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Therapeutic Efficacy of Adoptive Cell Transfer on Survival of Patients with Glioblastoma Multiforme: Case Reports

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Key Words
γδT cells · Glioblastoma · Immunotherapy · Overall survival

Abstract
Glioblastoma multiforme (GBM), which occurs mostly in individuals over the age of 40, accounts for 12–15% of all primary brain tumors. Patients with GBM have a poor prognosis, even after aggressive upfront therapies. The present study documents that in 5 of these patients, the use of a novel immunotherapeutic approach combined with standard initial therapies resulted in a prolonged survival of over 3 years, which is significantly longer than the expected survival time with conventional therapies. During the course of intravenous cell-transfer immunotherapy, axial magnetic resonance images of the tumor region were monitored for over 5 years. The discontinuation of adoptive transfer regimens resulted in the rapid deterioration of patients with development of Gd-enhancing regions, indicating the initiation of tumor recurrence. Among patients with recurrence, the reinstatement of adoptive cell regimens with more frequent cell-transfers resulted in an apparent re-regression of tumors. Significantly longer survival times were seen in patients receiving transferred autologous lymphoid cells which were expanded in vitro, and which had a considerable proportion of γδT cells. We conclude that immunotherapy, combined with standard treatment, plays a significant role in the management of GBM patients and provides patients with a better prognosis.

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Introduction

Glioblastoma multiforme (GBM), most commonly found in individuals over 40 years of age, accounts for 12–15% of all primary brain tumors. Without treatment, patients with recurrent GBM have a median survival time of 12–16 weeks, while after aggressive upfront therapies that include surgery, radiation, and chemotherapy, survival may increase to no more than 2 years [1, 2].

GBM is diffusely invasive, and highly infiltrative tumors spread into surrounding normal brain tissue. Because of this presentation, complete surgical resection of glioblastomas is not feasible without disrupting neurological function, invariably leaving residual microscopic disease [3]. It is often seen that surgical resection of GBM results in recurrence of tumors in a location close to the resection cavity, usually within a few centimeters of the original tumor bed [4]. Most patients die as a consequence of therapeutic failure followed by recurrence in this location. At the present time, the preferred standard treatment for glioblastomas is systemic radio-chemotherapy followed by surgical resection. Commonly prescribed chemotherapeutic regimens for GBM include a nitrosourea class of chemotherapy agents, specifically temozolomide. Data from recent chemotherapeutic regimens utilizing temozolomide plus radiotherapy following surgical resection resulted in minimal prolongation of survival time when compared to radiotherapy alone [5]. Systemic chemotherapy, followed by radiation therapy, improves the outcome for some patients with an additional 2–3 month increase in survival; however, effects on long-term disease control remain blurred. Lack of significant activity is attributable to the intrinsic chemoresistance of glioblastomas [6] as well as the physical isolation of brain tumors by the blood-brain barrier (BBB), or blood-tumor barrier (BTB), which prevents efficient delivery of systemically administered chemotherapeutic agents [7]. Conventional radiotherapy has limited success since malignant cells are also recalcitrant to current modalities of irradiation during the course of treatment, even in cases in which patients received a total permissible dose of 60 Gy [8]. However, studies examining the effects of radiation on the BBB reveal increases in gadolinium (Gd)-diethylenetriaminepentaacetic acid uptake in the initially nonenhanced tumor region during the course of radiation therapy, suggesting an opening of the BBB [6–8]. Thus, radiotherapy may have supplemental benefits for complementary and alternative therapy, such as immunotherapy, since a change in the permeability of the BBB or the BTB may also allow for infiltration of immune cells to the tumor site. In addition to this hypothesis, it has been shown that, in GBM patients, the BBB is considerably less effective and is a major reason for brain edema [9–11]. Additional evidence shows the presence of infiltrating T cells in the brain of patients with GBM that are not ordinarily seen in the normal brain [11, 12]. Needless to say, bulky established malignant GBM is physically more difficult for immune cells to eradicate [13]. Major obstacles to the development of clinical models in glioblastoma stem from the difficulties of producing high numbers of autologous tumor-reactive immune cells for infusion, and lack of MHC expression in most GBMs [14]. However, these obstacles are overcome by the use of γδT cells. γδT cells can recognize MHC-lacking GBM cells through less specific mechanisms that require no prior antigen exposure [14, 15]. Large amounts of autologous γδT cells can be produced in short-term laboratory cultures [16]. Thus, the use of immunotherapeutic approaches in conjunction with conventional therapy is reasonable and may result in more successful approaches to the treatment of GBM. The fact that brain tumors are uncommon limits large, randomized, controlled clinical studies, but there is still a significant role for a carefully performed and thoughtfully analyzed pilot study. The present study includes 5 GBM patients who received the initial therapy plus the adoptive cellular immunotherapy among 37 patients, all with a potentially poor prognosis if treated with a standard

therapeutic regimen. Immunotherapy remains a possibility for prolonging survival in patients after the completion of combination chemotherapy using nitrosourea and radiation followed by complete visual resection. The present study intended to not only establish the optimal combination regimens which would benefit from systemic adoptive transfer of lymphoid cells, but also to identify those patients who could benefit from a novel type of immunotherapeutic regimens.

