# Efficient lysis of rhabdomyosarcoma cells by cytokine-induced killer cells: implications for adoptive immunotherapy after allogeneic stem cell transplantation

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## ABSTRACT

### Background
Rhabdomyosarcoma is the most common soft tissue sarcoma in childhood and has a poor prognosis. Here we assessed the capability of *ex vivo* expanded cytokine-induced killer cells to lyse both alveolar and embryonic rhabdomyosarcoma cell lines and investigated the mechanisms involved.

### Design and Methods
Peripheral blood mononuclear cells from six healthy donors were used to generate and expand cytokine-induced killer cells. The phenotype and composition of these cells were determined by multiparameter flow cytometry, while their cytotoxic effect against rhabdomyosarcoma cells was evaluated by a europium release assay.

### Results
Cytokine-induced killer cells efficiently lysed cells from both rhabdomyosarcoma cell lines. Antibody-mediated masking of either NKG2D molecule on cytokine-induced killer cells or its ligands on rhabdomyosarcoma cells (major histocompatibility antigen related chain A and B and UL16 binding protein 2) diminished this effect by 50%, suggesting a major role for the NKG2D molecule in rhabdomyosarcoma cell killing. No effect was observed after blocking CD11a, CD3 or TCR$\alpha\beta$ molecules on cytokine-induced killer cells or CD1d on rhabdomyosarcoma cells. Remarkably, cytokine-induced killer cells used tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to activate caspase-3, as the main caspase responsible for the execution of apoptosis. Accordingly, blocking TRAIL receptors on embryonic rhabdomyosarcoma cell lines significantly reduced the anti-tumor effect of cytokine-induced killer cells. About 50% of T cells within the cytokine-induced killer population had an effector memory phenotype, 20% had a naïve phenotype and approximately 30% of the cells had a central memory phenotype. In addition, cytokine-induced killer cells expressed low levels of activation-induced markers CD69 and CD137 and demonstrated a low alloreactive potential.

### Conclusions
Our data suggest that cytokine-induced killer cells may be used as a novel adoptive immunotherapy for the treatment of patients with rhabdomyosarcoma after allogeneic stem cell transplantation.

Key words: CIK, NKG2D, lysis, rhabdomyosarcoma.

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## Introduction

Rhabdomysarcoma is the most frequent soft tissue tumor of childhood and accounts for 3-4% of all pediatric cancers.[1] During the last 30 years survival rates have increased as a result of multimodal therapy strategies. However, the chance of cure is very low for patients with primary metastatic or relapsed rhabdomyosarcoma or Ewing's tumors.[2-4] In the German multicenter studies CWS-81, -86, -91 and-96, patients older than 10 years at diagnosis with bone or bone marrow metastases had a 5-year event-free survival of 2%.[5,6] There were no survivors after relapse.

Allogeneic stem cell transplantation might provide a graft-*versus*-tumor effect, and successful induction and maintenance of remission have been reported in patients with solid tumors treated with such transplants.[7,8] To further evaluate treatment using allogeneic stem cell transplantation we started a prospective study in high-risk patients with rhabdomyosarcoma. Our preliminary data show that patients undergoing transplantation while in remission do have a realistic chance of being cured from their disease,[9] suggesting that the graft-*versus*-tumor effect may be of importance in the treatment of such tumors. Additional novel cellular therapies should, therefore, be further explored.

Cytokine-induced killer (CIK) cell therapy may become the key to successful treatment of rhabdomyosarcoma. The ideal therapeutic approach would be to generate CIK cells from the stem cell donor. CIK cells are *ex vivo*-activated and expanded non-MHC-restricted T cells. They can be prepared in large numbers from peripheral blood mononuclear cells within 14-21 days, by appropriate addition of interferon-γ, interleukin-2 and OKT3. CIK cells constitute a heterogeneous population, including a subset of NKT cells that express both CD3 and CD56 markers.[10-13] The antitumor effect of CIK cells was demonstrated particularly in lymphoma cell lines and leukemic blasts, both *in vitro* and *in vivo*,[14-16] but these cells also have antitumor activity against solid tumors such as osteosarcoma, hepatocellular carcinoma and glioblastoma.[17-19] Their cytotoxic effect is mediated by a perforin/granzyme-dependent mechanism.[20,21] Although their tumor recognition and killing properties are not fully understood, these seem to be mediated in part by NKG2D, an activating receptor on NK cells, and the adhesion receptor leukocyte function associated antigen-1 (LFA-1).[20,21] CIK cells also express CD94, part of the NK CD94/NKG2 family receptors with activating or inhibitory potential.[15] A good correlation between up-regulation of NK-activating C-type lectin NKG2C and NKG2E receptors and CIK cell cytotoxicity has been reported.[22]

Remarkably, CIK cells showed only limited graft-*versus*-host effects in various mouse models.[23,24] Two phase I clinical trials (one in patients with hepatocellular carcinoma and the other in patients with Hodgkin's disease or non-Hodgkin's lymphoma) showed significant improvement in survival of patients after reinfusion of autologous CIK cells.[25,26] Another phase I clinical trial investigated repeated administration of donor-derived CIK cells in an allogeneic transplantation setting:[27] symptoms of an acute graft-*versus*-host disease (grade I and II) were observed in 4/11 (36%) cases and complete responses in 3/11 (27%) cases.

