CREATING AN IN VITRO MODEL FOR ACUTE INFLAMMATION USING TISSUE-ENGINEERED BLOOD CLOTS

by

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A Thesis
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of
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Bioengineering

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Bachelor of Science
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DEDICATION

To my family and to my best friend, Mary Glenn, for always seeing my full potential.

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I would like to thank the many friends, relatives, and supporters who have made my graduate career successful. Most importantly, thank you to Caroline Hoemann, for being a great advisor and giving me a project that I am passionate about. This work would not have been possible without Remi Veneziano, Pat Gillevet, Masi Sikaroodi, Ginny Espina, and Barney Bishop allowing me to use their instrumentation and giving me help along the way. Jonathon Schug was instrumental in conducting RNA-sequencing and analyzing the data. Allan Doctor gave excellent insight for how to translate this work to a clinical environment. Manuel Carrasco was generous in giving his time, sharing lab materials, and helping me troubleshoot equipment. Ramya Chandrasekaran aided with experiments and gave support over the past two years. Megan Lawson was incredibly helpful with literature searches and blood collection. Lastly, this work would not have been possible without Dr. Hoemann's George Mason University start-up funds, which financed these experiments.

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LIST OF ABBREVIATIONS AND SYMBOLS

Acute Respiratory Distress Syndrome ..... ARDS
Adenosine Triphosphate ..... ATP
Aspirin ..... ASA
Damage Associated Molecular Pattern ..... DAMP
Dendritic Cells ..... DC
Deoxynucleotide Triphosphate ..... dNTP
Dexamethasone ..... Dex
Enzyme-linked Immunosorbet Assay ..... ELISA
Factor Inhibiting HIF ..... FIH
False Discovery Rate ..... FDR
First Generation Sequencing ..... FGS
Fragments Per Kilobase of transcript per Million reads ..... FPKM
Fresh Blood ..... FB
Gene Ontology ..... GO
Hypoxia Inducible Factor ..... HIF
Interleukin IL
Lipopolysaccharide ..... LPS
Log2 Fold Change ..... LFC
Monocyte Chemoattractant Protein-1 ..... MCP-1
Neutrophil Extracellular Traps ..... NET
Neutrophil-Platelet Aggregates ..... NPA
Next Generation Sequencing ..... NGS
Nitric Oxide ..... NO
Nuclear Factor Kappa B ..... NFκB
Pathogen-Associated Molecular Patterns ..... PAMPs
Pattern Recognition Receptor ..... PRR
Plasmacytoid Dendritic Cells ..... pDC
Polymerase Chain Reaction ..... PCR
Principal Component Analysis ..... PCA
P-selectin Glycoprotein Ligand 1 ..... PSGL-1
Radical Oxygen Species ..... ROS
Red Vacutainer ..... RV
Reverse Transcription ..... RT
RNA Integrity Number ..... RIN
Tissue Engineered ..... TE
Toll-like Receptor ..... TLR
Vascular Endothelial Growth Factor ..... VEGF
White Blood Cells ..... WBC

ABSTRACT

CREATING AN IN VITRO MODEL FOR ACUTE INFLAMMATION USING TISSUE-ENGINEERED BLOOD CLOTS

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George Mason University, 2021

Thesis Director: Dr. Caroline Hoemann

This work served to determine whether inflammatory responses induced in a disease state, such as Acute Respiratory Distress Syndrome (ARDS), can be modeled *in vitro* by tissue engineered whole blood clots. ARDS is characterized by hypoxia, immune cell dysfunction, sepsis, and mortality. Blood clots cultured at 37°C mimic sterile inflammation by exposing white blood cells WBC to damage-associated molecular patterns (DAMPs) that induce an innate immune response. Clots were cultured at 37ºC under hypoxia to determine whether hypoxia increases the expression of inflammatory mediators that mimic an ARDS disease state, including IL-8/CXCL8 and HIF-1α inducible genes. Transcriptomics of cultured clots and fresh blood was assessed using RT-PCR and RNA-sequencing while serum proteomics was carried out by ELISA. We used IL-8 as a marker of DAMP-induced inflammation and added LPS and IL-6 induction as a mimetic of sepsis. Whole blood (WB) was drawn under IRB-approved protocols on different occasions from 6 healthy consenting donors. Blood clots cultured at 37ºC elicited high IL-8 mRNA expression at 45m that was sustained over 4h. IL-8 was undetectable in baseline plasma

and progressively increased at 45m (60pg/mL), 2h (185pg/mL), and 4h (3000 pg/mL). Hypoxia elevated serum IL-8 (4700 pg/mL), but this was not significant compared to normoxia (N =6, p = 0.23). LPS had the greatest average IL-8 induction, at 7800pg/mL, which was significantly upregulated compared to normoxia (p = 0.03). Only LPS-spiked 4h cultured clots expressed IL-6 at the RNA and protein level. RNA-sequencing revealed over 2300 genes differentially expressed in the clot conditions compared to FB. All 3 cultured clot sample types showed HIF-1α and NFκB pathway inductions. The top expressed genes ($p < 0.05$) included the chemokines CXCL2, CXCL3, IL-8/CXCL8, VEGF and the chemokine receptor CXCR4. RT-PCR confirmed that VEGF expression was induced in most cultured blood clots compared to fresh blood. Only 28 genes were differentially expressed between cultured clots and cultured hypoxia clots. In a drug-screening model, cultured clots showed variable anti-inflammatory responses to aspirin and dexamethasone between N=2 healthy donors. Dexamethasone outperformed aspirin on reducing IL-8 and IL-6 levels in LPS-stimulated blood clots. This study showed that healthy blood from 6 donors was successfully engineered to induce an acute innate immune response within 4 hours using the cultured blood clot model. Cultured blood clots showed elevated VEGF, which is a pro-angiogenic factor induced by hypoxic environments. These results suggest the clots were inherently hypoxic and is consistent with the low number of differentially expressed genes between the cultured clot without and with hypoxia incubation. The cultured clot model also showed variable responses between 2 donors when treated with common anticoagulant medications. This has positive implications in

studying disease states such as ARDS and studying current medications on an individualized basis.

CHAPTER ONE. INTRODUCTION

The current gold standard for identifying differential transcriptomic expression of disease states is collecting fresh blood into Paxgene tubes for RNA purification and sequencing. This approach has led to countless discoveries of novel gene expressions. For example, Kangelaris et al. identified Lipocalin 2 and CD24 as peripheral blood biomarkers that predicted mortality of sepsis patients.[1] Englert et al. identified genomic differences between Acute Respiratory Distress Syndrome (ARDS) patients with and without stem cell transplants.[2] Ultimately, the current technology allows for extremely accurate and thorough analyses, discovering genomic "fingerprints" of diseases. However, without a disease state, transcriptomic analysis of fresh blood is unlikely to show abnormalities in an individual's innate immune response that could potentially lead to severe disease progression, like sepsis, because circulating leukocytes are not activated. To activate the innate immune response, coagulation and hypoxia, types of damage-associated molecular patterns (DAMPs), could be used in conjunction. These events are seen during wounding, infection and inflammation, serving as positive feedback loops for pro-inflammatory cytokine production.[3] In an abnormal response, these DAMPs induce a cytokine storm, characterized by a hyper-responsive initial state.[4]

This proposal serves to explore the transcriptomic and proteomic shifts in WBC after coagulation, as well as exploring how these shifts are amplified due to added hypoxia and LPS. It was previously reported that unmodified whole blood could be coagulated and cultured as a viable tissue at 37°C for up to 6h.[5,6] Serum assays on these tissue-engineered (TE) clots revealed upregulation of IL-8 and MCP-1, strong chemotactic factors that drive leukocytes to sites of inflammation. These results show promise that TE blood clots could be harvested from both healthy and unhealthy donors to explore the WBC response during sterile inflammation and how this is dysregulated in disease states. The null hypothesis is that fresh blood and coagulation will show no transcriptomic or proteomic shift. The alternative hypothesis is that coagulation will activate WBC, causing a detectable transcriptomic change. Clot priming agents such as hypoxia and the endotoxin LPS will further amplify this shift. Results from this study can be used in future work to identify novel differential gene expression in normal and abnormal innate immune responses to serve as prognostic information for disease progression.

Coagulation, Hypoxia, and the Immune Response

In vivo, coagulation and inflammation act as positive feedbacks for each other.[3] van Deventer et al. showed that in the presence of endotoxin in vivo, the coagulation system is activated in as little as 2h, while also promoting the pro-inflammatory factors TNF and IL-6.[7] At the beginning of coagulation, vWF exposed on damaged sub-endothelial surfaces binds with activated platelets, which release soluble vWF that forms platelet aggregates and induces thrombin formation to activate the innate immune response.[8] These activated platelets then further release pro-coagulant and inflammatory microparticles to activate other cells, including WBC and endothelial cells, along with thrombin formation.[9] Neutrophils bind to P-selectins expressed on an activated platelet surface via P-selectin protein ligand-1 (PSGL-1).[10] Induced by PSGL-1 binding and pathogen-associated molecular patterns (PAMPs), neutrophils release NETs that also upregulate thrombin generation and clot formation.[9,10] TLRs on leukocytes recognize PAMPs and initiate innate immunity by promoting signaling cascades and inflammatory cytokines.[11] Neutrophils account for the majority of leukocytes, but monocytes also play a major role in inflammation by expressing tissue factor that results in a coagulation cascade, as well as chemotactic interleukins.[12] Due to activation of white blood cells (WBCs) by thrombin, platelet factors, and activated platelet interactions, WBC cytokine expression is closely linked to coagulation. Previous studies reported that coagulation and culture of unmodified whole blood clots at 37°C for 4 to 6 hours induced the selective expression of IL-8/CXCL8 and MCP-1/CCL2 that was not seen in fresh whole blood.[5,6] Therefore, it is predicted that creating a coagulome will induce a unique inflammatory response visible at the

transcriptional and post-transcriptional level compared to fresh blood.

Rather than using harmful endotoxins, we hypothesize the key to stimulating healthy blood lies in creating a hypoxic environment, as tissue hypoxia and lactate build-up is an in vivo result of infection and trauma. Physiologic hypoxia is described as less than 1% or 7.5mmHg oxygen.[13] For comparison, normoxic conditions refer to 20%, or 150mmHg, oxygen.[13] Both wounds and infection are sites of physiologic hypoxia.[14] In both sterile and non-sterile inflammation, responding leukocytes flood to the site to begin their phagocytic attack, increasing the oxygen demand to generate radical oxygen species (ROS), and ultimately creating a hypoxic environment.[15] The hypoxic response is orchestrated by the HIF family of nuclear transcription factors, with HIF-1α playing the largest role. HIF-1α stabilization is controlled by the amount of the oxygen-dependent prolyl hydroxylase domain enzymes (PHD1, PHD2, PHD3) as well as by FIH, all of which destabilize HIF-1α.14 When oxygen levels decrease, PHD and FIH become inactive, and HIF-1α is stabilized. HIF-1α in turn activates a metabolic response to hypoxia to regulate cell homeostasis. For neutrophils, this change in activity includes increased expression of glucose transporters, increased aerobic glycolysis, ATP production, and generation of ROS, which allows for better survival, inflammatory cytokine expression, and phagocytosis.[16,17] For monocytes, hypoxia is known to regulate gene expression and cytokine production, including the upregulation of chemotactic factors like IL-8, CCL3, and CXCR4.[18] The hypoxic environment, specifically by activation of HIF-1α, ultimately plays a large role in the host response to infection and disease progression.[15] Hypoxia is not only a result of inflammation but can also serve as a DAMP during trauma, swelling,

compression, and thrombosis, activating neutrophils to begin an innate immune response.[19] Stimulating an in vitro hypoxic environment using whole blood results in increased expression of IL-8, which recruits neutrophils to the area, and macrophage secretion of proinflammatory cytokines.[19] Microarray analysis of mouse peripheral whole blood showed that *in vivo* hypoxia upregulated the average global gene expression by a 2-fold amount compared to a healthy control.[20] In vitro fibrin clots containing purified monocytes specifically have been shown to further stimulate a proinflammatory response in monocytes by upregulating IL-8 and MCP-1.[12] Due to this, hypoxia and coagulation are used in conjunction to induce and exaggerate inflammation in healthy clotted blood. LPS from E.coli (O111:B4, Sigma) at a concentration of 100ng/mL serves as a positive control. Previous literature suggests at this concentration LPS will elicit an exaggerated acute inflammatory immune response in all individuals.[21] Therefore, 100 ng/mL LPS from E.coli was used in this work as a positive control for inflammatory activation of blood clot cells.

RNA Sequencing

RNA sequencing is a promising way to analyze the global differences in gene expression between fresh blood and a tissue-engineered blood clot. Research of gene expression and biomarkers has quickly progressed in the last two decades because of First Generation Sequencing (FGS), such as Sanger dideoxy sequencing, and Next Generation Sequencing (NGS), like Ion Torrent and Illumina.[22,23] Compared to FGS, NGS allows researchers to study disease gene expressions at high throughputs. The sequencing is performed as parallel sequencing, rather than in a sequential manner like FGS, of a number of templates in a single run.[23] NGS also offers a more robust analysis, with higher amounts of detection of mutations, rare genes, and splice variants.[24] Ion Torrent can produce up to

80 million reads using a technique that detects hydrogen ions after a pair matching.[23] A single type of deoxynucleotide triphosphate (dNTP) is put in each microwell which contains a known nucleotide base. If the dNTP matches the base, then a hydrogen ion is released, which is measured by the change in pH. The process is repeated until each base has a matching dNTP.[23] A different sequencing methodology is Reverse Transcription (RT), used by Illumina technology, which can produce up to 5 billion single reads, depending on the machine and model. A fluorescently labelled RT nucleotide is put in each microwell. If it binds to a base pair, it is detected indirectly via fluorescence, and the process is repeated until all RNA fragments are sequenced.[23] Because of the improvements in high throughput sequencing methods, many researchers have used the technology on blood samples for diagnostics. However, this is not without limitations, including high amounts of fragmentation during RNA extraction and the predominance of globin mRNA in whole blood RNA.[25] Before sequencing, blood-derived RNA samples must be depleted of ribosomal RNA and globin mRNA, which make up 80-90% of total RNA and give little information on the transcriptome.[26,27] Globin mRNA can also make identifying low expression mRNA difficult, so depletion may better the depth of read of RNA sequencing. Although globin depletion is necessary, it can reduce the integrity of RNA due to the extra chemical processing and increase variability among technical replicates.[27] Ribosomal RNA can be removed via Poly-A+ tail selection during library preparation. Therefore, the correct choice of extraction, depletion, and sequencing kits are pertinent to maintain good RNA integrity.

