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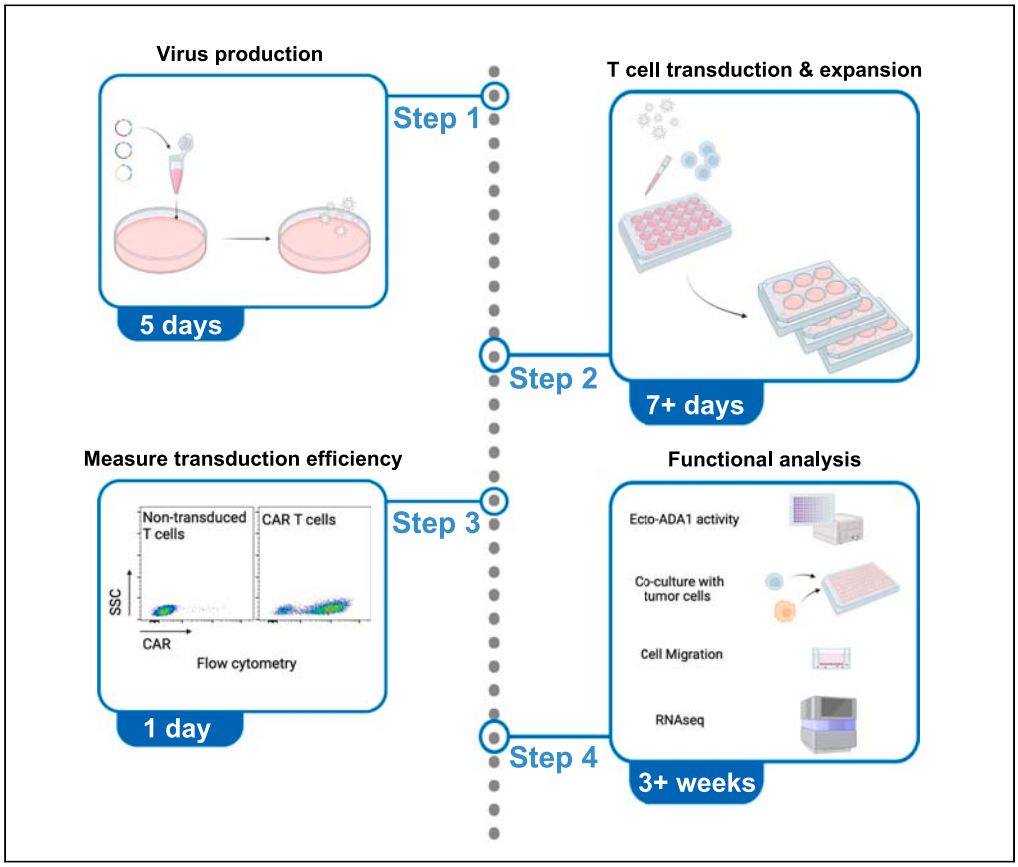
Protocol for preparing metabolically reprogrammed human CAR T cells and evaluating their *in vitro* effects



Chimeric antigen receptor (CAR) T cell therapy represents a cutting-edge cancer treatment, making the development and testing of CAR T cells crucial for advancing this therapeutic strategy. We present a protocol for creating and characterizing human epidermal growth factor receptor 2 (HER2)- and glypican-3 (GPC3)-metabolic reprogramming (MR)-CAR T cells by overexpressing adenosine deaminase 1 (ADA1) and CD26 (also known as dipeptidylpeptidase-4 or DPP4). This approach effectively converts immunosuppressive adenosine into inosine, which supports T cell survival in glucose-deficient tumor microenvironments. The protocol includes producing retroviral vectors, generating CAR T cells, and conducting ecto-ADA1 activity, cytotoxicity, cell migration, and RNA sequencing assays.

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Highlights

Transient production of retroviral vectors and generation of human CAR T cells from PBMC

Steps for assessing ecto-ADA1 activity in human CAR T cells

Steps for assessing cytotoxicity and migration capacity in human CAR T cells

Guidance on RNA-seq analysis of human CAR T cells

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Protocol for preparing metabolically reprogrammed human CAR T cells and evaluating their *in vitro* effects

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SUMMARY

Chimeric antigen receptor (CAR) T cell therapy represents a cutting-edge cancer treatment, making the development and testing of CAR T cells crucial for advancing this therapeutic strategy. We present a protocol for creating and characterizing human epidermal growth factor receptor 2 (HER2)- and glypican-3 (GPC3)-metabolic reprogramming (MR)-CAR T cells by overexpressing adenosine deaminase 1 (ADA1) and CD26 (also known as dipeptidylpeptidase-4 or DPP4). This approach effectively converts immunosuppressive adenosine into inosine, which supports T cell survival in glucose-deficient tumor microenvironments. The protocol includes producing retroviral vectors, generating CAR T cells, and conducting ecto-ADA1 activity, cytotoxicity, cell migration, and RNA sequencing assays.

For complete details on the use and execution of this protocol, please refer to Hu et al.[1]

BEFORE YOU BEGIN

This protocol outlines the preparation of metabolically reprogrammed human CAR T cells and their *in vitro* evaluation. CAR T cell therapy is a revolutionary approach in cancer treatment, utilizing genetically engineered T cells to target and eliminate cancer cells. The success of this therapy depends significantly on the metabolic state of CAR T cells, particularly in challenging tumor microenvironments characterized by nutrient deprivation.

In this protocol, we utilize human peripheral blood mononuclear cells (PBMCs) as the starting material for CAR T cell generation. It is crucial to obtain prior approval from the Institutional Review Board (IRB) for the collection and use of human samples. This ensures compliance with ethical standards and regulations governing research involving human participants. Before proceeding with the experiments, confirm that all necessary ethical approvals are in place. Familiarize yourself with the principles of CAR T cell therapy and metabolic reprogramming, as a strong understanding of these concepts will aid in the successful execution and interpretation of the protocol.

