

ORIGINAL RESEARCH



Human allogenic $\gamma\delta$ T cells kill patient-derived glioblastoma cells expressing high levels of DNAM-1 ligands

Haeyoun Choi^a, Yunkyung Lee^b, Soon A Park^c, Ji Hyeon Lee^c, Junseong Park^d, Jang Hyun Park^e, Heung Kyu Lee^e, Tai-Gyu Kim^{a,b}, Sin-Soo Jeun^f, and Stephen Ahn^b

^aDepartment of Microbiology, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea; ^bCatholic Hematopoietic Stem Cell Bank, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea; ^cDepartment of Biomedicine and Health Sciences, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea; ^dPrecision Medicine Research Center, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea; ^eGraduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Republic of Korea; ^fDepartment of Neurosurgery, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea

ABSTRACT

Adoptive transfer of $\gamma\delta$ T cells is a novel immunotherapeutic approach to glioblastoma. Few recent studies have shown the efficacy of $\gamma\delta$ T cells against glioblastoma, but no previous studies have identified the ligand–receptor interactions between $\gamma\delta$ T cells and glioblastoma cells. Here, we identify those ligand–receptor interactions and provide a basis for using $\gamma\delta$ T cells to treat glioblastoma. V γ 9V δ 2 T cells were generated from peripheral blood mononuclear cells of healthy donors using artificial antigen presenting cells. MICA, ULBP, PVR and Nectin-2 expression in 10 patient-derived glioblastoma (PDG) cells were analyzed. The in vitro cytokine secretion from the $\gamma\delta$ T cells and their cytotoxicity toward the PDG cells were also analyzed. The in vivo anti-tumor effects were evaluated using a U87 orthotopic xenograft glioblastoma model. Expression of ligands and cytotoxicity of the $\gamma\delta$ T cells varied among the PDG cells. IFN- γ and Granzyme B secretion levels were significantly higher when $\gamma\delta$ T cells were co-cultured with high-susceptible PDG cells than when they were co-cultured with low-susceptible PDG cells. Cytotoxicity correlated significantly with the expression levels of DNAM-1 ligands of the PDG cells. Blocking DNAM-1 resulted in a decrease in $\gamma\delta$ T cell-mediated cytotoxicity and cytokine secretion. Intratumoral injection of $\gamma\delta$ T cells showed anti-tumor effects in an orthotopic mouse model. Allogenic $\gamma\delta$ T cells showed potent anti-tumor effects on glioblastoma in a DNAM-1 axis dependent manner. Our findings will facilitate the development of clinical strategies using $\gamma\delta$ T cells for glioblastoma treatment.

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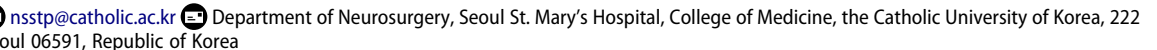
Introduction


Glioblastoma is the most common primary brain malignancy and has the most lethal prognosis; the median overall survival is still less than 2 years, and 5-year survival rates are lower than 10% despite aggressive multimodal standard treatments that include maximal surgical resection and chemoradiation followed by 6 cycles of chemotherapy.^{1,2} In the past two decades, the survival outcomes of several cancers, such as melanoma, non-small cell lung cancer, and leukemias, have improved dramatically as the result of targeted therapy and immunotherapy.^{3–5} However, the prognosis of glioblastoma has not improved much.^{6,7} Recent phase III clinical trials using an immune checkpoint inhibitor (ICI) for recurrent glioblastoma failed to improve survival outcomes.⁷ As an alternative approach, adoptive transfer of immune cells, such as T cells, natural killer (NK) cells, or $\gamma\delta$ T cells, with or without genetic engineering has recently been tried on ICI-resistant cancers, including glioblastoma.^{8–10}

Human $\gamma\delta$ T cells are a small subset of T cells and have been relatively unstudied compared with conventional $\alpha\beta$ T cells.^{11,12} Their immune surveillance function and ability to recognize transformed cells in a major histocompatibility complex-

unrestricted manner has been considered an advantage to their use in anti-tumor therapy.¹³ Along with the $\gamma\delta$ T cell receptor, various NK receptors are also involved in regulating $\gamma\delta$ T cell activation and its anti-tumor function.^{14–16} For example, NKG2D, which binds to MICA, MICB, and ULBP is known to activate $\gamma\delta$ T cells, along with DNAM-1, which binds to poliovirus receptor (PVR, CD155) and Nectin-2.^{14,16} In human peripheral blood, V γ 9V δ 2 T cells, which constitute 1–5% of peripheral blood lymphocytes,¹⁵ are the main subtype of $\gamma\delta$ T cells. V γ 9V δ 2 T cells can be expanded easily using phosphoantigens or artificial antigen presenting cells (aAPCs).^{17,18} Therefore, adoptive transfer of $\gamma\delta$ T cells has been suggested as a new immunotherapeutic approach for various cancers.¹⁹ A few recent studies have suggested that activated human V γ 9V δ 2 T cells can kill glioma and glioblastoma cells in vitro and in vivo.^{20–24} However, among the different ligand-receptor interactions, which ligand-receptor interaction plays an important role in the cytotoxicity of $\gamma\delta$ T cells has not yet been defined.

In this study, we demonstrate that allogenic human $\gamma\delta$ T cells activated by aAPCs can kill patient-derived glioblastoma (PDG) cells in vitro and in vivo. Interestingly, we found

CONTACT Stephen Ahn  nsstp@catholic.ac.kr 

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that the ligand expression of glioblastoma cells varied among patients and that among them the expression of DNAM-1 ligands could be significantly related to the anti-tumor effects of $\gamma\delta$ T cells in glioblastoma. The potent efficacy of $\gamma\delta$ T cells against PDG cells shown here and our identification of specific targets on glioblastoma could facilitate the development of clinical $\gamma\delta$ T cell immunotherapy for glioblastoma treatment.

