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Research paper

Visualization and quantification of NK cell-mediated cytotoxicity over extended time periods by image cytometry

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ABSTRACT

Natural killer (NK) cell-mediated cytotoxicity is traditionally measured using the chromium release assay, which measures the fraction of radioactive ^{51}Cr released from dying target cells co-cultured with NK cells. However, the time frame of ^{51}Cr release assays is limited to approximately 4 h due to spontaneous release of ^{51}Cr. In the tumor microenvironment, interactions between NK cells and tumor cells occur over extended time periods, and NK cell-mediated cytotoxicity is modulated by cytokines produced by tumor cells and other immune cells. Here we demonstrate that the interaction of NK cells and tumor cells can be imaged and quantified over an extended period of time using a novel image cytometry method. Specifically, we imaged killing of human ZsGreen$^+$ melanoma cells by primary human NK cells in the presence of an antibody targeting MICA and MICB on the tumor cell surface. The number of live ZsGreen$^+$ A375 cells was counted in 96-well plates over a three day time frame, and the results were used to first calculate % specific killing at the 4 h time point to compare to ^{51}Cr release assay. Analysis of data from the 4 h time point demonstrated that both ^{51}Cr and image cytometry enable sensitive detection of NK cell-mediated killing of tumor cells. Image cytometry demonstrated that the combination of the MICA/B antibody and IL-2 induced near-complete eradication of A375 melanoma cells by NK cells at later time points. This novel image cytometry based approach will be suitable for the discovery of combination therapies that enhance the cytotoxic function of NK cells against tumor cells.

1. Introduction

Natural killer (NK) cells are lymphocytes that recognize and kill tumor cells by releasing perforin, a molecule that creates pores in the tumor cell membrane and enables entry of granzymes that induce apoptosis (Herberman et al., 1979). The ability of NK cells to kill tumor cells is analyzed *in vitro* with cytotoxicity assays. The gold standard is the chromium (^{51}Cr)-release assay, in which tumor cells are labeled with radioactive ^{51}Cr and co-cultured with NK cells. The pores in the tumor cell surface created by perforin and subsequent apoptosis results in ^{51}Cr release into the supernatant, and radioactivity measured in the supernatant directly correlates with tumor cell death (Kim et al., 2007).

The percentage of cytotoxicity is calculated by comparing the levels of ^{51}Cr released into the supernatant of tumor cells co-cultured with NK cells (experimental release) *versus* tumor cells cultured alone (spontaneous release). In addition, the maximum release of ^{51}Cr is determined by tumor cell lysis in the presence of a detergent such as Triton X-100. Radioactivity levels measured from experimental, spontaneous, and maximum release are used to calculate the percentage of specific killing. However, there is only a short window of time (usually 4 h) that allows precise analysis of ^{51}Cr release because at longer time points (> 24 h), tumor cells spontaneously release a large fraction of ^{51}Cr, interfering with accurate assessment of cytotoxicity (Erskine et al., 2012; Packard et al., 2010; Zhu et al., 2006). Lactate dehydrogenase (LDH)- and calcein-based release assays are alternatives to the ^{51}Cr release assay, but they also require end-point measurements that are often shorter than 24 h (Korzeniewski and Callewaert, 1983; Somanchi et al., 2015). Luciferase-based ADCC assay is another method that uses the loss in luminescence to determine cytotoxicity. Similarly, the luciferase assay requires an end-point readout and measures the bioluminescence from the supernatant to indirectly determine ADCC activity (Bonsignori et al., 2012; Kramski et al., 2013). This method is not appropriate for analysis of cytotoxicity at multiple time points. Furthermore, most of the luciferase-based assays require transfection of target cells, which may not be appropriate for testing primary donor samples (Alpert et al., 2012; Lallemand et al., 2017; Parekh et al., 2012). The ability to monitor cytotoxicity over longer time periods may reveal novel aspects of NK cell-mediated cytotoxicity.

