# Trastuzumab derived HER2-specific CARs for the treatment of trastuzumab-resistant breast cancer: CAR T cells penetrate and eradicate tumors that are not accessible to antibodies

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## ARTICLE INFO

## ABSTRACT

HER2-targeted monoclonal antibodies improve the outcome for advanced breast cancer patients; however, resistance to therapy is still frequent. Epitope masking and steric hindrance to antibody binding through matrix components are thought to be the major mechanism. We asked whether tumors resistant to trastuzumab can still be eliminated by CAR T cells redirected by the same antibody domain. While saturating doses of trastuzumab in the presence of CD16.176V.NK-92 effector cells and trastuzumab derived CAR T cells equally well recognized and killed HER2-positive tumor cells in a monolayer, only CAR T cells penetrated into the core region of tumor spheroids and exhibited cytotoxic activity in vitro, whereas antibodies failed. In NSG mice treatment with trastuzumab and CD16.176V.NK-92 cells only transiently retarded tumor growth but did not induce regression of clinically trastuzumab-resistant breast cancer xenografts. In contrast, one dose of HER2-specific CAR T cells eradicated established tumors resulting in long-term survival. Data indicate that CAR T cells can successfully combat antibody resistant tumors by targeting the same epitope suggesting that CAR T cells can penetrate the tumor matrix which is a barrier for antibodies.

## 1. Introduction

Human epidermal growth factor receptor-2 (HER2)-specific monoclonal antibody therapies revolutionized treatment of HER2-positive breast cancer since the FDA approved the first of their kind, trastuzumab (Herceptin®), in 1998 [1]. Recent clinical studies show that the combination of pertuzumab, trastuzumab and docetaxel further improves progression free and overall survival [2]. However, a great percentage of patients still fail to respond to therapy or experience tumor relapse despite initial regression and persistent antigen expression [3]. The most intensively studied general mechanisms of trastuzumab resistance are obstacles for trastuzumab binding to HER2, up-regulation of HER2 downstream signaling pathways, signaling through alternate pathways and failure to trigger immune-mediated mechanisms to destroy tumor cells [1,4]. In this manuscript we focus on resistance to antibody therapy that occurs through epitope masking and steric hindrance to antibody binding by extracellular matrix components such as MUC4 or CD44/hyaluronan [5–7].

Epitope masking of HER2 is emphatic in advanced tumors of JIMT-1 xenografts [8] while trastuzumab can still bind to early stage tumors as well as circulating and disseminated tumor cells and induce antibody dependent cellular cytotoxicity (ADCC) [9,10]. In addition, targeting multiple epitopes by combined treatment with pertuzumab and trastuzumab further improves ADCC and recruits higher number of effector cells, thereby delaying tumor outgrowth [11]. These results suggest that in spite of intrinsic biological resistance of JIMT-1 cells, ADCC by NK cells can overcome primary resistance and hinder tumor outgrowth while the extracellular matrix is less abundant. However, at later stages the tumors become resistant due to a massive extracellular matrix that restricts access to antibodies [5–7].

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In contrast to passively diffusing antibodies, antibody-redirected immune cells can actively penetrate tumors. Immune cell therapy with adoptively transferred chimeric antigen receptor (CAR)-redirected T cells is an engaging option to improve outcomes for patients with advanced or metastatic breast cancer [12–14]. In vitro, HER2-specific CAR T cells recognized and efficiently killed trastuzumab sensitive SKBR3 or BT474 breast cancer cell lines [13,15]. However, we have no evidence for their therapeutic efficacy towards trastuzumab resistant tumors. Clinically, HER2-redirected CAR T cells are successful in the treatment of osteosarcoma and glioblastoma in early phase trials [16,17]; however, none of the trial participants received HER2 targeting monoclonal antibodies prior CAR T cell administration.

Here we addressed whether HER2-specific CAR T cells are efficacious against trastuzumab resistant, HER2$^+$ breast tumor lesions. We engineered T cells with a trastuzumab derived HER2-specific CAR that binds by the same binding domain and thereby recognizes the same epitope as trastuzumab. These T cells recognize HER2$^+$ breast cancer cells that are inherently resistant to trastuzumab in three dimensional cell cultures and successfully combat large trastuzumab resistant breast cancer xenografts that are not penetrated by trastuzumab to induce ADCC. Data indicate that CAR T cells in contrast to antibodies can be efficacious against antibody resistant tumors providing a significant survival advantage.

## 2. Methods

Cell lines and media conditions, flow cytometry, cytokine secretion assay, cytotoxicity assay, ex vivo tumor xenograft immunohistology, confocal microscopy and statistical analysis are described in detail in the supplementary methods section.

All materials were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

### 2.1. Retrovirus production and transduction of T cells

Retroviral particles were generated by transient transfection of HEK 293T cells with the CAR-encoding MSGV retroviral vector [12], Peg-Pam-e plasmid containing the sequence for MoMLV gag-pol, and pMEVSVg plasmid containing the sequence for VSVg, using jetPrime transfection reagent (Polyplus, Illkirch, France). The trastuzumab derived HER2-specific CAR, a kind gift from Dr. Richard A. Morgan at the NCI, NIH, is composed of the Ig kappa chain signal peptide; trastuzumab light chain variable region; the 218 peptide linker; trastuzumab heavy chain variable region; the hinge, transmembrane and cytoplasmic region of human CD28; and the cytoplasmic region of human CD3 zeta [12,18]. The retroviral vector with the irrelevant, p97 (melanotransferrin) specific CAR was generated in a similar fashion using the p97-specific scFv L49. Supernatants containing the retrovirus were collected after 48 h.

Experiments were carried out on human samples in accordance with the Declaration of Helsinki and approved by the Regional and Institutional Committee for Research Ethics (RKEB.5378/2019). To generate HER2-specific CAR T cells, human peripheral blood mononuclear cells were isolated by Ficoll gradient centrifugation and stimulated in non-tissue culture 24-well plates precoated with 1 μg/ml OKT3 (ThermoFischer, Waltham, MA, USA) and anti-CD28 (R&D Systems, Minneapolis, MN, USA) antibodies. On day 2, human interleukin-7 (IL7; 10 ng/ml) and human interleukin-15 (IL15; 5 ng/ml) (Miltenyi Biotec Bergisch Gladbach, Germany) were added to cultures. T cells were transduced with retroviral particles on RetroNectin-coated (Takara, Kusatsu, Japan) plates on day 3 in the presence IL7 (10 ng/ml) and IL15 (5 ng/ml). The expansion of T cells was subsequently supported with IL7 and IL15. OKT3/CD28 activated non-transduced (NT) T cells were expanded with IL7 and IL15 using the same protocol. Following 48 h incubation cells were used for further experiments.

