Title: CAR T cells targeting B7-H3, a Pan-Cancer Antigen, Demonstrate Potent Preclinical Activity Against Pediatric Solid Tumors and Brain Tumors


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Conflict of Interest Statement: C. Mackall and Y. Cui hold a patent comprising use of the B7-H3 CAR for cancer immunotherapy. D. Dimitrov, C. Mackall, and R. Orentas hold patents for anti-B7-H3 antibodies and single chain variable fragments. C. Mackall is a founder of and holds equity in Lyell Immunopharma. E. Bonvini is an employee of Macrogenics which has several agents targeting B7-H3 in clinical trials. R. Majeti is a consultant, equity holder, and member of the Board of Directors of Forty Seven Inc. D. Lee receives research funding from Kite Pharma and is a consultant for Juno Therapeutics. Other authors have no conflicts of interest.

Statement of translational relevance: We have undertaken the largest screen to date of B7-H3 expression on pediatric solid tumors and CNS malignancies. Using a previously described binder that preferentially binds tumor B7-H3 with restricted recognition on normal human tissues (MGA271, enoblituzumab), we generated a novel second generation chimeric antigen receptor (CAR). B7-H3 CAR T cells show significant in vivo activity against a range of xenograft models of lethal childhood cancers, including orthotopic models of osteosarcoma, Ewing sarcoma, and medulloblastoma. B7-H3 CAR T cells preferentially target tumor cells with high B7-H3 expression, demonstrating a possible therapeutic window for this novel agent. This work merits translation to the clinic where patients who have relapsed pediatric tumors have few therapeutic options, but will require carefully designed studies to mitigate potential toxicity.
Abstract:

Purpose: Patients with relapsed pediatric solid tumors and CNS malignancies have few therapeutic options and frequently die of their disease. Chimeric antigen receptor (CAR) T cells have shown tremendous success in treating relapsed pediatric acute lymphoblastic leukemia, but this has not yet translated to treating solid tumors. This is partially due to a paucity of differentially expressed cell surface molecules on solid tumors that can be safely targeted. Here, we present B7-H3 (CD276) as a putative target for CAR T cell therapy of pediatric solid tumors, including those arising in the central nervous system.

Experimental Design: We developed a novel B7-H3 CAR whose binder is derived from a monoclonal antibody that has been shown to preferentially bind tumor tissues and has been safely used in humans in early phase clinical trials. We tested B7-H3 CAR T cells in a variety of pediatric cancer models.

Results: B7-H3 CAR T cells mediate significant anti-tumor activity in vivo, causing regression of established solid tumors in xenograft models including osteosarcoma, medulloblastoma, and Ewing sarcoma. We demonstrate that B7-H3 CAR T cell efficacy is largely dependent upon high surface target antigen density on tumor tissues and that activity is greatly diminished against target cells that express low levels of antigen, thus providing a possible therapeutic window despite low-level normal tissue expression of B7-H3.

Conclusions: B7-H3 CAR T cells could represent an exciting therapeutic option for patients with certain lethal relapsed or refractory pediatric malignancies which should be tested in carefully designed clinical trials.
Introduction

Great progress has been made in the treatment of childhood cancer over the last forty years through the use of multimodal therapy, including combination chemotherapy, surgery, and radiotherapy (1). However, these gains have largely been realized in patients with leukemias, lymphomas, and localized sarcomas, while those with high risk and metastatic solid tumors and many CNS malignancies have seen few improvements (2,3). Furthermore, mortality rates are >90% for nearly all pediatric patients with relapsed sarcomas and brain tumors (4,5). Attempts have been made to integrate new targeted drugs into the treatment of metastatic or relapsed diseases, but thus far this approach has not improved outcomes (6,7). New therapies are desperately needed for children and young adults with high-risk and recurrent solid or CNS tumors.

Immunotherapy represents a growing field of oncology that has already shown impressive results in both children and adults. The use of T cell checkpoint inhibitors for diseases such as metastatic melanoma has resulted in long term remissions in previously incurable adult diseases by unleashing a native immune response (8). However, early trials of checkpoint inhibitors have not been promising in most pediatric cancers (9). Limited numbers of neoantigens in these tumors may preclude them from being effectively treated by checkpoint inhibition (10). Consistent with this, we have shown limited expression of PD-L1, a biomarker for response to anti-PD-1 checkpoint blockade, on pediatric solid tumors (11). In contrast, genetically engineered immunotherapeutics can mediate antitumor effects against cancers with low mutation burden, and thus may be more effective against pediatric solid tumors (10).

Chimeric antigen receptor (CAR) T cells are an exciting approach that draw on molecular biology to arm cytolytic T cells with a receptor that can recognize a surface protein on tumor
cells (12). CD19 CAR T cells have shown unprecedented results in the treatment of pediatric hematologic malignancies (13-15), but clinical results for solid tumors have thus far not been as remarkable (16,17). This may relate in part to the selection of antigens that have been targeted using CAR T cells for solid tumors. Unlike CD19 and CD22, lineage derived antigens that are highly expressed on cancer cells(13), many solid tumor antigens are expressed at lower levels on the surface of cancer cells(18). We and others have shown that low density antigen expression is insufficient for optimal CAR activation, raising the prospect that low levels of expression on normal tissue may be tolerable (18-21). Uncovering surface targets with differential expression between tumor and normal tissue has been a major focus for the application of CAR T cells outside of hematologic malignancies (22).

B7-H3 (CD276) is a checkpoint molecule expressed at high levels on pediatric solid tumors, including sarcomas and brain tumors (23-25). B7-H3 expression contributes to tumor immune evasion (26) and metastatic potential (27) and is correlated with poor prognosis (28). Two monoclonal antibodies targeting tumor-associated B7-H3, 8H9 and MGA271, are safe and have generated promising results in clinical trials (29-33). Given its role in tumor biology and its high level of expression across a wide range of pediatric cancer histologies, we hypothesized that B7-H3 would be a good target for CAR T cell immunotherapy. Here we present our results in constructing and testing a CAR targeting B7-H3, which demonstrates clear evidence of in vivo preclinical activity, regressing and clearing osteosarcoma, Ewing sarcoma (EWS), and medulloblastoma xenografts. Additionally, we demonstrate that B7-H3 CAR T cells exhibit a therapeutic window through which they may target high antigen expressing cells while leaving low antigen expressing cells largely intact.

**Materials and Methods**
Primary tumor samples

Archived samples representing a variety of pediatric tumor types were obtained from multiple sources (Children’s Hospital of Philadelphia, Seattle Children’s Hospital, Toronto Sick Kids, and the Children’s Oncology Group) as TMA unstained slides. Each TMA comprised of 0.6 mm or 1 mm cores in duplicate. Two slides containing patient DIPG samples from 22 patients were obtained from the Pediatric Oncology Branch at the NCI. Informed consent was obtained from all subjects or their guardians for use of their samples for research, and local Institutional Review Boards confirmed that this analysis did not constitute human subjects research.