The following key procedures are included in this protocol.

1. Transient Production of Retroviral Vectors and Generation of Human CAR T Cells from PBMCs: This section details the steps for creating retroviral vectors designed to express CAR constructs specific to HER2 and GPC3. The protocol outlines the transduction of PBMCs with these vectors, followed by expansion of CAR T cells.

2. Steps for Assessing Ecto-ADA1 Activity in Human CAR T Cells: This part of the protocol describes how to measure the enzymatic activity of ecto-ADA1 in CAR T cells. It includes procedures for substrate preparation, enzyme assays, and analysis of the resulting data to assess the metabolic activity of the engineered T cells.
3. Steps for Assessing Cytotoxicity and Migration Capacity in Human CAR T Cells: Here, we outline the methodologies for evaluating the cytotoxic effects of CAR T cells against target cancer cells, as well as their ability to migrate in response to chemotactic signals. The protocols provide details on co-culture experiments, viability assays, and migration assays.
4. Guidance on RNA-seq Analysis of Human CAR T Cells: This section offers a comprehensive guide to performing RNA sequencing on the generated CAR T cells. It includes sample preparation, sequencing protocol, and bioinformatics analysis to identify gene expression profiles and metabolic pathways relevant to CAR T cell function.

Ensure that you thoroughly understand each procedure and the rationale behind them before beginning the protocol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor 647 goat anti-mouse IgG, F(ab')$_2$ fragment specific; dilution, 1:100	Jackson ImmunoResearch	Cat# 115-605-006; RRID: AB_2338903
Anti-human CD3 antibody; dilution, 1:100	Miltenyi Biotec	Cat# 130-093-387; RRID: AB_1036144
Anti-human CD28 antibody; dilution, 1:1,000	BD Biosciences	Cat# 567117; RRID: AB_2916451
Goat IgG-AF647; dilution, 1:20	SouthernBiotech	Cat# 0109-31
Chemicals, peptides, and recombinant proteins		
Recombinant human ErbB2/Her2 Fc His Alexa Fluor 647 protein	Bio-Techne	Cat# AFR1129
Recombinant human IL-2	PeproTech	Cat# 200-02
RPMI 1640	Corning	Cat# 10-040-CV
DMEM	Corning	Cat# 10-013-CV
Stain buffer	BD Pharmingen	Cat# 554657
Fetal bovine serum	Gibco	Cat# 16140071
GeneJuice transfection reagent	Novagen	Cat# 70967-3
RetroNectin	Takara	Cat# T100 A/B
Critical commercial assays		
LDH Cytotoxicity WST Assay	Enzo Life Sciences	Cat# ENZ-KIT157
Adenosine Deaminase Assay Kit	GenWay Biotech	Cat# GWB-BQK080
Cell Migration/Chemotaxis Assay Kit (24-well, 5 μm)	Abcam	Cat# ab235696
Experimental models: Cell lines		
293T cells	ATCC	Cat# CRL-3216
A549 cells	ATCC	Cat# CCL-185
Calu3 cells	ATCC	Cat# HTB-55
HepG2 cells	ATCC	Cat# HB-8065
Huh-7 cells	Gift from Dr. Andras Heczey	N/A
Human peripheral blood mononuclear cells	STEMCELL	Cat# 70025
Recombinant DNA		
SFG GPC3-CAR	Gift from Dr. Andras Heczey	N/A
SFG HER2-CAR	Hu et al.[1]	N/A
SFG ADA1.CD3scFv/CD26	Hu et al.[1]	N/A
Peq-Pam 3 plasmid	–	N/A
RDF plasmid (encodes RD114 envelope)	–	N/A
Other		
Tissue culture plate 6-well	VWR	Cat# 10861-696

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tissue culture plate 12-well	VWR	Cat# 10861-698
Tissue culture plate 24-well	Falcon	REF# 353047
Tissue culture plate 96-well	Falcon	REF# 353072
Non-treated tissue culture plate 24-well	VWR	Cat# 10861-558
Tissue culture dish 10.0 cm	VWR	Cat# 10861-680
Polystyrene round-bottom tube	Falcon	REF# 352052
Millex-HV syringe filter unit 0.45 μm	Millipore	REF# SLHVR33RS
Transwell with 5.0 μm pore polycarbonate membrane insert	Corning	REF# 3421
Multiwell-plate carriers for SX4750 rotor	Beckman	N/A
Software and algorithms		
FlowJo 10.0	FlowJo, LLC	https://www.flowjo.com/
GSEA	Broad Institute	https://www.gsea-msigdb.org/gsea/index.jsp

MATERIALS AND EQUIPMENT

R10 medium		
Reagent	Final concentration	Amount
RPMI 1640	× 1	450 mL
Fetal bovine serum	10%	50 mL
Total	**N/A**	**500 mL**

Note: Store R10 medium at 4°C for up to 4 weeks.

D10 medium		
Reagent	Final concentration	Amount
DMEM	× 1	450 mL
Fetal bovine serum	10%	50 mL
Total	**N/A**	**500 mL**

Note: Store D10 medium at 4°C for up to 4 weeks.

STEP-BY-STEP METHOD DETAILS

Transient retrovirus production

Timing: 7 days

When working with viral particles, it is crucial to implement stringent safety precautions to protect personnel and the environment. Always wear appropriate personal protective equipment (PPE), including gloves, lab coats, and eye protection, and conduct all procedures within a Class II biosafety cabinet to contain potential aerosols. Ensure that all personnel are trained in biosafety practices and emergency response protocols.

All materials that come into contact with viral particles, such as gloves, pipette tips, and culture media, should be treated as biohazardous waste and disposed of in designated biohazard containers. Decontaminate surfaces and equipment with appropriate disinfectants, and follow established spill response protocols in case of accidental releases. Adhering to these safety measures will help ensure a safe working environment during experiments involving viral particles.

