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## Role of Natural Killer Cells in Antitumor Resistance

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### Abstract

Kaneno R. *Role of natural killer cells in antitumor resistance. ARBS Ann Rev Biomed Sci* 2005;7:127-48. Natural killer cells constitute a population of lymphocytes able to non-specifically destroy virus-infected and some kinds of tumor cells. Since this lytic activity was shown by non-immunized animals the phenomenon is denominated natural killer (NK) activity and contrasts with specific cytotoxicity performed by cytolytic T lymphocytes (CTLs) because it does not depends on MHC-restricted peptides recognition. In fact, the main feature of most functional receptors of NK cells (NKR) is their ability to be inhibited by different kinds of class I MHC antigens. In the middle of the 1950's, Burnet & Thomas forged the concept of tumor immunosurveillance and NK cells can be considered one of the main figures in this phenomenon both for effector and regulatory functions. In the present review the early studies on the biology of NK cells were revisited and both their antitumor activity and dependence on the activation by cytokines are discussed.

**KEYWORDS:** cell activation, cytotoxicity, natural killer, tumor immunology

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## Table of Contents

Abstract

1. Introduction
2. Phenotypic Markers of NK Cells
3. Activation-inhibition Balance of NK Effector Function
4. Role of NK Cells in Antitumor Resistance
5. Action of Cytokines on the Antitumor Activity of NK Cells

References

### 1. Introduction

In the middle of the 1970's Herberman *et al.*, (1975) and Kiessling *et al.*, (1975), showed that peripheral leukocytes of normal mice were able to lyse some lineages of tumor cells without a previous sensitization of the host. Since this lytic activity was shown by non-immunized animals the phenomenon was denominated natural killer (NK) activity as well as the effector cells (NK cells). It contrasts with specific cytotoxicity performed by cytolytic T lymphocytes (CTLs) because they are able to interact with target cells independently of peptides linked to major histocompatibility complex (MHC) antigens performing an MHC-unrestricted cytotoxicity.

Most natural killer activity is attributed to a population of cells morphologically defined as large granular lymphocytes (LGL) found in peripheral blood and lymphoid organs (Timonen *et al.*, 1979a, 1979b; Lotzova & Ades, 1988; Trinchieri, 1989). These cells are larger than typical small lymphocytes, showing a higher cytoplasm:nucleous ratio and large azurophilic cytoplasm granules (Timonen & Saksela, 1980). LGLs are non-T, non-B lymphocytes, since they do not express CD3 or TCR or any of the TCR chains ( $\alpha$ ,  $\beta$ ,  $\gamma$  or  $\delta$ ) (Saksela *et al.*, 1979; Timonen *et al.*, 1979a, 1979b; Lotzova & Ades, 1988). B lymphocyte markers CD19 and surface Ig (Nishikawa *et al.*, 1990; Natajarian *et al.*, 2002) are also absent and these cells are non-adherent leukocytes and do not show phagocytic activity although they share the expression of CD16 with macrophages and neutrophils (Trinchieri, 1989; Natajarian *et al.*, 2002).

NK cells show a heterogeneous tissue distribution, and it was observed that cells from mesenteric lymph nodes of BALB/c mice show the most prolific NK activity (53.9% of lytic activity against Yac-1 target cells), followed by inguinal lymph nodes (30.4%), spleen and blood (26.4 and 25.3%, respectively) peritoneum (7%) and bone marrow (3.8%) (Herberman *et al.*, 1975).

Reynolds *et al.*, (1981) observed that in rats the main NK activity is presented by blood and spleen (22.1 and 20.5%), whereas peritoneum and bone marrow cells show low lytic activity (6.4 and 1.1%). These authors also observed that NK activity decreases with age, although the number of peripheral LGL had not been altered. NK activity is also under the influence of the host's genetic background; and different inbred strains of

mice present different level of lytic activity. It was demonstrated by Herberman, *et al.*, (1975) that some inbred strains of mice like B6C3F1 present high NK activity (39.0% of specific lysis of RBL-5 cells), whereas others like C3H have shown very low activity (2.6%). They also observed that *nude* mice show higher NK activity than their heterozygous counterparts both against RBL-5 and Yac-1 target cells.

Besides the high correlation between LGL and MHC-unrestricted lytic activity, it must be noted that not all LGLs show cytotoxic activity and not all MHC-independent lytic cells are morphologically identified as LGL (Trinchieri, 1989). Reynolds *et al.*, (1981) observed earlier that there is no correlation between the number of LGL and NK activity of lung infiltrating cells, indicating that the cells from this organ do not present an effective lytic activity as happens with thymus and bone marrow cells, suggesting the existence of cells in distinct stages of activation.

