NK cells engineered to express a GD2-specific antigen receptor display built-in ADCC-like activity against tumour cells of neuroectodermal origin

Ruth Esser a, #, Tina Müller b, #, Dörthe Stefes b, #, Stephan Kloess a, Diana Seidel c, Stephen D. Gillies d, Christel Aperlo-Iffland e, James S. Huston e, Christoph Uherek b, Kurt Schömfeld b, Torsten Tonn f, Nicole Huebener g, Holger N. Lode h, Ulrike Koehl a, †, *, Winfried S. Wels b, †, *

a Pediatric Hematology and Oncology, University Hospital, Frankfurt am Main, Germany
b Chemotherapeutisches Forschungsinstitut Georg-Speyer-Haus, Frankfurt am Main, Germany
c Department of Pediatrics, Charité University Medicine Berlin, Berlin, Germany
d Provenance Biopharmaceuticals Corp., Waltham, MA, USA
e EMD Serono Research Institute, Inc., Billerica, MA, USA
f DRK-Blutspendedienst Ost and Center for Regenerative Therapies Dresden (CRTD), Medical Faculty Carl Gustav Carus, Technical University Dresden, Dresden, Germany
g TTUHSC, Department of Cell Biology and Biochemistry, Lubbock, TX, USA
h University Children’s Hospital, University of Greifswald, Greifswald, Germany

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Abstract

Treatment of high-risk neuroblastoma (NB) represents a major challenge in paediatric oncology. Alternative therapeutic strategies include antibodies targeting the disialoganglioside GD2, which is expressed at high levels on NB cells, and infusion of donor-derived natural killer (NK) cells. To combine specific antibody-mediated recognition of NB cells with the potent cytotoxic activity of NK cells, here we generated clonal derivatives of the clinically applicable human NK cell line NK-92 that stably express a GD2-specific chimeric antigen receptor (CAR) comprising an anti-GD2 ch14.18 single chain Fv antibody fusion protein with CD3ζ chain as a signalling moiety. CAR expression by gene-modified NK cells facilitated effective recognition and elimination of established GD2 expressing NB cells, which were resistant to parental NK-92. In the case of intrinsically NK-sensitive NB cell lines, we observed markedly increased cell killing activity of retargeted NK-92 cells. Enhanced cell killing was strictly dependent on specific recognition of the target antigen and could be blocked by GD2-specific antibody or anti-idiotypic antibody occupying the CAR’s cell recognition domain. Importantly, strongly enhanced cytotoxicity of the GD2-specific NK cells was also found against primary NB cells and GD2 expressing tumour cells of other origins, demonstrating the potential clinical utility of the retargeted effector cells.

Keywords: neuroblastoma • natural killer cells • chimeric antigen receptor • GD2 • adoptive immunotherapy

Introduction

Natural killer (NK) cells are part of the innate immune system and the body’s first line of defence against virally infected and malig-
about the target, cytotoxicity of NK cells can be activated rapidly and is regulated by a balance of signals from germline-encoded activating and inhibitory cell surface receptors [1, 2]. Over the last decade, significant progress has been made towards realizing the potential of NK cells for cancer immunotherapy [3, 4]. In addition to autologous or donor-derived primary NK cells, also clinically applicable, continuously growing cytotoxic cell lines such as NK-92 are being developed for adoptive cancer immunotherapy. NK-92 cells exhibit functional characteristics of activated NK cells and express typical NK-cell surface receptors, but lack Fc receptors. NK-92 cells exhibit functional characteristics of activated NK cells and are being developed for adoptive cancer immunotherapy. NK-92 cells display markedly enhanced cytotoxicity against established GD2-expressing NB tumour cells, whereas lysis of GD2- targets remained unaffected. Importantly, selective antitumoral activity was also observed against freshly isolated primary NB tumour cells, indicating that this strategy may be suitable for further development as a cell-based immunotherapy for NB.

Materials and methods

Cells and culture conditions

Human SK-N-SH, BE(2)-C, Kelly (ATCC, Manassas, VA, USA) and UKF-NB3 [28] NB cells were maintained in Iscove’s modified Dulbecco’s medium (Biochrom AG, Berlin, Germany), LAN-1 NB cells (kindly provided by Ralph A. Reisfeld, The Scripps Research Institute, La Jolla, CA, USA) in RPMI 1640 (HyClone Laboratories, Thermo Scientific, Logan, UT, USA). SK-Mel-23 [29] and NW1539 melanoma, and SK-BR-3 breast carcinoma cells (ATCC) were cultured in RPMI 1640 (Lonza, Cologne, Germany) containing 100 units/ml penicillin and 100 μg/ml streptomycin. NK-92 cells (ATCC) and genetically modified NK-92-scFv(ch14.18)-ζ cells were propagated in X-VIVO 10 medium (Lonza) supplemented with 5% heat-inactivated human serum (Red Cross Blood Donor Service Baden-Württemberg – Hessen, Frankfurt, Germany), 100 units/ml IL-2 (Proleneukin; Novartis Pharma, Nürnberg, Germany) and 0.6 mg/ml G418 (NK-92-scFv(ch14.18)-ζ).

