APPLICATION NOTE

Real-Time Monitoring of CAR T-Cell Mediated Killing of Suspension Tumor Cells

CYTO tronics™

INTRODUCTION

Immuno-oncology is a field of medical research and treatment that focuses on harnessing the body's own immune system to recognize, attack, and eliminate cancer cells. It has shown significant promise in the treatment of certain cancers, providing new avenues for therapeutic intervention and demonstrating durable responses in some patients. Research in this field continues to expand, and ongoing efforts aim to develop more targeted and effective immunotherapies for a broader range of cancer types. Of particular interest are adoptive cell therapies which involve modifying a patient's own immune cells, such as T-cells, to enhance their cancer-fighting capabilities before reintroducing them into the body.[1]

In this note, we demonstrate that CytoTronics' technology[2] can monitor CAR T-cell mediated killing of suspension target cells in real-time. Our approach involves the use of an optimized pair of target (T) and effector (E) cells, specifically employing CD19-targeting CAR T-cells in conjunction with K562 cells engineered to express CD19 (K562-CD19). Real-time measurements of confluence can precisely quantify effector cell activity even at low effector to target (E:T) ratios. This facilitates CAR T-cell testing under physiologically relevant conditions.

RESULTS

Precise Quantification of Target Cells for Immune Cell Killing Assays

Impedance measurements require cells to be in close proximity to the electrodes. In the case of suspension cells, this requires the cells to be brought closer to the electrodes for impedance measurements. To address this, CytoTronics' microplates were assessed for their effectiveness in measuring K562 cells using two methods: antibody immobilization tethering (with anti-CD71) and PDL coating. In the first method, CD71 antibodies immobilized on the electrodes bind CD71 on the K562 cells, thus tethering cells close to electrode surface. In the second, poly-d-lysine (PDL) promotes cell adhesion to the surface by enhancing electrostatic interaction between the cells and electrode surface. Figure 1 shows the measured confluence of K562 cells utilizing both approaches, as compared to bare electrodes. It was observed that PDL coating enables visualization of the maximum number of K562 cells with the expected increase in confluency with increased plating density, making it the method of choice for these cells.

Another crucial aspect in establishing an immune cell killing assay is the accurate quantification of the number of target cells. As target cells typically exhibit proliferative behavior, real-time assessment of their numbers becomes challenging. To achieve precision in estimating these numbers, K562-CD19 cells were seeded at a range of densities to generate a standard curve of confluence by cell number. Confluence measurements from these wells were assessed at 15 hours post cell seeding, ensuring that the cells had been immobilized, but had not yet begun proliferating (Figure 2).

For the immune cell killing assay, K562-CD19 cells were seeded at a density of 80,000 cells per well on CytoTronics' microplates. After 24 hours, confluence was measured, and the standard curve was used to calculate the true cell number. The cell number at 24 hours was calculated as 93,000 cells per well (Figure 2B), which served as the basis for determining the number of CAR T-cells

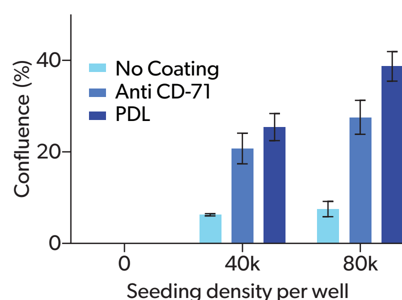

Figure 1. Confluence of K562-CD19 cells using two mobilization methods: PDL coating and antibody tethering at 0, 40,000, and 80,000 cells per well 24 hours after seeding.

