Application Note

Immuno-Oncology

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Automated Imaging-Based Method to Measure CAR T-Cell-Mediated Cytotoxicity

Author

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Abstract

The use of chimeric antigen receptor (CAR) T cells represents a promising cancer treatment modality, specifically engineered to recognize and destroy cancer cells. This application note details an automated imaging-based method to monitor and quantify immune-cell-mediated cytotoxicity in 2D cancer cell cultures. By employing fluorescent nuclear markers and advanced imaging techniques, this approach enables real-time, kinetic analysis of cell-mediated killing, providing significant advantages over traditional assays. This study demonstrates that epithelial cell adhesion molecule (EpCAM)-targeting CAR T cells exhibit enhanced cytotoxicity towards target cells, with variability observed across different cell lines based on EpCAM expression levels. Automation of the process ensures high throughput, minimal variability, and repeatable data, supporting the development and optimization of CAR T cell therapies.

Introduction

CAR T cell therapy is a form of cellular adoptive immunotherapy that is used to treat disease. Adoptive transfer of T cells expressing CARs is a promising anti-cancer therapeutic given that CAR-modified T cells can be engineered to target potentially any tumor associated antigen.[1,2] The basic concept underlying the design of CARs is to link an extracellular ligand recognition domain, typically a single-chain variable fragment (scFv), to an intracellular signaling module that includes CD3-δ to induce T cell activation upon antigen binding (Figure 1). Early CAR T cell research, which focused on blood cancers, has been remarkably successful in treating individuals with advanced refractory B cell malignancies.[3] The first approved treatments use CARs that target the antigen CD19, present in B-cell-derived cancers such as acute lymphoblastic leukemia (ALL) and diffuse large B-cell lymphoma (DLBCL).[4] Solid tumors have presented a more difficult target.[5] Identification of good antigens has been challenging, as the antigens must be highly expressed on most of the cancer cells, but largely absent on normal tissues.[6] CAR T cells are also not trafficked efficiently into the center of solid tumor masses, and the hostile tumor microenvironment suppresses T cell activity.[7]

Effective strategies for measuring cell-induced cytotoxicity are critical for the evaluation and development of CAR T cell therapies, as well as other cell-based cancer therapeutics, including natural killer (NK) cells, and bispecific T-cell engagers (BiTEs). However, conventional methods for measuring cell-mediated killing assays have considerable limitations. The chromium release assay, while widely used, is limited by its endpoint nature, its inability to measure cytotoxicity kinetics, and the use of harmful radioactive materials. ELISA assays, though valuable, rely on indirect measures of cytotoxicity, require complex workflows, and are limited to single end-point measurements. Despite their utility, these methods may not fully reflect the activation, proliferation, inhibition, and exhaustion of effector cells.

A robust automated cell imaging and analysis approach to quantifying cell-mediated cytotoxicity offers significant advantages over conventional methods. Fluorescence live cell imaging enables real-time monitoring of cell-to-cell interactions and treatment response, with sensitive detection of cell-mediated killing at relevant effector to target (E:T) cell ratios. Powerful image analysis tools ensure consistent and accurate measurements, providing detailed kinetic profiles of induced cytotoxicity. This straightforward automated approach reduces manual errors and allows for detailed kinetic analysis of cell-mediated cytotoxicity in various therapeutic contexts. By overcoming the limitations of traditional assays, automated imaging and analysis solutions can accelerate the development and optimization of cell-based therapies while supporting high-throughput applications.

