Supplementary Material

Intracranial injection of NK cells engineered with a HER2-targeted chimeric antigen receptor in patients with recurrent glioblastoma

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Supplementary Materials and Methods

HER2 Immunohistochemistry

Formalin-fixed and paraffin-embedded (FFPE) sections (4 µm thickness) of primary and recurrent tumor tissues were subjected to immunohistochemical staining of HER2 (polyclonal, dilution 1:250; Dako, Agilent, Santa Clara, CA) following established protocols by use of a LEICA BOND-III automated stainer (Leica, Wetzlar, Germany). For HER2 scoring, staining intensities and frequencies were used to calculate the Immune Reactivity Score (IRS) as described.[1] The staining intensity score ranged from 0 to 3 (no, low, moderate, strong staining), while the staining frequency ranged from 0 to 4 (0, 1-10, 11-25, 25-50, >50% of cells). The two scores were multiplied resulting in HER2 IRS values ranging from 0 to 12. Immunohistochemical stainings were independently evaluated by at least two experienced neuropathologists. A HER2 IRS value of 3/12 was initially required for study inclusion,[1] which was subsequently changed to any positive score (at least 1/12).

Determination of Serum Cytokine Levels

Serum samples collected at indicated time points before and after CAR-NK therapy were stored at -80°C until analysis. Cytokines were determined using ProcartaPlex Immunoassay Mix & Match panels (ThermoFisher Scientific, Darmstadt, Germany) comprising the cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-12p70, TNFα, IFNγ, MIP-1α (CCL3), IP-10 (CXCL10), MCP-1 (CCL2), granzyme B and perforin. Data acquisition was performed using a Luminex FLEXMAP3D (ThermoFisher Scientific). The data were analyzed using the ProcartaPlex Analyst 1.0 Software (ThermoFisher Scientific).

Detection of HLA-Specific Antibodies

To assess possible formation of anti-HLA antibodies induced by CAR-NK treatment, HLA-specific antibodies in serum samples were detected using the LABScreen Mixed Class I & II and the Single Antigen HLA Class I Kits (ThermoFisher Scientific). Tests were done according

to the manufacturer's instructions. Data acquisition was performed using a FLEXMAP3D (Luminex Corp., Austin, TX). HLA-Fusion software (Thermo Fisher Scientific) was used for data analysis. Complement activating antibodies were analyzed using commercial frozen cell trays (BAG Healthcare Diagnostics GmbH, Lich, Germany) according to the manufacturer's instructions. The cells were examined using UV microscopy.

Immunophenotyping of Peripheral Blood

Peripheral EDTA blood (2 x 7.5 mL) was collected at different time points ranging from day -1 to week 12 after NK-92/5.28.z intracranial application. To determine a detailed immune status, immune phenotyping was performed from either whole blood or from peripheral blood mononuclear cells (PBMCs) following density gradient centrifugation (Biocoll, Biochrom, Berlin, Germany) by multi-color flow cytometry (CytoFLEX S, Beckman Coulter, Krefeld, Germany) using 3 panels of fluorescence-labeled antibodies (see Supplementary Table S6). Briefly, 1 x 10^6 cells were blocked with human FcR binding inhibitor (eBioscience, Frankfurt am Main, Germany), incubated with antibodies specific for myeloid and lymphatic lineages (panel 1: CD45, CD3, CD14, CD11b, CD15, CD19, CD33, CD56, HLA-DR), T lymphocytes and activation markers (panel 2: CD3, CD4, CD8, CD25, PD-1, granzyme B, perforin), or regulatory T cells (panel 3: CD3, CD4, CD8, CD25, PD-1, FoxP3) after fixation and permeabilization (Fix & Perm, Nordic MUbio, Susteren, Netherlands, and FoxP3 staining set, Invitrogen, Frankfurt am Main, Germany) for panels 2 and 3. Next, a total of 100,000 $CD45^+$ cells were gated in case of panel 1, and of $CD3^+$ cells in case of panels 2 and 3, and data were analyzed by using the CytExpert software (Beckman Coulter) with the ratio of positively stained cells defined as the percentage of cells within a defined leukocyte phenotype gate from total $CD45^+$/$CD3^+$ cells. Absolute cell counts of lymphocytes and $CD56^+$ NK cells were derived by Sysmex analysis and by the BD Multitest™ 6-color TBNK staining kit (BD Biosciences, Heidelberg, Germany) on a FACS Canto II flow cytometer using FACS Canto software (BD Biosciences) according to the manufacturer's protocols.

Detection of CAR DNA

Primers specific for the FRP5 antibody fragment of the CAR (Fwd: 5'-GACAGTCAAGATCAGCTG-3'; Rev: 5'-AGGCTGAAGTCGAATCTG-3') and a TaqMan probe (5'-TCACCAACTACGGCATGAACTG-3') (Roche, Basel, Switzerland) were used to detect 5.28.z CAR DNA by qPCR. After erythrocyte lysis, DNA was extracted from peripheral blood mononuclear cells using QIAmp® DNA Mini Kit (Qiagen, Hilden, Germany). qPCR reactions were performed in triplicates using CFX Connect System (BioRad, Hercules, CA). An initial preheating step of 10 min to 95°C was followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. Data were analyzed with CFX Maestro Software (BioRad). To determine CAR copy numbers per µg of DNA, results were normalized to pre-treatment controls and a standard with serial dilutions of the CAR transfer plasmid. Genomic DNA of NK-92/5.28.z cells served as positive control.

