2020

Identification of Imiquimod as a Potential Combination for Anti-CD47 Antibodies in Cancer Therapy

Nicole Brittaney Pang
Scripps College

Identification of Imiquimod as a Potential Combination for Anti-CD47 Antibodies in Cancer Therapy

A Thesis Presented by
Nicole Brittaney Pang

To the Keck Science Department
Of Claremont McKenna, Pitzer, and Scripps Colleges
In partial fulfillment of
The degree of Bachelor of Arts

Senior Thesis in Biology
May 4th, 2020

Table of Contents

ABSTRACT 2

INTRODUCTION 3

Macrophage Mediated Phagocytosis of Cancer 4

CD47 Blockade: A Therapeutic Target 5

CD47-Sirpa Interaction 6

Imiquimod 8

Luciferase Assay 10

MATERIALS AND METHODS 12

RESULTS 16

DISCUSSION AND CONCLUSION 23

ACKNOWLEDGEMENTS 27

BIBLIOGRAPHY 28

ABSTRACT

The avenues of targeted immunotherapy offers a promise of less toxic treatment options for those battling different forms of cancer. Specifically, the process of hijacking a patient's own immune system to fight cancer from within versus using external treatments like chemotherapy which is extremely damaging to the patient. One such avenue includes the usage of monoclonal antibodies as an effective modality for immunotherapy. Cluster of Differentiation 47 (CD47), also known as the 'don't eat me signal', aids in cell proliferation and evasion of phagocytosis and has been found to be a target for stopping tumorigenesis. Previous research has been successful in combining CD20 antibody blockades with an FDA approved drug called rituximab to improve survival in B cell non-Hodgkin lymphoma patients. While the success of this clinical trial is an optimistic outcome, there has not been any research done with using adjuvant drug therapy on solid tumor masses and CD47 targets. In my thesis, I will be identifying Imiquimod as a potential drug to rationally combine with B6H12 monoclonal antibodies for human colorectal cancer therapy as well as improving the methodology for conducting a phagocytosis assay by using luciferase.

INTRODUCTION

Cancer treatment and management continues to be a prominent area of research for scientists because of the complex nature of the disease. Procedures such as chemotherapy, radiation therapy, and palliative care are all ways that have been found to eradicate or reduce the development of cancer. However, these treatments are far from being cures and many cancer cells are able to evade immune recognition and continue to proliferate within the body.

With this in mind, research looking at ways to hijack the immune system for the benefit of the patient has been ongoing. A critical advancement in the past couple of years has been the usage of targeted immunotherapy in oncological cases. The insurgence of immunotherapy research has opened up new perspectives on how to actively stop tumorigenesis using a patient's own immune system as a self-vaccine. In using this methodology, the host's immune system can eliminate unwanted malignant cells and reduce the amount of cancer cells evading immunosurveillance. So far, in the field of immunotherapy, the existing treatments range from activating effector immune cells with vaccination, going against inhibitory suppressive mechanisms, or neutralizing immune suppressor mechanisms. Antibodies have been extremely helpful in this as they can aid in reducing regulatory T cells and use them against immune-checkpoints. Prominent checkpoints that have been found to be critical in immunotherapy are CTLA-4 and PD1. CTLA-4 functions as a regulator of early immune response T-cell proliferation and PD1 functions in a later portion of the immune response in T-cell suppression (Rotte, 2019). These two checkpoints have proven to be incredibly essential in the process of tumorigenesis. Studies have evaluated that the combination of CTLA-4 and

PD-1 blockades increased response rates in metastatic melanoma patients and has increased efficacy in those cases. This study was critical in improving the prognosis of metastatic melanoma, advanced renal cell carcinoma and metastatic colorectal cancer patients in clinical trials and further in treatment (Rotte, 2019).

These strategies have paved an exciting new world of cancer immunotherapy and are advantageous to the race to cure cancer (Farkoma et al., 2016). With the identification of such checkpoints, more studies are being generated to find more checkpoints and functions that potentially can be blocked with monoclonal antibody therapy.

Macrophage Mediated Phagocytosis of Cancer

A process that has been largely impactful in the anti-tumorigenesis movement is phagocytosis. However, that process is considerably driven by inhibitory and stimulatory signals that could easily be disrupted by the tumor's microenvironment and needs to be considered in any therapeutic advancements. The process of phagocytosis by macrophages is elicited by the innate immune response in the presence of a foreign pathogen. Macrophages are classified as effector cells that have the ability to discern between self and foreign agents through receptors built into its mechanism (Aderem et al., 1999). They also function to clear apoptotic cells and functions in the absence of inflammation.

Aside from the tumor itself, the microenvironment plays a key role in tumorigenesis. The tumor's microenvironment contains blood vessels, immune cells like macrophages, fibroblasts, signaling molecules, and lymphocytes that form a cellular environment that a tumor can survive in. With a combination of non-cancerous cells and cancer cells, the microenvironment is made to

sustain the many hallmarks that cancer exhibits. Typically, the non-malignant cells that are present in the microenvironment are tumor-promoting at all stages of oncogenic change.

Although the tumor microenvironment has a vast number of different cell types and functions, it is a common understanding that each cell in that hypoxic environment contributes to the strong proliferation of tumor cells. This poses a challenge because, with the aid of the microenvironment, cancer cells can evade many different types of processes like phagocytosis. Evasion is achieved by a cell surface glycoprotein that is found on many healthy cells in the body. CD47 is the inhibitory receptor responsible for this process. It is part of the immunoglobulin superfamily (Ig) and is expressed on tumor cell surfaces, allowing the cell to escape from immunosurveillance from macrophages and other effector cells. In addition to this, the high expression of CD47 on the surface of these tumors can also aid with furthering metastasis. With the possibility of exosomes being secreted with CD47 expression, tumors are given an easier access to the rest of the body and the tumor microenvironment is expanded (Lian et al., 2019).

