DATE: 31st August 2018

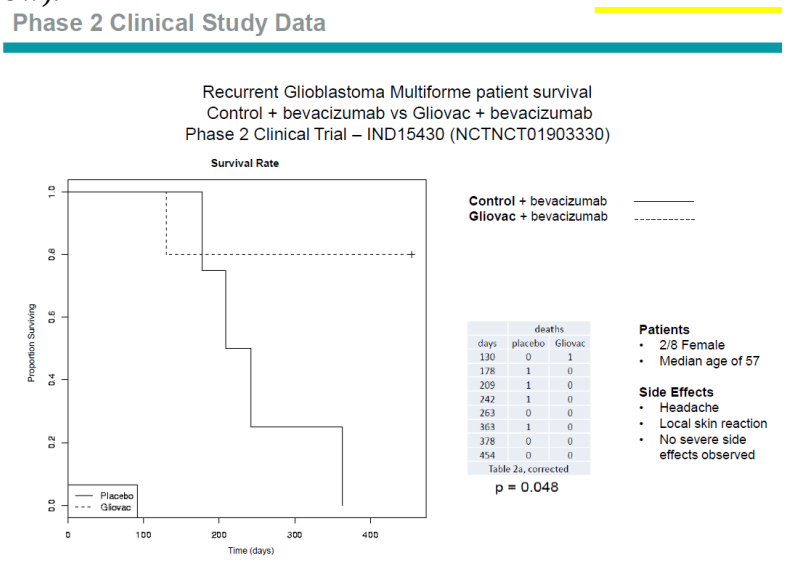
MEMO RE: Update on ERC Company Progress in past 12 months

INTRODUCTION:
ERC has made significant progress over the past 12 months in moving our experimental vaccine Gliovac (also know as ERC1671) toward commercialization. Major progress has been made in several areas:

1. **Clinical Trial - FDA (IND-15430)**
Daniela Bota, MD, Ph.D., Director, Neuro-oncology Program, UC Irvine Medical Center Orange, CA, 92868, received approval from the FDA on the 17th April 2013 for an investigator sponsored, 84 patient, double-blind, placebo controlled clinical trial of ERC-1671.

By the early 2017 Dr. Bota noticed that some patients participating in the trial experienced stable disease for an unusually long period which is normally not seen in recurrent refractory glioblastoma. As a result, a decision was made to unblind the first 9 patients who had completed the study.

The unblinding demonstrated a surprisingly promising separate distribution between patietns receiving placebo and those receiving Gliovac (ERC1671). While the numbers are very small (5 placebo controls and 4 patients, the results were highly promising (see graphical representation below).



2. **Regulatory Progress**
   a. **US Food and Drug Administration (FDA)**
   As a result of this promising data, ERC and Dr. Bota's clinical team had a call with the FDA Center for Biologics Evaluation and Research (CBER) to discuss the potential for Fast Track and Breakthrough Therapy designation of the Gliovac program. In general, the FDA team was receptive to the concept but suggested that we review the opportunity again when 24 or more patients had been accrued and unblinded.
   b. **European Medicines Agency (EMA)**
   The EMA's Committee for Medicinal Products for Human Use (CHMP) agreed to consider Gliovac for Conditional Marketing Autorization and ERC has had two meetings with the EMA's CHMP, on 9th February 2018 and on 9th July 2018 to further our applications for such authorization. If ERC's application for conditional approval is successful, we will be able to proceed with commercialization Gliovac within the EU and state insurance will be obligated to reimburse the cost of treatment.
   c. **UK Medicines and Healthcare Products Regulatory Agency (MHRA)**
   ERC has been approved to apply for a Promising Innovative Medicine (PIM) designation in the UK and the ERC team submitted its application and was invitied for a meeting with the MHRA on 29th July 2018. The meeting went very well and we will be submitting additional information to the UK authorities for PIM designation. If successful, this will allow ERC to market Gliovac in the UK, in the next 12 months
   d. **Right to Try Law in the US:**
   ERC informed the US FDA on 15th June 2018 that ERC intends to make Gliovac available to patient in the US under the new Right to Try Law of the US. The FDA acknowledged acceptance of our notification on 13th of July 2018. ERC is expecting to begin treating the first US patient shortly.

3. **Expanding the Clinical Trial:**
   Excellent progress has been made in adding Dana Farber Cancer Institute (DFCI) in Boston, a Harvard University hospital, as additional site for the clinical trial. DFCI is the largers brain cancer referral center in the United States. Once DFCI joins the clinical trial, we are confident that we will be able to complete the clinical trial in 12 months and begin the process of commercialization. All documents have been submitted to DFCI for a review by their IRB and ethics committees. DFCI is the largest referral center in the US for glioblastoma so, we expect that the rate of recruitment will dramatically accelerate during 2018

4. **Compassionate Use**
   The company has continued to make ERC-1671 available for compassionate use in Europe. By the end of 2018, more than 28 patients with recurrent, terminal (stage IV) GBM had been treated with ERC-1671 in Belgium, Germany, Colombia, South Africa and Australia.

5. **Manufacturing:**
   ERC's facility in Schaijk, The Netherlands, was inspected by the Dutch Health Authorities and was issued a new GMP certificate (attached) dated 26th July 2018 authorizing ERC-NL to continue to produce Gliovac for human use.

6. **Scientific:**
   ERC's academic collaborators continue to receive great attention. Prof. Daniela Bota, ERC's principal investigator in the Gliovac clinical trial in the US, presented our findings at the Society for Neuro-oncology (SNO) meeting in San Francisco in November 2017 and has been invited to present again at the 2018 meeting in November in New Orleans. Furthermore our clinical findings were published in the International Journal of Molecular Science in August 2018 (publication attached). In addition, the ERC team published an article discussing the mechanism of action of ERC1671 in the journal CNS Oncology on 29th August 2018 (publication attached).

CONCLUSION:
ERC has made significant progress over the past 12 months and our success continues to aggressively accelerate the development of Gliovac on commercial, scientific and regulatory fronts. With the inclusion of DFCI in Boston in our clinical trial we will be at the forefront clinical development at one of the world's leading cancer centers and will be well on the way to commercial success in the next 12 to 18 months.

Health and Youth Care Inspectorate – Pharmaceutical Affairs

CERTIFICATE NUMBER: ***NL/H 18/2003845A***

CERTIFICATE OF GMP COMPLIANCE OF A MANUFACTURER [1, 2]

Part 1

Issued following an inspection in accordance with :
Art. 15 of Directive 2001/20/EC

The competent authority of Netherlands confirms the following:
The manufacturer: ***ERC The Netherlands B.V.***
Site address: ***Nistelrooise Baan 3, SCHAIJK, 5374RE, Netherlands***

Has been inspected under the national inspection programme in connection with manufacturing authorisation no. ***5858 F*** in accordance with Art. 13 of Directive 2001/20/EC transposed in the following national legislation:
Art. 100 of the Medicines Act

From the knowledge gained during inspection of this manufacturer, the latest of which was conducted on ***2018-01-18*** , it is considered that it complies with :

- The principles and guidelines of Good Manufacturing Practice laid down in Directive 2003/94/EC [3]

This certificate reflects the status of the manufacturing site at the time of the inspection noted above and should not be relied upon to reflect the compliance status if more than three years have elapsed since the date of that inspection. However, this period of validity may be reduced or extended using regulatory risk management principles by an entry in the Restrictions or Clarifying remarks field. This certificate is valid only when presented with all pages and both Parts 1 and 2. The authenticity of this certificate may be verified in EudraGMDP. If it does not appear, please contact the issuing authority.

[1] *The certificate referred to in paragraph 111(5) of Directive 2001/83/EC and 80(5) of Directive 2001/82/EC, shall also be required for imports coming from third countries into a Member State.*

[2] *Guidance on the interpretation of this template can be found in the Help menu of EudraGMDP database.*

[3] *These requirements fulfil the GMP recommendations of WHO.*

Part 2

Human Investigational Medicinal Products

1 MANUFACTURING OPERATIONS	
1.1	**Sterile products**
	1.1.1 Aseptically prepared (processing operations for the following dosage forms) 1.1.1.4 Small volume liquids
	1.1.3 Batch certification
1.3	**Biological medicinal products (list of product types)**
	1.3.1 Biological medicinal products (list of product types) 1.3.1.3 Cell therapy products

Any restrictions related to the scope of this certificate :

This certificate wil expire on the first of January 2020.

Clarifying remarks (for public users)

This certificate wil expire on the first of January 2020.

2018-07-09

Name and signature of the authorised person of the Competent Authority of Netherlands

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Datum 26 juli 2018
Onderwerp GMP certificaat NL/H 18/2003845A

Ons kenmerk
V2003845

☐ Met het verzoek om advies

☐ Naar aanleiding van uw brief

☒ Ter kennisneming

☐ Volgens afspraak

☐ Met het verzoek voor verdere behandeling zorg te dragen

☐ Met dank voor inzage

☐ Om te behouden

Met vriendelijke groet,

Marjolein van Dijk-de Bruijn
Medewerker Toezicht IGZ[1]
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Bekijk het verhaal van IGJ: **Gezond vertrouwen**
.....

[1]*Bij ondertekening gebruiken wij de organisatienaam genoemd in de wetten op het terrein van de volksgezondheid en de jeugdhulp.*

International Journal of
Molecular Sciences

MDPI

Review

Therapeutic Immunization against Glioblastoma

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Abstract: Glioblastoma is the most common form of brain cancer in adults that produces severe damage to the brain leading to a very poor survival prognosis. The standard of care for glioblastoma is usually surgery, as well as radiotherapy followed by systemic temozolomide chemotherapy, resulting in a median survival time of about 12 to 15 months. Despite these therapeutic efforts, the tumor returns in the vast majority of patients. When relapsing, statistics suggest an imminent death dependent on the size of the tumor, the Karnofsky Performance Status, and the tumor localization. Following the standard of care, the administration of Bevacizumab, inhibiting the growth of the tumor vasculature, is an approved medicinal treatment option approved in the United States, but not in the European Union, as well as the recently approved alternating electric fields (AEFs) generator NovoTTF/Optune. However, it is clear that regardless of the current treatment regimens, glioma patients continue to have dismal prognosis and novel treatments are urgently needed. Here, we describe different approaches of recently developed therapeutic glioma brain cancer vaccines, which stimulate the patient's immune system to recognize tumor-associated antigens (TAA) on cancer cells, aiming to instruct the immune system to eventually attack and destroy the brain tumor cells, with minimal bystander damage to normal brain cells. These distinct immunotherapies may target particular glioma TAAs which are molecularly defined, but they may also target broad patient-derived tumor antigen preparations intentionally evoking a very broad polyclonal antitumor immune stimulation.

