Development of an Ex Vivo Human Whole Blood Platform for Quantifying Bacteria-Induced NETosis

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Burçin Tan

A Thesis in the Field of Biology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

May 2026

Abstract

This study focused on developing a human whole blood sepsis model using *S. aureus*. Methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA), obtained from a previous human abscess study, were first characterized through growth curve analysis and Protein A production, using an ELISA assay, across multiple media conditions.

We evaluated two outcome measures, red blood cell hemolysis and NETosis. S. aureus produces several hemolysins; however, it was not known if human red blood cells would be sensitive to them. There was no commercially available ELISA assay for these hemolysins, so we used a functional hemolysis assay. Despite optimizing the assay, we were not able to detect significant hemolysis. NETosis measurements were attempted using a commercially available H3.1 nucleosome-based ELISA kit. For unclear reasons this assay showed NETosis predominantly in the negative controls and not in response to the bacteria. We then switched to a kinetic fluorescence-based NETosis assay using SYTOX Green described by Zukas et al. We optimized the protocol by using hirudin as the anticoagulant and a SYTOX Green concentration of 5.0 uM. As expected, MRSA produced a greater degree of NETosis than MSSA. MRSA likely produces more Protein A than MSSA and we demonstrated that adding Protein A to MSSA, but not MRSA, increased the NETosis.

Overall, this work suggests that human whole blood, exposed to S. aureus, can be used to model NETosis which is seen in severe sepsis. The NETosis response needs to be

confirmed using complementary assays and that additional outcomes consistent with clinical sepsis modeled. If successful, the platform will provide a controlled framework for studying bacterial-induced sepsis response in a physiologically relevant ex vivo human model.

Acknowledgments

I would like to express my deepest and unwavering gratitude to my Thesis Director, Dr. Howard Smithline, for his mentorship, patience, and steady guidance throughout this project. I am especially thankful for the generosity of his time and attention, as he consistently provided a deep, thoughtful understanding and constructive feedback, often beyond the demands of his clinical responsibilities. His dedication to both clinical medicine and research has shaped the way I think about science and its responsibility beyond the laboratory. Although this work was not directly tied to our clinical hours together, watching the seriousness and care with which he approaches both patient care and investigation has influenced how I hope to practice medicine in the future. I am genuinely grateful for the opportunity to complete this thesis under his direction and for the trust he placed in me to grow not only as a researcher, but as an aspiring physician. His expectations pushed me to think more critically, work more deliberately, and approach uncertainty with curiosity rather than fear.

I am also very thankful to my Research Advisor, Dr. James R. Morris, for his thoughtful feedback and encouragement throughout the writing process. He was consistently responsive to my questions and always willing to clarify uncertainties, which made even the more complex stages of this work feel manageable and focused. His guidance strengthened not only the clarity of this thesis, but also my ability to articulate complex scientific ideas with confidence and structure. His steady support provided reassurance and refinement during moments of progress.

I would like to acknowledge Baystate Medical Center and its emergency department for providing an environment where clinical care and research intersect in meaningful and tangible ways. Conducting this work within a setting that directly serves patients, in which I directly work, reinforces the purpose behind every experiment, analysis, and revision. Being immersed in a space where urgency, compassion, and scientific research coexist has shaped my academic and professional development.

Lastly, I am deeply grateful to my family, my mother especially, and friends for their constant support, patience, and belief in me. They have stood beside me through every stage of this journey, through the long nights of writing or working, the early mornings of revision, the moments of stress, and the quiet persistence required to continue moving forward. Their encouragement was never loud or performative, but steady and unwavering. They believed in my ability to complete this work. Their reassurance, sacrifice, and understanding created a safe space for me to focus, to grow, and to persevere. That quiet confidence they placed in me has been a powerful foundation beneath every step of this process, and I carry it with me far beyond the completion of this thesis.

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Chapter I.

Introduction

Sepsis remains a leading cause of morbidity and mortality worldwide and is defined as the clinical response to infection that results in life-threatening organ dysfunction. A central component of this response is the activation of inflammatory pathways intended to eliminate invading pathogens. Inflammation is necessary for controlling infection; however, it can also cause damage to healthy tissues. To limit this collateral injury, counter-regulatory anti-inflammatory pathways are simultaneously activated. Under normal conditions, these pro- and anti-inflammatory responses remain balanced, allowing effective pathogen clearance while preserving tissue integrity. In sepsis, this balance can become disrupted. In some cases, the inflammatory response becomes excessive and hyperinflammatory, leading to widespread tissue injury and organ dysfunction.

Understanding how these dysregulated immune responses emerge during infection requires experimental systems capable of capturing the complex biological interactions that occur within human blood. Immune cells, plasma proteins, coagulation factors, and bacterial virulence determinants interact simultaneously within the vascular compartment, shaping the host response to infection. Studying these interactions in physiologically relevant systems is therefore essential for understanding the mechanisms that contribute to sepsis pathophysiology.

Clinical Burden of Sepsis

Sepsis represents one of the most significant challenges in modern critical care medicine. Rather than resulting solely from uncontrolled pathogen proliferation, sepsis arises from complex interactions between invading microorganisms and host immune, coagulation, and metabolic systems. Early hyperinflammatory responses may coexist with or transition into profound immune suppression, creating a dynamic and heterogeneous disease course. This duality has complicated the development of effective therapeutics and emphasizes the importance of mechanistically faithful experimental models (Bouwman et al., 2025; Nemzek et al., 2008).

Human studies have demonstrated that sepsis is characterized by widespread alterations in cytokine signaling, leukocyte function, complement activation, and coagulation cascades. These processes are tightly interconnected within the vascular compartment, making whole blood a uniquely relevant biological context for studying sepsis-associated immune phenomena. Ex vivo systems that preserve these interactions provide an opportunity to interrogate sepsis biology in a controlled yet physiologically meaningful manner (Barratt-Due et al., 2010; Brekke et al., 2008).

These complex immune dynamics highlight the need for experimental systems capable of modeling the connection between host immune response and invading pathogens in physiologically relevant environments.

Limitations of Existing Experimental Models

Traditional approaches to studying sepsis have relied heavily on murine models or simplified ex vivo systems using single immune cell populations. While these models have yielded mechanistic understanding, they are limited in their ability to capture the

complexity of human immune responses. Species-specific differences in immune signaling, coagulation pathways, and leukocyte behavior constrain the translational relevance of animal models (Nemzek et al., 2008).

Similarly, assays using purified neutrophils or monocytic cell lines remove critical components of the blood milieu, including platelets, erythrocytes, complement proteins, and soluble mediators that shape inflammatory responses. As a result, these simplified systems may fail to capture regulatory interactions that occur in vivo (Duffy et al., 2014).

Human whole blood models offer a promising alternative because they preserve the full cellular environment and plasma components that shape immune responses. However, developing a reproducible, high-throughput whole blood assay presents several technical challenges. Variability in experimental readouts—such as inconsistent signal intensity, high coefficients of variation, or limited separation between control and stimulated samples—can complicate interpretation. Determining which outcome measures most accurately reflect biologically meaningful immune responses therefore remains an important consideration when developing whole blood experimental systems.

Red Blood Cell Hemolysis in Severe Sepsis

Red blood cell (RBC) hemolysis has been described as a potential marker of severe bacterial infection and may reflect toxin-mediated host injury in the setting of sepsis. Hemolysis results from disruption of the erythrocyte membrane, leading to the release of free hemoglobin and intracellular contents into the extracellular space. In addition to serving as a marker of cellular damage, this process may contribute to

downstream inflammatory and vascular effects, including oxidative stress and endothelial dysfunction.

Staphylococcus aureus produces several hemolysins, including alpha, beta, and gamma toxins, which vary in their ability to lyse host cells. However, the susceptibility of erythrocytes to these toxins is species dependent. Many commonly used hemolysis assays rely on sheep or rabbit red blood cells, which are more sensitive to certain *S. aureus* hemolysins. As a result, these models may not accurately reflect hemolytic activity in human blood.

In this study, hemolysis was evaluated as a potential functional readout of severe sepsis within a human whole blood model. However, it was not known whether the bacterial strains used produced hemolysins at sufficient levels to induce detectable hemolysis in human RBCs. In addition, direct quantification of hemolysins was not performed due to the lack of a commercially available ELISA assay at the time of experimentation. Therefore, a functional hemolysis assay was used to assess red blood cell membrane disruption.

