Genetically engineered CAR NK cells display selective cytotoxicity against FLT3-positive B-ALL and inhibit *in vivo* leukemia growth

Sarah Oelsner[1], Anja Waldmann[1], Arne Billmeier[1], Jasmin Röder[1,2], Aline Lindner[1], Evelyn Ullrich[3,4], Rolf Marschalek[5], Gianpietro Dotti[6], Gundram Jung[7,8,9], Ludger Große-Hovest[10], Pranav Oberoi[1], Peter Bader[3] and Winfried S. Wels[1,2,9,11]

[1]Institute for Tumor Biology and Experimental Therapy, Georg-Speyer-Haus, Frankfurt, Germany
[2]Frankfurt Cancer Institute, Goethe University, Frankfurt, Germany
[3]Division for Stem Cell Transplantation and Immunology, Hospital for Children and Adolescents, Goethe University, Frankfurt, Germany
[4]LOEWE Center for Cell and Gene Therapy, Goethe University, Frankfurt, Germany
[5]Institute of Pharmaceutical Biology, Goethe University, Frankfurt, Germany
[6]Lineberger Comprehensive Cancer Center and Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC
[7]Department of Immunology, Eberhard Karls University, Tübingen, Germany
[8]German Cancer Consortium (DKTK), Partner Site Tübingen, Tübingen, Germany
[9]German Cancer Research Center (DKFZ), Heidelberg, Germany
[10]SYNIMMUNE GmbH, Tübingen, Germany
[11]German Cancer Consortium (DKTK), Partner Site Frankfurt/Mainz, Frankfurt, Germany

Chimeric antigen receptor (CAR)-engineered natural killer (NK) cells represent a promising effector cell type for adoptive cancer immunotherapy. Both, genetically modified donor-derived NK cells as well as continuously expanding NK-92 cells are currently under clinical development. To enhance their therapeutic utility for the treatment of pre-B-cell acute lymphoblastic leukemia (B-ALL), we engineered NK-92 cells by lentiviral gene transfer to express a FMS-like tyrosine kinase 3 (FLT3)-specific CAR that contains a composite CD28-CD3ζ signaling domain. FLT3 has primarily been described as a therapeutic target for acute myeloid leukemia, but overexpression of FLT3 has also been reported in B-ALL. Exposure of FLT3-positive targets to CAR NK-92 cells resulted in conjugate formation between NK and leukemia cells, NK-cell degranulation and selective cytotoxicity toward established B-ALL cell lines and primary blasts that were resistant to parental NK-92. In a SEM B-ALL xenograft model in NOD-SCID IL2R γ^{null} mice, treatment with CAR NK-92 but not parental NK-92 cells markedly inhibited disease progression, demonstrating high antileukemic activity *in vivo*. As FLT3 is known to be also expressed on precursor cells, we assessed the feasibility of incorporating an inducible caspase-9 (iCasp9) suicide switch to enhance safety of our approach. Upon addition of the chemical dimerizer AP20187 to NK-92 cells coexpressing the FLT3-specific CAR and iCasp9, rapid iCasp9 activation was observed, precluding further CAR-mediated cytotoxicity. Our data demonstrate that B-ALL can be effectively targeted by FLT3-specific CAR NK cells which may complement CD19-directed immunotherapies, particularly in cases of inherent or acquired resistance to the latter.

Introduction

Pre-B-cell acute lymphoblastic leukemia (B-ALL) typically affects children but can also be found in adolescents and adults.[1] While long-term survival rates for pediatric B-ALL patients approach 90%,[2] relapsed and chemotherapy-refractory disease still remains a significant cause of cancer-associated mortality. Even if treated

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Correspondence to: Winfried S. Wels, Institute for Tumor Biology and Experimental Therapy, Georg-Speyer-Haus, Paul-Ehrlich-Straße 42-44, D-60596 Frankfurt am Main, Germany, Tel.: +49-69-63395-188, Fax: +49-69-63395-189, E-mail: wels@gsh.uni-frankfurt.de

What's new?

Therapy-induced selection of antigen-loss variants can result in resistance of pre-B-cell acute lymphoblastic leukemia (B-ALL) to CD19-directed immunotherapies. To evaluate FLT3 as a target for B-ALL, here the authors engineered NK-92 cells to express an FLT3-specific chimeric antigen receptor (CAR). The CAR NK cells displayed high and selective cytotoxicity against established and primary B-ALL cells *in vitro* and markedly reduced leukemia burden in a human B-ALL xenograft model *in vivo*. The findings suggest FLT3-specific CAR NK cells as a promising alternative approach for adoptive immunotherapy of B-ALL.

with pediatric-based ALL regimens, 5-year overall survival for adult patients does not exceed 60%.[3] With the introduction of CD19-targeted immunotherapies such as the bispecific CD19xCD3 antibody blinatumomab and CD19-specific chimeric antigen receptor (CAR)-engineered T cells, promising treatment alternatives are now available which have demonstrated remarkable clinical efficacy against B-ALL in pediatric and adult patients.[4–11] Nevertheless, therapy-induced selection of leukemia cells that show altered CD19 expression or have lost the antigen is increasingly being recognized as a reason for treatment failure and relapse.[10,12,13] This has spurred the development of CAR T cells that recognize alternative B-ALL antigens such as CD22.[14] Similarly, FMS-like tyrosine kinase 3 (FLT3; CD135) may serve as a therapeutic target for B-ALL. Activating mutations of FLT3 represent the most common genetic aberration in acute myeloid leukemia (AML),[15] but overexpression of wild-type FLT3 and FLT3 mutations have also frequently been found in B-ALL.[16,17]

Here we employed genetically modified natural killer (NK) cells for specific targeting of FLT3-positive B-ALL. NK cells are cytotoxic lymphocytes of the innate immune system that can get activated rapidly upon encounter of virally infected, stressed or neoplastic cells, and play an important role in cancer immunosurveillance.[18–20] Unlike T cells, allogeneic NK cells do not carry a high risk of inducing graft-*vs.*-host disease, allowing to base clinical protocols on donor-derived primary NK cells or the clinically applicable NK cell line NK-92.[21,22] Both, primary NK cells and NK-92 engineered to express CD19-targeted CARs have shown antileukemic activity in preclinical models of B-ALL,[23–27] and early phase clinical trials with CD19-targeted NK cells for the treatment of B-ALL and other malignancies of B-cell origin are ongoing (NCT01974479, NCT03056339; clinicaltrials.gov). To investigate the activity of FLT3-targeted CAR NK cells against B-ALL, we transduced NK-92 cells with a lentiviral vector encoding a codon-optimized CAR encompassing an FLT3-specific single chain variable fragment (scFv) derived from monoclonal antibody 4G8,[28] fused to a composite CD28-CD3ζ signaling domain.[27,29] We analyzed *in vitro* activity of the resulting NK-92/4G8.28.z cells against established leukemia cell lines and primary B-ALL blasts, and dependence of cell killing on expression of FLT3 and CAR activation. Antileukemic effects of the CAR NK cells *in vivo* were investigated in a human B-ALL xenograft model in NOD-SCID IL2R γ^{null} (NSG) mice. To tightly control viability and cytotoxicity of the CAR NK cells, we evaluated co-expression of the FLT3-specific CAR together with inducible caspase-9 (iCasp9) as a safety switch.