Keywords: glioma tumor; brain tumor; immunotherapy; therapeutic vaccine; autologous; allogenic

1. Introduction

Glioblastoma (GBM) is the most common form of brain malignancy in adults. The annual incidence of this disease is about 3–4 cases per 100,000 individuals. The prognosis for late-stage glioblastoma (World Health Organization grade IV astrocytic glioma) is very poor. The median survival time of untreated tumors is only 3 months, with death mostly due to cerebral edema or increased intracranial pressure. Therapeutic interventions involve surgical resection (when safely feasible), followed by radiotherapy (RT), which has been the standard of care for decades. Since 2005, temozolomide (Tmz) chemotherapy has been added to the standard course of radiation [1], resulting in a median survival time of 14.6 months, an increase of about 2 months. However, the vast majority of patients relapse with limited treatment options left [2,3]. If safely feasible, repeat surgery may be considered, but tumor spreading into the brain and spinal cord will hinder adequate surgical resection. Moreover, the cells from the relapsing tumor are often more resistant to chemotherapy. In general, repeated treatment of recurrent tumor lesions may marginally extend overall survival in patients with good performance status [4,5]. The only treatment option left is the angiogenesis inhibitor bevacizumab, a humanized monoclonal antibody targeting vascular endothelial growth factor (VEGF), a molecule which promotes blood vessel growth into the tumor [6]. This antibody inhibits the growth of new blood vessels towards the tumor, but it is only approved in the USA. The latest therapy to be approved for GBM, in both the USA and in Europe, is the alternating electric fields (AEFs) generator NovoTTF/Optune [7], which may extend median overall survival by about 5 to 24 months [8]. A new treatment option for glioma tumors is the use of therapeutic vaccination. The aim of recently developed therapeutic brain cancer vaccines is to stimulate the patient's immune system to recognize tumor-associated antigens (TAA) on cancer cells, which results in an effective immune response eventually attacking and destroying the brain cancer cells, with minimal bystander damage to normal brain cells.

2. Glioma Tumors

Glioma tumors are debilitating and life-threatening brain cancers since they produce severe damage to the brain leading to a poor survival prognosis. Glioma tumors produce a combination of pathologies, which include focal neurologic deficits, resulting from compression and tumor infiltration into the surrounding brain tissue, compromised vascularization, and an increased intracranial pressure. Clinical symptoms include:

- Headaches are prevalent among 30–50% of patients. These headaches are non-specific and indistinguishable from tension headache. Intracranial pressure may increase as a result of tumor growth.
- Seizures may occur among 30–60% of patients. Depending on the tumor location, seizures may be simple partial, complex partial, or generalized.
- Focal neurologic deficits occur among 40–60% of patients. Patients who survive relatively long, may experience increasing cognitive problems, neurologic deficits resulting from radiation necrosis, communicating hydrocephalus, and occasionally cranial neuropathies and polyradiculopathies from leptomeningeal spread.
- Mental status changes are common among 20–40% of patients. With the advent of magnetic resonance imaging (MRI), brain tumors are increasingly diagnosed at an earlier stage and associated with subtle personality changes.

All of these conditions may result in chronic, debilitating symptoms, which negatively affect the patients' ability to function normally in work or family life and finally lead to a fatal outcome. Hence, there is a significant unmet clinical need for the therapy of malignant glioma, in particular for the late stage of the disease, where patients are faced with dismal prognosis. Advances in neurosurgery, radiation, chemotherapy and concomitant radiochemotherapy during the past decade have provided only small improvements in clinical outcome. The first-line treatment of glioblastoma is usually

surgery, both to confirm the diagnosis and to remove as much of the tumor as possible. Radiotherapy followed by adjuvant systemic temozolomide has produced a median survival of about 15 months, and this regimen is now the standard of care for GBM [1,9].

Despite these therapeutic efforts, the tumor returns in the vast majority of patients. When relapsing, statistics suggest an imminent death dependent on the size of the tumor, the Karnofsky Performance Status, and the tumor localization. A scale (ranging from 0 to 3 points) comprised of these three variables distinguishes patients with good (0 point), intermediate (1 to 2 points), and poor (3 points) postoperative survival and indicates that median survival times are respectively 10.8, 4.5, and 1 month, $p < 0.001$ (95% IC) [10]. The median time interval from re-operation after relapse to death for all patients is 7.4 months [10]. At this stage of progression, the patients may be treated with bevacizumab. Bevacizumab is an approved medicinal product in the US, but not in the European Union (EU). However, the response to bevacizumab is transient and short-lived. After 4–6 months, the patients typically develop progressive physical and mental debilitation, and succumb to the disease soon thereafter [11].

Based on the above, it is clear that regardless of current treatment regimens, glioma patients continue to have dismal prognosis and novel treatments are urgently needed.

2.1. Therapeutic Glioma Vaccines

The aim of therapeutic brain cancer immunization is to stimulate the patient's immune system to recognize tumor-associated antigens (TAA) on cancer cells which results in an effective immune response eventually attacking and destroying the brain cancer cells, with minimal bystander damage to normal brain cells. TAA are antigens expressed by tumor cells and not or less by normal healthy cells [12]. When aiming to develop a therapeutic vaccine to treat cancer patients, the prerequisites for the design of an effective cancer vaccine differ clearly from those for the design of a "conventional" prophylactic (often infectious disease) vaccine. First, it should be realized that the cancer patients who will receive the vaccines are immuno-compromised. Secondly, the tumor target antigens are often self-molecules from the patient and are, therefore, poorly immunogenic. Third, tumors develop mechanisms to escape and suppress the immune system. Thus, the design and the choice of immunomodulatory adjuvants for cancer vaccines, both require special attention, and differ relative to those for prophylactic infectious disease vaccines, which are mostly based on antibody responses. By contrast, cancer vaccines in general, need to be designed to generate T cell immune responses to destroy malignant cells, although not always, to be efficient. Nevertheless, a number of promising glioma brain cancer vaccines have been developed recently and will be discussed below.

2.1.1. Survivin-Targeting Vaccines

Survivin is a protein which is upregulated in a variety of human cancers. It is a family member of the inhibitor of apoptosis (IAP) family proteins, which is expressed during embryonic development, but absent in most normal adult cells [13]. Expression of survivin in tumors is associated with an aggressive phenotype [14], with increased resistance to chemotherapy [15]. One prototype product under study is the SVN53-67/M57-KLH peptide vaccine. It is a synthetic peptide vaccine, containing a 15-mer peptide (DLAQMFFCFKELEGW), with C to M alteration at amino acid position 57, derived from the anti-apoptosis protein survivin. The peptide is conjugated with keyhole limpet hemocyanin (KLH), with potential immunopotentiating and antineoplastic activities [16]. KLH may enhance immune recognition and may promote an enhanced response. As SVN53-67 is weakly immunogenic in humans, the M57 amino acid alteration may lead to greater affinity towards HLA-A*0201 and thus an enhanced anti-tumor immune response. Upon subcutaneous administration of SVN53-67/M57-KLH peptide vaccine, the synthetic peptide is able to bind both HMC class I and II molecules. It may, therefore, activate the immune system to mount both a cytotoxic T-lymphocyte (CTL) as well as a T-helper cell response against survivin-expressing cancer cells. This may result in decreased tumor cell proliferation

and ultimately tumor cell death. The study is active, but not recruiting in phase II. For detailed information please see ClinicalTrial.gov web site: NCT02455557.

Another viral vaccine approach involving survivin protein is a conditionally replicative oncolytic adenoviral (CRAd) vector that contains the tumor-specific survivin promoter (S) and a fiber protein polylysine modification (pk7), with potential antineoplastic activity. This is a neural stem cell-based virotherapy, which is based on infection of neural stem cells (NSCs) with the gliomatropic oncolytic adenovirus (OV) CRAd-S-pk7 [17]. This oncolytic virus preferentially replicates and destroys glioma tumor cells. This study is recruiting (in phase I study) according to clinical.gov; for detailed information please see ClinicalTrial.gov web site: NCT03072134.

2.1.2. Rindopepimut/CDX-110

The newly Food and Drug Administration (FDA)-approved vaccine-based therapy, rindopepimut/CDX-110, has demonstrated an extension of median survival. However, this vaccine is only applicable to those 30% of GBM patients who are positive for an epidermal growth factor receptor variant EGFRvIII [18]. Unfortunately, in a phase III study there was no significant difference in overall survival for patients with minimal residual disease (MRD): median overall survival was 20.1 months (95% CI 18.5–22.1) in the rindopepimut group versus 20.0 months (18.1–21.9) in the control group (HR 1.01, 95% CI 0.79–1.30; $p = 0.93$) [19].

2.1.3. DCVax Brain

DCVax is an autologous dendritic cell vaccine for newly diagnosed glioblastoma patients [20]. This vaccine showed an excellent safety profile and promising results in an interim result analysis of the latest phase III clinical trial against glioblastoma. In the trial, patients were randomized to receive temozolomide plus DCVax®-L (an autologous tumor lysate-pulsed dendritic cell vaccine) or temozolomide and placebo. The median survival of 331 treated patients was 23.1 months from surgery. In comparison, median survival for newly diagnosed glioblastoma patients with the standard of care (surgery, radiation and chemotherapy) is 15–17 months [21]. The trial is blinded and ongoing (NCT00045968).

2.1.4. ICT-107 (a Six Synthetic Antigen Peptide Vaccine)

ICT-107 is also an autologous dendric cell vaccine, which consists of a patient's own dendritic cells (DCs) loaded with six synthetic peptides from antigens (TAA) associated with glioblastoma tumor cells. The six tumor-associated antigens include: absent in melanoma 2 (AIM-2), melanoma-associated antigen 1 (MAGE-1), tyrosinase-related protein 2 (TRP-2), glycoprotein 100 (gp100), epidermal growth factor receptor 2 (HER-2), and interleukin-13 receptor subunit alpha-2 (IL-13Ra2) [22]. In 124 newly diagnosed GBM patients following surgery and chemoradiation, a randomized, double-blind, placebo-controlled Phase 2 study (NCT01280552) did not show an overall survival benefit [23]. However, the study showed two to three months progression-free survival that was statistically significant when compared with patients treated with DC vaccine that were not pulsed with antigens. In a subsequent randomized, double-blind, placebo-controlled phase 3 clinical trial, in newly diagnosed GBM patients following surgery and chemoradiotherapy (NCT02546102), preliminary analysis showed that four of the targeted antigens were associated with prolonged survival. However, the study was suspended in June 2017 for financial reasons [24].