Isolation of LGL enriched cell suspension by Percoll density gradients (Reynolds *et al.*, 1981; Trinchieri, 1989) helped to achieve much knowledge of NK biology but the lack of a specific antibody made difficult the obtainment of highly purified suspension of these cells. At that time the isolation of tumoral clones of NK cells was fundamental to amplifying the knowledge on functional and phenotypic features of these cells.

## 2. Phenotypic Markers of NK Cells

NK cells constitute a heterogeneous population of lymphocytes with a non-specific cytotoxicity presenting a variety of surface markers, which are shared with other cell populations. This heterogeneity reflects on the phenotypic expression of several cell markers, which are evolved in antigen recognition, triggering lytic activity and cell regulation (Trinchieri, 1989; Cooper *et al.*, 2001a; Natarajan *et al.*, 2002) and most NK cells can be quantified or isolated through the recognition of some of these markers (West *et al.*, 1977; Trinchieri, 1989; Cooper *et al.*, 2001b).

Among these surface markers, CD16 is expressed by virtually all of the NK cells (West *et al.*, 1977), however it is not exclusive to these cells, since it is found in neutrophils, mature eosinophils and tissue macrophages, but is scarce on monocytes (Trinchieri 1989). CD16 is a low affinity receptor for the Fc portion of IgG (Fc $\gamma$ RIII) but it cannot link to monomeric IgG. Fc $\gamma$ RIII has molecular weight of 50 – 70 kDa, is highly glycosylated and is structurally different from the high-affinity receptor (Fc $\gamma$ RI) expressed by monocytes and macrophages or Fc $\gamma$ RII (low affinity) expressed by B lymphocytes and PMN (Trinchieri 1989). In this way, whereas the receptors on macrophages and neutrophils enhance the phagocytosis of antibody-linked structures, the Fc-receptors on NK cells promote the antibody-dependent cell cytotoxicity (ADCC) (Kay *et al.*, 1977), interfacing with the adaptive and natural defense mechanisms. These receptors are able to signal transduction since NK can lyse the anti-CD16 producing hybridomas (Lanier *et al.*, 1988). Although almost all NK cells express CD16, it had been observed that there is a CD16<sup>-</sup> NK cell subset possibly able to destroy some target cells distinct from those sensitive to

CD16<sup>+</sup> ones (Masucci *et al.*, 1980).

The ganglioside asialo GM-1 is another classical marker of NK cells and the polyclonal antibody against it frequently was used for phenotypic characterization of both human (Herberman *et al.*, 1977) and murine (Santoni *et al.*, 1979) NK cells. This marker is also not exclusive to NK cells and can be expressed by CTLs and activated macrophages (Kasai *et al.*, 1980; Young *et al.*, 1980; Suttles *et al.*, 1987).

The development of a variety of monoclonal antibodies allows the identification of many other antigens, especially in human and murine cells. The main surface marker for human NK cells that is largely used in clinical studies is the CD 56, an isoform of neuronal adhesion molecule (Lanier *et al.*, 1989), the function of which in NK cells appears to be associated with adhesion to target cells (Nitta *et al.*, 1989; Suzuki *et al.*, 1991). CD56 has molecular weight of 200-220 kDa and is recognized in 90% of human NK cells and a small percentage of T lymphocytes (Griffin *et al.*, 1983). According to Robertson & Ritz (1990), CD56<sup>+</sup>/CD3<sup>-</sup> cells correspond to 15% of human lymphocytes and depending on the density of expression of this marker, they can be divided into 2 subsets identified as CD56<sup>dim</sup>/CD16<sup>bright</sup> or CD56<sup>bright</sup>/CD16<sup>dim</sup> (or CD16<sup>-</sup>). Lanier *et al.* (1986a) showed that resting lymphocytes are featured as CD56<sup>dim</sup> and show high cytotoxic activity. Other authors suggest that these two populations perform distinct regulatory roles through the production of different cytokines (Cooper *et al.*, 2001a, 2001b). In fact, Cooper *et al.*, (2001b) observed that fresh CD56<sup>bright</sup> cells produce higher levels of IFN- $\gamma$ , TNF- $\beta$  and GM-CSF than CD56<sup>dim</sup>, when stimulated with PMA or co-cultured with LPS-activated macrophages, suggesting that CD56<sup>bright</sup> NK cells are the main source of early IFN- $\gamma$  required for rapid macrophage activation in the initial phase of the immune response.

