

Chimeric PD-1 receptor redirects primary T cells against childhood solid tumors but not to PD-1 ligand-positive CD80-coexpressing cells

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Abstract. The clinical application of T cells engineered with chimeric antigen receptors (CARs) for solid tumors is challenging. A major reason for this involves tumor immune evasion mechanisms, including the high expression of immune checkpoint molecules, such as the programmed death 1 (PD-1) ligands PD-L1 and PD-L2. The inducible expression of PD-L1 in tumors has been observed after CAR-T-cell infusion, even in tumors natively not expressing PD-L1. Furthermore, numerous types of pediatric cancer do not have suitable targets for CAR-T-cell therapy. Therefore, the present study aimed to develop novel CAR-T cells that target PD-L1 and PD-L2, and to evaluate their efficacy against pediatric solid tumors. A novel CAR harboring the immunoglobulin V-set domain of the human PD-1 receptor as an antigen binding site (PD-1 CAR-T) was developed without using a single-chain variable fragment. PD-1 CAR-T cells were successfully manufactured by adding an anti-PD-1 antibody, nivolumab, to the *ex vivo* expansion culture to prevent fratricide during the manufacturing process due to the inducible expression of PD-L1 in activated human T cells. The expression of PD-L1 (and PD-L2 to a lesser extent) was revealed to be highly upregulated in various pediatric solid tumor cells, which displayed no or very low expression initially, on *in vitro* exposure to interferon- γ and/or tumor necrosis factor- α , which are cytokines secreted by tumor-infiltrating T cells. Furthermore, PD-1 CAR-T cells

exhibited strong cytotoxic activity against pediatric solid tumor cells expressing PD-L1 and PD-L2. Conversely, the effect of PD-1 CAR-T cells was significantly attenuated against PD-L1-positive cells coexpressing CD80, suggesting that the toxicity of PD-1 CAR-T cells to normal immune cells, including antigen presenting cells, can be minimized. In conclusion, PD-1 ligands are promising therapeutic targets for pediatric solid tumors. PD-1 CAR-T cells, either alone or in combination with CAR-T cells with other targets, represent a potential treatment option for solid tumors.

Introduction

The adoptive transfer of T cells engineered with chimeric antigen receptors (CAR-T) has shown promising effects against refractory hematological malignancies. In pediatric and adult patients with CD19-positive relapsed or refractory B-cell acute lymphoblastic leukemia (ALL), anti-CD19 CAR-T cell therapy achieved an overall survival (OS) rate of 76% at 1 year (1). In patients with refractory large B-cell lymphoma with extremely poor prognosis, anti-CD19 CAR-T cell therapy demonstrated a highly durable response, with an OS of 76% at 18 months (2). In patients with triple-class-exposed relapsed and refractory multiple myeloma, B-cell maturation antigen-directed CAR-T cell therapy demonstrated durable responses with a median progression-free survival of 13.3 months at a median follow-up of 18.6 months, compared with 4.4 months in the standard-regimen group (3).

However, the clinical translation of CAR-T cell therapy for solid tumors is limited for several reasons. First, finding suitable targets for CAR-T cell therapy is difficult, as potential target antigens are often heterogeneously expressed throughout solid tumor tissues, and they are sometimes expressed at low levels in healthy tissues. Furthermore, in CD19-positive ALL, loss of the target antigen CD19 occurs after anti-CD19 CAR-T cell attack (4). This phenomenon, termed 'antigen loss,' has been reported in CAR-T cell therapy targeting other

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antigens, including BCMA, CD22, and EGFR type 3 variant, in clinical settings (5-7). Second, in the tumor microenvironment, various immune escape mechanisms are observed, including the expression of immune checkpoint molecules such as programmed cell death 1 (PD-1) ligands (8).

PD-1 ligands, including programmed cell death 1 ligand 1 (PD-L1) and programmed cell death 1 ligand 2 (PD-L2), are critical antigens that allow cancer cells to evade host anti-tumor immunity and survive. PD-1 is expressed on the surface of T cells, suppresses immune responses, and prevents excessive immune activation by binding to PD-L1 and PD-L2 (9). PD-L1 and PD-L2 are expressed by various cancer cells and mediate escape from anti-tumor immunity by inducing cytotoxic T cell dysfunction (10,11). PD-1 blocking antibody can inhibit the binding of PD-1 to PD-L1 and PD-L2 and restore T cell cytotoxic activity against cancer cells, and the efficacy of PD-1 inhibitors has been confirmed in several malignant tumors (12,13). Furthermore, when used in combination with CAR-T cells, anti-PD-1 inhibitory antibodies have enhanced the effects of CAR-T cells in mouse models (14-16) and human clinical trials (17,18).

PD-L1 is expressed at low frequency in pediatric cancers (19). Anti-PD-1 antibody nivolumab and pembrolizumab do not exhibit significant activity in relapsed or refractory pediatric solid tumors (20,21). Therefore, PD-1 is considered to be a less important candidate for the treatment of relapsed and/or refractory pediatric cancers. However, even in tumors with weak PD-L1 and PD-L2 expression, both PD-L1 and PD-L2 are inducible in response to CAR-T cell attack and inactivate CAR-T cells (22).

As the expression of PD-L1 and PD-L2 on the surface of tumor cells may be induced by various stimuli, we hypothesized that PD-L1 and PD-L2 might be suitable targets in broad types of pediatric cancers. Few studies have investigated CAR-T cell therapy targeting PD-L1 and PD-L2. However, target tumors have been reported to have high expression of PD-L1, such as lymphoma (23) and ovarian cancer (24), although no studies have investigated its possible role in pediatric cancers. In this study, we created a novel CAR construct that uses the binding domain of the PD-1 receptor to target PD-L1 as well as PD-L2 (PD-1 CAR-T). Furthermore, we devised a novel method for manufacturing PD-1 CAR-T cells by suppressing fratricide in culture. Finally, we studied the effects of these CAR-T cells against pediatric solid tumors as well as CD80-coexpressing PD-L1 positive cells.

Materials and methods

Cell lines. The CD19-positive human Philadelphia-chromosome-positive B-lineage ALL cell line OP-1 was a generous gift from Dr. Dario Campana (St. Jude Children's Research Hospital, Memphis, TN, USA). The Burkitt lymphoma cell line Raji, myeloid leukemia cell line K562, T-lineage ALL cell line Jurkat, osteosarcoma cell lines U2OS and SaOS-2, cervical carcinoma cell line HeLa, and 293T were obtained from the American Type Culture Collection (Rockville, MD, USA). The neuroblastoma cell line, SK-N-SH, and rhabdomyosarcoma cell line, RMSYM, were obtained from the RIKEN BRC Cell Bank (Tsukuba, Japan). Neuroblastoma IMR32 cells and glioblastoma cell lines T98G, A172, and U251MG were obtained from the

JCRB Cell Bank of the National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan). The osteosarcoma NOS10 was a generous gift from Dr. Akira Ogoose (Division of Orthopedic Surgery, Niigata University Graduate School of Medical and Dental Sciences). HeLa and 293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS). Other leukemia and solid tumor cell lines were maintained in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS.

