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Alpha-type-1 Polarized Dendritic Cell-based Vaccination in Newly Diagnosed High-grade Glioma: A Phase II Clinical Trial

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Abstract. *Background/Aim: Glioblastoma multiforme (GBM) is an intractable tumor that has a very poor prognosis despite intensive treatment with temozolomide plus radiotherapy. Patients and Methods: Sixteen newly diagnosed patients with high-grade gliomas were enrolled in a phase II study of the α-type-1 DC vaccine. Briefly, DCs obtained from the culture of enriched monocytes in the presence of a cytokine cocktail, were pulsed with a cocktail of 5 synthetic peptides and cryopreserved until injection into patients. Results: The amount of IL-12 produced by activated DCs was higher than that previously reported. Among 15 evaluable patients, 10 showed positive CTL responses to any peptides in an ELISPOT assay. After 6 years of observation, five patients were still alive, and two of these patients were relapse-free. Moreover, a significant survival-prolonging effect was verified in DC-treated glioma patients. Conclusion: Peptide-cocktail-pulsed α-type-1 DC vaccines have a potential therapeutic effect on survival when used in combination with the standard regimen, which is partly based on IL-12-IFN-γ-mediated T-cell activation.*

Glioblastoma multiforme (GBM) is the most malignant and intractable brain tumor that has a dismal prognosis; the median progression-free survival for primary glioblastoma is 6.9 months, and the median overall survival is 14.6 months, with a 5-year survival rate of less than 5%, despite the use of intensive standard therapy consisting of radiation therapy and temozolomide (1-3). In addition, many diverse therapeutic regimens, such as cediranib (4), cilengitide (5), and rindopepimut (6), have been tested but they have failed to prolong overall survival. Bevacizumab and tumor-treating fields (TTField) had a marginal survival benefit in a randomized phase III trial (7, 8) but did not represent a therapeutic breakthrough against GBMs. Thus, novel therapeutic approaches to control GBM progression are urgently needed.

To date, many multiomics-based analyses of GBMs have been performed, and substantial literature regarding the specific genetic and biological characteristics of GBM tumors has accumulated including driver mutations (ERBB2, TP53, NF1, PTEN, mTOR, IDH1, *etc.*) (9, 10), activation of specific cancer signals (11, 12) and mesenchymal transformation signatures (c/CEBPb, STAT3). With advances in next generation sequencing (NGS) technologies, the specific association of the multiomic features of GBM tumors with the immune microenvironment has been examined; T cell dysfunction was associated with an exhaustion signature in GBM tissue, increased T cell infiltration was observed in mesenchymal type tumors and reduced infiltration of protumor macrophages was found in IDH1 mutant-harboring GBM tumors (13, 14).

In regard to immunotherapy of GBM tumors, immune checkpoint antibody-based clinical trials for the treatment of recurrent GBM tumors have indicated that nivolumab failed to greatly prolong the overall survival of recurrent GBM patients compared to bevacizumab (15). Additionally, a clinical trial using nivolumab for newly diagnosed GBM patients is ongoing (16).

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Moreover, DC-based vaccine studies against GBM have shown moderate effects in terms of a patient performance status (PS) or quality of life (QOL), and some have demonstrated a marginal survival benefit; however, the impact of such approaches is still not obvious because of the weak vaccine effect and small numbers of cases enrolled in the clinical trials (17, 18). Recently, Liau *et al*. have reported an ongoing large phase 3 clinical trial of a tumor lysate-based autologous dendritic cell vaccine (randomized and multicentered) in newly diagnosed GBM patients for the first time, and suggested that the patients in the phase 3 trial were living longer than expected (19).

Previously, we have reported a phase I clinical trial of HLA-A2 or A24-restricted, peptide cocktail-pulsed DC-based, immunotherapy against recurrent high-grade glioma and demonstrated that activated DC vaccines were feasible and safe (20). Alpha-type-1 polarized DCs, activated by a combination of maturation reagents such as TNF-α, IL-1β, IFN-α, IFN-γ and polyI/C, were manufactured in our cell processing facility.

In the present study, we performed a phase II study of an α-type-1 DC vaccine against high-grade gliomas to clarify the effects of DC vaccines on patient survival.

Patients and Methods

Patient characteristics and study design. Sixteen patients with newly diagnosed high-grade glioma were enrolled in a phase II clinical trial of a peptide cocktail-pulsed DC-based vaccine from 2013 to 2018 that was approved by the Institutional Review Board (IRB) of Shizuoka Cancer Center, Japan. The present study was registered in the Japanese clinical trial data base (Japan registry of clinical trial (JRCT) ID: JRCT c040190103). All patients gave written informed consent. The eligibility and exclusion criteria were similar to those used for the previous phase I trial (20) except that DC-based vaccines were given to newly diagnosed high-grade glioma patients in combination with the standard regimen consisting of TMZ and radiation therapy (Figure 1). The minimum doses of corticosteroid were permitted for patients with neurological deficits due to mass effects caused by the lesions. The patients received 3 intradermal DC vaccines in the posterior neck weekly, and then they received vaccines twice every 2 weeks; finally, they received them every month for 5 months. The dose range of the injected DCs was $1\sim5\times10^7$/patient/shot. Adverse effects were evaluated according to the National Cancer Institute (NCI) Common toxicity criteria. Measurable lesions and clinical responses were evaluated by response evaluation criteria in solid tumors (RECIST). DC vaccines were continued until progressive disease (PD) was determined by immune-related (ir)RECIST and imaging diagnosis. The primary and secondary endpoints were the relapse-free survival time (RFS) and overall survival (OS) time, respectively, starting on the enrollment date.

With regard to overall survival, 16 registered patients were followed for 6 years until Oct 2019, and for the retrospective study, survival data from 45 high-grade glioma patients treated with a standard regimen from 2006 to 2011 were utilized.

