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THE RHEOLOGICAL IMPACT OF CELL ACTIVATION ON THE FLOW BEHAVIOR OF NEUTROPHILS

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**THE RHEOLOGICAL IMPACT OF CELL ACTIVATION ON THE FLOW
BEHAVIOR OF NEUTROPHILS**

THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biomedical Engineering
in the College of Engineering
at the University of Kentucky

By

Nolan M. Horrall

Director: Dr. Hainsworth Y. Shin, Professor of Biomedical Engineering

Lexington, Kentucky

2016

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ABSTRACT OF THESIS

THE RHEOLOGICAL IMPACT OF ACTIVATION ON THE FLOW BEHAVIOR OF NEUTROPHILS

Previously, it was reported that the morphological changes (pseudopod projection) that circulating neutrophils adopt due to cell activation raises peripheral vascular resistance by disrupting microvascular rheology. Studies utilized murine muscle preparations to link neutrophil pseudopod formation to cell activation and a viscous impact on hemodynamic resistance. But because of the complexity associated with the organization of the vasculature and microvasculature in tissues, it was unclear whether the effects of neutrophil activation on hemodynamic resistance were associated with the macro-/micro- circulation. This research describes an in vitro analysis using viscometry and microvascular network mimics (microporous membranes) to assess the rheological impact of pseudopods on capillary-like flow. Suspensions of neutrophil-like HL-60 promyelocytes (dHL60's) and human neutrophils, stimulated with 10 nM fMLP were used, with/without hematocrit. Stimulation of dHL60s or human neutrophils with fMLP altered their flow behavior, which was detected as an increase in solution viscosity. Addition of hematocrit negated the effect of neutrophil activation on suspension viscosity. Moreover, cell activation increased the resistance of microporous membranes to flow of neutrophil suspensions with addition of hematocrit exacerbating this effect. Combined, the results of this study provided evidence that activated neutrophils influence microscale flow resistance via a rheological impact.

KEYWORDS: inflammation, viscosity, resistance, hematocrit, microcirculation

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1. Introduction

Tissue oxygenation, as well as the delivery of nutrients and signaling molecules, is regulated by the rate at which blood is delivered to the peripheral tissues. The exchange of gases, nutrients, and signaling molecules between the blood and the tissues predominantly occurs in the microcirculation. The regulation of this exchange takes place at many levels, from the heart all the way down to the capillaries. The importance of this regulation is exemplified by the many redundant mechanisms the body has in place to ensure blood transport takes place efficiently. One bodily response which can affect blood transport is inflammation.

During inflammation, the microcirculation responds in several ways, which are designed to enhance the delivery of inflammatory cells, isolate damaged regions from the surrounding healthy tissue, and set the stage for tissue reparative processes. These changes include altered vasomotor function, reduced capillary perfusion, activation of the coagulation cascade, increased vascular permeability, and increased expression of cell adhesion molecules [1]. This enhanced expression of adhesion molecules makes it easier for leukocytes to attach to the microvasculature and migrate to inflamed tissues. Increased affinity for adhesion to the vasculature can affect blood flow, especially in the microcirculation. These processes, mediated by the endothelial cells residing on the inner surfaces of blood vessels, however, are not the only ways that affect blood transport. Changes in the blood, in particular changes in its cellular constituents, have the potential to physically affect microcirculatory flow.

In this study, we developed a model to quantify the effects of changes in the cellular activation state of white blood cells on fluid flow in microvessels, typical of those found in the microcirculation. Implementation of this model served as an in vitro test, in support of ex vivo tissue data reported by others [2], that linked the flow behavior of white blood cells with hemodynamic resistance in microvascularized tissues. In effect, the present study sought to elicit further evidence that the activation state of the circulating white blood cells themselves may be determinants of hemodynamic resistance in the microcirculation, and thus, tissue perfusion.

1.1. Blood Composition and its effect on Rheological Flow

Blood consists of two main components: plasma and the formed elements. Plasma consists of a mixture of various functional macromolecules (enzymes, cytokines/growth factors, hormones, immunoglobulins, fibrinogen, fatty acids, etc.), nutrients, wastes, and gases dissolved in an aqueous (i.e., saline) solution. Rheologically, plasma is classified as a Newtonian fluid [3]. The viscosity of plasma at room temperature (37°C) typically ranges from 1.1 to 1.3cP depending on its content of water and its macromolecular make up. It can change depending on temperature and on the physiological/pathological state of the blood. For example, during the body's immune response to infection, immunoglobulin concentrations rise, which enriches protein concentrations in plasma and leads to an increase in blood viscosity [4].

Blood also contains the formed elements, which include the blood cells (erythrocytes and leukocytes) and platelets (also called thrombocytes). The cellular

composition of blood has a dramatic impact on its viscosity. The formed elements make up approximately 45% by volume of blood. A key component of blood that has a dominant effect on its viscosity is the hematocrit (i.e., the red blood cell count) [5]. Hematocrit refers to the concentration of erythrocytes, more commonly known as red blood cells (RBC's), in the blood. Erythrocytes are the most abundant of the formed elements, accounting for approximately 99% of cellular elements. They are highly deformable and prone to aggregation under low or no flow conditions, which increases apparent viscosity [6]. For example, under the effects of lower shear rates, and in the presence of fibrinogen, erythrocytes tend to “stack together” due to their disk-like shape and high surface area [7]. These “stacks”, called rouleaux are responsible for blood exhibiting solid-like behavior and higher viscosities. This phenomenon is only observed in low shear rate vasculature such as the venule network and some arterioles [8]. Under higher shear rate conditions, the RBC's do not aggregate to form rouleaux, because the fibrinogen-mediated binding between cells cannot withstand the shearing forces acting to pull apart the erythrocytes. Because of this, whole blood is a shear thinning non-Newtonian fluid, with its viscosity dependent on shear rate.

Red blood cell shape and stiffness also have a rheological influence on the apparent viscosity of blood, depending on the size of the vessels in which it is flowing. The Fåhræus–Lindqvist effect describes how flow behavior of erythrocytes can decrease the apparent viscosity of blood in small diameter microvasculature (10 and 300 μ m). Because the formed elements within the blood orient themselves within the center of the vessels, a thin lubrication layer of plasma forms at the vascular wall, separating the cells from the vessel intima. This layer of plasma is only about half the diameter of a red blood

cell thick [9], but since it is free of erythrocytes, its apparent viscosity is lower than that of the bulk blood volume flowing down the center of the vasculature and thus produces a “skimming effect” that lubricates the bulk movement of the blood. However, alterations in cell stiffness and shape can play a role in countering the Fåhræus–Lindqvist effect by disturbing the cell-free layer [9]. As seen in patients with sickle cell anemia, microvascular perfusion is impaired by sickle-shaped red blood cells, which have the effect of raising apparent blood viscosity by disrupting the cell free zone [10].

The degree of influence of the low-viscosity cell-free zone on overall apparent viscosity depends on the diameter of the vasculature [11-13]. In large vessels, this lubrication layer has little influence on the apparent viscosity of blood since its thickness is negligible in comparison to the vessel diameter. However, as the vessel diameter decreases, the influence of this lubrication layer on the apparent viscosity of blood becomes more noticeable. Moreover, in the smallest vessels (e.g., arterioles and capillaries), disturbance of this lubrication layer may have a significant impact on blood velocities and microvascular resistance, both of which are functionally dependent on apparent viscosity of the flowing fluid.

In addition to red blood cells, there are five varieties of leukocytes in circulation: granulocytes (neutrophils, eosinophils, and basophils), lymphocytes, and monocytes. Leukocytes, commonly known as white blood cells (WBCs), make up approximately 1% of the total number of cells in circulation. Neutrophils are the most abundant of white blood cells in circulation, accounting for more than 60% of the leukocyte population. Lymphocytes make up only 30% of the leukocyte population and are more commonly found in the lymphatic system than the blood. Monocytes make up only a small fraction

(3 - 8%) of circulating leukocytes [14]. This investigation focuses on the neutrophil, and its effect on apparent viscosity. Neutrophils were chosen not only for their prevalence, but also because of the dramatic changes in deformability and shape they undergo when activated under inflammatory conditions.

1.2. Neutrophil Activation and Its Impact on Microvascular Flow

Neutrophils are the first line of defense against tissue damage and pathogenic microorganisms making their way into the body. In their inactivated state, neutrophils flowing in the bloodstream are rounded, reasonably deformable, and don't easily adhere to surfaces. Inflammatory activation of these cells induces many biological changes. These include upregulation of surface receptors involved in cell adhesion, migration, and phagocytosis, as well as increased release of granular contents such as cytokines, proteases, and anti-bacterial agents. But cell activation during the body's acute inflammatory response to infection or tissue damage also has a mechanical effect [15]. Upon stimulation, neutrophils quickly alter their shape, size, and deformability. A hallmark morphological response of neutrophils stimulated by an agonist is pseudopod extension, which increases the surface area of the cell and changes the geometry from a regular, spherical shape to an irregular, amoeboid-like appearance. The activated cells also display an increased stiffness and adhesivity to vascular walls. These neutrophil activity-dependent changes have a fluid mechanical or rheological, effect on blood flow and tissue perfusion.

The rheological impact of neutrophil activation is highly dependent on vascular diameter. Neutrophils range from 10-15 μm in diameter, while the microvasculature is made up of arterioles (10-100 μm), capillaries (5-10 μm), and venules (7-50 μm). In larger diameter vessels, neutrophils have little impact on blood flow and vascular resistance. In the smaller vessels, diameters can be comparable to, and even less than, those of the red blood cells. However, red blood cells are able to easily pass through even the smallest of vessels due to their high degree of deformability and their lack of nuclei. Under non-inflammatory conditions, the neutrophils are also capable of deforming and passing through the microvasculature, albeit at a lower velocity. However, upon activation, the non-rounded, stiffer, and more adhesive neutrophils have increased difficulty in passing through the capillaries, a situation that may contribute to elevations in peripheral microvascular resistance.

Pseudopod-extending neutrophils may also block capillary flow by becoming lodged at the entrance to or within a capillary itself, a situation referred to as capillary plugging. It has also been suggested that adhesion of activated neutrophils, for example in postcapillary venules, may interfere with microvascular blood flow and enhance peripheral resistance. In this scenario, the already small venular diameter is further reduced by the adherent neutrophils. This reduction in area increases the vessel resistance to flow, and may even cause a “pile-up” of erythrocytes [16]. Thus, by either blocking capillaries or adhering to post-capillary venule walls, activated neutrophils are capable of reducing flow velocity [17-19]. However, these scenarios may not be the only causes for increased microvascular resistance.

1.3. Evidence that Neutrophil Activation Can Affect Microvascular Flow Rheology

Recent investigational evidence suggests that activated neutrophils, when traveling through the capillaries or smaller microvessels, reduce the velocities of trailing RBCs, disrupting their axial position in the flow field [16]. In an experiment involving leukocyte flow through hemodynamically isolated rat gracilis muscle [16], this effect on red blood cell velocity was observed, and largely attributed to the impact of increased neutrophil stiffness, which would accompany activation.

