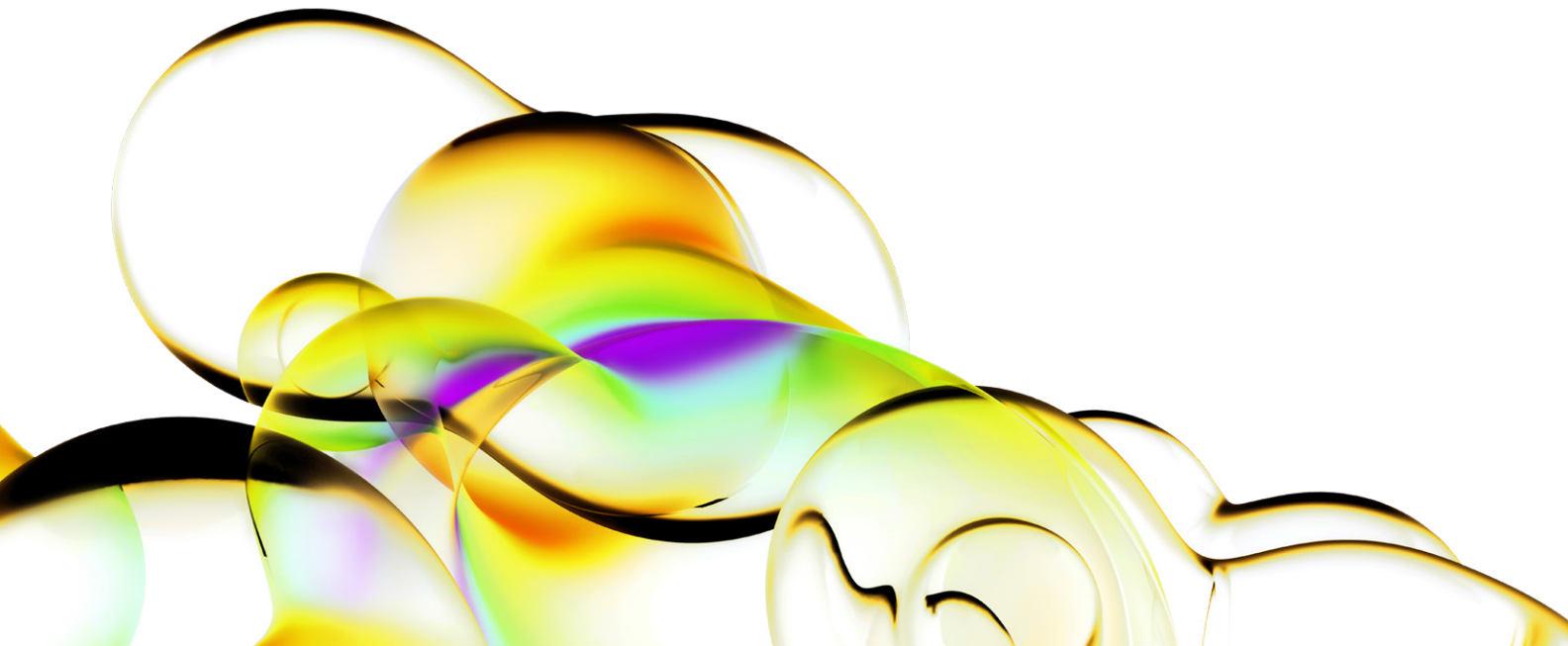
A novel NK cell-mediated cytotoxicity detection method using the Cellometer.

Introduction

As part of the innate immune system, natural killer (NK) cells are the primary form of defense against tumor cells and assorted pathogens [1]. A minor subset of NK cells ($CD56^{bright}CD16^-$) influence immune regulation via the secretion of cytokines interferon-γ and TNF-α [2]. The major subset of NK cells ($CD56^{dim}CD16^+$), however, directly lyse their targets [3]. Consequently, understanding the cytolytic functions of NK cells are key to understanding NK cell biology and function in adoptive immunotherapy.