Based on their antitumor activity and already reported low graft-*versus*-host disease potential, we investigated whether CIK cells generated from peripheral blood mononuclear cells from healthy donors show cytotoxic potential against embryonic and alveolar rhabdomyosarcoma cells. In addition, the molecular mechanisms mediating their cytotoxic effect were investigated. As adoptive immunotherapy is associated with the risk of graft-*versus*-host disease, we evaluated the expression of CD69 and CD137 as activation-induced markers, because depletion of T cells expressing these antigens results in selective removal of alloreactive T cells.[28-31]

## Design and Methods

### Generation of cytokine-induced killer cells from peripheral blood mononuclear cells

Fifteen to twenty milliliters of heparinized peripheral blood from six healthy volunteers were collected after written informed consent. Peripheral blood mononuclear cells were separated by Ficoll density gradient centrifugation (Biocoll, Biochrom, Berlin, Germany).

The freshly isolated peripheral blood mononuclear cells were resuspended at a density of $3\times10^6$/mL in culture medium and incubated with 1000 U/mL interferon-γ (Imukin, Boehringer, Ingelheim, Germany) at 37°C (day 0). On day 1 the concentration of the cells was adjusted to $1\times10^6$/mL and 50 ng/mL of Orthoclone monoclonal OKT3 antibody (Janssen-Cilag, Neuss, Germany) together with 500 U/mL of recombinant human interleukin-2 (Proleukin S, Novartis, Nuremberg, Germany) were added. Cell numbers were assessed every 3 to 4 days and cell density was adjusted to $1\times10^6$/mL by adding fresh culture medium and interleukin-2 at a concentration of 500 U/mL. CIK cells were further grown in RPMI 1640 supplemented with 10% fetal calf serum and 500 U/mL recombinant human interleukin-2, referred to as the culture medium. The input cells were characterized phenotypically and functionally at day 0, and the expanded cells at days 7, 14 and 21.

### Cell lines

Soft tissue sarcoma cell lines RMS13, Rh30, RH41 (alveolar rhabdomyosarcoma), TE671, RD (embryonic rhabdomyosarcoma), RH1 (Ewing's sarcoma) and erythroleukemia K562 were obtained from DSMZ (Braunschweig, Germany). RH1 was originally classified as a rhabdomyosarcoma cell line but was recently reclassified when gene expression profiling revealed that it expressed *EWS-FLI1*, a fusion transcript characteristic of tumors of the family of Ewing's sarcoma malignancies. All cell lines were maintained in culture according to instructions from the DSMZ.

### Multiparameter flow cytometric analysis

To characterize the antigen profile of CIK and rhabdomyosarcoma cells, multiparameter flow ctyometric analysis was performed following exposure of the cells to monoclonal mouse anti-human antibodies conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein or allophycocyanin. The antibodies used were CD1a, CD1b, CD1d, CD3, CD4, CD8, CD11a, CD40, CD45, CD54, CD56, CD69, CD94, CD102, CD137, CD154, CD161, CD337, granzyme A, granzyme B, perforin (BD Biosciences, Heidelberg, Germany), TCRVα24, TCRVβ11 (Beckman Coulter, Krefeld, Germany), CD314, MICA/B, and ULBP-2 (R&D Systems, Wiesbaden, Germany). Data were

acquired on a FACSCalibur (Becton Dickinson Biosciences, Heidelberg, Germany) and analyzed with CellQuest and CellQuest pro software (Becton Dickinson).

### *Europium release assay*

The lytic potential of CIK cell cultures was assessed using a europium release assay according to the manufacturer's instructions. Target cells were incubated with BATDA reagent (Perkin Elmer, Waltham, Massachusetts, USA) for 30 min at 37°C. After extensive washing the target cells were resuspended in medium, plated at a density of 5000 cells/well in a 96-well plate and effector cells were added at different effector to target (E:T) ratios. After incubation for 2 h at 37°C, supernatant was harvested and incubated with europium solution (Perkin Elmer) for 15 min under continuous rotation. Europium and TDA-ligand form a highly fluorescent and stable chelate complex (EuTDA) that can be measured in a time-resolved fluorometer (1420-018 Victor, Perkin Elmer). Background, spontaneous and maximal release were measured in the medium, from target cells alone and from cells following the addition of 5% Triton X (Sigma), respectively, and calculated by means of the following formula: specific cytotoxicity (%) = 100 x (experimental release - spontaneous release)/(maximum release - spontaneous release) and spontaneous release (%) = (spontaneous release - background)/(maximum release -background).

