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## Mechanotransduction of Leukocyte Transmigration

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MECHANOTRANSDUCTION OF LEUKOCYTE TRANSMIGRATION

by

CYNTHIA CELESTE AGUILAR  
B.A. University of Central Florida, 2017

A thesis submitted in partial fulfillment of the requirements  
for the degree of Master of Science  
in the Department of Interdisciplinary Studies  
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at the University of Central Florida  
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Major Professor: Robert Steward Jr.

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## **ABSTRACT**

The endothelium is among the most mechanically enriched environments in the body. It is exposed to a range of hemodynamic-induced and extracellular forces. Of these extracellular forces, the migration of leukocytes through the endothelium will contribute both to classic immune response and development of certain pathologies. While the path of migration across the endothelium will depend on leukocyte and vascular bed type, recent evidence has suggested that the intercellular mechanical microenvironment and forces are also equally as important to this process. Therefore, we present here a model that mimics specific physiological states of a stagnant hemodynamic flow in which we hypothesize that leukocytes will demonstrate attachment preferences to particular areas of differing intercellular stresses on the endothelial bed. Using a model such as this one, it may be possible to exploit these intercellular stresses when developing macrophage-targeted therapies.

**Keywords: Macrophage, Intercellular stresses, Endothelial cells, THP-1 Cells, Immunohistochemistry, Traction Force Microscopy**

## **ACKNOWLEDGMENTS**

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## CHAPTER 1 INTRODUCTION

White blood cells (WBC) or leukocytes are a diverse group of cell types that are distinguished by functional and physical characteristics that promote the body's immune response. Circulating through the blood and lymphatic system, leukocytes can exit the vasculature and penetrate into the tissues, either for patrolling in search for pathogens or to eliminate infection and activate the adaptive immune response. Leukocyte transcellular migration, however, may also be dependent on specific mechanotransduction processes of the surrounding endothelium.

There are several subsets of leukocytes that derive from hematopoietic stem cells that are distinguished by functional and physical characteristics. (Frederic Geissmann et al.) Neutrophils, the most numerous subset of leukocyte in the body, are the first line of defense at a site of infection or injury, killing and digesting bacteria and fungi. (Teng et al.) They are short-lived, often only living for a few hours. Neutrophils also contain granules that act as storage facilities for antimicrobial enzymes that produce chemical reactions in organic substances. (Liu and Sun) Basophils also contain granules and are the least numerous type of WBC. In contrast to neutrophils, these cells release histamines that mediate inflammation by widening the blood vessels and allowing other subsets of leukocytes to reach the site of infection. (Stone et al.) Eosinophils are the last of the granular type, releasing specific toxins, these cells are able to attack and kill particularly antibody coated parasites and other pathogens. (Fabre et al.) (Fulkerson and Rothenberg) Lymphocytes and monocytes are both classified as non-granular or agranular. Lymphocytes have specific antibodies with specialized surface receptors designed to combat specific invading pathogens. (Couture et al.) Monocytes in particular are advantageous in studies

because they are the largest and have longer lifespans than most white blood cells. They act as scavengers, digesting foreign organisms and other dead WBCs. (Riley and Rupert)

Macrophages further differentiate to employ a diverse set of functions, each having distinct fates. The addition of certain effectuates induces different differentiation pathways to M1-like macrophages or M2-like macrophages by using internal and external environmental cues with either type II interferon ( $\text{IFN}\gamma$ ) or interleukin 4 (IL-4), respectively. (Martinez and Gordon) Monocyte, specifically macrophages, migration to sites of inflammation and their subsequent accumulation are critical stages in the development of the inflammatory and immune response. (Cui et al.) Despite the importance of macrophage passage, the mechanisms of macrophage migration are still not fully comprehended.

The body's vasculature encounters a flow of blood that meets the thin sheet of cells which construct the tunica intima of the blood vessels comprised of endothelial cells. Endothelial cells have multiple functions, most notably their ability to regulate the passage of material between the bloodstream and the tissue, their role in blood flow regulation, and their vitality to vascular homeostasis. We find the endothelium throughout the body's vasculature; however, ECs organization differs depending on the location of the vasculature. In areas where blood solute permeability is less regulated, we find that the ECs are less tightly compact allowing for an easier passage of materials from the bloodstream through to the rest of the body. (Dewey et al.)

Leukocyte transendothelial migration (LEM) plays an essential role both in the normal immune response and the development of certain cardiovascular diseases, such as atherosclerosis and stroke. Arguably one of the most mechanically enriched environments in the body, the vascular endothelium is exposed to several physical forces, including shear stress from flow of blood and

intercellular contact from neighboring endothelial cells and normal stresses arising in regions where leukocytes breach the endothelial barrier. The effect of intercellular shear stress and intercellular normal stress on leukocyte adhesion and penetration of endothelial cells is particularly relevant to atherogenesis. Therefore, we present here a model that mimics specific physiological states of a stagnant hemodynamic flow in which we hypothesize that leukocytes will demonstrate attachment preferences to particular areas of differing intercellular stresses on the endothelial bed. Using a model such as this one, it may be possible to exploit these intercellular stresses when developing macrophage-targeted therapies.

### **1.1 Differentiation of Monocytes and Polarization of Macrophages**

The differentiation of monocytes to macrophages is specifically important to the response of inflammation. In 2000, a new classification of macrophages as either M1 or M2 was proposed. (Martinez and Gordon) The differentiation of macrophages into either an M1 or M2 state involves changing the intercellular signaling network of the individual macrophage at the transcriptional and translational levels. (Lee)

M1 and M2 macrophages differ in chemokine receptor profiles depending on the response to intracellular and extracellular cues. For example, the M1 secretes the Th1 cell attracting chemokines CXCL9 and CXCL10, while the M2 secretes CCL17, CCL22 and CCL24. (Tokunaga et al.)

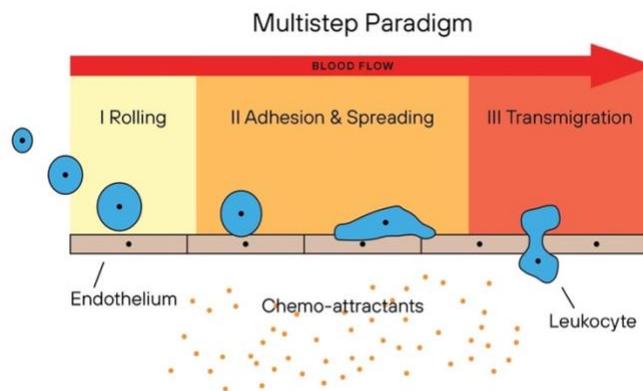
Activated M1 macrophages begin producing a proinflammatory cytokines as an immune response, while M2 macrophages function with an anti-inflammatory response. (Atri et al.) M1 macrophages are initiated by interferon gamma (IFN-g) or lipopolysaccharide (LPS) while M2

macrophages are activated by cytokines, such as IL-4, IL-10, or IL-13. (Dhananjay T. Tambe et al.)

## 1.2 Transmigration

LEM plays an essential role in a normal immune response and if this process occurs with abnormalities, certain pathological and physiological implications may ensue.

