Supplemental material

Prager et al., https://doi.org/10.1084/jem.20181454

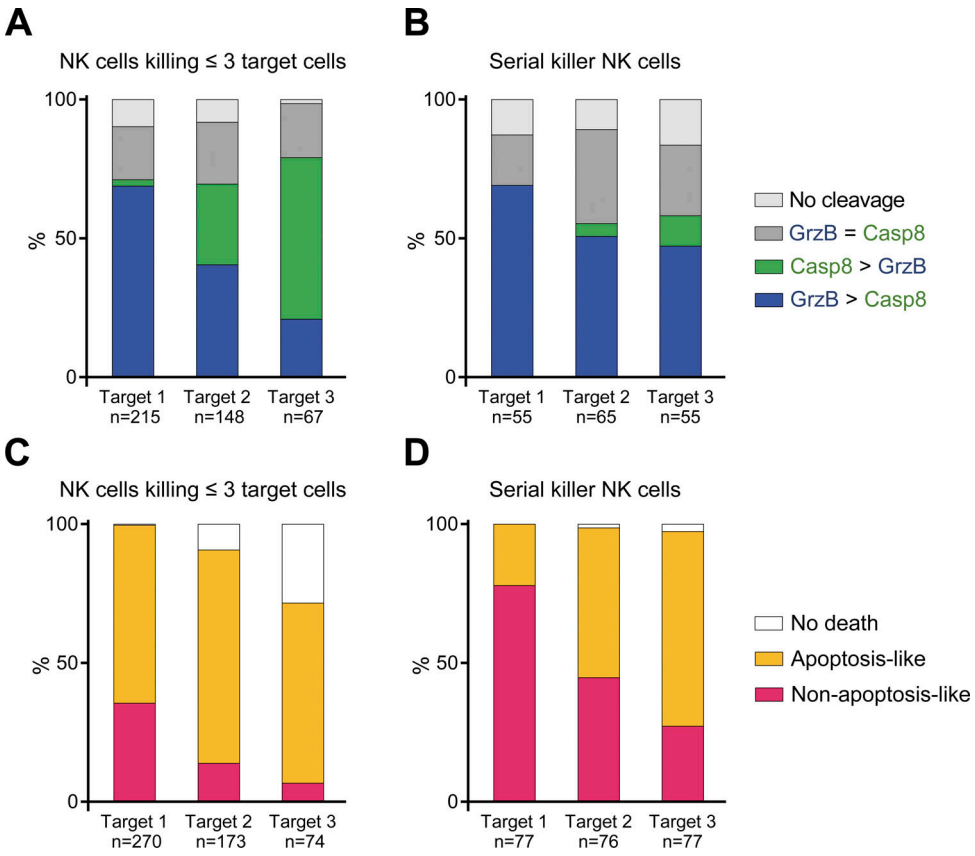


Figure S1. **Killing pathway and induced death phenotype differ between serial and nonserial killer NK cells.** Serial killing activity of primary human NK cells was evaluated as described in Fig. 3. NK cells were grouped according to the number of targets cells killed, where serial killers were defined as NK cells killing four or more target cells. **(A–D)** Comparison of the proportion of target cell deaths displaying activity of GrzB, Casp8, or absence of reporter cleavage (A and B) and the cell death morphology (C and D) for the first three killing events performed by either NK cells killing up to three target cells (A and C) or serial killer NK cells (B and D).