While functional assays provide a practical approach to measuring hemolysis, they may be limited in sensitivity, particularly when applied to human erythrocytes. These limitations highlight the challenges of using hemolysis as a surrogate marker of severe sepsis in human whole blood systems. Despite this, evaluating hemolysis remains important for understanding toxin-mediated effects and for determining whether it can serve as a complementary outcome measure alongside immune-based readouts such as NETosis.

Neutrophil Extracellular Traps in Sepsis Pathophysiology

Neutrophil extracellular traps (NETs) are web-like structures formed when neutrophils release decondensed chromatin into the extracellular space along with antimicrobial proteins such as myeloperoxidase and neutrophil elastase. The DNA backbone serves as a scaffold capable of trapping pathogens while associated proteases and antimicrobial proteins contribute to microbial killing.

NET formation, often referred to as NETosis, is intended to function as a host defense mechanism by limiting pathogen dissemination. However, in the setting of sepsis, excessive or uncontrolled NET formation can contribute to tissue injury. The extracellular DNA and associated histones are highly inflammatory and can activate nearby endothelial cells (Hoppenbrouwers et al., 2018).

NET-associated proteases and histones can also disrupt endothelial integrity, increasing vascular permeability. In addition, NET structures can provide a surface that supports platelet adhesion and activation of coagulation pathways, promoting microvascular thrombosis. When this process becomes widespread, it contributes to endothelial dysfunction, impaired tissue perfusion, and ultimately organ dysfunction.

NETosis is regulated by complex intracellular signaling pathways and is influenced by extracellular cues present in whole blood, including platelet activation, coagulation factors, and bacterial virulence determinants. These dependencies highlight the importance of experimental systems that preserve the physiological environment in which NET formation occurs.

NETs in Bacterial Sepsis

Bacterial pathogens vary in their ability to induce NET formation depending on their structural components and virulence factors. Gram-negative bacteria may stimulate NETosis through outer membrane components such as lipopolysaccharide, whereas Gram-positive organisms often rely on secreted toxins and surface-associated proteins to activate neutrophils.

In the context of Staphylococcus aureus bacteremia, NET formation has been associated with disease severity and vascular complications. Importantly, bacterial-induced NETosis is not solely dependent on soluble pathogen-associated molecular patterns. Direct contact between bacteria and neutrophils, as well as engagement of Fc receptors, complement receptors, and platelet-derived signals, contributes to NET induction (Hoppenbrouwers et al., 2018).

These interactions are naturally disrupted in plasma-free or cell-isolated systems, reinforcing the importance of whole blood-based experimental models.

Bacterial Determinants of NET Formation

Among bacterial pathogens, Staphylococcus aureus is a prominent cause of bloodstream infection and sepsis. This organism possesses multiple surface-associated and secreted proteins that modulate host immune pathways and neutrophil function (Hoppenbrouwers et al., 2018).

Protein A, a cell wall-anchored immunoglobulin-binding protein, has been identified as an important mediator of NET induction. Experimental studies have demonstrated that strains expressing high levels of Protein A elicit strong NET responses,

whereas strains with low endogenous expression induce minimal NETosis unless Protein A is supplemented exogenously.

This strain-dependent variability provides a biological axis for validating NETosis assays. A functional experimental platform should therefore be capable of distinguishing NET responses driven by endogenous Protein A from those requiring exogenous supplementation.

Whole Blood Models of Infection

Ex vivo whole blood models have been used to study cytokine production, complement activation, and bacterial survival. These systems preserve cellular diversity and soluble mediators, enabling more faithful recapitulation of in vivo immune responses (Duffy et al., 2014; Barratt-Due et al., 2010).

However, whole blood assays are sensitive to technical variables, including anticoagulant choice, incubation conditions, and readout methodology. Without careful optimization, these factors can introduce artifacts that obscure biological interpretation. Rigorous validation is therefore required to ensure reproducibility and physiological relevance.

Anticoagulants as Modulators of Immune Function

Anticoagulants are necessary to prevent clot formation during ex vivo blood assays but are not biologically inert. Chelating anticoagulants such as EDTA sequester divalent cations, disrupting calcium-dependent signaling pathways critical for leukocyte activation (Ashbrook et al., 2015; Akorsu et al., 2023).Heparin preserves divalent ions but can interact with extracellular chromatin structures and inflammatory mediators.

Hirudin, a direct thrombin inhibitor, prevents coagulation without chelating divalent ions and may therefore preserve physiological signaling pathways more effectively (Duvigneau et al., 2013).

High-Throughput NETosis Assays

There are several methods for measuring NETosis. The most common methods use fluorescence-based microscopy to image NETs in cell cultures. While this is a sensitive way to detect NETosis, it is challenging to accurately quantify the amount of NETosis. A commercial ELISA kit has been developed; but it has not been widely used in a whole blood model. Additionally, like most ELISA kits, it cannot be easily used to measure temporal kinetics.

Recent methodological advances have enabled kinetic, high-throughput quantification of NET formation using cell-impermeant DNA dyes. These assays facilitate systematic evaluation of experimental variables and allow measurement of NETosis dynamics over time (Zukas et al., 2024). When adapted appropriately, high-throughput NETosis platforms can provide efficient and reproducible measurement of NET formation under controlled conditions.

Rationale for a Whole Blood High-Throughput NETosis Platform

Fluorescence-based detection of extracellular DNA has enabled kinetic measurement of NET formation in a microplate format has been described by Zukas et al. utilized defined inflammatory and immune-modulating proteins to induce NETosis under controlled conditions.

While this approach provides a standardized method for quantifying NET kinetics, their model used downstream mediators of infection rather than the initiating

event itself. In clinical sepsis, NET formation occurs in response to live pathogens and their associated virulence factors, not isolated cytokines. Therefore, adapting this platform to incorporate bacterial stimulation allows evaluation of NETosis in a context that more closely reflects early host–pathogen interactions.

Reductionist in vitro systems that rely on purified neutrophils offer tight experimental control but remove plasma proteins, platelets, erythrocytes, and other immune cells that contribute to immune regulation. In contrast, in vivo animal models preserve physiological complexity but limit experimental control and make systematic parameter testing difficult. A whole blood, high-throughput system provides an intermediate approach: it maintains cellular and plasma components while allowing controlled manipulation of bacterial strain, concentration, and environmental variables within a reproducible format.

Implementation of such a system requires careful methodological consideration. Whole blood must be anticoagulated to prevent clot formation during incubation and measurement. However, anticoagulants can alter immune signaling pathways, affect coagulation–immune interactions, or influence baseline activation states. Similarly, bacterial growth conditions may influence virulence factor production, and fluorescent dyes must be used at concentrations that preserve cell viability while maintaining sufficient detection sensitivity. These variables are not simply technical details; they directly influence whether measured NET responses reflect biological activation or experimental artifact. Careful optimization is therefore essential for establishing a platform that can be used to study pathogen-induced NETosis and potentially inform strategies for modulating excessive NET formation in severe infection.

Objectives and Hypotheses

The overarching objective of this research was to determine if S. aureus can induce measurable changes in an ex vivo human whole blood model that are seen in clinical severe sepsis. Specifically, this work sought to:

1. Determine if S. aureus induces hemolysis in human whole blood by measuring cell-free heme using absorbance spectroscopy.
2. Determine if S. aureus induces NETosis in human whole blood using a commercially available ELISA assay.
3. Determine if S. aureus induces NETosis in human whole blood by adapting a published high-throughput kinetic model using a plate-based florescence assay.

It was hypothesized that S. aureus can induce changes in an ex vivo whole blood model that is similar to what is seen in severe sepsis.

Chapter II.

Materials and Methods

The following chapter describes the experimental design and methodological approaches used to develop and evaluate the human whole blood sepsis model. Emphasis was placed on establishing reproducible conditions for measuring bacterial growth characteristics, virulence factor production, and host response outcomes, including hemolysis and NETosis. Because whole blood systems are highly sensitive to technical variables, careful consideration was given to factors such as anticoagulant selection, bacterial growth conditions, and assay configuration to ensure that observed effects reflected biological responses rather than experimental artifact.

