|  |  |
| --- | --- |
| OUS_logo_RGB_eng | **Sébastien Wälchli Senior Researcher, Ph.D. Dept. Cellular Therapy, KKT Oslo University Hospital Radiumhospitalet** |
|  | Ullernchausséen 70  0379 Oslo  Norway  Tel +47 22781317  E-mail sebastw@rr-research.no  Org.no:  NO 993 467 049 MVA  www.oslo-universitetssykehus.no |
| To: the Editor-in-Chief  Jove  Oslo, 12 August 2018 |  |

**REBUTTAL LETTER**

Dear Editor,

Please find herein our response to the requests from the editorial board and the reviewers. We have answered them point-by-point and believe that the manuscript is now improved.

We hope that you will find it satisfactory for publication in Jove.

Yours sincerely,

On behalf of the co-authors

Sébastien Wälchli

**Editorial comments:**  
Changes to be made by the Author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.   
2. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file.   
3. Figure 2: Please include a space between all numbers and their time units (i.e., 20 h, 60 h, etc.).   
4. Please spell out each abbreviation the first time it is used.   
5. Please rephrase the Abstract to more clearly state the goal of the protocol.   
6. 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. For example: Incucyte, Falcon, etc.   
7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).   
8. 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.   
9. 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. Some examples:  
1.1: What is the concentration of trypsin used in this step?  
1.10: How to set up Incucyte scan? Please add more specific details (button press, etc.).   
1.11: What is observed for the growth of spheroids? How to measure the diameter?   
2.1.1: Please specify the conditions under which the T-cells are cultured.  
2.1.8, 2.2.4, 2.2.7, 2.2.10, etc.: Please indicate the specific steps that are repeated here.  
2.1.11: What is the temperature for freezing the cells?  
4.1-4.16: Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.   
10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.   
11. Please include single-line spaces between all paragraphs, headings, steps, etc.   
12. 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.  
13. 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.  
14. 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.  
15. 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.   
16. References: Please do not abbreviate journal titles.   
17. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.  
  
**Reviewers' comments:**  
  
Reviewer #1:  
  
Manuscript Summary:  
In this work, the Authors have described a method to evaluate cytotoxicity of CAR-T cells to target cells (colon carcinoma HCT116) transduced with the target (CD19) of CAR-T cells. This has been performed using spheroids of HCT116 cells.  
  
To follow the specific cytotoxic effect of HCT116 cell line, these cells were transduced with GFP (green) while the positivity with red annexin V of HCT116 incubated with CAR-T cells has been considered as a probe for cytolytic activity.  
  
  
Major Concerns:  
The methods described appear feasible and reliable. however, I would say that CD19 can be considered as a B cell markers not an epithelial cell marker. Thus, the use of spheroids of HCT116 carcinoma cells expressing CD19 antigen is quite unusual for an epithelial cell. If the Authors wanted to show the effect of CAR-T cells on epithelial cells they could chose another and more appropriate target of CAR-T cells.

The goal of this protocol is to offer an inexpensive, reliable and easy way to make spheroids and test effector cells potency (here we chose CD19 CAR). If we decided to go for a more adequate target for CD19 CAR based on B lymphoma cell lines, the protocol would be very different as the formation of spheroids based on B cells is very challenging. In addition, this type of “solid” tumour targeting combined with CD19 was already presented by Berahovich, R. *et al*. *Front. Biosciences.* **22,** 1644–1654 (2017).  
  
Minor Concerns:  
The amount of trypsin added for the area of flasks where HCT116 cells were cultured (lines 104-106) is quite low (0.250 microliters for 25cm2 flasks and 0.500 microliters for T75cm2 flasks). In my experience it is difficult that all the area of culture is covered with so low amount of trypsin solution. Please check or correct. **DONE** (l.111-l.112)  
  
Generally, to get spheroids of epithelial cells, these cells should be cultured in ultralow attachment plates. From the code indicated for the plates used it appears that these can be this kind of culture plates. This should be further stressed indicating this feature of the plates used in the text or in the table.

We are not using ultralow attachment plate, just regular U-well plates.  
  
The images are not really clear (probably because they are really little and the cells are cultured in U-well bottomed plates not as usual in flat bottomed plates). Perhaps, the Authors can improve the quality of details of images. **DONE**  
  
  
  
  
Reviewer #2:  
  
Manuscript Summary:  
This manuscripts describes the development of a spheroid imaging assay to measure CAR T mediated killing of tumor cells.  
  
  
Major Concerns:  
More detail is required with respect to the generation of CAR T for someone to reproduce these experiments. Section 2.1 refers to "stable expression" of CAR T. This makes the reader infer that this is viral transduction of T cells. Later the authors refer to electroporation of the CAR. Please describe protocol in more detail or include reference. **DONE**  
  
Where were the T cells obtained (patients, healthy volunteers) and how where they isolated? Was this done on PBMCs or isolated CD3+ cells? Which CD19 clone was used? FMC63? Please provide a reference to sequence and CAR architecture (hinge, transmembrane, costim) **DONE** (l. 150-l. 152).  
  
For T cell growth please specific optimal density for growing T cells. i.e. 1-5 x 10^6/mL **DONE** (l. 173)  
  
For Figure 1, please show the expression of the CAR on the T cells on the day (or day before-day 11 or 12) they are included in the spheroid assays. Additionally, please show the expression of CD19 on the HCT116 cell line in this figure. **DONE** (Modified Fig. 1)  
  
For Figure 2, please show a wider image, it is hard to see the spheroid. Generally, these are at the bottom of the well and one can not see this in these images. See this as an example: <https://www.youtube.com/watch?reload=9&v=kvgWPlKDoBg> **DONE**  
  
Consider using the parental HCT116 antigen negative (CD19-) cell line as a negative control. We agree with the reviewer that this is an essential control when checking the specificity of a CAR for its target. However, in the present study we aim at presenting a method to study CAR against a solid tumour and we therefore used a validated CAR. We have additional data on BLI-based killing showing the HCT116 are not killed by CD19 redirected Tc, but we did not perform this test in the present spheroid assay and have decided to restrict our negative control to mock Tc.  
  
Please describe how you identify dead tumor cells vs dead T cells in these assays.   
Exclusion of dead T-cells rely on two strategies:

* First, as suggested by the Annexin V manufacturer´s protocol, Tumor cells are pre-incubated alone with the Annexin V which is supposed to diminish drastically the potential marking of the T cells.
* Second and more important, all the analysis of the fluorescence signal rely on the detection of phase object (that are limited to the spheroids). Only the fluorescence signal emitted within the boundaries of the phase object *i.e.* the spheroid, is considered.  
    
  Minor Concerns:  
  Please clarify if mock is no T cells or if it is water electroporated T cells **DONE** (l-159-160) and the text was further modified.  
    
  The antibody and HCT116 cell line and antibody (from 2.2.5) should be included in the Table of Materials **DONE**.