Immunohistochemistry

Freshly cut tissue and FFPE TMA sections were analyzed for B7-H3 expression. All immunohistochemistry was performed using the Ventana Discovery platform. B7-H3 immunohistochemistry was optimized and performed with an R&D Systems antibody (catalog number AF1027, Goat polyclonal antibody) against B7-H3 with 1:1000 and 1:600 dilution. In brief, tissue sections were incubated in Tris EDTA buffer (cell conditioning 1; CC1 standard) or at 95°C for 36 minutes to retrieve antigenicity, followed by incubation with a respective primary antibody for 2 hours and no heat. Bound primary antibodies were incubated with Rabbit anti-Goat secondary antibodies (Jackson, catalog number 305-005-045, with 1:500 dilution), followed by Ultramap anti-Rabbit HRP and Chromomap DAB detection.

For staining optimization and to control for staining specificity, kidney tissue was used as a negative control and liver tissue was used as a positive control. Intensity scoring was done on a common four-point scale. Descriptively, 0 represents no staining, 1 represents low but detectable degree of staining, 2 represents clearly positive staining, and 3 represents strong
expression. B7-H3 expression was quantified as an H-Score, the product of staining intensity and % of stained cells.

Cells and culture conditions

Human cell lines used in these studies were supplied by the following sources: MG63.3 by C. Khanna (NCI, NIH), K562 by C. Thiele (NCI, NIH), EW8 by L. Helman (NCI, NIH), NALM-6-GL by S. Grupp (University of Pennsylvania), DAOY and D283 by A. Martin (Johns Hopkins University), D425 by S. Chesier (Stanford University), and 293GP and 293T by the Surgery Branch (NCI, NIH). SU-DIPG VI and SU-DIPG XVII were generated as previously described(34). STR-fingerprinting was conducted to verify the identity of all cell lines, and each cell line was validated to be Mycoplasma free by qPCR or MycoAlert (Lonza). The 293GP and 293T cell lines were cultured in DMEM. For the DIPG cultures, neurosphere-generating cultures were maintained in serum-free media supplemented with B27 (ThermoFisher), EGF, FGF, PDGF-AA, PDGF-BB (Shenandoah Biotechnology), and Heparin (StemCell Technologies). D425 cells were maintained in serum-free media supplemented with B27 (ThermoFisher), EGF, FGF (Shenandoah Biotechnology), human recombinant LIF (Millipore) and Heparin (StemCell Technologies). All other cells were cultured in RPMI-1640. DMEM and RPMI-1640 media were supplemented with 2 mM L-glutamine, 10 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 10% heat-inactivated fetal bovine serum (FBS).

PBMC and T cell isolation

Elutriated human peripheral blood mononuclear cells (PBMCs) from consenting, healthy donors were obtained from the Department of Transfusion Medicine, NIH Clinical Center, under an NIH IRB-approved protocol, and cryopreserved. Thawed PBMCs were cultured in AIM-V media (Invitrogen) supplemented with 2 mM L-glutamine, 10 mM HEPES, 100 U/mL penicillin,
100 μg/mL streptomycin, 5% heat inactivated FBS, and r-human IL-2 (Peprotech). PBMCs were used for the majority of experiments. For the D425 and EW8 in vivo experiments, isolated T cells were obtained by negative selection using a RosetteSep T cell isolation kit (Stem Cell Technologies) on buffy coats obtained from the Stanford Blood Center.

**Identification of B7-H3 single chain variable fragments**

A large yeast display naïve single chain variable fragment (scFv) human antibody library was used to generate the anti-human CD276 single chain variable fragments as previously described(35). The library was constructed using a collection of human antibody gene repertoires, including the genes used for the construction of a phage display Fab library and those from more than 50 additional individuals and contained a total of 1e10 scFv’s. In vitro selection of the yeast display library involved three rounds of sequential panning on biotinylated, purified recombinant CD276(ED)-AP (alkaline phosphatase) fusion proteins. For this, 10 μg of biotinylated hCD276(ED)-AP was incubated with approximately 5e10 cells from the initial naïve antibody library in 50 ml PBSA (phosphate-buffered saline containing 0.1% bovine serum albumin) for 2 hours, washed with PBSA and captured with streptavidin conjugated microbeads from Miltenyi Biotec using the AutoMACS system (Cologne, Germany). The sorted cells were amplified and the panning was repeated once with the human hCD276(ED)-AP, and once with the mouse mCD276(ED)-AP protein to enrich for cross-reactive binders. After characterizing several scFvs for binding specificity, cross-species reactivity, a panel of five binders (1-5) were sequenced and used for CAR construction.

**B7-H3 CAR Construct Synthesis**

B7-H3 scFv’s (five from the yeast display library and one provided by Macrogenics) were codon optimized for expression in human cells and synthesized by GeneArt (Life Technologies), and
then introduced into an MSGV.1 retroviral expression vector containing a CD8-α hinge-transmembrane domain, a CD137 (4-1BB) co-stimulatory motif, and a CD3ζ signaling domain. The best performing CARs were also tested in a construct containing both the 4-1BB and CD28 co-stimulatory motifs and a CD3ζ signaling domain (3rd generation CAR). Where indicated, the sequence of the human IgG1 constant domain (CH2-CH3) was inserted between the scFv and the transmembrane domain. Resultant B7-H3 CAR constructs were sequence verified (Macrogen USA) and used for downstream applications. Both the B7-H3 CAR and the CD19 CAR were fused to mCherry at the C terminus end for T cell trafficking experiments as previously described (36).

**Production of Retroviral Supernatant and CAR T Cell Transduction**

Retroviral supernatant for the B7-H3 CARs or CD19 CARs was produced by transient transfection of 293GP cells with the corresponding CAR plasmid and an RD114 envelope plasmid as previously described(18). T cell transduction was performed as previously described(18). Briefly, PBMCs were thawed and activated by culture for 2 or 3 days in the presence of 40 U/mL rhIL-2 (Peprotech) and anti-CD3/CD28 beads (Dynabeads, Human T-Activator CD3/CD28, Life Technologies) at a 3:1 bead:T cell ratio. Cells were exposed to retrovirus containing supernatants on days 2 and 3, or days 3 and 4, in media containing 300 U/mL rIL-2 on retronectin-coated non-tissue culture plates (on plate method as per manufacturer, Takara/Clonetech). Beads were magnetically removed on day 4 or 5, and cells expanded in AIM-V media containing 300 U/mL IL-2 until use in vitro and in vivo. For both in vivo and in vitro assays, CAR T cells were used on day 3–5 post bead removal.

**Lentiviral Engineering of Tumor Cell Lines**
NALM-6-GL cell lines stably overexpressing B7-H3 were produced by lentiviral transduction with supernatant containing the cDNA for B7-H3 (Origene, RC215064L1). The resultant bulk population was stained using B7-H3 antibody (R&D, clone MAB1027) and sorted into high-, medium-, and low-expressing lines using a FACS Aria (BD Biosciences). The bulk populations were then single-cell cloned on 96-well plates to create clones with distinct levels of expression.

