

## Induction of Cytotoxic T Lymphocytes Specific to Malignant Glioma Using T2 Cells Pulsed with HLA-A2-restricted Interleukin-13 Receptor $\alpha 2$ Peptide *in vitro*

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**Abstract** Interleukin-13 receptor  $\alpha 2$  (IL-13R $\alpha 2$ ) is a glioma-restricted cell-surface epitope not otherwise detected within the central nervous system. The present study is a report of a novel approach of targeting malignant glioma with IL-13R $\alpha 2$ -specific cytotoxic T lymphocyte (CTL) induced from the peripheral blood mononuclear cells of healthy donors by multiple stimulations with human leukocyte antigen (HLA)-A2-restricted IL-13R $\alpha 2$ <sub>345–353</sub> peptide-pulsed T2 cells. The induced CTL showed specific lysis against T2 cells pulsed with the peptide and HLA-A2<sup>+</sup> glioma cells expressing IL-13R $\alpha 2$ <sub>345–353</sub>, while HLA-A2 glioma cell lines that express IL-13R $\alpha 2$ <sub>345–353</sub> could not be recognized by CTL. The peptide-specific activity was inhibited by anti-HLA class I monoclonal antibody. These results suggest that the induced CTL specific for IL-13R $\alpha 2$ <sub>345–353</sub> peptide could be a potential target of specific immunotherapy for HLA-A2 patients with malignant glioma.

**Keywords** malignant glioma; interleukin-13 receptor  $\alpha 2$ ; human leukocyte antigen-A2; cytotoxic T lymphocyte

Malignant gliomas, the most common subtype of primary brain tumors, are aggressive, highly invasive and neurologically destructive tumors, and are considered to be one of the deadliest of human cancers. The standard management of malignant glioma involves cytoreduction by surgical resection, when feasible, followed by radiotherapy with or without adjuvant chemotherapy. Recent studies suggest that greater clinical efficacy may be obtained with the combined use of radiation and temozolomide [1]. Despite significant advances in modern treatments, the overwhelming majority of patients with malignant glioma experiences rapid tumor recurrence even after surgical resection or other treatments, and eventually dies. The reasons why current therapies have limited impact on the mortality of patients with malignant glioma include: (1) gliomas diffusely infiltrate the brain, render-

ing complete resection impossible; (2) gliomas are resistant to conventional tumoricidal agents; (3) the blood-brain barrier limits the penetration of chemotherapeutic agents into the tumor; and (4) the dose of irradiation that can be delivered to the brain is limited. Therefore, the development of new therapeutic strategies for these currently incurable tumors is essential. Although the central nervous system (CNS), and tumors that arise therein, reside in an “immunologically privileged” site [2], many data have successfully shown that effective anti-CNS tumor immune responses can be induced in preclinical models using syngeneic tumor and dendritic cell-based vaccines [3–5]. In addition, activated anti-tumor immune cells have the potential to migrate into the CNS and selectively destroy malignant cells that have infiltrated normal CNS tissues [6]. Therefore, immunotherapy for glioma is an attractive alternative treatment option.

The induction of antigen-specific cytotoxic T lymphocyte (CTL) has been suggested to be highly efficacious in the prevention and treatment of various types of tumor

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[7]. CTL recognizes “processed” peptides that are derived from endogenous proteins and are presented on the cell surface in association with major histocompatibility complex (MHC) class I molecules [7,8]. Peptides that bind to an MHC class I molecule have been shown to share common amino acid motifs, which are called major anchor motifs [8]. Hence, tumor-specific CTL can recognize and select the antigenic peptides, then kill tumor cells in an antigenic peptide-specific fashion. These tumor cells may have localized to sites that were inaccessible to traditional treatments. Interleukin (IL)-13 receptor  $\alpha 2$  (IL-13R $\alpha 2$ ) chain has been reported to be abundantly and specifically overexpressed in malignant glioma and not to be present in the normal brain [9–11]. Recently, the human leukocyte antigen (HLA)-A2.1-restricted CD8<sup>+</sup> T-cell epitope WLPFGFILI (referred to as pIL-13R $\alpha 2$ <sub>345–353</sub>) derived from the glioma-associated antigen (GAA) IL-13R $\alpha 2$  was identified [12].

