

# Indoleamine 2,3-dioxygenase and survivin peptide vaccine combined with temozolomide in metastatic melanoma

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**Background:** Indoleamine 2,3-dioxygenase (IDO) and survivin have been identified as potential targets for cancer vaccination. In this phase II study a vaccine using the peptides Sur1M2 and IDO5 was combined with the chemotherapy temozolomide (TMZ) for treatment of metastatic melanoma patients. The aim was to simultaneously target several immune inhibiting mechanisms and the highly malignant cells expressing survivin.

**Methods:** HLA-A2 positive patients with advanced malignant melanoma were treated biweekly with 150 mg/m<sup>2</sup> TMZ daily for 7 days followed by subcutaneous vaccination with 250 µg of each peptide in 500 µL Montanide solution at day 8. Granulocyte-macrophage colony-stimulating factor was used as an adjuvant and topical imiquimod was applied prior to vaccination. Treatment was continued until disease progression. Clinical response was evaluated by PET-CT and immunological outcome was assessed by ELISPOT and flow cytometry.

**Results:** In total, 17 patients were treated with a clinical benefit rate of 18% including one patient with partial tumor regression. Immune analyses revealed a vaccine specific response in 8 (67%) of 12 patients tested, a significant decrease in the frequency of CD4<sup>+</sup> T-cells during treatment, a tendency towards decreasing frequencies of naïve CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, and increasing frequencies of memory CD4<sup>+</sup> and CD8<sup>+</sup> T-cells.

**Conclusions:** These results demonstrate that vaccine-induced immunity towards survivin and IDO-derived peptides can be achieved in combination with TMZ in patients mainly suffering from grade M1c melanoma including patients with brain metastases. A significant clinical activity could not be proven in this small study and a larger setup is needed to properly assess clinical efficacy.

**Keywords:** Metastatic malignant melanoma; immunotherapy; peptide; vaccination; indoleamine 2,3-dioxygenase (IDO); survivin

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## Introduction

Peptide vaccination as therapy of cancer is able to induce robust CD8<sup>+</sup> T-cell responses towards a multitude of

antigens. However, despite multiple clinical trials, no peptide vaccines have yet reached approval for the treatment of human cancers. As reviewed by Aranda *et al.* (1), this might be due to low antigenicity of cancer cells,

insufficient immunogenicity of the peptide vaccines, and the immunosuppressive mechanism of cancer. To overcome the challenges associated with inducing clinical responses by peptide vaccination, one approach is to combine vaccination with other immune stimulatory treatments for augmented effect. Combining immunotherapy with chemotherapy may seem counterintuitive as cells of the immune system are sensible to chemotherapy, but recent studies indicate an immune component of chemotherapy's anti-tumoral effect (2). In Denmark, temozolomide (TMZ) is used as a standard chemotherapy in treatment of malignant melanoma (MM) and the lymphodepleting effect of TMZ has been correlated with clinical response in MM. Furthermore, we have previously shown that TMZ induces a shift from naïve to differentiated CD8+ T-cells and induces expansion of tumor associated antigen (TAA) specific CD8+ T-cells (3). TMZ has been tested in combination with other immunotherapies including interferon- $\alpha$  and IL-2 without increased clinical benefit (4,5).

In this study, we combined a human leukocyte antigen (HLA)-A2 specific peptide vaccine with epitopes from indoleamine 2,3-dioxygenase (IDO) and from survivin with TMZ. HLA-A2 was chosen as it is the most frequent tissue types expressed in melanoma patients (6). IDO is an enzyme that facilitates the degradation of tryptophan to kynurenine. In 2003, Uyttenhove *et al.* (7) linked expression of IDO to tumoral immune resistance, and later on IDO's immune inhibitory ability was coupled to several mechanisms: depriving T-cells of tryptophan initiates a GCN2 kinase mediated stress response resulting in proliferative arrest and anergy (8); inducing apoptosis by increasing the concentrations of kynurenine and inducing conversion of T helper cells (Th) 17 into T-regulatory T-cells (Treg) (9). In cancer patients, clusters of CD8+ cytotoxic T-lymphocyte (CTL) and CD4+ Th cells spontaneously recognizing peptides derived from IDO have been observed (10,11). These findings led us to initiate a vaccination trial for lung cancer patients using an IDO-derived peptide which resulted in two ongoing clinical responders and IDO specific CD8+ T-cell immunity in all patients (12).

Survivin is an anti-apoptotic protein first discovered in 1997 (13). It is overexpressed in a variety of cancers including MM (13,14) and its expression has been correlated to a poor prognosis and disease progression in, e.g., colon cancer and neuroblastoma (15,16). Survivin is expressed in a subpopulation of cancer cells with a high proliferative potential and as a consequence, targeting survivin means targeting tumor cells with the highest proliferative rate (17). Specific T-cells able to recognize a survivin-derived peptide have been observed both

in *in vitro* and in clinical studies (18,19).

When treating MM patients with a peptide vaccine with survivin and IDO in combination with TMZ, we aimed for an increased clinical benefit by targeting several immune inhibiting mechanisms in the tumor microenvironment as well as directly targeting the malignant cells.

## Methods

### Study design

Patients were enrolled in this phase II study at Herlev Hospital, Capital Region, Denmark. Major inclusion criteria were histological verified MM, metastatic disease, evaluable disease according to Response Evaluation Criteria In Solid Tumors (RECIST 1.1), a performance status equal to or lower than 2 according to Eastern Cooperative Oncology Group (ECOG) (20) and adequate hematological function. Major exclusion criteria were other malignant diseases, use of immune suppressive medication, severe medical disorders, infections, severe allergy and active autoimmune diseases. For a complete description of inclusion and exclusion criteria, we refer to [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT01543464). If the patients met all criteria, they were tested for HLA-A2 tissue type. Only HLA-A2 positive patients received experimental treatment with the vaccine in combination with oral TMZ. HLA-A2 negative patients would receive standard therapy with oral TMZ. The study was approved by Danish Health and Medicines Agency and the local Ethics Committee, Capital Region of Denmark and conducted in accordance with the Helsinki II declaration and good clinical practice (GCP). The study is registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT01543464) and <https://eudract.ema.europa.eu/> (EudraCT #2011-004577-91). Primary endpoint was clinical benefit rate (CBR) defined as complete responses (CR) plus partial responses (PR) and stable disease (SD) for at least 6 months. Secondary endpoint was immunological response and tertiary endpoint was toxicity. The planned number of patients to be treated was 30. However, due to changes in standard treatment, algorithm for metastatic melanoma accrual to the trial became increasingly difficult and it was therefore decided to close the trial before full enrollment.

### Treatment

HLA-A2 patients were treated with 150 mg/m<sup>2</sup> TMZ

daily for 7 days, biweekly, followed by subcutaneous (sc) vaccination with IDO5 and Sur1M2 peptide in Montanide solution at day 8. Topical 5% imiquimod cream (Medea, Alleröd, Denmark) was applied to the vaccine-site and kept under an occlusive bandage 6–12 hours before injection. Ten minutes prior to vaccination, patients received an injection of 75 µg granulocyte-macrophage colony-stimulating factor (GM-CSF) at the intended site of vaccine administration (Berlex Laboratories, Inc., USA).

Treatment was repeated biweekly for a period of up to 6 months. Patients still benefiting from treatment would continue TMZ biweekly and vaccination injections every 4 weeks.