Ex Vivo GB Cell Cultures

Tumor samples obtained during relapse surgery were dissociated to obtain single-cell suspensions.[2] Then, tumor cells were grown as primary GB cell cultures up to a maximum passage number of 4 in flasks pre-coated with 5 mg/mL laminin (Sigma-Aldrich, Munich, Germany) with DMEM/F12 medium (Lonza, Cologne, Germany) containing 20 ng/mL each of recombinant epidermal growth factor (EGF) and human recombinant basic fibroblast growth factor 2 (bFGF2) (ReliaTech, Wolfenbüttel, Germany), and 20% BIT Admixture Supplement (Pelo Biotech, Planegg/Martinsried, Germany). Surface expression of HER2 was determined by flow cytometric analysis of GB cells with Alexa Fluor 647-conjugated anti-human HER2 antibody (clone #24D2) (BioLegend, Koblenz, Germany). Alexa Fluor 647-conjugated mouse IgG1 antibody (clone #MOPC-21) (BioLegend) served as isotype control. Data were analyzed using FlowJo software (Version 10.0.7; FlowJo, Ashland, OR). Cytotoxicity of NK-92/5.28.z CAR-NK cells against primary GB cells was analyzed in a FACS-based assay as described previously.[3] Briefly, *ex vivo* cultured GB cells were labeled with calcein violet AM (CV) (Life Technologies, Darmstadt, Germany) and co-cultured for 2 hours at 37°C with CAR-NK cells at

an effector to target (E/T) ratio of 10:1. Then cells were collected by centrifugation and resuspended in 250 µL of a 1 µg/mL propidium iodide (PI) solution before flow cytometric analysis. Dead target cells were identified as CV and PI double positive. Spontaneous target cell lysis was subtracted to calculate specific cytotoxicity.

Whole Exome Sequencing

Whole exome sequencing (WES) was performed on all available tumor samples, including primary tumor, pre and post CAR-NK therapy and matching peripheral blood samples (control), using the Illumina NextSeq1000 platform (Illumina, San Diego, CA). DNA was extracted from FFPE GB tissues using the Maxwell RSC FFPE Plus DNA kit (Maxwell RSC Cultured Cells DNA Kit for blood samples), following the manufacturer's instructions (Promega, Fitchburg, WI). DNA concentration was determined using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific). WES libraries were generated from 750 ng of DNA (200 ng for blood samples), using the Illumina DNA Prep with Enrichment Kit (Illumina). In brief, the genomic DNA was tagmented, cleaned up and amplified. Clean-ups were performed with AMPureXP beads (Beckman Coulter, Brea, CA) with a 1.8 x volume ratio. The cleaned-up libraries were then pooled, hybridized with probes and amplified. The WES libraries were sequenced in 100 bp paired end reads on the Illumina NextSeq1000 machine using NextSeq1000 P2 200 cycles flow cells. Variant calling, tumor mutational burden (TMB) and microsatellite instability (MSI) calculations were performed using the DRAGEN somatic pipeline (v3.9.5; Illumina) in tumor normal mode. The TMB is the number of (filtered) non-synonymous variants divided by the eligible regions per million bases (Mb). Filtering parameters include removal of germline variants via normal sample, a minimum coverage of 40 and a minimum variant allele frequency of 5%. TMB values ≤ 5 are considered low, >5 and <20 intermediate, ≥20 and <50 high, and ≥50 very high.[4] MSI describes genetic hypermutability in short repetitive DNA sequences, which occurs due to defects in DNA mismatch repair. Elevated MSI is associated with certain types of cancer. The DRAGEN somatic pipeline collects counts from matching tumor/normal pairs for each microsatellite site, then calculates

the Jensen Shannon distance for each microsatellite site and identifies the unstable sites by performing chi square testing of tumor and normal distribution. A value of more than 20% unstable sites indicates microsatellite instability.

DNA Methylation Analysis and Reference-Based Tumor Deconvolution

All recurrent tumor samples were subjected to DNA methylation analysis by use of the Human Methylation EPIC array (Illumina). DNA was isolated, and bisulfite-converted for further processing and hybridization to the EPIC array BeadChip according to protocols provided by the manufacturer. BeadChips were scanned using the iScan (Illumina), and raw intensity data (idats) were acquired. The reference-based tumor deconvolution algorithm MethylCIBERSORT was used to deduce cellular composition and leukocyte infiltration in bulk tumors, which allows to estimate the proportions of tumor cells and immune cell subsets from DNA methylation data by comparison of sample methylomes to reference methylomes.[5,6] Reference methylomes were kindly provided by Tim R. Fenton, University of Southampton, Southampton, UK.[5] Briefly, idats were imported into the R minfi package for computation of quality checks and Noob normalization, as well as acquisition of beta values. Sample and reference beta value matrices were uploaded onto the CIBERSORT website and deconvoluted (https://cibersort.stanford.edu; provided by the Alizadeh Lab, Stanford University, USA).[6]

Supplementary References

1. Zhang C, Burger MC, Jennewein L, et al. ErbB2/HER2-specific NK cells for targeted therapy of glioblastoma. *J Natl Cancer Inst.* 2016;108(5):djv375.
2. Lee J, Kotliarova S, Kotliarov Y, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell.* 2006;9(5):391-403.
3. Sahm C, Schönfeld K, Wels WS. Expression of IL-15 in NK cells results in rapid enrichment and selective cytotoxicity of gene-modified effectors that carry a tumor-specific antigen receptor. *Cancer Immunol Immunother.* 2012;61(9):1451-1461.
4. Riviere P, Goodman AM, Okamura R, et al. High tumor mutational burden correlates with longer survival in immunotherapy-naïve patients with diverse cancers. *Mol Cancer Ther.* 2020;19(10):2139-2145.
5. Chakravarthy A, Furness A, Joshi K, et al. Pan-cancer deconvolution of tumour composition using DNA methylation. *Nat Commun.* 2018;9(1):3220.
6. Newman AM, Liu CL, Green MR, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods.* 2015;12(5):453-457.

