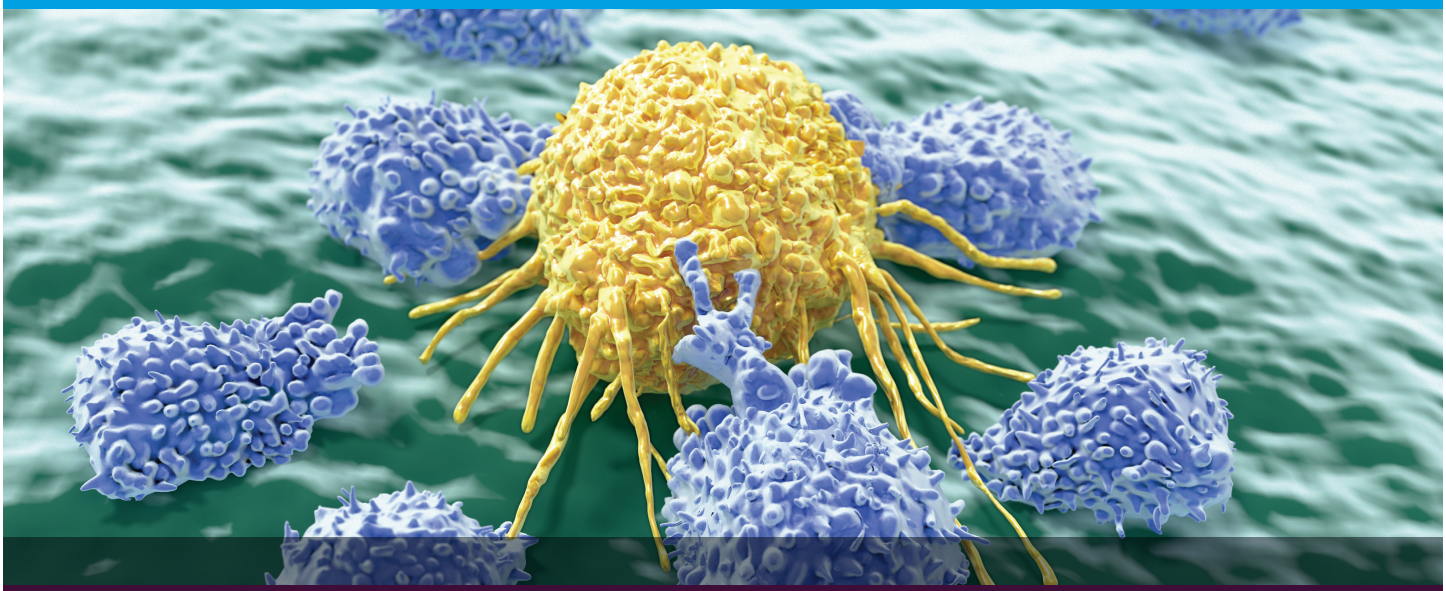
Agilent

Trusted Answers

Cancer Immunotherapy

Agilent xCELLigence RTCA handbook



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What is cancer immunotherapy?

Cancer immunotherapy consists of multiple approaches that harness and enhance the innate powers of the immune system to fight the disease. It is currently viewed as one of the most promising forms of cancer treatment with 12 cancer immunotherapies approved in recent years. In 2018, the Nobel Prize in Medicine was awarded to two researchers in the field.

Cancer immunotherapies can be divided into four major categories:

- Cytokines/immunomodulation agents
- Monoclonal antibodies
- Cell-based therapies
- Oncolytic viruses

Though monoclonal antibodies currently represent the largest class of commercialized cancer immunotherapies, cell-based therapies are rapidly making headway. This class of personalized therapies involves collecting immune cells from an individual, engineering them to recognize and kill cancer cells, before culturing, and reintroducing them into the same individual.

How can the immune system be harnessed to target tumors?

Immune cell-mediated tumor cell killing can involve components of both the innate and adaptive immune systems (Figure 1), including:

- Natural killer (NK) cells
- Cytotoxic T cells (MHC-dependent)
- Antibodies secreted by B lymphocytes
- Engineered antibodies such as bispecific antibodies and bispecific T cell engagers (BiTEs)
- Genetically engineered T cells targeting specific tumor antigens (for example, CAR-T, MHC-independent)
- Macrophage-mediated phagocytosis

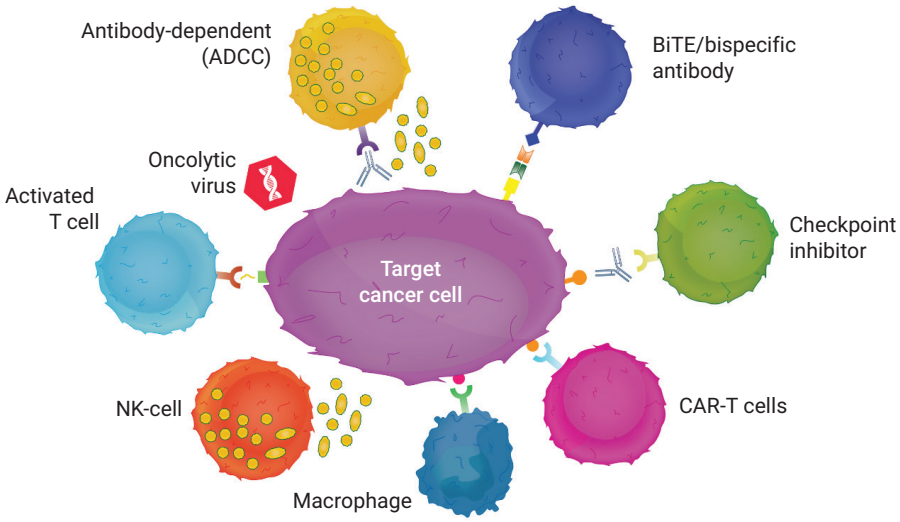


Figure 1. Various immunotherapy tumor-targeting schemes.

The need for a novel cancer immunotherapy assay

The most significant challenge faced by cancer immunotherapy researchers is the inability to predict treatment efficacy and response. While many methods have been developed to screen and evaluate the efficacy of immune cell-mediated killing, there is a need for a more robust *in vitro* assay to accurately predict the *in vivo* behavior of therapies. The ideal assay should be objective, simple to perform, provide quantitative kinetic results, and mimic physiologically relevant conditions. Other established methods, such as flow cytometry, can then provide extra data regarding immune cell phenotype, activation, and function.

The most commonly used method for measuring immune cell-mediated killing is the release assay, where effector cell-mediated disruption of the target cell membrane results in leakage of its cytoplasmic contents into the culture medium. Endogenous biomolecules (such as lactate dehydrogenase) or previously added exogenous labels (such as the radioisotope ^{51}Cr) that leak into the media are then measured as an indirect readout of the damage caused by effector cells. Alternative endpoint methods include ELISA-based granzyme measurement and morphometric analyses by microscopy. While the data provided by these assays help piece together an understanding of different facets of immune cell-mediated killing, it is important to note that the parameters being reported often do not correlate with target cell killing efficacy *in vivo*.

xCELLigence real-time cell analysis

Agilent xCELLigence real-time cell analysis (RTCA) instruments allow users to:

- Measure quantitative, real-time kinetics with exquisite sensitivity
  - Real-time cytolysis of target cells are measured at low effector-to-target ratios
- Easily study diverse effector cells and molecules
  - Measure cytotoxic effects of CAR-T cells or monoclonal antibodies, optimize potency of combination therapies, measure off-target effects, and much more
- Conduct experiments in label-free conditions
- Measure cytotoxicity with no ^{51}Cr, luciferase, or dyes

Diverse cancer immunotherapy applications

Thousands of xCELLigence instruments have been placed globally, resulting in more than 4,500 xCELLigence publications in peer-reviewed journals.

xCELLigence RTCA technology is being used extensively for cancer research in applications that include, but are not limited to:

- Compound-mediated cytotoxicity
- Cell-mediated cytotoxicity
- T cells
- NK cells
- CAR T cells
- Macrophages
- Antibody-dependent cell-mediated cytotoxicity (ADCC)
- Bispecific antibodies
- Bispecific T cell engagers (BiTEs)
- Checkpoint inhibitors
- Combination therapy
- Tumor microenvironment (cell–cell interactions)
- Cell adhesion/spreading
- Receptor activation
- Oncolytic viruses
- Autophagy
- Solid tumor killing assays
- Liquid tumor killing assays
- Immune cell activation
- Apoptosis
- Inflammation

How does the xCELLigence real-time cell analysis assay work?

xCELLigence RTCA instruments use gold biosensors embedded in the bottom of specialized microplate wells (Agilent E-Plates) to noninvasively monitor cell status including cell number, cell size, and cell-substrate attachment quality. The major distinguishing features of this technology include enhanced sensitivity, the exclusion of labels, and kinetic measurement of cell health/behavior.

Convenient and simple workflow

- Plate target cells, add effector cells, and start reading.
- Generate real-time killing curves for multiple conditions simultaneously, spanning seconds to days.
- Read an entire 96-well plate in 15 seconds and run up to six plates independently, with no scheduling conflicts.

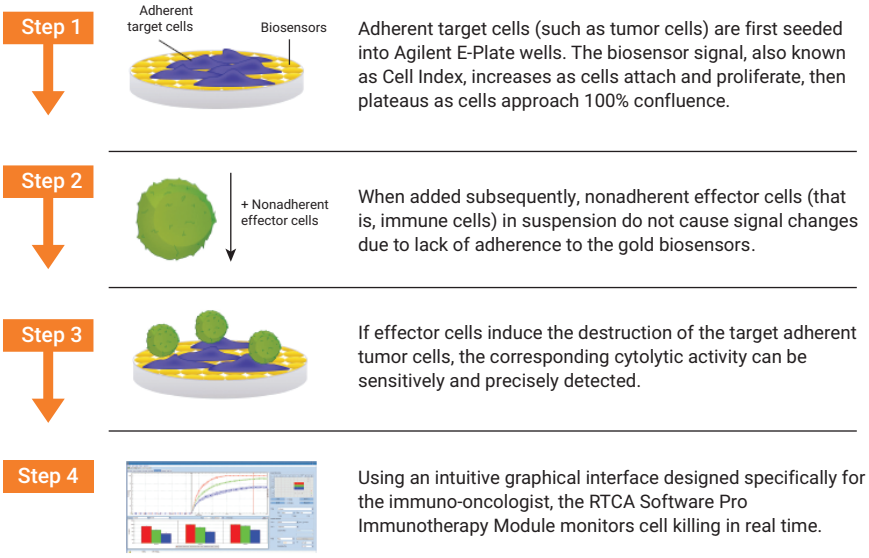


Figure 2. Overview of the Agilent xCELLigence RTCA assay.

Measuring cellular impedance with E-Plates

The gold biosensors in each well of Agilent electronic microplates (E-Plates) cover ~75% of the bottom surface area. The circular biosensors in each well of an E-Plate are linked to strands that form an interdigitating array (Figure 3). This proprietary design enables large populations of cells to be monitored simultaneously. The biosensors detect cellular impedance as cells adhere to and proliferate on the E-Plates, providing an extremely sensitive readout of cell number, cell size/morphology, and cell-substrate attachment quality in real time.

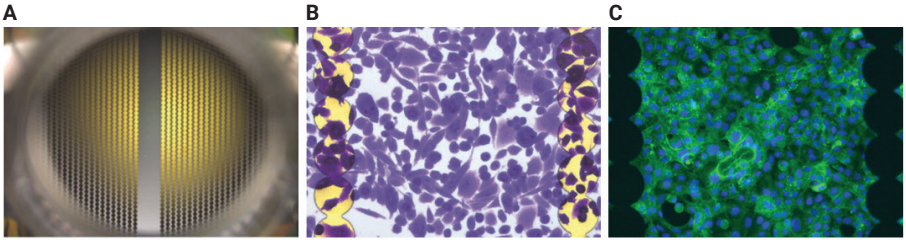


Figure 3. Biosensors measure cellular impedance on Agilent E-Plates. (A) Photograph of a single well in an E-Plate. Though cells can also be visualized on the gold biosensor surfaces, the region in the middle of the well facilitates microscopic imaging. (B) Crystal violet-stained human cells, as viewed in a compound microscope. (C) Immunofluorescence microscopy.

Real-time cellular impedance traces explained

The impedance caused by adherent cells is reported using a unitless parameter called Cell Index (CI), where:

$$CI = \frac{(\text{Impedance at n}) - (\text{Impedance without cells})}{(\text{Nominal impedance constant})}$$

Figure 4 illustrates an example of a real-time impedance trace throughout the course of setting up and running an apoptosis experiment:

1. **Rapid increase due to cell adhesion:** For the first few hours after cells have been added to a well, there is a rapid increase in impedance, which is caused by cell attachment and spreading.
2. **Slow increase due to cell proliferation:** If cells are subconfluent after the initial attachment stage, they will start to proliferate, causing a gradual yet steady increase in CI.
3. **Plateau due to cellular confluence:** When cells reach confluency, the CI value plateaus, reflecting the fact that the electrode surface area accessible to bulk media is no longer changing.
4. **Decrease due to cell death/detachment:** The addition of an apoptosis inducer at this point causes a decrease in CI back to zero. This is the result of cells rounding then detaching from the well bottom. While this generic example involves addition of the apoptosis inducer at the point of cellular confluence, impedance-based assays are flexible and can interrogate a wide variety of phenomena across the full spectrum of cell densities.