Originally introduced by Butcher (Butcher E C) and extended by Springer (Springer), the Multistep Paradigm (Figure 1) involves three steps that are widely accepted to occur during normal LEM. (Jaap D. Van Buul and Peter L. Hordijk)



*Figure 1 The Multistep Paradigm  
(Created by Author)*

The first of these steps is the rolling of leukocytes and involves several adhesion molecules such as selectins on the leukocyte or endothelial cell membrane attaching to the ligands on the membrane of their cellular counterpart. (Springer) Following the rolling of the leukocyte, a firm adhesion forms between the leukocyte and endothelium. This attraction is facilitated through the stagnant chemo-attractants passing through the endothelium from the point of infection or inflammation and is then reinforced by adhesion molecules such as integrins on the rolling leukocyte. (Jaap D. Van Buul and Peter L. Hordijk) Once the leukocyte forms a bond on the

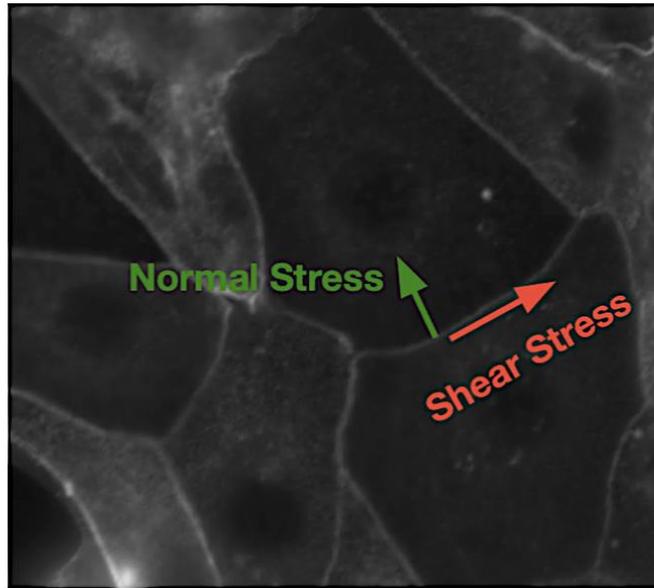
monolayer, they spread pseudopodia in order to select a point of entry. Once the leukocyte locates a point of entry, the diapedesis of the leukocytes begins.

Leukocyte stresses during transmigration have been thoroughly studied, however, the stresses in the endothelium may also affect this transmigration. The spreading and diapedesis of the leukocytes may be directly impacted by the cell derived intercellular stresses and affect where the leukocytes attach versus where they transmigrate.

### **1.3 Intercellular Derived Stresses**

For immune cells to travel from the blood stream to the tissues outside of the vessel, they must first cross the innermost layer of the blood vessel made of ECs. As leukocytes cross through this layer, forces are exerted both on the leukocytes and the ECs. Given their mechanical environment, there exists a constant normal and shear stress exerted on the ECs. In turn, this strain produces intracellular stresses in the endothelium from neighboring cells and matrix that act throughout the vasculature.

The intercellular stresses involved is comprised of two mutually independent components; normal stress, which acts perpendicular to the cell-cell junctions, pushing and pulling towards the center of the cell, and shear stress, which acts parallel to the cell-cell junctions (Figure 2).



*Figure 2 Normal and Shear Intercellular Stresses on Planarized Monolayer  
(Created by Author)*

Aside from the importance of biochemical interactions between cells for communication, intercellular mechanical forces, such as traction forces and intercellular stresses between cells on a monolayer are important for normal physiological activity.

#### **1.4 Intercellular Stresses**

Intercellular stresses are important since they are the forces that enable other cells to be pulled in unison to a particular direction. (Figure 2) That interplay between cell-cell forces maintains cell stability. Intercellular stress is important for all cells, but particular for those lining vasculatures as the endothelium facilitates a proper immune response. The local monolayer intercellular stress is comprised of two mutually independent components; normal stress that acts in lines perpendicular to the cell-cell junction in a pushing and pulling motion and the shear stress that acts parallel to the cell-cell junction in a sliding motion.

Intercellular shear stress in the endothelium stimulates cell mechanoreceptors and leads to the signal transduction changes in cell morphology and function and leads to the mechanotransduction of leukocyte migration and these stresses increase the further the cells are from the monolayer edge. (McKinney et al.)

### **1.5 Traction Forces**

Traction forces are local forces that a cell exerts on a substrate through the use of focal adhesions that provide the means for specific physiological processes to occur. Traction forces play a role in ECM reorganization and assembly.

In statistical mechanics, the root mean square displacement (RMS) is a measure of deviation of the position of a particle with respect to a reference position over time. In the case of measuring cell tractions, RMS displacement is measured over time to determine the contribution of an acquired force. (Michalet)

In order to understand how tractions are generated by each cell in question is to first localize the traction forces at the leading edge of each cell to assess it. Assessing the force at the leading edge of the cell and then comparing it to the other cells in the monolayer will demonstrate if large tractions will be exhibited by other cells that are independent of the lead cell.

### **1.6 Physiological and Pathological Implications of Improper Leukocyte Transcellular Migration**

Our objective to investigate the influence of intercellular stresses of endothelial cells during the leukocyte transmigration that takes place during inflammation is particularly relevant to

specific physiological and pathological implications. Therefore, the effect of intercellular shear and normal stresses on external cell adhesion to the endothelium is particularly relevant.

Inflammation is an essential mechanism for the development of several pathological implications, particularly cardiovascular and metabolic diseases. The outcome of these diseases depends heavily on the transmigration of WBCs and specifically the balance of migration and accumulation of pro-inflammatory (M1) and anti-inflammatory (M2) macrophages to damaged tissue. (Cui et al.)

Acute inflammation develops more swiftly than chronic inflammation, but it is the latter that plays an essential role in the initiation and progression of diseases including atherosclerosis, diabetes, obesity, and arthritis. (Parisi et al.) The subset of accumulated macrophages is critical for the progression and/or resolution of this chronic inflammation. (Cui et al.) Better understanding the mechanisms of the subsets of the immune response can help improve preventative healthcare and this idea is true for both endothelial cells and leukocytes.

Macrophages constitute the most numerous of the subset of infiltrating leukocytes correlated with solid tumors. The macrophages are recruited to the tumor site from the surrounding areas by the tumor through the secretion of chemotactic molecules. (Lee)

In general, macrophage recruitment into different organs has similarities to the multistep paradigm. The transmigration of macrophages into liver tissue, however, differs slightly. The recruitment into the hepatic sinusoids does not need to be initiated first by the rolling step, instead, the macrophages form attachments directly to the hepatic endothelium. (Wong et al.)

