

## Research Article

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## Adoptive Immunotherapy Against Malignant Glioma Using Survivin-specific CTLs Expanded by W6/32 Antibody-mediated Artificial Antigen-presenting Cells

Xiaoling Lu<sup>1,2,3\*</sup>, Jiaqi Shi<sup>4</sup>, Qin Yao<sup>5</sup>, Jian He<sup>1</sup>, Yuan Zhou<sup>6</sup>, Hongbo Cai<sup>7</sup> and Yongxiang Zhao<sup>1,2,3\*</sup>

<sup>1</sup>Biological Target Diagnosis & Treatment Center, Guangxi Medical University, Nanning 530021, China

<sup>2</sup>The First Affiliated Hospital, Guangxi Medical University, Nanning 530021, China

<sup>3</sup>Department of Immunology, Guangxi Medical University, Nanning 530021, China

<sup>4</sup>Department of Pathology, Arizona Cancer Center, University of Arizona, Tucson, AZ 85724, USA

<sup>5</sup>Zhongshan Hospital, Xiamen University, Xiamen, Fujian 361004, China

<sup>6</sup>Department of Pharmacology, Hunan Normal University Medical College, Changsha, Hunan 410013, China

<sup>7</sup>Department of Pathology, University of Pittsburgh, 200 Lothrop St, BST S-450, Pittsburgh, PA 15213, USA

### Abstract

Survivin is a bifunctional protein that acts as a suppressor of apoptosis and plays a central role in cell division. The protein is strongly expressed in the most common human neoplasms, has prognostic relevance for some of them and appears to be involved in tumor cell resistance to anticancer agents and ionizing radiation. Recently, survivin has been reported to be abundantly overexpressed in malignant glioma. The present study is a report of a novel approach of targeting malignant glioma with pSurvivin<sub>95-104</sub>-specific cytotoxic T-lymphocytes (CTLs). pSurvivin<sub>95-104</sub>-specific CTLs were induced from the peripheral blood lymphocytes (PBLs) of HLA-A2 positive healthy donors by multiple stimulations with W6/32 antibody-mediated artificial antigen-presenting cells (aAPCs) made by coating HLA-A2/pSurvivin<sub>95-104</sub> tetramer mediated by W6/32 antibody, anti-CD28 antibody, 4-1BBL and CD83 molecules to cell-sized latex beads. After multiple stimulations and sorting, the expanded CTLs were analyzed for tetramer staining, IFN- $\gamma$  production, CTL reactivity and adoptive immunotherapy experiments. Tetramer staining assay demonstrated the expanded CTLs specifically bound HLA-A2/pSurvivin<sub>95-104</sub> tetramer. The CTLs specifically produced IFN- $\gamma$  in response to W6/32 antibody-mediated aAPCs and exhibited specific lysis against T2 cells pulsed with the peptide and HLA-A2<sup>+</sup> glioma cells expressing pSurvivin<sub>95-104</sub> while HLA-A2<sup>-</sup> glioma cell lines that express survivin could not be recognized by the CTLs. The peptide-specific activity was inhibited by anti-HLA class I monoclonal antibody. Intravenous injection of the expanded pSurvivin<sub>95-104</sub>-specific CTLs into nonobese diabetic-severe combined immunodeficiency (NOD/SCID) mice harboring glioma cells resulted in glioma cells elimination, whereas transfer of control T-cells was ineffective. These results show the expanded CTLs specific for pSurvivin<sub>95-104</sub> peptide could be a potential target of specific immunotherapy for HLA-A2 patients with malignant glioma.

**Keywords:** Immunotherapy; Glioma; Survivin; CTLs

### Introduction

Malignant glioma patients usually undergo debulking surgery followed by radiation and aggressive rounds of chemotherapy. Despite the majority of the tumors being removed or killed, no lasting and effective antitumor immunity is generated and the patients die from the tumor cells escaping or resisting those therapies. These failures lead many to believe that an aggressive combination of standard therapies, along with other biologically based therapies, is needed to successfully treat this cancer. Although the central nervous system (CNS), and tumors that arise therein, reside in an “immunologically privileged” site [1], some glioma patients were successfully treated by immunotherapy [2-7]. Adjuvant immunotherapy modalities may be particularly useful for treating gliomas located next to critical brain control regions (brain stem or the thalamus) where surgery or radiation cannot be used, provided inflammation can be controlled. The advantage of generating an immune response toward the cancer cells is that the immunized T-cells can now seek and destroy the remaining tumor cells that resisted the therapies or were localized to sites that were inaccessible to the traditional treatments. Therefore, immunotherapy for glioma is an attractive alternative treatment option.

Adoptive transfer of large numbers of tumor-specific cytotoxic T-lymphocytes (CTLs) is currently being developed to treat cancer by changing the relative balance between tumor load and the immune

response [8,9]. CTLs recognize “processed” peptides that are derived from endogenous proteins and presented on the cell surface in association with major histocompatibility complex (MHC) class I molecules [10,11]. Peptides that bind to a MHC class I molecule have been shown to share common amino acid motifs, which are called major anchor motifs [11]. Hence, tumor-specific CTLs can recognize and select the antigenic peptides, then kill tumor cells in an antigenic peptide-specific fashion.

