Author's response to reviews

Title: Targeting and killing of glioblastoma with activated T cells armed with bispecific antibodies

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Version: 4 Date: 30 January 2013

Author's response to reviews: see over
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Version: 3

Date: January 30, 2013

Author's response to reviews: see over
Dear Dr. Rao:

Thank you for sending the comments of the reviewers on our manuscript entitled “Targeting and killing of glioblastoma with activated T cells armed with bispecific antibodies”. Authors have carefully reviewed the comments of the reviewers and addressed all the comments/suggestions in the revised manuscript. Please find enclosed a point-by-point response to the comments and a revised manuscript according to the reviewer’s suggestions/comments. All the changes in the revised manuscript are highlighted in bold and yellow.

I appreciate the time and effort of the reviewers in providing constructive critique and suggestions to improve the manuscript. We thank them for their careful and thoughtful evaluation.

Thank you in advance for taking time to review the enclosed revised manuscript. Please find enclosed a point-by-point response to the comments of the reviewers on the next page.

Sincerely,

Sandeep Mittal
Point-by-Point Explanation of Reviewer’s Comments:

We would like to thank the reviewers for their very thoughtful and constructive comments on our manuscript. We believe that the suggested revisions have enhanced the quality of the manuscript. In the revised manuscript, the changes in response to the reviewer’s comments are highlighted in yellow.

Reviewer’s report

Title: Targeting and killing of glioblastoma with activated T cells armed with bispecific antibodies

Version: 2 Date: 20 November 2012

Reviewer: Tiffany Doucette

Reviewer’s report:

Discretionary Revisions:

Comment #1. The Introduction of the manuscript could provide a little more background and data from previous studies using armed ATC with bispecific antibodies to show effectiveness of their use in other cancer models. Response: We have expanded the introduction section to provide data from previous studies that show effectiveness of ATC armed with bispecific antibodies in other cancer models.

2. In Materials and Methods, culturing of both primary and long-term cell lines was done in “DME-F12-based medium”. Is this the same as DMEM-F12 and if so, should be changed since it is commonly written as such. Response: We thank the reviewer for pointing out our typo, we corrected DME-F12 to DMEM-F12 under both subheadings ex vivo primary glioblastoma lines and long-term glioblastoma cell lines.

3. For consistency in the figures, show statistical significance if it is performed. Response: As suggested by the reviewer, we have now shown the statistical significance in the figures as appropriate.

Minor Essential Revisions:

1. On Title Page, it appears that the first name of author Lum is incomplete. Response: We have checked the Title Page, the full name of author Lum is given in the authors list as “Lawrence G. Lum”.

2. A description of the results for HER2Bi should be included under “Optimizing the Arming Dose Bispecific Antibody” in the Results section. Response: We have now incorporated the results for HER2Bi under “Optimizing the Arming Dose of Bispecific Antibody”.


3. Any time a statement is made about results that were obtained, there should be a reference to a figure showing the results or label it as “data not shown”. This occurs multiple times in the manuscript. **Response:** We have checked the manuscript carefully and the discussion of results have now been referenced to either a figure number or labeled as “data not shown”.

4. Figure 2: Please re-label the x-axis so that the E:T ratio is clearer (i.e. 1.5:1, 3:1, etc.). **Response:** X-axis of Figure 2 has been re-labeled to 1.5:1, 3:1, 6:1 and 12:1.

5. Please provide a reference describing proliferation of cells in the presence of ATC. **Response:** Reference has been provided in the methods section under “Generation and Expansion of Activated T Cells”.

6. Under Results in the section about the CD133 cells, the word “be” should be removed from the section title. **Response:** The word “be” has been removed from the section title.

7. Under Results, “Does chemoresistance confer…..?” 4th line in the paragraph, remove the word “that” and provide a reference for the other studies showing radioresistance of armed ATC effector function. **Response:** We have removed the word “that” and provided a reference for the other studies showing radioresistance of armed ATC effector function.

