Application Note

Topic:
CAR T,
Immuno-oncology
Cells:
A549, SKOV3
Tools:
AtlaZ

CAR T cell-mediated killing of cancer cells

Nanion Technologies GmbH, Munich.
TUM, Munich.

Summary

The US Food and Drug Administration has approved a number of chimeric antigen receptor (CAR) T-cell therapies. Due to the nature of CAR T cells as "living drugs", they display a unique toxicity profile. As CAR T-cell therapy is extending towards multiple diseases and being broadly employed in hematology and oncology, being able to reliably predict treatment efficacy and a quantification of responses are of high relevance. Furthermore, for continued breakthroughs, novel CAR designs are needed. This includes different antigen-binding domains such as antigen-ligand binding partners and variable lymphocyte receptors (1). Now, after amazing advances for treating blood cancers, CAR T cell therapy is showing promise for solid tumors.

In general, identifying T cells that kill cancer cells *in vivo* is critical to the development of successful cell therapies. The label-free AtlaZ immune cell killing assay can be used to measure rate of killing at Effector:Target (E:T) ratios to predict *in vivo* activity. In order to gain a deeper understanding of cancer cells, real-time and continuous monitoring is necessary to access kinetic and phenotypic information. Such monitoring captures also unique toxicity profiles of CAR T cells.

The platform used here, AtlaZ, is a quantitative live-cell analysis system and allows for cellular research on cell adhesion and proliferation, cytotoxicity, GPCR, morphology and barrier function, label-free and in real-time. Recordings can be performed in up to six 96-well plates simultaneously or independently. Electrical impedance spectroscopy (2, 3) as the methodology behind the AtlaZ system, in combination with the throughput of 6 x 96-wells allows for a so far unmet quantity and richness of information which can be gained from cells.

We investigated A549, adenocarcinomic human alveolar basal epithelial cells, and SKOV3 cells which are a human ovarian carcinoma cell line. In principle, addition of effector T-lymphocyte cells are co-cultured with the cancer cells, and cytolysis of the target cells can be measured. In our assay, were used CAR T-cells directed to the receptor EGFR on the target cells. Those CAR T-cells exhibit an antigen-induced cell-mediated cytotoxicity by triggering intracellular pathways leading to apoptosis of target A549 or SKOV3 cells. We observed a time- and ratio-dependent decrease of the Cell Signal, representing apoptosis of carcinoma cells.

The impedance of planar gold-film electrodes that are used as growth substrate for adherent cells reveals changes in electrode coverage or cell behavior, e.g. upon treatment with CAR T cells (Figure 1). Real-time impedance data provide

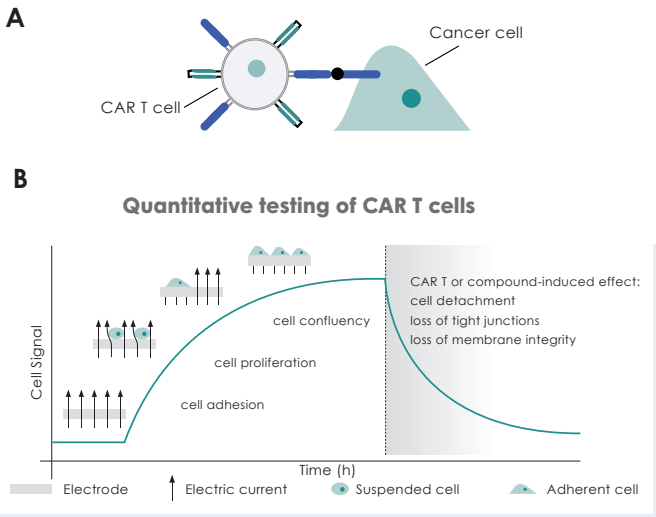


Figure 1: A *CAR T cells are genetically altered T cells which can locate and destroy cancer cells effectively.* **B** *The Cell Signal value offers information on cell adherence, proliferation or cell death of cancer cells upon treatment with CAR T cells.*

insights in various cell phenotypes, such as cell morphology, proliferation, lateral migration or cytotoxicity even over prolonged periods of time.

