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Quantification of tumor-associated macrophages following immunomodulation therapy in a murine allograft model of colorectal carcinoma

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1. Abstract

Colorectal Carcinoma (CRC) is one of the deadliest cancers in the world, with 150,000 new cases annually in the United States. Traditional treatments include chemotherapy and invasive surgery; however, research has shown that only 25% of patients that undergo traditional treatment have a positive result. Immunotherapy is an emerging form of cancer treatment that utilizes the patients' immune system to fight cancer cells by targeting inflammation, which plays a large role in the proliferation and metastasis of cancer cells.

Tumor-associated macrophages (TAMs) are immune cells that affect the inflammatory microenvironment of tumors. TAMs are M1 in the early stages of tumors, and are phagocytic and cytotoxic. However, these macrophages shift towards M2 as the tumors grow, which promote tumorigenesis, angiogenesis, and immunosuppression in CRC. M2 macrophage density may indicate the pro-tumor effects of TAMs, and it is expected that fewer M2 macrophages is correlated with a higher survival rate of patients with cancer.

The central hypothesis in this study is that blockade of TAM recruitment via immunotherapy would decrease M2 macrophages in the tumor microenvironment, resulting in a more treatable tumor. Monocyte chemoattractant protein-1 (CCL-2) is a cytokine secreted by tumors known to recruit monocytes that differentiate into TAMs. In our study, we examined the effects of CCL-2 blockade in a murine allograft model of CRC.

Thirty 10-week old Balb/c mice were injected subcutaneously with $1x10^5$ CT26 murine colon carcinoma cells. Fifteen mice received anti-CCL-2 immunotherapy, and 15 control mice received saline. Tumor allografts grew until they reached 75 mm^3. Tumor sections were stained for M2 macrophages and total cells, and then imaged to determine the M2 macrophage density. On Day 10, the tumors were 13.02 ± 3.04 and 11.03 ± 3.99 times their original sizes, and M2 macrophage densities were 593.3 ± 9.0 and 559.2 ± 7.0 M2 macrophages/mm^2 for the control and treated mice respectively.

2. Summary

The biomedical engineering department was started at the University of Arkansas in 2012, and I have been fortunate enough to do undergraduate research for the past 4 years under the supervision of one of the founding members of the department, Dr. Muldoon. During my freshman year, I took an undergraduate research class in which another student and I worked in Dr. Muldoon's biomedical engineering lab. We used a multi-photon microscope and computer programs for cell image analysis and presented at The Freshman Engineering Program's Honors Research Symposium. I remained in the lab and spent the next two years being an assistant to a graduate student and becoming familiar with the equipment. I assisted with the use of the multi-photon microscope to image live tissue for fluorescence, and collected preliminary data to begin a project on multispectral unmixing of images of live tissue. I also designed and constructed a blackout box to enclose the multiphoton microscope to block excess light in order to enhance the integrity and quality of acquired images.

In the Spring of 2017, I was awarded a State Undergraduate Research Fellowship to fund an original research project. I have been working with Dr. Muldoon and a graduate student to develop a protocol for observing immune cells on cancerous tumors in mice and using spectroscopy methods to correlate the findings with immune response. The specific cells I am focusing on are tumor-associated macrophages and their correlation to tumor progression and inhibition. I presented the preliminary findings at the Gulf Coast Undergraduate Research Symposium at Rice University in Fall 2017.

3. Introduction

3.1 Background

Colorectal cancer (CRC) is the 3rd deadliest cancer worldwide, and there are 150,000 new cases annually in the US. [1] Cancer progression is designated in stages. Progression through each stage involves the altering of genes that allow tumor cells to bypass growth control and signals for cell apoptosis. [2] **Figure 1** contains a representative diagram of a normal colon in comparison to one affected by CRC. Traditional treatment of locally advanced (stage II and III) CRC consists of chemotherapy followed by surgery. 5-fluorouracil (5-FU) is a pyrimidine that is among the most commonly used chemotherapy agents to treat CRC in both early and late stages. [2] However, only about 25% of patients achieve positive results from treatment with 5-FU alone. [3] Part of the low success rate can be attributed to patient-specific reaction to the chemotherapy.

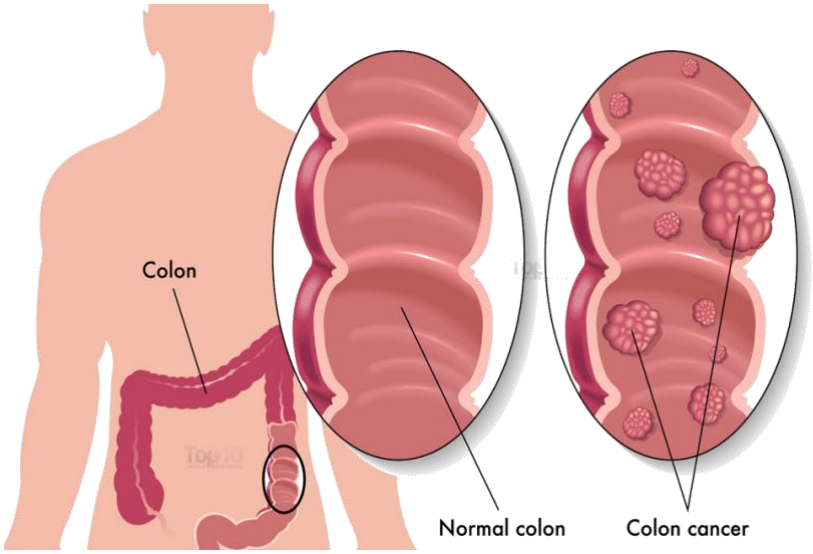

Figure 1. Representative diagram of a normal colon vs. one that contains cancer cells in the form of tumors. [18]

