Disulfide-directed multicyclic peptides for chimeric antigen receptors targeting solid tumors

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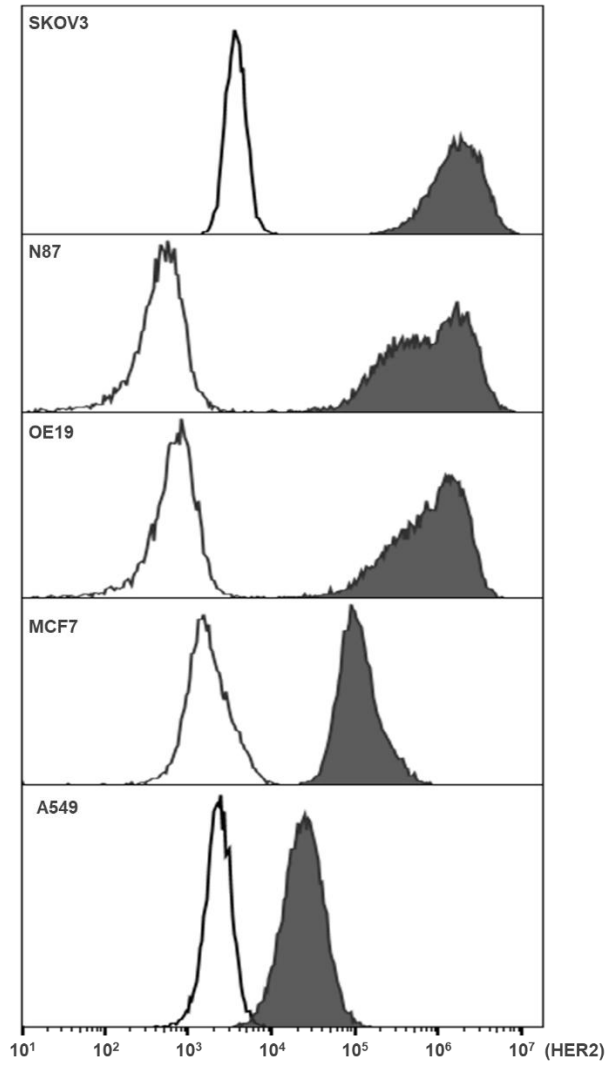


Figure S1. Quantification of cell-surface HER2 by flow cytometry.

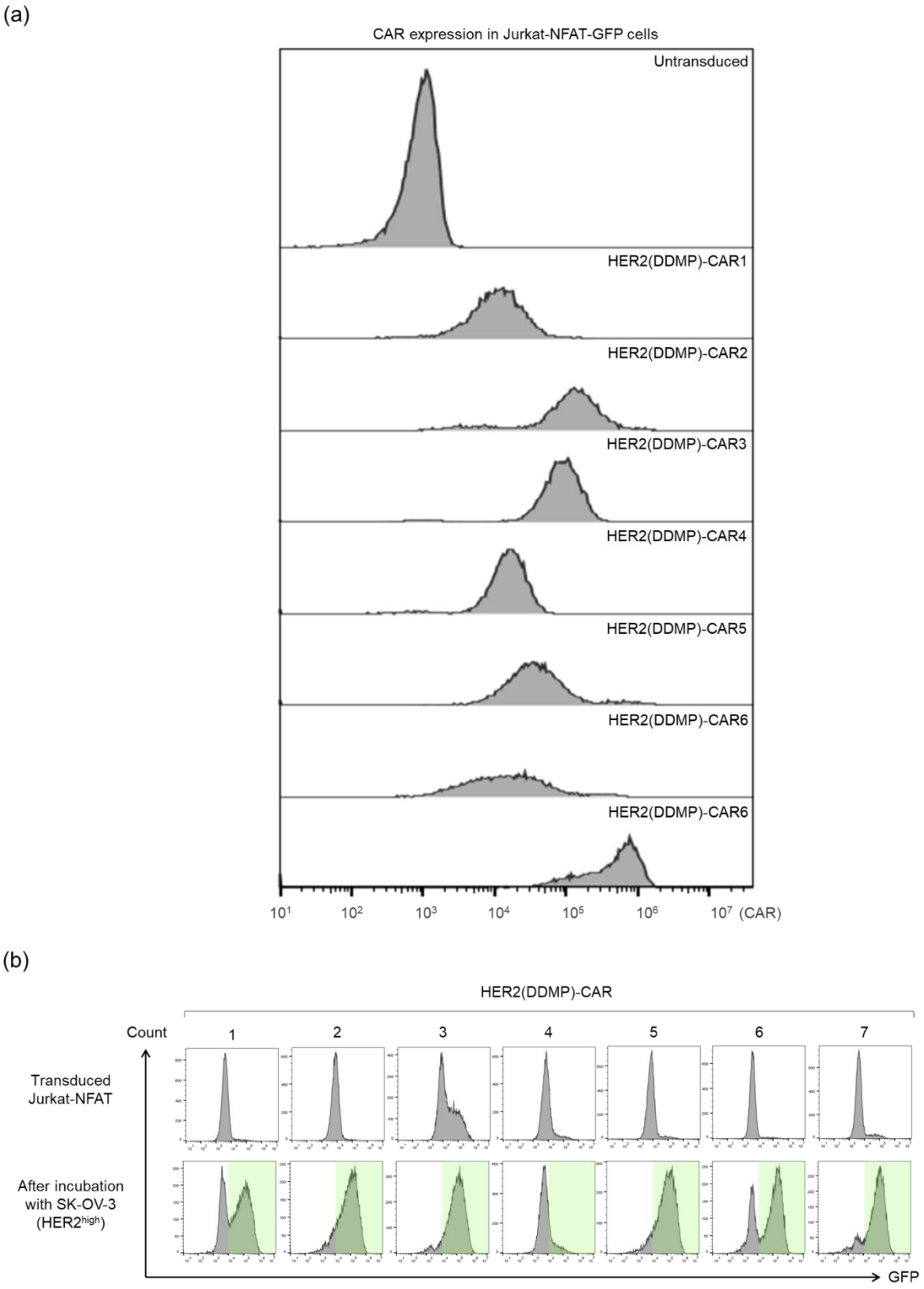


Figure S2. Functional evaluation of HER2(DDMP)-CAR variants in Jurkat reporter cell line.

(a) HER2(DDMP)-CAR1/2/3/5/6/7 expression in Jurkat-NFAT-GFP cells. (b) Jurkat-NFAT-GFP cells expressing HER2(DDMP)-CAR1/2/3/5/6/7 showed inducible GFP expression upon coculture with $HER2^{high}$ SK-OV-3 cells, indicating the functionality of these CAR constructs.

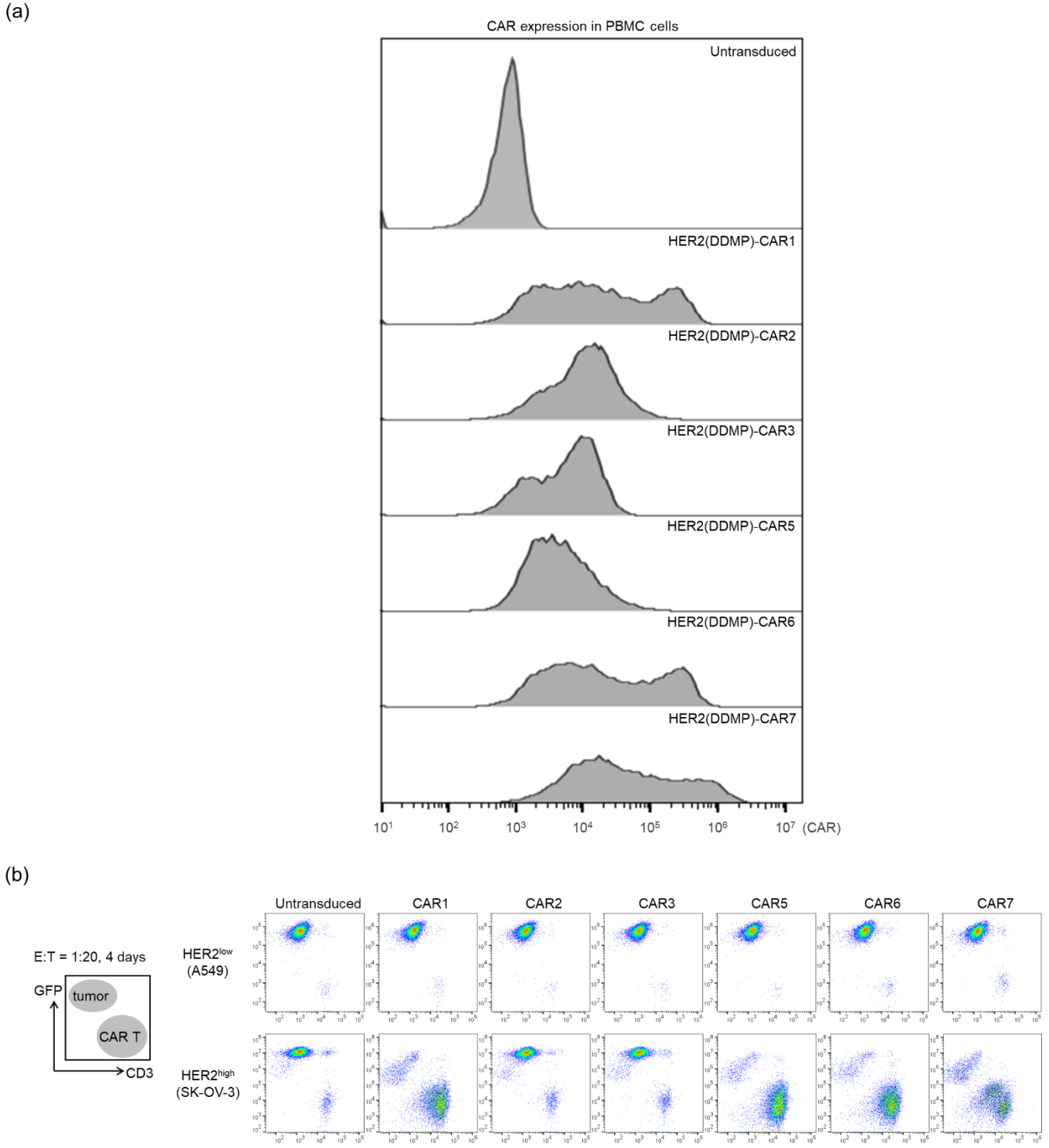


Figure S3. In vitro cytotoxicity of HER2(DDMP)-CAR T cells.

(a) HER2(DDMP)-CAR1/2/3/5/6/7 expression in human T cells (CD3+). (b) T cells were cocultured with A549-luc-GFP or SK-OV-3-luc-GFP cells for four days, followed by flow cytometry analysis. Gating strategy to distinguish tumor cells (GFP+) and T cells (CD3+) is shown in the left. The tumor cells stably express luciferase and GFP.

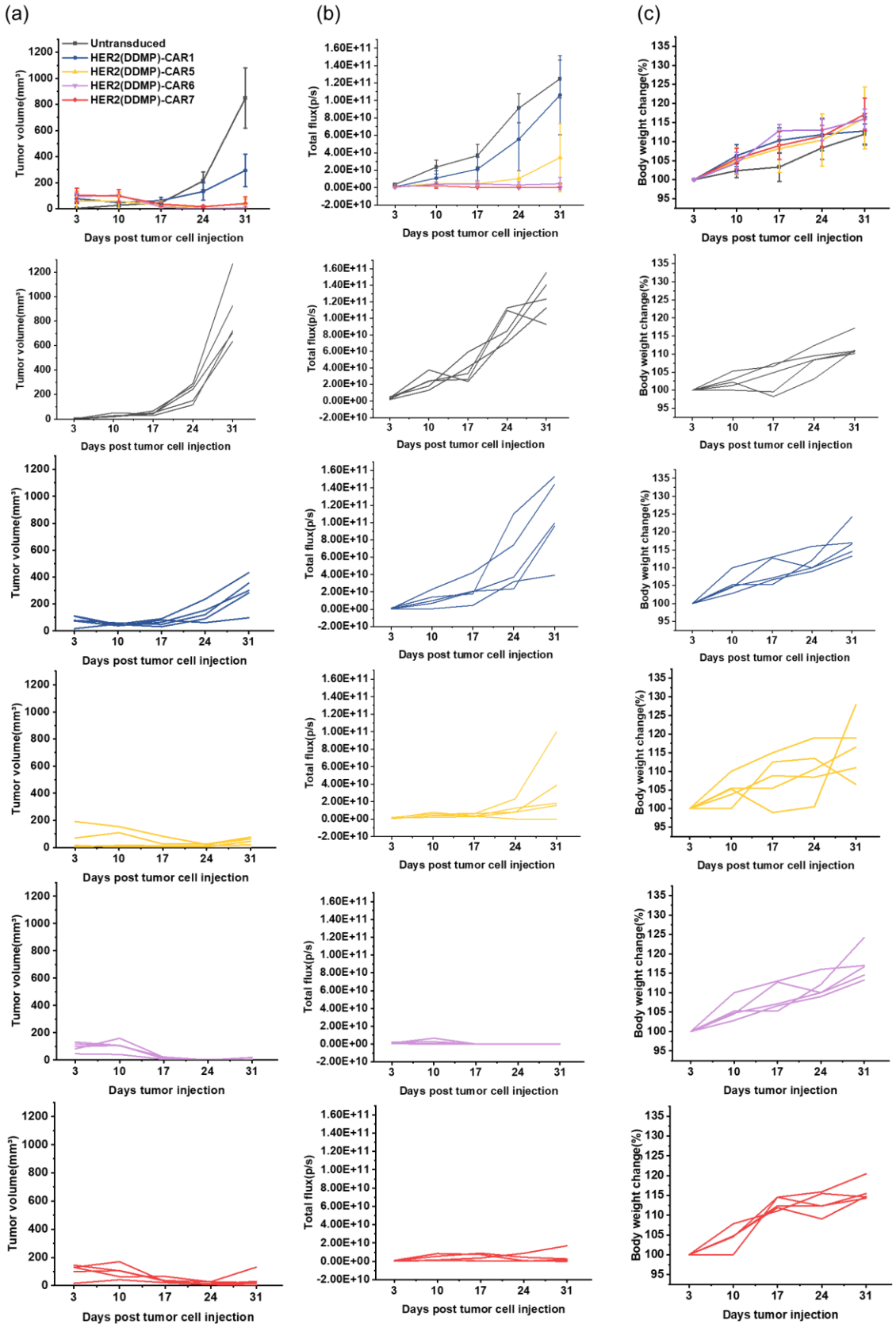


Figure S4. In vivo efficacy of HER2(DDMP)-CAR T cells in a SK-OV-3-luc xenograft model.