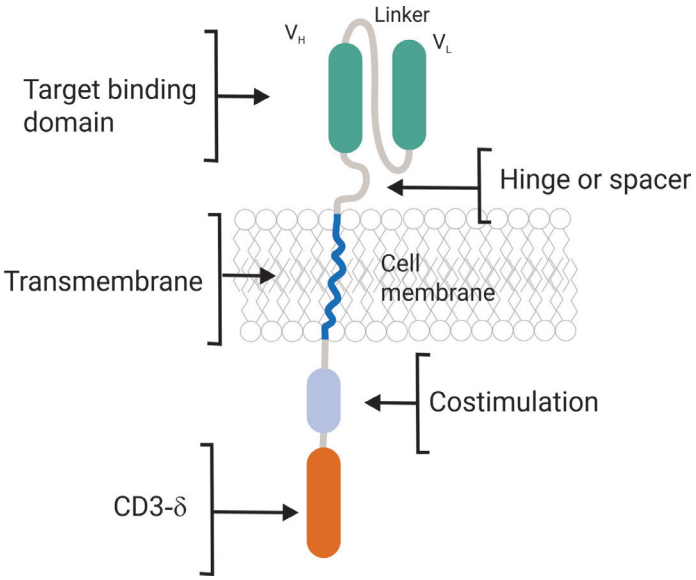

Figure 1. Schematic design of a CAR receptor.

In this proof-of-concept study, two target cancer cell lines expressing fluorescent nuclear labels were treated with EpCAM-targeting CAR T cells under various conditions and monitored over a multiday timecourse. Lentivirus-modified target cell lines expressing a fluorescent nuclear marker were used to avoid cytotoxic stains and enable low magnification detection. This facilitated capturing significant cell numbers in a single image. CAR-modified T lymphocytes demonstrated nearly double the cytotoxicity compared to unmodified T cells, influenced by the presence of target cell surface markers. This effect exhibited a strong dependency on E:T ratios tested. with significant target cell death detected at the lowest E:T ratio (1.25:1). The cytotoxicity varied across cell lines, correlating with EpCAM expression levels. Incorporation of automation strategies demonstrated the ability to run up to 8 microplates in the system concurrently for high throughput applications.

Experimental

T47D-mKate2 cells were cultured in RPMI 1640 (Life Technologies, part number 11875-093) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, and 0.2 U/mL insulin (Sigma Aldrich, part number I-1882) at 37 °C in 5% CO_2. HT1080-mKate2 and A549-mKate2 cell lines were cultured in A-DMEM (Life Technologies, part number 12491-015) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin. Cultures were routinely trypsinized (0.05% trypsin-EDTA) at 80% confluence.

CAR modified, and primary T lymphocytes were cultured in ImmunoCult-XF Cell Expansion media (STEMCELL Technologies, part number 10981) supplemented with 200 U/mL IL-2. (STEMCELL Technologies, part number 78036), normal lymphocyte were stimulated using 25 µL/mL of either ImmunoCult Human CD3/CD28 T cell activator (part number 10971) or ImmunoCult Human CD3/CD28/CD2 T cell activator (part number 10970) from STEMCELL Technologies. Cells were incubated for 3 days at 37 °C and 5% CO_2 and diluted 8-fold with fresh media. The cells were then rechallenged and incubated for another 3 days. Car T cells were stimulated in a similar manner except that 250 µg/mL of EpCAM was substituted for the T cell activator cocktails.

For each experiment, mKate2 expressing cell lines were trypsinized, counted and seeded into black-sided, clear bottom plates (Agilent part number 204626-100) at 5,000 cells per well in 200 µL of media. The following day, dilutions of CAR T or untransformed T lymphocytes were added in 100 µL of XF cell expansion media and imaging initiated as rapidly as possible.

Imaging

Wells were imaged kinetically using either an Agilent BioTek Lionheart FX automated microscope or an Agilent BioTek Cytation 5 cell imaging multimode reader in the widefield TRITC channel with a 4x objective. Cells were maintained at 37 °C in a humidified 5% CO_2 environment. Camera exposure settings were set manually with the Gen5 autoexposure routine on control wells prior to imaging the plate. 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, cell count analysis routines, based on a fluorescence intensity threshold in the TRITC channel and size discrimination were used to identify nuclei and report the relative changes in target cell number over time. Total nuclear area data for the kinetic assays were expressed as a percentage of the initial nuclear area determination.

