Construction and functional evaluation of cyclic peptide-based CAR T cells in tumor models

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Abstract

Cyclic peptide-based chimeric antigen receptor (CAR) T cells provide a compact, engineerable recognition modality that can mediate antigen-dependent cytotoxicity while exhibiting an attenuated cytokine secretion profile, supporting the development of potentially safer immunotherapies for solid tumors. Here, we present a comprehensive workflow spanning CAR construct design and generation through in vitro and in vivo functional evaluation. The protocol includes the generation of Jurkat NFAT reporter cell lines and luciferase-expressing tumor target lines, which are widely used in different assays. Together, these standardized readouts enable rigorous, objective comparison of CAR T cell efficacy and safety across tumor models.

Key features

- Modular pipeline to compare cyclic peptide-based CAR designs across in vitro and in vivo tumor models.
- Complementary functional readouts: NFAT activation, luminescence killing, flow cytometry cytolysis, and xenograft IVIS.

Keywords: chimeric antigen receptor, autologous CAR T, disulfide-directed multicyclic peptide, DDMP, NFAT reporter, cyclic peptide, HER2

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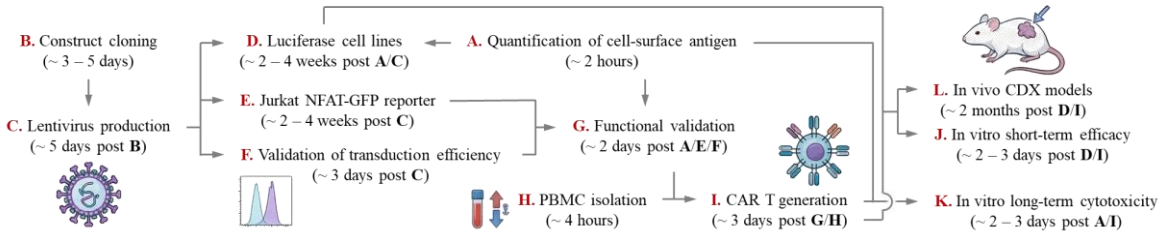
Graphical overview



Experimental workflow. Schematic illustration of the experimental pipeline described in this protocol, from construct design to functional evaluation in vitro and in vivo. Arrows indicate the logical progression of the workflow.

Background

Standardized workflows for CAR T-cell manufacturing and functional testing are widely used to benchmark receptor designs across diverse tumor models and experimental readouts. Building on reported procedures for T-cell isolation/activation, viral gene transfer, and downstream in vitro/in vivo assays [1-6], this protocol provides a beginner-friendly, end-to-end pipeline for preclinical evaluation of autologous CAR T therapy. The workflow includes (i) quantitative profiling of target-antigen density on tumor cells, (ii) generation of reporter cell lines for functional readouts, and (iii) multi-assay validation spanning reporter activation, cytolysis/cytokine secretion, and xenograft efficacy. A key principle for comparing efficacy between different constructs is to normalize by the number of CAR-positive T cells, enabling functional comparisons that are independent of transduction efficiency.

This protocol is exemplified using disulfide-directed multicyclic peptides (DDMPs) as compact, engineerable antigen-recognition modules. DDMPs are disulfide-rich multicyclic peptides whose oxidative folding is guided by disulfide-directing motifs (e.g., biscysteine motifs CXC, CPPC and CPXXC) [7]. In conventional disulfide-rich peptides, a sequence containing *2n* cysteine residues can theoretically form $\frac{(2n)!}{2^n\, n!}$ distinct isomers (e.g., 105 for $n = 4$), yielding complex mixtures. In contrast, incorporation of disulfide-directing motifs can drive highly efficient folding to a dominant product, which substantially simplifies isolation and characterization. Because DDMP folding is largely determined by these motifs, DDMP scaffolds also exhibit greater tolerance to extensive sequence manipulation, enabling highly diversified display libraries and systematic ligand discovery against a broad range of cell-surface receptors, including tumor-associated antigens and immune receptors [8-16]. Thus, the integrated workflow described here is well suited for iterative optimization and head-to-head benchmarking of DDMP-based CAR designs, linking molecular construction to rigorous functional evaluation across complementary in vitro and in vivo tumor models [17, 18].

Materials and reagents

Biological materials

1. HEK293T cells (Cell Bank of the Chinese Academy of Sciences, cat. no. GNHu17)
2. A549 cells (Cell Bank of the Chinese Academy of Sciences, cat. no. SCSP-503)
3. SK-OV-3 cells (Cell Bank of the Chinese Academy of Sciences, cat. no. SCSP-5214)
4. N87 cells (Procell, cat. no. CL-0169)
5. OE19 cells (Procell, cat. no. CL-0754)
6. Jurkat E6-1 cells (Cell Bank of the Chinese Academy of Sciences, cat. no. SCSP-513)
7. Human peripheral blood (MileCell Bio; healthy donors; origin as supplied)
8. NCG mice, 6–8 weeks (GemPharmatech, cat. no. T001475)

Reagents

1. DMEM (Gibco, cat. no. 21063029)
2. RPMI 1640 (Gibco, cat. no. 11875093)
3. Click's medium (Sigma, cat. no. C5572)
4. Opti-MEM (Gibco, cat. no. 31985-070)
5. FBS (TransGen Biotech, cat. no. FS401-02)
6. FBS (Gibco, cat. no. 10099141)
7. FBS (Sigma, cat. no. F8687)
8. Penicillin/streptomycin (Gibco, cat. no. 15140122)
9. Lentiviral packaging plasmid: psPAX2 (Addgene, cat. no. 12260)
10. Lentiviral envelope plasmid: pMD2.G (Addgene, cat. no. 12259)
11. Lentiviral transfer plasmid backbone: pCDH (Addgene, cat. no. 72265/72266)
12. Lentiviral transfer plasmid for generating luciferase-expressing stable Luc-GFP cell lines: pCCLc-MNDU3-Luciferase-PGK-EGFP-WPRE (Addgene, cat. no. 89608)
13. Lentiviral transfer plasmid for generating NFAT-GFP reporter cell line: FLX1.8NFATGFPpd2HS4 (Addgene, cat. no. 169096)
14. PEI (Polysciences, cat. no. 24765-1)
15. Ficoll (Cytiva, cat. no. 17544203)
16. CryoStor CS10 (Stemcell, cat. no. 100-1061)
17. Dynabeads Human T-Expander CD3/CD28 (Invitrogen, cat. no. 11141D)
18. Recombinant human IL-2 (Proteintech, cat. no. HZ-1015)
19. Retronectin (Takara, cat. no. T100A)
20. Polybrene (10 mg/mL; Solarbio, cat. no. H8761-5), store at –20 °C. Keep one tube as working solution at 4 °C.
21. Violet 450 (Tonbo, cat. no. 13-0863)
22. PE anti-HER2 (BioLegend, cat. no. 324406)
23. FITC anti-CD3 (4A Biotech, cat. no. FHF003-01-100)
24. PE anti-CD3 (ThermoFisher, cat. no. 25-0038-42)
25. APC anti-CD3 (BD, cat. no. 557832)
26. PE anti-V5 tag antibody (eBioscience, cat. no. 12-6796-42)
27. Anti-TROP2 antibody (Invitrogen, cat. no. 12-6024-42)
28. Cell stimulation cocktail (Invitrogen, cat. no. 00-4970)
29. Counting beads (eBioscience, cat. no. 01-1234-42)
30. D-luciferin sodium salt (MedChemExpress, cat. no. HY-12591)
31. IVISbrite D-Luciferin Potassium Salt (PerkinElmer, cat. no. 122799)
32. ELISA kits for IL-2 (Invitrogen, cat. no. 88-7025-88), TNF-α (Invitrogen, cat. no. 88-7346-77), IFN-γ (Invitrogen, cat. no. 88-7316-86), and GM-CSF (Invitrogen, cat. no. 88-8337-88) detection
33. Matrigel (Corning, cat. no. 356237)
34. PBS (Goonie Bio, cat. no. 251222)
35. Trypsin-EDTA (Gibco, cat. no. 25200072)

