Application Note

Cell Analysis

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Label-Free Assay for NK Cell-Mediated Cytolysis

xCELLigence real-time cell analysis

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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 due to its ability to kill a wide variety of tumor cells spontaneously, while sparing normal cells. While T cells must be educated by antigen-presenting cells before they recognize tumors, NK cells spontaneously lyse certain tumor targets *in vivo* and *in vitro* without requiring immunization or pre-activation. Several *in vivo* and *in vitro* studies have shown that, in addition to extravasation and the ability to infiltrate tumor tissues, NK cells have promising antitumor effects.

In addition, individuals lacking NK cells suffer from persistent viral infections and die prematurely as a consequence. The potency of uncontrolled or inappropriate NK cell responses is evident in disease conditions, such as allograft rejection, graft versus host disease, diabetes, various autoimmune and neurological diseases, and aplastic anemia/neutropenia. NK cells therefore play a prominent role in various physiological and disease states. The assessment of their cytolytic activity is important for monitoring immunocompetence in cancer, infectious diseases, and autoimmune diseases, and in determining the proteins that mediate the cytolytic effect.

The standard methods for measuring NK cell cytolytic activity are radioactive label release assays[1,2] using chromium (^{51}Cr), or indium (^{111}In).

In these assays, the target cells are radioactively labeled 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 four hours). Several nonradioactive labeling assays are also available, including flow cytometry, ELISA-based granzyme measurement, and morphometric analysis by microscopy.[3]

The Agilent xCELLigence system is an impedance-based real-time cell analysis (RTCA) technology. The attachment and interaction of adherent tumor cells with biosensors at the bottom of 96-well E-Plate leads to impedance changes, which correlate with cell number, size, and shape. In contrast, the addition of suspended NK cells to the wells results in negligible changes due to nonexistent or weak interaction with the biosensors. In this application note, we will summarize a series of experiments to demonstrate how the xCELLigence RTCA system can be applied to quantitatively, dynamically monitor NK cell-mediated cytolysis without labeling the target cells.

Materials and methods

Cells

The NK92, NIH 3T3, and all cancer cell lines used in these experiments were obtained from ATCC. The murine NK cell (mNK) was provided by Dr. Hui Shao of the University of Louisville. All the cell lines were maintained in a 37 °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 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 12.5% horse serum, 12.5% FBS, and 100 to 200 U/mL recombinant IL-2. Other cancer cell lines were maintained in RPMI media containing 5% FBS, 1% penicillin, and 1% streptomycin (Gibco). The NIH 3T3 cells were maintained in DMEM media containing 10% FBS, 1% penicillin, and 1% streptomycin.

Cytolytic analysis

Target cells were seeded (after establishing background with medium alone) into the wells of 96-well E-Plates in 100 µL of media. Cell growth was dynamically monitored with the xCELLigence RTCA system until they reached the log growth phase and formed a monolayer (24 to 34 hours, depending on the experiment). Effector NK cells at different concentrations were then directly added to the wells. Effector cells in wells without target cells are for the effector only background control. After the addition of the effector cells, the system continued to take measurements 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 the Cell Index (CI) dropped to 50% (relative to the control) after the addition of effector cells, cells were removed from the system, fixed in 80% methanol for five minutes, and stained with Giemsa blue. The morphology of the cells was examined under the microscope and imaged with a CCD camera.

Experiment data analysis

The integrated xCELLigence RTCA software displays the 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 were displayed in real time, so NK cell activity was monitored continuously. The measured impedance is expressed in arbitrary CI units. The CI at each time point was defined as $(R_n - R_b)/15$, where R_n was the cell-electrode impedance of the well when it contains cells, and R_b was the background impedance of the well with the media alone.

To quantify the lysis at specific time points, the data were exported to Microsoft Excel, and the percentage cytolysis at specific E/T ratios was determined by comparing with the control.

