In-Vitro Glioblastoma Treatment Focusing on Convection Enhanced Delivery

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ABSTRACT

Glioblastoma is a deadly brain cancer with discouraging standard of care. New methods like convection enhanced delivery and chimeric antigen receptor T cells (CAR-T) are promising treatments that can be translated to glioblastoma. In this study, CAR-T cell flow through a hydrogel was explored in the context of in-vitro convection enhanced delivery. A culture method to create large spheroids mimicking tumors from preexisting glioblastoma stem cell lines was fabricated, a convection enhanced delivery system for in-vitro testing was designed, and characterization of the CAR-T cells using the in-vitro system took place. The spheroid culture method was successfully optimized to produce spheroids large enough to act as a sufficient tumor in little time, the in-vitro set-up successfully administered treatment, and CAR-T cells were found to increase their velocities through a medium as their injection velocity increased. It was discovered that the density of the spheroid plays a crucial role in treatment delivery, often times driving how treatment will move through the spheroid. This system can be used in the future studies to test the killing potential of CAR-T cells to a tumor in-vitro.
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GENERAL AUDIENCE ABSTRACT

Glioblastoma is a deadly brain cancer with current treatments that are discouraging at best. New methods must be utilized to aid in patient recovery. Chimeric antigen receptor T-Cells (CAR-T) are a promising treatment that can be used in glioblastoma. In this study, CAR-T cell behavior is defined in the context of in-vitro convection enhanced delivery. A large spheroid, or sphere of cells, mimicking a tumor was created, a convection enhanced delivery system set-up for in-vitro testing was designed, and characterization of CAR-T cell behavior using the in-vitro system took place. The spheroids were successfully cultured to act as a sufficient tumor, the in-vitro set-up successfully administered treatment, and CAR-T cells were found to increase their velocities in a gel as their injection velocity increases. It was discovered that the density of the spheroid plays a crucial role in treatment delivery, often times driving how treatment will move through the spheroid. This system can be used in the future studies to test the killing potential of CAR-T cells to a tumor in-vitro.
Dedicated to Virginia Tech.
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This thesis is dedicated to my mother, Nancy Cook Brocke, who never saw this adventure. I hope that some of these findings can be used to help make a difference in the fight against cancer.
# Contents

List of Figures viii

List of Tables ix

List of Abbreviations x

1 Introduction 1

1.1 Glioblastoma Biology ............................................ 1
1.2 Glioblastoma Epidemiology ..................................... 2
1.3 Standard of Care .................................................. 3
1.4 CAR-T Cell Therapy ............................................ 4
1.5 Convection Enhanced Delivery ................................. 6
1.6 The Tumor Microenvironment ................................. 7

2 Methods 11

2.1 Standard Cell Culture ........................................... 11
2.2 3D Hydrogel System ............................................. 12
2.3 Large Spheroid Cell Culture ................................... 13
2.4 3D Spheroid Hydrogel System ............................... 15
List of Figures

1.1 Convection Enhanced Delivery .............................................. 6
1.2 The Tumor Microenvironment ............................................. 9

2.1 3D Hydrogel System Varying the TME and AMD3100 .................. 13
2.2 Large Spheroid Growth .................................................... 14
2.3 Spheroid in a Gel ........................................................... 16
2.4 In-Vitro Convection Enhanced Delivery System ....................... 17
2.5 Microbeads Moving Through a Spheroid .................................. 18
2.6 Average Velocities of Microbeads Moving Through a Spheroid ........ 19

3.1 Invasion of GSCs Varying the TME and AMD3100 .................... 23
3.2 Characteristics of Glial Stem Cell Spheroids with the Large Spheroid Culture Method .................................................. 25
3.3 Velocities of Microbeads Flowing Through a Spheroid and Their Variation .................................................. 29
3.4 Anomaly of Microbeads Flowing Through a Spheroid .................. 30
3.5 In-Gel Velocity Trends for CAR-T Cells in a Blank Gel Across Injection Velocities ................................................. 33
3.6 Flow Tracks of CAR-T Cell Average Velocity ........................... 34
List of Tables

3.1 Descriptive Statistics of G2 Cells Varying the TME and AMD3100 . . . . . . 23
3.2 Descriptive Statistics of G34 Cells Varying the TME and AMD3100 . . . . . . 24
3.3 Descriptive Statistics of the Average Velocity of CAR-T Cells . . . . . . . . . . 33
3.4 Descriptive Statistics of the Average Velocity of CAR-T Cells . . . . . . . . . . 35
List of Abbreviations

BBB  Blood Brain Barrier

CAR-T  Chimeric Antigen Receptor T-Cell

CED  Convection Enhanced Delivery

GSC  Glioblastoma Stem Cell

Gy  Gray, unit of absorption of radiation

IFF  Interstitial Fluid Flow

TME  Tumor Microenvironment
Chapter 1

Introduction

1.1 Glioblastoma Biology

Gliomas are brain tissue cancers. They emerge when a glial cell or ectodermal glial progenitor cell mutates. Gliomas differ from other central nervous system tumors because of their fast rate of mitosis, abnormal blood vessels, necrosis, and hypercellularity. Gliomas are divided into four grades, similar to stages in cancer. Grade I and grade II gliomas are slower-growing and easier to treat. Grade III gliomas are harder to treat and, therefore, more deadly. Grade IV gliomas are the hardest to treat and comprise 57.7% of all gliomas.[1] These gliomas are known as glioblastoma.[2]

Glioblastoma is further divided into two categories: primary glioblastoma and secondary glioblastoma. Primary glioblastomas appear without indication of evolution from a lower grade glioma. Secondary glioblastomas develop from grade II diffuse astrocytomas, gliomas that stem from astrocytes with ill-defined boundaries, or grade III gliomas.[3]

In primary glioblastoma, EGFR overexpression, PTN mutation, and loss of chromosome 10 can cause glioblastoma. In secondary glioblastoma, 19q loss, TP53 mutations, and IDH1 mutations lead to glioblastoma. Mutation in the IDH1 gene tends to be comparatively less severe since it is correlated with increased overall survival.[3]

Gliomas can occur in various parts of the brain and sometimes peripheral areas of the
CNS like the spinal cord. Sixty-one point four percent of gliomas develop in the occipital, parietal, frontal or temporal lobes of the brain. A small percentage of gliomas form outside of the brain.[1]

### 1.2 Glioblastoma Epidemiology

Glioblastoma is the most common brain cancer, accounting for 52% of brain cancers in the United States. The median survival rate is only 12 to 15 months, supporting the claim that it is the deadliest brain cancer.[4] Under 5% of patients reach 5 years after diagnosis. Every year in the United States, 14 in every 100,000 people develop Glioblastoma.[5] Even though it is the most common and most deadly brain cancer, the cancer is still rare. Only 0.0319% of people in the United States will obtain this cancer. Glioblastoma is 1.6 times more likely to occur in males as a whole. Primary glioblastomas incidence rates are higher in men by 303.03%. Interestingly, secondary glioblastoma incidence rates are higher in women by 152.85%. When it comes to ethnicity, glioblastoma is 1.99 times more likely to affect Caucasians than African Americans and 2.97 times more likely to affect Caucasians than Asian or Pacific Islanders. As one ages, the risk for glioblastoma increases. The median diagnosis age for glioblastoma between 2013 and 2017 was 65 years old. With that being said, the age group with the highest risk of glioblastoma is 75 and older.[1]

Other risk factors besides age include Li-Fraumeni syndrome, a congenital cancer syndrome that causes mutation in the TP53 tumor suppressor gene and Turcot syndrome, a mutation in the APC DNA repair gene. Mutations in the NF1 and NF2 genes from neurofibromatosis types I and II are also risk factors.6 Radiation is the only other known factor to put someone more at risk of glioblastoma.[6]

Symptoms of glioblastoma include fatigue, vomiting, dizziness, nausea, and neurocogni-
tive slowing.[7] One fifth of patients originally present with sensorimotor function symptoms and less than one third of patients initially present with headaches. Symptoms evolve over the course of the disease. With this being said, there is no clear progression of symptoms in glioblastoma. Symptoms vary with the location of the tumor, the amount of swelling present, and the degree of brain tissue destruction. Although glioblastoma affects few people, it is still necessary to study due to its deadliness and secrets it can unlock for cancer research.

