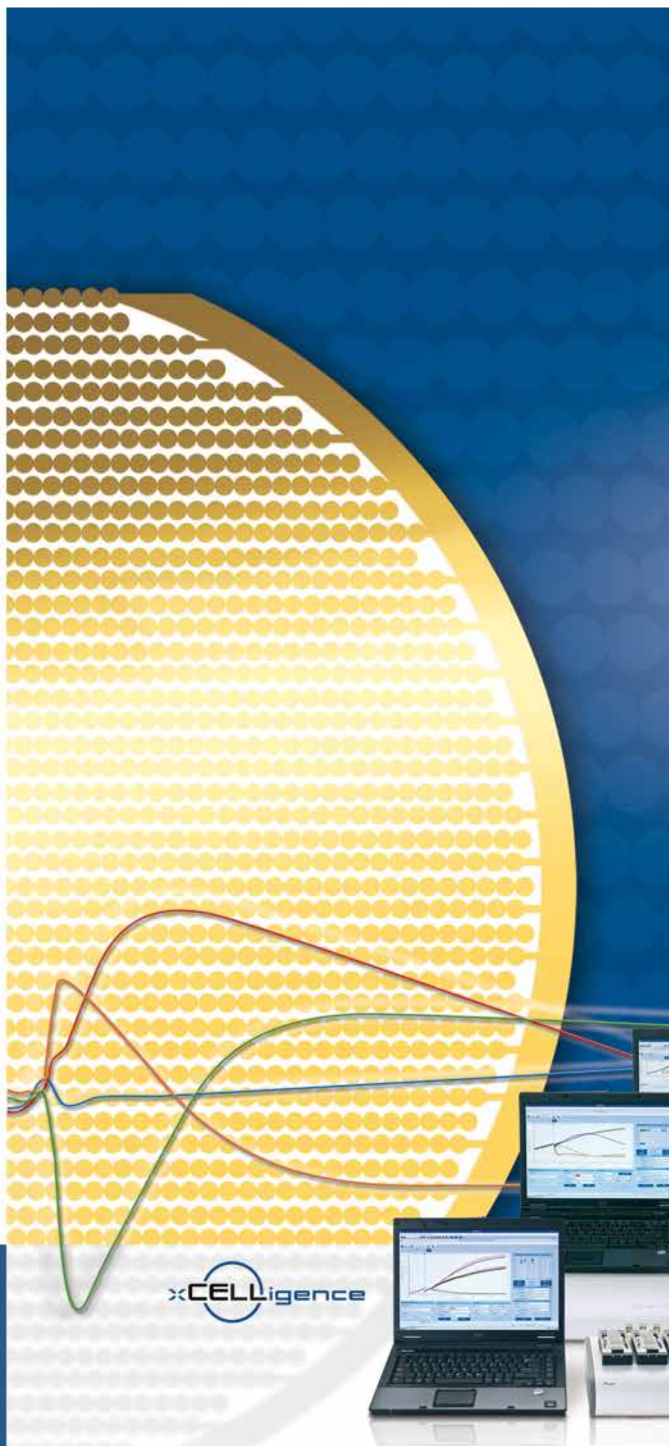
ACEA Biosciences, Inc.

xCELLigence System

Application Note No. 10 / January 2013

Real-Time, Label-Free Measurement of Natural Killer Cell Activity and Antibody-Dependent Cell-Mediated Cytotoxicity



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Real-Time, Label-Free Measurement of Natural Killer Cell Activity and Antibody-Dependent Cell-Mediated Cytotoxicity

Abstract

To determine if cell-mediated cytotoxicity, and specifically antibody-dependent cell-mediated cytotoxicity (ADCC), can be investigated using the xCELLigence System, the response of tumor cells (as target cells) to natural killer (NK) cell activity (as effector cells), in the presence or absence of immunoglobulin G isotype-specific antibody, was measured. Importantly, it is shown that the addition of NK cells in suspension, over a monolayer of adherent tumor cells, does not produce impedance or Cell Index (CI) changes, because the NK cells do not come in contact with the electronic sensor. However, the secretion of perforins and granzymes by these non-adherent NK cells does activate caspases inducing tumor cell apoptosis. Dysfunctional and dying tumor cells detach from the sensor electrode, reducing the number of viable and adhering cells on the electrode surface.

