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TITLE:

Generation of Chimeric Antigen Receptor T Cells Targeting Solid and Hematological Cancer Cells Assessed by a Real-Time Cytotoxicity Potency Assay

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SUMMARY:

We describe a quantitative real-time in vitro cytolysis assay system to evaluate the potency of chimeric antigen receptor T cells targeting liquid and solid tumor cells. This protocol can be extended to assess other immune effector cells, as well as combination treatments.

ABSTRACT:

Chimeric antigen receptor (CAR) T-cell therapy for cancer has achieved significant clinical benefit for resistant and refractory hematological malignancies such as childhood acute lymphocytic leukemia. Efforts are currently underway to extend this promising therapy to solid tumors in addition to other hematological cancers. Here, we describe the development and production of

potent CAR T cells targeting antigens with a unique or preferential expression on solid and liquid tumor cells in conjunction with a highly sensitive and real-time in vitro potency assessment that can inform the potency of the different CAR T cells. Specifically, the impact of different costimulatory signaling domains, such as glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related protein (GITR), on the in vitro potency of CAR T cells is examined. This report includes protocols to generate CAR T cells for preclinical studies using lentiviral gene transduction, to expand CAR T cells, to validate CAR expression, and to utilize impedance-based real-time cellular analyzer monitoring to assess the potency of CAR T cells in vitro.

INTRODUCTION:

In recent years, CAR T-cell therapy has been one of the most prominent breakthroughs in cancer immunotherapy for relapsed and refractory hematopoietic malignancies. With the recent U.S. Food and Drug Administration (FDA) approval of CD19-directed CAR T cells for acute lymphoblastic leukemia, non-Hodgkin lymphoma, and diffuse large B-cell lymphoma and the designation of breakthrough therapy for B-cell maturation antigen (BCMA)-directed CAR T cells for multiple myeloma, this technology has generated great excitement in the scientific community and has spun numerous basic, applied, and clinical studies worldwide[1–5]. In January of 2019, more than 700 clinical trials were registered in the clinical trial database (clinicaltrials.gov); about 450 of these trials were either about to start or actively recruiting patients. Most of the clinical trials are focused on hematological malignancies, and clinical trials utilizing CAR T cells targeting CD20, CD22, and BCMAs, in addition to CD19, are ongoing as well[6,7]. While most of the trials are using autologous CAR T-cell therapy, a significant number of them are also exploring the utility of allogenic CAR T cells[8–10]. Despite promising results with hematological malignancies, the application of CAR T cells directly targeting solid tumors has proven to be much more difficult in the clinic for a variety of reasons, including but not limited to the lack of good targets that are exclusively expressed in the tumor but are absent in other normal cells, the heterogeneity of solid tumors and tumor “escape”, and a difficult to access the tumor microenvironment by CAR T cells[11–15]. Therefore, there is a need for the development of CAR T cells targeting solid tumors, which can overcome the critical barriers to efficacy and on target-off tumor toxicity. While a multitude of in vitro and in vivo approaches are warranted to design and test CAR T cells, a robust and predictive in vitro potency assay is critical for the development of CAR T-cell therapies[16,17].

In order to assess the potency of CAR T cells, various in vitro methods have been developed. In general, these potency assays can be divided into two broad categories, depending on whether they directly measure the cytolytic activity of CAR T cells toward target tumor cell lines or surrogate markers released by CAR T cells, such as cytokine release. Techniques encompassing the measurement of direct cytolytic activity include radioactive-based methods such as the chromium-51 release assay (CRA) by target cells[18], an image-based assay which measures apoptosis of target cells via fluorescent probes[19,20], and a flow cytometry assay that detects apoptosis of target cells[21]. In these assays, typically, CAR T cells are cocultured with target cells which have been labeled by radioactive or fluorescent probes followed by appropriate measurement. The gold standard in the field is the CRA, which is considered to be a very sensitive assay. However, the CRA has certain drawbacks. First, it is an endpoint assay and does not provide

kinetic information. The target cells need to be labeled with chromium-51 (Cr51) which tends to leach out of the cells and can significantly increase the background noise[22]. Lastly, as a radioactive assay, it requires proper precautions and disposal of radioactive waste. Orthogonal assays, which measure byproducts of CAR T-cell interaction with target cells as an indication of potency, include the quantitation of various cytokines released by CAR T cells and using either flow cytometry-based methods or enzyme-linked immunosorbent assay (ELISA)-based methods. Once again, these assays are endpoint assays which measure the cumulative release of the cytokines at a given time point and, thus, may not necessarily be reflective of the actual cytolytic activity of the CAR T cells.

When developing any kind of potency assay, particularly for release criteria of cellular therapies such as CAR T-cell therapy, it is paramount to develop an assay which requires minimal reagents and hands-on time. Every interaction or addition of reagent is another variable that needs to be accounted for and can contribute to overall assay robustness and consistency. Furthermore, the interaction of CAR T cells with the tumor cells is a dynamic process, and any assay that is able to provide information related to this dynamic interaction, such as the rate of cytolysis, would be an important aspect of potency measurement. With these criteria in mind, we developed a label-free kinetic potency assay for CAR T cells. This assay utilizes the real-time cell analysis (RTCA) platform which is based on noninvasive impedance measurement. The principal of the RTCA platform is to utilize gold microelectrodes embedded in the bottom of microtiter wells (E-Plates) for measuring the impedance value of the adherent cells or suspension cancer cells tethered to the bottom of the plate well[17,23–28]. The measurement and data acquisition are programmable and user defined with respect to frequency and duration. The workflow is simple and involves, first, seeding the target cells into the wells of E-Plates, followed by the addition of the CAR T cells at different effector-to-target ratios (**Figure 1**). This step is basically similar to other current methods stated above. Then, impedance electrodes monitor the viability of the target cells in a temporal manner and the data is automatically displayed in real-time. Since the CAR T cells remain mostly in suspension, their contribution to the impedance signal is minimal and can be accounted for.

The RTCA assay has been validated for potency monitoring by measuring the cytolytic activity of natural killer (NK) cells, T cells, CAR T cells, checkpoint inhibitors, bispecific antibodies, oncolytic viruses, and some combinations[17,29–34]. Recently, the application of this potency assay integrated with T-cell receptor (TCR)-engineered T-cell manufacturing for clinical use has also been evaluated[35]. Here we report the application of this platform for the evaluation of the in vitro potency of CAR T cells designed to target solid tumors and liquid tumors for clinical therapies.

PROTOCOL:

1. Generation of CAR-encoding lentivirus

NOTE: Once the specific CAR T-cell plasmid construction is completed (CD47 and others), lentiviral CARs are generated by the standard procedure, using 293 FT cells, a lentiviral packaging mix, and transfection agents (see the **Table of Materials**) as described[29]. Subsequently, use a

quantitative reverse transcription polymerase chain reaction (RT-PCR) kit and the thermal cycler (see the **Table of Materials**) to determine the virus titer by measuring the lentiviral RNA amount according to the manufacturer's protocol. It is important that all the procedures should be carried out accordingly, following safety requirements.

1.1. Seed 15 x 10^6 HEK293FT cells in Dulbecco's modified Eagle's medium (DMEM) culture medium and incubate the cells overnight at 37 °C in one 150 mm dish inside a humidified 5% CO_2 incubator.