Results and discussion

Dynamic monitoring of NK cell-mediated cytolysis

An effector murine NK cell line (mNK), and a target mouse cell line (NIH3T3) were used to assess NK cell-mediated cytolytic activity. 5,000 NIH 3T3 cells were seated in wells of 96-welll E-Plate. An xCELLigence RTCA system was used to monitor the cell growth every 60 minutes until the cells reached the growth phase at 34 hours. The effector murine NK cells were then directly added to the wells at different E/T ratios. The NK cell-mediated cytolysis was continuously monitored afterwards. As shown in Figure 1A, Cell Index declined significantly after adding mNK effector cells at the E/T ratio of 15:1, but no notable change was observed in wells without effector cells and negative control wells with YAC cells, which is a T lymphocyte line. This indicates that the decrease in the CI was due to the addition of the mNK cells and most likely mediated by cytolysis. This mNK cell-mediated cytolysis is time-dependent (Figure 1B). To further confirm the cytolysis effect, target cells were stained at eight hours after the addition of the mNK cells when the cytolysis reached approximately 50%, then examined under a microscope. As shown in Figure 1C, in the presence of mNK cells, a portion of target cells were effectively cleared away by the cytolytic action of mNK cells, while adding the control YAC cells resulted in no change.

Furthermore, the kinetic analysis indicates that the mNK cell-mediated cytolysis can last longer than four hours, which is the standard incubation time for the radioisotope-based assay. The cytolysis was less than 30% after four hours, and can reach up to 70% by 12 hours. So, potentially, the standard incubation time of existing label-based assays could underestimate the NK cell's cytolytic activity. Thus, the xCELLigence RTCA system not only offers label-free detection, but also provides more accurate assessment of cytolysis.

In summary, the xCELLigence RTCA system is one of the few available assay formats that can directly monitor NK cell-mediated cytolysis without labeling the target cells or using any chemical reporters. It enables scientists to monitor the entire cytolysis process dynamically, a feature that would be difficult to replicate with any label-based, endpoint assay format.