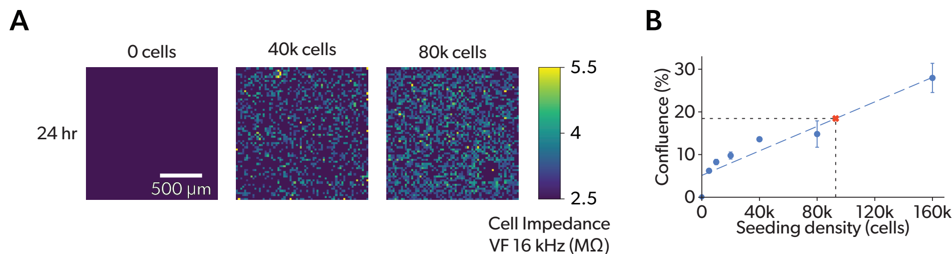

Figure 2. (A) Confluence images of K562-CD19 cells seeded on PDL-coated microplates at the 0, 40,000 and 80,000 per well 24 hours after seeding. (B) Standard curve of measured confluence to seeding density for a range of K562-CD19 cells (5,000-160,000, blue circles) 15 hours after plating. The red x indicates the calculated number of cells corresponding to the measured confluence 24 hours after seeding 80,000 K562-CD19 cells.

to be added in subsequent steps. This approach ensures the accuracy and reliability of the immune cell killing assay.

CAR T-Cell Mediated Killing

Existing assays employed for monitoring immune cell killing often require effector to target (E:T) ratios of 1:1 or higher. However, under physiological conditions, the E:T ratios are significantly lower, making it crucial to develop *in vitro* assays that are sensitive enough to detect target cell killing at low effector cell numbers.

To assess the spectrum of ratios at which immune cell killing could be detected, CD19-CAR T-cells were added to K562-CD19 cells at ratios of 3:1, 1:1, 1:3, and 1:6, 24 hours after target cell seeding (Figure 3). Measurements were captured at 15-minute intervals up to 24 hours post CAR T-cell addition and the confluence of cells was plotted over time.

Figure 3B illustrates the confluence of the target K562-CD19 cells throughout the entire experiment, encompassing both the growth phase (0-24 hours) and CAR T-cell mediated killing phase (24-72 hours). The confluence data was used to calculate the percent cytolysis, rate of killing, and the KT25, representing the time taken for 25% of the cells to be killed at each E:T ratio.

As shown in Figure 3, all wells exhibited comparable cell growth before the addition of T-cells. Twenty-four hours post-seeding, CD19 CAR T-cells were added to K562-CD19 target cells. As expected, higher E:T cell ratios correspond to an increased rate of

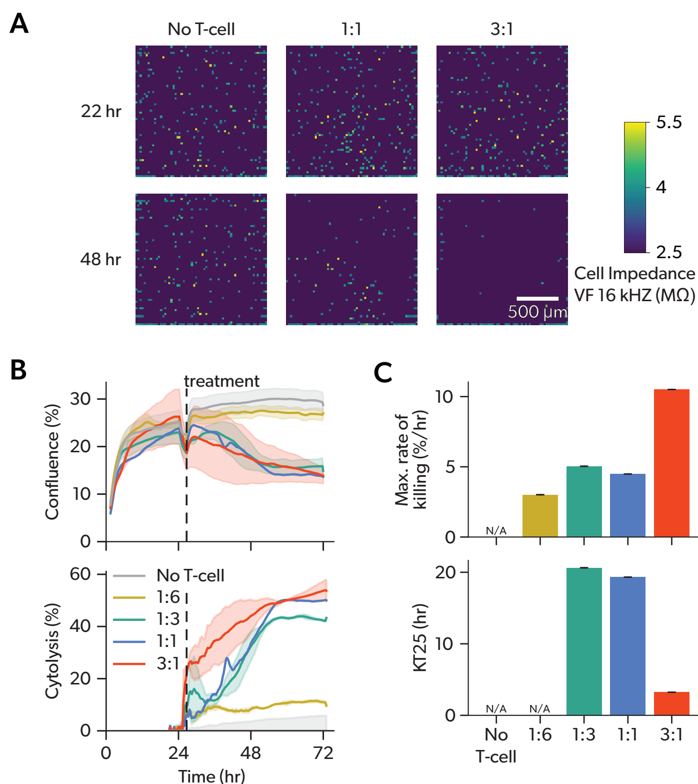

Figure 3. (A) Cell impedance images for K562-CD19 cells at 22 hours (prior to the addition of CAR T-cells) and 48 hours (24 hours post the addition of CAR T-cells) at the indicated E:T ratios. (B) Confluence (top) and percent cytolysis (bottom) of K562-CD19 cells across the entire time-course of experiment at the indicated E:T ratios. (C) Maximum rate of killing (top) and KT25 (bottom) for CAR T-cells at the indicated E:T ratios.