The vascular disease, atherosclerosis is one of the most common cardiovascular diseases that impact the vasculature throughout the body. It produces the hardening of the veins and the

formation of atherosclerotic plaque, constricting the blood vessels and reducing blood through the body. This plaque build-up is detrimental to vascular homeostasis. Oxidized LDL (oxLDL) in the tunica intima of the arterial wall triggers the recruitment of macrophages through the endothelium. The macrophages then absorb the oxLDL, and unable to process, then, they transforming into foam cells leading to plaque buildup. (Chen and Khismatullin)

## **CHAPTER 2 METHODOLOGY**

The assay conducted was accomplished over a time period of approximately two weeks. The culture of HUVEC and THP-1 cells took roughly one week to become confluent after cryopreservation. The polarization of THP-1 took four days. PA Gel and PDMS micropattern formation were accomplished simultaneously. Following the solidification of the PA gels, an ECM coating was done. HUVEC were then seeded to form a monolayer on the gels. After the monolayers became confluent, a proinflammatory state was induced. While this was happening, THP-1 cells were tagged, and an imaging assay was conducted immediately afterward, and derived data was processed.

### **2.1 Cell Culture and Preparation**

#### 2.1.1 THP-1 Human Monocytic Cell Line

THP-1 cells are a spontaneously immortalized human monocytic cell line derived from the peripheral blood of a childhood case of acute monocytic leukemia. These circulating monocytes contain the potential to differentiate into different subsets of tissue macrophages that aid against invading pathogens. Working with this type of cell provides a cost-effective and valuable tool for investigating monocytic structure and function. (Lee et al.)

This cell type is grown in RPMI 1640 medium purchased from ThermoFisher Scientific. To complete the growth medium, it was supplemented with 2-mercaptoethanol to a concentration of 0.05 mM and fetal bovine serum (FBS) to a final concentration of 10%. The FBS contains a large number of nutritional and macromolecular factors essential for cell growth and several smaller molecules such as amino acids, sugars, lipids, and hormones.

### 2.1.2 Human Umbilical Vein Endothelial Cells (HUVECs)

Immortalized human umbilical vein endothelial cells (HUVECs), purchased from ThermoFisher Scientific, were cultured in T25 flasks in sterile, liquid medium created for the culture of human large vessel endothelial cells (Medium 200). 50 mL of large vessel endothelial supplement (LVES) was added to 500 mL of this medium for culture. The cells were stored in an incubator at 37°C with 5% CO<sub>2</sub>.

### 2.2 Polarization of THP-1

THP-1 cells were treated for 72 hours with 50  $\frac{ng}{mL}$  phorbol 12-myristate 13-acetate (PMA) in three distinct t25 cell culture flasks with a cell suspension of 5x10<sup>5</sup> cells in each flask. Polarized, adherent cells were washed twice with phosphate buffered saline (PBS) to remove any non-adherent cells and was replaced with fresh media containing either (a) 50  $\frac{ng}{mL}$  PMA, (b) 100  $\frac{ng}{mL}$  LPS+ 50  $\frac{ng}{mL}$  PMA, or (c) 20  $\frac{ng}{mL}$  IL-4 + 50  $\frac{ng}{mL}$  PMA for a further 24 hours to generate PMA controls (M0-PMA), M1-like macrophages (M1-LPS/IFN $\gamma$ ) and M2-like macrophages (M2-IL-4).

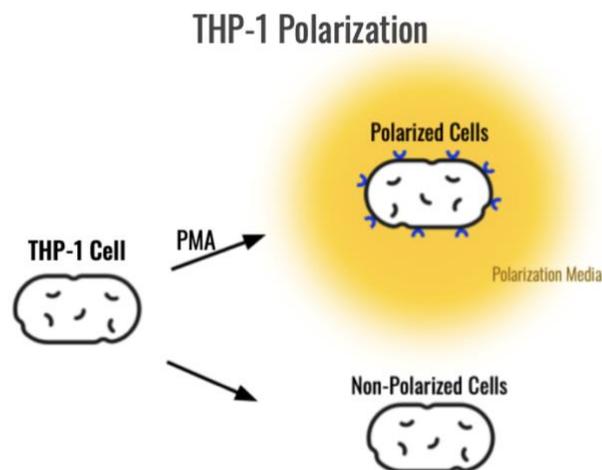
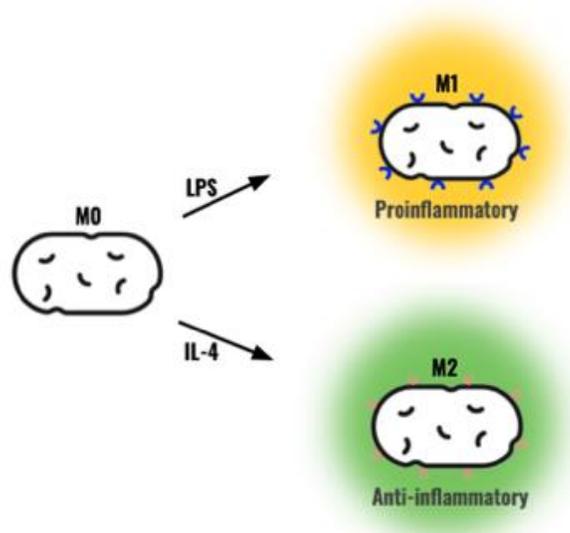


Figure 3 THP-1 Polarization  
(Created by Author)



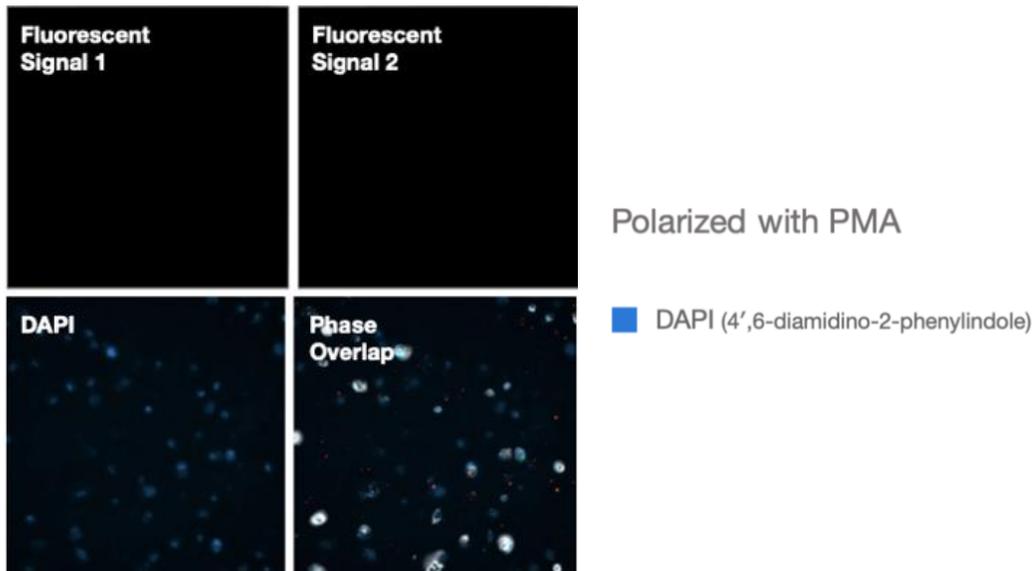
*Figure 4 Macrophage Polarization  
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### 2.2.1 Immunohistochemistry

