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RESEARCH ARTICLE

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Central memory phenotype drives success of checkpoint inhibition in combination with CAR T cells

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Abstract

The immunosuppressive microenvironment in solid tumors is thought to form a barrier to the entry and efficacy of cell-based therapies such as chimeric antigen receptor (CAR) T cells. Combining CAR T cell therapy with checkpoint inhibitors has been demonstrated to oppose immune escape mechanisms in solid tumors and augment antitumor efficacy. We evaluated PD-1/PD-L1 signaling capacity and the impact of an inhibitor of this checkpoint axis in an in vitro system for cancer cell challenge, the coculture of L1CAM-specific CAR T cells with neuroblastoma cell lines. Fluorescence-activated cell sorting-based analyses and luciferase reporter assays were used to assess PD-1/PD-L1 expression on CAR T and tumor cells as well as CAR T cell ability to kill neuroblastoma cells. Coculturing neuroblastoma cell lines with L1CAM-CAR T cells upregulated PD-L1 expression on neuroblastoma cells, confirming adaptive immune resistance. Exposure to neuroblastoma cells also upregulated the expression of the PD-1/PD-L1 axis in CAR T cells. The checkpoint inhibitor, nivolumab, enhanced L1CAM-CAR T cell-directed killing. However, nivolumab-enhanced L1CAM-CAR T cell killing did not strictly correlate with PD-L1 expression on neuroblastoma cells. In fact, checkpoint inhibitor success relied on strong PD-1/PD-L1 axis expression in the CAR T cells, which in turn depended on costimulatory domains within the CAR construct, and more importantly, on the subset of T cells selected for CAR T cell generation. Thus, T cell subset selection for CAR T cell generation and CAR T cell prescreening for PD-1/PD-L1 expression

Abbreviations: CAR, chimeric antigen receptor; EGFRt, truncated epidermal growth factor receptor; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; gRNA, guide ribonucleic acid; IFNG, interferon gamma; Ig, immunoglobulin; IL, interleukin; KO, knockout; NaCl, sodium chloride; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand 1; T_{CM}, central memory T cells.

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could help determine when combination therapy with checkpoint inhibitors could improve treatment efficacy.

KEYWORDS
central memory T cells, chimeric antigen receptor T cell therapy, microenvironment, PD-1, PD-L1

1 | INTRODUCTION

Despite the remarkable antitumor potency of CD19-targeting chimeric antigen receptor (CAR) T cells against various hematological malignancies, similar results have not yet been achieved against solid tumors with CAR T cells. Trafficking to the tumor site, tumor recognition, CAR T cell proliferation, and persistence as well as overcoming the immunosuppressive tumor microenvironment present hurdles to CAR T cell efficacy against solid tumors.[1] Programmed cell death 1 (PD-1, formally known as PDCD1) is a coinhibitory receptor expressed by T cells and member of the B7:CD28 family.[2] PD-1 factors into the immunosuppressive tumor microenvironment, as binding to its ligand, PD-L1 (formally known as CD274) leads to a reduction in T cell receptor downstream signaling and attenuates T cell activation.[3-5] Combining adoptive CAR T cell therapy with checkpoint inhibition enacted by monoclonal antibodies against PD-1 was demonstrated to augment tumor-specific T cell efficacy against in vitro and in vivo models of metastatic melanoma, mesothelioma, and prostate cancer.[6-8] Encouraging results have been achieved by other groups using CRISPR/Cas 9 to disrupt the endogenous PD-1 gene or by introducing either a chimeric PD-1:CD28 receptor or a truncated PD-1 receptor lacking the intracellular signaling domain into CAR T cells.[9-12] Research to date demonstrates that blockade of the PD-1/PD-L1 checkpoint axis shows promise to boost CAR T cell efficacy against solid tumors.

Less than half the children with high-risk or relapsed neuroblastoma, the most common extracranial solid tumor in childhood, survive disease despite increasingly aggressive multimodal therapeutic regimens.[13-15] Immunotherapy has joined the focus of investigators trying to improve combination therapies for these patient groups. Researchers currently focus on two different neuroblastoma surface antigens, namely, GD2 and L1CAM, for CAR T cell development.[16] Our group previously developed a CAR targeting L1CAM,[17] and has begun to use T cell fractions enriched for central memory T cells (T_{CM}) to generate CAR T cells, since in vivo studies have shown that T_{CM}-derived CAR T cells more actively proliferate and persist longer.[18-20] So far, CAR T cells targeting neuroblastoma and derived either from T_{CM} or bulk T cells have shown promising antitumor activity in preclinical in vitro and in vivo studies.[17,21,22] However, first results from on-going clinical trials show only modest or transient responses to both, mainly because CAR T cell persistence is limiting.[23,24] The observed low T cell infiltration into neuroblastomas classifies them as immunologically "cold" tumors[25] making a response to checkpoint inhibitors unlikely. In contrast, a melanoma microenvironment inflamed with T cells was demonstrated to indicate responsiveness to PD-1 checkpoint inhibitors.[26] Moreover, INFG-driven PD-L1 expression, previously described as adaptive tumor cell immune resistance,[27] was confirmed in three of four metastatic neuroblast samples derived from bone marrow aspirates from high-risk neuroblastoma patients.[28] An encounter with the neuroblastoma-targeting CAR T cell might cause an inflamed neuroblastoma microenvironment and induce the PD-1/PD-L1 checkpoint signaling axis. Thus, treatment with a checkpoint inhibitor to cause PD-1 blockade might augment CAR T cell efficacy and keep the "cold" neuroblastoma microenvironment immunologically "hot."

To date, additional to the standard CD3zeta cytoplasmic domain, four different costimulatory domains (CD28, 4-1BB, OX40, and CD27) have been used in second-generation CAR constructs targeting neuroblastoma.[16] The type of costimulatory domain incorporated is generally thought to influence checkpoint signaling through the PD-1/PD-L1 axis. However, recent data have described the contradictory impact of the CD28 and 4-1BB costimulatory domains on PD-1 expression in CAR T cells.[29,30] These findings make predicting the impact of the type of costimulatory domain expressed in CAR T cells on PD-1 expression in neuroblastoma-targeting CAR T cells difficult, and requires additional testing to identify CARs benefiting from PD-1 inhibition. For this reason, we included CARs using both the CD28 and 4-1BB costimulatory domains in our testing regimen to assess impact on PD-1/PD-L1 checkpoint signaling in in vitro models for neuroblastoma cell challenge with L1CAM-specific CAR T cells. Our in vitro testing regimen was designed to select the best candidate CAR targeting the neuroblastoma-specific antigen, L1CAM, for a combinational approach with PD-1 checkpoint inhibition. We aimed to assess the impact of the choices of (a) costimulatory domain and (b) starting material for T cell manufacture on both CAR T cell efficacy and PD-1/PD-L1 signaling induction to evaluate whether nivolumab could augment L1CAM-specific CAR T cell efficacy against neuroblastoma cells.

2 | MATERIALS AND METHODS

2.1 | Cell lines

The SK-N-BE(2) neuroblastoma cell line (ATCC CRL-2271) was lentivirally transduced with PDL1-T2A-CD19t_epHIV7.2 before immunomagnetic selection with CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to enrich for transduced cells. Parental and PD-L1-expressing SK-N-BE(2) cells, as well as SK-N-AS (ATCC CRL-2137) and SH-SY5Y (ATCC CRL-2266) neuroblastoma cells were transduced with GFP-ffLuc_epHIV7. GFP-expressing cells were

selected using fluorescence-activated cell sorting (FACS). Parental and transduced SK-N-BE(2) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA) and parental and transduced SK-N-AS and SH-SY5Y cells in Roswell Park Memorial Institute (RPMI) 1640 (Gibco Life technologies) base medium, supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma-Aldrich, St. Louis, MO). The TMLCL EBV-transformed lymphoblastoid cell line TMLCL was maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS and 2 mM/L of L-glutamine (Biochrom, Berlin, Germany). The 293T cell line, used for lentiviral construct propagation, was cultured in DMEM supplemented with 10% heat-inactivated FCS.

