Understanding NETosis priming and induction through Nucleosome changes and real-time monitoring of neutrophils

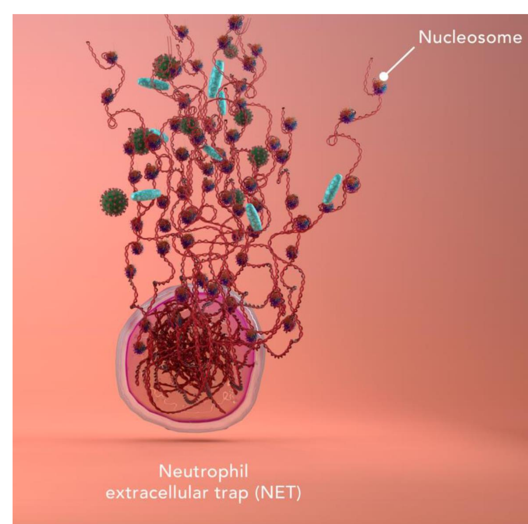
Justin Cayford[1], Kieran Zukas[1], Mark Eccleston[1], Theresa K Kelly[1]
[1]Volition America, 6086 Corte Del Cedro Carlsbad, CA 92011

Introduction

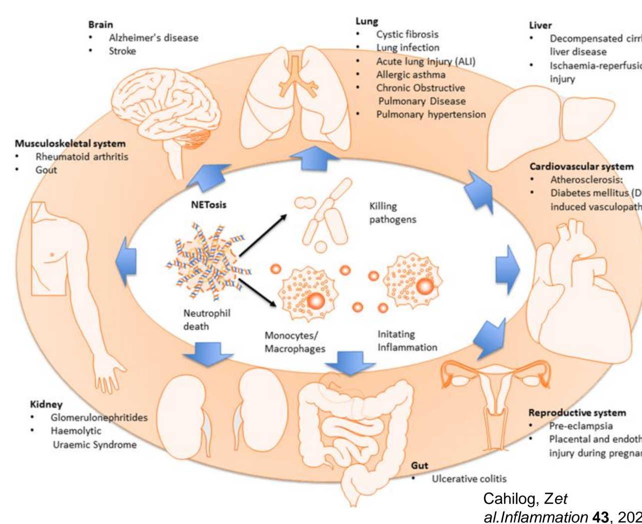
- NETosis is a form of cell death where neutrophils release their DNA to trap and kill pathogens, but it can also lead to inflammatory diseases
- NETs have become more relevant in disease but have not been widely characterized in humans
- Studies in NETosis have been mostly limited to isolated systems with unnatural stimulation
- Understanding natural NETosis activation and the underlying mechanisms could lead to new biomarkers and understanding of how these neutrophil associated diseases are regulated *in vivo*.

NETs and Disease

Neutrophil Extracellular Traps (NETs)



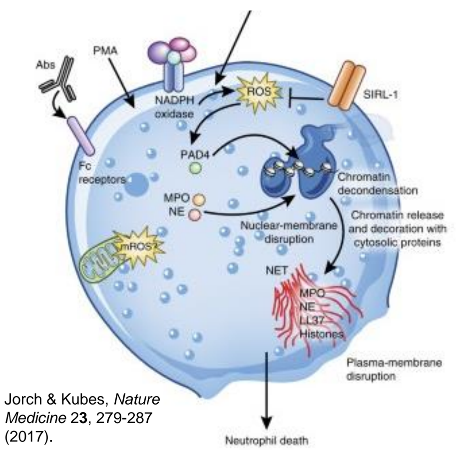
NETosis in Disease



- Neutrophil Extracellular Traps (NETs) are released by neutrophils upon various stimuli
- NETs are essential for trapping and neutralizing pathogens, but overproduction or uncontrolled formation can lead to tissue damage and disease progression during the inflammatory response.

