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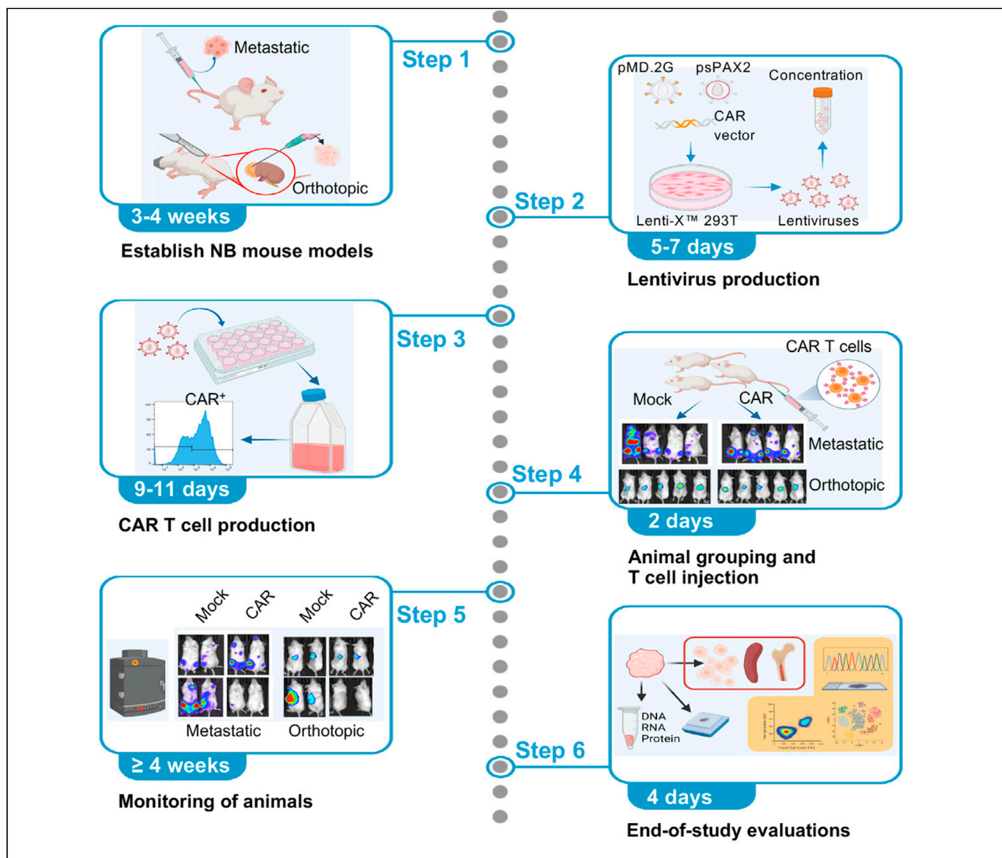
Protocol

Preclinical testing of chimeric antigen receptor T cells in neuroblastoma mouse models



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.

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Highlights

Establish neuroblastoma metastatic and orthotopic mouse models

Evaluate the tumor response to CAR T-cell therapy

Analyze the persistence of CAR T cells and T cell infiltration

Provide a platform for evaluation of experimental molecules for neuroblastoma

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Preclinical testing of chimeric antigen receptor T cells in neuroblastoma mouse models

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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

