>> GD2 CAR-T cells engineered using retroviral transduction or CRISPR editing exhibit strong cytolytic potency against glioma stem cells

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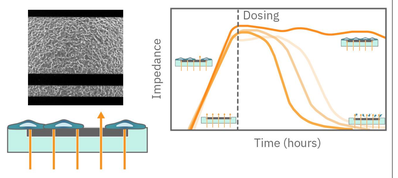
Maestro Z: Dynamic Cell Tracking

Impedance Technology

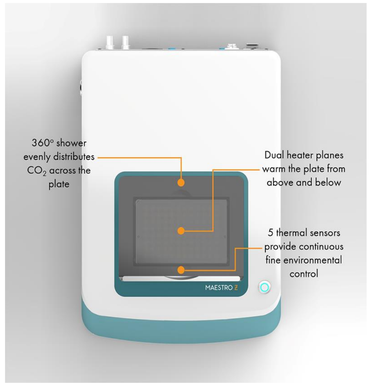
Glioblastoma (GBM) is an aggressive form of brain cancer that has no effective treatments and a prognosis of only 12-15 months. Immune effector T cells are a promising therapy due to their innate cytotoxicity. In addition, engineering chimeric antigen receptors (CAR) to target tumor-associated or neo-antigens can lend high specificity. Assessing the efficacy and potency of such T cell therapies label-free, *in vitro,* and at high throughputs is vital for the preclinical development of these promising therapies.

Axion BioSystems' Maestro Z platform offers impedance-based cell analysis for real-time, label-free monitoring of cell viability, morphology, cytolysis, and signaling. Here, we used the Maestro Z to compare the cytolytic potency and kinetics of retrovirally transduced (RV) vs virus-free CRISPR-edited (VFC) GD2 targeted CAR-T cells against glioma stem cells, a subpopulation of glioblastoma cells.



The impedance is measured from electrodes embedded in the bottom of each well. As cells cover more of the electrode, impedance increases in proportion to the number of viable cells. If a perturbation kills the attached cells, impedance decreases.

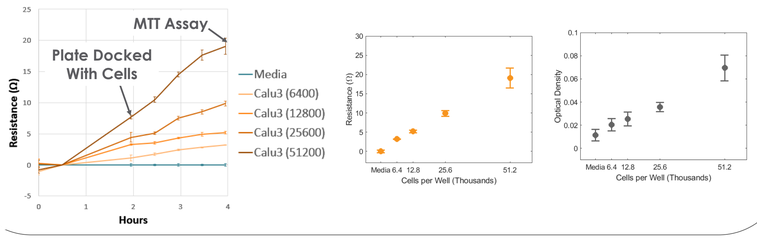
The Maestro Z Platform



- ***Label-free, non-invasive recording*** *of extracellular voltage from cultured electro-active cells*
- ***Integrated environmental control*** *provides a stable benchtop environment for short- and long-term toxicity studies*
- ***Automatic and continuous cell monitoring*** *from 96 or 384 wells simultaneously*
- ***"One button setup"*** *automatically docks the plate and adjusts temperature and CO2 levels*
- ***Powerful data analysis*** *to focus on the science, while AxIS Z handles the details with simple setup and automatic experiment tracking*
- ***See your cells*** *with the viewing window included in each well of the CytoView-Z 96-well plate*
- ***State-of-the-art electrode processing chip (BioCore v4)*** *offers stronger signals, ultra-low frequency content, and enhanced flexibility*

Impedance Assay is Directly Correlated with Cell Viability

To validate impedance-based monitoring of cell viability, Calu-3 cells were added to a CytoView-Z plate with varying number of cells per well and monitored for four hours on the Maestro Z platform. The change in resistance was correlated with the number of cells initially seeded, and the resistance continued to increase as the cells adhered and flattened on the surface. At four hours post-seeding, the plate was removed and an MTT assay was performed in the CytoView-Z plate. The resistance measured with the Maestro Z platform was linear with respect to cell number and directly correlated to the MTT assay readings from the same wells.

