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Label-Free Assay for NK Cell-mediated Cytolysis on the RT-CES™ System

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Introduction

Natural Killer (NK) cells are bone marrow derived lymphocytes originally identified by their large granular morphology. The NK cell lineage has been considered for cancer eradication owing to its ability to kill a wide variety of tumor cells while sparing normal cells. Importantly, NK cells spontaneously lyse certain tumor targets *in vivo* and *in vitro* without the need for immunization or pre-activation compared with T cells, which first require education by antigen-presenting cells. Using NK cells, several *in vivo* and *in vitro* studies have shown promising anti-tumor effects in addition to extravasation and infiltration of tumor tissues by NK cells. Mice with compromised NK cell function are more susceptible to carcinogen-induced cancers. In addition, individuals lacking NK cells suffer from persistent viral infections and as a consequence die prematurely. The potency of uncontrolled or inappropriate NK cell responses is evident in disease conditions such as allograft rejection, graft vs. host disease, diabetes, various autoimmune and neurological diseases, and aplastic anaemia/neutropia. Therefore, NK cells play a prominent role in various physiological and disease states and the assessment of its cytolytic activity is not only important for monitoring of immunocompetence in cancer, infectious diseases, and autoimmune diseases, but also in determining the proteins which mediate the cytolytic effect.

The standard methods used for measuring NK cell cytolytic activity are the radioactive Chromium (^{51}Cr) or the Indium (^{111}In) release assays [1,2]. In radioactive label-based assays, the target cells are labeled and then mixed with effector cells. The release of the radioactive isotope, which correlates with NK cell-mediated cytolysis is then measured at a given time point (less than 4 hours). Several non-radioactive labeling assays are also available, including flow cytometry, ELISA based granzyme measurement (BD Biosciences), and morphometric analysis by microscopy[3].

Unlike the label-based end point assays, ACEA Biosciences has developed a label free assay format allowing for dynamic monitoring of NK cell-mediated cytolysis. The assay is based on ACEA proprietary electronic sensor technology, and is conducted on the

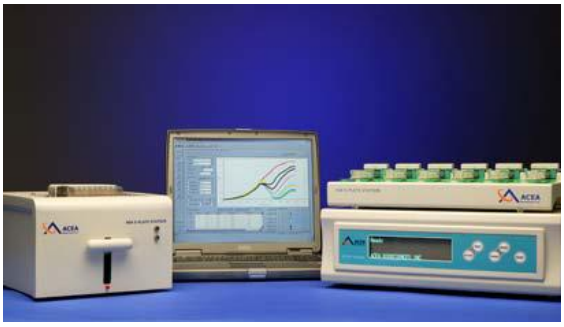


Figure 1. ACEA Biosciences RT-CES™ system. RT-CES Analyzer shown on bottom right, 16x E-Plate station on top right, and 96x E-Plate station on the left.

RT-CES system[5]. The RT-CES system is comprised of an analyzer, integrated software and 16 or 96-well electronic plate (e-plate), which has microelectrodes integrated in the bottom of the wells and measures cell-electrode impedance. The attachment and interaction of adherent cells with the surface of the electrodes lead to impendence changes, which correlate with cell number. In contrast, addition of suspended cells to the wells results in undetectable changes due to non-existent or weak interaction with the electrodes. Therefore, NK cell-mediated cytolytic effect on the cancer cell monolayer can be quantitatively and dynamically monitored on the sensor plate without labeling the target cells.

Methods and Materials

Cells The NK 92, the NIH 3T3 cell line, and most cancer cell lines used in these experiments were purchased from ATCC. The mouse NK cell line (mNK) was provided by Dr. Hui Shao of University of Louisville. All the cell lines were maintained at 37^{o}C incubator with 5% CO_2. The NK92 and mNK lines were maintained in Alpha MEM with 2 mM L-glutamine, 1.5 g/L Sodium bicarbonate, supplemented with 0.2 m inositol, 0.1 mM 2-mercaptoenthanol, 0.02 mM folic acid, 12.5% horse serum, 12.5% FBS, and 100-200 U/ml recombinant IL-2. Other cancer cell lines were maintained in RPMI media containing 5% FBS and 1% penicillin and streptomycin (GIBCO). The NIH 3T3 cells were maintained in DMEM media containing 10% FBS and 1% penicillin and streptomycin.

RT-CES system The system includes three components: the analyzer and e-plate station, the integrated software, and 16-well or 96-well e-plate (Figure 1) (www.aceabio.com). The e-plate station is placed inside

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the incubator and connected to the analyzer outside the incubator through a thin cable. The e-plate containing the cells is placed onto the e-plate station inside the incubator and the experiment data are collected automatically by the analyzer under the control of integrated software.