CHAPTER TWO. BLOOD COLLECTION AND MODEL DEVELOPMENT

Introduction

Previous work by Hoemann et al. saw blood coagulation within 30 minutes in sterile glass culture media tubes. After 2-6 hours at 37°C, cells within the clot released inflammatory factors detectable in the serum, including IL-8 and MCP-1.[6] This led to work exploring the clot not as an inert mass, but as a living tissue that can attract and activate immune cells, mimicking a disease state. The clot model challenges white blood cells by isolating them in a thrombotic environment, activating neutrophils and macrophages to induce an innate immune response. Neutrophils and macrophages are key determinants in disease outcome; these responding cells work to rapidly signal the immune response, as well as clear dead cells and infectious agents. Dysfunction in these roles can result in severe infections.[14] This gives promise of being able to use the clot model to compare disease states and explore what is deemed as a "healthy" immune response, and what is dysregulated. For example, during sepsis and ARDS, immune cell dysregulation results in initiation of the contact activation system by plasma kallikrein formation, which activates Factor XII, and results in thrombin formation.[28] This thrombosis could not only cause oxygen deprivation to tissues by blocking capillaries, but it can also result in further activating a hyper-inflammatory response,[29] also known as a cytokine storm.

To translate to clinical applications and a hospital setting, the goal was to determine whether clots incubated in a red vacutainer tube, rather than glass tubes, would result in the same inflammatory induction. Red vacutainer (RV) tubes are spray coated with silica

and typically used for serum analyses. Silica is a non-toxic, negatively charged material that activates coagulation via the contact pathway.[30] In the contact activation system, Factor XII binds to a surface, like silica, and begins a downstream signaling cascade resulting in coagulation.[28] This works much like the glass culture tubes; glass acts as a negatively charged surface that Factor XII binds to and activates coagulation. In both materials, clotting occurs within 30 minutes. The first objective of this model development was to determine via RT-PCR and ELISA (Figure 1) whether the clots in both glass culture tubes and RV had comparable IL-8 induction within 4 hours. The second objective was to analyze IL-8 secretion in clots cultured in a closed portable incubator at 37°C for 4 hours to test if this type of incubator can be used at a hospital bedside setting. Lastly, the third objective was to determine whether high quality RNA could be extracted from the clots for RNA-sequencing to explore the entire clot transcriptome.

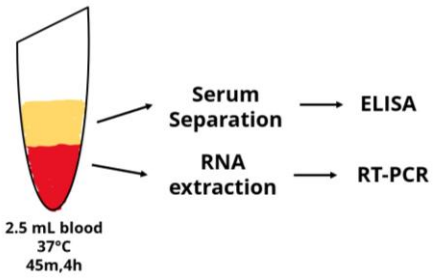


Figure 1. Tissue-engineered blood clot. Whole blood is incubated at 37°C in normoxia and hypoxia. The blood clot is preserved for transcriptomics. The serum is used for proteomics.

Methods

Blood Collection

Blood was collected from 13 healthy consenting donors following IRB #1322641. 8 conditions were tested:

1. Fresh Blood (FB), 2.5mL
2. 45m room temperature (RT), 0.5mL x 5, glass
3. Glass Normoxia, 0.5mL x 5, 37°C, 20% O_2, 45m and 4h
4. Red Vacutainer (RV), 3mL clot, 37°C
5. Glass Hypoxia, 0.5mL x 5, 37°C, 1% O_2, 45m and 4h
6. RV-LPS, 3mL clot, 37°C
7. Heparin, 4mL, 37°C, 4h
8. Heparin-LPS, 4mL, 37°C, 4h

Fresh blood was drawn directly into a Paxgene blood collection tube (Qiagen) and kept at room temperature following the manufacturer's instructions. For proteomics of fresh blood, blood was drawn into heparin, EDTA, or citrate tubes and placed at 4°C. For the remaining conditions, blood was drawn directly into a syringe (10mL), red vacutainer tubes (3mL), or heparin tubes (4mL). For the glass culture tubes, 0.5mL blood from the 10mL syringe were deposited in 5 depyrogenized glass tubes with vented steel caps for each condition. Glass normoxia tubes incubated for 45m and 4h at 37°C, 20% O_2. Glass hypoxia tubes incubated for 45m and 4h at 37°C, 1% to 8% O_2. The RV (2h, 4h) and heparin culture (4h) conditions incubated for at 37°C; and the RV-LPS and heparin-LPS conditions were

spiked with 100ng/mL LPS from E.coli (O111:B4, Sigma Aldrich) then incubated for 4 hours at 37°C. For 4 donors, a separate 3mL RV tube was placed in a portable Minitube incubator (Minitube) and kept at 37C for 4 hours. After 4 hours, all tubes were removed from incubation and immediately put on ice.

WBC Counts

WBC counts were determined for all donors (except donor H1) from anticoagulated EDTA or citrated blood. 5 µL of whole blood was pipetted into 95 µL 1% glacial acetic acid and cresyl violet solution to lyse RBC, then the stained nuclei counted on a hemocytometer.

Serum Extraction

Samples were centrifuged at 200g for 10 minutes. Serum was transferred to a clean Eppendorf tube then spun again at 1300 RCF to remove any remaining RBC and platelets within the serum. Serum was aliquoted into cryovials, flash frozen, and stored at -80°C.

RNA Extraction

Fresh blood and blood clots used Paxgene RNA kit (Qiagen) for RNA extraction. Fresh blood was drawn directly into Paxgene tubes. Blood clots were homogenized with the Paxgene buffer in 50mL conical tubes. All samples incubated at room temperature overnight then stored at -80C until RNA extraction. RNA extraction followed Paxgene manufacturer's protocols.

RNA Purification and Quality Control for RNA sequencing

RNA concentration and yield were assessed using a Nanodrop. Quality control was performed with Agilent Bioanalyzer. All RNA with RNA Integrity Number (RIN) > 7.0 were deemed good quality. For RNA-sequencing, RNA was concentrated using RNeasy

MinElute kit (Qiagen) then globin-depleted with Ambion GlobinClear (Thermo Fisher) following manufacturer's protocols.

RT-PCR

First strand cDNA was made using AMV-Reverse Transcriptase methods. Second strand amplification used Econotaq, with an initial heating to 95°C for 3 minutes, followed by 30 to 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, elongation at 72°C for 30 seconds, and a final elongation at 72°C for 5 minutes. RT-PCR tested for IL-6 and IL-8 mRNA expression. GAPDH and CD45 served as housekeeping genes. Primers purchased from Integrated DNA Technologies (IDT) are listed in Table 1 below.

Table 1. Primers for RT-PCR analyses

Gene	Forward Primer	Reverse Primer	Product Size
GAPDH	5' - ATG GGG AAG GTG AAG GTC GGA - 3'	5' - AGG GGC CAT CCA CAG TCT TCT -3'	573 bp
CD45	5' - CAA CTG ATG AAT GTG GAG CC - 3'	5' - AGG AAG AAT GTC AAC ATA AC - 3'	164bp
IL-6	5' - CCC CCA GGA GAA GAT TCC AA - 3'	5' - CGC AGA ATG AGA TGA GTT GT - 3'	496bp
IL-8	5' - AAC ATG ACT TCC AAG CTG GCC G - 3'	5' - CAG TTT TCC TTG GGG TCC AGA C - 3'	342bp

IL-8 and IL-6 ELISA

ELISA analyses followed the manufacturer's protocols (R&D Systems) using serum extracted from the blood clots. Citrated plasma, EDTA plasma, or uncultured heparin serum served as negative controls. LPS-spiked clot serum served as a positive control.

Statistical Analysis

A Student's T Test measured differences between the means for two groups for $n \geq 3$. Statistical analyses were conducted on ELISA experiments only.

Results

Donors 22 to 60 years old with diverse ethnicity, 3 males and 3 females, were included in ELISA analysis. All other donors were used for RNA extraction and validation. All donors declared themselves healthy, had no chronic blood borne diseases, were not on pain medications within 72 hours prior to blood draw, and had not received any vaccines two weeks before blood draw. WBC counts verified all but three donors were in the healthy WBC count range of 4.5-11.0 million cells/mL.[31] These donors were used for RNA extraction studies, not in the ELISA assays. The WBC were only slightly out of range and could not be deemed an "unhealthy" number, especially since counting was performed by hand, not in a clinical laboratory setting.

RT-PCR

Semi-quantitative RT-PCR analyses tested if clot conditions changed IL-6 and IL-8 chemokine induction in 3 donors (Figure 2). The clot conditions tested were glass normoxia, glass hypoxia, RV, and RV-LPS. CD45 and GAPDH served as housekeeping genes, although GAPDH did have stronger induction in hypoxic clots. Only the LPS clot condition strongly induced IL-6 across all donors. IL-6 induction began in as little as 45 minutes after exposure to LPS and stayed consistently high for 4 hours. IL-8 induction began almost immediately in all clots, with 45m cultured clots having strong induction that continued over the four hours. The 45m clot at room temperature did have IL-8 induction, but it was not as strong as clots incubated at 37C for 45m. LPS did not upregulate IL-8 RNA expression compared to clotting alone.

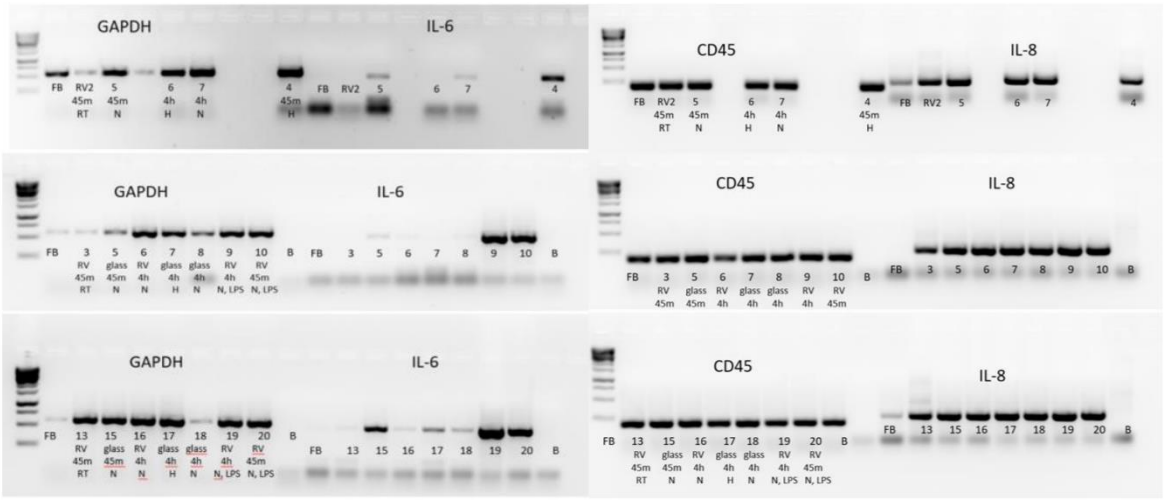


Figure 2. RT-PCR of GAPDH, IL-6, CD45, and IL-8 from 3 donors. cDNA was amplified for 35 cycles. GAPDH and CD45 served as housekeeping genes. B = blank. Lane 1: FB. Lane 2: RV 45m. Lane 3: Glass N 45m. Lane 4: 4h RV. Lane 5: 4h H. Lane 6: 4h H. Lane 7: 4h Normoxia. Lane 8: 4h RV-LPS. Lane 9: 45m H (Donor 1) or blank (Donors 2,3). Lane order is the same for all genes.

ELISA

Sandwich ELISAs were conducted on IL-8 and IL-6. The first ELISA analysis focused on a timescale assay of 45m, 2h, and 4h timepoints to determine when IL-8 proteins are detectable in the serum (Figure 3, N = 3-6). At 45 minutes, only small levels of IL-8 were detectable, approximately 60pg/mL in all conditions, and this was inconsistent between donors, with some having no detectable IL-8. By two hours, the average concentration was 185 pg/mL, still comparable with 45m time points. Strong IL-8 serum levels were not seen until the 4-hour time point, with an average concentration of approximately 3000pg/mL for normoxia conditions. Hypoxia elevated the average IL-8 concentration to 4800 pg/mL, but this was not deemed a significant increase compared to normoxia ($p = 0.37$). LPS significantly increased IL-8 compared to normoxia ($p = 0.03$).

However, it did not further elevate IL-8 compared to hypoxia ($p = 0.29$) due to the high standard deviation within groups. This ELISA validated the need to incubate the clots for 4h to have detectable protein in the serum.

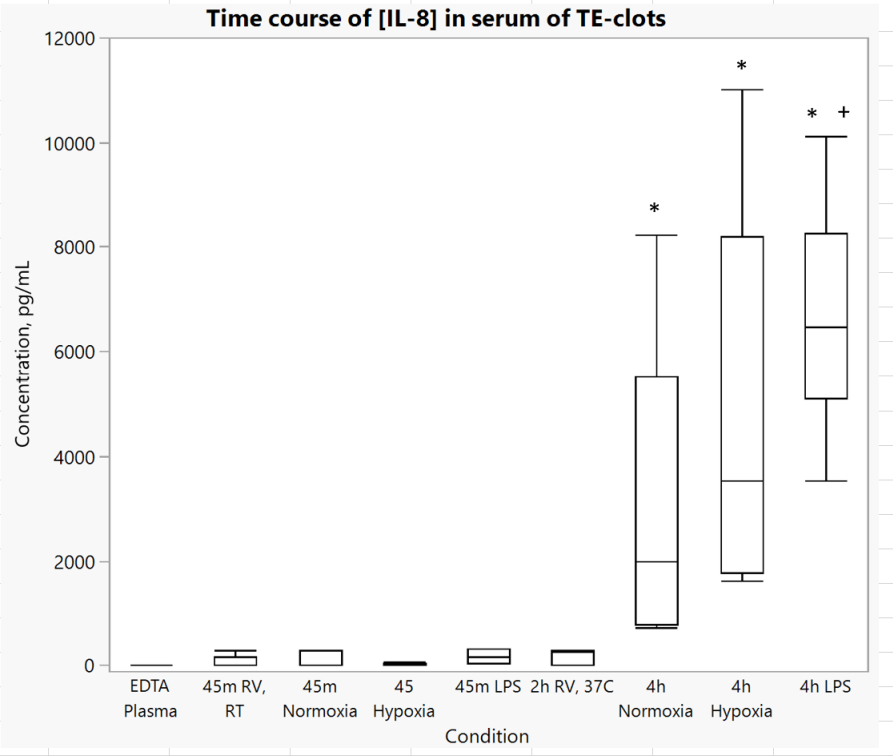


Figure 3. IL-8 in TE-clot serum, 45m and 4h. WB incubated for either 45m, 4h at RT, normoxia, or hypoxia. LPS spike served as a positive control. EDTA N = 4. 45m Hypoxia N = 3. 45m RV RT N = 5. 45m Normoxia N = 3. 45m LPS N = 2. 2h RV 37C N = 3. 4h Normoxia, Hypoxia, LPS N = 6 independent samples, 5 biological replicates. * = $p < 0.05$ compared to EDTA plasma. + = $p < 0.05$ compared to 4h normoxia.

