Analysis of Tyrosine Kinase Inhibitor Responses in Mixed Murine EML4-ALK-Driven Lung Tumors to Define Dominance of Their Tumor Immune Microenvironments

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For my thesis project, I was given the opportunity to conduct research at University of Colorado Anschutz Medical Campus. I worked under my two principal investigators, Dr. Lynn Heasley and Dr. Raphael Nemenoff. I completed my work over a two month period, although experiments are still ongoing.

Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States. In 2021, nearly 2.02 million lung cancer deaths were recorded. The cancer type that resulted in the second most deaths was about half of lung cancer cases at 1.04 million from colon and rectum cancers. In the past 40 years, lung cancer rates have dropped significantly due to increased public education due to the detrimental effects of smoking ("Lung Cancer Death Rates", 2021). Since the 1990's the stigma has remained that the majority of lung cancer diagnoses are a result of smoking, but it is becoming more common that these cases are rising more in non-smokers.

Chemotherapy has developed vastly in the last 30 years, where treatments are able to combat lung cancer precisely and without less harsh side effects. Precision oncologies are being used more and more to treat various cancer types. Unlike traditional IV chemotherapy, precision oncologies utilize a patient's genes to disrupt the cancerous cellular cascade in one way or another (Roser, 2020).

In recent studies, successes in precision oncology with tyrosine kinase inhibitors (TKIs) have yielded marked and durable tumor responses in tumor subsets defined by oncogenic mutations in the receptor tyrosine kinases (RTKs) EGFR, ALK, ROS1, and RET. Despite this progress, most patients show a partial response and these therapies fail to completely eliminate "drug tolerant persisters" also known as "residual disease". Thus, new therapeutic strategies are needed as presently there are no approved therapies after EGFR mutant lung cancer progression on the 3rd generation TKI, Osimertinib (Kleczko et al., 2023). This desperate need for additional precision therapies is crucial for 2nd and 3rd line therapies in oncogene-driven lung cancer patients. Anti-PD1/PD-L1 agents are approved treatments in lung cancers

that disrupt tumor-mediated immune suppression (Kleczko et al., 2023). However, these drugs are ineffective in patients whose tumors bear oncogenic RTK genes despite evidence that adaptive immune cells contribute to the anti-cancer activity of TKIs in these cancers. In other words, immune checkpoint inhibitors are not able to be utilized.

Cell Lines Driven from EML4-ALK Lung Cancer

The Nemenoff and Heasley labs have generated a panel of murine EML4-ALK cell lines (EA1, EA2) that can be orthotopically implanted in immune competent mice (Jinyang, et al., 2018). EML4 "Echinoderm microtubule associated protein like 4" are typically found in non-small cell lung cancers. ALK, "Anaplastic Lymphoma Kinase" is a specific TKI that shows a genetic alteration in lung cells' DNA that causes abnormal growth. The cell lines used in the lab are a fusion between the two, EML4-ALK most often detected in non-smokers. While these cells show similar inhibition of growth in vitro to ALK TKIs, the resulting tumors exhibit differences in the depth of response, with EA2 tumors showing a complete response and EA1 tumors showing a partial response with detectable residual disease. Also, the immune microenvironments observed in EA1 (partial response) and EA2 (complete response) tumors prior to therapy are distinct. EA1 tumors exhibit low amounts of CD8+ T cells restricted to the periphery of the tumor and high levels of immune-suppressive neutrophils. By contrast, EA2 cells generate orthotopic tumors with high levels of infiltrated CD8+ T cells and low neutrophil content.