Materials and methods

Generation of $\gamma\delta$ T cells

$\gamma\delta$ T cells were cultured as previously described.¹⁷ Briefly, peripheral blood mononuclear cells from five healthy donors were stimulated with 3 μ M zoledronic acid (Daewoong Pharmaceutical, Korea) and 1000 U/mL of IL-2 (Proleukin, Clinigen, United Kingdom). After 7 days, the cells were treated weekly with irradiated (100 Gy) aAPCs in a T cell: aAPC ratio of 2:1 in the presence of 1000 U/mL of IL-2. The cells were cultured until day 14 or 21 and then frozen using a controlled-rate freezer (Thermo Fisher Scientific, MA) and cryopreserved in liquid nitrogen until further use. For the *in vitro* and *in vivo* assays, the frozen $\gamma\delta$ T cells were thawed and rested overnight in the presence of IL-2 (1000 U/mL). The detailed information of generation of our $\gamma\delta$ T cells were presented in a previous study.¹⁷

Cells

All tissues were obtained from Seoul St. Mary's Hospital under the guidance of our institutional review boards. After freshly resected samples were obtained from glioblastoma patients, the tumor tissue was chopped and suspended in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, MA) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Thermo Fisher Scientific, MA). All cells were maintained at 37°C in humidified air with 5% CO₂. The clinical characteristics of the PDG cells are provided in the supplementary table.

Glioma cell lines (T98G, U87, U138, and U373) were purchased from the American Type Culture Collection and cultured in high-glucose DMEM (Thermo Fisher Scientific, MA) supplemented with 10% FBS (Thermo Fisher Scientific, MA) and 10,000 μ g/ml of penicillin and streptomycin (Thermo Fisher Scientific, MA). For luciferase expression in the mouse model, U87-luciferase cells were transduced using a lentiviral vector containing a luciferase gene.

Flow cytometry analysis

The expression of ligands on tumor cells was analyzed with flow cytometry. PDG cells were harvested when they reached optimal cell density with 0.25% trypsin-EDTA and PBS containing 2% FBS was used as a staining buffer. After staining with antibodies for 30 minutes at 4°C, cells were washed. Data acquisition was performed on BD FACS Canto (Becton Dickinson BD Biosciences, San Jose, CA, USA), and FlowJo software (v10.0.7, FlowJo LLC, Ashland, OR, USA) was used to analyze the data. The following antibodies were used for flow

cytometry: human MICA PE-conjugated antibody (clone 159227) and human ULBP-2/5/6 APC-conjugated antibody (clone 165903), which were purchased from R&D Systems; and APC/Fire 750 anti-human CD155 (PVR) antibody (clone SKII.4) and APC anti-human CD112 (Nectin-2) antibody (clone TX31), which were purchased from BioLegend. Isotype controls for each antibody were used according to each manufacturer's instructions.

Cytotoxicity and cytokine secretion

Cytotoxicity assay was performed using Calcein-AM (Invitrogen, MA). Target cells were stained with 5 μ M of Calcein-AM and incubated for 20 minutes at 37°C with 5% CO₂ in the dark. After incubation, the target cells were washed twice with phosphate-buffered saline (PBS) and suspended in DMEM with 20% FBS at 1×10^5 cells/mL. Effector cells and target cells were co-cultured in a 96-well V-bottomed plate at an E:T ratio of 20:1. For maximum release, Triton X-100 was added to a final concentration of 2%. For spontaneous fluorescence, only target cells were incubated. After 4 hours of incubation, the plates were centrifuged (2500rpm, 5 min), and the supernatants were carefully transferred to an opaque black 96-well plate. The fluorescence was measured with a microplate reader (Synergy H1, Biotek, VT). Specific release was calculated as (Experimental fluorescence) – (Spontaneous fluorescence)/(Maximum fluorescence) – (Spontaneous fluorescence) $\times 100$. For the cytokine secretion analysis, 1×10^5 $\gamma\delta$ T cells and 5×10^4 target cells were co-cultured for 24 hours, and then the supernatants were analyzed with a LEGENDplex Human CD8/NK panel (Biolegend, CA) according to the manufacturer's protocol. Cytokine concentration was calculated as (cytokine secretion from $\gamma\delta$ T cells co-cultured with tumor cell lines) – (cytokine secretion from tumor cells alone). For blocking assays, $\gamma\delta$ T cells were treated with mouse IgG1 κ isotype control antibody (clone MOPC-21), anti-human CD226 (DNAM-1) antibody (clone 11A8), or anti-human CD314 (NKG2D) antibody (clone 1D11) at the concentration of 2.5 μ g/mL for 15 min at room temperature before adding the target cells. For blocking PVR and Nectin-2, anti-human CD155 (PVR) antibody (clone SKII.4) and anti-human CD112 (Nectin-2) antibody (clone TX31) was used at the concentration of 20 μ g/mL.

3D spheroid generation and viability assay

3D spheroids of the U87 cell line and PDG cells were generated by seeding $5 \times 10^3/50$ μ l of cells to a low-adherent 96-well plate. On day 4, 2×10^6 $\gamma\delta$ T cells or culture medium was added to a final volume of 100 μ l. After 24 hours of co-culture, the viability of the 3D spheroids was analyzed using CellTiter-Glo (Promega, Madison, WI) following the manufacturer's protocol. Briefly, 100 μ l of CellTiter-Glo reagent was added to the wells after acclimating the spheroid and reagent to room temperature for 30 minutes. Wells were thoroughly mixed to lyse viable cells and luminescence was measured with Synergy H1 microplate reader (BioTek, VT). The luminescence of the 3D spheroids treated with $\gamma\delta$ T cells was calculated as (luminescence of 3D spheroids with $\gamma\delta$ T cells) – (luminescence of $\gamma\delta$ T cells alone).

Animals and adoptive $\gamma\delta$ T cell transfer

All procedures for the mouse experiments and determination of endpoints were carried out in accordance with guidelines approved by the animal institutional review boards of our institution. For the orthotopic intracranial glioblastoma-bearing mice, 7.5×10^4 U87 cells were stereotactically inoculated with 3 μ l of PBS into the right frontal lobes of NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{ct. m1Wjl}/SzJ, Jackson Laboratory) 2 mm lateral and 1 mm anterior to the bregma at 2.5 mm depth from the skull base using a Hamilton syringe (Hamilton Company) and a micro-infusion pump (Harvard Apparatus). After tumor implantation, tumor size was monitored in vivo by luciferase bioluminescence imaging using an IVIS Lumina XRMS (PerkinElmer), and the mice were randomly allocated into treatment arms. In the early model, 2×10^6 human $\gamma\delta$ T cells were injected on day 4 intracranially. In the advanced model, 2×10^6 human $\gamma\delta$ T cells were injected on day 7 and day 14 intracranially. In the control group, same volume of PBS was injected at the same site. We chose intracranial injection over intravenous injection according to previous studies showing the superior anti-tumor effects of intracranial injection of T cells in a glioblastoma orthotopic mouse model.^{10,25} Tumor size and survival were monitored every week. They were euthanized when they showed predetermined signs of neurologic deficits (failure to ambulate, weight loss >20% body mass, lethargy, hunched posture).

