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GD2-specific chimeric antigen receptor-modified T cells targeting retinoblastoma – assessing tumor and T cell interaction

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ABSTRACT

A novel disialoganglioside 2 (GD2)-specific chimeric antigen receptor (CAR)-modified T cell therapy against retinoblastoma (RB) were generated. GD2-CAR consists of a single-chain variable fragment (scFv) derived from a monoclonal antibody, hu3F8, that is linked with the cytoplasmic signaling domains of CD28, 41BB, a CD3ζ, and an inducible caspase 9 death fusion partner. GD2 antigen is highly expressed in Y79RB cell line and in several surgical RB tumor specimens. *In vitro* co-culture experiments revealed the effective killing of Y79RB cells by GD2-CAR T cells, but not by control CD19-CAR T cells. The killing activities of GD2-CAR T cells were diminished when repeatedly exposed to the tumor, due to an attenuated expression of GD2 antigen on tumor cells and upregulation of inhibitory molecules of the PD1 and PD-L1 axis in the CAR T cells and RB tumor cells respectively. This is the first report to describe the potential of GD2-CAR T cells as a promising therapeutic strategy for RB with the indication of potential benefit of combination therapy with immune checkpoint inhibitors.

Introduction

Retinoblastoma (RB) is the most common intraocular malignancy in children, and it is typically diagnosed at 2–3 years of age [1]. The incidence rate of this disease is 1 in 15,000 to 20,000 births worldwide, and it tends to increase each year [2]. RB patients in developing countries have a higher mortality rate than those in developed countries. Although RB patients tend to have a high survival rate, they experience increased occurrence of secondary malignancies, especially in hereditary RB cases [3,4]. These secondary malignancies include sarcomas, melanomas, and brain tumors. Systemic chemotherapy is increasingly being used as a primary intervention for intraocular RB in order to minimize the need for enucleation. A combination of two to three drugs is used to reduce the size of the tumor to the point where local therapy can be applied to destroy the remaining tumor. The limitations and risks associated with the currently available treatments for RB highlight the need for new, safer, and more effective therapeutic options.

Recent gene therapy advances have demonstrated clinical efficacy of chimeric antigen receptors (CAR)-engineered T cells, which have the ability to redirect T cells to target-specified tumor antigens via an MHC-independent antigen recognition process [5]. CARs were designed to couple the high-affinity binding of a single-chain variable fragment (svFv) antibody with the signaling domains of the T-cell receptor CD3ζ chain [6]. The CAR gene therapy strategy demonstrated clinical success

Abbreviations: CAR, chimeric antigen receptor; GD2, disialoganglioside 2; IHC, immunohistochemistry; LV, lentiviral vector; NB, neuroblastoma; PBMCS, peripheral blood mononuclear cells; PBS, phosphate buffer saline; PD1, programmed cell death 1; PD-L1, programmed cell death ligand 1; PHA, phytohaemagglutinin; RB, retinoblastoma; scFv, single-chain variable fragment.

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in the treatment of both CD19-positive B-cell leukemia and lymphoma [7]. A newer generation of CAR designs incorporated various types of intracellular costimulatory signaling domains, including CD28, OX40, CD27, and 4–1BB [8–10]. Individual or combinatorial T cell costimulatory signaling helps maximize T cell expansion, effector functions, and long-term survival [10]. A second-generation CAR incorporating the CD28 costimulatory signal demonstrated increased T cell expansion and persistence in patients [11–13]. A third-generation CAR incorporating 4–1BB (CD137) signaling demonstrated greater improvement in effector functions and persistence [14]. Although these later-generation CAR T cells demonstrated superior anti-cancer responses due to increased T-cell proliferation and expansion in vitro and in vivo [11,15], variable efficacy and outcomes are still being reported [16,17].

Critical to translational CAR T cell therapy is the selection of a target tumor-specific surface antigen that is expressed at high levels in tumor cells, but at low levels in normal cells. Disialoganglioside (GD2), which is a glycolipid onco-fetal antigen, is expressed in the fetus, stem cells [18], neurons [19], basal layer of the skin [20], and as a marker for breast cancer stem cells [21]. GD2 is overexpressed in many cancers, including neuroblastoma [22], glioma [23], Ewing's sarcoma [24], osteosarcoma [25–27], small cell lung cancer [28], melanoma [22], and RB. The high expression of GD2 in RB makes it an attractive target for CAR T cell therapy. GD2 is well-suited for use in targeted CAR T cell therapy for many reasons, the most important of which are its high density on tumor cells, and its restricted expression in normal tissues. Immunotherapy targeting GD2 antigen based on monoclonal antibodies has become an attractive strategy for eradicating tumor cells, such as neuroblastoma [29], osteosarcoma [27], Ewing's sarcoma [24], and potentially RB.

In this study, we generated T cells expressing a fourth generation GD2-specific CAR incorporating CD28–41BB-CD3ζ moieties with a safety switch (i.e., an inducible caspase 9 fusion domain, iCasp9) using lentiviral vectors (LV-4SCAR-GD2). We initially demonstrated high expression of GD2 in RB cells. The 4SCAR-GD2 T cells exerted evident anti-tumor activities against Y79RB, but extended target killing of the 4SCAR-GD2 T cells led to tumor escape. Further investigation revealed the immune evasion mechanisms of RB tumor cells. This is the first report demonstrating GD2-specific CAR T cells targeting RB. Moreover, the tumor escape mechanisms revealed by this study strongly suggest the need for a combined therapeutic strategy to prevent RB tumor immune escape.

Materials and methods

Cell culture

Human retinoblastoma cell line Y79RB and retinal pigmental epithelial cell line ARPE-19 were purchased from American Type Culture Collection (ATCC, HTB-18 and CRL-2302, respectively, Manassaas, VA, USA). Human neuroblastoma cell line SK-NAS was a gift from Dr. Naravat Poungvarin, Faculty of Medicine Siriraj Hospital, Mahidol University. The Y79RB cells were cultured in suspension in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco® Life Technologies, Thermo Fisher Scientific, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin (Mediatech; Corning, NY, USA). For ARPE-19, SK-NAS and primary NB cells, cells were maintained in Dulbecco's Modified Eagle Medium /Nutrient Mixture F-12 (DMEM/F12) (Gibco® Life Technologies, Thermo Fisher Scientific, CA, USA). All cells were maintained in a 37 °C incubator with 5%CO_2. Recombinant human cytokines, including interleukin (IL)−2 (250 µg/ml), IL-7 (250 µg/ml), and IL-15 (250 µg/ml), were purchased from PeproTech (PeproTech, Cranbury, CT, USA). AnnexinV-V450 and propidium iodide (PI) were purchased from BD Pharmingen (BD Biosciences, San Jose, CA, USA).