Quantification of tumor signal (a), tumor volume (b) and body weight (c) of individual mice corresponding to the experiment in Fig. 2e.

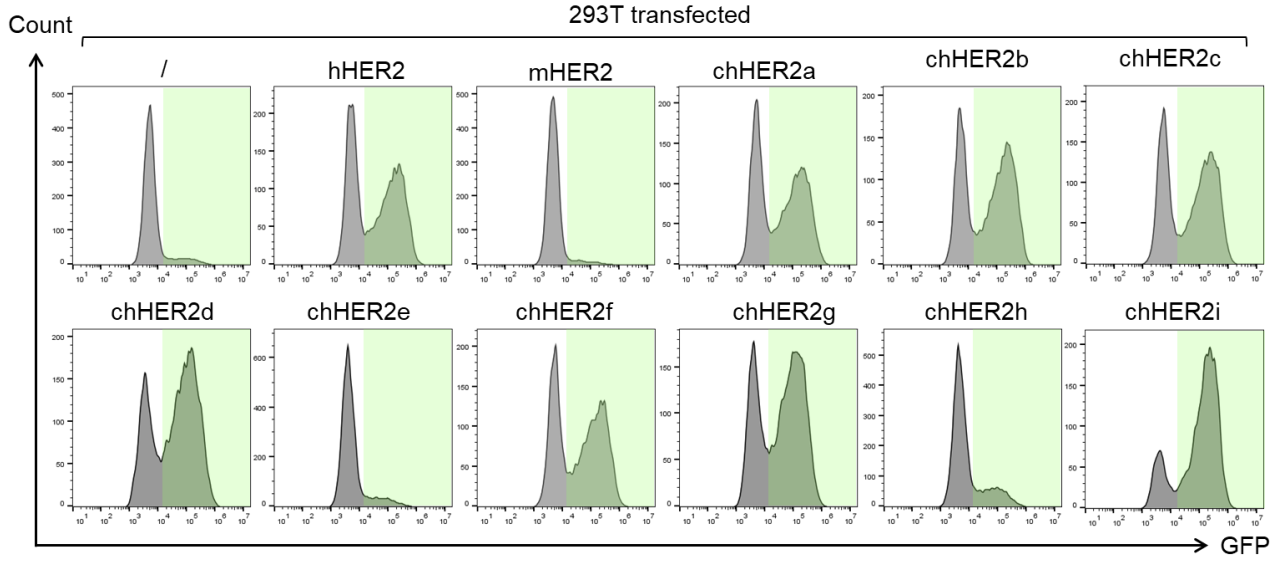


Figure S5. Functional evaluation of HER2(DDMP)-CAR variants in Jurkat reporter cell line.

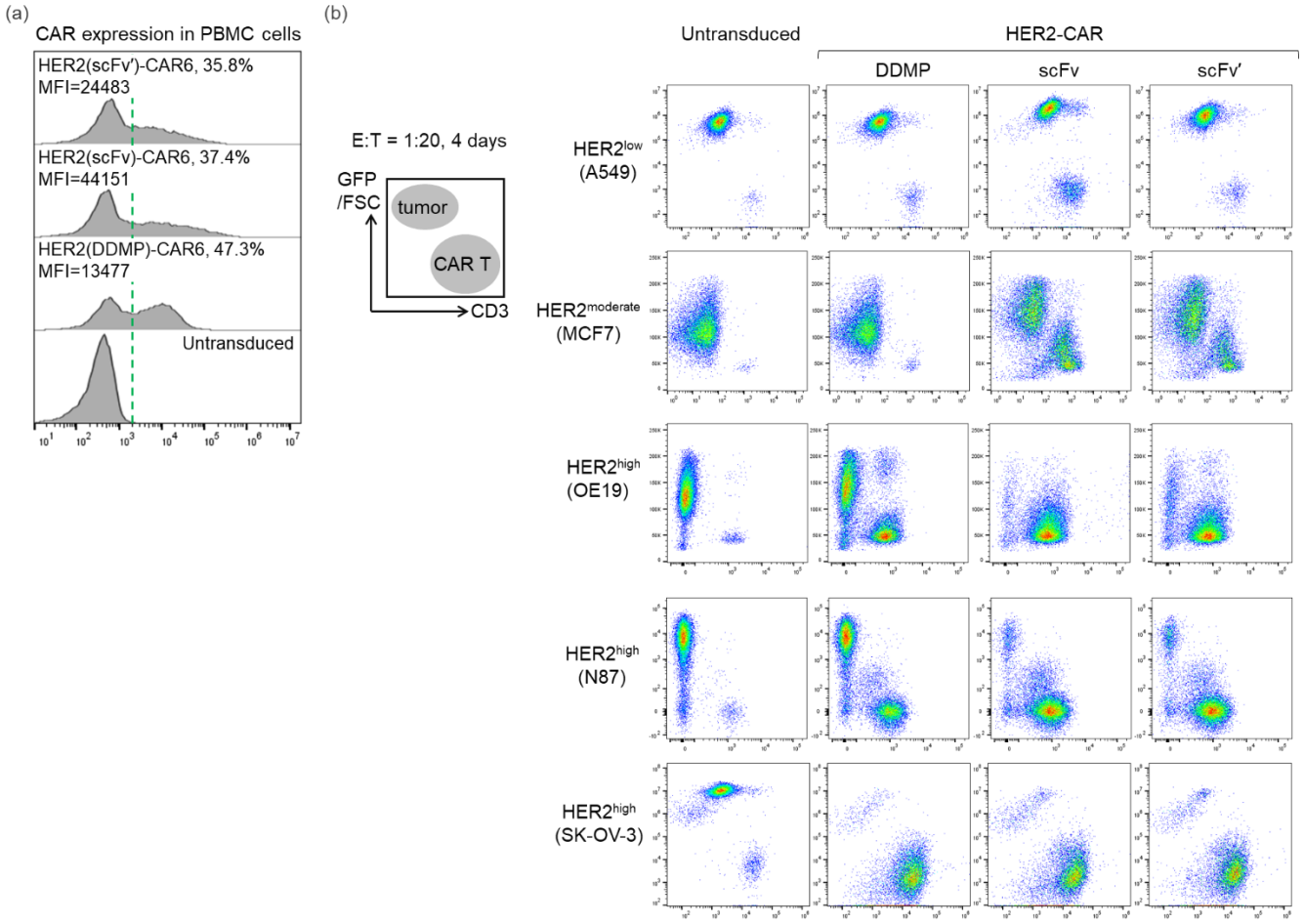


Figure S6. In vitro cytotoxicity of HER2-CAR T.

(a) HER2(DDMP)-CAR6, HER2(scFv)-CAR6 and HER2(scFv′)-CAR6 expression in human T cells (CD3+). (MFI represents mean fluorescence intensity of CAR^+ cells) (b) T cells were cocultured with A549-luc-GFP、MCF7-GFP、OE19-luc-GFP、N87-luc-GFP and SK-OV-3-luc-GFP cells for four days, followed by flow cytometry analysis. Gating strategy to distinguish tumor cells (GFP+) and T cells (CD3+) is shown in the left. The tumor cells stably express luciferase and GFP.

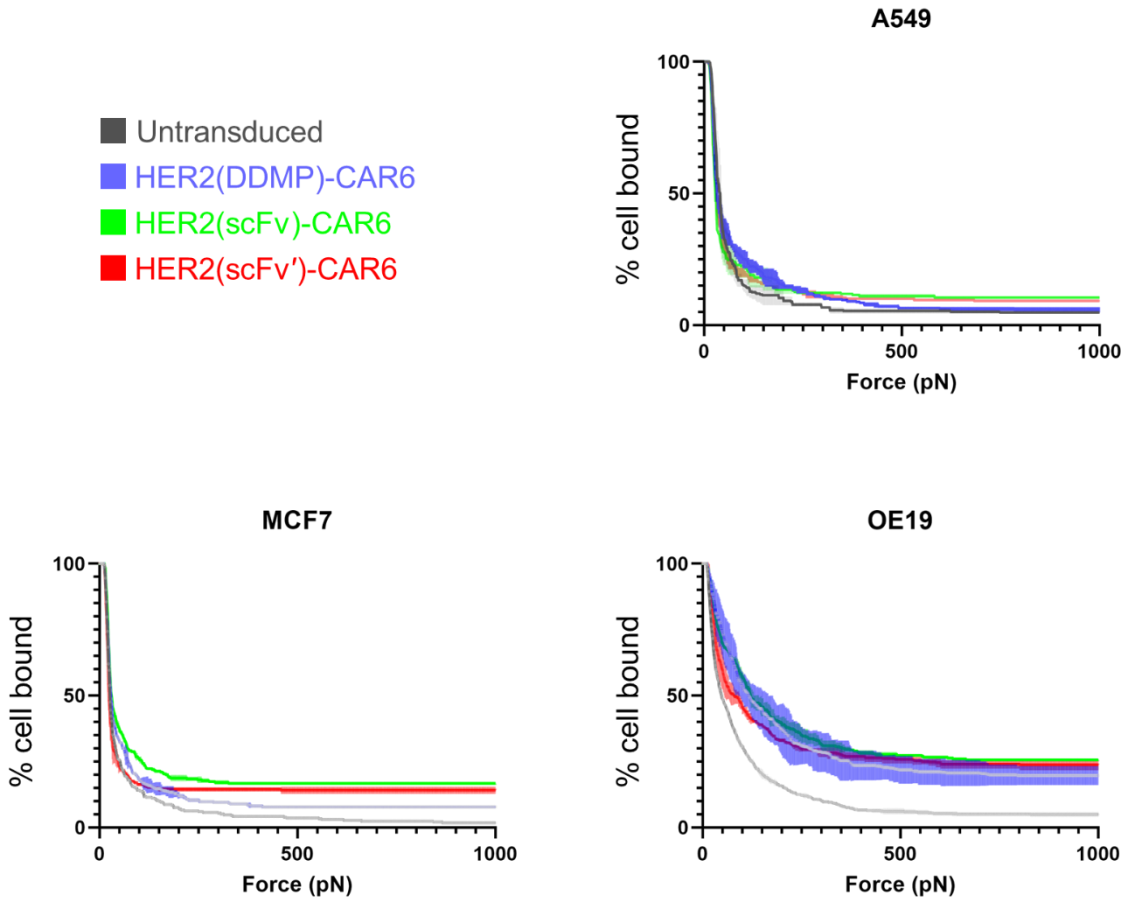


Figure S7. Cell avidity by z-Movi.

The percentage of T cells remaining bound as a function of acoustic force from 0 to 1000 pN.

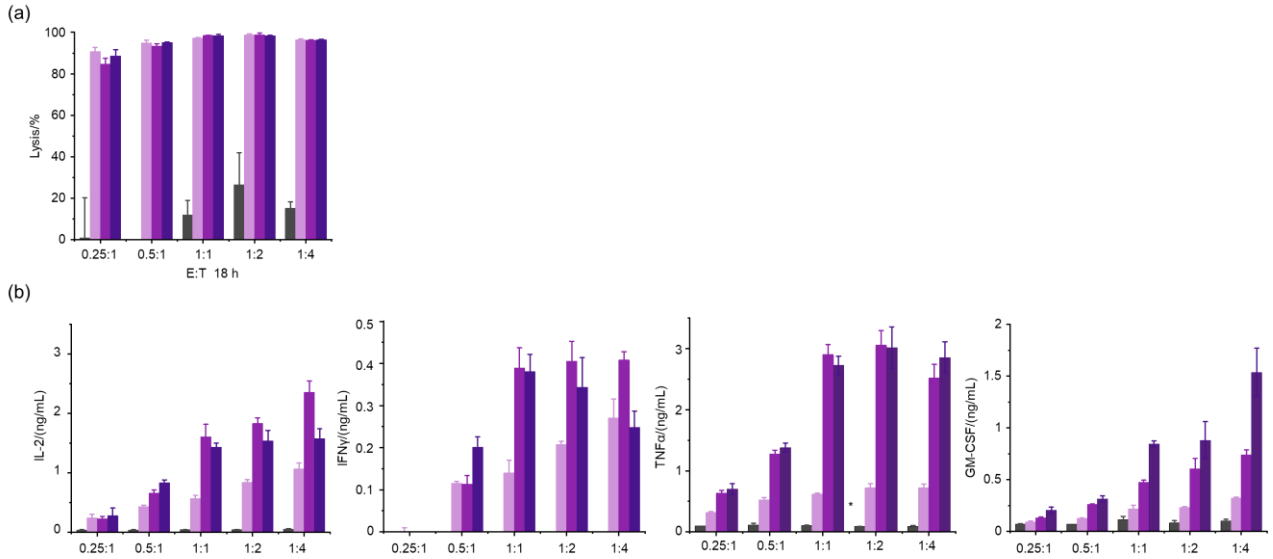


Figure S8. Cytotoxicity and cytokine secretion of HER2-CAR T cocultured with OE19.

