C: weiming.zheng@postgrad.manchester.ac.uk, milena.kalaitsidou@manchester.ac.uk, dgilham@celyad.com, robert.e.hawkins@manchester.ac.uk  
  
Dear Dr. Kueberuwa,  
  
Your manuscript, JoVE58492 A syngeneic mouse B-cell lymphoma model for pre-clinical evaluation of CD19 CAR T-cells, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.  
  
After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.  
  
Your revision is due by **Jul 10, 2018**.  
  
To submit a revision, go to the [JoVE submission site](https://outlook.manchester.ac.uk/owa/redir.aspx?C=4-IiaF699FoYRUNrnDsz1ahH5eAJ05eJXFO9dI6SYNcgbg9qktbVCA..&URL=http%3a%2f%2fwww.editorialmanager.com%2fjove) and log in as an author. You will find your submission under the heading "Submission Needing Revision".  
  
Best,  
  
Alisha DSouza, Ph.D.  
Senior Review Editor  
[JoVE](https://outlook.manchester.ac.uk/owa/redir.aspx?C=wPupQJNUavjwV2S5CMPRFnbdvXXRKT_MDgFuDxH5tNcgbg9qktbVCA..&URL=http%3a%2f%2fwww.jove.com%2f)  
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[About JoVE](https://outlook.manchester.ac.uk/owa/redir.aspx?C=JSVdTBIMKVEhwQT1WuHwnky8UF_IXXV7WmIrb1REWtMgbg9qktbVCA..&URL=http%3a%2f%2fwww.jove.com%2fabout)  
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**Editorial comments:**  
Changes to be made by the Author(s):  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

*GK: Proofread*

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

GK: A word document with a link and snapsjots of licence page, as well as a pdf showing communication with the EAGCT, in which they give explicit permission for use, is attached.

3. Figure 2: Please increase the size of the text in figures to make it easier to read.

*GK: Size of scale bar increased, as well as size of text. Time and date stamps removed.*

4. Figure 3: Please change “ml” to “mL”.

GK: changed  
5. Figures 3-7: Please define error bars and asterisk symbols in the figure legend.

GK: error bars and asterisks and statistical tests defined

6. Please shorten the figure legends. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

GK: Figure legends shortened. Additional descriptive text pertaining to figures has been moved to protocol text

7. Please provide an email address for each author.

GK: Provided on title page under “contact details”

8. Please define all abbreviations before use.

GK: checked

9. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

GK: edited

10. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

GK: edited

11. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

GK: edited

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

GK: checked

13. 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. For example: RetroNectin, Falcon, Zombie Violet, LSR Fortessa, Lumistart Omega, Xtreme II Bruker, etc.

GK: Commercial names removed and restricted to materials table

14. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

GK: ethics statement added

15. 1.1: Please write the text in the imperative tense in complete sentences.

GK: Explanatory elements are now under a “note”

16. 1.3, 1.4: Please mention the conditions for culturing cells.

GK: at 37 °C, 5 % CO2 added

17. 2.9: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.

GK: reference added

18. 2.10: What container is used in this step? What volume of PBS is used?

GK: additional description has been added

19. 2.14: What is centrifuged? Please add the missing information.

GK: added – resultant cell suspension overlay

20. 5.7: Please write the text in the imperative tense.

GK: altered

21. 5.16: Please add more details to this step or add references to published material specifying how to perform the protocol action.

*GK: added to description and referred to section 3*

22. What happens to mice after the experiment?

*GK: added instructions for schedule 1 method of euthenasia*

23. Please include single-line spaces between all paragraphs, headings, steps, etc. After that, 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.

*GK: implemented single spacing. In order to reduce to 2.75 pages removed some in vivo injections that will be easily found elsewhere. However, in order to maintain cohesion, about 3 pages are highlighted.*

24. 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.

*GK: N/A*

25. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included.

