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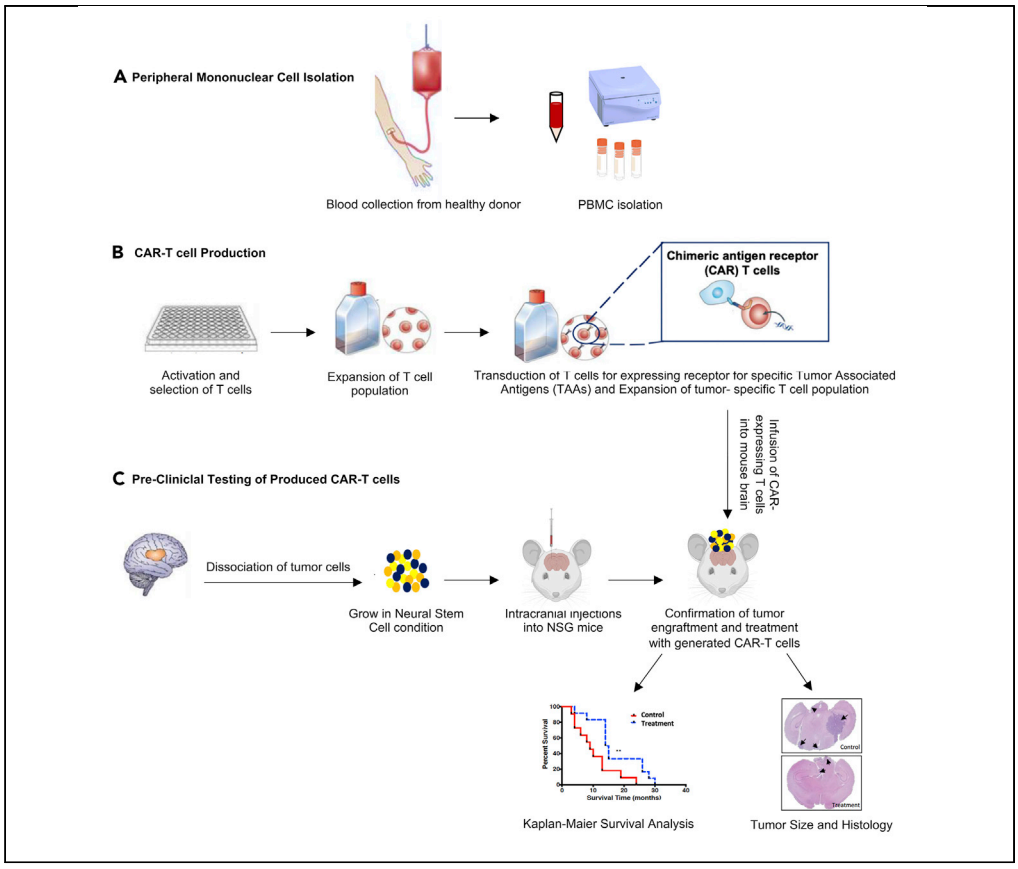
Protocol

Preclinical Testing of CAR T Cells in a Patient-Derived Xenograft Model of Glioblastoma



Glioblastoma (GBM) is the most common malignant adult brain tumor that is resistant to the standard care therapy. Advances in chimeric antigen receptor (CAR) T cell therapies have spurred renewed interest in developing CAR T cell therapies to target chemoradiotherapy-resistant brain tumor-initiating cells. This protocol shows how to isolate peripheral blood mononuclear cells from healthy donors and generate CAR T cells for the antigens of interest, and how to intracranially inject the CAR T cells into a patient-derived xenograft mouse model of GBM.

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HIGHLIGHTS

PBMC isolation from healthy donors for CAR T cell production

Production of tumor-associated antigen-specific CAR T cells

Generation of a PDX model of GBM for preclinical testing of CAR T cells

Tatari et al., STAR Protocols 1, 100174
December 18, 2020
https://doi.org/10.1016/j.xpro.2020.100174

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Preclinical Testing of CAR T Cells in a Patient-Derived Xenograft Model of Glioblastoma

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SUMMARY

Glioblastoma (GBM) is the most common malignant adult brain tumor that is resistant to the standard care therapy. Advances in chimeric antigen receptor (CAR) T cell therapies have spurred renewed interest in developing CAR T cell therapies to target chemoradiotherapy-resistant brain tumor-initiating cells. This protocol shows how to isolate peripheral blood mononuclear cells from healthy donors and generate CAR T cells for the antigens of interest, and how to intracranially inject the CAR T cells into a patient-derived xenograft mouse model of GBM.

For complete details on the use and execution of this protocol, please refer to Vora et al. (2020).

BEFORE YOU BEGIN

Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Human Whole Peripheral Blood Samples by Density Gradient Centrifugation

Timing: 1 day

Note: Ensure all the samples, reagents and centrifuge used in this part of the protocol are at room temperature (20°C–22°C).

1. Withdraw a total of 50 mL of peripheral blood from a consenting, healthy donors.
2. Add 15 mL of Lymphoprep (density gradient medium) to the 50 mL SepMate™ tube by gently pipetting it through the central hole of the SepMate™ insert.

Note: The Lymphoprep will stay above the insert in the SepMate™ tube. Also, some small bubbles might form in the Lymphoprep after pipetting. However, none of these will affect performance.

