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SYMPOSIUM PAPER

NK cell-mediated targeting of human cancer and possibilities for new means of immunotherapy

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Abstract Insights into the molecular basis for natural killer (NK) cell recognition of human cancer have been obtained in recent years. Here, we review current knowledge on the molecular specificity and function of human NK cells. Evidence for NK cell targeting of human tumors is provided and new strategies for NK cell-based immunotherapy against human cancer are discussed. Based on current knowledge, we foresee a development where more cancers may be subject to treatment with drugs or other immunomodulatory agents affecting NK cells, either directly or indirectly. We also envisage a possibility that certain forms of cancers may be subject to treatment with adoptively transferred NK cells, either alone or in combination with other therapeutic interventions.

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Abbreviations

AICD	Activation induced cell death
ADCC	Antibody mediated cellular cytotoxicity
AML	Acute myeloid leukemia
ATG	Anti-thymocyte globulin
ATM	Ataxia telangiectasia, mutated
ATR	ATM- and Rad3-related
CR	Complete remission
DC	Dendritic cell
DLI	Donor lymphocyte infusion
EBV	Epstein-Barr virus
Fas L	Fas ligand
Flt-3 L	Flt-3 ligand
GMP	Good manufacturing practice
GVH	Graft-versus-host
GVT	Graft-versus-tumor
GVHD	Graft-versus-host-disease
HLA	Human leukocyte antigen
IFN	Interferon
KIR	Killer-Ig-like receptors
LAK	Lymphokine activated killer
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptor
NK	Natural killer
SCF	Stem cell factor
SCT	Stem cell transplantation
TNF	Tumor necrosis factor
Treg	Regulatory T
TRAIL	TNF-related apoptosis-inducing ligand
UL	Unique long

Introduction

Natural killer (NK) cells represent a unique subset of lymphocytes, distinct from T and B cells. In an integrative manner with other immune cells, they contribute to host anti-microbial and anti-tumor immunity. NK cells were originally identified on a functional basis, because of their ability to lyse certain tumor cells in vitro without the requirement for prior immune sensitization of the host. This initial observation gave them their name [58]. Today, these cells are well characterized with respect to their origin, differentiation, receptor repertoire, and effector functions [53, 65, 77, 83]. Here, we discuss current insights into NK cell-recognition of human cancer, and discuss several possible future therapeutic modalities involving NK cells, either directly or indirectly.

Phenotype, numbers and tissue location of human NK cells

Natural killer cells account for 5–15% of human peripheral blood lymphocytes. In addition to peripheral blood, they are found in the bone marrow, spleen and lymph nodes as well as in specific organs such as the liver and lungs. They are activated by cytokines and/or by interactions with specific molecules expressed on target cells [13, 65, 83]. This leads to induction of effector functions, including cytokine production and cellular cytotoxicity [77]. Upon activation, NK cells produce cytokines and chemokines and can exert strong cytolytic effector functions [78, 94]. Human NK cells are broadly defined as $CD3^{-}CD56^{+}$ lymphocytes. They can be further sub-divided into two main functional subsets based on their surface expression of CD56. $CD56^{bright}$ NK cells have potent immunoregulatory properties, and $CD56^{dim}$ NK cells have potent cytotoxic functions [26, 37]. These functions are; however, not strictly confined to each respective subset; a certain degree of functional overlap exists. The $CD56^{dim}$ NK cells express high levels of FcγRIIIA (CD16) allowing them to mediate ADCC. Although insights into NK cell development has been obtained, it is still not fully clear at which stage of differentiation $CD56^{bright}$ and $CD56^{dim}$ NK cells separate from each other [30, 36].

NK cells in host responses against tumors

Since their discovery, a large number of studies have demonstrated NK cell killing of many different types of murine and human tumor cell lines in vitro [117, 131]. Several experimental studies in mice have demonstrated a role for NK cells in the eradication of grafted murine tumor cell lines [2, 68]. Furthermore, NK cells have been shown to be involved in rejection responses against experimentally induced and spontaneously developing tumors in mice [119]. Human NK cells adoptively transferred to mice also participate in the rejection of grafted human tumors [47]. Direct evidence for NK cell targeting of human cancer has come from studies of NK cell interactions with primary tumor cells tested for susceptibility to NK cells lysis ex vivo. Such approaches have recently been taken for, e.g., neuroblastomas, ovarian carcinomas, and multiple myelomas [18, 21, 33]. Evidence for NK cell targeting of human tumors has also come from clinical studies in settings of SCT and adoptive transfer of NK cells to cancer patients [70, 76, 99, 101]. It should be noted, however, that despite the large amount of studies that demonstrate the ability of NK cells to target tumor cells in vitro and in vivo in experimental models [117, 131], there is still only limited evidence for clinical efficacy of activated NK cells administrated to patients with cancer.