Study Design Overview

This study aimed to develop an ex vivo human whole blood sepsis model using bacterial stimulation. Experiments were designed to assess bacterial growth characteristics and quantify bacterial virulence factor production. Hemolysis and NETosis were chosen to be the primary readout measures. A series of experiments were conducted to evaluate different growth mediums, anticoagulants, and SYTOX Green concentration.

Human Blood Collection and Handling

Human whole blood samples were obtained from a single healthy volunteer. Blood was collected in anticoagulant-containing tubes to prevent clot formation during experimental procedures. Standard blood collection vacutainers containing ethylenediaminetetraacetic acid (EDTA), Acid Citrate Dextrose Solution B (ACD-B), and Heparin were used. Hirudin (Aniara Diagnostica) containing vacutainers were created by placing either 400 antithrombin units (ATU) or 525 ATU in empty blood collection vacutainers being careful not to disrupt their vacuum. Following collection, samples were gently mixed to ensure uniform anticoagulation and processed within a defined time window to preserve physiological cell function.

Blood samples were used directly in whole blood assays or diluted under defined experimental conditions depending on the requirements of individual experiments.

Bacterial Strains and Culture Conditions

Methicillin-sensitive Staphylococcus aureus (MSSA) and methicillin-resistant Staphylococcus aureus (MRSA) strains were used for bacterial stimulation experiments. The specific strains used were obtained from a previous clinical study of skin and soft tissue abscesses. As such, it is not known whether they are representative of strains seen in septic patients.

Bacterial cultures were grown on agar plates containing Colorex™ Staph aureus growth medium and Colorex™ MRSA growth medium (NELAnalytical) overnight to confirm that they were MSSA and MRSA strains. Single colonies were then grown in culture media. Growth media included tryptic soy broth (TSB, Teknova) and Iscove's modified Dulbecco's medium (IMDM, Aniara Diagnostica) buffered with 25 mM

concentration of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). IMDM containing two glucose concentrations were used – 2 g/L (IMDM-L) and 4.5 g/L (IMDM-H). These media were selected to evaluate the influence of growth conditions on bacterial growth kinetics and virulence factor production.

Standard laboratory culture techniques for Staphylococcus aureus were used to maintain bacterial strains and generate experimental cultures (Missiakas and Schneewind, 2013). Bacterial cultures were incubated at 37° C in room air overnight to reach plateau phase. 100 uL was then incubated in 10 mL of the final medium.

Experiment 1 – Bacterial Growth Kinetics and Protein A Production

The purpose of this experiment was to determine the best medium to grow the bacteria in terms of bacterial growth rate and amount of Protein A produced.

MSSA and MRSA were grown in human plasma, TSB, IMDM-L and IMDM-H. Samples were taken every two hours to measure their optical density at 600 nm (OD600). Results are shown in Figure 1. An aliquot from the MRSA culture was taken at 8-hours, centrifuged at 2500 × g for 10 minutes, filtered through 0.45 µm and 0.22 µm filters, and then frozen at -80º C for measurement of Protein A the following week.

Protein A was measured using a commercial ELISA kit (Cygnus Technologies, catalog F600). The frozen specimens were thawed on an ice bath and processed using the manufacturer's recommendations. The expected concentration was not known so 1:1 serial dilutions were performed to yield 4 dilutions: 100%, 50%, 25%, and 12.5%. The kit provides standards. A spike recovery was performed in TSB. Specimen measurements were performed in triplicate, standards were measured in duplicate.

Experiment 2 – Measurement of NETosis using Nu.Q® Discover H3.1

Fresh whole blood was collected in EDTA and ACD-B vacutainers and divided into 2 mL aliquots. MRSA cultures incubated in TSB for 12 hours. Undiluted and 1:9 diluted cultures were divided into 1 mL aliquots. 1 mL aliquots of TSB were used as negative controls. The 1 mL MRSA (or TSB) aliquots were added to the 2 mL whole blood aliquots and placed in a rotisserie incubator. At 1, 2, 4 and 10 hours specimens were taken out of the incubator, centrifuged at 2500 × g for 15 minutes and the supernatant was aspirated, being careful not to disturb the buffy coat, and frozen at -80º C. The NETosis assays were performed the following week.

NETosis was measured using a commercial ELISA kit, Nu.Q® Discover H3.1, (Volition, Inc). The standard protocol calls for centrifuging the specimens at 14,000 × g for 2 minutes. The purpose of this is to pellet out bacteria and debris. Our centrifuge does not spin that fast. So, we evaluated three methods: 1) centrifugation at 2500 x g for 11 minutes, 2) centrifugation at 9750 × g for 3 minutes, and 3) filtering the plasma through a 0.22 µM filter. The above times were calculated to be equivalent to 14,000 × g for 2 minutes. All specimens were assayed in duplicate.

Experiment 3 – Hemolysis

MRSA cell culture was grown overnight in IMDM-L and IMDM-H. TSB was not used because TSB has high absorbance at 405 nm which will interfere with the assay. The cultures were then centrifuged at 2500 × g for 10 min and passed through 0.45 and 0.22 µm filters. 1% Triton X-100 (Avantor) was used as a positive control. 100 uL of the supernatant (or Triton X-100) was then combined with 100 uL of a 0.5% RBC solution

on a 96-well plate and gently mixed. The plate was then incubated at 37 °C for 1 hour. Absorbance was then measured at 405 nm.

The experiment was then repeated using a higher percentage of RBC, 1%, to see if that would improve the signal to noise ratio (SNR). Only IMDM-H was used for this experiment.

This experiment assay was adapted from the Ridder at al. 2021 and Muñoz-Planillo at al. 2009.

Experiment 4 – NETosis Using the Method Described by Zukas

Our initial experiment was to find the best vacutainer for collecting the blood for this method. Blood was collected in EDTA, Heparin and Hirudin (400 and 525 ATU) vacutainers. Phorbol 12-myristate 13-acetate (PMA, DiagnoCine) in phosphate buffered saline (PBS, DiagnoCine) was used to induce NETosis. A final concentration of 320 nM and 160 nM of PMA were used (0 nM PMA was used as the negative control). A final concentration of 7.5 µM of SYTOX Green (Avantor) was used.

SYTOX Green was added to the whole blood and then combined 1:1 by volume with the PMA and plated on a 96-well plate. It was gently mixed on an orbital shaker and then centrifuged at 1200 × g for 10 minutes. It was then placed in a plate reader (37 °C, gain 1000, 485/520 nm with top reading measurements taken every 20 minutes). Each condition was duplicated.

The experiment was then repeated using two concentrations of SYTOX Green (5.0 µM and 7.5 µM). Only Hirudin (525 ATU) was used as the anticoagulant. A final concentration of 150 nM of PMA was used (0 nM PMA was used as the negative control). Each condition was in triplicate.

The experiment was then repeated using MRSA and MSSA strains of bacteria, instead of PMA, to induce NETosis. SYTOX Green (5 μM) and Hirudin (525 ATU) were used. Each bacterial culture using two concentrations (full strength and 1:9 dilution) and each prepared three ways: 1) standard culture (containing bacteria and culture medium-IMDM), 2) bacteria washed in IMDM x 3 (to remove soluble toxins) and diluted in IMDM to yield the same bacterial concentration, and 3) washed bacteria combined with Protein A (final concentration 10 ng/μL). All specimens were run in duplicate.

Chapter III.

Results

The following sections discuss the major findings of this study.

Experiment 1 – Bacterial Growth Kinetics and Protein A Production

Bacterial growth varied depending on the culture medium used. Both MSSA and MRSA strains demonstrated the highest growth in tryptic soy broth (TSB), while the lowest growth was observed in human plasma. Cultures grown in IMDM-L and IMDM-H exhibited intermediate growth patterns with minimal differences between the two media.

Growth curves demonstrated that both MSSA and MRSA entered stationary phase at approximately eight hours of incubation. MRSA cultures exhibited slightly slower growth kinetics compared to MSSA under the same experimental conditions.