Preparation of the DC vaccine. The methods used to produce the DC vaccine have been described previously (20). The procedures for preparing the DC vaccine were performed within a high efficiency particulate arrestance (HEPA)-filtered clean-air barriered good manufacturing practice (GMP) cell processing facility. A standard operation procedure (SOP) for DC vaccine production was established according to institutional GMP-based guidelines. Briefly, monocyte-enriched fractions were separated from leukapheresis products using OptiPrep™ and cultured in the presence of granulocyte macrophage-colony-stimulating factor (GM-CSF) and interleukin (IL)-4 in X-VIVO15 serum-free medium. On day 5 of culture, TNF-α, IL-1β, IFN-α, IFN-γ, and poly I/C were added to the culture to obtain mature DC-enriched cultures. On day 7, the harvested cells were pulsed with a cocktail of 5 glioma-specific synthetic peptides that were restricted to HLA A2 or A24, and then with keyhole limpet hemocyanin (KLH). The DC-enriched cells were washed and cryopreserved in a Cryocyte bag (Nipro Corporation, Osaka, Japan) until use. The percentage of DCs was determined as the lin-HLA-DR$^+$ population (lineage antibodies including CD3, CD14, CD16, CD19, CD20, and CD56; BD Biosciences, San Jose, CA).

IL-12p70 production assay. Cultured mature DCs were collected and incubated with the CD40 ligand-expressing mouse plasmacytoma cell line J558 for 24 h. Finally, the supernatants were collected and IL-12p70 levels were measured using an ELISA kit specific for human IL-12p70 (Endogen, Woburn, MA, USA).

Tumor antigen and HLA-class I expression detection in tumor tissues by immunohistochemistry (IHC). Formalin-fixed paraffin-embedded (FFPE) sections of primary high-grade gliomas were obtained from 16 patients. HLA class I and programmed death-ligand 1 (PD-L1) protein expression and tumor antigen expression were investigated using IHC as described previously (20). Monoclonal antibodies (mAbs) against human HLA class I (OriGene Technologies Inc., Rockville, MD, USA), PD-L1 (Abcam, Cambridge, UK), MAGE-A1 (Thermo Scientific, Flemont, CA, USA), MAGE-A3 (Abnova, Taipei, Taiwan, ROC), WT-1 (DakoCytomation, Glostrup, Denmark), HER1 (DakoCytomation) and gp100 (DakoCytomation) were used as the primary antibodies. The staining was evaluated according to the percentage of tumor cells showing positive membranous staining as follows: score 0, less than 1%; score 1, 1 to 5%; score 2, 5 to 50%; and score 3, more than 50%. Regarding tumor-infiltrating lymphocyte (TIL) staining, monoclonal antibodies against CD4 and CD8 (Thermo Fisher Scientific, Waltham, MA, USA), FoxP3 (Abcam, Cambridge, UK, and CD204 (TransGenic Inc., Kobe, Japan) were used.

Glioma-associated marker gene and cytokine gene expression analysis using a quantitative PCR. Real-time PCR analysis of glioma-associated marker genes and cytokine genes was performed using a QuantStudio 12K Flex (Applied Biosystems, Foster, CA, USA) as described previously (21). Total RNA was isolated from resected tumor tissues, FFPE specimens or peripheral blood mononuclear cells (PBMCs) derived from patients administered the DC-vaccine. Briefly, PCR primers for glioma-associated genes (EGFR, ESA, GFAP, KLF4, NANOG, NES, OLIG2, Oct3/4, CD133, SOX2, transforming growth factor (TGF) BR2, VIM, FOSL2, C/EBP, YKL-40 (CHI3L1), and podoplanin (PDPN) for glioma markers; BCL2, Bcl-Xl, Survivin, Cyclin D1, c-Myc, VEGFR2, MMP9, TGFB1, TP53, VEGFA, and HIF-1alpha for STAT3 target genes and glyceraldehyde-3-phosphate dehydrogenase

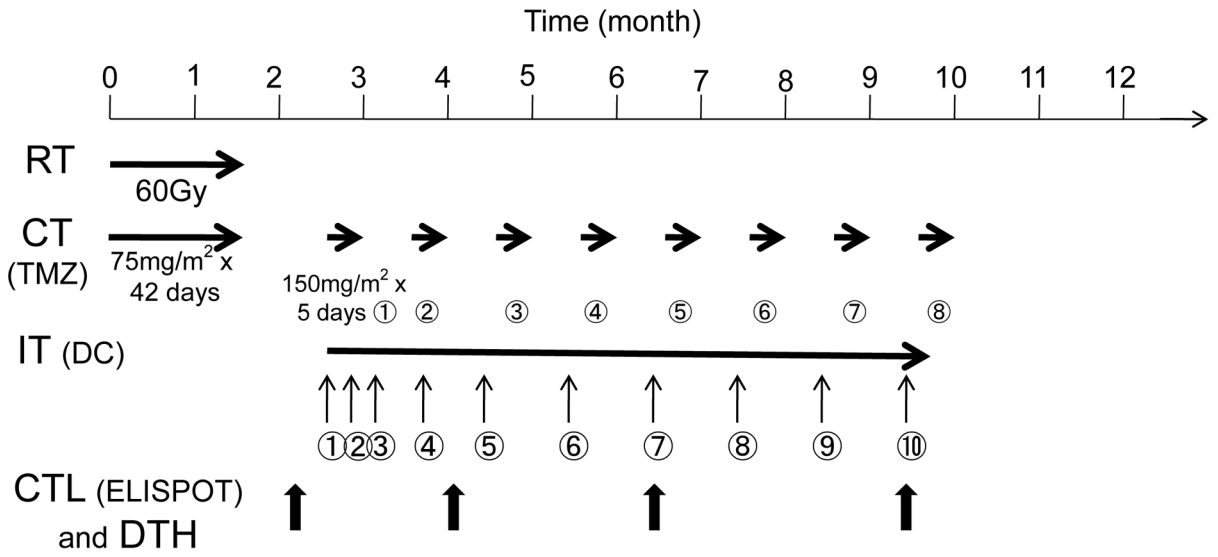


Figure 1. *Dendritic cell (DC) vaccine schedule. DC vaccines were prepared before the initiation of chemoradiotherapy through leukapheresis. After chemoradiotherapy (60 Gy plus concurrent temozolomide (TMZ) for 42 days at 75 mg/m^2), DC vaccines were administered intradermally 10 times during 7 months together with TMZ maintenance therapy. Immunological monitoring, such as ELISPOT assays or DTH, was performed during DC administration.*

(GAPDH) and PCR primes for cytokine and chemokine genes (IL-2, IL-6, IL-10, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-12A, TGFB1, VEGF-A, colony-stimulating factor (CSF)-1, Natural cytotoxicity triggering receptor (NCR)1, CCL3, CCL4, CCL20, CXCL10, CXCR3, CCR7, CD62L, PD-1, TIM3, TCF7, TOX, CD127, Arginase1 (ARG1) and CD11B) were used.