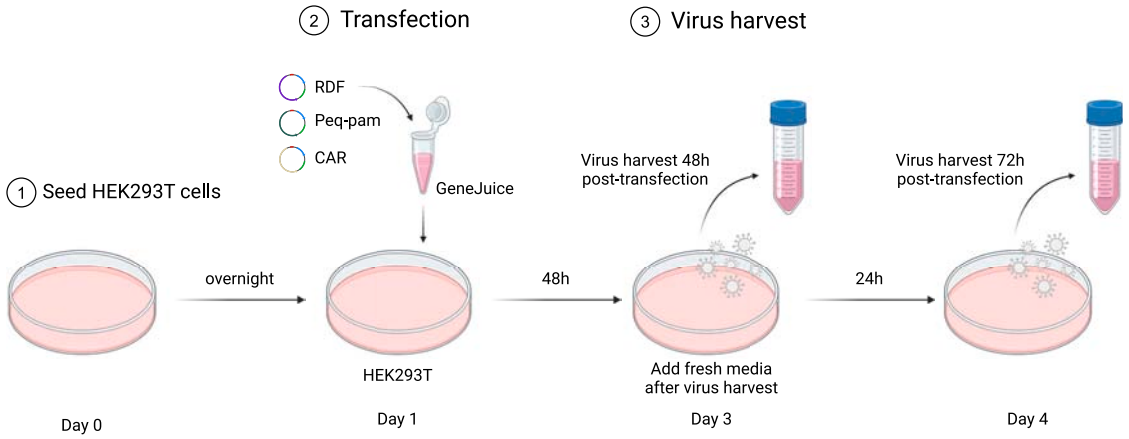

Figure 1. Production of Retroviral Vectors Expressing CAR, ADA1, and CD26

Step 1: Seed HEK293 cells. Step 2: Transfect cells with plasmids using GeneJuice. Step 3: Harvest and filter the supernatant. Figure created using Biorender.com.

Transfection involves the introduction of plasmid DNA into HEK293T cells using GeneJuice Transfection Reagent, a non-liposomal formulation. This method facilitates transient expression of the vector genome and key proteins essential for generating functional retroviral particles, which are replication incompetent (Figure 1).

1. Preparation of HEK293T Cells for Transduction (At least four days before transduction):
   a. Thaw a vial of HEK293T cells stored in liquid nitrogen (LN) at a concentration of 2–4 × 10^6 cells/mL in a 37°C water bath.
   b. Transfer the contents to 5 mL of pre-warmed D10 medium.
   c. Centrifuge at 300 × *g* for 5 min at 20°C–25°C.
   d. Discard the supernatant, then resuspend the cell pellet in 1 mL of D10 medium.
   e. Seed into a 10 cm tissue culture dish with 9 mL of D10 medium.
   f. Incubate at 37°C with 5% CO_2.

   Note: If significant cell death occurs, replace the medium after 24 h with 10 mL of fresh D10 medium.

   Note: Cells should reach 80% confluence in 2–3 days and are ready for passage.

   g. Remove and discard the culture medium.
   h. Rinse the dish with 3 mL of PBS to remove residual serum.
   i. Add 1 mL of 0.25% Trypsin solution to the dish and incubate at 37°C for 3–5 min to facilitate cell detachment.
   j. Confirm detachment of cells under an inverted microscope.
   k. Add 3 mL of D10 medium to neutralize trypsin.
   l. Split cells at a 1:6 ratio by transferring 0.66 mL of trypsinized cells into 9 mL of fresh D10 medium in a new 10 cm tissue culture dish.

   Note: HEK293T cells at lower passages typically yield higher titers of transient virus, resulting in higher transduction efficiencies.

   Note: HEK293T cells should be split at least twice to allow more T cell proliferation and improved viability before transduction.

Note: Due to HEK293T cells are very easy to detach from dishes, handling procedures for HEK293T cells, such as medium replacement, require particularly gentle execution.

2. Preparation one day before transfection.
   a. Prepare a suspension of HEK293T cells at a concentration of 3×10^5 cells/mL in D10 medium.
   b. Seed 10 mL of the HEK293T suspension into each 10 cm^2 tissue culture dish, totaling 3×10^6 cells per dish.

Note: Each construct requires one dish. A single 10 cm dish of HEK293T cells typically yields about 20 mL of viral supernatant (from 2 harvests), sufficient for transducing 9×10^6 human T cells. This step can be adjusted as needed.

   c. Gently swirl to distribute the cells evenly.
   d. Incubate the dishes at 37°C with 5% CO_2 for 18–24 h.
3. On the day of transfection.

Note: HEK293T cells should be 50%–70% confluency at the time of transfection, otherwise wait 24 h prior doing transfection.

   a. Prepare the transfection mixture by combining 420 μL of serum-free DMEM medium with 30 μL of GeneJuice in a 2 mL Eppendorf tube for each 10 cm dish of HEK293T cells.

Note: It is important that the GeneJuice reagent should be directly added into the medium to prevent adsorption of the reagent to the plastic.

   b. Tap the tube gently to thoroughly mix the GeneJuice/DMEM mixture.
   c. Incubate at 20°C–25°C for 5 min.
   d. Prepare a separate Eppendorf tube by adding 50 μL of serum-free DMEM medium and a total of 10 μg DNA (including 3.75 μg Peq-Pam3 plasmid, 2.5 μg RDF plasmid, and 3.75 μg of the appropriate retroviral vector plasmid).