2.2 | CAR construct

The previously described L1CAM-specific CE7-CAR[17] was cloned into the SIN epHIV7 lentiviral vector then propagated in 293T cells and isolated as previously described.[31] The single-chain variable fragment in the CAR construct was codon optimized and subsequently linked to a 229-amino acid spacer domain (long spacer) from the human immunoglobulin (Ig)G4 hinge. The long spacer domain was modified by substituting L235D and N297Q to reduce affinity to the IgG Fc gamma receptor (on natural killer cells and monocytes), which causes unintended CAR T cell activation via innate immune cell activation.[32] The spacer domain connects the antigen-binding domain to CD28 transmembrane domain followed by the signaling module containing the CD3zeta cytoplasmic domain and either the 4-1BB or CD28 (second-generation CAR) costimulatory domain. CAR constructs were linked downstream to a T2A self-cleaving peptide and a truncated epidermal growth factor receptor (EGFRt) allowing CAR T cell detection and enrichment.[33]

2.3 | L1CAM-specific CAR T cell generation

CAR T cells were generated from healthy donors (Charité ethics committee approval EA2/216/18) as previously described.[17] Briefly, apheresis products were obtained and peripheral blood mononuclear cells were isolated using Ficoll-Paque (GE Healthcare, Chicago, IL). $CD8^+$ T cells were obtained by positive selection using immunomagnetic microbeads (Miltenyi Biotec). If used for T_{CM}-derived CAR T cell generation, $CD8^+$ T cells were further enriched by CD45RA depletion and SELL positive selection using immunomagnetic microbeads (Miltenyi Biotec). Both bulk and T_{CM}-enriched $CD8^+$ T cells were activated with anti-CD3/CD28 beads at a bead-to-cell ratio of 1:1 (Life Technologies). On day 3, activated $CD8^+$ T cells were transduced with the lentivirus containing the CAR construct. The $EGFRt^+$ CAR T cell subset was enriched by immunomagnetic selection with biotin-conjugated cetuximab (Bristol-Myers Squibb, New York City, NY) and streptavidin microbeads (Miltenyi Biotec). T cells used as controls alongside CAR T cells in experiments were not lentivirally transduced. CAR and control T cells were cryopreserved until further use. Cryopreserved cells were thawed and stimulated with irradiated peripheral blood mononuclear cells, irradiated lymphoblastoid TMLCL cells, and 30 ng/mL antibody activating the CD3 complex (OKT3 clone, Miltenyi Biotec). For rapid expansion, T cells were maintained in RPMI 1640 media supplemented with 10% FCS, interleukin-15 (IL-15; 0.5 ng/mL, Miltenyi Biotec) and IL-2 (50 U/mL, Novartis, Basel, Switzerland) according to a rapid expansion protocol.[34] Functional in vitro assays were conducted between days 11 and 15 of culture or between days 11 and 25 for the serial tumor challenge.

2.4 | CRISPR-mediated generation of PD-L1 knockout in SK-N-BE(2) neuroblastoma cells

Guide RNA sequences were designed using Dr. Feng Zhang's online CRISPR design tool[35] (http://www.genome-engineering.org/crispr/?page_id=41), and targeted CD274 (formerly PD-L1) exons 2 and 3 to achieve a global knockout (KO) in the SK-N-BE(2) neuroblastoma cell line. Guide RNA sequences were TCTTTATATTCATGACCTAC (PD-L1-guide ribonucleic acid [gRNA1]) and TACCGCTGCATGATCAGCTA (PD-L1-gRNA2). Upon gRNA selection, respective oligonucleotides were annealed and ligated into the *BbsI*-digested CRISPR vector, pSpCas9(BB)-2A-Puro (PX459) V2.0 (donated by Feng Zhang to the Addgene nonprofit plasmid repository, Watertown, MA). Generated vectors were validated by sequencing before transfection into neuroblastoma cells using the Effectene transfection reagent kit (cat #301425, Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Briefly, parental and GFP-ffLuc_epHIV7-transduced SK-N-BE(2) neuroblastoma cells were plated 24 hours before transfection. Transfection reagents (100 µL Effectene buffer,[12] 1 µg of plasmid DNA, 2 µL Enhancer, and 7.5 µL Effectene) were added to the full media used for the media change of the plated cells. Selection was conducted using a full medium containing 2 µg/mL of puromycin on day 3 until day 6. SK-N-BE(2) cells with PD-L1 KO were first stimulated with 100 ng/mL recombinant human interferon-gamma (IFNG; PeproTech, Rocky Hill, NJ) in the medium for 24 hours before being enriched by immunomagnetic depletion using an APC-conjugated monoclonal antibody against PD-L1 (cat #329708, BioLegend, San Diego, CA) and APC microbeads (Miltenyi Biotec).

2.5 | In vitro tumor cell cytotoxicity assay in single and serial challenge

The parental SK-N-BE(2), SK-N-AS, and SH-SY5Y cell lines or derived models expressing high-level (transduced with a lentiviral vector) or no PD-L1 (CRISPR-based gene-KO) were stably transduced with the GFP-ffLuc_epHIV7 reporter plasmid to serve as tumor target cells. Target cells were cocultured in triplicate with

control T or CAR T cells at a 1:2 or 1:5 effector:target (E:T) ratio for singular tumor encounter and at a 1:1 E:T ratio during the serial tumor challenge. Nivolumab (Bristol-Myers Squibb) is a human monoclonal IgG-type antibody against PD-1. Lyophilized nivolumab was reconstituted in 0.9% sodium chloride (NaCl) for a stock solution, and used at 40 µg/mL culture medium for PD-1 blockade in functional in vitro assays. CAR T cell-induced cytotoxicity was quantified in a biophotonic luciferase assay in which the maximal biophotonic luciferase signal was defined by target cells plated with control T cells at the same densities (RLU_{max}, maximal relative light unit). After 24, 48, or 72 hours, 0.14 mg D-luciferin (PerkinElmer Inc, Waltham, MA) was added per mL medium in each well, and the biophotonic signal detected. The lysis was determined as $(1-[RLU_{sample}/RLU_{max}]) \times 100$ in relation to treatment with control T cells. Serial tumor cell challenge was conducted to mimic recursive antigen encounter on the basis of published experimental set-ups.[17,36] SK-N-BE(2) neuroblastoma cells and L1CAM-CAR T cells were cocultured at a 1:2 E:T ratio with nivolumab (or diluent, as control) added directly after plating. At 4, 8, and 12 days, L1CAM-CAR T cells were harvested and the living cells quantified using a hemocytometer and Trypan blue, before re-plating in fresh media containing added nivolumab or 0.9% NaCl with fresh SK-N-BE(2) neuroblastoma cells (E:T ratio of 1:2). Cytotoxicity assay and FACS-based marker analysis were conducted 24 hours after rechallenge at days 5, 9, and 12.

2.6 | In vitro cytokine release assay

For cytokine release assays, 2×10^5 T cells were seeded together with tumor cells at a 2:1 E:T ratio. After 24 hours, conditioned media was collected and stored at −80°C until analysis of IFNG using the OptEIA enzyme-linked immunosorbent assay (BD Biosciences) according to the manufacturer's instructions.