## Results

### Ex vivo *expansion and immunophenotyping of cytokine-induced killer cells*

Using the protocol described above we were able to expand CIK cells derived from peripheral blood mononuclear cells by 2.3-fold at day 7, 16.1-fold at day 14 and 22.7 fold at day 21 (Figure 1A). The majority of the cells had a $CD3^+CD56^-$ phenotype. Remarkably, the proportion of NKT ($CD3^+CD56^+$) cells in the culture increased over time from 4.82% at day 0 (range, 0.82-10.86%) to 9.36% at day 7 (range, 3.1-15.7%), 20.6% at day 14 (range, 9.1-32.9%) and 30.9% at day 21 (range, 15.9-46.1%) (Figure 1B, C).

### *Expression of T and NK cell receptors on cytokine-induced killer cells*

During cell culture the number of $CD3^+CD56^+$cells co-expressing CD8 antigen increased from 63.5±7.4% on day 0 to 77.3±3.4% on day 21, in contrast to the $CD3^+CD56^+CD4^+$ cell subpopulation which decreased continuously from 20±3.3% on day 0 to 5.3±1.3% on day 21 (*Online Supplementary Figure S1A*). The proportion of $CD3^+CD56^+$ cells co-expressing TCRαβ was highest on day 0 (77.3±6%) and remained fairly constant through to day 21 (67.5±5.9%). In contrast, the number of $CD3^+CD56^+$ cells co-expressing TCRγδ increased from 12.6±2.6% on day 0 to 19.9±5.1% on day 21 (*Online Supplementary Figure S1B*). These cells did not, however, express the invariant T-cell receptor which is usually composed of TCRVα24 α-chain and TCRVβ11 β-chain, suggesting that these NKT cells belong to type II NKT cells.

Most of the $CD3^+CD56^+$ cells (85.9±5.6%) expressed the activating NKG2D receptor (CD314) on day 0 and the percentage of the cells expressing this antigen increased to 96±3% by day 21. On day 0, only 43.8±7.4% of NKT cells expressed the natural cytotoxicity receptor CD337 (NKp30), responsible for NK cell activation in the process of natural cytotoxicity; by days 7 and 14 the number of cells positive for this antigen had roughly doubled (81.7±5.9%) although by day 21 the number had decreased to the initial level (*Online Supplementary Figure S1C*). The pattern of expression of killer inhibitory receptors (CD94 and CD161) was more or less the same, with an increase in the number of cells expressing these antigens at day 7 and then a gradual decrease to the initial expression levels (*Online Supplementary Figure S1C*).

Flow cytometric analysis of CIK cells demonstrated that these cells represent a mixture of T cells with naïve

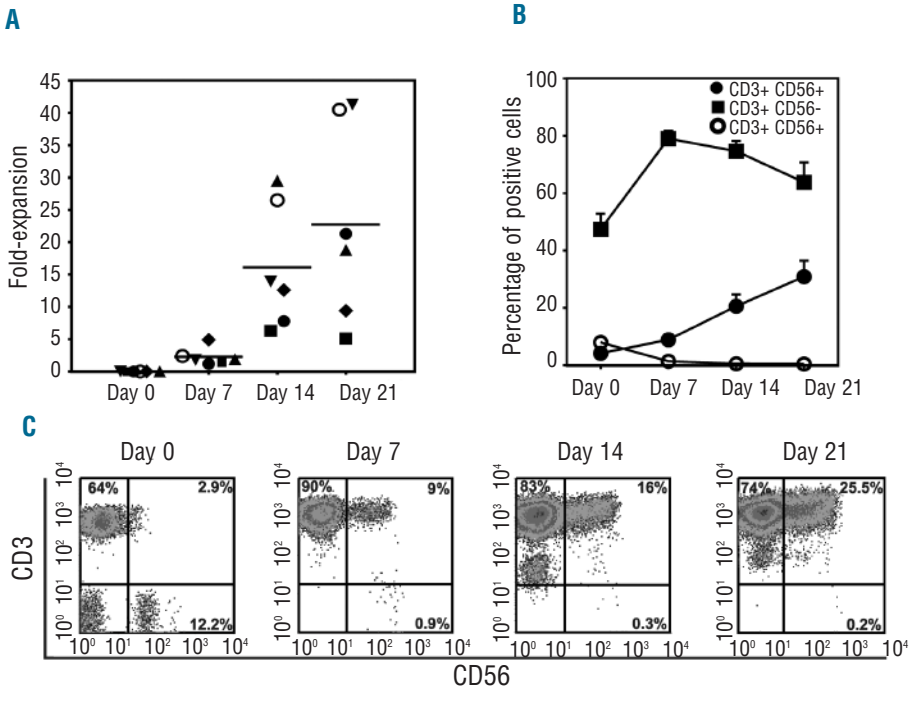


**Figure 1.** **CIK cell expansion out of peripheral blood mononuclear cells from healthy donors. (A) Freshly isolated peripheral blood mononuclear cells from six healthy donors were expanded for 21 days according to the protocol described in the *Design and Methods* section. (B) During culture CIK cells expanded and gave rise to three main cell subsets: T cells ($CD3^+CD56^-$), NKT cells ($CD3^+CD56^+$) and NK cells ($CD3^-CD56^+$). Flow cytometric analysis was performed on days 0, 7, 14 and 21 of cell culture. (C) Representative flow cytometric data of the CIK cell culture on days 0, 14 and 21.**

(CD62L$^+$CD45RA$^+$), central memory (CD62L$^+$CD45RA$^-$), effector memory (CD62L$^-$CD45RA$^-$) and effector memory RA$^+$ (EMRA) (CD62L$^-$CD45RA$^+$) phenotypes (*Online Supplementary Figure S1D*).

