The translation of chimeric antigen receptor (CAR) T cell therapy for pediatric solid tumors is limited by the lack of preclinical models that fully recapitulate solid tumor biology. We describe steps to implement neuroblastoma metastatic and orthotopic mouse models. We delineate an analysis pipeline to quantify the efficacy and determine the immunological characteristics of both CAR T and tumor cells in these models. Both mouse models can be applied to evaluate other experimental therapies for neuroblastoma.
SUMMARY
The translation of chimeric antigen receptor (CAR) T cell therapy for pediatric solid tumors is limited by the lack of preclinical models that fully recapitulate solid tumor biology. We describe steps to implement neuroblastoma metastatic and orthotopic mouse models. We delineate an analysis pipeline to quantify the efficacy and determine the immunological characteristics of both CAR T and tumor cells in these models. Both mouse models can be applied to evaluate other experimental therapies for neuroblastoma. For complete details on the use and execution of this protocol, please refer to Li et al. (2021).

BEFORE YOU BEGIN
Commonly used animal models
The protocol below describes the steps to create metastatic or orthotopic neuroblastoma (NB) models in immunocompromised mice for preclinical testing of exogenous chimeric antigen receptor (CAR) T cells. Metastatic relapse is the major cause of death in NB. Hence, a metastatic NB mouse model that shows similar pattern of experimental metastasis as occurs in NB patients should be employed when interrogating novel therapies. An established orthotopic NB mouse model allows for spontaneous metastatic spread as well as the formation of a tumor microenvironment, which poses physical barriers and paracrine immune suppression on immune effector cells. It is thus conceivable that orthotopic NB models may be more difficult to eradicate with CAR T-cell therapy and represent a more challenging experimental system for testing novel therapies.

Generation of luciferase-eGFP-expressing neuroblastoma cells
To track tumor growth using bioluminescence imaging, human MYCN-amplified IMR-5 cells are transduced with viral vectors to stably express luciferase and enhanced green fluorescent protein (eGFP). The IMR-5-luc-eGFP cell line is available under the material transfer agreement upon request from the lead contact.

Isolation of T cells from healthy donors
The isolation of human peripheral blood mononuclear cells (PBMCs) from buffy coat by Ficoll-Paque density gradient centrifugation is previously described (Fuss et al., 2009). T cells are then enriched
via immunomagnetic negative selection from PBMCs isolated from healthy donors and used for CAR T cell production. The ratios of CD4+/CD8+ T cells varies between 0.3 to 4.5 in healthy donors. T cells are frozen in liquid nitrogen in batches with 50 x 10^6 cells per vial.

**Production of CAR-expressing lentivirus**

Lentiviral CAR-expression vectors can be produced in research labs or obtained from commercial vendors. In our lab, we isolated a glypican 2 (GPC2)-specific monoclonal antibody, CT3, and demonstrated that CAR T cells harboring the single-chain variable fragment (scFv) of the CT3 antibody led to regression of NB in murine models. GPC2 is an oncofetal antigen selectively expressed on NB cells, and absent in normal tissues except testis (Bosse et al., 2017; Li et al., 2017; Orentas et al., 2012). Thus, this antigen is highly tumor specific and represents an ideal target for CAR T-cell therapy. We cloned the CT3 CAR transgene into a lentiviral vector, pWPT (Addgene #12255, a gift from Didier Trono), to construct the final CAR vector pMH303 that can be used for other targets-directed CARs. The amino acid sequence of the CT3 CAR transgene is shown in Table 1. In the present protocol, we use the second-generation CT3 CAR (Figure 1) transduced into human T cells to demonstrate the usefulness of our preclinical models. The CT3 CAR construct (pMH303) contains 4-1BB as a co-stimulatory factor and the truncated human epidermal growth factor receptor extracellular domains (hEGFRt) as a tag recognized by cetuximab. The pMH303 vector is available under a material transfer agreement upon request from the lead contact.