2.7 | Flow cytometric marker and antigen detection

Cell surface expression of CD8A (cat#301041, BioLegend), L1CAM (formerly CD171 cat #130-100-691, Miltenyi Biotec), PD-1 (PDCD1, cat #329922, BioLegend), PD-L1 (CD274, cat #329708, BioLegend), PTPRC isotopes CD45RO (cat #562641, BD Bioscience, Franklin Lakes, NJ) and CD45RA (cat #304105, BioLegened), SELL (formerly CD62L, cat #304821, BioLegend), and FAS (cat #305611, BioLegend) were detected by fluorophore-conjugated monoclonal antibodies on a Fortessa X-20 (BD Biosciences) 4-laser fluorescence-activated cell sorter (FACS). EGFRt expression was detected using biotinylated cetuximab (Bristol-Myers Squibb) and phycoerythrin (PE)-conjugated streptavidin antibody (cat #12-4317-87, BioLegend). QuantiBRITE PE calibration beads (BD Biosciences) were used to determine L1CAM antigen density on neuroblastoma cells according to the manufacturer's instructions. Briefly, QuantiBRITE PE calibration beads conjugated with four different amounts of PE molecules were used to generate a standard curve by comparing the geometric mean of fluorescence intensity and amount of PE. Neuroblastoma cell lines (200 000 neuroblastoma cells per well) were preincubated with PE-labeled monoclonal antibodies against L1CAM (Miltenyi Biotec) and the signal acquired in FACS analysis with the same settings as for QuantiBRITE PE beads. Antigen density at the cell surface for L1CAM was calculated from the fluorescence intensity of stained samples (geometric mean) using the standard curve. Flow cytometry data was processed using FlowJo software (Tree Star Inc, Ashland, OR).

2.8 | Statistical analysis

Differences in cytotoxic activity, cell surface marker expression, and intracellular cytokine expression between treatment groups and controls in experiments were tested using paired or unpaired Student *t* tests in GraphPad prism (GraphPad Software, La Jolla, CA). *P* values < .05 were considered statistically significant.

3 | RESULTS

3.1 | L1CAM-CAR T cell encounter upregulates PD-L1 on neuroblastoma cells and PD-1/PD-L1 expression in L1CAM-CAR T cells

The PD-1/PD-L1 axis usually plays no role in high-risk neuroblastomas due to low leukocyte infiltration of these immunological rather cold tumors. Most tumor cells upregulate PD-L1 surface expression in response to IFNG released by tumor-specific T cells (adaptive immune resistance).[27] We tested whether the encounter with neuroblastoma-specific L1CAM-targeting CAR T cells activates the PD-1/PD-L1 axis in neuroblastoma cells in vitro, providing a rational to combine CAR T cells with checkpoint blockade to treat neuroblastoma. To create a more challenging in vitro set-up, we selected three neuroblastoma cell lines expressing midrange or low surface densities of the targeted L1CAM antigen. The SK-N-BE(2) cell line expressed the median L1CAM level in the cell line panel examined using QuantiBRITE quantification, and low-antigen levels were expressed in the SH-SY5Y and SK-N-AS cell lines (Figure 1A). L1CAM-specific second-generation CAR T cells were generated from T_{CM}-enriched $CD8^+$ cells and comparable CAR expression was ensured by immunomagnetic selection for EGFRt (Figure S1). PD-L1 expression was significantly induced in all three cell lines after coculture with T cells expressing the L1CAM-targeting CAR but not control T cells (Figure 1B). We hypothesized that PD-L1 induction in neuroblastoma cells was driven by soluble cytokines released by activated L1CAM-CAR T cells after tumor encounter. Media conditioned by neuroblastoma cells alone, with L1CAM-CAR or control T cells for 24 hours was collected, then used to plate fresh neuroblastoma cells (Figure S2A). PD-L1 expression was only induced by

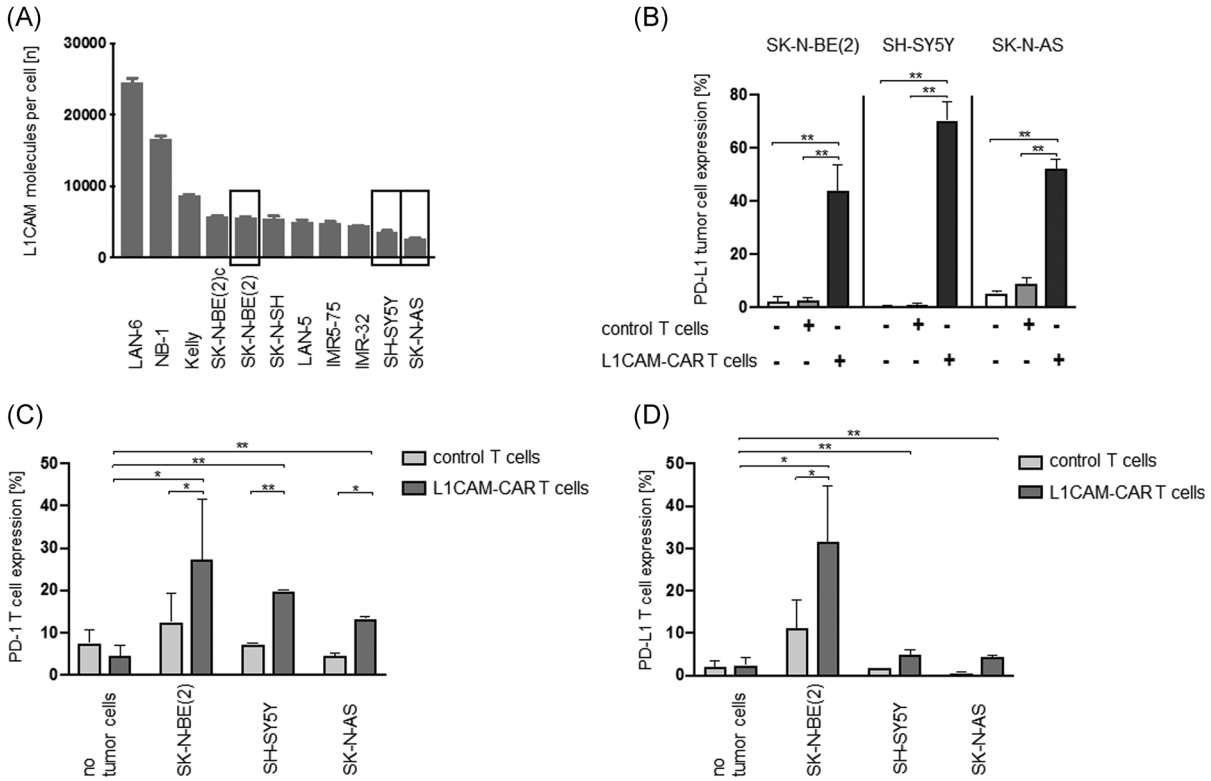

FIGURE 1 L1CAM-specific CAR T cell encounter activates the PD-1/PD-L1-axis in neuroblastoma cells. A, Cell surface L1CAM density of neuroblastoma cell lines obtained by flow cytometry. Chosen cell lines are framed. B, PD-L1 surface expression of SK-N-BE(2), SH-SY5Y, and SK-N-AS neuroblastoma cells alone and after coculture with either control or L1CAM-4-1BB-CAR T cells at a 1:5 effector:target (E:T) ratio for 24 hours. PD-1 (C) and PD-L1 expression (D) of central memory enriched control and L1CAM-4-1BB-CAR T cells before and after coculture with SK-N-BE(2) and SK-N-AS at a 1:1 E:T ratio or SH-SY5Y neuroblastoma cells at a 1:5 E:T ratio, respectively for 24 hours. Data shown depicts the mean expression from three independent experiments, $^{*}P < .05$ and $^{**}P < .01$ by unpaired (control compared with CAR T cells) or paired (control, CAR T or tumor cells alone compared with cocultured cells) Student *t* test. CAR, chimeric antigen receptor; PD, programmed cell death