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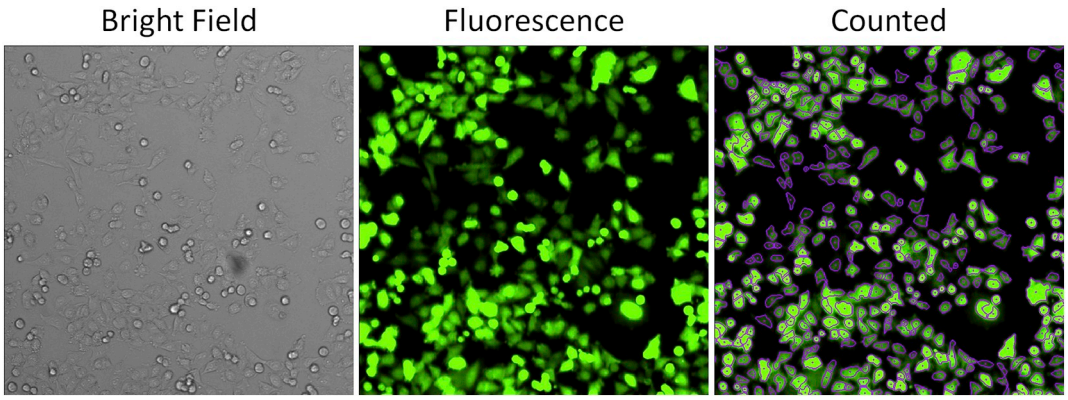

Fig. 1. Bright field and fluorescence images of ZsGreen-expressing A375 tumor cells counted in 96-well plates using Celigo Image Cytometry. Fluorescence signal (center) was used to define and count individual cells (purple outline of individual cells). The bright field image provides a second approach to identify adherent cancer cells.

Recent advances in image cytometry have improved the performance of complex assays for immune cell functions (Chan et al., 2016; Riedl et al., 2016). A high-throughput, plate-based image cytometer enables precise and specific counting of tumor cells expressing a fluorescent protein, even if the tumor cells are co-cultured with other non-fluorescent immune cells. We hypothesized that 1) fluorescent tumor cells could be counted by image cytometry when co-cultured with NK cells and 2) fluorescent tumor cell growth kinetics are associated with NK cell-mediated cytotoxicity. Tumor cells lose fluorescence when they die, which enables specific counting of live tumor cells over a time course. Here, we report a long-term assay of NK cell-mediating killing of ZsGreen$^+$ tumor cells in the presence of a MICA/MICB (MICA/B) antibody that enhances NK cell-mediated immunity *via* NKG2D receptor engagement and antibody-dependent cellular cytotoxicity (ADCC). MICA and MICB are stress proteins that are frequently expressed by different types of human cancer. MICA/B proteins are recognized by NK cells through the NKG2D receptor, enabling NK cell-mediated cytotoxicity (Andrade et al., 2018). The MICA/B antibodies we have generated induce NK cell-mediated killing by activation of NKG2D and CD16 receptors. The Celigo Image Cytometer precisely quantified live tumor cell numbers in co-culture with NK cells for up to 76 h. The proposed method analyses the kinetics of NK cell-mediated killing of tumor cells at longer time points than the standard ^{51}Cr release assay, which may provide novel insights into the interactions between NK cells and tumor cells.

2. Materials and methods

2.1. Antibody, target, and effector cell preparation

The MICA/B antibody 7C6-hIgG1 was expressed in mammalian cells as previously described (Andrade et al., 2018). The antibody targets the membrane-proximal domain of MICA and MICB and inhibits proteolytic shedding of these proteins; the Fc region of the mAb is derived from human IgG1. Interleukin (IL)-2 was purchased from PeproTech (Rocky Hill, NJ) and reconstituted in phosphate-buffered saline (PBS). NK cells (CD56$^+$ CD3$^-$ cells) were isolated from leukapheresis collars of healthy donors using a BD FACSARIA cell sorter (BD Biosciences, Franklin Lakes, NJ). NK cells were subsequently cultured in RPMI-1640, 10% fetal bovine serum (FBS), 1× Glutamax, and 1× penicillin/streptomycin (all from Gibco, Gaithersburg, MD), for 24 h in the presence of 1000 units/mL of IL-2 before use in the cytotoxicity assays.