Supplementary Tables

Table S1 MRI Protocol

Sequence	Orientation	Duration (min:sec)	FOV (cm)	Matrix	Slices	Slice thickness (mm)	TR/TE (ms)	Flip angle	Other
T2 TSE	transversal	2:36	230 x 230	448 x 448	25	5	4980/ 92	150°	
FLAIR	transversal	3:43	220 x 220	320 x 320	35	4	8500/ 81	150°	
DWI (EPI)	transversal; coronal	1:38; 1:39	220 x 220; 220 x 220	192 x 192; 192 x 192	26; 26	5; 5	3800/ 95; 3800/ 112		diffusion weightings: b 0/500/1000
SWI	3D	4:50	220 x 220	256 x 256	96	1.5	27/ 20	15°	
T1 SE	transversal	3:51	240 x 240	384 x 384	25	5	600/ 13	70°	acquisition both before and after administration of contrast agent
DSC PWI (EPI)	transversal	1:36	230 x 230	128 x 128	25	3	1790/ 30	90°	50 scans total, 10 baseline scans before administration of the contrast agent bolus
T1 MPRAGE	3D	5:21	240 x 240	256 x 256	192	0.9	2300/ 2.32	8°	acquisition after administration of contrast agent

Imaging was performed using a 3 Tesla MRI scanner Skyra® (Siemens, Erlangen, Germany). Application of a single bolus of gadolinium-based contrast agent during the DSC PWI measurement as specified above (Gd-DO3A-butrol, Gadovist®, Bayer Vital GmbH, Germany, 0.1 mmol/kg bodyweight; infusion rate, 4 mL/s followed by 21 mL of NaCl). Assessment was performed according to RANO criteria. Abbreviations: FOV, field of view; TR, repetition time; TE, echo time; TSE, turbo spin echo; FLAIR, fluid-attenuated inversion recovery; DWI, diffusion-weighted imaging; EPI, echoplanar imaging; SWI, susceptibility-weighted imaging; SE, spin echo; DSC PWI, dynamic susceptibility contrast perfusion-weighted imaging; MPRAGE, magnetization prepared rapid gradient echo.

Table S2 Antibody Panel for Multispectral Imaging

Antibody	Clone	Company	Fluorophore	Reference number
CD3	polyclonal	DAKO Agilent	Opal 690 o. 480	A0452
CD4	SP35	ThermoFisher Scientific	Opal 620	Ma5-16338
CD8	C8/144B	DAKO Agilent	Opal 520	M7103
CD163	BLR087G	Abcam	Opal 620	Ab265592
FoxP3	SP97	ThermoFisher Scientific	Opal 570	MA5-16365
Iba-1	polyclonal	FUJIFILM Wako	Opal 780	019-19741
Ki-67	MIB-1	DAKO Agilent	Opal 780	M7240
PD-1	EPR4877(2)	Abcam	Opal 690	Ab137132
vWF	polyclonal	DAKO Agilent	Opal 480	A0082

Table S3 Quality of Generated Cell Products and Conformity with Release Criteria

Patient ID	Dose level	Viability (%)	C56^{+} CD16^{-} (%)	CAR expression (%)	Specific cytotoxicity
CB001	1 x 10^{7}	95.7	99.4	97.3	90.1
CB003	1 x 10^{7}	93.6	99.5	99.6	96.8
CB004	1 x 10^{7}	93.7	99.4	98.6	92.5
CB005	3 x 10^{7}	93.3	99.4	98.5	89.8
CB006	3 x 10^{7}	94.5	98.8	98.9	92.6
CB007	3 x 10^{7}	94.7	98.7	98.5	98.3
CB009	1 x 10^{8}	93.4	96.7	96.9	76.6
CB010	1 x 10^{8}	96.2	98.0	97.6	95.5
CB011	1 x 10^{8}	95.7	98.7	98.6	80.0

Individual patient dosages of NK-92/5.28.z cells were expanded from a maintenance culture generated from a qualified GMP-compliant master cell stock. For each run, identity of NK-92/5.28.z cells (NK-92 immunophenotype: CD56^{bright}, CD16^{negative}) and CAR expression were confirmed by flow cytometric analysis with anti-human CD56-PE and CD16-FITC antibodies, or recombinant HER2-Fc fusion protein followed by goat anti-human-APC antibody, respectively[19]. Viability was determined using 7-AAD staining. CAR-mediated cell killing was analyzed on the final product after irradiation in a 2 hours Europium-TDA cytotoxicity assay using HER2-positive MDA-MB-453 breast carcinoma cells as targets at an effector to target ratio of 10:1. In addition, absence of mycoplasma, p24 antigen expression and endotoxin (< 3.0 EU/mL) were confirmed before release (data not shown). Initial release for sterility was based on an in-process control and confirmed on the final product after the time point of release. The final cell preparations were irradiated at 10 Gy, washed and provided as fresh cell suspension reconstituted in GMP grade single donor human serum containing 100 U/mL IL-2 for same day injections.

Table S4 Serum HLA Antibodies against NK-92 Pre and Post NK-92/5.28.z Treatment

	Complement activating antibodies against NK-92 cells and NK-92/5.28 cells		**HLA class I - complement activating antibodies (CDC)**		**HLA class I - Single Antigen Bead (SAB)**	
	pre treatment	post injection (week 4)	pre treatment	post injection (week 4)	pre treatment	post injection (week 4)
CB001	not detected	not detected	not detected	not detected	not detected	not detected
CB003	not detected	not detected	not detected	not detected	not detected	not detected
CB004	not detected	not detected	not detected	not detected	anti-HLA antibody specific for NK-92 HLA expression: B7 (MFI 3051)	anti-HLA antibody specific for NK-92 HLA expression: B7 (MFI 3045)
CB005	not detected	not detected	not detected	not detected	not detected	not detected
CB006	not detected	not detected	not detected	not detected	not detected	not detected
CB007	not detected	not detected	not detected	not detected	not detected	not detected
CB009	not detected	not detected	not detected	not detected	not detected	not detected
CB010	not detected	not detected	not detected	not detected	not detected	not detected
CB011	not detected	not detected	not detected	not detected	not detected	not detected

Complement activating antibodies (CDC) as determined by microlymphocytotoxicity test using SeraScreen FCT plates, parental NK-92 cells and NK-92/5.28.z cells (HLA *A03:01,*11:01; B*07:02,*44:03; C*07:02,*16:01). HLA-specific antibodies were determined by single antigen bead array. No patient had complement activating antibodies to either HLA-typed lymphocytes, parental NK-92 cells or NK-92/5.28.z cells. Patient CB004 was positive for HLA-specific antibodies reactive with NK-92/5.28.z cells (HLA B7), which were detected pre and post NK-92/5.28.z treatment with comparable signal intensity as a measure of antibody concentration. MFI: Mean fluorescence intensity (geometric mean).