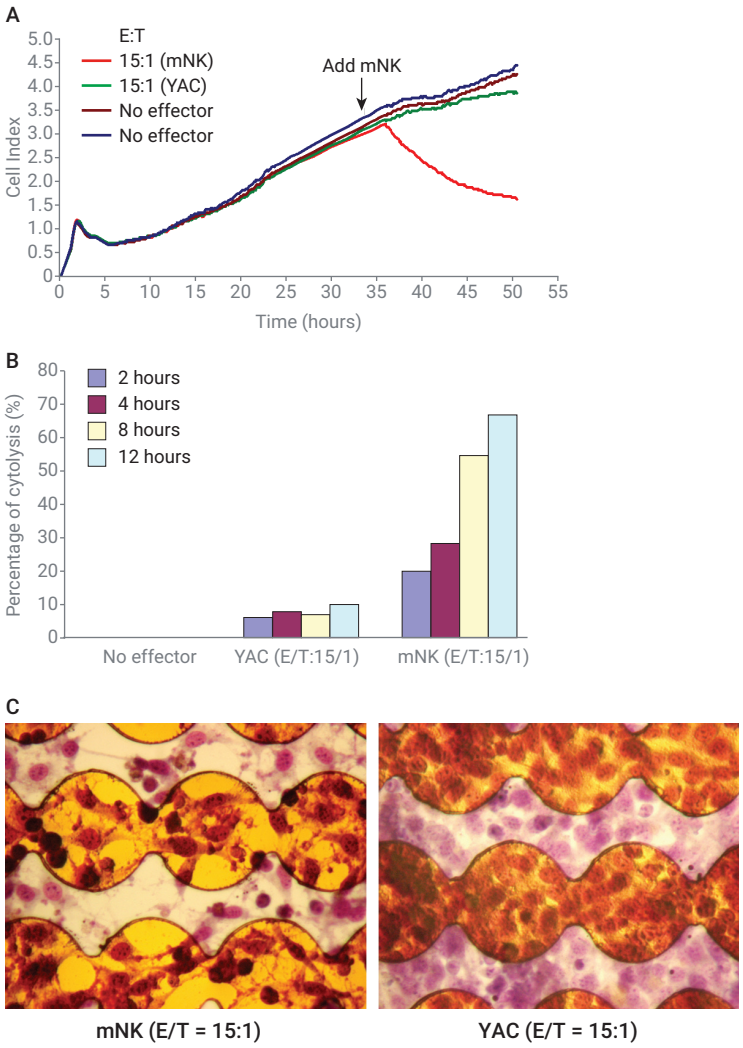

Figure 1. NK cell-mediated cytolysis. (A) Dynamic monitoring of NK cell-mediated cytolysis of NIH 3T3 cells. The NIH 3T3 cells were seeded in the 96-well E-Plate at 5,000 cells/well. Cell attachment, spreading, and proliferation were monitored in real time. 34 hours after seeding the cells, Cell Index values reached 3, which is equivalent to approximately 10,000 cells/well. 150,000 mNK cells or YAC (negative control) cells were added to wells in triplicate. (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 = ($CI_{no\ effector} - CI_{effector}$)/$CI_{no\ effector} \times 100$}. (C) NIH3T3 target cell morphology at eight hours after addition of mNK and negative control YAC cells at 15:1 E/T ratio. Cells were stained with Giemsa blue and imaged with a 10x Nikon objective.

Quantitative measurement of NK cell-mediated cytolysis

The CI correlates with cell number,[5] and has been used to quantitatively monitor cytotoxicity induced by chemical compounds, such as anticancer drugs, at different concentrations. To test whether NK cell activity can also be assayed quantitatively across a range of killing activity, cytolysis was monitored at different E/T ratios. We used both murine and human NK cell lines (mNK and NK92) as effectors. NIH 3T3 mouse cells and MCF7 cells (human breast cancer cells) were respective targets. As described previously, the target cells were first seeded to the 96-well E-Plate at 5,000 cells/well, and the cell growth was monitored with an xCELLigence RTCA system. When the target cells reached the growth phase, the NK cells were directly added to wells at different concentrations. The NK cell-mediated cytolysis at different E/T ratios was then monitored in real time.

As shown in Figure 2, the normalized Cell Index (NCI) declined relative to the "no effector" control after the addition of mNK or NK92 cells to its target cells. The decline in the NCI values was E/T ratio-dependent. In both cases, the higher the E/T ratio, the lower the CI value. The decline was caused by a decrease in cell/electrode interaction that occurred during cytolysis. This demonstrates that the xCELLigence RTCA system permits specific and quantitative measurement of NK cell-mediated cytolytic activity.

In addition, the dynamic monitoring of the cytolysis may also provide more insights into the underlying mechanisms of NK cell-mediated killing. In this study, the kinetic data show that NK92 cells are much more potent effectors than mNK cells. At E/T ratio of 4:1 or higher, NK92 cells killed more than 90% of MCF7 cells within four hours. Whereas for mNK cells, only 30% cytolysis occurs within that time. The difference in cytolytic kinetics of NK cells indicates that the nature of the interaction between effectors and targets is cell-specific and may involve factors such as expression of NK receptors and ligands, or different mechanisms of NK cell-mediated cytolysis.