Human skin malignant melanoma A375 cells were purchased from ATCC (Manassas, VA) and cultured in RPMI-1640, 10% FBS, 1× Glutamax, and 1× penicillin/streptomycin. Cells were negative for contamination with mycoplasma using the Universal Mycoplasma Detection Kit (ATCC® 30-1012K). For the long-term cytotoxicity assay, A375 cells were transduced with a pHAGE lentiviral vector to drive expression of cytoplasmic ZsGreen under the control of the EF1-alpha promoter. ZsGreen-positive A375 cells were isolated by FACS and cultured with the antibodies for 24 h prior to the cytotoxicity assay to inhibit proteolytic shedding of MICA and MICB. The image cytometer was also used to confirm that most of the A375 tumor cells were ZsGreen positive (> 99%, ZsGreen positive cells divided by the total number counted in bright field).

2.2. Celigo Image Cytometer instrumentation

The Celigo Image Cytometer utilizes one bright-field (BF) and four fluorescence (FL) imaging channels: Blue (EX: 377/50 nm, EM: 470/22 nm), Green (EX: 483/32 nm, EM: 536/40 nm), Red (EX: 531/40 nm, EM: 629/53 nm), and Far Red (EX: 628/40 nm, EM: 688/31 nm) with high-power light-emitting diodes (LEDs) to perform plate-based image cytometric analyses. The image cytometer allows auto-focusing in the well based on the image contrast or the thickness of the bottom surface (David et al., 2017; Fantini et al., 2018; Mazor et al., 2017; Mazor et al., 2016).

The Celigo software application "Target 1 + 2" was used to identify and count the number of ZsGreen$^+$ target cells (Green channel). Greiner 96-well microplates (655090) were used for ADCC experiments. The Celigo instrument was set up to acquire images in the Target 1 (BF) and Target 2 (Green), where the exposure time for ZsGreen was 10,000 µs. Next, hardware-based autofocus (HWAF) was used to focus in the BF channel, and the focus offset was applied for the Green (+26 µm) channel. The preset ANALYZE parameters were used to count ZsGreen$^+$ target cells above an intensity threshold of 8, which is the threshold value that enabled target cell counting directly in the fluorescent images after removal of background (Fig. 1). The counting results were used for further data analysis and graphing with GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Image acquisition and analysis were performed simultaneously.

2.3. Short-term NK cell-mediated ADCC assay using ^{51}Cr release

In the ^{51}Cr release assay, 100 µCi of ^{51}Cr were used to label 2 × 10^6 antibody-treated A375 target cells (ZsGreen negative) for 1 h. The tumor cells were subsequently washed with PBS and transferred to 96-well microplates at a density of 5,000 cells/well. Next, multiple effector-to-target (E:T) ratios of IL-2-pretreated NK cells were added to each well; the E:T ratios used were 1:1, 2:1, 5:1, and 10:1, which corresponded to 5,000, 10,000, 25,000, and 50,000 NK cells/well. The target and effector cells were co-cultured for 4 h before an aliquot of the supernatant was collected and analyzed using the MicroBeta2 Radiometric Microplate Counter (PerkinElmer, Waltham, MA). In

addition, negative (A375 cells only) and positive (lysed A375 cells with 1% Triton X-100) controls were set up to analyze spontaneous and maximum release, respectively; these control groups were used to calculate the percentage of specific killing with the following equation:

$$Specific\ Killing\% = \frac{Cr51_{Sample} - Cr51_{Spont}}{Cr51_{Max} - Cr51_{Spont}} \times 100$$

where $Cr51_{Sample}$, $Cr51_{Spontaneous}$, and $Cr51_{Maximum}$were ^{51}Cr release for the experimental, spontaneous, and maximum release, respectively.

2.4. Long-term NK cell-mediated ADCC assay using Celigo

For the long-term ADCC assay, ZsGreen^{+} A375 cells were cultured in the presence of the MICA/B antibody for 24 h. Next, A375 cells were transferred to a 96-well plate at a density of 5,000 cells/well. The plate was centrifuged for 5 min at 1,200 RPM and subsequently imaged with the Celigo instrument. Next, IL-2-pretreated NK cells were added to each well at E:T ratios of 1:1, 2:1, 5:1, and 10:1 (Supplementary Fig. 1). Negative control wells were also prepared (tumor cells without NK cells, or without NK cells but with antibodies). After plating the NK cells, each well was mixed uniformly using a multichannel pipette and centrifuged for 5 min at 1,200 RPM. The plate was imaged immediately after centrifugation (time 0) and subsequently at multiple time points (3, 4, 6, 24, 27, 30, 51, 74, and 76 h).

