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A Spheroid Killing Assay by CAR T Cells

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TITLE:

A Spheroid Killing Assay by CAR T Cells

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KEYWORDS:
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SUMMARY:

This protocol is designed to assess immunotherapeutic redirected T-cell (CAR T-cell) cytotoxicity against 3D structured cancerous cells (spheroids) in real time.

ABSTRACT:

Immunotherapy has become a field of growing interest in the fight against cancer otherwise untreatable. Among all immunotherapeutic methods, chimeric antigen receptor (CAR) redirected T cells obtained the most spectacular results, in particular with pediatric B-acute lymphoblastic leukemia (B-ALL). Classical validation methods of CAR T cells rely on the use of specificity and functionality assays of the CAR T cells against target cells in suspension and in xenograft models. Unfortunately, observations made *in vitro* are often decoupled from results obtained *in vivo* and a lot of effort and animals could be spared by adding another step: the use of 3D culture. The production of spheroids out of potential target cells that mimic the 3D structure of the tumor cells when they are engrafted into the animal model represents an ideal alternative. Here, we report an affordable, reliable and easy method to produce spheroids from a transduced colorectal cell line as a validation tool for adoptive cell therapy (exemplified here by CD19 CAR T cells). This method is coupled with an advanced live imaging system that can follow spheroid growth, effector cells cytotoxicity and tumor cell apoptosis.

INTRODUCTION:

Adoptive cell transfer (ACT) represents the next generation cancer treatment. It relies on the injection of effector cells (T- or NK-cells) into a patient. These cells can be genetically modified with a receptor that will guide them to their target, the tumor, and destroy it. Recently this approach was shown to be feasible when a Chimeric Antigen Receptor (CAR) directed against the B-cell marker CD19 was introduced into the patient T cells to kill his/her cancer[1]. In the case of CAR, which is an artificial receptor, the design consists of specific antibody fragments, the antigen binding domain reduced to an entity designated single chain variable fragment (scFv), linked to the T-cell signaling domains. Although there are several designs, the most commonly used versions referred to as second-generation CAR designs, consist of CD3z for TCR signaling and one co-stimulatory domain (CD28, 4-1BB, OX40, *etc.*)[1,2]. The immunotherapy field directed most of its attention to this new form of ACT when CD19 CAR-T cells efficaciously treated numerous patients with B cell malignancies[3,4]. Following this success, researchers tried to exploit the similar designs by targeting other epitopes for solid tumors with limited success. Unfortunately, the scarcity of tumor specific antigens and the harsher tumor microenvironments rendered CAR T cells less effective towards solid tumors[5].

Currently, the most commonly used *in vitro* validation strategies rely on two-dimensional (2D) systems that only address a fragment of the already mentioned solid tumor challenges. Classically, 2D *in vitro* systems involve a mixture of CAR T cells and target cancer cell lines as monolayers to assess the functionality and specificity of these effector cells. Although these strategies are important and vital parts of the studies, they do not take into consideration the complex morphology and three-dimensional (3D) structure of the cancer cells[6]. Cancer cells cultured in 3D systems, referred to as spheroids, acquire new phenotypic traits through changes in gene expression profile[7], which might influence the recognition by redirected effector cells. Birgersdotter and colleagues demonstrated that a Hodgkin lymphoma (HL) cell line when only grown in a 3D culture model acquires a gene expression profile that is similar to primary tumor samples[8]. Therefore, spheroids or similar 3D culture methodologies offer more relevant *in vitro* models as opposed to standard 2D systems. Such systems are also similar to *in vivo* studies which are seen as the final step in the validation process of a given CAR. Considering that 2D systems fail to mimic the morphology of cancer clusters, spheroids offer similar formations to assess the functionality of CAR T cells prior to *in vivo* models. In one study, Pickl *et al.* identified that a spheroid model of human epidermal growth factor receptor (HER2) overexpressing cancer cells demonstrated similar signaling profiles to *in vivo* models[9]. This further supports that spheroids offer more relevant and close-to-*in vivo* assessment of the CAR T cells. Additionally, CAR T-cell validation against spheroids might help assess their efficacy more critically and prevent some of the designs from moving to *in vivo* studies prematurely[10]; thus, contributing to ethically concerned research by sacrificing fewer animals. Moreover, protocols using spheroids are not more expensive than classical 2D systems and much faster as compared to classical *in vivo* studies. Taken together, one can predict that the inclusion of spheroid studies will soon become standard practice to link *in vitro* and *in vivo* studies.