1.3 Standard of Care

Standard of care of glioblastoma is maximal safe surgical resection of the tumor bulk, 60 Gray of radiation, and concurrent chemotherapy. After this, additional chemotherapy is applied to stop metastasis. Typically, temozolomide is used for chemotherapy because it has been proven effective and is a small enough molecule to pass through the blood brain barrier (BBB), a barrier in the brain that blocks large molecules from entering the parenchyma, and attack the tumor. Carmustine is sometimes used instead of temozolomide for localized therapy. Temozolomide aids radiation through temozolomide-mediated radiation enhancement to create a pro-apoptotic environment by increasing the amount of dsDNA breaks. The 2-year survival rate of temozolomide and radiation is 26.5% compared to just 10.4% with radiation alone.[8] The amount of radiation used can vary by age group. For the elderly, 50 Gy in 28 doses is typically used to optimize survival time, quality of life, and cognitive function. For all age groups, the maximum tolerated dose of radiation is 75 Gy.[9]

Unfortunately, the standard of care is poor. Resistance to radiation and chemotherapy can occur over the course of the treatment. Glioblastoma resistance is highly intrinsic and adaptive. Resistance happens because of intratumoral heterogeneity, molecular plasticity, the robust DNA-repair mechanisms of glioblastoma stem cells, and the ability of the tu-
mor cells to differentiate into vascular and stromal structures that help tumor growth.[9] Intratumoral heterogeneity is characterized by the vast array of distinct genotypes in the tumor. Since there are many different genotypes, there is a higher probability of intrinsically resistant cells to survive, grow, and metastasize to create a resistant tumor.[10] Molecular plasticity is the ability of the cancer cells to express multiple phenotypes for a single genotype given different environments. This gives glioblastoma an inherent advantage to survive in multiple environments. The robust DNA-repair mechanisms of glioblastoma stem cells compete with radiation and temozolomide, which specifically target the DNA. The cell mechanisms fight the DNA cleavage that happens with radiation and the DNA methylation in temozolomide.[11]

Standard of Care needs to be improved because it does not attack the high invasiveness of glioblastoma. The high invasiveness of glioblastoma leads to a recurrence rate of almost 100%. Tumor recurrence specifically occurs because invaded cancer cells tend to collect around blood vessels, below pial margins, and through white matter tracts, all places that are hard for a surgeon to operate on.[12] This is unfortunate because the amount of tumor left after resection is directly proportional to patient survival time and recurrence.[8] Leftover “hidden” cells, invade further into the brain parenchyma, and lead to metastasis to other parts of the brain. Fortunately, the metastasis of glioblastoma outside of the brain is low.[13]

### 1.4 CAR-T Cell Therapy

Chimeric antigen receptor T-cells (CAR-T) are an alternative to the standard of care. CAR-T cells are redesigned T-cells. First, T-cells are taken from a patient. Then, a gene is inserted into the T-cell via mRNA, viral vector transduction, or plasma transfection to create a receptor that specifically targets the cancer cell, creating a CAR-T cell. The CAR-T
cell is left to grow to create millions of CAR-T cells. They are then inserted into the patient so that the CAR-T cell can directly attack and kill the cancer cell. After attachment, they kill the cell through the release of cytokines and cytolytic degranulation.[14]

CAR-T cells have been successful in non-solid tumors like leukemia and lymphoma so far. The United States Food and Drug Administration have approved two CAR-T cell therapies. The first therapy is Tisagenlecleucel, which are CAR-T cells that attack CD19. This was approved for patients 25 years or older in relapse with B-cell precursor acute lymphoblastic leukemia. Axicabtagene ciloleucel is the second CAR-T cell therapy, which also targets CD19. It was approved for patents with large B-cell lymphoma that have had no success with at least 2 prior therapies. Given the success of CAR-T cells, researchers have shifted focus to see if CAR-T cells can treat solid tumors [14]. One solid tumor of interest is glioblastoma.[15]

CAR-T cells are promising for glioblastoma. For one, CAR-T cells can penetrate the central nervous system. They can readily pass through the BBB to infiltrate the brain if they utilize peripheral diffusion. This allows for promising drug delivery to the tumor. Second, CAR-T cells remove the need for antigen presentation. Since CAR-T cells are manufactured to seek out a specific antigen and bind to it, the number of neoantigens that the cancer cell produces to fight the cancer become less important. Lastly, CAR-T cells are promising for glioblastoma because they bypass glioblastoma stem cell (GSC) defenses. GSCs, part of the glioma tumor, tend to be very resistant to drugs and radiation. Fortunately, CAR-T cells have been known to kill GSCs in-vitro. This is promising for scale-up to in-vivo and eventually patient trials.[15]
1.5 Convection Enhanced Delivery

Convection enhanced delivery (CED) is a way to deliver treatments to the brain.[16] It utilizes a catheter and an infusion pump. The treatment is flowed through the catheter via a pressure gradient directly to the tumor, bypassing the BBB. A schematic of this is shown in Figure 1.1. CED is also advantageous because it utilizes bulk flow and imparts a pressure gradient, unlike more traditional diffusive flow. In CED, the velocity of the treatment molecule is dependent on the hydraulic conductivity and the pressure gradient, following Darcy’s Law. Therefore, one does not need a large concentration of treatment molecules for a large flow, like in diffusive flow. CED utilizes less treatment molecules, which is cost effective. Not to mention, in CED the size of the treatment molecule does not matter, allowing this system to be more universal. With diffusive flow, the size of the molecule is directly proportional to the delivery of the treatment.[17]

Figure 1.1: Convection Enhanced Delivery. The pump (green) is connected to the tube (blue), which connects to the catheter (black) that is inserted directly into the tumor (pink). Physicians use this method to inject a glioma with medication while bypassing the BBB.[17]

Although there are many benefits with CED, there are also pitfalls. Backflow and air
bubbles are substantial problems. Backflow is when fluid from the catheter exits the tip and flows back around the edge of the catheter. This happens when a space between the needle and the surrounding tissue forms from the inherent pressure created by the apparatus pushes against the tissue, tissue disruption when the catheter is inserted into the tissue, or from bumping the inserted needle. It can cause problems with consistent flow, cause leakage into unwanted areas, and cause a decrease in the amount of treatment reaching the target area.[18] To overcome this problem, soft, thin, porous-membrane, and/or valve-tip catheters are used. The soft and thin catheters decrease tissue disruption, while the porous-membrane and valve-tip catheters reduce the blockages at the bottom of the needle, and, thus, resultant backflow. Air bubbles tend to happen if the catheter is not primed. Air bubbles reduce the flow of the treatment. This can lead to erratic flow patterns and/or cause backflow. In surgery, it fortunately causes no health hazards. In a laboratory setting, air bubbles can alter the measured pressure at the end of the needle, the injection volume, and disrupt local tissue, changing the treatment delivery flow pattern.[18] For CED as a whole, edema, infections, seizures, hemorrhage, and neurological deterioration can be possible side effects of CED.

1.6 The Tumor Microenvironment

The brain consists of an array of structures and cells that work together harmoniously to function. Blood vessels carry nutrients to the cells and the extracellular matrix acts as the lattice the cells call home. The extracellular matrix consists of hyaluronan, proteins, and proteoglycans.[13] Not only is the extracellular matrix structural, but it also influences the responses of surrounding cells. Hyaluronan is a sugar chain made up of the disaccharide beta-4-glucuronic acid and the disaccharide beta-3-N-acetylglucosamine. Unlike other gly-
cosaminoglycans, HA is not bound to a protein.\[19\] On a more functional note, hyaluronan is the main component of some in-vitro models that represent the extracellular matrix.\[20\]

Neurons are abundant in the brain. They send electrical impulses for brain function. Glial cells are support cells. They include ependymal cells, oligodendrocytes, microglia, and astrocytes. Ependymal cells act as barriers between compartments and help create neural stem cells. Oligodendrocytes form the myelin sheaths that coat neuronal axons to protect them and speed up electrical activity. Microglia act as scavengers; they are like the immune cells of the brain. Lastly, astrocytes form tight junctions around the blood vessels to form the BBB so no foreign objects can enter the brain. Astrocytes also provide substrates for adenosine triphosphate production.\[21\]

When a tumor forms, the harmony of the microenvironment can change.\[21\] The tumor microenvironment (TME) is the localized environment around a tumor. It consists of the environment described above plus tumor cells. These tumor cells consist of glioblastoma stem cells that invade into the surrounding parenchyma. These stem cells can pull together to create a spheroid, or a ball of GSCs. In in-vitro cell culture, spheroids regularly form. An illustration of the tumor microenvironment is shown in Figure 1.2.

