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The Effect of the Elevated Circulating Levels of IL-33 on Metastasis of Colorectal Cancer

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Acknowledgements

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Tia Davis, Nikeya Tisdale, Danda Chapagai, Fides Elamparo

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Thesis Summary

In the United States, colorectal cancer serves as the third most commonly occurring cancer[1]. Approximately 143,000 new cases of colorectal cancer will be diagnosed this year in the United States, and about 51,000 of those cases will be fatalities[1].

If colorectal cancer is detected before stage III, the 5-year survival rate is almost 70%[4]. Previous research has shown that colorectal cancer development is a multistep process that evolves through a diverse molecular mechanism that can go undetected until metastasis occurs, which is often too late[4]. It is very important to identify the early genetic and molecular alterations that cause cancerous tumor cells to target specific organs. It is widely known that metastasis is not a random process and cancerous tumor cells that get into circulation don't always metastasize to target organs. Researchers found that certain genes expressed by the tumor cells direct the metastasis of those cells to specific target organs[11]. Therefore, subsequent research must be done in order to identify those specific genes and their genetic pathways.

The interleukin-33 (IL-33) gene encodes a newly identified pro-inflammatory, signaling cytokine that is secreted by colon cancer cells and apoptotic cells. The secretion of the IL-33 cytokine provides a link between

inflammation and cancer and plays a major role in tumor cells' resistance to apoptosis and chemoresistance in tumor cells. This indicated that the IL-33 signaling pathway plays a crucial role in the pathogenesis of colon cancer[5]. IL-33 is also an alarmin secreted by necrotic cells upon trauma and infection, which is necessary for proper virus control in the immune system. Using microarray analysis, Dr. Pena's laboratory identified IL-33 as the most highly secreted cytokine in the highly metastatic colon cancer cell line, CT26-FL3 as compared to the parental CT-26 cells. In this study, we will test our hypothesis that the increased circulating levels of IL-33 in serum of tumor bearing mice will increase colorectal cancer metastasis to the liver. The importance of IL- 33 in colon carcinogenesis and immune system responses is a widely accepted concept, but also a highly under-researched topic, which makes this particular project very unique and groundbreaking.

Abstract

In the United States, colorectal cancer is the third leading cause of cancer-related deaths in both men and women[1]. The pathogenesis of colorectal cancer begins with the development of polyps in the innermost colorectal lining and progresses to the final stage when metastasis occurs[2]. Metastasis is the spread of cancer cells to other organs, and has been shown to be organ specific rather than a random process. Genes that direct tumor cells to target specific organs have been identified. The primary tumor is thought to secrete molecules that promote the establishment of liver metastasis even before the arrival of cancer cells into this organ.

We have previously isolated a highly liver metastatic cell line, CT26-FL3 by in vivo selection of CT26 cells in balb/c mice. Microarray analyses showed that the CT26-FL3 cells expressed 34-fold higher levels of the Interleukin-33 (IL-33) cytokine as compared to the parental and less metastatic CT26 cells. Over-expression of IL-33 was shown to potently promote tumor proliferation and metastasis to the liver[5] indicating that IL-33 plays a crucial role in the pathogenesis of colon cancer.

In this study, we will use C57Bl/6 mice and the murine colon cancer cell line, MC38, to increase circulating serum levels of IL-33 using a gene therapy approach by in vivo electroporation of a plasmid expressing IL-33,

pV1J-IL33. We hypothesized that the increased secretion of IL-33 in MC38 tumor cells will increase cell metastasis to the liver, and that increasing circulating levels of IL-33 in mice bearing MC38 tumors will be sufficient to enhance its ability to metastasize to the liver.

Here we report the construction of plasmids expressing IL-33 and verification of the expression of IL-33 from these plasmids in transfected cells by Western Blot analyses. We also discuss the introduction of pV1J-IL-33 into C57Bl/6 mice by quadriceps injections and in vivo electroporation. Lastly, we report ELISA assays on mouse sera after splenic injections of MC38 tumor cells to determine the levels of secreted IL-33 and how long the IL-33 levels stayed up regulated after in vivo electroporation.

We concluded that the elevated serum levels of IL-33 increased tumor proliferation and their ability to metastasize to the liver in C57Bl/6 mice.

Introduction

Colorectal Cancer in Humans

The pathogenesis of colorectal cancer occurs in five stages beginning with Stage 0 where cancer cells are developing only in the innermost colorectal lining. Cell division and apoptosis are heavily regulated under normal conditions. Cancer cells have unchecked cell growth due to gene mutations. These mutations can cause accelerated cell division rates and inhibit control mechanisms such as apoptosis and cell cycle arrest.

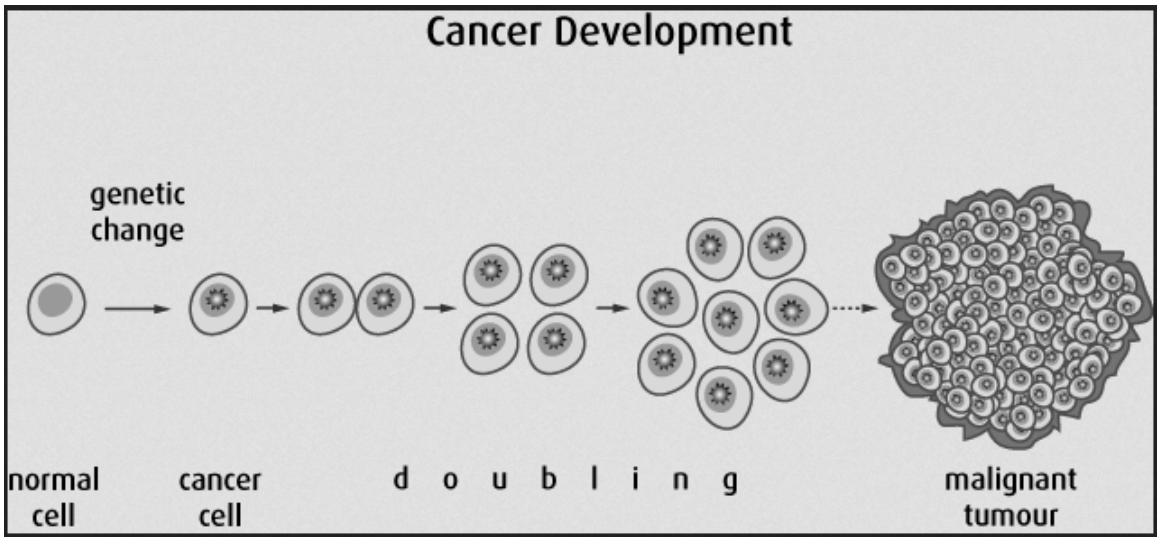

Figure 1. A normal cell developing into a cancerous tumor.

At Stage I, these cancerous cells collectively develop into a tumor on the inner colorectal walls, which then begin to extend deeper into the colorectal walls in Stage II. At Stage III, the cancerous cells begin to spread to the nearby lymph nodes and circulate through the blood stream. The final Stage IV of colorectal cancer is metastasis where the cancerous cells spread

to other parts of the body and invade other organs such as the liver and the lungs[2]. Unfortunately, colorectal cancer can develop without any symptoms and it usually isn't detected until a primary tumor is found or metastasis has already occurred in the patient. Once metastasis has occurred, the five-year survival rate decreases to about 6%[1].

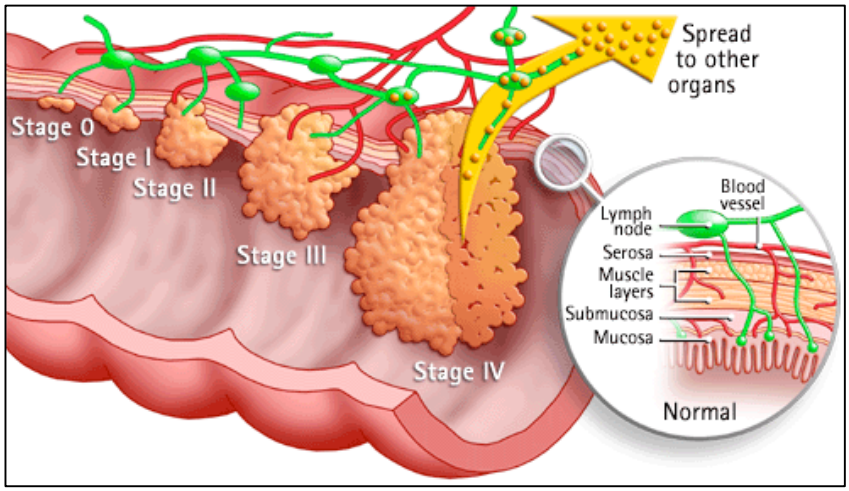

Figure 2. The pathogenesis of colorectal cancer from Stage 0 to Stage IV.