### Perforin, granzyme A and granzyme B expression in cultures of cytokine-induced killer cells

To characterize the cytotoxic potential of CIK cells, we evaluated their intracellular expression of effector molecules. Flow cytometry analysis revealed a continuous increased expression of granzyme A and granzyme B in CIK cells throughout cell culture. The mean percentage of CD3$^+$CD56$^+$CD8$^+$ cells expressing granzyme A increased significantly from 49.8±11.7% on day 0 to 73.4±9.5% on day 21, while the mean percentage of CD3$^+$CD56$^+$CD8$^+$ cells expressing granzyme B increased from 57.5±19.2% on day 0 to 74.1±0.6% (Figure 2A). In contrast to the increases of granzyme A and B, the intracellular expression of perforin decreased from 39.6±6.8% (day 0) to 2.9±1.1% (day 21) (Figure 2A).

### Cytotoxic activity of cytokine-induced killer cells against alveolar and embryonic rhabdomyosarcoma

The capacity of CIK cells to recognize and kill the standard target K562, alveolar rhabdomyosarcoma, embryonic rhabdomyosarcoma and Ewing's sarcoma cell lines was evaluated by using a europium release-assay.

At an E:T ratio of 50:1 CIK cells specifically lysed K562 cells (68.2±4.1% at day 7, 37.7%±7.6% at day 14 and 70.5±8.3% at day 21) (*data not shown*). The lytic capacity against the various rhabdomyosarcoma cell lines was less than that against K562 cells. The maximal lytic capacity against the Ewing's sarcoma cell line, RH1, was detected using CIK from day 7 of cell culture. At an E:T ratio of 50:1 these cells showed a specific lysis of 40%±8.3% (Figure 2B). Best results were achieved when using CIK from day 7 of cell culture against RMS13, and from day 14 against RH41. At an E:T ratio of 50:1, a maximum of 35.1±13.8% of RMS13 cells were lysed by day 7 CIK (Figure 2C), and 46.1±4.7% of RH41 cells were lysed by day 14 CIK cells (Figure 2D).

Maximal cytotoxic activity against cells of the embryonic rhabdomyosarcoma cell line TE671 was shown by day 7 CIK cells. At an E:T ratio of 50:1 such CIK cells lysed 35.6±7.83% of TE671 rhabdomyosarcoma cells (Figure 2E). Likewise, maximal cytotoxic activity against the other embryonic rhabdomyosarcoma cell line, RD, was also achieved by day 7 CIK. At an E:T ratio of 50:1, CIK cells lysed 33.2%±3.1% of RD cells (Figure 2F).

### Mechanisms of the cytotoxic activity of cytokine-induced killer cells against rhabdomyosarcoma cell lines

Antibody-mediated masking of the NKG2D-receptor on the surface of CIK cells led, in general, to their decreased cytolytic activity as effector cells. For example, at an E:T ratio of 50:1, the lytic capacity against the RH41 cell line, which expresses very low levels of MICA/B but very high levels of ULBP2 (Figure 3Aii, iii), decreased from 25.2±2.6% to 13.7±2.8% ($P$<0.001) (Figure 3Ai). As a consequence of this blocking, the cytotoxicity of CIK cells against embryonic rhabdomyosarcoma TE671 cells, which expresses almost no MICA/B antigens, but very high levels of ULBP-2 (Figure 3Bii, iii), decreased dramatically. Hence, the differences between the cytotoxic potential of CIK cells with blocked NKG2D receptor and unblocked CIK cells were highly significant at E:T ratios of 100:1 ($P$<0.008), 50:1 ($P$<0.003), 25:1 ($P$<0.004), 12.5:1 ($P$<0.01) and 6.25:1 ($P$<0.006) (Figure 3Bi).

The non-classical MHC molecules MICA/B and ULBP-2 are target molecules for the NKG2D receptor. Blocking each of these molecules separately on rhabdomyosarcoma cells led to a decrease in cytolytic activity of the effector cells (*data not shown*), while blocking both target cell ligands, which are highly expressed by the RH1 cell line