**Mouse strains**

We use 5 to 7-week-old female NOD-scid IL2rgnull (NSG) mice that are held in a specific pathogen-free environment. To obtain sex-independent results, a mix of male and female mice is highly

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**Table 1. The amino acid sequence of the CT3 CAR transgene**

| Fragment | Amino acid sequence |
|----------|---------------------|
| Signal sequence (SS) | MLLLLTSLELLCLPEAFLLIF |
| CT3 scFv | EVQLQQLGPGVLVQGSKMCSAASRFTPTTYNYHHVKQSPGKTLEMGYI NPNNDIFYQKFQHGAATLTINKSNTAYNRELSTLTSEDSAYVYCVRSSNI RVTFDFPFDVMVTQYVTVSSGQGSDLGQGQGNLINVQFPAIMASGL GEKVTMSCKRSSSVNYTQKSDFPGHLWVYTTSLAPFVPARFSGS GSWSYLLTISOMBEDSAATTYCQFSSPSAPTSGLLELA |
| CD8α hinge | TTTPAFPFPPAFTIASQPLSLREACRFAQAQAVYTRGGLFACD |
| CD8α transmembrane | IYINAPLAGTCOVLSSLVIT |
| 4-1BB | KRGRLKLLYTFQPFPFRFVQTQTEDGCSCRFPEREBQCEL |
| CD3ζ | RVKFSRSDADAQYPQQQIQNELMGRLKEEYDLVLDKRGDRPFGK PERKPQGLYNELQKDMAYESEIMKQGRRKGKHDGLQGLSTA TGVDYLALMQALPFR |
| T2A | EGRGSLLTCDGVEENPGP |
| hEGFRt | RKVCQIGIGEFPKDSIELSNATN1KHKFNCTSISGDLHIFVAFRQDSEFTI TPPDLQFQEDILKTVEITQFLLLQAMFENRTLDHAPFENELIIRGRTQHG GQFLAVSNITLGLRSLISDDIVQISQMNLCYANTINWKLPGF SQRTK11SNRENSCCKATQVCHALCSPGQMPKDCVSQCNKSRG KCVDKCMMLLEIGHPREPVENSEIQCHFECPLQAMNITCTGKPDNCIQA HYIDPHCVVCTPAVQEMBNTLVKAYDAGHVCHELHPNCTQCTQPG LEQCPPTNGKPIPSATQMVGAQALLLLVALGIGLIFM |

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**Figure 1. Schema of the second-generation CT3 CAR construct**

A truncated human epidermal growth factor receptor (hEGFRt) is added into the lentiviral construct to allow cell tracking by using the anti-EGFR monoclonal antibody cetuximab. SS: signal sequence. TM: transmembrane.
recommended. NSG mice are severely immunocompromised because they lack mature B, T, and NK cells, thereby rendering them tolerant to xenografts.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies** | | |
| Alexa Fluor® 700 anti-human CD3 Antibody (5 μL/test) | BioLegend | 317340 |
| PerCP/Cy5.5 anti-human CD8a Antibody (5 μL/test) | BioLegend | 300924 |
| APC anti-human CD4 Antibody (5 μL/test) | BioLegend | 317416 |
| RPE F(ab’)2 goat anti-human IgG antibody (1:200 dilution) | Jackson ImmunoResearch | 109-116-170 |
| Anti-GPC2 antibody CT3 (5 μg/mL) | This paper | N/A |
| Anti-human EGFR antibody cetuximab (1 μg/mL) | NIH Pharmacy | N/A |
| Anti-CD3-ζ Antibody (1:200 dilution) | Santa Cruz | sc-1239 |
| **Biological samples** | | |
| human buffy coat | Oklahoma Blood Institute | N/A |
| Age requirement: <45 years, no sex or race selection | NIH Blood Bank | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| DMEM | Gibco | 11965084 |
| RPMI-1640 | Gibco | 11875-085 |
| AIM-V CST Medium | Gibco | 087-0112BK |
| Phosphate buffered saline (PBS) | Gibco | 10010-023 |
| Fetal bovine serum (FBS) | HyClone | SH30071.03 |
| Bovine serum albumin (BSA) | Fisher Scientific | BP9703100 |
| Penicillin-Streptomycin | Gibco | 15140-122 |
| Trypsin/EDTA solution | Gibco | 25200-056 |
| Poly-D-Lysine | Gibco | A3890401 |
| HEPES | Gibco | 15630080 |
| Corning Matrigel Matrix | Corning | 356234 |
| Isoflurane | NCI Animal Facility | N/A |
| Normal saline | Intermountain Life science | Z1377 |
| Buprenorphine | NIH Pharmacy | N/A |
| Recombinant human IL-2 | NIH Pharmacy | N/A |
| Protamine sulfate | Sigma Aldrich | P3369 |
| Sodium azide | Sigma Aldrich | S-2002 |
| Formaldehyde | Sigma Aldrich | F-8775 |
| Human Glypican 2 / GPC2 Protein, Fc Tag | Acro Biosystems | GP2-H5255 |
| D-Luciferin | PerkinElmer | 122799 |
| **Critical commercial assays** | | |
| Lipofectamine™ 2000 Transfection Reagent | Thermo Fisher Scientific | 11668030 |
| Lenti-X™ concentrator | Takara | 631232 |
| EasySep™ Human T Cell Enrichment Kit | Stem Cell Technology | 19051 |
| Dynabeads™ human T-activator CD3/CD28 for T cell expansion and activation | Thermo Fisher Scientific | 11132D |
| Tumor dissociation kit, mouse | Miltenyi Biotec | 130-096-730 |
| **Experimental models: Cell lines** | | |
| IMR-5-Luc-eGFP | This paper | N/A |
| Lenti-X™ 293T Cell Line | Takara | 632180 |
| **Experimental models: Organisms/Strains** | | |
| NOD-scid IL2rgnull (NSG) mice | NCI CCR Animal Resource Program | N/A |
| **Recombinant DNA** | | |
| CT3 CAR (pMH303) | This paper | N/A |
| CD19-targeted CAR (pMH376) | This paper | N/A |
| psPAX2 | Addgene | 12260 |

