Mechanisms of Tumor and Viral Immune Escape from Natural Killer Cell-Mediated Surveillance

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Key Words

Natural killer cells · Immunosurveillance · Tumor immune escape · Viral immune evasion · Natural killer cell therapy · Ligand shedding · NKG2D · Neuroblastoma

Abstract

Human natural killer (NK) cells recognize and efficiently eliminate MHC class I low or negative malignant targets and virally infected host cells, without requirement for prior sensitization. However, viruses and various tumor cells display elaborate adaptations to evade and overcome immunosurveillance. The current review focuses on escape mechanisms of viruses and malignantly transformed 'stressed' cells to evade from NK cell cytotoxicity. A general overview of recent clinical studies using allogeneic donor NK cells is given, summarizing first data about a possible benefit for patients suffering from high-risk leukemia and solid tumors. Finally, the review discusses the future perspectives and hypotheses aiming to improve therapeutic NK cell strategies against tumor immune escape mechanisms.

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Introduction

Natural killer (NK) cells are important effector cells of the innate immunity against virus-infected and malignantly transformed cells. Recognition and killing of target cells is mediated by a balance of activating and inhibitory signals [1, 2]. NK cells kill their target cells by the polarized release of cytotoxic granules containing perforin and granzymes [3]. Killing of the target can also be achieved by ligation of the tumor necrosis factor (TNF) receptor family members Fas/CD95, TRAILRs and TNFR1 on tumor cells with their corresponding ligands (FasL, TRAIL and TNF) expressed on, or secreted by, NK cells [3, 4]. In combination with the adaptive immune response, NK cells are able to lyse targets by antibody-dependent cellular cytotoxicity, which is initiated after binding of the Fc portion of an IgG antibody to its receptor (Fc-γRIII, CD16) on NK cells (fig. 1a).

Activating signals (from receptor ligation of induced cellular ligands or viral proteins) triggers the cytotoxicity of NK cells, whereas predominance of inhibitory signals (from receptor ligation of MHC class I molecules) leads to tolerance [1–3, 5, 6]. The 'missing self' recognition theory describes the phenomenon that malignantly transformed cells, which lack expression of specific MHC class I molecules, trigger NK cell-dependent activation [7, 8].

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By contrast, erythrocytes are not lysed by NK cells although they lack MHC class I molecules on their surface, arguing for the existence of an 'induced self' component in decision making [9]. The majority of mature unstimulated NK cells express at least one receptor specific for self-MHC [10, 11], however, several NK cells lack receptors for self-MHC and are hyporesponsive [12–14].

Each NK cell expresses its own repertoire of activating and inhibitory receptors (average 2–9) [15, 16]. Human inhibitory receptors comprise both, killer cell immunoglobulin-like receptors (KIRs), which recognize HLA class I molecules, and the heterodimeric C-type lectin receptor CD94-NKG2A, which binds to HLA-E. The main activating receptors involved in the recognition and killing of malignant transformed cells include the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46, whose few ligands identified so far remain poorly characterized on the molecular level, as well as NKG2D, 2B4 and DNAM-1 receptors recognizing a variety of well-defined ligands [1, 2, 17, 18]. The importance of these activating receptors is underscored by the fact that the surface density of NCRs is correlated with the degree of NK cell-mediated cytotoxicity towards malignant transformed cells [19, 20].

In the current review, we will focus on the interaction between malignant transformed cells or virus-infected cells and NK cells as an essential part of the first line of immunosurveillance.

Tumor Escape from NK Cell-Mediated Immunity

The transformation of a normal cell into a malignant cell, which eventually escapes from immunosurveillance, requires a large number of intrinsic oncogenic events. To date, it is estimated that tumor transformation results from approximately 80 mutations within an individual cell, affecting the functionality of around 6–8 signaling pathways [21]. However, there are numerous intrinsic barriers that inhibit the development of cancer [22, 23]. There are two major challenges for immunosurveillance of malignant transformed cells: (1) tumor cells originate from 'self' as well as their biochemical properties and their behavior differs only subtly from their healthy counterparts, and (2) tumor cells employ many tricks to actively bypass detection and elimination by effector cells of the immune system (comprehensively reviewed by Vesely et al. [23]).

