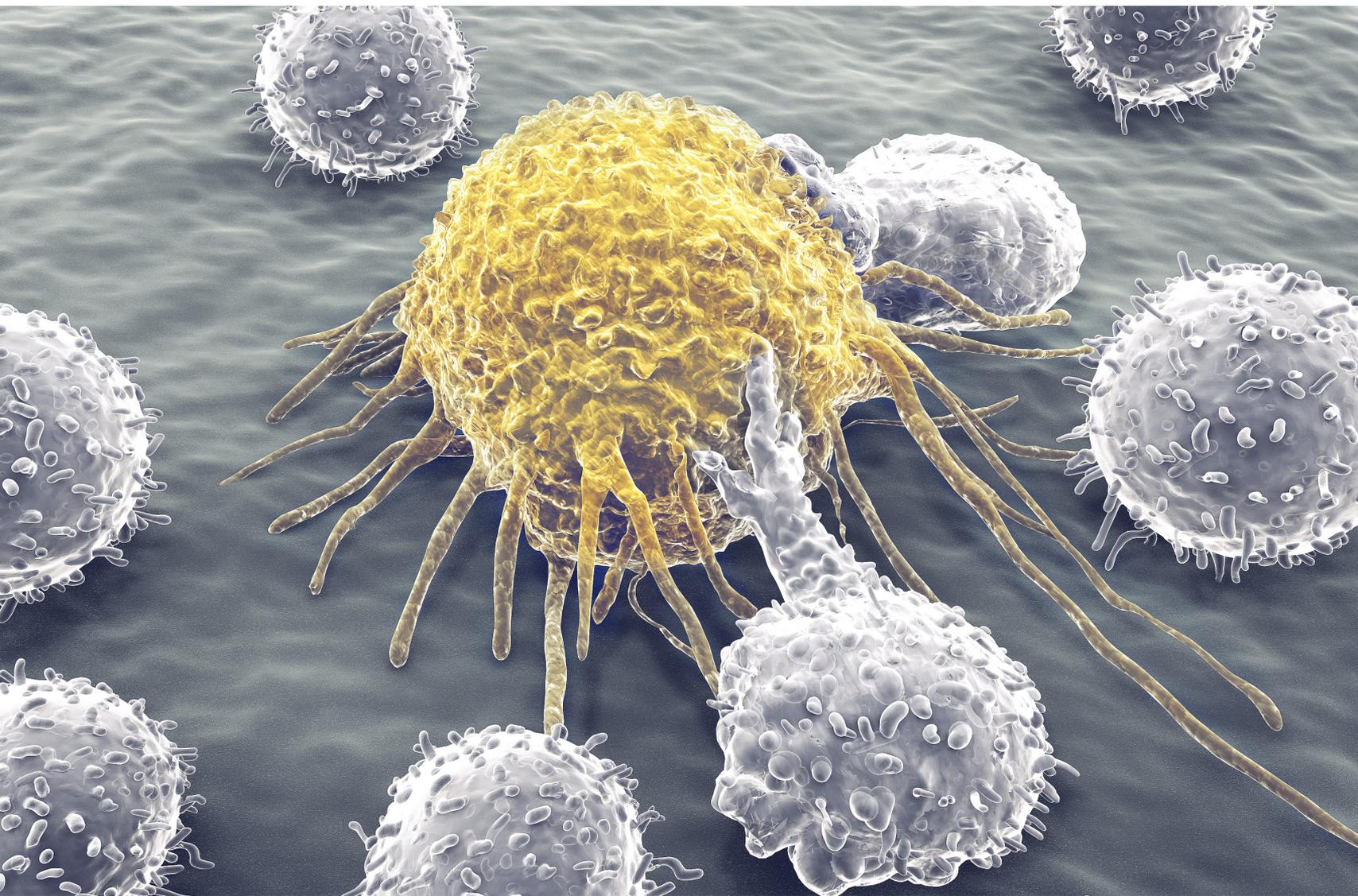


Cancer Immunotherapy Assays

Using xCELLigence Real-Time Cell Analysis



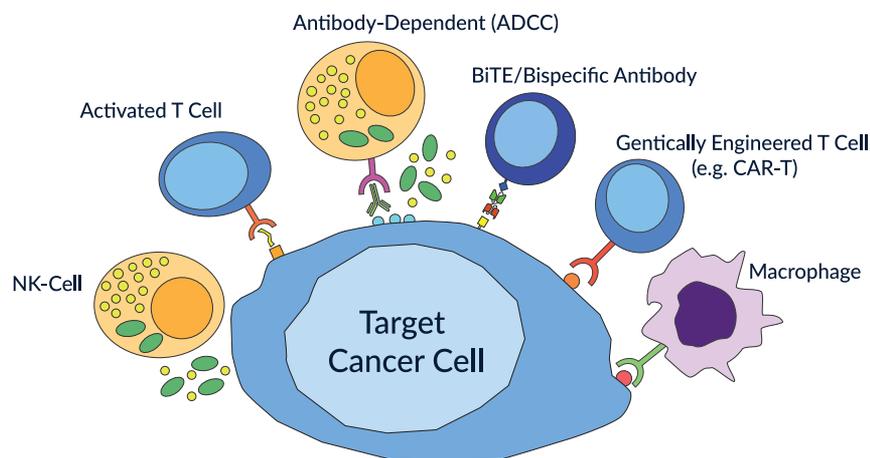
Overview

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?

As shown in the figure below, 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.