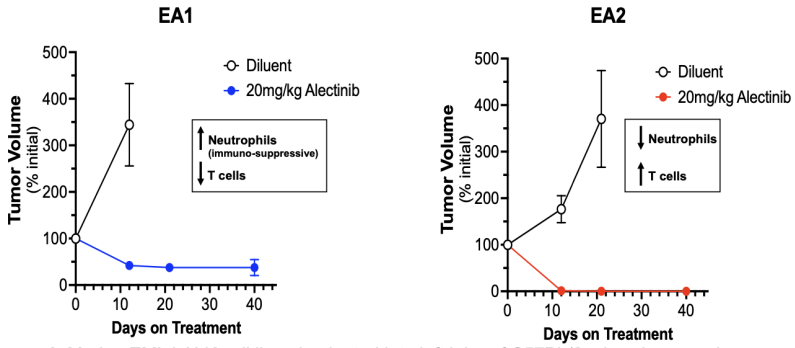


Figure 1. Murine EML4 ALK cell lines implanted into left lobe of C57BL/6 mice show varying response to ALK TKIs. EA1 (partial response) and EA2 (complete response) also show distinct immune microenvironments prior to therapy. EA1 exhibits low CD8+ T cells and high levels of immune-suppressive neutrophils while EA2 exhibits high CD8+ T cells and low neutrophil content.

Hypothesis

These findings support the hypothesis that the pretreatment immune cell composition of the lung tumor microenvironment could have a significant impact on the quality of the overall TKI response. This thesis will address the question of which baseline immune microenvironment is dominant in more heterogeneous ALK+ lung tumors that contain regions of anti-tumorigenic and immune suppressive microenvironments. This question is of importance as lung cancer patients that present with advanced tumors likely exhibit heterogeneity in the overall immune landscape. Thus, developing a "mixed" EML4-ALK tumor model may provide novel insight into how to develop improved therapies for these patients.

In order to address the question of immune dominance within a mixed EML4-ALK tumor, my project will deploy mixtures of EA1 and EA2 cell lines to intentionally establish heterogeneous tumors and assess the resulting immune microenvironment and the Alectinib response. The lab has generated EA1 and EA2 cells that incorporate specific fluorescent tags, allowing the assessment of each population in mice. In studying heterogeneous lung tumors composed of clones that avidly recruit anti-tumorigenic T cells and clones that recruit immune suppressive cells (neutrophils), we imagine that three different possibilities may be observed.

