	TGA AGG TCG GTG TGA ACG GAT TTG	Rat GAPDH	534 bp
G3PDH REV	TGA TGG CAT GGA CTG TGG TCA TGA		
iNOS FOR	CAC ATC TGG CAG GAT GAG AA	Rat iNOS	
iNOS REV	GAA GGC GTA GCT GAA CAA GG		

2.2.23 Statistical analysis

Student's t test was performed to analyze the significance of the differences between control and experimental groups. Differences were considered to be significant at $P< 0.01$.

CHAPTER 3

Target cell induced anergy in natural killer cells: suppression of cytotoxic function

3.1 INTRODUCTION

Natural killer cells play a major role in the early defense against viruses, intracellular bacteria, parasites and tumor cells by displaying direct cell mediated cytotoxicity as well as by producing various cytokines (Biron, 1994). Thus, patients with poor NK activity have lower resistance to infection and increased cancer metastases (Trinchieri, 1989). However, the mechanism responsible for the observed poor NK activity in patients with cancer and AIDS are not well understood.

The initiation and regulation of an immune response to antigen is dependent on the activation of helper T lymphocytes by the appropriate antigen / major histocompatibility complex ligand (Ag/MHC). In recent years, it has been observed that the engagement of T- cell antigen receptor (TCR) by its cognate ligand triggers a series of biochemical events within the cell, which can lead either to cellular activation i.e. lymphokine production and cell proliferation or to the induction of a state of functional anergy and apoptosis. Anergy has been defined as a cellular state in which a lymphocyte fails to respond optimally (in terms of both cytokine production and cell proliferation) when stimulated through its antigen receptor. The programmed cell death or apoptosis occurs in thymocytes and lymphocytes and is a mechanism of negative selection (King and Ashwell, 1993 & Debatin et al., 1994). This also can occur in mature T- cells and has been suggested to be a mechanism for tolerance. The precise mechanism in lymphocytes by which the 'choice' is made to proliferate or die is not well understood (Mapara et al., 1993; Falk et al., 1992; Lenardo, 1991; Boehme and Lenardo, 1993). It has been demonstrated that a high dose of antigen can lead to a paradoxical suppression of immune response that is termed as high zone tolerance. A proposed mechanism to explain this phenomena is based on the findings that T cells that have previously been exposed to IL-2 and then stimulated via their TCR undergo

apoptosis (Boehmeand and Lenardo, 1993). This has been termed propiocidal regulation and is thought to present feed back inhibition of T lymphocytes by which selective killing of specific T cell clone might occur. While NK cells fail to rearrange TCR subunit, nonetheless they share a number of features with T cells, including expression of surface molecules and secretion of some of the similar cytokines (Lanier et al., 1986 & Ortaldo et al., 1986). T and NK cells are also developmentally related however, unlike T cells, many of the details of NK cell development are poorly characterized. Like T cells NK cells also undergo functional anergy and apoptosis. A large body of data, mostly *in vitro*, indicate that anergy may develop in NK cells as a consequence of interaction with target cells or following cross-linking with specific antibodies to surface antigens. Tumor target cells also direct inactivation and IL-2 dependent reactivation of LAK cells. NK cells stimulated first with IL-2 and then ligated with anti CD16 antibody exhibited ligand-induced NK cell death (Ortaldo et al., 1995). Also target cell induced anergy of natural killer cytotoxic function is restricted to NK target conjugate subset (Jewett and Bonavida, 1995). Target induced inactivation and cell death by apoptosis in a subset of human NK cells is also reported (Jewett and Bonavida, 1996). Beside anti CD16 antibodies, other triggering receptors on NK cells such as CD44, anti NK receptor antibodies and pharmacological activation results in the generation of a cell death signal, which involves the CD95 pathway (Ortaldo et al., 1995). IL-2 and IL-12 induced IFN-γ production by human NK cell is followed by apoptosis in NK cells. The mechanism of cytokine induced apoptosis in human NK cells appears to involve the production of tumor necrosis factor alpha (Rose and Caligiuri, 1997). Anti CD16 monoclonal antibody treated naive NK cells acquired CD95 $^{+}$ phenotype and lost their cytotoxic function. A significant number of the NK cells underwent apoptosis and a selective induction of TNF-α synthesis and secretion was observed (Jewett et al., 1997).

AK-5 is highly immunogenic rat histiocytic tumor (Khar, 1986 & Khar, 1993) which regresses spontaneously in syngeneic animals when transplanted s.c. whereas it kills 100% animals when transplanted i.p. The AK-5 tumor follows a site-specific growth pattern when transplanted in syngeneic hosts. Upon s.c. transplantation, the tumor grows upto day 15 (growth phase), after which it starts getting rejected during the regression phase. This is followed by the healing phase when the animal is totally normal but immune to subsequent challenges of AK-5 tumor (Kausalya, 1994). However, the i.p. transplanted tumor cells divide rapidly and form a peritoneal bulge by day 2-3. These animals start dying by day 5. This short time duration with heavy tumor burden impairs and inactivates the immune system, whereas prolonged growth phase in s.c. transplanted animals leads to immune activation and tumor regression.

The specific events that take place subsequent to NK cell and tumor antigen interaction during inactivation at the cellular level remain obscure. In the present study, the phenotypic changes that take place in the NK cells after exposure to fixed tumor antigens were studied. The changes observed eventually lead to loss of cytotoxic function, TNF-α secretion and apoptosis of NK cells. These phenomena may be attributed to the observed anergy in the NK cell function following exposure to tumor antigen.

3.2 RESULTS

3.2.1 NK cell cytotoxicity after co-culture with fixed tumor cells

Antigen concentration may be a major regulator of effector cell function. Recent *in vitro* experiments have indicated antigen dose to be an important factor in immune response. We have studied the effect of formaldehyde fixed AK-5 cells (antigen) on naive rat splenocytes. A fixed number of splenocytes were co-cultured with fixed AK-5 cells at different effector to target cell ratios. After 20 h of incubation, NK cells were isolated from the co-cultured cell suspension by

mAb 3.2.3 coated Dynal immuno magnetic beads and were tested for their cytotoxic activity against ^{51}Cr labelled AK-5 and YAC-1 target cells. At higher antigen concentration (E: T = 100:100) cytotoxicity of fixed tumor cell treated NK cell was markedly lower than that of untreated NK cells. Addition of decreasing doses of antigen to splenocyte culture resulted in gradual increase in cytotoxic activity of NK cells. At lower antigen concentration (E: T = 100:1), NK cells showed a significant augmentation in their cytotoxic activity in comparison to untreated NK cells **(Fig. 3.1)**. The augmentation in the cytotoxic activity of NK cells is not due to the binding of mAb 3.2.3, which is a known activator of NK cell function, as the control cells were also isolated using the same procedure. Since AK-5 is a macrophage-like cell, we also cocultured splenocytes with formaldehyde-fixed naive macrophages at 100:100 ratio. There was no significant difference in the cytotoxic activity of macrophage treated and untreated NK cells. The concentration, which brought about inhibition of cytotoxic activity, will henceforth be referred to as "inhibitory cell number" and those at which a significant augmentation in cytotoxic activity was seen will henceforth be referred to as "stimulatory cell number". This bi-phasic dose response was retained from experiment to experiment though there were slight shifts in actual number of fixed AK-5 cells. These observations suggest that the interaction of splenocytes with fixed tumor cells either activate or suppress the NK cell cytotoxic function, which is regulated by the concentration of the antigen present during co-culture.

3.2.2 Purified NK cells undergo deactivation following co-culture with fixed AK-5 cells

We found that treatment of splenocytes with high antigen doses (100:100 to 100:25) results in deactivation of NK cells. In order to find out whether fixed tumor cells can induce anergy in pure NK cells by direct interaction or tumor antigen required other accessory cells for the induction of anergy in NK cells, we incubated purified naive NK cells (purified by 3.2.3

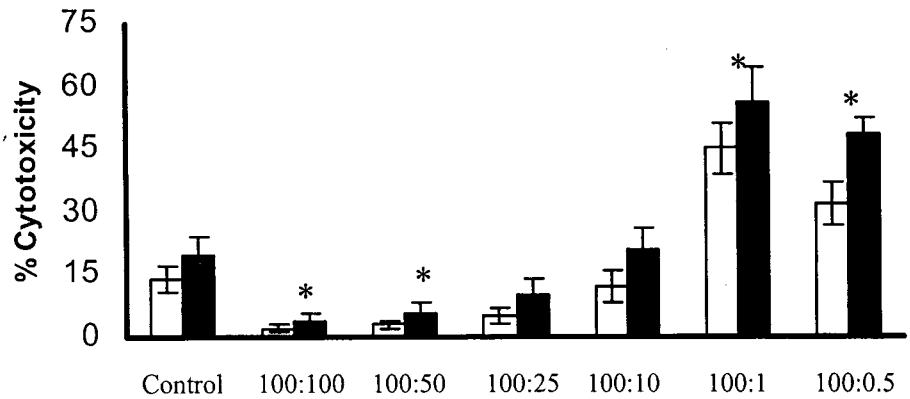

Fig. 3.1: Cytotoxicity of NK cells against AK-5 cells (open bar) and YAC-1 cells (closed bar). Naive splenocytes were co-cultured with fixed AK-5 cells at 37°C for 20 h at different splenocyte to fixed tumor cell ratios. Following incubation NK cells were isolated from co-culture cell suspension and checked for their cytotoxic activity. Data are expressed as the mean ± SD from one representative experiment in triplicate. Similar results were obtained in two other experiments.

* $p < 0.01$; control versus experimental group.

mAb coated immunomagnetic beads) with fixed tumor cells at different ratios. As shown in **Fig. 3.2**, coculture of fixed tumor cells with purified NK cells results in deactivation of NK cells at (100:100) to (100:25) ratios. At 100:100 ratio, NK cells showed negligible cytotoxic activity. With decreasing concentration of fixed tumor cells, increase in NK cell cytotoxic activity was observed. At 100:25 ratio, cytotoxic potential of NK cells treated with fixed tumor cells was not significantly different than that of untreated NK cells. These results indicate that induction of NK cell anergy can be a result of direct interaction of pure NK cell with tumor cells.

3.2.3 Effect of fixed AK-5 cells on the cytotoxic activity of immune NK cells

We examined the cytotoxic properties of immune NK cells obtained from animals that rejected the AK-5 tumor following their treatment with fixed AK-5 cells at higher antigen concentration. Purified immune NK cells were cocultured with fixed AK-5 cells at different ratios. Following incubation, NK cells were separated from fixed AK-5 cells and tested for cytotoxicity against YAC-1 and AK-5 target cells. As compared to the untreated NK cells, fixed tumor cell treated NK cells (100:100) exhibited significantly less cytotoxic activity. Addition of decreasing concentrations of tumor antigen results in decrease in anergy induction in immune NK cells. At 100:25 ratio tumor antigen did not show much inactivation in immune NK cells **(Fig. 3.3)**. Thus, the interaction between immune NK cells and fixed AK-5 cells resulted in the inactivation of NK cell cytotoxic potential. We have also done control experiments with formaldehyde fixed macrophages, which did not show any suppression of NK cell cytotoxicity.

3.2.4 Effect of fixatives used for fixation of AK-5 cells on NK cell deactivation

Experiments were done to clarify whether NK cell inactivation was achieved after co-culture with fixed AK-5 cells even when AK-5 cells were fixed with different fixatives. AK-5 cells were fixed with methanol, 95% ethanol, methanol acetic acid mixture (2:1), formaldehyde

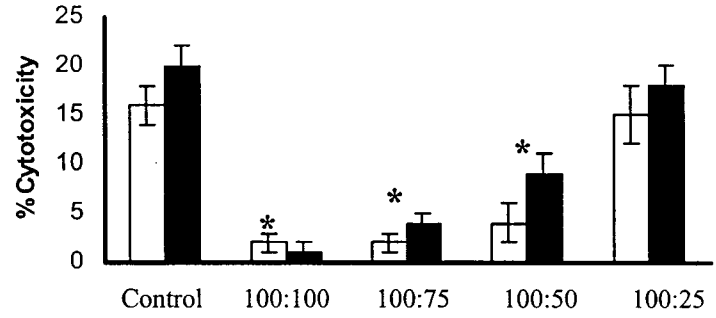

Fig. 3.2: Cytotoxicity of Naive NK cells against AK-5 cells (open bar) and YAC-1 cells (closed bar). Naive NK cells were co-cultured with fixed AK-5 cells at 37°C for 20 h at different NK cell to fixed tumor cell ratios. Following incubation NK cells were isolated from co-culture cell suspension and checked for their cytotoxic activity. Data are expressed as the mean ± SD from one representative experiment in triplicate. Similar results were obtained in two other experiments.

* $p < 0.01$; control versus experimental group.

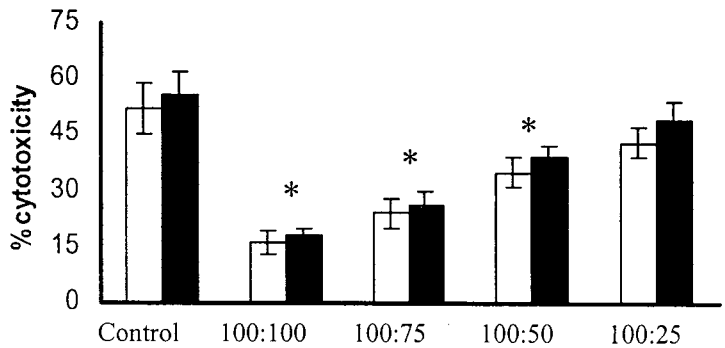

Fig. 3.3: Cytotoxicity of immune NK cells against AK-5 cells (open bar) and YAC-1 cells (close bar). Immune NK cells were co-cultured with fixed AK-5 cells at 37°C for 20 hrs at different immune NK cell to fixed tumor cell ratios. Following incubation NK cells were isolated from co-culture cell suspension and checked for their cytotoxic activity. Data are expressed as the mean ± SD from one representative experiment in triplicate. Similar results were obtained in two other experiments.

* $p < 0.01$; control versus experimental group.

(3%), paraformaldehyde (3%) and periodate-lysine-paraformaldehyde (PLP). It was found that NK cells cocultured with AK-5 cells that had been fixed with formaldehyde, paraformaldehyde and PLP solution exhibited inactivation against AK-5 and YAC-1 cells (**Fig. 3.4**). On the other hand, AK-5 cells that have been fixed with methanol, ethanol and methanol-acetic acid mixture failed to induce significant deactivation in NK cells. Same observations were made when immune NK cells were treated with AK-5 cells, which were fixed by different fixative agents (**Fig. 3.5**). Fixation with PLP is frequently used for immunocytochemical analysis of glycoproteins on cell surface because PLP can preserve the antigenicity of glycoproteins. It may be glycoprotein(s) on the AK-5 cells, which could be responsible for the activation of NK cells.

3.2.5 Production of soluble molecules after co-culture of NK cells and tumor cell

It is shown that interaction of NK cells with fixed tumor cells caused deactivation of both naive and immune NK cells at higher antigen concentration. In order to study whether the production of any soluble molecule(s) during the co-culture of NK cells and fixed tumor cells is responsible for the induction of anergy, we tested the effect of the culture supernatants on the cytotoxic activity of freshly isolated naive and immune NK cells. Culture supernatants were harvested following 24 h of incubation of naive and immune NK cells with fixed AK-5 cells separately and then concentrated two fold by ultrafiltration. Thereafter, the concentrated culture supernatants were added to freshly isolated immune and naive NK cells following dilution with complete tissue culture media at different ratios. Cytotoxic activity of NK cells decreased gradually with increase in concentration of culture supernatant. At higher concentrations of culture supernatants, the cytotoxicity of NK cells was significantly reduced (**Fig. 3.6**). These observations suggest that the interaction between NK cells and fixed AK-5 cells trigger the secretion of soluble molecule(s), which are involved in the induction of anergy in NK cells.

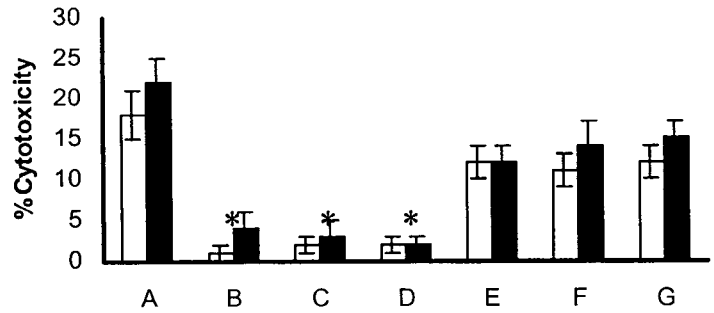

Fig. 3.4: Cytotoxicity of naive NK cells against AK-5 cells (open bar) and YAC-1 cells (closed bar). AK-5 cells were fixed by using different fixatives and then cocultured with NK cells (100:100). A, NK alone; B, formaldehyde (3%); C, paraformaldehyde (3%); D, P.L.P.; E, 80% methanol; F, 95% ethanol; G,methanol and acetic acid (2:1). Data are expressed as the mean ± SD from one representative experiment in triplicate.

* $p < 0.01$; control versus experimental group.

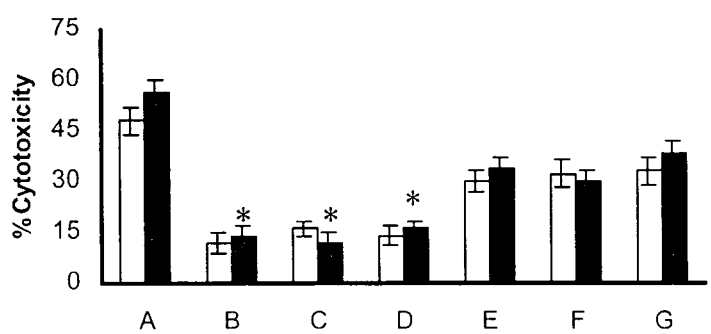

Fig. 3.5: Cytotoxicity of Immune NK cells against AK-5 cells (open bar) and YAC-1 cells (closed bar). AK-5 cells were fixed by using different fixatives and then co-cultured with NK cells (100:100). A, NK alone; B, formaldehyde (3%); C, paraformaldehyde (3%); D, P.L.P; E, methanol; F, 95% ethanol; G, methanol and acetic acid (2:1). Data are expressed as the mean ± SD from one representative experiment in triplicate.

* $p < 0.01$; control versus experimental group.

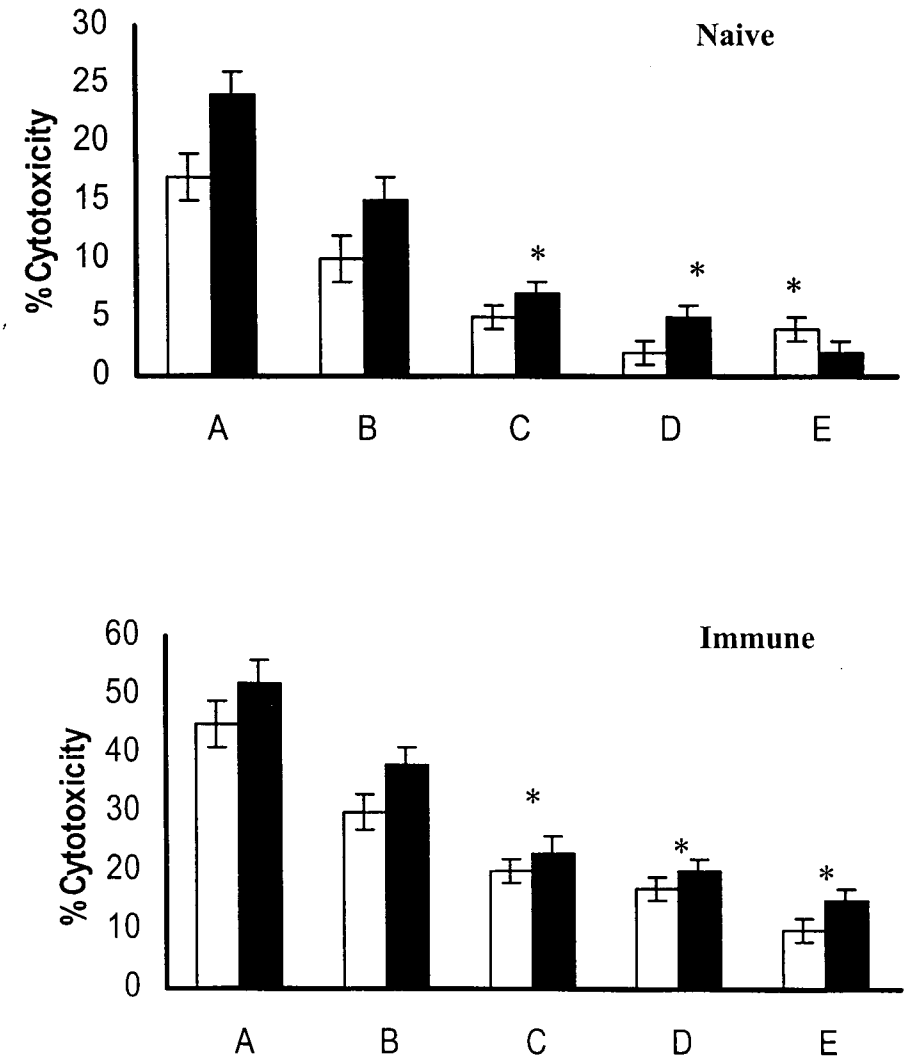

Fig. 3.6: Cytotoxity of naive and immune NK cells against AK-5 cells (open bar) and YAC-1 cells (closed bar). NK cells were incubated with spent culture supernatant for 24 h (at different dilutions with fresh culture medium) obtained from fixed AK-5 cell treated NK cells (100:100). A, control NK; B, NK + culture supernatant (1:2); C, NK + culture supernatant (1:1); D, NK + culture supernatant (1: 0.5); E, NK + fixed AK-5 cells.

* $p < 0.01$; control versus experimental group.

3.2.6 TNF-α secretion by splenocytes after co-culture with fixed AK-5 cells

The effect of fixed AK-5 cells on naive and immune NK cells were investigated for the secretion of cytokines and interleukins. NK cells are known to synthesize and secrete several cytokines, including GM-CSF, TNF-α and IFN-γ. NK cells were co-cultured overnight in the presence or absence of fixed AK-5 cells. The supernatants were harvested and analysed by ELIFA for the presence of cytokines. Addition of fixed AK-5 cells to NK cells induced significant secretion of TNF-α. TNF-α was secreted in the coculture supernatants by both immune and naive NK cells following overnight incubation with fixed AK-5 cells at 100:100 ratio (**Fig. 3.7**). No TNF-α production was observed at lower antigen concentration. In contrast to TNF-α secretion, treatment of NK cells with fixed AK-5 cells at 100:100 ratio did not stimulate the secretion of IFN-γ (**Fig. 3.9**). TNF-α levels secreted by immune NK cells were higher than the TNF-α levels secreted by naive NK cells (**Fig. 3.7**). In case of untreated NK cells very low levels of TNF-α and IFN-γ secretion were observed. These findings demonstrate that the treatment of naïve or immune NK cells with fixed AK-5 cells selectively triggered the secretion of TNF-α, which may be an important molecule for the induction of anergy in NK cells.

