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Local Delivery of CTLA-4 Blockade Inhibits Growth of Pancreatic Tumors

Jack Baltz
University of Arkansas, Fayetteville

Local Delivery of CTLA-4 Blockade Inhibits Growth of Pancreatic Tumors

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by

Jack Baltz

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University of Arkansas

Dr. David Zaharoff
Thesis Director

HANNA JENSEN
Committee Member

KARTIK BALACHANDRAN
Committee Member

Committee Member

Committee Member

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Abstract

Immune checkpoint blockade has demonstrated great potential in activating antitumor immunity. Ipilimumab is a monoclonal antibody which targets cytotoxic T-lymphocyte antigen-4. CTLA-4 belongs to the CD28 class of receptors and is found on the surface of $CD4^+$ and $CD8^+$ T cells. CTLA-4 acts to suppress the immune system when bound to CD80 and CD86 receptors on antigen presenting cells. Ipilimumab, or anti-CTLA-4, has shown to be effective in significantly extending the survival of patients with metastatic melanoma. However, systemic delivery of Ipilimumab also induces significant side effects such as: colitis, dermatitis, uveitis, and hypophysitis. In order to minimize toxicity, we and others have hypothesized that intratumoral administration of anti-CTLA-4 at a lower dose can have the same antitumor efficacy as systemic delivery but without the toxicity. This work begins with an investigational pilot study to determine the efficacy of anti-CTLA-4 by delivering 60 μg of anti-CTLA-4 to a group of mice and measuring the tumor growths when compared to an untreated control group. Once efficacy had been demonstrated, a dosing study was conducted to identify an optimal intratumoral dosage delivered to murine models. The groups were given doses of either 30 μg, 60 μg, or 120 μg. From this study, the 60 μg group had the lowest average tumor size of 300 mm^3. Our lab has previously demonstrated that IL-12 co-formulated with chitosan has demonstrated prolonged intratumoral retention therefore, 60 μg of anti-CTLA-4 was co-formulated with a chitosan solution to investigate the efficacy in a delivery vehicle. Finally, 60 μg of anti-CTLA-4 was delivered in a proprietary hydrogel alone and with Interleukin-12 to examine the effects of controlled release.

1. Introduction

A mutation in the sequence of DNA in a gene can lead a cell to become cancerous. Cancer can be quickly summarized as uncontrolled cell division. This cell division that occurs in a cancerous cell will lead to the development of a solid tumor within the body. Definitively curing this disease has proved troublesome as scientists have attempted surgery, radiation, and chemotherapy as the main treatment routes for patients. Currently, cancer trails only cardiovascular disease as the largest cause of death among individuals in the world[1]. Pancreatic cancer has a mortality rate near 85%. In addition to being characterized as one of the most aggressive cancers, about 44,000 new cases of pancreatic cancer are diagnosed every year. Pancreatic cancer has a 5 year survival rate of less than 5% while the median time of patient survival after diagnosis barely reaches six months. Pancreatic cancer is highly metastatic and resistant to drug treatments which helps to explain the poor survival rate[2,3].

Recently, a new method of cancer treatment has emerged in the form of immune checkpoint inhibitors. This treatment utilizes the machinery found in the immune system to attack and destroy cancer cells. Anti-CTLA-4 is a monoclonal antibody which targets cytotoxic T-lymphocyte antigen-4. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is an immunoglobulin protein found on the surface of $CD4^+$ and $CD8^+$ T cells and is part of the CD28 class of protein receptors. CTLA-4 delivers an inhibitory signal to T cells when bound to CD80 and CD86 expressed on antigen presenting cells. Anti-CTLA-4, or Ipilimumab, is an immune checkpoint inhibitor currently approved to treat metastatic melanoma. By blocking this interaction, anti-CTLA-4 essentially eliminates the ability of CTLA-4 to send inhibitory signals to T cells and thus activates T cell function[4,5]. Yet, anti-CTLA-4 therapy has shown to induce severe autoimmune effects in patients such as colitis, dermatitis, uveitis, and hypophysitis making