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

Fragment	Amino acid sequence
Signal sequence (SS)	MLLLVTSLLLCELPHPAFLLIP
CT3 scFv	EVQLQQSGPELVKPGASVKMSCKASRFTFTDYNIHWVKQSPGKTLEWIGYI NPNNGDIFYKQKFNGKATLTINKSSNTAYMELRSLTSEDSAVYYCVRSSNI RYTFDRFFDVWGTGTTVTVSSGGGGSGGGGSGGGGSENVLTQSPAIMSASL GEKVTMSCRASSSVNYIYWYQQKSDASPKLWIYYTSNLAPGVPARFSGSGS GNSYSLTISSMEGEDAATYYCQQFSSSPSTFGTGTKLELK
CD8α hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD
CD8α transmembrane	IYIWAPLAGTCGVLLLSLVIT
4-1BB	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
CD3ζ	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGK PRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALPPR
T2A	EGRGSLLTCGDVEENPGP
hEGFRt	RKVCNGIGIGEFKDSLSINATNIKHFKNCTSISGDLHILPVAFRGDSFTH TPPLDPQELDILKTVKEITGFLLIQAWPENRTDLHAFENLEIIRGRTKQH GQFSLAVVSLNITSLGLRSLKEISDGDVIISGNKNLCYANTINWKKLFGT SGQKTKIISNRGENSCKATGQVCHALCSPEGCWGPEPRDCVSCRNVSRGR ECVDKCNLLEGEPREFVENSECIQCHPECLPQAMNITCTGRGPDNCIQCA HYIDGPHCVKTCPAGVMGENNTLVWKYADAGHVCHLCHPNCTYGCTGPG LEGCPTNGPKIPSIATGMVGALLLLLVVALGIGLFM

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 a liquid nitrogen tank in batches with 50×10^6 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 IL2rg*^{null} (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

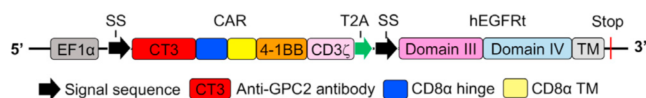

Figure 1. Schema of the second-generation CT3 CAR construct
A truncated human epidermal growth factor receptor (hEGFRt) is added into the lentiviral construct to allow cell tracking by using the anti-EGFR monoclonal antibody cetuximab. SS: signal sequence. TM: transmembrane.

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/Cyanine5.5 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 Age requirement: <45 years, no sex or race selection	Oklahoma Blood Institute 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 BRB Frederick	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 IL2rg*^{null} (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*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
pMD2.G	Addgene	12259
Software and algorithms		
Living image	PerkinElmer	N/A
FlowJo 10.0	FlowJo, LLC	N/A
Others		
IVIS Lumina III imaging system	PerkinElmer	CLS136334
BD LSRFortessa flow cytometer	BD	SN H17700008
Nightsea fluorescence viewing system	Nightsea	SFA-RB
Bullet blender tissue homogenizer	Next Advance	BT24M
Falcon® 150 mm TC-treated Cell Culture Dish	Corning	353025
0.45 μm filter system	CELLSTEAT	229303
Cell culture T75 flask	SARSTEDT	83.3911.002
Cell culture T175 flask	SARSTEDT	83.3912.002
15 mL Conical tube	CELLSTEAT	229411
50 mL Conical tube	CELLSTEAT	229421
Cell culture 6-well plate	CELLSTEAT	229106
Cell culture 12-well plate	CELLSTEAT	229112
Cell culture 24-well plate	CELLSTEAT	229124
Dynamag magnet-15 mL tube	Life Technologies	12301-D
27-gauge needle	BD	305109
29-gauge needle	Exelint International	26016
1 mL syringe	BD	309659
Insulin syringe	BD	329461
Forceps	NCI Animal Facility	N/A
Scissors	NCI Animal Facility	N/A
Clips	Kent Scientific	427631
Vicryl sutures	Ethicon	J392H
Anesthesia chamber with nose cone	NCI Animal Facility	N/A
Heating lamp/pad	NCI Animal Facility	N/A
40 μm strainer	Falcon	352340

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.

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.		

Note: This is used for culturing IMR-5-luc-eGFP.

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.		

Note: This is used for culturing CAR T cells.

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.		

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 2.5×10^7/mL (for the injection of 5.0×10^6 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.

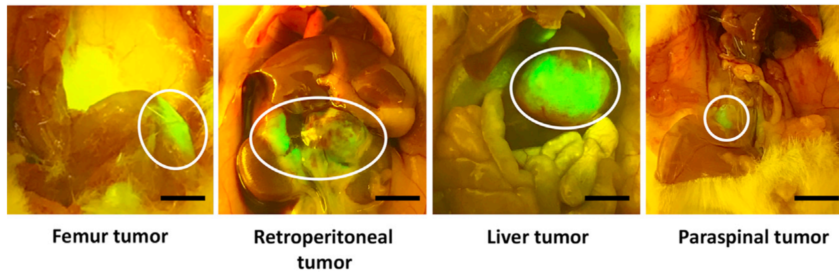

Figure 2. Metastatic IMR-5 NB cells in mice
The metastases are found in femur, abdomen, liver, spine and other sites. The white circled areas indicate tumors. The eGFP signal was detected by using the Nightsea fluorescence viewing system. Scale bar, 1 cm.

