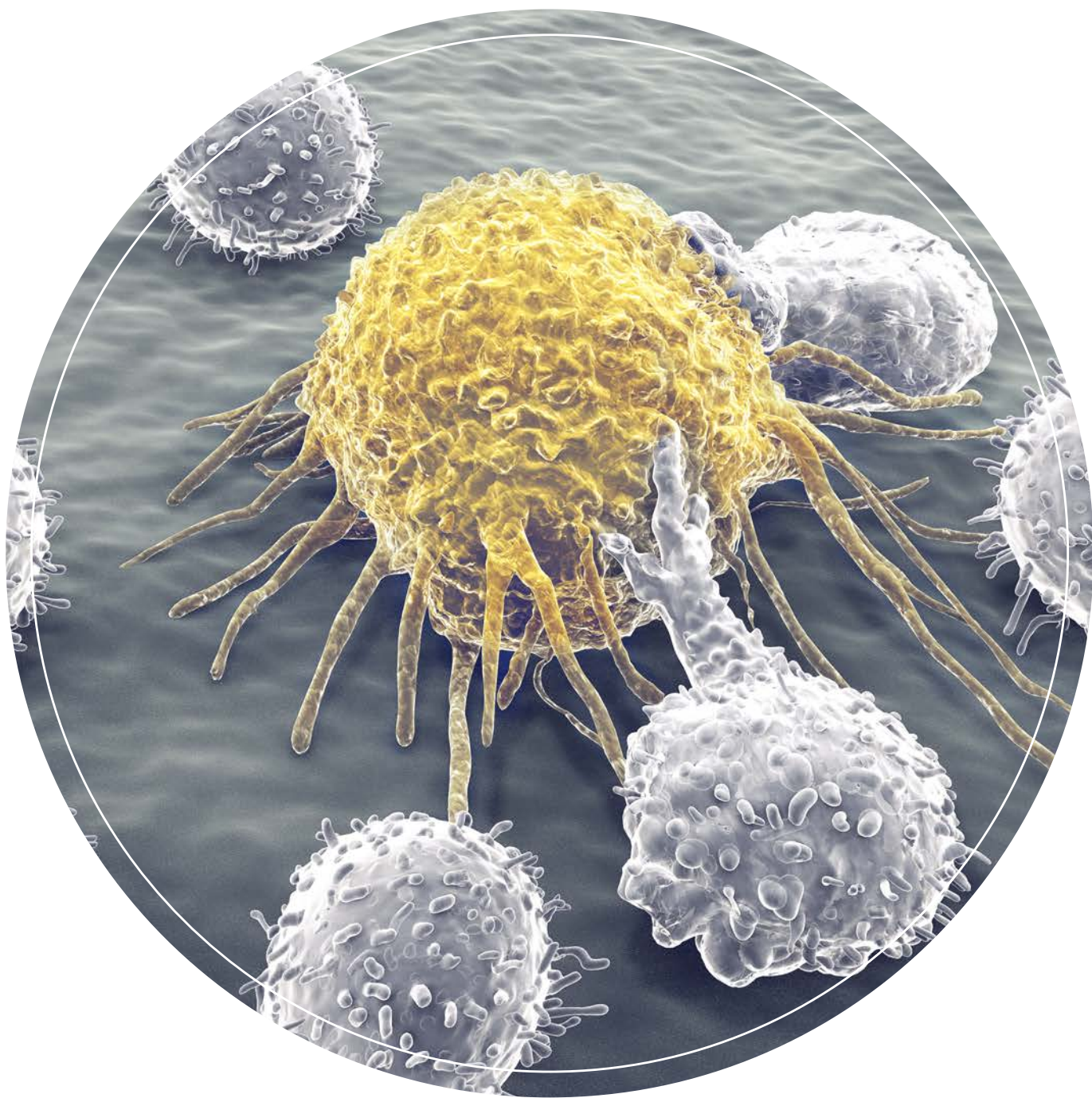


xCELLigence® RTCA Handbook

# Cancer Immunotherapy

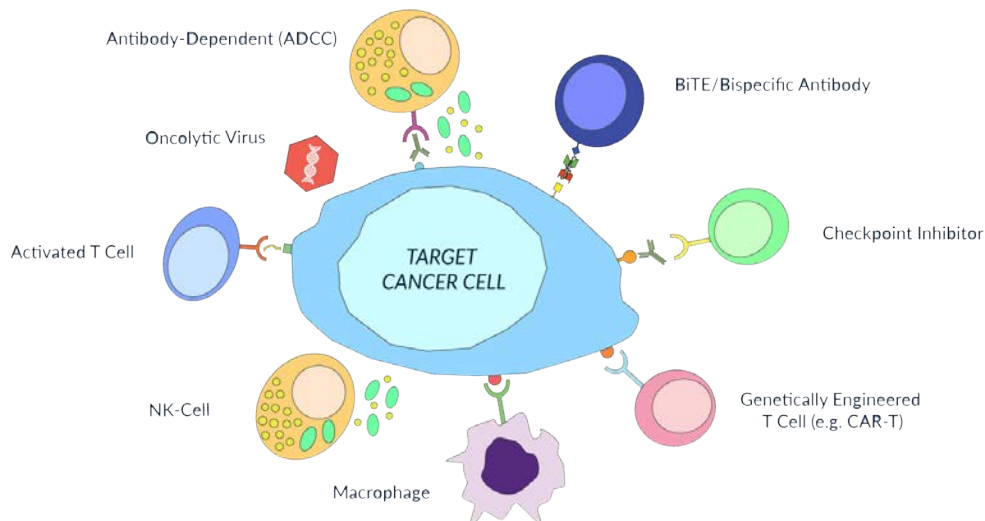


## What is Cancer Immunotherapy?

Cancer immunotherapy consists of multiple approaches that focus on harnessing and enhancing the innate powers of the immune system to fight cancer. While traditional small molecule chemotherapy continues to play a critical role in cancer treatment, immunotherapy is rapidly gaining traction; in 2014, immunotherapies constituted ~50% of the overall oncology pharmacopeia. Cancer immunotherapies can be divided into four major categories: (1) cytokines/immunomodulation agents, (2) monoclonal antibodies, (3) cell-based therapies, and (4) oncolytic viruses. Though monoclonal antibodies currently represent the largest class of commercialized cancer immunotherapies, cell-based therapies are rapidly making headway. This class of patient-specific therapies involve collecting immune cells from a cancer patient, engineering them (via genetic manipulation or peptide/adjuvant stimulation) to recognize and kill cancer cells, growing large numbers of these, and reintroducing them into the same patient.

## 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 including: (1) natural killer (NK) cells, (2) cytotoxic T cells (MHC-dependent), (3) antibodies secreted by B lymphocytes, (4) engineered antibodies such as bispecific antibodies and bispecific T cell engagers (BiTEs), (5) genetically engineered T cells targeting specific tumor antigens (e.g., CAR-T; MHC-independent), and (6) macrophage-mediated phagocytosis.