Before cancerous colon cells metastasize to the liver, the primary tumor sends signals to the liver to recruit bone marrow derived cells that create a pre-metastatic niche. This pre-metastatic niche provides a fertile environment in the liver that promotes the growth of these cancerous colon tumor cells. Therefore, it is also important to identify the specific signals that are sent from the primary tumor to the target organ in order to prepare the pre-metastatic niche for the cancerous colorectal cells to home in.

Previous Work by Dr. Peña's Lab

Highly Metastatic Mouse Model. Dr. Peña's lab created a mouse model of colon cancer using balb/c mice. They injected a parental line of CT26 colon cancer cells into the wall of the cecum of these balb/c mice in order to develop a primary tumor that spontaneously metastasizes to the liver. They found that only 8% of the balb/c mice injected with these CT26 cells had liver metastasis. They made a single cell suspension from these tumor cells, injected those cells into the cecum of the balb/c mice, and increased liver metastasis to 50%. They took the liver metastasis cells, grew them in culture, and injected those cells into the cecum. By repeating this procedure three times, the level of metastasis was increased to 90% and this new cell line was called the CT26-FL3 cell line. This created a CT26 cell line with a low level of metastasis and a CT26-FL3 cell line with a high level of metastasis.

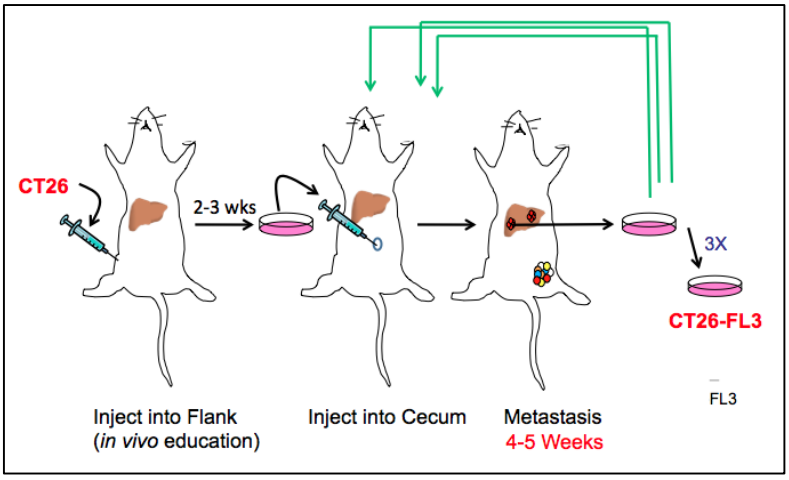

Figure 3. Development of CT26-FL3 cell line with 90% liver metastasis.

Elevated Circulating Levels of Interleukin-33. Microarray analysis shows all 44,000 genes expressed in a mouse on one slide, which allows you to analyze the expression levels of those genes at the same time. This allowed them to look at the genes that were highly expressed in the CT26 parental cell line versus the genes expressed in the highly metastatic CT26-FL3 cell line. After real time PCR was performed, it was observed that one of the most highly up-regulated genes was Interleukin-33 (IL-33). It was up-regulated by the CT26-FL3 cells almost 34 fold when compared to the parental CT26 cells, while the protein expression levels remained the same between the two cell lines. This implies that the IL-33 is being sent out of the CT26-FL3 cells to affect other tissues and organs, which raises the question of what role the IL-33 gene is playing in metastasis and why it is being up-regulated and excreted from the cells.

Study Objectives

The major objective of my project is to determine the role of Interleukin-33 (IL-33) in the metastasis of colorectal cancer to the liver. I will test the hypothesis that the elevated serum level of IL-33 is sufficient to enhance tumor growth and liver metastasis of tumors derived from MC38 cells. Because IL-33 can also be secreted by hematopoietic cells in the tumor microenvironment that are involved in colorectal cancer metastasis, the

results from my experiments will complement those that have been described above.

Materials and Methods

The cell lines used in the experiments were CT26 colon carcinoma cells and MC38 murine colon carcinoma cells. The mice used were balb/c mice and C57Bl/6 mice. Splenic injections of CT26 cells and CT26-FL3 cells were performed on balb/c mice. Splenic injections of MC38 cells were performed on C57Bl/6 mice. All procedures were approved by the Institutional Animal Care and Use Committee.

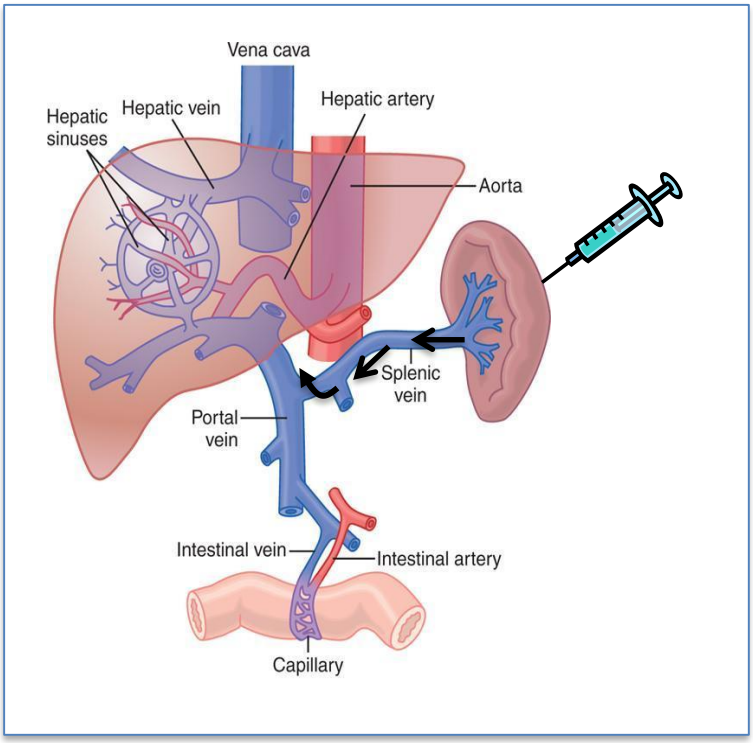

Figure 4. A schematic model of splenic injection of cancer cells.

Cloning IL-33 into pV1J Plasmid

A plasmid expressing IL-33 was previously constructed in the Peña lab through DNA recombination. To start, restriction enzymes Kpn I and Eco RV digested the pCMV6-IL33 plasmid and the pV1J plasmid. Through gel electrophoresis, the restriction fragments were separated based on size leaving a pCMV6 plasmid fragment, a pV1J plasmid fragment, and an IL-33 protein fragment. The pV1J and IL-33 fragments were ligated using T4 DNA ligase and ligation buffer to form the pV1J-IL33 plasmid to be used in the remainder of the experiments.

Plasmid Isolation Using Pure Yield Plasmid Miniprep System

Two flasks were prepared, each containing 25mL of Luria broth and 50μL of Kanamycin Marker. A sample of the pV1J-IL33 plasmid selected from colony 11 of the IL-33 stock plate was mixed into each flask. The flasks were stored in the 37°C incubator overnight. The following day, the samples were aliquoted into 4 tubes and centrifuged for 5 minutes at 3500rpm. Supernatants were discarded and 600μL of Molecular Biology Grade Water was added. The solutions were resuspended and aliquoted into 4 1.5mL Eppendorf tubes for lysate preparation.

100µL of Cell Lysis Buffer was added to the solution, the tubes were then inverted 15 times, and the solution processed for 5 minutes. 350µL of Neutralization Solution was added and the tubes were inverted until a homogenous yellow mixture was formed. The samples were centrifuged at 13,000rpm for 3 minutes and the supernatants were transferred into the Pure Yield Minicolumns, which were then placed into a collection tube. The collection tubes were centrifuged for 1 minute at 13,000rpm and the excess flow through was discarded.

To remove protein, RNA, and endotoxin contaminants from the purified plasmid DNA, 200µL of Endotoxin Removal Wash was added to the Minicolumns and they were centrifuged for 1 minute at 13,000rpm. 400µL of Column Wash Solution was added to the Minicolumns and they were centrifuged for 1 minute at 13,000rpm.