effective delivery of dosage and treatment difficult in clinical trials[4,5]. In clinical trials for Ipilimumab, Bristol Myers Squibb released a Phase III trial that showed improved life expectancy of over six hundred patients diagnosed with metastatic melanoma. Ipilimumab was given at 3 mg/kg early on during investigation only to show little efficacy, however, reevaluation at 10mg/kg of Ipilimumab systemic delivery with immune factors showed an increased survival by over 2 months, a significant increase[5]. Sandin *et al.* locally delivered anti-CTLA-4 to pancreatic tumors in murine models and have demonstrated that lower dose peritumoral injections of the antibody performed similar to that of the standard method of treatment of anti-CTLA-4 by higher dose systemic delivery[5]. In a paper published by Fransen *et al*., a slow release delivery system of Montanide ISA-51 was delivered with anti-CTLA-4 subcutaneously in murine models to treat MC-38 tumor cells. The results of this experiment show that the slow release Montanide solution with anti-CTLA-4 was effective in activating antitumor activity while minimizing autoimmune side effects[6]. Both of these studies are unable to effectively eliminate the respective tumors completely[5,6].

In this study, we hypothesize that direct intratumoral injections of anti-CTLA-4 will prove more effective in restricting tumor growth. Additionally, we believe that delivering this antibody intratumorally in a retentive vehicle will allow for a prolonged presence at the tumor site and will enhance the antitumor response. We have also delivered anti-CTLA-4 in combination with interluekin-12 (IL-12) in a hydrogel intratumorally. IL-12 is a pro-inflammatory cytokine produced by dendritic cells and macrophages. Our lab has demonstrated the efficacy of IL-12 delivered intratumorally in a chitosan hydrogel delivery vehicle. Our lab has also demonstrated tumor rejection in mice previously treated with IL-12 in chitosan solution[7]. Chitosan is a non-

toxic, biodegradable polysaccharide which can be derived from the exoskeletons of shellfish[7]. We believe that the combination therapy will inhibit the growth of pancreatic tumors while the IL-12 component will provide an additional level of immune activation.

The goal of this study is to determine the effectiveness of anti-CTLA-4 as a potential immunotherapy when injected intratumorally in concert with IL-12 in a slow release controlled hydrogel. The lower dose of intratumoral delivery not only will reduce autoimmune side effects, but also reduce cost for the patient. Ipilimumab treatment currently costs $120,000 for a four dose injection schedule[8]. By demonstrating that local delivery is just as effective in eliminating tumors, Ipilimumab treatment could be administered at lower doses and thus lower costs.

2. Materials and Methods

2.1 Cell Line Preparation and Maintenance

The Panc02 cell line was thawed from liquid nitrogen storage. Panc02 cells were then cultured in a T-25 culture flask with McCoy's 5A media supplemented with 10% fetal bovine serum and antibiotics at 37°C and 5% CO_2. Cells were passaged twice weekly or once 80%-90% confluency was reached in the flask. One week prior to beginning of animal studies, cell culture would be expanded into a T-75 culture flask to ensure sufficient cell numbers. Panc02 cells were counted using a 1:10 dilution of cell suspension and Trypan Blue. The mixture would then be placed onto a hemocytometer for cell counting. The four corner squares of the hemocytometer were used for cell counts. Final cell number calculations are as follows in Equation 1.

$$\frac{Cells}{mL} = \left(\frac{Cells\ counted}{4} * 10\right) * 10^4 \qquad \text{(Equation 1)}$$

Viable cells counted did not take up the Typan Blue stain and cells overlapping only the top and left boundaries of a square were included in the count. Panc02 cells were passaged at a dilution of 1:10 in a new T-25 culture flask.

2.2 Inoculation and Tumor Measurements

For tumor inoculation, all mice were anesthetized by administering a 50 μL intraperitoneal injection of 1:1:4.6 mixture of Ketamine, Xylazine, and deionized water, respectively. Mice in each study were given 2.5×10^5 Panc02 cells in 100 μL of PBS subcutaneously to the shaven right flank on day 0. After formation of palpable tumors, usually day 5 after implantation, treatment regime began. All intratumoral injections that were given were administered in a subcutaneous fashion, however, treatment was injected once syringe needle penetrated the tumor. In all experiments, two-week old female C57BL/6J mice were ordered from Jackson Laboratories for use. Tumor measurements and examination of health were made twice weekly.

