THE PENNSYLVANIA STATE UNIVERSITY
MILLENNIUM SCHOLARS PROGRAM

DEPARTMENT OF BIOMEDICAL ENGINEERING

DEVELOPMENT OF FEASIBILITIES FOR IMMUNE CELL-MEDIATED NANOPARTICLE TRANSPORT THROUGH THE ENDOTHELIAL BARRIER UNDER FLOW CONDITIONS

NMACHI ANUMBA
SPRING 2018

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biomedical Engineering

Reviewed and approved* by the following:

Cheng Dong
Distinguished Professor and Department Head of Biomedical Engineering
Thesis Supervisor

Scott Medina
Assistant Professor of Biomedical Engineering
Reviewer

* Signatures are on file in the Millennium Scholars Program office.

ABSTRACT

The blood-brain barrier (BBB) has consistently proved itself to be a roadblock in the development and administration of drugs for central nervous system (CNS) disorders and brain tumors. The cellular barrier is notorious for its highly selective permeability which limits the number of effective CNS drugs that have direct access to the brain. For this reason, study was done on the feasibility of using T lymphocyte Jurkat cells as a carrier to uptake Biodegradable Photoluminescent Polylactic Acid (BPLP-PLA) nanoparticles as they migrate through the BBB, carrying these nanoparticles to the other side of the tight endothelial barrier. This study builds on previous work in which this drug delivery mechanism was studied under static conditions by adding a flow component to the experiments.

A new bottom flow chamber plate was designed and made to accommodate for the use of suspensive Jurkat cells and facilitate a more accurate data collection process through use of flow cytometry. A reliable protocol was also developed for making a tight, confluent *in vitro* BBB model comprised of Bovine Brain Microvascular Endothelial Cells (BBMVEC). The migration of Jurkat cells was studied statically in the newly designed chamber plate at a concentration of 2×10^5 cells/mL and a CXCL12 chemoattractant concentration of 500 ng/mL. Flow migration studies of the Jurkat cells were then performed under a shear rate of 50 sec^{-1}. Tumor Necrosis Factor – α (TNF- α) was used to treat the endothelial cells and upregulate the expression of ICAM-1 in an effort to increase the migration of the Jurkat immune cells under flow conditions. However, the increase in migration shown by the use of TNF- α was proven to be statistically insignificant by way of the t-test.

The BPLP-PLA nanoparticles were successfully conjugated to the Jurkat cells and an experiment comparing nanoparticles being carried by Jurkat cells to a control experiment in which Jurkat cells were not used was performed. The results of this experiment showed an increase in migrated molecules with the assistance of Jurkat cells, however there was little difference in the sample fluorescence reading of the two experiments. This indicates that a significant amount of nanoparticles crossed the barrier in both cases and that further research needs to be done in the optimization of this process for more accurate results.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr. Cheng Dong for the opportunity to work in his lab and his guidance over the past three years. I would also like to sincerely express my gratitude to Virginia Aragon for her continued help, support, and encouragement throughout this project. A huge thank you to Gene Gerber for his help with making the bottom flow chamber plate and to the Millennium Scholars Program for exposing me to research and motivating me throughout my undergraduate career.

Finally, I would like to thank my parents for their unrelenting faith and confidence in me, and to all my friends and family who have made these past few years so great.

Chapter 1

Introduction

1.1 Motivation and Background

The brain is the body's most complex organ, being responsible for a multitude of functions that keep us alive. While there has been a vast increase in brain study initiatives over the past few years, there exists an extensive amount of information that is yet to be understood about the organ. There are a tremendous number of Central Nervous System (CNS) disorders which we know very little about, including neurodegenerative diseases, cerebrovascular diseases, and brain cancer [1]. Consequently, when it comes to treatment, many of these disorders are deemed untreatable or are treated with medications that are low in effectiveness and efficiency. Restricted accessibility to the brain via the skull and Blood-Brain Barrier (BBB), a selective diffusion barrier that separates blood from cerebrospinal fluid (CSF), is considered to be a major reason for the limited efficacy of brain drugs [2]. These protective structures are helpful in ensuring the blockage of potentially harmful substances from entering the CSF and consequently gaining access to the brain, however, they also make it difficult for needed drugs and therapeutic substances to treat the brain when necessary. The restriction of the BBB specifically, has been a constant consideration in the development of non-invasive brain-targeting drugs and is an issue that this research aims to address. Due to the high selectivity of the BBB it is incredibly difficult for drugs to cross through this barrier from the blood stream alone, therefore, this research investigates the feasibility of conjugating, or attaching, these drugs to immune cells that already

have the capability of crossing the barrier. In this way, once conjugated, immune cells can carry the drugs across the BBB and bring them through to the other side where they can effectively reach the brain. This study focuses specifically on the use of Jurkat cells as the immune cells of choice to transport Biodegradable Photoluminescent Polylactic Acid (BPLP-PLA) nanoparticles, used to portray the medical drugs, across an endothelial barrier.

1.1.1 The Blood Brain Barrier

The Blood Brain Barrier (BBB) is the physiological barrier made up of endothelial cells, neurons, astrocytes, pericytes and other biological components that separates blood from CSF in the head. The BBB is known to maintain homeostasis in the CNS and acts as a highly selective barrier. The neural environment of the brain is very strictly regulated and the brain can only undergo normal function when these specific parameter needs are met [3]. For this reason, it is imperative that the BBB actively monitors and regulates the transport of molecules, cells, and ions out of the blood and into the brain. The characteristic tightness of the BBB is incredibly effective in carrying out this purpose due to the specific anatomic and physiological elements that make up the barrier.

The primary elements of the BBB are the endothelial cells, pericytes, astrocytes, microglia, and the basement membrane as shown in **Figure 1-1**.

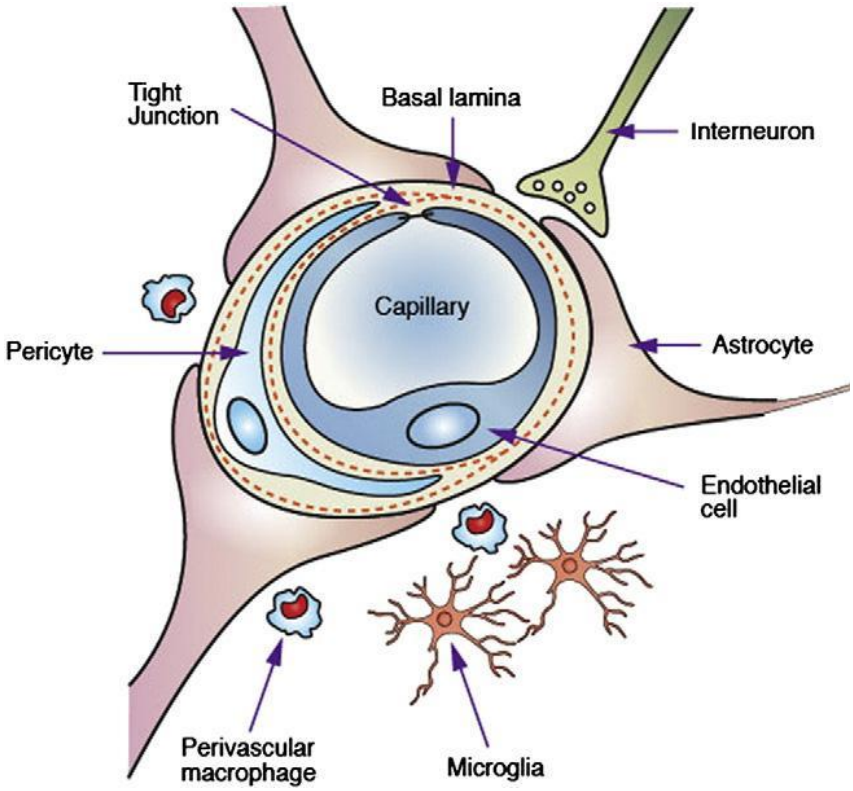


Figure 1-1: Physiological Components of the Blood-Brain Barrier [4]. The Blood-brain barrier is comprised of various elements that each contribute to the barrier's highly selective permeability. These elements include, but are not limited to, endothelial cells, the basal lamina or basement membrane, pericytes, microglia, and astrocytes.

The Brain Microvascular Endothelial Cells (BMECs) are the immediate lining of the brain's microvasculature and hold a key role in regulating the biochemical and biomechanical input from the vascular system. BMECs are also an integral part in upholding the tightness of the cellular barrier. Between endothelial cells, tight junctions and adherens junctions are formed, both of which are largely responsible for the blockage of molecules and ions from passing through the BBB. Pericytes cover the outside of the microvessels in the brain with only a layer of the basal lamina separating them from the BMECs and astrocyte end-feet. They communicate with other cells and neurons and are known to control blood flow to the brain through the

adjustment of the capillary diameter. Astrocytes are known to do a variety of things such as, monitor the concentrations of ions and water in the brain, regulate the number of neural synapses, and strengthen the formation of tight junctions between endothelial cells. Astrocyte protrusions extend out from the cell body and connect to the BBB through the basal lamina [1]. The microglia are a key part of the brain's immune response and become activated during brain injury. Many believe that these immune cells play a role in BBB permeability and the advancement of neurological diseases [5]. The basal laminar, typically about 30-40 mm in thickness, separates the different cells involved in the BBB and includes various extracellular matrix proteins such as collagen type IV and fibronectin [3].

All of these components contribute to the tightness of the BBB and its highly selective permeability. Common Transendothelial Electrical Resistance (TEER) values for the BBB range between 1000-5000 Ω cm^2, which is up to 500 times more resistive than capillaries in other parts of the body [2]. This increased impedance in the cellular barrier serves as a great protection mechanism in keeping harmful substances away from the brain, however, it also has served as a major stumbling block in drug development as it hinders helpful medication from getting through.

The *in vitro* model of the BBB used in these experiments is extremely simplified as it only contains endothelial cells and lacks many integral components needed for the model to be realistic. The best endothelial cells used in an accurate *in vitro* model of the BBB are BMECs, however, as these cells difficult to obtain and can be unreliable, bovine cells are seen as a satisfactory substitute and are used in these experiments [1].

1.1.2 Current Drug Delivery Methods

The blood-brain barrier is infamously known for complicating the development and administration of much needed medication to patients of various CNS disorders. It is known that well over 80 million people in the U.S. suffer from a type of CNS disorder and that the type of drugs currently being developed are insufficient and require an enormous increase in efficacy [6]. Given that 15% of all cardiac output goes to the brain [2], it makes sense for the shift in non-invasive neurotherapeutics to move toward using blood as a transport mechanism for these drugs to get to the brain. However, there appeared to be a large disconnect in the world of CNS pharmaceuticals as a lot of CNS drugs selected were based only on the premise that they work and not necessarily also on their ability to permeate the BBB. Of the readily available neurotherapeutics, ~100% of large molecule therapeutics and ~98% of small-molecule neurotherapeutics are blocked by the BBB from ever even entering the brain [7]. It is estimated that more than 99% of CNS drug development is dedicated to the making of these drugs, while less than 1% is focused on the actual delivery of these medications to the brain [6]. Seeing that the small percentage of drugs that are both lipid-soluble and weigh less than 400-600 Daltons are the only drugs able to easily pass through the BBB [6], it is evident that effective drug delivery across the Blood-Brain Barrier is a problem. This, in addition to the growing commonality of CNS disorders, proves the tremendous need for more effective, non-invasive drug delivery methods.

Many invasive and non-invasive methods of delivering drugs to the brain have been theorized and tried. It has been thought to inject drugs directly into the cerebrospinal fluid (CSF) due to its fast rate of convection from the brain into the blood stream. However, the process to get the drugs from the CSF into the brain is governed by diffusion and takes much longer. When

drugs are injected directly into the CSF, the concentration of drug diffused into brain tissue, for both small and large molecules, decreases significantly with distance away from the brain surface. The injection of a sufficient amount of drug into the CSF of a human brain runs the risk of exposing the ependymal surface of the brain to toxic amounts of the drug [8]. People have also tried to use this mechanism of getting drugs into the CSF by introducing transnasal drugs in which the drugs enter the olfactory CSF compartment through the nose. This mechanism runs into the same issues as the one stated above, and the additional obstacle of the olfactory region in humans only constituting 3-5% of the nasal mucosa which makes it hard to measure the amount of drug moving directly into the CSF [8]. These methods, as well as others, have all tried to address the issue of low representation of CNS drugs in the brain, but with little progress. For these reasons, it is important that a new mechanism be developed that allows CNS medication to pass through the BBB and effectively reach the brain for treatment. These studies investigate using immune cells to uptake the CNS drugs, having them migrate across the BBB, and therefore transporting them to the other side where they can effectively target the brain.

1.1.3 Previous Work

Past work in the lab has already looked into the mechanism of having immune cells uptake nanoparticles in order to target cancer cells [9]. These studies took advantage of immune cell's natural response to gravitate towards tumor cells and used this characteristic to study a targeted drug delivery system. In these studies, Biodegradable Photoluminescent Polylactic Acid (BPLP-PLA) nanoparticles were used to carry cancer specific drugs because of their biodegradability, fluorescent properties, and their non-cytotoxicity. These same nanoparticles

were also used in this study as this same immune cell carrier drug delivery system was used to study the transport of the nanoparticles across an endothelial barrier under flow conditions.

Previous studies in this area have done a lot of work in studying how immune cells are involved in physiological responses and how they react under flow conditions. Leukocytes, or white blood cells, are known to be a part of the inflammatory response in humans by binding to endothelial cells [10], making these immune cells a suitable target for the cell mediated drug delivery mechanism. Much study has also been done into the biomechanics of how these immune cells behave in attaching to an endothelial barrier, with some of the key factors involved being the shear flow of the blood, the adhesion of the cells to the endothelium, and the deformability of these cells as they attach [11]. These studies go into detail about the balance of these different forces and how they each contribute to the different stages of a cell's adhesion to the endothelium. The shear stress plays a significant role in this process and is important to pay attention to when doing these experiments. The equation for the wall shear stress (τ_w) is shown below:

$$\tau_w = \frac{6\mu Q}{w(h^2)}$$

Where μ signifies the fluid viscosity, Q is the volumetric flow rate, w is the flow field width, and h is its height. Most of the flow experiments done in this study were done at a shear rate of 50 sec^{-1}, or 2 mL/min, and performed in the flow chamber box shown in **Figure 1-2**. The flow chamber box is controlled by a motor that controls the volumetric flow rate of the circulating media in mL/min. The reason that the shear rate could be controlled by changing the volumetric

flow rate is because the flow field, height, width, and fluid viscosity were taken to be constant throughout these experiments [10].