Cytolytic analysis Target cells were seeded into the wells of either 16-well or 96-well e-plate in 100 µl of media. Cell growth was dynamically monitored using the RT-CES system for a period of 24 -34 hours, depending on the experiment until they reached log growth phase and formed a monolayer. Effector cells at different concentrations were then directly added into individual wells containing the target cells. For background control, effector cells were added to a well without target cells. After addition of the effector cells, the e-plate was returned to the e-plate station and the measurements were automatically collected by the analyzer every 15 minutes for up to 20 hours.

Cell morphology analysis by microscopy The effect of NK cell-mediated cytolysis on target cells was examined using a Nikon upright microscope. When Cell Index reduced to 50% of the control upon addition of effector cells, cells were fixed in 80% methanol for 5 minutes and stained with Giemsa blue. The morphology of the cells was examined by microscopy and photographed using an accompanying CCD camera.

Experiment data analysis The integrated software is able to display entire history of the experiment from seeding the cells to the end of cytolysis. The time- and effector to target ratio (E/T) -dependent curves can be displayed in real time allowing for dynamic monitoring of NK cell activity. The electronic readout, cell-electrode impedence is displayed as an arbitrary unit called Cell Index. Cell Index at each time point is defined as Rn-Rb/Rb where Rn is defined as the cell-electrode impedance of the well with the cells and Rb is defined as the background impedance of the well with the media only.
To quantify the lysis at specific time points, the data was exported to excel and percentage cytolysis at specific E/T ratio was determined by comparison to the control.

Results and Discussion

Dynamic monitoring of NK cell-mediated cytolysis

To assess NK cell-mediated cytolytic activity using the RT-CES system, a murine NK cell line (mNK line), and a target cell line, NIH 3T3 cells were used. The target NIH3T3 cells were seeded in the wells of 96-well e-plate at 5,000 cells per well and cell growth was continuously monitored on RT-CES system every 60 minutes until the cells reached growth phase, 34 hours later. The effector murine NK cells were then directly added to the well at E/T ratio of 15:1, and the NK cell-mediated cytolyses was dynamically monitored on the RT-CES system. As shown in the figure 2a, a significant decline of the Cell Index was seen in NIH 3T3 cells after the addition of mNK cells at the E/T ratio of 15 to 1, compared to the Cell Index from the media control. Furthermore, no significant decline of the Cell Index was seen in the effector control wells using YAC cells, a T lymphocyte line without cytolysis effect, indicating the decrease in the Cell Index due to addition of the mNK cells is specific and is most likely mediated by cytolysis. A time-dependent cytolysis of the NIH 3T3 cells was also seen in the presences of mNK cells but not in the presence of YAC cells (figure 2b). To further confirm the cytolysis effect, target cells were stained at 8 hours after addition of the mNK cells to target cells, when the cytolysis was approximately 50%, and then examined under microscope. As shown in figure 2c, in the presence of mNK cells, the target cells were effectively cleared away by the cytolytic action of the mNK when compared to control YAC cells. In summary, RT-CES system offers the only assay format so far to directly monitor NK cell mediated cytolysis without labeling the target cells and without using any chemical reporters. In addition, the entire history of the cytolysis can be dynamically monitored on the RT-CES system, which is difficult to perform by any label-based and endpoint assay format. Analysis of the kinetics of cytolysis indicates that for the mNK cells, a slow cytolysis was detected. The cytolysis is detected within 2 hours of mNK cell addition and interestingly, after 4 hours only less than 30% cytolytic activity is detected. Four hour incubation time is the standard incubation period for radioisotope based endpoint assays. Data obtained by the RT-CES system clearly shows that cytolytic activity can reach up to 70%, which occurs after 12 hours of adding the mNK cells. Such cytolytic activity which occurs after the standard incubation time of the assay can be easily missed by existing label-based, endpoint assays. Therefore, the RT-CES system not only offers label free detection but, more importantly, also allows more accurate assessment of cytolytic activity by dynamically monitoring the entire history of cytolysis.

