CELLPLY

APPLICATION NOTE

Characterization of NK cell donors for allogeneic cell therapy through single cell serial killing

HIGHLIGHTS

- A single-cell serial killing method can provide greater insight into a heterogenous population of immune cells than bulk analyses
- Serial killing can be used to identify subpopulations of best killer NK cells
- Optimal donors against various target cells were tested and selected for highest frequency of serial killers
- Good reproducibility is a achieved with only 7,000 effector cells per condition

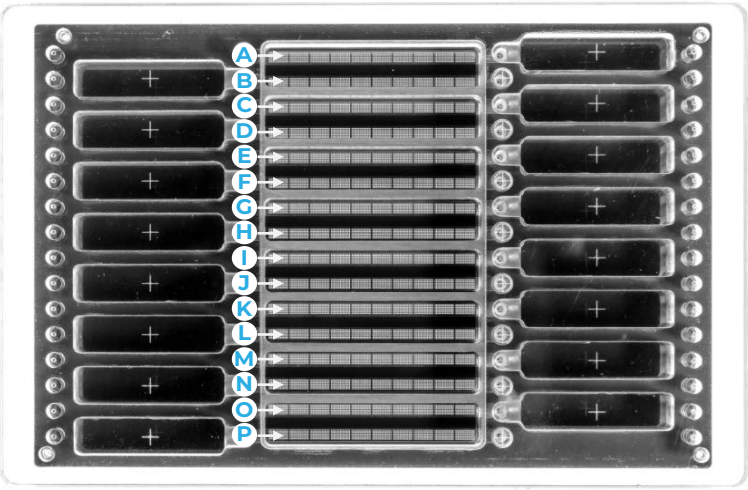
INTRODUCTION

Allogeneic cell therapies provide a simpler manufacturing solution for oncology-based cell therapies in comparison to autologous approaches. One key facet of allogeneic therapies is to reproducibly identify sustainable and potent cell sources. In addition to HLA screening, potency screening identifies donors which provide optimized cell
killing and cytokine profiles.

Current approaches to identifying optimal donors also include potency analysis of cytokine profiling and cytotoxicity assays within a heterogenous sample. In addition, serial stimulation can be used to assess long-term killing potential of the donor sample. While these assessments of potency are commonly performed there is not a simple methodology to evaluate supercharged subpopulations within a heterogeneous donor supplied sample which retains a phenotype capable of serial killing. By identifying such sub-populations, it may be possible to develop a process which favors expansion of these phenotypes thus providing the opportunity to lower dose requirements with potentially lesser risk of adverse effects or to better understand the biological activity of a manufactured batch to achieve more reproducible clinical results.

The Cellply VivaCyte™ system enables multi-parallel and high throughput analysis of single cell killing, serial killing and phenotypic characterization of the same set of cells within a heterogenous population.

This application note will demonstrate how NK cells have been analyzed by the Cellply VivaCyte using a unique single cell serial killing assay capable to rank order optimized donors relative to bulk cytotoxicity, or cytokine analysis profiling. The utility of single-cell serial killing analysis is to uncover hidden activity of a subset of cells within a population and by combining this analysis with a phenotypic characterization, to reveal which subset of cells might have a



Channel	cell types	
	Target	Effector
A	K562 or THP-1	NK (donor A)
B	K562 or THP-1	NK (donor A)
C	K562 or THP-1	NK (donor A)
D	K562 or THP-1	NK (donor A)
E	KG-1a	NK (donor A)
F	K562 or THP-1	NK (donor B)
G	K562 or THP-1	NK (donor B)
H	K562 or THP-1	NK (donor B)
I	K562 or THP-1	NK (donor B)
J	KG-1a	NK (donor B)
K	K562 or THP-1	NK (donor C)
L	K562 or THP-1	NK (donor C)
M	K562 or THP-1	NK (donor C)
N	K562 or THP-1	NK (donor C)
O	KG-1a	NK (donor C)
P	K562 or THP-1	-

Figure 1. The microfluidic device containing 16 channels and scheme showing the configuration of the channels utilized in this application note. Each channel runs an independent condition characterized by effector/target cell types.

dominant role in anti-tumor activity.