To elute the plasmids, the Minicolumns were transferred to 4 1.5mL Eppendorf tubes and 30µL of nuclease free water was added to the Minicolumns. They were processed for 1 minute and centrifuged at 13,000rpm for 1 minute. The Minicolumns were discarded and the Eppendorf tubes were stored at -20°C.

Restriction Enzyme Digestion

After the pV1J-IL33 plasmid DNA was purified, restriction enzyme digestion with 4 replicate samples was used to confirm the presence of the IL-33 protein in the pV1J plasmid. The following was added to each of the 4 1.5mL Eppendorf tubes in order: 10.5µL of Molecular Biology Grade Water, 1.5µL of NEB Buffer 3.1, 0.5µL of restriction enzyme EcoRV, 0.5µL of Bgl2, and 2µL of pV1J-IL33 plasmid. The Eppendorf tubes were incubated at 37°C for 2 hours. Gel electrophoresis was run for 25 minutes at 150V, which separated the pV1J plasmid and the IL-33 protein in the 4 replicate samples.

Transient Transfection of pV1J-IL33 Plasmid into CT26 Cells and MC38 Cells Using Lipofectamine 2000

Cell Culture. CT26 stock cells, MC38 stock cells, and cell media containing Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 1% Penicillin-Streptomycin were thawed at 37°C. 4 cell plates were prepared under the hood – 2 plates for CT26 cells and 2 plates for MC38 cells. 10mL of media and 1mL of cells were added to each plate, respectively. The cell plates were incubated at 37°C for 72 hours.

After 72 hours, the cell cultures were split. The previously used cell media and Trypsin-EDTA were thawed at 37°C. Under the hood, 5mL of Trypsin-EDTA was added to each cell plate and processed for 5 minutes. After 5 minutes, 5mL of cell media was added to each plate and mixed thoroughly. Each of the 10mL cell solutions were added to 15mL tubes and centrifuged for 5 minutes at 2,000rpm. The supernatants were discarded, and 5mL of cell media was used to resuspend the pellets. Onto 4 new cell plates, 5mL of cell media was added along with 2.5mL of the resuspended cells solutions. The new cell plates were incubated at 37°C.

After 96 hours, the cells were split again using the same procedure. The MC38 cells were split into a cell plate and 2 6-well plates. The CT26 cells were split into a cell plate and 2 6-well plates. The cell plates contained 10mL of cell media and 1 drop of cells. Each well of the 6-well plates contained 2.5mL of cell media and 250μL of cells. Each cell plate and 6-well plate was thoroughly mixed and incubated at 37°C.

Transient Transfection. The 6-well plates of MC38 Cells and CT26 Cells were used for transient transfection. On each plate - 2 wells served as sample controls with no plasmid, 2 wells served as vector control samples with pV1J plasmid, and 2 wells served as experimental samples with pV1J-

IL33 plasmid. Spectrophotometry determined the concentrations of the purified pV1J plasmid and the purified pV1J-IL33 plasmid to be used in the transient transfection.

Under the hood, the old media was suctioned off and 2mL of cell media containing DMEM and 10% FBS was added to each well. 6 tubes were labeled as Control A, Control B, pV1J A, pV1J B, IL-33 A, and IL-33 B. The Control A tube contained 400µL of DMEM. The pV1J A tube contained 400µL of DMEM and 18.1µL of pV1J vector. The IL-33 A tube contained 400µL of DMEM and 21.62µL of pV1J-IL33 plasmid. The Control B tube, the pV1J B tube, and the IL-33 B tube all contained 400µL of DMEM and 80µL of Lipofectamine 2000. After 5 minutes of processing, the A and B tubes of each respective sample type were combined. The combined control solution, the combined pV1J solution, and the combined pV1J-IL33 solution processed for 15 minutes. Each solution was evenly distributed and mixed into their respective wells of the 6-well plates and incubated for 72 hours at 37°C.

Protein Isolation

A 6-well plate from both the MC38 transfected cells and CT26 transfected cells were used for protein isolation from these cell samples.

500µL of Trypsin was added to each well and processed for 5 minutes. After 5 minutes, 500µL of media was added to each well and the 1mL solution from each well was aliquoted into 1.5mL Eppendorf tubes. The tubes were centrifuged for 5 minutes at 3,000rpm and the supernatants were removed. 500µL of PBS was added to each Eppendorf tube, the tubes were centrifuged for 5 minutes at 3,000rpm, and the supernatants were discarded.

100µL of Protein Extraction Buffer was added and mixed into each cell pellet. After 10 minutes of incubation on ice, the tubes were centrifuged for 40 minutes at 11,000rpm. The supernatants were collected into new Eppendorf tubes and the protein concentrations in the samples were determined using Gen5 Data Analysis Software. The protein samples were stored in -80°C.

Western Blot Analysis

The intracellular levels of IL-33 in the CT26 cells transiently transfected with pV1J and pV1J-IL33 were measured using a Western Blotting technique. The isolated protein samples were mixed with 6X SDS and Molecular Biology Grade Water and heated for 10 minutes at 95°C. The proteins were separated through agarose gel electrophoresis at 150V for 60 minutes. The proteins were then transferred to a nitrocellulose membrane at

100V for 100 minutes.

The membrane was blocked for 1 hour with a 5% milk solution comprised of 2.5g of Bio-Rad Blotting Grade Blocker Nonfat dry milk and 50mL of PBST. The MAB3626 anti-mIL-33 primary antibody was diluted 1:500 with PBST and added to the membrane for overnight incubation at 4°C. The membrane was washed 3 times for 10 minutes with PBST. The PAI-28636 Thermo goat anti-rat secondary antibody was diluted 1:2500 with PBST and added to the membrane for 1 hour of incubation at room temperature. The membrane was washed 3 times for 10 minutes with PBST. 500µL of each ECL Western Blot Detection reagent was mixed and added to the membrane for 2 minutes. Nitrocellulose membrane images were quantified using chemiluminescence.

RNA Isolation Using Bio-Rad RNA Isolation Kit

The two remaining 6-well plates from the MC38 transfected cells and CT26 transfected cells were used for RNA isolation from these cell samples. 500µL of Trypsin was added to each well and processed for 5 minutes. After 5 minutes, 500µL of media was added to each well and the 1mL solution from each well was aliquoted into 1.5mL Eppendorf tubes. The tubes were centrifuged for 5 minutes at 3,000rpm and the supernatants were removed.

500µL of PBS was added to each Eppendorf tube, the tubes were centrifuged for 5 minutes at 3,000rpm, and the supernatants were discarded.

350µL of a TRK Lysis Buffer and BME mixture was added and mixed into each cell pellet. The cells were homogenized using a needle and syringe. 350µL of 70% ethanol solution was added to the tubes and mixed well. The solutions were poured through spin columns and centrifuged for 1 minute at 10,000rpm and the flow through liquid was decanted. 500µL of Wash Buffer I was added to each spin column. The columns were centrifuged for 30 seconds at 10,000rpm and the flow through liquid was decanted. 500µL of Wash Buffer II was added to each spin column. The spin columns were centrifuged for 1 minute at 10,000rpm and the flow through liquid was decanted. The Wash Buffer II step was repeated. The spin columns were then centrifuged for 2 minutes at 10,000rpm and the flow-through liquid was decanted.

cDNA Preparation Using Bio-Rad iScript cDNA Synthesis Kit and Real-Time Polymerase Chain Reaction (RT-PCR)

The spin columns from the RNA isolation procedure were then placed into blue RNA columns. 40µL of DEPC water was poured directly onto the white filters in the spin columns and the columns were centrifuged for 2

minutes at 10,000rpm. The RNA concentrations of the flow through liquid were determined using Gen5 Data Analysis Software. The RNA samples were then mixed with 4µL of 5x iScript Reaction Mix, 1.0µL of iScript Reverse Transcriptase, and Nuclease-Free water. Using a Thermocycler, the reaction mix was incubated for 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, then held at 4°C. The results were quantified using Microsoft Excel.