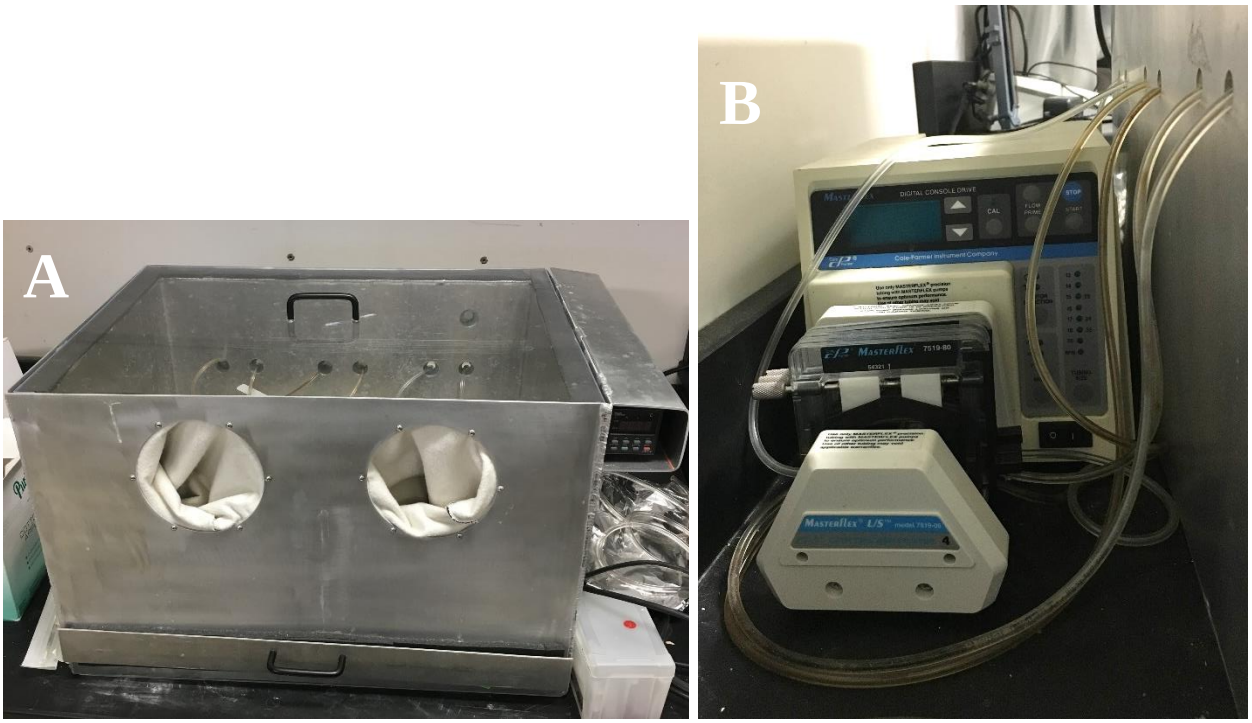


Figure 1-2: Flow Chamber Box and Motor. (A) This image shows the flow chamber box used to perform all of the flow migration experiments in this study, (B) and the motor installed behind the flow chamber box, through which the volumetric flow rate was modified and altered.

1.2 Objectives

The objectives of this thesis were to perform a preliminary investigation into the study of how the migration of immune cells conjugated with nanoparticles would be affected by an additional flow component. This added flow was used to mimic blood flow in the brain, in order to make the results of these experiments more accurate to realistic biological conditions. In order to study this proposed mechanism of drug delivery, many modifications had to be made to the protocol and equipment used to adjust for the change in type of experiment as well as the change

of cell lines from previous experiments. This study aimed to prepare a more realistic *in vitro* experiment with the goal of analyzing how the transport of nanoparticles across the blood brain barrier via immune cells is affected by blood flow conditions.

1.3 Thesis Organization

This thesis is comprised of 8 different chapters, the first of which provides an introduction to the problem in question, various techniques used, and a brief explanation of the biology. Chapter 2 goes into detail about the materials and methods used to carry out each experiment, Chapter 3 summarizes the thought process and results of the design and development of a new bottom chamber plate, and Chapter 4 describes the needs and results of developing a new protocol for producing a confluent endothelial monolayer to mimic the blood-brain barrier. Chapter 5 describes the study of the migration of Jurkat cells using the new flow chamber apparatus in static, and later, dynamic conditions, while Chapter 6 describes the migration of the Jurkat cells strictly under dynamic flow and explains the process of using TNF-α to increase cell migration under these circumstances. Chapter 7 talks about the findings of the experiments in which BPLP nanoparticles were conjugated to the Jurkat cells and expected to pass through the endothelial barrier, and the thesis ends with a conclusion and a discussion of future work in Chapter 8.

Chapter 2

Materials and Methods

2.1 Cell Culture Protocol

All cell types were treated in polystyrene petri dishes and kept in an incubator at 37 ˚C and 5% CO_2. Bovine Brain Microvascular Endothelial Cells (BBMVEC) (Cell Applications, San Diego, CA) were cultured in Bovine Brain Endothelial Growth Medium (Cell Applications, San Diego, CA) with 1% Penicillin Streptomycin (Corning, Manassas, VA). Petri dishes for BBMVECs were first treated with 3mL of Attachment Factor Solution (Cell Applications, San Diego, CA) for at least 20 minutes before any cells were added.

Once BBMVECs were deemed confluent, the media was aspirated from the petri dish and the cells were washed with 5 mL of Dulbecco's Modified Phosphate Buffer Saline (DPBS). After the DPBS had been aspirated, the cells were treated with 1 mL of 0.25% Trypsin-EDTA (Gibco, Grand Island, NY) and placed in the incubator for 5 minutes. The detached cells were then mixed with 3 mL of the BBMVEC cell culture media and placed in the centrifuge for 5 minutes at 10 ˚C and 1500 rpm. After this, cells would be resuspended in media and either passaged or used in an experiment.

Jurkat cells (ATCC, Manassas, VA) were cultured in RPMI 1640 Media solution (Gibco, Grand Island, NY) with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals, Flowery Branch, GA) and 2% Penicillin Streptomycin. When deemed confluent, these cells would be centrifuged

for 5 minutes at 10 ˚C and 1500 rpm. The media would then be aspirated, the cells would be resuspended in new media, and then either passaged or used in an experiment.

2.2 New Bottom Chamber Plate

The new flow chamber device was made out of Polycarbonate and was cut to size using a Milling Machine. The wells were made using an End Mill and the screws, dual threaded shoulder bolts, were recycled from previous chamber plates that were no longer in use.

2.3 Endothelial Monolayer

The endothelial monolayer grown to mimic the BBB for all experiments was made using BBMVECs. These monolayers were grown on Neuro Probe 8µm pore polycarbonate membrane filters. These filters were placed, shiny side up, in a petri dish with 3 mL of 90% ethanol and left under a UV light for 5-10 minutes. The remaining ethanol was aspirated and the filter was flipped over. A blocker (see **Figure 2-1**) was centered on top of the filter and 1 mL of Bovine Brain Endothelial Growth Medium without FBS was placed in the center hole on top of the filter. 30 µL of Fibronectin were also placed in the center hole and the petri dish was then placed in the incubator for at least 2 hours. After this incubation time, BBMVECs were counted using a Bright-Line® Hemocytometer so that the volume needed to obtain 250,000 cells was calculated. The petri dish with the filter was then taken out of the incubator and the media in the center hole was aspirated. Two silicon strips were placed on either side of the block to stop it from sliding. 7.5 mL of BBMVEC cell culture media were added to the petri dish on each side of the blocker for a total of 15 mL and 1.5 mL of media were added to the center hole. The volume of

BBMVEC solution needed to obtain 250,000 cells was also added to the center hole. The petri dish containing the filter, blocker, silicon strips, and media was then placed in the incubator for 5 – 6 days.

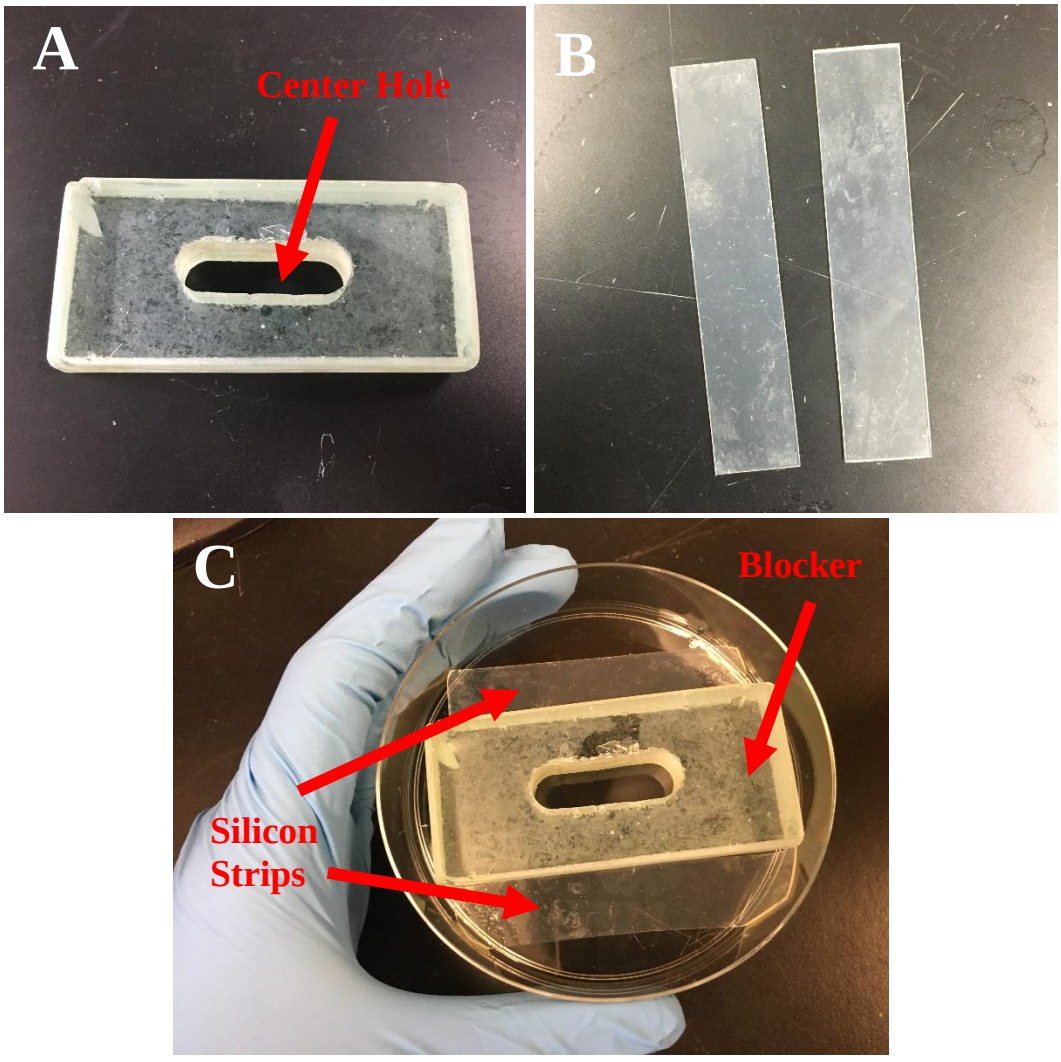


Figure 2-1: Blocker and Silicon Strips Used for Growing Endothelial Monolayer. This figure shows the tools used in growing the BBMVEC endothelial monolayers. (A) The blocker, placed on top of the filter to isolate the cells from the rest of the filter. Initially, fibronectin and media without FBS is placed in the center hole and then cells are added after an incubation time of at least 2 hours. (B) Silicon strips, placed on either side of the blocker to ensure that it does not move after media has been added. (C) Placement of blocker and silicon strips on top of filter, in petri dish as they would be placed while cell layer is growing.

2.3.1 Cell Imaging

All endothelial cells were imaged using a bright field microscope at a magnification of 10X. The cells were first labeled with the fluorescent dye Cell Tracker™ Green CMFDA (Life Technologies, Eugene, OR). The media was aspirated from the cells and 1 µL of dye for every 5 mL of media (without FBS) was mixed together for a total of 10 mL per plate of cells. The dye-media mixture was applied to the cells and the petri dish was placed in the incubator for 20-30 minutes. After this, the media was aspirated and the cells were fixed with 4% formaldehyde for 15 minutes before imaging. The 4% formaldehyde solution was made by adding 400 µL of 100% formaldehyde to 9.6 mL of DPBS.

2.4 Dextran Integrity Test

The experiment used to determine the integrity of the BBMVEC endothelial monolayer was done by allowing a solution of 40 kD Fluorescein Isothiocyanate (FITC)-Dextran (Sigma-Aldrich, St. Louis, MO) to circulate in the flow chamber and then quantifying the amount of FITC-Dextran that was able to pass through the monolayer. A media solution of 1% RPMI was made by supplementing RPMI 1640 Media with 1% FBS and 2% Penicillin Streptomycin. A control solution of dextran was made by adding 25 mg/mL of FITC-Dextran to 1 mL of dH_2O and 40 mL of a 0.25 mg/mL solution of FITC-Dextran were made by adding 10 mg of Dextran to 40 mL of 1% RPMI media. The experimental dextran solution was then split by putting 10 mL of the solution into a 50 mL centrifuge tube and the other 10 mL in a 15 mL centrifuge tube. The centrifuge tubes were placed in the flow chamber box and allowed to run through a chamber containing a fully grown BBMVEC monolayer for 30 minutes at a shear rate of 1 mL/min or 25

sec^{-1}. After 30 minutes, the motor of the flow chamber box was turned off. The solution in the 3 wells of the bottom chamber part was collected separately and placed into separate wells of a 96 well plate. The plate was then put in a Spectrofluorometer and the relative fluorescence of the samples were measured and compared to those of the control Dextran solution.

2.5 Flow Migration Tests

All blockers and silicon strips were autoclaved before being used for any experiment. The monolayers used were grown as described above (see Section 2.3). The chamber parts were washed with soap and hot water and then rinsed with DI water. The chamber parts were then each submerged in boiling DI water for various amounts of time according to their specific part (see **Figure 2-2**). The bottom plates were submerged for 30 seconds each, the top plates were submerged for 20 seconds each, and the silicon gaskets were submerged for 10 seconds each. The parts were then left to dry.

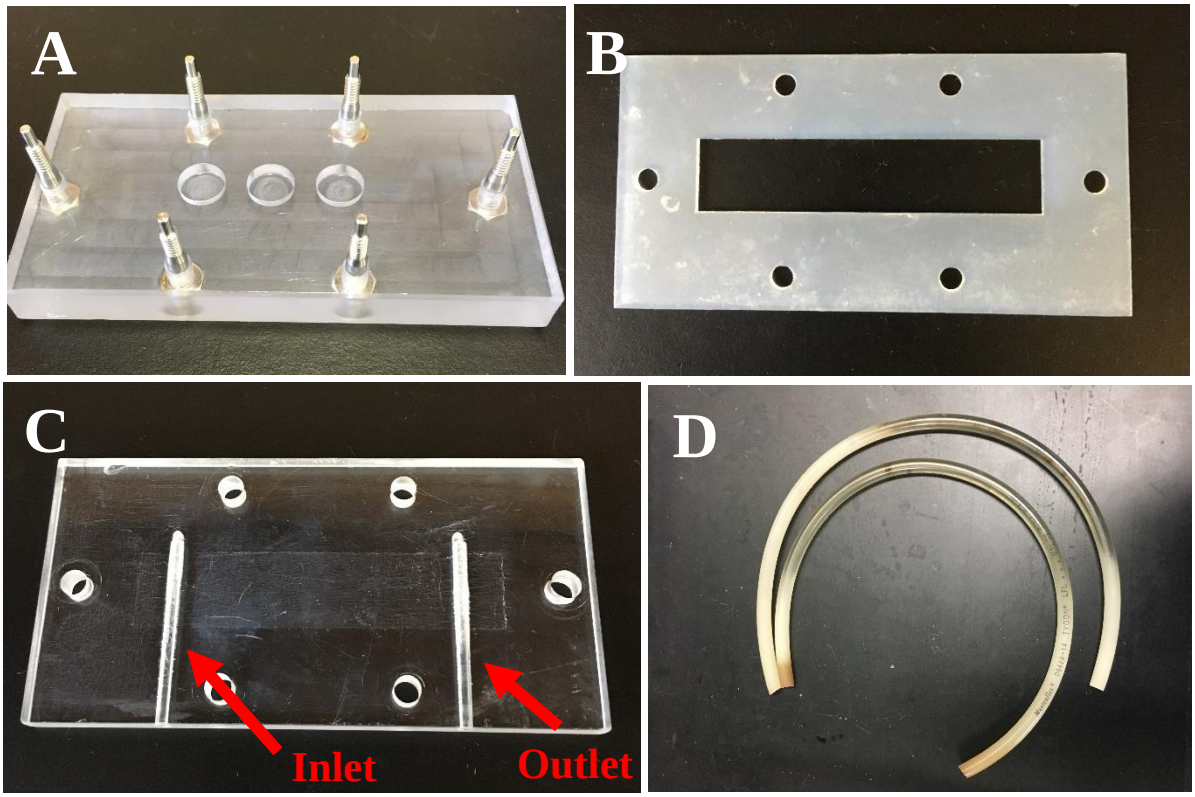


Figure 2-2: Parts of Flow Chamber. This figure shows the four major components that comprise the flow chamber. (A) The bottom chamber plate. The media containing the chemoattractant is placed in the three centralized wells and the filter supporting the monolayer is placed on top. (B) The silicon gasket. The gasket is placed on top of the filter to create a gap through which media can flow across the endothelial barrier. (C) The top chamber plate. This part is placed on top of the silicon gasket and contains an inlet and an outlet for the media to enter and exit the cavity containing the BBMVEC monolayer. (D) Tubing used to connect flow chamber to the flow chamber box.