CAR constructs and gene transduction. The MSCV-IRES-GFP, pEQ-PAM3(-E), and pRDF vector plasmids were obtained from St. Jude Vector Development and Production Shared Resource (Memphis, TN, USA). To ligate gene fragments to create the PD-1 CAR genes, we used the splicing by overlap extension-PCR method. The construct contains the extracellular and hinge domains of PD-1 cloned from human T cell cDNA and does not use a single-chain variable fragment (scFv) derived from an anti-PD-1 monoclonal antibody. The CD19 CAR gene (CD19-BB- ζ) was generated at St. Jude Children's Research Hospital (25). To generate RD114-pseudotyped retroviruses, lipofection was used to transfect 293T cells (Fugene HD, Promega, Madison, WI, USA). Retroviral titres were measured using HeLa cells (1×10^5). This study was approved by the ethics committee of Niigata University, Niigata, Japan (approval #2015-2686). Peripheral blood mononuclear cells were collected from healthy volunteers after obtaining written informed consent, and T cells were isolated using RosetteSep™ Human T Cell Enrichment Cocktail (STEMCELL Technology, Vancouver, BC, Canada). T cells were incubated for 48-72 h with Dynabeads human T-activator CD3/CD28 (Thermo Fisher Scientific, Waltham, MA, USA) in the presence of 200 IU/ml recombinant human interleukin-2 (rhIL-2; Peprotech, Cranbury, NJ, USA) in RPMI-1640 with 10% FBS. Activated T cells were then transduced with Retronectin (Takara, Otsu, Japan) according to the manufacturer's instructions, with slight modifications. To prevent fratricide of transduced cells, we added 1.4 μ g/ml of anti-PD-1 blocking antibody (Selleck Biotech, Yokohama, Japan) every 2 days until 1 week after transduction. The transduced cells were maintained in RPMI-1640 supplemented with 10% FBS, and 200 IU/ml rhIL-2 was added every 2-3 days until use. The surface expression of PD-1 CAR in T cells was detected using phycoerythrin (PE)-conjugated anti-PD-1 antibody (BioLegend, San Diego, CA, USA). Antibody staining was analyzed using the FACSCalibur flow cytometer (Becton Biosciences). The surface expression of anti-CD19 CAR in T cells was detected as previously reported (25). HeLa cells were transduced with MSCV-IRES-GFP vector harboring human CD19 cDNA, and were then subjected to single cell cloning by a standard limiting dilution method to produce HeLa expressing human CD19 (19-HeLa). 19-HeLa cells were further modified to express human CD80 by MSCV-IRES-GFP vector with human CD80 cDNA.

Detection of PD-1 ligand upregulation in cell lines after cytokine stimulation. To induce PD-L1 and PD-L2 upregulation,

100 IU/ml interferon (IFN)- γ and/or 10 ng/ml tumor necrosis factor (TNF)- α was added to each cell line for 24 h. To mimic and reproduce PD-L1 and PD-L2 upregulation after CAR-T cell attack, we collected supernatants from the co-culture of CD19-CAR-T cells and Raji Burkitt lymphoma cells and stimulated each cell line with conditioned medium (CM) for 24 h. To detect surface PD-L1 and PD-L2 expression in the cell lines, cultured cells were harvested using trypsin-EDTA (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and washed once with phosphate-buffered saline. The expression of PD-L1 and PD-L2 was detected using PE anti-human PD-L1 and PD-L2 antibodies (BioLegend, San Diego, CA, USA) and analyzed using a FACSCaliber flow cytometer (Becton Dickinson). PE Mouse IgG1, κ Isotype Ctrl Antibody (BioLegend) was used as the control.

Cytotoxicity assay. U2OS cells were transduced with an MSCV-IRES-GFP vector containing the luciferase gene (26), and the cytotoxicity of PD-1 CAR-T cells against U2OS cells was measured using a Luciferase Assay System (Promega, Madison, WI, USA). The other cell lines were measured using the MST assay and the CellTiter 96[®]Aqueous One Solution Cell Proliferation Assay (Promega). Target cells were seeded in a 96-well flat-bottom plate and allowed to adhere for 12–24 h. PD-1 CAR-T cells were added to the plate at effector to target (E:T) ratios of 2:1, 1:1, and 0.5:1. After 4 or 24 h of co-culture, residual tumor cells were analyzed by the Luciferase Assay System or CellTiter 96[®]Aqueous One Solution Cell Proliferation Assay.

Real-time killing assay. Target cells (1×10^4) were seeded in an iCELLigence[™] E-plate (ACEA Biosciences, San Diego, CA, USA) and allowed to adhere for 12–24 h. The next day, PD-1 CAR T cells were added at E:T ratios of 0.25:1 in the presence of low-dose rhIL-2 (10 IU/ml). The cellular impedance was measured continuously every 1 h for up to 7 days using a real-time cell analyzer iCELLigence[™] (ACEA Biosciences), according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were conducted using the EZR software (version 1.62) as previously described (27). One-way analysis of variance followed by Tukey's post-hoc test was used to evaluate the difference between three or more groups. All experiments were performed using three technical replicates, and the data are shown as mean $\pm$ standard deviation (SD). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PD-1 ligand expression in pediatric solid cancers. To explore whether PD-1 ligands could be CAR targets, we first investigated the cell surface expression of PD-L1 and PD-L2 in osteosarcoma (SaOS2, NOS10, and U2OS), neuroblastoma (IMR32 and SK-N-SH), glioblastoma (A-172, T98G, and U251MG), and rhabdomyosarcoma (RMS-YMR) cell lines. Most cell lines do not express or rarely express PD-L1 and PD-L2. However, in the osteosarcoma cell line SaOS2, PD-L1 (and PD-L2 to a lesser extent) expression was slightly induced by IFN- γ or TNF- α stimulation and was highly upregulated by

stimulation with a combination of IFN- γ and TNF- α (Fig. 1A). To investigate whether a similar upregulation of PD-L1 and PD-L2 was observed in an *in vitro* model of microenvironmental stimuli after T-cell infiltration, we utilized CM, a culture supernatant that presumably contained several cytokines released from T cells upon exposure to target cells, to stimulate cancer cells and found that the induction of PD-L1 and PD-L2 expression was strongest when stimulated with CM (Fig. 1B). In other cell lines, including NOS10, U2OS, IMR32, SK-N-SH, A-172, T98G, U251MG, and RMS-YMR, PD-L1 and PD-L2 were also highly upregulated upon CM stimulation (Fig. 1C). These observations suggest that PD-L1 and PD-L2 can be upregulated upon T cell infiltration and may be targeted by PD-1-ligand-specific CAR-T cells.