Cells of the adaptive immune system may detect tumor antigens in the context of MHC class I antigen pre-

sentation. In contrast, NK cells kill malignant transformed cells after interaction of induced or overexpressed ligands with their activating receptors. This killing may occur, provided that MHC-mediated inhibition is lowered by the downregulation of MHC class I molecules from the surface of malignant transformed cells. Among the ligands of NKG2D, the MHC class I chain-related proteins A and B (MICA/B) and the unique long 16-binding proteins (ULBPs) have been studied in molecular detail. MICA and MICB are highly polymorphic and resemble the MHC class I heavy chain in structural organization, but fail to associate with β 2-microglobulin and peptide. The ULBPs are either transmembrane or glycosylphosphatidylinositol anchored and comprise only the MHC class I-like α 1- and α 2-domains [17].

In healthy adult cells, the expression of NKG2D ligands is restricted to the thymic epithelium and to the gastrointestinal mucosa [24]. However, MICA/B and other ligands are upregulated on the surface of many cell types after malignant transformation and under conditions of stress such as heat shock [24], oxidative stress [25], genotoxic stress and stalled DNA replication, which activate a major DNA damage checkpoint controlled by ATM or ATR protein kinases [26]. Ligand overexpression has been detected in solid tumors of multiple origins and in lymphoproliferative malignancies. Some oncogenes may directly upregulate the expression of NKG2D ligands. As an example, the BCR/ABL fusion oncoprotein induces the expression of MICA on leukemia cells in chronic myeloid leukemia, whereas it is absent on healthy donor hematopoietic CD34⁺ stem cells [27]. However, transformation alone has shown to be insufficient to induce NKG2D ligand transcription, since the expression of the oncogenes (*K-ras* and *c-myc* or *Akt* and *c-myc*), or the lack of tumor suppressor p53 alone do not induce NKG2D ligand expression on primary ovarian epithelial cells [26].

The regulated expression of ligands of the activating NK cell receptors might have several consequences: (1) upregulation of NK cell ligands might be a cell intrinsic protective mechanism in order to render altered cells susceptible to killing, (2) reduced ligand expression is beneficial to tumor cells in order to prevent NK cell activation, and (3) shedding of ligands might interfere with NK cell activation. A selection of NKG2D-dependent immune evasion strategies employed by malignant transformed cells is detailed below.

Fig. 1. Strategies of tumor immune escape from NK cell-dependent immunosurveillance. NK cell recognition is mediated by a fine-tuned balance of activating and inhibitory signals. **a** NK cells kill their target cells by the combined release of cytotoxic granules containing perforin and granzymes, by the expression or release of TRAIL, TNF and FasL and by antibody-dependent cellular cytotoxicity, resulting in apoptosis of the target cell. Tumor cells may escape NK cell dependent immunosurveillance by several pathways: alterations in DNA modifying enzymes such as HDACs or microRNAs, involved in epigenetic gene regulation, repress the expression of MICA/B (**b**), persistent expression of activating ligands and sustained triggering of NKG2D leads to hypo-respon-

siveness and decreased cytotoxicity due to decreased NKG2D expression and reduced IFN- γ production (**c**), tumor-released cytokines such as TGF- β and IFN- γ repress MICA/B expression, downmodulate NKG2D expression and IFN- γ production in NK cells and promote the conversion of CD4 $^{+}$ T cells into regulatory CD4 $^{+}$ CD25 $^{+}$ FOXP3 $^{+}$ T cells, which suppress immunosurveillance (**d**) and upregulation of MMPs and ADAMS, which promote the shedding of activating ligands such as MICA, which binds to NKG2D on CD4 $^{+}$ T cells and NK cells, resulting in hypo-responsiveness in NK cells due to degradation of NKG2D and conversion of CD4 $^{+}$ T cells into regulatory CD4 $^{+}$ CD25 $^{+}$ FOXP3 $^{+}$ T cells (**e**).

Persistent Expression of NKG2D Ligands

Persistent NKG2D ligand expression and the sustained triggering of receptor signaling by appropriate cellular ligands lead to profound downregulation of surface NKG2D from NK cells and activated CD8 $^{+}$ T cells. Consequently, NK cell cytotoxicity is reduced, and aggressiveness of tumor growth is increased in transgenic mice in vivo [28, 29] (fig. 1c). Importantly, cytokine stimulation of NK cells, which were chronically exposed to NKG2D ligands in vitro, could restore NKG2D expression [29]. Thus, ex vivo stimulation of NK cells with cytokines might improve immunotherapy (see below).

