Author's response to reviews

Title: Transcriptional profiling provides insights into metronomic cyclophosphamide-activated, innate immune-dependent regression of brain tumor xenografts

Authors:

Joshua C Doloff (djosh@bu.edu)
David J Waxman (djw@bu.edu)

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**Doloff and Waxman:** BMC Cancer manuscript, MS: 6731681211504137
“Transcriptional profiling provides insights into metronomic cyclophosphamide-activated, innate immune-dependent regression of brain tumor xenografts”

**Author Responses to Reviewer Comments:**

**Reviewer #1**

Reviewer general comments: …The identification of responsive cytokines, chemokine and immune regulatory genes linked to the immune response, as well as several immunosuppressive factors that may contribute to tumor escape, is very important for a successful implementation of this work into clinic. Very few scientists and even fewer clinicians are familiar with the many varied mechanisms in which metronomic chemotherapy works and this study sets out numerous pathways for future investigations. Authors rightfully suggest that the work may lead to discovery of biomarkers to evaluate therapeutic efficacy and to optimization of treatment schedules. The paper is well written, and the analysis is thorough. I would recommend it for publication. **Response:** We thank Reviewer 1 for the strong positive review of our study.

**Reviewer #2**

Reviewer general comments: ...This is a very interesting study, which is also very timely. Indeed, metronomic chemotherapy is gaining considerable interest in the clinic, having demonstrated its efficacy in a variety of human tumours. In-depth mechanistic studies are now warranted to unravel the molecular factors involved in its anti-tumour effects and ultimately identify patients that are most likely to respond to this type of treatment.

This study has two major weaknesses. The first one is the use of heterotopic implantation of tumours. Indeed, the focus of the study is on the tumour micro-environment and especially anti-tumour immune response. Therefore, orthotopic (i.e intracranial) implantation of tumour cells would have been much more appropriate to investigate the impact of treatment on local immune response. Having said that, the authors have previously published several articles using this sub-cutaneous model of glioma and have characterized its response to metronomic cyclophosphamide. This allowed them to correlate the changes in gene expression observed in the present study, with tumour regression and immune system activation previously reported in the same model. The second weakness of the study is that it is purely descriptive and crucially lacks functional validation. The authors describe at great length the 1,447 human and 614 mouse genes whose expression was significantly altered as a result of metronomic cyclophosphamide treatment, alongside their associated pathways and upstream regulators identified by bioinformatics analysis. However, none of these changes in gene expression and activation/inhibition of upstream regulators are confirmed at the protein level (except for some rare, retrospective
validation like for the NKG2D activating ligand MICB). Furthermore, none of these changes have been functionally validated either by RNA interference or using pharmacological inhibitors/activators. Key molecular events such as activation of the interferon pathway, increase in CXCL9-11 cytokines or inhibition of MAPK1 and ERK1/2 could be quite easily validated by ELISA or western blotting. This would considerably strengthen this study. Despite these obvious limitations, this study provides numerous insights into the mechanism of anti-tumour immune response induced by metronomic cyclophosphamide.

Response: While we have not carried out functional analysis on the very large number of dysregulated genes and pathways identified in this study, we have previously reported results from several functional studies, including antibody-based immune cell depletion, as well as transgenic knock-out models, to evaluate the roles of key immune cell players, including NK cells, CD8+ T cells, and the cytotoxic effector protein Perforin. Furthermore, we have also validated several key targets at the protein level by FACS and/or immunohistochemistry, including: NK1.1, Perforin (Prf1), Gr1/Ly6g, CD11b, CD68, and CD74, as detailed in the 3rd Discussion paragraph. To make this more clear, we have added the text “as seen both at the level of RNA and protein” (line 425).

MAJOR COMPULSORY REVISIONS

1) The title of the article should be revised to better reflect the content of the manuscript. Indeed, descriptive studies do not “elucidate” a mechanism. Elucidation requires more than correlative studies: it needs functional validation. I suggest using the wording “provides insights” instead of “elucidates”.

Response: We have changed the manuscript title as the Review suggests (line 1).

2) The structure of the discussion is somewhat tortuous and sometimes sounds like a catalogue of genes involved in anti-tumour immune response. The authors should work on their discussion to make it flow better and to avoid repetitions (e.g results obtained on MICB are briefly discussed page 16 and then again at great length page 19).

Response: We have made extensive revisions and many edits to the Discussion to make the text flow better, to eliminate redundancies, and to address comments 3 an 4, below, by the addition of two new Discussion paragraphs (lines 469-486, and lines 488-501). These changes include the following. We have removed mention of MICB on page 16, and discuss it exclusively in the later section. Furthermore, the two last sections on death factors and PPARg resulting in immune activation were moved to earlier in the Discussion, just following the section of apoptosis/cell death, and just prior to the now-final paragraph on NK cell surface ligands/receptors. As such, the Discussion now moves from microarray-validating earlier targets, establishing new targets, first at the level of immune cells, then subsequent downstream functions (cytolysis, interferon, and complement activation), and ultimately discussing factors we hypothesize originate in the metronomic cyclophosphamide-damaged tumor cells themselves, such as DNA damage/stress/death pathway activation, leading to tumor-cell presentation of immune-activating receptors. We hope that the Reviewer agrees that this streamlines the linearity
of the presentation and makes the overall Discussion more informative for the reader.