(Continued on next page)
Note: Buprenorphine should be stored in a securely locked cabinet according to federal regulations.

MATERIALS AND EQUIPMENT

This protocol uses the IVIS Lumina III imaging system for in vivo bioluminescence imaging, BD LSRFortessa flow cytometer for flow cytometry (any cytometers with lasers detecting the fluorophores R-PE and Alexa Fluor® 700 can be used), Nightsea fluorescence viewing system for visualizing eGFP, and Bullet blender tissue homogenizer for tissue homogenization.

DMEM complete medium

| Reagent             | Final concentration | Amount   |
|---------------------|---------------------|----------|
| DMEM                | N/A                 | 1,000 mL |
| FBS                 | 10%                 | 110 mL   |
| Penicillin-streptomycin | 1%               | 11 mL    |

The medium can be stored at 4°C for 1 month.

Note: This is used for culturing Lenti-X™ 293T cells.
**STEP-BY-STEP METHOD DETAILS**

**Establishment of an experimental metastatic neuroblastoma xenograft mouse model**

| Timing: | 3–4 weeks |

This section describes how to establish an experimental metastatic NB xenograft mouse model and monitor tumor growth in vivo. The human MYCN-amplified NB cell line, IMR-5-luc-eGFP, is used.

1. Preparation of IMR-5-luc-eGFP cells
   a. Seed IMR5-5-luc-eGFP cells at low passages (4–10) in T175 flasks at approximately 30% confluency in RPMI-1640 complete medium.
   b. At approximately 80%–90% confluency, trypsinize and collect cells in conical tubes.
   c. Centrifuge cells for 5 min at 300 × g.
   d. Remove supernatant, resuspend cell pellet at a density of 2.5 × 10⁷/mL (for the injection of 5.0 × 10⁶ cells per mouse) in PBS. Prepare an excess of 20% of cells.
   e. To ensure single cell suspension, filter resuspended cells through a 40 μm cell strainer before counting cells is recommended.

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### RPMI-1640 complete medium

| Reagent       | Final concentration | Amount   |
|---------------|---------------------|----------|
| RPMI-1640     | N/A                 | 1,000 mL |
| FBS           | 10%                 | 110 mL   |
| Penicillin-streptomycin | 1%                 | 11 mL    |

The medium can be stored at 4°C for 1 month.

### AIM-V complete medium

| Reagent           | Final concentration | Amount   |
|-------------------|---------------------|----------|
| AIM-V CST Medium  | N/A                 | 1,000 mL |
| FBS               | 10%                 | 110 mL   |
| Penicillin-streptomycin | 1%                 | 11 mL    |
| HEPES             | 15 μM               | 15 mL    |

The medium can be stored at 4°C for 1 month.

### Flow cytometry staining buffer (FACS buffer)

| Reagent     | Final concentration | Amount |
|-------------|---------------------|--------|
| PBS         | N/A                 | 500 mL |
| BSA         | 5%                  | 25 g   |
| Sodium azide| 0.1%                | 0.5 g  |

The FACS buffer can be stored at 4°C for 6 months.

### D-luciferin is dissolved into sterile deionized water to make a 15 mg/mL stock solution

| Reagent     | Final concentration | Amount |
|-------------|---------------------|--------|
| D-luciferin | 15 mg/mL            | 1 g    |
| Sterile deionized water | –       | 66.7 mL|

The D-luciferin solution can be aliquoted and stored at −80°C for 6 months.
f. Keep cells on ice until inoculation.

CRITICAL: A single-cell suspension is crucial for avoiding cell emboli and vessel occlusion in mice (Troubleshooting 1).