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 (5.0×10^6 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: ≈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 0.25×10^6/mL cells per mouse (plus an excess of 20%). The cells are then resuspended in ice-cold Matrigel matrix equaling 30 µL per injection and kept on ice.

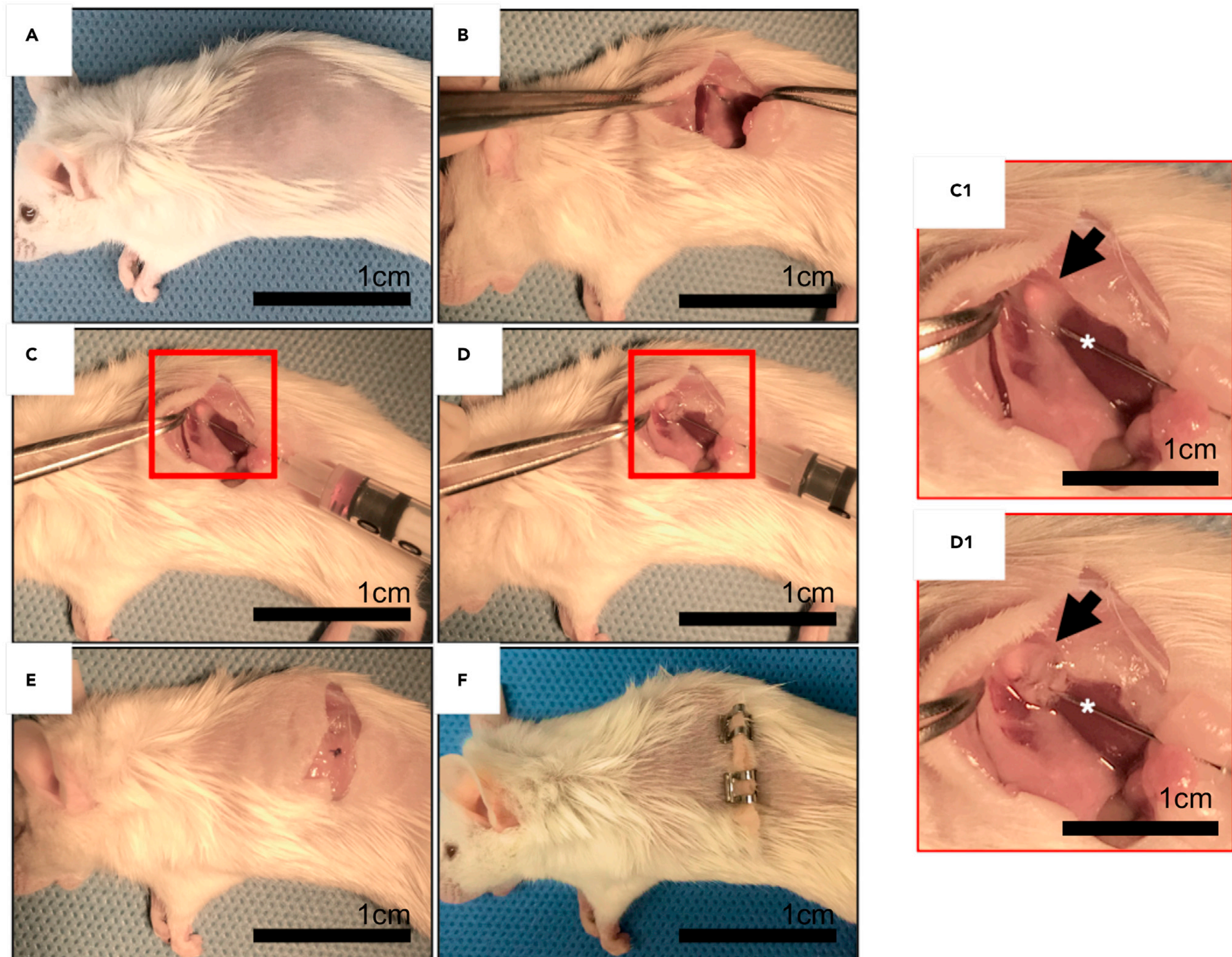

Figure 3. Orthotopic implantation of NB cells
(A) Mice are shaved in preparation for surgery.
(B) A transverse cut is made and dissected down to visualize the spleen.
(C and D) (C) The adrenal gland is visualized (black arrow) and (D) tumor cells are injected into the periadrenal fat pad. Images (C1) pre- and (D1) post-injection demonstrate swelling of the fat pad after cells are injected. The star indicates the tip of the injection needle.
(E) The peritoneum is closed with one basic surgical knot.
(F) The skin is closed with clips. Scale bar, 1 cm.

