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Research Article

Cancer Immunology Research

CAR T Cells Targeting Podoplanin Reduce Orthotopic Glioblastomas in Mouse Brains

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Abstract

Glioblastoma (GBM) is the most common and lethal primary malignant brain tumor in adults with a 5-year overall survival rate of less than 10%. Podoplanin (PDPN) is a type I transmembrane mucin-like glycoprotein, expressed in the lymphatic endothelium. Several solid tumors overexpress PDPN, including the mesenchymal type of GBM, which has been reported to present the worst prognosis among GBM subtypes. Chimeric antigen receptor (CAR)–transduced T cells can recognize predefined tumor surface antigens independent of MHC restriction, which is often downregulated in gliomas. We constructed a lentiviral vector expressing a third-generation CAR comprising a PDPN-specific antibody (NZ-1–based single-chain variable fragment) with CD28, 4-1BB, and CD3ζ intracellular domains. CAR-transduced peripheral blood monocytes were immunologically evaluated by calcein-mediated cytotoxic assay, ELISA, tumor size, and overall survival. The generated CAR T cells were specific and effective against PDPN-positive GBM cells *in vitro*. Systemic injection of the CAR T cells into an immunodeficient mouse model inhibited the growth of intracranial glioma xenografts *in vivo*. CAR T-cell therapy that targets PDPN would be a promising adoptive immunotherapy to treat mesenchymal GBM. *Cancer Immunol Res; 4(3); 259–68. ©2016 AACR.*

Introduction

Glioblastoma (GBM) is the most common and lethal primary malignant brain tumor in adults. After maximal surgical resection, the current standard of care is concurrent radiotherapy and the alkylating agent temozolomide (TMZ), followed by adjuvant TMZ (1). Despite the improvement in outcomes with this combined chemoradiotherapy approach, the median survival is 14.6 months and 5-year overall survival (OS) rates are less than 10% (1). Thus, novel therapies are required to improve patient survival.

In recent years, immunotherapy has emerged as a promising strategy for the treatment of GBM (2). The first generation of chimeric antigen receptors (CAR) were recombinant receptors that consisted of an extracellular domain derived from a single-chain variable fragment (scFv) taken from a tumor antigen–specific monoclonal antibody (mAb), a transmembrane domain, and a cytoplasmic signaling domain from the CD3ζ chain, a subunit of the T-cell receptor complex (3). CAR-transduced T cells can recognize predefined tumor surface antigens independent of the major histocompatibility complex (MHC) restriction, which is often downregulated in gliomas (4). CARs can be designed to bind not only to proteins, but also to carbohydrate and glycolipid structures (5). Second-generation CARs, which incorporate a single costimulatory signaling domain such as CD28 (6, 7), CD137 (4-1BB; refs. 6, 8), or CD134 (OX40; refs. 6, 8), have also been generated. T cells transduced with a CAR containing the CD28 signaling domain could enhance IL2 production (7), sustain T-cell proliferation (7), and resist immune suppression mediated by transforming growth factor-β (TGFβ) and regulatory T cells (Treg; ref. 9). The inclusion of domains derived from the tumor necrosis factor receptor family members 4-1BB and OX40 into CARs has also been shown to enhance the cytotoxicity of CAR-transduced T cells (8). Third-generation CARs, combining two costimulatory domains, such as CD28 and 4-1BB, have been described and are highly likely to lyse tumor cells (10). Adoptive transfer of CAR-transduced T cells has been clinically applied in the treatment of CD19-positive leukemia and lymphoma (11, 12) as well as GD2-positive neuroblastoma (13). These data provide a strong rationale for the pursuit of CAR T-cell therapy for other types of cancers, such as GBM. We and other groups have generated several CARs against the antigens expressed in GBM, including epidermal growth factor receptor variant III (EGFRvIII; ref. 14), human epidermal growth factor receptor 2 (HER2; ref. 15), IL13 receptor alpha 2 (IL13Rα2; ref. 16), and ephrin

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type A receptor 2 (EphA2; ref. 17). In addition, CAR-transduced T cells can migrate through the microvascular walls and penetrate tumors (14, 16). Clinical trials are ongoing using CAR T-cell therapy against GBM targeting EGFRvIII and HER2 (18, 19). Patient enrollment is completed for clinical trials targeting IL13Rα2 (20).

Podoplanin (PDPN) is a type I transmembrane mucin-like glycoprotein expressed in the lymphatic endothelium. It is overexpressed in several solid tumors, such as squamous cell carcinoma (21), malignant mesothelioma (22), Kaposi sarcoma, angiosarcoma (23), testicular seminoma (24), and brain tumors (25). PDPN expression is associated with malignant progression (25–27), epithelial–mesenchymal transition (27), and metastasis (26). In glial tumors, PDPN expression is positively correlated with tumor malignancy (25). PDPN is primarily expressed in the mesenchymal type of GBM, which presents the worst prognosis among GBM subtypes (28, 29). We previously produced a highly reactive mAb to PDPN, called NZ-1 (30), and its recombinant scFv (31).

In the current study, we generated a third generation of CAR that targeted PDPN, by using the NZ-1-based scFv. We report the generation of human CAR T cells that were specific and effective against PDPN-positive GBM cells *in vitro* and that, when infected systemically, inhibited the growth of intracranial glioma xenografts *in vivo*.

Materials and Methods

Cell lines

The human GBM cell lines LN319, U87MG, U251MG, T98, YKG-1, and SK-MG-1 were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2. U251MG, T98, and SK-MG-1 cells were purchased from American Type Culture Collection in 1995. YKG-1 and LN319 cells were gifts from Drs. H. Kanno (Yokohama City University, Yokohama, Japan) in 2005 and W. K. Cavenee (Ludwig Institute for Cancer Research, San Diego, CA) in 2002, respectively. The cell lines were authenticated by the letters when they were provided. The GBM-initiating cells GIC0222 were cultured in Neurobasal Medium (Life Technologies) supplemented with 2 mmol/L of L-glutamine (Sigma-Aldrich), N-2 and B-27 supplements (Life Technologies), recombinant human FGF basic and EGF (16.7 ng/mL each; R&D Systems), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2. Primary cultured GBM cells (pcGBM) were from the GBM tissue sample obtained from a patient undergoing surgery at the Nagoya University Hospital, Japan, after obtaining written informed consent. The study was approved by our institutional review board. The dissociation procedures have been described elsewhere (32). pcGBM cells were maintained in DMEM at 37°C in a humidified atmosphere of 5% CO_2.

