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Systemic Intravenous Adoptive Transfer of Autologous Lymphokine-activated αβ T-Cells Improves Temozolomide-induced Lymphopenia in Patients with Glioma

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Abstract. *In this clinical study, we investigated the safety and clinical usefulness of systemic adoptive immunotherapy using autologous lymphokine-activated αβ T-cells (αβ T-cells), combined with standard therapies, in patients with malignant brain tumors. Twenty-three patients with different malignant brain tumors, consisting of 14 treated with temozolomide (TMZ group) and 9 treated without temozolomide (non-TMZ group), received systemic intravenous injections of αβ T-cells (mean=10.4 injections/patient for the TMZ group, and 4.78 for the non-TMZ group). No significant adverse effects associated with the αβ T-cell injection were observed, and the total lymphocyte count (TLC) improved significantly in the TMZ group after five injections. Furthermore, CD8-positive or T-cell receptor V gamma -positive cells were increased with TLC in three patients with glioblastoma multiforme. These findings suggest that systemic αβ T-cell immunotherapy is well tolerated, and may help restore an impaired and imbalanced T-cell immune status, and temozolomide- and/or radiotherapy-induced lymphopenia. Future prospective study is needed to clarify the clinical merits of this immunotherapy.*

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Primary and metastatic malignant brain tumors are intractable cancers for which there are limited treatment options and an extremely poor prognosis. New therapeutic strategies, including chemotherapies involving novel targets, and more effective radiotherapies, need to be continuously developed (1-5). Glioblastoma multiforme (GBM) is one of the most aggressive and highest-grade primary brain tumors. The current standard therapy for GBM includes daily chemotherapy using temozolomide, an orally available DNA-alkylating agent, combined with standard focal irradiation (RT), followed by cyclic temozolomide chemotherapy; this treatment significantly prolongs the survival of these patients (6). However, despite many advances in standard chemoradiotherapy, the prognosis for patients with GBM remains poor.

Many new approaches are currently being investigated for treating malignant brain tumors including GBMs, among which immunotherapy is very promising and attractive (1-4). Adoptive immunotherapy using lymphokine-activated killer (LAK) cells (4, 7-10), and active immunotherapies involving the vaccination of peptides related to tumor-associated antigens or dendritic cells (1-4) have been examined. Recently, immune checkpoint inhibitors (2, 3), and the adoptive transfer of chimeric antigen receptor

(CAR)-transduced T-cells (3, 11) have been intensively studied. These immunotherapies have been applied to patients with brain tumors, alone or in combination with other agents or therapies, with promising results in many cases. It is expected these immunotherapies will make significant contributions to the treatment of various malignant brain tumors in the future. However, the appropriate protocols for immunotherapies in combination with conventional standard therapies are not well established, and the *in vivo* treatment-induced immunological improvements have not been fully verified.

In this clinical study, we investigated the safety and clinical usefulness of systemic adoptive immunotherapy using autologous lymphokine-activated αβ T-cells (αβ T-cells) in combination with standard therapies in patients with malignant brain tumors to develop a new therapeutic strategy for malignant brain tumors.

Patients and Methods

Patient population and eligible criteria. This clinical protocol was carried out in accordance with the principles of the Helsinki Declaration, and approved by the ethical committee (no. 75) and the Institutional Review Board (no. 16109) of Osaka National Hospital. Written informed consent was obtained from all patients before they were entered into the study and data for each patient was coded.

Eligibility criteria were as follows: patients aged ≤75 years with clinically or pathologically proven primary or recurrent malignant brain tumors, a life expectancy 2 months or more, Eastern Cooperative Oncology Group performance status (ECOG PS) of 0 to 3, normal bone marrow, liver, kidney, heart, and lung function, no other serious complication, and no serious drug allergies. Patients serologically positive for hepatitis B virus surface antigen, or with antibodies to hepatitis C virus, human immunodeficiency virus, human T-cell leukemia virus type 1, or syphilis were excluded.

Generation of autologous αβ T-cells. Autologous αβ T-cells were generated as previously described (12-14). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from the patient's peripheral blood using BD Vacutainer® (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The autologous plasma was simultaneously collected and stored at 4˚C until use. PBMCs were activated in a culture flask with an immobilized monoclonal antibody (Ab) to CD3 (Orthoclone OKT®3 Injection; Janssen Pharmaceuticals, Japan, or MACS GMP CD3 pure; Miltenyi Biotec, Bergisch Gladbach, Germany) in an interleukin-2 (IL2)-containing culture medium (ALyS203; NIPRO, Osaka, Japan) supplemented with autologous plasma for 4 days. The cells were then transferred to a culture bag containing ALyS203 medium and cultured for 14 days. After culture, the αβ T-cells were harvested and suspended in 100 ml of saline for intravenous injection.

For quality control, bacterial and fungal tests by standard culture methods, mycoplasma detection by polymerase chain reaction, and endotoxin detection using Endosafe®-PTS™ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were performed on the intermediate product and on the final product before each administration. Real-time quantitative polymerase chain reaction-based genetic polymorphism analyses were performed for individual identification before use (15).

Flow cytometric (FCM) analysis. The phenotypes of αβ T-cells were characterized by staining using a fluorescein isothiocyanate (FITC)-conjugated CD3 Ab, R-phycoerythrin(PE)-conjugated CD4 Ab, Peridinin-chlorophyll protein(PerCP)-Cyanin(Cy)5.5-conjugated CD8 Ab, and Allophycocyanin (APC)-conjugated CD56 Ab for 30 min at 4˚C. The patients' PBMCs before and during immunotherapy were also reacted with the following primary antibodies for 30 min at 4˚C: FITC-conjugated CD3 Ab, PerCP-Cy5.5-conjugated CD45 Ab, PE-conjugated CD4 Ab, PerCP-Cy5.5-conjugated CD8 Ab, APC-conjugated CD56 Ab, APC-conjugated CD69 Ab, and PE-conjugated T-Cell receptor V gamma (TCRVγ) 9 Ab. After being washed, the stained samples were analyzed by a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). All of the Abs used in the FCM analyses were from BD Biosciences.

Adoptive αβ T-cell immunotherapy. In patients receiving or not receiving cyclic chemotherapies, the autologous αβ T-cells were intravenously transferred five times at 2-week intervals in the first course. Thereafter, continual injections were performed at intervals of 2 or ≥4 weeks. Conventional standard therapies were continued during the αβ T-cell immunotherapy.