Downregulation of NKG2D Ligands by Cytokines

Due to their diverse immunomodulatory functions, the individual effects of cytokines on NK cell-dependent immunosurveillance of tumor cells are not precisely determined. Among these, the transforming growth factor- β (TGF- β), which is upregulated in many cancer types, has been studied intensively. TGF- β is one of the key immune regulators in tumor initiation and progression. TGF- β functions both as a suppressor and as a promoter of tumor formation, but the underlying molecular mechanisms remain unclear. TGF- β downmodulates Nkp30 and NKG2D receptor expression, thereby suppressing NK

cell cytolytic activity (fig. 1d) [30, 31]. As an example, release of TGF- β by human glioma cells decreases NKG2D levels in CD8⁺ T and NK cells and at the same time represses the expression of MICA and ULBP1 [32]. Neutralization of TGF- β enhances the antitumor response of NK cells (and CD8⁺ T cells) by accumulation of IFN- γ -producing NK cells with IFN- γ preservation of surface NKG2D [33]. Additionally, TGF- β plays an important role in the conversion of activated CD4⁺CD25⁺ T cells into regulatory CD4⁺CD25⁺FOXP3⁺ T cells (Tregs), a subpopulation that is commonly found in high numbers in the tumor microenvironment. Regulatory T cells reduce NKG2D expression and suppress NKG2D-mediated NK cell cytotoxicity, thereby promoting tumor progression [34].

Recently, a role of IFN- γ in MICA/B and ULBP expression was demonstrated. In glioma and melanoma, IFN- γ was shown to downregulate MICA mRNA and consequently MICA surface levels, suggesting an interesting therapeutic intervention possibility (fig. 1d) [35].

Epigenetic Repression of NKG2D Ligand Expression

Many cancer types display alterations in epigenetic gene regulation. Histone deacetylases (HDACs), that control the accessibility of chromatin for transcription factors and repress tumorigenesis, are frequently over-expressed in cancer cells [36]. As recently shown, the expression of MICA/B is influenced by HDAC activity, since inhibitors of HDACs have been shown to induce the expression of MICA/B on epithelial and leukemic cells. The observed hypoacetylation of histones within the promoter regions of MICA/B suggests an epigenetic repression of these promoters. Subsequent treatment with HDAC inhibitors markedly increased the expression of MICA/B in these cells, whereas the expression level of MICA/B in mononuclear cells from healthy donors was unchanged [37, 38]. Recently, HDAC3, was identified as a key repressor of ULBP expression in epithelial cancer cells, highlighting HDAC inhibitors as putative tools to restore immunogenicity of certain tumors [39]. Another approach to block MICA/B expression might be the use of microRNAs [40] (fig. 1b).

Ligand Shedding and Downregulation of NKG2D

Soluble NKG2D ligands were initially detected in sera from patients suffering from different types of cancer. The soluble variants of MICA/B are generated by shed-

ding from the surface of tumor cells, diffusing into the tumor periphery and dampening NKG2D-dependent immunosurveillance [41–44]. Due to its high concentrations in patients' sera, soluble MICA is a prognostic marker for cancer at early stages. Moreover, MICB serum levels correlate with disease progression and formation of metastases [43].

The consequences of NKG2D ligand shedding are manifold: (1) the number of ligands on the target cell surface is reduced and, consequently, the number of interaction sites for the NK cell is limited, (2) soluble NKG2D ligands can bind to NKG2D on the NK cell and promote its endocytosis as well as degradation, and (3) shedding of MICA/B stimulates the expansion of regulatory NKG2D⁺CD4⁺ T cells, which play a major role in immune suppression [45].