PDPN knockout (KO) LN319 and U87 (PDIS-6 and PDIS-7, respectively) cells were established using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system. CRISPR/Cas plasmids, which target the sequence GACACTGAGACTACAGGTTTGG of human PDPN, were obtained from Sigma-Aldrich. The CRISPR/Cas plasmid was transfected into LN319 and U87MG cells using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories Inc.). Single-cell cloning was performed by limiting dilution in 96-well plates using 10% FBS/DMEM medium, and PDPN expression was assessed by flow cytometry using the NZ-1 mAb. The transfection-cloning cycles were repeated until the complete lack of NZ-1 reactivity was reached. The stable nature of the knockout cells was confirmed by the lack of NZ-1 reactivity after more than 10 passages.

PDPN expression in public databases

PDPN mRNA expression data from a microarray of normal tissues were obtained from a public database, BioGPS Dataset Library (http://biogps.org/dataset/; ref. 33). *PDPN* expression in GBM subtypes was obtained from the UCSC Cancer genome browser (https://genome-cancer.ucsc.edu/; ref. 34).

PDPN immunohistochemical staining

The 10% formalin-fixed, paraffin-embedded surgical samples from 79 patients newly diagnosed with GBM were collected for immunohistochemical analysis. Immunohistochemical staining was performed as previously described (35). An anti-PDPN mAb (1:5,000, clone NZ-1.2, rat IgG_{2a}) was used to detect PDPN. For each immunostained slide, the percentage of positively stained GBM cells on a given slide was evaluated by two pathologists (R. Watanabe and I. Ito). The tumors in which stained tumor cells made up more than 50% of the tumor were graded as positive.

PDPN immunofluorescent staining

GBM and GIC0222 cell lines were plated on a 24-well plate containing BD BioCoat poly-L-lysine cellware 12-mm round coverslips with 10% FBS/DMEM and incubated for 24 hours. The coverslips were rinsed twice with phosphate-buffered saline (PBS; Life Technologies) and placed in 4% paraformaldehyde phosphate buffer solution (PFA; Wako) for 15 minutes. Blocking was performed in PBS containing 0.1% Triton X-100 (Sigma-Aldrich; PBST) with 1.5% goat serum for 1 hour at room temperature with shaking. The coverslips were incubated with the primary antibody NZ-1 diluted to 1 μg/mL in blocking solution for 1 hour at room temperature with shaking followed by three washes with PBS. The coverslips were stained using a secondary antibody, Alexa Fluor 488 Goat Anti-Rat IgG (H+L; Life Technologies), and DAPI solution (Dojindo) at 1:200 dilution in the blocking solution for 30 minutes in the dark, followed by three washes with PBST. They were then mounted onto slides. Stained cells were observed under a FV1000 confocal laser scanning biological microscope (Olympus), and pictures were taken.

PDPN expression analysis by quantitative real-time RT-PCR

PDPN expression was examined using quantitative RT-PCR. Total RNA was prepared from GBM cells using an RNeasy Mini kit (Qiagen). The purity was confirmed with an A260/A280 ratio greater than 2.0. The first-strand complementary DNA (cDNA) was synthesized using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo). The sequences of the primers used to detect *GAPDH* mRNA and *PDPN* mRNA were as follows: *GAPDH* forward primer (5′-AGCCACATCGCTCAGACAC-3′), *GAPDH* reverse primer (5′-GCCCAATACGACCAAATCC-3′), *PDPN* forward primer (5′-AGAAGGAGCCAGCACAGG-3′), and *PDPN* reverse primer (5′-CGCCTTCCAAACCTGTAGTC-3′). RT-PCR was

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performed by using the LightCycler 480 instrument II (Roche Diagnostics GmbH) and THUNDERBIRD SYBR qPCR Mix (Toyobo). The reaction solution (20 μL) consisted of 2 μL of the template, 10 μL of THUNDERBIRD SYBR qPCR Mix (Toyobo), 1 μL of each primer (10 μmol/L), and 6 μL of distilled water. The PCR conditions used were denaturation for 5 minutes at 95°C, followed by 60 PCR cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. Respective expression levels of *PDPN* were normalized to that of *GAPDH* in each sample using the $\Delta\Delta C_T$ method.

Construction of self-inactivating (SIN) lentiviral vector

The mAb NZ-1 was previously established (30). Its scFv was then produced (31). The scFv portion in the pELNS-3C10-CAR (36) was changed to the NZ-1–based scFv to generate pELNS-NZ-1-CAR by gene synthesis (Genscript). In this construct, the EF1α promoter drives the CAR fusion protein containing the NZ-1–based scFv targeting PDPN, CD28, 4-1BB, and CD3ζ domains. The mock vector was designed to harbor scramble sequence of the scFv portion that have shown no functional activity against glioma, breast cancer, colon cancer, and pancreatic cancer cell lines.

Preparation of NZ-1-CAR T and 3C10-CAR T cells

NZ-1-CAR T and 3C10-CAR T (targeting EGFRvIII; ref. 36) cells were prepared by production and transduction of lentiviral vectors. HEK293T cells (8×10^6) were plated on 175-cm^2 flask at 37°C in a humidified atmosphere of 5% CO_2. At 24 hours, the SIN vector, pMDLg/pRRE, pRSV-Rev, and pMD2.G were cotransfected by using X-tremeGENE 9 DNA transfection Reagent (Roche Applied Science). The supernatant was collected at 48 hours, mixed with PEG-it Virus Precipitation Solution (5×; System Biosciences), and incubated for 24 hours at 4°C. The supernatant/PEG-it mixture was then centrifuged at 1,500 × *g* for 30 minutes at 4°C. The pellet was resuspended in 1 of 10 of the original volume using cold, sterile medium at 4°C and stored at −80°C.

Freshly harvested heparinized peripheral blood mononuclear cells (PBMC) from healthy donors were separated over a monolayer of Ficoll-Paque PLUS (1,000 × *g* for 20 minutes at 20°C; GE Healthcare Bio-sciences AB). PBMCs were cultured in 4 mL of AIM-V Medium (Life Technologies) with 2.5% human serum per well of a 6-well plate coated with the anti-human CD3 mAb (eBioscience) in the presence of IL2 (50 units/mL; PeproTech) at 37°C in a humidified atmosphere of 5% CO_2 for 24 hours. Next, the medium (3 mL/well) was gently removed without disturbing the clustering PBMCs, and the lentiviral pELNS-NZ-1-CAR vector supernatant (×10; 3 mL/well) was added followed by culturing of the cells for 24 hours. The medium was then replaced by fresh medium, and the cells were cultured for 48 hours.