Here, we present the preparation of spheroids from the colon cancer cell line HCT 116. This cell line was modified to express the human CD19 molecule to render it sensitive to CD19 CAR T cells and to provide a clear assessment of the killing using a clinically validated CAR construct.

PROTOCOL:

1. Generation of Spheroids from Colorectal Cancer Cell Line

1.1. Wash HCT 116 (stably transduced to express Cluster of Differentiation 19 (CD19) and Green Fluorescence Protein (GFP)) cell monolayers with phosphate buffered saline (PBS; 5 mL for a 25 cm^2 or 10 mL for a 75 cm^2 flask). Add trypsin (0.5 mL for a 25 cm^2 or 1 mL for a 75 cm^2 flask) and incubate cells at 37 °C for 5 min.

1.2. Check cell detachment under a microscope and neutralize cell dissociation enzyme with complete Roswell Park Memorial Institute 160 medium (RPMI 1640) (RPMI 1640 + 10% FCS + Gentamycin; 10 mL for a 25 cm^2 or 20 mL for a 75 cm^2 flask).

1.3. Centrifuge cell suspension at 500 x g for 5 min. Remove supernatant using a pipette and resuspend by pipetting up and down several times with 5 mL of complete RPM1 1640 medium.

1.4. Count cells using Trypan blue exclusion on a compatible cell counter.

1.5. Centrifuge cell suspension at 500 x g for 5 min. Remove supernatant using a pipette and resuspend in RPMI medium to obtain 5x10^3 cells/mL.

1.6. Transfer the cell suspension to a sterile reservoir and dispense 200 µL/well into a 96-well round bottom plates using a multichannel pipette.

1.7. Transfer the plate to the automated imaging apparatus inside an incubator (37 °C, 5% CO$_2$, 95% humidity).

1.8. Log into the acquisition software, select **Schedule To Acquire** | **Launch Add Vessel** | **Scan On Schedule** | **Create Vessel: New**.

1.9. Select **Scan Type: Spheroid**. Select the channels of interest: Phase + Brightfield (to follow spheroids growth), Green (to follow tumor signal, acquisition time 300 ms) and Red (to follow apoptosis, acquisition time 400 ms).

1.9.1. Select the desired magnification: 10x.

1.9.2. Pick the plate model and its position in the drawer. Select the position of wells to image. Enter the description of the experiment: name, type of cells, number of cells.

1.10. For the analysis setup, select **Defer Analysis Until Later**. Right click on the timeline and select **Set Selected Scan Group Interval** option and set **Add scans every** to 4 hours and **For a total of** to 24 hours. Set the desired starting time (at least 1 h after incubation in the automated imaging apparatus).

1.11. Check every 2 days for the growth of spheroids by logging into the imaging software.

1.11.1. Pick the **View Recent Scans** option and double-click on the desired experiment. Select **Brightfield** in the image channels panel and then use the **Measure image features** tool to measure the diameter of the spheroids. It takes 6 days for a spheroid to reach the desired size: 0.5 mm of diameter. Add 50 µL of complete RPMI medium per well at day 4 to limit medium evaporation effect.

2. Generation of CD19 CAR T cells

2.1. Expansion of CD19 CAR T cells

Note: Stable expression of CD19 CAR T cells was acquired by bulk retroviral transduction of the healthy donor PBMCs as previously described[16]. The retroviral construct coding for CD19 CAR is a second-generation CAR and consists of fmc63 scFv chain, CD8 hinge and transmembrane domain, a 4-1BB co-stimulatory domain and finally a CD3ζ domain.

2.1.1. To expand, culture the transduced T cells in the presence of anti-CD3/28 magnetic beads with a cell to bead ratio of 1:1 for 10-11 days. During the expansion, cells are in complete medium (X-VIVO 15, 5% Serum Replacement, and 100 U/mL recombinant human IL-2).

Note: The ideal density for an efficient expansion is 1-2.10^6 cells/mL. Depending on the initial number, cells can be expanded in flasks (25 cm^2 flasks to 20 mL, 75 cm^2 flasks to 40 mL of total volume) in a cell culture incubator (37 °C, 5% CO_2, 95% humidity).

2.1.2. As a negative control group for the following assays, include non-transduced PBMCs (will be referred as Mock) to the expansion protocol parallel to the CD19 CAR T cells.

2.1.3. On day 3 and onwards, add fresh medium every day and divide the cells into more culture flasks if necessary.

2.1.4. On day 10-11, centrifuge cells at 500 x g for 5 min. Remove supernatant and combine all the cells into one 50 mL tube and resuspend the cells in ~30 mL of fresh complete media.