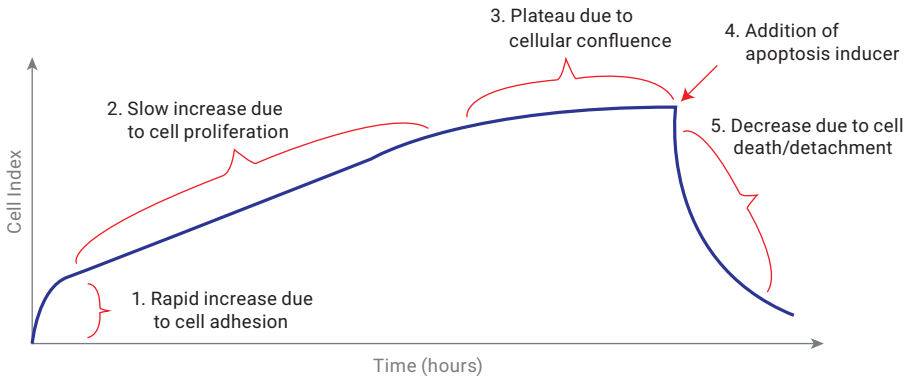


Figure 4. Generic real-time impedance trace for setting up and running an apoptosis assay. Each phase of the impedance trace, and the cellular behavior it arises from, is explained in the text.

xCELLigence instruments for immunotherapy

To control the temperature, humidity, and atmospheric composition of RTCA assays, xCELLigence instruments are housed inside standard tissue culture incubators or hypoxia chambers, except for the HT model (Figure 5). The instruments connect through a cable with analysis and control units that are housed outside the incubator. User-friendly software allows for real-time control and monitoring of the instrument, including real-time data display and analysis functions.

Of the nine xCELLigence RTCA instruments, those best suited for immunotherapy assays are the dual purpose (DP), single plate (SP), multiple plates (MP), high throughput (HT), and eSight models (Table 1). While each instrument monitors cell number, cell size, and cell-substrate attachment quality through cellular impedance in an identical manner, they differ from one another in plate configuration and throughput. The DP model has the additional capability to quantitatively monitor cell invasion/migration by using a specialized plate that functions as an electronic Boyden chamber. The eSight incorporates imaging in three colors, plus brightfield. Finally, though the HT model can be run as a standalone instrument, four of these can be linked to a single control unit to provide a total of 1,536 wells. HT instruments can also be integrated with a robotic liquid handler to maximize throughput.

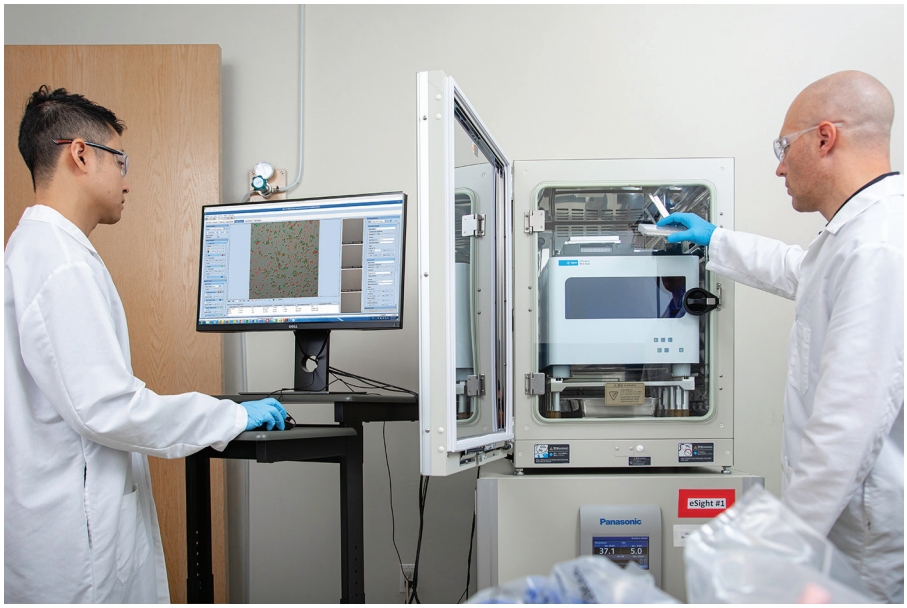


Figure 5. An Agilent xCELLigence RTCA eSight instrument and its control unit are housed inside and outside an incubator, respectively.

Table 1. Overview of Agilent xCELLigence RTCA instruments.

Immunotherapy Applications	Dual Purpose (DP)	Single Plate (SP)	Multiple Plates (MP)	High Throughput (HT)	eSight
Applicable to both liquid and solid tumor target cells					
Cell-mediated cytotoxicity	✓	✓	✓	✓	✓
ADCC	✓	✓	✓	✓	✓
Checkpoint inhibitors	✓	✓	✓	✓	✓
Combination therapies	✓	✓	✓	✓	✓
Antibody-drug conjugates	✓	✓	✓	✓	✓
Immune cell activation	✓	✓	✓	✓	✓
Cell invasion and migration	✓				
Live cell imaging					✓
Specifications					
Format	3 × 16 wells	1 × 96 wells	6 × 96 wells	1 × 384 wells	3 × 96 wells impedance 5 × 96 wells imaging
Maximum throughput	48 wells	96 wells	576 wells	Up to 4 × 382 wells (1,536 wells total)	288 wells impedance Up to 480 wells total for imaging

Antibody-dependent cell-mediated cytolysis (ADCC)

Using xCELLigence to study antibody-dependent cell-mediated cytolysis (ADCC)

Though the innate and adaptive branches of the immune system are typically described as being distinct and separate from one another, they often work in concert to afford protection and combat diseases. When a pathogen is encountered, cells of the innate immune system typically release cytokines that cross-talk with components of the adaptive immune system, causing them to expand and become activated. Many cells involved in the innate immune response (including NK cells, neutrophils, and eosinophils) also express CD16 (Fc receptor), which is a low-affinity receptor for immunoglobulins such as IgG. Immunoglobulin binding by CD16 targets innate immune cells to the immunoglobulin-bound target cell and triggers target cell destruction. This prophylactic mechanism is known as antibody-dependent cell-mediated cytolysis (ADCC) and is the basis of many monoclonal antibody therapies.

As shown in Figures 6 and 7, scientists have used the xCELLigence platform to measure the ability of mononuclear cells (MNCs) from blood to kill different breast cancer cell lines in the presence or absence of trastuzumab (also known as Herceptin). Trastuzumab recognizes the tumor cell through its antigen (HER2), resulting in the specific killing of the tumor cells.

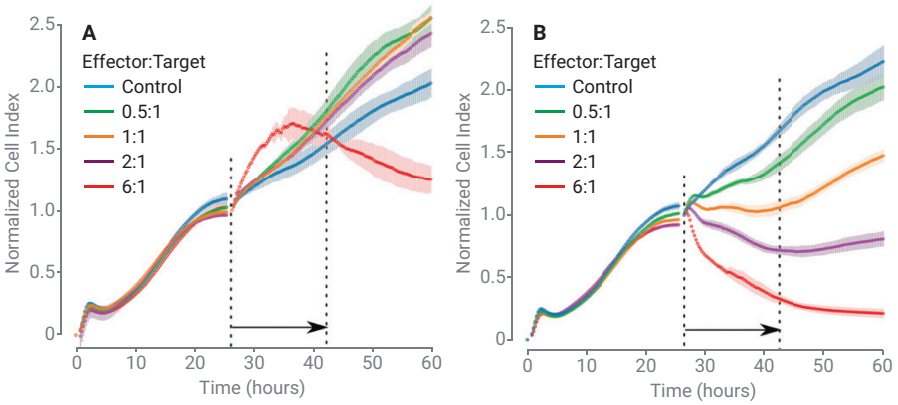
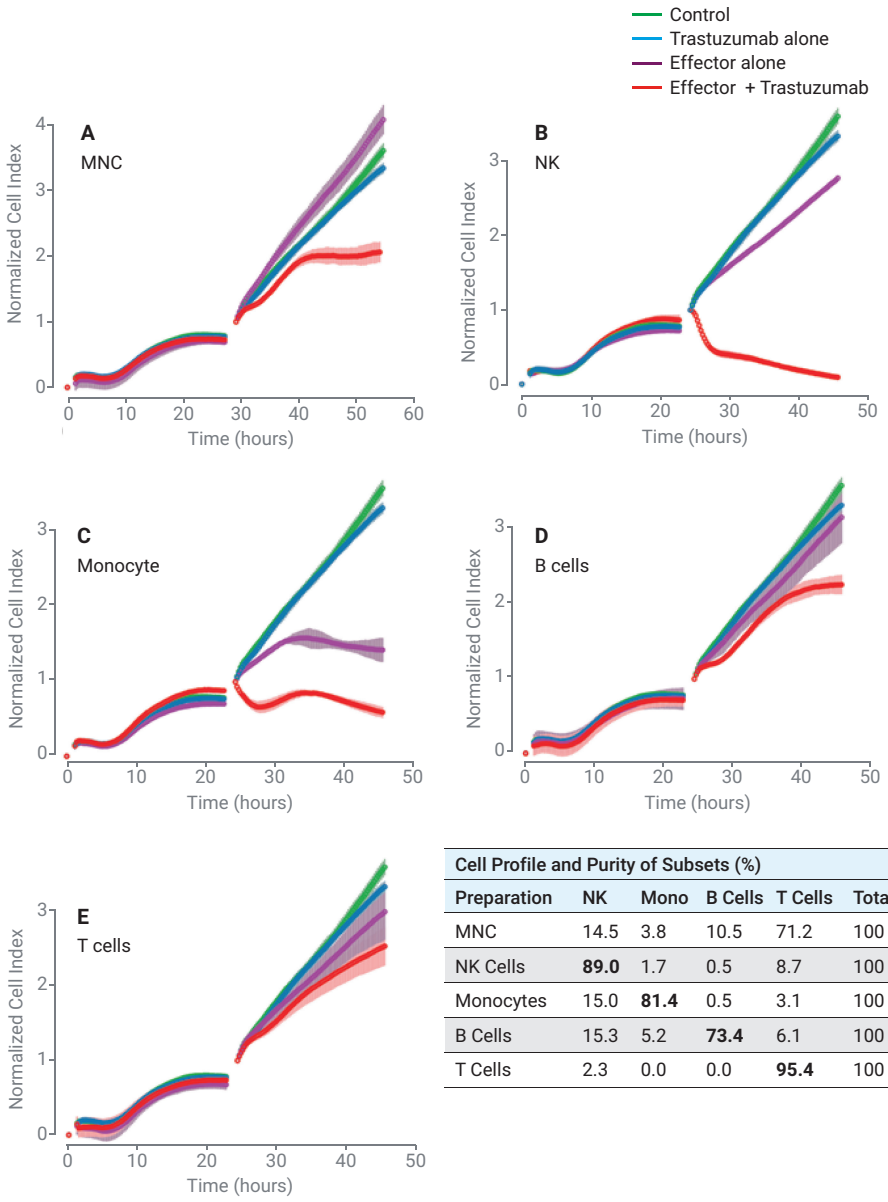


Figure 6. CI values are proportionally reduced with increasing effector-to-target (E:T) ratios in the presence of trastuzumab. BT474 clone five cells were maintained for 26 hours, then treated with media alone (control) or with media plus mononuclear cells (MNCs) isolated from human blood (A). Cells were treated in an identical fashion in (B) except for the inclusion of 0.1 µg/mL trastuzumab. CI values were normalized at the time of addition. Blue represents growth with no MNCs (control) while green, orange, purple, and red represent growth in the presence of MNCs at E:T ratios of 0.5:1, 1:1, 2:1, and 6:1, respectively. Vertical dashed lines indicate the 16 hour window of time after treatment used to determine AUC values. Normalized CI values are plotted in 15-minute increments as the average of three replicates with the standard deviation. This figure has been reproduced with permission from: Understanding Key Assay Parameters that Affect Measurements of Trastuzumab-Mediated ADCC Against Her2 Positive Breast Cancer Cells. Kute, T. *et al.* Oncoimmunology. **2012** Sep 1, *1(6)*, 810–821.[15]



Cell Profile and Purity of Subsets (%)					
Preparation	**NK**	**Mono**	**B Cells**	**T Cells**	**Total**
MNC	14.5	3.8	10.5	71.2	100
NK Cells	**89.0**	1.7	0.5	8.7	100
Monocytes	15.0	**81.4**	0.5	3.1	100
B Cells	15.3	5.2	**73.4**	6.1	100
T Cells	2.3	0.0	0.0	**95.4**	100

Figure 7. Natural killer cells exhibit the greatest ADCC activity among subpopulations of mononuclear cells. MNCs were tested for ADCC killing effect (A) or were separated into subpopulations, then tested. (B) NK cells, (C) monocytes, (D) B cells, (E) T cells. Green lines represent the control, blue shows 0.1 µg/mL of trastuzumab alone, violet shows MNCs or subpopulations at E:T = 6:1, and red shows MNCs or subpopulations at E:T = 6:1 in the presence of 0.1 µg/mL trastuzumab. The flow cytometry results showing the distribution of immune subtypes among purified cells and MNCs are given in the table. This figure has been reproduced with permission from: Understanding Key Assay Parameters that Affect Measurements of Trastuzumab-Mediated ADCC Against Her2 Positive Breast Cancer Cells. Kute, T. *et al.* Oncoimmunology. **2012** Sep 1, *1(6)*, 810–821.[15]