Materials and Methods

Cells and culture conditions

HL-60 acute promyelocytic leukemia cells, and NALM-16 and SEM B-cell precursor ALL cells were propagated in RPMI 1640 medium (Lonza, Cologne, Germany). HEK 293T cells were grown in DMEM (Lonza). Media were supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Darmstadt, Germany). Primary B-ALL blasts were cultured in Iscove's modified Dulbecco's medium (Lonza) supplemented with 1 μg/ml bovine insulin (Sigma-Aldrich, Taufkirchen, Germany), 50 μM β-mercaptoethanol, 200 μg/ml Fe^{3+}-saturated human apo-transferrin (Invitrogen, Karlsruhe, Germany), 0.6% human serum albumin (Sanquin, Amsterdam, The Netherlands), 2.0 mM L-glutamine and 20 μg/ml cholesterol.[30] NK-92 cells (kindly provided by NantKwest, Inc., Culver City, CA) were cultured in X-VIVO 10 (Lonza) containing 5% heat-inactivated human plasma (German Red Cross Blood Donation Service Baden-Württemberg - Hessen) and 100 IU/ml IL-2 (Proleukin; Novartis Pharma, Nürnberg, Germany).

Generation of CAR-expressing NK-92 cells

CAR 4G8.28.z consists of an immunoglobulin heavy chain signal peptide, a scFv fragment of FLT3-specific antibody 4G8,[28] a Myc-tag and a modified CD8α hinge region, followed by transmembrane and intracellular domains of CD28 and the intracellular domain of CD3ζ. A codon-optimized CAR sequence was synthesized (GeneArt, Invitrogen) and inserted into lentiviral transfer plasmid pHR'SIN-cPPT-SIEW (pSIEW) upstream of IRES and EGFP sequences,[31] yielding the vector pS-4G8.28.z-IEW. As a control, the CAR signaling domains were removed from the construct by digestion with *Nae*I and religation, with the resulting pS-4G8.TM-IEW vector containing a premature stop codon after the transmembrane domain of CD28 (CAR 4G8.TM). The integrity of all constructs was verified by restriction analysis and DNA sequencing. VSV-G pseudotyped vector particles were produced using HEK 293T cells and NK-92 cells were transduced as described.[27] Transduction efficiencies were 8% for pS-4G8.28.z-IEW (MOI of 24) and 18% for pS-4G8.TM-IEW (MOI of 42). EGFP-expressing NK-92 cells were enriched by flow cytometric cell sorting with a FACSAria fluorescence-activated cell sorter (BD Biosciences, Heidelberg, Germany). CAR surface expression in sorted single cell clones was confirmed by flow cytometry with AF647-conjugated Myc-tag-specific antibody (9E10; Santa Cruz Biotechnology, Heidelberg, Germany) using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed with CellQuest Pro software (BD Biosciences).

For generation of NK-92 cells coexpressing iCasp9 and the FLT3-specific CAR, a sequence encoding codon-optimized iCasp9 was *de novo* synthesized (GeneArt, Invitrogen),[32,33] linked in frame to the CAR 4G8.28.z sequence *via* a *Thosea asigna* virus self-cleaving peptide and inserted into lentiviral transfer plasmid pSIEW, yielding the vector pS-iCasp9-2A-4G8.28.z-W. NK-92 cells were transduced with VSV-G pseudotyped lentiviral particles as described above, and CAR-expressing single cell clones were obtained by flow cytometric cell sorting with AF647-conjugated Myc-tag-specific antibody.

FLT3 expression, degranulation assay and conjugate analysis

FLT3 expression on the surface of NK-92 and leukemia target cells was analyzed by flow cytometry using PE-coupled FLT3-specific antibody (4G8; BD Pharmingen, Heidelberg, Germany). Degranulation of NK cells upon exposure to SEM leukemia cells at an E/T ratio of 1:1 for 5 hr at 37°C was analyzed as described,[34] detecting surface expression of lysosomal-associated membrane protein LAMP-1 (CD107a) using BD FastImmune CD107a APC antibody (BD Biosciences) according to the manufacturer's instructions. As controls, NK cells were stimulated with 1 μg/ml phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml ionomycin (Sigma-Aldrich). Conjugate formation and redistribution of cytotoxic granules were assessed by confocal laser scanning microscopy. NK cells and target cells were mixed at a 1:1 ratio and kept on poly-L-lysine-coated cover slips (Life Technologies) for 1 hr at 37°C. Then the cells were fixed for 10 min with phosphate-buffered 4% formaldehyde solution and permeabilized for 5 min with 0.1% Triton X-100 in phosphate-buffered saline (PBS). After washing and blocking of unspecific binding sites for 30 min with 10% FBS in PBS, the samples were incubated for 1 hr with perforin-specific antibody (δG9; Santa Cruz Biotechnology) and Alexa Fluor 594-coupled anti-mouse secondary antibody (Invitrogen) in blocking buffer at room temperature. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (Life Technologies). After washing with PBS, cells were embedded with Mowiol 4-88 (Roth, Karlsruhe, Germany) and analyzed using a Leica SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

Cytotoxicity assays

Cytotoxicity of NK-92 cells toward established leukemia cell lines and primary human B-ALL blasts was analyzed in FACS-based assays as described.[35] Target cells were labeled with calcein violet AM (CV) (Molecular Probes, Invitrogen) and incubated with effector cells at various effector to target (E/T) ratios for 4 hr at 37°C. Then 150 μl of a 1 μg/ml propidium iodide (PI) solution were added to each sample before flow cytometric analysis in a FACSCanto II flow cytometer (BD Biosciences). Dead target cells were identified as CV and PI double positive. Spontaneous target cell lysis in the absence of NK cells was subtracted to calculate spe-cific cytotoxicity. Data were analyzed using FACSDiva software (BD Biosciences). Likewise, activity of NK-92/4G8.28.z cells against mobilized $CD34^+$ hematopoietic stem cells (HSCs) from healthy donors was analyzed.

In vivo leukemia model

SEM B-ALL cells were transduced with VSV-G pseudotyped SEW-luc2 lentiviral vector encoding firefly luciferase and EGFP linked *via* a 2A peptide,[29,36] and EGFP-positive cells were enriched by flow cytometric cell sorting. Six- to eight-week-old male NSG mice were intravenously (i.v.) injected with 1×10^6 SEM/Luc cells. Beginning 7 days after leukemia induction, animals were treated by i.v. injection of 1×10^7 NK-92/4G8.28.z or parental NK-92 cells once per week for 4 weeks. Control mice received PBS. Disease development was monitored by imaging with an IVIS Lumina II *in vivo* imaging system (Perkin Elmer, Rodgau, Germany) 10 min after intraperitoneal injection of 75 mg/kg of D-luciferin (Promega, Mannheim, Germany). For endpoint analysis, mice were sacrificed, spleens were removed, weighed and immediately analyzed by bioluminescence imaging (BLI), or subjected to immunohistochemical analysis as described below. For *in vivo* experiments all applicable guidelines for the care and use of animals were followed. All animal experiments were approved by the responsible government committee (Regierungspräsidium Darmstadt, Darmstadt, Germany).

Immunohistochemistry

Spleen tissues of sacrificed mice were fixed with phosphate-buffered 4% formaldehyde solution (Roth) and paraffin-embedded. Sections of 3 μm thickness were deparaffinized and hydrated, and then stained using a standardized staining protocol (Bond Polymer Refine IHC protocol, IHC-F; Leica Microsystems) with FLT3-specific (C-20, 1:50; Santa Cruz Biotechnology) or CD19-specific antibody (HIB19, 1:100; eBioscience, Frankfurt, Germany) and polymeric HRP-conjugated anti-mouse secondary antibody (DAB Polymer Refine Detection Kit; Leica Microsystems). Slides were then stained with hematoxylin and embedded in mounting medium.