### 2.2. Three-dimensional cell culture

JIMT-1 cells ($1 \times 10^5$ cells/ml in 200 μl) were placed into 96-well U-bottom plates. The suspensions were centrifuged at $500 \times g$ for 5 min, and the cell pellet was mixed in a 1:2 ratio with Matrigel (BD Biosciences, San Jose, CA, USA) and cultured for 10 days. Some spheroids were treated with 10 μg/ml trastuzumab or 10 μg/ml Alexa Fluor 488 conjugated trastuzumab for 24 h.

### 2.3. Propidium iodide incorporation assay

Cytotoxic activity of T cells and CD16.176V.NK-92 cells in three-dimensional cell cultures was determined by propidium iodide incorporation assay [19]. Spheroids of equal size were cocultured with $1 \times 10^5$ effector cells in the presence or absence of 10 μg/ml trastuzumab. After 24 h, 3D cocultures were labeled with 1 μg/ml propidium iodide and target cell killing was quantitatively analyzed by confocal microscopy. A quantitative digital image processing pipeline, created in ImageJ [20], was used to calculate specific PI incorporation of JIMT-1 cells in the entire spheroid and in the inner core. First, we measured the optical slice and defined two regions. Total area represented the whole slice, the central area with a 50% radius was defined as core. PI incorporation was determined for both regions as integrated PI fluorescence intensity above a common threshold in all background-corrected images. Three spheroids and at least five 2-μm-thick optical slices per sample were analyzed.

### 2.4. Xenograft tumors and in vivo antibody treatment

NSG (NOD.Cg-Prkdcscid/Il2rgtm1Wjl/SzJ) mice were purchased from The Jackson Laboratory and housed in a specific-pathogen-free environment. All animal experiments were performed in accordance with FELASA guidelines and recommendations and DIN EN ISO 9001 standards. Each seven-week-old female NSG mouse participating in the study was given a subcutaneous injection in both flanks, each containing $3 \times 10^6$ MDA.ffLUC, MDA-HER2.ffLUC or JIMT-1 cells in 100 μl PBS mixed with an equal volume of Matrigel (BD Biosciences, San Jose, CA, USA) JIMT-1 tumor volumes were derived as the product of the length, width and height. The growth of MDA.ffLUC and MDA-HER2.ffLUC xenografts was monitored with an IVIS Spectrum CT instrument (PerkinElmer, Waltham, MA, USA). Before measurement, isoflurane-anesthetized animals were injected IP with D-luciferin (150 mg/kg). A bioluminescence image was obtained and analyzed after 10 min using Living Image software Version 4.0 (Caliper Life Sciences, Waltham, MA, USA). A region of interest of the same size was drawn over the tumor region and the intensity of the signal measured as total photons per second per square centimeter per steradian (p/s/cm$^2$/sr) was obtained.

The trastuzumab group was treated with 100 μg antibody i.p. In 100 μl PBS twice weekly starting with the day of tumor cell injection. The untreated control group was injected with 100 μl PBS i.p. twice weekly. In the CD16.176V.NK-92 group, $5 \times 10^6$ CD16.176V.NK-92 cells were injected i.v. on the day of tumor cell injection which was repeated every second week. The CD16.176V.NK-92 + trastuzumab group received both of these treatments. In HER2-CAR T cell and NT T cell groups, mice received a single dose of $5 \times 10^6$ HER2-specific CAR or unmodified T cells i.v. on day 14 post tumor cell inoculation. (Fig. 3A). Experiments were approved by the National Ethical Committee for Animal Research (# 5-1/2018/DEMÁB).