Interestingly, there is some supportive evidence for a rheological impact of neutrophil activation on microvascular flow, for example in hypertension. Due to a chronically-inflamed blood state, leukocytes from spontaneously hypertensive rats (SHRs) have a higher propensity to display markers of cell activation, i.e. pseudopod projection, in comparison to cells from normotensive rates [20]. This sustained state of leukocyte activation is also associated with pathological elevations in hemodynamic (i.e., microvascular) resistance [21]. Notably, neutrophils from SHRs do not display an increase in surface expression of cell adhesion molecules (e.g., CD18 integrins), which is another index of inflammatory cell activation [20]. These observations suggest the possibility that elevations in microvascular resistance are linked to the sustained projection of pseudopods by neutrophils, rather than microvascular adhesion [16].

One possible mechanism to explain how leukocyte activation may lead to increased microvascular resistance without pathological elevations in neutrophil adhesion, as reported for SHRs [20], is via capillary plugging. However, research does not support the phenomenon of capillary plugging as a large contributor to increases in

blood flow resistance [16]. Locally, capillary plugging has been identified as a contributor to increased resistance within individual capillary networks. However, there are so many capillaries, and this phenomenon is so infrequent, that on its own, it has not been linked to increasing flow resistance on a larger scale [22]. If capillary plugging cannot account for increases in microvascular resistance in SHR's, an alternate explanation must be examined.

As discussed, inactivated neutrophils possess a rounded morphology that allows for “less disruptive” rheological behavior with minimal impact on the surrounding flow [23, 24]. In contrast, activated leukocytes, which project pseudopods and thus adopt an irregular shape, likely behave differently in the non-linear velocity gradient of a microvascular flow field than the rounded inactivated neutrophils. The hypothesis is that the pseudopod-extending neutrophils become irregularly shaped, causing them to ‘tumble’ more erratically than inactivated spherical cells in the vessel lumen. This tumbling likely increases stochastic interactions with the many erythrocytes and other cells of the bloodstream [2, 16]. Notably, this increase in collisions between cells increases hemodynamic resistance. This resistance increase is likely the result of disturbing the cell-free circumferential zone which leads to a rise in the apparent viscosity [2, 16, 25]. This potential scenario may explain how sustained pseudopod projection may have an effect on peripheral hemodynamic resistance.

1.4. Study Rationale

This investigation focuses on the possibility, based on ex vivo muscle tissue data, that sustained neutrophil activation may substantially influence hemodynamic resistance

by way of increasing apparent blood viscosity within the microvasculature. Worthen and Downey investigated the degree to which neutrophils become lodged in capillary diameter micropores, and how this phenomenon leads to a reduction in microcirculation flow rate [26]. But as previously explained, capillary plugging may not be a significant contributor to changes in whole organ blood flow resistance due to neutrophil activation.

The present study uses an *in vitro* approach focused on how neutrophils may impact microscale flow of blood-like cell suspensions in the microcirculation without capillary plugging or cell adhesion. Helmke suggested that pseudopod-extending neutrophils likely tumble more in the bloodstream when in the presence of erythrocytes [2]. He based this hypothesis on flow resistance studies using *ex vivo* spinotrapezius muscle preparations [2]. However, since the circulation in tissues is made up of a complex network of large and small arterioles as well as capillaries, his work did not specifically determine in which vessels the microvascular resistance increases occurred. This study sought out *in vitro* affirmation that pseudopod extension directly leads to increases in resistance within capillary-like microvessels in the absence of plugging. To this end, this study will employ capillary mimics to compare pseudopod-extending neutrophils to non-pseudopod-extending neutrophils. The system will determine if the mechanical disturbance of tumbling pseudopod-extending neutrophils is enough to raise microvascular resistance.

2. Materials and Methods

2.1. Cell Lines, Culture Conditions, and Passaging

Suspension cultures of HL-60 promyeloblast cells, derived from an acute promyelocytic leukemia patient, were purchased from the American Type Culture Collection (ATCC®). The cells were received frozen and suspensions were rapidly thawed in a 37°C water bath with gentle agitation. The cell solution was then centrifuged at 200xG for 5 min, and the resulting supernatant was aspirated with a vacuum to leave a cell pellet. These cell pellets were rinsed two consecutive times with phosphate buffered saline (PBS). Following each rinse the cell suspensions were spun down at 200xG for 5 min at 25°C and the PBS was aspirated under sterile conditions in a laminar flow hood. HL-60 cells were cultured in suspension within either 25 cm² (T-25) or 75 cm² (T-75) vented cell culture flasks (Corning®) in RPMI 1640 Media (HyClone®) supplemented with 10% v/v fetal bovine serum (FBS) (HyClone®) and 1% v/v penicillin/streptomycin/Lglutamine solution HyClone®) as described [27]. This media formulation will, from this point forward, be referred to as ‘complete media’. Cells were maintained under standard tissue culture incubator conditions composed of a humidified, 37°C, 5% carbon dioxide/95% air environment. Following routine cell culture practice, the media was replaced every 2 to 3 days. HL-60 cultures in T-25 or T-75 flasks were maintained at concentrations between 200,000 and 500,000 cells/mL of media.

2.2. HL-60 Storage

Excess suspension cultures of HL-60 cells in T-75 or T-25 flasks were cryopreserved for later use following standard cell culture procedures. Cell suspensions were transferred from the culture flasks to 15-mL centrifuge tubes (BD Falcon®) then pelleted by centrifugation at 200xG for 5 min at 25°C. The cell pellet was then resuspended in 1.5 mL of freezing solution which consisted of 10% dimethyl sulfoxide (DMSO) (Sigma®) in FBS. For freezing, the cells were concentrated between 3 and 5 x10⁶ cells/mL and transferred into 2-mL cryogenic vials (BD Falcon). These sealed tubes containing the cells were either stored at -80°C until needed or submerged under liquid-nitrogen for long term cryogenic storage.

As needed, frozen cell suspensions in cryogenic vials were thawed and cultured as described in section 3.1.

2.3. HL-60 Neutrophil-like Differentiation

HL-60 promyeloblasts in suspension cultures were counted using a hemocytometer. The suspensions of cells were then transferred from the T-75 cell culture flasks to 15 mL centrifuge tubes and pelleted at 200xG for 5 min at 25°C. Once the supernatant was aspirated, the remaining pellet was resuspended to cell densities of 500,000 – 1,000,000 cells/mL in complete media supplemented with 1.25% Dimethyl Sulfoxide (DMSO) under standard tissue culture incubator conditions for 3 days. Following the first 72 hours of differentiation, the cells were again pelleted at 200xG for 5 min at 25°C and resuspended in fresh DMSO-containing complete media. After a total of 6 days in the DMSO media, the cells were rinsed twice with Hank's buffered saline

solution (HBSS) and then resuspended in complete media overnight. Differentiated HL-60's were used on the 7th day after the addition of DMSO. Neutrophilic differentiation was verified by visual inspection following previously reported guidelines [28].

2.4. Purified Neutrophil Isolation

Human neutrophils were purified from 30 mL of fresh human donor blood from asymptomatic volunteers using informed consent and phlebotomy procedures that had been approved by the University of Kentucky Institutional Review Board. Briefly, heparinized blood was mixed with 3 mL of 6% dextran (a stock solution of 6g of Dextran 229 in 100 mL of PBS), and the erythrocytes were allowed to sediment for between 30 and 40 min. The buffy coat above the RBCs was gently pipetted onto 5 mL of Histopaque-1077 within a fresh 50-mL centrifuge tube and centrifuged for 20 min at 600xG and 25°C to yield a pellet of granulocytes and a small number of erythrocytes. The pellet was then resuspended in 5 mL of PBS and deposited on top 3 mL of 55% percoll in isotonic saline which had been layered on top of 3 mL of 74% percoll in a 15-mL centrifuge tube. This gradient solution was centrifuged for 15 min at 600xG and 25°C. The resulting band of neutrophils that formed between the two percoll layers was harvested and added to a fresh 15-mL tube containing 12 mL of PBS. The tube was inverted several times in order to mix the solution and then centrifuged one final time for 10 min at 600xG and 25°C. Afterwards, the cells were resuspended in the experimental buffer of choice.

2.5. Neutrophil Activation and Fixation

The protocol for activating and fixing cells was the same for both HL-60 cells and for isolated human neutrophils. The concentrations of either differentiated HL-60's or the isolated human neutrophils in stock cultures were determined using a hemocytometer in order to prepare cell suspensions at a cell density of 1×10^7 cells/mL. The cells were then either left untreated or stimulated with 10 nM N-formyl methionyl-leucyl-phenylalanine (fMLP) for 5 min. At that time, the cell solutions were combined with an equal volume of 2% paraformaldehyde in 0.2 M phosphate buffer and allowed to incubate for 5 min before rinsing in HBSS. In preparation for experiments, the cells were resuspended in fresh HBSS at desired cell densities (500,000 – 1,000,000 cells/mL).

2.6. Morphometric Analysis

Once samples of activated and inactivated neutrophils were generated, aliquots (100 μ L) of each solution were pipetted onto glass slides. Using an IX-71 microscope (Olympus) and Simple PCI software, photographs of random fields of cells were visualized and recorded under brightfield illumination, at a magnification of 400x. The images were imported into publically available Image-J software (NIH) and the imaged cells were individually traced until at least 50 random measurements were documented per image. Image-J was then used to estimate perimeter (p) and projected area (A) of the cells. From the area and perimeter measurements, circularity was calculated using the equation $C=4\pi A/p^2$. Circularity values were averaged from at least n=3 independent experiments and used to evaluate the morphological response of the neutrophils to stimulation. Data was expressed as a mean \pm SEM.

2.7 Viscometry

Viscometric analyses were used to assess the contributions of pseudopod extension on the rheological behavior of cell suspensions. Viscosity measurements of cell suspensions were conducted using a digital DV-II+Pro Brookfield cone-plate rheometer according to the manufacturer's instructions. Prior to analyses of experimental samples, the accuracy of the rheometer was verified and, if needed, calibrated using solutions of known viscosities as prescribed by the manufacturer. At the beginning of the analyses, 500 μL of cell solution was pipetted into the center of a stainless steel sample cup that was then incorporated onto the rheometer. For experimental measurements, the cone-plate viscometer (cone angle: 0.8°) was set to subject cell suspensions to a shear rate of 450sec^{-1} . The sample cup, in which the cell solutions were sheared, was also connected to a Cole Parmer peristaltic pump to circulate water through the water jacket for the purpose of maintaining the cell suspensions at 25°C . Wingather software (Brookfield®) was used to control the rheometer and acquire viscosity measurements every 30 seconds for a duration of 5 min. For data acquisition, the rheometer was allowed to run for 30 seconds before initiating the software to start recording the viscosity. After measurements, the raw data was exported as a Microsoft Excel® file. The plate and cone were then cleaned with deionized water in preparation for subsequent cell suspension analyses. In this analysis, solutions of isolated HL-60's were examined (both activated and inactivated), as well as solutions of purified human neutrophils (activated and inactivated). The study also examined activated human neutrophils in the presence of 10, 20, and 40% hematocrit.