8. Under Results, “Does chemoresistance confer…..?” 3rd sentence in the second paragraph should read “Radiation alone had no effect on the viability of either …….”. **Response:** The sentence has been corrected it reads “Radiation alone had no effect on the viability of either HER2Bi- and EGFRBi-armed ATC...”.

9. Under Results, “Do glioma cells exhibit suppressive or contact-dependent inhibitory effects on ATC?”, the 3rd sentence, “did not inhibit the ability of ......” to do what? Why was U188MG used only for that small test? **Response:** This sentence has been reworded, it now reads “Exposure of ATC or armed ATC to conditioned medium from normal astrocytes or gliomas did not inhibit their cytotoxic activity towards U118MG or U251MG target cells compared to the ability of control effectors that were not treated with conditioned medium (data not shown)”.

In addition to U118MG, we also tested U251MG target cells that has been added in the above sentence.

**Major Compulsory Revisions:**
1. If Supplementary Figure S1 showing MTT responses of the glioma cell lines, then it needs to be clearer. In the Results section, there is more description about other cell lines and the results when subjected to the armed ATC. It is stated that the authors have employed the 51Cr release assay in previous work with armed ATC but there is no reference or indication to show comparison of the results. If both assays are utilized in the study, then proof of validation for both should be included or at least referenced to previous work. **Response:** In Supplementary Figure S1 (upper panel), we showed a representative data of a linear correlation between OD and cell numbers of U251MG glioma cell line. This has now been clearly stated in the figure legend. In addition, we have added the figure showing the correlation between counts per
minute (CPM) and specific cytotoxicity using $^{51}$Cr release assay in U251MG glioma cell line (Figure S1, lower panel).

2. Figure 1 (top panel) is incomplete. There should be an unarmed control and the results of the other two target cell lines should be shown at least in supplementary data. How was the E:T ratio of 5:1 determined to be used? The data for the irradiated armed ATC should be shown as well since it is stated that there was “minimal effect on their activity”. Part of your goal was to show that irradiation doesn’t affect the ability of the armed ATC to target and kill glioma cells. One solution could be to make separate graphs for the MTT assay and the $^{51}$Cr assay and plot all cell lines (irradiated and radiated) on each. Also, why does the viability increase in cells treated with HER2Bi at the higher arming dose? **Response:** As suggested by the reviewer, we have now shown the data for irradiated or non-irradiated unarmed ATC (arming dose 0ng represent ATC) and armed ATC at various arming doses (5, 50 and 500ng) of EGFRBi and HER2Bi using one cell line U118MG cells at E/T of 5:1. As suggested, data has been plotted separately for the MTT and the $^{51}$Cr release assay (Figure 1A). In Figure 1B, we have now presented the data for all three cell lines using unarmed ATC or armed ATC at one arming dose of 50ng for EGFRBi, HER2Bi and CD20Bi at E/T of 5:1.

3. Figure 1 (lower panel): Authors do not comment on why there is a slight reduction in the U87MG cell line when treated with CD20Bi but actually state that it “showed no reduction of tumor cell viability”. Another statement was made to results from separate experiments with no reference or data not shown implicating where the results came from. Not sure why the last sentence in the Specific Killing of Long-Term Glioma…… Results is included. If it is believed that examining the effects of soluble antibodies on the glioma cells is important, then this should be included in the methods, a figure and the discussion. **Response:** We apologize for the misleading statement “showed no reduction of tumor cell viability”. We have corrected the results section, it now reads “CD20Bi-armed ATC (irrelevant control) showed no reduction of tumor cell viability for U118 and U251 cells but showed reduced viability (80%) for U87. CD20Bi-armed ATC mediated reduction in the viability against U87 cells could be due to the nonspecific binding of armed ATC to target cells resulting in effector target interaction induced cytotoxicity”. We have removed the statements “In separate experiments.....” and “Furthermore overnight incubation of the glioma cells with soluble antibodies...” made in the results section of “Specific Killing of Long-Term Glioma Cell Lines by Armed ATC”.

