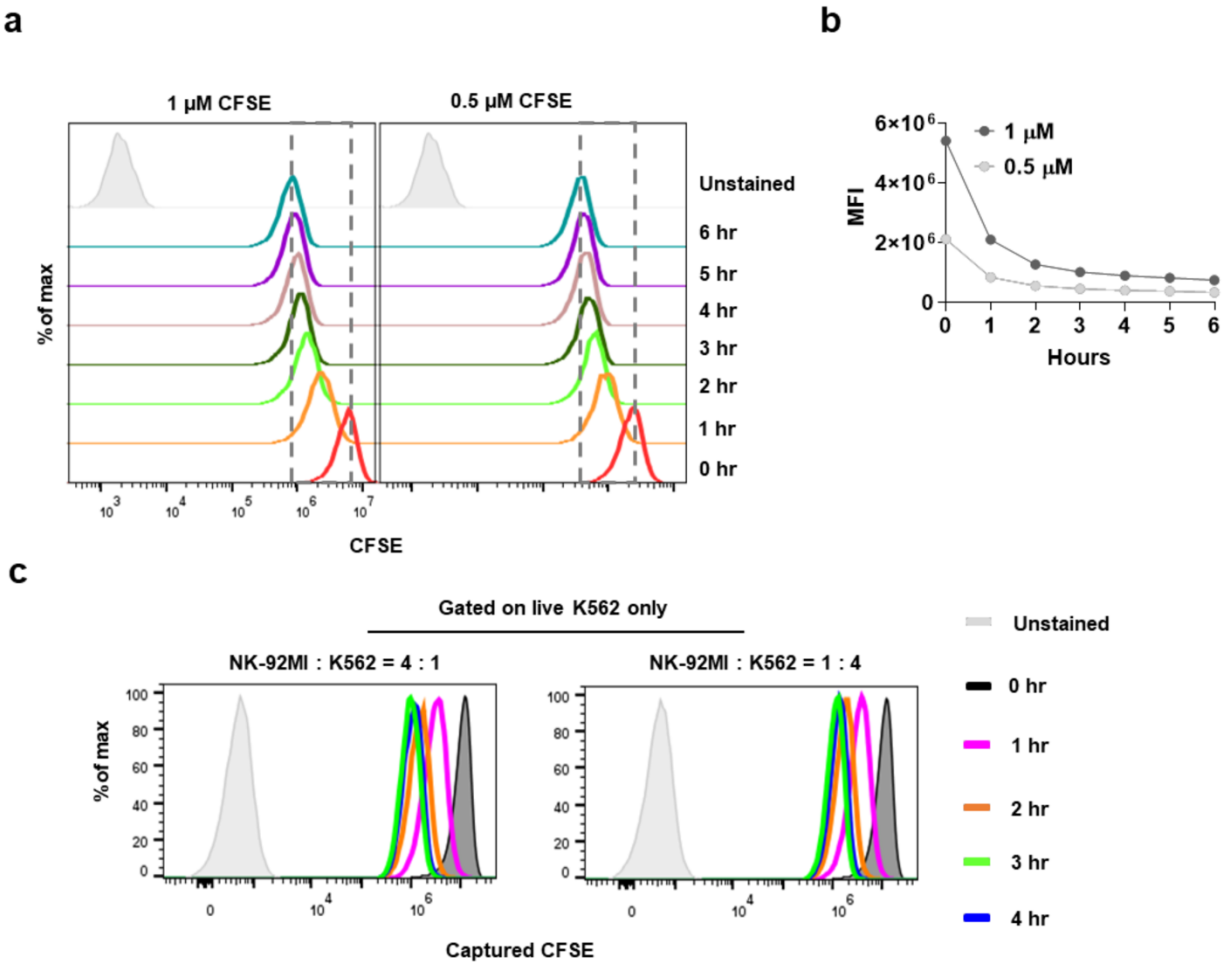
nature biomedical engineering

Article