The TME can affect the invasion of these cells. There are two main pathways that are affected: the CXCR4/CXCL12 pathway and the CD44 receptor mediated hyaluronic acid mechanotransduction (HA-mediated mechanotransduction) pathway. The CXCR4/CXCL12 pathway is made of the CXCL12 protein ligand and the CXCR4 receptor on cells. CXCL12 is secreted by cells in the tumor microenvironment like astrocytes and microglia, and CXCR4 is found on many glioblastoma stem cells.\[20\] CXCL12 attaches to CXCR4 and causes a cascade of downstream effects that change migration and chemotaxis, which ultimately increases cancer cell invasion. AMD3100 is a treatment that is traditionally used to block CXCR4.\[22\] HA-mediated mechanotransduction happens when a CD44 receptor on a glioblastoma stem
1.6. The Tumor Microenvironment

cell attaches to the hyaluronan in the extracellular matrix. The cell then “walks” along the hyaluronan polymer to invade. This pathway is important in interstitial fluid flow stimulated invasion.[20]

Interstitial fluid flow (IFF) is a phenomenon produced by a tumor. A pressure gradient forms from the high interstitial pressure tumor bulk and the normal interstitial surrounding environment. This pressure gradient drives fluid flow from the tumor to the surrounding tissue.[23] IFF can increase superficial velocity by a factor of 10 to 100, altering invasion.[20]

My research focuses on designing an in-vitro CED system that injects treatments into an environment that can be altered to mimic the TME. There are three purposes for this experiment. The first is to create a platform that others can easily use to add CED to their experiments to explore its useful effects. The second is to characterize the behavior of fluid flow through a tumor in the TME to understand in-vitro, in-tumor fluid flow dynamics for this system. The third is to characterize the flow of CAR-T cells to see how they behave.
in a CED system alone to gain useful insight into CAR-T cell invasion dynamics for future studies. To do this, I performed a gel experiment with a treatment called AMD3100 to see if I could create the standard tumor microenvironment. Next, I created a cell culture method to grow large spheroids to represent the tumor of the TME. I designed the culture method to take little time, little user effort, and use minimal material while maintaining a large spheroid size. I created a methodology to input the spheroid in the gel. Then, I designed an in-vitro CED system and characterized its flow with microbeads. Lastly, I used the system to understand how fluid flow from CED acted in a tumor and how CAR-T cells migrated after CED injection.
Chapter 2

Methods

2.1 Standard Cell Culture

Patient-derived human glioblastoma stem cells generously gifted to the lab from Jakub Golewski and Ichiro Nakano. Lines G2 and G34 were cultured. Both cell lines were maintained in Neurobasal medium (ThermoFisher Scientific) enriched with 0.01% fibroblast growth factor (FGF), 0.1% endothelial growth factor (EGF), 0.3% l-Glutamine, 0.5% N-2 Supplement, and 1% B27 Supplement without vitamin A. All GSCs were grown in non-treated optically clear USP VI crystal class virgin polystyrene cell culture flasks from Genesee Scientific. Human SV40-immortalized microglia were obtained through Applied Biological Materials, Inc. They were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; ThermoFisher Scientific) enriched with 10% fetal bovine serum (FBS). Human primary cortical astrocytes were bought from Sciencell and cultured following the company’s protocol. Both the astrocytes and microglia were cultured tissue culture-treated optically clear USP VI crystal class virgin polystyrene flasks from Genesee Scientific. All cell lines were maintained in a humidified incubator containing 21% oxygen and 5% carbon dioxide at 37°C and tested annually for mycoplasma (negative).[24]
2.2 3D Hydrogel System

The 3D Hydrogel system from Kingsmore et al. was utilized to explore how cancer cells act in a cellular environment mimicking the brain, with interstitial fluid flow, or when a treatment is applied. It is a base model for the exploration of glioblastoma treatment. This system was specifically utilized to explore how GSC’s act in the presence of the TME and AMD3100 in static conditions. This experiment was conducted with 8 µm membrane pore sized tissue culture inserts in a 96 well plate (Sigma Aldrich). One well was used to create one gel. All cells were fluorescently labeled with Cell Tracker dyes (Life Technologies) prior to the experiment following Life Technology protocol for color differentiation. If not, cells were dyed after the experiment with Vybrant dyes (Life Technologies) following Life Technology protocol. Astrocytes, microglia, and GSCs were seeded at 8.0x10^4, 8.0x10^4, and 5.0x10^5 cells respectively according to cell ratios obtained from human sections into a 75 µL gel to mimic the brain. The astrocytes and microglia represented the cells in the TME. The gel consisted of 0.2% hyaluronan (R&D Systems) and 0.12% rat tail collagen I (Corning). Five mg/kg of AMD3100 was added into the gels. After construction, gels were set in a humidified incubator containing 21% oxygen and 5% carbon dioxide at 37°C for 30 minutes to begin to solidify. They were rehydrated with 75 µL of Astrocyte Basal (Sciencell) enriched with 0.5% N2 and 1% B27 and replaced in the incubator for 3 hours to allow for all components to acclimate to the gel matrix. One hundred and twenty-five microliters of the same enriched medium was added to the bottom of the tissue culture insert at varying levels depending on the experiment. The top of the insert was given twenty-five microliters of media enriched with 0.5% N2, 1% B27, and 5 mg/kg of AMD3100. An illustration of this set-up is show in Figure 2.1. The cells were set to invade for 17 hours before invasion was quantified.
A new culture method was created for spheroids to maximize their size and minimize culture time so they could efficiently be inserted into a gel in the 3D hydrogel system. Both G2 and G34 were grown for 1 week to create spheroids. The GSC’s were seeded 68,000 cells per well in a non-treated polystyrene flat bottom 96 well plate (Zoro) with 200 µL of Neurobasal medium (ThermoFisher Scientific) enriched with 0.3% l-Glutamine, 0.5% N2 Supplement, 1% B27 Supplement without vitamin A, 0.2 µL of EGF, and 0.2 µL of FGF. After 48 hours, 0.15 µL of EGF and 0.15 µL of EGF were added per well in a solution of 50 µL of neurobasal and enrichment solution (GSC Media). After another 48 hours, 0.1 µL of EGF and 0.1 µL of FGF were added to each well in a solution of 50 µL of GSC media. The plate was subsequently placed on a VWR® microplate shaker at 500 rpm for 48 hours. After this time, undifferentiated spheroids were formed in the center of the well. This process is shown in Figure 4. From there, spheroids were placed in an upright oriented
T-25 non-treated optically clear USP VI crystal class virgin polystyrene cell culture flask from Genesee Scientific until they were needed for experimentation. The spheroids were maintained in 4 mL of neurobasal medium (ThermoFisher Scientific) enriched with 0.01% FGF, 0.1% EGF, 0.3% l-Glutamine, 0.5% N2 Supplement, and 1% B27 Supplement without vitamin A. Images from the culture method are shown in Figure 2.2.

Figure 2.2: Large Spheroid Growth. The spheroids were grown with the described culture method. On day 1, day 3, day 5, and day 7, photos were taken of them to mark their progress. On day 1, day 3, and day 5, an EVOS FL2000 was used. On day 7, the ZEISS Axio Zoom V16 for Biology was used. The top row shows the growth of G2 spheroids. The bottom row shows the growth of G34 spheroids.