Our findings provide compelling evidence for how the xCELLigence System can be used for the dynamic real-time monitoring of cell-mediated cytotoxicity and the impact of specific antibodies.

Introduction

Cell-mediated cytotoxicity is an important means by which the body protects itself from pathogenic attacks, providing an essential defense mechanism against viruses, bacteria, or parasites, as well as transformed and dysfunctional cells. It also plays an important role in maintaining homeostasis of the immune system. Cell-mediated cytotoxicity specifically involves leukocytes that recognize and destroy other cells or invaded microbes. Two types of leukocyte-effector cells carry out this activity:

- Lymphoid cells known as cytotoxic T-lymphocytes, and natural killer (NK) cells
- Myeloid cells such as macrophages, eosinophils, and neutrophils

The most important role of cytotoxic T-lymphocytes is the elimination of cells infected with viruses. In addition to expressing their own cell-specific peptides on their cell surface, infected cells also exhibit processed pathogen-specific antigens in association with the major histocompatibility complex (MHC) class I molecules. Virus-infected cells present these pathogen-specific antigens to cytotoxic T-lymphocytes, which are capable of distinguishing between *self* and *non-self* peptides, and can mobilize an anti-viral response, accordingly.

Cytotoxic T-lymphocytes and NK cells act in a complementary way to protect the body, because NK cells specifically recognize and kill cells that exhibit reduced or have lost MHC class I expression. Importantly, tumor cells also commonly express reduced levels of MHC class I molecules, which provides the basis for an effective anti-tumor effect of NK cells.

Cell-mediated cytotoxicity is a result of the following complex cell-cell interactions ultimately killing the targeted cells:

1. Direct cell-cell interactions producing internalization and phagocytosis of infected cells or pathogens by macrophages and neutrophils
2. Secretion of cytokines, such as Fas ligand and tumor necrosis factors, by cytotoxic T-lymphocytes
3. Release of granule proteins, such as perforin and granzymes, by both cytotoxic T-lymphocytes and NK cells
4. Release of toxic molecules, such as reactive oxygen intermediates and lysosomal enzymes, by myeloid cells

Cellular degranulation is also facilitated by a process known as 'opsonization' of the target cells by cross-linking antibodies. In this complex event, antibodies first specifically attach via their Fab fragments to antigens on the surface of the target cells to be killed. Opsonization confers the required spatial proximity between target and effector cells, by specifically binding the Fc fragments of the target cells directly to the Fc receptors of the corresponding effector cells (*i.e.*, cytotoxic T-lymphocytes and NK cells). The subsequent cross-linking of Fc receptors is an essential part of this immune response leading to target cell death known as antibody-dependent cell-mediated cytotoxicity (ADCC).

Rationale for a New Method to Measure NK Cell Activity and Additive Antibody-Dependent Cytotoxicity

Monitoring and quantifying the lytic activity of effector cells, such as cytotoxic T-lymphocytes and NK cells, is very important for defining physiological and pathophysiological states, such as immunocompetence during cancer development, response to infectious viral agents, and autoimmune reactions. These types of investigations are also essential for characterizing the lysis process itself and for identifying lysis mediators. The phenomenon of cell-mediated cytotoxicity via ADCC can be studied *in vitro*, using either fresh lymphocytes isolated from blood or natural killer cell lines as effectors. Appropriate *in vitro* targets include pathogen-infected eukaryotic cells and tumor cell lines.