- Recently, NETosis has been implicated in many diseases
- A fundamental understanding of the mechanism *in vivo* has been challenging to date.
- Most studies rely on isolated primary neutrophils, which misses many potential cross-talk events which would happen in whole blood

NETosis General Pathway

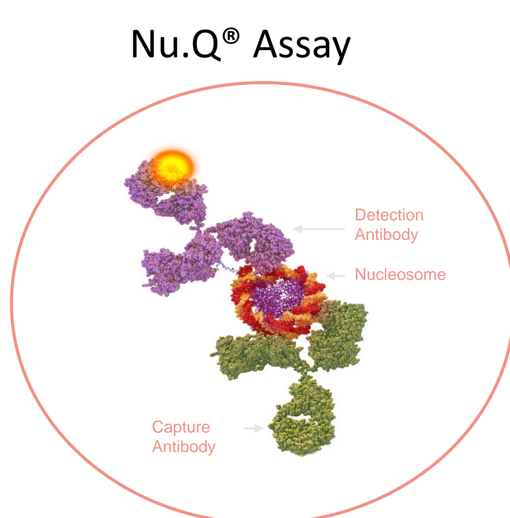


- NETosis is triggered by a variety of signals, including unnatural activation of protein kinase C (PKC) by PMA (Phorbol 12-myristate 13-acetate), or more natural triggers which activate through NADPH oxidase, calcium influx, ionophores, and bacterial toxins (LPS).
- These signals lead to the activation of enzymes like PAD4 (peptidylarginine deiminase 4) and the production of reactive oxygen species (ROS), which promote chromatin decondensation and the release of NETs in the matrix.
- During NETosis, neutrophil granules containing myeloperoxidase (MPO) and neutrophil elastase (NE) are degranulated after the activation of PAD4 and subsequent chromatin decondensation. The presence of MPO and NE in the extracellular matrix is a hallmark of NET formation and is indicative of the release of granular contents during the process.

Methods

- Nucleosomes were measured using Volition's Nu.Q® H3.1 Assay
- Neutrophils were isolated using MACSxpress® Whole Blood Neutrophil Isolation Kit
- Real-time Netosis was measured by inclusion of fluorescent dyes and continual fluorescent measurements using a SpectraMax plate reader