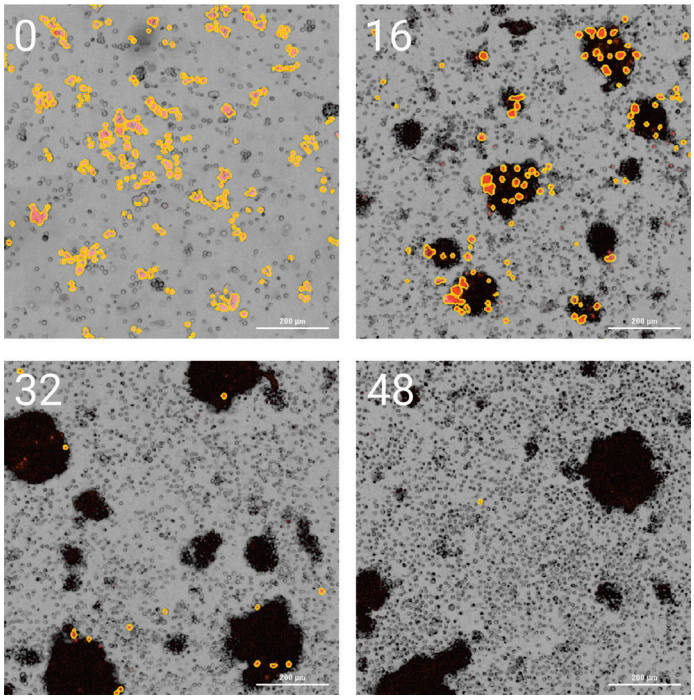

Figure 2. Brightfield and fluorescent images of T47D-mKate2 cells exposed to activated CAR T cells. Images (4x) were captured from the same well in the brightfield and TRITC channels every 16 hours. Images reflect a digitally zoomed region of the same well, with object masks (yellow) applied around TRITC fluorescence detected cell nuclei.

Results and discussion

Kinetic image-based analysis provides detailed insight into cell-dependent killing

The effect of activated CAR T lymphocytes on T47D-mKate 2 cells can be monitored using image-based analysis. As seen in Figure 2, where the same well is depicted every 16 hours for 48 hours, the number of fluorescent objects in the TRITC channel diminishes with time.

The cytotoxicity towards T47D-mKate2 cells is proportional to the number of activated CAR T- cell present (Figure 3). Different ratios of CAR T cells to target cells result in markedly different total nuclear areas in the population over time. Target cells treated with activated CAR T cells at a ratio of 20:1 exhibited near complete loss of signal within 24 hours.

Negative control conditions in which 100 µL of T cell media alone was added to T47D-mKate2 target cells resulted in no detectable cell killing. Under these conditions, target cells proliferated at a consistent rate, with the total nuclear area increasing in accordance with a population doubling within 48 hours.

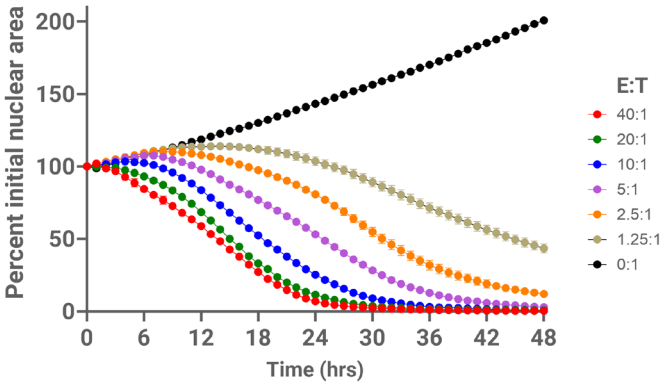

Figure 3. Total nuclear area of T47D-mKate2 cells exposed to different ratios of activated CAR T cells to target cells. Dilutions of activated CAR T cells were added to T47D-mKate2 cells. Images were captured every hour for 48 hours and fluorescent objects were identified and the total area calculated based on TRITC labeled nuclei. Data is expressed as the percentage of the initial kinetic determination. Each data point represents the mean and standard deviation of 4 replicate data points.