Survivin is a newly identified member of the inhibitor of apoptosis

**\*Corresponding authors:** Yongxiang Zhao, Biological Target Diagnosis & treatment Center, Guangxi Medical University, Nanning 530021, China, Tel: +86 771 535 0964; Fax: +86 771 531 2916; E-mail: [yongxiang\\_zhao@126.com](mailto:yongxiang_zhao@126.com)

Xiaoling Lu, Biological Target Diagnosis & treatment Center, Guangxi Medical University, Nanning 530021, China, Tel: +86 771 535 0964; Fax: +86 771 531 2916; E-mail: [luwuliu@163.com](mailto:luwuliu@163.com)

**Received** September 06, 2011; **Accepted** September 22, 2011; **Published** September 24, 2011

**Citation:** Lu X, Shi J, Yao Q, He J, Zhou Y, et al. (2011) Adoptive Immunotherapy Against Malignant Glioma Using Survivin-specific CTLs Expanded by W6/32 Antibody-mediated Artificial Antigen-presenting Cells. J Cell Sci Ther 2:110. doi:10.4172/2157-7013.1000110

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(IAP) gene family that has been implicated in suppression of apoptotic cell death and regulation of cell division [12,13]. Survivin expression has been found to be undetectable in normal adult tissues. However, it has been found to be abundantly expressed in fetal tissues and a wide variety of human malignancies [14]. The fact that survivin overexpression may provide a survival benefit for tumor cells and that its enhanced expression is almost completely restricted to malignant tissues makes survivin an interesting target for the development of immunotherapeutic strategies.

Recently, survivin has been reported to be abundantly overexpressed in malignant glioma [15,16]. The human leukocyte antigen (HLA)-A2.1-restricted CD8<sup>+</sup> T-cell epitope ELTLGEFLKL (referred to as pSurvivin<sub>95-104</sub>) derived from the tumor-associated antigen (TAA) survivin was identified [17]. In the present study, we used W6/32 antibody-mediated artificial antigen-presenting cells (aAPCs) made by coating HLA-A2/pSurvivin<sub>95-104</sub> tetramer mediated by W6/32 antibody, anti-CD28 antibody, 4-1BBL and CD83 molecules to cell-sized latex beads to generate specific CTL against malignant glioma from the peripheral blood lymphocytes (PBLs) of healthy donors in vitro, in which these CTL lysed HLA-A2<sup>+</sup> glioma cells that expressed pSurvivin<sub>95-104</sub> and could eliminate glioma cells in NOD/SCID mice.

## Materials and Methods

### Cell line and cell culture

T2 cells bear the HLA-A\*0201 gene, but express a very low level of cell surface HLA-A2.1 molecules, and are unable to present endogenous antigens due to a deletion of most of the MHC class II region, including the transporter associated with antigen processing, and genes encoding immuno-proteasomal subunits. This cell line was maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, USA) and 1% antibiotics (penicillin / streptomycin; 100 U/ml). Glioma cell lines U251 (HLA-A2<sup>+</sup>, survivin<sup>+</sup>) and A172 (HLA-A2<sup>-</sup>, survivin<sup>+</sup>) were cultured in our laboratory. These glioma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM)-supplemented with 10% FBS and 1% antibiotics (penicillin / streptomycin; 100 U/ml). NOD/SCID mice, 6 to 8 weeks of age, were obtained from Vital River Laboratories (Beijing, China) and were maintained under a specific pathogen-free condition.

### Synthetic peptide

Survivin-derived peptide pSurvivin<sub>95-104</sub> ELTLGEFLKL [17], and the control peptide HIV-Gag peptide SLYNTVATL (pHIV) [18] were used in this study; they were synthesized by standard solid-phase chemistry and characterized by mass spectrometry. The purity of the synthetic peptides was more than 95%, as indicated by analytical HPLC. Lyophilized peptides were dissolved in dimethylsulfoxide and stored at -80°C after dilution in phosphate-buffered saline (PBS).

### HLA class I typing

HLA class I typing was performed with microcytotoxicity by Lambda antigen tray class I (One Lambda, Canoga Park, USA).

### Detection of survivin gene expression in glioma cell lines by reverse transcription (RT)-polymerase chain reaction (PCR)

The expression of the survivin gene in glioma cell lines U251 and A172 was detected by RT-PCR. The primers used for amplification of human survivin and  $\beta$ 2-microglobulin ( $\beta$ 2m) were as follow: survivin,

5'-CGACCCCATAGAGGAACATAAA-3' (sense), and 5'-GGAATA-AACCCTGGAAGTGGTG-3' (anti-sense);  $\beta$ 2m, 5'-GGGTTTCATC-CATCCGACAT-3' (sense), 5'-GATGCTGCTTACATGTCTCGA-3' (anti-sense). For the surviving and the  $\beta$ 2m cDNA the PCR temperature profiles were as follows: 2 minutes pretreatment at 94°C and 30 cycles at 94°C for 30 seconds, annealing at 59°C for 30 seconds and 72°C for 60 seconds with a final extension at 72°C for 7 minutes.

### Preparation of HLA-A2-pSurvivin<sub>95-104</sub> tetramer

Synthesis of HLA-A2-pSurvivin<sub>95-104</sub> tetramer was carried out according to the protocol of Altman et al. [19]. Briefly, plasmids encoding HLA-A\*0201 (heavy chain) molecules with a C-terminal biotinylation site and human  $\beta$ 2m molecule were constructed by insertion of the target genes into pET28a. The heavy chain and  $\beta$ 2m molecules were expressed in *Escherichia coli*, and purified from inclusion bodies, then refolded in the presence of excess pSurvivin<sub>95-104</sub> to form HLA-A2-pSurvivin<sub>95-104</sub> monomeric product. The folded product was then subjected to enzymatic biotinylation by BirA enzyme (Avidity, Denver, USA) at 25°C for 12 h. This biotinylated HLA-A2-pSurvivin<sub>95-104</sub> monomer was used for the preparation of the HLA-A2-pSurvivin<sub>95-104</sub> tetramer, which was produced by mixing the purified biotinylated monomer with phycoerythrin (PE)-labeled streptavidin (Sigma, St. Louis, USA) at a molar ratio of 4:1. HLA-A2-pHIV tetramer was also prepared according to the protocol.