CD47 Blockade: A Therapeutic Target

Current approaches in immunotherapy include research on CD47 as a promising immunotherapeutic target. Otherwise known as Cluster of Differentiation 47, CD47 is categorized under an immunoglobulin family (IgSF) and aids in cell functions like proliferation, adhesion, apoptosis, and most importantly, phagocytosis (Russ et al., 2018). Discovered originally as a plasma membrane molecule, CD47 is also part of the family of receptors for thrombospondin and is inhibitory in nature (Lian et al., 2019). It is expressed on normal cell

surfaces and found to be highly expressed on tumor cell and cancer stem cell surfaces (Kong et al., 2016).

One of the main functions that CD47 takes on is what has been called a "Don't Eat Me Signal" (Russ et al., 2018). When it is expressed on cell surfaces, CD47 functions as a ligand of signal regulatory protein-1 (SIRP-1), delivering an inhibitory signal to stop phagocytosis (Kong et al., 2016). SIRPα can be found on specific early hematopoietic progenitors of myeloid cells like macrophages. The specific binding of CD47 to SIRPα results in the phosphorylation of immunoreceptor tyrosine-based inhibition motifs which then triggers the recruitment of two phosphates: SHP1 and SHP2. These two phosphates inhibit myosin II from gathering at the phagocytic synapse and prevents phagocytosis from occurring (Russ et al., 2018). As the first phagocytosis checkpoint that researchers found in cancers, the CD47–SIRPα axis is important to continue to build upon (Feng et al., 2019). Studying this interaction can lead to more answers on how to prevent malignant cells from escaping immune cells like macrophages and also aid in preventing more migration and metastases in cancer patients.

CD47-Sirpa Interaction

The interaction between CD47 and SIRPα on macrophages is important because of its role in tumorigenesis. Macrophages are critical components of the innate immune system that recognize and eliminate any foreign pathogens that enter the body (Kong et al., 2016). They typically phagocytose foreign cells and avoid normal cells because SIRPα sits on the surface of macrophages. In normal cells, CD47 will be expressed and the macrophage will receive a negative signal telling it to ignore that cell and move on to the next. These immune checkpoints

are critical in cancer cell development and is one of the many reasons tumors can grow so rapidly. The binding of SIRPα with transmembrane protein CD47 establishes a signal transduction cascade which enables the obstruction of phagocytosis (Chao et al., 2010).

Tumor cells have a characteristic ability to evade immunosurveillance. CD47 overexpression has been found to be a major factor in why tumor cells are able to protect themselves from phagocytosis and contribute to the progression of tumor growth (Kong et al., 2016). The abundance of CD47 expressed on tumor cell surfaces tricks macrophages into thinking that they are normal cells and therefore don't need to be phagocytosed.

Current research suggests that monoclonal antibodies (mAbs) are effective in aiding in tumor immunotherapy (Kong et al., 2016). B6H12 mAb is a monoclonal antibody that is able to interfere with the CD47 and SIRPα interaction because it is a specific anti-CD47 mAb. Recent studies have shown that anti-CD47 monoclonal antibodies eliminated selective tumor cells and evaded normal cells. Although the mechanism is not completely understood, studies have found that the addition of anti-CD47 antibodies significantly changed the outcome for acute myeloid leukemia and tumors in the bladder by initiating phagocytosis on leukemic stem cells and cancer stem cells, respectively (Kong et al., 2016).

Immunotherapy and specifically targeting of CD47 are topics that have been researched in the past, but now we are interested in the possibilities of including adjuvant therapies to help speed up and increase the efficacy of the anti-CD47 blockades. One way to do this is to add an additional drug onto existing monoclonal antibody treatments. Prior research has found that the co-administration of rituximab, an anti-CD20 antibody, and CD47-blocking antibody significantly inhibited the tumor growth in xenotransplanted models of human B cell

non-Hodgkin lymphoma in NSG mice and demonstrated a successful therapeutic effect (Feng et al., 2019). Other preclinical studies have also found similar effects with combination therapy with CD47-blocking antibodies in multiple different forms of cancers, such as anti-HER2 antibody in breast cancer, anti-CD271 antibody in melanoma, and a handful more (Feng et al., 2019).

Imiquimod

Imiquimod is an FDA approved drug that activates macrophages and cells through attachment to their cell surface receptors. In order to induce proinflammatory cytokine signalling, Imiquimod will bind to Toll-like receptor 7 and that process will signal the release of interferon (IFN)-a, interleukin (IL)-12, and tumor necrosis factor (TNF)-a (Stanley, 2002). It has also been shown in previous studies that the presence of imiquimod plated *in vitro* with peripheral blood mononuclear cells, induced a release of interferon at high concentrations, making it an efficient inducer of cytokines (Stanley, 2002).

Because macrophages hold such a central role in fighting tumorigenesis, the potential of enhancing their function with a TLR-7 agonist could be of great therapeutic value (Stanley, 2002). From the clinical standpoint, Imiquimod has also been administered as a topical cream and has shown success with treatment of some basal cell carcinomas (Stanley, 2002). The combination of imiquimod as an adjuvant drug therapy could potentially increase the efficacy of monoclonal antibody defense in immunotherapy.