2.8. Micropore Analysis of Cell Suspension Rheology

The present study used a custom microfiltration setup (Figure 1) adapted from a previous study [18] to quantify the effect of pseudopod extension on the flow of neutrophils through capillary-like microvessels. This setup consisted of a microporous Isopore membrane (10- μm pore diameter; Millipore) housed in a re-useable stainless steel filter holder. Cell suspensions to be tested were placed within a 60 mL syringe along with a small magnetic stir bar. The syringe was connected to the inlet of the Micropore filter holder using 0.04 inch inner diameter Tygon® tubing. During perfusion experiments, a Harvard apparatus syringe pump (model 2000) was used to drive cell suspensions through the micropore filter. During this time, the stir bar was gently agitated using a magnetic stir plate to ensure cells remained in a homogeneously mixed state in solutions. Cell solutions were allowed to drip into a waste beaker after passing through the membranes.

The readout of interest was how neutrophil pseudopod activity affected perfusion pressures across micropore membranes during flow experiments. To measure the pressure across the micropore filter, a Statham pressure transducer was placed between the inlet of the micropore filter and the syringe containing test cell suspensions. Labview Signal Express computer software (National Instruments®) interfaced to a Model MC1-3 signal conditioner (Validyne®) was used to record pressure readings from the pressure transducer. Before each experiment, the pressure transducer was calibrated against a manometer setup. Flow experiments consisted of activated and inactivated neutrophils suspended (at a density of 500,000 cells/mL) in isotonic PBS containing 10% FBS.

Experiments were also conducted with neutrophils in the presence of 10% autologous hematocrit.

Prior to perfusion experiments, the Isopore® filters were pre-incubated with 100% FBS for at least 30 min, after which they were incorporated into the stainless steel filter holder. Before perfusion of cell suspensions, fresh PBS containing 10% FBS was used to purge the system for 10 min. Then, the syringe containing this purge solution was replaced with test cell suspensions and the cell solution was perfused through the microfilters. Perfusion pressure (across the micropore membrane) was recorded for 20 seconds before turning on the syringe pump (to get a baseline pressure reading) and during the duration of the flow experiment. Time-dependent perfusion pressure data was exported from labview in the form of a text document and analyzed using Matlab (MathWorks®) software.

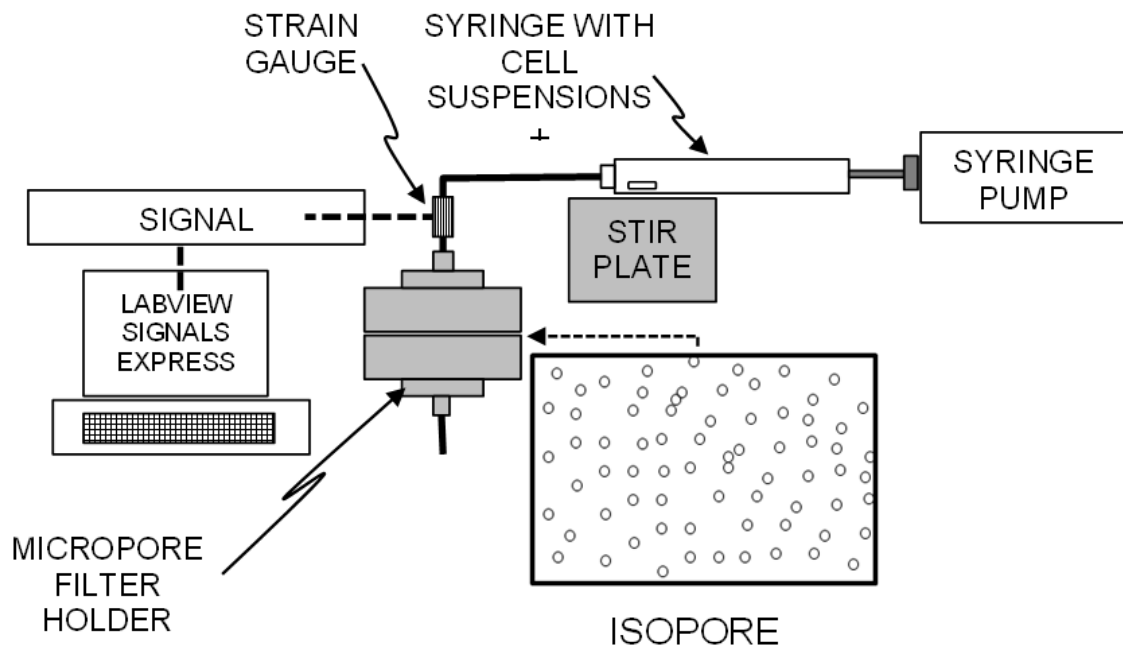


Figure 1 - Representative image of the micropore filtration setup

2.9. Microscopic Analysis of Micropore Clogging

Following micropore flow analyses, neutrophils remaining in the micropores of the Isopore® membranes were labeled with 10nM 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) fluorescent nuclear stain for a duration of 10 min. Random images of micropore filter surfaces showing the distribution of pores were inspected for retained neutrophils using an IX-71 fluorescence microscope under 200x magnification and DAPI illumination. Pores exhibiting positive nuclear staining were considered to be clogged with neutrophils. The percentage of blocked pores for the different cell populations tested was quantified by counting the total number of pores with stained nuclear material in them and then dividing that number by the total number of pores analyzed. On any individual filter, at least 100 pores were examined for the presence of nuclear material.

3. Results

3.1. HL-60 Neutrophil Differentiation

Transformation of promyelocytic leukemic HL-60 cells into mature dHL60 neutrophil-like granulocytes was induced with 1.25% dimethyl sulfoxide (DMSO). Figure 2 illustrates the morphologies of un-differentiated HL-60 promyelocytes and HL-60-derived neutrophilic cells.

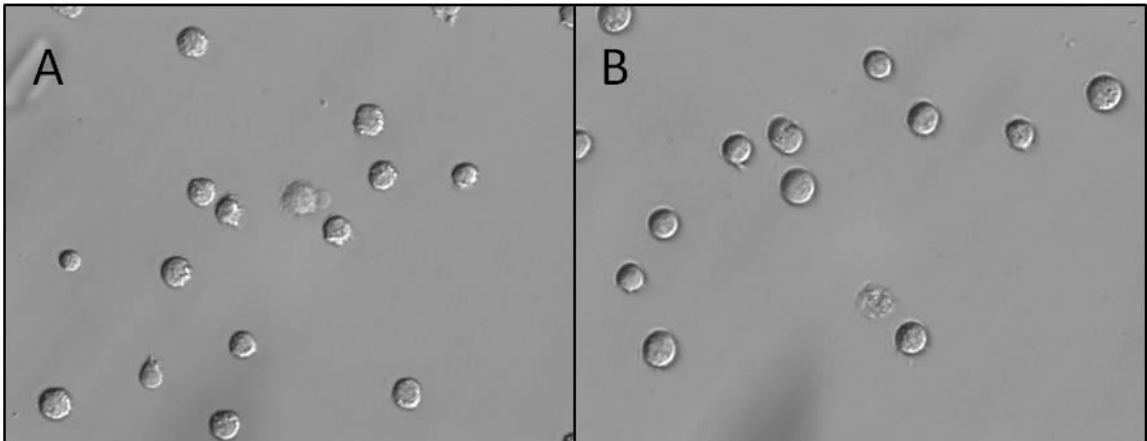


Figure 2 - Representative micrographs of HL-60 cells before and after differentiation with 1.25% dimethyl sulfoxide for 7 days. Panel A: differentiated HL-60 neutrophil-like cells. Panel B: naive HL-60 cells.

3.2. Morphometric Analysis of HL-60 Neutrophil-like and Human Neutrophil Activation

Morphometric analyses were conducted to verify that our dHL-60 neutrophilic cells displayed cell shape changes consistent with enhanced pseudopod activity in response to fMLP stimulation. Compared to untreated dHL-60 cells, similar neutrophil-like cells stimulated with 10nM fMLP for 5 min exhibited more irregular and non-uniform shapes characterized by increased pseudopod formation (Figure 3). Average circularity (our index of cell shape change) significantly ($p < 0.05$) decreased from 0.867 ± 0.01 ($n=3$) for untreated cells to 0.819 ± 0.02 ($n=3$) for cells stimulated with fMLP (Figure 4). DHL60's were used in this exploratory study to test the hypothesis that cell morphology due to cell activation affects the apparent viscosity of blood cell suspensions.

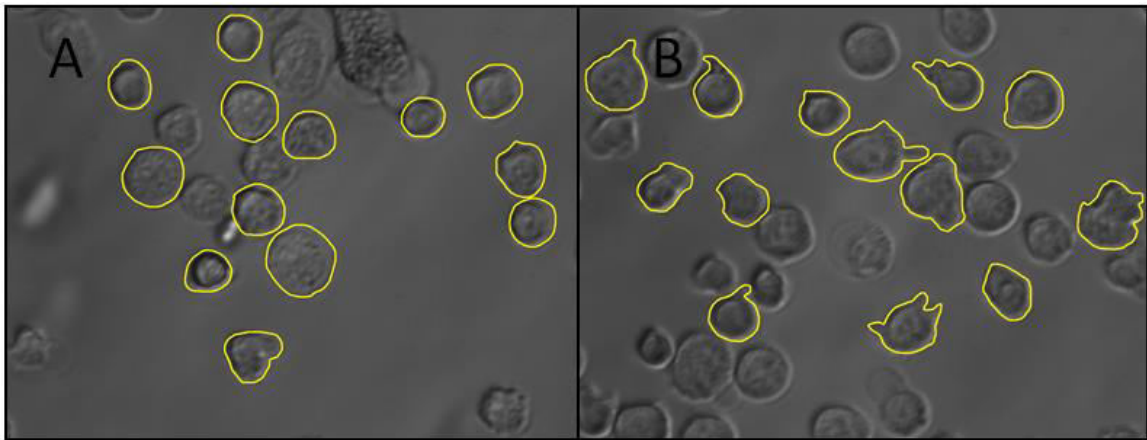


Figure 3 - Representative micrographs of dHL-60 neutrophil-like cells stimulated with fMLP. Panel A: untreated differentiated HL-60 neutrophil-like cells. Panel B: differentiated HL-60 neutrophil-like cells stimulated with 10nM fMLP for 5 minutes. Representative tracings used for semiquantitative morphometric analyses of individual cells are depicted in yellow outline.