⚠ 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 committee 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 primary 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 tumor 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 lentivirus at own institution.

11. Lentivirus production
    a. Seed Lenti-X™ 293T cells at low passages (4–10) at a density of 20 × 10^6 cells per Poly-D-lysine–coated 15 cm dish.
    b. The following day, transfect Lenti-X™ 293T cells with the CT3 CAR plasmid (pMH303), envelope (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 $1/20^{th}$ to $1/100^{th}$ of the original volume using AIM-V complete medium.

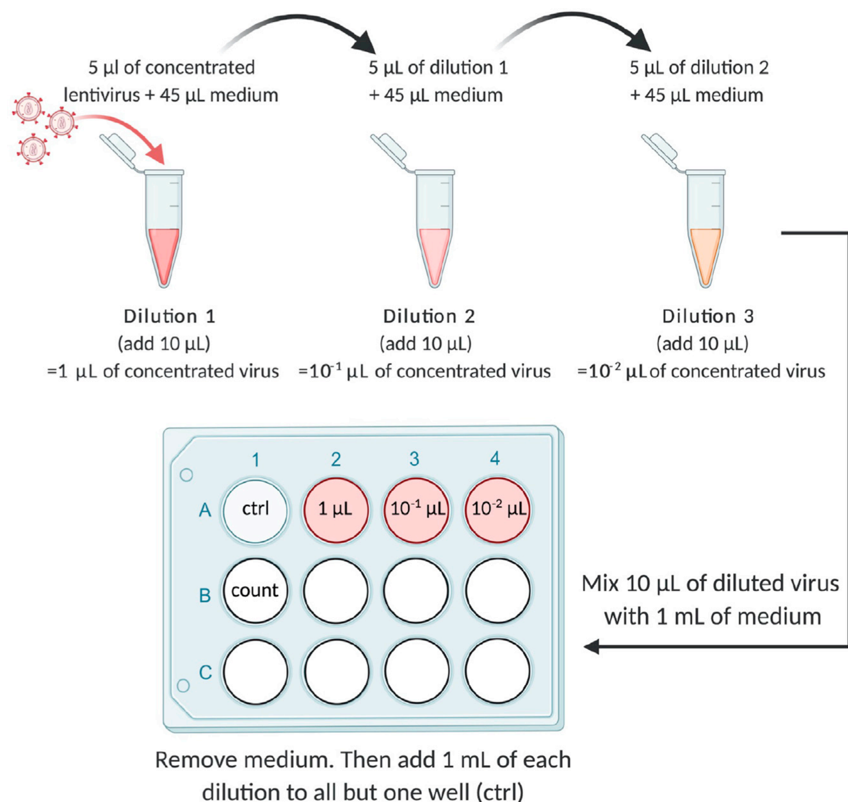

Figure 4. Graphical example of lentivirus titration in a 12-well plate format
One well of cells is used for counting the cell number. One well of non-lentiviral transduced cells is included as control (ctrl). Three dilutions of concentrated lentivirus are used in this protocol.

