nature biomedical engineering

PEER-REVIEW INFORMATION

Identification, sorting and profiling of functional killer cells via the capture of fluorescent target-cell lysate

Corresponding author: Lih Feng Cheow

Editorial note

This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

Correspondence

Sun 14 Aug 2022
Decision on Article NBME-22-1625A

Dear Dr Cheow,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "Tracking, catching and profiling killer cells with PAINTkiller assay". The manuscript has been seen by three experts, whose reports you will find at the end of this message.

You will see that the reviewers appreciate the work. However, they all express concerns about the degree of support for the claims of advantageous use and robustness of the assay, and provide useful suggestions for improvement. We hope that with significant further work you can address the criticisms, further characterize the assay, and convince the reviewers of the merits of the study. In particular, we would expect that a revised version of the manuscript provides:

* Evidence of the performance of the assay with primary NK cells.

* Thorough comparisons of performance with established assays.

* Further evidence of the degrees of robustness and reproducibility of the assay.

* Thorough methodological reporting.

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 16 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*.

We hope that you will find the referee reports helpful when revising the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

The manuscript describes a cellular assay to identify highly cytotoxic NK cells from bulk populations. While the concept is somehow interesting, I am not sure about the resolution and broad applicability of the current assay. The authors need to characterize their assay carefully and compare it with other available approaches to justify the novelty of their manuscript. In addition, the quality of biological findings is suboptimal – their single-cell data is not convincing due to the low number of positive cells.

Below are some comments to improve the novelty and breadth of the manuscript.

1. The proposed assay is not the first cytometry-compatible cytotoxicity assay (for example, Wong, STAR Protocols, DOI: 10.1016/j.xpro.2020.100262, and Kandarian et al, JOVE, DOI: 10.3791/56191). It is not even the first assay to use CFSE for cytotoxicity purposes (See Wu et al, Cyto Part A 2020, DOI: cyto.a.24242).

2. Given the fact that similar assays have been developed, the authors should clearly state the advantages and disadvantages (if any) of their assays compared to the existing approaches.

3. The proposed assays rely on the release of CFSE after lysis to paint the nearby effector cells. Therefore, it is important to make sure that the released CFSE only paints the effector cell that performed lysis, not other nearby non-cytolytic cells with capture probes. The authors need to carefully design an experiment to characterize the resolution and possible leakage of their assay.

4. CFSE is a regular assay for checking cell proliferation – it divides equally during cell division. Hence, after a certain time of culture, the CFSE load on target cells will vary significantly due to cell division. This will impact the principle of painting effector cells. K562 and NALM-6 are both fast dividing cells (doubling time 16 – 20 hrs). Hence, a significant portion of cells would undergo division during the 4hr co-incubation. The authors need to perform additional experiments to characterize the impact of cell division on CFSE density and downstream cell painting.

5. The authors used NK-92MI, an immortalized, genetically engineered lymphocyte cell line for the study. The authors need to validate the utility of their assay in primary NK cells.

6. The % of CFSE+ cells is extremely low in single-cell experiments (Fig. 7). The authors only got ~20 CFSE+ cells from the whole plot. I am not confident about the omics from this low number. I suggest the authors re-run this experiment to acquire at least 200 CFSE+ cells for better comparison. In addition, it is better to replace NK-92MI with primary NK cells for more physiologically relevant data.

7. The explanation on page 13 regarding the large difference between sequencing and FACS needs additional data to support it.

8. Representative genes in the upregulated list (Fig. 7f), need to be validated using mRNA or CRISPR knock-in at the protein and functional levels.

9. High-level cell lysis in the short-term (<= 4 hrs) does not necessarily define a good population for cell immunotherapy. For example, many studies suggest that stem-like T cells, which are not highly cytotoxic in the short term, are the main contributor to durable tumor management. The authors need to tone down some claims in their discussion accordingly.

10. Overall, the level of details in the method section is not sufficient.

Reviewer #2 (Report for the authors (Required)):

Luah et al provide initial data on a new assay (PAINTKiller assay) to identify cytotoxic killer cells. The concept to label (PAINT)killer cells using a dye released by lysed cells is interesting and attractive. However, the data presented in the manuscript a too preliminary to draw strong conclusions. In many of the figures, data from single (N = 1) experiments are demonstrated. Furthermore, studies were performed using an NK cell line (NK-92M), and data using primary NK cells are lacking. Overall, the preliminary results using the PAINTKiller assay are promising, but additional experiments demonstrating (a) reproducibility of findings and (b) relevance for the studies of primary NK cells are required. Finally, the new assay has to be benchmarked against existing assays. and the data using CD107a (supplemental figure 3) are not making a lot of sense.

Comments specific to figures:
* Figure 2: it appears if data from a single experiment are shown.

* Figure 3: it is difficult to identify the CAR-T cells that are positive for anti-CFSE. Also, in this (again) single experiment a new population of CD56dim CFSE+ cells appears (bottom right panel) - what is that population?

* Figure 4: the reduction in live K562 cells is minimal over 4 hrs (18% to 12%) - this is less than one would expect from NK-92M cells. Only data from 2 experiments (n = 2) are shown, but error bars are shown. How is that possible?

* Figure 5 - results from a single experiment are shown - it is unclear, how reproducible the findings are.

* Supplemental Fig. 3 - it is important to compare the PAINTKiller assay to established assays, such as the DC107a degranulation assay that identifies activated NK cells. The CD107a data presented in supplemental figure 3 are however not acceptable. It seems that ALL NK-93 cells express CD107a already after 1 hour after adding target cells (compared to NK control). Was the antibody against CD107a added to the NK control?

Reviewer #3 (Report for the authors (Required)):

In this manuscript the authors describe a novel technology to identify cytotoxic cells based on CSFE which is release from the dying target cells and which is captured by the effector cell. Overall, this is an interesting concept and may be very useful to identify, isolate and characterize certain immune cell subsets with high or low cytotoxic activities. However, the characterization of the method is currently underdeveloped, and the manuscript needs much more work to demonstrate the practical application of the technology.

Major points:
(1) Almost all experiments are done with an NK cell line (NK92). Many of the experiments will need to be repeated with primary NK cells to show that this technology can be applied to primary cells.

(2) Many figures seem to show a single experiment and there is no indication about biological and technical replicates. Also, statistical analyses are missing.