Flow cytometric analysis of NZ-1-CAR expression

NZ-1-CAR expression on the cell surface of PBMCs was examined using a FACSCalibur equipped with the CellQuest Pro software (BD Biosciences). PBMCs were washed twice with PBS containing 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and 2 mmol/L EDTA (Dojindo). PBMCs were then incubated with biotin-AffiniPure $F(ab')_2$ fragment-specific goat anti-mouse IgG (Jackson Immuno Research Laboratories) at 4°C for 30 minutes. After washing twice, PBMCs were stained with streptavidin (SA)–phycoerythrin (PE; BD Biosciences) at 4°C for 30 minutes in the dark. After washing twice, PBMCs suspended in 1% PFA were analyzed by FACSCalibur. The data were analyzed using the WinMDI version 2.9 software (http://en.bio-soft.net/other/WinMDI.html).

Analysis of IFNγ production of effector cells

IFNγ production by effector cells was measured by ELISA using human IFNγ ELISA Ready-SET-Go! (eBioscience). Effector cells (1.0×10^6 NZ-1-CAR–transduced PBMCs or mock-transduced PBMCs) were cocultured with 1.0×10^4 target cells (LN319, U87MG, or T98) in each well of a 96-well plate for 24 hours. The cell culture supernatants were then harvested and used for ELISA. PDPN is highly expressed in LN319 cells, but not in T98 cells (30). Therefore, these cells were used as positive and negative controls, respectively.

Intracellular cytokine staining

Intracellular cytokine staining was performed using the Cytofix/Cytoperm plus GolgiStop kit (BD Biosciences). Effector cells (2×10^5 cells of NZ-1-CAR–transduced PBMCs or mock-transduced PBMCs) were incubated with 4×10^5 target cells (LN319 or LN319 PDPN KO) in 200 μL RPMI-1640 (Life Technologies) along with GolgiStop in a round-bottom, 96-well plate. Following a 4-hour incubation at 37°C, the cells were incubated with biotin-SP-AffiniPure $F(ab')_2$ fragment-specific goat anti-mouse IgG (Jackson Immuno Research Laboratories) at 4°C for 30 minutes. After washing, cells were stained with SA-PE, allophycocyanin (APC)-Cy7–conjugated mAb to human CD8, PerCP-Cy5.5–conjugated mAb to human CD4, and APC-conjugated mAb to human CD107a (BD Biosciences) at 4°C for 15 minutes in the dark. After permeabilization and fixation, the cells were stained intracellularly with V450-conjugated mAb to human IFNγ, PE-Cy7–conjugated antitumor necrosis factor (TNF) mAb, and fluorescein isothiocyanate (FITC)-conjugated mAb to human IL2 (BD Biosciences) at 4°C for 20 minutes and then washed with BD Perm/Wash solution. After washing, the labeled cells were suspended in 1% PFA and analyzed by FACSCanto II (BD Biosciences).

Cytotoxicity assay

Target cells (LN319, PDPN-KO LN319, U87MG, PDPN-KO U87MG, and pcGBM) were suspended at a final concentration of 1.0×10^6 cells/mL and incubated with 10 μmol/L (100 times) calcein-AM solution (Dojindo) at 37°C for 30 minutes with occasional shaking. After washing twice, the cells were adjusted to 1.0×10^5 cells/mL and 1.0×10^4 cells (100 μL) were placed into a well of a round-bottom, 96-well plate. Effector cells (NZ-1-CAR–transduced PBMCs, 3C10-CAR–transduced PBMCs, or mock-transduced PBMCs) were incubated for 48 hours. The cells were then harvested and added to each well at an appropriate effector: target ratio (50:1, 25:1, 12.5:1, 6:1, and 3:1) and incubated at 37°C for 5 hours. After centrifugation at 300 × *g* for 2 minutes, 75 μL of supernatant were aspirated carefully and loaded into a 96-well white/clear flat-bottom plate (BD Biosciences). The absorbance was then measured to evaluate the tumor-killing efficacy. Target cells in the medium with 3 μL of 10% sodium dodecyl sulfate (SDS; Wako) were used to determine the maximum release, and target cells alone were used to measure spontaneous release. The percentage of specific lysis was calculated as follows:

$$100\times(\text{experimental release}-\text{spontaneous release})/(\text{maximum release}-\text{spontaneous release})$$

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Intracranial glioma xenograft model

Immunofluorescence and RT-PCR analyses indicated that PDPN is highly expressed in LN319 cells. However, this cell line is not tumorigenic *in vivo* (37). Thus, we utilized LN319 cells as a positive control only in *in vitro* experiments. U87MG cells that are tumorigenic presented the second highest level of PDPN expression, whereas other cell lines such as GIC0222 cells grew slowly and expressed relatively little PDPN. Thus, U87MG cells were used in animal experiments. All animal experiments were approved by the Nagoya University ethics committee and performed according to our institutional animal care and use guidelines. Five- to 6-week-old NOD/Shi-scid, IL2Rr KO Jic (NOG) female mice (Central institute for Experimental Animals, Kawasaki, Japan) were used for the experiments. Mice were anesthetized intraperitoneally (i.p) with 25 mg/kg body weight (BW) pentobarbital sodium (somnopentyl, Kyoritsu). After fixing the head and incising the scalp, a burr hole was made using an 18-gauge needle in the right side of the skull, 2 mm lateral to the midline and 3 mm posterior to the lateral angle of the eye. Using a 26-gauge Hamilton syringe (Hamilton), 5.0×10^4 U87MG cells/mouse suspended in 2-μL PBS were stereotactically injected 3.5 mm below the dura matter through the burr hole by using a stereotactic apparatus. Seven days after tumor inoculation, NZ-1-CAR–transduced PBMCs or mock-transduced PBMCs (2×10^6) were suspended in 200 μL PBS and infused intravenously (i.v.) via the tail vein. The nontreated mice were infused with PBS alone. Survival was monitored following the tumor inoculation.