Table S5 Tumor Mutational Burden (TMB) and Microsatellite Instability (MSI)

Patient	Status	TMB (Mut./Mb)	MSI (% of unstable sites)
CB001	P	1.96	7.71
	R (pre treatment)	1.74	4.98
CB003	P	1.14	4.75
	R (pre treatment)	4.46	10.51
CB004	P	1.20	4.71
	R (pre treatment)	2.30	6.86
CB005	P	1.35	3.33
	R (pre treatment)	45.91	7.46
	R (post treatment)	52.85	29.36
CB006	P	0.75	3.33
	R (pre treatment)	1.22	4.42
CB007	P	2.50	7.46
	R (pre treatment)	2.59	5.92
	R (post treatment)	2.05	8.71
CB009	P	1.99	4.72
	R (pre treatment)	5.42	4.60
CB010	P	1.55	3.2
	R (pre treatment)	2.15	4.96
CB011	P	1.76	5.20
	R (pre treatment)	1.79	4.01
	R (post treatment)	1.98	5.12

TMB is the number of somatic mutations per megabases of investigated genomic sequence. It is calculated by dividing the number of non-synonymous variants (mutations, Mut.) by the eligible regions per million bases (Mb). TMB values <5, low; 5-10, intermediate; 20-15, high; >50, very high. MSI describes genetic hypermutability in short repetitive DNA sequences occurring due to defects in DNA mismatch repair. This was calculated using the DRAGEN somatic pipeline. A value of more than 20% indicates microsatellite instability. P, primary tumor tissue; R, recurrent tumor tissue collected before (pre) or after (post) CAR-NK treatment.

Table S6 Antibody Panel for Flow-Cytometric Analysis of Peripheral Blood Mononuclear Cells

Antibody	Clone	Company	Fluorophore	Reference number
CD3	SK7	BD Biosciences	PerCP-Cy5,5	332771
CD4	SK3	BD Biosciences	V450	651849
CD8	SK1	BD Biosciences	APC-H7	641400
CD11b	D12	BD Biosciences	PE	333142
CD14	MφP9	BD Biosciences	APC-H7	641394
CD15	MMA	BD Biosciences	FITC	332778
CD19	SJ25C1	BD Biosciences	PeCy7; PC7	341113
CD25	2A3	BD Biosciences	BV510	740198
CD33	P67.6	BD Biosciences	APC	345800
CD45	2D1	BD Biosciences	V500	655873
CD56	MY31	BD Biosciences	BV650	742660
PD-1 (CD279)	eBioJ105	eBioscience	PeCy7; PC7	25-2799-42
Perforin	dG9	BD Biosciences	FITC	556577
Granzyme B	GB11	BD Biosciences	PE	561142
FoxP3	236A/E7	eBioscience	APC	17-4777-42
HLA-DR	L243	BD Biosciences	V450	655874

Supplementary Figures

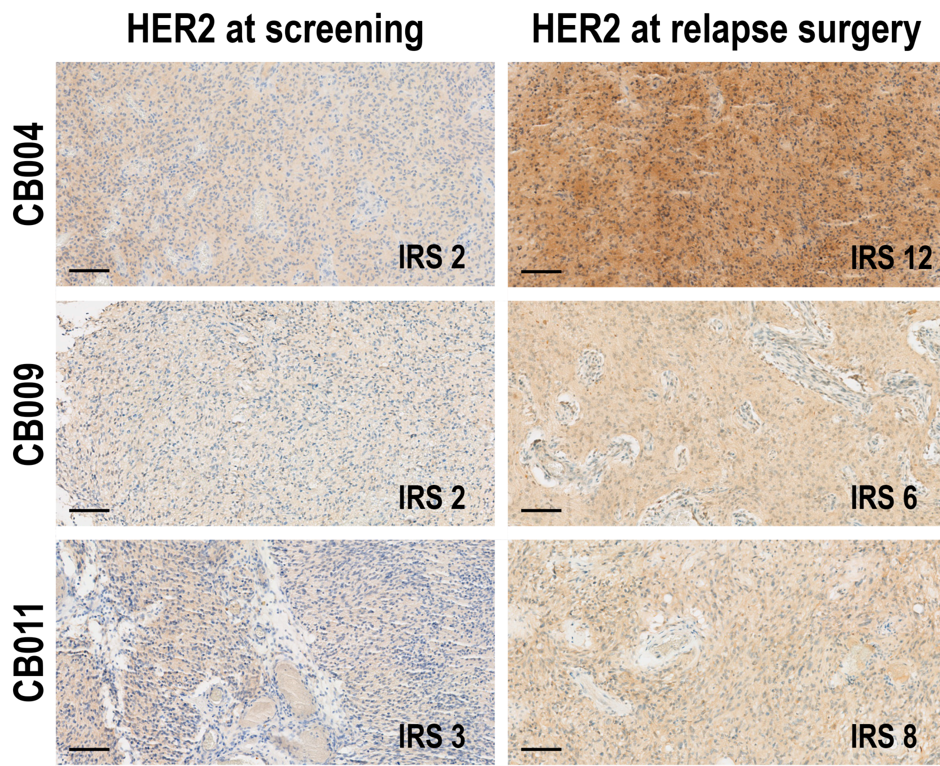

Fig. S1 Analysis of HER2 expression by immunohistochemistry. Tumor sections were stained with HER2-specific antibody as described in Supplementary Methods. The scoring system applied takes staining intensity (0 to 3: no, low, moderate, strong staining) and staining frequency (0 to 4: 0, 1-10, 11-25, 25-50, >50% of cells) into account. The two scores were multiplied resulting in an immune reactivity score (IRS) ranging from 0 to 12. Examples of primary (HER2 at screening) and recurrent glioblastoma tumors at the time of treatment (HER2 at relapse surgery) from patients CB004, CB009 and CB011 are shown. Scale bars: 100 µm.