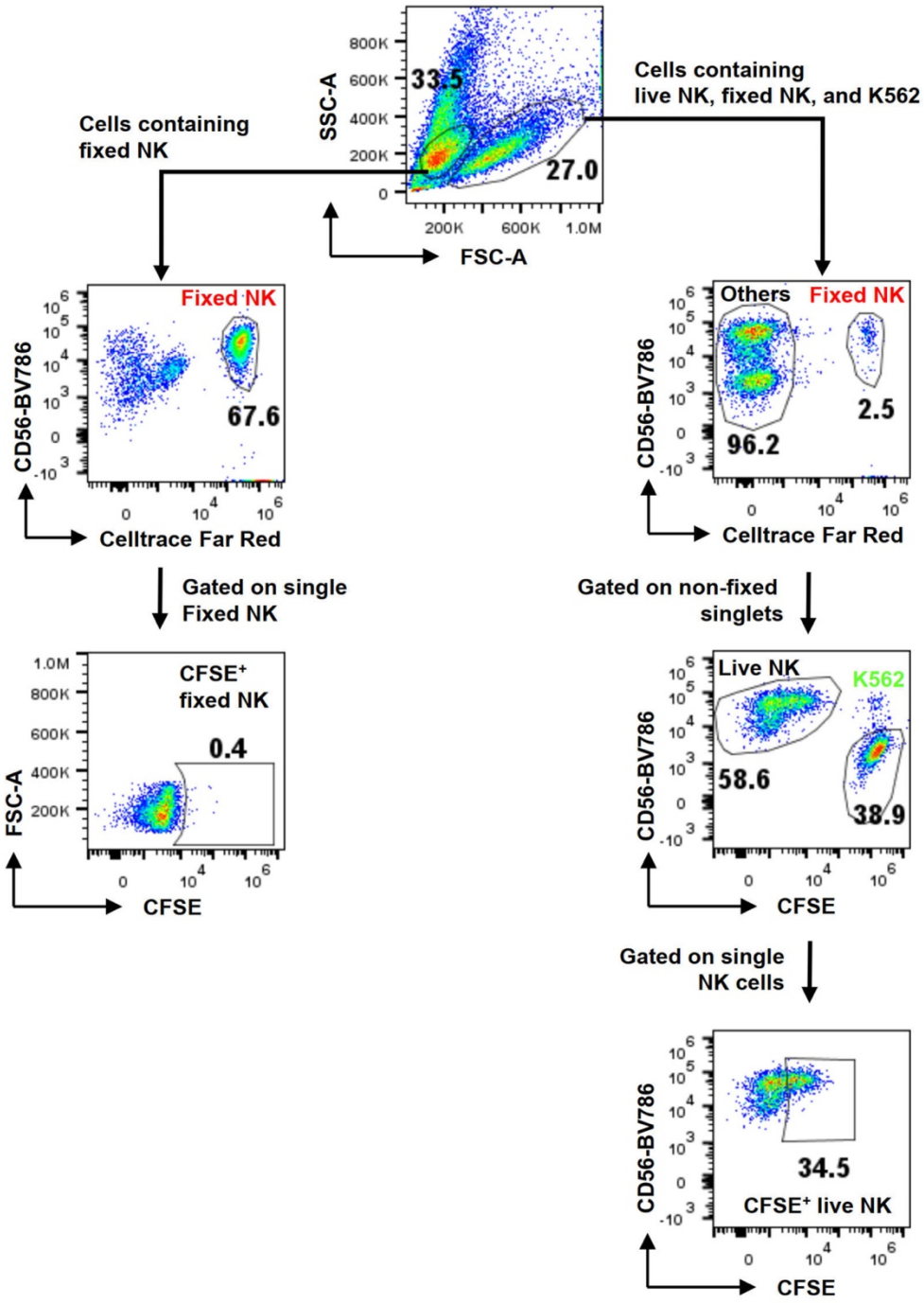
https://doi.org/10.1038/s41551-023-01089-z