Tumor imaging

The growth of intracranial tumors was measured by 3T magnetic resonance imaging (MRI; MRS 3000; MR Solutions) every other week. Each mouse was placed on an animal holder and anesthetized with 1% to 2% isoflurane at a rate of 1.5 L/minute of air. During the examination, the respiratory rate was monitored using a respiratory sensor connected to a monitoring and gating system and kept at 80 to 100 breaths per minute to the extent that was possible. Each mouse received i.p. injection of 0.05 mmol/kg BW gadolinium-diethylenetriamine penta-acetic acid (Gd-DTPA; Magnevist, Bayer) for

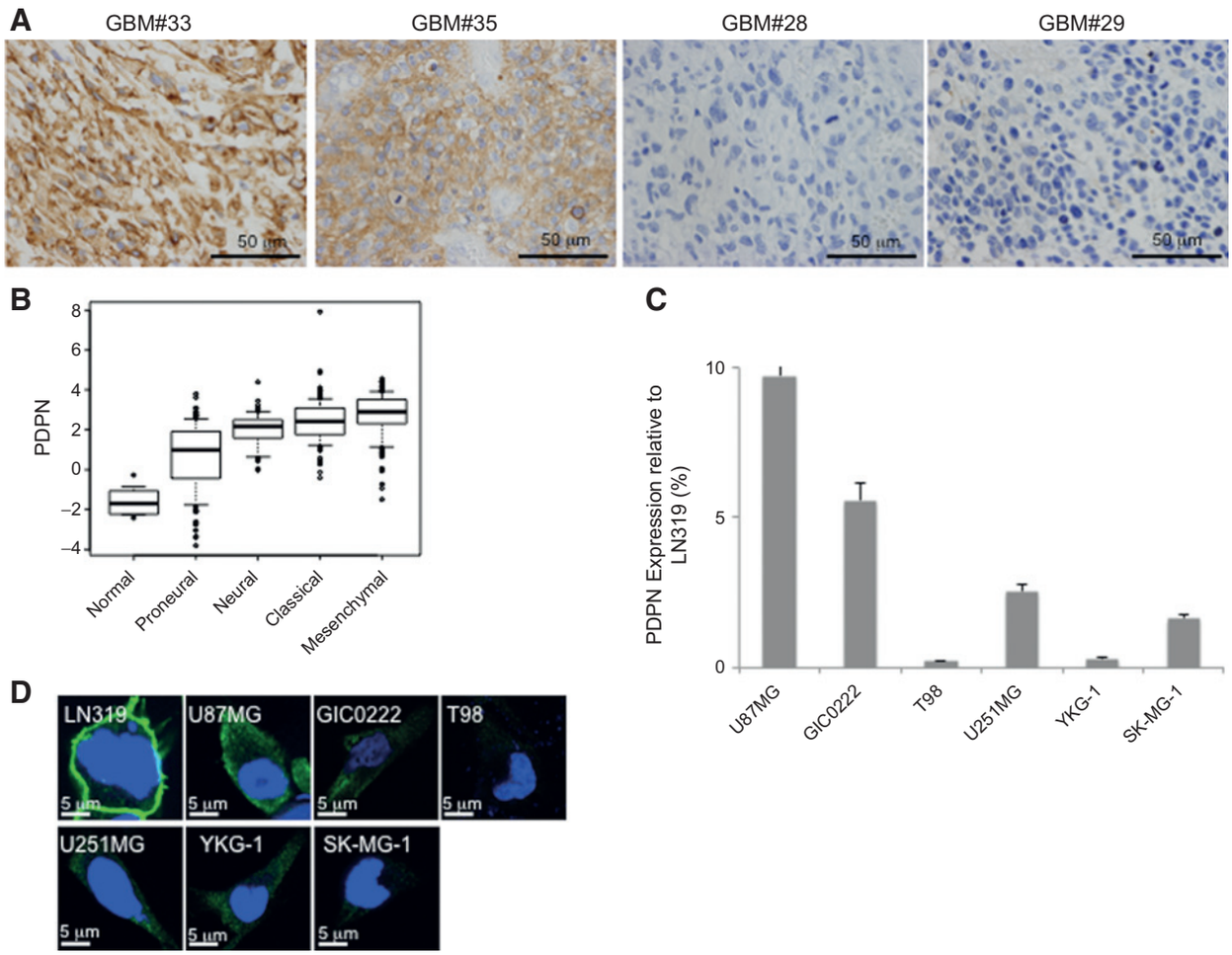

Figure 1.
The expression of PDPN in GBMs and cell lines. Out of 79 newly diagnosed GBM cases, 22 cases (27.8%) showed robust staining. A, 4 representative cases (2 positive and 2 negative) are shown. B, PDPN expression was the highest in GBM mesenchymal subtypes from UCSC Cancer Genome Browser (https://genome-cancer.ucsc.edu). C, PDPN expression in U87MG and GIC0222 was approximately 10% of that of LN319, which was used as a positive control for PDPN, as determined by quantitative RT-PCR. T98 was almost negative. D, immunofluorescence for PDPN (green) is consistent with the results of quantitative RT-PCR.

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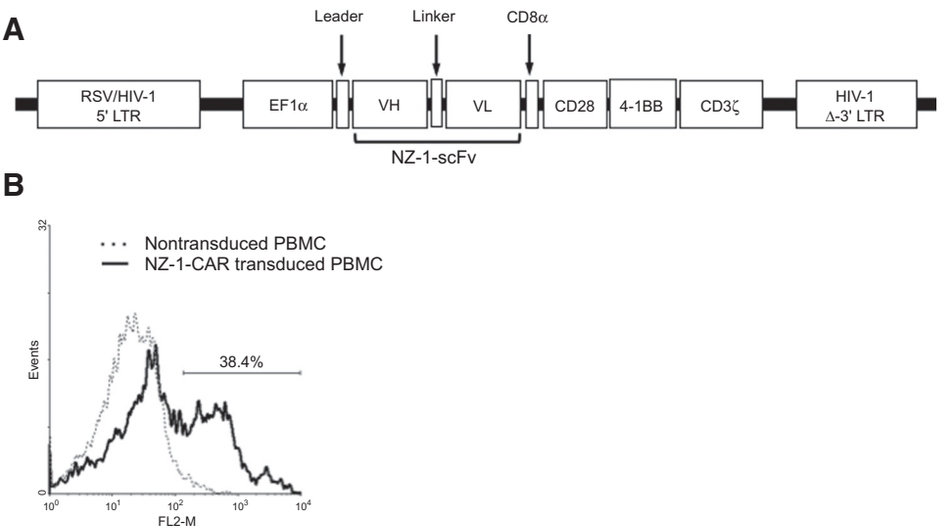

Figure 2.
Construction of NZ-1-CAR T cells. A, a lentiviral vector construct with the EF1α promoter followed by the leader sequence, NZ-1–based scFv, CD28, 4-1BB, and CD3ζ. The transduction efficiency was examined by flow cytometry with mouse-derived F(ab′)$_2$ recognizing biotin antibody. B, the efficiency was reproducibly 35% to 40%.

contrast-enhanced MRI. Tumor volume was estimated using the ABC/2 method as follows: An MRI slice with the largest area of tumor was identified in the contrast-enhanced MRI. The largest diameter (A) of the tumor on this slice was measured. Next, the width perpendicular to (A) on the same slice was measured (B). Finally, the approximate number of slices with tumor multiplied by the slice thickness (1 mm) was calculated (C). Then, A, B, and C were multiplied and the product divided by 2, which yielded the tumor volume in cubic millimeters.