In the present study, we report a simple and efficient method to induce specific CTL from the peripheral blood mononuclear cells (PBMCs) of healthy donors using T2 cells pulsed with HLA-A2-restricted IL-13R $\alpha 2$ <sub>345–353</sub> peptide *in vitro*, in which these CTL lysed HLA-A2<sup>+</sup> glioma cells that expressed IL-13R $\alpha 2$ <sub>345–353</sub>.

## Materials and Methods

### Cell line

T2 cells were kindly provided by Prof. ZAVAZAVA (Kiel University, Kiel, Germany). These 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 genes encoding 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 RPMI 1640 medium (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, USA) and 1% antibiotics (penicillin and streptomycin; 100 U/ml). Glioma cell lines U251 (HLA-A2<sup>+</sup>, IL-13R $\alpha 2$ <sup>+</sup>) and A172 (HLA-A2<sup>-</sup>, IL-13R $\alpha 2$ <sup>+</sup>) were cultured in our laboratory. These glioma cell lines were cultured in DMEM supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin; 100 U/ml).

### Synthetic peptide

IL-13R $\alpha 2$ -derived peptide pIL-13R $\alpha 2$ <sub>345–353</sub> WLPFGFILI [12], and the control peptide HIV-Gag peptide

SLYNTVATL (pHIV) [13] 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 90%, as indicated by analytical HPLC. Lyophilized peptides were dissolved in dimethylsulfoxide and stored at  $-80^{\circ}\text{C}$  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 *IL-13R $\alpha 2$* gene expression in glioma cell lines by reverse transcription-polymerase chain reaction (RT-PCR)

The expression of the *IL-13R $\alpha 2$*  gene in glioma cell lines U251 and A172 was detected by RT-PCR. The primers used for amplification of human *IL-13R $\alpha 2$*  and *GAPDH* were as follows: *IL-13R $\alpha 2$* , 5'-TGGTCAGAA-GTGTGCCTGTC-3' (sense), and 5'-TCTGCCAG-GAACTTTGAAC-3' (anti-sense); *GAPDH*, 5'-ACAGTCAGCCGCATCTTCTT-3' (sense) and 5'-TTGATTTTGGAGGGATCTCG-3' (anti-sense). PCR reactions using *Ex Taq* DNA polymerase (Life Technologies, Gaithersburg, USA) were subjected to 30 amplification cycles; conditions consisted of denaturation at  $94^{\circ}\text{C}$  for 30 s, primer annealing at  $57^{\circ}\text{C}$  for 30 s, followed by primer extension at  $72^{\circ}\text{C}$  for 1 min.

### Preparation of HLA-A2-pIL-13R $\alpha 2$ <sub>345–353</sub> tetramer

Synthesis of HLA-A2-pIL-13R $\alpha 2$ <sub>345–353</sub> tetramer was carried out according to the protocol of Altman *et al.* [14]. Briefly, plasmids encoding HLA-A\*0201 (heavy chain) molecules with a C-terminal biotinylation site and human  $\beta$ -microglobulin ( $\beta 2\text{m}$ ) molecule were constructed by insertion of the target genes into pET28a. The heavy chain and  $\beta 2\text{m}$  molecules were expressed in *Escherichia coli*, and purified from inclusion bodies, then refolded in the presence of excess pIL13R $\alpha 2$ <sub>345–353</sub> to form HLA-A2-pIL-13R $\alpha 2$ <sub>345–353</sub> monomeric product. The folded product was then subjected to enzymatic biotinylation by BirA enzyme (Avidity, Denver, USA) at  $25^{\circ}\text{C}$  for 12 h. This biotinylated HLA-A2-pIL-13R $\alpha 2$ <sub>345–353</sub> monomer was used for the preparation of the HLA-A2-pIL-13R $\alpha 2$ <sub>345–353</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 this protocol.