## 3. Results

As tumor models, we have used two HER2$^+$ trastuzumab resistant cell lines, the clinic-derived JIMT-1 and an in vitro generated MDA-MB-468 variant, MDA-HER2, stably expressing ectopic HER2 [21]. MDA-MB-468 (MDA for short) served as HER2-negative, SK-BR-3 and N87
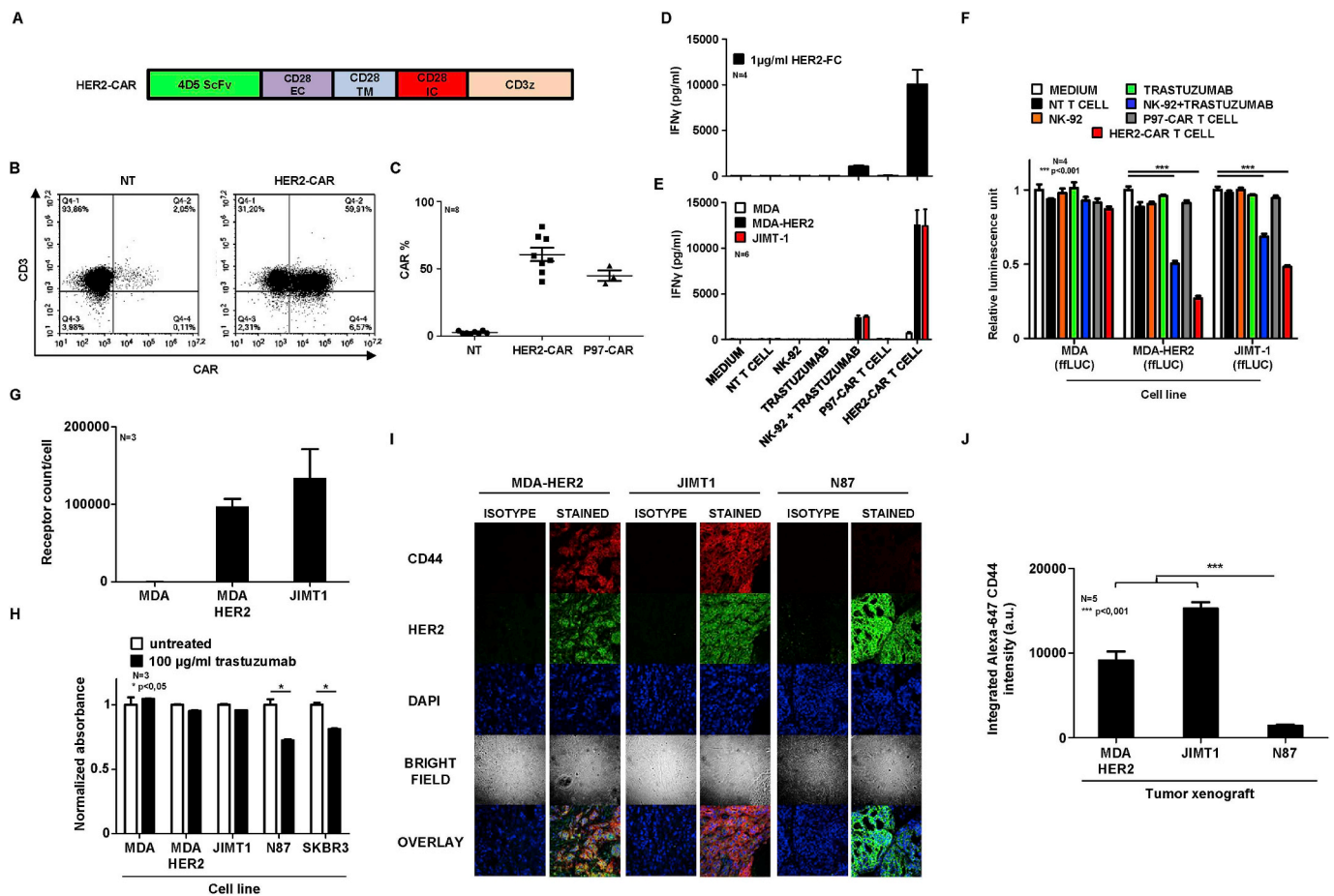
**Fig. 1.** Generation of trastuzumab-derived HER2-specific human CAR T cells, characterization of their targets and their in vitro anti-tumor function.
(A) Schematic diagram illustrating the modular composition of the HER2-specific CAR. (B and C) Representative flow cytometry dot-plot and summary data (HER2-CAR T cells [n = 6] and non-transduced T cells [n = 6]). (D and E) $5 \times 10^5$ CD16.176V.NK-92 cells $\pm$ 10 µg/ml trastuzumab or HER2-CAR or NT T cells were incubated with 1 µg/ml HER2-Fc protein (D) or cocultured with MDA-HER2 or JIMT-1 (HER2 positive) or MDA (HER2 negative) target cells in 1:1 T cell to tumor cell ratio (E). After 24 h, IFN$\gamma$ was determined in the culture supernatant by ELISA (n = 4, assay performed in duplicates). (F) firefly-Luciferase-based cytotoxicity assay using CD16.176V.NK-92 cells $\pm$ 10 µg/ml trastuzumab or HER2-CAR or NT T cells against MDA-HER2 or JIMT-1 (HER2 positive) or MDA (HER2 negative) as targets at 0.5:1 T cell to tumor cell ratio (n = 4; assay was performed in duplicates; Histograms show mean $\pm$ SEM; HER2-CAR or CD16.176V.NK-92 + trastuzumab vs. medium: ***p < 0.001). (G) Quantitative analysis of HER2 expression on MDA-HER2, JIMT-1 (HER2$^+$) and MDA (HER2$^-$) cell lines. (H) In vitro analysis of direct growth inhibitory effect of 100 µg/ml trastuzumab on MDA (HER2$^-$), MDA-HER2 and JIMT-1 (HER2$^+$) trastuzumab resistant cell lines; HER2$^+$ SKBR3 and N87 served as trastuzumab sensitive controls. (I) Cryostat sections from trastuzumab resistant MDA-HER2 (left column) and JIMT-1 (middle column) xenografts were fluorescently labeled for HER2 (green), CD44 (red), and DNA (DAPI, blue). N87 tumors served as trastuzumab sensitive control (right column). (J) CD44 expression was determined as integrated pixel intensity (A647 channel; intensity $\times$ area). Histograms represent the mean $\pm$ SD (n = 5; ***p < 0.001).

lines as HER2$^+$, trastuzumab sensitive controls. Also, ffLUC expressing variants of JIMT-1, MDA and MDA-HER2 were used where appropriate. The efficacy of human T lymphocytes engineered with a trastuzumab-derived HER2-CAR [12] was compared to ADCC mediated by trastuzumab through CD16.176V.NK-92 cells, a stably transfected variant of NK-92 [22] that express the high affinity (176V) variant of the Fc$\gamma$ receptor (CD16) [23,24].

## 3.1. Generation of trastuzumab-derived HER2-specific human CAR T cells and their anti-tumor function in vitro

First we asked whether CAR T cells redirected towards the same epitope as trastuzumab can control advanced tumors where trastuzumab treatment fails. In order to compare the impact of CAR T cells as living drugs with the impact of passively diffusing antibodies on tumor eradication, we have generated human T cells that express a CAR with an scFv binding domain derived from trastuzumab (humanized 4D5), stalk, transmembrane and cytoplasmic regions of human CD28 and a CD3$\zeta$ signaling domain (Fig. 1A). Mean transduction efficiency by VSVg pseudotyped $\gamma$-retroviruses in the CD3 positive human lymphocyte

population was 60.48% as judged by flow cytometry on day 4 post transduction (Fig. 1B Q4-2 quadrant and C). We made certain that CARs are stably expressed and re-confirmed CAR expression on day 10 (Suppl. Fig. 1). P97 melanotransferrin specific CAR T cells served as irrelevant controls (Fig. 1C).

To evaluate the HER2-specific CAR-redirected T cell activation we recorded the release of IFN$\gamma$ and cytotoxicity towards target cells as key effector functions. Incubation with HER2-Fc chimera proteins or with HER2$^+$ target cells (MDA-HER2 or JIMT-1) induced increase in IFN$\gamma$ release by HER2-specific CAR T cells indicating T cell activation. T cells with a CAR of irrelevant (p97) specificity or without a CAR did not release IFN$\gamma$. In the presence of 10 µg/ml trastuzumab, CD16.176V.NK-92 cells also recognized HER2 and were activated to secrete IFN$\gamma$ (Fig. 1D and E). There was no cytokine secretion in the absence of effector cells, target antigen (either recombinant or on the cell surface) or trastuzumab (in the case of CD16.176V.NK-92 cells) indicating that activation is strictly dependent on targeting the antigen.

