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Activated Human γδ T Lymphocytes Exhibit Cytolytic Activity against Glioma Cells

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Summary. **We investigated the cytotoxic activity of γδ-receptor bearing T cells against glioma cells. Highly enriched γδ-receptor bearing T cells were prepared from the peripheral blood mononuclear cells (PBMC) of three healthy donors and six patients with malignant glioma. After culturing with phytohemagglutinin and recombinant interleukin (rIL)-2, 46-92% of PBMC came to bear the γδ T cell receptor, which hereafter shall be referred to as gamma delta riched (GDR) cells. GDR cells from one of the healthy donors showed a wide ranging killer activity against glioma cell lines as well as Raji cells. GDR cells which lysed autologous tumor cells also showed strong cytotoxic activities against the allogeneic glioma cell line U251 in a non-major histocompatibility complex restricted manner. The data suggest that γδ T cells from patients with malignant glioma could be used as new effector cells in adoptive immunotherapy for patients with malignant glioma.**

Key words—adoptive immunotherapy, γδ T cell, LAK cell, malignant glioma.

INTRODUCTION

Many vigorous efforts have been made to improve the morbidity and the mortality of patients with malignant glioma. In spite of the combination of recent therapeutic modalities such as microsurgical resection, irradiation and chemotherapy, the prognosis for these patients remains very poor, as their best median survival periods have been reported to be only about 50-60 weeks.[1] In addition to the initial treatments of surgical debulking followed by the radiochemotherapy, we have tried the local adoptive immunotherapy (AIT) of intratumoral administration of lymphokine-activated killer cells (LAK) cells and high doses of reconbinant interleukin 2 (rIL-2), particularly in patients suffering from recurrent malignant glioma.[2,3] LAK cells could exhibit non-specific, non-major histocompatibilty complex (MHC) restricted, strong cytolytic activity against various tumor cells including autologous tumor cells *in vitro*.[4,6] Although inhibition of tumor growth or even regression of the tumor has been observed in some cases by this therapy, over-all results of this new therapeutic method have not been satisfying yet. To date some factors which abrogate the cytotocixity of LAK cells are known. From our clinical experience, it was supposed that the neo-membrane on the tumor cavity might prevent the migration of LAK cells into the surrounding tumor. In addition, immunosuppressive cytokines such as TGF β[7-11] and IL-10[12,13] released from glioma cells can suppress the proliferation and cytolytic activities of LAK cells. We found that, in peripheral blood lymphocytes from patients with malignant glioma, suppressor T lymphocytes were more dominant with deteriorated NK activity than normal controls.[14] Though the brain is one organ suitable for local AIT, nevertheless, the idea of the local administration of effector cells that allows direct contact with glioma cells is still attractive.

Although most of the T cells in human peripheral blood express the αβ receptor, other T cells expressing γδ receptor have also been reported recently.[15] These cells show unique tissue distribution, residing predominantly in the surface epithelia.[16] Though the functional and biological roles of γδ T cells remain

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largely unknown, there is some evidence that these cells are involved in the first defense mechanism against the invasion of exogenous pathogens.[17] Recently, it has been reported that $\gamma\delta$ receptor-bearing T cells can show strong cytotoxicity against a variety of tumor cells in an MHC nonrestricted manner.[18-25] It has also been shown that $\gamma\delta$ T cells are not only restricted to the primary stimulating antigen but also show cytotoxic activities against certain tumor cells.[26] We therefore examined whether $\gamma\delta$ receptor-bearing T cells could be effector cells of AIT, investigating its cytotoxic activity against glioma cells.

MATERIALS AND METHODS

Preparation of $\gamma\delta$ T cell rich effector cells

Peripheral blood mononuclear cell (PBMC) were isolated from heparinized peripheral venous blood by the Ficoll-Conray gradient separation. PBMC were depleted of plastic-adherent cells by incubation at 37℃ for 90 min. Non-adherent cells were treated with 1μg of anti-CD4, anti-CD8, anti-CD16, anti-CD19 and anti-TCR-$\alpha\beta$ antibodies (Phamingen, San Diego, CA, respectively) for 30 min at 4℃. After being washed twice, these cells were co-incubated with immunomagnetic particles (Dynabeads M-450, Dynal, Oslo, Norway) for 30 min at 4℃ with gentle shaking, and antibody-binding cells were removed with a magnetic particle concentrator (Dynal, Oslo, Norway). The remaining cells were collected and cultured for three weeks as described later. Of these cells, 46-92% expressed the $\gamma\delta$ T cell receptor after this culture, and shall hereafter be referred to as gamma delta riched (GDR) cells.