The most common method for measuring cell-mediated cytotoxicity is the release assay based on the loss of target cell membrane integrity (Brunner et al., 1968). Within up to four hours following effector cell addition resulting in target cell lysis, either the radioactive release from target cells prelabeled with Chromium (^{51}Cr) or Indium (^{111}In) is measured, or the release of naturally occurring substances, such as lactate dehydrogenase (LDH), into the culture medium is assayed. Release of these substances thus serves as an indirect measure of the extent of cell damage due to effector cell-mediated target cell lysis (Brunner et al., 1968). Alternative methods also include flow cytometry, enzyme-linked immunosorbent assay-based granzyme measurement, and morphometric analyses by microscopy (de Meyer et al., 2003).

It is important to keep in mind that all these complex labor-intensive studies are endpoint assays, with usually a single measurement over a period of hours. Moreover, the use of radioactively labeled target cells is inconvenient and may also affect the cellular response that is being studied. In contrast, the xCELLigence System enables dynamic, label-free, and non-invasive analysis of cellular events in real time. This system is based on a microelectronic sensor measuring impedance (Solly et al., 2004).

The xCELLigence System

The xCELLigence product line includes the Real-Time Cell Analyzer (RTCA) Single Plate (SP) and Multiple Plate (MP) Instruments. Both the **RTCA SP Instrument** and **RTCA MP Instrument** consist of several components:

- RTCA Analyzer for real-time data acquisition
- RTCA Control Unit with integrated RTCA Software for running and analyzing experiments
- RTCA SP Station or RTCA MP Station

The RTCA SP and MP Stations fit inside a standard cell culture incubator, ensuring a controlled temperature, humidity, and CO_2 environment. RTCA SP and MP Stations accept either one or up to six 96-well plates (**E-Plate 96**), respectively.

Electronic sensors measure cell impedance via electrodes located at the bottom of each well. The measured electrode impedance is expressed in terms of a Cell Index (CI). The CI is a dimensionless value representing the impedance changes of a cell population in contact with the sensing electrode over time.

The electronic sensing principle underlying the xCELLigence System is that cells adhere and attach to the sensor surface located in the bottom of each cell culture well of the specially engineered E-Plates. In contact with the electronic sensor, adherent cells behave as insulators generating resistance to current flow. These small changes in impedance are continuously measured by RTCA Instruments, and spatially integrated and expressed over time by the instrument software as the CI.

The xCELLigence System also includes the **RTCA Dual Plate (DP) Instrument**, which supports up to three impedance-based plates in two formats: the sixteen-well **E-Plate 16** for cellular assays and the **CIM-Plate 16** for cell invasion/migration assays.

Introduction continued

Real-time Cell Index (CI) monitoring is a reflection of the following basic parameters:

- Cell proliferation kinetics
- Cell size
- Strength of cellular adherence
- Cell viability
- Cell morphology

The RTCA SP, MP, and DP Instruments are suitable for addressing how cells respond in a broad spectrum of research fields, including drug development, toxicology, cancer biology, medical microbiology, and virology (Solly et al., 2004). To date, the impedance-based technology of the xCELLigence System has been shown to work for a diversity of applications such as cell proliferation and cytotoxicity (Solly et al., 2004), cell adhesion and spreading (Atienza et al., 2005), cell culture quality control (Kirstein et al., 2006), receptor tyrosine kinase activation (Atienza et al., 2006), mast cell activation (Abassi et al., 2004), G protein-coupled receptor (GPCR) activation (Yu et al., 2006), and most importantly in the context of this application note, in detection of NK cell activity in combination with ADCC (Glamann and Hansen, 2006).