4. Figure 2: There are discrepancies in the figure legend and the results on how many experiments the data was pooled from and it is unclear about the number and use of the ATC donors. Please clarify. The figure depicts results from one ex vivo cell line showing a slight difference between killing by HER2Bi and EGFRBi. Is it known whether expression of HER2Bi was higher in these cells to account for this? **Response:** We thank the reviewer for pointing out the typographical error. We have now corrected the typo in both places—in the results section and also in the figure legends for Figure 2. In the bottom panel of Figure 2, we now show the HER2 (7.15%) and EGFR (9.99%) expression as percent positive cells in this ex-vivo cell line. Contrary to the HER2Bi mediated cytotoxicity being higher compared to EGFRBi mediated killing of target cells, expression of HER2 was slightly lower than the EGFR expression levels. HER2Bi armed ATC...
are known to kill the target cells that have only fewer HER2 receptors such as MCF-7 and SUM1315 breast cancer cell lines (Sen M, and Lum LG, J Hematother Stem Cell Res. 2001 Apr;10(2):247-60). This has now been incorporated in the introduction section.

5. Data on simultaneous use of BiAbs: The fact that primary glioma cells from only two patients were used in this experiment and this experiment wasn’t done in the three long-term glioma cell lines does not provide enough support in the conclusion that these BiAbs do have an additive effect. As the authors mentioned before, there may be varying levels of expression of HER2 and EGFR among different patients and in the long-term cell lines. The importance of the significant difference in the EGFRBi-armed cells compared to the double- or mixed single-armed ATC should not be downplayed with the limited sample size. It is important to know what the expression levels were for HER2 and EGFR in both of these primary glioma lines to see if one was substantially higher than the other. **Response:** Authors agree with the reviewer that we do not have enough support to conclude that these BiAbs do not have an additive effect. However, at lower E:T ratio (5:1), data show a significantly reduced viable cells in double- or mixed single-armed ATC compared to individually EGFRBi- or HER2Bi-armed ATC (Figure 3), suggesting some additive killing effect by dual antigen targeting. We have removed the sentence “Thus, we observed neither additivity nor synergy in killing when simultaneously targeting two antigens on the glioma cells” from the results section of “Does the Simultaneous Use of Two BiAbs to Arm ATC Improve Killing Glioma Targets”.

6. Figure 4: Explain why the E:T ratio of (3:1) was used instead of 5:1 used in the previous experiments. Also, where is the data on the effect of EGFRBi armed ATC on these cells? **Response:** We repeated the experiment from two primary cell lines to include the EGFRBi data and the E:T ratio of 5:1 (Figure 4, upper panel). In the bottom panel of Figure 4, we show the expression of HER2 and EGFR in CD133 enriched population from two primary glioma cells.

7. Figure 5: Again, explain the use of a different E:T ratio (this time 10:1). How many times was this experiment repeated? A cell line not resistant to TMZ should have been used as a control. Were the results similar in the other cell types including primary glioma cell lines? For consistency, please show significance symbols on the graph. It needs to be clearer about whether you are comparing treatments to unarmed effects or treatments to each group’s Rad-TMZ- column when you are reporting your results in the results section. The authors also suggest that TMZ may be potentially toxic to ATC. Was a viability assay done on unarmed ATC with or without treatment of TMZ to show this? **Response:** We did use the lower ratio of 5:1 in this experiment, however, we noticed that irradiated or TMZ treated armed ATC were at this ratio did not show significant difference in killing and therefore we have shown the data at 10:1 ratio, and experiment was repeated three times. The purpose of this experiment was to show a) whether armed ATC can target TMZ resistant cells, b) Since TMZ and radiation are generally given after surgery, we want to know whether armed ATC could kill glioma cells under comparable in vitro conditions by using either TMZ treated ATC or irradiated ATC or both. TMZ treated ATC or irradiated ATC showed effective killing comparable to long term or primary cell lines. As suggested by the reviewer, we have shown the significance symbols on the graph
We did the viability test on armed or unarmed ATC with or without TMZ treatment, in TMZ treated ATC or aATC the viability was reduced by 15-20%.