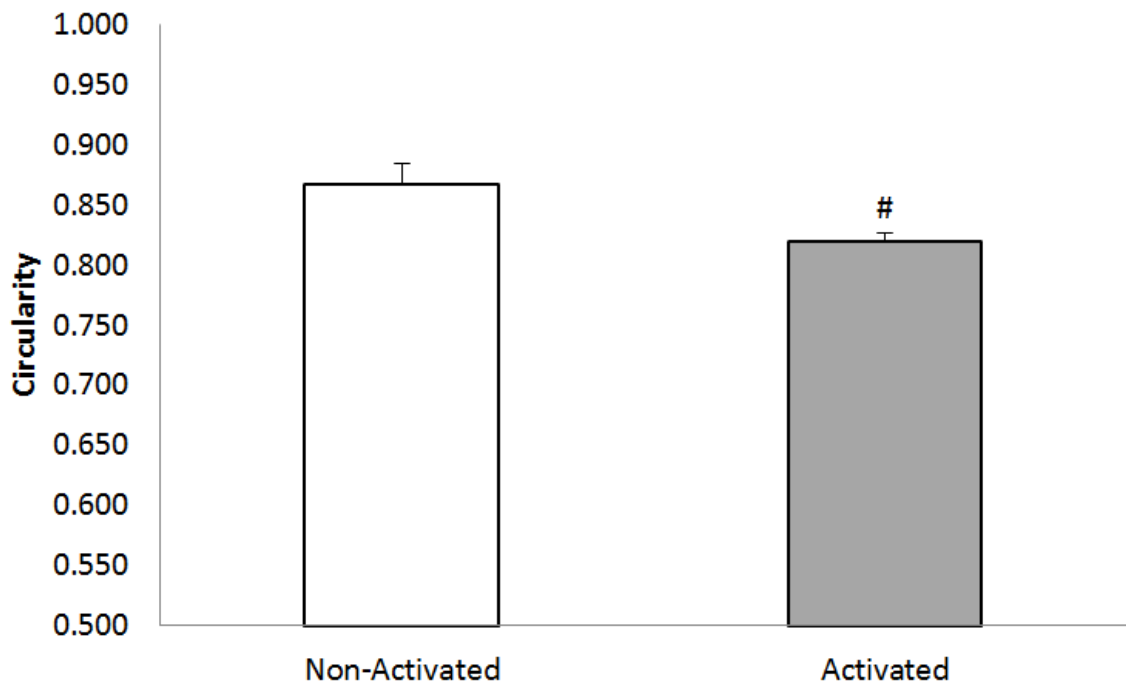


Figure 4 - Stimulation of HL-60-derived neutrophil-like cells with 10nM fMLP for 5 minutes reduces cell circularity. Bars are mean \pm SEM; n = 3 experiments. #p<0.05 compared to inactivated cells using Student's paired t-test.

Figure 5 displays morphological differences between purified human neutrophils that had been either left untreated or stimulated with 10 nM fMLP. Compared to untreated cells, fMLP-stimulated neutrophils exhibited significant ($p < 0.05$) decreases in circularity compared to untreated cells (Figure 6). Specifically, inactivated cells exhibited an average circularity of 0.913 ± 0.0021 ($n=3$) while their activated counterparts had an average circularity of 0.716 ± 0.0088 ($n=3$).

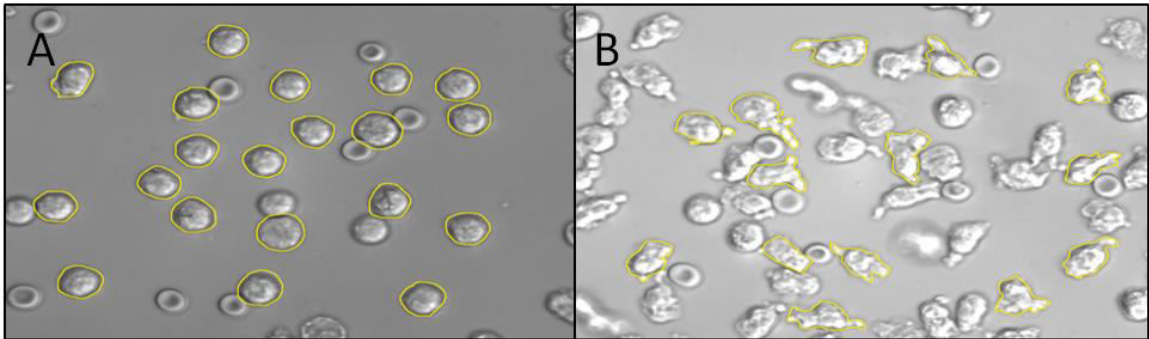


Figure 5 - Representative micrographs of purified human neutrophils stimulated with fMLP. Panel A: untreated human neutrophils. Panel B: human neutrophils stimulated to extend pseudopods with 10nM fMLP. Representative tracings used for semiquantitative morphometric analyses of individual cells are depicted in yellow outline.

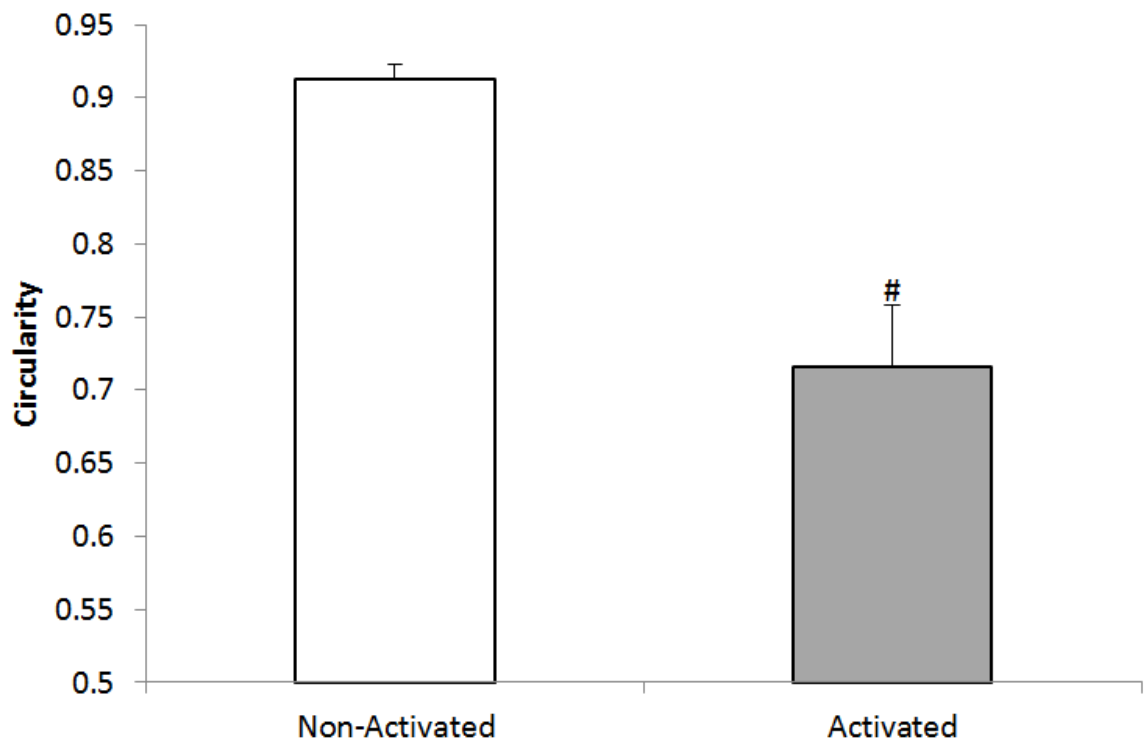


Figure 6 - Stimulation of purified human neutrophils with 10nM fMLP for 5 minutes reduces cell circularity. Bars are mean \pm SEM; n = 4. #p<0.05 compared to inactivated cells using Student's paired t-test.

3.3. Effect of fMLP Stimulation on Neutrophil-like Suspension Viscosity

The effects of cell morphological changes due to fMLP stimulation on the rheological behavior of dHL60 neutrophil-like cells in suspension was assessed using cone-plate viscometric analyses of cell suspensions. This relationship was examined for cell suspensions having cell densities of 1×10^6 or 1.5×10^6 cells/mL. At each of these cell densities, populations of dHL60 cells activated with fMLP exhibited significantly ($p < 0.05$) greater viscosities than untreated cell populations (Figure 5).

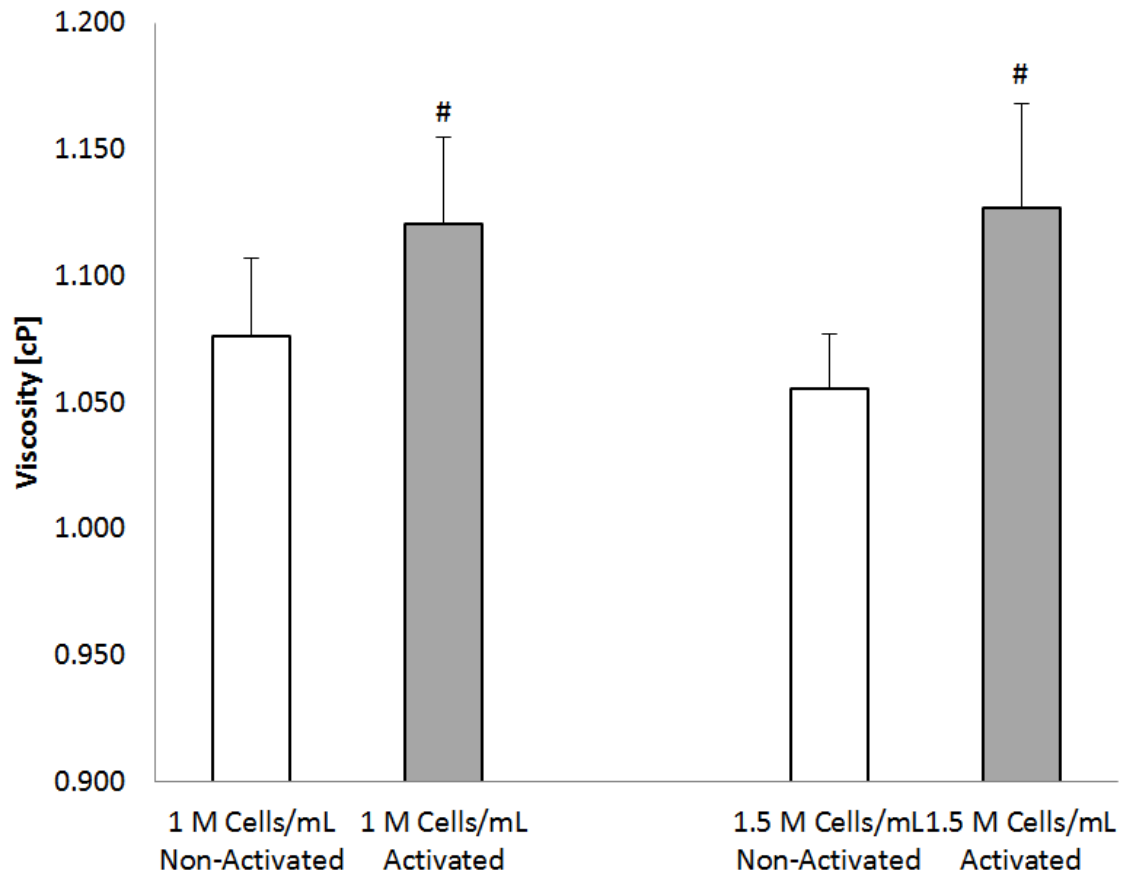


Figure 7 - Effect of fMLP stimulation on the rheological flow behavior of HL-60 neutrophil-like cell suspensions. The viscosities of suspensions containing 1×10^6 and 1.5×10^6 cells/mL were measured. Bars are mean \pm SEM; $n = 5$. # $p < 0.05$ compared to non-activated cells using Student's paired t-test.