### Generation of W6/32 antibody-mediated aAPC

The 4.5  $\mu$ m epoxy-activated magnetic beads (Dyna Beads, Lake Success, NY) were incubated with a mixture of W6/32 (2  $\mu$ g/10<sup>7</sup> beads; BD Pharmingen), anti-human CD28-specific antibodies (1 g/10<sup>7</sup> beads; BD Pharmingen, San Diego, CA, USA), 4-1BBL (0.5  $\mu$ g/10<sup>7</sup> beads; BD Pharmingen, San Diego, CA, USA) and CD83 (0.5  $\mu$ g/10<sup>7</sup> beads; BD Pharmingen, San Diego, CA, USA) for 1 h in 1 ml wash buffer per 10<sup>7</sup> beads at 4°C on a rotator, washed twice with PBS, then blocked with 0.4% BSA for 15 min and washed with PBS. Finally, the beads were incubated with 20  $\mu$ g HLA-A2/pSurvivin<sub>95-104</sub> tetramer or HLA-A2/pHIV tetramer for 24 h at 4°C on a rotator and washed with PBS. The W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs or W6/32 antibody-mediated pHIV-specific aAPCs were stored PBS at 4°C.

### Expansion of CTLs

PBLs from six healthy HLA-A2-positive donors (Informed consent was obtained from each donor). were separated using standard Ficoll-Hypaque (Sigma) gradient density centrifugation and PBL isolation kit (Miltenyi). The PBLs were stimulated with W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs in vitro using the protocol adapted from previous studies [20]. Briefly, The PBLs were used as responder cells and stimulated with W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs. Responder cells (3  $\times$  10<sup>6</sup>/well) were co-cultured with W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs (3  $\times$  10<sup>5</sup>/well) in 24-well plates in RPMI-1640 medium supplemented with 10% fetal bovine serum (1 ml/well). IL-7 (10 ng/ml, Pepro Tech EC, London, UK), IL-21 (10 ng/ml, Pepro Tech EC, London, UK) and IL-15 (10 ng/ml, Pepro Tech EC, London, UK) was added on day 1. IL-2 (50 units/ml, R&D, UK) was added on day 4. Medium, IL-2, IL-7, IL-21 and IL-15 were replenished twice a week. On day 7 and weekly thereafter, responder cells were collected, counted and re-plated at 3  $\times$  10<sup>6</sup> per well at the 24-well plate, together with 3  $\times$  10<sup>5</sup> antibody-mediated aAPC per well in RPMI-1640 medium with 10% fetal bovine serum, 10 ng/ml IL-7, 10 ng/ml IL-21, 10 ng/ml IL-15 and 50 units/ml IL-2. After four rounds of co-culture, pSurvivin<sub>95-104</sub>-specific CTLs were isolated

from the bulk cultures by fluorescence activated cell sorter (FACS) using HLA-A2/pSurvivin<sub>95-104</sub> tetramer. After sorting, the pSurvivin<sub>95-104</sub>-specific CTLs were restimulated and expanded with W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs in the same fashion for tetramer staining, IFN- $\gamma$  production, CTL reactivity and adoptive immunotherapy experiments. The pHIV-specific CTLs (as a control) were expanded by W6/32 antibody-mediated pHIV-specific aAPCs in the same fashion.

### Tetramer staining

Tetramer staining was carried out as previously described [21,22]. In brief,  $1 \times 10^6$  cells were incubated in 100  $\mu$ l fluorescence activated cell sorter (FACS) staining buffer (PBS supplemented with 1% BSA and 0.05% NaN<sub>3</sub>) with 20  $\mu$ g/ml HLA-A2-peptide tetramer at 37°C for 30 min. Cells were washed with PBS and subsequently incubated with FITC labeled anti-CD8 antibody (BD PharMingen, San Diego, USA) at 4°C for 30 min. All cells were washed with PBS twice after being stained, and then they were fixed in 1% formaldehyde. Stained cells were analyzed with FACScalibur (Becton Dickinson, Heidelberg, Germany).

### Enzyme-linked Immuno-spot assay

To determine the frequency of T-cells capable of responding to a specific stimulus by secretion of interferon (IFN)- $\gamma$ , an enzyme-linked immune-spot (ELISPOT) assay was carried out. The W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs, W6/32 antibody-mediated pHIV-specific aAPCs, Beads pulsed without HLA-A2/pSurvivin<sub>95-104</sub> tetramer (referred to uncoated aAPCs), K562, A172 (HLA-A2<sup>-</sup>, survivin<sup>+</sup>), U251 (HLA-A2<sup>+</sup>, Survivin<sup>+</sup>) were used as stimulating target cells. Other target cells T2pSurvivin<sub>95-104</sub> (the pSurvivin<sub>95-104</sub>-pulsed T2 cells) and T2pHIV (the pHIV-pulsed T2 cells) were prepared by incubating T2 cells with pSurvivin<sub>95-104</sub> (60  $\mu$ g/ml) and pHIV (60  $\mu$ g/ml) respectively for 3 h at 37°C. T2 cells pulsed without peptide were used as the negative control. Responder cells were co-incubated with the target cells at a ratio of 1:10 (stimulator cell : responder cell) at 37°C for 24 h in 96-well ELISPOT plates. The assay was performed and developed according to the manufacturer's instructions (BD PharMingen).

### Cytotoxicity assay

To analyze the cytotoxic activity of the expanded CTLs against various target cells, cytotoxic assays were performed by incubating <sup>51</sup>Cr-labeled (500  $\mu$ Ci) target cells with effector cells at various effector : target ratios at 37°C for 4 h. Glioma cell lines U251 (HLA-A2<sup>+</sup>, survivin<sup>+</sup>) and A172 (HLA-A2<sup>-</sup>, survivin<sup>+</sup>), T2pSurvivin<sub>95-104</sub>, T2pHIV, K562 and T2 cells pulsed without peptide were selected as target cells. The percentage of <sup>51</sup>Cr release was calculated according to the following formula:  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ .