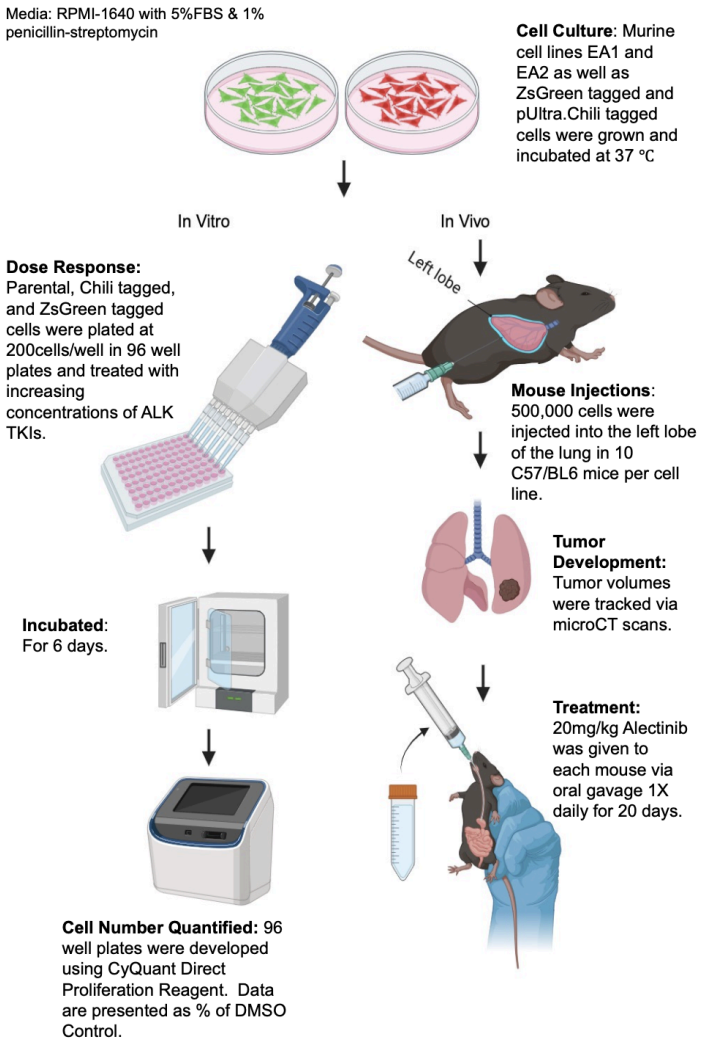
Methods for Single Cell Experiment

These experiments utilize wild type C57BL/6 female mice. Small incisions are made and the cells are injected into the left lung of the mouse. Following 10 to 14 days to allow the tumors to establish, the mice will be treated with Alectinib or diluent as control. Prior to and following treatment, microCT imaging will be performed to assess changes in total tumor volume in a noninvasive manner.
Figure 2 visually describes the procedure for the singular cell lines, as we perform dose response experiments. It should be noted that the dose response experiments were treated with Alectinib, Lorlatinib, and Crizontinib. The *in vitro* experiment verified that the fluorescently tagged cell lines of EA1

and EA2 showed similar sensitivities to TKI inhibitors like the parentals (Figure 3). The *in vivo* experiment using murine mice models also showed similar responses, as both EA1 and EA2 cell lines showed similar sensitivities to their parentals (Figure 4).

It's important to note that Keynance fluorescent imaging of mixed cells in vitro were also taken. 150,000 cells of both EA1 chili and EA2 ZsGreen were plated in 10cm dishes. 24 hours later cells were treated with 30nM Alectinib and incubated for 3 days before imaging was taken. DMSO acted as the control. Upon imaging, both cell lines were visible in the DMSO solution. The alectinib treatment had great effect on the cancer cells, as very few EA1 Chili cells were remaining.