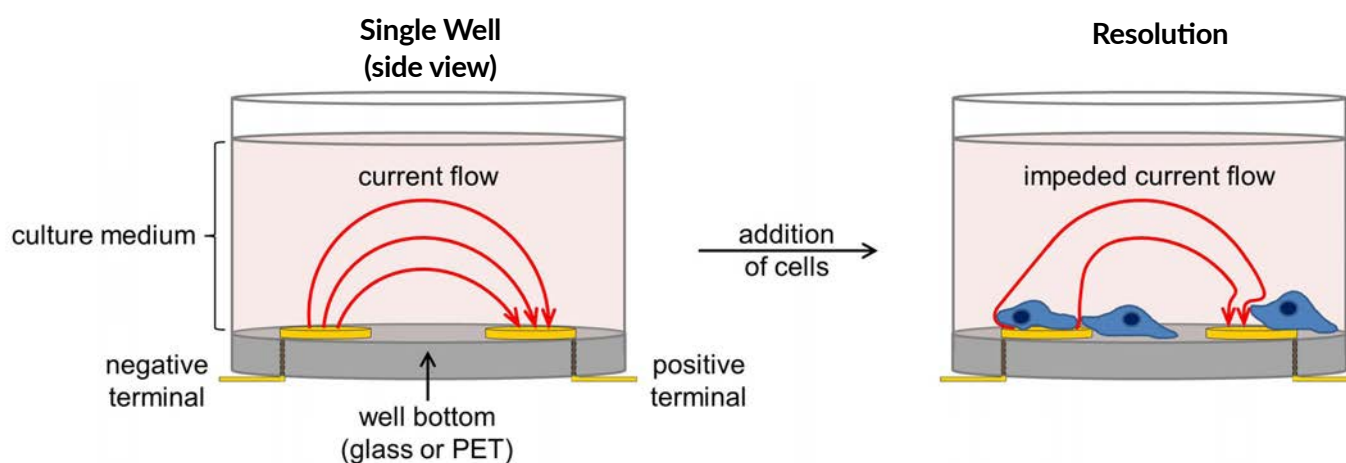


## The Need for a Novel Cancer Immunotherapy Assay

Many *in vitro* assays have been developed to screen and evaluate the efficacy of immune cell-mediated killing. The most common of these 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 <sup>51</sup>Cr) that leak into the media are then measured as an indirect readout of the damage caused by effector cells. Alternative endpoint methods include flow cytometry, ELISA-based granzyme measurement, and morphometric analyses by microscopy. While the data provided by these assays help piece together a reductionistic understanding of different facets of immune cell-mediated killing, it is important to acknowledge that the parameters being reported often do not correlate with target cell killing efficacy *in vivo*. There is an urgent need for *in vitro* assays that more accurately predict the *in vivo* behavior of therapies. The ideal assay should also provide quantitative results and be homogeneous, automated (requiring less hands-on time), and label-free.

## Cellular Impedance

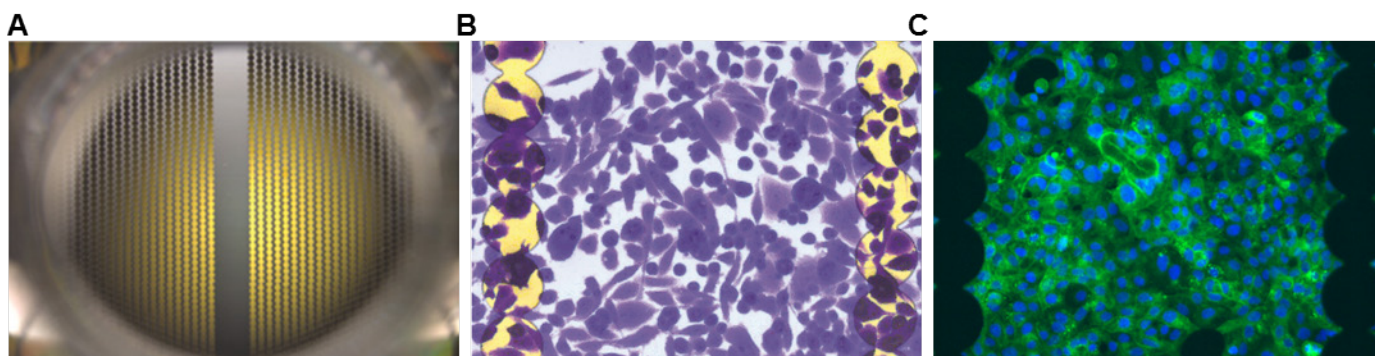
The functional unit of the cellular impedance assay that is run by the ACEA's xCELLigence® Real-Time Cell Analysis (RTCA) instrument is a set of gold microelectrodes embedded in the bottom surface of a microtiter plate well (below). When submerged in an electrically conductive solution (such as buffer or standard tissue culture medium), the application of a weak electric potential across these electrodes causes current to flow between them. Because this phenomenon is dependent upon the electrodes interacting with bulk solution, the presence of adherent cells at the electrode-solution interface impedes current flow. The magnitude of this impedance is dependent upon the number of cells, the size and shape of the cells, and the cell-substrate attachment quality. Importantly, neither the gold microelectrode surfaces nor the applied electric potential have an effect on cell health or behavior.



Overview of cellular impedance apparatus. A side view of a single well is shown before and after cells have been added. Neither the electrodes nor the cells are drawn to scale (they have been enlarged for clarity). In the absence of cells electric current flows freely through culture medium, completing the circuit between the electrodes. As cells adhere to and proliferate on the electrodes current flow is impeded, providing an extremely sensitive readout of cell number, cell size/morphology, and cell-substrate attachment quality in real time.

## E-Plates®

The gold microelectrode biosensors in each well of ACEA's electronic microtiter plates (E-Plates) cover ~75% of the bottom's surface area. Rather than the simplified electrode pair depicted in figure above, the circular electrodes in each well of an E-Plate are linked into "strands" that form an interdigitating array (below). This proprietary design enables large populations of cells to be monitored simultaneously and thereby provides exquisite sensitivity to the number of cells attached to the plate, the size/morphology of the cells, and the cell-substrate attachment quality.

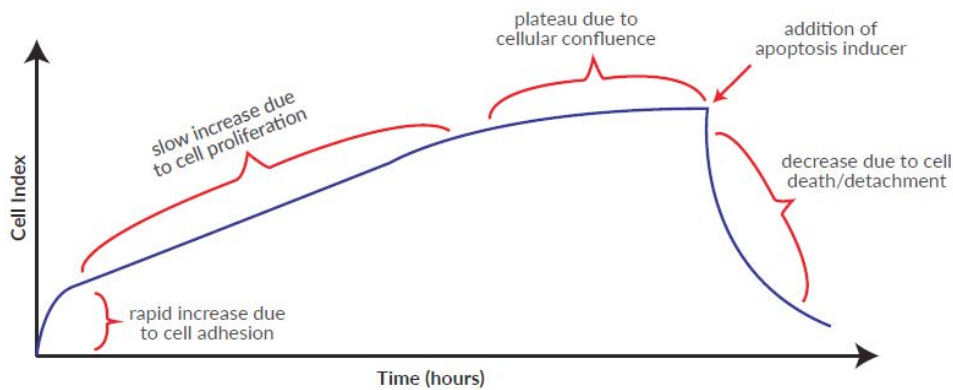


**Impedance electrodes on ACEA's E-Plates.** (A) Photograph of a single well in an E-Plate. Though cells can also be visualized on the gold electrode surfaces, the electrode-free region in the middle of the well facilitates microscopic imaging. (B) Gold electrodes and crystal violet stained human cells, as viewed in a compound microscope. (C) Immunofluorescence microscopy with gold electrodes silhouetted.



## Real-Time Impedance Traces Explained

The impedance of electric current that is caused by adherent cells is reported using a unitless parameter called Cell Index (CI), where  $CI = (\text{impedance at time point } n - \text{impedance in the absence of cells}) / (\text{nominal impedance constant})$ . The figure below provides a generic example of a real-time impedance trace throughout the course of setting up and running an apoptosis experiment. 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. If cells are sub-confluent after the initial attachment stage, they will start to proliferate, causing a gradual yet steady increase in CI. When cells reach confluency the CI value plateaus, reflecting the fact that the electrode surface area that is accessible to bulk media is no longer changing. The addition of an apoptosis inducer at this point causes a decrease in CI back down to zero. This is the result of cells rounding and then detaching from the well bottom. While this generic example involves addition of the apoptosis inducer at the point of cellular confluency, impedance-based assays are extremely flexible and can interrogate a wide variety of phenomena across the full spectrum of cell densities.