Analysis of iCaspase-9 activation

NK-92/iCasp9_2A_4G8.28.z and NK-92 control cells were incubated in the absence or presence of 10 nM of AP20187 (Ariad Pharmaceuticals, Cambridge, MA) for 5, 10 or 30 min. Then the levels of endogenous caspase-9, iCasp9 and their processed forms were analyzed by SDS-PAGE of whole cell lysates and immunoblotting with caspase-9-specific rabbit antibody (Thermo Scientific, Braunschweig, Germany), followed by HRP-conjugated secondary antibody (Sigma-Aldrich) and chemiluminescent detection. In addition, cytotoxicity assays with NK-92/iCasp9_2A_4G8.28.z or NK-92/4G8.28.z cells and leukemia target cells were performed in the presence or absence of 10 nM of AP20187.

Statistical analysis

For comparison of individual values, data were analyzed by two-tailed unpaired Student's *t*-test. For multiple comparisons of *in vivo* BLI data, two-way ANOVA with Tukey's multiple comparisons test was used. p Values <0.05 were considered

statistically significant. Prism 8 software (GraphPad Software, La Jolla, CA) was used for statistical calculations.

Results

Generation of FLT3-specific CAR NK cells

For targeting of NK cells to the FLT3 differentiation antigen, we generated a codon-optimized CAR (CAR 4G8.28.z) that harbors an immunoglobulin heavy chain signal peptide and a scFv derived from FLT3-specific antibody 4G8,[28,37] linked to a composite CD28-CD3ζ signaling domain *via* a Myc-tag and an optimized CD8α hinge region (Supporting Information Fig. S1*a*).[29,38] A corresponding truncated CAR (CAR 4G8.TM) containing the transmembrane domain of CD28 but lacking an intracellular signaling domain served as a control (Supporting Information Fig. S1*b*). The CAR sequences were inserted into the self-inactivating lentiviral vector pSIEW, where they are encoded under the control of the spleen focus forming virus promoter and coexpressed with an EGFP marker gene (Fig. 1*a*; Supporting Information Figs. S2*a* and S2*b*). VSV-G pseudotyped lentiviral vector particles were produced and used for transduction of human NK-92 cells. CAR NK cells were enriched by two rounds of flow cytometric cell sorting resulting in homogeneous EGFP-positive NK cell populations (Fig. 1*b*) with similar surface expression of full-length and truncated CARs by sorted NK-92/4G8.28.z and NK-92/4G8.TM single cell clones, respectively (Fig. 1*c*).

Activation of CAR NK cells upon encounter with FLT3-positive targets

To confirm the relevance of FLT3 as a therapeutic target for B-ALL, we investigated FLT3 expression on the surface of established B-ALL cell lines and primary B-ALL blasts by flow cytometric analysis with an FLT3-specific antibody. Thereby high FLT3 expression was found for the SEM B-ALL cell line, with more moderate FLT3 expression determined for the NALM-16 B-ALL cell line and primary B-ALL blasts (Fig. 2*a*). In contrast, HL-60 acute promyelocytic leukemia cells included for comparison displayed no significant FLT3 expression. Likewise, NK-92 cells themselves were FLT3-negative. To assess activation of CAR NK cells in response to FLT3-positive targets, we analyzed activation-induced degranulation of NK-92/4G8.28.z cells upon contact with SEM leukemia cells. Cells were coincubated at an effector to target (E/T) ratio of 1:1 for 5 hr, before surface expression of the lysosomal associated membrane protein LAMP-1 (CD107a) was measured by flow cytometry. CD107a levels were markedly increased on the surface of NK-92/4G8.28.z cells upon encounter of SEM cells, but no degranulation of parental NK-92 cells was observed under the same conditions. In contrast, both, NK-92/4G8.28.z

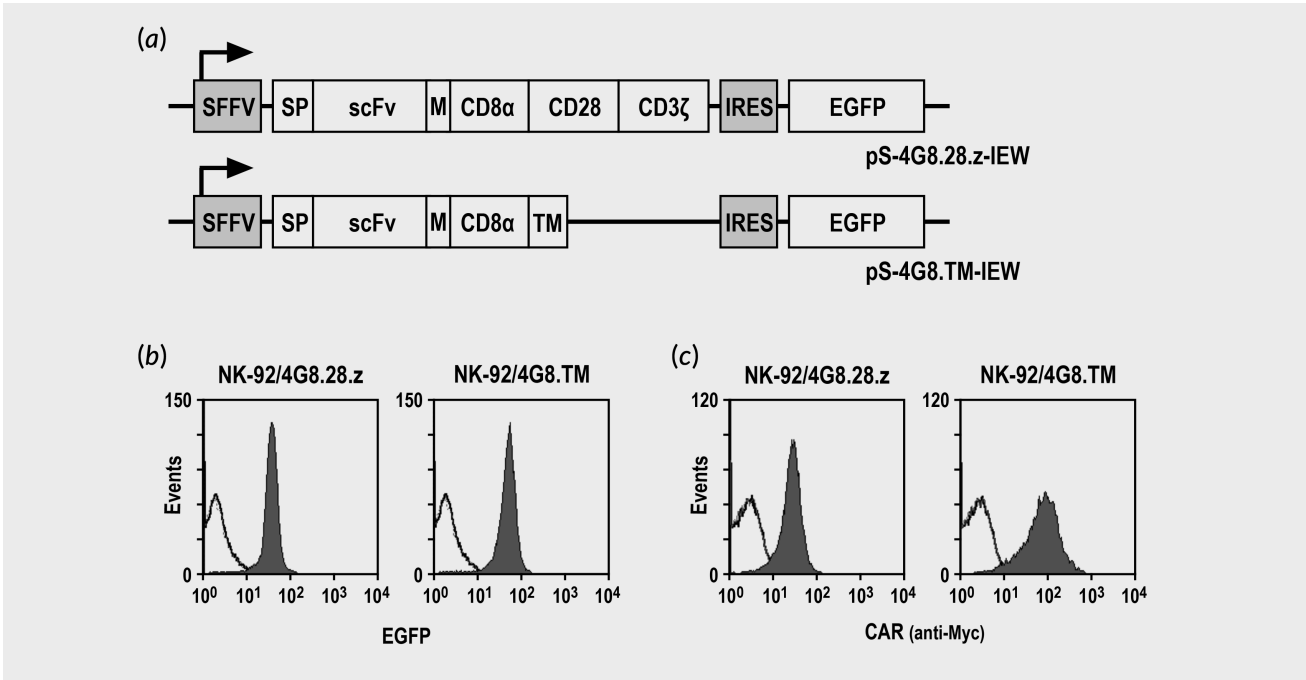

Figure 1. Generation of CAR NK-92 cells. (*a*) Lentiviral transfer plasmids encoding full-length or truncated FLT3-specific chimeric antigen receptors under the control of the spleen focus forming virus promoter (SFFV). The CAR sequences are followed by an internal ribosome entry site (IRES) and enhanced green fluorescent protein (EGFP) cDNA. In pS-4G8.28.z-IEW the CAR consists of an immunoglobulin heavy chain signal peptide (SP), a FLT3-specific scFv antibody fragment, a Myc-tag (M), a CD8α hinge region (CD8α), transmembrane and intracellular domains of CD28 and the intracellular domain of CD3ζ (CAR 4G8.28.z), whereas pS-4G8.TM-IEW encodes a truncated CAR without signaling capabilities that only harbors the transmembrane domain of CD28 (CAR 4G8.TM). (*b*) Enhanced green fluorescent protein expression of sorted NK-92/4G8.28.z and NK-92/4G8.TM single cell clones was determined by direct flow cytometry (filled areas). (*c*) CAR surface expression was analyzed by flow cytometry with Myc-tag-specific antibody (filled areas). Parental NK-92 cells served as controls (open areas).