Cells were cocultured at different E:T ratios for 18 h. (a) Cytotoxicity. (b) IL2, IFNγ, TNFα and GM-CSF.

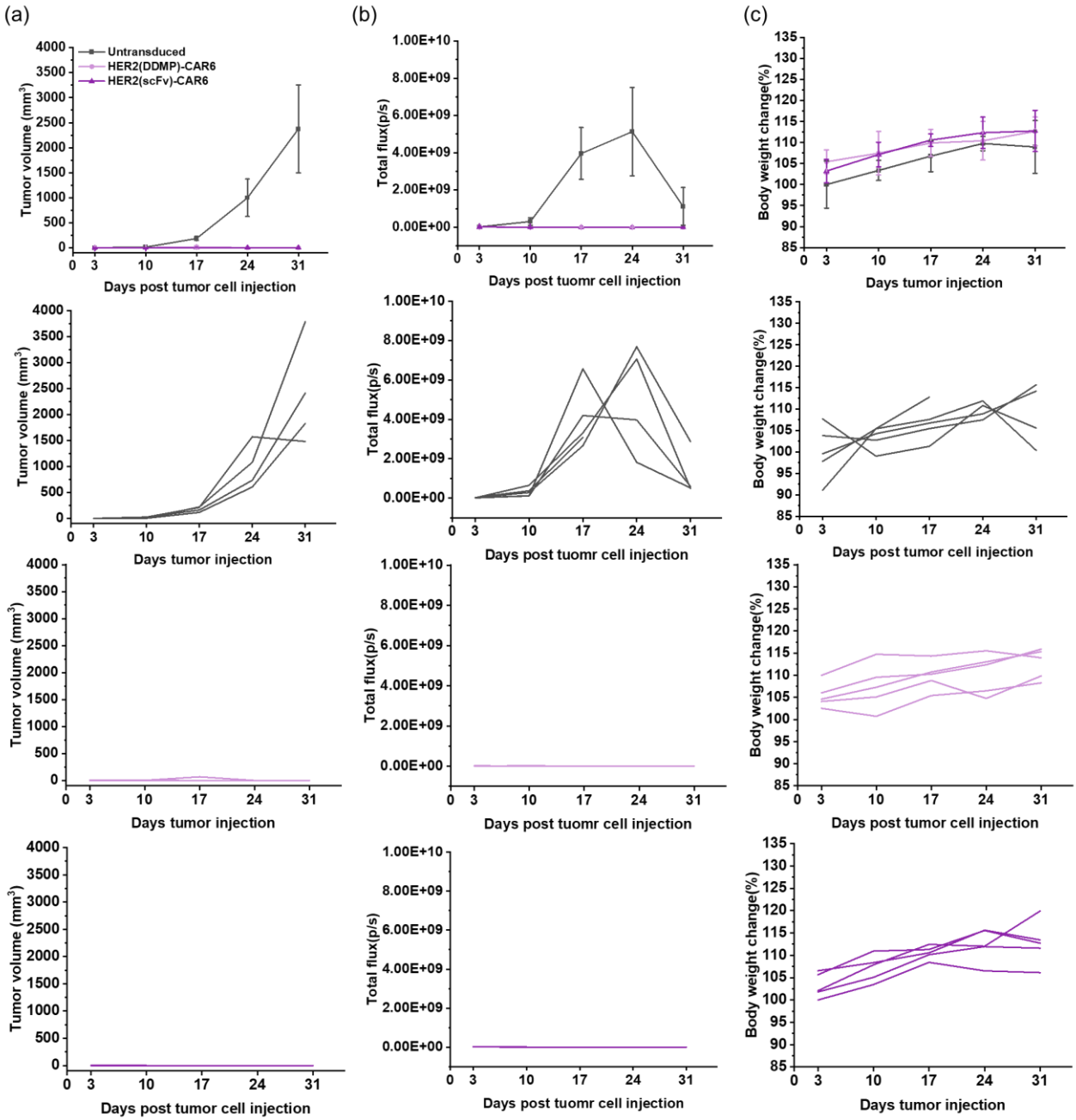


Figure S9. In vivo efficacy of HER2-CAR6 T cells in an OE19 xenograft model.

Quantification of tumor signal (a), tumor volume (b) and body weight (c) of individual mice corresponding to the experiment in **Fig. 5b**.

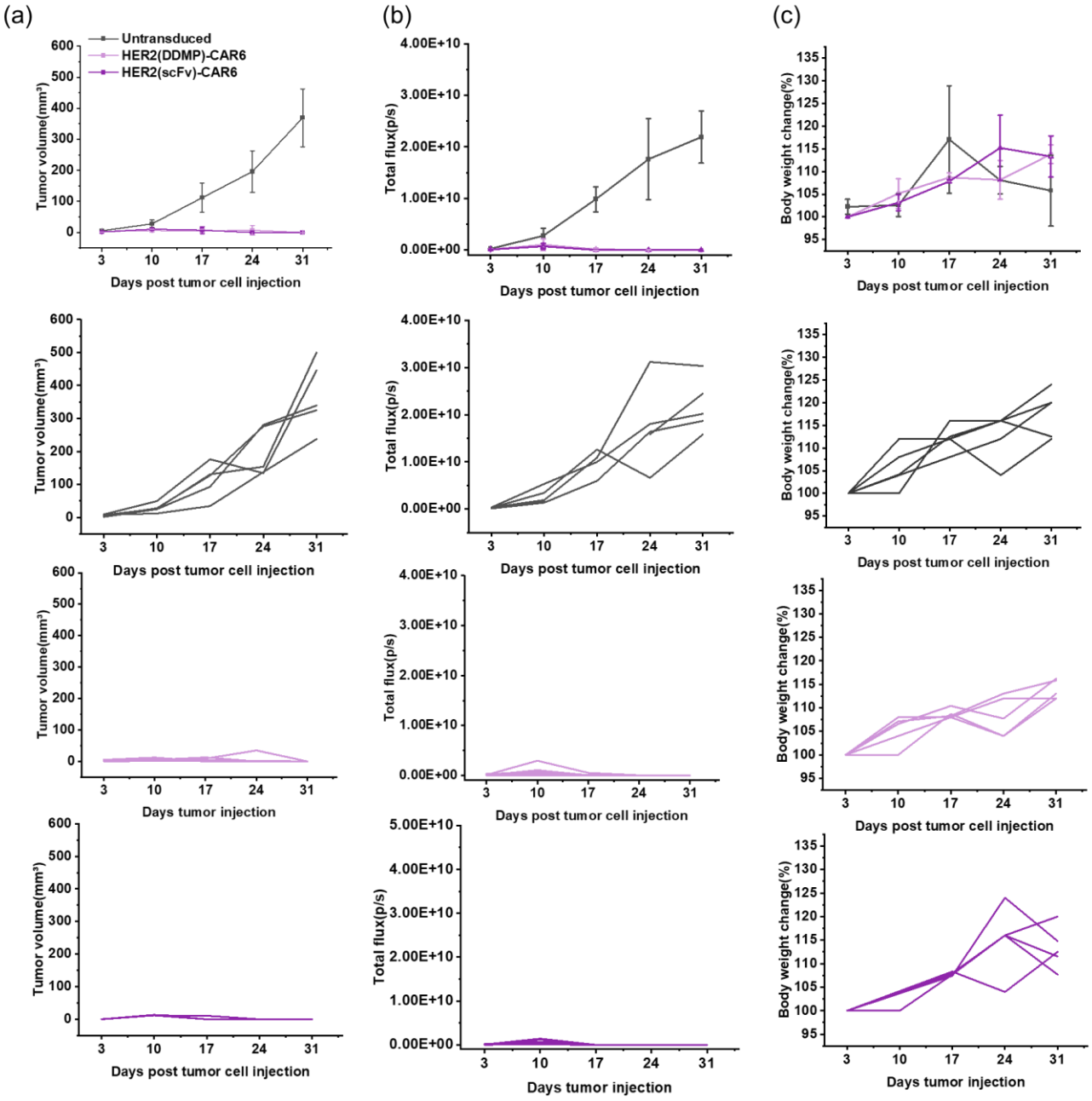


Figure S10. In vivo efficacy of HER2-CAR6 T cells in a N87 xenograft model.

Quantification of tumor signal (a), tumor volume (b) and body weight (c) of individual mice corresponding to the experiment in **Fig. 5c**.

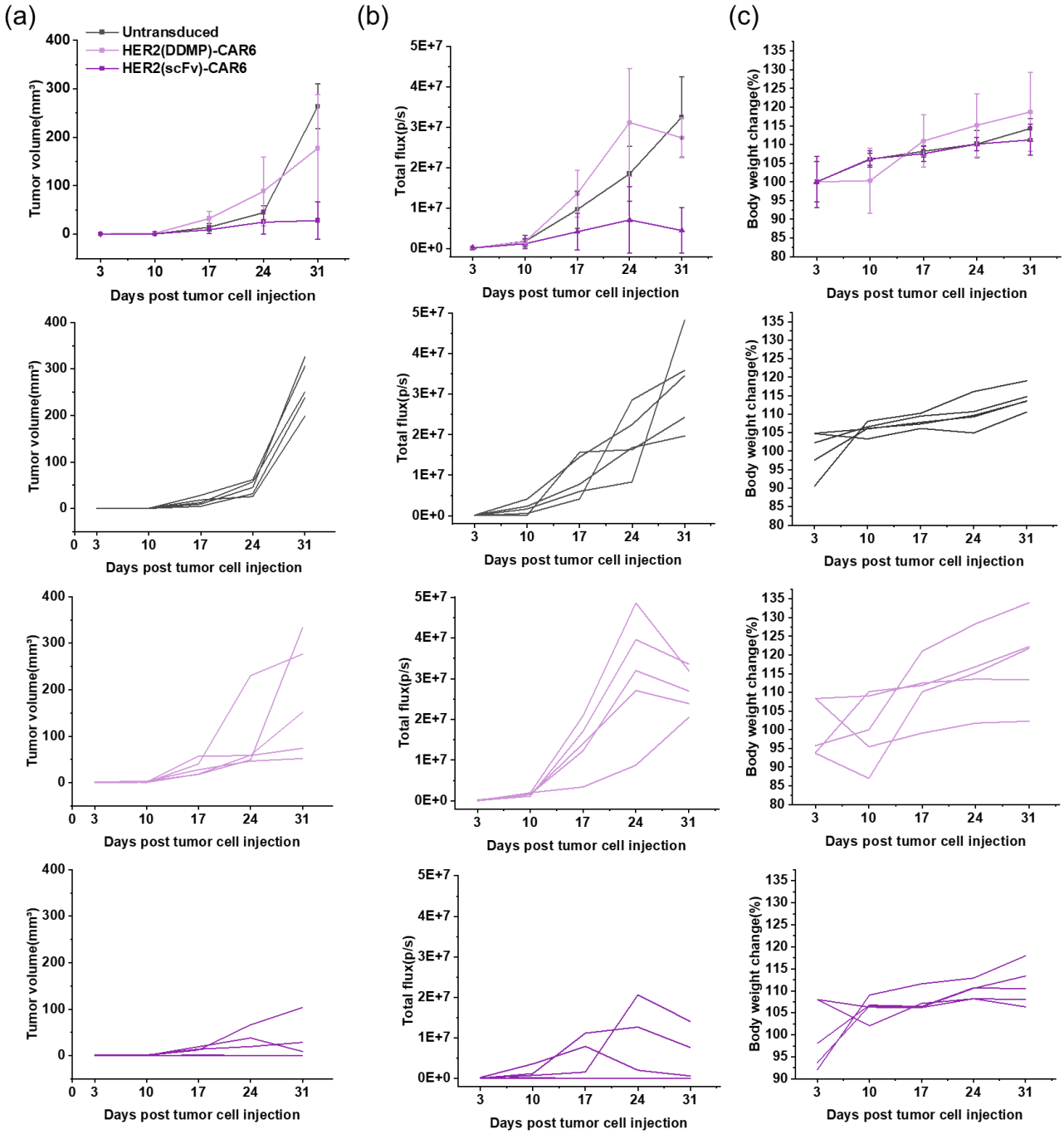


Figure S11. In vivo efficacy of HER2-CAR6 T cells in an A549 xenograft model.

Quantification of tumor signal (a), tumor volume (b) and body weight (c) of individual mice corresponding to the experiment in **Fig. 5d**.

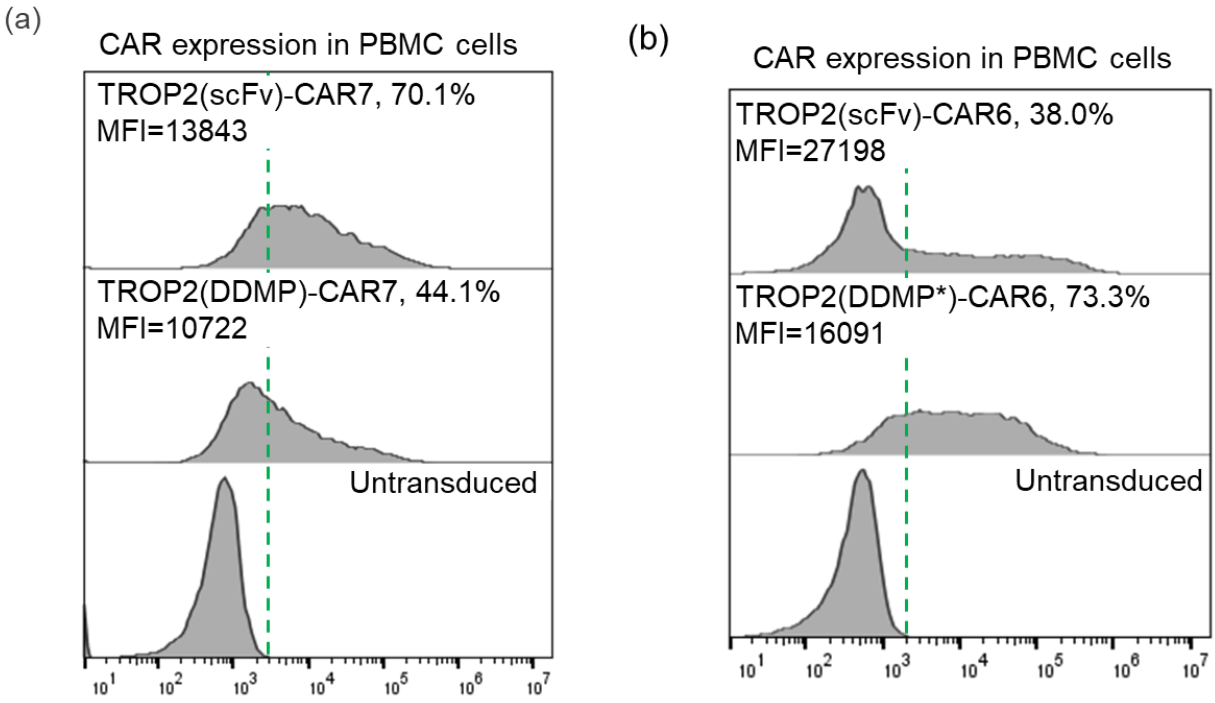


Figure S12. TROP2-CAR expression in human T cells.