A crucial advantage over standard assays, e.g. filter based methods using labels and either an optical or radiometric detection technique, is the continuity of cell monitoring.

Endpoint assays using dyes or labels quantify the potency of effector cells to kill target cells by either measuring target cell viabiliy (i.e. MTT or flow cytometry) or membrane integrity (i.e. LDH). Those assays are labor-intensive and lack kinetic information of the recorded effects.
Unique culture plates with integrated electrodes as used in the AtlaZ system enable long-term measurements over several weeks. Thus real-time data on cell adhesion, proliferation and compound effects can be acquired.

Results

In our assay, were used CAR T-cells directed to the receptor EGFR on the target cells A549 and SKOV3. Those CAR T-cells exhibit an antigen-induced cell-mediated cytotoxicity by triggering intracellular pathways leading to apoptosis of target cells. As a control, we used untransduced cells added to the target cells in the same ratio. We observed a time- and ratio-dependent decrease of the Cell Signal, representing apoptosis of carcinoma cells.

Figure 1 shows the lung adeno-carcinoma cell line A549 as adhered on the 96-well AtlaZ sensor plates on days 1, 2 and 4 post-plating.

The A549 cell seeding density was 40 k cells/well and 80 k cells/well for SKOV3. The cells then adhered and proliferated for the next 24 h. Next, at t = 24 h, CAR T cells were added at an effector T-cell to target A549 cell ratio of 1:1, 1:3 and 3:1. The added volume of CD8 T effector cells was 200 µl (after having completely removed the medium in which target cells have been seeded). The impedance signal of the cell monolayer was recorded every 2 minutes, and is plotted using the unitless parameter *Cell Signal* (Figure 2A). The data revealed that increasing E:T (Effector:Target) cell ratios induced a ratio-dependent apoptosis of A549 cells as well as of SKOV3 cells seen as a decrease of the *Cell Signal*. The lowest ratio of added CAR T cells, 1:3, had a larger killing effect on SKOV3 cells compared to A549 cells. Uninterrupted attachment of cells was observed in wells with only untransduced control cells or only medium (Figure 2A, grey lines).

The Kill Time 50 values were calculated to investigate at what timepoint and which ratios of effector cells killed the cancer cells to 50%. Figure 2B shows that 50% of A549 cells were killed after approx. 50 h (E:T = 1:1) or 9 h (E:T = 3:1). SKOV3 cells were killed to 50% after approx. 46 h (E:T = 1:1) or 8 h (E:T = 3:1).

The AtlaZ Control software calculates and displays Cytolysis in % (Figure 3). The data were retrieved at t = 73 h and showed that increasing E:T cell ratios induced a ratio-dependent apoptosis. The lowest amount (1:3) of CAR T cells that were added to A549 or SKOV3 cells did not induce a 50% cell death (Figure 2B), but they induced a cytolysis of only 10% (A549) or 20% (SKOV3) (Figure 3). Figure 3 shows as well that ratios 1:1 and 3:1 did induce a 84% and 100% cytolysis of A549 cells, and a 75% and 100% cytolysis in SKOV3 cells, respectively.

These results demonstrate the capability of CAR T cells to kill A549 and SKOV3 cancer cells in a ratio- and time-dependent way.

In summary, AtlaZ allows for label-free and real-time cellular research on cell adhesion, proliferation and cytolysis. Measurements can be performed in up to 6 x 96-well plates simultaneously or independently. The AtlaZ system provides a versatile tool for in vitro cell monitoring addressing the demands for versatility, physiological relevance and throughput.