Clinical assessment. Clinical assessment included clinical observations, blood tests, and magnetic resonance imaging (MRI). The target lesion size was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) ver 1.1(16). Adverse events were graded using the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. Overall survival (OS) time was defined as the interval from the first operation date to the date of death or the final assessment, and obtained using the Kaplan–Meier method.

Statistical analysis. Statistical analysis was conducted using the Mann–Whitney *U*-test or Kruskal–Wallis H-test. A value of $p<0.05$ was considered statistically significant.

Results

Patient characteristics. From February 2008 to July 2013, a total of 23 patients with various malignant brain tumors (16 gliomas, three intracranial germ cell tumors, three metastatic brain tumors, and one malignant transformation of an epidermoid tumor) underwent adoptive autologous αβ T-cell immunotherapy (Table I). Among them, 14 patients were treated in combination with standard temozolomide chemotherapy (TMZ group), and the other nine patients were treated without temozolomide chemotherapy (non-TMZ group) (Table I). The mean ages of patients in the TMZ and non-TMZ groups were 39.8 years and 42.9 years, respectively, which were not significantly different (Table II).

Representative cases

ONH-LAK20: A 43-year-old female developed decreasing spontaneous speech and appetite. MRI showed a gadolinium (Gd)-enhanced intramedullary mass in the right frontal lobe. Surgical excision was performed, and the pathological diagnosis was GBM, WHO grade 4 (Figure 1A). The patient then underwent standard adjuvant therapy combined with

Table I. *Summary of patient clinical characteristics*.

Case no.	Age, years	Gender	Diagnosis (WHO grade)	Location	Treatment history			KPS
					Surgery	Chemotherapy	Radiotherapy (dose, Gy)	
TMZ group (n=14)								
ONH-LAK3	57	M	GBM (G4)	Rt. parietal	BP	TMZ,IFN-β	Local (60)	90
ONH-LAK17	68	F	GBM (G4)	Lt. parietal	PR	TMZ	Local (60)	100
ONH-LAK20	43	F	GBM (G4)	Rt. frontal	TR	TMZ,IFN-β	Local (60)	50
ONH-LAK23	56	F	GBM (G4)	Rt. parietal	TR	TMZ	Local (60)	90
ONH-LAK24	49	M	GBM (G4)	Lt. frontal	PR	TMZ	Local (60)	90
ONH-LAK2	39	F	AOA (G3)	Lt. cerebellum	PR	TMZ	Local (60)	90
ONH-LAK26	37	M	AOG (G3) rec	Lt. frontal	PR	TMZ,DCvaccination, peptidevaccine	Local (60)	90
ONH-LAK6	34	M	Gliomatosis cerebri (lower grade glioma)	Lt. hemisphere	BP	TMZ,IFN-β	Local (56)	90
ONH-LAK18	56	F	Astrocytoma (G2) rec Secondary GBM (G4)	Rt. frontal	BP	1. ACNU(i.a.) 2. Procarbazine, ACNU,VCR 3. IFN-β	None	90
ONH-LAK19	55	F	DIPG	Brain stem (pons)	None	TMZ	Local (50)	80
ONH-LAK12	19	F	DIPG	Brain stem (pons, medulla)	None	TMZ	Local (40)	90
ONH-LAK16	8	F	DIPG	Brain stem (pons)	Cyst fenestration	CDDP,VCR,CPA, THP-ADR,TMZ	Local (50)	80
ONH-LAK15	30	M	DIPG	Brain stem	None	TMZ	Local (50.4)	70
ONH-LAK14	6	F	Anaplastic ependymoma (G3)	4thVentricle	STR	1. VCR 2. CBDCA,VCR, IFO,VP-16 3. CDDP,VP-16 4. TMZ,VP-16	WBI (40) Local (total 51.2) SRT (14, γ-knife)	90
Non-TMZ group (n=9)								
ONH-LAK21	34	M	GBM (G4)	Rt temporo-occipital	PR	TMZ	Local (34)	50
ONH-LAK5	42	M	Germinoma with STGC	Pineal gland	Total	IFO,CBDCA,VP-16	Local (50)	80
ONH-LAK8	12	M	Germinoma (yolk sac tumor)	Lt. basal ganglia	BP	1.VCR,CPA, VP-16,CDDP 2.TEPA,VP-16, CPA,PBSCT 3.IFO+VP-16+CBDCA, VCR+CPA+VP-16+CDDP CPT-11+CBDCA	Craniospinal (30) Local (30) SRT (CyberKnife)	80
ONH-LAK22	19	M	Germinoma (embryonal carcinoma)	Pineal gland	TR	1.CPA,VP-16, CDDP, VCR, DEX(i.t.), MTX(i.t.) 2.TPT,CBDCA,IFO,VP-16 3.TMZ,VP-16,CBDCA,PBSCT 4.TEPA,L-PAM,PBSCT 5.TMZ 6.PTX,GEM 7.CPT-11,CBDCA	Local (pineal: 50.4) Local (C6/Th7: 36)	90
ONH-LAK13	31	F	Ganglioglioma (G2)	Brain stem (medulla)	PR	IFN-β	Local	80
ONH-LAK9	59	F	Squamous cell carcinoma (malignant transformation of epidermoid)	Rt. cerebello-pontine angle	STR	None	Local (56, IMRT)	90
ONH-LAK7	60	F	MBT (breast cancer)	Rt. occipital	PR	None	SRT (γ-knife)	90
ONH-LAK10	57	F	MBT (rectal cancer)	Brain stem (pons)	None	None	SRT (γ-knife×2 times)	40
ONH-LAK11	72	M	MBT (lung cancer)	Lt. frontal, Rt. parietal	None	None	SRT (γ-knife)	100

GBM, Glioblastoma multiforme; AOG, anaplastic oligodendroglioma; DIPG, diffuse intrinsic pontine glioma; AOA, anaplastic oligoastrocytoma; STGC: syncytiotrophoblastic giant cells; MBT, matastatic brain tumor; rec, recurrence; BP, biopsy; PR, partial removal; STR, subtotal removal, TR, total removal; ETV, endoscopic third ventriculostomy; i.a., intra-arterial injection; i.t., intra-thecal injection; PBSCT, peripheral blood stem cell transplantation; DC, dendritic cell; TMZ, temozolomide; IFN-β, interferon-beta; ACNU, nimustine; VCR, Vincristine; CDDP, cisplatin; CPA, cyclophosphamide; THP-ADR, pirarubicin; CBDCA, carboplatin; IFO, ifosfamide; VP-16, etoposide; TEPA, triethylene thiophosphoramide; CPT-11, irinotecan; DEX, dexamethasone; MTX, methotrexate; TPT, topotecan; L-PAM, melphalan; PTX, paclitaxel; GEM, gemcitabine; WBI, whole-brain irradiation; SRT, stereotactic radiation therapy; IMRT, intensity-modulated radiation therapy; KPS, Karnofsky performance status.