Methods

Patients and Treatment Procedures

A total of 5 patients who were randomly selected, gave informed consent among 37 GBM patients, and who had received the initial therapy, were enrolled in an immunotherapeutic study approved by the ethics committee of Miyagi Cancer Center Hospital for the period between 1993 and 2007. Patients who received the initial therapy alone were aged between 24 years and 72 years (mean 45.0 years; 17 female, 15 male patients; table 1). Patients who received additional immunotherapy were aged between 17 years and 56 years (mean 39.8 years; 2 female, 3 male patients; table 1). Each patient underwent an initial evaluation consisting of a detailed history, physical examination, pre-operative cerebral magnetic resonance imaging (MRI) to determine the presence of metastases, and a review of pathologic material by a highly-experienced pathologist in this hospital. Patients provided written informed consent to be examined after the nature of the procedure had been fully explained to them. Patients with histologically confirmed glioblastoma (World Health Organization grade IV glioblastoma) were eligible for the study. Patients with prior chemotherapy, radiation therapy, or immunotherapy for at least 4 weeks before entry into the study were excluded.

Initial Therapy

Cerebral MRI was performed on a 1.5-T super-conducting system (Magnetom Vision, Siemens Medical Solutions). A standard circularly polarized head coil was used for all imaging procedures. The imaging protocol consisted of T1-weighted spinecho, with gadolinium (Gd), and T2-weighted gradient-refocused echo sequences in axial slices covering the entire CNS. Qualitative analysis included determination of the tumor location and detection of concomitant disorders such as hemorrhage and tumor necrosis. Necrosis was assumed in those patients in which histopathology revealed the presence of necrosis and imaging characteristics of necrosis were present. The initial therapy designated below before starting immunotherapy was as follows: firstly, the patient underwent a gross resection of the tumor, of which tumor volumes were delineated in Gd contrast-enhanced T1 imaging according to recommendations [17]. Secondly, patients indicating no evidence of the T1-enhancing residual tumor and the postoperative changes on MR images were subject to postoperative adjuvant chemotherapy. MR images were taken immediately following the completion of surgical resection. Additional delayed images were also acquired for the indicated time during chemotherapy. Adjuvant chemotherapy with continuous arterial infusion of nimustine hydrochloride (ACNU; 80 mg/m^2 body surface area) was initiated 10 days after surgery. Adjuvant chemotherapy was abandoned if there were hematologic toxic effects. Thirdly, after a 1 to 3-day break, the patient underwent 3-dimensional conformal radiotherapy of a total dose of 60 Gy in 30 fractions (2.0 Gy per fraction, once daily, 5 days per week for 6 weeks). The irradiation was performed to the high signal intensity of residual tumors and peri-tumoral edema on T2-weighted MR imaging. A course of the therapy, consisting of these series of treatments, was referred to as 'initial treatment' in this study. One month after the completion of the initial treatment, patients were enrolled in the following immunotherapy. The cells for intravenous infusion immunotherapy were prepared according to the methods described in the following section. All the patients received the initial therapy, and 5 of the patients received immunotherapeutic regimens.

Preparation of Cells for Adoptive Transfer, Immunotherapeutic Schedule and Follow-Up

Peripheral blood mononuclear cells (PBMCs) were isolated from 50 ml peripheral blood using Ficoll-Hypaque density gradient centrifugation as previously described [16, 18]. PBMCs were initially propagated at 37°C in a 5% CO_2 atmosphere, for 7 days in anti-CD3 mAb-coated (5 μg/ml; Janssen-Kyowa, Tokyo, Japan) culture flasks containing RPMI 1640 + 7 culture medium (Nikken Biomedical

Lab, Kyoto, Japan) supplemented with recombinant interleukin-2 (IL-2; 700 U/ml; Shionogi Pharmaceutical, Osaka, Japan) plus 2% pooled human plasma. Medium was changed every 3 days. Proliferating PBMCs were split into permeable culture bags for an additional 7 days in RPMI 1640 + 7 medium containing 1% pooled human plasma plus 175 U/ml of IL-2. After washing these expanded PBMCs, 1.0–1.7 × 10^{10} cells (table 1), which were predominantly γδT cells [14, 15], were resuspended in physiological saline containing 1% human albumin. Cells were administered intravenously to patients, once a month, or at indicated intervals, followed by the initial treatment, depending on individual patients as stated in the Results section, i.e., for either a weekly or a monthly administration. Patients were monitored for tumor recurrence using MRI every 2–3 months. Tumor recurrence, determined by contrast-enhanced T1-weighted MR images, was defined as a new or progressive increase in contrast-enhancing lesions, within the initial surgical resection site or in a remote location. Unless tumor recurrence was observed in patients during the subsequent 6 months after the initiation of adoptive immunotherapy, patients continued to receive adoptive transfer of autologous PBMCs for 5 years as scheduled on an outpatient basis.

Outcome Measures

The primary endpoint was overall survival. Toxic or adverse effects were not observed in the patients enrolled after the completion of immunotherapy (table 1).