Antibodies and reagents

Phycoerythrin (PE)-conjugated antibodies (Abs) to GD2 (clone 14.G2a) was purchased from BD Pharmingen™ (BD Biosciences, San Jose, CA, USA). PE-conjugated Abs to CD19 (clone SJ25-C1) and CD3 (clone 7D6) were obtained from Life Technologies (Thermo Fisher Scientific, MA, USA). PE-Cy7-conjugated Abs to CD279 or PD-1 (clone EH12.1) and CD274 (clone MIH1) were purchased from BD Pharmingen™ (BD Biosciences, San Jose, CA, USA), and eBiosciences (Thermo Fisher Scientific, CA, USA), respectively. Leaf™ Purified Anti-Human CD274 (PD-L1) was purchased from BioLegend, San Diego, CA, USA. Isotype-matched antibodies were included as a control for antibody staining.

Lentiviral vector construction and transduction

Lentiviral vectors (LVs) were generated using the NHP/TYF lentiviral vector system, as previously described [30,31]. The coding sequence of single-chain variable fragment (scFv) derived from hu3F8 monoclonal antibody, recognizing human GD2, was cloned into the self-inactivating pTYF transducing plasmid under the control of the *EF1α* promoter. The intracellular domain of the GD2-CAR construct contains CD28, 41BB and CD3ζ signaling molecules. To generate feeder cells, mouse fetal stromal cells were multiply transduced with LVs at 10–50 infectious units/cell in 12-well plates in a minimal volume of 0.3 ml per well. After 2 h, 0.5 ml of fresh media was added and cells were incubated at 37 °C overnight. To generate green fluorescent Y79RB tumor cells, cells were transduced with LVs containing a monomer green fluorescent protein (GFP) variant Wasabi gene until stable lentiviral transgene expression was confirmed.

Production of GD2-specific car t cells

Peripheral blood mononuclear cells (PBMCs) were prepared from buffy coats of healthy donors after receiving approval (507–1997) from the Institutional Review Board of the University of Florida (IRB-01). PBMCs were isolated by Ficoll-Hypaque density centrifugation (Sigma Aldrich, St Louis, MO, USA). PBMCs were activated with phytohemagglutinin (PHA; 5 µg/ml) for 2–3 days, after which they were maintained in TexMACS™ medium (Miltenyi Biotec Inc, San Diego, CA, USA). Activated T lymphocytes were transduced with CD19- or GD2-specific lentiviral particles, and were then maintained in TexMACS™ medium supplemented with human IL-2 (40 U/ml), IL-7 (20 U/ml), and IL-15 (10 U/ml) (PeproTech, Cranbury, CT, USA). Cells were expanded over 7–10 days in the presence of cytokines. After expansion, 1×10^5 cells were extracted for genomic DNA and subjected to real-time PCR analyses to determine the viral gene copy number.

Immunohistochemistry

The tumor and normal tissue paraffin block specimens were obtained under approval (507–1997) from the Institutional Review Board of the University of Florida (IRB-01). Eight samples of formalin-fixed paraffin-embedded tissues derived from RB patients were obtained from the Department of Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The paraffin-embedded tissue sections were prepared by the Department of Pathology, University of Florida, Gainesville, Florida, USA. Normal brain and brain tumor tissue sections were used as negative and positive controls for GD2 staining, respectively. The paraffin-embedded sections were washed in Histo-ClearII (National Diagnostics, Atlanta, GA, USA) for deparaffinization, and washed in a graded alcohol series and distilled water for rehydration. The sections were then blocked and heated in citrate buffer for antigen retrieval. Sections were blocked with 10% normal sheep serum and incubated with 1/25 (BD Biosciences, San Jose, CA, USA) GD2 antibody for three nights at 4 °C. After washing, sections were incubated with MACH 2 Mouse AP-Polymer (Biocare Medical, Pacheco, CA, USA) for