## Induction of CTL

PBMCs from five healthy HLA-A2 positive donors were separated using standard Ficoll-Hypaque (Sigma) gradient density centrifugation. PBMCs were stimulated with IL-13R $\alpha$ 2<sub>345-353</sub> peptide *in vitro* using the protocol adapted from previous studies [15]. Briefly, T2 cells were pulsed with pIL-13R $\alpha$ 2<sub>345-353</sub> peptide (referred to as T2pIL-13R $\alpha$ 2<sub>345-353</sub>; 50  $\mu$ g/ml) for 3 h at room temperature in the presence of 3  $\mu$ g/ml  $\beta$ 2m before co-culture with responder cells at a ratio of 1:10 (stimulator cell:responder cell). The peptide-pulsed T2 cells were then irradiated with 7500 cGy, washed once, and added to freshly isolated PBMCs in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. IL-7 (10 ng/ml; R&D Systems, Abingdon, UK) was added on day 1. IL-2 (50 units/ml; R&D Systems) was added on day 4. IL-2 and IL-7 were replenished twice a week. On day 7 and weekly thereafter, responder cells were collected, counted and re-plated at  $3 \times 10^6$  cells/well in a 24-well cell culture plate, together with  $3 \times 10^5$  T2pIL-13R $\alpha$ 2<sub>345-353</sub> per well in RPMI 1640 medium with 10% FBS, 10 ng/ml IL-7 and 50 units/ml IL-2. After a total of five rounds of stimulation *in vitro*, responder cells were tested for tetramer staining and assayed for cytotoxicity.

## Tetramer staining

Tetramer staining was carried out as previously described [16,17]. In brief,  $1 \times 10^6$  cells were incubated in 100  $\mu$ l of 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 PE-cy5 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, then they were fixed in 1% formaldehyde. Stained cells were analyzed with FACScalibur (Becton Dickinson, Heidelberg, Germany).

## Enzyme-linked immunospot assay

To determine the frequency of T cells capable of responding to a specific stimulus by secretion of interferon (IFN)- $\gamma$ , an enzyme-linked immunospot (ELISPOT) assay was carried out. The T2pIL-13R $\alpha$ 2<sub>345-353</sub> (the pIL-13R $\alpha$ 2<sub>345-353</sub>-pulsed T2 cell) was used as stimulating target cell. 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 T2pIL-13R $\alpha$ 2<sub>345-353</sub>-induced CTL 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>, IL-13R $\alpha$ 2<sup>+</sup>) and A172 (HLA-A2<sup>-</sup>, IL-13R $\alpha$ 2<sup>+</sup>), T2pIL-13R $\alpha$ 2<sub>345-353</sub>, K562 and T2 cells pulsed without peptide were selected as target cells. The other target cell T2pHIV (the pHIV-pulsed T2 cell) was prepared by incubating T2 cells with pHIV (50  $\mu$ g/ml) for 3 h at 37 °C. The percentage of <sup>51</sup>Cr release was calculated according to the following formula.

$$^{51}\text{Cr release} = 100\% \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$$

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

T2pIL-13R $\alpha$ 2<sub>345-353</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 G2 (IgG2; 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.

## Statistical analysis

All data in this study were analyzed using SPSS version 10.0 software (SPSS, Chicago, USA).  $P < 0.05$  was considered as statistically significant.

## Results

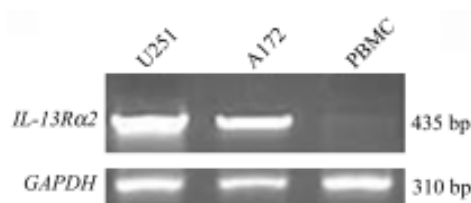
### Growth kinetic of co-culture bulk and phenotype of induced T cells

Following the first stimulation using T2pIL-13R $\alpha$ 2<sub>345-353</sub>, PBMCs expanded continuously. After 5 weeks of co-culture with T2pIL-13R $\alpha$ 2<sub>345-353</sub>, an approximate 40- to 50-fold increase of cell numbers in the culture bulk was achieved. The phenotype of the expanded cells was determined by flow cytometry. The percentage of CD4<sup>+</sup> cells gradually decreased while the CD8<sup>+</sup> cells

increased with the progression of the co-culture with T2pIL-13R $\alpha$ 2<sub>345-353</sub>. The phenotypes of the CTL for pIL-13R $\alpha$ 2<sub>345-353</sub> were CD8<sup>+</sup>, CD4<sup>-</sup>, CD3<sup>+</sup>, CD16<sup>-</sup> and CD56<sup>-</sup>.