1.2. Prepare two 15 mL tubes with transfection complex. The first tube contains lentiviral vector plasmid DNA (5 µg) and lentiviral packaging mix (22.5 µg) in 2.5 mL of transfection dilution solution. The second tube contains 82.5 µL of transfection reagent in 2.5 mL of transfection dilution solution (see the **Table of Materials**).

1.3. Pipet the contents of tube 1 into tube 2, and incubate the mixture at room temperature for 15 min.

1.4. Transfer the contents of the tube dropwise to the dish of HEK293FT cells and incubate the sample overnight at 37 °C in a humidified 5% CO_2 incubator.

1.5. Replace the existing medium with 19 mL of fresh DMEM culture medium and continue to incubate the cells overnight inside the humidified 5% CO_2 incubator at 37 °C.

1.6. Transfer the medium from the dish to a 50 mL centrifuge tube. Keep the tube with the virus-containing medium in the refrigerator.

1.7. Repeat the above procedure to add fresh DMEM and collect it again after 1 day.

1.8. Combine two collections of the media into one centrifuge tube. Centrifugate the tube at 2,000 x *g* for 30 min at 4 °C.

1.9. Transfer most of the lentivirus-containing supernatant to an ultraclear centrifuge tube. Leave a minimum volume, about 1 mL, of the supernatant to avoid disturbing the pellet.

1.10. Ultracentrifuge the supernatant at 110,000 x *g* for 100 min at 4 °C.

1.11. Remove the supernatant carefully and gently add 100 µL of DMEM medium to the pellet at the tube bottom. Leave the tube on ice for 15 min. Mix the solution gently and aliquot the lentivirus solution into prechilled sterile tubes. Store these virus stock tubes in a -80 °C freezer.

1.12. Use the quantitative RT-PCR kit to determine the titer of the lentivirus according to the manufacturer's protocol, which extracts and measures lentiviral RNA.

2. Generation and expansion of CAR T cells

2.1. Activate previously frozen human PBMCs (about 1 x 10^6 to 2 x 10^6 cells) in 1 mL of CAR T-cell medium with an equal number of CD3/CD28-coated microbeads (see the **Table of Materials**) and incubate the cells at 37 °C in a humidified 5% CO_2 incubator for 24 h.

2.2. Thaw an aliquot of the lentivirus stock on ice.

2.3. Add 1 µL of transduction enhance agent into the well with the cells and mix.

2.4. Add lentivirus to the cells at a multiplicity of infection (MOI) of 5:1 and mix gently. On the next day, repeat this step (24 h after the first transduction).

2.5. Monitor the T cell growth every 2–3 days. Add more fresh CAR T-cell medium to maintain the cells at the density of 1 x 10^6 to 2 x 10^6 cells/mL.

2.6. Freeze down the CAR T cells with a standard protocol using frozen solution (see the **Table of Materials**).

2.7. Thaw CAR T cells using the standard method and preculture them in the CAR T-cell medium for about ~2–4 h with IL2 (300 units/mL) before applying them to the assay.

3. Detection of CAR expression by flow cytometry

3.1. Transfer 3 x 10^5 CAR T cells and nontransduced T cells to two separate 1.5 mL microcentrifuge tubes.

3.2. Centrifuge the tubes at 300 x *g* for 2 min and resuspend the cells in 200 µL of fluorescence-activated cell sorting (FACS) buffer containing 1% human serum.

3.3. Pipet 100 µL of cell solution into two 5 mL polystyrene FACS tubes and keep the tubes on ice for 5 min.

3.4. Add 1 µL of biotinylated $F(ab')_2$ fragments of goat anti-mouse $F(ab')_2$ to one tube of each cell type. Then, add 2 µL of PE-labeled anti-tag antibody (see the **Table of Materials**) to the other tube of each cell type. Mix well and incubate them for 30 min on ice.

3.5. Wash the cells with 3 mL of FACS buffer in each tube and centrifuge the tubes at 300 x *g* for 5 min; discard the supernatants and vortex very briefly or shake the tubes briefly to resuspend the cells in the residual liquid.

3.6. Add 2 µL of APC anti-CD3 and 2 µL of 7-AAD antibody solution (see the **Table of Materials**) in each tube. In the tube of cells stained with anti-$F(ab')_2$ Ab, add 1 µL of PE-labeled streptavidin. Mix briefly and incubate the tubes on ice for 30 min more.

3.7. Use FACS buffer to wash the cells again as described in step 3.5 and add an additional 200 µL of FACS buffer to each tube.

3.8. Use flow cytometry to analyze the cells by first gating on the T cells in a forward scatter vs. side scatter plot and then gating on the alive cells (7-AAD-negative) in a CD3 vs. 7-AAD plot. The final step is to analyze anti-tag, anti-ScFv or anti-F(ab')$_2$ vs. CD3.

4. Real-time cytolysis potency assay

NOTE: Perform the RTCA assay according to the manufacturer's recommended conditions as described. In brief, first plate the target cells in the wells of the E-Plate, followed by the addition of CAR T cells on the next day. The cytolysis activity of CAR T cells against target cells is monitored in real-time. T cells and mock-transduced T cells (Mock CAR T cells) are used as negative effector cell controls. The following protocol describes an in vitro real-time cytolysis potency assay for adherent tumor cell lines.

4.1. Add 50–100 µL of target cell culture medium to each well to measure the E-Plate medium background in the RTCA system and bring the E-Plate out to add the cells.

4.2. Use a standard protocol to trypsinize target cancer cells from the culture device. Then, transfer the cells to a 15 mL centrifuge tube and add fresh culture medium, up to 15 mL. Pellet the cells by centrifugation for 5 min at 200 x *g*. Discard the supernatant and add 5 mL of fresh medium to gently resuspend the cell pellet by a serological pipette. Count the living cell density with a hemocytometer under the microscope.

4.3. Adjust the cell density and add the cell suspension to each well of the E-Plate 96 in a volume of 100 µL. The target cell number in each well is about 10,000 cells for adherent cell lines, BxPC3, Hela-CD19, and SKOV3, or 30,000 cells per well for Raji (see below for details to precoat the E-well).

4.4. Equilibrate the E-Plate at room temperature for about 30 min to allow the cells to settle evenly on the bottom of the well.

4.5. Place the E-Plate 96 to the RTCA system inside the cell culture incubator and start to measure impedance, displayed as cell index vs. time, automatically every 15 min overnight.

4.6. Prepare effector CAR T cells on the following day. Make sure to include the controls (i.e., Mock CAR T cells, nontransduced control T cells, and/or unrelated CAR T cells) ahead of time to ensure all the cells are ready at this step. Bring the effector cells and the controls to the proper concentration in several tubes for seeding the cells at proper E:T ratios in 100 µL of medium (i.e., 1,000,000 cells/mL for an E:T ratio of 10:1).

4.7. Pause the automatic acquisition in the RTCA system and bring the E-Plate 96 from the incubator to a cell culture hood.

4.8. Remove 50–100 µL of medium from each well so the leftover medium volume is 100 µL.

4.9. Add 100 µL of serially diluted effector CAR T cells or other control cells (i.e. Mock CAR T cells) at desired E:T ratios to the wells of all the treatments.