Intramuscular In Vivo Electroporation

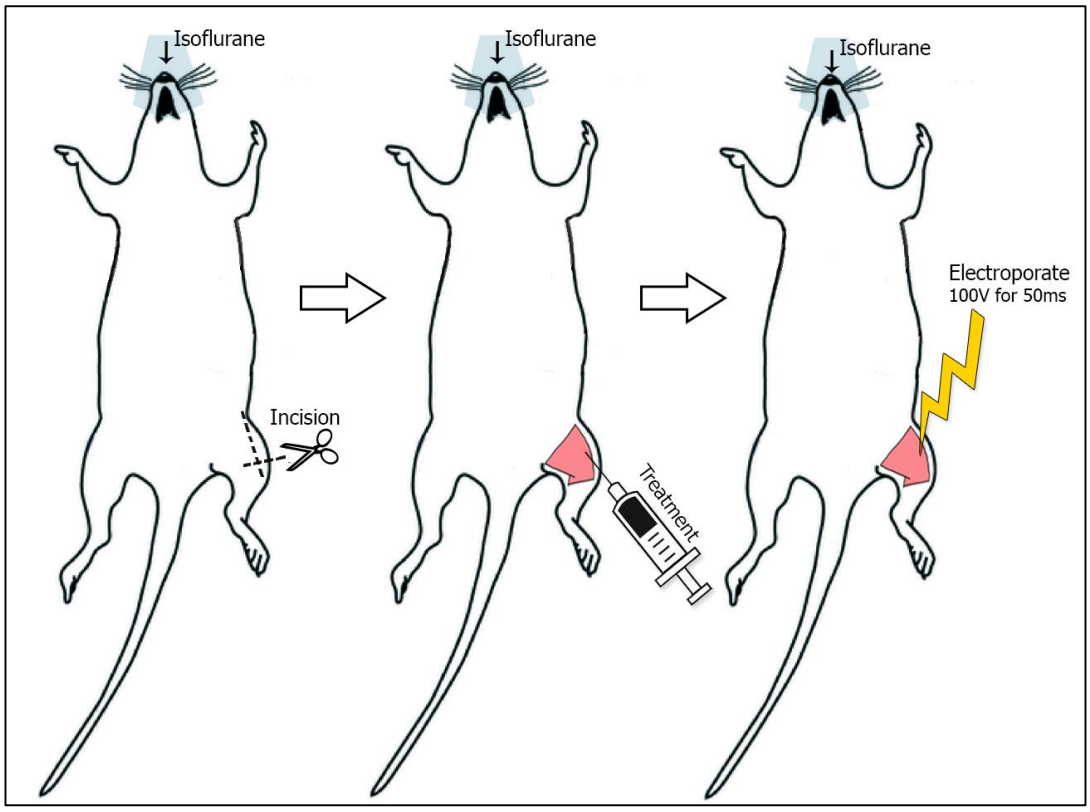

Figure 5. In vivo electroporation on C57Bl/6 mice.

To increase the serum levels of IL-33 in C57Bl/6 mice, intramuscular in vivo electroporation was performed using the pV1J-IL-33 plasmid purified under endotoxin free conditions.

50µg of the plasmid was injected into the surgically exposed quadriceps muscle of C57Bl/6 mice, and then electrodes were used to apply 8 pulses of 100 volts for 50 milliseconds each at the sight of injection, forcing the IL-33 plasmid DNA to be taken in by the muscle cells. The

muscles cells then expressed the IL-33 protein and secreted the protein into the bloodstream of the mice. Starting at 3 hours after electroporation, sera were collected by retro-orbital eye bleeding and then every 4 days up to 3 weeks post-electroporation.

Enzyme-Linked Immunosorbent Assay (ELISA)

In a separate experiment, an RnD Systems Quantikine Mouse/Rat IL-33 Enzyme-Linked Immunosorbent Assay (ELISA) was performed on the mouse sera to determine the levels of secreted IL-33 and how long the IL-33 levels stay up regulated after in vivo electroporation. A microplate pre-coated with an antibody for mouse/rat IL-33 was used for the assay. All sera samples and reagents were brought to room temperature. The mouse/rat IL-33 control was reconstituted with 1.0mL of deionized water, and 20mL of the Wash Buffer Concentrate was diluted with 480mL of deionized water. The Substrate Solution was prepared 15 minutes prior to usage by mixing 3.5mL of Color Reagent A with 3.5mL of Color Reagent B and protecting the solution from light. The mouse/rat Il-33 standard was reconstituted with 5.0mL of Calibrator Diluent RD5-17 and a 2-fold dilution series was performed.

50µL of Assay Diluent RD1-40, Standard, Control, and sera samples were added to the wells of the microplate and incubated for 2 hours at room temperature. Each well was aspirated and washed 5 times with 400µL of Wash Buffer. 100µL of mouse/rat IL-33 conjugate was added to each well and incubated for 2 hours at room temperature. Each well was aspirated and washed 5 times with 400µL of Wash Buffer. 100µL of substrate solution was added to each well and incubated for 30 minutes at room temperature protected from light. 100µL of stop solution was added to each well and mixed thoroughly. The optical density was determined with a microplate reader at 450nm, with wavelength correction at 570nm. All data analysis was conducted using Microsoft Excel.

Splenic Injections and In Vivo Electroporation

Based on the results of the previous intramuscular in vivo electroporation procedure, an injection of 50µg of plasmid produced the highest detected serum levels of IL-33 in C57Bl/6 mice. Using a sample of 12 C57Bl/6 mice, 6 mice received a quadriceps muscle injection of 50µg of pV1J and 6 mice received a quadriceps muscle injection of 50µg of pV1J-IL33. Following in vivo electroporation, approximately 200,000 MC38 tumor cells were injected into the spleens of the C57Bl/6 mice. To determine

the effect of the increased serum levels of IL-33 on liver metastasis of MC38 cells, the tumor cells injected into the spleen were allowed to grow and metastasize to the liver for 21 days. After 21 days, the C57Bl/6 mice were humanely sacrificed and the spleens and livers were harvested. The tumor growth and metastasis to the liver was determined by measuring the weights of the spleens and livers.

Results

Cloning of the pV1J-IL33 plasmid

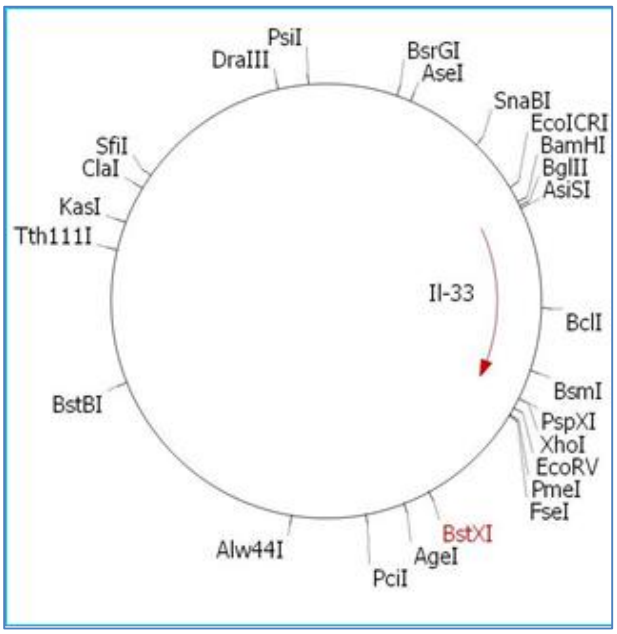

Figure 6. Map of pCMV6-IL33 plasmid which was the source of the IL-33 gene.

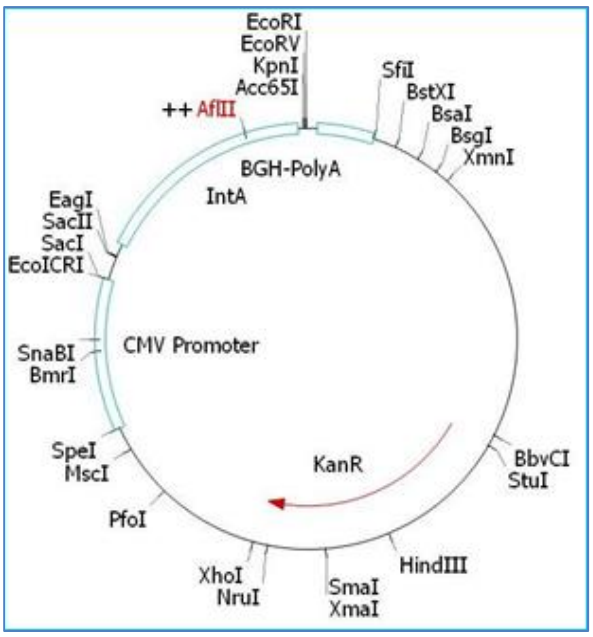

Figure 7. Map of the pV1J vector wherein the IL-33 gene was cloned into.

