Supplementary Information (SI) for Lab on a Chip.

Droplet Microfluidic Profiling of NK Cell Cytotoxicity with Machine Learning-Enabled Target-Cell Death Analysis

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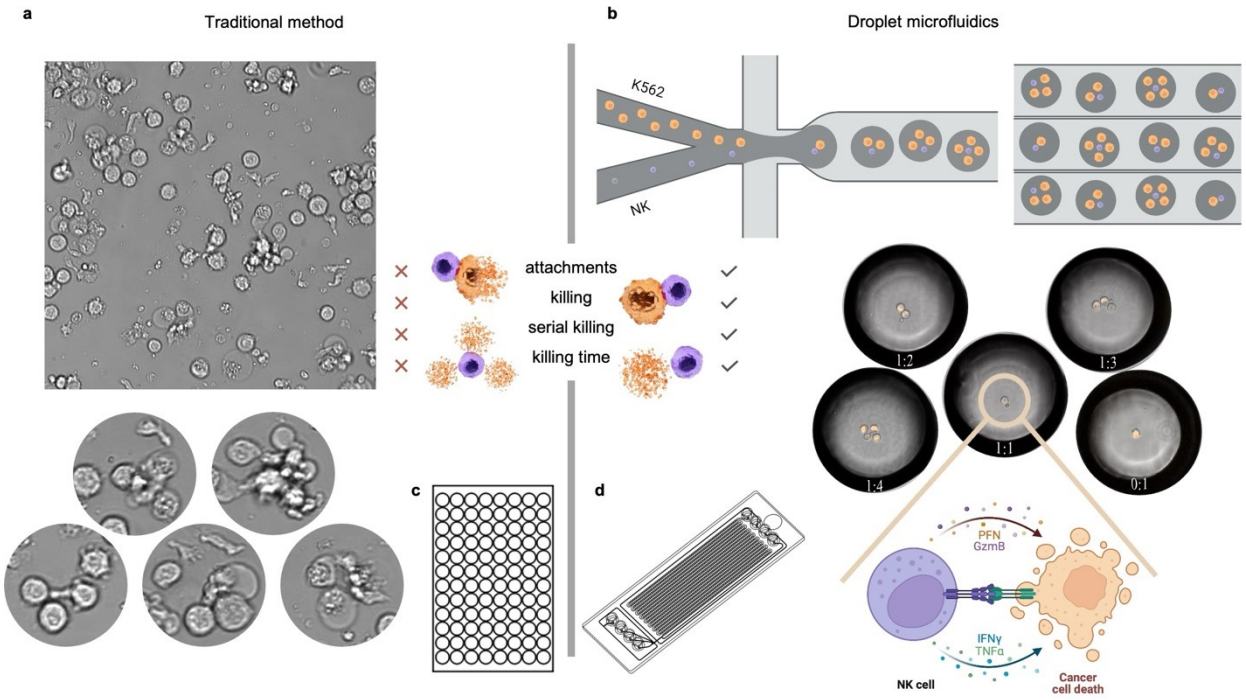
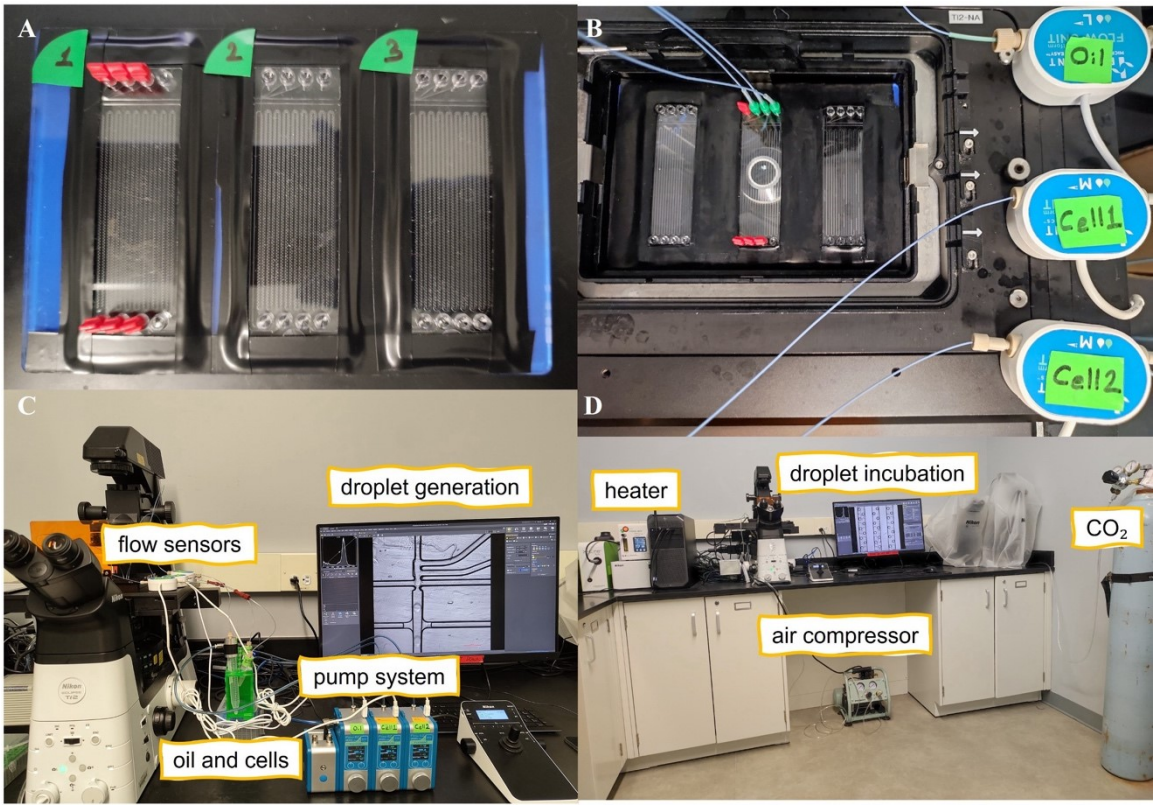
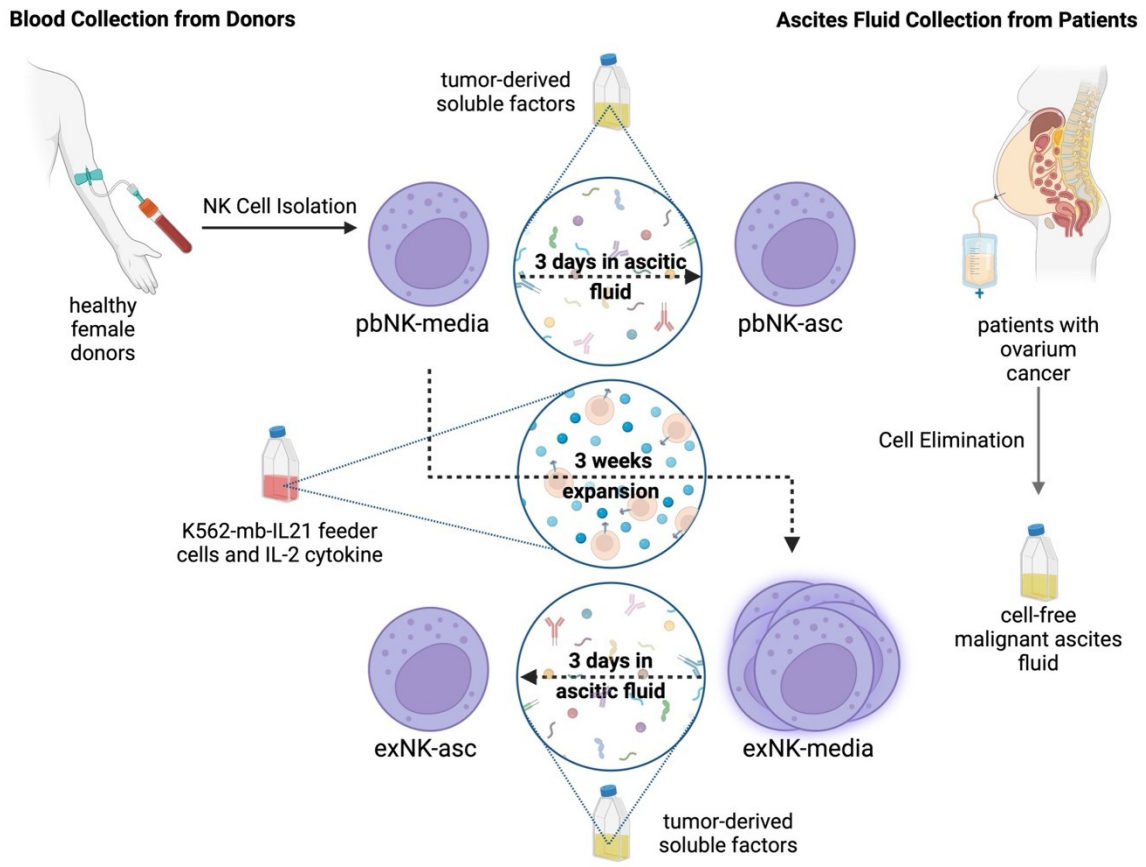