Note: For each 50 mL peripheral blood, three SepMate™ are required to be able to add the whole withdrawn blood.

3. Dilute peripheral blood sample with an equal amount of PBS + 2% FBS and mix gently. For example, if the sample volume is 50 mL, dilute it with 50 mL of PBS + 2% FBS.

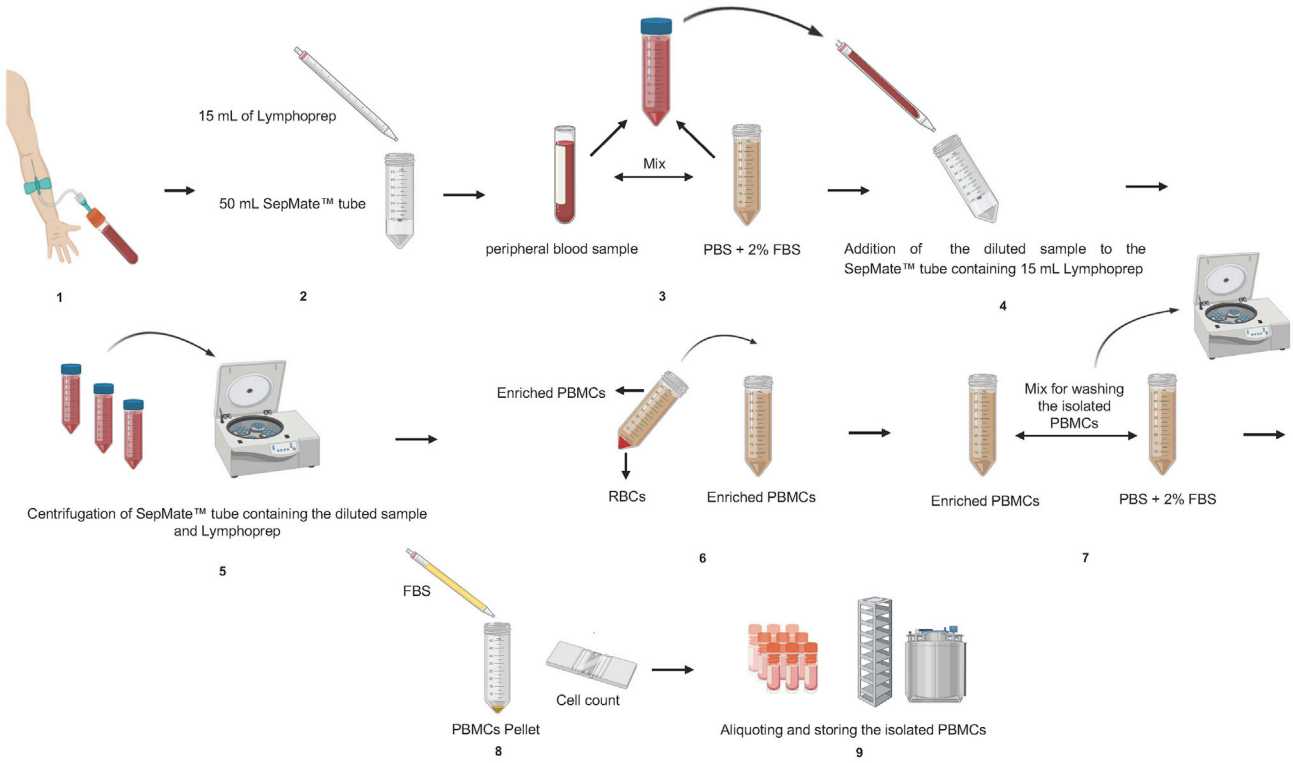

Figure 1. Workflow of Isolation of Human Peripheral Blood Mononuclear Cells

(1) Peripheral blood withdrawal. (2) Preparation of a SepMate tube by adding 15 mL of Lymphoprep into it. (3) Dilution of the peripheral blood sample with an equal amount of PBS + 2% FBS. (4) Transferring diluted sample into the SepMate tube. (5) Centrifugation. (6) Collecting the enriched PBMCs. (7) Washing the isolated PBMCs using FBS. (8) Resuspension of PBMCs in FBS and cell counting. (9) Aliquoting and storing the isolated PBMCs.

4. Add the diluted sample by pipetting it down the side of the tube while keeping the SepMate™ tube in a vertical position. The sample will mix with the density gradient medium above the insert.
5. Centrifuge the SepMate™ tube containing the diluted sample and Lymphoprep at 300 × *g* for 10 min at room temperature (20°C–22°C), with the brake on. ***Note:*** If the samples are older than 24 h, increase the centrifugation time to 20 min.
6. Very quickly (in 2 s) pour off the top layer into a new 50 mL tube. This part contains enriched peripheral blood mononuclear cells (PBMCs).
7. Add 25–50 mL PBS + 2% FBS to the enriched PBMCs and centrifuge it at 300 × *g* for 8 min.
8. Resuspend washed PBMC pellet in 10 mL FBS and obtain cell count/viability using Trypan Blue.
9. Aliquot PBMCs as desired, centrifuge at 300 × *g* for 5 min, resuspend pellet in PBMC freezing media (90% FBS and 10% DMSO), aliquot in cryovials and freeze slowly using CoolCell® Cell Freezing Containers at −80°C. Transfer vials to liquid nitrogen tanks for long term storage. ***Note:*** Recommended concentration for aliquoting PBMC is 2×10^6, 5×10^6, and 10^7.