2. Perform intravenous injection
   a. Use NSG female mice at the age of 5–7 weeks.
   b. Mix the cell suspension and intravenously inject 0.2 mL of the suspension (5.0 \( \times \) 10^6 cells) into the tail vein of each mouse.

3. Detection of tumor growth in mice 10 days after tumor inoculation.
   a. Inject 0.2 mL of the D-luciferin solution (15 mg/mL) into the peritoneal cavity of each mouse.
   b. Place the mice inside of an anesthetic induction chamber to sedate them with isoflurane gas (2.5% at a flow rate of 2 L/min for approximately 2–3 min).
   c. At ten minutes after D-luciferin injection, place the anesthetized mice into the IVIS imaging chamber.
   d. Acquire images for 1 min.
   e. Record the total photon flux in the region of interest to monitor tumor growth.

CRITICAL: The proper imaging time needs to be optimized for each cell line in each mouse model.

4. Metastatic tumors form 3–4 weeks post inoculation and are found in clinically relevant sites such as the femur, spine, brain and abdominal cavity (Figure 2). Mice are randomized based on their bioluminescence signal strength and grouped for CAR T cell treatment.

Note: Although NSG mice are used in this section, IMR-5-luc-eGFP cells also form metastatic tumors in athymic nude mice (Li et al., 2017).

Establishment of an orthotopic neuroblastoma mouse model

Timing: \( \approx \) 3–4 weeks

This protocol is adapted from (Khanna et al., 2002) and describes how to establish the orthotopic NB xenograft mouse model and monitor tumor growth in vivo.

5. Preparation of IMR-5-luc-eGFP cells: Prepare a single-cell suspension of IMR-5 to accommodate the injection of 0.25 \( \times \) 10^6/mL cells per mouse (plus an excess of 20%). The cells are then resuspended in ice-cold Matrigel matrix equaling 30 \( \mu \)L per injection and kept on ice.
CRITICAL: An accurate cell count is critical for a successful experiment. If too many cells are injected, the animals will develop too large a tumor burden that may not be amenable to CAR T-cell therapy.

6. Preparation of mice: shave the left flank of 5 to 7-week-old female NSG mice to expose a 1 cm × 1 cm area overlaying the spleen (Figure 3A).

7. Orthotopic implantation of IMR-5-luc-eGFP cells (approximately 15 min/mouse):
   a. Place mice under anesthesia with isoflurane and positioned in a lateral right recumbent position.
   b. Make a 1.0 cm transverse cutaneous incision at the height of the spleen and carefully dissect down.
   c. Upon penetration of the peritoneum, lift the spleen cranially to visualize the left adrenal gland (Figure 3B).
   d. Using a 29-gauge non-hub needle, inject 30 µL of the tumor cell suspension into the periadrenal fat pad (Figures 3C, 3C1, 3D, and 3D1). Troubleshooting 2.
e. Close the peritoneum with vicryl sutures (Figure 3E) and the skin with clips (Figure 3F).
f. Inject 30 μL buprenorphine extended-release subcutaneously for analgesia. For supportive
care, inject 0.5 mL of 0.9% normal saline subcutaneously. Troubleshooting 3.
g. Monitor post-operative animals for the occurrence of pain, bleeding, or other complications.
h. Remove clips 14 days after surgery.

**Note:** The timing of clips removal may vary as different institutions have own animal care com-
mittee requirements.

8. Detection of tumor growth in mice
   a. Inject 0.2 mL of the 15 mg/mL D-luciferin solution into each mouse.
   b. Place the mice inside an anesthetic induction chamber. The mice are sedated with isoflurane
gas (2.5% at a flow rate of 2 L/min) approximately 2–3 min after D-luciferin injection.
   c. Place the mice into the IVIS imaging chamber.
   d. Acquire images five minutes after D-luciferin injection for a total acquisition time of 60 s.
   e. The total photon flux in the region of interest is determined and used to monitor tumor
growth.

9. Tumors grow orthotopically within 3–4 weeks from the implantation date. In addition to the pri-
mary adrenal tumor, spontaneous distant metastasis can be found in orthotopic NB mouse
models (Khanna et al., 2002). NB tumor cells are also detected in bone marrow. Different NB tu-
mor cell lines and patient-derived xenografts (PDXs) can be utilized but tumor growth timelines
need to be rigorously established to ensure reproducibility.

10. Randomize and group mice for CAR T cell treatment based on their bioluminescence signal
strength.

△ CRITICAL: The use of young animals is important because older mice will develop involution
of their fat pad. Smaller fat pads will increase the risk of fatality due to puncturing the
aorta, which is located immediately medial and dorsal to the fat pad.

