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TITLE:

In vitro Tumor Cell Rechallenge For Predictive Evaluation of Chimeric Antigen Receptor T Cell Antitumor Function

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KEYWORDS:

immunotherapy, adoptive cell transfer, co-culture assay, effector potency, long-term function, t cell exhaustion

ABSTRACT:

Here, we describe an in vitro co-culture method to recursively challenge tumor-targeted T cells, which allows for phenotypic and functional analysis of antitumor T cell activity.

ABSTRACT:

The field of chimeric antigen receptor (CAR) T cell therapy is rapidly advancing with improvements in CAR design, gene-engineering approaches and manufacturing optimizations. One challenge for these development efforts, however, has been the establishment of in vitro assays that can robustly inform selection of the optimal CAR T cell products for in vivo therapeutic success. Standard in vitro tumor-lysis assays often fail to reflect the true antitumor potential of the CAR T cells due to the relatively short co-culture time and high T cell to tumor ratio. Here, we describe an in vitro co-culture method to evaluate CAR T cell recursive killing potential at high tumor cell loads. In this assay, long-term cytotoxic function and proliferative capacity of CAR T cells is examined in vitro over 7 days with additional tumor targets administered to the co-culture every other day. This assay can be coupled with profiling T cell activation, exhaustion and memory phenotypes. Using this assay, we have successfully distinguished the functional and phenotypic differences between $CD4^+$ and $CD8^+$ CAR T cells

against glioblastoma (GBM) cells, reflecting their differential in vivo antitumor activity in orthotopic xenograft models. This method provides a facile approach to assess CAR T cell potency and to elucidate the functional variations across different CAR T cell products.

INTRODUCTION:

Immunotherapy using chimeric antigen receptor (CAR)-engineered T cells has seen promising outcomes against B cell malignancies[1–4], while the potential of targeting other tumors continues to be under rigorous investigation[5–7]. Great progress has been made to optimize the CAR construct, manufacturing process and patient pre-infusion regimens[6-8], and novel synthetic biology approaches are expected to increase their persistence, safety and tumor specificity[9,10]. However, it has been difficult and labor-intensive to appropriately assess the therapeutic potential of CAR T cells in order to guide the best choice for clinical translation. The most established model thus far to evaluate CAR T cell function is in immunodeficient mice bearing human tumor xenografts, whereby CAR T cells are examined for antitumor efficacy and compared at titrated cell doses[11–15]. These in vivo murine studies are labor-intensive and time-consuming, especially when screening large numbers of parameters. Further, in vivo studies can be restrained by the accessibility of mouse strains, animal care facilities and animal-handling techniques. Therefore, there is a need to develop more convenient in vitro assays allowing for quick readouts of effector activity, which also faithfully reflect the in vivo antitumor function of these T cells.

Conventional methods to determine the cytotoxicity of T cells in vitro have focused on the detection of degranulation, cytokine production and the ability to lyse radioisotope-labeled target cells (i.e., chromium release assays). While these assays are informative for defining CAR T cell specificity and redirected target recognition, they often fail to reflect in vivo antitumor potential of engineered T cells[12,13,16]. In certain cases, in vitro killing activity in short term assays showed an inverse correlation with in vivo antitumor function[16]. Such inconsistency is likely the result of high effector:target (E:T) ratios used in these in vitro assays, and therefore the inability to differentiate CAR T cell products that are prone to exhaustion[17]. By contrast, during in vivo tumor eradication T cells usually respond against large tumor burdens, thereby requiring multiple rounds of killing and subsequently driving T cell differentiation and exhaustion[18–20], which is one of the major barriers against effective tumor clearance by CAR T cells[12,13]. Meanwhile, most short-term in vitro killing assays also do not readout differences in T cell proliferation, whereas in CAR T cell treated patients the capacity for CAR T cell expansion is strongly correlated with clinical responses[4]. Thus, the appropriate in vitro assay would need to recapitulate conditions of high tumor burden, induction of T cell exhaustion, and allows for the readout of T cell expansion.

Here we describe a strategy to evaluate CAR T cells for repetitive tumor killing potential, with a simple in vitro co-culture assay. Different T cell effector activity parameters can be simultaneously examined, including target cell killing, CAR T cell expansion and memory- or exhaustion-associated phenotypes. The results generated from this assay correlate well with the in vivo antitumor effect of CAR T cells, and can be exploited to assess the potency of CAR T

cell products. While we describe an assay to evaluate IL13Ra2-targeted CAR T cells against primary GBM lines[21], it can be readily adapted to any CAR T cell platform.

PROTOCOL

Our IRB does not require review of this protocol. We receive discarded fresh glioblastoma tumor samples from the City of Hope Pathology Department that are coded, and our laboratory cannot gain access to the key. We receive the specimens with data only on prior treatment and disease state at the time of biopsy/resection, with no identifying data.