Primary human NB cells were isolated after surgery from a tumour metastasis of a 4-year-old patient with NB stage IV, and from the bone marrow (BM) of another high-risk patient after relapse. The tumour metastasis from jejunum was minced, treated with papain for digestion and percolated through a 40 μm filter (Cell strainer; BD Biosciences, Heidelberg, Germany), 100 units/ml IL-2 (Proleneukin; Novartis Pharma, Nürnberg, Germany) to singularize the cells. Cells were resuspended in DMEM/F-12 medium (Invitrogen, Darmstadt, Germany) to singularize the cells. Cells were resuspended in DMEM/F-12 medium (Invitrogen, Darmstadt, Germany) containing 100 units/ml penicillin and 100 μg/ml streptomycin, and cultivated until analysis in Iscove’s modified Dulbecco’s medium with the same antibiotics. BM cells of healthy
donors and NB patients were obtained after informed consent. Mononuclear cells were applied for analysis after centrifugation with Ficoll Hypaque. Research use of anonymized samples of peripheral blood stem cells (PBSC) and CD34+ cells from healthy donors remaining after allogeneic transplantation was approved by the University Hospital Ethics Committee at the University of Frankfurt, Germany.

Construction of chimeric antigen receptors
cDNA of GD2-specific ch14.18 scFv antibody fragments was derived by PCR using plasmids encoding scFv(ch14.18)-Fc fusion proteins as templates (kindly provided by EMD Serono, Billerica, MA, USA) and oligonucleotide primers introducing 5’ Sfl and 3’ Not restriction sites. The resulting scFv sequences contain the variable domains of heavy (VH) and light chains (VL) of antibody ch14.18 connected by a synthetic (G4S)4 linker either in the orientation VH-linker-VL, designated scFv(ch14.18)HL, or VL-linker-VH, designated scFv(ch14.18)LH. For construction of GD2-specific antigen receptors, each scFv(ch14.18) fragment was assembled stepwise in frame with an immunoglobulin heavy-chain signal peptide sequence 5’ of the scFv, and sequences encoding a Myc-tag, the hinge region of CDB4, (amino acids 105–165) and CD3-ζ chain 5’ of the scFv in plasmid pGEM-1 (Promega, Mannheim, Germany). Complete CAR sequences were derived from the resulting pGEM-1-scFv(ch14.18)-ζ constructs as Sall, Sml fragments, and cloned into the Sall, HpaI restriction sites of a modified pLXSN retroviral vector [30] yielding pL-scFv(ch14.18)HL-ζ-SN and pL-scFv(ch14.18)LH-ζ-SN.

Production of amphotropic retroviral vectors and transduction of NK-92 cells
FLYA-JET packaging cells [31] were transfected with pL-scFv(ch14.18)HL-ζ-SN and pL-scFv(ch14.18)LH-ζ-SN constructs by electroporation using the Easyjet Optima electroporation system (Equibio, Ashford, UK) with the following parameters: 20 μg of plasmid DNA per 1 × 10^6 cells in 0.8 ml of MEM medium in a 0.4 cm cuvette, and ‘standard’ settings according to the manufacturer’s recommendations. Stable transfectants were selected for 1 week in DMEM growth medium containing 2.4 mg/ml G418. For production of amphotropic retroviral vector, selected packaging cells were grown over night in NK-92 medium. Culture supernatants were passed through a 0.2 μm filter and incubated with NK-92 cells in the presence of 8 μg/ml polybrene for 5 hrs at 37°C. Then NK-92 cells were grown over night in fresh X-VIVO 10 medium, before G418 was added to a final concentration of 0.6 mg/ml for selection of NK-92-scFv(ch14.18)-ζ cells.

Generation of clonal NK-92 cell lines expressing scFv(ch14.18)-ζ
After G418 selection, NK-92-scFv(ch14.18)-ζ cells expressing high levels of CARs were enriched by immunomagnetic cell separation. G418-resistant cells were incubated with Myc-tag-specific monoclonal antibody (mAb) 9E10 (Sigma-Aldrich, Deisenhofen, Germany) (1.5 μg/5 × 10^5 cells) and selected using goat antimouse IgG MicroBeads and MACS LS+ separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Then single-cell clones were derived from sorted cell pools by limiting dilution. During all steps, CAR expression was monitored by flow cytometry. For FACS analysis 5 × 10^5 NK-92-scFv(ch14.18)-ζ or NK-92 cells were incubated for 30 min. at 4°C with 1.5 μg of mAb 9E10. Cells were washed twice with phosphate-buffered saline and then treated for another 30 min. at 4°C with FITC-labelled goat antimouse IgG secondary antibody (BD Biosciences). Fluorescence of cells was analysed with FACSscan or FACScalibur cytometers (BD Biosciences).