**Lentivirus production**

**Timing:** ≈ 5–7 days

The second-generation of lentivirus production system is used in this protocol. Three plasmids
include the CAR-expressing transfer vector (pMH303), envelope vector (pMD2.G), and packaging
vector (psPAX2). Users need to follow the standard operating procedure for safe handling of lenti-
uirus at own institution.

11. Lentivirus production
   a. Seed Lenti-X™ 293T cells at low passages (4–10) at a density of 20 × 10⁶ cells per Poly-D-
lysine–coated 15 cm dish.
   b. The following day, transfect Lenti-X™ 293T cells with the CT3 CAR plasmid (pMH303), enve-
lope (pMD2.G) and packaging (psPAX2) plasmids at a ratio of 4:1:3. Use lipofectamine 2000
as the transfection reagent.
   c. Remove medium, replace with fresh DMEM complete medium 6–12 h after transfection.
   d. Harvest the lentivirus-containing supernatants 48–72 h post-transfection. Centrifuge at
300 × g for 5 min and then filter through a sterile 0.45 µm filter.
   e. To concentrate lentivirus, combine 1 volume of Lenti-X™ Concentrator with 3 volumes of
clarified supernatant.
   f. Incubate mixture at 4°C for 30 min to overnight (12–24 h). Centrifuge sample at 1,500 × g for
45 min at 4°C.
   g. Carefully remove supernatant, gently resuspend the pellet in 1/20th to 1/100th of the original
volume using AIM-V complete medium.
h. Immediately titrate lentivirus or store at –80°C in single-use aliquots.

**Note:** Though we use Lenti-X™ 293T cells, standard 293T cells can also be used for lentivirus production. In addition to lipofectamine 2000, other transfection reagents including Calfectin (SignaGen Laboratories) and Polyethylenimine are also highly efficient in producing lentivirus.

12. Lentivirus titration
   a. Seed Lenti-X™ 293T cells at a density of 1 × 10⁵ cells in 12-well cell culture plate. 
   b. The following day, use one well to count cells. It should be between 1–3 × 10⁵ cells/well. 
   c. Prepare dilution of concentrated lentivirus as shown in **Figure 4**. Remove medium, and transduce cells with 1 μL, 10⁻¹ μL, and 10⁻² μL of lentivirus in 1 mL of fresh DMEM complete medium. Keep one well as non-transduced control. 
   d. After 48–72 h of transduction, trypsinize and collect cells in microcentrifuge tubes. 
   e. Resuspend the pellet in FACS buffer containing 1 μg/mL cetuximab. The non-infected cells also need to be stained with cetuximab as Lenti-X™ 293T cells also express hEGFRt. Keep cells at room temperature (20°C–25°C) for 20 min. Wash cells once with PBS. 
   f. Resuspend the pellet in FACS buffer containing the RPE F(ab')2 goat anti-human IgG antibody (1:200 dilution). Stain cells for 12 min at room temperature in the dark, and wash them once with PBS. 
   g. Fix lentiviral-transduced cells in 1% formaldehyde in PBS and incubate for 5 min at room temperature. Wash cells once with PBS. 
   h. Resuspend the pellet in FACS buffer and analyze cells for CAR expression using a flow cytometer.
i. Calculate titer using the following equation: 

\[ \text{Titer (transduction unit/mL, TU/mL)} = \left( \frac{F \times Cn}{V} \right) \times DF; \ \text{F} = \% \text{ of hEGFRt positive cells/100; Cn=number of cells; V= volume of supernatant in (mL); DF= dilution fold. Chose dilutions yielding 1\%–10\% hEGFRt positives for titer calculations. The expected viral titer is } \geq 5 \times 10^7 \text{ TU/mL.} \]  

**Troubleshooting 4.**

**Note:** This protocol measures the functional lentiviral titer by flow cytometry. Other titration methods including measurement of p24 antigen by ELISA and quantification of the number of integrated DNA lentiviral copies by real-time PCR are frequently used. Different methods may result in titer difference.

### CAR T cell production

**© Timing: ≈ 9–11 days**

We use non-transduced T cells (mock T) as the control T cells in this protocol. For tumors lacking the expression of CD19, the CD19-targeted CAR in the same vector backbone (pMH376) is highly recommended as an irrelevant control.