Proliferation of PD-1 CAR-T cells. We created second-generation chimeric PD-1 receptors (PD-1 CAR) that contained an extracellular and hinge domain of PD-1, transmembrane and costimulatory domains of CD28, and CD3 ζ intracellular signaling domains (Fig. 2A). We also created a truncated PD-1 CAR (PD-1 TR) lacking the signaling domain, serving as the control receptor. T cells transduced with CAR or the control receptor showed strong cell surface expression, as detected using an anti-PD-1 monoclonal antibody (Fig. 2B). Although producing PD-1 CAR T cells was possible, obtaining a large number of CAR T cells was difficult. As mock-transduced T cells (expressing only GFP) or T cells transduced with PD-1 TR could proliferate exponentially in parallel experiments, we suspected that fratricide occurred during the manufacturing process; PD-L1 expression was significantly upregulated 24 h after T cell activation (Fig. 2C), although the cell surface expression of PD-L1 on T cells was modest 14 days after PD-1 CAR-T cell manufacturing. To block the interaction between PD-1 CAR and PD-L1 expressed by neighboring cells, we added anti-PD-1 blocking antibody nivolumab to the culture medium during the initial week. With the addition of nivolumab, we observed an improvement in cell expansion during the manufacturing process, with a growth curve similar to that of mock-transduced or non-signaling control CAR-transduced T cells (Fig. 2D).

PD-1 CAR-T cell-mediated cytotoxicity against cell lines. To test whether PD-1 CAR-T cells were functional, we used U2OS cells, an osteosarcoma cell line that modestly expresses PD-L1 at a steady state. PD-1 CAR-T cells demonstrated cytotoxic effects in a dose-dependent manner, whereas control T cells expressing non-signaling PD-1 CAR did not. Furthermore, when U2OS cells were pretreated with CM for 24 h, the cytotoxic effects were significantly enhanced (Fig. 3A). Next, we tested the cytotoxicity of PD-1 CAR-T cells against SaOS2, an osteosarcoma cell line that does not stably express PD-L1; PD-1 CAR-T cells had no cytotoxic effects. However, PD-1 CAR-T cells showed strong cytotoxicity against SaOS2 pretreated with CM for 24 h (Fig. 3B). Long-term anti-tumor effects against SaOS2 were measured using an iCELLigence real-time cell analyzer. Immediate cytotoxic activity of PD-1 CAR-T cells was observed in SaOS2 cells pretreated with CM (SaOS2-CM). By contrast, in SaOS2 without pretreatment, PD-1 CAR-T cells showed no effects during the early phase of co-culture

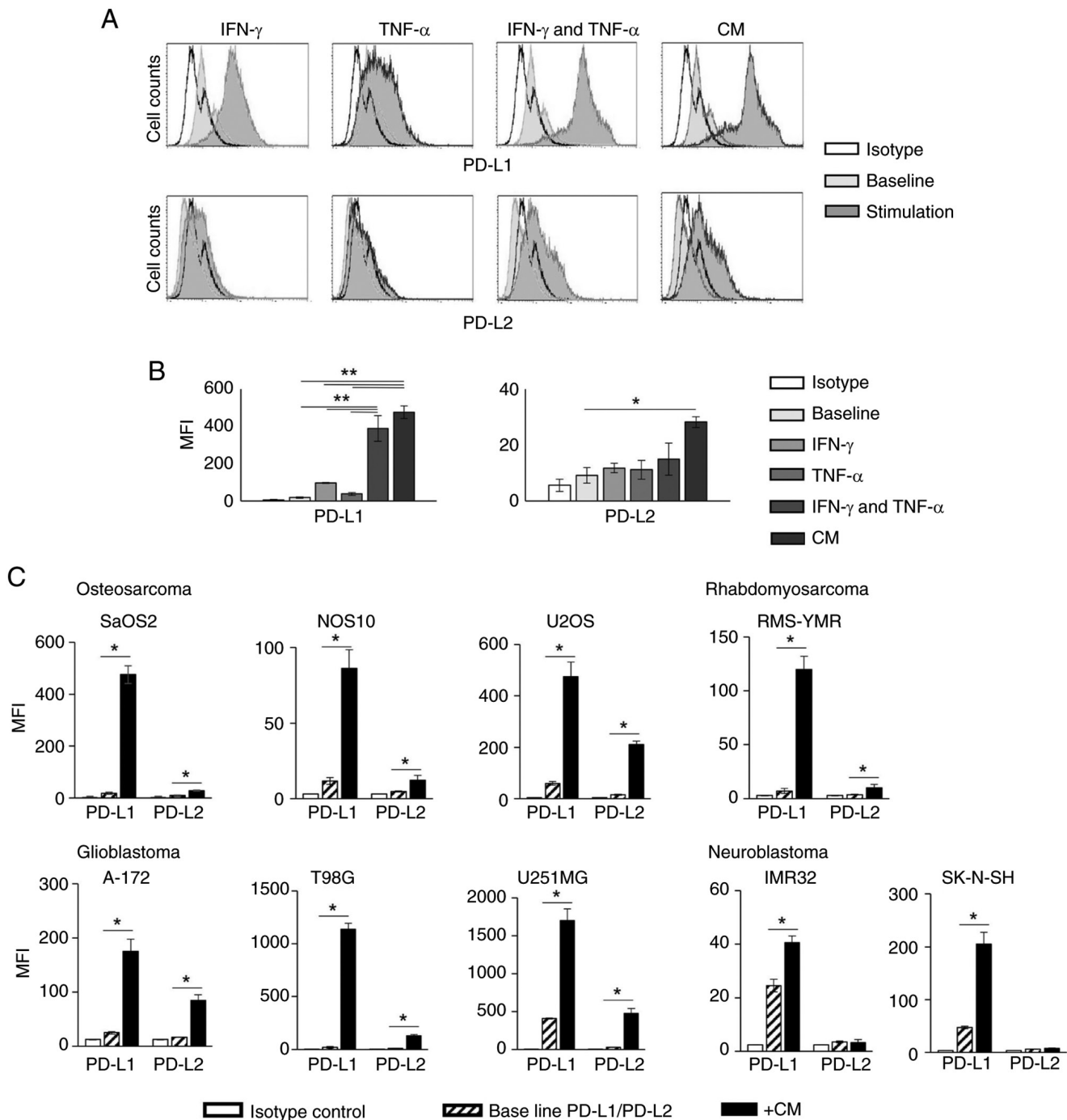


Figure 1. Expression of (PD-1) ligands in pediatric tumor cell lines. (A) Histogram plots showing the expression of PD-L1 (upper panels) and PD-L2 (lower panels) in SaOS2 cells with or without treatment with cytokines or CM. (B) Expression of PD-L1 and PD-L2 in SaOS2 with or without treatment with cytokines or CM. (C) Expression of PD-L1 and PD-L2 in various tumor cell lines treated with CM. * $P < 0.05$, ** $P < 0.01$. PD-1, programmed death 1; PD-L, programmed cell death 1 ligand; CM, conditioned medium; IFN- γ , interferon- γ ; TNF- α , tumor necrosis factor- α ; MFI, mean fluorescence intensity.

