High-Throughput Screening to Develop a Biologically Relevant Netosis Induction Model

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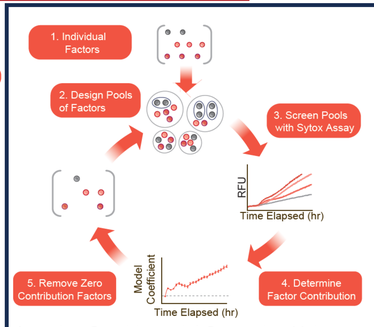
INTRODUCTION

- Neutrophils are a type of granulocyte known to undergo a specialized form of cell death that involves the release of decondensed chromatin and anti-microbial proteins.
- This structure is known as a Neutrophil Extracellular Trap (NET), and excess production of NETs is associated with inflammation, organ dysfunction, and cancer metastasis.
- In vitro models typically induce NETosis using non-biological molecules like PMA which bypass normal regulatory pathways
- Current models that detect NETosis in real-time are limited to isolated neutrophils, which does not reflect the complexity of real immune responses.

AIM

To Develop a HTS Scalable Human Whole Blood Ex-Vivo Model of NETosis Using Biologically Relevant Stimuli.

METHOD



Iterative Combinatorial Screening Using Experimental Design Statistics and Regression Analysis.

Novel Model to Measure DNA Release in Whole Blood using Real-Time Fluorescent Measurements of Sytox Green, a DNA Intercalating Dye.

RESULTS

Response of Whole Blood to Traditional NET-Inducing Stimuli

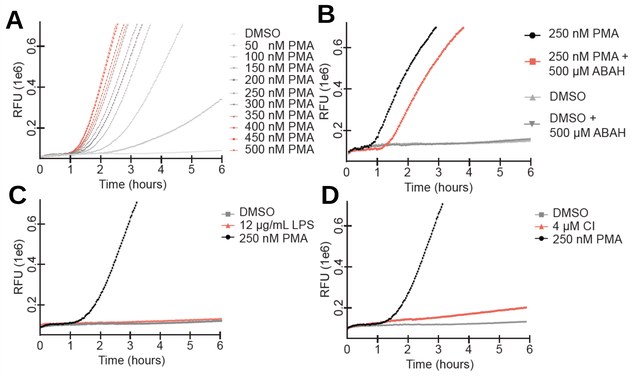


Figure 1. (A) Treatment of whole blood with varying concentrations of PMA, a commonly used NET-inducing factor, as measured by Sytox Green fluorescence. (B) Treatment of whole blood with ABAH, a myeloperoxidase inhibitor, followed by treatment with PMA (C,D) Treatment of whole blood with the commonly used NET-inducing factors, Lipopolysaccharide (LPS) and Calcium Ionophore (CI).

Effect of all Combinations of Eight Factors Between Donors

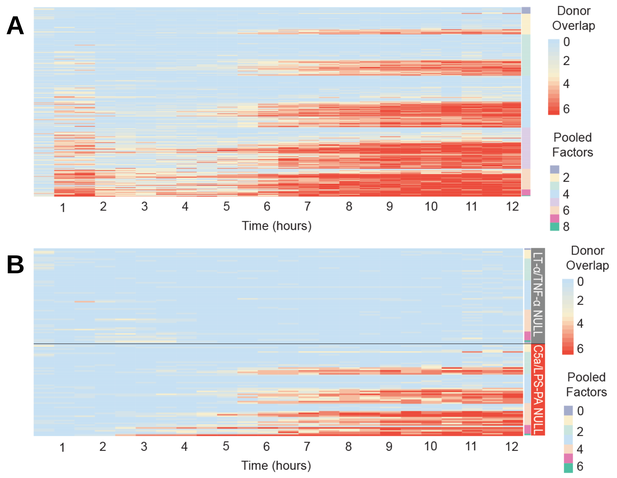


Figure 3. (A) Heat-Map representation of the observed response in whole blood obtained from six different donors when treated with every combination of the 8-factors identified. (B) Heat-Map representation of the observed response in whole blood from six donors, with LT-α and TNF-α excluded, or C5a and LPS-PA excluded.