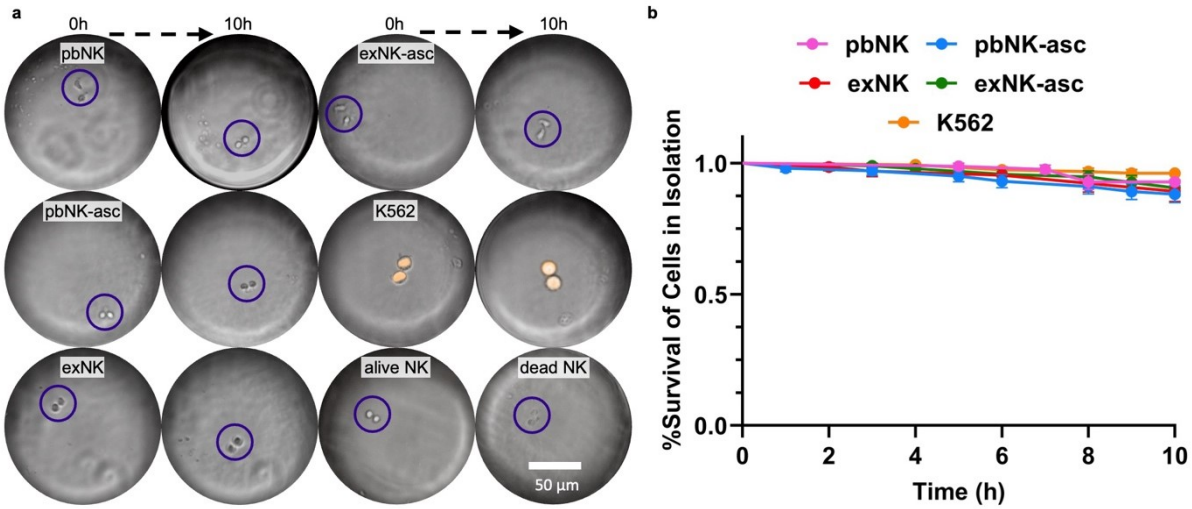
Supporting Figure 1: Comparative analysis of NK cell cytotoxicity assessment methods. *(a) The traditional method of using a bulk culture of effector and target cells with uncontrolled interactions and outcomes (b) Schematic of the droplet microfluidic method and corresponding droplet images illustrating the encapsulation and precise control of E:T cell ratios, enabling targeted observations of cytotoxic activity, serial killing, and secretions (c) Image of a well plate used in conventional bulk cytotoxicity assays (d) Image of Microfluidic ChipShop's Fluidic 719 Chip used for droplet generation and storage. Created with BioRender.com.*



