

Cancer Immunotherapy

Agilent xCELLigence RTCA handbook

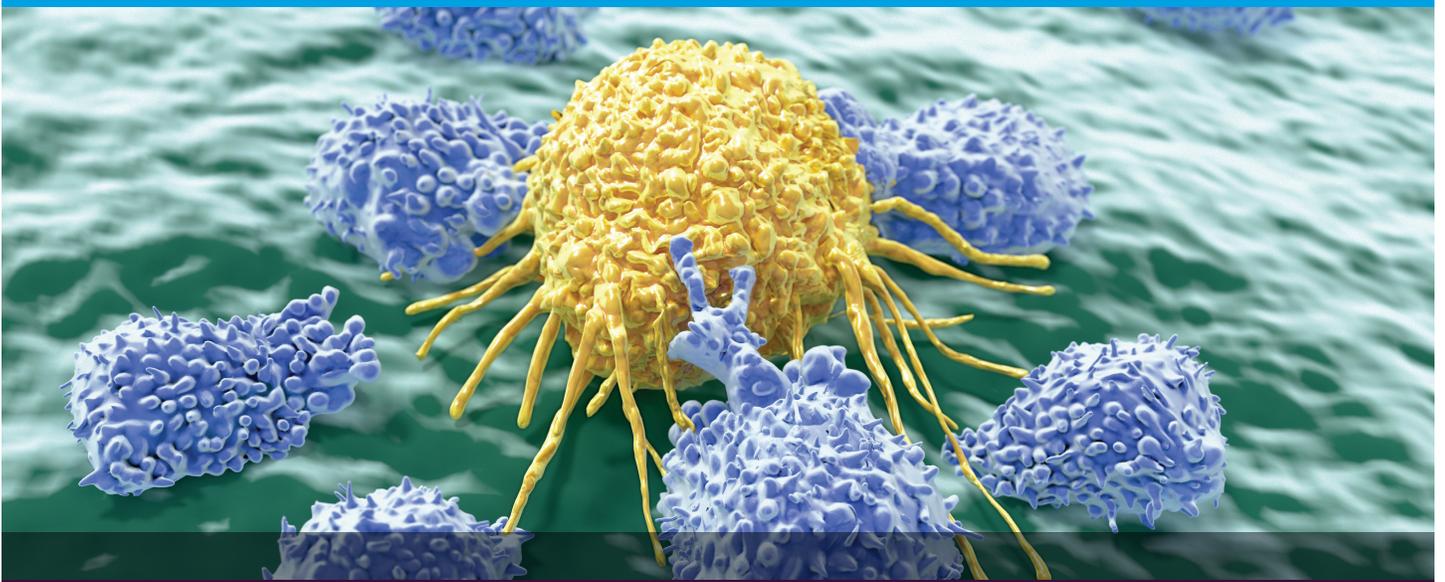


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What is cancer immunotherapy?

Cancer immunotherapy consists of multiple approaches that focus on harnessing and enhancing the innate powers of the immune system to fight cancer. Cancer immunotherapy is currently viewed as one of the most promising cancer treatments. The 2018 Nobel Prize in Medicine was awarded to two researchers in the field and 12 cancer immunotherapies have been approved in recent years.

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 cancer individual immune cells, engineering them (through genetic manipulation or peptide/adjuvant stimulation) to recognize and kill cancer cells, growing large numbers of these engineered cells, and reintroducing them into the same individual.

What are the different ways the immune system can be harnessed to target tumors?

Immune cell-mediated tumor cell killing can involve the 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

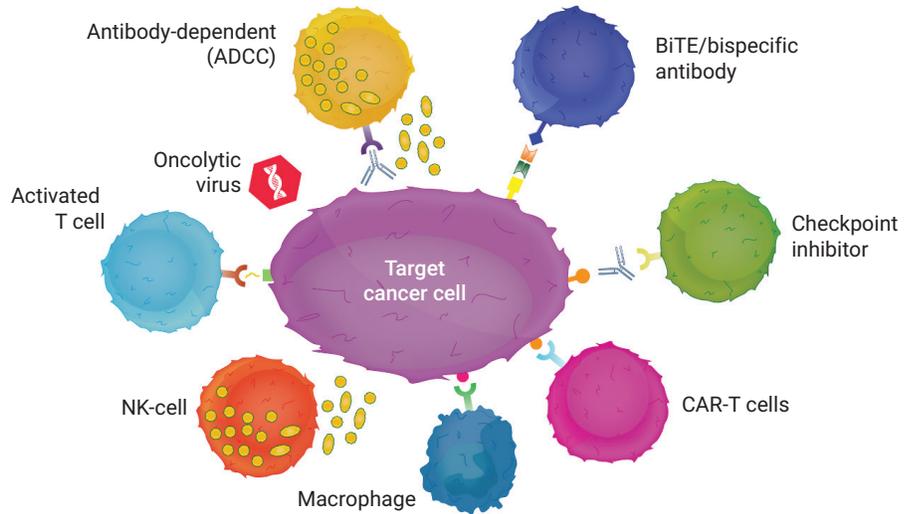


Figure 1. Various tumor-targeting schemes.

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 >1,800 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 preclusion 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.