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

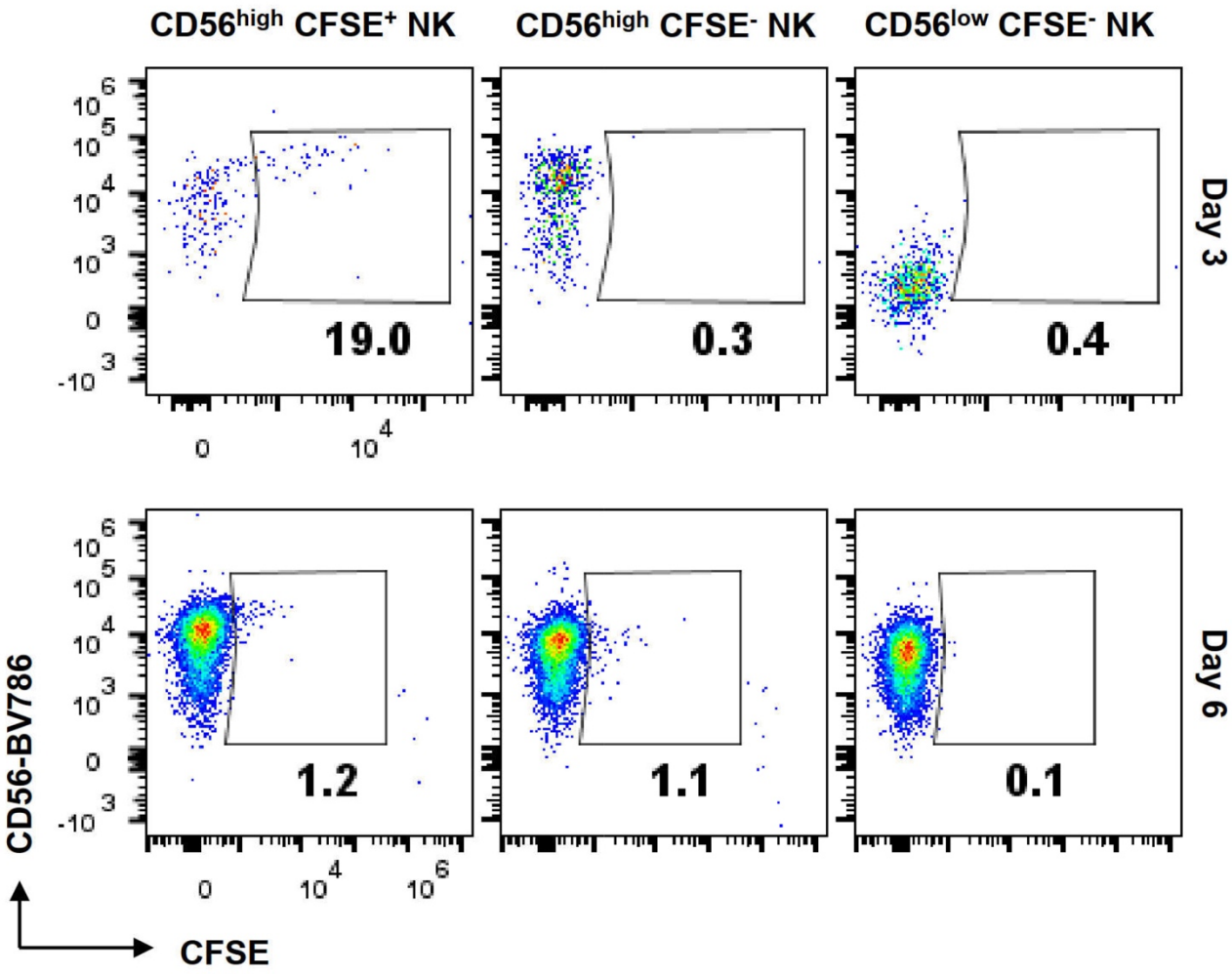
In the format provided by the authors and unedited