**T Cell Functional Assays**

Cytokine release was assayed by co-incubating 100,000 CAR$^+$ T cells with 100,000 tumor cell targets in complete RPMI-1640. At 24 hours, culture media were collected and cytokines were measured by a Human Proinflammatory Panel V-plex Human Tissue Culture multiplex assay (Meso Scale Discovery). In some experiments, IL-2 and IFN-$\gamma$ were measured by ELISA (Biolegend). Killing assays were performed by coculturing 50,000 CAR$^+$ T cells with 50,000 GFP-positive tumor cell targets in complete RPMI-1640 in a 96 well plate and acquiring images every 2-3 hours using an Incucyte (Sartorius). % Tumor cells remaining was calculated by dividing the Total Green Fluorescence Intensity at every time point by the same measurement at the first time point. CD69 and CD107a assays were performed by coculturing 100,000 CAR$^+$ T cells with 100,000 tumor cell targets in complete RPMI-1640 in the presence of monensin and CD107a antibody (BioLegend, Clone H4A3, BV605). After six hours, cells were harvested, washed, and then stained with Fixable viability dye (eBioscience, eFlour 780), huCD45 (eBioscience, Clone HI30, PerCP-Cyanine5.5), and CD69 (BioLegend, Clone FN50, BV421). Cells were gated on viable single cells that were GFP- (tumors expressed GFP) and huCD45+ in order to assess both the % CD69 and/or CD107a positive cells and the mean fluorescence intensity for both CD69 and CD107a. To determine B7-H3 expression on tumor cells after exposure to B7-H3 CAR T cells, 100,000 CAR$^+$ T cells or MOCK untransduced T cells were
coclultured with 100,000 tumor cell targets in complete RPMI-1640 for 80 hours. After 80 hours, cells were analyzed by flow cytometry for B7-H3 expression.

Assessment of soluble B7-H3

100,000 tumor cells were plated in triplicate for 24 hours in 200ul of complete RPMI-1640. Supernatant was collected and then used in a Human B7-H3 Quantikine ELISA Kit (R&D).

Antibodies and Flow Cytometry Analyses

Staining for B7-H3 expression on tumor lines was performed with PE or APC conjugated mouse anti-human B7-H3 antibody (R&D, clone MAB1027). B7-H3 surface molecule number was calculated the Quantibrite kit (BD Biosciences) according to the manufacturer’s protocol. CAR expression on transduced T cells was also measured by flow cytometry. CAR T cells were stained with Biotin-Protein L (Thermo Fisher Scientific), followed by fluorophore-conjugated streptavidin (BD Biosciences). All staining was performed in 0.1 mL FACS buffer (PBS + 2% BSA). Flow cytometry was performed using a FACS Fortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star). For the T cell exhaustion and phenotyping panel, T cells were stained with anti-human LAG-3 (eBioscience, clone 3DS223H, PE), PD-1 (eBioscience, clone J105, PE-Cy7), TIM3 (BioLegend, clone F38-2E2, BV510), CD4 (BD Biosciences, clone SK3, BUV395), and CD8 (BD Biosciences, clone SK1, BUV805). Cell phenotype of monocytes was confirmed by staining for CD11b (BD Biosciences, Clone ICRF44, PE-Cy7) and CD14 (BD Biosciences, Clone M5E2, BV605) and of MoDCs by staining for CD-80 (Biolegend, Clone 2D10, BV605), HLA-DR (Biolegend, Clone L243, BV421), and CD11c (Biolegend, Clone Bu15, PE-Cy7).

Mice
Immunodeficient NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl /SzJ) were purchased from The Jackson Laboratory or bred in house. Mice used for in vivo experiments were between 6 and 12 weeks old, and the ratio of male to female mice was matched in experimental and control groups. All animal studies were carried out according to NCI and Stanford University Animal Care and Use Committee-approved protocols.

*MG63.3 in vivo models*

Animal studies were carried out under protocols approved by the NCI Bethesda and Stanford University Animal Care and Use Committees. Cell lines were expanded under standard culture conditions (described above) and harvested with 2 mM EDTA (KD Medical) in PBS (Gibco, Thermo Fisher Scientific) or Trypsin (Gibco, Thermo Fisher Scientific). For MG63.3, 1e6 cells were injected periostal to the tibia. In the direct tumor shrinkage experiments, 1e7 B7-H3 CAR+ T cells or an equivalent number of CD19 CAR T cells (matched for total T cell dose) were injected intravenously into a tail vein two to three weeks after tumor inoculation (once the majority of tumors had an area greater than 75 mm²). Tumor growth was measured with digital calipers once to twice weekly, and the tumor area was calculated by multiplying the lengths of the major and minor axes. Mice were euthanized when the tumor exceeded a size set by institutional protocol. In the metastatic model experiments, once the bulk of the mouse tumors reached greater than 12.5 mm in one direction, the tumor bearing leg of all mice was amputated using sterile technique under isoflurane anesthesia. Buprenorphine 0.05 mg/kg was injected subcutaneously for pain control. Mice were randomized to B7-H3 CAR treatment or no treatment groups based on their pre-amputation tumor sizes and groups were statistically identical. Five days after amputation, experimental mice were treated with 1e7 B7-H3 CAR+ T cells.
**Ewing sarcoma in vivo models**

2e6 EW8 cells were injected periosteal to the tibia. 1e7 B7-H3 CAR+ T cells or an equivalent number of CD19 CAR T cells (matched for total T cell dose) were injected intravenously into a tail vein two weeks after tumor inoculation. Tumor growth was measured with digital calipers once to twice weekly, and the tumor area was calculated by multiplying the length of the major and minor axes. Mice were euthanized when the tumor exceeded a size set by institutional protocol.

**DAOY in vivo models**

As previously described(37), mice were anesthetized with 50 mg/kg ketamine and 0.5 mg/kg dexmedetomidine by intraperitoneal (IP) injection. The mice were immobilized in a mouse stereotaxic device (Stoelting). The head was shaved and scrubbed with 1% povidone-iodine, then a 1 cm skin incision was made along the midline and a burr hole was made using an 18G needle 2 mm anterior and 1 mm to the right of the lambda. Using a 28G needle mounted on a Hamilton syringe, 5e4 GFP-luciferase expressing DAOY cells in 5 ul were injected 3 mm deep from the surface of the skull over 5 minutes. The needle was slowly retracted and the incision closed using wound clips. Mice were treated with 1 mg/kg atipamezole IP to reverse the effects of dexmedetomidine. Buprenorphine 0.05 mg/kg was injected subcutaneously for pain control. Seven to ten days after tumor implantation and after confirmation of tumor formation by bioluminescence, mice were treated with 1e7 B7-H3 CAR+ T cells or an equivalent number of CD19 CAR T cells (matched for total T cell dose) intravenously by tail vein injection. Isofluorane-anesthetized animals were imaged using the IVIS system (Caliper Life Science) 10 minutes after 3mg D-luciferin (PerkinElmer, Waltham, MA, USA) was injected intraperitoneally. Living Image (Caliper Life Sciences) software was used to analyze the IVIS
data. Animals were imaged initially to confirm tumor implantation and then imaged twice weekly.