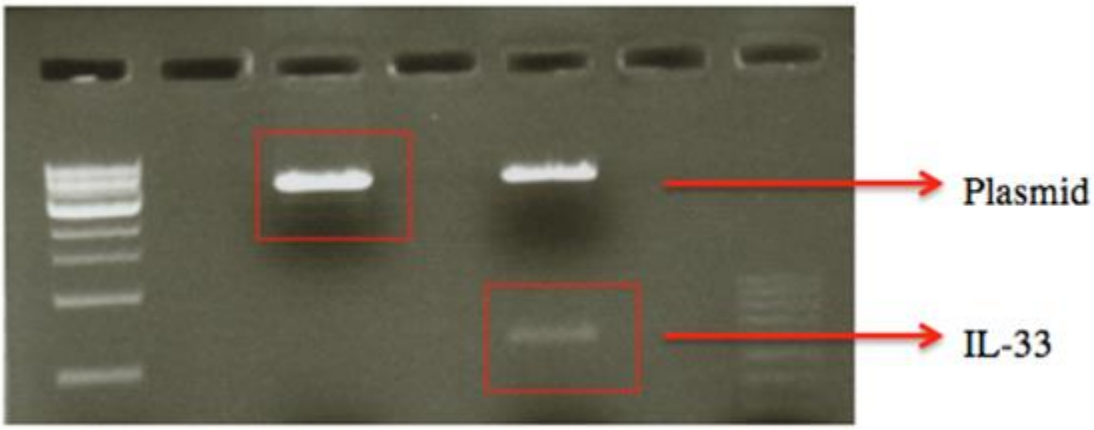

Figure 8. Agarose Gel electrophoresis of digested plasmids. pCMV6-IL33 and pV1J plasmids were digested with Kpn1 and EcoRV restriction enzymes. The digested fragments were separated by electrophoresis in a 0.8% agarose gel. Lane 1: 1 kb ladder, Lane 3: Digested pV1J plasmid, Lane 5: Digested pCMV6-IL33 Plasmid, Lane 7: 100 bp ladder. The fragments highlighted in the red squares were purified and ligated to generate the final plasmid.

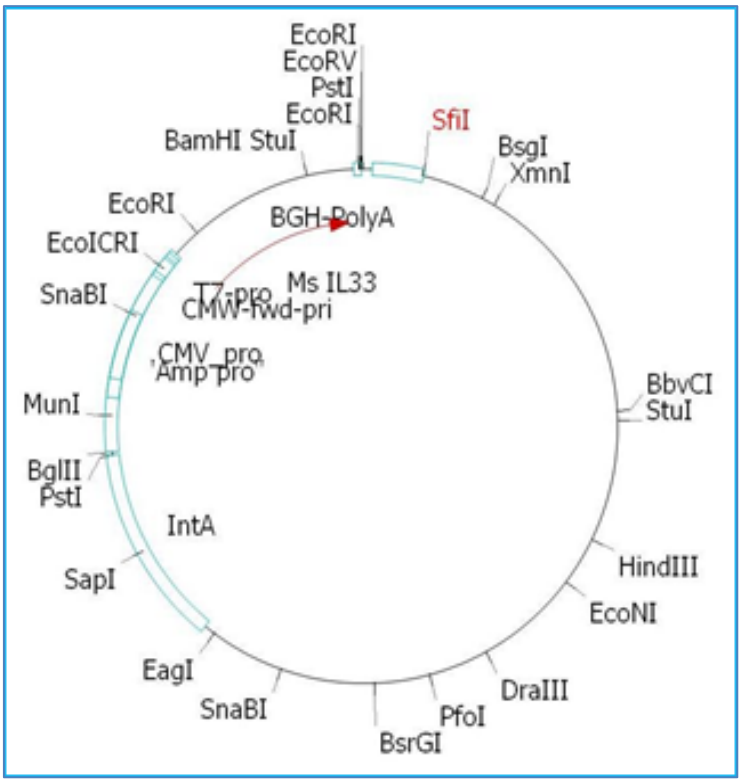

Figure 9. Map of the pV1J-IL33 plasmid that was constructed. This plasmid was used for in vitro transfection and in vivo electroporation experiments.

Intracellular mRNA in CT26 and MC38 Cell Lines

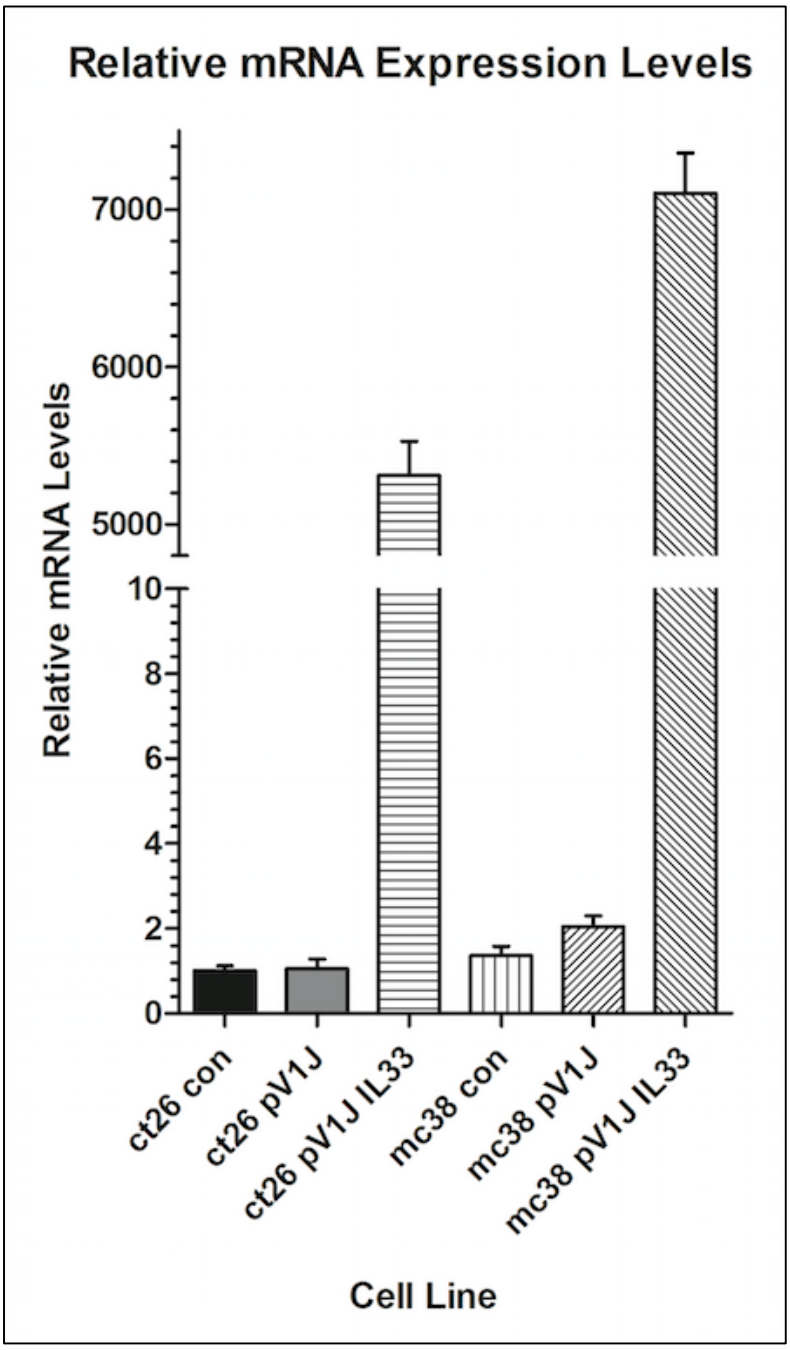

Figure 10. The pV1J and pV1L-IL33 plasmids were transfected into CT26 and MC38 colon cancer cell lines to determine expression of IL-33. Real time PCR analysis was used to determine the mRNA transcription levels of IL-33 in CT26 and MC38 derived cell lines.