Immunofluorescence

To verify tumor size using a histologic analysis, the mice were sacrificed on day 10 after tumor implantation. The mouse brains were perfused with 4% paraformaldehyde under deep anesthesia. Then, it was fixed within 100% methanol (chilled to -20°C) at room temperature for 5 min. Fixed tissues were cryo-sectioned (14- μ m sections) and stained with hematoxylin and eosin without antigen retrieval steps. For immunofluorescence staining, we used the following antibodies: for $\gamma\delta$ TCR (clone 5A6.E9, Thermo Fisher Scientific, MA), for PVR (clone D8A5G, Cell Signaling Technology, MA), Nectin-2 (clone AF2229, R&D Systems, MN), Alexa 488-conjugated goat anti-rat IgG (A-11006, Thermo Fisher Scientific, MA), Alexa 546-conjugated goat anti-mouse IgG (A-11010, Thermo Fisher Scientific, MA), and Alexa 546-conjugated goat anti-rabbit IgG (A-11057, Thermo Fisher Scientific, MA). Nuclei were counterstained with 40, 6-diamidino-2-phenylindole (DAPI; Sigma). Fluorescence images were acquired using an LSM900 confocal microscope (Carl Zeiss, Oberkochen, Germany).

RNA sequencing

All PDG cells were thawed and washed with PBS. Messenger RNA sequencing analysis was performed with 1×10^6 viable cells from Macrogen, Inc (Seoul, South Korea). RNA sequencing was performed using the TruSeq stranded RNA protocol on Illumina. Reads were trimmed based on the Fastp package and quality was checked by FASTQC. Trimmed reads were aligned on a Homo sapiens reference sequence (GRCh38) using HISAT2. Read counts were normalized and analyzed by StringTie. Differentially

expressed genes were defined by DESeq2 in R v 4.1.3. Normalized data were visualized using the plotPCA function.

Statistical analysis and software

The statistical significance of differences in cytotoxicity and cytokine secretion was assessed by paired t-testing. Correlations between ligand expression levels and cytotoxicity were analyzed by Pearson's correlation. The survival curve was calculated with the Kaplan-Meier equation, and the statistical significance of survival differences between groups was assessed by the log-rank test. All statistical analyses were performed and graphs produced using the R version 1.4.3 software program (R Foundation for Statistical Computing, Vienna, Austria) and Prism Software (GraphPad 9.0).

Results

Different susceptibility of patient-derived glioblastoma cells to $\gamma\delta$ T cells

To identify whether human glioma cell lines express ligands that bind to the activating receptors of $\gamma\delta$ T cells, we analyzed MICA, ULBP, PVR and Nectin-2 expression (Supplementary Figure 1). MICA was rarely expressed in the U87, U138, and U373 cell lines (0.8%, 1.51%, and 0.91% respectively). ULBP was highly expressed in U138 and U373 cells (91.4% and 98.6%, respectively), but ULBP expression in U87 cells was relatively low (32.8%). PVR and Nectin-2 were highly expressed in all those cell lines (94.5% and 89.0% for U87, 99.2% and 70.8% for U138, and 65.7% and 94.8% for U373, respectively) (Figure 1a). We next evaluated the cytotoxic effects of $\gamma\delta$ T cells toward the human glioma cell lines. U87 had the highest susceptibility to $\gamma\delta$ T cell-mediated cytotoxicity, with $60.2\% \pm 15.0$ (average \pm SD) of specific lysis, followed by U138 and U373 at $43.8\% \pm 6.9$ and $33.2\% \pm 5.0$, respectively (Figure 1b). $\gamma\delta$ T cells also showed cytotoxic effects on the U87 3D spheroid models (Supplementary Figure 2A, B).

Ligand expression levels were also analyzed in the 10 PDG cells. MICA was rarely expressed in the PDG cells (range 0.08 to 1.04%). The expression levels of ULBP, PVR, and Nectin-2 varied among the PDG cells. The mean expression level across all PDG cells of ULBP was 14.10% (range, 0.63% to 68.2%), that of PVR was 72.4% (range, 8.45% to 99.8%), and that of Nectin-2 was 40.6% (range, 7.66% to 66.5%) (Figure 1c and d). The cytotoxicity of $\gamma\delta$ T cells also varied among the PDG cells (Figure 1e). The highest tumor cell lysis ($76.9\% \pm 11.3$) was in PDG#6, and the lowest lysis ($9.6\% \pm 8.1$) was in PDG#8. $\gamma\delta$ T cells were also cytotoxic to the PDG#4- and PDG#6-derived 3D spheroids (Supplementary Figure 2C). Therefore, human allogenic $\gamma\delta$ T cells can kill human glioma cells and PDG cells, but the ligand expression levels and susceptibility to $\gamma\delta$ T cell cytotoxicity vary by cell line.

Cytokine secretion from $\gamma\delta$ T cells according to the susceptibility of PDG cells

Next, we sought to investigate whether cytokine secretion patterns from $\gamma\delta$ T cells varied upon co-culture with different PDG cells. The PDG cells were classified into two groups