(3) Currently, many labs are using CD107a externalization to detect cytotoxic cells. In principle, CD107a could also be used as a marker for identifying, isolating and characterizing cytotoxic cells. Therefore, it is imperative that the authors directly compare their method to the CD107a assay in more experiments than just to one shown in figure S3.

(4) For all flow cytometry experiments the gating strategy should be shown in the supplement.

(5) Figure 2: NK92 is a cell line with very limited heterogeneity. There are no CD56dim and CD56bright sub-populations within NK92 cells as there are for primary cells. Typically, NK92 can lose CD56 expression when cells have low fitness, are damaged and are about to die. Therefore, the CD56 staining in panel 2 likely identifies dying cells as CD56 low. Interestingly, in figure 3 the CD56 staining seems to be homogenous again – supporting this interpretation. A conclusion about the cytotoxic activity of CD56dim vs CD56bright cells can only be drawn from analyzing primary human NK cells.

(6) Figure 2b: I do not understand the use of the PE-labelled anti-CSFE antibody. Why do the authors not simply show the CSFE fluorescence? Why label a fluorescent molecule with another fluorescent molecule?

(7) Killing of K562 cells seems to be simply defined as percentage of K562 cells within the FACS gate. This is not sufficient. The authors need to use a specific live/dead cell marker to identify killing of K562 cells.

(8) Figure 3: One important question is if bystander cells can also pick up CSFE from dying cells in their vicinity, which would result in unwanted background in the assay. The experiment in figure 3 is not sufficient to address this. Titrations using different amounts of killing and not killing effector cells will need to be shown.

(9) Figure 3: The authors need to use a positive marker to identify the CAR-T cells (such as CD3). Relying on the lack of CD56 does not distinguish them sufficiently from the target cells. Why are the NALM6 target cells labelled by the PE anti-CSFE antibody? Should CSFE not only label intracellular proteins in these cells?

(10) Figure 4: The authors need to use an assay that can specifically detect serial killing NK cells if they want to claim that the CSFE bright NK cells are serial killers.

(11) Figure 7: As NK92 is a clonal cell line, I wander if the analysis of differential gene expression comparing degranulating and non-degranulating cells is useful. This needs to be demonstrated for primary NK cells.

Minor points:

(12) Figure 2a middle panel: This panel is supposed to show only NK92 cells. Why is there a faint CSFE positive population present? Are these K562 cells? How was the gating performed (see major point 4)? The same applies for figure S1.

(13) Figure S2: This titration does not show functional heterogeneity in NK92 cells (line 251) but is simply a

result of how many NK cells are exposed to how many target cells.

(14) Figure 5b: The gating for CSFE positive cells is completely different from the other figures. Please explain or correct. Please also include a NK-aIFNg only control.

(15) Figure 6e and S5: These figures seem to show repetitions of the same experiment. Please combine the results and include a statistical analysis.

(16) Figure S3a: The cells seem to be pre-gated but the gating strategy is not shown. In the 4h sample one can clearly see a diagonal for the CSFE/CD107a plot – suggesting that both markers correlate with each other (see major point 3).

Mon 03 Apr 2023
Decision on Article NBME-22-1625B

Dear Dr Cheow,

Thank you for your revised manuscript, "Tracking, catching and profiling killer cells with PAINTkiller assay". Having consulted with the original reviewers, I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*.

We will be performing detailed checks on your manuscript, and in due course will send you a checklist detailing our editorial and formatting requirements. You will need to follow these instructions before you upload the final manuscript files.

Please do not hesitate to contact me if you have any questions.

Best wishes,

Valeria

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Dr Valeria Caprettini
Associate Editor, *Nature Biomedical Engineering*

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Reviewer #1 (Report for the authors (Required)):

I have reviewed the revised materials and don't have further comments. I think the authors addressed the concerns adequately.

Reviewer #2 (Report for the authors (Required)):

The authors have addressed my principal concerns. This assay will add an interesting additional assay to the repertoire of assays that can determine NK cell function.

Reviewer #3 (Report for the authors (Required)):

The revised manuscript has been significantly improved. Primary NK cells were used, more controls for the specificity of the PAINTKiller assay were included and the comparison to the CD107a method is much improved. Therefore, this novel technology is very interesting and could be applied to various new questions and applications involving cytotoxic cells. I have no more comments.

Rebuttal 1

Response to reviewers' comments

We thank the reviewers for the detailed assessment of our paper. We are heartened that all the reviewers appreciate this work, and value the various helpful suggestions to improve on the manuscript. We have performed significant additional experiments as requested by the reviewers to further characterize the performance of the assay and demonstrate applications with primary NK cells. This revised manuscript provides convincing evidence on the performance and merits of the novel PAINTKiller assay. Below is a point-by-point detailed response to the reviewers' comments.

Reviewers' Comments:

Reviewer #1 (Report for the authors (Required)):
The manuscript describes a cellular assay to identify highly cytotoxic NK cells from bulk populations. While the concept is somehow interesting, I am not sure about the resolution and broad applicability of the current assay. The authors need to characterize their assay carefully and compare it with other available approaches to justify the novelty of their manuscript. In addition, the quality of biological findings is suboptimal – their single-cell data is not convincing due to the low number of positive cells.

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We acknowledge that PAINTKiller assay is not the only cytometry compatible cytotoxicity assay. There are other flow cytometry assays that measure the degree of target cell killing by measuring specific dead cell markers, nucleic acid stain, or live cell markers on the target cells. However, these assays cannot identify the effector cells that were responsible for the killing activities, and hence would not be able to provide information about the cytotoxic effector cell subpopulations.

The PAINTKiller assay is unique as it measures both the death of target cell (loss of CFSE) and identify the effector cells that performed the killing (transfer of CFSE-labelled protein to killer cells). Unlike existing target-cell-centric approaches, PAINTKiller assay is capable of tracking, sorting, and profiling the killer cells to have an in-depth understanding of the characteristic of killer cell subpopulation. To our knowledge, there are no other assays that can do this.

We have added additional comparisons of PAINTKiller assay with other cytometry-compatible cytotoxicity assays in the introduction of the revised manuscript to highlight its advantage.

2. Given the fact that similar assays have been developed, the authors should clearly state the advantages and disadvantages (if any) of their assays compared to the existing approaches.