The Jurkat cells were centrifuged for 5 minutes at 10 ˚C at 1500 rpm and resuspended in 1% RPMI media. The Jurkat cells were counted using a hemocytometer to get a concentration of 2 x 10^5 cells/mL in 41 mL. Of the total 41 mL, 20 mL were used in the experimental trial, 20 mL were used in the control trial, and 1 mL of cell solution was kept as the control for flow cytometry analysis. For each trial, 10 mL of the 20 mL solution were placed in a 50 mL

centrifuge tube and the other 10 mL were placed in a 15 mL centrifuge tube. A 500 ng/mL solution of C-X-C Motif Chemokine 12 (CXCL12) was made by adding 5 µL of a 100 ng/µL CXCL12 stock solution to 995 µL of 1% RPMI media. Afterward, 200 µL of the CXCL12 solution was placed in each of the three wells of the bottom plate of the flow chamber.

The filter supporting the BBMVEC monolayer was then placed on top of the bottom plate of the flow chamber so that the monolayer was directly on top of the three wells. The filter was then taped down to the bottom plate at the extreme sides of the filter so as to secure it during data collection. The silicon gasket was placed on top of the filter and the top plate on top of the gasket. The top plate was then screwed on to the bottom plate to complete the chamber, as shown in **Figure 2-3** below.

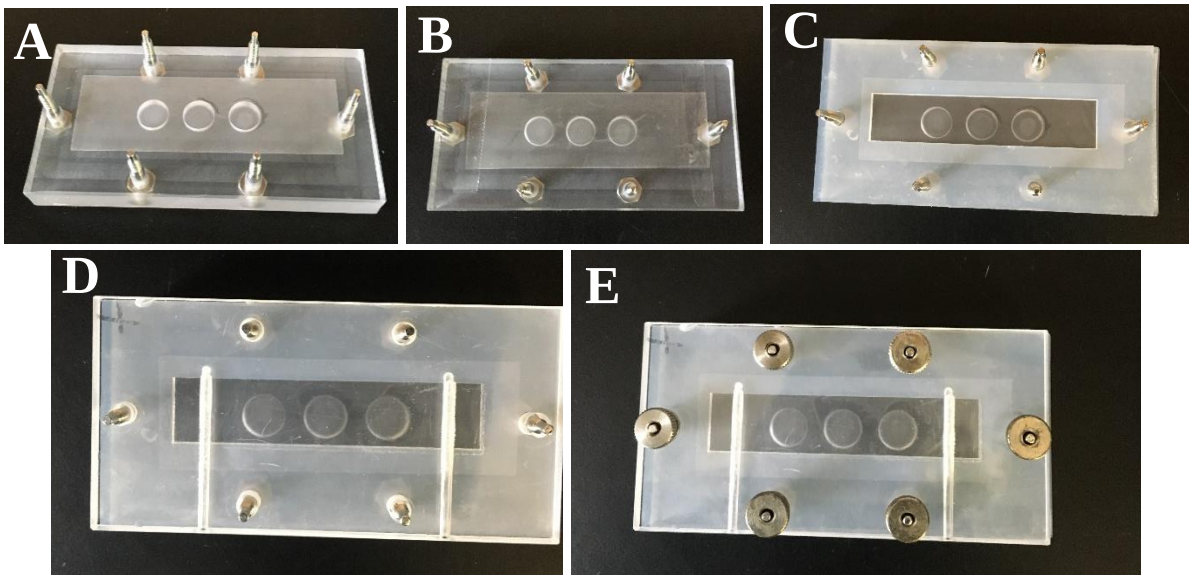


Figure 2-3: Assembly of Flow Chamber. This picture depicts the process of assembling the parts of the chamber to be a functional unit that can be used in experiments. (A) The filter containing the monolayer is placed on top of the wells of the bottom chamber plate. (B) A slither of tape holds the filter down and ensures that it is not lifted up in the data collection process. (C) The silicon gasket is placed on top of the filter. (D) The top chamber plate is placed on top of the gasket. (E) Screws are used to tighten and secure the unit and reduce the risk of leakage.

The chamber was connected to the flow chamber box by two tubes through which the cell solution was brought into the chamber and taken out from the chamber (see **Figure 2-4**). The motor of the flow chamber box was then turned on and the shear rate was set to 2 mL/min which corresponds to 50 sec^{-1} and left to run for 4 hours. The motor was then stopped and the chambers were removed from the flow chamber box. The media solution in each of the 3 wells in the bottom plate was collected as an individual sample and each sample was placed in the flow cytometer where a concentration of cells/µL was measured.

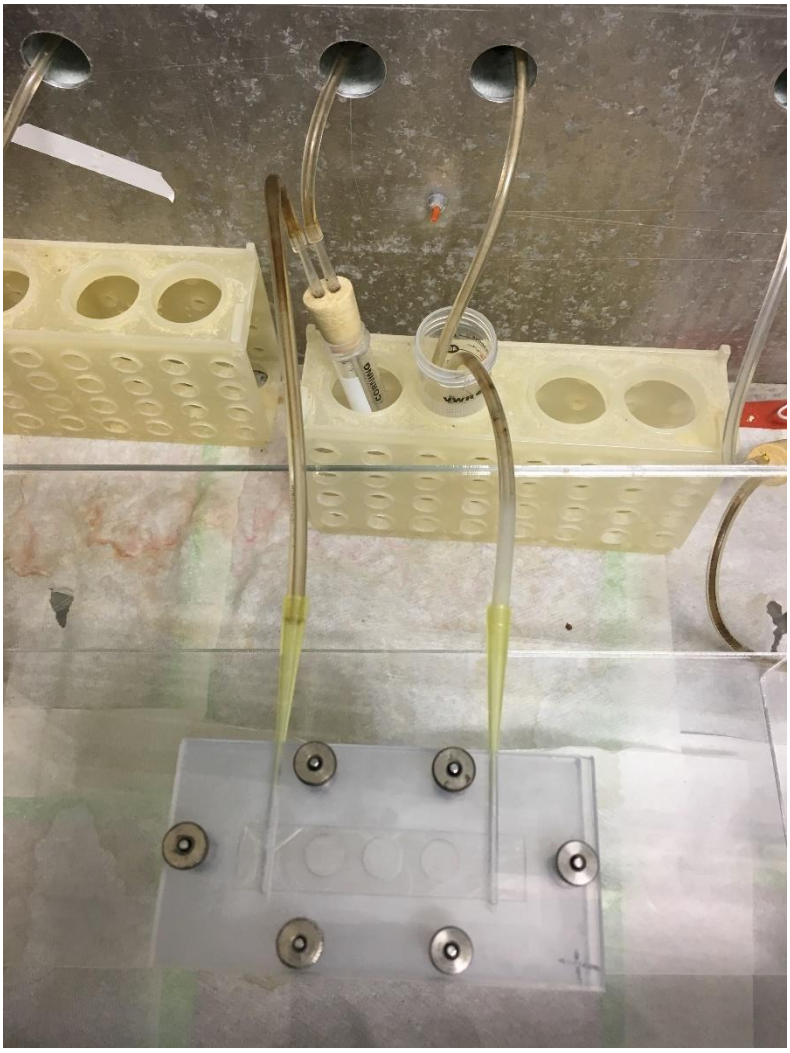


Figure 2-4: Flow Chamber Set-Up. The connection of the smaller flow chamber to the bigger flow chamber box is shown in this figure. The flow of media was controlled by the motor located behind the flow chamber box. Media would be pushed out from the 15 mL centrifuge tube on the left and through the inlet of the flow chamber. The media would then flow across the monolayer in the center of the flow chamber, out the outlet, and into the 50 mL centrifuge tube on the right. The fluid would then complete the circuit by flowing back into the 15 mL tube.

2.5.1 Treatment of TNF-α

For certain flow migration experiments, the BBMVEC endothelial monolayer was treated with TNF- α before the flow experiment. A TNF- α concentration of 25 ng/mL was made by adding 2.5 µL of a 100 ng/µL stock solution of TNF- α to 9.9975 mL of the BBMVEC cell

culture media. The media in which the endothelial monolayer was grown was aspirated and replaced with the TNF-α media solution. The monolayer was then left to incubate in this solution for 6 hours before the start of the experiment.

2.6 Static Migration Tests

The static migration experiments underwent the same protocol as the flow migration experiments with the exception of how they proceeded once inside the flow migration box. Instead of being left to run for 4 hours, the motor was turned on long enough to ensure that the entire cavity of the chamber that contained the monolayer had been completely covered by the Jurkat cell solution. A minute after this section of the chamber had been covered, the motor was shut off and the experiment was left for 4 hours. Afterwards, the media in the bottom plate wells was collected and sent through the flow cytometer where the concentration in cells/μL of each well was calculated.

2.7 Flow Cytometry Analysis

A Millipore Guava® easyCyte Single Sample Flow Cytometer was used to analyze the concentration of migrated cells in both the static and the flow migration studies. The flow cytometer was first cleaned by following the built-in procedure in the Guava® easyCyte program. After the first wash cycle, a new session was started in the program to collect the data from that experiment. The settings for data acquisition were then adjusted using a control sample and these settings were used for all subsequent samples in that particular experiment. Each sample that was retrieved from a bottom plate well was then placed into a 1.5 mL

microcentrifuge tube, which was vortexed before being put into the flow cytometer. The flow cytometer would then measure and display the cell concentration in the given sample.

2.8 Nanoparticle Conjugation

Biodegradable Photoluminescent Polylactic Acid (BPLP-PLA) nanoparticles were used to mimic drugs in these experiments. The Jurkat cells were first treated with a 50 µM solution of N-Azidoacetylmannosamine (Ac4ManNAz) and left in the incubator for 3 days. After this period of time, the Jurkat cells were washed twice with Dulbecco's Modified Phosphate-Buffered Saline (DPBS) in order to remove the Ac4ManNAz sugar compound from the cells. The Jurkat cells were resuspended in 500 µL of DPBS and counted using a hemocytometer to find the volume needed to collect 2 x 10^6 cells. The Jurkat cell solution was then diluted in DPBS by a factor of 20. A 0.1 mg/mL solution of CuCl was made by adding 5 mg of CuCl to 50 mL of DPBS and a 10 mg/mL solution of Na-Asc was made by mixing 100 mg of Na-Asc in 10 mL of DPBS. Then, 4 mg of BPLP-PLA nanoparticles were suspended in 4 mL of DPBS and sonicated until no large clumps could be detected in the solution. After sonication, the nanoparticle solution was filtered using a 1.5 µm filter and 3 mL syringe and then sterilized under ultraviolet light for 15 minutes. A total 2 mL solution was made by adding 830 µL of CuCl solution, 26.5 µL of Na-Asc solution, 1000 µL of the nanoparticle solution, the calculated amount of Jurkat cell solution, and the remaining volume being comprised of DPBS. This 2 mL solution was then placed on a rocker in the incubator for 1 hour. The cells were then washed twice with DPBS, resuspended in media and kept in the incubator until needed for the experiment.

2.9 Statistical Analysis

The migration results comparing the TNF- α treated experiment with the control were analyzed using a two-tailed, two-sample equal variance t-test. Results were proven to be statistically insignificant if these tests yielded a p-value that was greater than 0.05.

Chapter 3

Design of New Flow Chamber Plate

3.1 Motivation

The flow migration apparatus used in this study was comprised of many different parts (see **Figure 2-2**), all of which were necessary for the studies that were performed. Although the overall mechanism of how the flow migration assay was performed can mostly be translated to different cell types, alterations had to be made to some of the specific parts used in this process. Previously, the flow chamber had been used in studies that studied the behavior of adhesive cells such as MDA-MB-231 breast cancer cells [12]. The adhesive nature of these cells was accommodated for in the design of the bottom chamber plate that was used in the experiments (see **Figure 3-1**).

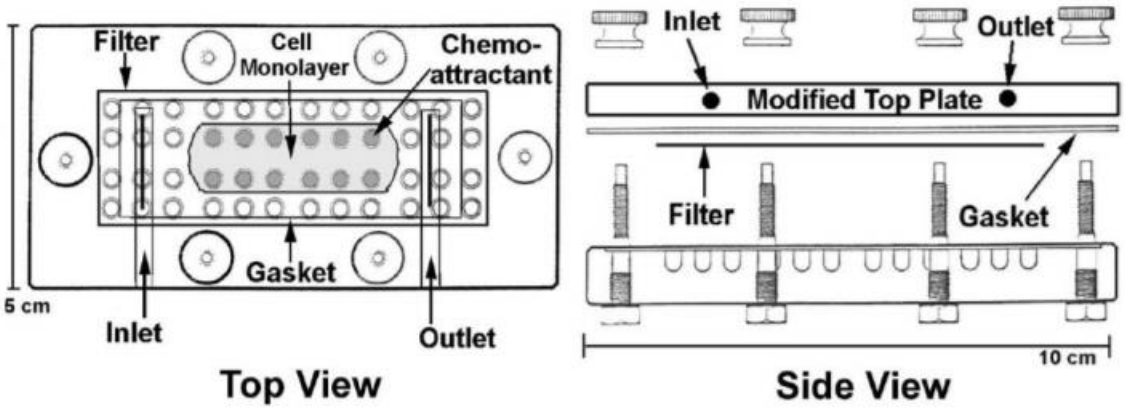


Figure 3-1: The Top and Side View of the Old Flow Chamber Parts [10]**.** This design involved a top and bottom polycarbonate plate, with 48 wells in the bottom plate for holding the chemoattractant and media. This design allows migrated cells to adhere to the bottom part of the filter and quantification of migration is not restricted by the small size of the wells.

With this design, cells were able to flow into the chamber and, in the presence of a chemoattractant, attach to a monolayer of cells grown on a filter, where, after having migrated through these cells, would maintain a position attached to the other side of the filter. Consequently, data collection for these experiments was done by dyeing the side of the filter to which the cells had migrated with a Hemacolor HARLECO dye kit, and then quantifying the migrated cells by averaging out a number representing cells/frame using a Brightfield microscope (see Appendix A, Step 36). Given that the immune cells of interest in the studies pertaining to this thesis were Jurkat T-cells, a white blood cell that is suspensive in nature, a new bottom chamber plate was needed. One that could be used in conjunction with a new method of data collection that accounted for the suspensive property of the cells. This new chamber plate needed to be able to somehow collect migrated cells as they would "fall" through the filter once they had migrated across the cell barrier. The chamber plate also needed to accommodate a new method of quantifying cells that would replace the filter dyeing procedure used in previous studies.