Phenotypic Analysis on Expanded PBMCs by Flow Cytometry

The analysis was made for the expanded cells immediately before the first infusion. Mouse FITC-conjugated anti-TCRαβ and anti-TCRγδ mAbs for analysis were purchased from Becton Dickinson (Mountain View, Calif., USA). Aliquots of cells expanded for 2 weeks were stained on ice for 30 min with 0.01 ml of FITC-conjugated mAbs. After washing, the cells were suspended in 0.5 ml of cold RPMI 1640 medium and analyzed using a FACScan flow cytometer (Becton Dickinson, Lincoln Park, N.J., USA). Isotyped-matched mAbs were used as negative controls.

Results

Clinical Outcome

All the patients (n = 32) who received the initial therapy but disagreed to receive the immunotherapy have died within 2 years after the initial therapy (16 ± 2 months; table 1). Among patients who received immunotherapeutic regimens, 2 of 5 evaluable patients (patient 1 and 2) had no more evidence of their disease over 10 years (table 1). Clinical results included a median survival time of 96.8 months longer for immunotherapy patients than 16 months for initial-therapy-alone patients (16 ± 2 vs. 96.8 ± 56.3), indicating significant difference in the survival time between the two treatment arms ($p = 0.001$). Time to progression (TTP) also indicates a marked difference for the two arms with a median TTP of 5 months for initial-therapy-alone patients and 88 months for immunotherapy patients ($p = 0.001$).

As shown in figure 1, a 37-year-old female diagnosed with GBM had a Gd-enhancing 5 × 4 cm tumor lesion, as seen on T1-weighted MR image, with necrosis within the right frontal lobe (fig. 1a). Axial T2-weighted MR imaging showed tumors extending evenly beyond the radiographic border of the lesion into the normal brain in the right frontal lobe (fig. 1b). The patient underwent a gross total resection of the tumor, which was histologically determined to be a GBM. Postoperatively, ACNU (80 mg/m^2) was administered as described in the Methods section. The patient underwent external beam radiotherapy of 60 Gy in 30 fractions. An MR image taken at the completion of the initial treatment demonstrated no histological evidence of neurological abnormalities (fig. 1b).

Thereafter, the patient received adoptive transfer of 1×10^{10}, expanded, PBMCs every month over an interval of 5 years. After completion of a 5 year-immunotherapeutic regimen, the patient had no progression of disease for a total of 14 years, including the 5-year immunotherapy period. Follow-up MR scanning showed no enhancement in the resection cavity (fig. 1c; T1), and regression of residual tumors around the cavity (fig. 1c; T2).

The second patient, a 17-year-old-female (patient 2) had a Gd-enhancing tumor lesion, accompanied by cystic components, measuring 6 × 5 cm in the right frontal lobe on axial MRI (fig. 2a; T1- and T2-weighted images). After completion of initial treatment, the patient underwent follow-up imaging, which revealed no evidence of primary or residual tumor (fig. 2b). The patient had no clinical symptoms such as seizures, headaches, or focal neurologic deficit. Cell-based adoptive immunotherapy (1.2×10^{10}) was administered as scheduled for 5 years on an outpatient basis. The patient had no signs of measurable disease at evaluation on MRI for 10 years after initial treatment was introduced, and after the initiation of immunotherapy (fig. 2c; T1- and T2-weighted images). However, the patient progressed with intratumoral hemorrhage 7 years after discontinuation of the 5-year-adoptive transfer (12 years after initial treatment was completed). The patient is currently receiving adjuvant therapy in another in-patient facility.

Of the 2 patients with partial response, 1 patient (patient 3) had no progressive disease, but a recurrence within 24 months after immunotherapy: at diagnosis, a 49-year-old-male had a Gd-enhanced 4.5 × 3.5 cm tumor, with the presence of necrosis, within the left temporal lobe on a T1-weighted MR image, which included high-density areas in the temporal lobe identified on T2-weighted MR images (fig. 3a; three slices in a lower row). No abnormal enhancement has been observed 22 months after the initiation of immunotherapy (fig. 3b; three slices), but on the MR images 24 months after undergoing immunotherapy, a strong enhancement consistent with tumor recurrence appeared locally around the resection cavity wall of the initial tumor in the left temporal lobe (data not shown). Although immunotherapy has been maintained, tumor recurrence at locations contiguous with the initial lesions were seen in the left frontal lobe, temporal lobe, and basal ganglia 30 months after the initiation of immunotherapy (fig. 3c; T1- and T2-weighted images in upper and lower rows). These images also indicate distant, recurrent lesions arising from the residual tumors around the resection cavity, and migrating to the left cerebral hemisphere. The process was characterized by hemiplegia, aphasia, and disturbance of consciousness, and eventually the patient died, 3 years and 9 months after the initiation of immunotherapy.

