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The endogenous danger signals HSP70 and MICA cooperate in the activation of cytotoxic effector functions of NK cells

Leslie Elsner [a, b], Perris F. Flügge [b], Jingky Lozano [c], Vijayakumar Muppala [a], Britta Eiz-Vesper [d], Sara Y. Demiroglu [a], Dörthe Malzahn [c], Thomas Herrmann [e], Edgar Brunner [f], Heike Bickeböller [c], Gabriele Multhoff [g], Lutz Walter [b, h], Ralf Dressel [a, b, *]

[a] Department of Cellular and Molecular Immunology, University of Göttingen, Germany
[b] Department of Immunogenetics, University of Göttingen, Germany
[c] Department of Genetic Epidemiology, University of Göttingen, Germany
[d] Institute for Transfusion Medicine, Hannover Medical School, Germany
[e] Institute of Virology and Immunobiology, University of Würzburg, Germany
[f] Department of Medical Statistics, University of Göttingen, Germany
[g] Department of Radiotherapy and Radiooncology, Technische Universität München and Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt, Germany
[h] Department of Primate Genetics, German Primate Center, Germany

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Abstract

Although natural killer (NK) cells are often described as first line defence against infected or malignant cells which act without the need of prior activation, it is known now that the NK cell activity is tightly regulated by other cells and soluble factors. We show here that the stress-inducible heat shock protein (HSP) 70 activates human NK cells to kill target cells expressing major histocompatibility complex class I chain-related molecule A (MICA) in a natural killer group 2 member D (NKG2D-) dependent manner. The HSP70-derived peptide TKD (TKDNNLLGRFELSG) was able to replace the full-length HSP70 and to exert the same function. Interestingly, the expression of the cytotoxic effector protease granzyme B in NK cells was increased after TKD stimulation. When MICA and MICB expression was induced in human tumour cells by a histone deacetylase inhibitor and NK cells were activated by HSP70 or TKD, both treatments jointly improved the killing of the tumour cells. Thus, the synergistic activity of two stress-inducible immunological danger signals, HSP70 and MICA/B, leads to activation and enhanced cytotoxicity of human NK cells against tumour cells.

Keywords: heat shock protein 70 • NKG2D ligands • natural killer cells • cellular cytotoxicity • cancer • immunotherapy

Introduction

Natural killer (NK) cells are often described as cytotoxic effector lymphocytes which can kill virus-infected and tumour cells without the need of prior activation or 'priming'. However, this picture has become more complex, because it is known that NK cells receive signals from other cells, which regulate their activity against infected or malignant target cells and also their self-tolerance [1–5]. NK cell effector functions such as cytotoxicity and cytokine production are often not easily detectable without providing naïve NK cells, especially naïve mouse NK cells, with additional signals for their activation. Thus, mice are frequently treated with cytokines, *e.g.* type I interferons (IFN), before NK cells are harvested or mouse and human NK cells are cultured *ex vivo* in the presence of cytokines such as interleukin (IL)-2 or IL-15, to obtain a NK cell population with detectable effector functions. Recent data indicate that the interaction with dendritic cells (DCs) is important for NK cell activation *in vivo* [6]. A number of signals, including IL-12, IL-15 and IFN-α, which can result in the activation of NK cells appear to be provided by DCs [7].

Interestingly, human NK cells have been shown previously to be activated by the stress-inducible heat shock protein (HSP) 70

*Correspondence to: Dr. Ralf DRESSEL,
Department of Cellular and Molecular Immunology,
University of Göttingen, Heinrich-Düker-Weg 12,
37073 Göttingen, Germany.
Tel.: 0049-(0)551-395884
Fax: 0049-(0)551-395852
E-mail: rdresse@gwdg.de

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to kill specifically tumour cells that express HSP70 at the cell surface [8, 9]. In accordance with this stimulatory property of HSP70, we observed in a xenograft model [10] that the growth of HSP70-overexpressing human melanoma cells was reduced in immunodeficient SCID mice, which lack B and T lymphocytes, compared to cells overexpressing control proteins. In SCID/beige mice, which lack also functional NK cells, the growth of the tumours was not different, indicating a crucial role of NK cells in the defence against the tumours. We could also show that cytotoxic cells were activated in SCID mice with HSP70-overexpressing tumours to kill target cells that expressed NKG2D ligands, but not HSP70 at the cell surface. NKG2D (natural killer group 2, member D) is an activating NK receptor [11, 12]. The HSP70-overexpressing melanoma cells released HSP70-containing exosomes and these exosomes were able to stimulate mouse NK cells *in vitro* to kill target cells expressing NKG2D ligands [10].

Now we wanted to analyse at the molecular level whether indeed the HSP70 protein itself is able to stimulate NK cells to kill target cells which express NKG2D ligands such as MICA (major histocompatibility complex class I chain-related molecule A). Furthermore, we addressed the question whether both stress-inducible danger signals, HSP70 and MICA/B, jointly augment the cytotoxicity of human NK cells.

We performed experiments with human NK cells as effectors and as targets MICA-transfected L cells. In addition, we analysed the human melanoma cells that we have studied previously in a mouse xenograft model [10] as targets for HSP70-activated human NK cells. The obtained results clearly confirm the relevance of the concerted action of HSP70 and MICA for activation of human NK cells against tumour cells. A combination of both danger signals, HSP70 and MICA, might be exploitable for immunotherapy of human cancer.

Materials and methods

Target cell lines and cell culture

The human melanoma cell lines Ge-Hsp70 (clone Ge-Hsp70-A) and Ge-con (clone Ge-TCR-C) [10, 13], human erythroleukaemia K562 cells, mouse fibroblast L cells, MICA and empty vector-transfected L cells were maintained under standard conditions described previously [10, 13]. To induce MICA/B expression, the melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10 μM of the histone deacetylase inhibitor suberoylanilide hydroxyamic acid (SAHA) (Qbiogene-Alexis, Grünberg, Germany) 20 hrs before being used for experiments [14]. Mouse L cells were co-transfected by electroporation with 30 μg of the cosmid A158 [15] containing the *MICA* gene of the rhesus macaque (*Macaca mulatta*) [16], and with 1 μg of the DsRed vector (Clontech, Heidelberg, Germany), which confers resistance to geneticin (G418, Invitrogen, Karlsruhe, Germany). After selection with geneticin (1 mg/ml), MICA expression was analysed in stable clones by flow cytometry. Four independent clones were tested as target cells for human lymphokine-activated killer (LAK) cells (data not shown) confirming susceptibility to human LAK cells due to MICA expression. Clone K43 (L-MICA) was selected for the further studies and compared to parental L cells and empty vector-transfected control cells.