The usage of monoclonal antibody (mABs) as cancer therapy has been tested in a clinical trial focused on B cell non-Hodgkin lymphoma. In this study, they found a link between increased expression of CD47 in non-Hodgkin lymphoma cells and identified that as a predictor for negative clinical outcomes. They used rituximab, an anti-CD20 antibody, in their trials as it has been proven to significantly improve survival when used as a therapy for CD20-positive B cell lymphomas (Chao et al., 2010). Treatment with rituximab was successful in their study as they found that the antibody engaged with Fc receptors on effector cells, such as natural killer cells and macrophages, which in turn stimulates phagocytosis. The mechanism of blocking anti-CD47 antibodies increased the efficacy of the therapy. Future directions that emerge from this clinical trial find that this blockade created by mABs may be an effective modality for different forms of cancer therapy. Because malignancies can appear in a multitude of forms, starting to search for other therapeutic antibodies will be beneficial (Chao et al., 2010).

Combining monoclonal antibodies in cancer therapy is worth studying further because of the positive outcomes it can achieve. This approach to cancer therapy reduces the toxicity rates of other therapy methods like chemotherapy and can increase the efficacy of the regimen.

However, despite the success of Chao's study, there are limitations that still need to be addressed. The study is primarily focused on non-Hodgkins lymphoma which is a form of liquid cancer or "blood cancer" and CD20 is specific to these types of B cell lymphomas. The efficacy of this sort of treatment on solid tumors has not been studied extensively and there is weak evidence of its efficacy.

Luciferase Assay

Typically, GFP fluorescence screening and flow cytometry methods are used to count and sort large numbers of cells in experimental procedures. However, these methods are not adequate enough when it comes to working with live cancer cells and calculating phagocytosis rates. For one, these traditional methods are costive and highly labor intensive. They are also not high throughput screening friendly. In order to enhance transfection efficiency in cancer cells, a promoter is generally combined to a reporter gene which can be easily quantified in highly sensitive assays (Smale, 2010). A reporter gene that has proven to work well with sensitive assays is the luciferase gene. Derived from Photinus pyralis, a firefly, luciferin emits a fluorescent light product which can be easily quantified and measured. The specific mechanism that it utilizes is that it oxidizes D-luciferin in ATP, oxygen, and Mg(++) environments and its interaction with coenzyme A provides a channel for sustained light reaction (Smale, 2010). Phagocytosis assays are highly sensitive, fast-paced, and specific procedures and there needs to be a high throughput long term method to help it function at its best. Based on research, using a luciferase assay can enhance the accuracy and feasibility of live cancer cell data. Luciferase assays can function under rapid pace, are inexpensive compared to past methodologies, and are best suited for sensitive assay types.

In my thesis, I will be studying the possibilities of rationally combining Imiquimod with CD47 monoclonal antibody blockades for human colorectal adenocarcinoma cancer therapy. As seen with previous clinical trials, adjuvant therapy is incredibly important in cancer treatment prognosis, and that is why it is increasingly important to study what ways targeted cancer antigen

specific regimens can be more refined. In addition to improving the efficacy of monoclonal antibody treatment, some methodologies for identifying phagocytosis rates are not adequate enough. This thesis will also address a high throughput alternative to GFP signalling for yielding better results.

MATERIALS AND METHODS

We looked for the efficacy of the drug Imiquimod in treating Colorectal Adenocarcinoma with human cell lines *DLD1* and *SW480.* Monoclonal antibodies B6H12 were used for the anti-CD47 blockades. Cells were harvested through trypsinization and washed using PBS. Cell lines were maintained at an incubation temperature of 37℃ and 5% CO_2. Serial dilutions were performed 10 times from $2.3x10^6$ to have a 100 cell count in 6 mL medium (3 mL conditional medium and 3 mL fresh DMEM medium).

Materials Preparation of Macrophages

Macrophages were obtained through extracting bones from mice models. Bone marrow was collected through centrifugation and 5 mL of ACK buffer was used to wash the pellet of tissue cells. Solution was combined with FACs buffer and strained through a 70 μm cell strainer and centrifuged at 8000 rpm. The cells isolated from the bone marrow were plated on a six well plate with 2.5 mL IMDM medium mixed with 20μL dethawed MCSF (macrophage colony stimulating factor). Plates were incubated for 5 days at 37℃ and at 5% CO_2. Additional 5μL MCSF was added on day four of incubation. Additional days of incubation were added if the differentiation of cells to macrophages was not enough.

Improving Methodology for Identifying Phagocytosis Rates

CMV-GFP-T2A-Luciferase Lentivector was used to infect cancer cell lines *SW480* and *DLD1*. GFP-positive cells were isolated using flow cytometry 48 hours after initial infection.

Cells were read for GFP and luminescence signals with a BioTek Gen3 microplate reader and cell number was counted manually.