24-hour exposure to medium conditioned by cocultured L1CAM-specific CAR T and neuroblastoma cells (Figure S2B). Furthermore, we detected IFNG in the conditioned media of neuroblastoma/L1CAM-CAR T cell cocultures underlining the finding that soluble factors rather than cell-cell interactions contribute to PD-L1 upregulation in neuroblastoma cells (Figure S2C). PD-1 expression also increased on the L1CAM-CAR T cell surface, and was most strongly induced by SK-N-BE(2) cells (Figure 1C). Interestingly, increased expression of the PD-1 receptor was accompanied by increased PD-L1 ligand expression on the L1CAM-CAR T cells after coculture with all three neuroblastoma cell lines (Figure 1D). PD-1-mediated inhibition of tumor-infiltrating T cells could not only be caused by tumor cells but potentially by PD-L1-expressing CAR T cells. Since we demonstrated PD-L1 expression in neuroblastoma cells was induced by the soluble cytokine IFNG, we next investigated if this was also true for PD-L1 expression in L1CAM-CAR T cells by adding either IFNG or anti-CD3/CD28 beads. Interestingly, only CD3/CD28 beads but not IFNG induced PD-L1 expression in L1CAM-CAR T cells excluding cytokine-mediated PD-L1 induction for CAR T cells (Figure S2D). Our data demonstrate that tumor/T cell encounter activated the PD-1/PD-L1 axis in L1CAM-targeting CAR T cells and neuroblastoma cells and caused PD-L1 upregulation on the CAR T cells themselves.

3.2 | Nivolumab enhances L1CAM-CAR T cell-directed neuroblastoma cell killing independent of PD-L1 expression on the tumor cells

Antibody-based blockade of PD-1 has been demonstrated to enhance CAR T cell efficacy in murine models for different solid tumors.[7,12] Having determined PD-1/PD-L1-axis activation in both L1CAM-CAR T and neuroblastoma cells, we assessed whether adding a monoclonal antibody against PD-1 (nivolumab), could boost L1CAM-CAR T cell efficacy in vitro. The SK-N-BE(2), SH-SY5Y, and SK-N-AS cell lines were transduced with a GFP-firefly luciferase reporter plasmid to quantify viable tumor cells in the luciferase-based reporter assay. L1CAM-specific CAR T cells were cocultured with SK-N-BE(2) reporter cells in a 1:2 and 1:5 E:T ratio with or without nivolumab. Cytotoxicity was assessed following 24, 48, and 72 hours of coculture by measuring the biophotonic signal released by the remaining viable tumor cells. Nivolumab

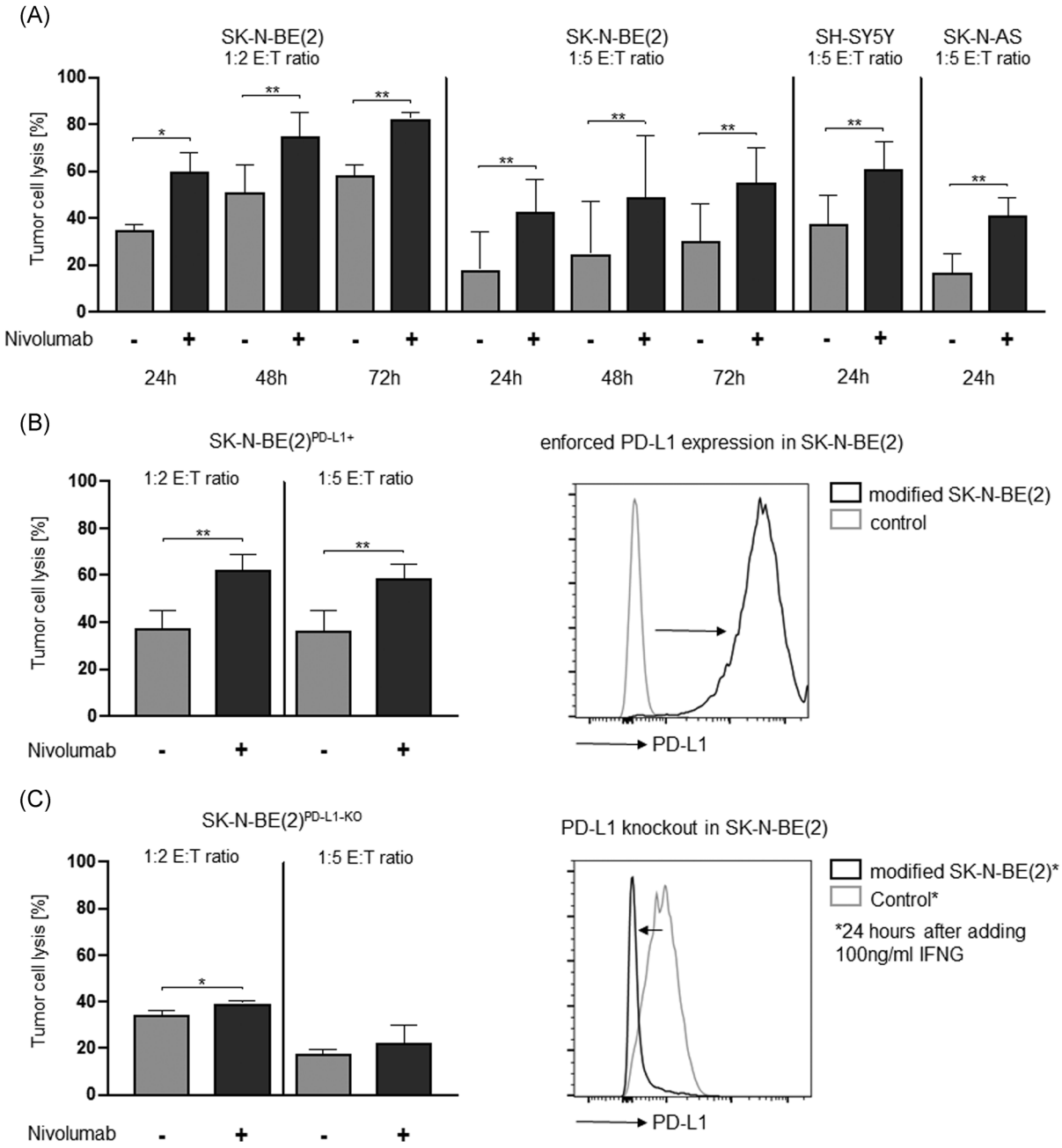

FIGURE 2 Nivolumab enhances neuroblastoma cell killing by L1CAM-4-1BB-CAR T cells independently of PD-L1 tumor cell expression level. A, Cytotoxicity of L1CAM-CAR T cells is determined by luciferase-based killing assay following a tumor coculture for 24, 48, and 72 hours at different E:T ratios and added nivolumab (or diluent, as control). Data shown depicts the mean cell lysis of three independent experiments, previously assessing the median of each experiment's triplicates. $^{*}P < .05$ and $^{**}P < .01$ by two-tailed paired *t* test. B, CAR T cell-induced cytotoxicity against SK-N-BE(2)$^{PD\text{-}L1+}$ after 24 hours at different E:T ratios ± nivolumab and flow cytometric quantification of PD-L1 expression on PD-L1-transduced SK-N-BE(2) cells. C, CAR T cell-induced cytotoxicity against SK-N-BE(2)$^{PD\text{-}L1\text{-}KO}$ after 24 hours at different E:T ratios ± nivolumab and flow cytometric quantification of PD-L1 expression on PD-L1-knock down SK-N-BE(2) cells 24 hours after adding 100 ng/mL IFNG in relation to parental SK-N-BE(2) cells. CAR, chimeric antigen receptor; E:T, effector:target; IFNG, interferon gamma; PD, programmed cell death