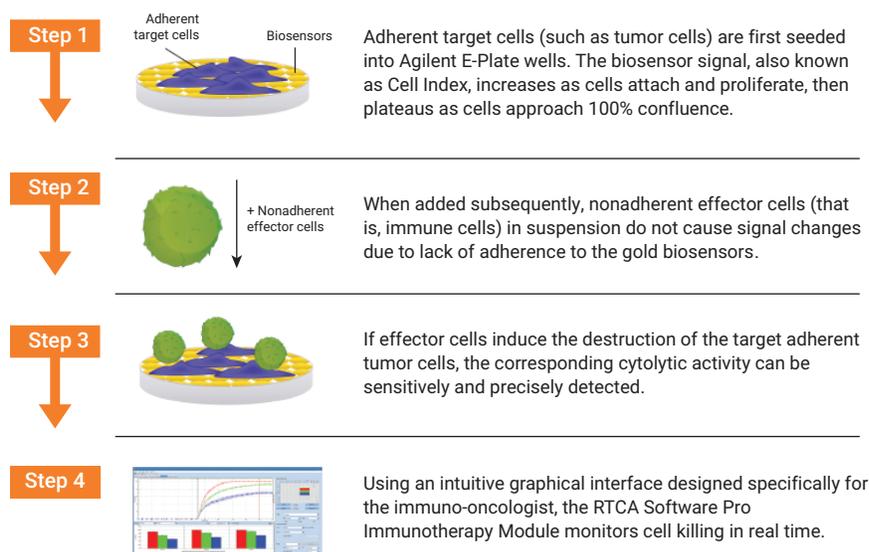


Figure 2. Overview of the Agilent xCELLigence RTCA assay.

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 into 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.

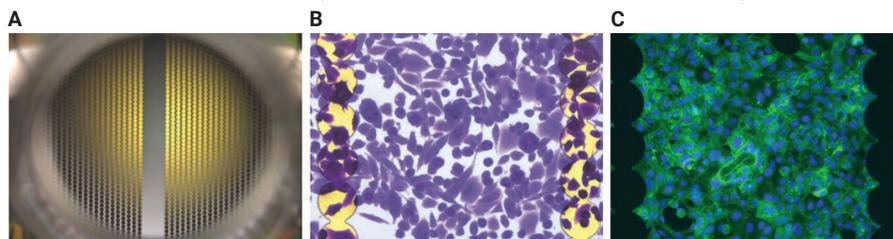


Figure 3. Biosensors measure cellular impedance on Agilent E-Plates. (A) Photograph of a single well in an E-Plate. Though cells can also be visualized on the gold biosensor surfaces, the region in the middle of the well facilitates microscopic imaging. (B) Crystal violet-stained human cells, as viewed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

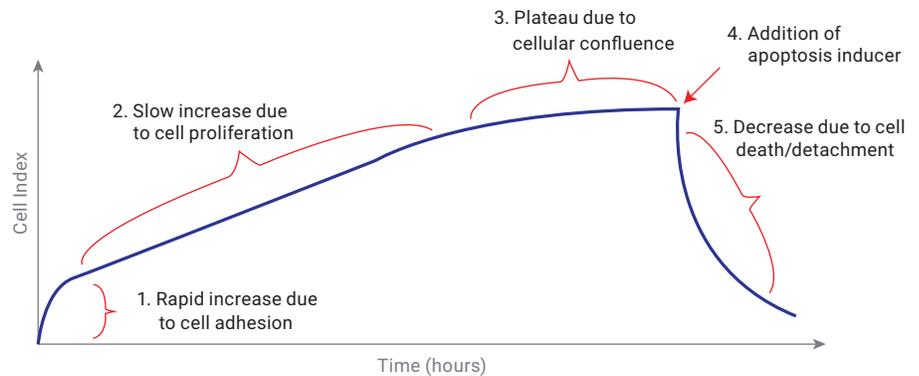


Figure 4. Generic real-time impedance trace for setting up and running an apoptosis assay. Each phase of the impedance trace, and the cellular behavior it arises from, is explained in the text.

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 (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 seven xCELLigence RTCA instruments, those best suited for immunotherapy assays are the dual purpose (DP), single plate (SP), multiple plates (MP), and high throughput (HT) models (Table 1). While each of these instruments monitors cell number, cell size, and cell-substrate attachment quality through cellular impedance in an identical manner, they differ from one another in their 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. 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 for maximizing 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 cytotoxicity (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 cytotoxicity (ADCC) and is the basis of many monoclonal antibody therapies.

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

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.

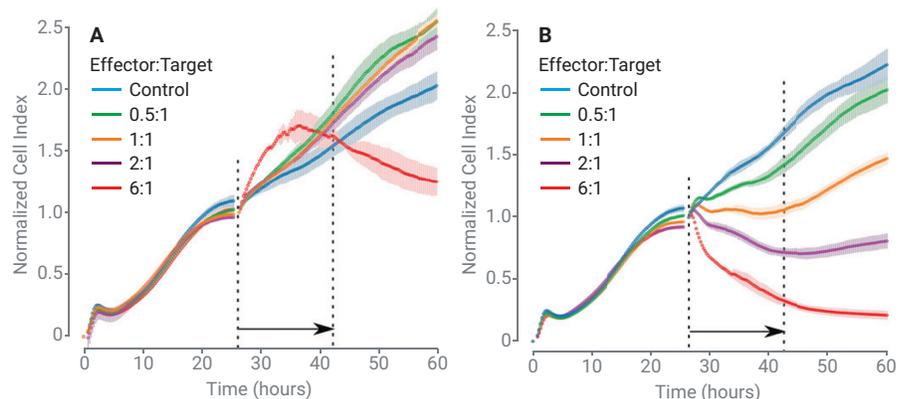


Figure 6. CI values are proportionally reduced with increasing effector-to-target (E:T) ratios in the presence of trastuzumab. BT474 clone five cells were maintained for 26 hours, then treated with media alone (control) or with media plus mononuclear cells (MNCs) isolated from human blood (A). Cells 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