The next IL-8 ELISA explored whether RV tubes could be used in place of normoxia glass tubes, as part of translation to a clinical setting, where vacutainers are almost exclusively used for blood collection. The hypothesis was that RV would not be

significantly different that normoxia. Here we show the 4h RV clot has no significant difference in IL-8 compared to normoxia ($p = 0.8$) or to hypoxia ($p = 0.23$) (Figure 4). From this study, we moved forward using RV instead of glass culture tubes in subsequent blood draws, as they had similar IL-8 concentrations.

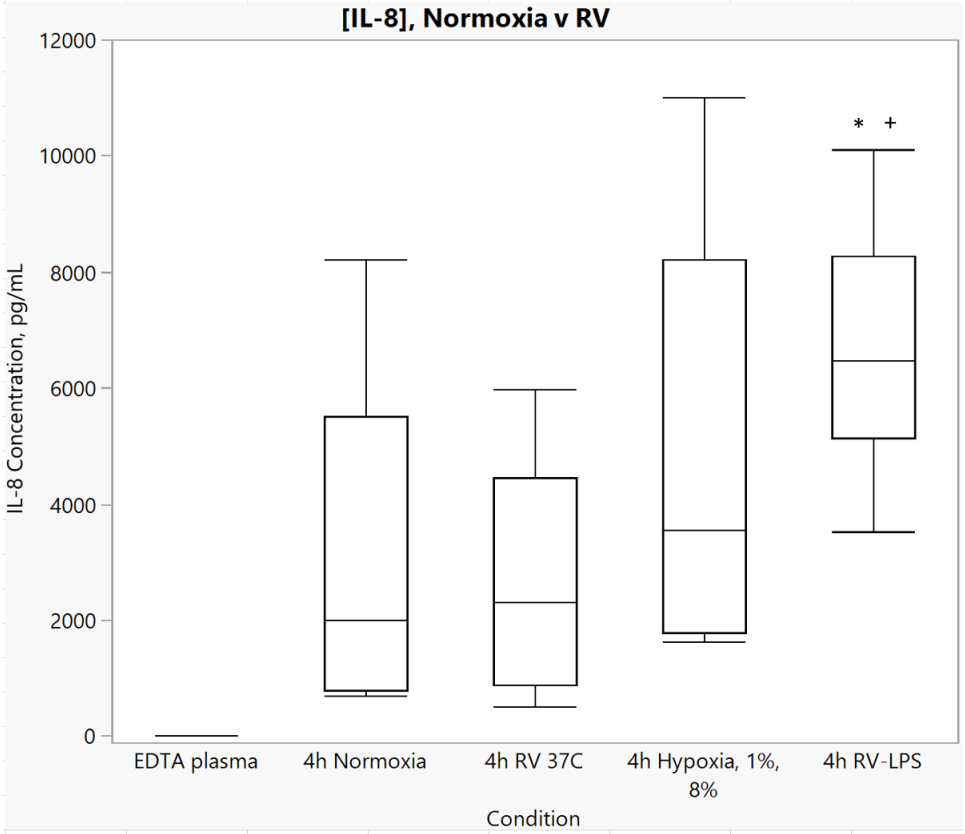


Figure 4. IL-8 ELISA, Normoxia v. RV. All clots were incubated at 37°C for 4h then serum extracted. EDTA N = 4. N = 6 all other conditions. * = $p < 0.05$ compared to normoxia. + = $p < 0.05$ compared to RV. EDTA serves as a baseline negative control, with all counts being 0. LPS serves as a positive control.

As a validation study to show that coagulation was necessary for inducing inflammatory factors, heparin blood was cultured for 4h at 37°C with and without LPS and compared to RV, RV-LPS for 3 donors. The hypothesis was that heparin culture alone would not have IL-8 expression. Spiking with LPS would induce an immune response, but IL-8 would not be as elevated as in RV-LPS. Heparin culture for 4 hours at 37°C had minimal IL-8 induction (Figure 5). Heparin with an LPS spike did induce inflammation, with an average concentration of 2500pg/mL between the three donors. Heparin-LPS had very similar IL-8 concentrations as the RV clot ($p = 0.8$). However, RV-LPS had a significantly amplified IL-8 concentration ($p = 0.02$) compared to uncoagulated blood spiked with LPS, supporting the hypothesis that clotting further elevates an LPS reaction.

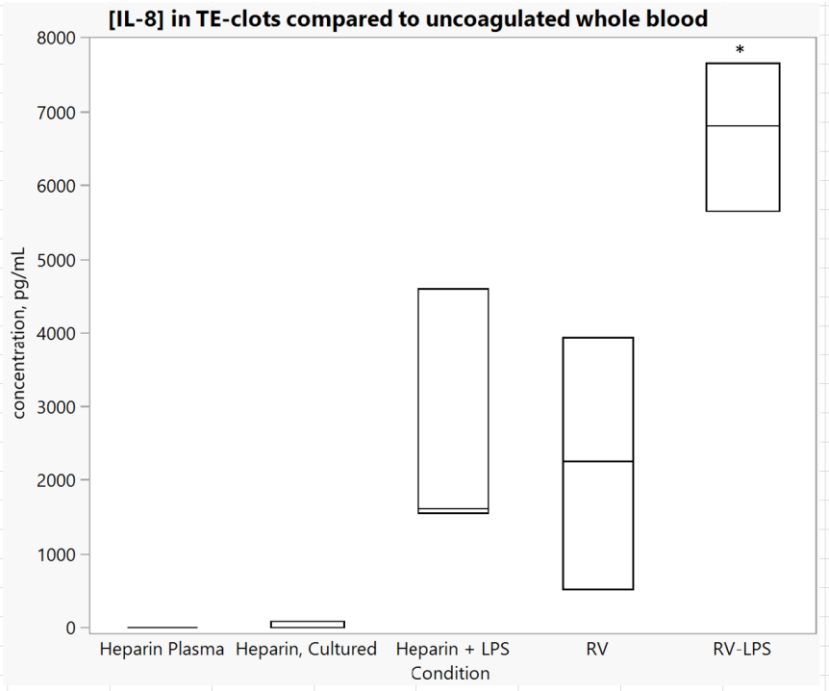


Figure 5. IL-8 ELISA, heparin blood v. TE-clots. N = 3 for all conditions. Heparin plasma was kept at 4C until serum extracted. All other conditions cultured for 4h at 37°C. * = $p < 0.05$ compared to Heparin + LPS.

The final ELISA experiment focused on comparing RV in the laboratory Heracell incubator to the portable Minitube incubator. The Minitube incubator is critical for translational research, as nurses cannot immediately transfer blood samples to the lab for incubation. The Minitube holds samples at 37°C for up to 12 hours, is light, and can be used at bedside. Both IL-6 and IL-8 levels were measured (Figure 6). IL-6 was only detectable in LPS-spiked clots. IL-8 levels were elevated in all clot conditions. RV clot in the Minitube incubator (RVM) had comparable, if not slightly elevated, IL-8 serum concentrations to RV incubated in the Heracell incubator (RV37) ($p = 0.55$). This study supported that the Minitube incubator could be used in a clinical setting.

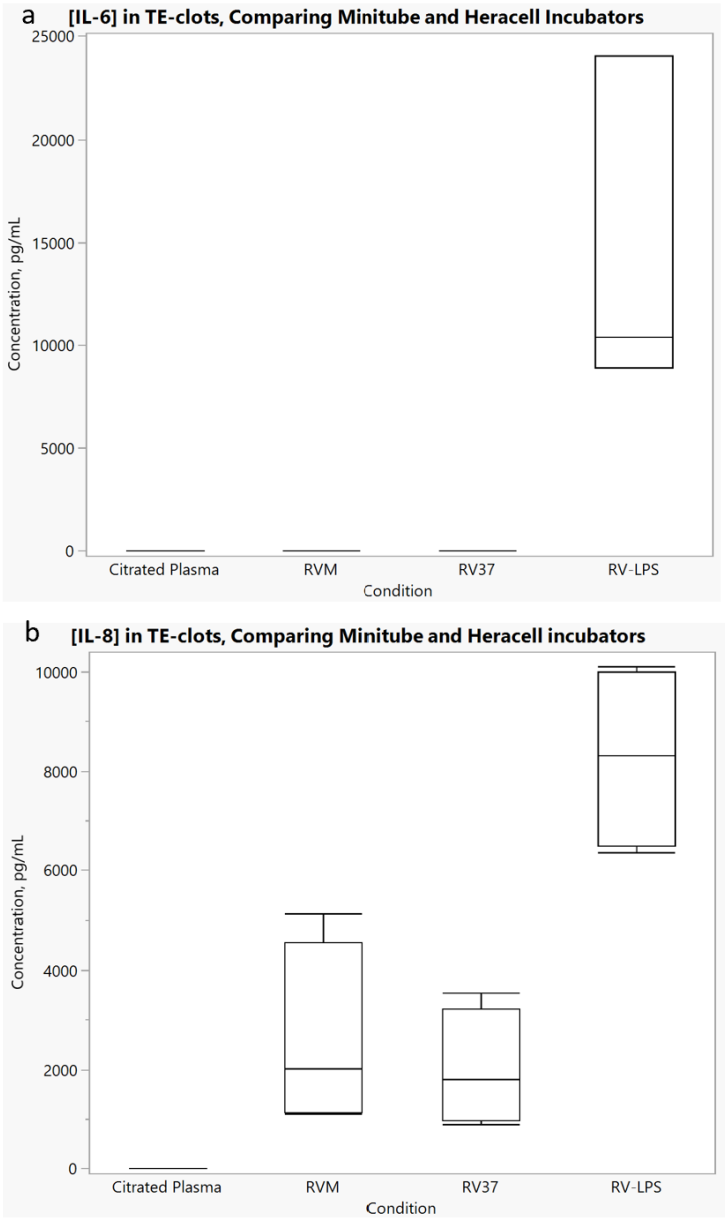


Figure 6. IL-6 (a) and IL-8 (b) ELISA, RVM v. RV37. RV was either incubated in a 37°C Heracell incubator or in a 37°C portable Minitube incubator. RVM = Minitube incubator. RVM = Heracell incubator. N =3 for IL-6. N = 4 for IL-8. Citrated plasma served as negative control. LPS served as a positive control. RVM and RV37 are not statistically different.

RNA processing and quality control

RNA was extracted from blood clots by homogenizing in Paxgene buffer than extracted following the manufacturer's protocols. A total of 65 RNA samples from 13 donors were extracted and tested for yield and quality using Nanodrop and Agilent Bioanalyzer. All samples had high quality RIN (> 7.0) that was not affected by condition (Figure 7a). Yields were highest for FB which was drawn directly into the Paxgene tube (Figure 7b). The clot resulted in roughly 30-70% reduction in RNA yield compared to fresh blood, but there was still at least 1μg RNA per total volume, which allowed for RT-PCR and RNA-sequencing. For RNA sequencing, RNA must be concentrated and globin depleted which reduces quality.[27] On 8 samples, the yield and quality were assessed after each step to determine the quality loss (Table 2). Average yield loss was 2μg, with the lowest yield being 0.67μg. An exception was H5 hypoxia, which had a higher yield after globin depletion. This could be attributed to inadequate dilution or a contaminated blank during Nanodrop analysis of original and concentrated RNA. Every sample except two had a reduced RIN score after processing, but none fell below the 7.0 cutoff. Although there were losses in both yield and quality, the Paxgene kit passed all necessary validations of original yield > 1μg and RIN > 7.0.

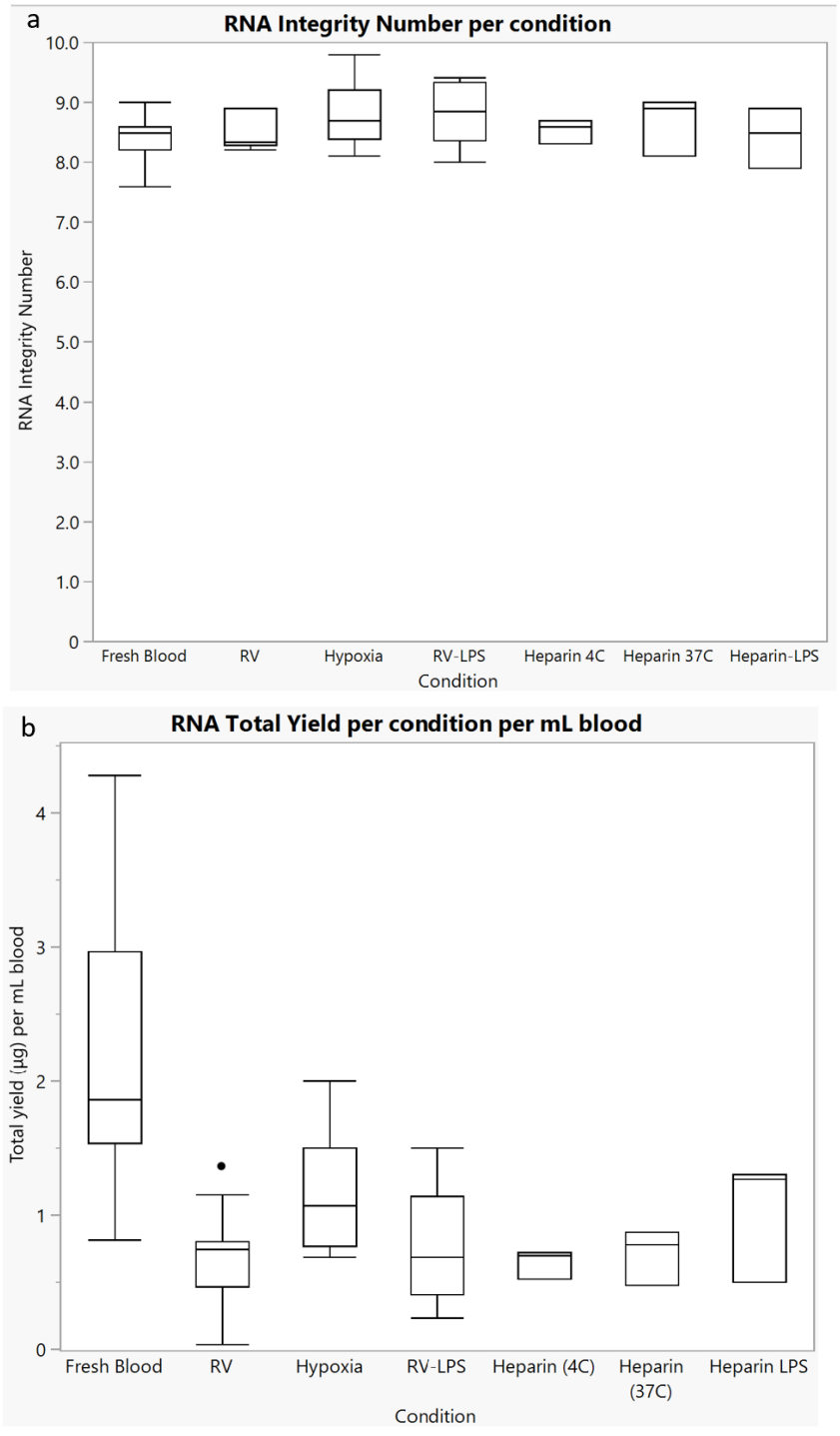


Figure 7. RNA RIN and yield per condition. A) Average RNA Integrity Number (RIN). B) Average total yield of RNA per mL of blood for each testing condition. RV, hypoxia, and RV-LPS are coagulated blood. Fresh blood, Heparin (4C), Heparin (37C), and Heparin-LPS are uncoagulated blood. RNA extracted with Paxgene. RIN determined with Agilent Bioanalyzer. Total Yield determined with Nanodrop. FB N = 17, RV N = 17, Hypoxia N = 6, RV-LPS N = 16, heparin 4C N = 3, heparin 37C N = 3, heparin-LPS N = 3. N = 65 total.