As was the case for HL-60 neutrophil-like cells, human neutrophil populations (1×10^6 cells/mL) treated with 10 nM fMLP displayed significantly ($p < 0.05$) greater apparent viscosities relative to inactivated cell suspensions (Figure 8).

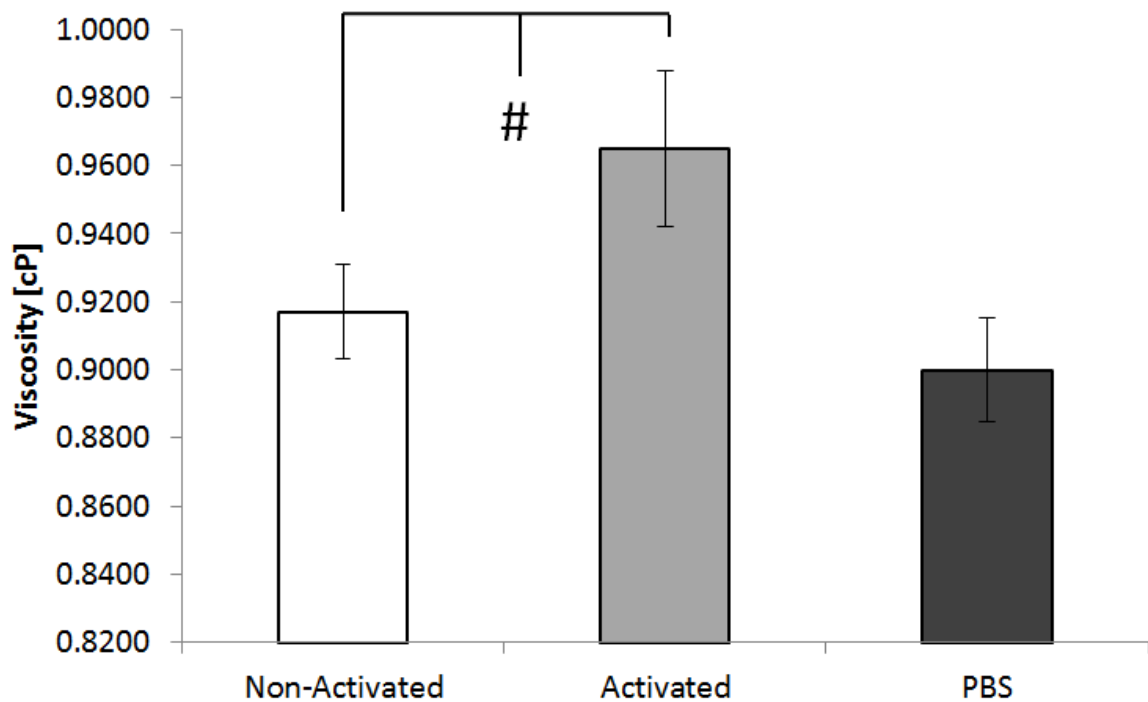


Figure 8 - Effect of fMLP stimulation on the rheological flow behavior of purified human neutrophil suspension. The viscosities of suspensions containing 1×10^6 cells were measured. Bars are mean \pm SEM; $n = 4$ experiments. # $p < 0.05$ compared to non-activated cells using Student's paired t-test.

Interestingly, there were no detectable differences in the apparent viscosities of untreated or fMLP-treated neutrophil suspensions supplemented with 10, 20, and 40% (v/v) hematocrit. However, viscosities of cell suspensions increased with increasing hematocrit, with 10% hematocrit corresponding to an average viscosity of 1.23cP, 20% being 1.58cP, and 40% equaling 2.61cP (Figure 9).

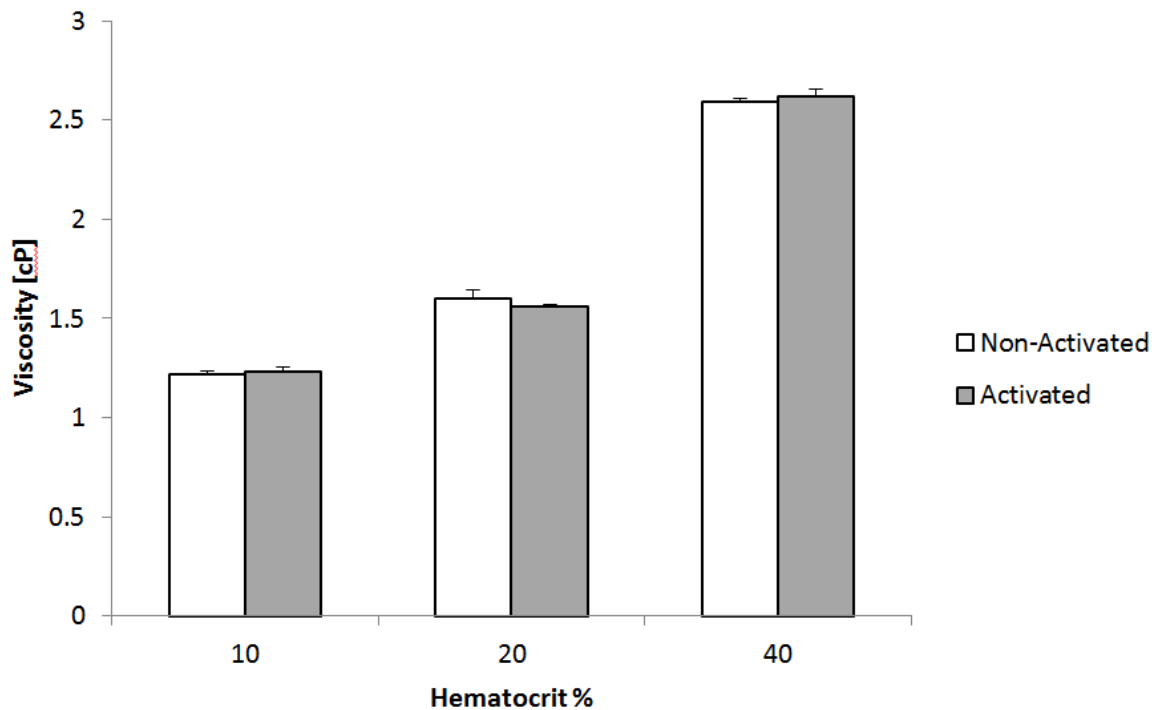


Figure 9 - Red blood cells mitigate the effect of cell activation on neutrophil rheological flow behavior. Apparent viscosities of human neutrophil suspensions (1×10^6 cells/mL) that either had (grey) or had not (white) been treated with 10 nM fMLP for 5 minutes were measured in the presence of 10, 20, and 40% hematocrit. Bars are mean \pm SEM; n = 3. #p<0.05 compared to non-activated cells using Student's paired t-test.

3.4. Effects of Neutrophil Activation on Microvascular Resistance Mimics

Results from micropore flow studies of human neutrophil populations indicate that cell activation influences their perfusion across microchannel networks. Figure 10 displays the perfusion pressures generated across Isopore® membranes (10- μm pore diameters) due to the flow of cell suspensions comprised of either unstimulated neutrophils or cells stimulated with 10 nM fMLP. The perfusion pressure versus time plots generated by the flow of both the activated and inactivated neutrophil suspensions displayed continuous rises in perfusion pressure over the course of the experiments. The injection of suspensions containing activated neutrophils through the micropore membranes eventually (after 450 seconds of flow and thereafter) resulted in significantly ($p < 0.05$) higher perfusion pressures.

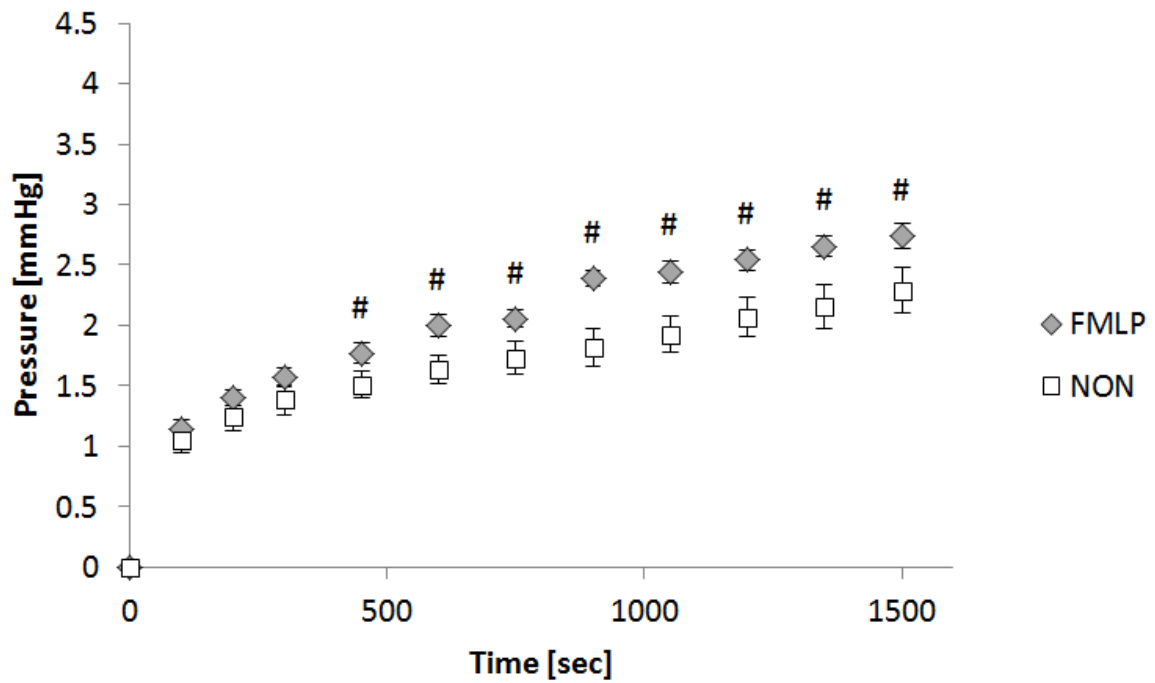


Figure 10 -Cell-activation dependent changes in perfusion pressure associated with micropore flow of neutrophil suspensions. Suspensions of neutrophils (1×10^6 cells/mL) that had (grey dots) or had not (white dots) been activated with 10 nM fMLP for 5 minutes were perfused through the micropore membranes. Points are mean \pm SEM; n = 4 experiments. #p<0.05 compared to inactivated cells using Student's paired t-test.

When 10% hematocrit was added to suspensions of non-activated neutrophils, perfusion pressures versus time plots were similar to those of neutrophil suspensions without red blood cells (Figure 11). However, addition of 10% hematocrit appeared to exacerbate the neutrophil activation-dependent increases in perfusion pressure (Figure 11). Differences in perfusion pressure for non-activated and activated cells in the presence of hematocrit were significantly different from the initial measured time point (Figure 11).