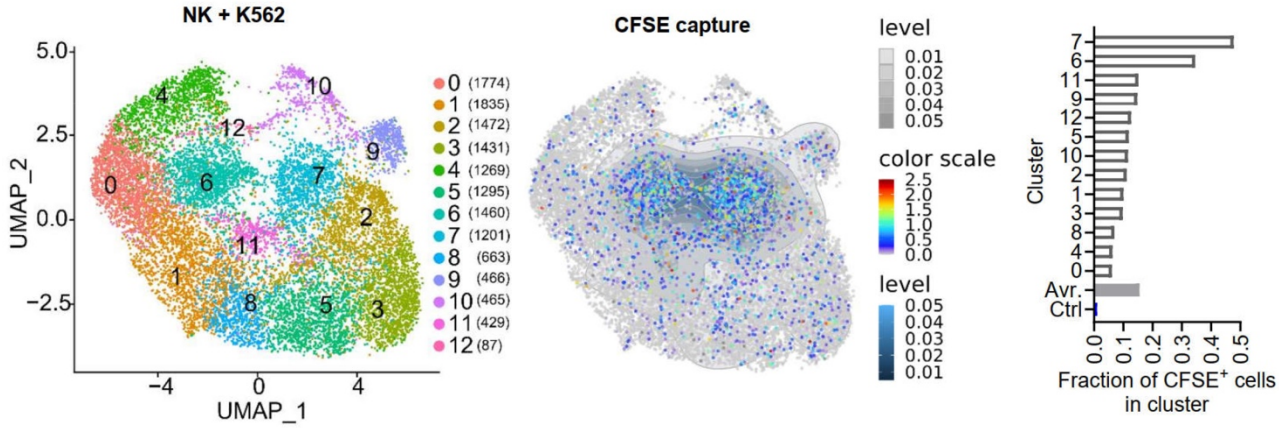
Supplementary Fig. 1 l Short-term incubation before co-culture assay help remove excess CFSE staining of K562 target cells. a,b, Shown were histogram plots (**a**) and change of medium fluorescence intensity (MFI) of CFSE in CFSE-loaded K562 cells over time (**b**). Briefly, 2 million of K562 cells were stained with CFSE (0.5 - 1 uM) in 1 ml PBS at 37oC for 20 min. The cells were incubated in 10 ml culture media and incubate for 10 min at room temperature to remove free dye. The cells were re-incubated in 3 ml medium at 37oC for additional 1, 2, 3, 4, 5, and 6 hours. At indicated time points, the cells were collected, briefly washed before analysed by flow cytometry. **c**, CFSE-loaded K562 cells, without pre-incubation at 37oC for 2 hours, were immediately co-cultured with NK-92MI at indicated ratios. CFSE intensity in K562 cells were detected by flow cytometry over time.