4.10. Equilibrate the E-Plate at room temperature for about 30 min and engage the E-Plate back to the system; then, resume the recording of the system.

4.11. Pause the monitoring of the system at any time to retrieve the E-Plate and take small samples for other orthogonal assays (i.e., measuring cytokine production by ELISA or analysis by flow cytometry).

4.12. In the EGFR-GITR-CD3 CAR T-cell experiment, measure INFγ yield with an ELISA kit based on the sample measuring value and the generated standard curve (follow the instructions of the manufacturer; see the **Table of Materials**).

NOTE: For testing hematological cancer cells—the B-cell lymphoma suspension cell line—Raji is tethered to the E-Plate first, using the (anti-CD40) liquid tumor killing assay kit (see the **Table of Materials**). Briefly, the E-Plate is coated with the anti-CD40 antibody before performing step 4.1. Below is the procedure to coat the plate.

4.12.1. Dilute the tethering reagent (anti-CD40) with tethering buffer at the concentration of 4 µg/mL.

4.12.2. Add 50 µL per well of the diluted tethering reagent to the E-Plate inside the cell culture hood and leave the E-Plate at room temperature or in a 37 °C incubator for 3 h.

4.12.3. Remove the tethering reagent and wash the E-Plate at least 2x with wash buffer. At this point, the E-Plate is ready for seeding the Raji target cells (30,000 cells/well).

4.12.4. Continue with step 4.1 to perform the rest of the procedure.

REPRESENTATIVE RESULTS:

CAR lentivirus preparation and CAR T-cell generation and potency assessment

The titers of the CAR lentivirus preparations were determined by quantitative RT-PCR kit (see the **Table of Materials**) according to the manufacturer's protocol. The titration protocol extracted virus RNA first and then measured the lentiviral RNA copy number, which indicated the amount of infectious viral particles. The titer of virus generated from one 150 mm dish from the above protocol usually ranges between 10^9–10^{10} viral copies/mL. **Figure 2A** shows the RT-PCR cycle number vs. the signal strength from a representative quantitative PCR result. Once the virus quality was satisfied—the titer was larger than 1 x 10^8 pfu/mL—it was frozen down for subsequent T cell transduction. After the CAR T cells were transduced with lentivirus, the T cells

were further cultured for about 12–14 days by maintaining their density around 1 x 10^6 to 2 x 10^6 cells/mL. The CAR T cells were then checked with anti-ScFv-specific antibody with a flow cytometer before a downstream application or freeze down. A good representative batch result is shown in **Figure 2B**. Using an anti-ScFv antibody, about 50% of the CAR T cells stained positive (Q2 50.6% vs. T cells 1%), indicating the expression of CAR in about 50% of the T cells. Subsequently, the RTCA potency assay was performed for cytolysis determination before each batch of CAR T cells was frozen down and ready for future application. One cycle of the CAR T-cell design, generation, and assessment procedure took about 1 month.

Killing of Raji lymphoma B cells by CD22-CAR T cells

The anti-CD40 liquid tumor tethering kit (see the **Table of Materials**) was used at a concentration of 4 µg/mL to coat an E-Plate for 3 h at 37 °C. After washing the wells with tethering buffer, B-cell lymphoma cells were added to the E-Plate at a density of 30,000 cells. The E-Plate was kept with the cells at ambient temperature for 30 min and then it was engaged to the RTCA system inside the incubator. Impedance measurements controlled by the presetting program and real-time cell index readings reflected the cell attachment and growth. The following day, CD22-CAR T cells were added to the treatment wells along with the controls of Mock CAR T cells and T cells without transduction. In **Figure 3**, an E:T ratio of 10:1 was used for all groups. Raji cells, CD22-positive, treated CD22-CAR T cells (curve in green) displayed significant killing in comparison with controls of T cells only and Mock CAR T cells.

Effective killing of pancreatic cancer cells by CD47-CAR T cells

CD47 is a transmembrane surface glycoprotein of the immunoglobulin superfamily. As an integrin-associated protein, it is highly expressed in both hematological cancers (leukemia, lymphoma, and multiple myeloma) and solid cancers (such as ovarian, small cell lung cancer, pancreatic, glioblastoma) and other types of cancers[36,37]. CD47 is also known as a do-not-eat-me signal to macrophages of the immune system which has made it a potential therapeutic target in some cancers. CD47-CAR T cells were produced and tested in BxPC3 pancreatic cancer cells which have a high-level expression of CD47[38,39]. BxPC3 cells were seeded in the RTCA system at day 1 and real-time monitoring showed that 10,000 cells per well of BxPC3 reached confluency after 16 h, and then CAR T cells were added at the E:T ratio of 10:1 per well (**Figure 4A**). Control effector cells like nontransduced T cells and Mock CAR T cells were added as well. The result showed that CD47-CAR T cells were selectively killing target BxPC3 cells[29]. Also, **Figure 4B** shows that the impedance signal from CD47-CAR T cells alone was significantly low when compared to target BxPC3 cells. The cell index value from wells with CD47-CAR T cells alone reached a maximum of 0.14. This was only slightly above the cell index signal from medium-alone wells, which read at 0.02. It means that the impedance signal resulting from suspension cells such as CAR T cells alone was minimal and was not contributing to the overall impedance signal when CAR T cells were added to the target cancer cells. The noise level signal from effector suspension cells enabled RTCA to measure, selectively, target cancer cells only within these mixed cell populations.

Costimulation of CAR T cells against cancer by GITR domain inside CAR

The GITR costimulatory domain has been previously reported to enhance the killing of EGFR-positive cancer cells SKOV3 but not EGFR-negative cancer cells MCF-7 when expressed in an

EGFR-GITR-CD3 CAR (data is not shown)[30]. To better clarify the role of the GITR domain, CAR constructs containing the full-length GITR domain or deleted or rearranged versions of the domain were generated and tested for the ability to coactivate CAR T cells (**Figure 5A**). **Figure 5B,C** shows results generated from the RTCA system in which the GITR costimulatory domain was proven to be more effective in enhancing CAR T cells killing against EGFR-positive target cells when compared to the original CAR construct without the GITR domain. Furthermore, a 10 amino acids deletion of the GITR domain (amino acids 184–193) failed to display the increase of this activity, while the relative shift in position between the CD3 and the GITR domain resulted in a reduced enhancement. The end time point data (**Figure 5B**) was comparable to IFNγ production as a surrogate of T-cell activation with similar results (24 h). The real-time cell index profiling obtained from the RTCA impedance system clearly displayed the killing kinetics and the difference between treatments (**Figure 5C**). The in vivo study also confirmed this result[30].

FIGURE LEGENDS:

Figure 1: The real-time cell analysis (RTCA) system detects the killing of target cells by effector cells. There are three main conceptional steps to measuring the killing activity of CAR T effector cells against target cancer cells. Step 1: Seed the target cells (i.e., tumor cells) into the well of one E-Plate. The cells will attach to the gold microelectrodes and this impedes the electric current flow between the electrodes. This impedance value is measured and plotted as a unitless parameter, or cell index. The cell index value increases as the cells grow and reaches a plateau as the cells approach confluency. Step 2: Effector cells—nonadherent immune cells—are subsequently added. Because these cells do not adhere to the gold microelectrodes, they do not directly cause an impedance change. Step 3: If the effector cells attack the target cancer cells, the destruction of the tumor cells is reflected by a decreasing of the cell index dynamically. This cytolytic activity of the effector cells, or the potency of the effector cells, can be sensitively and precisely monitored. The continuous acquisition of impedance data from the RTCA system enables the real-time killing kinetic analysis for multiple conditions simultaneously.