Results are shown in Figure 1

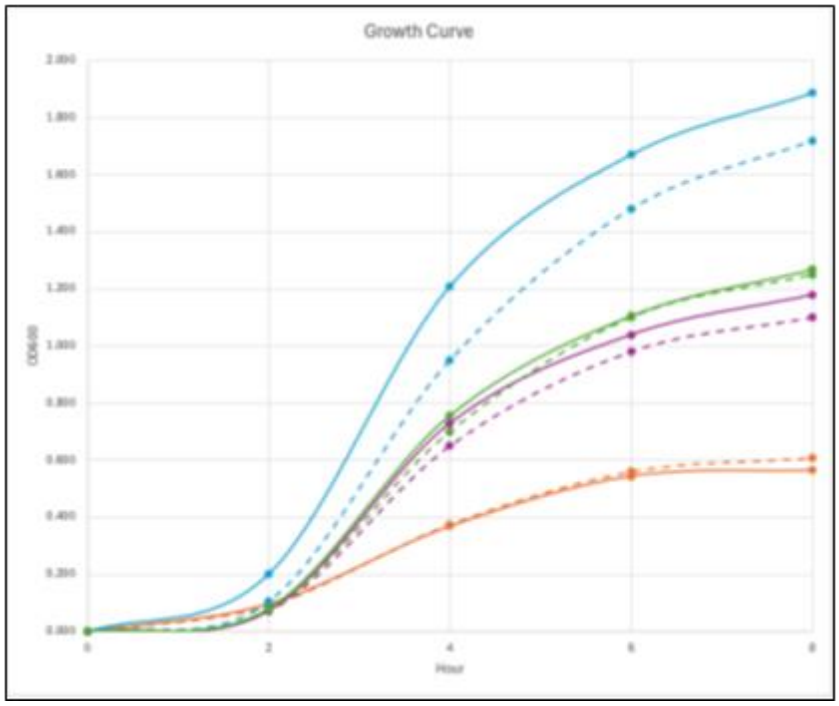

Figure 1. Bacterial Growth Curves of MSSA and MRSA in Different Media.

Optical density at 600 nm (OD600) was measured every two hours to evaluate growth kinetics of methicillin-sensitive Staphylococcus aureus (MSSA; solid lines) and methicillin-resistant Staphylococcus aureus (MRSA; dashed lines) cultured in tryptic soy broth (TSB; blue), IMDM-H (Green), IMDM-L (purple), and human plasma (orange). Both strains demonstrated highest growth in TSB and lowest growth in plasma. IMDM-H and IMDM-L supported intermediate growth with minimal difference attributable to glucose concentration. The exponential growth phase occurred between approximately 2 and 6 hours, with transition toward stationary phase by 8 hours. Examination of the growth curves demonstrated clear differences in bacterial proliferation across the tested media conditions. Both MSSA and MRSA exhibited the most rapid growth in tryptic soy broth (TSB), with optical density increasing sharply during the exponential growth phase between approximately two and six hours. Growth in IMDM-L and IMDM-H followed similar trajectories but with slightly reduced overall optical density values compared to TSB. In contrast, bacterial growth in human plasma remained substantially lower throughout the experiment, indicating limited proliferation under these conditions. While both strains followed comparable growth patterns, MRSA cultures displayed slightly slower growth kinetics relative to MSSA across most media conditions.

Protein A production was detected in bacterial cultures grown in all tested media. Standard curves generated for the ELISA assay demonstrated linearity across the tested

concentration range. Measured samples exceeded the highest standard concentration in several conditions, indicating high levels of Protein A expression.

At the 12.5% dilution level, cultures grown in TSB demonstrated higher relative Protein A levels compared to cultures grown in IMDM-L and IMDM-H. The lower measurement observed at the 100% dilution level in TSB cultures is consistent with a hook effect.

Results are shown in Figure 2 and Table 1.

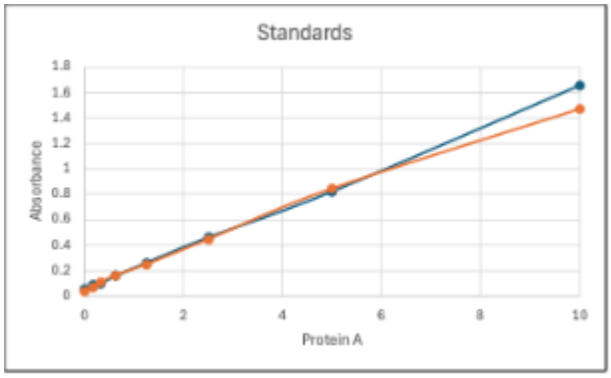

Figure 2. Protein A ELISA Standard Curve.

A standard curve was generated using known concentrations of Protein A according to the manufacturer's protocol. The Protein A ELISA standard curve demonstrated a consistent linear relationship between absorbance and Protein A concentration across the tested range. Replicate measurements produced minimal variability, indicating stable assay performance and reliable detection sensitivity. The linearity of the standard curve allowed relative quantification of Protein A levels in bacterial culture supernatants collected under different growth conditions

Table 1: Protein A in MRSA Specimen at 8-Hours

Dilutions	TSB			IMDM-L			IMDM-H		
	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
100%	2.57	0.07	3%	3.05	0.20	7%	3.03	0.11	4%
50%	3.12	0.04	1%	3.08	0.19	6%	3.01	0.07	2%
25%	3.13	0.02	1%	2.82	0.06	2%	2.86	0.15	5%
12.5%	2.82	0.06	2%	1.79	0.06	4%	1.77	0.02	1%

Protein A concentrations were measured in MRSA supernatants grown in TSB, IMDM-L, and IMDM-H at 8 hours. Samples were serially diluted (100%, 50%, 25%, 12.5%) and measured in triplicate. Mean absorbance, standard deviation (SD), and coefficient of variation (CV) are shown. All undiluted samples exceeded the upper limit of the standard curve, suggesting high Protein A concentrations. At the 12.5% dilution, relative absorbance values suggest higher Protein A levels in TSB compared to IMDM-based media. Variability remained low across replicates, as reflected by CV values generally below 10%. Protein A measurements demonstrated relatively consistent absorbance values across replicate wells, with coefficients of variation generally remaining below 10%. Undiluted samples frequently exceeded the upper limit of the standard curve, indicating high concentrations of Protein A in bacterial supernatants. Serial dilution of samples enabled measurement within the linear detection range of the assay and allowed comparison of relative Protein A levels across media conditions.

Experiment 2 – Measurement of NETosis using Nu.Q® Discover H3.1

NETosis measurements were evaluated using the Nu.Q® Discover H3.1 assay under multiple clarification conditions. Standard curves demonstrated linear behavior and replicate measurements showed consistent results across experimental replicates.

Results are shown in Figure 3.

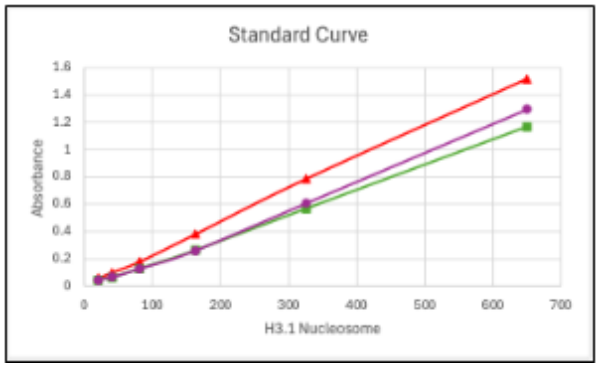

Figure 3. Standard Curve.

Standard curves for the Nu.Q® Discover H3.1 assay are shown following three sample clarification strategies: centrifugation at 2500 × g for 11 minutes (red), centrifugation at 9750 × g for 3 minutes (Green), and filtration through a 0.22 µm filter (purple). All three methods demonstrated strong linear relationships between nucleosome concentration and absorbance, with minimal variability between replicates. These findings indicate that the clarification method did not significantly affect standard curve performance.

Negative control wells without bacterial stimulation demonstrated higher absorbance values than several experimental conditions containing bacteria. Higher absorbance indicates greater NETosis. In addition, diluted bacterial cultures produced higher absorbance values compared to undiluted cultures.

Clarification methods including centrifugation and filtration produced similar measurement patterns across experimental conditions. Across all conditions tested, there was no consistent separation between negative control wells and bacterial stimulation conditions.

Results are shown in Figure 4.