1. Media preparation

1.1. Prepare neural stem cell media for culturing primary GBM cell lines: DMEM:F12, 1:50 B27, 5 µg/mL heparin, and 2 mmol/L L-glutamine; supplemented with 20 ng/mL epidermal growth factor (EGF) and 20 ng/mL basic fibroblast growth factor (FGF) twice a week (see **Table of Materials**).

1.2. Prepare T cell media: X-VIVO 15 containing 10% fetal calf serum (FCS); supplemented with 70 IU/mL rhIL-2 and 0.5 ng/mL rhIL-15 every 48 h (see **Table of Materials**).

1.3. Prepare co-culture media: take neural stem cell media without EGF and FGF supplement, and add 10% FCS.

1.4. Prepare FACS staining solution (FSS): HBSS, 2% FCS, NaN_3 (0.5 g/500 mL).

2. Preparation of GBM tumor cells

2.1. Harvest low-passage GBM tumor spheres (TSs) by centrifugation at 300 x *g* for 4 min and discard supernatant.

NOTE: GBM tumor spheres (TSs) are generated from resected tumors as described previously[22-24], and maintained in neural stem cell media, in incubators with 5% CO_2 at 37 °C.

2.2. Pre-warm co-culture media in a 37 °C water bath.

2.3. Add 1 mL of cold accutase to GBM TSs, dissociate TSs by pipetting for 30-60 s, and stop dissociation by adding 5 mL of warm co-culture media.

NOTE: GBM TSs should not be kept on accutase for more than 5 min.

2.4. Harvest GBM cells by centrifugation at 300 x *g* for 4 min, discard supernatant, and resuspend cells in 2 mL of co-culture media.

2.5. Determine cell concentration using a cell viability counter.

NOTE: Cell viability must be >70%.

3. Preparation of CAR T cells

3.1. Less than 24 h before the assay, take 100 µL of cultured CAR T cells into a flow cytometry tube and add 2 mL of FSS.

NOTE: CAR T cells were generated as described previously[11,21] and cultured in T cell media.

3.2. Centrifugation at 300 x *g* for 4 min and discard supernatant.

3.3. Add 2 mL of FSS to wash the cells, centrifuge at 300 x *g* for 4 min, and discard supernatant.

3.4. Stain the cells with appropriate antibody to indicate CAR expression at 4 °C for 30 min.

NOTE: For example, if an IL13Rα2-targeted CAR described previously[25] is used, then stain with anti-IL13 antibody.

3.5. Wash twice with 2 mL of FSS and analyze CAR exn using a flow cytometer.

3.6. Determine the CAR% on T cells using the gating strategy shown in **Figure 1B**.

3.7. At the day of co-culture, harvest all CAR T cells by centrifugation at 300 x *g* for 4 min, discard supernatant, and resuspend cells in 2 mL of co-culture media.

3.8. Determine cell concentration using a cell viability counter.

NOTE: Cell viability must be >70%.

4. Set up tumor–T cell co-culture

4.1. Dilute tumor cells to a concentration of 0.16 million/mL with co-culture media.

4.2. Based on CAR%, dilute CAR T cells to a concentration of 0.04 million CAR^+ cells/mL with co-culture media.

NOTE: For example, if the CAR is 50% and T cell concentration is 0.4 million/mL, then make a 1:5 dilution to get the final concentration of 0.04 million CAR^+ cells/mL.

4.3. Pipette 100 µL of diluted tumor cells into each well of a 96-well flat-bottom tissue culture plate.

4.4. Pipette 100 µL of diluted CAR T cells into each well that contains tumor cells and mix well.

NOTE: Each tumor-T cell co-culture will be analyzed at 4 time points. 4-6 replicates might be required at every time point based on different analysis, but <3 replicates per time point is not recommended; see **Table 1** for a representative platemap.

4.5. Maintain the plate in a 37 °C, 5% CO_2 incubator.

5. Tumor cell rechallenge

NOTE: Rechallenge takes place at 2, 4 and 6 days post the initial co-culture setup (**Figure 1A**).

5.1. Harvest and dissociate GBM TSs as described above (Steps 2.1-2.6).

5.2. Resuspend GBM cells at a concentration of 0.64 million/mL.

5.3. Determine the co-culture wells that need rechallenge (see **Table 1**) and carefully remove 50 µL media from the top of each well.

5.4. Add 50 µL of GBM cell suspension into each well and mix well, then put the plate back into a 37 °C, 5% CO_2 incubator.

6. Harvest samples and flow cytometric analysis

NOTE: Samples will be harvested at 1, 3, 5 and 7 days post the initial co-culture setup, with 1, 2, 3 and 4 rounds of tumor challenge, respectively.