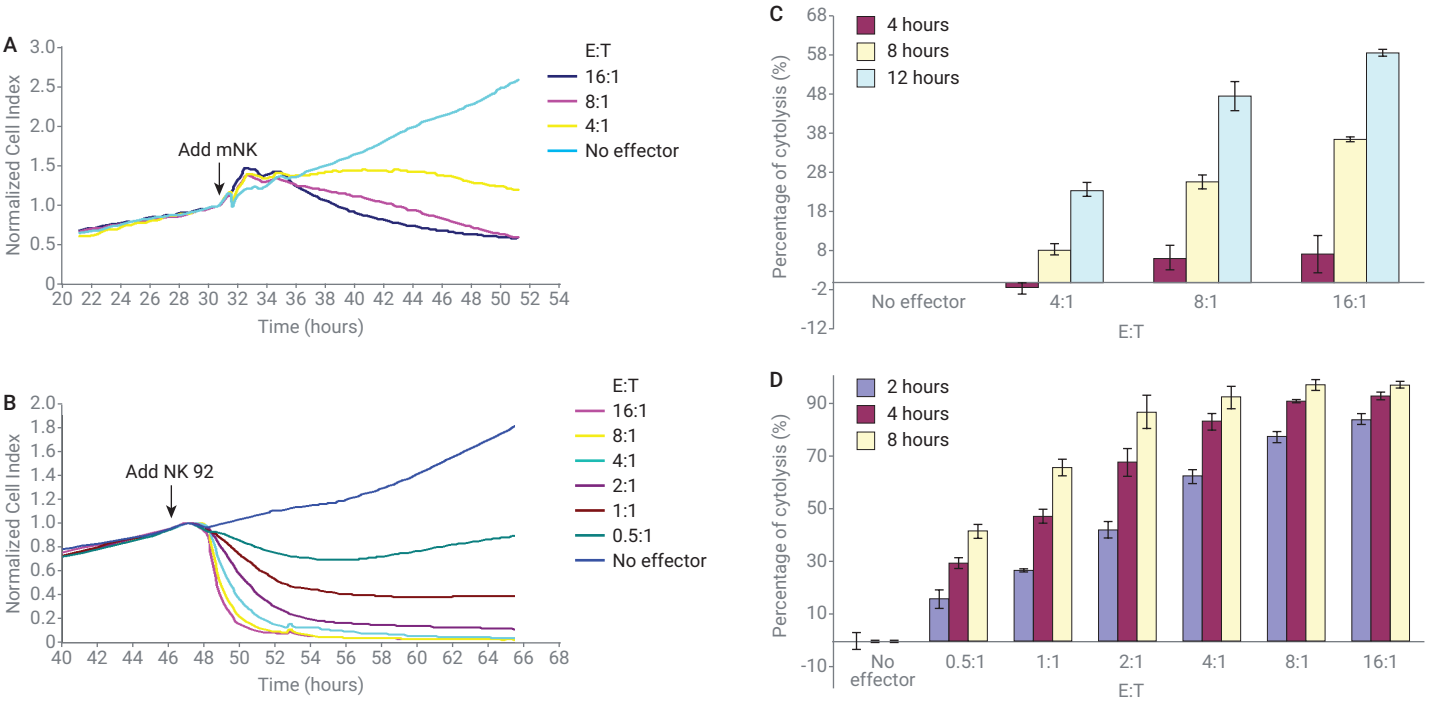

Figure 2. Label-free and quantitative measurement of cytolytic activity of mNK cells and NK92 cells. (A). Quantitative measurement of cytolytic activity of mNK cells. The NIH 3T3 cells were seeded to the 96-well E-Plate. Cell growth was monitored in real time on the xCELLigence RTCA system until the CI values reached 3, equivalent to 10,000 cells/well. The mNK cells were then added to target cells at different concentrations to generate a series of E/T ratios. The cytolysis of the target cells at different E/T ratios was dynamically monitored on the system. A normalized Cell Index was used, where the Cell Index values obtained after the addition of mNK cells were normalized against the Cell Index value from the same well before the addition of mNK cells. (C). 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 1. The time-dependent cytolytic activities are indicated by the curves. (B). Quantitative measurement of cytolytic activity of NK92 cells. The MCF7 target cells were seeded, and the cell growth was monitored on the system as described above. The NK92 cells were then added to each well at different concentrations to generate the series of E/T ratios indicated. The cytolytic activities of NK92 cells on MCF7 cells at different E/T ratios were dynamically monitored on the system. (D). Time-dependent cytolytic activity of NK92 cells at different E/T ratios. The percentage of cytolysis of the MCF7 cells by NK92 cells was calculated as described in Figure 1.

Label-free assessment of NK cell cytolytic activity in various target cell lines

The cytolytic activities of mNK and NK92 were tested on nine cell lines, including eight different human cancer cell lines and the NIH 3T3 mouse cell line. The susceptibility of different target cell lines to NK92-mediated cytolysis is summarized in Table 1. NK92 showed a broad spectrum of cytolytic activity on cancer cell lines. The cytolysis mediated by NK92 occurred quickly, and reached the maximum killing activity in less than eight hours. Among seven cell lines tested, four cell lines (H460, HepG2, MCF7, and MDA-MB-231) reached 90% cytolysis (Figure 3A). In contrast, mNK cell-mediated cytolysis appeared to be more selective than NK92 (Figure 3B). Only four target cell lines (NIH 3T3, A549, HeLa, and MDA-MB-231) showed 30% to 65% cytolysis after 12 hours. The remaining five target cell lines, HT108, H460, HepG2, MCF7, and OVCAR4, displayed no or weak cytolysis (10%). In addition, the cytolysis mediated by mNK was much slower than that mediated by NK92, reaching the maximum approximately 12 hours after the addition of mNK cells (data not shown).

