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NK cell-based immunotherapies against tumors

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Abstract: Natural killer (NK) cells provide the first line of defence against pathogens and tumors. Their activation status is regulated by pro-inflammatory cytokines and by ligands that either target inhibitory or activating cell surface receptors belonging to the immunoglobulin-like, C-type lectin or natural cytotoxicity receptor families. Apart from non-classical HLA-E, membrane-bound heat shock protein 70 (Hsp70) has been identified as a tumor-specific recognition structure for NK cells expressing high amounts of the C-type lectin receptor CD94, acting as one component of an activating heterodimeric receptor complex. Full-length Hsp70 protein (Hsp70) or the 14-mer Hsp70 peptide T-K-D-N-N-L-L-G-R-F-E-L-S-G (TKD) in combination with pro-inflammatory cytokines enhances the cytolytic activity of NK cells towards Hsp70 membrane-positive tumors. Based on these findings cytokine/TKD-activated NK cells were adoptively transferred in tumor patients. These findings were compared to results of clinical trials using cytokine-activated NK cells.

Keywords: Heat shock proteins, NK cells, cancer, immunostimulation, cell therapy

Abbreviations

AML Acute myelogenous leukemia
APC Antigen presenting cells
GrB Granzyme B
GvHD Graft-versus-host disease
GvL Graft versus leukemia

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HLA Human leukocyte antigen
HSP Heat shock protein(s)
MHC Major histocompatibility complex
NF-κB Nuclear factor-kappaB
NK cell Natural killer cell
IFN-γ Interferon-gamma
IL-2(15) interleukin-2
PBL Peripheral blood lymphocytes
TNF-α Tumor necrosis factor-alpha
TLR Toll-like receptor(s)
ULBP UL-16 binding protein(s)

1 Introduction

Heat shock proteins (HSP) are overexpressed in a broad range of human tumors and play crucial roles in tumor invasion, metastasis, cell proliferation, differentiation and death (for a review see ref [1]). In the past years HSP have attracted much attention as tumor-specific recognition structures for natural killer (NK) cells (summarized in refs [2, 3]). HSP are highly conserved proteins inhabiting nearly all subcellular compartments. Environmental stress as well as differentiation, proliferation, and maturation result in an increased HSP synthesis [4, 5]. Depending on their location, HSP either exert immune activation as danger signals in cancer immunity or protect cells from lethal damage induced by exogenous stress stimuli. Intracellularly, HSP function as molecular chaperones supporting folding and transport of a great variety of polypeptides and proteins under both, physiological conditions and following chemical or physical stress stimuli [6]. Hsp70, the major stress-inducible heat shock protein, is able to protect cells from a wide range of apoptotic and necrotic stimuli [7, 8]. This cell survival results from the inhibitory function of Hsp70 on lysosomal membrane permeabilization [9]. In contrast, extracellular or plasma membrane-bound HSP combined with pro-inflammatory cytokines have been found to elicit a potent anti-cancer immune response mediated either by the adaptive or innate immune system [10]. This review will focus briefly on the immunological roles of extracellular and membrane-bound HSP acting as potent stimulators of an immune response against cancer. We further present experimental evidence for the anti-tumoral activity of IL-2/TKD-activated NK cells in animal models. In a phase I clinical trial the adoptive transfer of *ex vivo* stimulated autologous NK cells in patients with progressive colorectal and non-small cell lung carcinoma, refractory to standard therapy, was found to be safe, feasible and well tolerated. Our data might thus have future clinical implications with respect to the development of an NK cell-based immunological approach as an adjuvant therapy for patients with progressive tumor disease and a high risk for developing distant metastases.

2 Extracellular heat shock proteins

Apart from their intracellular chaperoning functions, HSP have been found to play key roles in tumor immunity. Most immunotherapeutic approaches exploit the carrier function of HSP for tumor-derived antigenic peptides. Following cross-presentation of HSP-chaperoned peptides on MHC class I molecules [11–15], an antigen-specific CD8+ T cell response is initiated. Cross-presentation describes the transfer of exogenous peptides into the MHC class I pathway via an endosomal pathway. Uptake of HSP-peptide complexes by antigen presenting cells (APC) was found to be specific, saturable, and concentration-dependent [11, 22, 23]. Therefore, the existence of HSP receptors was hypothesized [14]. Toll-like receptors 2 (TLR2) and 4 (TLR4), either alone or in combination with the lipopolysaccharide (LPS) receptor CD14, were primarily identified as interacting partners for Hsp60 [24], Hsp70/Hsc70 [25, 26], and gp96 [27] on APC. Up to now, the list of putative HSP receptors has grown and actually includes the co-stimulatory molecule CD40 [28, 29], the scavenger receptor CD36 [14, 30], the LRP/α_2-macroglobulin receptor CD91 [13, 14, 31, 32], as well as other members of the scavenger receptor families, SR-A [33, 34] and LOX-1 [35, 36].

Even in the absence of immunogenic peptides, HSP act as danger signals for the host's cellular immune system [37]. APC and tumor cells have been identified as natural sources for extracellular HSP70. An active release of Hsc70 from tumor cells was observed following treatment of tumor cells with IFN-γ [38]. Exosomes have recently been discussed as export vehicles for HSP70 from the endosomal compartment into the extracellular milieu [39, 40].