6.1. Pre-warm 0.05% trypsin-EDTA solution in 37 °C waterbath.

6.2. Determine the wells that need harvesting and transfer the media into a new round-bottom 96-well plate.

6.3. Pipette 50 µL of trypsin-EDTA into the wells to digest remaining tumor cells at 37 °C for 5 min.

6.4. Under a microscope, confirm the cells have detached from the bottom.

6.5. Pipette around the well bottom to resuspend detached cells, then transfer trypsin-EDTA containing detached cells to the corresponding wells of the round-bottom 96-well plate.

6.6. Centrifuge the round-bottom 96-well plate at 300 x *g*, 4 °C for 4 min, then discard supernatant.

6.7. Add 200 µL/well of FSS to wash the cells, centrifuge at 300 x *g*, 4 °C for 4 min, then discard supernatant.

6.8. Resuspend cells in 100 µL/well FSS containing antibodies (see **Table of Materials**), and stain cells at 4 °C for 30 min.

6.9. Add 100 µL/well FSS to cells, centrifuge at 300 x *g*, 4 °C for 4 min, then discard supernatant.

6.10. Add 200 µL/well of FSS to wash the cells, centrifuge at 300 x *g*, 4 °C for 4 min, then discard supernatant.

6.11. Resuspend cells with 100-200 µL/well of FSS with 500 ng/mL DAPI, then analyze samples by flow cytometry.

7. Functional and phen otypic analysis of CAR T cells

7.1. Retrieve the data files from flow cytometer, and gate all live (DAPI-) cells (**Figure 1C**).

7.2. Quantify tumor cells by gating the $CD45^-$ population and CAR T cells by gating the $CD45^+$, CAR+ population (**Figure 1C**).

NOTE: If the tumor cells express CD45 (e.g. Raji lymphoma cells), anti-CD3 staining can be used to differentiate T cells from tumor cells.

7.3. Plot tumor cell and CAR T cell number through the time course.

7.4. Identify T cell activation by the co-expression of 4-1BB and CD69.

NOTE: These surface markers can be used to analyze T cell activation 6-24 h post initial co-culture.

7.5. Identify T cell exhaustion by the expression of PD-1, LAG-3 and TIM-3.

7.6. Identify T cell memory status by the expression of CD45RO and CD62L.

REPRESENTATIVE RESULTS:

Using the assay described above, we have distinguished the differential effector potency and killing dynamics mediated by $CD4^+$ and $CD8^+$ CAR T cells. The results presented here illustrate the difference between $CD4^+$ and $CD8^+$ CAR T cells, generated from a healthy donor independent of our previous publication[21].

Through a standard degranulation assay, we were able to observe that both $CD4^+$ and $CD8^+$ CAR T cells became equally activated against GBM cells that express the targeted antigen, as indicated by the expression of CD107a and intracellular cytokine (**Figure 2A**). However, using the repetitive tumor challenge assay, we found that $CD4^+$ but not $CD8^+$ CAR T cells exhibited the capability of multiple-round killing (**Figure 2B**). $CD4^+$ CAR T cells also achieved better expansion in comparison to CD8+ cells during this assay (**Figure 2C**). The difference of expansion between

CAR T cell subsets were only observed from D3 after two rounds of tumor challenge (1:12 E:T), while the difference of cytotoxicity was observed beginning at D5 after three rounds of tumor challenge (1:20 E:T), indicating that short-term assays failed to elucidate differences in the potency of different CAR T cell products. When the 1:1 mixed $CD4^+$ and $CD8^+$ CAR T cells were applied to this assay, they were found to outperform $CD8^+$ but not $CD4^+$ CAR T cells on long-term cytotoxicity (**Figure 2B**). The expansion of $CD8^+$ CAR T cells was induced by the co-applied $CD4^+$ cells, while $CD4^+$ CAR T cell expansion was inhibited in the presence of $CD8^+$ cells (**Figure 2C**).

To explain the mechanism that distinguishes the effector activity between $CD4^+$ and $CD8^+$ CAR T cells, we first confirm that 24 h after initial co-culture, both $CD4^+$ and $CD8^+$ CAR T cells were comparably activated (**Figure 3A**). Meanwhile, as indicated by CD45RO and CD62L expression, both $CD4^+$ and $CD8^+$ CAR T cells showed a transition from central memory ($CD45RO^+$, $CD62L^+$) to effector memory ($CD45RO^+$, $CD62L^-$) phenotype during repetitive tumor challenge (**Figure 3B**). Then we characterized the cells at D3 of this assay, a time before the CAR T cell subsets started to display functional difference. T cell exhaustion was marked by the co-expression of inhibitory receptors PD-1, LAG-3 and TIM-3[15,21], which showed that $CD8^+$ CAR T cells were more prone to exhaustion compared with $CD4^+$ CAR T cells (**Figure 3C**). Further, no difference was seen on $CD8^+$ CAR T cell exhaustion in the presence/absence of co-applied $CD4^+$ cells (**Figure 3C**), indicating that CD4-induced $CD8^+$ CAR T cell expansion is not associated with a better effector function.

