Changes in the Microbiome After Cancer Treatment: Are we Setting Patients Back Before They Begin?

by

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BS Microbiology, University of Pittsburgh, 2018

Submitted to the Graduate Faculty of the

School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2023

UNIVERSITY OF PITTSBURGH

SCHOOL OF PUBLIC HEALTH

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Colorectal cancer (CRC) is a deadly cancer that is becoming more commonly diagnosed in younger patients. One major issue with CRC is that it is largely unresponsive to new immunotherapies, such as PD-1 checkpoint inhibitor blockade. Individuals with microsatellite instability high (MSI-H) and mismatch repair deficiencies (dMMR) seem to be the only ones that have tumors that respond to PD-1, but this only accounts for about 10% of all CRC cases. The other 90% of patients who do not have these types of tumors will have no response to PD-1 therapy. Research has shown that the microbiome is involved with the regulation of the immune system, with previous results from patients who have received PD-1 found that patients who responded well had a more diverse microbiome compared to those who did not respond. Results from our mouse model show that groups who received chemotherapy in the form of 5-flurouracil along with anti-PD1 antibody had a lower tumor clearance rate compared to the anti-PD1 alone group, as well as the broad-spectrum antibiotics and anti-PD1 group. When looking at the tumors from these mice, flow cytometry analysis showed that mice who received 5-flurouracil along with anti-PD1 treatment had significantly increased numbers of CD8+ T cells that secreted higher levels of interferon gamma (IFNg). The anti-PD1 alone group had a large population of CD8+ T cells in the tumor that were double positive for IFNg and tumor necrosis alpha (TNFa). These results support that traditional cancer treatments, like chemotherapy, may lead to gut dysbiosis that changes how the immune system reacts to cancer in a patient and decrease the efficacy of anti-PD1 treatment.

It raises the question of how to proceed with treatments if the traditional ones that are currently used can hurt a person's ability to respond to immunotherapy.

Table of Contents

List of Tables

List of Figures

Acknowledgements

I want to thank Dr. David Geller and Dr. Samer Tohme, who took a chance on me very early in my science career and taught me countless skills that I still use to this day. In that lab, I also had the honor of working with Dr. Hamza Yazdani and Dr. Christof Kaltenmeier. Hamza and Christof were the first to introduce me to animal work, the world of surgery, and a deep love of the liver. I always looked forward to coming in to work with both of you every day and I will never be able to express how thankful I am for showing me that science and work can be fun and enjoyable.

My forever lab family, Nicole Martik Hays and Taylor Austin, have always supported me and pushed me to improve myself. I have been able to become a better person because of both of you. I am so lucky I get to talk to you every day and I will always look forward to our escapades out together. I cannot tell you how amazing the both of you are to me.

I also owe a debt of gratitude to the Delgoffe lab, especially Rachel Cumberland and Dr. Greg Delgoffe. Rachel has always been a great supporter as I have been trying new things in the lab and she has always made me feel heard and that someone is in my corner. Dr. Greg Delgoffe was also incredible and has always been supportive as we got started in our new lab space and a lot of my pilot experiments were able to be done with both Rachel and Dr. Delgoffe's help.

My committee members, Dr. Josh Mattila, Dr. Robbie Mailliard, and Dr. Amy Hartman have been endless help and support through my time in the program. Dr. Mattila, you really helped me when I needed a lot of support, and I will always appreciate the time and effort you put into your students. Dr. Mailliard, I cannot thank you for always checking in on me and being there

when I needed it. Dr. Hartman, you really instilled a great love of understanding viral pathogenesis in me and you made it super easy to be myself and share my thoughts.

I am thankful to my new lab members, Jess Jana and Katelyn Wolfgang, for always helping me with my big experiment days and always making me laugh when we work together at the bench.

There is one extremely important person that I must thank for all of this. She decided to hear me out after I sent a random email with my resume and my desire to get a project. I met my mentor, Dr. Abigail Overacre-Delgoffe at a particularly difficult transition period in my life and she very quickly helped me find my love of science again. She has trusted me to help her start her lab. She pushes me every day to become a better person and scientist. Her endless support and optimism inspire me every day to do better than the day before. Abby, thank you for everything. I can't put into words how thankful I am that I ended up here and that you are the best mentor anyone could ask for.

To my partner, Deven, thank you for always listening to me talk about my classes and letting me talk for hours about my experiments and my data. I love you dearly. To my dad, I love you and thank you for always hyping me up and being there when I need you. And to my mom, you were the first to make me love microbiology and science. You helped me find my way into a career in cancer research. I love you and I miss you.

1.0 Introduction

1.1 What is Cancer?

Cancer is one of the oldest documented diseases known to man, and this is partly why it has been dubbed “the emperor of all maladies”. Cancer often comes with a lot of fear as well including fear of aggressive treatments, the hospital bills, the stress put on loved ones, and even the fear of dying from cancer.

Cancer refers to a large group of diseases that all share the characteristic of uncontrolled cell growth (1). These cells can continue to mutate and travel to other parts of the body through the lymphatics and circulatory system (2). This can be caused by several environmental factors, like diet, level of activity, age, family history, and even the microbiome (2). Cancer can also arise by accident, as cells can have random mutations that compound on each other and give rise to cancer. These genetic mutations cause cells to grow and divide quickly, while also removing the mechanisms that stop these cells from continuing to replicate (3). In the United States, it is estimated that there will be over 1.9 million new cancer cases diagnosed this year and over 600,000 deaths due to cancer (4). While many cancers are curable when caught at an earlier stage, this still leaves many people who struggle with advanced disease.

Decades of research have been done and we are only starting to understand the mechanisms of how cancer develops and how it is able to grow and spread in a person. Scientists like Bert Vogelstein, who found that cancers can take years to develop and often involve a series of mutations that lead to the disease known as cancer, have paved the way for newer scientists to try and find more of the underlying mechanisms of cancer. Since then, it has been found that cancer

is a complex disease that involves many different processes and changes the environment around it to keep on surviving.

1.2 Epidemiology of Colorectal Cancer

Colorectal cancer (CRC) is the third deadliest cancer and second most diagnosed cancer that people face today (5). It was estimated that over 50,000 people died in 2022 (4) from the disease in the US, leaving patients with bleak outcomes when diagnosed. The current five-year survival rate of stage IV CRC is less than 15% (5) and this has not improved much, despite newer treatment options.

Colorectal cancer, like any other cancer, is a genetic disease. Random mutations can occur that can lead to the beginning stages of the disease. There are more risk factors that are being identified that can also help trigger the beginning mutations of the disease. Factors like a family history of CRC, older age, obesity, a sedentary lifestyle, inflammatory bowel disease, smoking, and a higher consumption of red meat (6) can all lead to a person increasing their risk of developing the disease.

Despite the incidence rates of CRC declining, one worrying trend has started to emerge. Early onset-colorectal cancer, which is when patients are diagnosed with CRC before the age of 50, has been on the rise for years (5). This may be due to having an increase in sedentary lifestyles and alarming increases in the obesity rate of Americans, which is now over 30% (7). This consistent increase in obesity rates and sedentary lifestyle has most likely contributed to the increase in early onset-colorectal cancer, as well as why the rates of overall CRC remain high

enough to be one of the most diagnosed cancers. Observational studies have shown that gut dysbiosis can be implicated in playing a role in early onset-colorectal cancer (8).

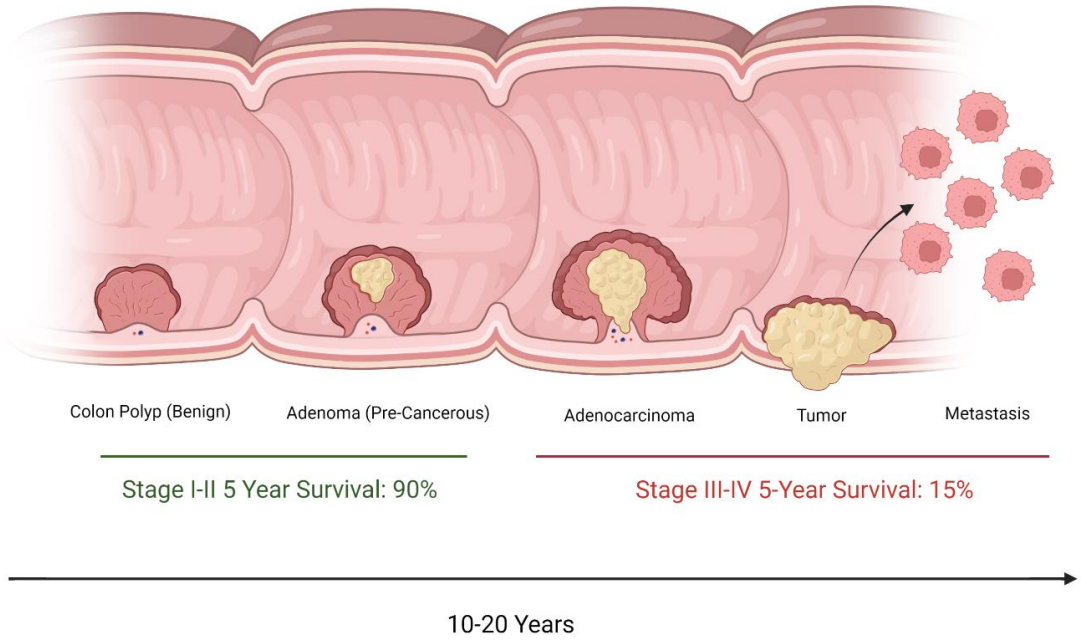

Figure 1: Representation of Colorectal Cancer Over Time. Created with *Biorender.com*.

