CAR-T Cell Screening in Tumor Spheroids using Corning® Spheroid Microplates and the KILR® Cytotoxicity Assay

Application Note

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Introduction

Chimeric antigen receptor (CAR)-T cells, which are engineered to recognize target cell surface antigens expressed on tumor cells, have shown promise to affect complete remission in patients with B-cell malignancies. However, applying this approach to target solid tumors has proven more challenging and, in some cases, resulted in adverse effects in clinical studies. Many of the surface antigens that are upregulated in solid tumor cells, and thus chosen as targets for CAR-T cells, are also present at significant levels in normal tissues[1]. This can not only cause toxicity of the normal tissues but may even result in death. Methods for testing different models of CAR-T cells *in vitro* can provide further insight into viable antigen targets before these models reach the clinical stage. Historically, two-dimensional (2D) cell culture models have been used in drug discovery for the development of cancer therapeutics due to their ease of use and established compatibility with high throughput screening. Recently, more elaborate, three-dimensional (3D) cell culture models have been developed, which can help bridge the gap between successful *in vitro* studies and success in clinical trials. 3D multicellular tumor spheroids develop hypoxic cores and demonstrate gradients of various soluble factors and a diffusion profile for drugs similar to tumors. However, conventional methods for 3D cell culture are often time consuming, display increased variability, and lack throughput. Corning spheroid microplates are multiple well, cell culture microplates with opaque walls and unique clear, round well-bottom geometry with Corning Ultra-Low Attachment (ULA) surface coating. The coating is hydrophilic, biologically inert, and non-degradable, which enables the rapid and highly reproducible formation of a single multicellular tumor spheroid centered in each well. To quantify the cytotoxic effects of CAR-T cells on tumor cells grown as spheroids, DiscoverX KILR® Cytotoxicity assay provides a non-radioactive, dye-free method to specifically measure target cell death in a co-culture. Target cells can be engineered to stably express a protein tagged with a β-gal reporter fragment that is released into media during cell death. The addition of detection reagents results in a chemiluminescent output that can be detected using a luminometer.

In this study, two KILR tumor cell lines of different EGFR expression status were cultured in 384-well Corning spheroid microplates to form spheroids or in 384-well flat-bottom microplates to form a 2D monolayer. Tumor-specific cytotoxicity was screened after treatment with EGFR scFv-CD28-CD3ζ CAR-T cells (ProMab Biotechnologies) that are engineered to target EGFR with a single chain variable fragment (scFv) and contain a CD3ζ antigen recognition domain and a CD28 co-stimulatory domain. The EGFR CAR-T cells contain affinity tuned scFvs, which exhibit higher anti-tumor efficacy to cells with higher expression of target receptor and no anti-tumor efficacy to cells exhibiting normal target receptor levels[2]. Cytotoxicity after treatment with negative "Mock" control T cells (ProMab Biotechnologies) was also monitored. Twenty-four hours after EGFR and Mock control CAR-T cell addition, prepared KILR detection reagent was added and the resulting luminescence in the microplates was detected with a plate reader. When combined with the KILR luminescent assay and ProMab Biotechnologies CAR-T cells, Corning spheroid microplates enabled a high throughput screenable CAR-T cell assay demonstrating CAR-T cells with EGFR-specific cytotoxicity.