A promising field called immunotherapy has emerged which involves optimizing the patients' specific immune system to combat cancer cells. Combination therapy including both conventional 5-FU and immunotherapy may improve overall clinical outcomes in patients with locally advanced disease. [4] Common targets of immunotherapy are the biochemical pathways involved in inflammation, which provides conditions that allow tumor cells to proliferate and

metastasize. [4] Therefore, immunotherapy which targets inflammation may have a beneficial role in treating CRC. **Figure 2** provides a visual aid to compare traditional cancer therapies with immunotherapy treatments. As can be seen in the diagram, traditional therapies like drugs and radiation kill both cancer cells and healthy cells, whereas immunotherapy treatment uses a patients' immune system to only kill cancer cells.

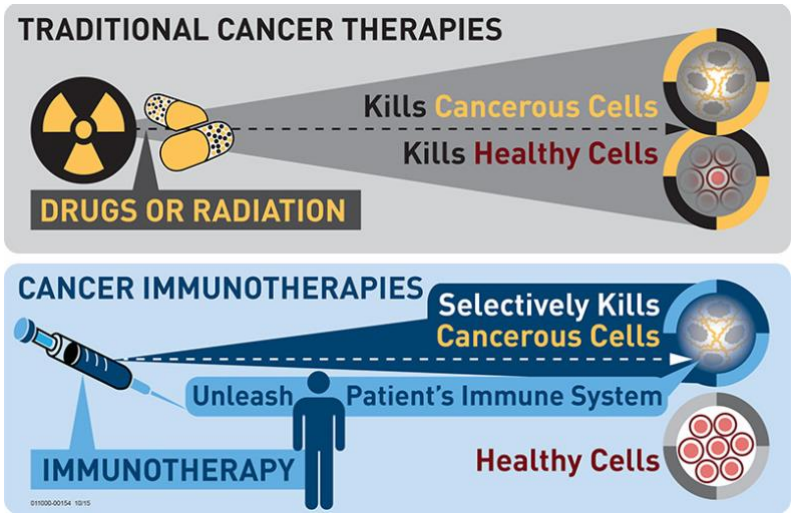

Figure 2. A diagram comparing traditional cancer therapies and immunotherapy. [19]

Inflammation is a complex cascade, but studies suggest that it is a contributing factor to tumor growth because it provides conditions that allow tumor cells to grow, survive, and become metastatic. [5] Inflammation can be initiated or encouraged by tumor cells, or by cells recruited to the tumor microenvironment which are required for the progression of tumors. [5] It is becoming more prevalently accepted that there is an immunosuppressive micro-environment within tumors, due to both cancer and immune cells, that limits the effectiveness of immunotherapy. [4] For successful treatments, it is common to involve a second agent to alter the tumor, such as combining other drugs with a chemotherapy agent to enhance its effect, or to combine chemotherapy with radiation. [4] Scientists are taking this knowledge and are considering alternative agents to find the most effective enhancers to allow the chemotherapy to have maximum effect. One method that

is being explored, and is the focus of the second phase of this experiment, is the effect of immunotherapy in addition to chemotherapy on M2 macrophage density in cancerous tumors.

Tumor-associated macrophages (TAMs) are primary immune cells that affect the micro-environment of tumors. TAMs in the early stages of tumors are known as M1 or "classically activated" type, and are phagocytic, cytotoxic, and present antigens well. [6] As the tumor continues to grow, however, macrophages change to a M2 or "alternatively activated" type. M1 and M2 TAMs differ based on receptor expression, antigen-presenting ability, function, and cytokine production. [7] M2 TAMs have properties that promote tumors, and are angiogenic and immunoinhibitory. [8] Similar to a wound healing response, macrophages enter the microenvironment and signal for other molecules to be recruited to increase the formation of new blood vessels. This, in turn, increases oxygenation and tumor size. [6] By determining the TAM phenotype, one could determine the density of M2 macrophages in a tumor, which could be used as a vital indicator of the effectiveness of immunotherapy treatment. Considering the differing functions of the macrophages, it is expected that a larger density of M2 macrophages would be correlated with a higher survival rate of patients with cancer. [9]

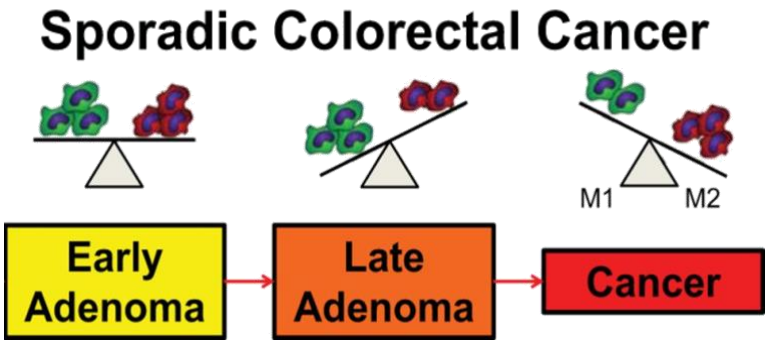

Figure 3. A diagram of the transition from M1 to M2 phenotypes as cancer progresses. [6]