Surface expression of GD2
Expression of GD2 on the surface of established cell lines and primary human cells was determined by flow cytometry using custom PE/Cy5 conjugated 14.62a anti-GD2 antibody and mouse IgG2a isotype control (BD Biosciences). For quantification of GD2 molecules, cellular antigen expression was measured as antibody binding capacity (ABC) units using the Quantum Simply Cellular kit (Bangs Laboratories, Indianapolis, IN, USA) according to the manufacturer’s recommendations. The ABC values calculated for GD2 after subtraction of the values of the appropriate isotype control were expressed as molecules per cell. Standard curves were set with the maximum at 250.000 ABC units, and the detection threshold was <1000 ABC units. The quantitative analyses were performed on an FC 500 cytometer and data were analysed using the CXP v2.2 software (Beckman Coulter, Krefeld, Germany) as described previously [27].

Cytotoxicity assays
Specific cytotoxic activity of NK cells towards target cells was analysed in europium (Eu(3+))-release, FACS-based and 51Cr-release assays. Europium release was measured as described previously [12]. Briefly, 5 × 10^5 target cells were incubated for 10 min. at 4°C in 800 μl of europium solution containing 50 mM Heps, pH 7.4, 93 mM NaCl, 5 mM KCl, 2 mM MgCl2, 10 mM Diethyleneetriamine-pentaacetic acid (Sigma-Aldrich, Munich, Germany), 2 mM europium(III)-acetate (Sigma-Aldrich) and electroporated at 200 μF, 960 V, 250 V using a GenePulser (Bio-Rad, Munich, Germany). Then the cells were washed and seeded in triplicates in 96-well tissue culture plates in X-VIVO 10 medium, followed by addition of NK-92 or NK-92-scFv(ch14.18)-ζ cells at different effector to target ratios (E/T) and incubation for 2 hrs at 37°C. To determine maximal lysis, europium-labelled target cells suspended in 100 μl of X-VIVO 10 medium were incubated in the absence of effector cells with 100 μl of lysis buffer (PerkinElmer Wallac, Freiburg, Germany) followed by repeated freezing/thawing. Cells were centrifuged for 5 min. at 500 g, 20 μl of culture supernatant were collected and added to 200 μl/well of enhancement solution (PerkinElmer Wallac) in FluorofNunc 96-well plates (Nunc, Wiesbaden, Germany). After incubation on a shaker at 50 rpm for 15 min. at room temperature, fluorescence was determined using an LKB-Wallac 1230 Arcus fluorometer (PerkinElmer Wallac). Specific cytotoxicity was calculated as: % cytotoxicity = (experimental lysis – spontaneous lysis) / (maximal lysis – spontaneous lysis).

For FACS-based cell killing assays, adherent tumour cells were harvested by treatment with Accutase (PAA Laboratories, Pasching, Austria) for 5 to 10 min. at 37°C and singualrized. To prevent unwanted cell clumping, the cells were then incubated with 50 μg/ml of DNase I (Roche Applied Science, Mannheim, Germany) in 1 × reaction buffer prepared from a 10 × stock solution (100 mM Tris-HCl, pH 7.6, 25 mM MgCl2, 5 mM CaCl2) (Roche Applied Science) for 15 min. at 37°C under constant gentle shaking.
The reaction was blocked by addition of 250 mM ethylenediamine-tetraacetic acid to a final concentration of 50 mM. Target cells were incubated with NK-92 or NK-92-scFv(ch14.18)-ζ cells for 2 and 4 hrs in X-VIVO 10 medium at different E/T ratios. Lytic activity was measured by single platform 5-colour flow cytometric analysis for effector cells alone, and for co-cultured effector and target cells on an FC 500 cytometer (Beckman Coulter) as described previously [32, 33]. Briefly, calculation of cytotoxicity was based on the loss of living propidium iodide (PI)− target cells (CD9+ CD81+ CD45neg PIneg). Absolute count-dedicated beads (Flow-Count Fluorospheres; Beckman Coulter) were used for absolute cell enumeration.

For GD2 blocking experiments, target cells were pre-incubated for 120 min. with 20 μg/ml of parental murine anti-GD2 antibody 14G2a prior to co-culture with NK-92-scFv(ch14.18)-ζ cells. Specific cytotoxicity was calculated as described above for europium 51Cr release. Target cells were loaded with 51Cr (0.125 mCi/5 x 10^5 cells in 500 μl) (PerkinElmer, Billerica, MA, USA) for 2 hrs at 37°C, and washed twice with X-VIVO-10 medium containing 5% human AB Serum. Then 5 x 10^5 target cells were co-cultured in quadruplicates for 6 hrs with NK-92 or NK-92-scFv(ch14.18)-ζ cells in 100 μl of medium at an E/T ratio of 6.3:1. Radioactivity in supernatants was measured using a Wizard 2 gamma counter (PerkinElmer).