Interaction of HSP70 with CD14 and/or TLR2/4 on APC results in the production and release of pro-inflammatory cytokines [25, 41, 42]. This effect is mediated by triggering the translocation of NF-κB into the nucleus and thus initiating an important factor in the signal transduction pathway of immune responses [25]. More recent data revealed that not only full-length HSP but also endotoxin-free HSP peptides can initiate an immune response. Depending on the sequence from which the HSP peptides were derived they exert either tolerogenic or immunogenic functions [42, 43].

3 NK cells and tumor cell killing

NK cells comprise 5-20% of the peripheral blood lymphocytes (PBL) and are well known players in the control of bacteria, parasites, viruses and cancer [44]. For a long period of time, the low affinity Fcγ receptor CD16 mediating the antibody-dependent cellular cytotoxicity (ADCC) [45], and the homophilic adhesion molecule CD56 were the only NK cell markers. More recently, it became obvious that the effector functions of NK cells are regulated by a number of killer cell inhibitory and activating receptors. These receptors either belong to the killer cell immunoglobulin-like (KIR), the immunoglobulin-like transcript (ILT), C-type lectin receptor [46], or the natural cytotoxicity receptor (NCR) families [47]. Depending on their intracellular immunoreceptor tyrosine-based inhibitory (ITIM)

or activation motifs (ITAM) these receptors mediate activating and inhibiting signals, respectively [47, 48]. A variety of different MHC class I allele groups including HLA-C were determined as regulatory ligands for NK cells. According to the "missing self" theory [49] tumor cells with altered or missing MHC expression pattern provide ideal targets for the cytolytic attack mediated by NK cells. However, evidence is accumulating that apart from "missing self" additional activating signals are necessary to enable an efficient activation of NK cells. For a group of NCRs including NKp30, NKp44, NKp46 and NKp80 with a varying expression pattern [47], tumor-specific activating ligands are discussed. For the homodimeric C-type lectin receptor NKG2D, non-classical stress-inducible MHC class I-related chain (MIC)A and MICB glycoproteins, the glycosylphosphatidylinositol-linked UL-16 binding proteins (ULBP), the retinoic acid early inducible-1 (RAE-1) protein and HA60, a minor histocompatibility antigen, provide target structures [46, 50, 51]. Figure 1 summarizes the current understanding of how inhibitory and activating C-type lectin receptors CD94/NKG2A, CD94/NKG2C, and NKG2D operate in NK cells. CD94/NKG2A as a heterodimeric receptor mediates inhibitory signals via the tyrosine phosphatase SHP-1. Following contact with HLA-E a negative signal is mediated through its SH2 domain which enables dephosphorylation of multiple targets in the ITIM-activating pathway. In contrast, the positively charged transmembrane domain of the ITAM-containing adaptor molecule DAP-12 for NKG2C or DAP-10 for NKG2D triggers the downstream activation cascade following contact with HLA-E, MICA/B, and ULBP, respectively. For more detailed information on the structure and function of NK cell receptors see refs [52–54].

Under physiological conditions non-classical HLA-E molecules presenting leader peptides of HLA-A, -B, and -C alleles serve as ligands for the inhibitory heterodimeric receptor complex CD94/NKG2A. Following stress, an HSP60-derived signaling peptide competes with HLA leader peptides for binding to HLA-E. HLA-E/Hsp60-peptide complexes are no longer recognized by the inhibitory receptor complex CD94/NKG2A [56, 57]. These data provide evidence that environmental stress modulates the immune response of NK cells.

In line with these findings, we identified the major stress-inducible Hsp70 in combination with low dose IL-2 as additional triggering factors for CD94-positive NK cells [58, 59]. Mapping of the Hsp70 sequence revealed that the 14-mer peptide TKDNNLLGRFELSG (aa 450-463), which is termed Hsp70 peptide TKD, represents a part of the C-terminal substrate binding domain of Hsp70. In combination with IL-2, TKD has an identical immunostimulatory capacity on NK cells like full-length Hsp70 protein [60, 61]. These findings are in line with observations of the group of Mario Colombo demonstrating that genetically engineered tumors secreting the inducible Hsp70 displayed an increased immunogenicity against cancer in a mouse model [62]. However, the genetic manipulation of tumor cells did not affect the chaperone activity of Hsp70. Tumor rejection in these mice was mediated on the one hand via an increased amount of dendritic cells (DC) inducing a robust CD8+ T cell response, and on the other hand by an enhanced susceptibility towards NK cells [63].

