Investigating the Role of Paracrine Signalling in the Secondary Release of Neutrophil Extracellular Traps

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BACKGROUND

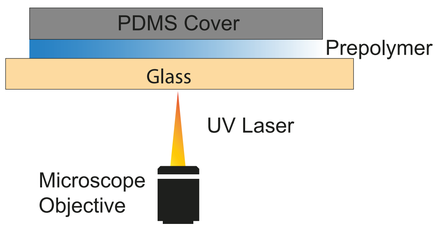
Neutrophil extracellular traps (NETs) are chromatin DNA released by activated neutrophils to trap and kill bacteria[1]. The release of NETs, a process known as NETosis, is an important step in host defence. However, excessive NETosis can damage host tissues and contribute to inflammatory disorders2,3.

Currently, the mechanisms of excessive NETosis are unknown, and we speculate that released NETs create a positive feedback loop by inducing secondary NETosis of neighbouring neutrophils, and further promotes excessive NETosis. Here, we developed (1) a nanowell-in-microwell array and (2) a propagation array to investigate the role of paracrine signalling in NETosis.

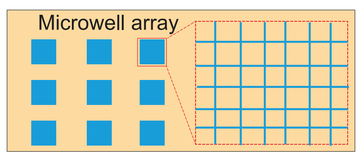
♦Neutrophil ♦NETosis ♦Paracrine Signalling ♦Nanowells