Recombinant HSP70 and HSC70

The His-tagged recombinant HSP70 protein derived from the rat *Hsp70–1* gene has been previously described [17] and is designated here as rHSP70. The rat *Hsc70* gene was amplified from rat lymphocyte cDNA by PCR (forward: 5′-GGATCCATGTCTAAGGGACCTGCAGTT-3′ and reverse 5′-GAATTCGACTTAATCGACCTCTTCAATGGT-3′). The forward primer included a *Bam*HI and the reverse primer an *Eco*RI restriction site at their 5′ ends. The amplification product was cloned into the pGEX-4T-2 expression vector (Amersham Pharmacia, Freiburg, Germany) *via Bam*HI and *Eco*RI. A *Bam*HI/*Sal*I restriction fragment was isolated from this vector and recloned into the pQE30–1 expression vector (Qiagen, Hilden, Germany) for expression of a His-tagged HSC70 protein designated as rHSC70. The construct was sequenced to exclude missense mutations. The *E. coli* strain M15 (Qiagen) was transformed with this construct and used as host for overexpression of the His-tagged proteins. Induction and purification of the proteins was done as described previously [17]. Endotoxin-free recombinant human HSP70 was produced as a secreted protein in an eukaryotic expression system as described elsewhere [18] and is designated here as Euk hHSP70.

Effector cells, effector cell culture and ^{51}Chromium release assays

Human effector cells were obtained from peripheral blood of healthy volunteers by centrifugation on Biocoll separating solution (Biochrom, Berlin, Germany). To obtain LAK cells the peripheral blood mononuclear cells (PBMCs) were cultured for 4 to 8 days in 5-ml Petri dishes for tissue culture (Sarstedt, Nümbrecht, Germany) at a density of 5 to 10 $\times$ 10^6 cells/ml in DMEM supplemented with 100 U/ml IL-2 (Proleukin, Chiron, Amsterdam, Netherlands). NK cells were isolated from PBMCs by magnetic-acitivated cell sorting (MACS) using a negative selection kit (NK cell isolation kit II, 130–091-152; Miltenyi Biotec, Bergisch-Gladbach, Germany) and cultured in 24-well plates for tissue culture (Sarstedt) at a density of 2 $\times$ 10^6 cells/ml. To some cultures 2 μg/ml recombinant HSP70 or heat shock cognate (HSC) 70 or peptides were added. Recombinant human 'low endotoxin' HSP70 (ESP-555, endotoxin concentration $<$50 EU/mg) was purchased from StressGen (via Biomol, Hamburg, Germany). The peptide TKD [19] is a GMP-grade 14-mer peptide of the C-terminal substrate-binding domain of human HSP70 (TKDNNLLGRFELSG, aa 450–463) and was purchased from Bachem (Bubendorf, Switzerland). TKD has been shown to augment in cooperation with IL-2 the cytotoxic activity of NK cells against tumour cells that express HSP70 at the cell membrane [19]. This sequence also generates the epitope that is present at the cell surface of HSP70 membrane-positive tumours [20] and can function as recognition structure for CD94-positive NK cells [21–23]. NGL (NGLTLKNDFSRLEG) is a scrambled peptide containing the same amino acids as TKD. Lipopolysaccharide (LPS) from *E.coli* (L4391) was from Sigma (Taufkirchen, Germany). ^{51}Chromium release assays were performed as previously described [17]. A recombinant human NKG2D-Fc chimeric protein (1299-NK, R&D Systems, Wiesbaden, Germany) and the anti-MICA monoclonal antibody (mAb) AMO1 (mouse IgG_1, Bamomab GmbH, Munich, Germany) were used to inhibit the lysis of target cells. An IgG_1 isotype control was purchased from Caltag (Caltag Laboratories, Hamburg, Germany).

Flow cytometry

Flow cytometry was performed with a FACScan™ flow cytometer (BD Biosciences, Heidelberg, Germany) and CellQuest™ software. Expression of intracellular HSP70 and TCRβ in Ge-Hsp70 and Ge-con clones, respectively, was regularly monitored by flow cytometry as described previously [13]. Cell surface expression of HSP70 on propidium iodide negative cells was examined using a mAb that has been reported [20] to detect HSP70 on the plasma membrane (cmHsp70.1, mouse IgG_1, multimmune GmbH, Munich, Germany). MICA/B cell surface expression was determined using the mAbs BAMO1 (mouse IgG_1, Bamomab), 6D4 (mouse IgG_{2a}, BD Biosciences), BAMO3 (mouse IgG_{2a}), and IIIC1 (the two latter kindly provided by Dr. A. Steinle, University of Tübingen, Germany) which all react with human MICA and MICB. Furthermore, the MICA specific mAb AMO1 (mouse IgG_1, Bamomab) and MICB specific mAb BMO2 (mouse IgG_{2a}, Bamomab) were used. To detect ULBP1, ULBP2, and ULBP3 expression the mAbs AUMO2 (mouse IgG_{2a}), BUMO1 (mouse IgG_1), and CUMO1 (mouse IgG_1) were purchased from Bamomab. Intracellular granzyme B expression was analysed using the B18.1 mAb (mouse IgG_1, Alexis) after permeabilization of the cells with 0.25% saponin as described before for HSP70 [13]. The binding of these unlabelled mouse IgG antibodies was revealed using a polyclonal fluorescein (FITC)-conjugated goat anti-mouse IgG Ab (115–095-062; Jackson Laboratories, Dianova, Hamburg, Germany). For the recombinant human NKG2D-Fc chimeric protein (1299-NK), which was used to detect cell surface expression of NKG2D ligands, a polyclonal FITC-conjugated goat anti-human IgG Ab (109–095-098; Jackson Laboratories) served as secondary reagent. Human PBMCs and NK cell enriched and depleted fractions were characterized using antibodies reactive against CD3 (clone MEM 57, mouse IgG_{2a}, FITC-conjugated, Immunotools, Friesoythe, Germany), CD4 (clone S3.5, mouse IgG_{2a}, phycoerythrin (PE)-conjugated, Caltag Laboratories), CD8 (clone 3B5, mouse IgG_{2a}, tricolor-conjugated, Caltag), CD14 (clone Tük4, mouse IgG_{2a}, PE-conjugated, Caltag), CD16 (clone 3G3, mouse IgG_1, tricolor-conjugated, Caltag), CD56 (clone MEM 188, mouse IgG_{2a}, PE-conjugated, Caltag), CD94 (clone HP-3D9, mouse IgG_1, FITC-conjugated, Becton Dickinson), and NKG2D (clone 149810, mouse IgG_1, PE-conjugated, R&D Systems). Isotype controls (mouse IgG_1 and IgG_{2a}) were purchased from Caltag.

Statistics

All data were analysed using the SAS version 9.1 software. ANOVA was used to analyse designs involving two or more factors. The different factors were incorporated into two-way or three-way ANOVA involving interactions. The t-test was used for the analysis of two paired and unpaired samples. A repeated measures ANOVA was carried out in all experimental designs with replicates. A significance level of $\alpha = 0.05$ was used. Adjustments for multiple comparisons were performed where appropriate due to subgroup testing.

Results

MICA-transfected L cells are susceptible to human LAK cells

Our previous findings suggested that HSP70 containing exosomes released by HSP70-overexpressing human melanoma cells activate *in vivo* mouse NK cells to kill human tumour cells that express NKG2D ligands, such as MICA/B [10]. Therefore, we wanted to test now whether the HSP70 protein itself can activate human NK cells to kill MICA/B expressing target cells. We had available mouse L cells transfected with a cosmid containing the *MICA* gene derived from the rhesus macaque (*Macaca mulatta*). These L-MICA cells express MICA at the cell surface and do not express HSP70 at the plasma membrane (Fig. 1). The transfected MICA functioned readily as target structure for human natural cytotoxic cells. In contrast to parental or vector transfected L-con cells, L-MICA cells were readily killed by IL-2-stimulated human LAK cells (data not shown). Similar results were obtained using further clones stably transfected with this cosmid or a *MICA* cDNA expression construct (data not shown). Control cells transfected with vectors only did not differ from parental L cells in these experiments (data not shown). The L-MICA cells transfected with the cosmid were selected for further experiments because the highest level of MICA expression were obtained in these transfectants (data not shown).