This part of the protocol allows for isolation of peripheral blood mononuclear cells (PBMCs) from donors for CAR T cell production (Figure 1).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-c-Myc antibody	Miltenyi Biotec	130-116-485

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Patient-derived GBM specimens	n/a	n/a
Human Peripheral Blood Mononuclear Cells	n/a	n/a
HEK293 T Lenti-X cells	n/a	n/a
Chemicals, Peptides, and Recombinant Proteins		
Ammonium Chloride Solution (RBC lysis buffer)	STEMCELL Technologies	07850
Antibiotic/Antimycotic Solution	Wisent Bio Products	450-115-EL
Avertin (2,2,2-Tribromoethanol)	Sigma-Aldrich	75-80-9
bFGF, Human, Recombinant	STEMCELL Technologies	78003.2
Bovine Serum Albumin (BSA)	Wisent Bioproducts	800-095-EG
Buprenorphine: Vetergesic multidose (0.3 mg/mL)	Ceva Animal Health	56492-01
CD271(LNGFR)-PE	Miltenyi Biotec	130-113-421
DMEM, high glucose with pyruvate	Thermo Fisher Scientific	11995073
DNase Vial (D2)	Worthington Biochemical Corporation	LK003170
EGF, Human, Recombinant	STEMCELL Technologies	78006
Fetal Bovine Serum (FBS)	Wisent Bioproducts	098-150
Formalin solution (10%), neutral buffered	Sigma-Aldrich	HT501128
Gibco Geneticin	Thermo Fisher Scientific	11811031
Heparin sodium	Sigma-Aldrich	H3393
Heparin Solution	STEMCELL Technologies	07980
Iodine (10%)	Teva	PUN510685
Iodine (7.5%) with detergent	Teva	PUN104257
Isoflurane USP	Fresenius Kabi	CP0406V2
Isopropanol (70%)	Atlas	LAT917986
Laminin, Mouse, 1 mg	Corning	354232
Liberase™ TM Research Grade	Millipore Sigma	5401127001
Lipofectamine 3000 Transfection Kit	Invitrogen	L30000015
MEM-non-essential amino acids	Thermofisher Scientific	11140050
MycoZapTM Prophylactic	Lonza	VZA-2031
NeuroCult™ NS-A Proliferation Kit (Human)	STEMCELL Technologies	05751
Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific	31985070
Optixcare sterile eye lube	Aventix	17873-1445
PBS pH 7.4, with calcium and magnesium	Wisent Bio Products	311-011-CL
Poly-L-ornithine solution	Millipore Sigma	P4957-50ML
Prime-XV T Cell Expansion Media XSFM	Irvine Scientific	91141
Recombinant hIL-2	Stem Cell	78036.3
Sodium Pyruvate	Thermo Fisher Scientific	11360070
Trypan Blue	Sigma-Aldrich	T8154-100ML
TrypLE™ Express Enzyme (1×), phenol red	Thermo Fisher Scientific	12605028
Other		
10 mL Syringe	BD Becton Dickinson	309604
25 µL Gastight Syringe Model 1702 RN	Hamilton	7654-01

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Alcohol Prep Pads	RED Mecial Supplies	211-MM-05507
Animal Heat Lamp	QC Supply	260038
Cell strainer (70 μm)	Millipore Sigma	CLS431751-50EA
Coated Vicryl Sutures 5-0	Ethicon	J493G
Coronal brain slicing matrix	Harvard Apparatus	72-5033
Countess Chamber Slides	Invitrogen	C10228
DynaMag™-2 Magnet	Thermo Fisher Scientific	12321D
Forceps	Fine Science Tools	FST 11053-10
General Surgery Pack	DRE Veterinary	13346
Gibco Dynabeads Human T-Activator CD3/ CD28	Life Technologies	11131D
Hematology K2EDTA 10.8 mg tubes	BD	367863
Insulin Syringes 0.5 mL/1.0 mL	ELI MEDICAL	U-40
LymphoprepTM	Stem Cell	07801
Needle driver	Fine Science Tools	FST 91201-13
Push Botton. Blood collection set	BD	367326
Regular bevel needles (23 gauge)	BD Becton Dickinson	305145
Saline Bag	Baxter	BAXJB1323
Saline Vials	Valuemed	HOS04888010
SepMateTM-50 (IVD) tubes	Stem Cell	85450
Scissors	Fine Science Tools	FST 14090-11
Sterile Cotton Swabs	Puritan	25-806 1WC
Stylo+ Versatile Craft Tool	DREMEL	2050-15
Surgical Glue	3M	1469SB
Tissue Embedding Cassettes	Kemtech America	1170Z96

MATERIALS AND EQUIPMENT

For preparation of activated T cells from frozen human PBMCs

- Prime-XV T Cell Expansion Media XSFM supplemented with 100 IU/mL stock recombinant hIL-2.
- Dynabeads Human T-Activator CD3/CD29 beads at a concentration of 4×10^4 beads/mL.
- Solution of PBS, 0.1% BSA, 2 mM EDTA.