Materials/Methods

KILR Retroparticles Transduction

HCC827 and NCI-H460 cell lines from the ATCC® EGFR Genetic Alteration Cell Panel (TCP-1027™) were cultured following recommended protocols using RPMI-1640 (Corning Cat. No. 15-040-CM) supplemented with 2 mM L-glutamine (Corning Cat. No. 25-005-CI) and 10% fetal bovine serum (FBS) (Corning Cat. No. 35-010-CV). All cell harvests were performed using Accutase® (Corning Cat. No. 25-058-CI). Cells were transduced using KILR Retroparticles for Adherent Cells (DiscoverX Cat. No. 97-003) following manufacturer's suggestions. Briefly, HCC827 and NCI-H460 cells were seeded at 4 x 10^5 cells/well in 2 mL/well into 6-well tissue culture (TC)-treated plates (Corning Cat. No. 3506) and cultured overnight to 40% to 60% confluence. Cell culture media (approximately 1.75 mL) was removed and replaced with 0.5 mL KILR Retroparticles for Adherent Cells that had been thawed at room temperature for 10 minutes. Cells were incubated for 5 hours in a humidified 37°C, 5% CO_2 incubator, then 4 mL of culture media was added per well, and cells were cultured for an additional 48 hours.

Transduced cells were cultured under antibiotic selection with G418 (Corning Cat. No. 20-234-CI) (250 µg/mL for HCC827 cells and 500 µg/mL for NCI-H460 cells) for >7 days and confirmed for expression using Total Lysis Control with the KILR detection reagents following recommendations from the KILR Retroparticles protocol. After confirmation of expression, the KILR transduced cells were scaled-up for assaying and freeze stocks were made in culture medium with 10% dimethyl sulfoxide (DMSO) (Corning Cat. No. 25-950-CQC).

KILR® Cytotoxicity Assay

KILR Retroparticles-transduced target cells were seeded in 20 µL assay medium (RPMI-1640 containing 5% FBS and 2 mM L-glutamine) per well in 384-well spheroid microplates (Corning Cat. No. 3830) at 5K cells/well, and cultured in a humidified 37°C, 5% CO_2 incubator for 48 hours for the formation of a single spheroid in each well for 3D assays. KILR Retroparticles-transduced target cells were also seeded into 384-well black/clear, flat-bottom microplates (Corning Cat. No. 3764) at 5K cells/well and incubated for 18 hours for the formation of a monolayer for 2D assays. All microplates were sealed with breathable sealing tape (Corning Cat. No. 3345) prior to incubation to help prevent evaporation.

After incubation, EGFR CAR-T cells (ProMab Cat. No. PM-CAR1021-10M) and Mock ScFv Control CAR-T cells (ProMab Cat. No. PM-CAR1000-1M) were thawed following vendor's recommendations and added in a range of effector to target (E/T) ratios varying from 40:1 to 0.04:1 in 10 µL of assay medium per well using a CyBi®-Well pipettor with mixing 3 times in the receiver microplates using an 8 µL/sec aspirate/dispense speed and a 1.5 mm tip height. Total Lysis Control from the KILR Cytotoxicity Assay kit was added to 3 wells of each cell type in each flat-bottom and spheroid microplate in a final dilution of 1 to 20. Microplates were sealed with new breathable sealing film and cells were cultured for an additional 24 hours prior to luminescent readout.

To detect the chemiluminescent signal, working detection solution was prepared by mixing KILR detection reagents 1, 2, and 3 provided in the KILR Detection kit (DiscoverX Cat. No. 97-0001M) following the vendor's protocol and 30 µL/well of this prepared working detection solution was added to the microplates. Microplates were foil-sealed (Corning Cat. No. 6569), agitated on a plate shaker for 1 minute, and incubated for 1 hour at room temperature in the dark until luminescence was detected with a PerkinElmer EnVision™ plate reader. Percent luminescence was calculated by comparing luminescence from sample wells to luminescence from total lysis control wells.

Confocal Imaging

For confocal imaging of 3D tumor cell spheroids and CAR-T cells, cells were washed 24 hours after CAR-T cell addition with 30 µL/well Dulbecco's Phosphate-Buffered Saline (PBS) (Corning Cat. No. 21-031-CM) and fixed in 40 µL/well 4% paraformaldehyde (Electron Microscopy Sciences Cat. No 157-SP) for 45 minutes. Cells were washed in PBS twice and permeabilized with 0.2% Triton™ X-100 (Integra Cat No. T756.30.30 diluted in PBS) for 1 hour. After fixation and permeabilization, cells were washed in PBS and blocked with PBS containing 2% BSA (Sigma-Aldrich Cat. No. A9576), 5% FBS, and 0.1% Triton X-100 for 45 minutes. Cells were then stained in 30 µL/well using the antibodies and final concentrations listed in Table 1 with the addition of 10 µM Hoechst 33342 (Thermo Fisher Cat. No. 62249) for nuclei counterstaining. Cells were incubated overnight at 4°C and washed with PBS prior to imaging using a Thermo Fisher CellInsight™ CX7 high-content screening platform.