METHODS

Hematopoietic Stem Cell Isolation and NK Cell Culture

Hematopoietic stem cells isolated from fresh umbilical cord blood of three different donors (Anthony Nolan, United Kingdom) were expanded and differentiated into NK cells as previously described (Spanholtz, 2010 and Saha, 2021).

In short, CD34+ cells were cultured in a six-well tissue culture–treated plate (Corning Incorporated) in Glycostem Basal Growth Medium (GBGM®, FertiPro) supplemented with 10% human serum (Sanquin, Netherlands), 25 ng/ml of TPO, IL7, Flt 3L, and SCF (Cellgenix), 1 ng/ml GM-CSF, 0.05 ng/ml IL-6 (Cellgenix), and 0.25 ng/ml Neupogen (G-CSF; Amgen BV). After 9 days, TPO was replaced by 20 ng/ml IL-15 (Cellgenix). After 14 days of expansion, differentiation medium was added, consisting of GBGM supplemented with 10% human serum, 20 ng/ml of IL-7, IL-15, and SCF, 1 ng/ml GM-CSF, 0.05 ng/ml IL-6, 0.25 ng/ml Neupogen, and 1000 U/ml Proleukin (IL-2; Novartis). The cells were cultured until day 28 and cryopreserved until further use. For usage, at day 28 cells were thawed and cultured for 1 week in differentiation medium.

Co-Culture Array creation on a CC-Array™ microfluidic device

NK cells produced as reported above and two different types of target cells, K562 and THP-1 (ATCC), were loaded onto a CC-array device based on the Open MicroWell (OMWTM) microfluidic technology (Bocchi, 2012). The CC-Array chip was then loaded in the Cellply VivaCyte instrument.

The CC-Array is a disposable miniaturized device with 16 independent microchannels for liquid perfusion along with inlet/outlet and waste ports **(Figure 1)**. Each microchannel features 1,200 microwells with a diameter of 70 um, for a total of 19,200 microwells per device. The design of the microwell permits rapid liquid exchange ensuring the rapid perfusion of the fluids in contact with the cells without removing the cells from the bottom of the microwell. The microdevices have been loaded according to the scheme reported in **Figure 1**. NK cells and target cells were tracked

using Calcein AM (Thermo Fisher) and CMAC (Thermo Fisher), respectively. In addition, NK cells were specifically marked with anti-CD336 (AF647, Biolegend) and anti-CD56 (BV421, BD Biosciences).

Cell delivery was made automatically by the liquid handling system integrated in the VivaCyte platform performing one delivery step for each cell type involved in the assay, i.e. effector and target cells. Cells were added in each microchannel and after stopping the fluid flow, cells were randomly deposited in the microwells by gravity. Cell concentration was determined empirically to achieve a significant number of co-cultures with single NK cells. NK cells were first loaded at a concentration of 240,000 cell/mL, using a volume of 30uL of suspension cells per microchannel, corresponding to about 7,000 cells per microchannel **(Figure 2a)**.

As a second step, target cells suspended in 30uL of medium were loaded at the final concentration of 3 x 106 cell/mL, corresponding to about 90,000 cells per microchannel (Figure 2b).

Finally, complete medium including a cell death marker (Propidium Iodide, PI) was added to the CC-Array wells **(Figure 2c)**. Fluorescent imaging was performed on a subset of 900 co-cultures per microchannel at time points 0h, 2h, 4h, 6h, 8h, 12h, 18h and 24h. Images were analyzed and co-cultures clustered based on the number of NK and target cells per microwell to carry out tumor killing analyses on co-cultures featuring similar patterns of NK and target cells, e.g. single NK cells and a number of target cells per microwell within a certain distribution.