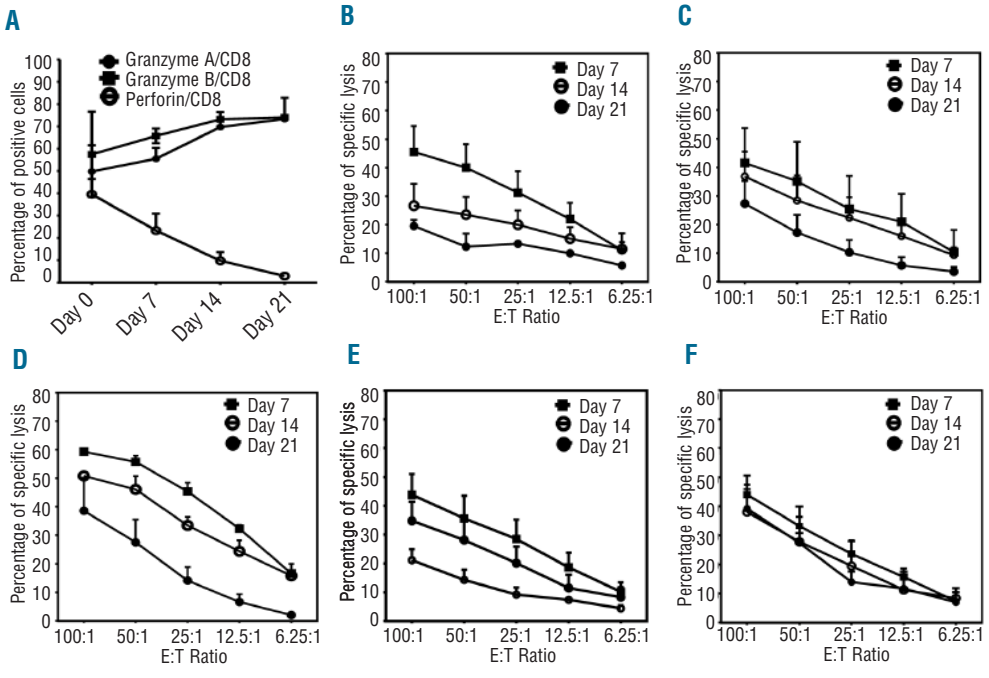


**Figure 2.** Cytotoxic activity of *in vitro*-generated CIK cells from healthy donors against rhabdomyosarcoma cell lines. (A) Intracellular expression of effector molecules in CIK cells. A continuous increase of granzyme A and B and simultaneous decrease of perforin over 21 days of cell culture was observed. Data from five experiments are shown as mean ± SEM. Cytotoxic activity of CIK cells generated from healthy blood donors against the Ewing's sarcoma cell line RH1 (B), alveolar rhabdomyosarcoma cell lines RMS13 (C) and RH41 (D) as well as embryonic rhabdomyosarcoma cell lines TE671 (E) and RD (F) was assessed by europium-release assays. Results represent data from three separate experiments and are shown as mean ± SEM for each cell line.