Solutions

1. 10% FBS in DMEM: 89% DMEM + 10% FBS (TransGen) + 1% penicillin/streptomycin
2. 10% FBS in RPMI 1640: 89% RPMI 1640 + 10% FBS (TransGen) + 1% penicillin/streptomycin
3. 20% FBS in RPMI 1640: 79% RPMI 1640 + 20% FBS (Gibco) + 1% penicillin/streptomycin. This is used for culturing Jurkat cells.
4. T cell culture medium: 44.5% RPMI 1640 + 44.5% Click's medium + 10% FBS (Sigma) + 1% penicillin/streptomycin
5. 1 μg/μL PEI solution in sterile water
6. 10 mM D-luciferin: Dissolve 4.53 mg D-luciferin in ddH_2O to the final volume of 1.5 mL. Sterile filtration with 0.22 μm filter, aliquot into 300 μL per tube, and store at –80 °C.
7. 2% BSA (w/v): Dissolve 10 g BSA in 1×PBS to the final volume of 500 mL. Sterile filtration with 0.22 μm filter. Keep at 4 °C.

Laboratory supplies

1. 1.5 mL microcentrifuge tube (Axygen, cat. no. MCT-200-C)
2. 15 mL centrifuge tube (LabSelect, cat. no. CT-002-15A)
3. 50 mL conical tubes (LabSelect, cat. no. CT-002-50A)
4. Tube for flow cytometry applications (Falcon, cat. no. 352235)
5. 300-mesh cell strainer (Solarbio, cat. no. YA0950)
6. Cryovials (ThermoFisher, cat. no. 377267)
7. Mr. Frosty freezing container (Stemcell, cat. no. 100-1061)
8. 96-well cell culture plate (LabSelect, cat. no. 11510)
9. 96-well plate, white bottom (Biosharp, cat. no. BS-MP-96W-CL)
10. 12-well plate, non-TC treated (LabSelect, cat. no. 11220)
11. 24-well plate, non-TC treated (LabSelect, cat. no. 11320)
12. 24-well cell culture plates (LabSelect, cat. no. 11310)
13. 0.45 μm PES filter (Biosharp, cat. no. BS-PES-45)

Equipment

1. Class II biosafety cabinet (Haier, HR40-IIA2)
2. CO_2 incubator (Haier, HCB-168)
3. 3D rotating mixer (Crystal, TR-02U)
4. Microcentrifuge (ThermoFisher, Pico 21)
5. High-speed centrifuge with a swinging-bucket rotor for 50 mL tubes (Eppendorf, 5910Ri)
6. Cell counter (DeNovix, CellDrop BF)
7. Flow cytometer (Beckman Coulter, CytoFLEX LX)
8. Cell sorter (Beckman Coulter, CytoFLEX SRT)
9. Microplate reader for bioluminescence detection (BioTek, Synergy Neo2)
10. In vivo imaging system (PerkinElmer, IVIS Spectrum)

Software and datasets

1. FlowJo (FlowJo, version 10.7)
2. Living Image (PerkinElmer, version 4.7.4)

Procedure

A. Quantification of cell-surface antigen expression

1. For each sample, prepare the staining solution by mixing 98 μL DMEM, 1 μL PE anti-HER2 antibody (1:100 dilution as per manufacturer's recommendation), and 1 μL Violet 450 viability dye (1:100 dilution as per manufacturer's recommendation). For example, to measure cell-surface HER2 on A549, NCI-N87, OE19, and SK-OV-3 in parallel, prepare 400 μL of staining solution in total.
   Note: HER2 (target antigen) is used here as an example; the same workflow applies to other cell-surface antigens by substituting the appropriate antibody.
2. Harvest 1.5×10^5 target cells into a 1.5-mL microcentrifuge tube, centrifuge at 140 rcf for 5 min at room temperature to pellet the cells and discard supernatant, then resuspend the pellet in 100 μL staining solution by gentle pipetting. Mix gently and incubate 15–30 min at room temperature in the dark.
3. Add 1 mL DMEM and centrifuge at 400 rcf for 5 min at room temperature. Discard the supernatant. Resuspend the cell pellet in 200 μL DMEM by gentle pipetting.
4. To remove cell clumps, place a small piece of 300-mesh cell strainer over a new 1.5 mL microcentrifuge tube and gently pass the suspension through the mesh. Collect the flow-through (typically ~150 μL) for acquisition.
5. Acquire samples on a flow cytometer (Violet 450, Ex 405 nm/Em 425-475 nm; PE, Ex 561 nm/Em 585 nm). Quantify

HER2 expression as mean fluorescence intensity (MFI) using unstained cells to set the gates. See the Data Analysis section for an example.

Note: For comparing HER2 levels across different cell lines (e.g., A549, OE19, and SK-OV-3), keep the PMT voltages and acquisition settings identical across samples.

B. Molecular cloning of reporter (NFAT-GFP, Luc) and CAR constructs

1. Choose a suitable lentiviral transfer plasmid backbone (e.g., pCDH) and clone the gene of interest into the vector. In our workflow, inserts for Luc-GFP, NFAT-GFP, and CAR constructs are obtained by custom gene synthesis. Alternatively, transfer plasmids for Luc-GFP and NFAT-GFP can be sourced directly from Addgene.
2. As an example, the HER2(DDMP)-CAR coding sequence encodes (from N- to C-terminus) a signal peptide, HER2(DDMP), a V5 tag (for quantifying CAR expression), a CD8 hinge, a CD28 transmembrane domain, a CD28 costimulatory domain, and the CD3ζ signaling domain. For cloning convenience, restriction sites are embedded at defined junctions and are indicated by underlining in the corresponding amino acid sequence (GS: BamHI, AAA: NotI, AS: NheI, GQKS: BoxI).

   MEFGLSWLFLVAILKGVQCGSCPWFCIYPCKVEPRCSEVYAEQCPQTCGSAAAGKPIPNPLLGLDSTASAKPT TTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDGQKSFWVLVVVGGVLACYSLLVTVAFIIFW VRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRR EEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD TYDALHMQALPPRDI*

   Note: Alternative sequences can also be used for individual CAR components. Examples include a signal peptide from human CD8 (MALPVTALLLPLALLLHAARP) or mouse immunoglobulin κ light chain (METDTLLLWVLLLWVPGSTG D); a hinge from CD28 (IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKP) or IgG-Fc (ESKYGPPCPPCPAP EFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFQSTYRVVSVLTV LHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESN GQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK); a transmembrane domain from CD8 (IYIWAPLAGTCGVLLLSLVIT); and a costimulatory domain from 4-1BB (KRGRKKLLYIFKQPFMR PVQTTQEEDGCSCRFPEEEEGGCEL).
3. Confirm each construct by Sanger sequencing across the full insert and all junctions. Archive plasmid maps, annotated sequences, and primer lists for ordering and reproducibility.

