

Enhanced anti-tumor effects of dendritic cells against glioblastoma by using cytoplasmic transduction peptide (CTP)-fused recombinant protein combined with anti-PD1

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Abstract

Background Recent clinical trials utilizing antigen-pulsed dendritic cells have demonstrated increased survival of the vaccinated cancer patients. Besides, the cytoplasmic transduction peptide has not only excellent transcellular efficiency but also a strong tendency to remain in the cytoplasm after transduction without migrating into the nucleus. In this study, we investigated the effectiveness of immunotherapy against malignant gliomas using DCs pulsed with cytoplasmic transduction peptide (CTP)-fused recombinant protein combined with programmed cell death protein 1 blockade. **Methods** The expression of tumor associated antigen (WT1 and BIRC5) on glioblastoma target cells was confirmed by western blot. The effect of CTP-rhWT1 and/or CTP-rhBIRC5 on DCs was determined. The immunophenotypes of VaxDCs pulsed with CTP-rhWT1 and/or CTP-rhSBIRC5 was confirmed by flow cytometry and the cytokine production levels of T helper polarization were measured by enzyme-linked immunosorbent assay. The IFN- γ -enzyme linked immunospot assay and lactate dehydrogenase release assay were done to estimate the cytotoxic activity of CTLs stimulated by CTP-fused recombinant protein pulsed VaxDCs along with PD1 blockade against malignant glioma cells expressing WT1 and BIRC5. **Results** The CTP-rhWT1 and CTP-rhBIRC5 enhanced activating markers of DCs. Besides, the CTP-rhWT1 and CTP-rhBIRC5 combination resulted in Th1 cytokine polarization. The increase in number of IFN- γ -secreting cells paralleled with enhanced cytotoxicity of CTLs-stimulated by CTP-fused recombinant protein pulsed VaxDCs against glioblastoma target cells. **Conclusions** Our study suggested that treatment of CTP-fused recombinant protein along with PD1 blockade, which enhances cytotoxicity of DCs, could be an effective immunotherapy strategy for glioblastoma.

Background

Glioma is a type of tumor derived from glial or precursor cells. It accounts for about 26.5% and 80.7% of all primary brain and malignant tumors, respectively. Glioblastoma accounts for the majority (56.1%) of gliomas [1]. The median survival of glioblastoma patients is about 11.3 months in the Korean population [2]. Cancer treatment by surgery, radiotherapy, or chemotherapy cannot significantly improve survival outcomes of these patients. Therefore, immunotherapy has become an attractive approach for cancer treatment, especially for patients with aggressive tumors [3]. Dendritic cells (DCs) are known as the most potent professional antigen-presenting cells (APCs) to initiate a T cell response, and the uptake of tumor antigens by DCs, as anti-tumor vaccines, was considered a potential strategy for cancer treatment [4].

Dendritic cells induce adaptive immune response mainly through the presentation and cross-presentation of antigen-derived peptide/MHC complexes to T-lymphocytes [5]. The present tumor-associated antigens, are mainly endogenous antigens in cytosol of DCs, enabling the MHC class I-presenting pathway, which is a key for tumor-specific cytotoxic T-lymphocytes CTLs response [6]. The cross-presentation of dendritic cells, exogenous antigens displayed by MHC class I molecules, stimulate CD8⁺ T cell responses has been shown in previous research [7]. Therefore, developing a method that directly delivers exogenous antigens into the cytosol of DCs, like the endogenous antigens, is critical in DC-based cancer immunotherapy. Using cytoplasmic transduction peptides (CTPs) is a newly designed approach for the effective delivery

of polymeric molecules across the cell membrane to the cytoplasmic compartment. This function of CTP is advantageous for the development of class I-associated CTL vaccines without causing any adverse effects on nuclear genetic material [8, 9]. The cytoplasmic transduction peptide (CTP)-fused recombinant protein demonstrated that when these recombinant antigens are delivered into the cytosol, they are recognized as endogenous antigens [10, 11]. Therefore, DCs pulsed with CTP-fused recombinant protein might be a promising vaccine candidate for cancer therapy, providing a new insight into DC-based immunotherapy design and future peptide therapy applications.

Early data indicated that the brain lacked dedicated lymphatic channels and considered that most glioblastomas are cold tumors [12]. The reasons that tumors are unresponsive to immunotherapy are likely multifactorial and include a highly immunosuppressive tumor milieu (PD1/PD-L1, IDO, TGF- β , IL-10, etc.), defects in tumor antigen presentation, and features of the physical microenvironment, such as hypoxia and necrosis. However, current research demonstrated that brain microenvironment can generate a robust immune response. Although the brain is an immunologically distinct site, the immune microenvironment offers adequate opportunities to implement immunotherapy for the treatment of brain tumors [13]. Hence, in this study, we used combination approaches between CTP-fused recombinant protein and PD1 blockade to enhance the function of dendritic cells with the aim of making these cold tumors hot, thus augmenting current immunotherapy strategies.

Methods

Cytoplasmic transduction peptide (CTP)-fused recombinant protein synthesis

CTP-fused recombinant human Wilm's tumor gene 1 (CTP-rhWT1) and CTP-fused recombinant human survivin (CTP-rhBIRC5) were synthesized at JW CreaGene (Seongnam, Korea). The purity of each protein was confirmed to be >95% by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Synthetic protein was dissolved in dimethyl sulfoxide (DMSO) according to the manufacturer's recommendations and stored at -70°C until use.