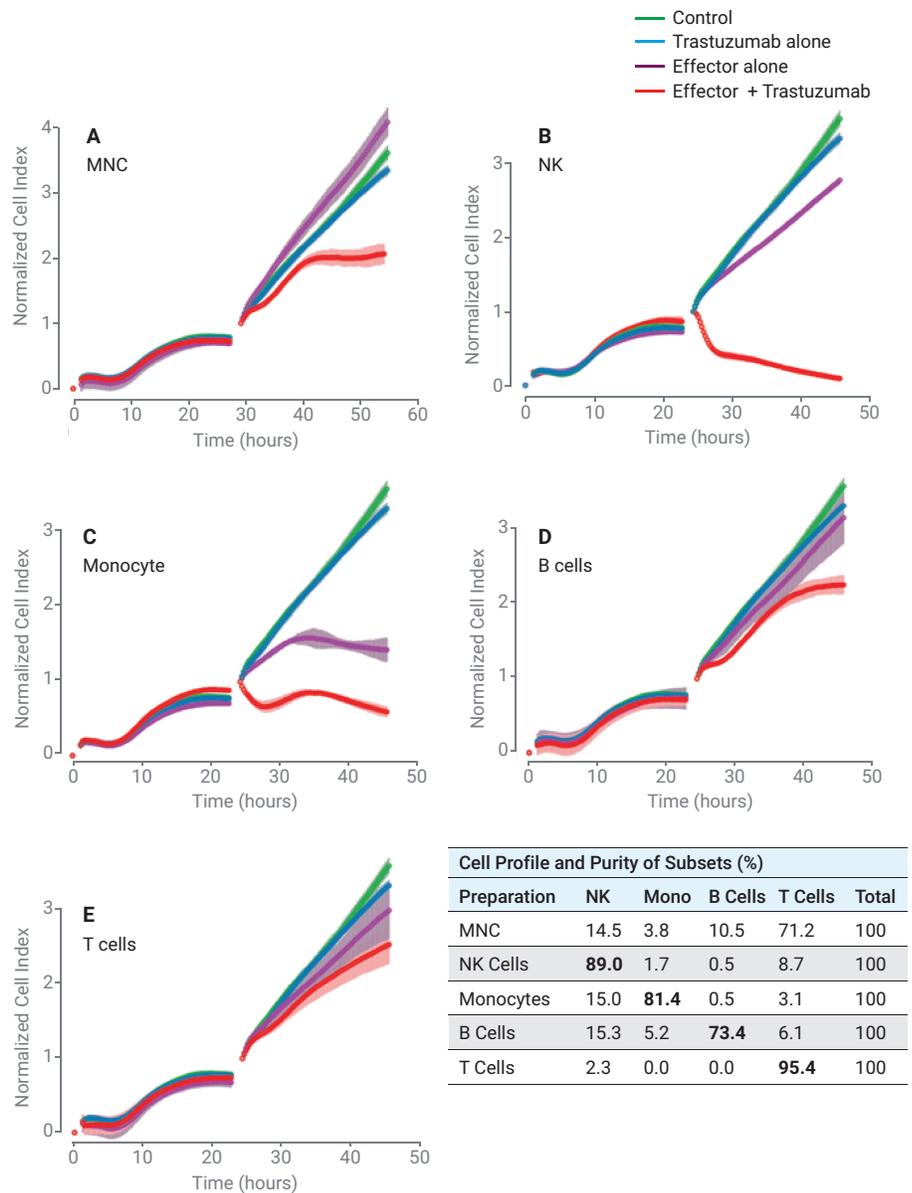


Figure 7. Natural killer cells exhibit the greatest ADCC activity among subpopulations of mononuclear cells. MNCs were tested for ADCC killing effect (A) or were separated into subpopulations, then tested. (B) NK cells, (C) monocytes, (D) B cells, (E) T cells. Green lines represent the control, blue shows 0.1 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$ 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

Bispecific T cell engagers (BiTEs) and bispecific antibodies

The therapeutic efficacy of the ADCC technique is mitigated by some immune cells expressing the CD16 antibody receptor, but not all. 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 that simultaneously (1) bind to specific antigens on the surface of tumor cells, and (2) tether and activate cytotoxic and helper T cells by binding the CD3 receptor that is 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 the utility of xCELLigence RTCA for characterizing BiTEs through the killing of adherent PC3 prostate cancer cells by PBMCs. This is studied in the presence of a BiTE that targets the EpCAM receptor (which is expressed on the surface of most cancer cells of epithelial origin, including PC3 cells).

These data show that in the absence of the BiTE, the 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.

