CLINICAL STUDY - PATIENT STUDY

Integration of autologous dendritic cell-based immunotherapy in the primary treatment for patients with newly diagnosed glioblastoma multiforme: a pilot study

Hilko Ardon · Stefaan Van Gool · Isabel Spencer Lopes · Wim Maes ·
Raf Sciot · Guido Wilms · Philippe Demaerel · Patricia Bijttebier ·
Laurence Claes · Jan Goffin · Frank Van Calenbergh · Steven De Vleeschouwer

Abstract Despite resection, radiochemotherapy, and maintenance temozolomide chemotherapy (TMZm), the prognosis of patients with glioblastoma multiforme (GBM) remains poor. We integrated immunotherapy in the primary standard treatment for eight pilot adult patients (median age 50 years) with GBM, to assess clinical and immunological feasibility and toxicity in preparation of a phase I/II protocol HGG-2006. After maximum, safe resection, leukapheresis was performed before radiochemotherapy, and four weekly vaccinations with autologous GBM lysate-loaded monocyte-derived dendritic cells were given after radiochemotherapy. Boost vaccines with lysates were given during TMZm. During the course of vaccination, immunophenotyping showed a relative increase in CD8+CD25+ cells in six of the seven patients, complying with the prerequisites for implementation of immunotherapy in addition to postoperative radiochemotherapy. In five patients, a more than twofold increase in tumor antigen-reacting IFN-γ-producing T cells on Elispot was seen at the fourth vaccination compared with before vaccination. In three of these five patients this more than twofold increase persisted after three cycles of TMZm. Quality of life during vaccination remained excellent. Progression-free survival at six months was 75%. Median overall survival for all patients was 24 months (range: 13–44 months). The only serious adverse event was an ischemic stroke eight months postoperatively. We conclude that tumor vaccination, fully integrated within the standard primary postoperative treatment for patients with newly diagnosed GBM, is feasible and well tolerated. The survival data were used to power a currently running phase I/II trial.

H. Ardon (✉) · J. Goffin · F. Van Calenbergh ·
S. De Vleeschouwer
Department of Neurosurgery, University Hospital Leuven,
Catholic University of Leuven, Herestraat 49, 3000 Leuven,
Belgium
e-mail: hilko.ardon@uz.kuleuven.ac.be

S. Van Gool · I. S. Lopes · W. Maes
Department of Experimental Medicine, Catholic University of
Leuven, Leuven, Belgium

R. Sciot
Department of Pathology, University Hospital Leuven, Catholic
University of Leuven, Leuven, Belgium

G. Wilms · P. Demaerel
Department of Imaging, University Hospital Leuven, Catholic
University of Leuven, Leuven, Belgium

S. Van Gool
Department of Child and Woman, University Hospital Leuven,
Catholic University of Leuven, Leuven, Belgium

P. Bijttebier · L. Claes
Centre for Clinical Assessment and Psychopathology, Catholic
University of Leuven, Leuven, Belgium

Introduction

Despite full, state-of-the-art oncological therapy, including maximum safe surgical resection, external beam radiotherapy, and chemotherapy, the prognosis of glioblastoma multiforme (GBM) remains poor with a median survival of 14 months [1]. Even after this maximum treatment, relapse is universal. At the time of relapse, the prognosis is even worse and virtually all patients are dead within 18 months after relapse [2–5]. There is a clear need for well-tolerated long-term treatments that are tumor-specific and able to kill

not only all (residual) tumor cells that infiltrate the adjacent areas of the brain [6], but also the recently described radio and chemoresistant CD133+ glioma cancer stem cells [7–9]. The immune system provides us with some promising tools in that regard, and further development of immune therapy and its integration in the current therapeutic concepts is warranted.

Brain tumors are located in the so-called immune distinct environment of the central nervous system and malignant gliomas display local and systemic immune suppressive characteristics [10–13]. Nevertheless, proof of the principle of dendritic cell (DC)-based vaccination strategies against high-grade glioma (HGG) has been documented by our group and several others both in vitro [14–16] and in animal models [17–20]. Early clinical data confirm the safety, feasibility and possible beneficial effects of DC vaccination in patients with this fatal disease (recently reviewed by De Vleeschouwer et al. [21] and Van Gool et al. [22]).

Considering the promising results of surgery and adjuvant autologous DC-based tumor vaccination in a large group of patients with relapsed malignant glioma [23], the next step in the clinical development of DC-based immunotherapy for HGG is to fully integrate immunotherapy within the standard first-line treatment, considering putative mutual beneficial effects of the combination of immunotherapeutic strategies with conventional therapeutic modalities such as radiotherapy and chemotherapy [24–30]. In these reports, immunotherapy was added to either radio or chemotherapy but was never fully integrated in a comprehensive schedule of radio–chemo–immunotherapy, as presented here. Following an initial pilot phase, a phase I/II clinical trial has to demonstrate feasibility, toxicity, and efficacy of such a multimodal treatment approach. As final step in the clinical development, a prospective double-blind randomized clinical trial is needed to demonstrate possible beneficial activity of immunotherapy integrated in the primary multimodal treatment for patients with HGG.

We summarize our experience in a pilot group of eight patients with newly diagnosed GBM treated with immunotherapy (autologous DC loaded with autologous tumor cell lysate) integrated in a standardized fashion in the multimodal standard therapy—surgical resection, radiochemotherapy (RCT), and maintenance temozolomide (TMZm) chemotherapy [1]. The emphasis of this report is on the clinical and immunological feasibility and toxicity of integration of DC vaccination in the conventional therapeutic modalities. The progression free survival data were used to power the currently running phase I/II trial (HGG-2006, EudraCT 2006-003881-20), integrating DC vaccination as an add-on therapy to standard postoperative RCT and maintenance chemotherapy in patients with newly diagnosed GBM.