Culture of GDR and LAK cells

Cells were cultured in RPMI 1640 supplemented with 5% heat-inactivated human AB serum, 200 U/ml penicillin G and 1 mg/ml streptomycin. GDR cells were incubated in U shaped 96-well culture plates, together with the same number of Mitomycin C treated autologous PBMC in the presence of 5 μg/ml phytohemagglutinin (PHA) (Burrough Wellcome, Greenville, NC). Fresh medium containing 100 U/ml of rIL-2 (kind gift from Shionogi Pharmaceutical, Osaka, Japan) was replaced every three days and cultured for three weeks. The LAK cells were generated from freshly isolated PBMC incubated with 200 U/ml of rIL-2, replacing the medium containing 200 U/ml of rIL-2 every three days for three weeks.

Flow cytometric analysis

Surface maker of lymphocytes were determined by flow cytometry. Cells were incubated with monoclonal antibodies (mAbs) for anti-CD4, anti-CD8, anti-CD16, anti-CD56, anti-TCR-$\alpha\beta$ and anti-TCR-$\gamma\delta$ (all from Becton-Dickinson, Mountain Veiw, CA) at 4℃ for 20 min and washed twice. After fixation with 1% paraformaldehyde, 5×10^3 variable cells were analysed with FACScan (Becton-Dickinson, Mountain Veiw, CA).

Preparation of target cells

Human glioma line U251 was purchased from Riken Cell Bank (Tukuba, Japan). NP1 and NP2 are human glioma cell lines established in our institute. Raji cells were from NK resistant human Burkkit's lymphoma cell line. These cell lines were used as target cells for cytotoxic assays of healthy donors. Fresh glioma cells were produced by preparation of single-cell suspensions from fresh tumor specimens digested enzymatically with RPMI 1640 containing 0.2% collagenase and 0.02% hiarunonidase at 37℃ in 5% CO_2 for 6h. Autologous tumor cells and U251 were used as target cells for cytotoxic assays of patients with malignant glioma.

Cytotoxic assays

Specific cytotoxic assay was performed by ^{51}Cr release assay. Target cells were labeled with 1μCi sodium chromium for 1 h and washed twice with serum free RPMI 1640. Then 5×10^3 target cells were added to the wells of 96-U bottom plates in which effector cells had previously been added at a volume of 100 μl as the effector, target (E:T) ratios ranging from 40:1 to 5:1. After co-incubation in U-shaped 96-well plates at 37℃ in 5% CO_2 for 4h, the supernatant was harvested and ^{51}Cr content was counted in a gamma scintillating counter. Specific cytotoxicity was calculated by the following formula: specific cytotoxicity = {experimental release-spontaneous release} /{maximal release-spontaneous release}.

Statistical analysis

Samples were tested in triplicate. Each point in the figures represents the mean of three values. The singnificance of the difference between groups was calculated by Student's *t-test*.

RESULTS

Phenotypic analysis of fresh PBMC, GDR cells and LAK cells

Initially examined were surface makers of fresh PBMC from healthy donors and patients with malignant glioma (Table 1). The population of γδ receptor bearing T cells of these cases ranged from 0.7 to 9.0%. Because only $0.5\text{-}1.0\times10^5$ of cells were recovered after immunomagnetic separation from 20ml of fresh venous blood, phenotypic analysis was difficult to assess at this point. After three weeks of culture, 46-92% of cells came to express TCR-γδ, but TCR-αβ bearing T cells and NK cells were also found. This indicates that the depletion of TCR-αβ bearing T cells and $CD16^+$ cells was incomplete and grew rapidly with the stimulations of PHA and rIL-2. On the other hand, there was no significant increase of TCR-γδ bearing T cells when PBMC were stimulated with rIL-2 alone for induction of LAK cells (Table 2 and Table 3).