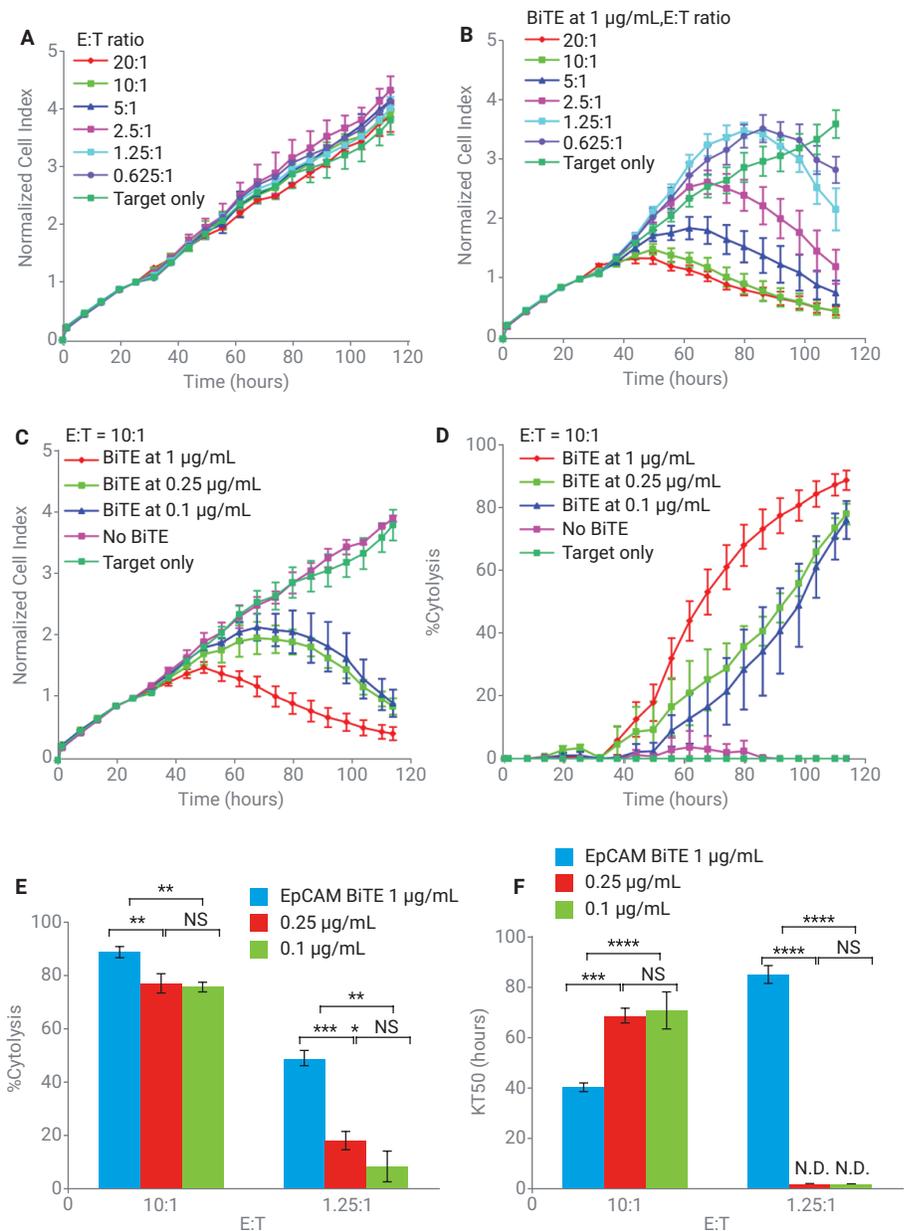


Figure 8. Impedance assessment of BiTE-mediated cytotoxicity. (A) Normalized CI plot for PC3 target cells incubated with PBMCs at different E:T ratios without the BiTE. (B) Same E:T ratios as (A) but with 1 $\mu\text{g}/\text{mL}$ anti-EpCAM/CD3 BiTE. (C) At E:T ratio of 10:1, different BiTE concentrations 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 Felix Bohne, Ph.D. (German Research Center for Environmental Health)
- **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

Checkpoint Inhibitors

Using xCELLigence to study checkpoint inhibitors

By disrupting the 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 you to efficiently answer your questions under conditions of maximal physiological relevance.

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 xCELLigence RTCA being used to monitor the impact of an anti-PD-1 antibody on the killing of prostate cancer PC3 cells by peripheral blood mononuclear cells (PBMCs). 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 10A, by themselves PBMCs display a modest capacity for killing PC3 cells (blue trace), but this 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 10B), 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.

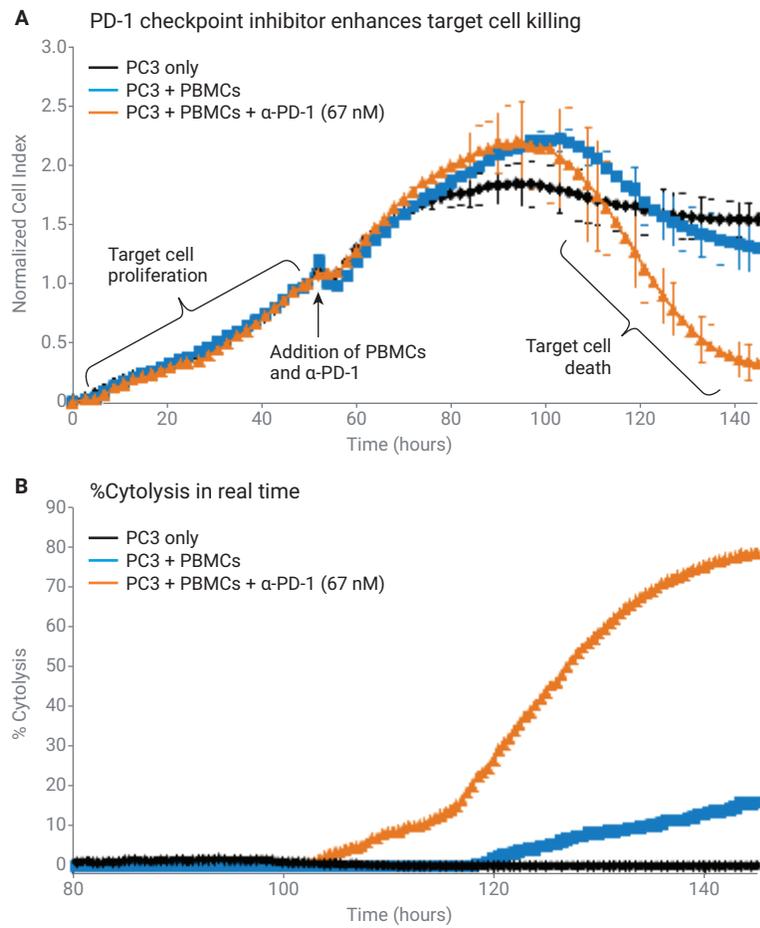


Figure 9. Using Agilent xCELLigence to monitor the impact of anti-PD-1 antibody on the killing of prostate cancer PC3 cells by PBMCs. PC3 cells were grown on an Agilent E-Plate. PBMC effector cells were added with or without the checkpoint inhibitor anti-PD-1 antibody. Impedance was monitored, and time-dependent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

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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 that a combination of PD-1 and CTLA-4 checkpoint inhibitors have on the killing of prostate cancer PC3 cells by PBMCs. 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 the target cells killing. However, adding anti-CTLA-4 along with anti-PD-1 promoted target cell killing in a dose-dependent manner (green and pink traces).