To measure the accuracy of the culturing method between cell lines, the spheroids’ lengths and cell number were measured. Growth of the G2 cell line and G34 cell line were compared with three technical replicates and five biological replicates each. The number of cells were counted using Trypan Blue Staining (ThermoFisher Scientific) and a .0025 mm² hemocytometer (Muhwa Scientific). The lengths were measured using a scale bar in a VWR® Trinocular Inverted Microscope. The spheroids grown with this culture method were used to represent a tumor in the gel. They were later placed in a gel in the spheroid hydrogel model and flow application.
2.4 3D Spheroid Hydrogel System

The 3D spheroid hydrogel system was created by varying the 3D hydrogel system. This was done to see if a spheroid could be placed in a gel to be treated by CED. The purpose of the spheroid was to represent a tumor to model surgical CED. To do this, an 8 µm membrane pore sized tissue culture inserts in a 96 well plate (Sigma Aldrich) were used. One well was used to create one gel. Astrocytes and microglia were fluorescently labeled with Cell Tracker Green and Cell Tracker Red (Life Technologies) respectively prior to insertion into the model for identification. Each gel was created with 0.2% hyaluronan and 0.12% rat tail collagen I (Corning). Each transwell received a total of 75 µL of this gel solution. Instead of inserting $5 \times 10^5$ GSCs, a spheroid was inserted into the gel. The insertion process utilized sterile P100 pipette tips (Genesee Scientific) with the edge of the tip cut off to allow for the suction of the spheroid from a T-25 culture flask. To insert the spheroid correctly, 37.5 µL of the required gel solution was aliquoted into the transwell and placed into a humidified incubator containing 21% oxygen and 5% carbon dioxide at 37°C for 10 minutes to begin to slightly solidify. The spheroid was then transferred directly into the gel from a T-25 Genesee Scientific culture flask. The culture media was directly removed from the transwell via a P200 so that only the spheroid remained on the gel. Another 37.5 µL of the gel solution was added directly on top of the spheroid. After construction, the gels were set in a humidified incubator containing 21% oxygen and 5% carbon dioxide at 37°C for 30 minutes to begin to solidify. Each gel was rehydrated with 75 µL of Astrocyte Basal (Sciencell) enriched with 0.5% N2 and 1% B2. The gels were returned to the incubator for 3 hours to allow for all components to acclimate to the gel matrix. Subsequently, 35 µL of media was added to the top of the tissue culture insert and 125 µL of media was added to the bottom of the tissue culture insert to prevent drying out. The gel was then rehydrated with 75 µL of Astrocyte Basal (Sciencell) enriched with 0.5% N2 and 1% B27 and placed directly into the humidified incubator for 17 hours to
invade. Once invasion was complete, all cells were fixed with 4% paraformaldehyde in 1:10 phosphate buffer saline to deionized water. All cells were co-stained using 4’,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; ThermoFisher Scientific). Lastly, the spheroids in the gels were imaged using the ZEISS Axio Observer for Life Sciences microscope. Figure 2.3 shows a G34 spheroid imaged in 3D and a G2 spheroid imaged in 2D and 3D.

Figure 2.3: Spheroid in a Gel. A) This is a 3D z-stack of G34 spheroid that was placed in a gel and let invade 17 hours. This picture represents a relatively dense spheroid. It accurately shows the topography of a spheroid. B – C) These pictures represent a 2D (B) and 3D (C) view of G34 spheroid. With the 3D picture, one can see a low density area of GSC’s in the frontal plane. These panel illustrate the variability of tumor shape and density.

### 2.5 Flow Application and Measurement

Bulk flow was applied to the gels to see flow patterns through the spheroids from Convection Enhanced Delivery. Protocol for the 3D hydrogel with a spheroid was used. After the gel was made, it was transferred into a non-treated 96 well plate (Zoro) where it was fixed with 4% paraformaldehyde in 1:10 phosphate buffer saline to deionized water and stained with DAPI. After it was stained, the gel was transferred into a sterile 8 chamber
polystyrene vessel tissue culture treated glass slide (Corning). The chamber slide was set on the ZEISS Axio Observer for Life Sciences. The injection set-up is shown in Figure 2.4. It consists of a 3D printed tube stand, the adjustment piece of the model 900 small animal stereotaxic instrument (Kopf), a syringe pump, and a 10 µL Hamilton syringe with a 1.0” 26G standard 12” beveled needle point.

Figure 2.4: In-Vitro Convection Enhanced Delivery System. This is a depiction of the in-vitro injection system. The catheter is inserted into a spheroid in a gel in the chamber slide. The catheter is attached to a pump that is attached to a stereotaxis part, which is attached to a 3D printed custom tube stand.

One micrometer yellow-green, fluorescent carboxylate-modified microspheres were injected into the spheroid at 1.0 µL/min to mirror the lab’s in-vivo CED protocol for 1.7905 minutes at a concentration of $1.82 \times 10^7$ microbeads/mL to characterize the system. This concentration allowed for a volume equal to that the average spheroid to be injected so the system did not become inundated. Microbeads flowing through the spheroid is shown in Figure 2.5. The velocity injected varied from 0.0 µm/s to 4.5 µm/s. The microbeads moving through the gel look to be flowing at a consistent speed as shown in Figure 2.6A. Higher flow rates can be seen around the breakage of the spheroid. This is because there is less mass for
the microbeads to travel through. This is shown in Figure 2.6B.

Figure 2.5: Microbeads Moving Through a Spheroid. This is a zoomed in picture of microbeads moving through a spheroid. Here, one can see that the microbeads prefer to move through less dense regions of the spheroid, denoted by the darker blue regions within the spheroid.

Next, the average velocity and the maximum velocity of microbeads flowing in a spheroid at four different injection velocities were compared. The injection speeds were 0.33 µL/min, 0.5 µL/min, 1.0 µL/min, and 2.0 µL/min. These speeds were chosen to vary around the lab-established in-vivo CED injection speed of 1.0 µL/min. Each injection speed had one biological replicate to eliminate error that would arise through spheroid variability. There were 3 trials. One million microbeads were injected into each spheroid for 5 minutes. The average and maximum velocity of each microbead was recorded every millisecond. The velocities of each frame were averaged over the path of a single microbead to get a representative “track” average velocity and “track” maximum velocity. The track average velocities between each injection speed were compared and the track maximum velocities between each injection speed were compared. Microbeads were also injected into a blank gel as a control.
2.6 Photo Hyaluronic Acid Gels for CAR-T Cells

Photo cross-linking hyaluronic acid (PhotoHA) is a substitution that can be used for the hyaluronic acid-collagen gels in the 3D hydrogel system. The gel is composed of 0.4% PhotoHA and 0.12% collagen. The gel solution was created in a non-treated polystyrene flat bottom 96 well plate (Zoro). There was 75 µL of gel per well. After construction, each gel was exposed to 50 mW/cm² of UV light for 30 seconds. Directly after, the entire place was set in a humidified incubator containing 21% oxygen and 5% carbon dioxide at 37°C for 30 minutes to finish solidification.
2.7 CAR-T Cell Flow

The flow of CAR-T cells in a blank gel was measured to see how CAR-T cells acted during injection in a gel by themselves. The injection speed and volume injected varied. There were three biological replicates per each condition. The injection velocities that were measured were 0.33 µL/min, 0.5 µL/min, 1.0 µL/min, and 2.0 µL/min because they are varied around the lab-established in-vivo CED injection volume of 1.0 µL/min. The volumes injected were 0.67 µL, 1.67 µL, and 3.33 µL. These were chosen because they seemed sufficiently different to note any possible correlation between injection velocity and in-gel CAR-T cell movement.

All CAR-T cells were fluorescently stained with Cell Tracker Orange (Life Technologies) following Life Technologies protocol. Gels remained in a non-treated polystyrene flat bottom 96 well plate (Zoro). One million CAR-T cells were injected directly into each gel using the CED apparatus. During injection, cells were imaged using a ZEISS Axio Observer for Life Sciences microscope. Photos were taken every millisecond. CAR-T cell velocities were subsequently analyzed using computer software.