We have expanded a paragraph in the introduction to highlight the limitations of existing approaches. The target-cell centric assays (e.g. chromium/LDH release, counting of life or dead target cells using FACS, optical or electrical means) cannot identify the effector cell subpopulations that are responsible for the killings. The degranulation marker CD107a has been used as a proxy for cell-mediated cytotoxicity, but this proxy marker is insufficient to capture all the conditions required for cell killing in view of the diverse mechanisms of cytolysis (e.g. death-receptor mediated vs lytic granule release). Similarly, Granzyme-ELISPOT assays can only enumerate cytotoxic effector cells that utilize the Perforin-Granzyme pathway, and is also unable to isolate and further characterize these cells.

In contrast to assays using proxy markers that are conditioned upon certain killing mechanisms (e.g. CD107a, Granzyme ELISPOT), PAINTKiller directly identifies killer cells that had been stained by fluorescent intracellular proteins released by the killed target cells. Thus it can identify killer cells regardless of the killing mechanism. Additionally, the intracellular protein-labelling dye (CFSE) used for target cells in PAINTKiller assay can serve as a viability marker, thus enabling simultaneous enumeration of killer cells and killed cells in a single experiment. Finally, the ability to sort and characterize the killer cells with PAINTKiller-sort and PAINTKiller-seq could allow detailed analysis of the killer subpopulations, which could be of interest for development of immunotherapy.

Currently, PAINTKiller assay has been validated for one kind of target cell lysis, due to the use of CFSE as target cell intracellular dye and anti-FITC as capture matrix on effector cells. However, in principle multiplexed PAINTKiller assay can be developed to measure and distinguish the killing of two or more kinds of target cells. A possible way to achieve this could be to transfect different target cells with distinct fluorescent proteins (e.g. GFP, YFP, RFP) and incubate them with effector cells functionalized with capture antibodies in PAINTKiller assay. Using this method, the killing specificities of multiple effector and target cells can be determined simultaneously, and may be of interest to many screening applications.

The PAINTKiller assay could have additional interesting applications beyond *in vitro* assays. Due to the “dual-modification” strategy (effector cells modified with capture matrix, target cells modified with intracellular fluorescence dye), the PAINTKiller assay can be used to measure very specific killing activities between defined cell types. This could be very useful for complex scenarios, such as *in vivo* experiments where background killing activities (e.g. against chronic viral infection) may mask the discovery of the desired effector cells (e.g. tumor-specific T cells). While complementary functional assays such as CD107a or intracellular cytokine staining assays would not distinguish between viral or tumor-specific T cells, a suitably designed PAINTKiller assay (only tumor cells labelled with fluorescence dye) would be able to achieve this objective. We anticipate that further adaptation of PAINTKiller technology for *in vivo* assays may uncover interesting biology of not just immune cell target recognition, but also their trafficking and interactions with the target cell microenvironment that ultimately determines their functionality.

At this stage, PAINTKiller assay has only been validated for short term killing experiments. There are certain points to take note if it is to be applied to long term killing assays. Although CFSE dye can be retained for long time in the cell and has

been used for *in vivo* tracing[1], the total CFSE in a cell would be diluted during target cell division. On the other hand, the CD45 surface antigen on effector cells has been shown to be remarkably stable and does not internalize for at least 19 hours[2]. The impact on sensitivity of these factors need to be carefully assessed to consider PAINTKiller assay for long term killing experiments.

We have expanded the discussion section of our revised manuscript to compare PAINTKiller assay with other approaches.

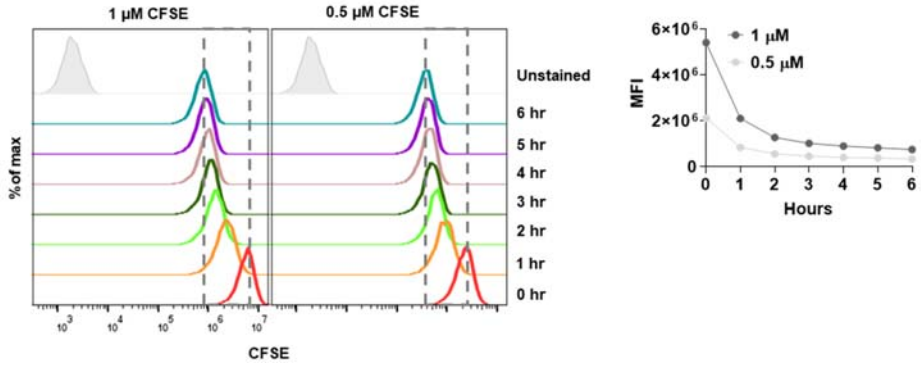
1. Parish CR. Fluorescent dyes for lymphocyte migration and proliferation studies. Immunol Cell Biol. 1999 Dec;77(6):499-508. doi: 10.1046/j.1440-1711.1999.00877.x. PMID: 10571670.

2. Zheng Y, Tang L, Mabardi L, Kumari S, Irvine DJ. Enhancing Adoptive Cell Therapy of Cancer through Targeted Delivery of Small-Molecule Immunomodulators to Internalizing or Noninternalizing Receptors. ACS Nano. 2017 Mar 28;11(3):3089-3100. doi: 10.1021/acsnano.7b00078. Epub 2017 Mar 1. PMID: 28231431; PMCID: PMC5647839.

3. The proposed assays rely on the release of CFSE after lysis to paint the nearby effector cells. Therefore, it is important to make sure that the released CFSE only paints the effector cell that performed lysis, not other nearby non-cytolytic cells with capture probes. The authors need to carefully design an experiment to characterize the resolution and possible leakage of their assay.

We thank the reviewer for the comment. We agree with this requirement, and thus we have optimized our experimental conditions and noted them in the manuscript. Below we highlight a few points that are important to ensure the specificity of PAINTKiller assay.