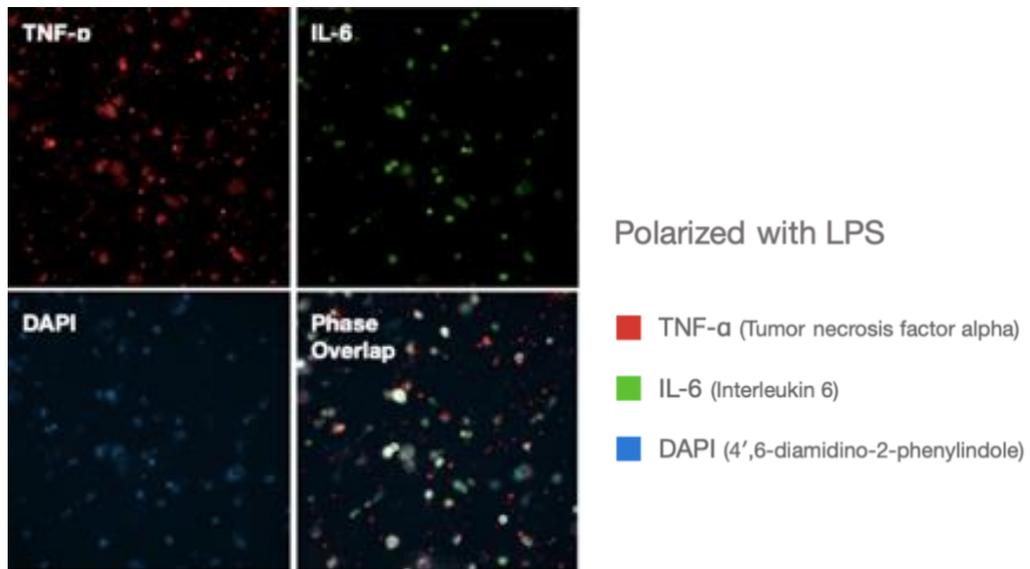
To confirm polarization, the THP-1 cells were immunostained after polarization protocol to indicate specific protein markers on M1 and M2 subsidiaries of macrophages; TNF-  $\alpha$  and IL-6 and IL-10 and MRC1, respectively. The samples were removed from the incubator after the four-day polarization protocol and were fixed with a 4% formaldehyde solution (5.26 mL formaldehyde and 44.74 mL PBS) [50 mL solution] for 15 minutes in the incubator. Immediately after fixing the cells with formaldehyde, samples were rinsed thrice with PBS and 0.2% Triton x-100 (2 mL), a no-denaturing and mild detergent, was added. Samples were incubated for 5 minutes in order to solubilize proteins. The Triton solution was then rinsed five times with PBS to ensure its removal and a Blocker™ BSA (10x) in PBS solution was added and incubated for 45 minutes in order to saturate excess protein-binding sites on cell membranes. While samples were permeabilizing, each respective primary antibody was prepared for an immediate application with a 5  $\mu$ L of the primary

antibody, 1 mL of PBS, and 1 mL of blocking solution. Primary antibodies used were TNF-  $\alpha$  and IL-6 for the M1 polarization state and IL-10 and MRC1 for the M2 polarization state. The antibody solution was let to sit for 12 hours at 4°C

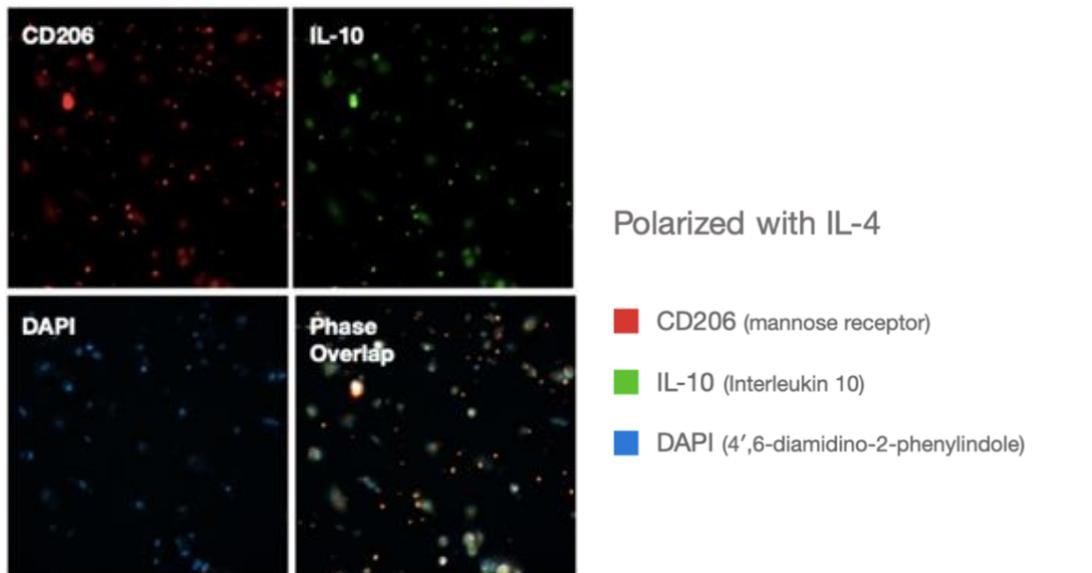
After 12 hours, the primary antibody solution was aspirated from samples and then they were rinsed four times with PBS and treated with a solution of 15  $\mu$ L secondary antibodies diluted in 1 mL of PBS. The secondary antibodies were used to correlate with the respective primary antibodies, were diluted in 1 mL of PBS, and displayed different wavelengths, allowing them to be distinguished; TNF-  $\alpha$  was marked with anti-rat (594), IL-10 was marked with anti-mouse (594), and IL-6 and MRC1 were both tagged with anti-rat (488). This solution was let to sit for four hours at room temperature covered with aluminum to prevent interference from ambient light. Following the four-hour exposure to the secondary antibodies, samples were fixed with the mounting solution Fluoromount-G and DAPI (a blue-fluorescent DNA stain, which binds to AT regions of double stranded DNA) to keep samples hydrated and a glass coverslip was placed over the sample. The images shown in Figure 1 depicts a sample of each macrophage polarization state. Imaging was conducted within three days after samples were fixed with the glass cover slides.



*Figure 5 Confirming Polarization of M0 after 16 Hours in HUVEC Media  
(Created by Author)*



*Figure 6 Confirming Polarization of M1 after 16 Hours in HUVEC Media  
(Created by Author)*



*Figure 7 Confirming Polarization of M2 after 16 Hours in HUVEC Media  
(Created by Author)*

## 2.3 Gel and Micropattern Formation

### 2.3.1 Cellular Micropatterning

A thin layer of Polydimethylsiloxane (PDMS) was manufactured in order to create circular micropatterns for cell culture. A combination of Sylgard® 184 silicone Elastomer Base, a silicone base, and Sylgard® 184 silicone elastomer curing agent, a curing agent, with a ratio of 20:1, respectively, was cured in 10mm petri dish. The cured PDMS was left overnight on a hot plate set to 36°C. Once polymerized, circular PDMS sections were extracted of a diameter of  $\frac{5}{8}$  inches to snugly fit on top of the 35 mm glass-bottom petri dishes. Holes were then perforated on the circular sections using a 1.5 mm diameter biopsy punch.