1.3 Cancer Immunotherapy

One area of cancer treatments that has been gaining traction and excitement is immunotherapy. Treatments like chimeric antigen receptor T-cells (CAR T-cells) and immune checkpoint inhibitors (PD1/PD-L1/LAG3/CTLA4) have helped cancer patients reach long lasting remissions.

PD1 immune checkpoint inhibitor therapy involves blocking PD1 (programmed cell death protein 1) (9). This protein is found on T cells and is involved with immune regulation. PD1 is involved with blocking T cells from attacking other cells, including cancer cells. By blocking the PD1 protein on T cells, this should allow the lymphocytes to attack and eradicate the cancer cells in the patient.

Despite PD1 therapy working in about 40% of melanoma tumors, the response rates are not as optimistic in other cancer types, including CRC (9). There are several common CRC mutations that can be used as treatment targets, but sometimes these mutations may make a difference in immunotherapy treatment. Immunotherapy does work in a small group of CRC patients. These patients have mismatch repair deficient (dMMR) mechanisms and high microsatellite instability (MSI-H) (10). These MSI-H tumors have higher neoantigen and mutational burden that leads to increased immune infiltration prior to treatment (11). Since anti-PD1 immunotherapy mainly works to revive lymphocytes and have them attack cancer cells, having lymphocytes already in the tumor would make for a better success rate.

Only about 15% of CRC cancers are found to be MSI-H dMMR tumors (12). These numbers show the unfortunate reality that most patients will have no response to immune checkpoint inhibitor therapy. Immunotherapy can work for patients, but more work needs to be done overall to find out the mechanism as to why it doesn’t work in all patients.

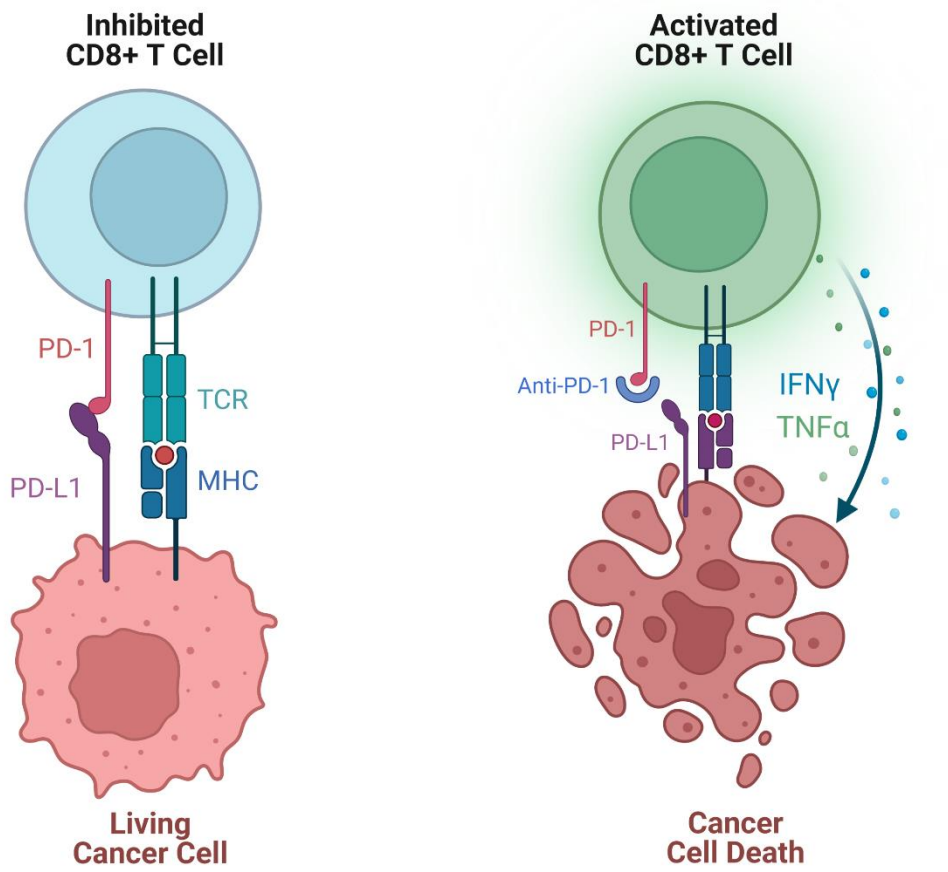

Figure 2: Representation of PD-1 Checkpoint Inhibition. Created with *Biorender.com*.

1.4 Gut Microbiome

The gut microbiome is a diverse makeup of numerous different types of bacteria and viruses that reside in and among the gut epithelium in humans. It has been found that these microbes also have a diverse set of functions that allow them to interact with the host. Serotonin is made in the gut as part of the gut-brain axis, which can give insight into how the gut can impact distant host sites. More recently, there has been progress in understanding how the gut plays a role in activating and regulating the immune system.

Studies have been focusing on the microbiome for a large variety of functions. Not only for understanding different treatments for cancer, but also for detection of cancer (13).

The gut microbiome was found to be greatly involved with immune checkpoint inhibitor success with the types and diversity of bacteria in the microbiome (14). Looking at melanoma patients who responded to anti-PD1 therapy and patients who did not respond had samples collected to compare the gut microbiome between the patients. Responders were found to have a great diversity of the types of bacteria in the microbiome compared to non-responders. Non-responders had higher amounts of *Bacteroides, E. coli,* and *Anaerotruncus* species.

The gut microbiome has been found to be involved with immune regulation. Murine studies looking at *Helicobacter hepaticus* (Hhep) enrichment of the gut microbiome saw reduced burden from colitis induced CRC (15). Hhep was found to be connected to Hhep-specific CD4+ T cells that were able to infiltrate tumors. This tumor infiltration allowed for increased rates of necrotic tumor cores and reduced tumor burden found in the colon. Hhep also helped increase the number of tertiary lymphoid structures around the tumor and increased lymphatics around and leading into the tumor. Overall, adding one microbe and not altering any other parts of the microbiome of the mice allowed for increased survival and decreased tumor burden that was associated with the microbe regulating parts of the immune system.

2.0 Specific Aims

Immune checkpoint inhibitor (ICI) therapy, namely PD1 immunotherapy, shows great promise and efficacy in the patients that it does work in. Compared to other treatments, ICI tends to be more tolerable for patients in terms of side effects. This makes it a great option for patients, as they can get a treatment that will clear the cancerous cells without the side effects that can make patients extremely ill. The issue that health professionals and scientists have at hand is *why* the treatment will work in some patients and not in others. Work with the gut microbiome and the immune system is still a new field, but it is already showing to be a great influence on how the immune system can respond to cancer. Previous work has suggested that the types and numbers of microbes matter in how the body responds to ICI, but there are still many questions into what makes a microbiome "responsive" or "unresponsive" to ICI and how traditional cancer treatments change the microbiome into one that would be considered "unresponsive". With these basic questions, we wanted to develop studies that could begin to uncover the effects these traditional treatments have on overall PD-1 efficacy. We propose to accomplish this with the following aims:

Aim 1: Determine whether having a microbiome that has been exposed to traditional cancer treatments affects anti-PD1 immunotherapy efficacy.

- Compare growth curves of murine MC38 tumors in mice that have been given traditional cancer treatments: chemotherapy and broad-spectrum antibiotics, as well as PD1 immunotherapy.
- Model the treatments to mimic a human patient's experience.
- Complete flow cytometry analysis on lymph nodes and tumors from the mice in each treatment group.

Aim 2: Investigate the changes in the microbiome caused by traditional cancer treatments.

- Collect stool at different time points from each treatment group to extract DNA and send out for 16S sequencing.
- Analyze the differences in microbial makeup to find any connections between immunotherapy response in different groups.

Aim 3: Use Data from Previous Bacterial Studies to Find a "Responsive" Microbiome.

- Narrowing down microbes that allow for a more responsive system for immunotherapy treatments and use that to "rescue" non-responders.
- Collect human patient data to compare CRC immunotherapy responders and non-responders.
- Put human microbiome into murine model and look at "rescue" studies in the mice.

3.0 Methods and Materials

3.1 MC38 Cell Culture

MC38 cells that were gifted from the Delgoffe and the Vignali labs were frozen at –80C in DMEM media and 20% DMSO at a concentration of 5 million cells per milliliter. Aliquots were thawed and grown in culture flasks in DMEM media with 10% fetal bovine serum, penicillin-streptomycin, L-glutamine, HEPES, sodium pyruvate, and beta mercaptoethanol. Cells were monitored for growth and passaged at 85-95% confluency. Trypsin is used to detach the cells from the flask and are centrifuged at 1250 rpm for 5 minutes. Cells were counted using a Cellometer and trypan blue staining. Cells were also evaluated for viability prior to injection and counts for injection were based off the viable cell number.