Although NK cells are CD3<sup>-</sup> they share some markers with T lymphocytes, so that 30% of human and 86% of rat NK cells express CD8 antigens (Reynolds *et al.*, 1981), whereas murine cells are CD8<sup>-</sup> (Young *et al.*, 1986; Trinchieri, 1989). These cells also express CD2 antigens, probably using it for cell adhesion and for activation of the effector function after interaction with LFA-3 (Fletcher *et al.*, 1998). Although  $\alpha$  and  $\delta$  chains of TCR (T $\gamma\delta$  lymphocytes) are also associated with the ability of MHC-unrestricted cytotoxicity, rearrangement of TCR genes is not observed in NK cells (Ritz *et al.*, 1985; Lanier *et al.*, 1986b), thus distinguishing them from T $\gamma\delta$  lymphocytes.

An important feature of NK cells is the expression of intermediary affinity IL-2 receptor (IL-2R). This IL-2R is a  $\beta\gamma$  heterodimer and is distinct from the low-affinity  $\alpha$  chain receptor and the high-affinity trimer receptor ( $\alpha\beta\gamma$ ) expressed by activated T lymphocytes (Nishikawa *et al.*, 1990). This feature allowed the exploitation of therapeutic potential of *in vitro* or *in vivo* stimulation of these cells by IL-2, as will be further discussed.

### 3. Activation-inhibition Balance of NK Effector Function

The main feature of most functional receptors of NK cells (NKR) is their ability to react with different kinds of class I MHC antigens, which results in inhibitory effect on the cytotoxic activity. This observation was the base of the missing-self hypothesis

(Ljunggren & Kärre, 1990). According to this, whereas CTLs are only activated by contact with MHC-associated antigens, NK cells are naturally activated and are potentially able to destroy any self cell. However, this destructive activity of NK cells is suppressed by interaction of their receptors with any class I histocompatibility antigen. So, only the cells that had lost the expression of class I MHC antigens are still susceptible to NK mediated lysis, as observed in some tumor cells and virus infected cells (Zeidler *et al.*, 1997; Orange *et al.*, 2002). In fact, many studies have demonstrated that that human class I-deficient cells are susceptible to NK lytic activity, but became resistant after their transfection with HLA A, B or C alleles, whereas a similar phenomenon was observed with murine cells (Shimizu & DeMars, 1989; Storkus *et al.*, 1989; Orange *et al.*, 2002).

Human NK cells interact with histocompatibility molecules through a variety of membrane receptors and, according to their structure, they can be classified as Ig-like receptors (KIR and LIR) or lectin-like receptors (CD94/NKG2 and NKG2D) (Cooper *et al.*, 2001b; Natarajan, 2002), whereas mice express the lectin-like Ly-49 family as their main receptors for class I MHC molecules (Lowin-Kropf & Held, 2000; Makrigiannis *et al.*, 2001; Natarajan, 2002). Each type of receptor shows a different level of affinity for molecules codified by the several loci of class I MHC genes and any NK cell expresses at least one ligand for these MHC molecules, a fact that constitutes a mechanism to avoid autoreactivity.

The killer cell Ig-like receptor (KIR), also known as CD 158 is a family of molecules that react with different class I MHC structure (Natarajan *et al.*, 2002). In general, KIR3D receptors recognize HLA-A and B, whereas those of KIR 2D recognize HLA-C alleles (Middleton *et al.*, 2002). Minimal variations in each receptor allow them to recognize allotypic differences in MHC molecules. Although it is believed that the main function of KIRs is to scan the normal expression of class I MHC, the cognitive process is not totally independent from the peptide linked to the histocompatibility molecule. In fact it has been demonstrated that the substitution of amino acids in positions 7 and 8 of a peptide nonamer promotes the recognition of MHC-peptide by other receptors (Malnati *et al.*, 1995 Lowin-Kropf *et al.*, 2000).

The mechanism of KIR-induced signal transduction is not completely known but it has been shown that these receptors frustrate cytotoxicity through the recruitment of tyrosine-phosphatase SHP-1 (Lowin-Kropf *et al.*, 2000) for the immunoreceptor tyrosine-based inhibition motifs (ITIM), inhibiting signal transcription (Burshtyn *et al.*, 1996; Campbell *et al.*, 1996).