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ADCC – adherent target cells tested

MCF-7, A431, BT-474, NCI-N87, SKOV3, PC8, PC9, PC11, PC12, PC13, HD9, HD10, HD11, H322, MCF-7- CD19tm, Colo38, and MDA-MB435

Supporting information

- **Agilent xCELLigence application note:** Real-Time, Label-Free Measurement of Natural Killer Cell Activity and Antibody-Dependent Cell-Mediated Cytotoxicity
- **Agilent xCELLigence technical overview:** Real-Time Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assays
- **Agilent xCELLigence RTCA protocols:** In Vitro Functional Assay Using Real-Time Cell Analysis for Assessing Cancer Immunotherapeutic Agents
- **Agilent xCELLigence eSight video:** Multiplexed ADCC with xCELLigence RTCA eSight

Bispecific T cell engagers (BiTEs) and bispecific antibodies

The therapeutic efficacy of the ADCC technique is decreased by expression of the CD16 antibody receptor on some, but not all, immune cells. In particular, cytotoxic and helper T lymphocytes do not express CD16 and, therefore, are not recruited to antibody-coated cells. To circumvent this constraint and mobilize the full capacity of the adaptive immune response against tumors, bispecific antibodies have been engineered to simultaneously (1) bind specific antigens on the surface of tumor cells to (2) tether and activate cytotoxic and helper T cells by binding the CD3 receptor expressed on their surface. This approach has the advantage of bypassing MHC-mediated activation of T cells and has the potential to target any antigen expressed on the surface of tumor cells. Though multiple variations of bispecific antibodies have been studied, BiTEs stand out as especially promising. BiTEs targeting the CD19 antigen on B cell malignancies were awarded "Breakthrough Therapy" status by the FDA.

Using xCELLigence to study BiTEs

Figure 8 exemplifies how the xCELLigence RTCA can be utilized to characterize BiTEs through the killing of adherent PC3 prostate cancer cells by PBMCs. The study is performed in the presence of a BiTE targeting the EpCAM receptor (which is expressed on the surface of most cancer cells of epithelial origin, including PC3 cells).

Data show that in the absence of BiTE treatment, PBMCs displayed no cytolytic activity at the E:T ratios tested in this experiment. At 1 µg/mL anti-EpCAM/CD3 BiTE and varying E:T ratios, the CI decreases in a dose-dependent manner, representing PBMCs killing PC3s. At a PBMC:PC3 ratio of 10:1, EpCAM/CD3 BiTE increases killing efficacy in a dose-dependent manner. Though PC3 cell killing is stimulated at the lower BiTE concentrations, the killing of PC3 cells is delayed. Normalized CI can easily be converted to %cytolysis. The data clearly show larger differences in cytolysis efficiency when fewer effector cells were used.

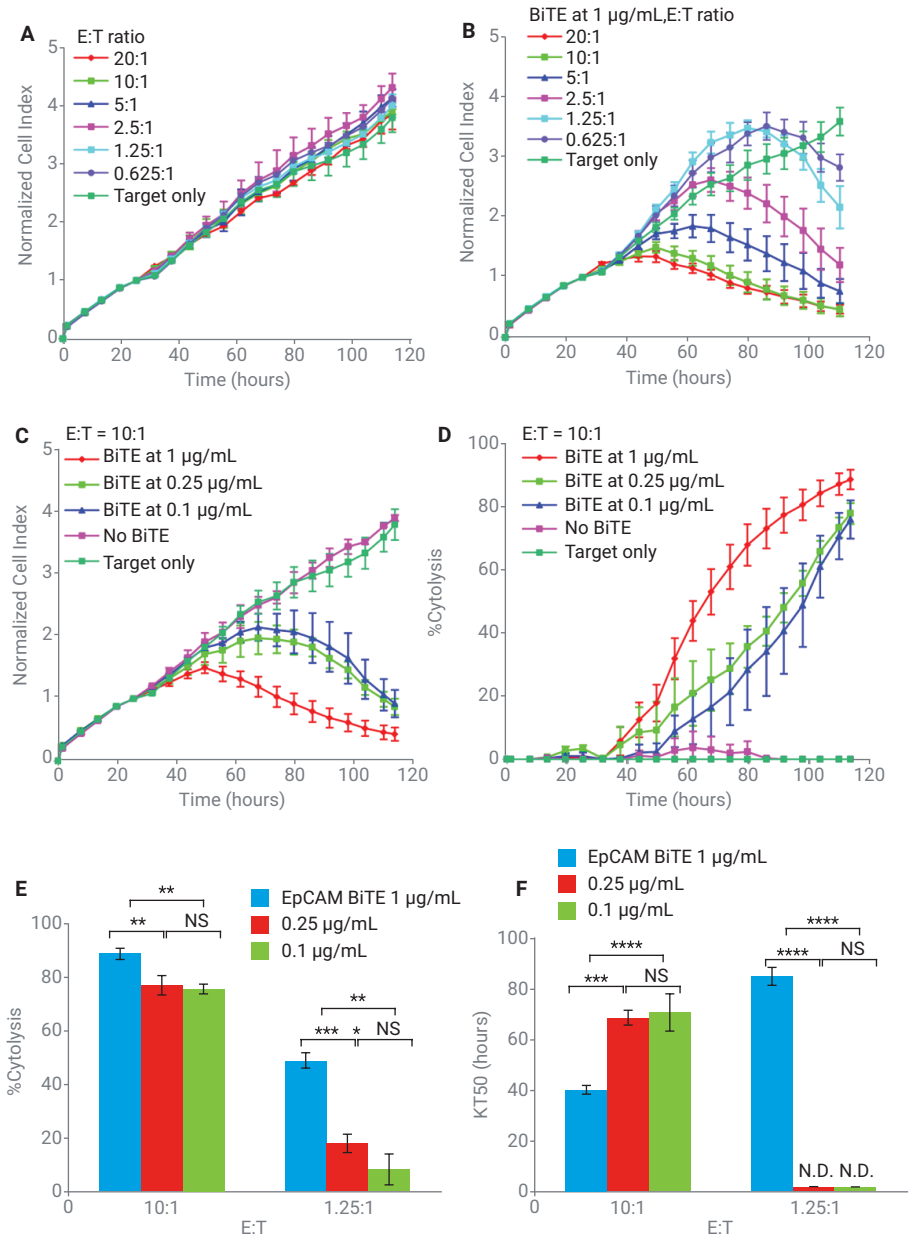

Figure 8. Impedance assessment of BiTE-mediated cytotoxicity. (A) Normalized CI plot for PC3 target cells incubated with PBMCs at different E:T ratios without the BiTE. (B) Same E:T ratios as (A) but with 1 µg/mL anti-EpCAM/CD3 BiTE. (C) At E:T ratio of 10:1, different BiTE concentrations resulted in varied dynamic cytolysis of the target cells. (D) Same result from (C) showed as %cytolysis. (E) Example of BiTE concentration depended %cytolysis from E:T ratio 10:1 and 1.25:1. (F) KT50 comparison for result from (E). Significance analysis performed by one-way ANOVA. (*** p< 0.001; ** p< 0.01; * p< 0.05; NS = Not Significant; N.D. = Not Detected). This figure has been reproduced with permission from Cerignoli, F. *et al. In vitro* Immunotherapy Potency Assays Using Real-Time Cell Analysis. *PLOS ONE* **2018**, *13(3)*, e0193498.

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BiTE and bispecific antibody mediated immune cell killing—adherent target cells tested

PC3 prostate cancer cells, Panc89, Colo357, PancTu-I, PDAC, Colo38, MDA-MB435, and HBV-transfected HuH7-S

Supporting information

- **Webinar recording:** Bispecific Antibody Constructs Mediate Immunotherapeutic Retargeting of Effector Cells Towards HBV Infected Target Cells
- **Webinar recording:** Bispecific Antibody Armed Metabolically Enhanced Headless CAR-T Cells - Safe, Effective Serial Killers of Solid Tumors
- **Agilent xCELLigence application note:** Tumor Cell Killing by T Cells - Quantifying the Impact of a CD19-BiTE Using Real-Time Cell Analysis, Flow Cytometry, and Multiplex Immunoassay
- **Agilent xCELLigence RTCA protocols:** In Vitro Functional Assay Using Real-Time Cell Analysis for Assessing Cancer Immunotherapeutic Agents

Checkpoint Inhibitors

By disrupting signaling pathways that normally suppress immune cell activation, checkpoint inhibitors enable immune effector cells to attack cancer cells more aggressively. From mechanistic validation of novel checkpoint targets to comparing the relative efficacy of two different checkpoint-modulating antibody constructs, xCELLigence RTCA instruments help answer your questions efficiently under conditions of maximal physiological relevance.

Using xCELLigence to study checkpoint inhibitors

Studies have shown that cancer cells are protected when PDL1 on the surface of cancer cells bind to PD1 expressed on activated cytotoxic T cells. This engagement leads to a decrease in cytotoxic activities and the production of cytokines such as interferon. PDL1/PD1-blocking antibodies are now being used as treatment to recover cytotoxic T cell activity and interferon production to inhibit tumor growth.

Figure 9 demonstrates the xCELLigence RTCA monitoring the impact of an anti-PD-1 antibody on PBMC killing of prostate cancer PC3 cells. Target PC3 cells are seeded in E-Plates and allowed to attach and proliferate. Frozen PBMCs are thawed, activated by incubation with Staphylococcus enterotoxin B (SEB) superantigen, then added on top of the PC3 cells in the presence or absence of anti-PD-1 antibody. The effector:target ratio was 5:1. As shown in Figure 9A, PBMCs display a modest capacity for killing PC3 cells (blue trace), but killing is much more robust in the presence of the anti-PD-1 antibody (orange trace). Using the xCELLigence RTCA software, the primary data are readily converted to %cytolysis (Figure 9B), which helps elucidate the checkpoint inhibitor's impact: earlier onset of target cell killing, increased rate of cytolysis, and a greater total extent of cell killing.

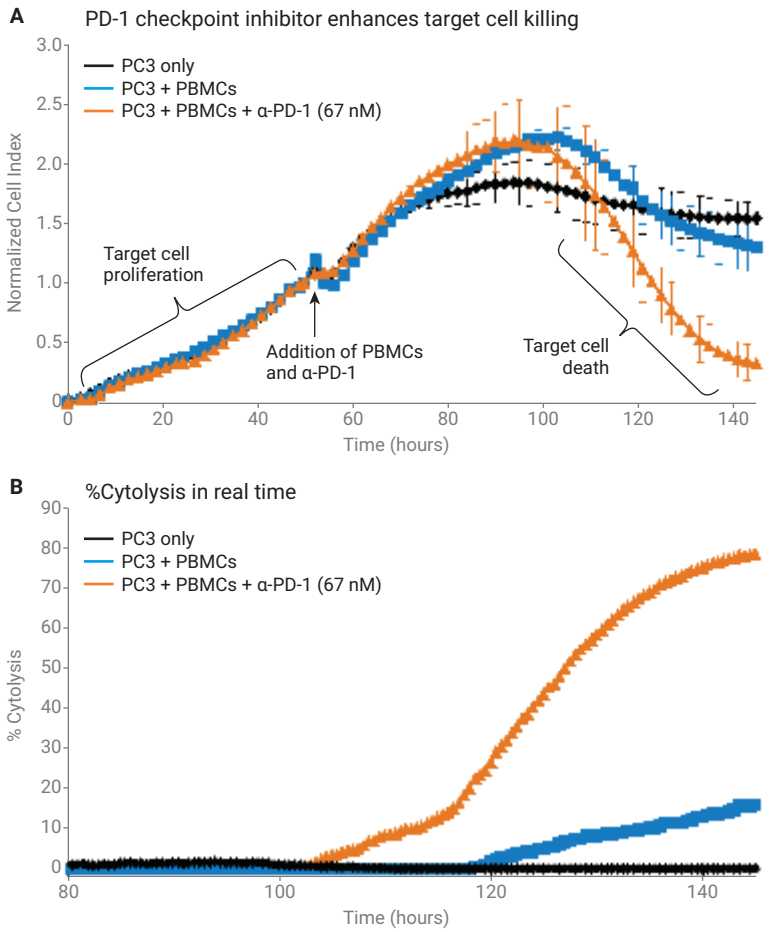

Figure 9. Using an Agilent xCELLigence to monitor the impact of anti-PD-1 antibody on PBMC killing of prostate cancer PC3 cells. PC3 cells were grown on an Agilent E-Plate. PBMC effector cells were added with or without the checkpoint inhibitor anti-PD-1 antibody. Impedance was monitored, and time-dependent cytolytic activity of the effectors was calculated.

