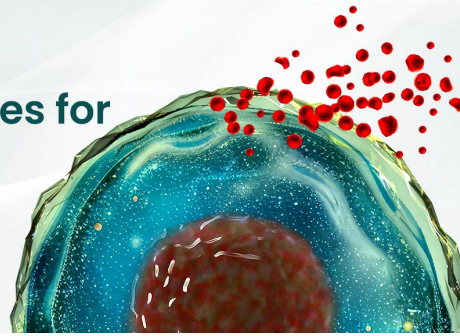




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BRIEF REVIEWS

NK Cells and Cancer¹

Loris Zamai,^{2*†§} Cristina Ponti,[¶] Prisco Mirandola,^{||} Giuliana Gobbi,^{||} Stefano Papa,^{‡#} Laura Galeotti,^{*†} Lucio Cocco,[#] and Marco Vitale^{2||}

In this review, we overview the main features and functions of NK cells, focusing on their role in cell-mediated immune response to tumor cells. In parallel, we discuss the information available in the field of NK cell receptors and offer a wide general overview of functional aspects of cell targeting and killing, focusing on the recent acknowledgments on the efficacy of NK cells after cytokine and mAb administration in cancer therapy. Since efficacy of NK cell-based immunotherapy has been proven in KIR-mismatch regimens or in TRAIL-dependent apoptosis, the ability to manipulate the balance of activating and inhibitory receptors on NK cells and of their cognate ligands, as well as the sensitivity of tumor cells to apoptosis, opens new perspectives for NK cell-based immunotherapy. The Journal of Immunology, 2007, 178: 4011–4016.

Natural killer cells have been initially identified as a lymphoid population representing the 10–20% of PBMC, able to lyse MHC class I (MHC-I)³-negative tumor and virus-infected cells and to orchestrate innate immunity of the organism. The majority of NK cells is localized in peripheral blood, lymph nodes, spleen, and bone marrow (1) but can be induced to migrate toward inflammation sites by different chemoattractants (2). NK cells constitutively express a lytic machinery able to kill target cells independently from any previous activation. These functional features have suggested since the time of their identification a role for NK cells in the control of tumor growth and metastasis diffusion in vivo.

For a long time, NK cells have been considered as a homogeneous subset of PBL. However, the intensity of CD56 expression (dim or bright) and the presence or absence of the CD16 Ag suggested functional differences among these subsets in terms of levels of cytotoxicity and cytokine production (3). Human CD56^{bright} NK cells have been recently described as the “cytokine responsive” NK cell subset that does not require “li-

censing” by host MHC-I molecules (4). A likely equivalent of the CD56^{bright} subset has been shown in the mouse thymus (5), where the NK cell development is IL-7 dependent and characterized by the expression of GATA-3 and CD127 (5). However, although the development of NK cells has been widely studied, it is still unclear at which stage of differentiation the two subsets of NK cells separate from one another (reviewed in Refs. 6 and 7).

NK cells express surface receptors (NK cell receptors (NKR)) that can be classified as inhibitory and activating (8, 9). There are several inhibitory receptors with different molecular structures and specificities for different alleles of class I molecules, the two main groups being the killer Ig-like receptors (KIR) (8), which bind HLA-class I, and the heterodimeric receptors CD94-NKG2A/B, which recognize HLA-E (10). The lack of even a single MHC-I allele, a frequent event in cancer cells, sensitizes them to NK cell cytotoxicity (8, 9). For the same reason, NK cells kill host lymphohemopoietic cells that, expressing different HLA-I molecules, mismatch NK inhibitory receptors (KIR-mismatch) (11). In the absence of inhibitory signals, however, NK cell cytotoxicity must be activated by a set of triggering receptors. Spontaneous cytotoxic activity is mainly triggered by NKG2D, leukocyte adhesion molecule DNAX accessory molecule 1 (DNAM-1) (CD226), and natural cytotoxicity receptors, whereas CD16, by binding the Fc portion of IgG, binds to opsonized cells mediating Ab-dependent cellular cytotoxicity (ADCC) (8, 9, 12). NKG2D and DNAM-1 recognize stress-induced ligands expressed by several tumor cell lines, such as MHC-I polypeptide-related sequence A, MHC-I polypeptide-related sequence B, and UL16-binding protein (NKG2D ligands), and poliovirus receptor (CD155) and Nectin-2 (CD112) (DNAM-1 ligands) (13–15). Natural cytotoxicity receptors include now three molecules specific for unknown host ligands: NKp46, NKp30, and NKp44 (8, 9), which mediate cell lysis of many cancer cells. Additional surface molecules have been implicated in NK cell activation and tumor cell lysis; these include 2B4, NTB-A, NKp80 coreceptors, CD18/CD11 (β_2