Using cytotoxicity assays based on ffLUC activity measurements (Fig. 1F) and real time cell impedance analysis (Suppl. Fig. 2), HER2-targeted CAR T cells killed specifically HER2$^+$ cells grown in

monolayers, but not HER2$^-$ (MDA) target cells (p < 0.001). These data are coherent with the pro-inflammatory activation upon CAR binding indicated by IFNγ release (Fig. 1E). In addition, trastuzumab-engaged CD16.176V.NK-92 cells induced HER2 specific killing of HER2$^+$ but not of HER2$^-$ target cells (p < 0.001). Real time analysis of killing HER2 positive target cells revealed that in the presence of saturating doses of trastuzumab CD16.176V.NK-92 cells executed faster killing than HER2-CAR T cells during the initial 3–4 h. However, their killing slowed down afterwards and became less efficient while CAR T cells remained potent killers during a period of 24 h (Suppl. Fig. 2).

We verified that native and ffLUC-transduced MDA-HER2 and JIMT-1 cells express similar amounts of HER2 antigens (Fig. 1G and Suppl. Fig. 3A), and are resistant to trastuzumab in vitro (Fig. 1H and Suppl. Fig. 3B). In these assays MDA cells served as HER2-negative, N87 and SKBR-3 cells as HER2-positive, trastuzumab sensitive controls [13,15]. We also confirmed that expression of CD44, the receptor for hyaluronan, a major ECM component, is significantly higher in the trastuzumab resistant JIMT-1 and MDA-HER2 tumor xenografts than in trastuzumab sensitive controls (Fig. 1I and J).

### 3.2. HER2-CAR T cells recognize and penetrate three dimensional tumor spheroids

The finding that trastuzumab-directed CD16.176V.NK-92 and HER2-specific CAR T cells likewise target JIMT-1 in monolayer cultures encouraged us to investigate their anti-tumor efficacy in three-dimensional cultures with an established extracellular matrix. We treated JIMT-1 spheroids with 10 μg/ml trastuzumab or Alexa Fluor 488-trastuzumab for 24 h and sectioned them to visualize HER2 and CD44 expression (Fig. 2A). Upon staining the sections, both Alexa Fluor 488-trastuzumab (Fig. 2A, column 2) and the Alexa Fluor 647-anti-CD44 antibody (Fig. 2A, columns 2–4) showed strong specific signals. In contrast, Alexa Fluor 488-trastuzumab added to intact spheroids as a treatment could not penetrate their three-dimensional structure (Fig. 2A, column 3). Incubation of spheroids with unconjugated trastuzumab and post-staining their sections with Alexa Fluor 488 anti-human IgG revealed a similarly low penetration of unlabeled trastuzumab as that of Alexa Fluor 488-trastuzumab into spheroids, excluding the antibody bound fluorescent dyes as the cause of low penetration. (Fig. 2A, column 4).

To explore whether spheroids are accessible to antibody and CAR T cell treatment, spheroids were cocultured with CD16.176V.NK-92 cells in the presence or absence of trastuzumab or with HER2-specific CAR T cells. Using confocal microscopy [19], we visualized (Fig. 2B) and analyzed (Fig. 2D and E) tumor cell killing indicated by incorporation of propidium iodide (PI) and quantified cell death by integrating PI fluorescence intensity (Fig. 2C). Both HER2-CAR T cells and trastuzumab bound CD16.176V.NK-92 cells showed substantial cytolytic activity in the peripheral zone of spheroids (Fig. 2B and D). However, CAR T cells penetrated far more efficiently into, and killed target cells in the inner spheroid core, than trastuzumab-guided CD16.176V.NK-92 cells (p < 0.001) (Fig. 2E).

### 3.3. HER2-CAR T cells eliminate HER2 positive tumor xenografts in vivo

To investigate whether the different tissue penetration capacities result in different efficacies in tumor elimination in vivo, we established subcutaneous HER2$^+$ MDA-HER2.ffLUC and JIMT-1 xenografts in NSG mice; HER2-negative MDA.ffLUC cells served as control (Fig. 3A). To test the in vivo cytolytic function of CD16.176V.NK-92 cells in the presence or absence of trastuzumab, 5 × 10$^6$ CD16.176V.NK-92 cells and trastuzumab were simultaneously administered starting from the day of tumor cell inoculation. We found that simultaneous administration of trastuzumab plus CD16.176V.NK-92 cells to trastuzumab resistant MDA-HER2.ffLUC and JIMT-1 cells yielded only partial improvement in overall survival (Fig. 3C and D right panels) by somewhat

delaying but not preventing tumor outgrowth from (p < 0.001; Fig. 3C and D left panels). JIMT-1 tumors treated with trastuzumab were delayed in growth with improved overall survival (p < 0.001; Fig. 3D), likely due to residual macrophage activity in those mice (Suppl. Fig. 4A and 4B), however, the tumors eventually grew out.

In contrast to the repetitive treatment with trastuzumab and CD16.176V.NK-92 cells, HER2-CAR T cells were applied in a single dose of 5 × 10$^6$ i.v. on day 14 post transplantation (Fig. 3A). We observed complete and long-lasting tumor regression of MDA-HER2.ffLUC and JIMT-1 tumors as opposed to all other treatments (p < 0.001; Fig. 3B, C and 3D). CAR T cells did not show anti-tumor activity against HER2-negative MDA.ffLUC tumors confirming the lack of allogeneic tumor recognition (p > 0.05; Suppl. Fig. 5A and 5B).

### 3.4. HER2-CAR T cells infiltrate HER2 positive xenografts

In animals treated with HER2-CAR T cells, higher number of infiltrating effector cells were localized in tumor sections than in animals treated with CD16.176V.NK-92 + trastuzumab, even though these animals have received two rounds of NK cell injections prior to tissue sectioning (Fig. 4A, B and 4C). We confirmed that the majority of tumor infiltrating lymphocytes are CD8 positive cytotoxic T cells (Suppl. Fig. 6) and that these cells are most likely of effector memory (EM) phenotype (CCR7 - CD45RA double negative, data not shown). This demonstrates that HER2-specific CAR T cells have the ability to penetrate trastuzumab resistant xenografts. In contrast, we did not detect HER2-CAR T cells in the absence of HER2$^+$ cancer cells (Fig. 4A and B). In the MDA-HER2.ffLUC group the rapid cytolytic effect of HER2-CAR T cells did not allow the excision of a tumor.