2.1.5. Place the 50 mL tube containing the resuspended cells on a magnetic stand to separate the magnetic beads from the culture medium.

2.1.6. Wait for 2-3 minutes for the beads to collect on the side of the tube.

2.1.7. Remove the culture medium with a pipette and transfer to a new tube without touching the magnetic bead collection zones.

2.1.8. Repeat steps regarding the bead removal (2.1.5-2.1.7) once more to limit the number of beads in the final culture medium.

2.1.9. Resuspend and count the cells, adjust the density to 1-2x10^6 cells/mL in complete medium.

2.1.10. Rest the cells for at least 4 hours up to overnight. Then directly freeze them down at -80 °C and transfer the vials to a liquid nitrogen tank on the following day for long-term storage. Alternatively, one can prolong the rest up to overnight for immediate use.

2.2. CD19 CAR expression control on primary T cells

2.2.1. Count the number of expanded T cells as the numbers might vary slightly after overnight culture or moderately after freeze/thaw.

2.2.2. Transfer 5x10^5 cells from both CD19 CAR and Mock primary T cells to separate flow cytometry tubes.

2.2.3. Wash the cells with 200 µL of Flow Buffer (2% FBS in PBS) and centrifuge the tubes at 500 x g for 5 min. Repeat the washing steps to get rid of any artifacts caused by the culture medium.

2.2.4. Prepare the primary antibody (Biotin Goat Anti-Mouse IgG, F(ab')$_2$ Fragment Specific) by performing 1:200 dilution in Flow Buffer.

2.2.5. Resuspend the cells in 100 µL of antibody mix per tube and incubate on ice for 15 minutes. Repeat the previous washing step twice to remove excess antibody.

2.2.6. Prepare the secondary antibody (Streptavidin-PE) as 1:400 dilution in Flow Buffer.

2.2.7. Resuspend the cells in 100 µL of antibody mix per tube and incubate on ice for 15 minutes. Repeat the previous washing step twice to get rid of excess antibody.

2.2.8. Resuspend the cells in 200 µL of Flow Buffer per tube and analyze it on a flow cytometer.

2.2.9. Use Mock T cells to set up the negative and positive gate and analyze CD19 CAR transduced T cells accordingly.

3. 3D Tumor Spheroid Killing Assay

3.1. After 6 days or once spheroids reach the desired size, remove the plate from the incubator. Using a multichannel pipette, gently remove 100 µL/well of complete RMPI 1640 medium from the spheroid plates.

3.1.1. For this step, angle the tips towards the inside wall of the 96-wells plate, avoiding contact with the bottom of the well in order to minimize disturbance of the spheroids. Remaining volume should be around 100 µL.

3.2. Prepare a 1:200 solution of Annexin V red by mixing 50 µL of Annexin V red with 9.95 mL of complete RPMI 1640 medium.

3.3. Add 100 µL/well of the 1:200 Annexin V red solution.

3.4. Transfer the plate to an incubator (37°C, 5% CO_2, 95% humidity) for 15 min.

3.5. Harvest the transduced CAR CD19 T cells in a 15 mL tube and centrifuge them at 500 x g for 5 min. Remove the supernatant using a pipette and resuspend by pipetting up and down several times with 2 mL of complete RPM1 1640 medium.

3.6. Count cells using Trypan blue exclusion on a compatible cell counter.

3.7. Centrifuge cell suspension at 500 x g for 5 min. Remove supernatant using a pipette and resuspend in RPMI medium to obtain $2x10^5$ cells/mL.

3.8. Transfer the cell suspension to a sterile reservoir and dispense 100 µL/well into a 96-well round bottom spheroid plate using a multichannel pipette.

3.9. Transfer the plate back to the automated imaging apparatus inside an incubator (37 °C, 5% CO_2, 95% humidity).

3.10. Log into the acquisition software, select **Schedule To Acquire**.

3.10.1. Right-click on the Scan timeline and select **Edit Timeline**. Right-click on the scan group and delete it. Right-click on the timeline and select **Set Selected Scan Group Interval** option and set **Add scans every** to 1.5 h and **For a total of** to 24 h.

3.10.2. Set the desired starting time (at least 1h after incubation in the automated imaging apparatus). Select **Save schedule scans**.

4. Automated Image Analysis

4.1. Log into acquisition, pick the **View Recent Scans** option and select **Launch Analysis** option. Select **Create New Analysis Definition | Analysis Type: Spheroid**. Tick the Image channels to analyze (Phase + Brightfield, Green and Red).