ELISPOT assay. The ELISPOT assay was performed using PBMCs drawn prior to vaccination and after 4 DC injections as described previously (20). Briefly, on day 1, PBMCs were incubated in a 24-well culture plate and divided into nonadherent and adherent cells. Nonadherent cells were stimulated with adherent cells treated with a peptide cocktail twice in the presence of IL-2 and IL-7. On day 15, the responder cells were stimulated overnight with each peptide in a 96-well culture plate coated with anti-IFN-γ antibody. Finally, the positive spots stained with anti-IFN-γ antibody were measured using the KS ELISPOT system (Carl Zeiss AG, Overkochen, Germany).

Delayed-type hypersensitivity (DTH) reactions. The HLA-A2 or A24 peptide solution and 50 μg/ml KLH were injected intradermally into the forearm and the immunological reactions at the injection site were measured regularly 4-times after the 1st DC injection.

Human plasma IFN-γ level after DC vaccine treatment. Human plasma IFN-γ levels were measured using a high-sensitivity IFN-γ-specific ELISA. Peripheral blood was drawn before and after the start of the DC vaccine trial (4-times in total) and IFN-γ levels were measured. The maximum IFN-γ level was determined after DC vaccine.

Statistical analysis. The relapse-free survival and the overall survival of high-grade glioma patients were examined by comparing the differences in the median survival time (MST) using the Kaplan-Meier method. A comparative analysis of the survival times between phase II patients and a retrospective historical control patient cohort was performed using the log rank test or the generalized Wilcoxon test. The association of clinicopathological factors and immunological factors with the overall survival data was analyzed using the log rank test. Additionally, the correlation of the genetic expression data obtained by real-time PCR of RNAs from glioma tumor tissues and PBMCs with the overall survival data was analyzed using a Pearson correlation test. Values of $p<0.05$ were considered statistically significant.

Table I. *Malignant glioma patients' characteristic in phase II study.*

Total no. enrolled	16
Age	56.9±13.6
Gender	M (11), F (5)
Performance status	PS0 (4), PS1 (8), PS2 (4)
HLA-typing	A2 (8), A24 (8)
Diagnosis	AA (1, G3), AO (1, G3), GBM (14, G4)

Results

Patient characteristics. The patient characteristics are summarized in Table I. The sixteen newly diagnosed glioma patients, who consisted of 14 GBM (WHO grade 4-5) patients, 1 anaplastic astrocytoma (AA) (grade 3) patient and 1 anaplastic oligodendroglioma (AO) (grade 3) patient, represented 8 HLA-A2$^+$ cases and 8 HLA-A24$^+$ cases (Table I).

Table II. *Phase II study of DC-based therapy against malignant gliomas.*

Case	Age	Gender	Pathology	DC number (times)	Side effect (grade)	DTH		Results
						Peptide*	KLH	(RFS/OS**)
GB-1	45	M	GBM	2.4×10^7 (10)	-	–	+	Dead (11/16)
GB-2	37	M	AA	9.2×10^6 (10)	-	–	–	Dead (54/65)
GB-3	53	M	GBM	1.4×10^7 (10)	Hepatic (I)	–	–	Alive (38/66)
GB-4	63	F	GBM	1.2×10^7 (10)	-	–	–	Dead (11/14)
GB-5	56	M	GBM	4.5×10^6 (3)	Hepatic (II)	ND	ND	Dead (4)
GB-6	71	M	GBM	2.6×10^7 (10)	Pruritus (I)	–	–	Dead (54/54)
GB-7	63	M	AO	3.6×10^7 (10)	-	+	+	Dead (11/16)
GB-8	52	M	GBM	5.2×10^7 (10)	-	–	+	Dead (11/21)
GB-9	72	M	GBM	1.4×10^7 (10)	-	–	+	Dead (12/21)
GB-10	77	F	GBM	1.8×10^7 (5)	-	–	+	Dead (5/17)
GB-11	69	F	GBM	3.9×10^7 (10)	-	–	–	Dead (18)
GB-12	59	F	GBM	1.8×10^7 (7)	–	+	+	Dead (7/9)
GB-13	38	M	GBM	1.4×10^7 (10)	Eosinophilia (I)	+	+	Alive (24/30)
GB-14	73	F	GBM	2.1×10^7 (10)	-	–	+	Alive (23/23)
GB-15	51	M	GBM	1.8×10^7 (10)	-	–	–	Alive (7/13)
GB-16	31	F	GBM	3.7×10^7 (10)	-	+	+	Alive (11/11)

*Any positive responses to five peptides from a cocktail rated as positive; **RFS/OST, relapse-free survival/overall survival. ND: Not done; GBM: glioblastoma multiforme; AO: anaplastic oligodendroglioma; AA: anaplastic astrocytoma; M: male; F: female.

Table III. *Immunological monitoring in malignant glioma patients (Phase II).*

Case	HLA typing	Tumor antigen expression*	HLA class I expression (score)	DC1/DC2 ratio**	IL-12 production (pg/ml)	ELISPOT
GB-1	A*0201	MAGE3	2	48.5	673	0
GB-2	A*0206	MAGE1	1	334	239	0
GB-3	A*2402	MAGE3, WT1	2	32.4	315	3 (WT1-1, MAGE-1, 3)
GB-4	A*0201	MAGE1,3, WT1	3	10.6	665	0
GB-5	A*2402	MAGE3, WT1	1	5.0	64.7	ND
GB-6	A*0201	MAGE1,3	3	16.2	638	2 (HER2, gp100)
GB-7	A*2402	MAGE1,3, WT1	1	252	2162	0
GB-8	A*0201	gp100, MAGE1,3, WT1	2	538	2838	1 (HER2)
GB-9	A*0201	MAGE1,3, WT1, HER2	3	42.8	2594	2 (WT1-2, gp100)
GB-10	A*2402	WT1	2	11.4	1768	3 (WT1-1, WT1-2, MAGE-3)
GB-11	A*2402	MAGE3, WT1	2	47.5	2298	3 (WT1-1, WT1-2, MAGE-3)
GB-12	A*2402	–	1	10.2	3660	4 (WT1-1, WT1-2, MAGE-1,3)
GB-13	A*2402	MAGE1,3, WT1	1	10.3	3355	4 (WT1-1, WT1-2, MAGE-1,3)
GB-14	A*0206	MAGE3, WT1	2	170	9016	0
GB-15	A*2402	MAGE3, WT1	3	10.6	11722	4 (WT1-1, WT1-2, HER2, MAGE-3)
GB-16	A*0206	MAGE3, WT1	2	48.0	11790	2 (HER2, gp100)

*Positively stained antigen in IHC; **shows the ratio of pre-vaccine DC1/DC2. ND: Not done.