To further investigate this hematocrit dependence, we determined the percent pressure increase in perfusion pressure due to addition of 10% hematocrit. In this case, we observed a significant ($p < 0.05$), time-invariant increase in perfusion pressure for suspensions of activated neutrophils relative to those of non-activated cells. These results verified our observations (Fig. 11) that hematocrit exacerbated the effects of cell activation on micropore perfusion pressure (i.e., micronetwork resistance). Figure 12 removes the time-dependent pressure increases seen in both solutions, and clearly displays the relative increase in pressure seen once red blood cells are added to both activated and inactivated neutrophil solutions.

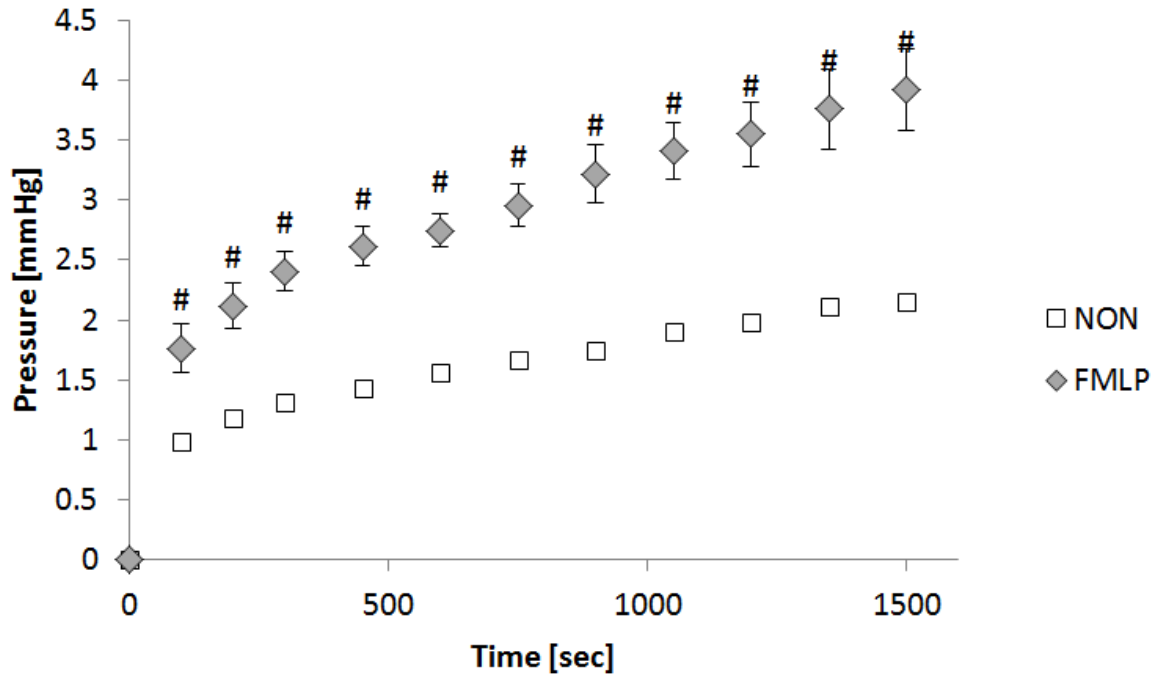


Figure 11 -Cell-activation dependent changes in perfusion pressure associated with micropore flow of neutrophil suspensions in the presence of hematocrit. Suspensions of neutrophils (1×10^6 cells/mL) that had (grey dots) or had not (white dots) been activated with 10 nM fMLP for 5 minutes were perfused through the micropore membranes. Points are mean \pm SEM; n = 4 experiments. #p<0.05 compared to non-activated cells using Student's paired t-test.

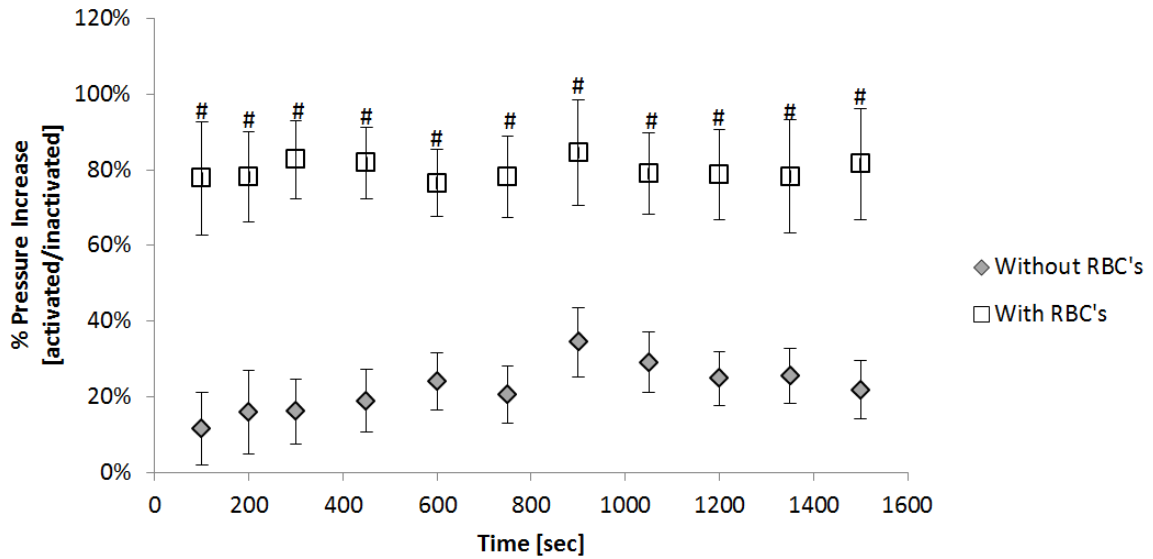


Figure 12 -Effect of 10% hematocrit on perfusion pressures of activated neutrophil solutions. Perfusion pressures of activated neutrophil suspensions were normalized to those of non-activated cells and expressed as a percent increase for the case of 0% and 10% hematocrit. Points are mean \pm SEM; n = 4 experiments. #p<0.05 compared to non-activated cells using Student's paired t-test.

3.5. Micropore Analysis

We analyzed micropore membranes (Figure 13) after subjecting them to the flow of either non-activated or activated neutrophil suspensions. This was carried out to investigate a potential explanation for the time-dependent response associated with perfusing cellular solutions through the micropore setup, (i.e., micropore clogging). Results of our micropore clogging analysis indicate that $6.0\pm 1.9\%$ and $7\pm 1.2\%$ of the micropores became clogged during micropore experiments with activated and inactivated neutrophil suspensions, respectively. Thus, flow of cell suspensions induced similar levels of clogging on micropores regardless of whether neutrophils were left untreated or stimulated with 10 nM fMLP.

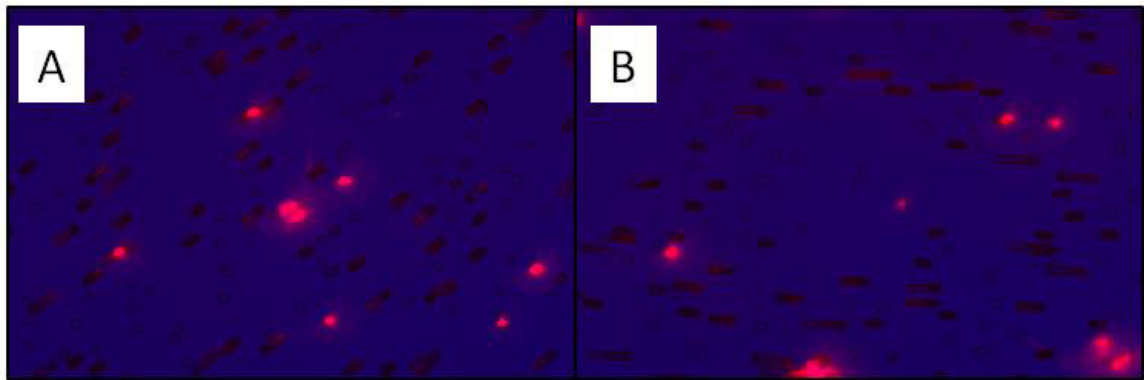


Figure 13 -Representative fluorescent micrograph of the 10-µm Isopore filters following perfusion of neutrophil suspensions. Panel A: micropore filter after being subjected to flow of inactivated \ human neutrophils. Panel B: micropore filter after being subjected to flow of purified human neutrophils stimulated with 10 nM fMLP for 5 minutes. Cell suspensions were injected through membranes at 500µL/min.

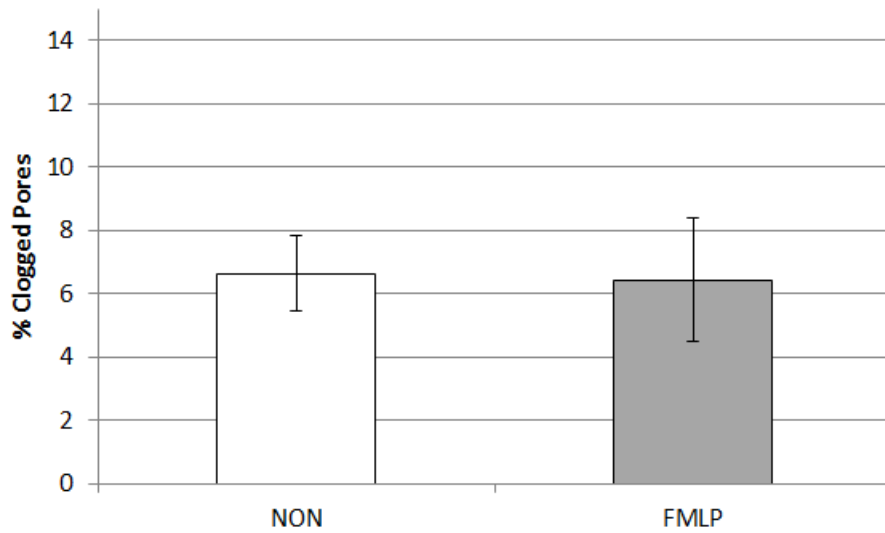


Figure 14 -Effect of fMLP stimulation on cell retention in micropores subjected to flow of neutrophil suspensions. Neutrophil suspensions (1×10^6 that had or had not been treated with 10 nM fMLP for 5 minutes were injected through micropores at 500 $\mu\text{L}/\text{min}$. Bars are mean \pm SEM; n = 5.

4. Discussion

The research presented in this thesis investigates the claim that sustained neutrophil activation influences hemodynamic resistance in the microvasculature. The study also evaluated the contribution of red blood cells to the effects of neutrophil activation (i.e., pseudopod extension) on microvessel flow resistance. This examination was conducted *in vitro* and *ex vivo* using viscometry and micropore flow analyses to link morphological changes of neutrophils (due to agonist stimulation) to changes in flow behavior within capillary-sized micropores (i.e., microvessels). The morphological effects of neutrophils on flow were measured by their impact on apparent viscosity of cell suspensions and their effect on micropore network perfusion pressure.