3.2.7 Western blot analysis of TNF-α secretion by NK cells

Secretion of TNF-α in culture supernatant following coculture of NK cells with tumor antigen was further confirmed by western blot analysis. Naive and immune NK cells were cocultured overnight with fixed AK-5 cells. The culture supernatants were harvested following overnight treatment and then concentrated by amicon ultra-filtration unit. The concentrated culture supernatants were used for western blot analysis. Addition of tumor antigen to NK cells at 100:100 ratio induced significant secretion of TNF-α by NK cells. TNF-α secretion was

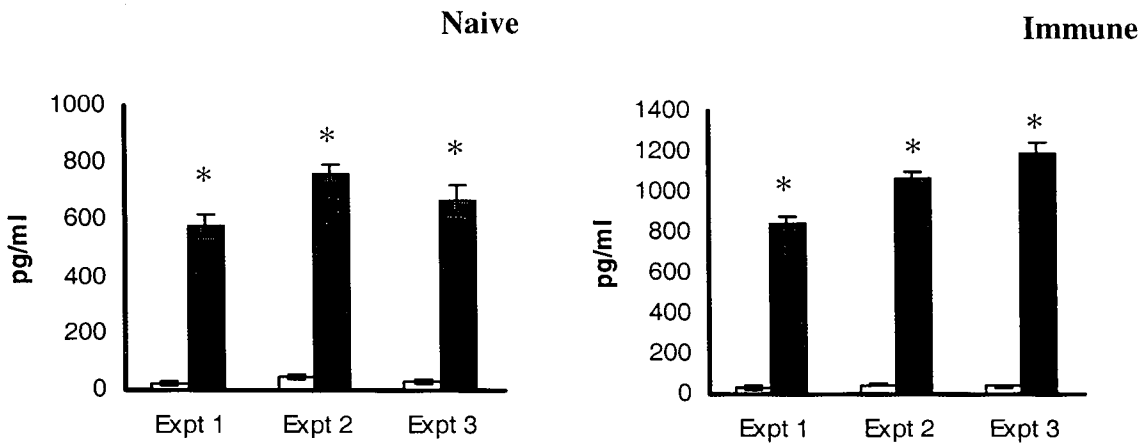

Fig. 3.7: Induction of TNF-α secretion by naive and immune NK cells after co-culture with fixed AK-5 cells (100:100). Supernatants were harvested from untreated NK cells (open bar) and co-cultured NK cells (closed bar) after 24 h and analyzed for the presence of TNF-α by ELIFA. Data are expressed as the mean ± SD from triplicate values.

* $p < 0.01$; experimental group versus control group.

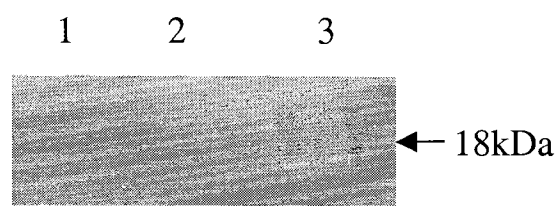

Fig. 3.8: Western blotting of TNF-α secretion by NK cells after 24h of incubation with fixed AK-5 cells (100:100). Lane 1, Naive NK cells; Lane 2, Naive NK cells + fixed AK-5 cells; Lane 3, Immune NK cells + fixed AK-5 cells.

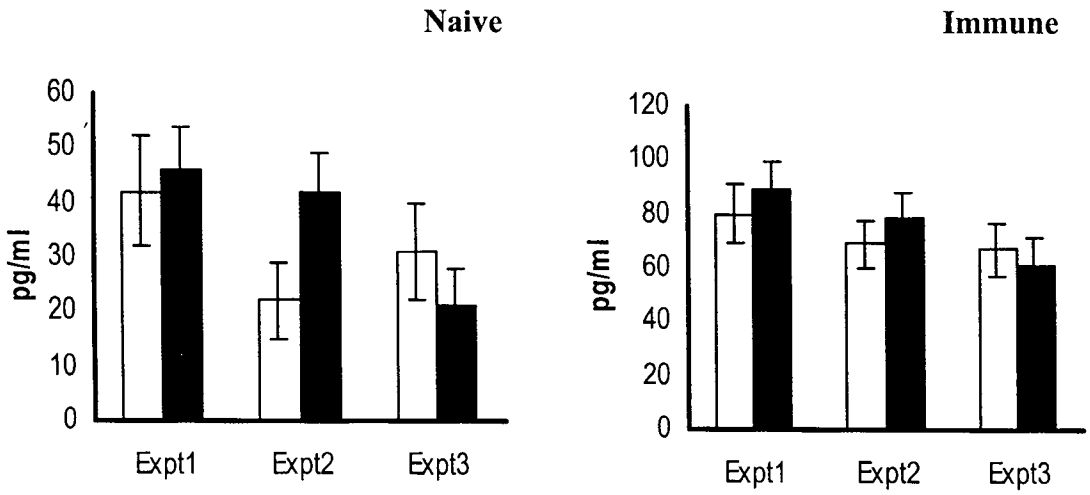

Fig. 3.9: Induction of IFN-γ secretion by naive and immune NK cells after co-culture with fixed AK-5 cells (100:100). Supernatants were harvested from untreated NK cells (open bar) and co-cultured NK cells (closed bar) after 24 h and analysed for the presence of IFN-γ by ELIFA. Data are expressed as the mean ± SD from three different experiments in triplicate.

* $p < 0.01$; experimental group versus control.

detected in the culture supernatant obtained from both naive and immune NK cells treated with fixed AK-5 cells but TNF-α levels secreted by immune NK cells were higher than that of TNF-α levels secreted by naive NK cells (**Fig. 3.8**). This experiment further confirmed the triggering of TNF-α secretion by NK cells following co-culture with fixed AK-5 cells.

3.2.8 Antigen dose-regulated TNF-α secretion by purified NK cells

To address the effect of antigen dose in influencing cytokine secretion, purified immune and naive NK cells were treated with fixed AK-5 cells at varying ratios of effector to fixed target cells (100:100 to 100:25). The supernatants were harvested following overnight incubation and then tested for the presence of TNF-α and IFN-γ by sensitive ELIFA. NK cells co-cultured with lower number of fixed tumor cells produced low levels of TNF-α. Addition of increasing doses of fixed AK-5 cells to NK cell culture resulted in the production of larger amounts of TNF-α. The administration of very high dose of antigen (100:100) however resulted in the induction of anergy in NK cells that produced large amount of TNF-α (**Fig. 3.10 & Fig. 3.11**). Control NK cells which were not treated with tumor antigen did not produce any TNF-α in the culture supernatant. IFN-γ secretion did not change much following treatment of NK cells with tumor antigen between concentration ranges of (100:100) to (100:25) ratio. This overall trend of cytokine secretion by NK cells at different antigen doses was consistent; however, a slight shift in absolute dose of antigen required for the production of a particular amount of TNF-α were observed in independent experiments.

3.2.9 Kinetics of TNF-α secretion by NK cells

We then studied the kinetics of TNF-α secretion by naive and immune NK cells following co-culture with fixed AK-5 cells for 72 h. NK cells were cultured in presence or absence of fixed AK-5 cells at 100:100 ratio. Following 24h, 48h and 72 h of incubation culture

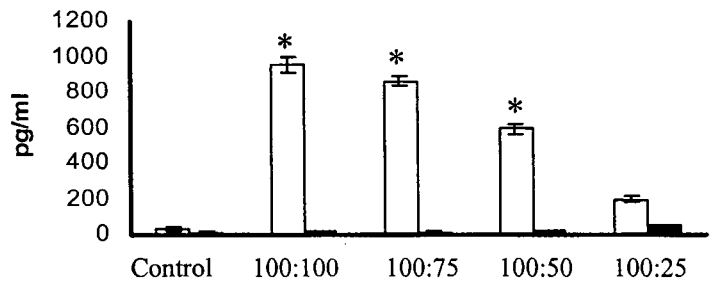

Fig. 3.10: Induction of TNF-α (open bar) and IFN-γ (closed bar) by naive NK cells. Naive NK cells were cocultured with fixed AK-5 cells at different NK cell to fixed AK-5 cell ratios. Following incubation supernatants were harvested and analysed for the presence of TNF-α and IFN-γ by ELIFA. Data are the mean ± SD from one representative experiment in triplicate. Similar results were obtained in two other experiments.

* $p < 0.01$; experimental group versus control group.

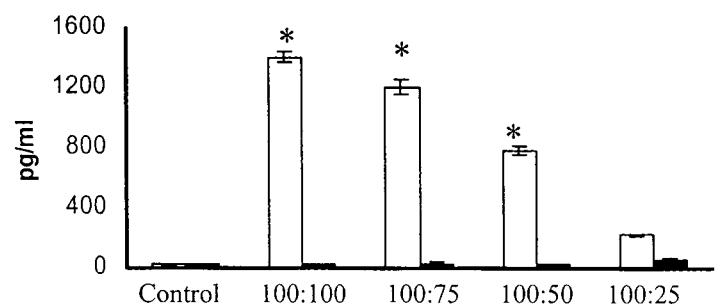

Fig. 3.11: Induction of TNF-α (open bar) and IFN-γ (closed bar) by immune NK cells. Immune NK cells were co-cultured with fixed AK-5 cells at different NK cell to fixed AK-5 cell ratios. Following incubation supernatants were harvested and analyzed for the presence of TNF-α and IFN-γ by ELIFA. Data are the mean ± SD from one representative experiment in triplicate. Similar results were obtained in two other experiments

* $p < 0.0$; experimental group versus control group.

supernatants were harvested and tested for the TNF-α content by ELIFA. After 24h of incubation, large amounts of TNF-α secretion were observed. Following 48h and 72h of incubation, a gradual increase in TNF-α secretion was detected (**Fig. 3.12**). Same kinetics was also followed by immune NK cells co-cultured with fixed tumor antigen at 100:100 ratio (**Fig. 3.12**). Secretion of TNF-α by immune NK cells was higher than that by naive NK cells in each time point.

3.2.10 Role of TNF-α in the suppression of NK cell function

The above findings demonstrated selective secretion of TNF-α following treatment of either naïve or immune NK cells with fixed AK-5 cells, suggesting a possible role for TNF-α in the various manifestations of fixed AK-5 treated NK cells. Accordingly, it was presumed that the neutralization of TNF-α activity would reverse the induction of anergy in NK cells. The addition of anti-TNF-α antibodies (1:100) to the NK cells cocultured with fixed AK-5 cells (100:100) resulted in significant inhibition of NK cell anergy (**Fig. 3.13**). Naive and immune NK cells, when treated with fixed AK-5 cells, showed decrease in NK cell cytotoxicity, as tested against YAC-1 and AK-5 cells (bars A and D). However, the suppression of cytotoxicity was significantly recovered after the addition of anti-TNF-α antibody during co-culture (bars B). The anti TNF-α antibody-mediated blocking of anergy was specific, as the addition of control isotype antibody had no effect (bar C). These observations suggest an important role for TNF-α in the induction of anergy in NK cells.

3.2.11 Reactivation of anergised NK cells by IL-2 and IFN-γ

The induction of anergy in NK cells following co-culture of tumor antigen was demonstrated. It was then examined whether the inactivated NK cells can be reactivated again by the NK cell activating cytokines, like IL-2 and IFN-γ. Purified NK cells were incubated with

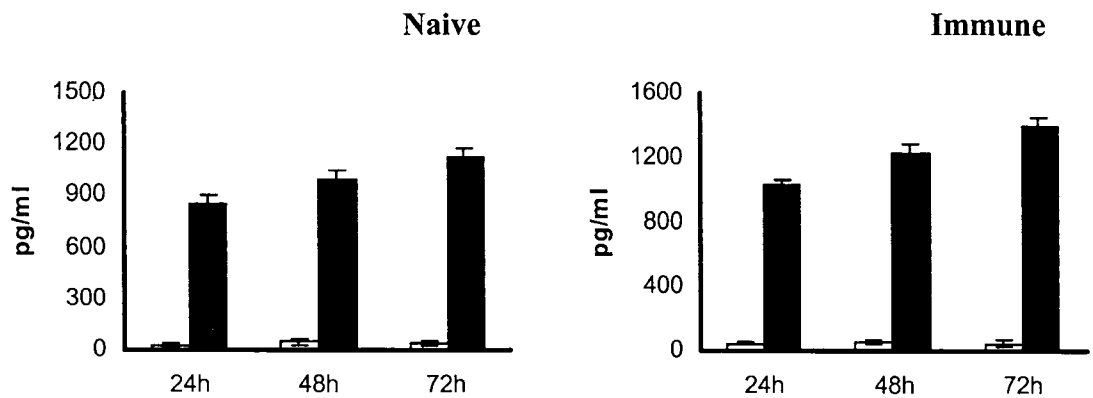

Fig. 3.12: Purified naive and immune NK cells were treated with fixed AK-5 cells (100:100) and incubated at 37°C. Culture supernatants were harvested at 24 h, 48 h and 72 h of culture and tested for TNF-α content by ELIFA. Open bar represents control and closed bar represents AK-5 treated culture supernatants. Results shown are mean ± SD from one representative experiment in triplicate. Similar results were obtained from two other experiments.

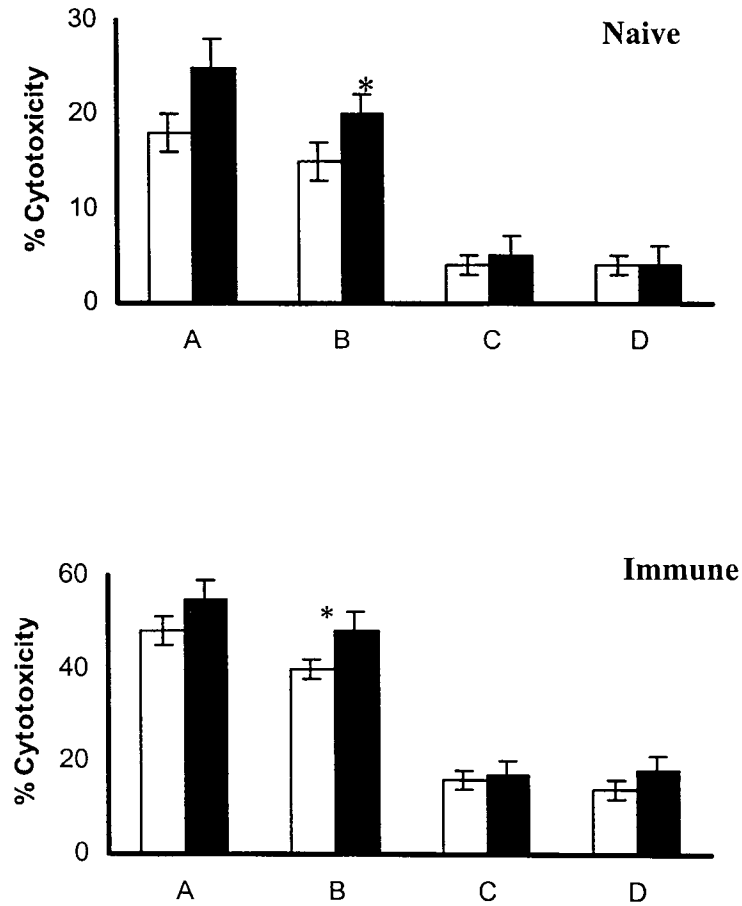

Fig 3.13: Cytotoxicity of NK cells against AK-5 (open bar) and YAC-1 (closed bar) target cells (E:T = 50:1). Naive and immune NK cells were cocultured with fixed tumor cells (100:100) in the presence or absence of anti-TNF-α antibody (1:100). A, NK alone; B, NK + anti-TNF-α antibody + fixed AK-5 cells (100:100); C, NK + control isotype antibody + fixed AK-5 cells (100:100); D. NK + fixed AK-5 cells (100:100). Data shown are the mean ± SD of one representative experiment in triplicate. Similar results were obtained in two other experiments.

*$p < 0.01$; fixed AK-5 cell treatment versus anti-TNF-α antibody treatment.

fixed tumor cells (100:100) for 24 h, after which, NK cells were isolated from the coculture cell suspension. These isolated anergised NK cells were then treated again with recombinant IL-2 (1000U/ml) and recombinant IFN-γ (1000U/ml) separately for the next 24h. Following IL-2 and IFN-γ treatment, NK cells were washed with PBS and tested for their cytotoxic activity against ^{51}Cr labelled YAC-1 and AK-5 target cells. Incubation with fixed AK-5 cells reduced the cytotoxic activity of naive NK cells drastically. But these anergised NK cells could be reactivated after overnight treatment with rIL-2 or rIFN-γ (**Table. 3.1**). Control NK cells which were not treated with fixed AK-5 cells were also activated by rIL-2 and rIFN-γ treatment. However, following treatment with rIL-2 and rIFN-γ, cytotoxic potential of control NK cells were much higher than that of fixed tumor cell-treated NK cells. Furthermore, increase in cytolytic activity of control NK cells following treatment with cytokines was also higher than the cytolytic activity of anergised NK cells. These results indicate that though inactivated NK cells can be reactivated but they cannot be activated to the same extent as untreated NK cells.

In case of immune NK cells, fixed tumor cells induced anergy, which was reflected in their reduced cytolytic potential. However, these anergised NK cells were again reactivated by treatment with recombinant IL-2 or IFN-γ (**Table. 3.2**). Control NK cells were not activated much by cytokine treatment because they were isolated from immune animals. Anergised immune NK cells were not reactivated to their initial cytotoxic potential following treatment with rIL-2 or rIFN-γ although rIL-2 or rIFN-γ treatment significantly enhanced the cytolytic activity of anergised NK cells.

3.2.12 Induction of apoptosis in anergised NK cells

Tumor antigen-mediated anergy induction in NK cells at high antigen dose was regulated by endogenous secretion of TNF-α by both naive and immune NK cells. Antigen-mediated

Table. 3.1

Role of IL-2 and IFN-γ in reactivation of naive NK cells

Treatment	YAC-1 cells		AK-5 cells	
	Cont	Exp	Cont	Exp
Fixed AK-5 cell treatment (100:100) (0 - 24 h)	20 ± 2	*4 ± 1	14 ± 2	*2 ± 1
IL-2 treatment (1000 u/ml) (24h - 48 h)	42 ± 5	*20 ± 3	43 ± 3	*18 ± 2
IFN-γ treatment (1000 u/ml) (24h - 48 h)	46 ± 6	*21 ± 3	44 ± 4	*19 ± 3

Purified NK cells were incubated with fixed AK-5 cells (100:100) for 24 h at 37°C. NK cells were separated by dynal beads and one part was tested for cytotoxicity against ^{51}Cr labelled target cells. Another part of NK cells was treated with IL-2 or IFN-γ separately for next 24 h. Following next 24 h incubation of NK cells with IL-2 or IFN-γ, NK cells were harvested and washed and tested for their cytotoxic activity against ^{51}Cr labelled YAC-1 and AK-5 cells.

*$p < 0.01$; Experimental group versus control group.

Table. 3.2

Role of IL-2 and IFN-γ in reactivation of immune NK cells

Treatment	YAC-1		AK-5 cells	
	Control	Exp	Control	Exp
Fixed AK-5 cell treatment(100:100) (0-24h)	55 ± 5	*21 ± 2	50 ± 4	*18 ± 2
IL-2 treatment (1000 u/ml) (24 h - 48h)	58 ± 4	*42 ± 4	54 ± 4	*37 ± 3
IFN-γ treatment (1000 u/ml) (24h - 48h)	60 ± 5	*40 ± 5	58 ± 5	*45 ± 3

Purified NK cells were incubated with fixed AK-5 cells (100:100) for 24 h at 37°C. NK cells were separated by dynal beads and one part was tested for cytotoxicity against ^{51}Cr labelled target cells. Another part of NK cells was treated with IL-2 or IFN-γ separately for next 24 h. Following 24 h incubation of NK cells with IL-2 or IFN-γ, NK cells were harvested and washed and tested for their cytotoxic activity against ^{51}Cr labelled YAC-1 and AK-5 cells.

*$p < 0.01$; experimental group versus control group.

anergy induction in lymphocytes often leads to cell death by apoptosis. In order to study whether fixed AK-5 cells can induce cell death, purified NK cells were co-cultured with fixed tumor cells and the frequency of cell death by apoptosis was determined by propidium iodide method. In the presence of fixed AK-5 cells, a gradual increase in NK cell death was observed when the cultures were incubated from 24 h to 72 h. Significant cell death by apoptosis was observed following 48 h and 72 h incubation of NK cells with fixed AK-5 cells (**Fig. 3.14**). A higher percentage of apoptotic cell death was seen in immune NK cells as compared to naive NK cells. In case of naive NK cells, almost 63 % cells showed apoptotic cell death after 72 h of incubation with tumor antigen at high dose (100:100) whereas in case of immune NK cells, 80% of the NK cells were apoptotic following exposure to tumor antigen at 100:100 ratio. These experiments suggest that induction of anergy in NK cells by fixed tumor antigen leads to NK cell death by apoptosis.

3.2.13 Role of endogenous TNF-α secretion in the initiation of apoptosis

This study demonstrated an important role for TNF-α in the induction of anergy by fixed tumor cell treated NK cells. Previous studies reported that TNF-α might activate or inhibit the function of NK cells depending on the nature of stimulus used. TNF-α induces cell death in tumor cells. In order to find out whether endogenously secreted TNF-α by NK cells was responsible for the induction of apoptosis in NK cells, TNF-α function was blocked in the co-culture of NK cell and fixed AK-5 cells (100:100) by addition of anti- TNF-α antibody (1:100) in culture media. The addition of polyclonal anti TNF-α antibody to NK cells co-cultured with fixed AK-5 cells resulted in significant inhibition of NK cell death (**Fig. 3.15.** bar B). Anti TNF-α mediated inhibition of induction of anergy was also observed in case of both immune as well as naive NK cells. The anti- TNF-α antibody mediated blocking of apoptosis was specific, as the

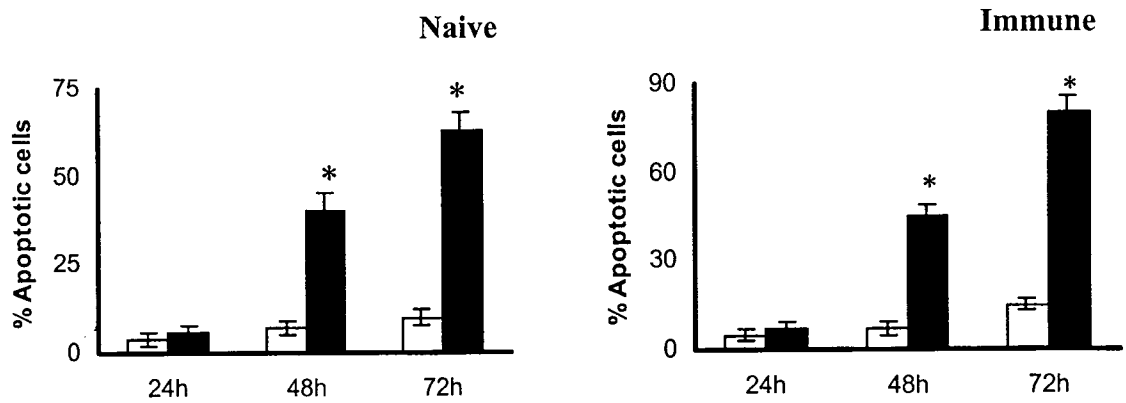

Fig 3.14: Fixed AK-5 cells mediated apoptosis of NK cells. Naïve and immune NK cells were incubated with fixed AK-5 cells (100:100) for 24 h, 48 h and 72 h. Following incubation, NK cells were isolated and frequency of apoptotic NK cells was obtained by PI staining and immunofluorescence analysis. Open bars represent control NK cells and closed bars represent AK-5 cell treated NK cells. Data shown are the mean ± SD of one representative experiment in triplicate. Similar results were obtained in two other experiments.
*$p < 0.01$; control versus experimental group.

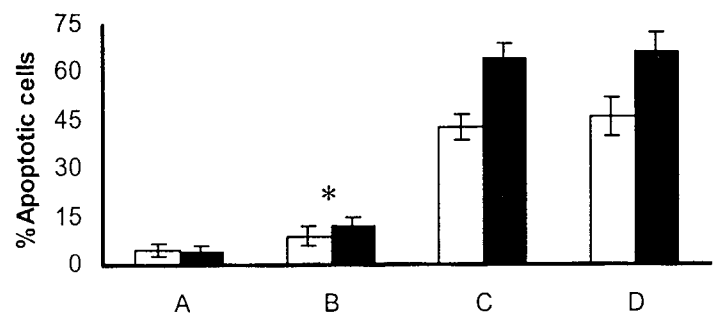

Fig 3.15: Inhibition of fixed AK-5 cell mediated apoptosis in NK cells by anti TNF-α antibody (1:100). Naive (open bar) and immune (closed bar) NK cells were cocultured with fixed AK-5 cells in presence or absence of anti-TNF-α antibody. A, NK alone; B, NK + anti TNF-α Ab+ fixed AK-5 cells (100:100); C, NK + control isotype Ab + fixed AK-5 cells (100:100); D, NK + fixed AK-5 cells (100:100). Following 48 h of incubation, NK cells were isolated from coculture and frequency of apoptotic NK cells were obtained by PI staining and immunofluorescence analysis.
*$p < 0.01$; fixed AK-5 cells treatment versus anti-TNF-α Ab treatment.

addition of control isotype antibody had no effect on the induction of apoptosis in NK cells (**Fig. 3.15** bar C).