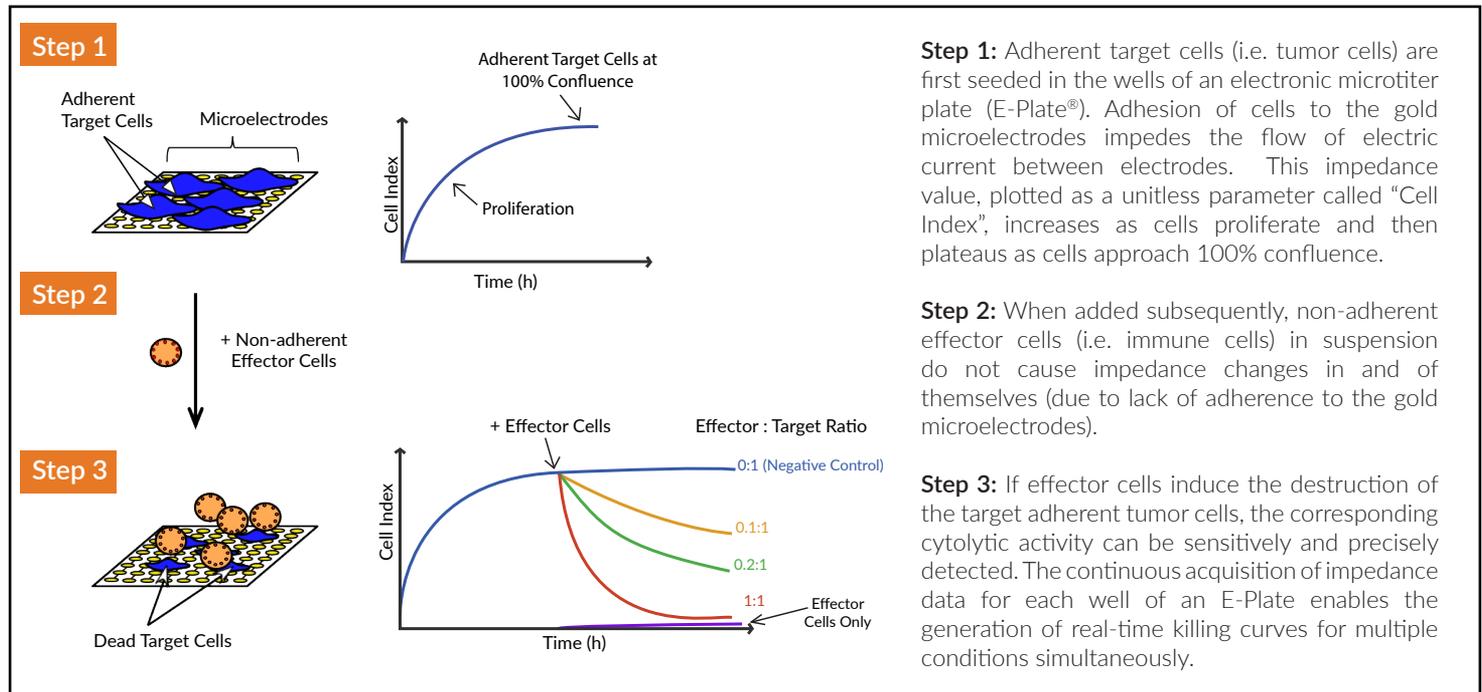
What assays are used to study immune cell-mediated killing?

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 ^{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 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.

Overview

What is the xCELLigence RTCA assay principle?

ACEA's xCELLigence® Real-Time Cell Analysis (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.



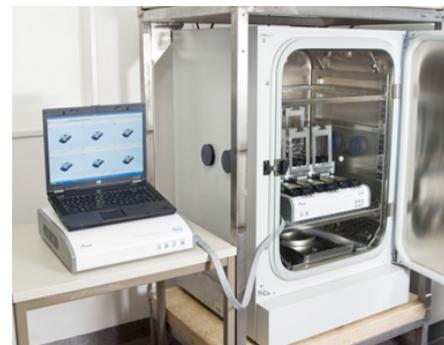
Key benefits of using xCELLigence to monitor immune cell-mediated killing:

1. **Label-Free:** Allowing for more physiological assay conditions; labeling or secondary assays aren't required.
2. **Real-Time:** Quantitative monitoring of both fast (hours) and slow (days) killing kinetics.
3. **Sensitive:** Capable of evaluating low effector cell to target cell ratios that are physiologically relevant.
4. **Simple Workflow:** Requires only the addition of effector cells to target cells (in the presence or absence of antibodies); homogeneous assay without additional sample handling.
5. **Automatic Data Plotting:** RTCA software enables facile data display and objective analysis, precluding the subjective data vetting that is common to imaging-based assays.

Overview

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, and includes 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 which are 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 on page 5. 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.

