Validation of High-Throughput Imaging Assays to Characterize CAR T-Mediated Migration and Targeted Cell Killing

Ernest Heimsath, Paul Held, Pete Brescia, and Joe Clayton
Agilent Technologies, Inc.

Cell Bio 2025
Poster # P1674

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Introduction

Adoptive cell therapies such as chimeric antigen receptor (CAR) T cells have ushered in a new paradigm of cancer immunotherapy. In this study, we use genetically modified T cells 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 and an imaging-based assay, we demonstrate that EpCAM-targeted CAR T cells exhibit dose-dependent cytotoxicity against T-47D tumor spheroids embedded in a three-dimensional extracellular matrix (3D ECM). The matrix environment required individual T cells to actively migrate through the ECM to reach their targets, resulting in a delayed cytopathic response compared to suspension models. This delay was reflected in higher IC_{50} values observed under embedded conditions. Additionally, we showed that antigen-stimulated CAR T cells not only bind to the spheroids but also infiltrate them, indicating deep penetration and engagement with tumor cells. These results highlight the utility of high-throughput 3D imaging for capturing dynamic cell interactions and quantifying therapeutic efficacy in physiologically relevant models, offering valuable mechanistic insights that can inform the development of more effective cell-based cancer therapies.

Experimental

Cell lines

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

T lymphocyte activation

EpCAM CAR T cells were stimulated twice with 500 ng/mL EpCAM epitope before the cell-killing assay initiation: 24 hours after initial thaw and on day four. Cells were counted daily and maintained at 1×10^6 cells/mL.

3D cell-killing assay setup

Spheroids of T-47D target cells expressing nuclear mKate2 were formed by seeding in a ULA U-bottom, 96-well plate (Corning part number 4520). Spheroids were either exposed directly to increasing ratios of activated EpCAM-engineered effector CAR T cells (E:T ratio) (Figure 1A and C) or embedded in a 1% collagen matrix within a flat-bottom, 96-well microplate (Agilent part number 204626-100), where activated T cells were top-seeded and allowed to migrate through the matrix (Figure 1B).

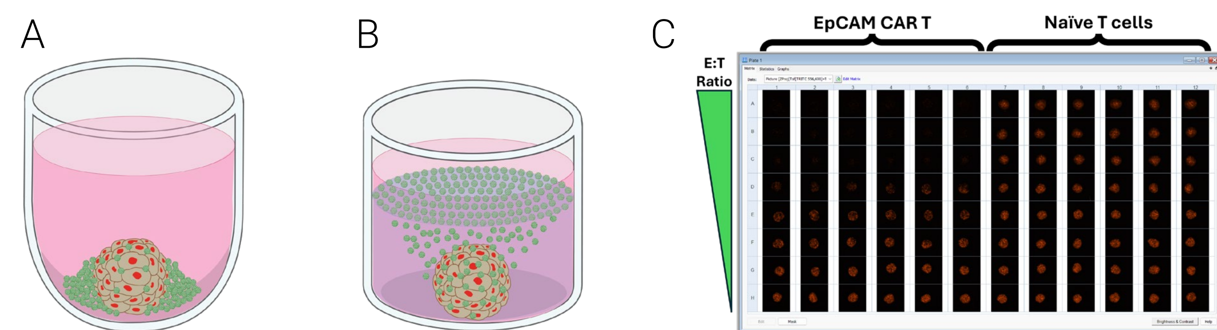

Figure 1. 3D cell-killing assay setup.

Imaging

Wells were imaged every three hours for a 48-hour period in the indicated channels using an Agilent BioTek Cytation C10 confocal imaging reader coupled with an Agilent BioTek BioSpa 8 automated incubator. A laser autofocus (LAF) routine was used for rapid focusing on each well. After the image capture, images were preprocessed to subtract background.

Image analysis and quantification

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. For time-lapse studies, fluorescence intensity values of experimental conditions (in the presence of T cells) are reported as percent change relative to the initial time point. For dose-response studies, values are reported as relative to control samples.