Target cells and western blotting

Surgical specimens from glioblastoma patients were obtained for research purposes following approval by the Chonnam National University Hwasun Hospital Ethics Panel. Samples were gathered by the Neurosurgery Department and kept at -80°C until used. The U87 human glioblastoma cell line (Gibco-BRL, Gaithersburg, MD, USA) was obtained for cell culture. These cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) at 37°C in a humidified atmosphere containing 95% air and 5% CO₂.

The bicinchoninic acid (BCA) assay kit (Thermo Scientific, USA) was used to measure protein concentration. Then, SDS-PAGE was used to separate the protein of interest which was transferred onto a polyvinylidene difluoride (PVDF) membrane and soaked in a blocking solution (5% non-fat dry milk in TBST (tris-buffered saline, Tween 20) for 1 h. The membrane was then probed with the primary

antibodies for Wilm's tumor gene 1 (WT1; Abcam, Cambridge, United Kingdom), survivin (BIRC5; Santa Cruz Biotechnology, CA, USA), programmed cell death ligand-1 (PD-L1; Santa Cruz Biotechnology, CA, USA), and β -actin (Santa Cruz Biotechnology, CA, USA) at 4°C overnight, and then incubated with horseradish peroxidase-conjugated a goat anti-rabbit or anti-mouse polyclonal IgG secondary antibodies (Ab frontier, Korea). Chemiluminescent detection was performed using immobilon western chemiluminescent HRP substrate (Millipore Corporation, Billerica, USA). The β -actin was used as an internal control. The expression levels of WT1, BIRC5 and PD-L1 were determined with Amersham Imager 600 (GE Healthcare).

Dendritic cell maturation and CTP-fused recombinant protein pulsed DCs

To confirm effect of CTP-rhWT1 and CTP-rhBIRC5 in combination with anti-PD1 in vitro, dendritic cells were used for checking the function of CTP-fused recombinant protein in stimulating cytotoxic T cells through pulsing CTP-fused recombinant protein with DCs. Human CD14⁺ monocytes, obtained from the peripheral blood of healthy human donors, were used for the experiment. Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS was used for cell culture with the addition of granulocyte-macrophage colony stimulating factor (GM-CSF; 50 ng/mL) and IL-4 (20 ng/mL). The immature DCs were differentiated from CD14⁺ monocytes after 6 days. Then, the immature DCs were differentiated into mature DCs (VaxDCs) by using a cocktail of cytokines such IFN- α (3,000 IU/mL), IFN- γ (10 ng/mL) and poly (I:C) (20 μ g/mL), LPS (1 μ g/ mL) and were loaded with CTP-rhWT1 and CTP-rhBIRC5 (5 μ g/ml) after 2hrs [9, 14]. On day 8, mature DCs were harvested and cryopreserved in liquid nitrogen until used.

Immunophenotyping and polarization cytokine production of DCs

The VaxDCs characteristics were evaluated by immunophenotyping. The expression of activating markers on the dendritic cells was compared using flow cytometry analysis at three stages: immature dendritic cells, dendritic cells after 48 hours maturation with and without CTP-fused recombinant protein (CTP-rhWT1 and CTP-rhBIRC5). Immature DCs were used as a negative control. At day 8, cells were obtained and stained for maturation markers, antigen presenting receptor and co-stimulatory molecules. Mainly, cell staining was performed using fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated monoclonal antibodies against CD40, CD80, CD83, CD86, CCR7, MHC I, and MHC II. All antibodies were purchased from eBioscience (San Diego, CA, USA).

The supernatant collected from the co-culture of DCs and CD40L-transfected J558 cells was subjected to ELISA. To evaluate DCs function after pulsing with CTP-rhWT1 and CTP-rhBIRC5, the cytokine levels for polarized helper T cells such as IL-12p40 and IL-10 were estimated. These cytokines were measured using ELISA kits following the manufacturer's protocols (BD Biosciences) [15].

Cytotoxic T lymphocytes (CTLs) generation

The cytotoxic T lymphocytes (CTLs) were generated as previously described with several modifications [16]. In general, the magnetic activated cell sorting system (MACs) was used to separate the CD3

lymphocyte populations. Then, these cells were stimulated by CTP-rhWT1 and CTP-rhBIRC5 pulsed VaxDCs with or without anti-PD1, respectively. On day 3, IL-2 (5 ng/mL) and IL-7 (10 ng/mL) cytokines were added. After that, the CTLs were harvested and re-stimulated with CTP-rhWT1 and CTP-rhBIRC5 pulsed VaxDCs at second times (day 15) and third times (day 22). Anti-PD1 (BioXcell, USA) was added during each stimulation. Two or three days from the last re-stimulation, an Enzyme-Linked ImmunoSpot (ELISPOT) assay and lactate dehydrogenase (LDH) release cytotoxicity assay were performed. The anti-PD1 was used for blockade in both DCs-CTLs interaction and CTLs-target cancer interaction.

Enzyme-Linked ImmunoSpot (ELISPOT)

Cytotoxic T lymphocyte activity was examined by measuring the secreted IFN- γ cytokine using an IFN- γ ELISPOT assay kit (BD Biosciences). The ELISPOT assay was performed as previously described with several modifications [17]. Practically, ninety-six-well microplates were coated with the capture-purified anti-human IFN- γ antibody overnight at 4⁰C. Then, RPMI medium supplemented with FBS was added to saturate the treated antibody. CTLs stimulated by VaxDCs pulsed with CTP-rhWT1 and CTP-rhBIRC5 were co-cultured with the target cells (U87 glioblastoma cell line and primary glioblastoma cells) at a ratio 1:10 with or without Anti-PD1. Co-cultured cells were added to triplicate wells in 10% FBS-RPMI medium and incubated 24 hours at 37⁰C in 5%CO₂. Then, cells were incubated for 2 hours with the biotinylated detection anti-human IFN- γ antibody and 1 hour with the streptavidin-HRP. After washing, spots were revealed by using an AEC substrate reagent set (BD Bioscience) and measured with an automatic CTL Immunospot Analyzer (Cellular Technology Ltd., USA).