Figure 2. Methods for Single Cell Experiments



Data From Single Cell Experiments

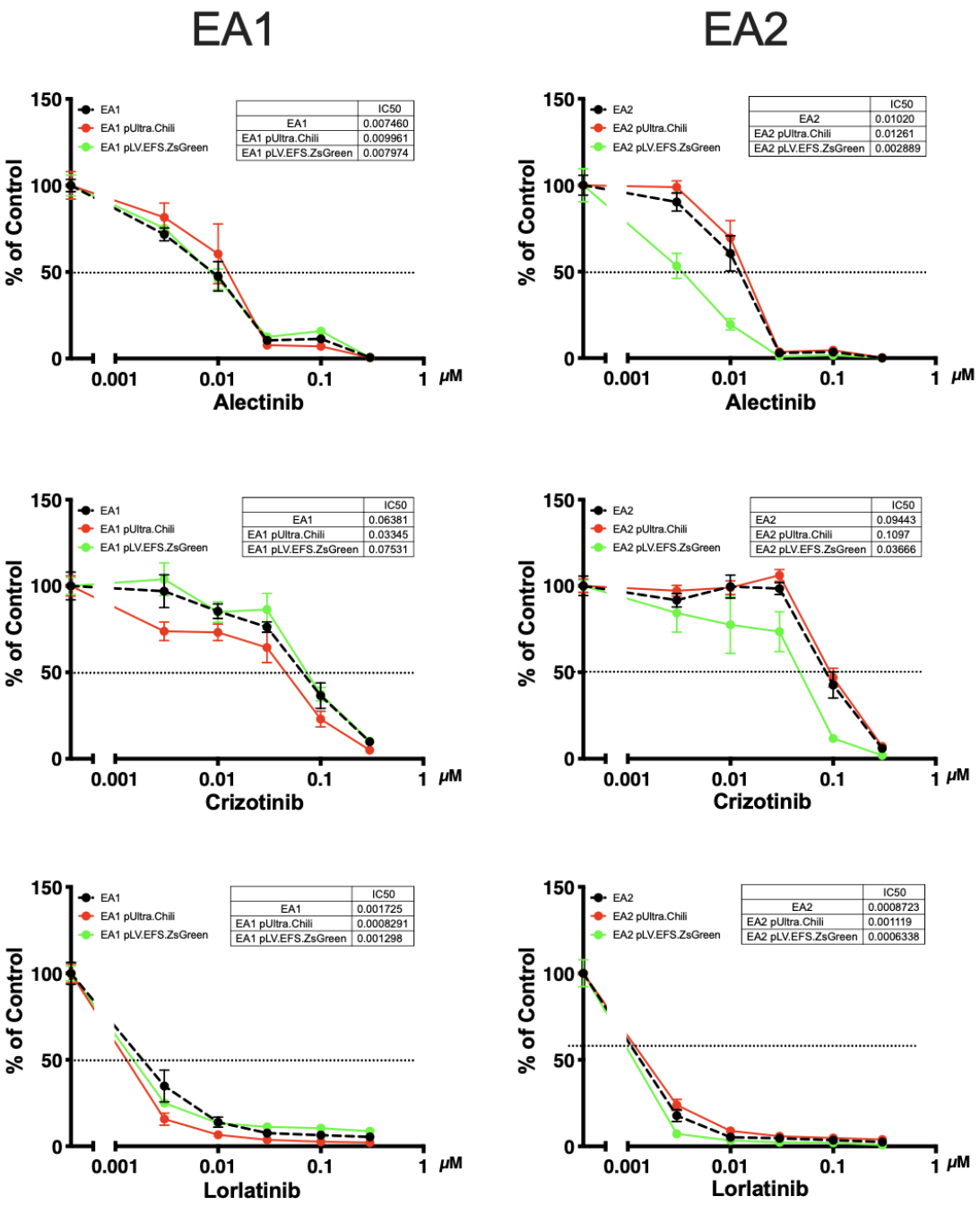


Figure 3. Dose response experiments were conducted for each cell line and treated with increasing concentrations of targeted ALK therapies: Alectinib, Crizotinib, and Lorlatinib. This verified that the fluorescently tagged cell lines of EA1 and EA2 showed similar sensitivities to TKI inhibitors like the parentals.

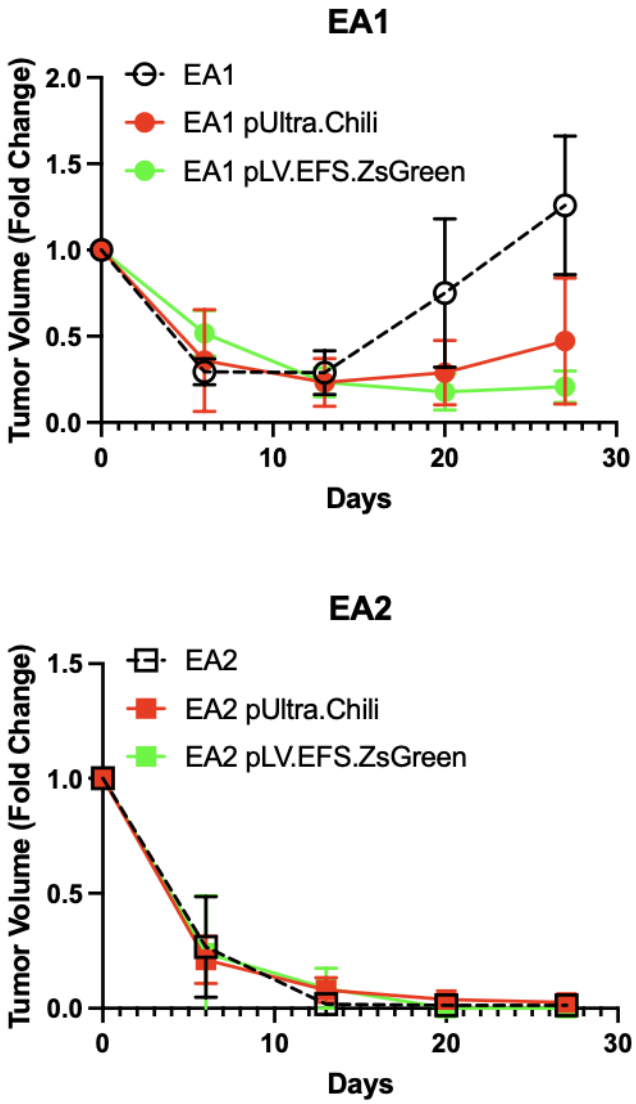


Figure 4. ZsGreen and Chili-labeled cell lines exhibit equal alectinib sensitivity as the parental lines. After 20 days of treatment on Alectinib, it was confirmed that the sensitivity of the EA1 mice was not as responsive as the mice with EA2 cells. Tumors in the EA1 mice still existed and even showed resistant tumor behavior. EA2 showed complete response.