2.1.5. GLIOVAC/ERC1671

GLIOVAC/ERC1671 is a vaccine based on surgically-resected tumor tissue. The exact active ingredients of gliovac are not defined at the molecular level. Instead the active ingredients are defined as a broad number of tumor-associated antigens, derived ex vivo post-surgery from a glioma tumor as confirmed by a histopathology. Hence, the tumor material isolated by surgery serves as the source of tumor-associated antigens that is required for induction of a pleiotropic anti-tumor immune effector

response by the patient's immune system. This immune response is directed against multiple targets in the non-resected remnants of the glioma tumor bed, that are either not removed by surgical ablation or evolve as new tumor outgrowth from these remnant tumor cells. The rationale, the preclinical and clinical development of this prototype vaccine will be described in more detail below.

2.2. The Rationale for a Therapeutic Vaccine Made from Resected Tumor Tissues

In the past researchers developed vaccine preparations by isolating and culturing pure cancer cells from the malignant mass, believing (hoping) they had extracted the essence of the cancer. But in doing so, they omitted connective tissue and other parts of the tumor's unique biological profile, helping to explain why the majority of cancer vaccines are unable to prevent immune escape of the non-removed tumor cells that evolve post-surgery and often acquire a different antigenic make-up that is not (sufficiently) recognized by the vaccine-induced immune cells. GLIOVAC/ERC1671 was designed to prevent immune escape [25]. GLIOVAC/ERC1671's active ingredients are from freshly resected, non-cultured glioma tumor cells, aiming to provide a broad set of antigens, which cover as much as possible both the antigenic make-up of patient's glioma tumor, as well as the antigenic profile of potential newly evolving tumor cells that are likely to appear post-surgery from non-ablated tumor tissue. Therefore, the GLIOVAC/ERC1671 treatment was designed to harbor not only antigens derived from autologous tissue but, in addition, also antigens formulated from allogeneic donated glioma tumor tissue of other patients. These allogeneic antigens further broaden the antigen target profile of in the vaccine. Moreover, the use of allogenic material also triggers a potent anti-'non-self', i.e., anti-allogeneic, immune response in the patient. The combination of the autologous with the allogenic antigen approach breaks immunotolerance, by enabling the patient's immune system to recognize the tumor cells which generally exhibit low immunogenicity. The inclusion of autologous cells allows for a complete personalized treatment for each patient (a single and specific treatment per patient), thereby avoiding the unspecified generic focus of the classical therapeutic immunization approaches. Hence, the allogeneic cells can be regarded as an immunostimulatory therapeutic vaccine 'adjuvant' which concomitantly increases the number of cancer-associated tumor antigens (TAA) to be recognized after vaccination by the patient's immune system.

2.3. Pre-Clinical Gliovac/Erc1671 Development

2.3.1. Proof-of-Concept, Composition, Dosing and Timing

Research in rodent (rat) models, designed by the scientific team of Epitopoietic Research Corporation (ERC), provided the proof-of-concept of the vaccine design and gave insight into the critical aspects of the therapeutic anti-tumor intervention strategies, helping to analyze the basic mechanisms of action. Pharmacokinetics and toxicology were then evaluated in mouse studies. The proof-of-concept of the vaccine design, based on the concept that allo-immune reactivity evokes anti-tumor immunity against an autologous tumor, was first observed in two syngeneic glioma tumor rat models. The anti-tumor effect was tested in a therapeutic tumor vaccine setting using two different rat glioma models. The 9L glioma tumor is autologous to Fisher 344 rats and allogeneic to the Sprague–Dawley (SD) rats, while C6 glioma tumor cells are autologous to SD rats and allogeneic to Fisher 344 rats. Therapeutic immunization with a combination of allogeneic cells and autologous lysates induces rejection of malignant autologous gliomas and offered a protective effect against a challenge with autologous tumor cells in both rat tumor models. The results confirmed a protective effect against challenge with autologous tumor cells [26].

Subsequently, the CNS-1 Lewis rat glioma model was used to explore the protective efficacy of various conditions in the vaccine preparation, as well as variations in the dosing and timing schedule. In addition, it was tested whether particular costimulatory agents were able to confer better immunity against CNS-1 tumor development when combined with the allo- and autologous tumor antigen preparation. The results showed that the prototype, consisting of a mixture of allogeneic and

autologous glioma cells and their lysates, is able to inhibit CNS-1 glioma growth in the autologous Lewis rats as published in Chapter 6 of the thesis of A. Stathopoulos [25].

Finally, in order to mimic the eventual human vaccine design, therapeutic CNS-1 tumor immunizations were evaluated in the Lewis rat model when combined with the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF), following a low-dose cyclophosphamide (Cy) treatment. The data show that the combination of GM-CSF and Cy with the vaccine was most effective in arresting glioma growth progression as published in Chapter 7 of the thesis of A. Stathopoulos, Vaccine antigen preparation used to evaluate therapeutic immunization when combined with GM-CSF [25]. As a result, this prototype formed the basis for further evaluation in glioma patients in a clinical study.

2.3.2. Pharmacology and Toxicology

As a next step a pharmacokinetic analysis of the human prototype vaccine was performed in 2012 and the results demonstrated that there was no human DNA detected after the vaccine injection when detected by PCR at 1 month after the treatment. Briefly, twenty-one (21) 6-week old, female immunocompromised NOD SCID mice were assigned to two treatment groups of 10 animals each, and one animal remained untreated. Group 1 was administered placebo (together with Cy and GM-CSF), and Group 2 was administered the vaccine (together with Cy and GM-CSF). The evaluation of the study results concluded that injection of the human vaccine substances was well tolerated by all animals. Furthermore, a histological analysis did not reveal any toxicity related to treatment (unpublished data). Histopathological analysis of the organs of each animal used in this study showed no histopathological difference between the placebo and human vaccine treated groups after two separate evaluations, confirming the absence of toxic effects of the human vaccine prototype.

2.4. GLIOVAC/ERC1671 Is a Vaccine Based on Surgically-Resected Tumor Tissue

The increased knowledge which had been collected from the animal experiments formed the basis for the composition of the vaccine in patients. For clinical evaluation, the production of the vaccine had to be adapted to adhere to good manufacturing production (GMP) standards for human use, according to the required GMP guidelines.

2.4.1. Background Information on Gliovac

The raw material for the vaccine is glioma tumor tissue obtained from brain surgery, the first step in the current standard treatment. Once resected, the tissue is immediately shipped in a sterile container to the Tumor Tissue Bank–The Netherlands (TTB–NL) and the manufacturing site in which both are situated in The Netherlands. The donated tissue, even though it is tumor tissue and normally discarded, is recorded at the Tumor Tissue Bank–The Netherlands, which is formally audited and approved by The Netherlands Authorities. Tissue release requires negative testing for transmissible infectious diseases, according to the laws of tissue donation. Upon release by the tissue bank and transfer to reception at the manufacturing site, the tissue immediately enters in a sterile production process, which mainly consists of tissue dissociation and cell extraction without a culture step. One part of the cells is stored in a sucrose medium, and one part is lysed by osmotic shock in water. Each donated tissue is manufactured by the same procedure. Quality controls regarding sterility of the product are performed at the end of the procedure, hence there is a theoretical risk that a finished product is not released at the end of the manufacturing process in case of non-conformity. Final release of the finished product is dependent on both the conformity of release of the starting material as well as the final product quality. Eventually, the finished product is irradiated in order to make the tumor cells' replication incompetent.

GLIOVAC/ERC1671 is a course of immunotherapy, where irradiated/replication-inactivated tumor cells are combined with tumor cell lysate for subcutaneous injection into the glioma patient. The treatment package consists of tumor cells and lysates that are derived from the patient to be treated

(autologous component), as well as from three other glioma patient donors (allogeneic component). This package of tumor antigens is administered in a particular treatment schedule (please see Figure 1). Each immunization is given together with GM-CSF (granulocyte-macrophage colony-stimulating factor) to support local immune system priming. In addition, a low dose of cyclophosphamide (Cy) is given a few days before each immunization cycle to deplete immune inhibitory cells in the patient (please see below the treatment details). By using this injection schedule, GLIOVAC/ERC1671 evokes an oligoclonal and partly allo-specific immune induction, based on the application of a broad set of tumor antigens derived from freshly resected glioma tumor tissues from the patient and three unrelated tumor tissue donors.

2.4.2. The Treatment

One treatment cycle is composed of 6 vials of material derived from 3 other patients/donors (allogeneic) and 4 vials of material derived from the patient (autologous) injected intradermally (see Table 1). For each patient the treatment is composed of 3 injections of allogeneic cells and lysates (Gliovac) A, B, C) plus 2 injections of autologous cells and lysate (Gliovac D) given with 3–4 day intervals (Figure 1). Each finished product batch (A or B or C or D) is produced by the same GMP manufacturing process. A full treatment is composed of GLIOVAC/ERC1671 administered with GM-CSF (Leukine®) as adjuvant, following a short three-day treatment period of a low-dose of cyclophosphamide (Endoxan®).

Table 1. Composition of one cycle of treatment.