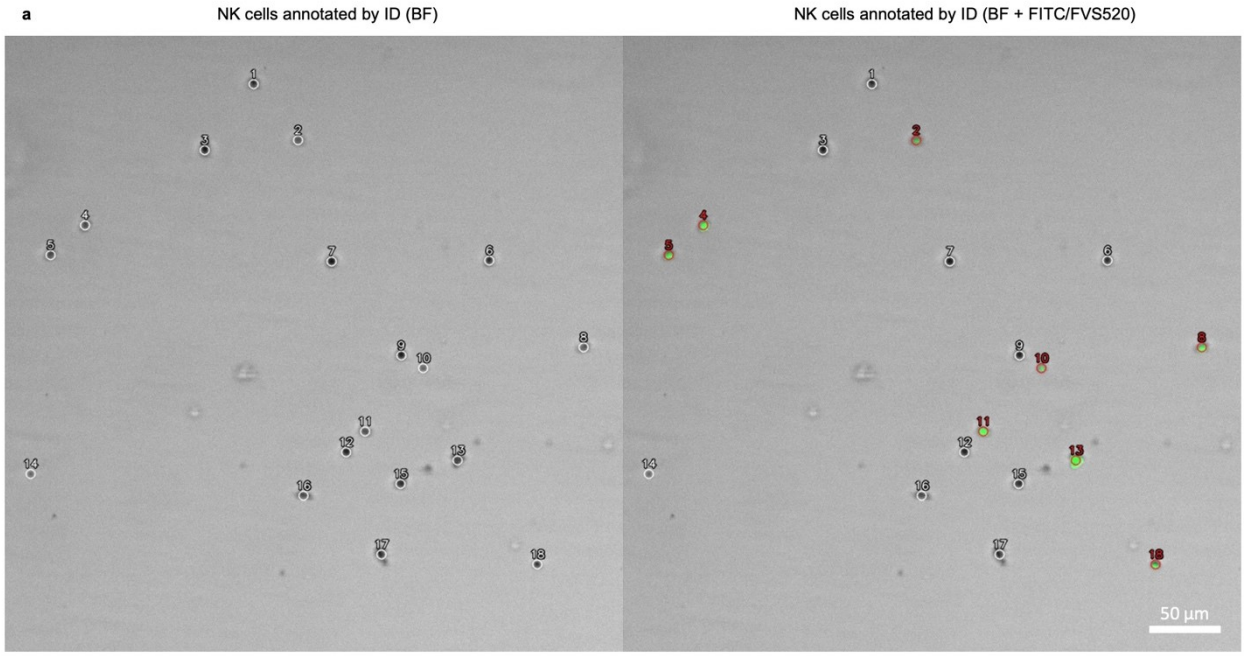
Supporting Figure 2: Droplet generation and capturing with Fluidic 719 chip*. (a) Three Fluidics719 droplet-generation/storage chips loaded in parallel for simultaneous processing. (b) A single chip mounted in the Fluigent flow sensors, with inlet tubing for oil and cell suspensions. (c) Droplet-generation workstation featuring a Nikon Eclipse Ti2-E inverted microscope, Fluigent Flow EZ pump system, and inline flow sensors, with reservoirs for dSurf oil and cell suspensions. (d) Droplet-incubation bench showing the heated microscope enclosure and CO_2 cylinder used to maintain 37 °C and 5% CO_2 during time-lapse imaging.*

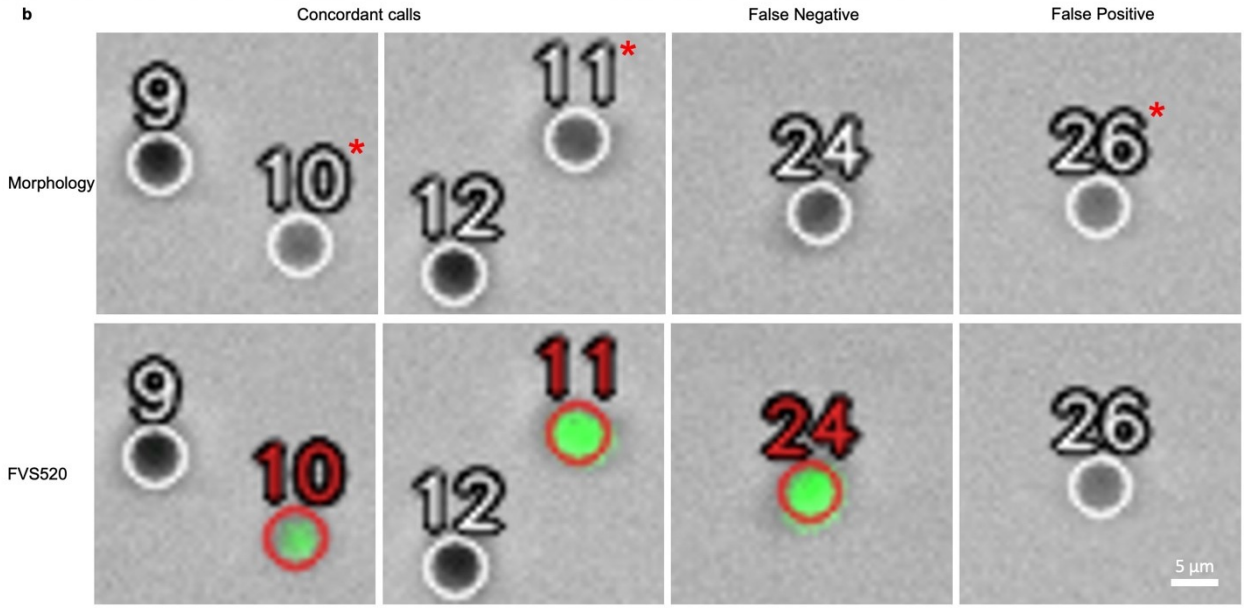


Supporting Figure 3: Illustration of the workflow for NK cell preparation. *NK cells are first isolated from the blood of healthy female donors. Cell-free malignant ascites fluid is collected from patients with ovarian cancer, representing the tumor-derived soluble factors that simulate the tumor microenvironment. Primary NK cells (pbNK-media) are cultured in ascites fluid for 3 days (pbNK-asc). In parallel, NK cells undergo a 3-week expansion using K562-mb-IL21 feeder cells and IL-2 cytokine to generate expanded NK cells (exNK-media). Expanded NK cells (exNK-asc) are also exposed to the ascites fluid for 3 days. Created with BioRender.com.*



Supporting Figure 4: Single-Cell Viability Validation. *(a) Single-cell viability controls in isolation. Representative microscopic images of individual NK cells (pbNK, pbNK-asc, exNK, exNK-asc) and K562 cells encapsulated alone in droplets at 0 h and 10 h. Purple circles indicate NK cell locations. Bottom right: examples of viable versus dead NK cell morphology showing characteristic morphological changes associated with cell death. Scale bar, 50 µm. (b) Ten-hour survival curves of cells in isolation demonstrating minimal spontaneous death in droplet environment. Lines represent different cell types: pbNK (n=83), pbNK-asc (n=101), exNK (n=65), exNK-asc (n=95), and K562 (n=156). n denotes individual single-cells across three biological replicates. Error bars represent SEM.*

