January 2023

CCl2 Suppression In Lewis Lung Carcinoma Model Enhances Host Survival And Immune Response In The Tumor Microenvironment

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CCL2 SUPPRESSION IN LEWIS LUNG CARCINOMA MODEL ENHANCES HOST SURVIVAL AND IMMUNE RESPONSE IN THE TUMOR MICROENVIRONMENT

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A Dissertation

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy in Biomedical Science

Grand Forks, North Dakota

May

2023

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Degree: Doctor of Philosophy

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LAND ACKNOWLEDGEMENT STATEMENT

Today, the University of North Dakota rests on the ancestral lands of the Pembina and Red Lake Bands of Ojibwe and the Dakota Oyate - presently existing as composite parts of the Red Lake, Turtle Mountain, White Earth Bands, and the Dakota Tribes of Minnesota and North Dakota.

We acknowledge the people who resided here for generations and recognize that the spirit of the Ojibwe and Oyate people permeates this land. As a university community, we will continue to build upon our relations with the First Nations of the State of North Dakota - the Mandan, Hidatsa, and Arikara Nation, Sisseton-Wahpeton Oyate Nation, Spirit Lake Nation, Standing Rock Sioux Tribe, and Turtle Mountain Band of Chippewa Indians.

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Nivedita Biswas
May 2 , 2023

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ACKNOWLEDGEMENT

I would like to express my sincere gratitude to all those who have supported and guided me throughout my PhD journey. First and foremost, I would like to thank my supervisor, Dr. David Bradley, for his invaluable guidance, support, and encouragement. Without his expertise, patience, and constructive feedback, this thesis would not have been possible. I am also grateful for his understanding and flexibility, which enabled me to pursue my research interests and develop my skills. I would like to extend my gratitude to the members of my thesis committee, Dr. Matthew Nilles, Dr. Colin Combs, Dr. Kumi Nagamoto-Combs and Dr. Peter Meberg, for their insightful comments and suggestions, which greatly enhanced the quality of this work. I appreciate their willingness to share their knowledge and expertise, and for their commitment to helping me succeed.

I am also grateful to my colleagues in the Department of Biomedical Sciences who have provided me with a stimulating and supportive environment to pursue my research. Their willingness to discuss ideas, share resources, and provide feedback has been invaluable. I am thankful for Mr. Nathan Riha's technical expertise and help collecting the data presented. I am also thankful for Mrs. Bernadette Meberg's constant support and encouragement. The flow cytometry data presented is thanks to the expert guidance and hardwork from Dr. Suba Nookala. I would like to extend my heartfelt gratitude and appreciation to my friends, Dr. Ashrifa Ali, Dr. Sema Oncel, Dr. Shefta Moula and Dr. Ayesha Abid for their kindness, generosity and understanding. Whether it was through sharing a meal, going for a walk, or simply having a conversation, my friends provided

me with a sense of community and belonging that was essential to my well-being during this time. Their presence in my life provided me with much-needed respite from the demands of academia, and helped me maintain a sense of balance and perspective. I would like to thank my family for their unwavering love and support. Their encouragement and belief in me has been a constant source of motivation throughout this journey. Thank you, Mom (Rakhi Biswas) and Dad (Snehasis Biswas), for your unconditional love, support, and guidance. I would not be where I am today without your unwavering belief in me, and I will forever be grateful for everything you have done for me. I am grateful to my Brother, Avhishek Biswas, for his love, friendship and unwavering support throughout my life and especially during my PhD journey. I am grateful for the time and energy that my therapist, Theresa Knudson, devoted to helping me overcome the challenges I faced during my doctoral journey both personal and professional. Her support and guidance were invaluable in helping me achieve this milestone. I will always be grateful for the positive impact people have had on my life and academic career. In conclusion, I acknowledge the support and contributions of all those who have helped me along the way, and without whom this thesis would not have been possible.

To my mother.

Rakhi Biswas

“I am out with lanterns, looking for myself.”

— Emily Dickinson

ABSTRACT

CCL2, a chemokine also referred to as monocyte chemoattractant protein 1 (MCP-1) and small inducible cytokine A, is chemotactic for monocytes. CCL2-induced recruitment of CCR2+Ly6C(hi) monocytes increases vascular permeability CCR2+ endothelium aiding the escape and migration of tumor cells. CCL2 is produced by cancers of varied immunogenicity and augments tumor proliferation and metastasis. Elevated CCL2 expression by tumor cells is linked to poor prognosis.

We have previously shown that two bacterially derived superantigens, Staphylococcal enterotoxin G and I (SEG/SEI) stimulate large numbers of T cells in an antigen-independent fashion and promote enhanced survival in the poorly immunogenic B16-F10 melanoma model. Conversely, the poorly immunogenic Lewis Lung Carcinoma (LLC) was not responsive to SEG/SEI and SEG/SEI stimulation did not change the time to death in LLC-implanted mice. All these studies were performed in humanized HLA-DQ8 (DQA1*0301 and DQB1*0302) transgenic mice.

Here we provide the background mechanistic differences between LLC and B16-F10 that may explain the disparate outcomes. Interestingly, the LLC cells secrete high levels of CCL2 *in vitro* whereas B16-F10 cells do not, which we analyzed via Flowcytometric Bead Array. We also show elevated levels of CCL2 in MC-38, an immunogenic murine colorectal cancer cell line. We hypothesized that the elevated CCL2 present in the LLC model, but lacking in the B16-F10 model, resulted in the influx of CCR2+Ly6C(hi) monocytes. We have used Crispr Cas9 as our genome editing tool to inhibit the expression of CCL2 and thereby investigate the effects of CCL2 inhibition on LLC tumor

proliferation and metastasis. We also provide data to support the successful creation of LLC MCP-1 KO's using CRISPR-Cas9.

These findings, taken *in toto*, suggest that combinatorial therapies inhibiting CCL2 with the addition of treatments that enhance the tumor-specific response, e.g. anti PD-1 antibodies, may alter the tumor environment of treatment refractory cancers that induce high CCL2 production, like LLC and MC-38, thus allowing a potent anti-tumor and anti-metastatic response and increased survival.

CHAPTER I

INTRODUCTION

A History of Cancer

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. The history of cancer dates back thousands of years and has been described in various ancient medical texts and documents. The word "cancer" itself is derived from the Greek word "karkinos," which means "crab." This term was used by the ancient Greek physician Hippocrates to describe tumors, as the swollen veins around some tumors resembled the legs of a crab. (Science Diction: The Origin of The Word 'Cancer', 2010).

Throughout history, cancer has been described and treated in a variety of ways. In ancient civilizations, cancer was often attributed to supernatural causes and was treated with a combination of spiritual and magical remedies. In more recent times, cancer has been studied and treated using a scientific approach (Blackadar., 2016).

In the 19th and early 20th centuries, significant progress was made in the understanding and treatment of cancer. The development of the microscope and the discovery of cells as the basic unit of life were important milestones in this process. In the latter half of the 20th century, major advances in cancer research and treatment, including chemotherapy, radiation therapy, and surgery, have led to significant improvements in cancer survival rates. Today, cancer is still a major public health challenge and research into the causes,

diagnosis, and treatment of cancer is ongoing. While significant progress has been made, much more work is needed to fully understand and effectively treat cancer (Blackadar., 2016).

During the past few decades, scientific research has clearly demonstrated the significance of oncogenes in human cancer. Since the discovery that human tumors contain activated oncogenes (Der *et al*, 1982; Goldfarb *et al*, 1982; Parada *et al*, 1982; Pulciani *et al*, 1982; Santos *et al*, 1982; Shih and Weinberg, 1982), many efforts have been made to understand their causal role in cancer development. The expression of oncogenes plays a vital role not only in the initiation of cancer but also in its sustenance, which has positioned oncogenes as the primary therapeutic targets for anti-cancer treatments. Oncogenes are genes that, when mutated or overexpressed, have the potential to cause cancer. These genes normally play a role in regulating cell growth and division, but when they are altered, they can lead to uncontrolled cell growth and the formation of tumors. There are several types of oncogenes, including:

Growth factor genes- These genes code for proteins that stimulate cell growth and division. Mutations in these genes can cause them to become overactive, leading to excessive cell growth.

Signal transduction genes- These genes are involved in transmitting signals within cells that regulate cell growth and division. Mutations in these genes can cause them to be activated in the absence of normal growth signals, leading to uncontrolled cell growth.

Cell cycle genes- These genes are involved in regulating the cell cycle, which is the process by which cells grow and divide. Mutations in these genes can disrupt the normal cell cycle and lead to uncontrolled cell growth. (Vicente-Dueñas *et al*., 2013)

Examples of oncogenes include the HER2/neu gene, which is overexpressed in certain types of breast cancer, and the BCR-ABL gene, which is involved in the development of chronic myeloid leukemia. Understanding oncogenes and how they contribute to cancer development is an important area of research in cancer biology and has led to the development of targeted therapies for some types of cancer.

Tumor suppressor genes function as normal genes that regulate cell division and promote programmed cell death, also known as apoptosis. Failure of these genes to function properly can result in uncontrolled cell growth and the development of cancer. For example, TP53 is an important tumor suppressor gene. It codes for the p53 protein, which helps keep cell division under control. Inherited changes in the TP53 gene can lead to Li-Fraumeni syndrome. Family members with this syndrome have an increased risk of several types of cancer, because all of their cells have this TP53 gene change (Cooper., 2000)

A crucial goal of cancer research is to understand how to counteract the mechanisms that underlie the ability of normal cells to become cancer cells in the first place.
The intricacy of the properties of cancer cells was distilled by Hanahan and Weinberg (2011) into ‘nine essential alterations in cell physiology that collectively dictate malignant growth'. Cancer cells are the groundwork of the disease: they initiate the tumors and drive cancer progression forward, and they are the ones carrying the oncogenic and tumor suppressor mutations that define cancer as a genetic disease

(Hanahan and Weinberg, 2011). However, we still do not comprehend sufficiently the underlying mechanisms leading to the origin of these cells, to have a sizable impact on cancer mortality (Jemal *et al*, 2009). Consequently, our advancements in the field of medicine are gradual and primarily based on practical experience, resulting in minor enhancements in treatments, surgical procedures, or radiation treatments. Although they may offer certain advantages, they appear to be insufficient in eradicating the disease itself.

Hanahan, D., & Weinberg, R. (2000) revised the concept of the hallmarks of cancer in 2000 referring to six fundamental traits or capabilities that are acquired by human tumors during their development. These hallmarks serve as a framework for understanding the intricate nature of cancer and provide a systematic approach for comprehending the disease. The six hallmarks of cancer include:

Sustained proliferative signaling: Cancer cells can sustain abnormal levels of cell growth and division, often by acquiring mutations in genes that regulate these processes.

Evading growth suppressors: Cancer cells can evade the body's mechanisms for inhibiting cell growth, often by inactivating tumor suppressor genes.

Resisting cell death: Cancer cells can resist programmed cell death, or apoptosis, which is a natural mechanism for removing damaged or abnormal cells from the body.

Enabling replicative immortality: Cancer cells can divide indefinitely, a process that is usually limited in normal cells.

Inducing angiogenesis: Cancer cells can stimulate the growth of new blood vessels to supply nutrients and oxygen, which is essential for their continued growth and survival.

Activating invasion and metastasis: Cancer cells can invade nearby tissues and spread to other parts of the body, a process known as metastasis. By understanding these hallmarks, researchers can develop targeted therapies that address the specific mechanisms driving cancer growth and progression (Hanahan and Weinberg., 2000).

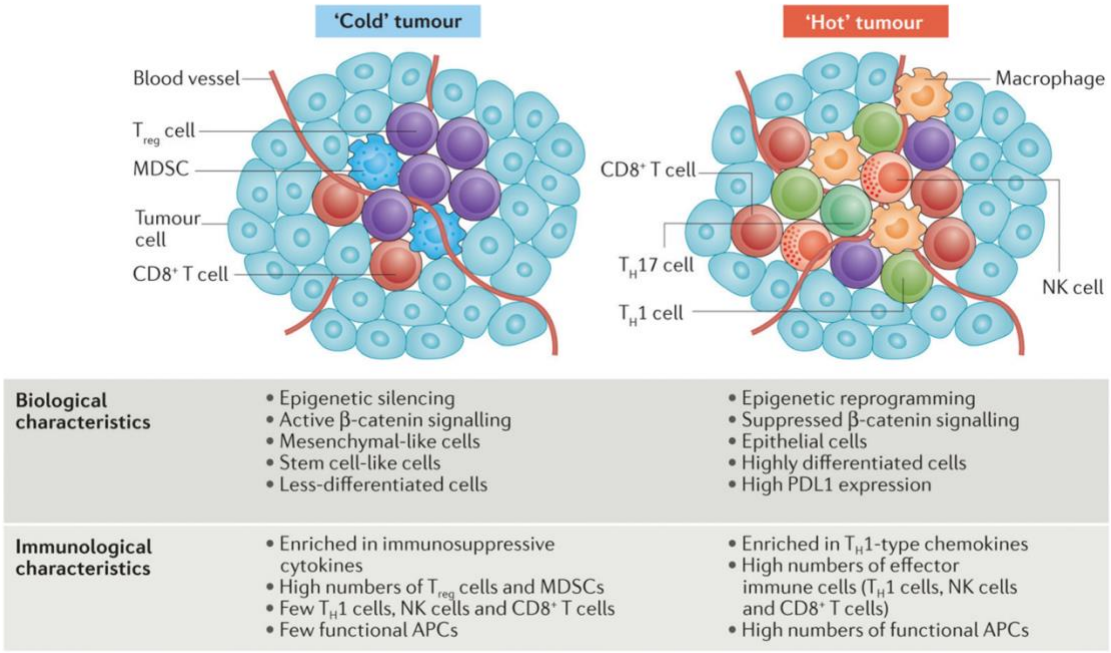
Numerous indications suggest that tumorigenesis in humans occurs through a series of successive steps that involve genetic changes, which propel the gradual evolution of normal human cells into highly aggressive cancerous forms. (Hanahan and Weinberg., 2000). To fully comprehend the cancer process, a comprehensive understanding of the mechanisms underlying neoplastic growth is crucial. This knowledge is not only necessary for understanding the origins of human cancer but also for identifying the molecular events responsible for cancer maintenance. However, the various aspects pertaining to the disruption of normal developmental regulatory mechanisms during carcinogenesis have not been given adequate attention in defining the hallmarks of cancer cells. The mechanisms that control the identity of tumor cells play a crucial role in the development of cancer and could potentially hold the key to eliminating it, as we will explore over the course of this dissertation.

Solid tumors are anomalous tissue masses that typically lack liquid areas or cysts, and they can occur in various regions of the human body. They represent more than 90% of all human cancer (cancer.org) cases in the United States and include sarcomas, carcinomas, and lymphomas. In addition to imaging techniques and tissue pathology analysis for initial diagnosis of solid tumors, molecular assays are available to provide a more detailed analysis of the cancer and its characteristics.

Solid tumors and liquid tumors are two broad categories of tumors based on their physical characteristics. Solid tumors are abnormal masses of tissue that form solid masses and usually do not contain any cysts or liquid areas. These tumors can develop in different parts of the body, including organs, bones, and tissues. Examples of solid tumors include sarcomas, carcinomas, and lymphomas (Nabi and Hohl 2009). In contrast, liquid tumors are cancers of the blood or bone marrow and are also known as hematologic malignancies. These tumors develop in the liquid or circulating parts of the body, such as the lymphatic system or blood vessels. Examples of liquid tumors include leukemia, lymphoma, and myeloma. The distinction between solid and liquid tumors is important because they often require different diagnostic and treatment approaches. Liquid tumors or hematologic malignancies are commonly treated with chemotherapy, stem cell transplantation, or immunotherapy while solid tumors are usually treated with surgery, radiation therapy, chemotherapy, or targeted therapy. Due to the complexity and heterogeneity of solid tumor cancers, ongoing research is focused on developing more effective and personalized treatment approaches, such as immunotherapy and precision medicine. (Nabi and Hohl 2009).

Tumor immunotherapy refers to active or passive tumor-specific responses to suppress cancer, including immune checkpoint blocks (ICBs), adoptive cell transfer (ACT), and tumor-specific vaccines (Zhang and Zhang, 2020) Despite immunotherapy marking the beginning of a new era in cancer therapy, it only works in a subset of cancers and a fraction of patients with cancer respond to immunotherapy (Yang, 2015). Meanwhile, the existence of immune escape makes the effect less than expected.

Hot vs Cold Tumors



	'Cold' tumour	'Hot' tumour
Biological characteristics	• Epigenetic silencing • Active β-catenin signalling • Mesenchymal-like cells • Stem cell-like cells • Less-differentiated cells	• Epigenetic reprogramming • Suppressed β-catenin signalling • Epithelial cells • Highly differentiated cells • High PDL1 expression
Immunological characteristics	• Enriched in immunosuppressive cytokines • High numbers of T_{reg} cells and MDSCs • Few T_H1 cells, NK cells and CD8⁺ T cells • Few functional APCs	• Enriched in T_H1-type chemokines • High numbers of effector immune cells (T_H1 cells, NK cells and CD8⁺ T cells) • High numbers of functional APCs

Figure 1: The relationship between, and mechanisms that underlie, tumor immune phenotype and biological phenotype. Nagarseth *et al*, 2017 (Used with permission)

A "hot" tumor refers to a cancerous tumor that is highly active and rapidly growing. It is often accompanied by higher levels of certain proteins or biomarkers, such as estrogen and progesterone receptors, and may be more responsive to certain types of treatment. A "cold" tumor, on the other hand, is a cancerous tumor that is less active and grows more slowly. It may have lower levels of certain proteins or biomarkers and may be less responsive to certain treatments. Hot tumors often undergo mutations resulting in the expression of molecules, called neoantigens, that make it easier for immune cells to recognize and attack

tumor cells. Conversely, a cold tumor is immunosuppressive, indicating it is unlikely to trigger an anti-tumor immune response. A "hot" tumor is one that is more active and aggressive, while a "cold" tumor is one that is less active and less aggressive. Hot tumors may grow and spread more quickly than cold tumors, and they may also be more resistant to treatment. In general, hot tumors are more difficult to treat than cold tumors. (Nagarseth *et al*, 2017)

Some studies like Nagarseth *et al* (2017) have suggested that hot tumors may produce higher levels of certain chemokines, which could contribute to their more aggressive behavior. However, the relationship between chemokines and hot tumors is not fully understood, and more research is needed to fully understand the role that chemokines play in cancer. In addition, chemokines may contribute to the development of blood vessels in tumors, which can provide a source of oxygen and nutrients for the cancer cells. Different immune cell subsets are recruited into the tumor microenvironment via interactions between chemokines and chemokine receptors, and these populations have distinct effects on tumor progression and therapeutic outcomes. Chemokine networks regulate lymphocyte recruitment into the tumor microenvironment. Chemokines play an important role in the progression of cancers. They are involved in tumor growth, senescence, angiogenesis, epithelial mesenchymal transition, metastasis, and immune evasion (Sarvaiya., 2013). The expression of chemokines and their receptors is altered in many malignancies and subsequently leads to aberrant chemokine receptor signaling. This alteration occurs due to inactivation of the tumor suppressor genes or constitutive activation of the oncogenes that play a role in the regulation of the chemokines. There is limited information available on the role of chemokines in cold tumors. It is possible that cold tumors may express different

chemokines than hot tumors, or that they may express lower levels of certain chemokines. However, more research is needed to fully understand the relationship between chemokines and cold tumors.

Three different phenotypes are associated with specific biological mechanisms. Tumors with the immune-desert phenotype may lack T-cell priming due to the absence of tumor antigens, defective antigen processing and presentation machinery, or impaired DC-T-cell interactions (Liu and Sun., 2021). Tumors with the immune-excluded phenotype may exhibit activation of oncogenic pathways, aberrant chemokines, aberrant vasculature and hypoxia, or an immunosuppressive tumor microenvironment (e.g., stromal barriers). Tumors with the immune-inflamed phenotype can be infiltrated by many immune cells, but these immune cells are suppressed due to checkpoint activation.

Liu *et al* (Liu and Sun., 2021) discuss driving T cells into the TME and that improving the infiltration of T cells into the tumor may help to "activate" the tumor and make it more responsive to treatment.

Effective anticancer immunity relies on the proper functioning and coordination between the innate and adaptive immune systems. CD8-expressing cytotoxic T cells are critical effectors in the immune response against cancer and are currently central to successful cancer immunotherapy treatments. (Raskov *et al*., 2020) CD8+ T cells are a type of immune cell that play a crucial role in the body's defense against cancer. These cells are also known as cytotoxic T cells because they have the ability to directly kill cancer cells.