The remaining patient (patient 4) had been disease-free for 39 months after immunotherapy but had a recurrence on 42 months: a 56-year-old-male had a Gd-enhanced 5.5 × 4.5 cm tumor with necrosis within the right frontal lobe at diagnosis (fig. 4a). It was confirmed that there was no enhancement on T1-weighted MR images after the resection (fig. 4b; T1-weighted images). Examination at the completion of initial treatment revealed no apparent focal neurological deficits or routine behavioral abnormalities. Eight months after the initiation of adoptive immunotherapy, a mild Gd-enhanced region appeared locally contiguous to the resection cavity wall, reflecting relapse (fig. 4c). Thereafter, the patient received cell infusions more frequently, i.e., on a weekly basis. This change in frequency of administration resulted in no further development of Gd-enhancement, indicating a significant regression of tumor growth around the resection cavity wall on T1-weighted MR images (fig. 4d; 11 months) and no further clinical deterioration. Two years and 4 months after the patient received more frequent transfer of cells (36 months after the initiation of adoptive transfer), the process

was characterized by no further development of tumor recurrence along the resection cavity wall (fig. 4e). Thirty-nine months after the initiation of immunotherapy, therapy was discontinued because of a lack of available cells. Three months after discontinuation (42 months after the initiation of immunotherapy), the patient deteriorated rapidly with development of a Gd-enhancing region, indicating the initiation of tumor re-growth (fig. 4f). This was followed by a rapid increase in the volume of Gd-enhancement evident on MRI. The patient experienced rapid consciousness disturbances, and was dead 6 months after tumor re-recurrence (4 years after the initiation of immunotherapy).

The remaining patient who had received more frequent adoptive transfer of cells (patient 5) has been disease-free over a period of 5 years, but experienced tumor recurrence after the immunotherapy has been suspended: at presentation, a 40-year-old-male had a Gd-enhanced 5.5 × 4.0 cm tumor, accompanied by necrosis in the central area on MRI (**fig. 5**a; T1-weighted images). Immediately after the initial treatment, there was no tumor recurrence (fig. 5b; T1-weighted images), focal neurological abnormalities, or routine life difficulties. Based on findings of patient 4, one month later, immunotherapy was initiated on a weekly basis for the first month, bi-weekly for the second to third month, followed by monthly immunotherapy thereafter. After 60 months of immunotherapy were completed, neither focal neurological deficits nor tumor recurrence was detected (fig. 5c). However, on follow-up MRI (**fig. 6**a), 4 months after the completion of 5 years of immunotherapy, the patient did have measurable recurrence of tumor, progressing from the edge along the resection cavity, which was followed by re-resection (fig. 6b). The patient died 6 years and 7 months after the onset of initial treatment with progressed tumor despite resection, and administration of temozolomide at 75 mg/m^2/day (fig. 6c).

Overall, in all of the enrolled patients, no toxic, hematologic effects of grade 2 or greater were seen during or after initial therapy and immunotherapy. There was no evidence of treatment-induced early or late toxic effects during or after the course of immunotherapy.

Defining Phenotypic Profiles of Populations Growing from PBMCs for Infusion into Patients

After 14 days in culture, patient PBMCs were analyzed for phenotypic expression. There was no significant difference in cell populations in the starting cultures when focused on the proportion of γδT cells (range 3–5% γδT cell positive in all patients). An increase in the proportion of γδT cells was a constant finding for all patients, i.e., after 2 weeks in culture, 2 patients showed a slight increase (3.2–8.8% in patient 3, 1–2.3% in patient 4, and 5.0–10.0% in patient 5; low responder), while in 2 other patients (patient 1 and patient 2), a greater increase in the proportion of γδT cells after expansion was observed (70–90%; high responder) (table 1). The remaining lymphoid cells represented CD4$^+$T cells, CD8$^+$T cells and a small portion of CD16$^+$ NK cells (data not shown).

Discussion

Although it is practical to undergo debulking surgery for GBM tumors which are often not enhanced on MRI, it is less feasible to extirpate tumors infiltrating into surrounding normal brain tissues, without neurological complications. The present study documents that following resection procedures, disease recurs largely around the resection cavity wall