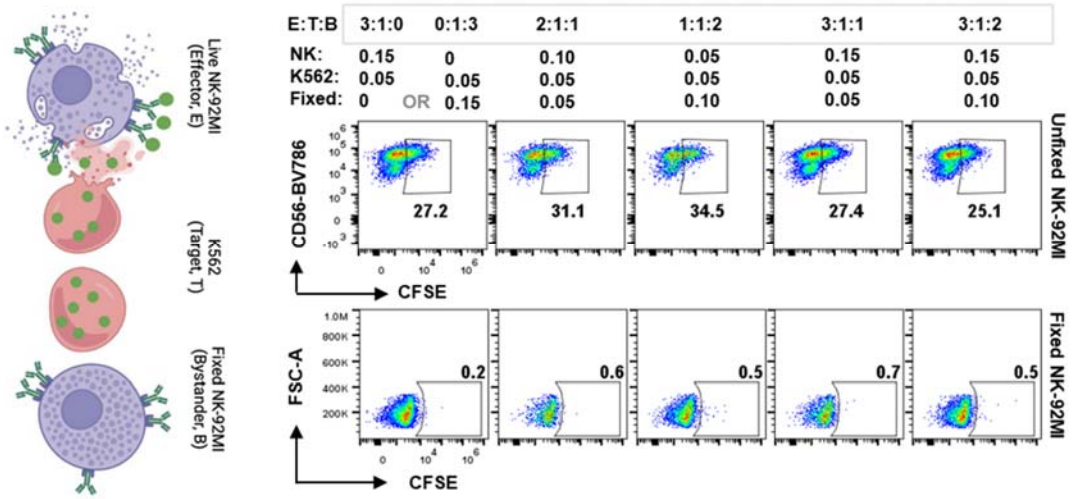
First, the target cells have to be rested for at least 2 hours after CFSE modification. After CFSE staining, the target cells undergo a rapid phase of releasing CFSE/CFSE-labeled protein from the cells (~2 hours) before final stabilization. This is shown in the figure below. If the target cells are incubated with effector cells immediately after CFSE modification, we observed considerable background "painting" of the effector cells. This is presumably due to the capture of nonspecific release of CFSE-labeled proteins from target cells. However, if the target cells are allowed to stabilize for 2 hours, followed by washing, before incubation with effector cells, we observed negligible background "painting" (Supplementary Fig. 1).



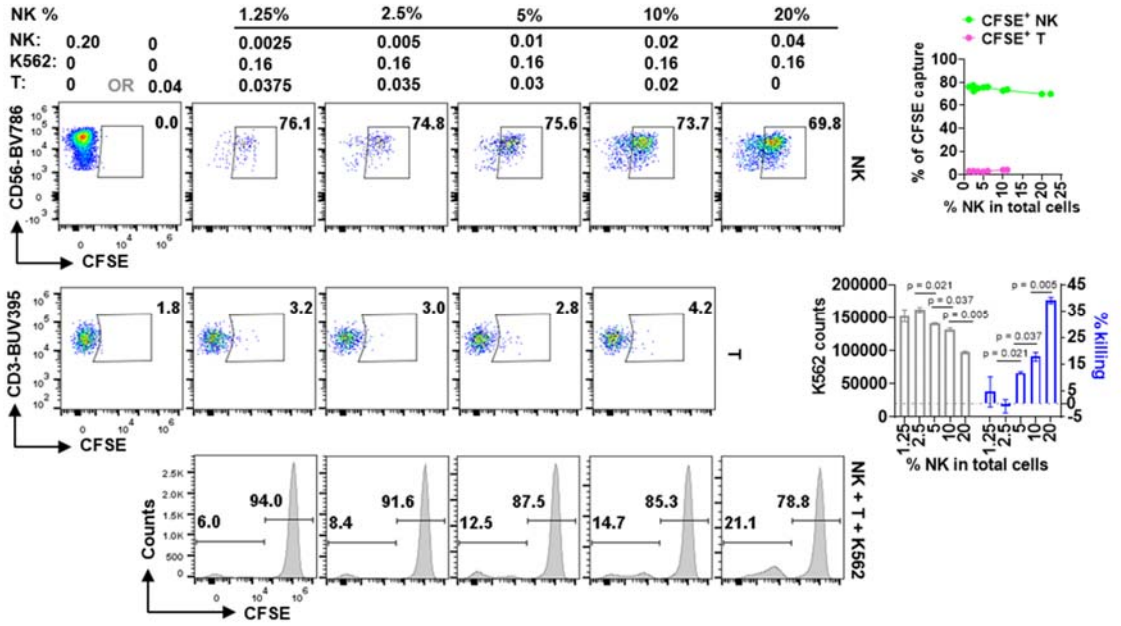
The final cell density in PAINTKiller assay is kept low (typically, a total of 0.2 million cells per well in 12-well cell culture plate). Killer and target cells are known to form conjugates for extended amount of time, compared to non-killer cells that may only enter the vicinity transiently. These conditions (low cell density and extended time for

specific CFSE capture on killer cells) ensures that capture of CFSE is much more effective between a killer-target pair compared to a bystander cell.

The specificity of the PAINTKiller assay is validated in two experiments. Firstly, we modified the surface of live-NK cells and fixed-NK cells (a model of non-effector cells) with capture antibodies. Then we co-cultured them together with CFSE-loaded K562 cells, at different live-NK, fixed-NK and K562 ratios. We observed that only a subpopulation of live-NK cells gained CFSE signal. None of the fixed-NK cells (non-cytolytic cells) gained CFSE signals. This experiment showed minimal leakage of CFSE to non-cytolytic cells (Fig. 2g,h).



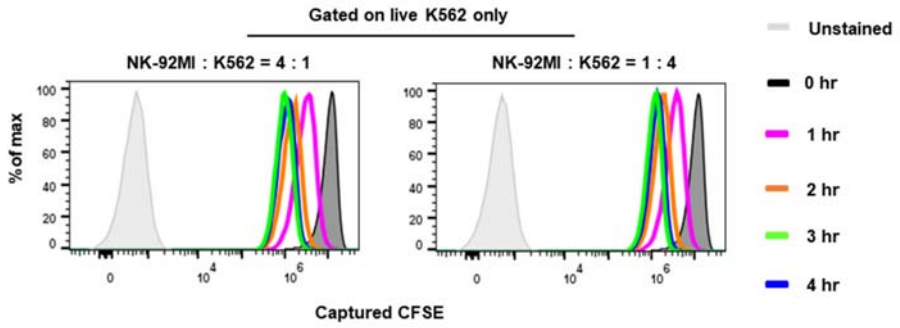
Finally, to characterize the specificity and potential leakage in primary cells, PAINTKiller assay was performed by co-incubating primary NK cells (effector cells), primary T cells (bystander cells), with CFSE-loaded K562 cells (target cells). Primary NK cells (but not T cells) are known to kill K562 cells due to their lack of MHC-1 expression. During the primary NK, primary T and K562 co-incubation experiments,



we found that K562 cells were killed but only the primary NK cells were painted with CFSE. The nearby T cells that were incapable of killing K562 cells were not painted with CFSE. This experiment once again demonstrates that the released CFSE only paints the effector cell that performed lysis, not other nearby non-cytolytic cells with capture probes (Fig. 4f-i).