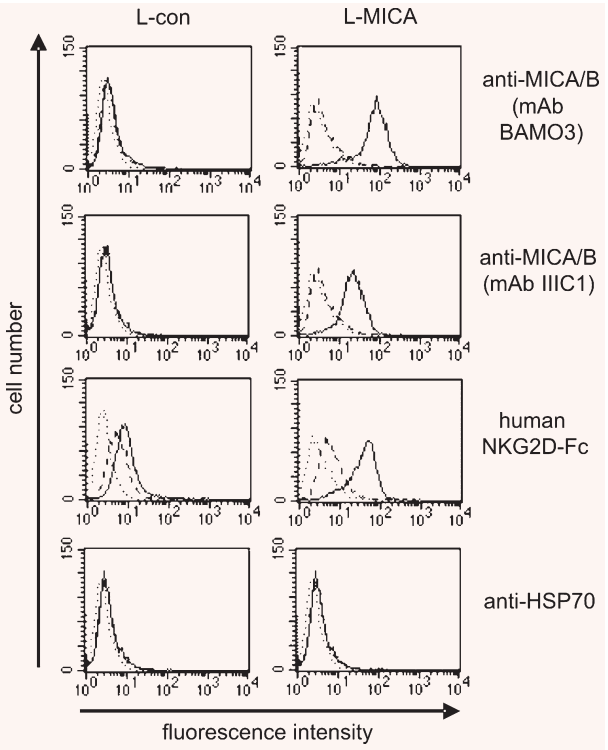


Fig. 1 L-MICA cell react with human NKG2D and MICA/B-specific antibodies. The expression of MICA and HSP70 was analysed by flow cytometry on MICA-transfected L-MICA and L-con cells. MICA cell surface expression is shown by staining with mAb against human MICA and MICB (BAMO3 and IIIC1) and by binding of a recombinant human NKG2D-Fc fusion protein. HSP70 cell surface expression was analysed using the mAb cmHsp70.1. Staining with the respective primary reagent (solid line) and FITC-labelled secondary reagent only (dashed line) is shown together with unstained cells (dotted line).

HSP70 but not HSC70 activates human PBMCs to kill MICA-expressing target cells

Human PBMCs were cultured for 8 days in the presence of low dose IL-2 (100 U/ml) and recombinant rat HSPs, either 2 μg/ml of the stress-inducible rHSP70 or the constitutively expressed heat shock cognate 70 (rHSC70). The MHC-encoded human and rat HSP70 proteins are 96.6% identical and the human and rat HSC70 proteins differ only in one amino acid (99.8% identity) [24]. The IL-2 treated PBMCs killed the MICA-expressing L cells, but not the MICA-negative L-con cells. In contrast to rHSC70 treatment, rHSP70 provided an additional stimulatory effect and enhanced the lysis of the L-MICA cells by IL-2 treated PBMCs as exemplified in Fig. 2A. A summary of six experiments is shown in Fig. 2B and confirms the different effects of rHSP70 and rHSC70 on the activity of LAK cells against L-MICA target cells ($P = 0.0227$, ANOVA). Thus, rHSP70 ($P = 0.0021$, ANOVA), but not rHSC70 ($P = 0.3241$, ANOVA) was able to further activate IL-2 stimulated LAK cells to kill MICA-expressing target cells. Both proteins, rHSP70 and rHSC70, were prepared as recombinant proteins in *E. coli* and can be expected to be contaminated with LPS. Therefore, we used additionally recombinant 'low endotoxin' (<50 EU/mg) human HSP70 and observed similar effects (data not shown). Furthermore, we tested the effects of rHSP70 and LPS on LAK cell stimulation in parallel. In these experiments, only the rHSP70 treatment significantly increased ($P = 0.0374$, ANOVA) the capacity of LAK cells to kill L-MICA cells (Fig. 2C). To avoid LPS contamination of HSP70 preparations completely, we used two strategies. First, endotoxin-free human HSP70 produced as secreted protein in eukaryotic cells (Euk hHSP70) was used to stimulate PBMCs together with IL-2. This Euk hHSP70 increased the relative cytotoxic activity of the effector cells compared to LAK cells that were stimulated only with IL-2 ($P < 0.0001$, ANOVA) in the same way as the recombinant rHSP70 produced in *E.coli* ($P < 0.0001$, ANOVA) (Fig. 2D). Secondly, we used the human HSP70-derived peptide TKD (TKDNNLLGR-FELSG), which was produced by chemical synthesis. The TKD peptide has previously been shown to be equivalent to the full length HSP70 in its ability to stimulate human NK cells to kill HSP70 cell surface positive target cells [19]. We found that the relative lysis of L-MICA was significantly increased when the effector cells were stimulated with IL-2 and TKD (2 μg/ml), compared to effector cells cultured with IL-2 only ($P < 0.0001$, ANOVA). Similarly the relative cytotoxic activity was augmented by IL-2 plus TKD compared to IL-2 plus the scrambled peptide NGL (NGLTLKNDFSRLEG) ($P < 0.0001$, ANOVA) (Fig. 2E). We compared the effects of IL-2, IL-2 plus HSP70, and IL-2 plus TKD on the proportion of various effector cell types in the LAK cell cultures at days 0, 4 and 8. We did not observe differences of the proportion of $CD3^+CD4^+$, $CD3^+CD8^+$, $CD16^+$, $CD56^+$, and $CD94^+$ cells (data not shown). The percentage of NKG2D positive cells increased in the time course of the culture in presence of IL-2 ($P < 0.0005$, ANOVA) but no additional effect of HSP70 or TKD was observed and the absolute number of cells was not different after culture with IL-2 only compared to IL-2 plus HSP70 or IL-2 plus TKD (data not shown).

NK cells are the effector cells that are activated by the HSP70 peptide TKD

We assumed from our previous data in the mouse model [10] that NK cells are the cytotoxic effector cells among the PBMCs that are activated by IL-2 plus HSP70 or IL-2 plus TKD. However, other cells such as $CD8^+$ T cells or γδT cells can express the MICA and MICB receptor NKG2D [11, 12] and might therefore contribute to the effects observed in the human PBMC cultures. Thus, we isolated NK cells from the peripheral blood of voluntary donors by MACS before the *in vitro* culture (Fig. 3A). In parallel to the stimulated NK cells, we tested the cytotoxic activity of the stimulated but NK cell-depleted cell population. The NK cell-enriched fraction killed L-MICA cells much better than L-con cells (Fig. 3B), and TKD plus IL-2 stimulation was able to significantly improve the relative activity of the NK cells against L-MICA cells when compared with IL-2 only ($P < 0.0001$, ANOVA) or with NGL plus IL-2 ($P = 0.0005$, ANOVA) (Fig. 3C). Stimulation of NK cell-depleted PBMCs by IL-2 or IL-2 plus TKD did not result in a cell population that was able to kill L-MICA cells (Fig. 3B). Even at high effector to target ratios (up to 200:1), the lysis of L-MICA and L-con cells remained very low (data not shown). Similar to TKD, also recombinant rHSP70 was able to stimulate isolated human NK cells to kill L-MICA targets (data not shown). Thus, NK cells mediate the HSP70 or TKD-induced cytotoxicity against MICA-expressing target cells. Furthermore, these experiments suggest that the isolated NK cells can be stimulated directly by HSP70 or TKD.