Materials and methods

Patient population

Eight patients (five males and three females) presented with newly diagnosed primary GBM, confirmed on central review histopathology. Patients were included for DC therapy, if they met the inclusion criteria as summarized in Table 1. Patients' characteristics are described in Table 2. Their median age was 50 years (range 31–62 years). All patients were operated upon and were off steroids and nonsteroidal anti-inflammatory drugs at the time of leukapheresis (as determined by the exclusion criteria) and during vaccination. Approval by the local ethics committee was obtained, and patients' written informed consent was obtained before the start of immunotherapy.

Assessment of the extent of tumor resection before vaccination

Total resection was defined by the neurosurgical report and the absence of any residual tumor mass on early postoperative MRI (T1 weighted spin-echo images before and after gadolinium enhancement) performed within 72 h after surgery. Any resection leaving a measurable contrast-enhancing tumoral mass less than 2 cm^3 was considered subtotal. All solid residual tumor of a measurable size ≥ 2 cm^3 was classified as partial resection.

Tumor cell lysate

Tumor tissue was transported from the operation room into the laboratory and snap-frozen at $-80°C$ without additives. The tissue was kept frozen at $-80°C$. For further preparation, the tissue was thawed and put into NaCl 0.9% with 1% human serum albumin (HSA), and was homogenized mechanically. Afterwards, six snap freeze–thaw cycles between liquid N_2 and 56°C were performed. The lysate was filtered with a 70 μm Falcon filter (BD Biosciences Europe, Erembodegem, Belgium). The amount of protein was measured using the Coomassie blue staining method and spectrophotometry at 595 nm [31]. After irradiation (60 Gy), the lysate was kept frozen in liquid N_2 until use.

Preparation of autologous DC vaccines

In all patients peripheral blood mononuclear cells (PBMC) were obtained from a single leukapheresis and kept frozen in liquid N_2 until use. For each vaccination, part of the PBMC was thawed, and adherent cells were differentiated to immature DC as described elsewhere [32]. Immature DC were loaded with 200 μg tumor proteins per million DC as described elsewhere [14]. For the loading procedure,

Table 1 Inclusion and exclusion criteria

Inclusion criteria

Age >18 and <70 years

First diagnosis of high-grade glioma, histologically proved

Total or subtotal resection of tumor mass, confirmed by assessment of neurosurgeon and postoperative MRI within 72 h

Availability of enough tumor tissue, kept dry in a sterile vial at −80°C

Perioperative administration of corticosteroids tapered within 7 days postoperatively

Life expectancy >3 months

Histology confirmed by reference pathology

Written Informed consent by patient

Exclusion criteria

Pregnancy

Postoperative Karnofsky index <70

Simultaneous treatment according to other clinical trials

Virus serology positive for hepatitis, syphilis, or HIV

Blood counts: leukocytes <3,000/μl, lymphocytes <500/μl, neutrophils <1,000/μl, hemoglobin <9 g/100 ml, thrombocytes <100,000/μl 2 days prior to leukapheresis

Documented immune deficiency

Documented autoimmune disease

Mandatory treatment with corticosteroids or salicylates in inflammatory dose

Other active malignancy

0.01% autologous plasma was used during the first 2 h, 0.1% for the next 4 h, and finally 1% for the last 20 h. At time of loading, rTNF-α (Strathmann Biotec, Dengelsberg, Germany), rIL-1β (Strathmann Biotec) and PGE2 (Prostin®; Pfizer, Brussels, Belgium) were added at final concentrations of 120, 120 and 20 ng/ml, respectively. After 24 h, early mature loaded DC were resuspended in PBS with 0.5% HSA at a concentration of $2–6 \times 10^6$/ml. Tuberculin syringes were filled with the loaded DC in

suspension (400 μl), containing 1–2 million mature DC per syringe. The phenotype of the cells (Table 3) was determined by FACS, using FITC-labeled and PE-labeled mAb purchased from BD Biosciences Pharmingen (San Jose, USA).

Treatment schedule: fully integrated radio–chemo–immunotherapy

Patients underwent maximum safe surgical resection of the tumor. Peri-operative corticosteroids were withdrawn within one week after resection. Leukapheresis was performed after histological diagnosis was obtained and inclusion criteria were met (Fig. 1). After leukapheresis, patients were treated with limited field external beam radiotherapy (30×2 Gy) and concomitant chemotherapy with TMZ (75 mg/m^2) during six weeks as outlined by Stupp et al. [1]. After radiochemotherapy, tumor lysate-loaded DC were injected weekly for four weeks. Following these four weeks, TMZm chemotherapy was started. The maintenance 28-day cycles consisted of five days oral intake of TMZ (150 mg/m^2 for the first and 200 mg/m^2 for the following cycles). During the first, second, third, and sixth cycles, further boost vaccinations with tumor lysate (without DC) were administered at day 8 of the cycle. At the time of progression possible rescue therapy was at the physician's discretion.