Another family of NKRs is the leukocyte immunoglobulin-like receptor-1 (LIR-1), also known as ILT-3 or CD58h (Cella *et al.*, 1997; Fanger *et al.*, 1999), which recognizes most class I MHC structures without discriminating among allotypic variations. Hence, any NK cell expressing LIR-1 is inhibited by any target cell expressing any class I molecule and this effector cell only will be able to destroy target cells totally depleted of HLA antigens (Cella *et al.*, 1997; Fanger *et al.*, 1999).

Receptors CD94/NKG2 are lectin-like receptors, in which subunits CD94

and NKG2 are linked by disulphide bonds. These receptors are specific to non-classic class I MHC antigens that are codified by HLA-E locus (Soderstrom *et al.*, 1997). The subunit CD 94 is an invariant chain (Chang *et al.*, 1995) whereas the NKG2 subunit constitutes a multigenic family with at least 5 proteins named NKG2A (with a B variant chain), NKG2C and NKG2E, whose intracellular structure share a high degree of homology (Chang *et al.*, 1995; Soderstrom *et al.*, 1997) and are responsible for inhibitory signaling after interaction with MHC molecules.

Mice NK cells express Ly 49 (C-type lectin-like receptors) as the main ligand for class I MHC molecules, which perform the same role as human KIR molecules (Lowinkropf & Held, 2000; Makrigiannis *et al.*, 2001; Natarajan, 2002) when reacting with H-2K and H-2D structures (mouse class I antigens). Some KIR and Ly 49 receptors are linked to adaptor molecules with immunoreceptor tyrosine-based activation motifs (ITAM) for signal transduction. These receptors show short cytoplasmic chains that are associated with homodimeric adaptor molecules DAP 12 or KARAP, that contain cytoplasmic ITAM sequences, which promote cell activation via Syk and ZAP-70 (Tomasello *et al.*, 1998; Wu *et al.*, 2000). Therefore, some KIR and Ly 49 receptors can play an activating role after interaction with some MHC molecules.

NKG2D is the main activation C-type lectin-like receptors, showing just 20-34% homology with other NKG2 (A/B, C, E) chains (Diefenback *et al.*, 2000). NKG2D is a homodimeric structure expressed by NK, CD8<sup>+</sup> T cell, and T $\gamma$  $\delta$  cells (Bauer *et al.*, 1999) that is bound to DAP-10 adaptor molecule that triggers tumor cell lysis (Wu *et al.*, 1999). In contrast with CD94/NKG2 that recognizes HLA-E antigens, NKG2D interacts with MIC A and MIC B that are class I-homologous structures (Menier *et al.*, 2002). MIC A and MIC B show the domains  $\alpha$ 1,  $\alpha$ 2 and  $\alpha$ 3 of class I MHC molecules but fail to express both  $\beta$ 2-microglobulin and peptides bound to the a chain (Wu *et al.*, 1999). MIC A and MIC B are scarce on normal cells but are presented in high density by epithelial tumor cells and, therefore, are important targets for NK cytotoxic function (Groh *et al.*, 1999; Natarajan *et al.*, 2002).

Since class I MHC antigens are able to be bound by both inhibitory and activator ligands of NK cells, the lytic activity of these cells appears to be the result of a fine balance of positive and negative signals triggered by such receptors. After the interaction between effector and target cells and if activator signals are more prevalent than the inhibitory ones, immunological synapses are formed between the cell surfaces and NK cell release the contents of cytoplasmatic granules. These granules are specialized lysosomes found only in NK and CTLs (Griffiths & Isaaz, 1993) and are full of enzymes and other compounds that have roles in the killing of target cells (Lieberman, 2003; Trambas & Griffiths, 2003).

Similarly to CTLs, in the early phase of cell:cell interaction, perforin monomers are released by the granules, which polymerize on target cell membrane forming transmembrane pores in the cell surface. Perforin shows strong homology with complement C 9 molecule (Shinkai *et al.* 1988) as well as the resulting pores (Sauer *et al.*, 1991), allowing

the loss of cell membrane integrity, influx of water, and cell death by osmotic lysis. During the degranulation of NK cells, they also release a series of many other compounds such as granzymes, calreticulin, cathepsins and granulisins in the immunological synapses. These products penetrate the target cell through the perforin pores triggering its apoptosis by several mechanisms, according to the compound involved.