HSP70 peptide (TKD) stimulation of NK cells increases granzyme B expression

To further analyse the effect of the HSP70 peptide TKD on NK cells we determined the expression of the NK cell markers CD56, CD94, CD16 and of NKG2D on NK cells that had been cultured for 5 days with IL-2, or IL-2 plus TKD, or IL-2 plus NGL (Fig. 4A). The proportion of NKG2D positive cells did not increase by TKD treatment (Fig. 4A) and similarly the mean fluorescence intensity (MFI) of NKG2D was not significantly altered (Fig. 4B). We then speculated that TKD treatment might augment the expression of cytotoxic effector molecules in the activated NK cells. Indeed, flow cytometric analysis revealed that IL-2 plus TKD stimulation results in an increased expression of intracellular granzyme B (Fig. 4C) compared to IL-2 stimulation alone ($P < 0.01$, paired t-test). Thus, the HSP70 effects on NK cells that we observed is, in part at least, explainable by an increased expression of the cytotoxic effector molecule granzyme B. However, the increased granzyme B expression in HSP70 or TKD-stimulated effectors resulted in increased killing only if the cytotoxicity was sufficiently triggered by MICA on the targets (see Figs 2 and 3).

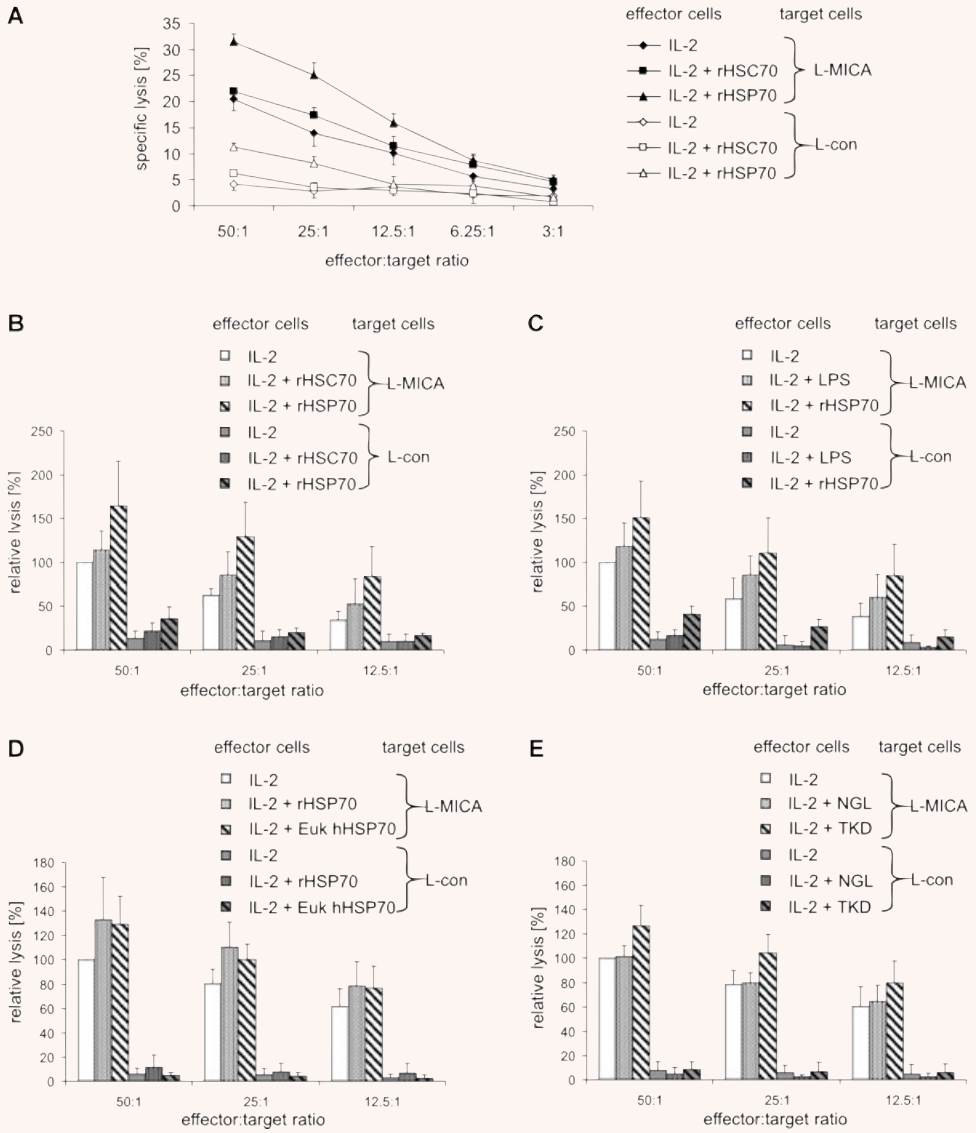
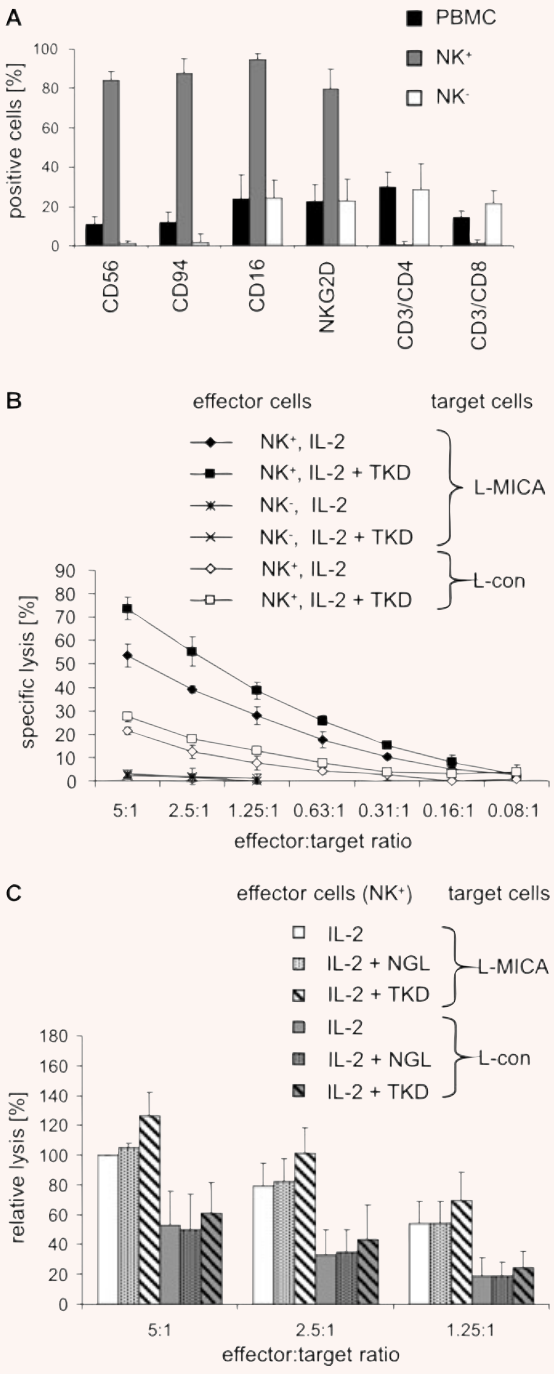