## 4. Discussion

HER2-targeted antibodies have been successful in treatment of HER2-positive breast cancer [1], however, a great percentage of patients still fail to respond to therapy despite persistent antigen expression [3]. Overexpression of ECM components such as MUC4 or CD44/hyaluronan forms a steric barrier that masks HER2 antigen and inhibits recognition [5–7]. HER2-redirected CAR T cells have shown great potential to eliminate HER2$^+$ trastuzumab sensitive tumor cell lines (SKBR3 and BT474) in pre-clinical models [13,15] or cure trastuzumab naïve patients in clinical trials [16,17], however we still have no evidence about their anti-tumor function in trastuzumab resistant tumors. In this study, we aimed to challenge trastuzumab derived HER2-specific CAR T cells with trastuzumab resistant tumor cells starting with cells grown in monolayer cultures, progressing to in vitro three dimensional spheroids, and reaching the highest tissue complexity with in vivo xenografts.

First, we demonstrated that trastuzumab-redirected CD16.176V.NK-92 and HER2-CAR T cells recognize and specifically kill HER2$^+$ target cells in monolayer cultures. This observation is consistent with our previous findings that trastuzumab binds to its targets and induces ADCC if the ECM is under-developed [9,10].

Next, we investigated whether trastuzumab-directed CD16.176V.NK-92 and HER2-CAR T cells are likewise efficacious in three-dimensional cultures with an established extracellular matrix [25]. To obtain this goal we established trastuzumab-resistant JIMT-1 spheriods and confirmed the well-developed ECM by confocal microscopy. In correlation with our previous results [5,9,10] overexpression of CD44/hyaluronan resulted in reduced binding of trastuzumab to HER2 in JIMT-1 spheroids and consequentially limited the penetration of CD16.176V.NK-92 cells. In contrast, HER2-specific CAR T cells could penetrate into the spheroid cores and induced potent anti-tumor response, as judged by propidium iodide incorporation [19].

While the lack of penetration of antibodies into tumors with a massive ECM could in itself limit the antibody-dependent persistence of NK cells in the tumor, we also need to highlight that the specificity,
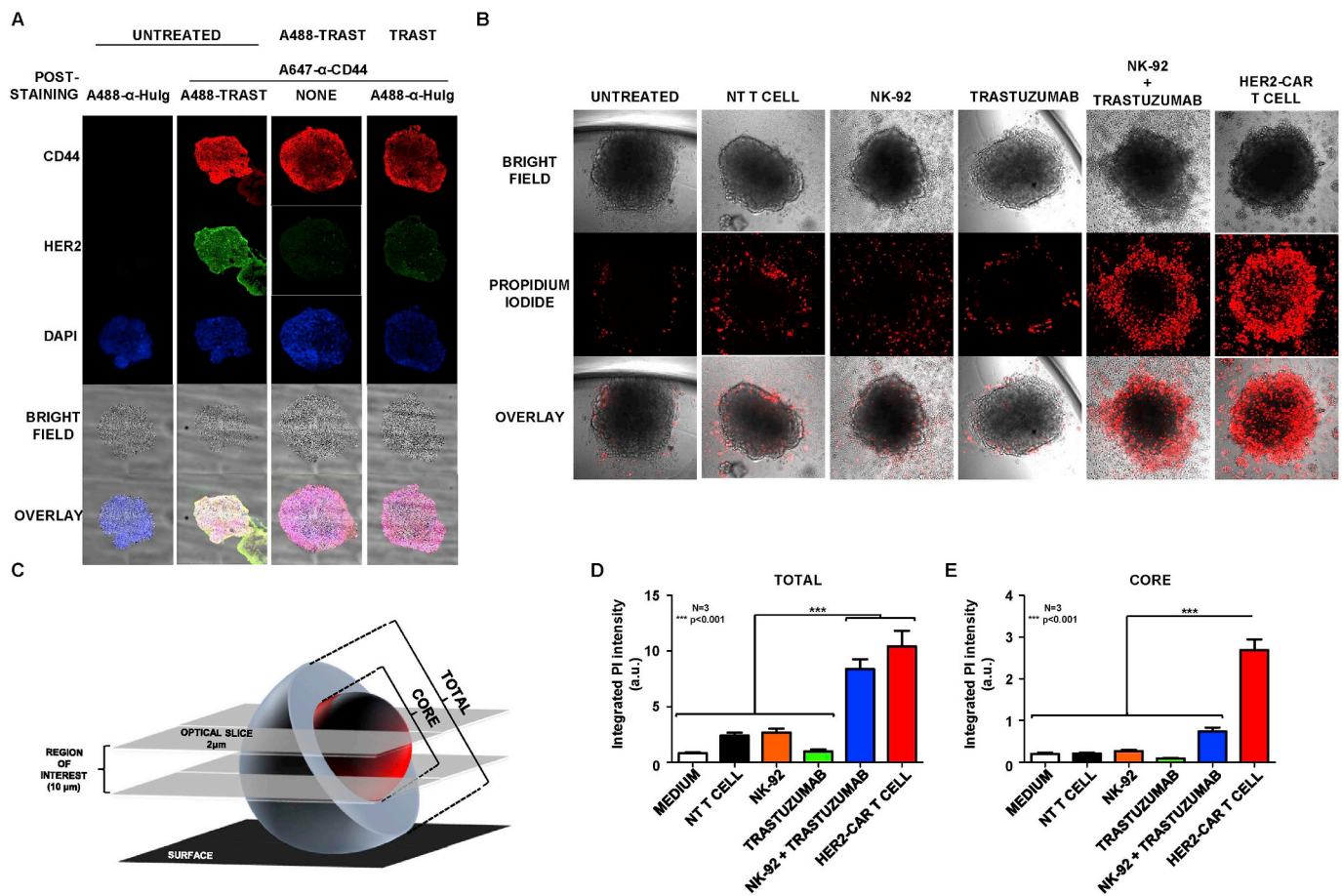
**Fig. 2.** Confocal microscopic analysis of HER2-CAR T cell and NK cell mediated killing of tumor spheroids.
(A) Representative frozen sections of JIMT-1 spheroids treated with 10 μg/ml trastuzumab or Alexa Fluor 488 conjugated trastuzumab (A488-TRAST) and controls. Sections were post-stained with 10 μg/ml Alexa Fluor 647-anti-CD44 to reveal CD44 (A647-α-CD44; columns 2–4, red, first row), Alexa Fluor 488-anti-human IgG (A488-α-HuIg) to detect trastuzumab bound to HER2 (columns 1/negative control/and 4, green, second row) or Alexa Fluor 488-trastuzumab to directly visualize HER2 (A488-TRAST; column 2, green, second row), as well as DAPI for DNA (blue). (B) Representative images (at 24 h) for detection of cytolytic activity of CD16.176V.NK-92 or T cells against JIMT-1 spheroids. Dead cells were visualized by PI uptake (red). (C) Schematic representation of the optical sections through the spheroid. (D) Tumor cell killing was determined as integrated pixel intensity (PI channel; intensity × area). Each well contained one spheroid and 200,000 effector cells. The histograms represent PI incorporation in the entire (D) spheroid and in the CORE region (E) of the spheroid in the presence of media, NT T cells, CD16.176V.NK-92 cells, 10 μg/ml trastuzumab, CD16.176V.NK-92 cells plus 10 μg/ml trastuzumab, and HER2-CAR T cells. Histograms show mean ± SD (n = 3; ***p < 0.001).