significantly increased L1CAM-CAR T cell-directed killing of SK-N-BE(2) cells in both effector to target ratios at all three-time points (Figure 2A). Cytotoxicity was more strongly enhanced by nivolumab with the 1:5 E:T ratio, demonstrating an increasing benefit of PD-1 blockade with enforced stress on L1CAM-CAR T cells. Nivolumab also significantly increased L1CAM-CAR T cell-directed killing (1:5 E:T ratio after 24 hours) of SH-SY5Y and SK-N-AS cells, which expressed low-antigen levels. We created neuroblastoma cells expressing high PD-L1 levels to test whether nivolumab required PD-L1 expression to enhance cytotoxicity. SK-N-BE(2) cells were

lentivirally transduced with PD-L1 to generated the SK-N-BE(2)$^{PD\text{-}L1+}$ neuroblastoma cell model (Figure 2B). Nivolumab significantly increased L1CAM-specific CAR T cell-directed killing of PD-L1-transduced SK-N-BE(2) cells in 1:2 and 1:5 E:T ratios after 24 hours. However, cytotoxicity was similar in SK-N-BE(2) cells with or without enforced PD-L1 expression in the presence of nivolumab, speaking against improved checkpoint inhibition response when tumor cells express high PD-L1 levels. PD-L1 KO was generated using CRISPR/Cas 9 in the SK-N-BE(2)$^{PD\text{-}L1\text{-}KO}$ model. PD-L1 expression levels in SK-N-BE(2)$^{PD\text{-}L1\text{-}KO}$ were below 8% after IFNG stimulation for 24 hours (Figure 2C). Nivolumab increased L1CAM-specific CAR T cell-directed killing in the SK-N-BE(2)$^{PD\text{-}L1\text{-}KO}$ model at the 1:2 but not 1:5 E:T ratio after 24 hours, indicating the benefit of PD-1 blockade remained despite minor PD-L1 tumor expression. Our data demonstrated that nivolumab-enhancement of L1CAM-CAR T cell efficacy in vitro did not strictly correlate with PD-L1 expression levels on neuroblastoma cells as a ceiling effect could be observed with enforced PD-L1 expression. These findings imply PD-1 mediated inhibition and its blockade foremost depend on PD-1 CAR T cell expression.

3.3 | Central memory derivation drives strong expression of PD-1/PD-L1-axis in L1CAM-CAR T cells

T_{CM}-derived CAR T cells persist longer in vivo,[18,19] and when an option, T_{CM} are used as a source. However, the T_{CM} cell fraction is lower in pediatric than adult patients, making T_{CM}-derived CAR T cell generation less often an option. Most ongoing pediatric CAR T cell studies use bulk-derived T cell products as starting material. L1CAM-CAR T cells were generated from the central memory fraction in experiments conducted so far in this study, and to bring preclinical testing closer to the clinical reality, we also compared L1CAM-CAR T cells from T_{CM} stocks with bulk-derived L1CAM-CAR T cells using our functional assays. Interestingly, PD-1 blockade with nivolumab did not enhance the cytotoxic activity of bulk-derived L1CAM-CAR T

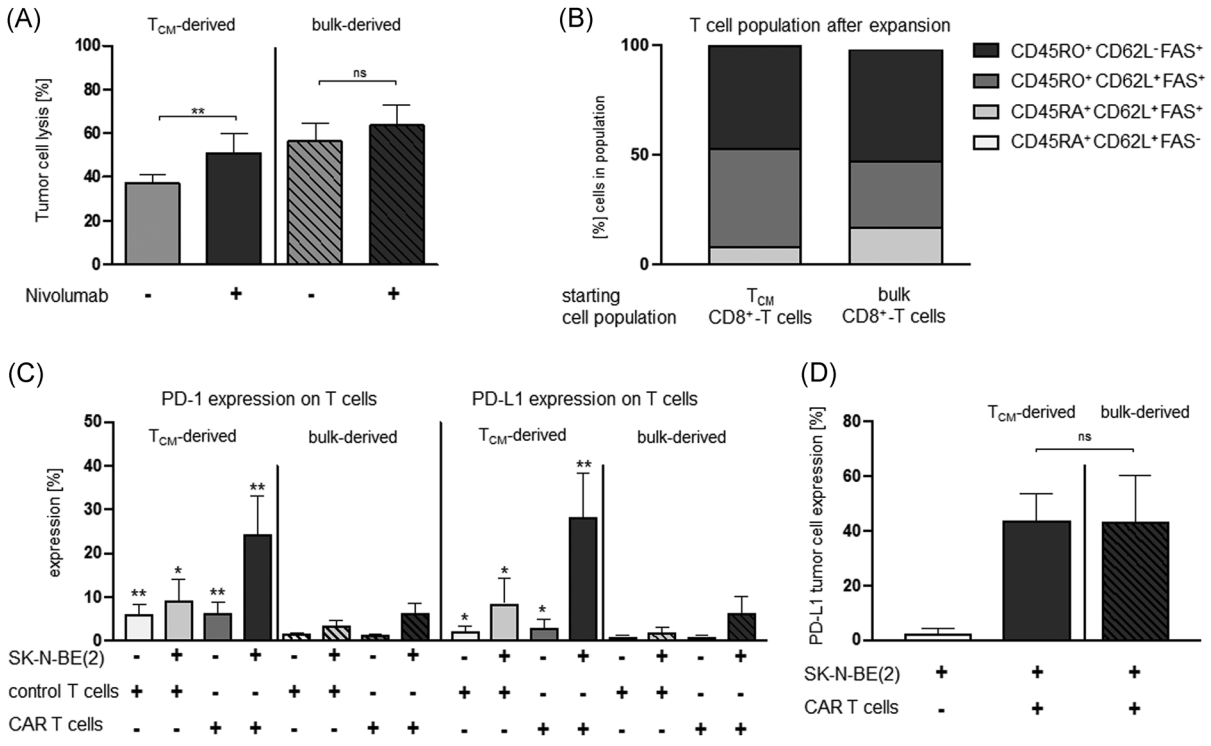

FIGURE 3 Starting material for CAR T cell generation impacts nivolumab efficacy. A, Cytotoxicity of L1CAM-CAR T cells derived from either bulk- or T_{CM}- cells is determined by luciferase-based killing assay following a tumor coculture for 24 hours at a 1:2 E:T ratio and added nivolumab (or diluent, as control). Cell lysis was determined and statistical analysis was performed as described in Figure 2. B, T cell subsets in bulk- or T_{CM}-derived L1CAM-4-1BB-CAR T cell populations, assessed on day 12 of rapid expansion by flow cytometry. Data depicts the mean of three donors for bulk and T_{CM}-derived CAR T cells each. CD45RO$^+$ CD62L$^-$ FAS$^+$ for T_{EM}-like, CD45RO$^+$ CD62L$^+$ FAS$^+$ for T_{CM}-like, CD45RA$^+$ CD62L$^+$ FAS$^+$ for T_{SCM}-like, CD45RA$^+$ CD62L$^+$ FAS$^-$ for naïve-like T cells. C, PD-1 and PD-L1 expression of bulk- or T_{CM}-derived control and L1CAM-4-1BB-CAR T cells before and after coculture with SK-N-BE(2) at a 1:1 E:T ratio for 24 hours. D, PD-L1 expression of SK-N-BE(2) neuroblastoma cells alone and after coculture with either bulk- or T_{CM}-derived L1CAM-4-1BB-CAR T cells at a 1:5 E:T ratio for 24 hours. Data depicts the mean of two donors for bulk and three donors for T_{CM}-derived CAR-T cells, respectively, with three independent experiments assessed for each donor, $^*P < .05$ and $^{**}P < .01$ were assessed by two-tailed unpaired *t* test, comparing T_{CM}-derived in relation to bulk-derived control and CAR T cell PD-1 and PD-L1 expression. CAR, chimeric antigen receptor; E:T, effector:target; PD, programmed cell death; T_{CM}, central memory T cells