4.1. Selection of Cell Type and Neutrophil Purification

Initially, we examined the effects of cell activation on the rheological behavior of dHL-60 neutrophil-like cell suspensions sheared in a cone-plate viscometer. Populations of differentiated HL-60 neutrophil-like cells were used due to their commercial availability as well as their extensive use as an *in vitro* model of neutrophils [29]. Using the same differentiation procedure as Tsai [28], reduced cellular diameters were observed for our HL60 cells after culture for 6 days in DMSO, consistent with differentiation into neutrophil-like cells [28]. Following differentiation, the cells were stimulated to alter their near-circular morphology by causing them to extend pseudopods.

We also conducted studies with primary neutrophils harvested from fresh donor blood. These studies were conducted to support results obtained using dHL60

neutrophil-like cells. In comparison to HL-60's, purified human neutrophils are smaller in diameter, suggesting that the effects of dHL60 activation on cell suspension viscosity may be different for human neutrophils. But it should be noted that primary neutrophils are also more rounded in their inactivated state, and after activation, display a greater degree of pseudopod projection (i.e., cell shape irregularity) than dHL60 neutrophilic cells. Finally, we also used purified human neutrophils since they more accurately mimic the in vivo state.

4.2. Chronic Inflammation and Relevance to Cardiovascular Physiological Pathology

Traditionally, inflammation is viewed as an acute response which spontaneously resolves itself. In 2011, the Center for Disease Control and Prevention stated that “Of the ten leading causes of mortality in the United States, chronic, low-level inflammation contributes to the pathogenesis of at least seven. These include heart disease, cancer, chronic lower respiratory disease, stroke, Alzheimer’s disease, diabetes, and nephritis” [30]. This recent revelation identified sustained inflammation to be a major contributor to the progression of many serious diseases. It is not fully understood how or why chronic inflammation is present in so many different diseases, or even how damaging the inflammatory symptom can be.

Inflammation is a complex biological response aimed at protecting the body by removing harmful stimuli such as pathogens, irritants, and damaged cells. Not only do neutrophils remove these harmful stimuli as the first responders of the innate immunity, but they also play an important role in initiating/directing the repair of damaged tissue.

Because neutrophils contain potent antimicrobial agents that are degradative to tissue, it is very important for the body to regulate not only the activation of neutrophils, but also their deactivation once their task is complete [31]. There are many mechanisms to “turn-off” or restrict inflammatory activation of these cells during the resolution stage of inflammation [32]. It is when these mechanisms fail, that the body experiences sustained levels of neutrophil activity, which may contribute to chronic inflammation.

In addition to its biological/biochemical impact, neutrophil activation in the blood has a physical “side effect” mainly derived from the associated morphological changes, such as pseudopod extension [15]. The non-uniform, non-circular shape of activated cells can alter the rheological flow behavior of blood by tumbling end over end through the vasculature. This tumbling increases the likelihood of disturbing the thin cell-free layer of the flow field, effectively leading to an increase in apparent viscosity. Increases in apparent viscosity may impact hemodynamic resistance in peripheral tissues because higher viscosity translates to increased resistance to flow. This investigation explored the specific phenomenon of pseudopod projection and the effects it can have on flow behavior of blood in the microvasculature.

4.3. fMLP Neutrophil Simulation Mimics Pseudopod Extension Response

To activate cells and promote pseudopod extension, dHL-60 cells and primary neutrophils were stimulated with fMLP. fMLP is a peptide which is derived from bacterial proteins [33]. The binding of this peptide to N-formyl peptide receptors expressed on leukocyte surfaces results in activation of the cell [33]. The initial response

of the leukocyte to fMLP stimulation is the rapid polymerization of G-actin into cytoskeletal F-actin. The maximum response of a cell to fMLP can be initiated by a concentration exceeding that of 1 nM, which roughly produces a 100% increase in F-actin content within 2 min of exposure [34]. A short while later, F-actin rich pseudopods begin to form from a cross-linked network of microfilaments. Studies by Worthen and Kawaoka [34] demonstrated not only all of these behaviors, but also that by increasing the fMLP concentration, there is an accompanying increase in cellular stiffness [34]. The studies of Frank et al. (1990) also demonstrated that fMLP activation is a heterogeneous phenomenon in that not every cell is always affected [34]. This explained the observation that not every HL-60 or human neutrophil within a given population displayed pseudopods in response to fMLP stimulation.

The association between pseudopod extension following fMLP activation and rheological behavior of leukocytes is the main focus of this investigation. Because neutrophils were immediately fixed after stimulation with fMLP, but before viscometry and micropore flow analyses, pseudopod extension during these experiments remained constant. Morphological analyses were conducted to ensure that the fMLP-activated neutrophils did indeed display enhanced pseudopod extension relative to inactivated cells. Circularity was used to gauge neutrophil activation status with a value of 1 representing a rounded morphology consistent with cell inactivity. On the other hand, elongated cells, or cells with pseudopods, would have circularities that were less than 1 but greater than zero. The closer to zero a circularity measurement is, the more

“non-circular” it is. Quantification of the “non-roundedness” after fixing the sample populations ensured that results of the viscosity and microvessel resistance experiments were examining populations of activated vs. non-activated neutrophils.

4.4. Effects of Cell Activation on Neutrophil Flow Behavior

Viscosity is a measure of a fluid’s resistance to flow. Blood is unique because it is a combination of plasma and formed elements. Apparent viscosity of blood decreases in small diameter tubes as shear rates increase due to the Fåhræus–Lindqvist effect, yet, apparent viscosity dramatically increases as hematocrit concentrations increase [17]. The relationships between viscosity, shear, and hematocrit are highly dependent on cell deformability, concentration, RBC aggregation, and white blood cell interactions [17]. What was unclear, however, was specifically how white blood cell activation may affect flow behavior and then in turn, how flow behavior affects apparent viscosity and microvascular resistance.

The results of the present investigation provided evidence that neutrophil activation may indeed be capable of impacting the flow behavior of blood. We draw this conclusion from the observation that activation was capable of raising the viscosity of a solution of neutrophils. One explanation for the increased solution viscosity of an activated neutrophil solution when compared to the inactivated solution would be the increased interactions between the pseudopod extending neutrophils. In comparison to rounded inactivated leukocytes, the pseudopod-extending cells likely spin, or ‘tumble’ more erratically due to their irregular shape and to the uneven velocity profile of the

surrounding flow field. The extended pseudopods may cause the neutrophils to experience different velocities on different regions of their surface, causing a force imbalance, and resulting in their ‘tumbling’. This tumbling probably increases incidences of bumping interactions between cells, as well as possibly increases their tendency to bump into the container walls. It should be noted that different cell densities were not examined, however, that would be an area for further research because more pseudopod-extending cells in the same volume would result in a greater number of collisions, and potentially a greater increase in viscosity.

With the addition of hematocrit into the cell suspensions, the impact of cell activation on apparent viscosity differences were abrogated. The rheological properties of these RBC-enriched cellular solutions likely model the rheology of macroscale blood flow because the cone and plate viscometer dimensions vary from millimeters to hundreds of microns. As discussed earlier, in larger vessels ($>100\ \mu\text{m}$) the red blood cells influence viscosity changes to the point that increased interactions between activated neutrophils cannot be detected on this scale. It is likely that the presence of a large number of highly deformable red blood cells dampened the ability of the pseudopod extending white blood cells to interact with each other and the wall.

Having seen that pseudopod extension associated with neutrophil activation is capable of affecting viscosity in the absence of hematocrit, we wanted to determine the effects of neutrophil activation within capillary sized microvessels. Shear rates in this environment can alter flow behavior differently than in the viscometer due to the Fåhræus–Lindqvist effect. These flow resistance experiments were carried out within capillary diameter microvascular mimics.

4.5. Isopore® Filters For Microvascular Modeling

The micropore network analysis was conducted using a syringe pump in conjunction with a stir plate to keep the cellular solution mixed. This stir bar setup was a necessary component of the system to ensure the white and red blood cells were homogeneously distributed in suspensions prior to their perfusion through micropores. There is evidence that shear stress causes neutrophils to retract their pseudopods [35]. In order to prevent this with our studies, the neutrophils were fixed with paraformaldehyde so their morphology would be maintained. This was a necessary step because our solutions lacked agonists which would normally be responsible for maintaining pseudopod projection in a sustained activation state. Fixation was the method of choice to ensure that the cells retained an activated morphology from the start of each experiment to its end.

The 10- μm Isopore® filters were chosen to mimic capillary vasculature. From the syringe pump, the solution was driven through the filter which was located in parallel with a pressure transducer (figure in section 2.8a). Several experiments have already been conducted to examine perfusion through microvessels using small pores as an in vitro mimic. In one such study, Worthen investigated neutrophil retention within pulmonary microvascular-like porous networks [26]. He postulated that some combination of three factors lead to this potential neutrophil retention. These factors included geometric constraints imposed by the small vasculature, the deformability of the neutrophil, and the hydrodynamic characteristics of blood. He found that the critical pore size for neutrophil retention was between 5 and 8 μm [26], and that in a 5- μm filter, retention was about

75%. However, when pore size was increased to 10- μm , the retention rate dropped to only about 10%. The object of this investigation was not to examine neutrophil plugging in the smallest capillaries. Instead, the present study was focused on examining how the morphology of activated neutrophils could affect microvascular resistance by increasing apparent viscosity. So, to decrease the retention rate and allow flow behavior to dominate the reasons for pressure changes, 10 μm micropore filters were chosen. Had the pore size been reduced to the diameter of smaller capillaries, we would have expected neutrophil plugging to have been more prevalent. This would have potentially led to pressure differences being the result of filter clogging and/or cells piling up behind the filter.

For the present study, perfusion pressure within the filter chamber was used as a metric to assess how the rheological behavior of neutrophil suspensions flowing through 10- μm pores impacted micropore flow resistance. We expected to observe changes in the perfusion pressure depending on whether or not the neutrophils perfusing through the pores were activated. It is likely that pseudopod-extending neutrophils would exhibit more erratic tumbling behavior in the pore channels, which would then cause them to disturb the cell-free layer and, in doing so, increase apparent viscosity. This increase in apparent viscosity would manifest as an increase in flow resistance of fluid through the pores and would be measured as an increase in perfusion pressure across the entire filter. Our expectation in this regard is based on the direct relationship between apparent viscosity and pore/microchannel resistance, according to classical Poiseuille flow.

A final important design aspect for this study was choosing the flow rate at which to pump the cell solutions through the micropore filter. The decision to use the 500 $\mu\text{L}/\text{min}$ flow rate was in an attempt to match the slower rates associated with

capillary diameter vasculature [26]. This flow rate matched the lowest flow rate Worthen investigated in his microvessel mimic experiments [26].