Figure 2: Titer determination of the lentivirus and flow cytometry evaluation of the CAR T cells. (**A**) Follow the instructions from the lentivirus quantitive RT-PCR kit, prepare the RNA, and perform the quantitative RT-PCR. The different color lines are representative samples in cycle number vs. delta Rn. The low cycle numbers indicate the relatively high amount of the RNA template present in the samples. (**B**) After transduction, CAR T cells were cultured and maintained at a density of less than 2 x 10^6 cells/mL and then subjected to flow analysis. The *y*-axis shows the staining for T cells, the positive value reflected in the areas of Q1 and Q2. Both of the samples show 100% positive for T cells. The expression of CAR scFV is determined by FACS with scFv-specific Ab (*x*-axis). The result shows that more than 50% of the T cells are scFv positive in CD22-CAR T cells.

Figure 3: Killing dynamics of CD22-CAR T cells against Raji Burkitt's lymphoma cells. The red curve is the Raji cells alone and the green curve is the Raji cells treated by CD22-CAR T cells. The pink curve is the Mock CAR T-cell treatment and the blue curve is the T-cells-only treatment. The E:T ratio is 10:1. For all figures with RTCA results, the standard deviation bar is labeled in each

treatment curve. The time scale is set up at 2 h intervals for easy display although more data points data are available.

Figure 4: Efficacy of CD47-CAR T cells against pancreatic solid tumor cells. (**A**) Pink is the target BxPC3 cells alone, while red is BxPC3 with CD47-CAR T cells added. Green is BxPC3 with the addition of nontransduced T cells only and blue is with Mock CAR T cells. The E:T ratio is 10:1. (**B**) In the same setting, red is control wells with CD47-CAR T cells only and green is the medium only.

Figure 5: GITR domain increases CAR T cells cytotoxic activity against EGFR-positive tumor cell lines. (**A**) The construction of the CAR. (**B**) The bar graphs of % cytolysis at 4 h (left) and 24 h (middle) resulted from different CAR constructions, along with the IFNγ production at 24 h (right). (**C**) Overtime RTCA killing kinetics for all treatments. The black curve is the growth curve of BxPC3 ovarian cancer cells only. The green curve is the target cells treated with T cells only, the blue curve is the target cells treated with Mock CAR T cells, the red curve is the target cells treated with EGFR-GITR-CD3-CAR T cells, the pink curve is the target cells treated with EGFR-ΔGITR-CD3-CAR T cells, and the brown curve is the target cells treated with EGFR-CD3-GITR-CAR T cells.

DISCUSSION:

Chimeric antigen receptors are multidomain proteins composed of an extracellular single-chain variable fragment (scFv region) derived from the variable domain of one antibody which recognizes a specific antigen, a hinge region, a transmembrane region, and a cytoplasmic domain composed of TCR signaling domains and additional costimulatory domains from receptors such as CD28 and OX40[11,40]. To design safe, selective, and efficacious CARs, it is imperative that the various permutations in the design of the CARs are thoroughly tested using in vitro potency assays and, eventually, animal models. In this study, we have provided a protocol and a workflow of how a real-time in vitro potency assay can inform on the design of efficacious CARs.

In designing any type of potency assay, particularly for manufacturing purposes, it is imperative that the assay should be sensitive, robust, consistent, and as close to the mechanism of action as possible[16,17,41]. The real-time potency assay described here is designed to measure the cytolytic activity of the CAR T cell directly rather than a surrogate marker such as cytokine release. Importantly, the assay does not require any additional components such as dyes and reagents other than the assay plate (E-Plate) and the recommended media for maintaining the cells. Additionally, the assay is exquisitely sensitive with quality data compared to other label-based assays[42–44] and can be used to assess very low effector-to-target ratios which is ideal for the assessment of specific cytolysis.

In order to demonstrate the flexibility and utility of the RTCA system, we have focused on two tumor types, namely tumors of hematological origin and solid tumors. In order to assess the potency of CD22-directed CAR T cells, Raji cells (i.e., a B-cell lymphoma cell line) are tethered to the E-Plates using an anti-CD40 antibody. The tethering of Raji cells to the bottom of the E-Plates results in an impedance signal which reflects the viability and number of Raji cells in the well. Following the addition of CD22-CAR T cells, the Raji cells are selectively killed in a time- and effector-dependent manner, culminating in a time-dependent decrease in the impedance signal.

The drop in impedance signifies cytolysis or loss of viability of the Raji cells[17]. The selective tethering approach using antibodies can be extended to other liquid tumor cell lines. An alternative strategy to using tumor cell lines of hematological origin is to use adherent cancer cells that are engineered to stably express the tumor antigens, such as CD19 expressed in HeLa cells. The advantage of this approach is that the parental negative-control HeLa cells are readily available and can be used as a negative control for specificity. Such an approach has already been validated with CHO-CD22 vs. CHO cells and CHO-BCMAs vs. CHO cells[29]. Using such different approaches, CAR T-cell design and efficacy can be easily tested, and the assay conditions can be modified in an appropriate manner to mimic physiological conditions.

One major advantage of the potency assay we described here is that it is a simple functional assay and can be used in conjunction with genetic engineering techniques to design optimal and efficacious CARs in a high-throughput fashion. As we have shown for CAR T cells designed to target the EGFR-positive cancer cells, the assay can be used to evaluate the relative activity of the various other mutations in designing CARs. For example, we show that when the GITR domain synthesized N-terminal to the CD3 domain and expressed in T cells, it shows much more robust cytolytic activity (as measured by RTCA) and is confirmed by a cytokine release assay and in vivo test. In the past, cytokine measuring has been the standard assay to evaluate the potency of CAR T cells, although it has not fully been linked to the in vivo CAR T-cell activity as a reliable parameter. It is important to note that the assay described here primarily informs on the potency of CAR T cells in vitro and provides the user with a quantitative way to assess the quality of CAR T cells that are being produced during manufacturing or before releasing for clinical application. However, the relevance of in vitro potency to in vivo efficacy remains to be fully established and verified. In vivo efficacy depends on a host of factors and variables that may not be captured by in vitro assays such as the homing of the CAR T cells to the site of the tumor, the stimulation and activation of the CAR T cell and its ability to persist within the patient, and especially the tumor microenvironment. Further refinement of this assay is needed to be able to model these complex processes in vitro.

The protocol provided here applies to most adherent cancer cell lines and some of the liquid tumor cell lines. Clinical samples such as primary cancer cells, however, need to be further tested and optimized due to the complexity of the tumor types and phases. It is certainly worth noting that the in vitro potency assay system described here uses cancer cell lines only to reflect the potential activity of the CAR T cells. The real tumor situation inside the human body is much more complex, especially when a solid tumor is targeted, due to the dynamic tumor environment and development. Therefore, the potency evaluation result may not translate very well into the clinical efficacy of the CAR T cells tested.