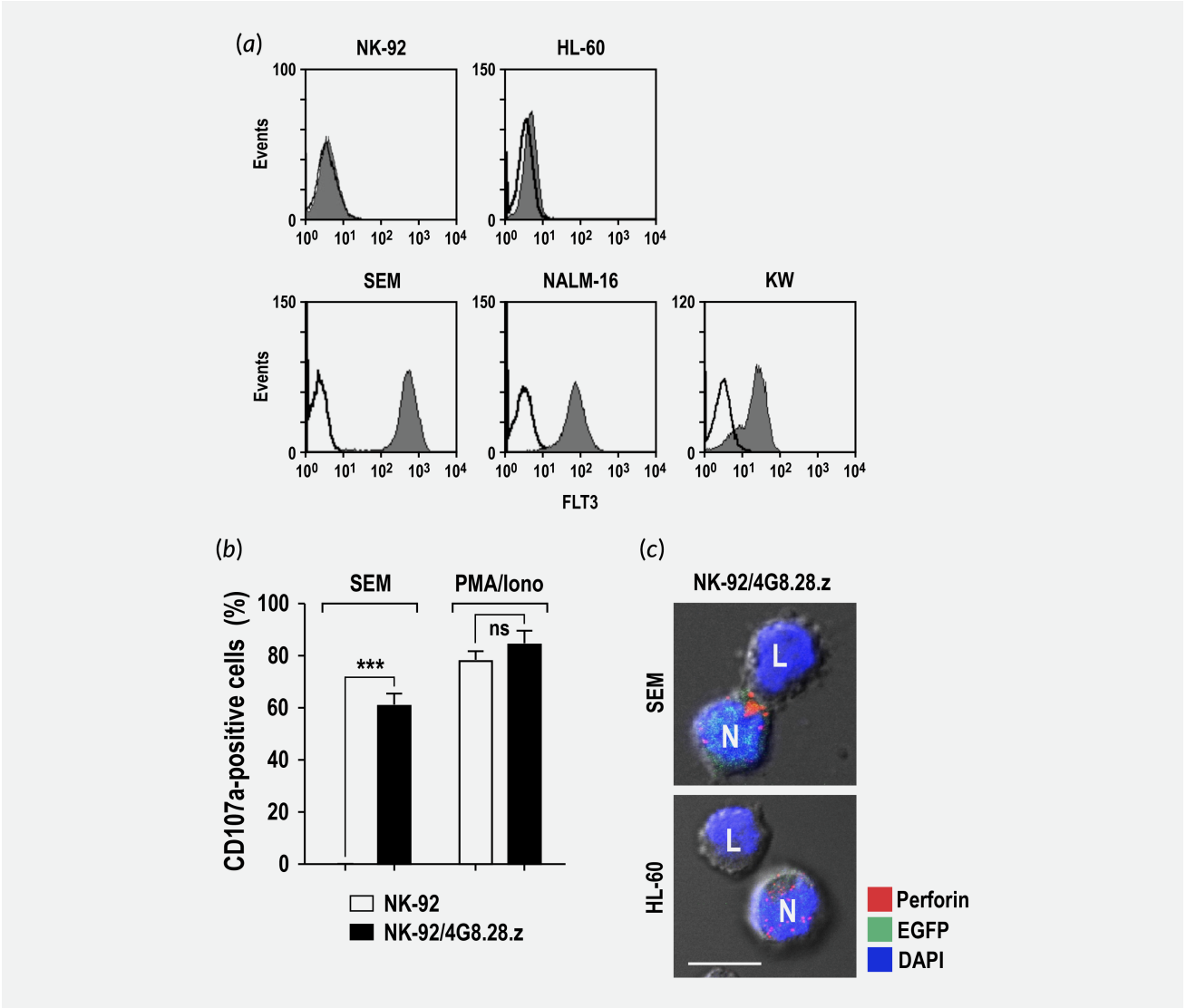

Figure 2. Activation of CAR NK cells by FLT3-positive targets. (*a*) Expression of FLT3 on the surface of NK-92 cells, HL-60 acute promyelocytic leukemia, SEM and NALM-16 B-ALL cell lines and KW primary B-ALL blasts was determined by flow cytometry with FLT3-specific antibody (filled areas). Control cells were stained with an irrelevant antibody of the same isotype (open areas). (*b*) Degranulation of NK-92/4G8.28.z cells was analyzed by flow cytometry determining CD107a surface expression after 5 hr of coculture with FLT3-expressing SEM leukemia cells at an E/T ratio of 1:1. Parental NK-92 cells were included for comparison. NK cells stimulated with 1 µg/ml phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml ionomycin (Iono) served as controls. Mean values ± SEM are shown; $n = 3$. p Values were calculated by pairwise analysis using two-tailed unpaired t-test. ***, $p < 0.001$; ns: not significant ($p > 0.05$). (*c*) Conjugate formation between NK-92/4G8.28.z cells and SEM (upper panel) and HL-60 (lower panel) leukemia cells was investigated by confocal microscopy. Leukemia (L) and EGFP-positive CAR NK (N) cells were co-incubated for 1 hr, fixed, permeabilized and stained for perforin (red) to identify cytotoxic granules. Cell nuclei were labeled with DAPI (blue). Scale bar: 10 µm.

and NK-92 cells responded strongly to unspecific activation by PMA and ionomycin (Fig. 2*b*). To further confirm specific activation of NK-92/4G8.28.z by FLT3-positive targets, we investigated conjugate formation between CAR NK and leukemia cells and polarization of cytotoxic granules to the site of contact by confocal laser scanning microscopy with mixed cultures of NK-92/4G8.28.z and SEM or HL-60 cells. Cells were cultured at an E/T ratio of 1:1 for 1 hr, and then stained with perforin-specific antibody to visualize cytolytic granules. While no stable conjugates were found between NK-92/4G8.28.z and FLT3-negative HL-60 cells, NK-92/4G8.28.z readily formed contacts with FLT3-positive SEM cells, which triggered reorientation of perforin-containing granules toward the interface with the target cells and release of cytotoxic effector molecules, indicated by membrane blebbing and formation of apoptotic bodies by the leukemia cells (Fig. 2*c*).

Cytotoxicity of NK-92/4G8.28.z against established and primary B-ALL cells

To assess antileukemic activity of CAR NK cells in a quantitative manner, *in vitro* cell killing experiments were performed by

coincubating established leukemia cells and primary B-ALL blasts with NK-92/4G8.28.z cells at different E/T ratios. NK-92/4G8.TM and parental NK-92 cells were included as controls. After 4 hr of coincubation, NK-92/4G8.28.z cells efficiently and selectively killed FLT3-positive SEM and NALM-16 B-ALL cell lines and primary B-ALL blasts, with the degree of cytotoxicity correlating with the level of FLT3 expressed by the targets and the applied E/T ratio (Fig. 3). CAR signaling was required for enhanced cell killing, indicated by the observed resistance of SEM leukemia cells and primary B-ALL blasts to NK-92/4G8.TM cells that carry a FLT3-specific but signaling-deficient CAR, which mirrored the cells' resistance to parental NK-92. FLT3-negative HL-60 acute promyelocytic leukemia cells were moderately sensitive to unmodified NK-92 cells, with NK-92/4G8.28.z and NK-92/4G8.TM cells not displaying enhanced cell killing. Human HSCs capable of long-term repopulation have been described to uniformly express FLT3,[39] which could make them a target for FLT3-specific CAR NK cells and result in undesired effects in a human host. To address this question, we tested cytotoxic activity of NK-92/4G8.28.z cells against mobilized $CD34^+$ HSCs derived from healthy donors. Thereby no cytotoxicity of the CAR NK cells toward HSCs was observed at the tested E/T ratios of up to 10:1. Similarly, parental NK-92 cells only displayed marginal background cytotoxicity toward HSCs (Fig. 3, bottom panel).