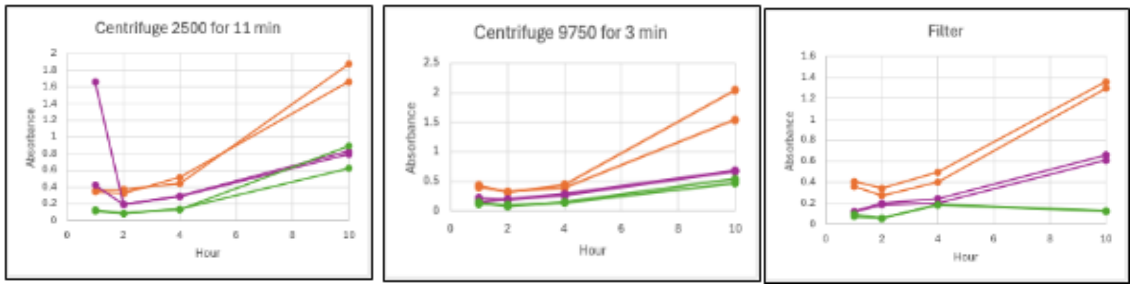

Figure 4. NETosis.

Whole blood samples incubated with no bacteria (orange), 1:9 diluted bacteria (purple), or undiluted bacteria (Green) were analyzed at 1, 2, 4, and 10 hours following clarification by centrifugation at 2500 × g for 11 minutes (left), centrifugation at 9750 × g for 3 minutes (center), or 0.22 µm filtration (right). Across all clarification methods, negative controls exhibited higher absorbance than bacterial conditions, and diluted bacteria demonstrated higher absorbance than undiluted bacteria. Replicates were consistent within each condition, and standard curves were linear. Despite technical consistency, the lack of expected biological separation indicates that this assay configuration did not reliably detect NETosis in this whole blood model.

Experiment 3 – Hemolysis Assay

Hemolysis assays were performed to evaluate red blood cell stability under experimental conditions. At a red blood cell concentration of 0.5%, hemolysis levels were approximately 2% in both IMDM-L and IMDM-H conditions. Coefficients of variation ranged between 6% and 13% across replicates. Signal-to-noise ratios ranged between 6 and 8.

The results are in Table 2.

Table 2. Hemolysis of Red Blood Cells Induced by MRSA Supernatants in IMDM-L and IMDM-H.

	IMDM-L	IMDM-H
% Hemolysis	2%	2%
CV	6%	13%
SNR	8	6

Hemolysis was measured by absorbance at 405 nm following incubation of filtered MRSA supernatants with a 0.5% RBC suspension. Percent hemolysis, coefficient of variation (CV), and signal-to-noise ratio (SNR) are shown. Both IMDM-L and IMDM-H conditions demonstrated low percent hemolysis (2%) with moderate variability and limited signal-to-noise separation. Neither growth medium produced hemolysis levels sufficient to meet predefined performance criteria.

When the red blood cell concentration was increased to 1%, hemolysis levels increased modestly to approximately 5–8%. Coefficients of variation remained near 8%, while signal-to-noise ratios decreased to approximately 1.5–1.7.

Increasing the red blood cell concentration did not substantially improve signal-to-noise separation between experimental conditions.

The results are in Table 3.

Table 3. Effect of Red Blood Cell Concentration on Hemolysis Assay Performance.

	RBC 0.5%	RBC 1%
% Hemolysis	8%	5%
CV	8%	8%
SNR	1.5	1.7

Hemolysis was measured following incubation of MRSA supernatant with either 0.5% or 1% RBC suspensions. Percent hemolysis, coefficient of variation (CV), and signal-to-noise ratio (SNR) are presented. Increasing RBC concentration did not improve assay

sensitivity or signal-to-noise ratio. Variability remained similar between conditions, and overall dynamic range remained limited, indicating that modification of RBC concentration did not enhance assay performance.

Experiment 4 – NETosis Using the Method Described by Zukas

Anticoagulant selection influenced NETosis kinetics observed in whole blood assays. Samples collected using EDTA demonstrated elevated baseline fluorescence in negative control conditions. Heparin-treated samples also exhibited increased background fluorescence relative to other conditions. In contrast, samples collected using hirudin demonstrated minimal baseline signal and clear fluorescence increases following stimulation with PMA.

NETosis kinetics demonstrated peak fluorescence at approximately 540 minutes following stimulation.

Results are shown in Figures 5.

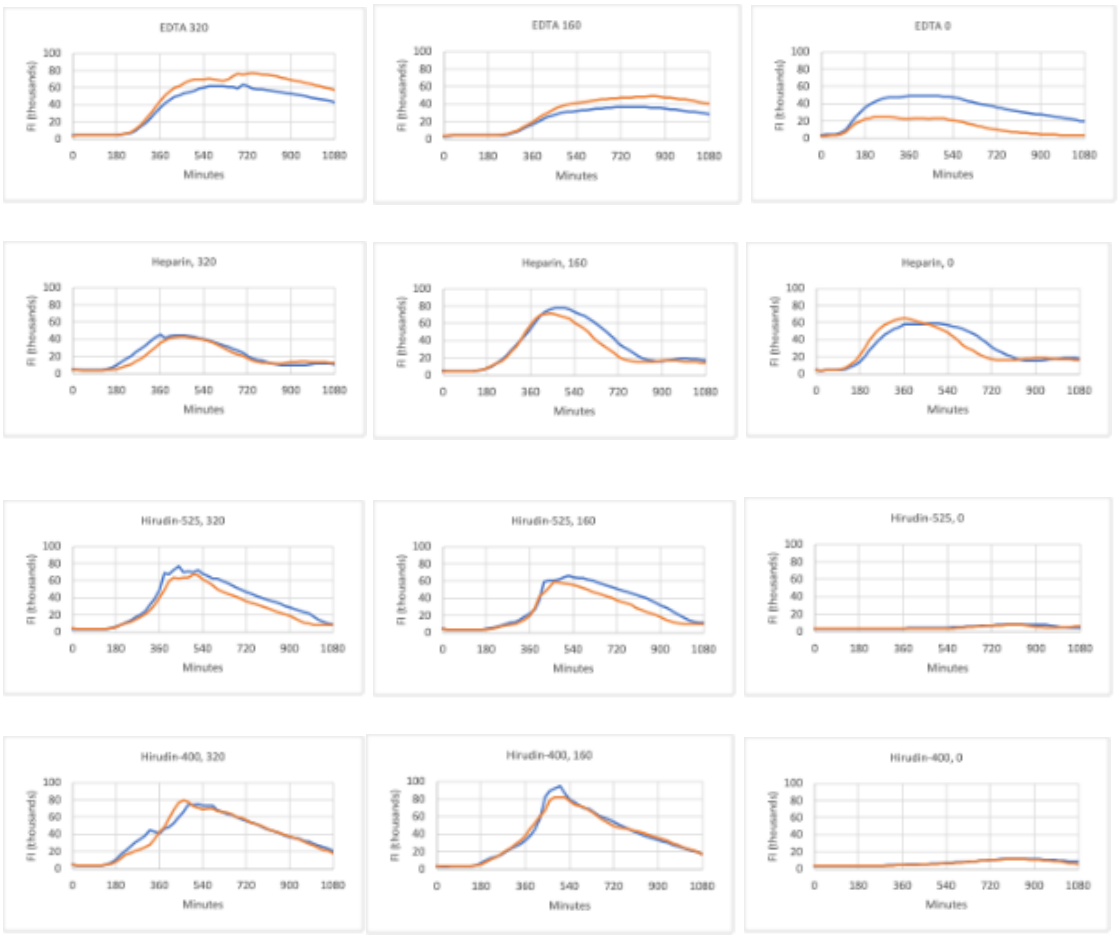

Figure 5. PMA Induced NETosis using Different anticoagulants.

Whole blood was stimulated with PMA and monitored for NET formation over 18 hours using SYTOX Green fluorescence. Samples were collected in EDTA, heparin, and hirudin (400 and 525 ATU) anticoagulants. EDTA-supported conditions demonstrated sustained NETosis peaking at approximately 540 minutes (9 hours), followed by a gradual decline; however, measurable NETosis was also observed in the absence of PMA. Heparin conditions similarly demonstrated elevated fluorescence in negative controls. In contrast, hirudin-treated samples showed minimal NETosis in negative controls and clear PMA-dependent induction, with peak fluorescence at approximately 540 minutes followed by a more rapid decline. These findings indicate that anticoagulant choice significantly influences both baseline signal and NETosis kinetics.