Granzymes (a name derived from granule enzymes) are the second most important of granular contents and they constitute a family of highly specific serine-proteases that still inactive in acidic medium of the granules (Lieberman, 2003) being activated after release in the target cell cytoplasm. Granzyme A and B are the more abundant compounds of granules and appear to be the main elements involved in the target destruction. Granzyme B has a large spectrum of substrate and can induce apoptosis of target cells by activation of the caspases cascade (Wolf *et al.*, 1999; Sharif-Askari *et al.*, 2001), lesion of mitochondria and cleavage of BID to cytochrome C (Heibein *et al.*, 2000; Alimonti *et al.*, 2001), DNA fragmentation due to derepression of CAD (Thomas *et al.*, 2000) or even by cleavage of nuclear membrane (Browne *et al.*, 2000; Zhang *et al.*, 2001).

Granzyme A induces cell death through a caspase-independent mechanism, promoting lesions on single-strand DNA and rapid loss of integrity of the plasmatic membrane (Irmeler *et al.*, 1995; Suidan *et al.*, 1996). This enzyme can also destroy the nuclear membrane by acting on laminin and can destroy DNA by acting on histone (Lieberman & Fany, 2003). Other members of the granzyme family include granzymes C, D, E, F, G, H, K, and M; all of them are involved in cell destruction (Lieberman, 2003; Trambas & Griffiths, 2003).

Calreticulin and cathepsins protect the effector cells from self-destruction. Calreticulin inhibits perforin and therefore is able to inactivate perforin polymerization on its own membrane, whereas the cathepsins family has both granzyme-activating and -inactivating molecules, preventing self-destruction (Lieberman, 2003; Trambas & Griffiths, 2003). Granulisins released by NK cells appear to function especially against microorganisms whose cell walls are susceptible to this molecule (Lieberman, 2003); but this activity against cancer target cells remains unclear. The close interaction of target and effector cell membranes can vary from a few minutes up to hours and is called lethal hit, since even after the cell detachment the signals for programmed cell death are still in action (Lieberman, 2003; Trambas & Griffiths, 2003).

Other NK-induced cell-destruction mechanisms include the expression of FasL (Berke, 1997; Screpanti *et al.*, 2001), since many kinds of tumor cells frequently express higher density of Fas than normal cells. Binding of FasL to Fas molecules on tumor cells induces apoptosis signaling in these target cells by DNase activation. TNF-related apoptosis-inducing ligand (TRAIL) is also able to induce cell death in a similar way (Kayagaki *et al.*, 1999). Although apoptosis-induction is accepted as the main mechanism for destruction of target cells, perforin appears to be essential to the effector function of any cytolytic cell, since animals knocked out for perforin are not able to destroy target cells even if the other granule compounds are normal (van den Broek *et al.*,

#### 4. Role of NK Cells in Antitumor Resistance

In the middle of the 1950's, Burnet & Thomas forged the concept of immunosurveillance, establishing that any normal host is able to recognize and eliminate rising tumor cells even if it had not been immunologically manipulated (Burnet, 1970). Since then many immunologists have asked if the immune system can, in fact, recognize naturally rising tumor cells to prevent their development. Although some authors disagree on this concept, recent advances have added fuel to this discussion, giving new evidence of an effective antitumor surveillance. In this way, studies with experimental model of cancer have shown that animals depleted of some specific populations of cells (T lymphocytes, NK or NKT, for instance) as well as specific molecules such as INF- $\gamma$ , TNF- $T\alpha$ , IL-12, perforin and others, unequivocally show higher incidence of a variety of tumors. Conversely, the administration of some cytokines or induction of their *in vivo* production reduces the incidence of primary malignancies.

The antitumor role of NK cells was originally described due to their ability to lyse cells of some tumor lineages maintained in culture such as Yac-1 (Moloney virus-induced murine lymphoma) and K-562 (human chronic myeloid lymphoma) (Ortaldo *et al.*, 1977). The more relevant question was whether these cells were able to effectively play an *in vivo* role in antitumor activity, which had been partially answered through experimental models showing that naturally NK-lacking or artificially NK-depleted mice are less efficient than normal animals in eliminating transplantable tumor cells and show difficulty in preventing metastasis (Harning *et al.*, 1989; Arisawa *et al.*, 1990; Shibuya *et al.*, 1990; Chiodoni *et al.*, 2001).