C. Preparation of lentivirus for transduction

1. Plate 6.5 × 10^6 HEK293T cells in 10 mL 10% FBS in DMEM per 10 cm dish.

   Note: The cells must be mycoplasma free so that the resulting lentivirus suspension would not contain mycoplasma. We concentrate the lentivirus from a 10 mL dish into 500 µL and use for transducing 5 × 10^5 primary T cells. For difficult to transduce constructs, we do a second transduction using 250 µL lentivirus, so that for 5 × 10^5 T cells, we need virus from 1.5 dishes. For new constructs, we initially produce virus from two dishes for validation. After validation, the production scale is adjusted according to experimental needs.
2. About 18 h after plating, prepare the transfection mix for each 10 cm dish as follows. In tube A, mix 500 µL Opti-MEM with 60 µL PEI by pipetting up and down. In tube B, mix 500 µL Opti-MEM with 12 µg transfer plasmid, 7.2 µg packaging plasmid psPAX2, and 6 µg envelope plasmid pMD2.G by pipetting up and down. Add the contents of tube A to tube B, mix thoroughly by pipetting up and down, and incubate at room temperature for 15 min.

   Note: For validation of a new construct, prepare the transfection mix for two 10-cm dishes by doubling all components (tube A: 1 mL Opti-MEM + 120 µL PEI; tube B: 1 mL Opti-MEM + 24 µg transfer plasmid + 14.4 µg psPAX2 packaging plasmid + 12 µg pMD2.G envelope plasmid).
3. Add the transfection mix dropwise across the dish, distributing it as evenly as possible over multiple locations.

   Note: If using a master mix for multiple dishes, add the same volume to each dish.
4. After 6-8 h, replace the medium in each dish with 10 mL of pre-warmed 10% FBS in DMEM.
5. After an additional 72 h, collect the virus-containing supernatant into a 15 mL or 50 mL centrifuge tube. Centrifuge at 860 rcf for 10 min at 4 °C to remove cell debris, then filter the clarified supernatant through a 0.45 µm PES filter. Concentrate lentiviral particles by centrifugation at 30,910 rcf for 2 h at 4 °C. Resuspend the viral pellet from each dish in 500 µL RPMI 1640 and store at −80 °C.

D. Generation of stably expressed luciferase target cell lines

1. Plate 8×10^4 SK-OV-3 cells per well in a 24-well plate. Prepare 12 wells.
   Note: SK-OV-3 is used here as an example. The same workflow can be adapted to other cells.
2. About 18 h after plating, prepare a transduction mix by adding 12.5 µL of 10 mg/mL polybrene to 10.6 mL 10% FBS in DMEM. Add 850 µL of this transduction mix to each well, so that each well contains 849 µL 10% FBS in DMEM and 1 µL of 10 mg/mL polybrene.
3. Test a virus dose range by adding 150, 100, 50, 25, 10, or 5 µL of concentrated Luc-GFP lentivirus to each well. Gently pipette up and down to mix evenly. Use duplicate wells for each condition. Top up each well with 10% FBS in DMEM to a final volume of 1 mL, yielding a final polybrene concentration of 10 µg/mL.
4. Incubate overnight. The next day, carefully remove 0.5 mL of transduction medium from each well and replace it with 0.5 mL of pre-warmed 10% FBS in DMEM, maintaining a total volume of 1 mL per well.
5. At about 48 h post-transduction, harvest cells from one well per condition for flow cytometry. Record the percentage of live cells (viability dye), the percentage of GFP-positive cells, and the MFI of GFP-positive population.
6. Select the condition that uses the lowest virus input while maintaining high viability and robust GFP intensity. Expand cells from the remaining replicate well of the selected condition into a T25 flask, then scale to a T75 flask as cells proliferate.
7. When the T75 reaches near confluence, harvest cells and resuspend in ~300 µL DMEM for cell sorting.
8. Sort the top 5% GFP-high population as single cells into 96-well plates. Typically, sort at least three 96-well plates. Include one “focus/control” well per plate containing ~100 cells, and sort single cells into all remaining wells.
   Note: The 100-cell well reliably grows and can be used to locate/focus the plate under a microscope.
9. As single-cell clones expand, transfer each clone to a fresh 96-well plate. Once confluent, transfer to a 24-well plate. If many clones are available at this stage, prioritize the top six clones with the highest GFP fluorescence (assessed by fluorescence microscopy) for expansion in a 6-well plate.
10. When cells in the 6-well plate reach confluence, validate luciferase signal linearity. For each clone, seed 1,000, 5,000, or 10,000 cells per well (in triplicate) into a white-bottom 96-well plate.
11. After ~18 h, aspirate the medium. Dilute 10 mM luciferin stock in PBS to 1 mM. Add 100 µL of 1 mM luciferin to each well and incubate 1–2 min at room temperature.
12. Measure bioluminescence using a plate reader. Confirm that luminescence scales linearly with cell number. Retain up to three clones with the highest luminescence signals (and good growth characteristics).
13. Expand the selected clones for cryopreservation and subsequent experiments.

E. Generation of Jurkat NFAT-GFP reporter cells

1. Plate 3.5×10^5 Jurkat E6-1 cells per well in a non-TC treated 12-well plate. Prepare 3 wells. For each well, resuspend the cells in 0.5 mL of 20% FBS in RPMI 1640 containing 2.5 µL of 10 mg/mL polybrene.
   Note: Prepare a master mix of 20% FBS in RPMI 1640 containing polybrene at the required concentration, then aliquot 0.5 mL per well to resuspend 3.5 × 10^5 Jurkat E6-1 cells.
2. Add unconcentrated NFAT-GFP lentivirus to each well at different doses (e.g., 2.0, 1.5, or 0.5 mL per well; as a reference, an unconcentrated virus harvest from a 10 cm dish is typically 10 mL total). Top up each well with 20% FBS in RPMI 1640 to a final volume of 2.5 mL, yielding a final polybrene concentration of 10 µg/mL.
3. At about 48 h post-transduction, harvest cells from one well per condition for flow cytometry. Record the percentage of live cells (viability dye), the percentage of GFP-positive cells, and the MFI of GFP-positive population.
4. Sort weakly GFP-positive cells into 96-well plates for single-cell cloning. Typically, sort at least three 96-well plates. Include one “focus/control” well per plate containing ~100 cells, and sort single cells into all remaining wells.
   Note: The 100-cell well reliably grows and can be used to locate/focus the plate under a microscope.
5. As single-cell clones expand, transfer each clone to a fresh 96-well plate. Once confluent, transfer to a 24-well plate for further expansion.
6. When clones in the 24-well plate reach near confluence, split each clone into three portions:
   a) One portion for continued culture/backup
   b) Two portions for functional testing (stimulated vs unstimulated)
7. For functional induction, add 1 µL of 500× cell stimulation cocktail to 499 µL of 20% FBS in RPMI 1640. Incubate for 5–18 h at 37 °C.
8. Analyze stimulated and unstimulated samples by flow cytometry. Select clones that exhibit strong GFP induction upon stimulation but minimal basal GFP in the unstimulated condition (i.e., the highest signal-to-background ratio). Expand the selected clones for cryopreservation and subsequent experiments.