Dose Sequence	Composition
[Gliovac] A	Vial 1: $1 \times 10^{5-6}$ allogeneic n°1 glioma tumor cells Vial 2: $1 \times 10^{5-6}$ allogeneic n°1 glioma tumor lysates
[Gliovac] D	Vial 3: $1 \times 10^{5-6}$ autologous glioma tumor cells Vial 4: $1 \times 10^{5-6}$ autologous glioma tumor lysates
[Gliovac] B	Vial 5: $1 \times 10^{5-6}$ allogeneic n°2 glioma tumor cells Vial 6: $1 \times 10^{5-6}$ allogeneic n°2 glioma tumor lysates
[Gliovac] C	Vial 7: $1 \times 10^{5-6}$ allogeneic n°3 glioma tumor cells Vial 8: $1 \times 10^{5-6}$ allogeneic n°3 glioma tumor lysates
[Gliovac] D	Vial 9: $1 \times 10^{5-6}$ autologous glioma tumor cells Vial 10: $1 \times 10^{5-6}$ autologous glioma tumor lysates

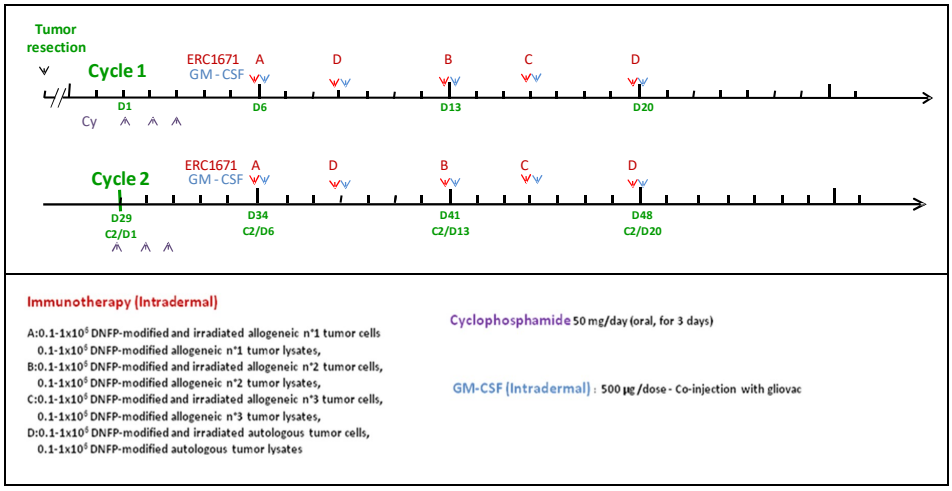

Figure 1. Time schedule of administrations of the treatment. Cyclocphosphamide (CY). ERC1671 doses A, B, C are allogeneic components. ERC1671 doses D are autologous components. Cycle 1 starts on day 1 (D1) with CY administration. ERC1671 is administered on day 6 (D6) with ERC1671 A, day 9 with ERC1671 D, day 13 with ERC1671 B, day 16 with ERC1671 C and day 20 (D20) with ERC1671 D.

2.5. Clinical Development

In 2015 a report [27] described the use of GLIOVAC/ERC1671 in a clinical setting. It described the immunotherapy's effect in one recurrent glioblastoma patient who previously had failed second-line standard of care. Although such patients are generally moribund within a few weeks, the GLIOVAC-treated patient survived for 10 months without any other adjuvant therapy [27]. In 2015, Schijns et al. [28] first described the experience with GLIOVAC/ERC1671 in recurrent glioma patients who were treated with Gliovac in an individual hospital exemption protocol. Six-month survival on the GLIOVAC regimen was 100%, and 12-month survival was 40%, providing initial evidence of low toxicity and promising activity of this new therapy with a highly significant overall survival (OS) increase [28], when compared to historic control patients. Please see below for detailed clinical data from the updated cases report [28].

2.5.1. Clinical Data of Individual Compassionate Use Treatments-Cases Report

The GLIOVAC formulation, as deduced from the animal models, has been used for clinical evaluation in individual patients. A total of 10 patients with a Karnofsky performance status (KPS) above 60 that were treated with GLIOVAC/ERC1671 in a compassionate/single program on a named case basis. Median age was 53 (26–62), with 6/10 female patients (see Table 2). The average KPS was 80 (60–100). Out of these 10 patients, all of whom were in terminal stages of disease, 6 had not received bevacizumab (bvz) during their disease treatment (before, during or after Gliovac treatment). Out of the 4 patients that had received bvz, 2 had bvz treatment until GBM progression, and stopped the bvz prior to surgery and the start of GLIOVAC/ERC1671. One patient had received bvz until disease progression, followed by surgery and 4 cycles of GLIOVAC/ERC1671, and was continued on bvz after the 4 cycles of GLIOVAC/ERC1671 were completed, due to concerns of possible disease progression. One patient received bvz until disease progression and continued on both GLIOVAC/ERC1671 and bvz for an additional 2 months. No significant side effects potentially attributable to the combination were witnessed. To properly assess the results from this study, patients have to be compared to the outcomes of currently used treatment regimens for recurrent GBM patients, which are as follows.

Table 2. Compassionate/single-name patient Characteristics.

Patient Anonymization Code	Clinical Site	Age at Diagnosis	Sex	KPS	OS (weeks)
ERC-B-G2012-54-005	CSL	58	F	80/100	28
ERC-B-G2012-50-011	CSL	62	M	80/100	41
ERC-UCI-40-JS-04031986	UCI	44	M	80/100	42
ERC-B-G2012-51-017	CSL	62	F	70/100	46
ERC-L-G2012-65-019	VIL	49	F	70/100	35
ERC-G-G2012-63-020	UKS	50	M	80/100	69
ERC-B-G2013-58-023	CSL	55	F	80/100	48
ERC-CO-G2014-87-033	FIRE	28	M	100/100	115
ERC-CO-G2014-88-042	FIRE	26	F	95/100	95
ERC-ZA-G2015-62-048	GVI	53	F	70/100	55

KPS is Karnofsky performance status; OS is overal survival.

The expected OS for those GBM patients with a good KPS (more than 70), after failing radiation and Tmz, and without access to bvz (as is the case with many patients in the EU), is 5–8 months, and their 6-month progression-free survival (PFS) is around 30% [29]. For patients that have already failed bvz, predicted OS is reported at 4–5.8 months, and 6-month PFS rate is from 4.4% to 16%, depending on the study [30,31].

In comparison, our dataset of recurrent GBM patients treated with Gliovac shows the following: 6-month OS is 100%, 12-month OS is 40%, and median OS is 46 weeks (10.5 months). Historic controls (data from [32]) have 6-month OS of 33% and median OS of 23 weeks (5.3 months) (Figure 2). Thus,

this dataset reveals a striking improvement over current clinical practice. As shown in Figure 2, the results showed a highly significant (log rank test, $p < 0.0001$) increase in the OS of patients when treated with Gliovac. Hence, these results are supportive of the benefit of the combined treatment schedule, administering an immunization package of allogeneic and autologous cells and lysates in repeated cycles of treatment, with minimal toxicity.

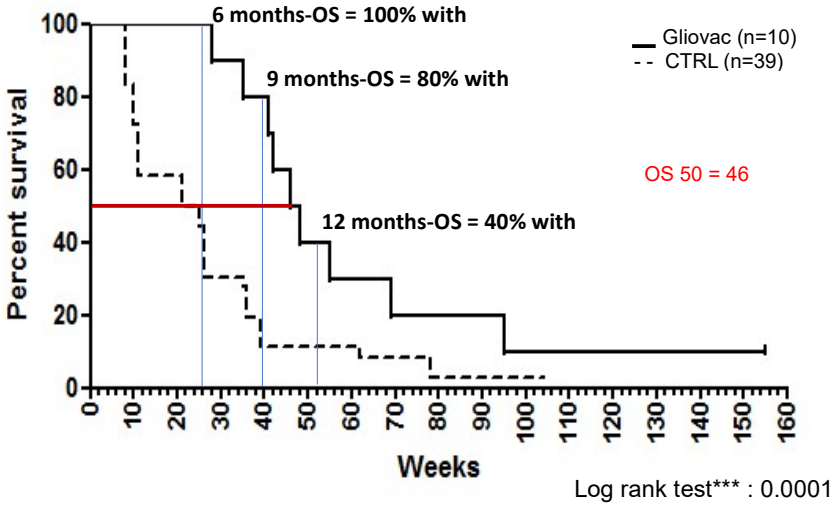

Figure 2. Results obtained from patients treated under compassionate/single-name program. Although data need to be confirmed in a stringent clinical trial, results show a highly significant (log rank test, *** p-value = 0.0001) increase of the overall survival (OS) of late stage relapsing patients when treated with Gliovac when compared to historic control patients (study published by Barker et al., 1998 [32]).

2.5.2. Clinical Study Data from a Phase II Food and Drug Administration (FDA) Trial

To further investigate and validate safety and effectiveness of GLIOVAC/ERC1671, an FDA-approved double-blinded, placebo-controlled phase 2, clinical study (NCT01903330), was initiated at the University of California, Irvine. This phase II clinical study, entitled "*ERC1671/GM-CSF/Cyclophosphamide for the Treatment of Glioblastoma Multiforme*" (NCT01903330), is designed to show the anti-tumor efficacy of Gliovac plus GM-CSF plus Cy with bevacizumab (bvz), as compared to placebo injection (instead of Gliovac/GM-CSF) plus placebo pill (instead of Cy) with bvz, in patients with recurrent grade IV malignant gliomas, including GBM. As mentioned before, both treatment arms include bvz. Therefore, this study aims to further validate superior activity of GLIOVAC/ERC1671 as compared to bvz alone. GLIOVAC/ERC1671 data from the compassionate use treatment, without bvz, already shows beneficial 6-, 9- and 12-month OS data in comparison to published bvz monotherapy studies (Table 3) from Taal et al., 2014 [33], Field et al., 2015 [34], Heiland et al., 2016 [35]. Currently, the clinical phase 2 study has started and is recruiting.

Table 3. OS data of gliovac in comparison to bevacizumab studies. Published Bevacizumab monotherapy studies from: Taal et al., 2014 [33], Field et al., 2015 [34], Heiland et al., 2016 [35].

		6 mo OS	9 mo OS	12 mo OS
Study	**Treatment**	**All Pts (%)**	**All Pts (%)**	**All Pts (%)**
ERC 1671	ERC 1671	100	80	40
Taal (BELOB)	BEV Monotherapy	62	45	26
Field (CABARET)		61	39	24
Heiland (Freiburg, Germany)		18	12	10

3. Discussion

Therapeutic immunization against brain cancer, aiming to stimulate the patient's immune system to (better) recognize and destroy tumor-specific antigens on malignant brain cells would provide a formidable new treatment option for the brain cancer patients for whom little new treatment progress has been made in decades. As described above, several encouraging new vaccine candidates have recently been developed, which showed an effective immune response, eventually attacking and destroying the brain cancer cells with clinically meaningful efficacy and an acceptable safety profile, with minimal bystander damage to normal brain cells.

For both the DCVax treatment and the Gliovac therapy, the tumor target antigens are not defined and characterized at the molecular level (and do not need to be). As a result, both approaches induce a strong and broad polyclonal immune response to multiple tumor antigens present in the antigen preparation, thereby reducing the risk of tumor immune escape following the loss of particular TAAs.

During its evolution, from drawing table to a prototype vaccine for human application, the GLIOVAC product was composed and tested in preclinical studies to harbor a number of characteristics for optimal immune induction. The prototype of GLIOVAC/ERC1671 was designed to contain as much as possible of the "original" tumor cells. Indeed, the isolated glioma tumor cells are not cultured, but directly filtered and conditioned from the surgically resected tumor tissue. This product aspect is particularly important, because in ex vivo tumor cultures only a percentage (about 20%) of the tumor cells survive the switch towards a culture medium environment. Hence, a culture procedure reduces considerably the mutation variety and quantity of TAAs that is normally present in freshly isolated tumor cells. So, avoidance of an in vitro cell culture step maintains the broadness of tumor target antigens in the final vaccine preparation and, hence, the broadness of induced immune power against the target tumor.