(up to 60 h after the initiation of co-culture). However, a delayed emergence of cytotoxic effects was observed (Fig. 3C), suggesting the induction of PD-L1 during co-culture possibly due to cytokines secreted by CAR-T cells; Indeed, we observed induced expression of PD-L1 on the surface of residual tumor cells after a 24- or 72-h exposure to PD-1 CAR-T cells, whereas no expression was observed at 4 h (Fig. 3D).

Dual-targeting CAR-T cells. Based on these results, we hypothesized that PD-1 CAR-T cells may be effective when

combined with other CAR-T cells that target disease-specific constitutively-expressed cell surface antigens. To test this hypothesis, we created dual-targeting CAR-T cells that co-expressed PD-1 CAR and anti-CD19 CAR (Fig. 4A and B) and tested them against CD19-transduced HeLa cells (cervical carcinoma) as a target (19-HeLa). As shown in Fig. 4C, HeLa cells showed high PD-L1 expression after CM stimulation, validating it as a target for both anti-CD19 and PD-1 CAR-T cells. To confirm the efficacy of the dual-targeting CAR-T cells, we co-cultured CAR-T cells expressing CAR with CM-pretreated

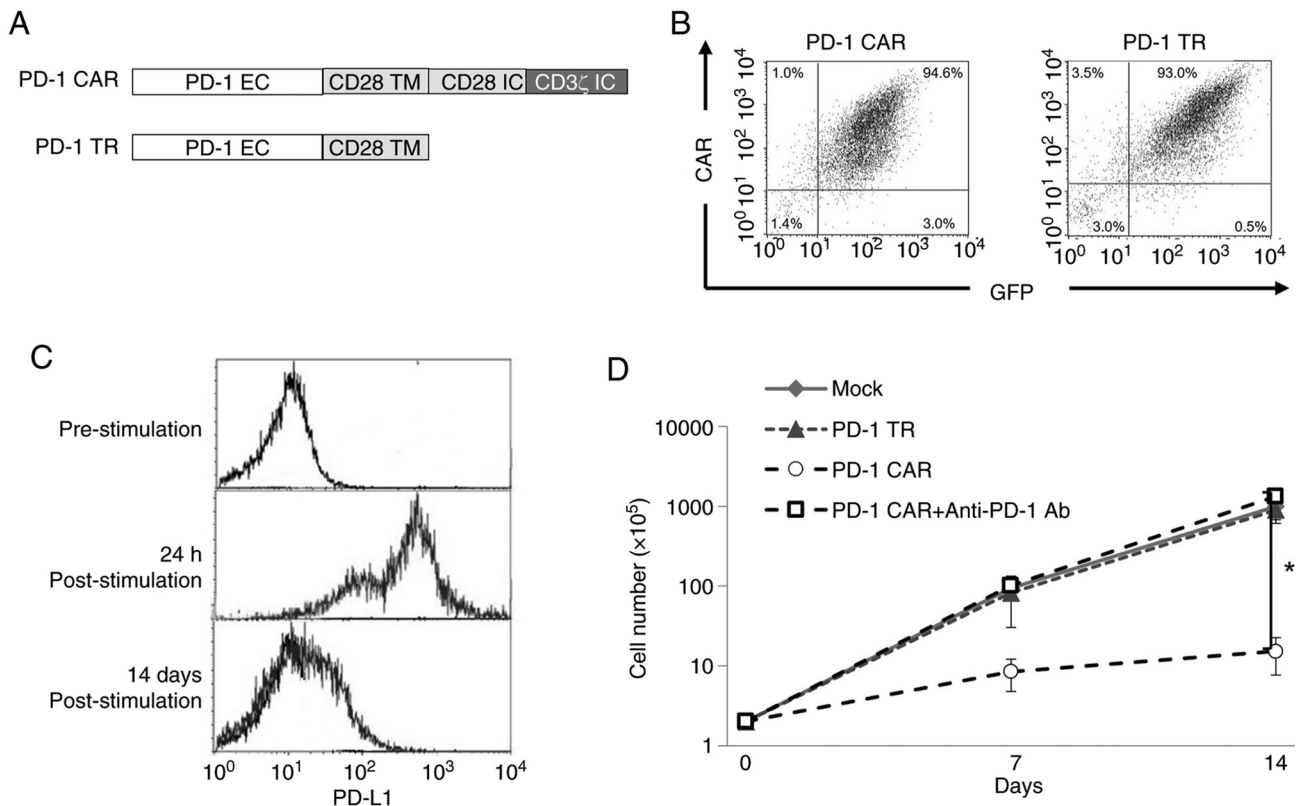


Figure 2. Generation of PD-1 CAR-T cells. (A) Gene constructs of PD-1 CAR. PD-1 TR represents a chimeric receptor lacking signaling domain to serve as a non-signaling control receptor. (B) Cell surface expression of PD-1 CAR and PD-1 TR in retrovirally transduced T cells. (C) Expression of programmed cell death 1 ligand 1 (PD-L1) in T cells before and after activation with CD3/CD28 beads. PD-L1 was not expressed before activation; however, expression was significantly upregulated 24 h after T cell activation and declined 14 days. (D) Absolute cell counts of CAR-T cells in the expansion culture. PD-1 CAR-T cells exhibited significantly poorer growth compared with mock-transduced T cells or PD-1 TR T cells that proliferated exponentially. By adding the anti-PD-1 Ab nivolumab to the culture during the first week, we were able to produce PD-1 CAR T cells with growth rates comparable to mock or PD-1 TR control T cells. * $P < 0.05$ vs. PD-1, programmed death 1; CAR, chimeric antigen receptor; PD-1 TR, truncated PD-1 CAR; Ab, antibody; EC, extracellular domain; TM, transmembrane domain; IC, intracellular signaling domain; GFP, green fluorescent protein.

19-HeLa cells. T cells expressing anti-CD19 CAR were used as controls. Long-term cytotoxicity against SaOS2 was measured using the iCELLigence real-time cell analyzer. Each CAR-T cell showed immediate cytotoxicity after co-culture, and the dual-targeting CAR-T cells showed similar but slightly higher cytotoxicity after several hours (Fig. 4D).