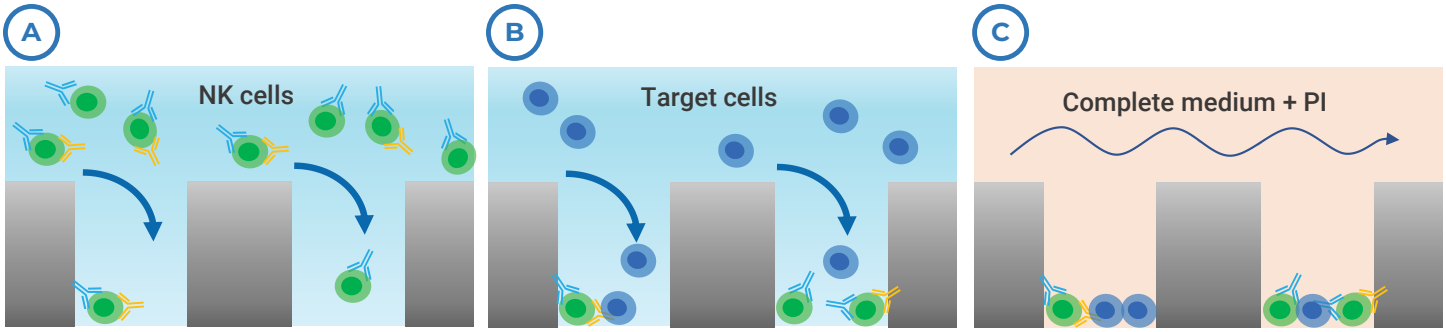


Figure 2. Workflow showing (A) loading of NK cells tracked in DAPI, FITC and CY5 channels followed by (B) loading of the target cells tracked in DAPI to create co-cultures and (C) staining of the co-cultures in time-lapse using PI as cell death marker.

RESULTS & DISCUSSION

Time-lapse visualization of killing and serial killing activity at the single cell level

We loaded NK and target cells treated as reported above into the CC-Array microdevice to create different combinations of co-cultures inside each of the microwells. Three types of co-cultures were obtained into the microwells using the same experimental condition within one channel: a) target cells without NK cells, b) target cells co-localized with a single NK cell, and c) target cells co-localized with multiple NK cells.

The three classifcations of co-cultures were determined by automated image processing counting the number of cells of each type localized in each microwell and allowed to measure a) spontaneous target cell death, b) serial killing capacity of the NK cells in terms of number of target cells killed by a single NK at a specific time and c) total NK cell killing ability of all targets in bulk.

When concentration is optimized for single NK cell occupancy, cell delivery can be approximated by a Poisson distribution and cell distribution in microwells is controlled by adjusting the input concentration.

In 3 experiments using the THP-1 as a target cell line an average of 667, 797, 732 microwells with a single NK cell were obtained per experiment for donor A, B and C, respectively. In the experiment using the K562 cell an average of 814, 956 and 717 microwells with a single NK cell were obtained per experiment for donor A, B and C, respectively.

Figure 3 shows an example of images analyzed to investigate the interaction among single NK effector cells and multiple target cells. Specifically, it is shown how the system enables time lapsed imaging of activity of a single NK cell and to determine its ability to kill the target cells and count the number of target cells killed at a specific time point. In addition, the platform allows monitoring of the ability of the same NK cells to kill none, one, two or more target cells (Figure 3) and consequently classify each NK cell as inactive, killer or serial killer. The example shows an inactive NK cell on the first row and a serial killer cell on the second row which successfully kills 4 target K562 cells in 12 hours and 7 target K562 cells in 24 hours.