Vaccination

Vaccination was performed by intradermal (i.d.) injection of 1–12 million (median 4.1×10^6) DC per lymph node region in the upper third of the arms (left and right) at weeks 1, 2, 3, and 4 (induction vaccines). Further vaccinations were given with tumor lysate with a median of 1,500 μg (range 1,500–4,000 μg) proteins per vaccine

Table 2 Patients' characteristics

Patient	Age at surgery (years)	Sex	Resection	Site of tumor	Reference histology	DC Therapy	RPA class
1	56	M	Subtotal	Left frontal	GBM	V1–V7	IV
2	48	M	Subtotal	Left frontal	GBM	V1–V15 (V3 + V4 contaminated—not given)	IV
3	31	M	Subtotal	Left frontal	GBM	V1–V15	IV
4	46	F	Total	Left temporal	GBM	V1–V14 and V1–V7[a]	IV
5	60	F	Subtotal	Left temporal	GBM	V1–V9	IV
6	51	F	Subtotal	Left parietal	GBM	V1–V6	IV
7	49	M	Subtotal	Right occipital	GBM	V1–V11	III
8	62	M	Total	Right parietal	GBM	V1–V8 and V1–V13[a]	IV

V, vaccine

[a] Re-operation at relapse followed by immunotherapy

Table 3 DC phenotype characteristics

	Median	IQR
CD86%	81.06	14.18
HLA DR%	93.58	6.4
CD14%	2.04	1.44
CD3%	0.82	2.36
CD1a%	9.8	13.94
CD25%	5.7	5.96
CD83%	22.96	12.66
CD80%	38.42	16.78
CD19%	0	0.35

CD, cluster of differentiation; HLA DR, human leukocyte antigen DR; IQR, interquartile range

injected in two syringes each containing a final volume of 400 µl (boost vaccines).

Immune monitoring

Immune monitoring was performed at three different levels. First, delayed type hypersensitivity (DTH) reaction was tested at the first and fourth vaccinations. For this, 100 µl crude tumor lysate and 100 µl control PBS/HSA were injected intradermally. After 48 and 72 h redness and induration were assessed. DTH reactions were judged as positive if the average perpendicular measurement of the induration exceeded 5 mm.

Blood samples were obtained at times of leukapheresis, vaccine 1, vaccine 4, and vaccine 7. In each whole blood

sample, the phenotype of circulating T cell populations was determined by FACS: total CD3+ population, the CD4+ and CD8+ subpopulations, and the activation markers HLA-DR on CD3+ cells and CD25 on both subpopulations. For this, FITC-labeled and PE-labeled mAb were purchased from BD Biosciences Pharmingen.

Finally, PBMC from each blood sample were cryopreserved and thawed together at the end of the immunotherapy for use in an Elispot assay. A positive response was defined as an at least twofold increase in the number of antigen-specific spots after the fourth and seventh vaccinations, as described by Bancherau et al. [33].

The protocol was adapted, based on the manufacturers' instructions (Mabtech, Nacka Strand, Sweden). In brief, 96-well polyvinylidene difluoride membrane plates (MAIPSWU10; Millipore, Bedford, MA, USA) were treated with 70% ethanol (50 µl per well) for 1 min and washed with PBS before coating. Next, plates were coated overnight (4°C) with coating antibody (1-D1K, 15 µg/ml, Mabtech). After blocking, 2×10^5 viable PBMC per well were seeded in the presence of PHA (1 µg/ml), serum-free medium (CTL-test™, Cellular Technology, Aalen, Germany) or autologous tumor lysate or protein extracted by ethanol precipitation and incubated for 24 h (37°C, 5% CO_2) in a final volume of 100 µl. Cells were washed away and detection antibody (7-B6-1-biotin, 1 µg/ml, Mabtech) was added (overnight, 4°C). Streptavidine-ALP (Mabtech) was added for 1 h after which substrate solution was added (AP conjugate substrate kit, Bio-Rad, Nazareth Eke, Belgium). Spots were counted with Immunoscan and Immunospot software (CTL).

Fig. 1 Treatment schedule. DC-based immunotherapy was integrated in the state-of-the art postoperative radiochemotherapy. Leukapheresis to harvest autologous monocytes is performed once, at least seven days after weaning off steroids and immediately before the start of the concomitant radiochemotherapy. After the radiochemotherapy, but before the maintenance chemotherapy with TMZ, four weekly induction vaccines are administered intradermally to the patient. Afterwards, maintenance chemotherapy (5/28 days) is started and one week after the start of the 1st, 2nd, 3rd, and 6th cycles of TMZ, a boost vaccine is administered
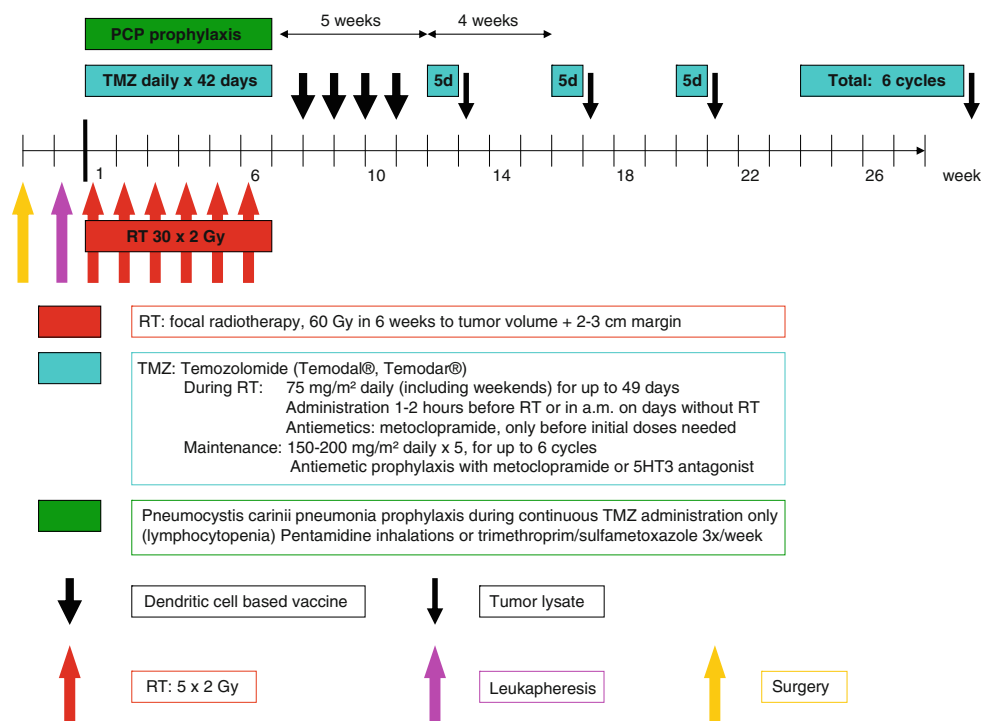
Antigen-specific spots were calculated after subtracting the background spots of unstimulated PBMC. All measurements were obtained in triplicate.