Results/Discussion

The cytotoxic effect of CAR-T cells on tumor cells can be measured in a co-culture system using Corning spheroid microplates and the KILR assay. Two lung tumor cell lines from the ATCC® EGFR Genetic Alteration Cell panel, HCC827 and NCI-H460 cells, were transduced using KILR Retroparticles to stably express a protein tagged with a β-gal reporter fragment that was released into media during cell death, enabling the detection of target cell death through a luminescent output. The HCC827 tumor cell line contains EGFR gene copy number amplifications, whereas the NCI-H460 cell line does not. CAR-T cells that target EGFR expressing cells (EGFR CAR-T cells) and mock control CAR-T cells engineered with an empty vector (Mock CAR-T cells) were used as effector cells against the two lung tumor cell lines cultured in a monolayer (2D) or as spheroids (3D) with E/T ratios of 40:1 to 0.04:1.

As visualized using confocal microscopy (Figure 1), the EGFR CAR-T cells encompassed and began to penetrate the HCC827 spheroid within 24 hours of CAR-T cell addition. As E/T ratio is increased from 10:1 to 40:1, invasion of the CAR-T cells into the HCC827 tumor spheroid and subsequent tumor cell lysis is visible. This cell lysis is also detected using the KILR Detection kit (Figure 2).

Twenty-four hours after CAR-T cell addition, HCC827-KILR cells and NCIH460-KILR cells cultured in 2D and 3D were assayed using KILR detection reagents. EGFR CAR-T cells displayed dose-dependent targeting of HCC827 cells with maximum 93% lysis in 2D and 54% lysis in 3D at an E/T ratio of 40:1 (Figure 2). The EGFR CAR-T cells did not display detectable cytotoxic effects in the NCI-H460 cells that lacked EGFR gene copy number amplification in 2D or 3D, which demonstrates the EGFR amplification target specificity of the ProMab CAR-T cells. Target cell cytotoxicity was absent or minimally detected in both cell lines upon the addition of mock control CAR-T cells, which further supports that EGFR CAR-T cell induced cytotoxicity in the HCC827 cells is target specific.

Table 1. Stains used for Confocal Imaging

Antibody/Isotype Control	Supplier/Cat. No.	Concentration (µg/mL)
Rabbit IgG, anti-Cytokeratin 7 antibody [EPR1619Y] - Cytoskeleton Marker (Alexa Fluor® 488)	abcam/ab185048	5
Rabbit IgG, monoclonal [EPR25A] - Isotype Control (Alexa Fluor 488)	abcam/ab199091	5
Human CD3 epsilon Alexa Fluor 594-conjugated Monoclonal Mouse IgG1 Antibody	R&D Systems/FAB100T-025	8
Mouse IgG1 Alexa Fluor 594-conjugated Isotype Control	R&D Systems/IC002T	8

