Despite a surge in the preclinical development of immunotherapies, current models are unable to predict putative toxicity, particularly the “on-target, off-tumor” effects of these therapeutics. To address this gap, we used a humanized mouse model of hematopoiesis to examine the toxicity profile of CAR-Ts targeting brain tumor-antigens that are also expressed in the hematopoietic system. In assessing the safety of cell-based therapies, we aim to develop and integrate a preclinical evaluation protocol as a necessary step in the clinical development pathway.
Protocol
Assessing the Safety of a Cell-Based Immunotherapy for Brain Cancers Using a Humanized Model of Hematopoiesis

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SUMMARY
Despite a surge in the preclinical development of immunotherapies, current models are unable to predict putative toxicity, particularly the “on-target, off-tumor” effects of these therapeutics. To address this gap, we used a humanized mouse model of hematopoiesis to examine the toxicity profile of CAR-Ts targeting brain tumor-antigens also expressed in the hematopoietic system. In assessing the safety of cell-based therapies, we aim to develop and integrate a preclinical evaluation protocol as a necessary step in the clinical development pathway. For complete details on the use and execution of this protocol, please refer to Vora et al. (2020).

BEFORE YOU BEGIN
Isolation of Lineage-Negative Mononuclear Cells (MNC) from Cord Blood Samples

@ Timing: 4 h

1. Primary cord blood samples are obtained with informed consent from patients and performed with the approval of the Research Ethics Board or Institutional Review Board.
2. Human pathogens may be present in cord blood samples. Adhere to appropriate biosafety protocols during handling. All instruments/containers used for processing should be decontaminated with freshly made 10% bleach after use.

Preparing PEF Buffer and PBS-EDTA

@ Timing: 5 min

3. PEF: In 1 × D-PBS, add FBS (to 2%) and EDTA (to 2 mM).
   a. eg. in 500 mL D-PBS, add 10 mL of FBS and 2 mL of 0.5 M EDTA, pH 8.0
4. PBS-EDTA: In 50 mL of 1x D-PBS, add 200 µL of 0.5 M EDTA, pH 8.0 and mix well to make a 200 mM solution of PBS-EDTA.

Transplantation of Human HSPCs into NSG Mice

© Timing: 2 h (depending on the number of mice)

5. All animal work should be performed with the approval of Animal Care Committees.
6. 6–8 week old immunocompromised NOD/Lt-scid/IL2Rγnull (NSG) mice were used for the xenotransplantation, which need to be housed in proper barrier conditions. All cages, food, bedding, and water should be autoclaved before entering the facility. All handling of animals should occur in Biological Safety Cabinets (BSCs) disinfected with Clidox (or similar disinfecting agents). All instruments should be autoclaved or disinfected with 70% ethanol prior to use.
7. Xenotransplantation of HSPCs requires preconditioning irradiation. Guidelines and safety precautions of the institution should be followed when operating an irradiator.

Assessing for Chimerism through Flow Cytometry

8. A panel of fluorophore conjugated antibodies, in compatible channels with your cytometer, should be selected prior to the experiment.

Isolation of MNCs from Bone Marrow and Spleen Samples

9. All surgical instruments should be autoclaved and opened in the BSC. Instruments required include: sharp scissors, forceps, and mortar+pestle.

Thawing Cord Blood MNCs/HSPCs

© Timing: 10 min

10. Prepare one 50 mL conical centrifuge tube with 5 mL of filtered FBS. Warm to 37°C in a water bath.
11. Retrieve one cryovial of cryopreserved sample (MNCs, lineage-depleted MNCs, or CD34+ HSPCs) from −150°C storage. Gently thaw in 37°C water bath, mixing occasionally to ensure even thawing. Remove the vial immediately after the sample has entered the liquid phase.
12. Gently transfer the contents with a micropipette to the sample tube prepared in step 1.
   a. Wash the cryovial tube with an additional 1 mL of room temperature D-PBS and combine with sample in the 50 mL tube.
13. Slowly add 30 mL of room temperature D-PBS dropwise to the 50 mL conical centrifuge tube.
14. Spin down the cells at 450 x g for 5 min.
15. Carefully remove the supernatant and resuspend the cells in 1 mL of PEF.
   a. If desired, count the cells manually with a hemocytometer.

Surgical Procedures

16. Gaseous anesthesia machine with an isoflurane chamber and nose cone attachment is necessary for transplantation.
17. All procedures should be carried out in a sterile BSC.
18. Analgesics (eg. 0.05–1 mg/kg buprenorphine) and saline are required for invasive procedures such as intracranial and intrafemoral injections.
Preparation of CAR-T Cells for Injection

© Timing: 10 min

Fresh or cryopreserved, CAR-T cells, or any cell-based therapy equivalent, should be prepared prior to use in this protocol. Depending on the dosing regimen of choice, the absolute number of CAR-Ts required for this experiment may be slightly adapted.

19. Collect cultured CAR-T cells into an appropriate volume centrifuge tube.
   a. Spin cells at 300 × g for 3 min.
20. Remove the supernatant and resuspend the pellet in 1 mL of PBS. Count the cells using an automated cell counter.
21. Adjust the volume of PBS accordingly to have the appropriate cell number in 10 μL for intracranial injections, and 100 μL for intravenous injections. Keep cells on ice prior to injection.