3.2 Rationale

The central hypothesis in this study is that blockade of TAM recruitment via immunotherapy techniques would decrease the number of M2-polarized macrophages in the tumor microenvironment, potentially resulting in a more treatable tumor. A hypoxic microenvironment is common to malignant tumors. [10] As a result of the lack of oxygen, the cancer cells will activate the transcription of downstream target genes. [11] TAM recruitment into tumors is modulated via a complex series of cytokines and other signaling molecules. Pyruvate kinase M2 (PKM2) is an enzyme that catalyzes the last step of glycolysis, and is highly expressed in CRC. [11] In CRC cells, PKM2 aids macrophage recruitment via nuclear factor-$\kappa\beta$ (NF-$\kappa\beta$) signaling pathway-mediated chemoattractant protein-1 (CCL-2) expression. [11] CCL-2 is a cytokine that is normally secreted by cancerous tumors and is known to recruit monocytes in peripheral blood via chemotaxis, which subsequently differentiate into TAMs. [13] The polarization of TAMs into M1 or M2 is dependent on transcriptional regulators. [12] Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that promotes a change in energy metabolism from oxidative phosphorylation to glycolysis and increases oxygenation. [14] HIF-1 acts as a regulator and is linked to the proliferation, migration, and metastasis of tumor cells. [15] M2 macrophages aid in tumor growth via inflammation, angiogenesis, extracellular matrix formation, and immunosuppression by contact with other cells or by producing growth factors, chemokines, and angiogenic substances such as inteleukin (IL)-10, IL-8, IL-6, IL-1ß, and tumor necrosis factor-α (TNF- α), and tumor growth factor-ß (TGF- ß). [16] In our study, we examined the effects of using anti-CCL-2 to block monocyte infiltration in the tumor microenvironment of a murine allograft model of CRC. An allograft model consists of mouse cells in a mouse tumor. The cancer cells are of the mouse species to have the mice remain immunologically intact for the introduction and development of the CRC.

3.3 Significance of Work

This project is important because there are some aspects of TAMs that are unknown, such as how M2 macrophage density affects prognostic outcomes and how the relative number and types of TAMs in a tumor affect response to immunotherapy. The results of this experiment will be used to guide immunotherapy in CRC by quantifying the effects that blocking TAM recruitment via CCL-2 blockade has on the M2 macrophage density. Future work will investigate the effects of combining immunotherapy with traditional 5-FU chemotherapy.

4. Materials and Methods

4.1 Murine Models

Thirty nine-week-old female Balb/c mice (strain: 000651, The Jackson Laboratory, ME, USA) were purchased by a graduate student for this study. This study was approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC #18060). The mice were housed in groups of three in five cages at 23°C ± 1°C and 40-60% humidity on a 12:12 hour light-dark cycle. Food (8640, Teklad) and water was provided *ad libitum*. All thirty mice were allowed to acclimate for seven days after arrival prior to the start of the study. After one week of acclimation, the left flanks of the now 10-week old Balb/c mice were shaved and Nair was applied for one minute to locally remove hair.

4.2 Tumor Model and Therapy

The 30 Balb/c mice underwent tumor allotransplantation by a graduate student. A colon carcinoma cell line derived from the Balb/c mouse strain, CT26 (ATCC®, CRL-2638™), was kept in Roswell Park Memorial Institute (RPMI)-1640 medium (ATCC®, 30-2001™) in addition to

10% fetal bovine serum (ATCC®, 30-2020), 1% antibiotic antimyocotic solution (Sigma-Aldrich, A5955-100ML), and 0.2% amphotericin B/gentamicin (Thermo Fisher Scientific, R015010) at 37°C and 5% CO_2. CT26 cells were brought to the third passage (P3), and $1x10^5$ CT26 cells in sterile saline were injected subcutaneously into the left flank by the graduate student. It took an average of 12.9 ± 1.9 days to reach $75mm^3$, which was designated as Day 0.

Fifteen mice received intraperitoneal (IP) administration of anti-CCL-2 at a concentration of 6.0 mg/kg/dose given on days 0 (tumor = 75 mm^3), 3, 6, and 9 (average of 2 mg/kg/day), and 15 control mice received equivalent saline by a graduate student.

4.3 Preparation of the Tissue

4.3a Preservation

At each endpoint, each mouse was euthanized by a graduate student and the tumor was subsequently surgically removed and flash frozen in liquid nitrogen. Anesthesia is typically used in experiments involving animals because it reduces stress, and allows for easier manipulation of the animals so injections of cancer cells and anti-cancer agents and any surgical procedures are carried out unhindered. [17] Anesthetic agents used in mouse studies can be delivered via inhalation or injection [17]. In this experiment, the mice were anesthetized via isoflurane inhalation, which is a halogenated anesthetic gas. After being flash frozen, the bulk tumors were embedded in OCT (Optimal Cutting Temperature compound) and frozen in a -80°C freezer for long term preservation.