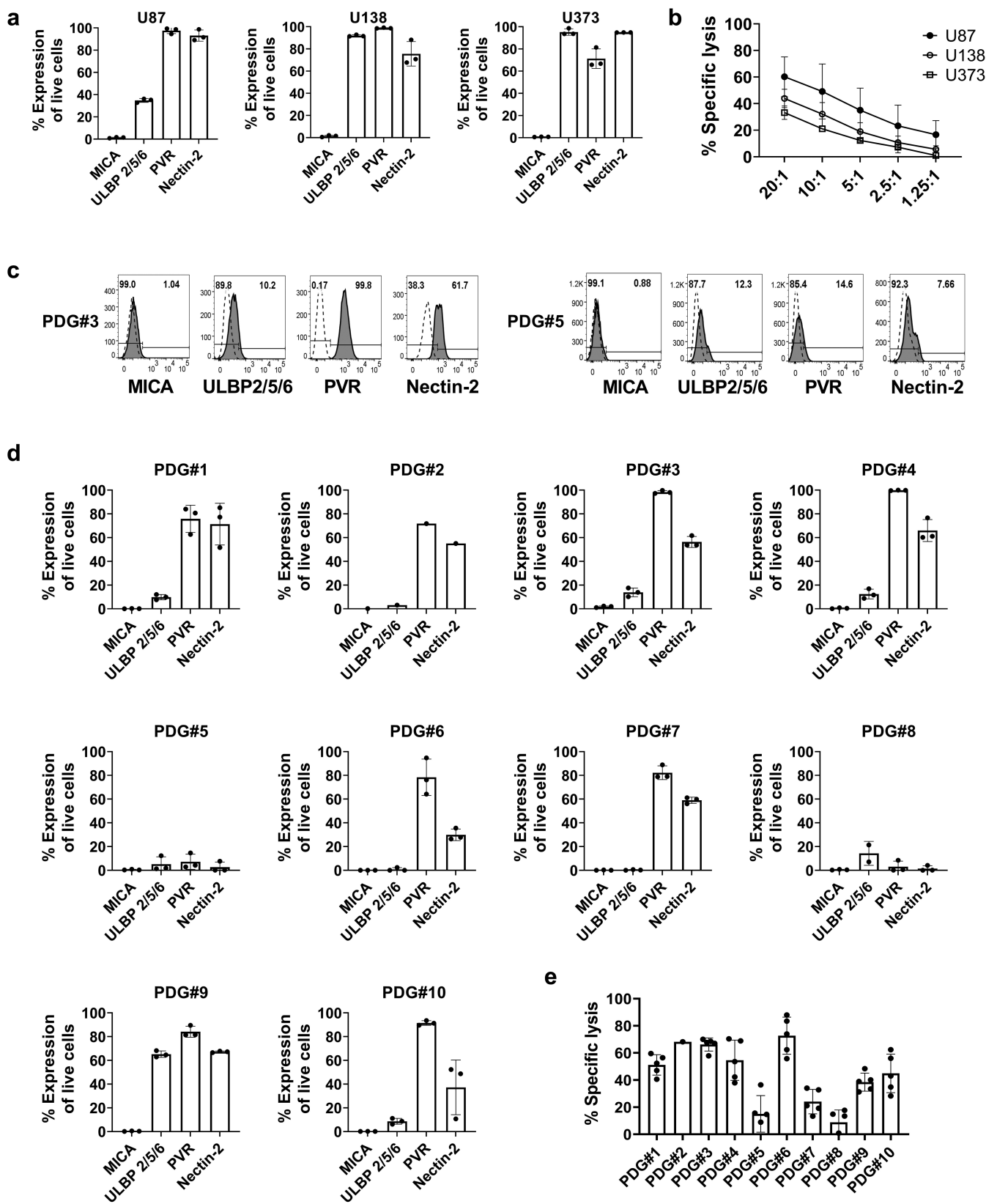


Figure 1. Expression of various ligands on glioblastoma tumor cell lines and $\gamma\delta$ T cell-mediated cytotoxicity. (a) The expression of MICA, ULBP 2/5/6, PVR, and Nectin-2 on the glioblastoma cell lines U87, U373, and U138 was analyzed three times by flow cytometry. (b) The cytotoxic effect of $\gamma\delta$ T cells toward glioblastoma cell lines were analyzed. Expanded $\gamma\delta$ T cells were co-cultured with Calcein-AM-labeled tumor cell lines for 4 hours at indicated E:T ratio. The % specific lysis was calculated as [(Experimental Release-Spontaneous Release)/(Maximum Release-Spontaneous Release)] \times 100. Experiments were performed in triplicate with $\gamma\delta$ T cells derived from five different healthy donors. (c, d) PDG cells were cultured and the expression of MICA, ULBP 2/5/6, PVR and Nectin-2 was analyzed by flow cytometry. Experiments were performed three times independently. Representative flow cytometry data of the expression of ligands on PDG#3 and PDG#5 are shown. (e) Cytotoxicity of $\gamma\delta$ T cells toward PDG cells was analyzed by Calcein-AM assay in duplicate, using $\gamma\delta$ T cells from five healthy donors at the ratio of E:T=20:1. Dots represent data from each $\gamma\delta$ T cell. Bars and error bars indicate mean and SD.

according to their susceptibility to $\gamma\delta$ T cells; PDG cells with specific lysis above 50% were classified as the high-susceptibility group, and those with specific lysis lower than 50% were considered the low-susceptibility group. Then, two PDG cells were selected from each group, and $\gamma\delta$ T cells from five different healthy donors were co-cultured with those tumor cells. As expected, the secretion levels of cytokines showed different patterns according to the susceptibility of the PDG cells (Figure 2). Specifically, the secretion levels of IFN- γ and granzyme B were significantly higher in the high-susceptibility group than the low-susceptibility group.

Expression of DNAM-1 ligands on PDG cells correlates with susceptibility to $\gamma\delta$ T cells

Because the PDG cells showed varying ligand expression levels and susceptibility to $\gamma\delta$ T cell-mediated cytotoxicity, we analyzed the correlations between the ligand expression levels and $\gamma\delta$ T cell-mediated cytotoxicity (Figure 3). Ligands which were investigated were MICA, ULBP2/5/6, PVR and Nectin-2 in which the former two ligands bind to the receptor NKG2D and the latter two to the receptor DNAM-1. Therefore, the correlations between ligand expression levels and $\gamma\delta$ T cell-mediated cytotoxicity were grouped according to the receptors. MICA and ULBP 2/5/6 were grouped as NKG2D ligands while PVR and Nectin-2 were grouped as DNAM-1 ligands.

Interestingly, the expression of DNAM-1 ligands by PDG cells correlated significantly with the degree of $\gamma\delta$ T cell cytotoxicity ($r = 0.5090$, $p = 0.0219$), but the expression of NKG2D ligand did not correlate with the cytotoxicity of $\gamma\delta$ T cells. In other words, $\gamma\delta$ T cells efficiently killed PDG cells with high expression of DNAM-1 ligands.

DNAM-1 axis is crucial to $\gamma\delta$ T cell-mediated lysis of PDG cells

Next, we examined whether the DNAM-1 axis played a crucial role in the anti-tumor effects of $\gamma\delta$ T cells. Four PDG cells with high DNAM-1 ligand expression were co-cultured with expanded $\gamma\delta$ T cells in the presence of an isotype control antibody or a DNAM-1 blocking antibody. The cytotoxicity of the $\gamma\delta$ T cells was significantly reduced in the presence of the DNAM-1 blocking antibody (Figure 4a). In addition, when PVR and Nectin-2, ligands of DNAM-1 were blocked simultaneously, the cytotoxicity of $\gamma\delta$ T cells was significantly reduced (Supplementary Figure 3). To determine whether the secretion levels of cytokines from $\gamma\delta$ T cells change on blocking DNAM-1 signaling, representative cytokines involved in the cytotoxicity of $\gamma\delta$ T cells such as IFN- γ , TNF- α , Granzyme and Perforin were analyzed. When cytokine secretion levels were analyzed, IFN- γ , Granzyme B, Perforin, and Granulysin secretion levels also decreased significantly in the presence of the