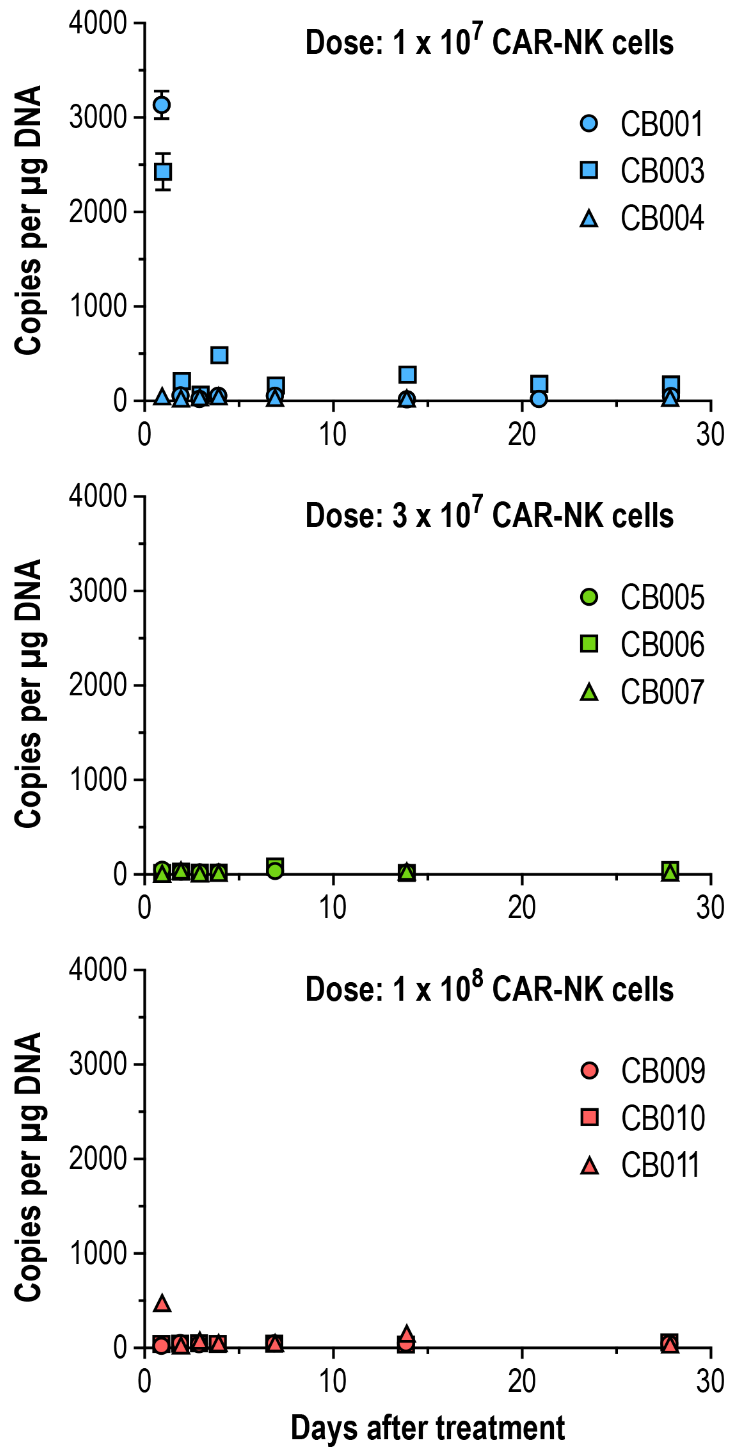

Fig. S2 Detection of CAR-NK cell DNA by TaqMan qPCR in peripheral blood to assess potential persistence of CAR-NK cells.