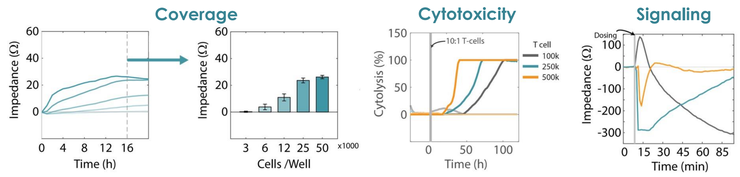


Dynamic Cytotoxicity Assay

Impedance Assay Measures Diverse Cell Properties

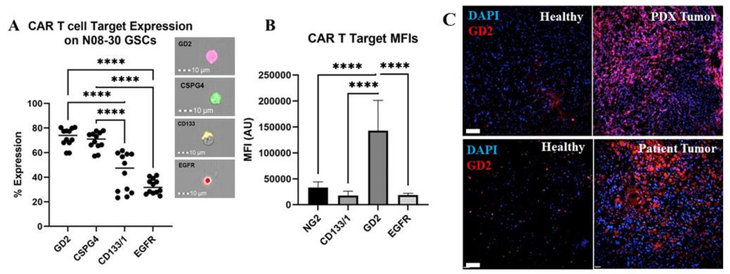
The Maestro Z records impedance at multiple frequencies simultaneously, enabling a thorough characterization of cell behavior, including:

- **Coverage** – the change in impedance is directly related to the number of cells covering the electrode.
- **Cytotoxicity** – dynamic monitoring of cell viability provides measures of the degree and speed of cell death.
- **Morphology** – cell size, shape, and intercellular tight junctions significantly impact the measured impedance.
- **Signaling** – small changes in cell shape or cytoskeleton organization are detected in response to intracellular signaling events



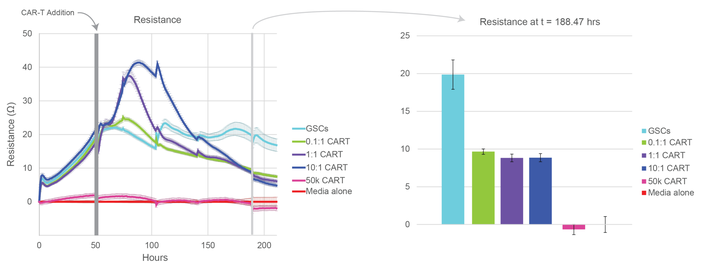
GD2 as a CAR T Target for Glioblastoma

Patient-derived N08 Glioma Stem Cells (GSCs) have high expression of CAR-T target antigen GD2 compared to other popular CAR-T targets for solid tumors as seen in imaging flow cytometry (A). This was further seen in MFI values taken from the same samples, showing that GD2 expression on N08 GSCs is significantly higher than other CAR-T target antigen expression (B). High GD2+ expression is retained in rodent xenograft tumors inoculated using N08 GSCs (C, top). GD2 is also abundantly expressed in human GBM patient samples as seen in human patient tissue arrays compared to healthy control tissue (C, bottom).



Continuous Monitoring of Glioma Stem Cell Viability

As the changes in impedance are correlated with cell attachment and viability, continuous, label-free monitoring with the Maestro Z can be used to track the kinetics of cytotoxicity over time. Patient-derived N08 glioma stem cells (GSCs) were plated at 50k cells per well on CytoView-Z 96-well plates, and their impedance was continuously monitored on the Maestro Z. After 48 hrs, retroviral (RV) transduced GD2 targeted CAR T cells were added at Effector:Target (E:T) ratios ranging from 0.1:1 up to 10:1. Impedance and cytolysis were subsequently monitored for 7 days. In addition, some wells were left untreated (GSC, teal) to serve as a No Treatment Control, while others received 50k CAR T cells alone (pink).



As expected, non-adherent CAR T cells alone (pink) showed little change in impedance as they do not attach to the surface. Untreated GSCs (teal) continued to exhibit an impedance of ~ 20 Ohms. All ratios of GD2 CAR T cells induced a decrease in impedance reflecting significant GSC cell death (green, purple, blue). Higher ratios of GD2 CAR T cells induced an initial increase in impedance, likely reflective of cell swelling or inflammation, before a subsequent decrease in impedance. To the right, impedance at ~ 6 days post-addition is shown across groups.