Patient assessment

All patients were followed by clinical examination and MRI scanning (12 weeks after surgery and then every 3 months). Upon specific indication methionine PET was performed. At the time of each vaccination, quality of life (QOL) was assessed using the QLQ-C30 and the Fertigkeitenskala Münster-Heidelberg (FMH) [34–36]. Karnofsky performance scale (KPS) was assessed by the patients and registered at each visit [37].

The QLQ-C30 is a 30-item questionnaire composed of multi-item scales and single items. It is designed by the European Organization for Research and Treatment of Cancer (EORTC) for use in international clinical trials in oncology. It is used to assess functional disability, somatic symptoms, global health, and overall quality of life. In this study, only the indices for general health and overall quality of life (single items 29 and 30) were used. The rating scale ranges from 1 (very bad) to 7 (excellent).

The FMH is a 56-item questionnaire used to assess the patients' ability to carry out daily life activities. In this study, a 53-item version of the instrument was used and a total score ranging from 0 to 53 was calculated.

The KPS was used for assessment of the patients' level of physical ability; it consists of one rating on a scale from 0 (dead) to 100 (normal functioning).

Results

Feasibility: preparation and characterization of vaccines

The patients received a median of 10 vaccines (range 6–16 vaccines). The details of the vaccination for each patient are described in Table 4. Patients #4 and #8 underwent a re-operation at time of relapse with postoperative inclusion in the HGG-IMMUNO-2003 cohort comparison trial (adjuvant DC-based immunotherapy in patients with relapsed GBM). The median yield of loaded mature DC from freshly isolated PBMC was 8.2×10^6 per vaccination session (range 2–24×10^6; $n = 30$). Two vaccinations (both in patient #2) could not be administered because the release criteria of the cellular product were not fulfilled.

Feasibility: immune response

An increase in the proportion of CD8+CD25+ T cells was noted during the course of vaccination in six of the seven patients (Table 5). Two of those patients (#3 and #7) showed

a positive skin test, and an increase in IFN-γ-producing tumor antigen-reacting T-cells was observed in five patients (#1, #3, #5, #6 and #7).

In six patients a DTH skin test with autologous tumor homogenate was performed at the time of the first and fourth vaccinations (Table 5). In patient #2 the skin test was only performed at the first vaccination and in patient #5 there was not enough tumor material available for the skin test (all tumor lysate was used for vaccination). Two patients (#3 and #7) had a positive reaction within 72 h after i.d. injection at the first and fourth vaccinations. In these patients the control i.d. injections with PBS/HSA were negative. Patient #3 was progression free at the end of the follow-up (FU) period. Patient #7 had a recurrence of the primary tumor at 17 months FU. In patient #8, a delayed positive reaction at multiple injection sites occurred 6 months after the first vaccination. This patient had a recurrence of the primary tumor at 26 months FU.

The results of the Elispot assay ($n = 8$) showed an increase in IFN-γ-producing tumor antigen-reacting T-cells between the first and fourth vaccinations in five of the eight patients (Table 5; Fig. 2). In three of these five patients (#1, #3 and #5) a more than twofold increase in tumor-specific IFN-γ production persisted after three cycles of TMZm (Table 5; Fig. 2). Two of the five patients with an increase in tumor specific IFN-γ production at the fourth vaccination, had positive skin tests (#3 and #7).

Toxicity

The clinical data during vaccination are listed in Table 6, including adverse events graded according to the National Cancer Institute (NCI) common toxicity criteria (CTC).

In patient #7 lymphopenia ($650/mm^3$) was diagnosed during TMZm chemotherapy, which did not require cessation or delay of treatment. In patient #2 a focal epileptic insult occurred between the second and third vaccinations. Three patients (#2, #4 and #8) experienced (transient) dysphasia postoperatively and in patient #8 there was a transient recurrence of the dysphasia after the second cycle of adjuvant TMZ chemotherapy. Four patients (#2, #6, #7 and #8) complained of fatigue during the treatment, general malaise and myalgia were each mentioned by one patient (#3 and #6, respectively).

In patient #5, a sudden onset right hemiplegia and aphasia occurred at 8 months FU (after the third boost with tumor lysate). MRI showed hyperintensities in T2 weighted images and heterogenic signals in T1 weighted images at the posteromedial side of the left temporal lobe, near the site of tumor resection (Fig. 3a, b). These MRI data were most compatible with ischemic changes and the episode of hemiplegia and aphasia was diagnosed as an ischemic event. Inflammation, in terms of auto-immunity, could not