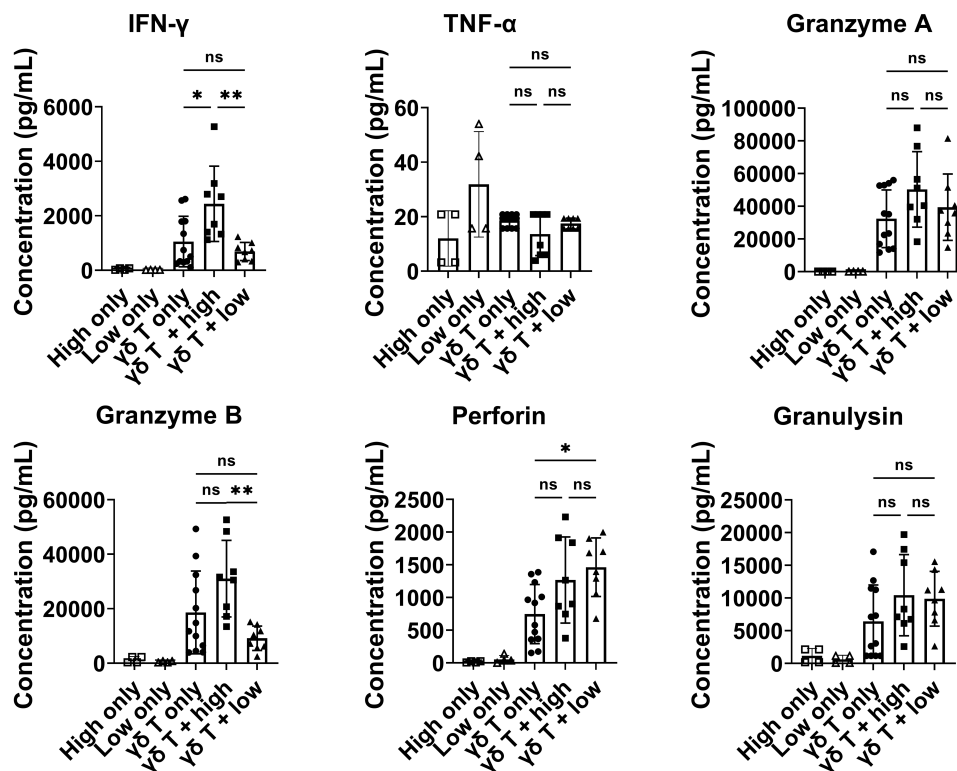


Figure 2. Cytokine secretion patterns of $\gamma\delta$ T cells according to the target PDG cells. Cytokine secretion patterns were analyzed upon co-culture with high-susceptibility or low-susceptibility patient GBM PDG cells. Two high-susceptibility (PDG#3 and PDG#4) and two low-susceptibility PDG cells (PDG#5 and PDG#8) were co-cultured with or without expanded $\gamma\delta$ T cells from 5 healthy donors at an E:T ratio of 2:1. After 24 hours of co-culture, the supernatants were harvested, and cytokine secretion levels were analyzed with a LEGENDplex Human CD8/NK panel according to the manufacturer's protocol. Cytokine concentration was calculated as (cytokine concentration from $\gamma\delta$ T cells co-cultured with tumor cell lines)-(cytokine concentration from tumor cells alone) and cytokine secretion level from only tumor cells is also presented. Cytokine secretion levels from high-susceptibility PDG cells are denoted as "High only" and those from low-susceptibility PDG cells are denoted as "Low only". Experiments were performed in duplicate using $\gamma\delta$ T cells from five different healthy donors. Bars and error bars indicate the mean and SD, respectively. Paired t test; * $P < .05$, ** $P < .005$.

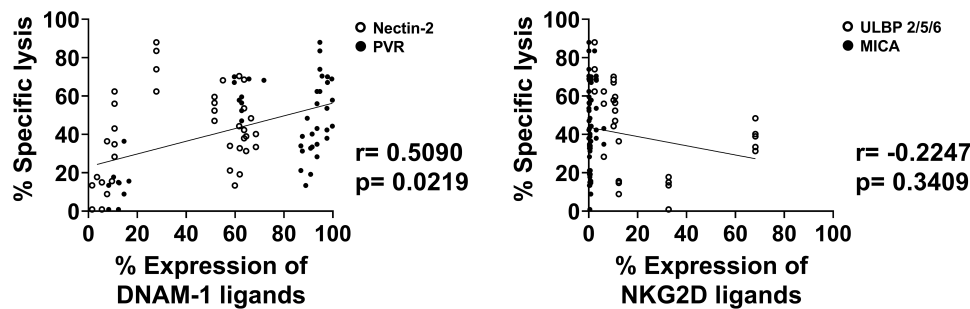


Figure 3. Correlation between the cytotoxic effects of $\gamma\delta$ T cells and ligand expression on patient GBM tumor PDG cells. Correlations were analyzed between the cytotoxicity of $\gamma\delta$ T cells toward PDG cells at a 20:1 E:T ratio and the ligand expression of the patient GBM cell lines. The ligands were grouped according to the receptors they bind to. PVR (black circle) and Nectin-2 (white circle) were grouped as DNAM-1 ligands (left panel) while MICA (black circle) and ULBP2/5/6 (white circle) were grouped as NKG2D ligands (right panel). Experiments were done with GBM tumor cells PDG cells from 10 patients and $\gamma\delta$ T cells from 5 healthy donors and cytotoxicity assays were done as triplicates. Data were analyzed by Pearson's correlation.

DNAM-1 blocking antibody (Figure 4b). This data demonstrates the role of DNAM-1 signaling on the secretion of various cytokines involved in cytotoxicity of $\gamma\delta$ T cells. In sum, the DNAM-1 axis plays a critical role in $\gamma\delta$ T cell-mediated cytotoxicity toward glioblastoma cells.