cell killing and a lower KT25. Notably, cell killing was observed at the lowest ratio (1:6) employed in this assay.

The minimum detectable cell killing threshold is influenced by various factors, including the growth rate of the target cells and the effectiveness of the CAR T-cells. In this instance, the growth rate of K562 cells is marginally lower than the death rate at the 1:6 ratio. Consequently, at the 1:6 ratio, cells reach a stable plateau without experiencing a pronounced decline in cell confluency as observed at higher ratios. A target cell type with slow growth could result in enhanced sensitivity of cell killing.

CONCLUSION

CytoTronics' high-resolution impedance assay provides an exceptional real-time evaluation of cell viability for suspension (and adherent) cells. It offers accurate measurements of confluency and thus facilitates the real-time monitoring of cell number and viability changes, making it the ideal tool for optimized immune cell killing assays in a variety of cell types. Furthermore, due to our superior single-cell resolution, we have the added capability to monitor cell killing in heterogenous cell populations.

MATERIAL AND METHODS

Cell Lines

All cell lines were maintained in a humidified incubator at 37°C and 5% CO_2. K562-CD19 (CSC-RO0252) cells were obtained from Creative Biogene and maintained in RPMI medium supplemented with 10% FBS and 100 U/mL Penicillin-Streptomycin. CD19-CAR T-cells (PM-CAR1002) were obtained from ProMab and maintained in CAR T-Cell Medium with 10% FBS and 300 U/mL IL2.

Treatment and Measurement

Impedance measurements were taken inside a humidified incubator at 37°C and 5% CO_2 every 15 minutes throughout the experiment.

For the mobilization experiment, CytoTronics' microplates were coated with Poly-D-Lysine (2 µg/mL) or Anti CD-71 (5 µg/mL, R&D Systems, MAB24741) prior to cell seeding. K562-CD19 cells were seeded at densities of 40,000 and 80,000 cells per well and then cultured for 24 hours.

Based on the mobilization optimization, CytoTronics' microplates were subsequently coated with Poly-D-Lysine (2 µg/mL) prior to cell seeding. For standard curve generation, cells were seeded at densities ranging from 5,000-160,000 cells per well. For the cell killing experiment, K562-CD19 cells were seeded at a density of 80,000 cells per well and cultured for 24 hours prior to treatment with CAR T-cells. CD19-CAR T-cells were added at a range of ratios (3:1, 1:1, ,1:3 and 1:6) after determination of the actual cell number via the standard curve.

Data Analysis

The well median of the confluence measurement was plotted over time, with the standard error calculated across three technical replicates. Confluence was calculated as a percentage of electrodes occupied by cells. To determine occupancy, impedance response of electrodes without cells was measured. When impedance response increases above the bare electrode due to attachment of cells, the electrode is considered occupied. Cytolysis is calculated as the percent change in confluence of the relevant CAR T-cell treatment condition relative to the control at a given time point. KT25 is defined as the time for cytolysis to reach 25% for the relevant condition after CAR T-cell addition.

REFERENCES

1. Zhang, P., Zhang, G. & Wan, X. Challenges and new technologies in adoptive cell therapy. *J Hematol Oncol* **16**, 97 (2023).
2. Chitale, S. et al. A semiconductor 96-microplate platform for electrical-imaging based high-throughput phenotypic screening. *Nat Commun* **14**, 7576 (2023).

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