KEY RESOURCES TABLE

| Reagent or Resource | Source | Identifier |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| CD3-PECy7           | BD Biosciences | Cat#557851, RRID:AB_396896 |
| CD19-APC            | BD Biosciences | Cat#555415, RRID: AB_398597 |
| CD33-PE             | BD Biosciences | Cat#347787, RRID: AB_400350 |
| CD34-FITC           | BD Biosciences | Cat#555821, RRID: AB_396150 |
| CD38-PECy7          | BD Biosciences | Cat#335790, RRID: AB_399969 |
| CD45-BV421          | BD Biosciences | Cat#563879, RRID: AB_2744402 |
| CD133/2-PE          | Miltenyi Biotec | Cat#130-113-186130-080-081Cat c |
| Compbeads           | BD Biosciences | Cat#552843 |
| Goat Anti-Human IgG AF488, F(ab’)2 fragment specific | Jackson ImmunoResearch | Cat#109-546-097, RRID:AB_2337849 |
| Goat Anti-Human IgG, F(ab’)2 fragment specific | Jackson ImmunoResearch | Cat#109-005-006, RRID: AB_2337533 |
| Human IgG           | Millipore Sigma | Cat#I4506 |
| Myc-FITC            | Miltenyi Biotec | Cat#130-116-653, RRID:AB_275132 |
| M13-HRP             | Abcam | Cat#50370, RRID:AB_881599 |
| Mouse Fc block      | BD Biosciences | Cat#553142 |
| NGFR-PE             | Miltenyi Biotec | Cat#130-112-790, RRID:AB_2725887 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| 7AAD Viability dye  | Beckman Coulter | Cat#A07704 |
| Ammonium Chloride (RBC Lysis Buffer) | STEMCELL Technologies | Cat#07850 |
| DMEM                | ThermoFisher | Cat#11995073 |
| EDTA                | Millipore Sigma | Cat#20158 |
| Fetal Bovine Serum, heat inactivated (FBS) | Wisent | Cat#098-150 |
| Ficoll-Paque Plus   | GE Healthcare | Cat#17144003 |
| IMDM                | GE Healthcare | Cat#SH30228FS |
| Isoflurane USP      | Fresenius Kabi | Cat#CP0406V2 |

(Continued on next page)
### Continued

| Reagent or Resource                  | Source                  | Identifier       |
|-------------------------------------|-------------------------|------------------|
| PBS                                 | ThermoFisher            | Cat#10010049     |
| Proviodine Solution, USP 10%        | Teva                    | PUN510685        |
| Proviodine Detergent, USP 7.5%      | Teva                    | PUN104257        |
| Trypan blue                          | ThermoFisher            | Cat#15250-061    |

### Critical Commercial Assays

| Assay                                | Source                      | Identifier       |
|--------------------------------------|-----------------------------|------------------|
| EasySep™ Human Progenitor Cell       | STEMCELL Technologies       | Cat#19356        |

### Software

| Software | Source                  | Website                                      |
|----------|-------------------------|----------------------------------------------|
| BD FACSDiva | BD Biosciences             | https://www.bd.com/instruments/research-instruments/flow-cytometry-acquisition/facsdiva-software |
| FlowJo   | FLOWJO LLC                | https://www.flowjo.com/                      |
| GraphPad Prism 5.0 | GraphPad Software Inc.    | http://www.graphpad.com/scientific-software/prism/ |

### Experimental Models: Cell Lines

| Model | Source                  | Identifier       |
|-------|-------------------------|------------------|
| CAR-T cells | Please refer to Vora et al. (2020) | N/A              |
| Cord Blood | This protocol | N/A              |

### Experimental Models: Organisms/Strains

| Organism/Strain | Source                  | Identifier       |
|-----------------|-------------------------|------------------|
| NOD-scid IL2Rgammanull (NSG) Mouse | Jackson Laboratory | Stock # 005557  |

### Other

| Item                                        | Source                  | Identifier       |
|---------------------------------------------|-------------------------|------------------|
| 1/2 mL U-100 insulin syringe                | BD                      | Cat#CABD329461   |
| 1.5 mL microcentrifuge tube                 | Fisher Scientific       | Cat#05-408-129   |
| 10 mL Hamilton syringe                      | Hamilton                | Cat#76350-01     |
| 12-well plates                              | Coming                  | Cat#353043       |
| 100 mm Ultra-Low Attachment Culture Dish    | Coming                  | Cat#431110       |
| 27.5G needle                                | BD Biosciences          | Cat#305109       |
| 250 mL PETG bottle                          | ThermoFisher            | Cat#2019-0250    |
| 3 mL syringe                                | BD Biosciences          | Cat#309657       |
| 3 x 3 inch Sterile Gauze, 4-ply             | CanMedDirect            | Cat#84133        |
| 40 μm Cell strainer                         | Falcon                  | Cat#352340       |
| 5.0 Dexon Sutures                           | Ethicon                 | J493G            |
| 5 mL round-bottom polystyrene tubes         | Coming                  | Cat#CA60819-820  |
| 5 mL round-bottom polypropylene tubes       | Coming                  | Cat#CA60819-761  |
| 5 mL round-bottom polystyrene tubes with 35 μm strainer lid | Coming | Cat#CA21008-948  |
| 50 mL Conical Tube                          | FroggaBio               | Cat#TB50-500     |
| 5810 Centrifuge                             | Eppendorf               | Cat#022625004    |
| 70 μm Cell strainer                         | Falcon                  | Cat#08-771-2     |
| Cryogenic tubes                             | Thermo Fisher           | Cat#03-337-7Y    |
| Dialysis Membrane                           | Spectrapor              | Cat#08-667A      |
| Fisherbrand Porcelain Mortar                 | Fisher Scientific       | Cat#FB961A       |
| Fisherbrand Porcelain Pestle                 | Fisher Scientific       | Cat#FB961K       |
| Forceps                                     | VWR                     | Cat#89259-940    |