### Inhibition of the cytotoxicity with HLA class I-specific monoclonal antibody

T2pSurvivin<sub>95-104</sub> and U251 target cells were incubated with anti-HLA class I monoclonal antibody (mAb) W6/32 (American Type Culture Collection [ATCC], <http://www.atcc.org/>) [18] and a control isotypic mAb of irrelevant specificity, immunoglobulin G2a (IgG2a; BD PharMingen) at a final concentration of 30  $\mu$ g/ml for 40 min at 4°C before cytotoxicity assay. After incubation, the target cells were mixed with effector cells for the <sup>51</sup>Cr release assay.

## Adoptive immunotherapy experiments

NOD/SCID mice were inoculated with  $4 \times 10^6$  human glioma U251 cells in 0.5 ml PBS intravenously. Mice were divided into three groups (n = 10 each). For adoptive transfer experiments, mice were treated 3 times on days 5, 15, 25 after tumor inoculation with i.v. injection of induced pSurvivin<sub>95-104</sub>-specific CTLs (up to  $3 \times 10^6$  cells per injection). Mice with injection of PBS or pHIV-specific CTLs were served as the control groups. All mice received two injections of rhIL-2 ( $2 \times 10^5$  IU/mouse) i.p. on days 5, 15, 25. Tumor volumes were calculated using Vernier calipers at 5-day intervals after tumor cells inoculation according to the formula:  $d1 \times (d2)^2 \times 0.5$  (d1 = largest diameter, d2 = perpendicular diameter). The survival time of each group of mice was monitored and recorded on a regular basis. They were sacrificed when any single or combined tumor linear measurement exceeded 20 mm. Three independent experiments were performed.

### Statistical analysis

All data in this study were analyzed using version SPSS 11.0 software (SPSS, Chicago, USA). P<0.05 was considered as statistically significant.

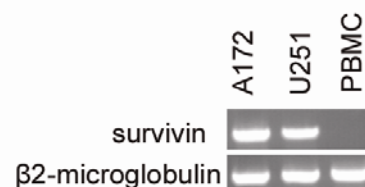
## Results

### Survivin gene expression and HLA class I typing in glioma cell lines

The mRNA expression of the survivin gene was detected in U251 and A172 cell lines. As shown in Figure 1, the expression level of the survivin gene was high in U251 and A172 cell lines, whereas there was no expression in PBMC. The results of HLA class I typing detection showed that U251 was an HLA-A2<sup>+</sup> cell line and A172 was an HLA-A2<sup>-</sup> cell line.

### Frequency of pSurvivin<sub>95-104</sub>-specific CTLs as determined by tetramer staining

A flowcytometric analysis of PBLs was performed before or after co-culture with the W6/32 antibody-mediated aAPC. Before stimulation using W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs, the frequency of CD8<sup>+</sup> T-cells stained with HLA-A2/pSurvivin<sub>95-104</sub> tetramers was 0.63%. However, after sorting and expansion, FACS analysis revealed that 95.1% of CD8<sup>+</sup> T-cells were stained with HLA-A2/pSurvivin<sub>95-104</sub> tetramers, which was not observed when staining with the control tetramers (HLA-A2/pHIV tetramer) (Figure 2). The pSurvivin<sub>95-104</sub>-specific CTLs expanded by W6/32 antibody-



**Figure 1: Glioma cell lines express message for survivin.** Expression of mRNA encoding survivin was analyzed in two glioma cell lines by RT-PCR. The origin of the RNA is indicated at the top of each lane. The amount of the template cDNA was adjusted by the 30-cycle amplification with primers for  $\beta$ 2m as a housekeeping gene, then the adjusted amount of cDNA template was used for a 30-cycle amplification with primers for survivin. The results shown are from one experiment that has been repeated at least three times.

mediated aAPC from the other five normal donors showed similar results (Table 1).

### Enzyme-linked immuno-spot assay

The Enzyme-linked immuno-spot assay was used to determine the frequency of individual antigen-specific IFN- $\gamma$  producing T-cells. As shown in Figure 3, priming with U251 cells, T2pSurvivin<sub>95-104</sub> or W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs resulted in the generation of peptide-specific IFN- $\gamma$ -producing CD8<sup>+</sup> T-cells after the stimulation. The average spot number of responding lymphocytes induced by U251 cells, T2pSurvivin<sub>95-104</sub> or W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs (~ 95 peptide-specific spots / 10<sup>2</sup> cells) were significant higher than that of responding lymphocytes induced by K562, T2pHIV, T2, A172 or uncoated-aAPCs (0 peptide-specific spots / 10<sup>2</sup> cells) ( $P < 0.05$ ).

### Cytotoxicity of pSurvivin<sub>95-104</sub>-specific CTLs

The cytotoxic activity of the expanded CTLs against various target cells was tested using the <sup>51</sup>Cr-releasing assay. CTLs showed approximately 75% specific lysis against the T2pSurvivin<sub>95-104</sub> and U251 (HLA-A2<sup>+</sup>, survivin<sup>+</sup>) at an effector : target ratio of 8:1. However, CTL showed approximately 1% cytolysis against the T2pHIV, A172 (HLA-A2<sup>+</sup>, survivin<sup>+</sup>), K562 and T2 without pulsed peptide at any tested effector : target ratio (Figure 4). The specific killing activity of the CTLs

Donor	tetramer staining (%)		cytotoxicity (%)		
	HLA-A2/pHIV	HLA-A2/pSurvivin <sub>95-104</sub>	T2/pSurvivin <sub>95-104</sub>	U251	T2pHIV
1	0.56	94.2	72.8	70.9	0.7
2	0.43	96.4	79.7	74.9	1.4
3	0.67	97.9	80.1	72.9	1.6
4	0.73	92.1	74.3	73.7	1.3
5	0.62	95.8	76.2	75.1	1.2