Unactivated CAR T cells have little effect on T47D-mKate2 cells over a 48-hour period (Figure 4). However, one could surmise that over time the presence of the EpCAM protein on the surface of the T47D-mKate2 cells would eventually activate the CAR T cells. There are indications of this within the wells treated with a high E:T ratio. While the percentage of cells relative to the initial determination is approximately 100% in the 40:1 ratio sample set, the nuclear area is not increasing to the same extent as observed with the samples that did not receive CAR T cells (Figure 4). Further incubation would likely lead to activation and cytotoxicity.

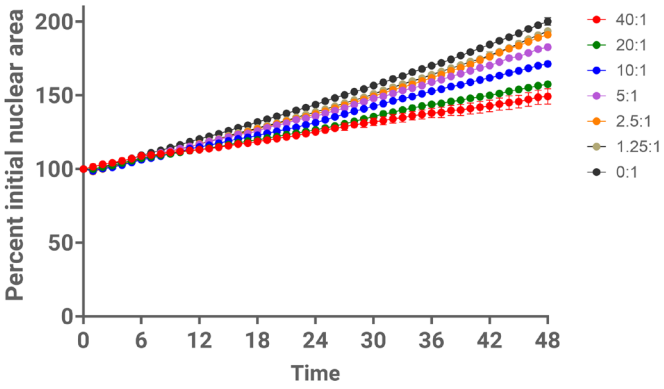

Figure 4. Total nuclear area of T47D-mKate2 cells exposed to different ratios of unactivated CAR T cells to target cells. Dilutions of unactivated CAR T cells were added to T47D-mKate2 cells. Images were captured every hour for 48 hours and fluorescent objects were identified and the total area calculated based on TRITC labeled nuclei. Data is expressed as the percentage of the initial kinetic determination. Each data point represents the mean and standard deviation of four data points.

Integral analysis of kinetic data for dose-response determination

The kinetic determination of nuclear area can be exploited further by using an integral or area under the curve (AUC) calculation of the kinetic data. Agilent BioTek Gen5 data reduction software can determine the integral or AUC for each kinetic plot (Figure 5). Samples where target cells are more effectively eliminated would exhibit a lower integral or AUC value, while samples that are subjected to less cell-induced killing would have higher values. High concentrations of CAR T cells demonstrated effective killing of T47D target cells in a short period of time, whereas lower concentrations of cells do not demonstrate killing over much longer periods of time. We used 48 hours as the run time for all AUC based experiments to capture cell-mediated killing resulting from low concentrations of activated T lymphocytes.

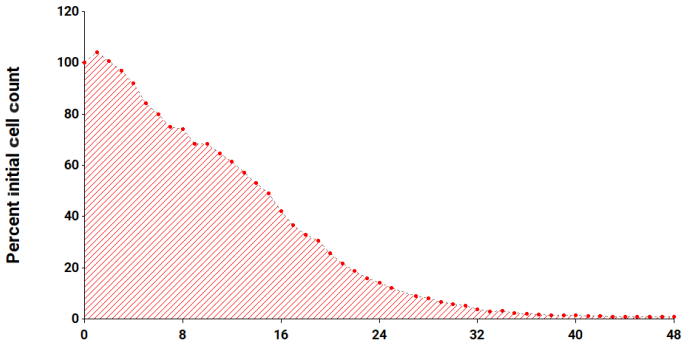

Figure 5. Integral calculation from kinetic data. The nuclear area for T47D-mKate2 cells exposed to a 20:1 ratio of CAR T cells was determined kinetically every hour for 48 hours. The percentage of initial nuclear area was calculated and plotted as a function of time. The red-shaded area represents the area under the curve (AUC) analysis of the kinetic data.

The AUC values for different CAR T E:T ratios can be plotted as a function of cell-to-cell ratio (Figure 6). There is a sigmoidal relationship between cytotoxicity of the target T47D-mKate2 cells and an increase in the CAR T cell to target cell ratio that can be described using a 4-parameter logistic fit of the data. Unactivated Car T cells were ineffective in killing T47D-mKate2 cells regardless of their concentration.