F. Validation of lentiviral transduction efficiency using Jurkat cells

1. Plate 1.8×10^5 Jurkat E6-1 cells per well in a non-TC treated 24-well plate. Prepare 1 well per lentiviral construct for validation. For each well, resuspend the cells in 0.8 mL of 20% FBS in RPMI 1640 containing 1 µL of 10 mg/mL polybrene.
   Note: Prepare a master mix of 20% FBS in RPMI 1640 containing polybrene at the required concentration, then aliquot 0.8 mL per well to resuspend 1.8 × 10^5 Jurkat E6-1 cells.
2. Add 200 µL of concentrated lentivirus to each well.
   Note: A concentrated virus harvest from a 10 cm dish is typically 500 µL total.
3. Incubate overnight. The next day, carefully remove 0.5 mL medium from each well using a pipette (avoid aspirating cells), and replace with 0.5 mL of pre-warmed 20% FBS in RPMI 1640, to restore a final volume of 1.0 mL per well.
4. At approximately 48 h post-transduction, collect cells for flow cytometry analysis.
5. Transfer each well's suspension to a 1.5 mL microcentrifuge tube and centrifuge at 400 rcf for 5 min at room temperature. Discard the supernatant and resuspend the pellet in 100 µL staining solution. Mix gently and incubate for 15–30 min at room temperature in the dark. For each sample, prepare the staining solution by mixing 98 µL DMEM, 1 µL PE anti-V5 tag antibody (1:100 dilution as per manufacturer's recommendation), and 1 µL Violet 450 viability dye (1:100 dilution as per manufacturer's recommendation). If staining multiple samples, prepare a master mix and aliquot 100 µL per sample.
   Note: Anti-V5 is used because the CAR constructs include an extracellular V5 tag. If a different tag or reporter is used, substitute the appropriate detection reagent.
6. Add 1 mL DMEM and centrifuge at 400 rcf for 5 min at room temperature. Discard the supernatant and resuspend the pellet in 200 µL DMEM by gentle pipetting (or gentle flicking of the tube).
7. To remove clumps, place a small piece of 300-mesh cell strainer over a new 1.5 mL microcentrifuge tube and gently pass the suspension through the mesh. Collect the flow-through (typically ~150 µL) for acquisition.
8. Acquire on a flow cytometer. As a benchmark for batch quality, ~100% V5-positive cells are typically achieved under these conditions. If V5 positivity is lower, prepare a new viral batch or troubleshoot the transduction conditions. Ideally, the V5 signal should be ~2 orders of magnitude above the background signal from untransduced Jurkat cells stained with anti-V5 under identical settings.

G. Validation of CAR function using Jurkat NFAT-GFP reporter cells

1. Plate Jurkat NFAT-GFP cells in a non-TC treated 24-well plate. Prepare a master cell suspension in pre-warmed 20% FBS in RPMI 1640 and polybrene such that each well receives 1.8×10^5 cells in a total volume of 900 µL (899 µL medium + 1 µL of 10 mg/mL polybrene). Set up two wells for each construct.
2. Add 100 µL of concentrated lentivirus to each well.
   Note: A typical concentrated harvest from one 10 cm dish yields ~500 µL in total.
3. After 18 h, collect cells of the same construct into a 15 mL centrifuge tube. Centrifuge at 400 rcf for 5 min at room temperature. Discard the supernatant. Resuspend in 1 mL 20% FBS in RPMI 1640. Measure the cell density.
4. Seed 1.5×10^5 SK-OV-3 cells in 500 µL 10% FBS in DMEM per well in TC-treated 24-well plate. Then add 1.5×10^5 transduced Jurkat NFAT-GFP in 500 µL 20% FBS in RPMI 1640 to the same well (final volume 1 mL; E:T = 1:1).
   Note: SK-OV-3 cells, which express high levels of HER2, are used to validate HER2(DDMP)-CAR function. For CARs targeting other antigens, use an appropriate antigen-high target cell line. Because activation of Jurkat NFAT-GFP cells induces GFP expression, we avoid using SK-OV-3-Luc-GFP in this assay to minimize potential fluorescence interference.
5. After 24 h, gently pipette the culture up and down to resuspend and detach Jurkat cells from the bottom of the well. Transfer the supernatant to a 1.5 mL microcentrifuge tube, centrifuge at 400 rcf for 5 min at room temperature, and discard the supernatant.
6. While centrifuging (Step 5), prepare a staining solution containing PE anti-V5 tag antibody (final 1:100) and Violet 450 viability dye (final 1:100) in DMEM. Prepare 100 µL per sample. For example, for 10 samples, prepare at least 1 mL of staining solution.
7. Resuspend each cell pellet in 100 µL staining solution, mix gently, and incubate for 15–30 min at room temperature in the dark.
8. Add 1 mL DMEM and centrifuge at 400 rcf for 5 min at room temperature. Discard the supernatant and resuspend the pellet in 200 µL DMEM by gentle pipetting (or gentle flicking of the tube).
9. To remove clumps, place a small piece of 300-mesh cell strainer over a new 1.5 mL microcentrifuge tube and gently pass the suspension through the mesh. Collect the flow-through (typically ~150 µL) for acquisition.
10. Acquire samples on a flow cytometer. If the sample contains both Jurkat and adherent target cells (e.g., SK-OV-3), use GFP to distinguish populations when applicable, and gate on Jurkat NFAT-GFP cells for analysis. Quantify the fraction

of V5 (PE) and GFP double-positive Jurkat cells after co-culture with target cells. A functional CAR should produce a clear increase in the V5 and GFP double-positive population compared to the no-target control. If the no-target control already shows a high double-positive fraction (often due to tonic signaling/self-activation), repeat the transduction using a lower virus input.

H. PBMC isolation and assessment of CD3+ cells in PBMC

1. Transfer peripheral blood (ca. 17 mL of red solution containing 1×10^9 white blood cells) from the collecting bag into a 50 mL centrifuge tube.
2. Rinse the bag with sterile, room-temperature PBS to recover residual blood, and combine the rinse with the transferred blood. Adjust the volume to achieve approximately a 1:1 (v/v) ratio of blood:PBS. Mix gently by inversion or slow pipetting to avoid foaming.
3. Aliquot the diluted blood into six 50 mL centrifuge tubes (typically 5–6 mL per tube).
4. Bring each tube to 25 mL with PBS and mix gently.
5. In a separate set of six 50 mL centrifuge tubes, add 25 mL Ficoll per tube. Carefully layer 25 mL of the diluted blood (from Step 4) on top of the Ficoll.
   Critical: Add the first ~5 mL dropwise along the tube wall (e.g., using a P1000 tip), then slowly add the remaining volume using a serological pipette, maintaining a clear interface.
6. Centrifuge at 400 rcf for 30 min at 18 °C with low acceleration (speed up = 1) and no brake (speed down = 0).
7. After centrifugation, the sample should separate into three layers: plasma (top, light yellow), a buffy coat/PBMC layer at the interface (middle, whitish), and erythrocytes/granulocytes (bottom, red). Using a 10 mL pipette, remove most of the plasma layer. Using a 2.5 mL pipette, carefully collect the PBMC layer and transfer it to a new 50 mL tube, avoiding carryover of the Ficoll and the red cell layer.
6. Fill each PBMC tube to 50 mL with PBS and centrifuge at 550 rcf for 20 min at 18 °C (speed up = 5, speed down = 4). Discard the supernatant and repeat the PBS wash for a total of three washes. Before the final wash, take an aliquot for cell counting. To quantify the T cell percentage, stain 2×10^5 cells with APC anti-CD3 and determine the CD3-positive fraction by flow cytometry. Values vary across donors; as a general guide, the lymphocyte gate (defined by FSC/SSC) often accounts for ~30–70% of events, and CD3+ T cells typically comprise ~50–80% of that gated population. See the Data Analysis section for an example.
8. Resuspend PBMCs in CryoStor CS10 to a final density of 2.5×10^7 cells/mL. Aliquot 1 mL per cryovial. Freeze overnight at −80 °C in a Mr. Frosty container, then transfer vials to liquid nitrogen for long-term storage.