Comparison of SYTOX Green concentrations at 7.5 µM and 5.0 µM produced similar fluorescence profiles and did not substantially alter detection sensitivity.

Results are shown in Figures 6.

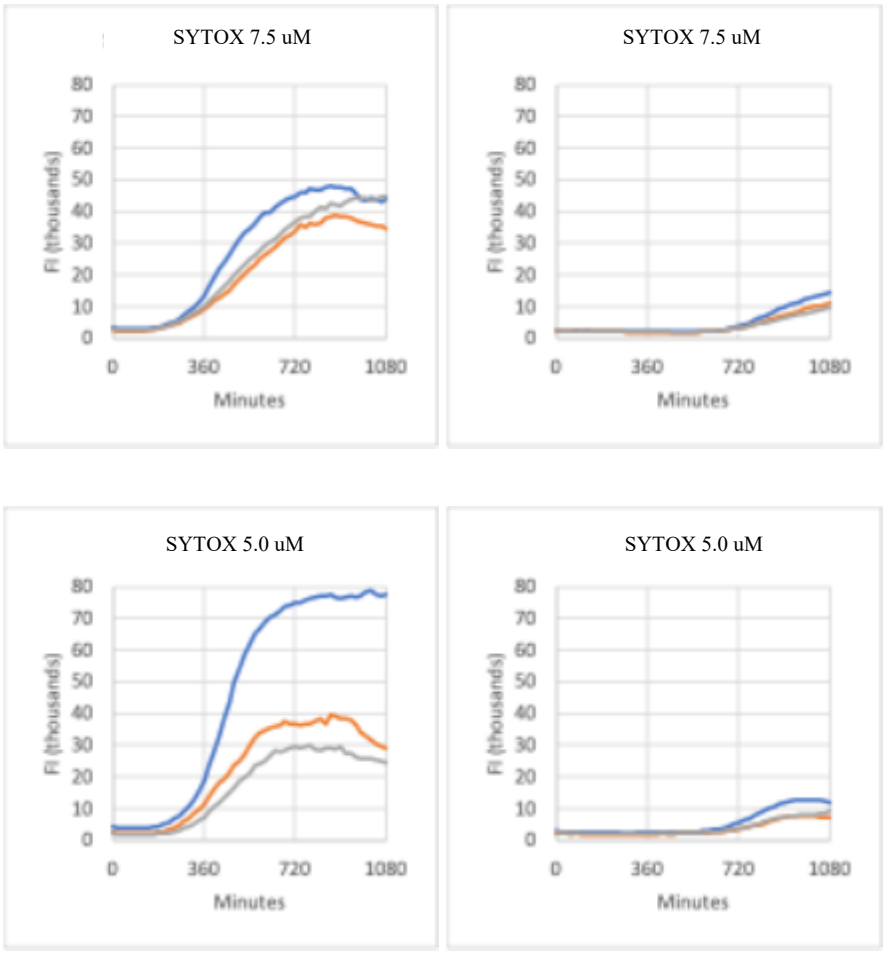

Figure 6. PMA-Induced NETosis Using 5.0 µM and 7.5 µM SYTOX Green in Hirudin Anticoagulated Whole Blood.

Whole blood collected in hirudin was stimulated with PMA and monitored for NET formation using either 7.5 µM or 5.0 µM SYTOX Green. PMA concentration was 150 nM or 0 nM. Each condition was performed in triplicate. Fluorescence intensity increased over time in PMA-stimulated samples, with minimal signal in negative controls. No substantial difference in NETosis kinetics or peak fluorescence was observed between the two SYTOX concentrations. Unlike prior experiments, fluorescence did not rapidly decline following peak signal. Variability between replicates was minimal, with one outlier likely attributable to technical handling. These findings suggest that 5.0 µM

SYTOX Green provides comparable NET detection while potentially reducing DMSO-associated toxicity.

MRSA stimulation produced stronger NETosis signals compared to MSSA when whole culture suspensions were used. Dilution of bacterial cultures reduced NETosis signal intensity. Washed bacterial preparations produced lower NETosis signals compared to whole culture suspensions.

Addition of exogenous Protein A increased NET formation under several experimental conditions.

Results are shown in Figures 7.

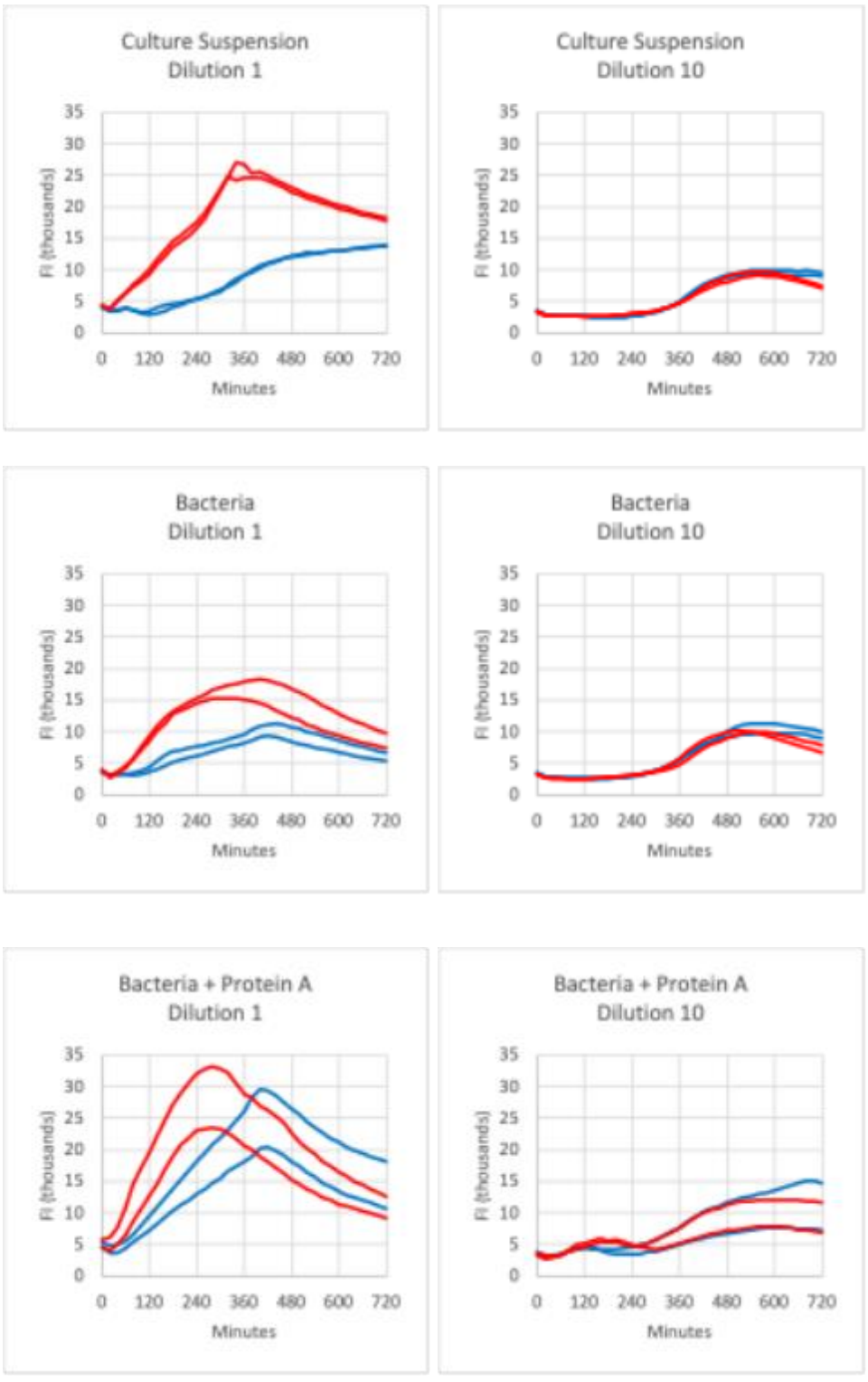

Figure 7. Bacteria Induced NETosis

Whole blood was stimulated with MRSA (red) or MSSA (blue) using culture suspension, washed bacteria, or washed bacteria supplemented with Protein A at two concentrations (Dilution 1 and Dilution 10). MRSA induced greater NETosis than MSSA when whole culture suspension was used, with reduced signal upon dilution. Washed bacteria produced lower NETosis compared to culture suspension, while addition of Protein A increased NET formation. These findings are consistent with prior reports identifying Protein A as a contributor to NET induction.