Table 1. NK92 cell-mediated cytolysis of seven cell lines.

Cell Name	Cell Type	Species	Maximum Cytolysis (%) at 12 Hours
HT1080	Fibrosarcoma	Human	42.2
H460	Nonsmall cell lung cancer	Human	95.4
HepG2	Hepatoma	Human	94.1
MCF7	Breast cancer	Human	96.5
A549	Nonsmall cell lung cancer	Human	52.2
HeLa	Cervix cancer	Human	51.0
MDA-MB-231	Breast cancer	Human	97.0

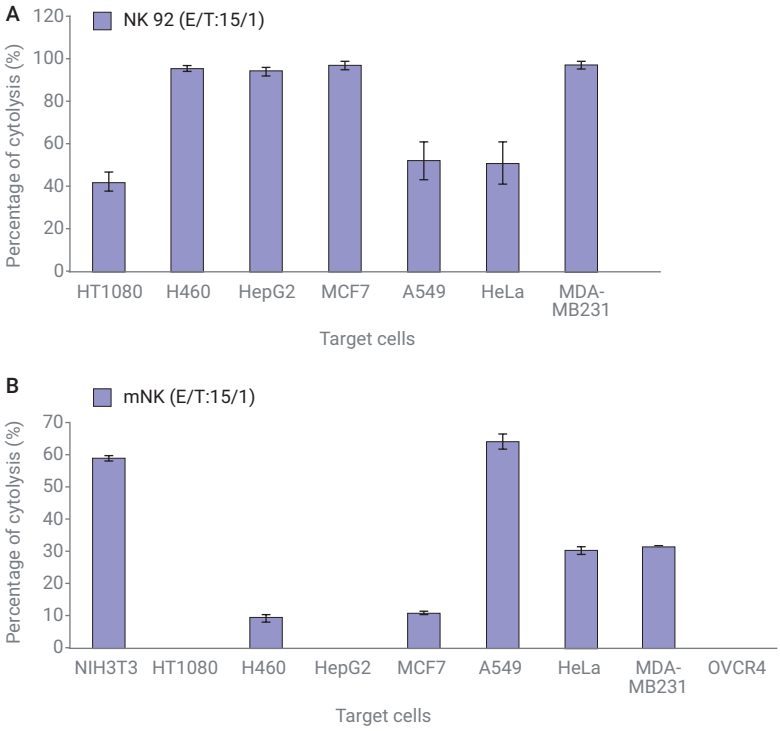

Figure 3. Label-free assessment of NK cell-mediated cytolysis using various cell lines. (A) The NK92-mediated cytolysis of seven different cancer cell lines. The percentage of cytolysis indicated for each cell line is calculated based on the Cell Index value of individual wells eight hours after NK92 cells were added. (B) The mNK-mediated cytolysis of nine different cancer cell lines. The percentage of cytolysis indicated for each cell line is calculated based on the Cell Index values of individual wells 12 hours after mNK cells were added. This was when the cytolysis reached maximum.

In summary, both human and murine NK cell lines were tested for their cytolytic activities on nine different target cell lines, including human cancer cell lines commonly used in the field. The experiments demonstrate that the xCELLigence RTCA system is capable of quantitatively assessing the NK cell-mediated cytolytic activity in real time, but without any labeling steps or additional reagents. This new technology also offers a full, automated solution, which could enable a large-scale screening of chemical compounds or genes responsible for the regulation of NK cell-mediated cytolytic activity.

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RA.0923726852

Printed in the USA, February 2, 2021
5994-1694EN

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