MFI represents mean fluorescence intensity of CAR^+ cells.

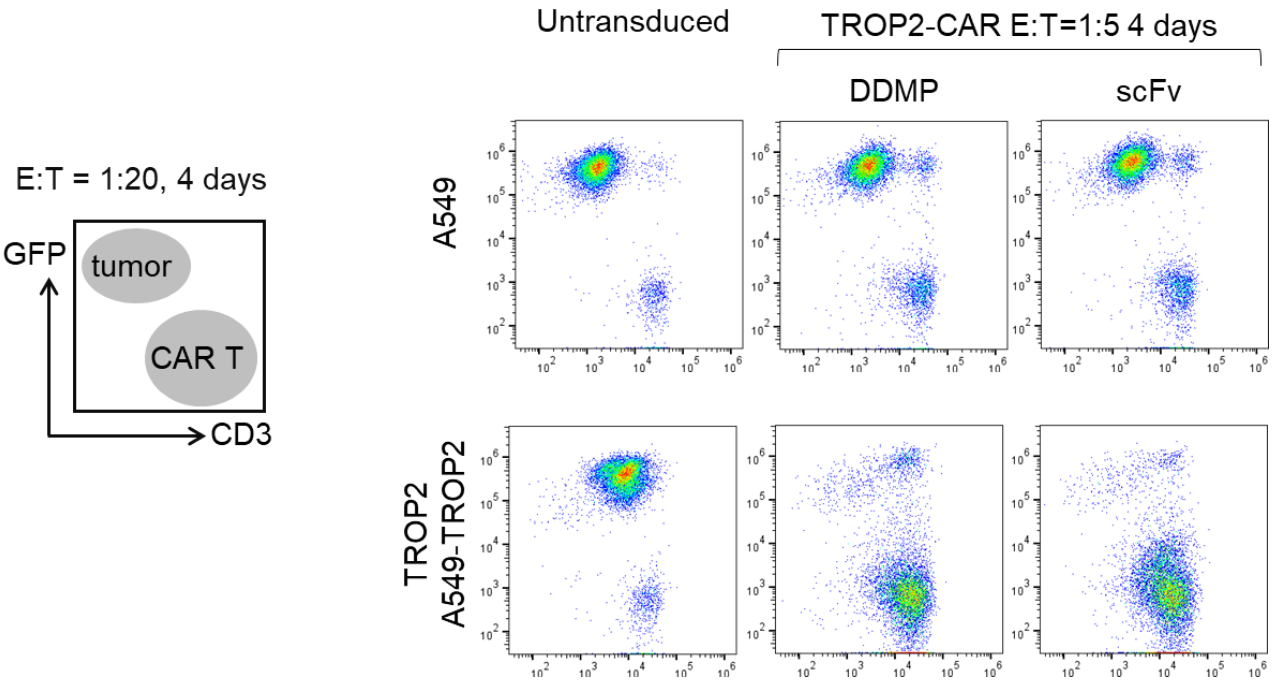
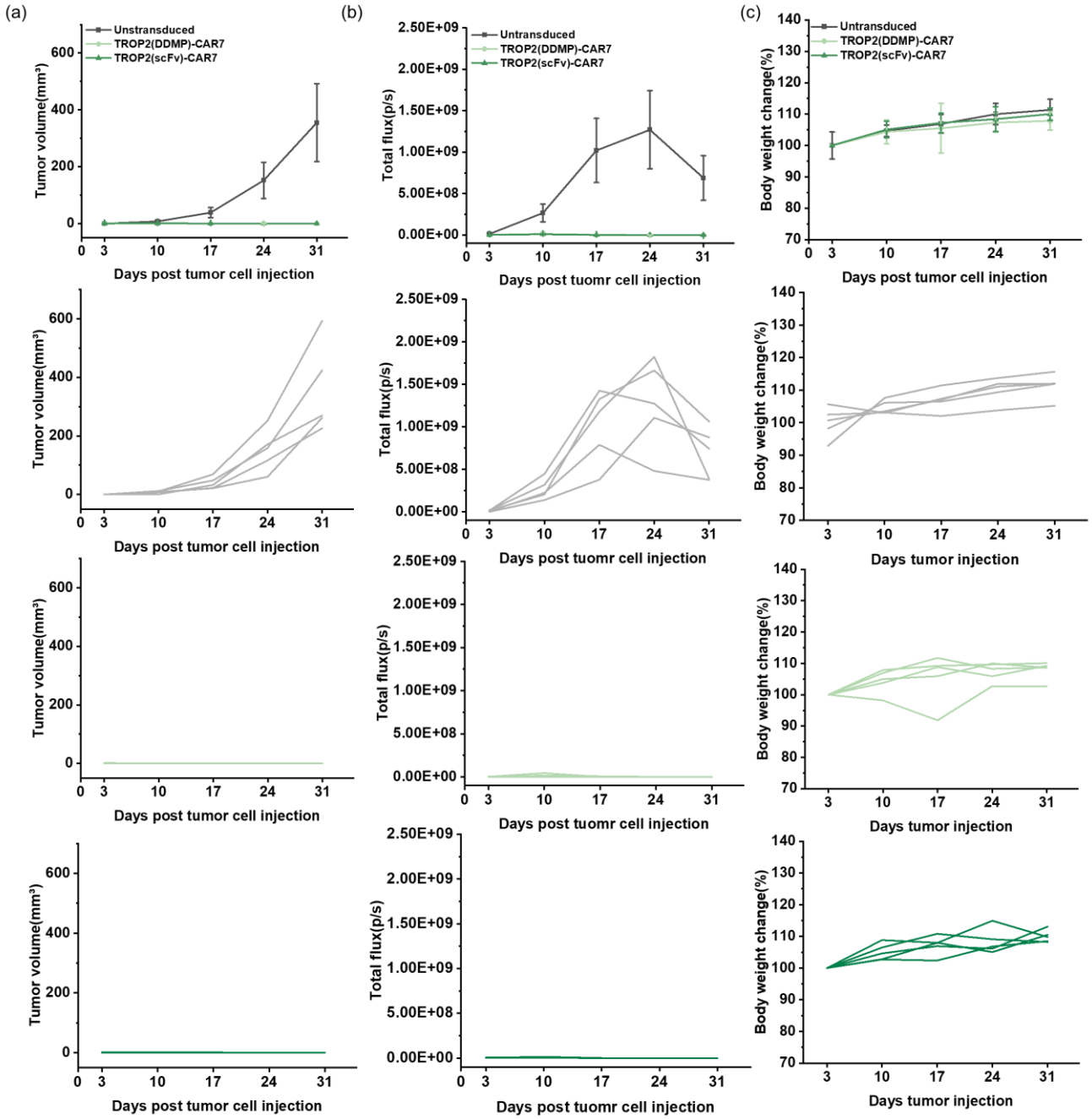


Figure S13. In vitro cytotoxicity of TROP2-CAR7 T cells.

T cells were cocultured with A549 or A549-TROP2 cells for four days, followed by flow cytometry analysis. Gating strategy to distinguish tumor cells (GFP^+) and T cells ($CD3^+$) is shown on the left. The tumor cells stably express luciferase and GFP.


(a)
(b)
(c)
Unstransduced
TROP2(DDMP)-CAR7
TROP2(scFv)-CAR7
Tumor volume(mm³)
Total flux(p/s)
Body weight change(%)
Days post tumor cell injection
Days post tuomr cell injection
Days tumor injection

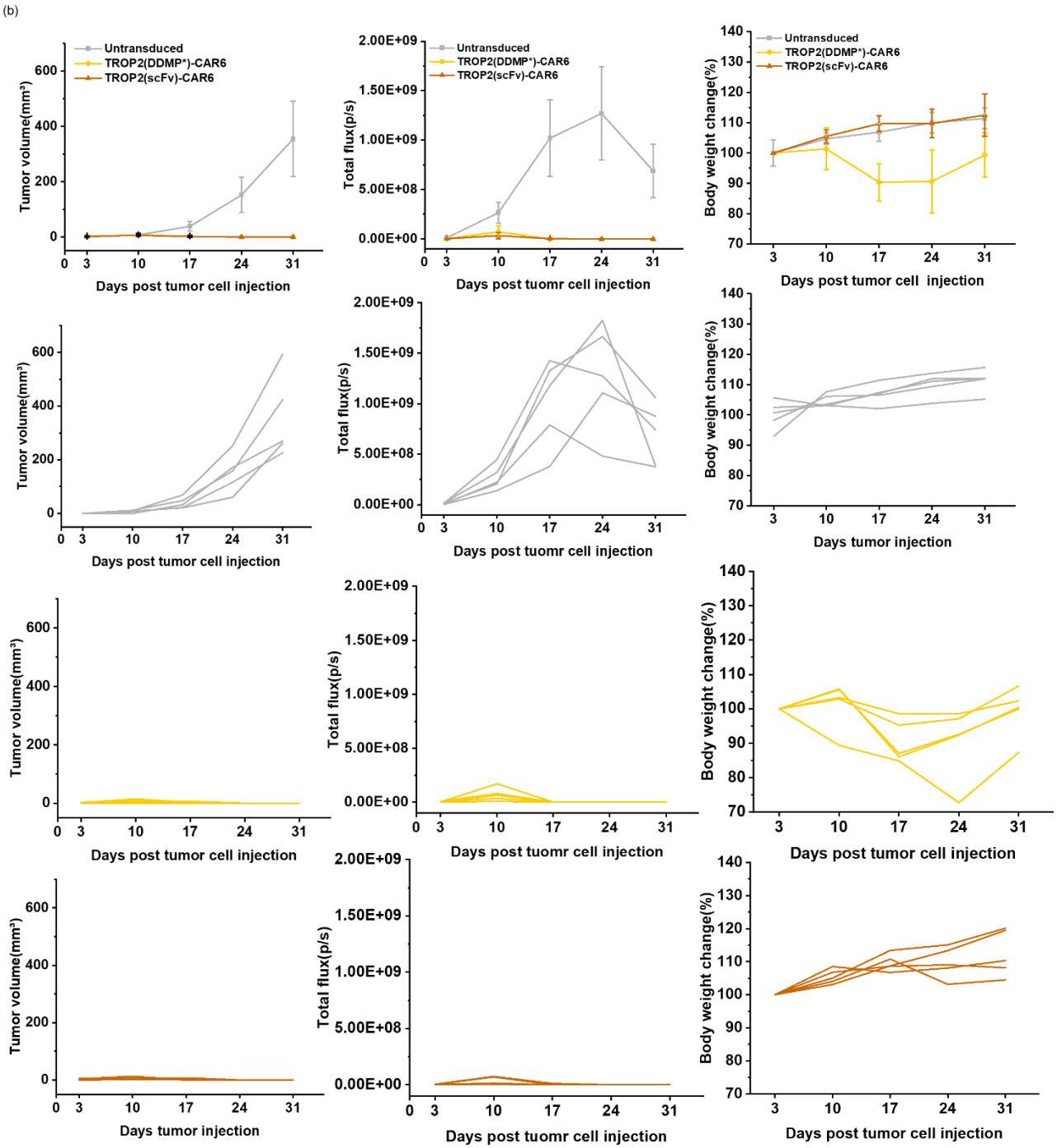


Figure S14. In vivo efficacy of TROP2(DDMP)-CAR T cells in an A549-TROP2 xenograft model.

Quantification of tumor volume (left), tumor signal (middle) and body weight (right) of individual mice corresponding to the experiment in **Fig. 6e**.