Table 2. Yield and RIN for original, concentrated, and globin depleted RNA. 500-1000ng RNA was reserved from each sample before concentration for future RT-PCR. RIN measured via Agilent Bioanalyzer

Donor	Tube Type	Time	Condition	Total Yield (ug)	RIN
H5	Paxgene	0	FB	8.8	7.6
H5	Paxgene	0	FB - conc.	6.2	7.8
H5	Paxgene	0	FB - conc, GD	3.9	7.6
H5	RV	4h	37C	4.1	8.3
H5	RV	4h	37C - conc	3.5	8.2
H5	RV	4h	37C - conc GD	2.0	8.3
H5	RV	4h	37C - LPS	1.6	9
H5	RV	4h	37C - LPS - conc	0.6	6.7
H5	RV	4h	37C - LPS, conc, GD	0.6	8.1
H5	Glass	4h	hypoxia	1.7	8.7
H5	Glass	4h	hypoxia - conc	1.1	8
H5	Glass	4h	hypoxia - conc, GD	3.1	8
H6	Paxgene	0	FB	7.4	8.6
H6	Paxgene	0	FB - conc	3.7	8.4
H6	Paxgene	0	FB - conc, GD	3.1	7.6
H6	RV	4h	37C	3.4	8.3
H6	RV	4h	37C - conc	2.6	7.9
H6	RV	4h	37C - conc, GD	2.0	7.5
H6	RV	4h	37C -LPS	4.5	8.5
H6	RV	4h	37C -LPS - conc	2.5	7.8
H6	RV	4h	37C -LPS - conc, GD	1.8	8
H6	Glass	4h	hypoxia	3.1	8.7
H6	Glass	4h	hypoxia - conc	1.5	8.4
H6	Glass	4h	hypoxia - conc, GD	1.3	7.8

Discussion

RT-PCR and ELISA results suggest WBC become activated due to clotting at 37°C, and endotoxin exposure exaggerates that response. IL-6 was inconsistently induced in normoxia and hypoxia, 45m and 4h. It was only highly upregulated in LPS exposure. This result is expected from previous work.[5,6] High IL-6 levels in infectious states can serve as a marker for increased morbidity, cytokine storms, and immune dysregulation.[32,33] Given

all donors were healthy, no immune dysregulation was anticipated unless challenged by a high-dose PAMP. On the other hand, cultured clotting elicits high IL-8 expression and secretion in all conditions. RT-PCR verified IL-8 mRNA expression within 45 minutes of TE-clots, but it was not detectable in the serum for all donors until the 4-hour time point. This supports previous work by Hoemann et al. which detected IL-8 protein secretion from blood clots at the 4 hour time point.[6] By 4 hours, normoxia had approximately 3000pg/mL IL-8. Both hypoxia and LPS further increased IL-8 concentration levels to 4800pg/mL and 7800pg/mL, respectively. Normoxia and RV had similar IL-8 secretion, with RV at an average of 2700pg/mL IL-8, validating the use of RV. Incubation at 37°C was not enough to induce an inflammatory response in uncoagulated blood, which is expected as this models the *in vivo* healthy environment. These results support moving forward with the TE-clot model.

The next step of model development focused on the translation of the blood clot system to a clinical setting. In a hospital, the incubator would need to be portable to ensure samples are quickly put at 37°C, without having to transfer to the hospital lab. The ELISA results found that the Minitube incubator was excellent at clot activation and produced similar, if not elevated, levels of IL-8 compared to the laboratory incubator. It is hypothesized that this is due to the metal beads within the incubator, which help heat the blood sample faster. Ultimately, the clot system passed all validations necessary for the clinical environment.

The last objective was to determine whether high-quality RNA could be extracted from blood clots for RNA sequencing of the transcriptome. The Paxgene kit was tested off-

label on the clots, as it is only approved for fresh blood purposes. Because of this, there was a yield loss in the blood clots of up to 70% compared to fresh blood. This loss could be due to multiple reasons: the fibrin clot encapsulating cells and preventing full Paxgene saturation or from removing the serum prior to adding Paxgene. The latter hypothesis is supported by heparin samples still having significant losses compared to FB although the blood is uncoagulated. However, the kit still produced high quality RNA that was consistent across all conditions. Further processing with RNeasy MinElute and GlobinClear also reduced yield and quality, but all samples had at least 500ng for RNA sequencing and RIN > 7.0. An mRNA sequencing library can be made with as little as 100ng total RNA. The Paxgene kit, RNeasy MinElute kit, and Ambion GlobinClear can be used moving forward for RNA extraction and processing, but RNA from two blood clots may need to be combined for RNA sequencing to have adequate yields. This is especially a concern for unhealthy donors who may have low white blood cell counts.

The biggest limitation to this study is the rather small sample size with a heterogeneous population. At this size, we cannot correlate responses to demographic features known to influence innate immune responses, such as age, ethnicity, and comorbidities. As well, it was out of the scope of this project to include platelet counts, which are instrumental in neutrophil activation during trauma and infection. Future work with large sample sizes and platelet counts could address the large standard deviations we see in IL-8 responses between donors.

Conclusion

The clot model can be successfully translated to a clinical setting by drawing blood into an RV tube then incubating for 4h at 37°C in a handheld incubator. From these clots, both serum and high-quality RNA can be extracted for ELISA, RT-PCR, and RNA-sequencing. These results could reveal novel insights about WBC in disease states, such as sepsis, ARDS, and coagulopathy.

CHAPTER THREE. RNA SEQUENCING

Introduction

The inflammatory factors IL-8 and IL-6 alone cannot show the full extent of WBC RNA shifts due to clotting and additional PAMPs. Cells undergo a shift into both recruiting other WBCs to the inflamed area via cytokines and switching to a "survival mode" which changes metabolic, transcriptional, and translational processes.[34] The hypoxic adaptive response is orchestrated through the Nuclear Factor kappa B (NFκB) pathway, which mediates HIF-1α.[14] NFκB is a transcription factor that regulates hundreds of genes once activated, including regulating cytokines and chemokines as a pro-inflammatory response.[35,36] As well, it controls adhesion, apoptosis, cell cycle, and angiogenesis.[36] *In vivo*, TLRs bound to cell surface receptors activate the NFκB pathway in response to both hypoxic environments and infection, with studies showing NFκB induction after LPS exposure.[14,36] RNA sequencing (RNA-seq) on whole blood clots compared RV and hypoxia, with LPS-spiked clots serving as the positive control to determine if clotting induces NFκB-associated genes, mimicking a disease state. The hypothesis of this study was that a TE clot incubated in hypoxic conditions (1% O_2, 37°C) results in a unique genomic fingerprint, specifically through greater activation of NFκB-regulated transcripts, compared to a clot incubated at 37°C.

To compare the full transcriptomic shift between RV, hypoxia, and LPS, RNA-seq sequenced all conditions for N =2 donors using Illumina NovaSeq, via reverse transcription methods and fluorescently-labelled nucleotides.[23] With this low of a sample size, statistical analyses used EdgeR and DESeq2 software, both of which take into account the low sample sizes in high throughput sequencing studies, and thus a low statistical power.[37,38] The algorithms apply shrinkage methods to reduce the dispersion parameters, or within-group variance. To do this, a linear model assumes genes of similar read counts will have similar dispersions. Outlying gene reads are shrunk to the curve to effectively reduce variance. Shrinkage methods are also applied to the log2 fold change (LFC) to reduce the noise created by genes with low read counts having high fold changes.[37] Finally, the p-value is adjusted to a false discovery rate (FDR) value to reduce the chance of discovering false positives.[39] These methods ultimately allow significance testing at low sample sizes, although increasing replicates will always strengthen the results of the study. In this way, we can identify significantly up or down regulated genes in the clot model and find unique transcript clusters for each condition with only two donors.

Methods

Blood Collection

Blood was collected from 2 healthy consenting donors following IRB #1322641. 4 conditions were tested: FB (2.5mL), RV (3mL), hypoxia (2.5mL), and RV-LPS (3mL). FB was drawn directly into a Paxgene (Qiagen) blood collection tube and kept at room temperature following the manufacturer's instructions. For RV, 3mL blood was drawn directly into red vacutainer tubes and incubated for 4 hours at 37°C. For hypoxia, blood

drawn into a syringe was deposited in 0.5mL aliquots into 5 sterile glass culture tubes with steel vented caps. These incubated for 4 hours at 37°C, 1% O_2 and then were pooled together. The RV-LPS condition was drawn directly into an RV tube and immediately spiked with 100ng/mL LPS from E.coli (O111:B4, Sigma Aldrich) then incubated for 4 hours at 37°C.

Library Preparation

RNA-seq on total RNA was performed on an Illumina instrument to identify novel transcriptomic shifts on 8 samples (2 donors, 4 conditions). Blood clots were homogenized in Paxgene (Qiagen) and RNA extracted following manufacturer's protocol. After extraction, RNA was concentrated using the RNeasy MinElute kit (Qiagen) and globin mRNA depleted (Ambion GlobinClear, ThermoFisher). Agilent Bioanalyzer and Nanodrop assessed RNA quality and concentration. All RIN were greater than 7.0 (Table 4). 100bp paired-end libraries were generated by University of Pennsylvania Core Facilities. Poly-A+ RNA was selected with oligo-dT, fragmented, reverse transcribed to form cDNA, annealed with sequencing primers carrying indexed barcodes for identification, and PCR enriched. The indexed libraries were combined at 5nM, simultaneously sequenced by MiSeq to verify the library is balanced, adjusted if necessary, then sequenced on a flow cell of an Illumina Bioanalyzer. The 8 samples were processed as one library on two separate lanes of an Illumina NovaSeq 6000. Read depth was 30M reads for each sample, 15M per lane.

Data analysis

Sequencing data were processed in three ways. All methods pooled reads from both lanes together, for a total of 30M reads per sample. The first method used Rsubread to align and count the reads to the reference genome GRCh37 (Ensembl). Reads with a PHRED score less than 30 were filtered out. Counts were normalized following the Fragments Per Kilobase of transcript per Million reads (FPKM) method in Matlab. Log2 fold changes were calculated following a matched pairs design. The Matlab Bioinformatics toolbox performed unsupervised clustering to create heatmaps comparing each condition to fresh blood.

Data were also processed by University of Pennsylvania sequencing core facilities. Counts were aligned to the Refseq reference genome hg19 and analyzed in EdgeR comparing each condition to FB. Reads with PHRED < 30 were excluded. At n=2, EdgeR could not conduct a matched pairs design. Gene ontology (GO) analyses determined which pathways were activated in each condition. K-means clustering performed unsupervised clustering to group genes with similar inductions.

Lastly, data were again processed following Illumina Basespace Dragen RNA pipeline. The pipeline aligned and counted reads then performed DESeq2 comparing all conditions. At n=2, DESeq2 could not conduct a matched pairs design.

RT-PCR Validation

First strand cDNA was made using 500-1000ng RNA and AMV-Reverse Transcriptase methods. Second strand amplification used Econotaq, with an initial heating to 95°C for 3 minutes, denaturation at 55°C for 30 cycles, annealing at 72°C for 30 seconds, and a final

elongation at 72°C for 5 minutes. RT- PCR tested for VEGF mRNA induction in glass normoxia, RV, and glass hypoxia. CD45 served as a housekeeping gene. Primers from IDT are listed in Table 3.

Table 3. Primers for RT-PCR validation

Gene	Forward Primer	Reverse Primer	Product Size
CD45	5' - CAA CTG ATG AAT GTG GAG CC - 3'	5' - AGG AAG AAT GTC AAC ATA AC - 3'	164bp
VEGF	5' - TCG GGC CTC CGA AAC CAT G - 3'	5' - CTC CTG CCC CGC TCA CCG CCT CGG - 3'	165, 189 bp

Statistical Analyses

All statistical analyses were conducted through EdgeR (UPenn Core Facilities) or DESeq2 (Illumina Basespace) for n=2 biological replicates. Both software packages apply a linear model and shrinkage of the dispersion to limit within-group variance. Samples were normalized using median-of-ratios method, which accounts for variation in sequencing depth. The software calculated the log2 fold change per gene between groups, then applied a shrinkage model to reduce the fold change of low-expressed genes. A linear model shrunk the dispersion parameter, or variance in counts, to fit the model and reduce variance within groups. The p-value was calculated from the adjusted values. Both softwares used a Benjamini-Hochberg approach to minimize false discovery rates (FDR) of significantly altered genes.