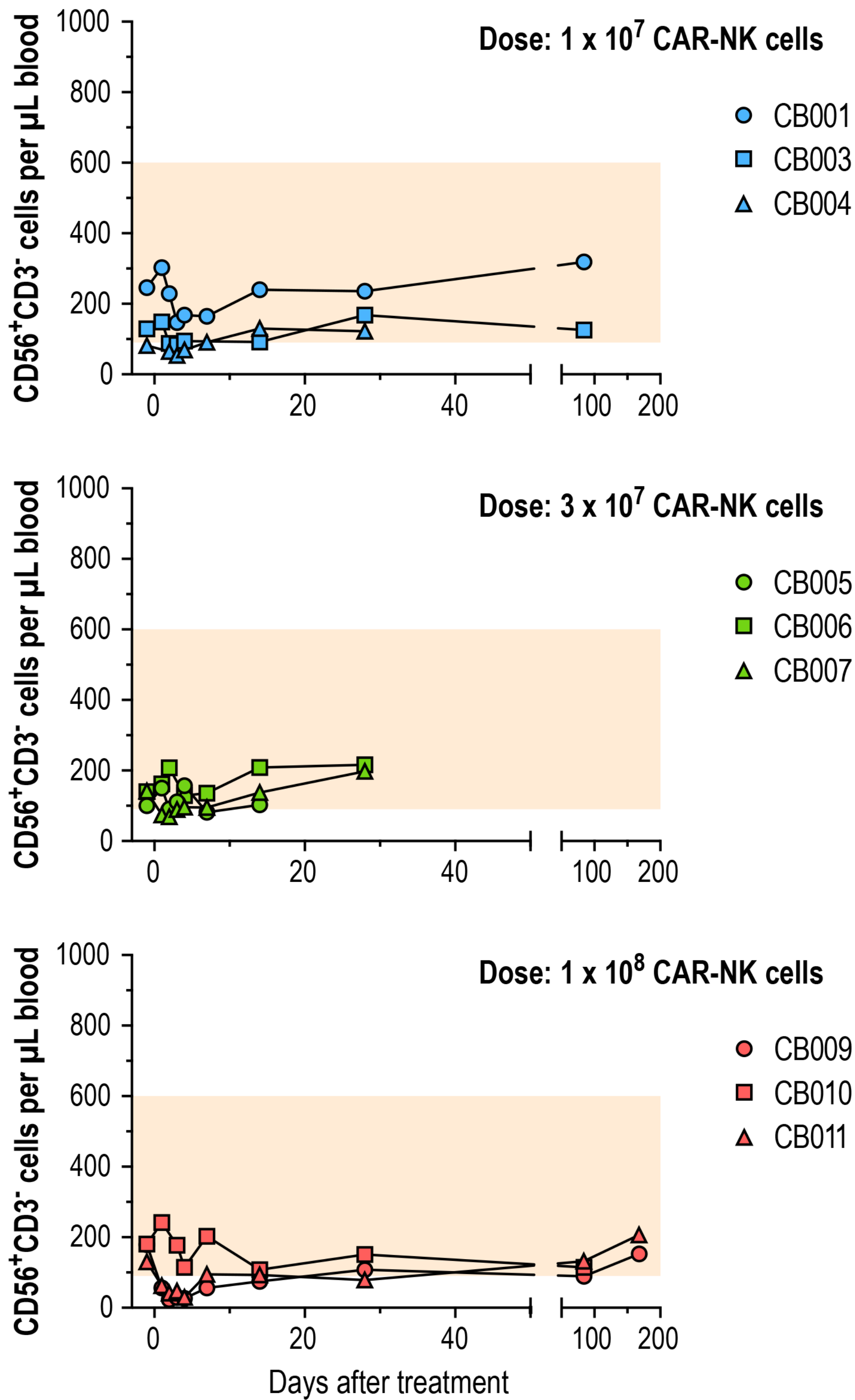

Fig. S3 NK cells in peripheral blood. Absolute numbers of NK cells were determined by flow cytometry in peripheral blood of patients one day before injection of NK-92/5.28.z cells and at the indicated time-points after treatment (normal range: 90-600 cells/µL).

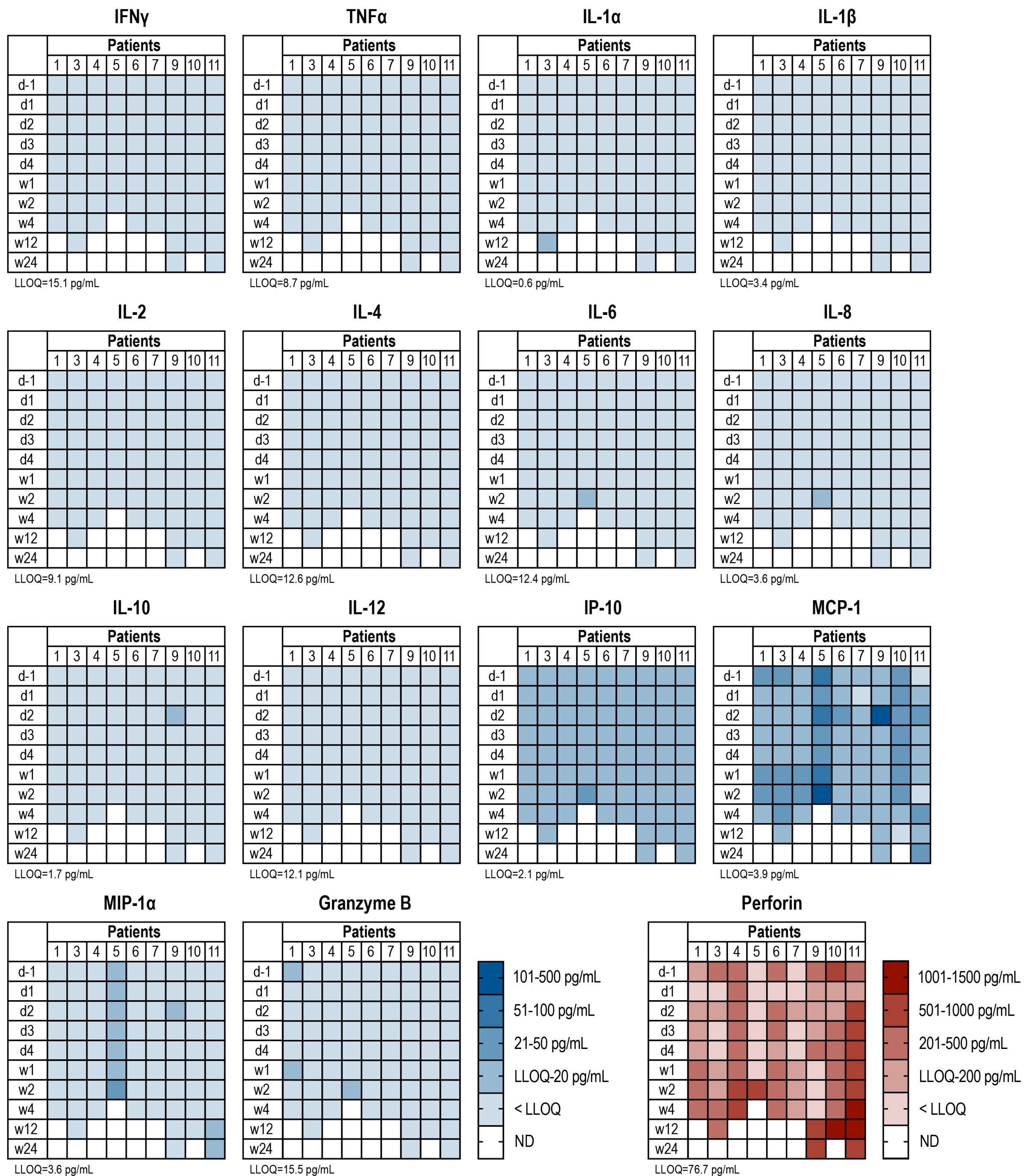

Fig. S4 Serum cytokine levels after CAR-NK cell therapy. Concentrations of a broad panel of cytokines including IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFNγ, IP-10, MCP-1, MIP-1α and TNFα as well as granzyme B and perforin were measured using a Luminex assay in peripheral blood of patients one day before injection of NK-92/5.28.z cells and at the indicated time-points after treatment. LLOQ: Lower limit of quantification.