Antileukemic activity of NK-92/4G8.28.z cells in a B-ALL xenograft model

Next we evaluated the *in vivo* activity of FLT3-specific NK-92/4G8.28.z cells in an aggressive leukemia xenograft model in NSG mice. Animals were intravenously injected with 1×10^6 luciferase-expressing SEM cells. One week later, the mice were treated by intravenous injection of 1×10^7 parental NK-92 or CAR-expressing NK-92/4G8.28.z cells once per week for 4 weeks (Fig. 4*a*). Control animals received PBS. Leukemia development was monitored at frequent intervals by *in vivo* BLI. Parental NK-92 cells had no significant effect on leukemia development in comparison to PBS treatment, resulting in marked leukemia growth in bone marrow, joints, lymph nodes, spleen, liver, brain and along the spinal cord (Fig. 4*b*). In contrast, therapy with NK-92/4G8.28.z cells was effective and significantly delayed disease development, with two out of five animals only showing very moderate leukemia burden at Day 35 (Figs. 4*b* and 4*c*). This was confirmed by examination of excised spleens collected that day for endpoint analysis, which demonstrated significantly higher spleen weights and BLI signals for mice treated with parental NK-92 or PBS in comparison to animals treated with NK-92/4G8.28.z (Figs. 5*a* and 5*b*). Likewise, immunohistochemical analysis showed heavy infiltration of the spleen with FLT3-positive leukemia cells despite treatment with parental NK-92 cells, while spleen tissue from an NK-92/4G8.28.z-treated animal displayed only moderate leukemia growth (Fig. 5*c*). Similar data were obtained upon staining of spleen sections with CD19-specific antibody for detection of SEM B-ALL cells,[40] demonstrating that reduced FLT3 signals were indeed due to reduced leukemia growth and not caused by selection of antigen-loss variants by treatment with NK-92/4G8.28.z.

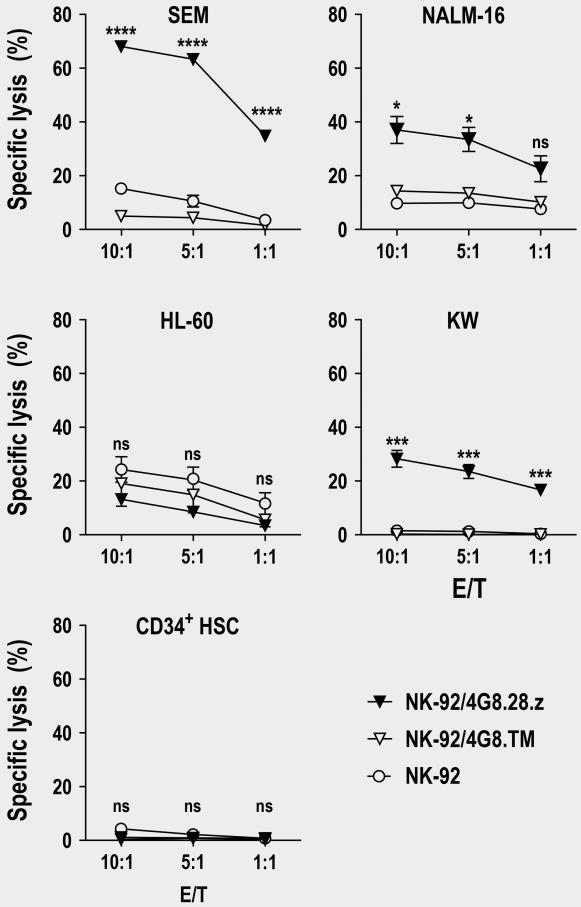

Figure 3. Cytotoxicity of CAR NK cells against established and primary leukemia cells. Cell killing by CAR-engineered NK-92/4G8.28.z cells (filled triangles) was investigated in FACS-based cytotoxicity assays after coincubation with established SEM, NALM-16 and HL-60 leukemia cells lines and KW primary B-ALL blasts as targets at different effector to target ratios (E/T) for 4 hr. Parental NK-92 (open circles) and NK-92/4G8.TM cells expressing a truncated CAR (open triangles) were included for comparison. Similar experiments were performed to investigate activity of NK-92/4G8.28.z and parental NK-92 cells against sorted $CD34^+$ hematopoietic stem cells from healthy donors (bottom panel). Mean values ± SEM are shown; $n = 3$. *p* Values for differences between treatments at the indicated E/T ratios were calculated by pairwise analysis using two-tailed unpaired *t*-test (shown for NK-92/4G8.28.z *vs.* NK-92/4G8.TM). ****, $p < 0.0001$; ***, $p < 0.001$; *, $p < 0.05$; ns: not significant ($p > 0.05$).

Control of NK-92/4G8.28.z activity by an inducible safety switch

At present, unmodified and CAR-engineered NK-92 cells are irradiated before clinical application as allogeneic cell therapeutics to prevent potential uncontrolled expansion of the immortalized cells in patients.[22,41] This results in loss of effector cells and declining antitumor activity over time,[38,42] likely requiring higher cell doses and repeated treatments to achieve efficacy. To test whether safety of FLT3-specific CAR NK-92 cells could also be enhanced