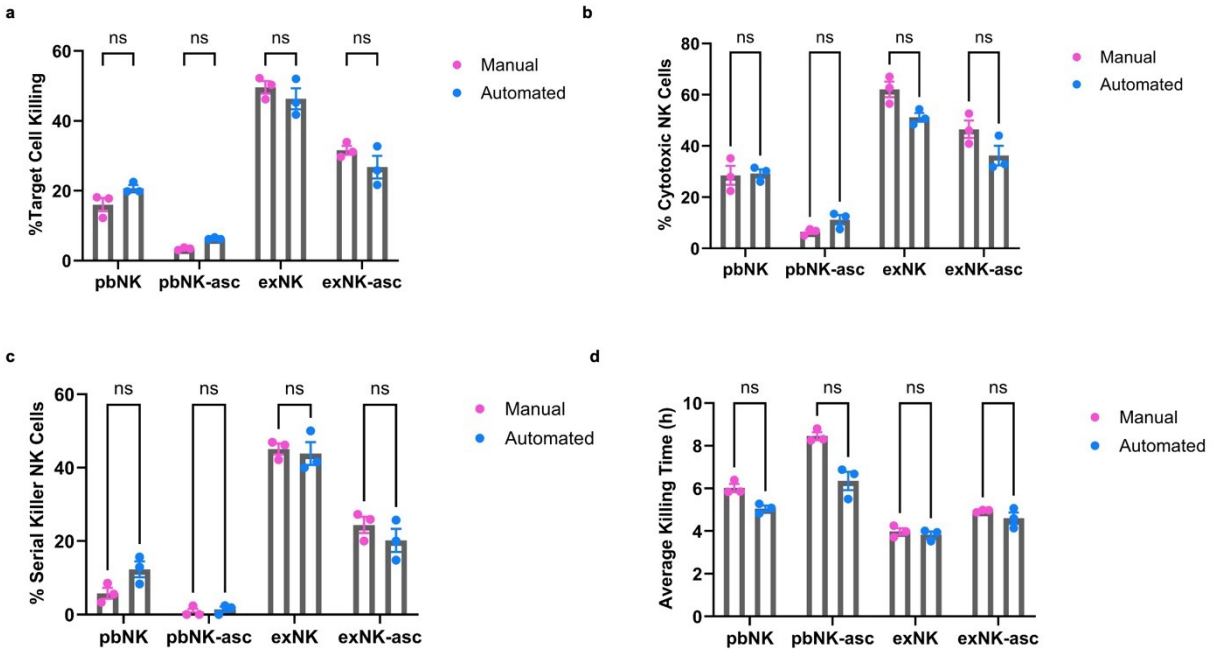




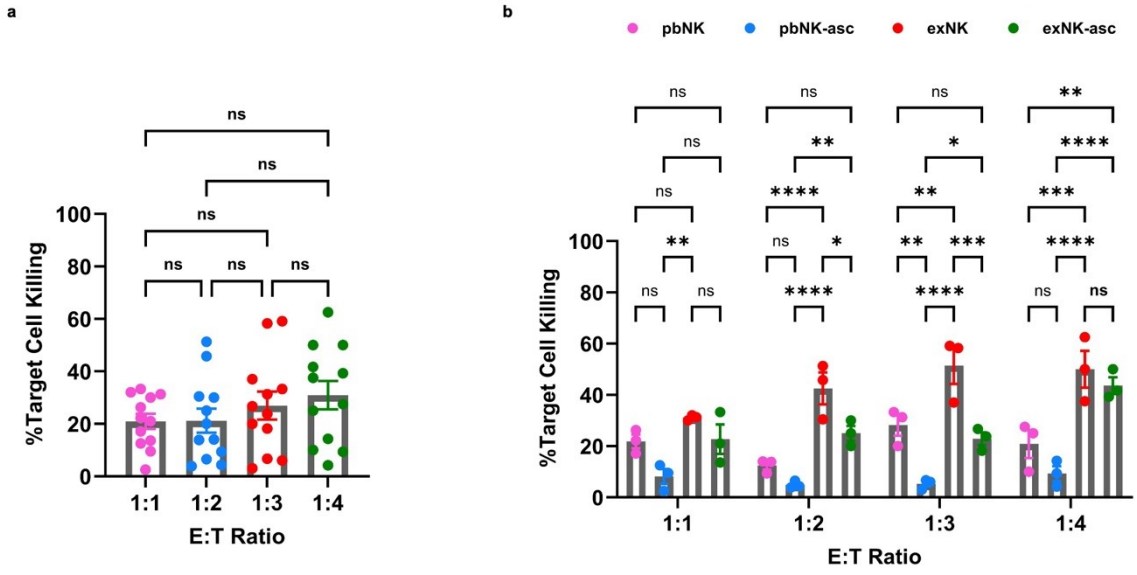
c	FVS520− (Live)	FVS520+ (Dead)	Accuracy	Sensitivity (Dead)	Specificity (Live)	PPV (Dead)	NPV (Live)	n
Morph-live	92	23	83.1%	76.5%	89.3%	87.2%	80.0%	201
Morph-dead	11	75						

Supporting Figure 5: Orthogonal validation of morphology-based NK viability scoring using FVS520 live/dead staining. *(a) Representative brightfield (BF) field of NK cells annotated by cell ID (left) and the corresponding BF image overlaid with FITC/FVS520 signal (right). The BF image was used for morphology-based viability scoring, and compared to the corresponding FVS520 dye status in the matched overlay image. FVS520 fluorescence (green) indicates membrane-compromised (non-viable) cells. Scale bar: 50 µm. (b) Example single-cell comparisons between morphology-based calls (BF; top) and FVS520 readout (bottom). Asterisks mark cells classified as dead by morphology, and red circles mark FVS520-positive (dead). Representative concordant examples are shown alongside mismatch cases defined relative to FVS520 as the reference standard: false negatives (morphology live, FVS520-positive) and false positives (morphology dead, FVS520-negative). Scale bar: 5 µm. (c) Confusion matrix and performance metrics*