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³ Abbreviations used in this paper: MHC-I, MHC class I; AICD, activation-induced cell death; ADCC, Ab-dependent cellular cytotoxicity; DC, dendritic cell; FasL, Fas ligand; Flt3-L, Flt3-ligand; KIR, killer Ig-like receptor; LAK, lymphokine-activated killer; MEL, melanoma; RCC, renal cell carcinoma; SCF, stem cell factor; DNAM-1, DNAX accessory molecule 1.

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integrins), CD2 adhesion molecules, and TLR (8, 9, 12, 16, 17). In particular, viral and bacterial products can trigger NK cell responses directly binding to surface TLR3 and TLR9 (16, 17). More in general, NK cells can be activated by various stimuli such as contact with dendritic cells (DC), MHC-I-negative cells, binding of IgG immunocomplexes, direct engagement of NKR by stress-induced tumor-associated molecules or pathogen-derived products, and several cytokines such as IL-1, IL-2, IL-12, IL-15, IL-18, IL-21, and type I IFNs (8–25). Upon cytokine stimulation, NK cells become lymphokine-activated killer (LAK) cells that proliferate, produce cytokines, and up-regulate effector molecules such as adhesion molecules, Nkp44, perforin, granzymes, Fas ligand (FasL), and TRAIL (8, 9, 12, 25–29). Thanks to this new pattern of protein expression, LAK cells enhance their ability to adhere to and recognize target cells, leading to a broader killing activity against tumor cells that essentially takes place as (30): 1) perforin/granzyme-dependent necrosis of target cells, involving cell adhesion, NKR triggering, and granule release (31, 32); and 2) apoptosis of target cells, which involves cell adhesion and is mediated by surface TNF ligand family members a) FasL, b) TNF- α , and c) TRAIL, each of which interacts with specific receptors on the target cell surface (26–29). For this reason, it is likely that TNFR-mediated apoptosis is dependent on adhesion molecules but NKR independent. It has in fact been shown in mice that TRAIL- and FasL-mediated apoptosis is NKG2D independent (33).

Given the ability of TRAIL to kill many cancer cell types, while sparing normal tissues, the use of recombinant TRAIL has been proposed in clinical trials (34). Increased incidence of primary tumors and metastases has been described in TRAIL-deficient mice (35). However, TRAIL is present in the bone marrow, a site of NK cell as well as erythromyeloid differentiation (29, 36). Since it has been demonstrated that erythroid cell differentiation is negatively regulated *in vitro* and *in vivo* by recombinant TRAIL (36–38), its use in therapy should be cautious. Activated NK cells themselves express different death receptors, such as TRAIL-R2 and CD95, that are generally seen as implicated in the termination of NK cell response and in tumor responses to specific immune activities (immune counterattacks) (28). However, differently from erythroid cells, NK cells are usually protected from TRAIL-induced apoptosis thanks to cytokine-dependent c-FLIP induction (28). Interestingly, the immature CD161⁺/CD56⁻ NK cell subset expresses functional, membrane-bound TRAIL.