LDH release cytotoxicity assay

For in vitro experiment, the CytoTox 96 nonradioactive cytotoxicity assay (CytoTox 96, Promega, USA) was performed to estimate the cytotoxic activity of CTLs according to the manufacturer's instructions. CTLs stimulated with CTP-rhWT1 and CTP-rhBIRC5 pulsed VaxDCs acted as the effector cells. The U87 cell line and primary cells (5×10³ cells/well) were used as the target cells. The W6/32 monoclonal antibody (1 μg/mL) (mAb; a gift from Dr. Bin Gao, ICH, London, United Kingdom) was used to block MHC-A, B, C antigen presentation on the target cells. The stimulated CTLs were co-cultured with the target cells at a ratio 1:10 in the 96-well uncoated plates (Costar, USA) for 4h in 37°C and 5% CO₂. Anti-PD1 (10 μg/mL) was added during co-culture. Then, supernatants were collected for lactate dehydrogenase concentration determination. The mean percentage of specific lysis was calculated as following: % Cytotoxicity = [(Experimental - Effector Spontaneous - Target Spontaneous) / (Target Maximum - Target Spontaneous)] × 100

Statistical analysis

All statistical analyses were performed with SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to across multiple groups. P < 0.05 was considered statistically significant.

Results

Expression of tumor associated antigens and PD-L1 in glioblastoma cells.

The expressions of the two tumor-associated antigens (WT1 and BIRC5) and PD-L1 were investigated in the target cells (human glioblastoma U87 cell line and human primary glioblastoma cells). As shown in **fig.1**, these targets cells display a significant signal for the WT1, BIRC5, and PD-L1 protein. The U87 cell line showed high expression in BIRC5 while primary glioblastoma cells showed strong expression with WT1 and PD-L1. The figure is made up of multiple gel images. Full-length blots are presented in **fig. S1** for U87 cell line and **fig. S2** for primary cells.

Characterization of CTP-fused recombinant human protein pulsed dendritic cells.

Immunophenotyping characteristics of VaxDCs. The expression of DC markers such as CD40, CD80, CD83, CD86, CCR7, MHC I and MHC II on VaxDCs is shown in **fig.2**. Although there was no significant difference between these markers after maturation into VaxDCs, there was a higher expression tendency of these markers observed on VaxDCs compared to immature DCs. Interestingly, pulsing VaxDCs with CTP-rhWT1 or CTP-rhBIRC5 showed a significant increase in most marker. With CTP-rhWT1, there was enhanced markers in CD83 ($p=0.012$), CD86 ($p=0.022$), CCR7 ($p=0.011$), MHC I ($p=0.006$). Moreover, CTP-rhBIRC5 was also caused increase in CD40 ($p=0.044$), CD80 ($p=0.041$), CD86 ($p=0.025$), CCR7 ($p=0.01$), MHC I ($p=0.002$). Both CTP-rhWT1 and CTP-rhBIRC5 did not showed enhanced markers in MHC II. In general, CTP-rhWT1 and CTP-rhBIRC5-pulsed VaxDCs caused enhanced in activated markers of mature DCs (VaxDCs). There was no significant difference was observed for the expression of these markers between CTP-rhWT1 pulsed VaxDCs and CTP-rhBIRC5 pulsed VaxDCs.

Activated cytokines secreted by dendritic cells pulsed with CTP-recombinant protein. The secretion of cytokines, such as IL-12p40 and IL-10 by activated DCs after stimulating with CD40L-transfected J558 cell line was also estimated. The results are shown in **fig.3**. These cytokines were produced at higher levels after maturation of DCs into VaxDCs compared to the immature DCs. After VaxDCs pulsing with CTP-rhWT1 or CTP-rhBIRC5, there was no significant difference compared with VaxDCs alone. However, combination of CTP-rhWT1 and CTP-rhBIRC5 showed enhanced in IL-12p40 while IL-10 was stable compared with control. It showed that combination of CTP-rhWT1 and CTP-rhBIRC5 enhanced Th1 polarization.

IFN- γ secretion of CTP-recombinant human protein-specific CTLs against human glioblastoma cells

The U87 glioblastoma cell line and primary glioblastoma cells were used as the target cells for ELISPOT assay. Western blot showed expression of WT1, BIRC5, and PD-L1 in both U87 cell line and primary glioblastoma cells (**fig.1**). The IFN- γ secretion against glioblastoma cells was studied at 10:1 E/T ratio (**fig.4**) and CTL alone was used as the negative control. The CTLs stimulated by CTP-rhWT1 or CTP-rhBIRC5 alone showed stable in number of IFN- γ spots against both U87 cell line and primary glioblastoma cells while combination of CTP-rhWT1 and CTP-rhBIRC5 showed enhanced in IFN- γ spots

against both U87 cell line ($p=0.05$). Interestingly, IFN- γ spots against both U87 cell line and primary glioblastoma target cells increase after treatment with anti-PD1. While CTP-rhWT1 caused no change in IFN- γ spots with anti-PD1, CTP-rhBIRC5 and combination of CTP-rhWT1 and CTP-rhBIRC5 showed increase in IFN- γ spots with anti-PD1. With CTP-rhBIRC5, IFN- γ spots enhanced compared with CTL alone against U87 cell line ($p=0.012$) and primary cells ($p=0.026$) respectively. Moreover, combination of CTP-rhWT1 and CTP-rhBIRC5 in the present of anti-PD1 showed more increased compared CTL alone than CTP-rhBIRC5 only in IFN- γ spots with U87 cell line ($p=0.013$) and primary cells ($p=0.002$). Therefore, combination of CTP-rhWT1 and CTP-rhBIRC5 with anti-PD1 enhanced function of CTLs through IFN- γ secretion.

