Application Note

Immunotherapy

Automated Assay for Measuring CAR T Cell Killing in 3D Cancer Models

High-throughput imaging and analysis using the BioTek Cytation C10 confocal imaging reader

Authors

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Abstract

Adoptive cell therapies such as chimeric antigen receptor (CAR) T lymphocytes have ushered in a new paradigm of cancer immunotherapy. In this study, we use T lymphocytes genetically modified to express the variable chain fragment specific for the epithelial cell adhesion molecule (EpCAM), also known as CD326, on their surface. With this CAR T line, we demonstrate the feasibility of high-throughput imaging assays where antigen-stimulated CAR T lymphocytes exhibited their cytotoxic effects in a dose-dependent manner using a 3D model: target T-47D spheroids. Confocal imaging reveals that activated CAR T lymphocytes infiltrate target T-47D spheroids and this automated functional assay enables high-throughput mechanistic characterization of candidate CAR T-cell lines.

Introduction

By directing the exquisite potency and molecular specificity of the immune system towards cancer cell destruction, immunotherapy in its various forms has permanently changed the landscape of clinical oncology. Among the frontrunners ushering this new paradigm are adoptive cell therapies such as chimeric antigen receptor (CAR) and T cell receptor (TCR) T cells, genetically engineered effector cells that drive T lymphocyte activation, proliferation, and target cell killing. The ability to engineer T lymphocytes and endow them with novel receptors to direct cytotoxicity towards cancer cells heralds a new era in cancer treatment. Optimization of receptor chimeric structures along with introduction of genes to enhance T cell function and persistence within the tumor microenvironment are necessary for T lymphocyte-based therapies to realize their full potential.

In this application note, we use genetically modified T lymphocytes which have the variable chain fragment specific for EpCAM, also known as CD326 on its cell surface. EpCAM is a transmembrane glycoprotein that mediates cell-to-cell adhesion in epithelial cells. First discovered in 1979, EpCAM was initially described as a dominant surface antigen on human colon carcinoma. EpCAM is expressed in many human epithelial tissues, carcinomas, and progenitor and stem cells. It is not found in nonepithelial cells or cancers of nonepithelial origin. These attributes make this T lymphocyte more amenable for use in treating solid tumors.

With this engineered CAR T line, we demonstrate the feasibility of high-throughput 3D imaging assays where antigen-stimulated CAR T effector lymphocytes exerted their cytotoxic effects on target T-47D spheroids in a dose-dependent manner. We further demonstrate that activated CAR T effector lymphocytes infiltrate a target T-47D spheroid to exert its cytotoxic effect. This kind of functional assay enables high-throughput mechanistic characterization of candidate CAR T cell lines.

Experimental

Cell lines and culture conditions

EpCAM+ T-47D breast ductal carcinoma cells (ATCC, part number HTB-133) stably expressing nuclear-localized mKate2 (Agilent eLenti-Red reagent, part number 8711011), EpCAM-transformed CAR T lymphocytes, and untransformed T lymphocytes were cultured in RPMI containing 10% FBS and 1x primocin (InvivoGen, part number ant-pm-1). Cells were supplemented with either 0.2 U/mL insulin (T-47D) or 200 U/mL IL-2 (T lymphocytes).

Spheroid formation

T-47D spheroids were formed by seeding 500 cells/well in ultralow attachment U-bottom 96-well plates (ThermoFisher, part number 174929) and allowed to culture for seven days. On the day of assay initiation, a cell count was conducted on a sample of spheroids to determine their final cell counts, which was used to calculate effector:target cell ratios (E:T ratio).

T lymphocyte activation

EpCAM-transformed CAR T lymphocytes were stimulated twice with 500 ng/mL EpCAM epitope (SinoBiological, product number 10694-H41H-B): 24 hours after the initial thaw (day one, post thaw) and on day four, post thaw. Cells were counted daily and maintained at 1×10^6 cells/mL.