Among activatory cytokines, IL-15 is believed to be responsible for NK cell development *in vivo* and acts as a survival factor protecting lymphocytes from IL-2-activation-induced cell death (AICD) (39–41). Recent evidence suggests a nonredundant unique role for IL-15 in the differentiation, proliferation, survival, and activation of NK cells (6, 7, 39–41). This cytokine synergizes with Flt3-ligand (Flt3-L) (and stem cell factor (SCF)) in inducing the human CD56^{bright} NK cell subset (6, 7). Indeed, the mouse counterpart of this subset develops in the thymus under the control of IL-7 (5), another NK activatory cytokine potentially useful to expand and activate NK cells. In humans, IL-7 would be an early-acting cytokine responsible for the generation of a pool of immature (stage 3 NK cells) CD56^{bright} NK cells that, when necessary, promptly respond to the differentiating/activating action of IL-15 (Ref. 6 and L. Zamaï and S. Papa, unpublished data).

Sharing receptor (R) signaling components with IL-15, IL-2 is a NK activatory cytokine that was used *in vivo* against tumors (42). IL-2 acts as growth factor for NK cell progenitors and mature NK cells, and induces the production of NK effector molecules, enhancing NK lytic activity. IL-12 and IL-18, NK activatory cytokines active during late NK cell differentiation, have been demonstrated to synergistically enhance cytotoxicity against tumor targets and IFN- γ production by NK cells (43–45). IFN- γ induces type I immune response and directly acts on cancer cells. Finally, IL-21, another cytokine binding the common γ -chain (shared with IL-2, IL-4, IL-7, IL-9, and IL-15), has been demonstrated to favor the onset of the most cytotoxic CD56^{dim}CD16⁺ NK cell subset and to enhance its cytotoxicity (18, 19).

NK cell-based immunotherapeutic strategies against cancer

Early 1980s clinical trials started introducing IL-2-activated NK cells in the treatment of heavily tumor-burdened patients with solid primary or metastasized cancers (42). Subcutaneous injections of NK-stimulating doses of IL-2 or administration of preactivated NK cells (adoptive transfer of LAK cells) showed a 15–30% positive effect in patients with advanced renal cell carcinoma (RCC) or melanoma (MEL) (42). However, both MEL and RCC show a variable susceptibility to apoptosis induced by TNF ligand members (46–48), and the clearance of murine renal cancer cells *in vivo* often does not even require the perforin-mediated pathway (49). Thus, antitumor response following IL-2 treatment also involves the NKR-independent pathways of NK (and T) cell cytotoxicity. Unfortunately, IL-2 treatment is associated with life-threatening toxicity, essentially represented by capillary leak syndrome (50). Another limitation of this approach is the fact that IL-2, but not IL-15, activated NK cells increase their sensitivity to apoptosis when in contact with vascular endothelium (41), likely causing a decrease in NK cell migration toward the cancer area. IL-15 appears as more efficient than IL-2 in expanding the NK cell compartment because it promotes the survival of NK cells and protects NK cells from AICD (40, 41). Unfortunately, extremely high doses of IL-15 are necessary to observe meaningful antitumor effects *in vivo*; thus recently, strategies favoring IL-15 *trans*-presentation to NK cells have been proposed (51). Alternatively, early-acting cytokines such as Flt3-L, SCF, and IL-7 can be used to enhance NK cell numbers. Flt3-L induces an expansion not only of mature nonactivated NK cells but also of DC (52, 53).

This numerical increase of DC and NK cells provides a possible explanation for the antitumor effect of *in vivo*-administered Flt3-L in some murine models (54). Despite these promising data, however, Flt3-L administration in cancer patients did not produce any desirable long-lasting effect.