of the initial tumor (table 1; patients 2, 3, 4 and 5 in figures 2–6). Despite tumor recurrence in most patients, the present study emphasizes that systemic cell-infusion contributed to a prolongation of survival for all patients with glioblastoma, although with significant variation in survival time over 3 years (table 1). Focusing on recurrence, patient 2 completed the 5-year protocol but had recurrence after 7 years (see Results and table 1). The recurrence sites, which were situated slightly remote from the resection cavity, indicated the potential presence of infiltrated tumors, which were suppressed by adopted cells during the course of immunotherapy. Patient 3 had recurrence after completing 2 years of cell administration but survived an additional 1 year and 9 months after the time of recurrence, indicating a slow progression of tumors and the beneficial effects of immunotherapy in terms of slowing tumor growth. Patient 4 also had recurrent tumors spreading along the resection cavity shown on MR imaging 8 months after resection (fig. 4c). However, tumor progression was significantly slower (fig. 4d) after more frequent administration of cells from a monthly to a weekly basis, and eventually was initiated 8 months after resection (table 1). After weekly-administration, recurrent tumor structures changed slightly on MRI but remained regressed (fig. 4e). Taking into account that the recurrence occurred after discontinuation of treatment (fig. 4f), it seems likely that the adoptive transfer of cells induced a dormancy rather than a cytotoxicity for the tumor cells in this patient. This supposition is validated by the fact that patient 5 had no signs of recurrence during the 5-year immunotherapy but had recurrence shortly after its discontinuation (fig. 5, table 1). The gradual diminution of the resection cavity is not attributable to immunotherapy, but to the removal of compression in the resection cavity, resulting in a return to the initial volume of a normal brain. These results indicate that immunotherapy has regressive effects on tumor development but not absolute cytotoxic effects on the tumor cells. As a result, the present study significantly enhanced survival in patients receiving LAK cells expanded in immobilized anti-CD3 and 700 U/ml of IL-2 when compared to survival seen with conventional 'initial therapy' (more or less 16 months of survival in 32 patients vs. >45 months of survival in 5 patients, table 1), as well as with immunotherapeutic regimens containing LAK cells (a median survival of 53 weeks to 1 year or more) and regimens containing both expanded $CD4^+$ and $CD8^+$T cells (a median survival of 1 year or more) as previously described [9, 13, 19]. In our patients with a longer survival time (patient 1 and patient 2), there were higher proportions of γδT cells in cell populations expanded in our culture system (70–90% γδT cells). It is believed that γδT cells may home tumor cells, and specific destruction in vitro of these freshly-isolated autologous glioblastomas (which are all devoid of HLA expression at the time of isolation) has been observed [14, 15]. Although the available data is limited in the present study, there may be a possibility that the higher proportion of γδT cells in expanded PBMCs for infusion may correlate with prognosis of patients by its static effects against tumor progression, i.e., high responder versus low responder. This suggests a difference in the γδT cell numbers available for homing tumors once infused. The precise reason for differences in the proportion of γδT cells in each patient is unknown. Although more patients should be examined for a clear-cut conclusion, it should be noted that the proportion of γδT cells might be a prognostic factor for patients with GBM. It is also considered that the difference of survival time in patients may reflect numbers of residual tumors situated along the cavity after resection of original tumors. It may be important to perform histopathological examination, such as the analysis of MIB-1, to draw an explicit conclusion. There is no evidence indicating that BBB or BTB opening by irradiation may result in the infiltration of γδT cells peripherally infused which otherwise may be directed to the intracerebral recurrent tumor cells.

The physical isolation of brain parenchyma from systemic circulation by the BBB prevents chemotherapeutic drugs from accessing tumor parenchyma [4]. Because of this

and the fact that the drugs used in our protocol rarely pass the endothelial junctions in the potentially unresected tumors [5], chemotherapy is likely to be ineffective when systemically administered as done in the present study. One study indicates a possibility that there is a detectable disruption of the BBB post-irradiation, characterized by diffuse vasculature leakage, severe loss of the capillary network, cortical atrophy and white matter necrosis [7]. In the present study, irradiation was confined to occult tumor areas (2 cm from the edge of original tumors) that were found beyond the MRI-defined abnormalities as shown in the Methods section. While it is not possible to eradicate recurrent tumor cells by irradiation at acceptable doses, it may be possible to partially disrupt the BBB or BTB without any neurological complications or adverse effects in the patients. Nonetheless, a protocol which infuses chemotherapeutic agents followed by irradiation would be reasonable, and thus chemotherapy should be followed by radiation for synergistic or additive effects. The presence of an intact BBB in the brain parenchyma may contribute to cell-based therapeutic failures, indicating that tumor cells remain isolated from immunological attack [20]. Although the integrity of the BBB is preserved during early tumor development, it has been suggested that lymphocyte infiltration parallels that of glioma growth, and specific tumor infiltrating lymphocytes are produced peripherally and then traffic into CNS tumors [21, 22]. In addition, increased infiltrating lymphocytes within gliomas have been shown to be significantly associated with longer survival [23, 24]. It is unrealistic to speculate that humoral factors released from the expanded lymphoid cells after infusion may affect the progression of an intracerebral tumor, because once the cytokines produced have passed the BBB, they may be diffused through a network of interstitial channels in the brain parenchyma [19].

The present study provides strong evidence that concomitant therapy significantly prolongs survival in patients, with generally more than a 3-year progression-free survival. Unlike most previous studies, the present study included patients who were infused with a novel type of LAK cells containing γδT cells either in the high-responder groups and low-responder groups. The study also included patients with successful debulking to the extent that tumors are not identified on enhanced MRI. These criteria and the infusion of expanded LAK cells containing a novel type of cells may have served to exclude patients with poor prognosis, who probably would not benefit from conventional therapy. In conclusion, the present study convincingly suggests that this novel type of immunotherapy plays a significant role in the management of GBM and provides patients with a better prognosis.