Together, the results from this assay identified that $CD4^+$ CAR T cells outperformed $CD8^+$ cells specifically in long-term cytotoxicity. Despite the similar short-term cytotoxicity (1-3 days, once rechallenged), $CD4^+$ CAR T cells sustained effector function upon repetitive tumor challenge, while $CD8^+$ cells became exhausted and failed to control tumor cell growth. When the two subsets were mixed, $CD4^+$ CAR T cells facilitated the expansion of $CD8^+$ CAR T cells, but the exhaustion status of $CD8^+$ cells were not ameliorated, resulting in the lack of synergistic effect between the two groups. Similar difference between $CD4^+$ and $CD8^+$ CAR T cells was seen in an in vivo model as previously described[21]. Indeed, $CD8^+$ CAR T cells were able to mediate short-term tumor clearance but followed with antigen-positive recurrence; in contrast, $CD4^+$ CAR T cell treatment resulted in long-term tumor eradication[21], which is reminiscent of their repetitive killing potential using the in vitro rechallenge assay.

FIGURE AND TABLE LEGENDS:

Figure 1. Schema and analysis strategy of repetitive challenge assay. (**A**) Schema and timeline of repetitive tumor challenge assay. For each well, CAR T cells were first co-cultured with PBT030-2 GBM cells (4,000 CAR+ cells, 16,000 tumor cells) and re-challenged with 32,000 tumor cells every other day (D2, D4 and D6). Analysis of tumor cell and CAR T cell number, as well as CAR T cell phenotype is carried out at D1, D3, D5 and D7. (**B**) Gating strategy to determine CAR% in T cells before setting up the co-culture. (**C**) Gating strategy of live cells, tumor cells and CAR T cells from the repetitive challenge assay. (**D**) Tumor cells number at different times of the rechallenge assay, co-cultured with untransduced T cells. Error bars = ±SEM.

Figure 2. Repetitive challenge assay reveals differences in killing and proliferative potency between CD4$^+$ and CD8$^+$ CAR T cells. (**A**) Degranulation and intracellular cytokine staining of CD4$^+$ and CD8$^+$ CAR T cells after 5 h of co-culture with GBM cells (E:T=1:1). (**B**) CD4$^+$, CD8$^+$ or mixed (CD4:CD8=1:1) CAR T cells were applied to repetitive challenge assay, and viable tumor cells were quantified. *p < 0.05, **p < 0.01, ***p < 0.001 compared with CD4$^+$, using one-way ANOVA with Bonferroni's Multiple Comparison Tests. (**C**) CD4$^+$ and CD8$^+$ CAR T cell expansion during repetitive tumor challenge assay. Comparison of (left to right) CD4$^+$ vs CD8$^+$, single subset; CD4$^+$ vs CD8$^+$, within the "mixed" group; CD4$^+$, single vs mixed; CD8$^+$, single vs mixed. *p < 0.05, **p < 0.01, ***p < 0.001 using an unpaired Student's t test. Error bars = ±SEM.

Figure 3. Analysis of T cell phenotype during repetitive challenge assay. (**A**) 4-1BB and CD69 staining on CAR T cells at D1 of the assay. (**B**) CD45RO and CD62L staining of CAR T cells before applying to rechallenge assay (input), and at D3 and D7 of the assay. (**C**) Co-expression of PD-1, LAG-3 and TIM-3 on CAR T cells at D3 of the assay. (Top) Gating strategies to identify T cells expressing 1, 2 or 3 inhibitory receptors. (Bottom) Comparison of: CD4$^+$ cells (single subset), CD8$^+$ cells (single subset), CD8$^+$ cells (within the "mixed" group).

Table 1. Representative plate map of rechallenge setup

DISCUSSION:

This repetitive tumor cell challenge assay provides a convenient approach to evaluate CAR T cell functional potency, using an in vitro setup that recapitulates the high tumor burden associated with in vivo tumor models. During this 7-day assay, CAR T cells undergo four rounds of tumor challenge (initial co-culture and 3x rechallenge), creating an environment where exhausted T cells may lose their capability to respond to tumor challenge despite a potent initial response. Tumor cell elimination and CAR T cell expansion represent two fundamental readouts that can be acquired from this assay, while the phenotypes of CAR T cells can be easily analyzed by staining surface or intracellular markers that indicate T cell activation, exhaustion and/or memory. This assay is also convenient to combine with non-biased analysis of CAR T cells transcriptome, proteome or phosphor-proteome[21,26], and T cell polyfunctionality which has been adopted to predict clinical responses[27]. Furthermore, tumor cells can also be tested for their adaptive response against T cell immunity such as cytokine secretion[28]. Since samples are harvested at multiple time points, this assay allows for not only static, but also dynamic analysis of CAR T cell behavior when responding to excess number of tumor cells.