Table II. *Summary of adoptive αβ T-cell immunotherapy*

	TMZ group	non-TMZ group	*p*-Value*
Number of cases	14	9	-
Mean age (years)	39.8±18.9	42.9±20.4	0.567
Male: female ratio	5:9	5:4	-
Number of injections			
Mean	10.4±9.5	4.78±1.7	0.052
Max	39	8	-
Minimum	1	2	-
Median	8	5	-
Completion rate of first course (%)	78.6	66.7	-
αβT-cells			
Cell number per injection ($\times 10^9$)	7.43±1.91	7.70±1.17	0.527
$CD3^+$ cells (%)	87.94±6.91	89.57±2.18	0.878
$CD3^+/CD4^-/CD8^+$ cells (%)	53.12±12.76	55.40±15.63	0.781
$CD3^+/CD4^+/CD8^-$ cells (%)	25.31±11.45	23.43±11.79	0.734
$CD3^-/CD56^+$ cells (%)	3.37±6.23	1.33±1.26	0.600
CD4/CD8 ratio	0.63±0.45	0.54±0.44	0.600

*Mann–Whitney *U*-test; Values are the mean±S.D.

chemotherapy using temozolomide plus interferon-beta and focal irradiation (60 Gy) (Figure 1B), followed by cyclic temozolomide chemotherapy every 4 weeks (Figure 1C) (6). However, MRI at 9 months after the initial operation showed Gd-enhanced lesions that had increased in size (Figure 1D); the patient visited our hospital to undergo adoptive αβ T-cell immunotherapy.

In the first course, she received five initial injections of αβ T-cells at 2-week intervals in combination with standard temozolomide chemotherapy. A blood test showed that her total lymphocyte count (TLC) was less than 500 cells/mm^3 and the neutrophil/ lymphocyte ratio (NLR) was 7.56 before immunotherapy. After receiving several injections of αβ T-cells, her TLC gradually increased, exceeding 500 cells/mm^3 by the end of the first course (Figure 1E). The interval between injections was then increased to every 4 or 8 weeks. She received a total of 19 αβ T-cell injections (Figure 1E). Her TLC was maintained at more than 1,000 cells/mm^3 for the first year after starting immunotherapy, and did not fall below 500 cells/mm^3 during immunotherapy. Her total white blood cell (WBC) count and neutrophil count also increased, and the NLR decreased and was maintained at around 4 during immunotherapy (Figure 1E). For one year after starting immunotherapy, her neurological and neuroradiological conditions were stable, and no serious adverse event caused by the αβ T-cell immunotherapy was observed (Figure 1F-M). However, the patient's neurological condition gradually worsened, and her family wished to pause the immunotherapy after the 19th injection. The patient's consciousness gradually deteriorated, and she died 3 years and 7 months after the initial operation.

ONH-LAK24: A 49-year-old male developed headache and amnesia, Gd-enhanced MRI showed a ring-enhanced mass lesion in the left frontal lobe (Figure 2A). The lesion was partially resected, and its pathological diagnosis was GBM, WHO grade 4. The patient underwent standard chemoradiation therapy with temozolomide and focal irradiation, followed by cyclic temozolomide chemotherapy every 4 weeks. However, the size of the enhanced lesion continued to increase, and its mass effect and surrounding brain edema continued to worsen. The patient then visited our hospital to undergo αβ T-cell immunotherapy.

In the first course, this patient received five initial injections of αβ T-cells at 2-week intervals with standard cyclic temozolomide chemotherapy. Blood tests showed that the TLC was stable at almost 1,000 cells/mm^3; however, the NLR gradually increased and stayed at over 5.0 during the first course of therapy (Figure 2B). Gd-enhanced MRI showed that the residual ring-enhanced mass lesion in the left frontal lobe was growing, with strong brain edema and mass effects spreading to the contralateral side (Figure 2C and D). Although minor right hemiparesis was suspected to be caused by the growing tumor, no serious adverse events caused by the αβ T-cell immunotherapy were observed in the first course. Immunotherapy was continued, and the patient received 5 additional injections at 2-week intervals in the second course. During the second course, the TLC gradually increased, and NLR also gradually decreased, finally to around 4 (Figure 2B). MRI at the end of the second course showed decreases in the lesion size, mass effect, and brain edema (Figure 2 E and F). The immunotherapy was assessed as being effective, and four more injections at 4-week intervals were performed in the third course. The TLC remained stable at over 1,000 cells/mm^3 continuously from the second course, and the NLR finally fell and stayed below 4. MRI at the end of the third course showed significant decreases in the lesion size and its mass effects (Figure 2G and H), therefore we evaluated the efficacy of immunotherapy as partial response (PR) according to the RECIST criteria. The patient's neurological symptoms were well improved, and no serious immunotherapy-related adverse events had been observed. The immunotherapy was finished, and the patient was kept on cyclic temozolomide chemotherapy every 4 weeks. One hundred days after the last αβ T-cell injection, the patient received a single maintenance injection. At this point, his immunological status was stable, and he had received a total of 15 αβ T-cell injections.

Adoptive injections of αβ T-cells. The mean number of αβ T-cell injections received by the TMZ and non-TMZ groups was 10.4 and 4.78, respectively (Tables II and III). The TMZ group tended to have more injections, but the difference did not reach statistical significance (Table II). The completion rate of the first course was higher in the TMZ group (Table II).