8. Supplementary Figure S2: Why was only HER2Bi tested and data shown for only one cell line? Authors state that co-culture with glioma cells did not suppress the ability of armed ATC to kill glioma cells but do not show the data or reference “data not shown”. No statistics are shown for this figure. Additionally, it appears that is some reduction in killing of HER2Bi/U251 cells after first contact with SKBR3 even though the authors state that there was no reduction. **Response:** We have now mentioned “data not shown” for the experiment where the ATC or aATC were incubated with the conditioned medium followed by co-culture with glioma cells. Since we used SK-BR-3 (expresses high levels of HER2 receptor) as a positive control in a cell-cell contact dependent experiment, we chose to use HER2Bi. We acknowledge that there is some reduction in 2nd killing, we have mentioned this in the relevant results section under “Do Glioma Cells Exhibit Suppressive or Contact-Dependent Inhibitory Effects on ATC”.

9. Figure 6: Again no explanation on difference in E:T ratio used. The authors only show data for EGFRBi-armed ATC and not HER2Bi-armed ATC. Only one cell line used and no explanation for why. No statistics shown for the results. **Response:** Since in all previous experiments and published data, we used 24h co-culture at 25:1 E/T ratio, we used the same ratio here with glioma cell lines in order to see whether cytokine profile is consistent with previous studies. We did use all three cell lines with EGFRBi armed ATC but showed a representative data for U251 in Figure 6. Similar cytokine profiles were obtained with U87 and U118 cell lines (data not shown), this now been mentioned in the results section. Since we used two normal donor ATC and did only one experiment, statistical analysis cannot be done for this figure.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**
I declare that I have no competing interests.
Reviewer's report

Title: Targeting and killing of glioblastoma with activated T cells armed with bispecific antibodies

Version: 2

Date: 30 November 2012

Reviewer: Randy Jensen

Authors would like to thank the reviewer for the comment that this is an “Excellent and interesting paper”.

Minor essential revisions:
Figure S1 needs some means to describe the symbols for each line and what that line represents. Response: Each symbol represents an OD at a specific number of cells. A line connecting round symbols represent an actual line while a straight line is just a trend line meaning that if the symbols are aligned on the trend line would show a 100% correlation between OD and specific number of cells.

Discretionary Revisions.
Figure 1 is somewhat hard to interpret with MTT viable cells % on the left side and 51Cr cytotoxicity on the right axis. If this were broken into two panels it might be easier to understand the appropriate dose response. Response: As suggested by the reviewer, we have made the separate panels for MTT and \(^{51}\text{Cr}\) release cytotoxicity assays in Figure 1.

Knowing the status of EGFR and HER2/neu on the U87MG, U118MG, and U251MG was really convincing, doing something similar on any of the neurospheres would also strengthen this paper. Response: We have now shown the data of EGFR and HER2 expression by flow cytometry on glioma cell lines and ex vivo glioma cells in Figure 1C (lower panel), Figure 2 (lower panel) and Figure 4 (lower panel).

Agree that next step of animal experiments will be the true test of the validity of this treatment modality. Response: Authors agree that the next step would be the animal experiments.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I declare that I have no competing interests of any kind.
Reviewer's report

Title: Targeting and killing of glioblastoma with activated T cells armed with bispecific antibodies

Version: 2

Date: 7 December 2012

Reviewer: Jun Wei

Reviewer's report:

Major Compulsory Revisions

1. Bi-specific antibody (BiAb) conjugated T cell therapeutic efficacy against glioblastoma should be validated in xenograft immunodeficient mice model and included in this study.

Response: We have previously shown the therapeutic efficacy of bispecific antibody (BiAb) armed T cell against prostate cancer and numerous other xenograft models (Please see the references below). In all these preclinical studies, we have shown an excellent association between the in vitro cytotoxicity of tumor cells and in vivo killing of tumors in xenograft models. These studies have now been discussed in the introduction section.