Target cell killing assay

For media-suspension killing assay, preformed T-47D spheroids were transferred to a fresh 96-well plate U-bottom, in order to minimize cellular debris (Corning, part number 4520) (Figure 1A), whereas for matrix-suspended killing assays, spheroids were embedded in 1% collagen (Corning, part number 354236) in a flat-bottom Agilent 96-well plate, part number 204626-100 (Figure 1B). In both formats, target spheroids were seeded at one spheroid per well. Activated EpCAM CAR T or naïve untransformed T lymphocytes were then seeded into each well at increasing ratios of activated EpCAM-engineered effector CAR T cells and imaged every three hours for 48 hours using an Agilent BioTek BioSpa 8 automated incubator (Figure 1C).

Image aquisition

Wells were imaged kinetically with a 10x 0.3 NA objective in the indicated channels using an Agilent BioTek Cytation C10 confocal imaging reader in widefield mode coupled with a BioSpa 8 automated incubator. A laser autofocus (LAF) routine was used for rapid focusing on each well. After image capture, images were preprocessed to subtract background.

Image analysis

After background subtraction, T-47D nuclei were identified using integrated fluorescence intensity in the tetramethylrhodamine isothiocyanate (TRITC) channel. The threshold value was determined based on complete identification in untreated controls. Fluorescence intensity values of experimental conditions are reported as percent change relative to the initial time point for kinetic studies, or as relative to control samples for dose-response studies.

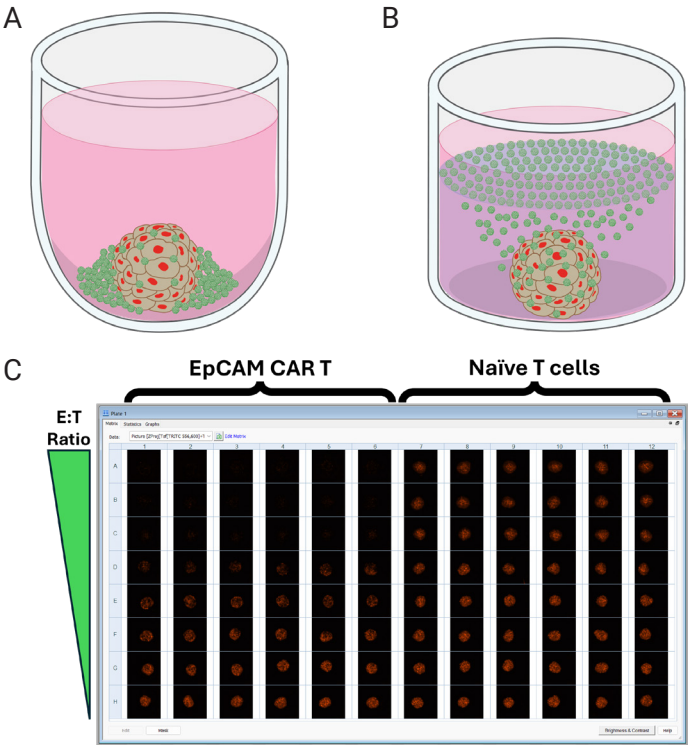

Figure 1. 3D cell-killing assay setup. Preformed T-47D spheroids expressing nuclear mKate2 were seeded in either (A) U-bottom 96-well microplates for media-suspended 3D spheroids, or (B) flat-bottom 96-well microplates for collagen-embedded 3D spheroids where activated CAR T lymphocytes seeded on top of the matrix are allowed to migrate towards the target cell spheroid, and exposed to increasing ratios of activated EpCAM-engineered effector CAR T cells (E:T ratio), and continuously imaged for the duration indicated for each assay. (C) Example layout of a 96-well U-bottom plate where media-suspended 3D spheroid killing assay is conducted.

Results and discussion

High-throughput imaging enables kinetic evaluation of CAR T-mediated target spheroid killing

The T-47D epithelial lines used in this study have been transduced with eLenti-Red reagent to constitutively express a nuclear localized mKate2 fluorescent protein. Thus, cytotoxicity is reported as the loss of signal in the TRITC channel. The BioTek Cytation C10 confoical imaging reader increases imaging throughput by using a 96-well microplate format. This enhancement improves statistical robustness when analyzing T-47D spheroids exposed to varying concentrations of either activated EpCAM CAR T cells or naïve, untransformed T lymphocytes (Figure 1C). This potency can be illustrated by temporally evaluating cell toxicity of target T47D spheroids (Figure 2A). Multiday high-throughput imaging of target spheroids in a 96-well microplate format is possible with the Cytation C10 confocal imaging reader when coupled with the BioSpa 8 automated incubator. As shown in Figure 2B, increasing E:T ratios of EpCAM CAR T cells increase the rate at which maximal cell death is achieved in a spheroid model, whereas nonactivated (naïve), nonengineered T lymphocytes have no effect on target spheroids (Figure 2C).