3.2 Results

The finalized bottom plate was designed as shown in **Figure 3-2**. This plate was designed with 3 large centralized wells instead of 48 smaller wells dispersed throughout the plate (see **Figure 3-1**). These three larger wells were designed so that when Jurkat cells migrate through the endothelial barrier, the majority of them would fall into one of the three wells. Each well is a cylindrical shape with a 9.52 mm diameter and a depth of 2.80 mm, resulting in a volume of

about 200 µL each. The overall block for the chamber maintained its dimensions of 101.80 mm x 51.11 mm since the size and location of the wells were the only major modifications that were made. Instead of dyeing the bottom side of the filter after the migration assays, the media with the migrated cells in each of the wells would be collected with a pipette and run through a flow cytometer that would measure the concentration of the samples.

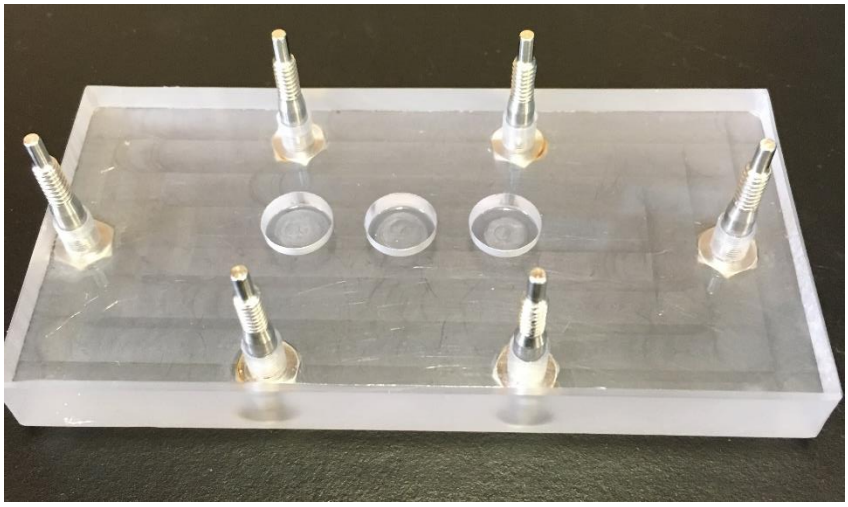


Figure 3-2: Newly Designed Bottom Chamber Plate. The new flow chamber plate designed for suspensive cell use was made out of polycarbonate and with the dimensions 101.80 mm x 51.11 mm as used with the previous bottom chamber plates. The three central wells were each 9.52 mm in diameter with a depth of 2.80 mm.

When first made, the chamber parts were tested for leakage by filling the bottom wells with 200 µL of left over MEF freezing media. A filter was placed over the wells and 20 mL of the same media was left to circulate through the chamber system for 1 hour at a shear rate of 75 sec^{-1}. There was no leakage seen after the course of 1 hour and the chamber plates were deemed ready for use.

3.3 Discussion

The newly designed chamber plate proved to be an integral part of analyzing the results of the flow migration experiments done in this study. In use, they were effective, reliable and greatly facilitated the process of quantifying migrated Jurkat immune cells. The ability to collect the suspended cells and run flow cytometry analysis on the samples yielded accurate results that were used to direct the course of future experiments.

Chapter 4

Endothelial Monolayer

4.1 Motivation

In order for these experiments to be as accurate as possible, it was ideal that the monolayer of endothelial cells be as confluent as possible. In this way, the monolayer could present a decent model of the Blood-Brain Barrier (BBB) in mimicking its tightness. It is important to note that the endothelial monolayer that was used in these experiments was an extremely simplified representation of the BBB, given that it did not include astrocytes, pericytes, or other integral components of the BBB, and the existence of tight junctions was not verified due to the novel geometry of flow model. Although simple, it was imperative that the monolayer of BBMVECs grown to mimic the BBB was a tight and continuous layer of cells, so as to ensure that circulating cells and particles were not able to "fall" through holes in the barrier. The absence of gaps in the monolayer would force the Jurkat cells to migrate through to the other side, as they would have to *in vivo*.

4.1.1 Previous Work

It was found that previous flow migration studies in the lab used an incomplete protocol to grow the endothelial monolayer with little to no confirmation of confluence. In said protocol, a cleaned filter would be coated with fibronectin for at least two hours, the filter would then be seeded with a various number of endothelial cells depending on when the experiment in question was set to take place. The filter would be seeded with 500,000 cells if the experiment were to be

done the following day and 250,000 cells if the experiment were to be done two days later (see Appendix A, Step 24). However, this protocol was found to be insufficient when held against the current standards being discussed in literature, all of which stated that at least 4 days is needed to grow a fully confluent monolayer [13]. This, in conjunction with the staining protocol previously used to quantify migrated cells (see Appendix A, Steps 31-36), made analyzing the efficacy of this process incredibly difficult. A reliable protocol for growing the monolayer has been used for the static migration experiments in the lab, however, the apparatus used for these experiments is significantly smaller than those used for the flow migration experiments. The static migration protocol could not be easily translated to the flow migration studies due to the size difference of the monolayers needed for each study. For these reasons, a new protocol that ensured the growth of a fully confluent monolayer of cells needed to be developed.

4.1.2 Necessary Improvements

The newly developed protocol needed to provide two specific things. The monolayer grown from this protocol needed to be fully confluent, meaning that there were no gaping holes between endothelial cells through which circulating cells could possibly fall, and this monolayer needed to be provide a tight barrier between one side of the filter and the other. That is to say that not only did the cells need to be close to each other but they had to be packed tightly so that any cells trying to get through the monolayer were forced migrate. There proved to be a fine line between confluence and overconfluence, a state in which the cells are packed so tightly together that it starts to affect their health and functions. Therefore, various experiments were performed that adjusted parameters, such as the initial number of cells seeded on the filter and the length of

time for which they were allowed to grow, in order to determine the best protocol for growing the endothelial monolayer.

4.2 Results

The initial preparation of the filters (explained in Section 2.3) was maintained and varied slightly from the previous protocol. The time needed to clean the filter was altered from 30 mins to 5 minutes under UV light. This change was made after consideration with another lab member who agreed that a length of 30 minutes was not needed to thoroughly clean the filters. The optimum combination of the number of initially seeded cells and the growth duration were tested by first altering the number of cells and afterward monitoring monolayer growth at different day intervals. Monolayers were seeded with 500,000 cells and 250,000 cells initially and observed after 5 days of growth. **Figure 4-1** shows the results of these trials.

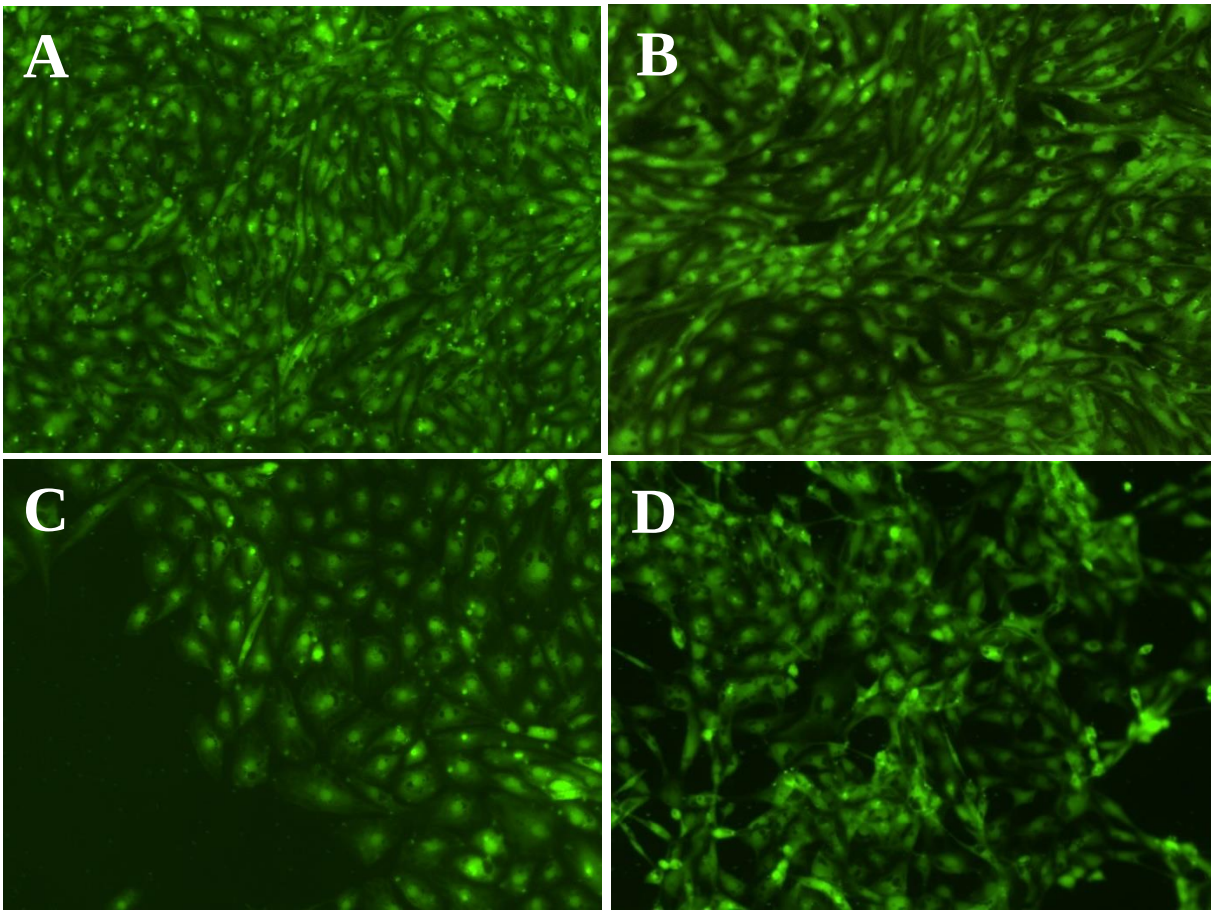


Figure 4-1: Endothelial Monolayers Seeded with 250,000 and 500,000 BBMVECs after 5 Days. This figure shows various bright field images of BBMVEC monolayers taken at a magnification of 10X. (A) Monolayer seeded with 250,000 cells, (B) Monolayer seeded with 500,000 cells, (C) Boundary of monolayer seeded with 250,000 cells, (D) Outer edge of a monolayer seeded with 500,000 cells.

After 5 days, both filters had grown to varying levels of confluency with the 500,000-cell monolayers having considerably more gaps than the 250,000-cell monolayers. The 250,000-cell monolayers were seen to have a distinct boundary between the cell layer and the rest of the filter (**Figure 3-1C**), indicating that the blockers being used to isolate the growing cells were effective. The outer edge of the 500,000-cell monolayer however, exhibited an increasingly large number of holes and gaps between cells (**Figure 3-1D**).

Monolayers were subsequently grown in the same manner and observed after a period of 8 days. After this amount of time it was observed that both the 250,000-cell monolayers and the 500,000-cell monolayers were more uniform with considerably fewer gaps being present. Overall, the BBMVECs looked less healthy after 8 days with the 500,000-cell monolayer cells looking worse than those of the 250,000-cell monolayer. A prolonged incubation and growth time can lead to cells within the monolayer dying [13] which could account for the observed decrease in cell health. These observations led to the conclusions that 8 days is too long of an incubation period and that monolayers appear to do better when filters are initially seeded with 250,000 cells as opposed to 500,000.

These conclusions led to multiple trials in which filters were seeded with 250,000 cells and monitored after 5, 6, and 7 days. The monolayers were observed to have reached a level of confluency, in which no gaps were observed, without any observable adverse effects at day 6. **Figure 4-2** shows images taken of the monolayer after 6 days of growth.

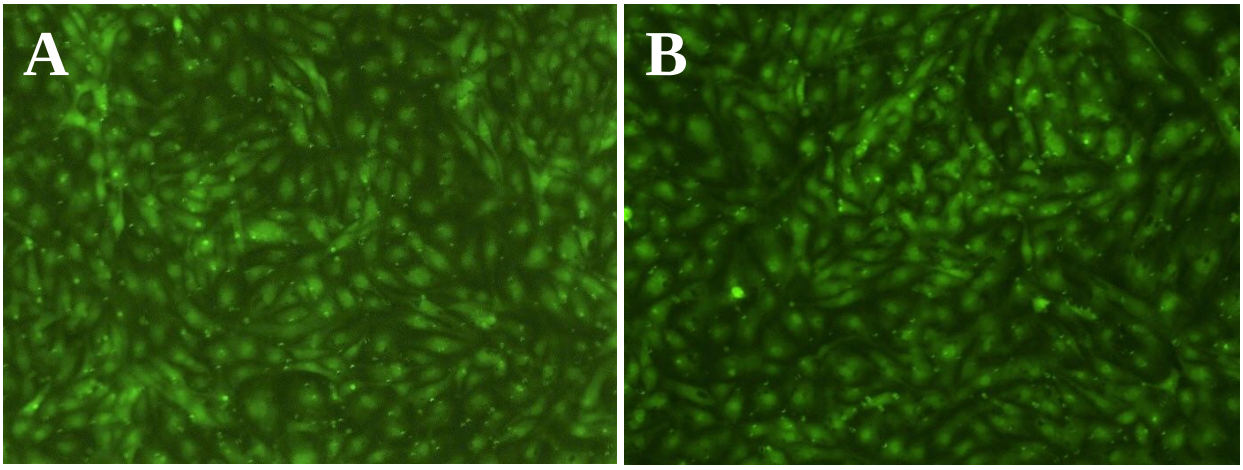


Figure 4-2: Endothelial Monolayers Seeded with 250,000 BBMVECs After 6 Days. The BBMVEC endothelial monolayers were shown to be in best condition when filters were seeded with 250,000 cells and left in the incubator for 6 days. Cell layers showed no gaps or holes and the cells also looked healthy. Images were taken with a bright field microscope at a magnification of 10X.

4.2.1 Final Protocol

The final protocol, determined to be the most efficient, involved initially seeding the filters with 250,000 cells and allowing them to grow for 6 days. A growth time of 6 days appeared to be optimal for confluence when analyzing the fluorescent images taken at various time intervals. The full protocol is explained at length and in detail in Section 2.3.

4.2.2 Monolayer Integrity Test

Once a final protocol had been chosen based on confluence of the monolayer, this monolayer had to be tested in order to make sure that the layer of cells was tight enough to block substances from easily passing through it. To do this, molecules of 40 kD Fluorescein Isothiocyanate (FITC)-Dextran were allowed to circulate in the migration chamber for 30

minutes through a bare filter, that acted as a negative control, and a filter with an endothelial monolayer grown on top of it. The aim of performing this test was to make sure that the amount of dextran that passed through the cell monolayer was significantly lower than the amount that passed through the bare filter. If this was the case, it could be concluded that the cell monolayer was sufficiently tight enough and able to form a firm barrier for the purposes of these experiments. The amount of dextran that passed through the filter was quantified by measuring the fluorescence in the samples taken from the wells after the migration period had ended. **Figure 4-3** shows the results in measured fluorescence of the samples taken from the trails with and without an endothelial monolayer. It should be noted that a specific threshold in fluorescence was not being targeted, rather, a comparative reading between the negative control and the trial experiment was done to determine the efficacy of the protocol in this area.