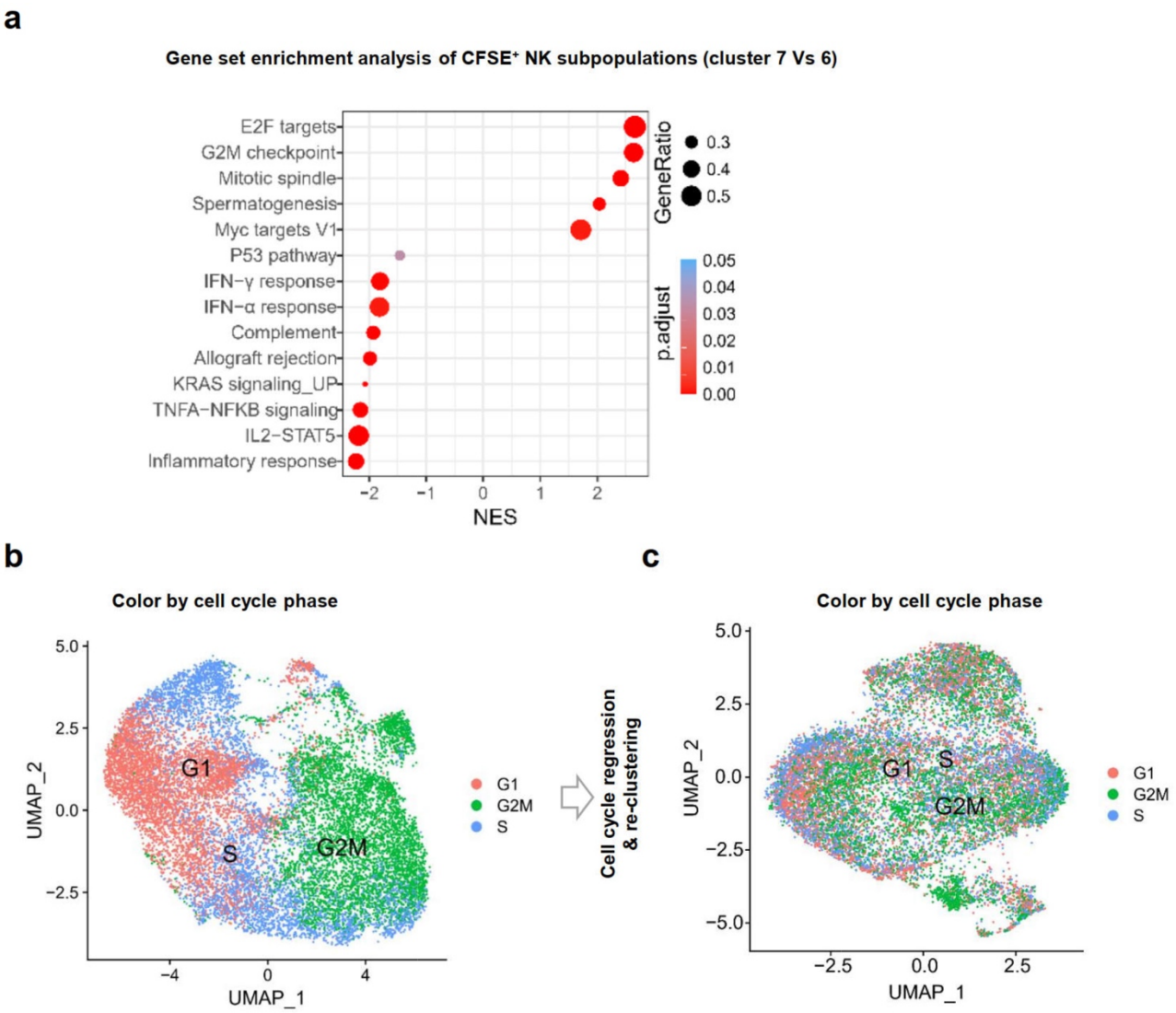
Supplementary Fig. 2 l Gating strategy to identify live and fixed NK-92MI cells from mixed culture cells. Part of NK-92MI cells were labelled with Celltrace Far Red dye (Invitrogen, C34564) before fixed/permeabilized with CytoFix/Cytoperm reagent (BD Biosciences, 554714) at 4°C for 10 min. Both live NK-92MI and fixed NK-92MI were labelled with CFSE capture antibodies and mixed together with CFSE-loaded K562 cells for secificity test of PAINTKiller assay. The fixed NK-92MI can be identified by its high staining of Celltrace Far Red dye compared to remained cell population containing live NK-92MI and K562, as well as a minor population of fixe NK-92MI. The live NK-92MI and K562 can be further identified by the CFSE intensity and CD56 expression.