(Continued on next page)
STEP-BY-STEP METHOD DETAILS
Processing Cord Blood Samples for Lineage-Negative MNCs [SP1]

© Timing: 4 h

This section describes the steps necessary to isolate mononuclear cells (MNCs) from fresh cord blood samples and further enrich for hematopoietic stem and progenitor cells (HSPCs) used in xenotransplantation.

1. Prepare one 50 mL conical centrifuge tube with 15 mL of Ficoll-Pacque for every 12 mL of cord blood sample.
2. Dilute cord blood samples at a ratio of 1:2 with D-PBS. For large volumes, this can be done in sterile 250 mL/500 mL PETG bottles.

Note: Use of an anticoagulant during blood collection is highly recommended, and samples should be stored at 4°C to preserve cells. Samples should be processed as soon as possible, although we have found samples less than 72 h old may still be used with minimal impact on cell viability.

Note: Diluting with a higher ratio of D-PBS (1:3–1:5) can be adopted to increase cell yield, but is inconvenient when processing large volumes of cord blood.

3. Very slowly layer the diluted cord blood onto the surface of the pre- aliquoted Ficoll-Pacque by inclining the tube and running sample down the side. In doing so, two distinct layers should be formed; 15 mL Ficoll-Pacque and 35 mL diluted cord blood. Special care should be taken to avoid mixing.

Note: Use of a Pipet-Aid on a slow setting is recommended.

4. Centrifuge the tubes at room temperature at 450 x g for 20 min on the lowest acceleration/deceleration settings (on a 5810 centrifuge, set to “0”).
5. Once completed, you should see a white cloudy layer at the plasma/Ficoll interface, as well as a large red blood cell (RBC) pellet at the bottom. Harvest the white cloudy layer, which represents the mononuclear cells (MNCs), into a fresh 50 mL conical centrifuge tube (see Figure 1). Do not combine tubes.
Prior aspiration of most of the plasma layer can be done to reduce platelet contamination in your final sample. Transfer of the Ficoll layer should be minimized as it may interfere with downstream yield.

6. Dilute the MNCs 1:2 with D-PBS and centrifuge at room temperature at 500 × g for 5 min on half brake (on a 5810 centrifuge, set to “5”). Carefully aspirate the supernatant. At this point you should see a large red pellet.

7. Resuspend the pellet in 5 mL of 0.8% ammonium chloride to lyse RBCs and incubate for 10–15 min at 4°C. Tubes from the same cord blood sample may be combined at this step.

8. Centrifuge samples at room temperature at 500 × g for 10 min on half brake (on a 5810 centrifuge, set to “5”). At this point you should see a thick white pellet. Aspirate the supernatant and resuspend MNCs in 1–3 mL of PEF.

9. Count the number of viable cells.

10. For enrichment of HSPCs, the EasySep Human Progenitor Cell Enrichment Kit with Platelet Depletion is used according to the manufacturer’s EasySep Magnet protocol. Aliquot 100 million MNCs, resuspended in 2 mL of PEF, into 5 mL (12 × 75 mm) polystyrene round-bottom tubes and follow manufacturer’s instructions.

11. Pellet and resuspend the lineage-depleted sample (Lin−) in PEF and count viable cells. Samples are now ready for FACS or cryopreservation.

Note: For cryopreservation, cryovials should be frozen in a Mr. Frosty or other freezing container at −80°C and transferred to long-term storage at −150°C within 24 h.

Pause Point: Lin− cord blood samples may be cryopreserved for use at a later date in 90% FBS/10% DMSO. However, one freeze-thaw cycle can decrease cell viability by >50%. Thus it is highly recommended to use fresh cells whenever possible.

Enrichment of HSPCs through FACS [SP2]

Timing: 2 h

This section describes the steps for preparation and enrichment of CD34+ HSPCs for xenotransplantation. The use of such HSPCs enables a robust long-term multilineage graft.

12. Thaw a vial of previously cryopreserved cells or use freshly prepared Lin− cells (as in [Before You Begin: SP10]). Resuspend up to 1 × 10^7 cells in 100 μL of PEF, and aliquot a small number of cells (~10,000) separately to use as a negative staining control.
**Note:** Count viable cells to ensure sufficient numbers. For calculations and expected FACS yields, see expected outcome section.

13. Fc block by adding 1 μg of human IgG from serum to every 100,000 cells. Incubate at room temperature for 10 min.

14. Add anti-CD34 APC antibody (1:100) and 7AAD (1:75) to the samples and incubate at 4°C for 20 min.

**Note:** To prevent photobleaching, the BSC light should be turned off for all procedures involving fluorescent antibodies. All stained samples should be covered (eg. with aluminum foil) whenever possible to minimize exposure to light.