Nanowell-in-microwell Array: Methods

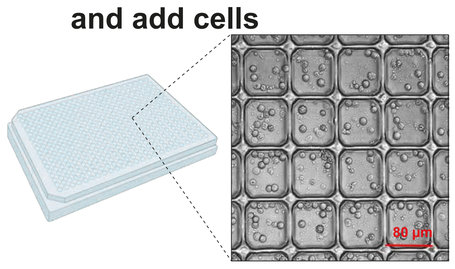
Step1: Laser micropatterning



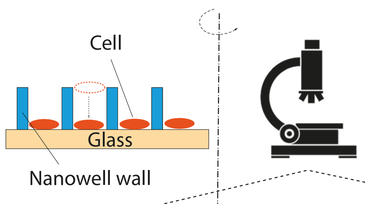
Step2: Clean the glass



Step3: Assmeble the well plate and add cells



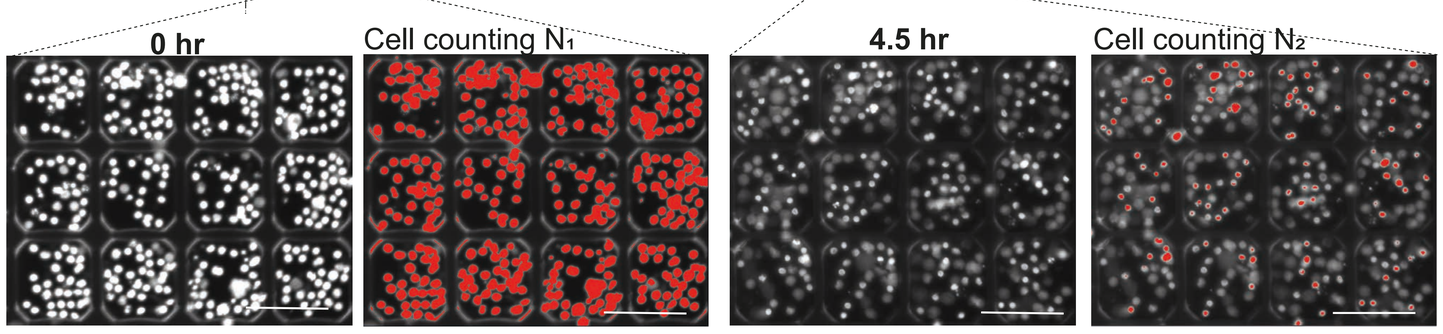
Step4: Centrifuge cells down & Image



Incubate ~4.5h

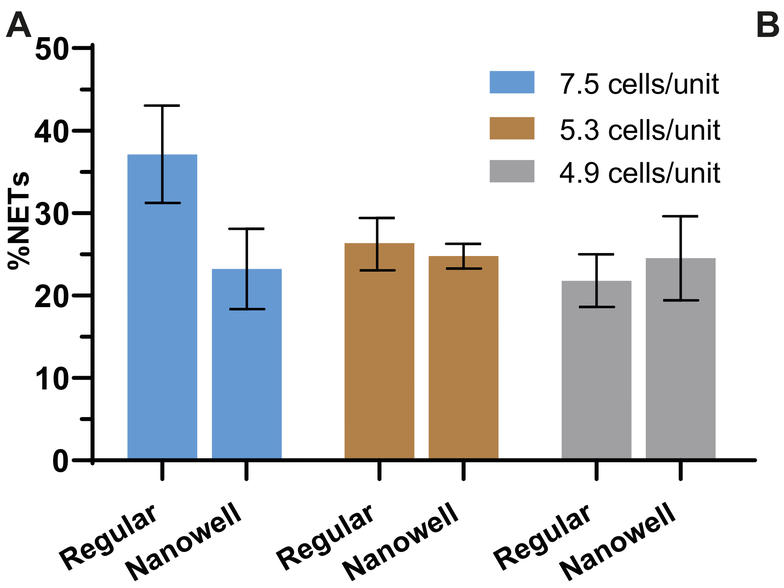
Step5: Image again

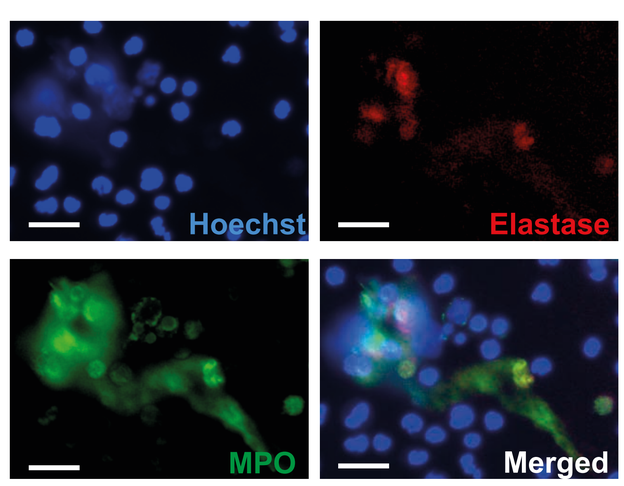
$\%NETs = (N_1-N_2)/N_1*100\%$



- Nanowell units are ~80×80 μm with the wall heights 26-35 μm.
- Cells are stained with a DNA stain (Hoechst) before imaging.
- Cells are cultured with NETosis stimuli or pre-stimulated cells for 4.5 hours.
- Intact cells have been highlighted in red based on the fluorescent intensity of DNA. Cell counting of intact cells is based on area, shape/circularity, and fluorescent intensity using ImageJ. (scale bar: 80 μm)