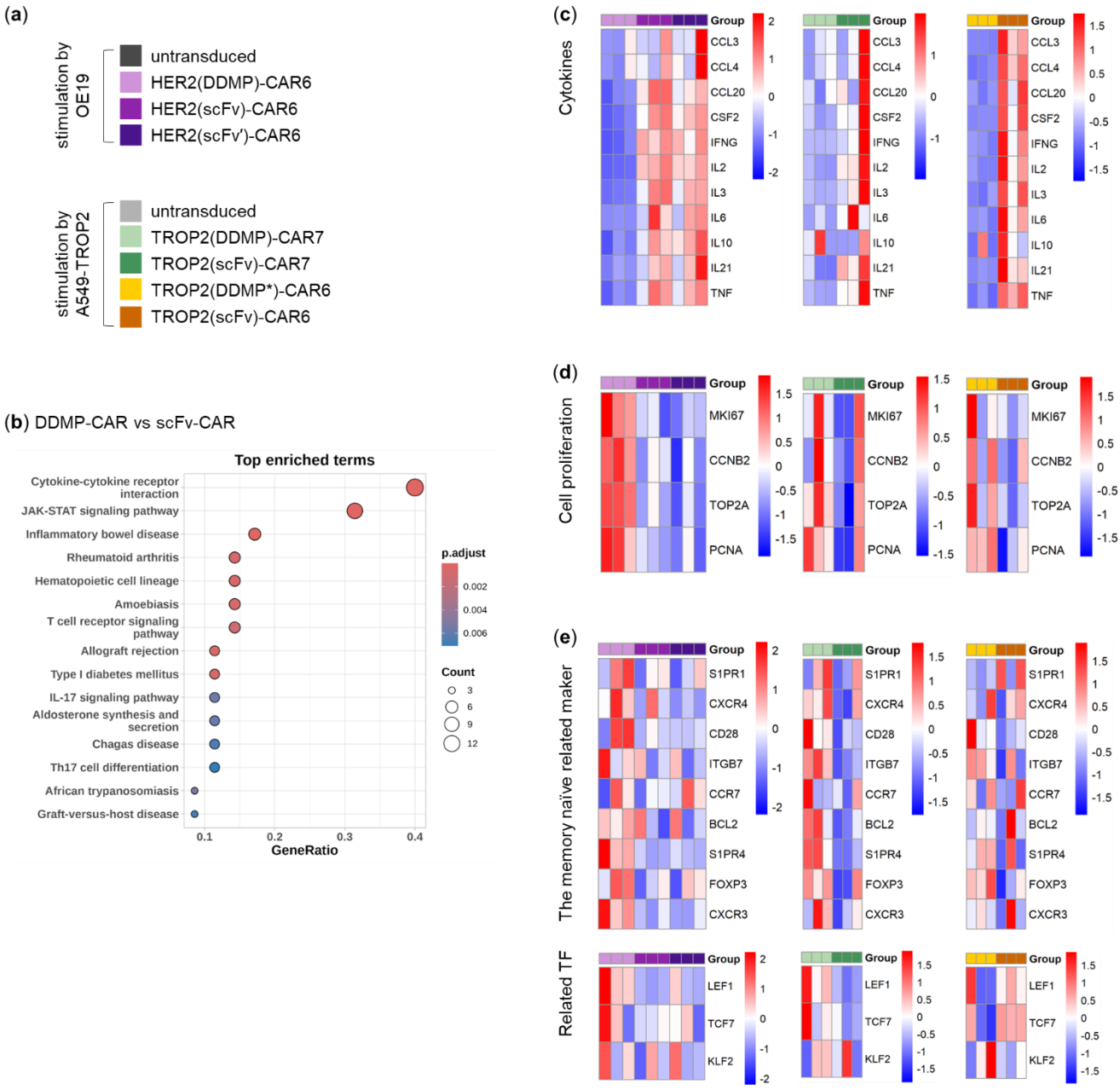


Figure S15. Transcriptomic profiling of DDMP- and scFv-CAR T cells after antigen stimulation.

(**a**) Legend of HER2- and TROP2-targeting CAR constructs used in RNA-seq. HER2- and TROP2-targeting CAR T cells were stimulated with OE19 and A549-TROP2 cells, respectively, for 18 hours at E:T = 2:1. (b) KEGG pathway analysis of top 500 DEGs reveals distinct signaling signatures. All data are based on T cells from three donors. (**c**–**e**) DEG expression heatmaps for cytokine genes (**c**), proliferation markers (**d**), and memory/naïve markers and transcription factors (**e**).

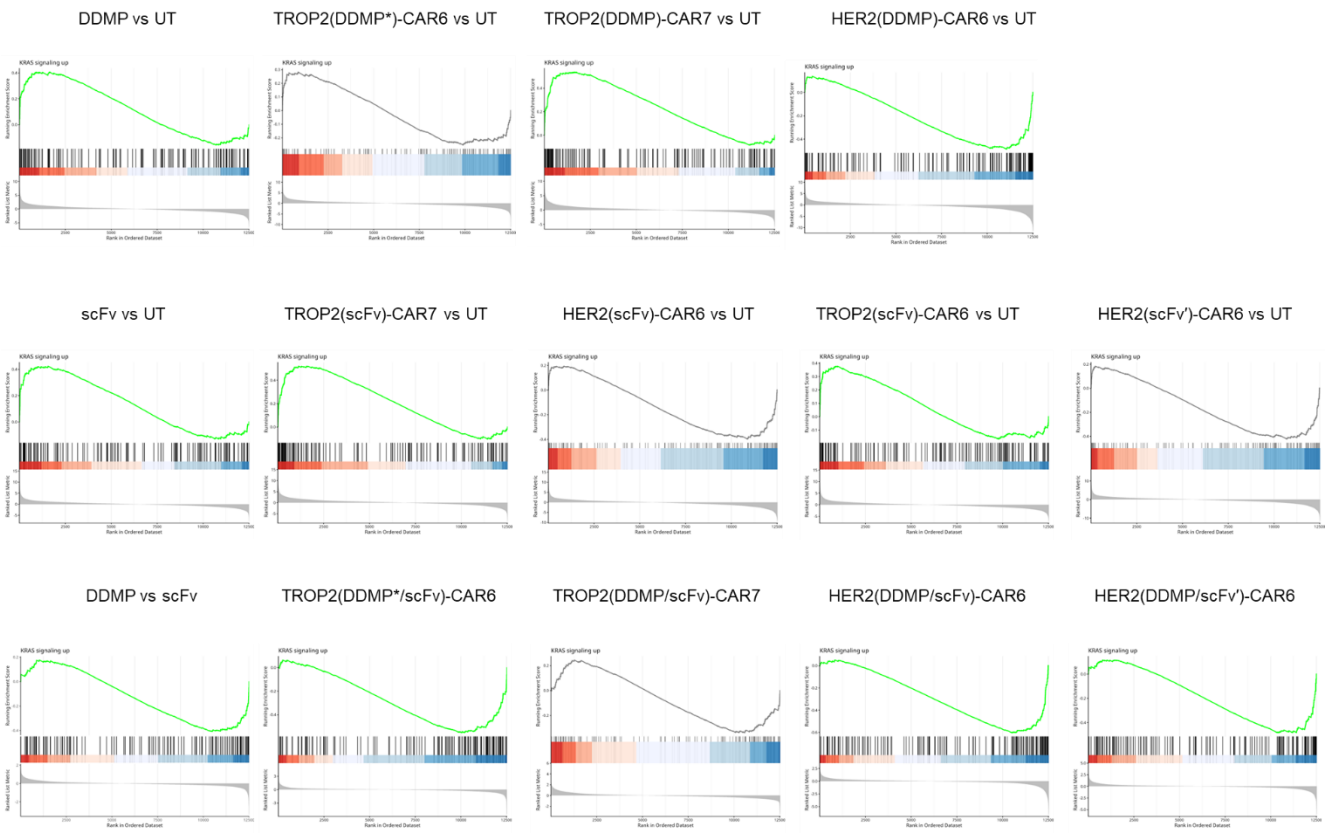


Figure S16. GSEA of Hallmark: KRAS.

Green lines indicate gene sets with significant positive or negative enrichment; non-significant results are shown in gray.

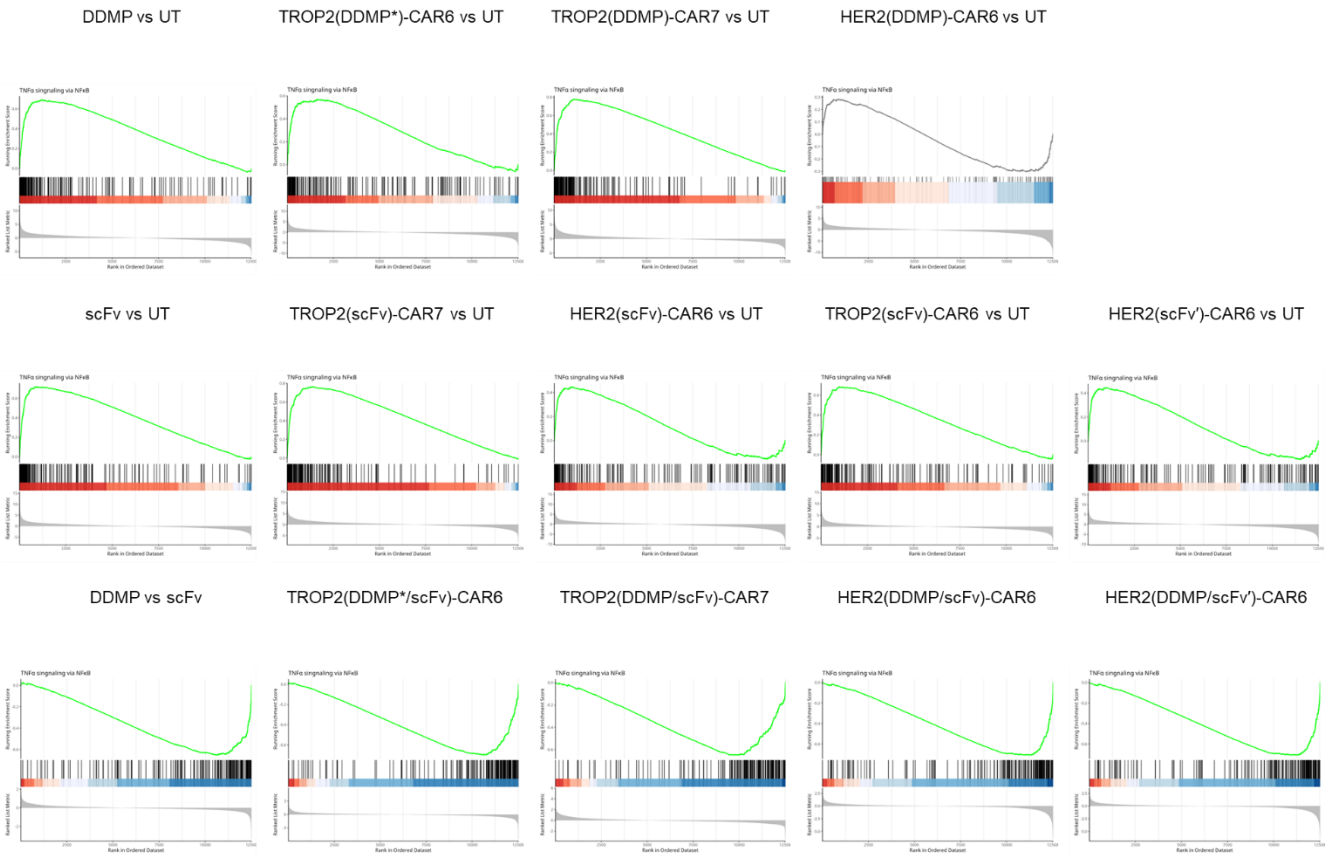


Figure S17. GSEA of Hallmark: TNF signaling via NF-κB.

Green lines indicate gene sets with significant positive or negative enrichment; non-significant results are shown in gray.

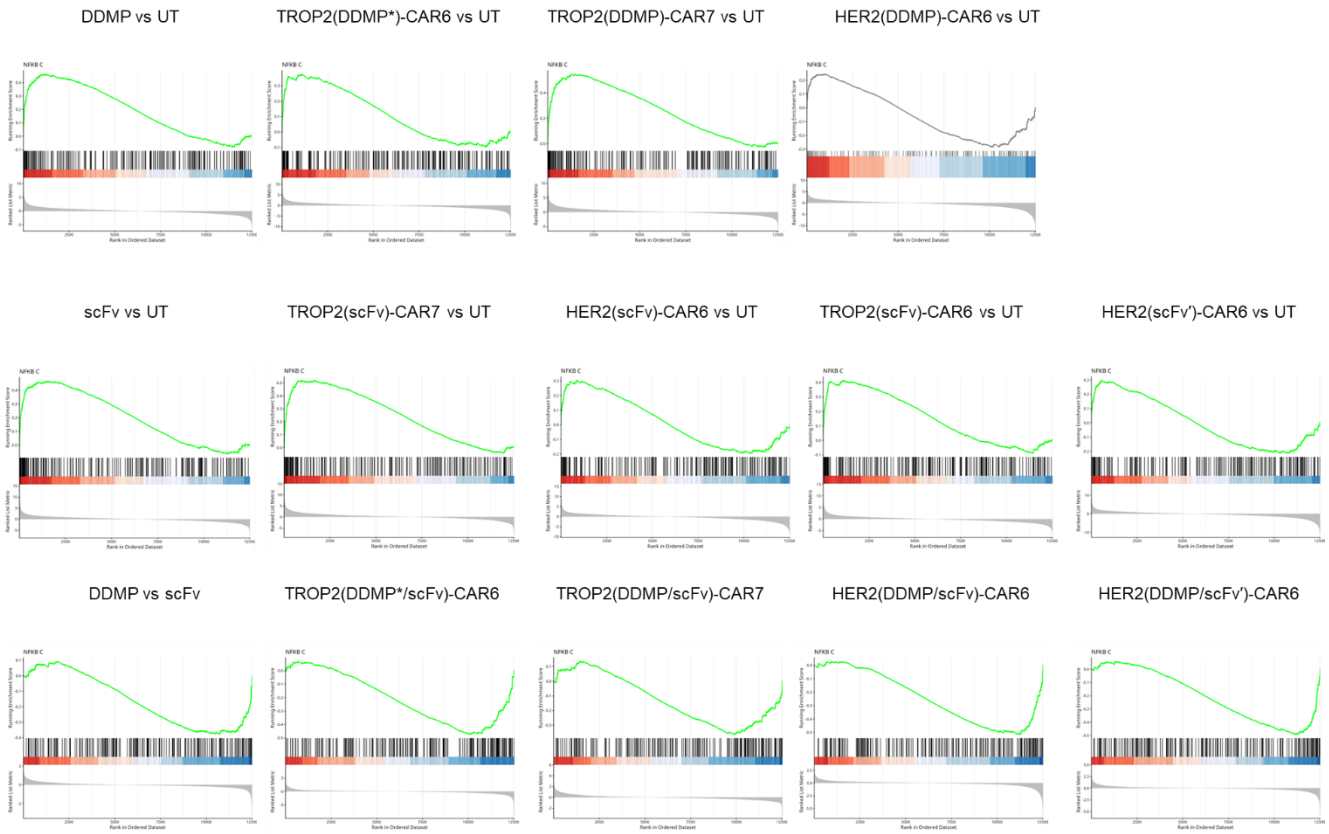


Figure S18. GSEA of TFT: NF-κB.