CD8+ T cells are activated when they recognize cancer cells as foreign or abnormal, and they then migrate to the site of the tumor to eliminate it. (Durgeau *et al*., 2018)

CD8+ T cells can also enhance the immune response to cancer by releasing cytokines, which are signaling molecules that activate other immune cells to attack the cancer. CD8+ T cells are essential in destroying both pathogens and neoplastic cells due to their cytotoxic capabilities. Meanwhile, CD4+ T cells also play a crucial role in supporting the function of CD8+ T cells and preventing exhaustion. Additionally, these cells can form long-term memory, which enables them to recognize and attack the cancer if it returns. (Durgeau *et al*, 2018)

However, cancer cells can sometimes evade the immune system by downregulating or altering the expression of molecules on their surface that are recognized by CD8+ T cells. This can result in a weakened immune response and allow the cancer to grow and spread. Therefore, strategies to enhance CD8+ T cell function are an important area of research in cancer immunotherapy. Approaches such as immune checkpoint blockade, which involves blocking proteins that inhibit the immune response, and adoptive T cell transfer, which involves isolating and expanding CD8+ T cells outside the body before re-infusing them into the patient, are currently being used in the clinic to boost the anti-cancer activity of CD8+ T cells. (Kalos and June., 2013)

According to the spatial distribution of cytotoxic immune cells in the tumor microenvironment (TME), a tumor is classified into one of three basic immunophenotypes: immune-inflamed, immune-excluded, and immune-desert phenotypes (Chen & Mellman, 2017). Immune-inflamed tumors, also named "hot tumors", are characterized by high T-cell infiltration, increased interferon-γ (IFN-γ) signaling, expression of PD-L1 and high tumor mutational burden (TMB) (Hegde P *et al*, 2016). Tumors with an inflamed phenotype tend to be more responsive to Immune Checkpoint inhibition. (Galon *et al*, 2019). Immune-excluded tumors and immune-desert tumors can be described as "cold tumors". There is growing evidence showing that the activation of tumor cell oncogenic pathways is related to the "cold tumor" phenotype and the potential for immunotherapy resistance. As the gene with the most common mutations associated with cancer progression, RAS can lead to the activation of multiple signaling pathways, such as MAPK and PI3K, driving tumorigenesis. In addition, oncogenic K-RAS mutations mediate inflammation and crosstalk with the TME. For example, oncogenic K-RAS mutations induce tumor-promoting inflammation through the production of inhibitory cytokines (e.g., IL-6 and IL-8), the activation of NLRP3 inflammasome, and the release of chemokines (e.g., CCL5 and CCL9) (Hamarsheh *et al.*, 2020)

Lung Cancer

Lung cancer is a type of cancer that affects the cells of the lung, typically the cells lining the airways. It is one of the most common types of cancer (12.3% of all cancers) and a leading cause of cancer-related deaths worldwide. (Minna *et al*., 2002)

There are two main types of lung cancer in humans: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (Zappa and Mousa 2016). SCLC is a more aggressive form of lung cancer that tends to grow and spread quickly. NSCLC is a more common form of lung cancer, accounting for 85% of all lung cancers, that grows more slowly and can be divided into several subtypes, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. (Zappa and Mousa 2016)

The most common risk factor for lung cancer is tobacco smoke, which is responsible for the majority of cases of lung cancer. Other risk factors include exposure to certain chemicals and pollutants, and a family history of lung cancer (Minna *et al*., 2002). Symptoms of lung cancer can include a persistent cough, chest pain, shortness of breath, and coughing up blood. Early diagnosis and treatment of lung cancer can improve the chances of successful treatment, but lung cancer is often not diagnosed until it is in an advanced stage, which can reduce the chances of a successful outcome. (Minna *et al*., 2002)

Treatment for lung cancer typically involves a combination of surgery, chemotherapy, and radiation therapy (Thai *et al.*, 2021). Current practice in Lung cancer treatment, based upon the results of non-comparative studies, is to use different forms of chemotherapy as well as immunotherapy but has only been successful in very small subsets of patients due to the aggressiveness of the disease, high relapse rate and lack of specialized approach leading to our overall objective of investigating a better therapeutic modality. Despite recent advances in understanding the molecular pathways that trigger cancer and its progression, the successful development of cancer therapies has been hampered by the complexity of these pathways and the existence of alternate or bypass pathways that foster drug resistance. Consequently, the duration of effectiveness for targeted therapies is finite; however multidimensional approaches are not, which offer the potential for improved effectiveness. Tumor heterogeneity may lead to an increase in the number and diversity of potential target sites for therapy; therefore, multimodal combination therapeutics may allow for a more specific targeting spectrum and greater impact on tumor subclones, potentially increasing the probability of therapeutic effectiveness. (Thai *et al.*, 2021). Lung cancer initiation and progression depends not only on the evolving genomics and molecular properties of cancer cells but also on their interaction with the tumor environment, specifically with the immune system (Forde *et al*, 2014).

Survival rates for metastatic lung cancer including non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) are poor with 5-year survival of less than 5%. The use of molecular targeted therapies has improved median overall survival (OS) in a limited group of NSCLC patients. The checkpoint inhibitors targeting cytotoxic T-lymphocyte-

associated antigen 4 (CTLA-4) and the programmed death-1 (PD-1) pathway have shown durable clinical responses with manageable toxicity. However, more studies are needed to understand the optimal combination of immunotherapeutic agents with chemotherapy and radiation therapy for the treatment of NSCLC and SCLC (Massarelli *et al*., 2014).

NSCLC can be divided into several subtypes, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Adenocarcinoma is the most common subtype of NSCLC and tends to occur in the outer part of the lung. Squamous cell carcinoma tends to occur in the central part of the lung and is more common in smokers. Large cell carcinoma is a more aggressive subtype that can occur anywhere in the lung (Thai *et al*., 2021).

Symptoms of NSCLC can include a persistent cough, chest pain, shortness of breath, and coughing up blood. Early diagnosis and treatment of NSCLC can improve the chances of successful treatment, but NSCLC is often not diagnosed until it is in an advanced stage, which can reduce the chances of a successful outcome. Treatment for NSCLC typically involves a combination of surgery, chemotherapy, and radiation therapy. Combination of chemotherapy and/or radiation therapy with immunotherapy and the timing of administration need to be further investigated. Finally combination of the two large groups of immunotherapy, antigen-specific vaccines and immunomodulatory agents, may have synergistic effects in augmenting the anti-tumor immune response. (Massarelli *et al*, 2014)

Lung cancer is the leading cause of cancer-based mortality worldwide (Molina *et al*., 2008). Non-small cell lung cancer accounts for 85% of all lung cancer cases in the United States (Molina *et al*., 2008). Most patients present with advanced and metastatic form of the disease at the time of diagnosis and hence surgical resection which remains the most successful option for cure falls short. Even though chemotherapy has increased the success of metastatic lung cancer treatment its limitations have not extended life significantly. Unfortunately, this poor survival rate has not improved in the past decades, although dramatic progress has been achieved in understanding the mechanism of action of lung carcinogenesis. Therefore, understanding the molecular genetics of Lewis lung carcinoma for the development of immune therapeutic intervention has remained an imperative approach in increasing the survival outcomes for non-small cell lung carcinoma. Cancer immunotherapy uses components of the immune system to treat cancer patients. Previously, clinical approaches to using the immune system against cancer focused on vaccines that intended to specifically initiate or amplify a host response against evolving tumors (Molina *et al*., 2008). Although vaccine approaches have had some clinical success, most cancer vaccines fail to induce objective tumor shrinkage in patients (Molina *et al*., 2008). Hence other novel avenues of therapy demand to be explored.

Lewis lung carcinoma

The Lewis lung carcinoma is a transplantable metastatic tumor of the C57BL mouse strain. A carcinoma discovered by Dr. Margaret R. Lewis of the Wistar Institute in 1951

(Rashidi *et al*, 2000).. This tumor originated spontaneously as a carcinoma of the lung of a C57BL mouse. The tumor is not grossly hemorrhagic and the majority of the tumor tissue is a semifirm homogeneous mass.

Mouse tumor models are helpful to the understanding of cancer biology and for the potential lifesaving development of therapeutics against cancer. Lewis lung carcinoma (LLC, ATCC CRL-1642) was derived from a spontaneous lung carcinoma from C57BL/6 and is a reliable model for human non-small cell lung carcinoma (Sugiura and Stock, 1955). This highly tumorigenic cell line is widely used in the study of metastasis and mechanisms of cancer immunotherapy because when implanted the cells are immunologically compatible with the mouse immune system unlike widely used xenograft models for human cancer. The cell line grows as a monolayer in a culture of complete DMEM medium supplemented with 10% fetal bovine serum and has a doubling time of about 22-24 hours. Lewis Lung carcinoma is a highly immunogenic cell line both in C57BL/6 and HLA-DQ8 Mice and produce primary tumors along with lung metastasis indistinguishable from the original non-small cell lung cancer tumor line. This lung cancer model employs the injection of lung cancer cell lines intravenously into a mouse to induce tumor growth either orthotopically or heterotopically.

LLC Genetics

Polyploid cancer cell lines i.e., tumor cells with a higher genomic content contribute to the rapid growth and evolution of the cells. Tetraploid cells representing an abnormal karyotype is an important intermediate on route to malignancy (Coward and Harding,

2014). Biological and genetic characteristics of tetraploid cells contribute to the unstable properties of human malignancies. Therefore, it is an extremely important part of cancer biology, and it is highly imperative that it be studied. This facilitates the formation of therapy resistant phenotypes in cancer patients and calls for new therapeutic strategies against these polyploid primary tumors (Coward and Harding, 2014). When the kinetic features of Lewis lung carcinoma cells were analyzed via flow cytometry it was found to have a tetraploid DNA content (Starace G *et al*, 1982).

Human cells contain two copies of each chromosome i.e., they are diploid in nature. Oncogenic tetraploid cells like LLCs are chromosomally unstable and contribute highly to tumorigenesis. Due to this tetraploid nature Lewis lung carcinoma cells are highly resistant to genetic manipulation. The exact mechanism by which they achieve such defiance is not known. Therefore, studying the genetic manipulation of tetraploid cells and establishing a fool proof protocol will allow us to fully comprehend how they promote tumor progression.

Lewis lung carcinoma cells produce increased levels of CCL2/MCP-1 and CCL2 was found at high concentrations in patients with multiple tumor types, including NSCLC, and high concentrations usually correlated with poor clinical outcomes (Kishimoto *et al.*,2019). MC-38 murine colon adenocarcinoma cells have been shown to behave similarly (Chun *et al.*,2015). Previous data from our lab have highlighted the role of Superantigens G and I (SEG/SEI) as immuno-stimulatory molecules which launch an enhanced T cell response

against melanoma and Lewis lung carcinoma illustrated by the prolonged survival of HLA-DQ8 tg mice (Knopick *et al,* 2002).

HLA-DQ8 transgenic mice

Human leukocyte antigen or HLA-D molecule is responsible for antigen presentation to CD4+ T cells. HLA-D is polygenic, with HLA-DP, DQ, and DR molecules co-expressed, and with each being polymorphic. HLA-DQ8 (DQA1*0301/DQB1*0302) is one of the HLA-DQ alleles has been demonstrated to be promiscuous in the range of antigens that can be presented, resulting in humans and transgenic (tg) mice being generally more resistant to infectious diseases, susceptibility to autoimmune diseases (Bradley *et al*., 1998), and more resistant to some cancers.

HLA-DQ8 tg mice have shown similar pathological response as non-human primates especially pro inflammatory responses to super antigens (Roy CJ *et al,* 2005). HLA tg mice used as disease models bring us a step closer to the human immunological response system than most conventional animal models, because the MHC molecules in these mice are the same ones involved in human disease.

The generation of mice expressing HLA-DQ8αβ (DQA*0301/DQB*0302) transgenes in the absence of endogenous MHC class II expression (Aβ0) was previously described (Bradley DS *et al*, 1998), and the mice were originally provided by Dr. Chella David (Mayo Clinic, Rochester, MN, USA). MHC class II alleles define the T cell selection in the thymus by presentation of self-peptides (Taneja and David, 1998). Knopick *et al*, 2020 have previously shown that this strain is a good model for hot tumors like Lewis lung carcinoma.

Transgenic mice that express functional HLA molecules have been an integral step as a model for increasing our understanding of the process of cancer induction and progression. These unique transgenic mice provide an opportunity to use an animal model that is genetically more closely linked to humans than any other that have been used before. Analyzing the role of a chemokine, CCL2, response of a highly immunogenic cancer model combined with a 'humanized' mouse model was imperative for our study in its capacity for translation.

CCL2 and cancer

Chemokines are a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes. They were first discovered as chemotactic factors in leukocytes during inflammation. CCL2, a chemokine also referred to as monocyte chemoattractant protein 1 (MCP-1) is chemotactic attractant for monocytes. CCL2 is the first discovered human CC chemokine. Located on chromosome 17 (chr.17, q11.2), human CCL2 is

composed of 76 amino acids and is 13 kDa in size (Van Coillie *et al*, 1999). CCL2 is the strongest chemoattractant responsible for macrophage recruitment and it is a powerful initiator of inflammation. CCL2 can also attract other host cells in the tumor microenvironment acting to the detriment of the host. Presence of a high level of CCL2 has a detrimental effect on prognosis in solid tumor patients because of its ability to attract and accumulate immune suppressive cell sub types (Kishimoto *et al*., 2019). Evidence suggests that CCL2 increases the antitumor effects of certain cell types such as inflammatory monocytes and neutrophils (Jin *et al*, 2021). However, the exact mechanism involving this process is currently unknown.

CCL2 is a chemokine that plays a key role in the migration of monocytes, a type of white blood cell, to sites of inflammation or tissue damage. CCL2 has also been shown to have several other important functions in the immune system and in various physiological processes. CCL2 has also been implicated in the development and progression of cancer and has been investigated as a potential therapeutic target for the treatment of cancer.

In recent years, CCL2 has also been implicated in the development and progression of cancer. Studies have shown that CCL2 is expressed at higher levels in several different types of cancer, including breast cancer, lung cancer, and pancreatic cancer (Kishimoto *et al*., 2019). It has been suggested that CCL2 may contribute to cancer progression by promoting the recruitment of monocytes and other immune cells to the tumor microenvironment, which can facilitate tumor growth and metastasis.

CCL2 is also involved in increasing vascular permeability of the endothelium aiding the escape and migration of tumor cells. CCL2 is produced by cancers of varied immunogenicity and augments tumor proliferation and metastasis (Kishimoto *et al*., 2019). Elevated CCL2 expression by tumor cells is linked to poor prognosis in lung cancer patients (Kishimoto et al., 2019). We have seen a robust increase in CCL2 in culture supernatants of poorly immunogenic Lewis lung carcinoma cells (Supporting data available). CCL2 is a chemokine that attracts and activates mononuclear cells and is also recognized to exert direct immuno-inhibitory (pro-tumorigenic) effects on T-cell function, such as inhibiting T-cell effector functions and switching T-cell differentiation toward Th2-like cells. CCL2 is one of the factors responsible for growth, proliferation, and metastasizing capabilities of tumor cells (Kishimoto et al., 2019).

As high CCL2 is considered an integral factor of poor prognosis and blockade of the CCL2/CCR2 axis presents a unique potential target for treatment of lung carcinoma (Teng Kun-Yu *et al*, 2017) either standalone or in tandem with robust immunotherapeutic interventions like checkpoint blockers (Wang Yue *et al*, 2018) etc. and its blockade can potentially augment cancer immunotherapy (Fridlender *et al*, 2010).

CCL2 expressed in tumors is a monocyte chemotactic protein and high levels of CCL2 in the tumor microenvironment correlates with high levels of tumor associated macrophages (TAMs). In general, TAMs acquire M2-like properties and resemble ‘tolerant’ macrophages, though there is diversity in pathways and phenotypes in different tumors

(Mantovani & Sica, 2010). TAMs can also express antitumor activity. Thus, in response to microenvironmental signals, TAMs can exert dual influence on tumor growth and progression (Mantovani & Sica, 2010). Macrophages and some of their products (IL-1; TNF; IL-6) have long been known to increase metastasis. TAMs are a major component of leukocytic infiltrate of tumors and have served as a paradigm for cancer-related inflammation (Kawai and Akira, 2007). CCL2 is responsible for high TAM infiltration and directly impacts tumor progression and metastasis thus leading to a poor prognosis (Mantovani & Sica, 2010). We are using crispr to manipulate cells into arresting the expression of CCL2 and studying its impact in cancer progression.

CCL-2 exerts its effects mainly via the CCL2/C-C motif chemokine receptor 2 axis and leads to the activation of classical signaling pathways, such as PI3K/Akt/mTOR, ERK/GSK-3β/Snail, c-Raf/MEK/ERK and MAPK in different cells. (Wang *et al,* 2021).

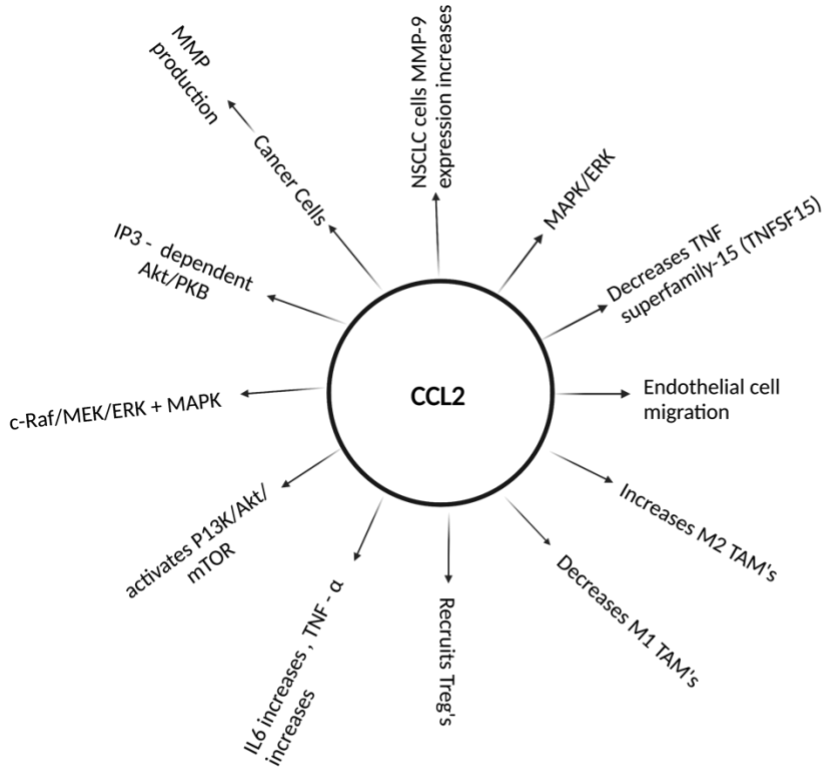


Figure 2: CCL2 and the pathways it affects. MCP-1 or CCL2 is a chemokine that can influence the development of tumors through its effects on various cell types, including monocytes, Treg cells, endothelial cells, and fibrocytes. It can also directly affect tumor cells. Created with Biorender.com

The CCL2 gene (SCYA2) is located on human chromosome 17q11.2-q21.1 (Mehrabian *et al,* 1991). CCL2 is a chemokine that is produced as a precursor protein with a hydrophobic signal peptide at the N-terminus. The signal peptide is cleaved during the process of protein synthesis, resulting in the release of the mature CCL2 protein, which consists of 76 amino acids. The signal peptide is important for the proper folding and transport of the CCL2 protein to its destination within the cell or secreted from the cell. The precursor CCL2 comprises 99 amino acids, with 23 amino acids at the N-terminal, as the hydrophobic signal

peptide, whereas the mature protein is comprised of 76 amino acids, after cleavage of the signal peptide. (Wang *et al,* 2021).

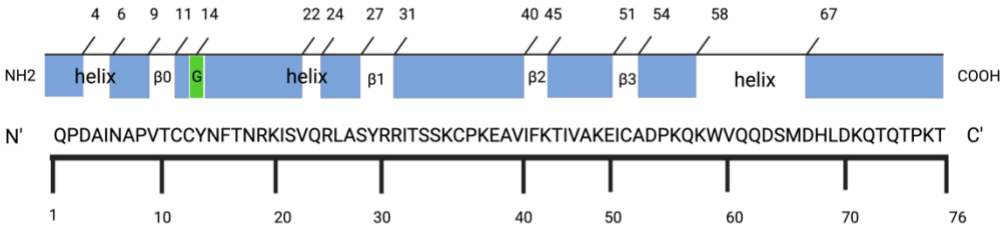
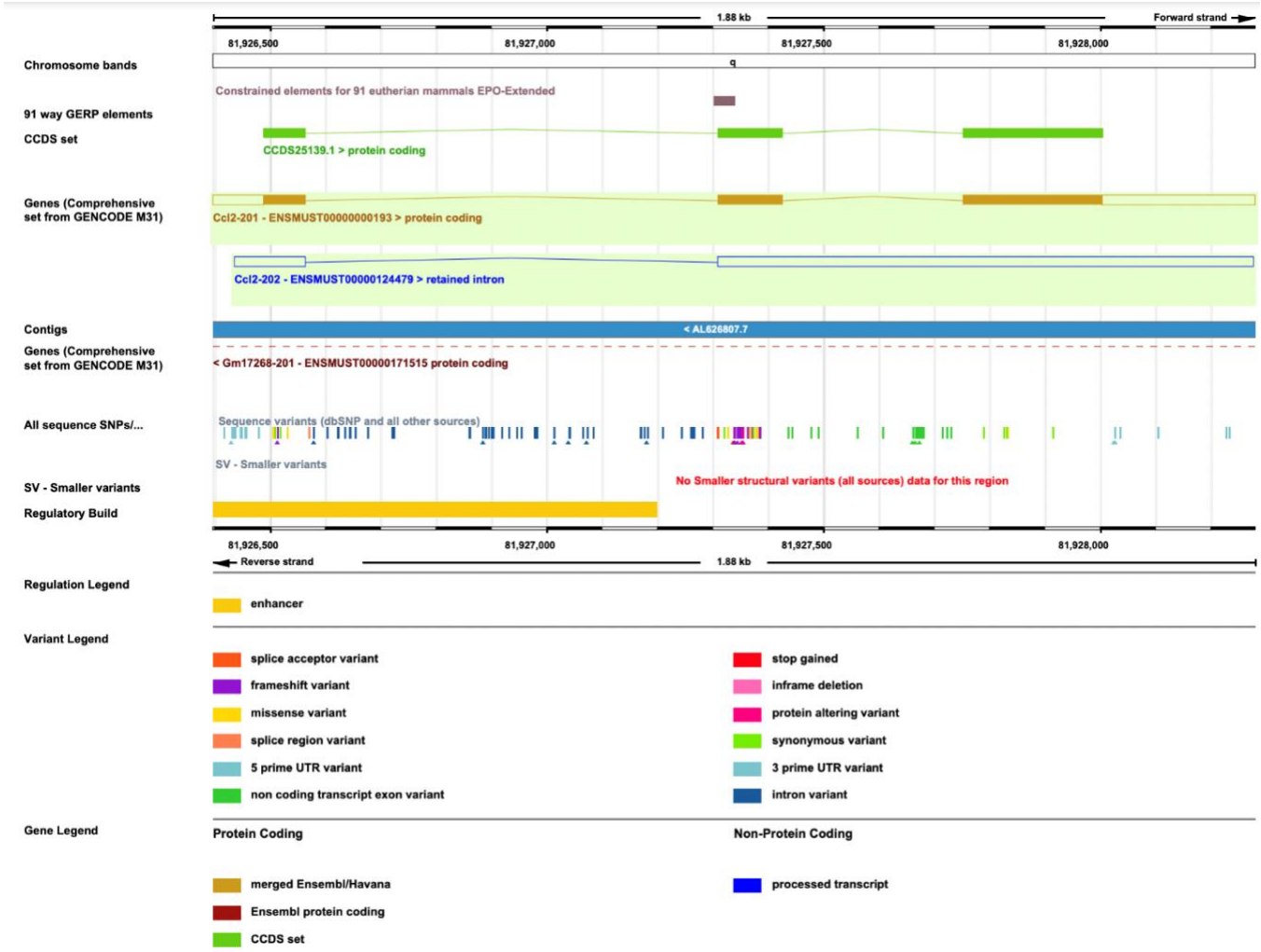


Figure 3: Diagram of CCL2 structure. Created with BioRender.com

The structural illustration of MCPs (chemokines) shows that the first six amino acids at the N-terminus are essential for their chemoattractant activity and the first amino acid is necessary for binding to receptors. CCL2 is composed of 76 amino acids, and the secondary structure of CCL2 consisted of one α-helix and four regions of β-sheet (the grey label), including residues 9–11 (β0), residues 27–31 (β1), residues 40–45 (β2), residues 51–54 (β3), which is little different from the data in PDB protein bank. The last one shows that CCL2 has three α-helix (the grey sections). Residue 14 can be glycosylated (the green section), which can slightly decrease the potency of the chemotactic activity of CCL2. Source (Wang *et al,* 2021).