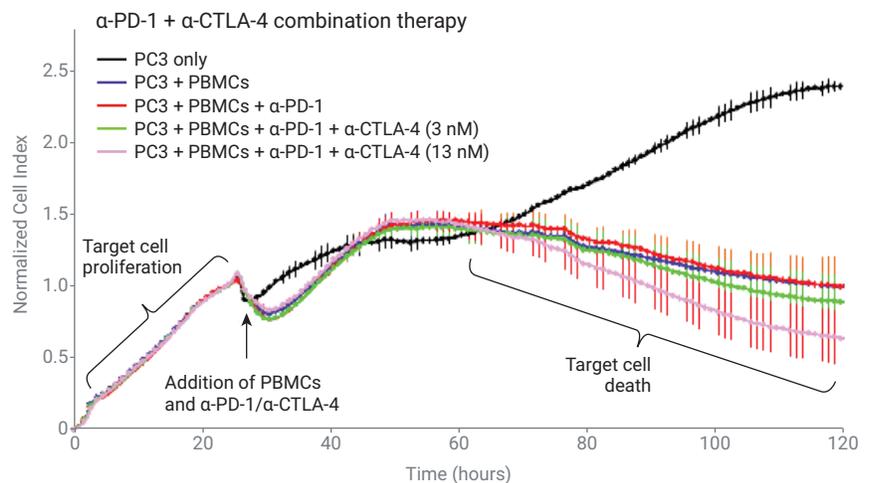


Figure 10. Anti-PD-1 and anti-CTLA-4 antibodies combination therapy. By analyzing cancer cell killing with high sensitivity and without the need for labels/modifications, the Agilent xCELLigence RTCA instruments allow effector and target cells to be studied under conditions that approximate human physiology 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.

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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.

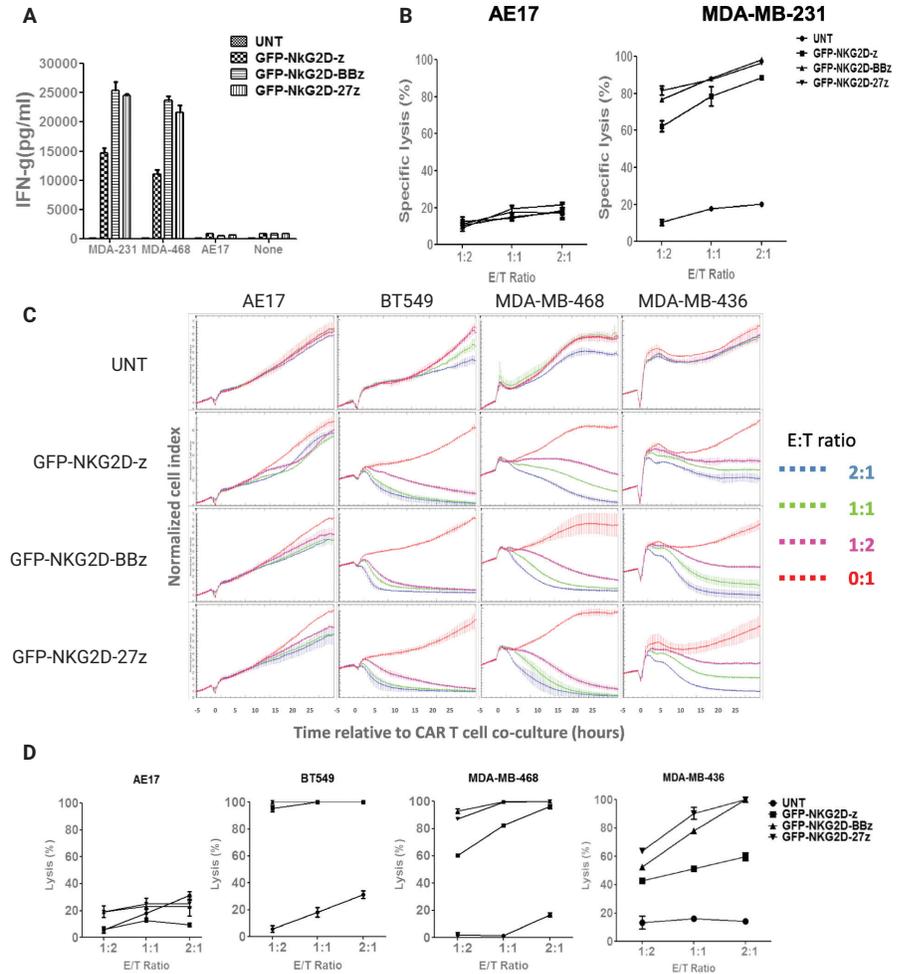


Figure 11. Recognition of human TNBC cells by NKG2D CAR T cells *in vitro*. (C) Normalized CI plot for target cells (AE17, BT549, MDA-MB-436, and MDA-MB-468) incubated with UNT or NKG2D CAR T cells at different E:T ratios for 24 hours. When seeded alone, target cells adhere to the plate and proliferate, increasing the CI readout (red lines). When T cells are added to target cells, NKG2D 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 \pm 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

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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 IL13Ra2 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.