Table 1: Expansion of cytotoxic T lymphocytes (CTLs) specific for pSurvivin<sub>95-104</sub> in five human leukocyte antigen (HLA)-A2 positive healthy donors. The CTLs expanded by the W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs were tested for tetramer staining and cytotoxic activity. Tetramer staining cells indicate the percentage of viable CD8<sup>+</sup> HLA-A2/pSurvivin<sub>95-104</sub> tetramer-positive or CD8<sup>+</sup> HLA-A2/pHIV tetramer-positive lymphocytes in the CD8<sup>+</sup> population of viable lymphocytes; lysis indicates the percent lysis of pSurvivin<sub>95-104</sub>-pulsed T2 cells (T2pSurvivin<sub>95-104</sub>), U251 (HLA-A2<sup>+</sup>, Survivin<sup>+</sup>) or pHIV-pulsed T2 cells (T2pHIV). Results are shown for an effector / target ratio of 8:1.

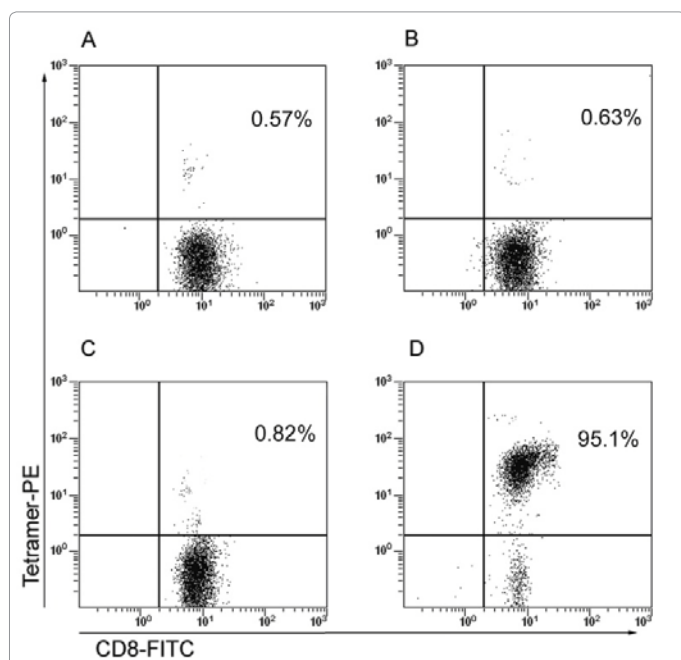


Figure 2: of the pSurvivin<sub>95-104</sub> cytotoxic T lymphocytes by HLA-A2-peptide tetramer staining. The frequency of HLA-A2-peptide tetramer-binding CD8<sup>+</sup> T lymphocytes before and after expanding with W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs was shown. The following HLA-A2-peptide tetramer was used: HLA-A2-pSurvivin<sub>95-104</sub> tetramer and the control tetramer HLA-A2-pHIV tetramer. A: The frequency of HLA-A2-pHIV tetramer-binding CD8<sup>+</sup> T lymphocytes before stimulation was 0.57%; B: The frequency of HLA-A2-pSurvivin<sub>95-104</sub> tetramer-binding CD8<sup>+</sup> T lymphocytes before stimulation was 0.63%; C: The frequency of HLA-A2-pHIV tetramer-binding CD8<sup>+</sup> T lymphocytes after stimulation was 0.82%; D: The frequency of HLA-A2-pSurvivin<sub>95-104</sub> tetramer-binding CD8<sup>+</sup> T lymphocytes after expansion was 95.1%. These results are representative of three experiments, and demonstrate that the co-culture of W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs and HLA-A2 positive PBLs can induce the pSurvivin<sub>95-104</sub>-specific CTLs.

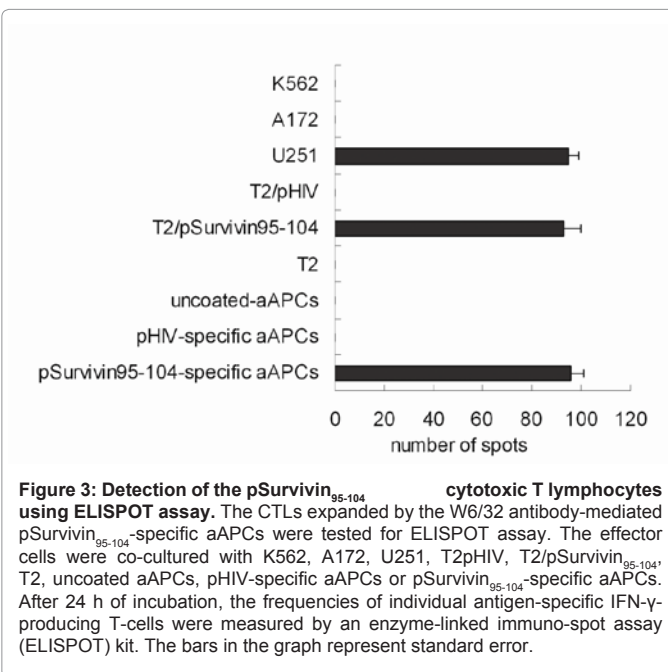


Figure 3: Detection of the pSurvivin<sub>95-104</sub> cytotoxic T lymphocytes using ELISPOT assay. The CTLs expanded by the W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs were tested for ELISPOT assay. The effector cells were co-cultured with K562, A172, U251, T2pHIV, T2/pSurvivin<sub>95-104</sub>, T2, uncoated aAPCs, pHIV-specific aAPCs or pSurvivin<sub>95-104</sub>-specific aAPCs. After 24 h of incubation, the frequencies of individual antigen-specific IFN- $\gamma$ -producing T-cells were measured by an enzyme-linked immuno-spot assay (ELISPOT) kit. The bars in the graph represent standard error.

expanded by the W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs against specific target cells was much more obvious than in any other control group ( $P < 0.05$ ). The specific CTLs for pSurvivin<sub>95-104</sub> expanded by the W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs from the other five normal donors showed similar specific lysis ability (Table 1). These results showed that the cytotoxicity of the CTLs expanded by W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs is pSurvivin<sub>95-104</sub>-specific.