All tumors are, however, not susceptible to NK cell-mediated lysis. A variety of reasons may underlie this resistance, including specific properties of some tumor cells themselves as well as effects imposed by certain tumors on NK cells. Data from both experimental models and from studies of human cancer have demonstrated tumor cell evasion from NK cells [50, 73, 74]. This includes the intriguing observations of, e.g., high MHC class I expression of some metastasizing human tumors and/or loss of ligands for NK cell activation receptors on other tumors [28, 55, 103]. This may argue for selection of tumor cell mutants or tumor cell modulation during an ongoing NK cell-mediated response. Down-regulation of NK cell-activating receptors can be mediated by, e.g., TGF-β, which selectively down-regulates the surface expression of some NK cell-activating receptors [20]. Likewise, NK cell interactions with tumor target cells may specifically down-regulate the expression of activating receptors, e.g., down-regulation of DNAM-1 upon recognition of PVR-expressing ovarian carcinomas ([33]; our own unpublished results). Finally, tumor cells may also restrain NK cell effector function by promoting the expansion of $CD4^{+}CD25^{+}$ Treg cells. Recent evidence points to a critical role for Treg cells in dampening NK cell immune responses by suppression of homeostatic proliferation, cytotoxicity, and IL-12-mediated IFN-γ production [43, 118].

Molecular interactions involved in NK cell targeting of tumors

The identification of NK cell germ-line-encoded activation and inhibitory receptors has in large uncovered the molecular mechanisms used by NK cells to recognize tumor cells (Table 1). This knowledge emerges from early observations that NK cell cytotoxicity is triggered by tumor cells lacking expression of certain (or all) self-MHC class I molecules [44, 52, 57, 68], a phenomenon

Table 1 Activation and inhibitory receptors expressed on freshly isolated human peripheral blood NK cells (Adopted from ref [13]. For some receptors, ligands are not known or not well characterized)

Receptor[a]	Type of receptor	Signaling motif	Ligand
CD16 (FcγRIIIA)	Activation	ITAM[b]	IgG
NKp30 (CD337)	Activation	ITAM	
NKp46 (CD335)	Activation	ITAM	Viral hemaglutinin
KIR2DS1-2	Activation	ITAM	HLA-C (low affinity)
KIR2DS3-6	Activation	ITAM	
KIR3DS1	Activation	ITAM	
NKG2C (CD94/159c)	Activation	ITAM	HLA-E
NKG2D (CD314)	Activation	Non-ITAM	ULBPs, MICA, MICB
2B4 (CD244)	Activation	Non-ITAM	CD48
CD2	Activation	Non-ITAM	LFA-3 (CD58)
CRACC (CD319)	Activation	Non-ITAM	CRACC (CD319)
NTB-A	Activation	Non-ITAM	NTB-A
DNAM-1 (CD226)	Activation	Non-ITAM	PVR (CD155), CD112
CD7	Activation	Non-ITAM	SECTM1, Galectin
CD59	Activation	Non-ITAM	C8, C9
BY55 (CD160)	Activation	Non-ITAM	HLA-C
KIR2DL4 (CD158d)	Activation	Non-ITAM	HLA-G (soluble)
CD44	Activation	Non-ITAM	Hyaluronan
LFA-1 (αLβ2, CD11α/18)	Activation	Integrin	ICAM-1-5
MAC-1 (αMβ2, CD11β/18)	Activation	Integrin	ICAM-1, iC3b, Fibrinogen
CD11c/18	Activation	Integrin	ICAM-1, iC3b
VLA-4 (α4β1, CD49d/29)	Activation	Integrin	VCAM-1, Fibronectin
VLA-5 (α5β1, CD49e/29)	Activation	Integrin	Fibronectin
KIR2DL1 (CD158a)	Inhibitory	ITIM[c]	HLA-C group 2
KIR2DL2/3	Inhibitory	ITIM	HLA-C group 1
KIR2DL5	Inhibitory	ITIM	
KIR3DL1	Inhibitory	ITIM	HLA-B alleles
KIR3DL2	Inhibitory	ITIM	HLA-A alleles
LIR-1/ILT2 (CD85j)	Inhibitory	ITIM	Multiple HLA class I alleles
NKG2A (CD94/CD159a)	Inhibitory	ITIM	HLA-E
KLRG1	Inhibitory	ITIM	E/N/P-cadherin
NKR-P1 (CD161)	Inhibitory	ITIM	LLT1
Siglec-7 (CD328)	Inhibitory	ITIM	sialic acid
Siglec-9 (CD329)	Inhibitory	ITIM	sialic acid
Irp60 (CD300a)	Inhibitory	ITIM	