CD80 inhibits PD-1 CAR binding. PD-L1 is constitutively expressed in immune cells, including monocytes/macrophages and dendritic cells (9) and can be targeted and destroyed by T or natural killer (NK) cells expressing CAR using anti-PD-L1 scFv. However, the situation may be different in T cells expressing PD-1 receptor-modified chimeric receptors that use the immunoglobulin domain of PD-1 as an antigen-binding site, as used in the present study. As the simultaneous expression of CD80 (and cis-interaction with PD-L1) masks PD-L1 from PD-1 binding (28), we created CD80-transduced 19-HeLa as a model (Fig. 5A) to determine the effect of PD-1 CAR-T cells against target cells expressing both PD-L1 and CD80. CD80-transduced 19-HeLa cells simultaneously expressed CD80 and CD19 on their surfaces. In this cell line, however, PD-L1 expression after CM stimulation was relatively attenuated compared to the original 19-HeLa (without CD80 transduction) and non-transduced (GFP-negative) fractions in the same culture, possibly due to reduced binding of

anti-PD-1 antibody to the epitope by CD80/PD-L1 cis-interaction (Fig. 5B). Next, we tested the cytotoxicity of anti-CD19 or PD-1 CAR-T cells cocultured with CD80-transduced 19-HeLa or 19-HeLa cells (Fig. 5C). As in the previous experiment, both PD-1 and anti-CD19 CAR-T cells exhibited cytotoxic effects against 19-HeLa cells pre-stimulated with CM. However, when we used CD80-expressing 19-HeLa cells pre-stimulated with CM as a target, the cytotoxicity of PD-1 CAR-T cells was abrogated, whereas that of CD19-CAR T cells was unaffected in both the short-term and long-term real-time killing assays (Fig. 5D and E).

Discussion

In this study, we confirmed that the expression of PD-L1 and PD-L2 in various tumor cells derived from pediatric solid tumors was induced upon CAR-T cell attack and could be targeted by primary T cells expressing a chimeric PD-1 receptor. Although PD-L1 and PD-L2 expression in pediatric cancers is reportedly weak and scarce (19,21), conflicting studies have shown the expression of PD-L1 and PD-L2 in a subset of pediatric solid tumors, such as osteosarcoma (29) and neuroblastoma (30). In our study, even in cells having very weak expression of PD-L1 and PD-L2, their expression was upregulated by stimulation with IFN- γ and/or TNF- α .

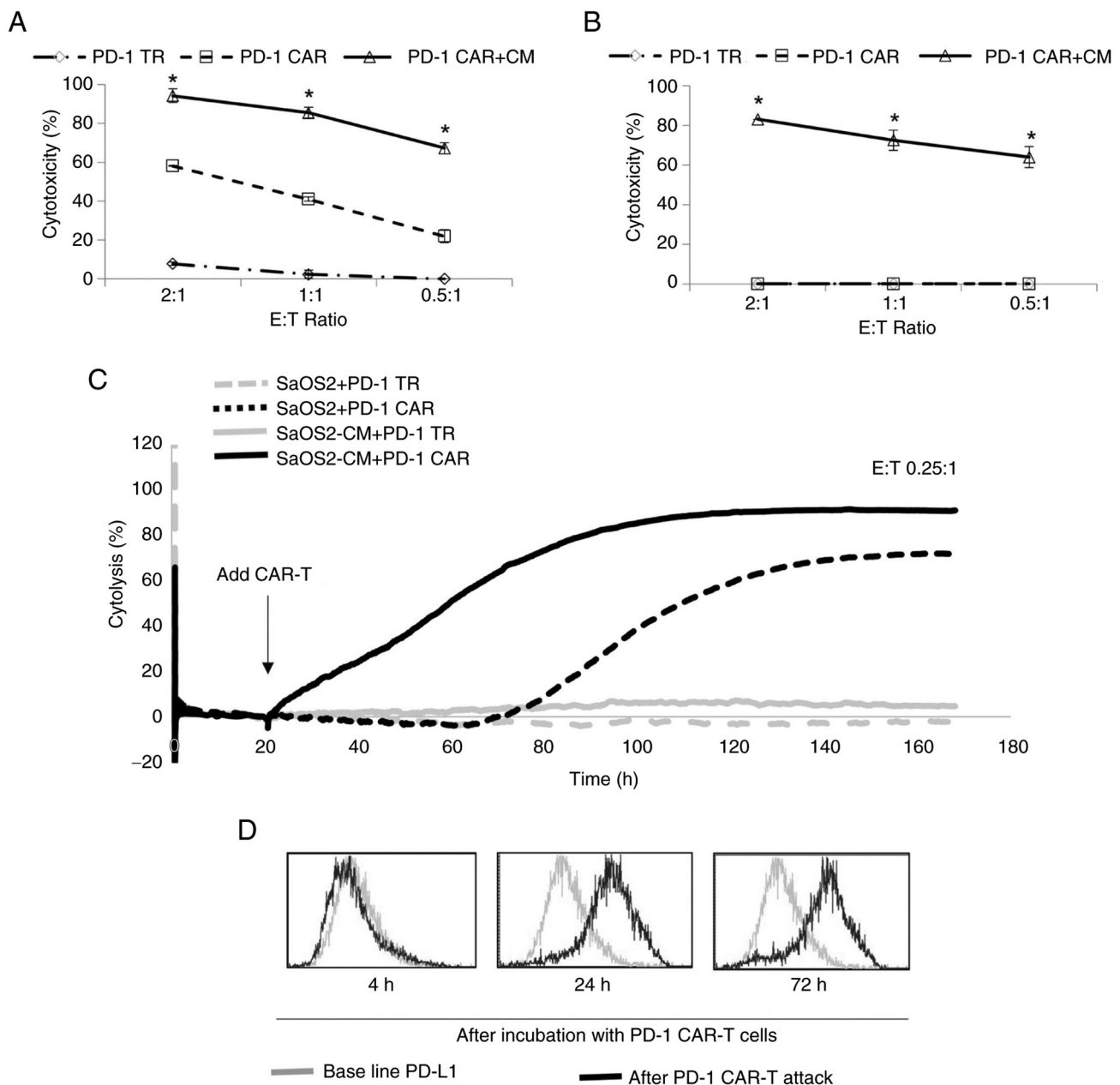


Figure 3. Cytotoxic effects of PD-1 CAR-T cells against tumor cell lines (A) 4-h cytotoxicity of PD-1 CAR-T cells against osteosarcoma cell line U2OS with modest expression of PD-1 ligands, with or without pretreatment with CM. (B) 4-h cytotoxicity of PD-1 CAR-T cells against osteosarcoma cell line SaOS2 that does not express PD-1 ligands, with or without pretreatment with CM. (C) Real-time monitoring of SaOS2 cytolysis. SaOS2 cells were incubated with PD-1 CAR-T at a low E:T ratio (E:T=0.25:1). SaOS2 cells were compared with SaOS2 cells with pretreatment with CM (SaOS2-CM). (D) Time series of histogram plots of PD-L1 expression in SaOS2 by flow cytometry. After co-culture with PD-1 CAR-T cells, PD-L1 expression of residual SaOS2 was gradually upregulated. * $P < 0.05$ vs. PD-1 CAR. PD-1, programmed death 1; CAR, chimeric antigen receptor; CM, conditioned medium; E:T, effector-to-target; PD-L1, programmed cell death 1 ligand 1; PD-1 TR, truncated PD-1 CAR.

