Supporting Information

Droplet-based cytotoxicity assay: implementation of time-efficient screening of natural killer cells antitumor activity

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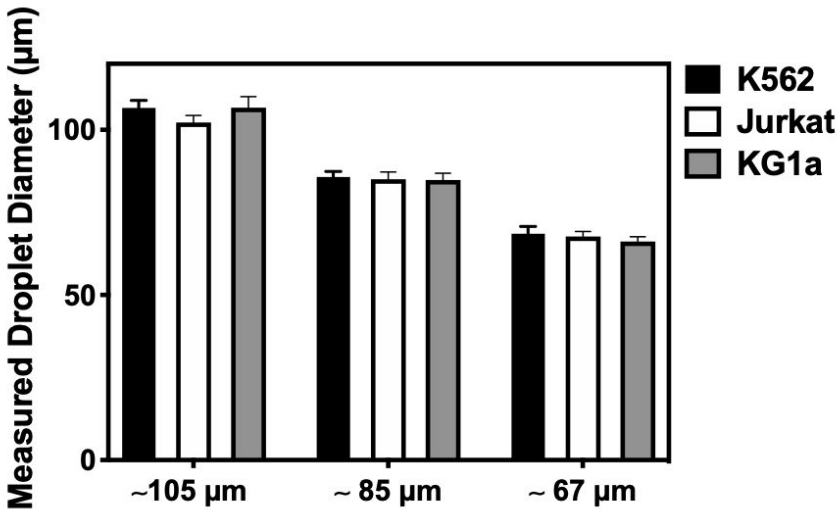
Defining the droplet diameters for the NK-92 cytotoxicity assay

In order to define the minimum droplet diameter necessary to perform an effective droplet-based cytotoxicity assay, several conditions like cell-size, NK cell motility and effector-target transient scanning must be considered. The diameter of a NK cell is roughly around 10-15 μm and the diameter of a tumor cell (e.g.: K562) is approximately 15-17 μm. Therefore we can estimate the approximate size of effector/target cell complexes containing a NK-92 : target cell ratio of 1:1, 1:2, or 1:3 to have an overall size of roughly 25-30 μm, 40-45 μm, or 50-55 μm, respectively. Because cells sediment at the bottom of the droplets after encapsulation, the available volume for the effector cell to meet and engage with his target(s) could be considered a bit smaller than the actual full volume of the droplet (**Figure 1**). In a setting of 1 effector cell : 1 target cell, we expect that a droplet diameter ≤ 50 μm would provide a reduced space for a NK-92 cells to maintain their motility and physically "examine" the surface of the tumor target cell(s). Transient conjugation between the effector and target cell, in some cases, revealed to be necessary to ensure the cytolytic hit.[1] Hampering NK-92 cell motility and scanning over the target cell, could potentially compromise NK-92 cytotoxicity. Droplets with a diameter ≥ 65 μm provide a balance between generating physical proximity among effector and target cells whilst guaranteeing enough space for the conjugation phase to take place. Accordingly, when a higher number of target cells was added to the droplets (1:2 and 1:3 effector : target cell ratio), optimal droplet size would be scaled up to a diameter ≥ 85 μm.



	K562 (µm)	Jurkat (µm)	KG1a (µm)
~ 105 µm	106.6 ± 2.2	102.2 ± 2.0	106.6 ± 3.3
~ 85 µm	85.7 ± 1.6	85.0 ± 2.1	84.8 ± 2.0
~ 67 µm	68.5 ± 2.2	67.7 ± 1.55	66.2 ± 1.4

Figure S1. *Assessment of Droplet Size.* Measured droplet diameters for droplets containing NK-92 cells and different target cells (K562, Jurkat and KG1a). On average, the measured droplet diameters consist of ~ 105, 85 and 67 µm droplets. Droplet size was quantified by measuring n ≥ 30 droplets using Fiji platform. Bars depict mean values ± s.d. As summarized in the table, for K562 target cells, droplet diameters had a mean size of 106.6 ± 2.2, 85.7 ± 1.6 and 68.5 ± 2.2 µm. For Jurkat target cells, droplet diameters had a mean size of 102.2 ± 2.0, 85.0 ± 2.1 and 67.7 ± 1.5 µm. For KG1a target cells, droplet diameters had a mean size of 106.6 ± 3.3, 84.8 ± 2.0 and 66.2 ± 1.4 µm.