cells against SK-N-BE(2) cells at a 1:2 E:T ratio after 24 hours (Figure 3A). CAR T cell phenotype was flow cytometrically assessed before their exposure to neuroblastoma cells in experiments to show that the original phenotype was maintained during ex vivo expansion of the bulk- or T_{CM}-derived CAR T cells. T cell subsets were defined following the previously published approach by the Riddell group.[37] Central memory-like ($CD45RO^+/CD62L^+$ [SELL]/FAS^+) $CD8^+$ T cells remained the biggest subset in T_{CM}-derived CAR T cells as expected (Figure 3B). Bulk-derived CAR T cells consisted mostly of effector memory-like ($CD45RO^+/CD62L^-/FAS^+$) $CD8^+$ T cell subsets. Induction of the PD-1/PD-L1 axis after 24 hours exposure to SK-N-BE(2) cells (1:1 E:T ratio) was significantly higher in T_{CM}-derived L1CAM-CAR T cells than in their bulk-derived counterparts (Figure 3C), and PD-1/PD-L1 expression was also higher in T_{CM}-derived T cells before tumor cell challenge. In addition to $CD8^+$ L1CAM-CAR T cells, higher PD-1 induction after 24 hours exposure to SK-N-BE(2) cells was also seen for $CD4^+$ T_{CM}-derived L1CAM-CAR T cells compared with their $CD4^+$ bulk-derived counterparts (Figure S3A). However, the induction of PD-L1 in SK-N-BE(2) caused by CAR T cell encounter was similar for bulk-derived L1CAM-CAR T cells and their T_{CM}-derived counterparts (Figures 3D and S3B). Taken together, nivolumab did not enhance the cytotoxic activity of bulk-derived L1CAM-CAR T cells, most likely because PD-1 and PD-L1 induction in CAR T cells themselves was only moderate and high PD-1 and PD-L1 CAR T cell expression limited to the T_{CM}-derived subset of effector cells.

3.4 | CAR T cell subset but not CAR costimulatory domain determines PD-1 and PD-L1 expression levels

Since the costimulatory domain of a CAR largely impacts CAR T cell properties such as activation or persistence, we investigated its effect on PD-1/PD-L1 upregulation in the L1CAM-CAR T cells. The L1CAM-CAR T cells we have used so far harbor the 4-1BB costimulatory domain, and we will compare them with L1CAM-targeting CAR T cells harboring the CD28 costimulatory domain, which we refer to here as L1CAM-CD28-CAR T cells. Similarly to what we observed for L1CAM-CAR T cells harboring the 4-1BB costimulatory domain, induction of the PD-1/PD-L1 axis was significantly higher after tumor cell encounter in T_{CM}-derived L1CAM-CD28-CAR T cells compared with bulk-derived cells, and PD-1/PD-L1 was also higher in T_{CM}-derived L1CAM-CD28-CAR T cells before tumor cell challenge (Figure 4A). The PD-L1 upregulation in SK-N-BE(2) was also similarly induced by coculture with T_{CM}- or bulk-derived L1CAM-CD28-CAR T cells, and comparable with PD-L1 expression levels after coculture with 4-1BB-harboring L1CAM-CAR T cells (Figure 4B). Although L1CAM-CD28-CAR T cells expressed higher PD-1 levels than the 4-1BB-harboring L1CAM-CAR T cells before tumor cell encounter, and independently of their source T cell subset ($P < .01$, data not shown), no significant difference in the expression of the PD-1/PD-L1 axis was detected in L1CAM-CAR T cells harboring either costimulatory domain after tumor cell encounter. Our data confirms the subset-specific enhancement of PD-1/PD-L1 upregulation in T_{CM}-derived L1CAM-CAR T cells and shows it is independent of the type of costimulatory domain used in the CAR.

3.5 | Recursive tumor cell challenge reveals enhanced L1CAM-4-1BB-CAR T cell-directed neuroblastoma cell cytotoxicity by nivolumab

The PD-1/PD-L1-axis is known to contribute to CAR T cell exhaustion when recursive rounds of tumor-specific T cell activation are necessary to achieve tumor eradication. We investigated how PD-1 checkpoint inhibition might assist in this scenario. Recursive

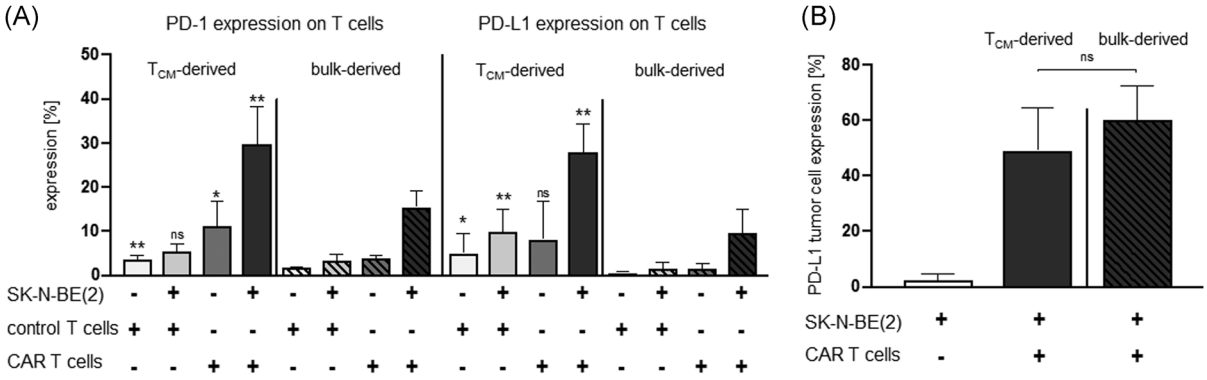

FIGURE 4 Enhanced PD-1/PD-L1 expression in T_{CM}-derived CAR T cells was independent of the costimulatory domain used. A, PD-1 and PD-L1 expression of bulk- or T_{CM}-derived control and L1CAM-CD28-CAR T cells before and after coculture with SK-N-BE(2) at a 1:1 E:T ratio for 24 hours. Data depicts the mean of two donors of bulk-derived CAR T cells and two donors of T_{CM}-derived CAR T cells, respectively, with three independent experiments assessed for each donor. $^*P < .05$ and $^{**}P < .01$ were assessed by two-tailed unpaired *t* test, comparing T_{CM}-derived in relation to bulk-derived control and CAR T cell PD-1 and PD-L1 expression. B, PD-L1 expression of SK-N-BE(2) neuroblastoma cells alone and after coculture with either bulk- or T_{CM}-derived L1CAM-CD28-CAR T cells at a 1:5 E:T ratio for 24 hours. Data depicts the mean of two donors each for bulk- or T_{CM}-derived CAR T cells, with three independent experiments assessed for each donor. CAR, chimeric antigen receptor; E:T, effector:target; PD, programmed cell death; T_{CM}, central memory T cells

