Supplementary Materials: Audencel Immunotherapy Based on Dendritic Cells Has No Effect on Overall and Progression-Free Survival in Newly Diagnosed Glioblastoma: a Phase II Randomized Trial

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Figure S1. Influence of extent-of-resection (EOR) on overall survival (OS). Patients of the Audencel cohort and the SOC cohort were stratified into three groups based on the EOR that could be achieved (100% vs 90-99% vs 70-90%). A minimal EOR of 70% was an inclusion criterion for the study, so no study patient was below that threshold. Kaplan-Meier analysis shows that EOR did not have an influence on the OS outcome in the Audencel cohort compared to the SOC cohort (p = 0.762).

Figure S2. Relation of DC vaccine quality control and overall survival (OS). One main quality control parameter measured during the production of the personalized, autologous vaccine was the IL-12
production capacity of every single vaccine. When analyzing a possible correlation with OS, no such connection could be made \((a, p = 0.891)\). Similarly, the T cell proliferation capacity of the DC vaccine did not have a connection to survival — for three different DC: T cell ratios tested: 1:5 \((b)\), 1:10 \((c)\), 1:20 \((d)\). Overall, vaccine quality did not have an influence on OS.

**Figure S3.** Analysis of potential influence of number of vaccinations received on overall survival (OS). In a Pearson correlation calculation, a non-significant trend towards better OS based on the number of vaccinations received can be registered \((p = 0.081)\).

**Figure S4.** CONSORT 2010 Flow Diagram.
| Section/Topic     | Item No | Checklist item                                                                 | Reported on page No |
|------------------|---------|--------------------------------------------------------------------------------|---------------------|
| Title and abstract | 1a      | Identification as a randomised trial in the title                              | 1                   |
|                  | 1b      | Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts) | 1–2                 |
| Introduction     |         |                                                                                  |                     |
|                  | 2a      | Scientific background and explanation of rationale                             | 2–3                 |
|                  | 2b      | Specific objectives or hypotheses                                               | 2–3                 |
| Methods          | 3a      | Description of trial design (such as parallel, factorial) including allocation ratio | 2, 11–13            |
|                  | 3b      | Important changes to methods after trial commencement (such as eligibility criteria), with reasons | 3, 12               |
| Participants     | 4a      | Eligibility criteria for participants                                          | 11                  |
|                  | 4b      | Settings and locations where the data were collected                           | 11                  |
| Interventions    | 5       | The interventions for each group with sufficient details to allow replication, including how and when they were actually administered | 11–13               |
| Outcomes         | 6a      | Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed | 11–13               |
|                  | 6b      | Any changes to trial outcomes after the trial commenced, with reasons          | -                   |
| Sample size      | 7a      | How sample size was determined                                                  | 13                  |
|                  | 7b      | When applicable, explanation of any interim analyses and stopping guidelines    | -                   |
| Randomisation:   | 8a      | Method used to generate the random allocation sequence                          | 11                  |
| Sequence generation | 8b  | Type of randomisation; details of any restriction (such as blocking and block size) | 11                  |
| Allocation       | 9       | Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned | 11                  |
| concealment      |         |                                                                                  |                     |
| mechanism        |         |                                                                                  |                     |
| Implementation   | 10      | Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions | 11–13               |
| Blinding         | 11a     | If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how | - (open-label)      |
|                  | 11b     | If relevant, description of the similarity of interventions                      | -                   |
| Statistical methods | 12a | Statistical methods used to compare groups for primary and secondary outcomes | 13                  |
|                  | 12b     | Methods for additional analyses, such as subgroup analyses and adjusted analyses | 13                  |
Results

Participant flow (a diagram is strongly recommended) 13a For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome 3–4

13b For each group, losses and exclusions after randomisation, together with reasons 3–4

Recruitment 14a Dates defining the periods of recruitment and follow-up 11, 4

14b Why the trial ended or was stopped 11

Baseline data 15 A table showing baseline demographic and clinical characteristics for each group 3–4

16 For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups 3–4

Outcomes and estimation 17a For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval) 4–6

17b For binary outcomes, presentation of both absolute and relative effect sizes is recommended 4–6

Ancillary analyses 18 Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory 7–9

Harms 19 All important harms or unintended effects in each group (for specific guidance see CONSORT for harms) 6–7

Discussion

Limitations 20 Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses 9–11

Generalisability 21 Generalisability (external validity, applicability) of the trial findings 9–11

Interpretation 22 Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence 9–11

Other information

Registration 23 Registration number and name of trial registry 11

Protocol 24 Where the full trial protocol can be accessed, if available 11

Funding 25 Sources of funding and other support (such as supply of drugs), role of funders 13

* We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming for those and for up to date references relevant to this checklist, see www.consort-statement.org.
Supplementary Materials and Methods

**Immunotherapy: Description of the Audencel DC vaccine**

Audencel is a DC-based autologous cancer vaccine. It is comprised of DCs charged with tumor-derived antigens that are matured via LPS and IFNγ and that are characterized by the secretion of IL-12. After production, the final DC vaccine product is 1-5 x 10^6 autologous, “semi-mature” DCs in a DMSO-containing freezing medium (CryoStore CS2/Lite, STEMCELL Technologies, Vancouver, CA).

