Author's response to reviews

Title: Alpha-type-1 polarized dendritic cell-based vaccination in recurrent high-grade glioma: A phase 1 clinical trial

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Author's response to reviews: see over
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Prof Adam Dicker  
Associate Editor  
BMC Cancer  
Biomedical Central

Dear Prof. Dicker,

We are pleased to re-submit our paper (Manuscript MS:1126226850792141) entitled “Alpha-type-1 polarized dendritic cell-based vaccination in recurrent high-grade glioma: A phase I clinical trial”. We thank the reviewers for their valuable comments and criticisms. We have revised the manuscript and addressed all concerns point-by-point (revised parts are underlined).

Referee 1

Major issues

Q1. It is unclear how “Alpha-type-1 DCs” are defined, manufactured and validated.

Answer. We carelessly forgot to describe the addition of cytokines other than GM-CSF and IL-4. We apologize for the inconvenience. The “alpha-type-1 DC” production protocol is concisely described below. Kalinski’s group reported that alpha-type-1 DCs were produced using a combination of TNF-α, IL-1β, IFNα, IFNγ and PolyI/C with GM-CSF and IL-4 and showed a large amount of IL-12p70 compared to conventional DCs.

1. Enrichment of monocyte using OptiPrep-based centrifugation
2. Culture of enriched monocytes in the presence of GM-CSF and IL-4 on day1
3. On day6, TNF-α, IL-1β, IFNα, IFNγ and PolyI/C are added to the culture
4. On day8, cells are harvested, and pulsed with a cocktail of 5 synthetic peptides restricted to HLA A2 or A24 and KLH
5. Finally, DC-enriched cells are washed and cryopreserved in Cryocyte bags

Kalinski’s group reported that alpha-type-1 DCs were produced using a combination of TNF-α, IL-1β, IFNα, IFNγ and PolyI/C with GM-CSF and IL-4 and showed a large amount of IL-12p70 compared to conventional DCs. We compared our method to other DC production methods using IL-12 production and CTL induction assays as shown below. Preliminary results suggest that
alpha-type-1 DCs should have a significantly greater capacity for IL-12 production and CTL induction than DCs produced with other combinations of cytokines.

**Data-1**

IL-12 production from DC stimulated with various cytokine cocktails

- J558 (-) (open bars)
- J558 (+) (closed bars)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cytokine cocktails</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>GM-CSF+IL-4</td>
</tr>
<tr>
<td>G2</td>
<td>GM-CSF+IL-4+TNF-α</td>
</tr>
<tr>
<td>G3</td>
<td>GM-CSF+IL-4+TNF-α +IL-1β+IL-6+PGE2</td>
</tr>
<tr>
<td>G4</td>
<td>GM-CSF+IL-4+TNF-α +IL-1β+IFN-α+IFN-γ+PolyI:C</td>
</tr>
</tbody>
</table>

**Data-2**

Impact of α-type1 polarized DC on CTL induction from melanoma patient PBLs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>A24-MAGE1</td>
<td>G1: GM-CSF+IL-4</td>
</tr>
<tr>
<td></td>
<td>G2: GM-CSF+IL-4+TNF-α</td>
</tr>
<tr>
<td></td>
<td>G3: GM-CSF+IL-4+TNF-α +IL-1β+IL-6+PGE2</td>
</tr>
<tr>
<td></td>
<td>G4: GM-CSF+IL-4+TNF-α +IL-1β+IFN-α+IFN-γ+PolyI:C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>A24-Tyrosinase</td>
<td>G1: GM-CSF+IL-4</td>
</tr>
<tr>
<td></td>
<td>G2: GM-CSF+IL-4+TNF-α</td>
</tr>
<tr>
<td></td>
<td>G3: GM-CSF+IL-4+TNF-α +IL-1β+IL-6+PGE2</td>
</tr>
<tr>
<td></td>
<td>G4: GM-CSF+IL-4+TNF-α +IL-1β+IFN-α+IFN-γ+PolyI:C</td>
</tr>
</tbody>
</table>

We added sentences regarding the use of additional cytokines as follows:

Page 7 lines 4-8 in Materials and methods

On day 6, cells were activated by the addition of TNF-α at 10 ng/ml (CellGenix).
IL-1β at 10 ng/ml (CellGenix), IFN-α at 3000 U/ml (Dainippon Sumitomo Pharma Co. Ltd, Osaka, Japan), IFN-γ at 1000 U/ml (Shionogi & Co. Ltd, Osaka, Japan), and poly I/C at 20 µg/ml (Amersham Biosciences Corp., Piscataway, NJ). On day 8, harvested cells

Q2. The manuscript needs extensive linguistic revisions.

Answer. English proofreading by a native speaker was performed prior to submission of the revised manuscript.

Q3. It is unclear how the criteria for positive response were set for immunological assay, such as ELISPOT.

Answer. The ELISPOT assay was performed using PBMCs drawn prior to vaccination and after 4 DC injections. The spot number per well of peptide-stimulated CTLs was compared to that of a negative well without peptide using Student’s paired two-tailed t-test, and a significant increase was found. We revised Fig.2 and indicated statistically significant results. The vertical line shows the spot number per well. We added the following:

Page 10 lines 8-9 in Materials and methods
The ELISPOT assay was performed using PBMCs drawn prior to vaccination and after 4 DC injections.

Page 10 lines 18-20 in Materials and methods
The spot number per well of peptide-stimulated CTLs was compared to that of a negative well without peptide using Student’s paired two-tailed t-test.