CAR Lentivirus Production and Collection

- HEK293T cells, preferably low-passage Lenti-X HEKs for greatest yield, plated at 11 million cells (95% confluency) per T75 cm^2 culture flask, left to attach overnight (12–18 h).
- HEK293 Media: DMEM, high glucose with pyruvate, supplemented with 10% FBS, 1% non-essential amino acids.
- Lentiviral packaging media (LVPM): Opti-MEM™ I Reduced Serum Medium supplemented with 5% FBS and Sodium Pyruvate.
- Solution of 10 μg CAR lentiviral plasmid (in this case, expressing scFv recognizing the antigen of interest with a c-myc tag and truncated NGFR), 6.5 μg psPAX2, 4.5 μg pMD2.G, 40 μL P3000, 4 mL Opti-MEM; Solution of 45 μL lipofectamine 3000 and 4 mL Opti-MEM.
- Ultracentrifuge tubes.

Lentiviral Transduction of CAR Constructs into Human T Cells

- Freshly prepared Prime-XV T Cell Expansion Media XSFM supplemented with 100 IU/mL stock recombinant hIL-2.
- 96-Well round-bottom plate.

Materials Required for CAR T Cell Expansion

- Freshly prepared Prime-XV T Cell Expansion Media XSFM supplemented with 100 IU/mL stock recombinant hIL-2.
- 96-, 24-, and 6-Well plates.

Materials Required for Characterization of CAR T Cells

- Trypan Blue, cell counter such as the Countess II Automated Cell Counter (Cat. # AMQAX1000).
- Anti-NGFR antibody and anti-c-Myc (or other appropriate antibody for identifying successfully transduced anti-Tumor-Associated Antigen (TAA) CAR T cells).

STEP-BY-STEP METHOD DETAILS

Lentivirus Production

Timing: 8 days

1. Four days prior to target transfection date, thaw cryopreserved HEK293T Lenti-X cells in a 37°C water bath.
2. Plate cells on 2 10 cm dishes at 1.5–2 million cells per plate in 10 mL of HEK media (DMEM, high glucose with pyruvate, supplemented with 10% FBS and 1% non-essential amino acids).
3. Three days after plating HEK293T Lenti-X cells (24 h prior to transfection), harvest cells from 10 cm plates using 1.5 mL warm TrypLE digest (leave in incubator for 5 min). Wash plate with PBS to collect any remaining cells.

   Note: If cells remain adherent to plate, add an additional 1 mL of TrypLE and incubate for additional 3–5 min, then collect cells.

4. Spin HEK293T Lenti-X cells down at 300 × *g* and resuspend them in 1 mL of PBS. Mix 10 μL of cells with 10 μL of Trypan Blue, take 10 μL of mixture and count by Cell Countess.

   Note: Cells should ideally have a viability of 90%–95%.

5. Spin down remaining cells and resuspend in 1 mL of HEK media. Take required volume to plate cells at 11 million cells per T75 flask, and top up to 15 mL with HEK media, then add to T75 flask. One flask for the control virus and one flask for the anti-TAA lentivirus is sufficient, this can easily be scaled up to create more virus.

   Note: Be sure to coat plates evenly with HEK293T Lenti-X cells.

6. Incubate overnight (12–18 h) at 37°C. Ensure cells are 95% confluent prior to transfection.

   Note: Gently handle cells to ensure cells remain adhered to the plate.

7. For transfection of HEK293T cells, prepare two series of 15 mL tubes for each flask as outlined in Figure 2.

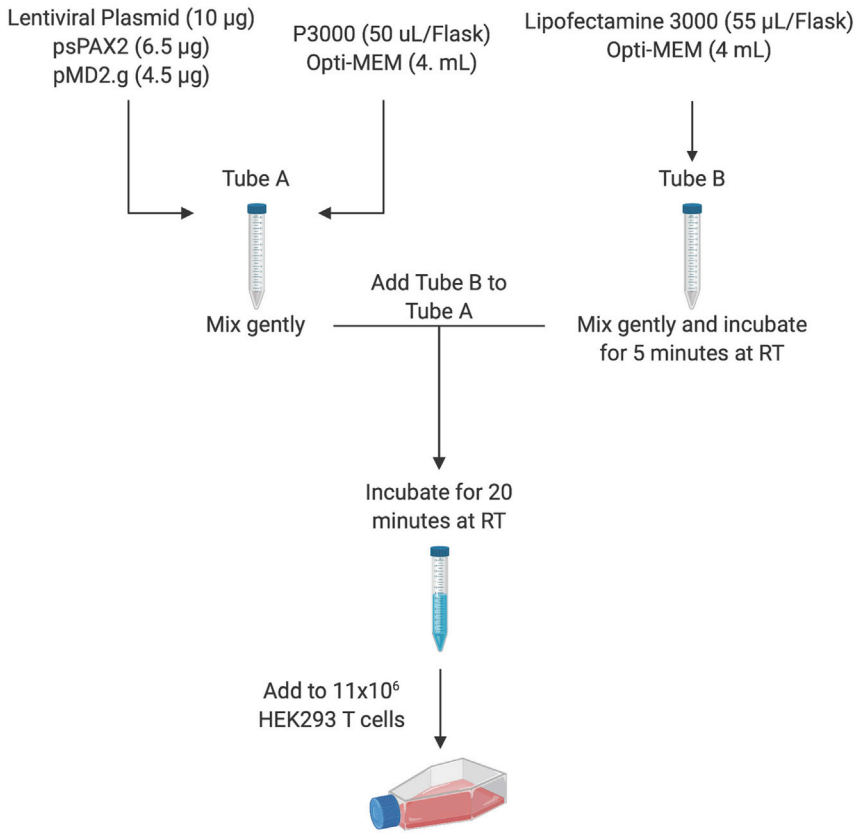

Figure 2. Workflow and Preparation of Reagents for Transfection of Plasmids into HEK293T Cells