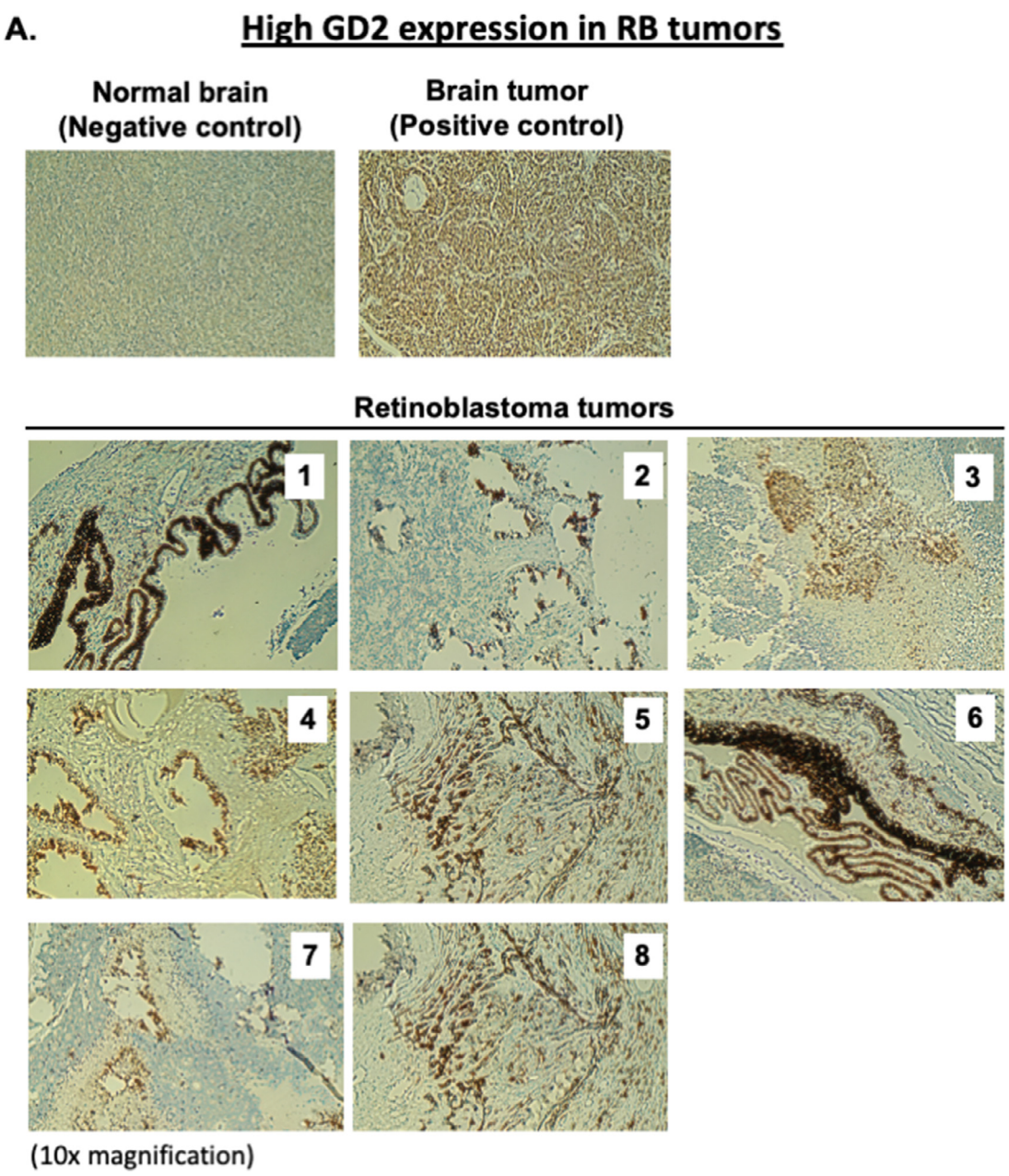

Fig. 1. Expression of GD2 antigen in RB patient tumor tissues and Y79 RB cell line.
a) Immunohistochemistry analyses of GD2 expression in tumor samples from RB patients. Paraffin-embedded retinal tumor samples were stained for GD2 expression using an antibody against human GD2, followed by staining with an enzyme-conjugated specific antibody. The IHC stained samples were visualized under a fluorescence microscope (Zeiss Axiovert 25, Maple Grove, MA, USA) using 10x magnification with 1/80 s exposure time.
b) Surface staining and flow cytometric analysis for GD2 and CD19 (black line) or an IgG isotype control (filled histogram) on the surface of Y79 RB cells, primary NB cells, human neuroblastoma cells SK-NAS, and normal human retinal pigment epithelial cell line ARPE-19. Upper right number indicates cell count, and the normalized mean fluorescence intensities (nMFI) are shown in parentheses.

2 h. The sections were developed using an AP Substrate Kit (ZSGB-BIO, OriGene Technologies Inc, Beijing, China), counterstained with hematoxylin, dehydrated, and then covered with a coverslip. Stained IHC samples were visualized under fluorescence microscope (Zeiss Axiovert 25, Maple Grove, MA, USA) using 10 x magnification.

In vitro killing assay

Green fluorescence-expressing Y79RB cells [target (T) cells (1×10^5 cell/well)] were co-cultured with effector (E) T cells, including CD19- and GD2-specific CAR T cells, at effector:target (E:T) ratios of 3:1, 1:1, 1:2, and 1:4 in the wells of a 96-well flat-bottom plate at 37 °C for 24 h. Target cell death was monitored under a fluorescence microscope (Zeiss Axiovert 25, Maple Grove, MA, USA) using 10 x magnification, after which the cells were collected to examine cell apoptosis and necrosis by AnnexinV/ PI staining (BD Bioscience, San Jose, CA, USA) and flow cytometry using BD LSRII Flow cytometer (BD Bioscience, San Jose, CA, USA). Specific cell death was calculated using the following formula: [(% cell death target - spontaneous cell death)/ (100 - spontaneous cell death)] x 100. Spontaneous target cell death was defined as the percent-

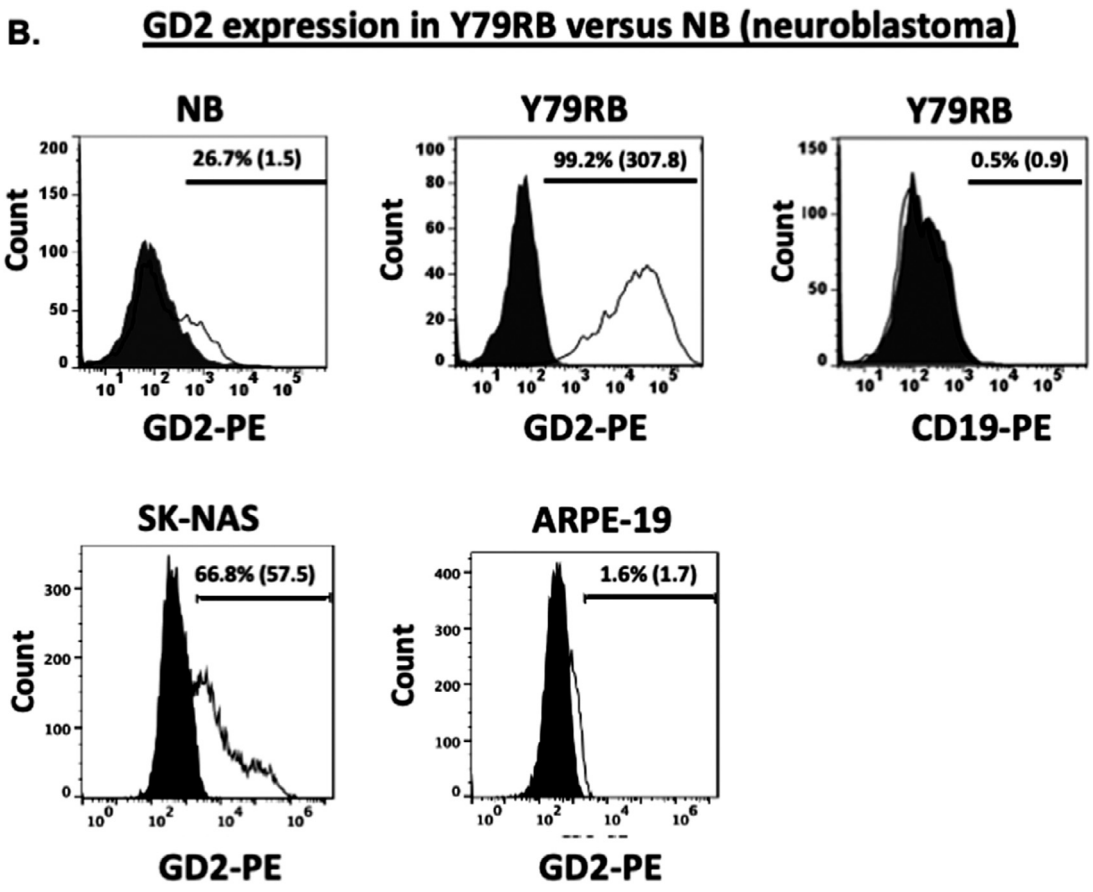

Fig. 1. Continued

age of target cell death that cultured in T cell media without effector T cells. The results are representative of three independent experiments.