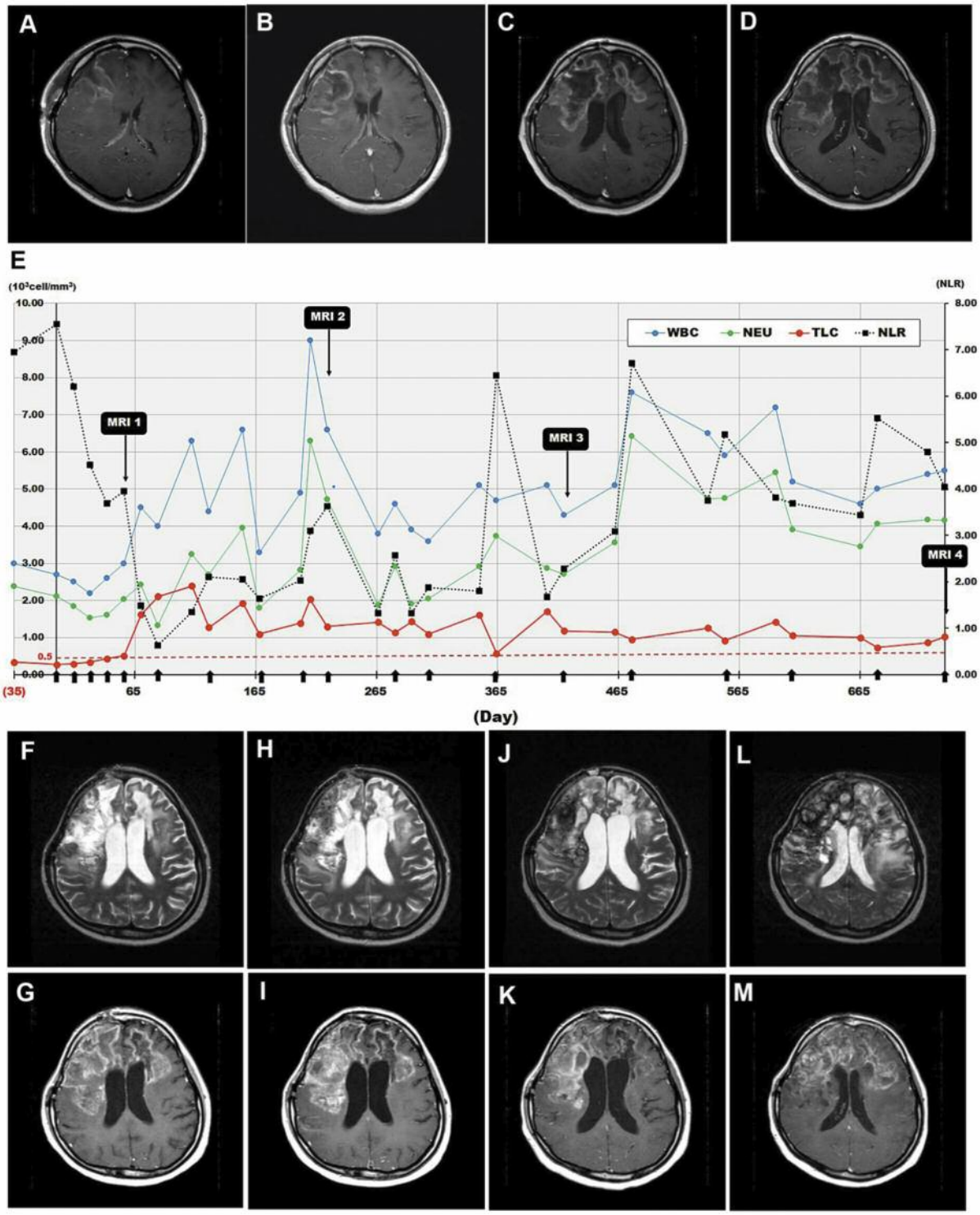

Figure 1. *Clinical course of patient ONH-LAK20. Gd-enhanced magnetic resonance images after surgical resection (A) and initial temozolomide/irradiation (B) showed a Gd-enhanced lesion in the right frontal lobe. Five months later, Gd-enhanced lesions were identified in the bifrontal lobes (C), and showed gradual enlargement at 9 months after the initial operation (D). Line graph in (E) shows the clinical course of the αβ T-cell immunotherapy. Black arrows on the x-axis indicate the time points of αβ T-cell injection. total white blood cell (WBC) count, neutrophil (NEU) count, and total lymphocyte count (TLC) during immunotherapy are shown using the left y-axis), neutrophil/lymphocyte ratio (NLR) is shown using the right y-axis. T2-Weighted (F, H, J, and L) and Gd-enhanced (G, I, K, and M) magnetic resonance images showed that the lesions were stable at the 5th (F and G), 10th (H and I), 14th (J and K), and 19th (L and M) αβ T-cell injection.*