2.8 Cell Tracking of CAR-T cells

To track the microbeads and the CAR-T cells, TrackMate, a function of the image processing software called FIJI, was used[25]. This program outputted the flow lines, velocity of each microbead per frame, average velocity of a microbead over a flow line, and displacement of each microbead. When using the program, Laplacian of Gaussian (LoG) segmentation Tracking Settings were chosen. When implementing LoG, a 15 µm bead diameter was selected because it most accurately captured the diameter of the beads with their
given fluorescence. Subsequently, a 2 µm threshold was chosen because it accurately filtered out background noise in this setting. LoG was implemented so maxima too close to each other would be suppressed, meaning there is less of a chance to double count a microbead as it moves. The segment in channel 2 with the medium filter and the subpixel localization was checked. Channel 2 was used so the program would select the microbeads in the front z-plane that were clearly seen. Subpixel localization was chosen to acquire a more accurate tracking than pixel localization can achieve. The median filter was chosen because it helped eliminate background noise from microbeads in much higher z planes than the one in view. Next, the initial threshold scale was toggled until no background microbeads were selected. Then, the standard hyperscape displayer was used to display the microbeads. This was selected because it allowed for manual manipulation of what the program considers a microbead. So, if the program mislabeled a frame for a microbead, it could have been manually fixed. No manual editing was necessary. No excess filters were added because they were not necessary for this application. In the next stage of TrackMate, The Simple Linear Assignment Problem (LAP) tracking system was used because it did not allow for branching tracks. In this application, there was no branching tracks because there was no cell division taking place. The default settings of 15.0 µm linking distance, 15.0 µm gap-closing max distance, and a gap-closing gap frame maximum of 2 were chosen. They seemed to give accurate results upon inspection and no change in default settings was necessary. The track settings were set to show a scale of mean velocities. Higher velocities were closer to red while lower velocities were closer to violet. Lastly, the “Analysis” function was used to obtain mean and maximum velocity data for each microbead.
Chapter 3

Results

3.1 3D Hydrogel System Varying the TME and AMD3100

When looking at the G2 cell line, multiple trends appeared. First, invasion in the presence/absence of the TME was within 0.11% (AMD3100-: (P=.980; P=.981), as shown in Figure 3.1A. The AMD3100- condition without the TME (Mean=0.046) had 8.7% less invasion (P=.89) than the AMD3100- condition with the TME (Mean=0.042). The AMD3100+ condition without the TME (Mean=0.018) had 18.18 % less invasion (P=.78) than the AMD3100+ condition with the TME (Mean=0.022). The variation of the data in all G2 conditions was large with the AMD3100-/TME+ condition having the largest (SD=0.023).

In this study, AMD3100 decreased invasion about the same amount regardless of TME presence. This is summarized in Table 3.1. After implementation of a two-tailed unpaired t-test with an alpha value of 0.05, no significant difference of invasion between AMD3100 presence without TME found in G2’s (P=0.13). Furthermore in G2’s, no significant difference of invasion between AMD3100 presence with TME was found (P=0.43).

When looking at trials with G34 cells, few trends appeared. As shown in Figure 3.1B, the TME increased invasion by 20.0% for the AMD3100- condition (P=.64) and increased invasion by 28.3% for the AMD3100+ condition (P=.73). The descriptive statistics for these trends are shown in Table 3.2. A two-tailed unpaired t-test with an alpha value of 0.05
was performed for this dataset. For G34 cells, there was no significant difference of invasion between AMD3100 presence without TME (P=0.88) and no significant difference of invasion between AMD3100 presence with TME (P=0.97).

Figure 3.1: Invasion of GSCs Varying AMD3100 and TME. A) This is the percent migration that G2 cells experienced varying AMD3100 and TME presence. There is no significant difference in each metric between trials B) This is the percent migration G34 cells experienced varying AMD3100 and TME presence. There is no significant difference in each metric between trials C) This shows the G2 and G34 data together. There is no significant difference of invasion between cell type.

<table>
<thead>
<tr>
<th>Treatment Presence</th>
<th>AMD3100 -</th>
<th>AMD3100 -</th>
<th>AMD3100 +</th>
<th>AMD3100 +</th>
</tr>
</thead>
<tbody>
<tr>
<td>TME Presence</td>
<td>TME -</td>
<td>TME +</td>
<td>TME -</td>
<td>TME +</td>
</tr>
<tr>
<td>Mean (% Migration)</td>
<td>0.046</td>
<td>0.042</td>
<td>0.018</td>
<td>0.022</td>
</tr>
<tr>
<td>Standard Deviation (% Migration)</td>
<td>0.026</td>
<td>0.045</td>
<td>0.018</td>
<td>0.013</td>
</tr>
</tbody>
</table>

When looking at G2 and G34 cells together, overall invasion was relatively consistent and invasion tended to increase across the TME despite AMD3100 presence, except for in
G2 AMD3100- condition, which from the G2 analysis was not significant. These trends are depicted in Figure 3.1C. After a 3-way ANOVA with an alpha value of 0.05 was utilized, it was discerned that there was not significant difference in invasion between G2 and G34 cells (P=0.46)

Table 3.2: Descriptive Statistics of G34 Cells Varying the TME and AMD3100

<table>
<thead>
<tr>
<th>Treatment Presence</th>
<th>AMD3100 -</th>
<th>AMD3100 -</th>
<th>AMD3100 +</th>
<th>AMD3100 +</th>
</tr>
</thead>
<tbody>
<tr>
<td>TME Presence</td>
<td>TME -</td>
<td>TME +</td>
<td>TME -</td>
<td>TME +</td>
</tr>
<tr>
<td>Mean (% Migration)</td>
<td>0.022</td>
<td>0.028</td>
<td>0.020</td>
<td>0.029</td>
</tr>
<tr>
<td>Standard Deviation (% Migration)</td>
<td>0.014</td>
<td>0.018</td>
<td>0.019</td>
<td>0.040</td>
</tr>
</tbody>
</table>

3.2 Large Spheroid Cell Culture

The number of cells in a spheroid were obtained to see if the culture method was consistent. G2 and G34 cells were compared to see if they acted significantly different in this culture method. Figure 3.2A shows the cell numbers of G34 and G2 spheroids grown with this culture method. The median number of G2 cells was 167,500. The mean closely resembled the median with 163,214 cells. There was a standard deviation of 52,462.7 cells in the G34 data. One hundred and ninety thousand cells was the most common number reported, appearing twice. There was a large range of 175,000 cells throughout the data set. The median amount of G34 cells was 165,000 cells. The mean was 160,667 cells, which was close to the median. The G34 data set had a standard deviation of 50,316 cells, which was relatively similar to the G2 standard deviation. The mode was 170,000 with the datapoint appearing twice in the dataset. The range of the data was 180,000, which is large, but similar to the range of the range in the G2 dataset.

The lengths of the spheroids were also compared and displayed in Figure 3.2B. Length was defined as the longest distance from one side of the spheroid to the other following a
Figure 3.2: Characteristics of Glial Stem Cell Spheroids with the Large Spheroid Culture Method. A) The number of cells in each G2 and G34 spheroid grown with the new culture method across all trials to compare how dynamics differ across cell lines. B) The cell lengths of both G2 and G34 spheroids grown with the new culture method across all trials to compare spheroid shape consistency. C) The number of cells in G2 spheroids across three trials of the new culture method to compare method consistency between trials. D) The length of each G2 spheroid across three trials to compare shape consistency between trials. E) The number of cells in G34 spheroids across three trials of the new culture method to compare if growth is consistent between trials. F) The length of each G34 spheroid across three trials to compare shape consistency between trials.
straight line. The mean length of G2 was 2,313.2 µm. The median was close to the mean with a length of 2,195.3 µm. The standard deviation of the dataset was 829.9 µm and the range was 2,736.79 µm. The mean length of G34 spheroids was 2,410.6 µm, which was only slightly larger than the mean length of G2 spheroids. The standard deviation was 1,022.3 µm. The median of the G34 data was 1,988.7 cells, which was within one standard deviation of the mean. This G34 median was dissimilar to the G2 median. The G34 dataset stretched 2,802.2 µm, making it only slightly larger than the G2 dataset.

Histograms, Shapiro-Wilk tests, and Cullen and Frey graphs were constructed to test normality of the datasets. Once a gamma distribution was determined, a generalized linear model was used to determine significance. The null hypothesis was that there was no significant difference between the metric in question between the groups. The alpha value was set at 0.05. Between G2 and G34 spheroids, there was no significant difference between the cell numbers (P =0.90). The lengths between the two cell lines were also not significantly different (P=0.78).