DC processing and characterization. The CD14 percentage increased from 14.4±5.3% to 42.2±12.9% after Optiprep™ density-gradient centrifugation. The mean percentage of DCs identified as lin-CD11c$^+$HLA-DR$^+$ and the DC1/DC2 ratios were 50.6±12.9% and 99.2±147, respectively, which were not different from the values in the phase I trial (20). The percentages of DC markers, including CD83, CD80, CD86, DC sign, DEC205, CMRF56, *etc*. did not differ from those in the previous phase I report (20), which indicated that the obtained DCs showed a fully mature phenotype.

Regarding the IL-12 production assay, the amount of IL-12 produced from activated DCs was 3362±3798 pg/ml per 10^5 cells on average, which was significantly greater than that observed in the previous phase I study (20).

Table IV. *Potential prognostic factors involved in malignant glioma DC vaccines-1.*

Factors	Cases	Mean±SD or case#	Statistical analysis (RFS)	Statistical analysis (OS)
PS status	16	PS0,1 (12) *vs*. PS2 (4)	***p*=0.0258***	*p*=0.078
MGMT status	16	Pos. (11) *vs*. neg. (5)	***p*=0.0032****	***p*=0.0036****
HLA-DNA typing	16	A2 (8) *vs*. A24 (8)	*p*=0.9028	*p*=0.7508
The number of positive antigens	16	High (5) *vs*. low (11)[a]	*p*=0.4056	*p*=0.6615
HLA-class I expression level	16	11/16 (pos. 69%)[b]	*p*=0.671	*p*=0.7329
PD-L1	16	4/16 (pos. 25%)[b]	p =0.0547	*p*=0.1232
pSTAT3	16	6/16 (pos. 38%)[b]	*p*=0.4083	*p*=0.4665
$CD4^+$ T cell	16	3/16 (pos. 19%)[b]	*p*=0.2096	*p*=0.3429
$CD8^+$ T cell	16	12/16 (pos. 75%)[b]	*p*=0.2382	*p*=0.4255
CD204	16	14/16 (pos. 87%)[b]	*p*=0.782	*p*=0.6792
ELISPOT assay	15	1.9±1.6[c] (cut off 2.0)	*p*=0.8716	*p*=0.9718
DTH (KLH)	15	Pos. (9) *vs*. neg.(6)	*p*=0.3285	*p*=0.5402

[a]Positive antigen number in IHC; ≥3 high, <3 low; [b]IHC score; 0-1 negative, 2-3 positive; [c]Mean ±SD of positive peptide number in ELISPOT assay. RFS: Relapse-free survival; OS: overall survival. Pos: Positive; neg. negative. Significant *p*-Values are shown in bold.

Table V. *Potential prognostic factors involved in malignant glioma DC vaccines-2.*

Factors	Cases	Mean±SD	Groups (case#)	Statistical analysis (RFS)	Statistical analysis (OS)
DC numbers ($\times10^7$)	15	2.2±1.2	<2 (8) *vs*. ≥2 (7)	*p*=0.6174	*p*=0.6271
DC ratio (%)	15	50.6±12.9	<50 (7) *vs*. ≥50 (8)	*p*=0.5497	*p*=0.4352
DC1/DC2 ratio	15	99.2±147	<100 (11) *vs*. ≥100 (4)	*p*=0.4304	*p*=0.3739
CD40 (%)	15	85.3±20.3	<90 (4) *vs*. ≥90 (11)	*p*=0.2289	*p*=0.2701
CD83 (%)	15	52.0±27.7	<60 (7) *vs*. ≥60 (8)	*p*=0.1544	*p*=0.0738
$CD83^+$ DC ($\times10^6$) numbers	15	13.1±10.0	<10 (8) *vs*. ≥10 (7)	*p*=0.3162	*p*=0.282
CCR7 (%)	15	8.6±8.7	<7 (8) *vs*. ≥7 (7)	*p*=0.6102	*p*=0.6088
IL-12 production	16	3.4 ±3.8[a] (cut off 2.0 ng/ml)	<2 (7) *vs*. ≥2 (9)	*p*=0.724	*p*=0.8747
Plasma IFN-γ level	14	cut off (10 pg/ml)	<10 (8) *vs*. ≥10 (6)	*p*=0.6771	*p*=0.7268

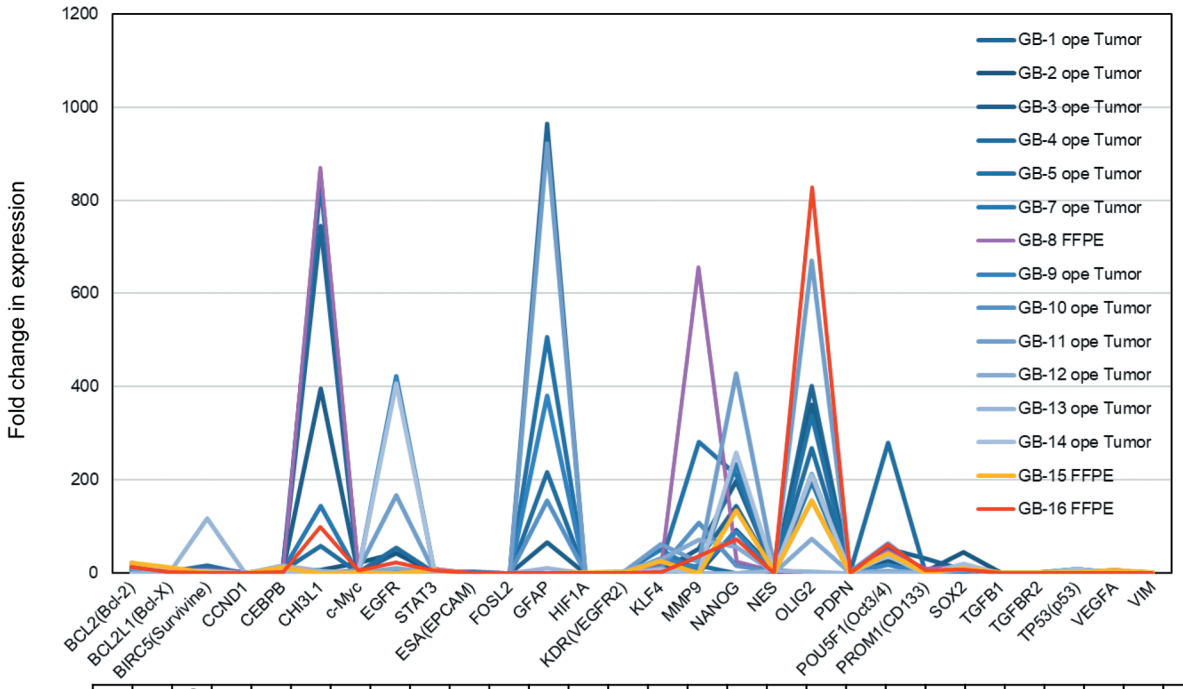
[a]Mean±SD value of IL-12 level produced from alpha type-1 DCs. RFS: Relapse-free survival; OS: overall survival.