Methods for Mixing Cell Experiment

The last experiment conducted was the mixing cell experiment where we injected EA2 ZsGreen cells and EA1 Chili cells into 30 different mice (Figure 5). More specifically, I was trying to determine when two cell types are mixed together (EA2 Z.s.Green and EA1 pultra.Chili), which cell line will "win"? Will there be a complete or partial response to the various therapies?

Figure 5.
Schematic shows the method of mixing EA2 ZsGreen and EA1 pUltra. Chili cells in a dish. These cells were grown and then injected into left lobe of the lung. Mouse tumors grew for one week, after which some were put on treatment and 3 were harvested for FLOW Cytometry.

Data from Mixing Cell Experiment

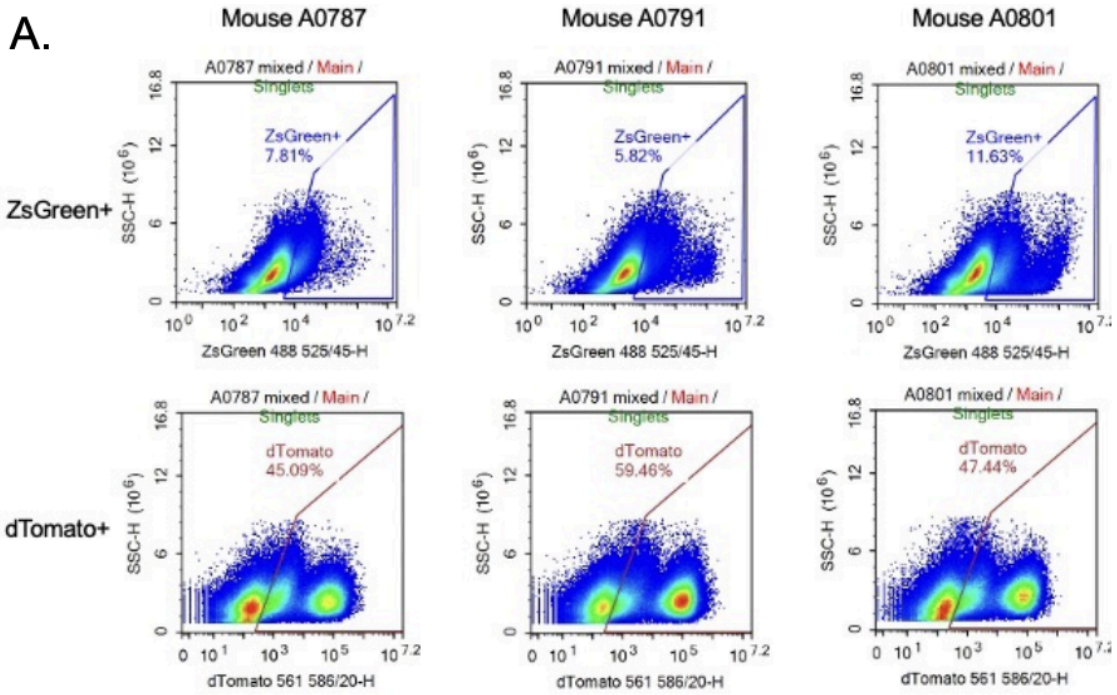
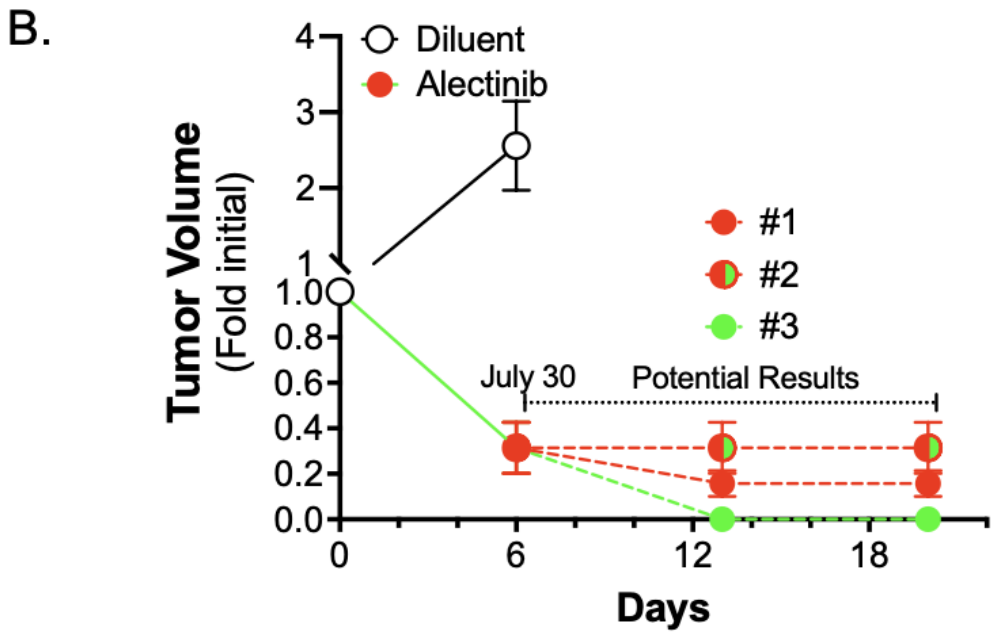