### IL-13R $\alpha$ 2 gene expression and HLA class I typing in glioma cell lines

mRNA expression of the *IL-13R $\alpha$ 2* gene was detected in U251 and A172 cell lines. As shown in **Fig. 1**, the expression level of the *IL-13R $\alpha$ 2* gene was high in U251 and A172 cell lines, whereas there was no expression in PBMCs. The results of HLA class I typing detection



**Fig. 1** Glioma cell lines express message for interleukin-13 receptor  $\alpha$ 2 (IL-13R $\alpha$ 2)

Expression of mRNA encoding the *IL-13R $\alpha$ 2* gene was analyzed in two glioma cell lines by reverse transcription (RT)-polymerase chain reaction (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 *GAPDH* as a housekeeping gene, then the adjusted amount of cDNA template was used for a 30-cycle amplification with primers for *IL-13R $\alpha$ 2* that were designed to generate the 435 bp product. The results shown are from one experiment that was been repeated at least three times. PBMC, peripheral blood mononuclear cell.

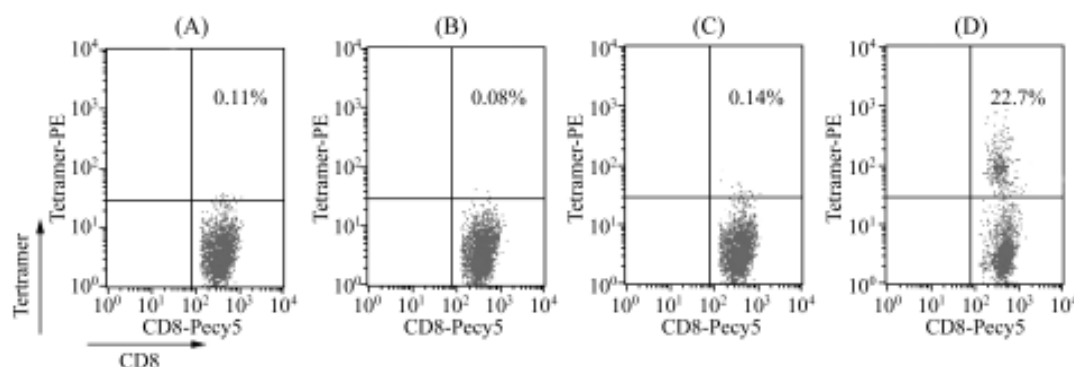
showed that U251 was an HLA-A2<sup>+</sup> cell line and A172 was an HLA-A2<sup>-</sup> cell line.

### Frequency of pIL-13R $\alpha$ 2<sub>345-353</sub>-specific CTL increased by co-culture with T2pIL-13R $\alpha$ 2<sub>345-353</sub>, determined by tetramer staining

A flow cytometric analysis of PBMC was performed before or after co-culture with the T2pIL-13R $\alpha$ 2<sub>345-353</sub>. Before the five rounds of stimulation using T2pIL-13R $\alpha$ 2<sub>345-353</sub>, the frequency of CD8<sup>+</sup> T cells stained with HLA-A2-pIL-13R $\alpha$ 2<sub>345-353</sub> tetramer was 0.08%. However, after stimulation, FACS analysis revealed that 22.7% of CD8<sup>+</sup> T cells were stained with HLA-A2-pIL-13R $\alpha$ 2<sub>345-353</sub> tetramer, which was not observed when staining with the control tetramer (HLA-A2-pHIV tetramer) (**Fig. 2**). The pIL-13R $\alpha$ 2<sub>345-353</sub>-specific CTL expanded by T2pIL-13R $\alpha$ 2<sub>345-353</sub> from the other four normal donors showed similar results (**Table 1**).

### Enzyme-linked immunospot assay

ELISPOT assay was used to determine the frequency of individual antigen-specific IFN- $\gamma$ -producing T cells. As shown in **Fig. 3**, priming with peptide-pulsed T2 cells 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 T2pIL-13R $\alpha$ 2<sub>345-353</sub> cell ( $143 \pm 24$  peptide-specific spots/ $10^5$  cells) was significant higher than that of responding lymphocytes induced by T2 cells without peptide ( $31 \pm 6$  peptide-specific spots/ $10^5$  cells) ( $P < 0.05$ ).