In this aspect, Arizawa *et al.*, (1990) observed that nude BALB/c mice show higher resistance to metastasis of colon carcinoma cells (C26) than heterozygous counterparts. In their experiment, the authors observed that 2 days after the inoculation of tumor cells all the animals of heterozygous control group (BALB/c *nu*/+) had developed hepatic metastasis whereas any BALB/c *nu/nu* presented metastasis. Moreover, they observed that 66% of nude animals treated with anti-asialo GM-1 became susceptible to hepatic development of tumor cells. These data were corroborated by Chiodoni *et al.* (2001) who worked with the same experimental model and supported *in vivo* the early suggestion of Herberman *et al.*, (1975) that the higher NK activity of nude mice is associated with antitumor resistance.

Harning *et al.*, (1989) have demonstrated that i.v. inoculation of melanoma B16F10 in C57BL/6J beige mice that show low NK activity results in the appearance of a great number of pulmonary metastases, whereas normal mice are resistant to the phenomenon. However, this resistance is broken by treatment of normal animals with anti-NK monoclonal antibody (PK 136). Using the same model, Shibuya *et al.*, (1990) observed that administration of batroxobin, an analogue of trombin that induces metastasis of these cells, was able to significantly inhibit the occurrence of pulmonary metastasis.



They also observed that stimulation of NK activity with poly I:C resulted in a more intense level of inhibition of metastasis, whereas depletion of NK cells with anti-asialo GM-1 blocked the antimetastatic treatment.

Although experimental data have shown that NK activity can be important to inhibiting the occurrence of colon cancer metastasis, their efficiency in the immunosurveillance of this kind of cancer in humans cannot be very easily demonstrable. Aparicio-Pages *et al.*, (1989) observed that NK cells from patients with colorectal carcinoma show the same level of lytic activity as normal donors. However, cells obtained from the tumor mass show reduced lytic activity when compared with NK cells from peripheral blood or from intestinal mucous lymphoid tissue of the same patient. These observations suggest a local suppressive state, due to factors produced or induced by the tumor cells themselves.

Analyzing the melanoma infiltrating cell subsets, Simony *et al.*, (1991) observed numerous CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes as well as NK cells in all evaluated samples (Golub *et al.*, 1982a, 1982b; Hersey *et al.*, 1982) suggesting the role of these cells in the antitumor responsiveness. Recent clinical studies by Ruggeri *et al.*, (1999, 2002) and Giebel *et al.*, (2003), have demonstrated that NK cells play an important antileukemia role in patients with acute myelogenous leukemia submitted to bone marrow transplantation from haploidentical donor. They observed that in some cases there is incompatibility between donor and recipient KIRs, that is, when recipient does not express inhibitory ligands for KIR molecules of donor NK cells, these effector cells show *in vivo* expansion in the host. Since they are not inhibited by leukemia cells they are able to substantially reduce the risks of tumor recidivism.

Although colorectal (Horny & Horst 1987; Adachi *et al.*, 1990), breast (An *et al.*, 1987; Balch *et al.*, 1990) and lung cancer tissues (Ishigami *et al.*, 2000; Villegas *et al.*, 2002) show a low number of infiltrating NK cells, their presence appears to have a prognostic value for patients with squamous cell lung carcinoma (Villegas *et al.*, 2002), gastric carcinoma (Ishigami *et al.*, 2000) and colorectal carcinoma (Coca *et al.*, 1997). Tissues obtained from metastasis also show reduced frequency or even absence of NK cells, whereas the patients submitted to treatment with cytokines show a marked increase in these effector cells (CD56<sup>+</sup>/CD3<sup>-</sup>), in the adjacent or infiltrating tumor. (Vujanovic *et al.*, 1996).

Considering that the action of NK cells results from the balance between the stimulatory and inhibitory signals triggered by their interaction with class I MHC molecules, it should be remembered that, similarly to other regulatory systems, inhibitory signals are more potent than the stimulatory ones. Because of this phenomenon, in some situations the NK cells need to receive additional stimulation by cytokines, whose *in vivo* production can determine quantitative increase in these cells and/or enhanced lytic activity, which can be fundamental to their antitumoral role. On the other hand, NK cells by themselves constitute an important source of a variety of cytokines, whose production can influence the systemic behavior of the immune system.