Figure 5. A. FLOW data of 3 distinct EA1 Chili + EA2 ZsGreen mixed tumors. Tumors were harvested and single cell dissociated 14days after implantation and submitted to FLOW cytometry analysis. The mixed tumors contained on average more EA1 Chili cells than EA2 ZsGreen cells.



B. Tumor volume (fold initial) after 6 days of alectinib treatment (July 30). EA1 Chili + EA2 ZsGreen mixed tumors were treated with either H_2O diluent or 20mg/kg alectinib. Tumor sizes on treatment predicted by potential outcomes #1-3 are graphically depicted (see box to right).

Three Potential Outcomes

Although this experiment is still ongoing and tumor volumes are being recorded, three potential outcomes are possible:

1. **EA1 cells will remain and EA2 cells will be eliminated.** This result would indicate that EA1 and EA2 cells are differentially recognized by CD8+ T cells and their distinct TMEs are not related to this differential response.
2. **When mixed, both EA1 and EA2 cells remain.** This result would support that the immunosuppressive microenvironment established by EA1 is dominant over the CD8+ T cell-rich TME generated by EA2 cells.
3. **When mixed, both EA1 and EA2 cells are completely eliminated.** In this case, the CD8+ T cell-rich TME in EA2 tumors is dominant over the immune suppressive

microenvironment generated by EA1 cells. This finding would potentially be the most rewarding for the development of new therapies. (Figure 6)

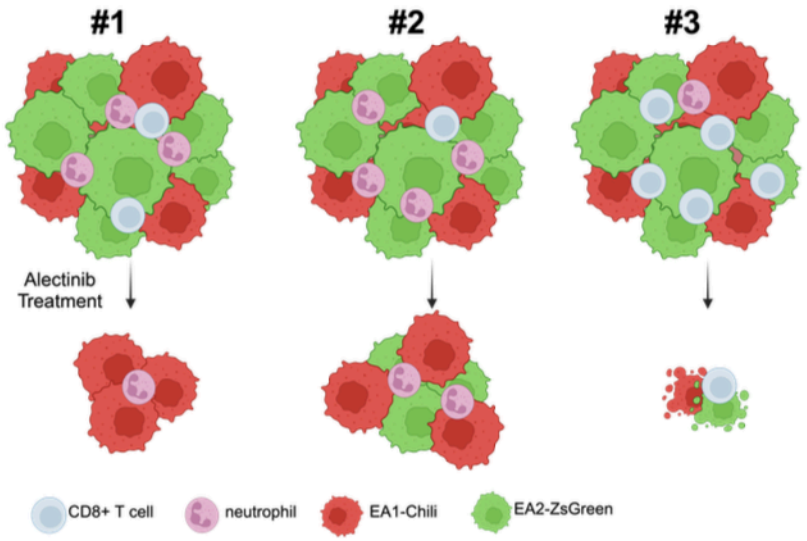


Figure 6. Three possible outcomes of the mixing experiment with EA2 ZsGreen and EA1 Chili cells.