The proteases which promote shedding of MICA from the plasma membrane of tumor cells are ill defined, however, they are members of the family of matrix metalloproteinases (MMPs) and the ADAM family as shown by inhibition studies with several generic inhibitors (fig. 1e) [42]. MMPs are structurally related endopeptidases which can degrade proteins of the extracellular matrix [46]. Shedding of MICA ligands from the cell surface is regulated by interaction with a chaperone called endoplasmic reticulum protein 5. Endoplasmic reticulum-resident chaperones are upregulated in many cancer types and translocated to the plasma membrane in response to cellular stress. On the cell surface, endoplasmic reticulum protein 5 mediates the reduction of a disulfide bond in the $\alpha 3$ domain of MICA and a subsequent conformational change that is essential for proteolytic cleavage of MICA and metalloproteinases of the ADAM family [47]. Among these, ADAM10 and ADAM17, which are known to cleave proteins within the membrane-proximal stalk region, were shown to be involved in the shedding of MICA and ULBPs [48, 49]. The contribution of other metalloproteinases such as ADAM9 and MMP14 has also been reported [50]. MICA/B are highly polymorphic and thus the shedding mechanism employed differs among the different alleles. As an example, full-length MICA*008 is released from cells on exosomes by an ADAM-independent mechanism [51]. Of note, similar observations have been made for the different ULBPs [52].

Ligand shedding by tumor cells via MMPs is a common immune evasion mechanism that opens up several possibilities for intervention. Many efforts are directed towards inhibition of MMP activity, and several MMP inhibitors are studied in clinical cancer trials. However, the first clinical trials show no statistically significant

benefit on survival. Moreover, side effects of MMP inhibition were observed since MMPs are involved in ligand shedding but also in other processes, such as tissue morphogenesis, tissue repair and angiogenesis.

Virus-Mediated Immune Escape from NK Cells

The straightforward strategy to subvert the immune response of NK cells is to infect and destroy the killer itself. Many viruses such as poxviruses, influenza, HIV, EBV and HSV have been reported to interfere directly with NK cell function [53]. Several strategies are pursued: (1) inhibition of NK cell activating receptors, (2) virus-induced expression of cytokine-binding proteins or cytokine receptor antagonists, (3) inhibition of apoptosis, (4) selective down- or upregulation of HLA alleles from the host cell surface, (5) virus-induced expression of MHC I homologues in host cells and (6) infection of NK cells.

Alteration of NK Cell Receptors under the Influence of Viral Infections

In the context of virus immunosurveillance, the NKG2D receptor and the NCRs play a major role [54, 55]. There are several other stimulatory NK receptors, yet, their immunological function and signaling pathways are poorly understood [1].

Herpes viruses employ sophisticated mechanisms to reduce the overall level of surface MHC class I molecules on the host cell to escape from killing by cytotoxic T lymphocytes (CTLs). At the same time they maintain the level of certain HLA alleles, which serve as ligands for inhibitory NK cell receptors to escape from killing by NK cells. Human cytomegalovirus (HCMV), which causes life-threatening infections in immunocompromised individuals such as AIDS patients or organ transplant recipients, employs several tricks to evade recognition. At least 4 HCMV proteins (US2, US3, US6 and US11) promote a drastic reduction in MHC class I antigen presentation to CTLs by interfering with MHC class I processing, leading to escape from immunosurveillance and persistence within the host [56]. In a similar way, the HIV-1 protein Nef and the K3 and K5 proteins of the Kaposi's sarcoma-associated herpes virus (KSHV) downregulate the cell surface expression of MHC class I molecules by redirecting them to the endosomal pathway [55, 57]. Nef decreases HLA-A and HLA-B molecules on the cell surface of HIV-infected cells. Interestingly, the HLA-C and

HLA-E alleles are not affected and are therefore still available for binding to the inhibitory KIR or CD94/NKG2A receptors. As a result, HIV achieves inhibition of both, CTL- as well as NK cell-mediated killing of the host cell.

Based on the 'missing self hypothesis' [7, 8], the loss of surface MHC class I molecules leads to NK cell-mediated killing of the target cell, which is in accordance with the fact that US2-11 deletion mutants of HCMV conferred resistance to NK cell lysis when compared to wild-type HCMV [58]. In order to circumvent elimination by NK cells, HCMV employs the proteins UL18, UL142 and UL40. UL18 mimics MHC class I molecules and serves as decoy ligand for inhibitory NK cell receptors [59]. UL40 provides a leader peptide, which serves as an efficient ligand for presentation on HLA-E molecules. These peptide::HLA-E complexes bind to the inhibitory NK cell receptor CD94/NKG2A thereby preventing the activation of NK cells [60].