Maximum 51Cr release was induced by addition of 100 μl of 1% SDS solution to 100 μl of target cell suspension. Specific cytotoxicity was calculated as described above for europium release. For CAR blocking experiments, 10 μg/ml of anti-idiotype antibody 1A7 (kindly provided by M. Bhattacharya-Chatterjee, University of Cincinnati Medical Center, Cincinnati, OH, USA) were added during coculture of cells. Mouse IgG1 clone X40 (BD Biosciences, San Jose, CA, USA) served as an isotype control.

Cytotoxic activity of NK cells upon prolonged co-culture with target cells at low E/T ratios was visualized by microscopy. Microscopic images of cells were taken after 18 hrs of co-culture using an Axiosvert 135 microscope (Carl Zeiss, Göttingen, Germany) and a Sony 3CCD camera (Sony, Berlin, Germany).

Microscopic control of the interaction between NK-92 and primary NB cells

Primary NB cells were seeded at low density on 20 mm chamber slides (Nunc, Langenselbold, Germany) and grown for 24 hrs. Then adherent NB cells were co-cultured with gene-modified NK-92 or NK-92-scFv(ch14.18)-ζ or parental NK-92 cells for 4 hrs at an E:T ratio of 5:1. NK-92 cells were identified with CD45-specific antibody followed by FITC-conjugated secondary antibody. CAR expressing NK-92 cells were detected with mAb 9E10 followed by PE-conjugated secondary antibody. All cells were counter-stained with DAPI. Fluorescence microscopy was performed with an Olympus IX71 microscope (Olympus, Hamburg, Germany), and microscopic images were acquired with a charge-coupled device camera.

Statistical analysis

Kruskal–Wallis test with Dunn’s multiple comparison and Wilcoxon matched pairs test were applied to assess statistical significance of differences between groups. P values <0.05 were considered as significant. Data were analysed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Results

Generation of NK cells carrying GD2-specific chimeric antigen receptors

GD2-specific scFv(ch14.18) antibody fragments were derived from constructs encoding scFv(ch14.18)-Fc fusion proteins that carry heavy and light chain variable domains of the chimeric mAb ch14.18 [34, 35]. To address potential differences in the functionality of scFv(ch14.18) molecules that depend on the orientation of the variable domains, we employed scFv fragments where VH and VL of antibody ch14.18 were either assembled in the orientation Vh-linker-Vl, or Vl-linker-Vh, with the synthetic (G4S)4 sequence serving as a flexible linker. Chimeric antigen receptors were constructed by inserting the scFv fragments designated scFv(ch14.18)HL and scFv(ch14.18)LH between a sequence encoding an N-terminal immunoglobulin heavy-chain signal peptide, and sequences encoding a Myc-tag, the CD8α hinge region (amino acids 105–165) and the CD3-ζ chain in the retroviral transfer vector pLXSN [30] (Fig. 1A).

Amphotropic retroviral vector particles were produced by stable transfection of FLYA-JET packaging cells [31], and used for transduction of human NK-92 cells. After selection with G418, expression of scFv(ch14.18)HL-ζ and scFv(ch14.18)LH-ζ receptor proteins on the cell surface was analysed by flow cytometry. At this step the majority of cells in the selected cell pools displayed low or undetectable expression of the CARs (Fig. 1B, left panels). To enrich NK-92 cells that express more homogenous receptor levels, cells were sorted with Myc-tag specific mAb 9E10 and immunomagnetic beads (Fig. 1B, middle panels), followed by limiting dilution to obtain single cell clones. This yielded stable NK-92 cell clones consistently expressing high levels of CARs (Fig. 1B, right panels). We did not observe a difference in expression levels between clones carrying scFv(ch14.18)HL-ζ or scFv(ch14.18)LH-ζ (Fig. 1B and data not shown), indicating that the orientation of VH and VL within scFv(ch14.18) had no influence on the overall expression or surface display of the receptors.

Surface expression of GD2 on NB cells

As a prerequisite for the analysis of CAR functionality and activity of retargeted NK-92 cells, first surface expression of GD2 by established NB cell lines and primary NB cells was investigated by flow cytometry using fluorochrome-labelled GD2-specific murine mAb 14.G2a. Control cells were treated with an irrelevant isotype-matched antibody. Established human UKF-NB3, Kelly, BE(2)C and LAN-1 NB cells displayed intermediate to high levels of GD2 on their surface, whereas only a very weak signal was determined with anti-GD2 antibody for SK-N-SH cells (Fig. 2A). Analysis of primary NB cells from the BM of 12 relapsed NB patients revealed markedly enhanced GD2 expression in these samples when compared to established GD2− NB cell lines (data not shown).

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To illustrate this pronounced difference exemplarily, BM with NB cells from one of these patients was mixed with established UKF-NB-3 cells, and GD2 expression was determined by flow cytometry (Fig. 2B).