Quantitative measurement of NK cell-mediated cytolysis

The Cell Index readout of the RT-CES system is correlated with cell number[5], and has been used previously to quantitatively monitor cytotoxicity induced by chemical compounds such as anticancer drugs. To test whether mNK cell activity can be quantitatively assayed in the RT-CES system, we monitored cytolysis at different ratios of effector/target. Both murine and human NK cell lines (mNK and NK92) were used as effectors, and the NIH 3T3 line and the MCF7 line (human breast cancer cells) were used as the targets for each of the effectors, respectively. As described above, the target cells were seeded to the 96-well e-plate at 5,000cells/well, and cell growth was monitored on the RT-CES system. When the target cells reached growth phase, the NK cells were directly added

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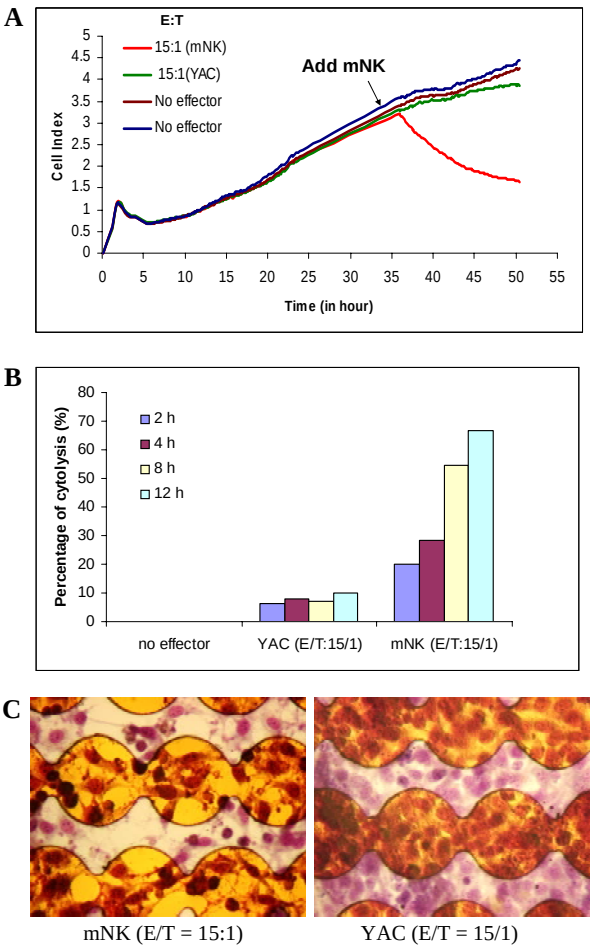


Figure 2. Dynamic monitoring of NK cell-mediated cytolysis on the RT-CES system

(**A**) Dynamic monitoring of NK-cell mediated cytolysis of NIH 3T3 cells. The NIH 3T3 cells were seeded in the wells of the 96-well e-plate at 5000 cells/well, and cell attachment, spreading and proliferation were monitored in real time. 34 hours after seeding of the cells, Cell Index values reached 3, which is equivalent to approximately 10,000cells/well. 150,000 mNK cells or YAC cells that function as the negative control were added to each well in triplicates. The mNK cell-mediated cytolysis was then dynamically monitored. (**B**) Time-dependent cytolytic activity of mNK cells. The cytolytic activity at a given time point was calculated and presented as the percentage of cytolysis, { % of cytolysis = ($CI_{no\ effector} - CI_{effector}$)/$CI_{no\ effector}$ X 100}. (**C**) The morphological examination of cytolysis by mNK cells. The specific cytolysis of NIH 3T3 cells by mNK cells was examined by microscopy after staining the cells with Giemsa blue.

to wells at different concentrations. The NK cell-mediated cytolyses at different E/T ratios were then monitored in real time on RT-CES system. As shown in figure 3, subsequent to the addition of mNK or NK92 cells to its target cells, the Cell Indices declined compared to the no effector control. The decline in the Cell Index values is E/T ratio dependent, and occurs due to the loss of cell/electrode interaction due to cytolysis. For either case, the higher the E/T ratio, the lower Cell Index value was obtained. This strongly indicates that RT-CES system allows for specific and quantitative measurement of NK cell-mediated cytolytic activity. Moreover, the dynamic monitoring of the cytolysis may provide more insights with respect to the underlying mechanisms of NK cell-mediated killing. For example, analysis of the dynamics of cytolysis indicates that the NK92 cells are much more potent effectors than mNK cells. At the E/T ratio of 4 to 1 or higher, greater than 90% cytolysis of the MCF7 can be achieved within 4 hours after addition of NK92 cells, whereas as for mNK cells it is only about 30 %. The different cytolytic kinetics of NK cells indicates the distinct nature of interaction between effector and target, such as expression of NK receptors and ligands, and mechanisms underlying the NK cell-mediated cytolysis.