Flow cytometry

The surface expression of GD2 was analyzed by flow cytometry with PE-conjugated anti-human GD2 mAb clone 14.G2a (BD Biosciences, San Jose, CA, USA) and isotype mouse $IgG2_{a}\kappa$ (BD Biosciences, San Jose, CA, USA). For PD-L1 expression, cells were stained with anti-human CD274 or PD-L1 conjugated with PE-Cy7 monoclonal antibody clone MIHI (eBiosciences, Thermo Fisher Scientifics, Waltham, MA, USA)and isotype $IgG_1\kappa$ (BD Biosciences, San Jose, CA, USA). For PD-1 expression, co-cultured cells were collected and stained with PE-conjugated anti-CD3 together with PE-Cy7-conjugated anti-PD1 antibodies (BD Biosciences, San Jose, CA, USA). For flow cytometric staining, cells were collected and washed with PBS containing 2% FBS, and then blocked with mouse and human serum at 4 °C for 30 min. Cells were incubated with antibodies according to the manufacturer's instructions. For each fluorochrome-labeled Ab used, appropriate isotype controls were included to establish a negative gate. After antibody staining, the cells were washed twice and fixed with 2% paraformaldehyde/PBS. Data acquisition and analysis were performed on an BD LSRII flow cytometer (BD Biosciences, San Jose CA, USA) or BD Accuri^{TM} C6 Plus flow cytometer (BD Biosciences, San Jose CA, USA) using FACSDiva software (BD Biosciences, San Jose, CA, USA) or FlowJo software (version 7.1.3.0, Tree Star Inc., Padadena, TX, USA).

AnnexinV/ PI staining

After the co-culture experiment, cell samples were collected to analyze tumor cell death by AnnexinV-V450/PI staining (BD Biosciences, San Jose, CA, USA) and flow cytometry using BD LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) as previously examined [32]. According to the manufacturer's instructions, cells were incubated in the dark with staining buffer containing 5 μl of Annexin V-V450 and 5 μl of propidium iodide (PI) and analyzed by flow cytometry on an BD LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). Each test condition was analyzed in triplicate. Early and late apoptotic cells were defined as $AnnexinV^{+}/PI^{-}$ and $AnnexinV^{+}/PI^{+}$ cells, respectively, while the necrotic or dead cells were stained with PI^{+} only. Percent cell death includes early apoptosis, late apoptosis, and necrotic cells. Percent specific death was calculated using the following formula: (% cell death target –% spontaneous cell death)/ (100 -% spontaneous cell death) × 100%. Flow cytometry data was analyzed by using FlowJo software (version 7.1.3.0, Tree Star Inc., Padadena, TX, USA).

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). The significance of the difference between groups was evaluated by unpaired *t*-test using GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA). Significant differences (*p*-values) represented on graphs are indicated, as follows: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

Results

High GD2 expression in Y79RB tumor cell line and surgical specimens from RB patients

GD2 is highly expressed on the surface of many cancer cells. Retinal tissue samples from eight RB patients were examined for GD2 expression by immunohistochemistry staining. Normal brain and brain tumor

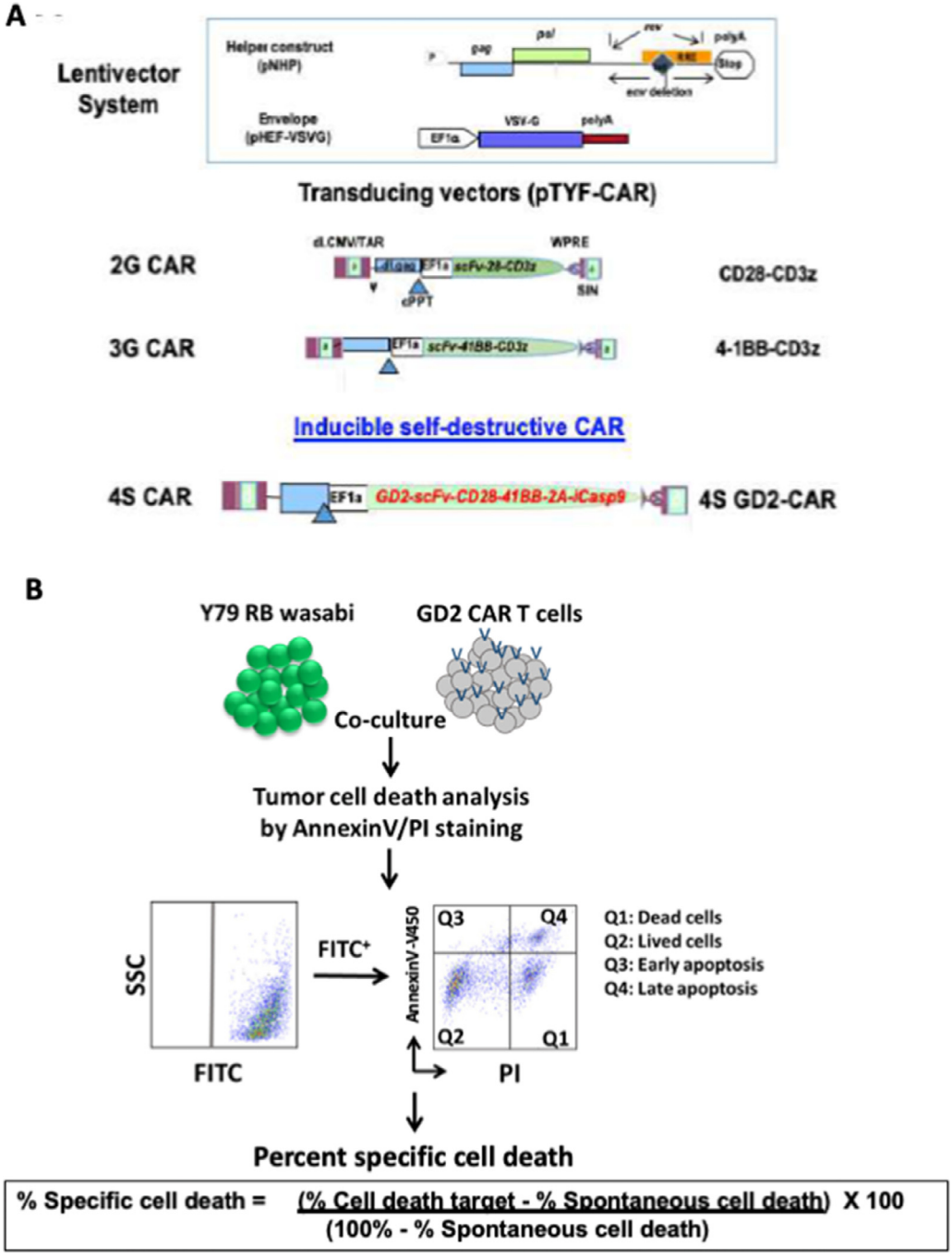

Fig. 2. Y79 RB cells transduced with lentiviral vectors (LVs) expressing Wasabi (a green fluorescent protein), and T cells transduced with LVs expressing scFv-GD2 chimeric antigen receptor.
a) Schematic diagram of the LV expressing different generations of GD2-specific CAR constructs. The diagram illustrates the self-inactivating *bi*-cistronic lentiviral vector pTYF-CAR expressing scFv-GD2 and costimulatory molecules under human *EF1α* promoter control.
b) Diagram of the GD2-specific CAR T cell killing assay. Y79RB-Wasabi cells were co-cultured with CD19 CAR T cells or GD2 CAR T cells. Twenty-four hours after co-culture, tumor cell death was monitored by fluorescence microscopy and staining by AnnexinV/PI followed by flow cytometric analysis.