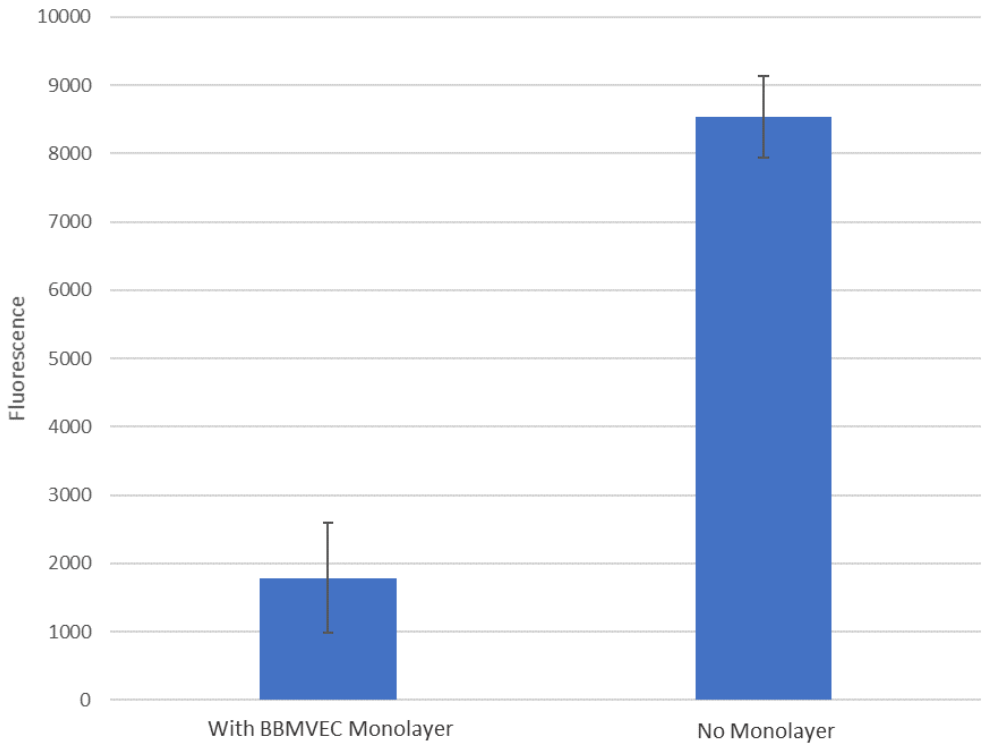


Figure 4-3: Results of the Dextran Integrity Test. This figure shows the difference in fluorescence measured after FITC-Dextran was allowed to run through a bare filter and an endothelial monolayer. The strength in fluorescence corresponds to the amount of dextran in the sample. The BBMVEC monolayer was effective in blocking a significant amount of dextran molecules as seen by the lower fluorescence reading obtained from this trial.

As **Figure 4-3** shows, the amount of dextran able to cross the filter with the endothelial monolayer in place is significantly less than the amount passed without it. This significant difference in fluorescence shows that the monolayer was able to block the passage of a substantial amount of dextran from falling through it. With these results, the newly developed protocol was accepted as having a satisfactory level of confluence and integrity to mimic the Blood-Brain Barrier.

4.3 Discussion

This much needed research on the endothelial monolayer helped increase the accuracy and reliability of future migration experiments. The finalized protocol was used in all future experiments and assumed to provide the same results described in Section 4.2. The monolayer integrity test was a good indicator of how well the endothelial cells blocked the passage of molecules, however it was not the most precise method that could have been used. The dextran test could be considered to be somewhat subjective since the fluorescence seen in the samples was not quantified but rather compared to control samples. For the purposes of these experiments, the differential value was acceptable, however, better methods of verifying monolayer integrity exist. One of the most common methods used is the Transepithelial/Transendothelial Electrical Resistance (TEER) method which measures the impedance across a cell layer and is favored for its compatibility with live cells and accepted resistance values [14]. TEER tests could not be done for the experiments performed in these studies due to the size of the monolayers being grown and the setup of the flow migration apparatus. The isolated system in which the monolayers are grown makes it difficult to place the electrodes in the two separate media compartments needed to effectively measure TEER (**Figure 4-4**). The chopstick electrodes depicted in **Figure 4-4** are unable to produce a uniform current density across a large membrane as it leads to an overestimation of the TEER value [14]. Inaccurate TEER values have been reported across membranes spanning an area of about 452 mm^2 and the monolayers grown in this experiment were often measured to be around 341 mm^2 in area, an area dangerously close to the one tested. In future experiments however, it would be helpful to devise a way in which TEER testing could be done with flow experiments to get an accurate reading for the endothelial monolayer integrity.

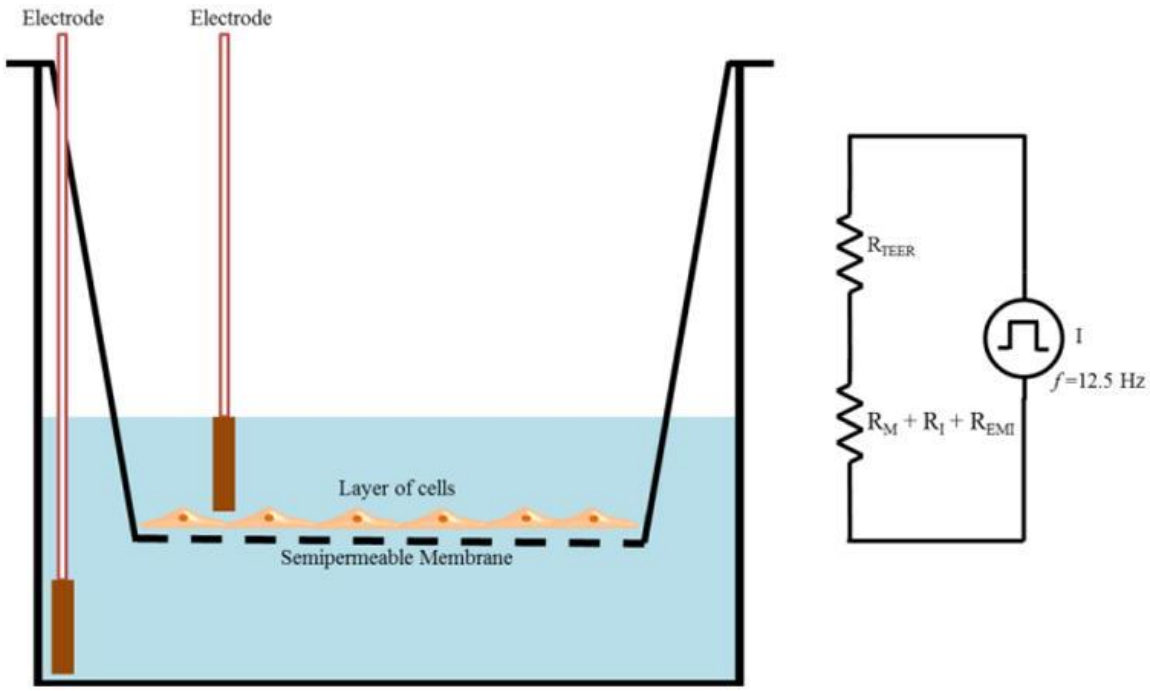


Figure 4-4: Common TEER Test Setup [14]**.** In measuring the TEER value of a cellular monolayer two chopstick electrodes are placed in the medium, one above the cell layer and one in the media below it. The resistance of the bare semipermeable membrane without cells is first measured (R_{BLANK}). The value recorded with the cells (R_{TOTAL}) includes the resistance of the cell layer (R_{TEER}), the resistance of the medium (R_M), the resistance of the semipermeable membrane (R_I), and the resistance of the electrode-medium interface (R_{EMI}). The TEER value, in ohms (**Ω**), of the cell layer (R_{TISSUE}) can be calculated by the following equation: $R_{TISSUE} = R_{TOTAL} - R_{BLANK}$.

Again, it is worthy to note that in the future, a more realistic model of the BBB should be used in these experiments to increase their reliability. The inclusion of astrocytes, pericytes, and other feasible BBB components, in addition to the use of TEER testing as a quantitative tool, would lead to a significant increase in reliability of the results produced by these experiments.

Chapter 5

Migration of Jurkat Cells in Flow Chamber

5.1 Motivation

As previously stated, the goal of this project is to investigate the possibility of eventually producing a better way to facilitate drug delivery to the brain. In order to do this through the use of immune cells, the migration of immune cells needs to be understood. Previous studies in the lab have studied the migration of Jurkat cells through an endothelial membrane under static conditions. However, this proposed methodology of using immune cells to carry nanoparticles across the BBB, when implemented in living creatures, will not take place under static conditions, but rather, in a dynamic environment. The immune cells being used will already be flowing in the blood stream when they approach the BBB, meaning that they will somehow need to stop, attach to the BBB, and then migrate through to the other side. Therefore, the next step in developing this mechanism of drug transport is to conduct migration experiments under dynamic conditions that mimic blood flow.

5.2 Results

The Jurkat cells were used in various experiments involving the newly designed chamber plate to ensure that the plate was fully functional for use in flow migration experiments and to study the state of migration of the Jurkat cells themselves.

5.2.1 Static Migration Experiments

Initially, flow migration experiments were performed without the use of an endothelial monolayer to verify that the chemoattractant being used, CXCL12, was effective in its ability to attract Jurkat cells towards the filter. A chemoattractant was used in these studies, not only because of its capability to affect the ability of cells to adhere to the endothelium, but because certain types of cancer cells are known to excrete different types of chemokines and chemoattractants have also been linked to inflammatory responses [10]. Experiments were done so that a concentration of 2×10^5 cells/mL was made to circulate at a shear rate of 75 sec^{-1} through one chamber that contained no chemoattractant and one that held a 25 ng/mL concentration of CXCL12 in the bottom wells. The average number of migrated cells in the trial without the chemoattractant was measured to be 9741.749 cells and the average number of migrated cells without the use of CXCL12 was 6216.499 cells. Although the chemoattractant proved to increase the migration of Jurkat cells under flow conditions, when these results were compared to those of the static experiments, the difference in migration under flow was not as large as was expected. Due to this, it was decided that static migration experiments be done with the flow chamber apparatus, to make sure that the chemoattractant is properly functioning and to see if the decrease in migration difference was due to the added flow component or if a stronger concentration of CXCL12 was needed to account for the variation in experimental conditions. After further investigation and the emergence of new discoveries, it was determined that all migration experiments in the lab use a CXCL12 concentration of 500 ng/mL.

Initially it was thought that for the static experiments, the concentration of Jurkat cells should be increased to account for the loss of flow. So, when the static migration experiments were performed, the concentration of circulating Jurkat cells was arbitrarily increased from 2 x

10^5 cells/mL to 20 x 10^5 cells/mL. However, the results of multiple experiments conducted with a Jurkat cell concentration of 20 x 10^5 cells/mL indicated that the migration of Jurkat cells is *decreased* with the use of chemoattractant (see **Figure 5-1**).

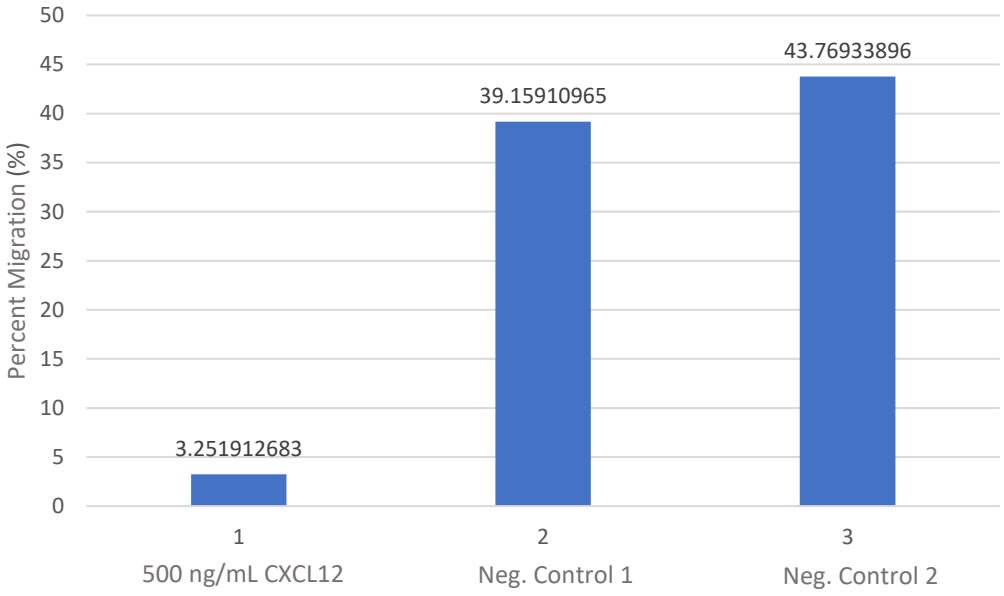


Figure 5-1: Static Cell Migration as a Percentage of the Control. This figure displays the results of three experiments each conducted at a shear rate of 75 sec^{-1} and using a Jurkat cell concentration of 20 x 10^5 cells/mL. Two of these experiments contained no chemoattractant and the other one used a CXCL12 concentration of 500 ng/mL. The outcomes of these experiments were confounding since they suggested that the use of a chemoattractant decreased the number of migrated immune cells. After further investigation it was deemed that this was untrue and that the concentration of Jurkat cells being used was unreasonably high.

Seeing as this did not make sense based on previous observations, the parameters of these experiments were looked into and discussed. A review of the protocol being used led to the conclusion that the dramatic increase of cells was the cause of such confounding results. The concentration of Jurkat cells was brought back down to 2 x 10^5 cells/mL and the results began to realign with the hypothesis that an increase in the use of chemoattractant will result in an

increase of migration. A static negative control (no chemoattractant) and trial experiment were done with a chemoattractant concentration of 500 ng/mL and a Jurkat cell concentration of 2 x 10^5 cells/mL. The results of this experiment can be seen in **Figure 5-2**.

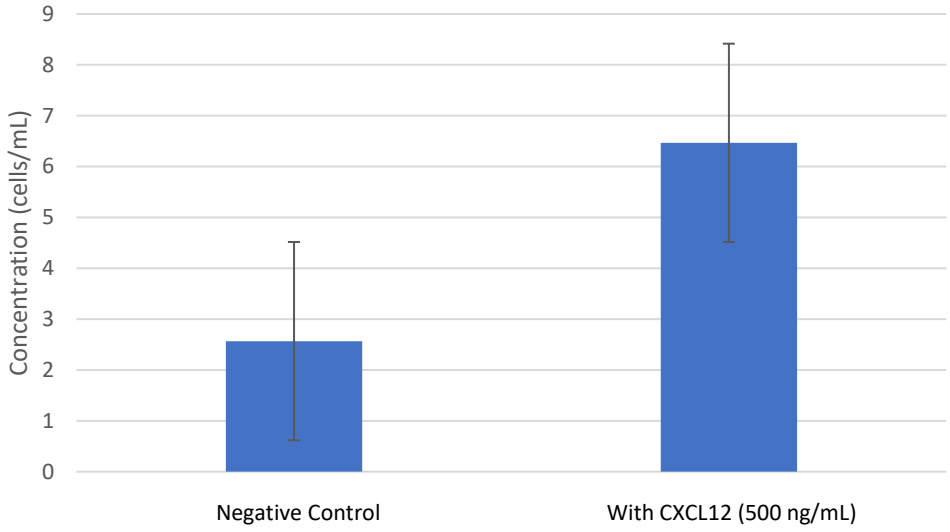


Figure 5-2: Static Cell Migration with CXCL12. This figure shows the averaged results of two experiments in which the concentration of circulating Jurkat cells was brought back down to 2 x 10^5 cells/mL. The results of these experiments support the hypothesis that the use of a chemoattractant increases the migration of immune cells.

5.2.2 Dynamic Migration Experiments

The results of the last static experiment described in the previous section served as proof that the Jurkat cells were able to migrate and that the 500 ng/mL concentration of CXCL12 was an appropriate concentration of chemoattractant to use. The next step was to reproduce these experiments under flow conditions, however, it was thought to introduce a change in protocol that could help account for the predicted decrease in migration by nature of the dynamic

component being added. For this reason, the use of Tumor Necrosis Factor α (TNF- α) was introduced. The details and results of this change will be explained in the next chapter.