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Table 1. Characteristics and survival of glioblastoma patients who received initial therapy alone and initial therapy combined with immunotherapy

Patient No.	Age/sex	Diagnosis[a]	Initial therapy[b]	Cell dose/ %γδT cells	Survival (months)[c]	Adverse events	TTP (months)[a]
Initial therapy (n = 32)	24–72 (median 45 years; F, 17: M, 15)	Glioblastoma multiform	RT+ACNU	N/A[d]	16±2	None	5±1
Immunotherapy (n = 5)	17–56 (median 39 years; F, 3: M, 2)				96.8±56.3		88±64.4
1	37/F	Glioblastoma multiform	RT+ACNU	1.0×10^{10}/78.2	≥168	None	≥168
2	17/F	Glioblastoma multiform	RT+ACNU	1.2×10^{10}/90.1	≥144	None	≥144
3	49/M	Glioblastoma multiform	RT+ACNU	1.5×10^{10}/8.8	45	None	22
4	56/M	Glioblastoma multiform	RT+ACNU	1.7×10^{10}/2.3	48	None	42
5	40/M	Glioblastoma multiform	RT+ACNU	1.6×10^{10}/10.0	79	None	64

[a] TTP, median time to progression from diagnosis (in months). [b] RT(radiation therapy; total of 60 Gy) plus ACNU (80 mg/m^2 for 4 weeks) in addition to surgical resection. [c] Months after completion of initial therapy (median survival time). [d] Not applicable.

Fig. 1. Contrast-enhanced axial MR images in patient 1 with GBM showing the extent of the tumor prior to resection, 1 month after resection, and 13 years after the initiation of immunotherapy. Axial enhanced MR images obtained prior to resection (**a**) show irregular ring-enhancing tumor region, with the presence of necrosis, within the right frontal lobe (upper; T1-weighted MR images with Gd). Axial MR images after resection show the resection cavity into the right frontal lobe 1 month after the completion of initial therapy (**b**). In three axial slices 13 years after the initiation of immunotherapy (**c**), no enhancement appears in the resection cavity on T1 images (upper left to right).

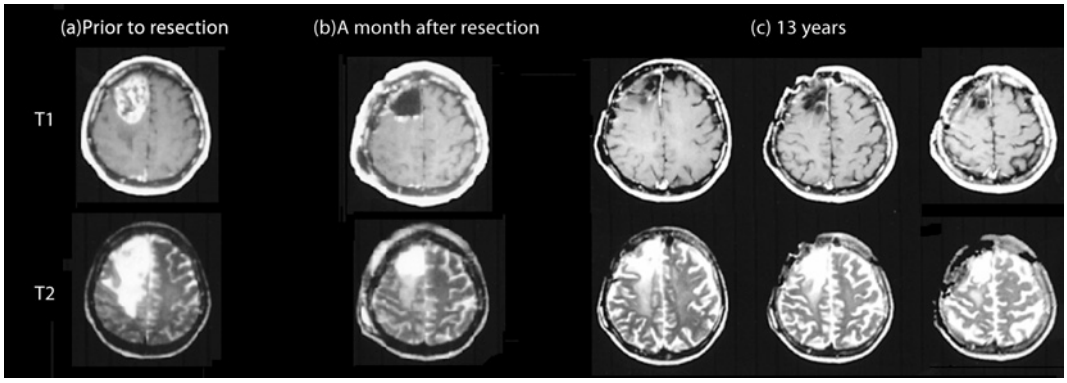

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Fig. 2. Contrast-enhanced axial MR images in patient 2 with GBM showing the extent of the tumor prior to resection, after resection, and 10 years after the initiation of immunotherapy. Axial MR images obtained prior to resection show the tumor region, accompanied by cystic components, situated within the right frontal lobe (**a**; upper; Gd-enhanced T1 image) in patient 2. There were no enhancements seen in the resection cavity on MR images (**b**; upper and lower) immediately after resection, and no recurrence on both T1- (upper 3 slices from left to right), and T2-images (lower 3 slices from left to right) 10 years after the initial therapy, followed by a 5-year immunotherapeutic regimen (**c**).

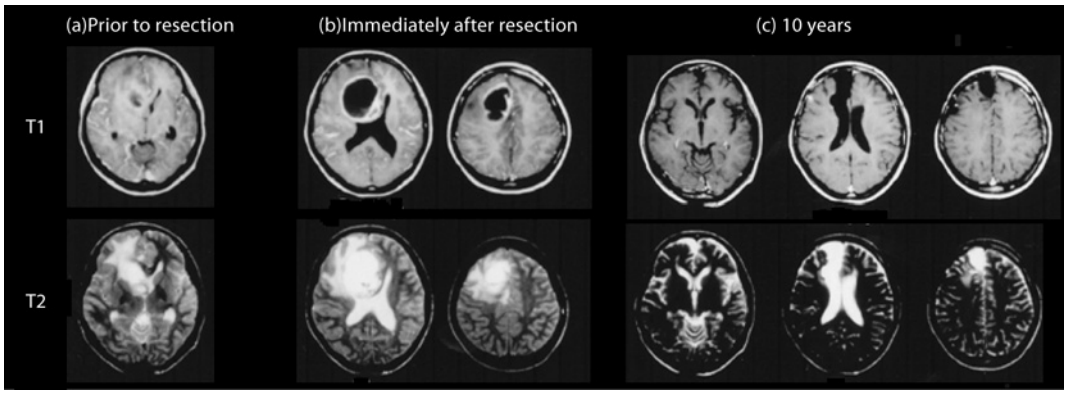

Fig. 3. Contrast-enhanced axial MR images prior to resection, 22 months, and 30 months after the initiation of immunotherapy in patient 3. Contrast-enhanced axial MR images obtained prior to resection (**a**) show the tumor region in the left temporal lobe (three slices on T1- and T2-images) in patient 3. Three slices on MR images on 22 months after the initiation of the initial therapy show no abnormal enhancement (**b**; upper three slices from left to right), but enhancing mass lesions consistent with tumor recurrence were seen in the left frontal lobe, temporal lobe and basal ganglia as shown on two slices from left to right of both T1- and T2-MR images, respectively, of the same patient 30 months after the initial therapy (**c**).