The number of ZsGreen^{+} target cells was counted at each time point, and the results were exported into EXCEL to generate time-course and end-point plots. The 4-h % specific killing was calculated by the equation, $Specific\ Killing\% = \left(\frac{\Delta Count}{Count_{t=0}}\right) \times 100$, where $\Delta Count = Count_{t=0} - Count_{t=4}$, which calculates the reduction in the number of live target cells over 4 h with respect to cell count at time 0. Results were normalized to control samples by directly subtracting the % Specific Killing calculated from control wells. Therefore, the NK cell-mediated ADCC effects were accurately represented by change in cell count over time, as well as baseline tumor cell death.

3. Results

3.1. Short-term assays of NK cell-mediated killing of tumor cells

Individual human A375 melanoma cells could be imaged and counted in a 96-well plate format using the Celigo Image Cytometer (Fig. 1). We first performed a direct comparison of NK cell-mediated killing of A375 melanoma cells over a 4 h time period using either the Celigo Image Cytometer assay or the standard ^{51}Cr release assay. These cytotoxicity assays were performed with an antibody specific for MICA/B, a stress protein expressed in many human cancers. This antibody inhibits MICA/B shedding and therefore enhances activation of NK cells through the NKG2D receptor; the mAb is also equipped with the Fc region of human IgG1 to enable simultaneous signaling through the CD16 receptor (Andrade et al., 2018). As shown in Fig. 2, both methods enabled sensitive detection of NK cell-mediated cytotoxicity over a wide range of effector to target ratios. These data demonstrate that image-based cytometry is a suitable non-radioactive alternative to the widely utilized ^{51}Cr release assay. It is important to note that the Specific Killing % generated by ^{51}Cr release method seemed to plateau around at a ratio of approximately 5:1, whereas the image cytometer method showed an increase in killing efficiency at higher E:T ratios with a slight smaller separation from the Isotype Control. We hypothesized that the differences could be caused by the methods of detection measuring either released ^{51}Cr or directly counting cells. For example, measuring ^{51}Cr release may be appropriate for short-term assays, while direct cell counting may produce a more accurate representation of cell numbers over longer time points.

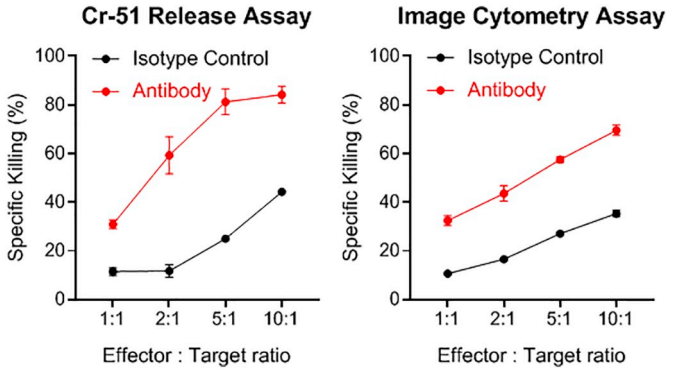

Fig. 2. Comparison of NK cell-mediated cytotoxicity by ^{51}Cr release and image cytometry. Human NK cells were co-cultured with A375 melanoma cells at effector to target ratios of 1:1 to 10:1 over a 4 h time period. Specific killing percentages were calculated for the isotype control and MICA/B antibodies, which showed significant differences. In addition, the ^{51}Cr release and image cytometry assays showed similar trends for short-term NK cell-mediated killing.