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 1×10^5 cells in 12-well cell culture plate.
   b. The following day, use one well to count cells. It should be between 1–3 $\times$ 10^5 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: Titer (transduction unit/mL, TU/mL)= (F×Cn/V) × DF; F= % 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 $\geq 5\times10^7$ 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).
    b. Thaw T cells and seed 1×10^6 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 (4×10^7 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 μg/mL and replenish the medium with 100 IU/mL of IL-2.
    b. Add CAR-expressing lentivirus with a titer above 5×10^7 TU/mL into activated T cells at the multiplicity of infection (MOI) of 5. Calculate the volume of desired lentivirus using the following equation: Multiplicity of Infection (MOI)= Titer (TU/mL) × Volume (mL) / Cell Number
    c. Centrifuge the cells at 1000 x *g* for 120 min.
    d. Maintain the culture in 37°C, 5% CO_2 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 x *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 0.5×10^6 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.

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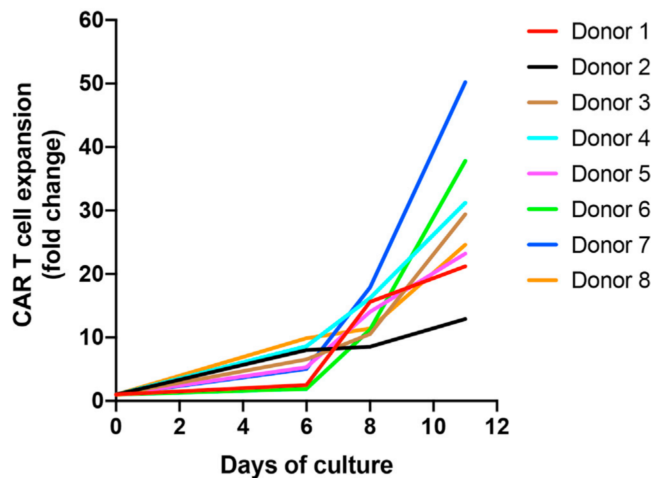

Figure 5. Proliferation of CT3 CAR T cells from eight healthy donors

⚠ CRITICAL: Excessive T cell density decreases cell viability.

16. Assessment of transduction efficiency by detecting either GPC2 binding or hEGFRt expression.
    a. Resuspend 1×10^6 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.

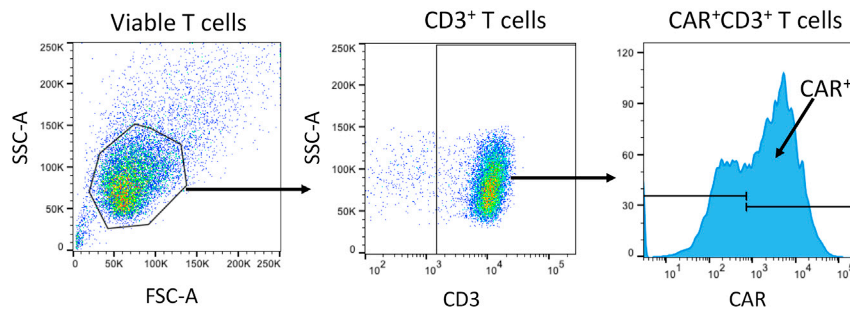

Figure 6. Measurement of transduction efficiency by flow cytometry
From the live cell gates, gates are drawn on $CD3^+$ T cells and populations plotted for CAR expression. The CAR expression can be detected by its binding to GPC2 or hEGFRt expression (detected by cetuximab). Of the transduced T cells, nearly 65% are CAR-positive.

19. The following day, count mock and CAR T cells. Each mouse receives 5–10 × 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 × 10^6 total T cells contain 5 × 10^6 CAR T cells. Accordingly, 7.7 × 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

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

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.