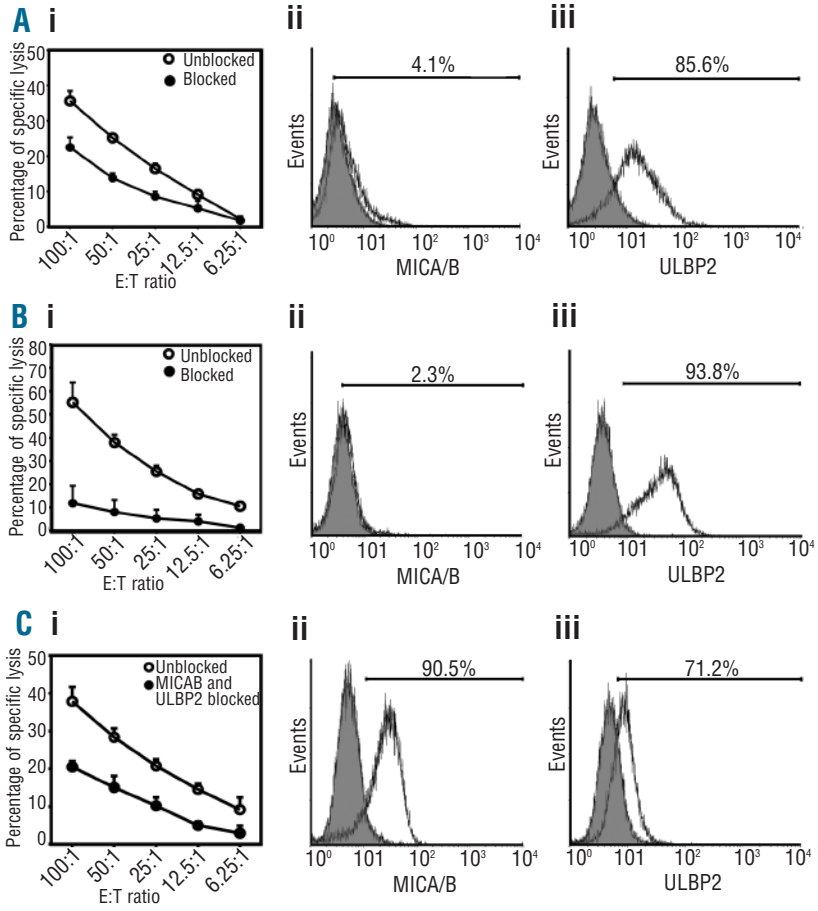


**Figure 3.** CIK-cell cytotoxicity after antibody-mediated masking of NKG2D receptor or NKG2D ligands. (A) Cytotoxicity against the alveolar rhabdomyosarcoma cell line RH41 was measured after blocking NKG2D receptor by means of a monoclonal antibody on CIK cells (n=4). These cells express low levels of MICA/B and high levels of ULBP2 (Aii,iii). Blocking the NKG2D receptor significantly decreased the cytotoxicity of CIK cells against RH41 rhabdomyosarcoma cells at ratios of 100:1 ($P$<0.02), 50:1 ($P$<0.001), and 25:1 ($P$<0.009) (Ai). (B) Antibody-mediated masking of NKG2D receptor on CIK cells (n=4) significantly decreased their cytotoxic potential against embryonic rhabdomyosarcoma TE671 cell line at all ratios e.i. 100:1 ($P$<0.008), 50:1 ($P$<0.003), 25:1 ($P$<0.004), 12.5:1 ($P$<0.01) and 6.25:1 ($P$<0.006) (Bi). This cell line expresses low levels of MICA/B and high levels of ULBP2 (Bii,iii). (C) To verify whether non-classical MHC molecules MIC A/B and ULBP2 represent the main target on rhabdomyosarcoma cells recognized by NKG2D of CIK cells these molecules were blocked on the RH1 cell line (n=3) that expresses high levels of both molecules (Cii,iii). Blocking both of these molecules on the RH1 cell line significantly decreased the killing efficiency of CIK cells against this target at E:T ratios of 100:1 ($P$<0.02), 50:1 ($P$<0.04), 25:1 ($P$<0.03), and 12.5:1 ($P$<0.02) but not at an E:T ratio of 6.25:1. (Figure 4Ci). Colored histograms represent the isotype control, whereas white histograms represent the percentage of rhabdomyosarcoma cell lines positive for MICA/B or ULBP2 antigens.

(Figure 3Cii, iii), induced a further impairment in the killing efficiency of CIK cells such that the killing efficiency of CIK cells against this target was significantly decreased at E:T ratios of 100:1 ($P$<0.02), 50:1 ($P$<0.04), 25:1 ($P$<0.03), and 12.5:1 ($P$<0.02) but not at an E:T ratio of 6.25:1. (Figure 3Ci). Inhibition of CD11a, CD3 or TCRαβ on the cell surface of CIK effector cells by means of blocking monoclonal antibodies did not influence the cytotoxic effect of the CIK cells (*data not shown*).

### Cytokine-induced killer cells and their alloreactivity potential

The lytic capacity of CIK cells against allogeneic peripheral blood mononuclear cells was analyzed on days 7, 14 and 21 of cell culture. There were no major differences in the specific cell lysis at the different E:T ratios (Figure 4A). The expression of CD69 and CD137, cell activation-induced markers, was related to low reactivity of CIK cells against allogeneic peripheral blood mononuclear cells. We found that the expression of CD69 by $CD3^+CD56^+CD8^+$ cells decreased from 28.9±9.8% on day 0 to 3.2±1.1% on day 21, whereas CD137 antigen expression showed an increase on day 7 and then fell to the low initial expression levels (0.8±0.1%) (Figure 4B).

### Apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand and caspase 3

In addition to the granule-dependent exocytosis pathway demonstrated by CIK cells, we investigated whether CIK cells use an additional mechanism to induce apoptosis of target rhabdomyosarcoma cells. After co-incubation of CIK cells with rhabdomyosarcoma cells for 4 h we observed the expression of caspase-3 by the latter as a sign of ongoing apoptosis. CIK cells were visualized by staining them with anti-CD45 antibody (Figure 4C), because rhabdomyosarcoma cells lack CD45 antigen. In order to determine whether caspase-3 is activated by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) as a result of cell-cell contact, we counterstained CIK cells with monoclonal antibody against this molecule. Analysis by fluorescence microscopy revealed the presence of TRAIL molecules on the surface of CIK cells (red fluorescence) (Figure 4D). In addition, blocking TRAIL receptors on rhabdomyosarcoma cells led to a significant reduction of the cytotoxicity of CIK cells against the embryonic rhabdomyosarcoma cell line TE671 at E:T ratios of 100:1 ($P$< 0.001), 50:1 ($P$<0.005), 25:1 ($P$<0.01), and 12.5:1 ($P$<0.05), but not at the ratio of 6.25:1 (Figure 4F). In contrast, blocking TRAIL receptors on the alveolar rhabdomyosarcoma cell line RMS13 did not affect the cytotoxic activity of CIK cells against these malignant cells (Figure 4E).