3.2. Long-term assay for NK cell-mediated killing of tumor cells using image cytometry

A 4 h time period represents an arbitrary choice for cytotoxicity assays and may underestimate bidirectional signals between NK cells and tumor cells (for example by cytokines) that are highly relevant in an *in vivo* microenvironment. We therefore used the Celigo instrument to image NK cell-mediated killing at multiple time points over a duration of 76 h by acquiring both whole-well BF and FL images (0, 3, 4, 6, 24, 27, 30, 51, 74, and 76 h). A subset of co-cultures was supplemented with IL-2 to support NK cell survival and compared against co-cultures without IL-2. A kinetic profile of NK cell-mediated cytotoxicity is illustrated in Fig. 3 which shows BF/FL overlay images for wells representing control, IL-2 as well as IL-2 plus MICA/B antibody conditions. Additional time-dependent images of A375 only in the presence of Ab are shown in Supplementary Fig. 3. Counting of cells in wells across all conditions enabled quantitative kinetic analysis of the data (Fig. 4). In the absence of NK cells (0:1 effector to target ratio), tumor cells grew quickly. Even in the absence of IL-2 or MICA/B antibody, NK cells exerted substantial control of tumor cell outgrowth. This result was particularly obvious at high effector to target ratios (such as 10:1) at later time points. Such NK cell-mediated killing may be difficult to observe in a standard 4 h assay (Fig. 4), while longer periods of co-culture can reveal additional killing characteristics such as the ability of NK cells to completely eliminate target cancer cells and prevent regrowth of residual cancer cells. Addition of the MICA/B antibody induced strong NK cell-mediated killing of A375 melanoma cells, resulting in eradication of most tumor cells at high effector to target ratios (5:1 and 10:1). The addition of IL-2 plus MICA/B antibody was synergistic and resulted in elimination of most live tumor cells, a result that can also be appreciated in the example shown in Fig. 3 as well as Supplementary Figs. 1 and 2. The synergistic effects are apparent for 1:1 and 2:1 ratios at time points longer than 6 h. It is important to note that Specific Killing % was not calculated at longer time points because tumor cell number is influenced by both killing and tumor cell proliferation (calculation of % specific killing incorrectly assumes that there is no tumor cell proliferation). Therefore, the results were represented as the number of live target cells to simplify the interpretation of cytotoxicity data over time.

4. Discussion

A reliable assay for NK cell-mediated cytotoxicity of tumor cells is needed to accurately analyze NK cell functions and develop therapeutic agents that enhance the potency of this cytotoxic lymphocyte population. The traditional ^{51}Cr release method limits investigation of NK cell-mediated killing to a short time period (typically 4 h) because