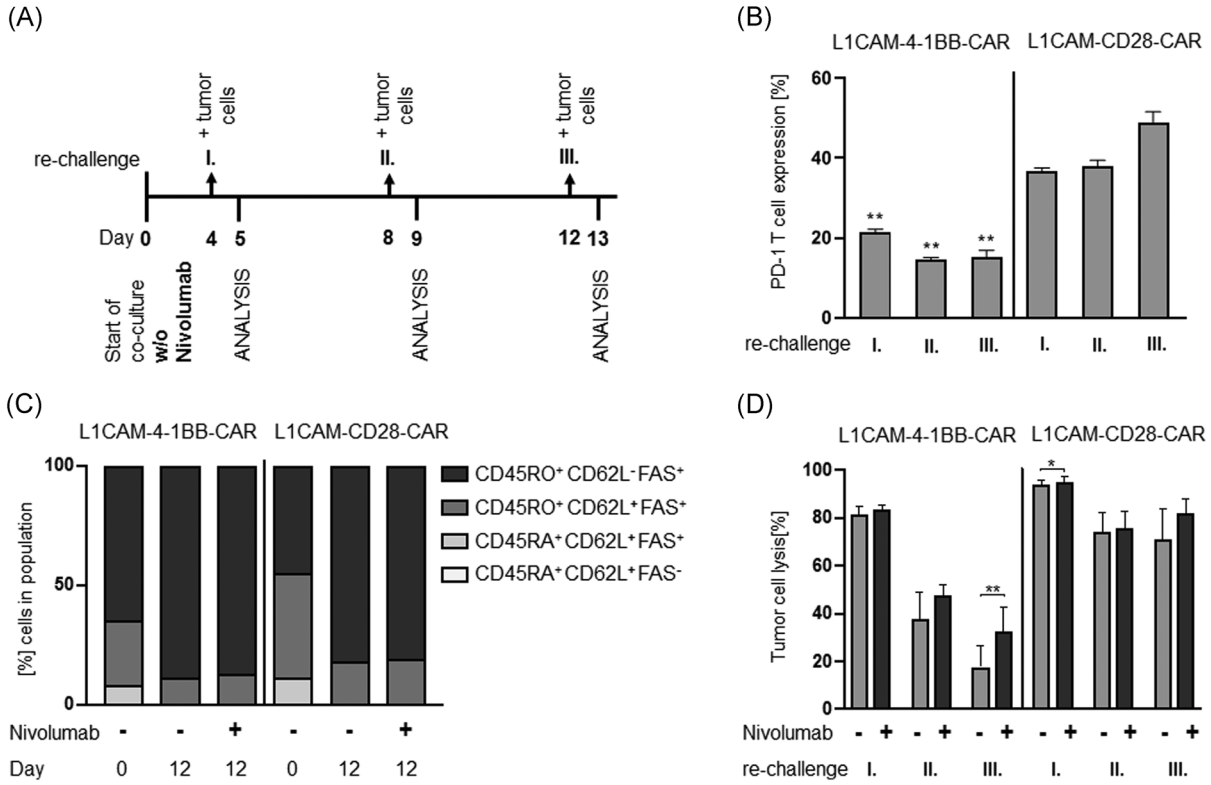

FIGURE 5 Nivolumab requires CAR T cell exhaustion additional to high PD-1 and PD-L1 CAR T cell expression. A, A schematic overview of the serial tumor challenge with three rechallenges of freshly added SK-N-BE(2) neuroblastoma cells at a 1:2 E:T ratio. B, PD-1 expression of T_{CM}-derived L1CAM-4-1BB- and CD28-CAR T cells 24 hours after rechallenge. $^{**}P < .01$ were assessed by two-tailed unpaired *t* test, comparing T_{CM}-derived in relation to bulk-derived control and CAR T cell PD-1 expression. C, T cell subsets of T_{CM}-derived L1CAM-4-1BB- or CD28-CAR T cell populations before and at day 12 of the serial tumor challenge. $CD45RO^+$ $CD62L^-$ FAS^+ for T_{EM}-like, $CD45RO^+$ $CD62L^+$ FAS^+ for T_{CM}-like, $CD45RA^+$ $CD62L^+$ FAS^+ for T_{SCM}-like, $CD45RA^+$ $CD62L^+$ FAS^- for naïve-like T cells. D, Neuroblastoma cell killing of T_{CM}-derived L1CAM-4-1BB- or CD28-CAR T cells 24 hours after rechallenge with SK-N-BE(2) neuroblastoma cells at a 1:1 E:T ratio with added nivolumab (or diluent, as control). Cell lysis was determined and statistical analysis was performed as described in Figure 2. $^{*}P < .05$. CAR, chimeric antigen receptor; E:T, effector:target; PD, programmed cell death; T_{CM}, central memory T cells

tumor encounter was mimicked by maintaining CAR T and tumor cell interactions throughout 13 days. CAR T cells were re-challenged with fresh SK-N-BE(2) cells three times during the 13-day period at a 1:2 E:T ratio following a previously published strategy[17,36] (Figure 5A). Only T_{CM}-derived CAR T cells were used in these experiments because of their stronger expression of the PD-1/PD-L1 axis following tumor cell challenge. PD-1 expression was significantly induced by all three tumor cell rechallenges in both second-generation L1CAM-targeting CAR T cells. In contrast to our previous findings, where after single tumor encounter no significant difference in PD-1 expression levels between L1CAM-CAR T cells with either CD28 or a 4-1BB costimulatory domain was observed, the PD-1 expression levels in L1CAM-CD28-CAR T cells were persistently higher during recursive tumor cell encounter (36.8%, 38.0%, and 49.0% in L1CAM-CD28-CAR T cells vs 21.3%, 14.6%, and 15.1% in L1CAM-4-1BB-CAR T cells, respectively; Figure 5B). To see if cotreatment with nivolumab has an influence on the CAR T cell phenotype, we cocultured and re-challenged the CAR T cells in the presence or absence of nivolumab and analyzed the cells after 12 days for expression of CD45RO, CD45RA, CD62L, and FAS using flow cytometry. Both L1CAM-CAR T cell populations, either with CD28 or 4-1BB as costimulatory domain, shifted phenotypically towards an effector-memory-like type over the course of the serial tumor challenge irrespective of nivolumab (Figure 5C). Next, we wanted to investigate PD-1 blockade mediated enhancement of T_{CM}-derived L1CAM-CAR T cell killing in the context of T cell exhaustion mimicked by our serial tumor challenge. Cytotoxic activity of L1CAM-CAR T cells was determined by the luciferase-based reporter assay when subsequently cocultured with new SK-N-BE(2) at a 1:1 E:T ratio after each rechallenge. Despite the higher PD-1 expression observed in L1CAM-CD28-CAR T cells, tumor cell lysis was only significantly enhanced by PD-1 blockade for L1CAM-4-1BB-CAR T cells after the third rechallenge (Figure 5D). Notably, the killing efficacy of L1CAM-4-1BB-CAR T cells decreased by almost 50% with each tumor encounter, which we did not observe for L1CAM-CD28-CAR T cells to that extent. The nivolumab-enhancement of neuroblastoma cell killing was not reflected by increased numbers of cytokine-producing T cells or lower apoptosis rates at the end of the serial tumor challenge (data not

shown). Nonetheless, cotreatment with nivolumab during recursive tumor cell encounters enhances L1CAM-CAR T cell-directed neuroblastoma cell killing especially in CAR T cells harboring the costimulatory domain 4-1BB.