Results and Discussion

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

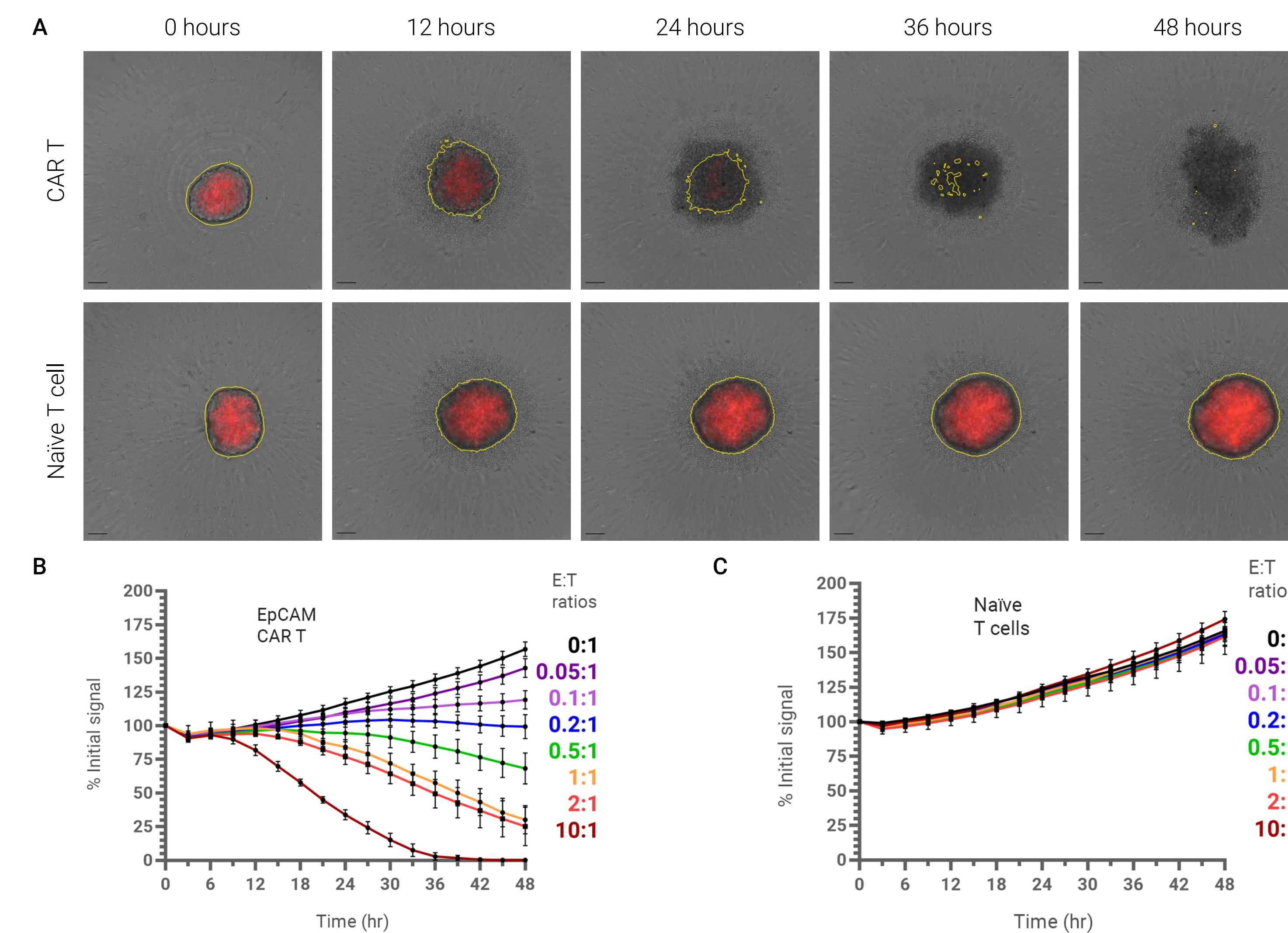

Figure 2. (A) T-47D spheroid (red, nuclear mKate2) exposed to activated CAR T lymphocytes (10:1 E:T, top row) or naïve untransformed T lymphocytes (10:1 E:T, bottom row) over a 48-hour period. Fixed mKate2 signal threshold (yellow) was set based on control signal and used to quantify loss of target cell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