Results

RNA for RNA-seq was extracted from FB and TE-clots using the Paxgene RNA kit and processed with RNeasy Minelute and Ambion GlobinClear to reduce globin mRNA counts. A total of 8 samples, 4 per donor, were sequenced. Table 4 shows the high quality and yield of each sample for sequencing. The library was made by UPenn core facilities using 250ng RNA, due to H5-LPS clot having a low total yield. At this amount, sequencing still generated 30 million reads per sample over 2 lanes.

Table 4. RNA quality results from Agilent Bioanalyzer.

Donor	Condition	Total Yield (µg)	RIN
H5	Fresh blood	3.9	7.6
H5	RV	2	8.3
H5	hypoxia	3.1	8
H5	LPS	0.7	8.1
H6	Fresh blood	3	7.6
H6	RV	1.9	7.5
H6	hypoxia	1.3	7.8
H6	LPS	1.8	8

Differential Analysis

DESeq2 differential analyses compared each condition (Table 5). All TE-clots had greater than 2300 differentially expressed genes compared to fresh blood. The clots themselves had more similar gene expressions, as shown in the generated heat map (Figure 8), with RV and hypoxia having only 28 differentially expressed genes. LPS had a difference in 237 genes to RV and 333 genes to hypoxia. Across all clots, transcripts were

more likely to be upregulated compared to fresh blood (Figure 9-11), with inflammatory cytokines being the most significantly upregulated transcripts.

Table 5. Number of differentially expressed genes between conditions. Analysis performed using Illumina Basespace DESeq2 software. N =2.

Sample	Number of genes expressed	Number of genes differentially expressed
FB v RV	16362	2392
FB v Hypoxia	16475	2581
FB v LPS	16364	2321
RV v Hypoxia	16283	28
RV v LPS	16147	237
Hypoxia v LPS	16297	333

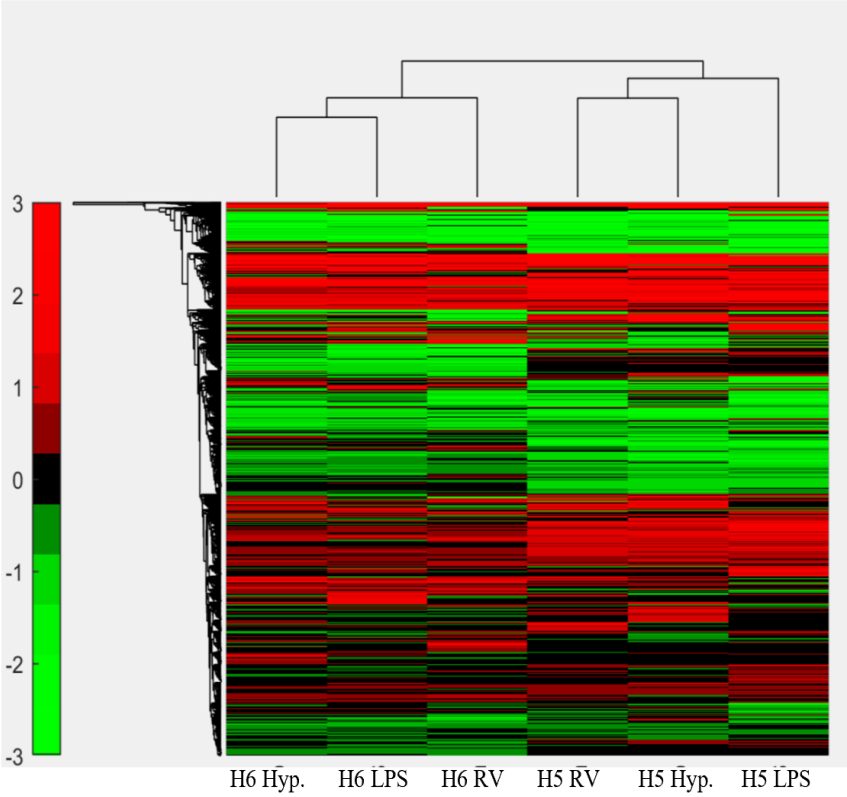


Figure 8. Heatmap of TE-clot transcriptome compared to FB. Data was analyzed in a matched pairs design. Reads were aligned and counted using Rsubread. Clustering and heat map generation used Matlab Bioinformatics Toolbox. FB served as the baseline for log fold change analysis.

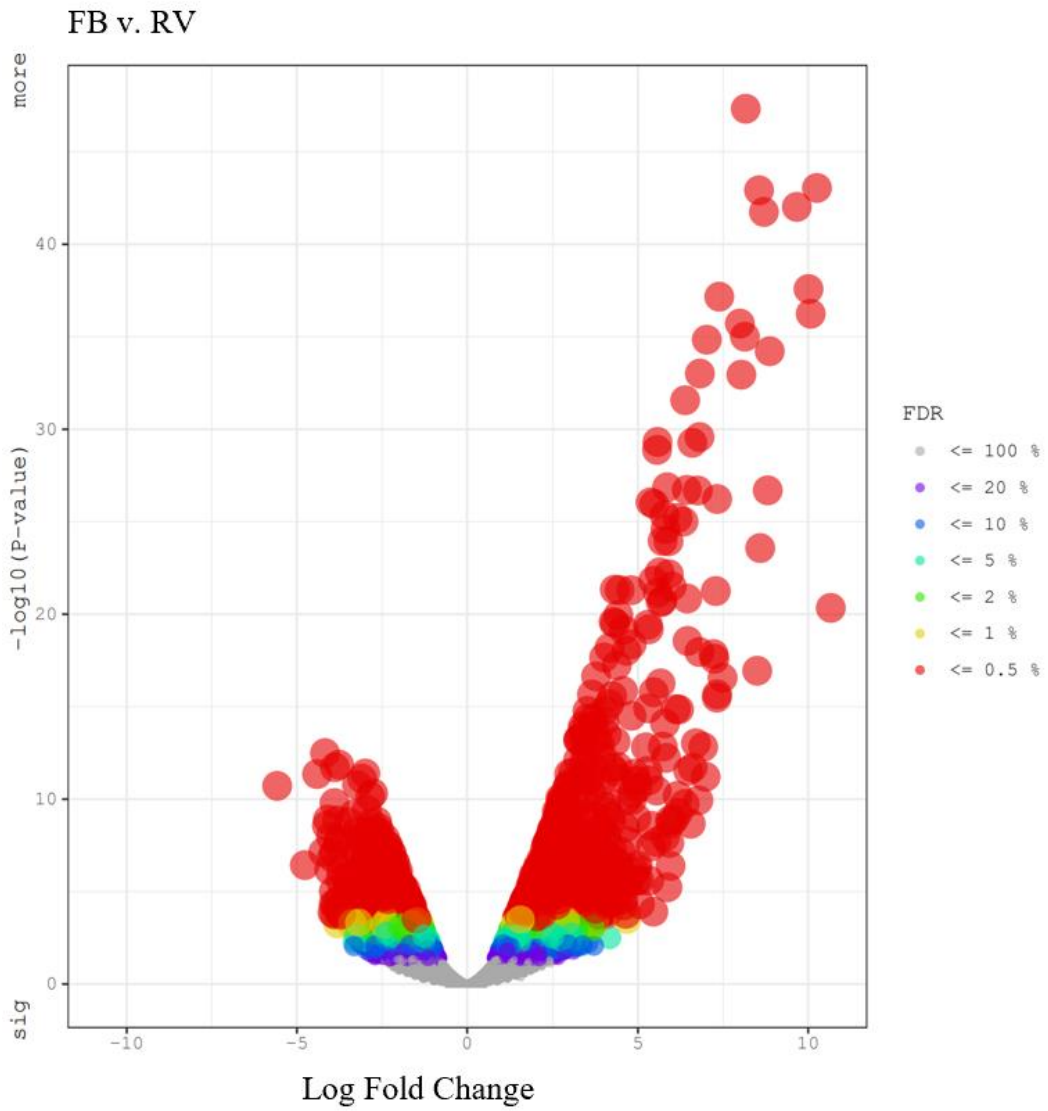


Figure 9. Volcano plot, FB v RV. Generated by EdgeR. Significance is based on log2 fold change and the false discovery rate. N =2.

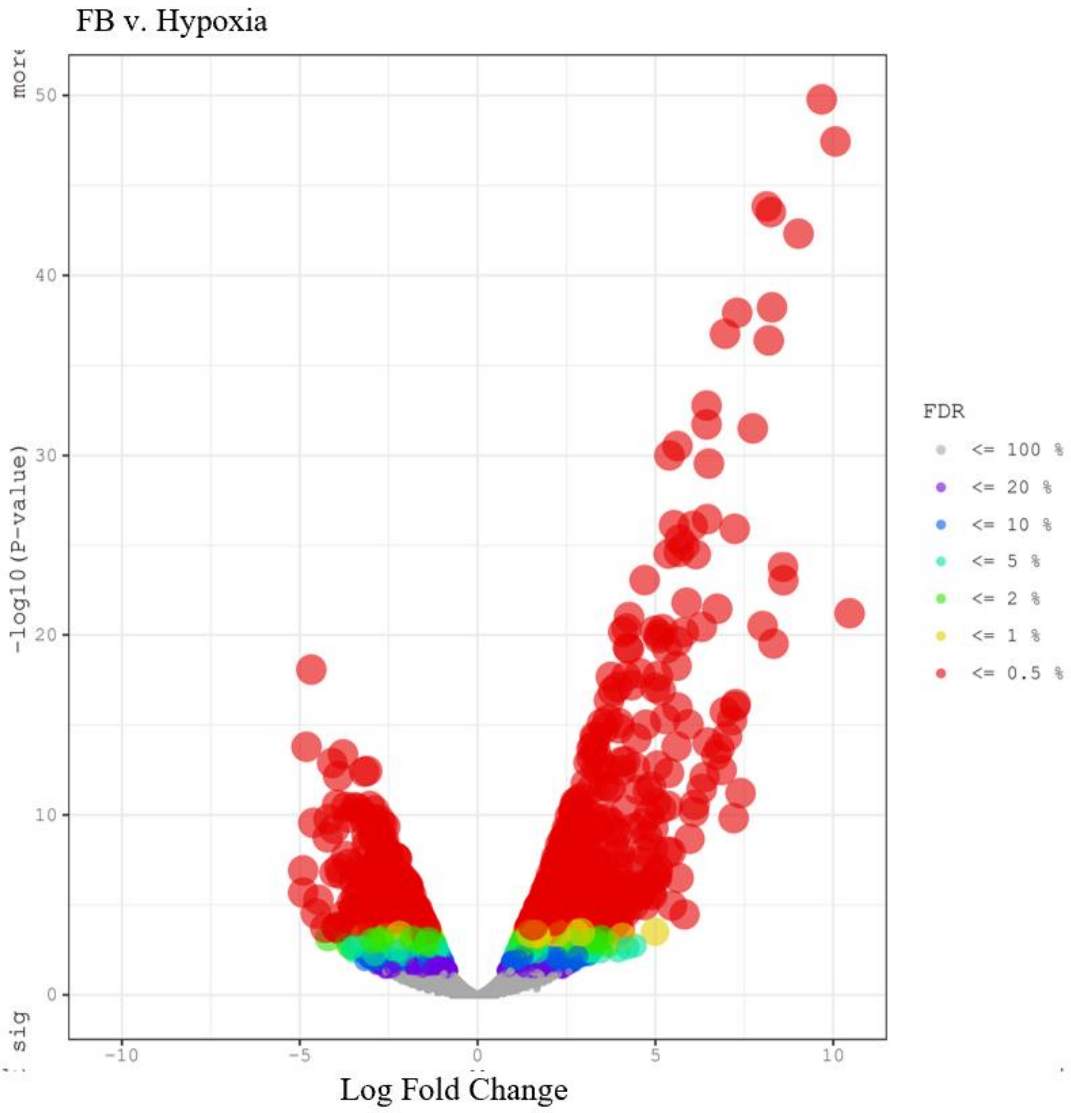


Figure 10. Volcano plot, FB v. Hypoxia. Generated by EdgeR. Significance is based on log2 fold change and the false discovery rate. N = 2.

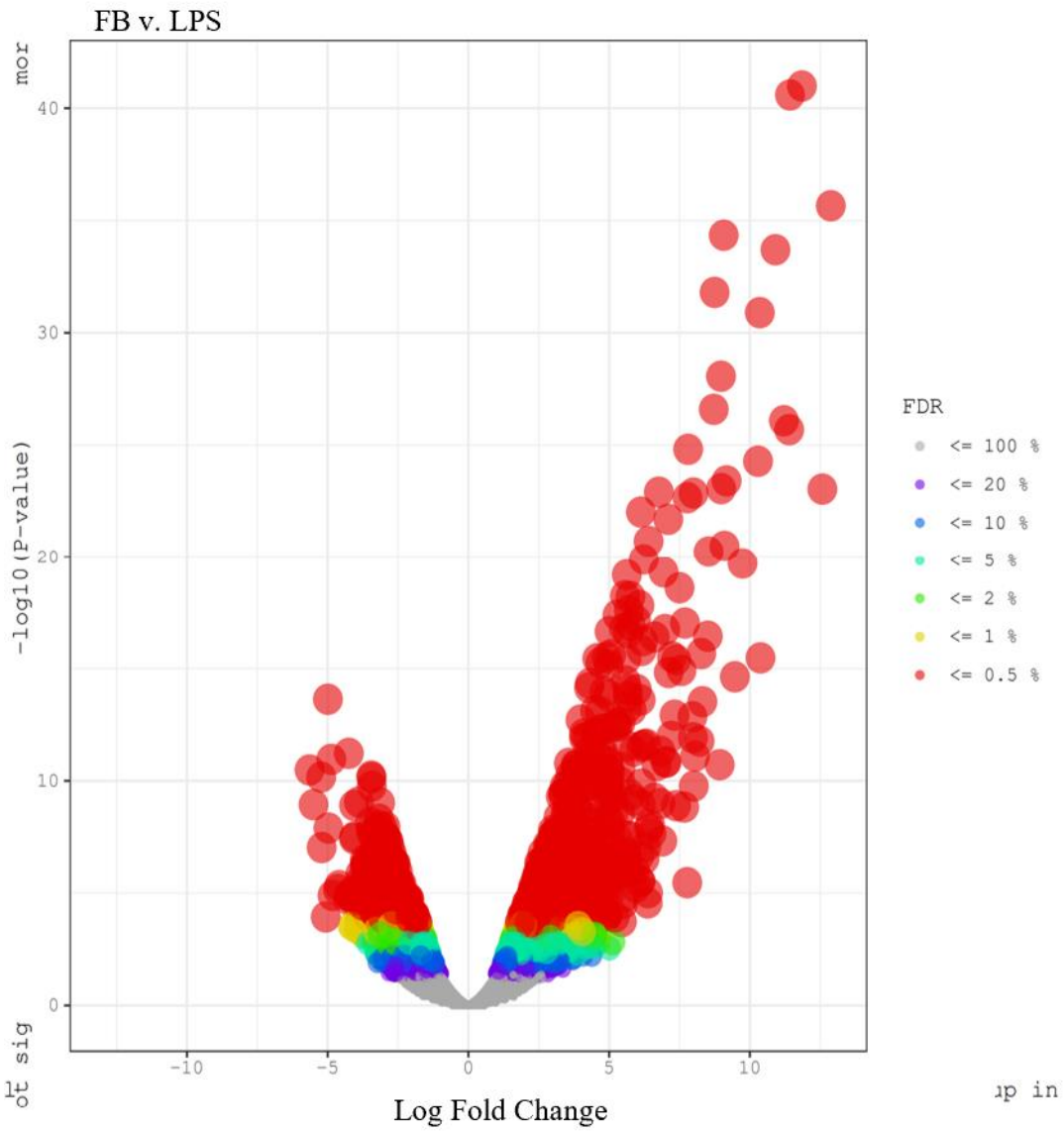


Figure 11. Volcano plot, FB v. LPS. Generated by EdgeR.. Significance is based on log2 fold change and the false discovery rate. N = 2

Principal Component Analysis (PCA) was conducted on EdgeR RNA-sequencing data by UPenn (Figure 12). PCA showed the greatest variation between FB and all other conditions. Clot conditions were more similarly grouped. Hypoxia and RV were highly overlapping. RV-LPS had more transcriptome differences compared to the other clotting conditions. PCA also revealed some donor-specific groupings. PCA results are in support of both DESeq2 and Matlab analyses on clotting inducing similar transcriptomics shifts compared to FB.