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

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Overview

xCELLigence Instruments for Immunotherapy



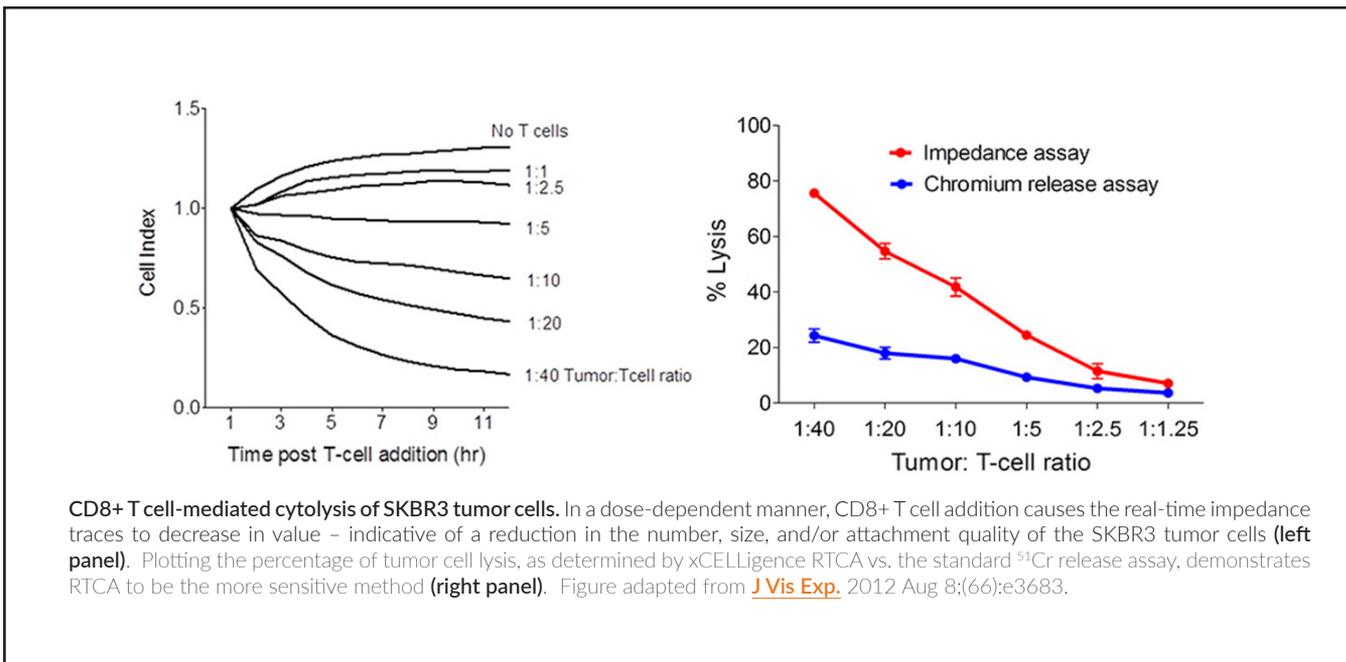
	Immunotherapy Applications	DP (Dual Purpose)	SP (Single Plate)	MP (Multi Plate)	HT (High Throughput)
Applicable to Both Liquid & Solid Tumor Target Cells	Cell-Mediated Cytotoxicity	✓	✓	✓	✓
	Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)	✓	✓	✓	✓
	Checkpoint Inhibitors	✓	✓	✓	✓
	Combination Therapies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

T Cell-Mediated Cytolysis

By seeking out and destroying infected cells directly, the CD8+ subgroup of T cells 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 through this antigen: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. 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+ 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 release assay has long been the gold standard for evaluating CD8+ T cell responses. In the assay shown below, SKBR-3 breast cancer cells expressing the HER2/Neu protein were pre-labeled with ^{51}Cr . They were subsequently co-incubated with increasing amounts of a CD8+ T cell clone that expresses a TCR targeting an antigenic peptide of HER2/Neu, and target cell killing was detected by release of ^{51}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+ T cells against the SKBR-3 target cells in a manner that was dependent on both time and number of CD8+ T cells added (**left panel**). 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 (**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.



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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, HCC1419

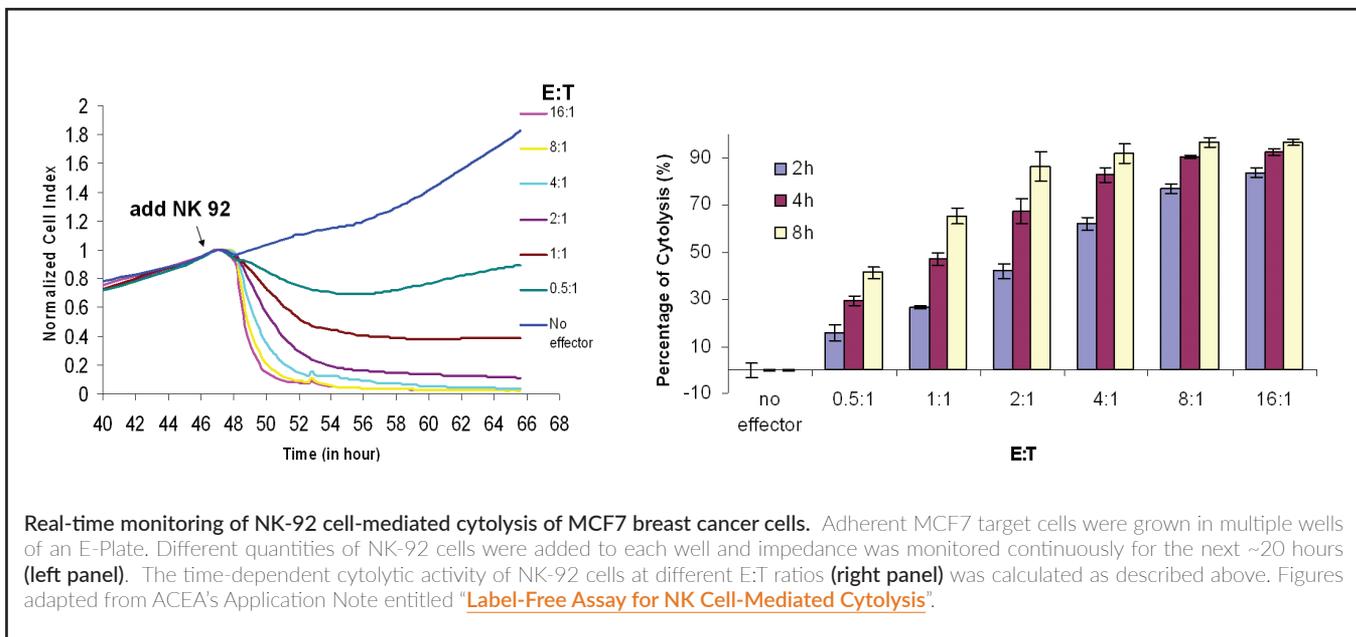
Supporting information:

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)

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 (**left and right panels**). 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: Percentage of cytolysis = $\frac{(\text{Cell Index}_{\text{no effector}} - \text{Cell Index}_{\text{effector}})}{\text{Cell Index}_{\text{no effector}}} \times 100$



NK Cell-Mediated Cytolysis

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12. **Phenotypic and Functional Dysregulated Blood NK Cells in Colorectal Cancer Patients Can Be Activated by Cetuximab Plus IL-2 or IL-15.** Rocca YS, Roberti MP, Juliá EP, Pampena MB, Bruno L, Rivero S, Huertas E, Sánchez Loria F, Pairola A, Caignard A, Mordoh J, Levy EM. [Front Immunol](#). 2016 Oct 10;7:413. (Centro de Investigaciones Oncológicas CIO-FUCA, Argentina)

NK cell-mediated cytolysis – adherent cell lines tested:

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

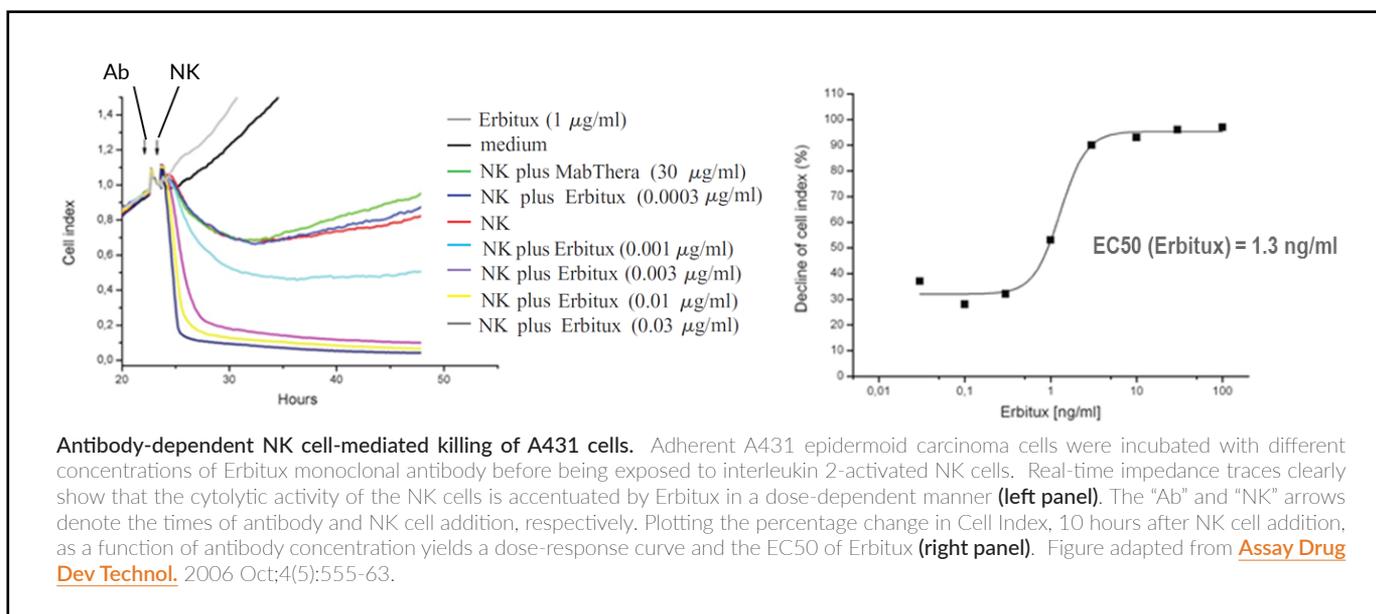
Supporting information:

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](#)

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 disease. 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 EC50 of Erbix was calculated.



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

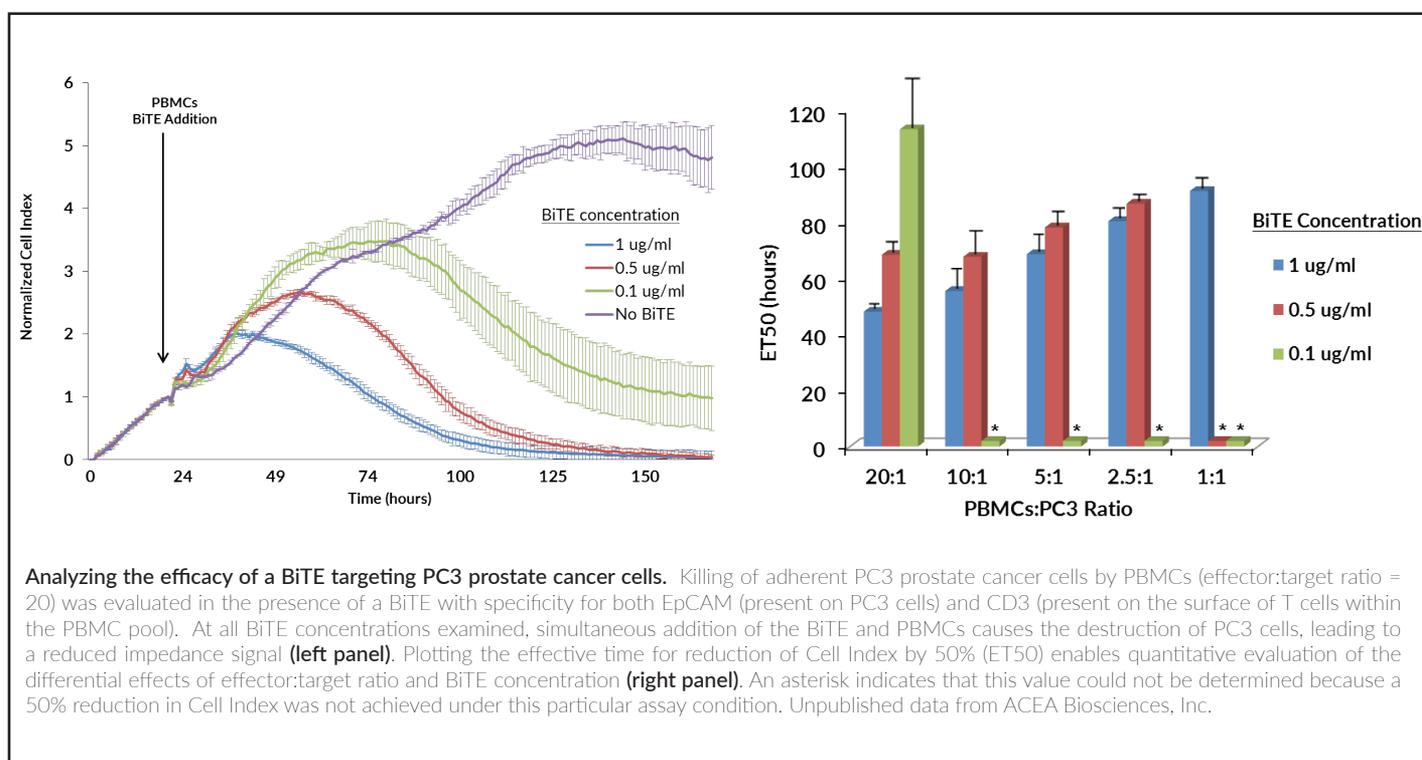
Supporting information:

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.



Bispecific T Cell Engagers (BiTEs) and Bispecific Antibodies

References:

1. **Novel bispecific antibodies increase $\gamma\delta$ T-cell cytotoxicity against pancreatic cancer cells.** Oberg HH, Peipp M, Kellner C, Sebens S, Krause S, Petrick D, Adam-Klages S, Röcken C, Becker T, Vogel I, Weisner D, Freitag-Wolf S, Gramatzki M, Kabelitz D, Wesch D. [Cancer Res.](#) 2014 Mar 1;74(5):1349-60. (Christian-Albrechts-University Kiel, Germany)
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3. **Committing cytomegalovirus-specific CD8 T cells to eliminate tumor cells by bifunctional major histocompatibility class I antibody fusion molecules.** Schmittnaegel M, Levitsky V, Hoffmann E, Georges G, Mundigl O, Klein C, Knoetgen H. [Cancer Immunol Res.](#) 2015 Jul;3(7):764-76. (Roche Pharma Research and Early Development, Germany)
4. **A New Class of Bifunctional Major Histocompatibility Class I Antibody Fusion Molecules to Redirect CD8 T Cells.** Schmittnaegel M, Hoffmann E, Imhof-Jung S, Fischer C, Drabner G, Georges G, Klein C, Knoetgen H. [Mol Cancer Ther.](#) 2016 Sep;15(9):2130-42. (Roche Innovation Center Basel, Switzerland)

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:

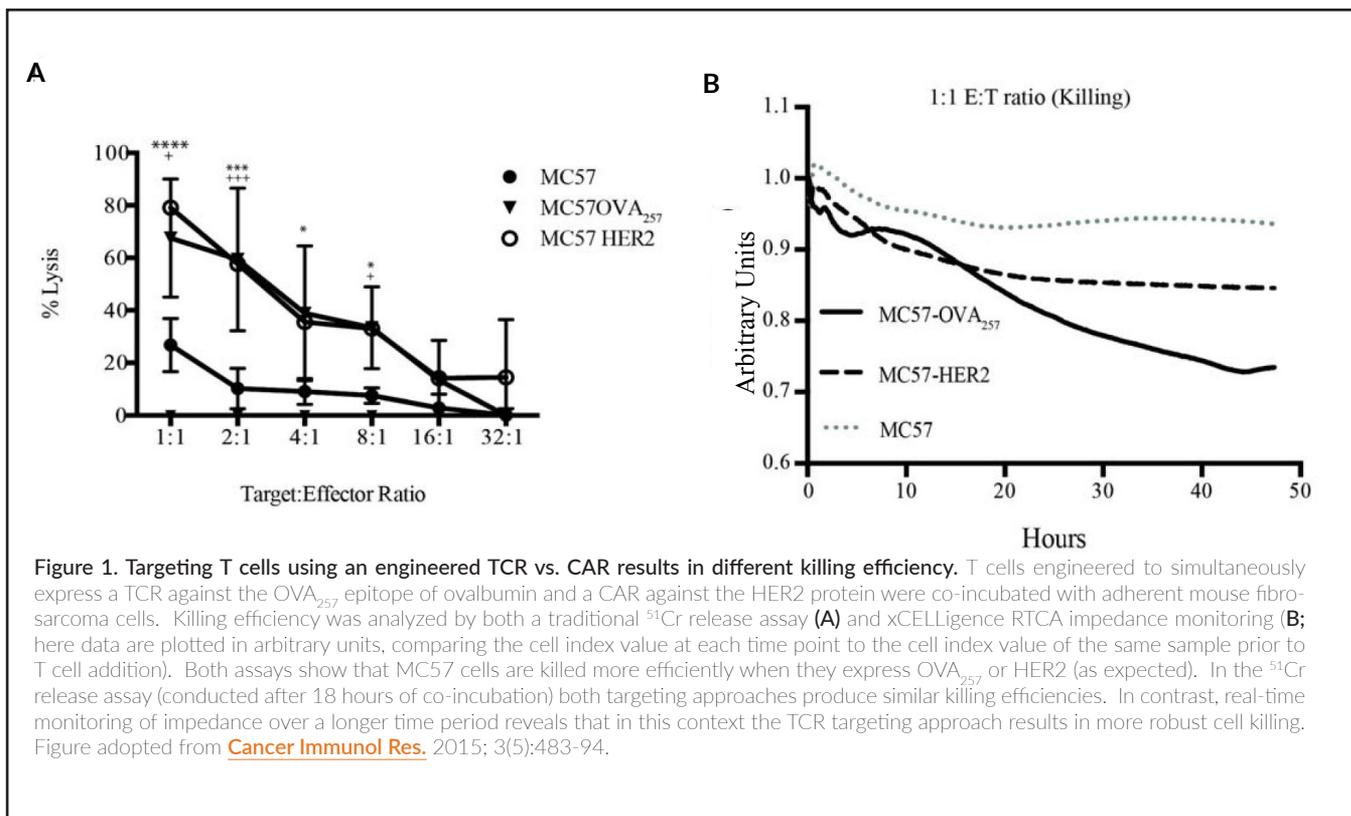
Webinar Recording: [Bispecific Antibody Constructs Mediate Immunotherapeutic Retargeting of Effector Cells Towards HBV Infected Target Cells](#)