DNA mix

DNA	Amount
Peq-pam 3	3.75 μg
RDF	2.5 μg
CAR plasmid and/or CD26-ADA1 plasmid	3.75 μg

Note: The vector plasmid expresses transgenes, including either the HER2-CAR or GPC3-CAR plasmid, as well as an MR plasmid that expresses the membrane protein CD26 and the ADA1-CD3scFv. 3.75 μg of the CAR plasmid or MR plasmid were used to generate retroviral supernatant separately.

   e. Slowly add the GeneJuice/DMEM mixture drop by drop to the tube containing the DNA.
   f. Tap the tube gently to ensure thorough mixing of the GeneJuice/DMEM/DNA mixture.
   g. Incubate at 20°C–25°C for 15 min.
   h. Carefully add the GeneJuice/DMEM/DNA solution dropwise to the cells while gently shaking.
   i. Incubate the cells at 37°C with 5% CO_2 for 48 h.
4. 48 h after transfection.
   a. Collect the supernatant (virus-containing media) from HEK293T cells and transfer it to a 50 mL Falcon tube.
   b. Add 10 mL of pre-warmed D10 medium to each dish.
   c. Return dishes to the incubator.

d. Filter the harvested supernatant using a 0.45 μm sterile filter.
e. Aliquot 1.5 mL of supernatant into labeled tubes.
f. Store at −80°C directly after aliquoting until needed.

5. 72 h after transfection.
   a. Harvest the HEK293T cell culture supernatant that contains virus particles into a 50 mL Falcon tube.
   b. Using 0.45 μm sterile filter to filter the harvested supernatant.
   c. Aliquot 1.5 mL of supernatant into labeled tubes.
   d. Store at −80°C until needed.

Note: Freshly harvested viral supernatant can be immediately used for T cells transduction. It is important to note that retrovirus titers gradually decline when stored at 4°C. Therefore, it is recommended to minimize storage at this temperature to no more than one day before use or freezing. We typically use the viral supernatant within six months of storage at −80°C, and there is no significant decrease in transduction activity during this time.

Note: There is no difference in transduction efficacy between using a 48-h collection and a 72-h collection.

Activation of human PBMCs

Timing: 2–3 days

In this step, PBMCs are stimulated and expanded by employing anti-CD3/anti-CD28 antibodies to activate T cells. The goal of this step is to enrich the activated T cells that are suitable for retroviral transduction.

6. One day prior to activation, preparation of anti-CD3/anti-CD28 antibody coated plates.
   a. Add 1 μg of each CD3 and CD28 antibody into 1 mL sterile water.
   b. Plate 1 mL of anti-CD3/anti-CD28 antibody suspension into each well of 24 well non-tissue culture plate.
   c. Incubate at 4°C for 18–24 h.

Alternatives: Alternatively, the anti-CD3/anti-CD28 antibody coated plate can be prepared on the same day as T cell activation by changing incubation to 37°C for 4 h at least.

7. On the day of activation.
   a. Take out anti-CD3/anti-CD28 antibody coated plate from 4°C.
   b. Warm in hood (at 20°C–25°C) for about 10 min.
   c. Thaw a vial of human PBMC cells in a 37°C water bath.
   d. Centrifuge the tube contents in 5 mL of pre-warmed R10 medium at 300 × *g* for 5 min at 20°C–25°C.
   e. After centrifugation, carefully remove the supernatant.
   f. Resuspend the cell pellet in R10 medium to achieve 0.5 × 10^6/mL concentration.
   g. Add IL-2 to cell suspension to achieve 100 IU/mL concentration.
   h. Aspirate 0.9 mL of anti-CD3/anti-CD28 antibody suspension from plate without touch the well bottom with the pipette tips.

Note: Do not let the well dry out.

   i. Plate 2 mL of cell suspension into each well (number of cells should not exceed 1 × 10^6 /well).
   j. Incubate at 37°C and 5% CO_2 for 48 h.

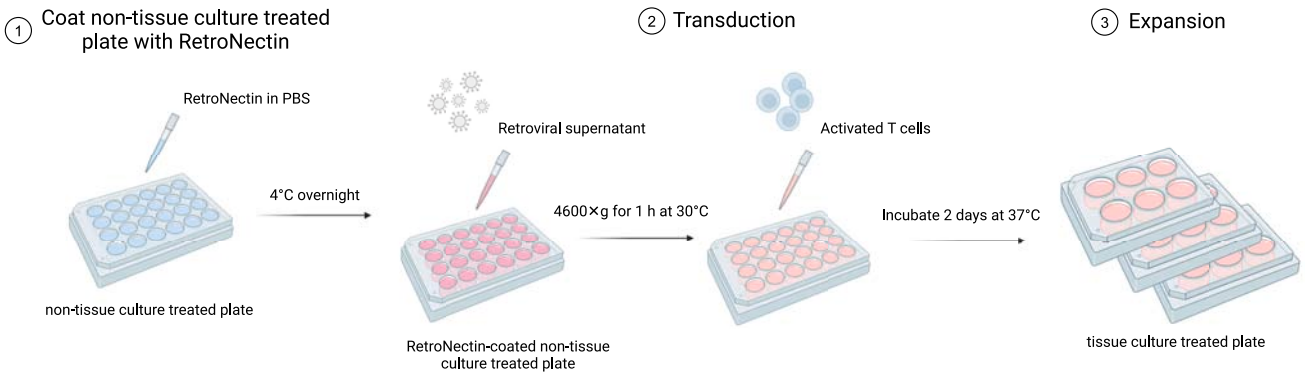

Figure 2. Human CAR T Cell Generation and Characterization

Step 1: Coat a non-tissue culture treated plate with RetroNectin. Step 2: Transduce activated human PBMCs with retroviral vectors. Step 3: Expand retroviral vector-transduced human CAR T cells. Figure created using Biorender.com.

Retroviral transduction of T cells

Timing: 3–4 days

In this step, retroviral vectors are transduced into human PBMCs to express the transgene including CAR, CD26, and ADA1 (Figure 2 steps 1–2).

8. One day prior to transduction, preparation of RetroNectin Coated Plates.
   a. Add 10 μL RetroNectin (1 mg/mL stock solution) to 1 mL sterile PBS.
   b. Add 1 mL of RetroNectin suspension (10 μg/mL) to each well of non-tissue culture 24-well plate.
   c. Incubate at 4°C for 18–24 h.