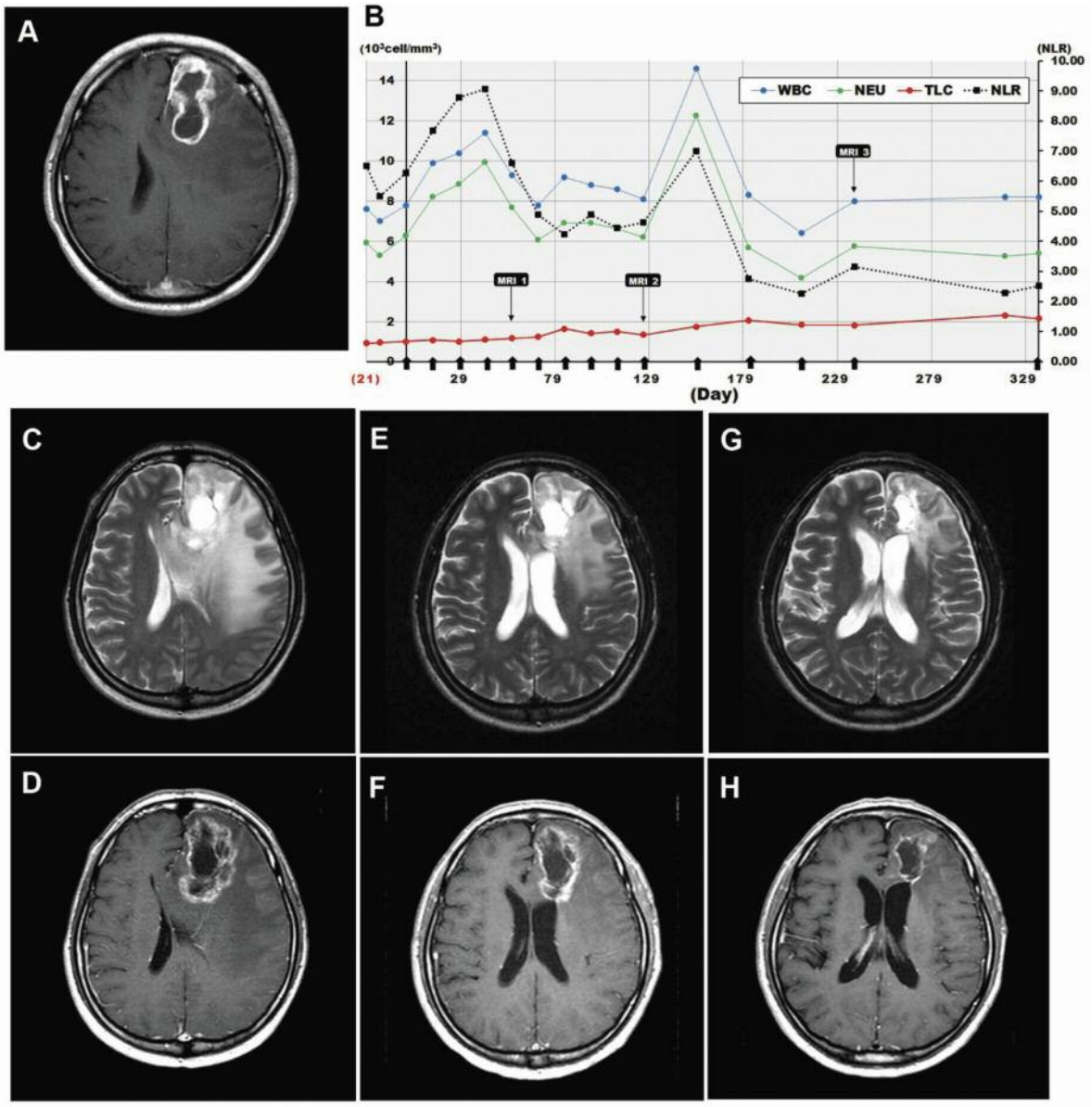

Figure 2. *Clinical course of patient ONH-LAK24. Gd-enhanced magnetic resonance images before surgical resection (A) showed a Gd-enhanced lesion in the left frontal lobe. Line graph (B) shows the clinical course of the αβ T-cell immunotherapy. Black arrows on the x-axis indicate the time points of αβ T-cell injection. total white blood cell (WBC) count, neutrophil (NEU) count, and total lymphocyte count (TLC) during immunotherapy are shown using the left y-axis, neutrophil/lymphocyte ratio (NLR) is shown using the right y-axis. T2-Weighted (C, E, and G) and Gd-enhanced (D, F, and H) magnetic resonance images showed decreasing lesions at the 5th (C and D), 10th (E and F), 14th (G and H) αβ T-cell injection.*

The cell number per injection was similar between the two groups, and FCM analysis of the αβ T-cells showed that the numbers of T-cells ($CD3^+$ cells), $CD4^+$ cells, $CD8^+$ cells, and $CD56^+$ cells, and the CD4/CD8 ratio were also almost the same between the two groups, without any statistically significant differences (Table II). These findings indicated that the TMZ and non-TMZ groups received activated αβ T-cell treatments of equivalent quality and quantity.

Adverse events and safety. Several neurological (grade 1 or 2), and hematological/investigational (grade 1 to 3) adverse events were observed in both groups during the αβ T-cell immunotherapy (Table III). However, they were all thought to be caused by progression of the disease or side-effects of the anticancer agents, including temozolomide, used with the immunotherapy; thus, it was ascertained that no serious neurological or hematological/investigational complication