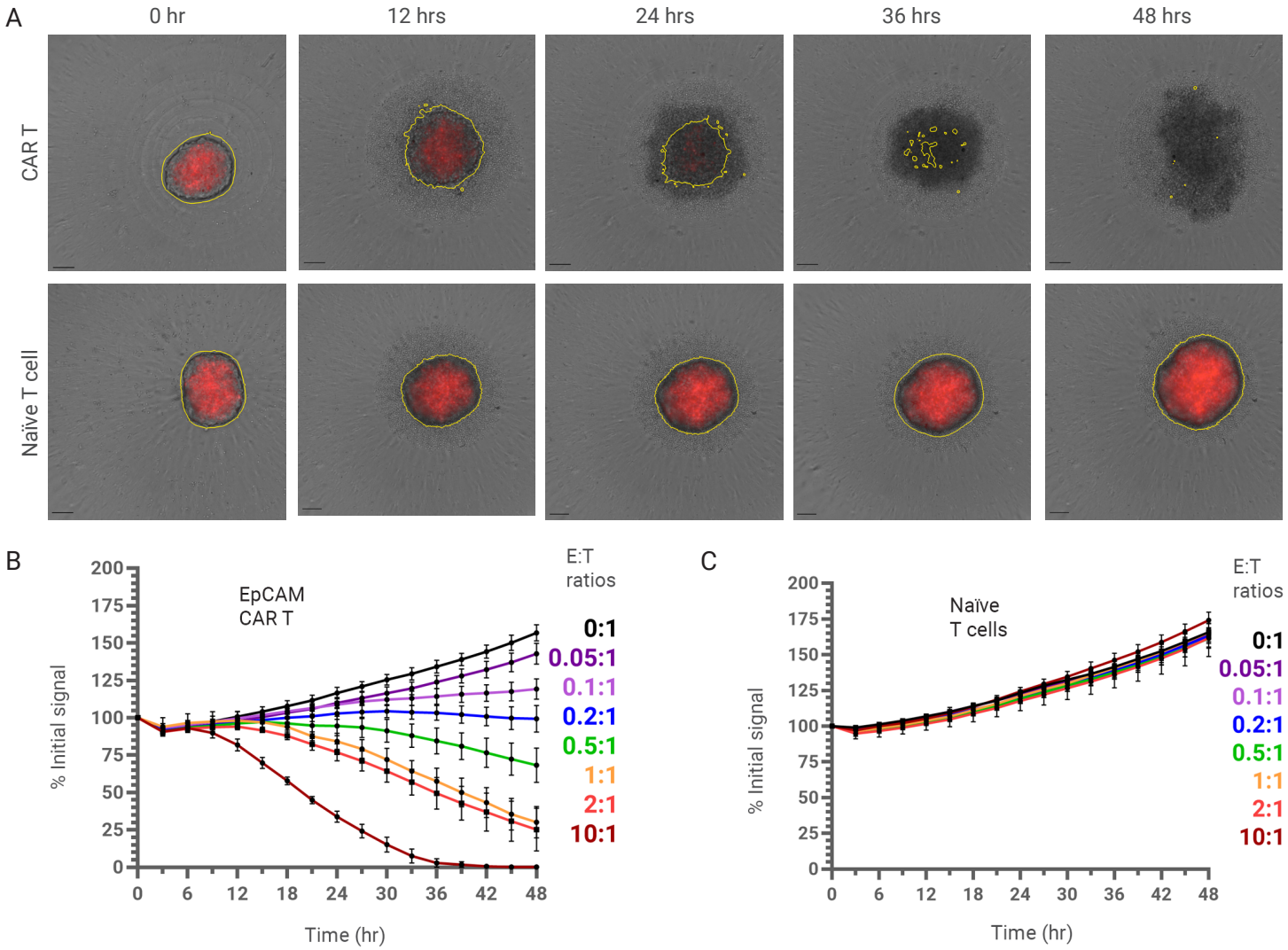

Figure 2. High-throughput imaging enables kinetic evaluation of CAR T-mediated target spheroid killing. (A) T-47D spheroid (red, nuclear mKate2) exposed to activated CAR T lymphocytes (10:1 E:T, top row) or naïve untransformed T lymphocytes (10:1 E:T, bottom row) over a 48-hour period. Fixed mKate2 signal threshold (yellow) was set based on control signal and used to quantify loss of target cell fluorescent signal, indicating toxicity associated with (B) activated EpCAM CAR T cells, or (C) naïve, nonengineered T lymphocytes. Scale bar = 200 µm.

Statistically robust and quantitative dose response calculations of target spheroid killing

CAR T potency can be quantified using either defined time points from the kinetic dataset or using a single end-point assay approach. Dose-response calculations are derived from target signal values associated with increasing E:T ratios, which can be directly compared at various E:T ratios and contrasted with the effects of naïve, nonengineered T lymphocytes (Figure 3A). This effect, in turn, enables an EC_{50} potency value for CAR T cells to be derived (Figure 3B).