4.2. Select at least 10 representative images: typically, 1 per condition and at least 3 time points (beginning, middle and end of the acquisition).

4.3. Preview the default analyze procedure on the whole image stack.

4.4. Modify the parameters for brightfield mask. Typical parameters are: Sensitivity 10, Hole fill 1000 µm^2, min area 1000 µm^2. Preview on the whole image stack and check that the selected parameters detect the spheroids accurately.

4.5. Modify the parameters for green mask (GFP). Typical parameters are: Top hat segmentation with radius 200 µm and threshold 3 GCU, Edge split off, Hole fill 5000 µm^2, Adjust size -2 pixels, Area min 3000 µm^2. Preview on the whole image stack and check that the selected parameters detect the spheroids accurately.

4.6. Modify the parameters for red mask (Annexin V). Typical parameters are: Top hat segmentation with radius 150 µm and threshold 2 GCU, Edge split off, Hole fill 5000 µm^2, Adjust size 0 pixels, Area min 1000 µm^2. Preview on the whole image stack and check that the selected parameters detect the spheroids accurately.

4.7. Launch the analyzer.

4.7.1. Once the analysis is done, extract the measurement of interest. Select the analyzed file and then the **Graph Metrics** option. Select the metrics of interest, the scan and the well. Typically, total red and green intensity within brightfield boundaries give the most accurate measurements by restricting the signal of fluorescence to the spheroids boundaries determined by the brightfield mask (**Figure 3**). Extract selected metrics in several file format by clicking on "**Export Data**".

4.8. Proceed to the extraction of the images and movies by selecting the analyzed file and then select the **Export Images and Movies** option. Two options are available, either "As Displayed" to retrieve images and movies as seen on the imaging software (usually composite images), either "As Stored" to retrieve raw data for external analysis through third-part software.

REPRESENTATIVE RESULTS:

As can be seen in **Figure 1**, it is crucial to check by flow cytometry the level of expression of CD19 CAR on T cells (**Figure 1A**) and the level of CD19 on HCT116 tumor cell lines (**Figure 1B**). **Figure 2** exemplifies the outcome of a typical spheroid experiment. The automated imaging apparatus takes pictures in four different channels: bright field, phase, green and red fluorescence. Phase channel is used to assert the formation of spheroids by displaying highly contrasted boundaries near the spheroids (which is a sign of spheroid formation). Bright field is used during the analysis process to detect the size of the spheroids and restrict the green signal (tumor) and red signal (apoptosis) to the aforementioned boundaries. It allows one to not consider events directly unrelated to spheroids (for example isolated tumor cells and their apoptosis). Green and red outlines represent respectively green and red signals considered in the metrics calculation. In **Figure 2**, bright field, phase, green and red channels were extracted through the “As stored” option whereas Composite images were extracted through the “As displayed” option and are representative of the metrics displayed in **Figure 3**. As can be seen in **Figure 2**, unlike Mock T cells, CD19 CAR T cells were able to specifically kill the spheroids and greatly diminish the number of live tumor cells.

Figure 3 displays the evolution of total green and red signal measured within the spheroid boundaries over time. As can be seen, shortly after CD19 CAR T cell injection, the spheroids’ size shrinks quickly (as measured by green fluorescence signal) and the apoptosis signal increases quickly (as measured by red fluorescence signal).

FIGURE AND TABLE LEGENDS:

Figure 1: **Surface expression of the CD19 CAR and CD19.** A. Surface expression of CD19 CAR on retrovirally transduced T-cells. Cells were analyzed by flow cytometry prior to the spheroid assay *via* anti-mouse-Fab biotinylated as primary and anti-Streptavidin-PE as secondary antibody. B. Surface expression of CD19 on retrovirally transduced HCT116 cells.

Figure 2: **Assessment of CD19 CAR T-cell cytotoxicity against tumor spheroids.** Time lapse of HCT116 spheroid growth; at t=138 h, Mock or CD19 CAR T cells were introduced at a density of 20000 cells/well. Green signal corresponds to HCT116 GFP+ cells and green outline corresponds to the detected spheroid. Red signal corresponds to apoptosis as monitored by Annexin V and red outline corresponds to the detected apoptosis events.

Figure 3: **Measurements of total red and green signal within brightfield mask boundaries over time.** Total red and green signals within brightfield mask metrics were obtained after analyzed and plotted as a function of time. Dashed line corresponds to the introduction of effector cells (CD19CAR or Mock T-cells). Measure corresponds to the mean ± SEM (n=12).