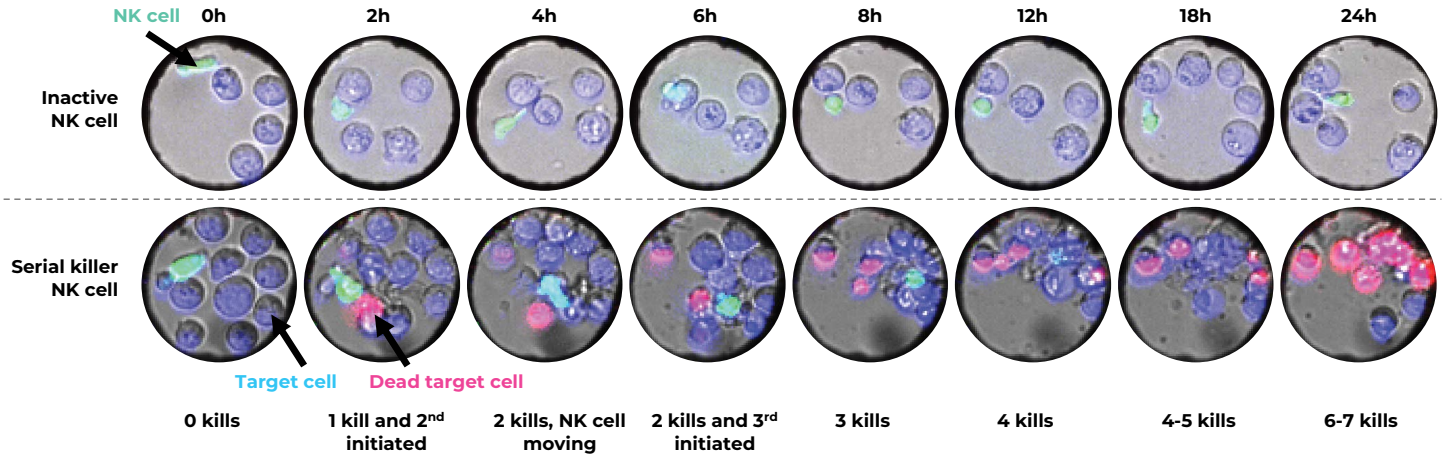


Figure 3. Time-lapse visualization of the cytotoxic activity of NK cell at the single cell level. Examples of co-cultures generated inside the microwells are represented. The images show a microwell containing an inactive NK cell (top) and a serial killer NK cell (bottom) obtained from the same donor sample, using K562 as a target cell line. The inactive NK cell (green) touched the target cells (blue) at different time points without inducing any cytotoxicity on the target cells, leaving target cells alive and growing as reported in the duplication of a target cell observed between time 12h and 18h. The serial killer NK cell (green) repeatedly gets in contact with more than one target cells (blue) leading the death of almost all target cells in the microwell (pink).

NK cell serial killing activity

We quantified the ability of single NK cells in a population of NK cells belonging to the same donor to kill one or more target cells by counting the co-cultures according to the number of NK and target cells after 12 hours of co-culturing inside the Cellply VivaCyte.

The analysis was then carried out on the microwells where one NK cell was delivered **(Figure 4)**. The experiment was repeated 3 times in 3 different days comparing in each experiment the different activity of NK cells from 3 different donors and thus determining the reproducibility of the measurement.

Differently from conventional bulk analyses that measure the number of target cells killed at a certain effector:target ratio, this type of analysis determines the number of active NK cells. Specifically, NK cells were classified according to the ability to kill 0, 1, 2, 3, 4, 5, 6 or more target cells. We performed this type of analysis using two different target cell lines, K562 and THP-1, known to be differentially sensitive to NK cell killing. A highly resistant cell line, the KG1-a was also included into the CC-Array device as reported in **Figure 1** as a control.

Data shown in Figure 4 demonstrates that, independently from the donor, many of the single NK cells have limited capacity to kill under those challenging conditions. For K562 target cells, an average of 70.61% (range 65.37-78.91) of the single NK cells analyzed among the three donors has been detected to have limited activity in this assay. Instead, for THP-1, the average was 82.44% (range 77.64-84.95) confirming that K562 cells are more susceptible to NK cell lysis. For KG1-a, a cell line known to be strongly resistant, almost all the NK cells had limited effect on the target under single