### *Peptide vaccine*

The vaccine consists of a 9-amino acid long peptide from survivin denoted Sur1M2, one-letter amino acid code LMLGEFLKL, and a 9-amino acid long peptide from IDO denoted IDO5, one letter amino acid code ALLEIASCL (Polypeptide Laboratories, France SAS). The 250 µg of each peptide were dissolved in 2% dimethyl sulfoxide (The Hospital pharmacy, Capital Region of Denmark) and 98% PBS and mixed with 500 µL Montanide ISA-51 (Seppic Inc., Air Liquide Healthcare specialty ingredients, Paris, La Defence, France).

### *Clinical and toxicity assessment*

Patients were eligible for response evaluation after 12 weeks of treatment. Clinical efficacy was assessed using positron emission tomography-computed tomography (PET-CT) scans <sup>18</sup>F-deoxyglucose-PET/CT (<sup>18</sup>F-FDG-PET/CT) scans at baseline (up to 28 days before treatment initiation) and every third month thereafter until progression. CT scans were generally evaluated according to RECIST 1.1 (21).

All patients receiving one or more treatment courses were evaluable for toxicity. Toxicity was assessed using Common Terminology Criteria for Adverse Events (CTCAE version 3.0).

### *Blood samples*

Peripheral blood mononuclear cells (PBMC) were purified from heparinized blood using lymphoprep™ (StemCell Technologies) density gradient centrifugation in LeucoSep™ tubes (Greiner Bio-One). After processing, cells were frozen in Nunc 1.8-mL Cryotube (Thermo

Scientific) at a concentration of 10<sup>6</sup> cells/mL and stored at -140 °C in 90% human AB serum (HS) (Sigma Aldrich) and 10% dimethyl sulphoxide (DMSO) (Herlev Hospital Pharmacy). Serum was separated by centrifugation of full-blood collected in an 8-mL Vacuette® gel-tube containing clot activator (Greiner Bio-One), aliquoted in Nunc 1.8-mL Cryotube (Thermo Scientific) and stored at -80 °C until analysis. For PBMC, 100 mL heparinized blood were drawn per sampling and for serum 8 mL full blood were drawn per sampling. Blood samples were obtained at baseline, week 4, week 8 and week 12. Until progression, patient samples were drawn each month for the first 6 months and thereafter every third month.

### *Ex vivo enzyme-linked immunospot (ELISPOT)*

ELISPOT analyses were performed in accordance with the Association for Cancer Immunotherapy immunoguiding program (CIP) guidelines ([http://cimt.eu/cimt/files/dl/cip\\_guidelines.pdf](http://cimt.eu/cimt/files/dl/cip_guidelines.pdf)). Vaccine responses were first assessed directly, *ex vivo*, and later indirectly, *in vitro*, see below. Cryopreserved PBMC were thawed in 37 °C RPMI medium 1640 + GlutaMAX (Life Technologies), washed twice in RPMI medium 1640 + GlutaMAX and resuspended in X-vivo 15 (Lonza Bioscience) containing 5% HS (Sigma Aldrich) and rested overnight in 24-well plates. Nitrocellulose bottomed 96-well plates were coated overnight with primary monoclonal antibodies (Mab) against human gamma interferon (IFN-γ) clone 1-D1K (Mabtech). The wells were washed in PBS (Sigma Aldrich) and blocked with X-vivo 15 media for 2 hours in 37 °C humidified atmosphere supplemented with 5% CO<sub>2</sub>. Cells were added to wells in 100 µL at concentrations of 5<sup>6</sup> cells/mL and 1.5<sup>6</sup> cells/mL along with 100 µL of 0.01 mM peptides in a X-vivo dilution. Peptides used were IDO5 (KJ Ross-Petersen APS, Klampenborg, Denmark), Sur1M2 (KJ Ross-Petersen APS, Klampenborg, Denmark), a negative control, C20, an HIV-derived epitope, amino acid one-letter code ILKEPVHGV (KJ Ross-Petersen APS, Klampenborg, Denmark) and a pool of human cytomegalovirus, Epstein-Barr virus and influenza virus epitopes (Mabtech) were used as a positive control (22). The plates incubated overnight at 37 °C in a humidified atmosphere supplemented with 5% CO<sub>2</sub>. Medium was discarded the following day and after washing in PBS, biotinylated anti-human IFN-γ, 7-b6-1 Mab (Mabtech) was added and plates were incubated 2 hours at room temperature, before washing with PBS and subsequently

adding streptavidin alkaline phosphatase (Mabtech). Plates were incubated 1 hour at room temperature then washed in PBS before BCIP/NBT substrate system (Mabtech) was added to induce a color reaction. Reaction was terminated at the emergence of dark purple spot by washing thoroughly with de-mineralized water. The spots were counted using the ImmunoSpotSeries 2.0 Analyzer (CTL Analyzers). ELISPOT assay was interpreted using the method of distribution free resampling (DFR) as described by Moodie *et al.* (23). Experiments were done in triplicates and results are presented as the mean number of spots in C20 stimulated wells subtracted.

### *In vitro* ELISPOT

After thawing, as described above, PBMC were resuspended in X-vivo 15 and seeded in a 24-well plate with  $4 \times 10^6$  cells per well. The cells were pulsed with IDO5 for 1 hour at room temperature and then Sur1M2 for 1 hour at room temperature both at a concentration of 5  $\mu\text{g}/\text{mL}$ . This was done because of Sur1M2's relative higher affinity than IDO5. After pulsing, 1.5 mL of X-vivo 15 with 5% HS were added to the cells and the plates incubated overnight at 37 °C in a humidified atmosphere supplemented with 5% CO<sub>2</sub>. On day 1, 240 international units (IU) of IL-2 (Novartis, Denmark) were added to each well. After 7 days, the cells were washed in X-vivo 15 and used in an ELISPOT assay as above. During incubation, the condition of the cells and the color of the media were regularly checked and new media was applied when needed.

### *Enrichment for antigen specific T-cells*

PBMC were pulsed with IDO5 and Sur1M2 in X-vivo 15 in a 24-well plate for 2 hours at room temperature at a concentration of 20  $\mu\text{M}$  and  $3\text{--}4 \times 10^6$  cells per well. Then 1.5 mL of X-vivo 15 with 5% HS were added and the cells incubated at 37 °C in a humidified atmosphere supplemented with 5% CO overnight. The day after peptide stimulation, 240 IU of IL-2 (Novartis, Denmark) were added to the wells. The cells were in culture for 3 weeks and were re-stimulated with peptide in log10 decreasing concentration of peptide each week. The cells were checked every day and new media was added every 10 days or when needed, assessed by cell number and color of the media. After 3 weeks, the cells were sorted using the TNF- $\alpha$  secretion assay (Miltenyi Biotec) according to the manufacturer's instructions and expanded in a rapid expansion protocol

(REP), see below. Prior to sorting, the cells were re-stimulated with 20  $\mu\text{M}$  peptide.