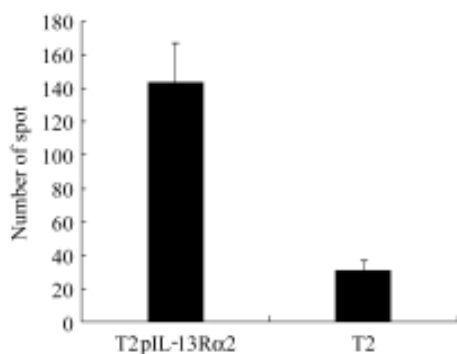
**Fig. 2** Identification of the T2pIL-13R $\alpha$ 2<sub>345-353</sub>-induced specific cytotoxic T lymphocytes (CTL) by human leukocyte antigen (HLA)-A2-peptide tetramer staining

The frequency of HLA-A2-peptide tetramer-binding CD8<sup>+</sup> T lymphocytes before stimulation and after five rounds of stimulation with T2pIL-13R $\alpha$ 2<sub>345-353</sub> is shown. The following HLA-A2-peptide tetramer was used: HLA-A2-pIL-13R $\alpha$ 2<sub>345-353</sub> tetramer and the control tetramer HLA-A2-pHIV. (A) HLA-A2-pHIV tetramer-binding CD8<sup>+</sup> T lymphocytes before stimulation (0.11%). (B) HLA-A2-pIL-13R $\alpha$ 2<sub>345-353</sub> tetramer-binding CD8<sup>+</sup> T lymphocytes before stimulation (0.08%). (C) HLA-A2-pHIV tetramer-binding CD8<sup>+</sup> T lymphocytes after stimulation (0.14%). (D) HLA-A2-pIL-13R $\alpha$ 2<sub>345-353</sub> tetramer-binding CD8<sup>+</sup> T lymphocytes after stimulation (22.7%). These results are representative of many experiments, and demonstrate that the co-culture of T2pIL-13R $\alpha$ 2<sub>345-353</sub> and HLA-A2 positive peripheral blood mononuclear cells can expand the pIL-13R $\alpha$ 2<sub>345-353</sub>-specific CTL.

**Table 1** Induction of cytotoxic T lymphocyte (CTL) specific for pIL13R $\alpha$ 2<sub>345-353</sub> in four human leukocyte antigen (HLA)-A2 positive healthy donors

Donor	Tetramer staining (%)		Cytotoxicity (%)		
	HLA-A2-pHIV	HLA-A2-pIL-13	U251	T2pIL-13	T2pHIV
1	0.15	21.9	59.4	66.9	9.4
2	0.17	25.4	65.7	63.8	8.7
3	0.07	22.8	61.6	61.7	10.8
4	0.09	23.1	67.8	64.3	10.2

The CTL induced by the T2pIL-13R $\alpha$ 2<sub>345-353</sub> for 35 d were tested for tetramer staining and cytotoxic activity. Tetramer staining cells indicate the percentage of viable CD8<sup>+</sup> HLA-A2-pIL-13R $\alpha$ 2<sub>345-353</sub> tetramer-positive or CD8<sup>+</sup> HLA-A2-pHIV tetramer-positive lymphocytes in the CD8<sup>+</sup> population of viable lymphocytes; cytotoxicity indicates the percent lysis of pIL-13R $\alpha$ 2<sub>345-353</sub>-pulsed T2 cells (T2pIL-13R $\alpha$ 2<sub>345-353</sub>), U251 (HLA-A2<sup>+</sup>, IL-13R $\alpha$ 2<sup>+</sup>) or pHIV-pulsed T2 cells (T2pHIV). Results are shown for an effector:target ratio of 50:1.