Lactate dehydrogenase release induced by CTP-fused recombinant human proteins specific CTLs against human glioblastoma cells

The ability of CTP-fused recombinant protein-specific CTLs to kill the U87 cell line and the primary glioblastoma cells with and without anti-PD1 was evaluated by using a standard LDH release assay at E/T ratio of 10:1 (**fig.5**). CTP-fused recombinant protein displayed an enhanced cytotoxicity in combination with anti-PD1. Especially, addition of anti-PD1 presented an increase cytotoxicity of CTP-rhWT1 or CTP-rhBIRC5 alone and combination group. Although the cytotoxicity was increased in both CTP-rhWT1 or CTP-rhBIRC5 alone and combination group with anti-PD1, this trend was greater in combination group. Particularly, cytotoxicity increased 26.63% and 11.6% with CTP-rhWT1 combined anti-PD1 (**fig.5a, c**) or 16.8% and 5.64% with CTP-rhBIRC5 combined anti-PD1 (**fig.5b, d**) with U87 cell line and primary glioblastoma cells, respectively. There was more increase in the CTP-rhWT1 and CTP-rhBIRC5 combined anti-PD1 with 43.91% for U87 cell line and 36.55% for primary glioblastoma cells (**fig.5e, f**). Interestingly, the blocking of MHC I pathway also showed decreased in all groups.

Discussion

Glioblastomas are the most frequent type of brain cancers. Despite progress in therapies, the mean survival is approximately 15-20 months [18, 19]. Immunotherapy has become a promising therapeutic option for cancer treatments. Many clinical trials for cancer immunotherapies have been performed. In the immunotherapy approach, the primary focus is on the development of a method where an antigen presenting cells induces CD8⁺ cytotoxic T cell response through MHC I stimulation because these cells can be able to lyse antigen-specific tumor cells. The development of a method for the enhancement of T-cell cross-priming by dendritic cells will help improve cancer immunotherapy [20]. Developing a new antigen delivery tool, based on cross-presentation mechanism, for exogenous antigens in DCs by using CTP has been investigated [9, 21]. The present data also indicated that CTP-fused recombinant protein not only increased the percentage of antigen-specific CTLs but also enhanced the function of these CTLs through Th1 immune response.

The antigenic effect of the single tumor-associated antigens (BIRC5 and WT1) has been mentioned in previous studies. In a clinical trial, WT1 vaccination was shown to trigger WT1-specific CTLs that

suppressed cancer without damaging the normal tissues [22]. WT1 was recommended as the most promising cancer antigen [23]. Similarly, the functions of BIRC5, such as regulation of apoptosis, cell division, chemo-resistance, and tumor progression have also been explored [24], and this antigen has also been investigated for malignant glioma in the clinical study [25]. Therefore, these two antigens were chosen for our study. In our study, the presentation of WT1 and BIRC5 on target cells (U87 cell line and primary cells) was observed.

The phenotype of mature DCs (VaxDCs) after pulsing with CTP-fused recombinant protein remained constant for most of the activated markers. However, there was an enhanced in most marker such as CD40, CD80, CD83, CD86, CCR7, and MHC I expression after pulsing with CTP-rhWT1 or CTP-rhBIRC5. It showed potential in stimulating DCs of CTP-rhWT1 and CTP-rhBIRC5. Besides, IL-12 has multiple biological activities, and it is a key factor that drives Th1 responses and IFN- γ production. Thus, IL-12 immunotherapy could be of importance in the treatment of diseases where a Th1 response is desirable [26]. In our study, combination of CTP-rhWT1 and CTP-rhBIRC5 showed an increased secretion in IL-12p40. While IL-12 plays an important role in Th1 polarization, IL-10 acts an inhibitor of Th1 polarization. An enhanced IL-10 production by antigen-stimulated CD40L-transfected J558 cell line resulted in the reduction of antigen-specific IFN- γ production [27]. Our data indicated a stable IL-10 level for CTP-rhWT1 combined CTP-rhBIRC5. Combining, our CTP-fused recombinant protein showed a potential differentiation of Th1 effectors.

Immune checkpoint inhibitors played an important role in anti-tumor efficacy of the immune response. The PD1/PD-L1 interaction was shown to be a limitation in T cell reactivity even long after the initial activation, and its blockade could restore immune function [28, 29]. Importantly, clinical trials using anti-PD1 have suggested that tumor-specific PD-L1 expression may be an important biomarker of anti-PD1 efficacy [30]. A subsequent study demonstrated that the expression of PD-L1 was associated with glioblastoma (50–90%) [31]. In our study, target glioblastoma cells also showed high expression of PD-L1. Stimulated CTLs showed a significant effect with anti-PD1 blockade after treatment. Especially, anti-PD1 resulted in increased IFN- γ production, leading to a stronger anti-tumor effect with single CTP-rhBIRC5 or combination of CTP-rhWT1 and CTP-rhBIRC5 against U87 cell line and primary glioblastoma cells. Moreover, blocking PD1 receptor in our study also implied the function of CTP-fused recombinant protein-specific CTLs against U87 cell line and primary glioblastoma target cells.