1.88 kb
Forward strand
81,926,500
81,927,000
81,927,500
81,928,000
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q
Constrained elements for 91 eutherian mammals EPO-Extended
91 way GERP elements
CCDS set
CCDS25139.1 > protein coding
Genes (Comprehensive set from GENCODE M31)
Ccl2-201 - ENSMUST00000000193 > protein coding
Ccl2-202 - ENSMUST00000124479 > retained intron
Contigs
< AL626807.7
Genes (Comprehensive set from GENCODE M31)
< Gm17268-201 - ENSMUST00000171515 protein coding
All sequence SNPs/...
Sequence variants (dbSNP and all other sources)
SV - Smaller variants
SV - Smaller variants
No Smaller structural variants (all sources) data for this region
Regulatory Build
81,926,500
81,927,000
81,927,500
81,928,000
Reverse strand
1.88 kb
Regulation Legend
enhancer
Variant Legend
splice acceptor variant
frameshift variant
missense variant
splice region variant
5 prime UTR variant
non coding transcript exon variant
stop gained
inframe deletion
protein altering variant
synonymous variant
3 prime UTR variant
intron variant
Gene Legend
Protein Coding
merged Ensembl/Havana
Ensembl protein coding
CCDS set
Non-Protein Coding
processed transcript

Figure 4: The sequence map details of CCL2 region Chr11:81926403-81928278 bp, + strand.

Ensembl Mus Musculus version 108. 39 (GRCm39) Chromosome 11: 81,926,397-81,928,279 - Region in detail - Mus_musculus - Ensembl genome browser 108. (2023). Retrieved from: http://useast.ensembl.org/Mus_musculus/Location/View?=ENSMUSG00000035385;r=11:81926397-81928279;db=core

Role of CCL2 in solid tumor cancer progression

The elevation in CCL2 expression has generated increasing interest in recent years due to its association with the progression of cancer and drug resistance (Kishimoto *et al*., 2019). CCL2-induced recruitment of CCR2+Ly6C(hi) monocytes increases vascular permeability of CCR2+ endothelium aiding the escape and migration of tumor cells (Kishimoto *et al*., 2019). CCL2 is produced by cancers of varied immunogenicity and augments tumor proliferation and metastasis. Elevated CCL2 expression by tumor cells is linked to poor prognosis (Jin *et al.*, 2021). Although first described as a chemotactic molecule with physiological roles in regulating inflammation, recent studies have revealed a pro-tumorigenic function for CCL2 in favoring cancer development and subsequent metastasis (Jin *et al.*, 2021). CCL2 binds the cognate receptor CCR2, and together this signaling pair has been shown to have multiple pro-tumorigenic roles, from mediating tumor growth and angiogenesis to recruiting and usurping host stromal cells to support tumor progression. The importance of CCL2-CCR2 signaling has been further championed by the establishment of clinical trials targeting this signaling pair in solid and metastatic cancers. (Li *et al*, 2013).

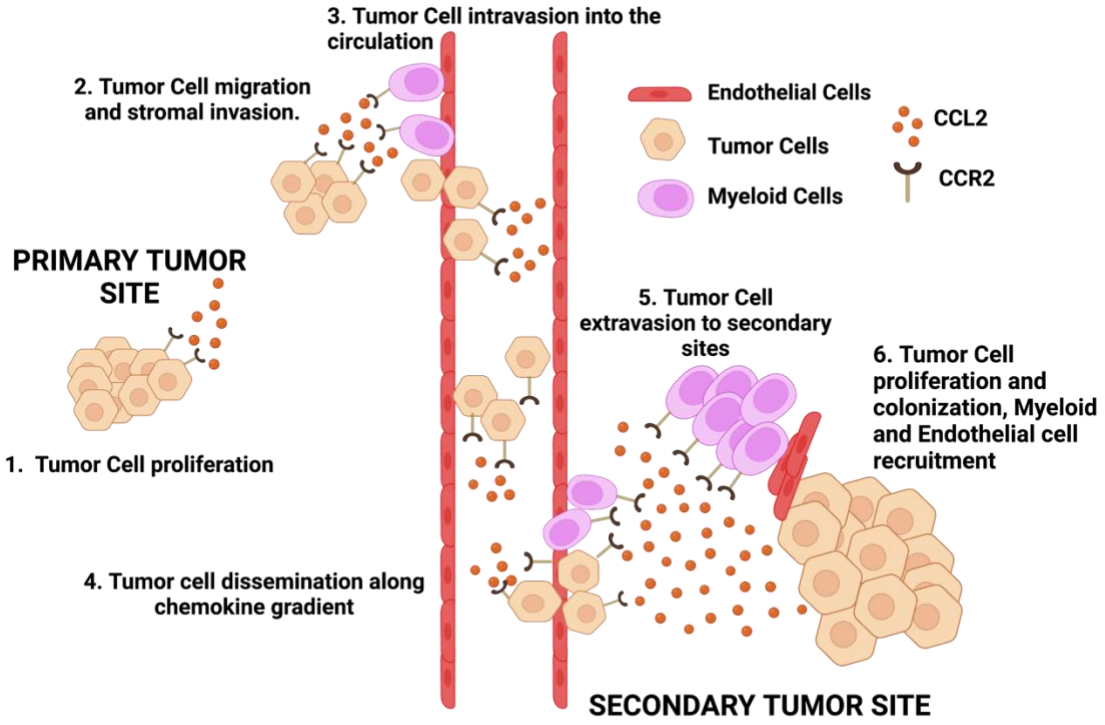


Figure 5: The role of CCL2-CCR2 signaling during the metastatic process. Created with BioRender.com

CCL2 is a chemokine protein that is produced by both cancer cells and stromal cells in the tumor microenvironment. **Figure 5** shows the crucial role it plays in various stages of tumor progression, including tumor growth, invasion, and metastasis.

1. CCL2 induces tumor cell proliferation at the primary tumor site: CCL2 has been shown to stimulate the proliferation of tumor cells at the primary tumor site, leading to increased tumor growth.
2. CCL2 stimulates tumor cell migration and invasion: CCL2 also promotes the migration and invasion of tumor cells into the surrounding extracellular matrix, which is a critical step in the metastatic process.

3. CCL2 promotes tumor cell intravasation: CCL2 recruits host myeloid cells, which facilitate tumor cell intravasation into the circulation. Intravasation is the process by which tumor cells enter the bloodstream and travel to distant sites in the body.
4. CCL2 directs the dissemination of cancer cells: Once in the circulation, CCL2 may direct the dissemination of cancer cells along a chemotactic gradient towards the metastatic site.
5. CCL2 initiates tumor cell extravasation: Tumor cells become trapped in small capillaries during their journey through the bloodstream, and CCL2 supports their extravasation, which is the process by which tumor cells leave the bloodstream and enter the surrounding tissue.
6. CCL2 promotes tumor growth and colonization: Finally, CCL2 promotes tumor growth at the metastatic site by recruiting additional myeloid and endothelial cells, which support tumor colonization and growth.

In summary, CCL2 plays a critical role in tumor progression, and targeting CCL2 signaling may provide a potential therapeutic strategy to inhibit tumor growth, invasion, and metastasis. (Li *et al*, 2013).

We aim to overcome the pro-tumorigenic effects of CCL2 by using CRISPR-Cas9 to edit the gene responsible and inhibit its expression. B16 melanoma is a spontaneous melanoma derived from C57BL/6 mice and MC-38 is a murine colon carcinoma model. We present data to show the difference in CCL2 levels in LLC vs B16 vs MC-38 *in vivo* and *in vitro*.

We also show data in support of creating a CCL2 knock-out Lewis lung carcinoma cell line to dampen its pro-tumoreigenic effects, suppress its growth and proliferation, and increase survival in LLC-KO mouse.

CRISPR-Cas9

CRISPR-Cas9 is a powerful tool for editing the genome, or genetic material, of cells. It consists of two components: the Cas9 enzyme, which acts as "molecular scissors," and a small piece of RNA, called a guide RNA, that directs the Cas9 enzyme to a specific location in the genome. CRISPR-Cas9 mediated genome editing technology brings a great promise for inhibiting migration, invasion, and even treatment of tumors. (Chen *et al*., 2022)

CRISPR-Cas9 technology has been widely used in cancer research to investigate the role of specific genes in cancer development, progression, and treatment. One of the major advantages of CRISPR-Cas9 is its ability to precisely target and edit specific genes in cancer cells. By deleting or knocking out specific cancer-associated genes using CRISPR-Cas9, researchers can identify the function of these genes in cancer development and progression. This information can be used to develop new targeted therapies that specifically target these genes or their associated pathways (Ishibashi *et al*, 2020)

In addition, CRISPR-Cas9 can be used to insert or "knock in" specific genetic changes that may be beneficial in the treatment of cancer. For example, researchers have used CRISPR-

Cas9 to activate tumor suppressor genes that are normally turned off in cancer cells or to inactivate oncogenes that drive cancer growth. These genetic changes can potentially be used as therapeutic strategies to treat cancer. (Katti *et al*, 2022)

Overall, CRISPR-Cas9 has revolutionized cancer research by providing a powerful tool for investigating the role of specific genes in cancer and for developing new targeted therapies for cancer treatment. In addition to its use in the study of cancer genetics, CRISPR-Cas9 has also been explored as a potential tool for cancer immunotherapy. For example, CRISPR-Cas9 has been used to modify T cells, a type of immune cell, to make them more effective at targeting and killing cancer cells (Dimitri *et al*, 2022). It has also been used to delete or knock out genes in T cells that may inhibit their ability to recognize and attack cancer cells. (Dimitri *et al*, 2022)

Another potential application of CRISPR-Cas9 in cancer therapy is the use of gene editing to repair DNA damage that may contribute to the development of cancer. For example, CRISPR-Cas9 has been used to repair genetic mutations in cells that may lead to the development of cancer, such as TP53 mutations in ovarian cancer cells (Walton *et al*., 2016)

Additionally, CRISPR-Cas9 has been explored as a potential tool for the delivery of cancer therapies (Doudna & Charpentier, 2014). For example, researchers have developed CRISPR-Cas9-based "gene drives" that can spread a specific genetic change through a population of cells. This technology has been used to deliver cancer therapies directly to

cancer cells, potentially increasing their effectiveness and reducing the side effects of treatment. (Douglas *et al*, 2017)

It is important to note that while CRISPR-Cas9 has shown great promise in the study of cancer and the development of new cancer therapies, these approaches are still in the early stages of development and much more research is needed to fully understand their potential and limitations.

One common application of CRISPR-Cas9 is the deletion or "knocking out" of specific genes. This is known as a CRISPR-Cas9 mediated knockout.

To perform a CRISPR-Cas9 mediated knockout, researchers first design a small piece of RNA called a guide RNA that directs the Cas9 enzyme to a specific location in the genome. The guide RNA is then introduced into cells along with the Cas9 enzyme, which cuts the DNA at the targeted location. The cell's repair machinery then attempts to repair the DNA damage, which can result in the deletion or modification of the targeted gene.

CRISPR-Cas9 mediated knockouts are used in a variety of research and clinical applications, including the study of gene function, the identification of potential therapeutic targets, and the development of new therapies for genetic diseases. (Doudna and Charpentier, 2014)

The main function of immunotherapies is to relieve tumor-induced immunosuppression and re-boost anti-cancer immunity (O'Donnell *et al*, 2019) Exploiting that mechanism, investigating the pathways, and finding out the vulnerabilities in the steps is where the cure

lies. Using CRISPR-Cas9 (Doudna and Charpentier, 2014) to manipulate a highly aggressive cancer cell line like Lewis lung carcinoma is our attempt at exploiting that vulnerability.

Since alterations in cellular DNA could lead them to become cancer cells scientists have been trying to modify and manipulate the cellular genetic material to control their division, metastases, and progression into tumors. Even though many techniques of gene manipulation were well researched and studied, most lacked precision. CRISPR changed that, a simple bacterial defense mechanism became a versatile gene-editing tool to be used by scientists all over the world. CRISPRs, or clustered regularly interspersed short palindromic repeats when attached to an identifying RNA (called guide RNA) guides the Cas9 protein to an exact location in the DNA and instructs it to splice it up. Varied genomic mechanisms then take over to repair that cut. This process either replaces harmful mutations with the desired DNA sequence or like in our case disrupt the gene to impede the protein (CCL2) production. (Doudna and Charpentier, 2014)

CRISPR-Cas9 systems have shown a substantial degree of efficacy, specificity, and flexibility to target the specific locus in the genome for the desired applications yet tetraploid cancer cells launch a formidable resistance to being genetically manipulated. tetraploid cells are commonly found in tumors, particularly in the early stages; and the number of chromosomes in tumor cells is often very high, which is difficult to explain by a repeated accumulation of chromosomes at each division. Tetraploid cells that can

propagate can trigger cell transformation and tumor formation. Other mutations in established oncogenes were recently linked to tetraploidization and its tumorigenic potential. (Storchova and Kuffer, 2008)

Due to having twice the number of diploid chromosomes gene expression in these cells are not absolute: they either revert to the parental combination or have a 50% editing efficacy. We overcame this problem by using three different guide RNAs (gRNA) to target the three different exons responsible for the production of CCL2 protein. We therefore contribute a robust editing technique which overcomes the limitations of CRISPR-Cas9 in immortalized cell lines.

We have designed and evaluated CRISPR based CCL2 knockout LLC cell lines which blocks this CCL2 expression restricting the abundant proliferation and metastasis of LLC cells and could be a potential target to revolutionize treatment for advanced stage lung cancer. The two principal methods that has been used in this study is Crispr-Cas9 and Flow cytometry, along with various supporting *in vitro* and *in vivo* experiments like cell cycle analysis, histology, transgenic mice survival, etc. My overall CRISPR-Cas9 knockout approach was to select gRNAs that would target the Cas9 endonuclease to the N-terminal coding exon unique to the desired CCL2 variant. I expected that Cas9 induced double strand breaks would undergo repair via Non Homologous End Joining, in an error prone manner, introducing indels, resulting in a frame shift mutation, a premature stop codon, and nonsense-mediated mRNA decay.

We illustrate the basis of our mechanism of CCL2 blockade via CRISPR in the figure below.

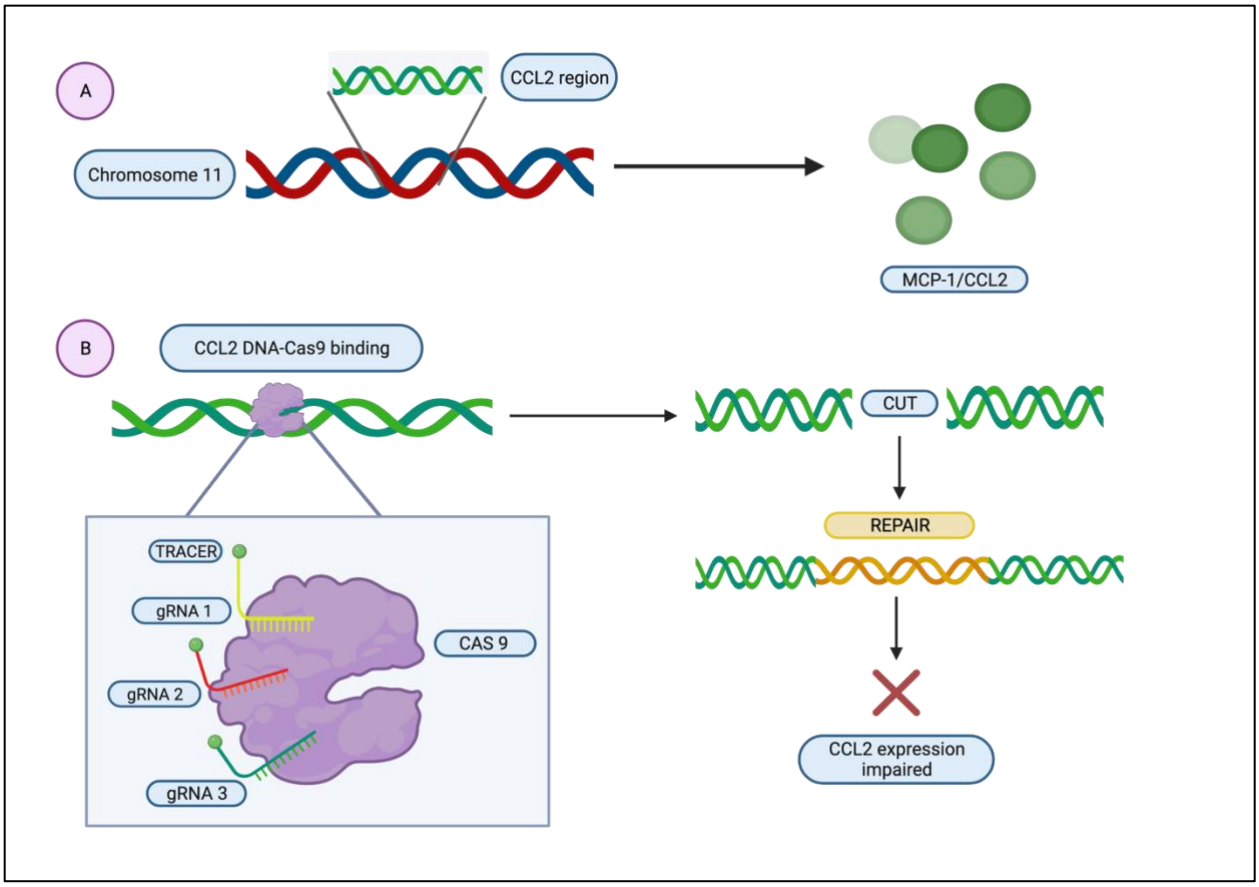


Figure 6: A. The CCL2 region is expressed into CCL2 protein. B. Cas 9 enzyme with the help of guide RNA (+ tracer molecule) binds to PAM site of CCL2 region to initiate a cut, that leads to mismatched repair thus inhibiting CCL2 expression. Created with BioRender.com

gRNA had been designed and attached to the Cas9 molecule with a flow cytometry-detectable marker/tracer ATTO488. This gRNA has been reverse-transfected into LLC cells, sorted via Flow cytometry and grown from single cells into pure CRISPR positive colonies. Reverse transfection combines RNA, transfection reagent, and cells in an altered sequence compared to traditional Lipofectamine transfection protocols; in short, a different gRNA is put in each well prior to transfection and combined with diluted Lipofectamine to form complexes in each well; cells are added directly to the Lipofectamine gRNA complexes and transfection occurs while cells are attaching to the well. The cells after multiplication were screened via PCR/T7EI endonuclease tests for positive clones. PCR with designed primers was followed by Gel electrophoresis to confirm the homology directed repair of gene-edited and reprogrammed LLC to inhibit CCL2. HLA-DQ (transgenic) mice were implanted with LLC IP. Mice were monitored daily; Blood was collected on Day 3, 6, 9 and 13 and the mice were sacrificed when moribund. The serum was extracted from blood samples and assessed via cytometric bead array by Flow cytometry for the presence/absence of Cytokines/chemokines. The ND Flow cytometry core supported by INBRE and Host-Pathogen COBRE has been integral in the success of this project.

Combinatorial therapies inhibiting CCL2 with the addition of treatments that enhance the tumor-specific response with therapies like anti PD-1 antibodies, may alter the tumor

environment of treatment refractory cancers that induce high CCL2 production, like LLC and MC-38, thus allowing a potent anti-tumor and anti-metastatic response and increased survival. While we would predict that CCL2-/- LLC may be less aggressive without a therapeutic modality, blocking CCR2 expression would allow therapies, e.g. SEG/SEI or anti-PD1 antibody treatment, to be able to induce the clearance of the tumor. Regardless, we provide here a CCL2 -/- LLC line that will be invaluable in finding putative therapeutic candidates for established LLC and work going forward for lung and other tumor types.

Tumors establish pro-tumorigenic and immunosuppressive environments to support their growth and promote immune evasion. Central to building an immunosuppressive tumor microenvironment are oncogenes and aberrant signaling pathways that lead to the production of cytokines and chemokines with potent effects. CCL2 is one such pro tumorigenic cytokine that needed to be studied in more detail like its over expression, its impact on the growth, survival and metastasis of this highly aggressive tumor, the impact of its presence or absence in contributing to survival outcomes. Our study and the results have attempted to answer these questions and uncover these responses.

We have previously shown and have recently published (Knopick *et al*, 2020) results that a cocktail of two bacterially derived superantigens, Staphylococcal enterotoxin G and I (SEG/SEI) stimulate large numbers of T cells in an antigen-independent fashion and promote enhanced survival in the poorly immunogenic B16-F10 melanoma model

(Knopick *et al*, 2020). Conversely, the poorly immunogenic Lewis Lung Carcinoma (LLC) was not responsive to SEG/SEI and SEG/SEI stimulation did not change the time to death in LLC-implanted mice (Knopick *et al*, 2020). All these studies were performed in humanized HLA-DQ8 (DQA1*0301 and DQB1*0302) transgenic mice. Here we investigate background mechanistic differences following SEG/SEI stimulation between LLC and B16-F10 that may explain the disparate outcomes. Interestingly, the LLC cells secrete high levels of CCL2 *in vitro* whereas B16-F10 cells do not. We also show elevated levels of CCL2 in MC-38, an immunogenic murine colorectal cancer cell line. We are using CRISPR-Cas9 as our genome editing tool to inhibit the expression of CCL2 and thereby investigate the effects of CCL2 inhibition on LLC tumor proliferation and metastasis. We also provide data to support the successful creation of LLC CCL2 KO's using CRISPR-Cas9. We investigate the process and explore a potential solution to this problem by knocking out the chemokine CCL2 which is a major factor responsible for the growth and metastasis of lung carcinoma. These findings, taken *in toto*, suggest that combinatorial therapies inhibiting CCL2 with the addition of treatments that enhance the tumor-specific response, for instance SEG/SEI or possibly other therapies like anti PD-1 antibodies, may alter the tumor environment of treatment refractory cancers that induce high CCL2 production, like LLC and MC-38, thus allowing a potent anti-tumor and anti-metastatic response and increased survival. Here, we present a CCL2 -/- LLC cell line that will be extremely useful in identifying potential therapeutic agents for established LLCs, as well as future research for lung and other types of tumors.