Besides restoration of inhibitory signals, the most common viral evasion strategies interfere with activating receptor functions by downregulation of their corresponding ligands in infected cells. As an example, certain strains of HCMV increase the resistance of their host cells to NK cell killing by downregulation of LFA-3 [61], which interferes with binding of LFA-3 to the NK cell-activating receptor CD2. Another example is provided by K5 of KSHV. The gene product MIR1, an E3 ubiquitin ligase, inhibits NK cell cytotoxicity by downregulation of the ligands ICAM-1 and B7-2 by ubiquitylation and reduced surface expression of NK cell receptors [1, 62].

Another mechanism to avoid NK cell activation is to inhibit binding of activating receptors to its corresponding ligand. A prominent example is the HCMV-encoded protein UL16, which binds to ULBPs and MICB in virus-infected cells leading to retention and sequestration of these ligands as well as inhibition of their interaction with NKG2D [63]. In a similar way, HIV reduces cell surface expression of the ligands MICA, ULBP-1 and ULBP-2 via the Nef protein [64]. A second way viruses interfere with activating receptor function is secondary modification of the ligand on the target cell surface. HIV and the human T cell lymphotropic virus I or II sialylate cell surface molecules, and although binding is not impaired, the cytotoxicity of NK cells is abrogated [65].

Another strategy by which viruses interfere with activating receptor function is the inhibition of downstream signaling following ligation of activating receptors. In this context, the Tat protein of HIV can block recognition of LFA-1 by activating receptors on the NK cell. Adhesion

to the target cell is not affected, but an L-type calcium channel is bound. The binding blocks calcium influx and subsequently the induction of the calcium-calmodulin kinase II in NK cells required for cytotoxicity [66]. Recently, the HCMV tegument protein pp65, one of the dominant antigens for HCMV-specific CTL responses [67], has been found to block NK cell cytotoxicity through interactions with NKp30 [68]. The authors propose that binding of pp65 results in dissociation of NKp30 and the CD3 ζ chain, which is required for immunoreceptor tyrosine-based activation motif-dependent signaling. However, it remains unclear how viral pp65 and cell surface NKp30 on the NK cell interact.

Beside viral proteins, also virus-derived noncoding regulatory RNAs, such as microRNAs are effectors in immune evasion strategies. As an example, HCMV-derived miR-UL112 can prevent translation of MICB. A similar observation was made for other viruses such as KSHV and EBV [69].

Modulation of Cytokines and Chemokines

Cytokines play a key role in the initiation and regulation of the innate and adaptive immune responses. In this context, viruses interfere with NK cell responses through virus-encoded proteins that counteract or modulate the interactions between cytokine or chemokine molecules and their corresponding receptors. These pathways are not exclusive to NK cells, and are therefore not strictly separated. Many viruses block signal transduction by ligands of the TNF family, whereas others induce cytokine pathways such as the EBV latent membrane protein 1 that recruits components of the TNF receptor and CD40 transduction machinery to mimic a cytokine response that could be beneficial to the virus. A fascinating mechanism is the mimicry of cytokines (virokines) and cytokine receptors (viroceptors) by large DNA viruses such as herpes viruses and poxviruses [70]. The functions of these viral homologues are diverse. Soluble viral receptors neutralize cytokine activity and viral cytokines might antagonize cytokines such as IL-12, IL-18, TNF- α , IL-1 α , IL-1 β and IL-15, which participate in the stimulation of IFN- γ production and cytotoxicity by NK cells. Alternatively, viruses facilitate overproduction or encode homologues of cytokines such as IL-10 that have an inhibitory effect on NK cells. Viral chemokines, including MIP-1 α , MIP-1 β , MCP-1, MCP-2, MCP-3 and RANTES, can affect NK cell chemotaxis. Moreover, chemokines can affect the recruitment of other immune cells [71].