In most situations, we agree with the reviewer’s perspective. In this case, it was not clear whether additional animal studies would add to the knowledge base of whether infusions of armed ATC would predict clinical responses in human beings. Based on our earlier preclinical studies as listed below, we respectfully request that repeating a series of these studies would not add additional or helpful information.

2. The data of EGFR and HER2 expression by FACS on glioma cell lines and ex vivo glioma cells need to be presented in the manuscript in order to make a clear view for the correlation of sensitivity of glioma cells to the BiAbs with EGFR and HER2 expression.

Response: We have now presented the data of EGFR and HER2 expression by flow cytometry on glioma cell lines and ex vivo glioma cells in Figure 1C (lower panel), Figure 2 (lower panel) and Figure 4 (lower panel).

3. Validation of MTT assay in Fig.1 is problematic, because absorbance measurement of residual viable cells consists of not only glioma cells, but also includes armed ATCs conjugated to the glioma cells. Inconsistency between HER2Bi/MTT and HER2Bi/51Cr at higher BiAb concentration in Fig.1 supports it.

Response: We understand the reviewer’s concern that residual viable cells may not only consist of glioma cells, but may also include ATC or armed ATCs that would be measured as viable cells by absorbance. Residual viable cells are usually in small numbers as we first collect the culture medium that contains ATC/aATC followed by 2-3 washings on plate shaker before adding the MTT reagent. Moreover, we use targets only or targets+ATC as control wells, and difference between two readouts is 5-12%. As the Figure 1A shows that viability in MTT assay is increased and the cytotoxicity in 51Cr release assays is reduced at higher arming dose of HER2Bi, indicating the consistency in both assays. Decreased cytotoxicity by ATC at higher arming dose of BiAb is a known phenomenon that could be due to the receptor saturation induced desensitization of ATC.

4. In Fig.4 histogram, majority of CD133+ cells and CD133- cells still overlap, and such CD133 purity is not acceptable and suitable for further study. Response: We repeated the experiment all three target populations (unfractionated cells, CD133 negative cells and CD133 enriched cells) from two primary ex vivo cells. Number of primary cells were the limiting factor in achieving the purity or enrichment, in our hands we have seen that passing through 2-3 columns can achieve ~80% pure cells. However, with 2nd or 3rd enrichment steps we loose a sizable number of cells that are needed for cytotoxicity assay and phenotyping. We have used two ex vivo primary cells with varying degree of CD133 enrichment (we now define them as CD133 enriched population instead of CD133+ cells). In the bottom panel of Figure 4, we show the expression CD133 and the expression of HER2 and EGFR in CD133 enriched population in ex vivo primary cells.

Minor Essential Revisions

1. The highest E:T ratio (12.5:1) is used in Fig.3, and this may saturate killing activity for each BiAb armed ATCs. Improved killing might be achieved when titrating down E:T for simultaneous use of two BiAbs. **Response:** Authors agree with the reviewer, we now show the cytotoxicity data at lower E:T ratio (5:1). Data show a significantly reduced viable cells in double- or mixed single-armed ATC compared to individual EGFRBi- or HER2Bi-armed ATC (Figure 3), suggesting some additive killing effect by dual antigen targeting.

2. It would be interesting to test BiAb armed ATCs killing on fresh ex vivo glioma stem cells (GSCs). **Response:** In Figure 4, we show the BiAb armed ATC mediated killing of both *ex vivo* CD133 enriched cell (putative stem cells) and bulk tumor cell populations.

3. It is not clear that ATCs are from healthy donor PBMCs or glioma patients. If they are generated from healthy donor, the possible allogeneic responses or GVHD side effects need to be discussed. **Response:** All ATC were from healthy donor PBMC. Since our cytotoxicity assay is only a short term assay (18h incubation), we do not anticipate allogeneic responses. Moreover, ATC are also from the same donor as armed ATC and they do not kill the target cells further excluding the possibility of allogeneic responses.

**Level of interest:** An article of limited interest

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare that I have no competing interests.