4. CFSE is a regular assay for checking cell proliferation – it divides equally during cell division. Hence, after a certain time of culture, the CFSE load on target cells will vary significantly due to cell division. This will impact the principle of painting effector cells. K562 and NALM-6 are both fast dividing cells (doubling time 16 – 20 hrs). Hence, a significant portion of cells would undergo division during the 4hr co-incubation. The authors need to perform additional experiments to characterize the impact of cell division on CFSE density and downstream cell painting.

We thank the reviewer for this insightful comment. Yes, cell painting could be impacted if there is significant cell division and dilution during the experimental timeframe. We have performed an experiment comparing the fluorescence intensity in K562 cells that are incubated up to 6 hours (Supplementary Fig. 1) after CFSE modification. Our results showed that there is minimal dilution of CFSE due to division (2-fold dilution of CFSE dye if any) during the time frame of co-culture assay (after 2 hours immediate pre-incubation before co-culture), which confirms the validity of the experiments presented in the manuscript.



However, we understand that there could be circumstances where significant division-coupled CFSE is significant, or when PAINTKiller assay is to be performed over extended time. A suggestion to avoid the effects of cell division would be to irradiate target cells before the experiment[1] so that they are not capable of cell division. As an alternative to irradiation, mitomycin-C, a DNA crosslinking agent, can also be used to prevent target cells from proliferating[2].

1. Llames S, García-Pérez E, Meana Á, Larcher F, del Río M. Feeder Layer Cell Actions and Applications. Tissue Eng Part B Rev. 2015 Aug;21(4):345-53. doi: 10.1089/ten.TEB.2014.0547. Epub 2015 Mar 24. PMID: 25659081; PMCID: PMC4533020.

2. Ponchio L, Duma L, Oliviero B, Gibelli N, Pedrazzoli P, Robustelli della Cuna G. Mitomycin C as an alternative to irradiation to inhibit the feeder layer growth in long-term culture assays. Cytotherapy. 2000;2(4):281-6. doi: 10.1080/146532400539215. PMID: 12042037.

5. The authors used NK-92MI, an immortalized, genetically engineered lymphocyte cell line for the study. The authors need to validate the utility of their assay in primary NK cells.

We thank the reviewer for the suggestion. We have performed additional PAINTKiller assays with primary NK cells and presented the results in the revised manuscript.

6, 7. The % of CFSE+ cells is extremely low in single-cell experiments (Fig. 7). The authors only got ~20 CFSE+ cells from the whole plot. I am not confident about the omics from this low number. I suggest the authors re-run this experiment to acquire at least 200 CFSE+ cells for better comparison. In addition, it is better to replace NK-92MI with primary NK cells for more physiologically relevant data. The explanation on page 13 regarding the large difference between sequencing and FACS needs additional data to support it.

Upon further testing, we found that the original barcoded-anti-FITC monoclonal antibody (Biolegend, clone FIT-22) had binds poorly to cell-surface CFSE. We also found report on the poor affinity of this clone binding to CFSE in another publication[1]. That could be the main reason for the poor sensitivity of our original PAINTKiller-seq assay. We overcome this problem with a two-step approach. Cell surface CFSE was first labelled with a high-affinity PE-conjugated-anti-FITC polyclonal antibody (the same clone that is used as capture antibody). Subsequently, the cells were labelled with barcoded-anti-PE antibody (PE) for signal readout in sequencing.

As suggested by the reviewer, we have performed PAINTKiller-seq with the new detection antibody and primary NK cells in the revised manuscript. Using this approach, we obtained a proportion of $CFSE^+$ cells more comparable between sequencing and FACS. >2000 $CFSE^+$ cells were obtained for further analysis in this experiment.

1. Good Z, Borges L, Vivanco Gonzalez N, Sahaf B, Samusik N, Tibshirani R, Nolan GP, Bendall SC. Proliferation tracing with single-cell mass cytometry optimizes generation of stem cell memory-like T cells. Nat Biotechnol. 2019 Mar;37(3):259-266. doi: 10.1038/s41587-019-0033-2. Epub 2019 Feb 11. PMID: 30742126; PMCID: PMC6521980.

8. Representative genes in the upregulated list (Fig. 7f), need to be validated using mRNA or CRISPR knock-in at the protein and functional levels.

We have now performed PAINTKiller-seq experiment with primary NK cells, which is a heterogeneous population. We have identified genes that are enriched in killer cells. Most of these genes are related to cell activation, while others may be associated with particular subpopulations of NK cells. These genes collectively described the cell state, rather than being the determinant of killing activity. In the revised manuscript, we have included references relating to the function of the differentially expressed genes. In view of the scope of this work, which is focused on the demonstration of a novel assay, we have not performed in depth biological characterization of candidate genes at the protein and functional level.

9. High-level cell lysis in the short-term (<= 4 hrs) does not necessarily define a good population for cell immunotherapy. For example, many studies suggest that stem-like T cells, which are not highly cytotoxic in the short term, are the main contributor to durable tumor management. The authors need to tone down some claims in their discussion accordingly.

We thank the reviewer for the comment and agree that multiple aspects need to be considered to define a good population for cell immunotherapy. We have revised the claims in our discussion section.

10. Overall, the level of details in the method section is not sufficient.

We have added detailed methods in the revised manuscript.