Central Hypothesis

Use of CRISPR-Cas9 for attenuation of localized chemokine CCL2 expression by Lewis lung carcinoma cells result in arrest of its growth, proliferation and metastasis leading to increased survival of DQ8 transgenic mice implanted with LLC.

CHAPTER II

MATERIALS AND METHODS

Ethics

All research performed, including animal and tissue collection, was conducted in accordance with the Animal Welfare Act and with the approval of the University of North Dakota's Institutional Animal Care and Usage Committee (IACUC). All mouse experiments were performed with 8–12-week-old C57Bl/6J male and female mice purchased from the Jackson Laboratory (Bar Harbor, ME, USA) or bred in-house at the University of North Dakota, with similar results. Mice were acclimatized to laboratory conditions for a week before starting the experiments. Mice were bred and housed in temperature-controlled rooms with a 12:12 h light–dark cycle at 23 ± 0.5 °C.

Murine Models

Mice were bred and maintained in within the Center for Biological Research at the University of North Dakota. A laboratory inbred colony of C57BL/6 mice and HLA-DQ8 tg mice were used for experiments. HLA-DQ8 (DQA*0301/DQB*0302) tg mice was used, originally a gift from Dr. Chella David (Mayo Clinic, Rochester, MN). The generation of these mice were described previously (Taneja and David, 1998; Rosloniec *et al,* 1997). DQ8 mice were generated on H2b haplotype mice lacking H2A (Petersson *et al,* 2002) and inherent H2E expression, thus making them devoid of murine class II.

Cell lines and culture conditions

Lewis lung carcinoma (LLC1) (CRL-1642^{TM}) and B16-F10 (CRL-6475^{TM}) murine melanoma cells and were obtained from American Type Culture Collection (ATCC), MC-38 (ENH204-FP ^{TM}) obtained from Kerafast were maintained according to manufacturer recommendations in complete Dulbecco's Modified Eagle's Medium ((DMEM) Gibco containing 10% heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals), 50 IU/ml Penicillin and 50 μg/ml Streptomycin (MP Biologicals), and HEPES. Cells were maintained under conditions humid in a 37°C incubator with 5% CO_2. atmosphere Frozen 1 ml aliquot stocks were made by freezing ~1x10^6 cells in complete DMEM supplemented with 50% vol/vol sterile dimethyl sulfoxide (DMSO). Cells were maintained in -80°C and liquid nitrogen vapor phase.

Cytokine analysis

Human and mouse innate cytokines Mentioned in Table. 1 were measured by DuoSet enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN) or mouse inflammation panel cytometric bead analysis (CBA) kit (BioLegend, San Diego, CA). CBA samples were measured by flowcytometry (LSR II, Becton Dickinson, San Jose, CA), and analyzed with software provided by the manufacturer (Biorad LEGENDplex online analysis tool).

Analyzing the Tumor Microenvironment by Flow Cytometry

Tumor cells were isolated by passing them through a 70 μm cell strainer (Falcon) with bottom plunger of 5ml or 10ml syringe. Cells were washed with DMEM, ACK lysed, washed and strained again before counting. Cell yield varies but averaged between 50-100 million total cells in naïve mice. Cells were washed with DMEM and strained again before counting.

Flow Cytometry

The flow cytometry staining protocol was a set of steps used to prepare cells or particles for analysis by flow cytometry. The following steps were involved in our Flow Cytometry procedure:

1. Sample preparation: We collected and prepared the sample for analysis. This involved isolating cells from a tissue samples like tumor and spleen, and purifying cells or particles from a complex mixture.

2. Sample labeling: This involved attaching fluorescent labels to specific proteins, and other molecules within the cells. These labels allow the flow cytometer to detect and measure specific characteristics of the cells or particles.

3. Staining: This involved adding the labeled cells or particles to a solution containing one or more reagents, FACS buffer that interact with the labels to produce a detectable signal.

4. Analysis: This involved running the labeled cells or particles through the flow cytometer and collecting data on their characteristics. This data was then analyzed to gain insights into the biology of the cells or particles.

Table1 : Antibodies used for flow cytometry experiments.

Fluorophore	MARKER	Vendor	Clone
bv405	viability	invitrogen	
	Fc block	Tonbo	2.4g2
Violet Fluor 500	CD45	Tonbo	30-F11
BV650	CD8a	Biolegend	53-6.7
BV 605	TCR b	Biolegend	H57-597
FITC	HLA DQ	Invitrogen/ebio	SK10
Per cp cy 5.5	CD4	Tonbo	RM4-5
PE CY7	CD127	BD	SB/199
PE CF594	CD49b	BD	DX5
PE	CD25	Tonbo	PC61.5
Apc Cy7	CD3	Tonbo	17A2
APC	FOX P3	ebioscience	FJK16S
BV786	CD86	BD	GL1
PE CF594	CDIIb	BD	M1/70
Violet Fluor 500	CD45	Tonbo	30-F11
BV 605	CD69	Biolegend	H1.2F3
BV711	CD196	BD	140706
BV786	CD279	BD	J43
FITC	Ki-67	Biolegend	11F6
Per cp cy 5.5	CD152	Biolegend	UC10-4B9
PE CF594	CD44	BD	IM7
PE CY7	CD192	Biolegend	QA18A56
PE	CD274	BD	MIH5
APC	MCP-1	Biolegend	2H5
Apc Cy7	CD3	Tonbo	17A2

Flow cytometry Cell Staining

Cells were washed with HBSS, stained with Ghost Dye for viability (TONBO), Fc blocked and stained for extracellular antigens via standard protocols. Cells were fixed and permeabilized using Foxp3 staining buffer kit (TONBO) for intracellular cytokine and transcription factor analysis. See Table 1 for list of antibodies used in this manuscript. Fluorescence minus one (FMO) and single stained controls were used for gating and compensation. Gating strategies are indicated within each experiment. In general, doublets and cell debris were excluded with only Ghost Dye negative cells being used for analysis. Samples were analysed using a BD Symphony A3 flow cytometer in the North Dakota Flow Cytometry and Cell Sorting (ND FCCS) Core. Data was analysed using FlowJo software.

Cell Cycle Assay

The cell cycle analysis was conducted using propidium iodide staining (PI) and a flow cytometric method. Each cell type was seeded in a 6 well plate in triplicate at sub-confluent cell density (2x 10^5 cells/per well) and incubated at 37^{o}C and 5% CO_2 for 3 days. On day 3 the cells were trypsinized, pelleted (450 g for 5 mins), and resuspended in PBS. A sample of each suspension was collected for cell counting. The suspension was pelleted again and resuspended in cold 70% EtOH (made in water, not PBS) and left to fix overnight at 4^0C. After cell fixation, EtOH was removed by two consecutive washes with 1X PBS (850g for 10 mins) and the cells were resuspended in 300 μL of FxCycle PI/RNase Staining Solution

(Invitrogen Cat No. F10797). The samples were run on a BD Symphony flow cytometer using PE or PE-CF594 channels. Collected data were gated using FLOWJo software to identify single cells which were then analyzed using the cell cycle feature in the software. Percentage number of events from 3 independent experiments was used to compare cell cycle differences between the CCL2 knockout isolates and WT using One-Way and Two-Way ANOVA.

Generation of tumors

Tumors were implanted intraperitoneally that generated palpable solid tumor masses in the peritoneal cavity at approximately 10 days.

Cells for tumor implant were grown in T75 tissue culture flasks and harvested for use at < 80% confluency. Phosphate buffer saline (PBS) was used to rinse the cells before adding trypsin without Ethylenediamine tetra acetic acid (EDTA). The flasks were kept in the incubator for 3-5 minutes at 37°C for overall detachment of cells. cDMEM was used to neutralize trypsin and cells were washed twice with PBS by Centrifugation and pelleting. Cell pellets were resuspended at $2.5x10^6$ cells/ml in PBS. The stock was aliquoted into 1ml microcentrifuge tubes for individual dosages to ensure consistent tumor burden among mice.

Mice were injected with $2.5x10^6$ cells/ml Lewis Lung carcinoma cells IP in 100µl HBSS on day 0. Mice were challenged on day 0 as described above. Blood was collected for serum CBA analysis on days 9 and 13 respectively. Mice were continuously evaluated and sacrificed when moribund.

Construction and Transfection of Triple Guide RNA (gRNA)

Guide sequences followed by protospacer adjacent motifs (PAMs) were designed using an online design tool developed by Dr. Feng Zhang at the Massachusetts Institute of Technology (https://zlab.bio/guide-design-resources) or a design tool provided by Integrated DNA Technologies on its website (www. Idtdna.com/site/order/designtool/) and selected based on the scores for highest ‘on target’ and lowest ‘off target’ events/scores.

Table 2. gRNA sequences targeting Exon 1, 2 and 3 generated using MIT and IDT algorithms.

Target Region	Position	Sequence	PAM	On Target Score
Exon 1	82035707	CAACACGTGGATGCTCCAGC	CGG	73
Exon 2	82037012	TTGTAGGTTCTGATCTCATT	TGG	60
Exon 3	82036498	ATGAGTAGCAGCAGGTGAGT	GGG	56

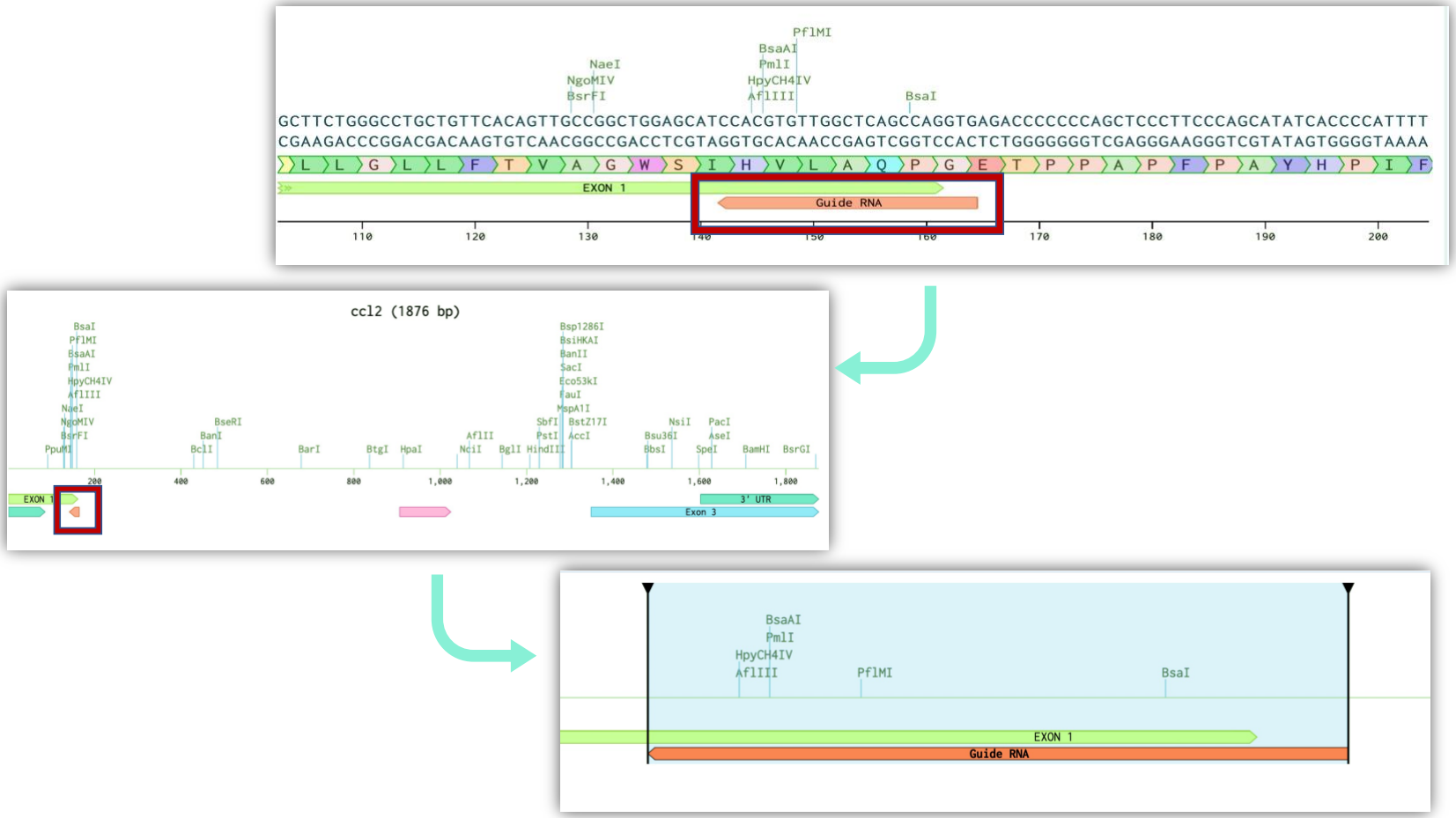


Figure 7: One guide RNA hit for Exon 1.

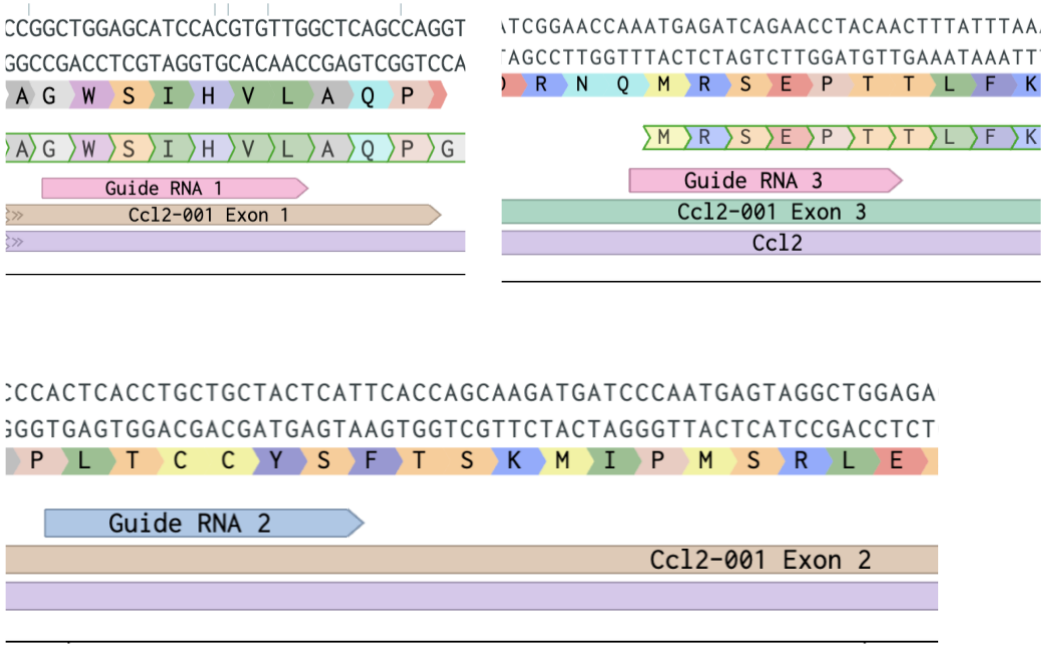


Figure 8: Three guide RNA hit for each different exon respectively

Preparation of Cas9-gRNA ribonucleoprotein complex

ATTO 488 is a new hydrophilic fluorescent label with excellent water solubility. The dye exhibits strong absorption, high fluorescence quantum yield and exceptional thermal and photo-stability. The fluorescence is excited most efficiently in the range 480 - 515 nm.

RNA oligos were resuspended in nuclease free IDTE Buffer (10 mM Tris, 0.1 mM EDTA). The gRNA is made up of two parts: crispr RNA (crRNA), a nucleotide sequence complementary to the target DNA, and a tracr RNA (ATTO-488), which serves as a binding scaffold for the Cas nuclease. To make the guide RNA, 2 nmol of crRNA was mixed with 5 nmol of tracrRNA and heated at 95ºC for 5 minutes then cooled to room

temperature. Alt-R S.p. Cas9 nuclease V3 (IDT cat# 1081058) was combined with guideRNA oligos to create a ribonucleoprotein (RNP) complex. The 3 RNP complex (one for each guide RNA) was reverse transfected into LLC cells. Confluent cultures were transfected using Lipofectamine CRISPRMAX transfection reagent (Invitrogen Cat.CMAX00003) two days after seeding. Cells were diluted to 400,000 cells/ml using complete media without antibiotics. To perform the transfection, cells were incubated in transfection solution containing RNP complex. 500ul of RNP complex (gRNA AA+AC+AE) was combined with 500ul of crisprmax reagent solution and incubated for 20 minutes at room temperature. 50ul of this solution was added to 100ul of cells in each well of a 96 well plate at 37 ^{o}C and 5% CO_2.

Fluorescence-Activated Cell Sorting (FACS)

For FACS, cells are first labeled with fluorescent dyes or other markers that are specific to certain cell types or molecules of interest. The labeled cells are then passed through a flow cytometer, which measures the fluorescence of each cell as it passes through a laser beam. Based on the fluorescence of the cells, the flow cytometer can sort the cells into different categories or "gates."

FACS can be used to sort cells based on a wide range of characteristics, including size, shape, surface marker expression, and intracellular marker expression. It is a fast and

efficient way to purify cells and can be used to isolate specific cell types or subpopulations of cells for further analysis or experimentation.

Twenty-four hours after transfection with the sgRNA plasmids, ATTO-488 positive cells were selected using a fluorescence-activated cell sorter (BD F.A.C.S.Aria III) and sorted as single cells into sterile 96 well flat bottom plates (corning) with one cell per well target and into 15 ml Falcon tubes containing media. Un-transfected LLC's were sampled first to control for autofluorescence prior to sorting the gRNA-ATTO transfected LLCs. Stringent gating was done to pick out the cells with the highest fluorescence to increase the fidelity of CRISPR-Cas9 events in the selected cells.

Selection and Cloning Knockout Cell Lines

Two different ways were used for selection of knockout cell lines: One from a few wells of the 96 well plates they were sorted into. Two, from $150m^3$ dishes where the transfected cells were grown, via cloning discs. Cloning discs were used to generate clonal cell lines in four steps. First, ATTO-488 positive cells from the CRISPR-Cas9 transfected cell suspension were allowed to recover and reach confluence. Next, the confluent cells were trypsinized with 2-5 µl. Trypsin without EDTA, serially diluted, and 1: 1000 and 1:100 dilutions were plated on 10 cm dishes for culturing and observation for 10 days. These single-cell colonies were sub-cultured into a 48 well plate using clonal discs soaked in trypsin. Finally, the wells that reached 80% confluence were passaged 1:2 to be screened for CRISPR-Cas9 induced mutations.

Gene sequence for CCL2 and the specific regions selected for primer-based PCR amplification:

CCTGGAAACACCCGAGGGCTCTGCACTTACTCA**GCGGATTCAACTTCCACT**
TTCCATCACTTATCCAGGGTGATGCTACTCCTTGGCACCAAGCACCCTGCCTG
ACTCCACCCCCCTGGCTTACAATAAAAGGCTGCCTCAGAGCAGCCAGAAGTG
CAGAGAGCCAGACGGGAGGAAGGCCAGCCCAGCACCAGCACCAGCCAACTC
TCACTGAAGCCAGCTCTCTCTTCCTCCACCACCATGCAGGTCCCTGTCATGCT
TCTGGGCCTGCTGTTCACAGTTGCCG**GCTGGAGCATCCACGTGTTG**GCTCA
GCCAGGTGAGACCCCCCCAGCTCCCTTCCCAGCATATCACCCCATTTTTGAAT
TGTCGTGGATTGTGATAGCATAGTCTCACATGGTCAGGTACTTTTTTTTTTTTC
TTTAACCAAGATAAGGAGCATAAAGAAGGAAGGACAAAGAGCCAACCCAAT
TACAAGATTGCTTCTGGAAAGCAACTAGAATTTTAATTGTTAGATCTAAATTT
GGAATCACACCTTCATATAGTTCCTGTTCCAGTTACTTCCCTCAGTATTTGGG
AACCTGGGTGATCAAACAGAGGCTTGGGTTGGTGCCTTTTTCCAGATAGAGG
AGAAAGGGGAAGAGATCCAAGATCCGAGCTGTGTTTCACCCAGCCCTGCTTC
CAGAGATAGCAGCTTAGCGGAGGTGGTTGGGATCAGAGATACTCATGATTTG
ATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCAGCCTACAGTAATGTACTC
AGGTAATCTTCTCAGGTCATAGTAATTTGACTTCTAACTCCCCCAAATGACAG
TCCCCAGAGTCACATAGTTTTAATGGCATCCCTCTACCCAAGACTGTGAGCCT
ACTTTAAGCTGCAAATAACTGAGTCTGTTGTCAAAGATCACATCCCAGATCTG
ATGTATTGGCAT**TTATATCCCATCCTGCTGAAACT**GCCTTCTCCCCGTGGTC
CTTCTCTTCTCTAAGGTCAGAAGCATCTTTCCTGTCCTAATGTGCTTCTCTTTT
ACTCTCCAGATGCAGTTAACGCCCCACTCACCTGCTGCTACTCATTCACCAGC
AAGATGATCCCAATGAGTAGGCTGGAGAGCTACAAGAGGATCACCAGCAGC
AGGTGTCCCAAAGAAGCTGTAGTGTGAGTTACATACCCCGGCCCTCCCTGGT
CCAAAGGTTTTTCCTTAAGAACAAGGGATGGTCCTCATATACTTATAGTCAGT
CACACACTCAGATCCAATGGGGAAACCAAGGCCAAGAAGGCAAAGGCAGT

PCR assay to amplify genomic DNA regions surrounding guide RNA

Mm.Cas9.CCL2.1.AA: CAACACGTGGATGCTCCAGC CGG

Amplicon Size: 948 bp

Cut products (after T7EI digestion): 253/695

Mm.Cas9.CCL2.1.AA_For: GCGGATTCAACTTCCACTTTC

Mm.Cas9.CCL2.1.AA _Rev: AGTTTCAGCAGGATGGGATATAA

Primer Tm: 61.6ºC

BLAST: No predicted off-target amplification in the mouse genome

Secondary structure: good

Analysis of Genomic of DNA for CRISPR-Cas9 Modifications

A T7EI assay was used to identify clones positive for a CRISPR-Cas9 induced edit. For this assay, total DNA from isolated clones of GFP positive cells was extracted using QuickExtract™ (QE09050), amplified using primers spanning the gRNA target region, digested with the respective restriction enzyme, and run on 2% agarose gel.

gRNA induced edits were screened by amplifying this region using NEB Hot Start Taq kit (M0495S) and forward and reverse primers (5' GCGGATTCAACTTCCACTTTC and AGTTTCAGCAGGATGGGATATAA 5', respectively). This 948 bp amplicon was run on a 2% agarose gel. After T7 Endonuclease I digestion, the resulting products are 253 bp and 695 bp respectively. Clones positive for an indel were expected to be digested compared to wild type (WT) controls. gRNA induced edits were screened by amplifying this region using the Roche Kappa HiFi kit (KK2601) and forward and reverse primers (respectively).