### *REP*

T-cells enriched for IDO/Sur1M2-reactivity were expanded, and approximately  $1\text{--}2 \times 10^5$  cells were used initially. Cultures were initiated in upright T25 flasks (Nunc) containing 20 mL X-vivo 15 (Lonza Bioscience) supplemented with 10% HS, 0.6  $\mu\text{g}$  anti-CD3 (OKT3, Janssen-Cilag), 6,000 IU/mL IL2 (Proleukin, Novartis), 1.25  $\mu\text{g}/\text{mL}$  Fungizone (Squibb), 100+100 IU/mL penicillin-streptomycin (Gibco, Life Technologies) and  $2 \times 10^7$  feeder-cells. As feeder cells, allogeneic PBMC mixed from three donors were used. The feeder cells were thawed separately from each donor, washed in RPMI-1640 (Gibco, Life Technologies),  $\gamma$ -irradiated with 30 Gy and mixed together. The feeder cells were mixed with anti-CD3 and added to the T-cells with the rest of the media components. The media was changed after 5 days by carefully removing 10 mL media from the flasks and adding 10 mL of fresh media. Media was changed when needed assessed by cell number and media color. The cells were first moved to a T80 (Nunc) and later to a T175 (Nunc) as the number of cells increased. After 14 days, cells were harvested and analyzed for IDO/Sur reactivity or cryopreserved in 90% HS with 10% DMSO.

### *Intracellular cytokine staining (ICS)*

ICS was performed in 96-well plates (Nunc, Fischer Scientific) with  $5 \times 10^5$  cells/mL in X-vivo 15. PBMC were stimulated with 5  $\mu\text{g}/\text{mL}$  of peptide in the presence of 1  $\mu\text{L}/\text{mL}$  GolgiPlug<sup>TM</sup> (BD Biosciences). After 5 hours, cells were stained for surface antigens and dead cell markers with the following reagents: anti-CD3-PerCP, anti-CD8-FITC and LIVE/DEAD Fixable Near-IR Dead Cell Stain 7 (Life Technologies). Cells were fixated and permeabilized with BD Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer's instructions. After permeabilization, cells were stained for intracellular antigens with the following reagents: anti-IL2-PE, anti-IFN $\gamma$ -APC, anti-TNF $\alpha$ -PE-Cy7 and anti-CD4-HV500. CD4 staining was done intracellular as stimulation of T-cells can induce internalization of CD4 protein (24). A minimum of 200,000 cells were acquired with the FACSCanto II (BD Biosciences) using FACSDiva software version 6.1.3 (BD Biosciences). FlowJo software version 10 (Tree Star,

**Table 1** Clinical comparison of control and treatment group at baseline

Characteristics	Temozolomide plus vaccine (n=17)
Age at baseline (mean) (years)	64.7
M-stage [n (%)]	
M1a	0 (0.0)
M1b	4 (23.5)
M1c	13 (76.5)
Brain metastasis [n (%)]	8 (47.1)
Sex (male) [n (%)]	9 (52.9)
Lactate dehydrogenase above normal [n (%)]	4 (23.5)
Number of patients without prior treatment [n (%)] <sup>†</sup>	11 (65.0)

<sup>†</sup>, treatments before study participation included: anti-CTLA-4 monoclonal antibodies, anti-PD-1 monoclonal antibodies, interleukin-2 plus pegylated-interferon alpha, B-Raf inhibitors, adoptive T-cell therapy and dendritic cell vaccination. M-stage, metastatic stage according to American Joint Committee on Cancer classification.

Ashland, USA) was used to analyze the data.

### Flowcytometric analysis

PBMC samples were thawed in 37 °C RPMI medium 1640 + GlutaMAX (Life Technologies). Samples were washed in RPMI and stained in PBS (Lonza Bioscience) containing 0.5% bovine serum albumin. Regulatory T-cells cells were stained with the following antibodies: CD127-FITC, FoxP3-PE or IG2a-PE (purchased from BD Biosciences), CD4-APC (purchased from BD Biosciences), CD25-BV421 (purchased from Biolegend) and CD3-HV500 (purchased from BD Horizon). For intracellular staining of transcription factor FoxP3, we used Foxp3/Transcription Factor Staining Buffer set (eBioscience) according to guidelines issued by the manufacturer. For phenotyping of CD3+ T-cells, the following antibodies were used: CD45RA-FITC, CCR7-PE-Cy7, CD4-APC (purchased from BD biosciences), CD27-PerCP, CD8-BV421 (purchased from Biolegend) and CD3-HV500 (purchased from BD Horizon). To evaluate myeloid-derived suppressor cells (MDSCs), the following antibodies were used: CD33-FITC, CD124-PE, CD11b-APC (purchased

from BD Pharmigen), HLA-DR-PerCP, lineage markers in the PE-Cy7 channel (CD19, CD3, CD56), CD14-BV421 (purchased from BD Biolegend) and CD15-HV500 (purchased from BD Horizon). Cells were acquired on a FACSCanto II using FACSDiva Software version 6.1.3 (BD Biosciences). Analysis of flow data was done using FlowJo software version 10 OSX (Tree Star, Inc., Ashland, USA). Phenotype data was additionally analyzed using SPICE (Bioinformatics and Computational Biosciences Branch, Rockville, USA).

### Statistical analysis

Statistical calculations were performed using either GraphPad Prism (GraphPad Software, La Jolla, CA, USA) or R (a language and environment for statistical computing (R Core Team, Vienna, Austria). Groups were evaluated in intention-to-treat analysis. All tests were performed two-sided with P values below 0.05 considered significant.

## Results

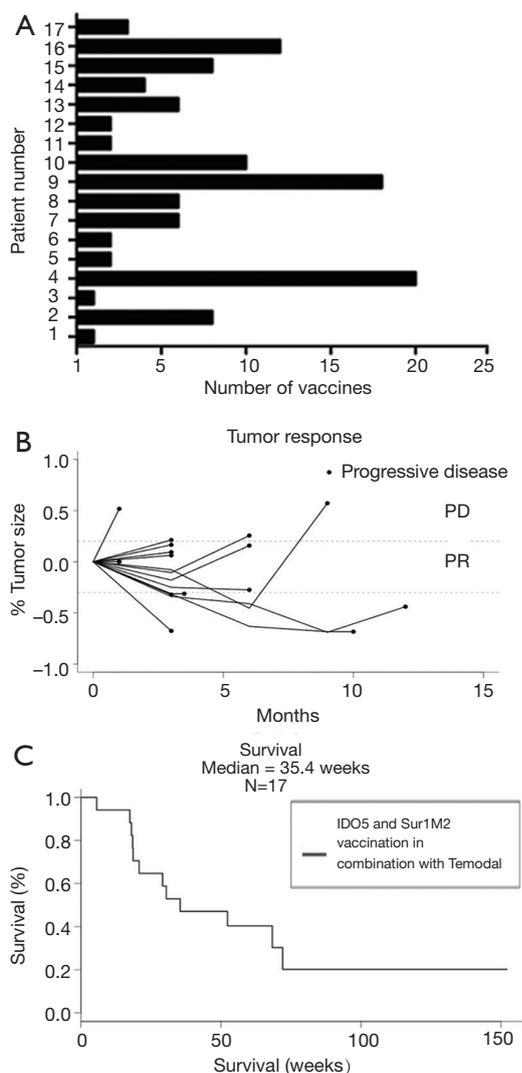
### Demography

In total, 41 patients were screened for HLA-A2 tissue type in the period from February 2012 to August 2015; 17 patients were HLA-A2<sup>+</sup> and assigned to the vaccine group. Patient characteristics are presented in *Table 1*. A total of 13 patients (73.5%) were staged as having M1c disease according to American Joint Committee on Cancer (AJCC) grading system for malignant melanoma. Screening for brain metastasis was not performed routinely at inclusion, but 8 patients (47%) had confirmed brain metastasis upon inclusion.