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6. Wang, H. *et al.* ROS/JNK/C-Jun Pathway is Involved in Chaetocin Induced Colorectal Cancer Cells Apoptosis and Macrophage Phagocytosis Enhancement. *Front. Pharmacol.* **2021**, *12*, 729367. doi: 10.3389/fphar.2021.729367 (Sun Yat-sen University)
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8. Verdura, S. *et al.* Resveratrol targets PD-L1 Glycosylation and Dimerization to Enhance Antitumor T-Cell Immunity. Aging (Albany NY). **2020**, *12(1)*, 8–34. 10.18632/aging.102646 (Catalan Institute of Oncology; Girona Biomedical Research Institute)
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Supporting information

- **Agilent xCELLigence RTCA protocols:** In Vitro Functional Assay Using Real-Time Cell Analysis for Assessing Cancer Immunotherapeutic Agents
- **Agilent xCELLigence RTCA video:** Targeting Checkpoint Inhibitors for Cancer Treatment: The Possibilities
- **Agilent xCELLigence RTCA video:** Checkpoint Inhibitor Research
- **Agilent xCELLigence RTCA video:** xCELLigence – A Transformative Technology in Cancer Research

Combination therapy

The traditional oncology pharmacopeia of small molecules is rapidly being supplemented with biologics such as checkpoint inhibitors. It will soon also include cellular therapies, such as CAR-T cells. With this expanding repertoire comes the possibility of boosting cancer killing efficacy by combining different modalities. The optimization of combination therapies would benefit from an assay platform that, by maintaining high sensitivity under physiologically relevant conditions, yields *in vitro* data that are predictive of *in vivo* behavior. Other desirable characteristics include an easy workflow and a high-throughput format to enable diverse permutations of combination therapies so they can be analyzed simultaneously. xCELLigence RTCA meets all of the above criteria.

Using xCELLigence to study combination therapies

Figure 10 illustrates use of the xCELLigence RTCA to monitor the impact a combination of PD-1 and CTLA-4 checkpoint inhibitors have on PBMC killing of PC3 cells. In this experiment, target PC3 cells are seeded in E-Plates and allowed to attach and proliferate. Frozen PBMCs are thawed, activated by incubation with Staphylococcal enterotoxin B (SEB) superantigen, then added on top of the PC3 cells in the presence or absence of 38 nM anti-PD-1 antibody and two different concentrations of anti-CTLA-4 antibodies. The effector:target ratio was 5:1. The killing efficacy of PBMCs varies dramatically from donor to donor, and, for this particular batch of cells, adding 38 nM anti-PD-1 did not enhance target cell killing. However, adding anti-CTLA-4 along with anti-PD-1 promoted target cell killing in a dose-dependent manner (green and pink traces).

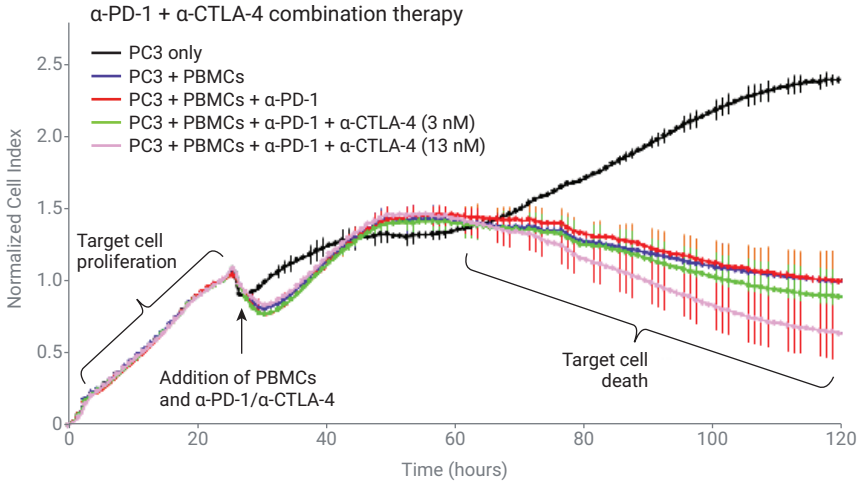

Figure 10. Anti-PD-1 and anti-CTLA-4 antibodies combination therapy. By analyzing cancer cell killing with high sensitivity and without the need for labels/modifications, the Agilent xCELLigence RTCA instruments allow effector and target cells to be studied under conditions that approximate human physiology more closely than other *in vitro* techniques. By monitoring combination therapy-induced target cell killing continuously, these instruments also do away with laborious endpoints, readily yielding cell killing data under many different conditions simultaneously.

Selected publications

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2. Sonzogni, O. *et al.* T-SIGn Tumor Reengineering Therapy and CAR T Cells Synergize in Combination Therapy to Clear Human Lung Tumor Xenografts and Lung Metastases in NSG Mice. *Oncoimmunology* **2022**, *11(1)*, 2029070. doi: 10.1080/2162402X.2022.2029070 (PsiOxus Therapeutics Limited)
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9. Feliz-Mosquea, Y. R. *et al.* Combination of Anthracyclines and Anti-CD47 Therapy Inhibit Invasive Breast Cancer Growth While Preventing Cardiac Toxicity by Regulation of Autophagy. *Breast Cancer Res. Treat.* **2018**, *172(1)*, 69–82. doi: 10.1007/s10549-018-4884-x (Wake Forest School of Medicine)
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13. Ruf, B. *et al.* Combination of the Oral Histone Deacetylase Inhibitor Resminostat with Oncolytic Measles Vaccine Virus as a New Option for Epi-Virotherapeutic Treatment of Hepatocellular Carcinoma. *Mol. Ther. Oncolytics* **2015**, *2*, 15019. doi: 10.1038/mto.2015.19 (University Hospital Tuebingen)

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15. Kasukabe, T. *et al.* Cotylenin A and Arsenic Trioxide Cooperatively Suppress Cell Proliferation and Cell Invasion Activity in Human Breast Cancer Cells. *Int. J. Oncol.* **2015**, *46(2)*, 841–8. doi: 10.3892/ijo.2014.2760 (Shimane University)
16. Chang, H. Y. *et al.* Combination Therapy Targeting Ectopic ATP Synthase and 26S Proteasome Induces ER Stress in Breast Cancer Cells. *Cell Death Dis.* **2014**, *5*, e1540. doi: 10.1038/cddis.2014.504 (National Taiwan University)
17. Von Mallinckrodt, B. *et al.* Dianthin-EGF is an Effective Tumor Targeted Toxin in Combination with Saponins in a Xenograft Model for Colon Carcinoma. *Future Oncol*. **2014**, *10(14)*, 2161–75. doi: 10.2217/fon.14.164 (Charité - Universitätsmedizin Berlin)
18. Li, J. *et al.* Synergistic Combination of Valproic Acid and Oncolytic Parvovirus H-1PV as a Potential Therapy Against Cervical and Pancreatic Carcinomas. *EMBO Mol. Med*. **2013**, *5(10)*, 1537–1555. doi: 10.1002/emmm.201302796 (Laboratory of Oncolytic Virus Immuno-Therapeutics, German Cancer Research Centre)
19. Tsamis, K. I. *et al.* Combination Treatment for Glioblastoma Cells with Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand and Oncolytic Adenovirus Delta-24. *Cancer Invest.* **2013**, *31(9)*, 630–8. doi: 10.3109/07357907.2013.849724 (University of Ioannina)

Supporting information

– **Agilent xCELLigence RTCA video:** RTCA Software Pro for Immunotherapy Applications | Agilent

Genetically engineered T cell-mediated cell killing

T cells can be genetically engineered to express a tumor antigen-specific T cell receptor (TCR) or a chimeric antigen receptor (CAR), composed of an intracellular signaling domain linked to an extracellular domain derived from a tumor-specific antibody. The primary motivation for genetically modified T cells is to avoid the immune tolerance issues associated with nonautologous therapies and to produce T cells that efficiently target tumors without the need for *de novo* activation in people. The efficacy of this approach is highlighted by the convincing clinical research data that have emerged in recent years (for example, see Clin. Transl. Immunology **2014**, *3(5)*, e16).

Using xCELLigence to study genetically engineered T cells

In Figure 11, the antitumor activity of NKG2D CAR-T cells on triple-negative breast cancer cells (TNBCs) is evaluated by xCELLigence RTCA *in vitro*. Results show that a time- and E:T ratio-dependent cytotoxicity for 4-1BB or CD27 costimulated NKG2D CAR-T cells against NKG2DL (+) MDA-MB-468, and MDA-MB-436 cells. As a negative control, untransduced T cells did not inhibit the growth of these cells.

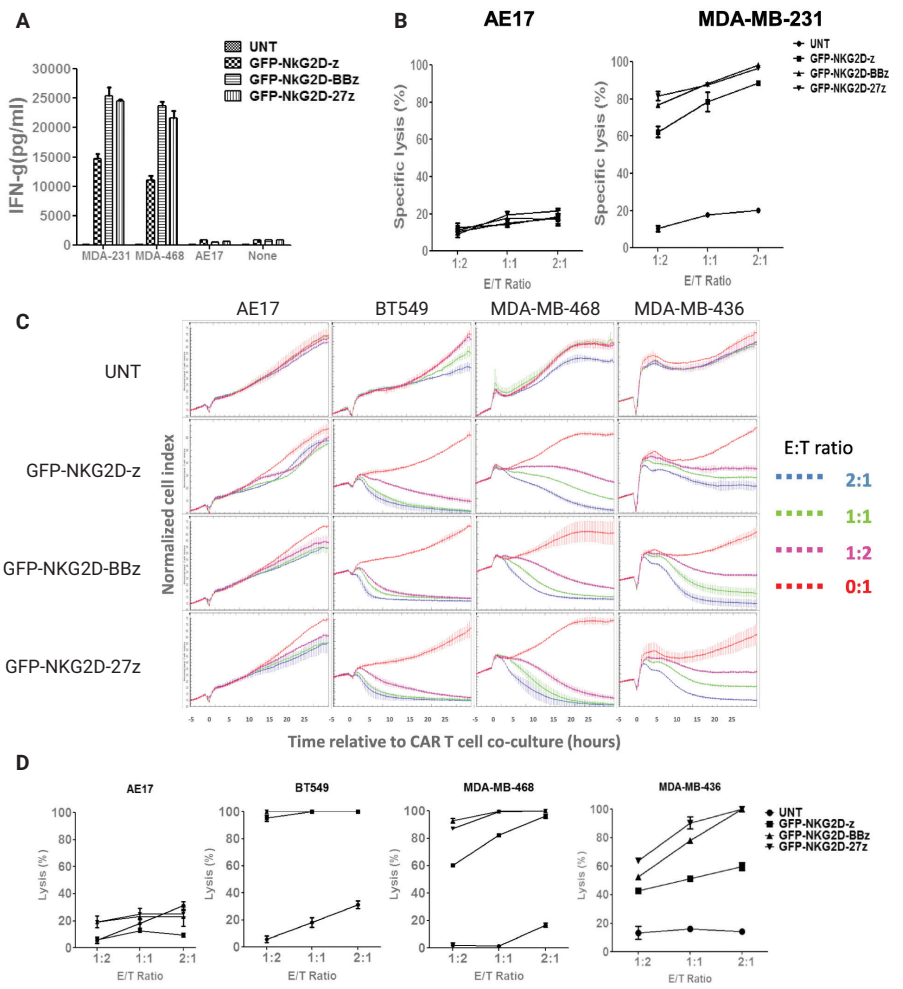

Figure 11. Recognition of human TNBC cells by NKG2D CAR T cells *in vitro*. (C) Normalized CI plot for target cells (AE17, BT549, MDA-MB-436, and MDA-MB-468) incubated with UNT or NKG2D CAR T cells at different E:T ratios for 24 hours. When seeded alone, target cells adhere to the plate and proliferate, increasing the CI readout (red lines). When T cells are added to target cells, NKG2CD CAR-T cells cause cell cytolysis and then a progressive decrease in CI. The Y-axis is the normalized CI generated by the RTCA software displayed in real time. The X-axis is the time of cell culture and treatment time in hours. Mean values of the CI were plotted ± standard deviation. (D) The CI plot is converted to %lysis by the Agilent xCELLigence immunotherapy software.

This figure has been reproduced with permission from: Hali, Y. et al. Control of Triple-Negative Breast Cancer Using *Ex Vivo* Self-Enriched, Costimulated NKG2D CAR T Cells. *Journal of Hematology & Oncology* **2018**, *11*, 92.[1]

Heterogeneous antigen expression within a cancer cell population can lead to an incomplete response to CAR-T cell therapy. While cancer cells that express the targeted antigen are killed off, cells that lack the antigen continue propagating undeterred. To minimize this phenomenon, known as antigen/tumor escape, there is growing interest in targeting multiple tumor cell antigens simultaneously.

Figure 12 compares different scenarios where CARs targeting the HER2 and IL13Rα2 antigens were expressed in separate T cells (CARpool), as distinct proteins within the same T cell (biCAR), or as a single fusion protein within T cells (TanCAR). When incubated with glioblastoma target cells, each of these CART approaches displayed differential killing capacity and kinetics (Figure 13). These nuances in serial killing behavior are readily elucidated by continuous impedance monitoring but would go undetected in traditional endpoint assays.

