Supporting Information for:

**Synaptic secretion from Human Natural Killer cells is diverse and includes supramolecular attack particles**

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**Materials and Methods**

*Cell isolation and culture*

Peripheral blood was acquired from the National Health Service blood service (Ethics license: 05/Q0401/108). Peripheral blood mononuclear cells were purified by density gradient centrifugation (Ficoll-Paque Plus; GE Healthcare) with NK cells and CD8⁺ T-cells isolated using negative selection microbeads (Miltenyi Biotec). NK cells were cultured (37°C/5% CO₂) with 200 U/mL rhIL-2 (Roche), but used when resting, 6 days later. CTLs were used immediately after isolation.

*Preparation of coated slides and bilayers*

Eight chamber glass slides (1.5 Lab-Tek II; Nunc) were coated with 0.01% poly-L-lysine and dried at 60°C for 1 hr. Slides were coated with either His-ICAM-1 (2.5 µg/mL; produced in-house) alone or with His-MICA (2.5 µg/mL; Sino Biological), B7-H6-Fc (2.5 µg/mL; R&D systems), αNKG2A (5 µg/mL; R&D systems) or αNkp30 (10 µg/mL; P30-15; Biolegend or 210845; R&D systems) in PBS overnight (4°C). Bilayers were prepared as previously described (1) and functionalised with His-ICAM-1 (2.5 µg/mL) alone, or with His-MICA (2.5 µg/mL), biotinylated-αNkp30 (10 µg/mL; P30-15) or biotinylated-αCD3 (5 µg/mL; OKT3; a gift from Andy Shepherd, GSK).

*IFNγ ELISA*

NK cells (1x10⁵) were incubated on coated slides for 16 hrs (37°C/5% CO₂), supernatants aspirated, and cells pelleted at 1000 g for 10 mins (4°C). IFNγ concentration was measured by ELISA (DuoSet, R&D systems) according to manufacturer’s instructions.
Imaging NK cell secretions

NK cells (1x10^5) were incubated on coated slides for 1 hr (37°C/5% CO₂), then detached with non-enzymatic cell-dissociation solution (Sigma-Aldrich) for 20 min (37°C) and washed with PBS. Where indicated, to inhibit exosome secretion, cells were pre-incubated with 100nM Cambinol (Sigma-Aldrich) for 1 hr (37°C). Slides were blocked with 1% BSA (Sigma-Aldrich) and 1% human serum (ThermoFisher Scientific) in PBS for 1 hr (RT) and stained for 1 hr (RT) with mAbs: αThrombospondin-1-AF647 (10 µg/mL; A6.1), αSerglycin-AF647 (10 µg/mL; C-11), αGalectin-1-AF647 (10 µg/mL; C-8) (all Santa Cruz Biotechnology), αCD63-AF647 (10 µg/mL; HSC6; BioLegend), αGranzyme B-AF647 (10 µg/mL; GB11; BioLegend) or αPerforin-AF488 (2.5 µg/mL; dG9; Biolegend). For coordinate based colocalization (CBC) positive controls, slides were first stained with αPerforin-AF488 (2.5 µg/mL; dG9; Biolegend) for 1 hour at RT, washed with PBS and then stained with a Goat anti-Mouse IgG2b secondary antibody conjugated to AF647 (ThermoFisher Scientific) for 1 hour at RT. Wheat germ agglutinin (WGA) conjugated to CF568 (2 µg/mL; Biotium) or AF647 (2 µg/mL; ThermoFisher Scientific) was used to stain glycoproteins and DiD (1 µM; ThermoFisher Scientific) to mark membrane phospholipids. Samples were washed with PBS and imaged with 488/561/647 nm lasers on an Eclipse Ti inverted Microscope (Nikon) using an Apo TIRF 100x 1.49 NA oil objective or using 488/642 nm lasers on a SR GSD (Leica Biosystems) using a 160x 1.43 NA oil objective. Images were analysed within ImageJ (2).

Shadow imaging

NK cells were prepared as before, but prior to cell detachment, slides were washed with PBS, stained with αICAM-1-AF488 (HCD54; 5 µg/mL; BioLegend) for 1 min and washed with PBS. Cells were detached and blocked as before then stained with αPerforin-AF4647 (2.5 µg/mL; dG9; Biolegend) for 1 hr and washed with PBS (all RT). Cells were imaged by total internal reflection (TIRF; Leica SR GSD microscope) using a 160x 1.43 NA oil-objective.

STORM

Stochastic optical reconstruction microscopy (STORM) was performed in TIRF mode on a Leica SR GSD on slides stained as above, using 488 nm or 642 nm lasers for 7000 frames (11 ms/frame). STORM datasets were analysed using ThunderSTORM (3) as previously described (4).

Statistical analysis
Statistical analysis was performed using Prism (GraphPad Software; v8.4.2), with specific analyses detailed in figure legends. All data presented as mean ±SD, unless stated.

Data Availability

All data is included within the manuscript.

References

1. T. J. Crites, et al., Supported Lipid Bilayer Technology for the Study of Cellular Interfaces. Curr. Protoc. Cell Biol. 68, 549–562 (2015).
2. C. A. Schneider, W. S. Rasband, K. W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675 (2012).
3. M. Ovesný, P. Křížek, J. Borkovec, Z. Švindrych, G. M. Hagen, ThunderSTORM : a comprehensive ImageJ plugin for PALM and STORM data analysis and super-resolution imaging User ‘ s Guide. Bioinformatics, 1–2 (2014).
4. A. Oszmiana, et al., The Size of Activating and Inhibitory Killer Ig-like Receptor Nanoclusters Is Controlled by the Transmembrane Sequence and Affects Signaling Article The Size of Activating and Inhibitory Killer Ig-like Receptor Nanoclusters Is Controlled by the Transmembr. Cell Reports 15, 1–16 (2016).