### Clinical beneficial rates and survival estimates

Patients were treated for a median of 56 days (range: 28–70 days) and received a median of 6 (range: 2–8) vaccines. CBR was defined as patients having CR, PR or SD for a minimum of 6 months after treatment initiation evaluated according to RECIST (*Figure 1A*). According to this definition, 3 patients (18%) experienced clinical benefit, 1 patient had a confirmed PR and 2 patients showed SD as depicted in the spider plot (*Figure 1B*). Additionally, three patients demonstrated SD lasting less than 6 months. Imaging examples of tumor regression are shown in *Figure S1*.

During treatment, 9 patients (53%) required treatment with glucocorticoids, either orally or intravenously,



**Figure 1** Clinical effect of treatment. (A) Number of vaccines received by each patient; (B) spider plot showing change in target lesion diameter generally measured according to RECIST for HLA-A2<sup>+</sup> vaccinated patients (n=15). Progressive disease markers, black dots, identify patients progressing based on scans or clinical evaluations; (C) survival plot for HLA-A2<sup>+</sup> vaccinated patients. PD, progressive disease; PR, partial response.

mainly due to symptomatic brain metastasis and they were therefore excluded from the trial. Eight additional vaccine patients (47%) discontinued treatment before first response evaluation due to rapid disease progression.

As depicted in *Figure 1C*, we have observed a median survival of 35.4 weeks.

Four patients had a survival above 150 weeks. The only

similarity among these four patients was treatment with an immune checkpoint inhibitor either prior to or post the vaccination trial. As recent publications have shown significant effect on survival when treating with immune checkpoint inhibitors, this could be the cause of their prolonged survival and it highlights the heterogeneity of the patient group.

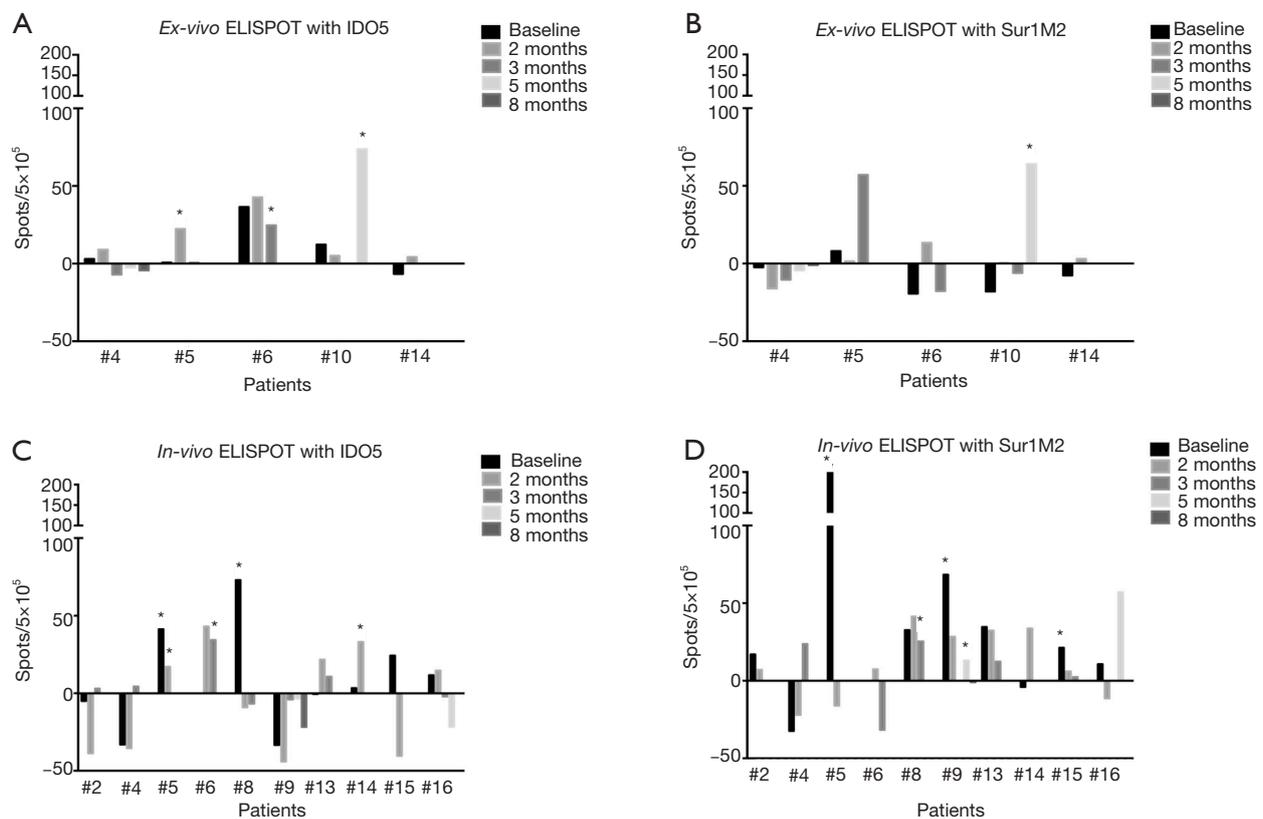
### Toxicity

Toxicity was scored in vaccinated patients. No grade 4 toxicity was observed as shown in *Table 2*. Grade 3 CTCAE toxicities were fatigue (15%), lymphopenia (15%), and leucopenia (8%), which are commonly observed during treatment with TMZ and leading to dose reduction. Nausea was observed in relation to brain metastases. Vaccine injection was associated with mild local reactions in the majority of patients due to the usage of two adjuvants. One patient developed arterial thrombosis in the left leg eventually leading to amputation. The event was assessed as unrelated to the temozolomide or vaccination. No severe local or systemic adverse events related to the vaccine were observed.

### T-cell reactivity towards vaccine peptides

It has previously been shown that vaccination with the peptides IDO5 and Sur1M2 can induce expansion of peptide specific T-cells (12,19). In addition, for Sur1M2 the reactivity was associated with a longer overall survival. In this study, we did an initial screening for IFN- $\gamma$  producing peptide specific T-cells with *ex vivo* IFN- $\gamma$  ELISPOT in blood samples from five patients, reactivity was only demonstrable in three patients. Therefore, on the remaining patients, we performed *in vitro* IFN- $\gamma$  ELISPOT to increase the sensitivity of the assay. For 12 of 17 patients, sufficient PMSCs material were available. Of these 12 patients, 9 patients remained in the study for 1st clinical evaluation point after 3 months. In total, we were able to demonstrate reactivity against IDO5 in 5 (42%) patients and reactivity against Sur1M2 in 5 (42%) patients of the 12 patients tested in both *in vitro* and *ex vivo* assays. *Ex vivo* ELISPOT responses are shown in *Figure 2A* for IDO5 and *Figure 2B* for Sur1M2 and *in vitro* ELISPOT responses are shown in *Figure 2C,D*. Of the 9 patients still included after 3 months, reactivity was demonstrated against Sur1M2 in 4 (44%) patients and against IDO5 in 3 (33%) patients.