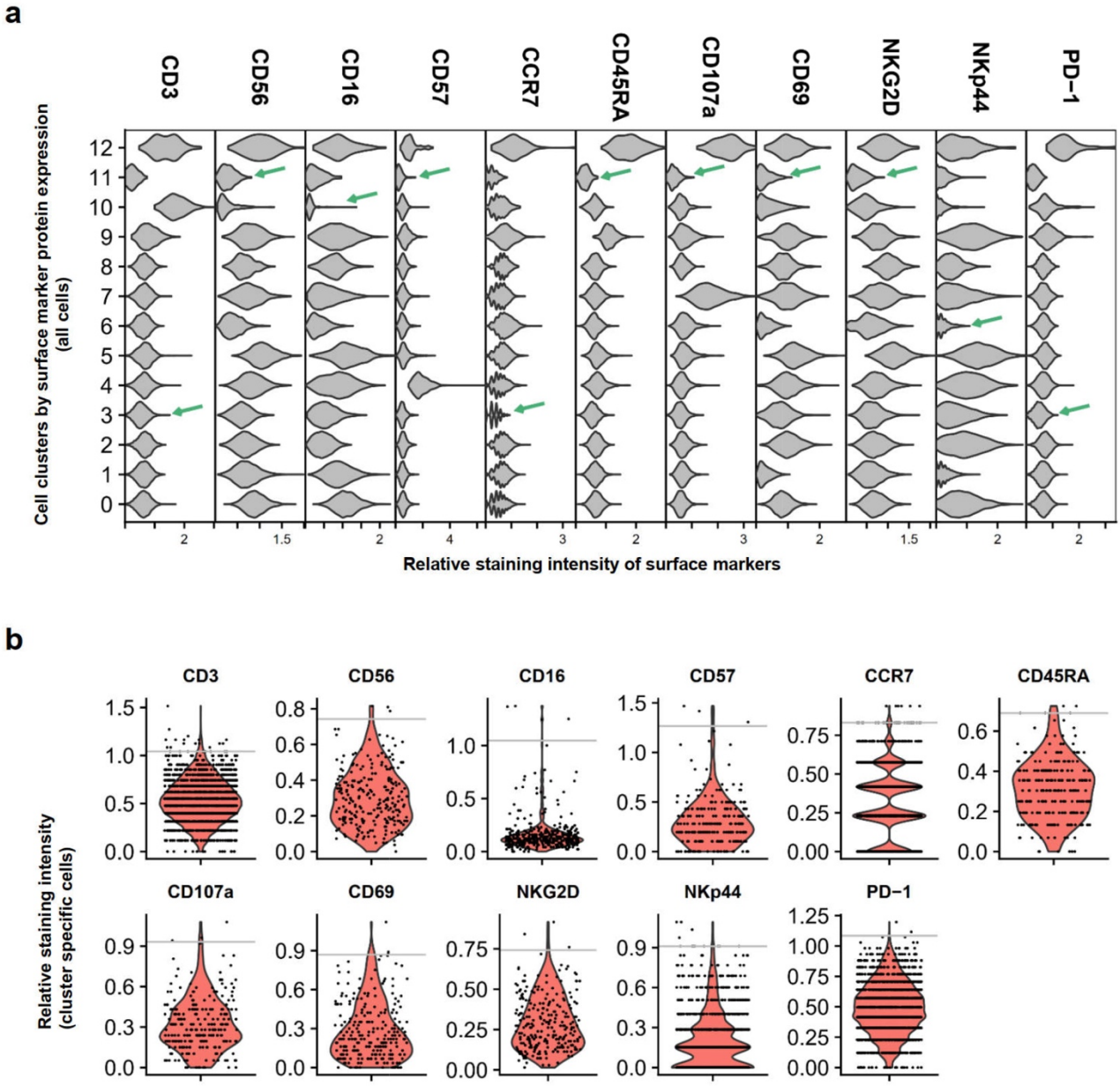
Supplementary Fig. 3 l Residual CFSE in sorted NK cells after expansion in vitro. As demonstrated in **Fig. 3b**, NK-92MI were co-cultured with CFSE-loaded K562 cells (E:T = 4:1). After 4 hours, cells were harvested, stained, and sorted into 3 populations (CD56^{high} CFSE^{+} NK, CD56^{high} CFSE^{-}, and CD56^{low} CFSE^{-} NK cells) based on the intensities of CD56 staining and CFSE capture. The sorted NK cells were cultured for additional 3 and 6 days. At indicated time points, cells were collected and stained with CD56-BV786 to determine the expression of CD56 as well as fraction of residual CFSE in cell over time. Shown were representative flow cytometry data of day 3 and day 6 post sorting.