**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.

## Applications

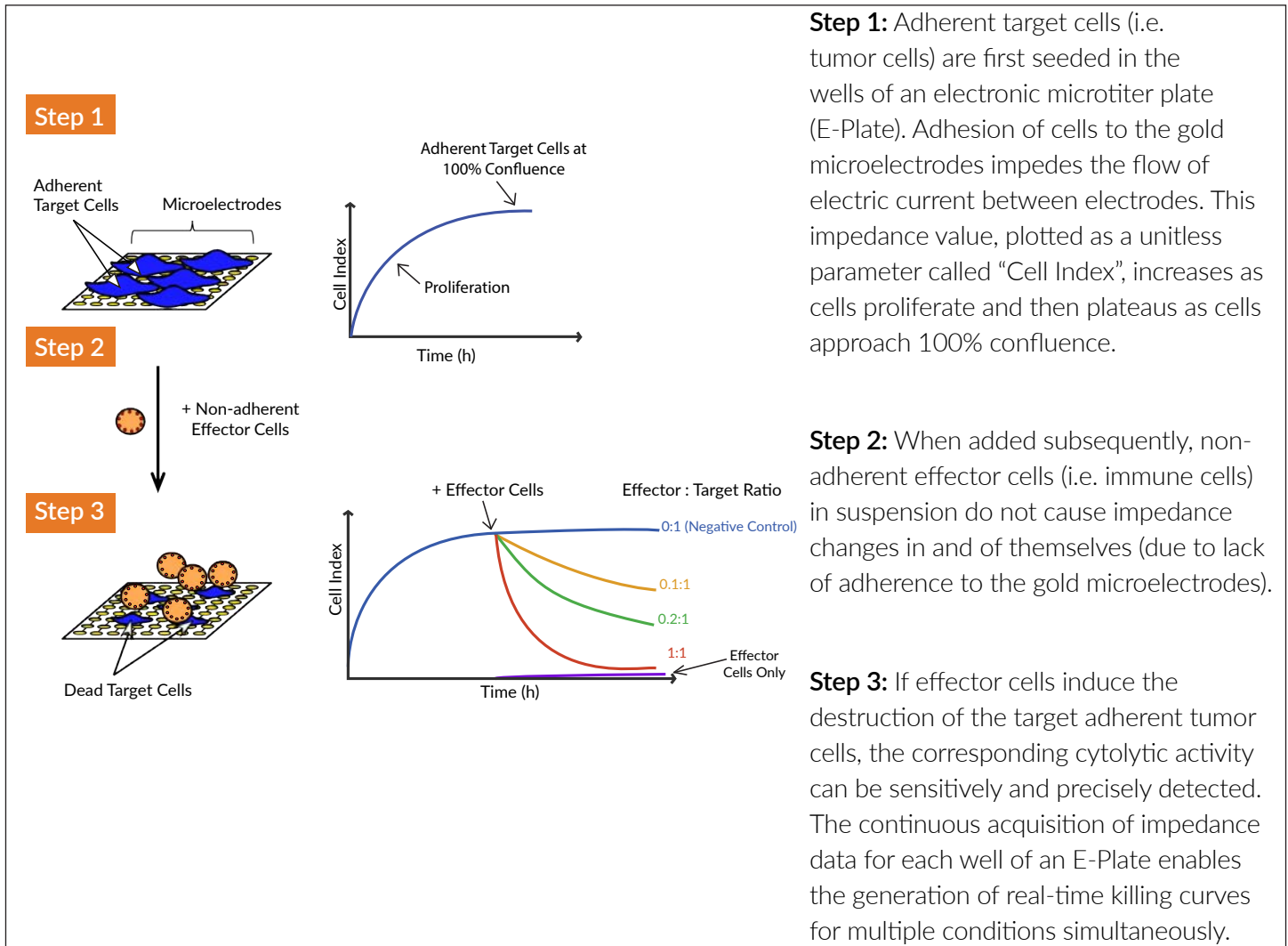
To date, thousands of xCELLigence instruments have been placed globally in labs that span everything from academia and biotech startups to contract research organizations and big pharma. This has resulted in >1400 xCELLigence publications in peer-reviewed journals.

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

- 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)
- Drug mechanism of action
- 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
- Checkpoint inhibitors

## What is the xCELLigence RTCA Assay Principle?

ACEA's xCELLigence RTCA instruments utilize gold microelectrodes embedded in the bottom of microtiter wells to non-invasively 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, importantly, kinetic measurement of cell health/behavior.



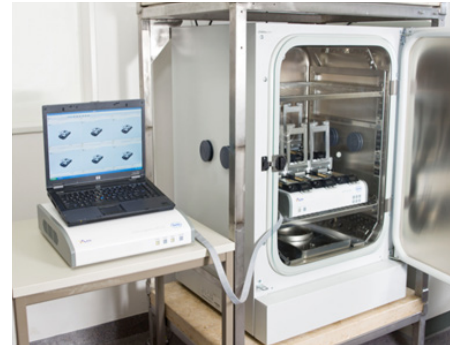
## Is Your Current *in vitro* Assay Truly Predictive of *in vivo* Outcomes?

Reap the benefits of game-changing predictivity with xCELLigence RTCA instruments:

- Label-Free: No <sup>51</sup>Cr, no luciferase, no dyes...NO PROBLEM
- Astonishingly Simple Workflow: Plate target cells, add effector cells, and start reading
  - Read an entire 96-well plate in 15 seconds
  - Run up to 6 plates independently, with no scheduling conflicts
  - Monitor target cell killing continuously from seconds to days
- Exquisite Sensitivity: Monitor target cell killing at low, physiologically relevant effector:target ratios
- Diverse Effector Cells and Molecules: TIL, NK, CART, TCR, checkpoint inhibitors, BiTEs, etc.
- Quantitative, Real-Time Kinetics: Highly accurate and highly reproducible

## xCELLigence Instruments for Immunotherapy

In order to control the temperature, humidity, and atmospheric composition of RTCA assays, the xCELLigence instruments are designed to be housed inside standard tissue culture incubators or hypoxia chambers. The instruments interface, via a cable, with analysis and control units that are housed outside the incubator (see figure to the right). User friendly software allows for real-time control and monitoring of the instrument, including real-time data display and analysis functions.



An xCELLigence RTCA instrument and its laptop control unit are housed inside and outside an incubator, respectively.

Of the seven xCELLigence RTCA instruments that are currently being produced by ACEA, those best suited for immunotherapy assays are the DP (dual purpose), SP (single plate), MP (multi plate), and HT (high throughput) models which are profiled in the table below. While each of these instruments monitors cell number, cell size, and cell-substrate attachment quality via cellular impedance in an identical manner, they differ from one another in their plate configurations/throughput. The DP model has the additional capability of quantitatively monitoring cell invasion/migration through the use of a specialized plate that functions as an electronic Boyden chamber. Finally, though the HT model can be run as a stand-alone instrument, four of these can be linked to a single control unit to provide a total of 1536 wells. HT instruments can also be integrated with a robotic liquid handler for maximizing throughput.



	Immunotherapy Applications	DP (Dual Purpose)	SP (Single Plate)	MP (Multi Plate)	HT (High Throughput)
Applicable to Both Liquid & Solid Tumor Target Cells	Cell-Mediated Cytotoxicity	✓	✓	✓	✓
	Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)	✓	✓	✓	✓
	Checkpoint Inhibitors	✓	✓	✓	✓
	Combination Therapies	✓	✓	✓	✓
	Antibody-Drug Conjugates	✓	✓	✓	✓
	Immune Cell Activation	✓	✓	✓	✓
	Cell Invasion & Migration	✓			
<b>Specs</b>					
	Format	3x16 wells	1x96 wells	6x96 wells	1x384 wells
	Maximum Throughput	48 wells	96 wells	576 wells	Up to 4x382 wells (1536 wells total)

## Applications Table of Contents

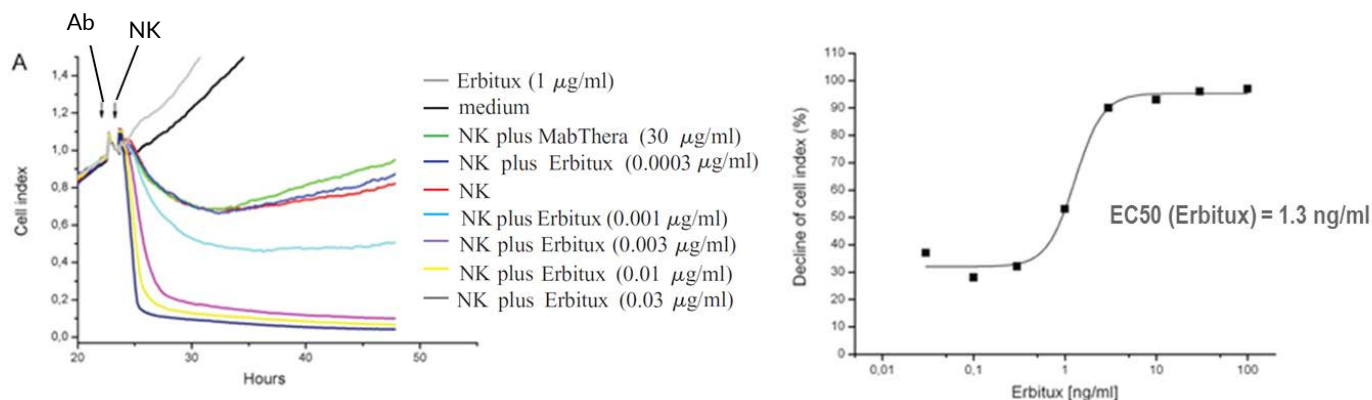
Though not exhaustive, the below table lists the different types of immunotherapy assays that can be run on xCELLigence RTCA instruments.