We also stimulated the cells with CM to reproduce accurately the microenvironmental cytokine production by CAR-T cells when exposed to target cells and found that the expression was induced more strongly than with IFN- γ or TNF- α alone. This suggests that cancer cells with no or very weak PD-L1 and PD-L2 expression at a steady state may have strong expression of PD-L1 and PD-L2 in the tumor microenvironment following CAR-T cell attack and can be targeted by PD-1 CAR-T cells. Indeed, in a clinical trial of epidermal growth factor receptor variant III-targeting CAR-T cells in patients with relapsed or refractory glioblastoma multiforme, tumor tissues that did not express PD-L1 in the diagnostic biopsy specimen expressed

high levels of PD-L1 in the post-infusion specimens obtained a few weeks after CAR-T cell infusion (7). In this trial, a loss or decrease in the target antigen of the CAR-T cells was also observed, suggesting the importance of a dual-targeting strategy.

In the present study, PD-1 CAR-T cells were effective against pediatric solid tumors lacking high steady-state expression of PD-L1. However, as the expression of the PD-L1 is further enhanced by cytokines released after CAR-T cell attack, PD-1 CAR-T cells will be more effective when co-administered with or sequentially administered after CAR-T cells that target constitutively expressed

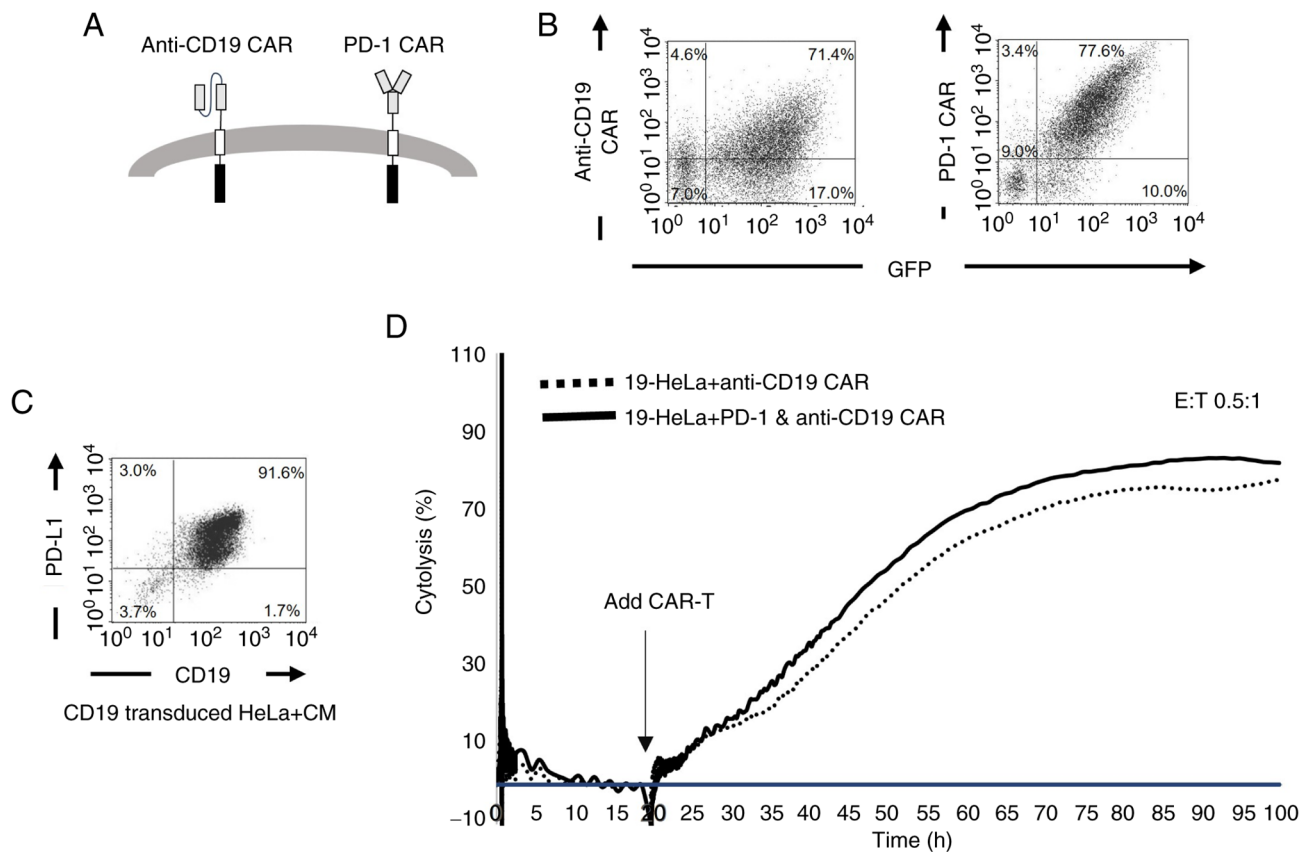


Figure 4. Dual targeting CAR-T cells. (A) The proposed scheme for dual-targeting CAR. PD-1 CAR and anti-CD19 CAR were transduced in the same T cells. (B) Surface expression of anti-CD19 CAR and PD-1 CAR in transduced T cells. T cells showed strong expression of both PD-1 CAR and anti-CD19 CAR. (C) Surface expression of CD19 and PD-L1 in HeLa cells retrovirally transduced with CD19 gene (19-HeLa). 19-HeLa cells stimulated with CM expressed both CD19 and PD-L1. (D) Long-term cytotoxicity of dual-targeting CAR-T cells against 19-HeLa with pretreatment with CM. Anti-CD19 CAR-T cells were used as control. PD-1, programmed death 1; CAR, chimeric antigen receptor; CM, conditioned medium; PD-L1, programmed cell death 1 ligand 1; GFP, green fluorescent protein; E:T, effector-to-target.

antigens, such as CD19, HER2, or GD2, than when used alone. Alternatively, PD-1 CAR may be expressed simultaneously with another CAR that targets another antigen, as in our experiments using CAR-T cells expressing two CARs that targeted different antigens. In this setting, PD-L1 and PD-L2 expressed in tumor tissues, as one of the mechanisms of evasion from immune attack, theoretically potentiate CAR-T cell therapy. In addition, PD-1 CARs expressed on a T cell are expected to compete with the wild-type PD-1 receptors co-expressed on the same cell, delivering strong activation signals (via CD28 and CD3z) instead of inhibitory signals. However, our study showed that CAR-T cells simultaneously expressing two different CARs, anti-CD19 and PD-1, exhibited slightly stronger anti-tumor effects. This concept needs to be validated in a future study using an *in vivo* tumor model in which the adaptive response by tumors inhibits CAR-T cells. Combinations of multiple CARs have also been reported. In particular, dual targeting of CAR for CD19/CD22 has been tested in patients with relapsed and refractory B-cell ALL (31). Serial administration or co-administration of CD19 and CD22 CAR-T cells has also been tested in clinical trials (32,33). Dual CARs described previously have aimed to enhance anti-tumor effects by targeting multiple antigens and overcoming the loss of one of the targets due to the immune evasion mechanisms of

the tumors. Targeting PD-L1 and PD-L2 with CAR-T cells offers two key advantages: first, broad applicability to pediatric cancers that lack abundant target antigens, and second, the ability to directly counteract tumor-driven immune evasion. Immune evasion from chimeric PD-1 CAR-T cells might result in the loss of expression of PD-L1 and PD-L2, which would lead to the restoration of the T cell-inhibiting microenvironment.