3.2.14 Upregulation of CD95 in the NK cells treated with tumor antigen

The interaction between Fas (CD95) and its ligand is involved in the induction of apoptosis in the immune cells to maintain selection, development and to limit lymphoid expansion. CD95 is involved in the apoptotic pathway of lymphocytes. CD95 and TNF receptors have similarity in protein structure. We have reported triggering of TNF-α secretion and induction of apoptosis by NK cells following co-culture of NK cells with tumor antigens. So it was important to study whether fixed tumor cell mediated anergy induction occurs through the involvement of CD95. Purified NK cells were treated with fixed AK-5 cells (100:100) for 48h. Following incubation, NK cells were isolated and the expression of CD95 antigen on the surface of NK cells was determined by staining with anti CD95 monoclonal antibody. Treatment of NK cells with fixed AK-5 cells induced overexpression of CD95 in NK cells, which was confirmed by immunofluorescence (**Fig. 3.16**). CD95 expression was higher in immune NK cells as compared to naive NK cells following treatment with fixed AK-5 cells. In case of untreated NK cells, expression of CD95 was very low in both immune and naive NK cells. These results indicate that anergy induction in NK cells is associated with enhancement of CD95 expression on the surface of NK cells, which can also be recognized as a marker of anergy induction in NK cells.

3.2.15 Blocking of CD95 upregulation in NK cells by anti TNF-α antibody

Tumor antigen mediated anergy induction and apoptosis seems to be mediated by the endogenous secretion of TNF-α by NK cells. In order to study whether there is a direct correlation between TNF-α secretion and CD95 up-regulation, TNF-α activity in the NK cells

cocultured with fixed AK-5 cells was blocked by addition of anti TNF-α antibody (1:100). Both naive and immune NK cells upregulated the CD95 expression on their surface following treatment with fixed AK-5 cells (**Fig. 3.17.** bars A & D). However, addition of polyclonal anti TNF-α antibody significantly reduced the expression of CD95 on NK cells (bar B). This inhibition of Fas upregulation in NK cells following treatment with tumor antigen was specific to anti TNF-α antibody, as control isotype antibody had no effect on the CD95 expression on NK cells (bar C).

3.2.16 Status of NK cell activation markers

We also tested the expression of different molecules like CD16 and perforin, which are generally associated with activation of NK cell function. The cell surface expression of CD16 and perforin by NK cells following treatment with fixed AK-5 cells was checked by immunofluorescence and cytofluorimetric analysis by staining NK cells with specific anti perforin and anti CD16 antibodies. There were no significant differences in the expression of CD16 and perforin by NK cells before and after treatment with fixed AK-5 tumor cells (**Fig. 3.18**).

3.4 DISCUSSION

This study provides evidence for the role of endogenously secreted TNF-α in mediating functional inactivation and apoptosis in NK cells when they are co-cultured with fixed AK-5 cells at 1:1 ratio. In addition, a change in the phenotype from $CD95^{dim}$ to $CD95^{bright}$ was observed in NK cells co-cultured with target cells. The up-regulation of CD95 expression on the NK cells after co-culture with fixed AK-5 cells may play an important role in signaling NK cells for functional anergy and apoptosis (Haux et al., 1999).

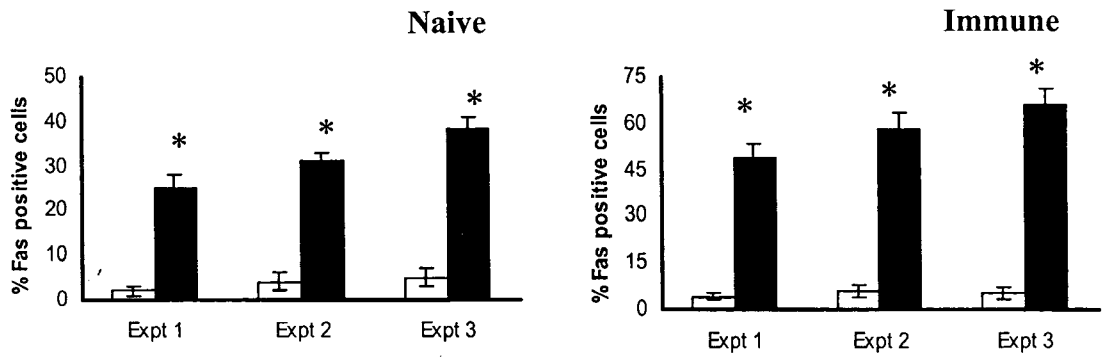

Fig 3.16: Upregulation of Fas in NK cells. Naive and immune NK cells were cocultured with fixed AK-5 cells (100:100). Following 48 h of incubation, NK cells were isolated and the expression of Fas antigen on NK cell surface was determined by staining with anti- Fas antibody. Open bar represents control NK cells and closed bar represents AK-5 cells-treated NK cells. Data shown are mean ± SD.
*$p < 0.01$; control versus experimental group.

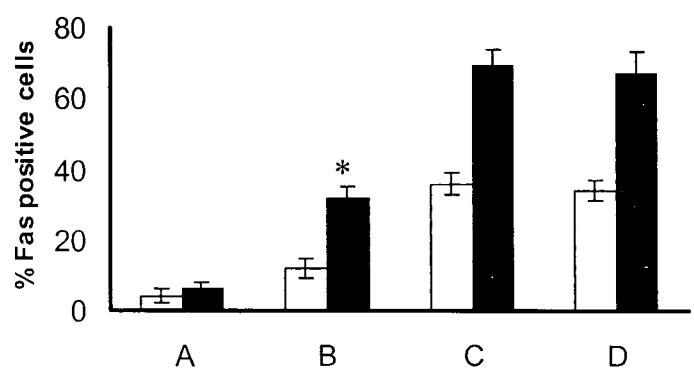

Fig 3.17: Inhibition of fixed AK-5 cell mediated Fas upregulation in NK cells by anti TNF-α antibody. Naive (open bar) and immune (closed bar) NK cells were cocultured with fixed AK-5 cells in presence or absence of anti-TNF-α antibody. A, NK alone; B, NK + anti TNF-α ab + fixed AK-5 cells (100:100); C, NK + control isotype Ab + fixed AK-5 cells (100:100); D, NK + fixed AK-5 cells (100:100). Following 48 h of incubation, NK cells were isolated from coculture and frequency of Fas positive NK cells were obtained by staining with anti- Fas antibody and immunofluorescence analysis.
*$p < 0.01$; fixed AK-5 cells treatment versus anti-TNF-α Ab treatment.

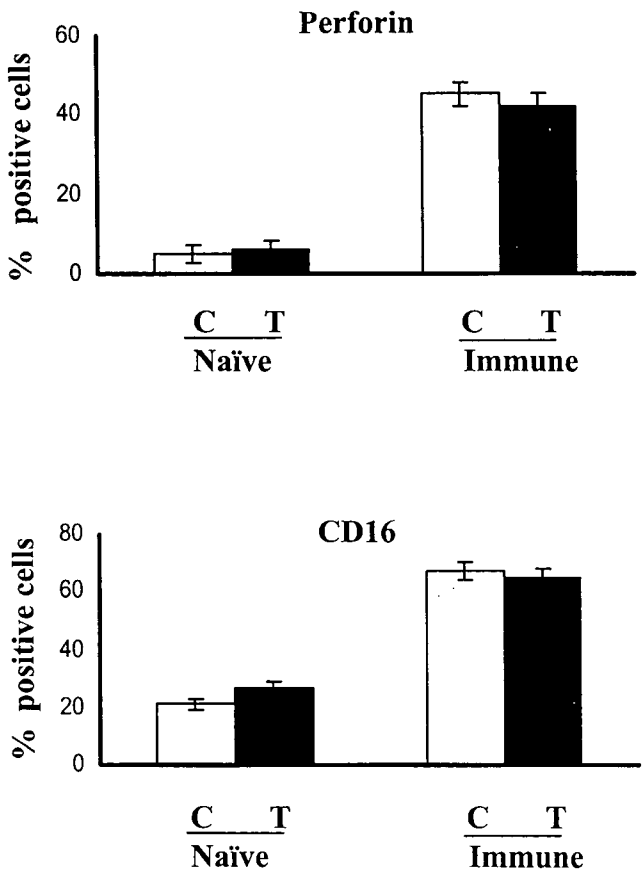

Fig 3.18: Expression of perforin and CD16 in NK cells. Naive and immune NK cells were cocultured with fixed AK-5 cells (100:100). Following 24 h of incubation, NK cells were isolated and the expression of perforin and CD16 antigen on NK cell surface was determined by staining with anti-perforin and anti-CD16 antibody. Open bar represents control NK cells and closed bar represents AK-5 cell-treated NK cells. Data are expressed as mean ± SD for three different experiments.

NK cells when treated with fixed AK-5 cells lost their cytotoxic function as compared to the untreated NK cells. The loss of cytotoxic function was observed for both normal and immune NK cells. Functional inactivation could be due to the depletion of the lytic factors or due to the impairment of the activation pathway of the lytic machinery. However, no change in perforin expression was observed between treated or untreated immune NK cells suggesting that anergy induction in the tumor cell treated NK cells is not due to the depletion of the lytic factors. The other possibility is that the treatment of NK cells with fixed AK-5 cells caused the impairment of the activation pathway of the lytic machinery, finally leading to the apoptosis of NK cells. Addition of IL-12 and IFN-γ to the NK cells, following co-culture with fixed AK-5 cells for 20 h, restored partially their cytotoxic function, suggesting their recovery from the anergic state. These results indicate that the reduction in cytotoxic function is more likely to be due to the functional inactivation of NK cells. There is also a possible involvement of IL-12 and IFN-γ in the reactivation of these inactivated NK cells.

Furthermore, the treatment of NK cells with fixed AK-5 cells triggered the secretion of TNF-α, which was found to play a critical role in the induction of NK cell inactivation and death. The role of TNF-α was corroborated by demonstrating that the blocking of TNF-α function by anti TNF-α antibody abrogated partially the AK-5 cell mediated NK cell inactivation. The addition of anti-TNF-α resulted in inhibition of the induction of anergy in normal and immune NK cells. These observations suggest that TNF-α may be one of the molecules involved in the NK cell inactivation. In addition, other molecules and cellular interactions may also participate in NK cell inactivation. Nonetheless, addition of culture supernatants harvested from AK-5 treated NK cells to the normal and immune NK cells caused inactivation of NK cells. This clearly indicated that the soluble molecules secreted by the NK

cells following co-culture with fixed AK-5 cells are responsible for the inactivation of NK cell function.

CD16 has been implicated in signal transduction pathway of NK cells (Nagrer et al., 1989; Salcedo et al., 1993; Ting et al., 1991). Lanier et al. (1988) showed that $CD16^{dim}$ cells had significantly lower cytotoxic activity than the $CD16^{bright}$ fraction. In our system, we did not observe any changes in the expression of CD16 levels on the surface of treated and untreated NK cells, thereby suggesting that the lower cytotoxic function of treated NK cells may not be due to the reduced expression of CD16 on these cells. No significant differences in the levels of perforin expression in treated or untreated NK cells were observed in our studies. These observations indicated that the lower cytotoxic function in the tumor-treated NK cells is not due to the downregulation of either CD16 or perforin expression. However, a change in the phenotype from $CD95^{dim}$ to $CD95^{bright}$ was observed in NK cells treated with fixed tumor cells. The upregulation of CD95 may be playing an important role in the induction of anergy in NK cells. CD95 is involved in the induction of apoptosis in immune cells to maintain selection and development and to limit lymphoid expansion (Ogasawora et al., 1995 & Azzoni et al., 1988). We have also observed increased apoptotic death in the NK cells, which were co-cultured with AK-5 cells. Thus, our results suggest that the up-regulation of CD95 may be an indication of the anergic state in NK cells.

There was a selective secretion of TNF-α, but not IFN-γ in tumor antigen treated naive and immune NK cultures. In a previous study, cross-linking of CD16 on NK cells was shown to elevate the secretion of both TNF-α and IFN-γ. The difference between these two studies may be due to the type of inducers used and the state of activation of NK cells. This study demonstrates that TNF-α plays an important role in the induction of anergy in NK cells. TNF-α has been reported to play a pivotal role in the activation of NK cells by IL-2 (Jewett and Bonavida, 1993). Thus endogenous TNF-α is able to activate and inhibit the function of NK cells depending on the

nature of the stimulus used (Jewett and Bonavida, 1993; Jewett et al., 1997; Ortaldo et al., 1995). Such dual roles for TNF-α are consistent with other findings (Aggarwal et al., 1996). Clearly, different pathways have been reported to lead either to cell death by apoptosis or to activate NF-κB and inhibit the cell death (Salcedo et al., 1993 & Trinchieri, 1989). We have observed up-regulation of CD95 on NK cells that were treated with fixed AK-5 cells, and its regulation by TNF-α could represent a mechanism by which TNF-α contributes towards inactivation of NK cells.

Thus, our findings demonstrate that interaction of NK cells with fixed AK-5 cells at high antigen concentration caused decrease in cytotoxic function of both normal and immune NK cells. The upregulation of CD95 expression and induction of TNF-α secretion is coupled with negative signaling for cytotoxic function and triggers induction of apoptosis in NK cells. In this context, it is conceivable that when the concentration of target cells is high, the NK cells are constantly interacting with the target cells, either directly or indirectly via the FcR, by ADCC or Ag-Ab complexes. Such interactions might result in the loss of cytotoxic function in NK cell. Further studies directed towards inhibiting the processes leading to NK cell inactivation might help in restoring the function of NK cells in immune surveillance against infection and cancer.

CHAPTER 4

Target cell induced activation of NK cells in vitro: Cytokine production and enhancement of cytotoxic function

4.1 INTRODUCTION

Natural killer cells are large granular lymphocytes distinguishable from T and B lymphocytes by their surface phenotype, cytokine profile and their ability to mediate spontaneous cytotoxicity against a broad range of targets including MHC class I negative tumor cells (Trinchieri, 1989). The exact mechanism of NK cell mediated oncolysis remains ill-defined, but involves a complex array of events such as (i) recognition and conjugation of effector and target cells, (ii) NK cell activation and delivery of the lethal hit and (iii) target cell disintegration and death (Herberman et al., 1986). Despite the differences between tumors, it has been established that the initiation of the entire lytic cascade starts with the recognition and effector : target conjugation, which is determined by the functional status of the effector cells and the susceptibility of the targets (Karre et al., 1991). Necrosis and apoptosis have been the two proposed modes of CTL /NK mediated target cell death (Young, 1989). The pore forming protein perforin has been recognized as the prime molecule in necrotic cell death (Duke et al., 1989). Upon stimulation with target cells, functional CD95-L is induced on NK cells in appropriate configuration, which cross-links with CD95 on the target cells and induces their death (Arase et al., 1995). Several cytokines such as IL-2, IFN-γ and IL-12 have been shown to augment NK cell proliferation and activation (Herberman et al., 1986). Activated NK cells produce a spectrum of cytokines such as TNF-α, IFN-γ, GM-CSF, lymphotoxin and IL-8 (Trinchieri, 1989). The production of IFN-γ by NK cells has been shown to be enhanced by IL-12 (Cui et al., 1997), which favors a Th1 type cellular response.

A lymphocyte subpopulation called NKT cells expresses both NK cell receptor (CD161) and a single invariant T cell receptor encoded by the Vα14 and Jα281 gene segments (Imai et al., 1986 & Lantz et al., 1997 & Masuda et al., 1997). Although anti-tumor effect was thought to be

mediated through NK cells and T cells, Vα14 NKT cells were found to be an essential target of IL-12. NKT cells mediated their tumor cell cytotoxicity by an NK-like effector mechanism after activation with IL-12 (Cui et al., 1997).

AK-5, a highly immunogenic rat histiocytic tumor (Khar, 1986) regresses spontaneously in syngeneic animals when transplanted s.c., whereas it kills 100% animals when transplanted i.p. (Khar, 1993). The NK mediated death of AK-5 cells is achieved through apoptosis (Kausalya et al., 1995) and necrosis (Bright et al., 1995). We have observed naive NK cells to be ineffective in the killing of AK-5 tumor cells *in vitro*. But NK cells isolated from animals transplanted with AK-5 tumor cells exhibited cytotoxic activity against the tumor cells, suggesting the requirement for some sort of priming / activation of NK cells before they induce death in the target cells. The present study aimed to analyze the direct and indirect effects of fixed AK-5 cells on rat NK cells. Our results show that fixed AK-5 cells at low ratio activate NK cells, which in turn secrete IFN-γ in the absence of professional APCs, and that T cells mediate further augmentation of the cytolytic potential of NK cells. The effector cells after coculture with fixed tumor cells, showed enhanced expression of perforin and CD95-L.

4.2 RESULTS

4.2.1 Fixed tumor cell mediated activation of NK cells from spleen

To investigate whether tumor antigen affect the lytic capacity of NK cells present in splenocytes, several parameters of NK cell activity were studied. A fixed number of splenocytes isolated from naive rats were treated with different concentrations of fixed AK-5 cells (100:10 to 100:0.5). Following 20 h of incubation, NK cells were isolated from the cocultured cell suspension and were tested for their cytotoxic potential against ^{51}Cr labeled YAC-1 and AK-5 target cells (**Fig. 4.1**). It was observed that with decreasing concentration of tumor antigen, there

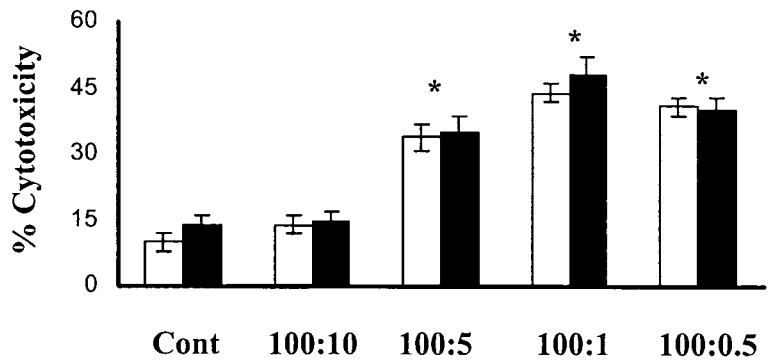

Fig. 4.1: Cytotoxicity of NK cells against AK-5 cells (open bar) and YAC-1 cells (close bar) (E:T 50:1). Naive splenocytes were cocultured with fixed AK-5 cells at 37°C for 20 h at different splenocytes to fixed tumor cell ratios. Following incubation, NK cells were isolated and checked for their cytotoxic activity. Data are expressed as the mean ± SD from one representative experiment in triplicate. Similar results were obtained in two other experiments.

* $p < 0.01$; control versus experimental group (n=3).

was an enhancement of cytotoxic activity of NK cells upto 100:1 ratio. Further decrease in tumor antigen concentration results in decrease in cytotoxic activity of NK cells. It is seen that fixed AK-5 cells induce cytotoxic activity of NK cells in a dose dependent manner with (100:1) ratio being the optimal dose. Difference in lytic potential between cells incubated with and without fixed tumor cells were statistically significant and the augmentation index observed in assays of splenocytes incubated with fixed tumor cells ranged from 1.12 in 100:10 ratio to 3.2 in 100.1 ratio. NK cell isolation from splenocytes was performed by using dynal beads and monoclonal antibody against rat CD161 antigen. Anti CD161 has been reported to be an activator of NK cell function. We have isolated both control NK cells and treated NK cells by using the same procedure. Hence, activation of NK cells in splenocytes is not due to the isolation process. The activation was specifically due to fixed AK-5 cells, since formaldehyde fixed macrophages did not show any enhancement of cytotoxic potential of NK cells in comparison to the NK cells isolated from untreated splenocytes.

4.2.2 Treatment of splenocytes with fixed tumor cells caused the secretion of Th1 cytokines

We next asked the questions as to what type of cytokines and interleukins were secreted by splenocytes in the coculture supernatant, after treatment with fixed AK-5 cells at (100:1) ratio, which were involved in the activation of NK cell cytotoxic function. Splenocytes were cocultured in presence or absence of fixed AK-5 cells. Following 24 h of incubation, the culture supernatants were harvested and analysed by ELIFA for the presence of cytokines and interleukins. Addition of fixed AK-5 cells to splenocytes induced significant secretion of IL-2, IFN-γ and IL-12 in the culture supernatant (**Table. 4.1**). The levels of IL-2, IL-12 and IFN-γ in the culture supernatant isolated from splenocytes after treatment with fixed AK-5 cells were

Table 4.1

AK-5 cell mediated cytokine production by splenocytes

		Cytokines (pg/ml)		
		IL-2	IL-12	IFN-γ
Exp-1	Control	40 ± 2	42 ± 4	52 ± 13
	Treatment	572 ± 25*	452 ± 22*	891 ± 22*
Exp-2	Control	30 ± 3	21 ± 5	42 ± 12
	Treatment	681 ± 39*	422 ± 45*	991 ± 52*
Exp-3	Control	53 ± 4	29 ± 5	46 ± 10
	Treatment	880 ± 40*	621 ± 32*	1022 ± 48*

Splenocytes (2 x 10^5/well) were plated with fixed AK-5 cells (100:1). Following 24 h of incubation, the culture supernatant was harvested and the cytokine levels were quantified by ELIFA. The values shown are mean ± SD in triplicate.

*$p < 0.01$; control versus experimental group.

much higher in comparison to the culture supernatants isolated from untreated splenocytes. These observations demonstrated that interaction of splenocytes with fixed tumor cells triggered the secretion of Th1 type of cytokines, which in turn participate in the activation of NK cell cytotoxicity.

4.2.3 Effect of cytokine-specific neutralizing antibodies on the activation of NK cells

Neutralizing antibodies were added to fixed AK-5 cell treated splenocyte cultures to block cytokines known to upregulate NK cell activity. Each neutralising antibody was titrated for its ability to block NK cell activation and the optimal dilution of each was determined. Each neutralizing antibody was then used at the predetermined optimal concentration. Antibody to IL-2 consistently blocked a portion of NK activity and suppression ranged from 45% to 56% (**Table 4.2**). Neutralising antibody to IFN-γ blocked NK cell activation, with suppression ranging from 61% to 77%. Suppression of NK activation was also observed when anti IL-12 antibody was used for blocking NK cell activation, and suppression was ranged from 40% to 70%. The differences in NK lytic activity between splenocytes treated with fixed AK-5 cells and splenocytes treated with fixed AK-5 cells along with anti IL-2 or anti IL-12 or anti IFN-γ antibodies were statistically significant. Isotype control antibody used at similar concentrations did not affect NK activation significantly.

4.2.4 NKT cells are the main effectors in cytotoxicity function

Recent studies indicate that there is a distinct lymphoid lineage of cells expressing several characteristics that demarcate them from T cells and natural killer cells, defined as NKT cells. NKT cells express surface antigens CD3 and CD161, which are characteristic of T-cells and NK cells, respectively. Most $CD161^+$ positive cells are $CD3^-$ cells; however, a minor subset of $CD3^+$, $CD161^+$ cells are also present. In the previous experiments, we separated effector cells

Table 4.2

Inhibition of NK cell activation by anti cytokine antibodies

	% Cytotoxicity			
	Exp-1		Exp-2	
	YAC-1	AK-5	YAC-1	AK-5
Control splenocytes	12 ± 3	9 ± 3	14 ± 2	10 ± 2
Splenocytes + AK-5 cells (100:1) (+ve control)	61 ± 4	52 ± 4	53 ± 4	49 ± 3
Splenocytes + AK-5 cells (100:1) + anti IL-2 Ab	39 ± 4* (45)	28 ± 4* (56)	32 ± 3* (54)	31 ± 3* (46)
Splenocytes + AK-5 cells (100:1) + anti IL-12 Ab	41 ± 4* (40)	32 ± 3* (70)	29 ± 3* (61)	32 ± 4* (43)
Splenocytes + AK-5 cells (100:1) + anti IFN-γ Ab	21 ± 3* (61)	19 ± 3* (77)	25 ± 3* (71)	21 ± 3* (72)
Splenocytes + AK-5 cells (100:1) + IgG isotyope control	62 ± 4 (0)	49 ± 3 (7)	45 ± 4 (20)	51 ± 3 (0)

Splenocytes were cocultured with fixed AK-5 cells in presence of anti IL-2 (10 μg/ml), anti IFN-γ (15 μg/ml) and anti IL-12 (12 μg/ml) antibodies. Following incubation, NK cells were isolated and assayed for their cytotoxic activity against target tumor cells. Values shown are mean ± SD in triplicate wells. Values shown in parentheses indicate SDA (materials and methods).