13. T cell activation (Day 1)
   a. Isolate T cells from PBMCs using the EasySep™ Human T Cell Enrichment Kit according to the manufacturer’s instruction ([https://www.stemcell.com/easysep-human-t-cell-enrichment-kit.html](https://www.stemcell.com/easysep-human-t-cell-enrichment-kit.html)).
   b. Thaw T cells and seed 1 \times 10^6 cells per well in a 24-well cell culture plate in 1 mL AIM-V CST complete medium supplemented with 40 IU/mL human IL-2.
   c. Wash Dynabeads human T-activator CD3/CD28 once with PBS and then resuspend beads in complete medium.
   d. Add Dynabeads human T-activator CD3/CD28 (4 \times 10^7 beads/mL) to T cells at a bead-to-cell ratio of 2:1. Cells are activated 24 h before transduction.

**Note:** In addition to purified T cells, PBMCs can be used to produce CAR T cells.

14. Lentiviral transduction of T cells (Day 2–3)
   a. Add protamine sulfate into each well to maintain a final concentration of 10 \mu g/mL and replenish the medium with 100 IU/mL of IL-2.
   b. Add CAR-expressing lentivirus with a titer above 5\times10^7 TU/mL into activated T cells at the multiplicity of infection (MOI) of 5. Calculate the volume of desired lentivirus using the following equation: 

\[ \text{Multiplicity of Infection (MOI)} = \frac{\text{Titer (TU/mL)} \times \text{Volume (mL)}}{\text{Cell Number}} \]

c. Centrifuge the cells at 1000 \times g for 120 min.
   d. Maintain the culture in 37°C, 5% CO₂ incubator for 24 h (Day 2).
   e. Repeat the transduction in 24 h (Day 3).

**△ CRITICAL:** High-quality CAR-expressing lentiviruses are necessary to achieve good transduction efficiency.

15. CAR T cell expansion (Day 4–11)
   a. On Day 4, collect the T cells in a 15 mL conical tube.
   b. Remove Dynabeads from cells with DynaMag magnet.
   c. Centrifuge at 300 \times g for 5 min and discard supernatant.
   d. Resuspend CAR T cells in complete T cell medium supplemented with 100 IU/mL of IL-2 at a density of 0.5 \times 10^6 cells/mL in a 6-well cell culture plate.
   e. Assess CAR T cell growth every other day with fresh medium/IL-2 added as required. CAR T cells are usually expanded into a T75 flask from Day 6–7. 10 to 50-fold of T cell expansion is expected at the end of culture (Figure 5). **Troubleshooting 5.**
CRITICAL: Excessive T cell density decreases cell viability.

16. Assessment of transduction efficiency by detecting either GPC2 binding or hEGFRt expression.
   a. Resuspend $1 \times 10^6$ T cells in FACS buffer containing 1 μg/mL human GPC2-Fc protein or 1 μg/mL anti-EGFR antibody cetuximab. Stain cells for 20 min at room temperature and then washed once with PBS.
   b. Resuspend the pellet in FACS buffer containing the RPE F(ab')2 goat anti-human IgG antibody (1:200 dilution) to detect GPC2-Fc binding or hEGFRt expression together with other fluorochrome-conjugated antibodies to mark CD3, CD8, and CD4.
   c. After incubating the samples for 12 min at room temperature in the dark, wash cells once and analyze them using a flow cytometer. Add DAPI right before the analysis to stain dead cells.
   d. Draw the live cell gate, then draw the gate of CD3+ T cells, subsequently plot a histogram for CAR expression. Among the transduced cells, typically >50% have CAR expression (Figure 6).

17. Continue to assess CAR T cell growth until the expected cell number is reached.

Note: The CAR transduction efficiency varies significantly among T cell donors. Therefore, it is recommended to test the efficiency in a small-scale experiment first before committing to a donor for in vivo experiments.

Randomization tumor-bearing mice and administration of experimental molecules
When bioluminescence signals reach the enrollment threshold (usually >$10^7$ photons/seconds), tumor-bearing mice are randomized into different groups with comparable tumor sizes as defined by total photon flux and used for experimental testing (Figure 7). For example, small molecules, antibodies, recombinant immunotoxins, antibody drug conjugates (ADCs), bispecific antibodies, or CAR T cells can be tested. In this protocol, CAR T cells are used as an example.

Preparation of T cell product for injection

© Timing: ≈ 2 days

18. The day before CAR T cell infusion, randomize mice into the different experimental groups based on the bioluminescence signal of their tumors. These signals are also used as the baseline measurement.
19. The following day, count mock and CAR T cells. Each mouse receives $5 \times 10^6$ CAR T cells. This is to say the total T cell number is adjusted to encompass the respective CAR T cell number. The number of mock T cells used for the injections corresponds to the total T cell number. E.g., the measured transduction efficiency is 65%. Thus, $7.7 \times 10^6$ total T cells contain $5 \times 10^6$ CAR T cells. Accordingly, $7.7 \times 10^6$ Mock T cells are injected into control animals.