Fig. 2 LAK cells are stimulated to kill MICA-expressing target cells by HSP70 and the HSP70-derived peptide TKD, but not by HSC70. (**A**) The mean of specific lysis ± S.D. of triplicates of L-MICA (closed symbols) or L-con (open symbols) target cells by PBMCs stimulated *in vitro* for 8 days with IL-2 only (100 U/ml) or IL-2 plus recombinant rHSP70 (2 μg/ml) or rHSC70 (2 μg/ml) are shown. The results shown here were obtained in an individual experiment that is included in the summary shown in panel B. (**B**) The mean of relative lysis + S.D. of L-MICA and L-con target cells by LAK cells stimulated for 8 days with IL-2 (100 U/ml), IL-2 plus rHSC70 (2 μg/ml), or IL-2 plus rHSP70 (2 μg/ml) was determined in four experiments. The percentage of specific lysis of L-MICA cells by IL-2 stimulated PBMCs at the highest effector to target ratio (50:1) was adjusted to 100% in each test and the relative lysis of the target cells by various effector cells at different effector to target ratios was calculated in this panel and the following panels (**C**–**E**). (**C**) The mean of relative lysis + S.D. of L-MICA and L-con target cells by LAK cells stimulated for 8 days with IL-2 (100 U/ml), IL-2 plus rHSP70 (2 μg/ml), or IL-2 plus LPS (10 ng/ml) was determined in six experiments. (**D**) The mean of relative lysis + S.D. of L-MICA and L-con target cells by LAK cells stimulated for 8 days with IL-2 (100 U/ml), IL-2 plus rHSP70 (2 μg/ml), or IL-2 plus Euk hHSP70 (2 μg/ml) was determined in eight experiments. (**E**) The mean of relative lysis + S.D. of L-MICA and L-con target cells by LAK cells stimulated for 8 days with IL-2 (100 U/ml), or IL-2 plus TKD (2 μg/ml), or IL-2 plus the scrambled peptide NGL (2 μg/ml) was determined in 15 (TKD) or 8 (NGL) experiments.



MICA/B induction on Ge melanoma cells improves their killing by LAK cells

Are the results obtained with the MICA-transfected targets and human NK cells relevant for the killing of human tumour cells? We have previously shown that in SCID mice with HSP70-overexpressing tumours derived from the human melanoma cell line (Ge-Hsp70) mouse NK cells were activated by released HSP70 to kill tumour cells that expressed MICA/B [10]. Now we wanted to test whether these melanoma cells can also become targets for HSP70 or TKD-activated human NK cells. Therefore, we induced MICA/B expression on control Ge-con and HSP70-overexpressing Ge-Hsp70 cells by the histone deacetylase inhibitor SAHA (Fig. 5A). The proportion of Ge-con and Ge-Hsp70 cells positive for NKG2D ligands and specifically MICA/B was significantly increased in these experiments by SAHA treatment as shown in Fig. 5A ($P < 0.0001$, ANOVA). In addition, also the MFI for NKG2D ligands and MICA/B significantly increased ($P < 0.0001$, ANOVA) (Fig. 5A). No significant differences were observed for the expression of NKG2D ligands and specifically MICA/B on Ge-con *versus* Ge-Hsp70 cells either before or after SAHA treatment. Staining with MICA and MICB specific mAbs indicated that both molecules were induced by SAHA (Fig. 5A). Importantly, we did not find HSP70 to be expressed at the cell surface before or after this treatment (Fig. 5A). In addition to MICA and MICB, the NKG2D ligands ULBP2 and ULBP3 were found to be slightly induced after SAHA treatment (Fig. 5B).

MICA/B induction on target cells and TKD activation of NK cells act synergistically

In the following experiments, isolated human NK cells were cultured for 5 days without IL-2, with IL-2, or with IL-2 plus

Fig. 3 NK cells are the effector cells that are stimulated to kill MICA-expressing target cells by the HSP70-derived peptide TKD. (**A**) Flow cytometric analyses of PBMCs (before MACS separation) and NK cell-enriched (NK^+) as well as NK cell-depleted (NK^-) cell populations was performed. The mean of the proportion of marker positive cells + S.D. of 17 experiments is given. (**B**) The mean specific lysis ± S.D. of triplicates of L-MICA and L-con target cells by isolated NK cells (NK^+) or NK cell-depleted PBMCs (NK^-) stimulated *in vitro* for 5 days with IL-2 (100 U/ml) or IL-2 plus TKD (2 μg/ml) is shown. The result shown is an individual experiment out of 3 similar tests with NK^- and 17 with NK^+ cells as effector cells. (**C**) The mean of relative lysis + S.D. of L-MICA and L-con target cells by NK cells stimulated for 5 days with IL-2 (100 U/ml), or IL-2 plus TKD (2 μg/ml), or IL-2 plus the scrambled peptide NGL (2 μg/ml) was determined in 17 (TKD) or 8 (NGL) experiments. The percentage of specific lysis of L-MICA cells by IL-2 stimulated NK cells at the highest effector to target ratio (5:1) was adjusted to 100% in each test and the relative lysis of the target cells by various effector cells at different effector to target ratios was calculated.