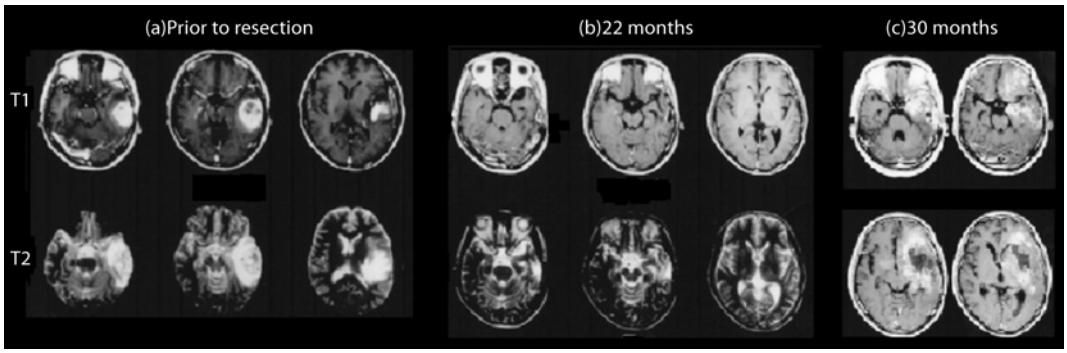

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Fig. 4. Contrast-enhanced axial MR images obtained prior to resection, 2 months after the completion of the initial therapy, and sequentially after the initiation of immunotherapy in patient 4. Contrast-enhanced T1-weighted axial MR images obtained at diagnosis (prior to resection) (**a**), 2 months after the initial therapy (**b**), and 8, 11, 36 and 42 months (**c–f**) after the initiation of immunotherapy (2 slices each). Prior to resection, the MR images show the presence of strong enhancement with necrosis in the right frontal lobe (**a**), and postoperative images show the resection cavity with nearly complete disappearance of tumor lesion (**b**). A strong flare developed 8 months after resection of mild enhancing tissue within the right frontal lobe (**c**), but frequent transfer of cells at this time resulted in mild enhancement around the resection cavity comparable to that in 8-month immunotherapy, indicating no further development of tumor growth in the frontal lobe (**d**, **e**). After the discontinuation of cell transfer, there was a significant increase in enhancement attributable to tumor-regrowth (**f**).

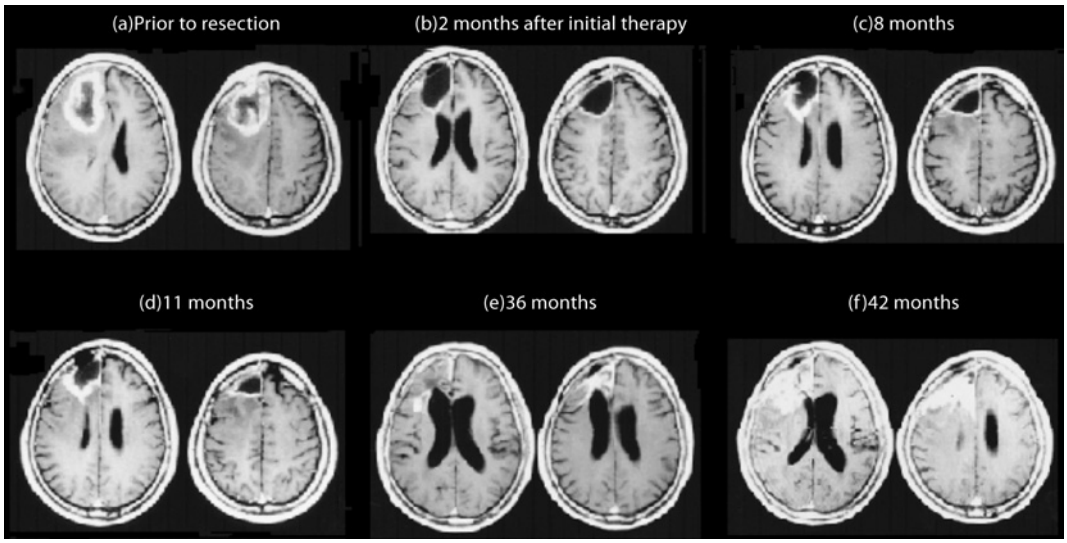

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Fig. 5. Axial MR images obtained prior to resection, immediately after the completion of the initial therapy, and 60 months after the initiation of immunotherapy in patient 5. Axial T1-weighted MR images (upper) obtained preoperatively (**a**; 3 slices) show ring-enhancing lesions, with the presence of necrosis. T1-weighted MR images (upper) immediately after the initial therapy (**b**; 3 slices) show no enhancing lesions. Both T1-weighted and corresponding T2-weighted images obtained at the end of a 5-year immunotherapy period show no enhancement in the surgical bed (**c**; T1, upper) indicating no tumor recurrence at this stage (60 months).