Reagents should first be prepared in two separate 15 mL screw cap tubes and mixed gently prior to combining the two solutions and incubating at room temperature (20°C–22°C) for 20 min. Optimization of volumes and masses may be required depending on the plasmid being used.

8. Once Lipofectamine 3000 and DNA have been added to the cells, gently swirl flasks to ensure even distribution of reagents and place into incubator at 37°C.
9. Six hours post transfection, remove media in flasks and replace with 15 mL of warm Lentiviral Packaging Media (LVPM) per flask.

By following the above-mentioned steps, HEK293T cells will be prepared and transfected, resulting in the production of lentiviral particles containing the desired CAR construct.

Lentiviral Harvest and Resuspension

Timing: 2–3 days

Note: You are working with viral particles from this stage. Ensure that all the disposable plastic ware used such as flasks, pipettes etc., are disposed into 20% bleach solution and kept in bleach for at least 30 min before proper disposal.

10. 24 h post transfection, collect viral supernatant in individual 50 mL falcon tubes and wrap the cap with paraffin film. Store at 4°C. Replace media in flasks with 15 mL of warm LVPM per flask, and place back in the incubator for 24–48 h.
11. Pre-cool ultracentrifuge buckets and rotor at 4°C.

12. 24–48 h post media change, harvest viral supernatant from flasks in individual 50 mL falcon tubes.
13. Centrifuge 50 mL falcon tubes containing viral supernatant at 300 × *g* for 5 min to sediment out any collected cells.
14. Add 30 mL of harvested media containing lentivirus to 38.5 mL ultracentrifuge tubes.

⚠ CRITICAL: This volume is required to prevent tube collapse during centrifugation.

15. Centrifuge samples at 42,000 × *g* for 2 h at 4°C with minimal deceleration.
16. Once centrifuged, remove supernatant from tubes and dispense into 20% bleach, being careful to preserve the viral pellet.
17. Resuspend lentivirus in 200 μL DMEM, being sure to wash down the walls of the tube. Homogenize the viral pellet and resuspend at a final concentration of 200 μL/flask, avoiding bubbles.
    a. Transfer virus to 1.5 mL viral cryovial aliquots and place in a −80°C freezer.
    b. Disinfect ultracentrifuge buckets and anything else that has come into contact with virus-containing tubes using 70% ethanol.

By performing this step of the protocol lentivirus particles containing the CAR construct will be collected and ready for immediate or future transduction.

Preparation of Activated T Cells from Frozen Human PBMCs

Timing: 2 h

18. Pre-warm supplemented XSFM media in a 37°C water bath and aliquot 7 mL of media into a 15 mL Falcon tube.
19. Thaw PBMCs and add them dropwise to the media. Spin down at 450 × *g* for 5 min.
20. Aspirate off media without disturbing the pellet and gently resuspend it in 1 mL of supplemented XSFM. Mix 10 μL of cells and 10 μL of Trypan blue, count cells, and record viability.
21. Take the required number of cells, dilute to 1 × 10^5 cells/100 μL with cytokine supplemented XSFM media.
22. Activate T cells with Gibco Dynabeads Human T-Activator CD3/CD28 beads that are in a concentration of 4 × 10^4 beads/μL.
    a. Resuspend beads by vortexing.
    b. Transfer beads to a 1.5 mL eppendorf tube containing 1 mL of PBS with 0.1% BSA, 2 mM EDTA (2 μL of beads for each 100,000 cells and scale up accordingly). Mix gently.
    c. Place beads on DynaMag magnet for 1 min and aspirate the supernatant without disturbing the beads. Remove tube from magnet and resuspend beads in 100 μL per 2 μL of beads of supplemented media.
23. Aliquot 100 μL of T cells per well into a 96-well round-bottom plate and add 100 μL of Dynabead suspension into each well. Incubate at 37°C and 5% CO_2.

This section of the protocol results in isolation and activation of T cells from human PBMCs.

Lentiviral Transduction of T Cells

Timing: 1.5 days

24. After 18–24 h (day 1), carefully remove 110 μL of media from the wells of the 96-well plate containing the PBMCs and the Dynabeads so as not to disturb the pellet of cells.
25. Add 10 μL viral suspension to transduce at a MOI of 1.
26. Incubate overnight (12–18 h) at 37°C and 5% CO_2.