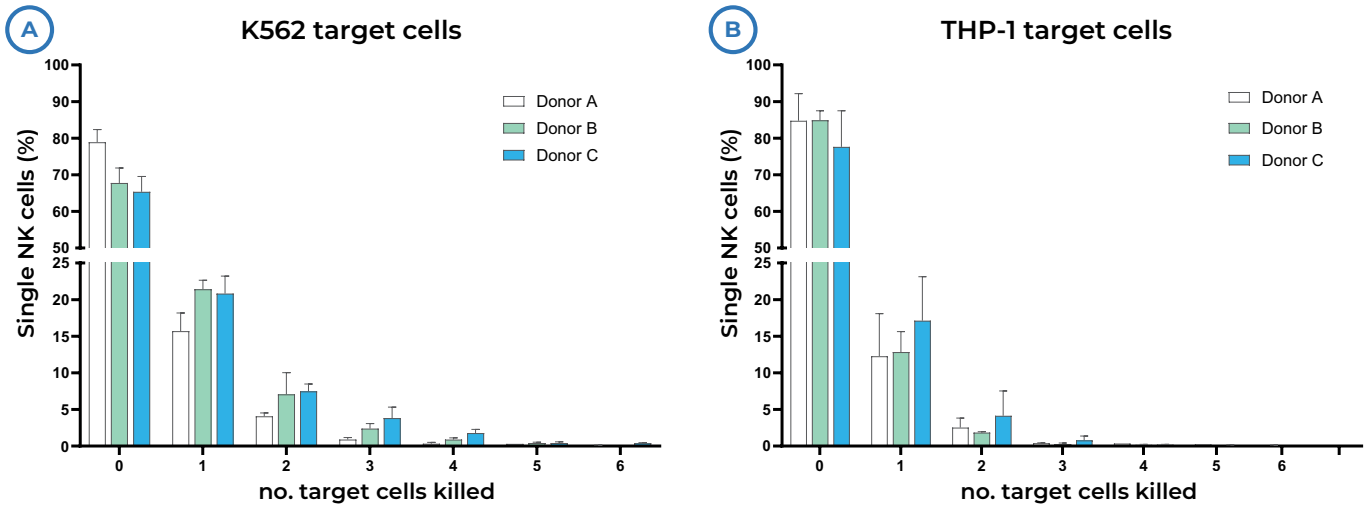
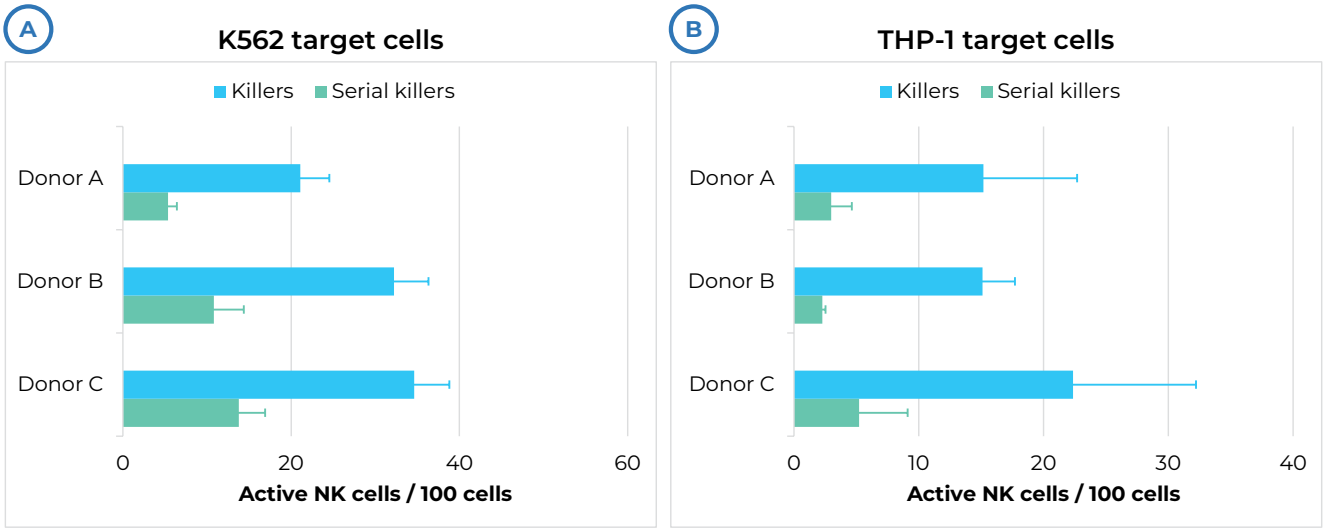