**D425 in vivo models**

As previously described(38), mice were anesthetized with 3% isoflurane (Minrad International, Buffalo, NY, USA) in an induction chamber. Anesthesia on the stereotactic frame (David Kopf Instruments, Tujunga, CA, USA) was maintained at 2% isoflurane delivered through a nose adaptor. D425 medulloblastoma cells were injected at co-ordinates 2mm posterior to lambda on midline and 2 mm deep into 6-10 week old NOD-SCID gamma mice using a blunt-ended needle (75N, 26s/2”/2, 5 μL; Hamilton Co., Reno, NV, USA). Using a microinjection pump (UMP-3; World Precision Instruments, Sarasota, FL, USA), 2e4 D425-GFP-Luc cells were injected in a volume of 3 μL at 30 nL/s. After leaving the needle in place for 1 minute, it was retracted at 3 mm/min. Tumor formation was followed by bioluminescence imaging on an IVIS spectrum instrument (Caliper Life Science) and quantified with Living Image software (PerkinElmer, Waltham, MA, USA). Four days after tumor implantation and after confirmation of tumor formation by bioluminescence, mice were randomized and treated with 1e7 B7-H3 CAR⁺ T cells or an equivalent number of CD19 CAR T cells (matched for total T cell dose) intravenously by tail vein injection. Isoflurane-anesthetized animals were imaged using the IVIS system (Caliper Life Science) 10 minutes after 3mg D-luciferin (Perkin-Elmer) was injected intraperitoneally. Living Image (PerkinElmer, Waltham, MA, USA) software was used to analyze the IVIS data.

**Immunofluorescence in D425 models**

Mice were deeply anesthetized with tribromoethanol (Avertin) before being perfused transcardially with cold PBS (two mice per group at two time points, Day +7 and Day +12 after CAR T cell infusion). Brains were fixed overnight in 4% PFA/PBS and transferred to 30%
sucrose for 2 days. Serial 40 micron coronal sections were then cut on a freezing microtome. Serially-sampled sections (1:12 series) were sampled and counterstained with DAPI. Mounted samples were imaged by confocal microscopy (Zeiss LSM710), and acquired Z stacks were flattened by maximum intensity projection (ImageJ). Tile images were acquired on an upright epifluorescence microscope with motorized stage (Zeiss AxioImager M2) and tile scanning software (MBF Bioscience).

*Leukemia in vivo models*

For K562, 1.5e6 tumor cells were transferred to NSG mice by tail vein injection. Five days later, 1e7 B7-H3 CAR⁺ T cells or an equivalent total number of mock/untransduced T cells were transferred intravenously. For NALM6-GL-B7-H3, 1e6 tumor cells were transferred to NSG mice by tail vein injection. Three to four days later, 1e7 B7-H3 CAR⁺ T cells or an equivalent total number of mock/untransduced T cells were transferred intravenously. NALM6-GL leukemia burden was evaluated using the Xenogen IVIS Lumina (Caliper Life Science). Mice were injected i.p. with 3 mg D-luciferin (PerkinElmer, Waltham, MA, USA) and then imaged 4 minutes later with an exposure time of 30 seconds. Luminescence images were analyzed using Living Image software (PerkinElmer, Waltham, MA, USA).

*Confirmation of antigen expression of tumor cell lines in vivo*

Indicated cell lines were engrafted into a mouse as was performed in the therapeutic experiments. Tumors were allowed to grow until mice became morbid and then were harvested (K562 from liver; NALM6-B7-H3 lines from bone marrow; EW8 and MG63.3 from orthotopic solid tumors). Single cell suspensions were made from tumor samples and red blood cells were lysed with ACK lysis buffer. Cell suspensions were stained for B7-H3 alongside cell lines with a fixable viability dye (eBioscience, eFlour 780). Tumor cells were gated by GFP except in the
case of EW8 in which cells were also stained for human HLA-ABC (BD Biosciences, Clone G46-2.6, FITC) and K562 in which cells were also stained for human CD45 (Biologend, Clone HI30, PE).

Monocyte and dendritic cell acquisition

PBMC were isolated from peripheral blood of healthy donors by Ficoll-Paque PLUS (GE Healthcare) gradient centrifugation. T cells were purified using the EasySep Human CD3 Positive Selection Kit (Stemcell Technologies). Monocytes were purified from the residual fraction of the same sample using the EasySep Human CD14 Positive Selection Kit II (Stemcell Technologies) and were cultured at 1 x 10^6 cells/mL in RPMI containing 10% FBS supplemented with 1000 U/mL human recombinant GM-CSF (Peprotech) and 500 U/mL human recombinant IL-4 (Peprotech) to generate monocyte-derived dendritic cells (moDCs). MoDCs were cultured for seven days with replacement of half of the culture volume with fresh media on days 3 and 5. CAR T cells were generated as described above from the same donors as the monocytes.

Graphs and Statistical Analysis

Data were visualized and analyzed using GraphPad Prism software. Graphs represent either group mean values ± SEM or individual values. All inset graphs are the averages of individual values seen. The p values were calculated with log rank statistics for survival analyses, the repeated-measures ANOVA for tumor growth curves, and student’s t tests for in vitro cytokine data and ANOVA for CD69/CD107a expression. p < 0.05 was considered statistically significant, and p values are denoted with asterisks as follows: p > 0.05, not significant, NS; * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001.

Results
**B7-H3 is highly and homogenously expressed on pediatric solid tumors**

Tumor microarrays (TMAs) were stained by immunohistochemistry (IHC) for expression of B7-H3. Of 388 tumor samples, comprising pediatric solid tumors and brain tumors, 325 (84%) were positive for B7-H3, with 70% demonstrating high intensity staining of 2+ or 3+. Representative images are shown in Figure 1a. Expression was typically homogeneous, with 291 (90%) of the positive samples staining positive for B7-H3 in 100% of the tumor cells on the core. A complete description of the IHC results is provided in Table 1. Greater than 90% of pediatric sarcomas tested expressed B7-H3, with rhabdomyosarcoma (alveolar and embryonal) and Ewing sarcoma samples demonstrating consistently high staining intensities and H-scores, defined as the product of staining intensity and % of stained cells (Figure 1b and Table 1). B7-H3 expression was also highly expressed in Wilms tumor and neuroblastoma (Figure 1c). Ganglioneuroblastoma and ganglioneuroma, more differentiated forms of neuroblastoma, demonstrated lower intensity staining and less frequent expression (Figure 1c). Pediatric brain tumors were also analyzed, including medulloblastoma, high grade gliomas (anaplastic astrocytoma and glioblastoma multiforme), and diffuse intrinsic pontine glioma (DIPG, recently re-classified by WHO as diffuse midline glioma, H3K27M mutant). Again, the majority of samples were positive, including 100% of the DIPG samples, albeit with lower mean intensity than the other two CNS tumor types (Figure 1d).