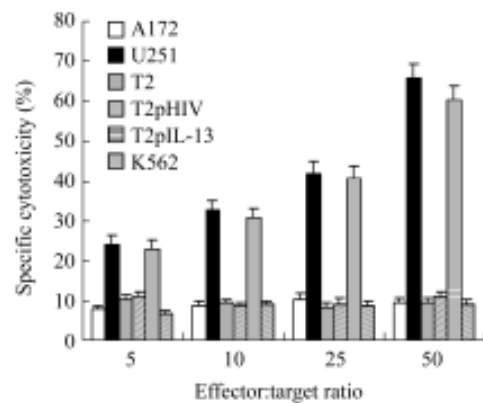


**Fig. 3** Detection of the T2pIL13R $\alpha$ 2<sub>345-353</sub>-induced specific cytotoxic T lymphocyte (CTL) using enzyme-linked immunospot (ELISPOT) assay

The CTL induced by the T2pIL13R $\alpha$ 2<sub>345-353</sub> for 35 d were tested by ELISPOT assay. The effector cells were co-cultured with T2 cells pulsed with or without peptide pIL13R $\alpha$ 2<sub>345-353</sub>. After 24 h of incubation, the frequencies of individual antigen-specific interferon- $\gamma$ -producing T cells were measured using an ELISPOT assay kit. The bars in the graph represent standard deviation.

**Cytotoxicity of T2pIL-13R $\alpha$ 2<sub>345-353</sub>-induced pIL-13R $\alpha$ 2<sub>345-353</sub>-specific CTL**

The cytotoxic activity of the T2pIL-13R $\alpha$ 2<sub>345-353</sub>-induced CTL against various target cells was tested using the <sup>51</sup>Cr-releasing assay. CTL showed approximately 65% specific lysis against the T2pIL-13R $\alpha$ 2<sub>345-353</sub> and U251 (HLA-A2<sup>+</sup>, IL-13R $\alpha$ 2<sup>+</sup>) at an effector:target ratio of 50:1. However, CTL showed approximately 10% cytolysis against the T2pHIV, A172 (HLA-A2<sup>-</sup>, IL-13R $\alpha$ 2<sup>+</sup>), K562 and T2 without pulsed peptide at any tested effector:target ratio (Fig. 4). The specific killing activity of the CTL induced by the T2pIL-13R $\alpha$ 2<sub>345-353</sub> against specific target cells was much more obvious than in any other control



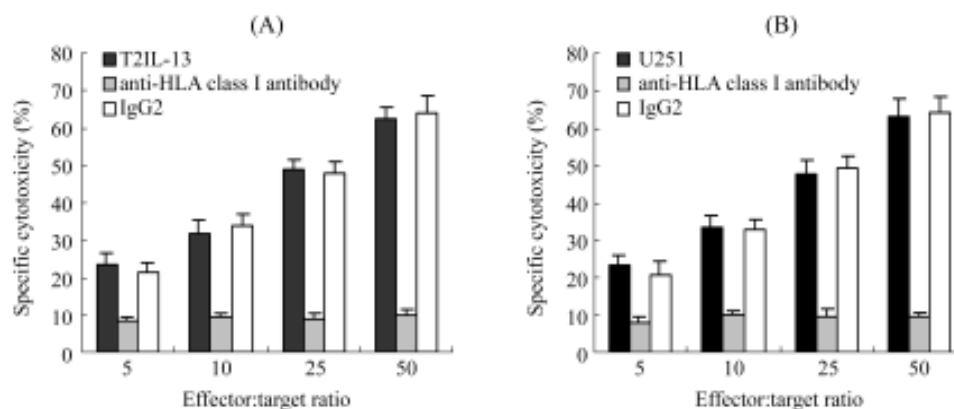
**Fig. 4** Lysis of various target cells by T2pIL13R $\alpha$ 2<sub>345-353</sub>-induced cytotoxic T lymphocyte (CTL)

The CTL were induced by T2pIL13R $\alpha$ 2<sub>345-353</sub> for 35 d. The cytotoxic activity of one individual's CTL is shown. The cytotoxic activity of the CTL was assessed against U251 (HLA-A2<sup>+</sup>, IL13R $\alpha$ 2<sup>+</sup>) and A172 (HLA-A2<sup>-</sup>, IL13R $\alpha$ 2<sup>+</sup>), T2 cells pulsed without a peptide, T2pHIV (the pHIV-pulsed T2 cells), T2pIL13R $\alpha$ 2<sub>345-353</sub> (the pIL13R $\alpha$ 2<sub>345-353</sub>-pulsed T2 cells), and K562 at various effector : target ratios. These results show that the cytotoxicity of the T2pIL13R $\alpha$ 2<sub>345-353</sub>-induced CTL is pIL13R $\alpha$ 2<sub>345-353</sub>-specific. The bars in the graph represent standard deviation.