5.3 Discussion

Through these results an appropriate concentration of CXCL12 chemoattractant was found, and it was shown that the Jurkat cells are able to migrate toward the chemoattractant. These findings provided direction for the future flow migration experiments and proved that the new flow chamber device was able to function correctly when used as intended.

Chapter 6

Jurkat Migration Under Flow Conditions

6.1 Motivation

Previous studies in the lab have led to many useful discoveries in the context of studying lymphocyte migration across an endothelial barrier under static conditions. While this information has been helpful, these experiments lack a critical physiological component, an atmosphere of flow. When looked at from an *in vivo* perspective, these immune cells will already be flowing in the blood stream when they come into contact with the Blood-Brain Barrier (BBB). This means that in order to carry nanoparticles across the BBB, the cells will need to somehow adhere to the endothelial cells while circulating in order migrate. To fully understand the complexity of this process, the study of the Jurkat cells adhering to the endothelial layer and *then* migrating while under flow conditions is crucial. This process of leukocyte extravasation has been studied in great detail [11] and the findings of these studies were taken into account while performing these experiments. This necessary step not only mimics the flow of blood that would be present in an *in vivo* study but it also provides the necessary considerations that will need to be taken into account when the time for *in vivo* studies arrives.

6.1.1 Use of TNF-α

Due to the nature of adding the flow component to these studies, it was expected that the migration of Jurkat cells in these conditions would decrease. Instead of simply being placed on the endothelial monolayer and migrating their way to the other side, the Jurkat cells would now need to form weak initial tethers to the endothelial cells that will allow them to roll along the barrier and eventually stop. Once they have stopped rolling, the cells would need to arrest, or form firm adhesive bonds, to the monolayer so that they can then migrate through it [15]. These factors and the additional work to be done by the cells led to the proposal that a decrease in migrated cells would be seen after the flow component was added. To help account for this decrease in migration, it was thought to incorporate a small change in protocol that would in some way increase the ability of the Jukrat cells to attach to the endothelial surface while moving. Tumor Necrosis Factor α (TNF- α) is a cytokine that acts as a host defense mechanism for cells during inflammatory responses [16]. When used on an endothelial surface, TNF- α is known to cause an upregulation of adhesion molecules on the endothelial cells, leading to an increase of cell binding to these treated cells. The treatment of the BBMVECs used in this study with TNF- α was thought to lead to increased regulation of the Intercellular Adhesion Molecule type 1 (ICAM-1) on the surface of these cells. It is known that Jukrat cells express Lymphocyte Function-associated Antigen 1 (LFA-1), a transmembrane glycoprotein [17], and that the LFA-1 on Jurkat cells binds to the ICAM-1 ligand on endothelial cells [18]. **Figure 6-1** provides a visual representation of the binding between Jurkat and endothelial cells.

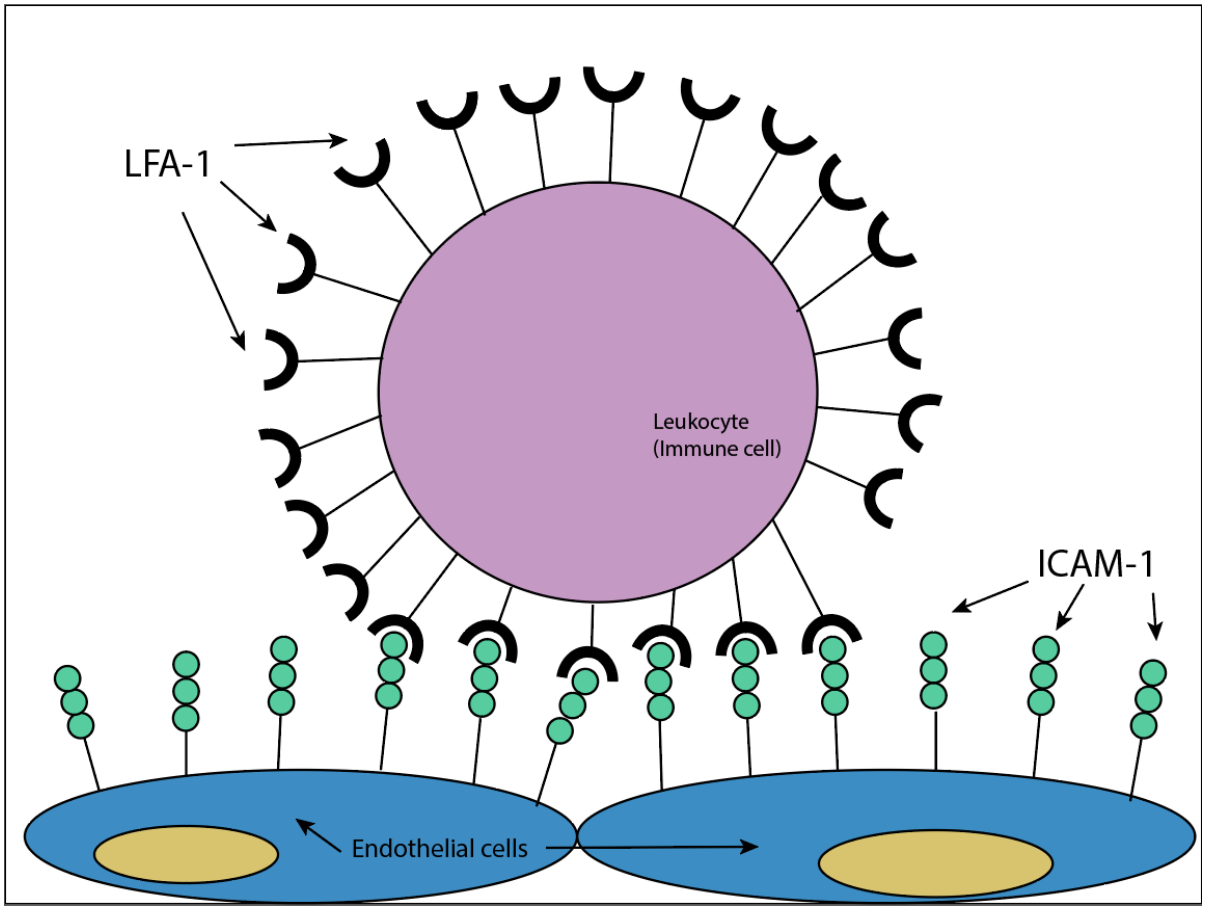


Figure 6-1: Immune Cell Binding to Endothelial Cells Through LFA-1 and ICAM-1. This figure shows an artistic rendition of the attachment of a leukocyte, such as a Jurkat cell, to an endothelial surface through the molecule Lymphocyte Function-associated Antigen 1 (LFA-1) and the ligand, Intercellular Adhesion Molecule type 1 (ICAM-1).

This interaction between LFA-1 and ICAM-1 has been seen to increase the arresting of immune cells on the endothelium and therefore helped the migration of these immune cells across an endothelial barrier [15]. For this reason, it was thought that treating the endothelial monolayer with TNF- α beforehand could potentially increase the migration of Jurkat cells under flow conditions.

The endothelial monolayer was treated with a 25 ng/mL concentration of TNF- α for 6 hours prior to the execution of the experiment as it was seen that ICAM-1 expression on

endothelial cells reaches a maximum at 6-8 hours of exposure to TNF- α [16]. After the 6 hour period had ended, the experiments were performed as described in Section 2.5.

6.2 Results

Three dynamic flow experiments were performed in which the endothelial monolayer was treated with TNF- α 6 hours beforehand. These experiments were performed at a shear rate of 50 sec^{-1}, with a Jurkat cell concentration of 2 x 10^5 cells/mL, and a CXCL12 concentration of 500 ng/mL. Each of the three experiments were performed as described in Section 2.5. The reading from each well in the chamber was taken to be a single data point and a statistical t-test was done on the results. These three data points were averaged and the averaged results for each of the three flow migration experiments can be seen in **Figure 6-2**.

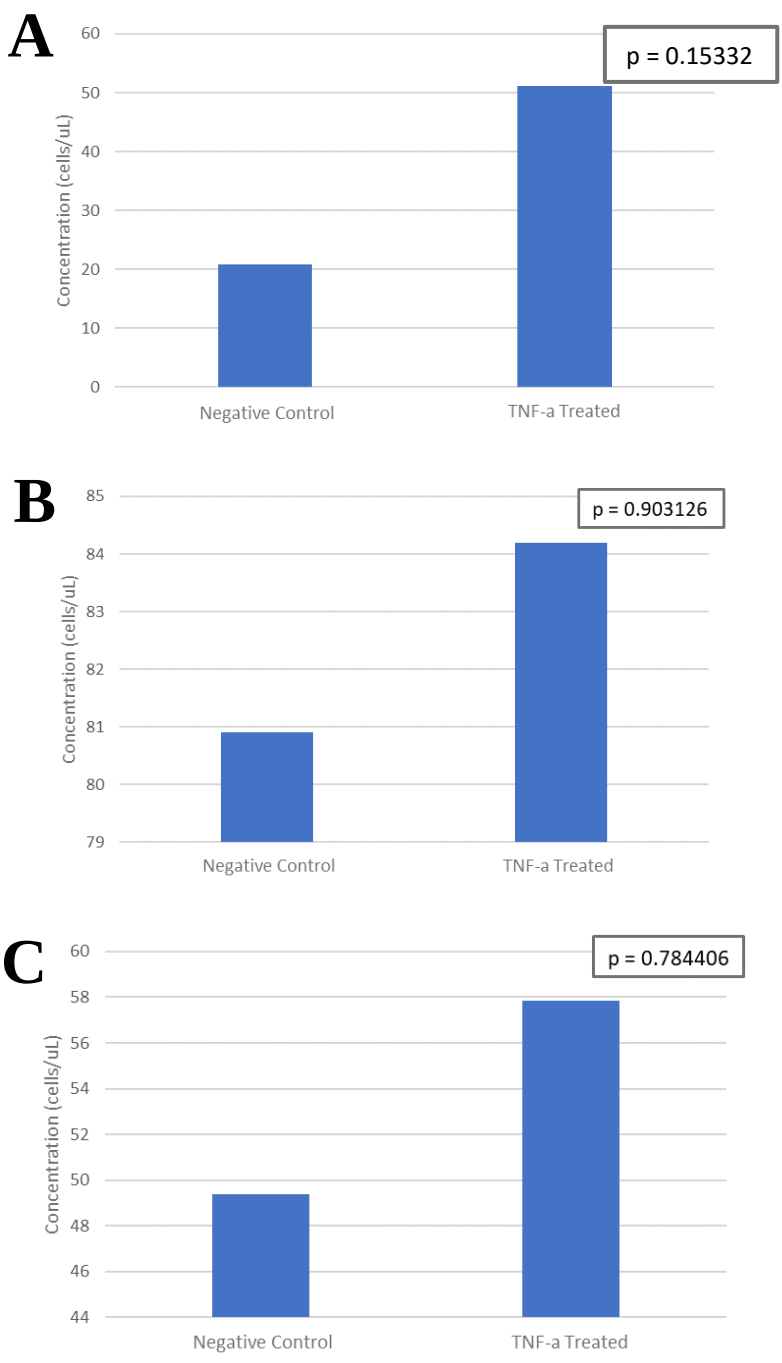


Figure 6-2: Results of TNF- α Migration Experiments. This figure shows the difference that the treatment of the endothelial barrier with TNF- α made and the respective p values of the results. (A), (B), and (C) represent the data of each experiment in chronological order. It can be seen that in each trial there was a noticeable increase in cell migration with the use of TNF- α, however the statistical p values, all greater than 0.05, prove that the difference was not large enough to be considered significant.

The values displayed by these charts were obtained by averaging the concentration of migrated cells found in each well. The respective p-values produced by the t-test for each trial were 0.15332, 0.903126, and 0.784406. Given that for this specific t-test, only p-values less than 0.05 were considered to be statistically significant, none of the results from these experiments proved to show a statistically significant difference between treating the endothelial cells with TNF- α and leaving it untreated. An additional t-test was taken on the averages of the three trials to see if the averaged results of each trial should be treated as one data point. However, with a p-value of 0.522624 it was still determined that the use of TNF- α does not increase the migration of Jurkat cells enough to be the sole solution to this problem.

6.3 Discussion

Through these experiments, the migration of Jurkat cells under flow conditions was observed and quantified. The use of TNF- α was introduced in order to try and increase the migration of Jurkat cells as a decrease in migration was anticipated due to the added flow component. However, through analyzing the results of the use of TNF- α it was shown that activating ICAM-1 on the endothelial cells does not provide a statistically significant increase in immune cell migration, even though a slight increase was shown. This means that while TNF- α does increase the migration of immune cells under flow, TNF- α alone is not enough to significantly increase these numbers.

The reason for the lack of significant difference in these experiments could be due to the fact that the LFA-1 molecule on Jurkat cells is most active in arresting, or making firm adhesions to the endothelial surface so that the cells can migrate through [19]. This means that while the

Jurkat cells are able to firmly adhere to the endothelial surface, they are not efficient in making the initial weak bonds needed to stop on the endothelial surface so that these strong bonds can take place. Therefore, in the future, more work will need to be done on the initial rolling and stopping of the Jurkat cells to ensure that they can then arrest on the endothelial cells and migrate under flow conditions.

Even though the use of TNF- α did not yield statistically significant results, it was continually used in following experiments due to the slight increase seen in migration and its noticeable use in literature.

Chapter 7

Conjugation of Nanoparticles to Jurkat Cells

7.1 Motivation

For the application of these findings to drug delivery research, it is important that we not only understand how immune cells attach to and migrate through an endothelial barrier, but also how they are able to take up drugs and carry them across the BBB. To do this, Biodegradable Photoluminescent Polylactic Acid (BPLP-PLA) nanoparticles were used to mimic the drugs that would utilize this type of drug delivery mechanism. In these experiments it was shown that the nanoparticles can be successfully conjugated to Jurkat cells and that these Jurkat cells were able to increase the transport of the nanoparticles across the endothelial barrier.

7.2 Results

The clicking, or conjugation, of the BPLP nanoparticles to the Jurkat cells was done using the protocol described in Section 2.8. This protocol was followed and two samples of Jurkat cells were put in the flow cytometer, one with BPLP and one without, to ensure that the clicking process was effective. **Figure 7-1** shows the results of this experiment.