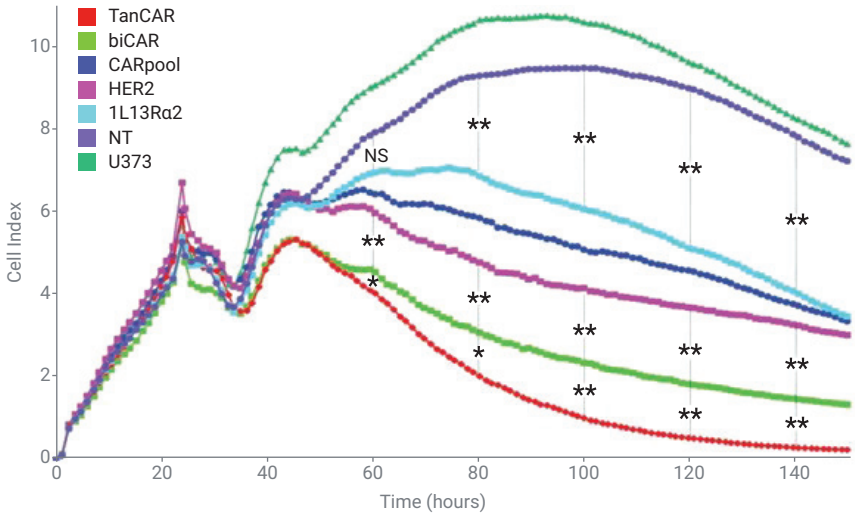

Figure 12. Using Agilent xCELLigence to monitor killing of the glioblastoma cell line U373 by CAR-T cells targeting either one or both of the antigens HER2 and IL13Rα2. In the figure legend: U373 = target cell line alone; NT = target cells treated with nontransfected T cells (not expressing a CAR); IL13Rα2 = target cells treated with T cells expressing a single CAR targeting IL13Rα2; Her2 = target cells treated with T cells expressing a single CAR targeting Her2; see the text for descriptions of CARpool, biCAR, and TanCAR.

Selected publications

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4. Brummer, A. B. *et al.* Dose-Dependent Thresholds of Dexamethasone Destabilize CAR T-Cell Treatment Efficacy. *PLoS Comput. Biol.* **2022**, *18(1)*, e1009504. doi: 10.1371/journal.pcbi.1009504 (Beckman Research Institute, City of Hope National Medical Center)
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13. Hu, M. *et al.* Discovery of the First Potent Proteolysis Targeting Chimera (PROTAC) Degrader of Indoleamine 2,3-Dioxygenase 1. *Acta Pharm. Sin. B* **2020**, *10(10)*, 1943-1953. doi: 10.1016/j.apsb.2020.02.010 (Sichuan University and Collaborative Innovation Center of Biotherapy)
14. Sureban, S. M. *et al.* DCLK1 Monoclonal Antibody-Based CAR-T Cells as a Novel Treatment Strategy against Human Colorectal Cancers. *Cancers (Basel)* **2019**, *12(1)*, 54. doi: 10.3390/cancers12010054 (University of Oklahoma)
15. Berahovich, R. *et al.* Hypoxia Selectively Impairs CAR-T Cells In Vitro. *Cancers (Basel)* **2019**, *11(5)*, 602. doi: 10.3390/cancers11050602 (ProMab Biotechnologies)
16. June, C. H. *et al.* Improving CAR T-Cell Therapy of Solid Tumors with Oncolytic Virus-Driven Production of a Bispecific T-cell Engager. *Cancer Immunol. Res.* **2018**, *6(5)*, 605–616. doi: 10.1158/2326-6066.CIR-17-0314 (University of Pennsylvania)
17. June, C. H. *et al.* Pancreatic Cancer Therapy with Combined Mesothelin-Redirected Chimeric Antigen Receptor T Cells and Cytokine-Armed Oncolytic Adenoviruses. *JCI Insight.* **2018**, *3(7)*, e99573. doi: 10.1172/jci.insight.99573 (University of Pennsylvania)

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19. Davenport, A. J. *et al.* CAR-T Cells inflict Sequential Killing of Multiple Tumor Target Cells. *Cancer Immunol. Res*. **2015**, *3(5)*, 483–94 doi: 10.1158/2326-6066.CIR-15-0048 (University of Melbourne)
20. June, C. H. Serial Killers and Mass Murderers, Engineered T Cells Are Up to the Task. *Cancer Immunol. Res.* **2015**, *3(5)*, 470–2 doi: 10.1158/2326-6066.CIR-15-0075 (University of Pennsylvania)
21. Lengagne, R. *et al.* T Cells Contribute to Tumor Progression by Favoring Pro-Tumoral Properties of intra-Tumoral Myeloid Cells in A Mouse Model For Spontaneous Melanoma. *PLoS One* **2011**, *6(5)*, e20235 doi: 10.1371/journal.pone.0020235 (Institut Cochin)

Genetically engineered T cell-mediated cell killing– adherent target cells tested

A375, SW480, MC57, MC57-HER2, U-251MG, and 13-06-MG

Supporting information

- **Webinar recording:** The Next Generation of CAR T Cells: New Techniques to Improve Functionality
- **Agilent xCELLigence RTCA protocol:** A Human In Vitro T Cell Exhaustion Model for Assessing Immuno-Oncology Therapies
- **Agilent xCELLigence application note:** Real-Time Specificity and Potency Assessment of Human Papilloma Virus Specific Engineered T Cells
- **Agilent xCELLigence application note:** Real-Time Potency Assay for CAR T Cell Killing of Adherent Cancer Cells
- **Agilent xCELLigence eSight video:** Car T Killing Cancer Cells with xCELLigence RTCA eSight
- **Agilent xCELLigence technical overview:** Real-Time Potency Assay for CAR T Cell Killing of Adherent Cancer Cells
- **Agilent xCELLigence case study:** Tmunity Therapeutics - Building a CAR T Toolbox for More Comprehensive Assessment of Cell Therapies
- **Agilent xCELLigence application note:** Ex Vivo Phenotyping and Potency: Using a combined flow cytometry and impedance-based real-time cell analysis workflow
- **Webinar recording:** Optimizing B7-H3-CAR T Cells for Targeting Solid Tumors
- **Webinar recording:** Cell Impedance as a Tool to Measure Potency of Chimeric Antigen Receptor T cells
- **Webinar recording:** Novel Bispecific CAR T Cells Against Hematological Cancers
- **Webinar recording:** An Effective, High Throughput Impedance-based Assay to Evaluate Potency of Immunotherapy Products
- **Webinar recording:** Comprehensive Real-Time Workflow: T-cell Potency and Metabolic Fitness

- **Webinar recording:** Modeling Anti-tumor Function of Human T Cells with xCELLigence RTCA eSight
- **Agilent xCELLigence application note:** Metabolic Preconditioning Improves Engineered T Cell Fitness and Function
- **Agilent xCELLigence brochure:** Measure Cell Movement, Health, and Function with xCELLigence RTCA eSight

Macrophage-mediated phagocytosis

Using xCELLigence to study macrophage-mediated phagocytosis

Macrophages are important effector cells of innate immunity. Depending on the tissue microenvironment, tumor-associated macrophages (TAMs) can differentiate into either cytotoxic (M1) or tumor-promoting (M2) states.

While cytotoxic M1 macrophages are typically induced by IFN-γ alone or in concert with microbial products, tumor-promoting M2 macrophages are induced by IL-4, IL-13, IL-10, IL-21, TGFβ, immune complexes, or glucocorticoids.

A recent study has shown the secreted glycoprotein thrombospondin 1 (TSP1) is a positive modulator of innate antitumor immunity by increasing M1 macrophage recruitment and stimulating reactive oxygen species (ROS)-mediated tumor cell killing (Figure 13). These conclusions are drawn, in part, using xCELLigence RTCA impedance monitoring to evaluate the effect of TSP1 on macrophage/monocyte activity when cocultured with MDA-MB-231 breast adenocarcinoma target cells. The %cytolysis data clearly indicate that the tumoricidal activity of both differentiated U937 human monocytes (A) and activated ANA-1 murine macrophages (B) are enhanced in the presence of TSP1.

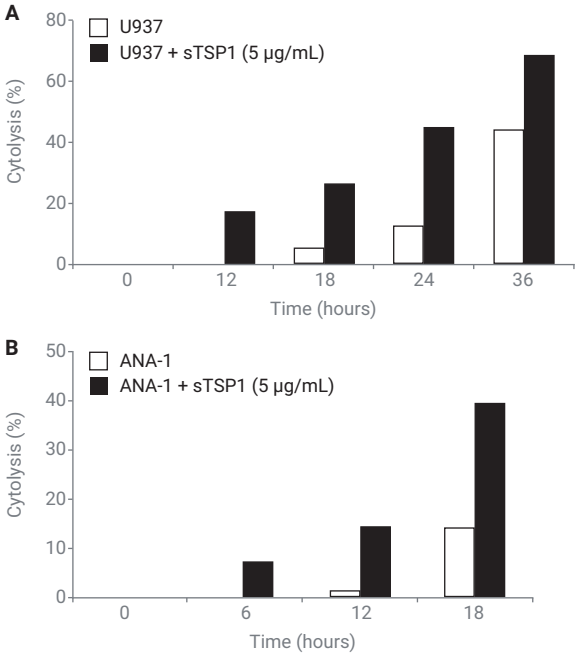

Figure 13. Secreted glycoprotein TSP1 increases macrophage/monocyte-mediated tumoricidal activity. MDA-MB-231 breast adenocarcinoma target cells were seeded in Agilent E-Plates and incubated for up to 24 hours. Differentiated U937 human monocytes (A) or activated ANA-1 murine macrophages (B) were then added in the presence or absence of soluble TSP1. This figure was adapted from: Cancer Res. **2008**, *68(17)*, 7090–9.[4] Note that the RT-CES described in this publication was Agilent's first-generation RTCA system, and has been rebranded as Agilent xCELLigence RTCA.

Selected publications

1. Baris, E. *et al.* Varenicline Prevents LPS-Induced Inflammatory Response via Nicotinic Acetylcholine Receptors in RAW 264.7 Macrophages. *Front. Mol. Biosci.* **2021**, *8*, 721533 doi: 10.3389/fmolb.2021.721533 (Izmir University of Economics)
2. Cheok, Y. Y. *et al.* Podoplanin Drives Motility of Active Macrophage via Regulating Filamin C During Helicobacter pylori Infection. *Front. Immunol*. **2021**, *12*, 702156 doi: 10.3389/fimmu.2021.702156 (University of Malaya)
3. Liu, C. *et al.* Danlou Tablets Inhibit Atherosclerosis in Apolipoprotein E-Deficient Mice by Inducing Macrophage Autophagy: The Role of the PI3K-Akt-mTOR Pathway. *Front. Pharmacol.* **2021**, *12*, 724670 doi: 10.3389/fphar.2021.724670 (University of Macau)
4. Rodriguez-Garcia, A. *et al.* CAR-T Cell-Mediated Depletion of Immunosuppressive Tumor-Associated Macrophages Promotes Endogenous Antitumor Immunity and Augments Adoptive Immunotherapy. *Nat. Commun.* **2021**, *12(1)*, 877 doi: 10.1038/s41467-021-20893-2 (University of Pennsylvania)
5. Guo, L. *et al.* Lipopolysaccharide-Anchored Macrophages Hijack Tumor Microtube Networks for Selective Drug Transport and Augmentation of Antitumor Effects in Orthotopic Lung Cancer. *Theranostics* **2019**, *9(23)*, 6936–6948 doi: 10.7150/thno.37380 (Sun Yat-sen University)
6. Barrett, T. J. *et al.* Platelet Regulation of Myeloid Suppressor of Cytokine Signaling 3 Accelerates Atherosclerosis. *Sci. Transl. Med.* **2019**, *11(517)*, eaax0481. doi: 10.1126/scitranslmed.aax0481 (New York University School of Medicine)
7. Recio, C. *et al.* Activation of the Immune-Metabolic Receptor GPR84 Enhances Inflammation and Phagocytosis in Macrophages. *Front. Immunol.* **2018**, *9*, 1419 doi: 10.3389/fimmu.2018.01419 (University of Oxford)
8. Cooks, T. *et al.* Mutant p53 Cancers Reprogram Macrophages to Tumor Supporting Macrophages Via Exosomal miR-1246. *Nat. Commun.* **2018**, *9(1)*, 771 doi:10.1038/s41467-018-03224-w (National Institutes of Health)
9. Soto-Pantoja, D. R. Unfolded Protein Response Signaling Impacts Macrophage Polarity to Modulate Breast Cancer Cell Clearance and Melanoma Immune Checkpoint Therapy Responsiveness. *Oncotarget* **2017**, *8(46)*, 80545–80559 doi: 10.18632/oncotarget.19849 (Wake Forest School of Medicine)
10. Hoefert, S. *et al.* Altered Macrophagic THP-1 Cell Phagocytosis and Migration in Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ). *Clin. Oral Investig.* **2016**, *20(5)*, 1043–54 doi: 10.1007/s00784-015-1584-3 (University Hospital Tuebingen)
11. Pirilä, E. *et al.* Macrophages Modulate Migration and Invasion of Human Tongue Squamous Cell Carcinoma. *PLoS One* **2015**, *10(3)*, e0120895 doi: 10.1371/journal.pone.0120895 (University of Oulu)
12. Shi, Y. *et al.* Trastuzumab Triggers Phagocytic Killing of High HER2 Cancer Cells In Vitro and In Vivo b Interaction with Fcγ Receptors on Macrophages. *J. Immunol.* **2015** May 1, *194(9)*, 4379–86.