### Inhibition of cytotoxicity of pSurvivin<sub>95-104</sub>-specific CTLs by HLA class I specific mAb W6/32

To determine whether the CTLs expanded by W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs could recognize the specific target cells in an HLA class I-restricted manner, anti-HLA class I mAb W6/32 was utilized to block the cytotoxicity of the expanded CTLs. The cytotoxic activity against the T2pSurvivin<sub>95-104</sub> and U251 was significantly eliminated by W6/32. As shown in Figure 5, W6/32

inhibited target cell lysis, whereas mouse IgG2a, used as an isotype control, showed no effect in T2pSurvivin<sub>95-104</sub> and U251 cells. These results suggested that the expanded CTLs lysed the specific targets in an HLA class I-restricted manner.

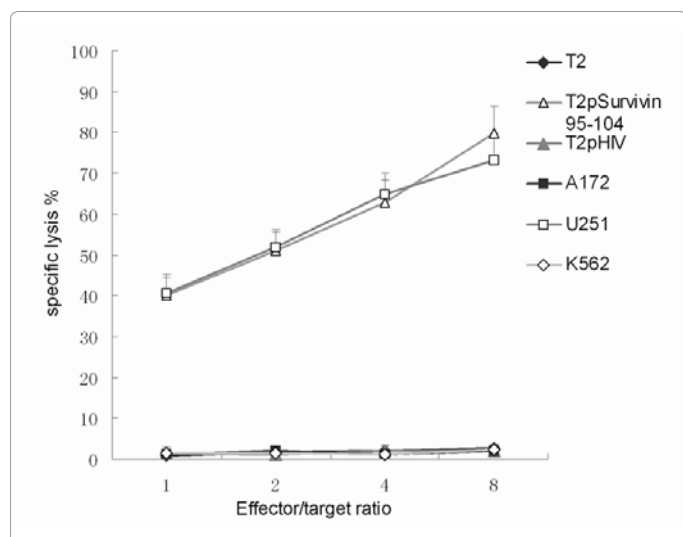
### Adoptive transfer of the expanded pSurvivin<sub>95-104</sub>-specific CTLs can mediate effective therapy of human glioma in NOD/SCID mice

To evaluate the therapeutic effects of pSurvivin<sub>95-104</sub>-specific CTLs expanded by W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs, NOD/SCID mice bearing U251 cells were treated by adoptive transfer of the expanded CTLs. Tumor volumes of PBS group or pHIV-specific CTLs group expanded as determined by measuring every 5 days. In contrast, the rate of tumor growth in animals treated with pSurvivin<sub>95-104</sub>-specific CTLs was significantly decreased as compared with that in animals treated with PBS or pHIV-specific CTLs ( $P < 0.05$ ) (Figure 6A). In addition, as shown in Figure 6B, none of treatment control mice survived longer than 40 days. However, treatment with the expanded pSurvivin<sub>95-104</sub>-specific CTLs was effective and cured 100% of the mice (> 60 days). These results demonstrated that adoptive transfer of the expanded pSurvivin<sub>95-104</sub>-specific CTLs can mediate effective therapy of human glioma in NOD/SCID mice.

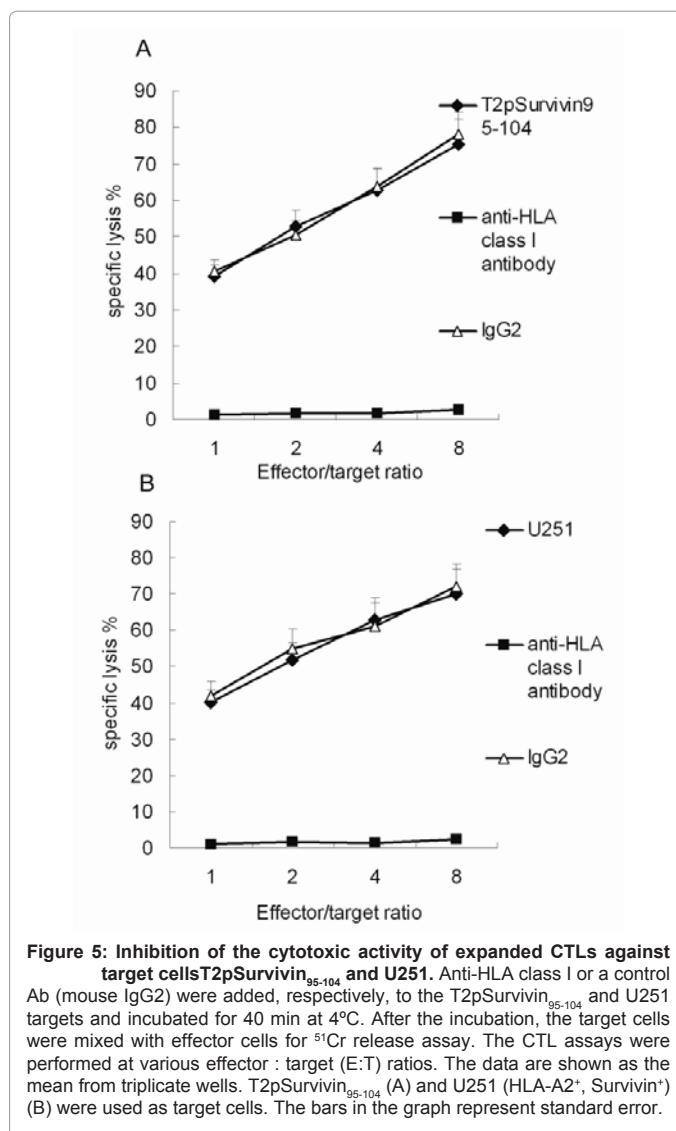
### Discussion

Malignant gliomas are the most common tumors in the central nervous system. When treated with conventional therapy such as surgery, radiation, or chemotherapy, the prognosis for patients with malignant glioma is poor [22]. Clearly, novel therapeutic strategies are necessary.

