Development of High-Throughput 2D and 3D Imaging Assay to Characterize CAR T-Mediated Cell Killing

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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 T cells 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 effector cells exhibited their cytotoxic effects on target T-47D adherent cultures (2D) as well as spheroids (3D) in a dose-dependent manner. Confocal imaging reveals that activated CAR-T effector cells infiltrate target T-47D spheroids. This automated functional assay enables high-throughput mechanistic characterization of candidate CAR T-cell lines.

Experimental

Cell lines

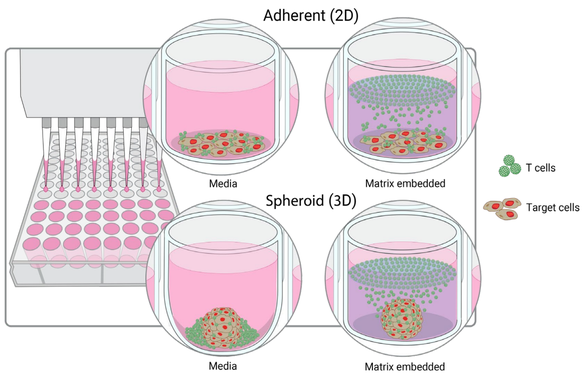
EpCAM+ T-47D breast ductal carcinoma cells (ATCC, part number HTB-133) stably expressing nuclear-localized mKate2 (eLenti-Red; Agilent part number 8711011), EpCAM CAR T cells, and primary T lymphocytes were cultured in RPMI containing 10% FBS and 1x primocin. Cells were supplemented with either 0.2 U/mL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

T lymphocyte activation

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

2D and 3D cell-killing assay setup

Adherent T-47D target cells expressing nuclear mKate2 were seeded in a 96-well plate (Agilent part number 204626-100 for 2D adherent, Corning part number 4520 ULA U-bottom for 3D spheroids), exposed to increasing ratios of activated EpCAM-engineered effector CAR T cells (E:T ratio), and continuously imaged for 48 hours.



Imaging

Wells were imaged kinetically in the indicated channels using either an Agilent BioTek Lionheart FX automated microscope or 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 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

T lymphocyte activation and clustering can be monitored and quantified with brightfield image analysis.

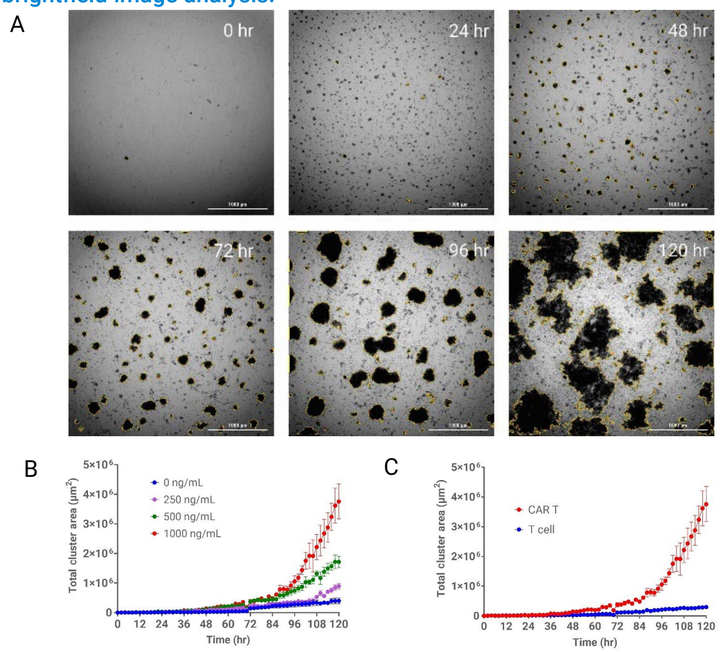


Figure 1. (A) Brightfield images of T lymphocytes activated with CD3/CD28 cocktail. Yellow masking indicates cluster area identified with automated image analysis. Scale bar = 1 mm. (B) Kinetic profiles of total cluster area for CAR T-cell activation as a function of EpCAM concentration and (C) CAR T versus T-cell lymphocytes treated with EpCAM protein.

2D CAR T-lymphocyte-mediated cell killing can be monitored and quantified using image-based analysis of a fluorescent cell marker.

