A High-Throughput Screening Platform to Identify Novel Inhibitors of Neutrophil Extracellular Trap Formation (NETosis)

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1 Targeting NETosis in inflammatory diseases

NETosis is a specialised mechanism of cell death, achieved through the formation of neutrophil extracellular traps (NETs).[1] NETs can contribute to the pathogenesis of numerous diseases, including rheumatoid arthritis and COVID-19.[1,2] Developing inhibitors that directly target NETs or inhibit upstream activation and signalling events provide an attractive therapeutic approach.[1-3] Ongoing commercial activity in this field includes the Phase 1 trial of a first-in-class anti-histone therapeutic CIT-013 (Citryll), and Brensocatib (Insmed Inc.), a DDP-1 inhibitor, undergoing Phase 3 trials for non-cystic fibrosis bronchiectasis. Development of novel NETosis inhibitors will rely on robust high-throughput screening assays to advance drug discovery. To this end, NETosis screening assays using primary human neutrophils and differentiated HL-60 (dHL60) cells were developed.

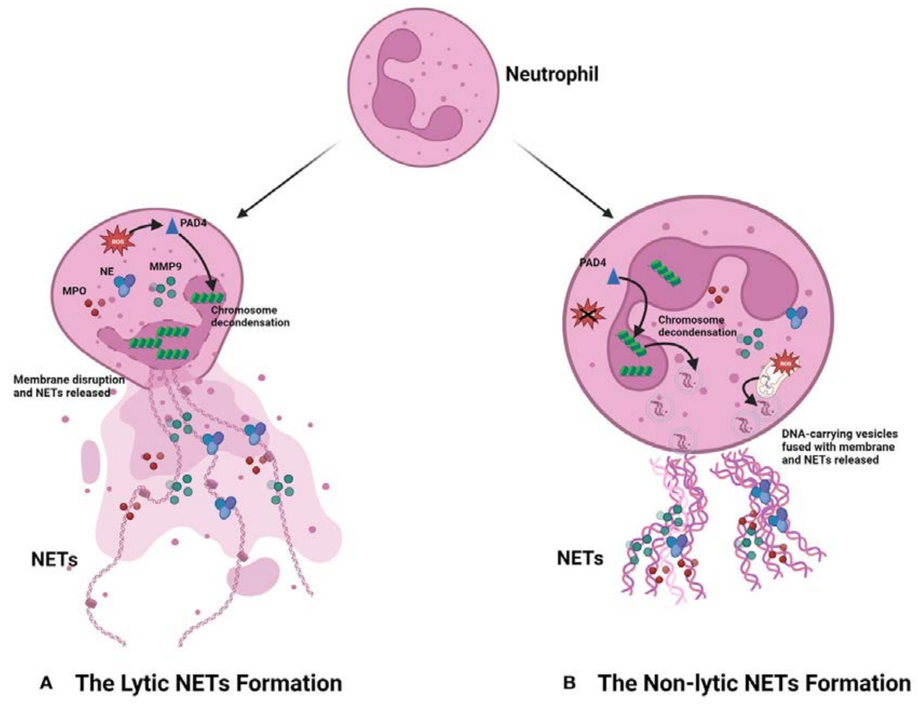

Figure 1: NETosis mechanism. NETs consist of DNA lattices coated in antimicrobial proteins and histones. There are two key pathways of NETosis. A) Lytic NET formation is mediated by NADPH-oxidase dependant reactive oxygen species (ROS) production and results in cell death. B) In Non-lytic NET formation NETs are released in vesicles.
Image: Zhong W, Wang Q, Shen X and Du J (2023) The emerging role of neutrophil extracellular traps in cancer: from lab to ward. Front. Oncol. 13:1163802, doi:10.3389/fonc.2023.1163802 (CC BY).

2 NETosis can be differentiated from other cell death mechanisms

Live cell imaging was used to distinguish NETosis from other cell death mechanisms, such as apoptosis or necrosis in primary human neutrophils. Of the stimuli tested, PMA alone induced NETosis, which was demonstrated by an increase in extracellular DNA and a loss of nuclear integrity.