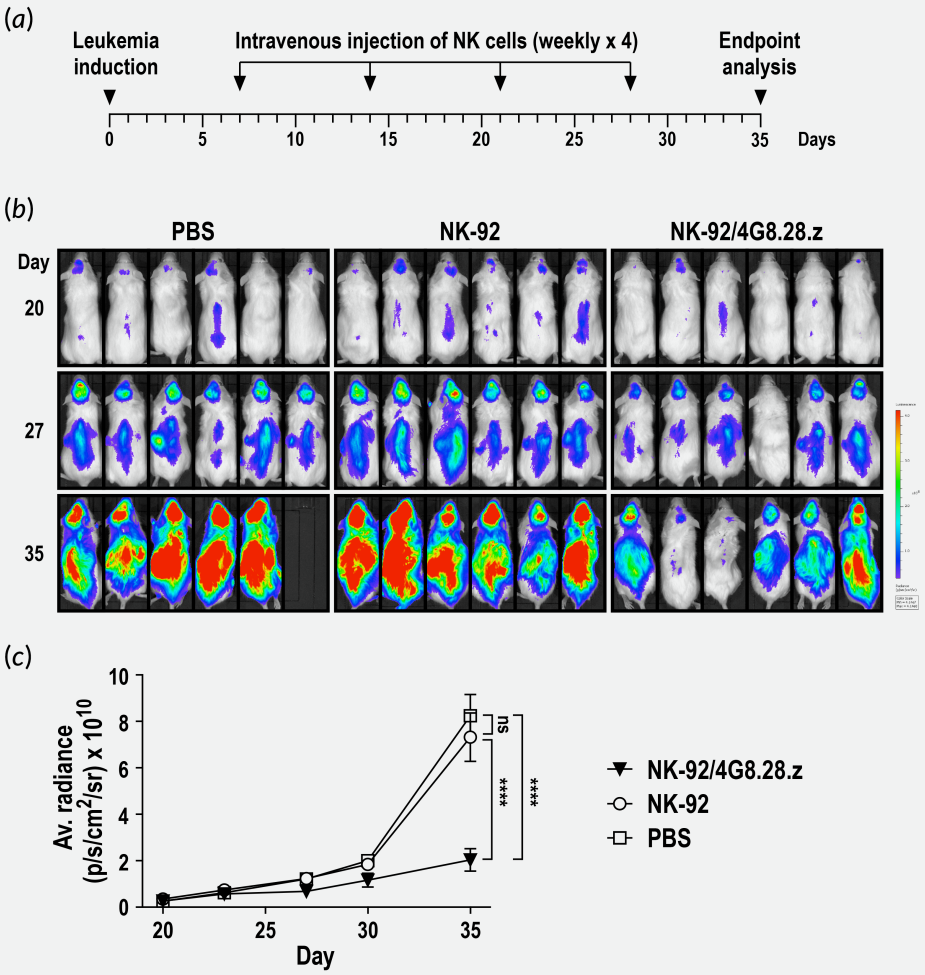

Figure 4. Inhibition of *in vivo* leukemia growth by FLT3-specific CAR NK-92 cells. (*a*) NSG mice were intravenously injected with 1×10^6 SEM/Luc leukemia cells. Starting 7 days after tumor cell inoculation, animals were treated by intravenous injection of 1×10^7 parental NK-92 or CAR-expressing NK-92/4G8.28.z cells once per week for 4 weeks. Control mice received PBS. (*b*) Leukemia development was monitored by *in vivo* bioluminescence imaging. Images taken in dorsal view at Days 20, 27 and 35 are shown (exposure time of 60 sec). (*c*) Kinetics of leukemia development. Average radiance values of 60 sec exposure time images recorded in dorsal and ventral positions were used for calculations. Mean values ± SEM are shown; $n = 6$. p Values for differences between treatment groups were calculated using two-way ANOVA with Tukey's multiple comparisons test. ****, $p < 0.0001$; ns: not significant ($p > 0.05$).

by incorporation of a suitable suicide gene, we generated NK-92 cells coexpressing CAR 4G8.28.z and iCasp9. This molecule represents a fusion of modified FK506 binding protein FKBP12 harboring an F36V mutation, and truncated caspase-9 lacking the caspase activation and recruitment domain.[33] iCasp9 and CAR sequences were combined in a lentiviral transfer plasmid, connected by a 2A *Thosea asigna* virus self-cleaving peptide (Fig. 6*a*; Supporting Information Fig. S2*c*). NK-92 cells were transduced with VSV-G pseudotyped lentiviral particles, and CAR-expressing cells were enriched by repeated flow cytometric cell sorting with Myc-tag-specific antibody recognizing the respective peptide epitope included in the extracellular CAR domain (Fig. 6*b*). The sorted CAR-positive NK-92/iCasp9_2A_4G8.28.z single cell clone selected for subsequent experiments also expressed high levels of iCasp9, revealed by immunoblot analysis of whole cell lysates with caspase-9-specific antibody (Fig. 6*c*). Interestingly, similar to parental NK-92 cells, NK-92/iCasp9_2A_4G8.28.z also expressed large amounts of endogenous pro-caspase-9. While unmodified NK-92 cells remained unaffected, addition of 10 nM of AP20187 to facilitate homodimerization of the FKBP12 F36V domain rapidly induced iCasp9 activation, with marked cleavage of both, iCasp9 and endogenous pro-caspase-9 already observed after 10 min and almost complete processing after 30 min (Fig. 6*c*). To test the effect of iCasp9 activation on the functionality of the CAR NK cells, *in vitro* cytotoxicity of NK-92/iCasp9_2A_4G8.28.z cells against FLT3-expressing SEM and NALM-16 leukemia cells in the absence or presence of AP20187 was investigated. Most likely due to minor clonal differences, without the homodimerizer

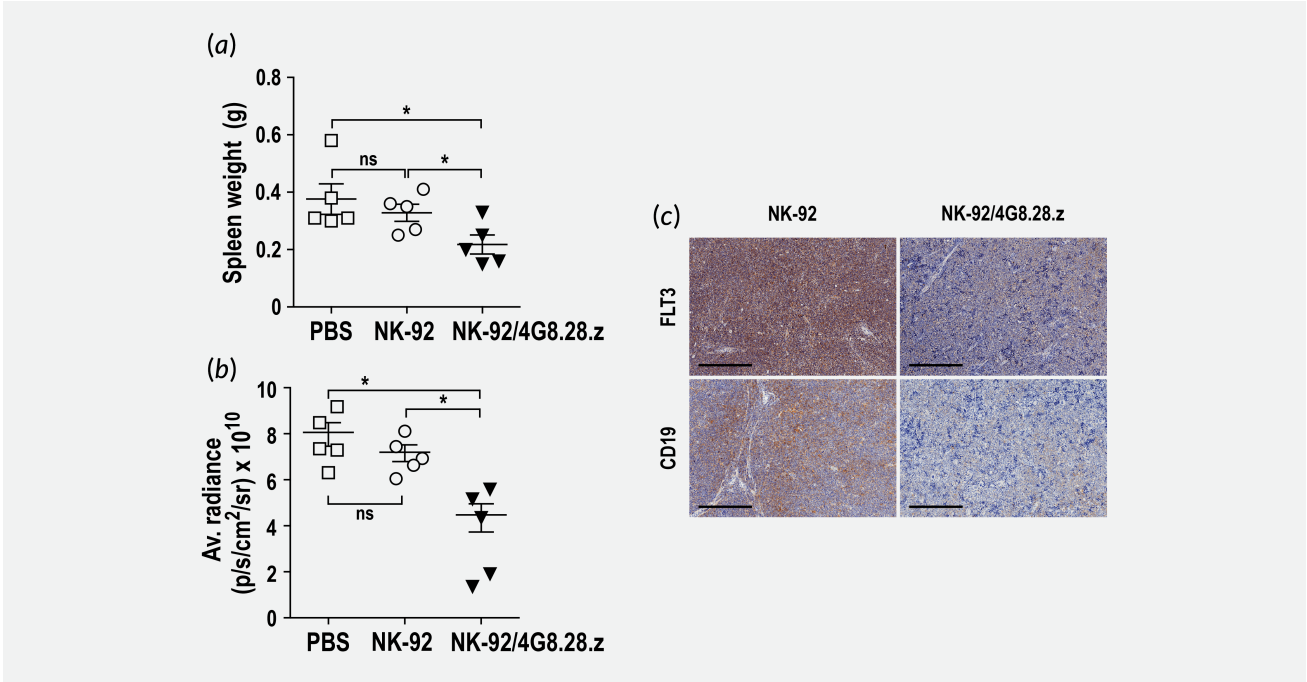

Figure 5. Endpoint analysis of leukemia-bearing mice after treatment with FLT3-specific CAR NK-92 cells. Animals from the experiment shown in Figure 4 were sacrificed for endpoint analysis at Day 35. Spleens were extracted from mice treated with NK-92 or NK-92/4G8.28.z cells, weighed (*a*), and immediately analyzed by bioluminescence imaging (*b*). Data points for individual animals and mean values ± SEM are shown; $n = 5$. p Values for differences between treatment groups were calculated by pairwise analysis using two-tailed unpaired t-test. *, $p < 0.05$; ns: not significant ($p > 0.05$). (*c*) Sections of spleen tissues from mice treated with NK-92 or NK-92/4G8.28.z cells were stained with FLT3- and CD19-specific antibodies for detection of leukemia cells. Scale bars: 300 μm.

specific cell killing of NK-92/iCasp9_2A_4G8.28.z was slightly higher than that of NK-92/4G8.28.z cells, but this was statistically significant only with NALM-16 target cells at an E/T ratio of 1:1 ($p < 0.05$). In contrast, when AP20187 was added, cytotoxicity of NK-92/iCasp9_2A_4G8.28.z cells was completely abrogated (Fig. 6*d*). As expected, AP20187 did not affect cell killing by NK-92/4G8.28.z cells which lack iCasp9. The proportion of viable NK-92/iCasp9_2A_4G8.28.z cells was reduced to around 11% after 6 hr and 2% after 26 hr of exposure to AP20187, further supporting suitability of iCasp9 as a safety switch for the CAR NK-92 cells (Supporting Information Fig. S3).