Chapter IV.

Discussion

The overall objective of this study was to develop an ex vivo human whole blood model that has measurable cellular changes, similar to what is seen in septic patients, when exposed to Staphylococcus aureus. The specific objectives of the initial experiments were to determine the best culture medium for growing bacteria and to produce soluble toxins. The specific objects of the subsequent experiments were to determine if we could detect two potential outcome measures, hemolysis and NETosis, for our model after optimizing anticoagulant type as well as percentage of red blood cells for the hemolysis assay and concentration of SYTOX Green for the NETosis assay. The central goal was to develop a human model that can be used to study bacterial and host characteristics responsible for the development of severe sepsis and to evaluate novel therapeutics.

Whole blood experimental systems preserve interactions between immune cells, plasma proteins, and microbial products that contribute to host immune responses during infection. Previous studies have shown that whole blood stimulation models provide an effective framework for studying inflammatory responses in physiologically relevant conditions (Duffy et al., 2014; Barratt-Due et al., 2010). By maintaining the interactions between leukocytes, complement proteins, and circulating mediators, these systems allow investigation of immune responses that are often difficult to reproduce in simplified single-cell culture environments.

Together, the findings from this study demonstrate that careful methodological optimization is essential when designing whole blood platforms intended to measure complex immune responses such as NET formation.

It is important to interpret our results cautiously. These were preliminary proof-of-concept experiments. They were not designed to detect statistically significant differences.

Overview of Findings

The findings of this study demonstrate that a human whole blood model can be used to detect bacterial-induced NETosis in response to *Staphylococcus aureus*, while hemolysis was not observed to a meaningful degree under the tested conditions. MRSA consistently produced a greater NETosis response compared to MSSA, and this response was influenced by the presence of soluble bacterial components, including Protein A. These findings support the use of NETosis as a measurable and biologically relevant readout of host response in this model.

In contrast, hemolysis did not provide a sensitive or reliable outcome measure in this system. Despite optimization of assay conditions, including red blood cell concentration and bacterial growth medium, only minimal hemolysis was detected. This suggests that either the bacterial strains used did not produce hemolysins at sufficient levels, or that human red blood cells are less sensitive to these toxins under the experimental conditions used.

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Together, these findings demonstrate that NETosis can be reliably measured in a human whole blood system using *S. aureus* as a stimulant, while hemolysis may not serve as a practical readout under these conditions. These results also highlight the importance of careful methodological optimization when designing whole blood platforms intended to measure complex immune responses.

Interpretation of Bacterial Growth and Protein A Expression

Characterization of bacterial growth kinetics and virulence factor production represented an important preliminary step in the development of the model. Bacterial growth conditions influence metabolic activity, toxin production, and surface protein expression, all of which can directly influence host immune activation. Factors that support bacterial growth may suppress other biochemical pathways such as toxin production; however, more bacteria should yield more toxin [REF].

S. aureus produces several toxins including a variety of hemolysins as well as Protein A. We only had access to a Protein A ELISA kit. There is a pre-built ELISA kit for alpha hemolysin but it was not available at the time we were running the experiments.

Both MSSA and MRSA strains demonstrated robust growth in tryptic soy broth, and poor growth in human plasma. Intermediate growth patterns were observed in IMDM-based media. There did not appear to a difference between the low-glucose

IMDM and high-glucose IMDM media. These findings are consistent with previous observations that bacterial growth conditions strongly influence virulence gene expression and host–pathogen interactions (Missiakas and Schneewind, 2013).

Protein A expression was detected across all tested culture conditions, although relative abundance varied depending on growth medium. Protein A is a cell wall-anchored immunoglobulin-binding protein known to interact with host immune components and modulate inflammatory signaling pathways. Prior studies have shown that Protein A can influence neutrophil activation and NET formation during Staphylococcus aureus infection (Hoppenbrouwers et al., 2018).

The experiment confirms that our MRSA strain produces Protein A. However, Protein A levels were above the highest standard even when diluted 8-fold. It is difficult to estimate how much greater we would need to dilute the specimens because there is a known hook effect at very high levels with this assay. Additionally, we only measured Protein A in bacterial strain, MRSA, and not MSSA. We also only measured Protein A at one time point. Finally, we did not measure Protein A when using plasma as the growth medium. To better understand the toxicity of our bacteria we will need to repeat the experiment under these additional conditions.

Measuring NETosis in Whole Blood

We attempted to measure NETosis using two methods, the commercial Nu.Q® Discover H3.1 ELISA kit and SYTOX Green. The H3.1 ELISA kit measures cell-free chromatin fragments (DNA wrapped around histone H3.1) in plasma. SYTOX Green is an impermeant fluorescent dye that binds to extracellular, cell-free, DNA.

We were unable to get a signal using the ELISA kit in specimens with inoculated with high concentrations of bacteria. This may occur if the nucleosome is degradation by nucleases. The high readings in our negative controls may reflect cell damage due to handling the blood specimens. The specimen handing technique for the SYTOX Green experiments was very different from the specimen handling for the H3.1 ELISA experiments.

We did not measure other markers in the same specimens used for the H3.1 ELISA which could have helped explain these results. However, the experiments using SYTOX Green were positive. This is the pattern that one would expect with high nucleosome degradation seen with nucleases. This could have been further confirmed by measuring myeloperoxidase (MPO) and/or neutrophil elastase (NE). Under conditions of nucleosome degradation, one would expect elevated MPO and NE in addition to cell free DNA.

Additional assays could have been added to experiments that used the SYTOX Green such as either the H3.1 ELISA, citrullinated histones and/or MPO–DNA ELISA, in addition to MPO and NE, to confirm that SYTOX Green was detecting NETosis.

Hemolysis as a Measure of Severe Sepsis

Hemolysis was evaluated in this study as a functional readout of bacterial toxin activity and as a potential marker of severe sepsis. Hemolysis is not a universal feature of all bacterial infections but has been associated with more severe forms of disease, particularly in infections caused by toxin-producing organisms such as *Staphylococcus aureus*. The production of hemolysins is often linked to the accessory gene regulator (*agr*) system, which coordinates the expression of multiple virulence factors. As a result,

the presence of hemolytic activity may reflect a broader profile of toxin-mediated pathogenicity and could serve as an indicator of more severe infection.

In this study, we aimed to assess whether *S. aureus* produced sufficient hemolysins to induce detectable red blood cell lysis in a human whole blood system. Direct quantification of hemolysins was not performed, as a commercially available ELISA for alpha hemolysin was not accessible at the time of experimentation. Instead, a functional hemolysis assay was used to evaluate erythrocyte membrane disruption. This approach is commonly used in the literature; however, it is important to note that many hemolysis assays utilize sheep or rabbit erythrocytes due to their increased sensitivity to *S. aureus* hemolysins. In contrast, this study used human red blood cells, which may be less sensitive to these toxins and therefore limit the detectability of hemolytic activity.

MRSA cultures used for the hemolysis experiments were grown in IMDM-based media. Tryptic soy broth (TSB) was not used in these experiments due to its high background absorbance at 405 nm, which interferes with accurate measurement of free heme. Despite optimizing assay conditions, including modification of red blood cell concentration to improve signal detection, hemolysis remained minimal under all tested conditions. Decreasing red blood cell concentration did not improve assay sensitivity or signal-to-noise separation. (Ridder at al. 2021; Carpenter and Van Hank et al. 2024)

These findings suggest that either the bacterial strains used did not produce hemolysins at levels sufficient to induce measurable hemolysis in human erythrocytes, or that the assay lacked sensitivity in this biological context. While hemolysis may represent a marker of severe toxin-mediated infection, it may not be a reliable or broadly applicable

readout in human whole blood systems, particularly when modeling moderate or early-stage sepsis.