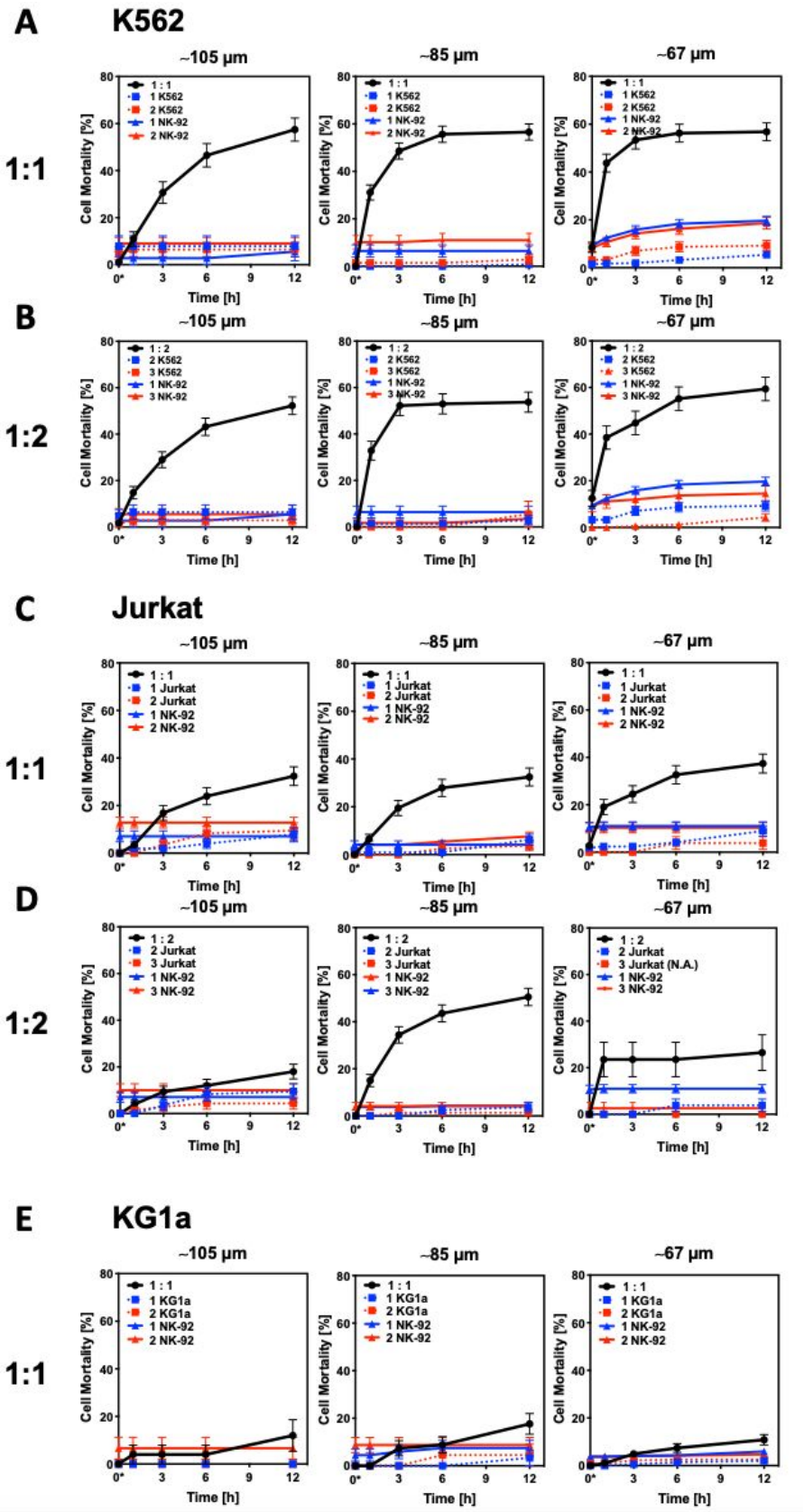


Figure S2. *Characterization of Cell Mortality in Droplets.* A) Percentage of dead K562 cells encapsulated within differently sized droplets (left: ~ 105 µm, middle: ~ 85 µm, right: ~ 67 µm) over time (12 h) under different conditions: one K562 together with one NK-92