For G2, the number of cells in the spheroid between trail 1 and trail 2 were not significantly different (P=0.86). The same held true between trail 1 and trail 3 (P=0.40). The lengths between trail 1 and trail 2 were significantly different (P=0.048). Lengths between trail 1 and trail 3 held the same trend (P=0.00194). Data for G2 trials is shown in Figures 3.2C and 3.2D. Figure 3.2E and Figure 3.2F show the variability of G34 dynamics between trials. For G34, the number of cells between trail 1 and trail 2 and trail 1 and trail 3 were non-significant (P=0.347; P=0.924). The lengths between trail 1 and trail 2 followed suit (P=0.33). Interestingly, the length of spheroids between trail 1 and trail 3 were significantly different (P=1.55e-05).
3.3 Microbead Flow

A ROUT outlier test with $Q = 1\%$ was performed on both the blank average velocity and blank maximum velocity. This test was performed to account for the chance that TrackMate omits a bead in a frame and uses a farther bead as a reference, raising the velocity. This is possible if the density of the injectate is low. Even though this was accounted for when analyzing velocities with TrackMate, the implementation was not 100\% accurate. Twenty-three outliers were removed from the average velocity dataset and 12 were removed from the maximum velocity dataset. Next, a one-way ANOVA test was performed for each dataset. The null hypothesis for both is that the means between all injection velocities are equal. The alpha value is 0.05 for both. In the average velocity dataset, the ANOVA model accounted for 45.3\% of the data. The average velocity variation between each injection velocity sample relative to the variation within each injection velocity was 586 and there was a significant difference between the average velocities of microbeads between each injection speed ($P<1e^{-4}$). The .33 µL/min condition had the slowest average velocity (Mean=0.17 µm/s), the 0.5 µL/min condition had the second slowest average velocity (Mean=0.44 µm/s), 1.0 µL/min condition had the second fastest average velocity (Mean=0.59 µm/s), while the 2.0 µL/min condition had the fastest average velocity by far (Mean=2.92 µm/s). As the injection velocity increased, the average velocity steadily increased with very little variability in a linear-like fashion across the 0.33 µL/min, 0.5 µL/min, and 1.0 µL/min injection speeds. The highest standard deviation was 0.14 µm/s in the 1.0 µL/min injection speed condition, the middle was 0.319 µm/s in the 0.5µL/min injection speed, and the lowest was 0.323 µm/s in the 0.33 µL/min condition. The 2.0 µL/min injection velocity condition had the largest variability (SD=2.33 µm/s). The velocities and variability are shown in Figure 3.3A. The 1.0 µL/min condition had an anomaly. It had a spheroid that had a shape that allowed for microbeads to flow in a regular path while also catching a large amount of mi-
crobeads, as shown in Figure 3.4. The standard error of mean comment on if the samples are representative of the population. In order of increasing injection speed, the standard error of mean were: 0.015 µm/s, 0.0083 µm/s, 0.016 µm/s, and 0.22 µm/s. Within each condition, the distributions of average velocities were not necessarily symmetrical. For the 0.33 µL/min condition (Skewness=1.77) and the 2.0 µL/min condition (Skewness=1.08), the average velocities in the spheroid were highly positively skewed. The 0.5 µL/min condition was moderately positively skewed (Skewness=0.53) and the 1.0 µL/min condition was distributed fairly symmetrically (Skewness=-.03). This is shown in Figure 3.3B.

In the maximum velocity data, the one-way ANOVA test accounted 56.0% of the dataset. There was a significant difference between each injection speed (P<1e-4) and the ratio of variance between samples to inside samples was 907. A similar trend to the average velocity is shown in Figure 3.3 with the maximum velocity. The 0.5 µL/min condition had the slowest maximum velocity (Mean=0.60 µm/s), differing from the average velocity data, the 0.33 µL/min condition had the second slowest maximum velocity (Mean=0.62 µm/s), the 1.0 µL/min condition had the second fastest velocity (Mean=0.70 µm/s), while the 2.0 µL/min condition had the fastest maximum velocity (Mean=5.38 µm/s). The 0.33 µL/min (SD=0.89 µm/s), 0.5 µL/min (SD=0.35), and 1.0 µL/min (SD=0.033 µm/s) injection speeds had very little variability, while the 2.0 µL/min condition had large variability (SD=3.78 µm/s). The velocities and variability is shown in Figure 3.3C. The standard error of mean of all injections in order of increasing injection speed were: 0.089 µm/s, 0.0091 µm/s, 0.016 µm/s, and 0.35 µm/s. Distribution of the maximum velocity within each condition was not necessarily uniform. For the 0.33 µL/min condition, there was high positive skew (Skewness=1.46). For the 0.5 µL/min condition, there was approximate symmetry (Skewness=.003). The 1.0 µL/min condition (Skewness=-.31) and the 2.0 µL/min condition (Skewness=.17) both had a approximate symmetry as well. This is seen in Figure 3.3D.
Outliers were removed from the spheroid microbead data also. A ROUT outlier test with $Q = 1\%$ was performed on the average velocity and maximum velocity datasets. This test was performed to account for the chance that the TrackMate omits a bead in a frame and
Figure 3.4: Anomaly of Microbeads Flowing Through a Spheroid. This shows how some microbeads became trapped in tears in the 1.0 µL/min spheroid or possibly became stuck on the plastic bottom of the chamber well due to user error, while many flowed around the spheroid. This caused a higher standard deviation and higher average velocity for the condition.

use a farther bead as a reference, possibly raising the velocity. Even though this is accounted for when analyzing velocities with TrackMate, the implementation is not foolproof. Twelve outliers were removed from the average velocity dataset and 41 were removed from the maximum velocity dataset. A one-way ANOVA test was performed for each dataset with an alpha value of 0.5. The null hypothesis was that there was no significant difference between the velocities produced with each injection speed.

The average velocity of the microbeads injected into the spheroid produced different results. The fastest condition was the 1.0 µL/min injection speed (Mean=9.99 µm/s) and
3.3. Microbead Flow

the slowest condition was the 0.5 µL/min injection speed (Mean=6.40 µm/s). The second slowest was the 0.33 µL/min injection speed (Mean=6.60 µm/s) and the second fastest was the 2.0 µL/min injection speed (Mean=6.57 µm/s). The ANOVA test accounted for 69.5% of the dataset. There was a significant difference between each injection speed (P<1e-4) and the ratio of variance between each sample to inside each sample was 369.5. The 1.0 µL/min injection speed has the largest variance (SD=7.03 µm/s), followed by the 2.0 µL/min injection speed (SD=5.99 µm/s), the 0.33 µL/min injection speed (SD=4.81 µm/s), and, lastly, the 0.5 µL/min injection speed (SD=3.13 µm/s). These trends are shown on Figure 3.3E. The standard error of mean of all injections were, in order of increasing injection speed: 0.066 µm/s, 0.043 µm/s, 0.13 µm/s, and 0.15 µm/s. The distributions of microbead track average velocities within each condition were varied. The 0.5 µL/min condition (Skewness=.13) and the 1.0 µL/min condition (Skewness=.26) were fairly symmetrical. The 0.33 µL/min condition (Skewness=.61) and the 2.0 µL/min condition (Skewness=.68) were moderately positively skewed. This is shown in Figure 3.3F.

The maximum velocity of the microbeads injected into the spheroid had similar results. The overall velocities were higher, as expected. The 1.0 µL/min injection condition had the highest maximum velocity (Mean=15.15 µm/s), followed by the 2.0 µL/min condition (Mean=10.96 µm/s), then the 0.33 µL/min and 0.5 µL/min conditions (Mean=9.51 µm/s, Mean=5.91 µm/s) as shown in Figure 3.3G. Only 9.4% of data was covered in the ANOVA test. There was significant difference between each injection speed (P<1e-4) and there was more variability between samples than within samples (F=509.5). Each condition had high variability within itself. The 1.0 µL/min condition had the highest variation (SD=9.560 µm/s), followed by the 1.0 µL/min condition (SD=9.134). Although the last two conditions had equal means, they had different standard deviations. The 0.33 µL/min condition had more variability (SD=6.43 µm/s) and the 0.5 µL/min condition had the less variability.
(SD=3.71 µm/s). The distributions of maximum velocities of each microbead varied per injection speed. The 0.33 µL/min condition (Skewness=.25), 1.0 µL/min condition (Skewness=.07), and 2.0 µL/min condition (Skewness=.39) were all fairly symmetrical. The 0.5 µL/min condition was moderately negatively skewed (Skewness=-.70). This is shown in Figure 3.3H.

### 3.4 CAR-T Cell Flow

The injection volume and the injection speed into a blank PhotoHA gel were varied. The 0.67 µL, 1.67 µL, and 3.33 µL injection volumes all were subjected to four injection speeds: 0.33 µL/min, 0.5 µL/min, 1.0 µL/min, and 2.0 µL/min. The average velocity and maximum velocities were analyzed. No outlier test was performed for the CAR-T cell data because the density of the injected CAR-T cells was high. The TrackMate code tended to run optimally in these conditions and not mistrack any cells.