Nu.Q® Assay



Results

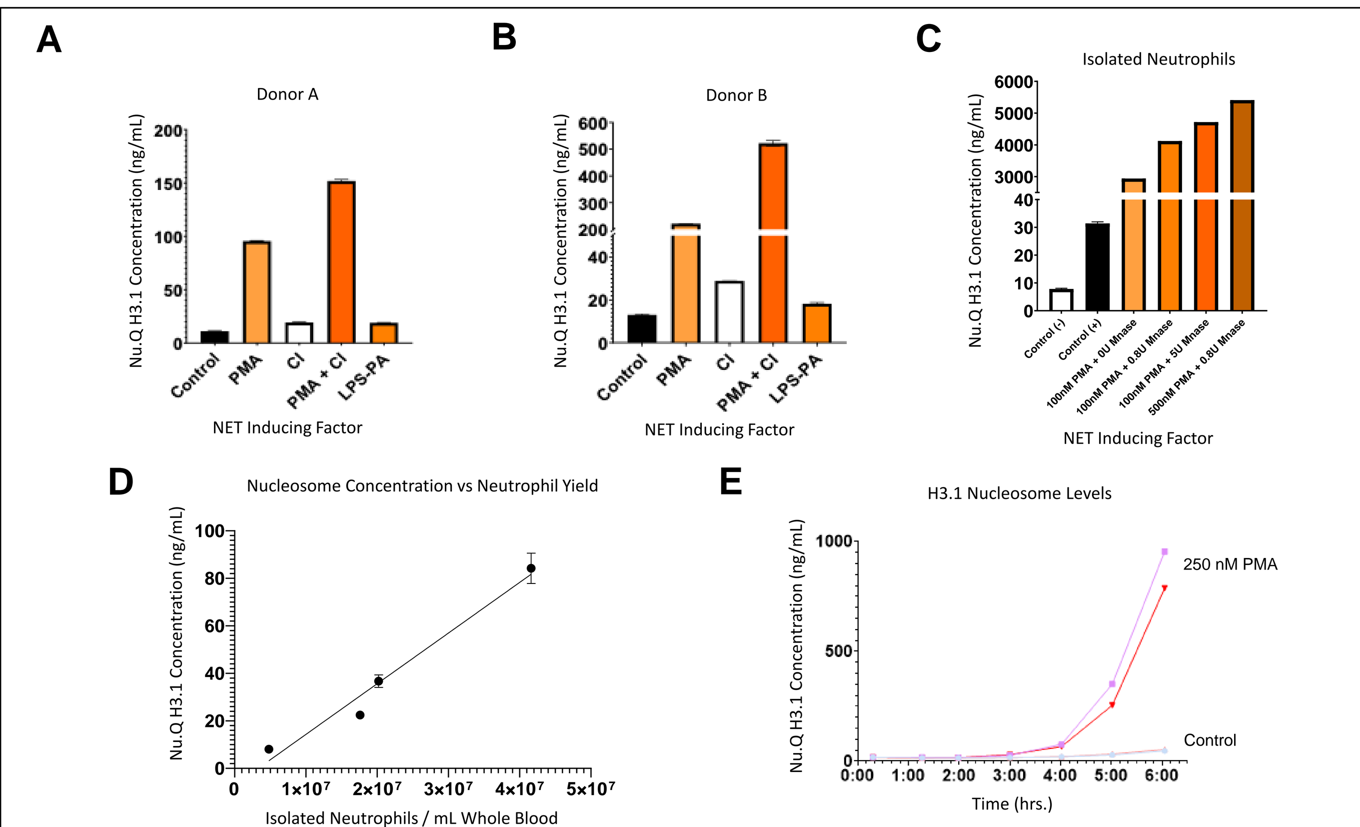


Figure 1: Nu.Q® H3.1 Results in both whole blood and primary isolated neutrophils. **(A & B)** Nucleosome levels as measured by Nu.Q® H3.1 demonstrate that nucleosomes are elevated when whole blood is treated with (PMA), with or with the co-addition of CI. Pre-treatment with CI and LPS-PA alone have marginal effects of Nu.Q® H3.1 levels. The extent to which H3.1 containing nucleosomes levels is elevated is donor dependent, likely reflecting a different number of Neutrophils in the sample. **(C)** End-point (3.5 hour) analysis of nucleosome levels as measured by Nu.Q® H3.1 assay across a variety of PMA and MNase concentrations demonstrating that H3.1 nucleosome levels are increased following NETosis induction of isolated Neutrophils. **(D)** Correlation of the number of neutrophils isolated from whole blood samples and the corresponding nucleosomes levels. **(E)** Time course of NET release in response to 250 nM PMA in duplicate
(PMA: Phorbol 12-myristate 13-acetate; CI: Calcium Ionophore; LPS-PA: Lipopolysaccharide from pseudomonas aeruginosa)

