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

Selim Kuçi,[1] Eva Rettinger,[1] Bernhard Voß,[1] Gerrit Weber,[1] Miriam Stais,[1] Hermann Kreyenberg,[1] Andre Willasch,[1] Zyrafete Kuçi,[1] Ewa Koscielniak,[2] Stephan Klöss,[1] Dorothee von Laer,[3] Thomas Klingebiel,[1] and Peter Bader[1]

[1]University Children's Hospital III, Department of Hematology, Oncology and Hemostaseology, Frankfurt am Main, Germany; [2]Olga Hospital, Stuttgart, Germany, and [3]Biopharmaceutical Institute Georg-Speyer-Haus, Frankfurt am Main, Germany

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Online appendix: Design and Methods

Detection of effector molecules (perforin, granzyme A and granzyme B) in cytokine-induced killer cells

In parallel to cytotoxicity assays, the percentages of cytokine-induced killer (CIK) cells containing effector molecules such as perforin, granzyme A and granzyme B were assessed by flow cytometry on days 7, 14 and 21 of culture. The CIK cells were incubated with monoclonal antibodies against cell surface CD3-PE, CD8-PerCP and CD56-FITC or -APC, then fixed and permeabilized according to the instructions of the manufacturer (BD Perm/Wash buffer and Cytofix/Cytoperm solution kit from BD Becton Dickinson Biosciences). The cells were then stained with intracytoplasmatic monoclonal antibodies (anti-perforin FITC, anti-granzyme A FITC and anti-granzyme B APC (BD Becton Dickinson Biosciences). The percentages of positive cells were determined by FACSCalibur and evaluated using CellQuest software.

Blocking of effector molecules on the surface of cytokine-induced killer cells or ligand molecules on rhabdomyosarcoma cells.

To gain more insights into effector-target interactions, CIK cells were adjusted to a concentration of 4×10^6/mL and incubated with monoclonal antibodies against the cell surface molecules CD11a-FITC, CD161-APC, CD337-PE (Becton Dickinson Biosciences) and unconjugated CD314 (NKG2D) at a concentration of 10 μg/4×10^6/mL for 30 min at 37°C. After incubation, effector cells were washed and their cytotoxicity further tested in a europium release assay. To verify whether non-classical MHC molecules MICA/B (major histocompatibility antigen related chain A and B) and ULBP2 (UL16 binding protein 2) represent the main targets on rhabdomyosarcoma cells recognized by NKG2D of CIK cells, we blocked these molecules on rhabdomyosarcoma cell lines with monoclonal antibodies (R&D Systems, Wiesbaden, Germany) and used them as the target cell population in the europium release assay.

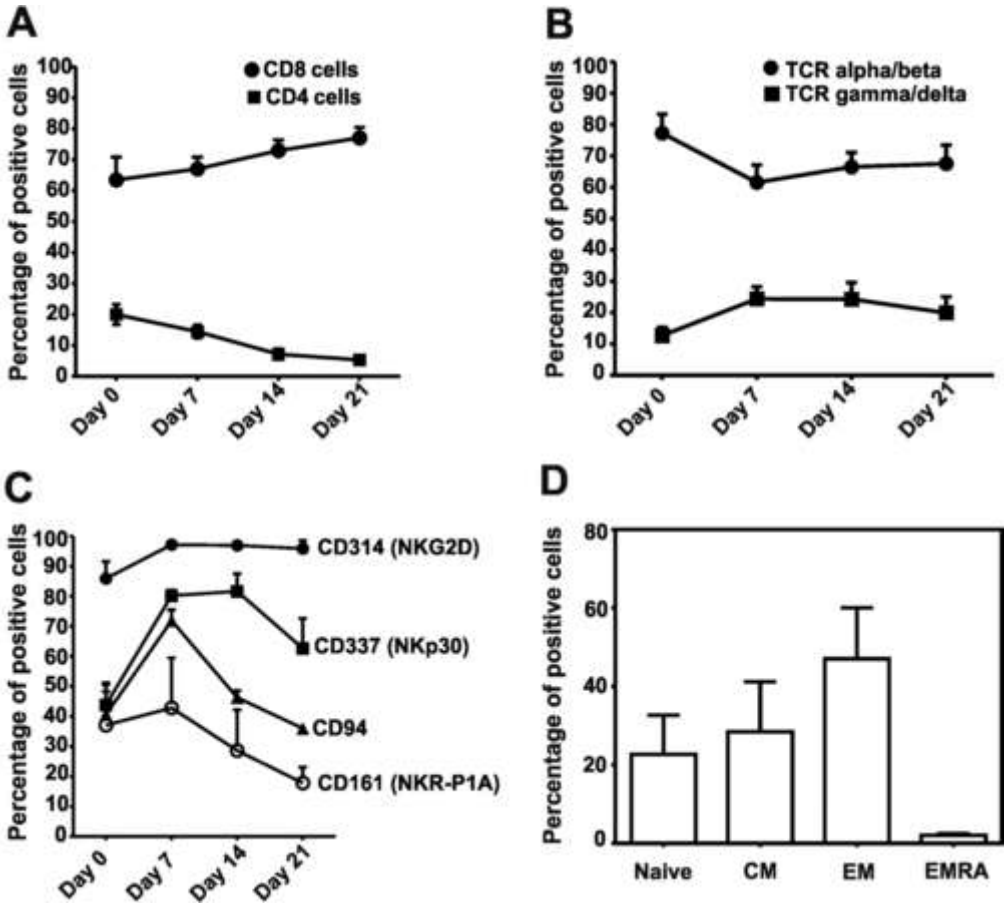
Detection of cytokine-induced killer cell-induced apoptosis in rhabdomyosarcoma cells