Tumors were measured in two dimensions and the following equation was used when calculating volume:

$$V = \frac{1}{2} l_{short}^2 l_{long} \quad \text{(Equation 2)}$$

The previous equation does assume the tumors are of ellipsoid shape. Mice were sacrificed by cervical dislocation after tumor volumes eclipsed 1000 mm^3. Anti-CTLA-4 antibody (clone 9D9) was ordered from Bio X Cell.

2.3 Pilot Study

In the initial pilot study to investigate the antitumor activity of anti-CTLA-4, two groups of five mice were used. Treatments began on day 5 after implantation and repeated on days 8 and 11 and given twice weekly until the conclusion of the experiment. Control mice were given PBS in 50 μL. Antibody treatment group was administered at 30 μg of antibody in 50 μL of PBS solution.

2.4 Dosing Study

The dosing study was expanded to four treatment groups. Intratumoral treatments occurred on days 5, 8, and 11. The control group of 3 mice was given 50 μL of PBS. The next group was given 30 μg of anti-CTLA-4 in 50 μL of solution to four mice. Five mice participated in both the 60 μg and 120 μg groups.

2.5 Chitosan Co-Formulation

In the first co-formulation study, five mice per group were used. This study consisted of three groups of mice: A control, anti-CTLA-4 only, and anti-CTLA-4 co-formulated with a chitosan hydrogel. Treatments were administered on days 5, 12, and 19. Again, control group mice were administered intratumoral PBS injections. Anti-CTLA-4 only mice received a 60 μg treatment and finally anti-CTLA-4 co-formulated with chitosan received a 50 μL intratumoral injection of

a chitosan hydrogel and 60 μg of anti-CTLA-4. The chitosan hydrogel was prepared by dissolving purified lyophilized chitosan in PBS to 1% weight by weight. Anti-CTLA-4 was then added to appropriate concentration.

2.6 Combination Immunotherapy

In the final study, six groups of 5 mice were used to investigate the effects of combination immunotherapy of anti-CTLA-4 and IL-12. Again, a control group consisting of 5 mice were administered 50 μL of PBS intratumorally. Intraperitoneal injections of 60 μg of anti-CTLA-4 were given to the next group. An additional group new to any of the studies was a hydrogel only group. 50 μL of hydrogel was administered intratumorally to mice. Additionally, a group 60 μg of anti-CTLA-4/hydrogel and a group of 1 μg of IL-12/hydrogel were given intratumoral injections. Finally, a combination group of 60 μg of anti-CTLA-4, 1 μg of IL-12, and hydrogel were delivered intratumorally in combination. Treatments were given on days 7, 14, and 21 or once weekly due to the retention of the hydrogel. This was done to ensure accurate tumor measurements.

3. Results

3.1 Demonstration of Efficacy

Since anti-CTLA-4 is new to our lab, a pilot study was performed to demonstrate the efficacy of anti-CTLA-4 treatment. After conclusion of the study, growth curves (Figure 1A) and average tumor volumes (Figure 1B) were constructed between the two groups: anti-CTLA-4 treated and untreated.