Table 4 Vaccination details

Patient	PBMC ($\times 10^9$)				DC$_i$ ($\times 10^6$)				Loaded DC$_m$ ($\times 10^6$)				Tumor lysate	
	V1	V2	V3	V4	V1	V2	V3	V4	V1	V2	V3	V4	# boosts	Amount/boost (µg)[a]
1	0.998	1.043	0.867	0.961	10.8	7.6	15.2	12.7	5.6	3.5	5	3.5	3	1,500
2	1.088	1.075	1.024	1.034	26.4	9	30.6	na	12	4.5	na	na	11	1,500
3	1.007	1.194	1.086	1.072	36	39	33	40	20.8	15.3	17	24	12	1,500
4[b]	1.099	0.92	1.186	1.191	14.7	20.6	16.8	15	7.4	9	5	8.4	10	1,500
5	0.823	0.922	0.597	0.916	31	32	37	29	13	13	16	14.3	5	1,500
6	1.025	0.892	1.062	1.05	16	18	17.5	26	10	8	13.5	13.5	2	1,500
7[c]	0.652	0.575	0.608	0.605	18	8.06	20.46	14	7.5	6.76	9.75	5	7	L1–2: 4,000 and L3–7: 1,500
8[b,c]	0.507	0.565	0.581	0.633	8	11.5	8.3	7.25	5.75	8	2	5	4	L1–3: 1752.5 and L4: 1,500

DC$_i$, immature dendritic cells; DC$_m$, mature dendritic cells; na, not available (contamination); V, vaccine; L, lysate (boost) − (L1 = V5, L2 = V6, etc.)

[a] Differences in amount/boost are irrelevant biologically

[b] Vaccination details after re-operation (immunotherapy for recurrence) not included in table

[c] In patients #7 and #8 less PBMC were obtained by the single leukapheresis and therefore less PBMC were thawed for each vaccination; enough loaded mature DC could be generated in both patients

Table 5 Immune response

Patient	Skin test	Elispot (V1 compared with V4)	Elispot (V4 compared with V7)	CTL[a] (V1 compared with V4)
1	Negative (V1 + V4)	Increase in IFN-γ-producing cells	Persistent increase	Increase in CTL
2	Negative (V1)	No increase	No increase	Not done
3	Positive (V1 + V4)	Increase in IFN-γ-producing cells	Persistent increase	Increase in CTL
4	Negative (V1 + V4)	No increase	No increase	Increase in CTL
5	Not done[b]	Increase in IFN-γ-producing cells	Persistent increase	Increase in CTL
6	Negative (V1 + V4)	Increase in IFN-γ-producing cells	Not done	Increase in CTL
7	Positive (V1 + V4)	Increase in IFN-γ-producing cells	No increase	Increase in CTL
8	Delayed positive[c]	No increase	No increase	No increase

V, vaccine

[a] CTL = CD8+CD25+ cytotoxic T-lymphocytes

[b] Not enough tumor lysate

[c] Positive reaction at injection sites 6 months after V1

be ruled out as no biopsy was performed. A repeat MRI three months after the described event showed the same changes at the posteromedial side of the left temporal lobe without any evolution (Fig. 3c–e). We concluded that these changes were most likely to be ischemic, although inflammation or post-radiotherapy sequellae could not be ruled out pathologically in this young patient; the clinical evolution was favorable without administration of steroids. In the same patient the TMZm chemotherapy was stopped because of hematological toxicity (grade 3) and at one year FU this same patient had a status epilepticus which was controlled with fenytoin. Tumor status at that time remained unchanged. No other serious adverse events have been reported.

Quality of life

The results of the QOL and disability assessments revealed that the patient-assessed KPS during the course of vaccination was equal to or exceeded 70 for six patients (median 85; range 70–100) (reflecting a physical condition that enables the patient to care for him/herself). Patients #2 and #5 had KPS scores that varied between 50 and 60 (median 60). Moreover, for all patients the KPS remained stable or even tended to increase throughout the treatment phases.

The FMH score also remained quite high during the course of vaccination: it exceeded 45 (median 52; range 46–53) for all but one patient (#5), indicating that the patients were capable of most daily life activities and did
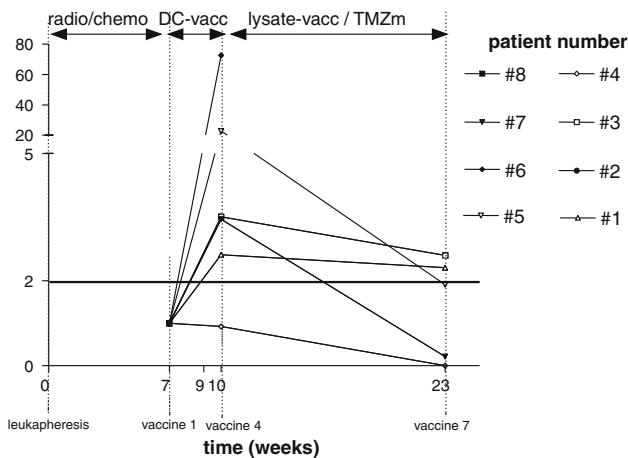
Fig. 2 Assessment of the number of specific tumor-antigen reacting T cells using Elispot. The number of IFN-γ producing tumor antigen-reacting T cells is depicted during the vaccination schedule in six of the eight patients. Two patients (#2 and #8) had more spots in the control condition than in the experimental condition at all time points (results not shown). In five patients an increase in tumor-specific IFN-γ production can be seen between the first and fourth vaccinations. In three of them a more than twofold increase in tumor-specific IFN-γ production persisted after three cycles of TMZm (vaccine 7). The fold increase was calculated by use of the formula: ((SFC at time x) $-$ (SFC in control condition at time x))/((SFC before treatment) $-$ (SFC in control condition before treatment))

not lose functional ability throughout the treatment phases. In patient #5 median FMH score was 36 (range 30–37).

Six out of seven patients evaluated their general health (item 29) and overall QOL (item 30) on the EORTC QLQ-C30 questionnaire as good (median 5 for both items; range 3–7 for both items) and these evaluations remained stable throughout the treatment phases. Only patient #2 had lower evaluations for general health (median 3; range 2–4) and overall QOL (median 3; range 3–4) during the course of vaccination.