To further investigate the vaccine response, we established cultures specific for IDO5 and Sur1M2. Cells were cultured in the presences of a log10 decreasing



**Figure 2** Enzyme-linked immunosorbent assay results. Enzyme-linked immunospot (ELISPOT) results with mean number of spots in negative control wells subtracted from stimulation wells. (A,B) *Ex vivo* results from patients #4–6, 10, and 14 after stimulation with either IDO5 or Sur1M2; (C,D) *in vitro* results from patients #2, 4, 6, 8, 9 and 13–16 after stimulation with either IDO5 or Sur1M2. \*,  $P < 0.05$  calculated using the distribution free resampling method.

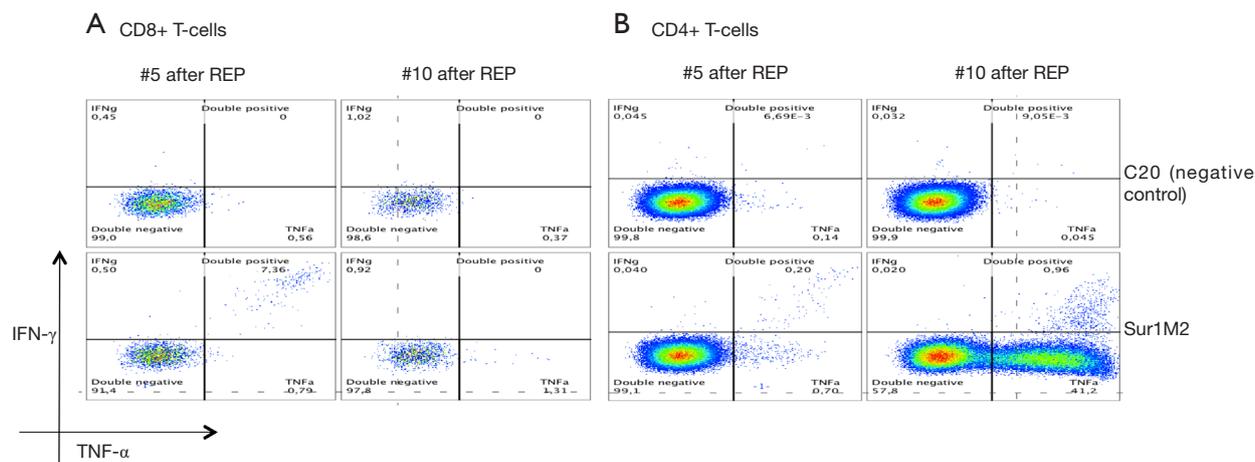
stimulation with peptides once each week for 3 weeks. Afterwards, cells were stimulated with a high concentration of peptide and cells secreting TNF- $\alpha$  were positively sorted and expanded in a REP. We were able to establish a Sur1M2 specific culture with PBMC from patients #5 and #10, as depicted in *Figure 3A,B*. Patient #5's culture demonstrated both Sur1M2 specific CD8+ and CD4+ T-cells, while patient #10's culture demonstrated a major Sur1M2 CD4+ specific population (41.2%). Hence, the modified survivin-epitope Sur1M2, used in many preclinical and clinical studies, is not only restricted to HLA-A2 but is in addition able to induce survivin-specific CD4+ T-cells. This specific CD4 response in patient #10 was further investigated and we found that such T-cells were functionally relevant as they recognize cells with intracellular expression of survivin as published elsewhere (25). We tested if vaccine responses at any time point were correlated to a prolonged survival

but we found no association, data not shown.

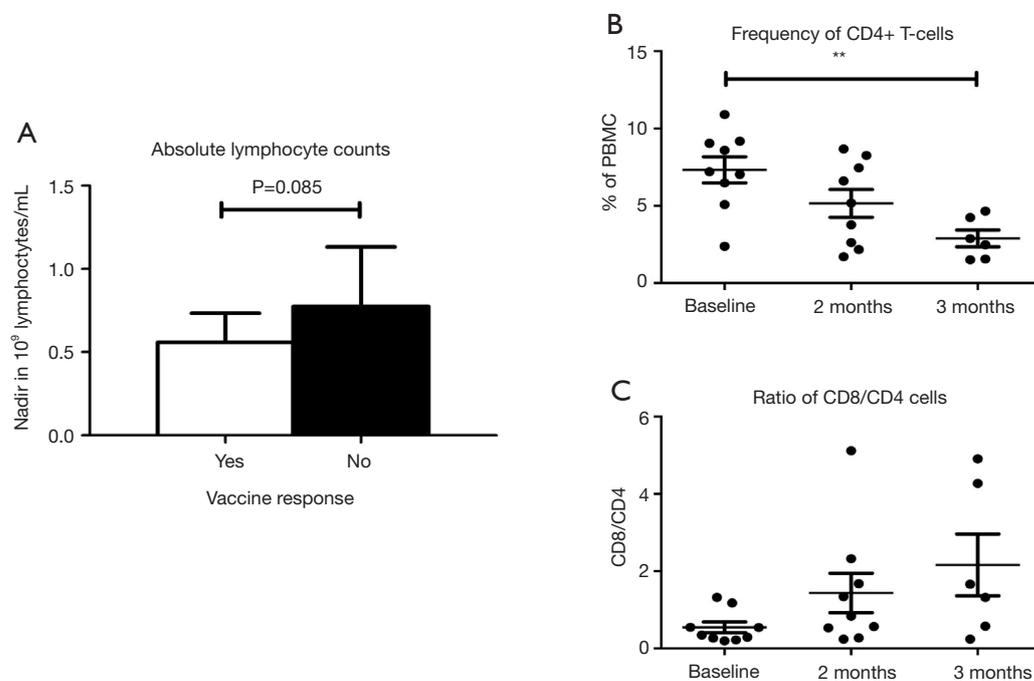
Lymphocyte counts were routinely obtained during therapy with TMZ and lymphopenia is linked to TMZ treatment. We assessed a possible correlation between TMZ-induced lymphopenia and vaccine responses by comparing lowest measured absolute lymphocyte count (ALC) in patients with or without a vaccine response and found a trend without statistical significance towards lower ALC in patients with detectable reactivity ( $P = 0.085$ ) as seen in *Figure 4*.

#### CD4+ reduction during treatment

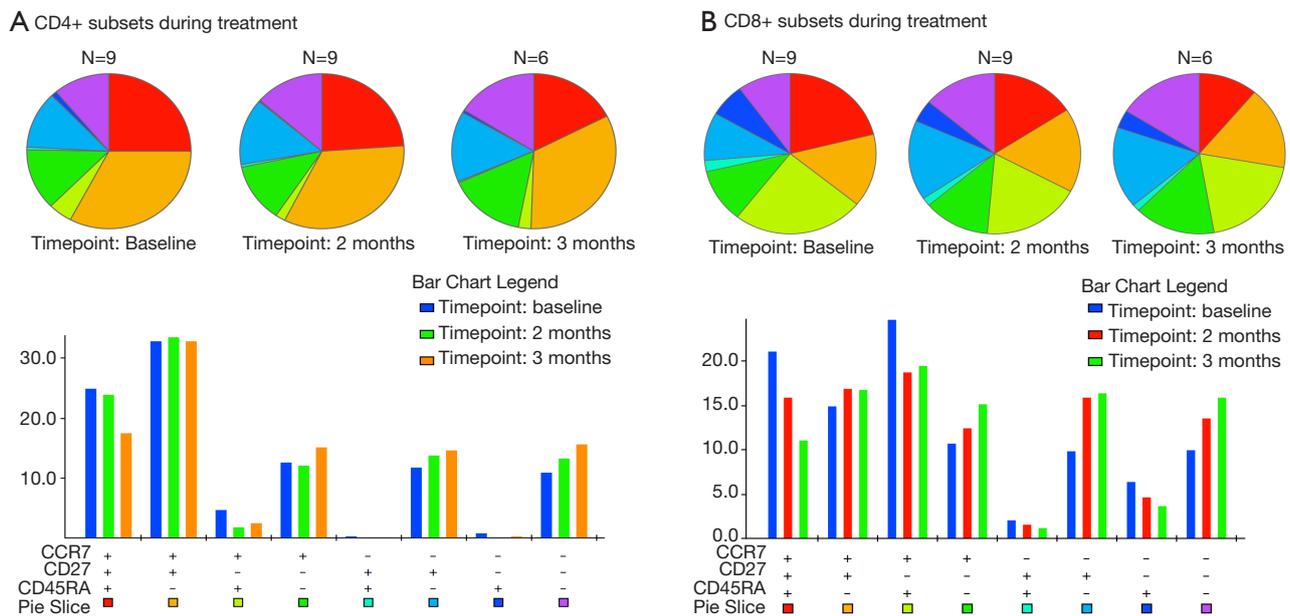
TMZ has been shown to decrease the relative number of CD4+ T-cells, increase the relative number of CD8+ T-cells and induce a shift towards a relatively more differentiated phenotype of both CD4+ and CD8+ T-cells (3). To