Furthermore, the Gliovac treatment not only includes autologous TAAs from the patient, but also includes TAAs from three allogenic glioma tumor donors. These allogenic antigens evoke an immunological phenomenon that is comparable to a "graft rejection" due to the presentation of allogeneic cells which are HLA-incompatible. The allogenic vaccines are prepared from donated tumor tissue in a process identical to the vaccine preparation for the autologous antigens. Upon recognition of and "immune rejection" of the injected allogeneic tumor antigen preparation, the immune system of the patient will develop an immune response recognizing cancer cell associated-proteins, including the so-called tumor associated antigens (TAA) overlapping with those from the patient, and consequently reject the patient's own tumor.

Hence, allorecognition and allo immune-induction is a key ingredient in the mechanism responsible for allograft immune rejection (reviewed in Fabre, 2001; also in Gervais, 2009) [36,37]. It is well known that unprimed T lymphocytes from one individual react with unusual strength against HLA antigens of other members of the same species, a phenomenon called "allo-agression". This process is based on the direct T-cell allorecognition. It reflects the capacity of T lymphocytes to recognize intact allogeneic HLA molecules on the surface of foreign cells. It is a powerful mechanism of T-cell activation, since about 1–10% of an individual's T lymphocytes will respond to the foreign HLA antigens of another individual. By comparison, the frequencies of T-cell precursors for "normal" environmental antigen (e.g., a virus protein) are of the order of only 1/10,000 or 1/100,000.

The injection of autologous and allogeneic glioma tumor antigens has the advantage that it exposes the patient's immune system to a larger variety of tumor antigens, which increases the chances to trigger essential immune effector cell populations. In addition, donated allogenic tumors warrant the availability of a critical quantity of active substance. In contrast to immunizations based on cell lines only, the autologous/allogeneic biopsy-based immunizations depends on the size of the tumor isolated during surgery from the patient and the donor. However, by using allogeneic donor tumor tissue, a theoretical limitation in active substance is partially circumvented, since a large part of the finished product can be obtained from a tumor tissue bank.

Both CD8 and CD4-positive T-cells are implicated in the allorecognition phenomenon. CD4 T-cells activated by direct recognition of HLA class II molecules during immunization may act as providers of T-helper activity, triggering and sustaining a TAA-specific immune response against the patient's own tumor cells. In organ transplant rejection, this powerful activation of T-helper cells is responsible for an early antibody response against the transplant. This is particularly important in tumor therapy as it could theoretically bypass the need for presentation of TAA within self HLA class II molecules to activate T-helper cells and could induce a powerful cellular and humoral immune reaction against tumor cells displaying TAA within HLA class I antigens.

In keeping with the above, it is worth mentioning that CD4 T-cell responses are not only necessary, but may also be sufficient for allograft destruction. Allogeneic responses have the potential to generate a milieu rich in cytokines sustaining both an innate immune reaction and promoting a T-cell response by providing T-cell costimulatory ligands. The exact number of CD4+ T cells, or other relevant immune cells, required for effective immunotherapy is currently subject of various clinical studies and remains to be determined. This may be especially relevant for immunotherapies in patients with depressed immune cell numbers as a result of chemotherapy. The use of Gliovac as an upfront treatment before cytotoxic therapy would likely circumvent this problem. Collectively, it is apparent that allorecognition can be used as a potent mechanism to stimulate a glioma TAA-specific immune reaction against a patient's tumor cells.

It should be kept in mind that immune cells triggered in the periphery by therapeutic immunization are undoubtedly able to cross the blood–brain barrier (BBB), which is "disrupted" in gliomas. Malignant gliomas actively degrade previously intact endothelial tight junctions of the BBB by secreting soluble factors, eventually leading to BBB disruption within invaded brain tissue as confirmed in neuroradiological examination [29,30].

Unfortunately, the evaluation of the patient using classical radiological imaging techniques becomes (more) complex. When using immunotherapy it is difficult to discriminate between progression of tumor growth versus pseudoprogression resulting from immune cell infiltrates. Therefore, the advent of immunotherapies implies the use of other evaluation techniques such as clinical parameters and overall survival of the patient.

4. Conclusions

The composition and regimen of GLIOVAC/ERC1671 appear to offer unique and compelling advantages. In comparison to historical controls, GLIOVAC therapy in individual late-stage relapsing patients resulted in significantly increased OS-6 month (100%) and greater median OS (46 weeks compared to 23 weeks). Moreover, current clinical data show that the product is safe, with no severe adverse effects (AE) observed. Related toxicities were mainly limited to low grade headaches and local skin reactions.

Moreover, after GBM recurrence following standard care treatments, about 10% of the case-reported patients treated with GLIOVAC/ERC1671 showed a total recovery and survived longer than 3 years in the compassionate/single-name program. Importantly, spontaneous remissions have never been observed in the relapsed GBM patients showing tumor progression. Despite the limited number of patients, the overt remission of patients, to our knowledge, is the best example of the product's efficacy.

The primary results in clinical evaluation based on combined allogenic/autologous antigen preparation, which has been developed in an animal model, are supportive of the product's rationale. The meaningful efficacy against relapsing high-grade glioma combined with very low toxicity of GLIOVAC/ERC1671, and promising clinical data of other vaccine candidates, indicate that therapeutic immunization against glioblastoma comes within reach for patients suffering recurrent refractory disease with no therapeutic option or choice left.

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F.P.K.H.), or performance of experiments and analysis and interpretation of the data (all authors). V.E.J.C. wrote the paper.

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Abbreviations

Bvz	bevacizumab
Cy	cyclophosphamide
Dc	dendritic cell
ERC	Epitopoietic Research Corporation
GM-CSF	granulocyte-macrophage colony stimulating factor
TAA	tumor-associated antigen
Tmz	temozolomide

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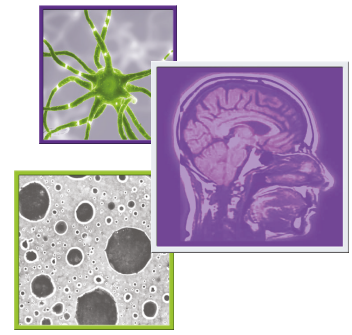

Phase II study of ERC1671 plus bevacizumab versus bevacizumab plus placebo in recurrent glioblastoma: interim results and correlations with CD4$^+$ T-lymphocyte counts

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Aim: ERC1671 is an allogeneic/autologous therapeutic glioblastoma (GBM) vaccine – composed of whole, inactivated tumor cells mixed with tumor cell lysates derived from the patient and three GBM donors. **Methods:** In this double-blinded, randomized, Phase II study bevacizumab-naive patients with recurrent GBM were randomized to receive either ERC1671 in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Leukine® or sargramostim) and cyclophosphamide plus bevacizumab, or placebo plus bevacizumab. **Interim results:** Median overall survival (OS) of patients treated with ERC1671 plus bevacizumab was 12 months. In the placebo plus bevacizumab group, median OS was 7.5 months. The maximal CD4$^+$ T-lymphocyte count correlated with OS in the ERC1671 but not in the placebo group. **Conclusion:** The addition of ERC1671/GM-CSF/cyclophosphamide to bevacizumab resulted in a clinically meaningful survival benefit with minimal additional toxicity.

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Keywords: allogeneic • autologous • bevacizumab • CD4+ T lymphocyte • ERC1671 • GBM • GBM vaccine • glioma surgery • immunotherapy

Glioblastoma (GBM, WHO grade IV astrocytic glioma) is the most common and most aggressive form of brain cancer in adults. The annual incidence is about 3.19 cases per 100,000 population, resulting in approximately 10,000 new cases each year in the USA [1]. Prognosis for GBM is very poor. For decades, the mainstay of therapeutic intervention was based on surgical resection (when safely feasible), followed by radiotherapy (RT). In 2005, data from the landmark European Organisation for Research and Treatment of Cancer – National Cancer Institute of Canada (EORTC-NCIC) trial changed the standard of care treatment for GBM. This Phase III trial demonstrated a survival advantage for concomitant and adjuvant temozolomide (TMZ) chemotherapy when added to the standard course of radiation [2]. In the group of patients assigned to radiation plus TMZ, median survival improved from 12.1 (RT alone) to 14.6 months. 2-year and 5-year survival was 27 and 9.8%, respectively, as compared with 10.9 and 1.9% for radiation alone [2,3]. The latest therapy to be approved for GBM in the USA and Europe is the

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alternating electric fields generator NovoTTF/Optune® [4], which may extend median overall survival (OS) by about 5–24 months [5].

However, despite all advancements in GBM care, the vast majority of patients relapse. At the time of recurrence after the first-line therapy, further treatment options are limited [6,7]. Repeat surgery is often considered, but tumor cells infiltrating the brain and spinal cord many times prevent a significant surgical resection. At the same time, invasive tumor cells appear to be more resistant to cytotoxic drug therapy and to have a higher proliferative potential. In general, the treatment of recurrent GBM by repeat surgery, re-irradiation and further chemotherapy may increase the symptom-free interval and moderately extend OS, primarily in patients with good performance status [8,9].

The only US FDA targeted treatment approved for recurrent GBM patients is the angiogenesis inhibitor bevacizumab, a humanized monoclonal antibody targeting VEGF [10]. When used alone or in combination with a cytotoxic agent, it improves imaging parameters for most patients, but duration of benefits is transient and short lived. Its impact on prolonging OS appears limited, especially when used outside the clinical trial settings [11–13]. Although, bevacizumab is approved for recurrent GBM in the USA and Canada, it did not receive market authorization by the EMA.

In recent years, immunotherapy of cancers has garnered much increased attention as a new pillar of cancer treatment, with the potential to assume a place alongside surgery, RT and chemotherapy [14,15]. We have been developing ERC1671 (Gliovac™) as a novel approach to GBM therapy. ERC1671 is a course of vaccines, where irradiated/inactivated tumor cells are combined with tumor cell lysate for subcutaneous injection. The tumor cells and lysates are derived from the GBM patient to be treated (autologous component), as well as from three other GBM patient donors (allogeneic component). This mix is administered together with cyclophosphamide and granulocyte-macrophage colony-stimulating factor (GM-CSF) to support immune system priming.