**Derivation of a novel B7-H3 CAR**

Given the promising high and homogeneous expression of B7-H3 on pediatric tumor tissues, we generated B7-H3 directed CAR T cells. Six anti-B7-H3 CARs were initially generated. Five fully human single chain variable fragments (scFv) (CD276.1, CD276.2, CD276.3, CD276.4, and CD276.5) were obtained by screening a yeast display library and a sixth
humanized scFv (CD276.MG) was obtained as a collaboration with Macrogenics (Rockville, MD). Of note, the scFv from MacroGenics contains the same DNA sequence found in enoblitzumab (MGA271), a humanized antibody that recognizes an epitope of B7-H3 with high tumor reactivity and restricted expression on normal human tissues (30). Second generation CARs with a 4-1BB costimulatory domain and a short hinge region were generated (Figure S1a). Using a gamma retrovirus, the CARs were expressed on primary T cells (Figure S1b) and screened for antigen induced interferon gamma production. Two of the six constructs, CD276.MG-4-1BB-ζ and CD276.3-4-1BB-ζ, showed superior function against cell lines expressing B7-H3 and were thus selected for further study (Figure S1c). The CARs that expressed at lower levels on the surface of T cells (CD276.1, CD276.2, and CD276.5) did not produce significant amounts of interferon gamma (Figure S1b-c), in line with our previously published work demonstrating that adequate CAR expression is required for efficacy(18). In order to determine whether other CAR structures could impart superior functionality, the CARs were re-engineered to contain either an extracellular spacer domain (CH2-CH3 from IgG1, Figure S1d) or an additional costimulatory domain (CD28-41BB-ζ, third generation CAR, Figure S1e). After co-culture with B7-H3 expressing cell lines, we found that these re-engineered CARs produced less cytokine than the original CARs with 4-1BB costimulatory domains (Figure S1f). Additionally, we found that the MGA271-based CAR, CD276.MG-4-1BB-ζ, hereafter referred to as the B7-H3 CAR, produced the most cytokine in response to tumor and thus it was chosen for further testing both in vitro and in vivo.

We previously demonstrated that tonic signaling can drive T cell exhaustion and contributes to failure of certain CAR constructs (39). In order to test if the B7-H3 CAR was superior to CD276.3 due to T cell exhaustion, we evaluated CARs transduced with each
construct for the expression of exhaustion markers PD-1, TIM-3, and LAG3, but we did not see major differences between the two that could account for their differential activity (Figure S1g). CD4 vs CD8 ratios for the B7-H3 CAR versus untransduced (MOCK) T cells are shown in Figure S1h.

_B7-H3 CAR T cells eradicate osteosarcoma and Ewing sarcoma xenografts in vivo_

We first tested the B7-H3 CAR in vivo against MG63.3, a xenograft model of osteosarcoma with strong metastatic potential (40). When co-cultured with this tumor line, B7-H3 CAR T cells specifically produced interferon-γ, TNF-α, and IL-2 (Figure 2a). NSG mice were orthotopically injected with 1e6 MG63.3 cells in the hind leg. Two to three weeks later, when all or most of the mice had measurable tumor with an area greater than 75 mm², animals received 1e7 intravenously administered B7-H3 CAR T cells or negative control CD19 CAR T cells (CD19 is not expressed by these solid tumors; Figure 2b). The B7-H3 CAR T cells mediated complete regression and eradication of xenografts (Figure 2c), leading to a significant survival advantage compared to control CAR T cell-treated mice (Figure 2d).

We also took advantage of the metastatic potential of this aggressive tumor line to evaluate the potential for B7-H3 CAR T cells to mediate activity against metastatic disease (Figure 2e). MG63.3 was clonally derived from the MG63 cell line because of its propensity to metastasize to the lungs (40). Mice were orthotopically injected with 1e6 MG63.3 tumor cells in the hind limb. Tumors were allowed to grow until they were approximately 12.5 mm in the longest dimension, at which point the hind leg and tumor were amputated. Five days later, mice were treated with 1e7 B7-H3 CAR T cells (Figure 2e). Because irrelevant CD19 control or mock transduced T cells cause xenogeneic GVHD and complicate long-term survival analysis, we compared B7-H3 CAR T cells to no treatment in this metastatic model (discussed below).
All untreated mice died within 50 days of amputation whereas 9/10 mice who received B7-H3 CAR T cells survived longer than five months (Figure 2f). At the time of death for the control mice, lungs of some mice were harvested in order to confirm the presence of metastasis, which could be grossly seen replacing most of the lung tissue. Therefore, B7-H3 CAR T cells mediate activity against both established and metastatic osteosarcoma xenografts.

In order to confirm activity in an additional model of pediatric sarcomas, we elected to test the B7-H3 CAR against a Ewing sarcoma xenograft. Two weeks after orthotopic tumor inoculation of 2e6 EW8 cells in the hind limb, NSG mice were treated with 1e7 intravenously administered B7-H3 CAR T cells or control CD19 CAR T cells (Figure 3a). The B7-H3 CAR T cells eradicated tumor (Figure 3b), leading to a significant survival advantage compared to control treated mice (Figure 3c).

**B7-H3 CAR T cells mediate activity against pediatric CNS tumors**

When co-cultured with medulloblastoma cell lines or DIPG patient derived cultures (34), B7-H3 CAR T cells specifically produced interferon-γ, TNF-α, and IL-2 (Figure 4a). In order to test if the B7-H3 CAR can effectively cross the blood-brain barrier and eradicate CNS tumors, we intravenously administered 1e7 B7-H3 CAR T cells to mice bearing DAOY medulloblastoma xenografts (engineered to express GFP-luciferase) in the posterior fossa (Figure 4b). B7-H3 CAR T cells eradicated the autochthonous xenografts as measured by bioluminescent imaging (BLI, Figures 4c-d). We then tested the CAR against a more aggressive c-MYC amplified group 3 medulloblastoma xenograft, D425, that often metastasizes to the leptomeninges and spinal cord(41) (Figure 4e). Again, intravenously administered B7-H3 CAR T cells cleared the disease by BLI (in 4/6 mice, Figure 4f-g), leading to significantly prolonged survival (Figure 4h). In order to better illustrate CAR T cell trafficking to the CNS, we fused both the B7-H3 CAR and
the CD19 CAR to the fluorescent protein mCherry and visualized mouse cerebellum at two time points after treatment in the D425 model by confocal microscopy. Confocal microscopy demonstrates that B7-H3 CAR T cells enter the CNS within seven days of treatment to eradicate tumor while control CAR T cells are not found in significant numbers in the CNS (Figure 4i).

**B7-H3 CAR T cells demonstrate a therapeutic window that can be exploited**

B7-H3 expression has previously been reported on lymphoid and myeloid leukemias (42). K562, an erythromyeloid leukemia, expresses B7-H3. We intravenously injected NSG mice with 1.5e6 K562 cells and then treated the mice with 1e7 B7-H3 CAR or MOCK untransduced T cells five days later (Figure 5a). Although the B7-H3 CAR demonstrated activity and prolonged survival in this model (Figure 5b), the results were not as striking as in our models of sarcoma and medulloblastoma, which was surprising given the clinical success of CARs in hematologic malignancies versus solid tumors. We ruled out the possibility that some of our tumor cells could be secreting B7-H3 that inhibits CAR activity as we did not detect B7-H3 protein in supernatant from tumor cells by ELISA (Figure S2a). We also confirmed that B7-H3 expression was maintained on all cell lines engrafted into mice at similar levels to expression on the cell line in culture (Figure S2b). We hypothesized that the limited activity of the B7-H3 CAR in this model was due to lower expression of B7-H3 on K562 than the other cell lines against which the CAR demonstrated impressive activity (Figures 5c-d).