I. Generation of CAR T

1. Calculate the number of CAR-positive T cells required for downstream experiments. For example, for an in vivo study with CAR T injection on day 9 post-activation, one cryovial containing 2.5×10^7 PBMCs would yield an estimated CAR-positive output of: $2.5 \times 10^7 \times 0.2$ (CD3-positive fraction, donor dependent) × 0.5 (post-thaw recovery) × 20 (typical fold expansion by day 9) × 0.3 (CAR-positive fraction on day 9, construct and lentiviral batch dependent) ≈ 1.5×10^7 CAR-positive cells. Adjust these factors based on your donor and construct.
2. Thaw one vial of cryopreserved PBMCs in a 37 °C water bath. Immediately transfer the contents into a 15 mL centrifuge tube containing 7 mL pre-warmed T cell culture medium. Determine the total cell number and estimate the T cell input using the CD3% measured during PBMC isolation. For example, if counting shows 3×10^7 total PBMCs and 20% CD3-positive cells, then the vial contains ~6×10^6 T cells.
3. Prepare CD3/CD28 Dynabeads at a 1:1 bead:CD3-positive cell ratio. For 6×10^6 T cells, add 60 µL Dynabeads (1×10^8 beads/mL) to 2 mL T cell culture medium. Mix, place on a magnetic stand for ~1 min, and remove the supernatant. Resuspend the beads in ~0.5 mL of the PBMC suspension and return the bead-containing suspension to the main tube.
4. Incubate the tube on a 3D rotating mixer at room temperature (10 rpm) for 30 min to promote bead-cell contact.
5. Place the tube on a magnetic stand for 5 min and remove the supernatant.
6. Resuspend the bead-bound cells in 2 mL T cell culture medium supplemented with 100 U/mL IL-2. Take a small aliquot (e.g., 10 µL) for counting. At this stage, the majority of recovered cells should be T cells; from an initial 6×10^6 T cells, ~3×10^6 cells are typically recovered after bead enrichment.
7. Plate activated T cells in a non-TC treated 24-well plate at 1.5×10^6 cells per well. Add pre-warmed T cell culture medium supplemented with 100 U/mL IL-2 to a final volume of 2 mL per well.
8. After 18 h, carefully remove 1 mL medium per well and replace with 1 mL fresh, pre-warmed T cell culture medium supplemented with 100 U/mL IL-2.
9. At 48 h after bead stimulation, transfer cells to a 15 mL tube. Place the tube on a magnetic stand for 5 min to remove

beads, then transfer the supernatant (cells) to a fresh tube and count. A modest expansion is typically observed at this stage (e.g., ~1.2-fold).

10. Prepare a Retronectin-coated non-TC treated 24-well plate for transduction. Make 8 μg/mL Retronectin in PBS (e.g., 8 μL Retronectin at 1 μg/μL + 992 μL PBS). Add 1 mL of 8 μg/mL Retronectin solution per well and incubate for at least 4 h at 37 °C. Remove the Retronectin solution, block with 1 mL 2% (w/v) BSA per well for 20 min at room temperature, discard the BSA, then add 500 μL concentrated lentivirus per well. Centrifuge the virus-coated plate at 2,630 rcf for 1.5 h at 32 °C (speed up = 3, speed down = 0). Plan this step so that virus loading and spin occur before adding cells (typically start ⩾6 h before Step 11).
11. Near the end of the 1.5 h centrifugation, resuspend activated T cells at 5 × 10^5 cells/mL in T cell culture medium supplemented with 100 U/mL IL-2. Immediately after the virus spin, add 1 mL cell suspension (5 × 10^5 cells) to each well. Centrifuge at 135 rcf for 5 min at 32 °C (speed up = 3, speed down = 0), then transfer the plate to a CO2 incubator.
12. For constructs with low transduction efficiency, perform a second transduction the next morning. Remove 250 μL medium per well and add 250 μL concentrated virus. Incubate for 6 h, then remove 750 μL medium per well and replace with 750 μL pre-warmed T cell culture medium supplemented with 100 U/mL IL-2. If a second transduction is not required, skip the virus addition and proceed with the routine medium change.
13. At 48 h after the first transduction (day 4 post-activation), pool wells transduced with the same lentivirus into a 15 mL tube. Remove 100 μL per well (or an equivalent aliquot) for flow cytometry (CD3 and V5 staining). Centrifuge the remaining cells at 400 rcf for 5 min at room temperature (speed up = 9, speed down = 9), resuspend in T cell culture medium supplemented with 100 U/mL IL-2 to 1 × 10^6 cells/mL, and re-plate 1.5 mL per well in a fresh non-TC treated 24-well plate.
14. Passage the cells every 48 h using the same dilution strategy, maintaining IL-2 at 100 U/mL, until the desired time point (e.g., day 9) for downstream assays.

J. In vitro cytotoxicity and cytokine secretion

1. For short-term cytotoxicity and cytokine profiling, seed 1 × 10^4 SK-OV-3-Luc-GFP cells per well in 100 μL 10% FBS in DMEM in a white-bottom 96-well plate.
2. Measure the CAR-positive fraction of each CAR T sample by flow cytometry (e.g., V5 staining) and use this value to calculate the total T cell number required to deliver the desired number of CAR-positive effector cells. Prepare effector cells at E:T ratios of 0.5:1, 1:1, and 2:1 (based on CAR-positive cells). Include untransduced T cells at the same total cell numbers as controls, and include a target-only condition (SK-OV-3-Luc-GFP only). Prepare all conditions in triplicates.
   Note: If a CAR T preparation is 40% CAR-positive and the desired E:T is 0.5:1 with 1 × 10^4 targets, each well requires 5,000 CAR-positive cells, corresponding to 12,500 total T cells. To set up triplicates, prepare 400 μL of effector (i.e., CAR T) suspension containing 5 × 10^5 total T cells in T cell culture medium, then add 100 μL per well (12,500 total T cells per well).
3. After 18 h co-culture, collect 200 μL supernatant from each well. Split the supernatant into two portions and transfer to two new 96-well plates, then store at −80 °C for subsequent ELISA assays.
   Pause point: Supernatants can be stored at −80 °C for a few weeks before ELISA.
4. To quantify cytotoxicity by bioluminescence, dilute 10 mM D-luciferin stock in PBS to 1 mM. Add 100 μL of 1 mM D-luciferin to each well, incubate for 1–2 min at room temperature, and measure luminescence on a plate reader.
5. Calculate percent lysis by setting the background signal from empty wells as 100% lysis and the mean signal from target-only wells as 0% lysis, then normalizing each condition accordingly.
6. Perform ELISA according to the manufacturer's instructions. We typically use 10 μL supernatant per well. If cytokine concentrations are below the detection limit, increase the input volume to 50 μL (i.e., lower dilution) to improve sensitivity.
   Note: Our ELISA workflow starts with plate coating on the day before the assay and typically requires approximately one full day to complete, with several incubation steps. In practice, it is efficient to run two ELISA plates in parallel, either testing different sample sets for the same cytokine or testing the same samples for two different cytokines.

K. Flow cytometry–based cytolysis assay

1. For long-term cytotoxicity, we typically use an E:T ratio of 1:20 (E = effector, T = target). Seed 1 × 10^5 SK-OV-3-Luc-GFP cells per well in 1 mL 10% FBS in DMEM in a 24-well plate.
   Note: SK-OV-3 is used here as an example HER2-high target cell to evaluate antigen-specific cytotoxicity of DDMP(HER2)-CAR T cells. Parental SK-OV-3 cells without luciferase/GFP can also be used, but GFP-positive targets

simplify gating. For a more systematic assessment, we recommend including at least one HER2-low/negative control line (e.g., A549) and additional HER2-high targets (e.g., NCI-N87 or OE19).