Reviewer #2 (Report for the authors (Required)):

Luah et al provide initial data on a new assay (PAINTKiller assay) to identify cytotoxic killer cells. The concept to label (PAINT)killer cells using a dye released by lysed cells is interesting and attractive. However, the data presented in the manuscript a too preliminary to draw strong conclusions. In many of the figures, data from single (N = 1) experiments are demonstrated. Furthermore, studies were performed using an NK cell line (NK-92M), and data using primary NK cells are lacking. Overall, the preliminary results using the PAINTKiller assay are promising, but additional experiments demonstrating (a) reproducibility of findings and (b) relevance for the studies of primary NK cells are required. Finally, the new assay has to be benchmarked against existing assays. and the data using CD107a (supplemental figure 3) are not making a lot of sense.

Comments specific to figures:

* Figure 2: it appears if data from a single experiment are shown.
We agree with the reviewer that the characterization of the reproducibility of the assay needs to be improved. We have repeated PAINTKiller assay for the relevant experiments for 3 times whenever statistical calculation is required to establish the reproducibility. The FACS plots from one representative experiment is shown and the error bars for quantitative data in at least three independent experiments are shown in the graphs (Figures 2b-e, 3c-d, 4a-c).

* Figure 3: it is difficult to identify the CAR-T cells that are positive for anti-CFSE. Also, in this (again) single experiment a new population of CD56dim CFSE+ cells appears (bottom right panel) - what is that population?
The CAR-T and NK-92MI experiment was performed to establish the specificity of the PAINTKiller assay in the original manuscript. As the CAR-T cells have a GFP-reporter, It was harder to identify those that acquired CFSE.

To answer the second question, in our earlier experiments we occasionally find some residual cells from a previous experiment during the FACS run. This could be the $CD56^{dim}$ $CFSE^{+}$ cells that appeared. In our new set of experiments we have implemented additional washes between FACS run to eliminate carryover, and we have not seen this population appearing.

In the revised manuscript, we have replaced the CAR-T and NK-92MI experiment with two separate experiments using cells without GFP-reporter to better demonstrate specificity in both cell lines and primary cells. First, we co-cultured live NK-92MI (effector cells), fixed NK-92MI (non-effector cells) and K562 cells in PAINTKiller assay and demonstrate that only live-NK-92MI cells acquire CFSE signals (Figure 2g-h). Secondly, we co-cultured NK primary cells (effector cells), T cells (bystander cells) and K562 cells in PAINTKiller assay and demonstrate that only primary NK cells acquire CFSE signals (Figure 4f-i).

* Figure 4: the reduction in live K562 cells is minimal over 4 hrs (18% to 12%) - this is less than one would expect from NK-92M cells. Only data from 2 experiments (n = 2) are shown, but error bars are shown. How is that possible?

We have performed additional experiments and presented results of three experiments (with error bars) in the revised manuscript. As pointed out by other reviewers, our earlier calculation of K562 cells as a percentage of total cell population could be difficult to interpret as the total cell count is decreasing over time during killing. We have included count beads in FACS and report on the actual number of K562 cells in the revised manuscript. We have consistently observed in our experiment that our batch of NK-92MI cells kill rather slowly. In contrast, the additional experiments PAINTKiller that we performed with primary NK cells showed that they killed K562 cells much more efficiently.

* Figure 5 - results from a single experiment are shown - it is unclear, how reproducible the findings are.
We have performed additional experiments and presented results from five separate experiments for the combined killing and cytokine secretion assay in the revised manuscript. The representative FACS plot of one experiment was presented in the figure to illustrate the results. Quantitative results comparing the percentage of cells having orthogonal effector markers (CFSE, CD107a and IFNγ) were shown in plots with error bars.

* Supplemental Fig. 3 - it is important to compare the PAINTKiller assay to established assays, such as the DC107a degranulation assay that identifies activated NK cells. The CD107a data presented in supplemental figure 3 are however not acceptable. It seems that ALL NK-93 cells express CD107a already after 1 hour after adding target cells (compared to NK control). Was the antibody against CD107a added to the NK control?
We have performed multiple additional experiments similar to Supplemental Fig 3 and saw that apart from the small populations that gained CD107a signal, CD107a staining background in co-culture assay is largely similar to the NK-only controls. There could be a systematic error in that previous NK control data as the CFSE level was also unexpectedly low.

We thank the reviewer for the suggestion to compare PAINTKiller assay to CD107a assay in more instances. We have performed these additional experiments in both NK-92MI cell (Fig. 2) as well as primary NK cells PAINTKiller assays (Fig. 5). We observed a generally positive correlation between CFSE and CD107a signals (Fig. 2e, 5c). This was reassuring, as CD107a is a degranulation marker, and Perforin-Granzyme-mediated cytolysis is recognized as a major effector mechanism of NK cells. However, there are also differences between the two measures. PAINTKiller consistently identifies more killer cells than the CD107a approach (Fig. 2f), thus may indicate a better sensitivity or the ability to identify killer cells utilizing other killing mechanisms. In fact, it was a striking observation that in primary NK cells (but not NK-92MI cells), the percentage of $CFSE^+$ cells increases with time while CD107a signal increased much less or even decreased. In trying to reconcile these observations, we note that are previous work demonstrating the transient nature of CD107a[1]. Downregulation of CD107a occurred after a certain period. In this respect, CD107a measurements may represent short term degranulation activities, rather than being a stable marker for killing activities (e.g. over 4 hours). Another interesting possibility consistent with this observation is that primary NK cells could utilize different killing mechanisms at early and late phase. It has been reported that perforin-granzyme degranulation is the primary mechanism for early cell killing, after which many cells

switch to Fas-FasL/Trail-induced apoptosis as a killing mechanism[2]. In this case, direct measurement of killer cells with PAINTKiller could provide a more comprehensive portrait of killing activities in effector cells.

1. Liu D, Bryceson YT, Meckel T, Vasiliver-Shamis G, Dustin ML, Long EO. Integrin-dependent organization and bidirectional vesicular traffic at cytotoxic immune synapses. Immunity. 2009 Jul 17;31(1):99-109. doi: 10.1016/j.immuni.2009.05.009. PMID: 19592272; PMCID: PMC2740634.

2. Prager I, Liesche C, van Ooijen H, Urlaub D, Verron Q, Sandström N, Fasbender F, Claus M, Eils R, Beaudouin J, Önfelt B, Watzl C. NK cells switch from granzyme B to death receptor-mediated cytotoxicity during serial killing. J Exp Med. 2019 Sep 2;216(9):2113-2127. doi: 10.1084/jem.20181454. Epub 2019 Jul 3. PMID: 31270246; PMCID: PMC6719417.