Clinical results and adverse effects. The mean injected DC number was 2.2×10^7/patient, the DC injection time was 8.8 on average, and 11 cases involved 10 injections. No severe side effects higher than grade III were observed. After 6 years of observation since the initiation of the study, five patients were still alive, and two of these patients were relapse-free. The relapse-free time and overall survival time (median) were 11.0 and 19.0 months, respectively.

Immunological monitoring. Regarding the skin tests, positive DTH reactions against peptide and KLH were detected in 4 cases (27%) and 9 cases (60%), respectively, in vaccinated patients (Table II). Ten of the 15 evaluable cases (67%) showed positive ELISPOT reactions against any tumor antigen peptides (Table III), and 9 cases responded to more than 2 peptides (positive peptides: 1.9±1.6 per case). The serum IFN-γ concentration measured with a sensitive ELISA kit was specifically increased in 6 cases (43%) to more than 10 pg/ml after the initiation of the DC vaccine trial (data not shown).

Characterization of HLA and tumor antigen expression and TIL status by IHC. An analysis of tumor antigen expression by IHC demonstrated that 15 cases showed evidence of at least one tumor antigen and that 12 cases (80%) exhibited more than 2 tumor antigens (Table III). HLA-class I protein was positive in all cases and 11 cases (68%) showed high expression levels with scores greater than 2. Meanwhile, tumor-infiltrating lymphocyte (TIL) staining revealed a low percentage of $CD4^+$ T cells and a high percentage of $CD8^+$ T cells and $CD204^+$ macrophages (Table IV). PD-L1 and phosphorylated STAT3 expression were relatively low.

Glioma-associated gene expression in tumor tissues and cytokine gene expression in PBMCs detected by quantitative



Genes	BCL2(Bcl-2)	BCL2L1(Bcl-X)	BIRC5(Survivine)	CCND1	CEBPB	CHI3L1	c-Myc	EGFR	STAT3	ESA(EPCAM)	FOSL2	GFAP	HIF1A	KDR(VEGFR2)	KLF4	MMP9	NANOG	NES	OLIG2	PDPN	POU5F1(Oct3/4)	PROM1(CD133)	SOX2	TGFB1	TGFBR2	TP53(p53)	VEGFA	VIM
Positive case#	14	13	8	1	15	9	6	12	5	1	0	8	1	3	14	11	13	12	13	1	14	6	15	0	0	9	7	0
Positive rate (%)	93	87	53	7	100	60	40	80	33	7	0	53	7	20	93	73	87	80	87	7	93	40	100	0	0	60	47	0

Figure 2. *Glioma-associated gene expression in tumor tissues detected by real-time PCR. Total RNA was isolated from tumor tissues or formalin-fixed paraffin-embedded (FFPE) specimens. Each gene is presented as the summation of expression data from 15 cases and is indicated as the fold change in expression compared to that in normal astrocytes, which is rated as 1. A fold change of more than 2 was evaluated as positive, and the positive rate of the expression of each gene expression in 15 cases is shown at the bottom.*

PCR. A real-time PCR analysis demonstrated that most glioma tissues showed GB-stem cell and mesenchymal phenotypes with high expression of CEBPB, CHI3L1, NANOG, NES, OCT4 and SOX2 (Figure 2), and that PBMCs from DC-vaccine-treated patients showed increase in T-cell activating cytokines (IFN-γ, GZMB, CD127) and chemokines (CCL4, CCL5, CCR5, CXCL10, CXCR3) (Figure 3). Additionally, the expression of the IFN-γ mRNA gene in PBMC from DC-vaccine-treated patients indicated a positive correlation with overall survival (Figure 4). However, glioma-associated mRNA gene expression in tumor tissues did not show any significant relationship with survival (data not shown).

Association of survival with various clinical and immunological parameters. Besides PS and MGMT status, no clinical or immunological parameters showed any association with RFS or OS (Tables IV and V).

The correlation of the plasma IFN-γ levels with the IL-12 levels and the ELISPOT-positive peptide number. The correlation among three parameters (plasma IFN-γ level, IL-12p70 level and ELISPOT-positive peptide number) was investigated using a Spearman test. The maximum plasma IFN-γ level after DC vaccine administration had a positive correlation with IL-12 levels produced by activated DCs, but not the ELISPOT-positive peptide number (Figure 5). Moreover, the plasma IFN-γ levels showed no significant association with OS as shown in Table V.

Evaluation of RFS and OS in glioma patients registered in phase II study. After six years of observation since the initiation of the study, five patients were still alive, and two remained relapse-free. The RFS and OS (median) were 11.0 and 19 months, respectively; the RFS and OS (median) of the historical control group were 9.0 and 16.0 months, respectively. The RFS and OS analyses revealed a significant