Felix Bohne, Ph.D. (German Research Center for Environmental Health)

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 OVA₂₅₇ 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 OVA₂₅₇ epitope, HER2, or no exogenous protein. After 18 hours of co-incubation, T cell mediated cytolysis was analyzed using a traditional ⁵¹Cr release assay (Figure 1A). Though the engineered T cells are able to kill MC57 cells, killing efficiency increases dramatically when the MC57 cells are expressing either OVA57 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 (Figure 1A). When the same assay is repeated using xCELLigence real-time impedance monitoring, after 18 hours of co-incubation killing via OVA₂₅₇ 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 (Figure 1B).



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

Genetically Engineered T Cell-Mediated Cell Killing

targeting the HER2 and IL13R α 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 (**Figure 2**). These nuances in serial killing behavior are readily elucidated by continuous impedance monitoring, but would go undetected in traditional end point assays.

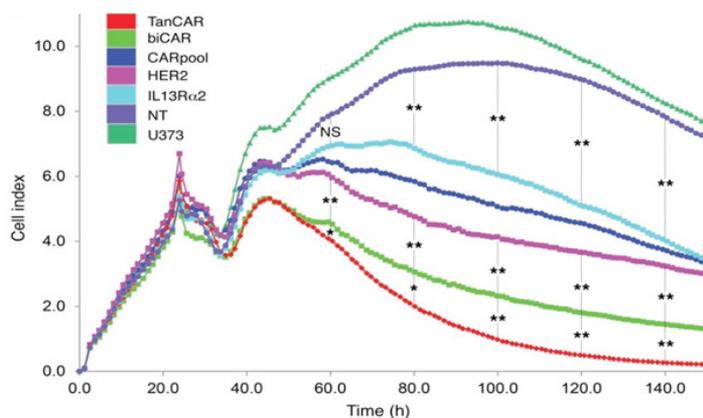


Figure 2. Using xCELLigence to monitor killing of the glioblastoma cell line U373 by CAR-T cells targeting either one or both of the antigens HER2 and IL13R α 2. In the figure legend: U373 = target cell line alone; NT = target cells treated with non-transfected T cells (i.e. not expressing a CAR); IL13R α 2 = target cells treated with T cells expressing a single CAR targeting IL13R α 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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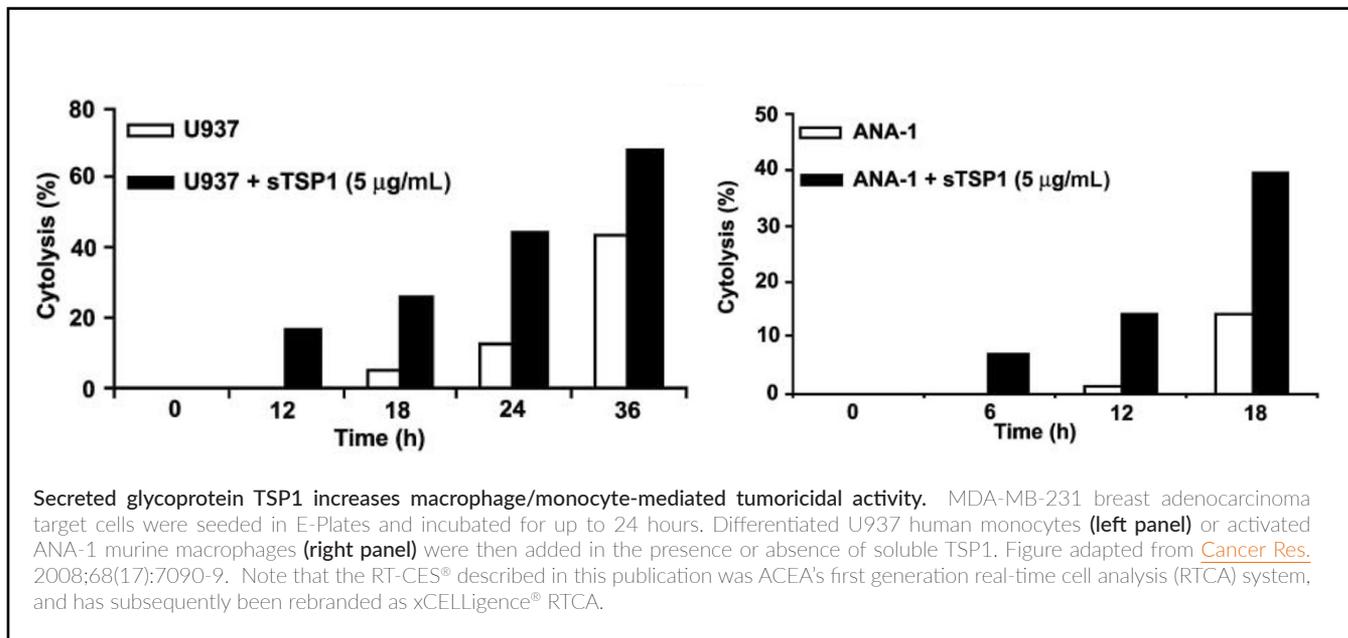
Genetically engineered T cell-mediated cell killing-adherent target cells tested:

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- γ alone or in concert with microbial products, tumor promoting M2 macrophages are induced by IL-4 or IL-13, IL-10, IL-21, TGF β , 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 % cytotoxicity 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.