As PD-L1 can be expressed in normal cells, the toxicity of PD-1 CAR-T cells in normal cells is a potential concern. PD-L1 is constitutively expressed on antigen-presenting cells, such as monocytes/macrophages and dendritic cells (9). These immune cells with an antigen-presenting capacity also express CD80, which inhibits the binding of PD-1 to PD-L1 via cis-interactions between CD80 and PD-L1 (28). Therefore, we hypothesized that PD-1 CAR-T cells would not recognize PD-1 ligands on cells expressing CD80. Thus, we established CD80-coexpressing tumor cells and found that the effect of PD-1 CAR-T cells was weak against cells expressing both PD-L1 and CD80. These observations suggest that in PD-1 CAR-T cells using the PD-1 extracellular domain developed in the current study, toxicity to normal cells, including professional antigen-presenting cells, would be minimized, while efficacy against tumor cells that usually lack CD80 would be retained (Fig. 6).

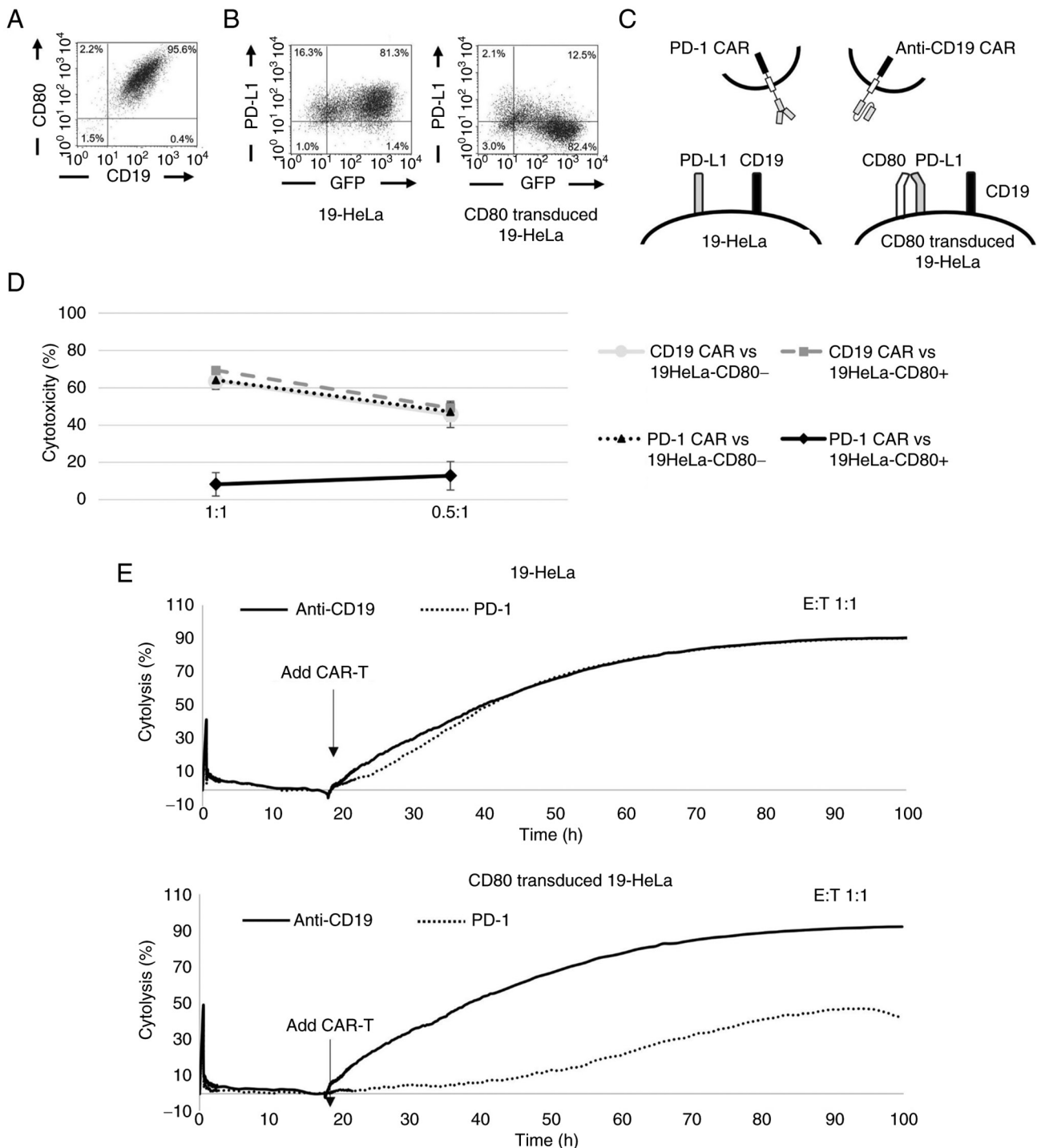


Figure 5. CD80 inhibits PD-1 CAR binding to PD-L1 and attenuates cytotoxicity. (A) Surface expression of CD80 in 19-HeLa cells after transduction of CD80. (B) Surface expression of PD-L1 after treatment with CM in 19-HeLa cells and CD80-transduced 19-HeLa cells. Anti-PD-L1 antibody binding was modestly attenuated in CD80-expressing 19-HeLa cells. (C) Schematic diagram of the experiment. Cytotoxicity of anti-CD19 CAR-T cells or PD-1 CAR-T cells against CM-pretreated, CD80-transduced 19-HeLa (that simultaneously expresses CD80, CD19, and PD-L1) were evaluated. (D) Cytotoxic effects of PD-1 CAR-T cells against CM-pretreated 19-HeLa with or without CD80 overexpression. Cells were cocultured for 24 h. (E) Long-term cytotoxicity of PD-1 CAR-T cells and anti-CD19 CAR-T cells against CM-pretreated 19-HeLa cells with or without CD80 overexpression (lower and upper panels). PD-1, programmed death 1; CAR, chimeric antigen receptor; CM, conditioned medium; PD-L1, programmed cell death 1 ligand 1; GFP, green fluorescent protein; E:T, effector-to-target.