For quantification of GD2 molecules, cellular antigen expression on the surface of established and primary NB cells as well as haematopoietic cells from healthy donors was determined in comparison to antibody-binding microbeads as a standard (Fig. 2C). We found very high expression of GD2 in the range of 2 to 4 x 10^5 antibody-binding capacity (ABC) molecules/cell for established BE(2)C and UKF-NB3 NB cells, 7 x 10^3 ABC molecules/cell for Kelly cells and 1.5 x 10^3 ABC molecules/cell for SK-N-SH (Fig. 2C), which is near the lower detection limit of 1 x 10^3 epitopes/cell for this assay. Malignant cells of non-NB origin such as K562 (erythroleukaemia) expressed low levels of GD2 (3.5 x 10^3 ABC molecules/cell). Similar results were obtained for A673 peripheral neuroepithelioma tumour (PNET) and rhabdomyosarcoma cells (data not shown). GD2 levels on primary NB cells isolated from a tumour metastasis and the BM of a relapsed NB patient with stage IV disease were above 2 x 10^6 ABC molecules/cells, which was the upper detection limit of the assay (Fig. 2C). Haematopoietic cells from healthy donors expressed very low GD2 levels (sorted CD34^+ cells), or yielded ABC signals below the lower detection limit of the assay (lymphocytes in PBSC and BM).

Cytotoxic activity of NK-92-scFv(ch14.18)-ζ against NB cells

Next we investigated whether expression of GD2-specific antigen receptors can augment cell killing activity of NK-92 towards established NB cells. Cytotoxicity of clonal NK-92-scFv(ch14.18)HL-ζ and parental NK-92 cells against UKF-NB3, Kelly and SK-N-SH cells during co-culture at different E/T ratios was analysed in FACS-based assays. Highly GD2^+ UKF-NB3 cells displayed already pronounced sensitivity to parental NK-92 cells (58% specific lysis at an E/T ratio of 10:1), but were more potently killed by CAR-expressing NK-92-scFv(ch14.18)HL-ζ cells (79% specific lysis; P < 0.01) (Fig. 3A). Similar results were obtained with NK-92-scFv(ch14.18)LH-ζ cells (data not shown). Kelly cells were relatively resistant to NK-92 (22% specific lysis at an E/T ratio of 5:1), but like UKF-NB-3 highly sensitive to NK-92-scFv(ch14.18)HL-ζ cells (58% specific lysis; P < 0.05). This cytotoxicity was strongly reduced when we blocked GD2 on the surface of Kelly cells with parental murine anti-GD2 antibody 14G2a prior to co-culture with NK-92-scFv(ch14.18)HL-ζ (18% specific lysis at an E/T ratio of 5:1), which demonstrates that the enhanced activity of CAR-expressing NK cells was dependent on the accessibility of GD2 on
the tumour cell surface. For SK-N-SH cells which do not overexpress the target molecule, similar lytic activity was found for parental NK-92 and retargeted NK-92-scFv(ch14.18) HL-\(\zeta\) cells (43% versus 41% specific lysis at an E/T ratio of 5:1; not significant).

Cytotoxic activity of NK-92-scFv(ch14.18)HL-\(\zeta\) towards established GD\(\text{2}\)\(^{+}\) LAN-1 NB cells was determined in \(^{51}\)Cr release assays at an E/T ratio of 6.3:1. Similar to Kelly cells, LAN-1 displayed resistance to parental NK-92 but were readily killed by NK-92-scFv(ch14.18)HL-\(\zeta\) cells (4% versus 70% specific lysis, respectively; \(P < 0.01\)) (Fig. 3B, left panel). When effector and target cells were co-cultured in the presence of anti-idiotypic antibody 1A7 which blocks the antigen binding site of ch14.18 [36], cytotoxicity of NK-92-scFv(ch14.18)HL-\(\zeta\) cells was inhibited by 81%, confirming dependence on the functionality of the target recognition domain of the CAR (Fig. 3B, right panel). Cell killing activity of NK cells at low E/T ratios was investigated with BE(2)C cells as targets. The NB cells were incubated for 18 hrs with clonal