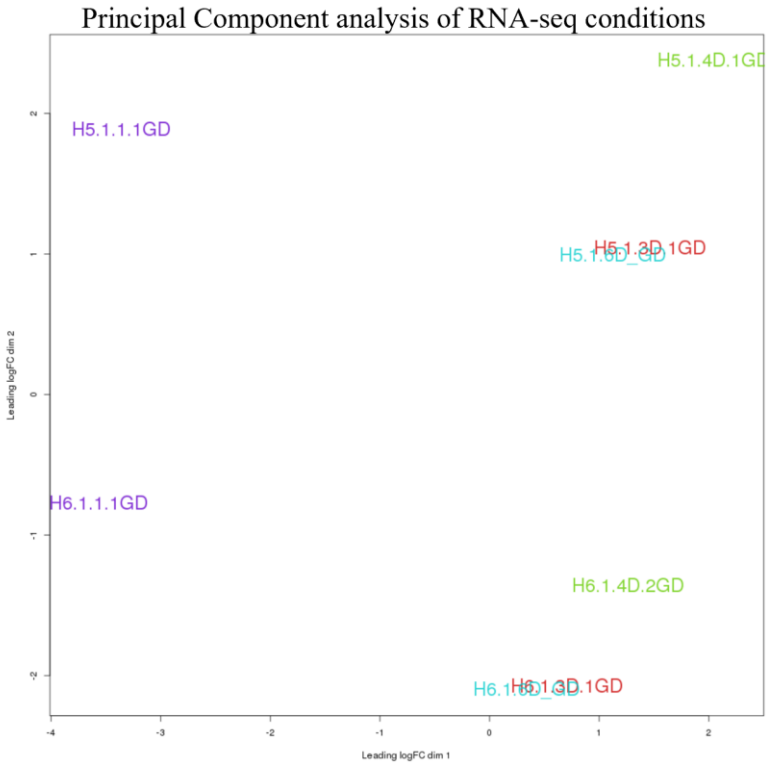


Figure 12. Principal Component Analysis. PCA was performed to compare each clot condition and determine the similarities within and between groups. 1.1.1GD (Purple) = FB, 1.3D.GD (Red) = RV, 1.6D.GD (Blue) = hypoxia, 1.4D.GD = RV-LPS. H5 and H6 are the labels for the two different donors.

Hypoxia

DEseq2 analysis showed only 28 differentially expressed transcripts between RV and hypoxia (Table 6), all of which were downregulated under hypoxia. The top 3 genes were all hemoglobin subunits, which may be an artifact of inadequate globin depletion across samples. Other notable genes include SGK1 and ATF3, which are involved in cellular stress responses.[40] PLPP3 is part of the VEGF family, involved in vascularization and pro-inflammatory cytokine induction, and is regulated by the NFkB pathway.[40,41] There were two novel proteins identified in this analysis, AC020909.1 and IGFN1. The unsupervised clustering analysis performed by UPenn did not identify a unique cluster to hypoxia. In all analyses conducted, the hypoxia transcriptome was not substantially different than RV.

All clots had more similarities than differences in hypoxia-related mRNA induction. Table 7 highlights genes known to be induced under hypoxic conditions and/or regulated by the NFκB pathway. Each TE-clot has similar fold changes across the genes listed. All transcripts were significantly upregulated compared to FB. CXCR4, which is induced by hypoxia and is involved in angiogenesis,[34] is one of the most significantly upregulated genes in all conditions. CXCL2, CXCL3, and IL-8, all known to be strong chemo-attractants, were also in the top-most upregulated genes. These chemokines participate in inflammatory signaling, WBC recruitment, and angiogenesis.[42] An interesting find is upregulation of the gene LDHA which regulates aerobic glycolysis, also known as the Warburg Effect, commonly seen in cancerous tissues.[43,44] All TE-clots also had overlapping GO analysis results with chemokine activity, cytokine binding, cytokine

activity, and regulation of immune response being some of the top expressed pathways. These results suggest all clots have hypoxic signatures that reflect *in vivo* hypoxic tissues.

Table 6. Genes differentially expressed between RV and hypoxia. RV serves as the control. Gene functions are from GeneCard.[40]

Gene	log2 Fold Change	P-adjusted value	Function
HBB	-3.30	5.46E-50	Oxygen transport
HBA2	-3.49	1.30E-21	Oxygen transport
HBA1	-3.72	3.43E-20	Oxygen transport
LHFPL2	-1.87	5.62E-09	Differences in male/female
PLPP3	-2.84	1.12E-08	vascular homeostasis, endothelial cell migration and adhesion, production of pro-inflammatory cytokines. VEGF family
CD109	-2.38	1.19E-07	Mediates TGFBR1 degradation to inhibit TGFB signaling
FNIP2	-1.91	5.61E-07	Cellular metabolism and nutrient sensing
GPNMB	-2.99	7.71E-07	Reduces metastatic potential
CCL7	-2.01	7.34E-06	Attracts macrophages
MMP19	-2.47	7.34E-06	Matrix degradation and remodelling
SGK1	-1.43	1.29E-05	Cellular stress response
ATF3	-2.04	1.30E-05	Cellular stress response
CCL2	-3.34	1.49E-05	Attracts monocytes and basophils
HSPA1B	-1.29	0.0001	Protects proteins from stress
EMP1	-2.67	0.0001	Novel protein
TM4SF19	-3.91	0.00046	proliferation, motility, adhesion
SDS	-2.54	0.0088	cell metabolism
CTSB	-1.01	0.0088	intracellular degradation and protein turnover
MT1X	-2.57	0.0088	possible anti-apoptotic signaling
ZMIZ1-AS1	-2.06	0.0088	Lnc RNA
RN7SKP176	7.59	0.0088	pseudogene
AC020909.1	7.54	0.010	Novel protein
CTSD	-1.45	0.012	Protein turnover
TBC1D2	-1.31	0.015	Vesicle-mediated transport
OLIG2	-2.86	0.019	chromosomal translocation
MT1G	-7.12	0.042	metal ion transporter
C3	-1.78	0.047	Activates complement system
IGFN1	-3.89	0.048	Novel protein

Table 7. Genes known to be induced by hypoxia. FB serves as the baseline control. All genes are significantly upregulated ($P < 0.05$) in TE-clots compared to FB.

Gene	L2FC FB v RV	L2FC FB v Hypoxia	L2FC FB v. LPS	Function	Author, Year
CXCL2	11.9	11.7	13.5	CXCL2 is a chemoattractant released by macrophages to recruit neutrophils. It is upregulated in injury and infection. Shown to be TLR4-dependent. It is induced by VEGF and IL-1β.	Filippo et al., 2013[45] Mohr et al.,2017[35]
CXCL3	10.6	10.6	12.3	CXCL3 is induced by VEGF, IL-1β. It is a pro-angiogenic chemokine. It is mediated by the NFκB pathway. Shown to be induced in monocytes under hypoxia.	Mohr et al.,2017[35] Korbecki et al., 2021[42]
CXCR4	3.3	3.2	3.0	CXCR4 is a receptor for the growth factor SDF1 (CXCL12), induced by hypoxia and involved in angiogenesis. It mediates LPS and TNF responses. It is important in wound healing for cell migration and vascularization.	Tirpe et al., 2019[34]
HIF-1α	2.5	2.2	3.3	HIF-1α protein is stabilized under oxygen deprivation and regulates metabolic and angiogenesis factors for cell survival. Infection upregulates HIF-1α transcription.	Nizet et al., 2009[14]
HILPDA	1.7	2.0	3.9	HILPDA is a HIF-1α target gene. Stimulates expression of cytokines including IL6 and VEGFA. Enhances cell growth and proliferation.	Bosco et al., 2006[18] The Human Protein Atlas[46]
IL-1α	8.5	8.7	14.4	IL-1α is cytokine regulated by the NFκB pathway. Upregulation seen in infected monocytes.	Li et al., 2008[47]
IL-1β	2.9	2.9	6.6	Hypoxia upregulates IL-1β, which is a pro-inflammatory macrophage cytokine.	Nizet et al., 2009[14]
IL-8 (CXCL8)	8.5	8.5	9.1	IL-8 is induced by VEGF, IL-1β, hypoxia, ROS, and bacteria. It is mediated by the NFκB pathway. It recruits macrophages to site of injury or infection during the acute immune response.	Mohr et al., 2017[35] Ha et al., 2017[48]
LDHA	2.5	2.2	2.3	LDHA is necessary for aerobic glycolysis (Warburg Effect) by catalyzing reduction of pyruvate to lactate. Inhibiting LDHA reduced glucose uptake	Pathria et al., 2018[43]
MCP-1 (CCL2)	10.3	7.0	11.6	Attracts monocytes to inflammatory sites. Increased MCP-1 has been highly reported in inflamed and cancerous tissues.	Yoshimura et al., 2018[49]
P4HA1	2.7	2.7	2.2	During hypoxia, cell modulates translation to conserve energy. HIF1a, VEGF, P4HA1 bypass this regulation and bind to internal ribosomal entry site.	Bosco et al., 2006[18], Lee et al., 2020[50]

				It stabilizes HIF-1α and is involved in nonglycolytic cell metabolism.	Xiong et al., 2018[51]
PDK1	1.5	1.5	1.6	Prolonged hypoxia results in upregulated of PDK1 to control ROS production and prevent cell death	Lee et al., 2020[50]
TINAGL1	9.2	9.3	8.3	TINAGL1is involved in cell survival, migration, proliferation, differentiation. Upregulated in metastatic tumors. It is dependent on VEGF, TGF-β pathways. Upregulation seen in intracellular hemorrhage.	Sun et al., 2019[52] Walsh et al., 2019[53]
VEGF	5.7	5.5	5.7	Cells in hypoxia have significant upregulation of VEGF within a few hours. VEGF induces vascularization to increase perfusion in the hypoxic area.	Shweiki et al., 1992[54]

Given the similar fold changes of hypoxic genes between all three clot conditions, a mathematical analysis of diffusion assessed whether oxygen was diffusing completely through the clot within 4 hours in an atmospheric condition, described as 20% oxygen. The equation for 3-dimensional diffusion is given in Equation 1.[55]

Equation 1. 3-Dimensional Diffusion

$$r^2 = 6Dt$$

Following the diffusion equation and solving for time, it would take 26 hours for ambient air to fully permeate to the center of the clot. By 4 hours, oxygen can permeate only 1.46cm. Assumptions made here are that diffusion is 3-dimensional and the clot has a diffusion coefficient similar to that of a 3D fibrin gel (D ~ 2.5x 10^{-5} cm^2/s).[56] As well, given the constraints of the clot by the plastic, air can only diffuse through the top of the clot. In the RV tube, the radius from the top to the center of the clot is approximately 3.8cm, and the residual air composition is unknown. Repeating these steps on the 0.5mL clot in the glass tubes and assuming a spherical shape, the distance to the center of the clot is 0.492cm. At

this size, it takes 27 minutes for air to diffuse to the clot center. Given these results, all clots incubated in an RV tube are at least partially hypoxic. However, the 0.5mL clots in glass tubes have full air permeation during incubation.

RT-PCR Validation

The next step was to further study whether the RV tube incubated at 37°C represented the glass normoxia condition, or if it could be considered a replicate of hypoxia. VEGF is known to be induced under hypoxic conditions, so it was not expected to be upregulated in a clot incubated in normoxic conditions. However, RNA sequencing revealed that it is significantly upregulated in RV, hypoxia, and LPS. RT-PCR analyses tested VEGF RNA expression in glass normoxia, RV, glass hypoxia, and LPS clots from 3 donors (Figure 13). RT-PCR results show all clot conditions have faint VEGF expression within 4 hours, although this was not consistent across donors (Figure 9). This suggests that in the clot environment, all clots may be inherently hypoxic, even when exposed to 20% oxygen. However, the RV tube may help further drive this hypoxic environment due to the size of the clot. More hypoxia-related transcripts will need validating before concluding all blood clots are hypoxic.