*GK: updated with paragraph to explain representative results.*

26. Discussion: Please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

*GK: paragraph two outlines the importance of T-cell transduction relative to tumour growth. Causes for poor transduction efficiency have been added, with recommended checks to reagents. Limitations are discussed in paragraph 4.*

27. Please include volume and issue numbers for all references.

*Two references are web links to FDA approval docs therefore no volume included*

28. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.

*GK: Multiple lot numbers used. RRID not available for antibodies, only available for Plat-E cell line which is now added.*   
  
**Reviewers' comments:**  
  
  
  
**Reviewer #1:**  
Manuscript Summary:  
Preclinical studies of CAR T-cells usually rely on the use of human CAR T-cells in immunodeficient mice that cannot recapitulate the immunosuppressive tumor microenvironment of human tumors. This makes the preclinical evaluation of strategies designed to overcome the tumor microenvironment almost impossible. Here is presented a strategy for the generation of murine CD19.CAR T-cells for use in syngeneic mice bearing murine lymphoma. This system can be modified for the evaluation of different CARs and modifying transgenes in different tumor systems. This system will be useful for evaluating concepts, but not for evaluating clinical CAR safety or CAR structure.  
  
Major Concerns:  
I would like to have seen the viability, recovery and subsequent proliferation of the murine T-cells after transduction using this method. It would also help to describe the results quantitatively in terms of transduction efficiency, phenotype, cytokine secretion and killing rather than simply referring to representative figures. The disadvantages of syngeneic mouse models as well as their advantages should be mentioned (clinical CARs cannot be tested, antigen distribution is often different, murine immune systems and tumors are different)

*GK: Viability and recovery is variable depending on which CAR T-cell construct. Those expressing IL-12 lead to much lower viability, possible dues to upregulation of Fas ligand signaling. Figure 2 includes representative results showing the proportion of live cells 4 days post transduction. These numbers vary greatly depending on donor cells.*

*The advantage/disadvantages of syngeneic models are discussed in several parts of this manuscript. Added description to ensure the reader appreciates the limitations has been added.*

\*Line 64, a major point of CARTs is that they do not have to break tolerance

*GK: this was referring to epitope spreading and general immune based approaches. Clarification added.*

\*5 GY of irradiation cannot be said to mimic the patient situation since Cytoxan and fludarabine are the standard means to produce lymphodepletion with a very different mechanism from irradiation that induces substantial inflammation.

*GK: The standard for human lymphodepletion in humans is now 5FU and fludarabine, however, several methods, including radiation have been used in the past. We have tested several methods for lymphodepletion in mice. We prefer total body irradiation as it is less invasive than an IV injection, more consistent and at the low dose rate used here, induces minimal discomfort to mice. There are several publications with differing opinions on the mechanisms of lymphodepletion. Our own work shows that TBI causes efficient lymphodepletion and a drastic enhancement of CAR T-cell engraftment – (shown in Figure 6 of this manuscript).*

\*Two paragraphs later, you state that you established a lymphoreplete model. This is confusingly presented, although later it becomes clear.

*GK: clarification added by describing standard method, and referring to patients ineligible for lymphodepletion.*

\*Please provide source of packaging plasmids and cell lines and more description of Plat-E cells

*GK: Plat-E and pCL-Eco added to materials list. Description of PlatE cell line added to section 1 of protocol.*

\*For how long do you rock the plates of Plat E cells with the CaCl2 DNA solution

*GK: added*

\*Not clear why parts of the text are highlighted?

*GK: highlighted for filming*

\*Step 2.19. What culture vessels are used to culture the murine T-cells?

*GK: this depends on the number of cells obtained – cells are kept at 5e6/ml. added text to say appropriate sized flask.*

\*Step 2.40. "Resuspend cells in 2.5 ml per well of transduction with 200 IU/ml and 4 ng/ml" (IL2 and IL7 is missing)

*GK: added*

\*2.45. What type of culture vessel and cell densities do you recommend? Wells or flasks?

*GK: cell density is stated, flasks now added to text for clarity*.

\*Centrifugation of splenocytes for 90 minutes at 1200 seems quite harsh. What is the viability and recovery of the cells after the second round of transduction?