Reviewer #3 (Report for the authors (Required)):

In this manuscript the authors describe a novel technology to identify cytotoxic cells based on CSFE which is release from the dying target cells and which is captured by the effector cell. Overall, this is an interesting concept and may be very useful to identify, isolate and characterize certain immune cell subsets with high or low cytotoxic activities. However, the characterization of the method is currently underdeveloped, and the manuscript needs much more work to demonstrate the practical application of the technology.

Major points:
(1) Almost all experiments are done with an NK cell line (NK92). Many of the experiments will need to be repeated with primary NK cells to show that this technology can be applied to primary cells.
We appreciate the reviewer's comments, which are echoed by other reviewers. We have repeated the PAINTKiller experiments with primary NK cells.

(2) Many figures seem to show a single experiment and there is no indication about biological and technical replicates. Also, statistical analyses are missing.
We have performed additional experiments to address this issue. We have performed at least three independent experiments for Figures 2-5, except Fig. 2h and 4f-I where multiple effector, target and bystander ratios are tested collectively to qualitatively show that specificity is high in all scenarios. FACS plot from a representative experiment was presented in the figures, while error bars from at least three experiments were shown in quantitative plots.

We have also added statistical analyses where relevant, and for PAINTKiller-seq.

(3) Currently, many labs are using CD107a externalization to detect cytotoxic cells. In principle, CD107a could also be used as a marker for identifying, isolating and characterizing cytotoxic cells. Therefore, it is imperative that the authors directly compare their method to the CD107a assay in more experiments than just to one shown in figure S3.
We have added comparison between PAINTKiller assay and CD107a staining in the NK-92MI cell line (Fig. 2) and primary NK cell experiments (Fig. 5). We have also performed these analyses in single cell PAINTKiller-seq (Fig. 6).

In general, we observed a positive association between CD107a staining and CFSE capture. This is reasonable as degranulation is a major mechanism for cell-mediated cytotoxicity in NK cells. However, we consistently detected more cells with CFSE than with CD107a (Fig. 2 and 5), especially at later times in primary NK cells (Fig. 5). As described in our reply to Reviewer 1 above, the observed differences could be due to the reported transient nature of CD107a surface expression, or could be due to the involvement of non-degranulation mechanisms for NK cell killing. Co-incidentally, in our PAINTKiller-seq experiment with primary NK cells, we observed that NK cells with CFSE-capture but without CD107a expression have higher levels of the death receptor *TNFSF10* (TRAIL) expression (Supplementary Fig. 9c). As PAINTKiller assay measures target cell killing independent of the killing mechanism, it could be useful as a comprehensive cytotoxicity assay.

We would also like to add on a few points regarding several situations where CD107a externalization would not be an accurate portrayal of cell cytotoxicity. Apart from target cell engagement, spontaneous degranulation could be triggered by certain chemical (e.g. PMA/Ionomycin)[1] or biological molecules (e.g. intravenous immunoglobulin)[2], without inducing target cell death. Studies on different subsets of CD8 T cells also revealed that despite similar kinetics of degranulation, effector cells result in much higher killing compared to memory subset due to the increased levels of cytolytic effector molecules in their lytic granules[3]. Hence, a method that directly measures target cell killing rather than using a proxy of effector cell degranulation would provide more accurate measurements in many circumstances.

1. Li Y, Yu M, Yin J, Yan H, Wang X. Enhanced Calcium Signal Induces NK Cell Degranulation but Inhibits Its Cytotoxic Activity. J Immunol. 2022 Jan 15;208(2):347-357. doi: 10.4049/jimmunol.2001141. Epub 2021 Dec 15. PMID: 34911773.

2. Jacobi C, Claus M, Wildemann B, Wingert S, Korporal M, Römisch J, Meuer S, Watzl C, Giese T. Exposure of NK cells to intravenous immunoglobulin induces IFNγ release and degranulation but inhibits their cytotoxic activity. Clinical Immunology. 2009 Dec 1;133(3):393-401.

3. Wolint P, Betts MR, Koup RA, Oxenius A. Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8+ T cells. J Exp Med. 2004 Apr 5;199(7):925-36. doi: 10.1084/jem.20031799. Epub 2004 Mar 29. PMID: 15051762; PMCID: PMC2211884.

(4) For all flow cytometry experiments the gating strategy should be shown in the supplement.
We have included the gating strategy for flow cytometry experiments in the revised manuscript.

(5) Figure 2: NK92 is a cell line with very limited heterogeneity. There are no CD56dim and CD56bright sub-populations within NK92 cells as there are for primary cells. Typically, NK92 can lose CD56 expression when cells have low fitness, are damaged and are about to die. Therefore, the CD56 staining in panel 2 likely identifies dying cells as CD56 low. Interestingly, in figure 3 the CD56 staining seems to be homogenous again – supporting this interpretation. A conclusion about the cytotoxic activity of CD56dim vs CD56bright cells can only be drawn from analyzing primary human NK cells.
We thank the reviewer for the suggestion. We have measured the viability of NK-92MI cells under our culture conditions and consistently saw high viability. In the PAINTKiller-sort experiment, we have also sorted and cultured NK cells with higher and lower CD56 levels and observed no statistically significant difference in their proliferation (Fig. 3c).

Viewed on its own, NK-92MI does not separate into distinct CD56 subpopulations. It looked like a single population. That is consistent with the reviewer's comments. However, when we performed the PAINTKiller assay we consistently saw that cells with higher levels of CD56 labelling gaining CFSE signal. A possible explanation for this could be the stochastic fluctuations of the CD56 expression in NK cells, leading to small variations in their protein levels rather than complete distinct subpopulations. It has been reported that CD56 expression in NK-92 cell line is required for cell cytotoxicity – abolishment of CD56 expression in NK-92 cells led to compromised cytotoxicity[1]. This is in contrast to primary NK cells where loss of CD56 does not lead to defect in cytotoxicity[1]. Thus, NK-92MI cells that happened to express higher CD56

could have improved ability to kill target cells. Supporting this hypothesis was our PAINTKiller-sort experiment. We had sorted cells with higher and lower CD56 levels and expanded them separately. We observed that the CD56 levels of these expanded populations did not remain as higher and lower as initially sorted, but reverted to a continuum from higher to lower levels just like the original population (Supplementary Fig. 3). This could imply that the CD56 levels are fluctuating, rather than defining a static and distinct subpopulation.