DISCUSSION:

The use of spheroids as an innovative tool to validate future cancer treatment has become a field of growing interest in the past years. Spheroids represent an intermediate step between classical 2D *in vitro* analysis and *in vivo* assessment. The method further holds a lot of promise regarding their potency in terms of tumor micro-environment mimicking as well as gene profiling[7]. The protocol presented in this publication was adapted from Saheen *et al.*[11] to the Incucyte S3 and represents an affordable and easy method to produce 3D tumor spheroids from colorectal cell lines. Coupling spheroid generation to a high-throughput imaging device permits reliable and fast screening of a wide range of anti-tumor agents and monitor the ability of the aforementioned agent to destabilize tumor structure.

There are several critical steps to take into consideration regarding this protocol. First, the starting number of tumor cells needs to be adjusted precisely. If the starting cell density is too high, the cells will degrade the PLL coating rapidly and will start to form an adherent monolayer. On the other hand, a low number of cells will introduce a high variability in terms of spheroid size and the number of spheroids per well. Second, the use of an automated imaging device is exposing cells to potential phototoxicity (especially if several fluorescence channels are used); it is recommended to adjust the image frequency to minimize the damage to the spheroids. It is a critical parameter to consider in order to capture the events of interest reliably (an image every 4 hours before effector cell introduction and one every 90 min after represents the best compromise to our knowledge). Third, we recommend being particularly careful to avoid medium evaporation that can occur a few days after effector cell introduction. Fourth, the number of effector cells needs also to be adjusted precisely: the desired number needs to be high enough to kill tumor spheroids efficiently but also as low as possible to allow the highest potential for optimal imaging process. Fifth, in a typical experiment, a well contains 2 to 4 spheroids that can coalesce into one; it is recommended to pay attention to potential spikes that may erupt in the metrics following this event and to disregard them. Sixth, on a 96-well plate, in average 20 to 50 wells contain spheroids in desired number and size; we recommend checking the wells to be considered for analysis before the introduction of the effector cells as it will save computation time. Finally, the analysis is partially automated but necessitates some inputs from the user regarding the different parameters. It is highly recommended to take particular care in this step and to focus on a balance between sensitivity and specificity. If the type of effector cells/drug treatment differs widely within one experiment, it might be necessary to run different types of analysis to select the one that suits the best.

This method is so far only limited to a certain type of adherent cell line (in this publication HCT 116); further development is required to generalize this protocol to every cancerous cell line. Although a wide range of methods is already available for a greater number of target cells[12-15], most of them rely on the use of expensive and/or complex procedures. Our method, although limited to a few types of target cells so far, is interesting as it requires very limited inputs from the experimenter and permits a fast and easy screen of various drugs and/or immunotherapy treatments (here CAR CD19 cells). Its reliability and its ability to be adapted to different kinds of treatments make this method a powerful tool in the context of anti-cancer treatment validation process.

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DISCLOSURES:

The authors have nothing to disclose.