*$p < 0.01$; positive control versus experimental group.

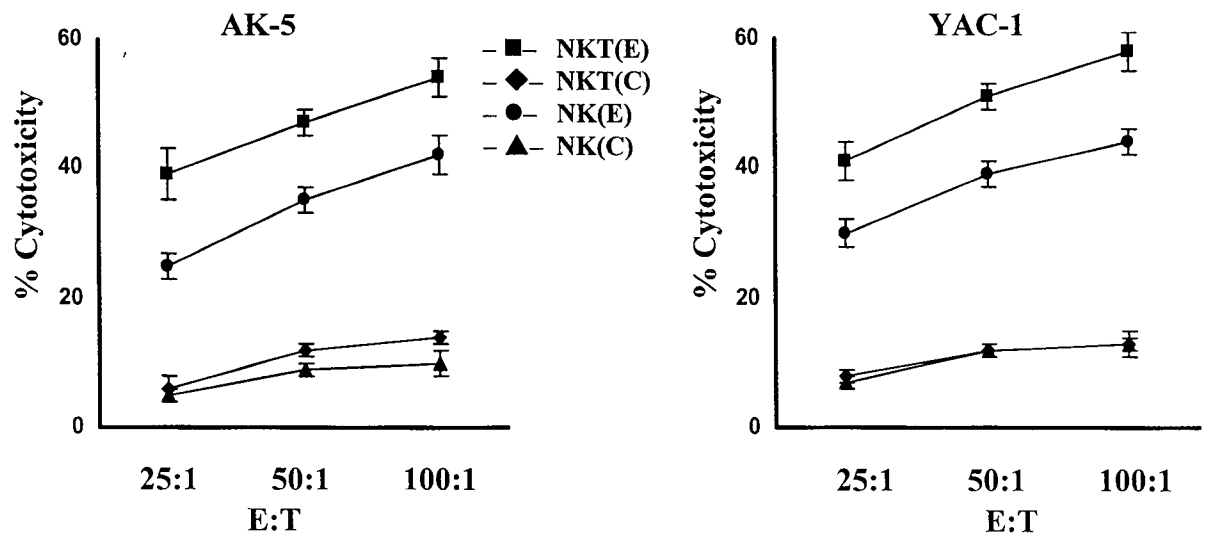

Fig. 4.2: Cytotoxicity of NKT and NK cells against AK-5 and YAC-1 cells at different effector to target ratios. Splenocytes were incubated with fixed AK-5 cells (100:1) for 20h. Following incubation, NK and NKT were isolated from cell suspension and assessed for their cytotoxicity. 'E' denotes fixed AK-5 cell treatment, and 'C' denotes without any treatment. Results shown are mean $\pm$ SD from one representative experiment in triplicate. Similar results were obtained in two other experiments.

$P < 0.01$; control NK cells versus AK-5 treated NK cells.

$P < 0.01$; control NKT cells versus AK-5 treated NKT cells.

from coculture by using anti CD161 (mAb3.2.3) monoclonal antibodies. Hence, it is unclear whether NKT ($CD161^+$, $CD3^+$) cells or NK ($CD161^+$, $CD3^-$) cells are responsible for the cytotoxic function against tumor target cells because both NKT and NK cells are $CD161^+$. The goal of this study was to know whether NKT or NK cells or both are activated following overnight incubation of splenocytes with fixed AK-5 cells. We cocultured splenocytes with fixed AK-5 cells at 100:1 ratio. After 20 hour incubation both NKT cells and NK cells were isolated individually and tested for their cytotoxic activity against ^{51}Cr labelled AK-5 and YAC-1 target cells. As shown in Figure 4.2, NK cells ($CD3^-$, $CD161^+$) which were stimulated by fixed AK-5 cells exhibited low levels of cytotoxic activity against AK-5 and YAC-1 target cells as compared to NKT cells. We also isolated $CD3^+$, $CD161^-$ lymphocytes (conventional T cells) from coculture and assayed them for cytotoxic activity. Fixed AK-5 cell contact had no effect on the cytolytic function of $CD3^+$, $CD161^-$ cells against YAC-1 and AK-5 target cells (data not shown). Thus $CD3^+$, $CD161^-$ T cells apparently do not account for the fixed AK-5-induced cytotoxicity that lacked CD161 antigen. In order to assess the role of NKT cells, ($CD3^+$, $CD161^+$), cells were isolated from coculture following stimulation with fixed AK-5 cells and tested for their cytotoxic activity against YAC-1 and AK-5 target cells. As shown in Figure 4.2, unstimulated NKT cells were less cytotoxic against target tumor cells. A significant increase in cytotoxicity value was achieved by NKT cells following stimulation with fixed AK-5 cells. Thus, our experimental data suggests that NKT cell population was activated more than the NK cell population following exposure of splenocytes with fixed AK-5 cells.

4.2.5 Role of co-stimulatory molecules in the activation of NK cells

Costimulation results from an interaction between the CD28 molecules on the T-cell surface and their ligands, which are members of the B7 family (B7.1 & B7.2), on the surface of

APC. There is evidence to suggest that B7 / CD28 interaction provides costimulatory signals not only for T cells but also for NK cells. In order to investigate the role of CD28, B7.1 and B7.2 molecules on the activation of NK cells by fixed tumor cells, we cocultured splenocytes with formaldehyde fixed AK-5 cells in the presence of anti CD28, anti B7.1 and anti B7.2 antibodies. Following 20 h of incubation, NK cells were isolated from the coculture cell suspension and tested for their cytotoxic activity against ^{51}Cr labeled YAC-1 and AK-5 cells. Each neutralizing antibody was titrated for its ability to block NK cell activation and the optimal dilution of each was determined. Each neutralizing Ab was then used at the predetermined optimal concentration. Antibodies to B7.1 blocked a large portion of NK cell activation and suppression achieved was upto 75% and 71% against AK-5 and YAC-1 target cells respectively (**Table 4.3**). Neutralizing antibodies to B7.2 also blocked NK cell activation with suppression reaching upto 40% and 56% against AK-5 and YAC-1 target cells respectively **(Table 4.4)**. Suppression of NK activity by anti CD28 ranged upto 53% against both YAC-1 and AK-5 target cells (**Table 4.5**). The differences in NK lytic activity of effector cells treated with fixed AK-5 cells and NK cells treated with fixed AK-5 cells along with neutralizing Abs were statistically significant for each neutralising Ab used. Isotype matched control Abs were used at similar concentrations but did not affect NK cell activation in a statistically significant manner. Thus, costimulatory molecules were involved in the induction of lytic activity of NK cells by fixed tumor cells *in vitro*.

4.2.6 Inhibition of NK cell cytotoxicity by anti LFA-1 and anti ICAM-1 antibodies

To investigate the effect of anti LFA-1 and anti-ICAM-1 on the NK cell mediated cytotoxicity, we cocultured splenocytes with fixed tumor cells (100:1) in the presence of anti-LFA-1 and anti-ICAM-1 antibodies. The cytotoxic activity of NK cells was significantly inhibited by these antibodies (**Table 4.6**). The combination of the two antibodies had a higher

Table 4.3

Inhibition of NK cell activation by anti B7.1 antibody

Group	% cytotoxicity	
	AK-5	YAC-1
Splenocytes (control)	12 ± 4	14 ± 2
Splenocytes + AK-5 (100:1) (+ve control)	52 ± 4	49 ± 4
+ anti B7.1 (2 μg/ml)	41 ± 3 (28)*	40 ± 3 (26)*
+ anti B7.1 (4 μg/ml)	25 ± 3 (68)*	28 ± 3 (60)*
+ anti B7.1 (6 μg/ml)	21 ± 4 (78)*	26 ± 3 (66)*
+ anti B7.1 (8 μg/ml)	22 ± 3 (75)*	24 ± 2 (71)*
+ IgG Isotype control (10 μg/ml)	49 ± 3 (8)	52 ±3 (0)

Splenocytes (2 X 10^6 / ml) were incubated for 20 h with fixed AK-5 cells (100:1) in presence of anti B7.1 antibody. Following incubation, NK cells were isolated and assayed for their cytotoxic activity against target tumor cells. Values in the parentheses indicate SDA.

*$p < 0.01$; as compared to the positive control.

Table 4.4

Inhibition of NK cell activation by anti B7.2 antibody

Group	% cytotoxicity	
	AK-5	YAC-1
Splenocytes	10 ± 2	12 ± 2
Splenocytes + AK-5 (100:1) (positive control)	48 ± 4	55 ± 4
+ anti B7.2 (1 μg/ml)	40 ± 3 (21)*	38 ± 3 (40)*
+ anti B7.2 (2 μg/ml)	33 ± 3 (40)*	32 ± 3 (53)
+ anti B7.2 (3 μg/ml)	32 ± 2 (42)*	29 ± 2 (60)*
+ anti B7.2 (5 μg/ml)	33 ± 2 (40)*	31 ± 3 (56)*
+ IgG isotype control (10 μg/ml)	46 ± 4 (5)	54 ± 5 (2)

Splenocytes (2×10^6 / ml) were incubated for 20h with fixed AK-5 tumor cells in presence of anti-murine B7.2 antibody. Following incubation, NK cells were isolated and assayed for their cytotoxic activity against target tumor cells. Values in the parentheses indicate SDA.

*$p < 0.01$; as compared to the positive control.

Table 4.5

Inhibition of NK cell activation by anti CD28 antibody

Group	% cytotoxicity	
	AK-5	YAC-1
Splenocytes	9 ± 3	11 ± 2
Splenocytes + AK-5 (100:1) (positive control)	41 ± 3	54 ± 4
+ anti CD28 (5 μg/ml)	32 ± 3 (28)*	41 ± 3 (30)*
+ anti CD28 (10 μg/ml)	29 ± 3 (38)*	32 ± 3 (51)*
+ anti CD28 (15 μg/ml)	25 ± 2 (50)*	30 ± 2 (56)*
+ anti CD28 (20 μg/ml)	24 ± 2 (53)*	31 ± 3 (53)*
IgG isotype control (10 μg/ml)	43 ± 4 (0)	50 ± 2 (9)

Splenocytes (2×10^6 / ml) were incubated for 20 h with fixed AK-5 cells (100:1) in presence of anti CD28 antibody. Following incubation, NK cells were isolated and assayed for their cytotoxic activity against target tumor cells. Values in the parentheses indicate SDA.

*$p < 0.01$; as compared to the positive control.

Table 4.6

Effect of anti LFA-1 and anti ICAM-1 antibody treatment on NK cells

Group	% Cytotoxicity	
	AK-5	YAC-1
Splenocytes	7 ± 2	9 ± 1
Splenocytes + AK-5 (100:1) (positive control)	39 ± 3	42 ± 2
+ anti LFA-1 (8 μg/ml)	14 ± 2 (78)*	22 ± 3 (64)*
+ anti ICAM-1 (10 μg/ml)	16 ± 2 (71)*	21 ± 3 (67)*
+ anti LFA-1 (8 μg/ml) and anti ICAM-1 (10 μg/ml)	12 ± 2 (84)*	14 ± 2 (90)*
+ isotype control antibody	36 ± 4 (12)	44 ± 4 (0)

Splenocytes (2 x 10^6 / ml) were incubated for 20 h with fixed AK-5 cells (100:1) in presence of anti LFA-1 and anti ICAM-1 antibodies. Following incubation, NK cells were isolated from the coculture and assayed for their cytotoxic activity against target tumor cells. Values in the parentheses indicate SDA.

*$p < 0.01$; as compared to the positive control.

inhibitory effect on the cytotoxic activity of NK cells than when tested individually. Addition of isotype-matched antibody did not inhibit the cytotoxic activity of NK cells.

4.2.7 Analysis of perforin and Fas-L (CD95L) expression by immunofluorescence and Northern analysis

The analysis of Fas-L and perforin expression at mRNA and protein levels in NK cells isolated from splenocytes following incubation with fixed AK-5 cells definitely established that enhanced cytotoxic activity of NK cells was due to induction of Fas-L and perforin at transcriptional and translational levels. The mRNA isolated from fixed AK-5 treated-NK cells for 20 hours resulted in high level of FasL and perforin mRNA expression. No evidence of perforin and Fas-L mRNA was observed in untreated NK cells (**Fig. 4.3**). The induction of Fas-L and perforin proteins in fixed AK-5 cell-treated NK cells was also confirmed by immunofluorescence and cytofluorimetric analysis by staining NK cells with specific anti FasL and anti perforin mAbs. The percentage of FasL and perforin positive NK cells greatly increased upon fixed AK-5 cell treatment and demonstrated that interaction of fixed target cells with splenocytes trigger the modulation of several surface antigens like FasL and perforin on NK cells as compared to untreated NK cells (**Fig. 4.4**). The results obtained above indicated that treatment of splenocytes with fixed AK-5 cells cause the induction of FasL and perforin expression by NK cells at transcriptional level followed by effective translation and transportation of FasL and perforin proteins to the NK cell surface, which are required for the cytolytic activity shown by NK cells.

4.2.8 Signal transduction mechanism utilised by fixed AK-5 cells to activate NK cell cytotoxic function

4.2.8.1 Phosphorylation

Phosphorylation of proteins at the amino acids, tyrosine, serine or threonine is known to be one of the most important signalling mechanisms in the eukaryotic cellular system. It is

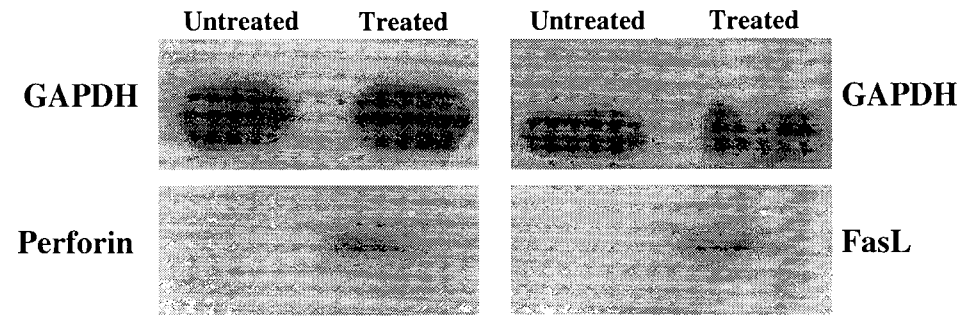

Fig. 4.3: Northern blot analysis of perforin and FasL expression. RNA from NK cells was hybridized to cDNAs corresponding to GAPDH, perforin and FasL. Untreated represents NK cells isolated from splenocytes, treated represents NK cells isolated from splenocytes after coculture with fixed AK-5 cells. Hybridization with GAPDH probe is done to test the integrity and loading of the RNA samples.

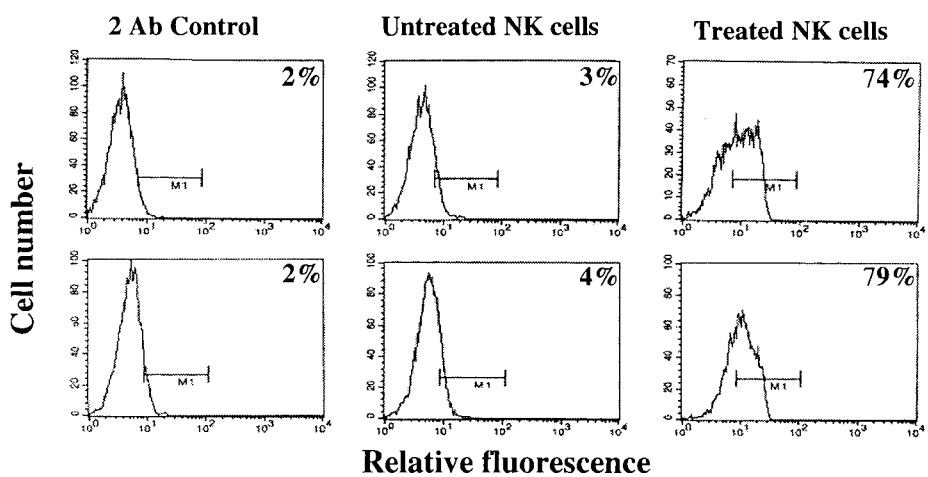

Fig. 4.4: Upregulation of perforin and FasL in NK cells isolated from splenocytes after coculture with fixed AK-5 cells for 20h. Top panel: NK cells stained for perforin. Bottom panel: NK cells stained for FasL

known that phosphorylation plays an important role in NK cell activation. Kinases which are of ser / thr class as well as tyr kinases play an important role in NK cell activation. We have studied the tyrosine phosphorylation in NK cells isolated from splenocytes following coculture with fixed AK-5 cells.

4.2.8.2 Tyrosine phosphorylation of NK cells after interaction with fixed tumor cells

The purpose of conducting these experiments was to understand the downstream pathways by which fixed tumor cells activate NK cells. Here, an attempt was made to identify the signaling pathways, which would provide an insight into the proteins in NK cells which are phosphorylated on their tyrosine residues following interaction with fixed AK-5 cells. The tyrosine phosphorylation experiments were done in vitro using different anti phosphotyrosine antibodies. Splenocytes were cocultured with fixed AK-5 cells overnight. Following incubation, NK cells from the coculture were isolated and used for western blot analysis to detected tyrosine phosphorylated proteins.

4.2.8.2.1 Using Promega anti-phosphotyrosine

As shown in Figure 4.5B, there is a difference in the tyrosine phosphorylation pattern of NK cells treated with fixed AK-5 cells as against untreated NK cells. Figure 4.5B indicates the differential tyrosine phosphorylation of three proteins of relative molecular sizes 30 kDa, 32 kDa and 57 kDa. The 57 kDa protein is not phosphorylated in untreated NK cells but is phosphorylated in activated NK cells. The 32 kDa protein is hyper phosphorylated in fixed tumor cell-treated NK cells in comparison to untreated NK cells. The 30 kDa protein also is hyper phosphorylated on activated NK cells compared to untreated NK cells. Figure 5A is the comassie stain of similar gel, which indicates the equal loading of protein sample.

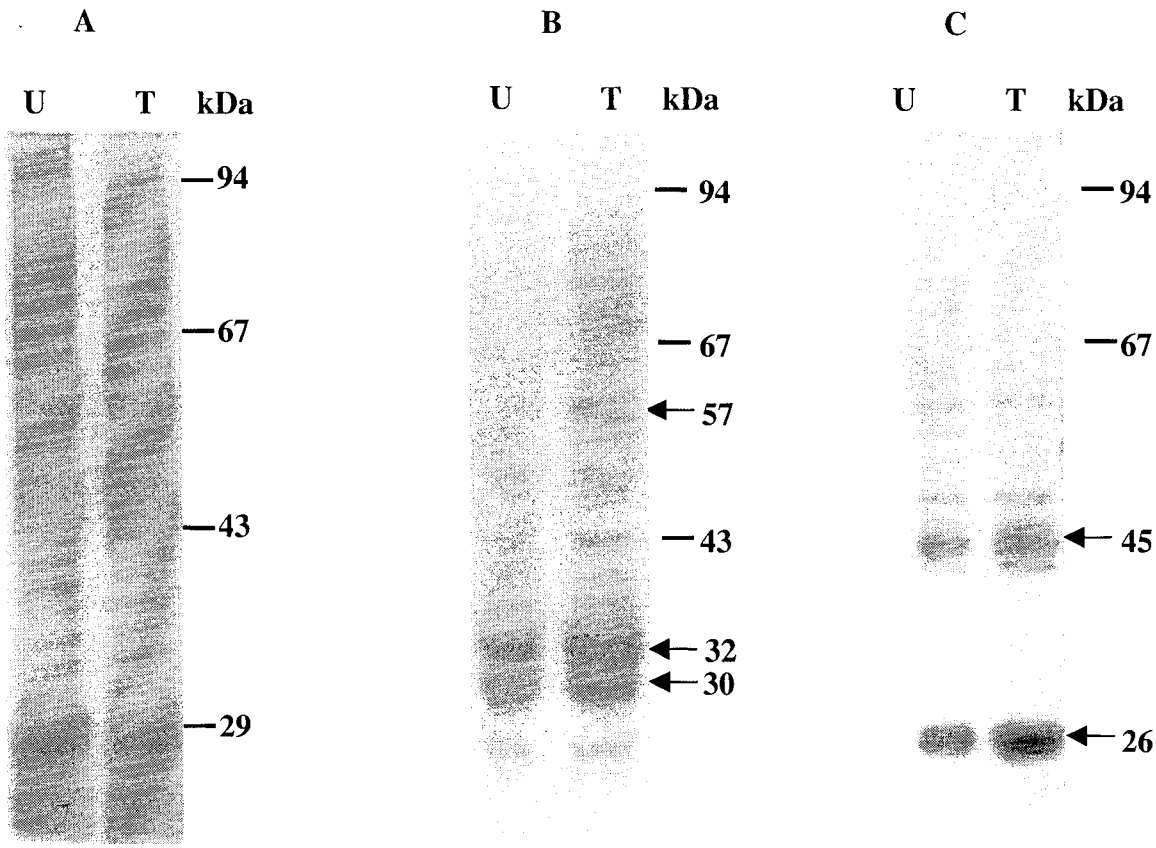

Fig. 4.5: Tyrosine phosphorylation of proteins expressed by control and fixed AK-5 cell-treated NK cells. Splenocytes were cocultured with fixed AK-5 cells for 20h. NK cell lysates were prepared following isolation of NK cells from control splenocytes and splenocytes treated with fixed AK-5 cells. Protein lysates were resolved in 10% SDS -polyacrylamide gel and subjected to western blotting using anti-phosphotyrosine antibodies.'U' denotes untreated NK cells and 'T' denotes fixed AK-5 cell-treated NK cells. A, Comassie stained gel; B, anti phosphotyrosine antibody (promega); C, anti phosphotyrosine antibody (hybridoma).

4.2.8.2.2 Using anti-phosphotyrosine (hybridoma)

Figure 4.5C shows the increase in tyrosine phosphorylation of a 26 kDa and 45 kDa proteins in NK cells treated with fixed AK-5 cells as compared to control NK cells. These results demonstrate that interaction of NK cells with fixed AK-5 cells directly or indirectly causes the differential tyrosine phosphorylation of 57 kDa, 45 kDa, 32 kDa, 30 kDa and 26 kDa proteins which may give signals for the activation of NK cell cytotoxic activity.

4.2.9 Analysis of CD25, CD16, iNOS, FasL and Vav-1 expressions by fixed AK-5 cell treated NK cells

Increased expression of CD16, CD25, iNOS, FasL and VAV1 is associated with the activation of NK cells. We therefore analysed the levels of mRNAs of these molecules in NK cells isolated from splenocytes following 24h treatment with fixed AK-5 cells (100:1) by using semiquantitative RT-PCR as described in methods (**Fig. 4.6 & 4.7**). The level of FasL mRNA was increased in NK cells treated with fixed AK-5 cells as compared to control NK cells which did not express FasL. The iNOS mRNA level showed 1.6 fold increase in treated NK cells compared to untreated NK cells. The CD25 and CD16 mRNA levels showed 1.9 fold and 1.8 fold increases in treated NK cells compared to untreated NK cells. However, the level of vav1 mRNA was not affected significantly by fixed AK-5 cell treatment.