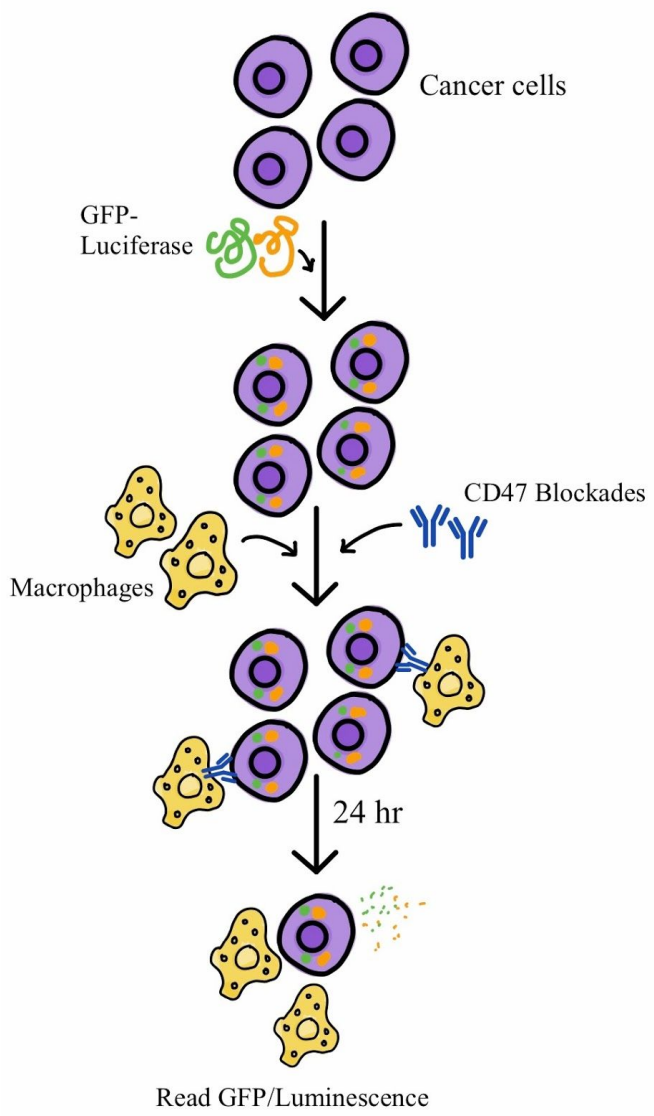

Figure 1. Scheme of fluorescent versus luminescence tagging with the addition of macrophages, CD47 blockades (B6H12) over the course of 24 hours.

Quantifying Phagocytosis Rates in Cell Lines

Visual analysis of a phagocytosis assay was done as a quick check for luminescence quality. Then, data from luciferin tagging with the addition of macrophages and B6 antibodies were compared to flow cytometry results through data analysis. A luciferase lentivector was added to colorectal adenocarcinoma cancer cell lines (*DLD1* and *SW480*). Macrophages (0.06 x 10^6 cells/well) and CD47 (B6H12) monoclonal antibody blockades at different concentrations (0, 0.01, 0.1, 1, 0, 0 μg/ml) were also added for an infection period of 24 hours to a 24 well plate for the visual assay. With 5 columns included the addition of macrophages and one control column with just cancer cells. This was then run through a flow cytometry system and the data was compared to luminescence assay reading on a BioTek Gen3 microplate reader.

Screening of FDA Approved Drugs for Highest Phagocytosis Rate Change

147 FDA approved anti-cancer small molecule drugs were screened and ranked. On day one, 5 μm of the drug compound with macrophage (0.06 x 10^6 cells/well) and 0.6 M cancer cells with 1 mL mannose DMSO medium were added to 96 well plates. Each compound had two wells each for two conditions. One condition was with the addition of B6H12 (anti-CD47) antibody (0.02 μg/mg) and the other was a control without the antibody (Figure 2). Four 96-well plates were left to incubate for 24 hours at 37°C and 5% CO_2. They were then read through the BioTek Gen3 microplate reader to analyse for final luminescence.

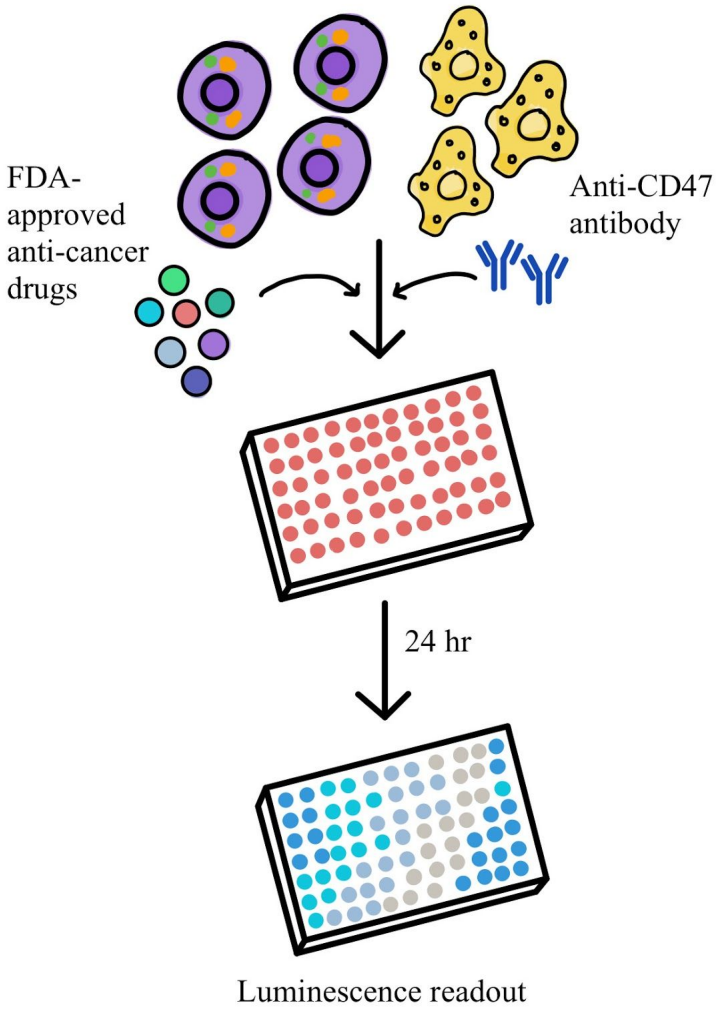

Figure 2. Scheme detailing the process for screening of 147 FDA-approved drugs with anti-CD47 antibodies, macrophages, and *DLD1* colorectal cancer cell lines.

RESULTS

GFP signalling is typically used as a way to quantify cell numbers but in prior experiments, GFP signalling was found to emit a large amount of background noise which causes inaccuracies with reading live cancer cell signals. To find out if an alternative solution would improve the methodology for identifying phagocytosis rates, cancer cells were combined with luciferin, which is a luminescence signal, and GFP to see which signals were clearer and stronger. We wanted to find a high throughput compatible long-term phagocytosis assay.