Applications Table of Contents	
Application Examples	Page
Antibody-Dependent Cell-Mediated Cytolysis (ADCC)	8-9
Bispecific T Cell Engagers (BiTEs) and Bispecific Antibodies	10-11
Checkpoint Inhibitors	12-13
Combination Therapy	14-15
Genetically Engineered T Cell-Mediated Cell Killing	16-17
Macrophage-Mediated Phagocytosis	18-19
NK Cell-Mediated Cytolysis	20-21
Oncolytic Viruses	22-23
T-Cell-Mediated Cytolysis	24-25
Liquid Tumor Killing	26-27

## 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. Upon encountering a pathogen, 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. Moreover, 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 current monoclonal antibody therapies.

Erbix (Cetuximab) is a therapeutic monoclonal antibody that binds specifically to the human epidermal growth factor receptor (EGFR) that is overexpressed in many tumor types. In the example shown below, real-time impedance monitoring with xCELLigence was used to evaluate the efficacy of Erbix-mediated NK cell killing. A431 human epidermoid carcinoma cells, which express high levels of EGFR, were first seeded in the wells of an E-Plate. 22 hours post seeding, Erbix was added at different concentrations. One hour after antibody addition, interleukin 2-activated NK cells were added at an effector:target cell ratio of 20:1. Neither Erbix nor medium alone have a substantial effect on the real-time impedance trace of the A431 cells (**left panel**). While NK cell addition alone induces a decrease in the number, size, and/or attachment quality of the adherent A431 cells, the prior addition of Erbix substantially increases this effect in a dose-dependent manner. The fact that MabThera (a monoclonal antibody against the CD20 protein which is not expressed in A431 cells) is ineffective highlights the specific role being played by Erbix in directing NK cell-mediated killing (**left panel**). In the **right panel** the percentage change in Cell Index (relative to untreated control) has been plotted as a function of Erbix concentration; the time point used for this analysis is 10 hours after NK cell addition. Using this dose response curve the EC<sub>50</sub> of Erbix was calculated.



**Antibody-dependent NK cell-mediated killing of A431 cells.** Adherent A431 epidermoid carcinoma cells were incubated with different concentrations of Erbix monoclonal antibody before being exposed to interleukin 2-activated NK cells. Real-time impedance traces clearly show that the cytolytic activity of the NK cells is accentuated by Erbix in a dose-dependent manner (**left**). The "Ab" and "NK" arrows denote the times of antibody and NK cell addition, respectively. Plotting the percentage change in Cell Index, 10 hours after NK cell addition, as a function of antibody concentration yields a dose-response curve and the EC<sub>50</sub> of Erbix (**right**). Figure adapted from Assay Drug Dev Technol. 2006 Oct;4(5):555-63.



# Antibody-Dependent Cell-Mediated Cytolysis (ADCC)

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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, MDA-MB435

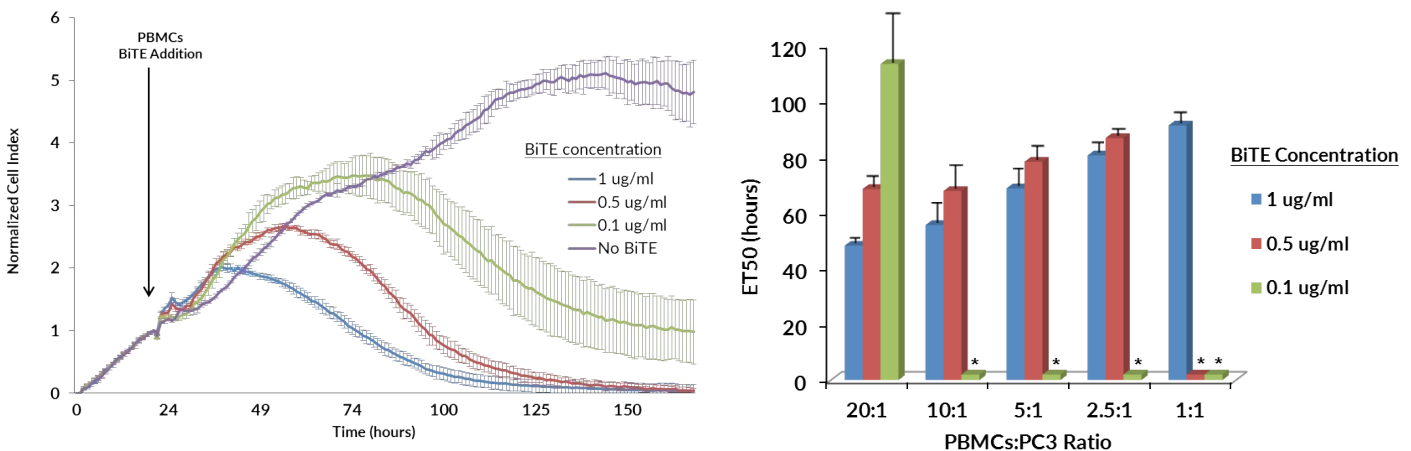
## Supporting information:

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 antibody-dependent cell-mediated cytotoxicity (ADCC) technique described in the previous section is mitigated by the fact that not all immune cells express the CD16 antibody receptor. In particular, cytotoxic and helper T lymphocytes don't express CD16 and therefore aren't recruited to antibody coated cells. In order to circumvent this constraint and mobilize the full capacity of the adaptive immune response against tumors, bispecific antibodies have been engineered which 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 that is expressed on the surface of tumor cells. Though multiple variations of bispecific antibodies have been studied, bispecific T cell engagers (BiTEs) stand out as being especially promising: BiTEs targeting the CD19 antigen on B cell malignancies were recently awarded "Breakthrough Therapy" status by the FDA.

To evaluate the utility of xCELLigence RTCA for characterizing BiTEs, killing of adherent PC3 prostate cancer cells by PBMCs was 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). At a PBMC:PC3 ratio of 20, EpCAM/CD3 BiTE increases killing efficacy in a dose dependent manner (**left panel**). Though PC3 cell killing is still stimulated at the lowest BiTE concentration, complete killing of the PC3 cells is delayed. Next, the BiTE's impact on PC3 killing by PBMCs was quantified. Using the "no BiTE" control (which has a cell index of ~4 at the 100 hour time point) as the reference, the effective time required to reduce the Cell Index by 50% (ET50) was plotted for different effector:target ratios at all three BiTE concentrations (**right panel**). As expected, ET50 values demonstrate PC3 lysis to be more efficient at higher effector:target cell ratios and at higher BiTE concentrations. The ability of xCELLigence RTCA to assess the effect of BiTEs on the cytolytic activity of effector cells in a continuous manner elucidates killing kinetics that would be impossible to capture with end point assays.



**Analyzing the efficacy of a BiTE targeting PC3 prostate cancer cells.** The Killing of adherent PC3 prostate cancer cells by PBMCs (effector:target ratio = 20) was evaluated in the presence of a BiTE with specificity for both EpCAM (present on PC3 cells) and CD3 (present on the surface of T cells within the PBMC pool). At all BiTE concentrations examined, simultaneous addition of the BiTE and PBMCs causes the destruction of PC3 cells, leading to a reduced impedance signal (**left**). Plotting the effective time for reduction of Cell Index by 50% (ET50) enables quantitative evaluation of the differential effects of effector:target ratio and BiTE concentration (**right**). An asterisk indicates that this value could not be determined because a 50% reduction in Cell Index was not achieved under this particular assay condition.