3) *It is pretty obvious that among the changes in gene expression reported in this study some will be beneficial to the anti-tumour effects of metronomic cyclophosphamide while others will be detrimental. Some might be direct effects, while others might be part of compensatory mechanisms. Although the authors presented a small selection of deleterious changes in Table 1, they failed to discuss any of those, although they did mention these detrimental changes in their overall conclusion to underline their importance. These changes and their potential therapeutic implications should be included in the discussion. For instance, has MMP13 up-regulation been previously implicated in chemo-resistance? Could it be targeted using pharmacological inhibitors and/or therapeutic antibodies to improve the efficacy of metronomic chemotherapy? Similarly for CEACAM1, IDO1…*

**Response:** We agree with the reviewer that such mechanisms, whether direct or compensatory, could potentially contribute to possible tumor cell escape or chemo-resistance. The subject of deleterious gene and upstream regulator changes is now discussed in a new Discussion paragraph (lines 488-501). However, we have not discussed in detail all of the potentially gene responses that we identified so as to not undermine our efforts to address the Reviewer’s concerns listed in comment 2, above, that the Discussion seems like a “catalogue of genes”. Based on our reading of the literature, we do not find that MMP13 is closely associated with cancer chemoresistance, certainly not resistance to CPA.

4) *Similar deleterious changes have been observed in the mouse array (e.g activation of SAMSNI, inhibition of CSF2…) and should be included in the discussion.*

**Response:** We now provide some clarifying text about these mouse responses at the end of the Results section: “By contrast, another activated upstream regulator, SAMSNI, which is highly expressed in glioblastoma, is associated with poor prognosis for survival [62]… Mouse upstream regulators inhibited by metronomic CPA in both tumor models include: CSF2 (GM-CSF), which induces differentiation of brain macrophages into M1 anti-tumor macrophages [64]…” Further, we have now provided comments about both targets and reasons why their inhibitions may or may not be relevant to the anti-tumor responses that we find. Comments about this are also included in the second new Discussion paragraph (lines 498-500).

**MINOR ESSENTIAL REVISIONS**

5) *Page 3 – the sentence “several cytokines and chemokines […] were also identified…” suggests that references #16 and #17 investigated the mechanism of action of metronomic chemotherapy, which is not the case. This should be rephrased or simply removed as it is confusing and the introduction would flow much better without. Response: We have rephrased the text to address this confusion (lines 76-78).*

6) *Page 4 – the sentences “Tumor regression is not, however, a secondary response […] rather it is a direct consequence of the mobilization of innate immune cells…” How was this demonstrated in references #20-23? This should
be stated. **Response:** We have rewritten the sentence to clarify what information is provided in Refs# 20-23 (lines 85-87).

7) Page 5 – How was the cyclophosphamide administered? Orally, by i.p, i.v injection? This should be clearly stated. **Response:** The revised text now specifies that cyclophosphamide was administered by i.p. injection (line 136).

8) Page 6 – in the sentence “Fluorescent labeling of RNA [...] in expression of host cell (mouse) RNAs” a verb is missing. This should be rephrased. **Response:** The sentence was edited to make it clearer (line 160).

9) Page 7 – The authors suggest that the low number of genes showing opposite regulation at the two time points (8) underlines the reliability of their approach. But does it really? If more genes showed such opposite regulation at the two time points, it would not necessarily mean that the microarray analysis was not significant. It could also mean that changes in gene expression are more transient and/or time-dependent. This sentence should be rephrased. **Response:** Text has been revised to address this point (line 236).

10) Out of the 1,447 genes whose expression was either up- (806) or down-regulated (641) at both treatment time points, only 19 are presented in Table 1. This suggests that only 19 of these genes could be classified as either beneficial or undesirable, which is not the case, I assume. The authors should explain the criteria that were used for this selection (e.g lowest p-value? Highest fold change?). **Response:** We did not systematically read the literature on all 1,447 genes to identify all genes whose responses to metronomic cyclophosphamide. Rather, we reviewed the literature for many of the top responsive genes (high fold-change, and low p-value, at both time points) and then listed those genes for which we felt that there was a clear consensus in the published literature of whether a gene expression change in the direction observed was either beneficial or undesirable. To make this point clear, we now specify that these represent select examples of genes whose responses are not beneficial (line 240, line 1036).

11) **The readers might not all be familiar with the IPA software. Therefore, the meaning of the different shapes (e.g circle, oval, diamond, square...) and lines (solid, broken, coloured...) should be explained in the figure legends (Figures 1 & 3).** **Response:** Sentences describing lines/arrows and shapes have been added to the Figure legends (lines 986-989; lines 1005-1008).

12) Page 13 – the sentence “the up-regulated gene cluster showing the second highest enrichment…” should be moved up to directly follow the sentence ending by “Table 4A”. **Response:** This change has been made. We thank the reviewer for making this suggestion (lines 332-337).

13) Page 20 - there is a typo in the second sentence: "distinguishing distinguish self". **Response:** This typo has been fixed (line 558).
14) Page 21 – ref #103 does not really support the statement that “CPA and other drugs that activate immunogenic cell death are increasingly being studied in combination with immunotherapies”. We and others have recently reviewed the combination of immunotherapy with metronomic chemotherapy (Andre et al., Nat Rev Clin Oncol 2014; Sheng Sow et al., Oncoimmunology; Nars et al., Int J Cancer 2013). Any of these references would be more appropriate to support this statement. **Response:** The references have been revised as suggested (line 582).

**DISCRETIONARY REVISIONS**

15) The authors evaluated the microarray probe specificity for the human and mouse genome by performing BLAT analysis. It could be interesting to undertake the same analysis with the rat genome to determine the overlap between the mouse array and the rat genome. **Response:** Unfortunately, we do not have that data available.

16) Page 19 – The authors mentioned that they have found that “CPA-damaged gliomas secrete HMGB1 protein” but this refers to unpublished experiments. Why was this not included in the present study? This is the kind of validation that would greatly enhance the significance of this work. **Response:** We have revised the text to refer to a corresponding published observation from another group, showing that CPA can induce HMGB1 release (line 525-526).