NETosis Induction Systems

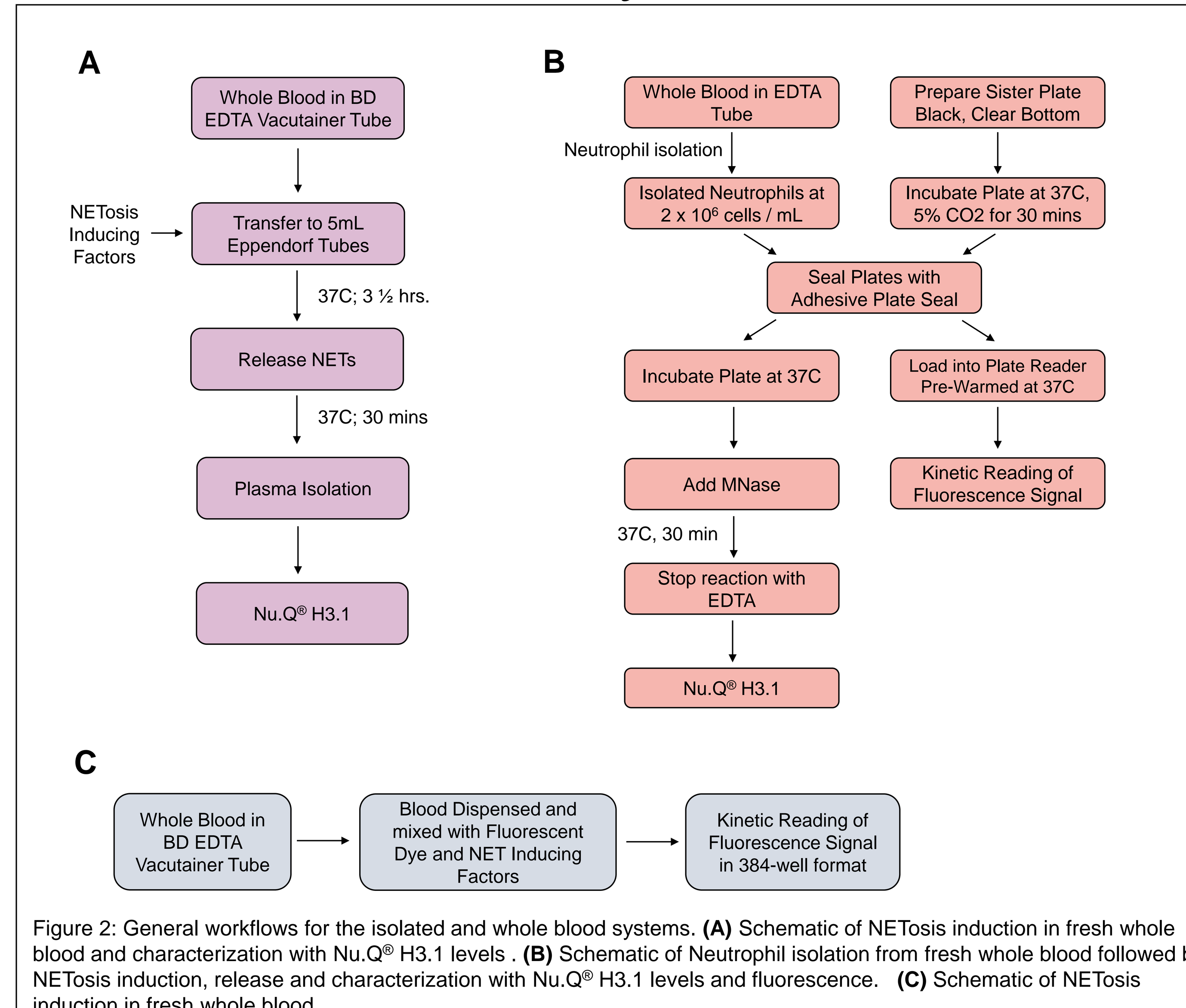


Figure 2: General workflows for the isolated and whole blood systems. **(A)** Schematic of NETosis induction in fresh whole blood and characterization with Nu.Q® H3.1 levels . **(B)** Schematic of Neutrophil isolation from fresh whole blood followed by NETosis induction, release and characterization with Nu.Q® H3.1 levels and fluorescence. **(C)** Schematic of NETosis induction in fresh whole blood.