*GK: This speed is required to have an effect on viral particles. A note on viability has not been added as in the first 2-3 days, all non T-cells die off under these tissue culture.*

\*What fold expansion do you achieve over the 4 days after transduction?

*GK: A statement about overall viability over first 4 days has been added to a note on point 2.46*

\*3.5. Wash twice with PBS and (missing centrifuge) 500 x g for 5 min

*GK: added*

\*4.0 What is total "n" in the experiments shown in Figure 3b and 3c

*GK: representative results. N added to figure legend.*

\*5.5. What unexpected toxicities have you observed and using what transgenes?

*GK: We have not observe toxicities from these CAR T-cells, however if adapting this protocol it is of vital importance to keep this caution in mind. Clarified in text*

\*It would be helpful to describe the results: What transduction efficiencies did you get (median and range, including n)

*GK: this varies based on length of CAR construct size. Ranges for first generation and large vectors now added.*

\*Is it surprising to see so little IFNg production from CART cell cultured with tumor cells. Very high background levels would be seen with human CART cells on day 5 or 6 after stimulation with CD3 and CD28.

*GK: Can’t comment without seeing protocol for human cells. Our lab stimulates human cells for much longer periods than mouse. This protocol is overnight before CD3 and CD28 antibodies are removed. It is noted in the manuscript that non transduced cells can often display non specific activity.*

\*Should the tumors fail to :"take", can the CART cells be cryopreserved for future use?

*GK: We have not validated this yet. The freeze thaw process causes substantial cell death and can have effects on the T-cell phenotype so it is avoided.*

\*Line 403 sense?

*GK: Clarified- concomitant production*

\*How do you measure epitope spreading?

*GK: there is not a standard measure, but presence is confirmed by lack of response to initial epitope. Not in the scope of this protocol.*  
**Reviewer #2:**  
Manuscript Summary:  
The manuscript describes the production and testing of mCD19-specific CAR T-cells. Testing is done in vitro and in vivo in a syngeneic tumor model using lymphodeplete and lymphoreplete recipients. The protocols are generally well described but there are concerns regarding the presentation of some data, lack of some details, and questions regarding the range of transduction efficiencies obtained.  
  
Major Concerns:  
1. CAR T-cell patients are typically given a lymphodepleting dose of cyclophosphamide (often with fludarabine) prior to CAR T-cell transfer. It therefore is not clear why the authors use TBI to lymphodeplete, especially since they wish "to mimic the patient setting" (lines 68-69). Lymphodepletion by chemotherapy administration prior to CAR T-cell transfer is more clinically relevant.

*GK: Similar comment to reviewer 1. Addressed above:*

*The standard for human lymphodepletion in humans is now 5FU and fludarabine, however, several methods, including radiation have been used in the past. We have tested several methods for lymphodepletion in mice. We prefer total body irradiation as it is less invasive than an IV injection, more consistent and at the low dose rate used here, induces minimal discomfort to mice. There are several publications with differing opinions on the mechanisms of lymphodepletion. Our own work shows that TBI causes efficient lymphodepletion and a drastic enhancement of CAR T-cell engraftment – (shown in Figure 6 of this manuscript).*

2. There are no measures of reproducibility or range. For example, what is the range of transduction efficiencies over a number of attempts? Does transduction efficiency vary between different CAR constructs?

*GK: Also similar comment to reviewer 1. Addressed above: range of efficiencies added, as well as an explanation that the larger inserts have reduced efficiency.*

3. The quality of the dot plots shown in Figure 1 is poor and the embedded text is illegible.

*GK: Text in figure 1 has been altered as per suggestion by editor.*

4. The number of animals in some figures (e.g. Figure 3C) is too low to inspire confidence in the results, despite the statistical significance.