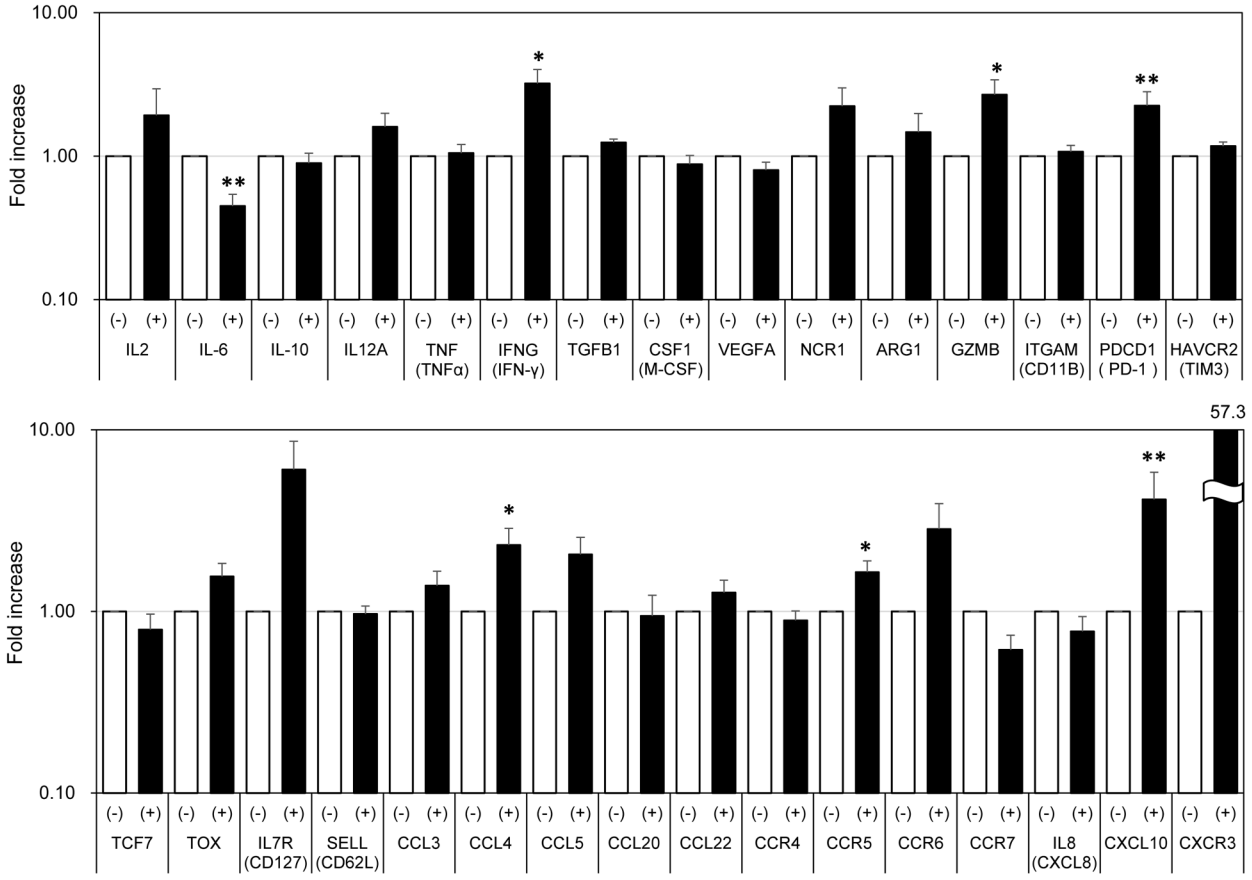


Figure 3. *Cytokine and chemokine gene expression in PBMCs from DC-treated patients using real-time PCR. Total RNA was isolated from PBMCs from patients [pre-DC vaccine and post-DC vaccine (4 times)]. The fold change in gene expression in the post DC vaccine PBMCs is shown compared to that in pre-DC PBMC, which is rated as 1. The open column shows pre-DC PBMCs (–); the closed column shows post-DC PBMCs (+). Each column represents the mean value of triplicate experiments. **p<0.01, *p<0.05, statistically significant.*

survival-prolonging effect in DC-treated glioma patients (p=0.024 in RFS, p=0.048 in OS, Figure 6).

Discussion

Since the development of immune checkpoint blockade therapy, novel effective combination immunotherapy with immune checkpoint inhibitors (ICIs) and tyrosine kinase inhibitors (TKIs), indoleamine 2,3-dioxygenase (IDO)-1 inhibitors or other small chemical compounds for the treatment of intractable solid cancers has been developed (22, 23). However, little success has been obtained. The next goal will be the maintenance or reinforcement of durable antitumor effects induced by ICIs for a long-term cancer control (23).

Dendritic cell-based cancer vaccines are one of the old technologies as cancer therapy regimens; however, they still show potential as immunotherapeutics despite the negative results obtained for sipuleucel-T (Provenge, Dendreon), an autologous cellular immunotherapeutic for treatment of prostate cancers, and for an anti-MAGE-A3 vaccine against advanced non-small cell lung cancers. Both have been evaluated in the context of phase III clinical trials.

Accumulating evidence has shown that IL-12p70-producing active DCs can have strong effects on TILs inside the tumor and exhibit very potent antitumor effect in an in vivo mouse model (24). Additionally, Okada *et al*. have demonstrated that IL-12p70-producing, HLA-A2 peptide-pulsed DCs, which are called α-type-1 polarizing DCs, showed antitumor effect and they obtained 2 objective responders and 9 patients with progression-free survival for at least 12 months in a phase I/II clinical trial involving 22 cases of HLA-A2$^+$ high-grade glioma (25). Our group also reported a phase I clinical trial of HLA-A2 or A24 peptide-pulsed α-type-1 polarizing DC vaccines for the treatment of high-grade malignant gliomas in 2012 (20).

PBMC

Target Name	PEARSON	PEARSON[p]
CCL4	-0.333	0.266
IL12A	-0.269	0.375
CCL20	-0.225	0.459
CCR6	-0.190	0.535
PDCD1 (PD-1)	-0.164	0.593
IL-6	-0.130	0.673
ARG1	-0.122	0.691
CSF1(M-CSF)	-0.102	0.739
PDCD1LG2 (PD-L2)	-0.097	0.752
IL2	-0.083	0.787
IL-10	-0.082	0.791
TGFB1	-0.080	0.794
VEGFA	-0.068	0.824
CXCL10	-0.066	0.829
TNF(TNFα)	-0.053	0.863
NCR1	0.012	0.969
GZMB	0.015	0.961
IL7R	0.024	0.937
TCF7	0.030	0.923
CXCR3	0.034	0.913
CCR7	0.045	0.883
CCR4	0.049	0.873
HAVCR2(TIM3)	0.118	0.700
TOX	0.146	0.633
CXCL12	0.150	0.625
CCR5	0.165	0.589
CCL5	0.198	0.517
IL8(CXCL8)	0.206	0.500
CCL3	0.235	0.439
ITGAM(CD11B)	0.240	0.430
CCL22	0.354	0.235
SELL(CD62L)	0.408	0.166
IFNG(IFN-γ)	0.576	0.039