Macrophage-Mediated Phagocytosis

References:

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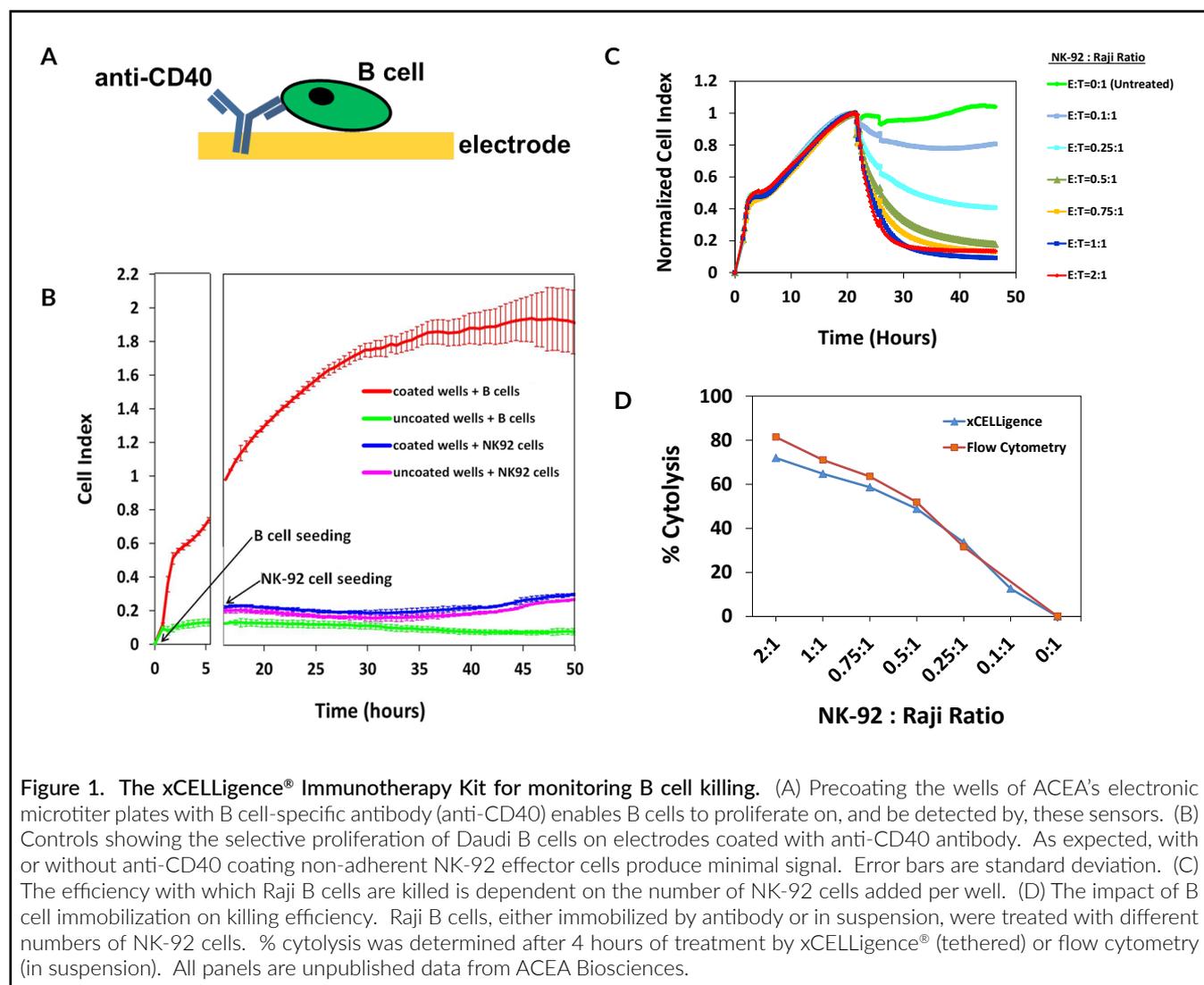
Macrophage-mediated phagocytosis – adherent target cells tested:

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

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, two 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 antibody (B cells) or anti-CD29 antibody (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 assays is illustrated below in Figure 1. 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 1 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 1B). Addition of NK-92 cells on top of immobilized B cells results in target