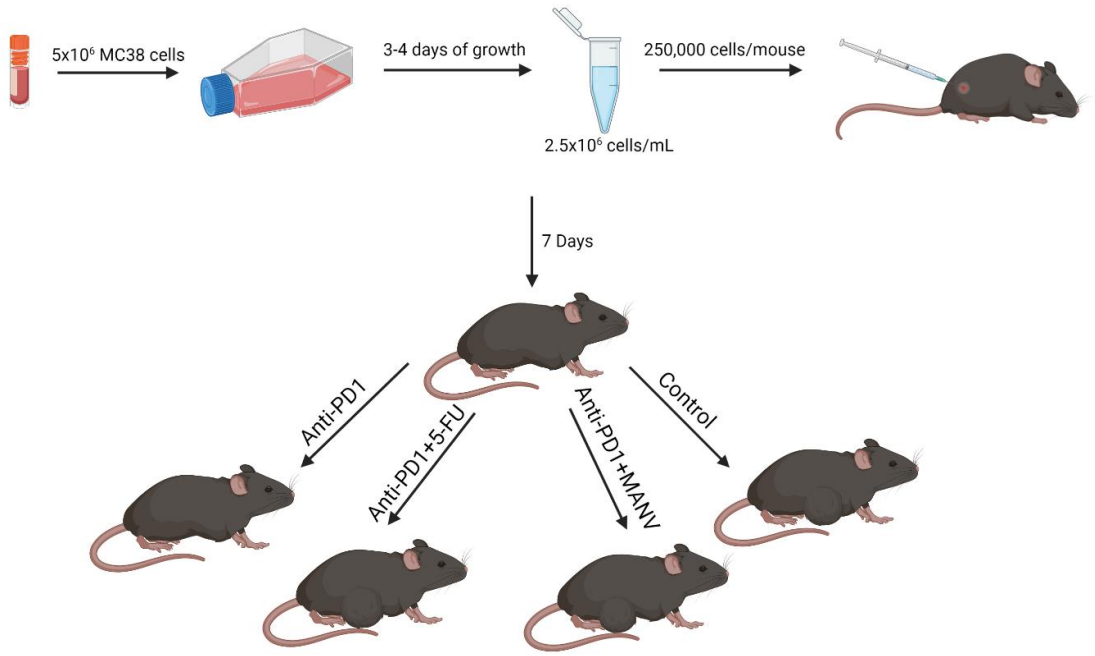

Figure 3: Murine Model Used to Evaluate Treatments on CRC Growth. Created with *Biorender.com*.

3.2 Mice

4–6-week-old female C57Bl/6 mice were purchased from Jackson Laboratories. These mice were housed in the UPMC Hillman Cancer Center Department of Laboratory Animal Resources (DLAR) Housing Facility and the University of Pittsburgh Department of Laboratory Animal Resources (DLAR) Ford Housing Facility. All mice were housed in immunocompromised conditions, as stated by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). These conditions involved the use of sterile water in autoclaved bottles and irradiated mouse chow to reduce the risk of mice encountering an unknown pathogen.

3.3 MC38 Subcutaneous Injections

MC38 cells were grown in culture (as previously described above in "MC38 Cell Culture") and suspended in sterile 1% PBS at a concentration of 2,500,000 cells/mL. Cells were kept on ice until ready for injection. Mice were anesthetized with isoflurane and checked for sedation using the pedal reflex. Each mouse had the right side shaved and ear punched to correspond to a number. 250,000 cells are then injected on the right side subcutaneously. The mice were then returned to their cage to monitor for recovery.

3.4 MC38 Tumor Measurement

Mice had tumors measured seven days out from initial injection. On day seven post injection, the mice are measured to ensure size consistency and are then divided into their treatment groups. Once initial measurements are taken on day 7, measurements were recorded of the tumors every 2-3 days using calipers. A cleared tumor was considered cleared if there was a tumor measured at any point along the timeline that was unable to be detected after. A tumor would be considered as a "recurrence" if a tumor cleared and then started to regrow. These recurrence tumors would not be a part of the clearance rate in analysis but would be included as a recurrence event. Any mice that had a tumor ulcerate and/or grow to be greater than 2 centimeters in diameter were euthanized in accordance with IACUC experiment endpoint protocol. Data analysis was performed in GraphPad PRISM software.

3.5 5-Flurouracil (5-FU) Treatment

5-flurouracil (Sigma A13456.06) is saved at a concentration of 10 milligrams per milliliter in aliquots. 5-FU is given through intraperitoneal injection at days 7, 8, 9, and 16 post MC38 injection. Each mouse is given 50 milligrams per kilogram of body weight. Signs of animal distress were monitored by looking for over 20% weight loss and lethargy.

3.6 Antibiotic Treatment

The broad-spectrum antibiotic water (MANV) is comprised of metronidazole (0.5 g/L), ampicillin (1 g/L), neomycin (1 g/L), and vancomycin (1 g/L) dissolved in sterile tap water. The water is sweetened with store bought saccharin packets (Sweet N' Low) at 6 g/L to entice the mice to ingest the antibiotics, as metronidazole has a foul taste to mice. The water is given to mice on days 7 through 10 post MC38 injection. Water consumption is monitored daily by checking water levels and examining the mice to make sure they are not showing signs of dehydration.

3.7 Anti-PD1 Treatment

Anti-PD1 (BioXCell BP0273) is saved at a concentration of 2 mg/mL in phosphate buffered saline (PBS) until use. Anti-PD1 is injected intraperitoneally to mice at a concentration of 200 ug/mL on days 9, 12, and 15 post MC38 injection. Anti-PD1 is given in conjunction with antibiotics and chemotherapy, as well as on its own.

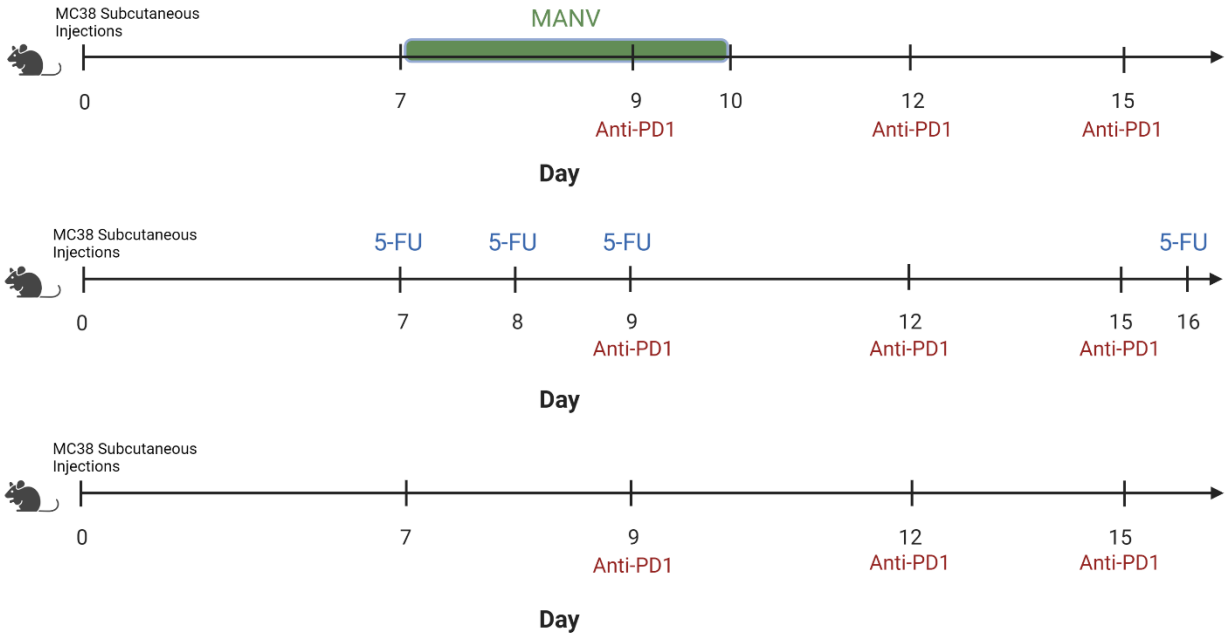

Figure 4: Timeline of Murine Model Used to Assess Anti-PD1 Efficacy. Created with *Biorender.com*.

3.8 Flow Cytometry

Tissues were collected for cytokine measurement when the mice were euthanized at day 15. Tissues were prepared and stained for surface markers before using BD CytoFix to fix and permeate the membranes to allow for intracellular staining. Cells were then analyzed on a Fortessa II at the UPMC Hillman Cancer Center Flow Cytometry Facility and further data analysis was done using FlowJo software. Splenocytes were taken from mice and used as single stains to compensate the machine for analysis.

Table 1: Antibodies and Fluorochromes Used in Cytokine Flow Cytometry Panel.

Fluorochrome	Antibody
BV421	IFNgamma
BV510	Live/Dead
BV650	CD90.2
BV780	CD8
PerCP-Cy5.5	TCRbeta
PE	IL-10
PE-Cy7	CD4
APC	IL-17
AF700	TNFalpha

3.9 Bacterial DNA Collection from Murine Stool

Stool was collected from mice at different timepoints and frozen in -80C before being processed to collect bacterial DNA. The bacterial DNA was collected using Qiagen's QIAamp® Fast DNA Stool Mini Kit. The DNA was then sent to Microbiome Insights© for large scale 16S sequencing.

4.0 Results

4.1 Pilot Experiments in UPMC Hillman Cancer Center DLAR

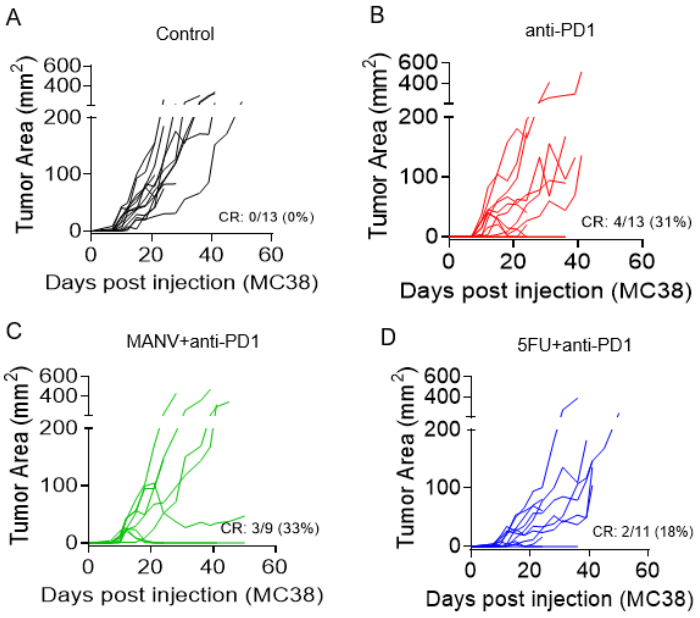

Figure 5: Pilot Tumor Growth Curves in UPMC Hillman Cancer Center. Control mice (A), mice treated with anti-PD1 alone (B), mice treated with antibiotics and anti-PD1 (C), and chemotherapy and anti-PD1 (D).