Since binding of Hsp70 protein as well as Hsp70 peptide TKD to NK cells was sat-

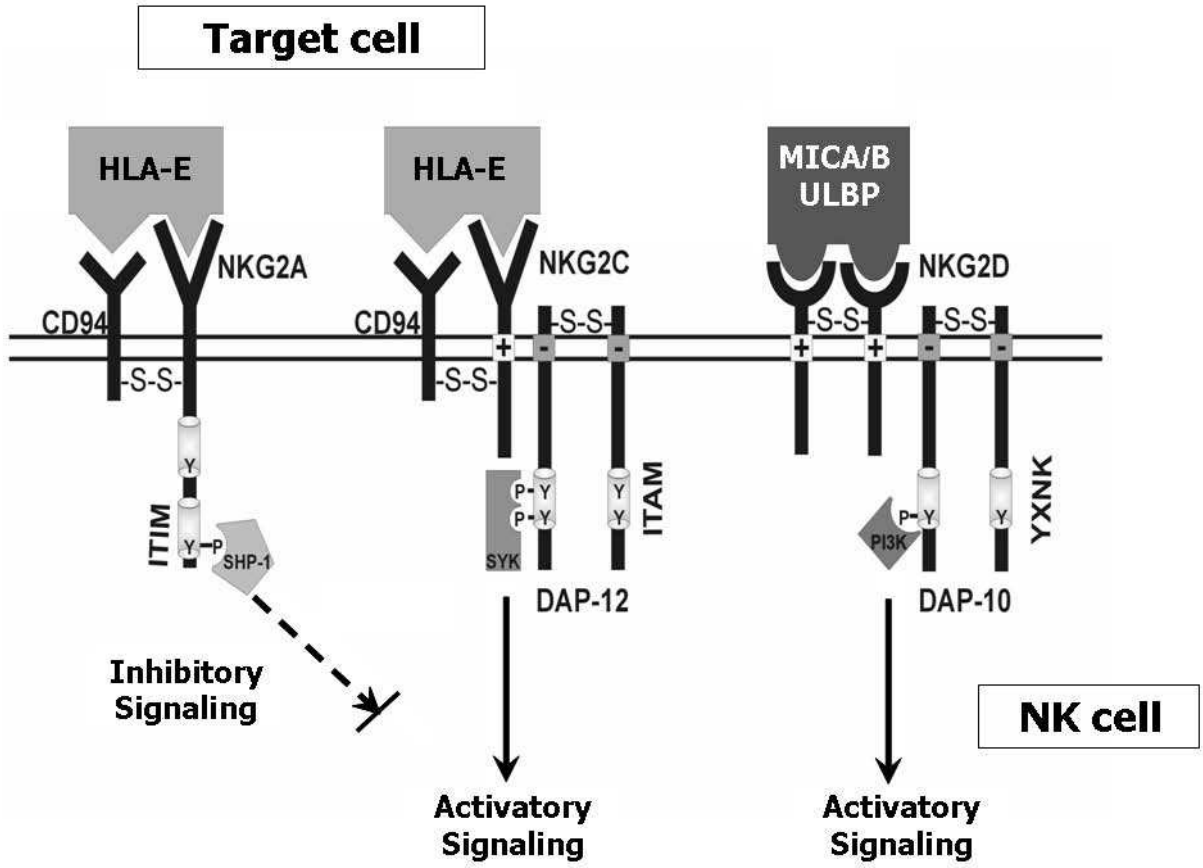

Fig. 1 Schematic representation of the operation mode of C-type lectin receptors in NK cells (adapted from [50, 55, 56]. C-type lectin receptors represent a heterogeneous group forming heterodimers of CD94 and certain members of the NKG2 family recognizing non-classical HLA-E molecules. CD94/NKG2A signals via tyrosine phosphatase SHP-1 through its SH2 domain enabling dephosphorylation of multiple targets in the ITIM-activating pathway resulting in negative signaling. NKG2C harbouring a positively charged transmembrane (TM) domain interacts with the negatively charged TM domain of the ITAM-containing adaptor molecule DAP-12 followed by binding and activation of the Syk family tyrosine kinases triggering the downstream activation cascade. In contrast, NKG2D forms homodimeric complexes to the exclusion of CD94 and binds MHC-like ligands MICA, MICB and ULBP family members. Activating signaling occurs via association with the adaptor molecule DAP-10 harboring a YXNK motif required for the binding of phosphatidyl inositol 3-kinase (PI3K).

urable and concentration-dependent [58, 59], a receptor-mediated interaction was hypothesized. In contrast to APC, the expression of HSP receptors including TLR and scavenger receptors were only weakly or not expressed on NK cells. In contrast, the cell surface density of the C-type lectin receptor CD94 was significantly up-regulated after co-incubation of NK cells either with Hsp70 protein or Hsp70 peptide TKD in combination with pro-inflammatory cytokines [64]. Concomitantly, the cytolytic and migratory capacity of NK cells towards Hsp70 membrane-positive tumor cells was found to be ini-

tiated [39]. Moreover, a CD94-specific antibody did not only block Hsp70 binding to NK cells but also the cytolytic activity towards Hsp70 membrane-positive tumor cells [59]. These data strongly suggest an involvement of CD94 in the interaction of NK cells with Hsp70 and Hsp70 peptide TKD.

A broad screening program of human tumor biopsies in our laboratory revealed that Hsp70, the major stress-inducible member of the HSP70 group, is frequently expressed on the plasma membrane of colon, lung, pancreas, mammary, head and neck and metastases derived thereof [65–67]. Also bone marrow-derived leukemic blasts from patients with hematological malignancies are frequently Hsp70 membrane-positive [68]. Interestingly, most metastases and relapsed tumors revealed an even enhanced density of Hsp70 on their cell surface as compared to the primary tumor (unpublished observations). Since the corresponding normal tissues and bone marrow of healthy human individuals always exhibited an Hsp70 membrane-negative phenotype, membrane-bound Hsp70 can be considered as a tumor-selective marker. It is worth mentioning that only the inducible Hsp70 and the Hsp70 peptide TKD in combination with pro-inflammatory cytokines, but not the highly homologous (84% sequence homology) constitutive Hsc70 were able to stimulate the activity of NK cells.

