**Re: Revision for JoVE59275 - [EMID:310a099d8b69baa3]**

**Dear JoVE editorial stuff,**

We appreciate the opportunity to provide the revised manuscript entitled “*In vitro* tumor cell rechallenge for predictive evaluation of chimeric antigen receptor T cell antitumor function”.

We are delighted that the reviewers realized the value of the described assay in the field of CAR T cell therapy. We also would like to thank the editors and reviewers for all the suggestions and comments, which have greatly improved the manuscript’s quality.

Below are the point-by-point responses to the editorial and reviewers’ comments.

**Editorial comments:**

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**We have proofread the entire manuscript.**

2. Title: Please avoid abbreviations.

**We have re-worded the title.**

3. Please provide an email address for each author.

**We have provided email addresses for each author.**

4. Please define all abbreviations before use.

**We have proofread the manuscript and confirmed that all abbreviations are defined first.**

5. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

**We have made all the edits.**

6. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

**We have converted all to x g.**

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

**We have adjusted all the numberings.**

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Irvine Scientific, Invitrogen, Abraxis Pharmaceutical Products, R&D Systems, Lonza, Hyclone Labs, accutase, Innovative Cell Technologies, MacsQuant, Miltenyi Biotech, EMD Millipore, etc.

**We have deleted all commercial languages.**

9. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

**We have re-formatted the protocol and moved the discussion-related contents to the Discussion section.**

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

**We have added details based on instructions below.**

11. Lines 82-83: Please specify at what conditions the GBM tumor spheres are maintained.

**We have added the information (Line#93-94 in the revised manuscript).**

12. Line 87: Please describe how to dissociate GBM TSs.

**We have added the method to dissociate GBM TSs (Lines#91-100 in the revised manuscript).**

13. Line 96: What volume of FSS is used to wash and how many times?

**We have added the information (Line#108 in the revised manuscript).**

14. Line 100: Please specify the gating strategies used.

**We have specified the gating strategy for CAR T cells (Line#113 in the revised manuscript and the new Figure 1B).**

15. Line 124: Please indicate the specific steps that are repeated here

**We have added the information (Line#133 in the revised manuscript).**

16. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

**We have combined shorter steps in the revised manuscript.**

17. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

18. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

**For comments 17-18, please refer to the highlights in the revised manuscript.**

19. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**The highlights include all relevant details.**

20. Figure 2: Please define error bars in the figure legend.

**We have defined error bars in Figure 2 legend (Line#232 in the revised manuscript).**

21. Table 2: Please remove manufacturer and catalog# information from the table. Such information should be provided in the Table of Materials. Please reference Table 2 in the manuscript.

**We have deleted Table 2 since all information is included in Table of Materials.**

22. Discussion: Please discuss critical steps and any limitation of the technique.

**We have added the information in Discussion (Lines#266-271 of the revised manuscript).**

23. References: Please do not abbreviate journal titles.

**We have changed the reference style.**

24. Table of Materials: Please provide lot numbers and RRIDs of antibodies, if available. Please remove trademark (™) and registered (®) symbols. Please sort the items in alphabetical order according to the name of material/equipment.

**We have edited the Table of Materials. It would be difficult to retrieve the lot numbers and RRIDs, but we included the clones of all antibodies used.**  
  
  
**Reviewers' comments:**  
  
Reviewer #1:  
  
In this manuscript, Wang et al. describes an in vitro assay to compare the functionality of different CAR-T cell lines via repeated challenge with target cells. The manuscript is clearly written, and describes an assay that would be of great value to the T-cell engineering community. A few minor changes could be made to improve the article.  
  
1. The authors state in the abstract that CAR-T cell activity levels quantified by this assay reflect "their differential in vivo antitumor activity in orthotopic mouse models." However, this manuscript does not actually provide any in vivo data for actual comparison. It appears the authors made this statement based on prior results published in JCI Insight in 2018 (reference 21). It would be helpful to add a sentence or two in the results section specifically stating that the results obtained from the assay corresponded to in vivo results previously reported in order to make this point clear.

**We appreciate the reviewer’s comment and have added the description of a previous *in vivo* study about differential antitumor effect between CD4+ and CD8+ CAR T cells (Lines#210-214 in the revised manuscript)**  
  
2. Centrifugation speeds should be reported in rcf (xg), not rpm, since readers of this article may use rotors whose rcf-to-rpm conversions are different than that of the authors' equipment.

**We appreciate the reviewer’s concern and have converted all centrifugation speeds to rcf (xg).**  
  
3. The authors recommended using CD45 as a marker to distinguish between T cells and tumor cells. This makes sense when the tumor cells are GBM or other CD45- cell lines, but not all CD45+ cells are T cells, and not all tumor cells are CD45-. Therefore, it would be wise to add a line of clarification (which may seem obvious) that the marker should be chosen depending on the identity of the tumor cell line.

**We share the reviewer’s concern about applying this assay to different tumor cells. If the tumor cells do not express CD45 (e.g. GBM cells), T cells in this co-culture can be identified as by anti-CD45 staining. If the tumor cells do express CD45 (e.g. Raji lymphoma cells), anti-CD3 staining can be used to distinguish T cells from co-cultured tumor cells. We have modified the protocol for clarification (Line#165-166 in the revised manuscript) and updated the Table of Materials.**

4. I assume the two plots in Fig. 3A are missing labels for CD4 vs. CD8.

**We appreciate the reviewer’s comment and have added labels in Fig.3A.**

Reviewer #2:

Using IL13Ra2 CAR T cells in a GBM tumor spheres culture system, the authors provide an In vitro tumor cell rechallenge for predictive evaluation of CAR T cell antitumor function, which could be potentially used for optimization of CART cell products in vitro. sufficient introduction of the protocol is sufficient and easy to follow. There are some issues need to be addressed.

1. The CAR T cells are targeting IL13Ra2, the expression of IL13Ra2 in GBM tumor should be tested.

**The expression of IL13Rα2 on the GBM cells used in this protocol has been shown in the previous study (PMID: 24204956).**  
  
2. In the co-culture system, at least a negative control is required, either non-transduced T cells or IL13Ra2 negative tumor.

**We share the reviewer’s concern about negative control and have included the viable tumor cell number when co-cultured with non-transduced T cells in the new Fig.1C.**

**However, the high tumor number in the negative control group, which is due to the multiple rechallenge steps, will diminish the functional difference between tested CAR T cell subsets if plotted on the same graph. It is thus recommended that the negative control not included when comparing the experimental groups.**

3. For Figure 3B, the starting T cell phenotype should be shown.

**We have edited Figure 3B to include the starting phenotype.**

4. For Figure 3C, it is better to show representing data of dot plot for single, double and triple positive T cells population.

**We appreciate the reviewer’s comment. Due to the difficulty in showing triple-positive in one single dot plot, we now provide the gating strategy to identify single, double and triple positive cells in Fig.3C**

Sincerely,

Dongrui Wang, PhD Candidate

And

Christine E. Brown, Ph.D, Heritage Provider Network Professor in Immunotherapy

T cell Therapeutics Research Laboratory

Department of Hematology/HCT

City of Hope, Duarte, CA, 91010