IL-33 protein levels in CT26 cells transiently transfected with pV1J-IL33 Plasmid Using Lipofectamine 2000

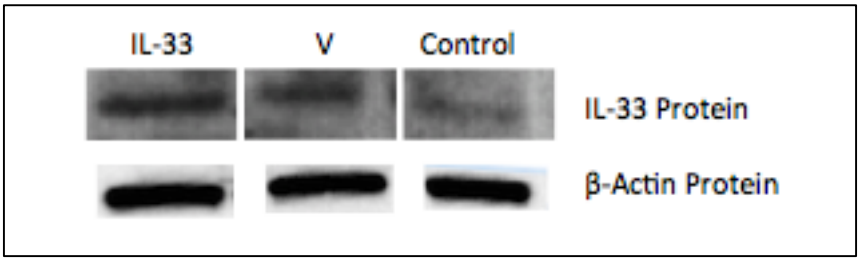

Figure 11. IL-33 protein expression in CT26 cells transiently transfected with pv1J and pV1J-IL33. (IL33: pV1J-IL33 Vector, V: pV1J Vector, Control: non-transfected CT26 cells)

Enzyme-Linked Immunosorbent Assay (ELISA)

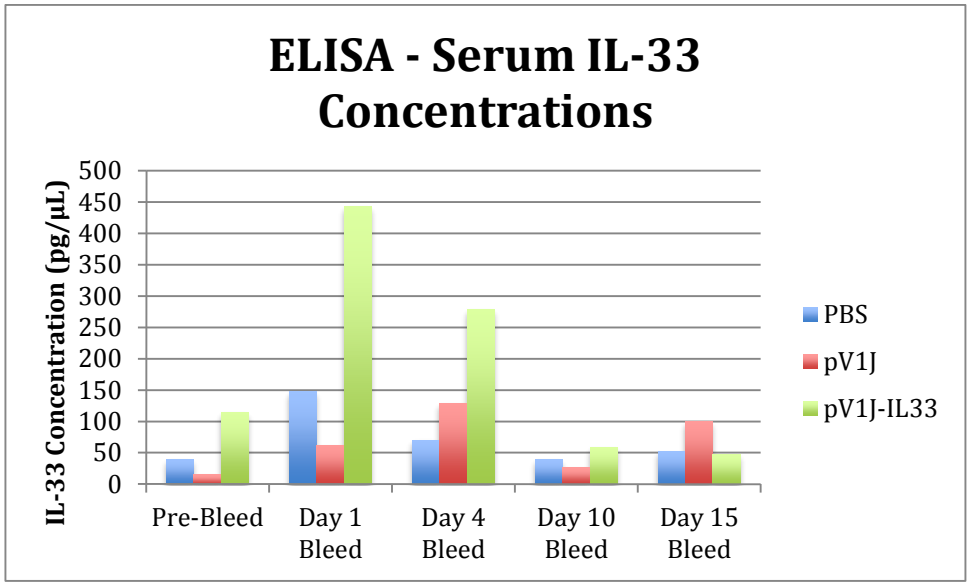

Figure 12. Concentration of IL-33 in the serum of C57Bl/6 mice following in vivo electroporation. Serum levels in mice injected with MC38 pV1J-IL33 cells were highest on Day 1 at 443 pg/µL and second highest on Day 4 at 279 pg/µL.

Assessment of the Rate of Metastasis in C57Bl/6 Mice

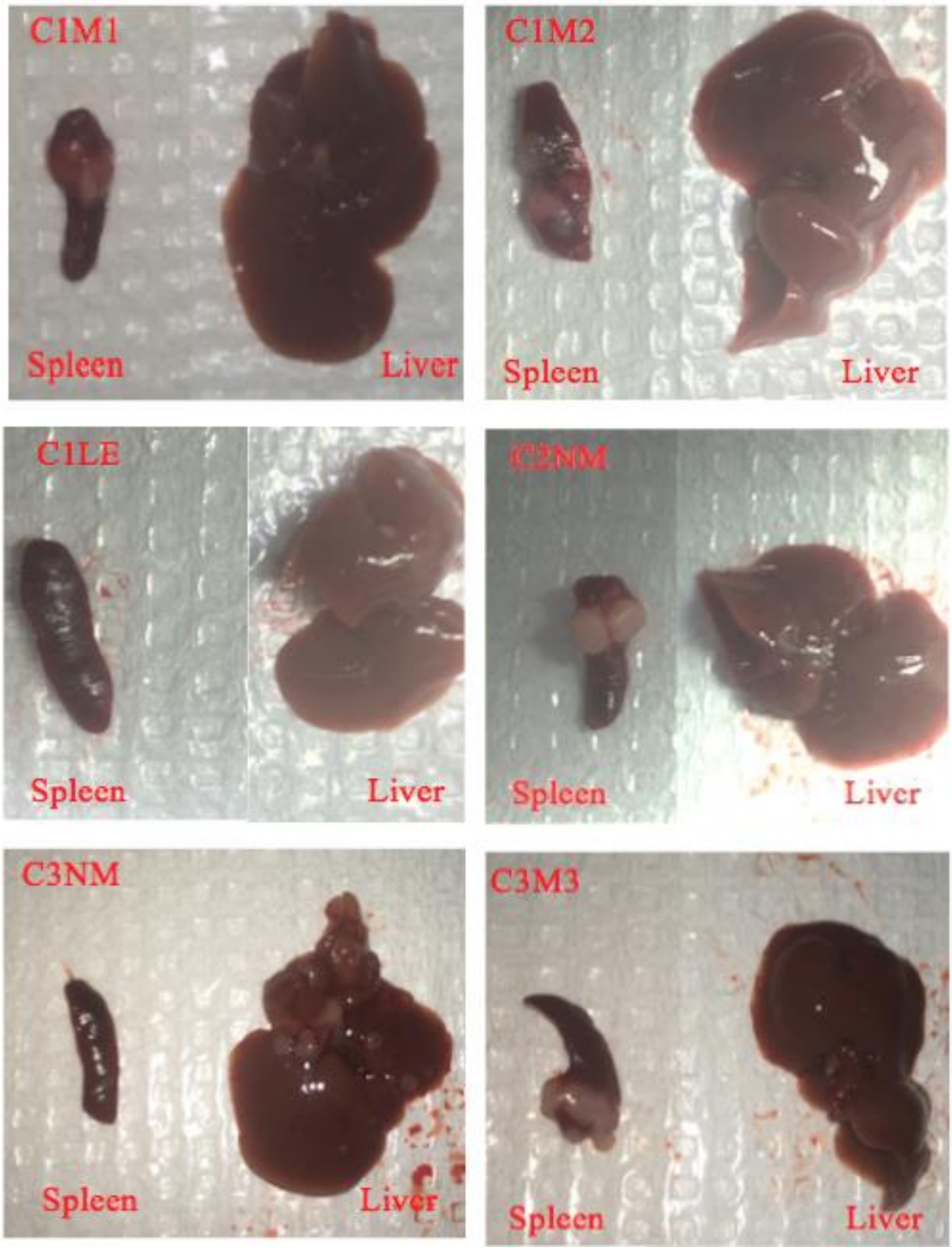

Figure 13. Primary tumors in the spleen and secondary tumors in the liver of C57Bl/6 mice injected with MC38 tumor cells and 50µg pV1J plasmid.