cell (1:1; black dots; 101-212 droplets analyzed per condition); one K562 cell alone (blue square; 38-126 droplets analyzed per condition); two K562 cells alone (red square; 31-92 droplets analyzed per condition); one NK-92 cell alone (blue triangle; 36-93 droplets analyzed per condition), two NK-92 cells alone (red triangle; 59-126 droplets analyzed per condition). B) Percentage of dead K562 cells encapsulated within differently sized droplets (left: ~ 105 µm, middle: ~ 85 µm, right: ~ 67 µm) over time (12 h) under different conditions: two K562 cells together with one NK-92 cell (1:2; black dots; 48-88 droplets analyzed per condition); two K562 cell alone (blue square; 31-92 droplets analyzed per condition); three K562 cells alone (red square; 6-54 droplets analyzed per condition); one NK-92 cell alone (blue triangle; 36-93 droplets analyzed per condition), three NK-92 cells alone (red triangle; 19-39 droplets analyzed per condition). C) Percentage of dead Jurkat cells encapsulated within differently sized droplets (left: ~ 105 µm, middle: ~ 85 µm, right: ~ 67 µm) over time (12 h) under different conditions: one Jurkat together with one NK-92 cell (1:1; black dots; 142-154 droplets analyzed per condition); one Jurkat cell alone (blue square; 100-168 droplets analyzed per condition); two Jurkat cells alone (red square; 26-42 droplets analyzed per condition); one NK-92 cell alone (blue triangle; 127-146 droplets analyzed per condition), two NK-92 cells alone (red triangle; 102-111 droplets analyzed per condition). D) Percentage of dead Jurkat cells encapsulated within differently sized droplets (left: ~ 105 µm, middle: ~ 85 µm, right: ~ 67 µm) over time (12 h) under different conditions: two Jurkat cells together with one NK-92 cell (1:2; black dots; 17-93 droplets analyzed per condition); two Jurkat cell alone (blue square; 26-42 droplets analyzed per condition); three Jurkat cells alone (red square; 23-25 droplets analyzed per condition); one NK-92 cell alone (blue triangle; 127-146 droplets analyzed per condition), three NK-92 cells alone (red triangle; 13-62 droplets analyzed per condition). E) Percentage of dead KG1a cells encapsulated within differently sized droplets (left: ~ 105 µm, middle: ~ 85 µm, right: ~ 67 µm) over time (12 h) under different conditions: one KG1a together with one NK-92 cell (1:1; black dots; 25-200 droplets analyzed per condition); one KG1a cell alone (blue square; 19-300 droplets analyzed per condition); two KG1a cells alone (red square; 11-160 droplets analyzed per condition); one NK-92 cell alone (blue triangle; 11-67 droplets analyzed per condition), two NK-92 cells alone (red triangle; 15-40 droplets analyzed per condition).The time point 0* indicates the beginning of the microscopy acquisition, corresponding roughly to 30 min after cell encapsulation and droplets entrapment within the microfluidic device. Error bars display s.e.m.

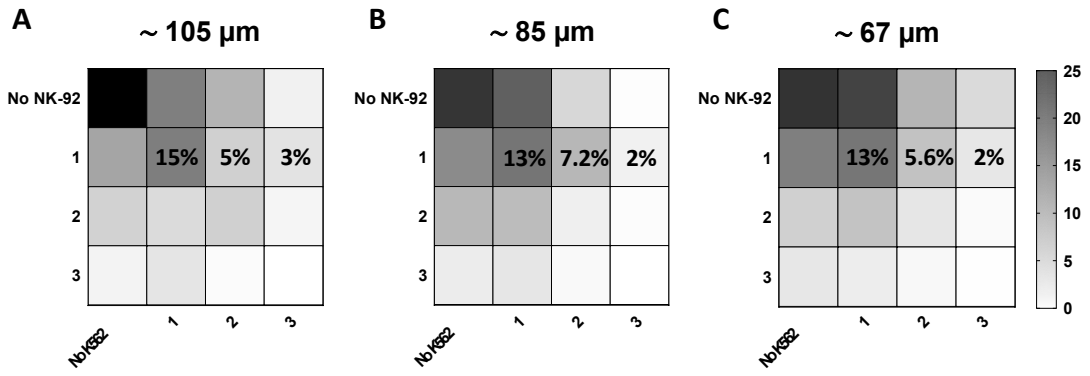


Figure S3. *Characterization of effector : target cell distribution in droplets.* A)-C) Heat maps visualizing the distribution of NK-92 and K562 target cells in percentage in differently sized droplets (~ 105, 85, or 67 µm diameter) after production. The color intensity in each panel (compare to the color key on the right) represents the percentage of cells with an effector : target cell composition specified on the row and column labels. Those panels showing the three desired compositions for our experiments (1 effector cell and either 1, 2, or 3 target cells) have the percentage value written on them.