[a] KIR, NKG2C, CD2, NKG2A, LIR-1, NKRG1, NKR-P1, Siglec-7, and Siglec-9 are only expressed by subsets of NK cells

[b] Immunoreceptor tyrosine-based activation motif

[c] Immunoreceptor tyrosine-based inhibition motif

referred to as "missing-self" recognition [69]. This observation led to the identification of NK cell inhibitory receptors that recognize MHC class I molecules [56, 79]. However, sensing the absence of self-MHC class I molecules is not sufficient to cause target cell killing by NK cells. NK cells also need stimulation by target cell-ligands to trigger activation via specific receptors. The identification of the latter remained elusive until rather recently [65, 83]. Thus, we now know that NK cell recognition of tumors is tightly regulated by processes involving the integration of signals delivered from multiple activating and inhibitory receptors [13, 65, 81, 83, 132].

NK cell activation receptors in tumor recognition

One important group of human NK cell activation receptors is the so-called NCRs: NKp46, NKp30, and NKp44 [90, 130]. Two of these, NKp46 and NKp30, are constitutively expressed on all peripheral blood NK cells, whereas NKp44 is induced and expressed by IL-2-activated NK cells and some T cells. Despite considerable effort to characterize their ligands, their constituents on tumor cells remain poorly defined [8, 92]. However, the nuclear factor HLA-B-associated transcript 3 (BAT3) was shown to be released from tumor cells and to bind NKp30. BAT-3 trig-

gered NKp30-mediated NK cell cytotoxicity and thus represents the first identified cellular ligand for any of the natural cytoxicity receptors [8, 92]. The NCRs have a major role in NK cell-mediated lysis of various human tumor cell lines including melanomas, carcinomas, neuroblastomas, and myeloid or lymphoblastic leukemias, as well as EBV-transformed B cells [8]. Other well-characterized activation receptors are NKG2D and DNAM-1 [4, 9]. NKG2D recognizes the stress inducible molecules MICA and MICB as well as the UL16-binding proteins. NKG2D ligands are expressed on a number of human epithelial tumor and leukemic cell lines and have a significant role in rendering these cells susceptible to NK cell-mediated lysis. DNAM-1 recognizes PVR (CD155) and Nectin-2 (CD122). These ligands are highly expressed in human carcinomas, melanomas, and neuroblastomas [24, 27, 80]. CD16 on NK cells binds the Fc-portion of IgG on opsonized cells, thus mediating ADCC. In addition, several other receptors, including 2B4 (CD244), NTBA, NKp80, CD2, CD11a/CD18, and CD59, have important co-activating or co-stimulatory functions in NK cell activation and tumor cell recognition [13].

NK cell inhibitory receptors in tumor recognition

Activation of NK cells is under control by inhibitory receptors [65, 81, 83]. Inhibitory receptors bind classical and/or non-classical MHC class I molecules. These molecules are normally expressed on most healthy cells in the body, but are often lost upon transformation or during tumor evolution [75]. In humans, KIR and CD94-NKG2A play major roles as HLA-class I-specific inhibitory NK cell receptors. KIR recognizes groups of HLA-A, -B, and -C alleles [82, 86, 124], whereas CD94-NKG2A/B receptors recognize HLA-E molecules [12]. Individuals differ in the number and type of inherited KIR genes, and specific KIR gene products are expressed on distinct subsets of NK cells. Thus, many NK cells express only a few of many possible types of receptors. Most NK cells, however, express at least one inhibitory receptor that is specific for a self-MHC class I ligand. The pattern of KIRs expressed creates a system allowing NK cells to detect cells lacking expression of single MHC class I alleles [86]. As discussed below, this diversity is potentially beneficial in settings of SCT and adoptive NK cell-based immunotherapy against cancer.