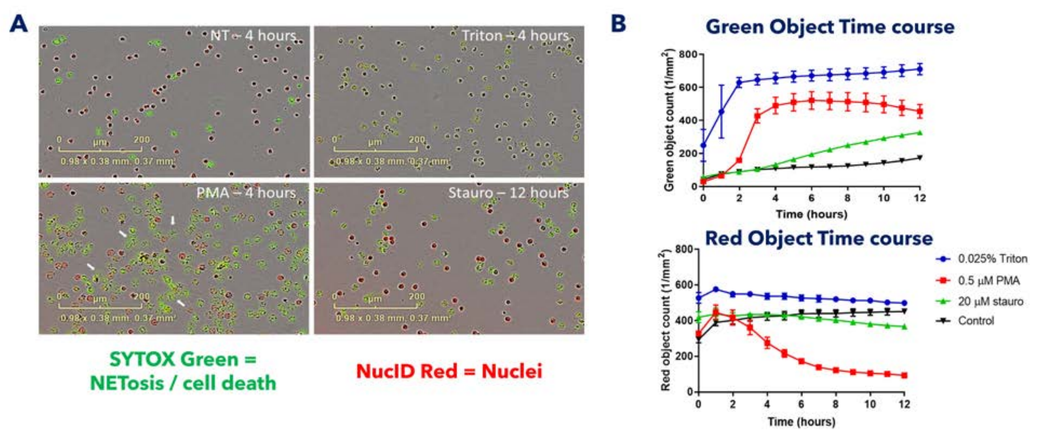

Figure 2: Comparison of cell death mechanisms identifies phorbol 12-myristate 13-acetate (PMA) as a NETosis initiator. (A) Neutrophils were stained with NucID Red and SYTOX Green, treated with known modulators of neutrophil cell death and monitored in real time using the Incucyte® Zoom and analysed using IncuCyte® software (B) Although all stimuli increase extracellular DNA, the concomitant loss of red fluorescent signal, indicating a loss of nuclear integrity, only occurs in cells stimulated to undergo NETosis with PMA and not with Staurosporine (apoptosis) or Triton-X (necrosis) (average of 3 wells ± SEM).

3 NETosis is observed in model cellular systems

To increase NETosis screening assay throughput & efficiency, we replaced primary human neutrophils with differentiated HL60 (dHL60) cells. Expression of neutrophil markers CD11b and CD66b were confirmed in dHL60 cells following 5 days of treatment with retinoic acid (data not shown). NETosis can be clearly identified in dHL60 cells stimulated with PMA, therefore allowing increased assay throughput and transfer to automation compared to relying on primary cells.

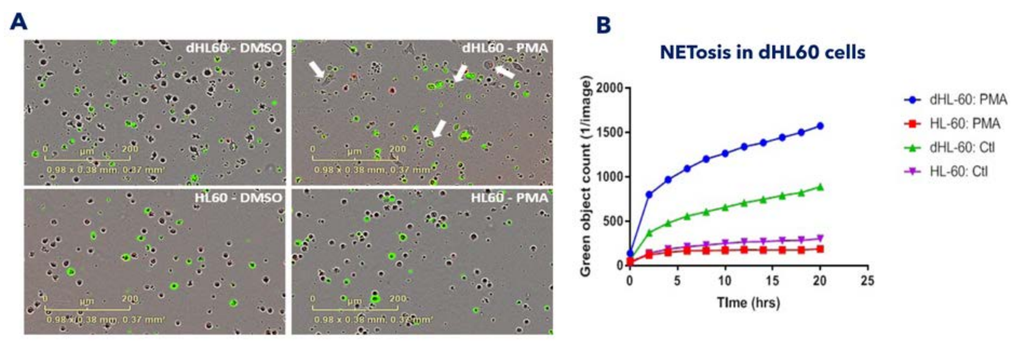

Figure 3: NETosis in dHL60 cells. HL60 Cells were differentiated for 5d with 1 µM retinoic acid (dHL60). Treatment with PMA induced a time-dependant increase in SYTOX Green fluorescence, which was not observed in undifferentiated HL60 cells (average of 3 wells ± SEM).

4 Using live-cell imaging to screen for inhibitors of NETosis

Development of robust high-throughput screening (HTS) assays are vital for the discovery of novel inhibitors of NETosis. Using live-cell imaging, as described previously, a range of literature inhibitors were explored using primary human neutrophils, in a 96-well format. PKC inhibitor Go6976, antioxidant Resveratrol and NADPH oxidase inhibitor DPI, displayed high levels of NETosis inhibition in vitro. Whereas Myeloperoxidase (MPO) inhibitor 4-Aminobenzoic Acid hydrazide (AMAH), did not greatly impact NETosis.