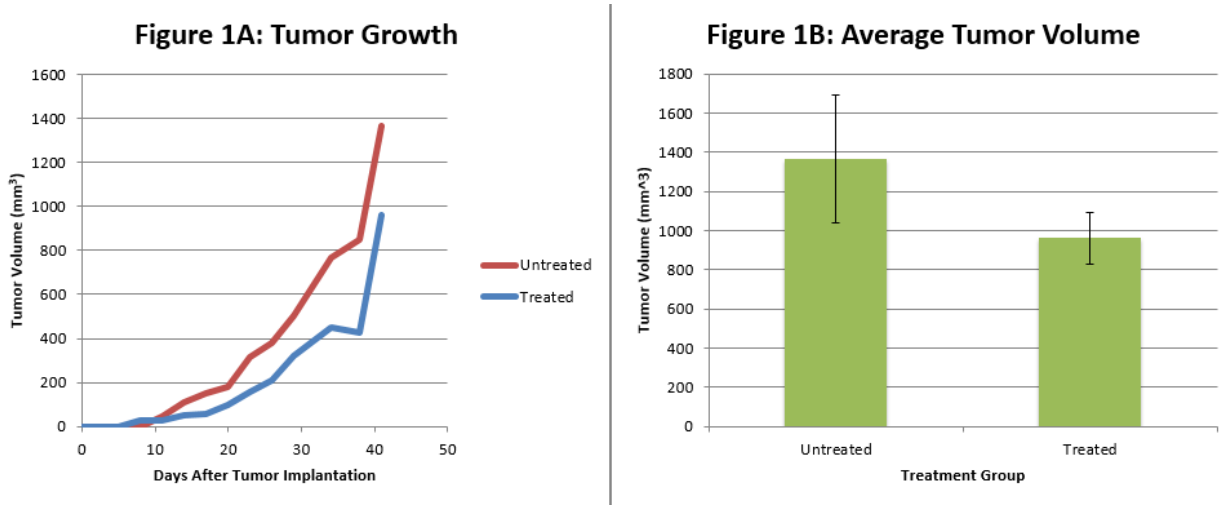

Figure 1: Tumor growths from initial pilot study of anti-CTLA-4 efficacy. n=4

Mice from the study were sacrificed on day 41 as tumor volumes had eclipsed 1000 mm^3 for the untreated group and the treated group approached that threshold. Additionally, anti-CTLA-4 treated mice had shown signs of minimal dermatitis. None of the mice exhibited any weakness or abnormal behavior. From Figure 1, the untreated group had an average tumor volume of 1364 mm^3 and the anti-CTLA-4 treated group showed reduced average tumor size of 960 mm^3. The standard deviations of the groups were: 412 mm^3 for the untreated group and 671 mm^3 for the anti-CTLA-4 treated group. Individual growth curves were also generated but are not shown here.

3.2 Anti-CTLA-4 Dosing Study

Following the pilot study, our lab was confident that anti-CTLA-4 had some antitumor efficacy. Because it was our first time using anti-CTLA-4, a dosing study was needed to determine the optimal dose. Previous studies that had delivered anti-CTLA-4 locally used doses ranging from 30 µg to 90 µg and used a dose of 200 µg of anti-CTLA-4 for systemic (intraperitoneal) injections. However, the study was not delivered directly into the tumor. Therefore, the dosing study consisted of four groups: a control (PBS), a 30 µg group, a 60 µg group, and a 120 µg group. Results from the dosing study are shown below in Figure 2.