In summary, the presented impedance-based platform allows label-free monitoring of cell killing for an extended period of time, namely up to 10 days. The availability of such a long temporal scale for data collection differentiates the technology from other assays that are currently in use and which require setting up multiple experimental replicates for time point collection and laborious sample manipulation. Furthermore, the minimal signal contribution of the effector immune cells, at the noise level, simplify data analysis. The software can process data

automatically and generate useful parameters (i.e., the percentage of cytolysis). The technology has already demonstrated high sensitivity (with E:T ratios as low as 1:20) and a large dynamic range (with E:T ratios from 20:1 to 1:20) which is not easily achieved with other assays. Overall, the implementation of this technology should allow a more accurate data analysis on a higher throughput scale that will enhance the development of CAR T-cell reagents, advancing the field at a much higher pace.

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DISCLOSURES:

The authors conduct research in CAR T-cell development and associated potency assays which are business interests of ProMab Biotechnologies and ACEA Biosciences, respectively. However, this does not alter the authors' adherence to JoVE's mission of increasing the dissemination of scientific knowledge. The terms of this publication have been reviewed and approved by ProMab Biotechnologies and ACEA Biosciences in accordance with its policy of research.

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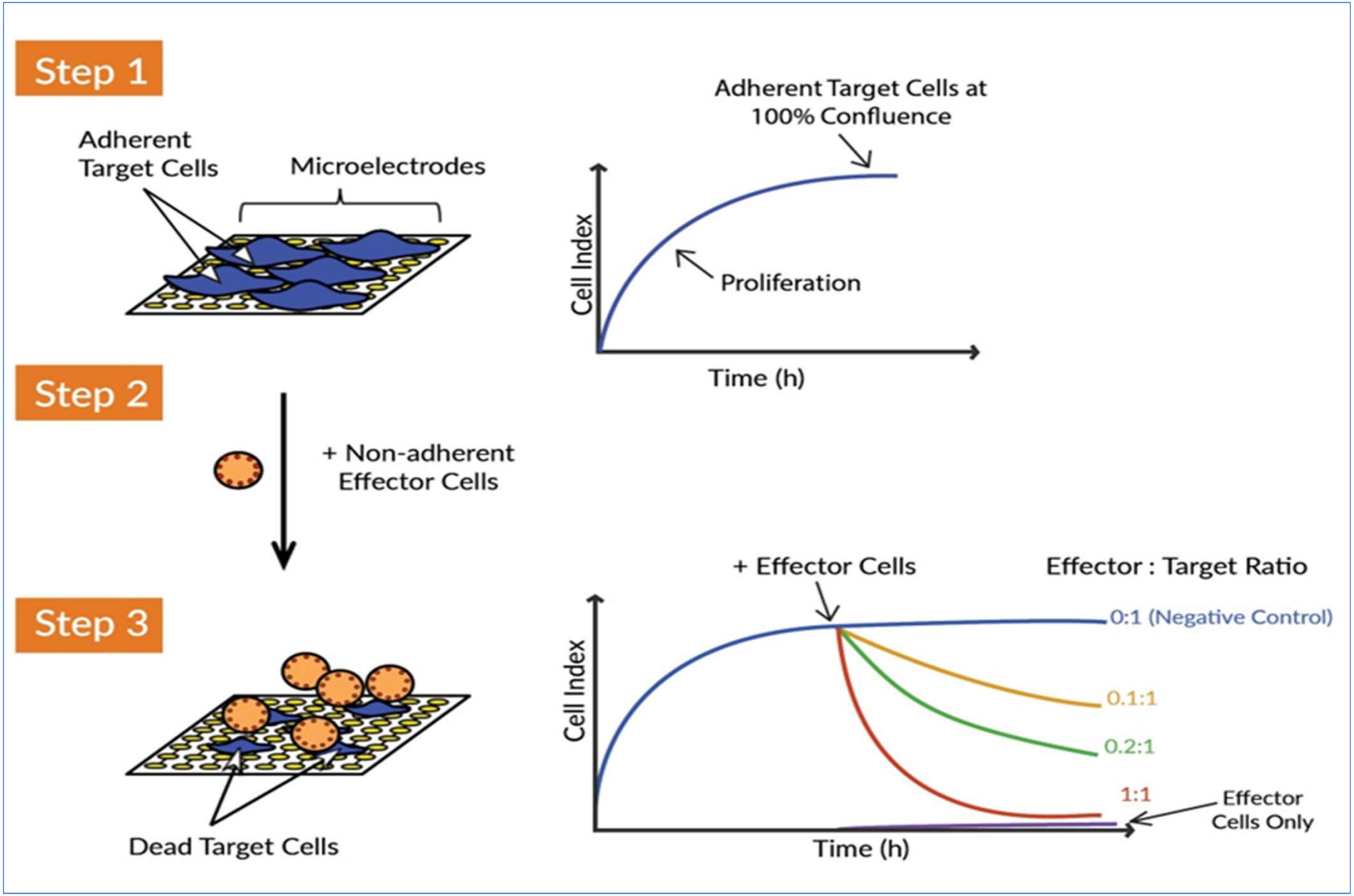

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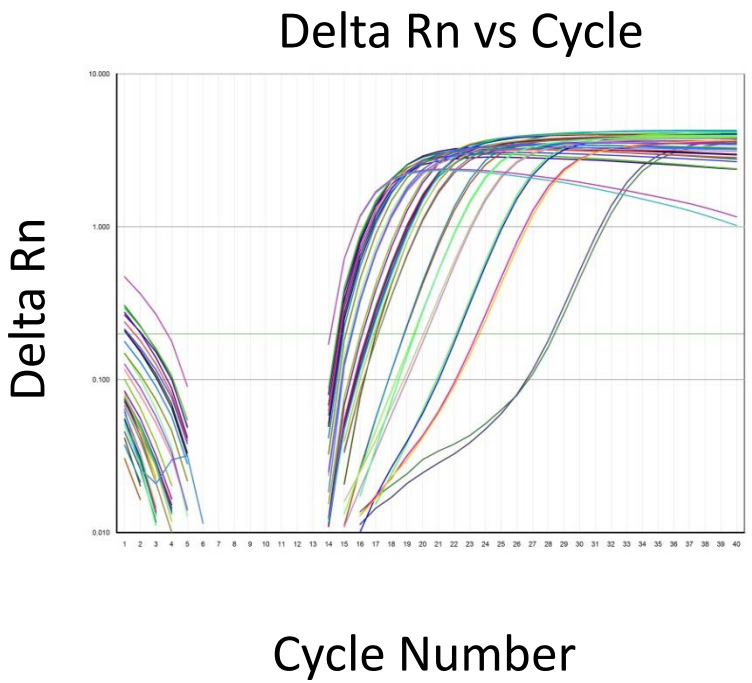