Clinical assessment

The details of the clinical results are given in Table 6. Three patients (37.5%) are still alive at last FU, with median FU of 35 months (34–44 months). The median overall survival (OS) for all patients is 24 months (range 13–44 months). Two patients underwent a total resection of the tumor, in six patients the resection was subtotal. One patient is still free from progression or recurrence at the end of the FU period at 34 months. The median progression-free survival (PFS) in all patients is 18 months (range 2–34 months). PFS at 6 months using this therapeutic regimen was demonstrated in six out of eight patients (75%). In Fig. 4a and b, Kaplan–Meier curves are shown for PFS and OS, respectively.

Discussion

We have summarized our observations in eight pilot patients with newly diagnosed GBM in whom vaccination with autologous DC loaded with autologous tumor cell lysate was fully integrated in the multimodal standard primary treatment, consisting of maximum safe neurosurgical resection, external beam radiotherapy with concomitant TMZ chemotherapy, and maintenance TMZ chemotherapy [1]. As such, this is the first report of a standardized radio–chemo–immunotherapy approach for patients with newly diagnosed GBM.

As shown in Table 4, DC characteristics and numbers varied substantially amongst patients. As we reported earlier, this is inherent in the nature of autologous cell therapy in an oncological patient population with highly variable baseline characteristics. Nevertheless, Liau et al. [38] did not find any argument for dose-related toxicity or efficacy in DC-based vaccination therapy. It is important to note that there were no serious adverse events (NCI CTC grade 4) except for one patient in whom an ischemic event occurred during the vaccination therapy. This patient made a partial recovery and the extent to which the ischemic event is related to the vaccination therapy remains a point of debate. Important, though, is the fact that post-radiotherapy sequellae or inflammation could not be ruled out completely as no biopsy was performed. At one-year FU the same patient had a status epilepticus which was controlled with fenytoin. Again, the relationship of this event to the vaccination therapy is unclear and the status epilepticus may be linked to the possible ischemic event that the patient suffered earlier. A favorable course of the patient's clinical symptoms was demonstrated even without the use of steroids, which makes an auto-immune reaction highly improbable. With regard to quality of life and disability assessment during treatment, one can conclude that six patients reported a physical condition that enabled them to take care for themselves and to perform daily life activities during treatment. Also general health and overall quality of life were rated moderate to good by these six patients.

The proof of the principle for DC-based immunotherapy for HGG has been demonstrated in in-vitro experiments [14–16], and in several rodent models [17–20], and justification for clinical use of DC-based immunotherapy for malignant gliomas has recently been reviewed [21, 22]. Several Phase I/II trials or case reports have been published in which patients with malignant glioma have been treated using slightly different variants of DC vaccination [23, 32, 38–50]. Safety, feasibility, and clinical and immunological bioactivity have been demonstrated in these publications. In none of the preclinical in-vivo models nor in patients treated thus far have auto-immune reactions been observed.

Table 6 Therapy-induced clinical effects

Patient	FU (months)	Radiological evolution	Clinical data during vaccination	NCI CTCAE (version 3.0)	PFS (months)	OS (months)	Alive
1	13	Recurrence (MRI)	Rescue chemotherapy[a]	None (grade 0)	2	13	No
2	23	Recurrence (MRI/PET)	Re-operation for recurrence after 11 months	Focal epileptic insult (grade 2)	11	23	No
			Rescue chemotherapy[b]	Fatigue (grade 1)			
				Dysphasia (grade 2)			
3	34	No recurrence (MRI)	No particular findings	General malaise after V2 (grade 1)	34	34	Yes
4	35	Recurrence (MRI)	Re-operation for recurrence after 25 months	Transient dysphasia postoperatively (grade 2)	25	35	Yes
			Immunotherapy[c]				
5	22	Inflammatory/ischemic changes after ischemic event (MRI	Stop of TMZ because of hematotoxicity	Transient confusion after V1 (grade 1)	18	22	No
				Ischemic event after V7 (partial recovery) (grade 4)[d]			
		Recurrence (MRI)		Hematotoxicity (grade 3)[d]			
				Status epilepticus (grade 4)[d]			
6	17	Recurrence (MRI)	Re-operation for recurrence after 6 months	Fatigue (grade 1)	6	17	No
			Rescue chemotherapy[a]	Myalgia after V1 + V2 (grade 1)			
7	25	Recurrence (MRI/PET)	Re-operation (twice)[e]	Fatigue during TMZ (grade 1)	17	25	No
			Rescue chemotherapy[f]	Lymphopenia during TMZ (grade 2)			
8	44	Recurrence (MRI)	Re-operation for recurrence after 26 months	Fatigue with transient confusion during TMZ (grade 1)	26	44	Yes
			Immunotherapy[c]	Discrete dysphasia (grade 2)			
			Rescue chemotherapy[b]				

NCI, National Cancer Institute; CTCAE, common terminology criteria for adverse events

[a] Cetuximab

[b] TMZ

[c] Immunotherapy, consisting of DC vaccinations, after re-operation

[d] As described in text

[e] Second-look operation for known residual tumor—re-operation for recurrence after 17 months

[f] TMZ and Gleevec–Hydrea

In a previous report, we already stressed the importance of minimum residual disease status after surgery before starting the adjuvant DC vaccinations for safety and efficacy reasons [32, 39]. Furthermore, a recently published manuscript from our group describes very promising long-term survival of patients with relapsed HGG treated with postoperative adjuvant DC vaccination [23].