Combinatorial Screening of Pro-Inflammatory Molecules Identifies Nine Potential Activators of NETosis

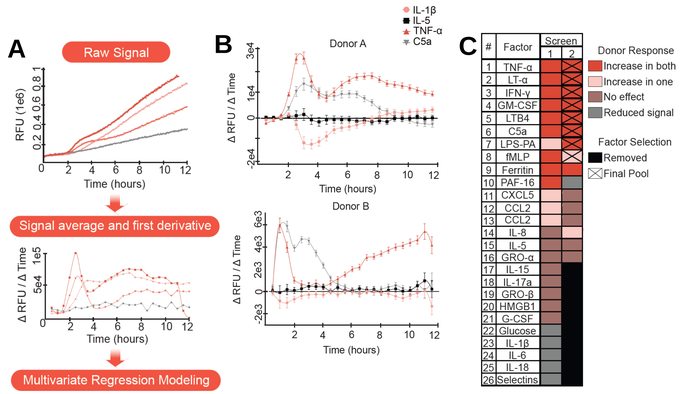


Figure 2. (A) Iterative combinatorial screening was conducted with 26 immune related molecules with signal analysis workflow shown. (B) Determination of the predicted contribution of each factor showed 4 distinct classifications; some factors contributing to an early increase in signal (e.g., C5a), some factors contributing to both an early and later increase in signal (e.g., TNF-α), some factors not contributing to the observed signal (e.g., IL-5), and some factors acting to decrease the observed signal(e.g., IL-1β). (C) Representation of predicted effect of each factor for two iterations of screening with two donors for each iteration, and selected factors for further deconvolution.

Identification and Validation of Minimal Factors Needed to Induce NETosis in Whole Blood

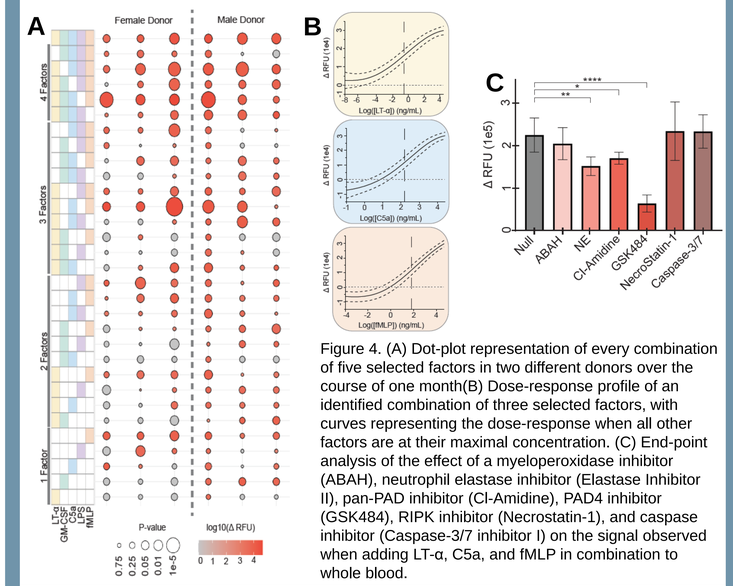


Figure 4. (A) Dot-plot representation of every combination of five selected factors in two different donors over the course of one month(B) Dose-response profile of an identified combination of three selected factors, with curves representing the dose-response when all other factors are at their maximal concentration. (C) End-point analysis of the effect of a myeloperoxidase inhibitor (ABAH), neutrophil elastase inhibitor (Elastase Inhibitor II), pan-PAD inhibitor (Cl-Amidine), PAD4 inhibitor (GSK484), RIPK inhibitor (Necrostatin-1), and caspase inhibitor (Caspase-3/7 inhibitor I) on the signal observed when adding LT-α, C5a, and fMLP in combination to whole blood.

CONCLUSIONS

- NETosis can be monitored in real-time in whole blood using Sytox Green, a DNA intercalating dye.
- Commonly used NET-inducing factors that induce NETosis in isolated neutrophils do not stimulate neutrophils to undergo NETosis in whole blood.
- Variability in response, between and within donors, is observed upon treatment with various pro-inflammatory factors.
- As the number of pro-inflammatory factors added to whole blood increases, the consistency in response between donors increases.
- At least three biologically relevant pro-inflammatory stimuli are necessary for NETosis to occur in whole blood.
- Utilization of this HTS whole blood model and pro-inflammatory stimuli can detect pharmacological inhibition of NETosis in a biologically relevant context.

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