When NKG2D was blocked, the cytotoxicity of $\gamma\delta$ T cells toward PDG#4 cells and IFN- γ was reduced, and Granzyme B secretion levels were also reduced (Supplementary Figure 4). However, $\gamma\delta$ T cell cytotoxicity was not affected by the tumor cell transcriptome (Supplementary Figure 5) and DNAM-1 ligand expression level measured by FACS in PDG cells did not correlate with mRNA expression (Supplementary Figure 6)

Intratumoral injection of human $\gamma\delta$ T cells elicits anti-tumor effects in orthotopic glioblastoma model

To investigate whether an intratumoral injection of human $\gamma\delta$ T cells show anti-tumor effect against glioblastoma cell lines expressing DNAM-1 ligand in vivo, an orthotopic NSG mouse model was established. First, an early tumor model was established to verify the anti-tumor effect of $\gamma\delta$ T cells toward glioblastoma in vivo. U87 cells with high DNAM-1 ligand expression (Figure 1) were injected intracranially on day 0. Then PBS (control group) or 2×10^6 $\gamma\delta$ T cells ($\gamma\delta$ T cell group) were injected intratumorally on day 4 (experiment 1) after tumor engraftment (Figure 5a). When tumor growth and survival were analyzed weekly, the $\gamma\delta$ T cell group showed

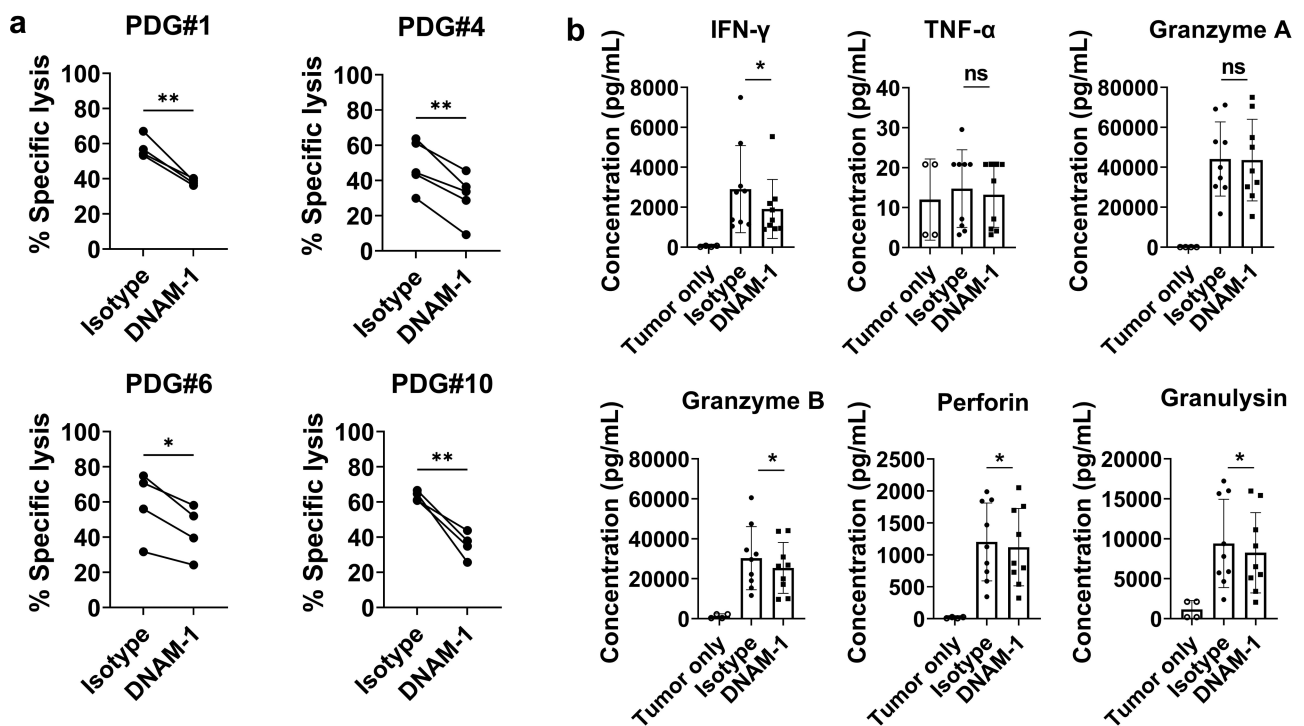


Figure 4. Blocking DNAM-1 significantly inhibits the cytotoxic effects and cytokine secretion of $\gamma\delta$ T cells. (a) Expanded $\gamma\delta$ T cells were co-cultured with Calcein-AM-stained target cells for 4 hours at an E:T ratio of 20:1 in the presence of an isotype control or DNAM-1 blocking antibody. The % specific lysis was calculated as [(Experimental Release-Spontaneous Release)/(Maximum Release-Spontaneous Release)] \times 100. Experiments were carried out in triplicates using $\gamma\delta$ T cells derived from 5 healthy donors. (b) The level of cytokine secretion from $\gamma\delta$ T cells was analyzed after co-culture with two tumor PDG cells with an isotype control antibody or DNAM-1 blocking antibody. Cytokine secretion from only tumor cells was subtracted from the data and is also demonstrated separately. Experiments were carried out in duplicate using $\gamma\delta$ T cells derived from five healthy donors and two PDG cells. Bars and error bars indicate mean and SD, respectively, paired t-test, * $P < .05$, *** $P < .005$.

reduced tumor size and significantly longer survival compared with the control group (Figure 5c, 5d, and 5e). Next, to verify the anti-tumor effect of $\gamma\delta$ T cells in an advanced glioblastoma model, PBS or 2×10^6 $\gamma\delta$ T cells were injected intratumorally on days 7 and 14 (experiment 2) after tumor engraftment (Figure 5b). The advanced tumor model also showed reduced tumor size and significantly better survival in the $\gamma\delta$ T cell group (Figure 5f, 5g, and 5h). $\gamma\delta$ T cell infiltration within the tumors (Figure 5i) and reduced PVR expression were shown in the immunofluorescence results (Figure 5j). These findings demonstrate that an intratumoral injection of human $\gamma\delta$ T cells show anti-tumor effects on DNAM-1 ligand expressing glioblastoma cells in vivo.

Discussion

Our findings demonstrate that human allogenic $\gamma\delta$ T cells expanded and activated by aAPCs can kill PDG cells in vitro. Interestingly, we found that the anti-tumor effects of the human $\gamma\delta$ T cells correlated significantly with the DNAM-1 ligand expression on the glioblastoma cells. Blocking the DNAM-1 receptor or ligands decreased the cytotoxicity of $\gamma\delta$ T cells to glioblastoma cells and decreased the cytokine secretion levels. However, $\gamma\delta$ T cell cytotoxicity was not affected by tumor cell transcriptome (Supplementary Figure 2) and DNAM-1 ligand expression level measured by FACS in PDG cells did not correlate with mRNA expression (Supplementary Figure 3). Most reports on mRNA and protein abundance showed a weak correlation, and this discrepancy could be influenced by some biological factors such as regulatory proteins, translational efficiency, and protein half-lives.²⁶ These findings may imply that DNAM-1 ligand expression on the surface of glioblastoma cells could affect the cytotoxicity of $\gamma\delta$ T cells, rather than PVR RNA expression levels. Glioblastoma is regarded to be a heterogeneous disease that can be broadly divided into different subtypes based on the molecular characteristics of the tumor cells. When the associations between DNAM-1 ligand expression in PDG cells and the subtype of GBM based on Verhaak's classification were analyzed, no significant correlations were found (data not shown). Further studies on the heterogeneous expression of DNAM-1 ligands between PDG cells are needed.