*GK: These are representative results. For simplicity, killing assay, IFN gamma ELISA and IL12 ELISA were taken from the same experiment. This can be edited if needs be.*

5. No indications of statistical significance are provided in some figures (e.g. Figures 5 and 7).

*GK: figures 5 and 7 contain tumour burden and survival curves.*

*Statistical analysis of tumour burden at the time of apparent therapeutic efficacy cannot be performed by classical two-way ANOVA because mice in control groups have been euthenised. Performing analysis without these mice skews results as clearly they attained high tumour burden before being euthenised.*

*have classical survival curve tails associated with immunotherapies. Analysis through classical comparison of median overall survival is therefore not suitable for this data. Analysis on an arbitrary level such as 80% rather than 50% could be performed, however it does not add value to statement of long term survival proportion.*

6. In addition to CD4 and CD8 expression, the transduced cells should be phenotyped for activation/exhaustion markers as different culture conditions yield different differentiated T-cell products.

*GK: This is beyond the scope of this protocol. Whilst T-cell phenotype is impacted upon by activation method, cell culture conditions, length of cell culture and transgenes added to CAR T-cell constructs. Previous research from our lab analysing phenotypic chnge with culture mmethods has been added to the text*

Minor Concerns:  
1. One line 149, a word is missing after, "Centrifuge the..."

*GK: changed*

2. The hybridoma clone designations for each monoclonal antibody should be added to the material/equipment table.

*GK: added to materials list*

3. The in vivo BLI data are presented incorrectly as counts (photons/sec); they should be presented as radiance units (photons/sec/cm²/sr). "Counts are a relative measure of the photons incident on the CCD camera and radiance is in absolute physical units that measure the photon emission from the  
subject." (<https://mbi-ctac.sites.medinfo.ufl.edu/files/2017/02/Concept-Tech-Note-2-Image-Display-and-Measurement.pdf>)

*GK: As described in the protocol, ROI and exposure time are constant for each mice and each time point. Distance from camera and steradian is equivalent throughout, therefore results of total flux are displayed, which is measured in photons/sec*

4. On line 285, the frequency of BLI measurements should be noted (i.e. q.a.d? weekly?)

*GK: added the description of weekly imaging.*  
  
  
**Reviewer #3:**  
Manuscript Summary:  
The authors present protocols for setting up a preclinical system for testing chimeric antigen receptor T cells in a syngeneic background. This includes the retroviral transduction of murine T cells and also the engraftment of B-cell lymphoma cells into BALB/c mice with and without lymphodepleting pre-conditioning.  
  
The protocol itself and experimental variables are well described. While these models have been published in the literature--and thus are not novel--the description here is concise and brings attention to the importance of studying CAR T cells in immunocompetent backgrounds, which can have significant effects on antitumor activity and the overall immunological milieu of the host.  
  
Major Concerns:  
The authors should comment on using target tumor lines that express luciferase, as it is a foreign protein and indeed is known to impact the tumorgenicity of several tumor lines. Certainly, an immune response against luciferase may play a role in the durability of antitumor responses. This should be addressed, for example by using experiments/cell lines without luciferase that monitor overall survival. In the very least, the appropriate caveats and the question of endogenous immunity to luciferase should be discussed so that readers are aware of this limitation when attempting to recapitulate this model.  
  
*GK: added in a description, reference to the molecular therapy oncolytics which contains examination of immune response to luciferase is added.*

**Reviewer #4:**  
Manuscript Summary:  
Kueberuwa et. al provide a detailed protocol on CD19 CAR T cell production and CAR T cell validation assays. They also show how to test their CAR T cells in lymphoreplete and lymphodepleted mouse models.  
  
Minor Concerns:  
2.23 - Takara (Clonetech) suggests the use of sterile 2% BSA to block non-specific binding instead of TCM.

*GK: we have used both BSA and media containing FCS. As T-cell media is used in the next step in the protocol it is more convenient.*

2.35 - Here you state that you reuse the Retronectin collected from the first and second transduction and save it for future use. Do you re-freeze the Retronectin at -20C after collection? Have you seen loss of transduction efficiency due to recycling Retronectin?

*GK: This point is addressed in the critical steps and modifications section of the discussion. Re-freezing leads to reduced transduction efficiency over time*

2.40 - Please clarify that the 200 IU/mL and 4 ng/mL are respective to IL-2 and IL-7

*GK: added*