15. Wash samples by topping up with 1 mL of fresh PEF and spin down at 450 x g for 5 min. Carefully aspirate the supernatant without disturbing the pellet and resuspend in 500 μL of PEF.

**Note:** To remove cell clumps that may clog the FACS sorter, transfer through a 5 mL round-bottom polystyrene tube with 35 μm strainer lid.

16. Prepare a collection tube with 500 μL of PEF added.

**Note:** Sample and collection tube format may depend on the specification and configuration of the FACS sorter. Most accept 5 mL round-bottom tubes or 1.5 mL microcentrifuge tubes. Softer material such as polypropylene is recommended for collection tubes, which may also be pre-coated with FBS prior to the addition of PEF to maximize cell viability following sorting.

17. Isolate HSPCs through FACS by gating on singlet live cells with CD34 positivity.

18. Centrifuge the sorted cells at 450 x g for 5 min, and resuspend in 1 mL of PEF. Count the viable cells using a hemocytometer.

19. Prepare HSPCs for intravenous injection by resuspending cells in IMDM with 2% FBS. Typically 15,000 to 20,000 sorted CD34+ HSPCs are sufficient to ensure robust engraftment within 8–12 weeks. To reduce variation between mice, all injections should be prepared in the same tube, accounting for 100 μL per mouse and an extra half dose to account for loss of sample through needle retention.

20. Keep the cell suspension on ice until injection.

**Note:** Alternatively, CD34 positive selection kits are commercially available (Stem Technologies #17856, #17879, #17896, #17897) that can directly select for CD34+ HSPCs. This method allows you to bypass FACS enrichment [SP2] and proceed directly to transplantation [SP3], which theoretically is more economic and exposes cells to less stress. However, in our hands we have found final cell yields from this approach are generally lower.

**Transplantation of Human HSPCs into NSG Mice [SP3]**

© Timing: 2 h (depending on the number of mice)

This section describes the steps for the conditioning of NSG mice and subsequent transplantation of cord blood HSPCs. The following steps are used to establish a cohort of mice with humanized hematopoietic systems.

21. Irradiate NSG mice at a dose of 315 cGy, a sublethal dose, to condition the hematopoietic system for xenotransplantation.

**Note:** Irradiation should be done 16–48 h prior to cell injection.
22. To facilitate efficient intravenous injection into the tail vein, place a heat lamp on top of the cage to stimulate vasodilation.

**Note:** Remove any bedding and enrichment structures to facilitate heating. Hyperthermia and burns are possible with the use of a heat lamp, so monitor mice for any signs of extreme discomfort throughout its use. Alternatively, a thermacage may be used which offers a higher degree of temperature control.

23. Load each cell suspension (100 μL) into a 1/2 cc insulin syringe prior to each mouse injection.

**Note:** It is important to mix the cell suspension by gently flicking the tube prior to loading each syringe. This ensures an even distribution of cells between mice. Loading each syringe immediately prior to injection prevents the cells from settling.

24. Place the mouse in the tail-vein restrainer, facing away, and wipe the tail with 70% ethanol and sterile gauze.

25. Identify the veins which run lateral to the tail on both sides, and can be visualized as a faint pink line.

26. Restrain the tail with one hand keeping the proximal section of the tail straight and the distal end angled to the side. Hold the needle in line with the tail vein with the bevel of the needle facing upwards (see Figure 2). Then slide the needle gently into the vein just under the skin. The placement of the needle in the vein can be confirmed by lightly applying negative pressure to the syringe and observing for a flash of blood in the needle hub.

27. Gently push the plunger and inject the cell suspension into the tail vein. If successful, there should be minimal resistance and the vein should blanch. Blebbing around the site of injection is a sign of accidental subcutaneous injection.

28. Withdraw the needle and apply firm pressure with dry sterile gauze until bleeding stops. Return the mouse to the cage and monitor for an additional 10 min to ensure bleeding has stopped.

**Bone Marrow Aspiration [SP4]**

© **Timing:** 1–2 h (depending on the number of mice)

This section covers the steps used to assess the engraftment level of human HSPCs in NSG mice, 8–12 weeks post xenotransplant. Bone marrow aspiration allows for the assessment of stem and progenitor cells within the graft, which are cell populations that are difficult to assess in peripheral blood. A video guide providing excellent visual aid for this procedure may also be found at: https://www.jove.com/t/51660/femoral-bone-marrow-aspiration-in-live-mice (Chung et al., 2014).

29. For each mouse, prepare a sample tube filled with 500 μL of PEF, and a 3 mL syringe with a 27.5G needle attached.

30. Anesthetize the mice in an isoflurane chamber. Remove one mouse and maintain under continuous flow of isoflurane with a nose cone during aspiration.

**Note:** Respiratory rate should be monitored throughout the procedure to ensure anesthesia is appropriately maintained. Ophthalmic ointment may be applied to prevent corneal drying.

31. Prepare the knee (aspiration site) with a three stage surgical prep. This consists of a wash with disinfectant soap (Proviodine detergent), followed by 70% ethanol to remove grease from the skin, and a final disinfectant paint (Proviodine disinfectant). Sterile gauze squares or cotton tipped applicators may be used, starting from the “injection” site and moving toward the periphery.
32. With the mouse lying supine, flex the leg to orient the femur and tibia in a vertical position with the foot resting flat on the surgical area. The hip should be abducted about 30 degrees. Brace the femur with your index finger for additional stabilization where needed (see Figure 2).