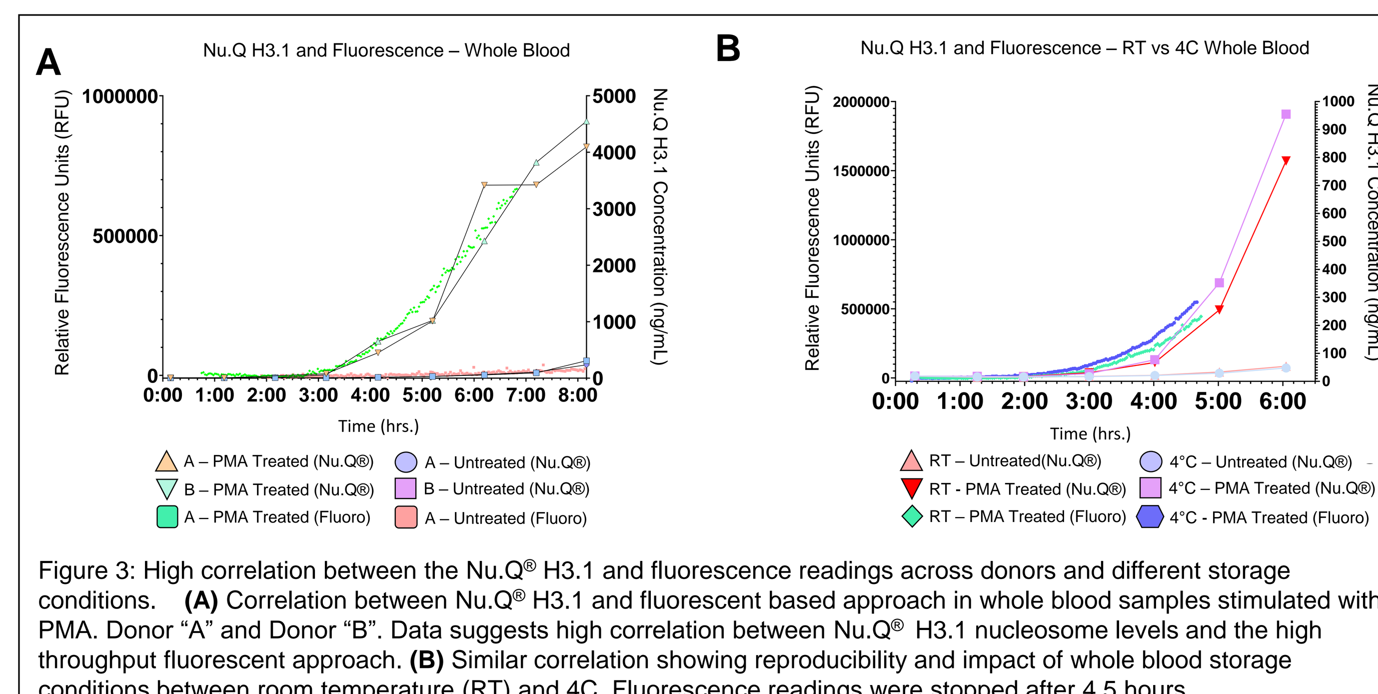


Figure 3: High correlation between the Nu.Q® H3.1 and fluorescence readings across donors and different storage conditions. **(A)** Correlation between Nu.Q® H3.1 and fluorescent based approach in whole blood samples stimulated with PMA. Donor "A" and Donor "B". Data suggests high correlation between Nu.Q® H3.1 nucleosome levels and the high throughput fluorescent approach. **(B)** Similar correlation showing reproducibility and impact of whole blood storage conditions between room temperature (RT) and 4C. Fluorescence readings were stopped after 4.5 hours