group ( $P < 0.05$ ). The specific CTL for pIL-13R $\alpha$ 2<sub>345-353</sub> induced by T2pIL-13R $\alpha$ 2<sub>345-353</sub> from the other four normal donors showed similar specific lysis ability (Table 1). These results showed that the cytotoxicity of the T2pIL-13R $\alpha$ 2<sub>345-353</sub>-induced CTL is pIL-13R $\alpha$ 2<sub>345-353</sub>-specific.

**Inhibition of cytotoxicity of T2pIL-13R $\alpha$ 2<sub>345-353</sub>-induced T cells by HLA class I-specific mAb W6/32**

To determine whether the induced CTL 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



**Fig. 5** Inhibition of the cytotoxic activity of effector cells [T2pIL13Rα<sub>2</sub><sub>345-353</sub>-induced cytotoxic T lymphocyte (CTL)] against specific target cells T2pIL13Rα<sub>2</sub><sub>345-353</sub> and U251 by anti-human leukocyte antigen (HLA) class I monoclonal antibody

Anti-HLA class I or a control antibody (mouse IgG2) was added, respectively, to the T2pIL13Rα<sub>2</sub><sub>345-353</sub> and U251 targets and incubated for 40 min at 4 °C. After incubation, the target cells were mixed with effector cells for <sup>51</sup>Cr release assay. The CTL assays were carried out at various effector : target ratios. The data are shown as the mean from triplicate wells. T2pIL13Rα<sub>2</sub><sub>345-353</sub> (A) and U251 (HLA-A2<sup>+</sup>, IL13Rα2<sup>+</sup>) (B) were used as target cells.

cytotoxicity of the T2pIL-13Rα<sub>2</sub><sub>345-353</sub>-induced CTL. The cytotoxic activity against the T2pIL-13Rα<sub>2</sub><sub>345-353</sub> and U251 was significantly eliminated by W6/32. As shown in **Fig. 5**, W6/32 inhibited target cell lysis, whereas mouse IgG2, used as an isotype control, showed no effect in T2pIL-13Rα<sub>2</sub><sub>345-353</sub> and U251 cells. These results suggested that the T2pIL-13Rα<sub>2</sub><sub>345-353</sub>-induced CTL lysed the specific targets in an HLA class I-restricted manner.

## Discussion

Malignant gliomas are a significant class of CNS tumor derived from the glial lineage. Despite recent advances in traditional treatments, the prognosis for patients with gliomas has not changed appreciably. The 5-year survival rate for patients with the most common class of glioma, glioblastoma multiforme, is less than 2% [19]. To improve the outcome for patients with brain tumors, there have been attempts to give adjunctive therapies consisting of radiation with or without chemotherapy. Thus far, research of the past three decades has failed to provide definitive evidence of improved outcome (*i.e.*, overall survival and disease-free survival) in patients. Clearly, modalities other than radiation and/or chemotherapy must be explored in an adjuvant setting. Immune-based treatments are a promising new class of therapy designed to harness the immune system to specifically eradicate malignant cells. However, immunotherapy for tumors located in the CNS has generally not achieved the results as for peripherally located tumors [20–23]. These findings

have historically been ascribed to the “immune privilege” of the CNS [24]. However, the immune privilege of the brain is not absolute but was originally used to describe the observation that tissue and tumor grafts survived better in the CNS than in any other peripheral site [25]. Effective anti-CNS tumor immune responses have been generated by immune-based treatments such as adoptive T-cell transfer [26–31], GAA-pulsed dendritic cells (DCs) [3–5,32], and cytokine-secreting gliomas or fibroblasts [33–35], lending further credence to the idea that the efficient induction of a cellular anti-tumor immune response can be targeted to antigens within the CNS.