4.6. Evaluating Time Dependent Micropore Pressure Results

The results of our viscosity analyses suggested that morphological changes associated with pseudopod extension have an effect on the rheological (tumbling) behavior of neutrophils in a flow environment. Previously we have already demonstrated that pseudopod extension increased the apparent viscosity of a neutrophil solution. The micropore experiments set out to demonstrate the rheological impact of cell activation on neutrophil flow behavior in microvessels using an approach similar to that of Worthen [26].

We first examined the effects of neutrophil activation on micropore resistance with cell suspensions devoid of hematocrit (i.e., red blood cells). The results of these analyses were that pseudopod extension (due to cell activation) affects perfusion pressure across the Isopore® membrane. Furthermore, changes in cell morphology due to agonist stimulation appeared to be a determinant of the pressure required to perfuse neutrophil suspensions through micropore membranes. Mechanistically, it is likely that the tumbling of the irregular shaped cells promoted increased stochastic interactions either with other cells or the cell-free zone of each pore, or likely some combination of the two. In the *in vivo* microvasculature, cellular collisions reportedly disrupt the thin cell-free zone separating the vasculature from the cell-rich vessel center [23, 24]. Disruption of this thin lubrication layer in the pores of the Isopore® membranes may have occurred. This disruption could have led to an increase in the apparent viscosity of cell suspensions, and

in turn, driven up micropore resistance. Thus, transport of leukocytes through the micropores required increased perfusion pressures.

For our studies with neutrophils in suspension with erythrocytes, we chose to use 10% hematocrit because this value is physiologically relevant to the microvasculature. Hematocrit in the microcirculation typically ranges between 10 and 15%[36]. Notably, the presence of red blood cells exacerbated the effects of cell activation on the pressure required to perfuse neutrophil suspensions through microfilter networks. Having used the 10- μm pore filters to minimize clogging of pores, the observed increases in perfusion pressures were likely due to enhanced stochastic cell to cell collisions. These collisions between the rigid, ‘tumbling’ leukocytes presumably disrupted the cell-free marginal layers in the micropores as the blood-like cell suspensions flowed across the membrane. Compared to suspensions of white blood cells alone, 10% hematocrit allows for additional collisions between the tumbling activated cells and the red blood cells. These ‘extra’ collisions at the moment of passing through the filters could have conceivably disrupted this layer even more than when the neutrophils were in suspension alone, causing greater increases in apparent viscosity. Another explanation for the increased resistance in the system is that RBC’s become destabilized and collides with the cell free layer as they pile up behind slower moving tumbling neutrophils. In either case, pseudopod extension has led to a significant increase in blood flow resistance in this capillary diameter vascular mimic.

Staining of used filters after flow experiments confirmed that activated neutrophils in either the absence or presence of hematocrit were not becoming lodged in the filter pores to a significantly higher degree than inactivated cells over the 1500-

second duration of the micropore flow experiments. Thus, cell clogging was not responsible for the observed effects of cell activation on neutrophil suspension perfusion pressure. The extent to which we observed cell clogging was similar to that observed previously by other investigators who studied microfiltration of neutrophil suspensions [26]. Furthermore, the clogging that we did observe during our studies appeared to be responsible for the gradual time-dependent increases in perfusion pressures observed for neutrophil suspensions under all conditions tested in this present study.

4.7. Summary and Concluding Remarks

In conclusion, the present study provided in vitro evidence that pseudopod extension by neutrophils may have a rheological impact on blood flow in pre-capillary diameter arterioles. These results are consistent with the findings first reported by Skalak and Helmke, [2, 16] who showed, using ex vivo studies of skeletal muscle tissues that leukocyte activation in the bloodstream enhances hemodynamic resistance in the microvasculature. This work has direct implications relating to the contribution of chronic inflammation in the blood to the pathogenesis and progression of cardiovascular disease. Chronic low level inflammation has been identified by the CDC as a common denominator for 7 out of 10 of the country's leading causes of morbidity. Thus, this study shows that cell-activation dependent changes in neutrophil flow behavior within the small microvessels of the arteriolar network can potentially impact hemodynamic resistance with serious consequences to health.

References

1. Granger, D.N. and E. Senchenkova. *Inflammation and the Microcirculation*. in *Colloquium Series on Integrated Systems Physiology: From Molecule to Function*. 2010. Morgan & Claypool Life Sciences.
2. Helmke, B.P., et al., *A mechanism for erythrocyte-mediated elevation of apparent viscosity by leukocytes in vivo without adhesion to the endothelium*. *Biorheology*, 1998. **35**(6): p. 437-448.
3. Késmárky, G., et al., *Plasma viscosity: a forgotten variable*. *Clinical hemorheology and microcirculation*, 2008. **39**(1): p. 243-246.
4. Kwaan, H.C., *Role of plasma proteins in whole blood viscosity: a brief clinical review*. *Clinical hemorheology and microcirculation*, 2010. **44**(3): p. 167-176.
5. Baskurt, O.K. and H.J. Meiselman, *Hemodynamic effects of red blood cell aggregation*. *Indian journal of experimental biology*, 2007. **45**(1): p. 25.
6. Cabel, M., et al., *Contribution of red blood cell aggregation to venous vascular resistance in skeletal muscle*. *American Journal of Physiology-Heart and Circulatory Physiology*, 1997. **272**(2): p. H1020-H1032.
7. Murata, T. and T.W. Secomb, *Effects of shear rate on rouleau formation in simple shear flow*. *Biorheology*, 1987. **25**(1-2): p. 113-122.
8. Brust, M., et al., *The plasma protein fibrinogen stabilizes clusters of red blood cells in microcapillary flows*. *Scientific reports*, 2014. **4**.
9. Caro, C., et al., *The Cell Free Layer*, in *The Mechanics of the Circulation*. 1978, New York: Oxford Medical. p. 396.
10. Toksvang, L.N. and R.M. Berg, *Using a classic paper by Robin Fåhræus and Torsten Lindqvist to teach basic hemorheology*. *Advances in physiology education*, 2013. **37**(2): p. 129-133.
11. Popel, A.S. and P.C. Johnson, *Microcirculation and hemorheology*. *Annual review of fluid mechanics*, 2005. **37**: p. 43.
12. Fåhræus, R. and T. Lindqvist, *The viscosity of the blood in narrow capillary tubes*. *American Journal of Physiology--Legacy Content*, 1931. **96**(3): p. 562-568.
13. Kim, S., et al., *The cell-free layer in microvascular blood flow*. *Biorheology*, 2009. **46**(3): p. 181-189.
14. Mescher, A.L., *Chapter 12. Blood*, in *Junqueira's Basic Histology : Text & Atlas*. 2010, McGraw-Hill Medical New York.
15. Schmid-Schönbein, G.W., *Analysis of inflammation*. *Annu. Rev. Biomed. Eng.*, 2006. **8**: p. 93-151.
16. Helmke, B.P., et al., *Mechanisms for increased blood flow resistance due to leukocytes*. *American Journal of Physiology-Heart and Circulatory Physiology*, 1997. **273**(6): p. H2884-H2890.
17. Lipowsky, H.H., *Microvascular rheology and hemodynamics*. *Microcirculation*, 2005. **12**(1): p. 5-15.
18. Worthen, G.S., et al., *Mechanics of stimulated neutrophils: cell stiffening induces retention in capillaries*. *Science*, 1989. **245**(4914): p. 183-186.

19. Eppihimer, M. and H. Lipowsky, *Effects of leukocyte-capillary plugging on the resistance to flow in the microvasculature of cremaster muscle for normal and activated leukocytes*. Microvascular research, 1996. **51**(2): p. 187-201.
20. Arndt, H., C.W. Smith, and D.N. Granger, *Leukocyte-endothelial cell adhesion in spontaneously hypertensive and normotensive rats*. Hypertension, 1993. **21**(5): p. 667-673.
21. Fukuda, S., et al., *Contribution of fluid shear response in leukocytes to hemodynamic resistance in the spontaneously hypertensive rat*. Circulation research, 2004. **95**(1): p. 100-108.
22. Warnke, K.C. and T.C. Skalak, *Leukocyte plugging in vivo in skeletal muscle arteriolar trees*. American Journal of Physiology-Heart and Circulatory Physiology, 1992. **262**(4): p. H1149-H1155.
23. Karnis, A., H. Goldsmith, and S. Mason, *Axial migration of particles in Poiseuille flow*. Nature, 1963. **200**: p. 159-160.
24. Pries, A., T. Secomb, and P. Gaehtgens, *Biophysical aspects of blood flow in the microvasculature*. Cardiovascular research, 1996. **32**(4): p. 654-667.
25. Mazzoni, M.C.a.G.W.S.-S., *Mechanisms and consequences of cell activation in the microcirculation*. Cardiovasc Res, 1996. **32**(4): p. 709-19.
26. Downey, G.P. and G.S. Worthen, *Neutrophil retention in model capillaries: deformability, geometry, and hydrodynamic forces*. Journal of Applied Physiology, 1988. **65**(4): p. 1861-1871.
27. Akenhead, M., X. Zhang, and H. Shin, *Characterization of the shear stress regulation of CD18 surface expression by HL60-derived neutrophil-like cells*. Biomechanics and modeling in mechanobiology, 2014. **13**(4): p. 861-870.
28. Tsai, M., R. Waugh, and P. Keng, *Changes in HL-60 cell deformability during differentiation induced by DMSO*. Biorheology, 1996. **33**(1): p. 1-15.
29. Hauert, A.B., et al., *Differentiated HL-60 cells are a valid model system for the analysis of human neutrophil migration and chemotaxis*. The international journal of biochemistry & cell biology, 2002. **34**(7): p. 838-854.
30. Hache, J., " *Today we Change the Practice of Medicine!*" Dr. M. Oz, MD.
31. Miles, K., et al., *Dying and necrotic neutrophils are anti-inflammatory secondary to the release of α -defensins*. The Journal of Immunology, 2009. **183**(3): p. 2122-2132.
32. Hainsworth Y. Shin, X.Z., Aya Makino, Geert W. Schmid-Schonbein, *Mechanobiological Evidence for the Control of Neutrophil Activity by Fluid Shear Stress*, in *Mechanobiology Handbook*. 2011, CRC Press.
33. Shin, M.K., et al., *N-formyl-methionyl-leucyl-phenylalanine (fMLP) promotes osteoblast differentiation via the N-formyl peptide receptor 1-mediated signaling pathway in human mesenchymal stem cells from bone marrow*. Journal of Biological Chemistry, 2011. **286**(19): p. 17133-17143.
34. Frank, R.S., *Time-dependent alterations in the deformability of human neutrophils in response to chemotactic activation*. Blood, 1990. **76**(12): p. 2606-2612.
35. Shin, H.Y., S.I. Simon, and G.W. Schmid-Schönbein, *Fluid shear-induced activation and cleavage of CD18 during pseudopod retraction by human neutrophils*. Journal of cellular physiology, 2008. **214**(2): p. 528-536.

36. Klitzman, B. and B.R. Duling, *Microvascular hematocrit and red cell flow in resting and contracting striated muscle*. American Journal of Physiology-Heart and Circulatory Physiology, 1979. **237**(4): p. H481-H490.

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