4 | DISCUSSION

Combining CAR T cell therapy with complementing immunotherapies such as PD-1 checkpoint blockade is a promising treatment approach for solid tumors. Here, we analyzed activation of the PD-1/PD-L1 axis during in vitro testing of neuroblastoma-specific CAR T cell products and assessed the impact of combining CAR T cell therapy and checkpoint blockade with nivolumab. We demonstrate that the PD-1/PD-L1 axis was induced in neuroblastoma cells after L1CAM-CAR T cell encounter. Furthermore, we show that nivolumab-enhanced L1CAM-CAR T cell-directed cytotoxicity relied on strong PD-1/PD-L1 expression in the CAR T cells themselves, which in turn depended on the type of costimulatory domain in the CAR construct and strongly on the T cell subset from which the CAR T cells were generated.

For PD-1 checkpoint blockade to assist CAR T cell therapeutic efficacy, PD-1 must be present on CAR T cells and its ligand, PD-L1, must be present on the neuroblastoma cells. PD-L1 expression in diagnostic neuroblastoma biopsies varies in recently published studies. Reports span the complete absence of PD-L1 in 18 neuroblastoma samples at diagnosis reported by Aoki et al[38] to PD-L1 expression in 14% of 118 mainly diagnostic neuroblastoma samples.[39] We demonstrate that neuroblastoma-specific CAR T cells induced PD-L1 expression in three neuroblastoma cell lines that expressed minor levels of PD-L1 ligand before CAR T cell encounter. We draw the conclusion that even if PD-L1 is often not expressed on the neuroblastoma cells at diagnosis, this may change at the time of treatment and is likely not to be the case after CAR T cell exposure, meaning that PD-L1 expression in diagnostic samples should not be used to determine whether to apply a combinational approach using CAR T cells and PD-1 checkpoint inhibition in patients.

To date, PD-1/PD-L1-axis disruption only proved advantageous for in vitro and in vivo preclinical testing in different solid tumor models if combined with CAR T cells strongly expressing PD-1.[6-8] First results from an ongoing phase 1 trial (NCT01822652) for high-risk and relapsed neuroblastoma showed no effect of additional PD-1 blockade on expansion or persistence of third generation iC9-GD2-CAR T cells. PD-1 expression in $CD8^+$-CAR T cells before transfusion was comparatively low.[24] Unfortunately, PD-1 expression in CAR T cells after transfusion was not measured by the authors precluding to call the low PD-1 expression to account for the missing benefit of additional PD-1 blockade. In a recent study, the benefit of combining PD-1 blockade with CAR T cell therapy for neuroblastoma lessened with incorporating IL-15 signaling into GD2-targeting CAR T cells likely due to reduced PD-1 expression levels in these modified CAR T cells.[40] Concordantly, we demonstrate that nivolumab only enhanced the cytotoxic activity of L1CAM-CAR T cells with high PD-1 expression.

Host immune cells such as myeloid-derived suppressor cells and tumor-infiltrating lymphocytes that express PD-L1 themselves have been shown to contribute to immune escape in solid tumor models.[41,42] In evaluating the response in patients with different cancers to a monoclonal antibody against PD-L1, Herbst et al[43] determined that PD-L1 expression on tumor cells positively correlated with treatment efficacy and that PD-L1 expression on tumor-infiltrating lymphocytes was predictive for higher response rates. We observed that tumor cell encounter increased PD-L1 expression on L1CAM-CAR T cells. In spite of minor tumor-expressed PD-L1 levels to inhibit CAR T cell cytotoxic activity by binding PD-1, a tendency towards nivolumab-enhanced L1CAM-CAR T cell efficacy persisted when cocultured with PD-L1 KO SK-N-BE(2) cells. We consider CAR-on-CAR and not only tumor-on-CAR inhibition to factor into the PD-1/PD-L1-conveyed inhibition of antitumor efficacy.

T_{CM}-derived CAR T cells have been demonstrated to persist longer and proliferate more in patients.[18-20] Comparing bulk and T_{CM}-derived CAR T cells, we found differences in PD-1 expression. Higher PD-1 expression in T_{CM}-derived L1CAM-CAR T cells was independent of the type of costimulatory domain used in the CAR construct and persisted over repeated tumor cell encounters. Enamorado et al[44] demonstrated that CAR T cells derived from T_{CM}-enriched cell subpopulations strongly expressed PD-1 expression, had antitumor activity and formed a resident memory T cell subset following tumor challenge. Notably, PD-1 blockade enhanced T_{CM} cell infiltration and augmented antitumor immunity. Rather than being a hindrance, an activated PD-1/PD-L1-axis in T_{CM}-derived CAR T cells is an innate quality on which we can capitalize to further augment their therapeutic efficacy.

How the CD28 costimulatory domain impacts CAR T cell function remains controversially discussed in the literature. Induction of PD-1 and PD-L1 after recursive tumor cell challenge was higher in our L1CAM-targeting CAR T cells with the CD28 instead of the 4-1BB costimulatory domain. Zolov et al[30] showed that the CD28 costimulatory domain rendered their CD123-targeting CAR T cells more susceptible to PD-1/PD-L1-mediated inhibition of T cell activation despite lower PD-1 expression. In line with these findings, the CD28 costimulatory receptor, as compared with the T cell receptor signaling cascade, was preferentially targeted by PD-1/PD-L1-mediated inhibition through downstream PD-1-activated Shp2 phosphatase.[45] In a mouse model for chronic infection, a proliferation of exhausted $CD8^+$ T cells induced by monoclonal antibody-based PD-1 blockade was dependent on CD28.[46] Contrarily, targeted CD28 deletion in coculture systems of T cells and antigen-presenting cells has demonstrated that the costimulatory receptor is unnecessary for PD-1/PD-L1-conveyed inhibition of T cell receptor signaling.[47] Furthermore, Mizuno et al[47] showed that CD28 costimulatory signaling rather attenuated PD-1/PD-L1-mediated inhibition of T cell activation. Similarly, the higher PD-1 expression in our CD28-harboring L1CAM-targeting CAR T cells did not translate to nivolumab-driven enhanced cytotoxic activity following neuroblastoma cell challenge. Contrarily, nivolumab significantly enhanced cytotoxic activity in our 4-1BB-harboring L1CAM-targeting CAR T cells during simulated

recursive tumor cell encounter despite the lower PD-1 expression. Since cytotoxic activity was lower and declined more quickly with recursive tumor encounter in these CAR T cells, exhaustion rendered them more susceptible to nivolumab-mediated enhancement. Our findings are in line with prior reports observing higher CD28-related PD-1 expression in CAR T cells, but question a straightforward understanding of the interplay between the costimulatory domain and PD-1/PD-L1 signaling axis.

Our data support that PD-1 blockade augments T_{CM}-derived L1CAM-CAR T cell efficacy against neuroblastoma. L1CAM-CAR T cell encounter induced PD-L1 expression on neuroblastoma cells, confirming adaptive immune resistance. However, PD-L1 expression on tumor cells did not strictly correlate with nivolumab-enhancement of L1CAM-CAR T cell cytotoxic activity. Therefore, PD-1 blockade did not solely depend on PD-L1 tumor expression levels in our model. T cell source, and to a lesser extent costimulatory domain type, for CAR T cell generation impacted PD-1/PD-L1 expression on L1CAM-CAR T cells, which in turn drove nivolumab-enhancement. Thus, T cell subset selection and prescreening CAR T cells for PD-1/PD-L1 expression, then combining only CAR T cells expressing higher PD-1/PD-L1 levels with nivolumab, could improve CAR T cell therapy treatment efficacy through more informed treatment combinations.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

Data will be available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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