To test this hypothesis, we lentivirally expressed B7-H3 on the surface of NALM-6, a B cell leukemia known to be susceptible to CAR T cells and obtained single clones expressing variable amounts of B7-H3 on their surface (Figures 6a-b). CAR T cells were tested in *in vitro* assays against NALM-6 lines expressing variable amounts of B7-H3. Both, tumor cell killing (Figure 6c) as well as CD69 (T cell activation) and CD107a (T cell degranulation) expression by...
T cells in response to tumor encounter (Figure 6d) were proportional to B7-H3 density on the tumor cells. Importantly, we detected minimal killing or T cell activation when tumor cells expressed low levels of B7-H3. There was no significant increase in the production of inflammatory cytokines by B7-H3 CAR T cells against low B7-H3 expressing NALM-6 as compared to the B7-H3 negative NALM-6 wildtype line. However, the B7-H3 CAR produced cytokines in response to NALM-6 cells expressing greater amounts of B7-H3 (Figure 6e-f).

NSG mice were then inoculated with 1e6 NALM6 cells expressing either low or medium amounts of B7-H3 and then treated with B7-H3 CAR T cells or control untransduced MOCK CAR T cells (Figure 6g). When leukemia cells expressed adequate B7-H3, the B7-H3 CAR mediated disease control (Figure 6h and 6j). However, when NALM-6 cells expressed lower levels of B7-H3, the CAR T cells demonstrated minimal in vivo activity (Figure 6i and 6k). Of note, no additional in vivo activity was seen when treating mice with leukemia expressing even higher levels of B7-H3 (Figure S3).

To confirm that antigen loss did not account for these findings, we assessed B7-H3 expression on tumor cells after an 80 hour period of coculture with B7-H3 or MOCK CAR T cells (Figure S2c). Antigen downregulation was not observed. We conclude therefore that the differences in tumor cell killing over time (Figure 6c) and in vivo activity (Figures 5b and 6h-6k) are due to differences in antigen driven T cell activation and proliferation. In summary, B7-H3 CAR T cells are promising for tumors with high level antigen expression and data are consistent with a therapeutic window in which low-level expression on normal tissue may not elicit toxicity.

**Conclusions/Discussion**
We present here preclinical results using a novel CAR targeting B7-H3, a pan-cancer antigen broadly expressed on many pediatric solid tumors. Additionally, we present the largest screen to date of pediatric tumor tissues for the expression of B7-H3. This molecule is highly and homogeneously expressed on numerous, common pediatric solid tumors, such as Ewing sarcoma, rhabdomyosarcoma, Wilms tumor, neuroblastoma, as well as CNS malignancies such as medulloblastoma. Homogeneous antigen expression is important for CAR T cell based therapies as tumor target heterogeneity is a limiting factor in treating malignancies with these powerful yet specific therapeutics (43,44), and the relatively low mutational burden in these cancers is expected to limit the degree of epitope spreading induced by CAR T cells (21). Other groups have similarly demonstrated expression of B7-H3 on DIPG (24), osteosarcoma (23), and neuroblastoma (45). The prognosis of the diseases tested here following relapse remains poor and this novel CAR could present a new therapeutic option. Additionally, we demonstrate expression on DIPG and high-grade gliomas, although in vivo efficacy remains to be tested in pediatric gliomas.

The exact function of this molecule is unclear, as its ligand(s) remain unknown. It was initially thought to be involved in T cell costimulation (46,47), however, there is substantial evidence for a model wherein B7-H3 mediates an immune-suppressive, checkpoint-like role (48,49). It has long been recognized as a potential target in cancer for antibody therapy (45) and, more recently, preclinical studies of an antibody-drug conjugate that targets both the tumor itself and tumor vasculature were published (35).

Despite initially drawing on a large yeast display Fab library to identify possible binders, ultimately our most efficacious CAR incorporated a binder from a previously published antibody (30). Though we have previously used a similar Fab library to generate a highly active CAR
against CD22 that is currently in clinical trials (50), this work serves as a reminder that antibodies that are readily available can be used to generate highly active CARs. Indeed, almost all versions of the CD19 CAR in clinical trials are based on previously described murine antibodies (51,52). Some of the differences in CAR efficacy between our binders appear to be related to lower CAR expression, which we previously reported can significantly impact CAR T cell function(18).

Our B7-H3 CAR based on MGA271 demonstrated impressive *in vivo* activity against pediatric bone sarcomas as well as medulloblastoma. Systemic administration of B7-H3 CAR T cells mediated regression and eradication of established osteosarcoma and Ewing sarcoma xenografts whereas no significant tumor control was observed with mock transduced cells. Additionally, drawing on a highly metastatic model of osteosarcoma with 100% lethality (40), we demonstrate near complete survival after treatment with the B7-H3 CAR, introducing the possibility that it could eventually be applied in an adjuvant setting for patients with high risk disease following standard therapy. Our metastatic model is limited by an inability to give mock T cells due to the universal development of lethal xenogeneic GVHD in mice given irrelevant or untransduced CAR T cells. However, the localized MG63.3 experiment demonstrates that the B7-H3 CAR anti-tumor effect is not xenogeneic. Additionally, administering unmanipulated naïve T cells has previously been shown to prevent osteosarcoma metastases in a murine model (50). However, this finding is unlikely to be clinically relevant given the failure of allogeneic stem cell transplantation to improve outcomes for children with solid tumors (51), while the B7-H3 CAR represents a novel, directly targeted approach that does not rely on GVHD for anti-tumor effect.
The B7-H3 CAR also mediates complete regression and clearance of two autochronous medulloblastoma xenografts (D425 and DAOY) when delivered intravenously. In a recent case report, intrathecally delivered CAR T cells mediated regression in an adult patient with glioblastoma multiforme (53), raising hopes that patients may be able to be treated with CAR T cells delivered regionally, potentially avoiding systemic toxicity. Whether local delivery would be more effective than systemic delivery utilized here is an area of ongoing study.

Activity against K562, a xenograft erythromyeloid leukemia line was more modest. While B7-H3 CAR T cells significantly extended survival compared to mice treated with untransduced T cells, the response was not universal or prolonged. We hypothesize that the activity of the CAR in this model is limited by lower B7-H3 antigen density on hematologic malignancies. Indeed, when we overexpressed the antigen on the NALM-6 B cell leukemia line at higher levels, the CAR mediated in vivo activity. When we expressed lower levels of the antigen on the same cell line, the CAR demonstrated minimal activity, indicating that a threshold of antigen density is required to mediate in vivo activity. This difference in in vivo functionality is supported by differences in antigen driven T cell activation and degranulation, tumor cell killing, and T cell cytokine production. We and others have previously demonstrated the importance of antigen density to CAR functionality (18,50).

