CAR-T Cell Screening in Tumor Spheroids using Corning® Spheroid Microplates

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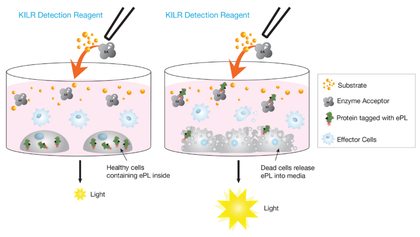
Abstract

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 resulted in adverse effects in clinical studies, as 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 cause toxicity of the normal tissues and may 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 better mimic the *in vivo* tumor microenvironment, to bridge the gap between successful *in vitro* studies and success in clinical trials. 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 that utilize Corning Ultra-Low Attachment 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 cytotoxicity of tumor cells grown in 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, KILR tumor cell lines were cultured in 384-well Corning spheroid microplates to form spheroids. Tumor-specific cytotoxicity was screened after treatment with ProMab Biotechnologies EGFR scFv-CD28-CD3ζ CAR-T cells, which 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 KILR detection reagent was added and the resulting luminescence in the spheroid microplate was detected with a plate reader. When combined with the KILR luminescent assay and ProMab Biotechnologies CAR-T cells, the Corning spheroid microplate enables a high throughput screenable CAR-T cell assay that targets tumor spheroids.

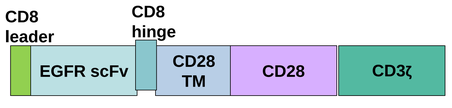
KILR® Cytotoxicity Assay from DiscoverX

- The KILR assay is a highly specific, non-radioactive measure of target cell death in a co-culture.
- KILR target cells are transduced to stably express a KILR reporter protein tagged with a β-gal fragment. This KILR reporter protein is released into the media upon cell death and lysis. Addition of detection reagents containing the other β-gal fragment results in a chemiluminescent output.



ProMab CAR-T Cells

- Second and third generation CAR-T cells that target a variety of cell-surface receptors are available from ProMab Biotechnologies.
- Affinity-tuned scFvs 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].
- In this study, a second generation CAR-T cell targeting epidermal growth factor receptor (EGFR) with the below construct was used to target breast and lung cancer cell lines.



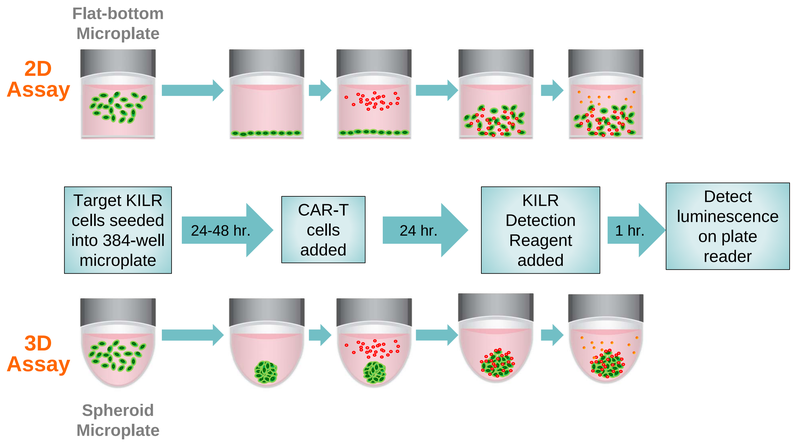
Spheroid Microplate

- 96- or 384-well cell culture microplates with round well-bottom geometry and Corning Ultra-Low Attachment surface coating enabling the rapid and highly reproducible formation of a single multicellular tumor spheroid, centered in each well. Clear bottom and opaque walls support spheroid visualization and luminescent and fluorescent assays directly in culture plate.