To test for this, we infected *DLD1* and *SW480* cancer cells with a lentivector containing both GFP and Luciferin. After 24 hours, the cells were sorted using flow cytometry and then manually counted. The results for after the GFP and Luciferin infected cells were separated show a difference in accuracy for signalling on cancer cell phagocytosis. In the graphs shown in Figure 3, the two large figures depict fluorescence and luminescence intensity separately and show a similar data set with similar trendlines. However, when zoomed in, a more significant difference is seen. The magnified portion is a small sampling size of approximately 700 cell count versus the 240 x 10^3 cell count. The data for GFP signalling at a small scale looks vastly different from its larger counterpart and shows a large amount of variation when there are less cells being evaluated. This is in comparison to the luciferase data which looks visually identical with less variation at large and small scale sample sizes. There is more validity in using a luminescence signal to quantify long-term phagocytosis based on the fact that luminescence signalling yields less variation and more accuracy when testing for small cell count size. It would be acceptable for GFP to be used as the method in which we test for phagocytosis in a large number of cancer

cells, but there is a significant loss of accuracy when presented with a small sample of cancer cells. Luminescence intensity is also measured at a higher quantity than in fluorescence. As shown in the graphs, luminescence intensity exceeds 35,000 which is around the maximum for the fluorescence intensity.

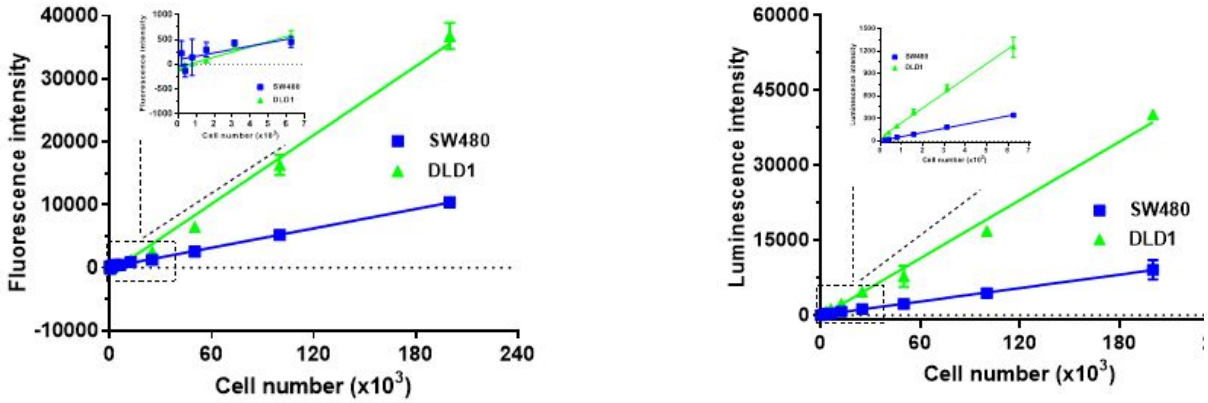

Figure 3. Fluorescence-GFP (right) and Luminescence-Luciferin (left) intensity assay on CMV-GFP-T2A-Luciferase Lentivector infected cancer cell lines *SW480* (blue line) and *DLD1* (green line). Small graphs within the larger graphs indicate a smaller cell sampling size of 700 cells.

Following this, we wanted to visually establish the phagocytosis assay and make sure that luminescence would yield strong results when macrophages, anti-CD47 antibodies and cancer cells were all combined. Different concentrations of B6H12 (anti-CD47 antibody) were added to each lane (0, 0.01, 0.1, 1, 0, 0 µg/ml) for two different cancer cell lines, *DLD1* and *SW480.* Five

of the well columns had both macrophage and cancer cells mixed together, and one with just cancer cells as a control. In Figure 4, there is a clear reduction of cancer cells when you include B6H12 in the wells which indicates the phagocytosis process with monoclonal antibodies is dose dependent. This can be seen by the stark difference between the dark blue and the bright colors in the cancer only control lane. The luminescence signal is strong in this phagocytosis assay, indicating that luminescence is a viable method and option to use in the rest of the experiment.

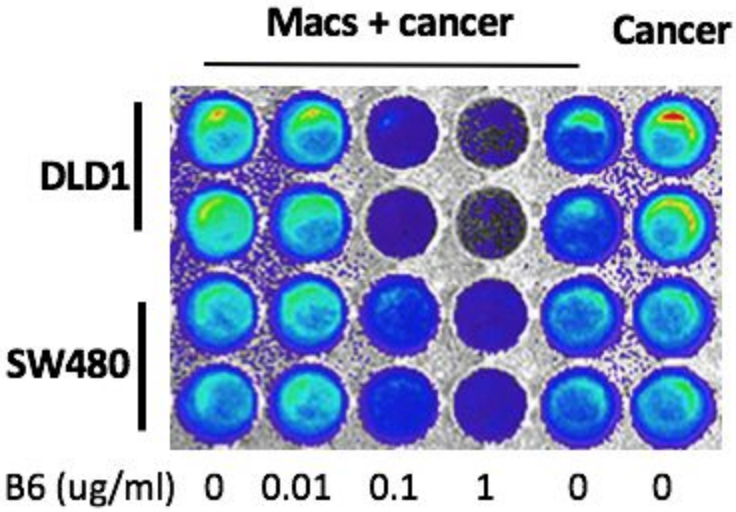

Figure 4. Phagocytosis assay check for luminescence validity. Macrophages (0.06 x 10^6 cells/well) and 0.6 M cancer cells were added to well columns 1-5. In column 6, control with no macrophage and only cancer cells tested. CD47 (B6H12) monoclonal antibody blockades at differing concentrations (0, 0.01, 0.1, 0 and 0 μg/mL) were also added for an infection period of 24 hours. Green and light blue luminescence indicated live cancer cell readings after phagocytosis, and dark blue indicates the cancer cells were phagocytosed within the time period. Column 6 with just cancer provides a reference for no phagocytosis over a 24 hour period.