Results

Dynamic Monitoring of NK Cell-Mediated Cytolysis of DU145 Cells

To assess cell-mediated cytolytic activity, the DU145 prostate cancer line was seeded as the target at a density of 5000 cells per well into an E-Plate 96. Cells were allowed to attach and proliferate for 20.5 hours. By then they had reached their logarithmic growth phase as evidenced by dynamic Cell Index (CI) monitoring of the DU145 cell population by the RTCA MP Instrument, every 15 minutes (Figure 1A). At 20.5 hours, real-time measurements were paused, medium was removed, and DU145 cells were exposed to NK92 cells stably overexpressing FcγR III (CD16). These effector cells were added to the target cells in NK cell medium at varying effector-to-target (E:T) cell ratios, ranging from 0.47:1 to 30:1 (a seven-fold doubling dilution series).

After addition of NK92 cells, CI measurements were restarted and changes in proliferation kinetics of the DU145 cells were recorded every 15 minutes for 67.5 hours. As shown in Figure 1A, there was an initial slight decline in CI values after adding the NK cells, due to medium and temperature changes. Within an hour after this decline in CI for all treated wells, there was a clear correlation between the number of added effector cells and the concomitant decrease in CI value (Figures 1A and 1B).

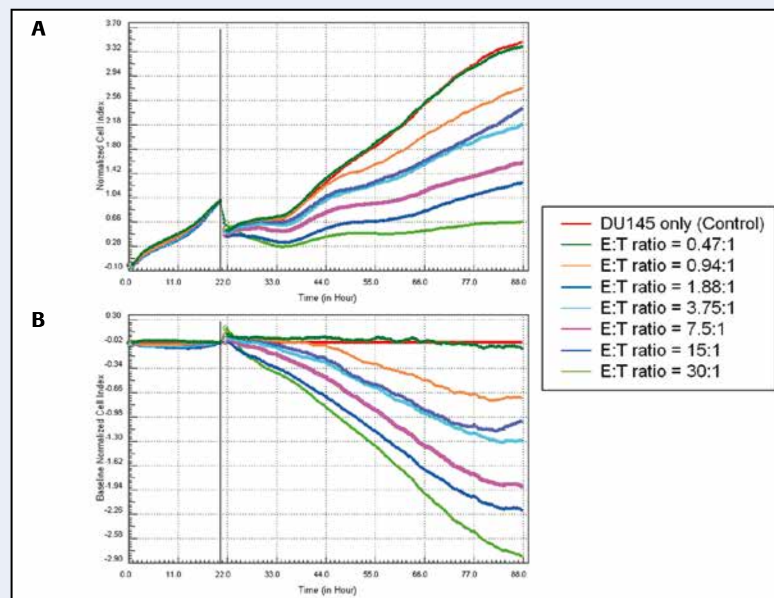


Figure 1: Real-time, label-free monitoring of NK92 cell-mediated cytolysis of DU145 cells. 5000 DU145 cells per well were seeded in triplicate into an E-Plate 96 and monitored every 15 minutes using the RTCA MP Instrument. At 20.5 hours, NK92 cells were added to the wells at E:T ratios ranging from 0.47:1 to 30:1. The effect of NK cell addition was monitored for 67 hours.
A. This plot shows data normalized to the last time point before NK cell addition.
B. Same data as above, but with non-treated control cells (red line) defined as baseline. Other curves are plotted in relation to this baseline. Plots were generated using RTCA Software 1.1.

Comparing the data between NK92 cells (E) to DU145 (T) cells with E:T ratios from 0.47:1 to 30:1, the CI values show that over time, the fewer NK cells added to the well, the fewer target cells are lysed, and the greater the increase of CI. Treatment with NK92 cells at an E:T-ratio of 30:1 appears not to allow recovery of the target cells, indicating that the majority of DU145 cells were damaged by NK cell-mediated cytolysis (Figure 1A, light green line).
This strong cell lytic effect is observed within a very short incubation time compared to control cells (Figure 1A, red line).

Target cells treated with lower numbers of NK cells (Figure 1A, dark green and other lines) reach distinctive plateau phases of growth at 88 hours, dependent on the number of NK cells introduced to the DU145 cells. Please note, when comparing plots normalized to either the last time point before NK cell addition (Figure 1A), or to the control cell baseline (Figure 1B), all E:T cell ratios, *except* the lowest E:T ratio of 0.47:1, produced a step-wise differential change in CI values between 33 to 88 hours of the experiment.