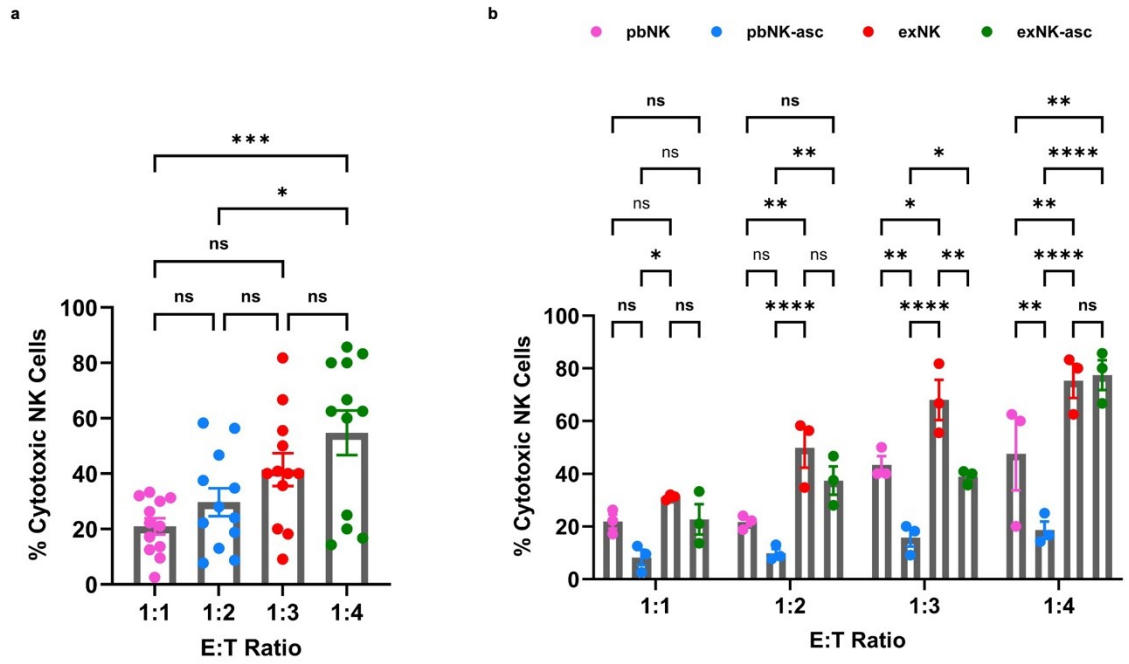
comparing morphology-based viability calls to FVS520 status across all analyzed cells (n = 201), with FVS520 used as the reference standard (FVS520− = live; FVS520+ = dead). Accuracy denotes overall agreement. Sensitivity (dead) is the fraction of FVS520+ cells correctly classified as dead by morphology (TP/(TP+FN)). Specificity (live) is the fraction of FVS520− cells correctly classified as live by morphology (TN/(TN+FP)). Positive predictive value (PPV, dead) is the fraction of morphology-dead calls that were FVS520+ (TP/(TP+FP)), and negative predictive value (NPV, live) is the fraction of morphology-live calls that were FVS520− (TN/(TN+FN)).



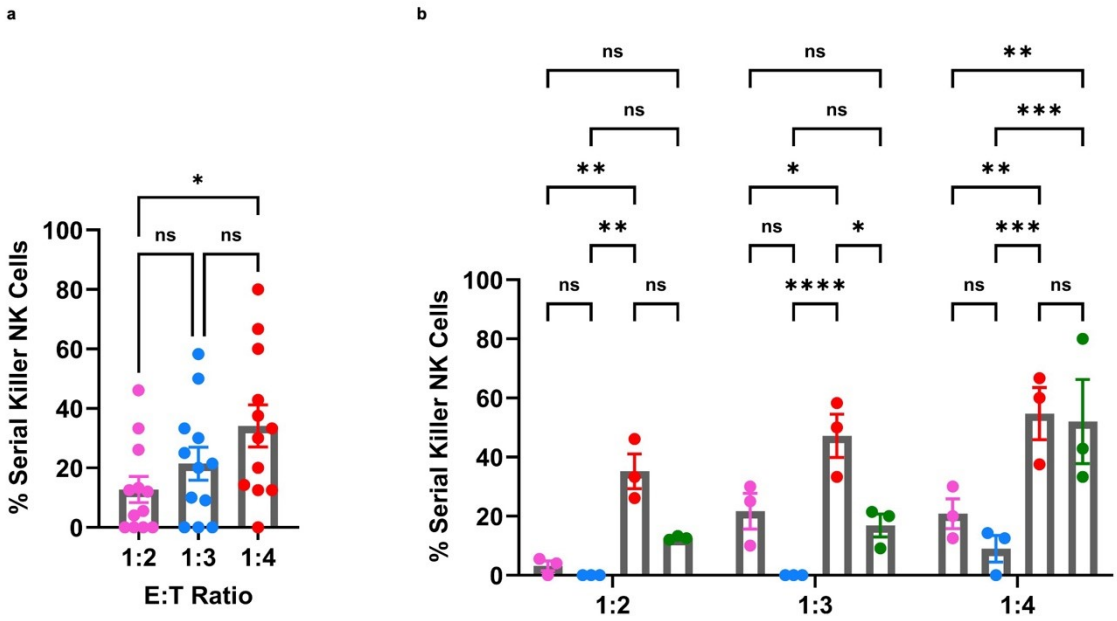
Supporting Figure 6: Comparison of manual and automated analysis methods for NK cell cytotoxicity assays. *(a) percentage of target cell killing, (b) percentage of cytotoxic NK cells, (c) percentage of serial killer NK cells, and (d) average killing time across different NK cell types (pbNK, pbNK-asc, exNK, exNK-asc). Data represent three independent biological replicates for each condition (n = 3), with individual replicate values shown as dots and bars representing mean ± SEM. Statistical comparisons were performed using paired t tests; all comparisons shown were not significant (ns, p > 0.05). Underlying sample sizes differed by metric. For panel (a), n = total target cells; for panel (b), n = droplets; for panel (c), n = multi-cell droplets (target cell ≥ 2); and for panel (d), n = kill events. Manual and automated sample sizes were: (a) manual 377, 391, 447, 365 and automated 390, 392, 451, 365; (b) manual 189, 193, 215, 170 and automated 190, 194, 216, 170; (c) manual 113, 118, 139, 112 and automated 118, 118, 145, 114; and (d) manual 62, 13, 225, 117 and automated 82, 25, 204, 95 for pbNK, pbNK-asc, exNK, and exNK-asc, respectively.*



Supporting Figure 7: Comparison of target cell killing across E:T ratios. *(a) Percentage of target cell killing across different droplet types (E:T ratios ranging from 1:1 to 1:4) regardless of NK cell type. Data represent three independent biological replicates across four NK cell groups (n = 12 plotted values) for each E:T ratio. The underlying sample size is total target cells pooled across all NK groups: 1:1 (n = 275), 1:2 (n = 480), 1:3 (n = 531), and 1:4 (n = 312). (b) Percentage of target cell killing by NK cell groups (pbNK, pbNK-asc, exNK, and exNK-asc) across varying droplet types. Data represent three independent biological replicates (n = 3) for each condition. The underlying sample size is total target cells within each NK-group/E:T-ratio condition: 1:1, pbNK (n = 72), pbNK-asc (n = 76), exNK (n = 71), exNK-asc (n = 56); 1:2, pbNK (n = 118), pbNK-asc (n = 118), exNK (n = 148), exNK-asc (n = 96); 1:3, pbNK (n = 108), pbNK-asc (n = 114), exNK (n = 156), exNK-asc (n = 153); 1:4, pbNK (n = 92), pbNK-asc (n = 84), exNK (n = 76), exNK-asc (n = 60). Asterisks represent "ns" ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$) from one-way or two-way ANOVA with Tukey's multiple comparisons test. All error bars represent SEM.*