4.2.10 NK and T cell population in splenocytes are enough to activate NK cell cytotoxic function following treatment with fixed tumor cells

Macrophage is one of the important arms in the immune system. B cell also has an important role in adaptive immune responses. In order to investigate whether macrophages and B cells have any immunoregulatory roles for the activation of NK cells isolated from splenocytes after treatment with fixed AK-5 cells, coculture experiments were performed by depleting

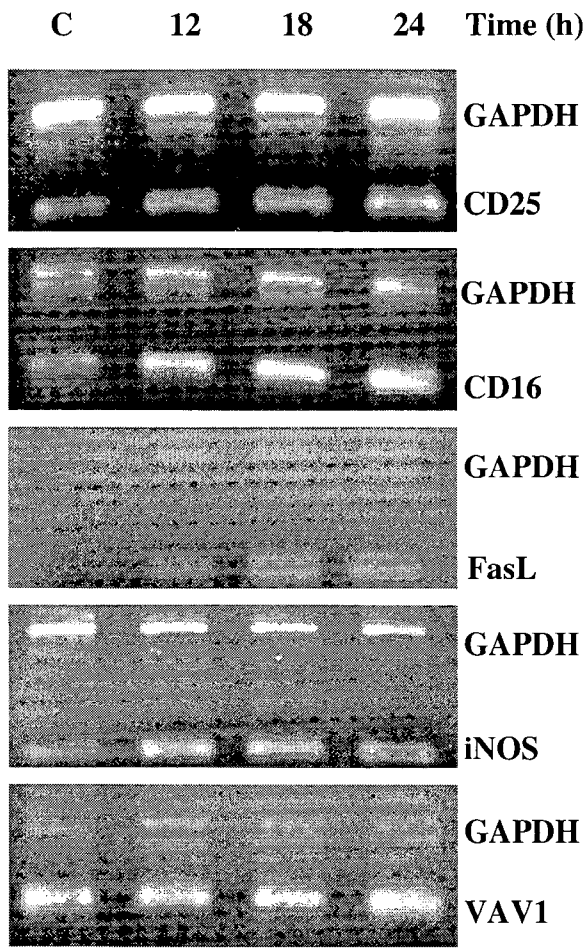

Fig. 4.6: The levels of mRNA expression by NK cells isolated from splenocytes following coculture with fixed AK-5 cells (100:1) at different time points. Specific primers were designed for CD25, CD16, fasL, inos, vav1 and semi-quantitative RT-PCR was carried out using GAPDH as an internal control. The PCR products were run on an agarose gel stained with ethidium bromide. RNA obtained from two independent pools were used in each experiment and PCR was done twice.

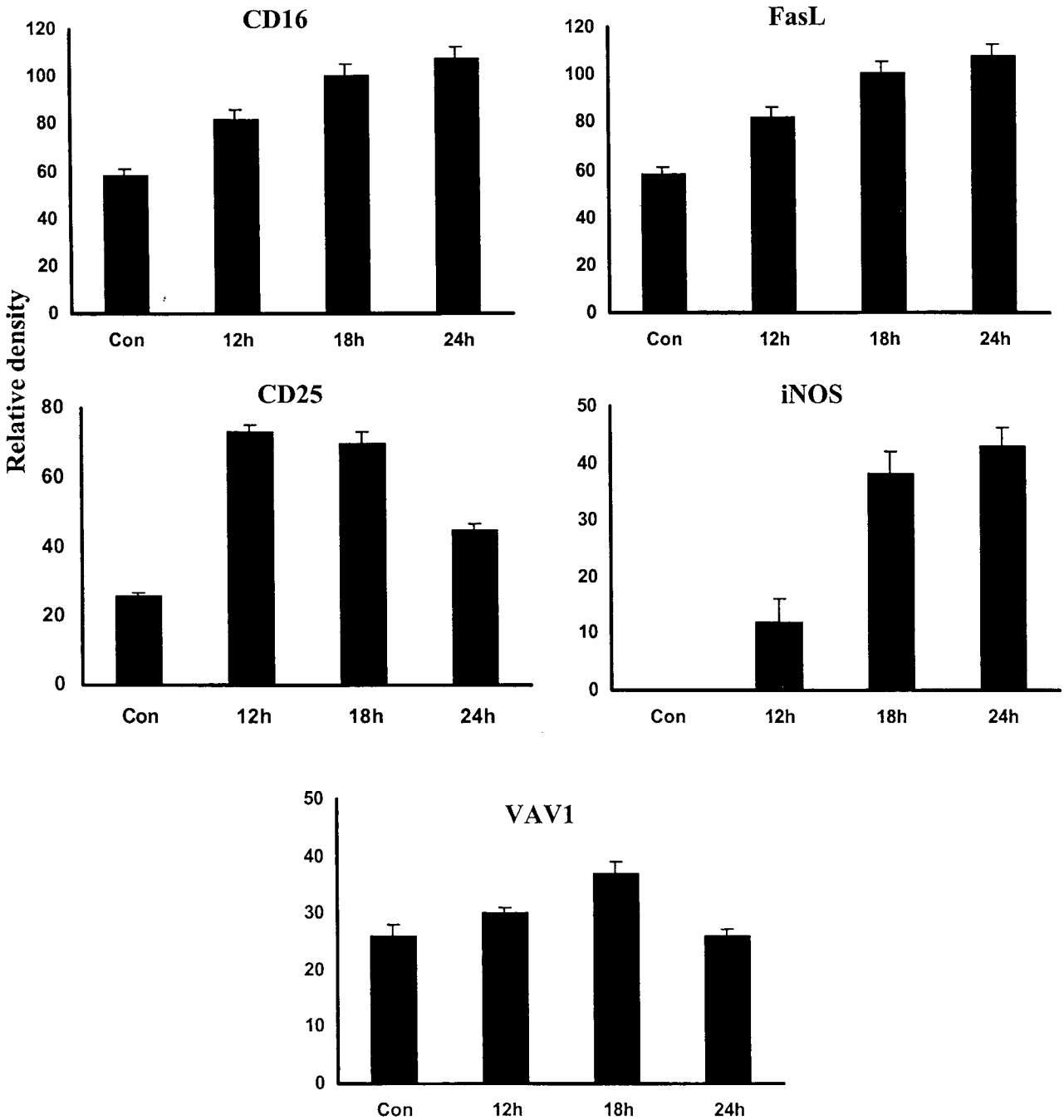

Fig. 4.7: The quantitation of PCR products using Gene Tool software (Syngene). Each bar represents mean ± SD of two quantitations done for each pool. X axis represents the relative density of the band in ethdium bromide stained gel.

macrophages and B cells from splenocytes. These macrophages and B cell depleted splenocytes were cultured in the presence of formaldehyde fixed AK-5 cells at different ratios (100:10) to (100:1). Addition of decreasing concentrations of tumor antigen to NK and T cell populations led to a dose dependent activation of NK cells with 100:2 ratio being the optimal dose (**Fig. 4.8**). Further decrease in antigen dose showed the decrease in cytotoxic potential of NK cells. The differences in cytotoxic potential of NK cells isolated from NK and T cell populations following treatment with fixed AK-5 cells, and NK cells isolated from total splenocytes after incubation with fixed tumor cells were not statistically significant. These results indicated that fixed AK-5 cells mediated enhancement of anti-tumor activity of NK cell does not require macrophages and B cells as accessory cells for the induction of lytic activity of NK cells.

4.2.11 Effect of fixed tumor cells on purified NK and NKT cell cytotoxicity

We have shown that fixed AK-5 cells can activate NK cells following coculture with splenocytes and macrophage-depleted splenocytes. To investigate whether fixed tumor cells affected the lytic capacity of pure NK cells upon coculture, several parameters of NK cell function were studied. We tested the ability of fixed AK-5 cells to up-regulate NK cell cytotoxicity in a dose-dependent manner against YAC-1 and AK-5 target cells and examined whether the effect was direct or mediated indirectly through cytokines secreted by these cells.

Purified NK cells were incubated with fixed AK-5 cells (50:1 to 10:1) for 20 h before being used as effector cells in a 4 h ^{51}Cr release assay. Differences in the lytic potential between NK cells incubated with or without fixed AK-5 cells were statistically significant and the augmentation index (AI) or fold increase observed when NK cells were incubated with fixed AK-5 tumor cells ranged from 1.0-8.5 as compared to the control (**Fig. 4.9A**). In addition, fixed AK-5 cells induced NK cell cytotoxicity in a dose-dependent manner.

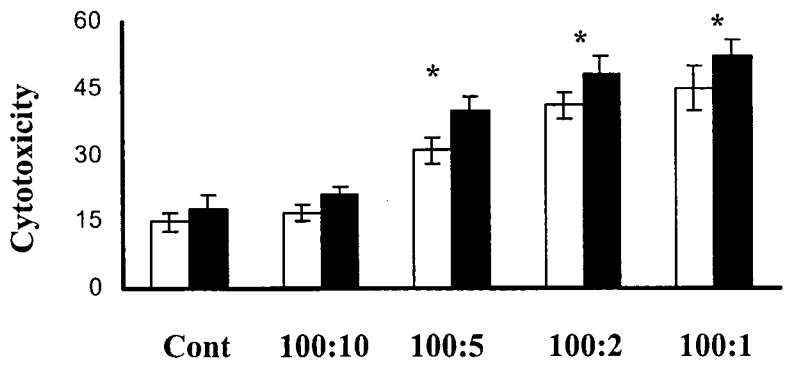

Fig. 4.8: Cytotoxicity of NK cells against AK-5 cells (open bar) and YAC-1 cells (closed bar) (E:T 50:1). Naive macrophage- and B cell-depleted splenocytes were cocultured with fixed AK-5 cells at 37°C for 20 h at different macrophage and B cell-depleted splenocytes to fixed tumor cell ratios. Following incubation, NK cells were isolated and checked for their cytotoxic activity. Data are expressed as mean ± SD from one representative experiment in triplicate. Similar results were obtained in two other experiments.

* $p < 0.01$; control versus experimental group.

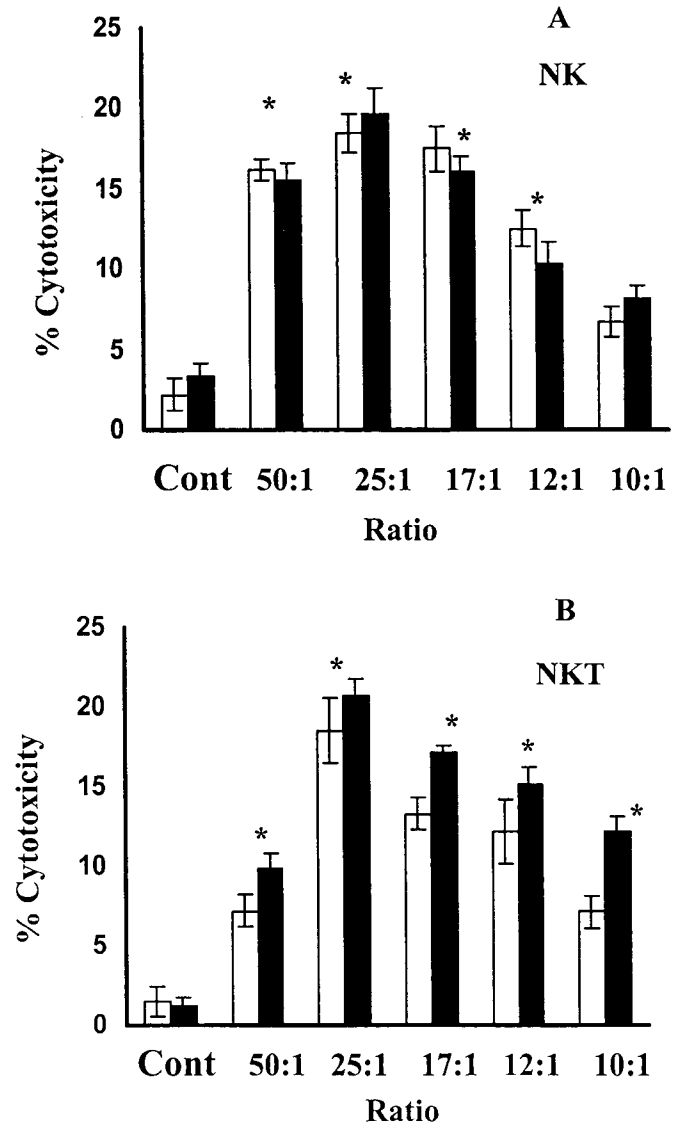

Fig. 4.9 Cytotoxicity of effector cells against AK-5 cells (open bars) and YAC-1 cells (closed bars) (E:T = 50). Naïve NK and NKT cells were cocultured with fixed AK-5 cells for 20h at different effector to fixed tumor cell ratios. Results are shown as mean ± SD from one representative experiment. Similar results were obtained in two other experiments.

*P< 0.01; control versus experimental group.

In order to study whether purified NKT cells were activated following 24 h incubation with fixed AK-5 cells, we fractionated the NKT cells using anti-CD3 and anti-CD161 mAbs. NKT cells were tested for their cytotoxicity against AK-5 and YAC-1 cells. As shown in Figure 4.9B, there was not much difference in the cytotoxic activities of NKT and NK cells. The augmentation in the cytotoxic activity of NK cells following exposure to fixed AK-5 cells was not due to the binding of mAb 3.2.3 which is a known activator of NK cell function, because the control NK cells were also isolated using the same procedure.

4.2.12 Role of T lymphocytes in NK cell activation

In order to investigate whether T cells have a possible immunoregulatory role in the augmentation of NK cell cytotoxic function, coculture experiments were performed. Purified NK cells were cocultured with purified T cells at different ratios (NK: T = 1:1 to 1:3) in the presence or absence of fixed AK-5 cells for 20 h and the cytotoxic potential of the isolated NK cells was tested against AK-5 and YAC-1 target cells. Addition of increasing number of T cells lead to a dose dependent increase in NK cell cytotoxicity against both YAC-1 and AK-5 cells (**Fig. 4.10**). In contrast, no activation was observed when formaldehyde fixed T-cells were cocultured with NK cells and fixed AK-5 cells as compared to the NK cells cocultured with fixed AK-5 cells. Moreover, in the absence of tumor cells, no activation of NK cells was achieved even when T cells were present.

4.2.13 Role of cytokines in NK cell activation

The activation of NK cells in the presence of fixed tumor cells required cell-cell interaction, thereby inducing the secretion of cytokines, which may induce activation of NK cells. IFN-γ being a major lymphokine produced by the NK and NKT cells, we have therefore tested the effect of fixed AK-5 cells on IFN-γ production. Purified NK and NKT cells were

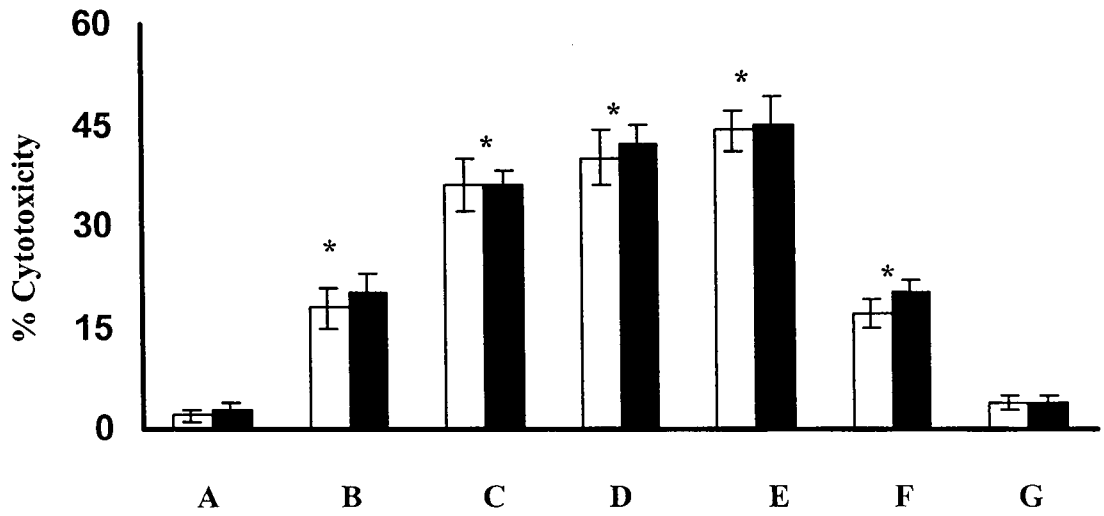

Fig. 4.10: Cytotoxicity of NK cells after coculture with T cells and fixed AK-5 cells (NK : AK-5 = 25 : 1), against AK-5 (open bars) and YAC-1 cells (closed bars) (E:T = 50:1). A: naive NK cells; B: NK cells plus fixed AK-5 cells; C: NK cells + T cells + fixed AK-5 cells (NK :T = 1:1); D: NK cells + T cells + fixed AK-5 cells (NK: T = 1:2); E: NK cells + T cells + fixed AK-5 cells (NK : T = 1:3); F: NK cells + fixed T cells + fixed AK-5 cells (NK : fixed T cell = 1:3); G: NK cells + T cells (1:3). Results are shown as mean $\pm$ SD from one representative experiment in triplicate. Similar results were obtained in two other experiments.

*$p<0.01$; control versus experimental group (n=3).

incubated with fixed AK-5 cells for 20 h and the culture supernatants were assayed for the presence of IFN-γ. The levels of IFN-γ in culture supernatants from cocultured cells were much higher than that in the supernatants from untreated control NK and NKT cells. But there was no significant difference in IFN-γ secretion between NK and NKT cells (**Table 4.7**).

We next asked the question as to what type of cytokines were secreted by the cocultured T and NK cells in the presence of fixed tumor cells, which further augmented the activation of NK cell cytotoxicity. The culture supernatants were assayed for the presence of IL-2 and IFN-γ. The levels of IL-2 and IFN-γ in the culture supernatants from co-cultured cells (fixed AK-5 cells, NK and T cells) were much higher than those in the supernatants obtained from the untreated NK and T cell populations (**Table 4.8**). These observations suggested that the interaction between NK and T cells with fixed tumor cells triggered the secretion of IL-2 and IFN-γ cytokines, which in turn participated in the augmentation of NK cell cytotoxicity.

4.2.14 Effect of cytokine specific neutralizing antibodies on NK cell activation

The secretion of IFN-γ following treatment of NK cells with fixed tumor cells, suggested a role for this cytokine in the activation process. Accordingly, it was expected that blocking of IFN-γ activity with specific antibody would reverse these changes. Addition of anti-IFN-γ antibody to cocultured NK cells with fixed tumor cells resulted in a significant inhibition of NK cell cytotoxicity (**Table 4.9**), thus demonstrating an important role for IFN-γ in the activation of NK cells in the presence of fixed tumor cells.

Neutralizing antibodies were added to fixed tumor cell treated NK and T cell cultures to block cytokines known to up-regulate NK activity. Each neutralizing antibody was titrated to determine the optimal dilution for its ability to block NK cell activation. Antibody to IFN-γ consistently blocked a large portion of the NK cell activity (**Table 4.10**). Similarly, neutralizing

Table 4.7

Cytokine production by NK and NKT cells

after coculture with fixed AK-5 cells

Ratio	IFN-γ (pg/ml)	
NK : AK-5	NK	NKT
Effector alone	14 ± 4	12 ± 5
50 : 1	130 ± 13*	98 ±19*
25 : 1	180 ± 19*	150 ± 12*
17 : 1	172 ± 26*	164 ± 17*
12 : 1	192 ± 11*	147 ± 21*
10 : 1	120 ± 21*	101 ± 25*

NK and NKT cells (1 x 10^6/ml) were incubated with fixed AK-5 cells at different ratios for 20 h. IFN-γ levels in the supernatant were quantified by ELIFA. The values shown are mean ± S.D from one representative experiment. Similar results were obtained in two other experiments.

*$p < 0.01$; control versus experimental group.

Table 4.8

AK-5 cell mediated cytokine production by NK and T cells

	Group	Cytokine (pg/ml)	
		IFN-γ	IL-2
Expt-1	NK + T	12 ± 9	20 ± 2
	NK + T + AK-5	442 ± 22*	200 ± 11*
Expt-2	NK + T	21 ± 5	15 ± 6
	NK + T + AK-5	765 ± 42*	210 ± 23*
Expt-3	NK + T	0	13 ± 8
	NK + T + AK-5	881 ± 43*	279 ± 17*

NK cells (1 x 10^6/ml) were plated with T cells at 1:3 ratio and cultured in the presence or absence of fixed AK-5 cells (NK : AK-5 = 25:1) for 20 h. Cytokine levels in the supernatant were quantified by ELIFA. The values shown are mean ± SD from one representative experiment. Similar results were obtained in two other experiments.

*$p < 0.01$; control versus experimental group.

Table 4.9

Inhibition of NK cell activation by anti IFN-γ antibody

Group	% Cytotoxicity	
	Experiment-1	Experiment-2
NK	7.8 ± 0.6	0.00
NK + AK-5 (positive control)	21.3 ± 0.9	13.3 ± 0.5
+ anti IFN-γ (1 μg/ml)	17.8 ± 0.7 (26)*	9.3 ± 0.4 (30)*
+ anti IFN-γ (2 μg/ml)	13.0 ± 0.8 (60)*	3.7 ± 0.3 (72)*
+ anti IFN-γ (3 μg/ml)	10.7 ± 0.9 (78)*	2.5 ± 0.6 (81)*
+ anti IFN-γ (4 μg/ml)	8.2 ± 0.2 (97)*	2.7 ± 0.7 (79)*
+ IgG Isotype control (5 μg/ml)	20.1 ± 1.1 (9)	14.5 ± 0.8 (0)

NK cells (1 x 10^6 cells/ml) were incubated with fixed tumor cells (25 :1), for 20 h in the presence of anti murine IFN-γ antibody. Following incubation, NK cells were isolated and assayed for their cytotoxic activity against target tumor cells. Values in the parentheses indicate SDA.

*$p < 0.01$; as compared to the positive control.

Table 4.10

Inhibition of NK cell activation in the presence of T-cells by anti-cytokine antibodies

Antibody	% Cytotoxicity			
	Experiment 1		Experiment 2	
	YAC-1	AK-5	YAC-1	AK-5
NK + T (control)	15.2 ± 0.9	11.7 ± 4.1	12.7 ± 2.1	9.5 ± 2.1
NK+T+AK-5 cells (positive control)	51.5 ± 2.5	42.5 ± 2.2	52.9 ± 3.9	32.6 ± 3.1
NK + T + AK-5 + anti IFN-γ	22.7 ± 2.3*	16.8 ± 2.1*	16.2 ± 2.6*	15.5 ± 3.2*
	(79)	(83)	(90)	(74)
NK + T+ AK-5 + anti IL-2	44.3 ± 3.7*	32.5 ± 2.9*	38.7 ± 5.3*	18.2 ± 3.1*
	(20)	(62)	(34)	(60)
NK + T + AK-5 + IgG Isotype control	48.5 ± 3.1	42.1 ± 2.0	49.0 ± 3.9	32.4 ± 2.6
	(8)	(2)	(2)	(0)

NK cells (1 x 10^6/ml) were cultured with T cells at a ratio of 1:3 and incubated with fixed tumor cells (25:1) in the presence anti-IL-2 (10 μg/ml) and anti-IFN-γ (15 μg/ml). Following incubation, NK cells were isolated and assayed for their cytotoxic activity against target tumor cells. Values shown in parentheses indicate SDA.

*$p < 0.01$; as compared to the positive control.

antibodies to IL-2 also blocked NK cell activation significantly. The differences in NK lytic activity of NK and T cell population treated with fixed cells along with neutralizing antibodies compared with that of controls were statistically significant for each neutralizing antibody used. Isotype matched control antibodies at similar dilutions did not affect NK cell activation significantly. These observations suggest that cytokines secreted by NK and T lymphocytes were involved in the activation of NK cells *in vitro*.

4.2.15 Role of preactivated NK cells on naive T cells

We have demonstrated that fixed AK-5 cells induced anti-tumor activity and IFN-γ secretion by NK cells and the cytolytic potential of NK cells was increased significantly in presence of T cells. We also investigated whether preactivated NK cells (after coculture by AK-5) were able to induce IL-2 and IFN-γ secretion by T cells. NK cells after 20 h, coculture with fixed AK-5 cells (25:1) were separated and incubated further either in the absence or presence of T cells at different NK: T ratios. In the absence of T cells, preactivated NK cells secreted low amount of IFN-γ and no IL-2 was detected. But in presence of T cells, the amounts of IL-2 and IFN-γ secreted were significantly higher. Cytokine secretion by NK and T cells increased with increasing number of T cells (**Table 4.11**).

4.2.16 Detection of RT16 (MHC-I) transcript in AK-5 tumor cells

MHC-class I antigen expression has been shown to regulate the lytic activity of NK cells. NK cells express a family of cell surface receptors for Class I antigens that have different specificity, which upon ligation inhibit NK cell-mediated cytotoxicity and cytokine secretion. In order to investigate the expression of RT16 by AK-5 tumor cells, we used primers which amplify RT16 transcript. RT-PCR analysis detected RT16 mRNA in normal rat splenocytes. However,

Table 4.11

Cytokine secretion by preactivated NK cells and naive T cells after coculture

NK : T	IL-2 (pg/ml)	IFN-γ (pg/ml)
p NK	ND	140 ± 10
p NK + n T (1:1)	115 ± 11*	210 ± 18*
p NK + n T (1:2)	189 ± 17*	488 ± 24*
p NK + n T (1:3)	222 ± 18*	572 ± 19*
n NK + n T (1:3)	20 ± 2	14 ± 2
nT	ND	ND

NK cells ($1x10^6$/ml) were cocultured with fixed AK-5 cells (NK : AK-5 = 25:1). After 20 h of incubation, NK cells were isolated. The preactivated NK cells thus obtained were incubated further with naive T cells at different ratios. Cytokine levels in the supernatant were quantitated by ELIFA. p = preactivated; n = naive; ND = not detectable.