Figure 4. NK cell activity quantification at the single cell level. Quantification of the activity of a single NK cell for three different donors after 12h incubation of NK cells with (A) K562 and (B) THP-1 target cells in the CC-Array. Each histogram represents the mean and standard deviation of three independent experiments carried out on three different days and with three different cell aliquots using the scheme reported in Figure 1. The X axes report the number of target cells killed by the single active NK cells for each donor.

NK cell conditions (data not shown).
Within the total population of cells, we identified that single active NK cells could be broken down into two subpopulations: the groups were defined as NK cells able to kill one or more target cells indicated as “killers” and NK cells being able to kill two or more target cells, indicated as “serial killers” **(Figure 5)**. Thanks to this classification, it is possible to obtain a highly detailed characterization of each donor at single-cell resolution including a detailed enumeration of the fraction of NK cells exhibiting serial killing activity.

For the sensitive K562 target cell line, considering the “killer” cell group, significant differences can be reported between donor A and C (p=0.0123) and donor A and B (p=0.0227) indicating that donor B and C have a higher killing activity than donor A. This data is confirmed also by analyzing the “serial killer” cell group with an additional consideration: the percentage of NK cells able to kill more than two K562 cells is higher for donor C which presents an even higher significant difference with respect to donor A. When using THP-1 target cells, at the time of the analysis (t=12h) no significant differences were observed in any of the groups.

The inter-experiment repeatability was also calculated as reported in **Figure 5**. The count of killer cells reported a coefficient of variation (CV) calculated in percentage between 24-48% for experiments with THP-1 target cells and between 11-16% for experiments with K562 target cells. The count of serial killer cells reported a CV of 11-72% for experiments with THP-1 cells and 20-33% for K562 cells. It should be observed that higher CV values have been reported for the serial killing analysis featuring a lower frequency of NK cells.



Count of killer cells: 12-16% CV
Count of serial killer cells: 20-33% CV
Avg. 814 single cells analyzed per Donor A per test
Avg. 956 single cells analyzed per Donor B per test
Avg. 717 single cells analyzed per Donor C per test

Count of killer cells: 24-48% CV
Count of serial killer cells: 11-72% CV
Avg. 667 single cells analyzed per Donor A per test
Avg. 797 single cells analyzed per Donor B per test
Avg. 732 single cells analyzed per Donor C per test

Figure 5. NK cell killing capacity quantification. Quantification of the ability of a single NK cell to kill one or more target cells (Killers) or two or more target cells (Serial Killers). Single NK cell activity is reported for each donor after 12h incubation of NK cells with (A) K562 and (B) THP-1 target cells in the CC-Array. Each histogram represents the mean and standard deviation of three independent experiments using the scheme reported in Figure 1. Under each graph, the reproducibility of the assay is also reported. In addition, the number of single NK cells analyzed for each donor is reported.

Time lapse serial killing activity

The analysis reported in the previous section can be applied to all the time points analyzed within the same experiment. The functional behavior of active NK cells inside a specific microwell of the CC-Array can thus be followed from time 0 up to 24 hours at multiple time points. **Figure 6** reports the data obtained for "killer" and "serial killer" cell groups as defined in the section above, with additional information regarding the kinetics of killing and serial killing. The number of active NK cells against THP-1 doubled in both the classes between 12h and 24 for all the three donors tested but confirming a non-significant difference in the functional activity of the NK between the three donors. Conversely, NK cells active against K562 target cells reported a consistent killing activity even after only two hours of incubation. This data all taken together indicated the ability of the analysis performed by the Cellply VivaCyte to not only identify subpopulations with different functional properties in terms of NK killing capacity, but also to observe different kinetics of killing among NK cells from different donors, which could be valuable information for the in vivo response to the therapy.