We understand that the common definition of of $CD56^{bright}$ and $CD56^{dim}$ in literature could differ from the higher and lower CD56 cells that we observed here. We have revised the manuscript to use $CD56^{high}$ and $CD56^{low}$ to avoid the confusion as well as included a note to distinguish these two states from what is known in primary NK cell.

1. Gunesch JT, Dixon AL, Ebrahim TA, Berrien-Elliott MM, Tatineni S, Kumar T, Hegewisch-Solloa E, Fehniger TA, Mace EM. CD56 regulates human NK cell cytotoxicity through Pyk2. Elife. 2020 Jun 8;9:e57346. doi: 10.7554/eLife.57346. PMID: 32510326; PMCID: PMC7358009.

(6) Figure 2b: I do not understand the use of the PE-labelled anti-CSFE antibody. Why do the authors not simply show the CSFE fluorescence? Why label a fluorescent molecule with another fluorescent molecule?
We have shown the CFSE fluorescence. The use of PE-labelled anti-CFSE antibody showed that CFSE is captured on the cell surface. The PE-labelled anti-CFSE antibody can become useful for performing PAINTKiller assay on originally-fluorescent cells (e.g. cells with GFP reporter). Detection of surface CFSE on effector cells with an antibody is also essential for PAINTKiller-seq experiment. Finally, the demonstration that effector cells that captured CFSE can be specifically labelled with antibodies could enable certain procedures such as immunomagnetic isolation of killer cells.

(7) Killing of K562 cells seems to be simply defined as percentage of K562 cells within the FACS gate. This is not sufficient. The authors need to use a specific live/dead cell marker to identify killing of K562 cells.
The PAINTKiller assay measures lysis of target cells as their fluorescence intracellular content spill out and stain the target cells. The lysed target cells became cellular debris, and they were subsequently gated out from FACS.

We understand the reviewer's concerns about defining the killing of K562 cells as a percentage of K562 cells within the FACS gate. Indeed, as more target cells are killed the total cell number decrease, changing the interpretation of the percentage of K562 left. In the new experiments, we have included count beads in FACS experiments to enable calculation of absolute cell counts, and use this to obtain an accurate estimate of percentage killing of target cells.

(8) Figure 3: One important question is if bystander cells can also pick up CSFE from dying cells in their vicinity, which would result in unwanted background in the assay. The experiment in figure 3 is not sufficient to address this. Titrations using different amounts of killing and not killing effector cells will need to be shown.
We thank the reviewer for the suggestion. We have performed additional experiments using different ratios of live NK-92MI (effector) and fixed NK-92MI (bystander) cells (Fig. 2g-h), or primary NK (effector) and primary T (bystander) cells (Fig. 4f-g) in K562 killing assay. Our experiments showed that background CFSE

staining is minimal, regardless of whether the effector or bystander cells are in excess.

(9) Figure 3: The authors need to use a positive marker to identify the CAR-T cells (such as CD3). Relying on the lack of CD56 does not distinguish them sufficiently from the target cells. Why are the NALM6 target cells labelled by the PE anti-CSFE antibody? Should CSFE not only label intracellular proteins in these cells?
Although CFSE is membrane permeable and mainly label intracellular proteins, we can actually detect some of them on the cell surface. We think that these could be labelled intracellular proteins that are externalized / transported to cell membrane.

To demonstrate that the PAINTKiller assay can be applied to primary cells, we have replaced the CAR-T/NK-92MI/K562 killing assay with Primary NK/Primary T/K562 killing assay. The primary NK and primary T cells are positively identified by CD56 and CD3 markers respectively.

(10) Figure 4: The authors need to use an assay that can specifically detect serial killing NK cells if they want to claim that the CSFE bright NK cells are serial killers.
We have attempted to perform additional experiments to further show serial killing of NK cells but the results were not very conclusive. We have removed this claim from the revised manuscript.

(11) Figure 7: As NK92 is a clonal cell line, I wander if the analysis of differential gene expression comparing degranulating and non-degranulating cells is useful. This needs to be demonstrated for primary NK cells.
We thank the reviewer for the suggestion. We have performed this analysis for primary NK cells in the new experiments. The differential gene expression comparing degranulating vs non-degranulating cells revealed significant upregulation of chemokine-encoding genes such as *CCL1* and *CCL4*, as well as cytokine-encoding genes like *CSF2* and *IFNG*. Gene set enrichment analysis showed enrichment in NFKB signalling, Myc-target genes as well as STAT3 and STAT5 signaling pathways.

Minor points:

(12) Figure 2a middle panel: This panel is supposed to show only NK92 cells. Why is there a faint CSFE positive population present? Are these K562 cells? How was the gating performed (see major point 4)? The same applies for figure S1.
We realized that this may have been caused by residual cells that were not completely removed from consecutive FACS experiments. In the new experiments we have implemented a stringent washing protocol in between FACS run and this issue is resolved.

(13) Figure S2: This titration does not show functional heterogeneity in NK92 cells (line 251) but is simply a result of how many NK cells are exposed to how many target cells.
We have corrected this in the revised manuscript.

(14) Figure 5b: The gating for CSFE positive cells is completely different from the other figures. Please explain or correct. Please also include a NK-aIFNg only control.

We have performed additional experiments and provided the appropriate gating. A NK-aIFN-γ control is added (Fig. 5b).

(15) Figure 6e and S5: These figures seem to show repetitions of the same experiment. Please combine the results and include a statistical analysis.
We have combined these figures and included a statistical analysis (Fig. 3c-d)

(16) Figure S3a: The cells seem to be pre-gated but the gating strategy is not shown. In the 4h sample one can clearly see a diagonal for the CSFE/CD107a plot – suggesting that both markers correlate with each other (see major point 3).
We have provided the gating strategy for FACS experiments in the revised manuscript. Yes, in this and out additional experiments we do see considerable correlation between CFSE and CD107a. This is reassuring as degranulation of Perforin-Granzyme is an established pathway for NK cell cytolysis. However, we also observed differences, particularly in the proportion of CFSE vs CD107a in primary NK cells at later times. Analysis of these may uncover novel biology about cell-mediated cytotoxicity.