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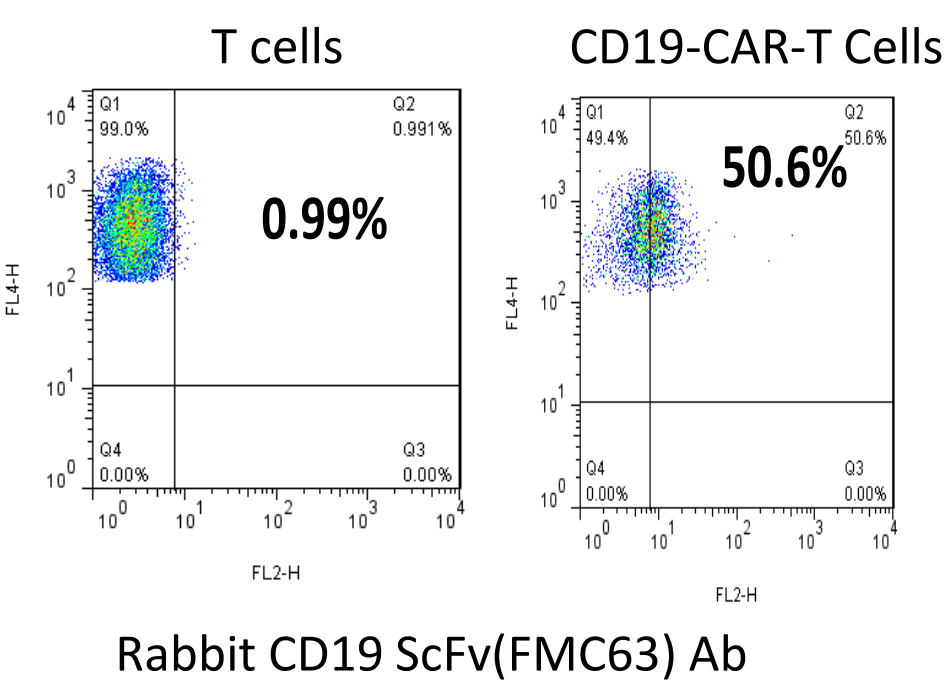