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14. Cook, K. L. *et al.* Hydroxychloroquine Inhibits Autophagy to Potentiate Antiestrogen Responsiveness in ER+ Breast Cancer. *Clin. Cancer Res.* **2014**, *20(12)*, 3222–32. doi: 10.1158/1078-0432.CCR-13-3227 (Georgetown University Medical Center, NIH)
15. Rietkötter, E. *et al.* Zoledronic Acid Inhibits Macrophage/Microglia-Assisted Breast Cancer Cell Invasion. *Oncotarget* **2013**, 4(9), 1449–1460. doi: 10.18632/oncotarget.1201 (University Medical Center Göttingen)
16. Iqbal, A J. *et al.* A Real Time Chemotaxis Assay Unveils Unique Migratory Profiles Amongst Different Primary Murine Macrophages. *PLoS One* **2013**, *8(3)*, e58744. doi: 10.1371/journal.pone.0058744 (University of Oxford)
17. Martin-Manso, G. *et al.* Thrombospondin 1 Promotes Tumor Macrophage Recruitment and Enhances Tumor Cell Cytotoxicity of Differentiated U937 cells. *Cancer Res.* **2008**, *68(17)*, 7090–9. doi: 10.1158/0008-5472.CAN-08-0643 (National Institutes of Health)

Macrophage-mediated phagocytosis – adherent target cells tested

MDA-MB-231, MDA-MB-435, and MCF-7

Supporting information

- **Agilent xCELLigence application note:** Real-Time Visualization and Quantitative Analysis of Macrophage Phagocytosis Using the xCELLigence RTCA eSight
- **Agilent xCELLigence eSight video:** Effectively Quantify Which Microorganisms Are Killed by Phagocytosis in Real Time
- **Agilent NovoCyte application note:** Examining the Kinetics of Neutrophil Phagocytosis by Flow Cytometry

NK cell-mediated cytolysis

NK cells are a type of cytotoxic lymphocyte that play a critical role in the innate immune system, primarily by recognizing and destroying virus-infected cells. NK cells express several activating and inhibitory receptors that work in concert to distinguish infected or diseased cells from normal cells. Once they bind to a target cell, NK cells become activated and secrete membrane-permeabilizing proteins (perforins) and proteases (granzymes), which collectively cause target cell death through apoptosis or osmotic lysis. NK cells also participate in a specialized type of cell killing known as antibody-dependent cell-mediated cytotoxicity (ADCC). In ADCC, the CD16 low affinity IgG receptor of NK cells enables them to recognize infected antibody-coated cells that need to be destroyed. These mechanisms used by NK cells to recognize and destroy infected cells are also critical for killing cancer cells. Unlike T cells, which must be activated by antigen-presenting cells before they recognize tumors, NK cells spontaneously lyse certain types of tumor cells *in vivo* and *in vitro* without requiring immunization or pre-activation. Similar to virally infected cells, tumor cells may also down-regulate their MHC-1 expression. Recognizing this change in expression, NK cells destroy such cancer cells through perforin/granzyme-mediated lysis. Owing to this capacity, NK cells are being investigated for the purposes of immunotherapy.

Using xCELLigence to study NK cell-mediated cytolysis

Figure 14 shows use of the xCELLigence RTCA to measure target tumor cell killing by NK cells at low, physiologically relevant E:T ratios *in vitro*. Results show that AZD1775, a small molecule inhibitor of WEE1 kinase, was able to sensitize tumor cells to NK cell lysis.

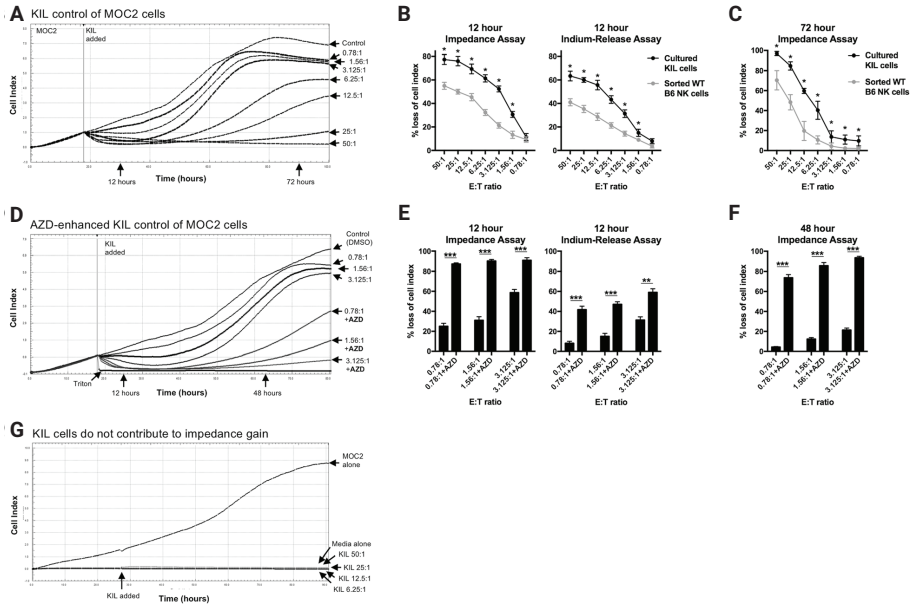

Figure 14. Target cell killing by KIL at low E:T ratios is enhanced following WEE1 kinase inhibition. (A) Loss of CI of MOC2 oral carcinoma cells following the addition of KIL at increasing E:T ratios was measured through real-time impedance analysis. The vertical line at 18 hours indicates the time at which KIL was added. Control cells were exposed to KIL media alone. (B) %Loss of CI at 12 hours after the addition of KIL (black line) or sorted WT B6 NK cells (gray line) quantified on the left. On the right, for comparison, KIL (black line) or sorted WT B6 NK cells (gray line) were used to induce indium release in a standard 12-hour radioactive compound release assay. (C) %Loss of CI at 72 hours after addition of KIL (black line) or sorted WT B6 NK cells (gray line) quantified. For B and C, * indicates significantly enhanced killing with KIL cells compared to sorted WT B6 NK cells. (D) Loss of MOC2 CI following the addition of KIL at low E:T ratios in the presence of AZD1775 (250 nM) or DMSO (volume equivalent). When AZD1775 was present, MOC2 cells were plated in drug at the start of the assay. Maximum loss of CI was achieved by addition of triton to some wells. %Loss of CI 48 hours after the addition of KIL to MOC2 cells is quantified on the right. (E) %Loss of CI of MOC2 cells in the presence (AZD1775 250 nM) or absence (DMSO volume equivalent) of WEE1 kinase inhibition 12 hours after the addition of KIL at the indicated E:T ratios quantified on the left. On the right, for comparison, the same was measured in a standard 12-hour radioactive compound release assay. (F) %Loss of CI of MOC2 cells 48 hours after the addition of KIL. (G) Impedance analysis of MOC2 cells alone (5×10^3 cells/well) compared to media or KIL cells alone up to an E:T ratio equivalent of 50:1 (2.5×10^5 KIL/well). Results presented are representative of three independent experiments with similar results. (*, $p < 0.05$; ***, $p < 0.001$).

This figure has been reproduced with permission from: Friedman, J. et al. Inhibition of WEE1 Kinase and Cell Cycle Checkpoint Activation Sensitizes Head and Neck Cancers to Natural Killer Cell Therapies. *J. Immunother. Cancer.* **2018**, *6*, 59.

In Figure 15, xCELLigence RTCA was used to quantitatively measure the cytolytic activity of NK cells in real time. After growing adherent breast cancer MCF7 cells in the bottom of E-Plate wells, NK-92 cells were added at different effector to target (E:T) ratios. The data clearly demonstrate NK-92 cell-mediated lysis of the MCF7 cells in a dose- and time-dependent manner. Real-time impedance monitoring by the xCELLigence system is sensitive enough to detect target cell killing even at low E:T ratios. For plotting purposes, %cytolysis is readily calculated using a simple formula:

$$\%\text{Cytolysis} = \frac{(\text{Cell Index}_{\text{no effector}} - \text{Cell Index}_{\text{effector}})}{\text{Cell Index}_{\text{no effector}}} \times 100$$

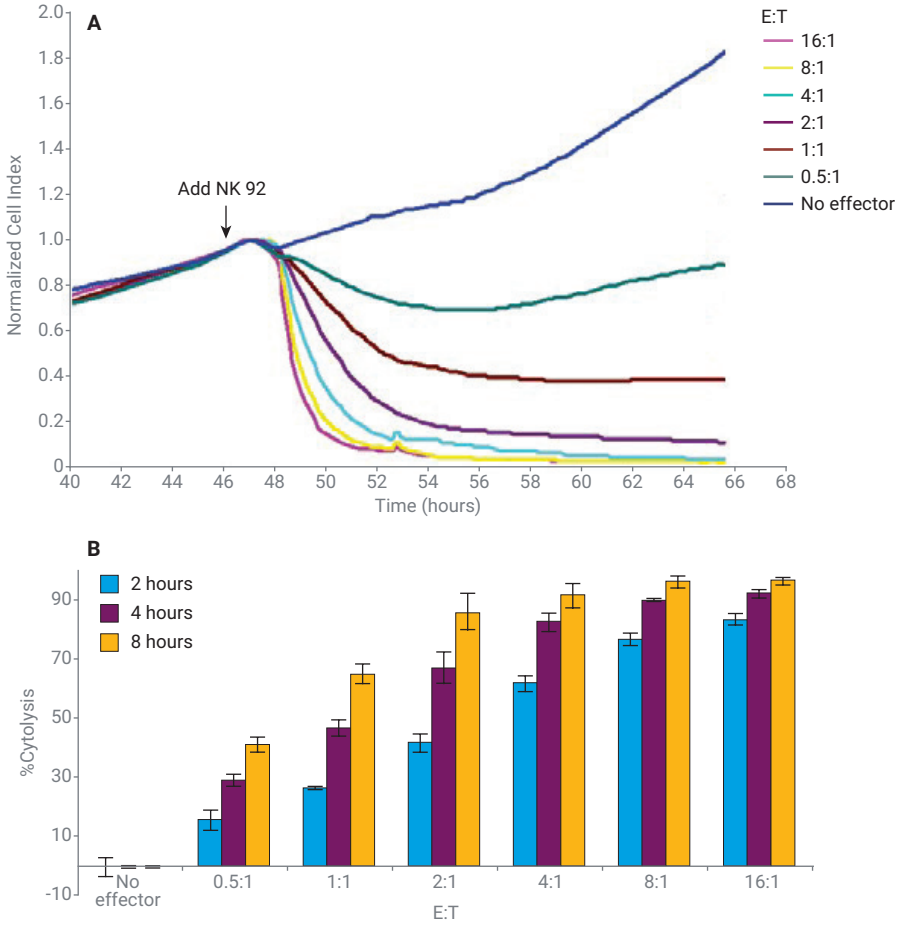

Figure 15. Real-time monitoring of NK-92 cell-mediated cytolysis of MCF7 breast cancer cells. Adherent MCF7 target cells were grown in multiple wells of an Agilent E-Plate. Different quantities of NK-92 cells were added to each well, and impedance was monitored continuously for the next ~20 hours (A). The time-dependent cytolytic activity of NK-92 cells at different E:T ratios (B) was calculated as described above. Figures adapted from Agilent application note Label-Free Assay for NK Cell-Mediated Cytolysis.