Discussion

In this work, we investigated NK cells engineered to express an FLT3-specific CAR as a novel approach to target pre-B-ALL in preclinical *in vitro* and *in vivo* models. While FLT3 has primarily been described as a therapeutic target for AML, overexpression of wild-type FLT3 and FLT3 mutations have also been found in B-ALL.[16,17] As effector cells we employed continuously expanding human NK-92 cells. In several early stage clinical trials, unmodified NK-92 cells and a CD33-specific CAR-engineered variant have been safely applied as allogeneic off-the-shelf therapeutics to cancer patients,[41,43–46] with clinical development of other CAR-expressing NK-92 derivatives ongoing (NCT03383978; clinicaltrials.gov).[47]

Here, NK-92 cells were transduced with a lentiviral vector encoding an FLT3-specific CAR encompassing a scFv fragment of monoclonal antibody 4G8,[28] fused to a CD8α hinge region, the transmembrane domain of CD28, and CD28 and CD3ζ signaling domains. CD28 and CD3ζ are both endogenously expressed in NK-92 cells, allowing CD28-CD3ζ CARs to readily link to the respective signaling pathways.[27,35,38,47] Indeed, contact with FLT3-positive leukemia cells triggered specific activation of CAR-expressing NK-92/4G8.28.z cells as evidenced by NK-cell degranulation and reorientation of perforin-containing cytolytic granules to the immunological synapse, which was not observed with untargeted NK-92 cells or FLT3-negative targets, respectively. In *in vitro* cell killing assays, FLT3-specific NK-92/4G8.28.z cells efficiently lysed at low E/T ratios FLT3-positive B-ALL cell lines and primary B-ALL blasts that were largely resistant to parental NK-92 cells, but showed no increase in cytotoxicity against FLT3-negative targets. Thereby CAR signaling was indispensable for enhanced cell killing, demonstrated by the absence of FLT3-dependent cell lysis in the case of NK-92/4G8.TM cells that carry a signaling-deficient CAR.

SEM B-ALL cells were chosen as a leukemia xenograft model to evaluate therapeutic activity of NK-92/4G8.28.z cells *in vivo*.[48] These cells carry high levels of activated wild-type FLT3, and are exceptionally aggressive and proliferative.[49] They readily express the pan-B-cell marker CD19, but have been described as biphenotypic, also displaying myeloid antigens and carrying a *t*(4;11) chromosomal rearrangement.[40,50] This is very similar to phenotype and genotype described for initially CD19-positive B-ALL, recurring after CD19 CAR-T cell therapy as CD19-negative disease.[13] NSG