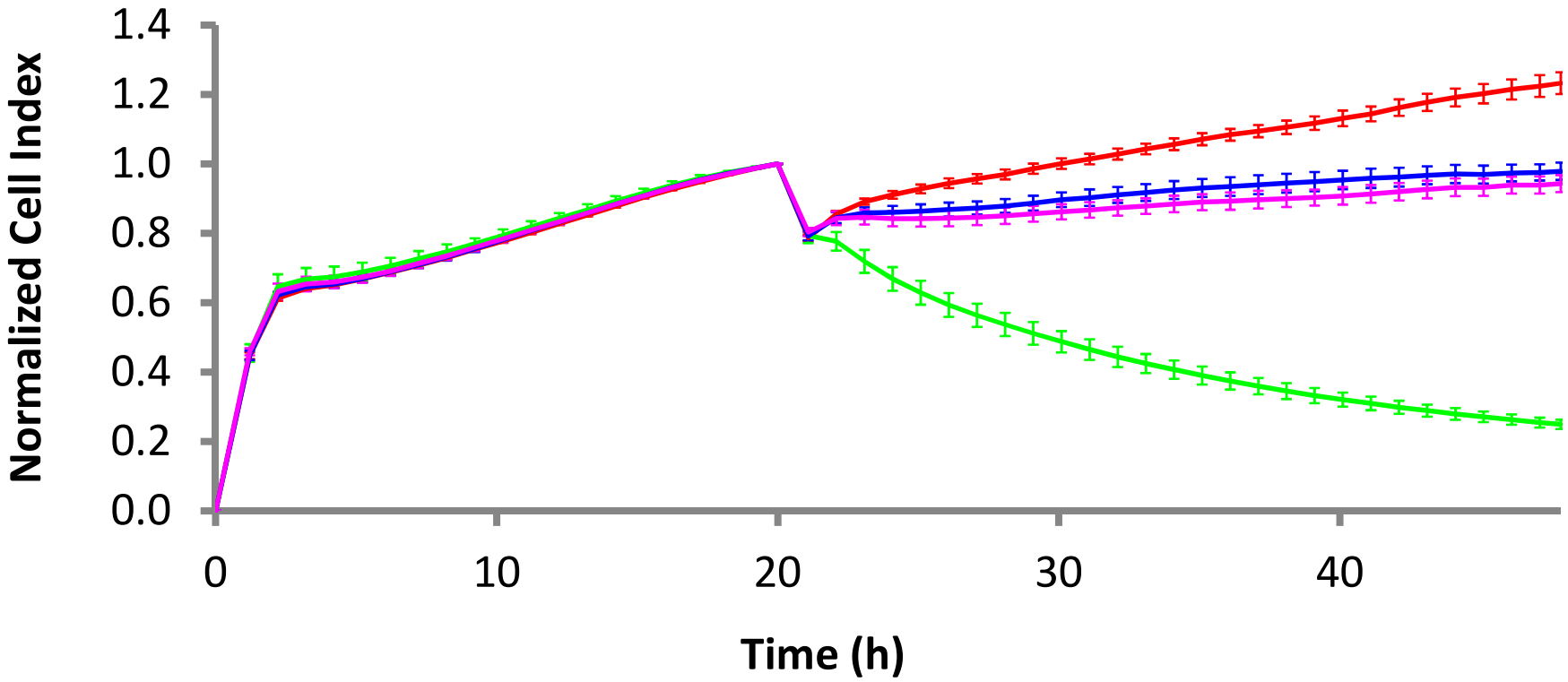

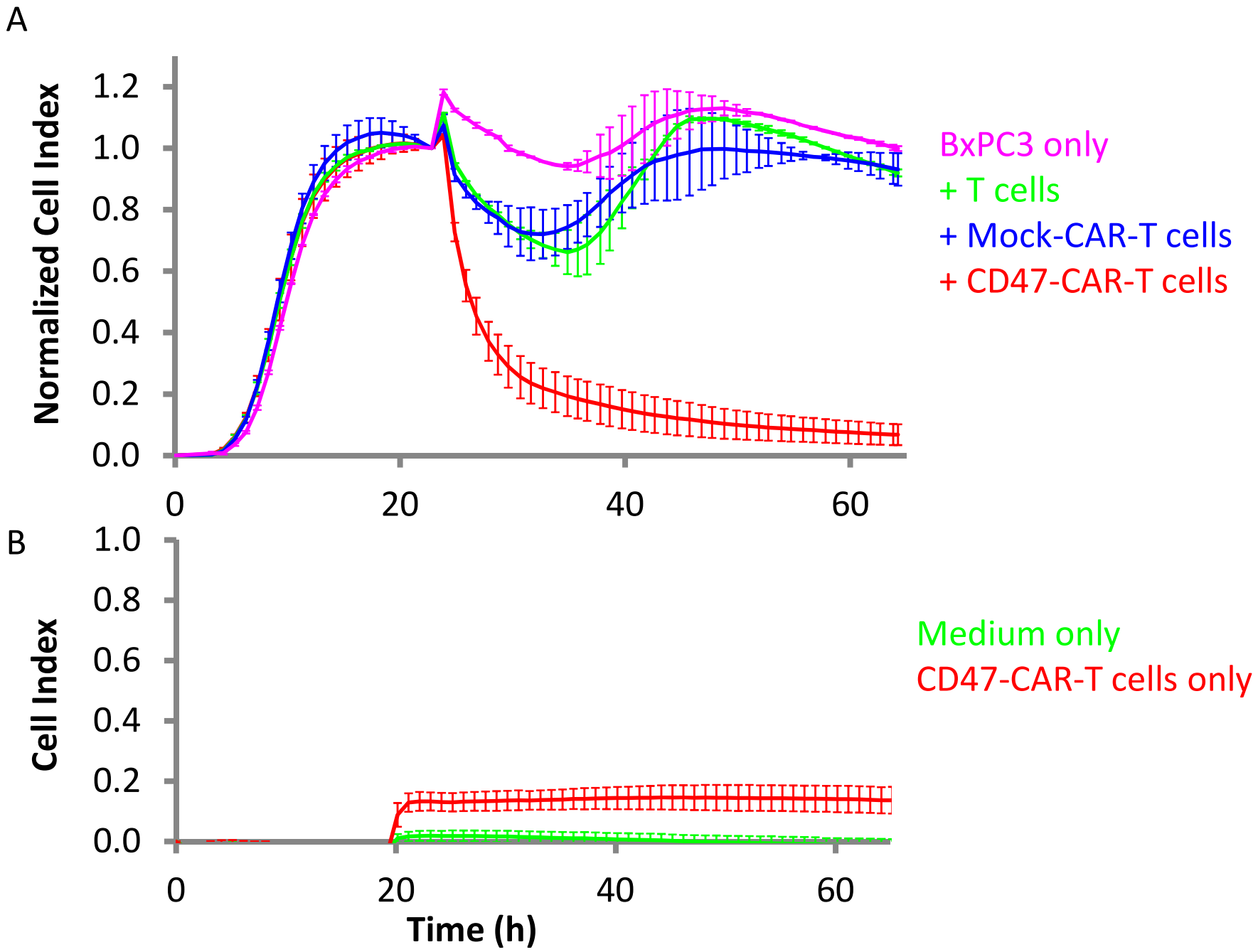

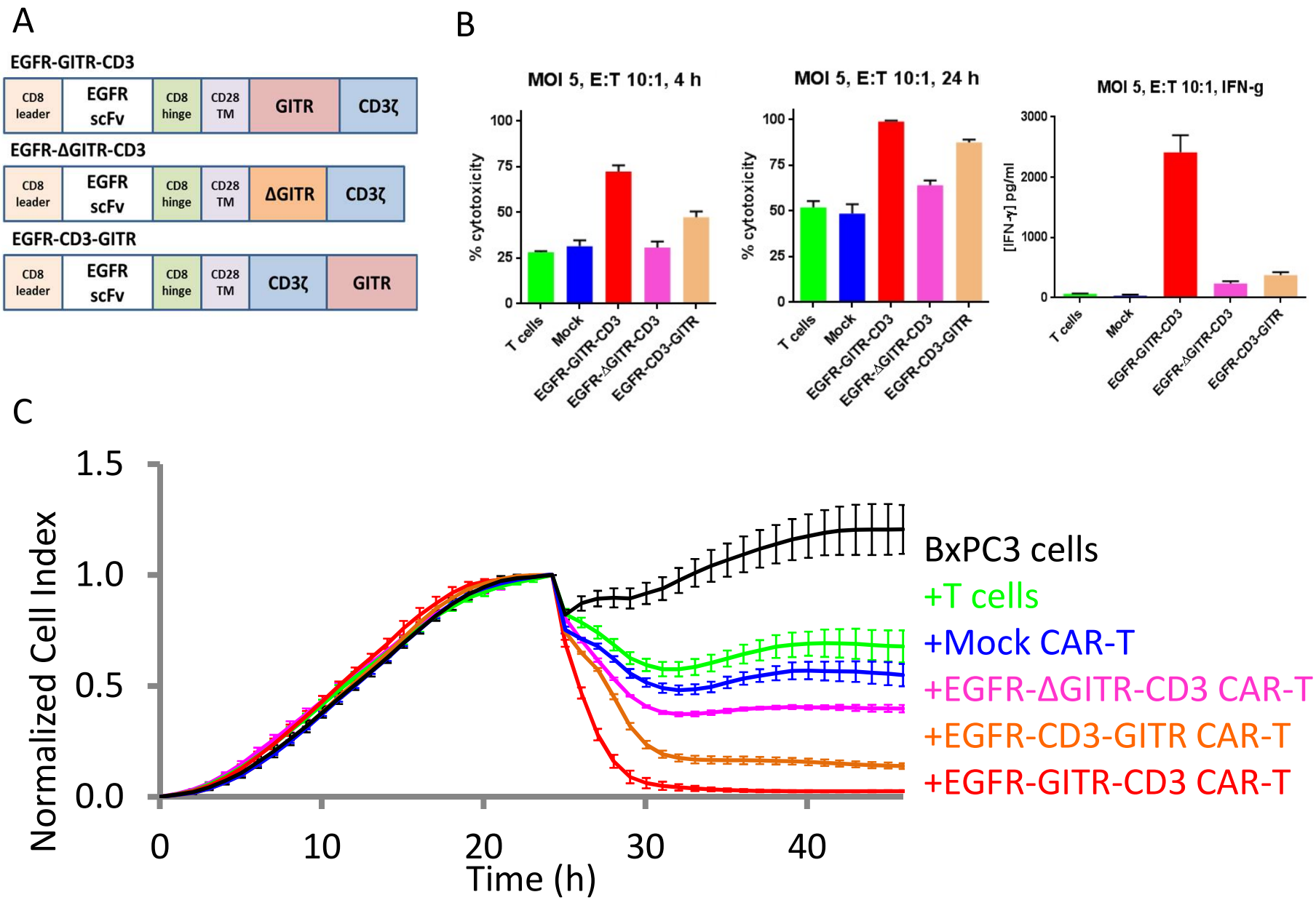

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
7-AAD	Biolegend	420404
Anti-CD40, liquid tumor killing assay kit	ACEA Biosciences	8100005
anti-human F(ab')2	Jackson Immunoresearch laboratories.	109-116-088
APC anti-CD3	Biolegend	317318
Assay medium RPMI1640	life technologies.Corp	11875-093
CAR-T cell frozen solution CryostorCS10	Stemcell technologies	#07930
CAR-T cell medium from ProMab	AIM-V+300IFU/ml IL-2	12055-091
CD3/CD28 coated microbeads, Dynabeads	Thermofisher	11131D
DMEM	GElifesciences.com	SH30243.02
FACS buffer	Promab made	
FBS	Lonza.com	14-503F
HEK293FT	Thermo Fisher	R70007
INFγ ELISA kit	Thermo Fisher	
Lentiviral Packaging Mix	System Biosciences	VP100
Lenti-X quantitative RT-PCR titration kit (Clontech)	Takara	631235
Promab medium for target cells	Varied with cell lines	
Real time Cellular Analyer	ACEA Biosciences	
Thermal cycler	Thermo Fisher	
Transduction enhance agent, Virus Transduction Enhancer (Alstem)	Transplus, Alstem	V020
Transfection dilution solution, Opti-MEM	Thermo Fisher	
Transfection reagent, NanoFect reagent	Alstem	NF100

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes a relatively novel approach to assess modified T cell killing utilizing the RTCA system which allows for real time killing assays to compare different CAR T cell constructs allowing for comparisons of the cytotoxic potential of these constructs. Top the credit of the authors, there is demonstration of the utility of this approach in the context of both solid (adherent) tumors as well as non adherent hematological malignancies. The protocols are well detailed and should provide the reader of this manuscript with a template of how to conduct these experiments. The manuscript is well written.