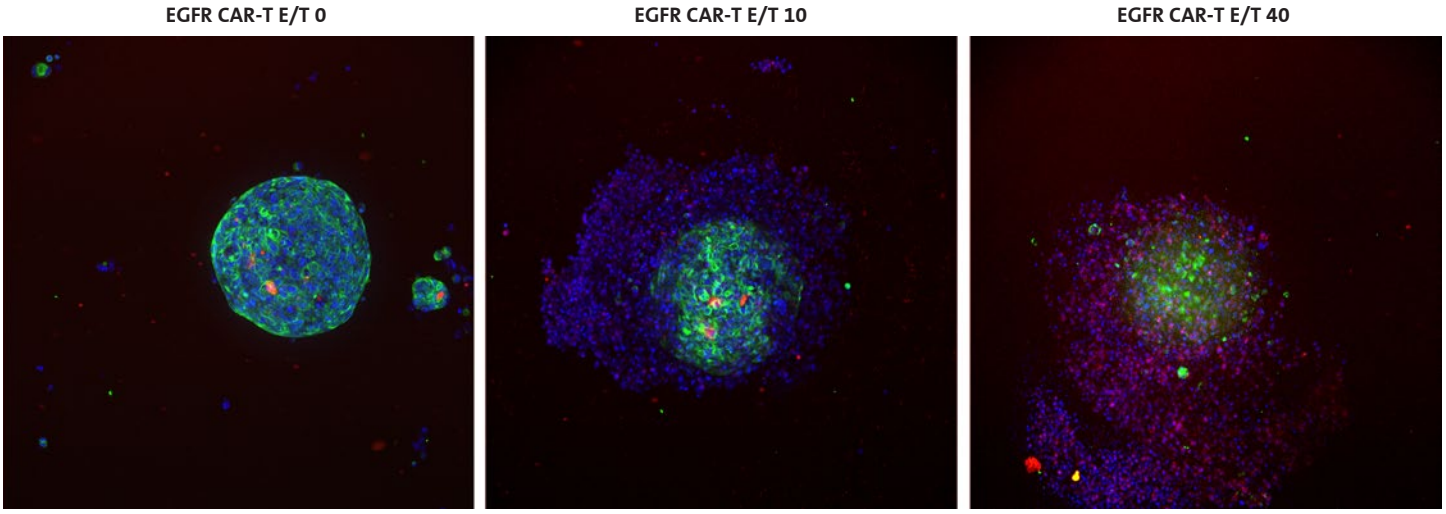

Figure 1. Representative confocal images of HCC827 KILR-transduced spheroids with CAR-T cell invasion. Twenty-four hours after CAR-T cell addition, HCC827-KILR cells were stained for cytokeratin-7 (green) and EGFR CAR-T cells were stained for CD3ζ (red). All cell nuclei were counterstained with Hoechst (blue). As effector to target ratio (E/T) is increased from 10:1 to 40:1, invasion of the CAR-T cells into the HCC827 tumor spheroid and subsequent tumor cell lysis is visible. Images obtained using a CellInsight™ CX7 in confocal mode using 10X objective.