### 2.3.2 Polyacrylamide Gel Fabrication

*Table 1 PDMS Stiffness Protocol  
(Created by Author)*

<b>Total solution (15 m)</b>	<b>1.2 kPa</b>
Ultra-pure water	12.49 mL
40% Acrylamide	2.062 $\mu$ L
2% BIS	375 $\mu$ L
Yellow-Green 0.5 $\mu$ L	80 $\mu$ L

Polyacrylamide (PA) gels of a stiffness of 1.2 kPa were prepared by first treating 35mm petri dishes with a bind saline solution for thirty minutes followed by three washes with de-ionized (DI) water. The glass-bottom dishes were then air-dried. The stiffness of the gel was accomplished by mixing the components in table 1, adding Flourospherestm (ThermoScientific) carboxylated, 0.5 $\mu$ m, yellow-green beads of a wavelength approximately between 505 and 515, and then de-gassing the solution for 45 minutes. After de-gassing 75  $\mu$ L of a 10% Ammonium persulfate solution dissolved in Ultra-pure water was added and then 8 $\mu$ L of TEMED was added to polymerize the gel. The solution was added in 20  $\mu$ L increments on the treated petri dishes. Quickly following this step, the PA gels were flattened to a height of 100  $\mu$ m by using 18mm glass circular cover slips. Ultra-pure water was added on top of the hydrogels to maintain its consistency. The gels were kept at 4°C for up to two weeks.

## 2.4 Extracellular Matrix Coating

A day before cells were to be seeded, the Ultra-pure water and glass cover slips were taken off of the gels. The micropatterns were then put on the previously made PA gels and treated with 1 mL of a SANPAH (sulfosuccinimidyl-6-(4-azido-2-nitrophenylamino) hexonate solution diluted with 10 mL of 0.1 M HEPES (Fisher Scientific). A SANPAH curing was performed by placing the PA gels under a UV lamp for six to ten minutes. This was then followed by rinsing the gels with once with HEPES and two times with PBS to remove any SANPAH remainders. Next, the patterned gels were treated with a freshly made 0.1  $\frac{mg}{mL}$  collagen I (advanced Biomatrix) solution in PBS. The gels were let to sit overnight at 4°C. The next day, excess collagen was removed from the gel and cells were seeded and incubated for an hour to allow attachment. After attachment, micropatterns were removed and HUVEC cells were allowed to form a confluent monolayer for 24 hours prior to experimentation.

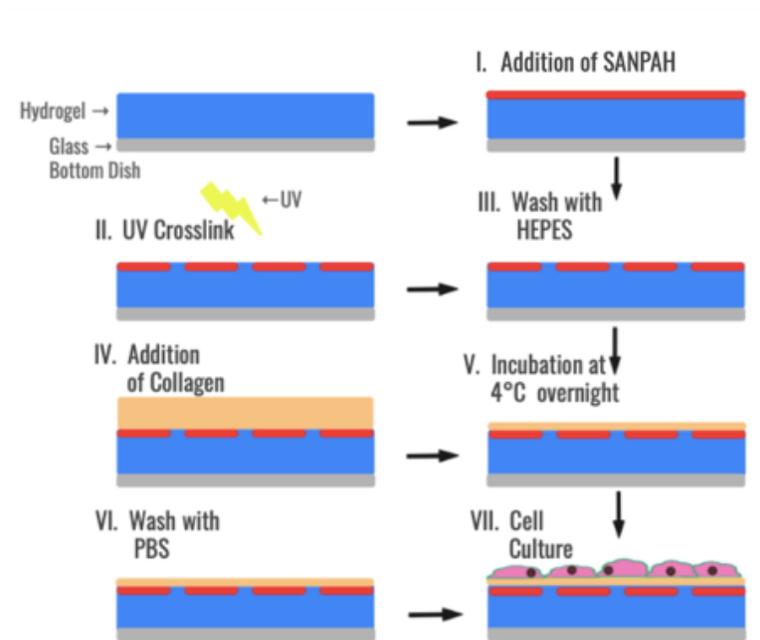
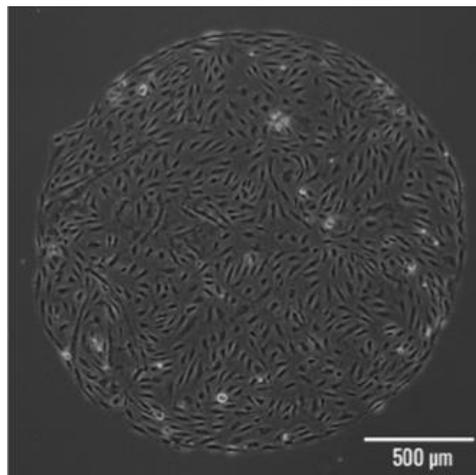


Figure 8 Extracellular Matrix Coating  
(Created by Author)

## 2.5 Monolayer Formation

HUVECs were added to PDMS micropatterns at a concentration of approximately  $1 \times 10^4$  cells per ml. The HUVECs were incubated for one hour to allow attachments to form on the PA gels underneath and then the micropattern was removed. This was done in order to prevent the cells from spreading onto the micropattern and then peeling off when eventually removed. The monolayers were then allowed to reach confluency in the constraints of the left by the removed micropattern.



*Figure 9 HUVEC Monolayer  
(Created by Author)*

## 2.6 Induction of Proinflammatory State

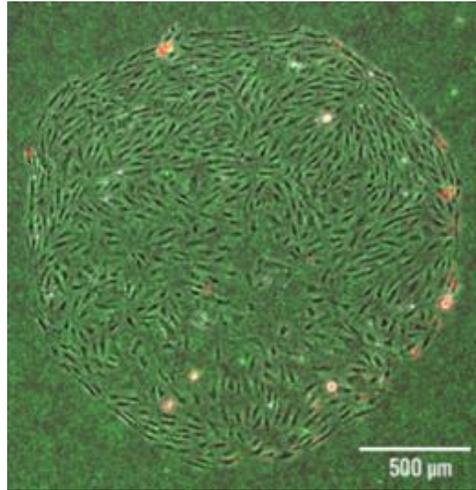
Once confluent, HUVEC monolayers were stimulated with TNF- $\alpha$  ( $20 \text{ ng}\cdot\text{mL}^{-1}$  in cell medium) for two hours at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Cell medium was then removed, wells were washed four times with PBS, and cell culture medium without TNF- $\alpha$  was added. (Huang et al.)

## 2.7 Experimental Setup

Primary HUVEC cells were used up to, and not exceeding, passage 14. They were placed in a PDMS circular micropatterns until confluency was reached (approximately 24 to 48 hours). While confluency was being reached, a macrophage-like state was obtained in THP-1 monocytes. Polarized THP-1 cells (M0, M1, and M2) were dyed and then detached from flasks. The cells were re-suspended in medium 200 and added in suspension to HUVECs wells at a concentration of approximately  $1 \times 10^4$  cells. A time lapse series was conducted over 12 hours with images taken at a ten-minute interval using a Zeiss epifluorescence microscope.