sensitivity and dynamics of NK cell mediated anti-tumor reactions are much less studied than those of T cells. NK cells as innate immune cells are one of the first responders at the sites of infection, are therefore much quicker than CD8$^+$ T cells in establishing a robust response [26]. However, a recent publication suggests that NK cells interact with the extracellular matrix that suppresses their anti-tumor response [27].

At the same time, several publications suggest that stimulation through the T cell receptor complex affects cell migration in response to chemokines, highlighting an interaction between the TCR and chemokine receptors [28]. Antigen recognition and consequential differentiation into effector memory phenotype downregulates CD62L and CCR7 expression to limit T cell homing to the lymph nodes and upregulates several molecules that enable them to migrate into the inflamed tumor environment, among them E- and P-selectins, and chemokine receptors such as CXCR3 which binds CXCL9 and CXCL10 [29]. Such regulatory mechanisms could well contribute to the enhanced persistence and cytolytic activity of CAR T cells in tumors.

In the preclinical setting, JIMT-1 and MDA-HER2 tumors growing in NSG mice were used to demonstrate in vivo the anti-tumor effects of trastuzumab-redirected CD16.176V.NK-92 and HER2-CAR T cells. To test the in vivo cytolytic function of CD16.176V.NK-92 cells in the presence or absence of trastuzumab we injected 5 × 10$^6$ cells i.v. bi-

weekly and simultaneously administered 100 μg trastuzumab twice-weekly starting from the day of tumor cell inoculation. Although this treatment was previously shown to effectively induce the total regression of established trastuzumab sensitive BT474 xenografts [30], it could not prevent the outgrowth of trastuzumab resistant MDA-HER2.ffLUC and JIMT-1 tumors in our pre-clinical model even when treatment started together with tumor inoculation. These results obtained with human tumor cells and human NK cells in NSG mice recapitulate our previous finding where trastuzumab treatment delayed but did not prevent the outgrowth of JIMT-1 xenografts in SCID mice having their own mouse NK cells if treatment was started at the time of tumor inoculation [9–11].