To understand how changes due to common cancer treatments change the microbiome and in response, affect anti-PD1 efficacy in CRC patients, we aimed to model the patient experience in mice and measure the tumor response and clearance rates (CR). Pilot experiments completed at UPMC Hillman Cancer Center (Figure 5 A-D) in BSL-2 immunocompromised housing conditions showed a slight difference between the treatment groups, but 5-fluoruracil and anti-PD1 (Figure 5 D) did have a lower clearance rate (18%) when compared to both the antibiotics and anti-PD1

(Figure 5 C) group (33%) and the anti-PD1 alone (Figure 5 B) group (31%). This was done with 15 mice per treatment group, but not all mice grew tumors after the MC38 injection on day 0. The clearance rates were based off the mice that grew tumors, but all mice are represented in the figure.

4.2 Tumor Growth Curve Analysis of Anti-PD1 and Traditional Cancer Treatments

The next group of experiments (Figure 6 A-D) to take place were done at the University of Pittsburgh Ford Building DLAR. These were once again taken place in BSL-2 immunocompromised conditions to ensure the microbiome stayed consistent and was not introduced to different bacteria through food or water sources. This round of treatments was completed to confirm the results seen previously in another building. Since conditions can differ between buildings and housing facilities, this can lead to differences in a mouse's baseline microbiome. It was important for us to make sure we saw a consistent tumor growth pattern to be able to understand how these treatments affect anti-PD1 treatments.

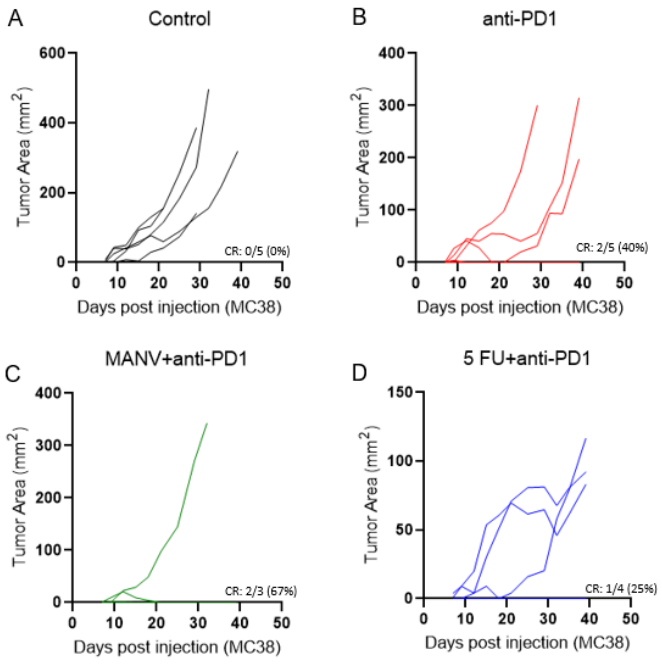

Figure 6: Tumor Growth Curve Analysis of Experiment 2 Mice. Analysis shows control (A), anti-PD1 treatment alone (B), antibiotics and anti-PD1 (C), and chemotherapy and anti-PD1 (D).

This round only had 5 mice per group and not all mice grew tumors, so clearance rates were only based off mice that did grow tumors. Despite this, the results were still following the same trend as seen at the UPMC Hillman Cancer Center DLAR facility. 5-flurouracil and anti-PD1 (Figure 6 D) had the lowest clearance rate of all the treatment groups, being only 25%. The anti-PD1 alone (Figure 5 B) group had a 40% clearance rate, and the MANV antibiotics and PD-1 (Figure 6 C) group were at 67% by the end of the study. By this point, it has also become noted that the tumors that received any anti-PD1 treatment at all had the tumors clear by around day 20 post tumor injection.

While a trend has started to emerge, several of the MC38 injections failed and would not allow us to get an accurate sense of what the average clearance rate of each condition looked like. To improve the overall number of mice that were positive for tumor growth, we looked for a new

source of MC38 cells. The new cells were grown as previously described in 3.1 and injected to measure tumors and improve the number of tumors overall.

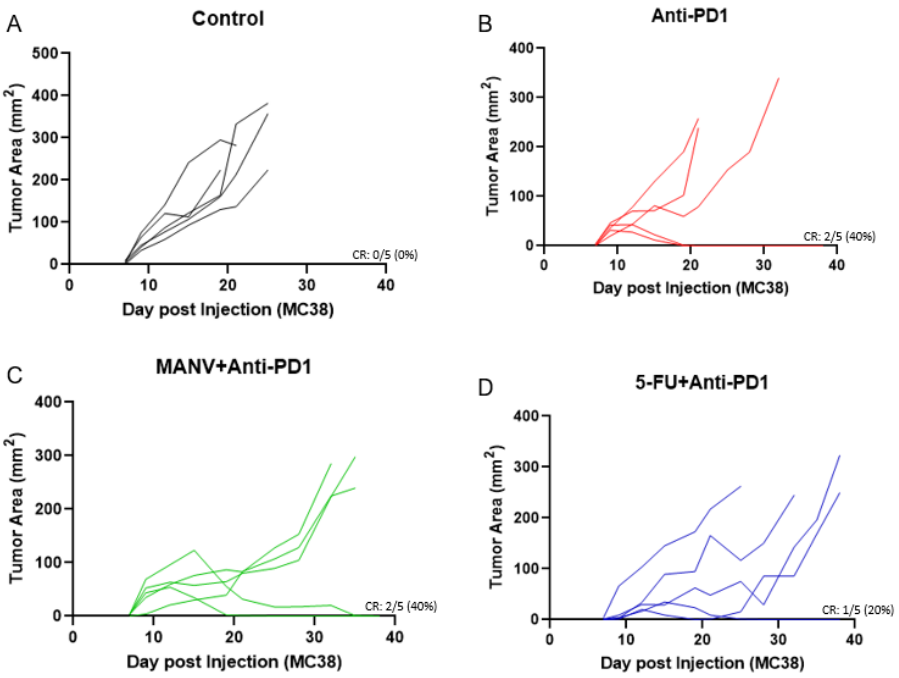

Figure 7: Tumor Growth Curve Analysis of Mice Injected with Newly Sourced MC38 Cells. Analysis shows control (A), anti-PD1 treatment alone (B), antibiotics and anti-PD1 (C), and chemotherapy and anti-PD1 (D).

The new cells were observed to have improved the number of mice that grew tumor nodules, which helped to improve the accuracy of the clearance rates in each group (Figure 7 A-D). Following the same protocol as seen in Figure 4, we observed that there is still a consistency between each experiment. Treating with anti-PD1 alone (Figure 7 B) has a tumor clearance rate of 40% overall, but when giving 5-flurouracil chemotherapy before starting anti-PD1 treatment (Figure 7 D) reduced the tumor clearance rate down to only 20%. The antibiotics and anti-PD1 group did have a 40% clearance rate, which is comparable to the anti-PD1 alone group. The anti-PD1 group also started to show that mice who had their tumors cleared by the immune system

started to show a decrease in tumor growth at around day 12 post injection. When comparing both antibiotics and anti-PD1 (Figure 7 C) and chemotherapy and anti-PD1 (Figure 7 D), anti-PD1 alone led to a faster immune response time to clear the tumor. Mice treated with either antibiotics or chemotherapy would have their tumors grow out, but they took longer to get to the study endpoint (35 days for chemotherapy and antibiotics compared to 30 days for anti-PD1 alone).

With the improvement in overall positive tumor growth in mice, we decided to also continue with manipulating the microbiome to understand how the immune system will react to cancer. An observation was made in human melanoma patients (14) that individuals who had a more diverse microbiome had better outcomes with their immunotherapy treatments.

The mice are normally kept under immunocompromised conditions, which keeps their food and water sterile to avoid introduction of unwanted microbes, but we wanted to see if there would be an improvement of anti-PD1 efficacy if the mice encountered more types of microbes.