# Bispecific T Cell Engagers (BiTEs) and Bispecific Antibodies

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2.  **$\gamma\delta$  T Cell-Mediated Antibody-Dependent Cellular Cytotoxicity with CD19 Antibodies Assessed by an Impedance-Based Label-Free Real-Time Cytotoxicity Assay.** Seidel UJ, Vogt F, Grosse-Hovest L, Jung G, Handgretinger R, Lang P. [Front Immunol.](#) 2014 Dec 2;5:618. (University Children's Hospital Tübingen, Germany)
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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, HBV-transfected HuH7-S

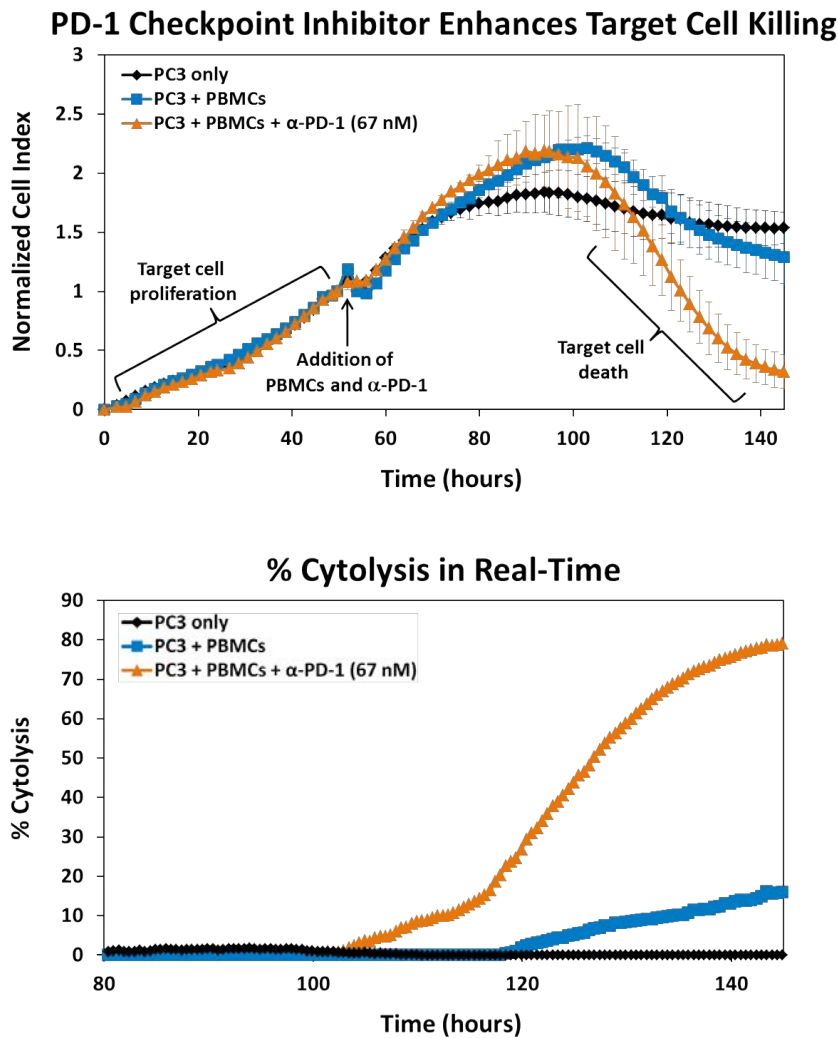
## Supporting information:

**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)

# 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 can help you to efficiently answer your questions under conditions of maximal physiological relevance.

In the example below, xCELLigence RTCA was used to monitor the impact of an anti-PD-1 antibody on the killing of prostate cancer PC3 cells by PBMCs. Target PC3 cells were seeded in ACEA's patented biosensor plate (E-Plate) and allowed to attach and proliferate. Frozen PBMCs were thawed, activated by incubation with Staphylococcus enterotoxin B (SEB) superantigen, and 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 seen in the upper figure, 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 is readily converted to a % Cytolysis plot (lower figure), which helps to elucidate the checkpoint inhibitor's impact: earlier onset of target cell killing, increased rate of cytolysis, and a greater total extent of cell killing.



Using 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 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.



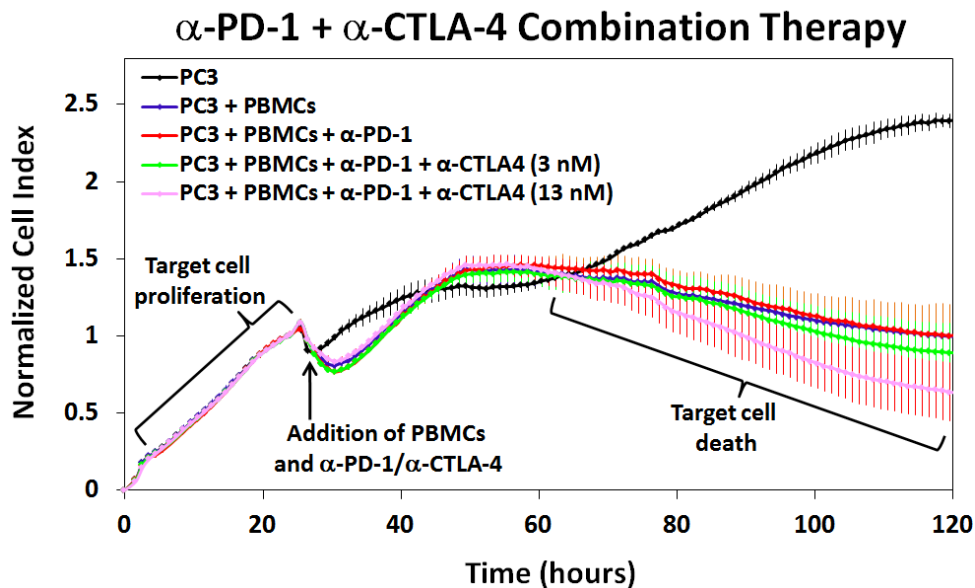
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## Combination Therapy

The traditional oncology pharmacopia of small molecules is rapidly being supplemented with biologics such as checkpoint inhibitors, and will soon also include cellular therapies (CAR-T cells, etc.). 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 is predictive of *in vivo* behavior. Other desirable characteristics include an easy workflow and a high throughput format, enabling diverse permutations of combination therapies to be analyzed simultaneously. xCELLigence RTCA meets all of the above criteria.

As demonstrated below xCELLigence RTCA was used 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. Target PC3 cells were seeded in ACEA's patented biosensor plate (E-Plate) and allowed to attach and proliferate. Frozen PBMCs were thawed, activated by incubation with Staphylococcal enterotoxin B (SEB) superantigen, and 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 killing of target cells. However, adding anti-CTLA-4 along with anti-PD-1 promoted target cell killing in a dose dependent manner (green and pink traces).



**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 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 end points and thereby readily yield cell killing data under many different conditions simultaneously.

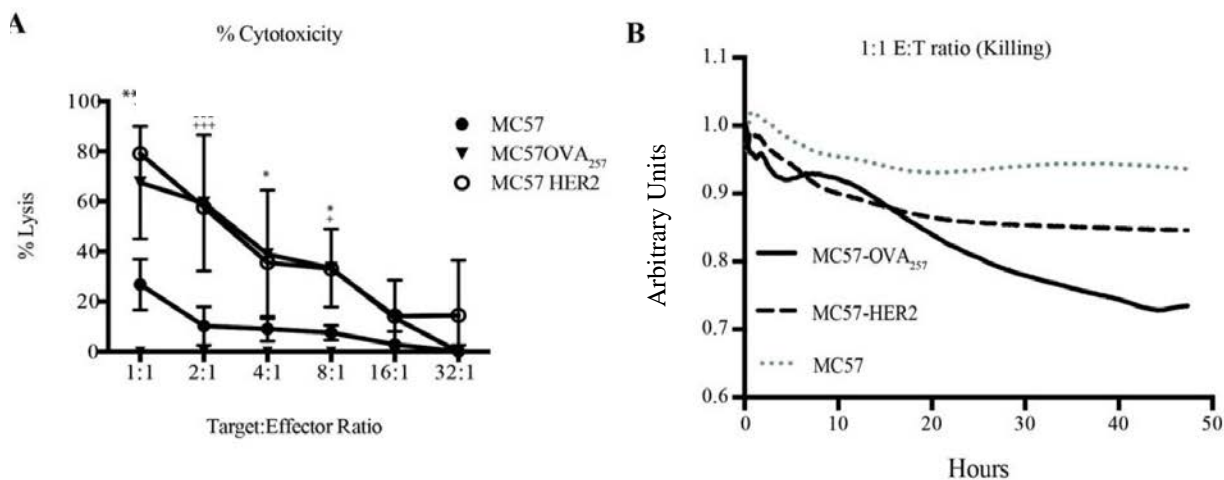
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## Genetically Engineered T Cell-Mediated Cell Killing

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 that is linked to an extracellular domain derived from a tumor-specific antibody). Avoiding the immune tolerance issues associated with non-autologous therapies and producing T cells that efficiently target tumors without the need for *de novo* activation in the patient are primary motivations for genetically modifying T cells. The efficacy of this approach is highlighted by the convincing clinical data that has emerged in recent years (as one example, see: [Clin Transl Immunology](#). 2014;3(5):e16.)