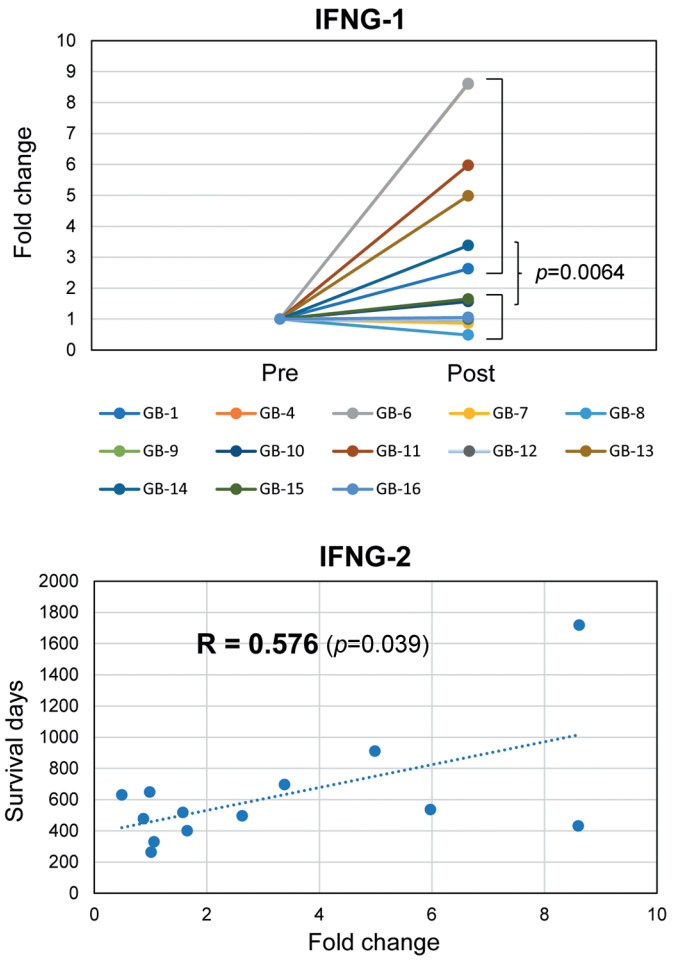


Figure 4. *Association of cytokine and chemokine gene expression in DC vaccine-treated patient PBMCs with overall survival. After α-type-1 DC vaccinations (4-times), the IFN-γ mRNA gene expression levels in PBMCs significantly increased by several times (IFNG-1). Importantly, upregulation of IFN-γ mRNA in PBMC from DC-vaccine-treated patients showed a positive correlation with overall survival using a Pearson correlation test (IFNG-2).*

Correlation of plasma IFN-γ protein level with IL-12 and ELISPOT-positive peptide number

Spearman co-efficient

Group	r value	*p*-Value
IFN-γ *vs.* IL-12	0.6586	**0.0104***
IFN-γ *vs.* elispot	0.4135	0.1417
IL12 *vs.* elispot	0.4157	0.1394

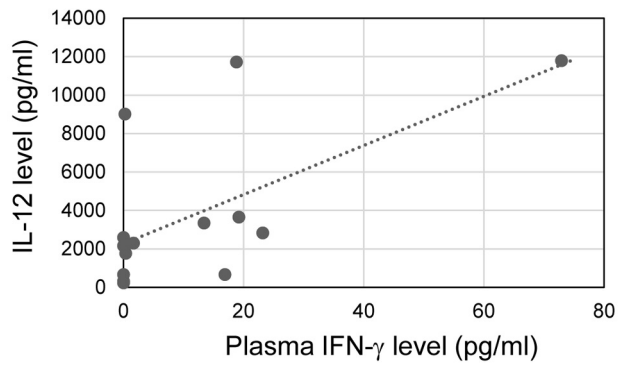


Figure 5. *Correlation of the plasma IFN-γ protein level with the level of IL-12 and the ELISPOT-positive peptide number. The correlation among the three parameters was analyzed using a Spearman correlation test. The levels of IL-12p70 in culture media and human plasma IFN-γ were measured using cytokine-specific ELISA kit. *p<0.05, statistically significant.*

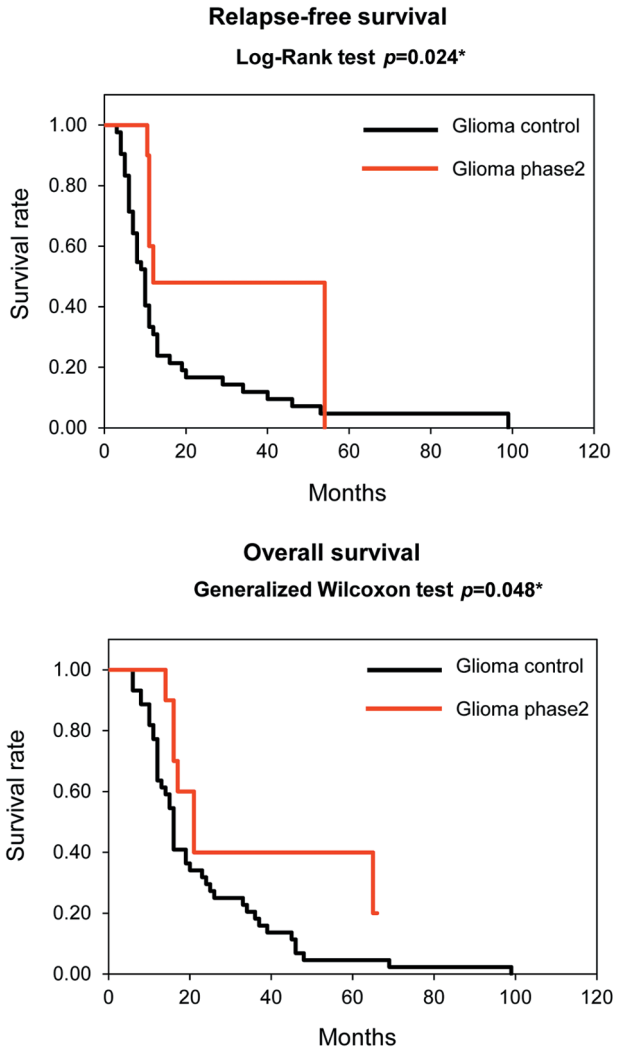


Figure 6. *Evaluation of RFS and OS in high-grade glioma patients registered in the phase II study. The relapse-free time and overall survival time (median) were 11.0 and 19 months, respectively, and were compared with those of the historical control group (9.0 and 16.0 months, respectively). A comparative analysis of the survival times between phase II patients (red line) and the retrospective historical control patient group (black line) was performed using the log rank test or generalized Wilcoxon test. The relapse-free and overall survival analysis indicated a significant survival benefit for DC-treated high-grade glioma patients (p=0.024 in RFS, p=0.048 in OS). *p<0.05, statistically significant.*