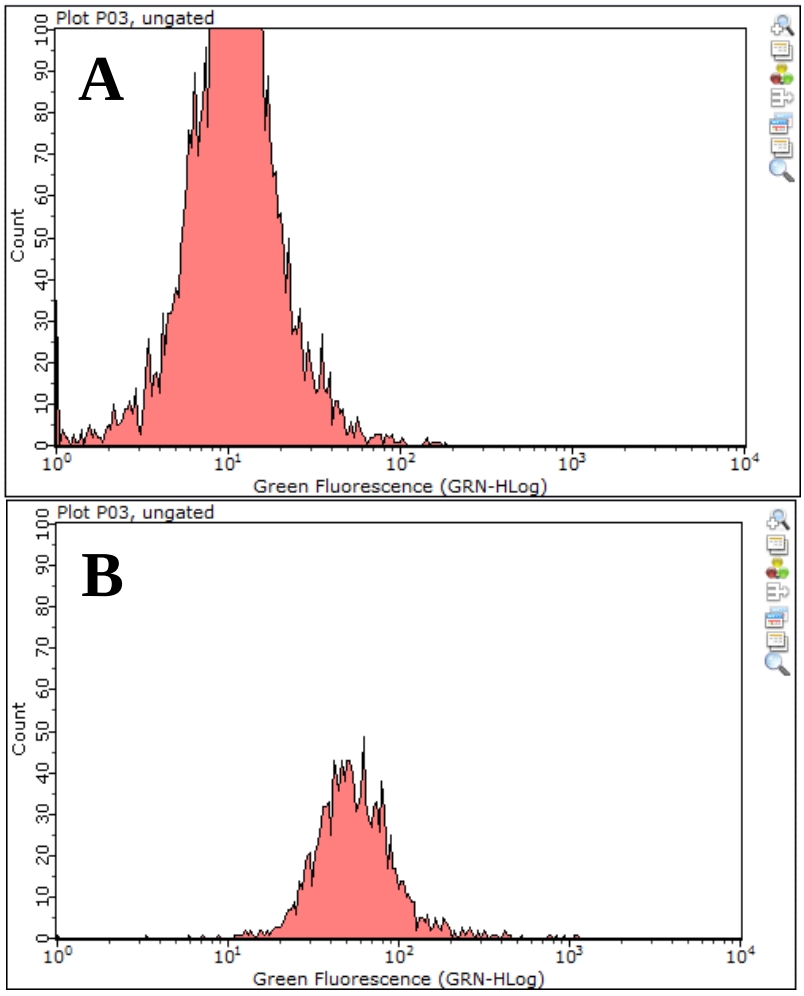


Figure 7-1: Fluorescent Proof of BPLP-PLA Nanoparticle Conjugation. This figure shows the shift in green fluorescence that serves as the proof of conjugation between the Jurkat cells and the BPLP nanoparticles. (A) shows the green fluorescence reading of the sample containing just Jurkat cells alone. (B) shows the green fluorescence reading of the Jurkat cells clicked with the BPLP nanoparticles. Since BPLPs are fluorescent in nature, a rightward shift in fluorescence was expected in the sample containing clicked Jurkat cells with BPLP. Disregarding the higher spike in (A) due to a higher concentration of cells in the sample, a shift to the right in fluorescence can be seen in (B) due to the presence of BPLP nanoparticles.

The positive shift of green fluorescence in **Figure 7-1** indicates that the Fluorescent BPLP nanoparticles were successfully clicked to the Jurkat cells, given that nanoparticles are fluorescent in nature and that their presence would be represented by an increase in fluorescence.

A flow migration experiment was then done in which the transport of nanoparticles was compared between a trial that had nanoparticles clicked to Jurkat cells and a trial that ran only nanoparticles (negative control). The results of this trial showed an increase of more than double the concentration of molecules collected from the wells when Jurkat cells were included and also reflected little change in fluorescence between the two trials. The results of this experiment are shown below in **Figures 7-2, 7-3**.

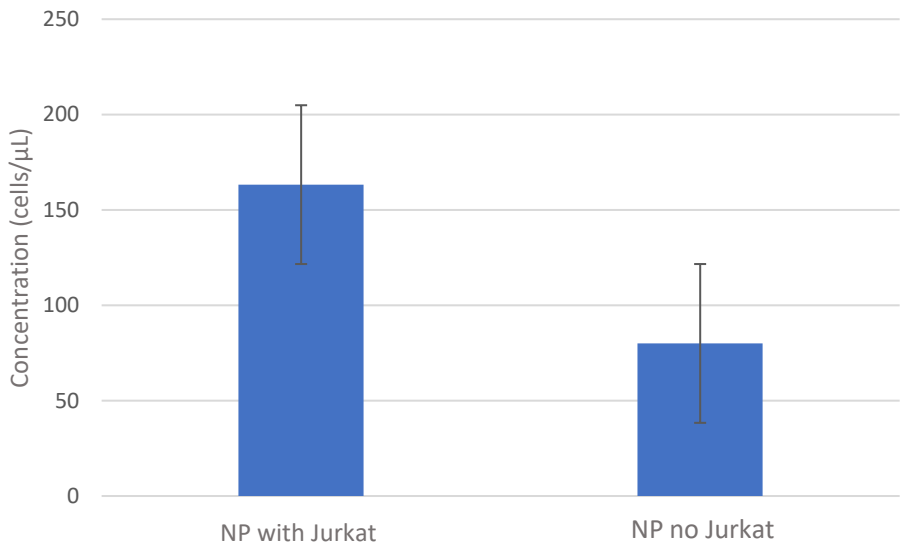


Figure 7-2: Concentration of Jurkat Mediated NPs Against Control. This figure shows that the sample concentration from the flow cytometer of the nanoparticles carried by the Jurkat cells is more than double that of the nanoparticles that circulated alone.

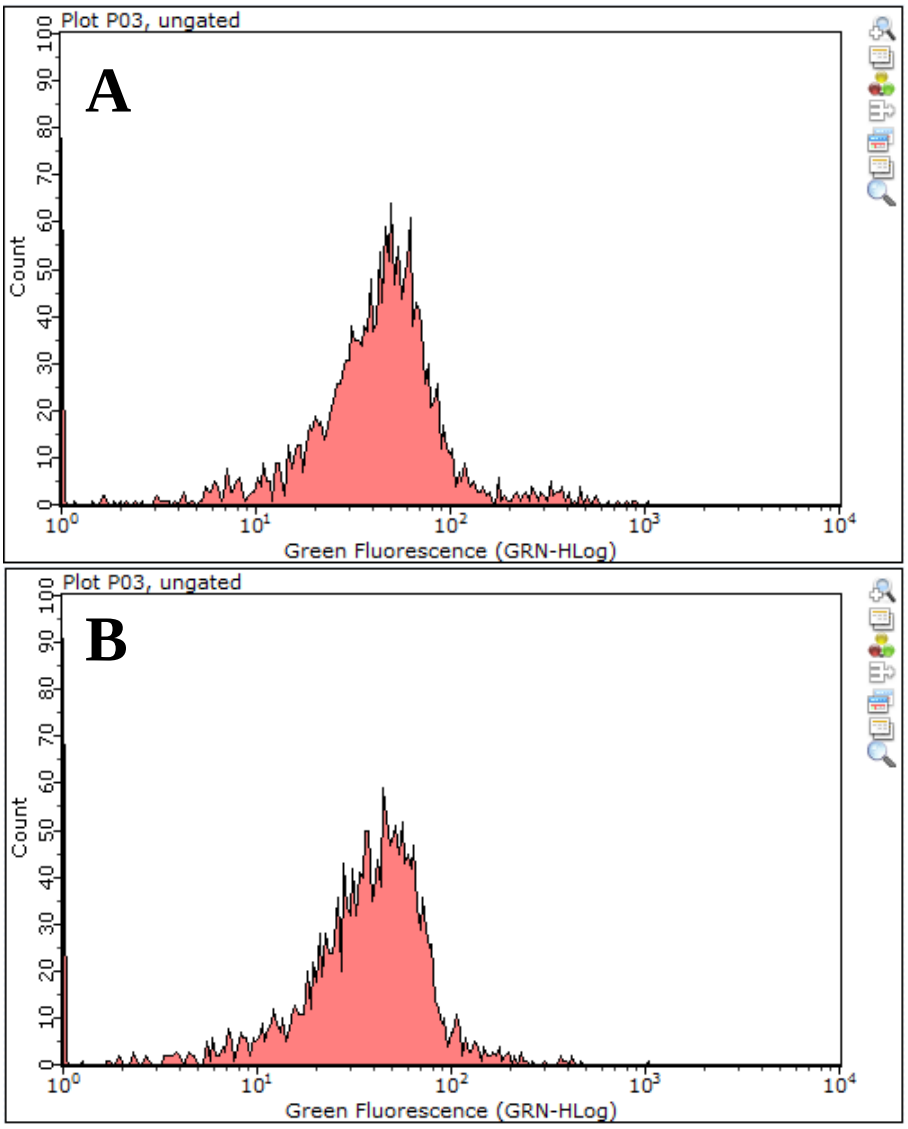


Figure 7-3: Fluorescent Reading of Jurkat Mediated NPs Against Control. This figure shows the lack of fluorescent shift between the trial containing Jurkat mediated BPLP nanoparticles and the control of just using nanoparticles. (A) Green fluorescence reading of nanoparticle only control. (B) Green fluorescence reading of Jurkat mediated nanoparticle trial.

The little change in fluorescence shown between the two experiments indicates that a decent amount of nanoparticles, most likely in clumps since they were detected by the flow cytometer under the set parameters, were able to pass through the barrier and into the wells.

Seeing that the nanoparticles hold the same intensity of fluorescence, if BPLPs were able to make it past the endothelial barrier, they would express the same level of fluorescence as the nanoparticles carried across through cells.

It should be noted that the concentration of "cells" that migrated into the wells was calculated based on a specific forward scatter threshold value that was set for migratory cells. The details of this setting are discussed in the following section.

7.3 Discussion

Through using the clicking protocol in Section 2.8 it was assumed that the clicking of the BPLP nanoparticles to the Jurkat cells was successful due to the Jurkat cell's observed increase in fluorescence after being conjugated to the nanoparticles. In collecting the data for the conjugated nanoparticle experiments, there was some discrepancy in how to analyze the amount of nanoparticles that had transported across the filter. In obtaining these results, the gain controls were set to common levels used for migration experiments. These gain settings can be seen in **Table 7-1** below.

FSC	SSC	GRN	YEL	RED	NIR	Thr - FSC
4.97	39.7	15.3	8.00	30.6	8.00	700

Table 7-1: Standard Gain Controls for Migratory Cells. These gain controls are the standard values used in the flow cytometer when analyzing samples from a cell migration experiment. FSC – Forward Scatter, SSC – Side Scatter, GRN- Green Fluorescence, YEL – Yellow, RED – Red Fluorescence, NIR – Near Infrared, Thr-FSC – Forward Scatted Threshold.

While the sample concentration was being looked at in the results, the fluorescence of the samples was also being considered. However, there was little change in fluorescence of nanoparticle samples that had conjugated Jurkat cells and those that didn't. This can be explained by the fact that had any nanoparticles made it through to the other side of the filter in the trial without Jurkat cells, they would exhibit the same strength of fluorescence as those that were collected with Jurkat cells. The fact that the strength of fluorescence was so similar for both trials indicated that there was still a fair amount of BPLP collected in the wells of the negative control.

The forward scatter threshold of 700 also posed a problem since this value is one commonly used to quantify migrated cells, not nanoparticles. Forward Scatter in flow cytometry reflects the size of the particle being quantified. Since the fluorescence alone was not a good indicator of transported nanoparticles, the concentration was also looked at as a possible indicator. The fluorescence had already shown that nanoparticles in the negative control were able to cross the barrier, however the quantity of nanoparticles that were included in the concentration count after the forward scatter threshold of 700 had been set indicates that these nanoparticles were most likely in clumps of considerable sizes as they crossed the endothelial barrier. When considering these results and the circumstances surrounding them, it is important to keep in mind that this flow migration assay still requires a lot of optimization before it can be held to a certain standard of accuracy. The areas in which this optimization needs to take place as well as the parameters that require further investigation will be discussed in Section 8.2.

Chapter 8

Conclusion

8.1 Summary of Findings

This study aimed to look into the possibility of using immune cells to carry drugs across the blood-brain barrier (BBB) and study this drug delivery mechanism under flow conditions. Before the direct study of these conditions could take place, some preliminary testing and modifications were made. The bottom flow chamber plate was redesigned to accommodate for the suspensive immune cells used in these trials and to facilitate a more accurate data collection process using flow cytometry.

A protocol was also made to verify the growth of a healthy, tight endothelial barrier made up of Bovine Brain Microvascular Endothelial Cells (BBMVEC). The finalized protocol that produced a confluent layer while maintaining healthy cells involved seeding a fibronectin-coated filter with 250,000 cells and allowing these cells to grow in an incubator for 6 days before use in an experiment. Work was also done into ensuring that the newly designed apparatus was able to function properly when used with Jurkat cells and that the appropriate concentration of chemoattractant, CXCL12, was being used in the experiments. The settled concentration of CXCL12 for use in the flow migration experiments was 500 ng/mL and it was determined that a concentration of 2×10^5 cells/mL was enough to produce conclusive results.

The endothelial barrier was treated with TNF- α in an effort to upregulate ICAM-1 and therefore increase the attachment of Jurkat cells to the endothelium in hopes that this would increase Jurkat migration under flow conditions. However, treating the BBMVECs with TNF-α

did not prove to be a sufficient way to increase immune cell migration, possibly due to the LFA-1 glycoprotein's roll in cell arresting and not necessarily in cell rolling/tethering.

BPLP-PLA nanoparticles were successfully conjugated to Jurkat cells and a comparative experiment was done to compare the use of Jurkat cells in carrying these nanoparticles across the endothelial barrier. The results of this experiment showed an increase in concentration from the Jurkat cell samples, however the fluorescence of these samples was essentially the same as the control samples. Meaning that there was a leakage of nanoparticles across the endothelial barrier without the help of Jurkat cells. This leakage is reflective of some of the shortcomings of this experimental process and may provide insight on how to gain more reliable results moving forward.

8.2 Future Work

Future work in this area will require that adjustments be made to the protocol and methodology of these experiments so that the results obtained can be both more realistic and more reliable. One of the biggest shortcomings of the current protocol is the *in vitro* blood-brain barrier model. Although a protocol was developed for a homogenous layer of endothelial cells, the monolayer is still lacking in other key BBB components (see Section 1.1.1). Additionally, a way of quantifiably measuring the tightness of these layers would be desirable in making sure that their resistance is on the level of other BBB models. An example of such a method would be TEER testing (see Section 4.3), however there are assembly constraints that would need to be taken care of in order for this to be a possibility.

Another area in which future studies could improve is in increasing the shear rate so that it corresponds to the physiological value seen along the blood brain barrier. The experiments in this study were performed at an incredibly low shear rate, 50 sec^{-1} or 2 mL/min, whereas the physiological cerebral blood flow shear rate has been noted to be in the range of 60-70 mL/100g per min [2]. Part of the reason that the flow rate was set to be so slow in these experiments is because of Jurkat cell's ineffectiveness in rolling and stopping on the endothelial barrier under flow conditions. This leads to another improvement for future studies. The direction of this project is set to move away from using Jurkat cells and toward using other T lymphocytes, especially ones that perform well in both initial tethering *and* arresting on endothelial cells under flow.

Bibliography

[1] A.D. Wong, M. Ye, A.F. Levy, J.D. Rothstein, D.E. Bergles, P.C. Searson, The blood-brain barrier: an engineering perspective, Front. Neuroeng. 6 (2013). doi:10.3389/fneng.2013.00007.

[2] E.H. Lo, A.B. Singhal, V.P. Torchilin, N.J. Abbott, Drug delivery to damaged brain, Brain Res. Rev. 38 (2001) 140–148. doi:10.1016/S0165-0173(01)00083-2.

[3] Y. Serlin, I. Shelef, B. Knyazer, A. Friedman, Anatomy and physiology of the blood-brain barrier, Semin. Cell Dev. Biol. 38 (2015) 2–6. doi:10.1016/j.semcdb.2015.01.002.

[4] A.K. Parashar, R.K. Nema, A Review on Novel Techniques for Drug Delivery to the Brain, Curr. Res. Pharm. Sci. 7 (2012) 134–141. https://www.researchgate.net/publication/282332393_A_Review_on_Novel_Techniques_for_Drug_Delivery_to_the_Brain.

[5] A.C.C. da Fonseca, D. Matias, C. Garcia, R. Amaral, L.H. Geraldo, C. Freitas, F.R.S. Lima, The impact of microglial activation on blood-brain barrier in brain diseases, Front. Cell. Neurosci. 8 (2014). doi:10.3389/fncel.2014.00362.

[6] W.M. Pardridge, Drug delivery to the brain., J. Cereb. Blood Flow Metab. 17 (1997) 713–731. doi:10.1097/00004647-199707000-00001.

[7] W.M. Pardridge, The blood-brain barrier: Bottleneck in brain drug development, NeuroRx. 2 (2005) 3–14. doi:10.1602/neurorx.2.1.3.

[8] W.M. Pardridge, Drug transport across the blood-brain barrier., J. Cereb. Blood Flow Metab. 32 (2012) 1959–72. doi:10.1038/jcbfm.2012.126.