tissues were used as negative and positive controls for GD2 staining, respectively. Paraffin-embedded tissue sections were stained with anti-human GD2 antibody and analyzed by peroxidase method. All eight RB tumor sections showed strongly positive signals for GD2 staining (Fig. 1A). Native expression of GD2 on RB cells was demonstrated in human RB cell line - Y79RB. Flow cytometry analysis revealed Y79RB cells to be strongly positive for GD2, but not for CD19, which is a B cell surface marker. Primary neuroblastoma (NB) cells and neuroblastoma cell line SK-NAS demonstrated a moderate level of GD2 expression (Fig. 1B). Moreover, surface GD2 expression did not observe on normal human retinal pigment epithelial cell line ARPE-19 cell line (Fig. 1B).

Generation of 4SCAR-GD2 T cells

A 4th generation GD2-specific CAR lentiviral vector was designed as described previously [27]. This vector encodes a GD2-specific CAR

(GD2-CAR) that consists of a sequence from the monoclonal anti-GD2 scFv clone hu3F8, a transmembrane region of CD28 molecule, costimulatory signaling moieties of CD28 and 4–1BB, and the cytoplasmic domain of a CD3ζ molecule – all of which were fused to an inducible caspase 9 sequence with a 2A peptide (Fig. 2A). PHA-activated lymphocytes of healthy donors were transduced with LV particles carrying 4SCAR-GD2 or 4SCAR-19, which is a control LV-CAR with CD19 scFv specificity [10]. To examine the cytolysis of GD2-specific 4SCAR T cells, a co-culture experiment consisting RB target cells and effector CAR T cells was performed, as shown in Fig. 2B. We created a green fluorescent RB cell line, namely Y79RB-Wasabi. This target cell line was used to monitor target cell killing under fluorescence microscope and by flow cytometry.

RB tumor cell lysis by 4SCAR-GD2 T cells

Cytolytic activity of 4SCAR-GD2 T cells was tested by using GD2-positive Y79RB-Wasabi cells as target cells. Y79RB-Wasabi was co-cultured with 4SCAR-19 or 4SCAR-GD2 T cells for 24 h. Target cell death was monitored by flow cytometry analysis of Annexin V and PI-stained green fluorescent RB cells, which were compared with 4SCAR-19 T cells. Y79RB-Wasabi cells co-cultured with 4SCAR-GD2 T cells demonstrated a significantly higher rate of tumor cell death (Fig. 3A-D). Close to 80% of tumor cells were killed by 24 h after co-culture (Fig. 3B-C), with the entire cell population had died by 3 days after co-culture (Fig. 3D). Thus, 4SCAR-GD2 T cells could effectively target and kill RB tumor cells with high specificity.

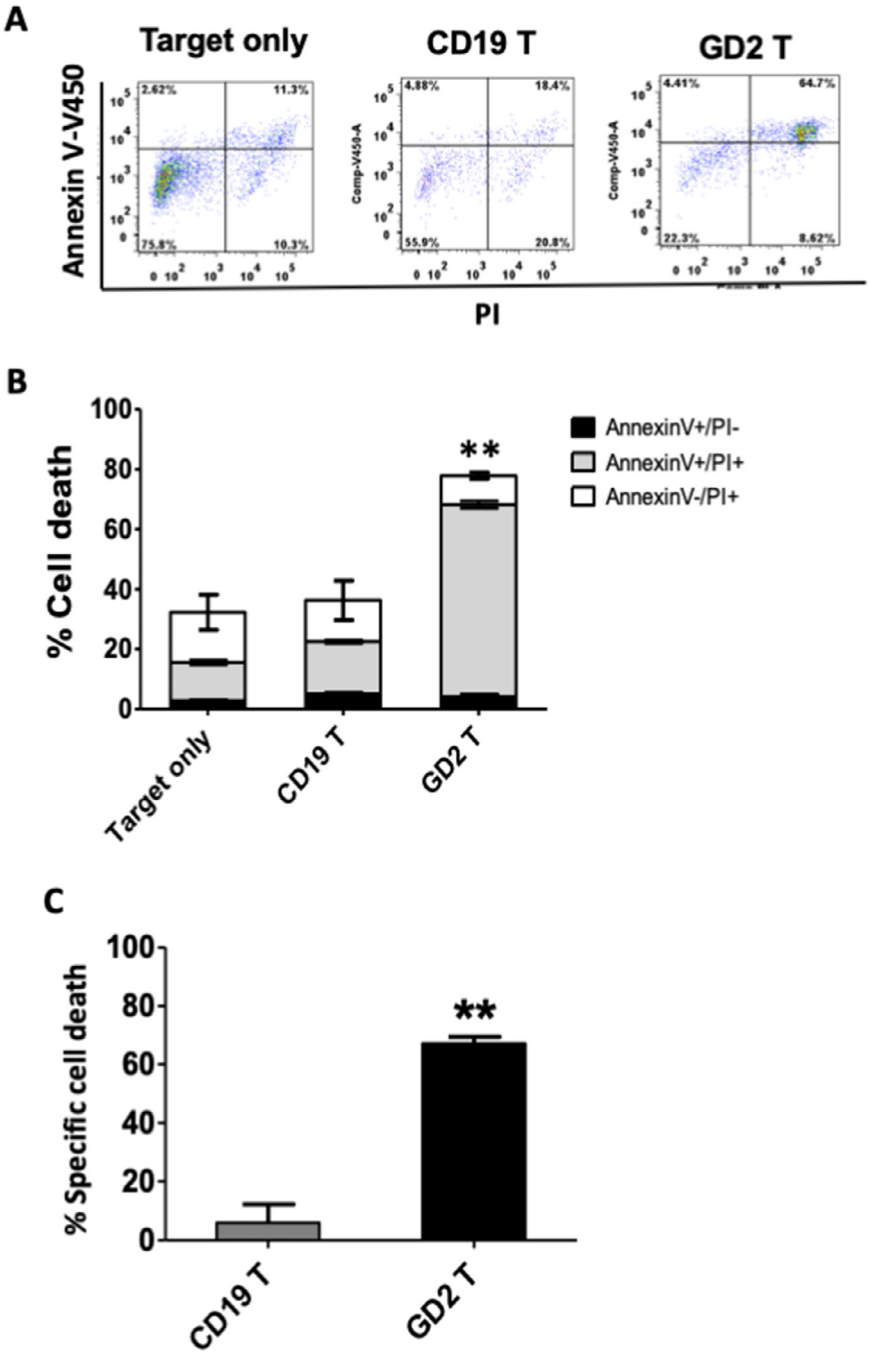

Fig. 3. Cytolytic activity of 4SCAR-GD2 T cells against Y79RB-Wasabi cell line.