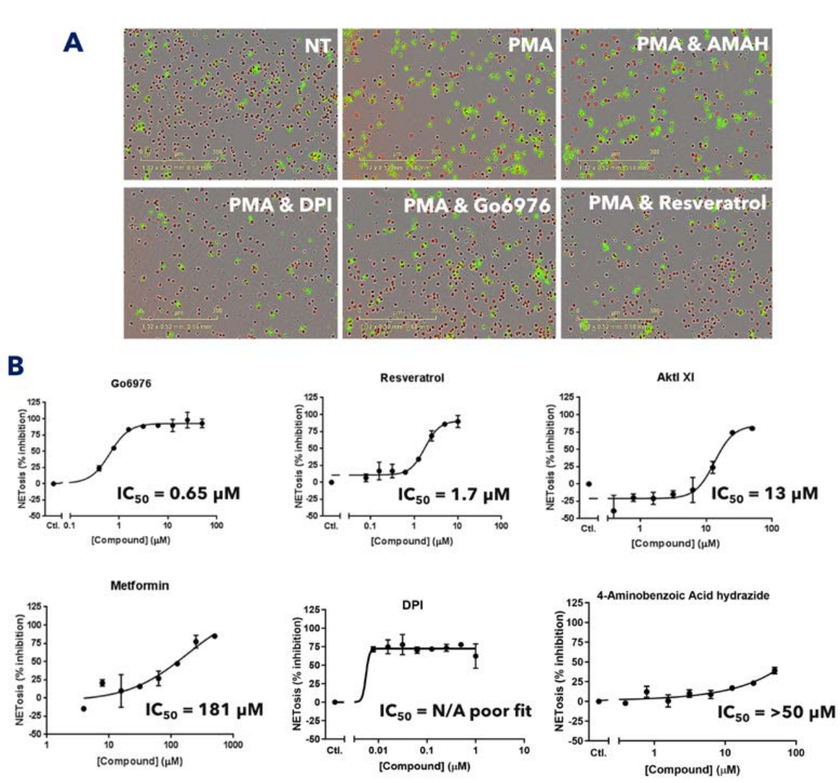

Figure 4: Inhibitors of NETosis detected using live cell imaging. Neutrophils were stained with NucID Red and SYTOX Green and stimulated for 4 hours with PMA and treated with Go6976, Reservatrol, DPI, Akt1 XI, Metformin, DPI or AMAH. Cells without PMA treatment were used as a negative control (NT). Images were captured using the Incucyte® Zoom and analysed using IncuCyte® software. NETosis inhibition was calculated as green/red object number and normalised to high (PMA) and low (DMSO) controls. The IC_{50} was not determined for DPI treatment. Data is representative of the mean of triplicate wells ± SEM.

5 High content IF imaging for NETosis

To complement live cell-imaging assays, a fixed end-point, immunofluorescence (IF) high-content imaging system was developed. IF imaging has advantages for HTS as it has increased NET specificity compared to SYTOX Green and enables storage and batching of plates prior to imaging and analysis. Here, we demonstrate NETosis quantification using nuclear staining (Hoechst) multiplexed with IF staining for abundant NET marker MPO.

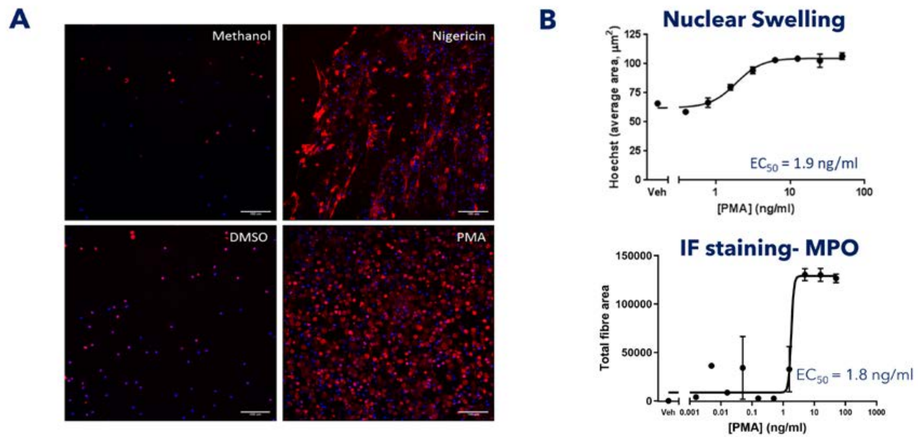

Figure 5: NETosis high content imaging. A) Neutrophils were treated with increasing concentrations of PMA or Nigericin for 3 hours in 96 well plates. Cells were fixed and stained with nuclear stain Hoechst and an anti-MPO antibody. Images were captured using the Image Xpress Confocal Microscope. B) The MetaXpress software was used to measure nuclear swelling and NET fibre area. There was a concentration dependant increase in these parameters following PMA treatment.

6 Summary

High throughput NETosis screening assays were developed using live cell imaging and IF staining for NET-associated proteins with primary human neutrophils and dHL60 cells.

These assays will enable large numbers of novel NETosis inhibitors to be screened efficiently, which will address the limited screening throughput often observed in this field.

References

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