Preclinical proof of principle of the concept underlying the ERC1671 vaccination approach was established 10 years ago in rat models, where it was shown that allogeneic GBMs can be used to vaccinate against an established syngeneic tumor [16]. The first published report [17] on ERC1671's use in the clinical setting described the vaccine's effect in a recurrent GBM patient who previously had failed second-line bevacizumab. Although similar patients generally are moribund within a few short weeks within bevacizumab failure, the ERC1671-treated patient survived for 10 months without any other adjuvant therapy, but eventually died of complications related to his previous chemotherapies [17]. In a related study [18], nine recurrent GBM patients were treated with ERC1671 on a compassionate use exemption protocol. The majority of these patients was from European countries and therefore did not receive bevacizumab either before or during the course of vaccination. 6-month survival on the ERC1671 regimen was 100%, and 12-month survival was 40%, providing initial evidence of low toxicity and promising activity of this new therapeutic approach [18].

To further investigate and validate safety and effectiveness of ERC1671, a Phase II, double-blinded, placebo-controlled clinical study (NCT01903330) was initiated at the University of California, Irvine. Because in the USA, bevacizumab is an approved treatment in the recurrent setting, bevacizumab was included, and so was cyclophosphamide and GM-CSF to support immune system priming. In this report, we are presenting interim results from this trial, based on nine patients who were unblinded as stipulated by the study protocol at the time of further progression.

Methods

Composition of vaccine

ERC1671 (Gliovac) is an immunological therapy composed of primary irradiated/inactivated whole tumor cells and lysates from allogeneic and autologous GBM patients, administered in combination with the immune system priming agents cyclophosphamide and GM-CSF. Specifically, the complete ERC1671 regimen consists of: inactivated tumor cells and tumor cell lysate from the patient to be treated (autologous component: ERC-D); inactivated tumor cells and tumor cell lysate from three other GBM patients (allogeneic components: ERC-A from donor X, ERC-B from donor Y and ERC-C from donor Z); cyclophosphamide to relax the immune-suppressive environment; and GM-CSF to enhance immune responses.

The ERC1671 vaccine is administered by intradermal vaccination. One dose of ERC1671 (i.e., ERC-A through D) consists of whole tumor cells (between 1×10^5 and 1×10^6 cells) combined with tumor cell lysate (between 1×10^5 and 1×10^6 cells). Immediately prior to injection, 500 µg GM-CSF (Leukine®) is added to each vaccine dose, and the combined volume is injected together. Cyclophosphamide (Cytoxan®) is given orally (2×25 mg

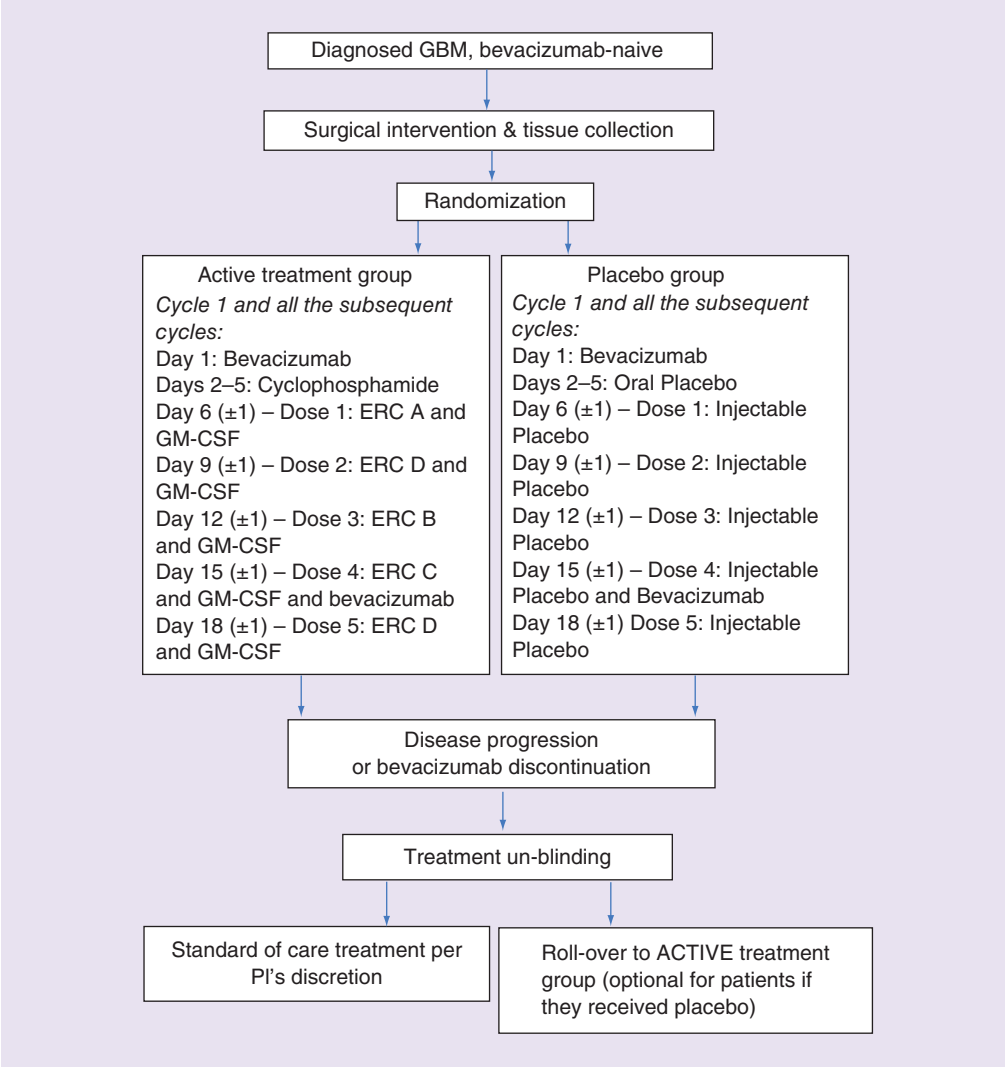

Figure 1. Study schema. Each treatment cycle is 28 days long. The first day of the first cycle is scheduled 29 days from surgery, which satisfies the US FDA mandated waiting time of 4 weeks between surgery and first use of bevacizumab, and allows for sufficient time to process the patient's tumor tissue for vaccine production. The timeline for days 1 through 5 (bevacizumab, followed by cyclophosphamide) is implemented strictly, whereas subsequent administration of each individual dose of vaccine (in combination with GM-CSF) is flexible by ±1 day. ERC-D is the autologous component, whereas ERC-A, -B and -C are allogeneic components from three different GBM patient donors. Both groups, active treatment group and placebo group, receive bevacizumab on day 1 and 15 (±1) of each cycle. This course of treatment is repeated every 28 days until disease progression or intolerance, at which time assignment of the respective patient will be unblinded.
GBM: Glioblastoma; GM-CSF: Granulocyte-macrophage colony-stimulating factor.

Cytoxan capsules per day) for 4 days (days 2–5) at the beginning of each cycle (see treatment scheme Figure 1). For the control patient group, all doses ERC-A through ERC-D are replaced by placebo treatment, which contains injectable freezing medium only, supplemented with sucrose and human albumin. No GM-CSF is added. Oral cyclophosphamide is replaced by oral placebo. Patients in both groups, active treatment group as well as placebo group, receive 10 mg/kg bevacizumab (Avastin®) infusion on day 1 and 15 of each 28-day cycle. Treatment cycles are continued until progression of disease or intolerance, at which time group assignments are unblinded.

Vaccine production

ERC1671/Gliovac is being manufactured under good manufacturing practice (GMP) approved aseptic conditions from surgically removed GBM tissues. These tissues are received and released by a tissue bank of human body mate-

rial, after testing for absence of transmissible infections, including HIV, HBV, HCV, CMV, HTLV and *Treponema pallidum*/syphilis. After coding by a suitable anonymization procedure, samples are sent under temperature-controlled conditions to the GMP manufacturing site. Cells are isolated by mechanical dissection and washed in Earl's balanced salt solution. Isolated cells are counted and haptenized with 1-fluoro 2,4-dinitrofluorobenzene to improve immunogenicity. Haptenized cells are divided into two equal parts. One part is preserved for freezing in a sucrose medium, and the other part is lysed by osmotic shock. Both parts are irradiated with 25 gray of gamma radiation to inactivate any replication competence. Thereafter, all preparations are stored at -80°C.

Patient characteristics & selection

For patients to be eligible for inclusion in the trial, they must have histologically confirmed WHO grade IV malignant glioma and documented treatment failure to standard of care treatment, including surgery followed by RT with concomitant and adjuvant TMZ chemotherapy. As well, these relapsed GBM patients must be bevacizumab-naive, aged ≥18 years, have measurable contrast-enhancing tumor on MRI and Karnofsky Performance Status of ≥70%. Patients must have normal organ and marrow function as defined by hemoglobin >9.0 g/dl, leukocytes >1,500/mcl, absolute neutrophil count >1,000/mcl, platelets >125,000/mcl; total bilirubin within normal institutional limits, serum creatinine >1.5 mg/dl and aspartate aminotransferase (AST or SGOT)/serum glutamic pyruvic transaminase (SGPT or ALT) <2.5× institutional upper limit of normal. Systemic corticosteroid therapy must be at a dose of ≤4 mg of dexamethasone or equivalent per day during the week prior to the first day of initiation of the first vaccination cycle.

Procedures

All patients described in the current report were enrolled and treated at the University of California Irvine, CA, USA, under an institutional review board approved protocol and after signing appropriate institutional review board approved informed consent forms.

Each treatment cycle is 28 days long. The first day of the first cycle (which starts the cycle by infusion of bevacizumab) is scheduled 29 days after the surgery. This satisfies the FDA-mandated waiting time of 4 weeks between surgery and first use of bevacizumab. In addition, it provides sufficient time to process the tumor tissue for vaccine production and ensures the availability of the ERC-D (autologous) component of the treatment regimen (which is administered for the first time on day 9 of the first cycle). The three allogeneic vaccine components (ERC-A, -B, -C) are in stock and readily available when needed. The treatment is repeated every 28 days until progression of disease or intolerance. Humoral immunologic response is measured at baseline and at selected times following vaccination and at the time of disease progression or end of treatment (EOT). Patients undergo brain MRI as part of standard care before starting cycle 1 and every 8 weeks until disease progression, and whenever progression is suspected based on clinical symptoms. Tumor response is assessed using both the Macdonald and the iRANO response criteria for high-grade gliomas [19,20], which considers radiologic imaging, neurological status and steroid dosing. Safety is evaluated throughout the trial by the incidence of adverse events (AEs), physical examination findings, vital signs and clinical laboratory test results. AEs are graded for severity using NCI Common Terminology Criteria for Adverse Events v.4.0 [21].