To further assess the validity of the luminescence assay, the results were compared to an established methodology for phagocytosis assays. The luminescence assay data was compared to traditional flow cytometry (FACS) data. The cells were prepared in the same way and only the reading method was changed. Luminescence assay was found to return similar results to the traditional flow cytometry results. The phagocytosis indexes were compared in Figure 5 and the data points matched up with the y=x axis depicting a direct linear relationship and all data points fell between the error lines (light and dark purple lines). The error margins were ± 10%, which is a relatively small error margin with no stark outliers seen. This emphasizes the feasibility of this method as a long term phagocytosis assay because it is comparable to older methods.

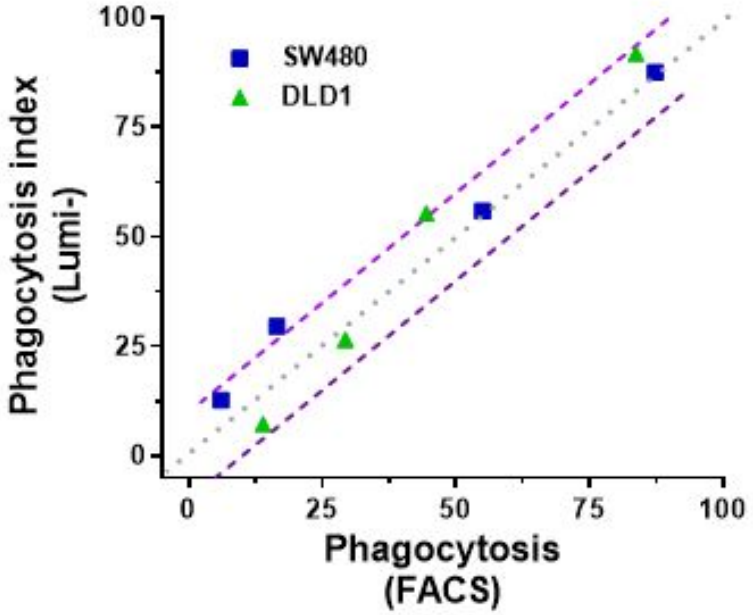

Figure 5. Comparison of flow cytometry data for phagocytosis in comparison to luminescence phagocytosis reading in cell lines (*SW480 and DLD1*) combined with Anti-CD47 antibodies (*B6H12*) and bone marrow derived macrophages.

The final step of the experiment was to test 147 different FDA approved anticancer small molecule drugs to find a potential adjuvant drug to monoclonal antibody immunotherapy. Using *DLD1* as our main cell line, we found that in screening all the FDA-approved drugs, imiquimod had the highest efficacy when plated with the monoclonal antibodies and the macrophages. Only the *DLD1* cancer cell line was used for this experiment because screening of 147 drugs was time consuming and expensive. Imiquimod had the highest phagocytic index change at 60% of all the drugs that were tested. Each green dot on the graph represents each different drug that was tested (Figure 6). The phagocytic index change was calculated by taking the number of phagocytosed cells and dividing them by a normalized value. The normalized value was found by calculating the amount of cells in the control well as there is no phagocytosis taking place in those. An example calculation is as follows: for one compound, the normalized value with no antibody is 100 and in the well with the compound and antibody, the value was 10 which yielded a 90% phagocytosis rate. This was then compared to a set of data in which mannose DMSO was used instead of the drug compound, and the phagocytosis rate for that was at a fixed 50%. The difference is then taken from those two values to get the phagocytosis index change amount, which in this example would be 40%. This is how we found a 60% index change for Imiquimod, indicating the efficiency of the drug in aiding in phagocytosis. Out of the 147 different drugs, half of them were found to have negative phagocytosis index change values as indicated by the green dots below the 0 line on the y-axis. This is because they were most likely helping with blocking phagocytosis. An example of why a compound would yield a negative phagocytosis index would be if the compound's phagocytosis rate is 10% and the difference between that and

the fixed 50% mannose DMSO value is -40%. Therefore, rather than enhancing phagocytosis, the compound induces anti-phagocytic effects instead.