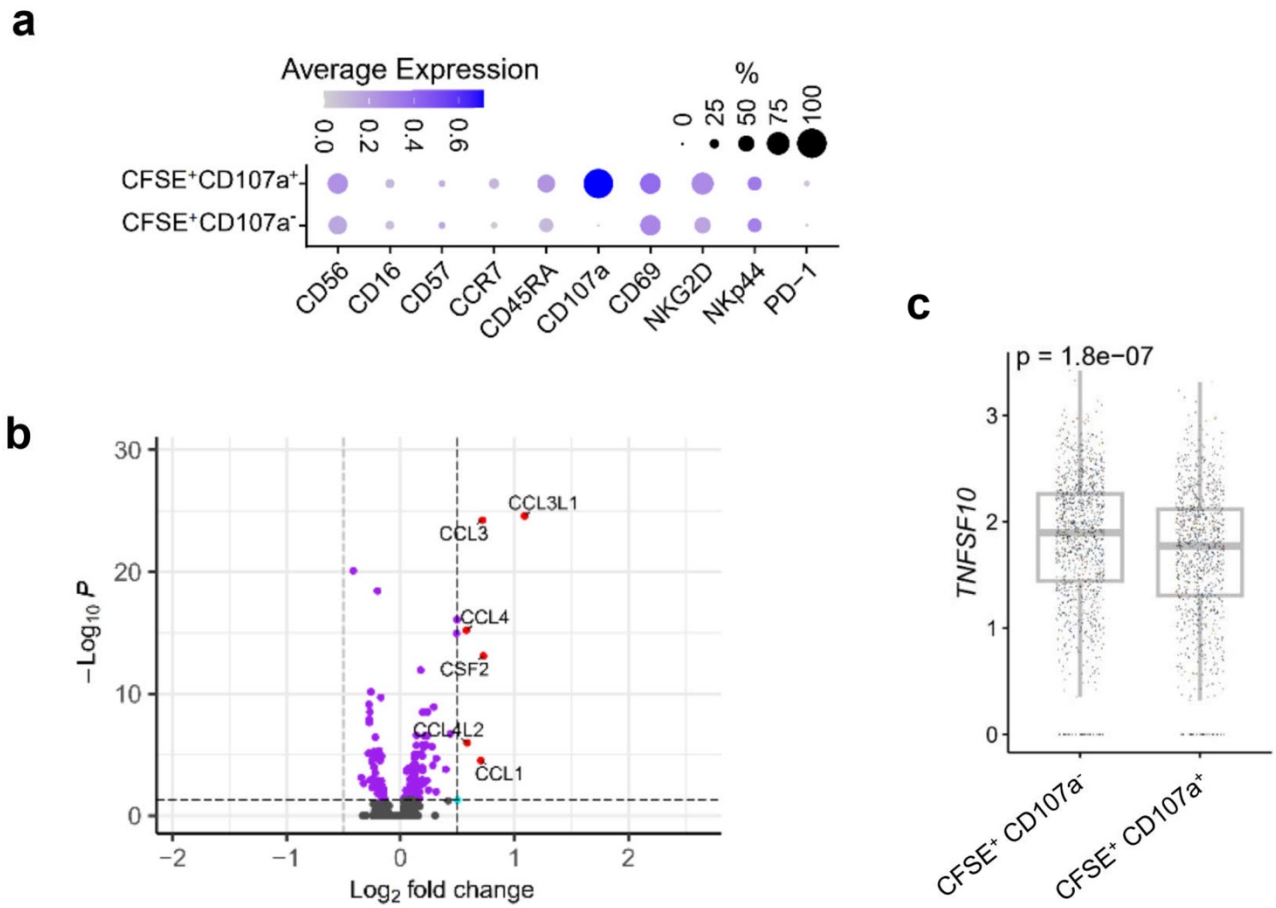
Supplementary Fig. 4 l UMAP plot showing the clusters of NK cells (from K562 co-culture group) with cell numbers indicated in bracket (**left**). CFSE capture in cluster was shown by projected density plot (**middle**) using Single-Cell Signature Explorer, and the fraction of CFSE⁺ NK cells were calculated (**right**).



Supplementary Fig. 5 l a, A list of differentially expressed genes (two-sided Wilcoxon Rank Sum test) by cells of cluster 7 over cluster 6 as indicated in Supplementary Fig. 5c were calculated using Seurat (v4.3.0) and imported for GSEA using R programs embedded in the clusterProfiler (v3.18.0). Shown were significantly enriched hallmark gene sets (adjusted P-value < 0.05) from the Molecular Signatures Database (MSigDB, v7.4). b, Cell cycle scoring was performed to mitigate cell-cycle effects on cellular heterogeneity characterization. The transcriptional profile-based clustering of NK cells as shown in Supplementary Fig. 5c was coloured by cell cycle phases. c, After cell cycle regression, the NK cells were re-clustered by their gene expression profiles and coloured by original cell cycle phases.