Differently from IL-2 and IL-15, IL-12 mainly enhances NK cell-mediated IFN- γ production, and IL-1 and IL-18 potentiate the effect of IL-12 by up-regulating the IL-12Rs expression on NK cells (16, 20–22, 25). The antitumor effects of IL-12 and IL-18 are essentially associated to IFN- γ production (16, 20–22, 25). IFN- γ has been shown to suppress tumor angiogenesis and to induce TRAIL- and FasL-mediated cellular susceptibility to apoptosis in a variety of tumor cells (34, 55). Only mature NK cells can produce IFN- γ , whereas immature NK

cells produce type 2 cytokines (56). Interestingly, IFN- γ induces type I immunity and counteracts the tumor escape mechanisms actively promoted by cancer cells and regulatory T lymphocytes through the secretion of type 2 cytokines (such as TGF- β and IL-10) (30, 57, 58). The induction of type I immune responses, as well as the terminal differentiation of NK cells, therefore appear relevant to an effective antitumor activity. To this regard, IL-21, a promising cytokine able to build up NK cell antitumor activity (59), has been found to promote both the expression of genes associated with type I response and the terminal differentiation of the highly cytotoxic CD56^{dim}/CD16⁺ NK cell subset, which can potentially direct ADCC against tumor cells via CD16-Fc ligation (18, 19, 60). NK cell-mediated ADCC response against tumor targets can be promoted by administration of mAbs to tumor-associated Ags (61, 62) (Fig. 1). Despite the lack of true specificity and the limited efficacy, this approach has a unique mechanism of action that does not produce cross-resistance or overlapping toxicities with conventional agents (62) and that can therefore be combined with cytokine-based immunotherapies. Indeed, several cytokines enhance NK cell response to mAb treatments (50, 63, 64). On the other hand, to reduce side effects of high-dose cytokine administration, recombinant fusion proteins to drive cytokines to tumor cells by tumor-specific Abs have been proposed (50) (Fig. 1). Alternatively, NK cells can be activated by the direct engagement of surface TLR3 and TLR9 (16, 17), and synthetic molecules able to mimic the immunostimulatory activity of viral and bacterial products via TLRs have been developed and tested against tumor cells in combination with cytokines (17).

Toxicity of systemic cytokine administration and cytokine-activated NK cell apoptosis are two important limitations of cytokine-mediated (and NK adoptive) immunotherapies for cancer treatment. In general, the sensitivity of NK cells to cell death seems to be related to the level of their activation since combinations of IL-2 or IL-15 with IL-12 or IL-18 synergistically increase NK cell apoptosis (65). Dose pulses of cytokine combinations with synergistic antitumor activities and incompletely overlapping toxicities have been suggested to reduce the negative effects of cytokine administration (toxicity and AICD) in the immunotherapy of cancer (50). In particular, a therapeutic strategy using low doses of IL-2 to expand NK cells, combined with high-dose pulses of IL-2 (or other activating cytokines) to activate expanded NK cells, has been pursued (50). Similarly, growth factors acting on early stages of NK cell differentiation (such as Flt3-L and SCF but also IL-7) in synergy with NK activatory cytokines have been proposed (50). As Flt3-L induces an increase in the number of both immature and mature NK

cells, treatment with Flt3-L (plus IL-7) followed by cytokines (or recombinant mAb-cytokine fusion protein) able to induce both differentiation of immature NK cells and activation of mature NK cells (like IL-21) and TLR agonists might provide a powerful strategy for NK cell-based therapies (Fig. 1).