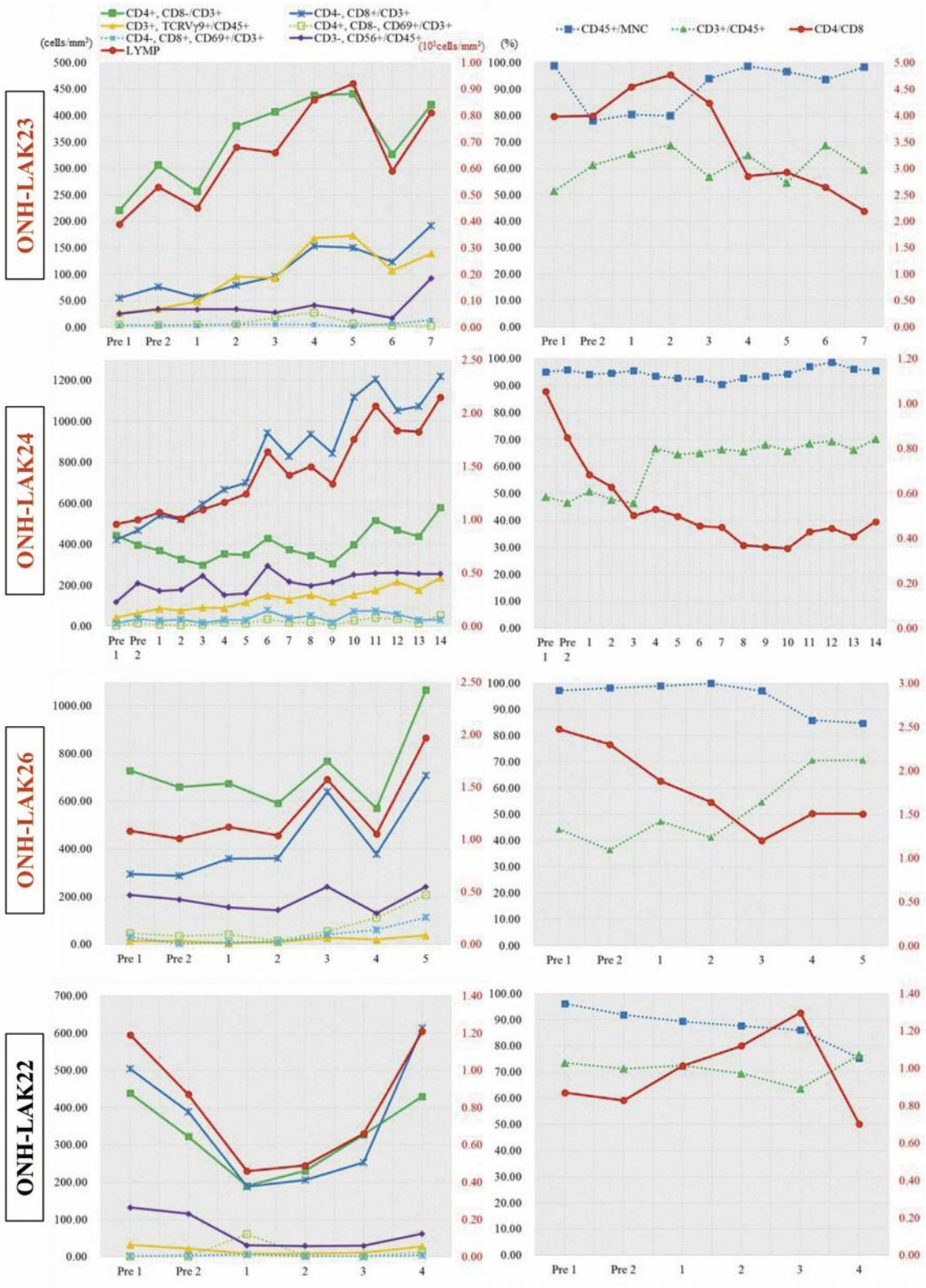

Figure 3. *Lymphocyte population analysis during αβ T-cell immunotherapy. Line graphs indicate the number of each sub-population of lymphocytes in peripheral blood mononuclear cells in four patients (three of the temozolomide group: ONH-LAK 23, 24, and 26; and one of the non-temozolomide group: ONH-LAK 22) during αβ T-cell immunotherapy (left y-axis). The total lymphocyte count (TLC) and CD4/CD8 ratio are shown in red (right y-axis). The x-axis shows the time points of αβ T-cell injection (pre injection point 1 to the 14th injection).*

was caused by injection of αβ T-cells. On the other hand, several grade 1 events, including fever (n=5; 21.7% of total), dizziness (n=5; 21.7% of total), and fatigue (n=4; 17.4% of total), were observed in both groups. These grade 1 adverse events may have been related to the αβ T-cell injections but all were very minor and not clinically problematic or serious. Taken together, these findings suggested that the adoptive injection of αβ T-cells in patients with malignant brain tumors was safe and caused no serious treatment-related complications, with or without combination temozolomide chemotherapy.

Hematological and immunological status. To evaluate the effects of αβ T-cell immunotherapy on the hematological and immunological status of patients, the blood test results of both groups were compared before and during the immunotherapy treatment. Before immunotherapy, the TLC of the TMZ group was significantly lower than that of the non-TMZ group (p=0.04, Mann–Whitney U-test, Table IV). However, there was no significant difference in any of the other characteristics between the two groups (Table IV). During immunotherapy, blood tests were performed twice in each group, at statistically equivalent time points (T1 and T2), and the TMZ group was further evaluated at one later time point (T3). Within the TMZ group, the TLC was significantly higher at T2 (p=0.009, Kruskal–Wallis H-test, TLC in Table IV); no significant differences in TLC between the TMZ and non-TMZ group were observed at this point (Table IV). There was also no significant difference in the other results between the TMZ and non-TMZ groups during immunotherapy. The NLR of the TMZ group was higher than that of the non-TMZ group before immunotherapy, and became smaller than that of the non-TMZ group at T2, but these differences were not statistically significant (Table IV).

To obtain a more precise analysis, we monitored some patients' lymphocyte population within PBMCs during immunotherapy (Figure 3). In all three patients in the TMZ group examined, the TLC increased, and increases in the number of $CD8^+$ or $TCRV\gamma^+$ cells were coupled with the TLC increase (Figure 3). Although the $CD4^+$ cells also increased in number, the CD4/CD8 ratio gradually decreased in the TMZ group, but not in patient ONH-LAK 22 (from the non-TMZ group) (Figure 3). These findings indicate that the injected αβ T-cells had some effect on increasing several sub-populations of T-cells *in vivo*, which might have contributed to an improvement in the patients' immunological status, especially in patients treated with temozolomide.

Clinical outcomes. In the TMZ group, three cases were PR, and seven were stable disease (SD) (Table III). The disease control rate (PR+SD/total cases) was 71.4%. On the other hand, in the non-TMZ group, one case was PR, three were SD, and the disease control rate was 44.4%. The median OS of the five GBM cases in the TMZ group treated with more than five αβ T-cell injections was 21.4 months (95% CI=79.3-not available).

Discussion

Adoptive αβ T-cell immunotherapy has been applied to patients with brain tumor using various methods. Historically, *ex vivo* IL-2-activated T-cells generated from PBMCs (7-9) or lymph nodes (10) were transferred into patients with malignant glioma by direct injection into the brain tissue surrounding the cavity remaining after operative tumor removal (7), by intracavitary infusion through the reservoir (8, 9), or by intravenous injection (10). In these studies, the αβ T-cells were injected alone or with IL-2. These previous studies showed that adoptive αβ T-cell immunotherapy can be administered safely to patients with brain tumor, and indicated some clinical merits for patient prognosis (7-9). However, most of these studies were carried out before temozolomide was available, hence the safety and efficacy of this immunotherapy in patients treated with temozolomide or other standard therapies have not been fully examined.

In this clinical study, we first carefully examined the safety of the intravenous systemic adoptive injections of αβ T-cells for patients with malignant brain tumors, and confirmed that the injected αβ T-cells caused no serious treatment-related complications when given alone or combined with temozolomide chemotherapy. The reported adverse events associated with immune-cell therapy for various malignancies include fever (grade 1 and 2) and fatigue (grade 1 and 2), observed in 2.7% and 13.8% of 484 activated αβ T-cell therapy procedures, respectively. No other serious treatment-related complication was observed, suggesting that αβ T-cell immunotherapy for cancer treatment is well tolerated (12). In our study, the adverse events fever, dizziness, and fatigue were observed in about 20% of the patients. While this frequency may be slightly higher than that reported previously, all were grade 1 with very minor symptoms, and it was possible that some of them were caused by the brain lesions themselves, or by concomitant drugs such as temozolomide. Taken together, we concluded that the adoptive injections of αβ T-cells were safe for patients with malignant brain tumors, and caused no serious treatment-related complications.