Dynamic Monitoring of anti-IGF-1R-Dependent NK Cell-Mediated Cytolysis of DU145 Cells

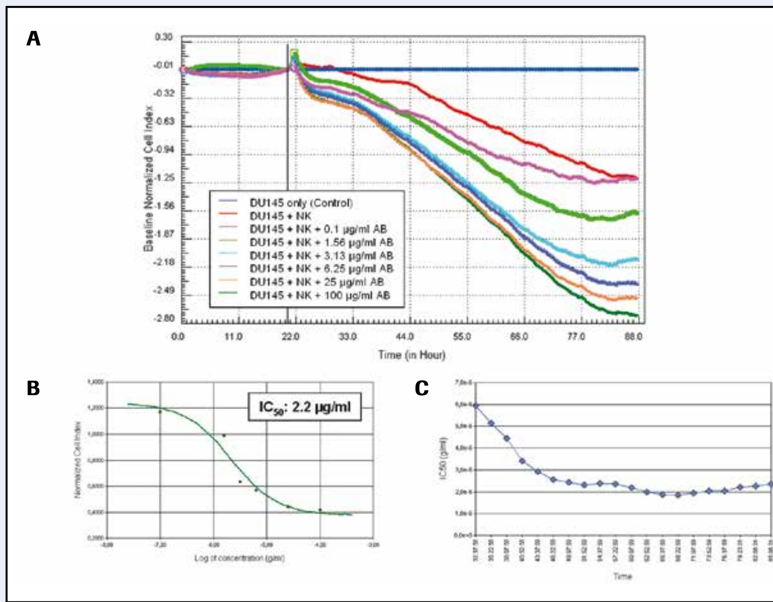
In the next experiments, cytolytic activity of NK92 cells on DU145 cells was examined in the presence of the anti-IGF-1R monoclonal antibody, which binds to both extracellular human IGF-1R (via its Fab fragments) and the Fcγ receptor III (FcγR III) on NK92 cells (via its Fc fragment).The insulin-like growth factor 1 receptor (IGF-1R) is a member of the tyrosine kinase receptor family, mediating the effects of IGF-1, a polypeptide similar to insulin. IGF-1 plays an important role during development and growth, and IGF-1 and IGF-1R are implicated in several types of cancer (Hartog et al., 2007). The development of therapeutic antibodies against IGF-1R, based on their involvement in NK cell-mediated lysis of tumor cells, may be a strategy to treat or prevent cancers (Stagg and Smyth, 2007).

Again, 5000 DU145 cells were seeded into each well of an E-Plate 96 and logarithmic growth was monitored every 15 minutes for 20.5 hours. NK cells in NK cell medium were added at E:T ratios of 3.75:1 (Figure 2A) and 1.88:1 (Figure 3A). DU145 target cells were pre-incubated with different amounts of anti-IGF-1R for 30 minutes, resulting in final concentrations between 0.1 and 100 µg/ml. The effect of NK cells without antibody (untreated control), as well as the effect of the varying amounts of antbody were recorded every 15 minutes for 67.5 hours.

In comparison to non-treated control cells, NK cells induced a moderate decline in CI values on DU145 cells without antibody pretreatment (red line), due to NK-mediated cytolysis of DU145 cells (compare controls to antibody-treated wells in Figures 2A and 3A). DU145 pretreatment with antibody clearly produced a concentration-dependent decrease in impedance and CI values (summarized in Table 1). Cell Index values of anti-IGF-1R-treated DU145 cells are lower than that of DU145 cells without antibody pretreatment, indicating that the presence of the antibody increases the cytotoxic effect of the NK cells (Figures 2A and 3A, Table 1).