The integration of immunotherapy within the standard postoperative therapy for patients with a newly diagnosed malignant glioma is based on the presumed mutually beneficial effect of the conventional treatment strategies and immunotherapy. Each aspect of the presented concept is believed to play a major role in the global results of this approach. First, maximum safe surgery is performed to induce a state of minimum residual disease as a starting point for subsequent therapy. Not only has it been shown that the extent of resection has a major effect on the benefit of postoperative radiochemotherapy in GBM [51], but the extent of resection is a strong, independent predictor of outcome for patients with relapsed malignant glioma treated with postoperative, adjuvant DC vaccination [23]. Many antitumoral strategies, for example radiotherapy kill tumor cells by apoptosis and the resulting apoptotic bodies form a good source of cross-presented antigens, which might further lead to cross-priming of T cells in an appropriate pro-inflammatory environment. In 1984 North
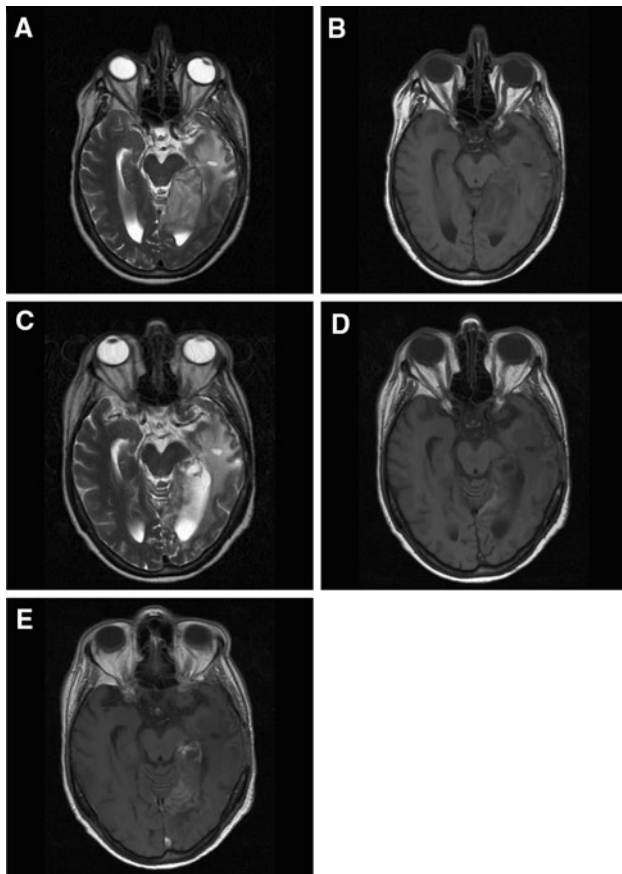
Fig. 3 Patient #5: MRI images at eight and eleven months follow-up (FU). Hyperintensities in T2 weighted images (**a**) and heterogenic signals in T1 weighted images (**b**) at the posteromedial side of the left temporal lobe, near the site of tumor resection, one day after sudden onset of right hemiplegia and aphasia (at eight months FU). A repeat MRI three months after the described event shows the same changes at the posteromedial side of the left temporal lobe without any evolution; **c** T2 weighted images; **d** T1 weighted images; **e** T1 weighted images with gadolinium

MHC molecules in tumor cells, thereby making them better targets for CTL.

Chemotherapy with TMZ during radiotherapy might be of benefit for subsequent immunotherapy. First, chemotherapy affects the size of the lymphocyte pool slightly, thereby enabling thymic-independent antigen-driven T cell regeneration within the context of T cell homeostasis [24, 29]. In this pilot group of patients, the lymphocyte counts were only moderately reduced. The concept of tumor-specific immunization at the time of immune reconstitution after chemotherapy has been demonstrated in several animal models [24, 29]. Specifically of importance to a regimen with six weeks TMZ, it has been shown that TMZ affects the attraction of tumor-specific regulatory T cells (Treg) into the tumor cavity by blocking CCL2 production by the tumor cells [52]. Although a relative enrichment of Treg in peripheral blood has been described at the end of radiochemotherapy, while the total CD4+ T cell population is diminished [53], one might assume that the pro-inflammatory condition induced by radiotherapy [54] counteracts Treg functionality in the periphery [55, 56].

Immunotherapy itself can increase the sensitivity of GBM tumor cells to chemotherapeutics such as TMZ. Wheeler et al. [30] has already suggested an increased susceptibility of GBM to chemotherapy in patients pre-exposed to DC-based vaccination. Their group even published a presumed molecular mechanism of this synergy based on preferential targeting of tyrosin-related protein 2 (TRP2), a chemoresistance mechanism, by cytotoxic T cells as an explanation [26]. The idea of combining these treatment modalities is not restricted to neuro-oncology [57, 58]. Recently Masucci et al. [59] showed an advantage of combining TMZ with restorative immunotherapy using IL-2 in melanoma patients.

Overall, it is hypothesized that the combination of radiotherapy, chemotherapy, and immunotherapy could potentiate the cumulative antitumoral activity when applied in a well designed strategy. Here, we advocate such a strategy of autologous DC-based immunotherapy as add-on therapy, but fully integrated in the multimodality treatment of surgery, radiotherapy, and chemotherapy in patients

et al. [27] reported elimination of local regulatory T cells (*avant la lettre*) in irradiated brain tumor areas. Recently, Kjaergaard et al. [25] showed that long-term survivors in a murine brain tumor model occurred only in the group of animals receiving both radiotherapy and DC-based vaccines. The data showed irradiation-induced upregulation of