Cells of the alveolar rhabdomyosarcoma line (RMS13) and CIK cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. RMS13 cells were seeded on glass coverslips and incubated overnight to become adherent. Next day, CIK cells were added (E:T=10:1) and co-incubated for 4 h. The cells were rinsed twice with phosphate-buffered saline and fixed with 1 mL 100% methanol (Sigma Aldrich, Munich, Germany) for 5 min at -20°C. The cells were washed three times and those from some co-cultures were permeabilized with 1 mL 0.2% Triton X-100 for 10 min at room temperature. Permeabilized cells were washed three times and exposed to a primary, rabbit anti-human caspase-3 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) diluted in phosphate-buffered saline (1:20). The cells were incubated overnight at 4°C and, after washing three times, were then incubated with a secondary, PE-conjugated goat-anti-rabbit IgG antibody (Jackson Immunoresearch Laboratories) (1:10) for 1 h at room temperature. Cells were washed and finally stained with a FITC-conjugated anti-CD45 (BD Biosciences) antibody (1:20) for 1 h at room temperature.

Some coverslips were stained with directly conjugated antibodies against CD45 (FITC) and PE-conjugated anti-hTRAIL (BD Biosciences Pharmingen) (1:20) for 1 h at room temperature. As additional controls, cells were stained with PE- and FITC-conjugated isotype antibodies (BD Pharmingen). After washing, DAPI with the mounting medium was added to the slides and the immunofluorescence was evaluated using a fluorescence microscope (Olympus IX 71, Hamburg, Germany) equipped with a Soft Imaging System F-View II camera and Cell^P imaging software.

Blocking of TRAIL receptors on rhabdomyosarcoma cell lines

Adherent TE671 and RMS13 rhabdomyosarcoma cells were trypsinized and washed twice with phosphate-buffered saline. The cell concentration was adjusted to 5×10^6 cells/mL and the cells were incubated for 2 h at 37°C in the presence or absence of monoclonal anti-human TRAIL/TNFSF10 antibody (5 μg/mL) (R&D Systems) for 2 h at 37°C. The cells were washed twice and used as a target cell population in the europium release assay.



Online Supplementary Figure S1. Phenotypic analysis of CIK cells at days 0, 7, 14 and 21 of cell culture. (A) The number of $CD3^+CD56^+$ cells expressing the CD8 cytotoxic phenotype increased over time in culture, while the number of $CD3^+CD56^+$ cells expressing CD4 antigen decreased. (B) The majority of $CD3^+CD56^+$ cells expressed TCR$\alpha\beta$ and only a smaller proportion of these cells expressed TCR$\gamma\delta$. (C) Activating NK-receptors such as NKG2D (CD314) showed a rather constant expression, while natural cytotoxicity receptor NKp30 (CD337), inhibitory receptors such as NKR-P1A (CD161) and CD94 demonstrated an increase at day 7 and then a decrease by day 21 in culture. (D) Flow cytometry analysis showed that CIK cells represent a mixture of T cells with a naïve ($CD62L^+CD45RA^+$), central memory (CM: $CD62L^+CD45RA^-$), effector memory (EM: $CD62L^-CD45RA^-$) and effector memory RA+ (EMRA: $CD62L^-CD45RA^+$) phenotype. Data are shown as mean values ± SEM of five independent experiments.