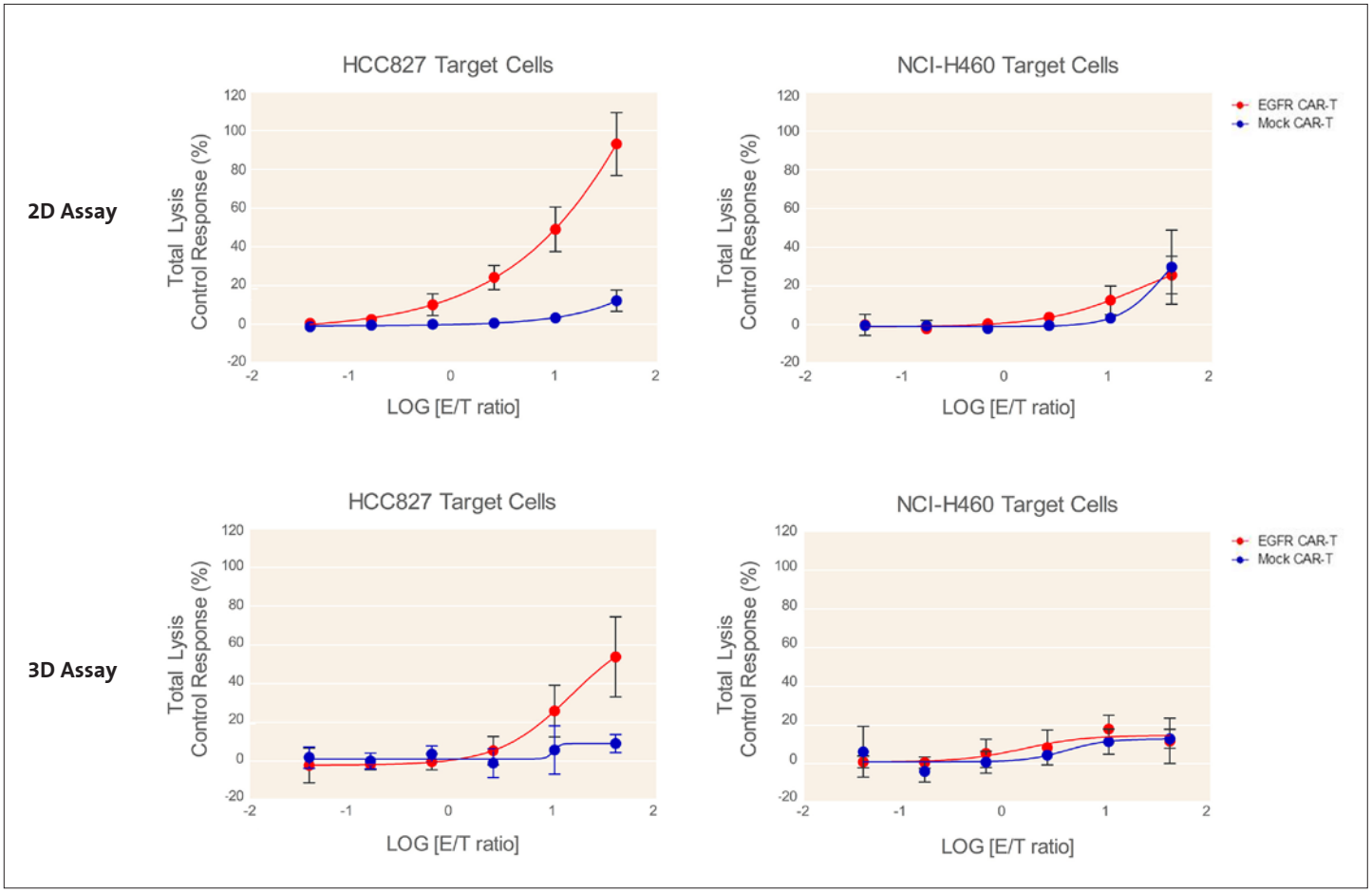

Figure 2. EGFR CAR-T cells targeted HCC827 cells in 2D and 3D. Twenty-four hours after CAR-T cell addition, HCC827-KILR cells and NCIH460-KILR cells cultured in 2D and 3D were assayed using DiscoverX KILR® detection reagents. EGFR CAR-T cells displayed dose-dependent targeting of HCC827 cells, with maximum 93% lysis in 2D and 54% lysis in 3D at an E/T ratio of 40:1. This cytotoxicity was absent or minimally detected in NCI-H460 cells and upon using mock control CAR-T cells. Assays were repeated 2 independent times, N = 4 for each data point.

Conclusions

- KILR® Cytotoxicity assays can be utilized to easily measure target-specific cell death with target cells cultured in a monolayer (2D) or as a spheroid (3D).
- CAR-T cells from ProMab Biotechnologies engineered to target EGFR demonstrate EGFR target-specific cytotoxicity as displayed using KILR assays with target cell lines cultured in 2D and 3D.
- In combination with KILR Cytotoxicity assays and ProMab CAR-T cells, the Corning spheroid microplate provides a high throughput platform for culturing and screening tumor spheroids with CAR-T cell assays.

References

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2. Liu X, et al. (2015). Affinity-tuned ErbB2 or EGFR chimeric antigen receptor T cells exhibit an increased therapeutic index against tumors in mice. Cancer Res. 75(17):3596-3607.

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