Conclusions

In our study, CTP-rhWT1 and CTP-rhBIRC5 showed not only an enhanced dendritic cell phenotype but also a potential in Th1 polarization. There were enhanced in CTLs functions against glioblastoma have upgraded after blocking with anti-PD1 in both CTP-rhBIRC5 and combination of CTP-rhWT1 and CTP-rhBIRC5. Our study suggested that treatment with CTP-fused recombinant proteins along with anti-PD1 brings a promise effect in boost dendritic cell functions that could be an effective strategy for immunotherapy targeting malignant gliomas.

Abbreviations

BIRC5: Survivin

DCs: Dendritic cells

CTLs: Cytotoxic T lymphocytes

CTP: Cytoplasmic transduction peptide

PD1: Programmed cell death protein 1

PD-L1: Programmed cell death ligand 1

WT1: Wilm's tumor gene 1

Declarations

Ethics approval and consent to participate

The blood from healthy donor and surgical specimens from glioblastoma patients were obtained for research purposes following approval by the Chonnam National University Hwasun Hospital Ethics Panel.

Consent for publication

No applicable

Availability of data and materials

All data supporting the findings are included in this publication and are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Young-Hee Kim designed and performed experiment. Data analysis and interpretation: Young-Hee Kim, Thi-Anh-Thuy Tran, Thi-Hoang-Oanh Duong. Young-Hee Kim, Tae-Young Jung, Thi-Anh-Thuy Tran contributed in writing the manuscripts. Shin Jung, In-Young Kim, Kyung-Sub Moon, Woo-Youl Jang, Hyun-Ju Lee, Je-Jung Lee, and Tae-Young Jung contributed intellectually to the research. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

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Figures

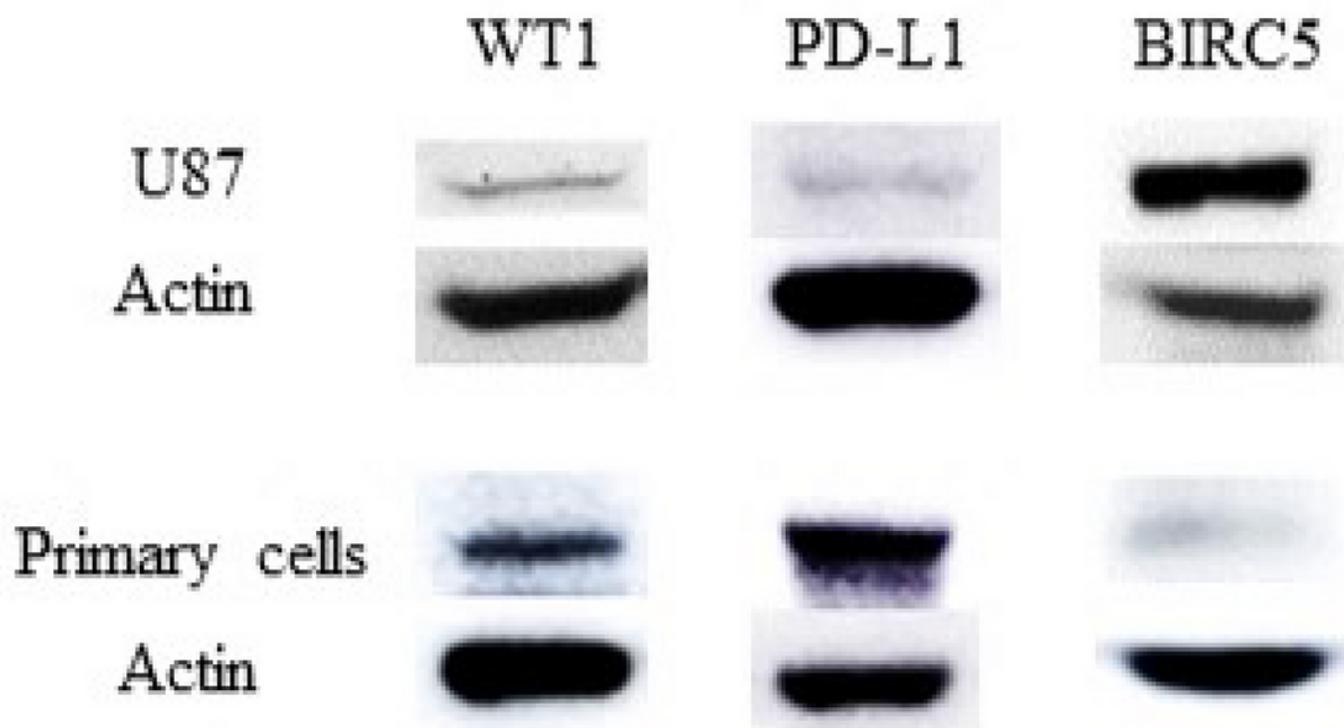


Figure 1

The expression of WT1, BIRC5 (survivin) and PD-L1 on human glioblastoma cells. The expression level of WT1, BIRC5 and PD-L1 on U87 cell line and primary cells were confirmed by western blot. As results, the WT1, BIRC5, and PD-L1 protein expressed on both U87 cell line and primary cells. The U87 cell line showed high expression of BIRC5 while primary cells expressed strong signal with WT1 and PD-L1. The β -actin (ACTB) was used as an internal control in all western blot experiments. The figure is made up of multiple gel images. Full-length blots are presented in supplementary figure. U87 cell line: human glioblastoma cell line; Primary cells: human primary glioblastoma cells.