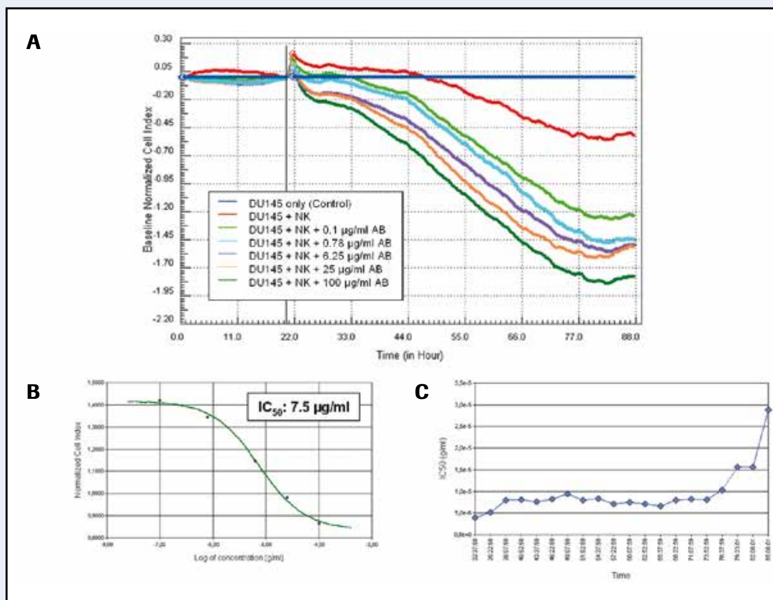
E:T ratio	anti-IGF1R antibody (µg/ml)	Cytolysis (%) after 48 h
0.47:1	–	0
0.94:1	–	21
1.88:1	–	35
	0.1	36
	0.78	42
	6.25	47
	25	52
	100	58
3.75:1	–	37
	0.1	40
	1.56	54
	3.13	69
	6.25	73
	25	79
	100	82
7.5:1	–	54
15:1	–	66
30:1	–	80

Table 1: NK cell-mediated cytolysis of DU145 cells 48 hours after NK cell addition and DU145 cell pretreatment with increasing amounts of IGF-1R antibody. NK92 cells were added to DU145 cells at different effector cell:target cell (E:T) ratios. The percentage of cytolysis was calculated based on the normalized CI values 48 hours after DU145 antibody pretreatment and NK cell addition. The last time point before addition of the NK92 cells was chosen for normalization of data.



A. Plot of normalized CI values of the entire 88 hours of the experiment. Data are normalized to the last time point before NK cell addition and curves were plotted with control wells (DU145 cells only) set as baseline.
B. Calculation of IC_{50} after 60 hours.
C. Calculation of time-dependent IC_{50} from 9 hours after NK cell addition to the end of the experiment at 88 hours. All plots were generated using the RTCA Software 1.1.

Figure 2: Effect of the anti-IGF-1R antibody on NK cell-mediated cytolysis of DU145 cells (E:T ratio = 3.75:1). 5000 DU145 cells per well were seeded in triplicate into an E-Plate 96 and monitored every 15 minutes using the RTCA MP Instrument. After 20.5 hours, NK92 cells were added to the wells at an E:T ratio of 3.75:1. Target cells were pre-incubated in advance with different concentrations of anti-IGF-1R antibody for 30 minutes. The effect of NK cell and anti-IGF-1R addition was monitored for 67 hours.



A. Plot of normalized CI values of the entire course of the experiment (88 hours). Data are normalized to the last time point before NK cell addition and curves are plotted with control wells (DU145 cells only) set as baseline.
B. Calculation of IC_{50} after 60 hours.
C. Calculation of time-dependent IC_{50} from 9 hours after NK cell addition to the end of the experiment at 88 hours. All plots were generated using the RTCA Software 1.1.