In the example below, multiple assays are used to evaluate the killing efficiency of T cells when they are directed at cancer cells using either an engineered TCR or a CAR. To facilitate this comparison, CD8+ T cells were engineered to express both a TCR (recognizing the OVA<sub>257</sub> epitope of ovalbumin) and a CAR (recognizing HER2). At different effector:target cell ratios, these T cells were incubated with adherent MC57 mouse fibrosarcoma cells that expressed the OVA<sub>257</sub> epitope, HER2, or no exogenous protein. After 18 hours of co-incubation, T cell mediated cytotoxicity was analyzed using a traditional <sup>51</sup>Cr release assay (**left panel**). Though the engineered T cells are able to kill MC57 cells, killing efficiency increases dramatically when the MC57 cells are expressing either OVA<sub>257</sub> or HER2 (as expected). At all effector:target cell ratios analysed, activating these T cells via their TCR or their CAR results in similar killing efficiency (**left panel**). When the same assay is repeated using xCELLigence real-time impedance monitoring, after 18 hours of co-incubation killing via OVA<sub>257</sub> targeting and HER2 targeting are very similar. Importantly, however, when analysed over a longer time period there are substantial differences in the killing efficiencies of these two targeting approaches (**right panel**).

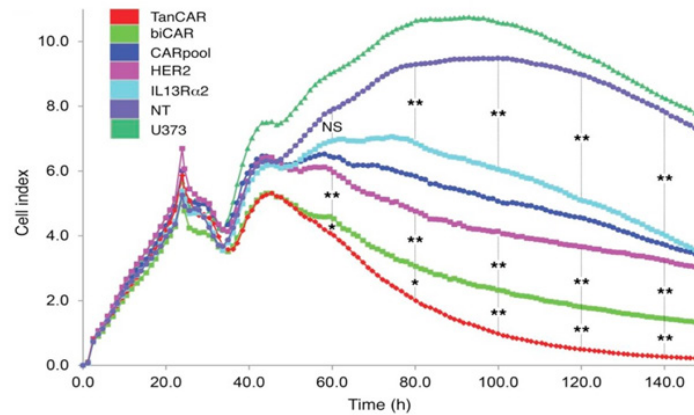


**Targeting T cells using an engineered TCR vs. CAR results in different killing efficiency.** T cells engineered to simultaneously express a TCR against the OVA<sub>257</sub> epitope of ovalbumin and a CAR against the HER2 protein were co-incubated with adherent mouse fibrosarcoma cells. Killing efficiency was analyzed by both a traditional <sup>51</sup>Cr release assay (**left**) and xCELLigence RTCA impedance monitoring (**right**; here data are plotted in arbitrary units, comparing the cell index value at each time point to the cell index value of the same sample prior to T cell addition). Both assays show that MC57 cells are killed more efficiently when they express OVA<sub>257</sub> or HER2 (as expected). In the <sup>51</sup>Cr release assay (conducted after 18 hours of co-incubation) both targeting approaches produce similar killing efficiencies. In contrast, real-time monitoring of impedance over a longer time period reveals that in this context the TCR targeting approach results in more robust cell killing. Figure adopted from [Cancer Immunol Res](#). 2015; 3(5):483-94.



# Genetically Engineered T Cell-Mediated Cell Killing

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 which 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. The below study compared different scenarios where CARs targeting the HER2 and IL13R $\alpha$ 2 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 (**below**). These nuances in serial killing behavior are readily elucidated by continuous impedance monitoring, but would go undetected in traditional end point assays.



Using xCELLigence to monitor killing of the glioblastoma cell line U373 by CAR-T cells targeting either one or both of the antigens HER2 and IL13R $\alpha$ 2. In the figure legend: U373 = target cell line alone; NT = target cells treated with non-transfected T cells (i.e. not expressing a CAR); IL13R $\alpha$ 2 = target cells treated with T cells expressing a single CAR targeting IL13R $\alpha$ 2; Her2 = target cells treated with T cells expressing a single CAR targeting Her2; see text for descriptions of CARpool, biCAR, and TanCAR. Figure adapted from *J Clin Invest*. 2016 Aug 1;126(8):3036-52.

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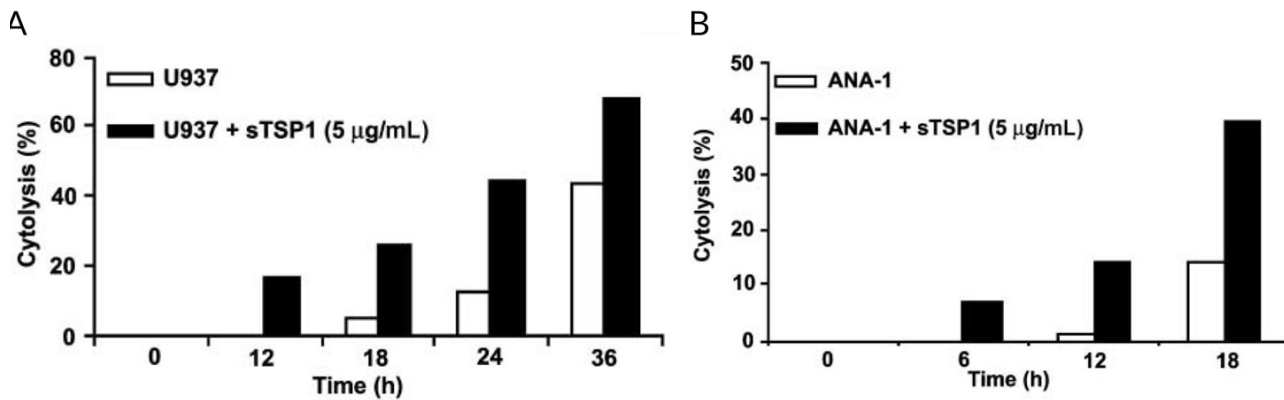
## Genetically engineered T cell-mediated cell killing-adherent target cells tested:

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

## Macrophage-Mediated Phagocytosis

Macrophages are important effector cells of innate immunity. Depending on the tissue microenvironment, tumor-associated macrophages (TAM) can differentiate into either cytotoxic (M1) or tumor-promoting (M2) states. While cytotoxic M1 macrophages are typically induced by IFN- $\gamma$  alone or in concert with microbial products, tumor promoting M2 macrophages are induced by IL-4 or IL-13, IL-10, IL-21, TGF $\beta$ , immune complexes, and glucocorticoids.

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



**Secreted glycoprotein TSP1 increases macrophage/monocyte-mediated tumoricidal activity.** MDA-MB-231 breast adenocarcinoma target cells were seeded in E-Plates and incubated for up to 24 hours. Differentiated U937 human monocytes (**left**) or activated ANA-1 murine macrophages (**right**) were then added in the presence or absence of soluble TSP1. Figure adapted from [Cancer Res.](#) 2008;68(17):7090-9. Note that the RT-CES<sup>®</sup> described in this publication was ACEA's first generation real-time cell analysis (RTCA) system, and has subsequently been rebranded as xCELLigence RTCA.

# Macrophage-Mediated Phagocytosis

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## Macrophage-mediated phagocytosis – adherent target cells tested:

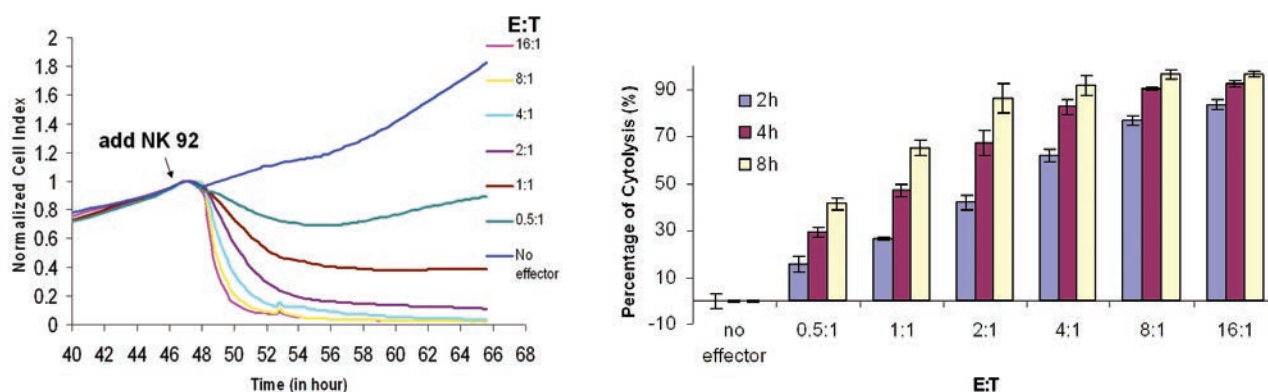
MDA-MB-231, MDA-MB-435, MCF-7

## NK Cell-Mediated Cytolysis

Natural killer (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 a number of 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 a membrane permeabilizing protein (perforin) and proteases (granzymes) which collectively cause target cell death via 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. The above mechanisms employed 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.