During the manufacturing process of PD-1 CAR-T cells, PD-L1 was unexpectedly expressed on CAR-T cells, causing fratricide and poor cell proliferation. No previous reports have reported PD-L1 expressed on T cells. Furthermore, CAR-T cells targeting PD-L1 have been reported; however, there have been no reports of fratricide resistance. Previous

reports on CAR-T cell-targeting PD-L1 have mostly focused on auxiliary purposes such as inhibiting PD-1 inhibitory signals (34) or converting inhibitory signals to activating signals (35) rather than directly targeting PD-L1. Therefore, fratricides may not pose serious problems. Several reports have described PD-1 CAR-engineered NK cells (36-38). In

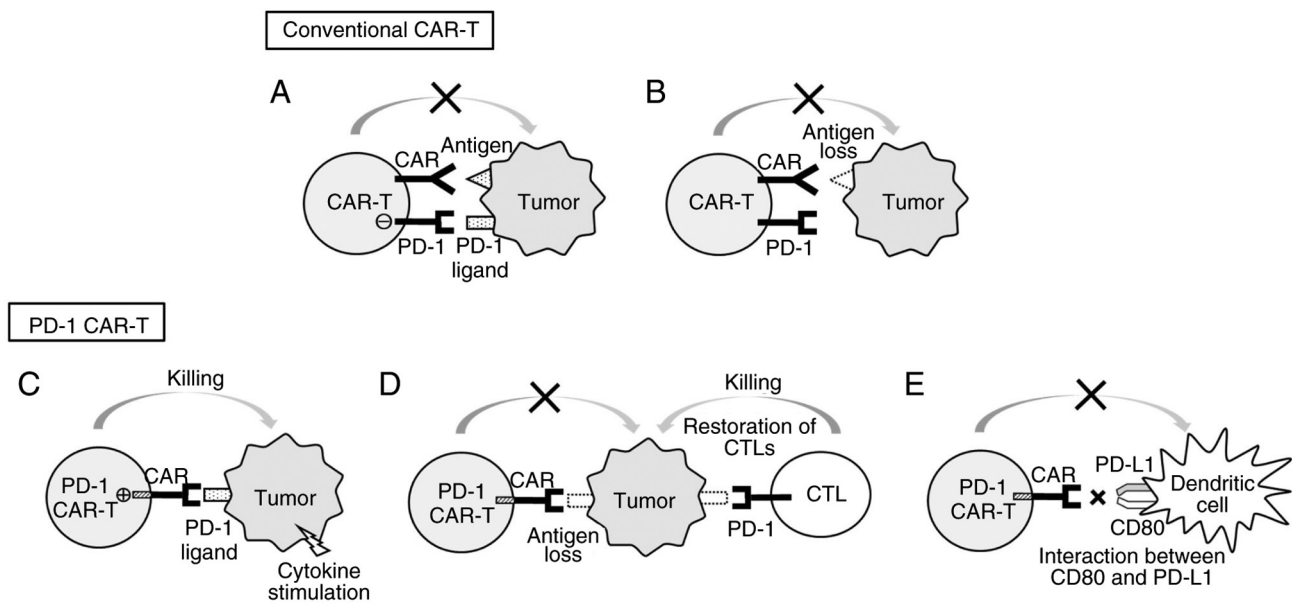


Figure 6. Proposed concept of the study. (A) Conventional CAR-T cells are inhibited by immune checkpoint molecules, such as PD-1 ligands. (B) Loss of the target antigen occurs after exposure to CAR-T cells as one of immune evasion mechanisms by cancer cells. (C) PD-1 CAR-T cells can target PD-1 ligands. (D) Loss of PD-1 ligands in cancer cells occurring as an immune evasion mechanism after attack by PD-1 CAR-T cells leads to the restoration of the T-cell inhibiting microenvironment. (E) PD-1 CAR-T cells using the PD-1 extracellular domain developed in this study have minimal impact on normal immune cells that express both CD80 and PD-L1 (e.g. dendritic cells). PD-1, programmed death 1; CAR, chimeric antigen receptor; PD-L1, programmed cell death 1 ligand 1.

these studies, PD-L1 was targeted by anti-PD-L1 scFv or PD-1 immunoglobulin domains; however, fratricide was not described in the manufacturing process. In our study, although CAR-T cells expressed PD-L1 for a short period after activation with CD3/CD28 stimulatory antibodies and IL-2, the poor efficiency in manufacturing CAR-T cells was successfully overcome by the short-term addition of the anti-PD-1 blocking antibody nivolumab. This observation also suggests that in a clinical setting, the use of anti-PD-1 antibodies such as nivolumab, which was shown in the current study to bind directly to our PD-1 CAR and inhibit the cytotoxic activity (fratricide) of CAR T cells, would be beneficial as a safety switch to treat severe and refractory adverse events such as cytokine release syndrome. A possible concern is that PD-1 CAR-T cells may upregulate PD-L1 expression upon activation through CAR engagement with cancer cells, rendering them susceptible to killing by neighboring CAR-T cells and thus compromising their persistence. However, the sustained cytotoxic activity of CAR-T cells targeting PD-L1 and/or PD-L2 in long-term coculture assays in the current study, along with *in vivo* evidence from previous reports, indicates that fratricide during the effector phase is not a concern (37,39).

This study has limitations that must be considered when interpreting the finding. Notably, we did not evaluate the function and safety of the PD-1 CAR-T cells in a preclinical xenograft model using severely immunodeficient mice; our investigation was limited to *in vitro* functional evaluation. However, previous research has reported that administration of PD-L1-targeting CAR-expressing T cells or NK-like cell line (e.g. NK-92) suppressed tumor growth and prolonged survival in immunodeficient mice bearing human cancer cells (37,39).

These findings support the potential safety and efficacy of our approach.

In conclusion, PD-1 CAR may be a novel candidate for the treatment of pediatric solid tumors. Despite their inhibitory role, PD-1 ligands may be suitable targets for CAR-T cell therapy owing to their importance and abundance in cancer cells. In addition, because PD-1 ligands are induced by cytokine stimulation, the PD-1 CAR is expected to be more effective in combination with other CAR-T cells and may be useful as a CAR for secondary attacks. Future research should test the efficacy of the simultaneous or sequential *in vivo* administration of PD-1 CAR-T cells in combination with conventional CAR-T cells (e.g. CD19, HER2, GD2 and mesothelin), which upregulate inhibitory immune checkpoint molecules, including PD-L1 and PD-L2. Exploring dual CAR-expressing T cells is also an important approach.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CS designed the methodology, performed the experiments, validation and formal analysis, analyzed the data, wrote the original draft and edited the manuscript. MI designed the study, acquired funding and analyzed the data. YK, YS, MB, NK, RH, HI and YM performed the experiments. AS provided supervision and analyzed the data. HK, AO and KM provided resources (study materials including cell lines and a plasmid) and analyzed the data. CI designed the study (conceptualization), designed the methodology, acquired funding, provided project administration and supervision, analyzed the data, and reviewed and edited the manuscript. CS and CI confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the ethics committee of Niigata University (approval #2015-2686). We obtained written informed consent before collecting peripheral blood from healthy volunteers.

Patient consent for publication

Not applicable.

Competing interests

CI reports patent royalties (patent title: Chimeric receptors with 4-1BB stimulatory signaling domain) from Juno Therapeutics and research funds and advisory fees from CURED, Inc. YS was an employee of CURED, Inc. from February 2022 to January 2024. The other authors declare that they have no competing interests.

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