Relative importance of different NK cell activation receptors in NK cell tumor targeting

The relative importance of different NK cell activation receptors and their ligands in recognizing primary human tumors is only partially known. Interestingly, efficient natural cytotoxicity by resting (e.g., not preactivated by cytokines) NK cells usually requires co-activation by several types of receptors [13, 14]. In contrast, engagement of LFA-1 is sufficient to induce cytotoxicity by IL-2 activated NK cells [14]. Translated to the context of killing tumor cells, these findings indicate that a tumor cell might elicit activation of an unstimulated NK cell by expressing array of ligands for several activating receptors, any one of which alone would be incapable of triggering a response. For an NK cell activated by, e.g., IL-2, fewer qualitatively distinct receptor–ligand interactions might suffice for tumor cell recognition and killing provided MHC class I molecules do not confer inhibition. In some situations, the activation signals may override the inhibitory signals mediated by MHC class I molecules, as has been demonstrated, e.g., for NKG2D-mediated triggering of some MHC class I expressing tumor cell lines [23, 31]. The final fate of the NK cells is also critically dependent—on not only—the type of ligands but also their relative density for the integration of signals from activating and inhibitory signals.

NK cell-effector functions involved in anti-tumor responses

Like cytotoxic T cells, NK cells possess different effector mechanisms by which they mount anti-tumor responses [127]. Many of them have been explored extensively in experimental models. NK cells use two major mechanisms to induce target cell apoptosis, the granule exocytosis and the death receptor pathway [114]. Granulae exocytosis involves the release of perforin and granzymes [121]. The death receptor pathway is largely mediated by members of the TNF superfamily, in which Fas L, TNF-α, and TRAIL are key apoptosis-inducing members [105, 114]. NK cells can also produce many different types of cytokines (e.g., IFN-γ, TNF-α, and GM-CSF) as well as chemokines, at least some of which have a direct effect on tumors. The best-studied cytokine in this respect is IFN-γ. IFN-γ can decrease tumor proliferation and metabolic activity and inhibit angiogenesis [116]. IFN-γ produced by NK cells might also play a role in the regulation of killing by death receptors, either by down-regulating anti-apoptotic proteins, or by up-regulating caspases that are essential for death receptor-mediated apoptosis. IFN-γ induces type-1 immunity and may by this means counteract tumor escape mechanisms actively promoted by cancer cells and Treg cells through the secretion of type-2 cytokines [118].

Activation of NK cells to enhance anti-tumor responses

Given the fact that NK cells in some situations can target human cancers, it may be of interest to activate endogenous NK cells to induce more potent anti-tumor responses. It may also advantageous to activate NK cells *ex vivo* prior

to adoptive immunotherapy. Several cytokines affect NK cell differentiation and activation such as IL-2, IL-12, IL-15, IL-18, IL-21, and type-1 IFNs [5, 25]. Upon cytokine stimulation, NK cells proliferate, produce cytokines, and upregulate effector molecules such as adhesion molecules, NKp44, perforin, granzymes, FasL, and TRAIL. IL-2 stimulates NK cell progenitors and mature NK cells, and induces the production of NK cell effector molecules, enhancing NK cytolytic activity. In addition, IL-2 augments NK cell degranulation via a syntaxin 11-independent pathway [15], and may reduce the dependency of co-activation for some receptors implicated in tumor cell lysis [14]. Recent evidence suggests a unique role for IL-15 in the differentiation, proliferation, survival, and activation of NK cells [84, 126]. IL-15 may also protect lymphocytes from IL-2 induced AICD [96, 126]. This cytokine synergizes with Flt3-L and SCF in inducing human $CD56^{bright}$ NK cells [30, 36]. IL-12 and IL-18 act late during the NK cell differentiation, and synergistically enhance cytotoxicity against tumor targets and IFN-γ production by NK cells [66]. IL-21 is of particular interest in its ability to stimulate cytotoxic $CD56^{dim}$ NK cells and to enhance NK cell cytotoxicity [10, 87].