Selected publications

1. Kararoudi, M. N. *et al.* Optimization and validation of CAR transduction into human primary NK cells using CRISPR and AAV. *Cell Rep. Methods* **2022**, *2(6)*, 100236. doi: 10.1016/j.crmeth.2022.100236 (Nationwide Children's Hospital; Ohio State University)
2. Klapdor, R. *et al.* NK Cell-Mediated Eradication of Ovarian Cancer Cells with a Novel Chimeric Antigen Receptor Directed against CD44. *Biomedicines* **2021**, *9(10)*, 1339. doi: 10.3390/biomedicines9101339 (Hannover Medical School)
3. Morimoto, T. *et al.* CRISPR-Cas9-Mediated TIM3 Knockout in Human Natural Killer Cells Enhances Growth Inhibitory Effects on Human Glioma Cells. *Int. J. Mol. Sci.* **2021**, *22(7)*, 3489. doi: 10.3390/ijms22073489 (Nara Medical University)
4. Li, H. K. *et al.* A Novel off-the-Shelf Trastuzumab-Armed NK Cell Therapy (ACE1702) Using Antibody-Cell-Conjugation Technology. *Cancers (Basel)* **2021**, *13(11)*, 2724. doi: 10.3390/cancers13112724 (Acepodia Biotech Inc.)
5. Lee, M.Y. *et al.* Chimeric antigen receptor engineered NK cellular immunotherapy overcomes the selection of T-cell escape variant cancer cells. *J. Immunother. Cancer* **2021**, *9(3)*, e002128. doi: 10.1136/jitc-2020-002128 (National Institutes of Health)
6. Banu, N. *et al.* B7-H6, an immunoligand for the natural killer cell activating receptor NKp30, reveals inhibitory effects on cell proliferation and migration, but not apoptosis, in cervical cancer derived-cell lines. *BMC Cancer* **2020**, *20(1)*, 1083. doi: 10.1186/s12885-020-07608-4 (Universidad de Guadalajara)
7. Robbins, Y. *et al.* Tumor control via targeting PD-L1 with chimeric antigen receptor modified NK cells. *Elife* **2020**, *9*, e54854. doi: 10.7554/eLife.54854 (National Institutes of Health)
8. Sayitoglu, E. C. *et al.* Boosting Natural Killer Cell-Mediated Targeting of Sarcoma Through DNAM-1 and NKG2D. *Front. Immunol.* **2020**, *11*, 40. doi: 10.3389/fimmu.2020.00040 (Nova Southeastern University)
9. Zhang, X. *et al.* Low-Dose Gemcitabine Treatment Enhances Immunogenicity and Natural Killer Cell-Driven Tumor Immunity in Lung Cancer. *Front. Immunol.* **2020**, *11*, 331. doi: 10.3389/fimmu.2020.00331 (University of Science and Technology of China)
10. Frazao, A. *et al.* BRAF inhibitor resistance of melanoma cells triggers increased susceptibility to natural killer cell-mediated lysis. *J. Immunother. Cancer* **2020**, *8(2)*, e000275. doi: 10.1136/jitc-2019-000275 (Université de Paris)
11. Clar, K. L. *et al.* Inhibition of NK Reactivity Against Solid Tumors by Platelet-Derived RANKL. *Cancers (Basel)* **2019**, *11(3)*, 277. doi: 10.3390/cancers11030277 (German Cancer Consortium (DKTK) and Research Center (DKFZ))
12. Klapdor, R. *et al.* Characterization of a Novel Third-Generation Anti-CD24-CAR against Ovarian Cancer. *Int. J. Mol. Sci.* **2019**, *20(3)*, 660. doi: 10.3390/ijms20030660 (Hannover Medical School)
13. Ao, X. *et al.* Anti-αFR CAR-engineered NK-92 Cells Display Potent Cytotoxicity Against αFR-positive Ovarian Cancer. *J. Immunother.* **2019**, *42(8)*, 284–296. doi: 10.1097/CJI.0000000000000286 (Third Military Medical University)
14. Du, X. *et al.* CD226 regulates natural killer cell antitumor responses via phosphorylation-mediated inactivation of transcription factor FOXO1. *Proc. Natl. Acad. Sci. USA* **2018**, *115(50)*, E11731–E11740. doi: 10.1073/pnas.1814052115 (Genentech, Inc.)

15. Friedman, J. *et al.* Inhibition of WEE1 Kinase and Cell Cycle Checkpoint Activation Sensitizes Head and Neck Cancers to Natural Killer Cell Therapies. *J. Immunother. Cancer* **2018**, *6(1)*, 59. doi: 10.1186/s40425-018-0374-2 (National Institutes on Deafness and Other Communication Disorders, National Institutes of Health; Johns Hopkins School of Medicine)
16. Fasbender, F.; Watzl, C. Impedance-Based Analysis of Natural Killer Cell Stimulation. *Scientific Reports* **2018**, *8(1)*, 4938. doi: 10.1038/s41598-018-23368-5 (Technical University Dortmund (IfADo))
17. Pantic, J. M. *et al* The Frog Skin Host-Defense Peptide Frenatin 2.1S Enhances Recruitment, Activation and Tumoricidal Capacity of NK Cells. *Peptides* **2017**, *93*, 44–50. doi: 10.1016/j.peptides.2017.05.006 (University of Kragujevac)
18. Fregni, G. *et al.* Phenotypic and Functional Characteristics of Blood Natural Killer Cells from Melanoma Patients at Different Clinical Stages. *PLoS ONE* **2013**, *8(10)*. 10.1371/journal.pone.0076928 (University Paris Descartes)
19. Park, K. H. *et al.* Evaluation of NK Cell Function by Flowcytometric Measurement and Impedance Based Assay Using Real-Time Cell Electronic Sensing System. *Biomed. Res. Int*. **2013**, 210726. doi: 10.1155/2013/210726 (Catholic University of Korea)
20. Moodley, K. *et al.* Real-Time Profiling of NK Cell Killing of Human Astrocytes Using xCELLigence Technology. *J. Neurosci. Methods* **2011**, *200(2)*, 173–80. doi: 10.1016/j.jneumeth.2011.07.005 (University of Auckland)

NK cell-mediated cytolysis — adherent cell lines tested

HT1080, H460, HepG2, MCF-7, A549, HeLa, MDA-MB-231, NIH3T3, MelC, MelS, astrocyte-like cell (NT2A), RCC6, RCC4, and mesenchymal stromal cells (MSCs)

Supporting information

- **Agilent xCELLigence application note:** Label-Free Assay for NK Cell Mediated Cytolysis
- **Agilent xCELLigence application note:** Real-Time, Label-Free Measurement of Natural Killer Cell Activity and Antibody-Dependent Cell-Mediated Cytotoxicity
- **Agilent xCELLigence application note:** Evaluating Functional Potency of Immunotherapies Targeting Liquid Tumors

Oncolytic viruses

Oncolytic virotherapy is a promising cancer treatment that uses a replication-competent virus to selectively infect cancer cells, cause cytotoxicity, and generate antitumor immunity. This approach has seen major advances in recent years using wildtype (WT) and genetically engineered viruses.

Analyzing cancer cell killing with high sensitivity and without the need for labels/modifications, the xCELLigence RTCA instruments allow the interaction between viruses and target cells to be studied under conditions that approximate human physiology more closely than other *in vitro* techniques. By monitoring target cell killing continuously, these instruments also eliminate laborious endpoints and readily yield cell killing data under many different conditions simultaneously.

Figure 16 shows the use of xCELLigence RTCA to monitor killing of A549 lung cancer cells by a chimeric adenovirus (Enadenotucirev, EnAd). This infects cells by binding to CD46 or desmoglein, which are widely expressed on many carcinoma cells. In a potency analysis, the cytotoxicity (killing kinetics) of EnAd at a range of concentrations is compared with WT adenoviruses Ad11p and Ad5. At the highest concentration (red, 500 particles per cell (PPC)), EnAd and Ad11p are seen to cause complete cell killing (cell index decreasing to zero) 36 to 48 hours after infection. However, at lower virus concentrations (0.8 to 20 PPC) EnAd is more potent than Ad11p, displaying an earlier onset of cytotoxicity and a more rapid completion of cytolysis. When compared with EnAd and Ad11p, WT Ad5 is much less efficient at killing the cancer cells, requiring five days to achieve full cell killing even at the highest virus concentration.

These data highlight the ability of xCELLigence RTCA assays to quantitatively capture differences in the potency of different oncolytic viruses.

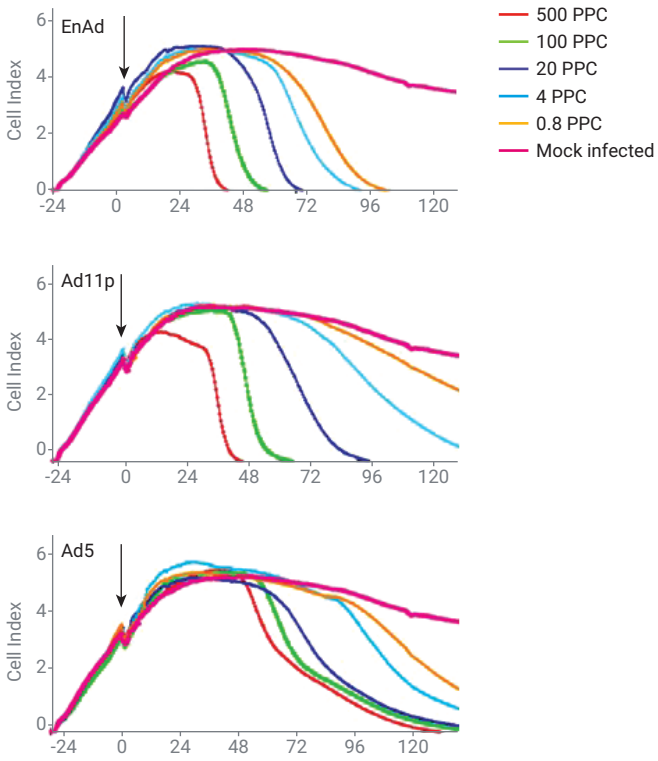

Figure 16. Killing of A549 lung cancer cells by different adenoviruses. The black arrows indicate the time of virus addition. Virus concentrations are listed as PPC. Figure adapted from: *Mol. Ther. Oncolytics* **2016** Dec 10, *4*, 18–30.[2]

Selected publications

1. Ohnesorge, P. V. *et al.* Efficacy of Oncolytic Herpes Simplex Virus T-VEC Combined with BET Inhibitors as an Innovative Therapy Approach for NUT Carcinoma. *Cancers (Basel)* **2022**, *14(11)*, 2761. doi: 10.3390/cancers14112761 (University Hospital Tuebingen)
2. Ferreira, T. *et al.* Oncolytic H-1 Parvovirus Hijacks Galectin-1 to Enter Cancer Cells. *Viruses* **2022**, *14(5)*, 1018. doi: 10.3390/v14051018 (Laboratory of Oncolytic Virus Immuno-Therapeutics, German Cancer Research Centre)
3. Tian, L. *et al.* Targeting Fc Receptor-Mediated Effects and the "Don't Eat Me" Signal with an Oncolytic Virus Expressing an Anti-CD47 Antibody to Treat Metastatic Ovarian Cancer. *Clin. Cancer Res.* **2022**, *28(1)*, 201–214. doi: 10.1158/1078-0432.CCR-21-1248 (City of Hope National Medical Center)
4. Zhang, C. *et al.* Oncolytic Adenovirus-Mediated Expression of Decorin Facilitates CAIX-Targeting CAR-T Therapy Against Renal Cell Carcinoma. *Mol. Ther. Oncolytics* **2021**, *24*, 14–25. doi: 10.1016/j.omto.2021.11.018 (Xuzhou Medical University)
5. Khalique, H. *et al.* Oncolytic Herpesvirus Expressing PD-L1 BiTE for Cancer Therapy: Exploiting Tumor Immune Suppression as an Opportunity for Targeted Immunotherapy. *J. Immunother. Cancer* **2021**, *9(4)*, e001292. doi: 10.1136/jitc-2020-001292 (University of Oxford)
6. Kulkarni, A. *et al.* Oncolytic H-1 Parvovirus Binds to Sialic Acid on Laminins for Cell Attachment and Entry. *Nat. Commun.* **2021**, *12(1)*, 3834. doi: 10.1038/s41467-021-24034-7 (Laboratory of Oncolytic Virus Immuno-Therapeutics, German Cancer Research Centre)

7. Hamdan, F. *et al.* Novel Oncolytic Adenovirus Expressing Enhanced Cross-Hybrid IgGA Fc PD-L1 Inhibitor Activates Multiple Immune Effector Populations Leading to Enhanced Tumor Killing In Vitro, In Vivo and with Patient-Derived Tumor Organoids. *J. Immunother. Cancer* **2021**, *9(8)*, e003000. doi: 10.1136/jitc-2021-003000
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9. Ammour, Y. *et al.* The Susceptibility of Human Melanoma Cells to Infection with the Leningrad-16 Vaccine Strain of Measles Virus. *Viruses* **2020**, *12(2)*, 173. doi: 10.3390/v12020173 (I.I. Mechnikov Research Institute for Vaccines and Sera)
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Supporting information

- **Agilent xCELLigence RTCA protocols:** In Vitro Functional Assay Using Real-Time Cell Analysis for Assessing Cancer Immunotherapeutic Agents
- **Agilent xCELLigence eSight video:** Imaging Oncolytic Virus-Mediated Killing on eSight
- **Agilent xCELLigence RTCA Handbook:** Vaccine and Virology Applications

T Cell-mediated cytolysis

By seeking out and destroying infected cells directly, CD8+ T lymphocytes play a critical role in adaptive immune response. Every CD8+ T cell clone expresses a unique variant of specialized receptor, the T cell receptor (TCR), that can recognize and bind to a specific antigenic peptide presented by MHC class I (MHC-I) molecules on the surface of target cells. Engaging infected or cancerous cells using MCH-1 complex causes CD8+ cells to secrete perforin and granzymes, leading to lysis of the target cell.

Tumor cells typically acquire extensive mutations in their genomes, including the genes of key regulatory and signaling proteins. When cleaved, processed, and presented by MHC molecules on the surface of antigen presenting cells, these mutated proteins can elicit a cellular immune response. This explains T lymphocytes being found inside tumors. Some cancer vaccines exploit this tumor targeting capacity of T cells by priming the cellular arm of the adaptive immune response to target cancer cells expressing proteins that are mutated or expressed at abnormal levels.

While in some contexts, quantifying the number of antigen-specific CD8+ T cells in samples using assays such as ELISpot or flow cytometry is useful, there is often a critical need to assess the functional cytotoxicity of these cells through killing assays. Measuring cytolytic activity through the chromium-51 (^{51}Cr) release assay has long been the gold standard for evaluating CD8+ T cell responses.