Page 27 lines 15-18 in Figure legends
The spot number per well of peptide-stimulated CTLs was compared to that of a negative well without peptide using Student’s paired two-tailed t-test. Statistically significant compared to no peptide, *, P < 0.05, **, P < 0.01.

As to the analysis of the Th1/Th2 balance, PBMCs (CD4+ T cells) drawn prior to vaccination were stimulated with PMA and ionomycin, and stained with FITC-anti-CD4 MoAb, and subsequently intracellular staining using PE-labeled anti-IFN-γ or anti-IL-4 MoAb was performed. Finally, the ratio of Th1 (IFN-γ+) and Th2 (IL-4+) was calculated, and the balance was evaluated.

Q4. The authors can revise to talk about high-grade gliomas in general with some
emphasis on GBM.

*Answer.* We have changed GBM to high-grade gliomas or high-grade gliomas including GBM.

Page4 line2 in Background
High-grade gliomas including glioblastoma multiforme (GBM)

Page4 lines7, 12 and 14 in Background, Page15 line4 in Discussion high-grade gliomas

Q5 There is no clear criteria determining DC1 vs DC2.

*Answer.* A description regarding the ratio of DC1 and DC2 was added as follows;

Page7 lines15-20 in Materials and methods

The frequencies of the DC-related markers were determined using various antibodies for CD1a, CD11c, CD33, CD40, CD54, CD80, CD86, CD123, CD205, CD207, CMRF44, CMRF56, E-cadherin CCR7, HLA-class I, and HLA-DR (BD Biosciences). The ratio of DC1 (CD11c^+HLA-DR^+) and DC2 (CD123^+HLA-DR^+) was calculated using a flow cytometric analysis reported by Ferrari et al [21].

Q6 Which patient demonstrated liver toxicity?

*Answer.* Patient No.4 (anaplastic astrocytoma grade III) showed a mild elevation of hepatic enzyme (grade 2) after 4 DC injections. DC vaccinations ceased after 5 injections owing to tumor progression, and liver dysfunction disappeared.

Q7 Eligibility-was the use of corticosteroid allowed?

*Answer.* Minimum doses of corticosteroid (dexamethasone up to 1 mg/day) were permitted for patients with neurological deficits due to mass effects by the lesions. We added the following sentence;

Page6 lines5-7 in Materials and methods

Minimum doses of corticosteroid (dexamethasone up to 1 mg/day) were permitted for patients with neurological deficits due to mass effects by the lesions.

Q8 Some conditions in the exclusion criteria do not seem to belong there.

*Answer.* Exclusion criterium i) was deleted to avoid confusion. Anaphylaxis to
synthetic peptides should be checked in the first skin test prior to DC vaccination. If an anaphylaxis reaction occurs, the registration should be excluded.

Q9 The method section for DC preparation does not include the use of additional cytokines

Answer. We apologize for any inconvenience. We added sentences regarding the use of additional cytokines in the Materials and methods as follows;

Page7 lines4-8 in Materials and methods
On day6, cells were activated by the addition of TNF-α at 10 ng/ml (CellGenix), IL-1β at 10 ng/ml (CellGenix), IFN-α at 3000 U/ml (Dainippon Sumitomo Pharma Co. Ltd, Osaka, Japan), IFN-γ at 1000 U/ml (Shionogi & Co. Ltd, Osaka, Japan), and poly I/C at 20 µg/ml (Amersham Biosciences Corp., Piscataway, NJ). On day8, harvested cells

Q10 Immunohistochemistry for the antigens is not convincing. Pictures with higher magnification should be provided.

Answer. We provided a revised picture (Fig.1) with higher magnification (x400), indicating that tumor cells, not infiltrating stroma and immune cells, are positively stained with anti-cancer antigen antibodies.

Q11 It is not clear why “recovery rate” is important. A better discussion should be provided.

Answer. Previously, we reported a phase II study of a DC vaccine against HLA-A24+ metastatic melanoma, and demonstrated that the number of DC injections was significantly associated with a good prognosis (Oshita et al., Oncol Rep, 2012). Specific data (Table 4) cited from the paper are shown below.
This result suggests the total dose of qualified DCs to be a key factor to a successful vaccination. The longer patients are given DC vaccines, the better their prognosis will be. Therefore, the yield of DCs, namely the recovery rate from a leukapheresis product, would be important. We added the following sentences to the discussion.

Page16 lines13-18 in Discussion
Previously, we reported a phase II trial of a DC vaccine against HLA-A24+ metastatic melanoma, and demonstrated that the number of DC injections was significantly associated with the prognosis [35]. This result suggests the total dose of qualified DCs to be a key factor to a successful vaccination. The longer patients are given DC vaccines, the better their prognosis will be. Therefore, the yield of DCs, namely the recovery rate from a leukapheresis product, is important.

Referee 2

Minor issues

Q1 It is unclear how the authors choose the tumor antigens for this trial.

Answer. We chose 5 tumor antigen (MAGE-A1, MAGE-A3, WT-1, HER2,
gp100)-derived peptides in the present study. MAGE-A1, MAGE-A3, and WT-1 have been reported to be expressed frequently in glioblastoma tissue by several researchers. Additionally, our group had already utilized MAGE-A1 and MAGE-A3 peptides in a clinical study of a DC vaccine against metastatic melanoma, and confirmed positive immunological responses. As to HER2 and gp100, our mRNA expression analysis using real-time PCR in glioblastoma cell lines revealed high and frequent expression.

I hope that the revised manuscript is now suitable for publication, and am looking forward to receiving your response.

Please send all correspondence to:

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