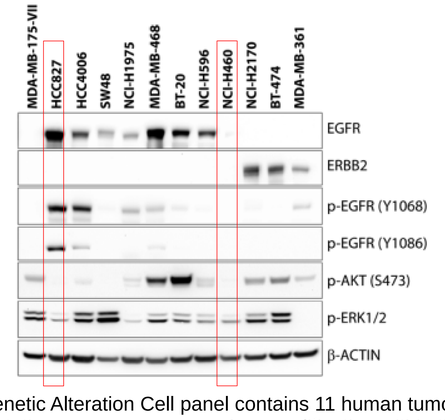
Spheroid CAR-T Assay Procedure

1. HCC827 and NCI-H460 cell lines from the ATCC® EGFR Genetic Alteration Cell Panel (TCP-1027™) were cultured following recommended protocols using Corning media and supplements. All cell harvests were performed using Accutase® (Corning Cat. No. 25-058-CI).
2. Cells were transduced using KILR Retroparticles for Adherent Cells (DiscoverX Cat. No. 97-003) following manufacturer protocols. Cells were cultured under antibiotic selection with 250 - 500 µg/mL G418 for ≥7 days and confirmed for expression using Total Lysis Control with the KILR detection reagents.
3. KILR transduced cells were seeded in 20 µL assay medium (RPMI-1640 with 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 transduced cells were also seeded into 384-well flat black/clear-bottom microplates (Corning Cat. No. 3764) at 5K cells/well and incubated for 18 hours for the formation of a monolayer for 2D assays.
4. 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 added in a range of effector to target 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. Cells were cultured for an additional 24 hours prior to luminescent readout.
5. 30 µL/well of prepared KILR detection reagents (DiscoverX Cat. No. 97-0001M) were added to the microplates, followed by agitation on a plate shaker for 1 min., and incubation for 1 hour at room temperature in the dark until luminescence was detected with a PerkinElmer EnVision® plate reader.
6. To obtain confocal images, cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences Cat. No 157-SP), permeabilized with 0.2% Triton X-100 (Integra Cat No. T756.30.30), and blocked with 5% FBS, 2% BSA, 0.1% Triton X-100 in PBS prior to staining for anti-cytokeratin 7 (Abcam Cat. No. ab185048) and CD3epsilon (R&D Systems Cat. No. FAB100T-025) and counterstaining with Hoechst 33342 (Thermo Fisher Cat. No. 62249). Cells were imaged using a Thermo Fisher CellInsight™ CX7 High-Content Screening Platform.

Assay Overview

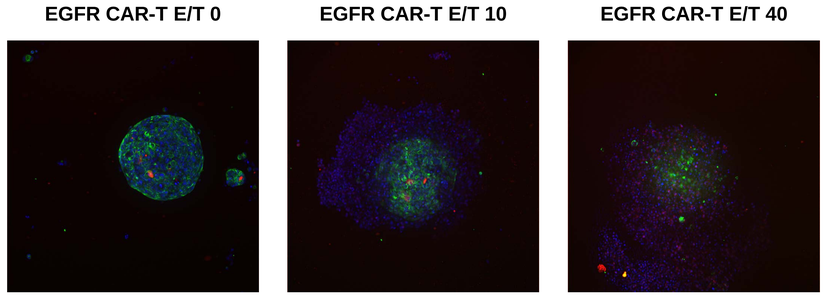


ATCC® EGFR Genetic Alteration Cell Panel



The ATCC EGFR Genetic Alteration Cell panel contains 11 human tumor cell lines that have hotspot mutations or gene copy number amplification within the EGFR or ERBB2 gene. Two cell lines from this panel were selected for this study: the HCC827 cell line, which contains EGFR copy number amplification and the NCI-H460 cell line, which does not contain EGFR copy number amplification.

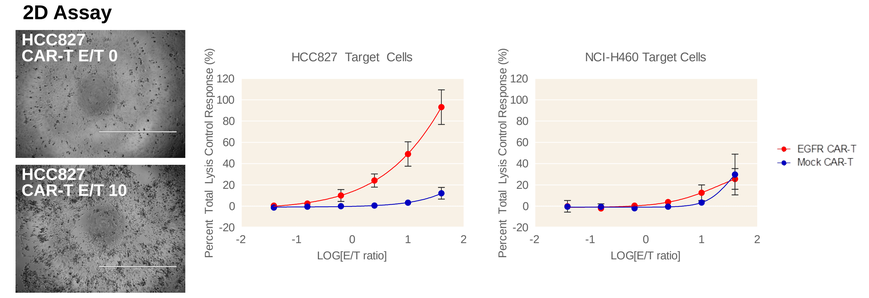
CAR-T Cell Invasion of Tumor Spheroids



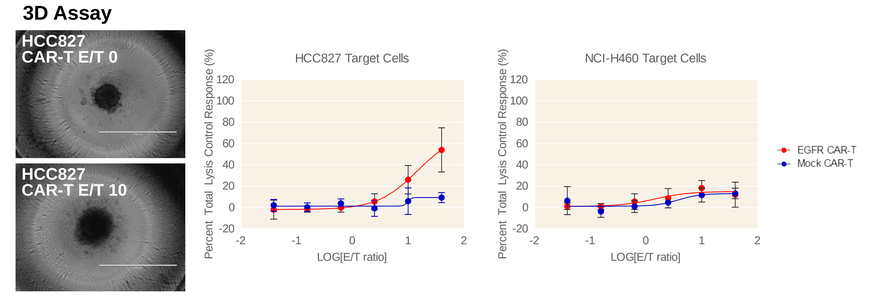
Representative photomicrographs of HCC827 KILR-transduced spheroids with CAR-T cell invasion. Twenty-four hours after CAR-T cell addition, HCC827-KILR cells were stained for cytokeratin-7 (green) and EGFR CAR-T cells were stained for CD3ε (red). All cell nuclei were counterstained with Hoescht (blue). 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. Images obtained using Thermo Fisher CellInsight CX7 in confocal mode using 10X objective.

KILR CAR-T 2D and 3D Assay Results

2D Assay



3D Assay



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 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. Representative images of HCC827 cells 24 hours post-EGFR CAR-T cell addition at E/T ratios of 0 or 10:1 captured using EVOS™ microscope. Scale bar = 1,000 µm.

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

1. Zhang BL, et al. (2016). Hurdles of CAR-T cell-based cancer immunotherapy directed against solid tumors. Sci China Life Sci, 59:340-348. doi: 10.1007/s11427-016-5027-4.
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.