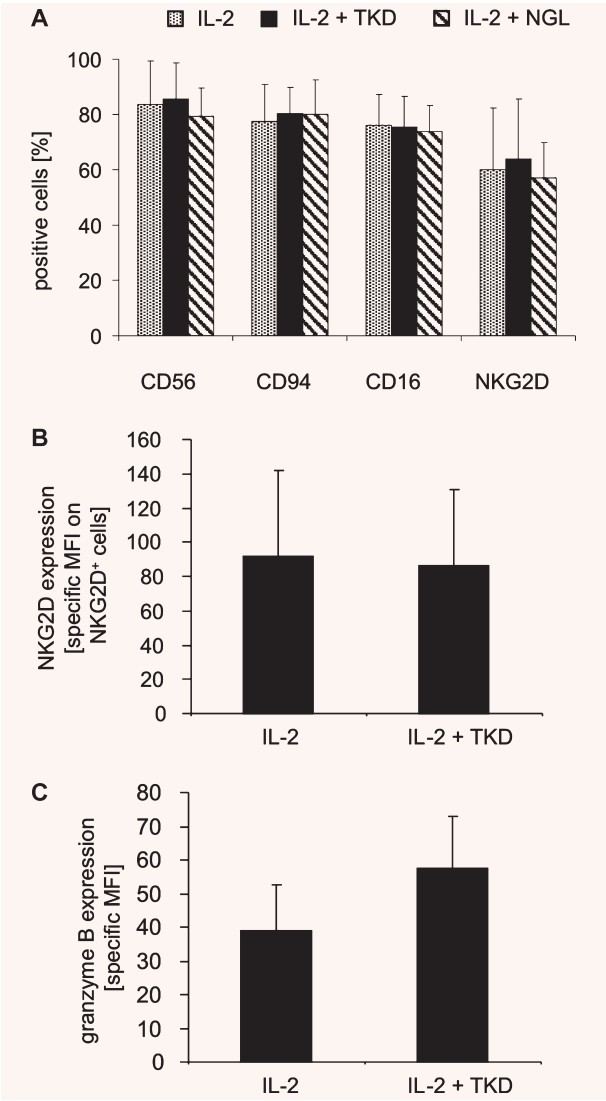


Fig. 4 NK cells express increased amounts of granzyme B after stimulation with TKD. (**A**) MACS-enriched NK cells after 5 days culture with IL-2 (100 U/ml), or IL-2 (100 U/ml) plus TKD (2 μg/ml), or IL-2 (100 U/ml) plus NGL (2 μg/ml) were analysed by flow cytometry for the expression of CD56, CD94, CD16 and NKG2D. The mean of marker positive cells + S.D. of 39 (IL-2), 34 (IL-2 plus TKD) or 10 (IL-2 plus NGL) experiments are given. (**B**) The mean + S.D. of the specific MFI of NKG2D (MFI for the specific staining minus MFI for the isotype control) on NK cells cultured in the presence of IL-2 or IL-2 plus TKD is shown. (**C**) The mean + S.D. of the specific MFI for granzyme B (MFI for the specific staining minus MFI for the staining with the secondary Ab alone) was analysed in eight experiments, and was determined by intracellular flow cytometry in NK cells cultured for 5 days with IL-2 (100 U/ml) or IL-2 (100 U/ml) plus TKD (2 μg/ml).

TKD before they were used as effector cells against Ge-con or Ge-Hsp70 target cells that had been cultured either under standard conditions, or for 20 hrs in the presence of a histone deacetylase inhibitor (10 μM SAHA) to induce MICA/B. As indicated in Fig 6A, IL-2 was indispensable for NK cell-mediated killing of Ge-con and Ge-Hsp70 melanoma cells. TKD treatment of NK cells increased their capability to lyse target cells. SAHA treatment of Ge target cells augmented their susceptibility to NK cells. The combination of TKD treatment of NK cells and SAHA treatment of target cells resulted in the highest killing (Fig. 6A). A summary of these results is given in Fig. 6B. TKD treatment of NK cells significantly augmented their cytotoxic activity against Ge-con and Ge-Hsp70 target cells (Ge-con: $P = 0.0055$ and Ge-Hsp70: $P = 0.0096$; three-way ANOVA as for all results reported in this paragraph). SAHA treatment of Ge target cells significantly increased their susceptibility to NK cells (Ge-con and Ge-Hsp70: $P < 0.0001$). Thus, the combination of TKD treatment of NK cells and SAHA treatment of target cells acted synergistically (*P*-value for interaction, Ge-con: $P = 0.0003$, Ge-Hsp70: $P < 0.0001$), and significantly increased target cell killing in these experiments. Similar results were obtained after stimulation of NK cells with IL-2 plus full-length recombinant rHSP70 compared to IL-2 plus LPS and testing them against both target cell lines Ge-con and Ge-Hsp70 (data not shown). Thus, in accordance with the mouse experiments [10], HSP70 and MICA/B can jointly augment killing of human Ge tumour cells by human NK cells.

Lysis of MICA/B expressing cells can be partially inhibited by soluble NKG2D

IL-2-activated human NK cells killed the human melanoma cells to some extent even if they did not express NKG2D ligands. However, the induction of NKG2D ligands further improved the killing. Therefore, we wanted to test whether this improvement of lysis was indeed dependent on the interaction between NKG2D and its ligands. The increase of killing of Ge-con cells by SAHA-mediated induction of NKG2D ligands was reversible by a soluble NKG2D-Fc construct (3 μg/ml) as exemplified in Fig. 7A. Similarly, the anti-MICA mAb AMO1 that blocks the NKG2D epitope of MICA was also able to partially inhibit the lysis of SAHA-treated melanoma cells in contrast to an isotype control (Fig. 7B). Thus, the expression of NKG2D ligands and the subsequent recognition of these ligands by NK cells *via* NKG2D appear to be responsible for the effects of SAHA-treatment of target cells.

Discussion

Numerous studies have evaluated tumour-derived HSPs, including glucose regulated protein (GRP) 94 (gp96, HSP96) and HSP70, for immunotherapy [25]. Although the focus was initially on the induction of CTL responses, it has been noticed that NK cell