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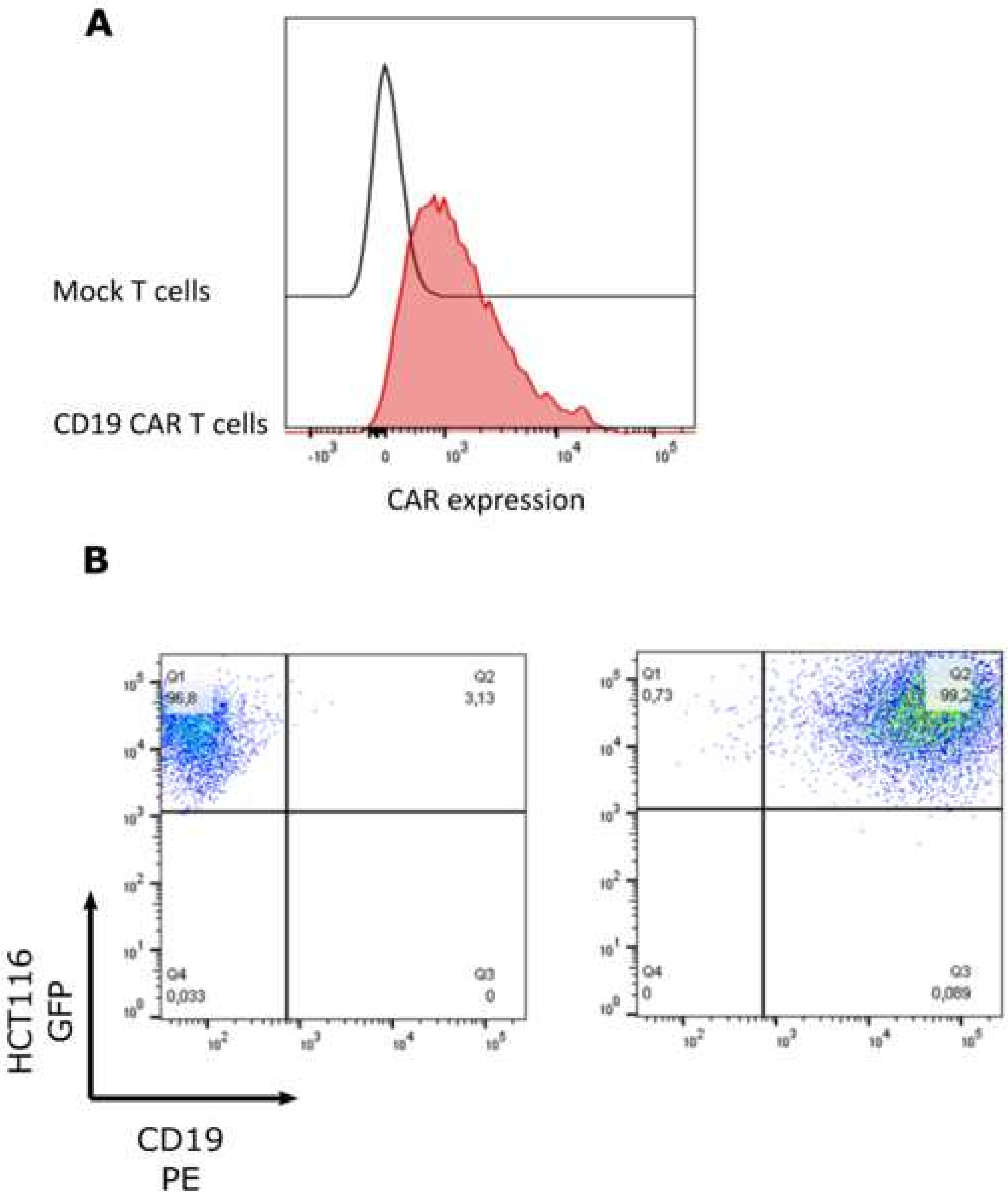
Figure 1


A
Mock T cells
CD19 CAR T cells
-10³
0
10³
10⁴
10⁵
CAR expression
B
Q1
96,8
Q2
3,13
Q4
0,033
Q3
0
10⁰
10¹
10²
10³
10⁴
10⁵
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Q2
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Q4
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Q3
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HCT116
GFP
CD19
PE