a) Representative flow cytometry dot plots showing significant percentage of tumor cell death stained by AnnexinV/PI dye after 24-h incubation with GD2-speciifc CAR T cells.

b) Tumor cell death after co-culturing with control T cells or CD19-specific CAR T cells or GD2 CAR T cells. Bar represents the mean of percent cell death. $^{*}p<0.05$ versus CD19 CAR T using unpaired *t*-test.

c) Percent specific cell death of tumor cell after co-culturing with control T cells or CD19-specific CAR T cells or GD2 CAR T cells. Bar represents the mean of percent specific cell death of tumor cells. Error bars represent the standard error of the mean (SEM) from three-independent experiments. $^{*}p<0.05$ versus CD19 CAR T using unpaired *t*-test.

Tumor cell death was monitored under fluorescence microscope. After co-cultivation of tumor and effector T cells for three days, almost 100% of the tumor cells were completely killed in the presence of GD2 CAR T cells, but none of the CD19 CAR T cells were similarly affected. The green tumor cell death was observed under a fluorescence microscope (Zeiss Axiovert 25, Maple Grove, MA, USA) using 10x magnification.

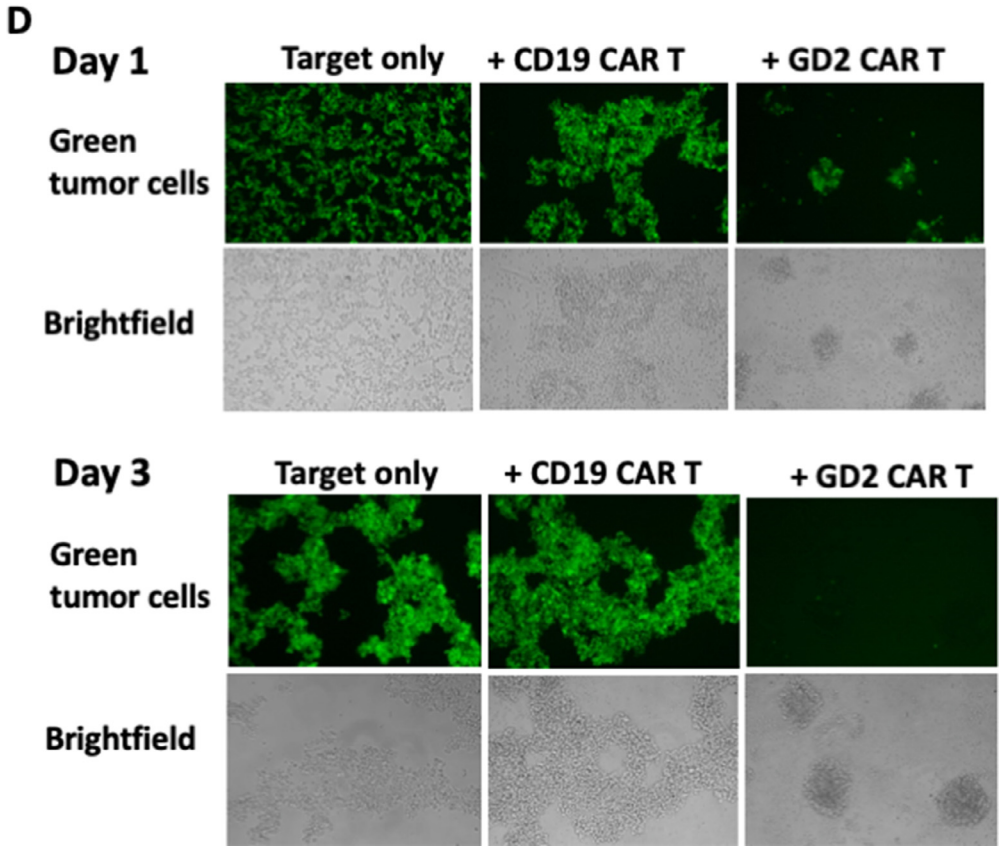

Fig. 3. Continued

Re-stimulation of CAR4S-GD2 T cells and tumor escape

To mimic *in vivo* high tumor burden condition, we added three times more target cells to the 4SCAR-GD2 co-culture after one-round of target killing. Surprisingly, after co-culture for six days, some tumor cells survived and proliferated under incubation with 4SCAR-GD2 T cells (Fig. 4A). Flow cytometry analysis revealed that the 4SCAR-GD2 T cell-incubated Y79RB cells expressed a reduced level of GD2 on their cell surface when compared with the original Y79RB cells (Fig. 4B, left panel). GD2 expression on the parental Y79RB cells was 93.2%, but the proportion decreased to 65.5% on the CAR T-resistant Y79RB cells (Fig. 4B, right panel). Immune checkpoint blockade is one of the known mechanisms of tumor escape from immune surveillance. Thus, we examined expression of programmed death ligand 1 (PD-L1) on tumor cells and programmed death protein 1 (PD-1) on T cells by immunostaining with specific antibodies, and by analysis with flow cytometry. The result clearly illustrated that parental Y79RB cells lacked PD-L1 expression on their cell surface; however, after co-culture with 4SCAR-GD2 T cells (but not 4SCAR-19 T cells), the Y79RB cells exhibited upregulated PD-L1 expression (Fig. 4C). At the same time, expression of PD-1 on the co-cultured 4SCAR-GD2 T cells (but not on the co-cultured 4SCAR-19 T cells) was significantly upregulated (Fig. 4C, right panel). These results suggest that after repeated antigen exposure, the PD1:PD-L1 axis of immune tolerance contributed to the survival of escaped tumor cells and suppression of effector CAR T cell function [33].

Discussion

RB patients require combined therapies that include chemotherapeutic drugs and focal treatment. Repeated chemotherapeutic treatment causes severe side effects and contributes to increased incidence of both tumor recurrence and secondary metastasis. RB cells may become drug-resistant after repeated treatments [34], which indicates that RB cells can be highly adaptive to not only chemotherapeutics, but also to immune cell therapy.