2. Determine the CAR-positive fraction of each CAR T sample by flow cytometry and calculate the total T cell input required to deliver the desired number of CAR-positive effector cells. Add effector cells in 1 mL T cell culture medium to each target well. Include the following controls: (i) target-only wells supplemented with 1 mL T cell culture medium, and (ii) untransduced T cells at the same total cell number as the CAR T condition.
   Note: If the CAR T product is 40% CAR-positive and the desired E:T is 1:20 with 1 × 10^5 targets, each well requires 5,000 CAR-positive cells, corresponding to 12,500 total T cells.
3. Incubate the co-cultures at 37 °C in 5% CO_2 for 4 days.
4. To harvest all cells (including adherent targets), carefully remove 1 mL medium from each well and retain the remaining volume (~800 µL) to resuspend cells by gentle pipetting. Transfer the suspension to a 1.5 mL microcentrifuge tube. Rinse the well with 200 µL PBS and combine with the same tube. Add 150 µL trypsin to the well and incubate at 37 °C for 5 min to detach adherent cells. Use ~400 µL of the collected suspension to wash the well thoroughly and recover the detached cells, then combine all fractions in the same tube. Centrifuge at 400 rcf for 5 min at room temperature and discard the supernatant.
5. Resuspend the pellet in 100 µL DMEM containing APC anti-CD3 (final 1:100) and Violet 450 viability dye (final 1:100). Incubate for 30 min at room temperature in the dark.
6. Add 1 mL DMEM and centrifuge at 400 rcf for 5 min at room temperature. Discard the supernatant and resuspend the pellet in 100 µL DMEM. Add 20 µL counting beads (1 × 10^6/mL) and mix gently.
7. To remove clumps, pass the suspension through a small piece of 300-mesh cell strainer placed over a new 1.5 mL microcentrifuge tube. Collect the flow-through (typically ~100 µL) for flow cytometry analysis. See the Data Analysis section for an example.

L. In vivo cytotoxicity (xenograft models with IVIS monitoring)

1. Obtain ethical approval before initiating any animal experiments.
   Note: Depending on institutional procedures, approval may take days to weeks to obtain.
2. For the SK-OV-3 CDX model, use female NCG mice (6–8 weeks old). SK-OV-3 is an ovarian cancer cell line, so female mice are used. For each mouse, prepare 2 × 10^6 SK-OV-3-luc-GFP cells in a total volume of 150 µL by mixing 75 µL PBS with 75 µL Matrigel. Matrigel should be kept cold to prevent gelling; thaw it in advance and keep it on ice. For example, for 10 mice, prepare 10 tubes containing 2 × 10^6 SK-OV-3-luc-GFP cells in 75 µL PBS on ice. Immediately before injection, mix the cell suspension with 75 µL Matrigel by pipetting up and down with a P200 tip, then perform subcutaneous injection using a 1 mL syringe with a 26G needle (0.45 × 12 mm).
3. On day 3 (72 h post tumor implantation), inject CAR T cells (corresponding to day 9 post activation in the example timeline). Measure the CAR-positive fraction by flow cytometry (typically ~30%) and use this value to calculate the total cell number required. For example, to inject 5 × 10^5 CAR-positive cells per mouse from a product that is 30% CAR-positive, prepare 1.67 × 10^6 total cells in 100 µL PBS per mouse. Add 40 µL of 30 mg/mL (200×) IVISbrite to the cell suspension, then perform intravenous injection using a 1 mL syringe with a 26G needle (0.45 × 12 mm).
4. Monitor mouse body weight regularly. Measure tumor size with calipers and assess tumor burden by IVIS bioluminescence imaging at defined time points. Calculate tumor volume as $\frac{(major\ axis) \times (minor\ axis)^2}{2}$.
5. Normalize IVIS images using a consistent scale bar with fixed minimum and maximum values across all groups and time points.

Data analysis

Quantification of cell-surface antigen expression

Flow cytometry data were analyzed in FlowJo. Events were gated sequentially to obtain a clean and comparable population for antigen quantification: (i) cells were identified on an FSC-A vs SSC-A plot to exclude debris, (ii) singlets were selected on an FSC-A vs FSC-H plot to remove doublets and aggregates, and, if a viability dye was included, (iii) live cells were gated by excluding Violet 450–high events (dead cells). **Fig. 1A** illustrates the gating steps in (i) and (ii). HER2 expression was quantified as the mean fluorescence intensity (MFI) in the PE channel for the final gated population. Unstained cells acquired using identical PMT voltages and acquisition settings were used to define the PE gate and establish background fluorescence (**Fig. 1B**). For comparisons across cell lines, all samples were acquired with the same instrument settings; if desired, background-corrected HER2 signal can be reported as ΔMFI = MFI(stained) − MFI(unstained). In the exemplar dataset, HER2 staining of SK-OV-3 cells produced a clear rightward shift in PE signal relative to the unstained control.

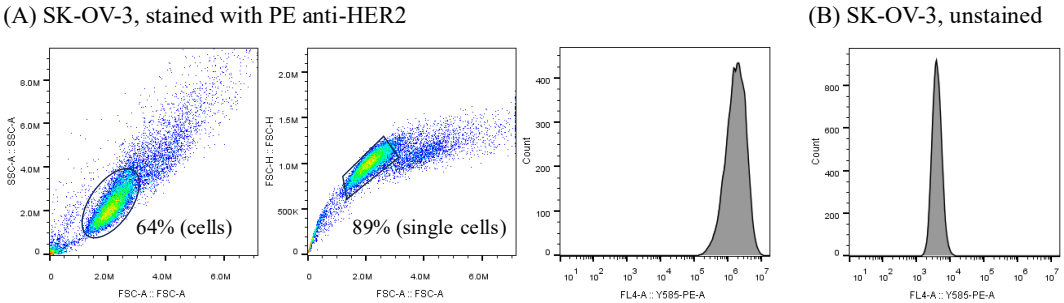


Figure 1. Representative gating strategy and exemplar HER2 staining for quantification of cell-surface antigen expression by flow cytometry. (A) SK-OV-3 cells stained with PE anti-HER2 were first gated on FSC-A vs SSC-A to exclude debris (64% of total events), followed by singlet gating on FSC-A vs FSC-H (89% of events within the "cells" gate). HER2 expression was then quantified as the mean fluorescence intensity (MFI) of the PE channel. (B) Unstained SK-OV-3 cells acquired with identical instrument settings were used to define the PE gate and background fluorescence for MFI calculation.

Quantification of T-cell ratio

Flow cytometry data were analyzed to estimate the T-cell fraction in isolated PBMCs (**Section H**). Events were first gated on FSC-A vs SSC-A to define the lymphocyte population and exclude debris and non-lymphoid cells. Singlets were then selected on FSC-A vs FSC-H to remove doublets and aggregates. The CD3 gate was defined using an unstained sample acquired under identical instrument settings, and the percentage of CD3+ cells (i.e., T cells) was quantified within the gated lymphocyte singlet population.

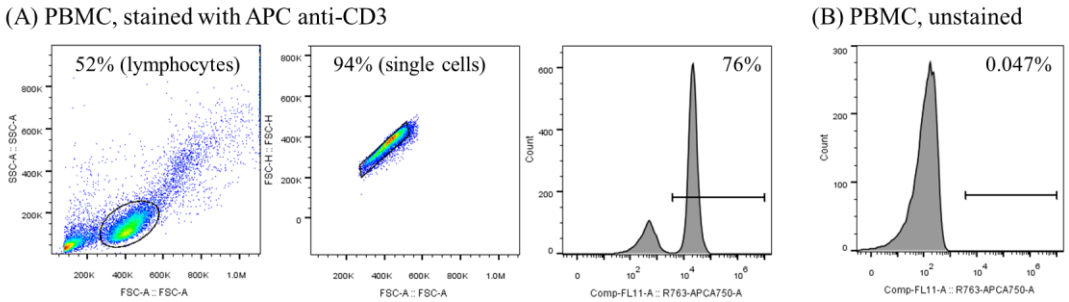


Figure 2. Representative flow-cytometry gating and exemplar CD3 analysis for estimating T-cell frequency in PBMC preparations. (A) PBMC stained with APC anti-CD3 were first gated on FSC-A vs SSC-A for lymphocytes (52% of total events), followed by singlet gating on FSC-A vs FSC-H (94% of events within the "lymphocytes" gate). T cells were accounted for those expressing CD3 (76%). (B) Unstained PBMC acquired with identical instrument settings were used to define the APC gate.