Immunohistochemical staining *in vivo*

The U87MG-bearing mice treated with NZ-1-CAR or mock-transduced PBMCs were euthanized on day 12, 22, or 38 after the PBMC injection. Brain tissues were harvested and embedded in optimum cutting temperature (OCTTM) compound (Sakura Fine Technical) and frozen in liquid nitrogen. Six-micrometer-thick frozen sections were prepared with a cryostat (CM3050S, Leica). After drying, the sections were fixed with 4% formaldehyde. The sections were then blocked with 1.5% normal goat serum (Vector Laboratories) in PBS containing 0.05% Tween 20 at room temperature for 1 hour, and were stained with rabbit anti-human CD3 antibody (1:100, Thermo Lab Vision) diluted to 1 μg/mL. The secondary labeled polymer from EnVision HRP kit (Dako) was applied, and sections were incubated for 30 minutes. The substrate-chromogen solution from the EnVision HRP kit (Dako) was applied for 10 minutes. After washing, sections were counterstained with hematoxylin and mounted in Multi Mount (Matsunami Glass Ind).

Statistical analysis

The statistical significance of differences between two groups was determined using the Student *t* test. A two-tailed *P* value of <0.05 was considered statistically significant. In mouse experiments, survival curves were obtained by using the Kaplan–Meier method and compared by using the log-rank test.

Results

PDPN expression in human GBM specimens and cell lines

PDPN is expressed in lymphatic endothelial cells and basal cells in normal tissues (Supplementary Fig. S1A). The analysis of public databases indicates that PDPN expression is the highest in the placenta with a 4-fold difference with that in the brain (Supplementary Fig. S1B). However, PDPN is overexpressed in several solid tumors (21–25). In particular, PDPN expression in GBMs is 16-fold higher than that in the brain (Supplementary Fig. S1C). First, we evaluated PDPN expression in human GBMs by immunohistochemistry with the PDPN mAb, NZ-1.2. Out of 79 newly diagnosed GBM cases, 22 cases (27.8%) showed robust, but heterogeneous, staining. Four representative cases (2 positive and 2 negative) are presented (Fig. 1A). Although most GBM specimens were not positive for PDPN, specimens presenting the mesenchymal type of GBMs predominantly expressed PDPN (Fig. 1B). The mesenchymal type has been reported to present the worst prognostic among GBM subtypes (28, 29). Likewise, PDPN was not highly expressed in all human glioma cell lines used in this study. However, quantitative RT-PCR indicated that *PDPN* expression in U87MG and GIC0222 cells was approximately 10% of that in LN319 cells, which was used as a positive control (Fig. 1C). The immunofluorescence analysis using the PDPN mAb confirmed that PDPN was expressed on the cellular membrane of LN319 and U87MG cells, whereas T98 cells were almost negative for PDPN (Fig. 1D).

Construction of NZ-1-CAR T cells

We constructed a lentiviral vector tandem linked with the EF1α promoter followed by the leader sequence (so that the product was able to protrude extracellularly), and NZ-1–based scFv, CD28, 4-1BB, and CD3ζ (Fig. 2A, the third generation). The lentiviral vector was used to infect human PBMCs. The transduction efficiency was examined by flow cytometry with a mouse-derived F(ab′)$_2$–biotin antibody. The percentage of transduced PBMCs with NZ-1 CAR was 35% to 40% (Fig. 2B). These results were reproducible.

Functional assay of NZ-1-CAR T cells *in vitro*

The calcein-based nonradioisotope cytotoxic assay indicated that PDPN-positive LN319 cells were significantly lysed by NZ-1-CAR–transduced PBMCs in an effector:target (E:T) ratio–dependent manner. Cytotoxic effects of NZ-1-CAR–transduced PBMCs on PDPN-positive U87MG cells and primary cultured

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GBM cells (pcGBM) were not as drastic as that on LN319 cells, but a significant specific lysis was still observed. In contrast, specific lysis was not observed against PDPN-KO LN319 and PDPN-KO U87MG cells (Fig. 3A). EGFRvIII-targeting (3C10)-CAR PBMCs did not lyse LN319 cells, suggesting that cells were not lysed nonspecifically (Supplementary Fig. S2A). These results suggest that NZ-1-CAR T cells present PDPN-specific cytotoxicity.

Coculture of LN319 or U87MG cells with NZ-1-CAR–transduced PBMCs resulted in the production of approximately 350 to 400 pg/mL of IFNγ, whereas mock-transduced PBMCs released significantly less IFNγ (Fig. 3B). Thus, we successfully generated functional active NZ-1-CAR T cells that recognize PDPN. $CD4^+$, and $CD8^+$ NZ-1-CAR T cells may secrete different sets of cytokines. T-cell functionality (production of IFNγ, TNFα, IL2, and CD107a) was separately analyzed for $CD4^+$ and $CD8^+$ CAR T cells by FACS (Fig. 3C). IFNγ, IL2, and CD107a were predominantly produced by $CD4^+$ CAR T cells cocultured with LN319 and U87MG cells, whereas $CD8^+$ CAR T cells mainly produced TNFα. The sorted CAR T cells were subjected to RT-PCR (Supplementary Fig. S2B). TNFα was significantly produced in NZ-1-CAR T cells that were stimulated by LN319 cells ($P < 0.01$), but IL2 and CD107a were expressed even in mock-CAR T cells, which suggested that the secretion of IL2 and CD107a was not specific to the PDPN antigen. The mock-CAR T cells did not produce IFNγ, TNFα, IL2, or CD107a (Supplementary Fig. S3).