Next, we examined the effects of the injected αβ T-cells on the patients' immunological status. It is reported that RT/temozolomide therapy sometimes causes serious treatment-related lymphopenia (17-19), and preferentially reduces the proportion of $CD4^+$ T-cells (17). In addition, radiation therapy with steroids induces a decrease in $CD4^+$ T-cells (20). Findings indicate that such temozolomide and/or radiation therapy-induced lymphopenia is related to a poor prognosis (17-20). An NLR >4 before treatment (21) or prior

Table III. *Summary of adverse events and clinical outcomes*.

Case no.	Number of αβ T-cell injections	Combined chemotherapy	Best clinical response	Adverse event (CTCAE v.4.0 Grade)			OS (months)	Outcome
				Neurological	Hematological/ investigational	Other		
TMZ group (n=14)								
ONH-LAK3	9	TMZ, IFN-β	PD	Gait disturbance (G2) Lt. hemiparesis (G2)	Hypoalbuminemia (G1) Anemia (G1) GGT increased (G1) ALP increased (G1) Platelet count decreased (G1)		18.5	Dead
ONH-LAK17	8	TMZ	SD	Diplopia (G1) Rt. hemiparesis (G1)	ALT increased (G1) AST increased (G1) GGT increased (G2)	Dizziness (G1)	13.1	Dead
ONH-LAK20	19	TMZ	SD	Gait disturbance (G2) Seizure (G2)	ALT increased (G1) AST increased (G1) Cholesterol high (G2)	Fever (G1) Lung infection (G1) Weight loss (G2) Anorexia (G3)	43.1	Dead
ONH-LAK23	8	TMZ	SD	Lt. paresthesia (G1)	-	-	21.5	Dead
ONH-LAK24	15	TMZ	PR	Rt. hemiparesis (G1)	ALT increased (G1), AST increased (G1) GGT increased (G1)	Dizziness (G1)	18.3	Alive
ONH-LAK2	4	TMZ	PR	-	-	-	5.1	Stopped
ONH-LAK26	6	TMZ	PD	-	-	-	148.1	Dead
ONH-LAK6	5	TMZ	SD	-	-	Fatigue (G1)	6.9	Stopped
ONH-LAK18	39	TMZ, IFN-β	PR	Dysarthria (G1) Lt. paresthesia (G1) Rt. facial nerve disorder (G2) Lt. hemiparesis (G2)	-	Fatigue (G1) Ear pain (G1) Nausea (G1)	205.7	Alive
ONH-LAK19	12	TMZ	SD	-	-	Fever (G1) Dizziness (G1) Urinary retention (G1) Dysphagia (G2) Anorexia (G3)	18.4	Dead
ONH-LAK12	10	TMZ	SD	-	-	Fever (G1)	9.1	Stopped
ONH-LAK16	5	TMZ, VP-16, IFN-β	SD	Diplopia (G1) Gait disturbance (G2)	-	Fatigue (G1) Dizziness (G1) Dysphagia (G2)	39.9	Dead
ONH-LAK15	1	TMZ, IFN-β	NA	-	-	-	17.9	Stop
ONH-LAK14	4	TMZ, VP-16	PD	-	Platelet count decreased (G3)	-	34.4	Stopped
Non-TMZ group (n=9)								
ONH-LAK21	2	None	NA	-	-	-	8.3	Stopped
ONH-LAK5	8	None	SD	-	-	-	98.6	Stopped
ONH-LAK8	4	None	PD	-	-	Edema lower limbs (G1)	30.5	Stopped
ONH-LAK22	5	CBDCA, MTX (i.t.)	PD	Gait disturbance (G2)	Anemia (G1) White blood cell decreased (G1) GGT increased (G1) Platelet count decreased (G3) Lymphocyte count decreased (G3)	Fever (G1) Nausea (G1) Anorexia (G2)	72.2	Stopped
ONH-LAK13	6	None	PR	-	-	Fever (G1) Fatigue (G1)	215.2	Stopped
ONH-LAK9	5	None	PD	Ataxia (G1)	-	Dizziness (G1)	26.0	Stopped
ONH-LAK7	5	None	SD	-	Hypokalemia (G1)	-	10.7	Stopped
ONH-LAK10	3	None	PD	-	-	Anorexia (G3) Lung infection (G3)	11.5	Dead
ONH-LAK11	5	None	SD	-	-	-	4.9	Stopped

TMZ, Temozolomide; IFN-β, interferon-beta; CBDCA, carboplatin; VP-16, etoposide; MTX, methotrexate; i.t., intra-thecal injection; GGT, γ-glutamyltransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate transaminase; PR, partial response; SD, stable disease; PD, progressive disease; NA, not available.

Table IV. *Hematological and immunological status before and during adoptive αβ T-cell immunotherapy.*