The assay is particularly powerful in comparing the effector potency of CAR T cells targeting the same antigen but with different designs and/or manufacturing processes. We have used this assay to identify that CD4$^+$ CAR T cells were able to mediate superior long-term effector function than CD8$^+$ cells[21]. It was also shown that in vivo CAR efficacy was correlated with the cytotoxicity at later time points (D5-D7) during this assay, further highlighting the necessity of using repetitive tumor challenge for in vitro CAR T cell evaluation. Although GBM cells were used as the example of target cells here, this assay could be easily adopted to a different T cell-tumor combination. Notably, CAR T cell behavior can also vary upon different antigen densities and engineering cancer cells to express various levels of targeted antigens provided the tool to

investigate sequential molecular events upon CAR recognition[29]. Therefore, this assay can also be exploited to examine the activation pattern of certain CAR T cells against different targets.

Since the target cell viability and CAR T cell expansion are two upfront readouts of this assay, it is critical that all the co-cultures start with viable (>70%) tumor and T cells. When performing the rechallenge processes, the action of aspirating media (step 5.3) should not disturb tumor and T cells in the bottom of the wells, in order not to introduce unnecessary variations of cells counts. When performing this assay on suspension target cell lines, it is recommended to harvest remaining cells by centrifugation before tumor cell rechallenge.

One limitation of this assay is that the setup parameters (E:T ratios of initial co-culture and rechallenge) needs to be adjusted based on different CAR-tumor combinations. For example, if the tumor cells express a considerable level of immuno-inhibitory molecules (e.g. PD-L1), a higher E:T ratio is recommended given that the overall killing efficiency is impaired compared with PD-L1-low tumor cells. Before applying the assay to new CAR-tumor combinations, a pilot study is recommended to determine the optimal conditions, which usually allows the most potent CAR T cells tested in the assay to eliminate >80% of tumor cells at the end point. Since direct cell-cell contact is needed for CAR T cell-mediated killing, if the tumor cells were fluorescence-labeled, then the panel of flow cytometric analysis must be adjusted accordingly (e.g. if the tumor cells were GFP-labeled, then any FITC-conjugated antibodies should not be used).

The clinical activity of CAR T cells against B cell malignancies has led to two FDA-approved drugs. However, the response has shown substantial variation across different patients[27,30,31], which was elucidated to correlate with the phenotypic properties of the CAR products[30]. This in vitro repetitive tumor challenge assay further provides an approach to functionally test the CAR effector potency in addition to the phenotypic characterizations and polyfunctionality cytokine production, and allows for higher-throughput screening of clinical products in comparison to in vivo tumor models while retaining the predictive fidelity. Overall, this assay could be used for T cell functional test for CAR T cell design, pre-clinical and clinical development.

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DISCLOSURES:

The CAR construct described in this work have been licensed by Mustang Bio., Inc., for which S.J.F. and C.E.B. receive royalty payments. All other authors declare no potential conflicts of interest.