NK cell cytolytic activity is typically evaluated either through a degranulation assay [4] or via a cytotoxicity assay. The former does not provide data on the response's outcome, such as the cytolysis of the tumor targets, making cytotoxicity assays fundamental to understanding the effect of NK cells and the sensitivity of a given tumor target for lysis. Currently, the ^{51}Chromium release assay (CRA) is considered the standard for analyzing NK cell and cytolytic T cell cytotoxicity [5-9]. Non-radioactive methods have been developed to avoid the toxicity and disposal concerns surrounding CRA. One such alternative employs Calcein AM [10], while others use flow-based assays [11-15], LDH release [16-18], or bioluminescence [19]. Several of these methods correlate well to target cell lysis data from CRA [15, 20, 21], while others report greater target cell lysis values than CRA [11, 19]. The calcein release method has demonstrated good correlation to CRA at evaluating percent specific lysis [21], and so that was the method we employed for our studies [22, 23].



We have noticed though that calcein has various loading efficiencies depending on cell line, and calcein has been shown to have a greater spontaneous release as compared to ^{51}Cr [21], which could lead to a smaller dynamic range and lessened assay sensitivity. Furthermore, an incomplete release of calcein from lysed target cells could lead to an underestimation of target cell percent lysis. Necrotic cell lysis can cause an emptying of cellular contents into the culture supernatant, but apoptotic death causes a formation of cellular "blebs" (apoptotic bodies) that could retain cellular contents without release into the supernatant. This phenomenon could cause an underestimation of target cell percent lysis when using the calcein release assay. Here, we use a Cellometer® Image Cytometer to overcome the limitations of variable loading, high spontaneous release, and incomplete release associated with the calcein release assay. We directly compare the efficiency of image cytometry to the previously published calcein release assay [22] and report that the image cytometry method demonstrates significantly higher percent specific lysis of tumor cell targets as compared to the standard calcein release assay. We therefore recommend image cytometry as a sensitive method for evaluating NK cell cytotoxicity that is more accurate than the standard calcein method.

Materials and methods

Cell cultures

Human tumor cell lines K562 (chronic myelogenous leukemia, CML), Jurkat cells (acute T leukemia), the 721.221 parent cells line (EBV transfected B cell line), and MOLM-13 were cultured in RPMI 1640 media. SK-N-BE(2), CHLA 155, and CHP134 (all neuroblastoma lines), as well as IMR32, were cultured in IMDM media. The buffy coat from five healthy donors was obtained and the NK cells were expanded from the peripheral blood mononuclear cells as described elsewhere [22].

Cytotoxicity assay

Target tumor cells were stained with calcein to determine the cytotoxic potential of NK cells by the calcein release assay and image cytometry. Samples of each NK cell serial dilution (2×10^5, 1×10^5, 0.5×10^5 cells) were put into 96-well U-bottom plates, in triplicate. Calcein-loaded tumor cells were added (1×10^5 cells/well) to create 2:1, 1:1, and 0.5:1 effector-to-target ratios (E:T ratio). Maximum and spontaneous release controls were created in six replicates using 1% Triton X-100 and plain media, respectively.

After 4 hours of incubation, the cells were mixed gently to distribute the released calcein evenly. For the calcein release assay, samples of the supernatant were collected and placed in a flat bottom plate. A BioTek Synergy 2 plate reader read fluorescence and the percent specific lysis was calculated using the following formula:

[(Test release – Spontaneous release)/
(Maximum release – Spontaneous release)] x 100.

For image cytometry, the cell pellet was resuspended in media and placed into a disposable counting slide. The Cellometer collected brightfield and fluorescent images of the sample using optics module VB-535-402 (Ex: 470nm/Em: 535nm) for calcein analysis. FCS Express™ 4 software analyzed the images. The specific lysis percentage was calculated using the following formula:

[(Live fluorescent cell count in spontaneous –
Live fluorescent cell count in test)/
(Live fluorescent cell count in spontaneous)] x 100

The two methods were also compared for evaluating the percent specific lysis of primary NK cells. MOLM-13 cells were stained with calcein and added to NK cell-containing wells to create E:T ratios from 10:1 through 0.6:1. Six replicates of maximum release and spontaneous release controls were included as before. Live imaging of NK cell cytotoxicity was carried out using Nikon Biostation IM-Q using two tumor targets. CHP134, loaded with calcein, was allowed to adhere in culture and NK cells were then added at an E:T ratio of 10:1 for imaging every 4-6 minutes. K562, loaded with calcein, were added to NK cells at an E:T ratio of 10:1 for imaging every 4-6 minutes. The dynamic range of the calcein release assay was calculated from the fluorescence data from maximum and spontaneous release controls from various calcein-loaded targets, after normalizing maximum release data to 100%.