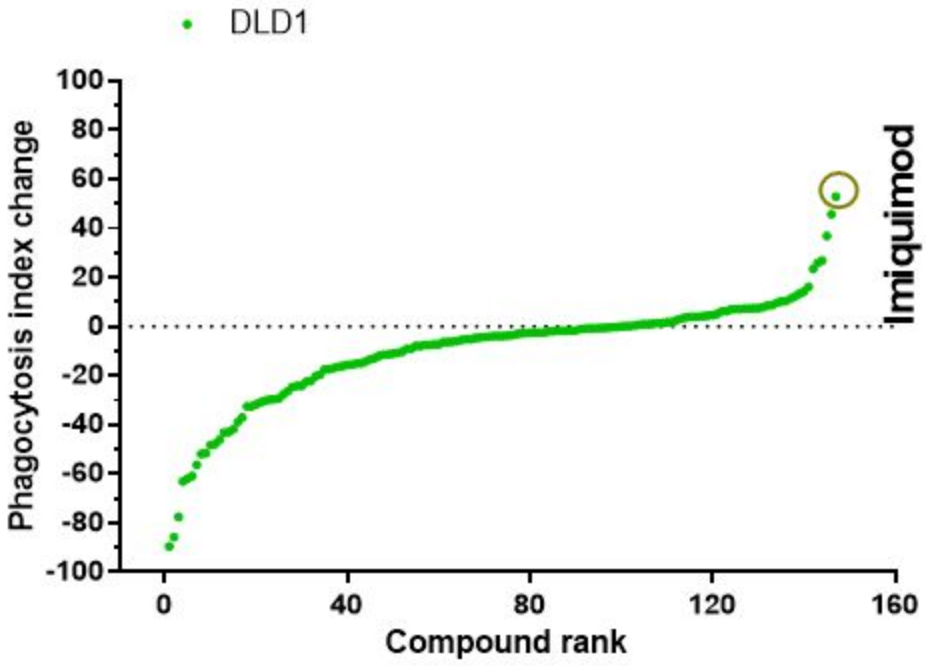

Figure 6. Testing of 147 FDA-approved anti-cancer small molecule drug compounds ranked for maximum efficacy in relation to phagocytosis index change. Drugs were only tested with the *DLD1* human colorectal adenocarcinoma cell line. Below the zero index change line (y-axis) are drug compounds that likely induce anti-phagocytic effects.

DISCUSSION AND CONCLUSION

The increase in avenues of monoclonal antibody immunotherapy creates more possibilities for non-toxic treatment for cancer. The targeting of CD47 in the field of immunotherapy has increased over the past years and continues to stay that way because of the progress seen in the research. Identifying a drug with the potential to aid in anti-CD47 blockade functions without inducing additional toxic effects has become essential for the efficacy of the clinical treatments for specific cancers. Using monoclonal antibody immunotherapy exclusively is not adequate enough for the eradication of a fast developing and unpredictable disease.

It was discussed in the earlier sections of this thesis that there are currently two major limitations regarding research on utilizing CD47 blockades as agents of immunotherapy that we wanted to focus on. The first major limitation was that the effects of targeted CD47 immunotherapy on solid tumor cases had not been proven as a success as of yet because only liquid type cancers (B cell non-Hodgkin's Lymphoma) had been tested extensively. In this experiment, we found that in addition to the combination of anti-CD47 antibodies (B6H12) and anti-SIRPα antibodies (P84), imiquimod as an adjuvant drug treatment can increase the efficacy of this cancer therapy based on it's phagocytic index change amount. Based on the results of our 147 FDA-approved anticancer small molecule drug screen, the difference between when imiquimod was added and when mannose DMSO was added showed a 60% increase in phagocytosis rates. This was the highest amount of all the drugs screened. We found this by specifically testing in human colorectal adenocarcinoma cancer cell lines *DLD1* as they are representative of solid tumor cancers.

The second limitation was that GFP signalling provided far too disruptive results because of the background noise and light emitted in the screening. In this experiment we were able to find an alternative solution to that through the usage of luciferin as a tagging signal and found that it drastically improved our outcomes. We found that GFP signalling was accurate at large scale cell counts but when we tested for cell numbers in the hundreds, the signals were disruptive and highly variable. For Luciferase signalling, the results were similar and not variable at both small and large scale cell counts. We verified our findings through both a visual screening of the efficacy when plated with anti-CD47 antibodies and through traditional FACS sorting. Our proposed method of using Luciferase proved to be more efficient than GFP signalling in phagocytosis assay experiments. Using this luminescence approach would provide more accurate results with less background noise or light signals when working with small sample sizes.

A large quantity of drugs were tested in the search for the best adjuvant treatment for monoclonal antibody immunotherapy and imiquimod came out as a top contender in *DLD1* cell lines. Now that we have sufficient data for the ranking of the compounds, we no longer need to test for all 147 drugs in solid tumor cell lines. We can immediately eliminate those that elicited a negative phagocytosis index range number and focus on either the top five found in the experiment, or any of the drugs found above the line. This way, we can do further testing on other cell lines like *SW480* or other solid tumor cell lines to identify whether or not it is entirely accurate or if those specific drugs work on just human colorectal adenocarcinoma cancer cell lines.

Potential weaknesses of this study was that we were only able to test these drugs in *DLD1* cancer cell lines. It is more than possible that imiquimod is only feasible and effective in this cell

line based on its mechanisms. However, this is not something that we can assure until more testing is done on other cell lines and models. Another potential weakness for where this study could go is based more on previous findings and is a potential weakness for anti-CD47 therapy in general. There are concerns that xenograft hosts can underestimate the effects of anti-CD47 therapy because of specific reagents in mice models that affect CD47 clustering and also the interaction of CD47-SIRPα (Huang, 2017). We may not be able to truly test the efficiency of imiquimod as an adjuvant drug until a more certain xenograft model is created. We have no idea of how imiquimod would work in vivo if the CD47 interactions in a different species could be variably different from human processes.

However, because of the success of Chao's findings with *Rituximab* and anti-CD20 antibodies, hopefully this study can further the studies into solid tumor treatments in the future. The clinical trials for B cell non-Hodgkin's lymphoma have been successful in its goals and these findings could potentially open up more avenues to increase the efficacy of treatment in both liquid and solid tumor cancers.

There are however concerns on the direction in which monoclonal antibody therapy will take in the future. It is important to clarify that there is much more research needed to be done in order for this finding to be significant. For example, because xenograft mice models are different in composition from humans, much more additional research will have to be done on how to precisely determine validity in in-vivo testing. Another concern would be, since CD47 is present in all normal cells, would monoclonal antibody treatment affect the normal cells in the body? Imiquimod would most certainly help guide macrophages in targeting cancer cells over normal cells, but it would not be surprising if normal cells that surround the tumor microenvironment

would be affected as well, maybe also eliciting an unwanted immune response. It is important that more research goes into specific things like that, and figuring out methods in which only the tumor is targeted in therapy. The point of immunotherapy is to reduce toxic effects to the rest of the body. In order for monoclonal antibody therapy to be the better option or the more effective option, these safety concerns must be addressed.