Cytokines and other immunomodulatory drugs in cancer treatment

Cytokines have successfully been applied in the treatment of several human cancers and, in some instances, the mechanisms of action are through direct or indirect activation of NK cells [35, 115]. Several clinical trials have assessed the effects of IL-2 administration on activation of NK cells in patients with cancer [97, 115]. Unfortunately, high doses of IL-2 are associated with significant toxicity [38]. Irrespectively, IL-2 is frequently used in lower doses to promote NK cell activity in vivo. This cytokine has more recently also been used together with monoclonal antibodies that mediate ADCC to enhance NK cell activity [95]. In many respects, IL-15 may be a better cytokine. IL-15 is more efficient than IL-2 in expanding the NK cell compartment [46, 85, 125]. It promotes survival of NK cells and protects NK cells from AICD. However, high doses of IL-15 may be needed in vivo to obtain effective anti-tumor effects [60, 125]. New insight into the role of early hematopoetic growth factors, such as c-kit ligand and flt-3 ligand, and their synergy with IL-15 in the development of human NK cells in the bone marrow, will likely permit studies of additional cytokine combinations for expansion of NK cells for clinical use. Nevertheless, better knowledge about how best to use cytokines for optimal activation of NK cells, either alone or in combination with other immune interventions [115], is clearly needed. It still remains to be studied whether the impressive results obtained in mice, using individual and combinations of cytokines to activate NK cells to kill tumor cells, can be translated to humans. Apart from specific cytokines and/or growth factors, broad activators of immune function implicated in anti-tumor immunity, may also stimulate NK cells. For instance, in myeloma, NK cell activity has been shown to increase in response to thalidomide and its analogue lenalidomide, explaining, in part, the mechanism of action of this drug [51]. Immunostimulatory DNA complexes have also been shown to enhance in vivo anti-tumor activity, mediated, at least in part, through the activation of NK cells [39]. Imatinib mesylate (Gleevec), a specific inhibitor of tyrosine kinase receptors may also lead to host-anti tumor effects mediated by the innate immune system and a new type of immune cell, referred to as IFN-producing killer dendritic cells (IKDC) that resemble natural killer cells [7, 113, 123]. Finally, NK cells may also contribute to the clinical efficacy of bacillus Calmette-Guerin (BCG) treatment of bladder cancer [11].

NK cell-mediated anti-tumor responses in settings of allogeneic SCT

Allogeneic hematopoietic SCT is an established treatment strategy for several hematological malignancies. An intentional mismatch between donor KIRs and recipient HLA ligands in hematopoetic SCT is predicted to allow for a graft anti-tumor effect by NK cells that develop in the recipient. In pioneering studies by Velardi and collaborators, adult patients with AML undergoing haploidentical SCT showed greatly improved disease-free survival time and low relapse rates when a KIR-ligand mismatch prevailed [100, 101]. This effect occurred in the absence of donor T cells, which had been removed prior to the transplant. Notably, hematopoetic grafts with NK cell alloreactivity in the GvH direction also had increased rates of bone marrow engraftment and reduced rates of clinically significant GvHD. The latter effect may be caused by donor NK cell lysis of host antigen-presenting cells, impairing alloreactive donor T cell priming [101, 109]. Since the initial report by Velardi and collaborators [101], numerous studies have addressed the role of KIR-ligand mis-match in different settings of transplantation [34, 102]. Some, but not all, studies have demonstrated a beneficial role of NK cell alloreactivity. Discrepancies in outcome among the studies may depend on the transplantation protocol including differences such as the type of preconditioning, dose of stem cells, degree of T cell depletion, and post-transplantation immunosuppression. Further studies are warranted to better understand the conditions that steer NK cell maturation and receptor-acquisition following SCT, particularly relative to alloreactivity and GvT effects.