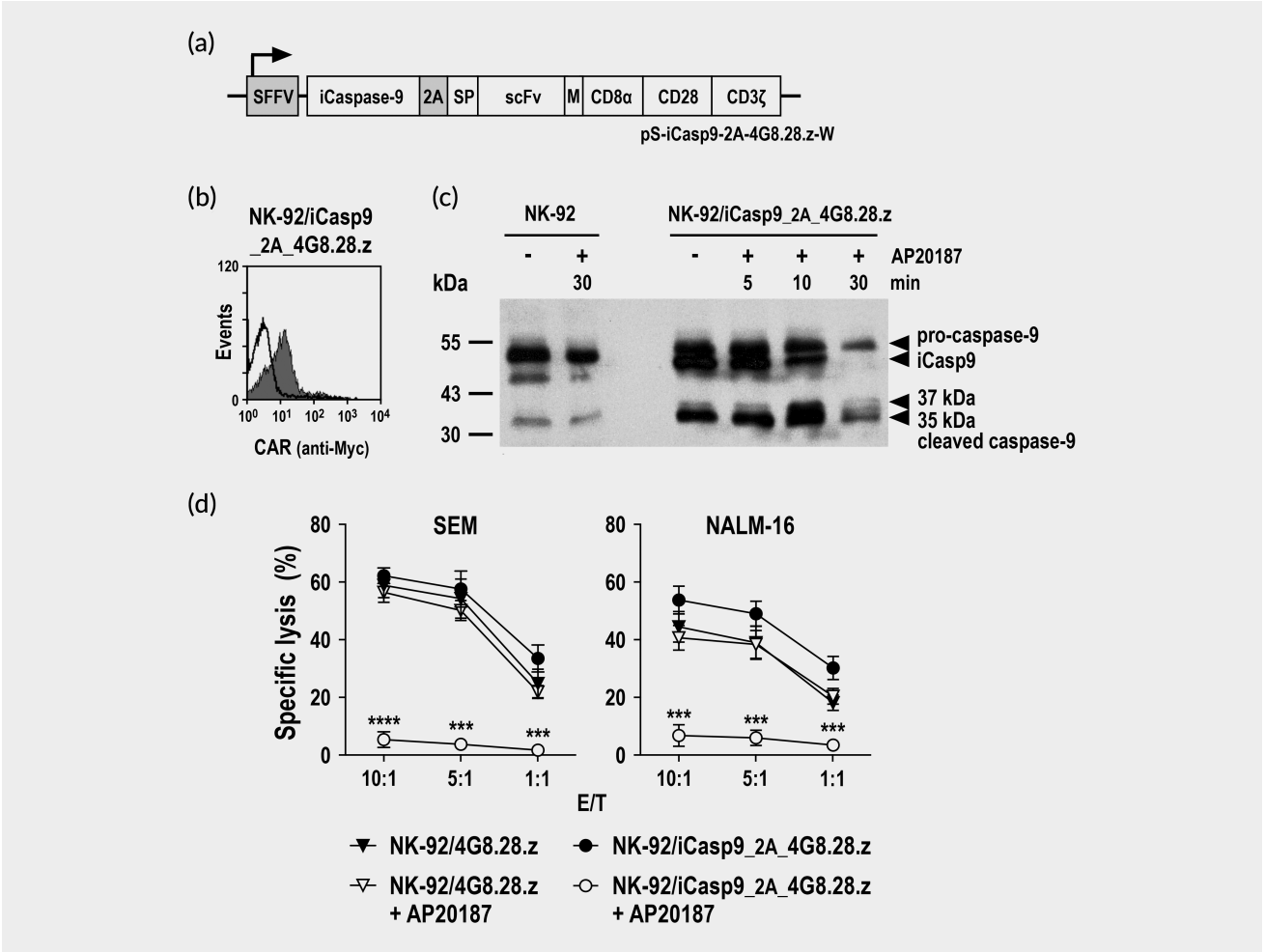

Figure 6. Inducible caspase-9 as a safety switch for FLT3-specific CAR NK cells. (*a*) Lentiviral transfer plasmid encoding under the control of the spleen focus forming virus promoter (SFFV) inducible caspase-9 (iCaspase-9) and FLT3-specific CAR 4G8.28.z as described in the legend of Figure 1*a*, separated by a *Thosea asigna* virus self-cleaving peptide (2A). (*b*) CAR surface expression by the selected NK-92/iCasp9_2A_4G8.28.z single cell clone was confirmed by flow cytometry with Myc-tag-specific antibody (filled area). Parental NK-92 cells served as control (open area). (*c*) NK-92/iCasp9_2A_4G8.28.z cells were incubated in the presence of 10 nM of the homodimerizer AP20187 for iCasp9 activation. Lysates of cells collected after 5, 10 or 30 min of exposure to AP20187 were subjected to SDS-PAGE and subsequent immunoblotting with a caspase-9-specific antibody. Lysates of NK-92/iCasp9_2A_4G8.28.z cells kept without dimerizer and NK-92 cells incubated in the absence or presence of AP20187 served as controls. (*d*) Cytotoxicity of NK-92/iCasp9_2A_4G8.28.z cells against FLT3-expressing SEM and NALM-16 leukemia cells was investigated in flow cytometry-based cytotoxicity assays after coincubation of NK cells and tumor cells at different effector to target ratios (E/T) for 4 hr in the absence (filled circles) or presence of AP20187 (open circles). NK-92/4G8.28.z cells without suicide gene were included for comparison in the absence (filled triangles) or presence of AP20187 (open triangles). Mean values ± SEM are shown; $n = 4$. p Values for differences between treatments at the indicated E/T ratios were calculated by pairwise analysis using two-tailed unpaired t-test (shown for NK-92/iCasp9_2A_4G8.28.z in the absence *vs.* NK-92/iCasp9_2A_4G8.28.z in the presence of AP20187). ****, $p < 0.0001$; ***, $p < 0.001$.

mice inoculated with luciferase-expressing SEM cells received four weekly treatments by intravenous injection of CAR NK cells starting 1 week after leukemia induction, and BLI was performed to monitor disease development. Therapy with parental NK-92 cells had no effect in comparison to PBS-treated control animals, with overt spreading of leukemia to lymphoid tissues, liver, brain and spinal cord. Encouragingly, treatment with four weekly doses of NK-92/4G8.28.z cells was effective and led to a significant reduction of leukemia burden in comparison to the controls, with two out of five animals in this group displaying only very moderate leukemia development until endpoint analysis at Day 35, indicated by significantly lower overall bioluminescence signals and markedly reduced leukemia infestation in the spleens. In contrast to human CAR T cells, NK-92 cells and their CAR-engineered variants do not engraft permanently in immunodeficient mice and do not expand upon encounter of target cells.[47] Hence, in the murine model application of higher doses of the CAR NK cells, more frequent treatment intervals, or further extension of the treatment period may be necessary to achieve even more effective leukemia control.

Prior to infusion into human cancer patients, NK-92 and CAR NK-92 cells have so far been irradiated with 10 Gy as a safety measure,[41,43–46] which prevents further proliferation. Irradiation has no short-term effect on CAR-mediated cytotoxicity (Supporting Information Fig. S4), but eventually results in a gradual loss of effector cell activity over time.[38,42] While this is not relevant in murine models, where nonirradiated CAR NK-92 do not proliferate and due to their limited life span in the xenogenic host do not display more enhanced antitumor activity than their irradiated counterparts,[38,51] it would most likely be different in a human patient, providing a more suitable environment for human NK cell survival and expansion. Accordingly, replacement of irradiation of NK-92 cells with a suicide gene-based approach appears warranted to extend *in vivo* functionality and enhance efficacy for potential clinical application without compromising on safety. Here we investigated co-expression of the FLT3-specific CAR 4G8.28.z together with iCasp9 in NK-92 cells. iCasp9 can be activated rapidly through addition of otherwise inert chemical inducers of dimerization of its modified FKBP12 domain such as rimiducid (AP1903) or the AP20187 compound used in our study,[33] and has been employed successfully to control development of graft-*vs.*-host disease after infusion of genetically modified donor-derived T cells in haploidentical HSC transplant patients.[52]

Indeed, sorted NK-92/iCasp9_2A_4G8.28.z cells coexpressed CAR and iCasp9 as evidenced by flow cytometry and immunoblot analysis of whole cell lysates. While most of iCasp9 and endogenous pro-caspase-9 were found as intact full-length proteins, a distinct signal corresponding to the p35 iCasp9/caspase-9 cleavage product was noted in the absence of chemical dimerizer suggesting some spontaneous iCasp9 cleavage. Nevertheless, the cells continued to expand, indicating that a limited degree of iCasp9 autoactivation is tolerable for NK-92. When iCasp9 was specifically activated by addition of AP20187, a large proportion of iCasp9 and pro-caspase-9 were fully processed already after 10 min, with no intact iCasp9 and only very little unprocessed pro-caspase-9 remaining after 30 min. iCasp9 activation precluded cell killing by NK-92/iCasp9_2A_4G8.28.z cells, evidenced by a complete lack of CAR-mediated cytotoxicity in the presence of AP20187. Also viability of NK-92/iCasp9_2A_4G8.28.z cells was strongly affected by iCasp9 activation, resulting in a progressive loss of viable cells over time, with only around 2% remaining after 26 hr of exposure to the chemical dimerizer (Supporting Information Fig. S3). These data demonstrate that iCasp9 can indeed be applied as an inducible safety switch for CAR-engineered NK-92 cells, which may not only bypass the need for γ-irradiation, but also provide a means to control possible adverse events as currently done in the case of CD19-CAR engineered NK cells derived from cord blood.[24]

FLT3 is expressed on normal human HSC and precursor cells,[39] raising the possibility of CAR-mediated cytotoxicity against these cell types. In a previous study parental antibody 4G8 on its own did not affect colony formation and was not cytotoxic toward CD34-positive human bone marrow cells.[28] Likewise, two prior studies with T cells carrying CARs based on FLT3-specific scFv antibody or FLT3 ligand reported normal human hematopoiesis in NSG mice transplanted with HSCs after treatment with the FLT3-specific CAR T cells, and undisturbed colony formation of cord blood derived HSCs in the presence of CAR T cells, respectively.[53,54] In contrast, in another recent report, a marked reduction in colony formation and HSC viability was observed upon exposure of HSCs to FLT3-targeted CAR T cells.[55] Here we did not observe CAR-mediated killing of mobilized human CD34-positive HSCs by NK-92/4G8.28.z cells at the tested E/T ratios. Nevertheless, given the diverging data with FLT3-specific CAR T cells, FLT3 expression by HSCs and sensitivity to FLT3-targeted agents may be different for individual donors or patients. Accordingly, potential negative effects of NK-92/4G8.28.z cells on normal hematopoiesis cannot be fully excluded at this point, underscoring the importance of employing a suitable safety measure such as iCasp9 in a clinical setting.

Taken together, our data demonstrate that B-ALL can be effectively targeted by continuously expanding NK cells engineered with an FLT3-specific CAR. This approach may complement CD19 CAR expressing effector cells, in particular in cases of acquired resistance to the latter due to treatment-induced selection of antigen-loss variants. We showed that iCasp9 can be readily employed as an inducible safety switch in FLT3-specific CAR NK-92 cells, where it may serve a dual function, allowing to bypass γ-irradiation as a safety measure for clinical application, but also to rapidly inactivate the effector cells in case of serious adverse reactions.

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