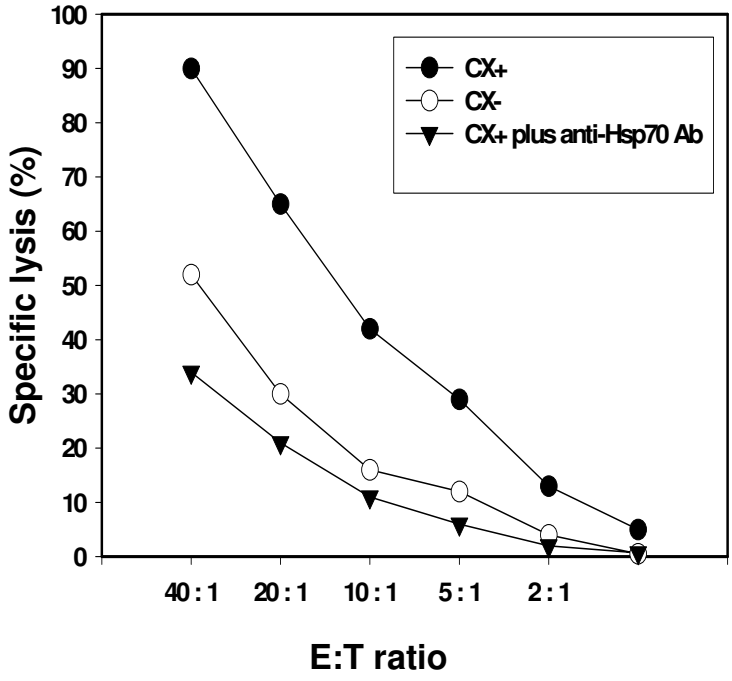

Fig. 2 Selective kill of Hsp70 membrane-positive CX+ tumor cells, mediated by NK cells, is blockable by an Hsp70-specific monoclonal antibody recognizing the Hsp70 peptide sequence TKD.

The cell surface density of Hsp70 on tumors could be further enhanced by clinically applied reagents and procedures including membrane-interactive alkyl-lysophospholipids [69], cytostatic drugs including taxoides and vincristinsulfate [70], cyclooxygenase (COX-

1/2) inhibitors, acetyl salicyl acid, insulin sensitizers [71], hyperthermia [72], and photodynamic therapy [73]. This increased Hsp70 membrane expression might render them more sensitive to the cytolytic attack mediated by TKD-activated NK cells. Although a variety of other chaperones were found to be present on the plasma membrane of tumor cells [74], predominantly cell surface-expressed Hsp70 and also gp96, an ER-residing member of the HSP90 family, are able to stimulate the immune system [65, 66, 75]. In concert with the pro-inflammatory cytokine IL-2, Hsp70 and Hsp70 peptide TKD were able to initiate an Hsp70-reactivity in NK cells.

The C-type lectin receptor CD94 was found to be essential for the cross-talk of NK cells with Hsp70 membrane-positive tumor cells [59]. Previous data indicated that Hsp70 protein as well as Hsp70 peptide TKD bind to CD94 on NK cells at 4°C and are internalized after a temperature shift to 37°C [58, 59]. Following internalization the density of CD94 on the plasma membrane of NK cells is highly up-regulated. An incubation of CD94-positive NK cells with Hsp70 peptide TKD plus IL-2 for 4 days also resulted in an up-regulated production of granzyme B, as determined by intracellular granzyme B flow cytometry (unpublished observation). Contact of these NK cells with Hsp70 membrane-positive tumor cells causes an increased kill of the Hsp70 membrane-positive colon carcinoma cell line CX+ as compared to Hsp70 membrane-negative CX- cells (Fig. 2). Following secretion of granzyme B by NK cells the intracellular granzyme B levels dropped (unpublished observation). It is not completely clear yet whether a second stimulation is able to restore the intracellular granzyme B levels or whether a new generation of NK cells are activated for the first time.

The enhanced cytolytic activity of NK cells against Hsp70 membrane-positive tumor cells is blockable by an Hsp70-specific monoclonal antibody recognizing the Hsp70 epitope TKD ([59], see also Fig. 2). From these data we hypothesize that membrane-bound Hsp70 is a relevant target structure for these NK cells. An incubation of effector NK cells with an anti-CD94 monoclonal antibody also results in blocking of lysis of Hsp70 membrane-positive tumor cells [58, 59]. These data indicate that CD94 most likely in combination with NKG2C is involved in the cytolytic activity of IL-2/TKD-activated NK cells. Receptors including TLR2 and TLR4 which have been described to mediate interaction of Hsp70 with APC [26] are unlikely to be important for NK cell/Hsp70 interaction, since irrespectively of the stimulation their expression was always less than 2% on NK cells [58]. We therefore conclude that they do not have an impact on the interaction of NK cells with Hsp70 peptide TKD.