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Figure 1

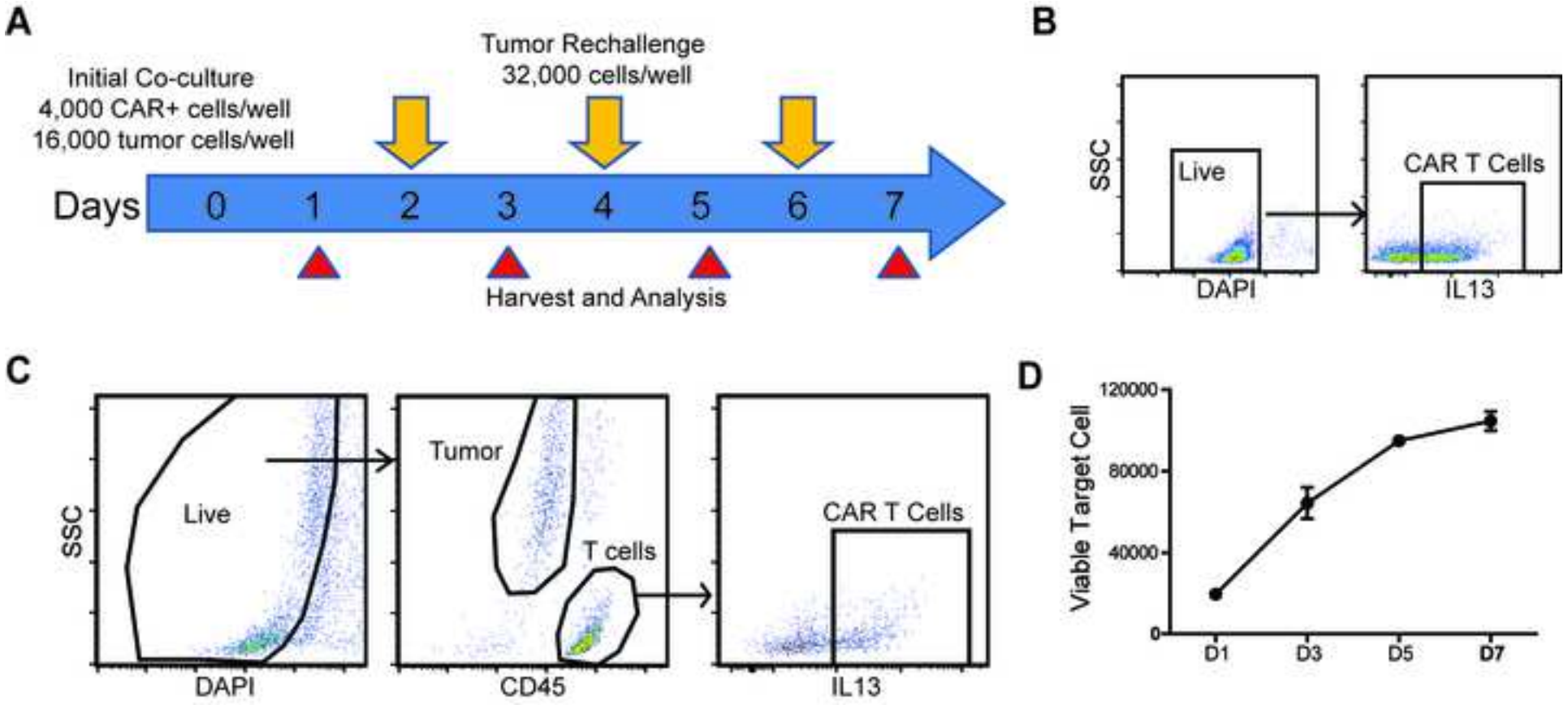

Figure 2

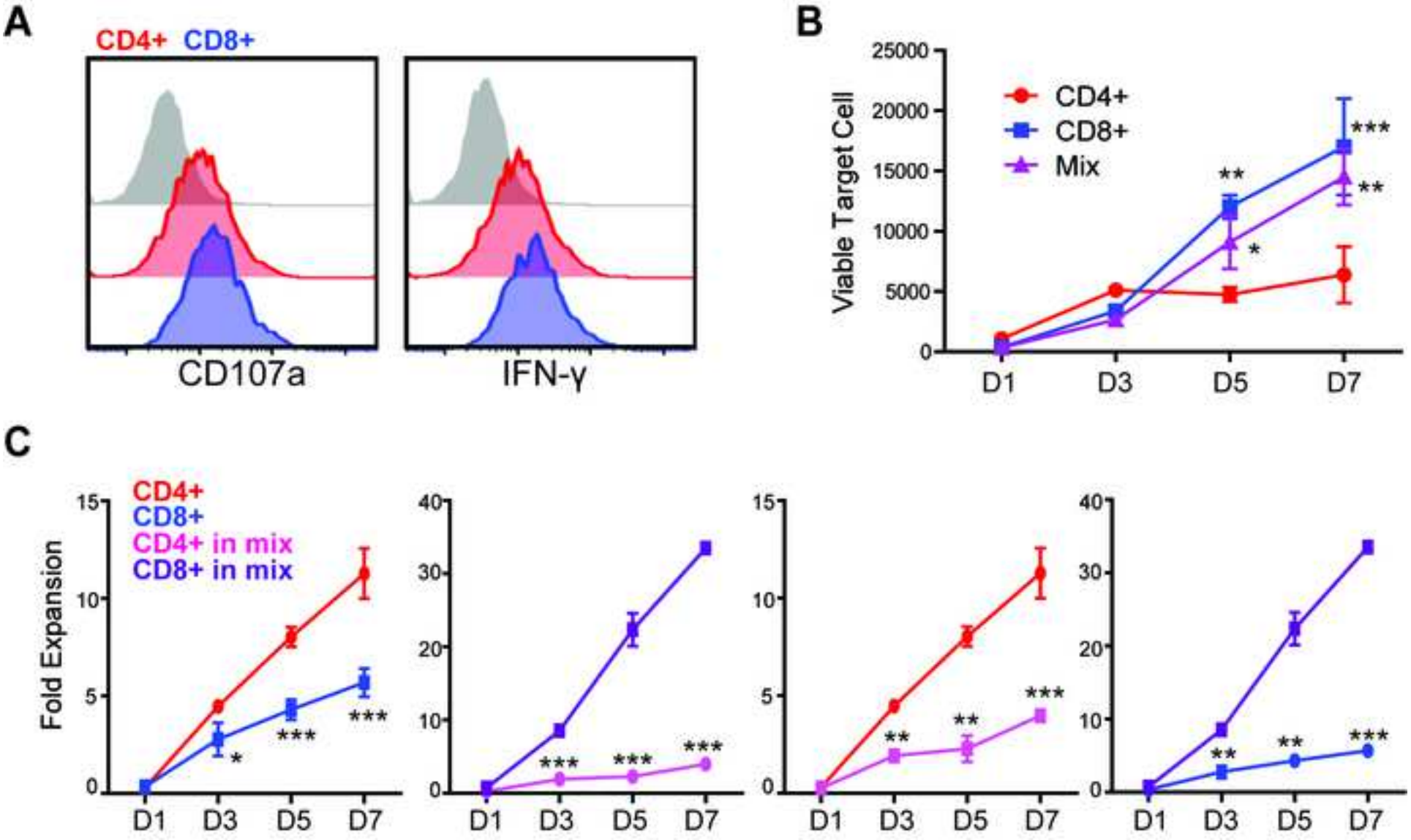

Figure 3

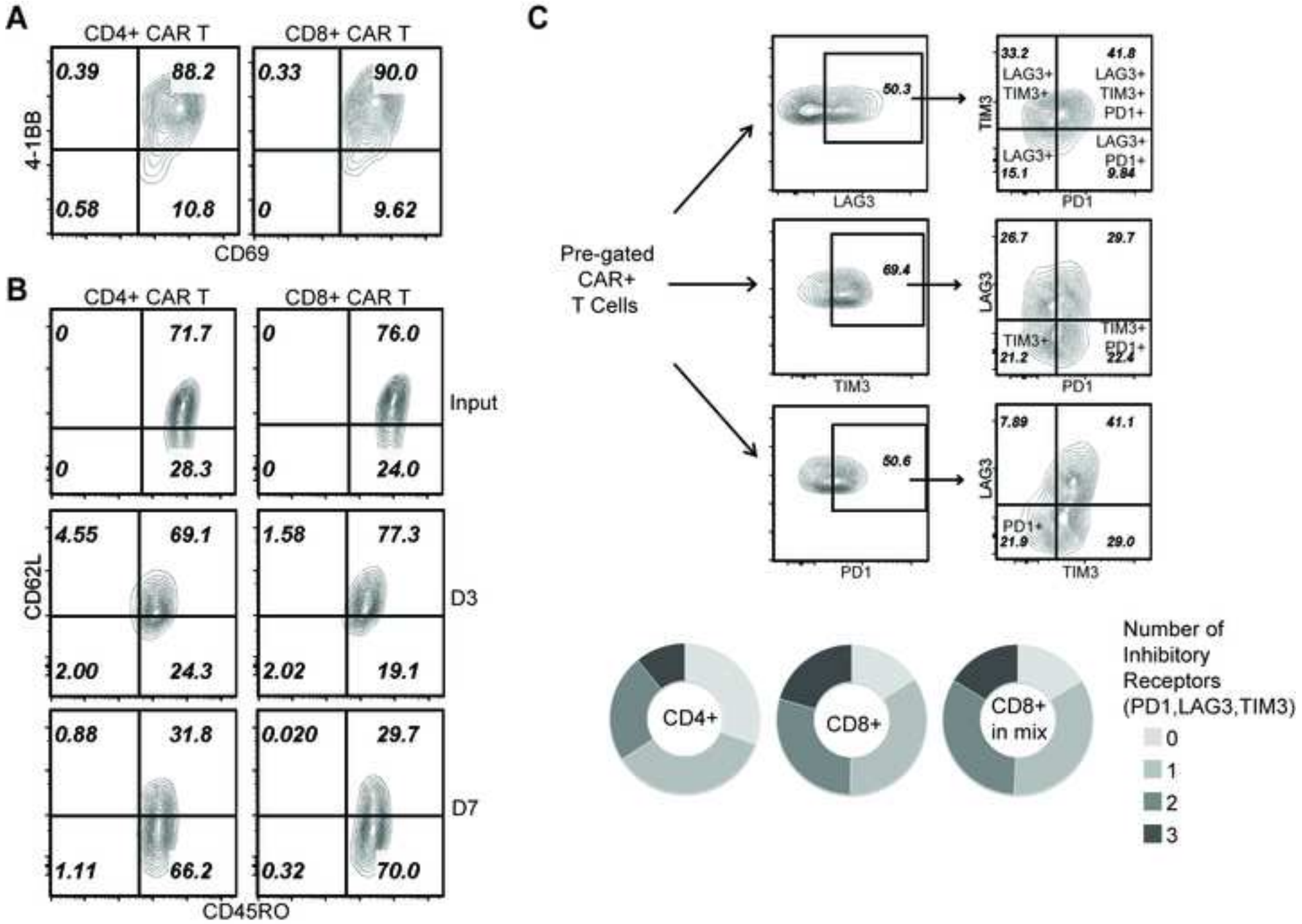

Table 1

	1	2	3	4	5	6	7
A							
B		CD4+ CAR	CD4+ CAR	CD4+ CAR	CD4+ CAR	CD8+ CAR	CD8+ CAR
C		CD4+ CAR	CD4+ CAR	CD4+ CAR	CD4+ CAR	CD8+ CAR	CD8+ CAR
D		CD4+ CAR	CD4+ CAR	CD4+ CAR	CD4+ CAR	CD8+ CAR	CD8+ CAR
E		CD4+ CAR	CD4+ CAR	CD4+ CAR	CD4+ CAR	CD8+ CAR	CD8+ CAR
F							
G							
H							

	No rechallenge; Analyze on D1
	Rechallenge on D2; Analyze on D3
	Rechallenge on D2, 4; Analyze on D5
	Rechallenge on D2, 4, 6; Analyze on D7

8	9	10	11	12
CD8+ CAR	CD8+ CAR			
CD8+ CAR	CD8+ CAR			
CD8+ CAR	CD8+ CAR			
CD8+ CAR	CD8+ CAR			

Name of Material/ Equipment	Company
0.05% Trypsin/0.53mM EDTA in HBSS w/o Calcium and Magnesium	Corning
1M Hepes	Irvine Scientific
200 mM L-Glutamine	Cambrex Bio Science
4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) - FluoroPure grade	Invitrogen
Accutase	Innovative Cell Technologies
Aldesleukin Proleukin (rhIL-2)	Novartis Oncology
Anti-CD137, PE	BD Biosciences
Anti-CD19, PE-Cy7	BD Biosciences