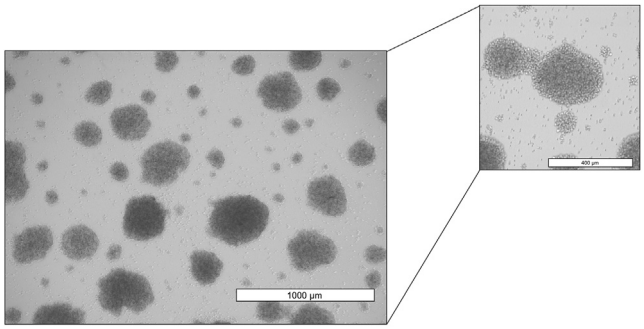

Figure 3. Activated Human PBMCs in Culture on Day 3 Post Thaw

This step results in human PBMC-derived T cells transduction with the desired CAR construct.

CAR T Cell Expansion

Timing: 5+ days (depending on required number of CAR T cells)

27. Check cells daily and assess growth (Figure 3 which shows day 3 of T cell expansion).
28. On day 2, top up 100 µL CAR T cells with XSFM media + hIL2 in each well.
29. On day 4, gently replace 100 µL of spent media with fresh XSFM media + hIL2. Be careful to remove the XSFM only from the top portion of each well without disturbing the cells.
30. On day 5, the CAR T cells should be ready to move to a larger plate (24-wells). However, the users' discretion should be used here to determine whether there are a sufficient number of cells for transfer. Move T cells from the 96-well plate to a single well in a 24-well plate containing 900 µL of supplemented XSFM+hIL2 media. Two wells from the 96-well plate should be combined in a single well in the 24-well plate.
31. Once ~95% of the 24-well is covered, transfer cells to a 6-well plate for continued expansion. Cells in a 6-well plate should be kept in a volume of 4–5 mL of XSFM media. Add fresh cytokine supplemented media on alternate days.

Note: It should be noted that T cells grow best when in close proximity to one another. This should be taken into account before transferring to a larger plate.

Note: Cells are best kept at a density of 100,000 cells/cm^2.

Following the above-mentioned steps in this part of the protocol leads to expansion of large number of CAR T cells, depending on length of expansion.

Characterization of CAR T Cells

Timing: 1–2 h

32. Remove CAR T cells transduced with both the control CAR construct and the anti-TAA CAR construct from wells and gently move them to individual 15 mL screw cap tubes. Spin down at 450 × *g* for 3 min.
33. Gently resuspend CAR T cells in 1 mL of PBS and mix 10 µL of cells with 10 µL of Trypan Blue. Count with Cell Countess.
34. Aliquot approximately 1 × 10^5 CAR T cells from both the control and the anti-TAA tubes, into round-bottom flow cytometry tubes.
35. Spin down tubes and aspirate off the supernatant, then resuspend cells in 100 mL PBS.

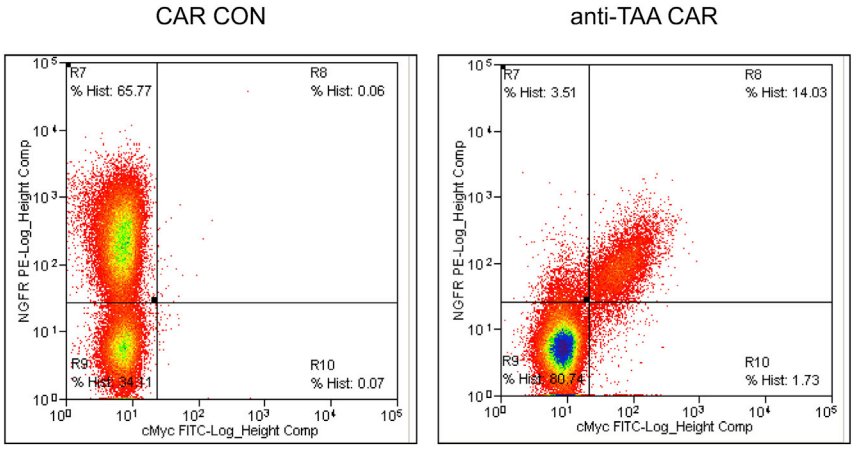

Figure 4. Sample Flow Cytometric Analysis of Control CAR T and Anti-TAA CAR T Tagged with NGFR and NGFR/c-Myc, Respectively

36. With the cells on ice, stain cells anti-TAA CAR Ts with anti-NGFR antibody for 15 min, add 2 mL of PBS, spin down, and aspirate off supernatant.
37. Then, stain both the control and anti-TAA CAR Ts with anti-c-Myc for 15 min. Add 2 mL of PBS, spin cells down, and add 7-AAD to all tubes. The manufacturer's recommended antibody dilution for flow cytometry should be used.
38. Analyze by flow cytometry (Figure 4).

This step allows for confirmation of successfully produced CAR T cells, as well as insights into transduction efficiency.

Intracranial Injections of Patient-Derived Brain Tumor-Initiating Stem Cells (BTICs) into NSG Mice

Timing: 1 day for injections; engraftment of tumor depends on the cell line used in the experiment

In order to preclinically test the anti-tumor effect of generated CAR T cells in GBM model, an *in vivo* patient-derived xenograft model of treatment-refractory GBM needs to be generated.

Generation of patient-derived xenograft model of treatment-refractory GBM:

39. Inject the patient-derived GBM BTICs into the right frontal lobe of mouse brain (Figure 5) (Chokshi et al., 2016).
40. Confirm the tumor formation using imaging depending on the engrafted tumor cells (ie., MRI if GBM cells are not labeled and IVIS imaging if GBM cells express luciferase).
41. Upon confirmation of tumor engraftment, mice will be treated with generated tumor-associated antigen (TAA) specific CAR T cells. The treatment with CAR T cells will start halfway to pre-established endpoint.