4.3b Sectioning

The tumors were sectioned using cryosectioning techniques. A Leica CM3050 S Research cryostat was used to section the tissue. A cryostat is a microtome inside a freezer that can be used to create thin sections of tissue for microscopy. The cryostat was kept at -20°C and the tissue was sliced in 6 µm sections. Once cut, the tissue sections were mounted on 22 x 40mm VWR Microscope Cover Glasses that have a lysine coating to make them positively charged. This positive charge allows for negatively charged tissue to adhere to the coverslip better. One slide was prepared per mouse, and each slide contained 2 tumor sections for a total of 60 tumor sections in this experiment.

4.3c Application of Antibody and Staining

Immunohistochemistry was performed at different time points (days 1, 4, 7, and 10). Alexa Fluor 594 anti-mouse CD206 antibody (M2 macrophage count) was applied to the prepared tissue sections, and the sections were washed with PBS-t (Phosphate Buffered Saline solution with Tween). The tissue sections were then stained for DAPI (total cell content and macrophage fraction). The primary antibody was already fluorescently tagged, and DAPI is a fluorescent stain that binds strongly to DNA found in cell nuclei. The stains aided in understanding how anti-CCL-2 immunotherapy affects total M2 count over time. An immediately adjacent tissue section of control tumors on days 4,7, and 10 and an experimental tumor on day 10 were also stained with Hematoxylin and Eosin to visualize the bulk tumors for size comparison and reference for imaging. This stains proteins in the cytoplasm pink and the nucleus purple. The antibody staining protocol used in this experiment can be found in **Table 1. Figure 4** includes a flowchart of the experiment from injecting the CT26 cells through the staining procedure.

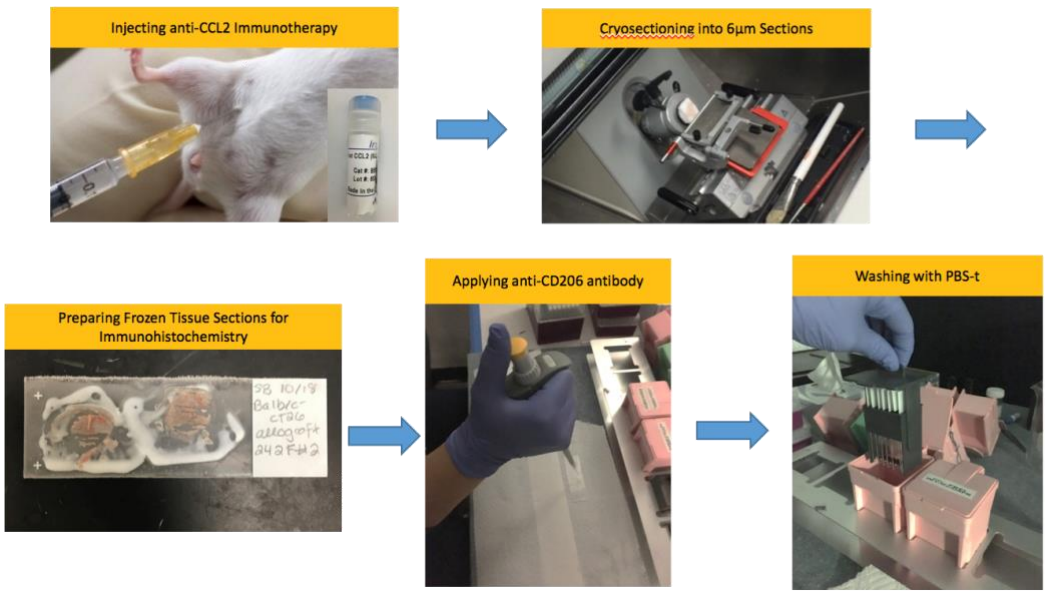

Figure 4. Flowchart of experimental methods from the injection of the immunotherapy to the application of the antibody.

4.4 Imaging

A Nikon Eclipse N*i*-U multi-color fluorescent imaging microscope with a Lumencor SOLA SM6-LCR-SB light engine lamp and a Nikon DS Qi1Mc cooled, digital monochrome camera were used to image the tumor sections and determine the M2 macrophage density. The objective on the microscope was a 20X magnification with a 0.50 numerical aperture. The two filter cubes that were used on the microscope were DAPI, and Texas-Red. **Figure 5** is an image of the microscope and camera setup, including the objective used.

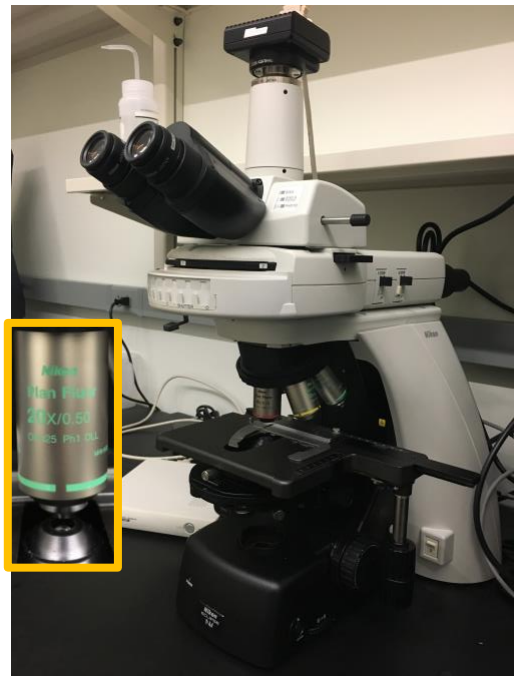

Figure 5. Nikon Eclipse N*i*-U microscope with a Lumencor SOLA Light engine lamp and a Nikon DS Qi1camera.