Anti-CD3, PERCP	BD Biosciences
Anti-CD4, FITC	BD Biosciences
Anti-CD45, PERCP	BD Biosciences
Anti-CD45RO, PE	BD Biosciences
Anti-CD62L, APC	BD Biosciences
Anti-CD69, APC	BD Biosciences
Anti-CD8, APC-Cy7	BD Biosciences
Anti-IL-13, PE	BD Biosciences
Anti-LAG-3, PE	eBiosciences
Anti-PD-1, APC-Cy7	BioLegend
Anti-TIM-3, APC	eBiosciences
B-27 Serum-Free Supplement (50X)	Invitrogen
Corning 96 Well Clear Flat Bottom Polystyrene TC-Treated Microplates, Individually Wrapped, with Lid, Sterile (Product #3596)	Corning Life Sciences

Defined fetal bovine serum,HI IR	Hyclone Labs
DMEM F-12 50:50 (1X) w/o Glutamine	MediaTech, Inc.
DMEM High Gluc w/o L-Glu, Na Pyr 1 L	Invitrogen
DMEM-Ham's F12 50:50 Mixture with L-glutamine and 15 mM HEPES	Fisher Scientific
HBSS	Irvine Scientific
Heat-Inactivated FCS	Hyclone
Heparin Sodium(1,000 U/ml)	American Pharmaceutical
MACSQuant	Milteni Biotech Inc.
PBS 1X W/CA & MG	Irvine Scientific
PBS with 1MM EDTA (No Ca2+ or Mg2+)	VWR Scientific Products
Recombinant human EGF	R&D Systems
Recombinant human FGF	R&D Systems
rhIL-15 Working Dilution (10 ng/µL)	CellGenix, US Operations
Sodium Azide (NaN_3)	Sigma