Fig. 2  Surface expression of GD\(\text{2}\) on established and primary NB cells. (A) Expression of GD\(\text{2}\) on established human UKF-NB3, Kelly, BE(2)C, LAN-1 and SK-N-SH NB cells was determined by flow cytometry using PE-Cy5-labelled GD\(\text{2}\)-specific mAb 14.G2a (open areas). Control cells were treated with isotype control (grey areas). (B) To illustrate high level expression of GD\(\text{2}\) on primary cells, BM with NB cells from a relapsed patient was mixed with established UKF-NB3 NB cells before analysis by flow cytometry as described in (A). (C) For quantification of GD\(\text{2}\) molecules on the cell surface, antigen expression was measured as ABC molecules/cell as described in the methods section. GD\(\text{2}\) expression on established NB cell lines (left panel, open bars), K562 erythroleukaemic cells (left panel, shaded bar), primary cells from an NB tumour metastasis (NB tumour) and BM infiltrating NB cells (NB BM) (right panel, filled bars) as well as haematopoietic cells (selected 34\(^{+}\) cells, CD34; lymphocytes from peripheral blood, PBSC; lymphocytes from bone marrow, BM) from healthy donors (right panel, shaded bars) is indicated. The graphs are shown with a logarithmic scale. Lower and upper detection limits of this assay were 1 \(\times\) 10\(^3\) and 2 \(\times\) 10\(^6\) ABC molecules/cell, respectively. Data are represented as mean \(\pm\) S.D.
Fig. 3 NK-92-scFv(ch14.18)-ζ cells display enhanced cell killing activity towards established GD2-expressing NB cells. (A) Cytotoxic activity of NK-92-scFv(ch14.18)HL-ζ cells towards UKF-NB3, Kelly and SK-N-SH NB cells was determined in FACS-based cytotoxicity assays at different effector to target ratios (E/T). Parental NK-92 cells were included for comparison. To investigate dependence of cell killing on GD2 recognition, GD2 on Kelly NB cells was blocked with parental murine anti-GD2 antibody 14G2a prior to co-culture with NK-92-scFv(ch14.18)HL-ζ as indicated. Data are represented as mean ± S.D. (B) Cytotoxic activity of NK-92-scFv(ch14.18)HL-ζ towards LAN-1 NB cells was determined in 51Cr-release assays at an E/T ratio of 6.3:1. Parental NK-92 cells were included for comparison (left panel). To block interaction of CAR with GD2 on the target cell surface, LAN-1 and NK-92-scFv(ch14.18)HL-ζ cells were co-cultured in the presence of 10 μg/ml of anti-idiotype antibody 1A7 (right panel). Data are represented as mean ± S.D. (C) Cytotoxic activity of NK-92-scFv(ch14.18)HL-ζ and NK-92-scFv(ch14.18)LH-ζ towards BE(2)C NB cells was investigated by microscopical analysis after 18 hrs of co-culture at low E/T ratios of 1:1 and 0.3:1. Parental NK-92 cells were included for comparison. Control cells were incubated in the absence of NK cells, or were treated with 8 μM of the apoptosis-inducing drug staurosporine as indicated. Representative fields are shown.
NK-92 variants, we tested the effects of irradiation on cytotoxic activity of GD2-specific NK-92-scFv(ch14.18)HL-ζ/H9256 cells towards established UKF-NB3 cells. We found that irradiated effector cells retained marked cytotoxicity, which was comparable to that of non-irradiated NK-92-scFv(ch14.18)HL-ζ cells (Fig. 4). These results demonstrate that expression of scFv(ch14.18)-ζ receptors on NK-92 triggers markedly enhanced cell killing towards NB cells, which is dependent on specific recognition of GD2 on the target cell surface. Thereby NK-92-scFv(ch14.18)HL-ζ and NK-92-scFv(ch14.18)LH-ζ cells were similarly active, indicating that the orientation of VH and VL in the scFv(ch14.18) antibody fragment does not influence target cell recognition and GD2-specific cytotoxicity.

**Enhanced cell killing activity of NK-92-scFv(ch.14.18)-ζ against primary NB cells**

To investigate cytotoxic activity of NK-92-scFv(ch14.18)-ζ towards primary tumour cells, human NB cells were freshly isolated from a tumour metastasis of a 4-year-old patient with NB stage IV, singularized and cultivated for several days. Quantitative flow cytometric analysis revealed very high levels of GD2 expression on the surface of these cells (Fig. 2C). The primary NB cells were co-cultured at different E/T ratios with NK-92-scFv(ch14.18)HL-ζ or parental NK-92 cells for 2 hrs before analysis of specific lysis in FACS-based assays. Primary tumour cells displayed intermediate sensitivity to parental NK-92 cells (43% specific lysis at an E/T ratio of 10:1), but were highly sensitive to CAR-expressing NK-92-scFv(ch14.18)HL-ζ cells, resulting in 82% specific lysis at an E/T ratio of 10:1 (P < 0.01) (Fig. 5A). This enhanced cytotoxic activity corresponded well with the high level of GD2 expression on the target cells. In accordance with the results of the cytotoxicity analysis, interaction between gene-modified NK-92 and adherent, primary NB cells was substantially enhanced when compared to untargeted parental NK-92 cells (Fig. 5B).