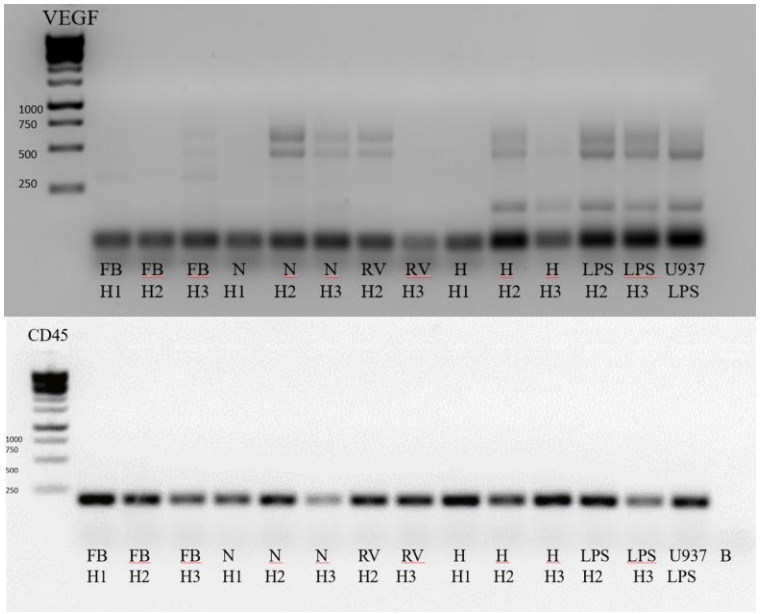


Figure 13. VEGF RT-PCR on blood clots from 3 donors. N = glass normoxia, H = hypoxia, B = blank. H1-H3 are the 3 biological replicates. RT-PCR cycled for 35 cycles. The bands represent different VEGF isoforms. U937 cells stimulated with LPS served as a positive control.

LPS Transcript Induction

LPS-stimulated blood clots created a unique genomic cluster of transcripts involved in dendritic cell (DC) activation and maturation (Figure 14a).[57,58] This cluster was identified from the k-means clustering performed by UPenn Core Facilities. It included CCL3, CCL4, CXCL10, IL-12β, IL-6, Rsad2, and TNFAIP6. CXCR4 and CD83 were not in this cluster, as they are expressed in all TE clots, but they were included in Figure 10 given their role in DC maturation. Gene functions in relation to DC maturation are highlighted in Table 8. These transcripts were upregulated in both donors, although one had a stronger response (Figure 14b). It is unknown if this is due to a greater response to LPS or to more circulating plasmacytoid dendritic cells (pDC). The healthy range of pDC

is 3,000-17,000 pDC/mL blood.[59] This large range could account for differences in DC transcript induction.

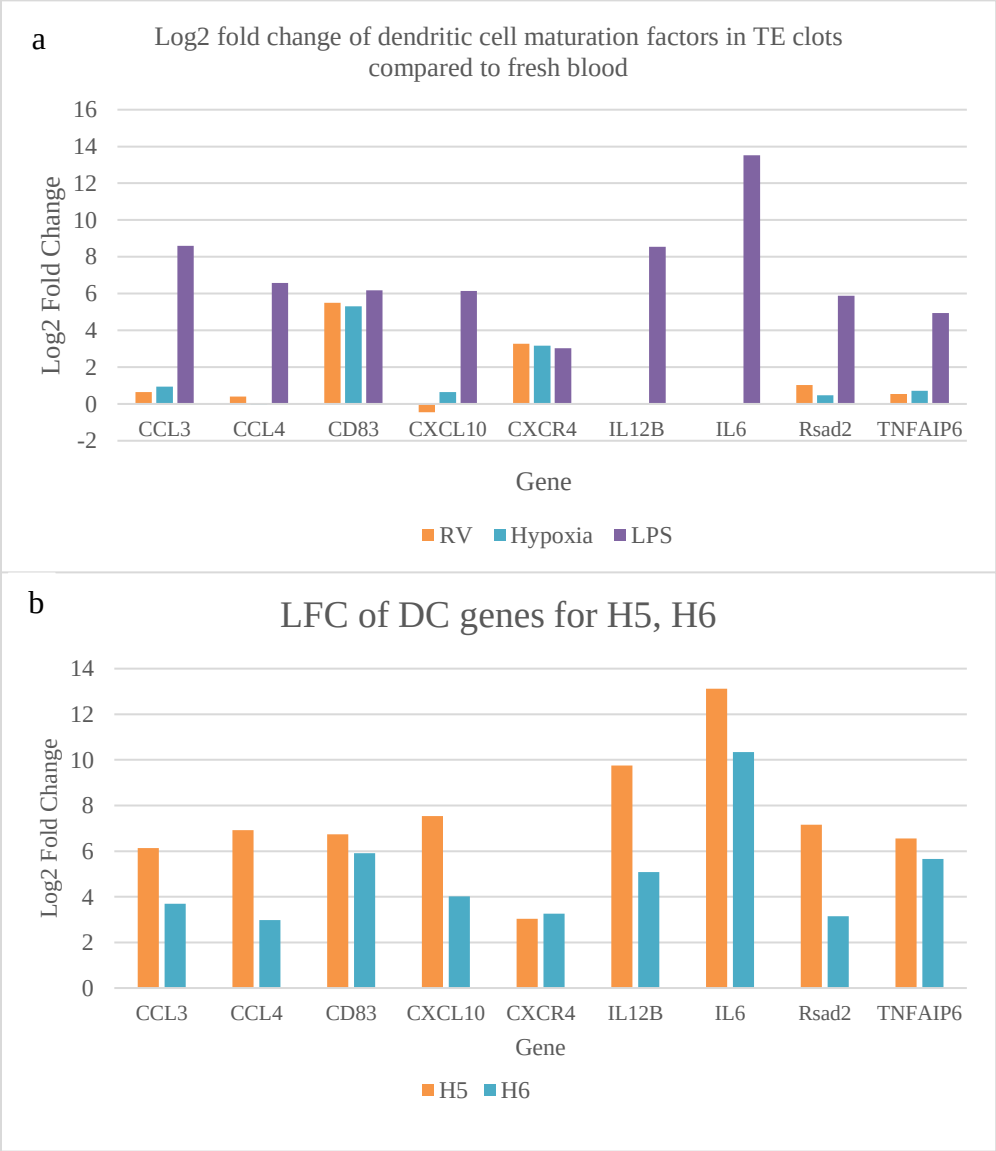


Figure 14. DC-related genes expressed in LPS clots. A) Log2 Fold Change of DC maturation genes in TE clots, . LFC calculated using DESeq2. B) Comparison of log fold change of DC maturation genes in LPS clots between donors. LFC calculated using Matlab, using a matched-pairs design and without shrinkage methods, accounting for the differences between LFC. N =2.

Table 8. Dendritic cell maturation genes and their function.

Gene	Role in DC activation and maturation	Reference
CCL3	Neutrophils release CCL3 to recruit DCs to areas of inflammation and infection. pDCs release CCL3 in response to endotoxin.	Charmoy et al., 2010[60] Megjugorac et al., 2004[61]
CCL4	Pro-inflammatory chemokine expressed by DCs in response to viral stimulation.	Megjugorac et al., 2004[61]
CD83	Marker most highly expressed by activated DC, it is also expressed by other immune cells including monocytes and neutrophils	Li et al., 2019[62]
CXCL10	Cytokine CXCL10 expression is upregulated by pDCs after viral stimulation	Megjugorac et al., 2004[61]
CXCR4	CXCR4 is a receptor for CXCL12. DC maturation by LPS results in high CXCR4 upregulation. The CXCL12/CXCR4 complex helps DCs migrate.	Kabashima et al., 2007[63]
IL12b	IL12b is a subunit that comes together with IL12a to form IL-12. IL-12 is a pro-inflammatory cytokine that DCs secrete for 8-12 hours after maturation. DCs produce high amounts of IL-12 after LPS stimulation.	Ma et al., 2015[64] Efron et al., 2003[57] Megjugorac et al., 2004[61]
IL-6	IL-6 is upregulated in the IRF7 pathway. It is highly expressed by pDC.	Laustsen et al., 2018[65]
Rsad2	Rsad2 is highly expressed in a murine model of dendritic cell maturation. It is mediated by the IRF7 pathway, which can be TLR4 dependent.	Jang et al., 2018[58] Ning et al.[66]
TNFAIP6	DC maturation upregulates TNFAIP6, which is an apoptosis regulator.	Schinnerling et al., 2015[67]

Discussion

The RV, hypoxia, and LPS blood clots had a massive transcriptional shift due to clotting at 37°C compared to FB, with over 2300 transcripts differentially expressed. Most of these transcripts were upregulated, the most significantly expressed genes including chemo attractants CXCL2, CXCL3, IL-8/CXCL8; NFκB-associated genes; and VEGF-associated genes. In this way, the clot tissue model mimics an *in vivo* sterile inflammatory environment that is characterized by immune cell signaling and migration via NFκB regulation.[35] RV and hypoxia had a very similar gene induction, leading us to question whether RV mimics a normoxic or hypoxic state. As well, it is estimated that the size of the RV clot did not allow full oxygen diffusion within 4 hours, while the normoxic clots had oxygen diffusion to the center in under 30 minutes, strengthening the RNA-seq results

that the RV clot is comparable to the hypoxia clot. To test for this, RT-PCR analyzed VEGF expression in normoxic, RV, hypoxic, and LPS clot conditions. VEGF was chosen because VEGF transcription is typically only upregulated in hypoxic environments to promote angiogenesis and restore oxygen supply.[35]All 4h clots had VEGF induction, although it was not consistent across donors. While more genes need to be studied, it indicates that a clot is inherently hypoxic. Campbell et al. saw monocytes increase inflammatory signaling in fibrin clots incubated at 37°C with no added hypoxia.[12] Inflammatory cytokines upregulated in the fibrin clots were also upregulated in *in vivo* thrombotic clots extracted from patients. *In vivo* thrombosis creates a hypoxic environment by reduced oxygen availability and increased WBC oxygen demand.[19] Because similar inflammatory cytokines were seen in normoxic fibrin clots and *in vivo* thrombotic clots, it supports the results that incubation in additive hypoxia has negligible effects. These results failed to support the original hypothesis that clots incubated in hypoxic conditions have amplified HIF1-α and NFκB gene expression compared to clots in normoxic conditions. However, this has further implications in how we look at coagulopathy and microthrombi. All clots within the body may be sending already-amplified stress signals, no matter the size of the clot, which may further disease progression. This is an important area of future research to address.

Addition of LPS to the clot created a unique genomic cluster compared to RV and hypoxia, with upregulation of genes associated with DC activation. This finding was not expected but has interesting implications. DCs are antigen-presenting cells. Immature DCs become activated upon recognition and capture of non-self antigens.[68] Once captured, DCs

undergo maturation so they can migrate back to lymph nodes to activate T cells and begin the innate immune response.[68] DCs orchestrate the body's response to infection, as well as allergies and even vaccines.[69] Currently, human blood DCs are difficult to isolate and culture, with only 50% surviving past 24 hours.[65] Because of this, most dendritic cell models are murine, leaving a gap in understanding of DC roles. This model shows promise of DC activation and maturation within 4 hours in response to endotoxin. From this, researchers could study responses to infection, medication, allergies, and vaccines quicker and on a personalized level. Even with n=2, RNA-sequencing revealed donor-specific responses to endotoxin, with one donor having a stronger pro-inflammatory response and greater upregulation of DC-related genes. As we increase sample size, we can better determine what constitutes as "normal" and "dysregulated" then apply to disease states from patients, or for diagnostics before determining medications.

Limitations to this study are the sample size of N=2 resulting in low statistical power and inability to determine whether a response was an outlier. Although the study design was to have a matched-pairs LFC analysis, EdgeR and DESeq2 were incapable of performing a matched-pairs design. Therefore, all counts were averaged for each gene, making it difficult to discern if donors had variable responses without individually comparing read counts. For the glass normoxic clots, more PCR validation needs to be done to conclude the glass normoxia clots have a hypoxic response. RT-PCR on VEGF alone, along with inconsistency across donors, cannot fully support this statement.

Conclusion

The clot model successfully stimulated healthy blood to show an immune response by allowing the unmodified blood to clot at 37°C. RNA-sequencing revealed over 2300 differentially expressed genes in the cultured blood clots compared to FB. The transcriptome shifts seen in WBC are supported by previous literature on inflammation and infection, which are characterized by hypoxic responses. This induction was consistent in all clots, with upregulation of VEGF and NFκB-associated transcripts. An additional endotoxin spike revealed a unique genomic fingerprint, with DC-related mRNA induction. Ultimately, RNA-sequencing validated that the tissue-engineered blood clot model has WBC signaling that mimics both *in vivo* inflammation and infection. Future work incorporating disease states could reveal dysregulation on an individual level.

CHAPTER FOUR. PERSONALIZED MEDICINE AND DIAGNOSTICS

Introduction

Severe COVID-19 increases the risk of coagulopathy and ARDS. 70% of non-survivors have evidence of coagulopathy, including heightened D-dimer levels and fibrinogen, compared to only 1% of survivors.[70] As well, severe COVID-19 patients have upregulated circulating inflammatory cytokines. High levels of IL-6 have been associated with increased mortality in COVID-19.[33] It is currently recommended that all hospitalized COVID-19 patients are put on anticoagulants.[70] Two common anticoagulants include Aspirin (ASA) and Dexamethasone (Dex).[71,72] Both drugs are well-established anti-inflammatory medications. ASA has a long history of being used to treat patients with chronic hypertension to reduce the risk of heart attack and stroke.[73] Dex is a glucocorticoid commonly used to reduce the pro-inflammatory factors seen in ischemia, infection, and trauma.[74] Although all hospitalized COVID-19 patients are recommended for anticoagulant treatment, these results have been controversial. Anticoagulant treatment did reduce the risk of mortality, but users were more likely to require mechanical ventilation and have major bleeding.[75] Now, researchers are calling for a more individualized diagnostic procedure to decide the best treatment option.[70,75,76] This may include limiting anticoagulant treatment if the risk of thrombosis is low or screening for the best medication and dosage on an individual basis.

This study serves as a proof-of-concept that anticoagulant medications can be studied on an individual level using the TE clot model. On the same two donors from RNA-

sequencing, blood was drawn and treated with high and low doses of ASA and Dex, with and without an endotoxin spike to mimic infection. Blood clotted for 4 hours at 37°C and serum extracted from the clots for ELISA to test for the inflammatory factors IL-8 and IL-6. The null hypothesis was that both drugs would reduce IL-6 and IL-8 inflammation by at least 50%. The results showed different mechanisms of actions between both drugs, as well as a donor-specific response.

Methods

Blood Collection

A citrate tube and a 10mL syringe of blood were drawn from two healthy consenting donors following IRB # 1322641. 0.5mL of blood was deposited into sterile glass culture tubes with steel-vented caps, 10 per donor. Blood was spiked with ASA or Dex at low and high doses. Half of the clots were LPS -spiked at a concentration of 100ng/mL. Conditions were as follows:

1. WB
2. WB + 0.1µg/mL ASA
3. WB + 30µg/mL ASA
4. WB + 10nM Dex
5. WB + 1µM Dex
6. WB + LPS
7. WB + LPS + ASA 0.1µg/mL
8. WB + LPS + ASA 30µg/mL
9. WB + LPS + 10nM Dex
10. WB + LPS + 1µM Dex

Blood incubated for 4 hours at 37C, 20% O_2. After incubation, tubes were immediately put on ice and serum extracted.