CONCLUSION

In this application note we presented a novel methodology enabling an in-depth profiling of the immune system by evaluating the potency of single NK cells through single-cell cytotoxicity assay.

The presented approach reveals complex serial killing patterns in specific NK cell subpopulations upon interaction with different type of target cells.

The innovative analytical method analyzes spatial information and evaluates the real action of each NK cell on target tumor cells, allowing the study of the impact of immune system activity in a microenvironment represented by multiple tumor cells surrounding individual NK cells. Such information, which is commonly ignored in more supportive conditions like bulk killing assays, might play a key role in the evaluation of the specific immune cells used to develop allogeneic cell therapies. With respect to donor selection, the identification of donors featuring higher serial killing activity and better killing kinetics can direct the use of a more potent batch requiring fewer immune cells to generate a response in the patient.

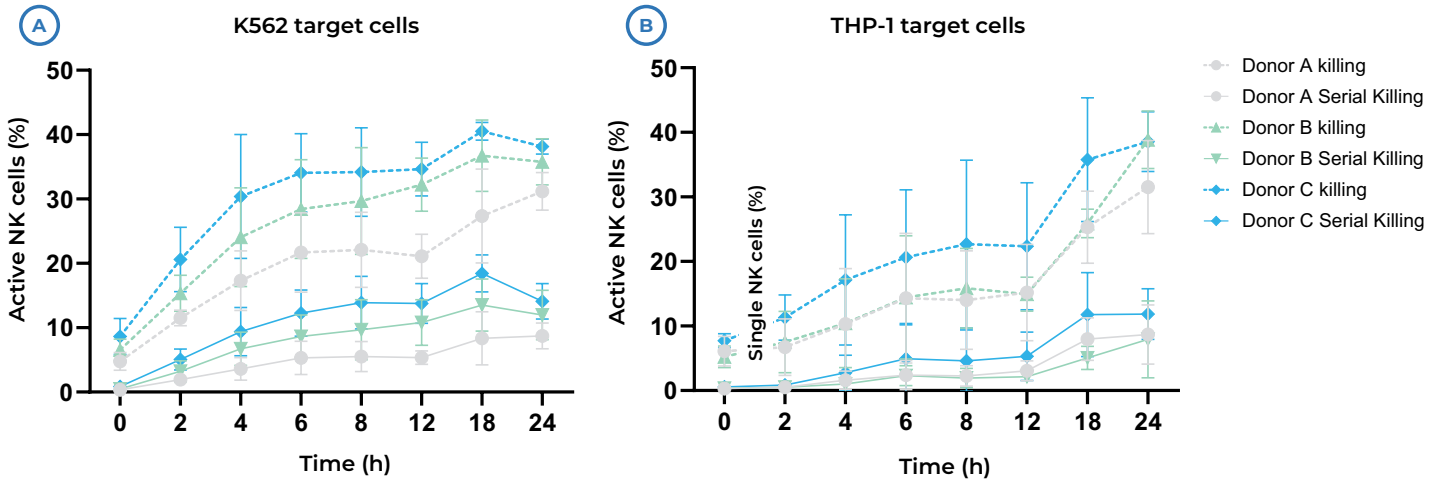


Figure 6. Single NK cell time-lapse killing capacity. Quantification of Killer and Serial Killer NK cells is reported at multiple time points for (A) K562 and (B) THP-1 target cell lines. Each point represents the mean and standard deviation of three independent experiments using the scheme reported in Figure 1.

The serial killing as described in this application note can be used either alone or in combination with different approaches: like cytokines release, bulk cytotoxicity as well as immunophenotypic characterization of NK cells, to support a final decision for selecting either an optimal donor or an optimal gene edit.

Here we demonstrated the opportunity to support the identification of the more active donors for the development of an NK cell-based allogeneic cell therapy with the analytical power of a single cell resolution platform.

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