33. Fill the 3 mL syringe with the previously prepared 500 μL of PEF. Align the syringe and needle with the angle of the femur and insert the needle through the patellar tendon. The needle should rest in the cavity between the condyles of the femur.

34. Rotate the needle clockwise and counter-clockwise in a drilling motion while applying gentle pressure. The needle should overcome an initial resistance and enter into the medullary cavity. Its location should be confirmed by gentle lateral movements that should feel restricted by the femur.

Figure 2. Injection and Aspirate Route Schematic
A visual guide to the injection sites for (A) Tail-vein injections used for HSPC xenotransplantation and intravenous CAR-T treatment; (B) intrafemoral visual for bone marrow aspirate; (C) intracranial injection site for CAR-T treatment.
35. While still inside the medullary cavity, create negative pressure by gently pulling up on the plunger then letting it depress, several times, to aspirate a small amount of bone marrow. The appearance of red marrow in the syringe indicates a successful aspiration.
   a. Transfer the contents of the syringe to the sample tube and use the syringe to aspirate and expel the contents several times to create a single cell and keep on ice until analysis.

   **Note:** After drilling, the needle can become obstructed by bone debris which can be dislodged by removing the needle, and gently pushing down on the plunger and expelling a small amount of PEF into a disposal tube. The needle can then be inserted back in the femoral cavity to continue the aspiration.

36. Repeat for all mice and monitor for recovery. Administer analgesics (e.g., 0.05–1 mg/kg buprenorphine) as per your animal care committee requirements.
37. Pellet cells by spinning at 450 × g for 5 min, and resuspend each in 2 mL of ammonium chloride to lyse RBCs. Immediately spin again at 450 × g for 5 min and aspirate ammonium chloride to be left with a white pellet. Resuspend cells in 1 mL of PEF.
38. Cells can be counted and are now ready for downstream analysis.

||Pause Point: See Troubleshooting 1 if no appreciable levels of engraftment after flow cytometric analysis [SP5].

### Assessing for Chimerism through Flow Cytometry [SP5]

© Timing: 2 h

This section outlines the steps used to assess engraftment of human HSPCs and recapitulation of a human hematopoietic system in the NSG mouse. This can be applied to a variety of sample types such as peripheral blood or bone marrow.

39. Resuspend desired number of cells in 100 μL of PEF.
40. Fc block with both IgG from human serum (1 μg/100,000 cells) and mouse FcBlock (1:75). Incubate at 4°C for 15 min. Set aside 10,000 cells as unstained control.
41. Prepare a 2× master mix of antibodies to assess cell surface marker expression. Panel listed below was used to assess the graft and HSPC populations before and after CAR-T treatment (Table 1).

| Antibody | 1× Stain | 2× Mastermix |
|----------|----------|--------------|
| Anti-human CD45-BV421 | 1:100 | 1:50 |
| Anti-human CD19-APC | 1:100 | 1:50 |
| Anti-human CD34-FITC | 1:100 | 1:50 |
| Anti-human CD38-PECy7 | 1:400 | 1:200 |
| Anti-human CD133-PE | 1:100 | 1:50 |
| 7AAD | 1:75 | 1:37.5 |

**Note:** Turn off light in the BSC and avoid direct exposure to light sources from this step onwards. Samples should be covered (e.g., with aluminum foil) whenever possible.

42. Add master mix 1:1 to the samples and mix thoroughly by gently pipetting up and down. Incubate at 4°C for 20 min.
Note: In addition, prepare single stains for compensation (and fluorescence minus one controls, if desired).

43. Wash the sample by topping up with 1 mL of fresh PEF and spin down at 450 × g for 5 min. Then carefully aspirate the supernatant without disturbing the pellet. Repeat the wash two times and resuspend in a final volume of 200 μL of PEF.

44. Transfer the sample to 5 mL round-bottom polystyrene tubes through 35 μm strainer tops to remove cell clumps.

45. Analyze the samples with a flow cytometer.

Assessment of Systemic Leakage of CAR-T [SP6]

© Timing: 4 weeks

In this section, the basal leakage of intracranial CAR-T treatment in non-tumor bearing, non-humanized mice will be determined in order to predict the systemic effects and/or toxicity on the hematopoietic system.

46. Divide mice into three cohorts to collect blood 24 h, 1 week, and 2 weeks post treatment. Prepare a cohort of untreated mice that undergo surgery as a negative control for later analysis.

47. In non-tumor bearing mice, inject CAR-T cells into the right frontal lobe of mice.
   a. Place mice in a chamber to be anesthetized in an isoflurane chamber and maintained under a continuous flow with a nose cone during the procedure. Confirm deep anesthetization by lack of toe-pinch reflex.
   b. Prepare the Hamilton syringe by rinsing it with 70% ethanol, and then PBS numerous times. Collect 10 μL of the prepared CAR-T mixture.
   c. Using scissors or a razor, remove fur from the head of the mouse to clear the area for incision.
   d. Disinfect the area by first swabbing the skin with disinfectant soap (Proviodine detergent), followed by 70% ethanol, and a final disinfectant paint (Proviodine disinfectant).
   e. Create a small, vertical midline incision and locate the lambda of the skull. Move along approximately two millimeters to the right of the lambda, and one millimeter rostrally. Approximately one millimeter caudal to the right coronal suture, create a small hole in the skull of the mouse using a Dremel tool with a small burr attachment (see Figure 2).
   f. Insert the Hamilton syringe approximately 3 mm deep into the brain and slowly inject the cells at a rate of 1 μL/s to reduce backflow.
   g. Wait 10 s, and then slowly remove the needle from the brain.
   h. Close the incision by suturing the skin together with 5.0 dexon sutures and seal it with tissue glue.