Despite the huge amount of studies that demonstrate the ability of NK cells to kill tumor target cells *in vitro* and *in vivo* in animal models, the clinical efficacy of activated NK cells in cancer therapy has proven effective only in few cases. To this respect, it has to be considered that most NK cell-based immunotherapy trials have been performed in heavily tumor-burdened patients for which conventional therapies were ineffective. It is possible that, at least in some cases, the engagement of MHC-I inhibitory receptors, necessary to generate fully competent self-tolerant NK cells during NK cell development (5, 66), could be exploited by selected autologous tumor cells to inhibit LAK cell activity. Indeed, strategies that use NK cell donors mismatched for inhibitory NK receptors and MHC-I ligands, present in some allogeneic settings, have been more successful (67, 68). An important antitumor role for alloreactive NK cells has been shown in patients with acute myeloid leukemia either after stem cell transplantation or adoptive transfer of haploidentical NK cells (Refs. 11, 67, 68; for review, see Refs. 3, 62, 69). Donor NK cells attack host hemopoietic cells but not other tissues. Thus, allogeneic stem cell transplantation or adoptive transfer of polyclonal or clonal NK cells with mismatch NK inhibitory receptors and HLA class I ligands would produce graft-vs-leukemia (graft-vs-host) in the absence of graft-vs-host disease. The absence of NK mediated graft-vs-host disease in KIR-incompatible transplantation settings is not yet completely understood, but it is presumed that most nonhemopoietic tissues lack the ligands for NK activatory receptors able to activate NK cells. In this situation, the signals transduced by MHC-I inhibitory receptors become superfluous and likely exploited by some tumor cells to elude NK immunosurveillance. Activated endogenous NK cells would be ineffective against cancer *in vivo*, likely because NK activity is blocked by inhibitory receptors; indeed, inhibitory NKR have been suggested to represent an important mechanism of specific immunosuppression (70). As discussed above, TNFR-mediated apoptosis of sensitive tumor cells should be NKR independent, suggesting that this mechanism should, however, work upon NK cell activation, independently from the KIR/MHC-I setting (Fig. 1). To mimic the antitumor effect of KIR-mismatched NK cells, Abs to block NK inhibitory interactions with MHC-I on tumor cells have been tested *in vivo*. Mouse models of leukemia have

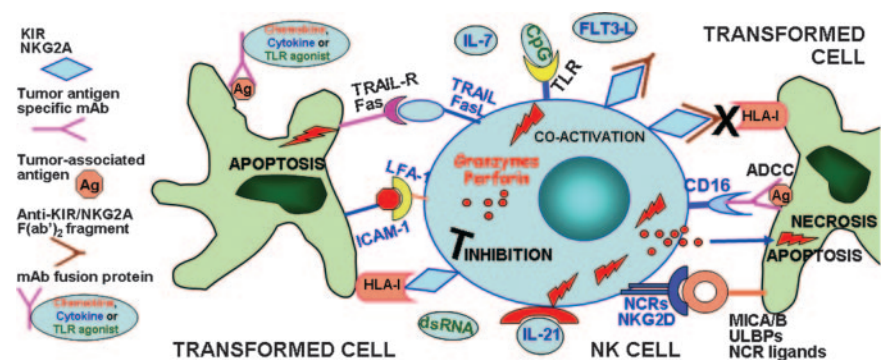


FIGURE 1. Effects of LAK cells against tumor cells. NKR-dependent and -independent mechanisms (for reference, see the text).

demonstrated efficacy of anti-KIR-blocking Abs without adverse effects on normal cells, indicating the feasibility of treatments with Ab fragments to prevent KIR/NG2A-MHC-I interactions in cancer therapy (71) (Fig. 1).