   Alternatives: RetroNectin coated plate can be prepared on the same day as transduction by changing incubation to 37°C for 4 h at least.

9. On the day of transduction.
   a. Warm RetroNectin coated plate at 20°C–25°C for about 10 min.

      Note: Or until it reaches room temperature.

   b. Thaw the retroviral supernatant from the previous steps (step 4 and step 5) on ice.
   c. Carefully aspirate 0.9 mL of the RetroNectin supernatant from the wells without touch the well bottom with the pipette tips.

      Note: Do not let the well dry out.

   d. Add 1 mL of retroviral supernatant to each well.

      Note: For the generation of CAR T cells, 1 mL of the CAR retroviral supernatant was used. To generate MR-CAR T cells, a mixed CAR retroviral supernatant and MR retroviral supernatant were used at a 1:1 ratio, totaling 2 mL. The transfection efficiency showed no difference, as both MR-CAR and CAR T cells expressed CAR on the T cell surface at similar levels.

   e. Add 1 mL D10 medium to each well of non-transduced control T cells control.
   f. Centrifuge at 4600 × *g* for 1 h at 30°C.
   g. Collect the supernatant containing stimulated T cells.

h. Resuspend and count the T cells.
i. Adjust T cell concentration to 0.25 × 10^6/mL in R10 medium.
j. Add IL-2 to cell suspension to achieve 100 IU/mL concentration.
k. Carefully aspirate 0.9 mL of the retroviral supernatant from the wells without touch the well bottom with the pipette tips.

Note: Do not let the well dry out.

l. Plate 2 mL of the T cell suspension into each well.
m. Centrifuge at 1000 × *g* for 10 min.
n. Incubate cells at 37°C and 5% CO_2 for 48 h.

Note: It is recommended not to wash activated T cells before transduction to avoid removing potentially stimulatory factors present in the conditioned medium. Discard 1.5 mL old medium from activated T cell wells and resuspend the stimulated T cells by adding fresh R10 medium.

Note: Reserve a portion of the activated T cells for subsequent experiments, such as the control for flow cytometry analysis and functional studies.

10. Replating CAR T cells.
    a. Remove transduced T cells from plate and collect them in 15 mL falcon tubes.
    b. Add 10 μL trypan blue to 10 μL PBMC suspension.
    c. Count cell number using hemocytometer.
    d. Spin and resuspend CAR T cells in R10 medium to achieve 0.5 × 10^6 to 1 × 10^6 per mL concentration.
    e. Add IL-2 to cell suspension to a final concentration of 100 IU/mL.
    f. Plate appropriately into tissue culture treated plate.
    g. Incubate at 37°C and 5% CO_2.

Expanding CAR T cells and assessing transduction efficiency

Timing: 5–14 days

This step generates CAR, HER2-MRCAR, GPC3-MRCAR, and control T cells for subsequent functional analysis (Figure 2 step 3).

11. Every two or three days.
    a. Monitor cell growth.
    b. Add fresh R10 media.

Note: Re-plate the T cells into larger plates if cells become over 100% confluence, otherwise only change top media if color has changed.

    c. Add fresh IL-2 into the cultures to achieve 100 IU/mL concentration.
    d. Incubate cells at 37°C with 5% CO_2.

Note: The T cells are cultured as needed based on the desired number of cells. Regular cell counting every 2–3 days is recommended, with adjustments made to maintain T cell concentrations between 0.5 × 10^6 cells/mL and 2 × 10^6 cells/mL using fresh R10 medium supplemented with IL-2.

12. Assess transduction efficiency via flow cytometry.

a. Transfer 2 × 10^5 T cells per CAR construct into individual 5 mL flow cytometry tubes.

Note: Additionally, it is necessary to prepare 2 × 10^5 non-transduced T cells as control.

b. Wash the cells with 2 mL of Flow Cytometry Staining Buffer.
c. Centrifuge the cells at 400 × *g* for 5 min.
d. Discard the supernatant.
e. Resuspend the cell pellet in 100 μL of Flow Cytometry Staining Buffer.
f. Add recommended quantity of fluorophore-labeled antibody to each tube.
g. Incubate at 4°C for 30 min in the dark.

Note: Anti-F(ab')$_2$ Alexa Fluor 647-conjugated antibody is used to detect GPC3-CAR expression. Alexa flour 647 conjugated HER2 protein is used to detect HER2-CAR expression.

h. Set aside one tube without antibody stain or label with IgG1 isotype control.
i. Wash the cells with 2 mL of Flow Cytometry Staining Buffer.
j. Centrifuge the cells at 400 × *g* for 5 min at 20°C–25°C.
k. Repeat Step f.
l. Discard the supernatant.
m. Resuspend the T cells in 200 μL of Flow Cytometry Staining Buffer.
n. Analyze samples by flow cytometry analyzer.

Note: Evaluation of gene transfer efficiency is typically conducted between day 5 and day 10 post retrovirus transduction.

Ecto-ADA1 activity assay

Timing: 2 days

This section describes how to measure the amount of ADA1 presented on the T cell membrane by using an ADA enzyme activity assay.

13. Resuspend the CAR T cells to a concentration of 2 × 10^6 cells/mL with R10 medium.
14. Plate 200 μL of the cell suspension in each well to 96 well cell culture plate.
15. Incubate plate at 37°C and 5% CO_2 for 24 h.
16. Centrifuge the plate at 250 × *g* for 2 min at 20°C–25°C to precipitate the cells.
17. Determinate ADA activity by Adenosine Deaminase Assay Kit.
    a. Discard the supernatant from wells.
    b. Add 180 μL of Reagent 1 (included in the Kit) to each well.
    c. Incubate plate at 37°C for 3 min.
    d. Add 90 μL of Reagent 2 (included in the Kit) to each well.
    e. Incubate plate at 37°C for 10–30 min.
    f. Transfer 200 μL of supernatant to each well of a new flat-bottom clear 96-well plate.
    g. Measure the absorbance at 550 nm by a microplate reader.