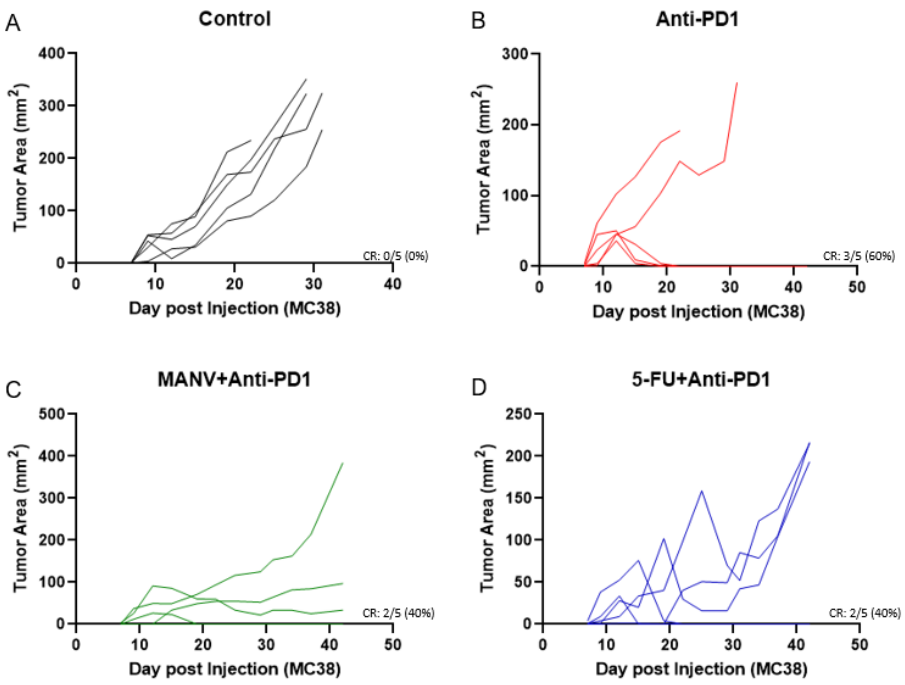

Figure 8: Tumor Growth Curve Analysis in Mice Housed with Diverse Bacteria Sources. Analysis shows control (A), anti-PD1 treatment alone (B), antibiotics and anti-PD1 (C), and chemotherapy and anti-PD1 (D).

The tumor growth results (Figure 8 A-D) were taken after animals were exposed to specific pathogen free (SPF) bedding on day 7. This was done to expose the mice and diversify their microbiomes compared to mice who are kept in sterile conditions. It was observed that the trends stayed consistent, whereas anti-PD1 alone (Figure 8 B) had a faster response and improved tumor clearance rate (60% compared to 40%). Tumor clearance for anti-PD1 alone occurred at around day 15, and the other groups that received treatment (antibiotics and immunotherapy and chemotherapy and immunotherapy) had slower tumor growth rates. The one newer observation was that Anti-PD1 alone had a higher clearance rate (60%) compared to previous experiment runs where the mice were kept in immunocompromised conditions. There was no change in clearance rate for the antibiotics and anti-PD1 group, but there was an increase to 40% tumor clearance for

the chemotherapy and PD-1 group, which may show that we might be able to rescue a microbiome and increase anti-PD1 efficacy.

4.3 Analysis of Survival of Mice Treated with Anti-PD1 and Traditional Cancer Treatments

Another important aspect of cancer treatment is looking at overall survival. Stage IV CRC patients only have an average 5-year survival of less than 15% (5), which also gives us the goal of trying to improve the overall survival of patients. While analyzing the overall growth rates of the tumors in the injected mice, we also looked at how survival was impacted by these treatments.

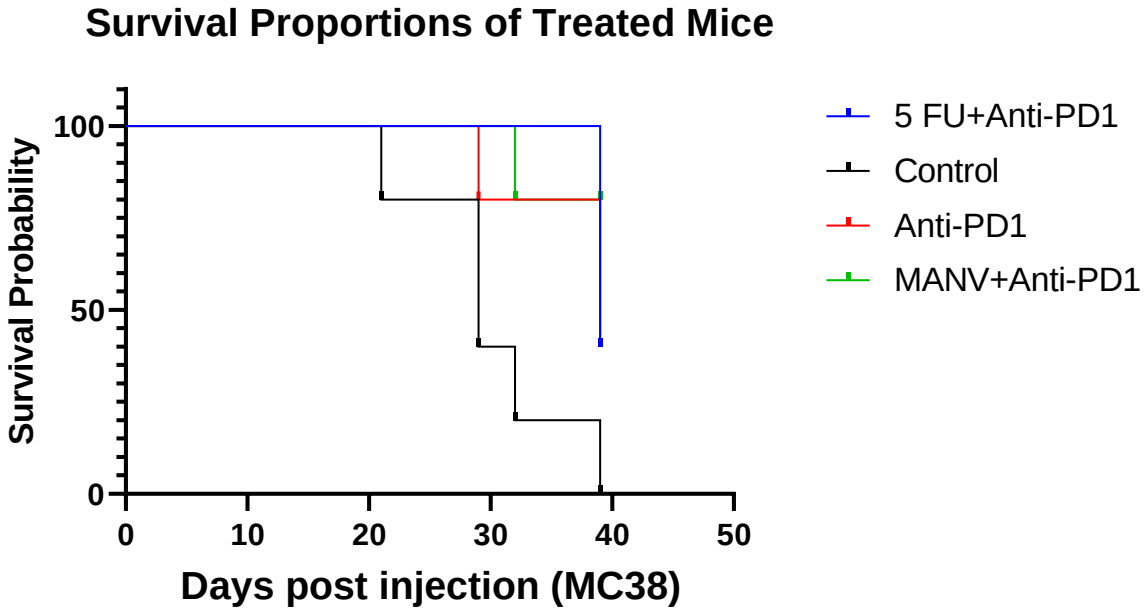

Figure 9: Survival Proportions of Mice with MC38 Tumors Treated with Anti-PD1 and Traditional Cancer Treatments.

We looked at the survival probabilities (Figure 9) of the mice represented in the experiment in Figure 6. Overall, receiving treatment did increase the lifespan of the mice who developed tumors. Mice who received no treatment in the control group had a less than 50% survival proportion at day 30 post injection, while the anti-PD1 alone, 5-flurouracil and anti-PD1, and antibiotics and anti-PD1 all had 75% survival at this time point. Mice in the control group were observed to have reached study endpoints starting at day 20 post injection, while the mice in other groups did not start to reach their endpoints until day 30 post injection.

With these encouraging results, we wanted to continue to look at the survival of mice that receive traditional cancer treatments and/or immunotherapy. We faced the same concerns with the survival proportions as we did with the tumor growth curve analysis, mice who did not develop tumors are injection would have affected the survival analysis. After observing tumors develop in mice who were injected with MC38 cells from a new source, we decided to move forward and analyze the survival proportions of the mice who all developed tumors in Figure 7. The main goal is to see if there are similar survival trends after changing MC38 cells.

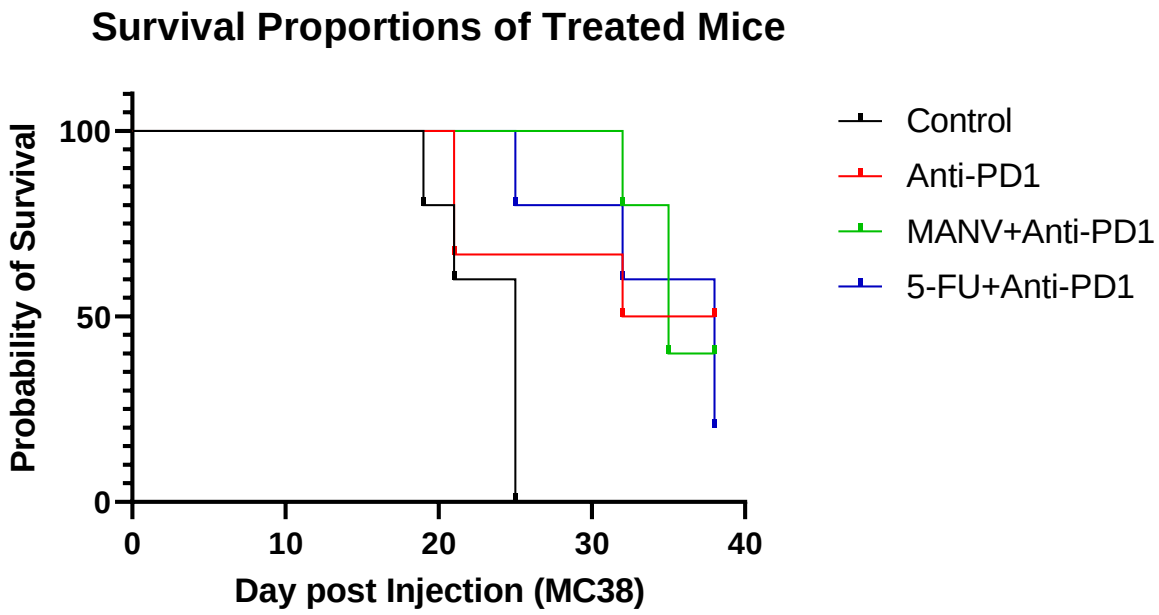

Figure 10: Survival Proportions of Mice with Newly Sourced MC38 Cell Tumors Treated with Anti-PD1 and Traditional Cancer Treatments.

The control group showed similar survival trends to the control group in Figure 9. Mice that receive treatment will live longer than those who do not. Although the group that received anti-PD1 alone had 40% of the group surviving to day 40, but only 20% of 5-flurouracil and anti-PD1 mice surviving to day 40. These numbers reflect the results seen above, as the mice who had their tumors clear survived past any others that had their tumors grow out. Although these treatments do prolong survival of the mice who receive it, the tumors do eventually grow out, which results in death. So far, it has become evident that animals who do not have any gut disturbances that would alter the baseline microbiome are more likely to have a better response to anti-PD1 immunotherapy for CRC.

4.4 Analysis of the Immune System Response in Mice Treated with Anti-PD1 and Traditional Cancer Treatments

It has become clear that there are changes taking place from the treatments that are affecting how the immune system is responding to the MC38 tumors in the mice. We decided our next step was to complete flow cytometry analysis on the immune cells found in the lymph nodes and the tumor to see how they have changed after the different treatments. It was decided that the model would be modified to do an analysis of the earlier stages of the immune system after the mice receive their respective treatments.

