**Author's response to reviews**

**Title:** Profound tumor-specific Th2 bias in patients with malignant glioma

**Authors:**

Shinji Shimato (shinji.shimato@gmail.com)  
Lisa M Maier (lisa.m.maier@googlemail.com)  
Richard Maier (richardmaier@chello.at)  
Jeffrey N Bruce (jnrb@columbia.edu)  
Richard CE Anderson (rca24@columbia.edu)  
David E Anderson (deanderson.phd@gmail.com)

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**Author's response to reviews:** see over
Reviewer 1: Dr. Silvano Ferrini

Minor essential revisions
1) **The HLA class II restriction profile of the peptides used should be indicated along with the HLA class II haplotype of the subjects included in the study. Otherwise, where the peptides promiscuous to multiple HLA-Class II alleles? In any case this aspect should be clearly defined.**

In contrast to binding of HLA class I restricted peptides, class II-restricted peptides are typically far more promiscuous in their binding to HLA class II alleles. That said, we used the ProPred HLA class II binding algorithm to predict which alleles were capable of binding the peptides used in our experiments. This new data is presented in table 1, and new narrative is found on page 6 in the revised manuscript.

2) **Clinical characteristics of the patients should be provided in a summary table.**

We have expanded on the clinical characteristics of the patients examined in this study on page 6 in the revised manuscript.

3) **The conclusion that future vaccination strategies should consider the possibility “to reverse the profound Th2 skewing” is rather obvious and is presently addressed by the use of different adjuvants in clinical trials. The authors should further comment on this point and suggest which approach they would pursue to achieve this goal (TLR agonists? Cytokines such as IL-12?).**

We agree that this point warrants further discussion and have expanded on it on page 14 in the revised manuscript.

Major Compulsory Revision
1) **The authors state that a Th2 profile has been induced by antigen stimulation on the basis of the IFN-g/IL-5 profile of secreted cytokines. This assay however is not sufficient to define a Th2 skewing. In particular, gliomas have been suggested to induce induction of Tr1 (regulatory type I) cells (Akasaki et al), which produce both IL-5 and high levels of IL-10 (reviewed in Wu et al). Therefore further efforts to characterize the Th2 or Tr1 skewing of the response should be attempted by measuring IL-10 and IL-4 (the prototypic cytokine produced by Th2 cells). In this context, the protocol used for the evaluation of cytokine secretion by Th cell lines seems not optimal. It appears that PBMC were cultured for 14 days with a single antigen pulse at the onset of cultures. Usually a second
**short-term stimulation is used before cytokine assay to avoid consumption of cytokines such as IL-4. Alternatively, attempts to assess the expression of the Th2-specific transcription factor GATA3 (mRNA or protein) in cultured T cells should be considered.**

Dr. Ferrini raises an important point that we insufficiently addressed in our manuscript. In initial studies that established measurement of cytokines in subjects with gliomas, we measured IL-10 secretion by ELISA in addition to IL-5 and IFN-γ, but failed to detect any IL-10. This was not due to technical problems with our ELISA, as secretion of IL-10 from primary GBM cell lines could readily be detected. We have added this point on page 8 in the revised manuscript. We understand that re-stimulation of T cell lines is often performed after 2 weeks of primary T cell stimulation, but were unable to do so because our IRB protocol allowed only 10mls of blood to be collected once from the subjects (at the time of surgical resection of tumor), sufficient to establish and test primary T cell line responses (as reported in our manuscript). We respectfully maintain that measurement of IFN-γ and IL-5 (now making clear no peptide-induced IL-10 was detected in our subjects) after primary (14 day) stimulation is an appropriate assay used by others (Viglietta *et al.*, reference 36), and that the ratios derived from measurement of these cytokines are a valid means of assessing the Th1/2 ratios in our subjects, having been used to assess the Th1/2 balance in a clinical trial of subjects with metastatic melanoma (Green *et al.*, reference 16). On page 8 of the revised manuscript, we now make reference to two papers that make clear that GATA-3 binds to the human IL-5 gene and that in the early stages of human Th2 cell differentiation, there is a positive correlation between GATA-3, IL-5, and IL-4 gene expression kinetics.