**Cytotoxicity of NK-92-scFv(ch.14.18)-ζ against melanoma and breast carcinoma cells**

To investigate cell killing activity of GD2-specific NK-92 against GD2-expressing tumour cells of origins other than NB, we analysed
the sensitivity of established melanoma and breast carcinoma cells for NK-cell mediated lysis. Flow cytometric analysis revealed high levels of GD2 on SK-Mel-23 and NW1539 melanoma cells, whereas SK-BR-3 breast carcinoma cells expressed only moderate GD2 levels (Fig. 6A). In europium release assays, SK-BR-3 cells were resistant to lysis by parental NK-92 cells, confirming previous findings [12]. In contrast, clonal NK-92-scFv(ch14.18)LH-ζ cells were able to kill SK-BR-3 cells achieving specific lysis of 44% at an E/T ratio of 10:1 (P < 0.01) (Fig. 6B). Although SK-Mel-23 and NW1539 melanoma cells displayed moderate sensitivity already to unmodified NK-92 cells, cell killing activity was markedly enhanced upon expression of GD2-specific scFv(ch14.18)-ζ receptors with specific lysis of SK-Mel-23 cells at an E/T ratio of 10:1 of 22% and 92%, respectively (P < 0.001) (Fig. 6B, C). At a low E/T ratio of 1:1 prolonged co-culture of NW1539 cells with NK-92-scFv(ch14.18)LH-ζ or NK-92-scFv(ch14.18)LH-ζ resulted in almost complete elimination of the target cells, comparable to exposure to the apoptosis-inducing reagent staurosporine. Even at an E/T ratio below 1:1 only very few viable melanoma cells remained, suggesting that individual NK-92-scFv(ch14.18)LH-ζ and NK-92-scFv(ch14.18)LH-ζ cells may sequentially attack several NW1539 targets as in the case of BE(2)C NB cells (Fig. 3C).
Discussion

High-risk stage IV NB is characterized by disseminated metastasis, and continues to be a therapeutic challenge in paediatric oncology. Despite intense multimodal treatment, prognosis remains poor with long-term survival in only around 30% to 40% of patients [20–22], justifying efforts to develop alternative, more efficient treatment strategies. In this study, we have redirected continuously growing human NK cells to GD2 expressing NB cells using CARs that harbour a GD2-specific scFv fragment derived from the ch14.18 antibody. This was linked to the CD3–ζ chain to trigger cytolytic activity upon target cell recognition. Gene-modified NK cell lines were derived by transduction with retroviral vectors followed by selection of single cell clones, which displayed high and stable CAR expression over several months in continuous culture. NK-92 cells carrying CAR that harboured scFv(ch14.18) antibody fragments either in V\textsubscript{H}-linker-V\textsubscript{L} or V\textsubscript{L}-linker-V\textsubscript{H} orientation both displayed high cytotoxic activity towards GD2 expressing NB and melanoma cells. A moderate cell killing activity was achieved against breast cancer cells with more limited GD2 expression.

Immunotherapeutic approaches that target GD2 have first focused on monoclonal antibodies. To date murine antibodies 3F8 and 14G2a, the chimeric human/mouse antibody ch14.18, and the humanized immunocytokine hu14.18-IL2 have been used in clinical trials for the treatment of NB to enhance antibody-dependent cell-mediated cytotoxicity (ADCC) against GD2\textsuperscript{+} tumour cells [18, 25, 26]. Yet there is no consensus about the benefit of these strategies [37]. Promising responses were seen in a recent phase III study combining ch14.18 with GM-CSF and IL-2 [25], and a recent phase II study demonstrating antitumoral activity of the immunocytokine hu14.18-IL2 in patients with relapsed or refractory NB [26]. Other reports described no advantage over conventional therapy [38]. Yu et al. attributed the difference in outcome to the addition of IL-2 and GM-CSF, which augments ch14.18-mediated ADCC \textit{in vivo} [25]. Combination of mAbs with different treatment modalities may be required to improve survival of NB patients, which includes utilization of cellular effector mechanisms [39]. Safety and feasibility of anti-GD2 antibody 3F8 together with haploidentical NK cells for the treatment of high-risk disease is currently being investigated in a phase I study (NCT00877110; clinicaltrials.gov). The lack or very low expression of MHC class I molecules on NB cells make them an ideal target for NK cells. Ex vivo stimulation of the effector cells with IL-2 resulted in increased expression of natural cytotoxicity receptors and NKG2D, and enhanced cytotoxicity towards NB cells [27]. Nevertheless, expression of soluble NKG2D ligands such as MHC class I related protein A by NB cells can have a marked inhibitory effect on NK cells [40, 41]. Furthermore, ADCC inducing activity of anti-GD2 antibodies may be affected by Fc receptor polymorphisms, as it has been described for other therapeutic antibodies [42, 43].