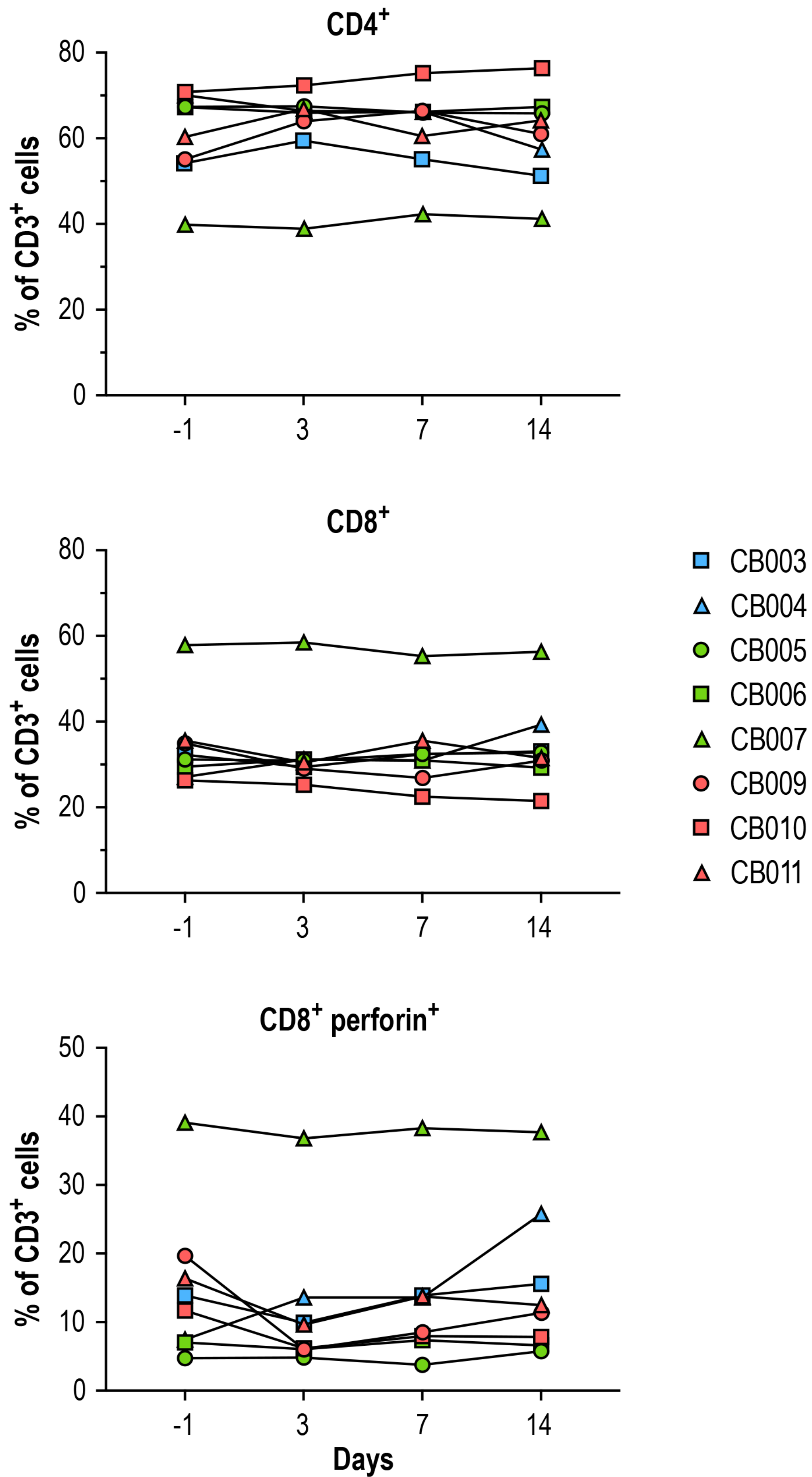

Fig. S5 T-cell subtype distribution in peripheral blood. $CD4^+$, $CD8^+$, and $CD8^+$perforin$^+$ T cell counts were determined by flow cytometry in peripheral blood of patients one day before injection of NK-92/5.28.z cells and at the indicated time points after treatment.

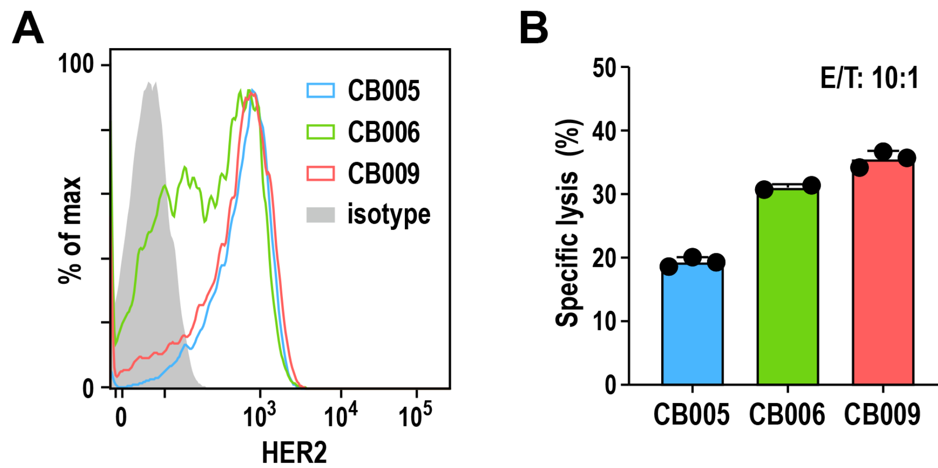

Fig. S6 Sensitivity of patient-derived glioblastoma cells to HER2-specific CAR-NK cells *ex vivo*. (A) Low-passage cultures were established from recurrent tumor tissues of patients CB005, CB006 and CB009 obtained immediately before CAR-NK injection and analyzed by flow cytometry with a HER2-specific antibody. Staining with an irrelevant isotype-matched antibody was included as control. (B) Specific cytotoxicity of NK-92/5.28.z cells against GB cells was determined after coculture for two hours at an effector to target (E/T) ratio of 10:1. Mean values ± SD are shown (n= 2-3 technical replicates).