Future studies should focus on directly measuring hemolysin production using commercially available ELISA-based approaches when accessible. In addition, alternative methods for assessing red blood cell membrane integrity may provide complementary information regarding toxin-mediated effects. However, it remains unclear whether these measurements would correlate with clinically relevant indicators of disease severity.

SYTOX Green Concentration and Potential Cytotoxic Effects

Optimization of SYTOX Green concentration demonstrated that both 7.5 μM and 5.0 μM concentrations produced similar fluorescence profiles. These findings suggest that lower dye concentrations may be sufficient for reliable detection while minimizing potential cytotoxic effects associated with solvent components.

Together, these results support the use of fluorescence-based extracellular DNA detection as a practical and reproducible approach for monitoring NETosis kinetics in whole blood.

Anticoagulant Choice as a Determinant of NETosis Outcomes

We observed that anticoagulant selection may have impacted NETosis measurements. Whole blood collected using EDTA demonstrated elevated baseline fluorescence even in the absence of stimulation. Heparin-treated samples also exhibited increased background fluorescence relative to other conditions. In contrast, hirudin-treated samples produced minimal baseline signal and clearer separation between stimulated and unstimulated conditions.

These findings are consistent with previous studies demonstrating that anticoagulants can influence immune signaling pathways and cytokine production in whole blood experimental systems (Ashbrook et al., 2015; Akorsu et al., 2023). EDTA functions as a chelating agent that binds divalent cations such as calcium and magnesium. Because these ions are essential for numerous cellular signaling pathways, their removal can disrupt neutrophil activation pathways and alter immune responses.

Heparin, while preserving divalent cations, interacts with several extracellular proteins and inflammatory mediators. These interactions may influence chromatin structure or inflammatory signaling, potentially contributing to increased background signal in NETosis assays.

Hirudin acts as a direct thrombin inhibitor that prevents clot formation without chelating divalent ions or broadly interacting with inflammatory mediators. Previous studies have shown that hirudin preserves immune signaling pathways more effectively in whole blood systems (Duvigneau et al., 2013). Consistent with these observations, hirudin-anticoagulated samples in this study demonstrated minimal baseline NETosis and clearer stimulus-dependent responses.

SYTOX Green was used in this study as a fluorescent marker for extracellular DNA to quantify NETosis. However, an important consideration when using SYTOX Green is that it is dissolved in dimethyl sulfoxide (DMSO), which is known to have cytotoxic effects at higher concentrations. Therefore, increasing the concentration of SYTOX Green may inadvertently affect cell viability and influence NETosis measurements.

The initial concentration of 7.5 µM SYTOX Green was selected based on the methodology described by Zukas et al. However, this concentration approaches levels at which DMSO-related toxicity may become significant. Reducing the concentration of SYTOX Green would decrease cellular exposure to DMSO and potentially minimize cytotoxic effects. At the same time, lower dye concentrations may result in reduced fluorescence intensity, thereby decreasing the sensitivity of NETosis detection.

To evaluate this trade-off, SYTOX Green concentrations of 7.5 µM and 5.0 µM were compared. No substantial differences in fluorescence signal or NETosis kinetics were observed between the two conditions. These findings suggest that, within this range, the effects of DMSO toxicity and reduced signal sensitivity are likely minimal and do not significantly impact assay performance.

Further optimization may be required to fully define the optimal SYTOX Green concentration. Future studies should evaluate a broader range of concentrations to better characterize the balance between minimizing cytotoxic effects and maintaining sufficient detection sensitivity in whole blood systems.

Protein A as a Functional Determinant of NET Induction

Staphylococcus aureus expresses numerous virulence factors that influence host immune responses. Among these factors, Protein A has been shown to play an important role in modulating neutrophil activation and NET formation.

Protein A is a cell wall-anchored immunoglobulin-binding protein capable of interacting with host immune components. Experimental studies have demonstrated that

Protein A can promote NET formation through interactions with neutrophil receptors and immune signaling pathways (Hoppenbrouwers et al., 2018).

The observation that NET formation increased following addition of Protein A supports the role of this virulence factor in regulating neutrophil responses to S. aureus infection.

NETs in Sepsis Pathology

Although NET formation serves an important antimicrobial function, excessive or dysregulated NETosis has increasingly been implicated in the pathophysiology of sepsis. NET structures consist of extracellular chromatin decorated with antimicrobial proteins such as neutrophil elastase and myeloperoxidase. These structures function as physical scaffolds capable of trapping pathogens and preventing their dissemination.

However, the extracellular DNA and histones associated with NET structures possess potent inflammatory properties. NET components have been shown to activate endothelial cells and promote inflammatory signaling pathways (Hoppenbrouwers et al., 2018). Activation of endothelial cells can increase vascular permeability and disrupt endothelial barrier integrity.

NET structures can also promote platelet adhesion and activation of coagulation pathways. Histones and extracellular DNA released during NET formation contribute to thrombin generation and fibrin deposition. When these processes occur within the microvasculature, they may lead to the formation of microvascular thrombi.

Microvascular thrombosis represents a hallmark of severe sepsis and contributes to impaired tissue perfusion and organ dysfunction. Accumulation of NET structures

within small vessels may therefore represent a mechanistic link between immune activation and coagulation dysregulation during infection.

These observations illustrate the dual role of NETs as both antimicrobial defense mechanisms and potential contributors to inflammatory vascular injury during severe infection.

Implications for Studying Host–Pathogen Interactions

The development of a whole blood NETosis assay platform provides an experimental system capable of investigating interactions between bacterial pathogens and host immune responses within a physiologically relevant environment.

Because the system preserves leukocytes, erythrocytes, plasma proteins, and coagulation factors, it allows investigation of immune responses that depend on these interactions. This environment more closely reflects the biological conditions encountered during bloodstream infection.

Such a platform may be particularly useful for studying the role of bacterial virulence factors in immune activation. The results observed in this study suggest that Protein A contributes to NET induction under certain conditions. Similar approaches could be used to evaluate additional virulence determinants expressed by bacterial pathogens.

Beyond mechanistic studies, high-throughput NETosis assays may also provide a framework for evaluating therapeutic strategies aimed at modulating excessive NET formation. Because dysregulated NETosis has been implicated in endothelial injury and thrombosis during sepsis, interventions that regulate neutrophil activation could represent potential therapeutic strategies.

In this context, the whole blood NETosis platform may serve as a useful translational research tool for investigating host–pathogen interactions and inflammatory responses associated with severe infection.

Study Limitations

In addition to the limitations discussed above, there are several other limittations. First the bacterial strains used came from skin and soft tissue abscesses. These strains may not be representative of strains that cause sepsis. This can be explored by either further typing the bacterial strains or collecting additional specimens from septic patients. Second, the blood cells, in particular neutrophils and platelets are very sensitive to handiling. Some of the findings we saw may be explained by accidental activation of these cells. This can be explored by more careful cell handling. To this end we plan to repeat the experiments using a robotic pipettor. The third major limitation is that we did not use a sufficient number of replicates for most of the experiments to determine if the findings are statistically significant.

Implications for Sepsis Research

Despite these limitations, the development of a reproducible whole blood NETosis assay provides an important tool for studying host immune responses to bacterial pathogens. Experimental platforms capable of measuring neutrophil activation within physiologically relevant environments may improve understanding of how immune dysregulation contributes to sepsis pathophysiology.

Whole blood systems allow investigation of interactions between immune cells, bacterial virulence factors, and circulating mediators that influence inflammatory

signaling during infection. Such models may therefore provide understanding into mechanisms of host defense and inflammatory injury.

Future Directions

Future studies may expand upon this work by applying the whole blood NETosis platform to additional bacterial species and virulence determinants. Investigating how different pathogens influence NET formation could help understand pathogen-specific immune responses during bloodstream infection.

Integration of additional experimental readouts, including cytokine production, complement activation, and platelet signaling, could further expand the utility of the system. Combining these measurements with NETosis assays may provide a more comprehensive evaluation of host immune responses during infection.

High-throughput NETosis assays may also serve as useful tools for evaluating therapeutic strategies aimed at modulating excessive neutrophil activation during severe infection.

Concluding Statement

This study was successful in demonstrating that extracellular DNA was detected in a human shole blood sepsis model using S. aureus as a stimulant. The release of the extracellular DNA is suggestive of NETosis which is a marker of severe sepsis.

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