Distribution and antitumor effect of NZ-1-CAR T cells on human glioma in the mouse brain

After confirming the presence of intracranial tumors by MRI, mock-transduced PBMCs or NZ-1-CAR–transduced PBMCs were injected in the tail vein of mice. Brain tissues were harvested and embedded in OCT compound, and the sections were stained with rabbit antibody to human CD3 on days 12, 22, and 38 after injection (Fig. 4A). Whereas human $CD3^+$ cells were not found in the tumors obtained from the mock-treated mice on day 12, $CD3^+$ cells were still observed on day 38 in the

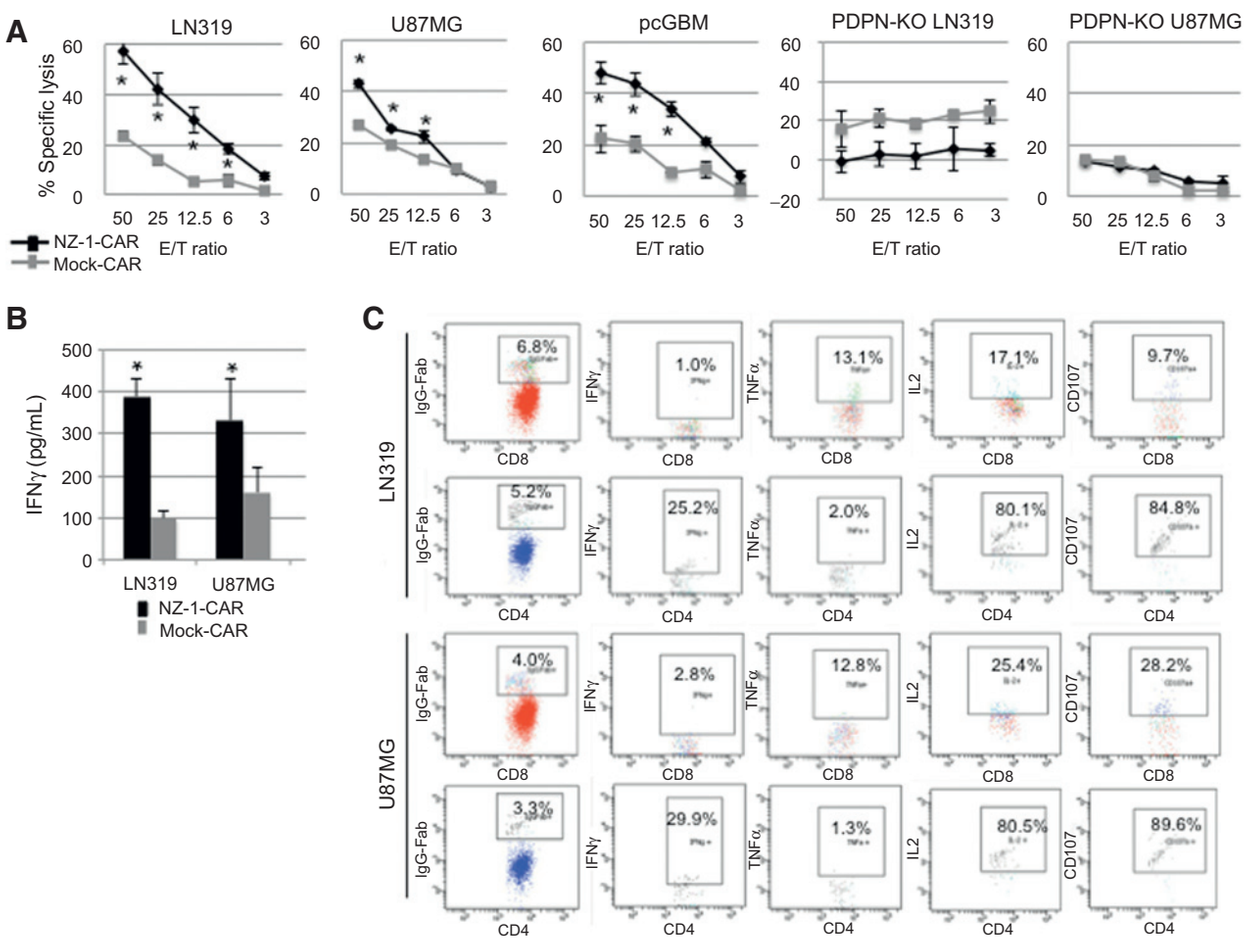

Figure 3.
Functional assay of NZ-1-CAR T cells *in vitro*. A, PDPN-positive glioma cells are lysed significantly by NZ-1-CAR–transduced PBMCs compared with mock-transduced PBMCs in an E:T ratio–dependent manner; however, specific lysis was not observed in PDPN-knockout glioma cells. *, $P < 0.05$. B, the coculture of LN319 or U87MG with NZ-1-CAR–transduced PBMCs produced approximately 350 to 400 pg/mL of IFNγ. The IFNγ levels were significantly higher than those from mock-transduced PBMCs. *, $P < 0.05$. Cytokine secretion profiles (IFNγ, TNFα, and IL2) and those of CD107a were separately analyzed for $CD4^+$ and $CD8^+$ CAR T cells by an intracellular cytokine assay using FACS. C, IFNγ, IL2, and CD107a were secreted predominantly by $CD4^+$ CAR T cells cocultured with LN319 and U87MG cells, whereas $CD8^+$ CAR T cells mainly produced TNFα.