Note: Prevent the formulation of bubbles when the reagent or sample is transferred into the microplate.

Note: Our lab used the Adenosine Deaminase Assay Kit (GenWay Biotech, Cat# GWB-BQK080). This kit is currently discontinued, but there are such kits available from different suppliers. For example, use the Adenosine Deaminase (ADA) Activity Assay Kit (Elabscience, Cat# E-BC-K197-M).

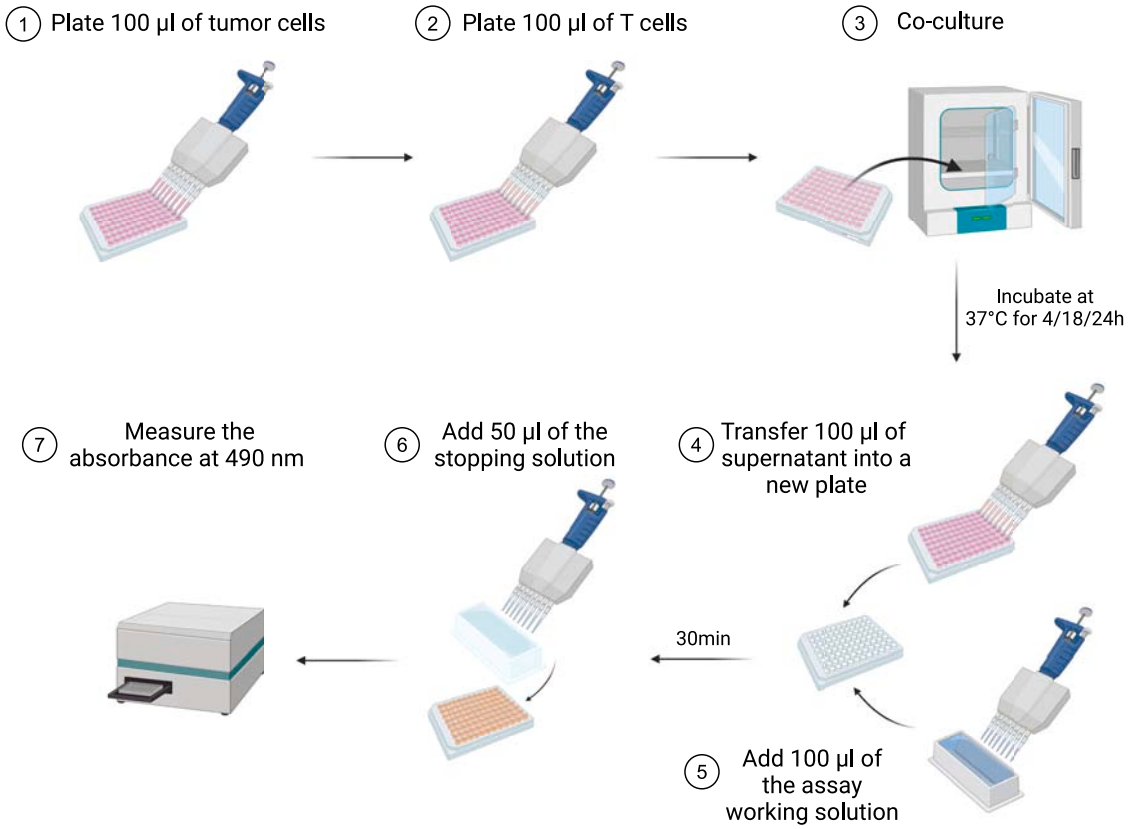

Figure 3. LDH CAR T cell cytotoxicity assay

Step 1: Plate 100 µL of tumor cell suspension to 96-well cell culture plate. Step 2: Plate 100 µL of T cell suspension at the indicated E:T ratio. Step 3: Co-culture CAR T cells and tumor cells for the specified time. Step 4: Transfer 100 µL of cell supernatant into a new 96-well plate. Step 5: Add 100 µL of the assay working solution to the wells. Incubate the plate 30 min in dark. Step 6: Add 50 µL of the stopping solution to each well of the plate. Step 7: Measure the absorbance at 490 nm by a microplate reader. Figure created using Biorender.com.

In vitro LDH cytotoxicity assay

Timing: 2 days

This method describes how to quantify T cell-mediated tumor cell lysis (Figure 3).

18. Re-suspend the tumor cells (HepG2, Huh7, Calu3 or A549) in D10 medium to a concentration of 10^5 cells/mL.
19. Plate 100 µL of the cell suspension in each well to 96 well cell culture plate.
20. Count CAR, MR-CAR and non-transduced T cells.
21. Resuspend each to a final concentration of 2 × 10^6 cells/mL, 1 × 10^6 cells/mL, 5 × 10^5 cells/mL and 1 × 10^5 cells/mL in R10 medium.
22. Add 100 µL of CAR, MR-CAR or non-transduced T cell suspension per well in triplicate.
23. Incubate cells at 37°C with 5% CO_2.
24. Repeat steps 17–20 to prepare three identical co-culture 96-well plates, designated for analysis at 4 h, 18 h, and 24 h.

Note: The GPC3-targeted CAR T cells are co-cultured with GPC3-positive HCC HepG2 or Huh7 cells, and the HER2-targeted CAR T cells are co-cultured with HER2-positive A549 or Calu3 cells at 1:1, 5:1, 10:1 or 20:1 E:T ratios.