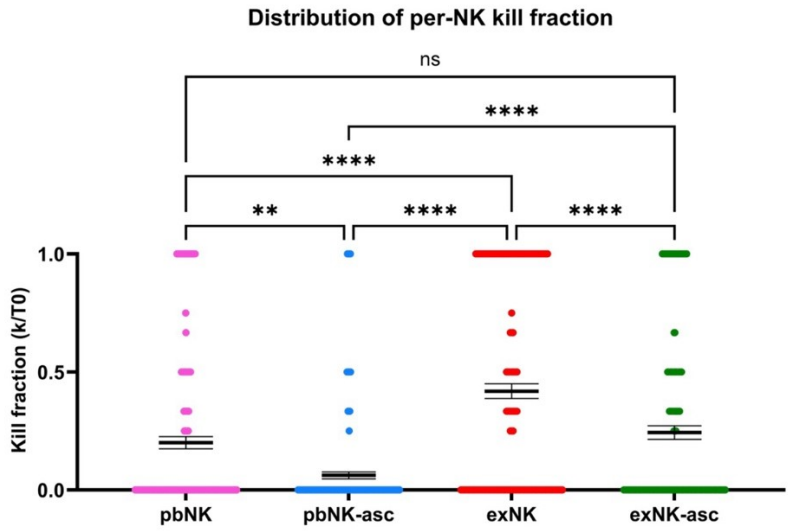


Supporting Figure 8: Comparison of cytotoxic NK Cells across E:T ratios. *(a) Percentage of cytotoxic NK cells across different droplet types (E:T ratios ranging from 1:1 to 1:4) regardless of NK cell group. Data represent three independent biological replicates across four NK cell groups (n = 12 plotted values) for each E:T ratio. The underlying sample size is the number of droplets pooled across all NK groups: 1:1 (n = 275), 1:2 (n = 240), 1:3 (n = 177), and 1:4 (n = 78). (b) Percentage of cytotoxic NK cells by NK cell groups (pbNK, pbNK-asc, exNK, and exNK-asc) across varying droplet types. Data represent three independent biological replicates (n = 3) for each condition. The underlying sample size is the number of droplets in each NK-group/E:T-ratio condition: 1:1, pbNK (n = 72), pbNK-asc (n = 76), exNK (n = 71), exNK-asc (n = 56); 1:2, pbNK (n = 59), pbNK-asc (n = 59), exNK (n = 74), exNK-asc (n = 48); 1:3, pbNK (n = 36), pbNK-asc (n = 38), exNK (n = 52), exNK-asc (n = 51); 1:4, pbNK (n = 23), pbNK-asc (n = 21), exNK (n = 19), exNK-asc (n = 15). Asterisks represent "ns" ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$) from one-way or two-way ANOVA with Tukey's multiple comparisons test. All error bars represent SEM.*

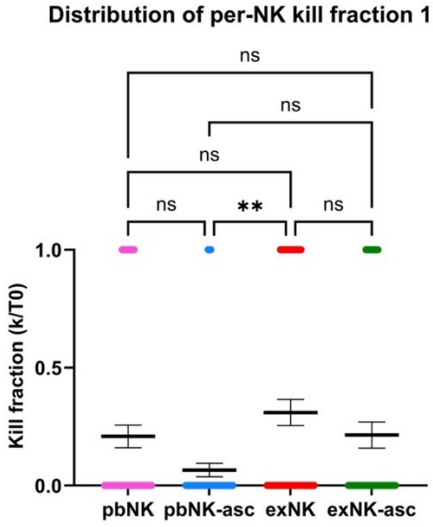


Supporting Figure 9: Comparison of serial killer NK cells across E:T ratios. *(a) Percentage of serial killer NK cells across different droplet types (E:T ratios 1:2, 1:3, and 1:4) regardless of NK cell group. Data represent three independent biological replicates across four NK cell groups (n = 12 plotted values) for each E:T ratio. The underlying sample size is the number of multi-cell droplets (CC ≥ 2) pooled across all NK groups: 1:2 (n = 240), 1:3 (n = 177), and 1:4 (n = 78). (b) Percentage of serial killer NK cells by NK cell groups (pbNK, pbNK-asc, exNK, and exNK-asc) across varying droplet types. Data represent three independent biological replicates (n = 3) for each condition. The underlying sample size is the number of multi-cell droplets (CC ≥ 2) in each NK-group/E:T-ratio condition: 1:2, pbNK (n = 59), pbNK-asc (n = 59), exNK (n = 74), exNK-asc (n = 48); 1:3, pbNK (n = 36), pbNK-asc (n = 38), exNK (n = 52), exNK-asc (n = 51); 1:4, pbNK (n = 23), pbNK-asc (n = 21), exNK (n = 19), exNK-asc (n = 15). Statistical significance was determined using one-way or two-way ANOVA with Tukey's multiple comparisons test. Asterisks represent * (p ≤ 0.05), ** (p ≤ 0.01), *** (p ≤ 0.001), and **** (p ≤ 0.0001), while ns indicates non-significant comparisons. All error bars represent SEM.*

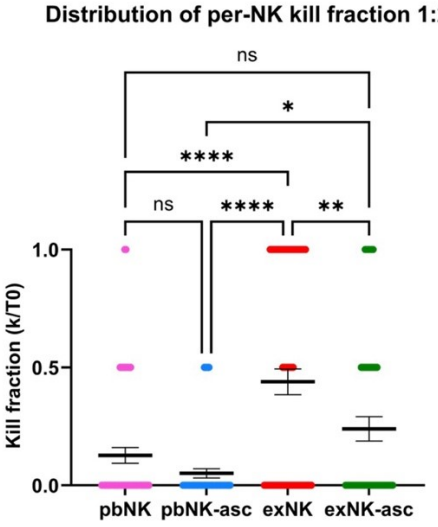
a


Distribution of per-NK kill fraction
ns
****
****
**
****
****
Kill fraction (k/T0)
1.0
0.5
0.0
pbNK
pbNK-asc
exNK
exNK-asc