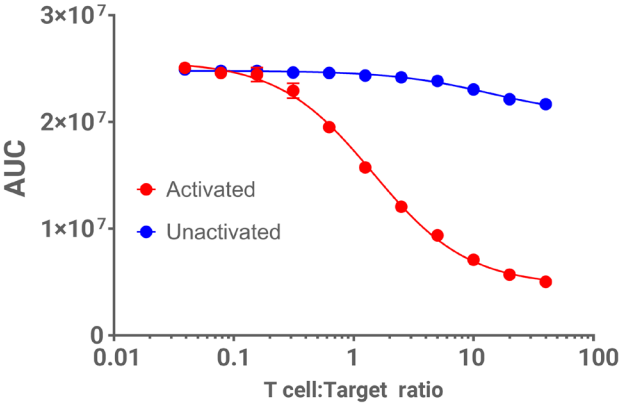

Figure 6. Comparison of activated and unactivated CAR T cells cell killing. Dilutions of activated CAR T or unactivated CAR T cells were added to T47D-mKate2 cells. Images were captured every hour for 48 hours and fluorescent objects were identified and the total area calculated based on TRITC labeled nuclei. Using kinetic analysis, the percentage of the total nuclear area compared to the initial measurement is plotted as a function of time. The subsequent AUC for each T cell concentration is calculated and plotted as a function of E:T ratio. Data represents the mean and standard deviation of eight replicates.

Similar comparisons can be made with untransformed yet activated T cells. Figure 7 depicts a relative potency determination made using Gen5 data analysis software. Using parallel line analysis, the EC_{50} of both activated CAR T cells and activated untransformed T cells can easily be compared. This analysis calculates the 4-parameter fit values of both data sets concurrently and constrains the slope, minimum, and maximum values to be the same value. The inflection point parameter or EC_{50} value can be used to determine relative potency. While both activated CAR T cells and activated T cells are cytotoxic towards T47D-mKate2 cells, activated CAR T cells are more potent than activated T cells.

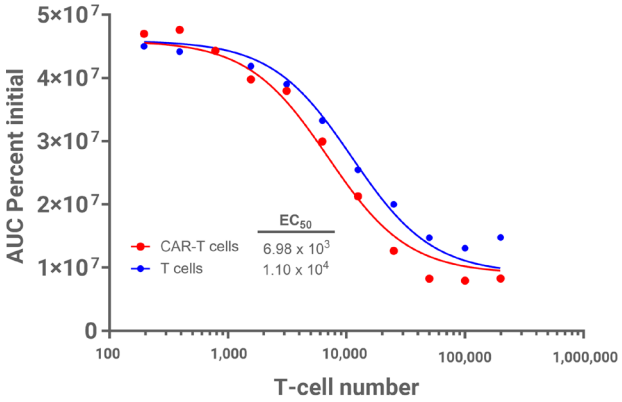

Figure 7. Potency of activated CAR T verses activated T cell killing. Dilutions of activated CAR T or activated untransformed T-lymphocytes cells were added to T47D-mKate2 cells that had been previously seeded in the microplate. Images were captured every hour for 48 hours and fluorescent objects were identified and their total area calculated based on TRITC labeled nuclei. Using kinetic analysis, the percentage of the total nuclear area compared to the initial measurement was plotted as a function of time. The subsequent AUC for each lymphocyte cell concentration is calculated and plotted as a function of cell number using Agilent BioTek Gen5 parallel line analysis tool. Data represents the mean and standard deviation of duplicate measurements.

Target cell killing correlates with EpCAM epitope levels

There is a marked difference in the effectiveness of the CAR T cell line in killing different cell lines. As seen in Figure 8, the CAR T cell line killed all the T47D-mKate2 cells within 48 hours, while HT1080-mKate2 cells were unaffected during the same time-period.