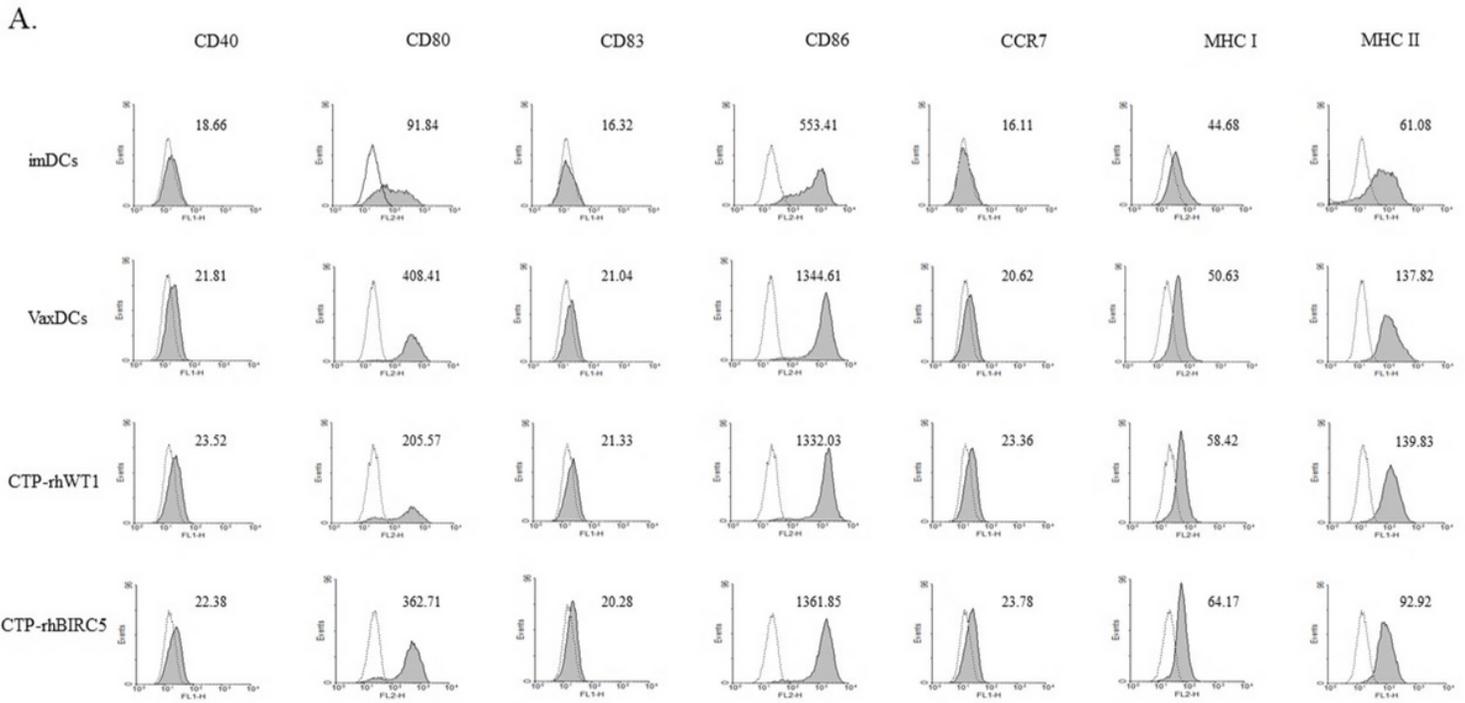


Figure 2

Immunophenotypes of cytoplasmic transduction peptides-fused recombinant protein pulsed VaxDCs. (A) Immunophenotypes of dendritic cells after maturation and pulsing with CTP-rhWT1 and CTP-rhBIRC5 were estimated by flow cytometry. (B-H) Data were summarized by bar chart. Mean fluorescence intensity of dendritic cells markers such as CD40, CD80, CD83, CD86, CCR7, MHC I, MHC II was estimated. The data represents the mean of triplicate from two independent experiments. imDCs: immature DCs, VaxDCs: cytoplasmic transduction peptides-unpulsed VaxDCs, CTP-rhWT1: CTP-rhWT1-pulsed VaxDCs, CTP-rhBIRC5: CTP-rhBIRC5-pulsed VaxDCs. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