Table 1. Percentage of TCR-γδ+T cells of freshly isolated peripheral blood mononuclear cells from healthy donors and patients with malignant glioma

		TCR-αβ	TCR-γδ	CD4	CD8	CD16
Healthy donors	No.1	74.2	4.5	44.0	32.9	15.1
	No.2	79.6	3.1	56.4	21.3	7.2
	No.3	70.5	6.8	43.1	28.1	11.0
Patients with glioma	No.1	58.2	0.7	26.7	51.9	16.3
	No.2	55.9	9.0	25.4	42.5	24.3
	No.3	75.8	4.6	27.5	48.2	13.9
	No.4	82.7	2.9	53.7	35.0	2.5
	No.5	71.3	2.1	38.9	30.9	22.0
	No.6	70.3	1.2	45.5	28.5	8.8

Data are percentage of positive cells determined by FACScan from freshly isolated peripheral blood mononuclear cells of eight healthy donors and seven patients with malignant glioma.

Cytotoxic activity of GDR cells from healthy donors

GDR cells derived from healthy donor 1 was studied for cytolytic activities against U251, NP1, NP2, and Raji. These GDR cells could show the cytotoxicity over a wide variety of cell lines, particularly against U251 and NP1 in an MHC non-restricted manner (Fig. 1). We then compared the cytotoxicity of GDR

Table 2. Phenotypic analysis of GDR cells and LAK cells of three healthy donors

Healthy donors	GDR					LAK				
	TCR-αβ	TCR-γδ	CD4	CD8	CD16	TCR-αβ	TCR-γδ	CD4	CD8	CD16
1	7.8	71.2	9.0	12.2	6.4	73.8	3.5	47.1	25.8	17.5
2	18.0	74.4	3.9	20.1	0.4	63.7	5.6	57.3	24.5	7.2
3	20.0	62.5	6.0	33.2	2.5	77.7	8.7	51.6	29.5	3.1

GDR cells are generated by the incubation of γδ T cell rich fractions with stimulations of PHA and rIL-2 for three weeks. LAK cells are generated with stimulations of rIL-2 alone for three weeks. Data are percentage of positive cells determined by FACScan.

Table 3. Phenotypic analysis of GDR cells and LAK cells from six patients with malignant glioma

Patients No.	1		2		3		4		5		6	
	GDR	LAK	GDR	LAK	GDR	LAK	GDR	LAK	GDR	LAK	GDR	LAK
TCRαβ	22.8	30.8	50.1	81.6	N.D.	81.6	36.5	90.4	5.9	92.4	0.9	93.0
TCRγδ	71.8	0.8	46.4	6.9	76.7	5.2	53.6	3.4	74.7	5.9	91.9	0.9
CD 4	0.1	11.8	22.7	56.4	2.7	42.1	42.2	42.2	1.0	83.5	0.2	86.4
CD 8	0.1	52.8	16.7	28.1	1.0	46.9	9.6	49.6	2.8	9.6	1.0	8.3
CD 16	1.4	67.5	2.5	8.4	1.5	5.8	0.1	0.3	14.6	4.4	0.7	0.8

GDR cells are generated by incubation of γδ T cells rich fraction with stimulations of PHA and rIL-2 for three weeks. LAK cells are generated with stimulations of rIL-2 alone for three weeks. Data are percentage of positive cells determined by FACScan.

cells with the LAK activities from three healthy donors against human glioma cell line U251. Although differing to some extent, all GDR cells from three healthy donors exhibited cytolytic activity against U251 as well as LAK cells (Fig. 2).

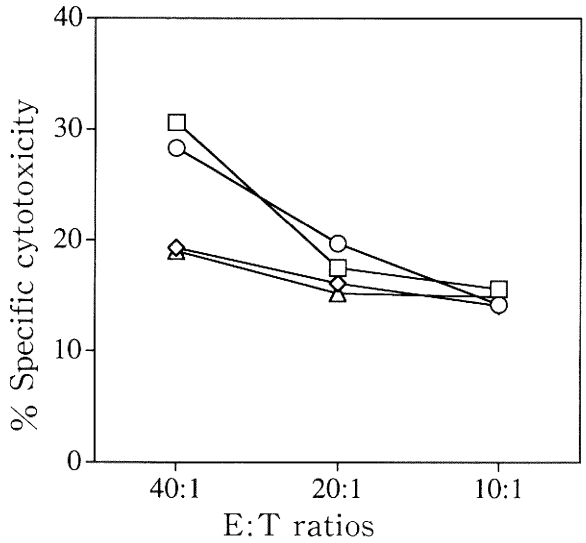

Fig. 1. Cytotoxic activity of GDR cells from healthy donor 1 against a variety of target cells. Each value represents the mean of triplicate cultures; SD was less than 10%. Targets □, U 251; ○, NP 1; ◇, NP 2; △, Raji.