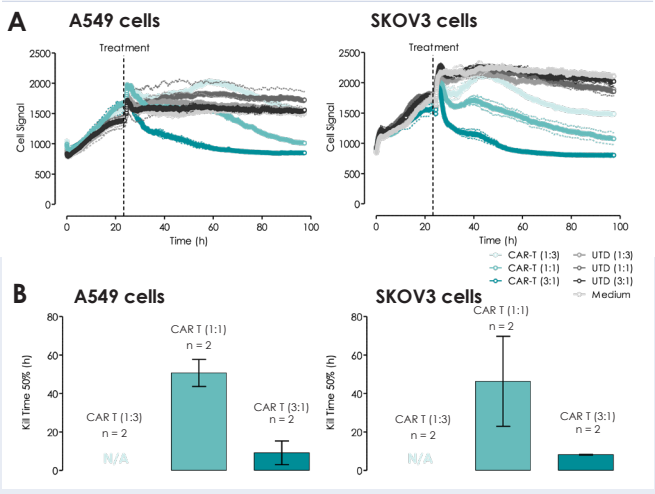
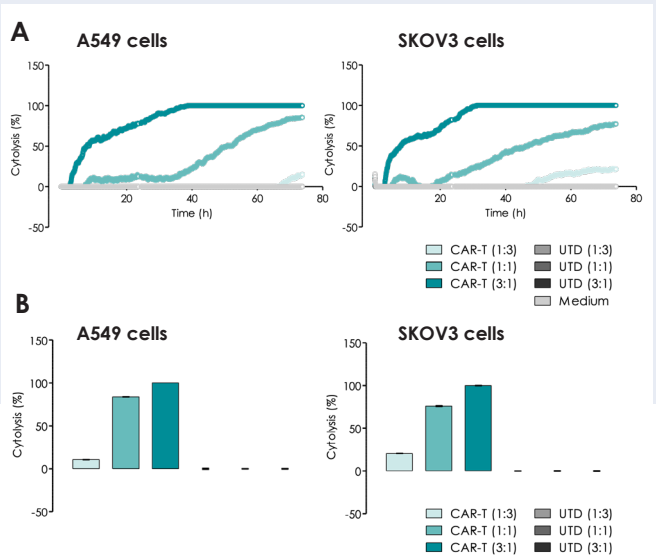


Figure 2: A *CAR T cells added in different ratios to A549 (left) or SKOV3 (right) cells at t = 24 h after plating.* **B** *Kill Time 50 values calculated from measurements as shown in A (+/- SD, n = 2 wells each. UTD = untransduced control cells).*

Figure 3: A *Cytolysis in percent (%) as a timecourse and as calculated from the timepoint t = 73 h (***B***). The ratios 1:1 and 3:1 did induce a 84% and 100% cytolysis of A549 cells, and a 75% and 100% cytolysis in SKOV3 cells, respectively. The ratio 1:3 induced a 10% (A549) or 20% (SKOV3) cytolysis.*



Methods

AtlaZ platform

The AtlaZ platform (Nanion Technologies) provides quantitative live-cell analytics by measuring the impedance (Ohm, Ω) of adherent cells as grown on 96-well plates with embedded planar gold-film electrodes (Nanion Technologies).

Real-time impedance data provide insights in various cell phenotypes, such as cell morphology, proliferation, lateral migration or cytotoxicity even over prolonged periods of time. A crucial advantage over standard assays is the continuity of cell monitoring. Continuous measurements reveal the kinetics of cell behavior and allow an in-depth mechanistic understanding without the need for time- and labor-intensive endpoint assays. For example, one of the crucial advantages of data derived from the AtlaZ platform is the possibility to analyse dose-responses at any time during the experiment.

Advanced information content is obtained by using multi-frequency impedance readouts, which is possible with the AtlaZ Control software. With impedance readouts at different frequencies (here a spectrum of 0.1 kHz – 100 kHz) it is possible to further dissect physiological response into deeper levels, and thus zooming in on changes in membrane topography, cell-cell or cell-matrix junctions. G-protein-coupled receptor (GPCRs) activation is one example here.

Immune-cell mediated killing assay

The *Cell Signal* values that are displayed were calculated by subtracting the impedance in empty wells with medium, measured at the beginning of the experiment just before cell addition. Data are presented in mean ± SD.