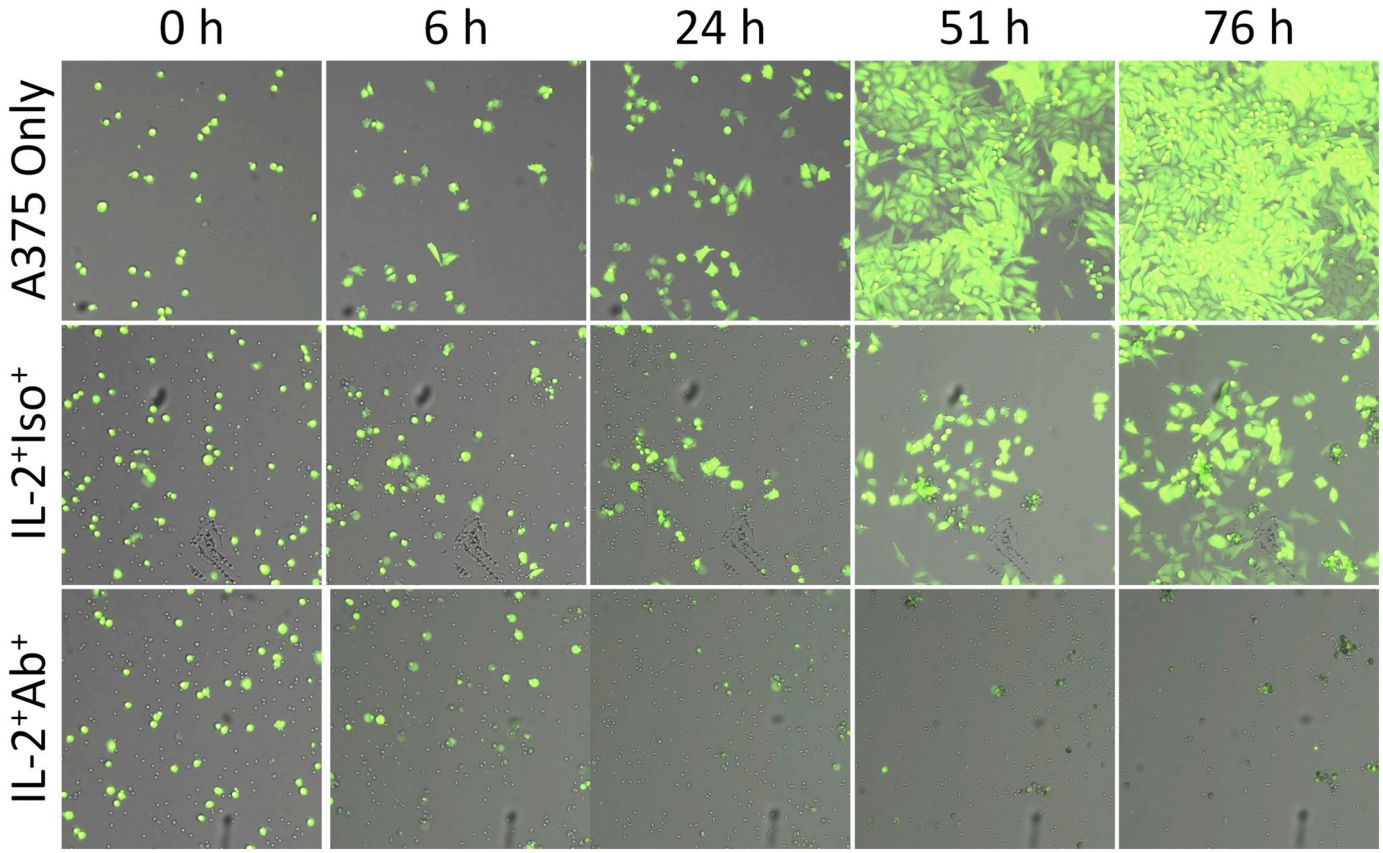

Fig. 3. Time-course of NK cell-mediated killing over a 76 h time frame. Wells were seeded with A375 tumor cells (control) or A375 tumor cells plus NK cells at 5:1 ratio (IL-2^+Isotype$^+$ and IL-2^+Ab$^+$ conditions). Both bright-field and fluorescence images were taken and the overlay of these images is shown. In control wells (no NK cells), continuous tumor cell proliferation was observed. In comparison, co-culture of NK cells and A375 melanoma cells in the presence of IL-2 showed growth inhibition over time. Co-culture of NK cells with A375 melanoma cells in the presence of IL-2 and MICA/B mAb resulted in complete elimination of intact tumor cells over time. An additional negative control condition (A375 tumor cells plus Ab) displayed the same growth pattern as the A375 cells grown in the absence of antibodies (not included in the panel).

spontaneous release of ^{51}Cr is high at later time points. In contrast, image cytometry offers many advantages, in particular the ability to quickly visualize and count target cells of multiple tested conditions over an extended period of time. One of the major advantages of long-term NK cell-mediated cytotoxicity assays is the analysis of cytokine-driven tumor cell killing. Interferon-gamma for example kills tumor cells, but the ^{51}Cr release assay measures only perforin-mediated killing Ni and Lu, 2018; Packard et al., 2010. In this report, we demonstrated that image cytometry is a viable non-radioactive alternative to the ^{51}Cr release assay. The assay is particularly valuable for examining combinatorial strategies for enhancing NK cell function. Specifically, we showed that the addition of IL-2 enhanced NK cell-mediated killing in the presence of the MICA/B antibody, and this combination resulted in near complete elimination of tumor cells at later time points. The synergistic effects of MICA/B and IL-2 were confirmed by statistical 2-Sample *T*-Test for each tested E:T ratio. Visually, the graphs showed observable differences only for the lower E:T ratios, however, there are significant statistical differences between conditions with and without IL-2 with p-values < 0.05 for time points > 6 h. Interestingly, the differences would not have been detected if the assay had been performed for only 4 h. These data thus illustrate how important information is gained by kinetic analysis of NK cell-mediated cytotoxicity.

Other non-radioactive assays based on LDH and calcein release are utilized to determine the level of ADCC. The methods are straightforward but such assays suffer from high background and provide killing data for only one time point. Also, they often require a significant number of target and effector cells, which is not always feasible when working with primary patient samples that are available in limited quantities. In contrast, image cytometry assays can be performed with small numbers of cells because individual target cells are imaged and counted.