Statistics and analysis

Each cytotoxicity assay was carried out in triplicate. Fluorescent intensities from image cytometry were analyzed using FCS Express™ 4. Percent specific lysis was calculated based on reported fluorescent live cell numbers. The average percent specific lysis data for the five NK cell donors from the calcein release and image cytometry assays were assessed and statistical significance was calculated using Wilcoxon matched-pairs signed rank, non-parametric, two-tailed test. Significance was determined at $p<0.05$.

Results

Limitations of calcein release assay

- Using maximum and spontaneous release data for the various cell lines examined here, we demonstrate the dynamic range for the assay to measure percent specific lysis (Figure 1a). K562 cells have the highest dynamic range amongst the cell lines tested.
- It is a possibility that calcein-loaded target cells could retain dye within cell debris upon NK cell-mediated necrotic or apoptotic cell death (Figure 1b). Using live imaging, we show that incomplete release of calcein from lysed cells (necrosis-like) and apoptotic bodies does occur during NK cell-mediated cytolysis.
  - Figure 1c illustrates sequential steps of CHP134 lysis in necrosis-like cell death by NK cells.
  - Figure 1d shows sequential steps of K562 apoptotic death by NK cells.
- The narrow dynamic range of the assay, as well as the dye's retention due to both necrotic and apoptotic modes of cell death, contribute to a significantly lower percent specific lysis when compared to complete lysis achieved by Triton X-100.