NK cell-mediated anti-tumor responses in settings of DLI following SCT

Unfortunately, many patients relapse after hematopoetic SCT. DLIs can induce a direct and potent GvT effect in some of these patients [63, 64, 112]. The major risk of DLI is GvHD, which may be a severe, even lethal complication. To minimize the risk of GvHD, modified strategies have been developed such as partially T cell-depleted DLI. In haploidentical SCT, studies have been initiated where purified donor NK cells have been used as DLIs with the aim to consolidate engraftment and induce GvL effects [88, 89]. In a related study, patients with solid tumors undergoing allogeneic SCT were infused with donor-derived *ex vivo* expanded NK cells (Dilber and collaborators, submitted). Although, no firm conclusions can be made regarding the clinical efficacy of NK cell-based DLI at this stage, available data indicate that NK cell infusions are safe and may generate anti-tumor responses. Since normal tissues do not generally express ligands for activating NK cell receptors, alloreactive NK cells should not normally cause GvHD [8, 45]. The development of NK cell-based DLI represents new possibilities for treating relapses in patients undergoing haploidentical or cord blood SCT.

The use of NK cells in pre-SCT conditioning

Experimental data have demonstrated that NK cells can be administered directly after conditioning but before SCT. The potential benefits are threefold. First, NK cell-mediated GvL effects could enhance anti-tumor activity and reduce risk for relapse. Second, NK cell-mediated depletion of recipient DC could prevent the development of acute GvHD and perhaps allow higher numbers of alloreactive T cells in the graft, thus avoiding death from infections in the early post-transplant period [72, 99, 101]. Finally, NK cells may facilitate engraftment and promote a fast immune reconstitution, thereby reducing the need for myeloablative regimens and shortening the neutropenic period.

Adoptive immunotherapy using short term ex vivo activated NK cells

Because of the lack of significant clinical effects with past protocols based on autologous “LAK” cells or NK cells [16, 67, 98], and because of the promising effects observed in haploidentical T cell-depleted SCT, focus shifted toward the potential of using allogeneic (potentially alloreactive) NK cells in adoptive cell therapy. In recent studies, Miller and collaborators infused haploidentical NK cells together with IL-2 to 43 non-transplanted patients with advanced cancer. Low and high intensity preparative regimens were tested. The high-dose lymphodepleting regimen resulted in long-term survival and expansion of donor-derived NK cells in vivo. The in vivo expansion was associated with increased levels of endogenous IL-15, possibly driving the proliferation of donor NK cells. Notably, one of these patients manifested a preferential expansion of the alloreactive NK cell subset. In general, these donor NK cell infusions were feasible and tolerated without unexpected toxicity. Moreover, 5/19 patients with AML achieved CR with this protocol. Only four of the 19 patients had donors with a predictable alloreactive NK cell repertoire. Interestingly, of these four patients, three achieved CR [76].

Adoptive immunotherapy using NK cell lines and ex vivo expanded NK cells

In parallel with the foregoing studies, adoptive transfers are also being done with the NK-92 cell line [120]. This cell line can be grown continuously under GMP-conditions, express many NK cell activation receptors and low levels of KIRs, and displays significant cytotoxicity toward many tumor targets. Following irradiation, more than 20 patients with advanced renal cell carcinoma and malignant melanomas have received NK-92 cells [59]. In general, these infusions are well tolerated and have yielded anti-tumor effects in some cases. Furthermore, this cell line is easily modified genetically which opens up interesting possibilities for future therapeutic trials. Whether adoptive immunotherapy with this cell line will produce more substantial clinical responses remains to be seen.

Several techniques have been developed for ex vivo expansion of NK cells [59]. A few of these protocols allow the expansion of NK cell-enriched cellular populations under GMP-conditions [17]. Using such protocols, it has been demonstrated that NK cells can be expanded ex vivo, also from tumor-bearing patients [3, 48]. The latter opens up for expansion of autologous NK cells for adoptive immunotherapy, as strategy that may be developed further despite the earlier disappointments with autologous LAK cell therapy in the 1980s to patients with advanced solid tumors [67, 98]. Expansion protocols provide greater numbers of NK cells to be used for adoptive therapy that might be desired in some situations. For such expansions to be effective, it is important that the expansion of NK cells ex vivo is not associated with phenotypic changes, linage deviation, and/or selective expansion of specific subsets, such that their anti-tumor function will be affected. Another aspect to consider, apart from consequences of activation and proliferation, is that in vitro manipulation does not alter the NK cells’ ability to mediate cell–cell interactions, trafficking, and homing to desired location. With respect to autologous NK cells, one may predict that they may be more effective in situations where tumors express low levels of MHC class I molecules.