In the experiment shown below, 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 (**below**). Importantly, 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, the percentage of cytolysis is readily calculated using a simple formula:

$$\text{Percentage of cytolysis} = ((\text{Cell Index}_{\text{no effector}} - \text{Cell Index}_{\text{effector}}) / \text{Cell Index}_{\text{no effector}}) \times 100$$



**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 E-Plate. Different quantities of NK-92 cells were added to each well and impedance was monitored continuously for the next ~20 hours (**left**). The time-dependent cytolytic activity of NK-92 cells at different E:T ratios (**right**) was calculated as described above. Figures adapted from ACEA's Application Note entitled "[Label-Free Assay for NK Cell-Mediated Cytolysis](#)".



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## 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, mesenchymal stromal cells (MSCs)

## Supporting information:

1. [xCELLigence Application Note: Label-Free Assay for NK Cell-Mediated Cytolysis](#)
2. [xCELLigence Application Note: Real-Time, Label-Free Measurement of Natural Killer Cell Activity and Antibody-Dependent Cell-Mediated Cytotoxicity](#)

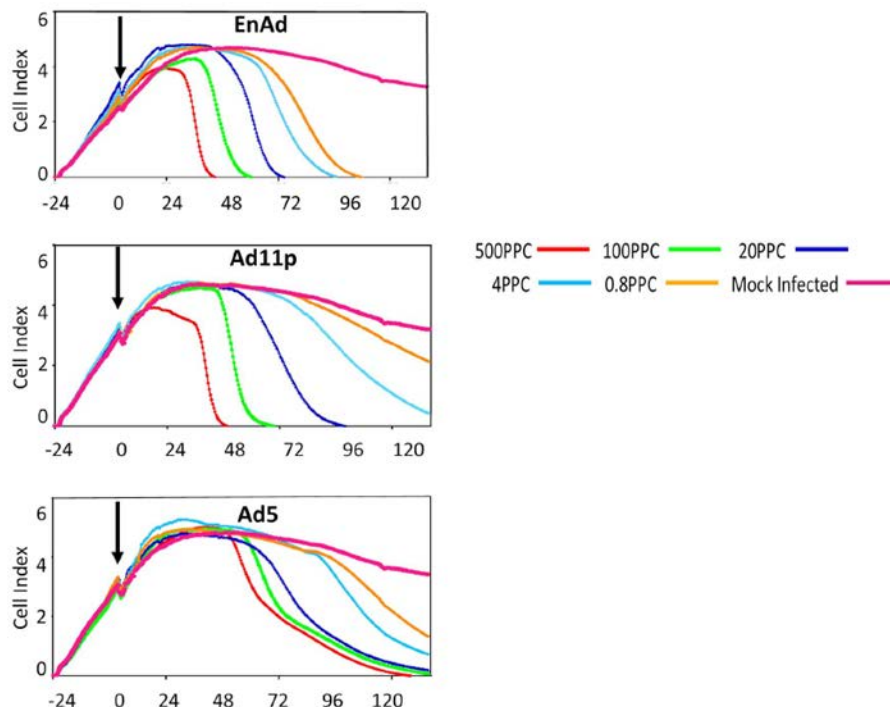
## Oncolytic Viruses

Oncolytic virotherapy is a promising cancer treatment that uses a replication-competent virus to selectively infect cancer cells, cause cytotoxicity, and generate anti-tumor immunity. This approach has seen major advances in recent years using both wildtype 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 thereby readily yield cell killing data under many different conditions simultaneously.

In the below example xCELLigence RTCA was used to monitor killing of A549 lung cancer cells by a chimeric adenovirus (Enadenotucirev, EnAd) which infects cells by binding to CD46 and/or desmoglein, both widely expressed on many carcinoma cells. In a potency analysis, the cytotoxicity (i.e. killing kinetics) of EnAd at a range of concentrations was compared with wild-type adenoviruses Ad11p and Ad5. At the highest concentration (red, 500 PPC (particles per cells)), EnAd and Ad11p caused complete cell killing (Cell Index decreasing to zero) between 36-48 hours post-infection. However, at lower virus concentrations (0.8-20 PPC) EnAd is substantially more potent than Ad11p, displaying both an earlier onset of cytotoxicity and a more rapid completion of cytolysis. When compared with EnAd and Ad11p, wildtype Ad5 is much less efficient at killing the cancer cells, requiring 5 days to achieve full cell killing even at the highest virus concentration.

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



**Killing of A549 lung cancer cells by different adenoviruses.** Black arrow indicates the time of virus addition. Virus concentrations are listed as particles per cell (PPC). Figure adapted from: [Mol Ther Oncolytics](#), 2016 Dec 10;4:18-30.

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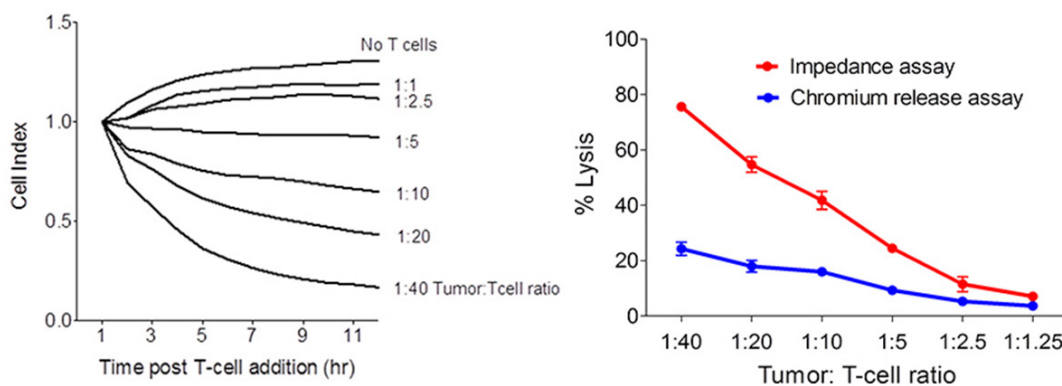
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## T Cell-Mediated Cytolysis

By seeking out and destroying infected cells directly, the CD8<sup>+</sup> T lymphocytes play a critical role in the adaptive immune response. Every CD8<sup>+</sup> 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 through this antigen:MHC-I complex causes CD8<sup>+</sup> 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. It is for this reason that T lymphocytes can be 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 that are expressing proteins that are either mutated or expressed at abnormal levels.

While in some contexts it is useful to quantify the number of antigen-specific CD8<sup>+</sup> T cells in samples using assays such as ELISpot or flow cytometry, it is often critical to assess the functional cytotoxicity of these cells via killing assays. Measuring cytolytic activity via the chromium-51 (<sup>51</sup>Cr) release assay has long been the gold standard for evaluating CD8<sup>+</sup> T cell responses. In the assay shown below, SKBR-3 breast cancer cells expressing the HER2/Neu protein were pre-labeled with <sup>51</sup>Cr. They were subsequently co-incubated with increasing amounts of a CD8<sup>+</sup> T cell clone that expresses a TCR specific for an antigenic peptide of HER2/Neu, and target cell killing was detected by release of <sup>51</sup>Cr into the medium. This same assay was concurrently performed using the xCELLigence RTCA system without pre-labeling of the target cells. The RTCA system quantitatively detected the cytolytic activity of CD8<sup>+</sup> T cells against the SKBR-3 target cells in a manner that was dependent on both time and number of CD8<sup>+</sup> T cells added (**left panel**). Side by side comparison with the <sup>51</sup>Cr release assay shows that the sensitivity and dynamic range of the xCELLigence RTCA assay surpasses that of <sup>51</sup>Cr (**right panel**). Moreover, the preclusion of radio-labeling, and the kinetic data provided by RTCA (including both the onset of cytolysis and the rate of tumor cell killing) make this assay especially attractive.