Cytotoxicity of GDR cells from patients with malignant glioma

GDR cells from patients 1, 4, 5 and 6 had strong cytolytic activity for up to 15% against autologous tumor cells at the E:T ratio of 20:1. As for GDR cells from patient 6, more than 90% of cells stained for TCR-$\gamma\delta$ mAb, showing the strongest cytolytic activity among six patients, which was up to 50% lysis against autologous tumor cells at the E:T ratio of 40:1. Because these GDR cells contained very small populations of TCR-$\alpha\beta^+$ $CD8^+$ cells and $CD16^+$ cells which usually mediate cell lysis, it was concluded that this strong cytotoxic activity of GDR cells in patient 6 was dependent upon TCR-$\gamma\delta$ bearing T cells. On the other hand, GDR cells from patients 2 and 3 showed modest cytotoxicity against autologous tumor cells (Table 4). Comparing the cytotoxicity of GDR cells with LAK activities from the glioma patients, three out of six GDR cells showed a stronger lytic activity than LAK cells (Fig. 3). Cell numbers of these three GDR cells increased about 100-fold with initial stimulations of PHA and repetitive administrations of rIL-2, while the other three GDR cells increased a modest 10-fold even after three weeks of culture (data not shown). When allogeneic human glioma cell line U251 was used as the target cells, GDR cells also exhibited profound cytolytic activity as well against autologous tumor cells in an MHC non-restricted manner (Fig. 4).

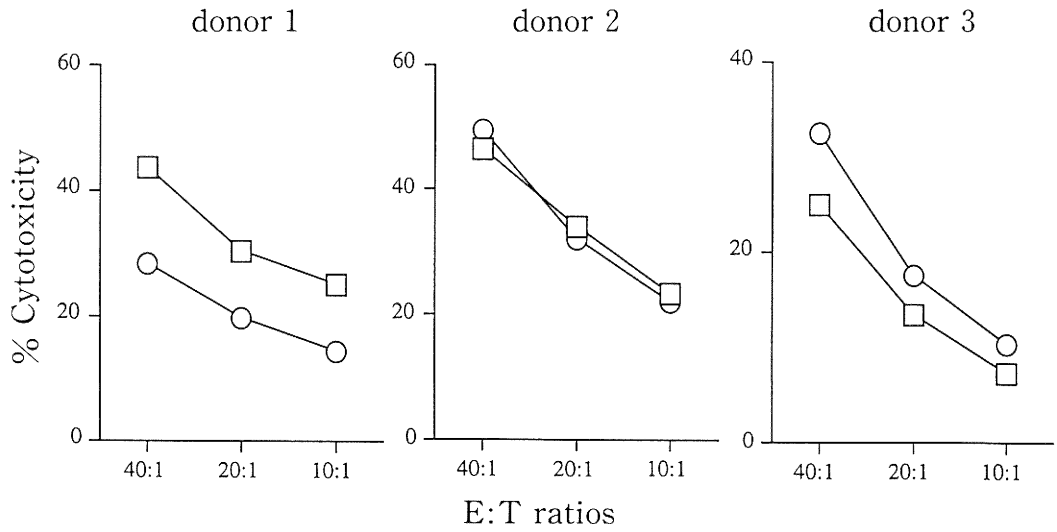

Fig. 2. Comparison of cytotoxic activity between GDR cells and LAK cells from three healthy donors against U251. donor 1, (*left*); donor 2, (*middle*); donor 3, (*right*); ○, GDR; □, LAK. Each value represents the mean of triplicate cultures; SD was less than 10%.