Sorvall ST40R	Thermo Scientific
TC-HEPES BUFFER (1M) SOLN 100ML	IRVINE SCIENTIFIC CORP
Tecnol Procedure Mask	Kimberly-Clark
X-VIVO 15	Lonza

Catalog Number	Antibody Clone
MT25051CI	
9319	
17-605E	
D21490	
AT104	
NDC 0078-0495-61	
555956	4B4-1
557835	SJ25C1
340663	SK7
340133	SK3
340665	2D1
561137	UCHL1
559772	DREG-56
340560	L78
348793	SK1
340508	JES10-5A2
12-2239-41	3DS223H
329921	EH12.2H7
17-3109-42	F38-2E2
17504-044	
3596	

SH30070.03IH	
15-090-CV	
11960051	
MT10092CV	
9224	
SH30070.03	
401811B	
BIS013220	
9236	
PB15009A	
236-EG-200	
233-FB	
tF0297	
S8032	
41693700	
6472	
47117	
BW04-744Q	

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Author(s):	Dongrui Wang, Renate Starr, Darya Alizadeh, Xin Yang, Stephen J. Forman, Christine E. Brown

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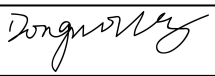
Signature:	
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Re: Revision for JoVE59275 - [EMID:310a099d8b69baa3]

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.

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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