Figure 2

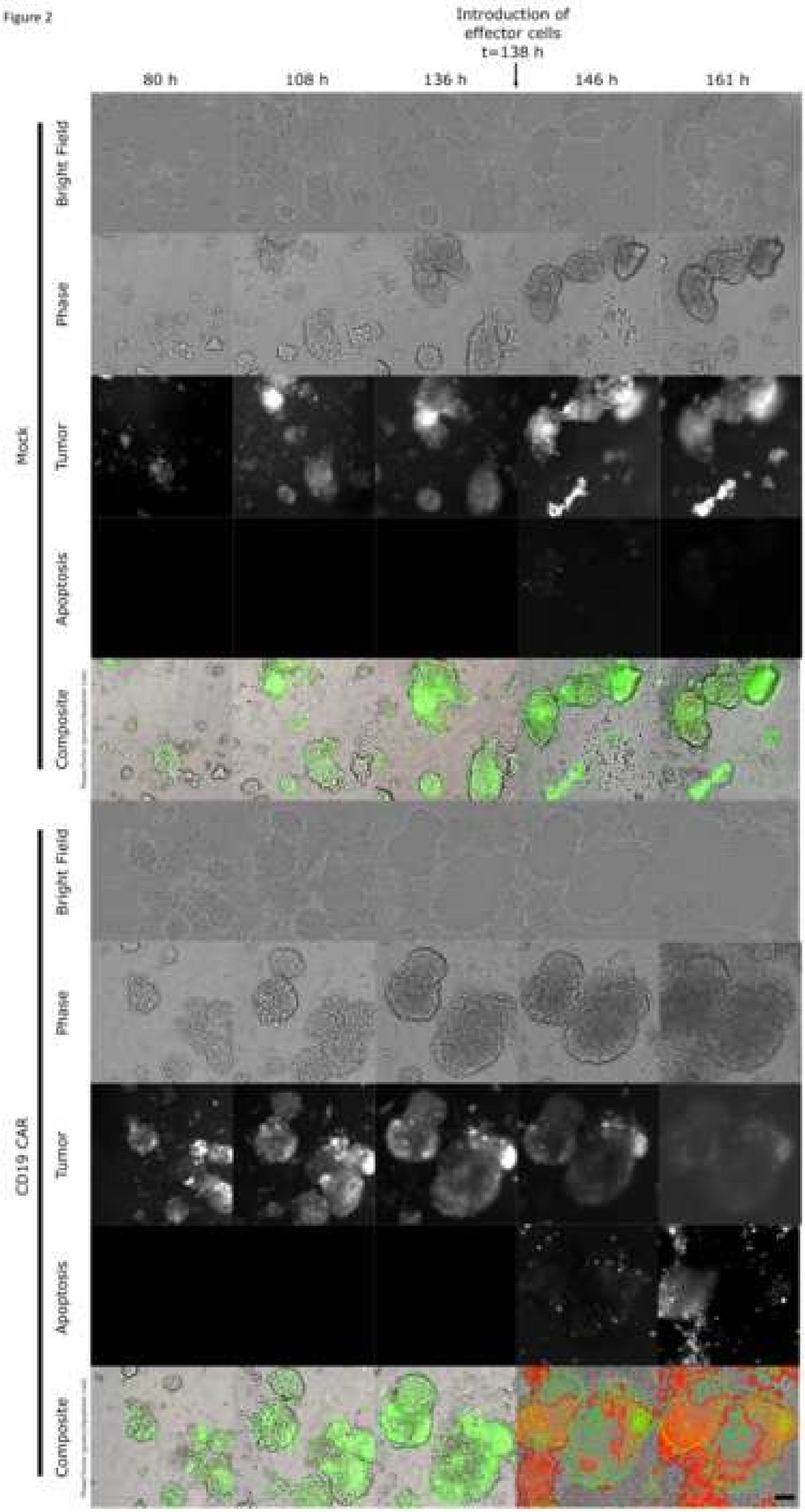
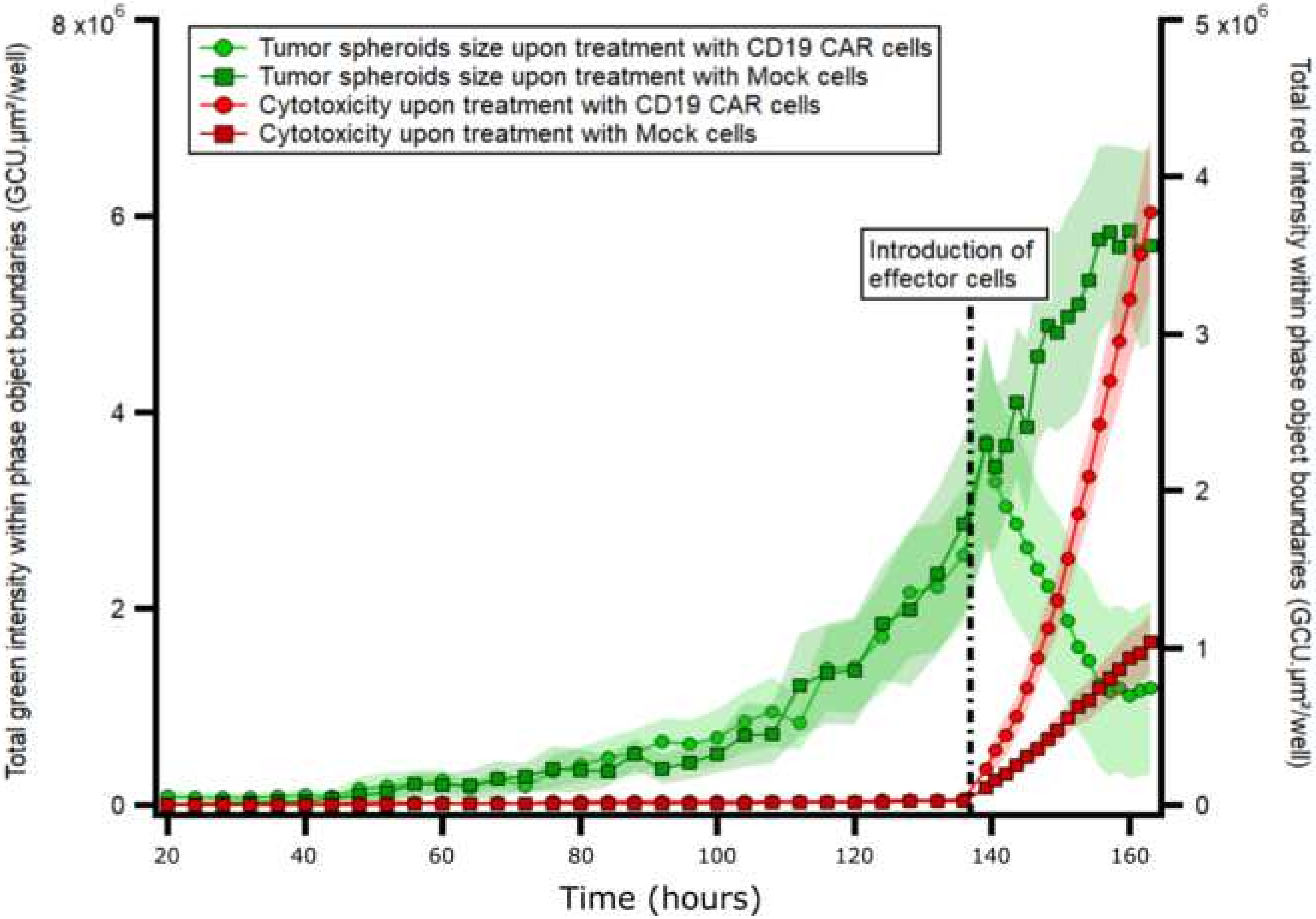