Supplementary Fig. 6 l a,b, Unsupervised cell clustering of total cells (without removal of non-T cells as shown in Supplementary Fig. 5a) based on the expression of a panel of surface protein markers as indicated above the violin plots (**a**). The “negative population” (pointed out by green arrow in a) was determined as the cluster with the lowest specific protein expression (**b**).



Supplementary Fig. 7 l a, Dot plot showing expression profiles of surface protein markers of CFSE$^+$ CD107a$^+$ and CFSE$^+$ CD107a$^-$ NK cells. **b,c**, Differential gene expression (Wilcoxon rank sum test) of CFSE$^+$ CD107a$^+$ and CFSE$^+$ CD107a$^-$ NK cells primed by K562 cells. Volcano plot showing differentially expressed genes with cut-offs of Bonferroni adjusted $P < 0.05$ (two-sided) and Log2FC ≥ 0.5 or ≤ -0.5 (**b**). Boxplot showing the differential expression of *TNFSF10*. Box plot shows the 25th to the 75th percentile with a line through the median and whiskers spanning 1.5x the interquartile range. Significance calculation by two-sided Wilcoxon rank sum test. (**c**). A total of 1002 CFSE$^+$ CD107a$^+$ double positive cells and 1148 CFSE$^+$ CD107a$^-$ single positive cells were included for analysis (a-c).

Supplementary Table 1 | Contingency table for association between CFSE and CD107a or CFSE and IFNG.

Contingency table for association between **CFSE** and **CD107a**

Observed (Expected)	CD107a+	CD107a-	Row total
CFSE+	1003 (358)	1149 (1794)	2152
CFSE-	1301 (1946)	10394 (9749)	11695
Column total	2304	11543	13847

Two-sided Chi-square is 1649.86, p-value < 0.00001

Contingency table for association between **CFSE** and ***IFNG***

Observed (Expected)	*IFNG*+	*IFNG*-	Row total
CFSE+	547 (226)	1605 (1926)	2152
CFSE-	907 (1228)	10788 (10467)	11695
Column total	1454	12393	13847

Two-sided Chi-square is 603.36, p-value < 0.00001

Supplementary Table 2 | Contingency table for association between CFSE and Cluster 2 subclusters or CD107a and Cluster 2 subclusters.

Contingency table for association between CFSE and Cluster 2 subclusters

Observed (Expected)	Cluster 2.0	Cluster 2.1	Row total
CFSE+	774 (827)	427 (374)	1201
CFSE-	1363 (1310)	540 (593)	1903
Column total	2137	967	3104

Contingency table for association between CD107a and Cluster 2 subclusters

Observed (Expected)	Cluster 2.0	Cluster 2.1	Row total
CD107a+	568 (712)	466 (322)	1034
CD07a-	1569 (1425)	501 (645)	2070
Column total	2137	967	3104

Two-sided Chi-square is 139.96, p-value <0.00001

Supplementary Table 3. Training of a Naïve Bayes classifier (70% data) and validation of the classifier (30% data) for classifying NK cells into clusters 0 and 1

Class counts for Cluster

Class:	0	1
Count:	4153	3458

Total count: 7611

Threshold to used for zero probabilities: 1.0E-4

Gaussian distribution for Cluster0.Score1 per class value

	0	1
Count:	4153	3458
Mean:	0.45997	-0.267
Std. Deviation:	0.37926	0.42117
Rate:	55%	45%

Gaussian distribution for Cluster1.Score1 per class value

	0	1
Count:	4153	3458
Mean:	0.36625	0.83623
Std. Deviation:	0.21037	0.27495
Rate:	55%	45%

Cluster/Prediction	0	1
0	1635	190
1	288	1150

Correctly classified: 2785

Wrongly classified: 478

Accuracy: 85.35%

Error: 14.649%

Cohen's kappa: 0.701