The image cytometry approach also offers substantial flexibility in terms of experimental design. A bright fluorochrome is required for accurate quantification of tumor cells, and we used tumor cells expressing ZsGreen. Fluorescent proteins targeted to the nucleus may be particularly useful for such assays because they could facilitate the counting of tumor cells in regions where cells are confluent. The image cytometry method can directly count fluorescent cells that are above the set intensity threshold. However, due to variable ZsGreen expression, cells with low fluorescence may not be identified. One way to overcome this issue is to optimize the intensity threshold to ensure that most of the cancer cells are counted, but in our case with a threshold of 8 the ZsGreen-low cells were not counted (these were just 2.5% of all tumor cells). It is likely that evaluation of other fluorescent proteins can be used to further improve the sensitivity of this approach.

The technology platform could also enable simultaneous analysis of NK cell markers, such as the degranulation marker CD107a, the activation marker CD69 or production of interferon-gamma. The presented image cytometry method for long-term measurement of NK cell-mediated ADCC can deepen our understanding of NK cell – tumor cell interactions and be used to develop novel strategies for enhancing their cytotoxic function against human cancers.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.04.001.

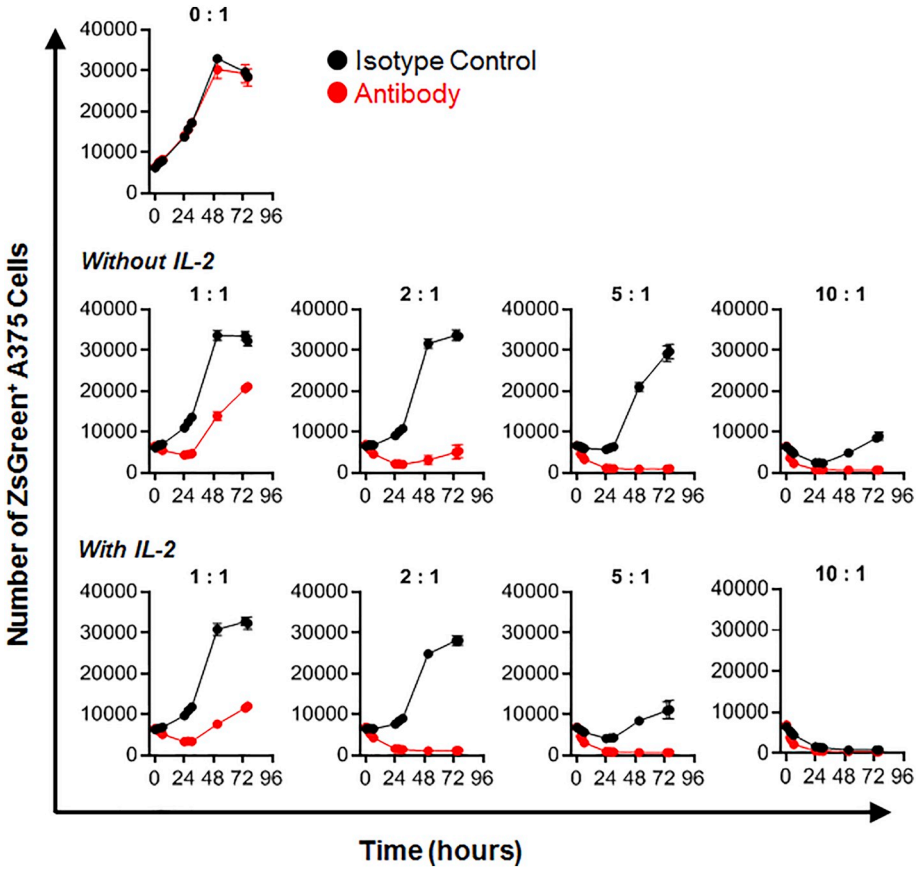

Fig. 4. Quantitative analysis of NK cell-mediated killing of tumor cells. Wells were seeded with 5000 ZsGreen positive A375 melanoma cells and NK cells were added at the indicated effector to target ratios. MICA/B or isotype control antibodies were added to wells, and a subset of wells was also supplemented with IL-2. The number of live A375 cells was counted using the Celigo. Time-dependent cell count averages and standard deviations were calculated and plotted.

Conflicts of interest

The author LLC declares competing financial interests. The research instrument used in this manuscript is a product of Nexcelom Bioscience, LLC. The work was performed to demonstrate a novel high-throughput method to measure long-term NK-cell mediated ADCC using the Celigo Image Cytometer.

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