In contrast to the repetitive treatment with trastuzumab and CD16.176V.NK-92 cells, a single i.v. dose of 5 × 10$^6$ HER2-CAR T cells on day 14 post tumor inoculation resulted in complete and long-lasting tumor regression of MDA-HER2.ffLUC and JIMT-1 tumors, even though at the time of treatment tumors were 250 mm$^3$ in size and had a well-stablished ECM. Similar to the case of in vitro grown spheroids, HER2-specific CAR T cells also penetrated trastuzumab resistant xenografts in vivo. However, no penetration and tumor lysis was seen in HER2-negative MDA.ffLUC control xenografts, which suggests that active invasion of and accumulation in the tumor of HER2-CAR T cells is driven by
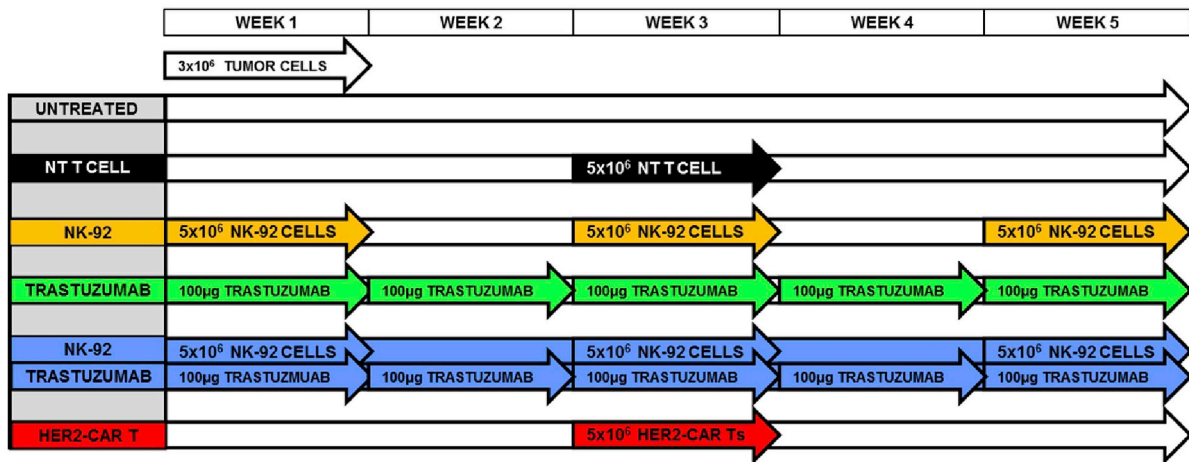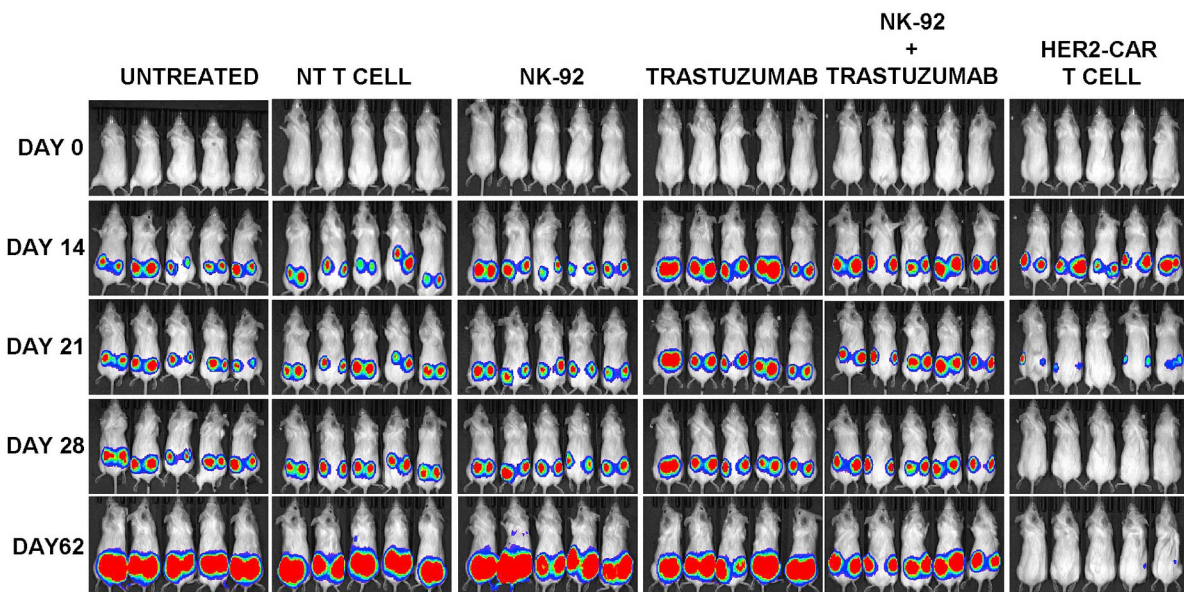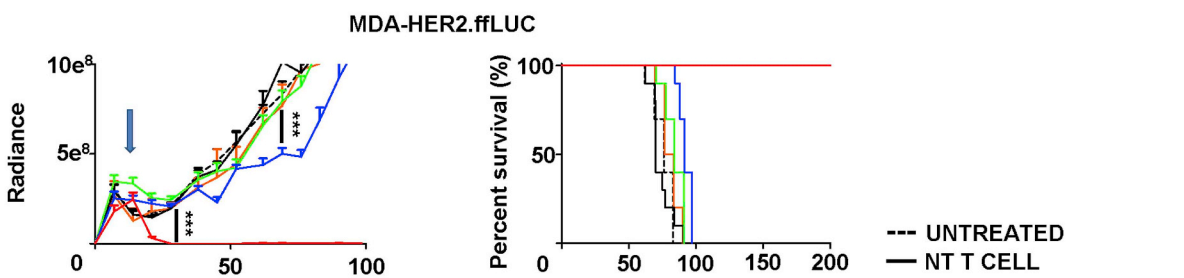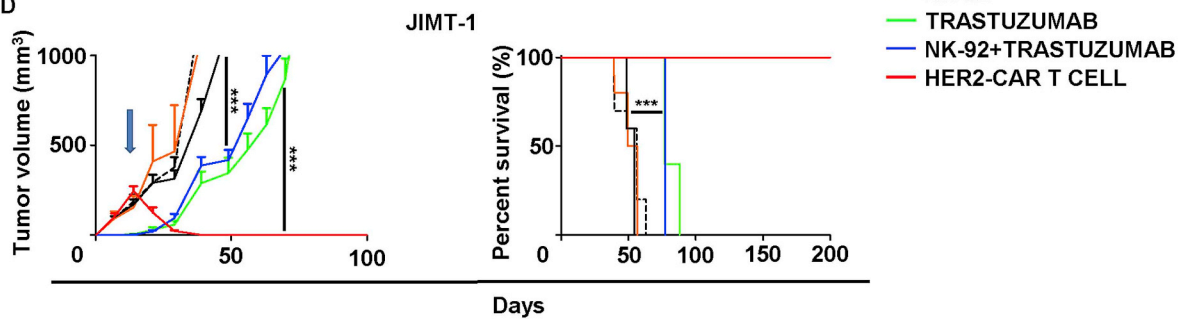
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**Fig. 3.** HER2-CAR T cells eliminate HER2 positive tumor xenografts in vivo.
Mice were injected s.c. with $3 \times 10^6$ MDA-HER2.ffLUC or JIMT-1 cells. Mice treated from the day of tumor inoculation received 100 μl PBS i.p. twice weekly (untreated, n = 10), or 100 μg trastuzumab in 100 μl PBS i.p. twice weekly (trastuzumab, n = 10, green), or $5 \times 10^6$ CD16.176V.NK-92 cells i.v. biweekly (CD16.176V.NK-92, n = 10, orange) or trastuzumab i.p. twice weekly plus CD16.176V.NK-92 cells i.v. biweekly (CD16.176V.NK-92 + trastuzumab, n = 10, blue). CAR T treated mice received on day 14 (arrow) a single i.v. dose of $5 \times 10^6$ NT T cells (NT T cell, n = 10, black) or HER2-CAR T cells (HER2-CAR T cell, n = 10, red). Tumor growth was followed by bioluminescence imaging (MDA-HER2.ffLUC) or by caliper measurements (JIMT-1, volume derived as the product of the length, width and height). (A) Outline of the treatment schedule. (B) Representative images of MDA-HER2.ffLUC injected animals. (C left panel) Quantitative bioluminescence imaging data of MDA-HER2.ffLUC xenografts (radiance = photons/s/cm²/sr; HER2-CAR vs. all other treatments: ***p < 0.001; CD16.176V.NK-92 + trastuzumab vs. all non-HER2-CAR groups ***p < 0.001). (C right panel) Kaplan-Meier survival curve (HER2-CAR vs. all other treatments: ***p < 0.001). (D left panel) Tumor volume of JIMT-1 tumors (mm³; HER2-CAR vs. all other treatments: ***p < 0.001; Trastuzumab ± CD16.176V.NK-92 groups vs. all non-HER2-CAR groups ***p < 0.001). (D right panel) Kaplan-Meier survival curve (HER2-CAR vs. all other treatments: ***p < 0.001; Trastuzumab ± CD16.176V.NK-92 groups vs. all non-HER2-CAR groups ***p < 0.001). Histograms represent mean ± SD.
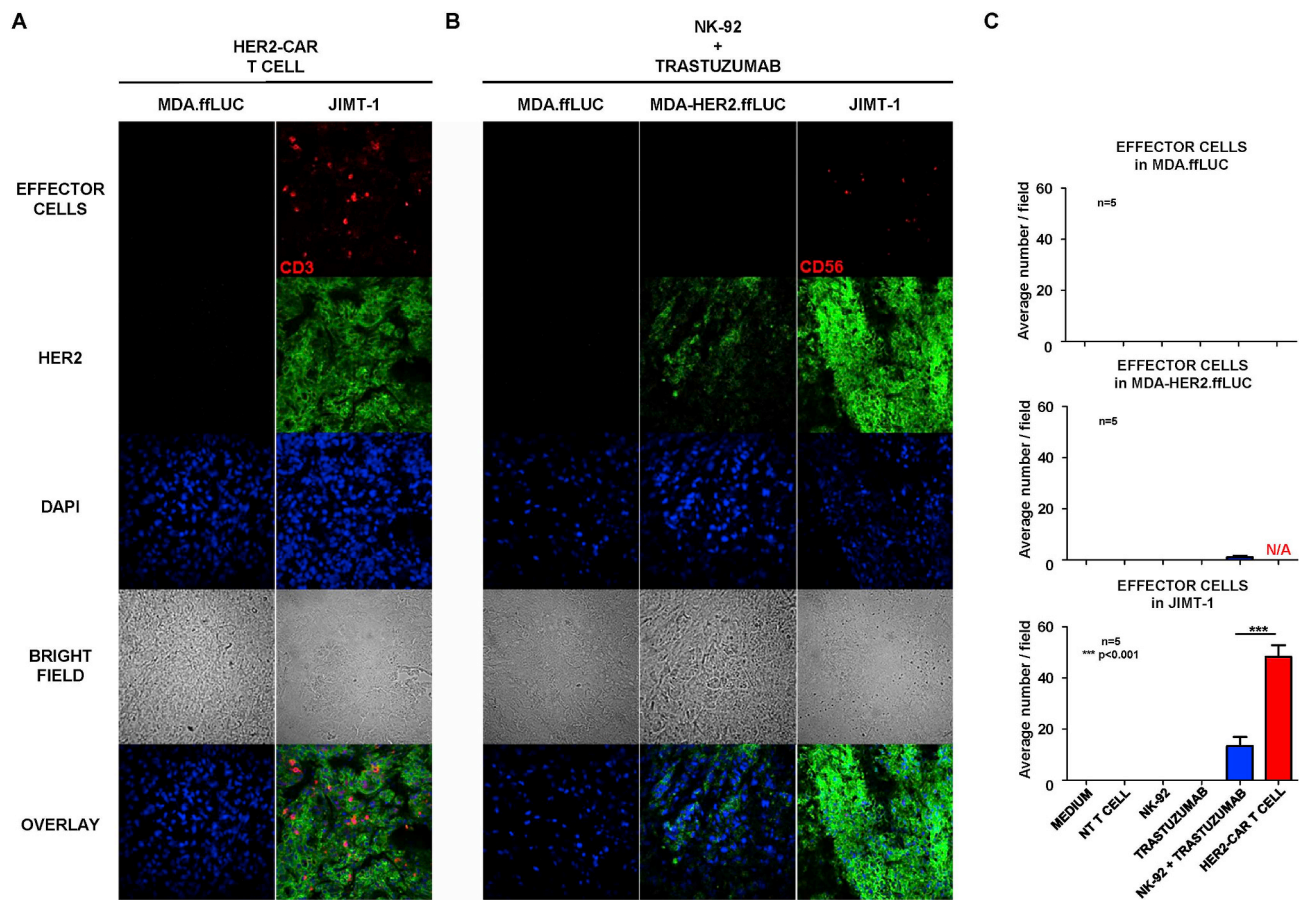