**CD8<sup>+</sup> T cell-mediated cytolysis of SKBR3 tumor cells.** In a dose-dependent manner, CD8<sup>+</sup> T cell addition causes the real-time impedance traces to decrease in value – indicative of a reduction in the number, size, and/or attachment quality of the SKBR3 tumor cells (**left**). Plotting the percentage of tumor cell lysis, as determined by xCELLigence RTCA vs. the standard <sup>51</sup>Cr release assay, demonstrates RTCA to be the more sensitive method (**right**). Figure adapted from [J Vis Exp.](#) 2012 Aug 8;(66):e3683.



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## T cell-mediated cytotoxicity – 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, HCC1419

## Supporting information:

1. **JOVE Video Protocol:** [Determining Optimal Cytotoxic Activity of Human Her2neu Specific CD8 T Cells by Comparing the Cr51 Release Assay to the xCELLigence System](#)
2. **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)

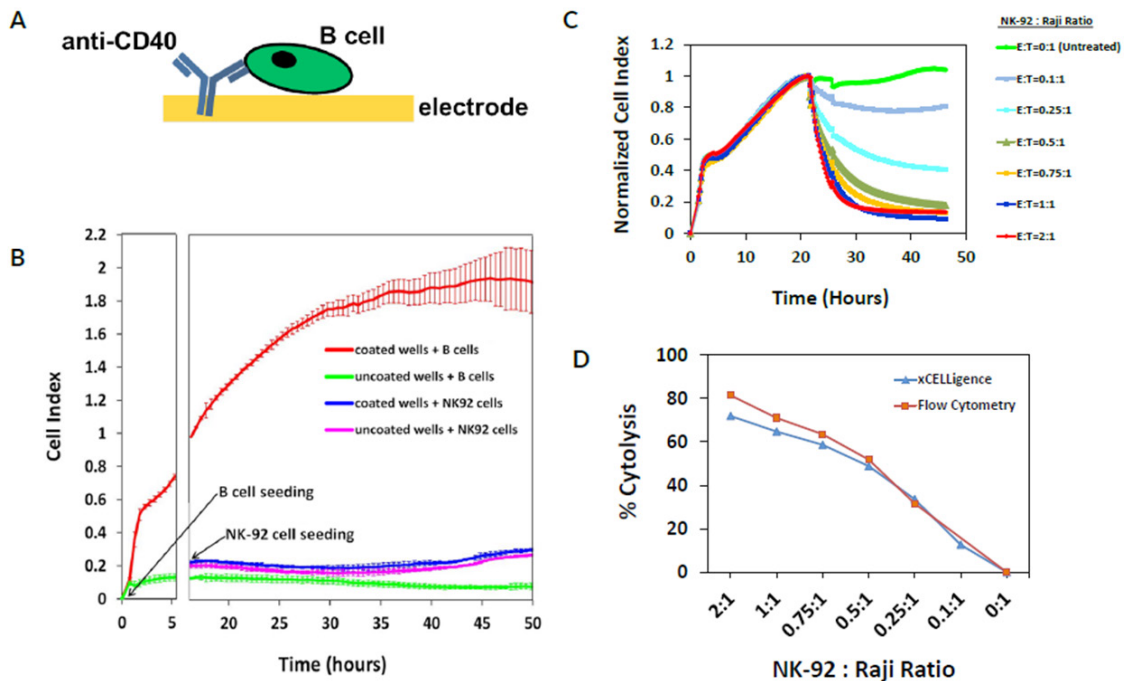
## Liquid Tumor Killing Assays

With dozens of peer-reviewed studies published over the past decade, the utility of xCELLigence RTCA for probing the efficacy of immunotherapies targeting solid/adherent cancers is firmly established. However, ~10% of all cancers are liquid in nature, are therefore non-adherent, and cannot be directly monitored by the standard impedance assay. Moreover, because they are readily accessible within the bloodstream and aren't confounded by the microenvironment complexities/heterogeneities associated with solid tumors, liquid cancers are prominent immunotherapy targets. To help accelerate research in this area, ACEA has developed xCELLigence RTCA Immunotherapy Kits which enable impedance-based killing assays to be performed with liquid tumor targets. At present, three kits are available, enabling either B cell lines or the K562 myelogenous leukemia line to be used as targets. For the purpose of these assays, the wells of ACEA's electronic microtiter plates (E-Plates) are precoated with anti-CD40 or anti-CD19 (for B cells), or anti-CD29 antibody (for K562 cells), enabling these cells to be immobilized on the plate bottom prior to treatment with effector cells, antibodies, small molecules, etc.

The utility of the xCELLigence RTCA Immunotherapy Kit for B cell killing (anti-CD40) assays is illustrated below. 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 A and B). Importantly, 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 B). Addition of NK-92 cells on top of immobilized B cells results in target cell death in a dose dependent manner (Figure C). Note that 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 which are not physiologically relevant. The tethering and killing behaviors seen in Figures B and C have been observed in all three of the B cell lymphoma lines tested (Daudi, Raji, and Ramos), for multiple effector cell types (NK, T, CART), and for combination therapies (CART + checkpoint inhibitors, etc.). Experiments looking at killing of patient-derived B cells by patient-derived effector cells are in progress.

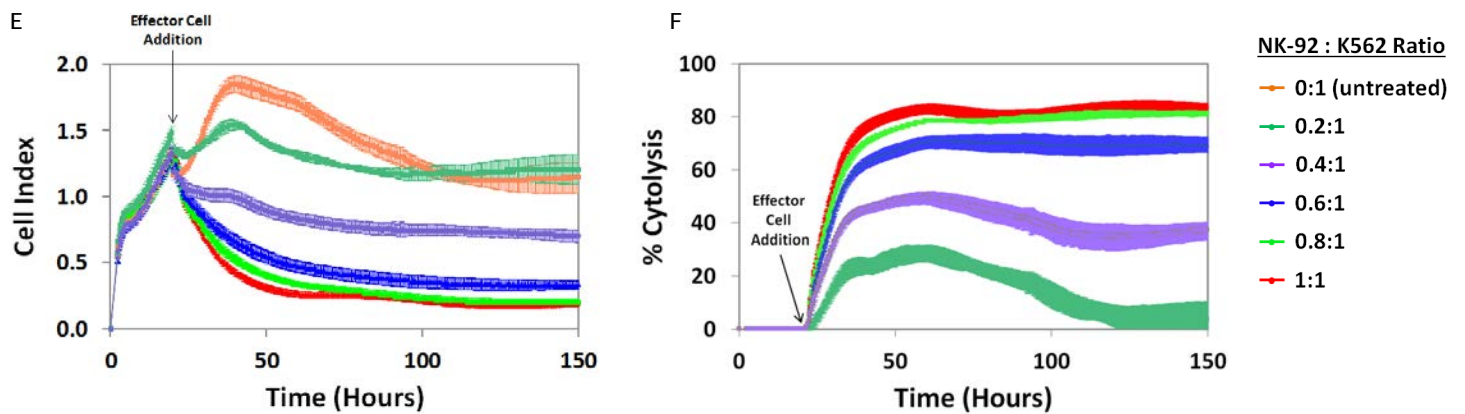
An important question is whether the physical immobilization of B cells via antibody tethering affects the efficiency with which they are killed. To assess this, side-by-side four hour assays were performed for NK-92 cell-mediated killing of Raji B cells that were either immobilized (analyzed by xCELLigence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

# Liquid Tumor Killing Assays



**The xCELLigence Immunotherapy Kit for monitoring B cell killing.** (A) Precoating the wells of ACEA's electronic microtiter plates with B cell-specific antibody (anti-CD40) enables B cells to proliferate on, and be detected by, these 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 non-adherent NK-92 effector cells produce minimal signal. Error bars are standard deviation. (C) The efficiency with which Raji B cells are killed is dependent 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. % cytotoxicity was determined after 4 hours of treatment by xCELLigence (tethered) or flow cytometry (in suspension).

In a second example of liquid tumor cell killing, Figures E and F 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, destruction of K562 cells is detectable even at low effector:target ratios.



**The xCELLigence Immunotherapy Kit for monitoring K562 cell killing.** (E) K562 cells, immobilized on the bottom of E-Plate wells by anti-CD29 antibody, are destroyed by NK-92 cells in a time- and dose-dependent manner. (F) Data from panel "A" re-plotted as % Cytotoxicity as a function of time. Error bars in both panels are standard deviation.

The liquid tumor killing assays described above are currently being used in both industrial and academic labs for evaluating/optimizing combination therapies, and for the development of adoptive cell therapies and engineered antibodies. Beyond the arena of R&D, we envision these liquid tumor killing assays being utilized for functional validation/quality control of manufactured immuno-oncology therapies.



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