**Immunotherapy: Prior studies on Audencel**

The DC vaccine technology behind Audencel has been investigated in prior preclinical studies and in a phase I clinical trial [1–4]. Preclinically, Felzmann et al. showed in human in vitro experiments that maturation of DCs with LPS and IFNγ leads to an immunostimulatory phenotype (characterized by IL-12 secretion) that can efficiently triggered cytolytic activity in autologous T lymphocytes. Importantly, this was only the case for co-cultures performed 2–6 h after maturation stimulus (LPS/IFNγ) but not for co-cultures performed at 48 hours [1]. The Audencel technology thus uses DCs matured for 6 hours, called “semi-mature”. Hüttner et al. studied the DC generation technique used for Audencel in a syngeneic murine in vivo model and found that the IL-12 secreting DCs used could reduce tumor growth (murine cell line K-Balb) [2]. The method for generating DCs from peripheral blood monocytes was evaluated in further human in vitro experiments by Felzmann et al [3]. Finally, the Audencel technology was also tested in a phase I clinical trial where pediatric cancer patients suffering from advanced solid pediatric malignancies were vaccinated by Dohnal et al [4]. This study established feasibility and safety. As a continuation of these early preclinical and clinical experiences, the here presented phase II trial of Audencel applied to patients suffering from glioblastoma was initiated.

**Immunotherapy: Production of the Audencel DC vaccine for the phase II clinical trial on glioblastoma**

Tumor samples were harvested through surgical resection, irradiated with 12,000 rad (in accordance with local guidelines for the irradiation of blood products for human transfusion) and then stored subsequently without further delay at 4°C and transported to our Good Manufacturing Practice (GMP) facilities under sterile conditions for the generation of autologous tumor lysate. For that, tumor tissue was kept in Phosphate Buffered Saline (PBS; Hyclone, ThermoScientific, Utah, USA), was disrupted mechanically via a scalpel, pressed through a nylon mesh and the resulting cells in single cell suspension were lysed by five freeze/thaw cycles (liquid nitrogen, −150°C) in distilled water resulting in tumor cell lysate ready for further use. Particulate components were removed by centrifugation. Protein concentration of each tumor lysate was determined by Bradford assay and the vials containing protein lysate were kept frozen at −80°C.

Peripheral blood mononuclear cells (PBMCs) were obtained by leukocyte apheresis (performed at the Transfusion Medicine departments of the respective treatment centers according to local protocols and yielding 4–10 × 10^9 mononuclear cells) followed by elutration (Eutra cell separator, Gambro BCT, Inc. Lakewood, Colorado, USA) for the selective enrichment of clinical-scale monocytes. Then, monocytes were cultured in vitro in Cellgro medium (CellGenix Technology, Freiburg, Germany) with the presence of (317U/ml) recombinant human interleukin-4 (IL-4, CellGenix Technology, Freiburg, Germany) and (1000U/ml) recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF, CellGenix Technology, Freiburg, Germany) at a density of 1x10^6 monocytes/cm². On day 3, fresh medium containing the same cytokines, at the same concentration was added. On day 6, immature dendritic cells were incubated with autologous tumor lysate (see above) together with the immunological adjuvant Keyhole Limpet Hemocyanin (KLH, Calbiochem, Darmstadt, Germany) for 2 hours prior maturation stimulus. Subsequently, the DCs were incubated with LPS (200U/ml), E. coli strain O111:B4, Calbiochem, San Diego, CA, USA) and IFNγ (50ng/mL, Boehringer Ingelheim, Vienna, Austria) for 6 hours to induce functional maturation.
“Semi-mature” DCs were then harvested, washed with Phosphate Buffer Saline (PBS, HyClone, ThermoScientific, Utah, USA), aliquoted to vials containing $1-5 \times 10^6$ DCs each and stored in a liquid nitrogen tank.