Our results show that expression of a GD2-specific CAR in NK cells directly couples antibody-mediated recognition of GD2 with the execution of cytotoxicity. This provides the effector cells with built-in ADCC-like activity, which can bypass limitations such as insufficient FcγRIII activation by antibodies and overcome the tumour cells’ endogenous resistance mechanisms as demonstrated for Kelly and LAN-1 cells. These NB cells were largely resistant to parental NK-92 cells, but were lysed by NK-92-scFv(ch14.18)-ζ with high efficiency. In the case of intrinsically NK-sensitive NB cell lines, we observed markedly increased cell killing activity of NK-92-scFv(ch14.18)-ζ cells. This enhanced activity was strictly dependent on specific recognition of the GD2 target antigen by the NK cells. Blocking of GD2 on the target cell surface with GD2-specific antibody or occupation of the antigen binding site of the scFv(ch14.18) domain of the CAR with an anti-idiotypic antibody abrogated cytotoxic activity of NK-92-scFv(ch14.18)-ζ towards GD2-expressing targets. In addition to GD2-dependent cell killing, retargeted NK-92-scFv(ch14.18)-ζ cells retained endogenous natural cytotoxicity of NK-92, demonstrated by similar activity of NK-92 and the GD2-specific variant against NK-sensitive SK-N-SH NB cells which express only very low GD2 levels. This may be of advantage for the treatment of tumours that consist of cells with heterogeneous GD2 expression. Importantly, strongly enhanced cytotoxicity of NK-92-scFv(ch14.18)-ζ cells was also found in the case of primary NB cells, indicating that the retargeted effector cells may be of clinical utility. GD2 has previously been established as a relevant cancer antigen for NB, and only minimal intra- or intertumoral heterogeneity of GD2 expression has been described [44, 45]. Moreover, persistent GD2 antigen expression after treatment with GD2-specific antibody was demonstrated. Only in 1 out of 62 NB patients the tumour lost GD2 expression after therapy, but in this case underwent phenotypic transformation into a pheochromocytoma-like tumour [45].

High level GD2 expression is restricted to NB, melanoma and other tumours of neuroectodermal origin [18, 19, 39]. We did not observe measurable GD2 expression on lymphocytes from BM and peripheral blood, and only very low GD2 expression on sorted CD34\textsuperscript{+} progenitor cells. Hence, haematological toxicities induced by treatment with GD2-specific NK cells appear unlikely. Nevertheless, limited antigen expression in other normal tissues can result in unwanted side effects of GD2–targeted therapies. Reported toxicities of treatment with anti-GD2 antibodies with or without GM-CSF included fever, nausea/vomiting, urticaria, hypotension, capillary leak syndrome, ocular symptoms, neuropathic pain and neurotoxicity [25, 26, 38, 46, 47]. In general, these adverse effects were considerable but manageable, and did not result in discontinuation of the treatment. Recent data from animal models suggest that such toxicities are to a large part due to complement-dependent cytotoxicity (CDC) induced by such antibodies, and may be reduced by limiting CDC [48]. Introduction of a point mutation in GD2-specific ch14.18 antibody which interferes with CDC while retaining ADCC activity, resulted in markedly reduced allopdynia in rats when compared to the parental molecule [48]. In the case of retargeted NK-92-scFv(ch14.18)-ζ cells, the antibody fragments within the GD2-specific CAR are restricted to the variable domains of antibody heavy and light chains, and do not contain the Fc portion that would be required to elicit CDC.
Hence, CAR-triggered cytotoxicity of the NK cells is limited to ADCC-like activity, which will likely circumvent CDC-dependent toxicities.

Similar to our results, selective cytotoxicity of GD2-specific primary NK cells and cytotoxic T lymphocytes (CTLs) was found in experimental models [49, 50]. Clinical application of GD2-targeted CTLs appeared safe, and was associated with tumour regression or necrosis in half of the NB patients treated [51]. In the latter study, Epstein-Barr virus-specific CTLs were employed for genetic modification with a GD2-specific CAR to minimize the risk of autoimmunity caused by reactivity of the endogenous T-cell receptors of the retargeted cells with normal tissue antigens. In studies evaluating untargeted NK-92 cells, the effector cells were irradiated as a safety measure prior to infusion into cancer patients. This prevented permanent engraftment, although cells remained viable and retained cytotoxicity for several days [10, 11]. This was also the case for irradiated retargeted NK-92 cells (this study and [12]).

The use of CAR-expressing primary cells for adoptive immunotherapy requires for each individual patient the isolation, expansion and genetic modification of the relevant T- or NK-cell populations. Clinically applicable cytotoxic cell lines such as NK-92 could complement these approaches, especially in cases where autologous effector cells cannot be employed and suitable donors are not available. Methodology for GMP-compliant large scale production of unmodified NK-92 cells is well established [10, 11], and could be readily applied for continuous expansion of retargeted derivatives such as GD2-specific NK-92-scfV(ch14.18)-ζ cells. NK-92 cells express HLA class I molecules. Nevertheless, in the clinical trials conducted so far with unmodified, parental NK-92 cells, only few patients developed anti-HLA class I antibodies upon repeated treatment with the allogeneic effector cells [10, 11]. This was most likely due to the impaired immune status of the patients following chemo- and radiation therapy. Hence, repeated intravenous NK-92 therapy appears feasible when performed under continuous crossmatch testing with the patients’ serum before infusion. Our results demonstrate that clonal NK-92-scfV(ch14.18)-ζ cells selectively and reliably eliminate established and primary NB cells and GD2 expressing tumour cells of other origins. Utilization of NK-92-scfV(ch14.18)-ζ cells could bypass the need for separate genetic modification of effector cells for each individual patient, justifying further development of this approach.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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