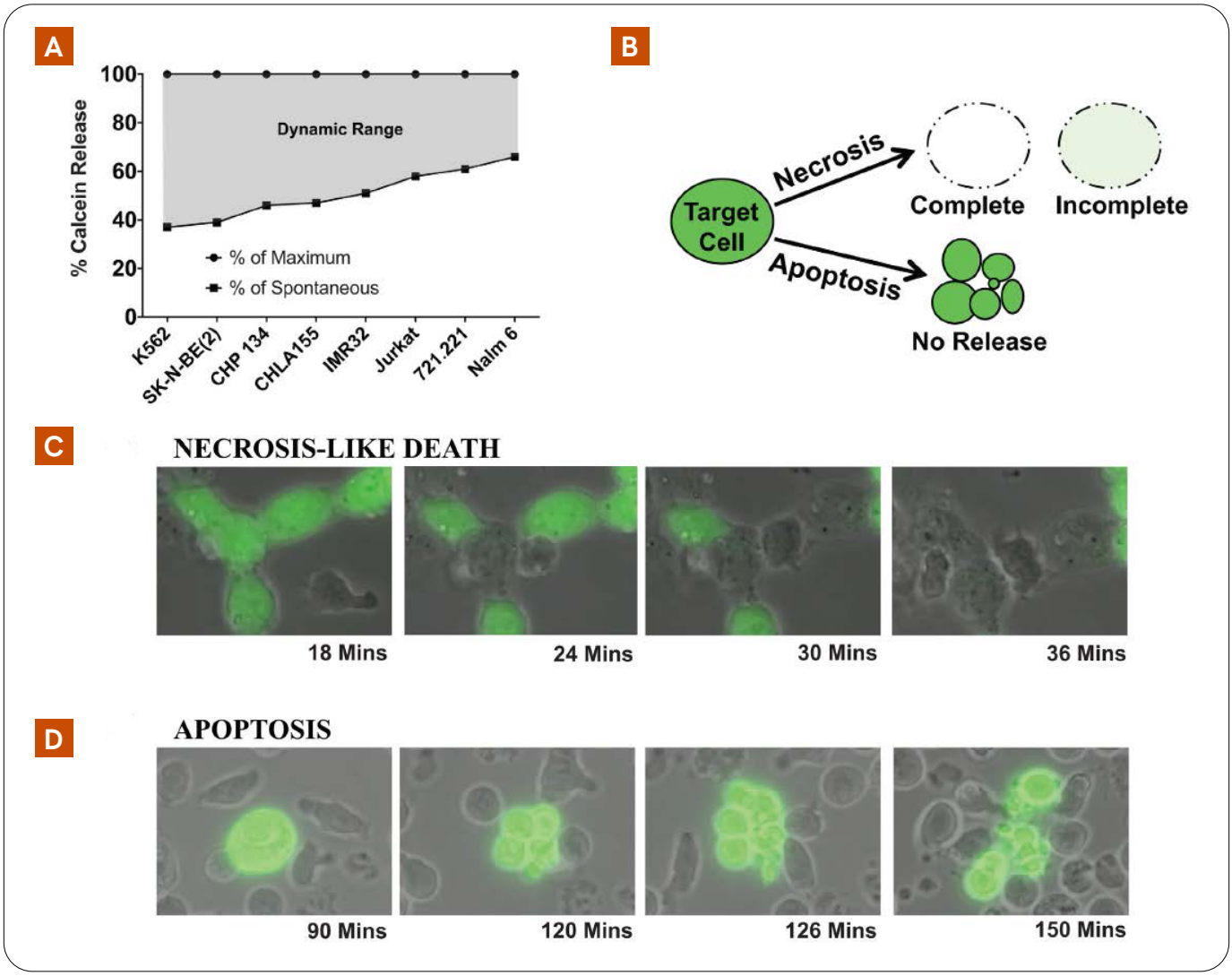


Figure 1A-D: Determining the sensitivity of the calcein release assay. (A) Assessing the dynamic range using various tumor cell targets. (B) Illustration of calcein release from target cells following necrosis-like and apoptotic death. (C) Brightfield and fluorescent overlay images of calcein release from CHP134 cells undergoing necrosis-like death. (D) Brightfield and fluorescent overlay images of calcein release from K562 cells undergoing apoptotic death.

Image cytometry for assessing NK cell cytolysis

- Brightfield and fluorescent images of K562, 721.221, and Jurkat cells were used to assess percent specific lysis. See Figure 2 for representative images at each E:T ratio.
- In the spontaneous release control (without NK cells), the cells were alive and brightly fluorescent, while live fluorescent target cells are seen with increasing NK cell numbers. An E:T ratio of 2:1 showed a nearly complete lysis of K562 cells, while 721.221 exhibited a nearly complete lysis at an E:T ratio of 1:1, and Jurkat showed the same at an E:T ratio of 0.5:1.
- The head-to-head comparison of percent cytotoxicity obtained by calcein release and image cytometry was compared.
- Figure 3 illustrates that image cytometry derived live cells counts through fluorescent intensities of various E:T ratios, with any object fluorescing at an intensity less than that of the targets cells in the spontaneous control being gated out. This gating out ensures that cells with incomplete calcein release and lower overall signal intensity are excluded from the cell counts.

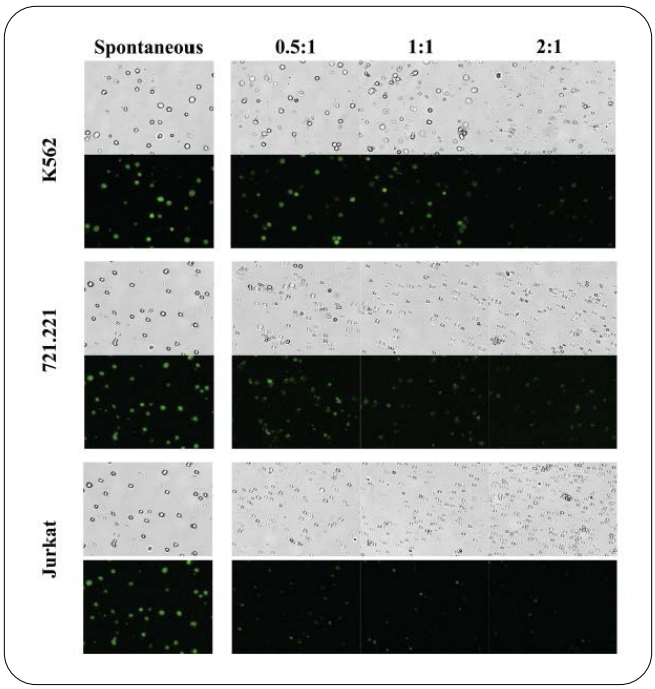


Figure 2: Image cytometry evaluation (brightfield and fluorescent images) of NK cell cytotoxicity for K562, 721.221, and Jurkat cell lines showing loss of live fluorescent cells as E:T ratio increases.