For the average velocity data, all injection volume conditions had similar trends across all injection speeds. All injection volumes had a net positive slope across all injection speeds. The average velocities varied slightly across volume for each injection speed. Their means interchanged as the injection velocity increased. These trends are shown in Figure 3.5A.

Each condition varied around the mean by around 2.0 µm to 2.5 µm. The means and standard deviations are located in Table 3.3. Note that there was no data for 0.67 µL volume with 2.0 µL/min injection speed because it was not analyzable. Given the number of frames and speed of the CAR-T cells, the code was unable to accurately detect the next frame’s CAR-T cell. When the parameter space was changed to account for the increased speed, the code still was unable to detect the correct CAR-T cell as the frame moved on. The code would often select for a CAR-T cell perpendicular to the path of flow. When manual
3.4. CAR-T Cell Flow

Figure 3.5: In-Gel Velocity Trends for Car-T Cells in a Blank Gel Across Injection Velocities. A) This shows how average velocity of each microbead track varies with injection velocity. Black represents the 0.67 µL injection volume, blue represents the 1.67 µL injection volume, and brown represents the 3.33 µL injection volume.

tracking was implemented, it was impossible to accurately tell where the CAR-T cell traveled to because with this concentration, volume, and speed, all CAR-T cells looked like streaks.

Table 3.3: Descriptive Statistics of the Average Velocity of CAR-T Cells

<table>
<thead>
<tr>
<th>Injection Velocity (µL/min)</th>
<th>0.33</th>
<th>0.33</th>
<th>0.5</th>
<th>0.5</th>
<th>1.0</th>
<th>1.0</th>
<th>2.0</th>
<th>2.0</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Volume (µL)</td>
<td>0.67</td>
<td>1.67</td>
<td>3.33</td>
<td>0.67</td>
<td>1.67</td>
<td>3.33</td>
<td>0.67</td>
<td>1.67</td>
<td>3.33</td>
</tr>
<tr>
<td>Average Velocity Mean (µm/s)</td>
<td>4.04</td>
<td>4.20</td>
<td>3.28</td>
<td>4.88</td>
<td>4.26</td>
<td>4.39</td>
<td>3.85</td>
<td>5.46</td>
<td>4.78</td>
</tr>
<tr>
<td>Standard Deviation (µm)</td>
<td>2.29</td>
<td>2.46</td>
<td>2.41</td>
<td>2.45</td>
<td>2.39</td>
<td>2.55</td>
<td>2.24</td>
<td>2.08</td>
<td>2.50</td>
</tr>
</tbody>
</table>

There was a significant difference in the average velocity across all injection velocities for 0.67 µL volume, 1.67 µL volume, and 3.33 µL volume (P<1e-4, P<1e-4, P<1e-4). Each one had more variation between injection volumes than within the injection volume itself (0.67 µL: F= 870.4; 1.67 µL: F=11084; 3.33 µL: F=16452). The one-way ANOVA test only accounted for a small amount of the data for each test (0.67 µL: R²=0.038; 1.67 µL: R²=0.078; 3.33 µL: R²=0.074). There was also a significant difference between each injection volume given across all injection velocities (P<1e-4, P<1e-4, P<1e-4, P<1e-4). A view of
the average velocity tracks throughout the gel as the injection velocity and volume changes is in Figure 3.6. The areas of higher average velocity tend to be clustered around the injection site.

Figure 3.6: Flow Tracks of CAR-T Cell Average Velocity. As the injection speed increases, the average velocity of the CAR-T cells per track increases. The tracks with higher average velocities tend to be clustered around the injection site.

The maximum velocity data followed the same trend, except all velocities were higher, as shown in Figure 3.5B. The order of the fastest average velocity within the injection speed was maintained, but the absolute value between the average and maximum velocity for each injection velocity was not the same. All absolute values hovered around 2.0 µm/s and slightly decreased as the injection velocity increased. In other words, the gap between the average and maximum velocity slightly decreased as the injection velocity increased. The means, absolute values, and standard deviations are located in Table 3.4. According to a one-way ANOVA test, there was a significant difference in the maximum velocity across all injection
3.4. CAR-T Cell Flow

velocities for 0.67 µL volume, 1.67 µL volume, and 3.33 µL volume (P<1e-4, P<1e-4, P<1e-4). Each one had more variation between injection volumes than within the injection volume itself (0.67 µL: F=4849; 1.67 µL: F= 5018; 3.33 µL: F= 8929). There was also a significant difference between the average velocities across injection velocities for each injection volume according to a one-way ANOVA test (P<1e-4, P<1e-4, P<1e-4, P<1e-4).

Table 3.4: Descriptive Statistics of the Average Velocity of CAR-T Cells

<table>
<thead>
<tr>
<th>Injection Velocity (µL/min)</th>
<th>0.33</th>
<th>0.33</th>
<th>0.33</th>
<th>0.5</th>
<th>0.5</th>
<th>0.5</th>
<th>1.0</th>
<th>1.0</th>
<th>1.0</th>
<th>2.0</th>
<th>2.0</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection Volume (µL)</td>
<td>0.67</td>
<td>1.67</td>
<td>3.33</td>
<td>0.67</td>
<td>1.67</td>
<td>3.33</td>
<td>0.67</td>
<td>1.67</td>
<td>3.33</td>
<td>0.67</td>
<td>1.67</td>
<td>3.33</td>
</tr>
<tr>
<td>Maximum Velocity Mean (µm/s)</td>
<td>6.12</td>
<td>6.40</td>
<td>5.52</td>
<td>6.77</td>
<td>6.31</td>
<td>6.62</td>
<td>5.12</td>
<td>7.42</td>
<td>6.79</td>
<td>N.D.</td>
<td>7.66</td>
<td>7.40</td>
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<tr>
<td>Avg Velocity - Max Velocity (µm/s)</td>
<td>2.08</td>
<td>2.21</td>
<td>2.23</td>
<td>1.89</td>
<td>2.05</td>
<td>2.23</td>
<td>1.27</td>
<td>1.96</td>
<td>2.00</td>
<td>N.D.</td>
<td>1.69</td>
<td>1.77</td>
</tr>
<tr>
<td>Standard Deviation (µm)</td>
<td>2.92</td>
<td>2.89</td>
<td>3.11</td>
<td>2.80</td>
<td>2.92</td>
<td>2.84</td>
<td>2.39</td>
<td>2.82</td>
<td>N.D.</td>
<td>2.27</td>
<td>2.47</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 4

Discussion

4.1 3D Hydrogel System Varying the TME and AMD3100

The 3D Hydrogel experiment was used as a basis for the 3D hydrogel spheroid model. For G2 cells, AMD3100 seemed to decrease invasion, as shown in Figure 3.1A. This makes sense because blocking the binding of CXCL12 decreases invasion in other cells. However, there was no significance with a one-way ANOVA test. The increase in invasion with the TME for G2 and G34 cells is also a trend that appeared in the Figure 3.1C. This could have happened because the increase in CXCL12 in the environment increased the proportion of binding to CXCR4 and caused invasion. With that being said, all increases of invasion for any condition was well within each condition’s error bars that represent one standard deviation. Since no significance was reached, no conclusions about this could be drawn.

No significant trends existed between the presence of AMD3100 and invasion. It cannot be concluded that AMD3100 significantly affects invasion in TME and non-TME conditions for G2 and G34 cells. This experiment should be repeated with at least 6 standard biological replicates, instead of 4, to explore these trends fully. The experimental set-up should be kept the same.
4.2 Large Spheroid Cell Culture

The similarity of G2 and G34 descriptive statistics point to the idea that the cell lines will grow similarly using the new culture method. For instance, the median of the number of cells per spheroid of each cell line only differed by 2500 cells. The mean number of cells differed by 2547 cells. This is a small difference in when using the trypan blue hemocytometer counting method. The standard deviations only differed between the two cell lines by 2146.7 cells. The ranges of each cell line only differed by 10000 cells, less than 7% of the average spheroid cell number for each cell line. This suggests a similar consistency across both cell lines.

The lengths of the two cell lines also had similar descriptive statistics. The means of the two datasets differed by only 97.36 µm and the medians by only 206.7 µm. This suggests that the center of the data is very similar and the culture method produces spheroids of approximately the same length. The ranges were also very similar and differed by a mere 65.41 µm. The standard deviations differed by 192.5 µm. With the range and standard deviation being so similar between the two cell lines, it suggests similar spread of data and similar consistency in the cell culture method.