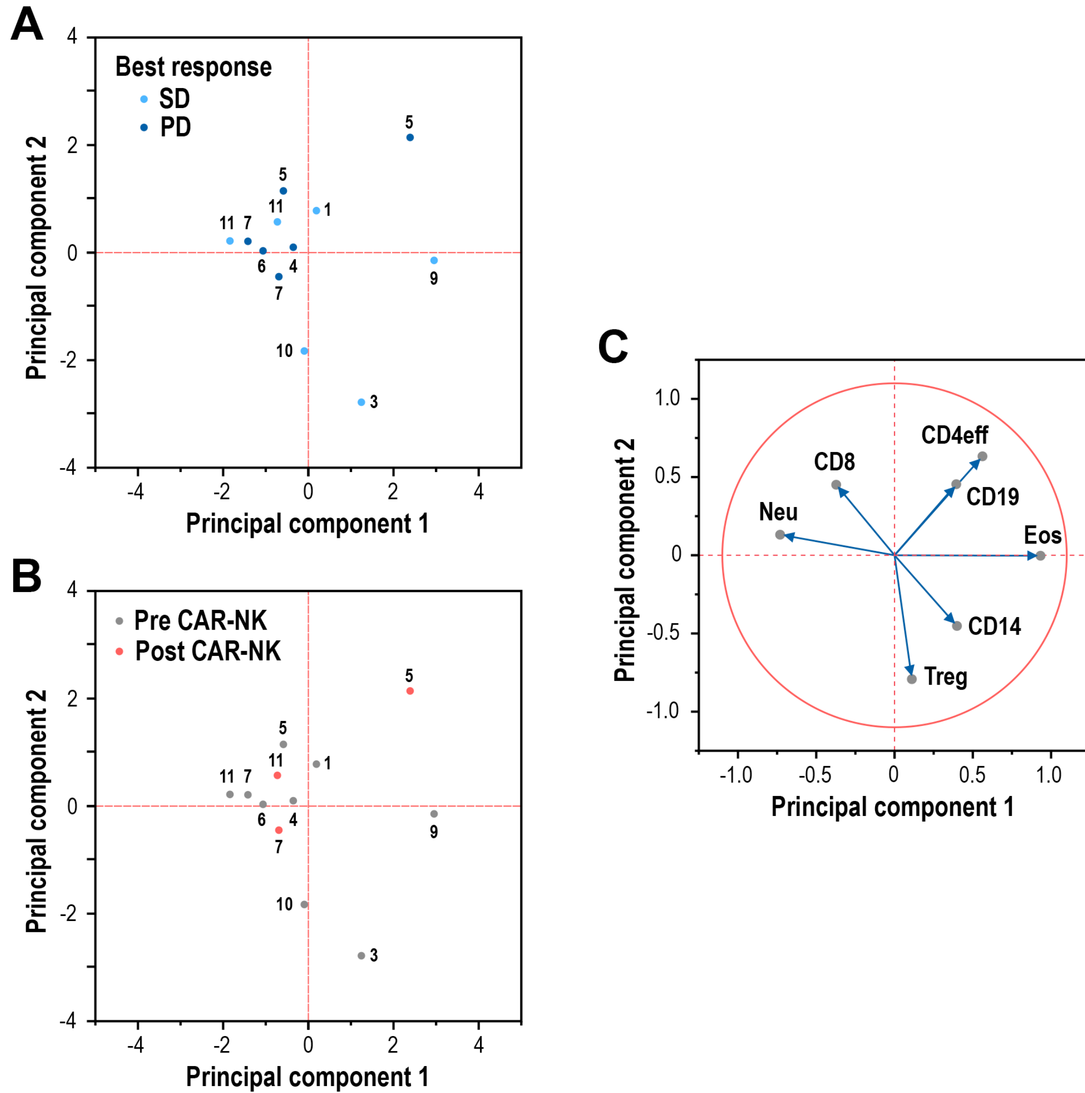

Fig. S7 Analysis of the immune cell composition of recurrent tumor samples based on DNA methylomes. The proportions of $CD8^+$ and $CD4^+$ effector T cells, $CD14^+$, $CD19^+$ cells, neutrophils (Neu), eosinophils (Eos) and regulatory T cells were computed by use of reference-based tumor deconvolution and considered for principal component analysis (PCA). For patients CB005, CB007 and CB011 both, recurrent tumor samples pre and post CAR-NK cell therapy were analyzed. (A,B) PCA of all available recurrent tumor samples. Best response after CAR-NK therapy is shown in (A), the type of sample (pre or post CAR-NK treatment) is shown in (B). Patient numbers are indicated. PD, progressive disease; SD, stable disease. (C) Positioning of immune cell subsets in the PCA.

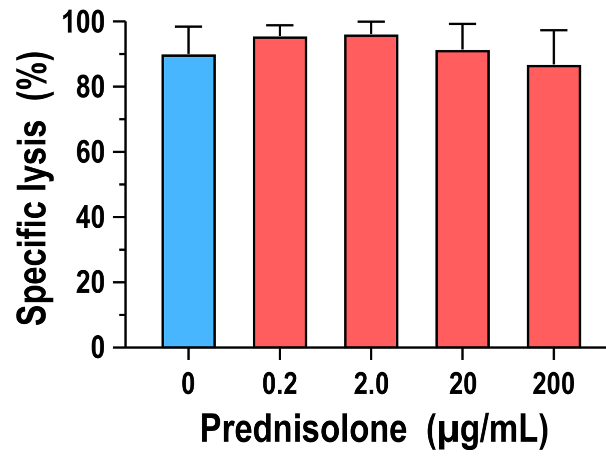

Fig. S8 CAR-mediated cytotoxicity of NK-92/5.28.z cells against HER2-positive MDA-MB453 cells after pre-treatment for 24 hours in the absence (blue bar) or presence of increasing concentrations of prednisolone (red bars). Mean values ± SD are shown (n=3 independent experiments).