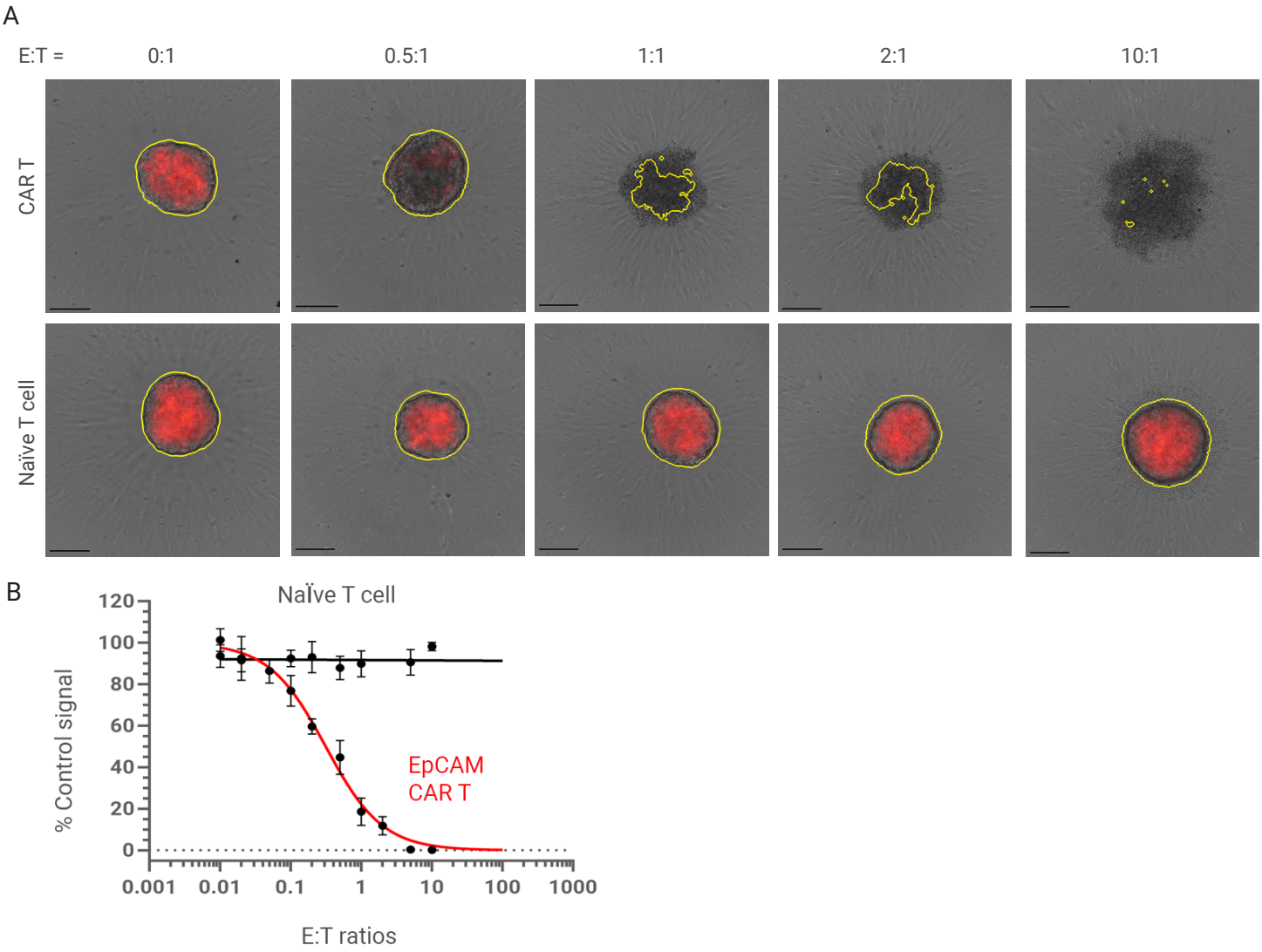

Figure 3. High-throughput imaging enables statistically robust and quantitative dose response of target spheroid killing. (A) T-47D spheroids (red, nuclear mKate2) exposed to increasing E:T ratios of activated CAR T lymphocytes (top row) or naïve, nonengineered T lymphocytes (bottom row) for 48 hours. (B) Fixed mKate2 signal threshold (yellow) was set based on control signal and used to quantify loss of target cell fluorescent signal, indicating toxicity. Scale bar = 200 µm.

Quantification of target cell killing in a matrix-embedded 3D tumor model

The complex tumor microenvironment in solid tumors regulates lymphocyte recruitment and function. An important challenge faced by lymphocytes in solid tumors is navigating the acellular space filled with structural scaffolding of the extracellular matrix (ECM) during migration or invasion. This ECM is heterogenous, and its components can modulate a wide array of cellular responses in both resident tumor cells and infiltrating lymphocytes. Using the high-throughput imaging capabilities of the Cytation C10, a kinetic killing assay can be conducted in a 96-well format, where activated EpCAM CAR T lymphocytes migrate towards T-47D target spheroids which are embedded in a 3D matrix (Figures 1B and 4A). Because this matrix-embedded assay requires individual T lymphocytes to migrate through the ECM to the target cells, the relative cytopathic effect elicited by a given E:T ratio is delayed relative to the suspension model (Figure 4A and 4B). This results in higher IC_{50} values observed across 3D embedded conditions compared to tumor spheroid models suspended in media (Figure 4C).