20. Resuspend T cells in a volume to accommodate an injection volume of 0.2 mL per mouse. Injections are administered via the tail vein.

Monitoring of animals

Ø Timing: ≈ 4–7 weeks

Mice are monitored bi-weekly for the occurrence of graft-versus-host disease (GVHD). GVHD can manifest as weight loss, scruffy appearance, decreased activity, and/or hair loss (Troubleshooting 6). The tumor growth is tracked longitudinally with weekly bioluminescence imaging.

21. Monitor animals by weekly bioluminescence imaging as shown in Figure 8. As part of their clinical assessment, weekly weights are obtained.

End-of-study evaluations

Ø Timing: ≈ 4 days

At the end of the experiments, correlative studies are conducted. For example, flow cytometry is used to assess T cell persistence in various organs. T cells can be retrieved from mouse tissues and used for T cell functional analysis. Tumors can be evaluated for their histologic appearance and topographic distribution of T cells in topographic relation to tumor cells (Troubleshooting 7).

Tissue isolation and preservation:

22. Euthanize the animals, and dissect their tumors, spleen, and femurs.
23. Weigh and photograph the tumors (Figure 8C).
24. For both spleen and tumor tissues, cut the tissue into three pieces. Fix one piece (no greater than 1.5 cm) in 4% paraformaldehyde (PFA) and use it for immunohistochemistry (IHC) analysis; snap-freeze one for downstream genomic or proteomic applications; and dissociate the last one into a single cell suspension for T cell isolation via fluorescence-activated cell sorting or magnetic bead isolation.
25. Fix one femur in 4% PFA, while flush the other one with PBS. Count the produced cells and use them for flow cytometry applications.

Flow cytometry analysis:

26. Count the single cells from mouse tissues and use $1 \times 10^5$ cells per flow cytometric test.
27. Analyze the cells isolated from tumor, spleen, and the bone marrow by flow cytometry for residual human mock or CAR T cells. We use anti-CD3, CD8, CD4, and GPC2-HFc detected by an anti-human Fc antibody to quantify GPC2-targeting CAR T cells. The cell counts can be normalized to the number of tumor cells and tumor weight.
28. Quantify the antigen persistence in isolated tumor cells. We use CT3 antibody and a secondary anti-mouse antibody to detect GPC2 on the tumor cells.

Genomic/proteomic analysis:

29. Homogenize the frozen tissues from step 24 using the Bullet Blender tissue homogenizer. Extract RNA, protein, and genomic DNA. T cells retrieved from mouse tissues (step 24) are similarly processed as the frozen tissues.
30. Use RNA and protein for analysis of gene enrichment and identification of CAR interaction partners, etc.
31. Use genomic DNA to analyze CAR integration sites and quantify CAR copy numbers in tissues.

Immunohistochemistry analysis:

32. Stain paraffin-embedded tumor sections from step 24 for T cell markers (e.g., CD3) as shown in Figure 9.

**EXPECTED OUTCOMES**

The data generated from these preclinical studies using the experimental metastasis and orthotopic with spontaneous metastasis NB models will provide information regarding the efficacy of tested CAR T cells against established tumors. Correlative studies can shed light on mechanisms of therapy failure, such as antigen down-regulation, limited persistence of effector cells, or physical barriers of CAR T cell homing, which can all contribute to immune evasion. Robust and reproducible results may accelerate a successful translation from the bench to bedside.

**LIMITATIONS**

Accurate counting prior to the injection of tumor cells is critical for the success of the experiment. If too many cells are injected, animals will develop large tumors that do not respond to CAR T-cell
therapy. This narrow therapeutic window can be logistically challenging if there are problems along the way of generating CAR T cells that delay T cell injection. Because patients with NB present with variable tumor burden, we are aware that our model does not reflect the quantitative heterogeneity encountered in the clinic. Moreover, CAR T cell reconstituted NSG mice lack other cells of the immune system, particularly innate immune cells that play a critical role in the tumor microenvironment (TME) and anti-tumor immunity. Thus, syngeneic animal models would be more suitable for comprehensive studies of immune interactions.

**TROUBLESHOOTING**

**Problem 1**
The mouse dies quickly after tail vein injection of CAR T cells or tumor cells (step 1).

**Potential solution**
There are two main reasons that could lead to an early death after cell injection. Reason 1: The cell suspension may contain aggregated cells, which form an embolus and occlude vessels in the mice, ultimately causing, for example, cardiac congestion or stroke. Reason 2: The cell suspension contains air bubbles that cause an air embolism leading to similar symptoms as a cell embolus. Therefore, the injection of a single cell and air-free suspension is critical to avoid lethality related to cell injections.