In conclusion, we were able to improve the methodology of conducting phagocytosis assays by comparing GFP fluorescent signalling to our proposed alternative which is luminescence signalling with luciferase. We were able to verify our luciferase assay for phagocytosis rate accuracy using flow cytometry and a visual check, and finally we screened FDA approved drugs for highest phagocytosis rate change and found that Imiquimod would yield the highest efficacy. The identification of imiquimod as a potential combination for anti-CD47 antibodies in cancer therapy provides a stepping stone for future studies on creating a treatment modality for cancer that is less toxic and life quality reducing. Through searching for more potential adjuvant drug combinations as well as more immunological targets, improvements to life quality and disease prognosis can increase positively.

ACKNOWLEDGEMENTS

First, I would like to thank my team at the City of Hope Beckman Research Institute for their continued support and kindness during my year long research process. To Dr. Mingye Feng, I am grateful to have had the opportunity to work in his lab. Throughout my undergraduate career, I have always been interested in the intersection of immunology and oncology and to be able to conduct research on macrophage-mediated immune surveillance was incredible. I would also like to thank Dr. Xu Cao for his immense support in guiding me through this research process and helping me understand the significance of the findings. I would also like to thank Bolei Li for taking time out of his busy schedule to train me in cell biology techniques and procedures, I learned a lot during this short period of time.

Next, I would like to thank Professor Emily Wiley for her support in navigating an off-campus thesis. The feedback and the advice was incredibly valuable to me. I would also like to thank Professor Mary Hatcher-Skeers for her guidance and input through my undergraduate experience. I am incredibly lucky to have had her as both a professor and my advisor.

Thank you to all my family and friends who pushed me to become the student that I am today. I have gained incredible insight and knowledge throughout these years and I couldn't have done it without the encouragement. Finally, thank you to my mom for her unwavering support throughout my college career.

WORKS CITED

Aderem et al. (1999). Mechanisms of Phagocytosis in Macrophages. *Annual Review of Immunology*, *17*, 593–623.

Chao, M. P., Alizadeh, A. A., Tang, C., Myklebust, J. H., Varghese, B., Gill, S., Jan, M., Cha, A. C., Chan, C. K., Tan, B. T., Park, C. Y., Zhao, F., Kohrt, H. E., Malumbres, R., Briones, J., Gascoyne, R. D., Lossos, I. S., Levy, R., Weissman, I. L., & Majeti, R. (2010). Anti-CD47 Antibody Synergizes with Rituximab to Promote Phagocytosis and Eradicate Non-Hodgkin Lymphoma. *Cell*, *142*(5), 699–713. https://doi.org/10.1016/j.cell.2010.07.044

Farkoma, S., Diamandis, E. P., & Blasutig, I. M. (2016).Cancer immunotherapy: The beginning of the end of cancer? | *BMC Medicine* | *Full Text*. (n.d.). Retrieved April 11, 2020. https://bmcmedicine.biomedcentral.com/articles/10.1186/s12916-016-0623-5

Feng, M., Jiang, W., Kim, B. Y. S., Zhang, C. C., Fu, Y.-X., & Weissman, I. L. (2019). Phagocytosis checkpoints as new targets for cancer immunotherapy. *Nature Reviews Cancer*, *19*(10), 568–586. https://doi.org/10.1038/s41568-019-0183-z

Huang, Y., Ma, Y., Gao, P., & Yao, Z. (2017). Targeting CD47: The achievements and concerns of current studies on cancer immunotherapy. *Journal of Thoracic Disease*, *9*(2), E168–E174. https://doi.org/10.21037/jtd.2017.02.30

Kong, F., Gao, F., Li, H., Liu, H., Zhang, Y., Zheng, R., … Jia, Y. (2016). CD47: A potential immunotherapy target for eliminating cancer cells. *Clinical and Translational Oncology*, *18*(11), 1051–1055. https://doi.org/10.1007/s12094-016-1489-x

Lian, S., Xie, X., Lu, Y., & Jia, L. (2019). Checkpoint CD47 Function On Tumor Metastasis And Immune Therapy. *OncoTargets and Therapy*, *12*, 9105–9114. https://doi.org/10.2147/OTT.S220196

Rotte, A. (2019). Combination of CTLA-4 and PD-1 blockers for treatment of cancer. *J Exp Clin Cancer*. Res 38, 255. https://doi.org/10.1186/s13046-019-1259-z

Russ, A., Hua, A. B., Montfort, W. R., Rahman, B., Riaz, I. B., Khalid, M. U., … Anwer, F. (2018). Blocking 'don't eat me' signal of CD47-SIRPα in hematological malignancies, an in-depth review. *Blood Reviews*, *32*(6), 480–489. https://doi.org/10.1016/j.blre.2018.04.005

Smale, S. T. (2010). Luciferase assay. Cold Spring Harbor Protocols, 2010 (5), pdb.prot5421. https://doi.org/10.1101/pdb.prot5421

Stanley, M. A. (2002). Imiquimod and the imidazoquinolines: Mechanism of action and therapeutic potential. *Clinical and Experimental Dermatology*, *27*(7), 571–577. https://doi.org/10.1046/j.1365-2230.2002.01151.x

Takimoto, C. H., Chao, M. P., Gibbs, C., McCamish, M. A., Liu, J., Chen, J. Y., … Weissman, I. L. (2019). The Macrophage 'Do not eat me' signal, CD47, is a clinically validated cancer immunotherapy target. *Annals of Oncology*, *30*(3), 486–489. https://doi.org/10.1093/annonc/mdz006