**Fig. 4.** Effector cell infiltration of HER2 positive tumors.
Frozen sections of excised tumors were fluorescently labeled for HER2 (green), T cells (CD3e; red) or NK cells (CD56, red), and DNA (DAPI, blue). (A) Sections from HER2⁻ control MDA.ffLUC tumors (left column) and HER2⁺ JIMT-1 tumors (right column) treated with HER2-CAR T cells. (B) Sections from HER2⁻ control MDA.ffLUC tumors (left column), HER2⁺ MDA-HER2.ffLUC tumors (middle column) and JIMT-1 tumors (right column) treated with CD16.176V.NK-92 cells plus trastuzumab are shown. (C) Effector cell penetration was quantitatively analyzed by counting CD3e+ and CD56⁺ nucleated cells in randomly selected 0.05 mm² areas of the section. The histograms represent mean ± SD of effector cells/field (n = 6). In mice with MDA-HER2.ffLUC tumors the rapid cytolytic effect by HER2-CAR T cells did not allow the excision of a tumor (N/A label). ***p < 0.001.

specific antigen recognition.

Taken together, we here demonstrate that established clinic-derived and in vitro generated trastuzumab-resistant tumors can be efficiently eliminated by HER2-CAR T cells but not by treatment with trastuzumab plus CD16.176V.NK-92 cells although both recognize the same HER2 epitope. Our previous data indicate that ECM components cause trastuzumab therapy resistance by steric hindrance of antigen recognition, which could also mitigate the success of emerging antibody-drug conjugate (ADC) therapies. CAR T cells obviously overcome the ECM barrier as trastuzumab resistant spheroids and established xenograft tumors are penetrated and destroyed by CAR T cells. We posit that clinical application of trastuzumab derived HER2-specific CAR T cells can be an option for the treatment of trastuzumab resistant tumors.

**Author contributions**

G.V. and A.Sz. designed the study. T.G. and A.Sz. performed experiments. B.Zs. provided JIMT-1 spheroids. V.Sz. performed ELISA expreiments. Z.E and H.A. provided reagents. All authors contributed to data analysis and manuscript preparation.

## Declaration of competing interest

The authors declare no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2020.04.008.

## Abbreviations

| | |
|---|---|
| ADCC | Antibody-dependent cell-mediated cytotoxicity |
| CAR | Chimeric Antigen Receptor |
| CD16 | Fc gamma receptor type III |
| CD44 | Receptor for hyaluronic acid |
| ECM | Extracellular matrix |
| EM | Effector memory |
| ffLUC | Firefly luciferase |
| GFP | Green fluorescent protein |
| HER2 | Human epidermal growth factor receptor 2 |
| IFN-γ | Interferon gamma |
| IL7 | Interleukin 7 |
| IL15 | Interleukin 15 |
| MUC4 | Mucin 4 |
| NK | Natural Killer cells |
| NSG | NOD scid gamma mouse |
| PBS | Phosphate buffer saline |
| scFv | Single chain variable fragment |
| SCID | Severe combined immunodeficiency |
| VSVg | Vesicular stomatitis virus G protein |

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