DISCUSSION

The precise biological function of γδ T cells is not well understood. Although only minor populations of peripheral blood $CD3^+$ T cells express TCR-γδ in humans, γδ T lymphocytes are abundantly found in certain epithelia such as the intestine, lung, and skin. According to their unique tissue distribution and reactivity to some kinds of mycobacterium, particularly to mycobacterial heat shock protein (HSP)[27,28] like *Mycobacterium tuberculosis* and *Mycobacterium leprae*, it has been suggested that these cells may represent a first line of defense against infections.[17] Recently, it has also been observed that activated γδ T cells or γδ T clones show a non-specific killing activity against a variety of tumor cells in a non-MHC restricted manner.[18-25] From these data, it is supposed that γδ T cells may be involved in immune surveillance against neoplastic cells. From a therapuetic point of view, investigations of cytotoxic activity of γδ T cells using clinical material are rare. Wang et al. showed that peripheral blood γδ T cells activated by sonicated antigens of *M. tuberculosis* exhibited strong cytolytic activity against two NK-resistent bladder carcinoma *in vitro*.[24] There are also some reports that γδ tumor-infiltrating lymphocytes from renal carcinoma[18] and lung carcinoma[25] exhibit selective lysis of autologous tumor cells. In this report, we demostrated that peripheral blood γδ T lymphocytes from patients with malignant glioma exhibited cytotoxic activity against not only autologous glioma cells but also against allogeneic glioma cell lines. As $CD8^+$ cells and $CD16^+$ cells were contaminated because of the incomplete negative selection of γδ T cells in many GDR cells from patients with glioma, it was speculated that tumoricidal effects of GDR cells might be partially influenced by $CD8^+$ cells and $CD16^+$ cells. However, in the GDR cells of patient 6, approximately 92% of GDR cells expressing TCR-γδ had the strongest cytotoxic activity against glioma cells among all effector cells including LAK cells. Moreover, these GDR cells had about a 10-fold greater killing activity

Table 4. Cytotoxic activity of GDR cells from six patients with malignant glioma against autologous tumor cells

Patients No.	E:T ratios			
	40:1	20:1	10:1	5:1
1	28.1	18.0	7.1	ND
2	15.0	10.8	10.0	8.0
3	ND	7.9	5.8	3.2
4	38.8	28.0	15.9	3.2
5	22.9	16.7	13.1	11.1
6	50.2	38.0	27.4	18.9

Cytotoxic activity was measured by ^{51}Cr release assay. Each value represents the mean of triplicate cultures; SD was less than 10%. ND, not done.

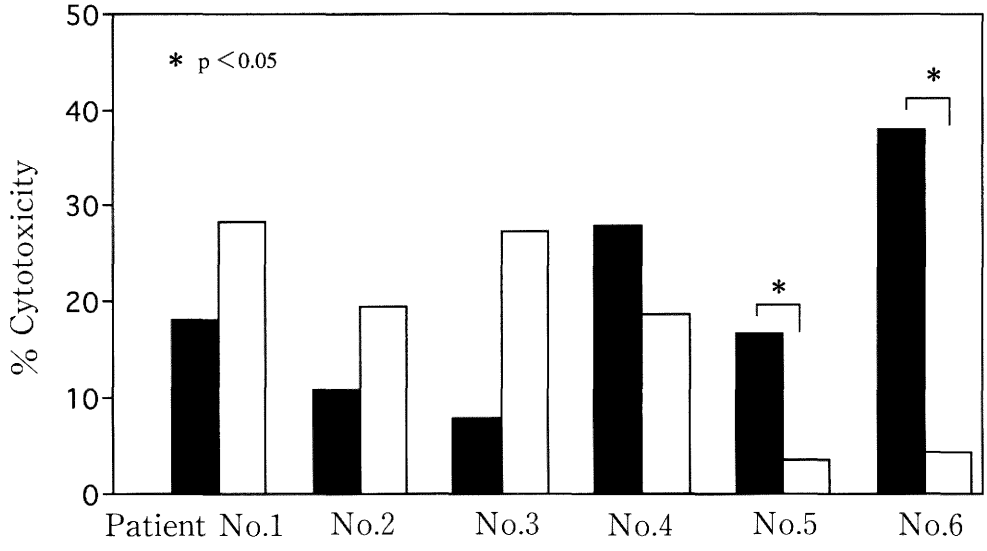

Fig. 3. Comparison of cytotoxic activity between GDR cells and LAK cells from six patients. % specific lysis is at an E:T ratio of 20:1, because data of an E:T ratio of 40:1 was not culled from patient 3. ■, GDR; □, LAK. Each value represents the mean of triplicate cultures; SD was less than 10%.