## Discussion

In the present study, we investigated the *in vitro* cytotoxic potential of CIK cells against embryonic and alveolar rhabdomyosarcoma cell lines as well as against a Ewing's sarcoma cell line (RH1). Our results demonstrated that CIK cells use a TCR-independent mechanism for their

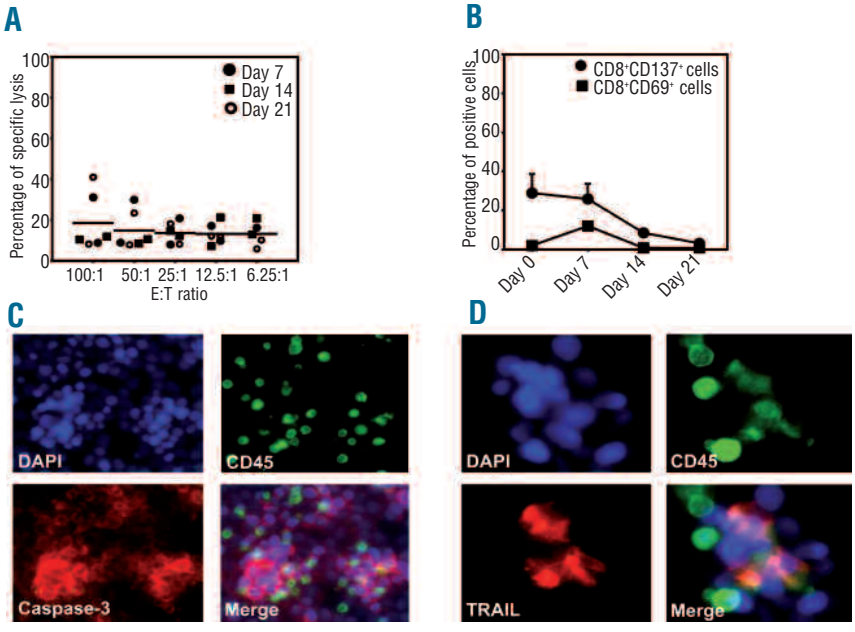


**Figure 4. Alloreactivity of CIK cells and their apoptosis-inducing mechanisms in target cells. (A) Alloreactivity of CIK cells was evaluated against allogeneic peripheral blood mononuclear cells as targets at different E:T ratios by means of the europium release assay. Results represent data from separate experiments for each measurement at days 7, 14 and 21 (n=10). (B) Alloreactivity of CIK cells was evaluated by expression of activating markers CD137 and CD69 during cell culture for 21 days. Results represent data from five experiments with five different donors and are shown as mean ± SEM. (C) Expression of caspase-3 by rhabdomyosarcoma cells (red fluorescence), as a main caspase responsible for the execution of apoptosis, was found 4 h after co-incubation with CIK cells (green fluorescence) (magnification: 100x). (D) CIK cells (green fluorescence) express TRAIL (red fluorescence) after co-incubation with rhabdomyosarcoma cells (not stained nuclei) (magnification: 200x). (E) Blocking of TRAIL receptors on alveolar RMS cell line RMS13 (n=3), did not affect the cytotoxic activity of CIK cells against these cells. (F) Blocking of TRAIL receptors on the embryonic rhabdomyosarcoma cell line TE671 (n=3) led to significantly reduced cytotoxicity of CIK cells at E:T ratios of 100:1 ($P<0.001$), 50:1 ($P<0.005$), 25:1 ($P<0.01$), and 12.5:1 ($P<0.05$) compared to the cytotoxicity of CIK against unblocked TE671 cells.**

cytotoxic effect, as previously reported.[13] We found that despite an increased TCRαβ expression by CIK cells, the cytotoxic effect of day 7 and 21 CIK cells decreased. In addition, blocking TCRαβ did not cause a decrease of lytic capacity. In the next step, we tried to determine whether CD11a (LFA-1) or NKG2D expressed on CIK cells or CD1d on rhabdomyosarcoma cells, as a target molecule, are involved in cytotoxicity against rhabdomyosarcoma cell lines as described for other tumor cells.[20,21] Neither blocking CD11a on CIK cells nor blocking CD1d on rhabdomyosarcoma cells showed an effect on the cytotoxicity of the CIK cells (*data not shown*). However, blocking NKG2D receptor on CIK effector cells or NKG2D ligands on rhabdomyosarcoma target cells did lead to a significant decrease in cytotoxicity. Despite antibody-mediated masking of NKG2D receptor on the surface of CIK cells, these cells were still effective in killing rhabdomyosarcoma cells, suggesting that CIK cell-mediated cytotoxicity is only partially mediated by this activating receptor.

Activating natural cytotoxicity receptors such as NKp30, NKp44 and NKp46 have been considered to be specific NK receptors, playing an important role in NK-cell mediated cytotoxicity against tumor cells.[32,33] The level of expression of the natural cytotoxicity receptor CD337 (NKp30) on CIK cells correlated very well with the cells' cytotoxic potential against rhabdomyosarcoma cells. On day 7 and 14 the number of CIK cells expressing this receptor increased, while on day 21 a decrease to the initial levels was observed.