Treatment of Patient-Derived Xenograft Mouse Model with Enriched TAA Specific CAR T Cells

42. Preparation of enriched CAR T cells for intracranial injections.
    a. Collect the CAR T cells in a 50 mL falcon tube and spin them down at 450 × *g* for 3 min.

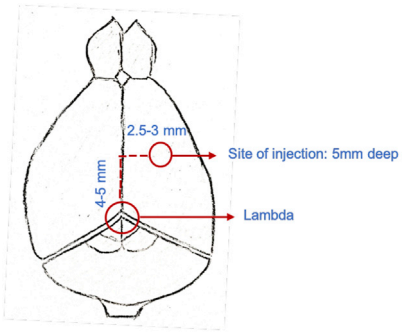

Figure 5. Landmarks for Intracranial Injection of Both Tumor Cells and CAR T Cells

The injection site is 4–5 mm anterior to the coronal suture, 3 mm lateral to the midline, 5 mm deep.

b. Remove the supernatant and resuspend the cells in 1 mL PBS and transfer them to an Eppendorf tube.
c. Place the Eppendorf tube in MagnaRack (magnetic separation rack) to remove the Dynabeads.
d. Count the CAR T cells and resuspend 1 × 10^6 in 10 μL PBS for treating each mouse.

Note: Keep the CAR T cells on ice throughout the injection.

43. Intracranial Injection of TAA specific CAR T cells.
    a. Wipe down all equipment and hood with Clidox prior to start.
    b. Bring the mice which were engrafted with GBM tumor into the surgical room.
    c. Put each animal into anesthetic chamber (2.5% isoflurane + 1 L/min O_2).
    d. Transfer each animal from the anesthetic chamber to the surgical stage.
    e. Put eye gel on each mouse eyes to protect them from drying.
    f. Remove the hair from the incision site and wipe the area with two different percentages of iodine (90% and 50%) and 70% ethanol using cotton swaps.
    g. Make a small incision on the skin and find the injection site.
    h. Drill a burr hole right on the site of tumor engraftment in the frontal lobe (4–5 mm anterior to the coronal suture, 3 mm lateral to the midline).

⚠ CRITICAL: Creating the burr hole can cause excessive bleeding and post-operative complications if not performed carefully. Make sure the drill is not hitting the blood vessels.

    i. Place the Hamilton syringe on the syringe holder and Rinse it with 70% ethanol and then PBS (3 times each).
    j. Load the Hamilton Syringe with 10 μL suspension of CAR T cells (1 × 10^6 CAR T cells in 10 μL of PBS).

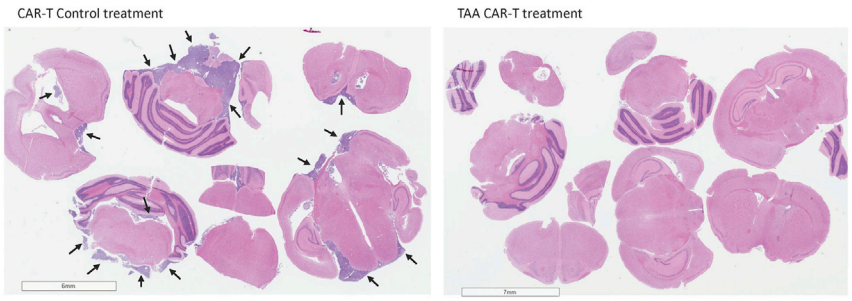

Figure 6. The Efficacy of TAA CAR T Treatment on an *In Vivo* Patient-Derived Xenograft Model

The tumor size is significantly reduced in the TAA CAR T treatment cohort compared with the control CAR T treated group.

k. Inject the cells into the drilled burr hole over 1 min (4–5 mm anterior to the coronal suture, 3 mm lateral to the midline, 5 mm deep as depicted in Figure 5).

⚠ CRITICAL: Take extra care to slowly push the plunger on the Hamilton syringe down
l. at very small intervals to give cells enough time to sit in the brain.

⚠ CRITICAL: After the injection is done, wait for 30 s. This step is necessary to prevent cell flushing out after the injection.
m. Pull out the needle slowly.
n. Close the incision using Maxon stitches and put a droplet of skin glue on it.
o. Record animals' weight and give them 1 mL saline and 0.5 mL Buprenorphine subcutaneously.
p. Put the animals into the cage and place them on a heating pad until they are fully recovered.

44. Perfusion and fixation of brain tumor samples (1 week post last CAR T cell treatment).
    a. Anesthetize mice by injecting 1 mL Avertin i.p. and then cull the mice by cervical dislocation.
    b. Cut the abdomen and the rib cage and then inject 10 mL saline by inserting the needle into the Apex of heart to wash out the blood. Repeat the same step but this time by injecting 10 mL formalin (10%) through the same route to fix the organs.
    c. Cut the skull and remove it slowly without touching the brain tissue. Take out the brain and put it in 10% formalin for histological analysis.