*$p < 0.01$;as compared to nNK + nT coculture.

AK-5 cells were negative for RT16, suggesting thereby the absence of expression of this antigen in AK-5 cells (**Fig. 4.11**).

4.2.17 T-cell proliferation

Treatment of T cells with stimuli such as anti CD3 Ab or antigen presented by APC results in T-cell activation, which is measured by proliferation and cytokine production by T-cells. We have shown that formaldehyde-fixed tumor cells induced anti tumor activity of NK cells, and in presence of T cells with cytotoxic potential of NK cells was increased significantly. In order to investigate whether T-cells directly responded to fixed AK-5 cells, we performed T cell proliferation assays and cytokine secretion following coculture of fixed tumor cells. Freshly isolated naive T cells were incubated with fixed AK-5 cells at 100:100 ratio to 100:1 ratio for 24 h. After 36 h of incubation, cells were pulsed with 0.5 μCi of [^{3}H] thymidine for 14 to 18 h. No significant proliferation of T cells following exposure to fixed AK-5 cells was observed (**Fig. 4.12**). In a similar manner, naive T cells were incubated with fixed AK-5 cells for 24 h. After incubation, the supernatants were collected and assayed for IL-2 production. Naive T cells treated with fixed AK-5 cells were unable to produce IL-2. This experiment was performed three times and in each instance, IL-2 production were virtually undetectable *in vitro* culture. This experiment confirmed that naive T cells cannot recognise fixed tumor cells as antigen presenting cells and T cell cannot be activated in presence of fixed tumor cells in absence of NK cells in the culture.

4.2.18 Status of NK cell activation markers

Coculture of purified NK cells with fixed AK-5 cells causes the activation of NK cell cytotoxic function. However, this activation was low compared to NK cells treated with fixed AK-5 cells in presence of T cells. We wanted to know whether coculture of pure NK cells with

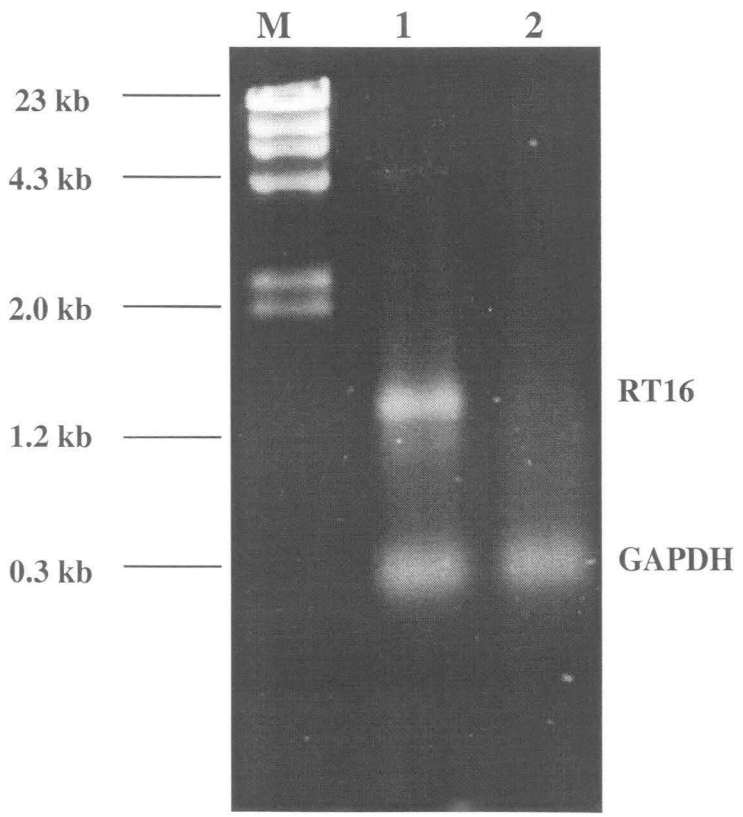

Fig. 4.11: Expression of RT16 and GAPDH mRNA in AK-5 cells and splenocytes as detected by semi-quantitative RT-PCR. M: Molecular weight marker; Lane 1: splenocytes; Lane 2: AK-5 cells. GAPDH was coamplified with RT16 in splenocytes and AK-5 cells for 23 cycles used as an internal standard. Lamda DNA digested with *Eco*RI and *Hind* III was used as marker.

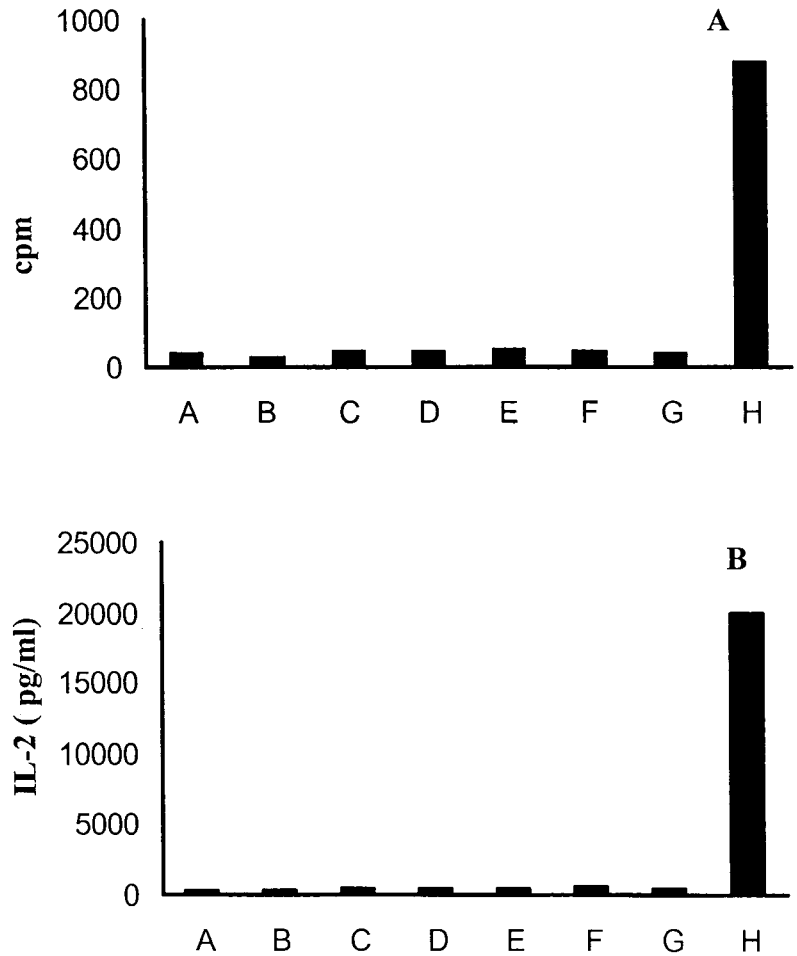

Fig. 4.12: Cytokine secretion and primary proliferative responses against fixed AK-5 cells. T cell were co-cultured ($2x10^5$ cells per well) in the presence of fixed AK-5 cells at different ratios. A, control; B, 100:100; C, 100:75; D, 100:50; E, 100:25; F, 100:10; G, 100:1; H, Con A (proliferation) and IFN-γ (cytokine secretion). T cell proliferation which was measured by pulsing the plates with 0.5 μci of[^{3}H] thymidine during the final 12 h of culture are shown in panel A. Cytokine levels were quantified by ELISA after 24h are shown in panel B. Results are the mean of triplicate wells. Similar results were obtained in two other experiments.

fixed AK-5 cells cause the upregulation of CD95-L and perforin expressions at protein level. The analysis of the expression of CD95-L and perforin in NK cells treated with fixed AK-5 cells established the enhanced cytotoxic activity of NK cells and its correlation with the induction of CD95-L and perforin expression. The induction of CD95-L and perforin in cocultured NK cells was confirmed by immunofluorescence and flow cytometric analysis after staining NK cells with specific anti-CD95-L and anti-perforin antibodies (**Fig. 4.13**). The percentage of CD95-L and perforin positive NK cells was significantly higher after coculture with fixed AK-5 cells, which was concomitant with their higher cytotoxic activity.

4.2.19 Activated NK cell mediated nitric oxide production and iNOS expression

Purified NK cells cocultured with fixed AK-5 cells have been found to produce nitric oxide as compared to NK cells cultured alone, as assessed by nitrite determination in culture medium (**Fig. 4.14A**). The Western and Northern analyses were performed to determine iNOS expression in NK cells following coculture with fixed AK-5 cells. The western blot revealed the iNOS expression in activated NK cells incubated with fixed AK-5 cells whereas control NK cells did not show any iNOS expression (**Fig. 4.14B**). The northern analysis showed that NK cells without treatment with fixed AK-5 cells did not show any iNOS expression, whereas significant iNOS expression was noticed in NK cells following overnight exposure with fixed AK-5 cells (**Fig. 4.14C**). The analysis of iNOS expression at protein and mRNA levels in fixed AK-5 treated NK cells definitely established that NO generation was due to the induction of iNOS at transcriptional as well as at translational levels.

4.2.21 Effect of anti LFA-1 and anti ICAM-1 on cell adhesion

To verify the role of LFA-1 and ICAM-1 in adhesion of effector cells to target cells, ^{51}Cr labeled NK cells were incubated with fixed AK-5 cells in the presence of anti LFA-1 and/ or anti

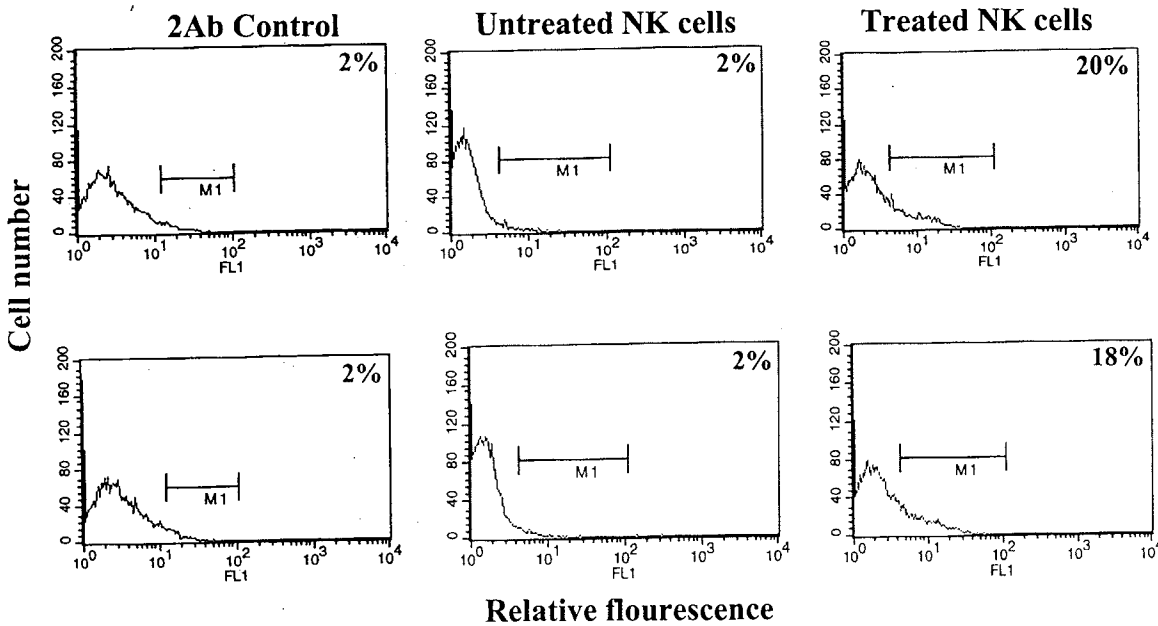

Fig. 4.13: Upregulation of perforin and FasL in NK cells isolated following coculture with fixed AK-5 cells for 20h. Top panel: NK cells stained for perforin. Bottom panel: NK cells stained for FasL.

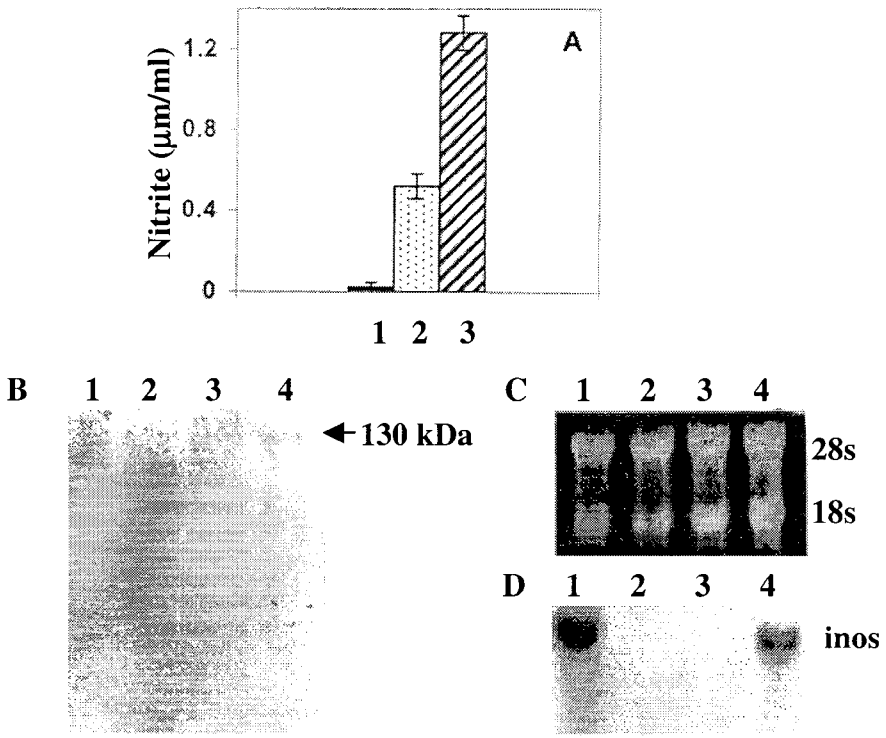

Fig. 4.14: Nitrite accumulation in culture supernatants of fixed AK-5 cells(1), naïve NK cells (2) and NK cell coculture with fixed AK-5 cells (3) are shown in panel A. Western analysis of iNOS protein levels is shown in panel B. Lane 1, fixed AK-5 cells; Lane 2, Naïve NK cells; Lane 3, naïve NK cells cultured for 20h; Lane 4, naïve NK cells cocultured with fixed AK-5 cells for 20h. Northern analysis of the expression of inos is shown in panel D. Lane1, immune macrophages; Lane 2, AK-5 cells; Lane 3, control NK cells; Lane 4, NK cells treated with fixed AK-5 cells. Panel C represents the integrity and loading of the corresponding RNA samples shown in panel D.

ICAM-1 monoclonal antibodies, and the adhesion of NK cells was analysed. There was comparatively less adhesion of NK cells to AK-5 cells in the presence of either anti LFA-1 or anti ICAM-1 mAbs and combination of these mAbs had a greater inhibitory effect on the cell adhesion. The adhesion of NK cells to AK-5 cells was higher in the absence of anti LFA-1 and anti ICAM-1 mAbs. Maximum adhesion was observed in presence of anti-AK-5 antibody, which represents the attachment of NK cells to the AK-5 cells through Fc-FcR (**Table 4.12**). Addition of isotype-matched antibody, either in the presence or absence of anti-AK-5 antibody, had no effect on NK cell adhesion to target cells, which eliminates the possibility of non-specific inhibition of the effector cell adhesion.

4.3 DISCUSSION

NK cells have been shown to be the effector cell during AK-5 tumor regression (Khar, 1993). This study demonstrates the induction of NK cell lytic function after their interaction with fixed tumor cells and the requirement for cell-secreted accessory signals for the augmentation of the lytic function. It also demonstrates that AK-5 cell mediated augmentation of NK cell cytotoxicity in the presence of T cells is mediated by lymphokines (IL-2 and IFN-γ) and that NKT cells possess higher cytotoxic potential than NK cells. In addition, we have also observed over-expression of CD95L and perforin in NK cells following exposure to fixed AK-5 cells.

Previous studies have shown that fixed BALL-1 cells enhanced the antitumor activity of NK cells through an IFN-γ independent pathway (Hirashima et al., 1998). In addition, tumor cell specific boosting of IL-2-induced activation of NK cells by paraformaldehyde fixed tumor cells is also reported (Dhillon and Saxena, 1998). In the present studies, we report the activation of NK cells by fixed tumor cells through the endogenous secretion of IFN-γ. The presence of exogenous IL-2 was not required for the initial activation of NK cells.

Table 4.12

Effect of anti LFA-1 and anti ICAM-1 antibodies treatment on the adhesion of NK cells to fixed AK-5 cells

Group	% Adhesion	
	30 min	60 min
NK + AK-5 (Control)	15 ± 2	32 ± 3
+ Anti ICAM-1 (10 μg/ml)	14 ± 2	24 ± 3*
+ Anti LFA-1 (8 μg/ml)	12 ± 2	22 ± 3*
+ Anti ICAM and anti LFA-1	8 ± 2	16 ± 2*
+ Anti AK-5 (5 μg/ml) (+ve control)	20 ± 2	45 ± 4*
+ Control isotype (10 μg/ml)	1.6 ± 2	35 ± 3

The adhesion assay was carried out as described in methods. The results are shown as the percentage of ^{51}Cr labeled NK cells attached to the tumor cells.

*$p < 0.01$; control versus experimental group.

The fixed tumor cell-mediated augmentation of NK cell cytotoxicity in the presence of T cells is mediated by T cell secreted accessory cytokines like IL-2 and IFN-γ, which contribute to the induction of NK cell cytotoxic activity. In order to find out whether T cells responded directly to fixed tumor cells, we performed T-cell proliferation assays after coculture of fixed tumor cells with T cells at different ratios. We were unable to detect any T cell response against fixed AK-5 cells. These observations support the hypothesis that NK cells produce IFN-γ following coculture with fixed tumor cells, which in turn activate T cells for the production of cytokines (IL-2 and IFN-γ). The T cell secreted cytokines are responsible for further augmentation of NK cell cytotoxic activity.

The interesting consequence of the interaction of fixed tumor cells with NK cells is the induction of IFN-γ secretion by NK cells. Early secretion of IFN-γ during an immune response may have profound effects on the pattern of cytokines secreted by activated T cells. The differentiation of the immune response is largely controlled by the type of lymphokines secreted by T cell subsets. The T helper 1 (Th1) lymphocytes are defined by their ability to secrete IL-2, IFN-γ and lymphotoxin whereas the T-helper-2 (Th2) cells produce IL-4, IL-5 and IL-6 (Mosmann and Coffman, 1989). A predominantly Th1 response results in IgG2a production, macrophage activation, and generation of delayed type hypersensitivity, whereas Th2 response leads to IgG1, IgG3 and IgE production (Powrie and Coffman, 1993). Whether an immune response will be primarily Th1 or Th2 in nature is controlled by cytokines such as IFN-γ and IL-4 respectively (Anegon et al., 1988 & Street and Mosmann, 1991). Since NK cell is a producer of IFN-γ, and we have shown that NK cells contribute to the production of IFN-γ after coculture with fixed tumor cells, therefore, NK cells may be participating directly in driving the immune system towards a Th1 type response (Ronagnani, 1992 & Gajeski et al., 1989).

We also present evidence regarding the absence of RT16 (MHC-1) gene expression in AK-5 cells. MHC class 1 molecules on target cells are known to provide inhibitory signals for NK cell cytotoxicity (Lohwasser et al., 1999 & Takei et al., 1997 & Vance et al., 1998). Recent studies have revealed that some tumor cells directly activate NK cell production of IFN-γ, and MHC-I molecules on tumor cells are able to inhibit this activation of IFN-γ production by NK cells (Kubota et al., 1999). Many tumor cell lines, including the NK sensitive target like YAC-1, are highly sensitive to NK cell cytotoxicity, and yet they are poor inducers of IFN-γ production. The differential regulation of NK cell cytotoxicity and cytokine production has been demonstrated in earlier studies. Human NK cell clones killed K-562 and 721.221 targets equally well, but they secreted more IFN-γ in response to K-562 (Kurago et al., 1998). These observations suggest that the activation signals required for NK cell cytotoxicity may be different from IFN-γ production. In our experiments we have used fixed tumor cells to boost NK cell cytotoxic activity, which could be either IFN-γ dependent or IFN-γ independent or both. Suppression of cytotoxic activity of NK cells by anti IFN-γ antibody clearly indicated that the activation of the lytic potential of NK cells by fixed tumor cells is probably due to the interaction of NK cell activation receptors with the ligand(s) present on tumor cells which gives signal for the induction of IFN-γ production by NK cells. NK cell cytotoxicity and IFN-γ production also differ in their time course. Cytotoxicity of NK cells is rapidly triggered on contact with susceptible target cells (Lohwasser et al., 1999) and can be detected within 3 h, whereas IFN-γ production is a slower process and takes at least 12 h to reach peak levels (Farar and Schreiber, 1993 & Farar et al., 1986). We also found that at least 16 h exposure to fixed tumor cells was required to induce cytotoxic activity of NK cells against AK-5 targets (data not shown). These observations also suggest that the activation of NK cells in our system is basically IFN-γ dependent. Recently Diefenbach *et al.* have reported the expression cloning of two murine

ligands for the lectin like receptor NKG2D that is an NK cell activation receptor. The two ligands, H60 and Rae1β are distant relatives of major histocompatibility class I molecules (Young, 1989). NKG2D ligands are not expressed by many normal cells but are upregulated in several types of tumor cells. Expression of either of the NKG2D ligands by the target cells triggered NK cell cytotoxicity and IFN-γ secretion by NK cells. Thus, through this interaction with NKG2D, H60 and Rae1β are newly identified potent stimulators of innate immunity. Since AK-5 cells are MHC-I negative target cells, they do not give inhibitory signals for NK cell activation and the activating ligand(s) present on tumor cells may give signal for IFN-γ secretion by NK cells which may ultimately be responsible for the enhancement of the cytotoxic activity against target cells.

Our present studies also demonstrate overexpression of CD95-L and perforin in NK cells, which were activated after coculture with fixed tumor cells. However, the role of CD95-L remains elusive since we have not been able to demonstrate the expression of CD95 by the AK-5 cells. In our earlier studies, we have shown the requirement of priming or activation of NK cells before they can participate in tumor cell killing. The present studies suggest a direct interaction between NK cells and tumor cells leading to the activation of NK cells, thereby, explaining the efficiency of tumor cell killing, leading to the spontaneous regression of the tumor in syngeneic hosts.

CHAPTER 5

Regulation of NK cell function by the dose of tumor transplanted in the peritoneum

5.1 INTRODUCTION

Natural killer cells are large granular lymphocytes distinguishable from T and B lymphocytes by their surface phenotype, cytokine profile and their ability to mediate spontaneous cytotoxicity against a broad range of targets including MHC class I negative tumor cells (Farrar et al., 1986). Necrosis and apoptosis have been the two proposed modes of CTL /NK mediated target cell death (Duke et al., 1989). Several cytokines such as IL-2, IFN-γ and IL-12 have been shown to augment NK cell proliferation and activation (Herberman et al., 1986). Activated NK cells produce a spectrum of cytokines such as TNF-α, IFN-γ, GM-CSF, lymphotoxin and IL-8 (Farrar et al., 1986). The production of IFN-γ by NK cells is enhanced by IL-12 (Cui et al., 1997), which favors a Th1 type cellular response. A lymphocyte subpopulation called NKT cells express both NK cell receptor (CD161) and a single invariant T cell receptor encoded by the Vα14 and Jα281 gene segments (Masuda et al., 1997). NKT cells mediate their tumor cell cytotoxicity by an NK- like effector mechanism after activation with IL-12 (Cui et al., 1997).