Serum Extraction

Samples were centrifuged at 200g for 10 minutes. Serum was transferred to a clean Eppendorf tube then spun again at 1300 RCF to remove any remaining platelets within the serum. Serum was aliquoted into cryovials, flash frozen, and stored at -80°C.

ELISA

ELISA analyses followed the manufacturer's protocols (R&D Systems) using serum extracted from the blood clots. Citrated plasma served as a negative control. LPS-spiked clot serum served as a positive control.

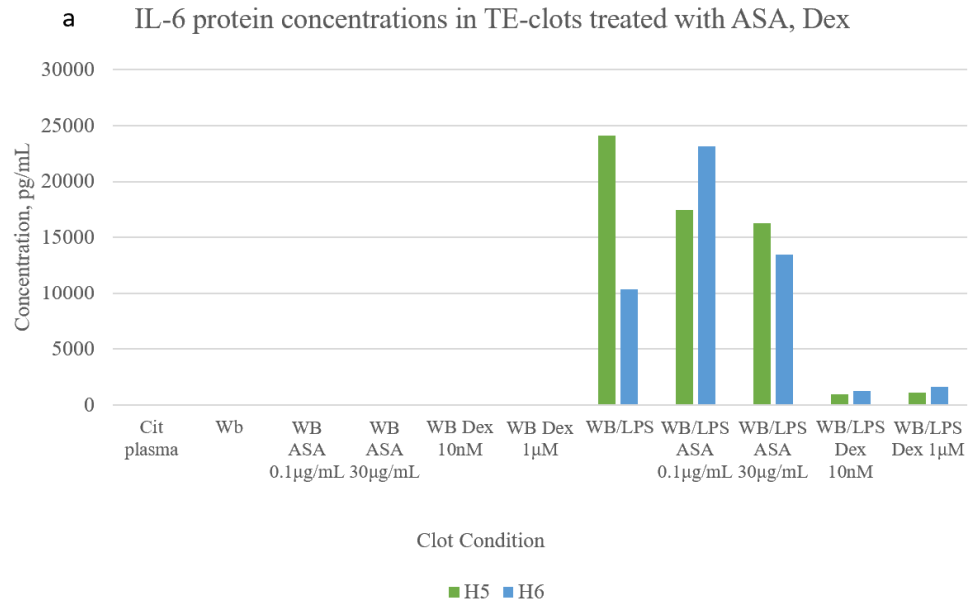
Statistical Analyses

No statistical analyses were conducted for N =2.

Results

Whole blood with and without LPS were treated with the anti-inflammatory drugs ASA and Dex and allowed to clot for 4h at 37°C. After incubation, clots were put on ice then serum extracted. The ASA-spiked blood failed to clot for both donors, while Dex-spiked clots had coagulation. Two ELISA analyses were performed to test whether ASA and Dex could inhibit the pro-inflammatory cytokines IL-6 and IL-8 compared to cultured clotted blood (Figure 15). IL-6 was only detectable in LPS-spiked clots (Figure 15a). LPS greatly increased IL-8 levels (Figure 15b), which agreed with previous IL-8 analyses. Both drugs could inhibit IL-8 by 50% in WB clots for both donors. This finding is significant because ASA most probably inhibited IL-8 by preventing coagulation, while Dex could inhibit IL-8 induction after coagulation. On the other hand, the LPS-spiked clots had donor-specific drug responses and an inconsistent dose-dependent response. Aspirin failed to

inhibit IL-8 induction in LPS-spiked clots for either donor but did reduce IL-6 levels for H5 LPS clots only. H6 LPS-spiked clots had greater IL-8 inflammatory suppression to low dose Dex than high dose, while Dex effectively suppressed IL-6 levels at both doses tested. These findings show the variability, even among two donors, of the anti-inflammatory effects of commonly prescribed medications.



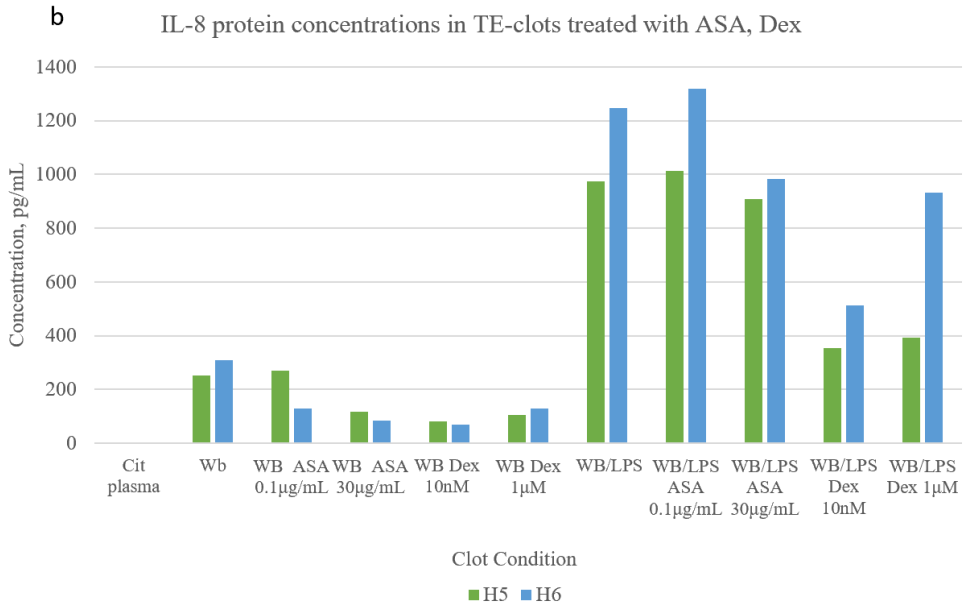


Figure 15. IL-6 and IL-8 ELISA ASA, Dex treated TE-clots. a) IL-6 serum concentrations in TE-clots treated with high and low dose ASA and Dex. b) IL-8 serum concentrations in TE-clots treated with high and low dose ASA and Dex. Citrated plasma served as the negative control. LPS-spiked clots, no treatment served as the positive control. N=2.

Discussion

TE-clots were treated with ASA and Dex as a proof of concept for using blood clots to determine a person's anti-inflammatory response to non-steroidal anti-inflammatory and steroidal anti-inflammatory medication. First, the model revealed how the drugs react differently when clots were unmodified or spiked with LPS. Both drugs worked well to reduce IL-8 levels in whole blood alone, potentially through different mechanisms, but this was not the case when an endotoxin was present. For the two donors there were variable effects in treatment for the LPS-spiked clots, with one donor having increased IL-8 and IL-6 concentrations in response to ASA, and the other having no remarkable change in inflammation. This could be due to potential dysregulation at high endotoxin levels. Sepsis-induced coagulopathy causes an increase in pro-inflammatory cytokines and dysfunction,[3] which the LPS-spiked clot mimics in this study. Only Dex consistently reduced the cytokine levels for both donors, with the low dose being more effective. This proof of concept effectively shows that users have variable responses to medications, and it is quantifiable via the TE-clot model.

Dex's outperformance on reducing IL-6 and IL-8 is due to the different mechanics of the drugs. ASA and Dex differ in that ASA is a non-narcotic pain reliever, while Dex is a steroid hormone mimetic. Both have anti-inflammatory effects. ASA works by inhibiting COX-1 and COX-2 to reduce downstream inflammatory processes and by inhibiting thromboxane, which is necessary for platelet activation.[73] ASA is 170 times more potent in inhibiting COX-1, which involves platelet activation, than COX-2, which inhibits cytokines.[77] Two different studies reported the IC50 needed for ASA to inhibit COX-2 at

5.67mM and 50 +/- 10 μg/mL.[78,79] Our high dose ASA was below both of these numbers at 30μg/mL (0.167mM). This could explain why we saw inhibited blood clotting in ASA, but ASA did not reduce cytokine levels. H6 having increased IL-6 and IL-8 levels at low dose ASA may not be a donor response but instead ASA reacting with the IkB pathway that upregulates NFκB.[78] In another study, macrophages incubated in LPS + low dose ASA had increased IL-6 and ROS than LPS alone.[80] On the other hand, Dex works by specifically inhibiting cytokine gene expression,[81] explaining why it was more efficient at IL-6 and IL-8 attenuation. Dex has been shown to trigger the anti-inflammatory state by reducing TNF-α, IL-6, and IL-8 and upregulating IL-10.[74] Although the cells may be shifting to anti-inflammatory responses, the blood still clotted in Dex samples. The different mechanisms in these drugs being used in COVID-19 could have profound effects on a patient's outcome. ASA may prevent coagulopathies but could increase systemic IL-6 levels that lower the chances of survival. Dex works in the opposite way, in that it seems to not prevent coagulopathies, but could lower the pro-inflammatory cytokine concentrations and lessen disease severity. Exploring both drugs' effects on thrombin and D-dimer may give more insight into if they are effective treatments for infection-induced coagulopathy. As well, RT-PCR and ELISA on IL-10 could show whether cells are shifting towards an anti-inflammatory response.

Limitations to this study include variation in some of the duplicates, perhaps due to inadequate stop solution in particular ELISA wells or inadequate mixing during diluting. As well, the small sample size was n=2 donors, which prevents statistical analyses and limits the ability to determine what a "normal" response is to each medication.

Conclusion

ASA and Dex are two medications being used to reduce infection-related coagulopathies, but their efficacies have been variable. This study served as a proof-of-concept that the TE-clot model can be used as a personalized diagnostic tool. Between two donors, there were variable responses to the drugs, and a dose-dependent effect was not seen. This shows promise of moving forward and studying ASA and Dex on more donors to understand why there are variable responses, and whether this model can be used in a clinical setting for drug screening before treatment.

CHAPTER FIVE. CONCLUSION

The goal of these studies was to determine whether blood clots incubated in hypoxic conditions further amplify an immune response compared to normoxic clots, with the long-term goal of translating this work to a clinical setting to examine disease states such as ARDS, which is characterized by hypoxia, cell dysfunction, sepsis, and mortality.[82] First, a translatable blood clot model had to be created. Previously, blood clots were incubated at 37°C in glass culture tubes. However, the glass tubes are not feasible in a clinical setting. Therefore, RV tubes were explored as an alternative. ELISA and RT-PCR analyses comparing RV, normoxic glass culture tubes, and hypoxic glass culture tubes revealed no significant difference in IL-8 upregulation. Within the TE-clot model, IL-8 transcription was upregulated in as little as 45 minutes, with IL-8 proteins being detectable in the serum within 4 hours. ELISA IL-8 concentrations were similar to what was previously reported.[6] Incubation in a portable incubator, which only regulates temperature and not oxygen or carbon dioxide levels, had comparable IL-8 levels to the RV clots cultured in a laboratory Heracell incubator, verifying a portable incubator could be used in a bedside setting. Because an RV tube is a closed environment, the incubator gases are irrelevant. The next step was to determine that high quality RNA with a sufficient yield could be extracted from blood clots without having to isolate the WBC first. Clots were homogenized in Paxgene buffer then RNA extracted and quality controlled. Results on 39 RNA-extracted clots showed high quality RIN of greater than 7.0, and at least 1µg RNA, sufficient for RNA sequencing. From these results, the study moved forward with RNA-sequencing.

RNA-sequencing revealed a blood clot is a living, functioning tissue rather than an inert clump of cells. All conditions had a massive transcriptomic shift of over 2300 differentially expressed genes compared to FB. The cells enveloped in the tissue released pro-inflammatory signals to recruit immune cells to the area. This is supported by previous work by Hoemann et al., and Campbell et al., which revealed WBC encapsulated in a clot or fibrin gel release the pro-inflammatory factors IL-8 and MCP-1 that can be detected on both the mRNA and protein level.[6,12] The blood clot condition did little to alter the transcriptomic shift seen due to clotting; blood clots incubated at 37°C, under hypoxic conditions, and with an LPS spike all showed upregulation of NFκB-associated genes. The NFκB pathway is induced *in vivo* under hypoxic environments created during inflammation and infection.[14] Clots incubated in a true normoxic environment had VEGF expression, supporting an argument that all clots are inherently hypoxic due to thrombin formation. Incubation in a hypoxic environment (1% O_2) did little to enhance the inflammatory effect already created via thrombin acting as a DAMP to immune cells. Ultimately, in the blood clot model healthy blood with otherwise undetectable IL-6 and IL-8 were able to be manipulated via clot formation and culture at 37°C to induce a large transcriptomic shift that mimics an *in vivo* inflammatory state.

The TE-clot model can be used for basic research or drug screenings of hypoxic disease states like ARDS and dendritic cell function. Comparing healthy blood clots to clots from a patient in disease state could reveal dysregulation patterns in true disease-state blood, as this study reveals endotoxin spiking creates a unique transcriptomic fingerprint in otherwise healthy adults compared to the cultured clots. LPS created a unique cluster of

DC-associated genes in response to endotoxin. This finding has positive implications in studying DC function, as it is currently difficult to isolate and culture human DC cells.[65] For diagnostics and therapeutics, it could be a practical drug screening tool to determine the best medications for an individual. For example, the proof-of-concept study examining two donors' responses to ASA and Dex revealed one donor had increased inflammatory factor levels with ASA treatment. In COVID-19, increased IL-6 concentration is associated with increased risk of mortality. The blood clot model could help address gaps in not only basic research, but also translational work.

Limitations to these studies are the small sample sizes, especially for RNA-sequencing and the ASA/Dex studies having only 2 donors. In the ELISA studies, N = 6 cannot explain the variation in donor IL-8 levels, as the study included donors of varying demographics and lifestyles. It will be important moving forward with disease states to have more of a matched-pairs design between healthy and disease state donors.

Future work will focus on increasing the sample size of RNA-sequenced clots to produce more meaningful data, especially with a heterogeneous population. Much of this work focused on upregulation of pro-inflammatory factors and did not address anti-inflammatory mediators that may have balanced out the large immune response. Future studies will need to look at both pro- and anti-inflammatory transcripts to get a better understanding of the acute response within the clot. As well, it has not been tested how the clot serum affects endothelial cells, and whether it may drive dysfunction in vasculature. Once these points are addressed in the healthy donors, these studies will shift to looking at

blood from hypoxic disease states, such as ARDS, and determining if the clot model truly mimics an *in vivo* hypoxic condition.

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