At the time of proven disease progression, the group assignment of these patients is unblinded as per protocol stipulation. At this time, patients from the active treatment group are offered standard or palliative care, whereas patients from the placebo group are offered the opportunity to roll over to the active treatment group. Patients who do not participate in the rollover option of the study return for an end-of-therapy visit where alternate treatment and/or care options are discussed. Patients continue to be followed for survival.

Outcomes

OS is measured from day 1 of Cycle 1 until death. Progression-free survival (PFS) is defined as the time from day 1 of Cycle 1 to the date of progression or death due to any cause. Immune monitoring in the peripheral blood (including, but not limited to $CD4^+$ T lymphocyte counts) was performed every 2 weeks.

Statistical analysis

The survival data were plotted as Kaplan–Meier survival curves and analyzed for significance using logrank test.

Table 1. Patient characteristics.

Characteristics	Active treatment group + bevacizumab (n = 5)	Placebo control + bevacizumab (n = 4)
Age (average [range])	59 (49–65)	57 (48–74)
Male (n [%])	4 (80%)	3 (75%)
KPS (average [range])	80 (70–100)	90 (70–100)
Relapses (n [%])		
1	5 (100%)	3 (75%)
IDH1/2 status (wild-type [%])	5 (100%)	4 (100%)
MGMT promoter	Unmethylated 4 (80%) Undetermined 1 (10%)	Unmethylated 3 (75%) Undetermined 1 (25%)

KPS: Karnofsky performance status.

Results

Study design

Presented here are interim results (as per 1 September 2017) of an ongoing, Phase II, double-blinded, placebo-controlled study of a novel cancer therapeutic vaccine, ERC1671/Gliovac, in patients with recurrent, bevacizumab-naive GBM. In the active treatment group, vaccination is combined with GM-CSF, cyclophosphamide and bevacizumab, whereas the control group receives placebo and bevacizumab only (Figure 1).

The key principle underlying this particular vaccination approach is the use of a broad set of tumor antigens, derived from freshly resected whole tumor tissue – not only from the patient under treatment, but expanded to include the same from three independent GBM tissue donors. This multivalent array of autologous and allogeneic antigens is expected to reduce the chance of immune escape, which can emerge from antigenic loss or active major histocompatibility complex (MHC) downregulation and is more likely to occur when using a single- or limited-antigen targeted immunotherapy. During each immunization cycle, the immune effector response is triggered by breaking tolerance to the patient's tumor antigens through first injecting one of the allogeneic components (i.e., ERC-A). This is facilitated by the fact that the injection of allogeneic preparation evokes a strong anti-nonself immune response. Thereafter, the second vaccine dose is patient-derived (autologous ERC-D) to focus the triggered immune reaction toward the patient's tumor antigens. This is followed by two additional (booster) injections of allogeneic material (ERC-B and -C) and a final injection of ERC-D (Figure 1).

For each cycle, the immunizations are preceded by a short regimen of low-dose, metronomic cyclophosphamide, based on the published literature which shows that low-dose cyclophosphamide stimulates dendritic cell expansion, contributes to the induction of antitumor cytotoxic T lymphocytes and depletes immune-inhibitory immune cells and stimulates the polarization of $CD4^+$ T cells into TH1 and/or TH17 lymphocytes eventually affecting the Treg/T-effector ratio in favor of tumor regression [22,23]. In addition, each dose of tumor antigens is accompanied by co-injection of GM-CSF. This growth factor has been shown to effectively and potently enhance the immune response in several different systems [24,25]. The rationale for also including bevacizumab is based on earlier observations that VEGF inhibits immune activity via inhibitory actions upon dendritic cells [26]. As such, using anti-VEGF therapy via administration of bevacizumab to cancer patients can enhance dendritic cell and T-cell responses to antigens [27], and potentially increase the level and specificity of the immunostimulation achieved by ERC1671.

As per treatment protocol, nine study participants were recently unblinded. Characteristics of these patients are summarized in Table 1. Unblinding revealed that four patients had received ERC1671 vaccine, four had received placebo. One patient was marked as nonevaluable due to discontinuation prior to completion of the first cycle.

Clinical safety

Clinical results for toxicity show an equal distribution of AEs between the active treatment and placebo groups, with no grade 4 or 5 toxicities (Table 2). Among documented grade 3 toxicities, headaches were the most common. Among all toxicities, injection site reactions (induration, erythema and ulceration) were most frequently noted. Although these skin reactions were mild, they indicated the development of immune responses. However, they were not consistently noted in all patients, and hence no clear correlation between efficacy and erythema response can be concluded. Similarly, other observed mild systemic reactions, including self-limiting fever and chills, represent expected outcomes related to the intended immune stimulation.

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Table 2. Toxicities (only the grade 3 toxicities or the toxicities reported at least as five separate events are included). No grade 4 or grade 5 toxicities were encountered in either group.

Adverse events (ERC1671 plus bevacizumab)	Grade 3	Total
Injection site reaction	0	67
Arthralgia	0	70
Gait disturbance/fall	1	4
Back pain	1	6
Headache	2	9
Anxiety	0	6
Total events of any grade	**4**	**162**
Adverse events (placebo plus bevacizumab)	**Grade 3**	**Total**
Gait disturbance/fall	1	38
Muscle weakness	2	4
Hydrocephalus	2	7
Delirium	1	1
Urinary incontinence	1	4
Thromboembolic event	1	4
Total events of any grade	**8**	**58**

Clinical efficacy: radiology data

The patients were monitored with imaging every 8 weeks (2 cycles) as stated by protocol. The overall response rate was higher for the ERC 1671 arm versus the control arm at 75% (3/4) versus 25% (1/4). The responders in the ERC 1671 group experienced durable responses – as exemplified in Figure 2. The patient in this case has achieved a partial response after cycle 1, and has maintained his response for more than 7 months. The patient's OS is now over 2 years.

Clinical efficacy: OS

Median OS of patients treated with ERC1671 plus bevacizumab was 12.1 months, with one patient surviving >2 years. In the group treated with placebo plus bevacizumab, median OS was shorter at 7.6 months, with all patients having succumbed within 1 year (Figure 3). Median PFS of the treated with ERC1671 + bevacizumab was 7.3 months (223 days), compared with the patients treated with placebo + bevacizumab, where median PFS was 5.4 months (164 days) (data not shown).

Clinical efficacy: immune correlations

$CD3^+/CD4^+$ helper T-lymphocytes counts were monitored in peripheral blood at the baseline and every 2 weeks during the study participation. The maximum count (cells/μl) is defined as the highest value measured in a patient during the study participation, while the EOT count is the $CD3/CD4^+$ helper T-lymphocytes count (cells/μl) at the EOT visit. Both the maximum count and the EOT count highly correlated with the OS in the patients treated with ERC1671 and bevacizumab, but not in the placebo and bevacizumab group (see Figure 4).

Discussion

The present study provides preliminary evidence that ERC1671/gliovac immunotherapy, combined with bevacizumab, is safe and potentially effective in recurrent GBM patients. A number of bevacizumab-based clinical trials were recently completed, all of them with a medium OS at 12 months between 10 and 26% [28–30], very similar with our control arm results (only one out of four patients survived 12 months). In comparison, our active treatment group patients had a 12 months OS of 50%, very similar with our previously published Phase 0 data [18].

Our study also suggests that the response to the ERC1671 vaccine treatment directly correlates with the $CD4^+$ helper T-lymphocytes counts in the peripheral blood. The role of $CD4^+$ T-cell response in antitumor immunity is well-described in the literature [31]. In animal models, the presence of $CD4^+$ helper T lymphocytes is essential for eliciting a response to cell-based vaccines [32,33]. In GBM, $CD4^+$ tumor infiltration after vaccination was reported for HSPPC-96 dendritic cell vaccine [34]. Also, a recent preclinical study has shown that, while $CD4^+$, $CD8^+$ T and NK-cell subsets are required early to establish anti-GBM immune responses, only $CD4^+$ T cells are continuously

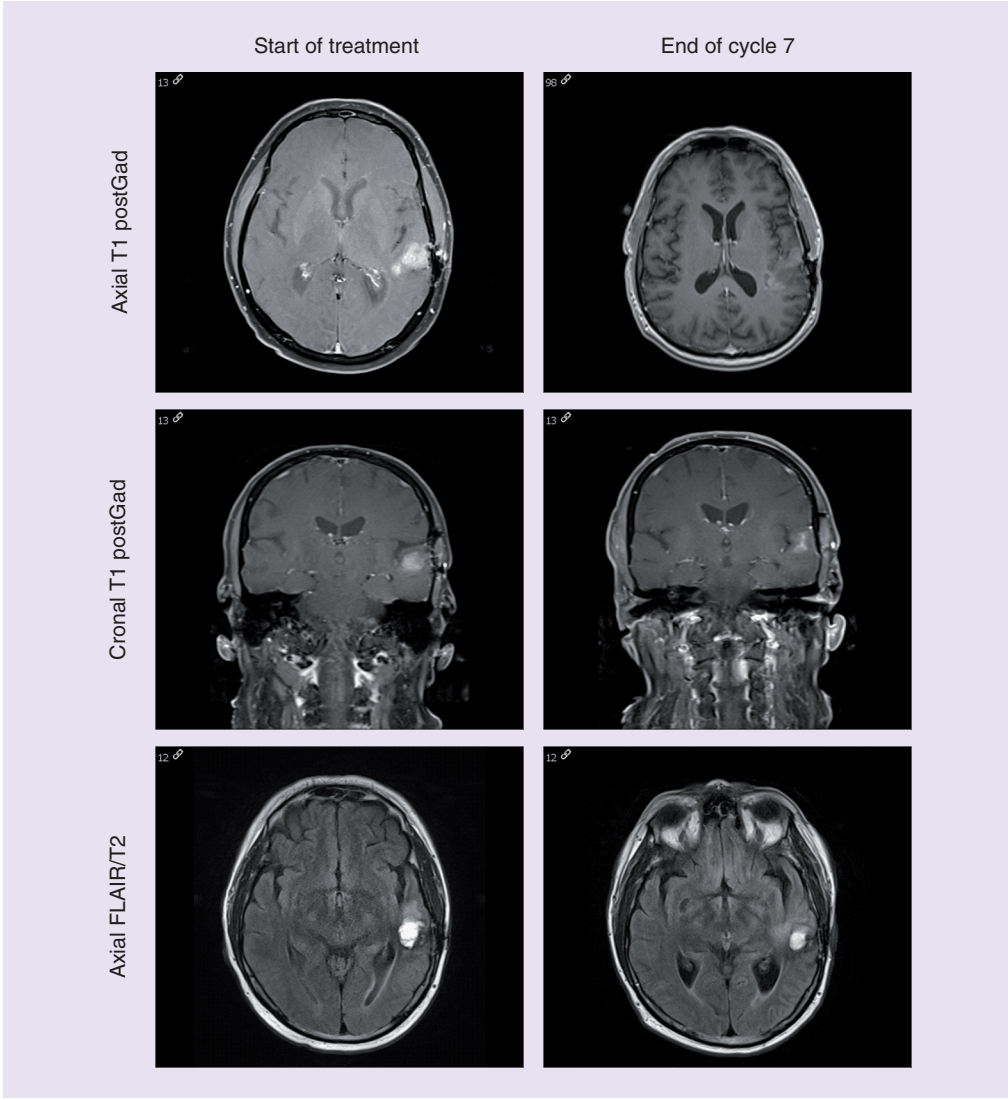
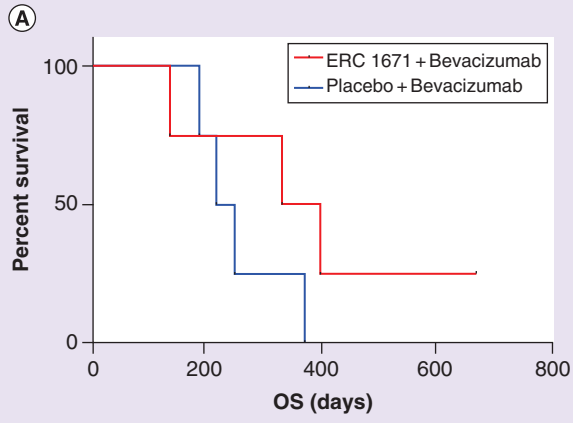