**Figure 3** Intracellular staining results. Lymphocytes were gated on live singlet CD3 positive cells. IFN- $\gamma$ , gamma interferon; TNF- $\alpha$ , tumor necrosis factor alpha; IDO5, indoleamine 2,3-dioxygenase derived epitopes; Sur1M2, survivin-derived modified epitopes; C20, HLA-A2 specific human immunodeficiency virus derived peptide; REP, rapid expansion protocol.



**Figure 4** Dynamics of circulating T-cells and lymphocytes during treatment. (A) Absolute lymphocyte counts tested for correlation to vaccine response with borderline significant association. Nadir refers to lowest measured lymphocyte count during treatment; changes in (B) CD4+ and (C) ratio of CD8/CD4 T-cell distribution during treatment. Flow cytometry analysis of nine patients at different time points. At baseline and after 2 months, patients #4–7, 9, 10 and 14–16 were available for analysis and after 3 months patients #4, 6, 9, 10 and 14–16 were evaluated. \*\*,  $P < 0.01$ . PBMC, peripheral blood mononuclear cells.



**Figure 5** Phenotyping of CD4+ and CD8+ T-cells. Phenotype characterization of (A) CD4+ and (B) CD8+ T-cells based on expression of CD27, CD45RA and CCR7. Results are depicted as pie charts showing all populations at three time points and as bar charts showing each population’s frequency at three time points.

**Table 2** Adverse reactions and events according to common toxicity criteria adverse events (CTCAE) version 4.0

Symptoms	Grade I+II	Grade III	Grade IV
Fatigue	77%	15%	0%
Weight loss	62%	0%	0%
Nausea	92%	0%	0%
Vomiting	31%	0%	0%
Diarrhea	15%	0%	0%
Oral mucositis	0%	0%	0%
Obstipation	46%	0%	0%
Dyspnea	46%	0%	0%
Fever	8%	0%	0%
Febrile neutropenia	0%	0%	0%
Reaction at injection	23%	0%	0%
Allergic reaction	16%	0%	0%
Neutropenia	0%	0%	0%
Thrombocytopenia	8%	0%	0%
Anemia	8%	0%	0%
Autoimmune disease	8%	0%	0%

investigate the influence of adding a vaccine to TMZ treatment, we performed flow cytometry analysis on PBMC at baseline, after 2 months of treatment and after 3 months of treatment. PBMC were gated for lymphocytes, live cells and singlets as shown in *Figure S2*. Sufficient PBMC samples were available for analysis in 9 patients, of these patients, 6 were still included in the study after 3 months. During treatment, the proportion of CD3+ cells decreased non-significantly, CD4+ T-cells showed a significant reduction ( $P=0.01$ ) after 3 months of treatment whereas CD8+ T-cells did not demonstrate any significant changes during treatment, as presented in *Figure 5*. To investigate the treatment-related dynamics of different phenotypes of CD4+ and CD8+, we assessed T-cell expression of CD27, CCR7 and CD45RA, as presented in *Figure 5A,B*. Though not significant, we observed a relative depletion in the CD27+CCR7+CD45RA+ cells ( $P=0.25$  for CD4+ and  $P=0.08$  for CD8+) and a relative induction of CD27-CCR7-CD45RA- cells ( $P=0.32$  for CD4+ and  $P=0.08$  for CD8+). CD27+CCR7+CD45RA+ cells are deemed as a naïve population, thus, during treatment there was a tendency towards decreasing frequencies of naïve CD4+ and CD8+ T-cells and increasing frequencies of memory CD4+ and CD8+ T-cells. This indicates, though without statistical significance, that the naïve subpopulation might be more

sensitive to TMZ treatment and that a drop in naïve CD4+ T-cells is the subset mostly responsible for the decrease of absolute CD4+ T-cells.

### *Changes in regulatory cells during treatment*

The frequency of regulatory T cells (Tregs) and monocytic myeloid-derived suppressor cells (moMDSCs) were assessed for treatment-associated changes as depicted in *Figure S3*. Nine patients were available for testing; of these 6 were still included after 3 months. Gating strategy is also shown in supplementary material.

Tregs defined as PBMC CD3+CD4+CD25<sup>high</sup>CD127-FoxP3+ did not change significantly during treatment, but remained at a stable level of mean 1.0–1.5% of PBMCs. MoMDSC defined as PBMCs negative for lineage markers CD3, CD19 and CD56 and HLA-DR<sup>-low</sup> CD14+CD11+CD124-CD33+ decreased in some patients during treatment but not in general.

### *Clinical effect of vaccination compared to standard care*

In an intention-to-treat manner, we compared the vaccinated HLA-A2<sup>+</sup> patients and HLA-A2<sup>-</sup> patients receiving standard temozolomide therapy at baseline and found no significant differences between the two groups, though we did observe a non-significant tendency towards a lactate dehydrogenase (LDH) above normal value in the HLA-A2<sup>-</sup> group as seen in *Table S1*. Furthermore, we compared the CBR and survival as seen in *Figure S4* and found no significant difference between the two groups.

## **Discussion**

Chemotherapy and vaccination have previously been regarded as conflicting treatment modalities. During the last decade, this hypothesis has been challenged by data showing that the immune modulating effects of chemotherapies can lead to increased immunity through e.g., inhibition of immune inhibitory cells (26). Hence, Dueland *et al.* treated metastatic melanoma patients with a peptide vaccine against telomerase in combination with TMZ (27) and reported an increased number of telomerase specific T-cell responses compared with other vaccine studies using the same peptides (28,29). In this present study, we investigated the clinical and immunological effect of treatment with an IDO5 and Sur1M2 peptide vaccine in combination with TMZ.