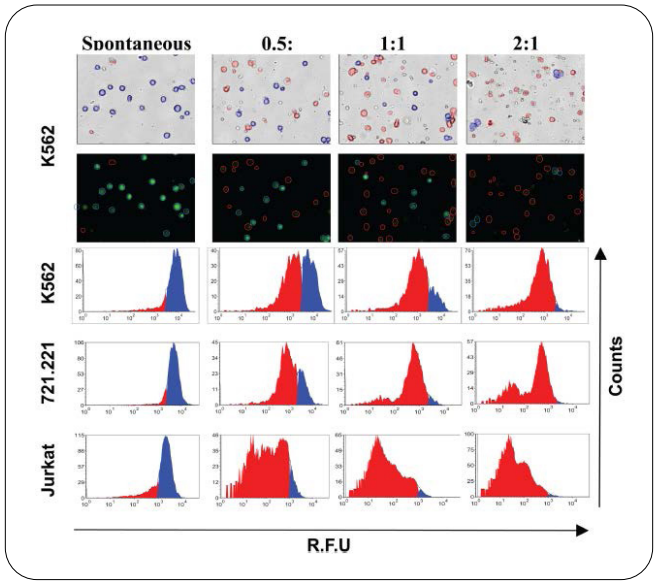


Figure 3: Images and analysis of target cell lysis by image cytometry. Live target cells in the culture was calculated by plotting fluorescent intensity of target cells from each E:T ratio as compared to spontaneous control. Live K562 cells are circled in blue, while lysed cells and apoptotic bodies are circled in red.

- Percent specific lysis of K562 as assessed by image cytometry was significantly higher than by standard calcein release assay at E:T ratios 2:1 ($p<0.0001$) and 1:1 ($p<0.03$). At E:T of 0.5:1, however, the data between the two methods were not significantly different ($p=0.67$).
- Percent specific lysis in 721.221 cells was significantly higher as determined by image cytometry than that reported by calcein release at all E:T ratios tested (2:1, $p=0.0006$; 1:1, $p<0.0001$; and 0.5:1, $p<0.0001$).
- Percent specific lysis in Jurkat cells showed the same trend at all E:T ratios ($p<0.0001$). (Figure 4).