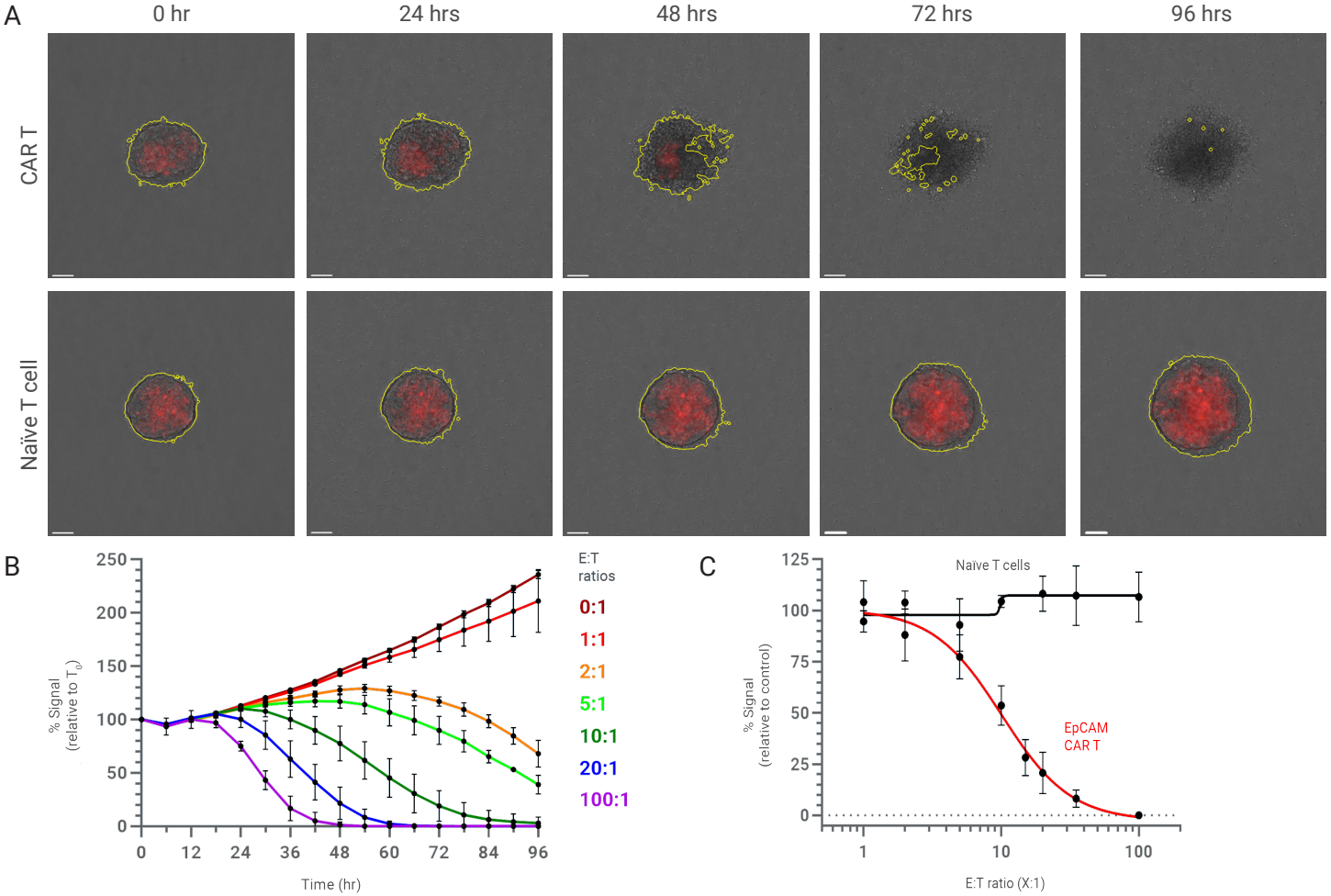

Figure 4. High-throughput imaging enables quantitative evaluation of spheroid killing in a 3D matrix. (A) T-47D spheroid (red, nuclear mKate2) embedded in a collagen matrix and exposed to activated CAR T lymphocytes (100:1 E:T, top row) or no exposure (bottom row) over a five-day period. Fixed mKate2 signal threshold (yellow outline) was set based on control signal and used to quantify loss of target cell fluorescent signal, (B) kinetically, and (C) potency at 48 hrs. Scale bar = 100 µm.

CAR T-mediated target cell toxicity requires that activated effector T lymphocytes directly interact with and form an immunologic synapse with target cells. In the context of a multicellular mass, such as the matrix-embedded tumor spheroid models used in this study, T lymphocytes have the capacity to interact not only with target cells superficial to the spheroid, but infiltrate and interact with target cells within the mass. The spinning disk confocal imaging mode on the Cytation C10 enables detailed characterization of labeled CAR T cell infiltration of 3D model targets (Figure 5A). Furthermore, available water immersion imaging at 60x provides an optically ideal configuration to capture deep cellular insight into activated CAR T engagement with 3D target cells (Figure 5B).