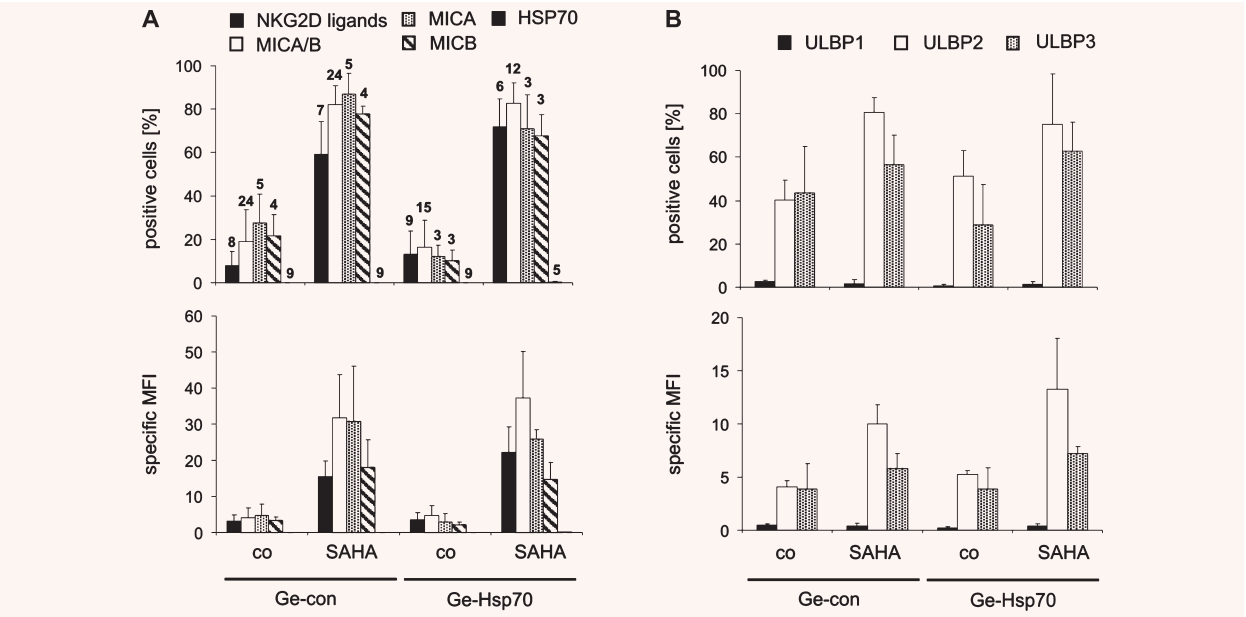


Fig. 5 Induction of NKG2D ligands MICA/B on human melanoma cells. (**A**) Ge-con and Ge-Hsp70 cells were analysed by flow cytometry for cell surface expression of NKG2D ligands (human NKG2D-IgG-Fc fusion protein), MICA/B (mAb BAMO1), MICA (mAb AMO1), MICB (mAb BMO1) and HSP70 (mAb cmHsp70.1). The cells were either cultured under standard conditions (co) or exposed to 10 μM SAHA for 20 hrs before the test. The mean percentage + S.D. of cells showing cell surface expression and the mean + S.D. of the specific MFI (MFI for specific staining minus MFI for the secondary reagent only) are shown. The number of independent measurements is given in the upper panel above the bars. (**B**) Ge-con and Ge-Hsp70 cells were analysed by flow cytometry for cell surface expression of ULBP1, ULBP2, and ULBP3 using specific mAbs. The cells were either cultured under standard conditions (co) or exposed to 10 μM SAHA for 20 hrs before the test. The mean percentage + S.D. of cells showing cell surface expression and the mean + S.D. of the specific MFI (MFI for specific staining minus MFI for the secondary reagent only) are shown as determined in three to four experiments.

depletion can also abrogate the efficacy of immunization with gp96 or HSP70 [26]. Furthermore, a perforin-dependent NK cell activity has been reported to be required to induce a CTL-mediated rejection of tumour cells engineered to secrete gp96 [27]. NK cells seem to be necessary for the adjuvant-like activity of HSP70 in the induction of CTL responses [28]. Consistent with these reports, it was recently observed that patients treated with autologous tumour-derived HSP70 or HSP96 undergo a significant boost of NK cell activity [29, 30]. Thus, an initial NK cell-mediated lysis of tumour cells and/or NK cell-released cytokines might contribute to an efficient priming of tumour-specific CTL by HSPs.

Importantly, not only T cells but also resting NK cells require activating signals to acquire their full effector functions [1–5]. HSPs might be a new component also in this system and contribute to the activation of NK cells. It has previously been described that HSP70 [9] and the HSP70-derived peptide TKD [19] can stimulate human NK cells to specifically kill tumour cells that express HSP70 at the plasma membrane [8]. HSP70 serves in this case as a stimulatory molecule and as a target structure for NK cells. In a SCID mouse model we previously have found evidence that HSP70 can stimulate mouse NK cells to kill human tumour cells that do not express HSP70 at the cell surface but MICA/B molecules, [10] which are known ligands for the well-defined activating NK receptor NKG2D [11, 12, 31].

In addition to MICA and MICB, human NKG2D ligands include the UL16-binding proteins (ULBP) 1 to 4 [11, 12]. NKG2D ligands appear to be up-regulated in response to stress, such as heat shock [32, 33], virus infection [11, 34], or genotoxic stress [35]. They signal the presence of potentially dangerous cells to the immune system [36, 37], because NK cells can distinguish between normal healthy cells and virus-infected or transformed tumour cells by scanning the expression pattern of NK receptor ligands [38]. Therefore, the combination of a stress response leading to HSP70 release and MICA/B expression in tumour cells might alert the innate immune system synergistically.

We have shown now that the two stress-inducible endogenous danger signals, HSP70 and MICA, indeed jointly augment the cytotoxic activity of human NK cells against tumour cells. Human PBMCs and isolated NK cells cultured in presence of recombinant HSP70 acquired an increased cytotoxic activity against MICA expressing target cells. In this model the cytotoxic activity was largely dependent on the MICA expression on the transfected L-MICA cells, because MICA-negative L-con cells remained resistant to lysis. Human NK cells were stimulated specifically by

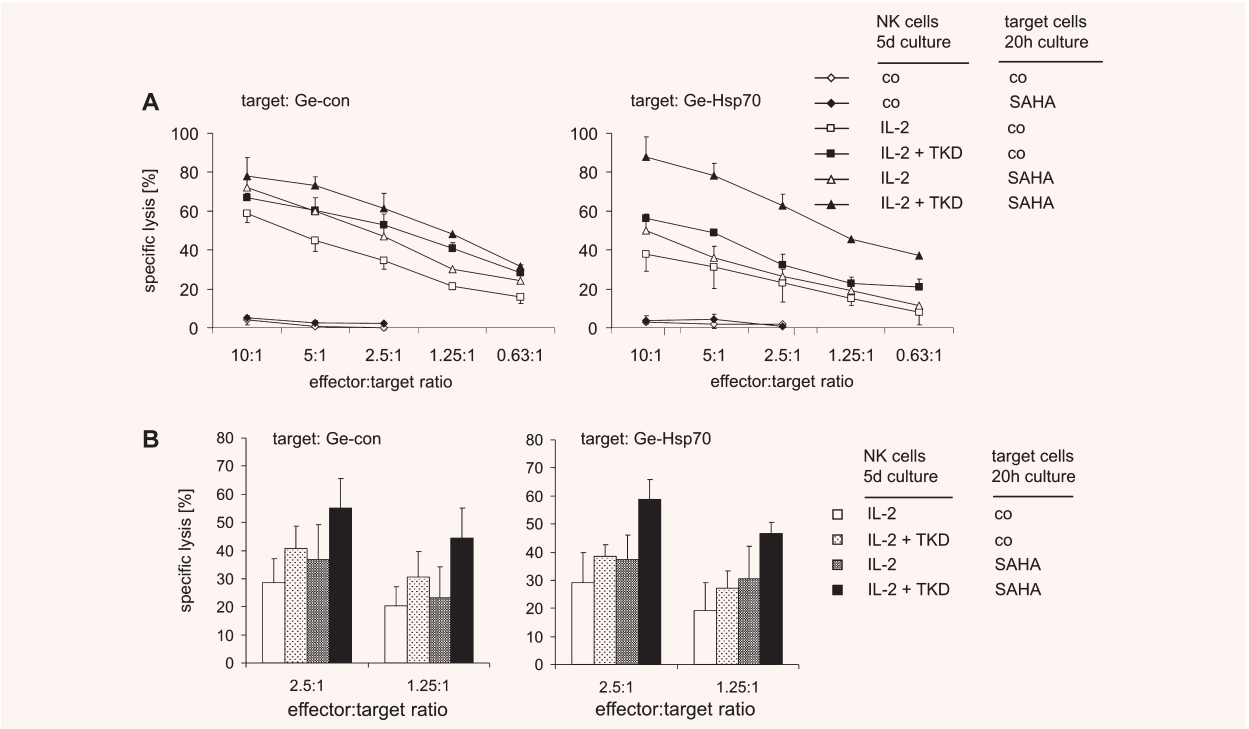


Fig. 6 The combination of TKD treatment and MICA/B induction synergistically augments killing of melanoma cells. (**A**) The mean of specific lysis ± S.D. of triplicates of Ge-con and Ge-Hsp70 target cells by NK cells cultured for 5 days with or without IL-2 (100 U/ml) in combination or not with TKD (2 μg/ml) is shown. The target cells were either cultured under standard conditions (co) or exposed to 10 μM SAHA for 20 hrs before the test. The results shown here were obtained in individual experiments that are included in the summary shown in panel B. (**B**) The mean of relative lysis + S.D. of Ge-con and Ge-Hsp70 target cells by NK cells stimulated for 5 days with IL-2 (100 U/ml) with or without TKD (2 μg/ml) was determined in four experiments. The target cells were either cultured under standard conditions (co) or exposed to 10 μM SAHA for 20 hrs before the test.