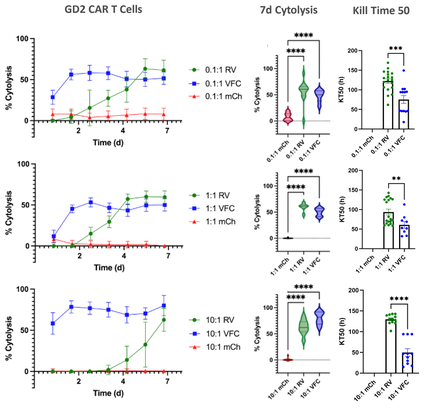
Immune Cell-Mediated Cytotoxicity

Kinetics and Potency of anti-GD2 CAR T Cells against GSCs

Impedance is valuable for evaluating both the potency and efficiency of immune cell-mediated cytotoxicity.

Here, percent cytolysis was used to compare the potency of targeted, retroviral (RV) transduced anti-GD2 CAR T cells to virus-free CRISPR-edited (VFC) anti-GD2 CAR T cells. VFC mCherry (mCh) CAR T control cells were edited via the same protocol, replacing the CAR domain with an mCherry fluorophore. Percent cytolysis was computed by comparing CAR T cell-treated wells to untreated GSC wells (No Treatment Control, 0% Cytolysis) and full lysis control wells (100% Cytolysis). Kill Time 50 was defined as time after dosing required to reach 50% cell death.

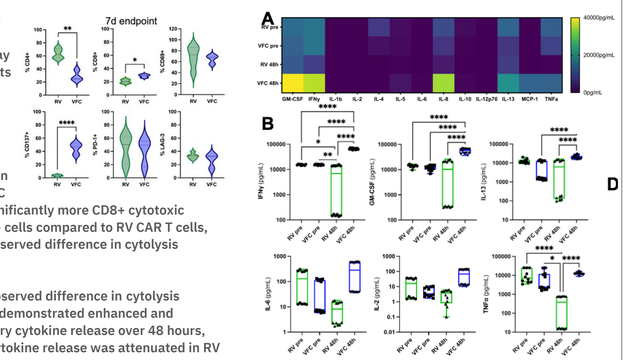
Both RV and VFC anti-GD2 CAR T cells exhibited similar cytotoxicity by 7 days, while no cytotoxicity was observed with VFC mCh CAR T cell treatment. All Effector:Target ratios (0.1:1, 1:1, and 10:1) of anti-GD2 CAR T cells induced > 50% cytolysis on average at the 7 day endpoint. Notably, although terminal cytolysis was similar between RV and VFC CAR T groups, the kinetics differed significantly. VFC cells killed faster than RV cells, evident both in the continuous cytolysis plots and quantification of Kill Time 50. The increased rate of cytolysis in the VFC group was consistent across all E:T ratios.



VFC CAR T Cells Exhibit Increased Activation Markers and Enhanced Cytokine Release Compared to RV CAR T Cells

Anti-GD2 CAR T cell state was evaluated with flow cytometry at day 2 and day 7. Both CAR T cell products expressed a high number of CD69+ and few exhausted cells (PD-1+, LAG-3+) after 48 h in co-culture with GSCs (data not shown). After 7 days in co-culture with GSCs, VFC CAR T cells contained significantly more CD8+ cytotoxic lymphocytes and CD137+ cells compared to RV CAR T cells, which may explain the observed difference in cytolysis kinetics.

Further supporting the observed difference in cytolysis kinetics, VFC CAR T cells demonstrated enhanced and continued proinflammatory cytokine release over 48 hours, while proinflammatory cytokine release was attenuated in RV CAR T cells at 48 hours.



Conclusions

- **VFC anti-GD2 CAR T cells exhibited greater potency against patient-derived glioblastoma compared to RV anti-GD2 CAR T cells, suggesting greater clinical potential.**
- **Dynamic cell tracking allowed quantification of the kinetics of immune-cell mediated cytolysis, including kill time 50, across CAR T lines and E:T ratios. While the final cytolysis did not differ between RV and VFC CAR T cells, the kinetic profile differed significantly, highlighting the importance of continuous monitoring over the course of cytolysis.**
- **Activation marker and cytokine analysis confirmed an increase in CD137+ cells and proinflammatory cytokine release by the VFC CAR T population.**
- **Overall, the Maestro Z platform enabled continuous, dynamic, label-free quantification of the potency, efficiency, and kinetics of immune-cell mediated cytolysis of glioblastoma.**

Acknowledgements

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