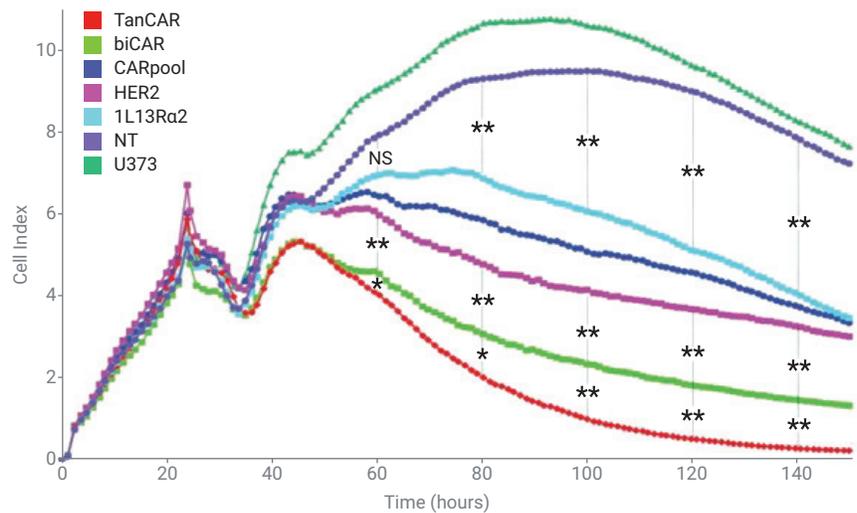


Figure 12. Using Agilent xCELLigence to monitor killing of the glioblastoma cell line U373 by CAR-T cells targeting either one or both of the antigens HER2 and IL13Ra2. In the figure legend: U373 = target cell line alone; NT = target cells treated with nontransfected T cells (not expressing a CAR); IL13Ra2 = target cells treated with T cells expressing a single CAR targeting IL13Ra2; Her2 = target cells treated with T cells expressing a single CAR targeting Her2; see the text for descriptions of CARpool, biCAR, and TanCAR.

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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 in Gene Editing and Rapid Assessment with Real Time Live Cell Analysis, Mark C Johnson (Precision Biosciences) and Cynthia Bamdad (Minerva Biotechnologies)

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, TGF β , 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.

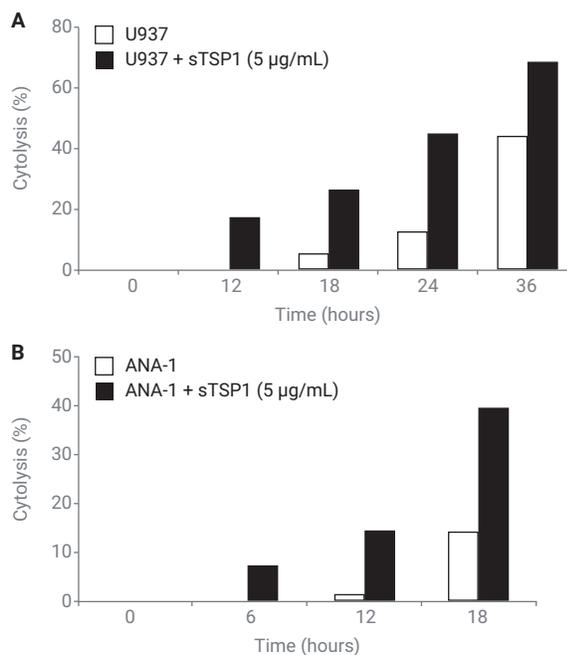


Figure 13. Secreted glycoprotein TSP1 increases macrophage/monocyte-mediated tumoricidal activity. MDA-MB-231 breast adenocarcinoma target cells were seeded in Agilent E-Plates and incubated for up to 24 hours. Differentiated U937 human monocytes (A) or activated ANA-1 murine macrophages (B) were then 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.

Reprinted by permission from the American Association for Cancer Research: Martin-Manso G, et al. Thrombospondin 1 Promotes Tumor Macrophage Recruitment and Enhances Tumor Cell Cytotoxicity of Differentiated U937 cells. *Cancer Res.* **2008** Sep 1, *68(17)*, 7090–9. DOI: 10.1158/0008-5472.CAN-08-0643

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Macrophage-mediated phagocytosis – adherent target cells tested

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

NK cell-mediated cytotoxicity

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 to 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 cytotoxicity

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.

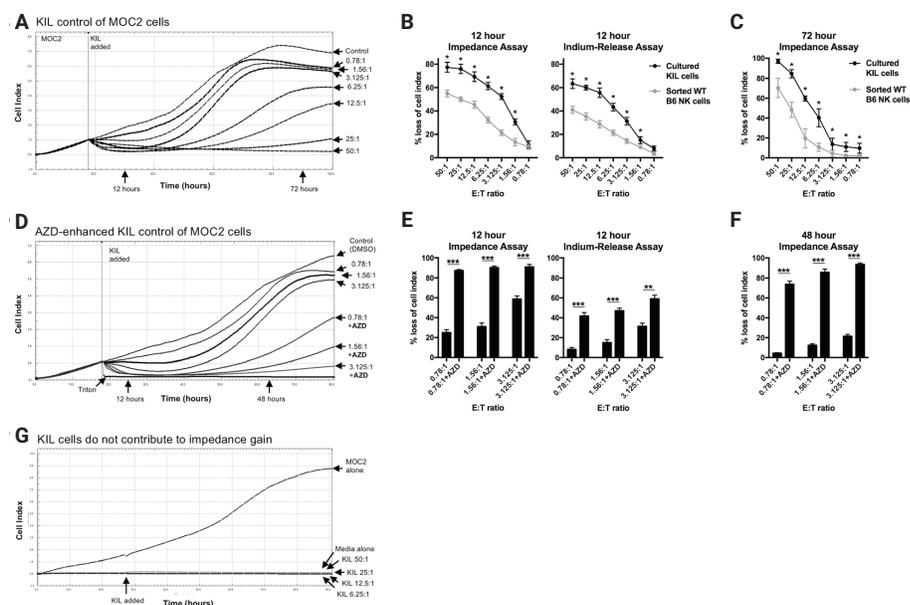


Figure 14. Target cell killing by KIL at low E:T ratios is enhanced following WEE1 kinase inhibition. (A) Loss of CI of MOC2 oral carcinoma cells following the addition of KIL at increasing E:T ratios was measured through real-time impedance analysis. The vertical line at 18 hours indicates the time 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.