[9] Z. Xie, Y. Su, G.B. Kim, E. Selvi, C. Ma, V. Aragon-Sanabria, J.T. Hsieh, C. Dong, J. Yang, Immune Cell-Mediated Biodegradable Theranostic Nanoparticles for Melanoma Targeting and Drug Delivery, Small. 13 (2017). doi:10.1002/smll.201603121.

[10] M.J. Slattery, C. Dong, Neutrophils influence melanoma adhesion and migration under flow conditions, Int. J. Cancer. 106 (2003) 713–722. doi:10.1002/ijc.11297.

[11] C. Dong, X.X. Lei, Biomechanics of cell rolling: Shear flow, cell-surface adhesion, and cell deformability, J. Biomech. 33 (2000) 35–43. doi:10.1016/S0021-9290(99)00174-8.

[12] V.L. Gonzalez, The effects of bioconjugation of calcium phosphosilicate nanoparticles on the delivery to breast cancer, The Pennsylvania State University, 2016. https://honors.libraries.psu.edu/files/final_submissions/3696.

[13] D.M. Wuest, K.H. Lee, Optimization of endothelial cell growth in a murine in vitro blood-brain barrier model, Biotechnol. J. 7 (2012) 409–417. doi:10.1002/biot.201100189.

[14] B. Srinivasan, A.R. Kolli, M.B. Esch, H.E. Abaci, M.L. Shuler, J.J. Hickman, TEER Measurement Techniques for In Vitro Barrier Model Systems, J. Lab. Autom. 20 (2015) 107–126. doi:10.1177/2211068214561025.

[15] A. Sigal, D. a Bleijs, V. Grabovsky, S.J. van Vliet, O. Dwir, C.G. Figdor, Y. van Kooyk, R. Alon, The LFA-1 integrin supports rolling adhesions on ICAM-1 under physiological shear flow in a permissive cellular environment., J. Immunol. 165 (2000) 442–452. doi:10.4049/jimmunol.165.1.442.

[16] F. Mackay, H. Loetscher, D. Stueber, G. Gehr, W. Lesslauer, Tumor necrosis factor alpha (TNF-alpha)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55., J. Exp. Med. 177 (1993) 1277–86. doi:10.1084/jem.177.5.1277.

[17] E.P. Wojcikiewicz, M.H. Abdulreda, X. Zhang, V.T. Moy, Force spectroscopy of LFA-1 and its ligands, ICAM-1 and ICAM-2, Biomacromolecules. 7 (2006) 3188–3195. doi:10.1021/bm060559c.

[18] S.D. Marlin, T.A. Springer, Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1), Cell. 51 (1987) 813–819. doi:10.1016/0092-8674(87)90104-8.

[19] J. V. Stein, A. Rot, Y. Luo, M. Narasimhaswamy, N. Hideki, M.D. Gunn, A. Matsuzawa, E.J. Quackenbush, M.E. Dorf, U.H. von Andrian, The Cc Chemokine Thymus-Derived Chemotactic Agent 4 (Tca-4, Secondary Lymphoid Tissue Chemokine, 6ckine, Exodus-2) Triggers Lymphocyte Function-Associated Antigen 1-Mediated Arrest of Rolling T Lymphocytes in Peripheral Lymph Node High Endothelial Venules, J. Exp. Med. 191 (2000) 61–75. http://jem.rupress.org/content/191/1/61.

Appendix A

Previous Flow Migration Protocol

FLOW MIGRATION PROCEDURE

Preparation Materials:

- Blockers (must be autoclaved before each use)
- Centrifuge tubes (15 mL and 50 mL)
- 90% ethanol
- Petri Dishes
- Filters with 8 um pore size (top left drawer in room 311)
- 1x DPBS
- 0.25% Trypsin/EDTA
- Fibronectin (30 uL for each chamber, 100uL/vial; vials are in a clear tip box with green ink labeling in the door of the freezer in 311)
- 2 silicon strips for each blocker (6 total)
- EI cells (500,000 or 250,000 cells for each dish)
- DMEM (for EI cells)
- DMEM + 10% FBS (for tumor cells)
- DMEM +10% FBS (for EI cells)
- Hemocytometer
- Microscope

Experimental Materials:

- Flow Chamber Box and Incubator (307)
- Chamber parts: plates, silicon gasket, tubing, screws (307; screws in drawers and rest in sink)
- Hot plate (307)
- Large beaker for boiling water bath
- Tumor cells (6 million, 2-3 confluent plates)
- 1x DPBS
- 0.25% Trypsin/EDTA
- Trypan Blue
- Hemocytometer
- Centrifuge tubes (15 mL and 50 mL)
- Albumin Bovine – BSA (fridge in 307; in white bottle)

- DMEM+10% FBS
- DMEM
- Collagen IV (frozen tubes in -80 degree freezer in hallway)
- Centrifuge
- Cell Rocker
- Tissue Culture Incubator
- Hemacolor HARLECO dye kit (307; white box)
- Petri Dishes
- DI water
- VWR Microscope Slides (drawer next to cytometer in 307)
- Brightfield Microscope

Procedure:

Filter Preparation

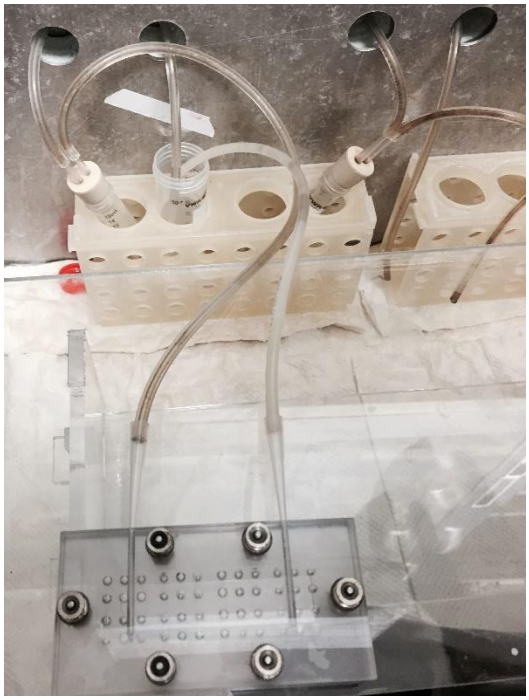
1. Autoclave the blockers / silicon strips before use
    a. Use blockers that have been washed with soap from the previous experiment
    b. Rinse with DI water
    c. Autoclave in a pipet tip box with autoclave tape
2. Inside of the hood: gather 90% ethanol and 3 petri dishes
3. Add 3mL ethanol to each dish and put one filter in each dish shiny side up
4. Soak the filters for 30 minutes under UV light within laminar hood (hood slightly open and blower on)
    a. Keep the lids of petri dishes off while under UV light
5. Meanwhile, defrost 1 fibronectin vial (fridge in 311; usually 100uL/vial) and warm up the media and trypsin
6. Turn UV light off and turn light on
7. Aspirate out ethanol and allow the filters to dry shiny side up in the hood
8. Flip filters over to un-shiny side using tweezers
9. Put dry blockers on top of the filters with the holes centered on the filters (put blockers shiny side up inside petri dishes)
10. Add 1mL DMEM without FBS to the hole of the blocker in each petri dish
11. Add 30uL fibronectin to the same hole (do not shake the petri dish)
12. Incubate the filters/petri dishes for 2 hours minimum (this can be done for longer or overnight as well)
13. At the end of incubation, prepare EI cells. Aspirate petri dishes and wash with DPBS. Lift EI cells with 0.25% Trypsin/EDTA, inactivate trypsin with 6 mL of RPMI+10% FBS, and transfer to centrifuge tube. Centrifuge at 1500 rpm for 5 minutes and resuspend in 6 mL of DMEM+10%FBS

14. Count cells using hemocytometer. You will need 500,000 cells per filter if you are running migration the next day and 250,000 cells per filter if you are running 2 days later
15. Gather the filters/ petri dishes from incubator without tipping the petri dishes (to prevent the blockers from moving) and move to the hood
16. Wash the filters and bottom of blockers with 10mL DPBS
17. Pick up the blockers, place them in the lids and let them dry
18. Transfer filters using tweezers to new petri dishes and let them dry
19. Add dried blockers centered on top of filters in the new petri dishes
20. Place one silicon strip on each side of the blockers using tweezers to prevent the blockers from moving
21. Add 7.5 mL media (DMEM with FBS) to each of the two sides of the blocker
22. Add 1.5mL media (DMEM with FBS) to hole
23. Add relevant volume for either 500,000 or 250,000 cells to hole from previous calculation
24. Incubate until experiment
    a. For 500,000 cells, incubate for 24 h
    b. For 250,000 cells, incubate for 48h

Running Flow Chamber

1. Turn on the hot plate with a large beaker filled with DI water. Bring water to a boil.
2. Turn on flow chamber. It takes about 45 minutes to warm up to 37 °C
3. Defrost collagen IV (in -86 °C freezer) and warm up media and trypsin
4. Clean all chamber parts (gaskets, top plates, bottom plates and tubing) with soap and hot water, and rinse them with DI water.
5. Dip parts in beaker one-by-one with boiling water using the large tweezers
    a. 30s for bottom plates, 20s for top plates, 10s for gaskets and 2s for tubing.
    b. Do not place tubes in the boiling water as they will melt.
6. Let the parts dry on paper towels. You may need to aspirate water out of the wells.
7. Make 1 w/v % BSA/DMEM. Add 0.4 g BSA to 40 mL DMEM without FBS in 50mL centrifuge tube. Make 2 tubes (80 mL total)
8. Prepare the tumor cells: Aspirate, wash with DPBS, and lift tumor cells with 0.25% Trypsin/EDTA as if passing
9. Inactivate cells with DMEM +10% FBS, transfer to centrifuge tube
10. Centrifuge the tumor cells for 5 minutes at 1500 rpm
11. Resuspend in DMEM +10% FBS media
12. Count cells using hemocytometer. You need 2 million cells for each migration chamber, so 6 million total cells usually requiring 2 or 3 confluent plates

13. Spin down and resuspend cells with BSA solution to get 6 million cells in the 15mL centrifuge tubes
14. Place tube on the rocker in the incubator for at least 45 minutes
15. Make 1 w/v% collagen IV solution in BSA/DMEM of 100ug/mL.
16. Fill middle 12 wells of each chamber with 23-24 uL of collagen IV solution (try to avoid bubbles)
17. Fill the remaining wells with 23-24 uL of 1% BSA/DMEM
18. Take the petri dishes out of the hood and take off the blockers
19. Grab the filter with the tweezers and scrape the shiny side (bottom) with the cell scraper to remove extra EI cells
20. Put the filters on the bottom plates of chambers with EI cells facing up (un-shiny side up) trying to center the EI cells over the collagen wells
21. Place the silicon gaskets on top of the filters (doesn't matter which side)
22. Screw on top plates of chambers (with the open holes facing downwards)
23. After the tumor cells have been incubated for 45 minutes, transfer the cell suspension to 50 mL centrifuge tube (2 million cells/tube), bring the volume to 20 mL with 1% BSA/DMEM and mix well
24. Transfer 10 mL of the 20 mL of cell suspension back to each 15 mL centrifuge tube
25. Connect centrifuge tubes, tubing, and chambers (see the photo). Place the rubber tubing from the pump inside of the 50 mL centrifuge tube, so that the tubing touches the bottom of the centrifuge tube. Place the outlet rubber tubing inside of the 50 mL centrifuge tube and make sure that it is not touching the liquid (to avoid bubbles)



26. Turn on the motor located behind the motor, set it to the desired shear rate, and start the flow. The numbers are in mL/min. 2mL/min corresponds to 50 sec^{-1}, 3mL/min corresponds to 75 sec^{-1} (low shear) and 8mL/min corresponds to 200 sec^{-1} (high shear).
27. After fluid moves into the chambers, you can add the particle (in 1x DPBS) treatment and the same volume of 1x DBPS to the nontreatment as a control in the 50 mL centrifuge tubes. The volume of particles is 4 times the volume used for viscometer experiments because you have 4 times as many cells.
28. Allow the experiment to run for 4 hours. Check on the chambers occasionally to be sure that no bubbles have formed. If bubbles form, tilt the chamber to the left so that the bubbles move to the exit tube.
29. Gather 3 petri dishes and add solutions 1, 2, and 3 of the Hemacolor HARLECO dye kit to the lids of the petri dishes. The solutions are clear (fixative), red, and blue respectively
30. Fill the 4th dish and the 5th dishes with DI water
31. When the experiment is complete, turn off the power of the migration box. Disconnect the tubes and unscrew the chambers.
32. Grab the filters with tweezers and scratch the un-shiny side (top) to remove the EI cells and some tumor cells. Rinse the filter with 1x DPBS if necessary (if the solution 1 is dirty after soaking the filters)
33. Dye the filter (un-shiny side up) by soaking in solutions 1, 2, and 3 for 30, 4, and 6 seconds respectively. Do not rinse in between steps
34. Using tweezers, rinse the filters in the petri dish with DI water last
35. Attach each filter un-shiny side up to a microscope slide and let it dry. Label the slide with date, shear rate, and treatment.
36. Use the microscope to take images of the migrated cells (20x, Brightfield). Take 5 images from the center of the filter. Average the total number of migrated cells to get your number representing cells/frame.
37. Place chamber pieces in beaker of hot, soapy water. Wash flow chamber machine by running 45 mL hot DI water (in 50mL tube) through connected tubing at 8 mL/min twice (see the photo)

38. Empty the tubes, stop the pump and turn off the machine.

ACADEMIC VITA

Nmachi Anumba

nca5078@psu.edu

EDUCATION

The Pennsylvania State University, University Park, PA
Bachelor of Science in Biomedical Engineering
Bachelor of Arts in Spanish
Millennium Scholars Program
Study Abroad - Universidad San Francisco de Quito, Ecuador **Spring 2016**

RESEARCH EXPERIENCE

University of California, Berkeley **Berkeley, CA**
Undergraduate Researcher, MikLab *June–August 2017*
Advisor: Dr. Michael Lustig

- Created various hydrogels for realistic, anatomically structured MRI phantoms
- Developed a protocol for 3D printing free-standing hydrogel structures

The Pennsylvania State University **University Park, PA**
Undergrad Researcher, Cellular Biomechanics Laboratory *June 2016 – Present*
Advisor: Dr. Cheng Dong

- Developed protocol for confluent endothelial monolayer to mimic blood-brain barrier
- Designed and built a novel flow chamber plate for suspensive cells to be used in flow migration assays
- Studied the migration of Jurkat cells and BPLP-PLA nanoparticles under flow conditions

Undergraduate Researcher, Medical Imaging Laboratory *June – July 2015*
Advisor: Dr. Steven Schiff

- Gathered data on MRI B_0 field homogeneity through electric field mapping
- Analyzed results of field mapping by using visual representations
- Determined solutions to compensate for inconsistency of electric field
- Calculated dimensions and material needed for new B_p coil

HONORS AND AWARDS

ABRCMS Students Travel Award	November 2017
UC Berkeley Best Poster Nomination	August 2017
Millennium Scholars Program	August 2014 – May 2018
Provost's Scholarship Award	August 2014 – May 2018

SKILLS

Computing:	MATLAB & Simulink, COMSOL, Mimics, Solidworks
Language:	Spanish (professional fluency)

LEADERSHIP EXPERIENCE

Global Engineering Fellow	September 2016 - Present
MSP Student Mentor, Millennium Scholars Program	August 2016 - Present
Secretary/Choreographer, Penn State International Dance Ensemble	August 2016 - Present
Assistant THON Chair, Penn State International Dance Ensemble	Sept.– Dec. 2015
Leader, Chi Alpha Campus Ministry	January 2015 – May 2017