In addition to in vitro experiments, we showed that intracranial injection of $\gamma\delta$ T cells reduced tumor size and improved overall survival in both early and advanced tumor models. However, in the advanced model, the anti-tumor effect seemed to be similar to that in the early tumor model despite additional injection of $\gamma\delta$ T cells. When comparing the early and advanced models, the degree of cancer progression at the point of injection seemed to be an important factor in the anti-tumor effect of $\gamma\delta$ T cells.

Previously, several preclinical studies suggested that human V γ 9V δ 2 T cells show anti-tumor immunity against glioblastoma cells.^{20–24} In a recent in vitro study, V γ 9V δ 2 T cells stimulated with (E)-4-hydroxy-3-methyl-but-enyl pyrophosphate were activated by glioblastoma cells and had cytotoxic effects.²⁴ In another recent preclinical study, stereotaxic administration of human V γ 9V δ 2 T cells in orthotopic

glioma-bearing mice treated with zoledronic acid showed anti-tumor effects.²² However, no one had yet determined which of the various possible ligands for $\gamma\delta$ T cell receptors expressed in glioblastoma cells play an important role in $\gamma\delta$ T cell cytotoxicity. To the best of our knowledge, our findings are the first to show that DNAM-1 axis could be the most important immunologic interaction in $\gamma\delta$ T cell therapy and that glioblastoma cells with high levels of DNAM-1 ligands could potentially be eliminated by an adoptive transfer of human $\gamma\delta$ T cells. Our findings are consistent with previous studies demonstrating DNAM-1 receptor mediated tumor cell killing of $\gamma\delta$ T cells.^{27–29} Specifically, DNAM-1 expression level on $\gamma\delta$ T cells and its interaction with its ligands were significantly related to the cytotoxicity of $\gamma\delta$ T cells against neuroblastoma and hepatocellular carcinoma models.^{27,29} In addition to solid tumors, DNAM-1-mediated $\gamma\delta$ T cell lysis of tumor cells was reduced in acute myeloid leukemic blasts when DNAM-1 was blocked.²⁸

PVR, a member of the Nectin-like family of proteins, has been known to have two critical roles in glioblastoma. It was originally known to be related to cellular adhesion processes, like other members of its family.³⁰ Cancer cells with high PVR expression tend to have more aggressive cellular invasion, migration, and progression than those with low PVR expression.^{31,32} In addition, PVR has an immunomodulatory function because it can bind to both DNAM-1 (activating) and TIGIT (inhibitory).³³ PVR overexpression has been extensively identified in numerous cancers, including glioblastomas, and it induces immune escape by binding to the inhibitory receptors of host T cells.^{34,35} Thus, PVR is an emerging target in cancer immunotherapy. Several studies have already demonstrated the presence of PVR in glioblastoma and other pediatric and adult gliomas.^{35,36} In addition, recent clinical trials using recombinant oncolytic poliovirus, which can target the PVR of glioblastoma cells, showed promising therapeutic potential.³⁷ Furthermore, a TIGIT-inhibitor, which reduces the inhibitory effects of PVR in the immune system, enhanced the anti-tumor effects of a PD-1 inhibitor in an in vivo mouse glioblastoma model.³⁸

Our study has several limitations. First, our data about PVR and Nectin-2 expression in the PDG cells might not be representative of whole tumor tissues, although several studies have shown frequent PVR expression in glioblastoma specimens.^{34,35} Further studies are needed to validate PVR and Nectin-2 expression in whole tumor specimens. Second, the importance of other interactions, including those of the NKG2D ligands and their receptors cannot be overlooked. In our study, only one out of ten PDG cells showed ULBP expression exceeding 50%, and the average expression level was $15.2\% \pm 20$. The MICA expression levels were even lower than that, with an average expression of $0.5\% \pm 0.3$. Low expression of MICA and ULBP have also been reported in other studies. Hypoxia in the tumor microenvironment and miRNA targeting NKG2D ligands were found to be involved in the low expression of those ligands.³⁹ This phenomenon is one mechanism by which GBM evades immunity. Furthermore, MICA and ULBP2 expression decrease as the WHO grade of malignancy increases.⁴⁰ Although expression of the NKG2D ligands is low, a partial decrease in the cytotoxicity of $\gamma\delta$ T cells