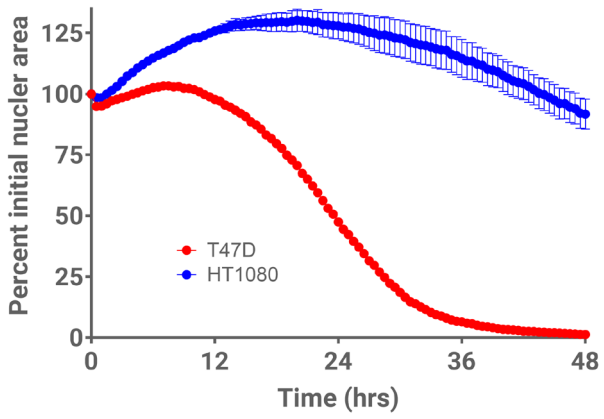

Figure 8. Comparison of activated CAR T killing with T47D-mKate 2 and HT1080-mKate2 human cell lines. 100,000 activated CAR T were added to T47D-mKate2, and HT1080 mKate2 cells that had been previously seeded. Images were captured every half-hour for 48 hours and fluorescent objects were identified and their total area calculated based on TRITC labeled nuclei. Using kinetic analysis, the percentage of the total nuclear area compared to the initial measurement was plotted as a function of time. Data represents the mean and standard deviation of four replicates.

Immunostaining of these target cell lines for the presence of EpCAM protein suggests the differential CAR-T cell-dependent killing is a function of EpCAM epitope levels. T47D-mKate2 cells show significant amounts of EpCAM fluorescent signal, which correlates well with the observed cytotoxicity of EpCAM directed CAR T cells. Likewise, HT1080-mKate2 cells, which were observed to be resistant to the CAR T cells, demonstrates very little EpCAM protein by immunostaining (Figure 9).

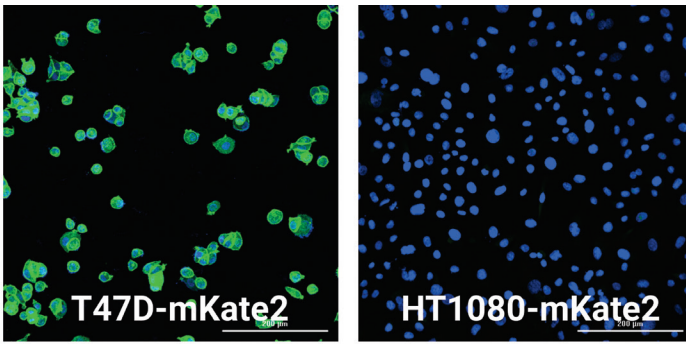

Figure 9. Immunostaining of EpCAM protein on the cell surface of T47D-mKate2 and HT1080-mKate2, cell lines. Fixed cells were incubated with anti-EpCAM antibody and counterstained with Hoechst 34580. Blue staining indicates cell nuclei, while green staining depicts labeled EpCAM surface protein. Cell lines were imaged with an Agilent BioTek Cytation C10 confocal imaging reader using a 20x objective.

Automation for high-throughput applications

Integrating the Agilent BioTek BioSpa automated incubator with the Cytation imaging system supports running the immune cell killing assay on up to 8 microplates concurrently. Multiple temperature zones and circulating fans ensure that optimal growth conditions are maintained uniformly for each microplate position. Figure 10 demonstrates plate-to-plate consistency across matched duplicate plates (plates 1 and 2) containing T47D-mKate2 cells treated with either positive or negative controls. A 48-hour timecourse recapitulated the previously observed CAR T-induced cell killing, with highly consistent values across duplicate plates 1 and 2 for each treatment condition.