GD2 is a surface antigen that is found to be highly expressed in RB tumor, with low or undetectable levels observed in normal tissues. GD2-specific CAR T cells have been investigated in several tumor models, including neuroblastoma [35,36]], osteosarcoma [27], Ewing's sarcoma [37], and melanoma [38]. We demonstrated that GD2 was abundantly expressed in the tumor specimens of many RB patients and highly expressed on surface of human retinoblastoma cell line Y79RB, but it was not detected on normal human retina pigment epithelial cell line, ARPE-19. In addition, it could be effectively targeted by a new generation of GD2-CAR T cells, 4SCAR-GD2. An earlier generation GD2 CAR T cells demonstrated moderate effect against GD2-positive neuroblastoma cells, with low cytotoxic activity against Y79RB cell line [35]. The first generation of GD2-CAR cells was reported to gradually decrease tumor-specific cytokine release during *in vitro* culture [35], which is similar to the lack of functionality of the first generation of CD19-specific CAR T cells *in vivo* [39]. Compared with the earlier CAR design, a later generation of GD2-specific CAR incorporating CD28 and OX40 signaling domains demonstrated superior killing efficiency, *in vitro* expansion potential, and cytokine secretion [14]. Incorporating the 4–1BB signaling domain increased 14.18 Ab-based GD2-CAR T cell persistence and reduced T cell exhaustion [38,40,41].

In the present study, we selected a humanized hu3F8 Ab-based GD2-CAR design incorporating the CD28 and 4–1BB cytoplasmic signaling domains and an inducible caspase 9 death domain (4SCAR-GD2), and we were able to demonstrate improvement in RB targeting function. The 4SCAR-GD2 T cells generated from this study showed very high cytolytic activity against RB cells as compared with a different GD2-CAR design at the same effector to target ratio [42]. Although the benefit of anti-GD2 treatment for NB is evident, there is scant evidence on anti-GD2 therapy for RB treatment. Furthermore, CAR T therapy has several

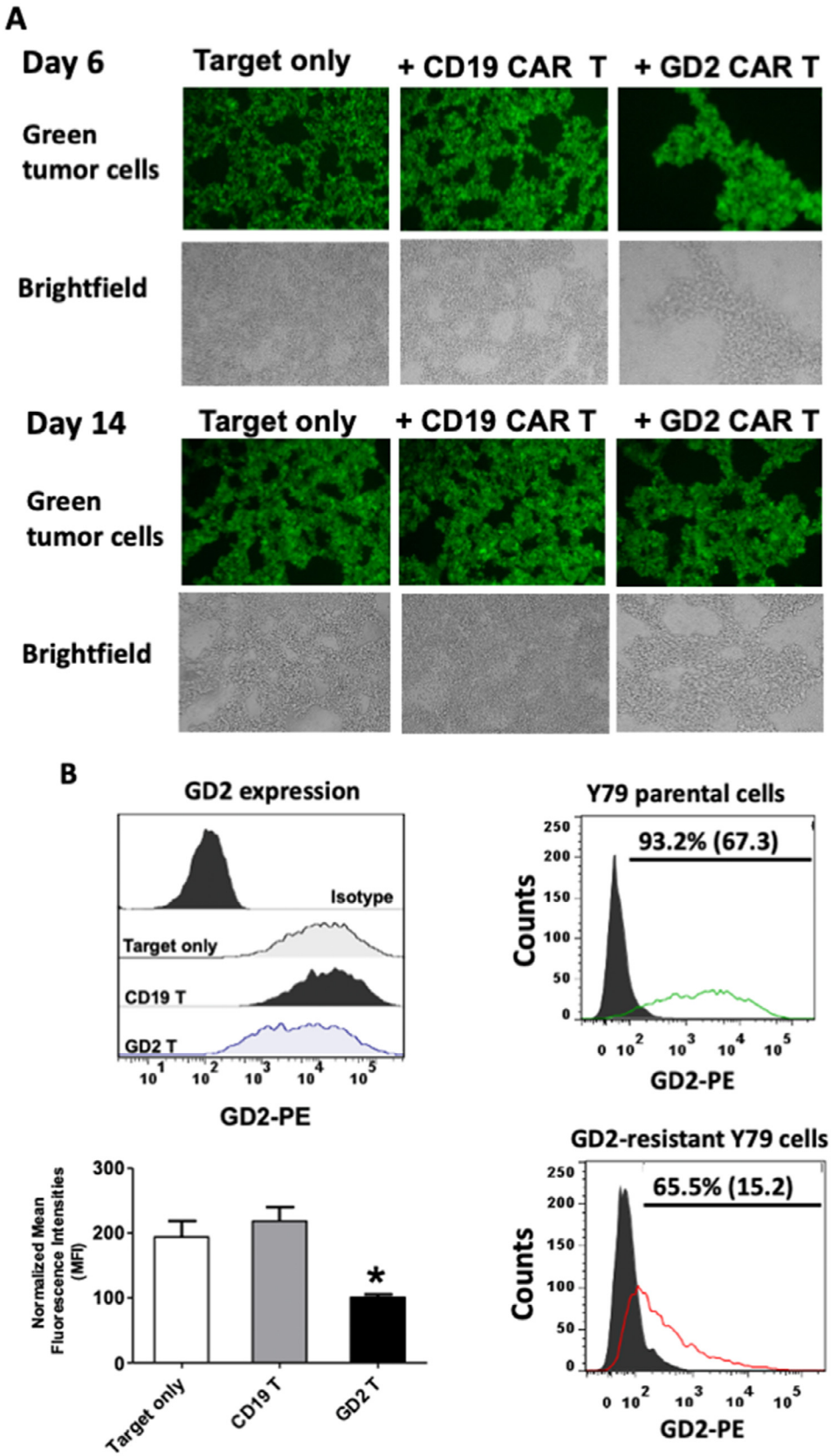

Fig. 4. Restimulation of GD2-specific CAR T cells promotes hypofunctional killing activity.
a) Restimulation of GD2 CAR T cells by adding more Y79RB-Wasabi tumor cells. Green tumor cells were monitored every day under fluorescence microscope. Photographic images taken at 10x magnification on day 6 and day 14 after co-culture are shown in the upper and lower panels, respectively.
b) Expression of GD2 on Y79RB tumor cells after re-stimulation. Green tumor cells after co-culturing with control T or CD19 CAR T or GD2 CAR T cells were collected for immunostaining with anti-GD2 antibody and analysis by flow cytometry. The results of GD2-positive staining in green tumor cells are shown as histograms (upper panel) and the bar graphs (lower panel). $^{*}p<0.05$ versus CD19 CAR T using unpaired *t*-test.
c) Expression of PD-L1 on tumor cell surface, and PD-1 on CAR T cells. Y79RB cells were co-cultured with control T or CD19 CAR or GD2 CAR T cells at an E:T ratio of 1:1. Flow cytometry histograms show surface expression of PD-L1 on Y79RB cells after co-culturing. The bar graph on the lower left shows the normalized mean fluorescence intensity (nMFI) for GD2 positive staining. The bar graph on the upper right panel shows percent of PD-1 expression on CD3+ *T* cells. The data are shown at mean ± standard error of the mean (SEM); $^{*}p<0.05$ using unpaired *t*-test.