## 5. Action of Cytokines on the Antitumor Activity of NK Cells

Many studies have been developed to evaluate the interaction between cytokines and NK cells both through *in vitro* and *in vivo* models. One of the main early findings in this area was that IL-2 stimulates the MHC-independent cytotoxicity. Henney *et al.* (1981) have observed that NK cells show variable toxicity against several target cells and that previous incubation with IL-2 or type I interferon resulted in potent stimulation of lytic activity. These cells were called lymphokine-activated killer cells (LAK) by Grimm *et al.* (1982) and it was further observed that this function is fulfilled essentially by NK cells (Phillips & Lanier, 1986). It was also largely demonstrated that IL-2 triggers antitumor activity in both experimental and clinical studies (Sacchi *et al.*, 1991; Hayakawa *et al.*, 1994; Ueda *et al.*, 1999).

From the 80s through the middle 90s several clinical trials were conducted to evaluate the efficiency of antitumor immunotherapy with LAK cells. Arienti *et al.*, (1993) have observed that passive transference of IL-2-activated tumor infiltrating lymphocytes (TIL) to melanoma patients resulted in complete or partial cure of 33% of them. Continuous i.v. or intrasplenic infusion of high doses of IL-2 and further transference of LAK in 9 patients with hepatic metastasis also resulted in complete or partial response in 1/3 of them, who showed longer survival time (26-36 months) (Keilholz *et al.*, 1992). This group also observed that the treatment of patients with hepatic metastasis of melanoma by intraportal injection of IL-2 and LAK cells showed better results than systemic treatment, indicating that the cells found *in situ* present a more differentiated behavior than do systemic cells. Patients with cerebral tumors receiving IL-2/LAK therapy also have shown total or partial regression of tumor in 30% of cases, as demonstrated by tomography and clinical signals (Ibayashi *et al.*, 1993).

Although IL-2/LAK therapy has shown good results in some patients with a variety of tumors, this proportion was considered low and many of the patients showed several important adverse effects such as pulmonary dysfunction (Villani *et al.*, 1993), thrombocytopenia by destruction of progenitor megacariocytes (Guarini *et al.*, 1991), alterations of clotting processes (Richard *et al.*, 1991), ischemia of the colon and severe diarrhea (Sparano *et al.*, 1991), besides minor symptoms such as cutaneous alterations (Gaspari, 1991). Considering these findings, IL-2/LAK-based immunotherapy proposed by Rosenberg's group (Grimm *et al.*, 1982; Barba *et al.*, 1989; Rosenberg *et al.*, 1989) was not considered a secure therapeutic method, due to the difficulty in achieving an efficient dose of IL-2/LAK with minimum adverse effects. Unfortunately, clinical trials for new therapeutic methods are usually tested in patients in an advanced stage of the disease who in general are resistant to conventional therapy. This fact makes it difficult to obtain conclusive data on the efficiency of experimental methods to establish secure and efficient doses for therapy.

Interleukin 15 (IL-15) is a cytokine whose structure shows some homology with IL-2 and has also been studied for its antitumor effect. This cytokine is a crucial

factor for the development of NK cells in the bone marrow (Mrozek *et al.*, 1996; Fehniger & Caligiuri, 2001) both in mice and humans, being produced by bone marrow stroma (Grabstein *et al.*, 1994; Mrozek *et al.*, 1996). IL-15 binds to a trimer receptor (IL-15R) expressed by 99% of NK cells, whose specific  $\alpha$  chain is associated with the same  $\beta$  and  $\gamma$  chains shared by IL-2R (Dunne *et al.*, 2001). *In vitro*, IL-15 drives the differentiation of progenitor cells towards NK (Mrozek *et al.*, 1996) and *in vivo* it is required for development and homing of NK, NKT, CD8<sup>+</sup> T, T $\gamma\delta$  and intestinal intraepithelial lymphocytes (IELs), as demonstrated through experimental studies with knockout mice (Willerford *et al.*, 1995; Ohteki *et al.*, 1997; Suzuki *et al.*, 1997). In addition, IL-15 induces proliferation and supports the *in vitro* survival and effector functions of NK cells (Carson *et al.*, 1994, 1995) more intensively than IL-2 (Dunne *et al.*, 2001). Similarly to IL-2, IL-15 is able to induce IFN- $\gamma$  production, enabling the possibility of its use in antitumor immunotherapy.