Green lines indicate gene sets with significant positive or negative enrichment; non-significant results are shown in gray.

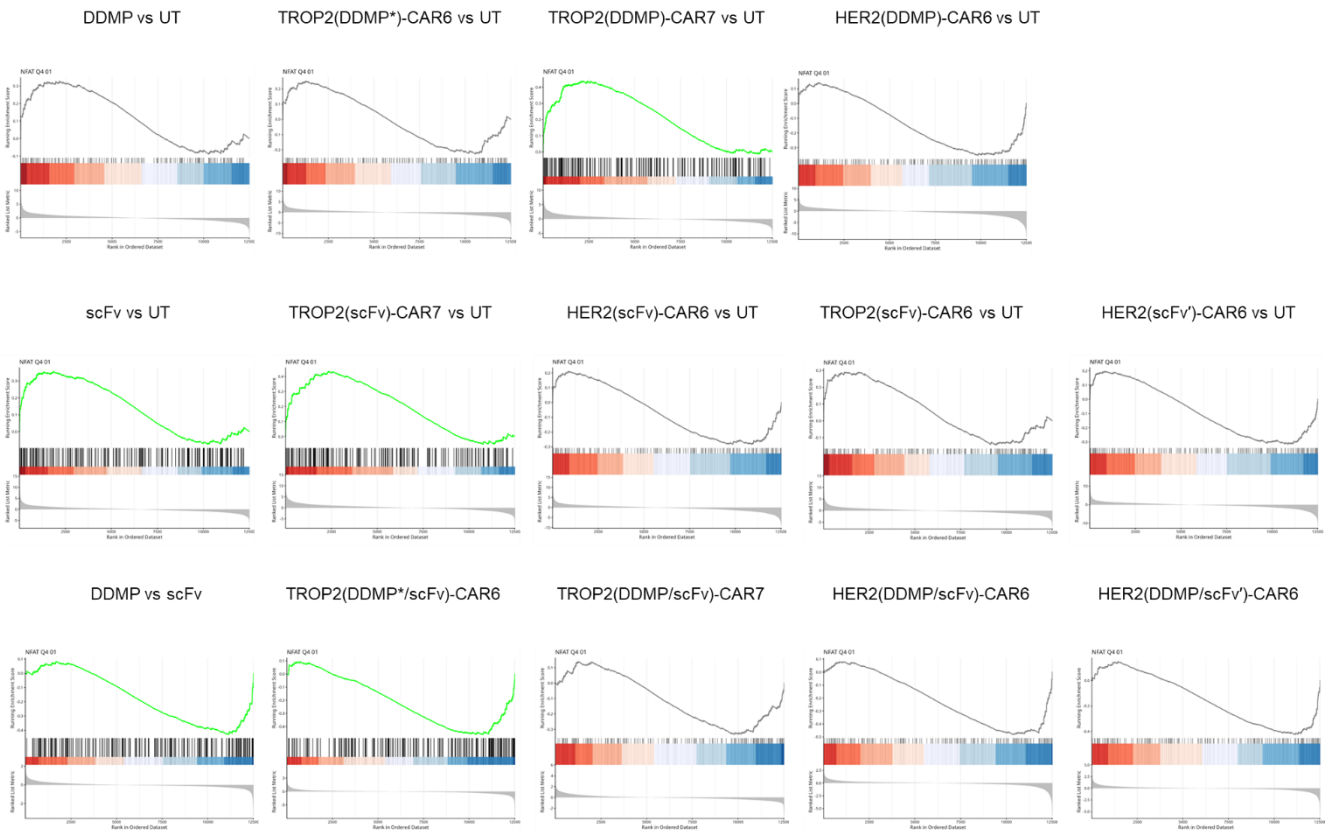


Figure S19. GSEA of TFT: NFAT.

Green lines indicate gene sets with significant positive or negative enrichment; non-significant results are shown in gray.

Materials and Methods

Cell culture

HEK293T cells (National Collection of Authenticated Cell Cultures, #GNHu17), A549 cells (National Collection of Authenticated Cell Cultures, #SCSP-503) and SK-OV-3 cells (National Collection of Authenticated Cell Cultures, #SCSP-5214) were cultured in DMEM (Gibco, #21063029) supplemented with 10% fetal bovine serum (FBS, TraNCGen Biotech, #FS401-02) and 1% penicillin/streptomycin (Gibco, #15140122). N87 cells (Procell, #CL-0169), OE19 cells (Procell, #CL-0754) were cultured in RPMI1640 (Gibco, #11875093) supplemented with 10% fetal bovine serum (FBS, TraNCGen Biotech, #FS401-02), 1% penicillin/streptomycin. Jurkat cells (National Collection of Authenticated Cell Cultures, #SCSP-513) were cultured in RPMI1640 (Gibco, #11875093) supplemented with 20% fetal bovine serum (FBS, TraNCGen Biotech, #FS401-02), 1% penicillin/streptomycin. The cells were cultured in a humidified atmosphere of 5% (v/v) CO_2 at 37 °C. Mycoplasma contamination was routinely monitored using PCR-based assays.

PBMC Isolation from Whole Blood by Density Gradient Separation

PBMCs were isolated from leukopaks of healthy donors (Milesbio) by Ficoll (Cytiva, #17544203) Density Gradient Centrifugation. The procedure mainly involves the following steps: (1) Dilute whole blood with sterile PBS at a 1:1 v/v ratio in 50 mL sterile conical tubes, and then mix the blood and buffer by gently inverting the tube or pipetting up and down; (2) Add 20 mL of Ficoll to a fresh 50 mL conical tube to prepare for layering. Gently and carefully overlay ~25 mL of diluted blood onto the Ficoll layer taking care not to mix the layers. Slowly dispense the first 5 mL of blood dropwise to avoid disturbing the separation medium and blood interface. (3) Centrifuge the tubes at 400 rcf at 18 °C for 30 minutes (the speed up is 1, the speed down is 0) to prevent disrupting the density gradient during deceleration. (4) Carefully remove the tubes from the centrifuge without disturbing any of the layers. After centrifugation, multiple layers are obtained in the following order: Yellowish layer of dilute plasma and platelets Fluffy white layer of PBMCs at the interphase Ficoll or cell-separation medium red blood cell and granulocyte layer. (5) Slowly remove the top diluted plasma layer (~25 mL) using a plastic Pasteur pipette. Carefully collect the PBMC layer using a sterile Pasteur pipette, starting at the periphery, and slowly moving the pipette tip over the entire cross-sectional area of the tube. Avoid aspirating too much Ficoll medium as this can be toxic to the cells. Transfer PBMCs to a fresh 50 mL conical tube. Discard the remaining Ficoll and RBC layers. (6) Wash PBMCs by bringing up the volume in the conical tube to 50 mL using PBS wash buffer. Centrifuge the tubes at 550 rcf at 18 °C for 30 minutes (the speed up is 1, the speed down is 0). Carefully discard the supernatant and loosen the cell pellet in the remaining solution, using a transfer pipette to mix large clumps. Continue to repeat this step 2 times until the supernatant is clear. PBMC counts were performed during the third wash step using an automated cell counter. (7) After isolation, PBMCs can be used immediately or cryopreserved to maintain sample integrity for future use. Cryostor CS10 (Stemcell, #100-1061) is used for cryopreservation. Discard the supernatant and re-suspend the pellet in cold Cryostor CS10 to obtain a cell density of 25 x 10^6 cells/mL. Quickly aliquot 1 mL of PBMC cell suspension in each cryovial (ThermoFisher, #377267). Place PBMC-containing cryovials in a Mr. Frosty freezing container in a -80 °C freezer overnight. Transfer the vials to liquid nitrogen for long-term storage the next day. In addition, 1.5 × 10^5 PBMC cell suspension were stained with FITC-labeled α-CD3 antibody (1:100 incubation at 25 °C for 15-30 min, then washed with 1 mL RPMI1640, and resuspended in 200 μL RPMI1640; 4A Biotech, #FHF003-01-100) to determine the percentage of CD3+ T cells in the sample by flow cytometry.

Quantification of cell-surface HER2 protein by flow cytometry

1.5×10^5 target cells (A549, SK-OV-3, N87 and OE19 cells) were resuspended with 100 μL DMEM (Gibco, #21063029), followed by the addition of 1 μL of PE-labeled α-HER2 antibody (BioLegend, #324406. The mixture is gently shaken to ensure homogeneity, then incubated either at room temperature in the dark for 15-30 minutes. Then the samples were washed with 1 mL DMEM. The cell pellets were resuspended in 200 μL DMEM. Then the HER2 expression on the surface of A549, SK-OV-3, N87 and OE19 cells were detected by flow cytometer using CytoFLEX LX (Beckman Coulter, Brea, CA, USA) instruments.

DNA constructs

All HER2(DDMP)-CAR constructs contain a HER2(DDMP) peptide, a V5 tag, CD28 transmembrane domain (Uniprot: P10747), CD28 costimulatory domain and cytosolic domains of CD3ζ (Uniprot: P20963), with different spacers including IgG4(EQ) containing two point mutations (L235E and N297Q) of human IgG4 Fc; ΔCH2, IgG4(EQ)-Fc with the CH2-domain deleted; hinge, human IgG4(EQ) hinge (S to P substitution); a GSGSGS short linker; CD28h and CD8h. The sequences of the spacers are shown below. HER2(DDMP)-CAR7 constructs contain a HER2(DDMP) peptide, a V5 tag, CD8h, CD8 transmembrane domain, 4-1BB (Uniprot: Q07011) costimulatory domain and cytosolic domains of CD3ζ. HER2(scFv)-CAR6 and HER2(scFv′)-CAR6 were constructed using VL and VH segments of the Herceptin[1] and 7-5.[2] Each scFv was connected to a CD8h domain, the transmembrane region and costimulatory domain of CD28, followed by the intracellular domain of human CD3ζ.

All TROP2-CAR7 constructs contain a V5 tag, CD28 transmembrane domain, a spacer including IgG4EQ [IgG4 with two point mutations (L235E and N297Q)], the transmembrane region and costimulatory domain of CD28, and cytosolic domains of CD3ζ(1XX).[3] The structure of TROP2(DDMP)-CAR is different from the TROP2(scFv)-CAR7 in the antigen recognition region. The former is composed of a TROP2(DDMP) peptide. The latter is composed of VL and VH segments of the hRS7.[4] All HER2 CAR and TROP2 CAR sequences were chemically synthesized and inserted into lentiviral vectors.

The ECD of all chimeric HER2 constructs (chHER2a-i), hHER2 and mHER2 were cloned respectively into a mCherry lentiviral expression vector under the CMV promoter using Gibson assembly.

Luc-GFP plasmid contains Luciferase (Luc) and green fluorescent protein (GFP) gene for expression of EGFP and luciferase in mammalian cells. TROP2-mCherry plasmid contains TROP2 and mCherry gene for expression of TROP2-mCherry fusion protein in mammalian cells.

All constructs were obtained by gene synthesis. NFAT-GFP plasmid was a generous gift from Dr. Chen Yu.

HER2(DDMP):
CPWFCIYPCKVEPRCSEVYAEQCPQTC

HER2(scFv):
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGT
DFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTGSTSGSGKPGSGEGSEVQLVESGGGLVQP
GGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQM

NSLRAEDTAVYYCSRWGGDGFYAMDVWGQGTL

HER2(scFv′):
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASNLYSGVPSRFSGSRSGT
DFTLTISSLQPEDFATYYCQQHATTPPTFGQGTKVEIKRTGSTSGSGKPGSGEGSEVQLVESGGGLVQP
GGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGATRYADSVKGRFTISADTSKNTAYLQMN
SLRAEDTAVYYCSRWGGDGFYAMDVWGQGTLVTV

TROP2(DDMP):
CPPCGRWLECYDFNECELIDWCPPC

TROP2(DDMP*):
CKPKGAPCSPLMYPCCTGPCPGAGWLMKDCLCC

TROP2(scFv):
DIQLTQSPSSLSASVGDRVSITCKASQDVSIAVAWYQQKPGKAPKLLIYSASYRYTGVPDRFSGSGSGTD
FTLTISSLQPEDFAVYYCQQHYITPLTFGAGTKVEIKRGGGGSGGGGSGGGGSQVQLQQSGSELKKPGA
SVKVSCKASGYTFTNYGMNWVKQAPGQGLKWMGWINTYTGEPTYTDDFKGRFAFSLDTSVSTAYLQIS
SLKADDTAVYFCARGGFGSSYWYFDVWGQGSLVTVSSGSA

IgG4(EQ):
ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHN
AKTKPREEQFQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQ
EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFS
CSVMHEALHNHYTQKSLSLSLGK

IgG4(EQ) ∆CH2:
ESKYGPPCPPCPGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK

IgG4(EQ) hinge:
ESKYGPPCPPCP

CD28h:
IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKP

CD8h:
AKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD

Lentivirus packaging

About 6.5×10^6 HEK293T cells in 10 mL of complete media (i.e., DMEM with 10% FBS) were added into each 10-cm dish. After 18 h, cells in each plate were transiently transfected with a mixture of 12 μg plasmid encoding the gene of interest, 7.5 μg packaging plasmid psPAX2 (Addgene, #12259) and 6 μg envelop plasmid pMD2.G (Addgene, #12259) using PEI (Polysciences, #24765-1, PEI was prepared with water to a concentration of 1 μg/μL.) according to the manufacturer’s protocol. After 6 h, the media was replaced

with fresh complete media. After another 72 h, supernatants containing lentivirus were collected, centrifuged at 400 rcf for 10 min at 4 °C to remove cell debris, and filtered through a 0.45 μm PES filter (Biosharp, #BS-PES-45). The lentiviral particles were concentrated by centrifugation at 19800 rcf for 2 h at 4 °C. Finally, the lentivirus pellets from each dish were resuspended with 500 μL RPMI1640 (Gibco, #11875093) and stored at -80 °C.