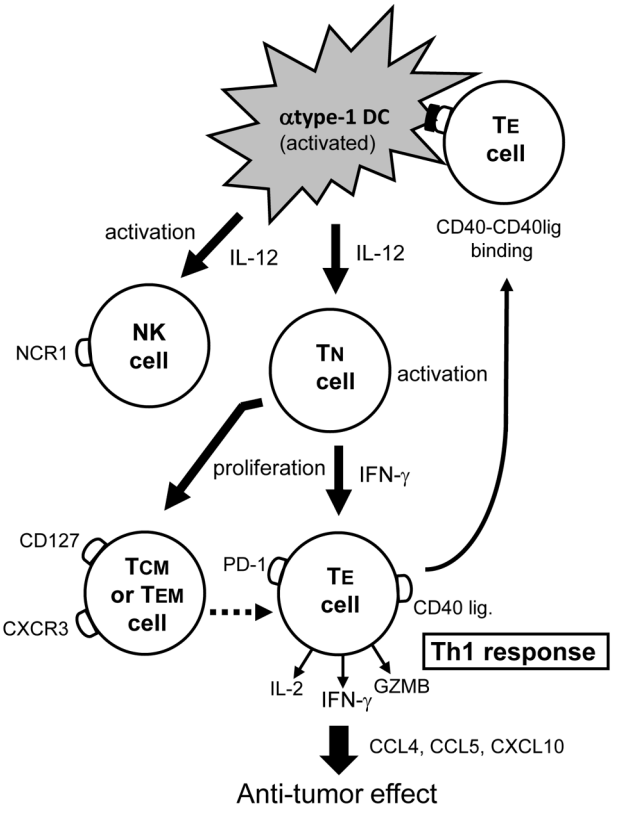


Figure 7. *Alpha-type-1 active DC-initiated T-cell activation cycle. Activated effector T cells expressing CD40 ligand can stimulate α-type-1 DCs, which can produce a high amount of IL-12 after CD40-CD40 ligand binding, and IL-12 can activate naïve T cells that differentiate into effector and memory T cells, and repeat the cycle each time DCs are administered. TN: Naïve T cells; TE: effector T cells; TCM: central memory T cells; TEM: effector memory T cells.*

Recently, as the important mechanistic role of ICI in the immunological effect was determined, IL-12 production by activated DCs has received extensive attention (26-28). Garris *et al*. have demonstrated that the activation of antitumor T cells by anti-PD-1 antibody was not direct, but rather involved T cell-DC interactions mediated by IL-12 and INF-γ production (26). Additionally, Algazi *et al*. have reported that intratumoral IL-12 plasmid transfection combined with anti-PD-1 blockade enhanced TIL infiltration and sustained the IL-12/IFN-γ feed cycle, resulting in a stronger antitumor response in a clinical phase II trial of poorly immunogenic melanomas (27). Meanwhile, Greaney *et al*. have investigated the effect of intratumoral plasmid IL-12 transfection and found that the number of intratumoral PD-1^{+}CD3^{+}CD8^{+} tumor-specific T cells increased after treatment of cutaneous melanoma patients (28). Surprisingly, this observation was very similar to the results obtained in a previous study; injection of a high amount of IL-12-producing DCs (retrovirus-mediated) intratumorally into mouse B16F10 melanoma tumors effectively induced high accumulation of TILs (29).

In the current study, we hypothesized that the IL-12/IFN-γ cascade (Figure 7) in activated DCs and effector T cells acts as a key factor in the triggering and maintenance of potent antitumor activity initiated by high amount of IL-12-producing DCs. In turn, activated effector T cells expressing

the CD40 ligand can stimulate α-type-1 DCs, which can produce a high amount of IL-12 after CD40-CD40 ligand binding, and IL-12 can activate naïve T cells that differentiate into effector and memory T cells to repeat the cycle each time DCs are administered.

As far as the IL-12/IFN-γ feed cycle reported by Algazi *et al*., which is a reasonable theory, the combination of α-type-1 DCs with anti-PD-1 blockade is considered to be worth investigating in a clinical trial of malignant glioma or other intractable solid tumors because our cascade theory is similar to the IL-12/IFN-γ feed cycle. IL-12 is well-known to stimulate antigen-specific effector T cells and promote antitumor response of Th1 type T cells. However, IL-12 may reduce the memory T cell population compared with the effector cell populations (30). Our quantitative PCR assay demonstrated that besides Th1-type effector T cell activation, $CD127^{+}CXCR3^{+}$ memory type T cells (central ~ effector memory T cells) were identified as shown in Figure 3. These results suggest that α-type-1 DCs might lead to the partial proliferation of central memory T cells and that some approach for maintaining memory T cells as precursors of effector T cells could be necessary for the reinforcement of the antitumor effect of DC vaccines (31).

Finally, long-term observation over 6 years revealed the survival benefit in terms of both relapse-free and overall survival in α-type-1 DC vaccine-treated glioma patients compared with those in glioma patients who did not receive DC vaccines as a retrospective historical control group. These observations are preliminary because of the study design and the small number of enrolled cases. However, secretion of a substantial amount of IL-12 from α-type-1 DCs probably induced the IFN-γ-mediated stimulation of Th1-type effector T cells involved in the tumor-specific ELISPOT response, which could contribute to the association with the overall survival benefit. Taken together, the results indicate that α-type-1 DC vaccines with the ability to induce high IL-12 production could be used in the future for combination immunotherapy with anti-PD-1/PD-L1 blockade for the treatment of intractable solid cancers including glioblastomas (32).

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

KM and Yasuto Akiyama participated equally in the design of the study and drafting of the manuscript and were responsible for completing the study. AI, HM, Yoshiaki Abe and Yasuto Akiyama carried out apheresis and cell processing and were responsible for DC production. SD, KM and NH were responsible for the clinical work of the study including the collection of clinical samples. CM, RK, AK, KW, and TA participated in the design of the experiments and performed the biological assays. II, TO and TS contributed to the pathological diagnosis. YN and KY reviewed the manuscript. All Authors read and approved the final draft.

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