48. One week later, inject a second dose of CAR-T cells in the same drill site created a week earlier.

Note: This technique was adapted off of the Kim et al. publication (Kim et al., 2006). A stereotaxic instrument may be used instead of manual injection.

f. Insert the Hamilton syringe approximately 3 mm deep into the brain and slowly inject the cells at a rate of 1 μL/s to reduce backflow.

Note: Respiratory rate should be monitored throughout the procedure to ensure anesthesia is appropriately maintained. Ophthalmic ointment may be applied to prevent corneal drying.

b. Prepare the Hamilton syringe by rinsing it with 70% ethanol, and then PBS numerous times. Collect 10 μL of the prepared CAR-T mixture.

c. Using scissors or a razor, remove fur from the head of the mouse to clear the area for incision.

d. Disinfect the area by first swabbing the skin with disinfectant soap (Proviodine detergent), followed by 70% ethanol, and a final disinfectant paint (Proviodine disinfectant).

e. Create a small, vertical midline incision and locate the lambda of the skull. Move along approximately two millimeters to the right of the lambda, and one millimeter rostrally. Approximately one millimeter caudal to the right coronal suture, create a small hole in the skull of the mouse using a Dremel tool with a small burr attachment (see Figure 2).

Note: This technique was adapted off of the Kim et al. publication (Kim et al., 2006). A stereotaxic instrument may be used instead of manual injection.

f. Insert the Hamilton syringe approximately 3 mm deep into the brain and slowly inject the cells at a rate of 1 μL/s to reduce backflow.

g. Wait 10 s, and then slowly remove the needle from the brain.

Note: Administer analgesics (eg. 0.05–1 mg/kg buprenorphine) as per your animal care committee requirements following each intracranial injection.
49. At each time point (Untreated control, 24 h, 1 week, 2 weeks post treatment), collect blood from the cohort of mice by cardiac puncture to analyze for systemic CAR-T leakage.
   a. At each time point, anesthetize the cohort of mice with 500 µL of (930 mg/kg) avertin by intra-peritoneal injection.
   b. Once anesthetized, lie the mouse on its back for access to the ventral side. Insert 1 mL, 27.5G needle syringe below the ribcage and into the left ventricle of the heart.
   c. Collect as much blood as possible per mouse and transfer into K₂-EDTA-coated tubes to prevent coagulation.

50. To prepare samples for flow cytometric analysis, standardize blood volume for each mouse by using the lowest amount of blood collected from a sample
   a. For example, if from three independent mice, you collect 400 µL, 600 µL and 700 µL, use 400 µL from each mouse sample. This will be important for extrapolating CAR-T counts.

51. Add 12 mL of ammonium chloride and incubate for 5 min at room temperature.

52. Top up with 12 mL of PBS and centrifuge at 300 × g for 5 min.

53. Resuspend pellet in 1 mL of 200 mM PBS-EDTA and filter into 5 mL round-bottom polystyrene tubes with 35 µm filter cap.

54. Re-centrifuge tube at 300 × g for 5 min and resuspend cells in 100 µL of PBS with 200 mM EDTA for antibody staining.

   Note: Depending on the CAR construct, stain with a tag or with human CD3 antibody as per manufacturer’s instructions. In this study, the CAR-T had a truncated NGFR tag. We thus stained the cell pellet with a human NGFR antibody at a 1:10 dilution.

55. Incubate antibody for 15 min at room temperature in a BSC and avoid contact with light.

56. Wash with 1 mL of PBS-EDTA and spin at 300 × g. Resuspend in a final volume of 100 µL of PBS-EDTA with 1:100 dilution of 7AAD viability dye.

57. Analyze the samples by flow cytometry.

Testing CAR-T Toxicity Systemically Using Dose Escalation [SP8]

© Timing: 1–2 weeks

In this section, the amount of CAR-T systemic leakage will be used as a starting dose in which to assess toxicity to the hematopoietic system. This will determine whether inherent leakage of the CAR-T can induce hematopoietic toxicity, as well as confirm at which dose this can be detected in the model system.

58. Generate the dosing regimen to be tested in the humanized mouse model.
   a. Using the 100 CAR-T cells/400 µL of murine blood as the minimal amount of systemic leakage, we tested the following doses:
      i. 100 cells/400 µL.
      ii. 10,000 cells/400 µL.
      iii. 100,000 cells/400 µL.
      iv. Untreated control (PBS).

59. Prior to injection of CAR-T doses, segregate humanized mice into triplicate cohorts in which average human chimerism, measured by CD45+ positivity from the bone marrow aspirates, is consistent between groups. This will account for initial variations in engraftment.