Nanowell-in-microwell Array: Results





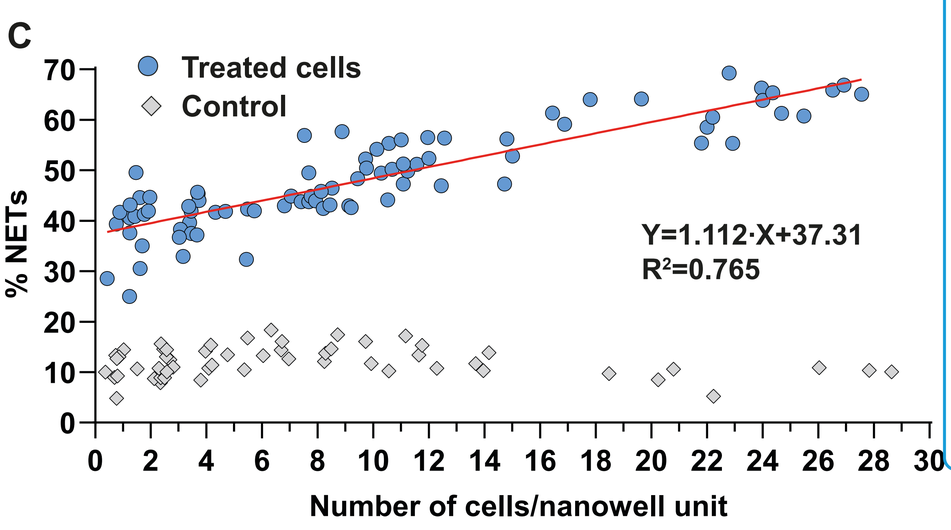


Figure 1. Neutrophils isolated in nanowells exhibited reduced %NETs compared to regular microwell plates (Fig. 1A) which was positively correlated with neutrophil cell density. (R2>0.76, Fig.1C). NETosis was initiated by ionomycin calcium sault (6 μM) treatment for 4.5 hours and was visible (Fig. 1B) by staining for DNA (Hoechst, blue), Neutrophil elastase (red) and Myeloperoxidase (MPO, green). Scale bar - 20 μm. **(R^2>0.76)** (Fig.C).

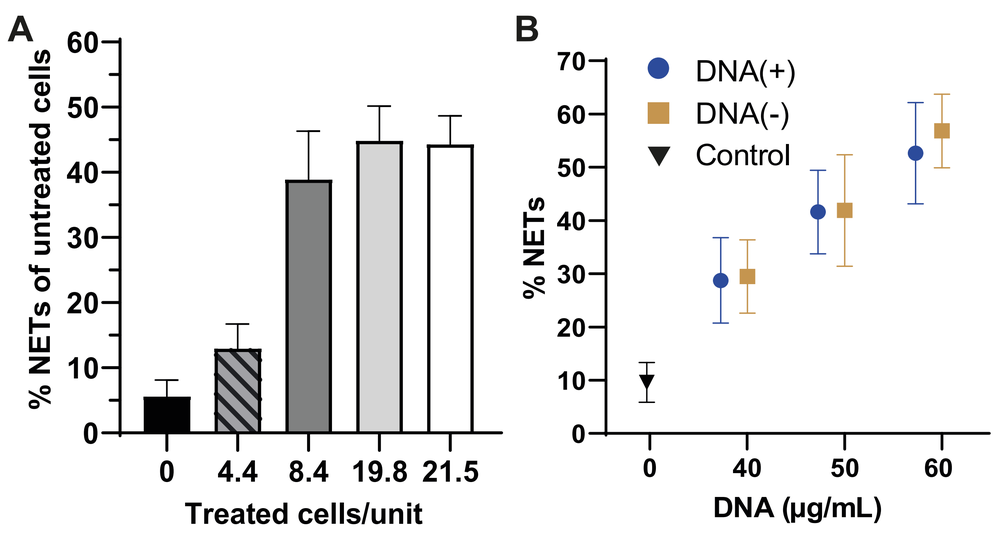
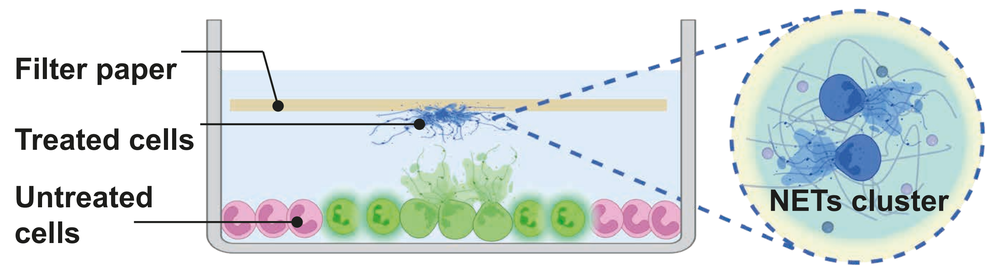