Besides IL-2, IL-12 is the main subject of study on antitumor effect, due to its potent NK stimulatory property (Kobayashi *et al.*, 1989; Ramani & Balkwill, 1989; D'Andrea *et al.*, 1992; Brunda *et al.*, 1993). Lieberman *et al.*, (1991) observed that IL-12 increases the NK and ADCC activity of human peripheral blood leukocytes against cells of human colon carcinoma. This cytokine acts on T lymphocytes and NK cells, enhances their generation and cytotoxicity and, in addition, induces the production of IFN- $\gamma$ , for activation of TH1 branch and consequently of CD8<sup>+</sup> cytolytic T cells. Using the murine melanoma B16 model, Kodama *et al.*, (1999) observed that IL-12 depends essentially on the participation of NK cells that kill the tumor cells in a perforin-dependent way. Martinotti *et al.*, (1995) have observed that tumor mass of IL-12 treated C26-bearing mice are infiltrated by NK and CD8<sup>+</sup> cells. Curiously the authors noted that depletion of CD4<sup>+</sup> cells with monoclonal antibody increases the NK and CTL infiltration, rather than depressing the antitumor response. Although they could not explain it at that time (1995), the current concepts on T reg cells suggest that this effect was due to elimination of these CD4<sup>+</sup>/CD25<sup>+</sup> regulatory cells.

Studies with experimental Burkitt's lymphoma show NK cells in the vicinity of infiltrating fine vessels (Yao *et al.*, 1999). Under treatment with IL-12 these cells became cytolytic for endothelial cells, probably due to release of IFN- $\gamma$  and TNF- $\alpha$ , indicating that NK cells and IL-12 can prevent neovascularization. The antiangiogenic action of IL-12 however, is not dependent on NK cells, since this cytokine inhibits angiogenesis of both murine and human tumors in NK-depleted SCID mice (Duda *et al.*, 2000).

Several clinical studies have also investigated the importance of IL-12 in the antitumor response: For example Robertson *et al.*, (1999) observed that i.v. administration of this cytokine in cancer patients induces increased NK activity and lymphoproliferative responsiveness of T cells. However, it had not been observed in many kinds of clinical cancer and the employment of this cytokine as antitumor therapy did not achieve the expectation suggested by experimental studies.

TNF- $\alpha$  and type I interferons (IFN  $\alpha/\beta$ ) inhibit the hepatic metastasis of colon carcinoma C 26 in mice and this effect is attributed to the capacity of these cytokines

to enhance NK activity, both in isolated form and synergistically (Sanada *et al.*, 1990). Some authors observed that human rINF- $\alpha$  inhibit lung metastasis from original carcinoma and also attributed the effect to NK activation (Ramani *et al.*, 1989).

Interleukin 23 (IL-23) is another cytokine that shows a significant effect on NK cells, discovered by Oppmann *et al.*, in 2000. This cytokine is a heterodimer formed by the subunits IL-12 p40 and a specific p19, the structure of which presents some similarity with IL-6, G-CSF and less homology with IL-12 p35 (Parham *et al.*, 2002). According to Parham *et al.*, (2002), IL-23 acts preferentially on memory T cells inducing their proliferation and production of IFN- $\gamma$  and in this aspect is different from IL-12 that acts mainly on naïve T cells. Lo *et al.*, (2003) evaluated the antitumor effect of IL-23 by using C26 carcinoma cells transfected with IL-23 genes and observed that the *in situ* production of this cytokine reduces the tumorigenesis and metastasis. However, antimetastatic effect of IL-23 was observed to be less efficient than that of IL-12 perhaps because IL-12 is more efficient in triggering specific cellular response to tumor antigens.

IL-21 produced by CD4 T cells also show homology with IL-2, IL-4 and IL-15 (Parrish-Novak *et al.*, 2000) and promote *in vitro* NK expansion and differentiation from bone marrow progenitor cells (Parrish-Novak *et al.*, 2000, 2002). Inoculation of plasmidial DNA with IL-21 genes induces constant production of this cytokine and supports its high serum levels that are associated with inhibition of growth of B16 melanoma and MCA-205 fibrosarcoma. *In vivo* depletion of CD4<sup>+</sup> or CD8<sup>+</sup> cells do not affect the antitumor effect of IL-21 but depletion of NK completely abolishes the resistance against tumor growth, indicating the importance of these cells in the fight against melanoma.

Although NK cells can be insufficient to avoid tumor growth by themselves, even under cytokine activation, it must be considered that after destruction of some target cells, apoptotic bodies are generated, which can be endocytized by dendritic cells of macrophages with subsequent presentation of tumor antigens for T lymphocytes. In addition, early production of IFN- $\gamma$  by NK cells could activate macrophages and dendritic cells, which in turn, can stimulate NK to play both the effector and regulatory roles in the antitumor response.

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