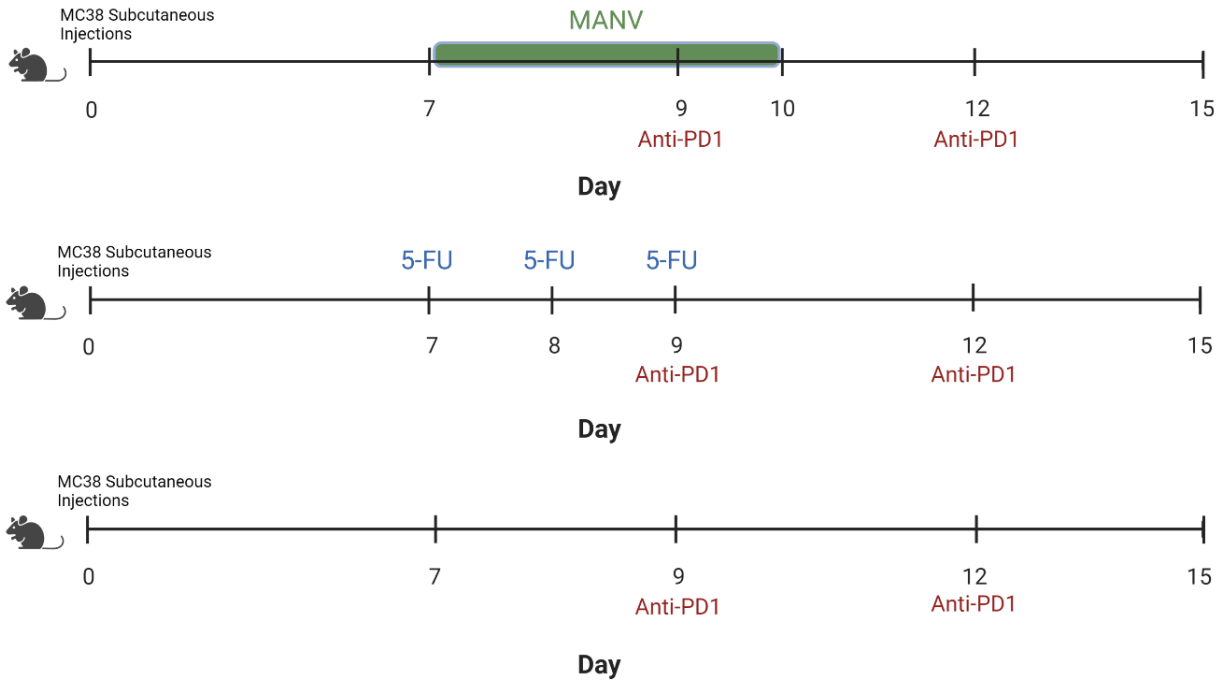

Figure 11: Modified Timeline of Murine Model Used to Assess Anti-PD1 Efficacy. Created with *Biorender.com*.

Mice were euthanized on day 15 post MC38 injection for organ and tumor harvest to process for flow cytometry. Results shown in figures 5, 6, and 7 showed that mice in the anti-PD1 alone group started to clear tumors around day 15. We hypothesized that this timepoint would be early enough in the model to be able to see how the immune system is responding to the tumor after being exposed to different treatments.

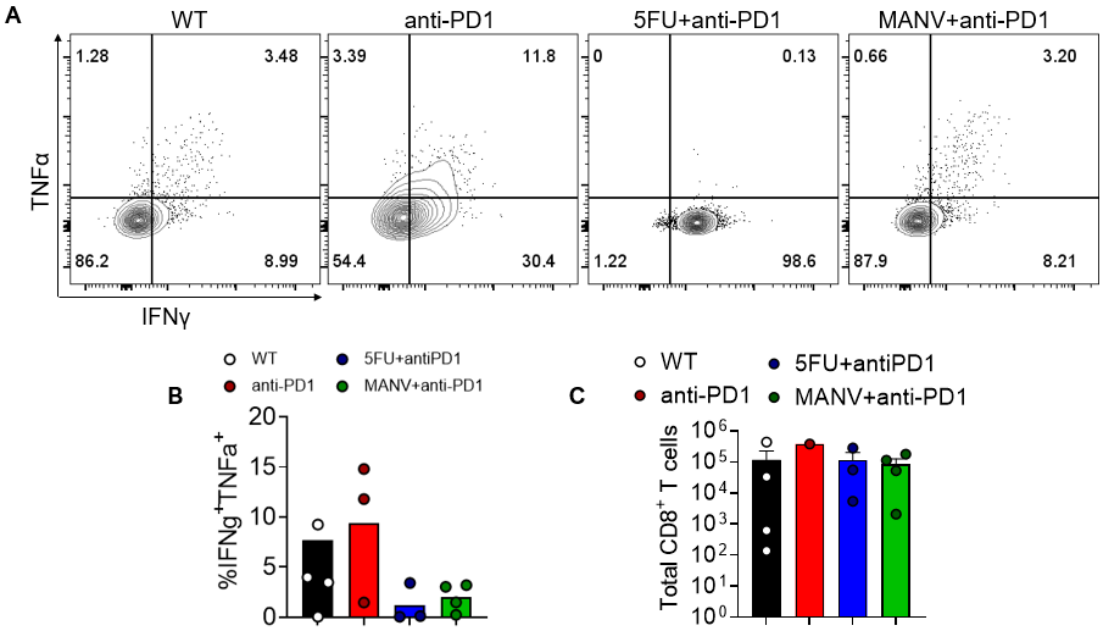

Figure 12: Flow Cytometry Results of Cytokine Production of CD8+ T Cells Found in MC38 Tumors. Flow cytometry plots showing levels of TNFa and IFNg (A) are shown with graphical representation of the double positive CD8+ cells (B) and the total number of CD8+ cells found in the tumor (C).

Along with the mice represented in Figure 6, mice were injected and treated on the same days that would follow a modified timeline (Figure 11) for flow cytometry analysis. The colons, tumor draining lymph node, tumor nondraining lymph node, mesenteric lymph nodes, and tumors were collected to be stained for analysis. The cells from each sample were stained (Table 1) for

cytokine markers that would help give insight into how CD8+ T cells are responding to the presence of cancer. The CD8+ T cells found in the tumors were expressing significantly different levels of cytokines between the different groups. The flow plots comparing levels of TNFa and IFNg (Figure 12 A) show that the anti-PD1 group alone had a larger population of CD8+ T cells that expressed both TNFa and IFNg, which we call polyfunctional T cells. Previous studies show that polyfunctional T cells are beneficial in clearing both infections and cancer (17, 18). The flow cytometry analysis also shows that almost all the CD8+ T cells in the chemotherapy and anti-PD1 group tumors express high levels of IFNg. T-cell exhaustion occurs over stages and usually involves losing the function of different cytokines, leaving and exhausted T cell with higher expression of IFNg (16). We do observe polyfunctional CD8+ T cells in the control group, but we hypothesize that there are not enough of these cells to effectively fight the cancer cells. The flow cytometry results match up with the tumor growth curve data, anti-PD1 alone has a greater population of polyfunctional T cells, and they had higher tumor clearance rates. Chemotherapy and anti-PD1 have evidence of exhausted T cells, which correlates with the lower clearance rates. When looking at the antibiotics and anti-PD1 group, there was a higher tumor clearance rate, but unlike anti-PD1 alone, they do not have the same population size of polyfunctional T cells.

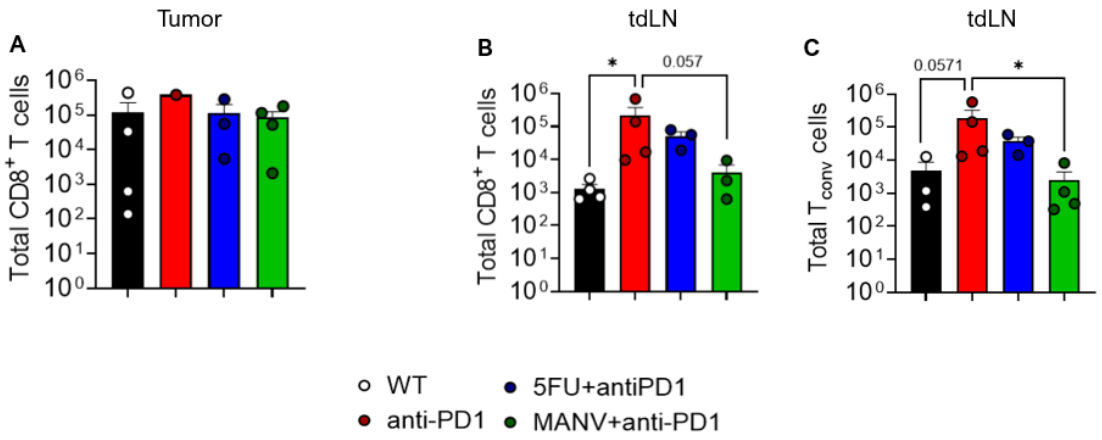

Figure 13: Cell Counts of CD8+ T Cells from Tumor and Draining Lymph Nodes. Total CD8+ T cells found in the tumor (A), total CD8+ T cells found in tumor draining lymph node (B), and total conventional T cells found in tumor draining lymph node (C).

These counts show that overall, there is no significant difference in the number of CD8+ T cells that were found in the tumors between each of the treatment groups. This shows that treatments may not affect the overall movement of the T cells into the tumor. It was found that there is a significant difference in the overall numbers of CD8+ T cells found in the draining lymph nodes found directly under the tumors. There were higher amounts of CD8+ T cells found in the lymph nodes of mice treated with anti-PD1 compared to the control group that had no treatment. The difference in T cells present in the tumor draining lymph node may indicate that blocking the PD1 protein with anti-PD1 allows for increased activation and proliferation of T cells. This increase in T cells from the increased proliferation would help to filter new T cells through the tumor and keep activated cells attacking the cancer cells present.

