Integrated Real-Time Impedance and Fluorescence Imaging to Characterize NETosis

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Abstract

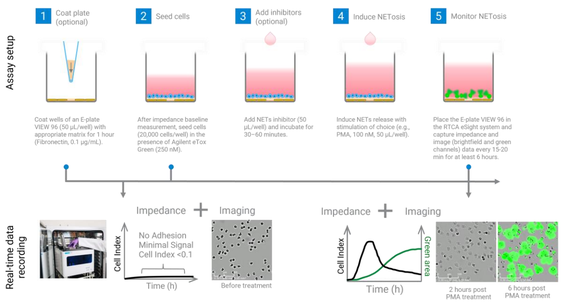
NETosis is a specialized form of neutrophil cell death characterized by the release of web-like DNA structures and associated proteins, known as neutrophil extracellular traps (NETs). Two main pathways mediate NETosis.[1,2] (1) classical "suicidal" NETosis, which involves cell lysis following NET release, typically occurs within three to four hours and depends on reactive oxygen species (ROS) generation via NADPH oxidase; and (2) "vital" NETosis, where NETs are extruded through vesicles within one to two hours while the cell remains intact. NETosis plays a dual role in host defense and disease, contributing to both antimicrobial immunity and pathological inflammation, thrombosis, and autoimmunity.[3]

In this study, differentiated neutrophil-like HL-60 (dHL-60) and primary human neutrophils were treated with a small set of compounds: PMA, which triggers suicidal NETosis; A23187, a pharmacological agent that induces rapid, vital NETosis; and camptothecin, an apoptotic compound. To visualize NET formation, the cells were cultured in media containing eTox Green, a membrane-impermeable DNA-binding dye. The morphological and physiological changes were continuously monitored using both impedance measurements (reported as Cell Index) and live-cell imaging simultaneously on the Agilent xCELLigence RTCA eSight system.

Our results demonstrate that (1) DNA-binding dye staining distinguishes apoptosis from NETosis, with PMA- and A23187-induced NETosis producing larger fluorescence areas than camptothecin-induced apoptosis, consistent with the greater size of extruded NETs; (2) suicidal NETosis induced by PMA differs from vital NETosis triggered by A23187 by ROS dependency, DPI completely blocked NET release after PMA but not A23187 treatment, with A23187 inducing earlier NET extrusion and neutrophil death occurring only after PMA stimulation; and (3) PMA-induced NETosis is accompanied by a suspension-to-adherent transition, quantified by impedance measurements.

Experimental

Brief workflow for the NETosis assay



Add 50 µL of cell-specific medium to the E-Plate and incubate for at least 30 minutes before baseline detection. Prepare neutrophils or dHL-60 cells at 200,000 cells/mL in medium with eTox Green (1.5 × 375 nM). Transfer 100 µL (20,000 cells/well) to each well, allow to settle for 30 minutes, and then warm at 37 °C for 15 minutes. Perform the first scan with a 20x objective, collecting impedance and four images per well every 5 to 10 minutes for at least six hours.

For the inhibitor assay, cells were pretreated for 30 to 60 minutes with 50 µL of medium containing 5x inhibitor and 1x eTox Green, then stimulated with 50 µL of 5x PMA and A23187 to yield final concentrations of 100 nM and 10 µM for NETosis induction. After adding the inhibitor, the user can scan one sweep on the RTCA eSight to observe the baseline effect of the inhibitor as needed.

For the induction assay, prepare the stimuli (PMA or A23187, or the apoptosis inducer camptothecin) at a 4x concentration, and prepare eTox Green at 1x in 50 µL of medium. Then add this mixture to 150 µL of neutrophils to obtain a final volume of 200 µL.

Results and Discussion

Induction of classic "suicidal" NETosis in neutrophil-like dHL-60 cells using PMA

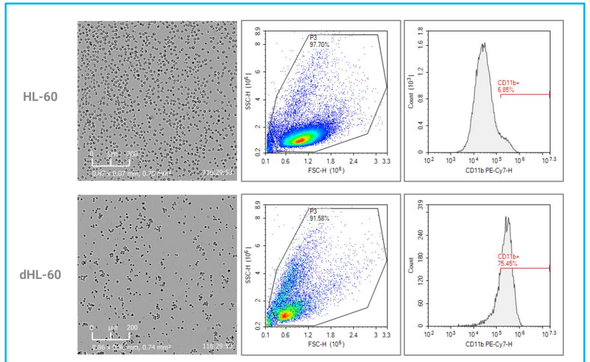


Figure 1. Successful differentiation of HL-60 cells into neutrophil-like dHL-60 cells using DMSO (1.25% v/v) and ATRA (1 µM) for five days. The Agilent xCELLigence RTCA eSight system is used to monitor the morphological changes of the HL-60 during five days of differentiation, and Agilent NovoCyte Penteon flow cytometer is used to detect the side scatter signal changes and CD11b expression before and after differentiation.