Label free assessment of NK cell cytolytic activity in a variety of target cell lines on the RT-CES system

Cytolytic activities of mNK and NK92 were tested using 9 cell lines, which include 8 different human cancer cell lines and the NIH 3T3 cell line. The susceptibility of different target cells lines to mNK or NK92-mediated cytolysis is summarized in Table 1, and 2. NK92 shows a broad spectrum of cytolytic activity on cancer cell lines. The cytolysis mediated by NK92 occurs fast and reaches the maximum killing activity prior to 8 hours after addition of NK92 cells. The comparison of susceptibility of 7 cancer cell lines to NK92 cells is shown in figure 4a. Among 7 cancer target lines, over 90% cytolysis of 4 target lines was achieved, including H460, HepG2, MCF7 and MDA-MB231. In contrast, mNK cell-mediated cytolysis appears to be more selective than NK92 (figure 4b). Among 9 target cell lines, 4 target cell lines showed cytolysis of over 30% after 12 hours of incubation with mNK cells, including NIH 3T3, A549, Hela, and MDA-MB231, while no cytolysis (0%) or weak cytolysis (10%) was found in 5 target lines, including OVCR4, HT1080, HepG2, H460 and MCF7. In addition, the cytolysis mediated by mNK was much slower than NK92, reaching the maximum after 12 hours of addition of mNK cells.

In summary, we introduce a microelectronic sensor-based platform system, the RT-CES (real time electronic sensing) system for label free assessment of NK cell-mediated cytolytic activity. A human and a murine NK cell lines were both tested for their cytolytic activities using 9 different target cell lines, including human cancer cell lines commonly used in the field. The quantitative and dynamic measurement of NK-cell mediated cytolysis was achieved on the RT-CES system without any labeling steps and reagents. Moreover, RT-CES system offers fully automated measurement of the cytolysis in real time, which enables a large scale screening of chemical compounds or genes responsible for the regulation of NK cell-mediated cytolytic activity.

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Table 1. mNK cell-mediated cytolysis of 9 cell lines

Cell Name	Cell Type	Species	Maximum Cytolysis (%) at 12 h
NIH 3T3	Fibroblast	Murine	58.8
HT1080	Fibrosarcoma	Human	0.1
H460	Non-small cell lung cancer	Human	9.46
HepG2	Hepatoma	Human	0
MCF7	Breast cancer	Human	11
A549	Non-small cell lung cancer	Human	64
HELA	Cervix cancer	Human	30.3
OVCAR4	Ovarian cancer	Human	0
MDA-MB-231	Breast cancer	Human	31.5

Table 2. NK92 cell-mediated cytolysis of 7 cell lines

Cell Name	Cell Type	Species	Maximum Cytolysis (%) at 8h
HT1080	Fibrosarcoma	Human	42.17
H460	Non-small cell lung cancer	Human	95.4
HepG2	Hepatoma	Human	94.1
MCF7	Breast cancer	Human	96.5
A549	Non-small cell lung cancer	Human	52.2
HELA	Cervix cancer	Human	51
MDA-MB-231	Breast cancer	Human	97