Fluorescent Microscope Imaging

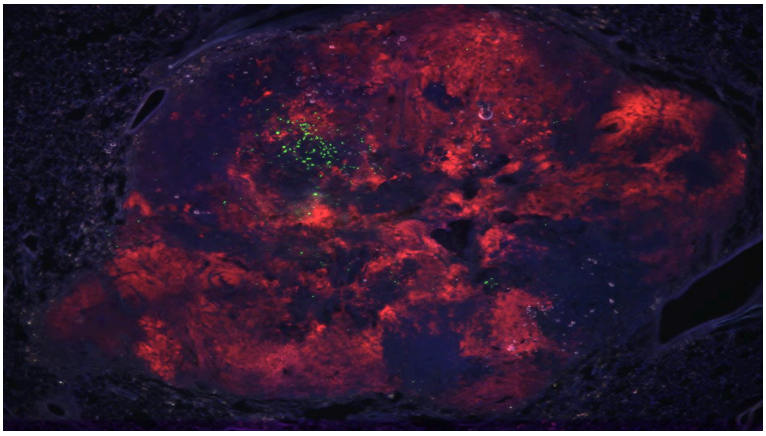


Figure 7a. Fluorescent microscope image of untreated mixed tumors (EA1 Chili and EA2 ZsGreen) exhibited T cell staining around boundary of tumor, expected like EA1 behavior. Intratumoral T cells are limited suggesting that mixed tumors behave more like EA1.

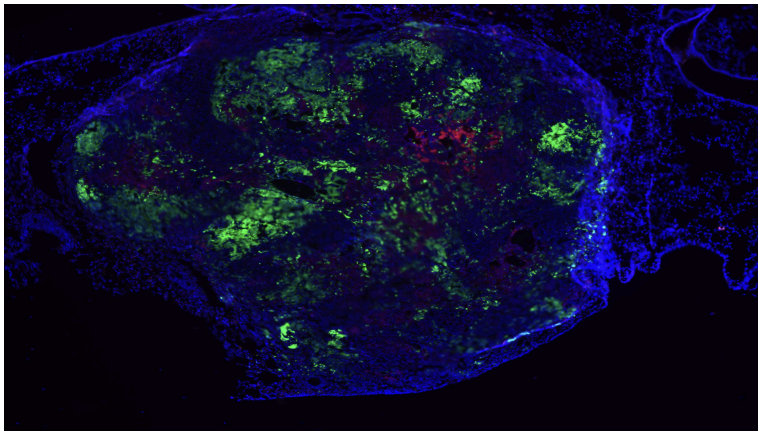


Figure 7b. Fluorescent microscope image of untreated mixed tumors (EA2 Chili and EA1 ZsGreen) EA1 still remaining around periphery of tumor.

Future Directions

For future improvements, the mixed *in vivo* experiment could be repeated with a lower cell count of EA1 Chili cells and a higher count of ZsGreen EA2 cells. This would give more of a 50/50 balance of the two cell lines in the tumor prior to treatment. Some tumors from the murine models will also be fixed and submitted to immunofluorescence analysis of tumor cells and immune cells (T cells and neutrophils). This will give more detailed input on the microenvironment of these cells.

Reflection

My Honors Thesis serves as a very personal chapter in my life. My dad was diagnosed with Stage 4 Non-Small Cell Metastatic Lung cancer when I was 16 years old. He was given 9 months to live and as the daughter of a cancer patient, it became important for me to understand what was happening in my dad's body and why. Understanding cancer biology was comforting to me because it offered me some kind of control on the situation that was out of my hands. Consulting various oncologists, attending my dad's monthly scans, his radiation therapy, failed surgeries, and watching his targeting therapy provides my family and I a temporary miracle by keeping him alive. This all led me to pursue my thesis topic on lung cancer research. I was given the opportunity to be on the front lines of cancer research that will eventually impact my dad and other patients like him. The research I conducted inspired me to pursue a career in cancer research and oncology. I gained valuable skills as a scientist as I was given the opportunity to complete this research. It taught me there's so much as researchers we actually do not know, and when we finally find what we're looking for, it should be celebrated. One of the most important lessons I will take with me is conversing with other like minds is one of the most important things you can do as a researcher. Even asking questions or getting opinions from those not directly in your field can offer you so much insight, it could change the trajectory of your thinking for your project. Lots of questions are still to be answered, but the future is promising as studies are ongoing like lives depend on it.

Acknowledgements

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