The adoptive transfer of tumor-specific CTLs provides a promising approach to the immunotherapy of cancer. Previous attempts to determine the impact of adoptive transfer in tumor immunotherapy



**Figure 4: Cytotoxic activity of pSurvivin<sub>95-104</sub> cytotoxic T lymphocytes (CTLs).** The CTLs were expanded by W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs. The cytotoxic activity of one individual's CTLs is shown. The cytotoxic activity of the CTLs was assessed against T2 cells pulsed without a peptide, T2pSurvivin<sub>95-104</sub> (the pSurvivin<sub>95-104</sub>-pulsed T2 cells), T2pHIV (the pHIV-pulsed T2 cells), A172 (HLA-A2<sup>+</sup>, Survivin<sup>+</sup>), U251 (HLA-A2<sup>+</sup>, Survivin<sup>+</sup>) and K562 at various effector:target (E:T) ratios. These results show that the cytotoxicity of the expanded CTLs is pSurvivin<sub>95-104</sub>-specific. The bars in the graph represent standard error.



**Figure 5: Inhibition of the cytotoxic activity of expanded CTLs against target cells T2pSurvivin<sub>95-104</sub> and U251.** Anti-HLA class I or a control Ab (mouse IgG2) were added, respectively, to the T2pSurvivin<sub>95-104</sub> and U251 targets and incubated for 40 min at 4°C. After the incubation, the target cells were mixed with effector cells for <sup>51</sup>Cr release assay. The CTL assays were performed at various effector : target (E:T) ratios. The data are shown as the mean from triplicate wells. T2pSurvivin<sub>95-104</sub> (A) and U251 (HLA-A2<sup>+</sup>, Survivin<sup>+</sup>) (B) were used as target cells. The bars in the graph represent standard error.

have been limited by the difficulty of isolating T-cells of known antigen specificity. Melanoma and renal cell carcinoma were the first tumors in humans to be treated with adoptive immunotherapy [23]. Infusions of polyclonal T-cell populations isolated from the tumor (tumor-infiltrating lymphocytes) and nonspecifically expanded in vitro with high concentrations of IL-2 suggested the therapeutic potential of this approach in cancer patients [23]. The recent characterization of glioma-associated antigens recognized by human CTLs has opened new possibilities for the adoptive transfer of T-cells in glioma immunotherapy.

Rimoldi et al. [24] were the first to document that melanoma associated antigen-specific CTL lines could recognize HLA-matched glioma cells in vitro. Then, Chi et al. [25], Scarcella et al. [26], and Sahin et al. [7] reported that gp100 and MAGE-1 mRNA was expressed in glioma tumor cells and tumor tissue by RT-PCR, and HER-2 was found in brain tumor by immunohistochemical staining [28,29]. Prins et al. [30] validated melanoma-associated antigen gp100 and TRP-2 as immunotherapeutic targets in a murine glioma model. Liu et al. [31] reported on TRP-2 as a CTL target in malignant glioma,

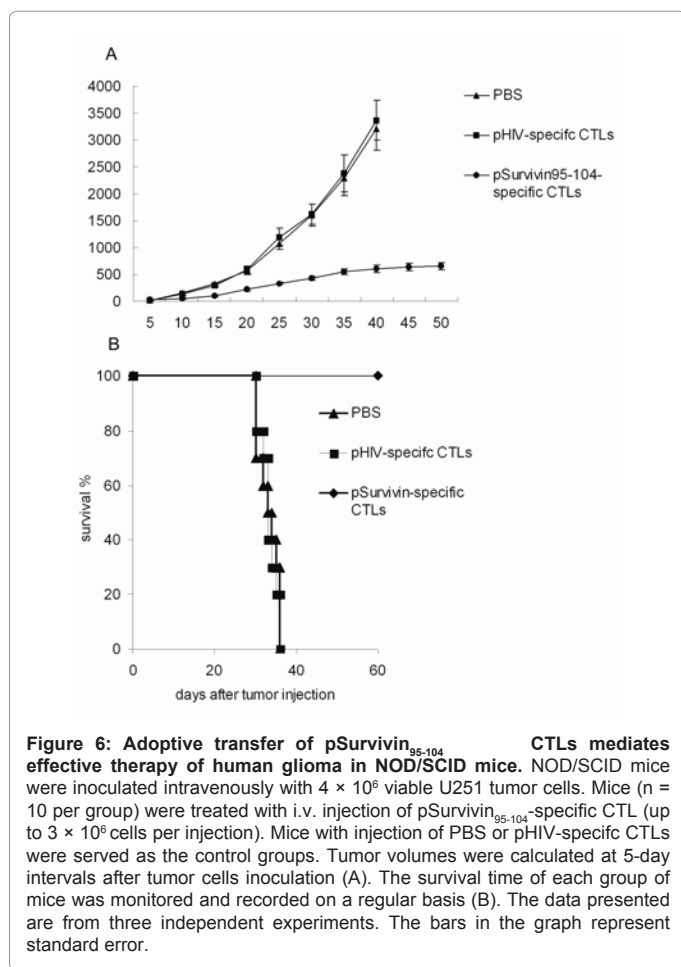
which demonstrated that TRP-2 antigen can be naturally processed and recognized by TRP-2-specific CTLs. They also found that TRP-2-specific cytotoxic T-cell activity was detected when PBMCs were stimulated with autologous DCs pulsed with irradiated GBM tumor cells in vitro and in patients' PBMCs after DC-pulsed autologous tumor lysate vaccinations. Very importantly, IL-13 receptor  $\alpha 2$  has been identified as a glioma-specific antigen [32], and a HLA-A2.1-restricted CTL epitope (WLPFGFIL) might serve as an attractive component of peptide-based vaccines to treat glioma [33,34]. Survivin is a member of the inhibitors of apoptosis family and is overexpressed in different types of malignancies [35]. Cytotoxic T-cells recognizing survivin epitopes can be elicited in vitro and by vaccination in patients with leukemia, breast cancer, and melanoma. Our results indicate that pSurvivin<sub>95-104</sub> epitope could be used as tumor antigen targets for surrogate assays for antigen-specific CTLs or to develop antigen-specific active immunotherapy strategies for glioma patients.