Our primary end point was CBR which was measured

to 18%, including one patient with confirmed PR, in the vaccination group. At the end of our study, four patients were still alive, but not receiving treatment because of disease progression. The trial was prematurely terminated due to recruitment difficulties as TMZ treatment moved from 1st or 2nd line to 2nd or later line of treatment with the approval of ipilimumab (30). As a consequence, the majority of available patients were less likely to respond to study treatment due to poor performance status or need of prednisone treatment. To evaluate the immunological effect of the vaccine, we screened patient PBMC samples using *ex vivo* and *in vitro* IFN- $\gamma$  ELISPOT assays and found responses against IDO5 in 5 (42%) of the patients tested with two responses being present at base line. Likewise, responses against Sur1m2 were detected in 5 patients (42%) with three responses being present at baseline. Previously spontaneous reactivity against IDO5 in PBMC from MM patients has been reported in 3/6 patients by *in vitro* ELISPOT assays by Sørensen *et al.* (11) and spontaneous reactivity against Sur1M2 in PBMC from MM was detected in 7/14 patients by *in vitro* ELISPOT assays by Andersen *et al.* (31). The baseline reactivity is lower in this study and this could have an impact on the ability of the vaccine to induce reactivity. For vaccine efficacy comparison, Iversen *et al.* (12) demonstrated vaccine specific T-cells in all treated patients when using IDO5 as vaccine monotherapy of metastatic lung cancer while Becker *et al.* (19) have reported vaccine specific T-cells in 13/28 patients using Sur1M2 for patients with MM. Stage M1c disease was found to correlate with impaired vaccine responses in that study, which could offer an explanation to the relatively low vaccine response level in our study as 76.5% of the patients had stage M1c disease. Iversen *et al.* (12) observed long-lasting disease stabilization for NSCLC patients with a IDO5 peptide reactivity prior to vaccination with IDO5. In our study, two patients had baseline reactivity against IDO5, but it was not associated with prolonged survival.

The moderate number of patients displaying vaccine specific immune responses could also be caused by TMZ-induced reduction in PBMC sample quantity and quality.

We further characterized vaccine reactive TNF- $\alpha$  secreting T cell in two patients by sorting and expansion and were able to demonstrate Sur1M2 reactive CD8+ T-cells producing both IFN- $\gamma$  and TNF- $\alpha$  suggestive of CTLs (32). Furthermore, we detected a CD4+ T-cell population producing mostly TNF- $\alpha$  (25). The clinical effect of the CD4+ vaccine specific T-cells likely depends on the functional subtype. In MM CD4+ Treg and Th17 have been

described to correlate to a poor prognosis (33,34). Th1 and Th2 CD4+ T-cells has, however, been described to possess anti-tumor effect either directly or indirectly by attraction or activation of other immune cells (35,36).

In a recent study (3), it was described that TMZ treatment induced a significant decrease in circulating CD4+ T-cells, a relative decrease of naïve T-cells and a relative increase in differentiated T-cells. These findings were confirmed in the present study but due to a smaller patient cohort the changes did not meet the level of statistical significance.

In the study by Iversen *et al.* (3), reactivity towards several known TAA were assessed before and after TMZ treatment. It showed that frequencies of CD8+ TAA specific T-cells either stabilized or increased after TMZ treatment and in some patients TMZ treatment even revealed TAA specific T-cells, which were not detectable before treatment. Here we assessed whether the lymphodepleting effects of TMZ could influence induction of vaccine specific responses and found a borderline significant association between reduced ALC and vaccine response.

As IDO expression can induce Tregs (9) and TMZ can reduce Treg levels (3), we hypothesized that vaccinating against IDO in combination with TMZ would have a synergistic effect on the Treg frequency in the blood. Such an effect would be interesting as it might contribute to an augmented vaccine efficacy and may affect disease prognosis (37). However, we observed no significant change in Treg frequencies during therapy. In some patients, we observed a fall in the frequency of moMDSC during treatment. The two patients, #5 and #10, with the largest relative fall in frequency of moMDSC also showed reactivity against IDO5. In another recent study from our group, patients were treated with Ipilimumab and a long peptide vaccine against IDO. Here, a significant fall in moMDSC frequency was observed (38).

In summary, we safely treated 17 patients with a Sur1M2 and IDO5 peptide vaccine in combination with TMZ. We were able to induce vaccine specific immunity in a significant number of patients; however, demonstration of clinical benefit was hampered by early and rapid disease progression including progressive brain metastases leading to need of high dose prednisone.

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## Footnote

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

**Ethical Statement:** The study was approved by Danish Health and Medicines Agency and the local Ethics Committee, Capital Region of Denmark (No. H-1-2011-136).