Results

NETosis Induction

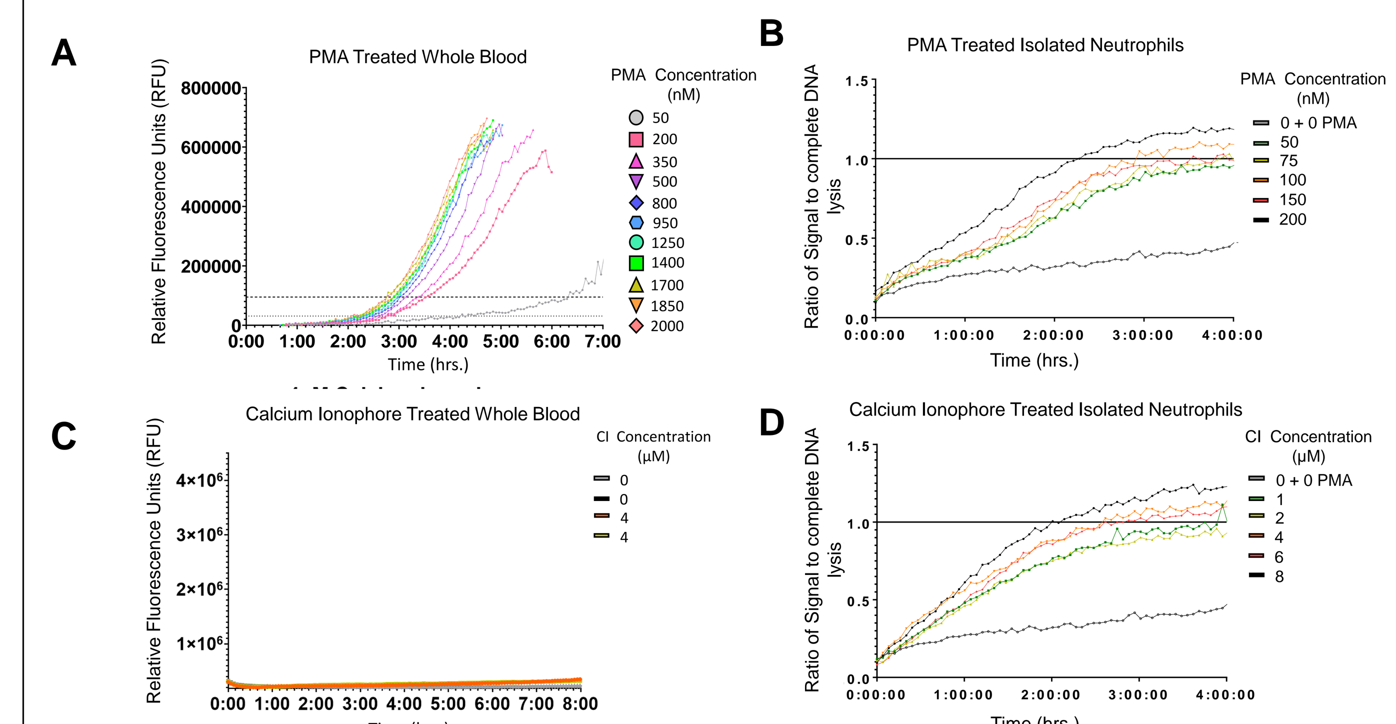


Figure 4: Time course of NET release in response to PMA in whole blood **(A)** and in isolated neutrophils **(B)**. Calcium Ionophore time course in whole blood **(C)** and in isolated neutrophils **(D)** as measured by fluorescence dye intercalation normalized to total DNA content.