In the exemplar dataset (**Fig. 2**), lymphocytes comprised ~52% of total events, and singlets accounted for ~94% of the lymphocyte gate. Within this final gate, CD3 staining identified a dominant CD3+ population (76%), while the unstained

control showed minimal background in the CD3 gate (0.047%). Therefore, the estimated T-cell fraction in this PBMC preparation was 0.52 × 0.94 × 0.76 ≈ 37%, corresponding to ~9.3 × 10^6 T cells in a cryovial containing 2.5 × 10^7 total PBMCs. This value was used to calculate the required number of CD3/CD28 activation beads for downstream activation.

In vitro cytotoxicity

Short-term cytotoxicity was quantified using the luciferase-based killing assay described in **Section J**. Luminescence was measured after adding D-luciferin, and percent lysis was calculated using background subtraction and normalization to the target-only control. Specifically, the background signal ($RLU_{background}$) was defined as the mean luminescence of at least three empty wells containing the same culture medium composition and D-luciferin but no cells. The target-only control (RLU_{max}) was defined as the mean luminescence of at least three wells containing luciferase-expressing target tumor cells without effector T cells. After subtracting $RLU_{background}$ from all wells, the background-corrected target-only signal ($RLU_{max} - RLU_{background}$) was set as 0% lysis, and decreased luminescence in test wells was interpreted as target cell killing. Specific lysis was calculated as:

$$\text{Specific lysis} = 1 - \frac{RLU_{sample} - RLU_{background}}{RLU_{max} - RLU_{background}}$$

where RLU_{sample} is the luminescence measured in each CAR T or control co-culture condition.

Long-term cytotoxicity was quantified using flow cytometry with counting beads, as described in **Section K**. Events were gated sequentially to obtain clean, comparable populations for absolute counting: (i) cells and counting beads were identified on an FSC-A vs SSC-A plot, allowing exclusion of debris while retaining both the "cells" and "beads" populations for downstream calculations; in the exemplar target-only sample (**Fig. 3A-i**), 45% of events fell within the cell gate and 5.9% within the bead gate. (ii) Singlets were selected on an FSC-A vs FSC-H plot to remove doublets and aggregates (94% singlets in **Fig. 3A-ii**). (iii) Target cells and effector T cells were then separated on a two-parameter fluorescence plot (e.g., FITC channel for SK-OV-3-Luc-GFP targets and by APC anti-CD3 for T cells); in the target-only control, 98% of events were assigned to the SK-OV-3 gate and 0% to the T-cell gate (**Fig. 3A-iii**), confirming gate specificity. These same gates were applied to co-culture samples to determine the absolute numbers of remaining target cells and T cells.

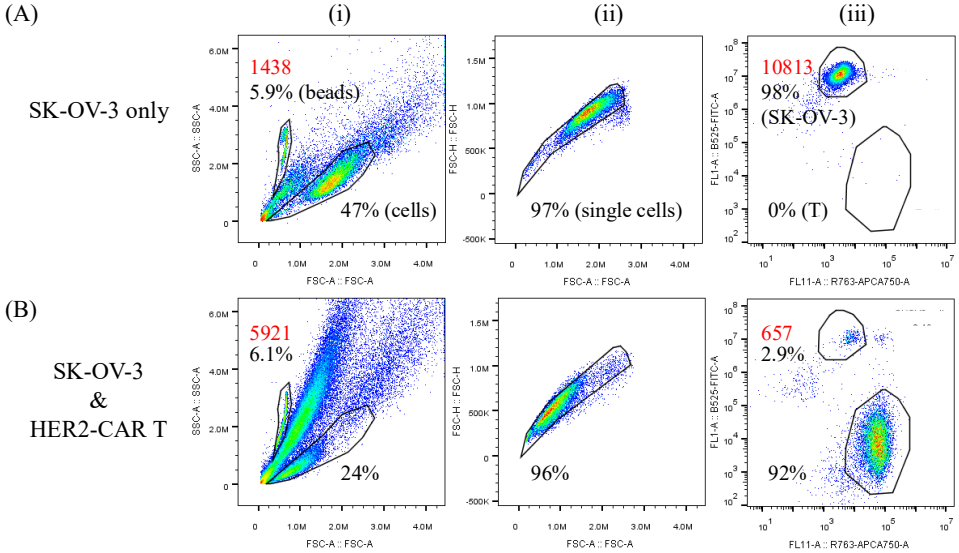


Figure 3. Representative flow-cytometry gating strategy for quantifying long-term cytotoxicity using counting beads. Left panels: FSC-A vs SSC-A was used to identify the main "cells" population and the "beads" population. Middle panels: singlets were selected using FSC-A vs FSC-H to exclude doublets and aggregates. Right panels: target tumor cells and effector T cells were separated based on marker expression (e.g., GFP-positive target tumor cells and CD3-positive T cells), enabling calculation of absolute counts for each population using the bead-based counting approach. Percentages shown in the plots indicate representative gate frequencies for the exemplar dataset (e.g., beads and cells in the FSC/SSC gate, singlets, and marker-defined populations). The event numbers required for the calculation are shown in red.

Absolute cell concentrations are calculated using the manufacturer-provided bead concentration (10^4 beads/μL) and the recorded bead and sample volumes according to:

absolute count = $\frac{event_{cell} \times vol_{bead}}{event_{bead} \times vol_{cell}} \times concentration_{bead} \times (vol_{bead} + vol_{cell})$

For **Fig. 3A**, total SK-OV-3 cells = $\frac{10813 \times 20\ \mu L}{1438 \times 110\ \mu L} \times \frac{10000}{\mu L} \times (20\ \mu L + 110\ \mu L)$ = 177732

For **Fig. 3B**, total SK-OV-3 cells = $\frac{657 \times 20\ \mu L}{5921 \times 110\ \mu L} \times \frac{10000}{\mu L} \times (20\ \mu L + 110\ \mu L)$ = 2622

Specific lysis for HER2-CAR T in **Fig. 3B** = $1 - \frac{2622}{177732}$ = 99%

Validation of protocol

This protocol has been used and validated in the following research article(s):

- Meng et al. [17] Disulfide-directed multicyclic peptides for chimeric antigen receptors targeting solid tumors. *J. Am. Chem. Soc.* **2026**, *148*, 400 (Figures 2–6, S1–S6 and S8–S14).
- Liu et al. [18] Proline-mediated enhancement in evolvability of disulfide-rich peptides for discovering protein binders. *J. Am. Chem. Soc.* **2025**, *147*, 24870 (Figures 6 and S15).