Construction of stable cell lines

The Jurkat NFAT-GFP reporter cells were constructed by transducing 3.5×10^5 Jurkat cells with 1 mL unconcentrated NFAT-GFP lentivirus. Single cell clones of low GFP expression were sorted by FACS (the GFP voltage of Beckman CytoFLEX SRT set to 164, cells with the fluorescence less than 1000 arbitrary units were considered negative, cells with the fluorescence at 1000-3000 arbitrary units were considered low GFP expression). All sorted single cell clones were cultured in RPMI1640 (Gibco, #11875093) supplemented with 20% fetal bovine serum (FBS, TraNCGen Biotech, #FS401-02), 1% penicillin/streptomycin with cell stimulation cocktail (Invitrogen, #00-4970) for 16 h for functional tests. A clone showing the strongest selective activation (i.e., high GFP expression after stimulation) was used for subsequent studies.

The A549-luc-GFP, SK-OV-3-luc-GFP, N87-luc-GFP and OE19-luc-GFP cells were constructed by transducing the corresponding cells with Luc-GFP lentivirus, making the target cells positive for GFP and luciferase. The cells were then checked for expression of GFP by FACS and sorted for stable expression (the GFP voltage of Beckman CytoFLEX SRT set to 164, cells with the fluorescence less than 2000 arbitrary units were considered negative, cells with the fluorescence at 800000-2000000 arbitrary units were sorted).

The A549-luc-TROP2-mCherry cell line was transduced with Luc-GFP lentivirus and TROP2-mCherry lentivirus, making this target cell line positive for GFP and mCherry. The cells were then checked for expression of GFP and TROP2-mCherry were verified by FACS through an α-TROP2 antibody (Invitrogen, #12-6024-42). Both high GFP and TROP2 expression cells were sorted for stable expression.

NFAT activation assay

For evaluation of CAR functionality, Jurkat-NFAT-GFP cells were transduced in complete media (i.e., RPMI1640 with 20% FBS) supplemented with 10 μg/mL polybrene (Solarbio, #H8761-5) using lentiviral vectors encoding the CAR. Typical transduction was performed by incubating 1.8×10^5 cells with 35 μL concentrated virus (i.e., virus produced by cells in a 10-cm dish = 500 μL). After 48 h, CAR expression was verified by flow cytometry through an α-V5 tag antibody (1:100 incubation at 25 °C for 15-30 min, then washed with 1 mL RPMI1640, and resuspended in 200 μL RPMI1640; eBioscience, #12-6796-42). CAR-transduced Jurkat-NFAT-GFP cells (1.8×10^5) were plated with target cells (A549 or SK-OV-3 cells) at a ratio of E:T=1:1 in 24-well plates. After 24 h, cells were collected to analyze both CAR expression through an α-V5 tag antibody (eBioscience, #12-6796-42) and the percent of activated CAR-transduced Jurkat (NFAT-GFP) cells (GFP+) using flow cytometry.

Elucidation of the cyclic peptide binding epitope on HER2: Jurkat (NFAT-GFP) cells were transduced with HER2(DDMP)-CAR1, and 293T cells were transfected with the indicated HER2 construct using Lipofectamine 2000 (Life Technologies, #11668-019). After 24 h, HER2(DDMP)-CAR1-transduced Jurkat

(NFAT-GFP) cells (1.8×10^5) were plated with target cells (chimeric HER2-transfected cells) at a ratio of E:T=1:1 for 24 h. Cells were collected to analyze both CAR expression through an α-V5 tag antibody (eBioscience, #12-6796-42) and the percent of activated CAR-transduced Jurkat (NFAT-GFP) cells (GFP+) using flow cytometry.

Primary human T cell activation, transduction and expansion

Primary human T cells were activated by Dynabeads Human T-Expander CD3/CD28 (Invitrogen, #11141D) at a 1:1 bead:CD3+ T cell ratio in T cell culture media, consisting of 45% RPMI1640 medium (Gibco, #11875093), 45% clicks medium (Sigma, #C5572), 10% FBS (Sigma, #F8687) and 1% P/S (Gibco, #15140122), supplemented with 100 U/mL IL2 (Proteintech, #HZ-1015). After 48 h, the CD3/CD28 Dynabeads were removed, and cells were transduced with a lentivirus encoding a CAR construct using Retronectin (Takara, #T100A) according to the manufacturer's protocol. CAR T cells were maintained in T cell culture media supplemented with 50 U/mL IL2. CAR expression was verified by flow cytometry through an α-V5 tag antibody (eBioscience, #12-6796-42).

In vitro cytotoxicity and cytokine secretion

Target tumor cells (SK-OV-3, N87, OE19, A549 or A549-luc-TROP2-mCherry) were seeded at the density of 1×10^4 cells in 100 μL complete media per well in 96-well plates (Biosharp, #BS-MP-96W-CL). After 4 h, CAR-T cells (i.e., effector) were added (100 μL per well) at different effector to tumor (E/T) ratios (i.e., 0.5:1, 1:1 or 2:1). After 18 h, the supernatants (E:T=2:1) were collected and stored at -80 °C for subsequent cytokine secretion analysis. The concentrations of IL2, TNFα, and IFNγ were measured using ELISA kits (Invitrogen, #88-7025-88, #88-7346-77, #88-7316-86) according to the manufacturer's instructions on 1:10 diluted supernatant samples.

For cytotoxicity analysis, 100 μL of 1 mM D-luciferin sodium salt (MedChemExpress, #HY-12591) was added into each well. Bioluminescence signal was measured at the indicated time points in relative luminescence units (RLU) using a microplate reader. Target cells alone were plated at the same cell density to determine the maximal luciferase expression (i.e., RLUmax). Specific lysis was calculated as 1-(RLUsample / RLUmax).

It is noteworthy that in all comparative experiments, we normalized by the number of CAR T cells. We used the CAR-positive ratio to calculate the total number of T cells needed to achieve the desired CAR T cell dose. Consequently, samples with lower CAR-positive ratios contained more untransduced T cells, which exhibited negligible cytotoxicity and cytokine secretion in our assays.

In vitro cytolysis assay using flow cytometry

Target tumor cells were seeded at the density of 1×10^5 cells in 500 μL complete media per well in 24-well plates. After 4 h, CAR-T cells (i.e., effector) in T cell culture media were added (500 μL per well) at indicated effector to tumor (E/T) ratios (i.e., 1: 20). After cocultured 4 days (with media replenishment every 2–4 days), and cells mixture were analyzed by flow cytometry. To prepare cell samples for flow cytometry, the remaining supernatant was transferred to 1.5 mL tubes to collect nonadherent cells. Adherent cells were then washed with PBS, lifted by trypsin, and combined with nonadherent cells. Cells were stained with α-human CD3 PE-Cy7 (ThermoFisher, #25-0038-42) at room temperature or 4°C for 15 to 30 min. Then re-

suspend with 15% counting beads (eBioscience, #01-1234-42) in PBS per well and analyzed by flow cytometry. All samples were acquired using a CytoFlex LX (Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo v.10.7 (FlowJo). The numbers of CAR-T cells and target cells were calculated by the following equation: Absolute Count (cells / μL) = (Cell Count × eBeads volume)/ (eBeads Count × Cell Volume × eBeads concentration). Target cells alone were plated at the same cell density to determine the maximal cell number. Tumor cell killing was calculated as 1- (Target cells of CAR-T treated / Target cells alone).

Avidity measurement of HER2-CAR T cells

A549, MCF7, and OE19 cells were seeded at a density of 60-80 x 10^6/mL per chip in a poly-L-lysine precoated z-Movi chips (LUMICKS) and allowed to adhere for 1 hour in serum-free RPMI-1640 medium at 37 °C. Then, the adherent cells were maintained in RPMI-1640 supplemented with 10% FBS, 20 mM HEPES, and 0.1% BSA till 2 hours before the avidity test.

HER2-CAR T cells and untransduced control T cells were harvested, washed, and stained with Celltrace Far Red Cell Proliferation Kit (ThermoFisher, #C34564) according to the manufacturer's protocol.

For each experiment, 5 x 10^6/mL labeled effector T cells were flushed into the chip containing adherent target cells and incubated for 3 minutes at 37 °C to allow immune synapse formation. After incubation, a linear acoustic force ramp (0–1000 pN over 150 seconds) was applied to detach bound T cells on the z-Movi Cell Avidity Analyzer System (LUMICKS). Fluorescence images were recorded continuously during force application to monitor detachment in real time.

Avidity was quantified as the percentage of T cells remaining bound to the target cell monolayer at each force increment. The resulting avidity curve was normalized to the baseline number of attached T cells and plotted against acoustic force to generate force-retention profiles. Statistical comparisons between CAR constructs were based on the percentage of T cells retained at 1000 pN, a force threshold shown to discriminate between low- and high-avidity interactions.

In vivo cytotoxicity

All animal procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Shenzhen Bay Laboratory (IACUC approval no. AECYX202301). For tumor establishment, about 2 × 10^6 SK-OV-3-luc-GFP cells in 150 μL of a 1:1 mixture of PBS and Matrigel (Corning, #356237) were subcutaneously injected into the right flank of 6-8 weeks female NCG mice (GemPharmatech, #T001475). Similarly, about 1 × 10^5 N87-luc-GFP cells or OE19-luc-GFP cells were injected into male NCG mice, and about 5 × 10^4 A549-luc-TROP2-mCherry cells into NCG mice of either sex, using the same PBS:Matrigel formulation.

Three days after tumor inoculation, engraftment was confirmed by IVIS imaging, and mice were randomly assigned to treatment groups using a computer-generated randomization scheme to ensure unbiased allocation. CAR T cells were administered intravenously via tail vein injection in 150 μL of PBS containing 500 U IL2 and 12 mg/mL IVISbrite D-Luciferin Potassium Salt (PerkinElmer, #122799) using a 26G needle. Group sizes were determined based on a priori power calculations using pilot data or published effect sizes, targeting 80% statistical power at a significance level of $\alpha = 0.05$. Additional animals were included

to compensate for potential attrition.

To minimize observer bias, investigators responsible for tumor measurements and bioluminescence analysis were blinded to treatment allocation through the use of coded sample identifiers until completion of data analysis. Tumor dimensions were measured regularly, and volume was calculated using the formula: (major axis) × (minor axis)^2 / 2. Tumor growth and CAR T cell efficacy were monitored by IVIS imaging at designated time points. Luciferase activity was quantified using Living Image Software (Caliper) by measuring photon flux within defined regions of interest encompassing the entire body or thoracic area of each mouse.

Inclusion criteria encompassed 6-8-week-old NCG mice: female mice for SK-OV-3-luc-GFP or A549-luc-TROP2-mCherry tumor models, and male mice for N87-luc-GFP, OE19-luc-GFP or A549-luc-TROP2-mCherry models. Only mice with successful tumor cell inoculation and confirmed tumor engraftment by IVIS imaging at day 3 post-injection were included for downstream analysis. Exclusion criteria included failure of tumor engraftment, injection-related technical errors (e.g., tail vein misinjection of CAR T cells or luciferin), severe illness, or euthanasia prior to study endpoint based on humane criteria. Imaging data were excluded if photon flux signals were insufficient or compromised by technical artifacts.

Attrition during the study was carefully monitored and documented. Of the total animals allocated to each experimental group, any mice were excluded from analysis if tumor engraftment failed to be confirmed by IVIS imaging three days after inoculation, if technical issues such as tail-vein injection failure or misadministration of CAR T cells or luciferin occurred, or if mice became ill or were euthanized early per humane endpoint criteria. Additionally, imaging or photon-flux data points were excluded if quality control thresholds were not met (e.g. insufficient signal, imaging artifacts, or software processing errors).

AlphaFold2 protein structure predictions

ColabFold [5] with default settings was used to generate AlphaFold2 [6] predicted structures of human HER2 complex with the cyclic peptide. Specifically, the amino acid sequences of the cyclic peptide and human HER2 extracellular domain (23-652) were entered, separated by a ':' to specify inter-protein chain breaks for modeling complexes (for example, heterodimers).

Bulk RNA-seq analysis

Primary human T cells were transduced with the different CAR constructs. After 19 days of transduction, 1 × 10^7 CAR T cells were stimulated 18 h with target cells at indicated effector to tumor (E/T) ratios (i.e., 2: 1). About 1,000,000 CAR T cells were sorted for CAR+ using the EasySep™ Release Human PE Positive Selection Kit (Stem cell, #17654). Then, CAR T cells were washed by 1×PBS and the cell precipitation was rapidly frozen in liquid nitrogen for 15 minutes, and the prepared samples were sent to Geneplus technology Co., Ltd. for RNA sequencing.

We performed differential gene expression analysis using the DESeq2 R package on raw read counts, comparing DDMP- and scFv-based CAR T cells and untransduced T (UT) cells. Differentially expressed genes (DEGs) were defined using an adjusted p-value < 0.05 and an absolute log2 fold change greater than the mean plus two standard deviations of all log2 fold changes. The top 100 DEGs ranked by adjusted p-value were visualized as a heatmap using the pheatmap R package. Gene Ontology (GO) and KEGG

pathway enrichment analyses were carried out with the clusterProfiler R package. For gene set enrichment analysis (GSEA), we used the msigdbr R package to obtain the H: Hallmark gene sets and the C3:TFT TFT_LEGACY transcription factor target gene sets.