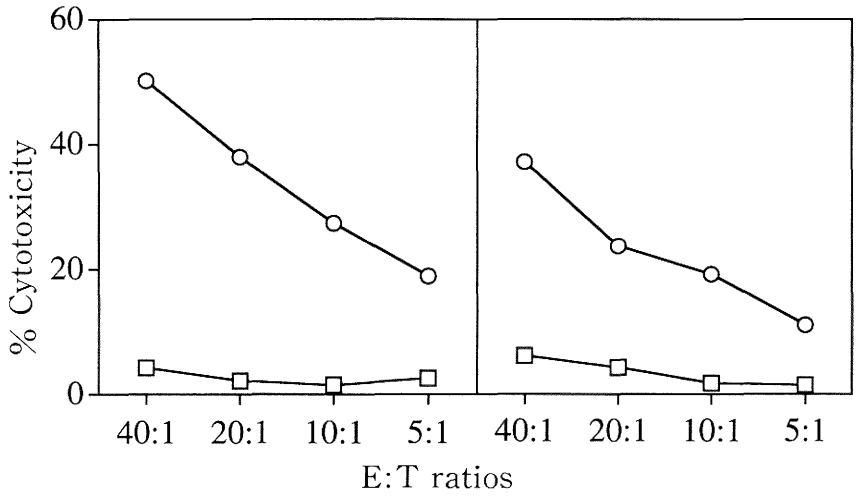

Fig. 4. Cytotoxic activity of GDR cells from patient 6 against different targets. Target is autologous tumor cells (*left*), target is glioma cell line U 251 (*right*). ○, GDR; □, LAK.

compared with that of LAK cells. This indicates that highly purified γδ T cells have the potential to lyse neoplastic cells efficiently.

There still remains the problem of the different proliferative response of γδ T cells with mitogens among patients with malignant glioma reflecting on their cytotoxic activity. This may be due to the abrogation of the reactivity of T lymphocyte by systemic anti-cancer therapies such as steroids, irradiation, and chemotherapy. Suppression of cellular immunity has long been observed in patients with malignant glioma.[29,30] To date, some candidates of potent immunosuppressive factors have been identified. The TGF-β family, initially described as a gliobalstoma-derived suppressor factor, inhibits the activation of cytotoxic T cells and LAK sells, and decreases production of interferon (INF), down-regulating the MHC complex expression on the surface of glioma cells. They can also inhibit the proliferation of LAK cells and antitumor cytotoxicity *in vitro*.[7-11] IL-10 is also proven to be secreted by human glioma cells. IL-10 inhibits the production of INF-γ, IL-1α, IL-1β, IL-6, IL-8, granulocyte colony stimulating factor and granulocyte-macrophage stimulating factor from lymphocytes, eventually inhibiting the cytotoxicity of the responding cells.[12,13] Immune suppression of these agents appears to impair partly, the proliferation of some GDR cells. Moreover, the negative selection of γδ T cells using many kinds of antibodies and immunomagnetic particle would damage residual γδ T cells because these reagents contain sodium azaids toxic to living cells. In this study, we used negative selection with immunomagnetic beads to enrich γδ T cells. This method may not be practical when considering clinical trials in the future because these procedures are complicated and require many kinds of mAbs including immunomagetic beads. For further trials we have to attempt other reagents to stimulate γδ T cells, such as a combination of immobilized anti CD-3 antibody and higher concentrations of IL-2.[31] IL-12, an essential cytokine for the induction of cytotoxic T cells, may also be a very good candidate for stimulation with IL-2.[32] However, extent concentrations of IL-12 are reported to downregulate cytolytic activities of γδ T cells.[33]

Futhermore, there seems to be some benefit to use γδ T cells as effector cells. Recently, some studies have reported on the responsiveness of human and murine γδ receptor bearing T cells with certain kinds of mycobacterial antigen such as *M. tuburculosis* and *M. Leprae*, and the antigen recognized by these cells was defined as HSP.[27,28,34] Several types of the HSP family, highly conserved proteins from prokaryotes to eukaryotes, have been considered as tumor associated or tumor rejection antigens.[35-37] In fact, T cells showing a response to HSP expressed on tumors are reported for $CD4^+$ T cells[38,39] and for $CD8^+$ T cells.[40] In addition, $CD4^-$ and $CD8^-$ double negative T cells are also reported to react to 70kDa heat shock cognate protein, which is expressed on the activated H-*ras* oncogene-transformed rat fibrosarcoma.[36] It has been also already proven that human peripheral γδ T cells recognize hsp60-related molecules on Daudi Burkitt's lymphoma cells.[41] It is not clear whether HSP is induced by some kinds of stress on

glioma cells. Moreover, if HSP on glioma cells could be recognized as a tumor associated antigen by TCR-γδ, one would anticipate that γδ T cells receive their specific killing activity mediated from interaction between TCR-γδ and HSP expressed on the target cells in addition to their non-specific cytotoxicity.

In conclusion, our data indicates that activated γδ T cells might be applied as new effector cells of local AIT for patients with malignant gliomas because malignant gloimas hardly ever metastasize outside the brain.

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