Our investigations identified an alternative mode of NK cell-mediated tumor cell killing. The mechanism of lysis of Hsp70 membrane-positive tumor cells was biochemically characterized as granzyme B-mediated but perforin-independent apoptosis [64]. A schematic representation of the mechanism how granzyme B can initiate apoptosis is shown in Figure 3. Classically, NK cell-mediated tumor cell killing involves the exocytosis of secretory granules (SG) containing perforin (PFN) and granzyme B (GrB) [76]. It was assumed that these vesicles enter the target cells either by endocytosis or by receptor-mediated uptake. The role of the mannose 6-phosphate receptor (MPR) in granzyme

B uptake was recently refuted, since MPR-negative cells were killed by granzyme B-mediated apoptosis [77–79]. After internalization of secretory vesicles, granzyme B is released via perforin, and rapid DNA fragmentation occurs within the target cell by a mechanism that is attributed to the apoptotic granzyme activity [80–85]. As summarized schematically in Figure 3, apart from a vesicular-mediated uptake of granzyme B which requires perforin, enzymatic active granzyme B can be taken up through an alternative pathway which is mediated through membrane-bound Hsp70. Full-length Hsp70 as well as the 14-mer Hsp70 peptide TKD (aa 450-463) which is exposed to the extracellular milieu of tumor cells [60], both have the capacity to bind granzyme B as determined by affinity chromatography [64]. Following interaction, granzyme B uptake is facilitated by Hsp70 molecules which appear to mediate a "channel activity" for granzyme B. The interaction of HSP with granzyme B is supported by work of the group of Judy Liebermann [86] showing that granzyme B-coupled columns precipitate Hsp70 and Hsp27 from tumor cell lysates. We further demonstrated that even in the absence of perforin, granzyme B is taken up by Hsp70 membrane-positive tumor cells and thus causes apoptosis of this cell type [64]. These data led us to the following working hypothesis: in a first step granzyme B binds to membrane-bound Hsp70 on tumors. After this interaction the uptake of granzyme B into tumors is facilitated and thus apoptosis is initiated. *In vitro* results from the group of Antonio DeMaio are supporting our hypothesis. They could show that HSP70 proteins spontaneously form channels in artificial lipid bilayers that mediate cation transfer [87]. Recently published data from different groups speculate about an interaction of Hsp70 in lipid microdomains, also termed lipid rafts [88]. Biochemical interaction of Hsp70 with phosphatidylserine has been shown by the group of Antonio DeMaio [89]. Although the function of Hsp70 in lipid bilayers and rafts remains to be elucidated in detail it became apparent that Hsp70 is residing within the plasma membrane as an integral protein. More information is available on the mechanism of release of Hsp70. An active, exosomal export of Hsp70 from viable tumor cells expressing Hsp70 on their plasma membranes has been determined by several laboratories [38–40].

4 From bench to bedsite

Previous reports on different animal models revealed that IL-2-activated NK cells are able to induce regression of established lung and liver tumors and metastases of different origin The control of tumors and metastases corresponded with an extended life expectancy. In contrast, the injection of IL-2 alone was much less efficient compared to an adoptive transfer of IL-2-activated NK cells. Previous investigations by the group of Lutz Uharek and Matthias Zeis identified allogeneic IL-2-activated NK cells as potent anti-leukemic effector cells after allogeneic bone marrow transplantation in mice [95–99]. More recently, the same group was able to identify CD56dim/CD16bright NK cells as being primarily responsible for the cytotoxic activity against tumors [100]. NK cells have also been identified as essential components in effective adjuvant intravesical bacillus Calmette-Guérin (BCG) therapy in the treatment of superficial bladder cancer