the stress-inducible HSP70 in contrast to the constitutively expressed HSC70. This finding confirms previous results obtained with human NK cells that were directed against HSP70 plasma membrane positive target cells [9]. In accordance with this observation, the peptide TKD, which is derived from HSP70 and is not present in the HSC70 protein due to a variation of two amino acids (TKDNNLLG**K**FEL**T**G) [19, 24], was able to substitute for the full-length HSP70 protein and to stimulate human NK cells to kill MICA expressing targets. Interestingly, TKD appears to increase the cytotoxic activity of NK cells by inducing the expression of the cytotoxic effector molecule granzyme B.

The experiments directly comparing recombinant rHSP70 and rHSC70 in stimulation assays were also important because there has been some speculation that the effects of recombinant HSPs on cells of the immune system results from contamination with LPS [39–41], especially in the case of *E.coli*-derived recombinant proteins. However, recombinant rHSC70 was produced under the same conditions as rHSP70, but did not have the same effect on the NK cells. Furthermore, commercially available 'low endotoxin' human HSP70 was still able to stimulate the NK cells, whereas low dose LPS did not significantly stimulate the NK cell cytotoxicity. Even more importantly, the endotoxin-free Euk hHSP70 produced in eukaryotic cells and the HSP70 peptide TKD, which was produced by chemical synthesis, both stimulated NK cells, thereby ruling out the possibility that the observed effects were simply mediated by endotoxin.

In the experiments in which we stimulated PBMCs by HSP70, it was not clear whether other cells, in addition to NK cells, were required for the stimulation. HSP70 might either act directly on NK cells, *e.g. via* C-type lectin NK receptors [21, 42], or on other cells which express HSP receptors [25] and cross-talk to NK cells, *e.g.* DCs [7]. Interestingly, MICA/B on DCs has been reported to be induced by IFN-α and subsequently stimulate NK cells [43]. Recently, it has been suggested that stimulatory effects of HSP70 on the IFN-γ response of NK cells entirely depends on the induction of NKG2D ligands on DCs [44]. Our experiments on isolated NK cells suggest that the cytotoxic response of human NK cells can be directly stimulated by HSP70. Given the limited efficiency of cell separation experiments we cannot completely exclude a role for another rare but potent cell type in the stimulation of the

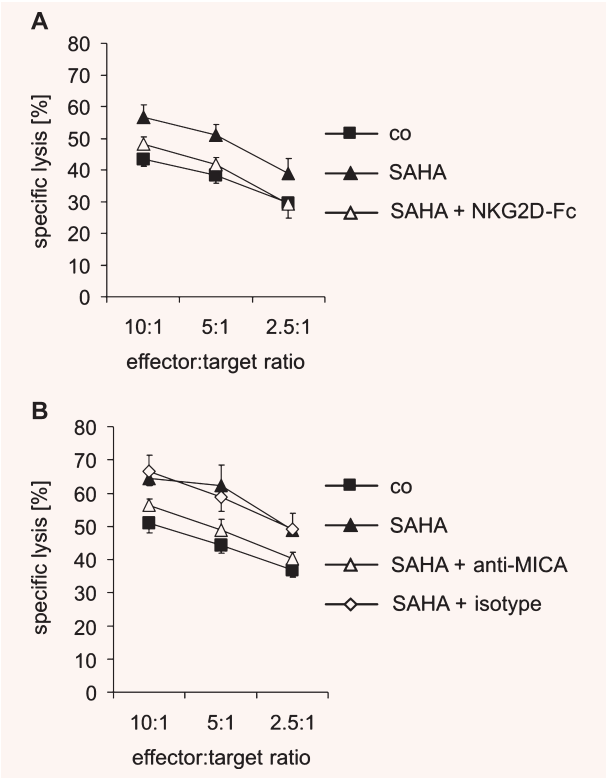


Fig. 7 Partial inhibition of NK cell cytotoxicity against SAHA-treated melanoma cells by soluble NKG2D and anti-MICA antibody. (**A**) The mean of specific lysis ± S.D. of triplicates of Ge-con target cells by NK cells cultured for 5 days with IL-2 (100 U/ml) plus TKD (2 μg/ml) is shown. The target cells were either cultured under standard conditions (co) or exposed to 10 μM SAHA for 20 hrs before the test. To inhibit the NKG2D dependent lysis a soluble human NKG2D-Fc fusion protein was added to the ^{51}Chromium release assay at a concentration of 3 μg/ml. The results of one individual out of three similar experiments are shown. (**B**) The mean of specific lysis ± S.D. of triplicates of Ge-con target cells by NK cells cultured for 5 days with IL-2 (100 U/ml) plus TKD (2 μg/ml) is shown. The target cells were either cultured under standard conditions (co) or exposed to 10 μM SAHA for 20 hrs before the test. To inhibit the NKG2D dependent lysis the anti-MICA mAb AMO1 or an isotype control (3 μg/ml) were added to the ^{51}Chromium release assay. The results of one individual out of three similar experiments are shown.

NK cell cytotoxicity by HSP70. However, it is also known that the generation of IFN-γ does not necessarily parallel the cytotoxic function of NK cells [5] and both pathways might be stimulated by HSP70 in different ways. A study on patients vaccinated with the HSP gp96 suggested that both indirect and direct pathways of NK cell stimulation by HSPs exist [30].

In the experiments shown here HSP70 and MICA/B jointly improved the cytotoxic activity of human NK cells also against the human Ge melanoma cells which we used previously as targets for mouse NK cells *in vitro* and *in vivo* [10]. We stimulated human NK cells with HSP70 or TKD and the melanoma target cells were induced to express MICA/B molecules by exposure to histone deacetylase inhibitors. These experiments indicated clearly that a combination of both treatments acted together and resulted in a significantly enhanced killing of target cells. In Ge melanoma cells MICA/B was not induced by heat shock but by chromatin-modifying reagents [10]. Similar induction patterns have been described also for other cell lines [35]. Therefore, the combination of a proteotoxic stress leading to HSP70 expression and release and a genotoxic stress leading to MICA/B expression might act together and alert the innate immune system synergistically.

The adoptive transfer of HSP70 or TKD-activated NK cells is a promising new immunotherapy for tumours that express HSP70 at the plasma membrane, and this has been successfully evaluated in preclinical animal models [45] and also in a phase-I-clinical trial [46]. Because not all tumours express HSP70 at the cell surface, our finding that HSP70-stimulated NK cells can use NKG2D ligands as target structures substantially increases the spectrum of patients who might profit from this kind of tumour immunotherapy.

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