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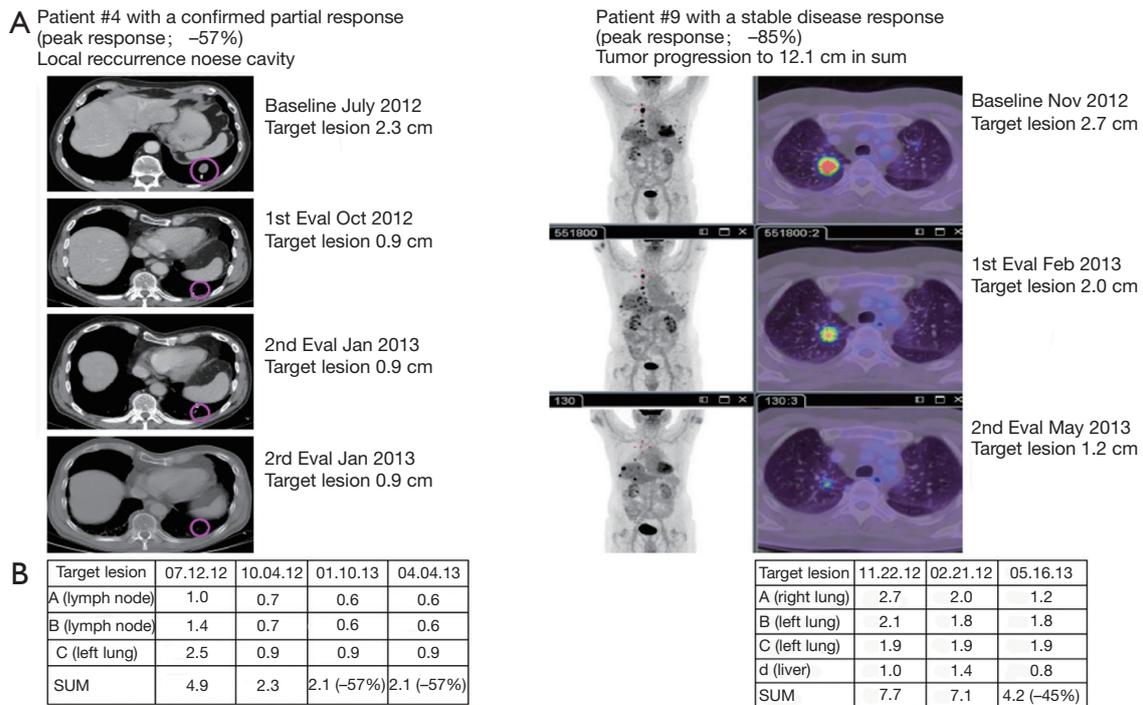
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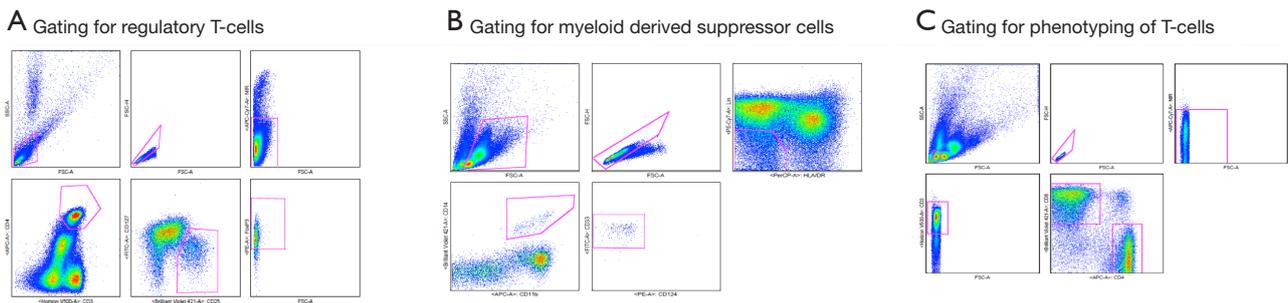
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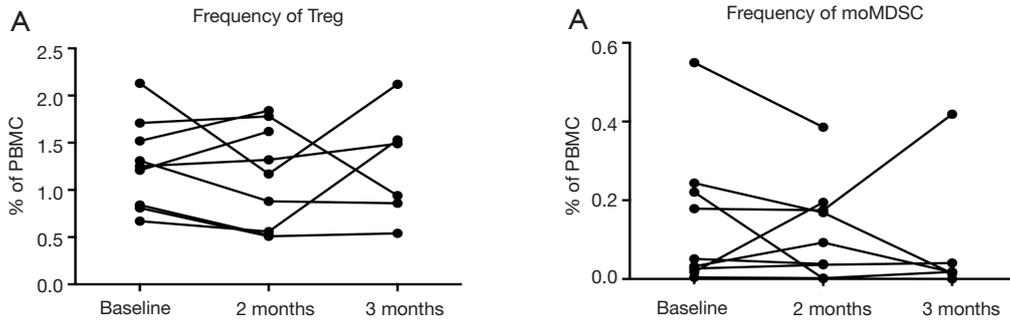
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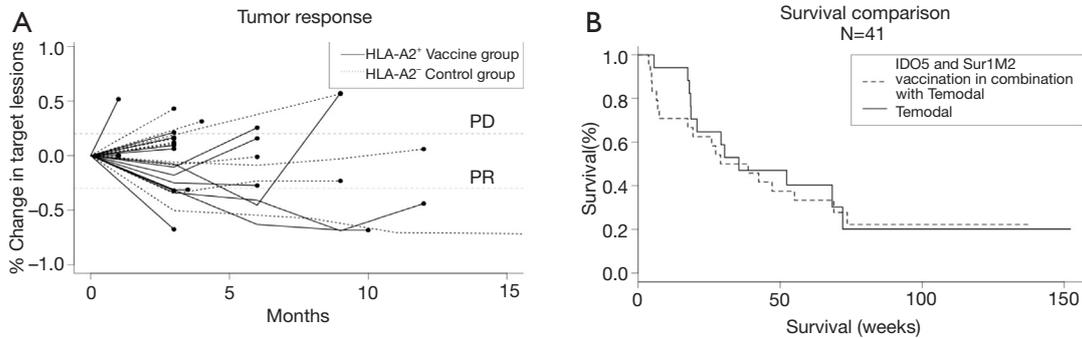
**Figure S1** Tumor responses to vaccine treatment in patients with metastatic malignant melanoma. (A) Examples of tumor responses on PET-CT with images from patients #4 and #9; (B) examples of tumor size responses from target lesions and as sums from patients #4 and #9.



**Figure S2** Examples of flow cytometric gating strategies. (A) Gating strategy for regulatory T-cells. Gated for lymphocytes (FSC-A, SSC-A), singlets cells (FSC-A, FSC-H), live cells (FSC-A, APC-Cy7A: NIR), CD3 and CD4 positive cells (Horizon V500-A: CD3, APC-A: CD4), then gated for CD25 positive CD127 low cells (Brilliant Violet 421-1: CD25, RTC-A: CD127) and finally FoxP3 positive cells (FSC-A, PE-A: FoxP3); (B) gating strategy for myeloid-derived suppressor cells. Gated for lymphocytes and monocytes (FSC-A, SSC-A), singlet cells (FSC-A, FSC-H), then lineage and HLA-DR negative cells (PerCP-A: HLA-DR, PE-Cy7-A: Lin), where lineage included CD19, CD3 and CD56, then gated for CD14 and CD11b positive cells (APC-A: CD11b, Brilliant violet 421-A: CD14) and finally CD33 positive and CD124 negative (PE-A: CD124, FITC-A: CD33); (C) gating strategy for phenotyping of T-cells. Gated for lymphocytes (FSC-A, SSC-A), singlets cells (FSC-A, FSC-H), live cells (FSC-A, APC-Cy7A: NIR), CD3 positive cells (FSC-A, Horizon V500-A: CD3) and then CD8 and CD4 positive cells were gated separately (APC-A: CD4, Brilliant Violet 421-A: CD8) and further analyzed.



**Figure S3** Flow cytometry analysis of moMDSC and Treg during treatment. (A) Treg cells were gated first on lymphocytes, live cells, CD3+CD4+ cells, CD125 low, CD27 high and FoxP3 expression; (B) moMDSC were gated on lymphocytes plus monocytes, negative for HLA-DR and lineage markers (CD19 for B-cells, CD3 for T-cells and CD56 for NK-cells), positive for CD14 and CD11b, CD33 positive and CD124 negative. At baseline and after 3 months, patients #4–7, 9, 10 and 14–16 were available for analysis and after 3 months patient #4, 6, 9, 10 and 14–16 were evaluated. MoMDSC, monocytic myeloid-derived suppressor cells; Treg, regulatory T-cells.



**Figure S4** Clinical comparison between HLA-A2<sup>+</sup> vaccination group and HLA-A2<sup>-</sup> control group. (A) Spider plot showing change in diameter of target lesions generally measured according to RECIST for HLA-A2<sup>+</sup> vaccinated patients (n=15) and HLA-A2<sup>-</sup> patients (n=9). Progressive disease markers, black dots, identify patients progressing based on scans or clinical evaluations; (B) survival curve comparing survival for HLA-A2<sup>+</sup> vaccinated patients and HLA-A2<sup>-</sup> control group. PD, progressive disease; PR, partial response.

**Table S1** Clinical comparison of control and treatment group at baseline

Characteristics	Temozolomide (n=24)	Temozolomide plus vaccine (n=17)	P value	Test
Age at baseline (mean) (years)	66.5	64.7	0.65	–
M-stage [n (%)]			0.67	Exact
M1a	1 (4.2)	0 (0.0)		
M1b	3 (12.5)	4 (23.5)		
M1c	20 (83.3)	13 (76.5)		
Brain metastasis [n (%)]	8 (33.3)	8 (47.1)	0.57	–
Sex (male) [n (%)]	10 (41.7)	9 (52.9)	0.69	–
Lactate dehydrogenase above normal [n (%)]	14 (58.3)	4 (23.5)	0.05	Exact
Number of patients without prior treatment [n (%)] <sup>†</sup>	16 (67.0)	11 (65.0)	1.00	–

<sup>†</sup>, treatments before study participation included: anti-CTLA-4 monoclonal antibodies, anti-PD-1 monoclonal antibodies, interleukin-2 plus pegylated-interferon alpha, B-Raf inhibitors, adoptive T-cell therapy and dendritic cell vaccination. M-stage, metastatic stage according to American Joint Committee on Cancer classification.