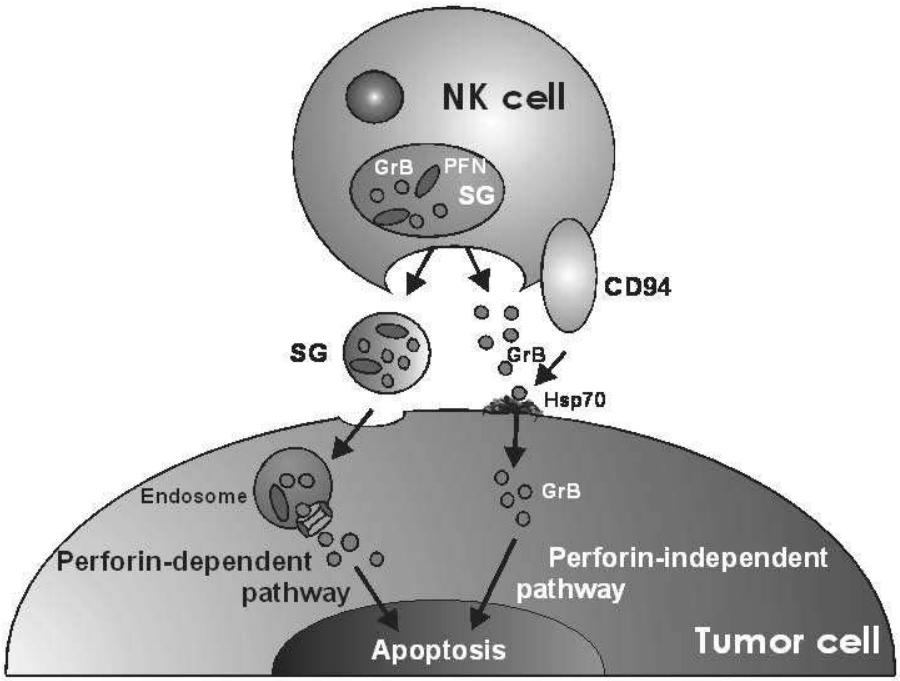

Fig. 3 Induction of apoptosis in Hsp70 membrane-positive tumor cells. Membrane-bound Hsp70 is selectively found on the surface of tumor cells but not on normal cells providing a target recognition structure for NK cells. Cross-talk between NK cells and Hsp70 membrane-positive tumor cells occurs via the C-type lectin receptor CD94 and the Hsp70 epitope TKD. On the one hand, the molecule stimulates production and delivery of granzyme B (GrB) by NK cells. On the other hand, it facilitates uptake of GrB selectively into Hsp70 membrane-positive tumors and thus causes apoptosis in a perforin-independent manner.
Abbreviations: SG, secretory granule; PFN, perforin.

in vitro and *in vivo* [101, 102]. The cytotoxic NK cell line NK-92 was shown to exhibit substantial anti-tumoral activity against a wide range of malignancies *in vitro* as well as in xenografted SCID mice. Tolerability, safety, and feasibility of repeated transfusions of irradiated NK-92 cells were shown in a phase I/II clinical trial in patients with advanced cancer [103]. Recently, the same group developed a protocol for clinical-use expansion of highly enriched and IL-2-stimulated NK cells. As demonstrated by the authors, *ex vivo* expansion of highly purified NK cells might be a new treatment option for pediatric patients with leukemia or solid tumors [104, 105]. The group of Theresa Whiteside and Ronald Herberman could convincingly demonstrate the beneficial effect of IL-2-activated NK cells in tumor cell killing [91, 106–108]. These data indicated that cytokine-stimulated NK cells exert beneficial effects on the control of tumors and distant metastases in immunocompetent and immunocompromised animals [109–111].

Cytokine-activated NK cells but not resting NK cells have been found to play an important role in the control of tumors and metastases [106, 112]. In the 1990s, Steve Rosenberg and Michael Lotze reported on a trend towards an increased survival in melanoma

patients treated with IL-2-activated NK cells [113]. A prospective randomized trial, performed six years later by the same group, in patients with metastatic melanoma comparing chemotherapy alone with an IL-2-based chemo-immunotherapy, did not reveal any survival benefit for patients receiving chemo-immunotherapy [114]. The administration of IL-2 alone to patients with acute myelogenous leukaemia (AML) in first complete remission was found to be tolerable, but only some AML patients showed clinical benefit [115]. A clinical trial in 23 patients with metastatic cancer demonstrated previously that the *in vivo* administration of low-dose IL-2 expanded the number of NK cells that could dramatically be augmented by additional IL-2 exposure *in vitro* [116]. However, the number of phase I/II clinical trials on adoptive immunotherapy using IL-2-activated NK cells is limited and does not allow firm conclusions, except to ascertain the feasibility and lack of toxicity of this therapeutic approach. A clinical phase I trial with 140 patients diagnosed for stage III colon carcinoma demonstrated a correlation between metastases-free survival rates and an enhanced NK cell activity *in vitro* [111]. These data indicate that the measurement of the NK cell activity might be relevant for the selection of patients at high risk for metastasis in advanced colon carcinomas.

As mentioned earlier, screening of primary tumors and metastases derived thereof revealed that Hsp70 is frequently expressed on the cell surface of malignant cell types. In contrast, the corresponding normal tissues were always found to be Hsp70 membrane-negative. Therefore, we hypothesized that membrane-bound Hsp70 acts as a tumor-specific recognition structure for the immune system. Since we observed that the cytolytic activity of NK cells *in vitro* could be further enhanced by incubation with IL-2 plus Hsp70 peptide TKD, we asked the question as to whether these NK cells might be superior in the eradication of tumors compared to NK cells that had been stimulated with IL-2 alone. In a xenograft SCID/beige mouse colon cancer model we studied the immunological effects of IL-2/TKD-activated NK cells [117]. A single injection of peripheral blood mononuclear cells containing about 10-20% of IL-2/TKD-activated NK cells resulted in a significant tumor regression. In the absence of the danger signal TKD, these effector cells were found to be significantly less efficient in the suppression of the growth of Hsp70 membrane-positive tumors [63].

We next studied the efficacy of IL-2/TKD-activated NK cells in eradication of pancreatic tumors in a xenograft SCID/beige mouse tumor model [118]. Pancreatic carcinoma is the fifth leading cause of cancer-related death in humans and refractory to conventional therapy. Phenotypical analysis revealed that Hsp70 is frequently present on the plasma membrane of pancreatic carcinomas including our model cell line Colo357. An orthotopic (o.t.) injection of Colo357 cells resulted in rapidly growing primary pancreatic tumors and in metastastic dissemination into the liver. In line with *in vitro* migration assays, IL-2/TKD-activated human NK cells had the capacity to infiltrate pancreatic tumors and liver metastases in tumor-bearing mice. These data are further supported by the group of Qin Yang who also showed the presence of cytokine-activated NK cells in lung metastases of immunocompetent mice [119].