Figure 2
Introduction of effector cells t=138 h
80 h
108 h
136 h
146 h
161 h
Mock
Bright Field
Phase
Tumor
Apoptosis
Composite
CD19 CAR
Bright Field
Phase
Tumor
Apoptosis
Composite

Figure 3


8 x10^6
6
4
2
0
5 x10^6
4
3
2
1
0
20
40
60
80
100
120
140
160
Time (hours)
Total green intensity within phase object boundaries (GCU.µm²/well)
Total red intensity within phase object boundaries (GCU.µm²/well)
Tumor spheroids size upon treatment with CD19 CAR cells
Tumor spheroids size upon treatment with Mock cells
Cytotoxicity upon treatment with CD19 CAR cells
Cytotoxicity upon treatment with Mock cells
Introduction of effector cells

Name of Material/ Equipment	Company	Catalog Number	Lot Numbers
Dulbecco’s Phosphate Buffered Saline	SIGMA-ALDRICH	D8537-500ML	RNBG7037
75 cm^2 growth area flasks	VWR	430639	2218002
75 cm^2 growth area flasks	VWR	734-2705	3718006
Trypsin-EDTA	SIGM-ALDRICH	T3924-100ml	SLBTO777
RPMI 1640 med L-glutamin, 10 x 500 ml	Life Technology (Gibco)	21875-091	1926384
Fetal Bovine Serum	Gibco	10500064	08Q3066K
Gentamicin	Thermo Fischer	15750060	1904924A
Trypan Blue Solution, 0.4%	Thermo Fischer	15250061	1886513
96 well plate, round bottom	VWR	734-1797	33117036
Dynabeads Human T-Activator CD3/CD28	Thermo Fischer	11132D	-
X-VIVO 15 with Gentamicin L-Gln, Phenol Red, 1 L	BioNordika	BE02-060Q	8MB036
CTS Immune Cell Serum Replacement	Thermo Fischer	A2596102	1939319
IL-2 Proleukin	Novartis		505938M
IncuCyte Annexin V Red Reagent	Essen Bioscience	4641	17A1025-122117
Reagent Reservoir	VWR	89094-672	89094-672
15 ml tubes	VWR	734-1867	19317044
anti-human CD19-PE	BD Biosciences	555413	4016990
Biotin-SP (long spacer) AffiniPure $F(ab')_2$ Fragment Goat Anti-Mouse IgG	Jackson ImmunoResearch	115-066-072	129474
Streptavidin-PE	BD Biosciences	554061	5191579
HCT 116 Colorectal Carcinoma Line	ATCC	CCL-247	-
Incucyte S3	Essen Bioscience		

RRID
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Title of Article: Assessment of cancer cells spheroids killing by CAR T cells

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To: the Editor-in-Chief
Jove
Oslo, 13 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.
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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**.