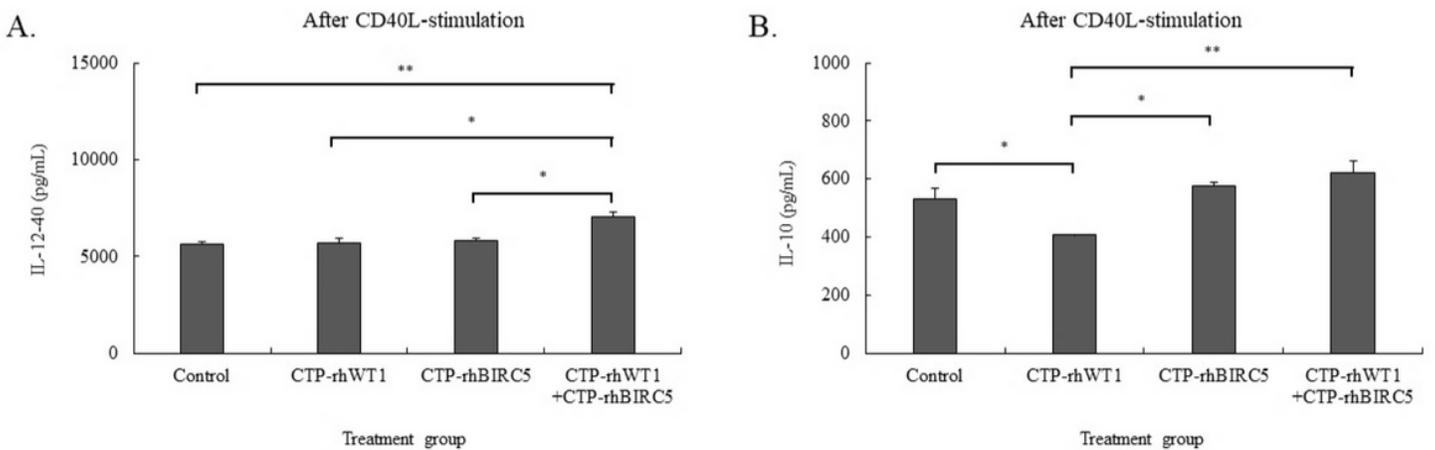


Figure 3

Cytokines secretion of CTP-fused recombinant protein pulsed VaxDCs. Cytokines secreted into the culture supernatants after stimulation with CD40 ligand-transfected J558 cell line were measured by ELISA. Secreted cytokine of IL-12p40 (A) and IL-10 (B) were summarized by bar chart. Control: CTLs stimulated cytoplasmic transduction peptides-unpulsed VaxDCs; CTP-rhWT1: CTLs stimulated CTP-rhWT1-pulsed VaxDCs; CTP-rhBIRC5: CTLs stimulated CTP-rhBIRC5-pulsed VaxDCs; CTP-rhWT1+CTP-rhBIRC5: CTLs stimulated (CTP-rhWT1 and CTP-rhBIRC5)-pulsed VaxDCs. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

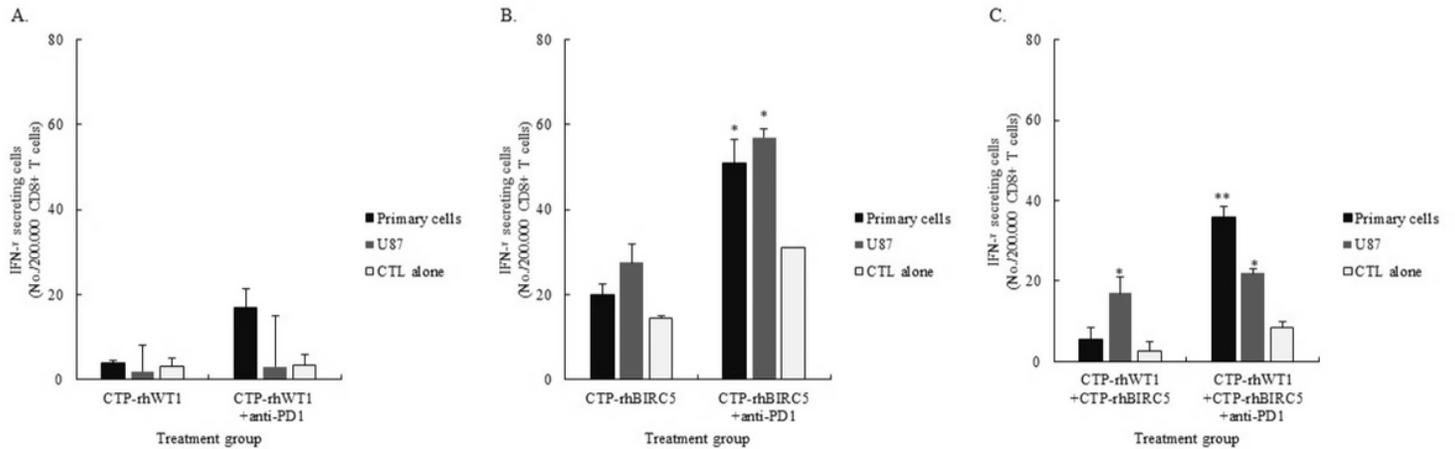


Figure 4

The IFN- γ ELISPOT assay of CTP-fused recombinant protein specific CTLs with or without anti-PD1 against human glioblastoma cells. IFN- γ secretion of (A) CTP-rhWT1-specific CTLs with and without anti-PD1, (B) CTP-rhBIRC5-specific CTLs with and without anti-PD1, and (C) (CTP-rhWT1 and CTP-rhBIRC5)-specific CTLs with and without anti-PD1 against human primary glioblastoma cells and U87 cell lines was summarized. The number of IFN- γ -secreting cells was increased with CTLs stimulated CTP-rhBIRC5 and CTLs stimulated CTP-rhWT1 combined CTP-rhBIRC5 against human primary glioblastoma cells and U87 cell lines after blocking PD-1. Human glioblastoma primary cells and U87 human glioblastoma cell lines were used as the target cells. The E/T ratio was 10:1. Primary cell: human glioblastoma primary cells, U87 cell line: human glioblastoma cell line, CTL alone: CTLs stimulated cytoplasmic transduction peptides-unpulsed VaxDCs; CTP-rhWT1: CTLs stimulated CTP-rhWT1-pulsed VaxDCs; CTP-rhBIRC5: CTLs stimulated CTP-rhBIRC5-pulsed VaxDCs; CTP-rhWT1+CTP-rhBIRC5: CTLs stimulated (CTP-rhWT1 and CTP-rhBIRC5)-pulsed VaxDCs. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