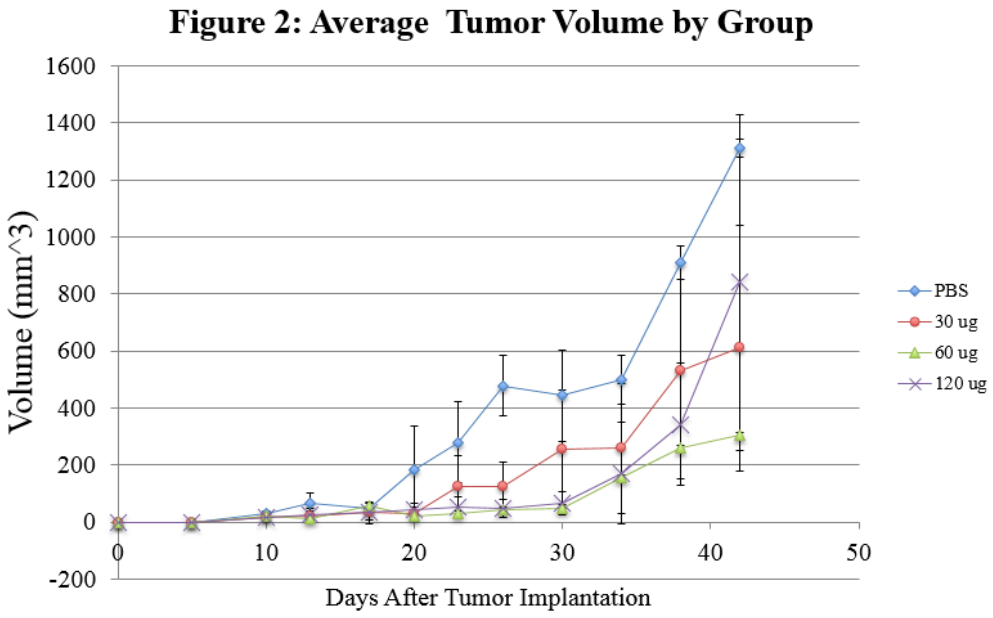

Figure 2: Growth curve of dosing study. Groups were given bi-weekly injections. n=3-5

All remaining mice were sacrificed on day 46 at the conclusion of the study. On day 17, a mouse in the PBS group was accidentally killed due to a bad injection resulting in an air embolism. The PBS group, as expected, had the highest average tumor volume at the conclusion of the study with an average size of 1311 mm^3. The 120 µg group had an average tumor size of 839 mm^3 followed by the 30 µg group at 609 mm^3. The 60 µg group yielded the smallest average tumor size at 304 mm^3, over half that size of the next closest group. Standard deviations

were 31.66 mm^3, 429 mm^3, 389 mm^3, and 587 mm^3 for the PBS, 30 µg, 60 µg, and 120 µg groups, respectively.

3.3 Co-Formulation of Anti-CTLA-4 with Chitosan

After determining that an optimal intratumoral dose of anti-CTLA-4 was 60 µg, formulation of the antibody with chitosan was investigated. Previous studies had used a Montanide emulsion as a delivery vehicle for anti-CTLA-4. However, this solution was delivered subcutaneously as opposed to intratumorally. Our lab has previously demonstrated that chitosan leads to increased retention at the tumor site[7]. The 60 µg dose was combined with a chitosan hydrogel and injected intratumorally. From the measurements the average and individual growth curves generated are shown in Figure 3 and 4.

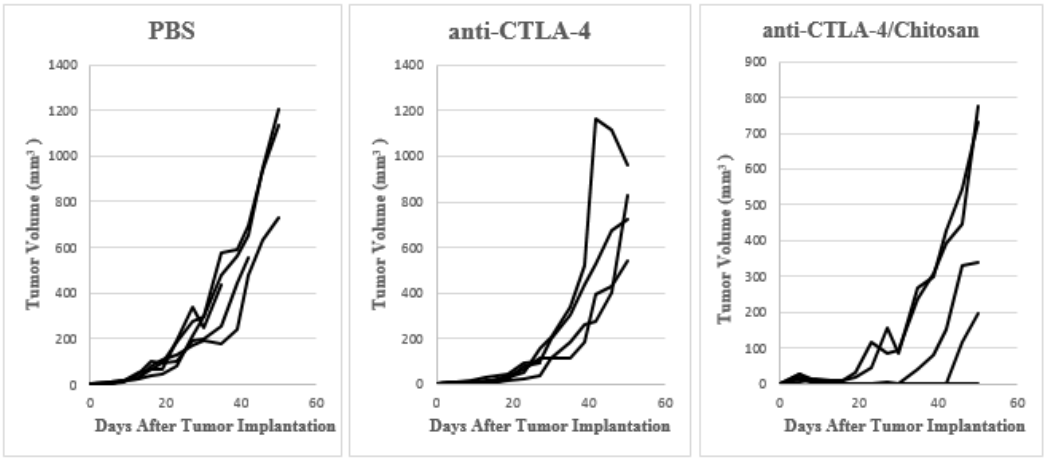

Figure 3: Individual growth curves of treatment groups. n=5

From these results, the anti-CTLA-4/chitosan treated group showed the smallest sized tumors. This group was the only to have at least one mouse eliminate its tumor. Earlier in the study at day 23, 60% of the anti-CTLA-4/chitosan group did not have any palpable tumor mass. Anti-

CTLA-4 treatment still shows to restrain tumor growth when compared to PBS treated mice. The average tumor volumes between groups were calculated and shown in Figure 4.