Figure 17 shows SKBR-3 breast cancer cells expressing the HER2/Neu protein prelabeled with ^{51}Cr. They are then co-incubated with increasing amounts of a CD8+ T cell clone. This expresses a TCR specific for an antigenic peptide of HER2/Neu and target cell killing is detected by release of ^{51}Cr into the medium. An xCELLigence RTCA system performs this assay without prelabeling the target cells. The RTCA system quantitatively detects the cytolytic activity of CD8+ T cells against the SKBR-3 target cells in a manner that depends on time and number of CD8+ T cells added (Figure 17A). Side-by-side comparison with the ^{51}Cr release assay shows that the sensitivity and dynamic range of the xCELLigence RTCA assay surpass that of ^{51}Cr (Figure 17B). The preclusion of radiolabeling and the kinetic data provided by RTCA (including the onset of cytolysis and the rate of tumor cell killing) make this assay especially attractive.

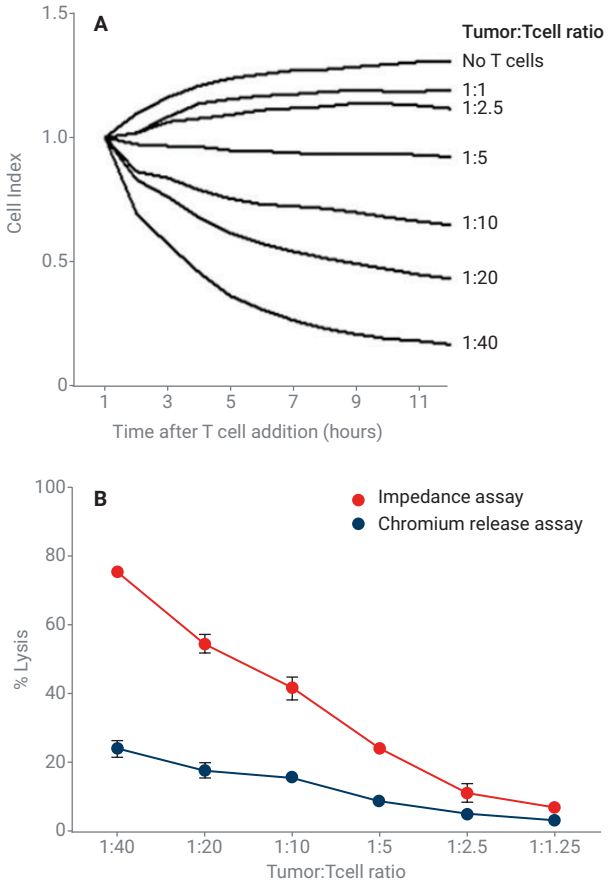

Figure 17. CD8+ T cell-mediated cytolysis of SKBR3 tumor cells. In a dose-dependent manner, CD8+ T cell addition causes the real-time impedance traces to decrease in value, indicative of a reduction in the number, size, or attachment quality of the SKBR3 tumor cells (A). Plotting the percentage of tumor cell lysis, as determined by an Agilent xCELLigence RTCA versus the standard ^{51}Cr release assay, demonstrates RTCA to be the more sensitive method (B). Figure adapted from: J. Vis. Exp. **2012** Aug 8, *(66)*, e3683.[10]

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T cell-mediated cytolysis – adherent target cells tested

TIII melanoma, SK-BR3, HCC1419, MCF-7, BT20, 15-12RM, OAW42, HLA-negative NCI-ADR-RES cells, murine 4T1 mammary gland tumor cells, BCSC (breast cancer stem cell), MSC (mesenchymal stem cell), BT20, and HCC1419

Supporting information

- **Agilent xCELLigence video:** Determining Optimal Cytotoxic Activity of Human Her2neu Specific CD8 T cells by Comparing the Cr51 Release Assay to the xCELLigence System
- **Webinar recording:** Using Impedance-Based Approaches for Measuring Antigen-Specific Cytotoxic T cell Activity
- **Webinar recording:** Modeling Anti-tumor Function of Human T Cells with xCELLigence RTCA eSight

Liquid tumor killing assays

Many peer-reviewed studies have been published over the past decade, establishing xCELLigence RTCA as a prime method of studying immunotherapies that target solid/adherent cancers. However, approximately 10% of all cancers are liquid in nature, nonadherent, and cannot be monitored directly by the standard impedance assay. Moreover, because they are readily accessible within the blood stream and are not confounded by the microenvironment complexities/heterogeneities associated with solid tumors, liquid cancers are prominent immunotherapy targets. To help accelerate research in this area, Agilent has developed xCELLigence RTCA immunotherapy kits that enable impedance-based killing assays to be performed on liquid tumor targets. Five kits are available, enabling either B cell lines or the K562 myelogenous leukemia line to be used as targets. In these assays, the wells of E-Plates are precoated with anti-CD40 or anti-CD19 (for B cells), or anti-CD29 or anti-CD71 antibody (for K562 cells), as well as anti-CD9 antibody (for NALM6, RPMI8226 cells). This enables these cells to be immobilized on the plate bottom before treatment with effector cells, antibodies, small molecules, and more.

Figure 19 illustrates the utility of the xCELLigence RTCA immunotherapy kit for B cell killing (anti-CD40) assays. Whereas antibody-immobilized B cells generate a robust impedance signal and proliferate to the point of confluence (resulting in a plateaued impedance signal), the growth of untethered B cells is essentially undetectable (Figures 18A and 18B). With or without anti-CD40 coating of the wells, effector cells such as the NK-92 cells used here produce minimal signal on their own (Figure 18B). Addition of NK-92 cells on top of immobilized B cells results in target cell death in a dose-dependent manner (Figure 18C). Killing is easily detected even at low effector:target ratios. This sensitivity greatly exceeds that of traditional release assays which require high effector:target ratios that are not physiologically relevant. The tethering and killing behaviors shown in Figures 18B and 18C have been observed in all three of the B cell lymphoma lines tested (Daudi, Raji, and Ramos), for multiple effector cell types (NK, T, and CART), and for combination therapies (CART + checkpoint inhibitors). Experiments looking at killing of human blood-derived B cells by the effector cells isolated from the same person are in progress.

An important question is whether the physical immobilization of B cells through antibody tethering affects the efficiency with which they are killed. To assess this, side-by-side four-hour assays are performed for NK-92 cell-mediated killing of Raji B cells that are immobilized (analyzed by xCELLigence RTCA) or in suspension (analyzed by flow cytometry). As shown in Figure 18D, the killing trends observed by these two methods show high correlation, with the magnitude of %cytolysis varying minimally. This is consistent with the large number of publications showing that xCELLigence data consistently recapitulate data obtained by traditional assays.

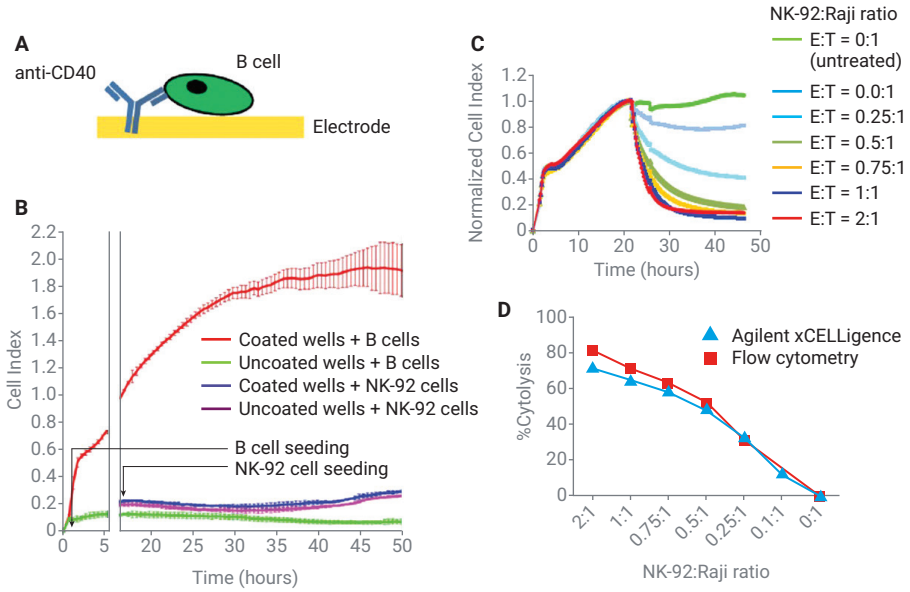

Figure 18. The Agilent xCELLigence immunotherapy kit for monitoring B cell killing. (A) Precoating the wells of Agilent E-Plates with B cell-specific antibody (anti-CD40) enables B cells to proliferate on, and be detected by, the sensors. (B) Controls showing the selective proliferation of Daudi B cells on electrodes coated with anti-CD40 antibody. As expected, with or without anti-CD40 coating nonadherent NK-92 effector cells produce minimal signal. Error bars are standard deviation. (C) The efficiency with which Raji B cells are killed depends on the number of NK-92 cells added per well. (D) The impact of B cell immobilization on killing efficiency. Raji B cells, either immobilized by antibody or in suspension, were treated with different numbers of NK-92 cells. %Cytolysis was determined after four hours of treatment by xCELLigence (tethered) or flow cytometry (in suspension).

In a second example of liquid tumor cell killing, Figures 19A and 19B show the destruction of K562 cells (tethered to E-Plate well bottoms using anti-CD29 antibody) by NK-92 cells. As expected, K562 killing increases as a function of time and effector cell concentration. Similar to the B cell killing assay, the destruction of K562 cells is detectable even at low effector:target ratios.

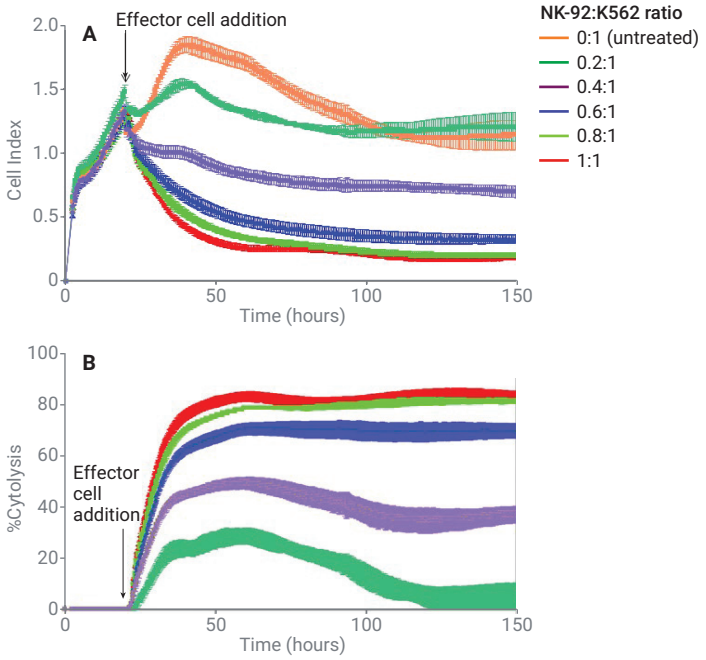

Figure 19. The Agilent xCELLigence immunotherapy kit for monitoring K562 cell killing. (A) K562 cells, immobilized on the bottom of Agilent E-Plate wells by anti-CD29 antibody, are destroyed by NK-92 cells in a time- and dose-dependent manner. (B) Data from (A) replotted as %cytolysis as a function of time. Error bars in both are standard deviation.

The liquid tumor killing assays described here are being used in industrial and academic labs for evaluating/optimizing combination therapies. They are also used for developing adoptive cell therapies and engineering antibodies. Beyond the arena of R&D, these liquid tumor killing assays may be used for functional validation/quality control of manufactured immuno-oncology therapies.

Selected publications

1. Zhang, C. *et al.* Novel CD19 Chimeric Antigen Receptor T Cells Manufactured Next-Day for Acute Lymphoblastic Leukemia. *Blood Cancer J.* **2022**, *12(6)*, 96. doi: 10.1038/s41408-022-00688-4 (Xinqiao Hospital, Army Medical University)
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3. Kararoudi, M. N. *et al.* Optimization and Validation of CAR Transduction into Human Primary NK cells Using CRISPR and AAV. *Cell Rep. Methods* **2022**, *2(6)*, 100236. doi: 10.1016/j.crmeth.2022.100236 (Nationwide Children's Hospital, Ohio State University)
4. Fousek, K. *et al.* CAR T-Cells that Target Acute B-Lineage Leukemia Irrespective of CD19 Expression. *Leukemia* **2021**, *35(1)*, 75–89. doi: 10.1038/s41375-020-0792-2 (Children's Hospital Los Angeles; University of Southern California Keck School of Medicine)

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9. Berahovich, R. *et al.* Hypoxia Selectively Impairs CAR-T Cells In Vitro. *Cancers (Basel)* **2019**, *11(5)*, 602. doi: 10.3390/cancers11050602 (ProMab Biotechnologies)
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12. Cerignoli, F. *et al.* In Vitro Immunotherapy Potency Assays Using Real-Time Cell Analysis. *PLoS One* **2018**, *13(3)*, e0193498. doi: 10.1371/journal.pone.0193498 (ACEA Biosciences)

Supporting information

- **Agilent xCELLigence application note:** Evaluating Functional Potency of Immunotherapies Targeting Liquid Tumors
- **Agilent xCELLigence brochure:** Agilent xCELLigence Immunotherapy Kits: Monitor liquid tumor cell killing in real time

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RA44903.6900925926

Published in the USA, January 28, 2023
5994-1303EN

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