**Problem 2**
The mouse succumbs during the orthotopic injection (step 7d).

**Potential solution**
Death during orthotopic implantation could be caused by internal bleeding after puncturing the aorta, which is located dorsal and medial to the adrenal fat pad. The use of mice aged 6 weeks or...
younger may facilitate the injection and avoid this complication because the fat pad has not involuted yet, which occurs with increasing age.

**Problem 3**
A mouse dies after the orthotopic implantation though there is no evidence of injury to the aorta (step 7f).

**Potential solution**
The mouse may not awake after the orthotopic implantation due to issues with the anesthesia. To avoid prolonged or excessive anesthesia, mice should be kept for <5 min in the anesthesia chamber. Since mice are also sensitive to body temperature changes, the use of a heating lamp or pad may maintain an adequate body core temperature during the procedure.

**Problem 4**
The recommended functional titer is $\geq 5 \times 10^7$ TU/mL, while a titer below $2 \times 10^7$ TU/mL is considered as low (step 12).

**Potential solution**
There are a few factors affecting lentivirus titer: 1) Lenti-X™ 293T cells need to be healthy and actively dividing at early passages. 2) High quality endotoxin-free transfer, envelope and packaging plasmids are important for optimal transfection efficiency. 3) Different transfer plasmids can affect titer, and the ratios of plasmids to transfection reagent may need to be optimized.

**Problem 5**
CAR T cell expansion is slow during the culture (step 15).

**Potential solution**
We observed that T cells enter in the phase of rapid expansion from day 6 post-activation. Supplementation of IL-2 is required at least every other day even if new culture medium is not added into the culture. If T cell expand less than 10-fold on day 9 of culture with appropriate IL-2 supplementation, selection of another T cell donor is necessary for CAR T cell production.

**Problem 6**
Mice may develop symptoms of GvHD (>15% weight loss, hunched posture, fur loss, and reduced mobility), thereby reaching a humane endpoint before the end of the experiment, which can add a variable of efficacy outcomes (step 21).

**Potential solution**
We observed that mice injected with both mock or CAR T cells can develop GvHD. The occurrence of GvHD correlates with the injected T cell dose. Thus, selecting a T cell donor that can yield high

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**Figure 9. Immunohistochemistry staining of tumor tissues**
(A) Densely packed small round blue NB cells (red arrow) are seen throughout the field of view.
(B) CAR T cell-treated tumors also have clusters of NB cells (red arrow) but the remaining light pink areas represent mostly necrosis.
(C) Staining for CD3⁺ T cells (black arrow) in the vicinity of tumor clusters. Scale bar, 200 μm, 5× magnification.
transduction efficiencies can delay the onset of GvHD because less total numbers of T cells are transferred for reconstitution.

Problem 7
The CAR T cell therapy shows a trend but no significant efficacy compared to controls (end-of-study evaluations).

Potential solution
We optimized the tumor and CAR T cell dose in this model to treat animals in the therapeutic window and yield a therapeutic effect. However, the therapeutic window may be missed if the tumor burden is too large at the time of therapy or the effector cell number is too low. Thus, the timing of CAR T cell injection may have to be moved up or vice versa, the dose of CAR T cells increased to observe a therapeutic effect.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mitchell Ho (homi@mail.nih.gov). Further requests for information on orthotopic neuroblastoma with spontaneous metastases modeling and neuroblastoma tumor biology should be directed to Rosa Nguyen (hongharosa.nguyen@nih.gov) and Carol J. Thiele (thielec@mail.nih.gov).

Materials availability
IMR-5-luc-eGFP cell line, CT3 CAR plasmid (pMH303), and the CD19-targeted CAR plasmid (pMH376) generated in this study can be made available under appropriate materials transfer agreement.

Data and code availability
The published article includes all datasets generated or analyzed during this study. The data is available upon request to the lead contact.

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AUTHOR CONTRIBUTIONS
N.L. and R.N. performed experiments; N.L. and R.N. wrote the manuscript; C.J.T. and M.H. supervised the project; and all authors approved the final version of this manuscript.

DECLARATION OF INTERESTS
M.H. and N.L. are inventors on international patent application no. PCT/US2019/045338, “High affinity monoclonal antibodies targeting glypican-2 and uses thereof” and international patent application no. PCT/US2018/059645, “Chimeric antigen receptors for targeting tumor antigens.” The authors declare no other competing interests.
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