Fig. 4 Progression-free survival (PFS) and overall survival (OS). Kaplan–Meier curves are shown for PFS (**a**) and OS (**b**). The median OS for all patients is 24 months (range 13–44 months). The median PFS in all patients is 18 months (range 2–32 months). PFS at 6 months using this therapeutic regimen was demonstrated in six of the eight patients (75%)
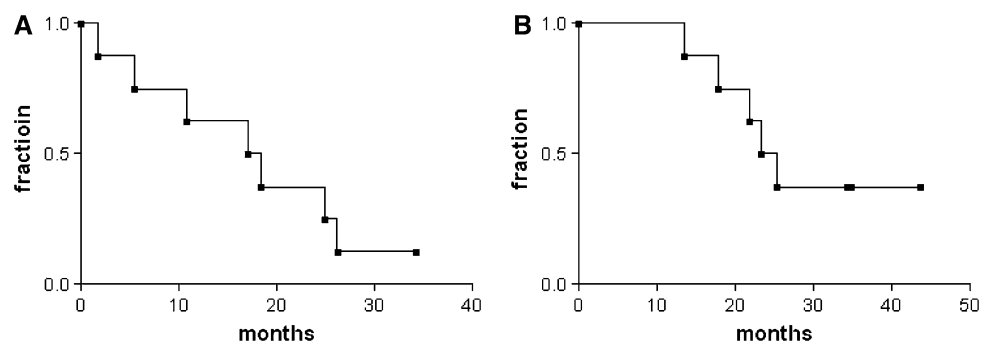
with HGG. For this radio-chemo-immunotherapy, we provide early feasibility data in terms of clinical and immunological responses and an acceptable QOL.

The use of boost lysates after the first four induction vaccinations with loaded DC, is based on the data from Jouanneau et al. [60]. They showed that multiple vaccinations with DC efficiently induced an immune response in an orthotopic mouse model, but did not elicit optimum long-term survival. In contrast, injection of DC for priming, followed by boosts with tumor lysate alone generated the most effective anti-tumor effects (CTL and humoral responses). Also, we have shown in relapsed GBM patients that vaccination with boost lysates can result in substantial numbers of long-term survivors after relapse [23].

Immune monitoring was performed with skin tests, provided that enough tumor material was available, Elispot and general immunophenotyping of circulating T cells by FACS analysis. Immune monitoring was used to assess the induction and/or maintenance of vaccine-induced anti-tumoral immunity in the described close temporal relationship with radiochemotherapy. Testing the DTH reaction to the antigen is one monitoring tool to indicate cellular immunity, although it remains controversial whether or not DTH to the autologous tumor can be a reliable correlate of clinical responses. In this small series, we could not find any positive correlation between immune reactivity and clinical outcome. In two of the six patients tested there was a positive skin test at the first and fourth vaccinations, suggesting that radiochemotherapy did not compromise the patients' specific immunity. One patient had a delayed positive reaction at the injection sites, six months after the first vaccination. The results of the Elispot showed a vaccine-induced increase in IFN-γ-producing tumor antigen-reacting T-cells in five of the eight patients. This points to the induction of a tumor-antigen-directed immune response. In three of these five patients a more than twofold increase in tumor specific IFN-γ production persisted after three cycles of TMZm. These data further support the notion that radiochemotherapy does not interfere negatively with immunotherapy and that these treatments can be combined without losing the therapeutic effect of either. An increase in the proportion of CD8+CD25+ cells within the CD8+ population was noted during the course of vaccination in six of the seven patients. This might suggest a re-expansion of activated CD8+ cytotoxic T cells, which is a prerequisite for implementing tumor vaccination. However, further functional studies should be performed on these cells. To which degree the different immune monitoring tests correlate with clinical responses remains a point of debate, and there was no intention to assess this for this pilot group of patients.

The discordance between clinical and immunological data is a known problem for this type of treatment and is illustrated here by the fact that the two patients with the longest survival (#4 and #8) are the only ones not showing increased IFN-γ production by Elispot. Both patients had had complete resections, in contrast with the other patients. In patient #4 an increase in CTL was seen; the skin test, however, was negative. In contrast, the skin test for patient #8 was delayed positive, but in this patient there was no increase in CTL. This shows the inherent shortcomings of immune monitoring for these types of treatment. Therefore, use of surrogate immunological endpoints as main data on which to build a treatment strategy does not seem to be ideal. The full nature of the estimated beneficial effects of DC vaccination is without any doubt much more complex than any immune monitoring tool at this stage can fully capture.

Three patients (37.5%) are still alive at last FU, with median FU of 35 months (34–44 months). The median OS for all patients is 24 months (range 13–44 months). Considering the fact that seven patients were in RPA class IV and 1 patient in RPA class III, these preliminary data compare favorably with RPA class-related survival estimates and RPA class-adjusted outcome in the EORTC26981/ 22981-NCIC CE3 trial [61, 62]. MGMT promoter methylation was not assessed in these patients, because it was not an inclusion or exclusion criterion. This warrants an even more cautious interpretation of these survival data.

Tumor vaccination integrated within the multimodal standard therapy, consisting of surgical resection, radiochemotherapy, and maintenance TMZ chemotherapy for patients with newly diagnosed malignant glioma is feasible both clinically and immunologically, is well tolerated, and is possibly beneficial for patients with minimum residual tumor burden. Our findings further underscore the importance of a proper assessment of the potentials of adjuvant DC vaccination in patients with HGG as such and as a fourth oncological treatment modality, integrated in the state-of-the-art conventional therapy for these patients in particular. To further investigate the potential of tumor vaccination as add-on therapy to postoperative radiochemotherapy for newly diagnosed malignant glioma, the PFS rate at 6 months was used to power a currently ongoing phase II clinical trial (HGG-2006). This trial might finally lead to a prospective double-blind randomized clinical trial with add-on vaccination as experimental arm, compared with standard primary treatment.

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