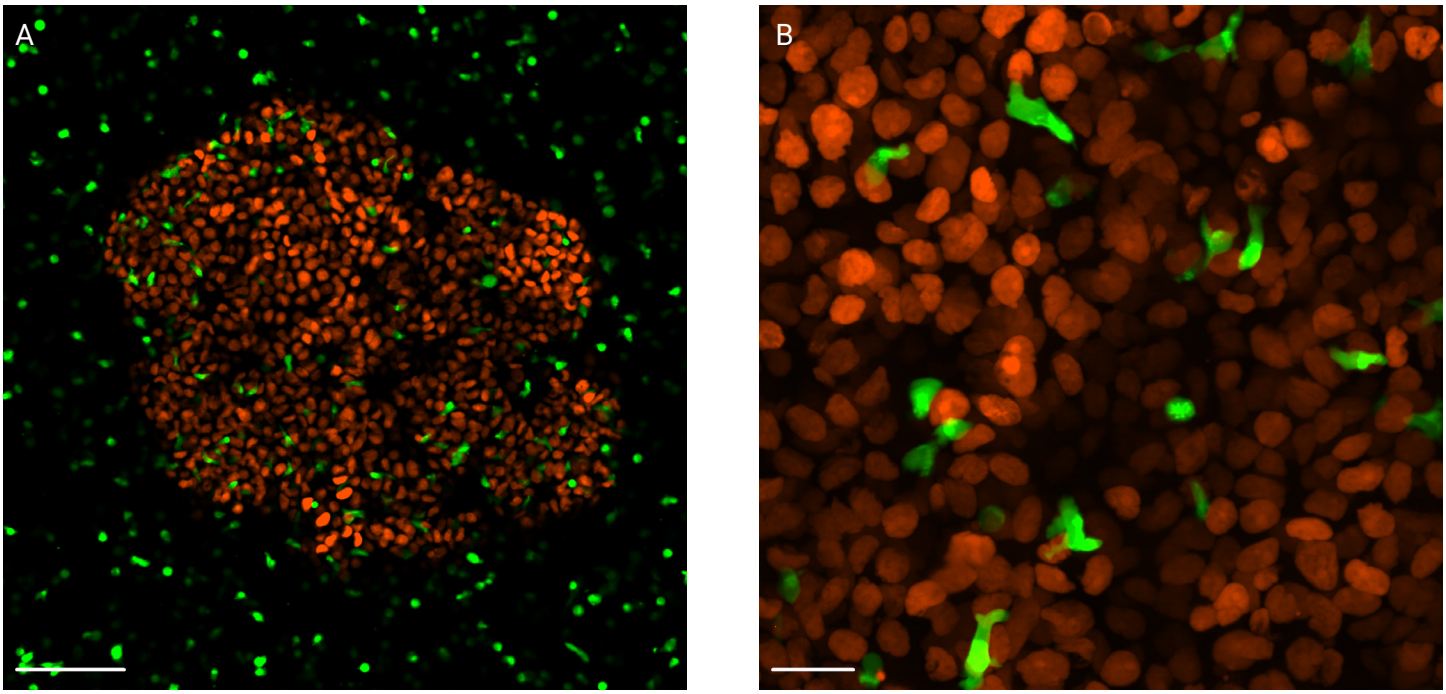

Figure 5. Confocal imaging provides insight to CAR T infiltration of target cell spheroids after 96 hrs of treatment. (A) A T-47D spheroid expressing nuclear-localized mKate2 embedded in a 1% collagen matrix and surrounded by activated CAR T lymphocytes loaded with CellTracker-Green. Image is at 96 hours post CAR T seeding and ~ 25 µm deep into the target spheroid with a 20x objective in confocal mode using the deep sectioning disc. Scale bar = 200 µm. (B) Region within the same spheroid and depth as A) but imaged with 60x water immersion confocal imaging with deep sectioning disc, which provides clearer insight to effector cell infiltration. Scale bar = 20 µm.

Conclusion

In this application note, we demonstratde that the Agilent BioTek Cytation C10 confocal imager reader provides a high-throughput kinetic 3D imaging solution that quantifies CAR T cell-mediated cell killing of target spheroids using fluorescence microscopy. Specifically, this application note highlights the effectiveness of image-based analysis in kinetically monitoring T lymphocyte-induced cytotoxicity of human cells, supporting the development and optimization of CAR T cell therapies.

Coupled with the Agilent BioTek BioSpa 8 automated incubator, the BioTek Cytation C10 confocal imager reader is ideally suited for conducting long-term immune cell killing assays. We find that EpCAM targeting CAR T cells exhibit enhanced toxicity towards target spheroids compared to nonengineered T lymphocytes. The integration of automated imaging systems with high-throughput capabilities provides a powerful tool for advancing cell-based cancer therapeutics, offering detailed kinetic profiles and consistent measurements that can accelerate research and applications.

Products used in this application

Agilent products

Agilent BioTek Cytation C10 confocal imaging reader

Agilent BioTek BioSpa live cell analysis system

www.agilent.com/lifesciences/biotek

RA250624.410

Published in the USA, June 27, 2025
5994-8428EN

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