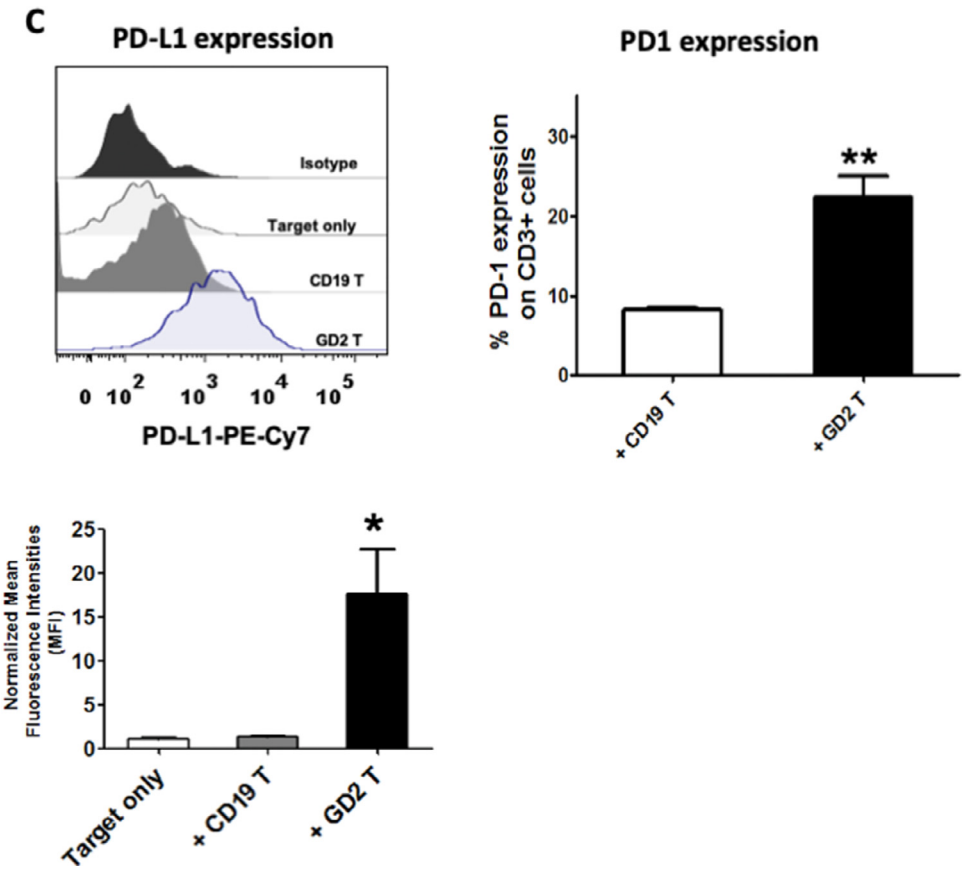

Fig. 4. Continued

benefits over the use of antibody therapy such as high potential of GD2 CAR T to cross blood-brain barriers, high persistence, and low toxicity. Recent data has shown that hydrogel encapsulated second generation GD2-CAR T secreting IL-15 can successfully eliminate RB tumor cells in a mouse model [43], supporting the potential use of GD2 CAR T cells including our 4SCAR-GD2 T cells for RB treatment.

We illustrated a high degree of RB tumor cell death after 4SCAR-GD2 T cell engagement, almost all tumor cells were eradicated after increased co-culture time. Upon 4SCAR-GD2 T cell interaction, the RB tumor cells showed increased late apoptotic markers (double positive for Annexin V/PI). The cytotoxicity assay can also be done using LDH enzyme quenching method to illustrate the degree of necrosis [44,45]. In our experience, the flow cytometry-based cytotoxicity assay is more sensitive and specific than other methods because individual cells were stained and detected, and both early and late apoptotic tumor cells could be detected in contrast to alternative methods such as chromium release assay and LDH activity assay, both of which detect overall lysis of cells. In addition, the stage of tumor cell death could be specified based on surface marker expression by flow cytometry.

The 4SCAR-GD2 T cells demonstrated persistence *in vitro* after repeated addition of increasing numbers of target cells. We have also observed the ability of 4SCAR-GD2 T cells to proliferate and survive after antigen engagement upon prolonged culture time (Data not shown). Importantly, we showed that residual RB cells became resistant to the 4SCAR-GD2 T cells via selective attenuated expression of GD2, and increased PD-L1 expression on tumor cells. This latter observation closely mimicked the reported downregulation of CD19 surface expression in B lymphoblastic leukemia after CD19 CAR T cell treatment [46]. Nevertheless, GD2 was reported to have stable expression on the preponderance of tumors throughout disease stage and evolution [47], and GD2-specific immunotherapy has not been shown to elicit GD2 expression loss in the bulk of treated patients [48]. The potential implications of our findings relative to *in vivo* treatment of RB using 4SCAR-GD2 T cells will be elucidated by future clinical investigations.

The results of our study indicate that CAR T cell treatment alone may be unable to completely eradicate tumor cells due to tumor cell immune evasion. Similar to our findings, upregulation of immune inhibitory HLA-G was recently reported as an evasion mechanism in Ewing's sarcoma from GD2-specific NK cell killing [37]. It is, therefore, essential to consider and investigate the combination of chemotherapeutic drugs and immune checkpoint inhibitors with CAR T cells to improve therapeutic outcomes in RB and other types of solid tumors.

In summary, this study demonstrated the potential of novel fourth-generation GD2-CAR T cells as a promising therapeutic strategy for treatment of RB. The combination of CAR T cells with immune checkpoint inhibitors may overcome the potential immune escape of RB cells.

Ethical approval statement

For the use of blood samples from unidentified healthy donors and unidentified tumor paraffin specimens, approval with waiver was obtained in accordance with the regulation of the Institutional Review Board of the University of Florida (IRB-01, 507-1997).

Declaration of Competing Interest

The authors declare no competing financial interests.

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CRediT authorship contribution statement

Jatuporn Sujjitjoon: methodology, investigation, validation, formal analysis, writing the original draft; **Elias Sayour:** resources, funding acquisition; **Shih-Ting Tsao:** resources, methodology; **Mongkol Uiprasertkul:** resources; **Kleebsabai Sanpakit:** resources; **Jassada Buaboonnam:** resources; **Pa-thai Yenchitsomanus:** conceptualization, supervision; **La-ongsri Atchaneeyasakul:** resources, project administration; **Lung-Ji Chang:** project administration, design of the study, conceptualization, resources, funding acquisition, writing-review& editing.

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