Since the p values for both length and cell number were not significant across cell type, it was concluded that the cell type did not affect the ending spheroid length and cell number. In other words, G2 and G34 spheroids had similar lengths and cell numbers at the end of this culture method, meaning both G2 and G34 were comparable in these metrics. This allowed for consistency when implemented in the resection and bulk flow models. From this point forward, it was assumed that the number of cells per spheroid grown with this method and their lengths were about the same, regardless of cell type, as long as the spheroids were grown in the same batch. With this being said, the significance between all of the trials within a
metric were different. For G2, the lengths between trial 1 and trial 2 and trial 1 and trial 3 were significantly different. That means per batch, the lengths of G2 spheroids differed. This was not concerning because tumors differ in shape,[26] making use of these spheroids in models more biologically accurate. The difference in lengths were also consistent with G2 spheroids grown over months using the laboratory’s standard GSC culturing protocol. The cell number between all of the G2 trials was not significantly different, meaning the G2 spheroids had roughly the same number of cells with every batch.

There was also a difference between trials for G34 cells. The lengths of G34 cells between trial one and trial 2 were not significant. The lengths of G34 cells between trial one and trial 3 were significant. More trials are needed to see if significance between trials does, in fact, hold. The cell number, on the other hand, was not significant between trials, meaning that every G34 spheroid had approximately the same number of cells. This made implementation into a resection or bulk flow model feasible since quantification could now be accurately calculated.

### 4.3 Microbead Flow

There was no discernible pattern observed in the microbead flow velocity as the injection speed was increased. This is most likely due to variance in spheroid shape and size. Although the spheroids had the same cell number, their lengths with the new culture method were significantly different. This was because some tumors were more dense or less dense. The density of the spheroid is critical for microbead velocity. If a spheroid has a large density, the microbead will meet more resistance and flow slower than one with a smaller density. Not to mention, the shape of each spheroid was different. Some had more patches of high density and some had more patches of low density. This can be seen in Figure 2.3. The spheroid
4.3. Microbead Flow

in photo A was very dense. It was a solid mass with protruding semi-spheres of mass. The
spheroid in photos B and C, on the other hand, had areas of low density. Although seemingly
uniform in the 2D view, one can see that the cells are held very tight together in the 3D
view. This can cause an easier time for the microbeads to pass through the spheroid. Each
spheroid was created through natural formation and then coagulation of smaller spheroids
with shear stress on day 5 through day 7 of growth. Even though every spheroid coagulated
with shear stress, there was variability in this coagulation which most likely led to this
variability in density. Specifically, one can predict which spheroids will be less dense than
others. Spheroids imaged in a 3D view with many defined or loosely connected spheres will
be less dense. This is because the intermingling of cells during collision during shear stress
was not as efficient. Microbeads preferred to take the path of least resistance between the
loose connected spheres, as seen during characterization in Figure 2.5. The proportion of
microbeads that find their way to this path could explain the skew in all microbead velocity
variations.

The difference in skew between blank gel average velocity, blank gel maximum velocity,
spheroid average velocity, and spheroid maximum velocity can be explained. All skewness
in the blank gels could be due to heterogeneity of the gel. During gel construction, cross-
linking may not have occurred in a homogeneous manner. Focusing on the difference in skew
between the average velocity and maximum velocity of microbeads in a spheroid, suppose
the maximum velocity of all microbead tracks are at the edge of the spheroid when it moves
from the dense spheroid to the less dense surrounding matrix. Here, there’s a decrease in
friction, which should correlate to an increase in flow. The decrease in friction, and therefore,
the change in velocity, is conserved across all microbeads since each microbead is in the same
environment. The skew difference between the average and maximum velocity is from the
variation of speed between the microbead of the time point before exiting the spheroid and
the mean. There is a correlation between the standard deviation of the average velocity and the amount of increase in negative skew from the average to the maximum velocity. If one compares the average velocity standard deviations, Figure 3.3F, and Figure 3.3H, one can see there is more of an increase in negative skew with lower standard deviations. A lower standard deviation means could mean microbeads took a lower resistance path in the spheroid or went through a space created as the needle was inserted. This would correlate to a large shift towards negative skew in the maximum velocities because most maximum velocities will be relatively the same while only a few will be small because they got stuck in the gel. A higher standard deviation happens if the spheroid had less access to low density pathways to the outside of the spheroid. More microbeads would be stuck in the gel and have lower maximum velocities. This correlates to a small negative shift in skew because if there’s more stuck microbeads, there’s more tracks with lower maximum velocities. The impediment to movement could be due to higher density spheroids or less breakage during catheter insertion. Namely, there is the greatest shift in the 1.0 µL/min condition, then the 2.0 µL/min condition, then the 0.33 µL/min condition, and, lastly, the 0.5 µL/min condition. This coincides with decreasing deviation. The 1.0 µL/min condition should be taken note of. This condition had a spheroid that had a shape that allowed for microbeads to flow in a regular path while also catching a large amount of microbeads. The topography of the spheroid, the various nooks and crannies, mixed with the placement of the catheter most likely lead to this. The catheter could have been placed through the spheroid and be touching the plastic. This cannot be completely discerned through inspection. A picture of this can be seen in Figure 3.4. This would lead to a high average standard deviation a high maximum velocity. From all of these observations, it can be postulated that the 1.0 µL/min microbeads had the most access to the outside of the spheroid, followed by 2.0 µL/min microbeads, then the 0.33 µL/min microbeads, and, lastly, the 0.5 µL/min microbeads, in accordance with a decreasing standard deviation. The 1.0 µL/min condition could possibly have broken in such
4.4 CAR-T Cell Flow

As the injection volume increased, the average velocity and maximum velocity both increased. This makes intuitive sense; the faster the CAR-T cells were pushed into the gel,
the faster they moved. As long as the environment is conserved throughout all samples, which it was, this theory is sound. Since the measured velocities, both average and maximum, between injection speeds for all volumes was significant, this trend can be welcomed. Also, the gap between the average and maximum velocity slightly decreased as the injection velocity increased. This means that the beads were moving more uniformly and the maximum velocity data points became less of an outlier. This could be because the inertia from injection carried beads farther at higher speeds during the imaging time frame. When looking at the areas of higher average velocity, as seen in Figure 3.6, one would notice that they are aggregated around the injection site, supporting this claim. It also makes sense that the CAR-T cells would be faster nearer to the needle than on the outside edge because they have experienced less friction in that location.

The injection volumes differ significantly and seem to cause a decrease in CAR-T cell velocity as the injection volume increased, as shown in Figure 3.6. Though, on Figure 3.5, they are within one micrometer per second of each other. No injection volume has clearly higher or lower velocities across every injection speed. There is a disconnect. The significance achieved from the ANOVA test may have only been achieved due to the large amount of microbead tracks that were analyzed. The large dataset skewed the ANOVA test towards significance. Therefore, nothing concrete can be said about the trend between injection volume and injection velocity within a blank PhotoHA gel.

Future studies could include flowing CAR-T cells into a spheroid and measuring the injection velocities. This would give insight on how CAR-T cells flow through a spheroid. The data of that experiment can be compared to this data to see how CAR-T velocities change in a tumor-rich environment. Taking this study a step farther and looking at the invasion of the spheroid after CAR-T cells are injected could give insight on how glioblastoma invasion patterns change with the CAR-T treatment. If the killing potential is also assessed,
both metrics can be compared and correlated. An exciting goal could be to use the 3D hydrogel system to incorporate biophysical forces like IFF, the TME, and other treatment options like temozolomide and AMD3100 to model how CAR-T cells act in an accurate tumor environment with any cocktail of treatment.
Chapter 5

Conclusion

In conclusion, the CED injection system was successfully optimized. Experiments exploring CED in an in-vitro setting can now be easily conducted. The new spheroid cell culture method successfully creates large spheroids that can act as a tumor in a gel to mimic the TME for experiments and produces spheroids with consistent cell numbers per batch. When microbeads were flowed into the in-vitro CED system, it gave valuable insight into how the shape of a tumor varies the transport of a potential drug. When the CAR-T cell treatment was loaded into the CED system with blank gels, a control for future CAR-T experiments using the system was created, opening up potential for discoveries to come.
Bibliography


[22] R Chase Cornelison, Caroline E Brennan, Kathryn M Kingsmore, and Jennifer M Mun-