For the B7-H3 CAR, a window in which some expression of the antigen on normal tissue may be below the threshold required for CAR efficacy could represent an advantage for targeting this pan-cancer antigen that is expressed at lower levels on normal tissue. Furthermore, while B7-H3 is expressed on some normal tissues, the antibody on which this CAR is based (MGA271) demonstrates minimal binding to normal tissues, as previously published in this journal by Loo et al. (30). MGA271 has been safely used in early phase clinical trials without
major toxicities and has resulted in clinically meaningful responses (31). A Phase I trial in children is ongoing (NCT02982941) as are trials of this agent in combination with a PD-1 inhibitors (NCT02475213). A CD3 x B7-H3 bispecific molecule that uses this same binder is in clinical trials in adults as well (NCT02628535). 8H9, another antibody targeting B7-H3, has also been safely used in clinical trials as a radioconjugate administered directly into the CNS of pediatric patients for more than ten years (29, 54) and has more recently shown clinical promise when directly administered into the abdomen of patients with desmoplastic small round cell tumor (33).

Of interest, previous work has shown that B7-H3 expression can be induced on normal tissue in response to inflammation (48), and safety of this novel therapeutic can only be accurately assessed in the context of a carefully designed clinical trial. Interestingly, when we differentiated monocytes to dendritic cells in vitro by culturing them in IL-4 and GM-CSF, we observed significant levels of B7-H3 expression and CAR reactivity (Figure S4). The level of B7-H3 on human dendritic cells in vivo has not been assessed and the clinical effects of CAR mediated targeting of dendritic cells is unknown. If toxicity is observed, logic gated CAR T cells (55) and/or probodies that mask a CAR except when the T cell is within the tumor microenvironment (56) could be developed to allow targeting of this broadly expressed tumor associated molecule.

In summary, we report on a CAR T cell directed at B7-H3 that shows strong activity against a wide array of xenograft pediatric cancer models including solid, liquid, and CNS tumors. We demonstrate that CAR T cell activity is dependent on antigen density, as has emerged for many CAR therapeutics. This CAR may provide a new therapeutic option for
children with incurable, metastatic or chemo-refractory disease and should be carefully studied in early phase clinical trials.
References


50. Fry TJ, Shah NN, Orentas RJ, Stetler-Stevenson M, Yuan CM, Ramakrishna S, et al. CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. Nat Med 2017 doi 10.1038/nm.4441.


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**Author contributions:** R.G.M., Y.K.C., R.J.O., and C.L.M. conceived of the study. R.G.M., and C.L.M. wrote and revised the manuscript. R.G.M., Y.K.C., A.N., S.H., J.T, S.R., M.H.L., E.S. C.R., P.X., L.L, and C.M. conducted the majority of experiments. C.M. and M.M. created and validated the DIPG patient derived cell cultures. M.Q. provided DIPG tissues for IHC. A.D., A.S., and P.S. performed the IHC and analysis. E.B. provided the antibody sequence (MGA271) on which the B7-H3 CAR is based. D.S.D., B.S.C, and Z.Z. derived the other single chain variable fragments that recognize B7-H3. D.W.L., S.M., J.M.M., R.M., M.M., and C.L.M. provided funding and/or supervision. All the authors contributed to the writing and editing of the manuscript.
**Figure Legends**

**Figure 1: B7-H3 is highly expressed on pediatric solid tumors.** (A) Pediatric tumor microarrays were stained by immunohistochemistry for the expression of B7-H3. Representative images of Ewing sarcoma (3+), glioblastoma multiforme (3+), medulloblastoma (2+), and alveolar rhabdomyosarcoma (3+, 2+, and 1+) samples are shown. H-scores were generated by multiplying the % cells positive x intensity seen for each core. H-scores are shown for (B) pediatric sarcomas, (C) neuroblastoma and Wilms tumor, (D) and pediatric CNS tumors.

**Figure 2: Systemically administered B7-H3 CAR T cells induce regression of osteosarcoma xenografts.** (A) B7-H3 CAR T cells produce IFN-γ, TNF-α and IL-2 following 24-hour in vitro co-culture with MG63.3 osteosarcoma. Representative results of 4 experiments with 3 different PBMC donors are shown. (B) Mouse model of orthotopic osteosarcoma:1e6 MG63.3 tumor cells were injected into the periosteum of the tibia in NSG mice. Two to three weeks later, following onset of measurable tumors, 1e7 B7-H3 CAR* T cells or irrelevant control CD19 CAR T cells were intravenously administered. (C) Tumor growth was measured biweekly by digital caliper and tumor area was calculated. Values for individual mice, as well as mean values per treatment group (inset) are shown. (D) Survival curves of mice treated as in (B). Representative results of 3 experiments with 3 different PBMC donors are shown. (E) Metastatic model of osteosarcoma: MG63.3-derived tumors were allowed to grow and metastasize before the mouse underwent amputation followed by administration of intravenous 1e7 B7-H3 CAR* T cells. (F) Survival curves of mice treated as in (E). Representative results of 4 experiments with 3 different PBMC donors are shown. All error bars represent SEM. P values were calculated as described in Material and Methods.

**Figure 3: Systemically administered B7-H3 CAR T cells induce regression of Ewing sarcoma xenografts.** (A) Mouse model of orthotopic Ewing sarcoma: 2e7 EW8 tumor cells were injected into the periosteum of the tibia in NSG mice. Two weeks later, 1e7 B7-H3 CAR* T cells or irrelevant control CD19 CAR T cells were intravenously administered. (B) Tumor growth was measured twice weekly by digital caliper and tumor area was calculated. Values for individual mice, as well as mean values per treatment group (inset) are shown. (C) Survival curves of mice treated as in (A). Representative results of 2 experiments with 2 different PBMC donors are shown. Error bars represent SEM. P values were calculated as described in Material and Methods.

**Figure 4: Systemically administered B7-H3 CAR T cells can clear medulloblastoma xenografts.** (A) B7-H3 CAR T cells were co-cultured in vitro with medulloblastoma cell lines and patient derived DIPG cell cultures and, 24 hours later, IFN-γ, TNF-α, and IL-2 were measured in the supernatant. Representative results of three experiments with three different PBMC donors are shown. (B) Orthotopic xenograft model of medulloblastoma: NSG mice were autochthonously injected with luciferase expressing DAOY medulloblastoma tumor cells. Following evidence of tumor engraftment by IVIS imaging, animals received 1e7 B7-H3 CAR* T cells or CD19 CAR T cells intravenously. (C) In vivo imaging of DAOY tumors treated with B7-H3 or CD19 CAR T cells. (D) Tumor progression was measured by bioluminescence photometry and flux values (photons per second) were calculated using Living Image software. Values for individual mice, as well as mean values per treatment group (inset) are shown.
Representative results of three experiments with three different PBMC donors are shown. (E) Orthotopic xenograft model of c-myc amplified medulloblastoma: D425 tumor cells expressing luciferase were autochthonously injected into NSG mice. Mice were treated with 1e7 B7-H3 CAR⁺ T cells or CD19 CAR T cells after 3-4 days, at which point tumor was detectable by IVIS imaging. (F) Tumor progression was measured by bioluminescence photometry and flux values (photons per second) were calculated using Living Image software. Values for individual mice, as well as average values per treatment group (inset) are shown. (G) In vivo imaging of D425 tumors treated with B7-H3 or CD19 CAR T cells. (H) Survival curves of mice shown in (G). Representative results of three experiments with three different T cell donors are shown. (I) Confocal images of brains from D425-GFP⁺ tumor bearing mice treated with B7-H3 CAR-mCherry or CD19 CAR-mCherry T cells, harvested at two different time points after T cell infusion. Representative image of two mice at two time points in one experiment. All error bars represent SEM. P values were calculated as described in Material and Methods.