On the other hand, three main groups of inhibitory receptors and their ligands are described for NK cells: (i) receptors of the KIR family (KIR2DL and KIR3DL), (ii) C-type lectin receptors such as CD94/NKG2A, and (iii) the Ig-like transcripts/CD85.[34-36] As NKp44 was described on activated γδ T cells,[37] and the activating NK heterodimers CD94/NKG2C and CD94/NKG2E are found on the surface of CIK cells, we investigated whether inhibitory NK receptors such as CD161 might also be expressed on CIK cells. We observed an increase of CD161 expression on day 7 of CIK cell culture and then decreases on days 14 and 21. To our knowledge, this is the first time the presence of CD161 on CIK cells has been described. Whether rhabdomyosarcoma cells upregulate LLT1 (CD161 ligand)[38] through autocrine production of transforming growth factor-β[39] in order to evade the antitumor activity of CIK cells remains to be elucidated in future experiments. In addition, CD94, a part of the C-type lectin receptor CD94/NKG2, showed a pattern of expression similar to that of CD161 antigen on the surface of the $CD3^+CD56^+$ subset of cells. It has been reported that upregulation of activating CD94/NKG2C and CD94/NKG2E receptors in the CIK cell population correlates very well with observed cytotoxicity.[22] However, according to our results expression of killer inhibitory receptors CD94 and CD161 seemed to play a less important role in mediating the cytotoxicity of CIK cells against rhabdomyosarcoma cells, because their expression did not directly correlate with the potential of CIK cells to kill the rhabdomyosarcoma cells.

Franceschetti *et al.*[40] reported that CIK cells are terminally activated cytotoxic T-EMRA lymphocytes. Flow cytometric analysis of CIK cells has demonstrated that these cells represent a mixture of T cells with naïve ($CD62L^+CD45RA^+$), central memory ($CD62L^+CD45RA^-$), effector memory ($CD62L^-CD45RA^-$) and effector memory $RA^+$ (EMRA) ($CD62L^-CD45RA^+$) phenotypes. In our analyzed samples of CIK cells the largest proportion of T cells had an effector memory phenotype, followed by T cells

with a central memory phenotype and naïve T cells, while the smallest proportion of T cells had an EMRA phenotype.

To further study the contribution of effector molecules to the cytotoxic potential of CIK cells, we evaluated the time-line of perforin/granzyme expression in CIK cell culture over 21 days. Remarkably, there was a continuous increase of granzyme A and granzyme B expression throughout the 21 days of cell culture, while at the same time the number of $CD3^+CD56^+$ cells expressing perforin decreased. Perforin, granzyme A and granzyme B are described as essential molecules for CIK cell cytotoxicity,[20] with perforin being absolutely necessary to deliver the granzymes to the cytoplasm of target cells.[41] Based on these results we, therefore, assume that the decrease in cytotoxicity of CIK cells against rhabdomyosarcoma cells over the time in culture may be a consequence of decreased expression of perforin by day 21 CIK cells. In order to increase perforin expression and consequently the cytotoxic effect of the CIK cells, we suggest modifying the expansion protocol of the CIK cells by adding interleukin-15, given that other studies showed that perforin expression and cytotoxicity of NK and lymphokine activated killer cell were significantly higher when cells were cultured with interleukin-15.[42]

As previously reported, the antitumor effect of CIK cells is not mediated through a Fas-Fas ligand interaction.[13,24] We, therefore, investigated whether CIK cells use the TRAIL-mediated caspase-3 activation pathway. Immunocytochemical analysis of CIK cells co-incubated with rhabdomyosarcoma cells indicated that CIK cells do express TRAIL molecules which may ultimately lead to activation of caspase-3 in rhabdomyosarcoma cells, as a main caspase responsible for the execution of apoptosis. Remarkably, blocking TRAIL receptors on rhabdomyosarcoma cell lines demonstrated that the embryonic rhabdomyosarcoma TE671 cell line, but not the alveolar RMS13 cell line, is apparently highly sensitive to the TRAIL-induced apoptosis pathway induced by CIK cells. To our knowledge, this is the first direct evidence that CIK cells use this apoptosis-inducing pathway in their antitumor activity.

To be suitable cells for transplantation and not to induce graft-*versus*-host disease, CIK cells should not be alloreactive. We, therefore, examined whether CIK cells express cell activation-induced antigens CD69 and CD137 during culture. In our study, we observed low levels of CD69 and CD137 expression by CIK cells. In addition, the CIK cells showed a low level of cytotoxicity against allogeneic peripheral blood mononuclear cells as target cells. Despite this low alloreactivity *in vitro*, in the first clinical studies some patients infused with CIK cells developed mild graft-*versus*-host disease (grade I and II).[27]

We conclude that CIK cells can be easily isolated and expanded from peripheral blood mononuclear cells in bulk quantities. Bearing in mind their low level of alloreactivity and efficient antitumor activity, CIK cells may be used as a novel therapeutic strategy in the treatment of patients with rhabdomyosarcoma after allogeneic stem cell transplantation.

## Authorship and Disclosures

SK, BV and ER designed and performed the research, interpreted the data and wrote the manuscript. GW, MS, HK, AW, ZK and SK performed the research, EK, DvL, TK and PB co-ordinated the research and approved the final version of the manuscript.

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