Tumor-specific CD8<sup>+</sup> CTL constitutes the most important effector cells for anti-tumor responses [8]. In the present study, we developed a simplified method for the induction of specific CTL by multiple stimulations with pIL-13Rα<sub>2</sub><sub>345-353</sub> peptide, in which CTL responses could be induced from PBMCs by stimulation with peptide-pulsed T2 cells as antigen-presenting cells (APCs) and using IL-7 for the primary culture. Following this procedure, we found that the induced CTL, derived from PBMCs in an HLA-A2<sup>+</sup> healthy donor, could kill the HLA-A2<sup>+</sup> pIL-13Rα<sub>2</sub><sub>345-353</sub><sup>+</sup> glioma cells when stimulated with T2pIL-13Rα<sub>2</sub><sub>345-353</sub> *in vitro*. The specific killing activity of CTL against specific target cells T2pIL-13Rα<sub>2</sub><sub>345-353</sub> and U251 was much more effective than that of any other control group. The results indicated the cytotoxicity of the T2pIL-13Rα<sub>2</sub><sub>345-353</sub>-induced CTL is antigen-specific; that is, the target cells bear the corresponding HLA-A2-pIL-13Rα<sub>2</sub><sub>345-353</sub> complexes.

The pIL-13Rα<sub>2</sub><sub>345-353</sub> epitope-specific CTL generated

from HLA-A2<sup>+</sup> healthy donors by using mature DCs have previously been described [12]. Our results further showed this pIL-13R $\alpha$ 2<sub>345-353</sub> epitope might serve as an attractive component of peptide-based vaccines to treat glioma and as a surrogate marker of T-cell immune responses in patients before and after therapy. Recently, Eguchi *et al.* reported that modified peptides (WLPFGFILV and ALPFGFILV) for IL-13R $\alpha$ 2<sub>345-353</sub> epitope can exhibit a superior capacity (versus the wild-type peptide, WLPFGFILI) to induce CTL capable of (cross-)reacting against the IL-13R $\alpha$ 2<sub>345-353</sub> epitope in HLA-A2<sup>+</sup> patients with glioma by using autologous DCs [36]. In our study, the pIL-13R $\alpha$ 2<sub>345-353</sub> epitope-specific CTL were generated from HLA-A2<sup>+</sup> healthy donors by using T2pIL-13R $\alpha$ 2<sub>345-353</sub> cells as APCs instead of freshly isolated PBMCs or DCs. The low level of peptides in this transporter associated with antigen processing (TAP)-mutant T2 cell line causes most MHC class I molecules to remain no peptide or to associate with low-affinity peptides. By the external addition of peptides, empty molecules can be stabilized and low-affinity peptides can be replaced [37,38]. The data show that T2 cells functioned efficiently as APCs in the induction of HLA-A2 binding peptide-specific CTL have its unique characteristic. One of these is ease of preparation of T2, which is not required for sterile culture cytokines, compared with preparation of DC, thereby reducing both the variability and expense associated with *ex vivo* expansion. The variability is particularly important when considering therapies for cancer, as there have been reported defects in DCs obtained from patients with malignancies [39]. Another advantage is good stability of T2 cells as APCs, unlike the biologic variability and patient-to-patient variation when producing DCs. For analysis of the antigen-specific CTL induced from HLA-A2<sup>+</sup> PBMCs using T2pIL-13R $\alpha$ 2<sub>345-353</sub>, T2 cells would be very convenient as APC for performing multiple experiments and confirming reproducibility of the results in future research. Malignant glioma is one of the most common brain neoplasms worldwide [30]. The pathogenic mechanisms responsible for malignant glioma are not well defined, and therapeutic means, particularly for inoperable malignant glioma, are still unsatisfactory and are expected to be improved. In malignant glioma patients in China, the proportion of HLA-A2 is higher than any other allele. Therefore, most of the patients with malignant glioma could potentially be candidates for specific immunotherapy with the adoptive transfer of specific CTL generated from PBMCs by stimulation with T2pIL-13R $\alpha$ 2<sub>345-353</sub>. In this regard, the results of this study should benefit most patients with malignant glioma in the future.

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