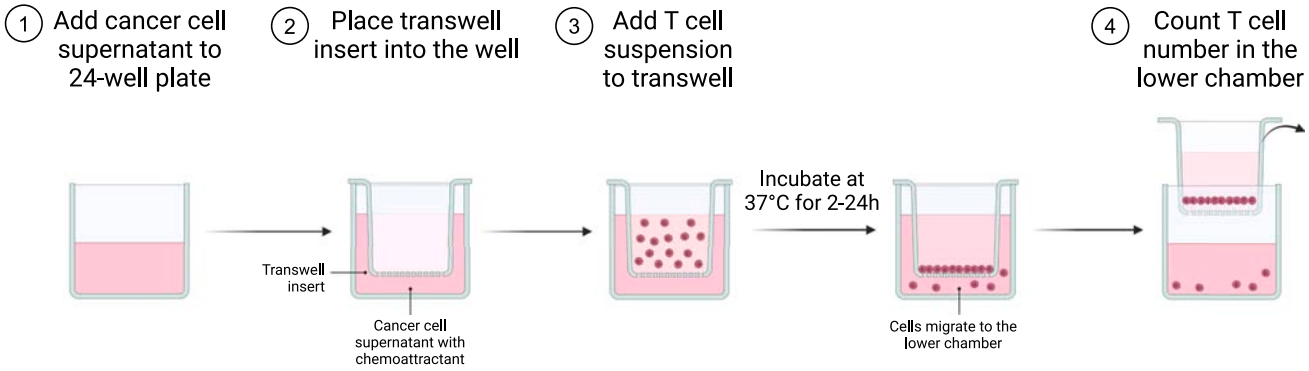

Figure 4. CAR T cell migration assay

Step 1: Add tumor cell culture medium to a 24-well cell culture plate. Step 2: Place the transwell insert into the well. Step 3: Add T cell suspension to the insert well (transwell) and incubate the plate at 37°C for 2–24 h. Step 4: Remove the transwell from the well and count the number of T cells in the lower chamber at different time point. Figure created using Biorender.com.

25. After 4/18/24 h, detection of the lactate dehydrogenase (LDH) is conducted as specified by the manufacturer using Enzo LDH cytotoxicity EST assay (Cat# ENZ-KIT157):
    a. Add 20 μL of lysis buffer into tumor alone wells in triplicate.
    b. Culture for 30 min as maximum load (high control).
    c. Centrifuge the cells at 250 × *g* for 2 min.
    d. Transfer 100 μL of cell supernatant into each well of a new flat-bottom, optically clear 96-well plate.
    e. Add 100 μL of the assay working solution to each well.
    f. Shield the plate from light.
    g. Incubate at 20°C–25°C for 30 min.
    h. Add 50 μL of the stopping solution to each well.
    i. Measure the absorbance at 490 nm by a microplate reader.
    j. Calculate the specific lysis using the following formula:

$$\text{Specific lysis} = (\text{experimental} - \text{spontaneous release}) / (\text{maximum load} - \text{spontaneous release}) \times 100\%$$

Note: 100 μL of supernatant from tumor alone wells (without lysis buffer) acts as spontaneous release (low control).

Note: The LDH cytotoxicity assay measures cytotoxicity by detecting lactate dehydrogenase (LDH) activity released from damaged cells. A limitation of this method is that dead effector cells can also elevate the enzyme concentration, affecting cytotoxicity measurements. Use effector cells with over 95% viability at the time of the experiment, and limit the co-culture duration to a short period, such as 4 h, to ensure measurement accuracy. At this time point, cytotoxicity efficacy can reach 10%–40% at an E:T ratio of 1:1 and up to 100% at an E:T ratio of 20:1.

Cell migration assay

Timing: 2 days

Cell migration assay Protocol is to check the migration of cells towards the chemoattractant (Figure 4). This assay is based on a cell-permeable membrane which is placed in the insert of upper

chamber. Cells of interest are placed in the upper chamber and chemoattractant is given into the lower chamber. After incubation of desired time, count the migrated cells.

Note: Number of cells in upper chamber and the concentration in the lower chamber and incubation time depend on the types and aim of the experiment.

26. Grow the cells (Transduced and Non-transduced T cells) to perform a cell migration assay in desired media and culture conditions.

Note: For chemoattractant split your desired tumor cell line one day prior to the assay so that it will be 80% confluent.

27. Collect the T cells.
28. Resuspend the cell in serum-free media.
29. Count the number of T cells using a hemocytometer or any cell counting machine.
30. Resuspend T cells in a serum-free media at a concentration of 2×10^6 cells/mL.

Note: Depending on the cell type make the cell as per your desired concentration.

31. Take Transwell insert and Plate (24-well, 5 μm) for the cell migration assay in the cell culture hood.

Note: Pore size of the transwell may vary depending on cell types/size or specific experiment.

32. In the bottom Chamber, add 235 μL of tumor cell supernatant (Chemoattractant) and 365 μL of serum free media to make it 600 μL.
33. Place the top chamber (Transwell, 5 μm) carefully into the bottom chamber.

Note: Ensure no air bubbles are trapped between the top and bottom chamber.

34. Add 5×10^5 cells (300–350 μL) of cell suspension to each top chamber.
35. Place the plate cover.
36. Incubate at 37°C and 5% CO_2 for 2–24 h.

Note: Make sure a smooth handling During moving the assay plate. Try to avoid any shaking of the plate.

37. Count the cells in the lower chamber using a hemocytometer at different time point (2, 4, 22/24 h).

Note: Make individual well for different time point counting.

RNA seq assay

Timing: 20–30 days

Bulk RNAseq is a technique to understand the patterns of gene expression across different cells or tissues (Figure 2 step 5). This protocol for analysis of bulk RNAseq from T cells shows the changes in signature gene expression levels across the different group of CAR T cells and non-transduced cells to explore the contribution of specific gene sets.

38. Grow different transduced CAR T cells and non-transduced cells for 6 days.
39. Isolate total RNA by RNeasy Plus mini kit (Qiagen).

40. Same day ship the samples (3 μg) with clear label in microcentrifuge tube in dry ice by Fedex overnight shipping facility.