### 2.7.1 CellTracker™ Dye

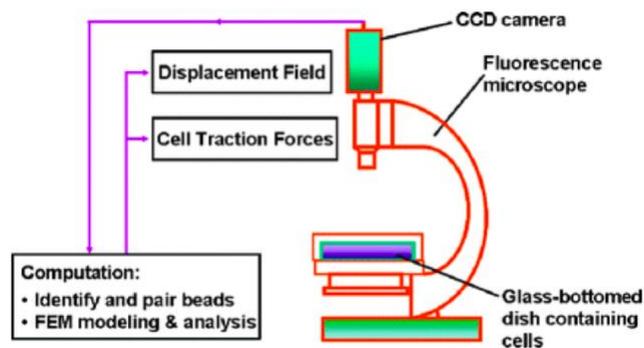
CellTracker™ (Molecular Probes) Red CMTPX was used to label polarized THP-1 cells during an experimental protocol. The dyed polarized cells are seen in red over a HUVECs monolayer on a PA gel of green florescent microbeads in Figure 10. The florescent label was diluted to a concentration of 10mm in DMSO in cell culture media and was added to the cells then allowed to incubate for 45 minutes at 37°C and 5% CO<sub>2</sub>. Cells were then washed with PBS to remove residual labels and then detached from the t25 flasks using trypsin 10X for seven minutes and then placed in a centrifuge for three minutes at 3000 RPM. Media was then aspirated, and cell pellet was broken apart and the cells were then re-suspended in cell culture medium. The wavelength of CellTracker™ used was chosen specifically to contrast the wavelength of the microbeads on the PA gels.



*Figure 10 Florescence Image of HUVECs Monolayer Overlaid with THP-1 Cells  
(Created by Author)*

### 2.7.2 Microscope Conditions

The experiment was conducted, and images were taken using a Zeiss epifluorescence scanning microscope with a 10X objective (Figure 11). Images of each HUVECs monolayer were taken at a consistent position throughout each sample; exposure time was the same for all images taken. Images were analyzed using ImageJ image-processing software.



*Figure 11 Scheme of Cell Traction Force Microscopy  
(Gavin)*

Cell culture medium was then removed from wells without removing them from the microscope stage to prevent unnecessary movement. Trypsin 10x was added in order to detach cells from gels and several images were taken of the unstressed fluorescence beads to later measure displacements.

## CHAPTER 3 RESULTS

### 3.1 Method of Analysis

The images produced during the 12-hour experimental assays were used to extract the data acquired. The fluorescence microbeads on each PA gel are used to track the cells as they migrate to and across the endothelial monolayers. As these cells move, they pull on the fluorescence beads. The fluorescent markers are used to derive the traction forces and intercellular stresses of the endothelial layer. The multi-paradigm numerical computing programming language, MATLAB, is used to calculate the traction and intercellular stresses from the images.

### 3.2 Traction Force Microscopy

There exists an intercellular stress force between the cell in the monolayer matrix for every unit area of contact. (Ladoux) The traction forces of ECs are represented by the deformation of substrate, in this case the PA gel, due to cell-generated stresses (Xavier Trepate et al.) (Tolic-Nørrelykke). The field of displacement is plotted by tracking the fluorescent microbeads embedded near the surface of a PA gel substrate. That deformation is then computed using the particle image velocimetry (PIV) optical technique.

Despite signals from the migrating macrophages that would otherwise tend to pull the cells in the monolayer apart, the ECs intercellular stress keeps them together. (Montgomery) This intercellular stress can be extrapolated from the traction forces generated from a monolayer of cells moving across a substrate by applying Newton's second law. Newton's second law states that the acceleration of an object depends on the net force acting on the object in question and the mass of the said object.

The distance of fluorescent bead displacement can be converted from pixels traveled in the image to microns. The time points from images incorporated with stiffness from gel are used to calculate the bead speed and then the intercellular stresses can be calculated from the force it took them to travel from point a to b during the ten-minute interval it took the image to be captured. All of these displacements are then compared to an image of the fluorescent beads in a relaxed state without adherent cells. (Figure 12)

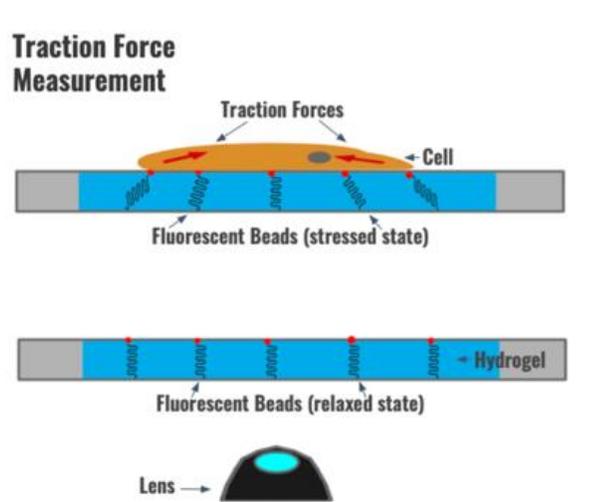
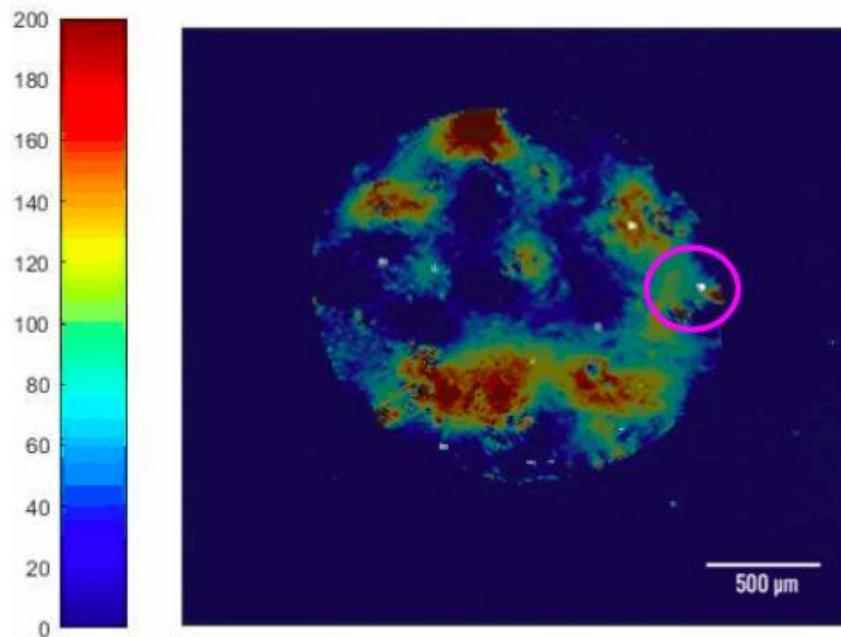