High-throughput imaging enables statistically robust and quantitative dose response of target spheroid killing

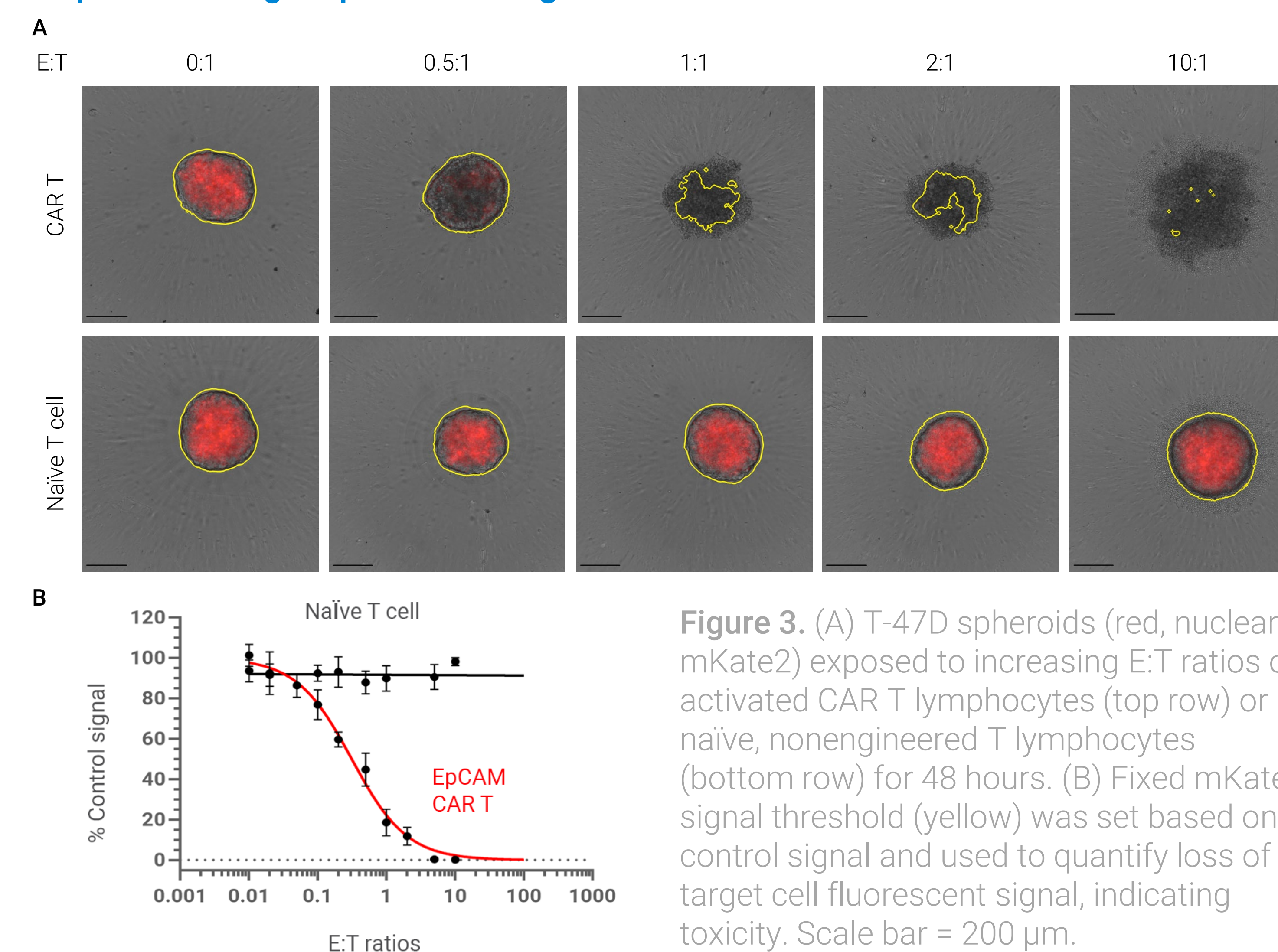

Figure 3. (A) T-47D spheroids (red, nuclear mKate2) exposed to increasing E:T ratios of activated CAR T lymphocytes (top row) or naïve, nonengineered T lymphocytes (bottom row) for 48 hours. (B) Fixed mKate2 signal threshold (yellow) was set based on control signal and used to quantify loss of target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

Results and Discussion

High-throughput imaging enables quantitative evaluation of spheroid killing in a 3D matrix