Figure 5: B7-H3 CAR T cells have limited activity against B7-H3 low expressing K562 xenografts. (A) Mouse model of K562 leukemia: NSG mice were inoculated with K562, a myeloid leukemia that expresses low levels of B7-H3, and then treated with 1e7 B7-H3 CAR⁺ T cells or mock transduced control T cells five days later. (B) Survival curves of mice treated as in (A). Representative results of five experiments with three different PBMC donors are shown. (C) Flow cytometric analysis of B7H3 expression on the surface of K562 (leukemia), MG63.3 (osteosarcoma), EW8 (Ewing Sarcoma), DAOY and D425 (medulloblastoma) human cell lines. (D) Number of B7-H3 surface molecules expressed by human tumor cell lines as estimated by Quantibrite kit.

Figure 6: B7-H3 CAR T cells require adequate antigen expression for in vitro and in vivo activity. (A) Flow cytometry analysis of B7-H3 expression on single cell clones derived from Nalm-6 expressing different amounts of lentivirally expressed B7-H3. (B) Number of B7-H3 surface molecules expressed by Nalm-6-B7-H3 cell lines as estimated by Quantibrite kit. (C) GFP⁺ Nalm6-B7H3 clones were co-cultured with B7-H3 CAR T cells and tumor cell killing was measured in an Incucyte assay over 72 hours. Representative data of three experiments with three different PBMC donors is shown. (D) Percentage of CAR T cells positive (left panel) and Mean Fluorescence Index (right panel) for T cell activation and degranulation markers CD69 and CD107a, as measured by flow cytometry 6h after co-culture of tumor cells expressing increasing amounts of B7-H3 and CAR T cells. Representative results of three experiments with three different PBMC donors are shown. (E, F) Cytokine production by CAR T cells co-cultured with tumor cells expressing increasing amounts of B7-H3. (G) Mouse model for Nalm6-B7H3: 1e6 NALM6 cells expressing either low or medium amounts of B7-H3 were engrafted into mice by tail vein injection. Three days later, mice were injected with 1e7 B7-H3 CAR⁺ T cells or untransduced control T cells (MOCK). In vivo imaging of mice bearing (H) Nalm6-B7-H3-Medium leukemia or (I) Nalm-6-B7-H3-Low Leukemia. (J, I) Tumor progression was measured by bioluminescence photometry and flux values (photons per second) were calculated using Living Image software. Values for individual mice are shown. Representative results of four (Nalm-6-B7-H3-Med) and two (Nalm-6-B7-H3-Low) experiments with two different PBMC donors are shown.
Table 1: Expression of B7-H3 on pediatric tumors by immunohistochemistry (IHC).

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<th># Stained</th>
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<td></td>
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<td>2+ (%)</td>
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<td>Alveolar Rhabdomyosarcoma</td>
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<td>35%</td>
<td>16%</td>
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<td>Diffuse Intrinsic Pontine Glioma</td>
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<tr>
<td>TOTAL</td>
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<td>84%</td>
<td>34%</td>
<td>25%</td>
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<td></td>
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</table>
Figure 1

A

1. **Ewing Sarcoma (3+)**
2. **Glioblastoma Multiforme (3+)**
3. **Medulloblastoma (2+)**
4. **Alveolar Rhabdomyosarcoma (3+)**
5. **Alveolar Rhabdomyosarcoma (2+)**
6. **Alveolar Rhabdomyosarcoma (1+)**

B

- Graph showing H-Score distribution for Alveolar RMS, Embryonal RMS, and EWS.

C

- Graph showing H-Score distribution for different tumor types: Ganglioneuroma, Neuroblastoma, Wilms Tumor.

D

- Graph showing H-Score distribution for Medulloblastoma, High Grade Glioma, and DPFG.
Figure 2

A. IFN-γ, TNF-α, and IL-2 levels measured in MG63 and NALM-6 cells following B7-H3 CAR and MOCK T-Cell treatments.

B. Timeline of experimental protocol: MG63.3 inoculation, CAR treatment (IV), and amputation 30 days later.

C. Area (mm²) over time for B7-H3 CAR (n=5) and CD-19 CAR (n=5) treatments, showing a significant difference (p=0.0067).

D. Kaplan-Meier survival analysis for B7-H3 CAR (n=5) and CD-19 CAR (n=5) treatments, with p < 0.0001.

E. Follow-up experiment timeline: MG63.3 inoculation, amputation, and CAR treatment (IV) 5 days later.

F. Percent survival analysis post-amputation, comparing no treatment (n=5) and B7-H3 CAR treatment (n=5), showing a significant difference (p < 0.0001).
**Figure 4**

- **A** IFN-γ, TNF-α, and IL-2 levels in different conditions.
- **B** DAOY Inoculation followed by IV CAR Treatment.
- **C** Comparison of B7-H3 CAR and CD19 CAR expression post-treatment.
- **D** Total Flux (p/s) over days post-CAR treatment.
- **E** DAOY Inoculation followed by IV CAR Treatment.
- **F** Total Flux (p/s) over days post-CAR treatment for B7-H3 CAR and CD19 CAR.
- **G** Time-lapse imaging of B7-H3 CAR and CD19 CAR.
- **H** Percent survival of treated groups.

**Legend:**
- B7-H3 CAR
- CD19 CAR
- MOCK

**Statistical Significance:**
- **NS** Not significant
- **p = 0.09**
- **p = 0.0029**
Figure 5

A. K562 Inoculation → IV CAR Treatment

B. Percent survival over days elapsed post-tumor inoculation:
- Blue line: B7-H3 CAR (n=5)
- Red line: MOCK (n=5)

C. Flow cytometry analysis of B7-H3 expression:
- K562
- MG63.3
- EW8
- DAOY
- D425

D. Tumor Line | Molecules B7-H3/Cell
---|---
K562 | 4,750
MG63.3 | 93,544
EW8 | 36,260
DAOY | 92,050
D425 | 77,932
CAR T cells targeting B7-H3, a Pan-Cancer Antigen, Demonstrate Potent Preclinical Activity Against Pediatric Solid Tumors and Brain Tumors


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