Figure 12 Scheme of Cell Traction Force Measurements  
(Created by Author)

### 3.3 Data Analysis

Once images of each HUVECs monolayer were processed through MATLAB and Image J, the intercellular stresses were graphed using Excel to average the samples taken. A compilation of the average intercellular stresses over three distinct monolayers in areas of 80 pixel cropped areas containing cells and areas containing no cells were compared. This process was completed for samples of pro-inflammatory, anti-inflammatory, and resting state macrophages. The tables shown

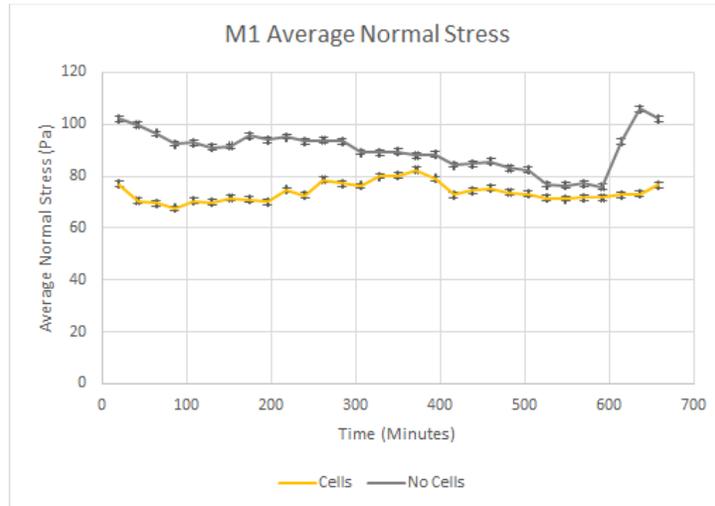
below exhibit the compilation of expressed data acquired adjusted to remove artifact inconsistencies and standard error bars of 5% were added.



*Figure 13 Intercellular Stresses with Overlapped Macrophage at Single Time Point  
(Created by Author)*

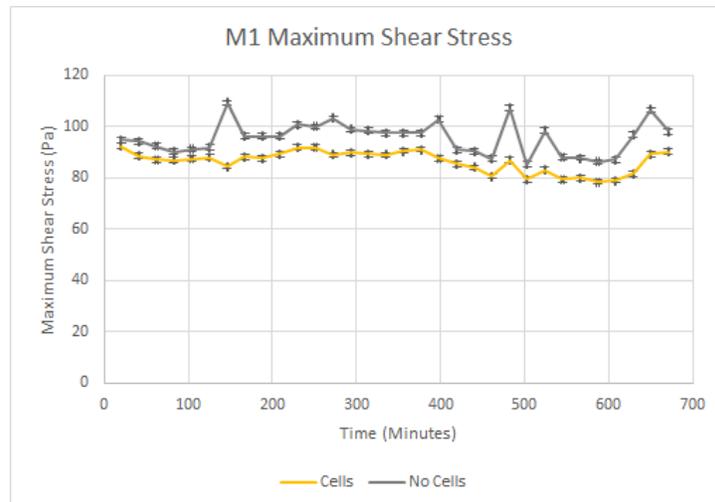
The range of intercellular stresses in Pascals are mapped on the bar to the left of Figure 13; a dark blue color represents low intercellular stress while the red represents a higher level of intercellular stress. The white cell encircled in pink marks one of the tagged macrophages observed on the EC monolayer.

Table 2 M1 Average Normal Stress



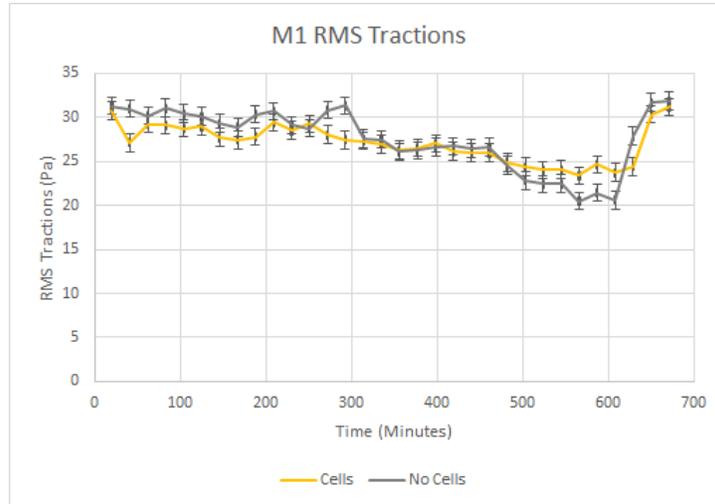
Polarized pro-inflammatory macrophages appear to have a preference to areas with lower normal stress. (Table 2)

Table 3 M1 Maximum Shear Stress



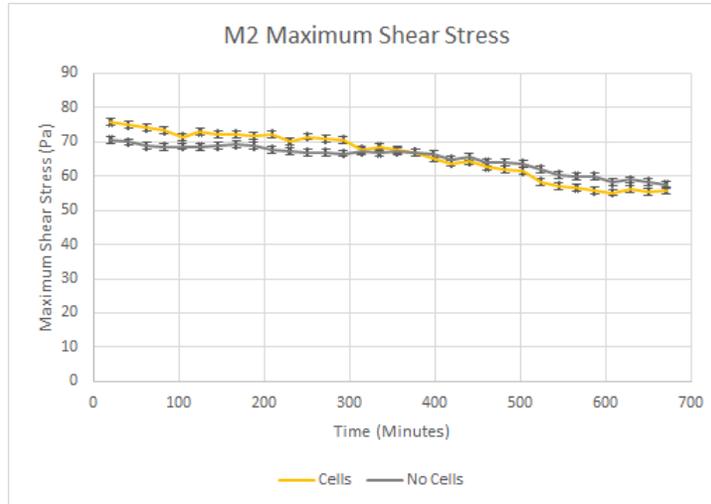
Polarized pro-inflammatory macrophages appear to have a preference to areas with lower shear stress. (Table 3)

Table 4 M1 RMS Traction



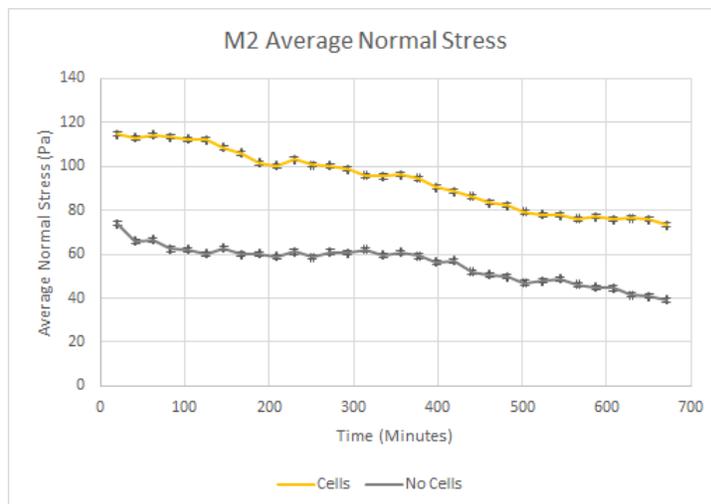
There appears to be no significant preference for tractions in the X and Y direction. (Table 4)