The AtlaZ assay is sensitive to adherent target cells, but not to the presence of non-adherent immune effector cells. This allows to specifically investigate target cell cytotoxicity and is an advantage over other assays where the presence of a second cell type like effector cells could induce background signals.

Workflow of AtlaZ immune cell-mediated killing assay

Target cells are seeded in AtlaZ sensor plates and they adhere on the surface with embedded gold electrodes (Figure4, 1). Each well of the 96-well plate contains 1 center gold electrode with 0.6 mm in diameter and one reference electrode. After the target cells being adhered, the population starts to proliferate. When the cells reach confluency, non-adherent effector cells (e.g. CAR T cells) are added (Figure 4, 2).

Derived from the *Cell Signal*, the software automatically calculates and plots Cytolysis (%) over time and as a bar graph at a specific time of interest. Furthermore, KT50 values are plotted automatically.

Calculation of cytolysis and Kill Time 50

Percent (%) cytolysis was used to quantify cell death and is calculated by AtlaZ Control software as follows:

$$Cytolysis\ (t,\ \%) = \left[\left(\frac{\Delta_{kt}}{\Delta_G} \right)_{\text{no effector cells}} - \left(\frac{\Delta_{kt}}{\Delta_G} \right)_{\text{target + effector cells}} \right] \cdot 100$$

where $\Delta_{k,t}=Z_t-Z_0$ and $\Delta_G = Z_0-Z_{min}$. Z_t is the impedance value after the addition of the treatment and Z_0 is the last impedance value before treatment addition. Z_{min} is the smallest impedance value in the region before treatment addition. This means that Cytolysis (t, %) is the effect of the effector cells on the target cells at a certain time t, displayed in percent. $\Delta_{k,t}$ (k=killing; t= time) contains information on the time-dependent cell death based on the impedance change before and after treatment and Δ_G considers the cell proliferation and growth (g = growth) post-seeding.

Next to Cytolysis (%) AtlaZ Control software also displays Kill Time 50 values which are as well determined through the impedance measurements.

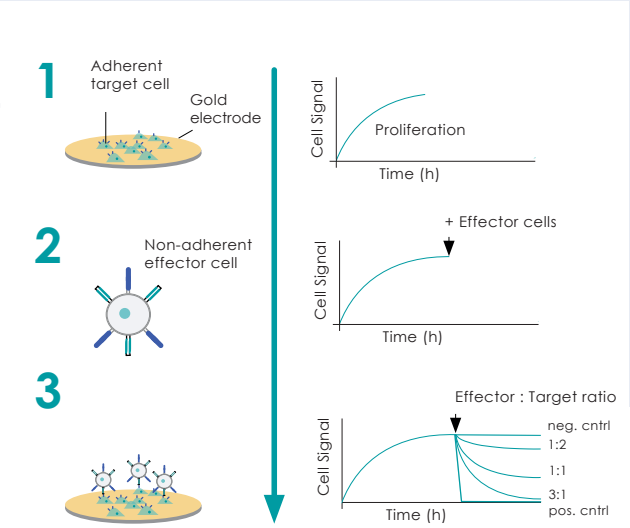


Figure 4: *Workflow of CAR T cell-mediated killing assay. Target cells seeded on AtlaZ sensor plates, adhere and proliferate (1). When they reach confluency, non-adherent effector (CAR T) cells are added (2). The cell death of target cells is measured and can be monitored as Cell Signal over time.*

References

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Key findings

1. Our results demonstrate the capability of CAR T cells to kill A549 and SKOV8 cancer cells.
2. AtlaZ allows for cellular research on cell adhesion, proliferation and cytotoxicity, label-free and in real-time.
3. Recordings can be performed in up to six 96-well plates simultaneously or independently.

Nanion Technologies GmbH I Ganghoferstraße 70a, 80339 Munich, Germany
+49 89 2190 95-0 I info@nanion.de I www.nanion.de

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