Mutation Detection Surveyor Assay- T7E1

The T7 endonuclease 1 (T7E1) mismatch detection assay is a widely used method for evaluating the activity of site-specific nucleases, such as the clustered regularly interspaced

short palindromic repeats (CRISPR)-Cas9 system. PCR was used to amplify the targeted genomic region. PCR products were denatured and reannealed in a thermal cycler to allow the potential heteroduplex formation between wild-type and CRISPR–mutated DNA. Digestion of reannealed PCR products with T7EI cleaved mismatched DNA heteroduplexes. The results were analyzed using gel electrophoresis as shown in fig. []

Heteroduplex controls were supplied by IDT mismatch detection assay kits (Alt-R® Genome Editing Detection Kit, Cat no. 1075931) and denoted as A and B.

Next Generation Sequencing

Next-generation sequencing (NGS) is a powerful and widely used technique for DNA and RNA sequencing. It allows scientists and researchers to quickly and efficiently generate large amounts of genetic data from a sample.

NGS involves breaking a sample of DNA or RNA into smaller fragments and then using enzymes to synthesize new DNA strands that are complementary to the original fragments. These new strands are then sequenced using specialized high-throughput sequencers. The resulting data can be used to identify genetic variations, identify gene expression patterns, and perform many other analyses of genetic material.

There are several different types of NGS technologies available, including Illumina, PacBio, and Ion Torrent. These technologies differ in terms of the specific enzymes and sequencers they use, as well as the scale and speed of the sequencing process.

NGS has revolutionized the field of genomics and has a wide range of applications, including disease diagnosis, drug development, and evolutionary biology. It has also played a critical role in the COVID-19 pandemic, with NGS being used to identify and track the spread of the virus.

NGS can be used in combination with CRISPR-Cas9 to analyze the effects of gene editing on a genome. After using CRISPR-Cas9 to edit a gene, NGS can be used to sequence the edited genome and identify any changes that have been made. This allows researchers to determine whether the CRISPR-Cas9 system has successfully edited the targeted gene and to assess any unintended effects on the genome.

NGS is also commonly used to guide the design of CRISPR-Cas9 experiments by identifying the most suitable target sequences for editing. In addition, NGS can be used to confirm the success of CRISPR-Cas9 editing by identifying any changes in the targeted gene or surrounding genome after the editing has been performed.

Overall, the combination of CRISPR-Cas9 and NGS has greatly expanded the capabilities of gene editing and has numerous potential applications in research and medicine.

Nanopore sequencing is a type of next-generation sequencing (NGS) technology that allows for the rapid and efficient sequencing of DNA and RNA. It is based on the principle of measuring changes in the electrical current passing through a narrow pore as individual nucleotides pass through it.

In nanopore sequencing, a sample of DNA or RNA is attached to a polymerase enzyme, which synthesizes a new strand of DNA that is complementary to the sample. This strand is then passed through a nanopore, which is a small, narrow opening in a thin membrane. As each nucleotide passes through the nanopore, it blocks the flow of ions and causes a change in the electrical current. These changes are measured and used to identify the sequence of the nucleotides in the sample.

One of the main advantages of nanopore sequencing is that it can be performed on a portable device, making it a potentially valuable tool for fieldwork and remote locations. It is also relatively low cost and can produce long reads, which can be useful for certain applications such as de novo genome assembly.

Nanopore sequencing has a wide range of applications, including genome sequencing, transcriptome sequencing, and metagenome sequencing. It has also been used in the study of ancient DNA and in the identification of new species.

Nanopore sequencing is also commonly used to confirm the success of CRISPR-Cas9 editing by identifying any changes in the targeted gene or surrounding genome after the editing has been performed.

For further verification, NGS was performed on the samples. NGS requires a larger segment to be sequenced than the smaller (1876bp) CCL2 fragment, therefore we designed PCR primers that amplify our experimental target site and adjacent sequences on either end. These set of primers (TAACCACCAAGTGGAGAGAATG and CCTTCCTATTCCAAACCCTGTAT) were used to amplify a region of about 3000bp. The CCL2 expressing region fell approximately in the middle of this sequence.

PCR assay to amplify genomic DNA regions surrounding guide RNA

Mm.Cas9.CCL2.1.AA: CAACACGTGGATGCTCCAGC CGG

Forward Primer: TAACCACCAAGTGGAGAGAATG

Reverse Primer: CCTTCCTATTCCAAACCCTGTAT

Primer Tails to be attached for NGS Seq detection:

Forward primer tail: 5’ TTTCTGTTGGTGCTGATATTGC 3’

Reverse primer tail: 5’ ACTTGCCTGTCGCTCTATCTTC 3’

These primer tails are attached to the primers so that different barcode sequences can be attached to them as a way of detecting different samples during next generation sequencing.

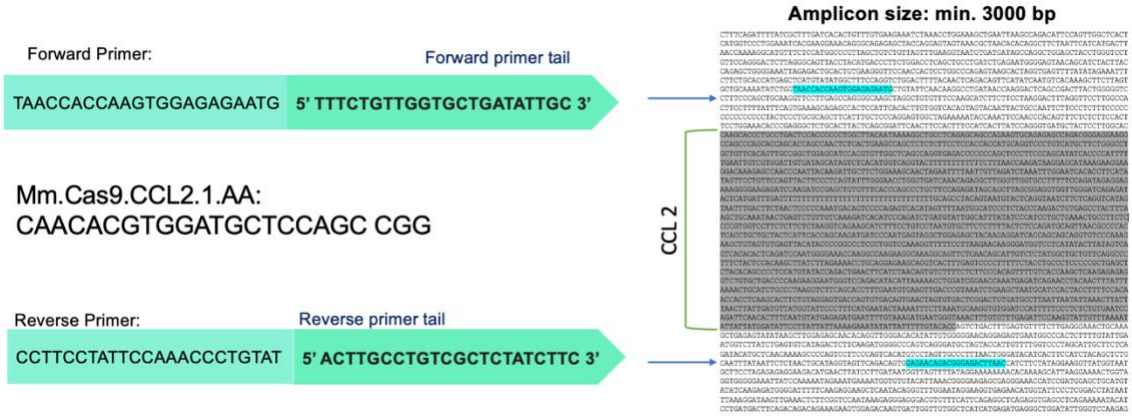


Figure 9: Primers with oligo tails attach to barcodes for sequencing

Table 3: Barcodes used for sequencing

Barcode position	barcode ID	Sequence
A1	BC01	AAGAAAGTTGTCGGTGTCTTTGTG
B1	BC13	AGAACGACTTCCATACTCGTGTGA
C1	BC25	GTAAGTTGGGTATGCAACGCAATG
D1	BC37	GCTTGCGATTGATGCTTAGTATCA
E1	BC49	ACTGGTGCAGCTTTGAACATCTAG
F1	BC61	AGAGGGTACTATGTGCCTCAGCAC
G1	BC73	AAGAAACAGGATGACAGAACCCTC
H1	BC85	AACGGAGGAGTTAGTTGGATGATC
A2	BC02	TCGATTCCGTTTGTAGTCGTCTGT
B2	BC14	AACGAGTCTCTTGGGACCCATAGA
C2	BC26	CATACAGCGACTACGCATTCTCAT
D2	BC38	ACCACAGGAGGACGATACAGAGAA
E2	BC50	ATGGACTTTGGTAACTTCCTGCGT
F2	BC62	CACCCACACTTACTTCAGGACGTA
G2	BC74	TACAAGCATCCCAACACTTCCACT
H2	BC86	AGGTGATCCCAACAAGCGTAAGTA
A3	BC03	GAGTCTTGTGTCCCAGTTACCAGG
B3	BC15	AGGTCTACCTCGCTAACACCACTG
C3	BC27	CGACGGTTAGATTCACCTCTTACA
D3	BC39	CCACAGTGTCAACTAGAGCCTCTC
E3	BC51	GTTGAATGAGCCTACTGGGTCCTC

F3	BC63	TTCTGAAGTTCCTGGGTCTTGAAC
G3	BC75	GACCATTGTGATGAACCCTGTTGT
H3	BC87	TACATGCTCCTGTTGTTAGGGAGG
A4	BC04	TTCGGATTCTATCGTGTTTCCCTA
B4	BC16	CGTCAACTGACAGTGGTTCGTACT
C4	BC28	TGAAACCTAAGAAGGCACCGTATC
D4	BC40	TAGTTTGGATGACCAAGGATAGCC
E4	BC52	TGAGAGACAAGATTGTTCGTGGAC
F4	BC64	GACAGACACCGTTCATCGACTTTC

Western Blotting

To perform SDS-PAGE western blotting, total protein from cultured cells was extracted by mammalian protein extraction reagent (Pierce, Rockford, IL, USA) and mixed with protease inhibitor cocktail (Sigma). Protein samples were fractionated by 10% SDS-PAGE gel and were transferred to PVDF membranes. samples were lysed in extraction buffer containing 0.02 M Tris pH-7.4, 0.15 M NaCl, 0.01 M EDTA disodium salt, 0.01 M Benzamidine HCl, 1% Triton-X100, 0.5% Tween-20, 1 mM PMSF, and 100 µg/ml leupeptin as previously described (Yan *et al*., 2009). Samples were prepared by mixing lysates with sample buffer [0.5 M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 10% (w/v) SDS. 5% (v/v) ß-mercaptoethanol] while on ice and then incubating the mixture at 100 ^{o}C for 5 mins and run on 15% SDS polyacrylamide gel for 3.5 hours at 30 mA using a gel electrophoresis system (mini protean II, Biorad, CA) and transferred to PVDF membranes. Due to the high isoelectric point of CCL2 (9.81) we used a Dunn carbonate buffer (10 mM NaHCO3, 3mM Na2CO3, pH 9.9, 20% methanol) as transfer buffer that produces higher efficiency transfers and improves the ability of antibodies to recognize and bind to proteins. (Dunn 1986). The membranes were blocked by TBST buffer with 5% skim milk for 2 h,

were then incubated overnight at 4ºC with primary antibodies CCL2 (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA). B-actin (1:2500, Sigma, USA) was used as loading control. Membranes were probed using a rabbit polyclonal anti-CCL2 antibody and immunoreactive bands were detected via chemiluminescence using an alkaline phosphatase-conjugated anti-rabbit IgG antibody. Beta actin was used as experimental control. Fluorescent secondary antibodies (1:15000, LI-COR, Nebraska, USA) and infrared imaging system (LI-COR) were used for visualization of the protein bands.

Wound Closure Assay

LLC (WT & Crispr modified) cells were seeded at 80% confluence into 6-well plates precoated with type I collagen type I (Wang Q *et al*, 2019; Oncel, S *et al* 2021). After monolayers reached 100% confluence (48–72 h after seeding), they were wounded with non-barrier autoclaved 200uL pipette tips. Wound images were captured using an inverted light microscope (OLYMPUS CK2, Center Valley, PA, USA) at 0 h, 12 h, 24 h, 36h, and 48 h after wounding. Wound areas were measured with Image J software.

CCR2 Antagonist Study

INCB3344 is a novel, potent and selective small molecule antagonist of the mouse CCR2 receptor. In vitro, INCB3344 inhibits the binding of CCL2 to CCR2 with nanomolar potency (IC50=10 nM) and displays dose-dependent inhibition of CCL2-

mediated functional responses. (Brodmerkel *et al.*, 2005). Mice were implanted with $2.5x10^5$ Lewis lung carcinoma cells. 1mg of INCB3344 in a 50mg/kg dose was given to HLA-DQ8 mice via IP every 3 days for 19 days.

Statistical Analysis

Mean and standard error values were computed for all continuous variables and frequency distributions were calculated for all categorical variables. Statistical comparisons were made using Student's t-test to compare the means between two groups or ANOVA to compare the means among three or more groups. Kaplan Meier curves and Mantel-Cox Test were used to evaluate survival data. Mice were assigned to experimental groups at random. Except where indicated, comparisons between groups in other experiments were performed with one-way ANOVA, followed by Bonferroni's post-hoc analysis, or with unpaired Student's t-test. Two tailed tests were used throughout. Significance was ascribed for $p<0.05$. Levels of significance are indicated by the number of symbols, e.g., *, $p = 0.01$ to <0.05; **, $p = 0.001$ to 0.01; ***, $p < 0.001$. Data are presented as mean ± S.E.M.

CHAPTER III

RESULTS

Due to the lack of targeted approaches for early diagnosis and treatment, the five-year survival rate of Non-Small Cell Lung Cancer patients remains dismal, 25%–30% death in less than 3 months (Guo H *et al.*, 2021). The poor outcome urges development of novel therapeutic strategies against this disease, which highlights the need for better understanding of the carcinogenesis of a robust model of lung cancer like Lewis Lung Carcinoma.

Rate of survival of mice implanted with Lewis Lung Carcinoma

Murine Lewis lung carcinoma cell line LLC was implanted ($1x10^4$ cells IP) in two strains of 6-8 week old male and female mice. In **Figure 10** the data did not show any significant disparity in survival outcomes between the different sexes. The two strains considered were HLA-DQ8 and C57BL/6. DQ8 mice were originally created using multiple strains of mice under the same MHC allotype, H2b. It is reasonable to hypothesize that tumor models using cells originating from C57BL/6 mice, MHC allotype H2b, would implant in HLA mice. **Figure 10** also highlights the aggressive nature of the Lewis lung carcinoma cell type due to the short survival range of mice implanted with it.

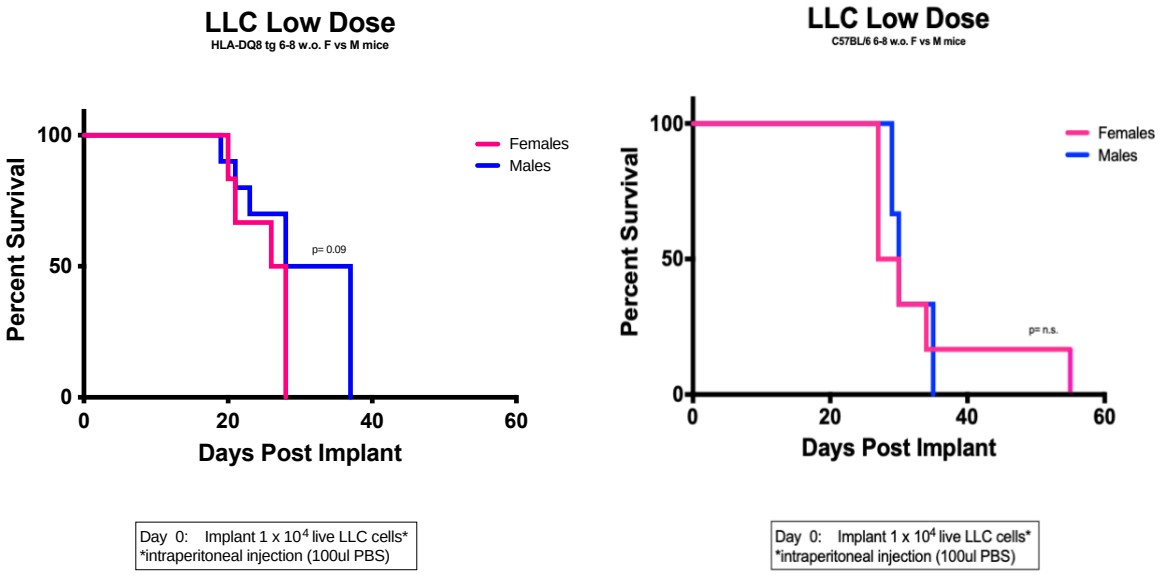


Figure 10: Survival pattern in HLA-DQ8 tg and C57BL/6 mice following LLC implant to establish tumor

Mice with an established tumor (6 days after IP implantation of 1 x10^4 LLC cells). Mice were monitored daily and sacrificed when moribund or significant tumor load. Tumor load never exceeded 20mm at the largest diameter. This data highlights the aggressive nature of LLC and the lack of any difference in survival between male and female mice. Kaplan-Meier survival curves comparing survival of mice challenged in varying conditions. Average of 6 mice/group.

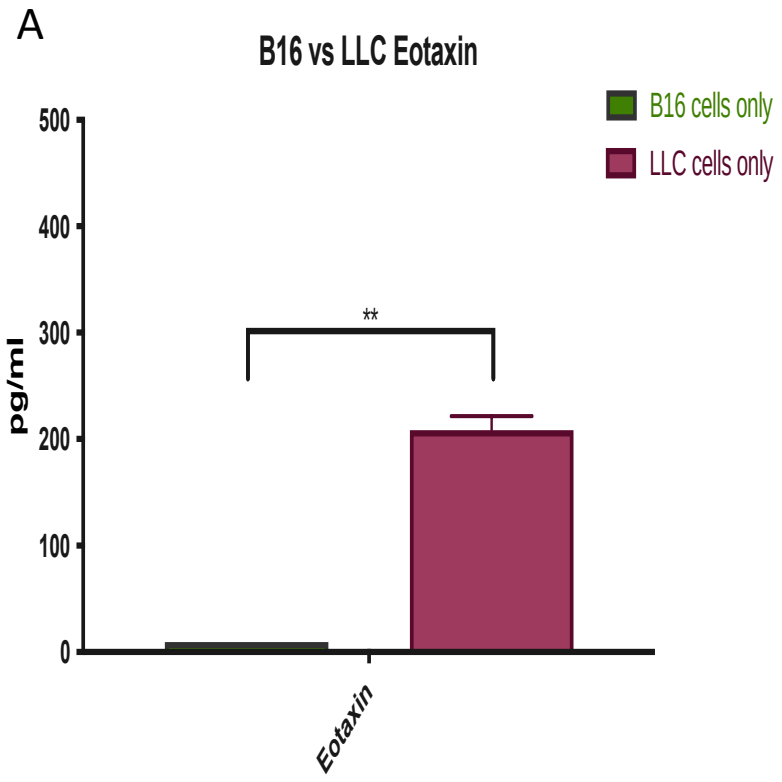
Contributing factors of the aggressive nature of LLC

We wanted to know the particular inflammatory response contributing to the aggressiveness of these tumors and therefore chemokine levels were measured as shown in **Figure 11** and **Figure 12** in B16 and LLC (and MC-38) cell supernatant using BioLegend's LEGENDplex™ and the samples were run on BD FACSymphony™ flow cytometer. Standard curves were evaluated for appropriate error and considered suitable for all parameters given. The data can be interpreted collectively that demonstrate a powerful CCL2/MCP-1 response. **Figure 11** shows the significant over expression of chemokines like Eotaxin, Lix and CCL2 where the chemokine CCL2 expressed by the gene CCL2 was the highest. This led us to conclude that CCL2 overexpression is a major contributing factor in the aggressive nature of LLC.

We wanted to know if the over expression of CCL2 was proportional to the number of cells plated. So, we plated cells along a fixed gradient of 50,000 to 6250 serially diluted. The cells received no external stimulation. The chemokine level was assessed after 24 hours of growth. **Figure 12** shows the secreted chemokine profile in unstimulated B16, LLC and MC-38 cells along the fixed gradient. There is a gradual decrease of CCL2 having a direct correlation with the gradual decrease in cell numbers. This shows that CCL2 production was directly proportional to the number of LLC cells.

Seeing this trend in CCL2 secretion in cells we wanted to know if this pattern holds up systemically. We implanted the LLC and MC-38 cells in HLADQ mice via IP. In **Figure 13** there was no evidence of CCL2 in sera of mice with established LLC or MC-38 tumors

on days 3, 6, 9, 13 pointing towards the fact that secreted CCL2 is being restricted to the tumor micro-environment. This also alluded towards the fact that the tumor cells secreting CCL2 might use this chemokine for its growth and proliferation.



