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.
Protocol
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Note: For each 50 mL peripheral blood, three SepMate™ are required to be able to add the whole withdrawn blood.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.
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          | Miltenyi Biotec | 130-116-485 |

(Continued on next page)
| 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: Vetersgec 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 Genetecin | 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 | CP0465V2 |
| Isopropanol (70%) | Atlas | LAT917986 |
| Laminin, Mouse, 1 mg | Corning | 354232 |
| Liberase™ TM Research Grade | Millipore Sigma | 5401127001 |
| Lipopectamine 3000 Transfection Kit | Invitrogen | L30000015 |
| MEM-non-essential amino acids | Thermo Fisher 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 V Cell Expansion Media XSFM | Irvine Scientific | 91141 |
| Recombinant hIL-2 | Stem Cell | 78033-3 |
| Sodium Pyruvate | Thermo Fisher Scientific | 11360070 |
| Trypan Blue | Sigma-Aldrich | T8154-100ML |
| TrypLE™ Express Enzyme (1x), 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)
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/CD28 beads at a concentration of \(4 \times 10^4\) 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\textsuperscript{TM} 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.

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### 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\textsuperscript{TM}-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 |
| Lymphoprep\textsuperscript{TM} | Stem Cell | 07801 |
| Needle driver       | Fine Science Tools | FST 91201-13 |
| Push Button. Blood collection set | BD | 367326 |
| Regular bevel needles (23 gauge) | BD Becton Dickinson | 305145 |
| Saline Bag          | Baxter | BAXJB1323 |
| Saline Vials        | Valumed | HOS04888010 |
| SepMate\textsuperscript{TM}-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 | 14695B |
| Tissue Embedding Cassettes | Kemtech America | 1170296 |
Lentiviral Transduction of CAR Constructs into Human T Cells

- Freshly prepared Prime-XV T Cell Expansion Media XSF 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 XSF 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 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.

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

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.
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% CO₂.

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% CO₂.
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 XSF media + hIL2 in each well.
29. On day 4, gently replace 100 μL of spent media with fresh XSF media + hIL2. Be careful to remove the XSF 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 XSF+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 XSF 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/cm².

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 × 10⁶ 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.
36. With the cells on ice, stain 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.
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 $1 \times 10^6$ 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 O₂).
   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 ($1 \times 10^6$ CAR T cells in 10 μL of PBS).

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.

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 at very small intervals to give cells enough time to sit in the brain.

l. 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 system in 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.

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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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