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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:

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

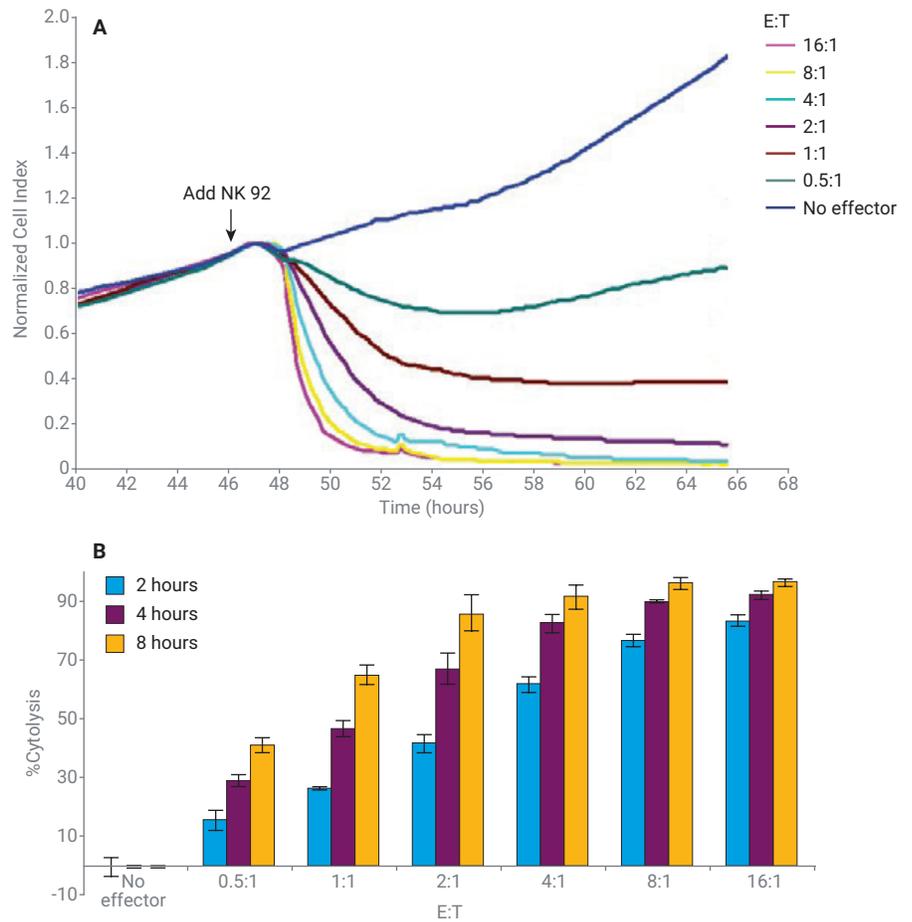


Figure 15. Real-time monitoring of NK-92 cell-mediated cytolysis of MCF7 breast cancer cells. Adherent MCF7 target cells were grown in multiple wells of an Agilent E-Plate. Different quantities of NK-92 cells were added to each well, and impedance was monitored continuously for the next ~20 hours (A). 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*.

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NK cell-mediated cytotoxicity – adherent cell lines tested

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

Supporting information

- **Agilent xCELLigence application note:** Label-Free Assay for NK Cell-Mediated Cytotoxicity
- **Agilent xCELLigence application note:** Real-Time, Label-Free Measurement of Natural Killer Cell Activity and Antibody-Dependent Cell-Mediated Cytotoxicity

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.

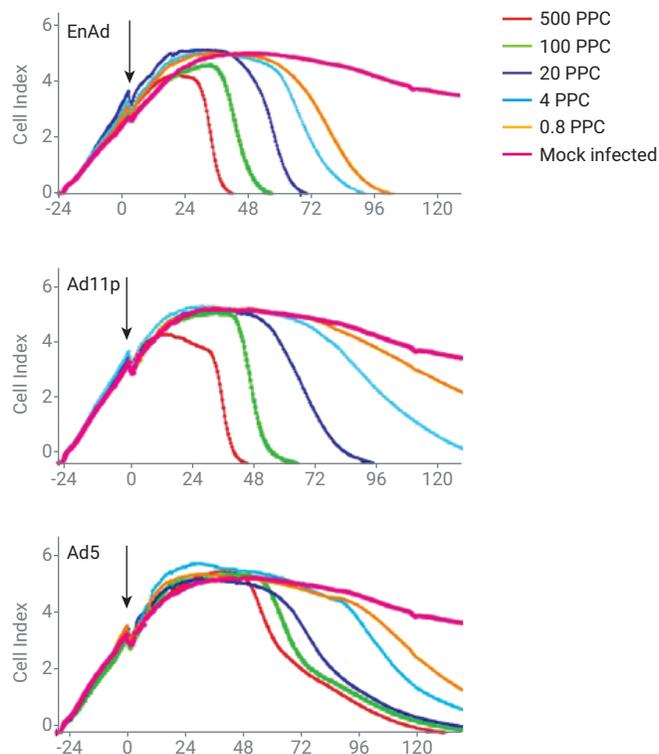


Figure 16. Killing of A549 lung cancer cells by different adenoviruses. The black arrows indicate the time of virus addition. Virus concentrations are listed as PPC. Figure adapted from: *Mol. Ther. Oncolytics* **2016** Dec 10, 4, 18–30.²

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T Cell-mediated cytotoxicity

By seeking out and destroying infected cells directly, the CD8+ T lymphocytes play a critical role in the adaptive immune response. Every CD8+ T cell clone expresses a unique variant of a 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 MHC-I 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 clone 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 18A). Side-by-side comparison with the ^{51}Cr release assay shows that the sensitivity and dynamic range of the xCELLigence RTCA assay surpasses that of ^{51}Cr (Figure 18B). 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.