Once the microscope, camera, and lamp were turned on, the microscope was switched to the 4X/.25NA objective, and the exposure was set to 100ms. The desired slide was then placed on the microscope stage, and the filter wheel was turned to the DAPI filter. The course focus was used to locate the top of the tissue, and the translation stage was moved so the first image taken was halfway between the left and right edge of the tumor. The fine focus was then used to bring the cells into focus. The image was then captured and saved as a ".tif" file. The filter was then changed to the Texas Red filter and the fine focus was adjusted until the M2 macrophages were clear. Another image was taken and saved as a ".tif" file. These steps were done for each field of view and were repeated until 20 high-powered fields-of-view were acquired.

After the slides were imaged, the M2 macrophages were manually counted from the raw images. Afterwards, ImageJ was used to process the images and obtain representative images. Colors were assigned to the raw images, and the images were merged to create a composite image overlaying the two-colored images. Red was chosen to represent M2 macrophages because the antibody that was used to stain the M2 macrophages was fluorescently tagged with a fluorophore that is excited from 590nm to 617 nm, which is in the red wavelength. Blue was chosen to represent DAPI, because it emits a blue fluorophore.

5. Results

This experiment resulted in a visual decrease in the number of M2 macrophages between the control mice and those treated with anti-CCL-2 immunotherapy. **Figure 6** shows the various stages of tumor growth on an untreated mouse, and **Figure 7** is a graph of the bulk tumor growth from day 0 to day 20. Day 0 was designated as when the tumor reached $75mm^3$.

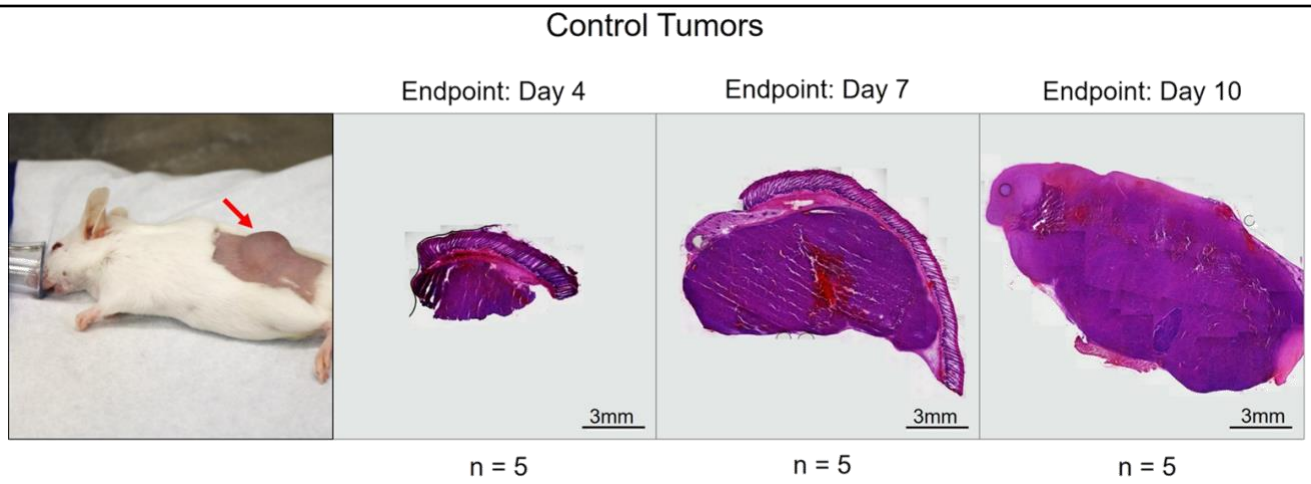

Figure 6. Control tumors sectioned and stained with Hematoxylin and Eosin, to show the growth of the untreated tumors from days 4 to 10.

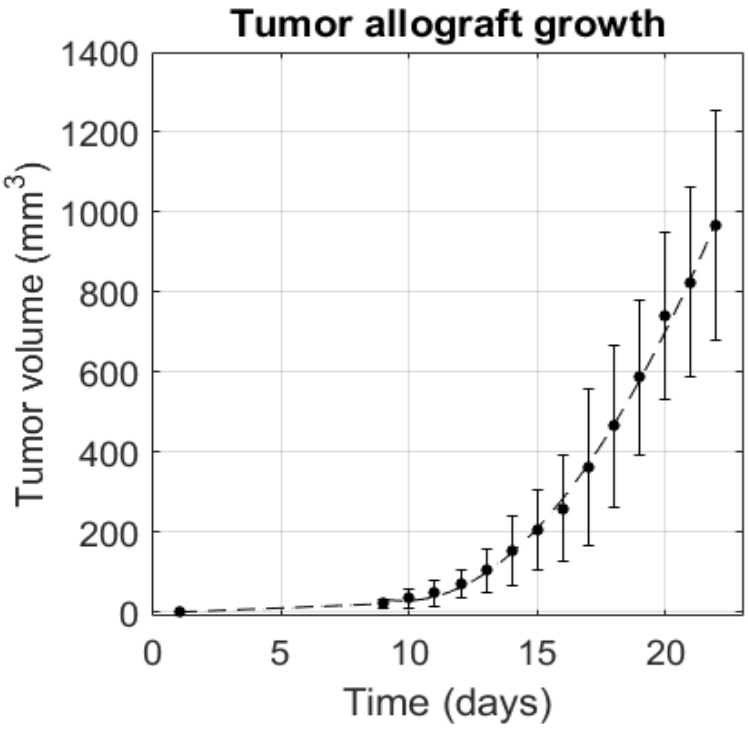

Figure 7. Graph of bulk tumor growth in an untreated, control mouse from day 0 to day 20.