**Immunotherapy: Quality control**

At the arrival of tumor material for vaccine production at the GMP facility, a sterility test was immediately conducted (BACTEC system, BD Biosciences, NJ, USA). Only if sterility could be proven, the material was processed further. Two aliquots of each final vaccine batch after production (see above) were again used for quality control that included tests for viruses, mycoplasma, and bacteria according to standard clinical guidelines. In addition, functional potency and the phenotype of the tumour lysate-loaded DCs was examined *in vitro.*

The purity and phenotype of each DC lot was determined by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA). Cells were stained with antibodies against CD45 (BD PharMingen, San Diego, CA, USA), CD14 (BD PharMingen, San Diego, CA, USA), CD1a (BD PharMingen, San Diego, CA, USA), CD83 (BD PharMingen, San Diego, CA, USA), CD80 (Immunotech, Marseille, France) and CD86 (BD PharMingen, San Diego, CA, USA). Release criteria for usage in clinical application were more than 70% viable dendritic cells and more than 60% CD86+/MHCI+/MHCII+/CD80+/CD83+ expression on these cells.

To ensure functional potency prior to application to patients, IL-12 production capacity and T-cell stimulation capacity were determined for each batch of the DC vaccine. For IL-12 measurement, an ELISA test system was used. Briefly, one vial of the vaccine was thawed, and the cells were used 24 hours after thawing. 96-well plates were coated with capture antibody (BD, San Jose, CA) diluted in PBS with 0.02% sodium azide. The next day, unspecific binding in the wells was blocked with 2% BSA in PBS. After blocking, IL-12 standard solutions (BD, San Jose, CA) of known concentration (30-1250 pg/ml) and diluted samples (1:2; 1:20; 1:50) were distributed into the wells. On the third day, captured cytokines were detected by primary incubation with a biotinylated detection antibody (BD, San Jose, CA) and secondary via incubation with alkaline phosphatase-conjugated streptavidin (Chemicon, Temecula, C). When phosphatase substrate at a concentration of 1 mg/ml in diethanolamine buffer was added, the respective yellow colour reaction developed. The diethanolamine buffer consisted of 1 M diethanolamine and 0.5 mM MgCl2 diluted in sterile water with a pH of 9.8. The optical density was measured with an ELISA reader (Anthos, Salzburg, Austria) at a wavelength of 405 nm and a reference wavelength of 690 nm. The cytokine concentrations was calculated using the WinRead V.2.3 software. The release criterion for application of the batch for human use in the trial was >100pg/ml IL-12.

For measurement of T-cell stimulation capacity, allogeneic mixed leukocyte reactions (alloMLR) were carried out. Briefly, allogeneic responder peripheral blood mononuclear cells (PBMCs) collected from healthy donors were isolated by gradient centrifugation from peripheral blood and recovered in AIM-V medium (ThermoFisher, Waltham, MA) supplemented with 2% human plasma (Octapharm, Vienna, Austria). Stimulating DCs (10,000, 2,000, or 400) were placed in triplicates (100 μl per well) on a 96 well round-bottom plate and 10⁵ responder cells in 100 μl medium were added to each well. For a positive reference 10⁵ responder cells were stimulated in 100 μl medium with Staphylococcal enterotoxin A/B (SEA/SEB, Toxin Technologies Inc., Sarasota, FL) at 100 ng/ml final concentration. On day 4 of the co-culture, 1 μCi of tritium thymidine solution (NEN Life Science Products, Boston, MA) was added to each well and the cells were incubated for another 18 hours. Finally, the cells were harvested with a Skatron harvesting device (Skatron, Liep, Norway) and the incorporated tritium thymidine was counted on a Trilux β-plate reader (Wallac Oy, Turku, Finland). The release criterion for application of the batch for human use in the trial was a T-cell proliferation of at least 30% of the reference SEA/SEB response (for the DC:T-cell ratios 1:5 and 1:10 and at least 15% for the DC:T-cell ratio 1:20).

Summing up, all batches of the DC vaccine that were released to the patient had shown IL-12 production capacity and T-cell stimulation capacity as well as a pre-defined stimulatory phenotype.
and absence of contamination with pathogens. After quality control, the personalized, autologous DC vaccine for each patient was then kept frozen until application. At the time of treatment an aliquot of the DC cancer vaccine containing approximately 1-5 million DCs was thawed and inoculated to the corresponding patient by ultrasound-guided injection intranodally into a tumor-free (cervical) lymph node.

Immunotherapy: Treatment schedule

All patients received the first line standard therapy for GBM: surgery, radiotherapy, and chemotherapy (Temozolomide). Randomization was done following surgery; patients in the treatment arm who received Audencel as an add-on to the standard treatment underwent leukocyte apheresis within 7-14 days after surgery. The first 4 immunizations were administered in weeks 7-10. Six more immunizations were applied in between the 6 blocks of maintenance chemotherapy. After completion of that schedule, patients received boost immunizations every 3 months. The vaccine was applied intranodally; each vaccine aliquot of Audencel contained 1-5 x 10^6 DC. The immunization schedule continued unaltered even if patients suffered disease recurrence and Temozolomide was withdrawn and replaced with an alternative therapy such as Bevacizumab. Patients of both groups received supportive care for acute or chronic toxicity whenever indicated.

References

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