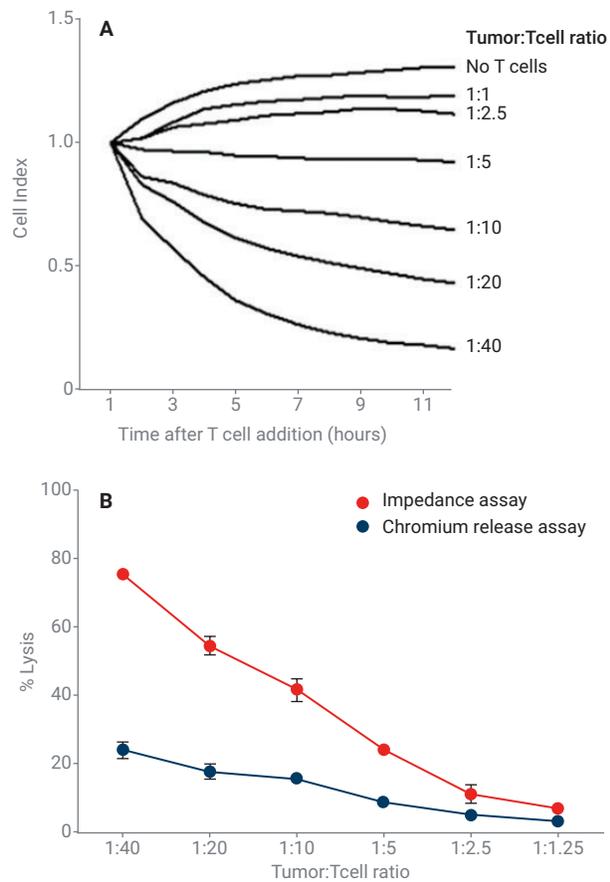


Figure 17. CD8+ T cell-mediated cytotoxicity of SKBR3 tumor cells. In a dose-dependent manner, CD8+ T cell addition causes the real-time impedance traces to decrease in value, indicative of a reduction in the number, size, or attachment quality of the SKBR3 tumor cells (A). Plotting the percentage of 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

- **JOVE video protocol:** 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 Keith L. Knutson, Ph.D. (Vaccine & Gene Therapy Institute of Florida)

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.

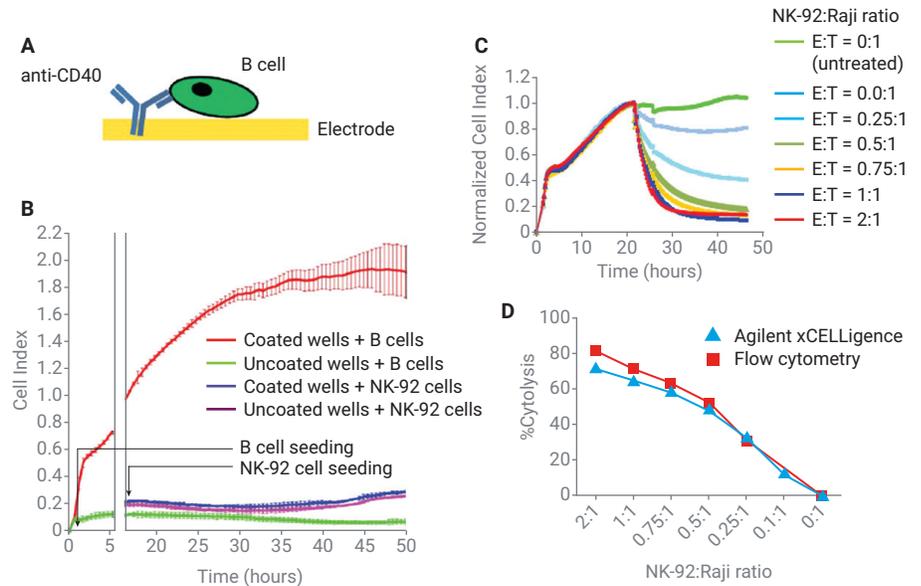


Figure 18. The Agilent xCELLigence immunotherapy kit for monitoring B cell killing. (A) Precoating the wells of Agilent E-Plates with B cell-specific antibody (anti-CD40) enables B cells to proliferate on, and be detected by, the sensors. (B) Controls showing the selective proliferation of Daudi B cells 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.

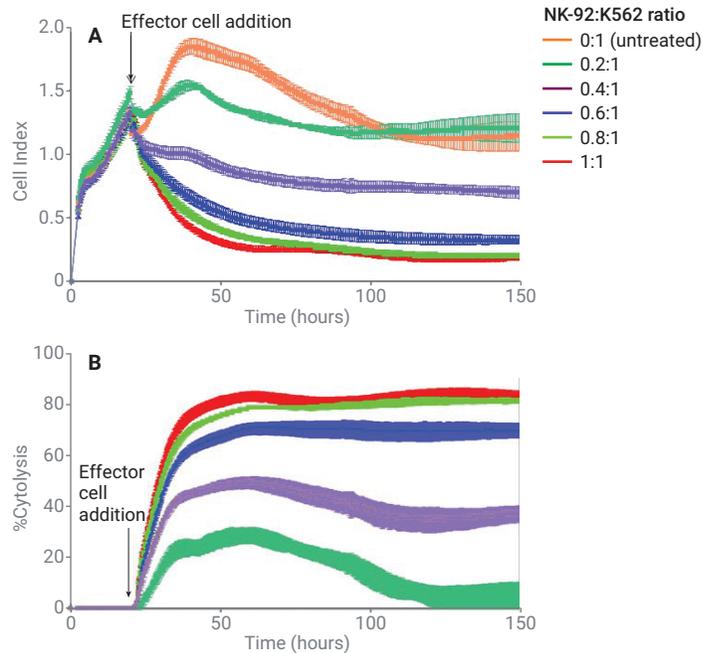


Figure 19. The Agilent xCELLigence immunotherapy kit for monitoring K562 cell killing. (A) K562 cells, immobilized on the bottom of Agilent E-Plate wells by anti-CD29 antibody, are destroyed by NK-92 cells in a time- and dose-dependent manner. (B) Data from (A) replotted as %cytolysis as a function of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

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