APPLICATION NOTE

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

INTRODUCTION

Immuno-oncology, also known as cancer immunotherapy, 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 adherent 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 (CD19-CAR T-cells) in conjunction with HeLa cells engineered to express CD19 (HeLa-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

A 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 a challenge. To achieve precision in estimating these numbers, HeLa-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 6 hours post cell seeding, ensuring that the cells were fully attached to the surface of the microplate, but had not yet begun proliferating (Figure 1).

For the immune cell killing assay, HeLa-CD19 cells were seeded at a density of 6,000 cells per well on CytoTronics' microplates and allowed to attach. 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 19,000 cells per well (Figure 1B), which served as the basis for determining the number of CAR T-cells to be added in subsequent steps. This approach ensures the accuracy and reliability of the immune cell killing assay.

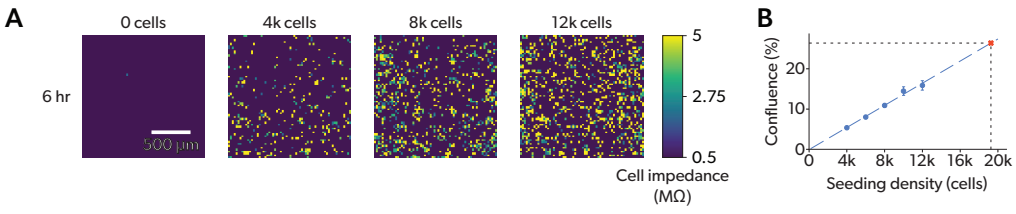

Figure 1. (A) Confluence images of HeLa-CD19 cells seeded at the indicated densities 6 hours after seeding. (B) Standard curve of measured confluence to seeding density for a range of HeLa-CD19 seeding densities (4,000-12,000, blue circles). The red x indicates the calculated number of cells corresponding to the measured confluence 24 hours after seeding 6,000 HeLa-CD19 cells.

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 HeLa-CD19 cells at ratios of 6:1, 3:1, 1:1, and 1:3, 24 hours after target cell seeding (Figure 2). 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 2B illustrates the confluence of the target HeLa-CD19 cells throughout the entire experiment, encompassing both the growth phase (0-24 hours) and CAR T-cell mediated killing phase (24-48 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 2A and 2B, all wells exhibited comparable cell growth before the addition of T-cells. Twenty-four hours post-seeding, CD-19 CAR or Mock T-cells were added to HeLa-CD19 target cells. As expected, the Mock T-cells also induced cell death, but required higher E:T ratios for efficacy. This difference is also evident in the overall lower rate of killing observed for the Mock T-cells as compared to the CD19-CAR T-cells. In the case of CD19-CAR T-cells, higher E:T cell ratios correspond to an increased rate of cell killing and a lower KT25. Notably, cell killing was observed at the lowest ratio (1:3) 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 scenario, HeLa-CD19 cells exhibit rapid growth. Thus, the rate of cell killing at lower ratios equals the growth rate, leading to cell replacement. Enhanced sensitivity can be achieved by employing a slower-growing target cell. Moreover, employing a more optimized effector to target (E:T) cell combination has the potential to attain lower sensitivities in the detection of cell killing.

CONCLUSION

CytoTronics' high-resolution impedance assay provides an exceptional real-time evaluation of cell viability for adherent (and suspension) 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.

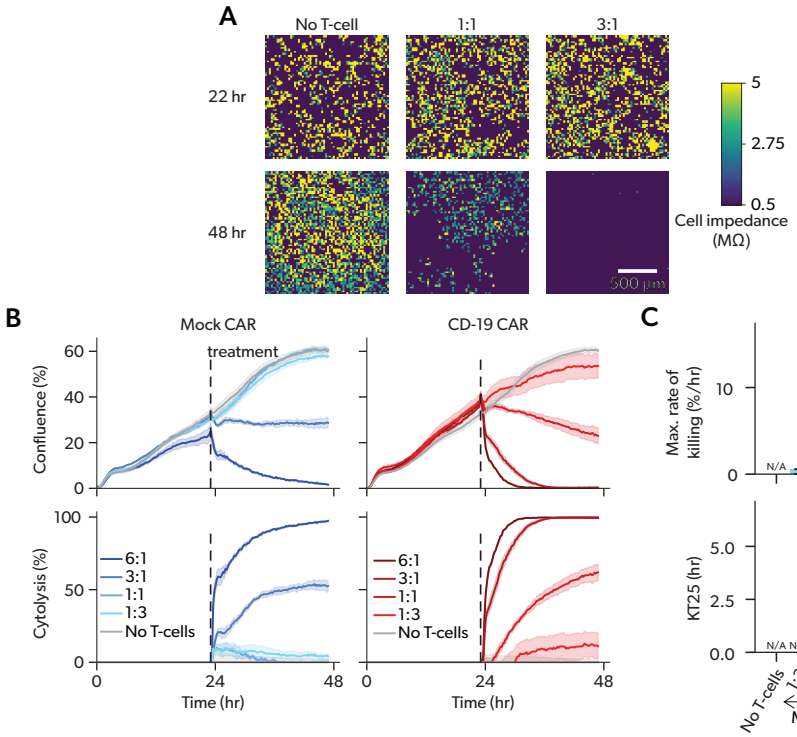

Figure 2. (A) Confluence images for HeLa-CD19 cells at 22 hours (prior to CAR T-cell addition) and 48 hours (24 hours post CAR T-cell addition) at the indicated E:T ratios. (B) Confluence (top) and percent cytolysis (bottom) of HeLa-CD19 cells across the entire time-course of the experiment. Mock T-cells (left) and CD19-CAR T-cells (right) were added at 24 hours. (C) Maximum rate of killing (top) and KT25 (bottom) for the Mock and CD19-CAR T-cells at the indicated E:T ratios.

MATERIAL AND METHODS

Cell Lines

All cell lines were obtained from ProMab and maintained in a humidified incubator at 37°C and 5% CO_2. HeLa-CD19 (PM-HELA-CD19) cells were maintained in DMEM supplemented with 10% FBS and 100 U/mL Penicillin-Streptomycin. Mock CAR T-cells (PM-CAR1086) and CD19-CAR T-cells (PM-CAR1002) were maintained in CAR T-Cell Medium (Promab PM-CAR2001).

Treatment and Measurement

Impedance measurements were taken inside a humidified incubator at 37°C and 5% CO_2 every 15 minutes throughout the experiment. Cytotronics' microplates were coated with Poly-D-Lysine (2 µg/mL, Gibco A3890401) prior to cell seeding. For standard curve generation, cells were seeded at densities ranging from 4,000-12,000. For the cell killing experiment, HeLa-CD19 cells were seeded at a density of 6,000 cells per well and cultured for 24 hours prior to treatment with CAR T-cells. Mock CAR and CD19-CAR T-cells were added at a range of ratios (6:1,3:1, 1:1, and 1:3) 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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