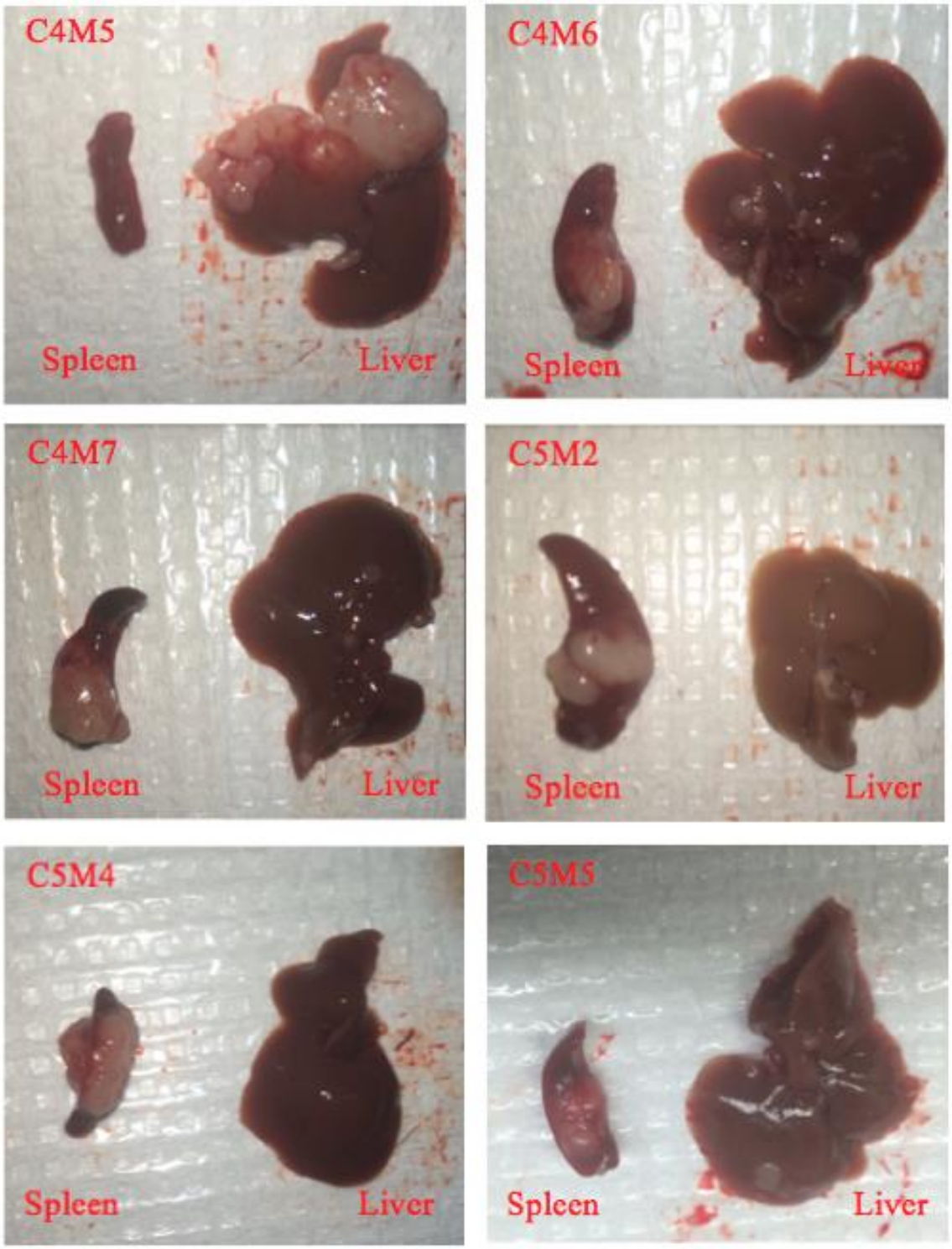

Figure 14. Primary tumors in the spleen and secondary tumors in the liver of C57Bl/6 mice injected with MC38 tumor cells and 50µg pV1J-IL33 plasmid.

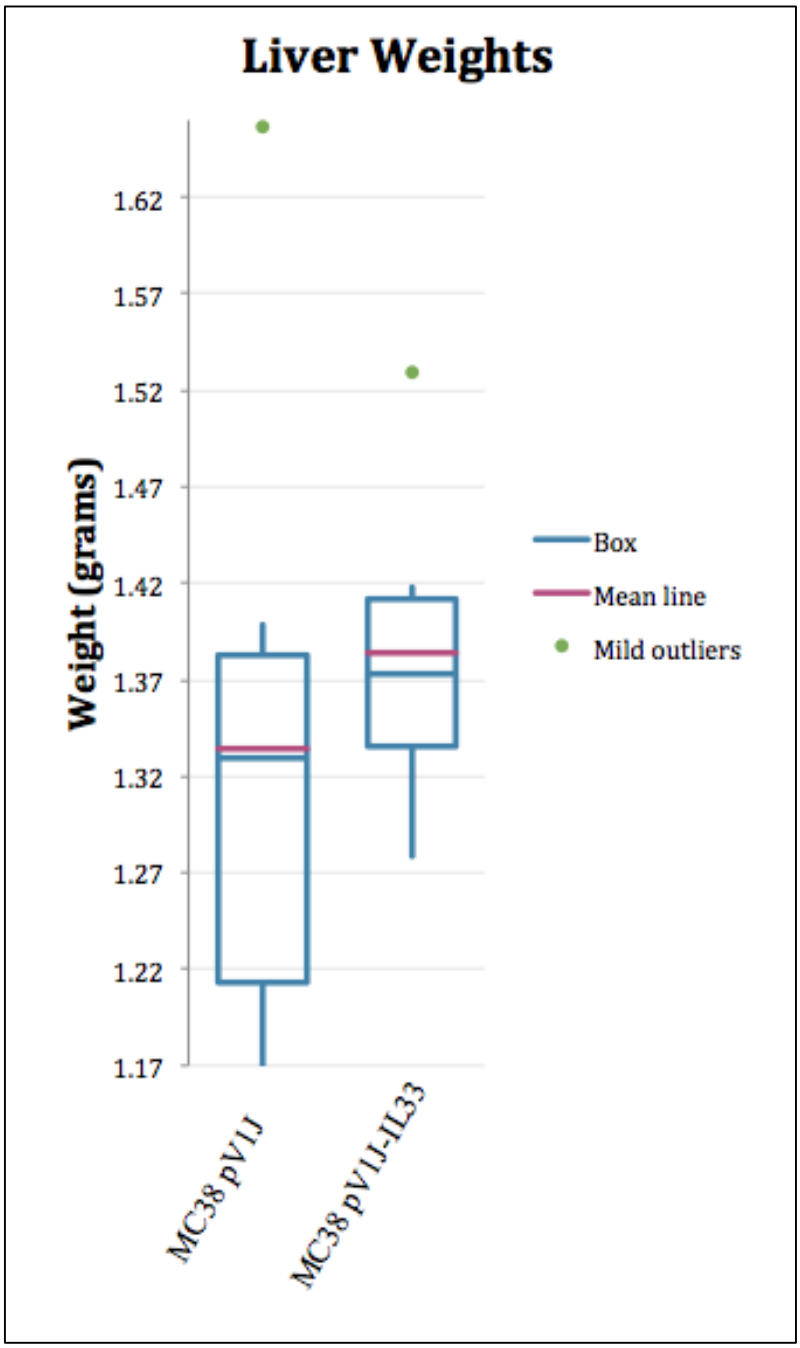

Figure 15. Assessment of the rate of metastasis in C57Bl/6 mice by measuring liver weights. The average liver weight for MC38 pV1J C57Bl/6 mice was 1.33 grams and the average liver weight for MC38 pV1J-IL33 C57Bl/6 mice was 1.38 grams.

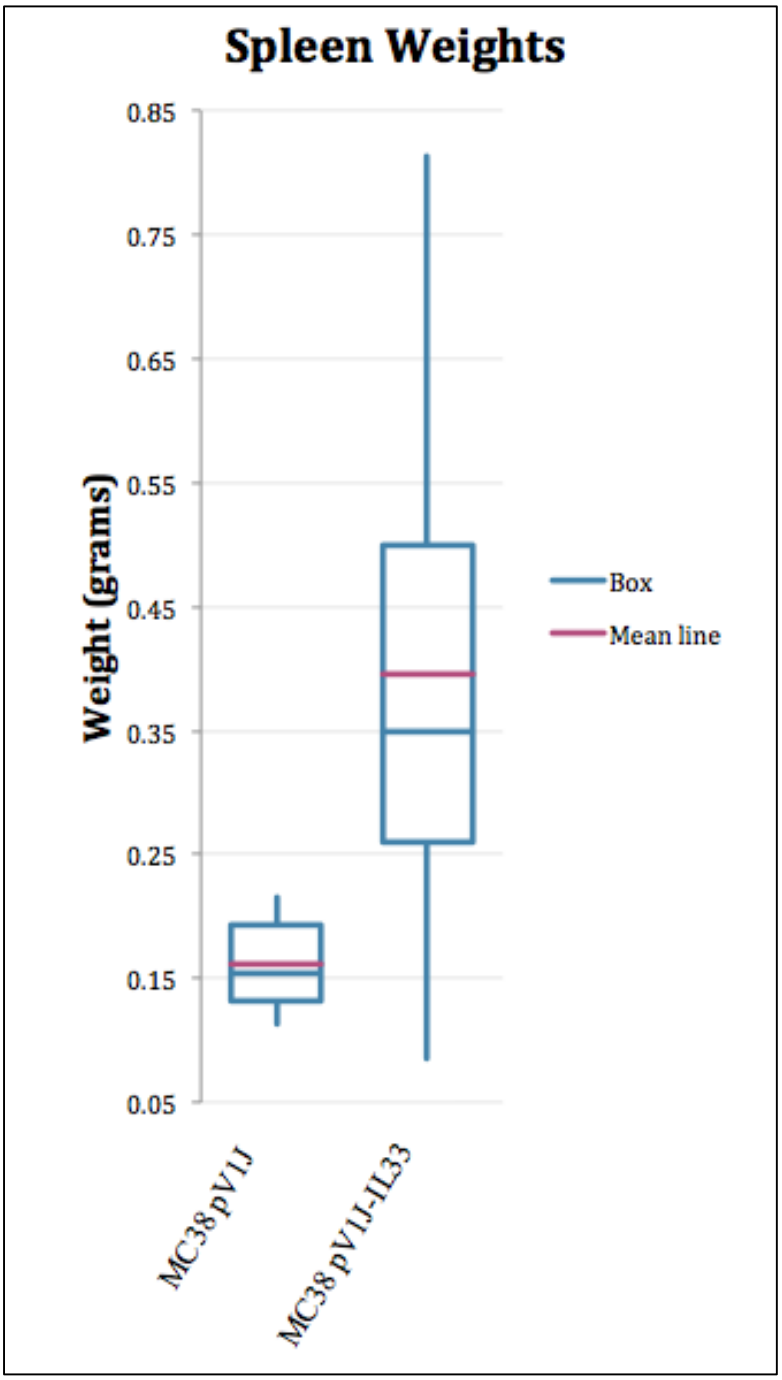

Figure 16. Assessment of the rate of metastasis in C57Bl/6 mice by measuring spleen weights. The average spleen weight for MC38 pV1J C57Bl/6 mice was 0.161 grams and the average spleen weight for MC38 pV1J-IL33 C57Bl/6 mice was 0.396 grams.