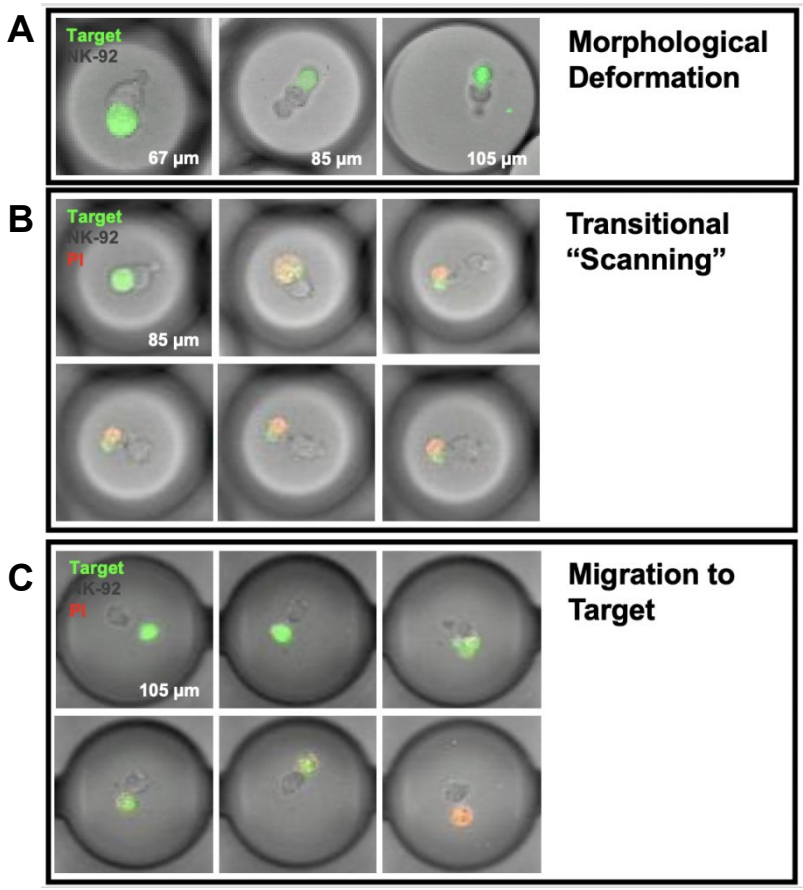


Figure S4. *Representative confocal fluorescence microscopy images of several feature characterizing the immune cell motility*. A) Representative confocal fluorescence microscopy images of NK-92 cells (unstained) undergoing morphological deformation when in conjugation with target cells. The droplets depicted in the images have a diameter of ~ 67, 85 and 105 µm (from left to right). B) Representative confocal fluorescence microscopy images of an NK-92 cell (unstained), co-encapsulated within a droplet of ~ 85 µm in diameter, transiently scanning over its target cell (green), over time (from left to right, top to bottom). C) Representative confocal fluorescence microscopy images of a NK-92 cell (unstained) migrating towards a target cell (green), within a droplet of ~ 105 µm in diameter, over time (left to right, top to bottom). All the images represent an overlay of brightfield, Cell Tracker Green (excitation/emission maxima of 492/517 nm) and propidium iodide (excitation/emission maxima of 535/617 nm).