There are several methods currently in use to activate and expand CD8<sup>+</sup> T-cells to the numbers required for a trial of adoptive immunotherapy. One of the most widely used approaches has been based on the use of autologous antigen-loaded dendritic cells (DCs) as antigen presenting cells (APCs). However, the generation and maintenance of DCs is expensive and cumbersome. Furthermore, variability in DC number and the quality of the DCs are affected by both pretreatment of the patient and the patient's disease. Therefore, a number of different systems have been developed in which aAPC

present antigen-HLA complexes in combination with the appropriate costimulatory molecules. The benefits of an aAPC system to expand T-cells include their consistency over time and over multiple donors, their "off-the-shelf" availability, and the lack of need for coculture of allogeneic or virally infected cells with the desired T-cells. Furthermore, the use of artificial APCs may be more attractive for clinical use because they are more amenable to manufacturing within the constraints of current Good Manufacturing Practices than are cellular APCs [36-38]. In the present study, we used W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs to induce specific CTL against malignant glioma from the PBLs of healthy donors. Following this procedure, we found that the expanded CTLs, derived from PBLs in an HLA-A2<sup>+</sup> healthy donor, could kill the HLA-A2<sup>+</sup> pSurvivin<sub>95-104</sub><sup>+</sup> glioma cells when expanded with W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs in vitro. The specific killing activity of CTLs against specific target cells T2pSurvivin<sub>95-104</sub> and U251 was much more effective than that of any other control group. The results indicated the cytotoxicity of the CTLs expanded with W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-specific aAPCs is antigen-specific, that is, against the target cells bearing the corresponding HLA-A2-pSurvivin<sub>95-104</sub> complexes. Intravenous injection of the expanded pSurvivin<sub>95-104</sub>-specific CTLs into nonobese diabetic-severe combined immunodeficiency (NOD/SCID) mice harboring glioma cells resulted in glioma cells elimination, whereas transfer of control T-cells was ineffective.

Proper cell signaling during the expansion phase is also an essential component in producing effective, functioning CTLs following infusion. Excessive stimulation and improper or absent co-stimulation could result in deficient cytolytic activity, lack of persistence or apoptosis. June et al. found the beads coated with HLA class II tetramers in an indirect fashion, via an anti-Class II antibody covalently bound to the bead, were more efficient activators of antigen-specific CD4<sup>+</sup> T-cells than beads directly and covalently coated with HLA monomers or tetramers [39]. Our results show the beads coated with HLA class I tetramers via antibody W6/32 covalently bound to the bead, were more efficient activators of antigen-specific CD8<sup>+</sup> T-cells than beads directly and covalently coated with HLA monomers or tetramers (data not shown). In addition, in this study, we used a combination of anti-CD28, 4-1BBL and CD83 for the generation of a costimulatory signal. Recent studies have demonstrated that combining B7.1/CD28 stimulation and 4-1BB signaling was required for optimal induction of CD25 and bcl-XL expression in CD8<sup>+</sup> T-cells, increased CTL activity and protective anti-tumor immunity [40-42]. In addition, CD83 molecule is important in: (a) priming naive CD8<sup>+</sup> T-cells; (b) driving their antigen-specific expansion; and (c) supporting their long-term survival and function when coupled to aAPCs [43,44]. As we learn more about the in vivo response and the effects of differing amounts and types of co-stimulation on CTL function post-infusion, future generations of the aAPC might prove to be essential because the bead can easily adapt to the new stimulatory requirements by adding or removing different co-stimulatory complexes.

In summary, we have presented data showing that the use of aAPC represents the state-of-the-art in generation of antigen-specific CTLs for adoptive immunotherapy. Thus, The W6/32 antibody-mediated artificial antigen-presenting cells (aAPCs) made by coating HLA-A2/pSurvivin<sub>95-104</sub> tetramer mediated by W6/32 antibody, anti-CD28 antibody, 4-1BBL and CD83 molecules to cell-sized latex beads could provide a useful tool for the reproducible expansion of peptide-specific CTLs in vitro and significantly advance the field of adoptive immunotherapy. The W6/32 antibody-mediated pSurvivin<sub>95-104</sub>-



specific aAPCs could be used as a standardized, “off-the-shelf” reagent to enrich pSurvivin<sub>95-104</sub>-specific CTLs for the treatment of patients with malignant glioma.

#### Disclosures

The authors have no financial conflict of interest.

#### Acknowledgements

This work was supported by the grants from the National Natural Science Foundation of China (No. 30801355, No. 81060183, No. 81072161 No. 30760248, No. 30660185) and Supported by Program for New Century Excellent Talents in University (No. NECT-06-0761) and International Cooperation Project of The Ministry of Science and Technology of the People's Republic of China (No. 2008DFA31380).

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