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 \textit{ex vivo} cytolytic activity against YAC-1 target cells, suggesting that it contains functionally mature NK cells. The CD117\textsuperscript{+} subset was capable of reconstituting \(\alpha\beta\)T 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\textsuperscript{+} population failed to give rise to \(\alpha\beta\)T 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\textsuperscript{+} population represents restricted progenitor cells for the T and NK lineages while the CD117\textsuperscript{−} 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\textsuperscript{+}, DX5\textsuperscript{−}, CD117\textsuperscript{+} 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\textsuperscript{+}/CD117\textsuperscript{low} population that was capable of giving rise to \(\alpha\beta\)T cells after intrathymic transfer. It was concluded that this population represents a restricted \(\alpha\beta\)T 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, NCAM. 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 \(\beta\) subunit (CD18, 95kD) and different \(\alpha\) 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 (e.g., 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 I (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'-oligoadenylate (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-γ J, 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 FcγR+ lymphocytes, after interaction with immune complexes, suppress the polyclonal B cell differentiation induced by pokeweed mitogen (PWM). E-rosetting Fcγ-R+ cells are now known to be almost exclusively CD2+, CD16+ NK cells. Lobo (1981) showed that non-E-rosetting FcγR+ 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-GM₁ 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-GM₁⁺ 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-GM₁⁺ 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 \textit{in vitro} generation of virus-specific CTLs (Burlington 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-\(\alpha\)-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 \textit{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 som-
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 \textit{in vitro} and have been proposed to be responsible for the \textit{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).