Figure 3: Effect of the anti-IGF-1R antibody on NK cell-mediated cytolysis of DU145 cells (E:T ratio = 1.88:1). 5000 DU145 cells per well were seeded in triplicate into an E-Plate 96 and monitored every 15 minutes using the RTCA MP Instrument. After 20.5 hours, NK92 cells were added to the wells at an E:T ratio of 1.88:1. Target cells were pre-incubated in advance with different concentrations of anti-IGF-1R for 30 minutes. The effects of NK cell and anti-IGF-1R pretreatment were monitored for 67 hours.

Anti-IGF-1R is thought to increase the interaction between DU145 and NK cells by cross-linking over-expressed FcγR III on NK92 cells. The CI findings show a clear antibody dose-response dependency and the degree of NK92-mediated cell lysis is also a function of the E:T ratio: The more NK cells administered, the stronger the additive effect of the antibody (Table 1). In contrast, DU145 cell treatment with anti-IGF-1R in the absence of NK92 cells showed no reduction in CI values compared to control levels. In fact, antibody amounts over 6.25 µg/ml produced a slight increase of CI values and corresponding DU145 proliferation curves clearly run above the curve representing non-treated control cells (Figure 4).

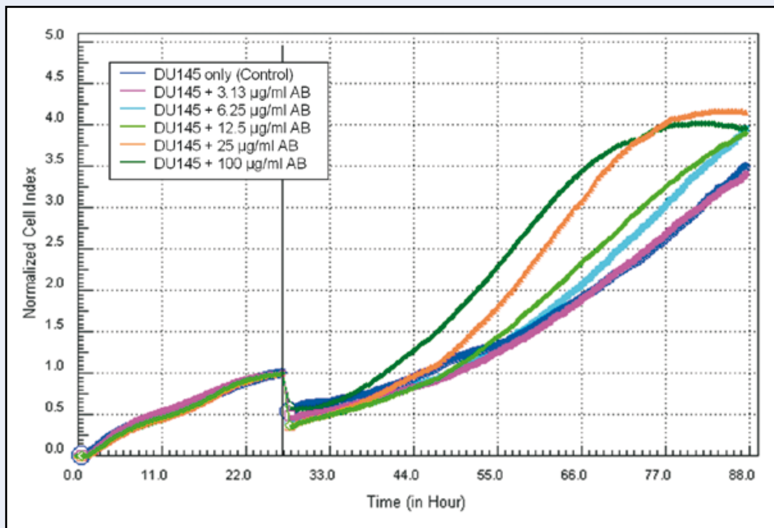


Figure 4: Real-time, label-free monitoring of the effect of anti-IGF-1R antibody on DU145 cells in the absence of NK92 effector cells.
5000 DU145 cells per well were seeded in triplicate into an E-Plate 96 and monitored every 15 minutes using the RTCA MP Instrument. After 27 hours, anti-IGF-1R was added to the wells at the indicated concentrations. The effect of the anti-IGF-1R addition was monitored for 60 hours. Plotted CI values were normalized to the last time point before the addition of antibody. Plots were generated using the RTCA Software 1.1.

Calculation of Half Inhibitory (IC_{50}) anti-IGF-1R Concentrations

To further quantify the effects of the anti-IGF-1R antibody on the NK92 and DU145 cytolytic cell-cell interaction, normalized CI values obtained from the antibody dose-responses were used to calculate IC_{50} values using the RTCA Software 1.1. Data obtained from experiments using either 3.75:1 or 1.88:1 NK92 to DU145 (E:T) cell ratios were used to calculate IC_{50} values of 2.2 µg/ml and 7.5 µg/ml, respectively (Figures 2B and 3B). The IC_{50} value is significantly higher for the lower number of NK cells added. This indicates that approximately 3.5 times more antibody is needed to induce the same extent of target cell lysis when only half of the NK cell amount is administered.

Time-dependent IC_{50} values for more than 50 hours of the experiment were calculated using another tool in the RTCA Software 1.1, showing the excellent reproducibility of IC_{50} values over this long period of time (Figures 2C and 3C).