Code for GO/KEGG/Heatmap

```
library(sysfonts)
library(showtextdb)
library(showtext)
library(tidyverse)
library(clusterProfiler)
library(org.Hs.eg.db)
library(ggsci)
library(ggtree)
library(ggfortify)
library(patchwork)
library(pheatmap)
library(ggplotify)
library(corrplot)
library(DESeq2)
library(readxl)
countData <- read_excel("sample.xlsx", sheet=1, col_names=TRUE)
countData$Symbol.1 <- as.character(countData$Symbol.1)
rownames(countData) <- countData$Symbol.1
countData_without_first_column <- countData[, -1]
rownames(countData_without_first_column) <- rownames(countData)
countData <- countData_without_first_column
group_list <- read_excel("Sample_info_compare.xlsx", sheet=1)
library(DESeq2)
condition = factor(group_list$Group, ordered = F)
coldata<-data.frame(row.names = colnames(countData),condition)
dds<-DESeqDataSetFromMatrix(countData = countData,
                            colData = coldata,
                            design = ~condition)
dds<-DESeq(dds)
resultsNames(dds)
res<-results(dds, contrast = c("condition","sample1","sample2"))
resOrdered<- res[order(res$padj),]
resOrdered=as.data.frame(resOrdered)
head(resOrdered)
logFC_cutoff = with(resOrdered, mean(abs(log2FoldChange)) + 2*sd(abs(log2FoldChange)))
resOrdered$Change=as.factor(ifelse(resOrdered$pvalue < 0.05 &
abs(resOrdered$log2FoldChange)>logFC_cutoff,
                                   ifelse(resOrdered$log2FoldChange > logFC_cutoff, 'up',
'down'),'noDEG'))
table(resOrdered$Change)
```

```
chs_x= resOrdered %>%
  slice_min(pvalue, n=500)
heat_matrix=countData[rownames(chs_x),]
legend_col=data.frame(row.names = group_list$Sample,
                      Group = group_list$Group)
bk = 2
brk<-c(seq(-bk, -0.01, by=0.01), seq(0, bk, by= 0.01))
heat1 = pheatmap(heat_matrix,
                 scale = "row",
                 annotation_col = legend_col,
                 color = c(colorRampPalette(colors = c("dodgerblue4","white"))(length(brk)/2),
                           colorRampPalette(colors = c("white","brown"))(length(brk)/2)),
                 legend_breaks = seq(-bk, bk,1),
                 breaks = brk,
                 treeheight_row = 40,
                 treeheight_col = 40,
                 border_color = NA,
                 cluster_cols = F,
                 show_rownames = F,
                 show_colnames = F,
                 annotation_colors = list(   # 设置分组颜色
                   Group = c("M234" = "#C00E0E", "M345" = "#5A57FF")
                 ))
p7=as.ggplot(heat1)+
  ggtitle("Top 500 DEGs")+
  xlab('Sample')+ ylab('Gene')+
  theme(
    plot.title = element_text(hjust = 0.4),
    plot.background = element_rect(fill = "transparent", color = NA)
  )
p7
ggsave("result/heatmap2.png", plot = p7, width = 5, height = 3, units = "in")
sig_genes <- subset(resOrdered, padj < 0.05 & abs(log2FoldChange) > 1)
gene_list <- rownames(sig_genes)
gene_df <- bitr(gene_list,
                fromType = "SYMBOL",
                toType = "ENTREZID",
                OrgDb = org.Hs.eg.db)
entrez_ids <- gene_df$ENTREZID
go_data<-enrichGO(OrgDb = "org.Hs.eg.db",
                  gene = entrez_ids,
                  keyType       = "ENTREZID",
                  # ont           = "BP",
                  pAdjustMethod = "BH",
                  pvalueCutoff  = 0.05,
                  qvalueCutoff  = 0.2,
```

```
                    ont = "ALL",
                    readable = TRUE)
kegg_data<-enrichKEGG(gene = entrez_ids,
                      keyType = "kegg",
                      organism = "hsa",
                      pvalueCutoff = 1,
                      qvalueCutoff = 1,
                      use_internal_data = F) %>%
  setReadable(., OrgDb="org.Hs.eg.db", keyType='ENTREZID') # %>%          #      ENTREZID 转换为
SYMBOL
dotplot(kegg_data,showCategory=20, title="Top enriched terms")+
  theme_light()
ggsave("result/KEGG.png",    width = 6, height = 6, units = "in")
```

Code for GSEA

```
exprset1 <- GeneExpression
library(org.Hs.eg.db)
exprset1 <- as.data.frame(exprset1)
setequal(Sample_info$Sample,colnames(exprset1))
summary(as.numeric(unlist(exprset1)))
table(is.na(exprset1))
table(exprset1<0)
hist(unlist(exprset1))
hist(unlist(log2(exprset1)))
zero_xx=apply(exprset1,1,function(xx){sum(xx==0)})
head(zero_xx)
table(zero_xx<5)
low_xx1=apply(exprset1,1,function(xx){sum(xx)})
head(low_xx1)
table(low_xx1>10)
low_xx2=apply(exprset1,1,function(xx){mean(xx)})
head(low_xx2)
table(low_xx2>3)
exprset2=exprset1[zero_xx<5 & low_xx1>10 & low_xx2>3, ]
dim(exprset2)
boxplot(exprset2)
boxplot(log2(exprset2))
library(ggsci)
library(ggfortify)
library(DESeq2)
exprset2 <- round(exprset2)
condition = factor(Sample_info$Group, ordered = F)
coldata<-data.frame(row.names = colnames(exprset2),condition)
dds<-DESeqDataSetFromMatrix(countData = exprset2,
                            colData = coldata,
```

```
                                design = ~condition)
dds<-DESeq(dds)
resultsNames(dds)
res_DDMP_vs_scFv <- results(dds, contrast = c("condition", "DDMP", "scFv"))
resOrdered_DDMP_vs_scFv <- res_DDMP_vs_scFv[order(res_DDMP_vs_scFv$padj),]
resOrdered_DDMP_vs_scFv <- as.data.frame(resOrdered_DDMP_vs_scFv)
resOrdered<-resOrdered_DDMP_vs_scFv
logFC_cutoff = with(resOrdered, mean(abs(log2FoldChange)) + 2*sd(abs(log2FoldChange)))
resOrdered$Change=as.factor(ifelse(resOrdered$pvalue < 0.05 &
abs(resOrdered$log2FoldChange)>logFC_cutoff,
                                   ifelse(resOrdered$log2FoldChange > logFC_cutoff, 'up',
'down'),'noDEG'))
table(resOrdered$Change)
top_upregulated<-resOrdered %>%
  filter(log2FoldChange > 0) %>%
  arrange(desc(log2FoldChange)) %>%
  head(10)
top_downregulated <- resOrdered %>%
  filter(log2FoldChange < 0) %>%
  arrange(log2FoldChange) %>%
  head(10)
top_genes <- bind_rows(top_upregulated, top_downregulated) %>%
  mutate(label = rownames(.))
top_genes <- top_genes %>%
  filter(!is.na(log2FoldChange) & !is.na(pvalue))
summary(resOrdered)
resOrdered_clean <- resOrdered %>%
  filter(!is.na(log2FoldChange) & !is.na(pvalue))
library(clusterProfiler)
symbol_to_entrez <- bitr(rownames(resOrdered_clean), OrgDb = "org.Hs.eg.db", drop = F,
                         fromType = "SYMBOL", toType = "ENTREZID")
symbol_to_entrez <- symbol_to_entrez %>%
  distinct(SYMBOL, .keep_all = TRUE)
degs_anno <- resOrdered_clean %>%
  mutate(symbol = row.names(.)) %>%
  left_join(symbol_to_entrez, by = c("symbol" = "SYMBOL"))
summary(degs_anno$baseMean)
table(is.na(degs_anno$pvalue))
table(degs_anno$pvalue==0)
degs_final = degs_anno %>%
  filter(!is.na(symbol)) %>%
  group_by(symbol) %>%
  mutate(mean = mean(baseMean)) %>%
  group_by() %>%
  filter(baseMean>=mean|baseMean>=mean(baseMean)) %>%
  arrange(pvalue) %>%
```

```
  distinct(symbol, .keep_all = T)
exprset3=exprset2[degs_final$symbol,]
identical(degs_final$ENTREZID,rownames(exprset3))
rownames(exprset3)=degs_final$symbol
summary(degs_final$baseMean)
summary(abs(degs_final$log2FoldChange))
qu1=quantile(degs_final$baseMean, probs=0.25)
fc1=mean(abs(degs_final$log2FoldChange))
qu1;fc1
degs_top=degs_final %>%
  filter(baseMean > qu1 & abs(log2FoldChange) > fc1 & pvalue < 0.05) %>%
  arrange(pvalue) %>%
  pull(symbol) %>%
  bitr(., fromType = "SYMBOL", toType = c("ENTREZID"),
       OrgDb = org.Hs.eg.db) %>%
  distinct(SYMBOL, .keep_all = TRUE) %>%
  slice_head(n = 13735)
btr_gsea_order = bitr(degs_final$symbol,
                      fromType = "SYMBOL", toType = c("ENTREZID"),
                      OrgDb = org.Hs.eg.db) %>%
  mutate(log2FC=degs_final$log2FoldChange[match(SYMBOL,degs_final$symbol)]) %>%
  arrange(desc(log2FC))
table(is.na(btr_gsea_order$ENTREZID))
table(is.na(btr_gsea_order$log2FC))
head(btr_gsea_order) ; tail(btr_gsea_order)
gsea_list=setNames(btr_gsea_order$log2FC,btr_gsea_order$ENTREZID)
head(gsea_list); tail(gsea_list)
library(msigdbr)
hallmark_gene_sets <- msigdbr(species = "Homo sapiens", category = "H")
tft_genesets <- msigdbr(species = "Homo sapiens",
                        category = "C3",
                        subcategory = "TFT:TFT_LEGACY")[, c("gs_name", "entrez_gene")]
combined_gene_sets <- rbind(hallmark_gene_sets[, c("gs_name", "entrez_gene")],
                            tft_genesets)
gsea_combined_res <- GSEA(
  gsea_list,
  TERM2GENE = combined_gene_sets,
  seed=42
)
gseaplot2(gsea_res, geneSetID = "HALLMARK_KRAS_SIGNALING_UP", title = "KRAS signaling up")
ggsave("GSEA_KRAS_DDMP_scFv.png", width = 8, height = 6, dpi = 300)
gseaplot2(gsea_res, geneSetID = "NFKB_C", title = "NFKB C")
ggsave("GSEA_NFKB_C_DDMP_scFv.png", width = 8, height = 6, dpi = 300)
gseaplot2(gsea_res, geneSetID = "NFAT_Q4_01", title = "NFAT Q4 01")
ggsave("GSEA_NFAT_Q4_01_DDMP_scFv.png", width = 8, height = 6, dpi = 300)
gseaplot2(gsea_res, geneSetID = "HALLMARK_TNFA_SIGNALING_VIA_NFKB", title = "TNFα signaling
```

```
via NFκB")
ggsave("GSEA_HALLMARK_TNFA_SIGNALING_VIA_NFKB_DDMP_scFv.png", width = 8, height = 6, dpi = 300)
```

References

(1) Hernandez-Lopez, R. A.; Yu, W.; Cabral, K. A.; Creasey, O. A.; Pazmino, M. D. L.; Tonai, Y.; De Guzman, A.; Makela, A.; Saksela, K.; Gartner, Z. J.; Lim, W. A. T cell circuits that sense antigen density with an ultrasensitive threshold. *Science* **2021,** *371* (6534), 1166–1171.
(2) Silman, I.; Zwaagstra, J. C.; Sulea, T.; Baardsnes, J.; Radinovic, S.; Cepero-Donates, Y.; Robert, A.; O'Connor-McCourt, M. D.; Tikhomirov, I. A.; Jaramillo, M. L. Binding and functional profiling of antibody mutants guides selection of optimal candidates as antibody drug conjugates. *Plos One* **2019,** *14* (12), e0226593.
(3) Feucht, J.; Sun, J.; Eyquem, J.; Ho, Y. J.; Zhao, Z.; Leibold, J.; Dobrin, A.; Cabriolu, A.; Hamieh, M.; Sadelain, M. Calibration of CAR activation potential directs alternative T cell fates and therapeutic potency. *Nat. Med.* **2019,** *25* (1), 82–88.
(4) Goldenberg, D. M.; Cardillo, T. M.; Govindan, S. V.; Rossi, E. A.; Sharkey, R. M. Trop-2 is a novel target for solid cancer therapy with sacituzumab govitecan (IMMU-132), an antibody-drug conjugate (ADC). *Oncotarget* **2015,** *6* (26), 22496–512.
(5) Mirdita, M.; Schütze, K.; Moriwaki, Y.; Heo, L.; Ovchinnikov, S.; Steinegger, M. ColabFold: making protein folding accessible to all. *Nat. Methods* **2022,** *19* (6), 679–682.
(6) Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Zídek, A.; Potapenko, A.; Bridgland, A.; Meyer, C.; Kohl, S. A. A.; Ballard, A. J.; Cowie, A.; Romera-Paredes, B.; Nikolov, S.; Jain, R.; Adler, J.; Back, T.; Petersen, S.; Reiman, D.; Clancy, E.; Zielinski, M.; Steinegger, M.; Pacholska, M.; Berghammer, T.; Bodenstein, S.; Silver, D.; Vinyals, O.; Senior, A. W.; Kavukcuoglu, K.; Kohli, P.; Hassabis, D. Highly accurate protein structure prediction with AlphaFold. *Nature* **2021,** *596* (7873), 583–589.