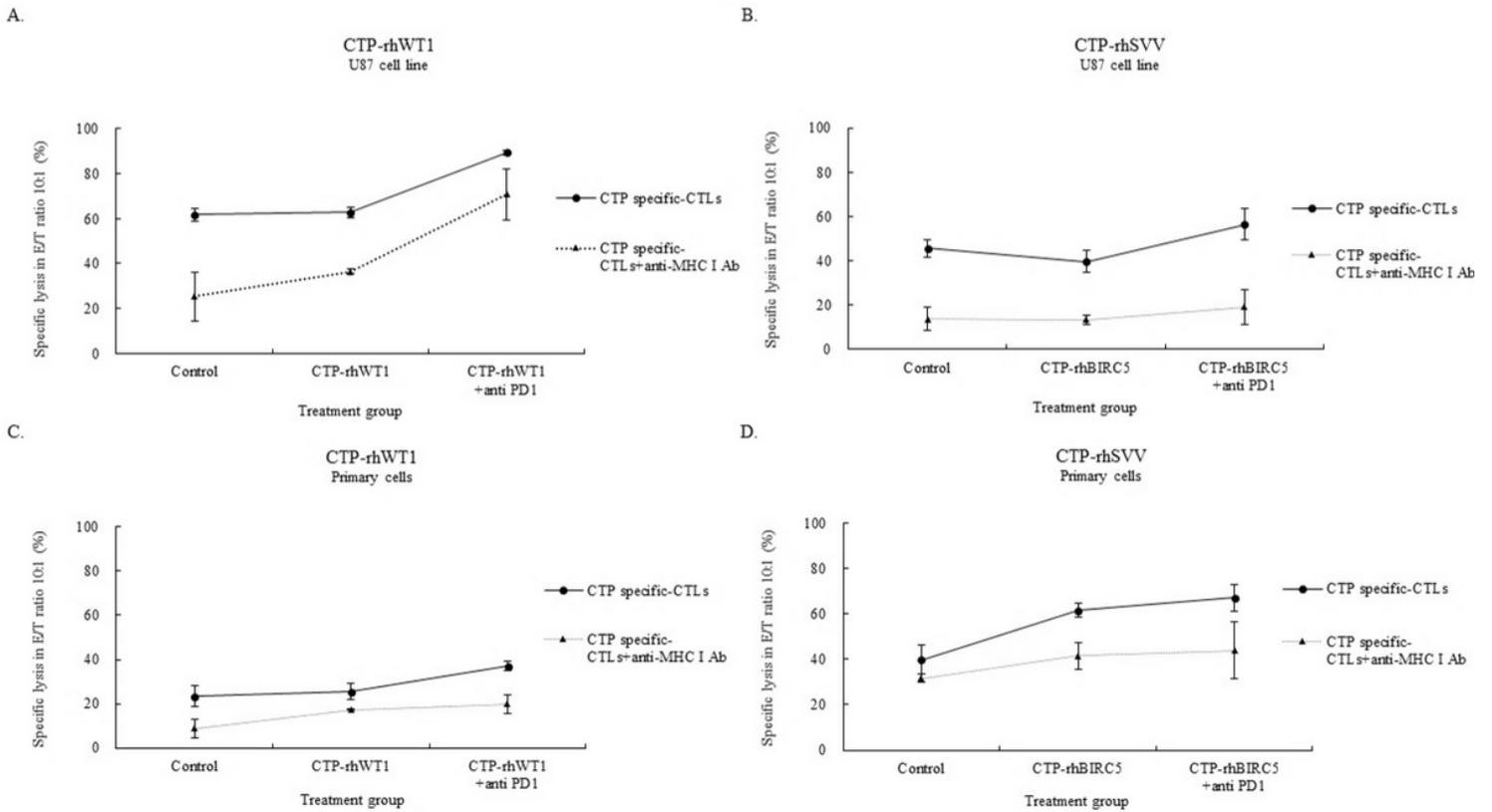


Figure 5

Lactate dehydrogenase (LDH) release to estimate the cytotoxic activity of CTLs stimulated by CTP-fused recombinant protein pulsed VaxDCs combined with anti-PD1. (A-F) We tested the ability of CTP-fused recombinant protein specific CTLs to kill primary cells and U87 cell lines by using a standard LDH release assay. The CTLs stimulated by CTP-rhWT1 or CTP-rhBIRC5 or combination of CTP-rhWT1 and CTP-rhBIRC5 along with anti-PD1 exhibited highest cytotoxic activity against both primary cells and U87 cell line. Human glioblastoma primary cells and U87 human glioblastoma cell lines were used as the target cells. The E/T ratio was 10:1. Primary cell: human glioblastoma primary cells, U87 cell line: human glioblastoma cell line, CTL alone: CTLs stimulated cytoplasmic transduction peptides-unpulsed VaxDCs; CTP-rhWT1: CTLs stimulated CTP-rhWT1-pulsed VaxDCs; CTP-rhBIRC5: CTLs stimulated CTP-rhBIRC5-pulsed VaxDCs; CTP-rhWT1+CTP-rhBIRC5: CTLs stimulated (CTP-rhWT1 and CTP-rhBIRC5)-pulsed VaxDCs. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

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