		TMZ group (n=10)	*p*-Value[a]	Non-TMZ group (n=5)	*p*-Value[a]	*p*-Value[b]
Number of injections at assessment						
	T1	2.10±0.32	3.41E-06	2.10±0.00	0.067	1.000
	T2	5.10±0.74		6.50±2.12		0.288
	T3	8.88±1.36		NA		
Population analysis						
WBC count (10^3/mm^3)	Pre	4.64±1.54	0.425	6.91±5.26	0.881	0.572
	T1	5.76±2.66		6.75±4.02		0.635
	T2	6.45±2.52		6.60±1.41		0.909
	T3	6.80±4.07		NA		
NEU count (10^3/mm^3)	Pre	3.32±1.43	0.570	5.11±4.58	0.751	0.594
	T1	3.32±1.43		4.87±3.13		0.733
	T2	3.32±1.43		5.28±1.54		0.606
	T3	5.10±3.78		NA		
TLC (10^3/mm^3)	Pre	0.79±0.45#	0.009*	1.34±0.50	0.601	0.040#
	T1	1.00±0.39		1.46±0.87		0.454
	T2	1.41±0.48*		1.06±0.08		0.485
	T3	1.18±0.40		NA		
MONO count (10^3/mm^3)	Pre	0.39±0.13	0.984	0.37±0.19	0.641	0.679
	T1	0.41±0.16		0.34±0.07		0.733
	T2	0.42±0.17		0.23±0.19		0.273
	T3	0.41±0.16		NA		
EOS count (10^3/mm^3)	Pre	0.11±0.13	0.934	0.07±0.07	0.479	0.768
	T1	0.10±0.10		0.05±0.07		0.197
	T2	0.10±0.14		0.02±0.01		0.182
	T3	0.09±0.05		NA		
BASO count (10^3/mm^3)	Pre	0.03±0.02	0.850	0.02±0.02	0.578	0.310
	T1	0.03±0.02		0.03±0.02		0.945
	T2	0.03±0.02		0.01±0.00		0.061
	T3	0.02±0.01		NA		
RBC count (10^6/mm^3)	Pre	3.96±0.54	0.981	3.84±0.44	0.694	0.679
	T1	3.88±0.48		3.70±0.24		0.635
	T2	3.90±0.47		3.99±0.02		1.000
	T3	3.95±0.50		NA		
HGB (g/dl)	Pre	13.1±1.4	0.931	12.3±1.0	0.881	0.296
	T1	12.8±1.2		12.3±1.7		0.611
	T2	13.0±1.2		12.9±0.9		0.879
	T3	13.1±1.3		NA		
HCT (%)	Pre	38.3±4.1	0.987	36.5±3.0	0.831	0.386
	T1	37.9±3.5		35.9±4.8		0.318
	T2	38.3±3.3		37.9±2.4		0.970
	T3	38.2±3.2		NA		
PLT count (10^3/mm^3)	Pre	218±70	0.996	196±61	0.468	0.594
	T1	223±70		209±65		0.733
	T2	222±98		154±35		0.349
	T3	219±75		NA		
NLR	Pre	5.23±2.6	0.544	3.47±1.9	0.633	0.310
	T1	4.54±2.7		3.50±1.7		0.733
	T2	3.54±2.3		4.92±1.1		0.273
	T3	4.36±2.7		NA		

WBC, White blood cells; NEU,neutrophils; TLC, total lymphocyte count; MONO, monocytes; EOS, eosinophils; BASO, basophils, RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; PLT, platelets; NLR, neutrophil/lymphocyte ratio; [a]Kruskal Wallis H-test, time: *$p<0.05$; [b]Mann–Whitney *U*-test, TMZ *vs*. non-TMZ: #$p<0.05$; NA, not available. Values are the mean±S.D.

to a second surgery (22) is also a poor prognostic factor for GBM. These reports suggest that it is important to maintain the TLC and NLR during RT/temozolomide therapy. Our present findings show that before immunotherapy, the TLC was significantly lower and NLR tended to be higher in the TMZ compared with the non-TMZ group (Table IV). These results were expected, considering the known toxicity of RT and/or temozolomide (17-20). However, we found that injections of αβ T-cells significantly improved the TLC and NLR of TMZ-treated patients after five injections. This TLC-increasing effect of αβ T-cell immunotherapy was previously observed in patients with advanced solid cancer, and it is a proposed mechanism by which αβ T-cell immunotherapy improves patient immune status (13). These findings indicate that αβ T-cell immunotherapy has the ability to restore T-cell numbers in RT/temozolomide-treated patients and to restore RT/temozolomide therapy-induced lymphopenia.

Our FCM analysis of patients' PBMCs also indicated that the injected αβ T-cells might improve the quality of the lymphocyte population *in vivo*. As mentioned above, RT/temozolomide therapy affects the $CD4^+$ T-cell population (17, 20). It is also reported that decreased γδT-cell levels are observed prior to tumor resection and throughout therapy in patients with GBM (23). Our present results showed increases not only in $CD4^+$ T-cells but also in $TCRV\gamma^+$ cells and CD8-positive T-cells in the TMZ group (Figure 3). In addition, although the $CD4^+$ T-cells increased, the CD4/CD8 ratio gradually decreased in the TMZ group. The αβ T-cells used were a heterogeneous population consisting of several phenotypes of lymphocytes (Table II); they also contained a minor population of $TCRV\gamma^+$ cells (data not shown). Although it is impossible to explain the detailed mechanism behind our results at present, these heterogeneous αβ T-cells might help restore the impaired and imbalanced T-cell immune status of patients treated with temozolomide.

Finally, we assessed the clinical merits of αβ T-cell immunotherapy for patients with malignant brain tumors. The median OS of patients with GBM treated with an intralesional injection of activated T-cells was reported to be 20.5 months (9). We were able to control tumor progression in 71.4% of the patients in the TMZ group, and the median OS of the patients with GBM in the present study was 21.4 months, which was close to the previously reported prognosis (9). Our results might show some clinical merits in the TMZ group. However, we assessed only five patients with GBM in the TMZ group, therefore it is difficult to evaluate the clinical usefulness of αβ T-cell immunotherapy for this group from the present results only. In addition, for the non-TMZ group, our study did not show significant immunological or clinical efficacy because the follow-up times for all of the patients in the non-TMZ group were very short, and this group was small and consisted of clinically and pathologically heterogeneous patients. αβ T-Cell immunotherapy is reported to have a significant additive effect with chemotherapy for adenocarcinoma (14). Thus, it is possible that αβ T-cell immunotherapy combined with other anticancer agents provides other clinical merits for the non-TMZ group. All these points should be addressed in a future prospective study using a larger cohort.

The effects of αβ T-cell immunotherapy on patients' immunological condition indicate that it may be an attractive adjuvant for treating the lymphopenia caused by RT/temozolomide therapy, or by other high-dose chemotherapy (24). In addition, the intentional use of αβ T-cell immunotherapy from the start of RT/temozolomide therapy may be a promising option for preventing treatment-related lymphopenia. A TLC under 1,200 cells/mm^3 before RT/temozolomide therapy predicts severe lymphopenia during such therapy (25). Therefore, patients with a low TLC might particularly benefit from αβ T-cell immunotherapy. It is also possible that the success rate of conventional standard therapies will improve when combined with αβ T-cell immunotherapy, and this approach is likely to be pursued as a future direction for treating malignant brain tumors

In conclusion, we performed intravenous systemic adoptive αβ T-cell immunotherapy on 23 patients with malignant brain tumors in combination with conventional standard therapies including temozolomide. The systemic αβ T-cell immunotherapy was well tolerated, restored patients' T-cells in both quantity and quality, and was able to reverse RT/temozolomide therapy induced-lymphopenia. Future prospective study is needed to clarify the clinical merits of this immunotherapy.

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