Figure 8 includes a visual representation of the location of the field of views on a bulk tumor, and highlights the location of an overlaid composite image after processing with ImageJ. These findings can be seen in **Figure 9.**

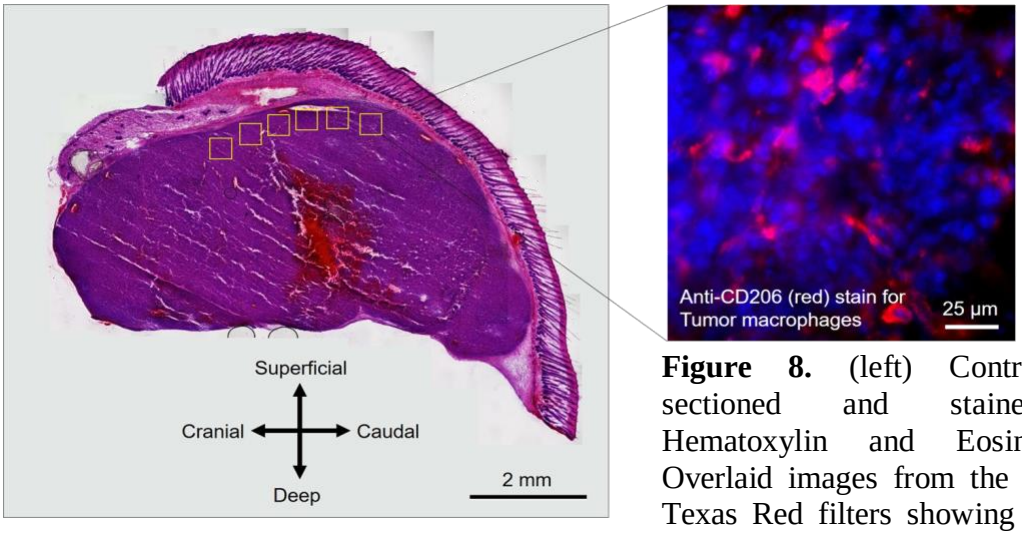

Figure 8. (left) Control tumor sectioned and stained with Hematoxylin and Eosin. (right) Overlaid images from the DAPI and Texas Red filters showing total cells (blue) and M2 macrophages (red). The field of view is .0152mm^2.

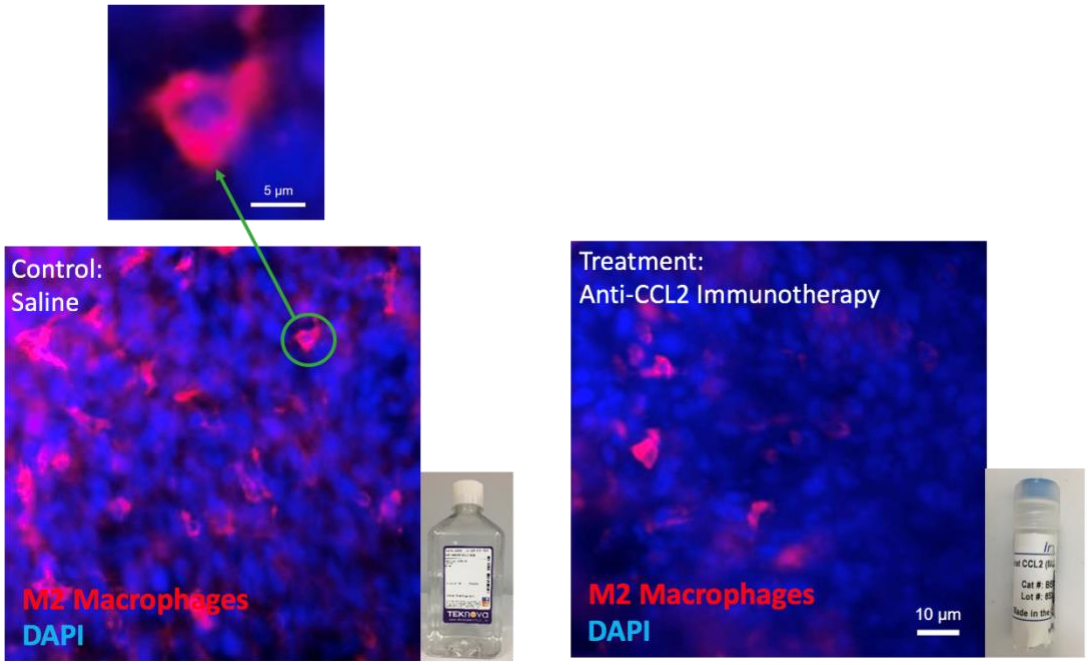

Figure 9. Stained tumor sections on Day 10 of treatment. M2 macrophages are colored red and total cells are colored blue. One can clearly see a decrease in the number of M2 macrophages present in the tissue section treated with immunotherapy treatment.