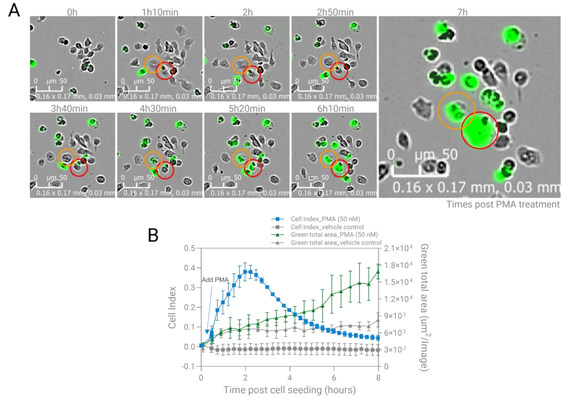


Figure 2. Evaluate the formation of "suicidal" NETosis in dHL-60 cells induced by PMA using the Agilent xCELLigence RTCA eSight system. (A) Representative images taken following the NETosis process induced by PMA (50 nM) in the presence of Agilent eTox green reagents. The red and orange circles highlight two cells going through the NETosis process. (B) Cell Index and green total area changes as a function of time. Each data point represents mean ± SD, n = 3 wells.

"Suicidal" NETosis formation in primary human neutrophils

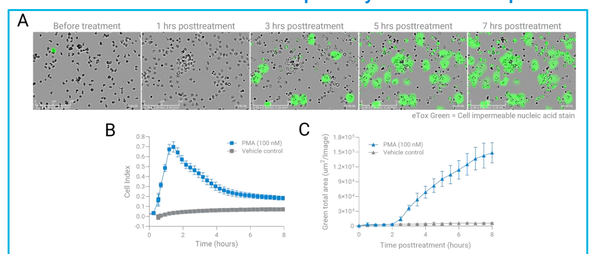


Figure 3. The Agilent xCELLigence RTCA eSight visualizes the NETosis formation in primary human neutrophils. (A) Representative images taken following the NETosis process induced by PMA (100 nM) in the presence of Agilent eTox green reagents. (B) Cell Index and (C) green total area changes as a function of time. Each data point represents mean ± SD, n = 3 wells.

Results and Discussion

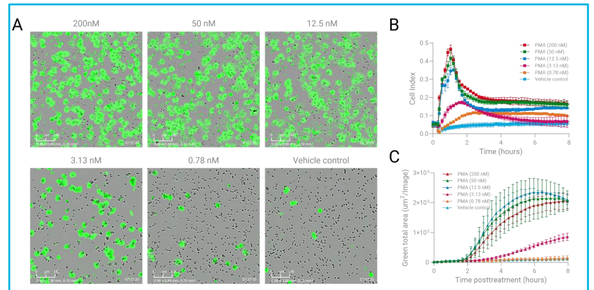


Figure 4. Impedance and imaging monitor PMA concentration-dependently induce "suicidal" NETosis formation. (A) Typical images with brightfield and green channels on at seven hours after treatment with various PMA concentrations. (B) The Cell Index over time plot. (C) The green total area over time plot.