Stimulation of T cells via their antigen receptor can induce either proliferation or unresponsiveness / cell death (anergy); however, the precise mechanism by which the 'choice' is made is not well understood (Lenardo et al., 1991). It has been demonstrated that high dose of antigen can lead to a paradoxical suppression of immune response that is termed as high zone tolerance. While NK cells fail to rearrange TCR subunit, nonetheless they share a number of features with T cells, including expression of surface molecules and secretion of similar cytokines (Lanier et al., 1986 & Ortaldo et al., 1986). T and NK cell are also developmentally related and they also undergo functional anergy and apoptosis. NK cells become inactive, lose their cytotoxic function following cross-linking of CD16 receptor or incubation with target tumor

cells after overnight incubation (Jewett et al., 1997 & Jewett and Bonavida, 1996). In patients with advanced cancers, NK cell cytotoxic activity is usually suppressed (Oh et al., 1987). Inactivation by tumor cells, reduced number of NK cells, reduced responsiveness to IFN-γ or IL-2, ability to produce IFN-γ or IL-2, presence of suppressor cells (including monocyte/macrophages acting through release of prostaglandins), presence of inhibitory substances such as glycoproteins and glycolipids, and other mechanisms have been described as responsible for NK cell suppression in cancer patients. AK-5 is a highly immunogenic rat histiocytic tumor (Khar, 1986 & Khar, 1993) which regresses spontaneously in syngeneic animals when transplanted s.c. The AK-5 tumor follows a site-specific growth pattern when transplanted in syngeneic hosts. Upon s.c. transplantation (5×10^6 cells) the tumor grows upto day 15 (growth phase), after which it starts getting rejected during the regression phase followed by the healing phase when the animal is totally normal but immune to subsequent challenges of AK-5 tumor (Kausalya et al., 1994). Tumor regression in s.c. transplanted animals is mediated by $CD8^+$, $CD3^-$ NK cells through antibody dependent cellular cytotoxicity (ADCC) and apoptosis (Bright et al., 1995). However, the i.p. transplanted tumors (5×10^6 cells/animal) divide rapidly and form a peritoneal bulge by day 3 and the animals start dying by day 5. The death of 100% animals in i.p. injected tumors was attributed to the rapid growth in the peritoneum, not giving sufficient time to the immune system to mount a strong immune response and to the immune escape strategies adopted by the AK-5 cells (Khar et al., 1998).

Recent experiments *in vitro* have implicated antigen dose as an important factor in immune response and a major regulator of effector cell function. Previous studies from our laboratory have shown that low dose of antigen (fixed tumor cells) activated NK cell cytotoxic function whereas high dose of antigen suppressed NK cell cytotoxicity and induced anergy *in*

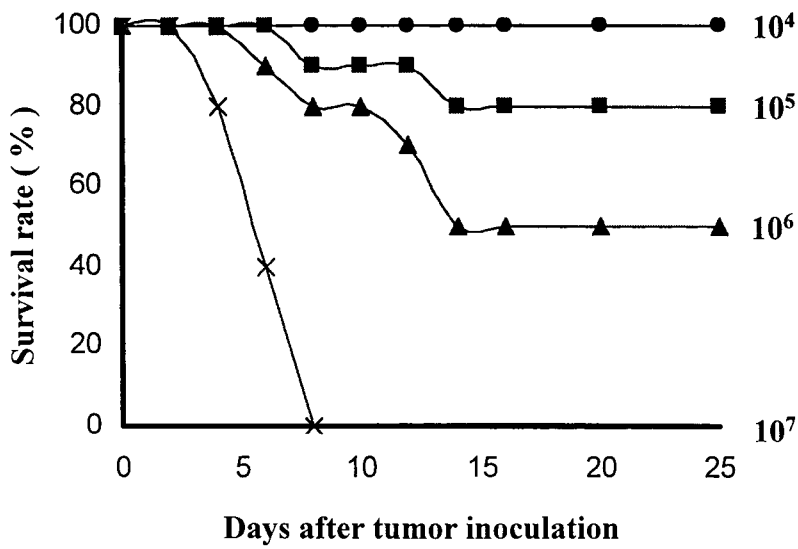

Fig.5.1: Effect of i.p. transplantation of different number of tumor cells on the survival rate of animals. Animals (20 per group) were injected with 10^4, 10^5, 10^6 and 10^7 AK-5 cells per animal on day 0. Survival rate was monitored regularly after tumor transplantation.

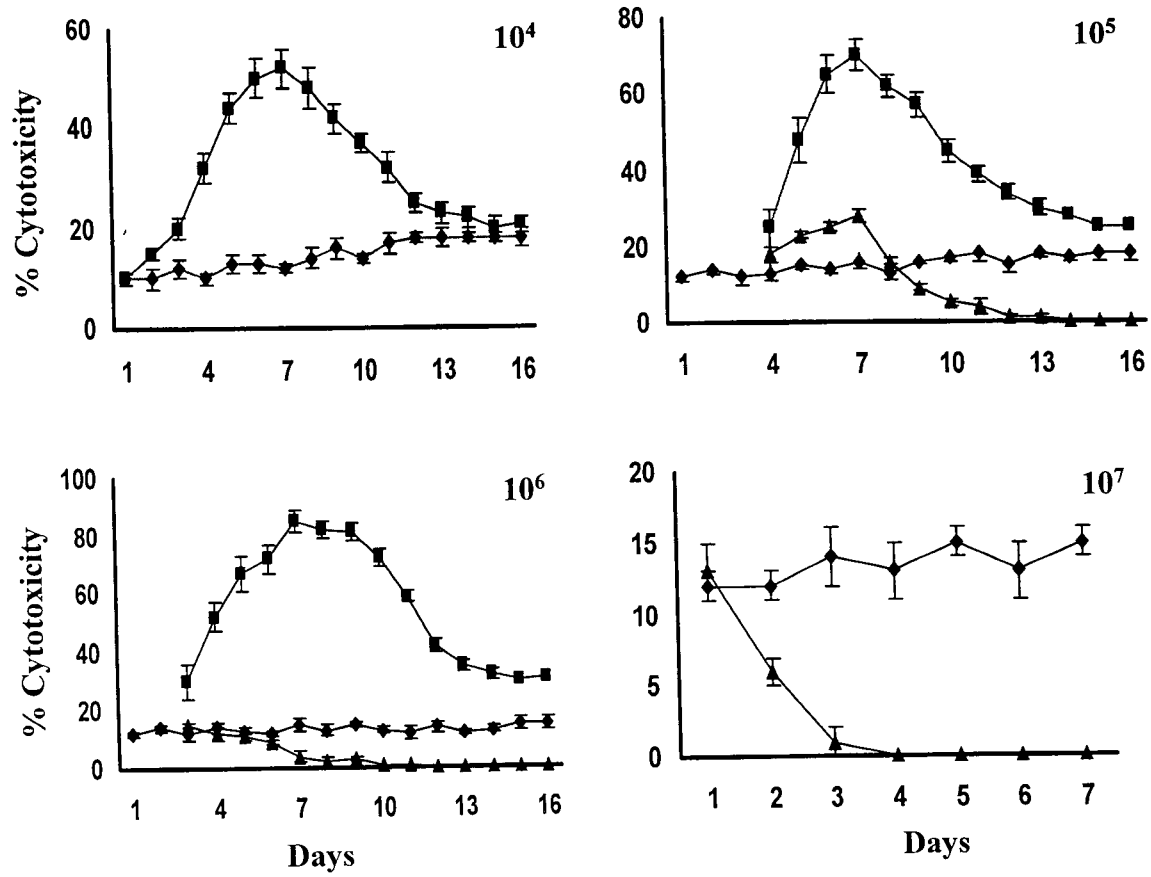

Fig.5.2: Effect of AK-5 tumor transplantation on the cytotoxic activity of NK cells. NK cells isolated from PEC on different days after i.p. tumor transplantation were tested for cytotoxicity against YAC-1 cells. Rejectors (–■–), non-rejectors (–▲–) and control animals (–●–), NK cells from normal animals of the same age group were used as controls and the bars represent ± SD.

vitro (Das et al., 2001 and Das et al., 2000). However, a direct correlation between dose of tumor cell inoculation (i.p.) and NK cell cytotoxic function in vivo has not been demonstrated. The aim of our present study was to find out whether AK-5 tumor cells can modulate NK cell cytotoxic function in a dose dependent manner following injection of tumor cells intraperitoneally.

5.2 RESULTS

5.2.1 NK cell cytotoxicity after i.p. transplantation of AK-5 cells

Antigen concentration could be a major regulator of effector cell function. In order to analyze whether the concentration of antigen (live AK-5 cells) regulates NK cytotoxic function, Wister rats were injected i.p. with 10^4, 10^5, 10^6 and 10^7 AK-5 cells per animal.

5.2.1.1 Inoculation of 10^4 AK-5 cells per animal

We investigated the effect of intraperitoneal injection of 10^4 AK-5 tumor cells on syngeneic rats. When 10^4 cells were injected per animal (20 per group) (**Fig. 5.1**), all animals survived upto day 25. Cytolytic activity of NK cells isolated on different days after tumor injection from the peritoneal cavity were checked against YAC-1 target cells. Three days after tumor transplantation, NK cells isolated from PEC demonstrated 20% cytotoxic activity, which increased steadily upto a maximum of 52% on day 7. Thereafter, there was a gradual decrease of cytotoxic activity of isolated NK cells to 20% on day 15, while NK cells isolated from the peritoneal cavity of PBS injected rats showed cytotoxic activity of 10% on day 0, reaching to a maximum of 18% on day 15 (**Fig. 5.2**).

Similarly, cytotoxic activity of NK cells isolated from splenocytes was checked against ^{51}Cr labeled YAC-1 cells. Splenic NK cells from animals bearing 3 days tumor demonstrated 15% cytotoxic activity, which increased gradually to a maximum of 34% on day 7. Thereafter, there was a slight decrease in cytotoxic activity of NK cells to 28% on day 15, while NK cells

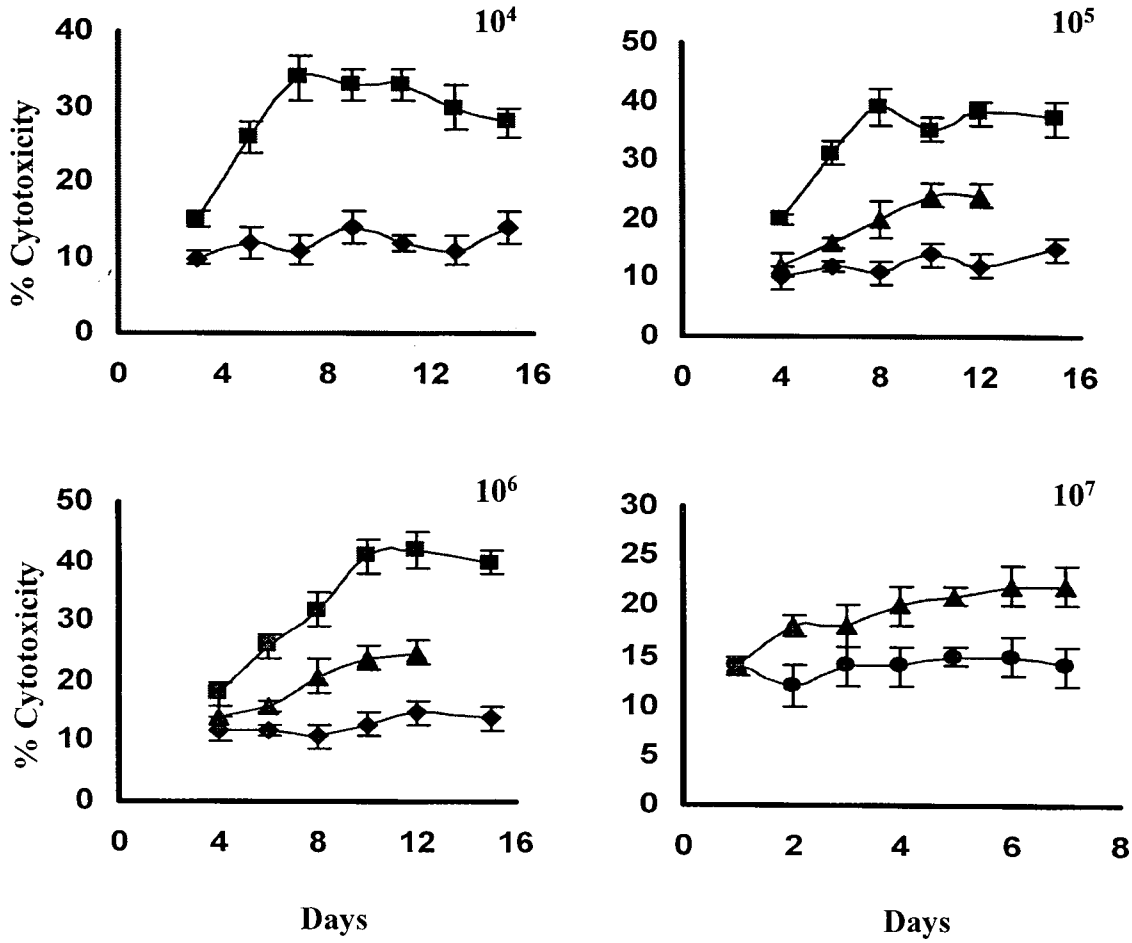

Fig.5.3: Effect of AK-5 tumor transplantation on the cytotoxic activity of splenic NK cells. NK cells isolated from the splenocytes on different days after i.p. tumor transplantation were tested for cytotoxicity against YAC-1 cells. Rejectors (–■–), non-rejectors (–▲–) and control animals (–●–), NK cells from normal rats of the same age group were used as controls and the bars represent ± SD.

isolated from splenocytes of PBS injected rats showed cytotoxic activity of 10% on day 3 reaching a maximum of 14% on day 15 (**Fig. 5.3**). These results demonstrate that i.p. injection of 10^4 AK-5 cells per animal resulted in augmentation of cytotoxic activity in NK cells from both splenocytes and PEC as compared to controls and the cytotoxicity of NK cells isolated from the peritoneum was higher than the splenic NK cells.

5.2.1.2 Inoculation of 10^5 AK-5 cells per animal

Similarly, 20 animals were injected i.p. with 10^5 AK-5 cells per animal. After three days, animals were monitored for tumor growth. Based on the survival pattern, the animals were categorized into two groups. 80% animals (16 / 20) were free of tumor development beyond 25 days, which survived (rejectors), whereas 20% animals died within 15 days (non-rejectors) (**Fig. 5.1**). Non-rejectors showed a peritoneal bulge from day 4 onwards and died within 15 days. We isolated the NK cells from the PEC of non-rejectors on different days and assessed their cytotoxic activity against YAC-1 cells (**Fig. 5.2**). Four days after tumor transplantation, NK cells isolated from PEC showed 18% cytotoxic activity: which increased gradually to 28% on day 7. Thereafter, there was a steady decrease in cytotoxic activity of NK cells to 1% by day 12. NK cells isolated from PEC of control animals showed cytotoxic activity from 12% on day 0 to a maximum of 18% on day 15. The other set of animals, which did not develop tumors (rejectors) showed a higher number of TIM present in the peritoneal cavity upto day 15. Four days after tumor transplantation NK cells isolated from PEC of rejectors demonstrated 25% cytotoxic activity, which increased sharply to 70% on day 7. Thereafter, there was a gradual decrease of NK cytotoxicity to 25% observed on day 15.

We also assessed the cytotoxic activity of splenic NK cells of non- rejectors on different days against YAC-1 cells (**Fig. 5.3**). Four days after tumor transplantation, splenic NK cells from

non-rejectors showed 12% cytotoxic activity, which increased gradually to 24% on day 12. However, in case of rejectors four days after tumor transplantation, splenic NK cells showed 20% cytotoxic activity, which increased gradually to 39% on day 8. Thereafter, it maintained the same activity upto day 15. These results indicate that following i.p. inoculation of 10^5 tumor cells, NK cells isolated from the splenocytes of both rejectors and non rejectors possessed higher cytolytic activity than control; however cytotoxic activity of NK cells isolated from rejectors was higher than those isolated from non-rejectors.

5.2.1.3 Inoculation of 10^6 AK-5 cells per animal

We also studied the effect of intraperitoneal injection of 10^6 tumor cells per animal. 50% of the animals did not develop the tumor in this group after 25 days and the other 50% animals died within 15 days (**Fig. 5.1**). Here again we categorized the animals into two groups, one set being the rejectors where the tumor did not develop and the other set as non-rejectors where the animals died within 15 days because they were unable to clear the tumor burden from the peritoneum. NK cell cytotoxicity was evaluated from non-rejectors. Three days after tumor injection, NK cells isolated from PEC of non-rejectors showed 15% cytotoxic activity which decreased gradually to 1% on day 10, whereas the NK cells from control animals showed cytotoxic activity from 10% on day 0, to a maximum of 16% on day 15. We also isolated the NK cells from the peritoneum of rejectors and checked their lytic activity. All the rejectors had higher number of TIM present in the peritoneal lavage upto day 18. Three days after tumor inoculation, NK cells isolated from PEC of rejectors showed 30% cytotoxic activity, which increased steadily to 85% on day 7. Thereafter, there was a gradual decrease of NK cell cytotoxic activity to 30% by day 15 (**Fig. 5.2**).

NK cells from the splenocytes of non rejectors were assayed for their cytotoxic activity. Four days after tumor injection, splenic NK cells showed 14% cytotoxic activity, which increased gradually to 25% on day 12 (**Fig. 5.3**). However in case of rejectors, splenic NK cells isolated after 4 days demonstrated 18% cytotoxic activity which increased gradually to 42% on day 12. These observations showed that NK cells from both rejectors and non-rejectors possess higher cytotoxic activity than the controls when 10^6 tumor cells are transplanted i.p.

5.2.1.4. Inoculation of 10^7 AK-5 cells per animal

We also investigated the effect of intraperitoneal injection of 10^7 tumor cells per animal. Injection of 10^7 cells led to a rapid development of ascites and mortality within 5-8 days. The i.p transplanted tumor cells divided rapidly and formed a peritoneal bulge by day 3 and the animals started dying from day 5 (**Fig. 5.1**). All the rats developed the ascites and died. NK cells from ascites were evaluated for their cytotoxic activity against YAC-1 target cells. There was a drastic increase in AK-5 cell number from day 3 with simultaneous depletion of NK cell population in the ascites. Cytotoxic activity of NK cells isolated from PEC against YAC-1 was completely lost by day 3. After day 3, no cytotoxic activity was observed although NK cells isolated from ascites of control animals showed cytotoxic activity of 12% on day 0 and a maximum of 15% on day 7 (**Fig. 5.2**).

Cytotoxic activity of NK cells isolated from splenocytes of non-rejectors following i.p. transplantation of AK-5 tumor was also checked. NK cell cytotoxic activity increased gradually from 14% on day 0 to 22% on day 7, whereas control animals did not show any increase in their cytotoxic activity (**Fig. 5.3**). These results indicate that though NK cells isolated from ascitic fluid showed suppression of their cytotoxic activity, there was a slight enhancement in the lytic potential of NK cells isolated from the spleens.

5.2.2 NKT cell cytotoxicity after inoculation of AK-5 cells

PEC were collected 5 and 10 days after injection of different doses of AK-5 tumor cells. NKT cells were isolated from PEC by Dynal immunomagnetic beads and the cytotoxic activity was checked against YAC-1 target cells (**Fig. 5.4**). NKT cells also show dose dependent activation and inactivation following AK-5 tumor injection. NKT cells from rejectors showed higher cytotoxic activity than from controls whereas NKT cells from non rejectors were inactivated and showed lower or negligible cytotoxic activity depending on the tumor dose injected.

5.2.3 AK-5 tumor cell dose modulates the ratio of NK and NKT cells

In normal rats, in addition to T cells approximately the same number of NK cells (5.4 ± 1.1%, n=5) and NKT cells (4.2 ± 0.9%, n=5) are present in PEC. Six days after tumor inoculation (10^4 to 10^7), PEC from rejectors and non-rejectors were isolated and stained with anti CD3 and anti CD161 and the percentage of NK and NKT cells present in PEC were analysed (**Fig. 5.5**). In tumor rejectors, both NK cells and NKT cells increased significantly ($p < 0.01$). However, in non rejectors, no significant increase in the percentage of NK and NKT cells was observed following inoculation of 10^5 tumor cells per animal. At higher tumor doses (10^6 and 10^7), the percentage of NK cell and NKT cells decreased significantly ($p < 0.01$). These results indicate that high doses of tumor reduced the survival time of animals and also depleted both NK and NKT cells from the peritoneal cavity.

5.2.4 Cytokine levels in serum and ascitic fluid of tumor rejectors and non-rejectors

When 10^6 tumor cells were injected per animal, 50% animals survived and were free from tumor after 20 days and the other 50% died within 15 days. Based on the intraperitoneal tumor development we classified the animals as rejectors (survived after 20 days with no

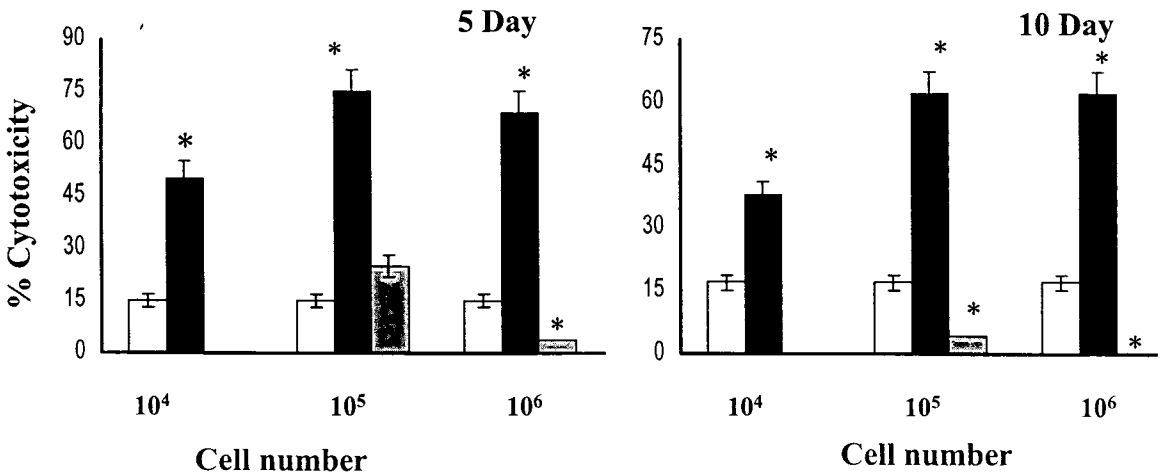

Fig.5.4: Effect of AK-5 tumor transplantation on the cytotoxic activity of NKT cells. NKT cells isolated from PEC on day 5 and 10 after AK-5 tumor transplantation (i.p.) were tested for cytotoxicity against YAC-1 cells. Open bars represent controls, closed bars represent rejectors and the shaded bars represent non rejector animals. *$p < 0.001$; control group versus experimental group.

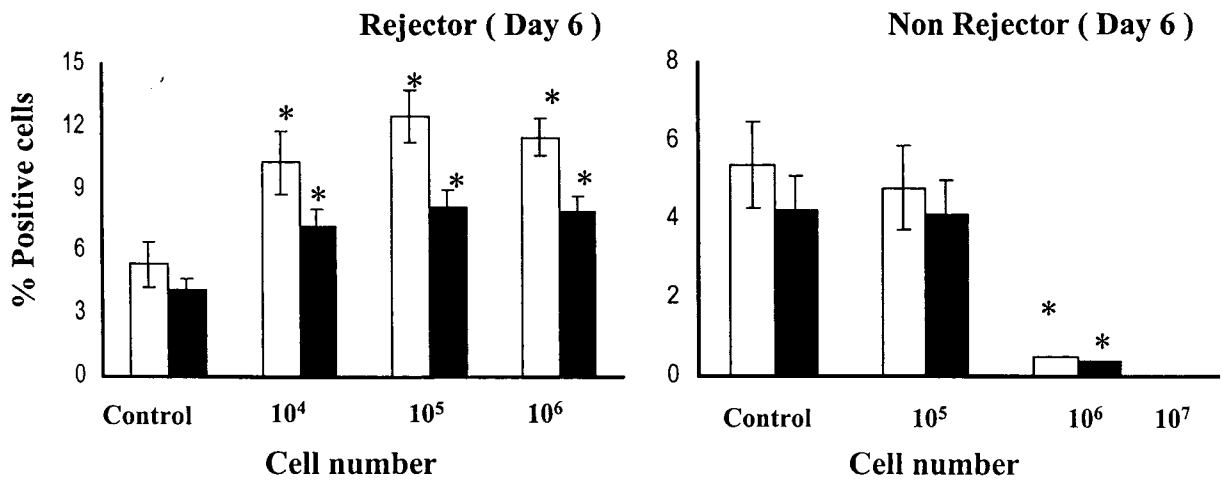

Fig.5.5: Number of peritoneal NK and NKT cells after injection of AK-5 tumor into the peritoneal cavity. AK-5 cells (10^4-10^7) were injected i.p. and the PEC from rejectors and non-rejectors were analyzed on day 6 after immunofluorescence staining. Open bars represent NK cells and the closed bars represent NKT cells.
*$p < 0.01$; control group versus experimental group.

peritoneal bulge) and non-rejectors (died within 15 days). Figure 5.6 shows the serum cytokine profile in rejectors and non-rejectors estimated on different days. Levels of IL-2, IL-12, IFN-γ and TNF-α were higher in rejectors than in non-rejectors. We also analysed the presence of IL-2, IL-12, IFN-γ and TNF-α in the cell free ascitic fluid from rejectors and non-rejectors on different days as shown in Figure 5.7. Ascitic fluid also showed higher cytokine levels in rejectors than in non-rejectors. These results suggest the requirement of higher levels of cytokines and their role in the activation of effector cell function leading to the inhibition of tumor growth.