   Note: Record the weight of each mouse. Due to the dosing regimen being a measure per set volume of blood, adjust the dosing according to the predicted blood volume, calculated by weight. The average reported blood volume in mice is 7.8 mL per 100 g (O’Connell et al., 2015). For example, if the mouse weighs 25 g, the expected blood volume will be 1.95 mL. If this mouse is expected to receive 100 CAR-T cells/400 µL of blood, then it should receive
a raw number of approximately 488 CAR-T cells. This is the total dosing regimen and should be divided over two treatments.

60. Treat each individual mouse via tail-vein injections with the absolute total dose over two weeks, one week apart.

   Note: In the above example, this mouse would receive 244 cells at treatment timepoint 1, and a week later, the remaining 244 cells.

61. Prepare cells and refer to steps 23–28 for tail-vein injection of samples.

62. One week after the completion of the total dosing regimen, refer to SP6 for isolation of MNCs from bone marrow and spleen, and subsequent flow cytometric analysis.

   Pause Point: See Troubleshooting 2 if no observed HSPC toxicity after analysis is completed.

Isolation of MNCs from Bone Marrow and Spleen [SP9]

   Timing: ~2 h

This section describes the techniques used to isolate cells from mouse bone marrow and spleen at experimental endpoints for assessment of toxicity to HSPCs. Isolated cells can then be used for analyses including flow cytometry.

63. Into a 12-well plate, add 2 mL of IMDM + 2% FBS into each well. Label wells for each spleen and set of bones (right and left tibia, femur, and pelvic bones) to be collected. Plates should be kept on ice to preserve samples.

64. Euthanize mice in accordance with your animal care committee guidelines.

65. Using forceps and scissors, remove the spleen (dorsal, below the ribcage on the left side within the peritoneal cavity) and place in the sample well.

66. Peel back the skin from the lower limbs and isolate each hind leg by cutting the pubic symphysis and muscles attaching to the hip. Remove the feet. Carefully remove all muscles from the legs and isolate the hip, femur, and tibia bones. Place the bones from both legs into a pre-labeled well and ensure all bones are submerged.

   Note: Mouse bones can be fragile, so special care should be taken to prevent fractures. Muscles on the bones can be more easily removed by cleaning with a sterile gauze soaked in 70% ethanol.

   Note: Other methods for bone marrow harvest such as flushing or centrifugation may be adopted in place of crushing. Please see Amend et al. (2014), and Liu and Quan (2015).

67. Once all samples have been collected, process accordingly:

   a. Bone Marrow
      i. Place all six leg bones, along with the media from that well, into a mortar and crush with a pestle. Bones will become finely fragmented and lose red traces of the marrow.
      ii. Filter the media containing marrow through a 40 μm strainer, and into a 50 mL conical centrifuge tube.
      iii. Repeat this process with an additional 2 mL of fresh media to collect any remaining marrow.

   Note: Between each sample, clean the mortar and pestle with 70% ethanol and wipe down with sterile gauze.
Note: Once processed, keep samples on ice until ready for the next step.

b. Spleen
   i. Crush each spleen within its well, using the back (flat end) of a syringe plunger. After crushing, cells will enter into media suspension with only the capsule and very small fragments of spleen parenchyma remaining.
   ii. Filter this suspension through a 40 μm strainer and into a 50 mL conical centrifuge tube.

Note: Once processed, keep samples on ice until ready for the next step.

68. Pellet the marrow and spleen suspensions at 450 × g for 5 min and resuspend each in 2 mL of ammonium chloride to lyse RBCs.
69. Immediately spin again at 450 × g for 5 min and aspirate ammonium chloride to be left with a white pellet. Resuspend cells in 2 mL of PEF.
70. Cells can be counted and are now ready for downstream analysis. For flow cytometric analysis, refer to SP5.

**Testing CAR-T Toxicity with Intracranial CAR-T Treatment [SP10]**

© Timing: 2 weeks

In this section, the safety of the CAR-T will be compared to a non-targeting CAR-T control in a humanized mouse model to determine whether intracranial treatment can induce toxicity.

71. Follow steps 47–48 to treat humanized mice with a total of 2 million CAR-T cells intracranially.

   Note: Administer analgesics (eg. 0.05–1 mg/kg buprenorphine) as per your animal care committee requirements following each intracranial injection.

72. One week after the completion of treatment, follow SP9 for isolation of MNCs from the bone marrow and spleen of mice. For subsequent flow analyses, follow SP5 to assess for hematopoietic toxicity from CAR-T treatment.

**EXPECTED OUTCOMES**

When processing cord blood, typical isolations of MNCs following use of the specified lineage-depletion kit contain 30%–80% CD34+ cells, verified by flow cytometry post depletion. For FACS of CD34+ HSPCs, expect ~5% lower yield than calculated by CD34+. If CD34+ is not available, a conservative estimate of 25% yield should be used in calculations. Excess cells may be cryopreserved for later use, but expect 50% cell death upon thaw.

BM aspirates typically yield around 0.5–1 × 10^6 cells. Engraftment (CD45%) by xenotransplanted HSPCs at the above mentioned cell doses, measured by BM aspirate at 8–12 weeks post transplantation, should reach ~50%. When harvesting MNCs from humanized mice, expect a total of ~5 × 10^7 cells from the bone marrow and ~1.5 × 10^7 cells from the spleen.