Future possibilities and strategies for adoptive NK cell therapy

We have recently described critical questions that must be considered for the development of successful NK cell-based adoptive immunotherapy [70]. Below, we briefly discuss some issues with respect to the possible advantages, but also difficulties, of using allogeneic NK cells in future settings of adoptive NK cell-based immunotherapy.

Since autologous NK cells are inhibited by self-MHC class I molecules, allogeneic NK cells may, in certain situations, represent a better cellular population for adoptive immunotherapy in vivo. The latter choice applies particularly to situations in which tumor targets express normal levels of MHC class I molecules in combination with low or only moderate expression of ligands for activating receptors. NK cell alloreactivity depends on "missing" KIR-ligands (MHC class I) in the recipient. However, although NK cell alloreactivity is predicted by genetic algorithms based on KIR- and HLA-genotyping, the numbers of alloreactive NK cells in a given donor vary significantly, from below 1 to 60 % of the NK cells (our unpublished observations). Predicting the effectiveness of therapy may thus be based on assessment of the NK cell repertoire and selection of a donor with the largest alloreactive NK cell subset. This is feasible in KIR haplotype-A donors who lack activating KIR; in such donors, genetic analysis can be combined with phenotypic analysis using multi-parameter flow cytometry.

A prerequisite for survival of the infused cells is that they are not rejected by the recipient's immune system. If donor-derived NK cells are infused at the time of transplantation they may engraft along with the stem cells because of the pre-transplant conditioning. However, rejection of allogeneic NK cells represents a major challenge for specific NK cell therapy in the absence of myeloablative conditioning. It is likely that some type of conditioning will be required for effective transfer of allogeneic NK cells. Apart from preventing rejection, such regimens may also eradicate regulatory T cells that could otherwise interfere with the proliferation and function of the donor derived NK cells [42]. Moreover, there is reduced competition for growth factors during the homeostatic proliferation that follows lymphodepletion and the surge of cytokines, including IL-15, may promote proliferation, in vivo survival, and expansion of the infused NK cells. Indeed, in the studies by Miller and collaborators [76], NK cell expansion was dependent on an intense preparative regimen (high-dose cyclophosphamide/fludarabin). The latter regimen is similar to that used recently by Rosenberg and colleagues to induce homeostatic proliferation of adoptively transferred T cells [32]. As understanding of the conditions required for engraftment of NK cells improves, dosing of the preparative regimen will be more precise and the risks associated with high-dose myeloablative treatments will decrease.

Strategies to activate and/or modulate NK cells in vivo in patients with cancer

An exciting possibility that has now reached the stage of clinical trials in humans is to block inhibitory KIRs with monoclonal antibodies, thereby augmenting tumor cell recognition by NK cells [108]. Such reagents could ideally be used, e.g., in the treatment of hematopoetic cancers that are not amenable to SCT. Preclinical evidence in mouse models has demonstrated that this strategy may enhance antitumor activity in autologous [61] and allogeneic settings [62]. Along the same lines, the modulation of activating ligands on tumor cells may also improve the efficacy of NK cell recognition. NK cells may also be genetically engineered prior to adoptive transfer to the patients. One interesting possibility is to stably over-express chimeric receptors recognizing ligands expressed by tumors combined with signaling components that trigger NK cell function [106]. For example, NK cells engineered to express chimeric anti-CD19-CD3 signaling receptors became highly cytotoxic against autologous leukemia cells [54]. Genetically modified NK-92 cells expressing a chimeric antigen receptor specific for the tumor-associated ErbB2 (HER2/neu) antigen specifically lysed ErbB2-expressing tumor cells that were completely resistant to cytolysis by parental NK-92 cells [122].

Which human cancers may be targeted by NK cells?