5.0 Conclusions and Discussion

Despite great advancements in medical technology and treatments, colorectal cancer (CRC) can evade these new medicines and remains one of the deadliest cancers. This is shown best through CRC's ability to evade the immune system and immune checkpoint inhibitors (ICI), save for a small population of tumors that do respond to therapy. These tumors, known as microsatellite instability-high (MSI-H) with a deficient mismatch repair mechanism (dMMR), respond extremely well to ICI treatments. This tells us that immunotherapy can work for CRC, but something is happening that is holding it back. This is the main question we decided to tackle in this project.

Beginning to understand what is going on in the tumor and the immune system to explain why ICI does not work in 90% of CRC tumors. Recent work has given us data to support that the gut microbiome has a large role to play in how the immune system responds to cancer. Most patients are given traditional treatments like radiation, chemotherapy, surgery, and broad-spectrum antibiotics to combat infections. Almost all these treatments cause gastrointestinal side effects including diarrhea, vomiting, and nausea. These side effects indicate that there is a fair amount of damage and inflammation of the gut that would cause changes in the microbiome. With all of this, we hypothesized that traditional cancer treatments change a responsive microbiome into an unresponsive microbiome. We define responsive as having a gut microbiota that interacts with the immune system to respond to ICI therapy. Unresponsive is defined as a gut microbiota that either does not interact with the immune system or even reacts in a negative manner that does not allow for a response to ICI therapy. The goal with the mouse models was to model the human patient

experience. The mice are given rounds of chemotherapy and rounds of antibiotics as a person would get if they were diagnosed with an infection or a cancer.

The tumor growth curves show consistent results between different cycles, which is optimistic for being able to repeat with different treatments and working closely with the microbiome. The pattern emerged showing that anti-PD1 treatment alone was able to clear more tumors, with clearance rates being anywhere from 40-60%, compared to only 20% for animals treated with 5-fluoruracil and anti-PD1. This gives insight into how gut dysbiosis starts to affect the immune system in a way that will have downstream effects on anti-PD1 immunotherapy efficacy. When also looking at the effects of broad-spectrum antibiotics, the clearance rates look very similar to anti-PD1 therapy alone, which was found to be a 40% clearance rate. Broad-spectrum antibiotics do cause some gut dysbiosis, but antibiotics reduce the overall numbers of the bacteria found in the gut. If there is no opportunity for more "pathogenic bugs" seen in non-responders (14) to grow and take their place, it may not have as much of a drastic impact on anti-PD1 therapy. We do not have much information on how chemotherapy changes the makeup of the gut microbiome, we just know that there is gut dysbiosis from observing that the small intestine and colons of these mice are inflamed and distended. The results from the tumor growth curves does support that gut dysbiosis from traditional parts of cancer treatment, such as the use of broad-spectrum antibiotics and chemotherapy, supports a non-responsive gut microbiome, leading to lower clearance rates in the mice.

With the data from the tumor growth curves being so consistent across different cycles and even between different DLAR facilities, the next question was what was happening to the T cells that are being exposed to these treatments. Cells from tumor non-draining lymph nodes, draining lymph nodes, mesenteric lymph nodes, and the tumor were collected and stained for cytokines.

Seeing the change in the CD8+ T cells that were expressing TNFa and IFNg was striking. We chose day 15 as the day for tissue harvest, as we were interested in seeing what was happening at the time when the immune system would most likely be activated and fighting the tumor cells. To see that on day 15 the chemotherapy and anti-PD1 group had almost exclusively CD8+ T cells that secreted high amounts of IFNg really showed that the gut dysbiosis did not take long to start and that the effects on the immune system were immediate. Day 15 is still early in the cycle, as they would have only gotten two doses of anti-PD1 and three doses of 5-flurouracil. To see that the CD8+ T cells that are in the tumor at this time point are exhausted can partly explain why we see lower clearance rates in the mice that get chemotherapy along with anti-PD1. When looking at the anti-PD1 only group, there is a larger population of CD8+ T cells that secrete TNFa and IFNg that are polyfunctional. These types of T cells have been found to be beneficial in cancer and some infectious diseases, so this can help to understand why there is a higher clearance rate in mice that only receive anti-PD1. While these two results are helpful in explaining what is going on with the immune system, it was observed that the group that was fed antibiotics and got anti-PD1 looked very similar to the control group. We would have expected more polyfunctional T cells in the tumor because the clearance rates of the antibiotics and anti-PD1 group are like the anti-PD1 alone group. It raises the question as to what is going on in the immune system to explain why the antibiotic and PD-1 mice can clear tumors, but the control group doesn't clear any despite the cytokine profile looking almost the same.

The experiment detailed in Figure 8 was inspired by previous work done with melanoma patients that showed patients were more likely to respond to anti-PD1 therapy if their gut microbiomes had a more diverse group of bacteria making it up. The mice are kept in more sterile conditions with immunocompromised housing protocol to keep from introducing unknown

bacteria or repopulating the microbiome after antibiotic treatment. We decided to take bedding from specific pathogen free (SPF) mouse cages and put it in with our mice on day 7 post MC38 tumor injection. These cages are exposed to more bacteria through food and central water supplies, but there is not enough bacteria in these sources and personnel must still keep the housing areas clean to prevent contaminating all the mice in the room. This bedding would have more types of bacteria and we wanted to see if these bacteria can become a part of the mice's microbiome during treatment and if it would be able to help anti-PD1 treatment. We did see this to be true, as the clearance rate of tumors in the anti-PD1 group alone increased to 60% and we saw that our chemotherapy and anti-PD1 group also increased up to 40%. The antibiotics group and anti-PD1 group did not change, but this may be because the bacteria introduced would have been reduced after the antibiotic treatment that started that day. While we do not know what microbes were present after the exposure, this early experiment does support that having a diverse gut microbiome helps to have a better response to anti-PD1 therapy.

The group receiving both antibiotics and anti-PD1 had similar tumor clearance trends with anti-PD1 treatment alone. To test and see if giving antibiotics benefits the anti-tumor response, mice were injected with MC38 cells and given either no treatment or antibiotics, but no anti-PD1 treatment. Antibiotics were given in the water from days 7-10 as previously described.

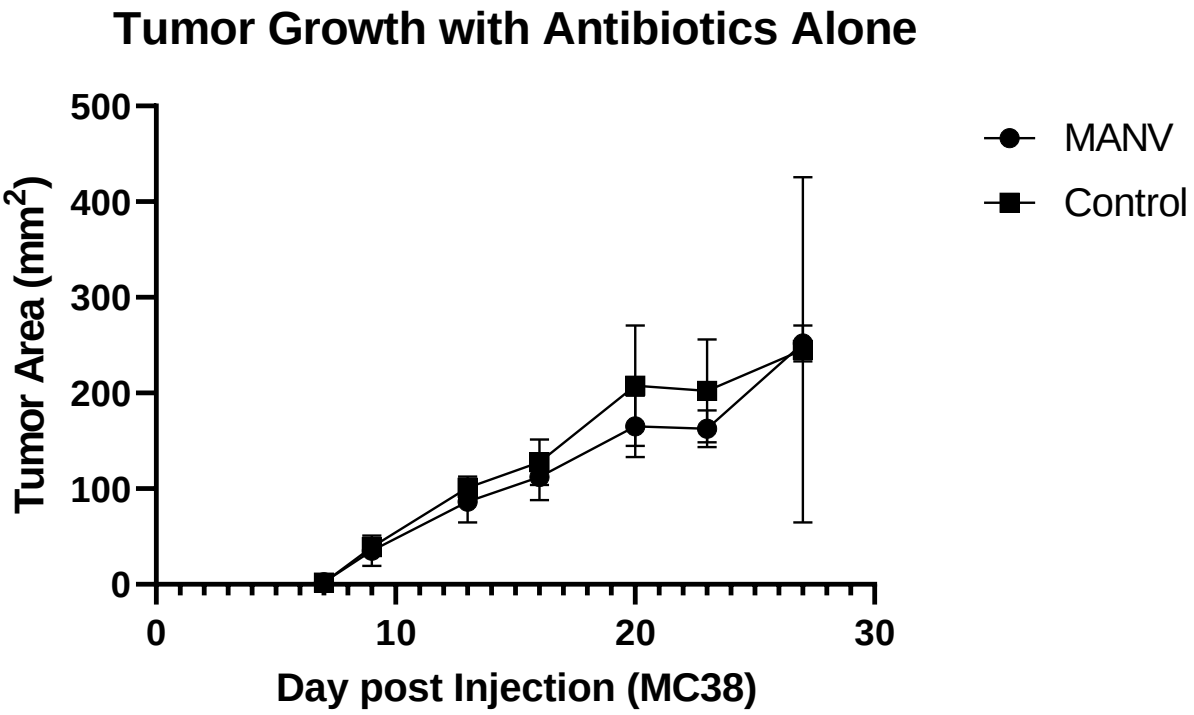

Figure 14: Tumor Growth Curves of Mice Receiving Antibiotics Alone.

Results from that trial (Figure 14) show that there is no difference between the group that received antibiotics and the group that had no treatment. This supports that antibiotic depletion of the microbiome does not benefit nor harm the anti-tumor response.