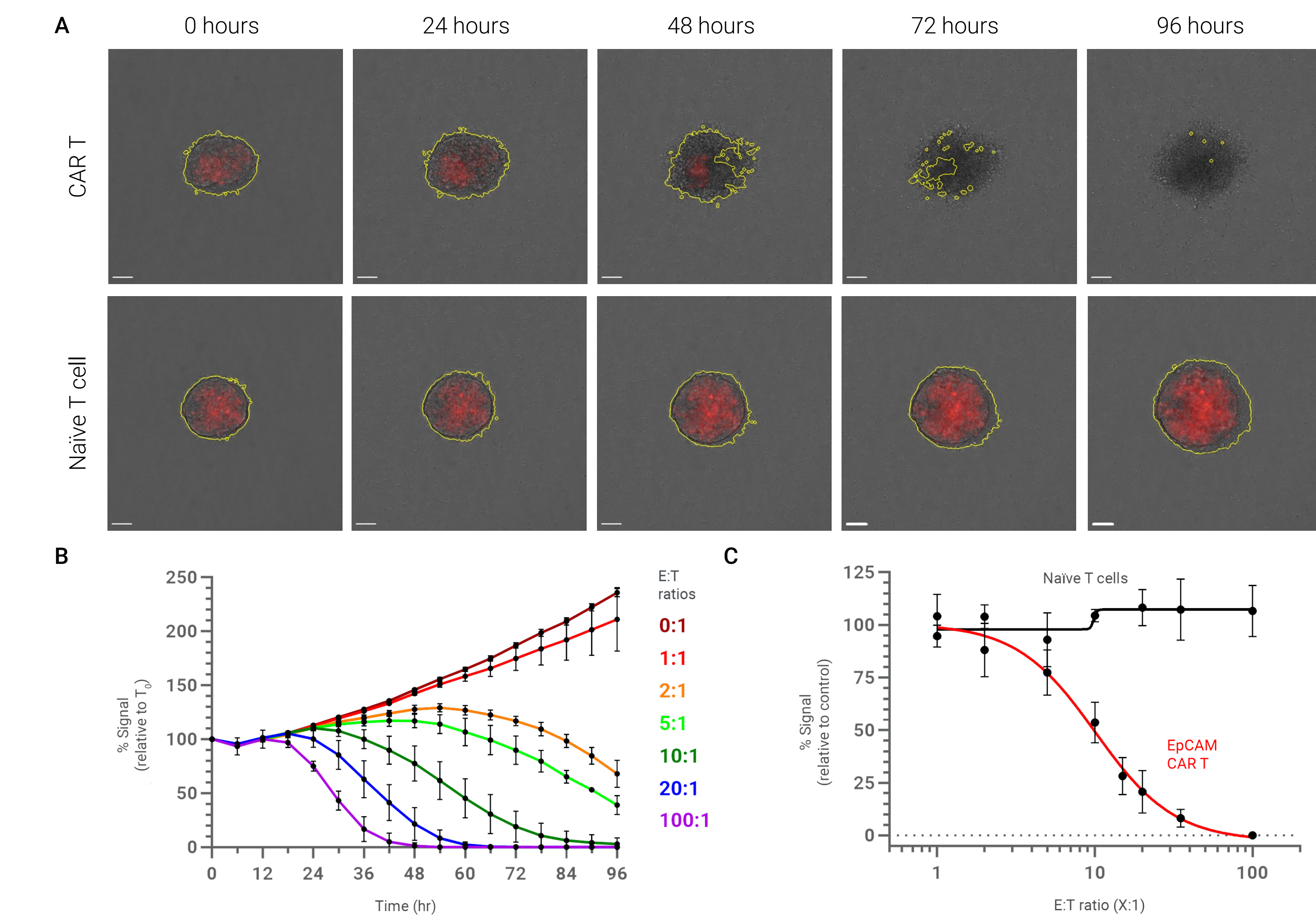

Figure 4. (A) T-47D spheroid (red, nuclear mKate2) embedded in a collagen matrix and exposed to activated CAR T lymphocytes (100:1 E:T, top row) or no exposure (bottom row) over a five-day period. Fixed mKate2 signal threshold (yellow outline) was set based on control signal and used to quantify loss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

High-resolution confocal imaging reveals CAR T infiltration into 3D sample

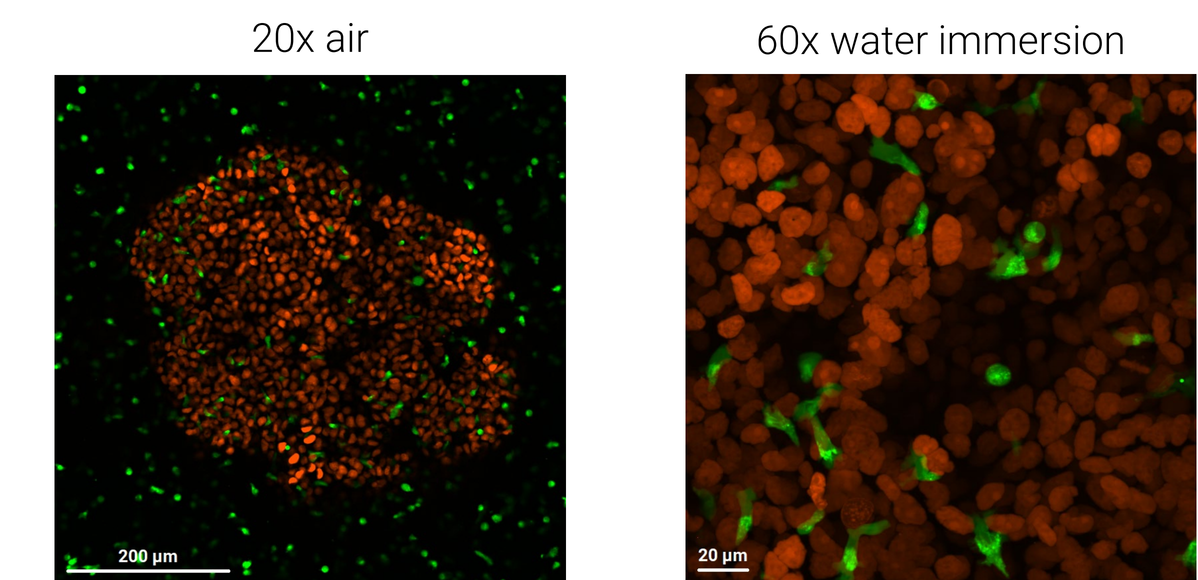

Figure 5. EpCAM CAR T lymphocytes infiltrating a T-47D spheroid embedded in a 1% collagen matrix. Confocal images taken at a Z depth of ~ 50 µm into the spheroid. Red: T-47D spheroid (mKate2-labeled nuclei). Green: EpCAM CAR T (CellTracker Green CMFDA).

Conclusions

High-throughput imaging-based methods of analysis enable temporal monitoring of T lymphocyte-induced cytotoxicity of human cells, supporting the development and optimization of CAR T cell therapies.

Scan the code for the application note.

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RA251117.559