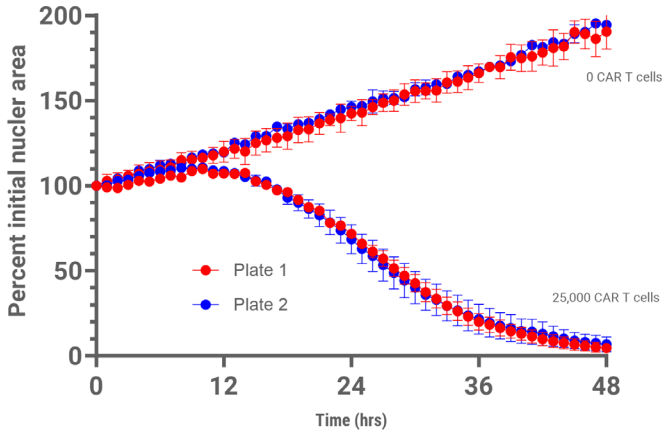

Figure 10. Percent Change of T47D-mKate 2 cell nuclear area exposed to activated CAR T cells. Activated CAR T cells or media only were added to T47D-mKate2 cells. Cells were imaged every hour for 48 hours and nuclear area determined based on TRITC fluorescence. Data is expressed as the percentage of the initial determination. Each data point represents the mean and standard deviation of two data points.

In this study, we describe a robust automated imaging-based solution for measuring immune cell killing, including CAR T-directed cell killing of adherent target cancer cells. This study utilized target cell lines modified with Lentivirus to include a fluorescent nuclear marker, enabling straightforward cell detection and quantification without exogenous stains. However, the same approach can be readily applied to alternative methods for labeling target cells. Low magnification imaging of samples in either 96- or 384-well microplates enables a statistically significant number of target cells to be captured in a single field of view for analysis while supporting high throughput applications. For this study, total object area was used as a readout for target cell number instead of the nuclear count due to the difficulty in segmenting tightly clustered cells, particularly T47D-mKate2 cells. Interestingly, discrete nuclei often coalesced into larger masses of poorly distinguished nuclei, yet these clusters encompassed an area equivalent to the total number of cells present.

The CAR modification of T lymphocytes enhanced their toxicity towards target cells. Activated CAR T cells were nearly twice as potent as unmodified activated T cells under these conditions. Several factors influence CAR T cell cytotoxicity, with the presence of the cell surface marker targeted by the CAR being the most significant. High concentrations of the target epitope increase cell line susceptibility. CAR-modified T cells can also recognize tumor cells via TCR-MHC/peptide interactions, with engineered signaling endodomains aiding in T cell activation and persistence. Other surface proteins

recognized by cytotoxic T cells can also make cells vulnerable, even without the specific CAR target. In this study, the cytotoxicity of CAR T cells varied with different human cell lines. The T47D cells express high levels of EpCAM, while HT1080 cells exhibit undetectable levels. The kill rates of EpCAM-directed CAR T lymphocytes for these cell lines correlate well with epitope immunostaining.

Conclusion

The automated imaging-based method described in this application note offers a robust and efficient approach to measuring CAR T-cell-induced cytotoxicity. By utilizing fluorescent nuclear markers and advanced imaging techniques, this method allows for real-time monitoring and quantification of cell-mediated killing, overcoming the limitations of traditional assays. The ability to capture significant cell numbers in a single image and the use of total object area as a readout provide a reliable measure of cell-mediated killing. The study demonstrates that EpCAM-targeting CAR-T cells exhibit enhanced toxicity towards target cells over non modified T cells, with cytotoxicity varying across different cell lines based on EpCAM expression levels. Automation further increases throughput, ensuring minimal variability and repeatable data across multiple microplates.

The Agilent BioTek Lionheart FX automated microscope and Agilent BioTek Cytation BioSpa live cell analysis system are both ideally suited for conducting immune cell killing assays. Integrated environmental controls support monitoring live cells over extended periods, while onboard Agilent BioTek Gen5 image analysis tools enable robust and sensitive detection of cell-induced target cell killing.

This application note highlights the effectiveness of image-based analysis in kinetically monitoring T cell lymphocyte-induced cytotoxicity of human cells, supporting the development and optimization of CAR T cell therapies. 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 clinical applications.

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Products used in this application

Agilent products

Agilent BioTek Lionheart FX automated microscope

Agilent BioTek Cytation 5 cell imaging multimode reader

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