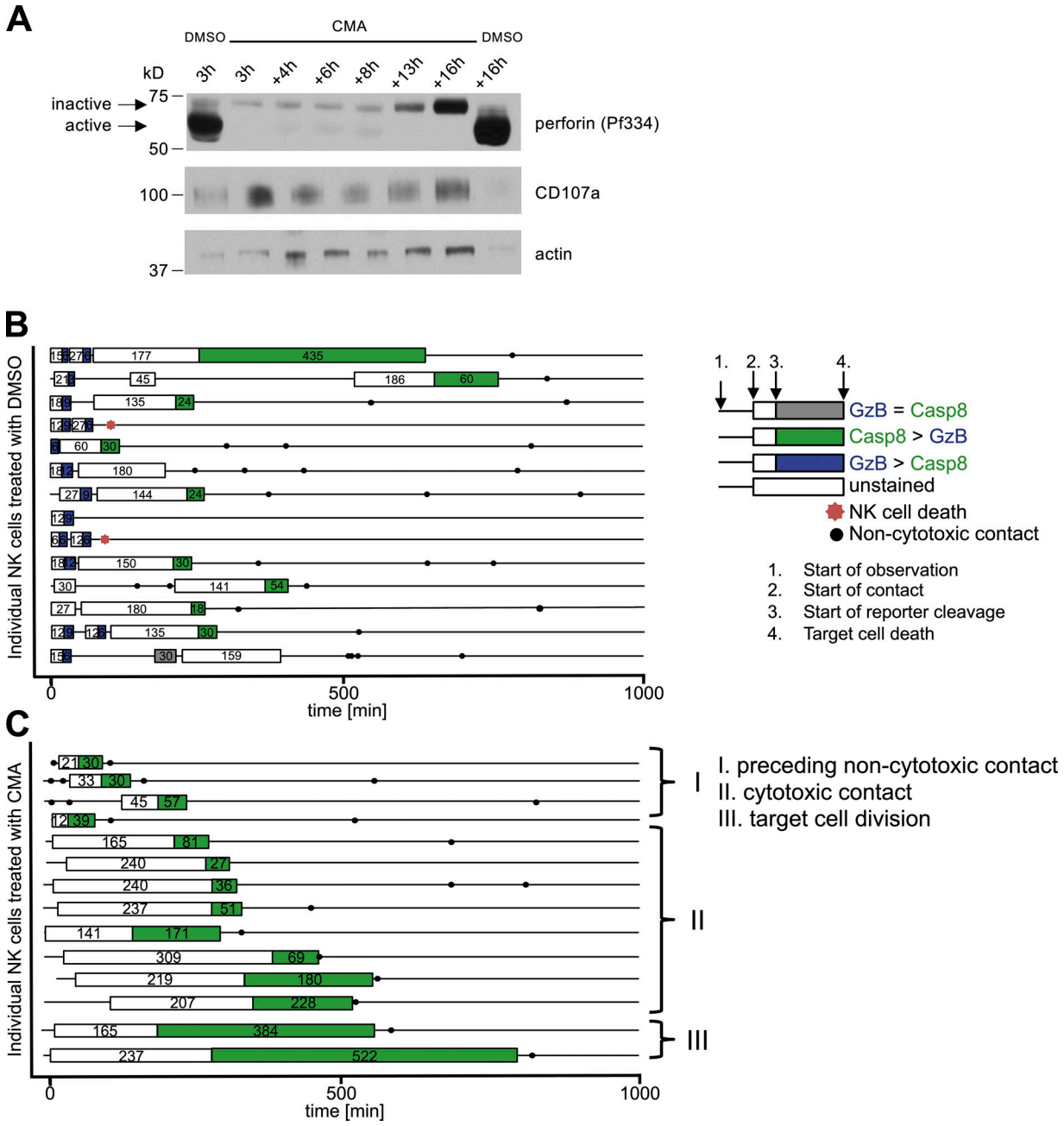


Figure S2. **Deletion of perforin by CMA prevents serial killing. (A)** Primary human NK cells were incubated with DMSO as control of 50 nM CMA for 3 h. CMA is an inhibitor for vacuolar H^+-ATPases. Inhibition of vacuolar type H^+-ATPase prevents the acidification of the lytic granules and in turn inhibits cathepsin L, which is needed for cleavage of immature perforin into its active form (Kataoka et al., 1996). Thus, treatment with CMA prevents the maturation of perforin and effectively depletes NK cells of active perforin. Cells were then washed and incubated in medium for ≤16 h. At the indicated time points, cells were analyzed by Western blotting for perforin, CD107a, and actin. **(B and C)** As the depletion of perforin was stable for ≥16 h, we pretreated NK cells with DMSO (B) or CMA (C) for 3 h, washed the cells, and analyzed their serial killing activity as described in Fig. 6. We observed differences in Casp8 cell death kinetics when NK cells had preceding noncytolytic contacts (C, I) or when the target cell divided during the NK cell contact (C, III).

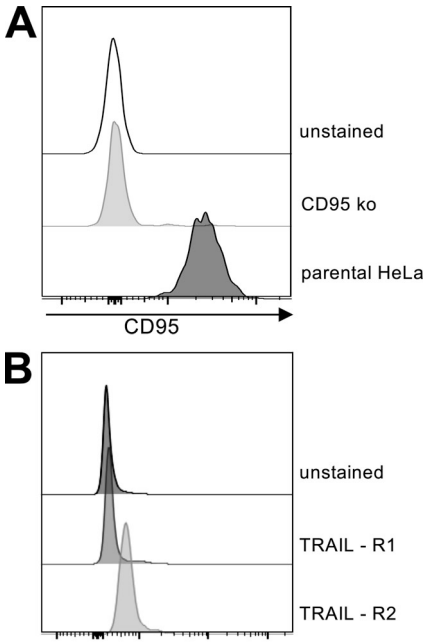
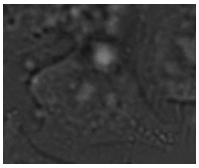
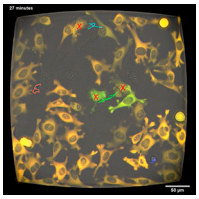


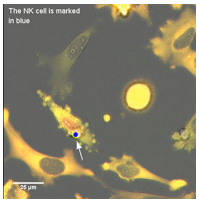
Figure S3. **CD95 and TRAIL-R expression in HeLa targets. (A)** The CD95 receptor was deleted via CRISPR/Cas9 in HeLa-CD48 cells stably expressing NES-ELQTD-GFP-T2A-NES-VGPD-mCherry. Cells were sorted for CD95 surface expression, and the CD95 staining of the resulting CD95KO cell line is shown in comparison to the parental HeLa-CD48-NES-ELQTD-GFP-T2A-NES-VGPD-mCherry. **(B)** HeLa-CD48 cells stably expressing NES-ELQTD-GFP-T2A-NES-VGPD-mCherry were stained for surface expression of TRAIL-R1 and TRAIL-R2 as indicated.



Video 1. **Example of the detection of GrzB and Casp8 activity during NK cell–mediated killing of a HeLa-CD48 target stably expressing NES-ELQTD-GFP-T2A-NES-VGPD-mCherry.**



Video 2. **Example of a 17-h time-lapse video showing a 350-µm-wide microwell, containing HeLa-CD48/NES-ELQTD-GFP-T2A-NES-VGPD-mCherry cells and four NK cells (not fluorescent).** NK cell tracks are marked by different colors, and killing events are indicated by red crosses. The time resolution is 3 min between frames.



Video 3. **Five consecutive killing events caused by a serial killer NK cell as shown in Fig. 3 A.** All time-lapse images have been background-subtracted and adjusted for brightness and contrast using ImageJ, and the video shows a region of a 350-µm-wide microwell. The time resolution is 3 min between frames.

Reference

Kataoka, T., N. Shinohara, H. Takayama, K. Takaku, S. Kondo, S. Yonehara, and K. Nagai. 1996. Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. *J. Immunol.* 156:3678–3686.