KSHV encodes for the broad-spectrum chemokine (including CC, CXC and CX3C) antagonist vMIP-II, which can block chemotaxis of monocytes to RANTES, MIP-1 α and MIP-1 β . In addition, vMIP-I binds to receptors on NK cells [72]. Moreover, HCMV and EBV possess IL-10 homologues, which have been found to inhibit the production of type 1 cytokines and to act directly on NK cells [73]. Specific interference with NK cell activation can also be mediated by cytokine-binding protein homologues. A principal target is IL-18, which is central in NK cell production of IFN- γ . Ectromelia poxvirus encodes a cytokine-binding protein, which is homologous to IL-18BP and inhibits IL-18 receptor binding as well as activity [74].

Inhibitors of Apoptosis

NK cells eliminate virus-infected cells by inducing apoptosis via secretion of cytokines such as TNF, the release of perforin and granzymes, or the ligation of Fas on the target cell. In order to prevent induction of apoptosis, viral proteins inhibit the activation of caspases and encode homologues of antiapoptotic proteins such as Bcl-2 or FLIP. Apoptotic signals triggered by the TNFR family members are blocked by death effector domain-containing proteins, which inactivate IFN-induced PKR and the tumor suppressor p53 [71].

These mechanisms developed by viruses highlight the importance of NK cells in defense against viral infection. Further studies of viral evasion strategies will provide insights into NK cell biology as well as potential therapeutic targets to support immune response.

Prospects for the Use of NK Cells in Immunotherapy Trials of Cancer

Various studies have revealed that NK cells are particularly efficient in the eradication of metastasizing tumor cells and small tumors, but fail to eliminate larger tumors [75]. Rosenberg et al. [81] pioneered NK cell-based immunotherapy by administration of autologous IL-2-activated killer cells to patients with advanced cancer. Although the authors could clearly demonstrate antitumor effects against different kinds of solid tumors in some of their patients, it is known that the cytotoxic potential of autologous NK cells is weak compared to allogeneic NK cells. Based on the fact that allogeneic, in particular haploidentical, NK cells can target human malignancies

nancies in a superior way, new protocols for allogeneic NK cell-mediated immunotherapy are being developed. In these trials, NK cell function could be improved by cytokine stimulation, especially treatment with IL-2, leading to an enhanced NK-cell cytotoxicity and enhanced antitumor response [20, 76]. There is good evidence that a combination of cytokines like IFN- α , IL-2, IL-12, IL-15, IL-18 and IL-21 may further increase the cytotoxic activity of NK cells [77] and possibly modify the NK cell receptor repertoire. In addition to antitumor response, a multitude of potentially beneficial effects, such as NK cells versus leukemia activity, NK cells versus residual host T cell activity and NK cells versus host dendritic cell activity with a reduced risk of graft-versus-host disease (GvHD), was observed by allogeneic NK cells in vivo [78].

Furthermore, drugs widely used in supportive care, such as ketamine, thiopental and morphine, suppress NK activity and increase the risk for pulmonary metastasis in rats [79]. This deleterious effect could be prevented by small doses of immunostimulators and underline the importance of a sufficient NK activity in anticancer immunity. A comprehensive overview of the prospects for the use of NK cells in immunotherapy of human cancer is given by Ljunggren et al. [80].

NK Cell-Based Immunotherapy of Solid Tumors and Leukemia

Cell therapy provides a promising treatment option for patients suffering from leukemia and solid tumors that harbor a high risk for relapse after allogeneic, especially haploidentical stem cell transplantation (SCT). Whereas the established T cell therapies (donor lymphocyte infusions) are known to cause GvHD, NK cells may mediate graft-versus-leukemia/tumor effects without induction of GvHD. Therefore, immunotherapy with highly purified NK cells in recipients of haploidentical SCT could serve as an attractive alternative cell therapy [82, 83].

After SCT, the stem cells of the donor reconstitute the immune cells and finally a new immune system in the patient. Although recovery time and velocity vary among patients, NK cells are the first population of developing lymphoid cells after SCT [84]. Due to their early reconstitution, NK cells might play a pivotal role in the lysis of malignantly transformed cells in patients. However, the early expansion phase after SCT is characterized by an unusually high percentage of immune-regulatory CD56^{bright} NK cells, which gradually decline but persist

for at least 1 year [85]. Another study demonstrated that early reconstituting NK cells are immature and display impaired cytotoxicity [86], thus illustrating the necessity for additional donor NK cell-based immunotherapy to enhance graft-versus-leukemia/tumor effects.