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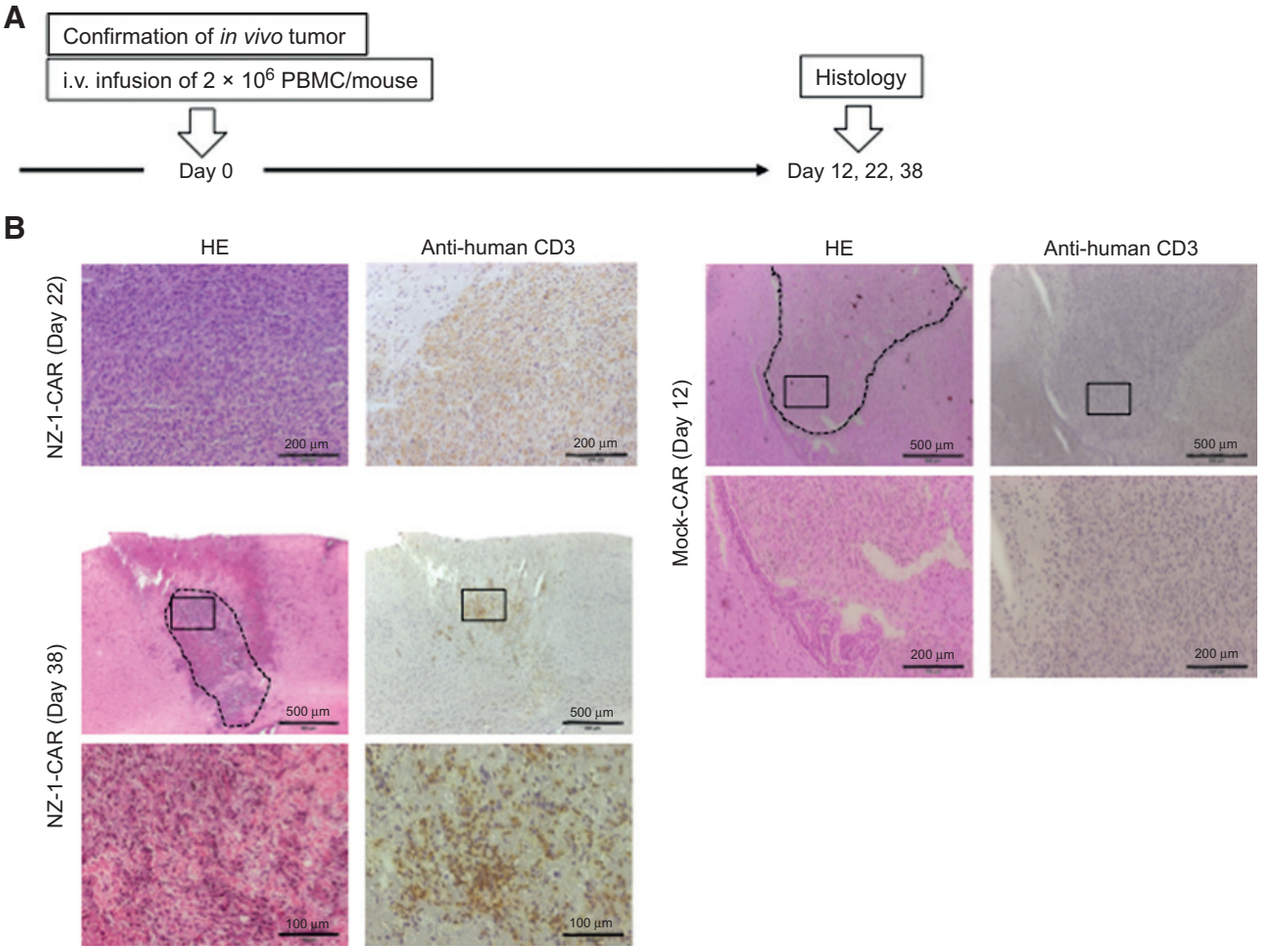

Figure 4.
Distribution of NZ-1-CAR T cells on human glioma in mouse brain. A, after the presence of intracranial tumors was confirmed by MRI, mock-transduced PBMCs, or NZ-1-CAR–transduced PBMCs were injected in the tail vein of mice. Brain tissues were harvested and embedded in an OCT compound, and the sections were stained with rabbit anti-human CD3 antibody on days 12, 22, and 38 after injection. B, while human $CD3^+$ cells were not found in the tumors obtained from the mock-treated mice on day 12, $CD3^+$ cells were still observed on day 38 in the tumors of NZ-1-CAR–treated mice. Dotted lines indicate the tumors. The higher magnification of images in the rectangular insets is also shown below.

tumors of NZ-1-CAR–treated mice (Fig. 4B). Thus, NZ-1-CAR T cells spread to the brain tumor and persisted for at least 38 days after injection.

The treatment design is summarized in Fig. 5A. On day 7 after intracranial implantation of tumor cells, PBS, mock-transduced PBMCs, or NZ-1-CAR–transduced PBMCs were injected in the tail vein of the mice. The volume of gadolinium-enhanced tumors was evaluated sequentially. In approximately 60% of the mice treated with NZ-1-CAR PBMCs, the tumor grew markedly more slowly than that in the other two groups (Fig. 5B and C). Median OS durations of the three groups were 59, 56.5, and 79 days, respectively. In addition, a log-rank test indicated that mice from the NZ-1-CAR group survived significantly longer (Fig. 5D). The survival curve of approximately 40% of the NZ-1-CAR mice overlapped with those of the two control groups. We repeated the animal experiment twice, and the results were reproducible. The tumor growth and effective CAR T-cell proliferation may be almost equivalent; thus, the adoptive transferred CAR T cells started to elicit an effect around 60 days. We speculate that a certain amount of time is required for effective T-cell proliferation, so that the number of cells is sufficient to eradicate an intracranial tumor through the blood–brain barrier (BBB).

Discussion

In this study, we report the construction of a third-generation CAR that targets PDPN and its successful lentivirus-mediated expression on human T cells. We showed that the generated T cells were specific and effective against PDPN-positive GBM cells *in vitro,* and systemic injection of the T cells significantly increased survival time *in vivo* ($P = 0.035$).

PDPN is especially expressed in the mesenchymal type of GBM, which presents the worst prognosis among GBM subtypes (Fig. 1B; refs. 28, 29). This study and previous reports showed that the expression of PDPN in GBM was observed in 27.8% (this study), 47% (25), and 31% of patients with GBM (28). Considering that PDPN is one of the factors associated with poor prognosis, PDPN-targeted CAR T-cell therapy would be useful in