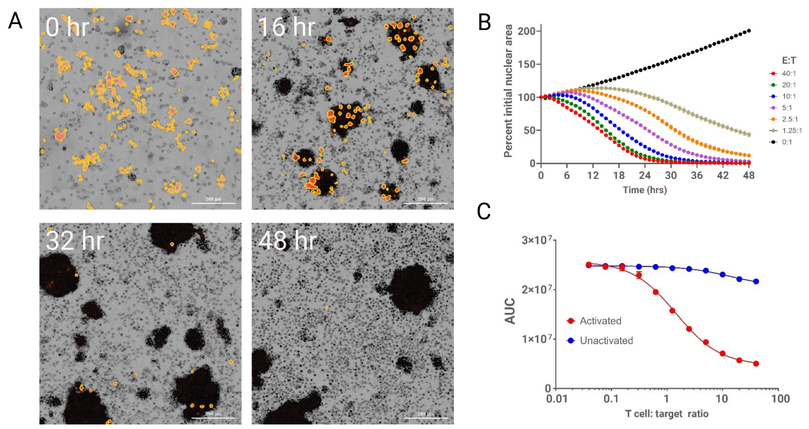


Figure 2. Loss of nuclear-localized fluorescent signal (red) in adherent T-47D target cells can be used as a readout for cell death. (A) Image-based analysis of T-47D-mKate2 target cells treated with activated CAR T cells. Images were captured with a 4x objective in the TRITC and brightfield channels over a 48-hour period. Yellow masking indicates mKate2-positive viable target cells for quantification. (B) Kinetic profiles of the total nuclear area of T-47D cells exposed to varying E:T ratios. (C) Dose response of activated versus nonactivated CAR T-cell-induced killing of T-47D target cells using AUC values from kinetic profiles.

Results and Discussion

CAR T-lymphocyte-mediated cell killing of T-47D spheroids is quantified with fluorescent image analysis.

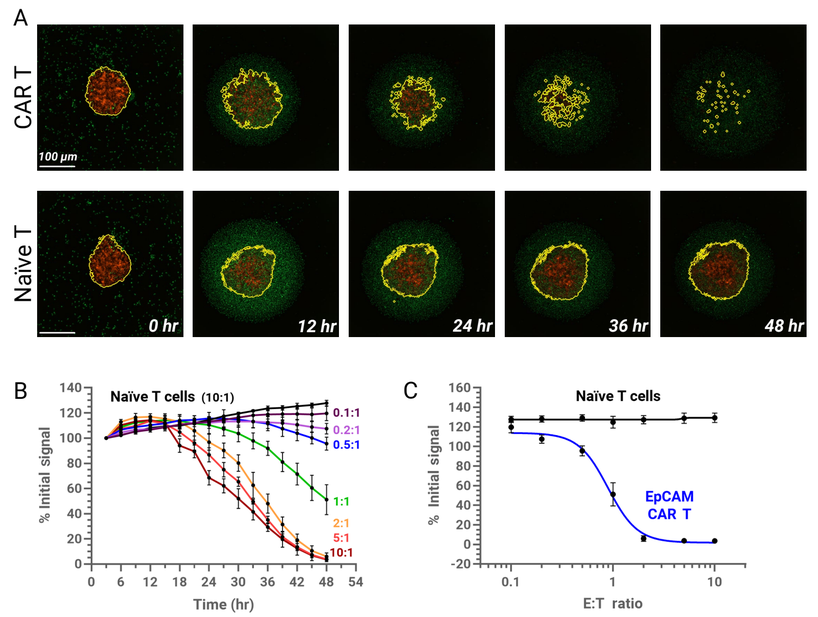


Figure 3. (A) Timelapse of T-47D spheroid killing by activated EpCAM CAR T or naïve T lymphocytes. (B) High-throughput quantification of integrated fluorescent signal of T-47D spheroids exposed to increasing ratios of activated EpCAM CAR T cells. (C) EC_{50} potency of activated EpCAM CAR T or naïve T cells against target T-47D spheroids.

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

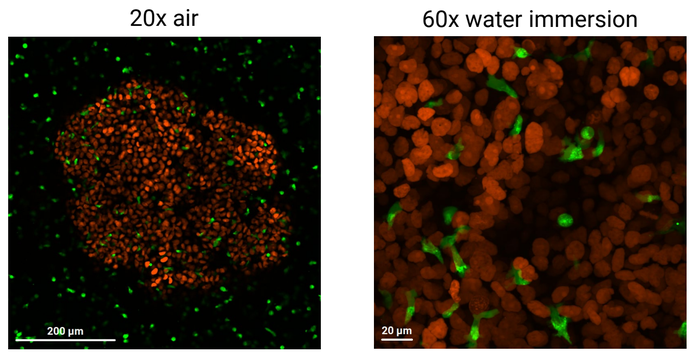


Figure 4. EpCAM CAR T lymphocytes infiltrating a T-47D spheroid embedded in a 1% collagen matrix. Confocal images taken at a Z-depth of ~ 50 µm into the spheroid. **Red:** T-47D spheroid (mKate2-labled nuclei). **Green:** EpCAM CAR T (CellTracker Green CMFDA).

Conclusions

High-throughput imaging-based methods of analysis enable temporal insight to targeted CAR T-mediated cell killing.

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