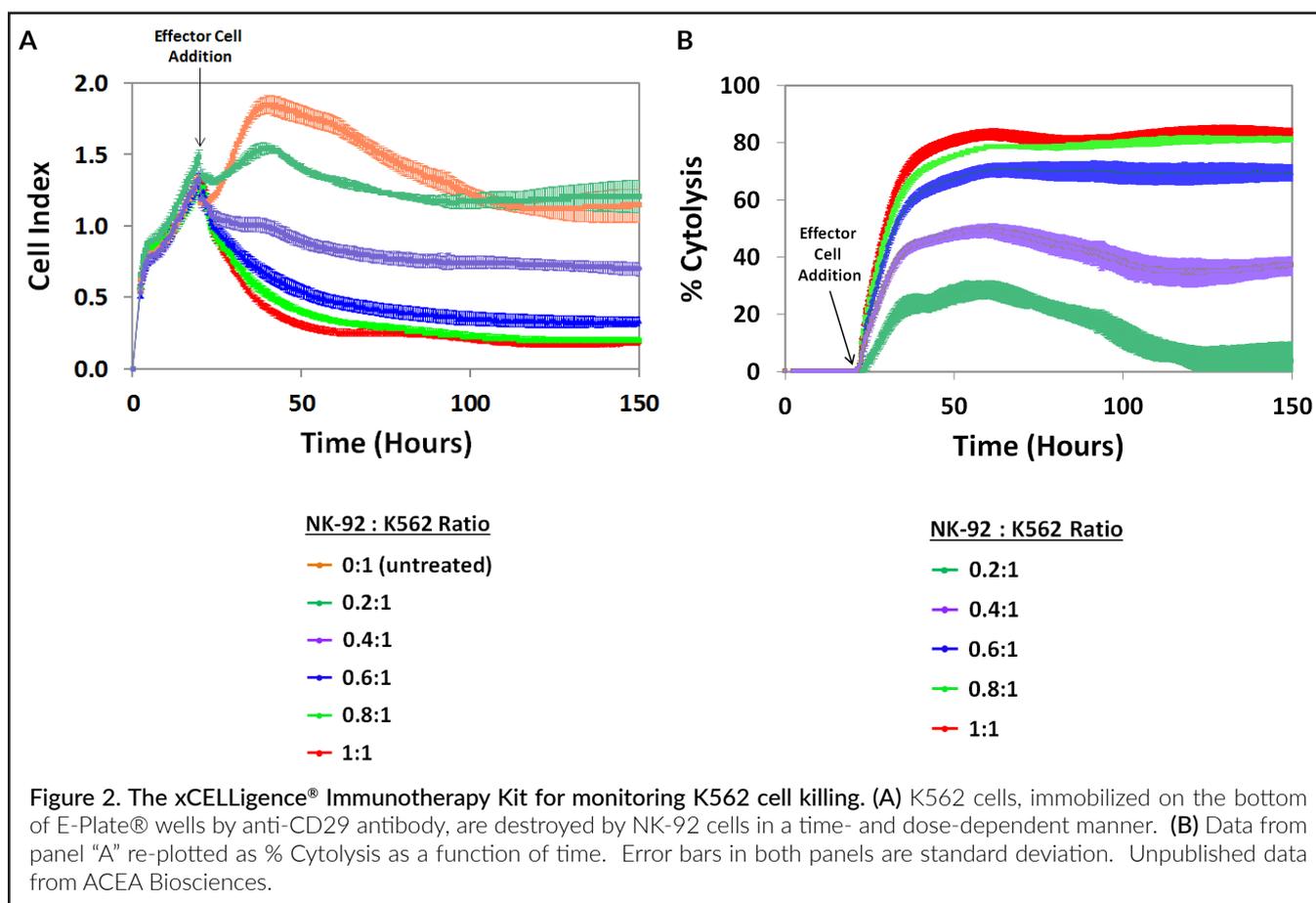


Liquid Tumor Killing Assays

cell death in a dose dependent manner (**Figure 1C**). 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 1 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 1D**, the killing trends observed by these two methods correlate perfectly, with the magnitude of % cytotoxicity varying minimally. This is consistent with a large number of publications showing that xCELLigence data consistently recapitulates data obtained by traditional assays.

In a second example of liquid tumor cell killing, **Figure 2** shows 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 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.

Ordering Information

Instruments	Cat. No.	Pack Size
xCELLigence RTCA DP Instrument	00380601050	1 Bundled Package
RTCA DP Analyzer	05469759001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
xCELLigence RTCA SP Instrument	00380601030	1 Bundled Package
RTCA Analyzer	05228972001	1 Instrument
RTCA SP Station	05229057001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
xCELLigence RTCA MP Instrument	00380601040	1 Bundled Package
RTCA Analyzer	05228972001	1 Instrument
RTCA MP Station	05331625001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
xCELLigence RTCA HT Instrument	00380601070	1 Bundled Package
RTCA Analyzer	05919843001	1 Instrument
RTCA HT Station	05919878001	1 Instrument
RTCA Control Unit	06200176001	1 Notebook PC
Consumables	Cat. No.	Pack Size
E-Plate 16	05469830001 05469813001	6 Plates 6 x 6 Plates
E-Plate VIEW 16	06324738001 06324746001	6 Plates 6 x 6 Plates
E-Plate 16 PET (Polyethylene Terephthalate)	00300600890 00300600880	6 Plates 6 x 6 Plates
E-Plate 96	05232368001 05232376001	6 Plates 6 x 6 Plates
E-Plate VIEW 96	06472451001 06472460001	6 Plates 6 x 6 Plates
E-Plate 96 PET (Polyethylene Terephthalate)	00300600910 00300600900	6 Plates 6 x 6 Plates
E-Plate 384	05867681001 05867673001	2 x 5 Plates 8 x 5 Plates
B Cell Killing Complete Kit	8100004	Reagents + 6 E-Plates + Software
B Cell Killing Sample Kit	8100006	Reagents + 2 E-Plates + Trial Software
B Cell Killing Tethering Kit	8100005	Reagents Only
Leukemic Cell Killing Complete Kit (anti-CD29)	8100007	Reagents + 6 E-Plates + Software
B Cell Killing Sample Kit (includes 2 E-Plate 96)	8100009	Reagents + 2 E-Plates + Trial Software
B Cell Killing Tethering Kit (reagents only)	8100008	Reagents Only
xIMT Software	310100190	1 Flash Drive

Published by:

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