Whether alloreactive KIR-mismatched NK cells were able to mediate antineoplastic effects against solid tumor cells is currently under investigation. A recent report (72) demonstrates that KIR-incompatible allogeneic NK cells have superior antitumor effects against fresh tumor cells isolated from different solid cancers, as well as against MEL and RCC established tumor cell lines (73). Interactions between solid tumor cells and the microenvironment *in vivo* create a context that promotes tumor growth, selection, and protection from immune attack, suggesting that the tridimensional architecture of solid cancer lesions is likely one of the tumor mechanisms to escape immunosurveillance (70). To this regard, another important mechanism to control NK cell activity is their ability to traffic to tumor sites. Chemokines are key regulators of NK cell migration and are required to drive NK cells to tumor sites. NK cells express chemokine receptors on the cell surface and migrate vigorously in response to CXCL12 and CXCL3L1 (2). The possibility to induce NK cell migration and infiltration of solid tumors should be considered in NK cell-based immunotherapy of cancer, and mAb-chemokine fusion proteins could be developed to ensure that immune effector cells reach the tumor site (Fig. 1). Chemokines are also involved in the control of NK cell interaction with other cells of the immune system such as DC. The cross-talk between NK cells and DC has been studied intensely in these recent years (for review, see Refs. 16, 20–22). DC express two types of receptors: Ag uptake receptors for Ag presentation by MHC and TLR for pathogen-associated molecules, crucial for maturation/activation of DC (16). TLR ligands induce not only higher MHC molecule expression and secretion of cytokines and chemokines but also up-regulation of chemokine receptors and ligands for NK-activating receptor and coreceptors (in particular, ligands for NKp30, NKG2D, and DNAM-1) (16, 20–22). Interestingly, engagement of DNAM-1, as well as TLR and cytokine receptors, has been shown to increase the Ag-presenting ability of maturing DC (74), suggesting that these molecular interactions stimulate both DC maturation and NK cell activation. An intriguing aspect for new approaches in NK cell immunotherapy is based on the observation that DC can directly trigger NK cell-mediated antitumor immunity (75). DC can promote NK cell survival, activation, and differentiation through DC-NK direct cell contact and secretion of cytokines, such as IL-12, IL-1, IL-18, and IL-15. NK cell activation and regulatory T lymphocyte functional suppression is produced by DC in response to TLR-mediated DC maturation/activation, which likely represents the molecular basis of anticancer mechanisms induced by microbial components (16, 76, 77). In particular, the bacillus Calmette-Guerin cell wall skeleton (BCG CWS), the active component of the Freund adjuvant, has been shown to induce *in vivo* antitumor effects via DC TLR2 and TLR4 (76). Recently, a number of degradation products of endogenous macromolecules (in particular, heat shock proteins), produced following tissue injury and/or remodeling, have been reported to be ligands of TLR (76). Heat shock proteins have been shown to be potent activators of the innate immune system (76). Thus, microbial, synthetic, and endogenous TLR ligands can potentially act as immunoadjuvants for the treatment of cancer, and the NK cell

antitumor activity recorded after vaccination with microbial or tumor-derived molecules (76, 78) would be mediated in part by the stimulatory effects on DC, which would in turn trigger NK cells through the local production of cytokines and the interaction with costimulatory molecules.

Conclusions

A proliferation of studies are currently unraveling the intimate mechanisms of target cell recognition and selection on one side and the multifaceted lytic machinery of NK cells on the other. Although the results of NK-based immunotherapeutic treatment of cancer are promising in the experimental models, to date, their clinical efficacy in human trials has been modest, presumably due to tumor escape by alteration of NK cell function and resistance to killing associated with tumor progression and chronic inflammation. Essentially, clinical efficacy of NK cell immunotherapy has been proven in cases in which either the inhibitory receptor control has been eluded by KIR mismatching or in activated NK cells that mediate TRAIL-dependent/NKR-independent cytotoxicity. This suggests that, to increase antitumor activity *in vivo*, cytokine combinations need to be used in association with other approaches. The ability to manipulate not only the balance of activating and inhibitory receptors on NK cells but also their cognate ligands, as well as the sensitivity of tumor cells to apoptosis, opens new perspectives in NK cell-based immunotherapy.

Both conventional therapies and immunotherapy kill tumor cells inducing programmed cell death, thus selection of tumor cells resistant to apoptosis would be the reason of cross-resistance of cancer cells to chemotherapy and immunotherapy (79). Therefore, sensitization of tumor cells to activated cytotoxic lymphocytes by up-regulating either TNF death receptors or effector-activating ligands on tumor cells combined with immunotherapy have been pursued to overcome tumor cell resistance and establish an effective antitumor response (80–85). Alternatively, an innovative treatment to sensitize resistant tumor cells to cell-mediated cytotoxicity might include intermittent low-dose/high-dose pulses of cytokines or Ab-chemokine/cytokine fusion proteins to attract and activate effector cells, combined with mAbs that prevent KIR/NG2A-MHC-I interactions (e.g., anti-KIR- or anti-NG2A-blocking Abs) or that promote ADCC (antitumor-specific Ags) (Fig. 1).

For the future, a good control upon NK cell activity based on a deep knowledge of their basic physiology at the bench is probably one of the more promising tools for the management of human cancer in clinical applications.

Disclosures

The authors have no financial conflict of interest.

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