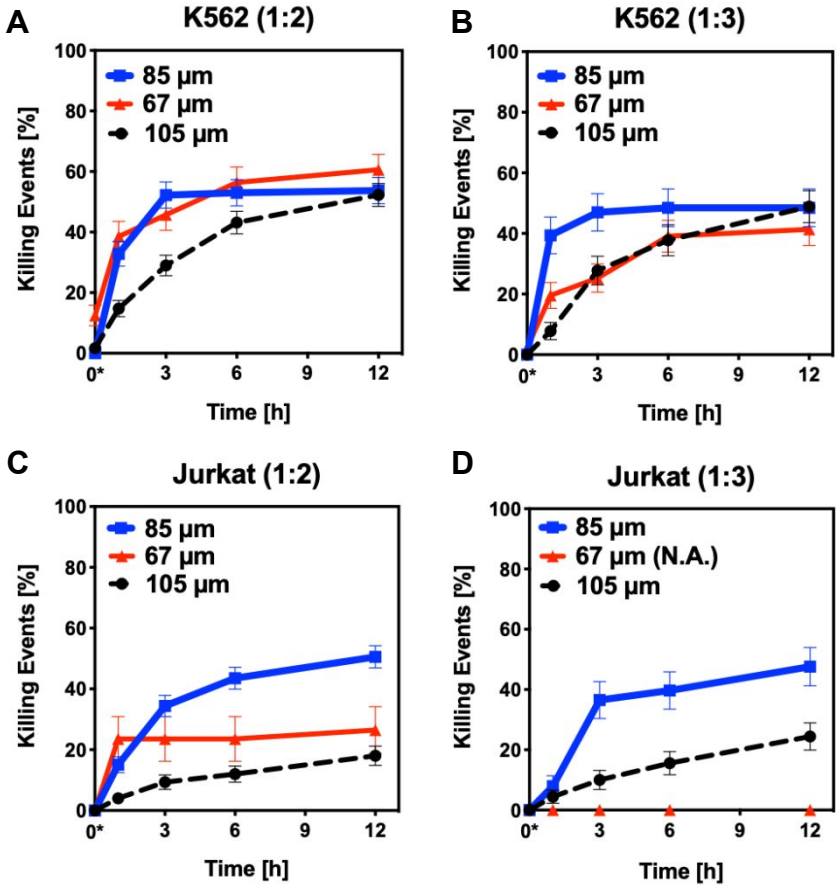


Figure S5. *Droplet-based microfluidic assessment of NK-92 cytotoxicity against two and three K562/Jurkat target cells*. A)-B) Percentage of K562 cells killed by NK-92 cells over time (12 h) in differently sized droplets (black: ~ 105 µm, blue: 85 µm, red: 67 µm). The analyzed droplets contain a ratio of 1:2 (A) and 1:3 (B) effector : target cells, respectively. For graph (A) and (B) 48-88 and 22-30 droplets have been analyzed for each condition, respectively. C)-D) Percentage of Jurkat cells killed by NK-92 cells over time (12 h) in differently sized droplets (black: ~ 105 µm, blue: 85 µm, red: 67 µm). The analyzed droplets contain a ratio of 1:2 (C) and 1:3 (D) effector : target cells, respectively. For graph (C) and (D) 17-93 and 21-30 droplets have been analyzed for each condition, respectively. The time point 0* indicates the beginning of the microscopy acquisition, corresponding roughly to 30 min after cell encapsulation and droplets entrapment within the microfluidic device. Error bars display s.e.m.