Figure 2. NETosis of untreated neutrophils was triggered by treated neutrophils in a density-dependent manner (Fig. 2A). It could also be triggered by DNA extracted from treated/untreated cells (DNA+/ DNA-, Fig. 2B). Treated cells were activated by ionomycin (6 μM) treatment for 25 min before co-culturing with untreated cells.

Propagation Assay: Methods



- Treated cells were cultured with ionomycin and Hoechst to form a stained NETs cluster (blue).
- The NETs cluster was washed with fresh media before being transferred onto a filter paper.
- Untreated cells were seeded on a 96-well plate with Sytox Green.
- The filter paper was introduced. Untreated cells were cultured with the NETs cluster for 3 hr.

Propagation Assay: Results

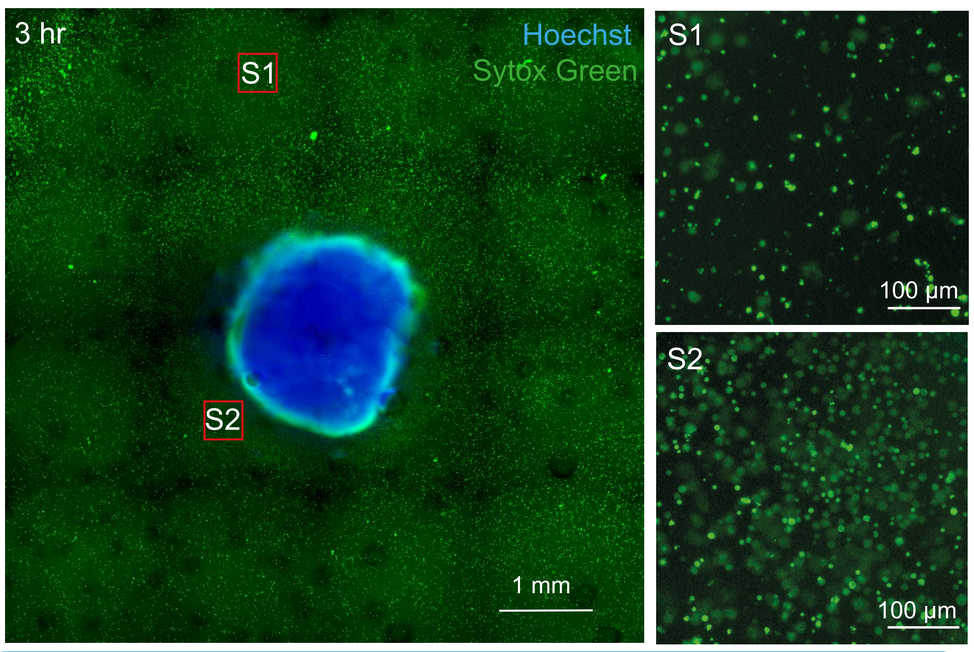


Figure 3. Secondary NETosis (green) propagates from the NETs cluster (blue), where there was increased secondary NETosis closer (S2) to the NETs cluster compared to further away (S1).

CONCLUTION

- We developed a **nanowell-in-microwell** technique to isolate and repeatedly image cells over the course of incubation.
- Using this technique, we provided evidence of **paracrine signalling** in neutrophils propagating the NETosis response in the population.
- We presented a **NETs propagation** assay to show the impact of **secondary NETosis** at swarming sites.

Acknowledgements

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References

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2. Gould, T. J., Z. Lysov, and P. C. Liaw. Journal of Thrombosis and Haemostasis 13 (2015): S82-S91.
3. Wang, Jun, et al. Frontiers in immunology (2020): 2063.