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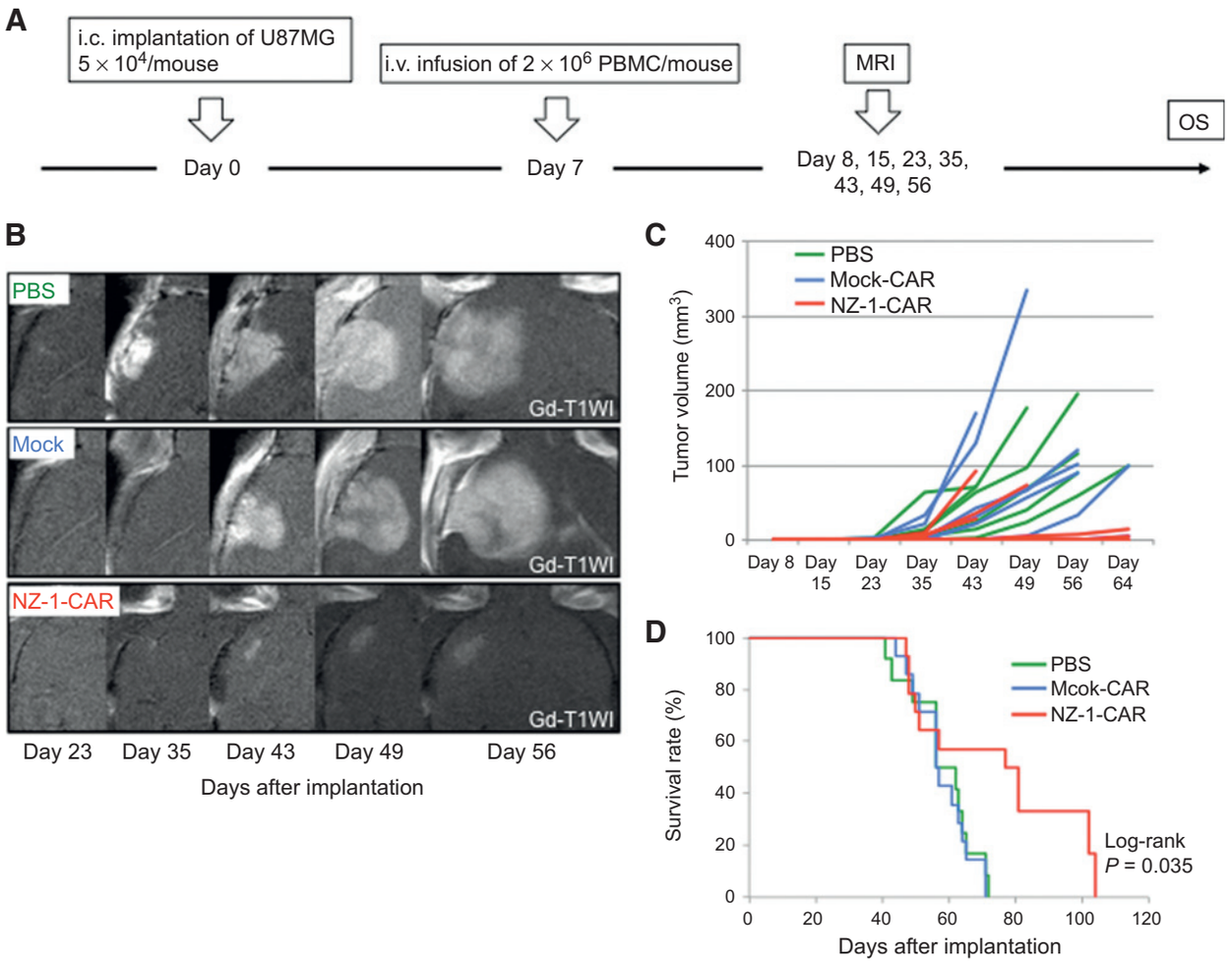

Figure 5.
Antitumor effect of NZ-1-CAR T cells on human glioma in mouse brain. A, the experimental design. B and C, in approximately 60% of the mice treated with NZ-1-CAR–transduced PBMCs, the tumor grew markedly more slowly than those from the other two groups. D, median OS durations of the three groups were 59, 56.5, and 79 days, respectively. $P < 0.05$ by a log-rank test i.c., intracranial.

the treatment of patients with relapsed/resistant tumors following first-line chemotherapy.

Recent studies focused on immune checkpoint targeting to cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1). CTLA-4 is expressed on T cells in which it regulates the amplitude of early stages of T-cell activation by counteracting the activity of the T-cell costimulatory receptor CD28 (38). Moreover, it enhances Treg immunosuppressive activity (39). In contrast, PD-1 limits the activity of T cells in peripheral tissues to avoid autoimmunity (38). The same mechanism is observed in tumor immune evasion (40). The major PD-1 ligand, PD-1 ligand 1 (PD-L1; known as B7-H1 and CD274), is overexpressed in various solid tumors, including malignant melanoma, ovarian cancer, lung cancer, and GBM (41). PD-L1 signaling inhibits T-cell proliferation and IFNγ secretion (42). One of the fully humanized antibodies to CTLA-4, ipilimumab, was clinically tested (43). John and colleagues demonstrated for the first time that the administration of an antibody to PD-1 can significantly enhance the therapeutic efficacy of CAR T cells (44). Combination of NZ-1-CAR T cells and ipilimumab (anti–CTLA-4) or nivolumab (anti–PD-1) would be another potential strategy. Such strategies may lead to an increase in effector T-cell proliferation *in vivo*, so that there are enough cells to penetrate the BBB.

One of the concerns with PDPN-targeted CAR therapy is that PDPN is expressed in normal tissues, including the lymphatic endothelium, lung type I alveolar cells, kidney glomerular podocytes, and mesothelium (21, 23). In the central nervous system, PDPN is expressed in the choroid plexus, ependyma, meninges (21), and Purkinje cells (45). We established a cancer-specific mAb (CasMab) to human PDPN (46). The newly established mAb clone LpMab-2 recognizes the cancer-type PDPN, which is aberrantly glycosylated, and was purified from a human PDPN-transfected GBM cell line. LpMab-2 can react with PDPN-expressing cancer cells, but not with normal cells such as lymphatic cells and type I alveolar cells. It may be useful to produce a new CAR using LpMab-2 for CAR therapy targeting PDPN.

Although there may be a number of issues to be addressed for clinical application, such as off-targets, slow responses, and short effectiveness, overall we successfully established CAR T cells

against a promising tumor antigen, PDPN, and provide new insights toward therapies targeting solid tumors that have failed other treatments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Wakabayashi, A. Natsume
Development of methodology: M. Ohno, T. Wakabayashi, Y. Kato, A. Natsume
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Shiina, M. Ohno, F. Ohka, A. Kato, K. Motomura, T. Yamamot, M. Hamaguchi, T. Wakabayashi, D.D. Bigner
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Ohka, A. Yamamichi, K. Motomura, T. Wakabayashi, M. Ohno, A. Natsume
Writing, review, and/or revision of the manuscript: M. Ohno, F. Ohka, T. Senga, D.D. Bigner, A. Natsume
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Ohno, A. Yamamichi, A. Kato, K. Tanahashi, T. Wakabayashi, M.K. Kaneko, D.D. Bigner
Study supervision: M. Ohno, T. Wakabayashi, A. Natsume
Other (contributed to histological and morphological evaluation): R. Watanabe
Other (contributed to microscopical or morphological appraisal): I. Ito
Other (provided the scFv targeting the podoplanin antigen): V. Chandramohan

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Cancer Immunology Research

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