**Reviewer 2: Dr. Kathrin Bauer**

**Major Compulsory Revisions**

1) *Why did the authors use PBMCs for stimulation and did not further isolate T cells, B cells and monocytes? This should be discussed. A stimulation of 14 days is too short to talk about T cell lines, particularly without phenotypic characterization of the cells, for example by FACS analysis.*

Our IRB protocol allowed us to collect on 10mls of blood from our subjects, which precluded isolation of individual cell types from the limited number of PBMCs obtained. As suggested, we have eliminated reference to “T cell lines” in the revised manuscript.

2) *The manuscript would clearly benefit from an additional ex vivo analysis of day 0 T cells to exhibit that the analyzed epitopes are relevant in the GBM setting and to reveal that the same bias towards*
Th2 responses can be detected regarding memory T cells.

Dr. Bauer raises an excellent point that we now aim to address with new data in the form of Figure 2. To verify that existing (memory) T cell responses exist in patients that react to our candidate GBM-associated peptides, we use a traditional IFN-γ Elispot assay that involves culture of PBMCs with peptides for only 48 hours. As demonstrated in Figure 2, responses could clearly be detected in this short period of time, demonstrating that detectable memory T cell responses can be measured ex vivo.

Minor Essential Revisions

1) In the section “Responses to HLA Class II-restricted peptide stimulation” on page 10, the significant p-value is given as 0.5. This should be corrected.

This correction has been made in the revised manuscript.

2) One aim of the study was to detect the CD4-positive T cells against the analyzed antigens in the peripheral blood of glioma patients. This should be included in the results part of the abstract. It would be helpful to mention the selection criteria for the analyzed peptides already in the Methods section (page 6).

We have revised the abstract to address this point in the revised manuscript.

3) Throughout the manuscript the authors are talking of glioma patients, however, only GBM patients have been included in the study. This should be clearly stated in the figures.

We have addressed this issue in revised figures, associated legends, and throughout the text.

4) The authors should explain why they added amino acid substitutions in the analyzed peptides and discuss the limitations that follow from these modifications.

We have more fully addressed the amino acid substitutions in the analyzed peptides, rationale, and implications on page 10 of the revised manuscript.

5) The authors should explain why they used the geometric mean not only for comparison of IFN-gamma/IL-5 ratios but also for
comparison of cytokine levels.
We discuss this issue on page 8 of the revised manuscript.

6) The differences between healthy subjects and glioma patients regarding the cut-off definition (page 10) should be explained. What was the cut-off for meningioma patients?

We define a positive T cell response for each patient as the amounts of IFN-γ or IL-5 that were > 50 pg/mL and two standard deviations above the mean cytokine levels secreted after stimulation of cells from that patient with negative control MBP peptide. The cut-off cytokine values differed quantitatively for each patients based on their “background” response to the MBP peptide (reflecting in part their state of activation when isolated, health of the cells, etc.) This was true for meningioma patients as well, though because so few responses were detected relative to the other patient cohorts, we have not emphasized the peptide-specific responses in these patients in the manuscript. Nevertheless, the limited number of responses detected according to the criteria above are now summarized in revised Table 2.

7) Meningioma patients should also be listed in table 2.

We now include data for patients with meningiomas in revised table 2.

8) The discussion and conclusion of the data are a little bit unbalanced and the conclusion that “glioma patients are unlikely to have the capacity of favourably respond to immunization against tumor antigens ....” is too general and is, stated like this, not supported by the data. These paragraphs should be adapted.

We have added additional discussion to our concluding statements on page 14, indicating that we believe co-formulation of a peptide-based vaccine such as ours with TLR agonists will likely be needed to both induce a strong Th1 response as well as attenuate pre-existing regulatory T cells known to exist in GBM patients and hamper efficacious tumor-specific immunity.