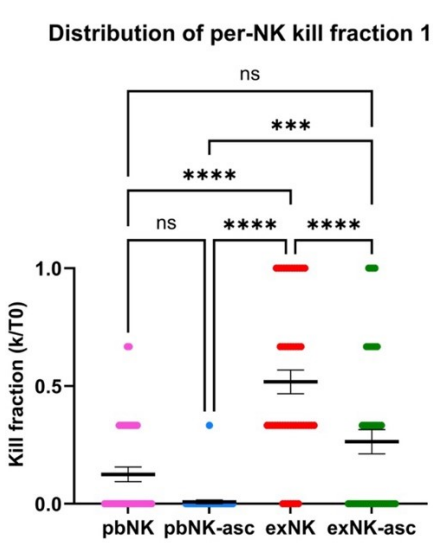
b


Distribution of per-NK kill fraction 1:1
ns
ns
ns
ns
**
ns
Kill fraction (k/T0)
1.0
0.5
0.0
pbNK
pbNK-asc
exNK
exNK-asc

c


Distribution of per-NK kill fraction 1:2
ns
*
****
ns
****
**
Kill fraction (k/T0)
1.0
0.5
0.0
pbNK
pbNK-asc
exNK
exNK-asc

d


Distribution of per-NK kill fraction 1:3
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***
****
ns
****
****
Kill fraction (k/T0)
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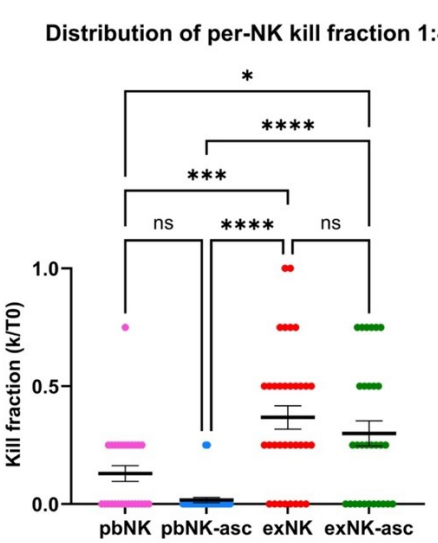
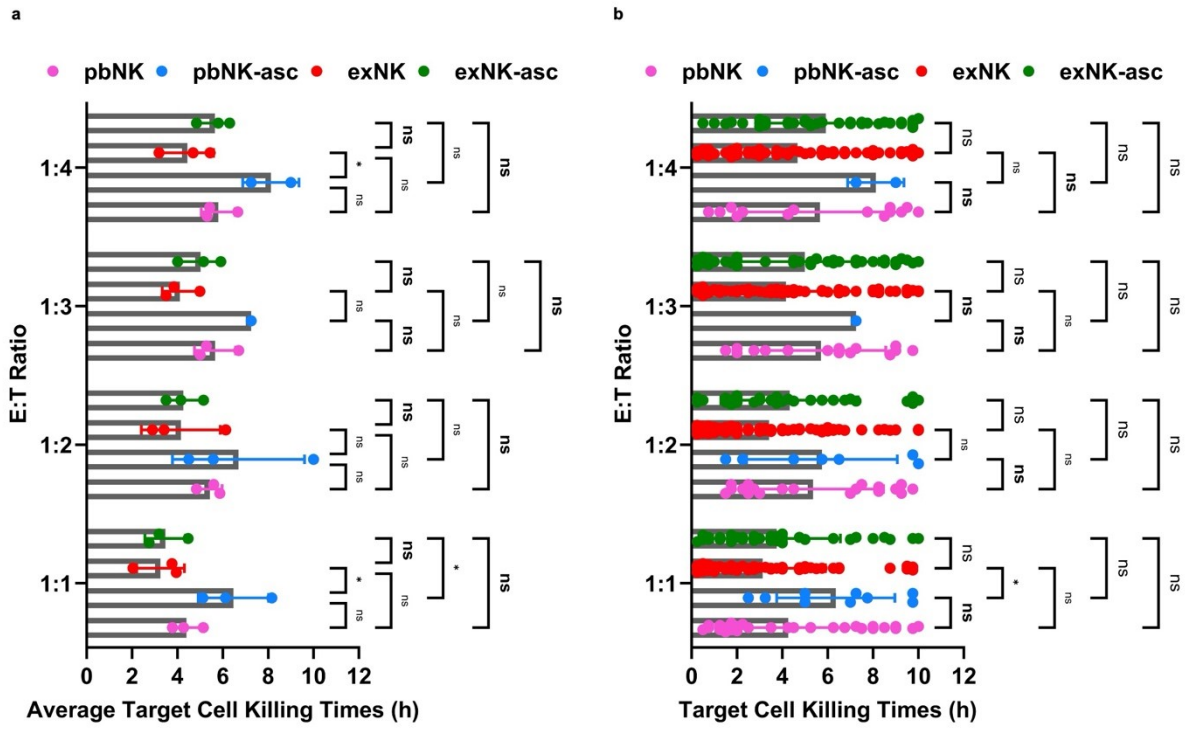
pbNK
pbNK-asc
exNK
exNK-asc

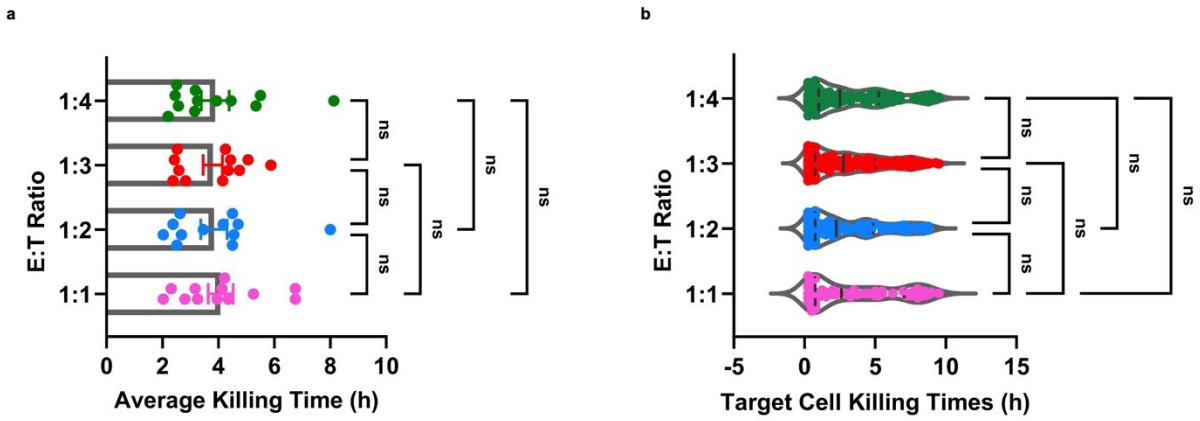
e


Distribution of per-NK kill fraction 1:4
*
****
***
ns
****
ns
Kill fraction (k/T0)
1.0
0.5
0.0
pbNK
pbNK-asc
exNK
exNK-asc