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Figures

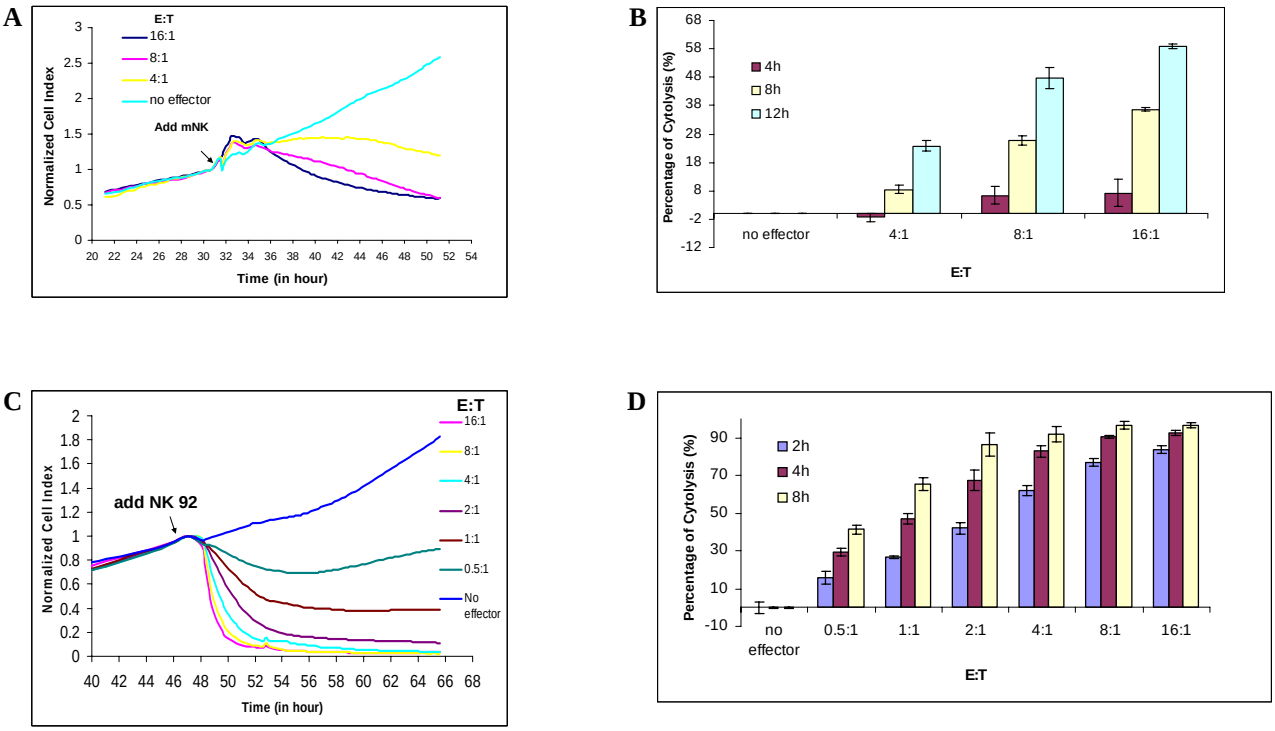


Figure 3. Label free and quantitative measurement of cytolytic activity of mNK cells and NK92 cells. (**A**). Quantitative measurement of cytolytic activity of mNK cells. The NIH 3T3 cells were seeded to the 96-well e-plate. Cell growth was monitored in real time on the RT-CES system until the CI values reached 3, equivalent to 10,000cells/well. The mNK cells were added to target cells at different cell concentrations, making a series of E/T ratios as indicated. The cytolysis of the target cells at different E/T ratios was dynamically monitored on the RT-CES system. The normalized Cell Index was used, in which the Cell Index values obtained from post addition of mNK cells were normalized against the Cell Index value from the same well before the addition of mNK cells. (**B**). Time-dependent cytolytic activity of mNK cells at different E/T ratios. The percentage of cytolysis of the NIH 3T3 cells by mNK cells was calculated as described in figure 2. The time-dependent cytolytic activities at different E/T ratios are indicated. (**C**). Quantitative measurement of cytolyic activity of NK92 cells. The target cells, MCF7 were seeded and the cell growth was monitored on the RT-CES system as described above. The NK92 cells were then added to each well at different concentration making the series of E/T ratios as indicated. The cytolytic activities of NK92 cells on MCF7 cells at different E/T ratios were dynamically monitored on the RT-CES system. (**D**). Time-dependent cytolytic activity of NK92 cells at different E/T ratios. The time-dependent cytolysis of the MCF7 cells at different E/T ratios was calculated as described in figure 2, and indicated.

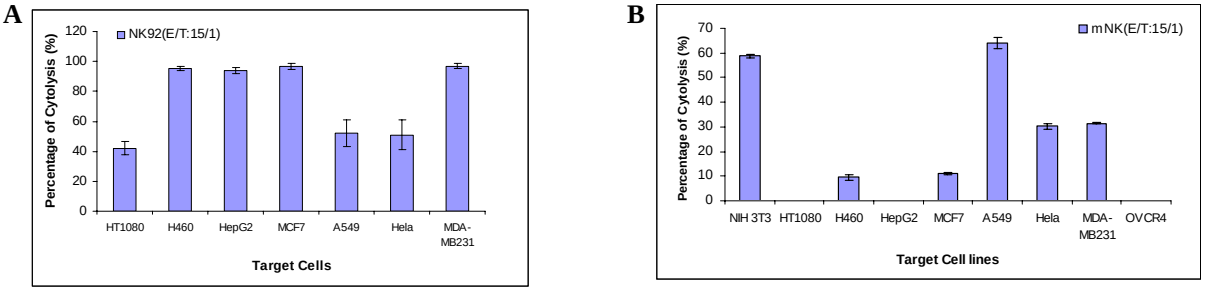


Figure 4. Label free assessing cytolysis of a variety of cell lines by NK cells on RT-CES system. (**A**) The NK92-mediated cytolysis of 7 different cancer lines. The percentage of cytolysis indicated for each line is calculated based on the Cell Index value of individual wells after 8 hours of addition of NK92 cells, which showed the maximum cytolysis. (**B**) The mNK-mediated cytolysis of 9 different cell lines. The percentage of cytolysis indicated for each line is calculated based on the Cell Index values of individual wells after 12 hours of addition of mNK cells, which showed the maximum cytolysis.

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