General notes and troubleshooting

General notes

1. Biosafety and contamination control. Perform all lentiviral work under approved institutional biosafety procedures. Use mycoplasma-free producer and target cells to avoid contaminating viral stocks and downstream cultures.
2. Normalization by CAR-positive dose. For comparisons across CAR designs, normalize functional assays by the number of CAR-positive T cells, not total T cells. Determine the CAR-positive fraction by flow cytometry (e.g., V5 staining) at the time of each assay and adjust cell inputs accordingly.
3. Target antigen stability. Surface antigen levels can vary with passage number and culture conditions. Quantify antigen expression by flow cytometry before key experiments and keep passage numbers consistent across comparisons.
4. Reporter and target line selection. Use well-validated Jurkat NFAT-GFP reporter clones with low basal GFP and high inducible GFP. For target cells, luciferase/GFP-expressing lines simplify quantification and gating, but consider potential fluorescence overlap with reporter readouts when designing co-culture assays.
5. Controls. Include target-only wells, untransduced T cell controls, and at least one antigen-low/negative target line for specificity assessment. For in vivo studies, predefine inclusion/exclusion criteria (e.g., engraftment threshold by IVIS on day 3).
6. Donor variability. PBMC yield, CD3-positive fraction, activation kinetics, and expansion rates vary across donors. Plan cell numbers conservatively and record donor-dependent parameters (CD3%, recovery after thaw, fold expansion) for reproducibility.
7. Cell clumping and acquisition quality. Clumping can distort flow cytometry quantification and sorting outcomes. Use gentle pipetting, filter suspensions before acquisition, and keep staining conditions consistent across samples.

Troubleshooting

Problem 1: Low transduction efficiency in Jurkat cells.
Possible cause: Viral titer is low, or viral activity has decreased due to repeated freeze–thaw cycles.
Solution: Use freshly thawed aliquots and avoid repeated freeze–thaw. If a batch consistently underperforms, prepare a new viral stock. When preparing new stocks, first verify the quality and relative concentration of the required plasmids by agarose gel electrophoresis. For example, load ~200 ng of each plasmid; samples should show comparable band intensity and the expected supercoiled plasmid pattern. If plasmid quality is poor or concentrations are inconsistent, re-prepare the plasmids before repeating virus production. In some cases, changing the lentiviral transfer vector backbone or the signal peptide sequence can also affect CAR surface expression and apparent transduction performance.

Problem 2: High background (tonic) activation in Jurkat NFAT-GFP cells (high GFP in the no-target control).
Possible cause: Tonic signaling due to high CAR expression, CAR design (e.g., signaling domain configuration), or excessive virus input leading to very high CAR density.
Solution: Reduce the virus dose during transduction. If tonic activation persists, test alternative hinge, transmembrane, and/or costimulatory domain configurations.

Problem 3: Poor single-cell outgrowth after sorting (luciferase target lines or NFAT reporter clones).
Possible cause: Sorting stress, suboptimal post-sort culture conditions, or overly stringent gating.
Solution: Use gentle sort settings, include a 100-cell "focus/control" well per plate, and expand from an early polyclonal GFP-high population before single-cell cloning. Sort cells in log-phase growth whenever possible.

Problem 4: Luciferase signal is not linear with cell number.
Possible cause: Uneven cell attachment, insufficient equilibration after luciferin addition, or signal saturation due to plate reader settings.
Solution: Standardize seeding density and attachment time, keep luciferin concentration and incubation time constant, and adjust integration time/gain to avoid saturation.

Problem 5: Low cytokine signal in ELISA.
Possible cause: Insufficient effector activation (low CAR-positive fraction, low antigen density, or suboptimal E:T ratio) and/or excessive dilution of supernatant.
Solution: Confirm CAR-positive fraction and antigen expression, increase the E:T ratio or co-culture duration, and reduce dilution by increasing the supernatant input volume per well (within kit limits).

Problem 6: Inconsistent tumor growth or weak IVIS signal in vivo.
Possible cause: Variable tumor cell viability at implantation, Matrigel handling issues (premature gelling), or inconsistent injection technique.
Solution: Keep Matrigel cold, mix immediately before injection, standardize injection volume and site, confirm engraftment by IVIS before randomization, and exclude non-engrafted mice using predefined criteria. Using a single-clone reporter line can also reduce variability in IVIS signal and tumor growth kinetics.

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Competing interests

The authors are inventors of a Chinese patent application on DDMP-CAR T.

Ethical considerations

Human peripheral blood from healthy donors were obtained from MileCell Bio with appropriate informed consent and ethics approval (LL-KT-2022055). Mouse experiments were approved by the Institutional Animal Care and Use Committee of Shenzhen Bay Laboratory (AECYX202301).

References

1. Q. Xiao, X. Su, Anti-tumor Efficacy of CD19 CAR-T in a Raji B Cell Xenografted Mouse Model. *Bio Protoc.* **2023**, *13*, e4655.
2. S. Prommersberger, M. Hudecek, T. Nerreter, Antibody-Based CAR T Cells Produced by Lentiviral Transduction. *Curr. Protoc. Immunol.* **2020**, *128*, e93.
3. M. M. Safont, et al., Protocol for the development of a bioluminescent AML-PDX mouse model for the evaluation of CAR T cell therapy. *STAR Protoc.* **2024**, *5*, 103522.
4. D. Zhang, et al., Protocol to generate traceable CAR T cells for syngeneic mouse cancer models. *STAR Protoc.* **2024**, *5*, 102898.
5. M. E. Selli, et al., Inducing T cell dysfunction by chronic stimulation of CAR-engineered T cells targeting cancer cells in suspension cultures. *STAR Protoc.* **2023**, *4*, 101954.
6. N. Tatari, et al., Preclinical Testing of CAR T Cells in a Patient-Derived Xenograft Model of Glioblastoma. *STAR Protoc.* **2020**, *1*, 100174.
7. C. L. Wu, J. C. Leroux, M. A. Gauthier, Twin disulfides for orthogonal disulfide pairing and the directed folding of multicyclic peptides. *Nat. Chem.* **2012**, *4*, 1045–1050.
8. C. Wu, Motif-Directed Oxidative Folding to Design and Discover Multicyclic Peptides for Protein Recognition. *Acc. Chem. Res.* **2025**, *58*, 1620–1631.
9. C. Xu, et al., Directed Evolution of Multicyclic Peptides Using Yeast Display for Sensitive and Selective Fluorescent Analysis of CD28 on the Cell Surface. *Anal. Chem.* **2025**, *97*, 4031–4040.
10. Z. P. Duan, et al., Triscysteine disulfide-directing motifs enabling design and discovery of multicyclic peptide binders. *Nat. Commun.* **2024**, *15*, 7799.
11. S. M. Lu, et al., Disulfide-directed multicyclic peptide libraries for the discovery of peptide ligands and drugs. *J. Am. Chem. Soc.* **2023**, *145*, 1964–1972.
12. J. Li, et al., De Novo Discovery of Cysteine Frameworks for Developing Multicyclic Peptide Libraries for Ligand Discovery. *J. Am. Chem. Soc.* **2023**, *145*, 28264–28275.
13. S. Yao, et al., De novo design and directed folding of disulfide-bridged peptide heterodimers. *Nat. Commun.* **2022**, *13*.
14. Y. P. Wu, et al., Structure-guided design of CPPC-paired disulfide-rich peptide libraries for ligand and drug discovery. *Chem. Sci.* **2022**, *13*, 7780–7789.
15. S. M. Lu, et al., Directed disulfide pairing and folding of peptides for the de novo development of multicyclic peptide libraries. *J. Am. Chem. Soc.* **2020**, *142*, 16285–16291.
16. Y. Zheng, et al., Artificial disulfide-rich peptide scaffolds with precisely defined disulfide patterns and a minimized number of isomers. *Chem. Sci.* **2017**, *8*, 2547–2552.
17. X. Meng, et al., Disulfide-Directed Multicyclic Peptides for Chimeric Antigen Receptors Targeting Solid Tumors. *J. Am. Chem. Soc.* **2026**, *148*, 400–413.
18. H. Liu, et al., Proline-mediated enhancement in evolvability of disulfide-rich peptides for discovering protein binders. *J. Am. Chem. Soc.* **2025**, *147*, 24870–24883.