There was also a significant reduction in tumor growth rate and a decrease in M2 macrophage density in the mice administered anti-CCL-2 immunotherapy as compared to the untreated control mice. On Day 10, the tumors were 13.02±3.04 and 11.03±3.99 times their original sizes, and M2 macrophage densities were 593.3±9.0 and 559.2±7.0 M2 macrophages/mm^2 for the control and treated mice respectively. Statistical analysis on the results was performed using a two-tailed t-test with p-values less than 0.1. Graphical representations of the results can be seen in **Figure 10** and **Figure 11**.

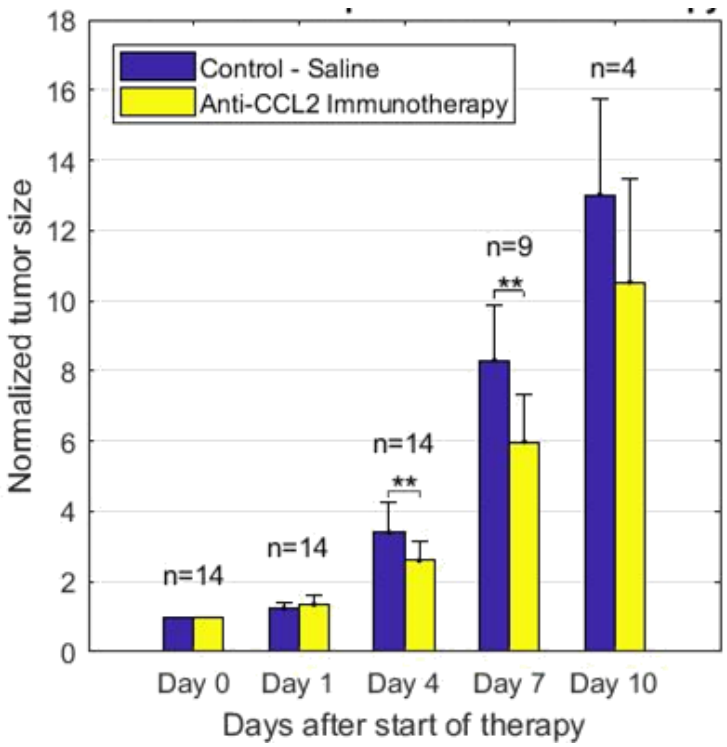

Figure 10. Graph of tumor size in response to immunotherapy. The data was normalized to the tumor on day zero ($75mm^3$). $^{**}p < .01$ via two-tailed t-test.

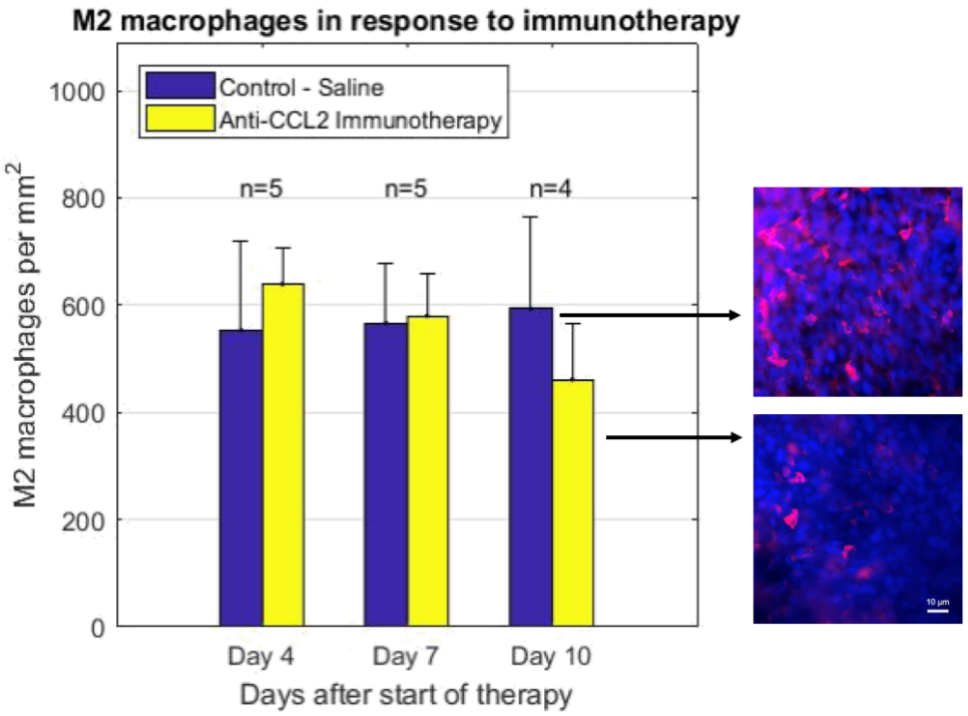

Figure 11. Graph of M2 macrophage density after immunotherapy. The data was normalized to the tumor on day zero ($75mm^3$). $^{**}p< .01$ via two-tailed t-test.

6. Discussion and Future Work

The results of this experiment will be used to guide immunotherapy in CRC by quantifying the effects that blocking TAM recruitment via CCL-2 blockade has on the M2 macrophage density. The second phase of this experiment is to determine the effects of traditional chemotherapy treatment with 5-flurouracil vs. a combination of traditional chemotherapy treatment with immunotherapy treatment on M2 macrophage density. **Figure 12** is a diagram of the two phases of this experiment. The intent behind this is to determine the best form of therapy by determining which treatment method has the smallest density of M2 macrophages after treatment.