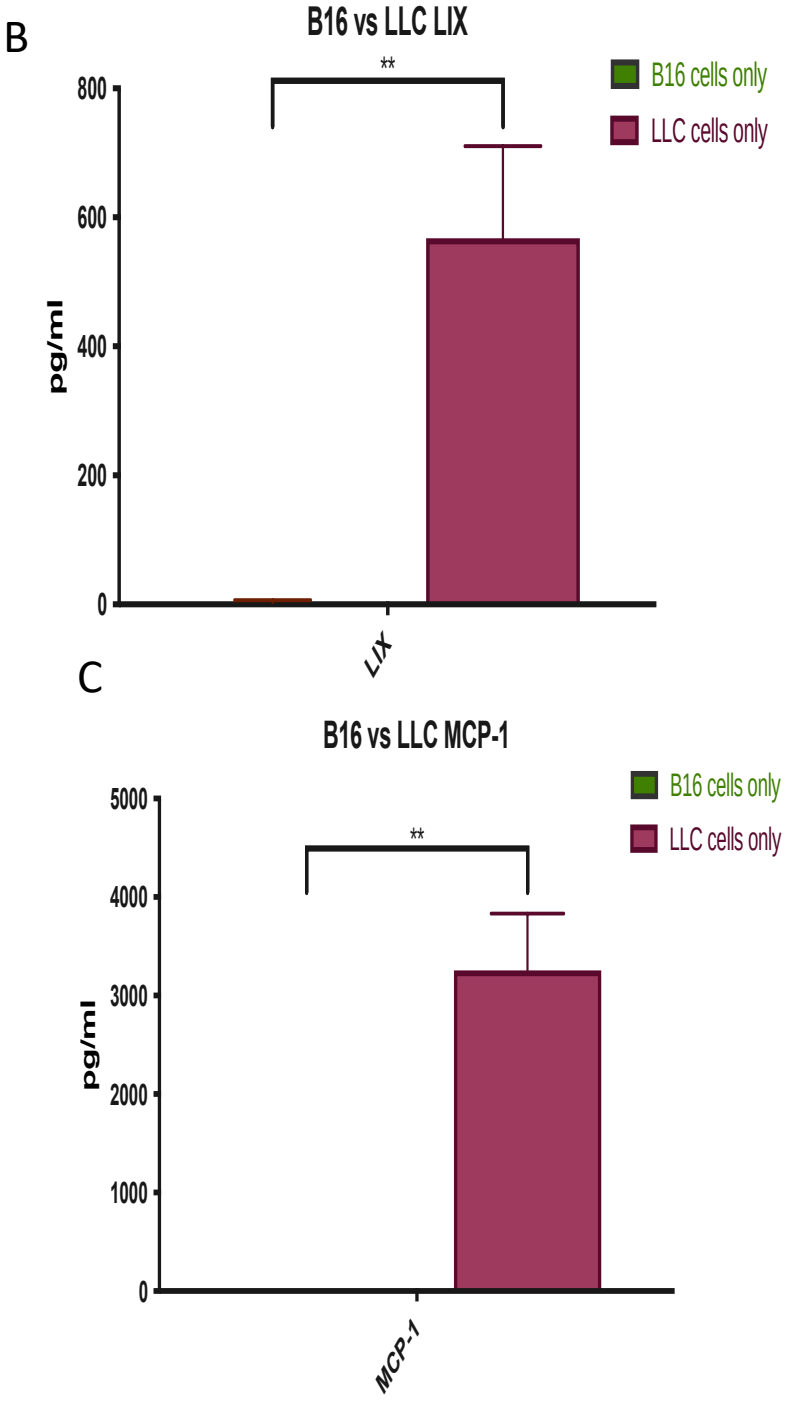
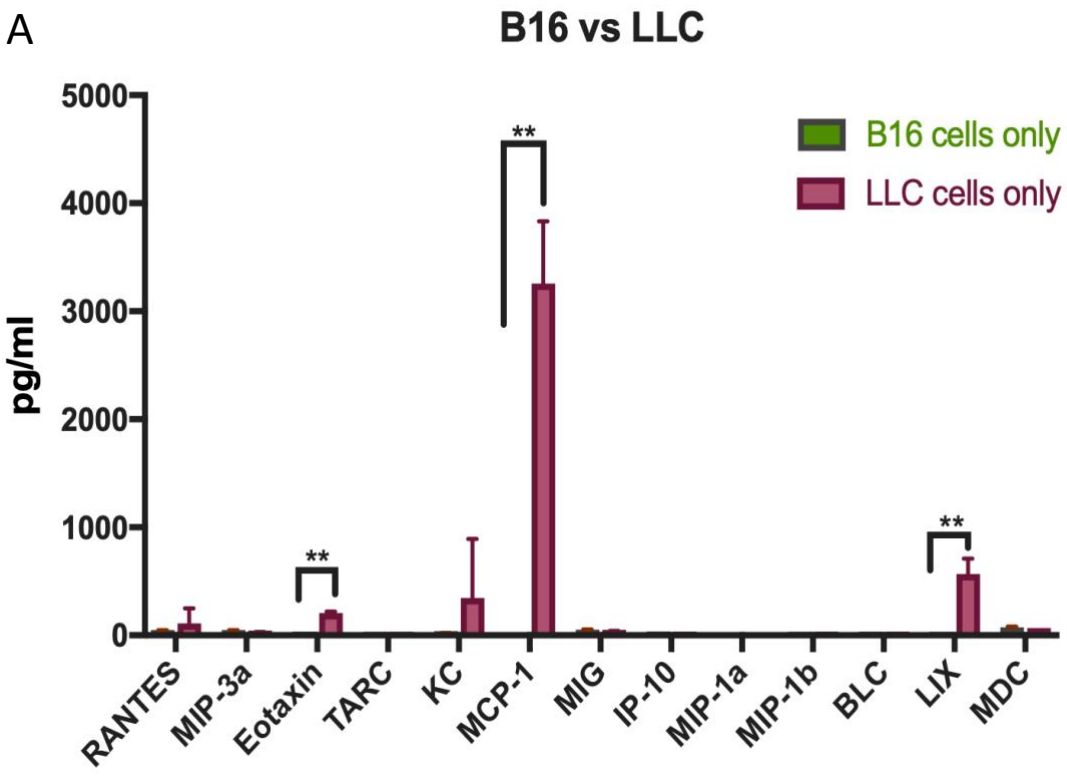


Figure 11: Elevated levels of Chemokines A. Eotaxin B. LIX and C. CCL2 was observed in Lewis lung carcinoma cells compared to B16 Melanoma cells

A


B16 vs LLC
B16 cells only
LLC cells only
pg/ml
5000
4000
3000
2000
1000
0
**
**
**
RANTES
MIP-3a
Eotaxin
TARC
KC
MCP-1
MIG
IP-10
MIP-1a
MIP-1b
BLC
LIX
MDC

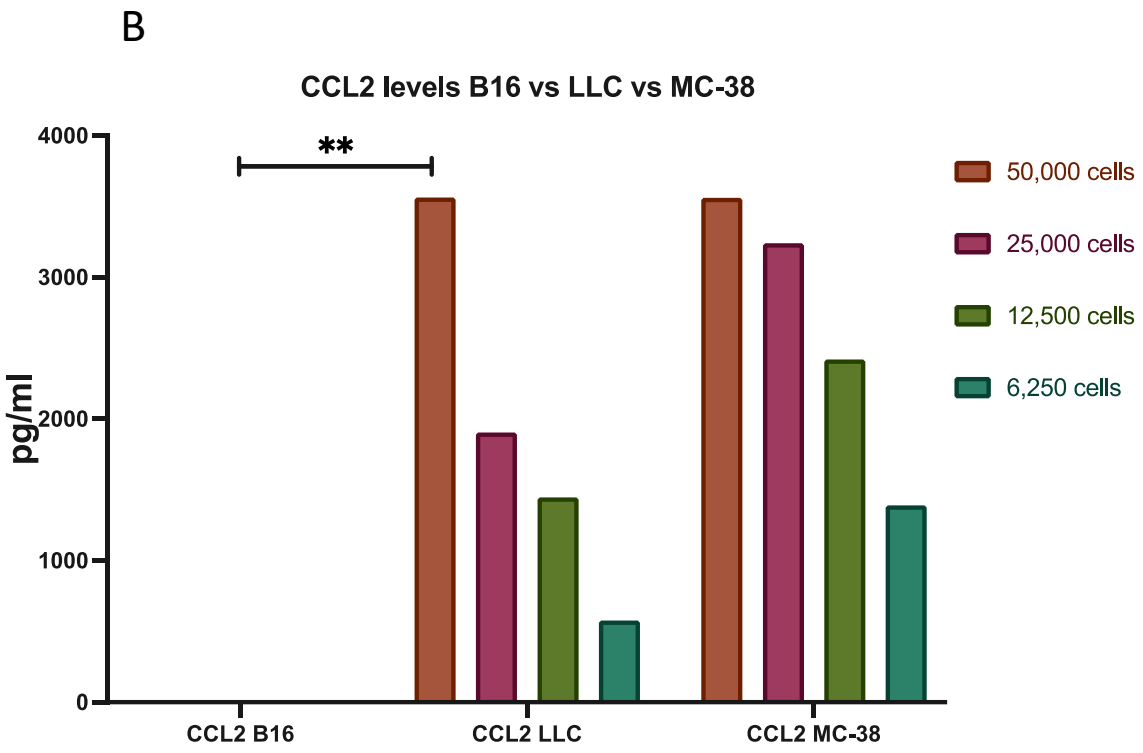


Figure 12: Secreted chemokine profile in unstimulated B16, LLC and MC-38 cells

Significantly elevated levels of CCL2 in LLC and MC-38 compared to B16F10 unstimulated cell supernatant. 12B shows the gradual decrease in secreted CCL2 with serially diluted gradient of Lewis lung carcinoma cells vs a basal level of B16F10 cells. (**p=0.0041)

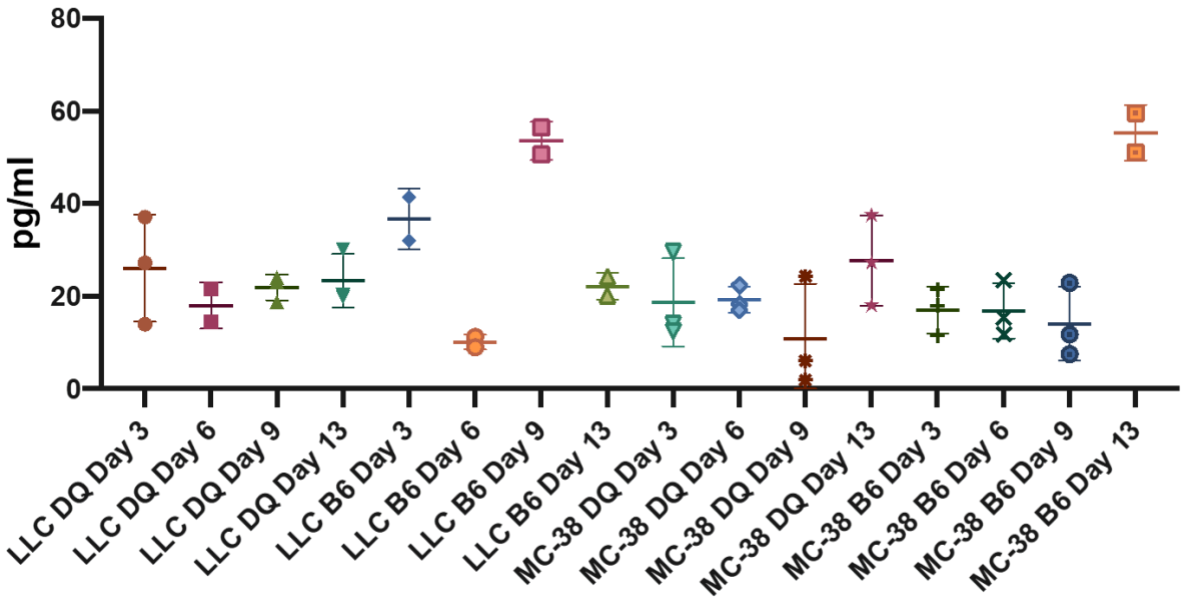


Fig 13: Time course of Serum CCL2 level in mice.

All mice were implanted with $2.5x10^5$ live LLC and MC-38 cells intraperitoneally. Blood was collected from the submandibular vein in EDTA tubes on days 3, 6, 9 and 13. All plasma samples were frozen and run together using BioLegend's LEGENDplex Mouse chemokine panel.

Surveyor Assay to detect CRISPR-Cas9-mediated gene editing

The T7 endonuclease 1 (T7E1) is a structure-selective enzyme that detects structural irregularities in heteroduplexed DNA. In using this assay to detect CRISPR-Cas9-mediated gene editing, reagents are transfected into cells, and the genomic DNA surrounding the target locus is amplified several days later by PCR (Sentmanat *et al*., 2018) and gel electrophoresis.

We performed targeted PCR and prepared the resulting amplicons for mismatch detection by T7E1. Although the T7E1 assay is error prone due to high background (e.g., excessive banding), predictable banding patterns are apparent in cell pools edited by high-performing sgRNAs.

As illustrated in **Figure 14**, screening of colonies transfected with the guide RNA's using a commercial Surveyor endonuclease kit (T7EI-IDT) designed to identify single base insertions and deletions identified multiple clonal isolates. The fragments were found at 253bp and 695bp showing the cuts exactly where we expected from a 948bp CCL2 sequence. CCL2 KO CRISPR detection with fragments at 253 bp and 695 bp showing cuts by multi-step T7EI endonuclease detection (IDT) test for CRISPR insertion/deletion of PCR products. T7EI controls. Multiple sets of CRISPR positive DNA. Four samples (1, 3, 6, 7) which had the most well-defined bands were chosen from this set and sent for sequencing.

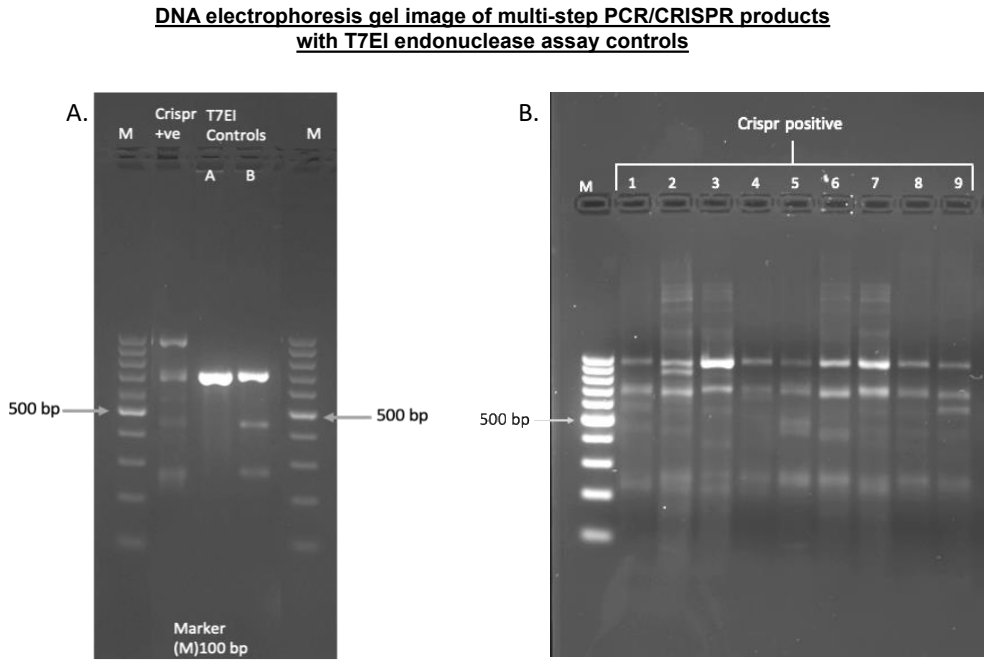


Fig 14. T7EI surveyor Assay for mutation detection

A. CCL2 KO CRISPR detection with fragments at 253 bp and 695 bp showing cuts by multi-step T7EI endonuclease detection (IDT) test for CRISPR insertion/deletion of PCR products. T7EI controls

B. Multiple sets of CRISPR positive DNA. Four samples (1, 3, 6, 7) which had the most well-defined bands were chosen from this set and sent for sequencing.

Next gen sequencing to confirm CRISPR-Cas9-mediated gene editing

NGS is the recommended method for full investigation of CRISPR edits. Highly precise and accurate, NGS allows identification of even small numbers of unintended edits at both the target site and at off-target sites. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) associated Cas9 (enzyme) system, adapted from the bacterial adaptive immune system, has made functional genomics more reliable by permitting precise editing of the genome to activate, inhibit

or alter a gene function. The first in vitro demonstration that CRISPR/Cas could be programmed to cleave targeted DNA was in 2012, by Jinek *et al*. (Jennifer Doudna and Emmanuelle Charpentier). Here, they showed that mature CRISPR RNAs (crRNAs), which were base-paired to trans-activating crRNA (tracrRNA) formed a two-RNA structure that directed the CRISPR-associated protein Cas9 to introduce double-stranded (ds) breaks in target DNA (Jinek et al., 2012).

The leading CRISPR papers described two main categories of genome edits. First is a simple CRISPR manipulation (gRNA and Cas9) which results in non-homologous end joining (NHEJ), where Cas9 induces a double-stranded break approximately 4 bp downstream of the PAM sequence and results in an error-prone repair process which introduces insertions/deletions (indels) at this site. The second method, homology-directed repair (HDR), uses a repair template with homology arms in addition to the CRISPR-Cas9 components so that the nicked DNA could be repaired according to this template allowing for precise gene editing. HDR uses modified Cas9 that functions as nickase instead of the endonuclease.

In this study I used the simpler CRISPR-Cas9 approach, non-homologous end joining (NHEJ), to create CCL2 knockout cell lines. The gRNA was selected such that it targeted the upstream region of the desired exon and had a score that reflected high on target and low off target efficiency. I anticipated that the Cas9 induced double-stranded break (DSB), downstream of the PAM sequence, would create an indel in this region as the DNA repaired itself via NHEJ. Since I tagged/labelled the guideRNA with ATTO-488 it was easy to pick out the clones using FACS. **Figure 15** shows the gating strategy we used to pick out clones that were CRISPR positive via Fluorescence Activated Cell Sorting (FACS). During this sorting process each single cell was

placed in each well of a 96-well plate that gave rise to distinct colonies. **Figure 16** shows the single cell colonies that proliferated from each well of the plate 5 days post sorting.

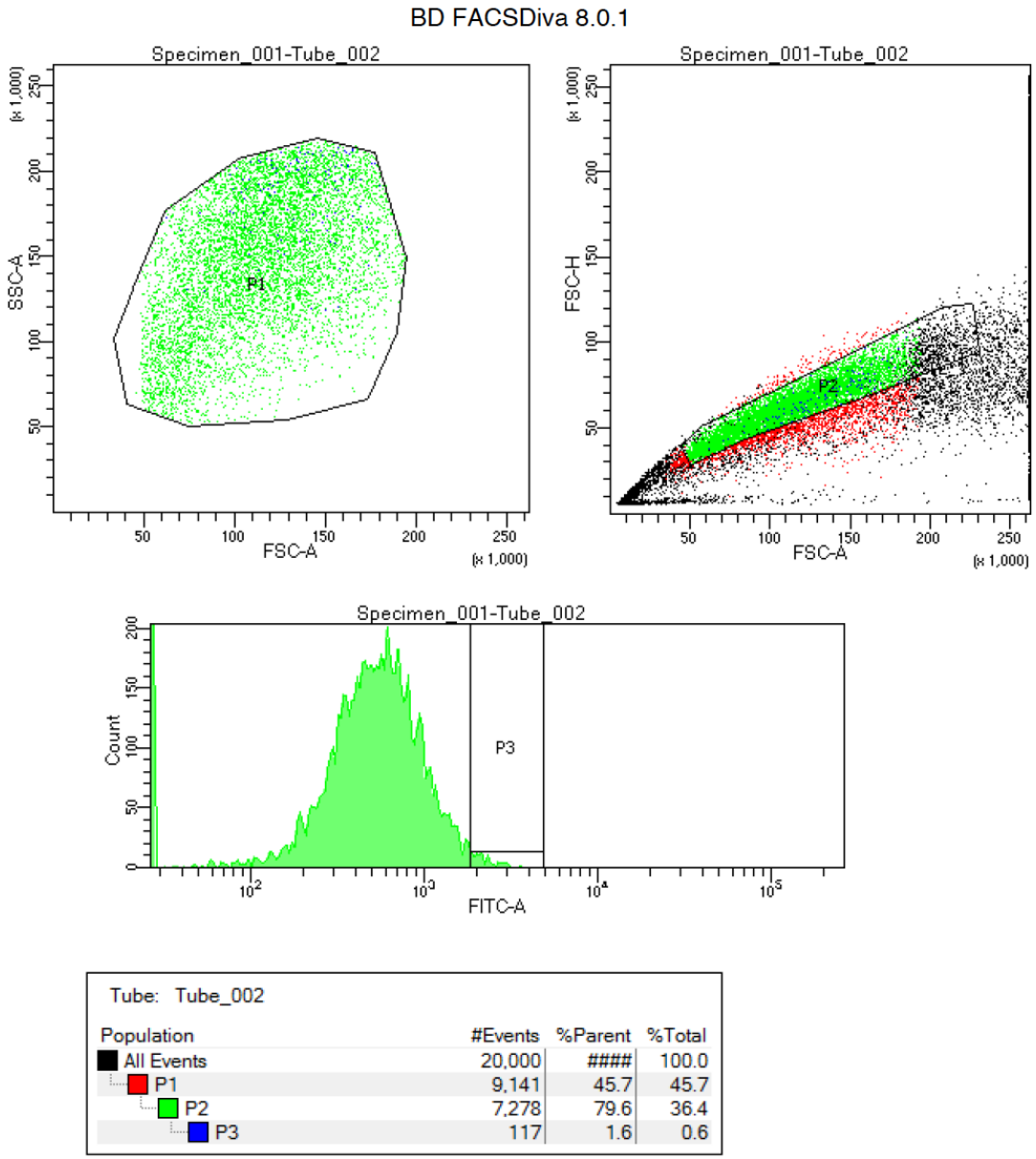


Fig 15: The gating strategy and selection of ATTO-488 positive clones by Fluorescence Activated Cell Sorting.