We additionally analyzed life expectancy of tumor-bearing mice after a single i.v. in-

jection of pre-activated effector cells [118]. As shown in Fig. 4, immunodeficient control mice showed first signs of tumor disease from day 18 onwards; the maximum survival rate was 35 days. Adoptive transfer of pre-activated CD3+/CD94- T cells only marginally improved life expectancy with all animals dying from progressive tumor disease on day 37. In contrast, a single injection of TKD-activated CD3-/CD94+ NK cells significantly prolonged the survival of the mice, with more than 60% of the mice still alive on day 72. Thus, our *in vivo* mouse data imply that IL-2/TKD-activated NK cells might provide a novel therapeutic strategy for the treatment of therapy-refractory, Hsp70-positive pancreatic tumors. As shown recently [118], activation of NK cells with IL-2 alone resulted in death of all mice on day 52, indicating that Hsp70 peptide TKD is essential for the benefit in overall survival. Unstimulated NK cells and TKD-activated NK cells in the absence of IL-2 did not cause significant kill of Hsp70 membrane-positive tumors *in vitro* and thus were not tested in the xenograft tumor mouse model (unpublished observation).

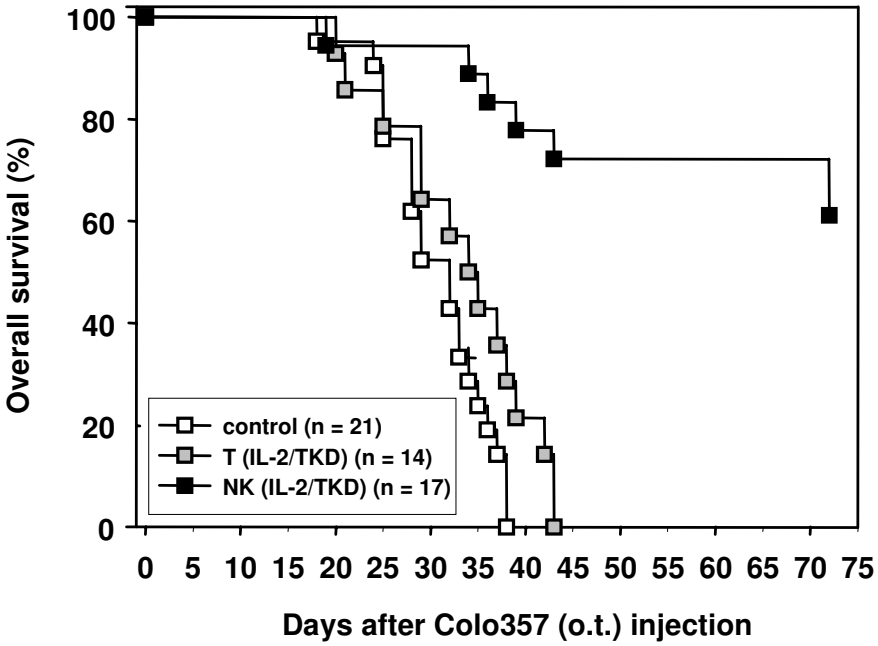

Fig. 4 Overall survival of tumor-bearing control mice and after treatment with TKD/IL-2-stimulated T and NK cells, respectively. 15 days after o.t. injection of Colo357 pancreas carcinoma cells, SCID/beige mice received a single i.v. injection of either medium (control), IL-2/TKD-activated CD3+/CD94- T or CD3-/CD94+ NK cells into the tail vein. The increased survival of mice injected with IL-2/TKD-activated NK cells was significant ($p < 0.01$) compared to control mice.

As mentioned before, a tumor-selective cell surface localization of Hsp70, the major heat-inducible member of the HSP70 group, could be correlated with an increased sensitivity to lysis mediated by IL-2/TKD-activated human NK cells, and therefore also might be of clinical relevance. We also could demonstrate that NK cells stimulated with IL-2/TKD have the capacity to eradicate Hsp70 membrane-positive tumors *in vitro* and

in tumor mouse models. These findings encouraged us to test these *ex vivo* IL-2/TKD-activated NK cells in a clinical trial. Patients with multiple metastasized colorectal and non-small cell lung carcinomas who failed standard therapy were enrolled into this phase I clinical trial [120].