NETosis exhibits larger eTox-positive areas and distinct impedance patterns vs apoptosis

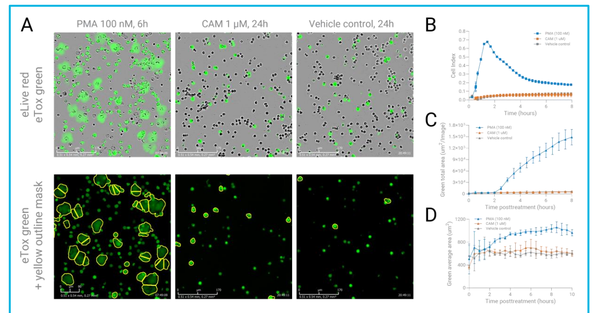


Figure 5. "Suicidal" NETosis can be distinguished from apoptosis by eTox green area and changes in Cell Index. Cell Index changes, eTox green dye are used to visualize NET release (extracellular DNA, PMA treatment) and cell death (intracellular DNA, camptothecin treatment). (A) Representative images were taken seven hours after treatment with 100 nM PMA and 24 hours after treatment with 1 µM CAM and 1% DMSO (vehicle control). The yellow outline is the green fluorescence mask. (B) Cell Index, (C) green total area, and (D) green average area changes over 8 or 10 hours post-treatment. CAM means camptothecin. Each data point represents mean ± SD, n = 3 wells.

Mechanism differences between "suicidal" NETosis and "vital" NETosis

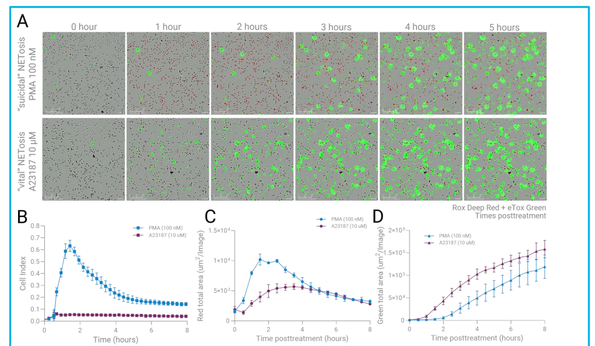


Figure 6. "Suicidal" NETosis and "vital" NETosis can be distinguished using the Agilent xCELLigence RTCA eSight system. (A) Typical images with brightfield and green channels during zero to five hours after PMA or A23187 treatment. (B) The Cell Index over time plot. (C) The normalized red total area over time plot. (D) The green total area over time plot.

Results and Discussion

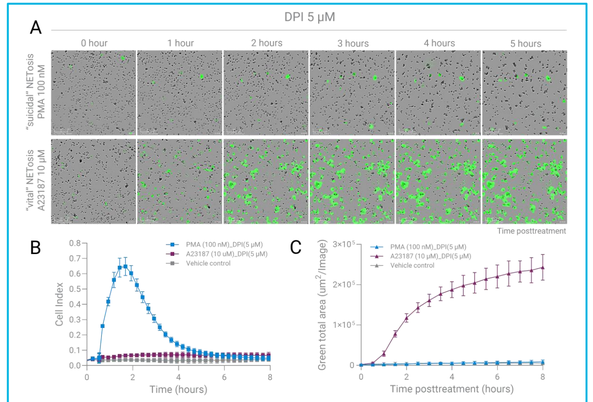


Figure 7. DPI inhibits NOX-dependent suicidal" NETosis. (A) Images and (B) curve show DPI inhibits PMA-induced "suicidal" NETosis but not A23187-induced "vital" NETosis. (C) The Cell Index over time plot indicates that DPI does not affect the morphology change after PMA treatment.

Conclusions

- Real-time monitoring of NETosis is enabled by combining brightfield and fluorescence imaging on the Agilent xCELLigence RTCA eSight system.
- Impedance-based Cell Index measurements of the RTCA eSight capture the suspension-to-adhesion transition characteristic of "suicidal" NETosis.
- Simultaneous impedance and imaging readouts of the RTCA eSight allow clear differentiation among "suicidal" NETosis, "vital" NETosis, and apoptosis.
- This integrated approach provides a robust platform for mechanistic NETosis studies, drug screening, and investigations of neutrophil-driven pathologies.

References

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2. Shafqat, A.; Abdul Rab, S.; Ammar, O.; Al Salameh, S.; Alkhudairi, A.; Kashir, J.; Alkattan, K.; Yaqinuddin, A. Emerging role of neutrophil extracellular traps in the complications of diabetes mellitus. *Front Med (Lausanne)*. **2022**, *9*, 995993. DOI:10.3389/fmed.2022.995993
3. Huang, J.; Hong, W.; Wan, M.; Zheng, L. Molecular mechanisms and therapeutic targets of NETosis in diseases. *MedComm (2020)*. **2022**, *3* (3), e162. DOI: 10.1002/mco2.162