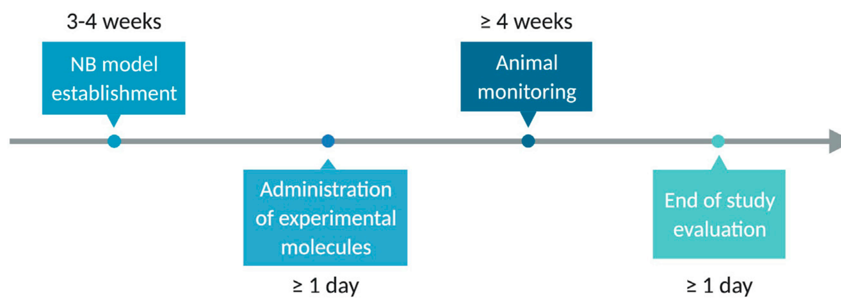

Figure 7. Experimental schematic of the IMR-5 metastatic and orthotopic mouse models
IMR-5 metastatic and orthotopic tumors are established 3–4 weeks prior to CAR T cell injection. Both models can be used to test various experimental therapies for NB. The treatment schedules vary based on the therapy type. Upon therapy initiation, mice are monitored for tumor growth, weight loss, and other symptoms of clinical change for at least 4 weeks. At the end of the experiment, tissues are harvested for further correlative studies.

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 1×10^6 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

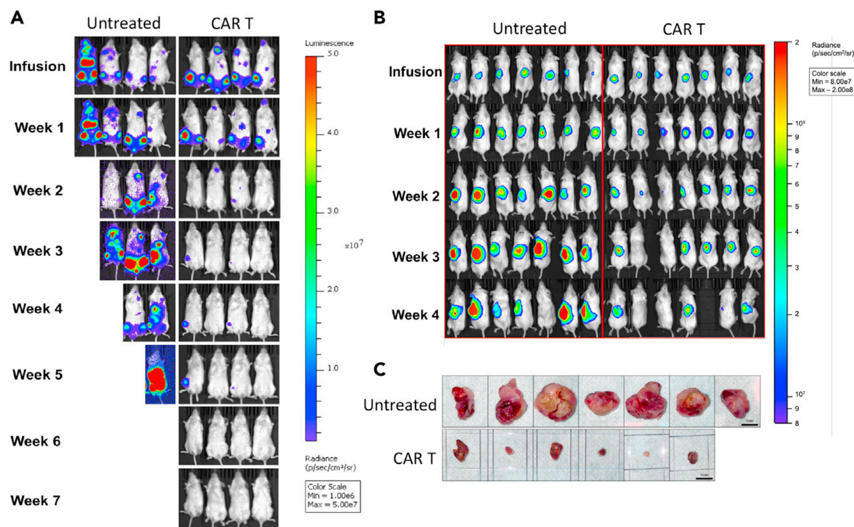

Figure 8. Weekly bioluminescence imaging of IMR-5-Luc-eGFP-bearing mice

(A) In the experimental metastasis model, CAR T cells regressed tumors in 100% of the mice and bioluminescence signal was undetectable, whereas the signal increased over time in the untreated group.

(B) Bioluminescence images of therapy cohorts using the orthotopic with spontaneous metastasis model. During week 4, there is one mouse in the untreated control cohort that lacks a signal, which was attributed to poor clinical status and decreased cardiac output, resulting in hypo-circulation of the D-luciferin. The signal intensity in the untreated cohort increased over time, while there were 3 animals in the therapy group that responded to therapy and showed almost undetectable levels of bioluminescence signal.

(C) The size of tumors in mice corresponding to Figure 8B. Scale bar, 1 cm.

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

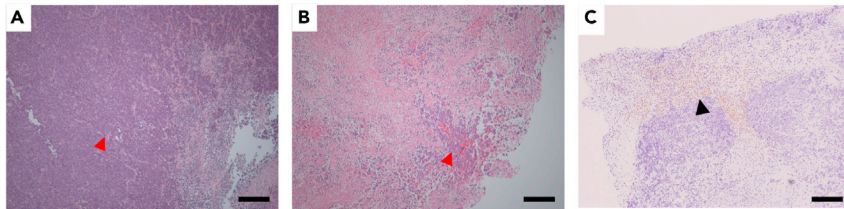

Figure 9. Immunohistochemistry staining of tumor tissues
(A) Densely packed small round blue NB cells (red arrow) are seen throughout the field of view.
(B) CAR T cell-treated tumors also have clusters of NB cells (red arrow) but the remaining light pink areas represent mostly necrosis.
(C) Staining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

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 2×10^7 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

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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