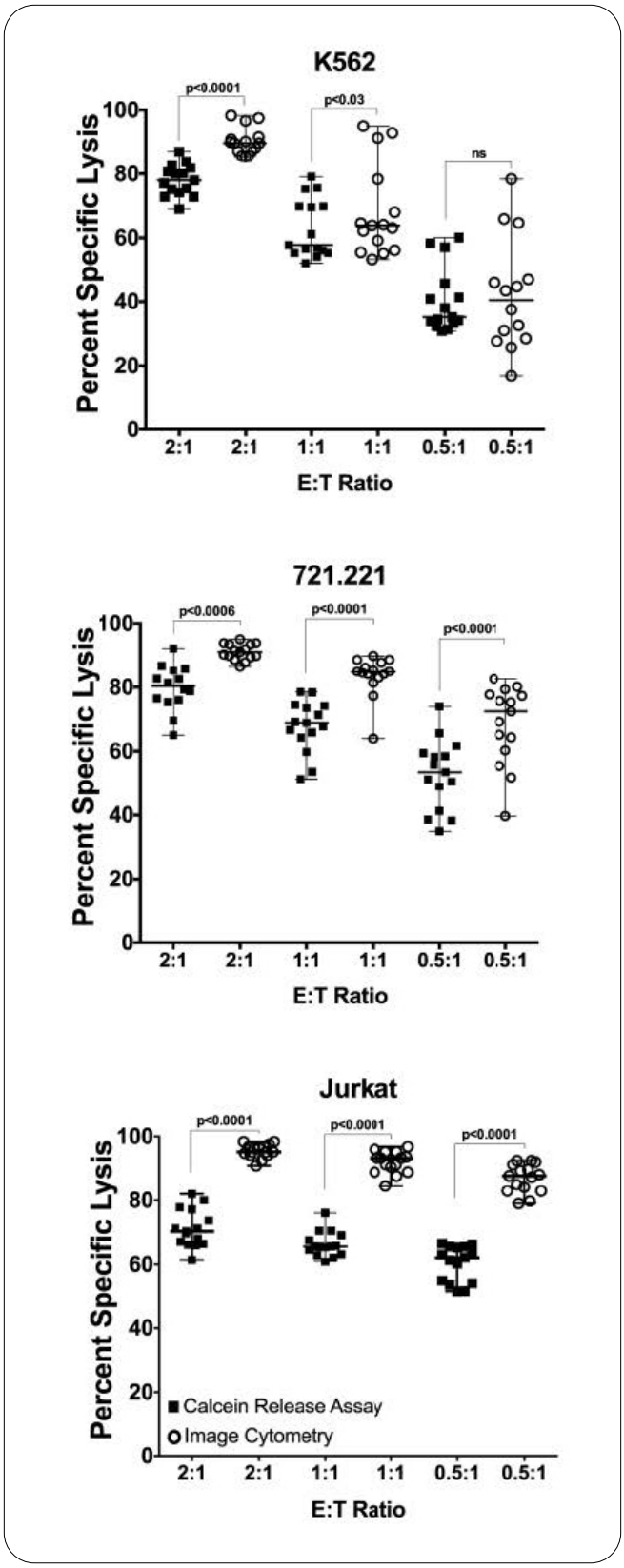


Figure 4: Comparison of percent specific lysis as determined by calcein release and image cytometry. Data are shown for each tumor cell line using NK cells expanded from 5 donors.

- To include instances of smaller sample size, we analyzed the image cytometry method with smaller numbers of target and effector cells such as the expanded NK cells donated by five donors against the K562 target cell line (Figure 5a).
- It was noted that by reducing the assay's cell density, a more significant improvement in percent specific lysis of K562 was seen by NK cells from the same donors (Figure 5b).
- Lastly, image cytometry was compared to calcein release for evaluating the cytotoxic potential of primary NK cells. The E:T ratios were increased to 10:1 as primary NK cells have lower cytolytic potential than activated and expanded NK cells.
- Live fluorescent target cells were imaged (Figure 6). The percent specific lysis of MOLM-13 cells by primary NK cells as determined by image cytometry was significantly higher across all E:T ratios as compared to that determined by standard calcein release.

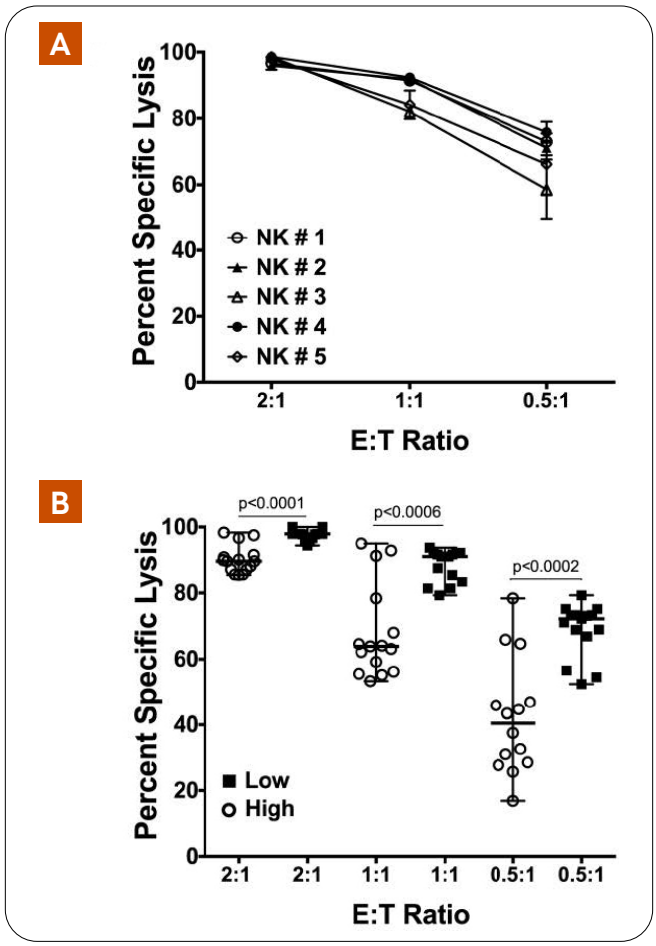


Figure 5A, B: Percent specific lysis of K562 cells with smaller sample size optimized for image cytometry. (A) Reduced target cell number (50,000 cells/well) at various E:T ratios using NK cells from donors. (B) Data demonstrating that reduced target cell density (low) showed significantly higher percent specific lysis than a higher target cell density (high).