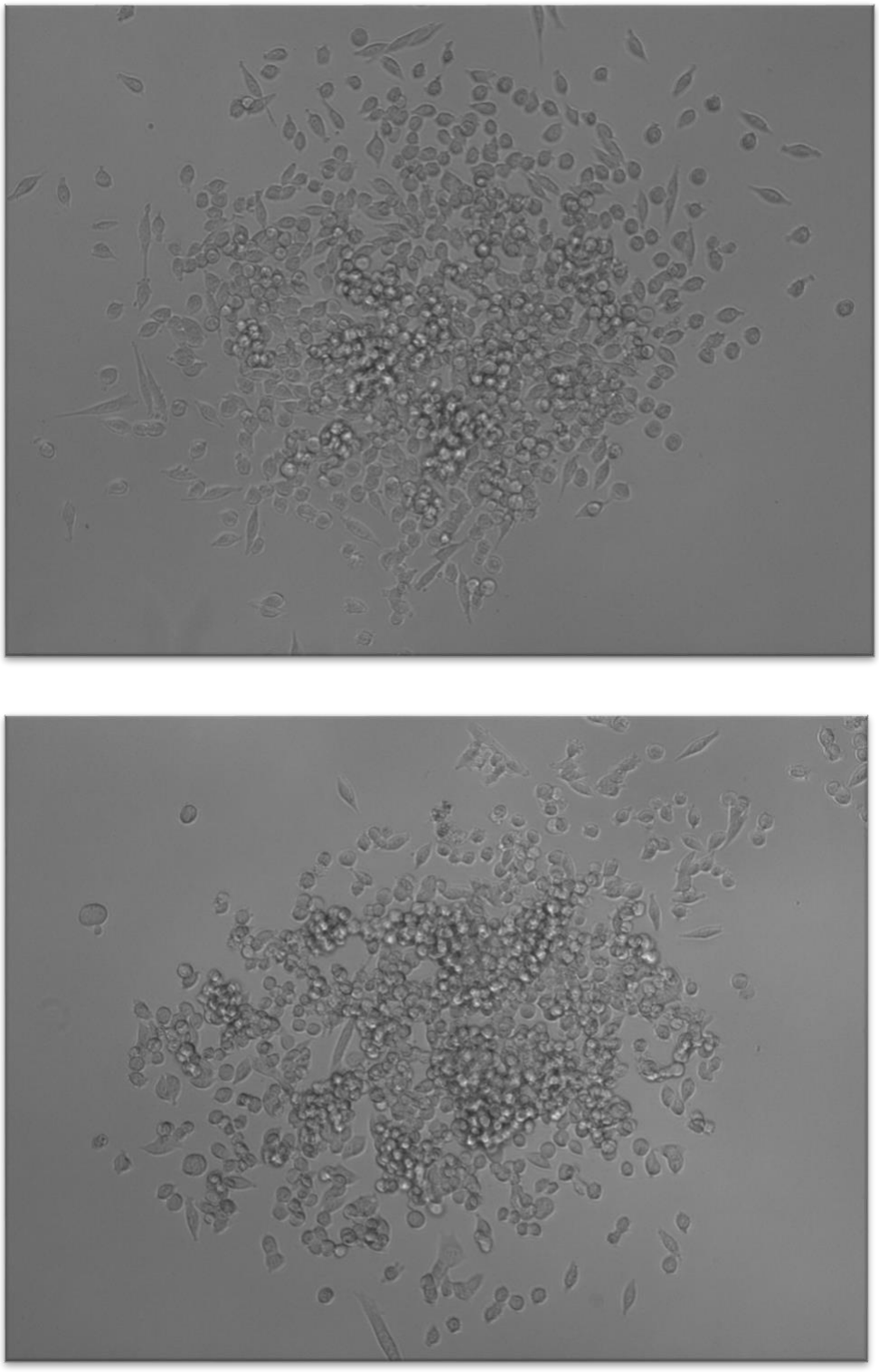


Figure 16: Colonies arising from single cells 5 days post sort.

Next Generation Sequencing data visualization is an essential component of genomic data analysis. However, the size and diversity of the data sets produced by today's sequencing and array-based profiling methods present major challenges to visualization tools. The Integrative Genomics Viewer (IGV) is a high-performance viewer that efficiently handles large heterogeneous data sets, while providing a smooth and intuitive user experience at all levels of genome resolution. The Integrative Genomics Viewer (IGV) was one of the first tools to provide NGS data visualization, and it provided us with a rich set of tools for inspection, validation, and interpretation of NGS datasets, as well as other types of genomic data. We used IGV in **Figure 17** precisely to reflect large motif deletions in our CRISPR positive cell lines and compare the coverage with our unaltered wildtype (WT-LLC) Lewis lung carcinoma. We used IGV for the detection of small insertions and deletions with respect to the CCL2 reference genome. Due to the 3 guide RNA for 3 exon hit we found large deletions in the IGV coverage leading us to conclude the success of our CRISPR based modification in knocking down CCL2.

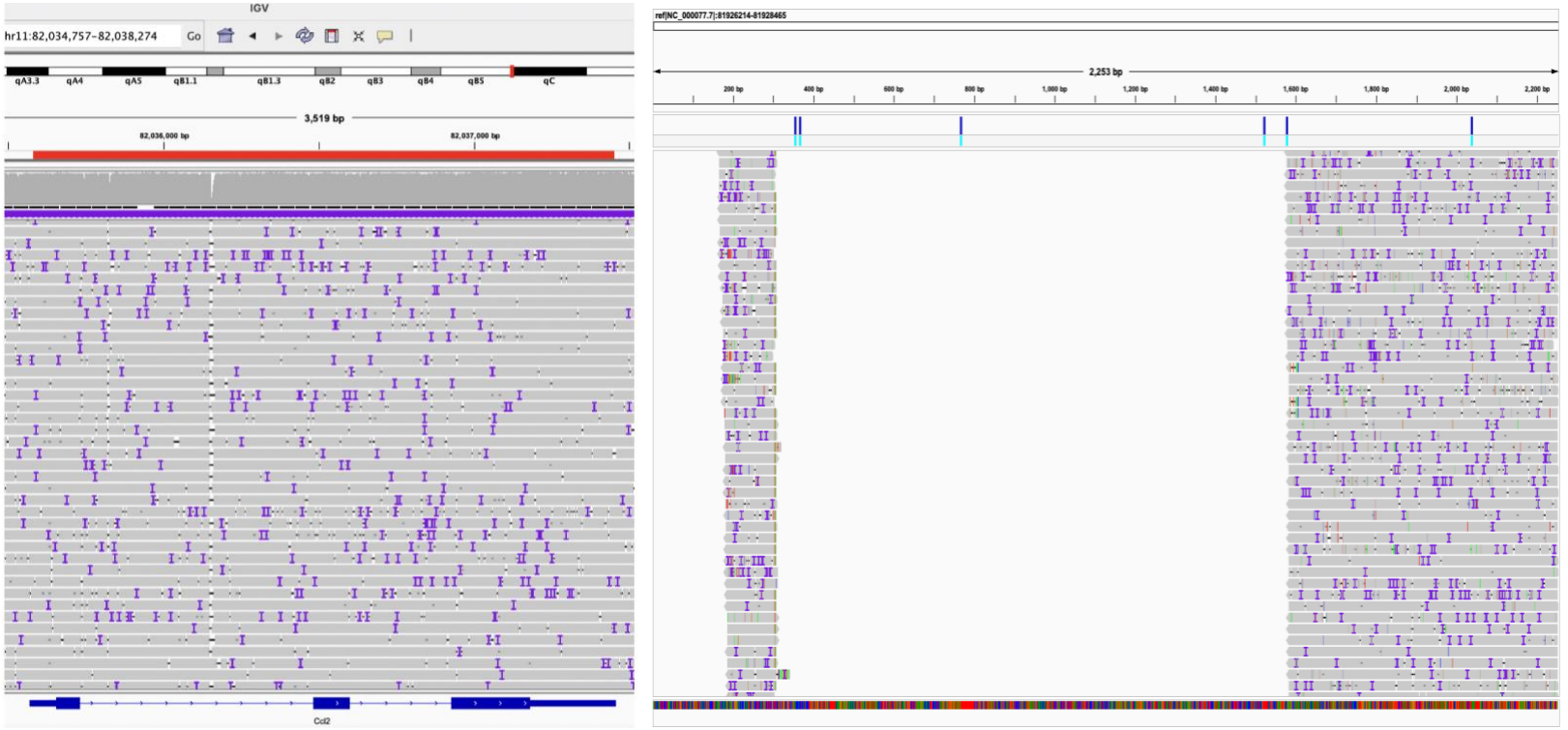


Fig 17: Base deletion difference between WT and 3 exon hit crispr modification in the IGV genomic browser analysis window.

Western Blot Assay to check CCL2 expression levels

We wanted to check the inhibition of CCL2 in the modified cells and to what extent they were being downregulated. To see the expression levels of CCL2 we performed a western blot assay. **Figure 18** shows the western blot image and the corresponding densitometric analysis of the CCL2 knockdown. Due to the tetraploid nature of Lewis lung carcinoma cells and its proclivity to revert to its parental configuration a complete KO of CCL2 was not possible as shown in the western blot image but a significant downregulation was achieved which is imperative for exceptional therapeutic outcomes as explored in this study.

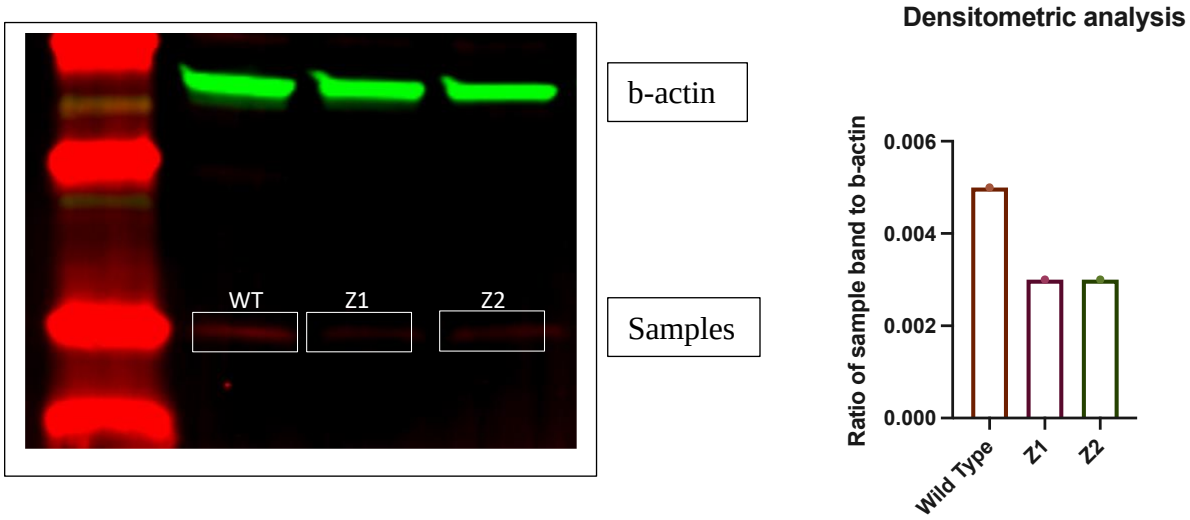


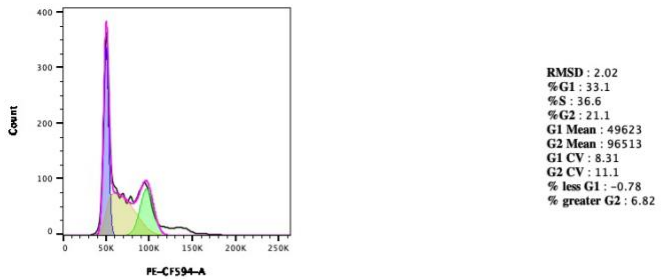
Fig 18: Western blot image and it's corresponding densitometric analysis

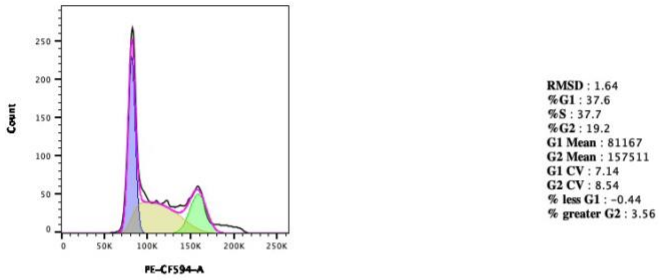
Green bands correspond to beta-actin used as control. Faint bands for Z1 and Z2 show significant downregulation in the production of CCL2 by CRISPR modified cells when compared to WT. The densitometric analysis further shows the fold difference in CCL2 downregulation.

Cell Cycle analysis to explore the impact of CCL2 in its modification

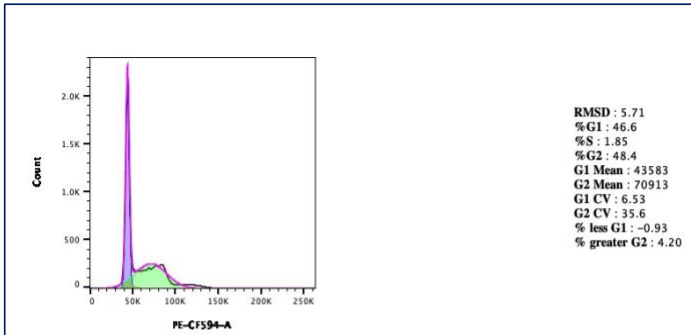
We hypothesized that downregulation of CCL2 would impact the cell cycle activity since CCL2 is an important factor in the growth and proliferation of tumor cells. To determine the impact of knocking out CCL2 on cell cycle, flow cytometry analysis of propidium iodide stained cells was performed 1 and 3 days after seeding at an initial cell density of 2×10^5 cell/cm^2. **Figure 19** shows the analysis of the experiments revealed that all cell types had different percentages of cells in G0/G1, S, and G2/M phases at day 3 suggesting that there was a significant difference in cell cycle between the CCL2 KO cell lines and WT cells. The inhibition of CCL2 expression in LLC cells dramatically altered the cell cycle of the LLC CCL2 KO cells, with significantly fewer cells in S phase between Days 1 and 3 in LLC CCL2 KOs compared to LLC CCL2 wildtype cells. An arrest in S-phase implies that the cell is unable to duplicate its DNA. As a result a decrease in M-phase is expected as well as an increase in G0/G1 phase.

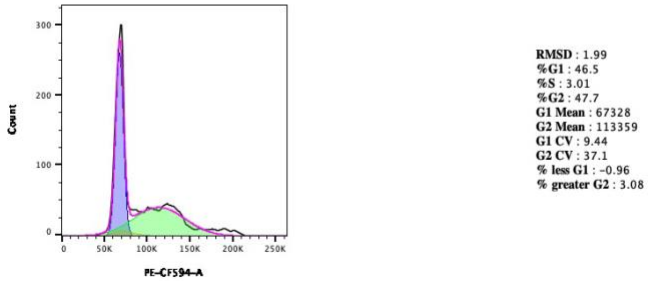
Cell Cycle Analysis





Day 1





Day 3

Fig 19: Propidium iodide-stained flow cytometric cell cycle analysis.

The inhibition of CCL2 expression in LLC cells dramatically altered the cell cycle of the LLC CCL2 KO cells, with significantly fewer cells in S phase between Days 1 and 3 in LLC CCL2 KOs compared to LLC CCL2 wildtype cells. Representative images illustrate different cell cycle phases for CCL2 KO isolate vs WT cells. 2.0×10^5 cells of each cell type were seeded into wells of a 6 well plate, harvested on day 3, and fixed and stained with Fxcycle PI/RNAse. Single cell suspensions were analyzed using a PE or PE-CF594 channel and quantified using cell cycle module of FlowJo software.

Wound Closure Study to detect changes in cell migration due to downregulation of CCL2

Our next hypothesis was that the downregulation of CCL2 would impact cell migration and we explored that in a wound closure study. The cell cycle study showed a lack of proliferation in CCL2 KO cells and based on that we wanted to study its impact in a migration assay with a wound closure model since CCL2 contributes to the growth, migration and metastasis of LLC cells. **Fig 20** depicts the analysis of wound experiments that showed the two CCL2 KO isolates migrated significantly slower than WT in 0-6 hours, albeit with some variability among them. **Figure 20 D** shows the significant disparity of WT vs KO cell migration where CCL2 KO's were much slower indicating the role of CCL2 in the growth, proliferation and migration of the tumor cell. Imaging studies were conducted in the UND Imaging Core facility supported by NIH grant P20GM113123, DaCCoTA CTR NIH grant U54GM128729, and UNDSMHS funds.

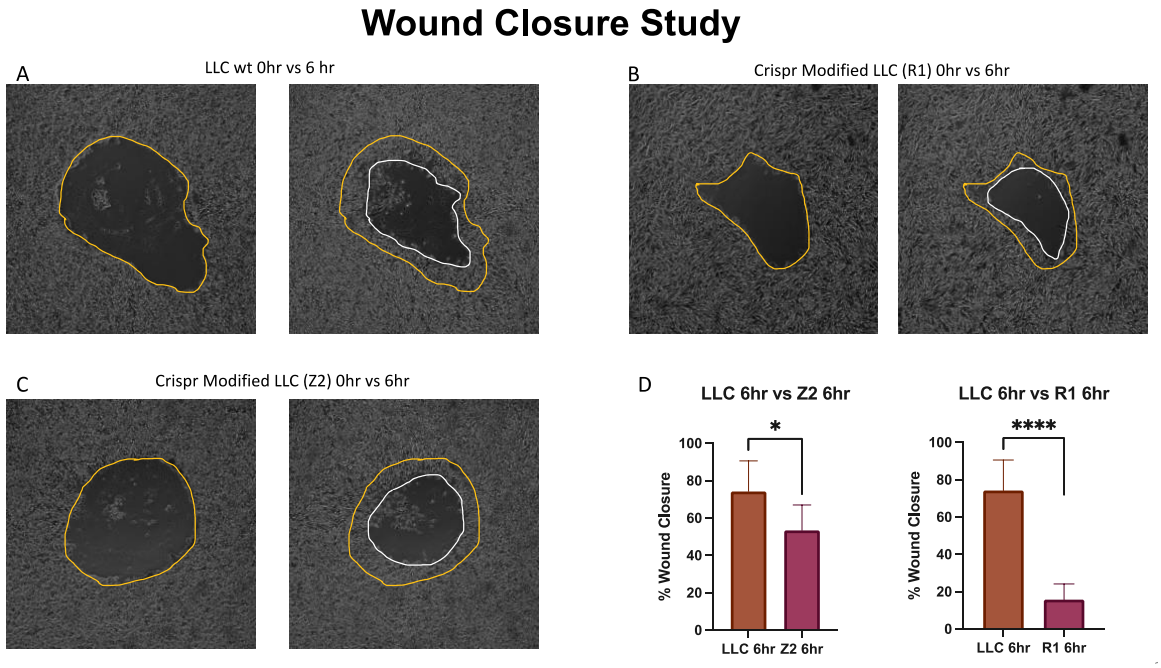


Fig 20: Typical wound closure study images for Lewis lung carcinoma cells, and CRISPR modified LLC cells (R1 and Z2 respectively). CRISPR modified LLC cells showed a decelerated rate of wound closure when compared with wild type LLC cells. Yellow represents the circumference of the 0-h wounds; white represents the circumference of the wounds 6 h later.

CCR2 Antagonist Study to assess the impact of a systemic CCR2 blockade

We wanted to check if blocking the CCL2-CCR2 axis via blockade of CCR2 receptor for CCL2 would incur a different survival rate when compared to wild type Lewis Lung Carcinoma cells. We used INCB3344 in our study**.** INCB3344 is a small molecule antagonist (MW 577.6) of the chemokine receptor, CCR2 which was used in our CCR2 Antagonist Study as shown in **Figure 21.** 1mg of INCB3344 in a 50mg/kg dose was given to HLA-DQ8 mice via IP every 3 days fo 19 days. **Figure 21** shows the lack of difference in survival between mice implanted with only wild type Lewis Lung Carcinoma and mice implanted with WT LLC treated with a CCR2 antagonist. One of the reasons for the lack of significant difference is the clearance of INCB3344 from the system within 8-12 hours making it inept at launching a robust systemic response against the LLC implant. Also in the absence of the CCR2 receptor CCL2 is still present abundantly in the tumor microenvironment continuously contributing to its growth, proliferation etc. Thus, a study with CCL2 knockout cells instead of a CCR2 bloackade is imperative to show a prolonged response in survival.

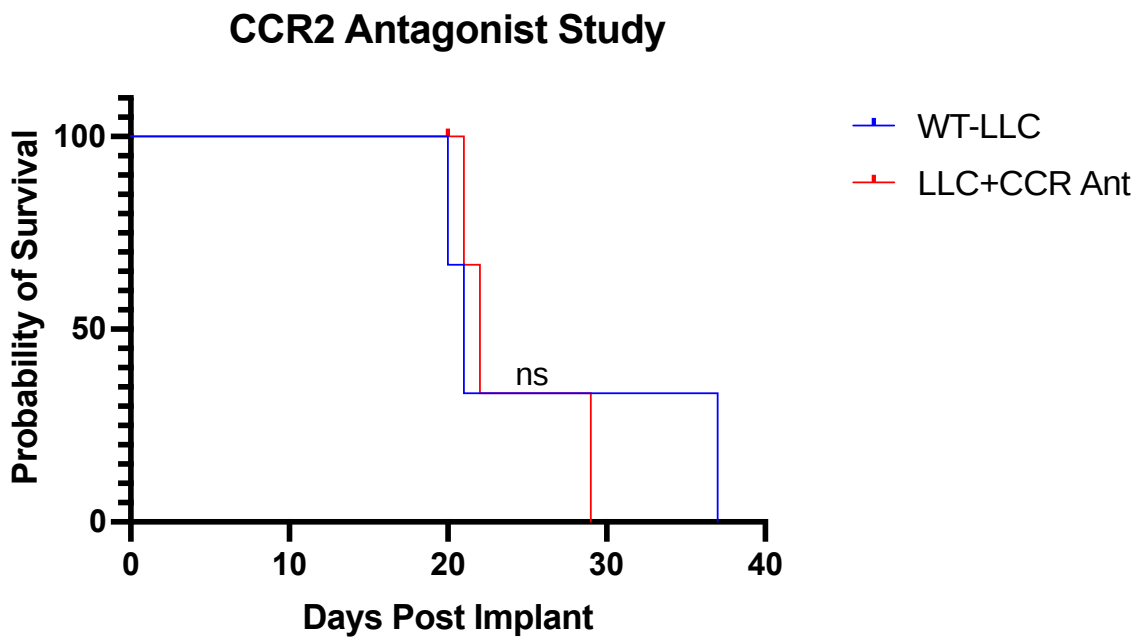


Fig 21: CCR2 Antagonist study

Mice with an established tumor (implanted with 2.5 x10^5 LLC cells IP). Mice were monitored daily and sacrificed when moribund. 1mg of INCB3344 in a 50mg/kg dose was given to HLA-DQ8 mice via IP every 3 days for 19 days. N=4-6

Assessment of survival in LLC and LLC-CCL2 KO implanted HLA-DQ8 tg mice

To evaluate our hypothesis that the paucity of CCL2 in the tumor microenvironment would increase survival in HLADQ8 mice we implanted WT LLC vs CCL2 downregulated cells intraperitonially in mice to check for survival. As hypothesized the data in **Figure 22** highlights the aggressive nature of LLC leading them to become moribund at the 20 day time point and the difference in survival between mice implanted with WT LLC and CRISPR modified LLC with downregulated CCL2. CRISPR modified LLC with downregulated CCL2 prolonged the survival of HLADQ8 mice.