Discussion and Conclusions

Colorectal cancer metastasis is a multistep process that oftentimes develops without any detectable symptoms prior to the fatal stages of the disease. Although colorectal cancer is the third leading cause of cancer-related deaths in the United States, the mechanisms involving the progression to the lethal stages of colorectal cancer where cancer cells have invaded the liver and other organs remains under-researched. It has been found by the Peña lab that there are particular biomarkers that are up regulated in colorectal cancer cells that could potentially represent significant aspects of the transition to the metastatic stages of the disease. In previous studies, a highly liver metastatic CT26-FL3 cell line showed extremely elevated levels of the IL-33 protein. Identifying biomarkers such as IL-33 can increase early detection of colorectal cancer and improve diagnostic and therapeutic procedures.

In order to study the role of IL-33 on the metastasis of colorectal cancer to the liver, several in vitro and in vivo experiments were performed. The first experiment involved cloning IL-33 into the pV1J plasmid to create the pV1J-IL33 plasmid to be used for the remainder of the study procedures. The pCMV6-IL33 plasmid and pV1J plasmid were purified and digested with restriction enzymes Kpn I and Eco RV. Gel electrophoresis confirmed

the presence of equally cut plasmids and the excised IL-33. Ligation of the digested pV1J plasmid and the excised IL-33 created the pV1J-IL33 vector used in the subsequent experimental procedures.

To create a colorectal cancer cell line containing the IL-33 protein, the pV1J and pV1J-IL33 plasmids were transiently transfected into CT26 cells and MC38 cells. To confirm the presence of the IL-33 in these cell lines, the protein and RNA were isolated. Western Blot analysis of the proteins isolated from the CT26 cells transiently transfected with IL-33 showed increased IL-33 protein expression in the cells containing pV1J and pV1J-IL33 compared to the control cells with no plasmid. The results of the Western Blot analysis of the MC38 cell line did not show visible results. To further confirm the up regulated expression of IL-33 in the CT26 and MC38 cell lines transiently transfected with IL-33 RNA was isolated, cDNA was synthesized, and RT-PCR was performed. After quantification using Excel, the relative mRNA expression levels in the CT26 cells transiently transfected with the pV1J-IL33 plasmid were shown 5400-fold when compared to the cells containing no plasmid and the pV1J vector. Similar results were seen in the MC38 cell line, where the cells transiently transfected with pV1J-IL33 expressed mRNA 7000-fold when compared to the MC38 cells containing no plasmid and the pV1J vector. Although the IL-

33 protein was not over-expressed drastically in the cells containing pV1J and pV1J-IL33, the IL-33 mRNA expression levels were extremely over-expressed in the cells containing pV1J-IL33. The over-expression of IL-33 mRNA levels did not correlate to the protein over-expression of IL-33, which indicates that IL-33 protein is being immediately secreted from the cells after synthesis. To confirm this hypothesis, the intracellular levels of the IL-33 protein in the cell media samples should have been analyzed through Western Blotting techniques.

After confirming that the MC38 transiently transfected cells containing the pV1J-IL33 had the highest up regulated levels of IL-33, subsequent in vivo experiments were performed to determine if muscle cells in C57Bl/6 mice would uptake the MC38 transiently transfected cells and secrete the IL-33 protein into the sera of the C57Bl/6 mice. Intramuscular in vivo electroporation was performed using the pV1J-IL33 plasmid in order to increase the serum levels of IL-33 in C57Bl/6 mice. 50μg of the plasmid was injected into the quadriceps of the C57Bl/6 mice and electrodes applied pulses of electricity to the injection site to force the uptake of the plasmid DNA into the muscle cells. These cells then expressed the IL-33 protein and secreted the protein into the bloodstream of the C57Bl/6 mice. Blood sera were collected through retro-orbital bleeding prior to any experimental

procedures. Blood sera were also collected post-electroporation, which was performed on days 1, 4, 10, and 15. The concentration of IL-33 in C57Bl/6 mice sera was extremely up regulated following electroporation on day 1 and day 4 in mice receiving the pv1J-IL33 plasmid. The sera levels were normal on day 10 and day 15. Since the IL-33 protein is an alarmin secreted by necrotic cells upon trauma and infection, the sera levels of IL33 2 weeks post-injection were mainly due to the invasive procedure. If the in vivo electroporations were carried out for an additional 3-4 weeks, then the observed sera levels of IL-33 would be produced by the injected pv1J-IL33 MC38 cells.

After confirming that the sera levels of circulating IL-33 protein in C57Bl/6 mice increased following in vivo electroporation, splenic injections were performed to analyze the effects of the elevated circulating levels of IL-33 on the metastasis of colorectal cancer cells to the liver. Following in vivo electroporation, approximately 200,000 MC38 tumor cells were injected into the spleens of the C57Bl/6 mice. Splenic injection of MC38 tumor cells facilitates rapid liver metastasis because of the splenic vein that is directly connected to the portal vein of the liver. The tumor cells injected into the spleen were allowed to grow under conditions of high circulating IL-33 protein and metastasized to the liver for a total of 21 days. After

humanely sacrificing the mice, the primary tumors in the spleen and the secondary tumors in the liver were examined and the organs were weighed. Quantification of the data through Excel showed that the average weight of the spleens in C57Bl/6 mice receiving the pV1J-IL33 plasmid was almost 3 times the average weight of the spleens in mice receiving the pV1J plasmid. A similar trend was seen in the average weight of the livers of C57Bl/6 mice, on a less extreme level. Images of the spleens and livers showed more visible tumors in both organs of the C57Bl/6 mice receiving the pV1J-IL33 plasmid than in the mice receiving pV1J.

Due to these findings, MC38 cells transiently transfected with pV1J-IL33 plasmids have increased IL-33 mRNA and protein expression. Subsequently, the injection of pV1J-IL33 MC38 cells into the quadriceps of C57Bl/6 mice and in vivo electroporation increases circulating sera levels of the IL-33 protein. The increased circulating levels of IL-33 protein were primarily due to the alarmin properties of the IL-33 protein following the injection and in vivo electroporation. Elevated circulating levels of IL-33 in addition to splenic injections of MC38 tumor cells can increase the metastasis of primary tumor cells to the liver to form secondary tumors.

Due to the limited experimental timeframe, there are several future experiments that can stem directly from these primary conclusions. The

protein expression levels can be further analyzed to determine why the extremely high mRNA expression levels don't manifest in the protein expression levels. The cell media protein expression levels can also be determined through Western Blotting techniques to confirm the excretion of IL-33 immediately following its translation. Furthermore, the experiments can be repeated with a larger sample size of mice in order to create statistically significant results. If carried out for a increased time span, researchers will be able to determine that the injected pV1J-IL33 MC38 cells caused the increased circulating sera levels of IL-33 rather than the alarmins produced following the surgical procedure. Also, an ELISA performed on the C57Bl/6 mice sera following splenic injections of MC38 cells would determine if the IL-33 protein levels are still elevated during tumor growth and metastasis to the liver. Additionally, in vivo experiments using balb/c mice and CT26 cells would also reconfirm the effects of the increased circulating levels of IL-33 on the metastasis of colorectal cancer. By deliberately increasing the IL-33 mRNA and protein expression levels and increasing the levels of MC38 colorectal cancer cells in C57Bl/6 mice, liver metastasis was heavily induced and the primary results supported the hypothesis. Collectively, the experiments performed in addition to the future directions will help to further understand the effects of certain biomarkers in

colorectal cancer cells in patients in order to improve the detection time and prognosis in patients diagnosed with colorectal cancer.

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