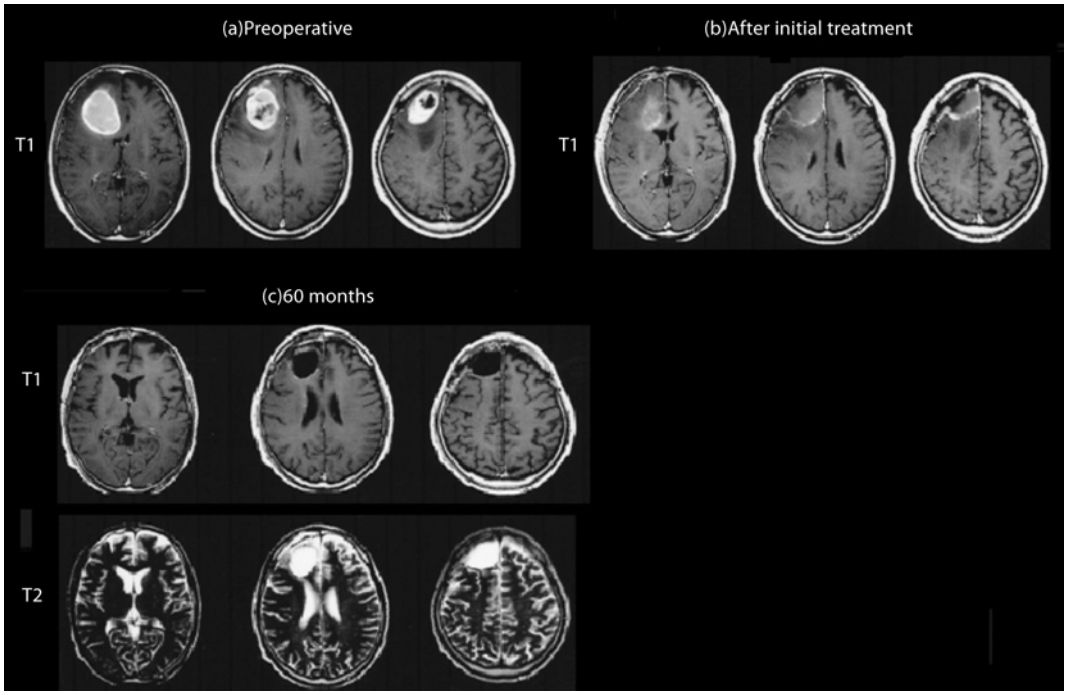

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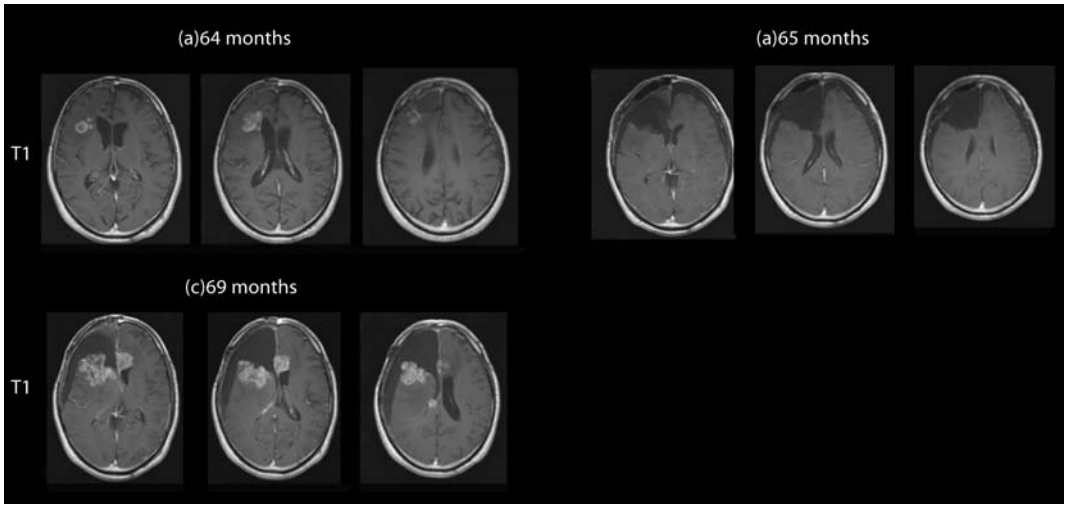
Fig. 6. Axial MR images obtained 64 months, 65 months, and 69 months after the initiation of immunotherapy in patient 5. Axial T1-weighted MR images obtained 4 months after completion of immunotherapy show complete resolution of abnormality (**a**; 64 months after initiation of immunotherapy; 3 slices) that was most likely due to re-progression of residual tumors. MR images obtained 1 month after second tumor resection (**b**; 65 months after initiation of immunotherapy) show the complete disappearance of the tumor bed, but 4 months after follow-up with chemotherapy according to the protocol described in the Methods section, MR images show a significant extension of the lesion in the right to left frontal lobe, compatible with tumor regression (**c**; 69 months after initiation of immunotherapy).



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