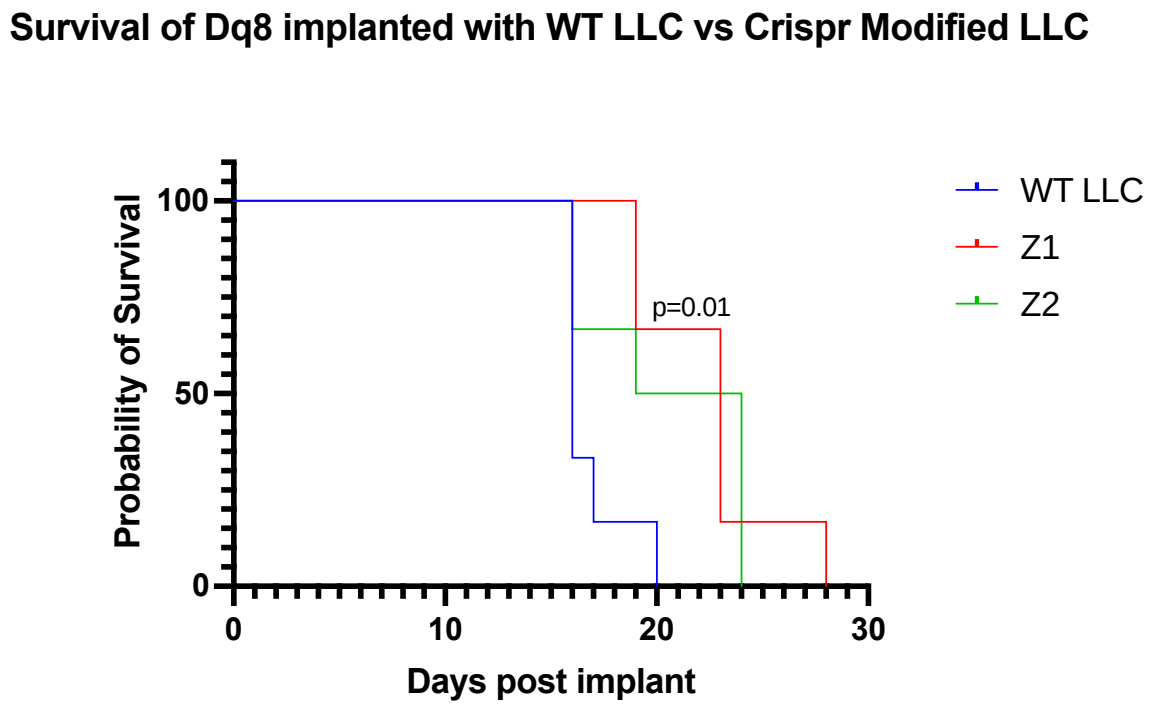


Fig 22: Survival pattern in HLA-DQ8 mice following LLC implant to establish tumor

Mice with an established tumor (implanted with 2.5 x10^5 LLC cells IP). Mice were monitored daily and sacrificed when moribund. This data highlights the aggressive nature of LLC and the difference in survival between mice implanted with WT LLC and Crispr modified LLC with downregulated CCL2. Kaplan-Meier survival curves comparing survival of mice challenged in varying conditions. Mice were monitored daily and sacrificed when moribund. Average of 6 mice/group. (**p=<0.01)

Assesment of the tumor immune profile

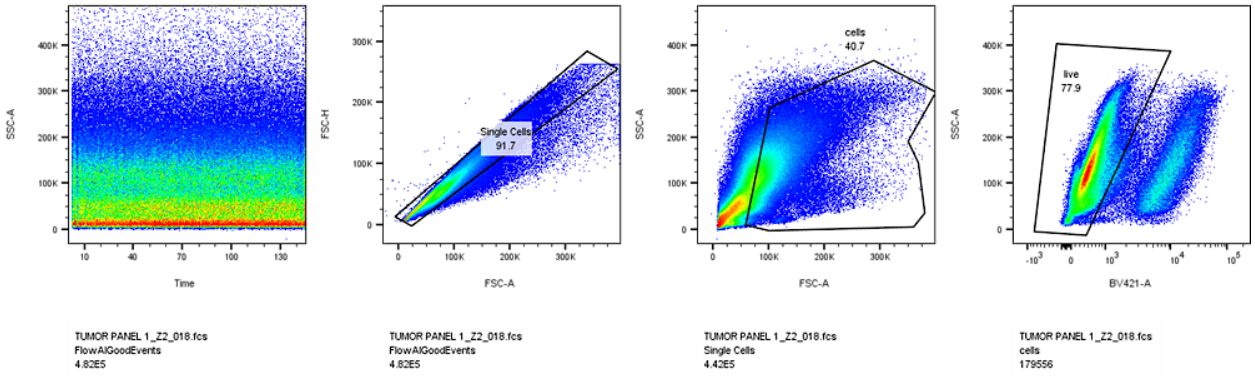


Fig 23: Gating strategy for assessing tumor immune profile Tumor cells were stained with respective antibodies and the responses were assessed via flowcytometer. Fluorescence minus one (FMO) and single stained controls were used for gating and compensation. Only good events were taken for analysis that were further distinguished based on separation via FSC vs SSC.

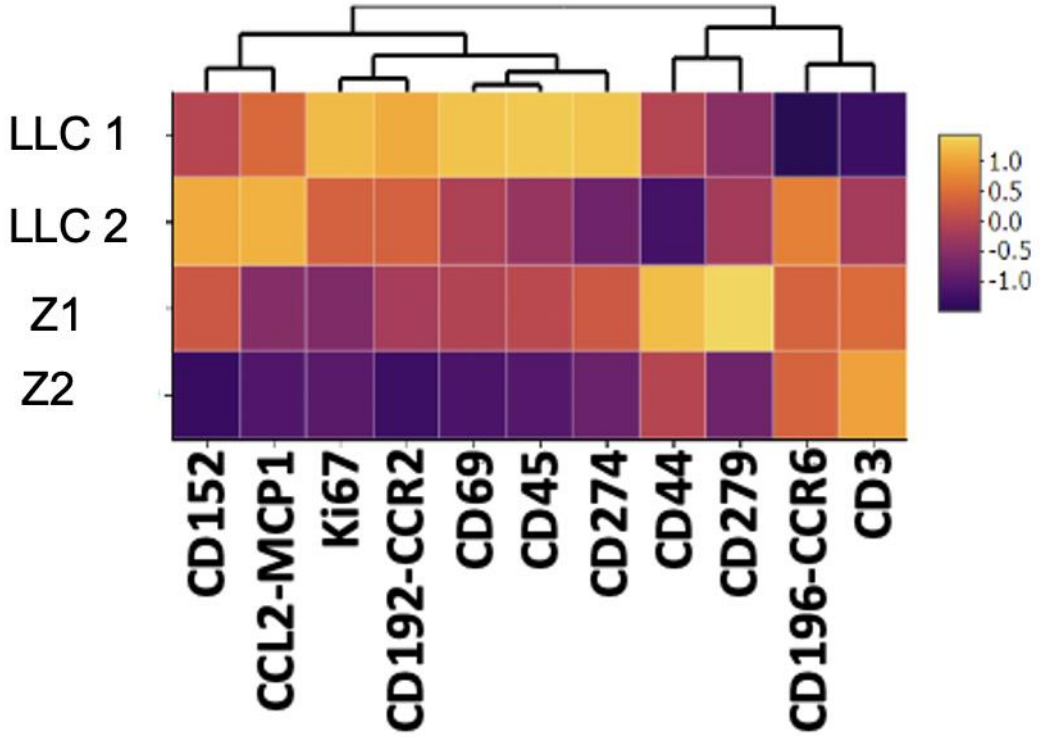


Fig 24: Immunosuppression based tumor immune profile. Heatmap analysis of the immune cell milieu of mice injected with LLC 1 & 2 (Lewis lung carcinoma WT cells) vs Z1 & Z2 (Crispr Modified CCL2 negative Lewis lung carcinoma cells) scaled per column low (yellow) to high (purple). N=2.

Immunosuppression based tumor immune profile

Here we show a heatmap on the immune cell milieu scaled per column. CD152/CTLA4 and CD 279 is PD1and they are immune checkpoint markers that negatively regulate T cell activation and cell mediated immune response. We see a substantial decrease in this immune suppressor as

a result of our ccl2 knock down. Since we aimed to deplete CCL2 we see a substantial decrease in CCL2 and interestingly a robust decrease of CCR2 which is the CCL2 receptor

CD69 is responsible for tumor immune escape via t cell exhaustion and we also see a decrease in its levels indicating that our KO might help in barring tumor immune escape. CD69 negatively regulates the effector function of intratumoral T cells and importantly controls the 'exhaustion' of CD8 T cells. We found a robust decrease of CD69 in our CCL2 lacking tumors. CD44 is a Treg marker that induces immunosuppression by inhibiting T cell proliferation. We also see a decrease in CD44 indicating an attenuation of immunosuppression.

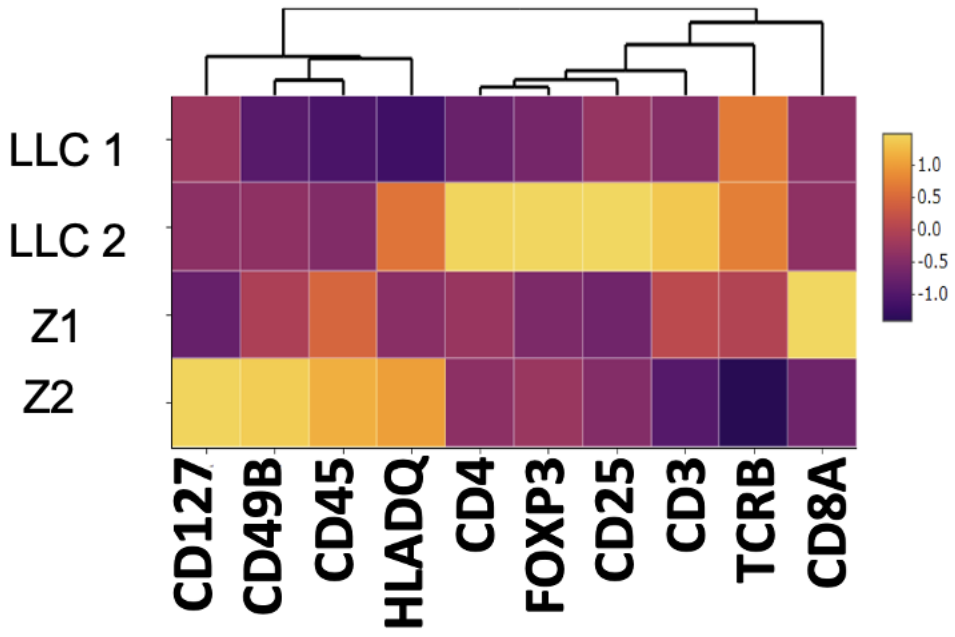


Fig 25: T cell based tumor immune profile. Heatmap analysis of the immune cell milieu of mice injected with LLC 1 & 2 (Lewis lung carcinoma WT cells) vs Z1 & Z2 (Crispr Modified CCL2 negative Lewis lung carcinoma cells) scaled per column low (yellow) to high (purple). N=2.

T cell based tumor immune profile

Increase in CD127 identifies an increased memory and effector T cell response which is imperative for curbing tumor growth and proliferation. We also see a robust increase in CD49b that indicates an increase in natural killer and NK T cells. We see an increase in CD45 also known as leukocyte common antigen that is a marker for T cell activation. The presence of CD45+ cells in the CCL2 negative tumors indicates an influx of T cells in the tumor milieu. These T cells help in curbing the tumor growth and proliferation. The decrease in FoxP3, CD25 also indicates a dampening of tumor suppressive response along with an influx of T cell repertoire. While the heatmaps were generated using FlowJo and its add on algorithms, we also checked each piece of data manually.

CHAPTER IV

DISCUSSION

Despite the significant progress made in understanding the biology of tumor cells over the last forty years, it has not translated into a corresponding improvement in our ability to cure the disease. The treatment of most cancers has remained largely unchanged during this time, and the decline in mortality rates has primarily been due to early detection and prevention measures, rather than the effectiveness of available therapies (Etzioni *et al*, 2003).

Even though cancer research and treatment has progressed in leaps and bounds there are still significant gaps and limitations in current cancer therapies. One of the major challenges is the heterogeneity of cancer cells, which can make it difficult to develop therapies that are effective for all patients or even for all cells within a single patient's tumor (Cagan and Meyer., 2017) Another challenge is the development of drug resistance, which can occur when cancer cells adapt to the presence of a specific treatment and continue to grow and spread despite treatment. This can occur through a variety of mechanisms, including mutations that allow the cells to evade the effects of the treatment, increased drug efflux, and alterations in signaling pathways. Another limitation in cancer therapy is the potential for off-target effects, which can occur when a treatment affects healthy cells or tissues in addition to cancer cells. This can lead to unwanted side effects and can limit the effectiveness of the treatment (Jemal *et al*, 2009).

Finally, access to cancer therapy is a significant issue in many parts of the world. Cancer treatments can be expensive, and not all patients have access to the latest treatments or to comprehensive cancer care. This can lead to disparities in cancer outcomes between different populations. Addressing these challenges will require continued research and innovation in cancer therapy, as well as improvements in healthcare infrastructure and access to care.

The swift developments and breakthroughs in CRISPR technology have already started to tackle many of the perplexing and fundamental queries surrounding cancer. CRISPR allows for a detailed understanding of the contribution of specific genes to cancer cell behavior, facilitates the development of advanced immunotherapies, assigns functional roles to repetitive coding variants, and unveils the elusive functions of noncoding and regulatory elements in tumor formation.

At present, there are various methods to treat tumors, such as conventional cancer therapy including operation, chemotherapy and radiotherapy, molecular targeted therapy, immunotherapy, and genetic therapy. Traditional methods pose a significant challenge to patients' tolerability and adherence due to toxicity (Mun *et al.*, 2018). In the past decade, CRISPR-Cas9 gene editing technology as a strategy for disease therapy successfully entered preclinical and clinical stages. With the continuous improvement of gene editing tools and the identification of new effective targets for diseases, the clinical translation and application research of gene editing technology has been expanded. Not only in insects and plants, but also in animals and even in humans, the CRISPR-Cas9 gene editing technology proves its powerful utility.

Tumor progression is regulated by various intrinsic and extrinsic (microenvironment) factors. It is now well recognized that cancer cells exist in a complex environment in which they interact with a plethora of stromal cells, including the multiple cell types that make up the immune system of the host. A large number of these interactions are mediated by chemokines. The roles of chemokines in tumorigenesis have been shown to be diverse, including both negative and positive regulation of inflammatory cells, chemoattraction of tumor cells to metastatic sites, regulation of angiogenesis, and direct regulation of proliferation of cancer cells (Ben-Baruch., 2006) the exact mechanisms of most are still unknown. It has been recognized that the development of cancer is influenced by interactions between tumor cells and host immune response (Chen and Mellman, 2013). We blocked the production of CCL2 in the tumor cells and observed its effects *in vivo*. We found that tumorigenesis was remarkably suppressed by the blockade of CCL2 in animals implanted with Lewis lung carcinoma.

In the tumor microenvironment, CCL2 interacts with C-C motif chemokine receptor 2 (CCR2) to mediate chemotaxis of monocytes and tumor associated macrophages (TAMs), which in turn contributes to the shaping of the tumor microenvironment and enables cancer progression (Qian *et al*., 2011; Murray and Wynn., 2011). In their study Wang *et al*, 2020 demonstrated the antitumor activity of CCL2-CCR2 blockade in esophageal carcinogenesis. Inhibition of CCL2-CCR2 signaling blocks the recruitment of inflammatory immune cells and inhibits cancer cells metastasis in tumor-bearing mice (Lim *et al*., 2016). Our study emphasizes the importance of the blockade of CCL2 in the treatment of Lung carcinoma.

Developing and optimizing the Cas9-based gene editing should promote the technology forward to therapeutic applications and offer a wide variety of treating strategies for human diseases, especially tumor. This study addresses challenges in immunotherapy against cancer by creating a path for clinical discovery and drives forward a revolutionary development of knocking down chemokines in hypotetraploid cells and contributes to the knowledge pool of immune mechanisms of cancer.

Yes, the progress and advances in CRISPR technologies have indeed provided a powerful tool for studying cancer biology and developing new cancer therapies. CRISPR technology allows researchers to make precise and targeted changes to the genome, enabling them to better understand the role of individual genes in cancer development and progression.

For example, CRISPR has been used to identify genes that play a key role in cancer cell behavior, and to develop new immunotherapies that can target specific cancer cells. CRISPR has also been used to study the functional effects of recurrent coding variants, which can provide important insights into the underlying causes of cancer (Doudna & Charpentier, 2014). CRISPR-Cas9 technology has the potential to be used to develop new targeted therapies for cancer. By targeting specific genes or pathways that are involved in cancer development and progression, CRISPR-Cas9 can potentially provide a more precise and effective way to treat cancer.

Cancer tetraploidy refers to a state in which cancer cells have four sets of chromosomes instead of the normal two sets. This can occur when a cell undergoes an error during cell division, resulting in the duplication of chromosomes. Tetraploidy can also occur when a cell fails to divide after undergoing DNA replication, resulting in the accumulation of chromosomes. Tetraploidy is relatively rare in normal human cells, but it is frequently observed in cancer cells. The presence of extra copies of chromosomes can lead to genomic instability and contribute to the development and progression of cancer. In some cases, tetraploid cancer cells can undergo additional rounds of cell division, leading to the formation of highly abnormal cells with even more sets of chromosomes, known as polyploidy. Research has suggested that tetraploidy may play a role in the resistance of cancer cells to chemotherapy and radiation therapy. In addition, some studies have suggested that tetraploidy may be a prognostic factor for certain types of cancer, indicating a poorer prognosis for patients with tetraploid tumors. (Lim and Ganem., 2014)

Understanding the mechanisms that lead to tetraploidy in cancer cells may help researchers develop new treatments and strategies for preventing or reversing the genomic instability associated with tetraploidy. An analysis of sequencing data from approximately 4,000 human cancers using computational methods has revealed that around 40% of all human tumors have experienced a tetraploidization event at some stage during their development. This finding suggests that tetraploidy is a common feature of cancer cells and may play a significant role in the development and progression of a wide range of tumor types. The analysis also revealed that tetraploidy is more prevalent in some cancer types than in others. For example, tetraploidization was found to be particularly common in liver, ovarian, and endometrial cancers, while it was less

common in lung and pancreatic cancers. Additionally, the study found that tumors that had undergone tetraploidization tended to have more complex and diverse genomic alterations than tumors that had not undergone this event (Zack *et al*., 2013).

These findings have important implications for our understanding of the biology of cancer and may help researchers develop new strategies for diagnosing and treating the disease. For example, targeting the mechanisms that lead to tetraploidy in cancer cells could be a promising approach for developing new therapies that can halt or reverse the progression of the disease.

The tetraploid nature of Lewis lung carcinoma contributes to its genetic instability making it a more difficult cell line for any kind of modifications. We show that this instability can be overcome by multiple gRNA hits to the multiple exons within CCL2 gene for a successful CRISPR based modification and downregulation.

The administration of Crispr cas9 modified Lewis lung carcinoma cells yielded a cancer distinct from the WT in shape, structure, and morphology in as much as being highly resectable. The tumors were localized instead of spreading out along the mesentery, smaller in size and easily removable, pointing towards the importance of a therapeutic intervention that prevents or minimizes CCL2 production. Our study also showed the restriction of metastasis and the slow growth of the cells and tumors in the absence of CCL2 thus leading to higher survival in the mice.

Our data reveal elevated levels of CCL2, in LLC and MC-38, which have important roles in cancer progression and metastasis. Our study reveals the serum cytokine profile of mice with established LLC and MC-38 shows a lack of CCL2 leading us to hypothesize that the expression of CCL2 is localized and restricted to the tumor micro-environment. We show our success in creating CRISPR LLC-CCL2 KO cells lines which no longer express CCL2, restricting the abundant proliferation and metastasis of LLC cells, and could be a potential target to revolutionize treatment for advanced stage lung cancer. Our data also shows the impact of CCL2 in cell cycle alteration and cell growth and proliferation. Our study showed that the levels of certain cytokines and chemokines like CCL2 produced by LLC *in-vitro* cells were far higher compared to B16-F10. This leads to the more aggressive nature of Lewis lung carcinoma and contributes to its increased metastasis. Chemokines like CCL2 leads to the diminished survival rate of mouse implanted with Lewis lung carcinoma. Our study shows that suppression of this chemokine CCL2 can lead to increased survival outcomes in DQ8 humanized mice.

CCR2 antagonist is a type of drug that blocks the activity of the chemokine receptor CCR2. Chemokines and their receptors play an important role in immune system function, including the recruitment of immune cells to sites of infection or injury. CCR2, in particular, is involved in the recruitment of monocytes and macrophages to sites of inflammation. Our objective was to determine whether inhibiting the CCL2-CCR2 axis by blocking the CCR2 receptor for CCL2 would result in a distinct survival rate compared to that of mice implanted with wild type Lewis Lung Carcinoma cells. The study indicates that there was no notable difference in survival between mice that were implanted with wild type Lewis Lung Carcinoma alone and mice that received treatment with a CCR2 antagonist after implantation. This lack of significant difference

may be attributed, in part, to the fact that INCB3344, the CCR2 antagonist used, is cleared from the system within 8-12 hours, making it ineffective at eliciting a strong systemic response against the LLC implant. Therefore, it was necessary for us to conduct a study using CCL2 knockout cells to demonstrate a sustained survival response.

Our study has already shown that the levels of certain cytokines and chemokines produced by LLC in-vitro cells were far higher compared to appropriate controls. Our preliminary data reveal elevated levels of CCL2 in LLC which have important roles in cancer progression and metastasis. Our data on the serum cytokine profile of mice with established LLC and MC-38 shows a lack of CCL2 leading us to hypothesize that the expression of CCL2 is localized and restricted to the tumor micro-environment

CRISPR has enabled us to study the role of regulatory elements in tumorigenesis like the chemokine CCL2, which has long been a challenging area of research. By allowing us to precisely manipulate CCL2 production, CRISPR has provided important new insights into the complex processes that drive cancer development and progression and has advanced our understanding.

Overall, the rapid progress and advances in CRISPR technologies are likely to continue to play a critical role in our efforts to understand and treat human cancers and hold significant promise for the development of new and more effective cancer therapies in the years to come.

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