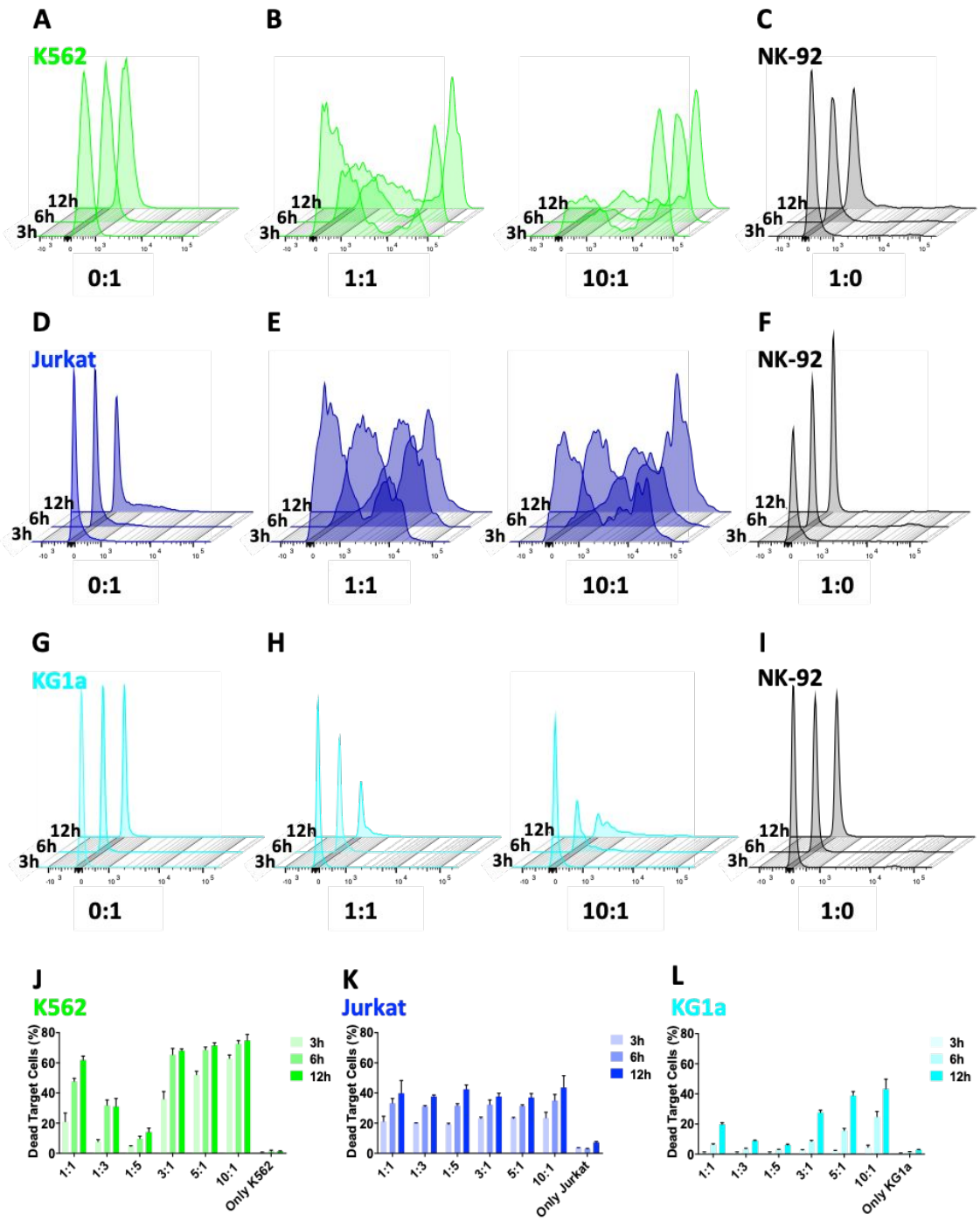


Figure S6. *Flow Cytometry Measurements of NK-92 Cell Cytotoxicity*. A)-C) Histogram overlays of cell mortality within a twelve-hour period of time. The label underneath the histograms give the ratio of effector : target cells in the sample; A) K562 cells cultured alone, B) K562 co-cultured with NK-92 at a ratio of 1:1 (left) or 1:10 (right) (1 K562 : 10 NK-92), and C) NK-92 cells culture alone. D)-F) The same experiments as shown in A)-C) performed with Jurkat target cells. G)-I) The same experiments as shown in A)-C) and D)-F) performed with KG1a target cells. Histogram counts are based on PI fluorescence intensity. For each data set 10,000 cells were analyzed. J)-L) Summarized results from the cell mortality experiments performed with different cell types and effector : target cell ratios over a time period of twelve hours. Effector : target cell ratios of 1:1, 1:3, 1:5, 3:1, 5:1, 10:1, and 0:1 (only target cells) are shown for K562 (J), Jurkat (K), and KG1a (L) cells.

Histogram bars represent mean values ± s.d. of the percentage of dead target cells from three technical replicates that included 10,000 cells each.

Supporting Video

Video S1: Confocal Fluorescence Microscopy Time-Lapse of NK-92 Cells Interacting with K562 Cells in Bulk. NK-92 cells are unstained, tumor cells are stained in green and dead cells are marked in red (PI staining). The scale bar is 100μm.

References

1. S. Sarkar, S. McKenney, P. Sabhachandani, J. Adler, X. Hu, D. Stroopinksy, J. Rosenblatt, D. Avigan and T. Konry, *Sensors and Actuators B: Chemical*, 2019, **282**, 580-589.