Enriched NK cells can be infused without additional manipulation, after overnight culture in high-dose IL-2 or after expansion with IL-2 for two weeks [87–91]. First clinical trials show the feasibility of freshly purified or IL-2-activated donor NK cells for the treatment of high-risk patients suffering from leukemia or tumors in both nontransplant settings and after haploidentical SCT as an additional immunotherapy [88, 90, 92, 93]. NK cells were applied in a single dose or several times with doses between 0.2×10^7 and 8.1×10^7 CD56⁺CD3⁻ NK cells/kg body weight with less than 2.5×10^4 CD3⁺ T cells/kg body weight [20, 83, 93]. NK cells were well tolerated by the patients without development of GvHD above grade II. However, a few cases of GvHD have been observed after NK cell infusion which might be partly associated with insufficient T cell depletion during cell preparation. In a pioneer study, it was demonstrated that patients with AML had a reduced rate of leukemia relapse, a lower rate of graft rejection and a paradoxical reduction in GvHD after haploidentical SCT if the NK cells possessed inhibitory KIRs for which the recipient had no ligand [78]. Recently, Rubnitz et al. [93] have described that NK cell application led to a 2-year event-free survival of all patients in a cohort of 10 pediatric patients with AML in first complete remission.

In solid cancers, the use of NK cells for immunotherapy is rare. We observed an increased cytotoxic activity of activated NK cells against high-risk neuroblastoma (NB) due to IL-2 mediated upregulation of the activating receptors NKp30, NKp44, NKp46 and NKG2D [20].

Taken together, these first clinical data show that the application of ex vivo purified donor NK cells with or without further IL-2 stimulation is feasible, well tolerated and holds a great potential for immunotherapy of patients suffering from various malignancies without severe side effects.

Escape from NKG2D-Dependent Immunosurveillance after Allogeneic NK Cell Transplantation in Neuroblastoma

In an ongoing clinical phase I/II NK cell study, we treat patients suffering from high-risk NB with allogeneic IL-2 stimulated donor NK cells after haploidentical

SCT in order to improve the clinical outcome [44]. Although NB cells frequently overexpress NKG2D ligands on their surface, growth of these malignantly transformed cells becomes progressive due to various tumor escape mechanisms [94]. In our study, we monitored the levels of soluble MICA in NB patients' plasma prior to and at several time points after NK cell application together with the expression level of NKG2D on donor NK cells. Elevated levels of tumor-released MICA in patients' plasma correlated significantly with impaired NK cell cytotoxicity and highlight the influence of shed ligand on tumor immune escape leading to impaired NKG2D-dependent cytotoxicity of ex vivo stimulated donor NK cells in vivo. Most importantly, this effect could be temporarily reversed by infusion of large numbers of activated NK cells expressing high NKG2D levels, which were able to scavenge shed MICA and retained cytotoxicity via non-occupied NKG2D receptors [44].

Future Perspectives

Future studies should improve NK cell immunotherapy by (1) increasing the cytotoxicity of NK cells against various malignancies, (2) optimizing the schedule of the NK cells and (3) developing strategies to overcome tumor immune escape mechanisms. Given the plausible benefit of IL-2-stimulated NK cells compared to freshly isolated, resting NK cells with regard to cytotoxicity, additional increase in cytotoxicity may be achieved by activation with cytokine combinations like IL-2/IL-15 or by cross-

talk with dendritic cells. In order to overcome immune escape, treatment options are: (1) monoclonal antibodies against soluble MICA, (2) genetically engineered NK cells with chimeric receptors for tumor targeting and improved recognition of tumor cells or (3) using small interfering RNA to downregulate inhibitory receptors. Open issues in clinical studies include the optimal number of infused NK cells, the injection scheme, the appropriate selection of donor/recipients and the type of disease, since certain types of malignantly transformed cells are more susceptible to NK cell therapy than others. Taken together, cellular immunotherapy of cancer is a very recent concept and only starts to provide evidence of selectivity, applicability and safety. The efficacy of cell therapy still has to be demonstrated but will probably emerge as a complementary strategy for actual therapies. Finally, combinatorial approaches using drugs, antibodies, cytokines and cells may become successful attempts limiting immune escape from tumors.

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