Table 5 M2 Maximum Shear Stress



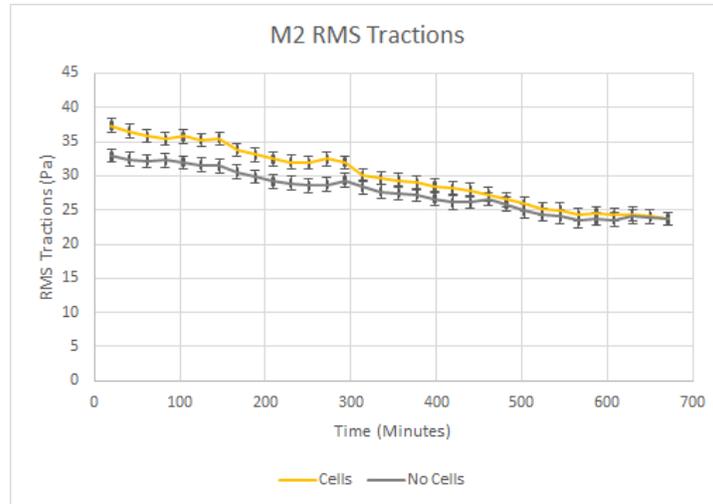
There appears to be no preference of anti-inflammatory macrophages between areas of high or low shear stress. (Table 5)

Table 6 M2 Average Normal Stress



Polarized anti-inflammatory macrophages appear to have a preference to areas of higher normal stress. (Table 6)

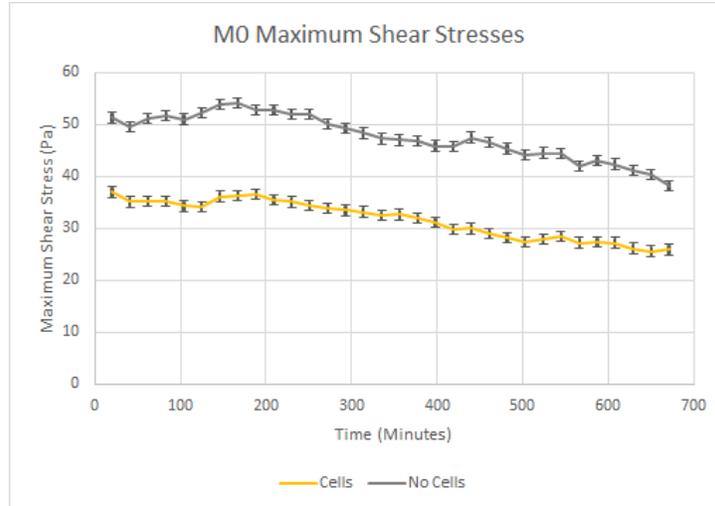
Table 7 M2 RMS Traction



There appears to be no significant preference for tractions in the X and Y direction. (Table

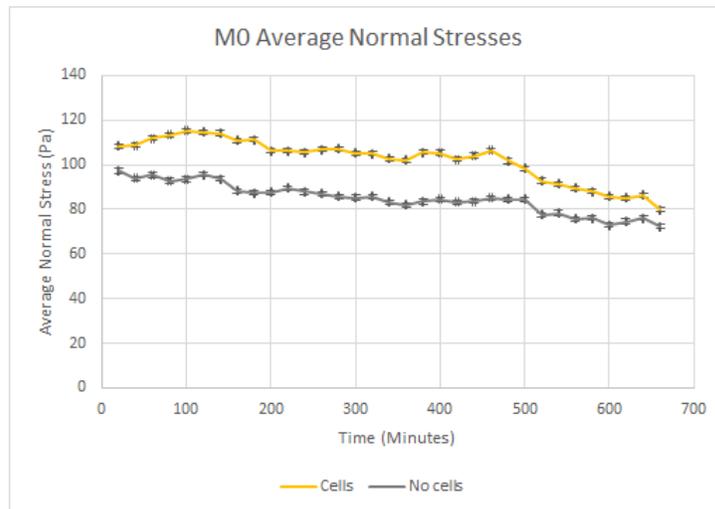
7)

Table 8 M0 Maximum Shear Stress



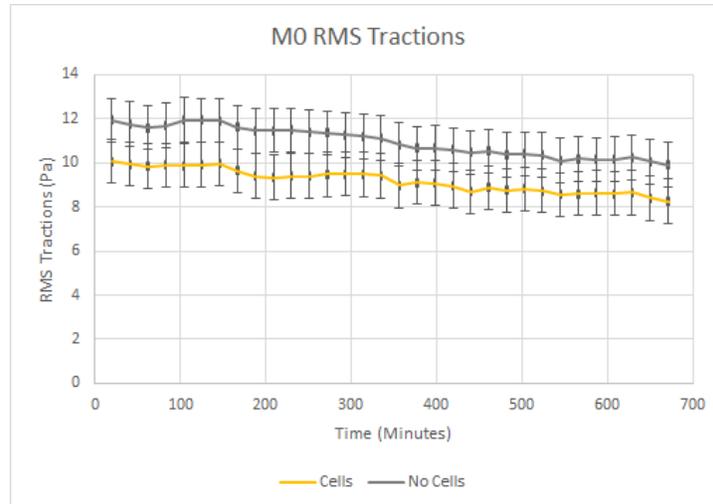
Polarized, resting state macrophages appear to have a preference to areas with lower shear stress. (Table 8)

Table 9 M0 Average Normal Stress



Polarized, resting state macrophages appear to have a preference for areas with higher normal stress. (Table 9)

Table 10 M0 RMS Traction



There appears to be no significant preference for tractions in the X and Y direction. (Table 10)

## CHAPTER 4 DISCUSSION

### 4.1 Potential Employment

Studies of macrophage functions, specifically transmigration, have been hampered by the lack of appropriate cells and models for comprehensive in vitro studies. What we present here, is a model that mimics specific physiological states of a stagnant hemodynamic flow in which we hypothesize that specific subsets of leukocytes will demonstrate attachment and migration preferences to particular areas of differing intercellular stresses on the endothelial bed. Using a model such as this one, it may be possible to exploit these intercellular stresses when developing macrophage-targeted therapies. Specific subsets of macrophages, particularly M1 and M2, appear to demonstrate a preference in attachment to areas of particular intercellular stresses.

Anti-inflammatory macrophages appear to show a preference for areas of high intercellular normal stress. Pro-inflammatory macrophages appear to show a preference for areas of low normal intercellular stress and low shear intercellular stress. (Table 11)

*Table 11 Compared Intercellular Stresses  
(Created by Author)*

Compared Intercellular Stresses	Intercellular Shear Stress	Intercellular Normal Stress	RMS Traction
M0	↓	↑	—
M1	↓	↓	—
M2	—	↑	—

Macrophage-targeted therapies can be made more specific knowing where on the endothelial bed they choose to attach and migrate through.

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