Figure 2. MRI of the brain for a patient randomized to the ERC1671/bevacizumab arm. Showing the tumor size before starting in the ERC1671/bevacizumab treatment and end of cycle 7. The MRI shows significant decrease in contrast enhancement over time and stable fluid-attenuated inversion recovery signal.

required to achieve maximal survival in this immunocompetent GBM model treated with radiation and PD-1 blockade [35]. We have also reported T-lymphocyte infiltration in our first ERC1671 publication (single-patient report) [17]. However, the importance of monitoring $CD4^+$ helper T lymphocytes in the peripheral blood is not well established in GBM clinical trials of cell-based vaccines, and might represent a biomarker of response for our therapeutic strategy.

The rationale underlying the ERC1671 vaccine is to evoke an oligoclonal and partly allo-specific immune induction, based on the use of a broad set of tumor antigens derived from freshly resected GBM tumor tissues from patient and three unrelated donors. This broad antigen-based approach differs from many other currently ongoing attempts at developing a tumor vaccine, some of which zero in on one or only a few individual, more or less tumor-specific targets, such as SurVaxM (aimed at surviving) or rindopepimut, a peptide vaccine aimed at the EGFR deletion mutation EGFRvIII [36,37].

In developing ERC1671, we preferred a multimodular approach that is based on syngeneic lysates and cells, mixed with lysates and cells from three different allogeneic tumor donors. This mixture of antigens is not defined, but expected to overlap to a large degree with the specific tumor antigens in the patient. Moreover, this strategy enables triggering of an immune response against a broad array of tumor antigens and also triggers nonself



	OS (days/months)
ERC 1671 + Bevacizumab	363/12.1
Placebo + Bevacizumab	229/7.6

B

Treatment	OS12
Bevacizumab only studies	10–26%
Taal (BELOB) [28]	26%
Field (CABARET) [29]	24%
Heiland (Germany) [30]	10%
ERC1671 compassionate use [18]	40%
ERC1671 plus Bevacizumab	50%

Figure 3. Overall survival and comparisons with previous studies. (A) Median OS was 363 days in the active treatment group (4 patients), compared with 229 days in the placebo group (4 patients). **(B)** A total of 12 months OS in the ERC1671/bevacizumab arm of the study is superior to previously published single-arm bevacizumab studies. OS: Overall survival.

triggered allo-immune reactivity, a classical allograft-directed immune response, typical for nonmatching major histocompatibility between graft cells and the host. Cyclophosphamide is used in this protocol because this drug was shown to diminish the Treg population, and thereby decrease the immune suppressive environment of the tumor [22]. GM-CSF has been used to effectively and potently enhance the immune response in several different systems and therefore will be administered as part of this protocol [38].

In summary, preliminary analysis of interim results from our study indicates that the addition of ERC1671/GM-CSF/cyclophosphamide to bevacizumab resulted in a clinically meaningful survival benefit with minimal additional toxicity. The study is ongoing with the anticipated addition of two other sites.

Future perspective

The last years have revolutionized the field on cancer immunotherapies, with novel treatments being approved almost every month for a variety of malignancies, including melanoma, lung cancer, bladder cancer, etc. However, in spite of multiple Phase III studies, no GBM immunotherapy has been able to show effectiveness and to obtain FDA approval. Our approach brings a different concept – namely the use of a broad antigen-based approach, including both allogeneic and autologous components and shows promise in activating a very important population of cells – namely the $CD4^+$ helper T lymphocytes. The future promise of our treatment might also rest in the ability to combine it with bevacizumab, and potentially with immune checkpoint inhibitors – an option that will allow more

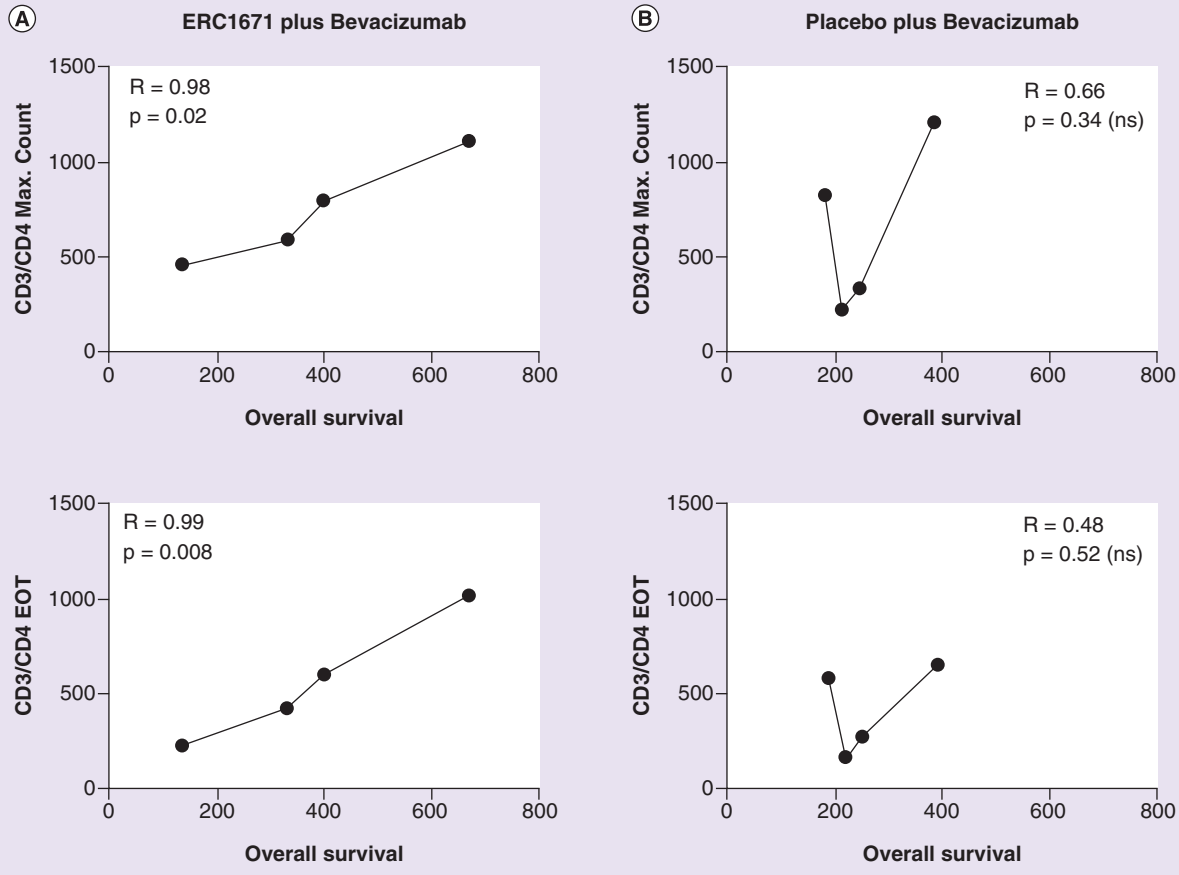


Figure 4. $CD3^+/CD4^+$ lymphocyte counts highly correlate with overall survival in the ERC1671/bevacizumab treatment arm. The maximal values, as well as end-of-treatment values, of $CD3^+/CD4^+$ mature helper/inducer T lymphocytes were determined in all patients from the ERC1671/bevacizumab group **(A)** and the placebo/bevacizumab group **(B)**. Cell numbers were plotted over overall survival time.

powerful immune activation in the periphery as well as more aggressive local tumor immunological targeting and destruction.

Summary points

- Immunotherapies for glioblastoma (GBM) are currently being developed.
- ERC1671 is a novel immunotherapy concept, combining allogeneic/autologous components – whole, inactivated tumor cells mixed with tumor cell lysates derived from the patient and three GBM donors, in addition to immune priming with Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Leukine®, Sanofi Genzyme, MA, USA) and low-dose cyclophosphamide.
- This experimental immunotherapy is very well tolerated, with minimal side effects.
- In the active treatment group (ERC1671 plus bevacizumab), overall survival is correlated with the maximal $CD4^+$ T-lymphocyte count in the peripheral blood, highlighting the role of $CD4^+$ T lymphocyte in achieving and maintaining immunologic tumor control.
- The combination of ERC1671 and bevacizumab had encouraging overall survival results, when compared with the bevacizumab alone group in our study and with recently published bevacizumab studies.
- Recurrent GBM patients might benefit from participation in personalized immunotherapy studies such as the one presented in this article.

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