5.2.5 Anti-IFN-γ and anti IL-12 injections shortened the survival time of animals

To study the role of IFN-γ and IL-12 produced by PEC in suppressing the tumor growth, either anti-IFN-γ mAb (1 mg Ig/injection) or anti-IL-12 mAb (1 mg Ig/injection) were injected i.p. on day 0 (just before the tumor inoculation) (10^5 cells/animal) and on day 2. The results show that treatment with either antibody significantly reduced the survival time of animals. The effect of anti IFN-γ mAb was stronger than that of anti-IL-12 mAb. (**Fig. 5.8**). Anti IL-2 had no significant effect on the survival time of animals.

5.2.6 Expression of CD95-L by AK-5 cells

Following i.p. transplantation of 10^7 AK-5 cells per animal, the tumor cells were isolated from the peritoneum on different days and analysed for the expression of CD95-L after immunostaining with specific antibody. CD95-L expression by AK-5 cells is maximum on day 3 and 4 after transplantation and decreased from day 5 onwards (**Fig. 5.9**). These observations suggest that AK-5 cells are able to regulate CD95-L expression on their surface, which may have a role in antitumor immune response.

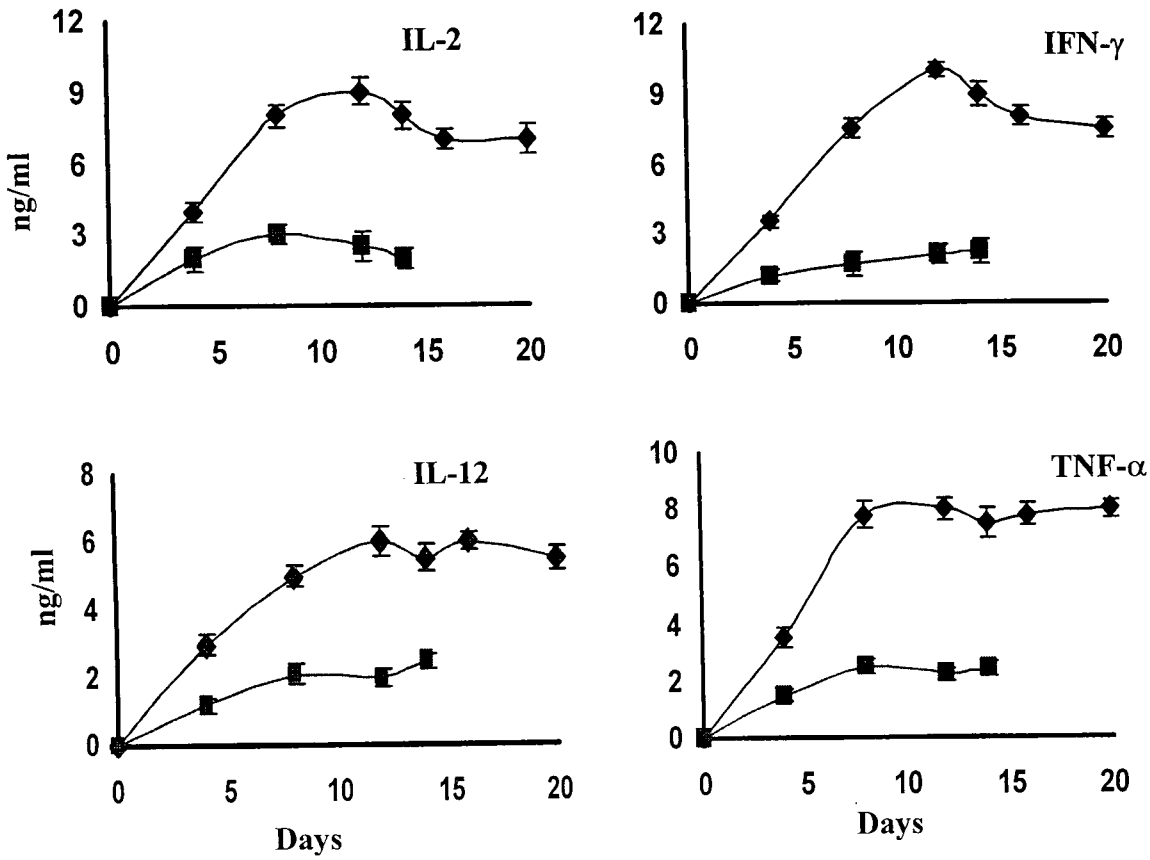

Fig.5.6: Cytokine levels in the sera of tumor rejectors (–◆–) and non-rejectors (–■–) on different days after i.p. tumor transplantation. The values represent mean ± SD of 3 animals. Cytokine levels in control animals on different days were very low (data not shown). The values were compared between rejectors and non-rejectors by Student's "t" test and were significant ($p < 0.01$) from day 4 onwards.

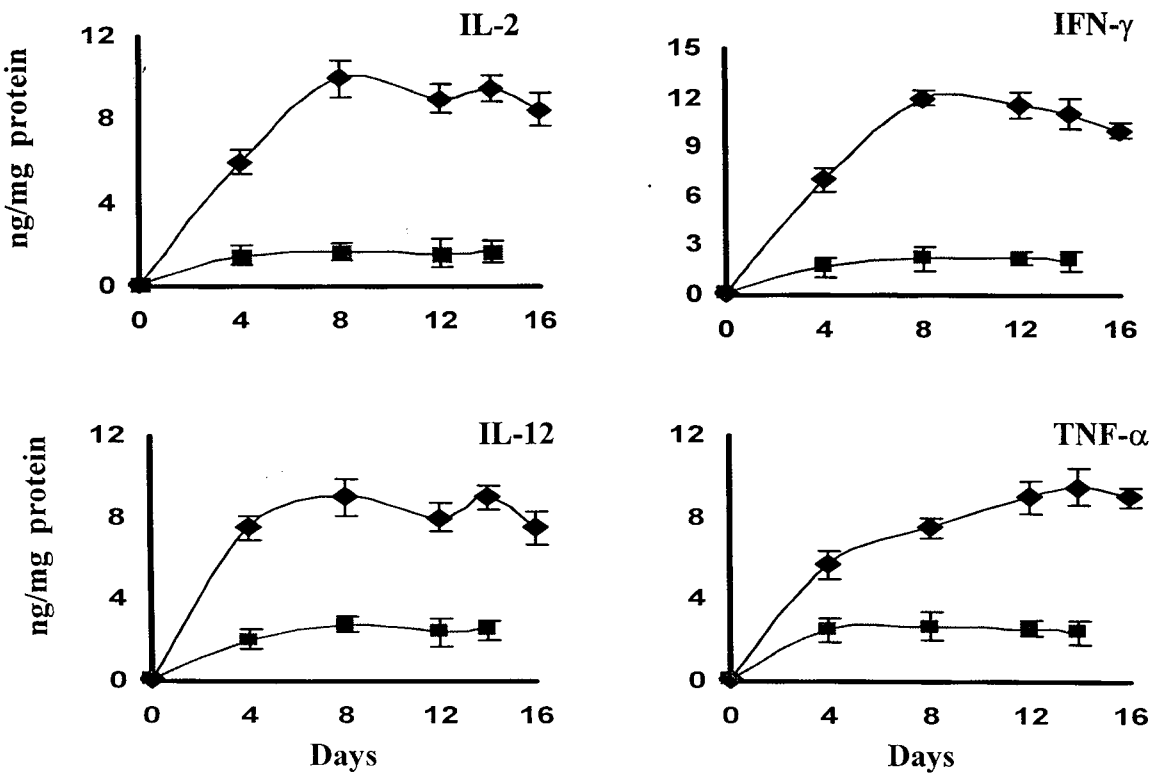

Fig.5.7: Cytokine levels in ascitic fluid of tumor rejectors (–◆–) and non-rejectors (–■–) on different days after i.p. tumor transplantation. The values represent mean ± SD of 3 animals. Cytokine levels in control animals on different days were very low (data not shown). The values were compared between rejectors and non-rejectors by Student's "t" test and were significant ($p < 0.01$) from day 4 onwards.

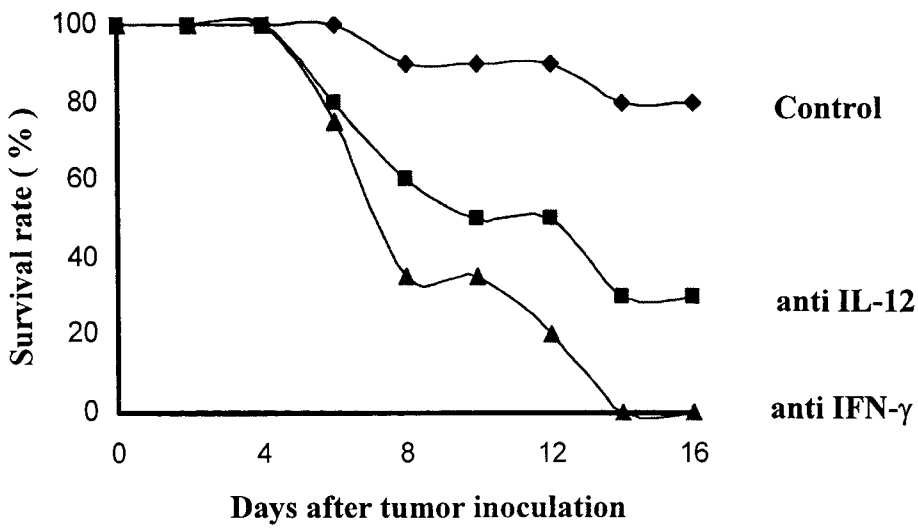

Fig.5.8: Anti IFN-γ or anti IL-12 antibody injections significantly shortened the survival time of rats transplanted i.p. with 10^5 AK-5 cells. 1 mg Ig / injection / animal was injected on day 0 and day 2 (i.p.). Control rats received only 10^5 AK-5 cells per animal. 10 animals were used in each group.

*$p < 0.001$; control group versus anti-IL-12 and anti IFN-γ treatment.

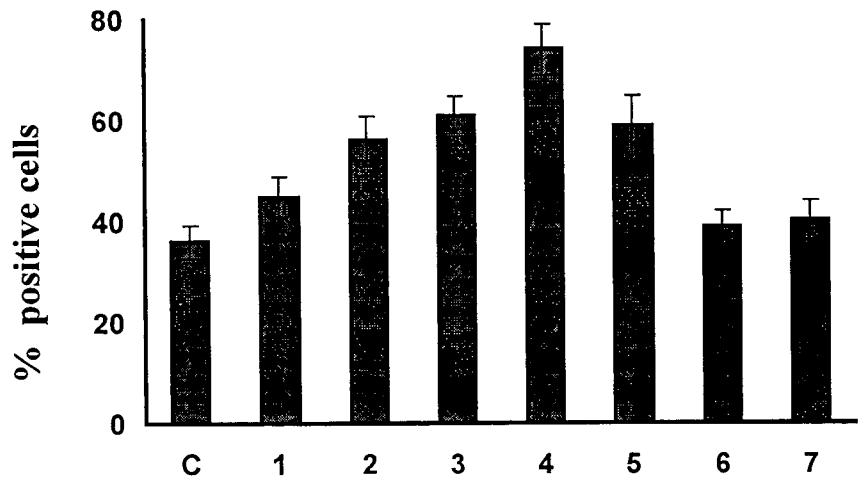

Fig.5.9: Differential expression of CD95-L by AK-5 cells (10^7cells / animal) transplanted i.p. on different days. The values shown are mean ± SD of triplicates and are representative of two similar experiments.

5.2.7 Expression of CD95 and perforin by NK cells

After transplantation of AK-5 tumor cells i.p. (10^6 cells / animal), we isolated NK ce from ascitic fluid of non-rejectors on different days and analysed for the expression of CD95 a perforin after immunostaining with specific antibodies. Three days after tumor transplantatic NK cells isolated from ascites showed 2% CD95 positive cells, which increased sharply to 72 on day 10 (**Fig. 5.10**). On the other hand, 5% NK cells showed perforin expression on day which increased gradually to 15% on day 10.

Similarly, NK cells from ascitic fluid of tumor rejectors were analysed for the expressi of CD95 and perforin on different days. Four days after tumor inoculation, 15% NK cells we perforin positive which increased steadily to 65% on day 12 (**Fig. 5.10**). However, only 4% N cells were positive for CD95 expression on day 4, which increased gradually to 10% on day These observations demonstrated that when the tumor burden was high, NK cells expressed mo of CD95 and less of perforin, whereas when the tumor load is low, NK cells expressed higl levels of perforin and low levels of CD95 on their cell surface, thereby suggesting CD95 a perforin as the markers of suppressed and activated NK cells respectively.

5.3 DISCUSSION

AK-5 is a rat histiocytoma which when transplanted s.c. grows as solid tumor a develops into ascites when injected i.p. upon transplantation of 5 x 10^6 cells. All the i.p. injec animals are killed by day 10, however, about 70% s.c. transplanted animals reject the tur spontaneously (Kausalya et al., 1995). We have demonstrated NK cell as the effector c involved in tumor cell killing (Bright et al., 1995). In the present study, we have evaluated activation of effector cell function based on the dose of tumor cells transplanted i.p.

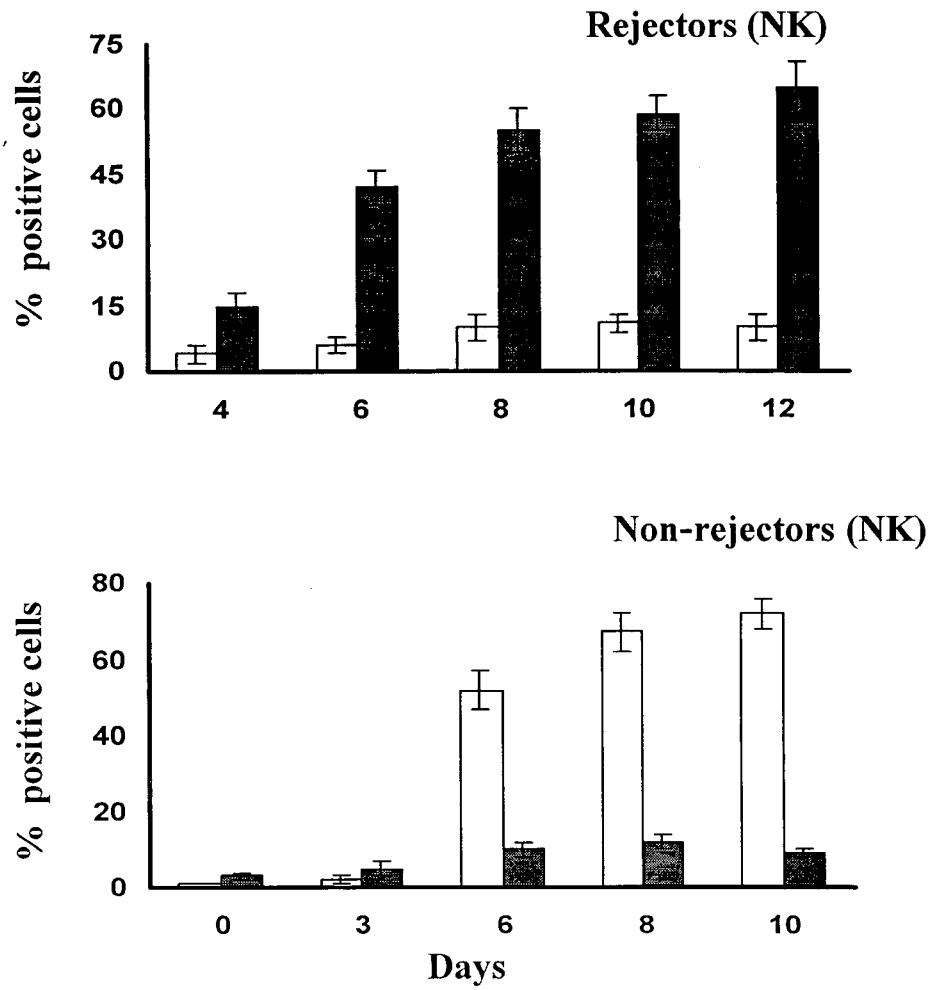

Fig.5.10: Differential expression of CD95 (white bar) and perforin (black bar) by NK cells isolated from ascitic fluid of rejectors and non rejectors on different days following i.p. transplantation of AK-5 cells (10^5 cells/animal). The values shown are mean ± SD of triplicate.

We have observed that a low dose of tumor cells mounts a high immune response which is manifested by increased number of NK and NKT cells in the peritoneum, higher cytotoxic activity and upregulation of perforin expression by NK cells followed by the rejection of AK-5 tumor from the peritoneal cavity. However at higher tumor doses, animals could not mount an effective immune response, which was reflected by the presence of low number of NK and NKT cells in the ascitic fluid, lower cytotoxic activity and upregulation of CD95 expression by NK cells. These observations indicate that the tumor cell load in the peritoneum is responsible for the regulation of NK cell activity and the surface phenotype on NK cells. Lower tumor dose also resulted in higher cytotoxic activity of splenic NK cells in comparison to control NK cells isolated from naive splenocytes. However, cytotoxic activity of NK cells isolated from PEC was higher than NK cells isolated from splenocytes when low dose of tumor cells was inoculated. NK cells in PEC are in direct contact with tumor cells, which might be responsible for the higher activity of NK cells present in ascites as compared to NK cells isolated from splenocytes, which had no direct contact with the AK-5 cells. Moreover, lower tumor cell dose also evoked an appropriate immune response followed by the secretion of several cytokines which are involved in the enhancement of cytotoxic activity of NK cells, whereas, in case of high tumor dose splenic NK cells showed higher cytotoxic activity than NK cells isolated from the peritoneal cavity. NK cells in the peritoneal cavity are exposed directly to the high tumor load and hence had undergone suppression of their cytotoxic activity. Since the NK cells in the spleen are not in direct contact with tumor cells, therefore they did not show any suppression of their cytotoxic function and the cytokines secreted by immune cells present in ascites following interaction with tumor cells were responsible for the activation of NK cells from the spleen. These observation demonstrate that when AK-5 tumor cells are in direct contact with NK cells, the tumor cell

could either activate NK cells at low dose, or suppress NK cell activity when the tumor load is high. Hence, this dose-dependent activation or inactivation of NK cell function determines the fate of the tumor growth and drives it either towards tumor rejection or tumor progression.

In the present study, we have detected IL-2, IL-12, IFN-γ and TNF-α in the sera as well as ascites from both rejectors and non-rejectors. IL-2 has been shown to trigger the activation and tumoricidal capacity of NK and LAK cells (Hara et al., 1995). Similarly, IFN-γ induces cytotoxic activity of CTLs, LAK and NK cells (Perussia et al., 1991). IL-12, a hetero-dimeric cytokine which was originally called natural killer cell stimulatory factor, augments spontaneous as well as antibody dependent cell mediated cytotoxicity of resting NK cells (Trinchieri and Perussia, 1985). Besides, contact between tumor cells and macrophages is known to release TNF-α without mediation of endotoxin (Carswell, 1975 and production of biologically active TNF-α has been demonstrated in malignancy (Zacherchule et al., 1983). Though we have detected all the above cytokines in both rejectors as well as non-rejectors, the levels of cytokines in the rejectors were higher than in non-rejectors. Therefore, high levels of TNF-α along with increased IL-2, IL-12 and IFN-γ levels in rejectors evokes a higher level of immune response which might be responsible for the rejection of AK-5 tumor. However, in case of non-rejectors, which secreted comparatively low levels of IL-2, IL-12, IFN-γ and TNF-α, an effective immune response could not be evolved against AK-5 tumor and hence could not cause rejection of the tumor in the peritoneum.

Tumor cells are known to have devised mechanisms of immune escape by masking or shedding of the antigens (Herlyn et al., 1987). In addition, a novel mechanism of immune evasion by tumor cells was demonstrated where the tumor cells expressed CD95-L, thereby keeping the activated immune cells which normally express CD95, away from the immune attack

(O'Connell et al., 1996 & Niehans et al., 1997). Tumor cells expressing CD95-L have been shown to deliver the death signal to CD95-expressing targets in vitro (O'Connell et al., 1996 & Niehans et al., 1997 & Strand et al., 1995). AK-5 cells express CD95-L on their surface. We have shown that NK cells isolated from ascites with a huge peritoneal bulge had higher expression of CD95 on their surface. Thus, it is possible that when the tumor burden is high, CD95-L positive AK-5 cells upon interaction with NK cells may cause upregulation of CD95 on NK cells. The CD95-CD95-L interaction between the effector cells and the target cells leads to the suppression of NK cell cytotoxicity and the depletion of NK cell population in the ascites. The present study suggests a direct interaction between NK cells and tumor cells in vivo, leading to the dose dependent activation or suppression of NK cell function that determines the fate of the tumor either towards progression or regression in syngeneic hosts.

Summary of the results

***In vitro* studies**

The role of endogenously secreted TNF-α in mediating functional inactivation and apoptosis in NK cells when they are co-cultured with fixed AK-5 cells at 1:1 ratio.

A change in the phenotype from $CD95^{dim}$ to $CD95^{bright}$ was observed in NK cells co-cultured with target cells.

The up-regulation of CD95 expression on the NK cells after co-culture with fixed AK-5 cells may play an important role in signaling NK cells for functional anergy and apoptosis.

The induction of NK cell lytic function after their interaction with fixed tumor cells and the requirement for cell-secreted accessory signals for the augmentation of the lytic function.

AK-5 cell mediated augmentation of NK cell cytotoxicity in the presence of T cells is mediated by lymphokines (IL-2 and IFN-γ) and that NKT cells possess higher cytotoxic potential than NK cells.

We have also observed over-expression of CD95L and perforin in NK cells following exposure to fixed AK-5 cells.

***In vivo* studies**

Inoculation of AK-5 tumor cells intraperitoneally modulate the cytotoxic function of NK and NKT cells present in the peritoneal exudates cells (PEC) in a dose dependent manner.

Low dose of tumor causes activation of NK and NKT cell cytotoxic function and enhanced NK and NKT cell population in PEC whereas high doses of tumor cause inactivation of NK and NKT cell cytotoxic function and depletion of the two sub-populations in the peritoneum.

Different doses of tumor inoculation in the peritoneal cavity did not suppress the cytotoxic function of NK cells from spleen suggesting that a direct interaction between NK cells and tumor cells is required for the suppression of NK cell cytotoxic function.

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2. Das S, Varalakshmi C, Kumari AL, Patel M, Khar A. Target-cell-induced activation of NK cells in vitro: cytokine production and enhancement of cytotoxic function. Cancer Immunol Immunother. 50(8) 2001.

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4. Alli R, Savithri B, Das S, Varalakshmi C, Rangaraj N, Khar A. Expression and reorganisation of activating receptor NKR-P2 by dendritic cells upon antigen contact:- TNF-α induced target cell apoptosis. Manuscript submitted.

5. Das S, Varalakshmi C, Mitra R, Savithri B, Khar A. In vitro studies of anti-tumor immune response by innate immune system against AK-5 tumor cells. Manuscript in preparation.

6. Das S, Kumari AL, Khar A. The role of the NKR-P2 receptor in dendritic cell maturation. Manuscript in preparation.