This section of the protocol allows for generation of PDX model for preclinical testing of CAR T cells and other therapeutic modalities

EXPECTED OUTCOMES

The effect of CAR T cell treatment will be assessed by comparing the tumor size and the survival advantage between the animals treated with control CAR T cells versus the group treated with TAA CAR T cell (Figure 6).

LIMITATIONS

Our protocol efficiently produces CAR T cells from healthy donor PBMCs. However, a limitation of the protocol is that T cell populations vary from donor to donor. Some studies suggest that T cell subpopulations vary from donor to donor that affects the efficacy of adoptive T cell therapy. These findings could potentially be extended to CAR T cell therapy (Klebanoff et al., 2012).

This protocol may require optimization of the lentiviral production workflow. Particularly the amount of lentiviral and packaging plasmids being used may vary depending on the selected target and the plasmids being used.

When considering limitations for *in vivo* intracranial injections, we must take into account that creating the burr hole can cause excessive bleeding and post-operative complications if not performed carefully. Additionally, an increase in intracranial pressure after tumor engraftment may cause difficulty when administering CAR T cell injection, resulting in backflow of the therapeutic solution out of the burr hole.

Given that the mice in the protocol being used are NSG mice (immunodeficient), there may be some differences in CAR T cell efficacy in humanized mouse models compared to immunodeficient models due to having an active immune systemin humanized mice.

TROUBLESHOOTING

Problem 1

In some cases, there are some red blood cells (RBCs) present in the isolated PBMCs.

Potential Solution 1

Throughout the PBMC isolation process, some RBCs might pass into the PBMC pellet. If there are RBCs present in the PBMC pellet, add 10 mL of RBC lysis buffer to the PBMC pellet and incubate it for 5 min at room temperature (20°C–22°C). After 5 min, top up the PBMC solution to 50 mL by adding PBS + 2% FBS and centrifuge tubes at 300 × *g* for 8 min.

Problem 2

Makeup of T cell subsets varies from donor to donor

Potential Solution 2

Many groups have shown that certain T cell populations are better suited for adoptive T cell therapy than others, demonstrating that they are less prone to exhaustion or differentiation into undesirable T cell subsets such as regulatory T cells (Davidson et al., 2019; Park et al., 2019). By sorting for specific T cell subpopulations, the variation from donor to donor may be mitigated. Users of the protocol would need to decide for themselves what subpopulation they think will be more efficacious for them. Generally naïve and memory T cell subsets are less capable of entering the tumor microenvironment.

Problem 3

Lentiviral production will require optimization depending on the construct.

Potential Solution 3

This is most likely to be problematic during the transfection into HEK293T cells and may result in lower transfection efficiency and viral titer. However, this problem can easily be overcome by consistently measuring viral titer, adjusting the amount of lentiviral plasmid and confirming the quality of the DNA used during the transfection accordingly.

Problem 4

Drilling of the burr hole for intracranial injection may result in excessive bleeding and post-operative complications.

Potential Solution 4

The location for drilling a burr hole in preparation for intracranial injections has been specifically selected as such as it has a limited number of surrounding blood vessels compared to other areas in the skull. As such, bleeding should be limited, provided the hole is not drilled too deep.

Problem 5

Increased intracranial pressure from the xenografted tumor may cause backflow when injecting CAR T cells.

Potential Solution 5

When administering the intracranial dose of CAR T cells, the user should take extra care to slowly push the plunger on the Hamilton syringe down at very small intervals, thus allowing the therapeutic solution to spread and settle within the brain, prior to pushing the plunger further. For the same reason, after the full volume of solution has been injected, the Hamilton syringe should not immediately be removed, but should instead rest within the brain for approximately 30 s. Gently twist the Hamilton syringe as it is removed, as this will also help prevent cells from flushing out.

Problem 6

Humanized mouse models may have further hurdles compared to NSG or immunodeficient models.

Potential Solution 6

This is a problem which many groups are working on, and there is no simple solution. Potential solutions are changing the dosage of CAR T cell therapy used, the specific subpopulations of CAR T cells, and polytherapy solutions. Allogeneic CAR T cells have also been proposed in order to overcome the recognition by the host immune system.

RESOURCE AVAILABILITY

Lead Contact

Dr. Sheila Singh, Professor, Department of Surgery, Division of Neurosurgery, Faculty of Health Sciences, Email: ssingh@mcmaster.ca

Materials Availability

Requests for cell lines and information of all other pertinent information can be directed toward Dr. Sheila Singh.

Data and Code Availability

No exclusive data or code was used or generated for this method.

ACKNOWLEDGMENTS

This work was supported through a program project grant from the Terry Fox Research Institute, the catalyst program grant from Bio CanRx and Canadian Institute of Health Research to S.S.

AUTHOR CONTRIBUTIONS

N.T., W.M., and S.S. drafted the protocol. W.M. and S.S. described the *in vitro* culturing of CAR T cells. N.T. described the isolation of PBMCs, T cell expansion, and preclinical testing of CAR Ts in PDX GBM models. D.M. provided the Key Resources Table. C.V. and S.S. edited the manuscript. N.T. and S.S. prepared the figures. N.T. prepared the graphical abstract.

DECLARATION OF INTERESTS

S.S. is a scientific advisor for Century Therapeutics Inc. and her role in the company has been reviewed and is supported by McMaster University.

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