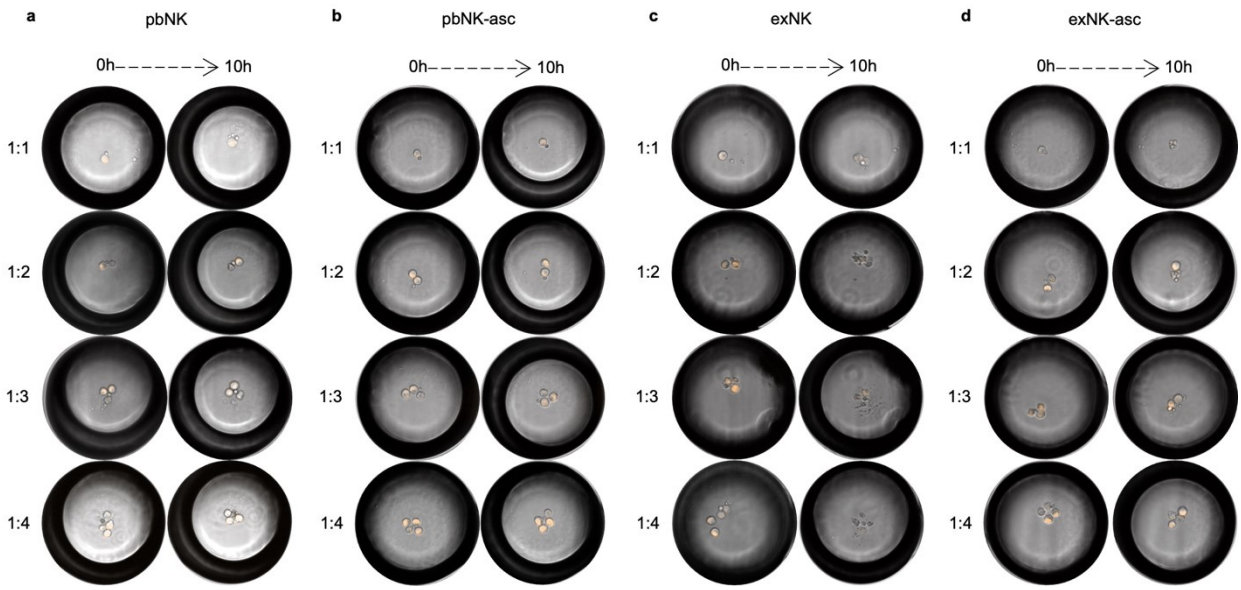
Supporting Figure 10: Functional heterogeneity of per-NK killing across NK cell states and target cell loads. *(a) Distribution of per-NK kill fraction (k/T_0) across all single-NK droplets, where k is the number of K562 target cells killed by an individual NK cell and T_0 is the initial number of K562 target cells in the droplet, for pbNK (n = 190), pbNK-asc (n = 194), exNK (n = 216), and exNK-asc (n = 170). (b-e) Distribution of per-NK kill fraction for droplets containing (b) T_0 = 1: pbNK (n = 72), pbNK-asc (n = 76), exNK (n = 71), exNK-asc (n = 56); (c) T_0 = 2: pbNK (n = 59), pbNK-asc (n = 59), exNK (n = 74), exNK-asc (n = 48); (d) T_0 = 3: pbNK (n = 36), pbNK-asc (n = 38), exNK (n = 52), exNK-asc (n = 51); and (e) T_0 = 4: pbNK (n = 23), pbNK-asc (n = 21), exNK (n = 19), exNK-asc (n = 15). n denotes individual single-NK droplets pooled across three biological replicates. Asterisks represent "ns" ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$) from one-way ANOVA with Tukey's multiple comparisons test. All error bars represent SEM.*



Supporting* Figure 11*: Comparison of target cell killing times across NK cells and E:T ratios. *(a) Average target-cell killing times for different NK-cell groups (pbNK, pbNK-asc, exNK, and exNK-asc) across varying droplet types (E:T ratios ranging from 1:1 to 1:4). Data represent three independent biological replicates (n = 3) for each condition. The underlying sample size is the number of kill events pooled across all NK groups: 1:1 (n = 54), 1:2 (n = 109), 1:3 (n = 151), and 1:4 (n = 92). (b) Distribution of individual target-cell killing times for each NK-cell group across E:T ratios. The underlying sample size is the number of kill events in each NK-group/E:T-ratio condition: 1:1, pbNK (n = 15), pbNK-asc (n = 5), exNK (n = 22), exNK-asc (n = 12); 1:2, pbNK (n = 15), pbNK-asc (n = 6), exNK (n = 65), exNK-asc (n = 23); 1:3, pbNK (n = 31), pbNK-asc (n = 6), exNK (n = 80), exNK-asc (n = 34); 1:4, pbNK (n = 21), pbNK-asc (n = 8), exNK (n = 37), exNK-asc (n = 26). Asterisks represent "ns" ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$) from one-way or two-way ANOVA with Tukey's multiple comparisons test. All error bars represent SEM.*



Supplementary Figure 12: Comparison of target cell killing times across E:T ratios. *(a) Average target-cell killing times across different droplet types (E:T ratios ranging from 1:1 to 1:4) for all NK cell groups combined. Data represent three independent biological replicates across four NK cell groups (n = 12 plotted values) for each E:T ratio. The underlying sample size is the number of kill events pooled across all NK groups: 1:1 (n = 54), 1:2 (n = 109), 1:3 (n = 151), and 1:4 (n = 92). (b) Distribution of individual target-cell killing times across varying E:T ratios. The underlying sample size is the number of kill events pooled across all NK groups: 1:1 (n = 54), 1:2 (n = 109), 1:3 (n = 151), and 1:4 (n = 92). Asterisks represent "ns" ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$) from one-way or two-way ANOVA with Tukey's multiple comparisons test. All error bars represent SEM.*



Supplementary Figure 13: Time-Lapse images of NK cell-mediated cytotoxicity in microfluidic droplets. *Illustration of the interaction dynamics of (a) pbNK, (b) pbNK-asc, (c) exNK and (d) exNK-asc cells with K562 over 10 hours at various E:T ratios, highlighting differences in cytotoxic engagement and the impact of cell conditioning on NK cell activity.*