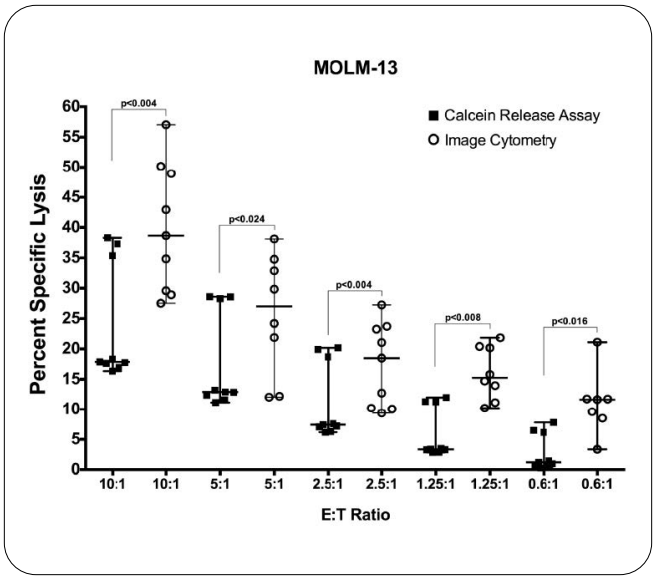


Figure 6: Comparison of the evaluation of percent specific lysis by primary NK cells against MOLM-13 target cells using calcein release and image cytometry. Image cytometry reported significantly higher target lysis at all E:T ratios examined.

Discussion

- We have used the calcein release assay to report cytotoxicity of expanded NK cells against various tumor targets due to its simplicity, ease of set up, and its use of a non-toxic dye [22, 23].
- Here, however, we have shown that the calcein release assay's dynamic range varies across different cells lines, which can affect the assay's accuracy in some tumor lines.
- Live imaging demonstrated that calcein is not completely released from all dead and dying target cells, whether they undergo necrosis-like or apoptotic cell death. Incomplete dye release results in an underestimation of percent lysis, a constraint that is shared by most release-based assays [16, 24–26].
- The Cellometer Image Cytometer showed significantly higher percent specific lysis of target cells than the calcein release assay within the same experimental paradigm.
- We believe image cytometry overcomes many of the constraints intrinsic to calcein release, such as incomplete release, because image cytometry allows for cells with incomplete release of calcein to be excluded from analysis based on fluorescent intensity and size.
- The only exception noted was for K562 cells at E:T ratio 0.5:1, where cytotoxicity values were not significantly different between the two methods, possibly due to K562 cells' higher dynamic range for the calcein release assay.
- NK cell cytotoxicity as determined by image cytometry improved when the total cells used were reduced, leading us to recommend the use of 50,000 target cells for a cytotoxicity against with the K562 cell line and image cytometry.
- With primary NK cells, image cytometry still remained more sensitive than calcein release at reporting percent specific lysis even at the lower tumor cell lysis achieved by primary NK cells.
- Here, we used suspension cells as targets and yet this method can easily be applied to adherent cells using trypsinization or non-enzymatic cell dissociation.
- We consider the heightened sensitivity and the ability to exclude cells of low fluorescent intensity and smaller size from the counts as great advantages to image cytometry over the standard calcein release assay, particularly in experiments that utilize chemotherapy drugs to sensitize tumors to NK cell killing. These assays generally result in increased apoptotic cell death, meaning that image cytometry would be better suited to accurately determine the increase in NK cell-mediated cytotoxicity than the calcein release assay.
- Many multi-parametric flow cytometry-based assays have been developed to analyze degranulation, cytokine production, and NK cell and target cell receptor expression [11, 27–30]. These tools excel at specialized inquiries but are less suitable for routine cytotoxicity assays.
- In conclusion, we propose image cytometry as an appealing option for carrying out routine cytotoxicity assays and potency assays for adoptive immunotherapy cell products.

Please see the supporting information online for additional figures.

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