Future work will investigate the effects of combining traditional 5-FU chemotherapy with immunotherapy, and tumor therapeutic response by quantifying tumor hemoglobin and oxygenation using Diffuse Reflectance Spectroscopy.

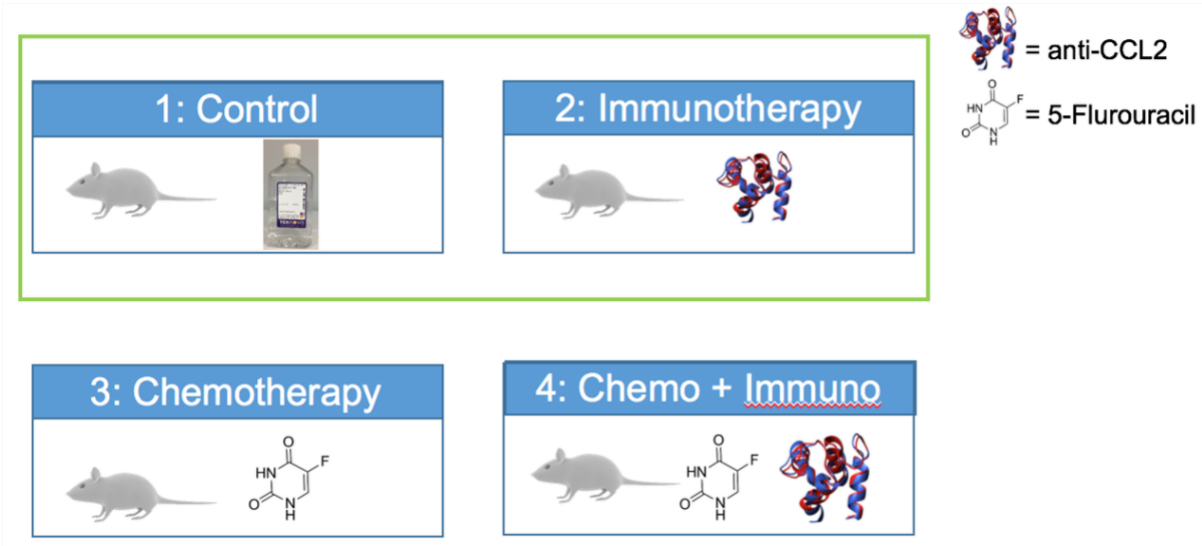

Figure 12. Diagram of the overall research project and future direction until completion.

7. Acknowledgements

This research would not have been possible without the guidance and support of my faculty mentor, Dr. Muldoon, and the graduate student I researched under, Gage Greening. I also want to thank Sandra Prieto, Haley James, and Shelby Bess for their contributions to my research project. Funding of this project was provided by the National Institutes of Health, National Science Foundation, and the Arkansas Department of Higher Education State Undergraduate Research Fellowship (SURF). I would also like to thank the Honors College at the University of Arkansas for allowing me to write and present my research in the form of an Honors Thesis. I began college in an honors undergraduate research course, and I would not have made the connections with Dr. Muldoon and had the experiences that I have had without being in that course my freshman year. Finally, I would like to acknowledge the University of Arkansas Biomedical Engineering Department for providing the equipment and lab facilities to conduct this research.

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9. Appendix

Table 1. A step-by-step breakdown of the staining protocol used in this experiment.

Step	Activity	Quantity	Contents	Light	Temp.	Time (min)
1	Prepare Blocking Solution	800µL	Goat-based blocking solution	Yes	Room	5
2	Prepare slides for staining	10	Slides containing tissue sections	Yes	-20˚C	20
3	Dispense Buffer and Antibody	1:125	Universal Antibody dilution buffer and Anti-CD206 mix	No	Room	5
4	Prepare Series of Solution Boxes	4 jars each with 90mL of solution	-Acetone -PBS-t (acetone) -PBS-t (M2) -PBS-t (M2/DAPI)	Yes	-20 ˚C Room Room Room	20
5	Fixation of Slides With Acetone	10	Slides in Acetone	Yes	0 ˚C	10
6	Washing Slides with PBS-t	10	Slides in PBS-t (acetone)	Yes	Room	3
7	Addition of Blocking Solution	80µL	Goat-based blocking solution	Yes	Room	3
8	Place Slides in Dark Humidified Chamber	10	Slides with goat-based blocking solution	No	Room	60

9	Addition of Buffer and Antibody Mix	100 µL	1:125 Universal Antibody dilution buffer and Anti-CD206 mix	No	Room	3
10	Place Slides in Dark Humidified Chamber	10	Slides with buffer and antibody mix	No	Room	90
11	Washing Slides with PBS-t	10	Slides in PBS-t (M2)	No	Room	Stable: 6 Moving: 1
12	Adding DAPI	1 drop per slide	DAPI	No	Room	1
13	Washing Slides with PBS-t	10	Slides in PBS-t (M2/DAPI)	No	Room	Stable: 6 Moving: 1
14	Mounting the Coverslips	10	-Stained slides -22x40 coverslip -Fluoromount G	No	Room	8
15	Sealing Slides	10	-Coverslipped slides -Nail Polish	No	Room	60