Note: Quality control check by Azenta NGS laboratory TapeStation system where RIN for RNA recommend ≥6.0 (We got RIN ≥9.9 for all samples) and sequencing by Genewiz form Azenta Life Sciences.

Note: The timing for isolating RNA from transduced T cells is influenced by the goals and design of the experiment. For example, if the objective is to assess immediate changes in gene expression, RNA may be isolated shortly after transduction. In contrast, if the goal is to observe longer-term effects or functional outcomes, RNA isolation may occur several days post-transduction. Typically, this timeframe can range from 1 to 7 days, depending on the specific experimental motif and the desired insights into T cell behavior and function.

41. Download the human reference genome sequence (GRCh38) and genome annotation gtf file from the GENCODE V22 database (https://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_22/gencode.v22.annotation.gtf.gz).

Note: Users can use the most recent gtf file version.

42. Generate STAR index files of the human reference genome.
43. Mark duplicate reads (150 bp) in BAM files of paired-end RNA-seq profiles using "bammarkduplicates" command of biobambam (version 0.0.191) tool.
44. Algin sequence reads with the human reference genome (GRCh38) using the STAR two-pass (version 2.7.2b) alignment algorithm.
45. Perform gene-level quantification of STAR-aligned sequenced reads using htseq-count (version 0.11.0).
46. Perform differential gene expressions study using DESeq2 (version 3.5) tool.
47. Rank differentially expressed genes based on fold-change values for pre-ranked Gene set enrichment analysis (GSEA) using the GSEA (version 4.0.3) tool.

Note: The GSEA tool can be downloaded from https://www.gsea-msigdb.org/gsea/login.jsp.

EXPECTED OUTCOMES

This experimental scheme offers detailed instructions for producing retroviral vectors and generating human CAR T cells *in vitro*. Following a 3-day retroviral vector transduction, over 80% of human CAR T cells exhibit homogenous expression of antigen-specific CARs. The characteristics of these human CAR T cells can be evaluated using flow cytometry, ELISA, ecto-ADA1 activity assay, cytotoxicity assay, and migration assay.

LIMITATIONS

The methodology described in this protocol can be applied to generate CAR T cells from human PBMCs. We have successfully used this protocol to generate CAR T cells from PBMCs of multiple healthy donors, with limited influence from inter-individual variability. However, validation is needed for using patient-derived PBMCs to generate CAR T cells, as the dysfunction of patient PBMCs may affect the generation, expansion, and function of the CAR T cells.

TROUBLESHOOTING

Problem 1

The potential drawback of the two-retroviral vector co-transduction approach is its potential to reduce transduction efficacy and produce some single viral vector-transduced or virus-free T cells (related to Step 3).

Potential solution

Following co-transduction with two retroviral vectors, flow analysis of the transgenes CAR (vector 1) and CD26 (vector 2) was conducted separately. We observed that each vector achieved at least 80% transduction efficacy, comparable to single retroviral vector transduction controls. Thus, we anticipate that at least 64% of cells were double-transduced, 16% were single vector-transduced, and only 4% of T cells remained vector-free. Given that double-transduced T cells are likely more CD3 activated, their expansion is expected to outpace that of less-activated T cells not susceptible to viral transduction. Consequently, the proportion of double viral vector-transduced T cells should increase with expansion. Given these dynamics, the two-vector transduction system may not pose any challenges for both *in vitro* and *in vivo* testing scenarios.[2,3]

Problem 2

RNA sequencing analysis of CAR T cells cultured *in vitro* may reveal heterogeneity in transcriptional profiles across experimental replicates (related to Step 8).

Potential solution

Variations in gene expression profiles among individual samples from the same donor can arise from inherent differences. T cells within the population display diverse cellular states, reflecting various phenotypes and differentiation stages despite their common origin.[4] This inherent heterogeneity contributes to distinct transcriptional profiles. Moreover, even with consistent CD3 antibody treatment and retrovirus transduction protocols, subtle differences in signaling dynamics can lead to varied gene expression patterns over time, influenced by the timing and strength of signaling cascades. Therefore, to discern differences between MR-CAR T cells and CAR T cells using cultured T cell populations, employing multiple experimental replicates is essential to mitigate natural heterogeneity within the T cell populations.

Problem 3

The ecto-ADA1 activity assay, designed to measure ADA1 on the surface of T cells, could be contaminated by ADA present in fetal bovine serum (FBS) used in the cell culture medium (related to Step 5).[5]

Potential solution

It is essential to wash the cells three times with PBS prior to conducting the ecto-ADA1 activity assay.

Problem 4

The LDH assay used to measure the cytotoxicity of CAR T cells in killing tumor cells is limited by its ability to detect both tumor cell death and CAR T cell death (related to Step 6).

Potential solution

The LDH assay is particularly limited in 24-h killing assays, as CAR T cells themselves may die during cell culture. Thus, while the LDH assay can differentiate between MR-CAR T cells and CAR T cells, its results do not specifically measure tumor cell death. Therefore, additional assays such as flow cytometry analysis of tumor cell death or other methods may offer a more accurate assessment of the cytotoxic capacity of human CAR T cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiaotong Song (xsong@tamu.edu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contacts, Yue Hu (huyue@tamu.edu), Abhijit Sarkar (abhijit.sarkar@uth.tmc.edu), and Xiaotong Song (xsong@tamu.edu).

Materials availability

This study did not generate any unique reagents.

Data and code availability

The published article includes all the datasets generated or analyzed using this protocol.[1]

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AUTHOR CONTRIBUTIONS

X.S., Y.H., and A.S. designed the experiments, analyzed the data, created the figures, and wrote/edited the manuscript. All authors critically read and approved the manuscript.

DECLARATION OF INTERESTS

X.S., A.S., and Y.H. are the inventors of the technology discussed in this work, and Texas A&M University has ownership of the technology and has filed a patent application for it.

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