Intracranial leakage may vary depending on the cell-based therapy and its capacity to bypass the blood-brain barrier into the system. Activated cells may have an increased frequency. Toxicity to HSPCs may be influenced by cell-therapy of choice, as well as target antigen.
QUANTIFICATION AND STATISTICAL ANALYSIS

This section provides a brief overview of the workflow for flow cytometric analysis using FlowJo software, as well as absolute cell quantification for leakage studies (SP7).

All cells analyzed on flow cytometry are gated on live singlet cells (see Figure 3) using FlowJo. Engraftment is quantified based on huCD45 positivity. Markers of HSPCs (CD34, CD38, CD133), and lymphoid (CD19) cells are sub-gated within the CD45+ graft compartment. Data are entered into GraphPad Prism to generate figures and carry out statistical analysis. Student's t tests are used to determine statistical significance between two groups. To quantify flow cytometric data of CAR-T cell leakage, take the raw counts of antigen-positive cells (ex: NGFR/huCD3). This is the amount of CAR-Ts in the system per amount of blood collected. To account for noise from false positives, use the untreated control to normalize the data by setting this value to 0 in analysis. Average the results between replicates of mice. Amalgamate the results from each time point to identify the time point in which there is maximal leakage of CAR-Ts in the system using GraphPad Prism 5.0. Statistical significance is determined by Student's t test.

LIMITATIONS

In this protocol, the safety of an immunotherapeutic strategy in the context of “on-target, off-tumor” effects is assessed.

Currently, this humanized model system is limited to non-tumor bearing mice. The lack of tumor may affect the expansion and activation of the CAR-T, thus affecting leakage kinetics into the system. However, this limitation is partially mitigated by the dose escalation portion of this protocol in which toxicity to varying degrees of putative CAR-T leakage may be predicted. Alternatively, CAR-Ts may be pre-activated, or injected alongside a surrogate antigen to mimic an antigen-positive tumor. This study also lacks direct CAR-T application on mice which bear both brain tumors and a hematopoietic system derived from the same patient, which would most accurately reflect the clinical scenario.

Additionally, umbilical cord HSPCs were used to humanize mice due to their enhanced ability for engraftment in comparison to adult bone marrow HSPCs. Thus it is important to note that the toxicity of CAR-T treatment is being extrapolated to adult hematopoietic cells. The use of immunodeficient (NSG) mice is also an imperfect model of the human hematopoietic system. Not all lineages are well
represented (eg. erythroid) and not at the same proportions as one would observe in endogenous human bone marrow. It is possible that some alterations may be masked or overlooked. However, immunodeficient mouse models remain the gold-standard in hematopoietic transplantation assays and their ability to support most lineages still offers valuable insight into off-target toxicity effects.

Despite these limitations, this protocol provides a thorough review of “on-target, off-tumor” safety in the context of CAR-T or other cell-based therapy treatment.

TROUBLESHOOTING

Problem 1
No appreciable level of engraftment is observed in the bone marrow aspirate (by flow cytometry) after 8 weeks.

Potential Solution 1
If no appreciable level is observed (greater than 5% huCD45), it is unlikely the graft will significantly increase and the mouse should be removed from the experiment.

Problem 2
No observed toxicity in the intravenous dose escalation study.

Potential Solution 2
Intravenous delivery is necessary to also validate that the model system is able to detect toxicity based on the antigen of choice. Consider increasing intravenous cell dosing until there is detection of toxicity.

RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Sheila K. Singh at ssingh@mcmaster.ca

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS
S.K.S., J.X., and N.W. drafted the protocol. J.X. and N.W. described the generation of the humanized mouse model through to assessment by flow cytometry. S.K.S. described the CAR-T toxicity assessment experiments. C.V., K.H., and S.S. edited the manuscript with input from all authors. S.K.S. completed the figures and graphical abstract.

DECLARATION OF INTERESTS
Dr. Sheila K. Singh is a scientific advisor for Century Therapeutics Inc. and her role in the company has been reviewed and is supported by McMaster University.
REFERENCES

Amend, S.R., Valkenburg, K.C., and Pienta, K.J. (2016). Murine hind limb long bone dissection and bone marrow isolation. J. Vis. Exp. 110, e53936.

Chung, Y.R., Kim, E., and Abdel-Wahab, O. (2014). Femoral bone marrow aspiration in live mice. J. Vis. Exp. 89, e51660.

Kim, S.K., Kim, S.U., Park, I.H., Bang, J.H., Aboody, K.S., Wang, K.C., Cho, B.K., Kim, M., Menon, L.G., Black, P.M., Carroll, R.S., et al. (2006). Human neural stem cells target experimental intracranial medulloblastoma and deliver a therapeutic gene leading to tumor regression. Clin Cancer Res. 12, 5550–5556.

Liu, X., and Quan, N. (2015). Immune cell isolation from mouse femur bone marrow. Bio. Protoc. 5, e1631.

O’Connell, K.E., Mikkola, A.M., Stepanek, A.M., Vernet, A., Hall, C.D., Sun, C.C., Yildirim, E., Staropoli, J.F., Lee, J.T., and Brown, D.E. (2015). Practical murine hematopathology: a comparative review and implications for research. Comp. Med. 65, 96–113.

Vora, P., Venugopal, C., Salim, S.K., Tatari, N., Bakhshinyan, D., Singh, M., Seyfrid, M., Upreti, D., Rentas, S., Wong, N., et al. (2020). The rational development of CD133-targeting immunotherapies for glioblastoma. Cell Stem Cell 26, 832–844.e6.