Major Concerns:

None

Minor Concerns:

There is a lack of statistical analysis is a short coming of this manuscript. One should show that differences in cytotoxicity using this RTCA system should be demonstrated highlighting the sensitivity of this approach and the ability of this approach to demonstrate statistically relevant differences in the presented assays. Statistical analysis should be applied to the data presented in this manuscript.

Authors:

We appreciate the comments and concerns especially regarding the data quality. In our figures, all the replicates from RTCA have been displayed with standard deviation bars . There are numerous publications which show the statistical significance of RTCA data. But we indeed made some change to reflect the data quality. We hope this paper primarily focused just on the protocol for CAR-T generation and development and validation of the cytolytic potency assay. By this simple introduction, this protocol may provide people alternative method to try and conclude in their own hand to avoid some commercial influence.

Reviewer #2:

Manuscript Summary:

This manuscript describes an in vitro real-time assay system for assessing the potency of CAR-T cells.

The system provides advantages in comparison to exiting methods, such as ease of use, higher signal to noise ratio, and the capability of real-time analysis over a relatively long period of time. Therefore it is recommended for publication, but a few critiques should be addressed to improve the clarity of the protocol.

Major Concerns:

None

Minor Concerns:

1. Passive past tense was used throughout the protocol, instead of imperative tense, which would be more proper for a protocol.

We appreciate reviewer's concerns and made the changes based on this suggestion.

2. For the protocol for the generation of CAR-encoding lentivirus and qRT-PCR, it would be more clear for readers if the protocol provided more specifics, i.e. seeding cell number, the size and kind of a plate to be used, lentiviral plasmids amounts, based on a desired virus titer as an example. This way, researchers who have limited experience with virus production can better assess their performance.

All the details have been specified based on the suggestion and hope the new version could be easily followed.

3. Generation and titration of CAR-encoding lentivirus would be more appropriate title.

Appreciate reviewer's suggestion. We hope we present a overall CAR-T generation and evaluation approach, not just a lentivirus production and indeed we focus on the CAR-T potency evaluation as preclinical quality control assay. In this way, we hope read can learn the whole procedure not just one step.

4. at step 3 the ts in tube one and tube two should be lower case.

Changed as suggested. Thanks!

5. Specify amounts of APC anti-CD3 and 7-AAD in the step 5 of detection of CAR expression by flow cytometry protocol.
Changed as suggested.

6. Figure 1 is not cited in the manuscript.
Changed as suggested.

7. There is no interpretation of the results shown in Figure 3.
Changed as suggested.

8. In the line 355, Figure 6 should be Figure 5.
Changed as suggested and we appreciated all the details suggestion from this reviewer.

9. Potential differences between in vitro and in vivo targeting should be discussed. Especially the limitations of the assay for determining the efficacy of CAR-T cells targeting solid tumor cells should

discussed as the in vitro assay condition is vastly different that of in vivo solid tumors.

The relevance of in vitro potency assay to in vivo efficacy is an important point and is certainly worth considering. We did insert a few sentences in the discussion part to mention the limitations of in vitro potency assay to ultimately predict in vivo outcome. More sophisticated assays that are able to model CAR-T homing, penetration of the tumor microenvironment and ultimately activation and persistence is needed to begin to address how the CAR-Ts ultimately perform within the patient. These points were discussed.

Reviewer #3:

Manuscript Summary:
The authors present a novel technique that selectively targets adherent or in-suspension cancer cells using chimeric antigen receptor T-cell therapy (CAR-T) that can be used in a clinical setting. A label free kinetic potency assay was developed for the use CAR-Ts using a real-time cell analysis (RTCA) platform. This uses gold E-plates onto which target cells can be seeded. The effector cells were then added under different effector to target ratios. The impedance electrodes monitor the viability of the cells real-time without the signal from the CAR-T cells which are mostly in suspension. Overall this is an interesting strategy with clear advantages over existing methods such as the CRA assay, which is mostly an end-point assay not providing real time kinetics. The manuscript is well-written.

Major Concerns:
1. What is the overall claim for this assay reducing the hands on time? How is it relevant in a clinical setting? Can the authors give as estimate of the time and explain why or why not this might be important?

Appreciate this critical question from reviewer. The assay is label-free and real-time and requires minimum hands on time compared to other assays. The whole procedure of our approach takes about 2 of hours to setup the experiment but this time is almost same to other current methods like ELISA and Cr51. However, RTCA requires minimum time in comparison to ELISA and Cr51 assay in later steps because no washing, fixation or other processing is required. Additionally, data analysis is very straightforward and calculation of key parameters such as percent cytolysis is automated. Obviously, less hand on procedure will significantly reduce the data variation. which is very useful and welcomed in clinical setting.

2. What tumor types or classes will this assay be best for?

Another great question. To this point, we think in vitro quality assessment of the CAR-T is universally important to all the CAR-T cells application regardless the tumor types or classes.

3. How is the impedance value measured in terms of cell index? Whats the value of cell index that can

be considered as a significant effector action? Can the authors comment on this?

Thanks for reviewer with this good question of the basics setup of RTCA system. We hope the short sentence explained in figure 1 is good enough since there are more than thousand reports about this technology and principal of the system already. Basically, we convert the impedance to an arbitrary number called Cell index, C.I.= impedance reading value/30. See literature 23~28 for more details.

Thanks again for another great question of what value of cell index is significant. After more than thousand researcher work, we think in general the Cell index should will be large than 0.5. But the absolute value is not as important as the kinetics which reflect the cells change. If not, even a relative high cell index may not necessary useful.

Minor Concerns:
Not sure what BCMA specified in the Introduction section is. It was not abbreviated.

BCMA is added to the Abbreviation. BCMA stands for B-cell maturation antigen, also known as tumor necrosis factor receptor superfamily member 17 (TNFRSF17). It was proved to be a very valuable target in clinical in recent years for B cell malignancy.

Reviewer #4:

The authors reported a method for the production of potent CAR-T cells targeting antigens expressed on solid and liquid tumor cells and also described a highly sensitive and real-time method for assessing the cytotoxicity potency of these cells. This is an interesting attempt to produce CAR-T cells and assess their potency. I think it can be accepted for publication after addressing the following comments.

1. All CARTs in the abstract and main text should be changed into CAR-Ts.

Thanks and changed applied.

2. In lines 134-138 from "Lastly" to "methods", this sentence should be divided into two separate ones,

or it will be difficult to understand for nonnative English speakers.

Thanks and changed applied.

3. In line 197, after centrifugation, was the supernatant discarded? If so, what buffer or medium was used to resuspend the pellet?

Changed applied.

4. In line 222, how many cells are transferred?

Changed applied.

5. In line 229, how can a 1.5-mL tube (from line 222) hold 3 mL of FACS buffer?

Thanks for pointing out and changed is applied.