Summary

The present study shows the feasibility for using the xCELLigence System to monitor both NK cell-mediated tumor cell cytolysis and antibody-dependent cell-mediated cytotoxicity (ADCC) in a label-free, non-invasive manner, corroborating earlier findings by Glamann and Hansen (2006). It was possible to show both the quantitative effect of adding different amounts of NK92 cells, as well as the additive cytolytic effect of introducing an NK92-dependent anti-IGF-1R monoclonal antibody.

The xCELLigence System is thus ideal for directly monitoring ADCC without the need for labeling the target cells or using a chemical reporter. Importantly, the entire cytolytic process can now be measured using impedance electronic sensing to detect both expected and unexpected cellular responses, an impossible task for previously described conventional end-point assay formats.

Materials and Methods

Cell Culture and Antibodies

DU145 target cells and NK92 effector cells were obtained from ATCC. NK92 cells were genetically modified to stably over-express the FcγR III. Both cell lines were grown in a standard cell culture incubator at 37°C and 5% CO_2. DU145 cells were maintained in RPMI 1640 Glutamax media (Gibco) with 10% FCS (Perbio Science), 1% Penicillin, and 1% Streptomycin (Roche Applied Science). NK92 cells were cultured in MEM alpha medium with L-Glutamine (Gibco), 10% FCS, 10% Horse serum (Invitrogen), 0.1 mM 2-Mercaptoethanol (Gibco), 0.2 mM Myo-Inositol (Calbiochem), 0.02 mM Folic Acid (Alfa Aesar), and 10 ng/ml Interleukin-2 (Cell Systems). The monoclonal antibody for the ADCC assay is specific for human IGF-1R, binding the human FcγR III with its Fc fragment.

Instrumentation

Impedance measurements and Cell Index (CI) determinations were performed using an RTCA MP Instrument including RTCA Analyzer, RTCA MP Station, RTCA Control Unit, and dedicated RTCA Software 1.1.

Real-Time Cytolytic Analysis

DU145 cells were seeded in 200 µl growth medium at a density of 5000 cells per well into E-Plates 96. Cell attachment and growth to the logarithmic growth phase were monitored using the RTCA MP Instrument. RPMI medium was removed, then NK92 cells in NK cell medium were added to the wells containing DU145 cells. For the antibody studies, NK cells were added at effector:target (E:T) ratios of either 3.75:1 or 1.88:1 to DU145 cells which had been pre-incubated with different concentrations of an anti-IGF-1R antibody for 30 minutes. Upon addition of the NK92 effector cells, the impedance measurements were made every 15 minutes for up to 88 hours.

Data Analysis

The integrated RTCA Software 1.1 displays the CI values measured in the E-Plate 96 from the time of seeding the DU145 cells, after applying the NK92 cells, up to the end of the experiment at 88 hours. Time- and Effector:Target-dependent proliferation curves are displayed in real time. To quantify the extent of cell lysis at specific time points, CI data was exported to Microsoft Excel, where percentage of lysis calculations at specific E:T ratios and antibody concentrations were calculated in reference to the control. IC_{50} calculations at specific time points or over a period of time were performed using the RTCA Software 1.1.

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Ordering Information

Product	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
E-Plate 16	05469830001	6 Plates
	05469813001	6 x 6 Plates
E-Plate VIEW 16	06324738001	6 Plates
	06324746001	6 x 6 Plates
E-Plate Insert 16	06465382001	1 x 6 Devices (6 16-Well Inserts)
CIM-Plate 16	05665817001	6 Plates
	05665825001	6 x 6 Plates
E-Plate 96	05232368001	6 Plates
	05232376001	6 x 6 Plates
E-Plate VIEW 96	06472451001	6 Plates
	06472460001	6 x 6 Plates
E-Plate Insert 96	06465412001	1 x 6 Devices (36 16-Well Inserts)
E-Plate Insert 96 Accessories	06465455001	6 Units (6 Receiver Plates + 6 Lids)

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