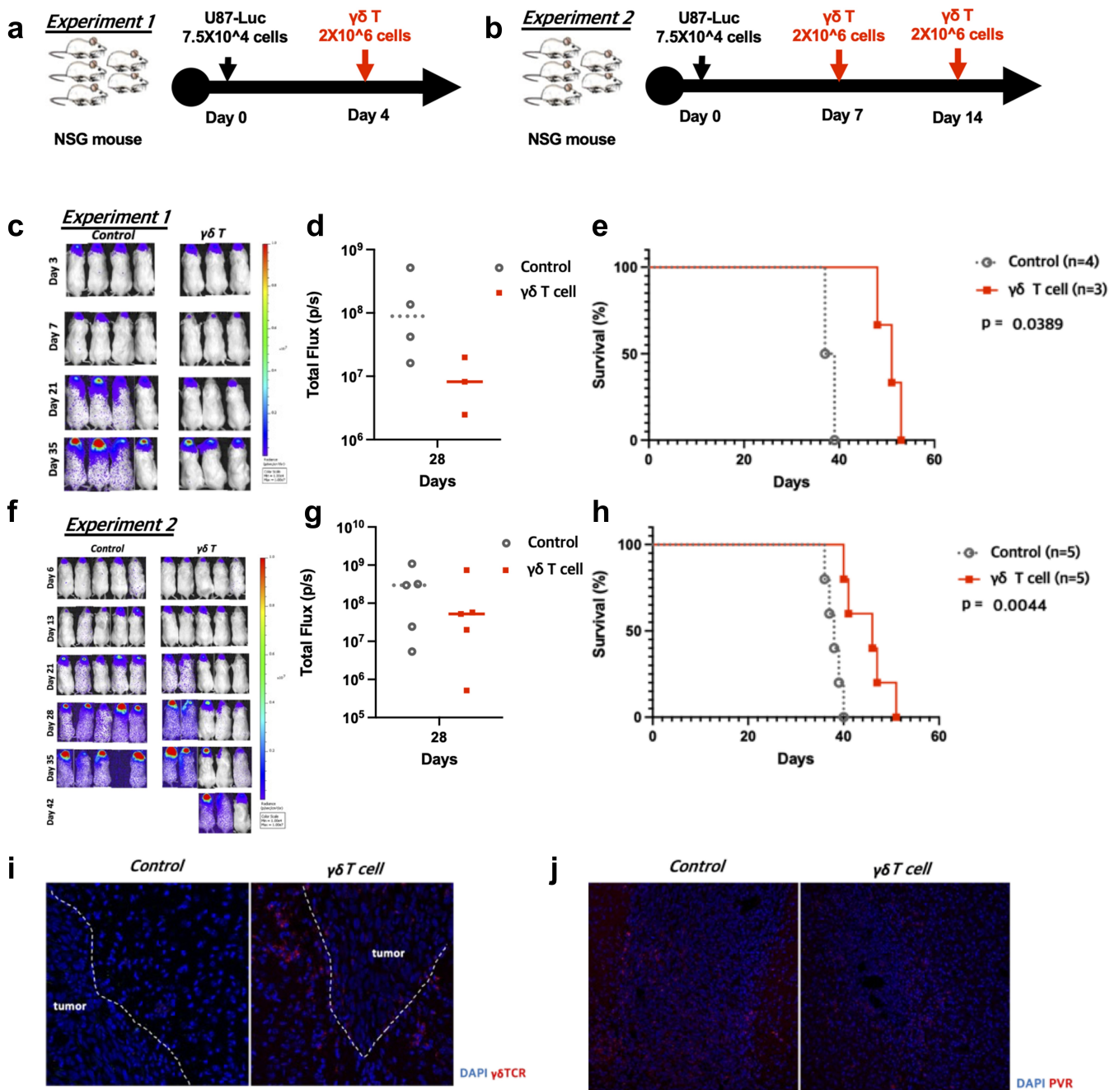


Figure 5. In vivo anti-tumor effects of human $\gamma\delta$ T cells in an intracranial U87 NSG mouse model. (a,b) Intracranial injections of 5×10^4 U87 cells were done on day 0, and the mice were randomized into two groups on the treatment day. We performed intratumoral injections of PBS (control group) or 2×10^6 human $\gamma\delta$ T cells ($\gamma\delta$ T cell group) following two schedules (experiment 1: on day 4, experiment 2: on days 7 and 14). (c,f) Tumor growth was weekly monitored by IVIS and the average radiance per mouse were analyzed, following treatments. (d,g) Total photons were calculated by IVIS Lumina XRMS. (e,h) A Kaplan-Meier survival curve of intracranial glioblastoma-bearing mice intratumorally treated with PBS (control) or human $\gamma\delta$ T cells ($\gamma\delta$ T cell) is presented. (i) Infiltrating $\gamma\delta$ T cells within tumors from each group are shown from day 10 after tumor implantation. (j) PVR expression in each group on day 10 after tumor implantation.

toward GBM cell lines expressing NKG2D ligands has been reported.²³ Also, in this study, blocking NKG2D decreased the cytotoxicity of $\gamma\delta$ T cells and the secretion levels of IFN- γ and Granzyme B upon co-culture with ULBP-expressing PDG#4 (Supplementary Figure 4). Third, the immune suppressive environment of glioblastoma was not investigated. In our findings, IFN- γ was the only cytokine that showed a significant increase upon co-culture with high-susceptibility PDG cells. Moreover, IFN- γ and Granzyme B from $\gamma\delta$ T cells tended to decrease upon co-culture with low-susceptibility PDG cells

compared to baseline secretion. This strongly suggests the immunosuppressive effect of GBM and the reverse correlation with DNAM-1 ligand expression, which should further be studied. Also, additional study of the strong immunosuppressive effect of low-susceptibility GBM tumor cells (e.g. expression of checkpoint inhibitors, inhibitory cytokine secretion) will help to maximize the anti-tumor effects of $\gamma\delta$ T cells. Fourth, since immunodeficient mice were used in this study, the immunoeediting process and tumor microenvironment could not be assessed directly. Further in vivo experiments

using a large number of humanized mice with DNAM-1 inhibition could demonstrate these processes and the importance of PVR-DNAM-1 axis in detail.

Taken together, our study results indicate that adoptive cell transfer of human V γ 9V δ 2 T cells can successfully eliminate glioblastoma cells expressing high levels of DNAM-1 ligands. Because PVR overexpression in glioblastoma has been established in several studies,^{35,36} the administration of allogenic human V γ 9V δ 2 T cells may provide clinical benefits for glioblastoma patients. In addition, combination treatments with a TIGIT inhibitor, which targets the PVR-TIGIT axis, could increase the therapeutic potential of human V γ 9V δ 2 T cell therapy. However, because not all glioblastoma cells express abundant DNAM-1 ligands, further strategies, including redirecting $\gamma\delta$ T cells to new targets and producing $\gamma\delta$ T cells that express a chimeric antigen receptor, are needed. When considering clinical trials of $\gamma\delta$ T cells that mainly target DNAM-1 ligands, safety validation is also essential. Although PVR and Nectin-2 is rarely expressed in normal brain parenchyma,³⁰ further studies are warranted to validate the safety of the intratumoral administration of $\gamma\delta$ T cells for glioblastoma.

Availability of data and material

Data are available only on request due to privacy/ethical restrictions.

Consent to participate

The requirement for informed consent to participate was met according to the policy of the IRB of our institution.

Ethics approval

The Institutional Review Board of Seoul St. Mary's Hospital approved this study design (ethical code: KC21TISI0793, KC22SISI0079).

Disclosure statement

Nothing to declare.

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ORCID

Stephen Ahn  <http://orcid.org/0000-0002-6854-1597>

Author contributions

Drafting the manuscript: Stephen Ahn and Haeyoun Choi
Acquisition, analysis, or interpretation of data for this study: Haeyoun Choi, Yunkyung Lee, Soon A Park, Ji Hyeon Lee, Junseong Park, Jang Hyun Park, and Heung Kyu Lee.
Conceptualization and supervision of this study: Stephen Ahn, Haeyoun Choi, Tai-Gyu Kim, and Sin-Soo Jeun.
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