Inhibition of NETosis

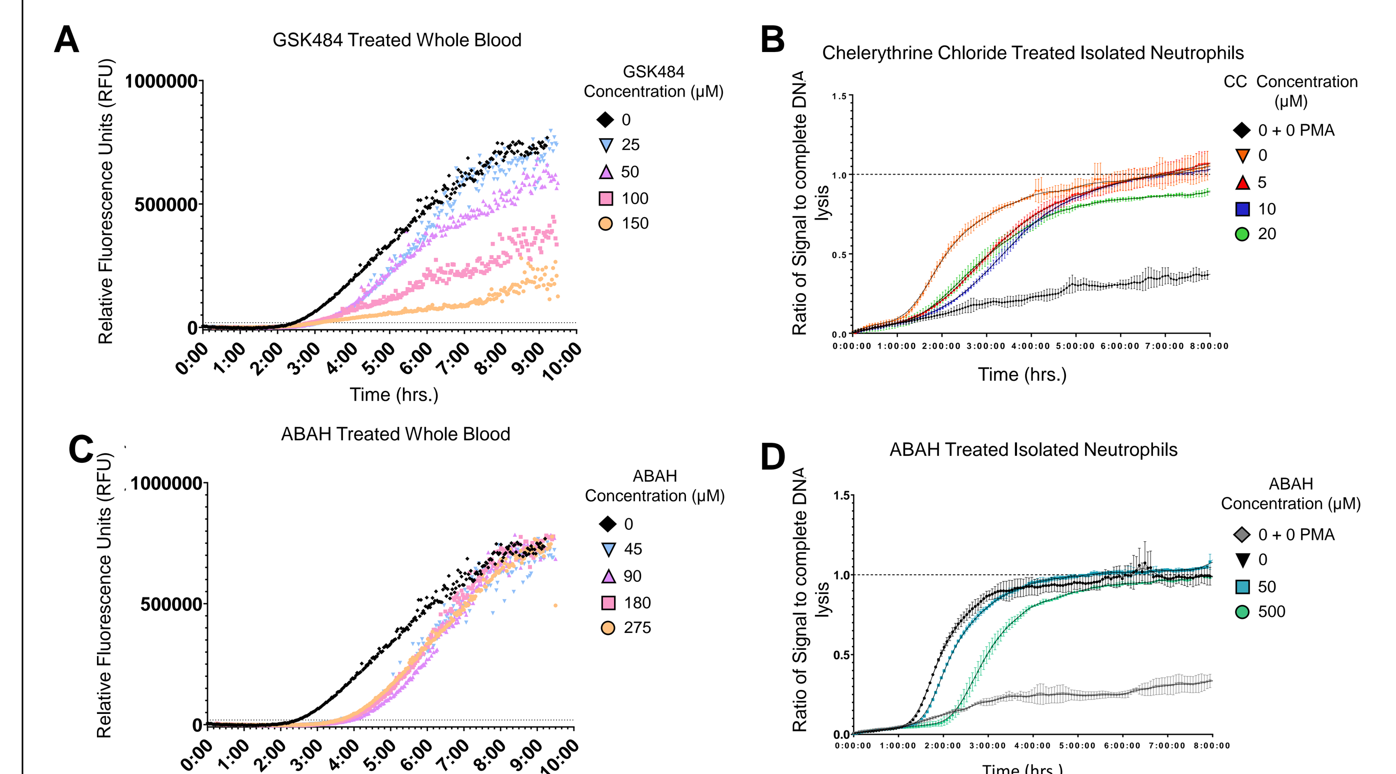


Figure 5: PMA Treated Whole Blood (A & C) or Isolated Neutrophils (B &D) with NETosis inhibiting factors. GSK484 is a reversible PAD4 inhibitor, Chelerythrine Chloride (CC) is a Protein Kinase C (PKC) selective inhibitor, and ABAH is an MPO inhibitor. **(A)** PMA treated whole blood with a titration of GSK484. **(B)** PMA treated Isolated Neutrophils treated with a titration of CC. **(C)** PMA treated whole blood with a titration of ABAH. **(D)** PMA treated Isolated Neutrophils treated with a titration of ABAH.

Summary & Conclusions

- Nu.Q® H3.1 levels in whole blood and primary isolated human neutrophils can be measured and correlate well with fluorescent based approaches.
- Using standard chemical inducers, there are clear differences between NETosis induction in whole blood and isolated neutrophils
- Using known NETosis inhibitors, both the isolated neutrophils and whole blood respond, indicating the observed signal is NETosis.
- Isolated neutrophils and whole blood react differently, and the whole blood system is much closer to an *in vivo* response.

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For more information
contact Justin Cayford
Innovation Group Leader
J.Cayford@volition.com