During the second experiment detailed in Figure 6, stool was also collected from each mouse in each group on days 1, 7, 11 and 19 post MC38 tumor injection to analyze the changes in the makeup of the gut microbiome over treatment cycle using 16S analysis. The stool was collected in a sterile method and frozen at -80C before being processed to extract bacterial DNA from the pellet. After all the DNA was extracted from each sample, the DNA was sent off to Microbiome Insights© for 16S processing and raw data extraction. We are currently waiting for the data to be sent back to us for further analysis.

6.0 Limitations

Early pilot experiments showed that there were issues with tumor growth in some of the mice that were injected with MC38. This skewed some of the results seen, an example being Figure 6, where only 3 mice developed tumors. 2 of those tumors cleared after anti-PD1 treatment, but may have falsely elevated the clearance rate, as it was 67%. We obtained these new MC38 cells from the Vignali lab and ran a cycle to see if the tumors grew in these mice. The original MC38 cells from the Delgoffe lab were observed to grow at a slower rate than normal MC38 cells, making us believe that something may have been wrong with the line. The new cells grew at a faster rate and all animals injected were able to grow a tumor. Because this was early enough in the project, we decided to switch out these cells as the main cell line used for injection. No testing had been done on the previous cells to look for any *Mycoplasma* species, so it is unknown if there are any underlying issues with the line.

Stool was collected and sent to Microbiome Insights©, but sequencing can take long periods of time before we receive the raw data back. In this case, it did not arrive in time for analysis for this work. These long wait times can slow down analysis and progress, so more planning must be done to ensure that samples are sent off in a timely manner after collection.

7.0 Future Directions

There are many plans for this project, as we want to keep looking into how the microbiome can be used to better immunotherapy treatments for more patients.

We are going to continue with the goals outlined in aim 1. We want to repeat the SPF bedding experiment and investigate the effects of adding more diverse bacteria to the mice and how that regulates the immune system and anti-tumor response. We are also working on repeating the previous flow cytometry panel and expanding the panels to include more markers, as well as increasing sample size and ensuring consistency. There are also plans to begin completing fecal microbiome transplants at the Pitt Gnotobiotic Core and work to see if we can rescue an "unresponsive microbiome".

Aim 2 goals will involve us analyzing the raw 16S data from Microbiome Insights© as well as collecting stool from mice who are being housed with SPF bedding. We want to keep investigating what changes are happening in the microbiome that are helping to better the anti-tumor response.

For our aim 3, we are currently partnering with UPMC Hillman Cancer Center clinicians to start a clinical trial to collect human samples to find a "responsive microbiome". We are also working to collect data to understand patterns in diet and how that affects the gut microbiome.

With the importance of the microbiome, as well as what changes the microbiome, we decided to investigate the sweetener that we add to our antibiotic water. A pilot experiment was completed comparing the tumor growth curves of mice who are fed different types of sweeteners. Results showed that sucralose had a negative impact and tumors grew faster in these mice. We

have now started a new project where we will investigate how sucralose negatively impacts the immune system and the gut microbiome to be pro-tumor.

8.0 Public Health Significance

Everyday, numerous people will be diagnosed with cancer. Cancer is a life-changing diagnosis and public health can play a great role in making it easier. Many cancers tend to be more curable when found early or when prevention measures are taken place to keep a cancer from ever forming. One example is how public health officials have worked to push the human papillomavirus (HPV) vaccine and have led studies to follow how the vaccine has changed the incidence of HPV caused cancers.

My work looks at how the gut microbiome regulates the immune system to help improve an anti-tumor response. We hope that my work can help to investigate a "responsive" and "unresponsive" microbiome that we can give to people to help them respond to more therapies and give more options.

We also want to investigate what makes an "anti-cancer" and a "pro-cancer" microbiome. We know that as people become unhealthier and develop gut dysbiosis, this may play a role in the development of cancer. We can use public health to better understand how we can use the microbiome to treat and prevent cancer in patients. We can also expand to understand how we can use the microbiome to better treat other types of health problems, like chronic viral infections and severe bacterial infections.

Bibliography

1. Institute NC. 2021. What is Cancer? https://www.cancer.gov/about-cancer/understanding/what-is-cancer. Accessed

2. Cooper G. 2000. The Development and Causes of Cancer, The Cell: A Molecular Approach. Sinauer Associates, MA.

3. Clinic M. 2022. Cancer. https://www.mayoclinic.org/diseases-conditions/cancer/symptoms-causes/syc-20370588. Accessed

4. Society AC. 2022. 2022 Cancer Facts and Figures. https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2022.html. Accessed

5. Rawla P ST, Barsouk A. 2019. Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors. Prz Gastroenterol doi:10.5114/pg.2018.81072.

6. Johnson CM WC, Ensor JE, Smolenski DJ, Amos CI, Levin B, Berry DA. 2013. Meta-analyses of colorectal cancer risk factors. Cancer Causes Control doi:10.1007/s10552-013-0201-5.

7. Wang Y BM. 2007. The obesity epidemic in the United States--gender, age, socioeconomic, racial/ethnic, and geographic characteristics: a systematic review and meta-regression analysis. Epidemiol Rev doi:10.1093/epirev/mxm007.

8. Yang Y DL, Shi D, Kong C, Liu J, Liu G, Li X, Ma Y. 2021. Dysbiosis of human gut microbiome in young-onset colorectal cancer. Nat Commun doi:10.1038/s41467-021-27112-y.

9. Yi M ZX, Niu M, Zhu S, Ge H, Wu K. 2022. Combination strategies with PD-1/PD-L1 blockade: current advances and future directions. Mol Cancer doi:10.1186/s12943-021-01489-2.

10. Ganesh K SZ, Cercek A, Mendelsohn RB, Shia J, Segal NH, Diaz LA Jr. 2019. Immunotherapy in colorectal cancer: rationale, challenges and potential. Nat Rev Gastroenterol Hepatol doi:10.1038/s41575-019-0126-x.

11. Motta R C-CS, Torres-Mattos C, Riquelme A, Calle A, Figueroa A, Sotelo MJ. 2021. Immunotherapy in microsatellite instability metastatic colorectal cancer: Current status and future perspectives. J Clin Transl Res.

12. Gatalica Z VS, Xiu J, Swensen J, Reddy S. 2016. High microsatellite instability (MSI-H) colorectal carcinoma: a brief review of predictive biomarkers in the era of personalized medicine. Fam Cancer doi:10.1007/s10689-016-9884-6.

13. Chen F DX, Zhou CC, Li KX, Zhang YJ, Lou XY, Zhu YM, Sun YL, Peng BX, Cui W. 2022. Integrated analysis of the faecal metagenome and serum metabolome reveals the role of gut microbiome-associated metabolites in the detection of colorectal cancer and adenoma. Gut doi:10.1136/gutjnl-2020-323476.

14. Gopalakrishnan V SC, Nezi L, Reuben A, Andrews MC, Karpinets TV, Prieto PA, Vicente D, Hoffman K, Wei SC, Cogdill AP, Zhao L, Hudgens CW, Hutchinson DS, Manzo T, Petaccia de Macedo M, Cotechini T, Kumar T, Chen WS, Reddy SM, Szczepaniak Sloane R, Galloway-Pena J, Jiang H, Chen PL, Shpall EJ, Rezvani K, Alousi AM, Chemaly RF, Shelburne S, Vence LM, Okhuysen PC, Jensen VB, Swennes AG, McAllister F, Marcelo Riquelme Sanchez E, Zhang Y, Le Chatelier E, Zitvogel L, Pons N, Austin-Breneman JL, Haydu LE, Burton EM, Gardner JM, Sirmans E, Hu J, Lazar AJ, Tsujikawa T, Diab A, Tawbi H, Glitza IC, Hwu WJ, Patel SP, Woodman SE, Amaria RN, Davies MA, Gershenwald JE, Hwu P, Lee JE, Zhang J, Coussens LM, Cooper ZA, Futreal PA, Daniel CR, Ajami NJ, Petrosino JF, Tetzlaff MT, Sharma P, Allison JP, Jenq RR, Wargo JA. 2018. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. Science doi:10.1126/science.aan4236.

15. Overacre-Delgoffe AE BH, Cillo AR, Burr AHP, Tometich JT, Bhattacharjee A, Bruno TC, Vignali DAA, Hand TW. 2021. Microbiota-specific T follicular helper cells drive tertiary lymphoid structures and anti-tumor immunity against colorectal cancer. Immunity doi:10.1016/j.immuni.2021.11.003.

16. Yi JS CM, Zajac AJ. 2010. T-cell exhaustion: characteristics, causes and conversion. Immunology doi:10.1111/j.1365-2567.2010.03255.x.

17. De Groot R VLM, Guislain A, Nicolet BP, Freen-Van Heeren JJ, Verhagen OJHM, Van Den Heuvel MM, De Jong J, Burger P, Van Der Schoot CE, Spaapen RM, Amsen D, Haanen JBAG, Monkhorst K, Hartemink KJ, Wolkers MC. 2019. Polyfunctional tumor-reactive T cells are effectively expanded from non-small cell lung cancers, and correlate with an immune-engaged T cell profile. Oncoimmunology doi:10.1080/2162402X.2019.1648170.

18. Rodrigues LS BA, Bomfim LGS, Gomes MC, Ferreira NLC, da Cruz GS, Magalhães LS, de Jesus AR, Palatnik-de-Sousa CB, Corrêa CB, de Almeida RP. 2021. Multifunctional, TNF-α and IFN-γ-Secreting CD4 and CD8 T Cells and CD8High T Cells Are Associated With the Cure of Human Visceral Leishmaniasis. Front Immunol doi:10.3389/fimmu.2021.773983.