With respect to tumor cell types, it is already evident from studies performed in vitro and even in some clinical studies that certain tumor types may be better suited than others for NK cell based immunotherapy. The presence on human tumors of ligands for activating receptors provides an important prerequisite for NK cell activation, and thus for the potential of achieving good clinical results [80]. An illustration of this is the inefficient NK cell killing of lymphoid compared to myeloid leukemias that may be caused, at least in part, by the absence of LFA-1 ligand expression [100]. Likewise, low expression of MHC class I molecules, particularly in situations where KIR-ligand mismatching ("missing-self"-reactivity) does not prevail, is also important. Most immunotherapy trials have been performed in patients with significant tumor burdens, where conventional therapies were ineffective. The best clinical setting for most cellular therapies including NK cell-based immunotherapy is probably when the tumor burden is small, i.e., in minimal residual disease [111]. NK cell therapy against large solid tumors represents special problems including not only the size of the tumor per se but also the presumed necessity of

NK cells to infiltrate the tumor. Despite the knowledge gained so far about the mechanisms that control trafficking of NK cells, we still know too little about the requirements for NK cell homing to and infiltration of tumors. It is known, however, that chemokines are required to attract NK cells to tumor sites. NK cells express a wide array of chemokine receptors on their cell surface. Different NK cell subsets can be identified based upon chemokine receptor expression and the pattern of expression is likely highly dependent upon the activation status of the NK cells [94].

Combination therapies involving NK cells may develop into promising treatment options for some human cancers

In all likelihood, combination therapies with NK cells representing one important mediator will become ever more important in the future. Ligands for the activating receptor NKG2D are up-regulated by genotoxic stress and stalled DNA replication, through activation of major DNA damage checkpoint pathways initiated by ATM or ATR protein kinases [40, 41]. Thus, the response to DNA damage alerts the immune system to the presence of potentially dangerous cells. Since several of the currently used chemotherapeutic drugs as well as ionizing irradiation act via the DNA damage response pathway, a mild preconditioning using these drugs and/or local ionizing irradiation might sensitize tumor cells to immune recognition, leading to synergistic anti-tumor effects. Similarly, new generation cancer drugs such as the proteasome inhibitors and the histone deacetylase inhibitors can upregulate the death receptor DR5, sensitizing tumor cells to TRAIL-mediated killing by NK cells [71, 104, 128]. Histone deacetylase inhibitors induce MICA/B expression [110]. Imatinib mesylate, previously discussed as a potential stimulator of innate immunity to tumors, was also shown to influence the expression and shedding of the activating NK cell ligand MICA on Bcr/Abl positive targets [22]. Thus, although NKG2D expression on NK cells is restored upon Imatinib treatment, this may lead to decreased tumor targeting because of reduced MICA expression [6, 22].

As has been discussed, NK cells are major effectors in mediating ADCC. Rituximab (Mabthera), a chimeric mouse/human antibody that recognize the CD20 antigen expressed on mature B lymphocytes [93] is currently given alone or combined with chemotherapy to patients with non-Hodgkin's lymphomas. One mechanism of this antibody's action is the induction of ADCC mediated by NK cells [29, 129]. Trials combining Rituximab with IL-2 to activate and expand the pool of NK cells available for ADCC are under way [91]. Several other antibodies are being evaluated in clinical practices and for many of them such as, e.g., Herceptin, at least part of their effector mechanism seem to be mediated by NK cells via ADCC [1, 19]. These and other findings suggest the possibility of using antibodies in conjunction with adoptive NK cell immunotherapy or NK cell stimulation-based protocols. A related therapeutic approach is the use of bispecific antibodies to promote NK cell targeting of tumors. Experimental and clinical data suggest that bispecific antibodies can be beneficial in tumor treatment. One approach is the use of antibodies specific for CD16 on NK cells and CD19 on B cell lymphomas or HER2/neu on breast cancers to target tumors expressing these, respective antigens [107]. Interestingly, clinical responses have been observed in patients with Hodgkin's lymphoma treated with bispecific antibodies against CD16 and CD30 [49].

Concluding remarks

We foresee a development where certain forms of cancer may become targets for therapeutic interventions involving NK cells. In addition to adoptive-based NK cell immunotherapy, we have highlighted different ways to enhance NK cell activity and the possibility of exploring combinatorial treatment strategies where NK cells mediate direct or indirect anti-tumor effects in future cancer treatments. In the latter respect, induction of NK cell activation ligands on tumor targets is one interesting aspect. Clearly, many questions and obstacles need to be met, and practical issues often play a central role in all strategies of human cell therapy. The effort required to translate advances at the bench to the clinic shall not be underestimated. Standardization of protocols will be needed to allow for future comparisons between different trials. Nonetheless, we believe that NK cell may likely find a role in future therapies against some human cancers, either alone or in combination with other therapies.

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