The majority of patients showed a significant increase in the membrane expression of CD94 following *ex vivo* stimulation with IL-2/TKD. Concomitantly, the cytolytic activity towards Hsp70 membrane-positive tumor cells was augmented. Concerning tumor response, one patient was in stable disease during therapy by formal staging and another patient showed stable disease in one pulmonal metastasis and progression in another. This finding was not expected since all patients were in a progressive tumor stage and refractory to standard chemotherapy before entering the clinical trial. Taken together, the adoptive transfer of TKD-activated NK cells was feasible, safe and very well tolerated. Immunological results and clinical responses in two of five therapy-refractory, multiple metastasized patients warrant additional studies in patients with lower tumor burden and an established Hsp70 membrane-positive tumor stage. Within this context it is interesting to note that in human gastric carcinomas HSP70 immunoreactivity was found to correlate positively with the number of intratumoral NK cells [121]. The same report further demonstrated that HSP70 immunoreactivity is associated with advanced tumor stages and might function as a prognostic factor. Our findings are confirmed by several groups including those of Lutz Uharek and Theresa Whiteside showing that purified NK cells may be administered to tumor patients without adverse events and without inducing graft-versus-host disease (GvHD) [122–127] rendering NK cell-based adoptive immunotherapy an useful tool especially in situations where infusion of T cells is impractical such as in recipients of haploidentical stem cell transplantation from haploidentical donors [128]. Adoptive immunotherapy using *ex vivo* activated NK cells is of advantage with respect to their potential in preventing the incidence and severity of GvHD, promoting hematopoiesis, and augmenting numerous anti-tumor effects after bone marrow transplantation implying their crucial role in mediating graft-versus-tumor effects [129].

Allogeneic stem cell transplantation combined with donor lymphocyte infusion (DLI) provides a promising strategy in the therapy of recurrent chronic leukemia. However, therapeutic efficacy is limited by myelosuppression and transplant-related complications mainly caused by donor T lymphocytes. Recently, stem cell-derived, alloreactive NK cells have been found to mediate graft-versus-leukemia (GvL) effects without inducing GvHD [130, 131]. Efficiency has been demonstrated even in patients with advanced refractory disease following myeloablative regimens eliminating leukemic cells. Despite a strong anti-leukemic effect mediated by alloreactive donor lymphocytes, leading to a low relapse rate [132], transplant-related complications including GvHD [133] and immunosuppression increase mortality rates in these patients. GvHD can be modified by reducing the dose or by depleting donor-derived CD8-positive T cells [134] thus indicating that T cells are major mediators of transplant-associated complications. Unfortunately, a complete T cell depletion resulted in increased relapse and rejection rates. Therefore, an adoptive transfer of specific donor T lymphocyte subsets, pre- and post-transplantation, was investigated

[134–140]. However, a specific T cell subpopulation mediating only GvL without GvHD could not be isolated so far.

Regarding these results it became obvious that there is an urgent need for new therapeutic strategies improving the clinical outcome of allogeneic transplantation. In a mouse model the cytolytic potential of NK cells in adjuvant immunotherapy of hematological malignancies has been shown to reduce the risk for relapse without increasing GvHD [97, 141]. We could induce an NK cell-mediated anti-leukemic response by addition of Hsp70 peptide plus low dose IL-2 to patient-derived PBL [68]. The group of Bertram Glass and Lutz Uharek demonstrated a GvHD-independent GvL effect in NK cells [142]. Recently, the group of Andrea Velardi convincingly showed the beneficial effects of stem cell-derived, alloreactive NK cells in the treatment of AML patients [54, 143–145]. These NK cells did not only mediate GvL but also improved engraftment by a selective elimination of host-derived dendritic cells that might activate graft-reactive T cells. The anti-leukemic effect was dependent on the HLA-mismatch of donor and recipients, affecting the repertoire of killer cell inhibitory receptors (KIR). KIR-epitope mismatching in the graft-versus-host direction may confer unique potential for GvL effects and for engraftment [144] suggesting that infusion of alloreactive NK cells in humans will not cause GvHD [144]. The benefits of NK cell alloreactivity in mismatched hematopoietic transplantation has currently been highlighted by Michael Caligiuri and Andrea Velardi [147, 148]. Moreover, recent experimental and clinical data show the possibility of exploiting NK activity as a cell-based immunotherapy to treat cancer [149–152].

5 Conclusion

Within the last decade it became evident that NK cells, which were historically considered as non-specific killers, are highly sophisticated in distinguishing normal from tumor cells. The characterization of the molecular nature of a variety of NK cell receptors belonging either to the immunoglobulin-like, C-type lectin, or natural cytotoxicity receptor families and mediating either activatory or inhibitory signals provides an explanation for their mode of action. Together with the finding that NK cells provide the first line of defence it is very likely that NK cells have a high potential to become key players in the immunotherapy of cancer. A goal in the near future will be the development of protocols allowing an expansion of specific NK cell lines with anti-tumor activity. Another aim will be the elucidation of additional tumor-specific ligands that are recognized by activating NK cell receptors. Apart from non-classical, stress-inducible MHC molecules, recent studies indicated that a tumor-specific plasma membrane expression of Hsp70 serves as a recognition structure for NK cells that had been activated with Hsp70 peptide plus low dose IL-2. Feasibility, tolerability, and safety of *ex vivo* IL-2/TKD-activated, autologous NK cells was demonstrated in a phase I clinical trial in patients with locally advanced, metastasized colorectal and non-small cell lung carcinomas. Clinical responses were observed in patients receiving more than 4 repeated infusions of IL-2/TKD-activated NK cells. Therefore, we hypothesize that after surgical resection of an Hsp70 membrane-

positive tumor, patients with a high risk for metastatic dissemination might profit from an adoptive transfer of IL-2/TKD-activated NK cells.

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