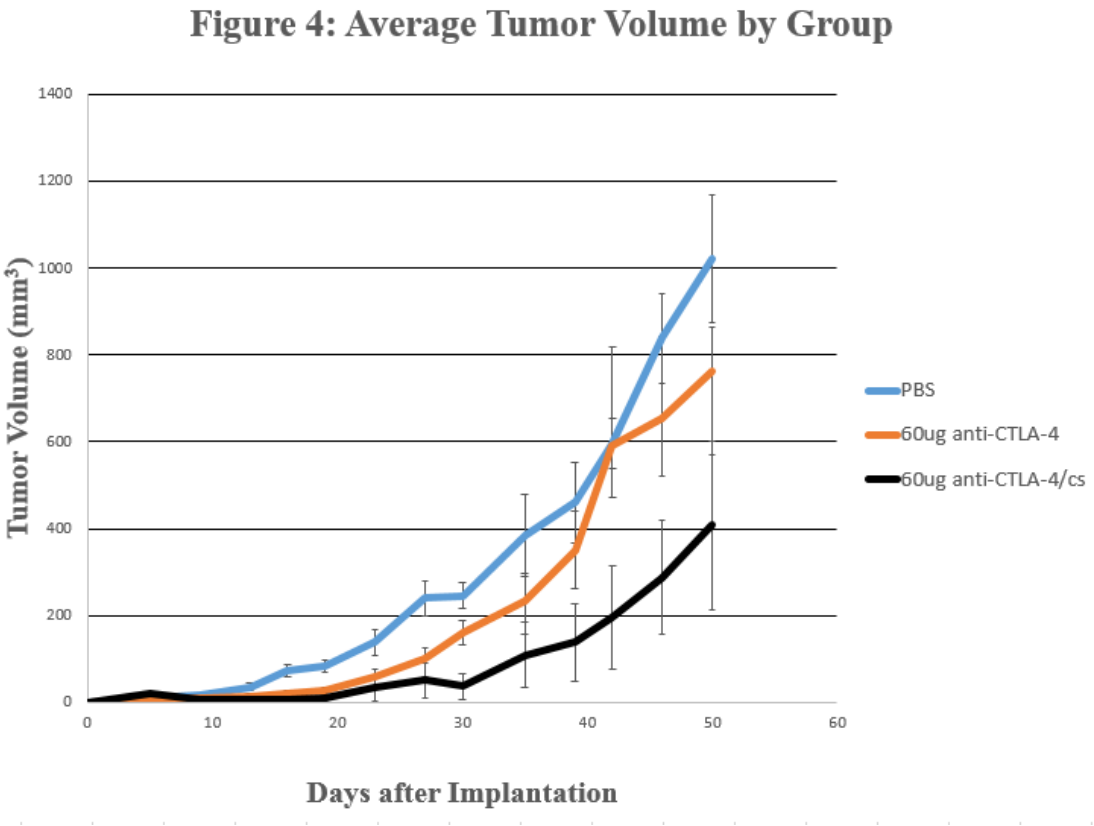

Figure 4: Average tumor volume by group. n=5

The average tumor size for the PBS mice once again eclipsed the 1000 mm^3 barrier with the final average tumor size of 1021 mm^3. The 60 µg anti-CTLA-4 only group had a final average tumor volume of 763 mm^3 while the 60 µg anti-CTLA-4/chitosan group had the lowest tumor sizes at an average of 407 mm^3. It is important to note that one mouse from the co-formulated group eliminated its tumor and two additional mice were lacking tumors until day 23 and day 42 of the 50 day study. The mice were examined periodically during the study and all mice seemed to be free of dermatitis and abnormal behavior. The co-formulation group did develop sores at the

tumor site after injections that eventually healed. Selected mice from each group are shown below in Figure 5.

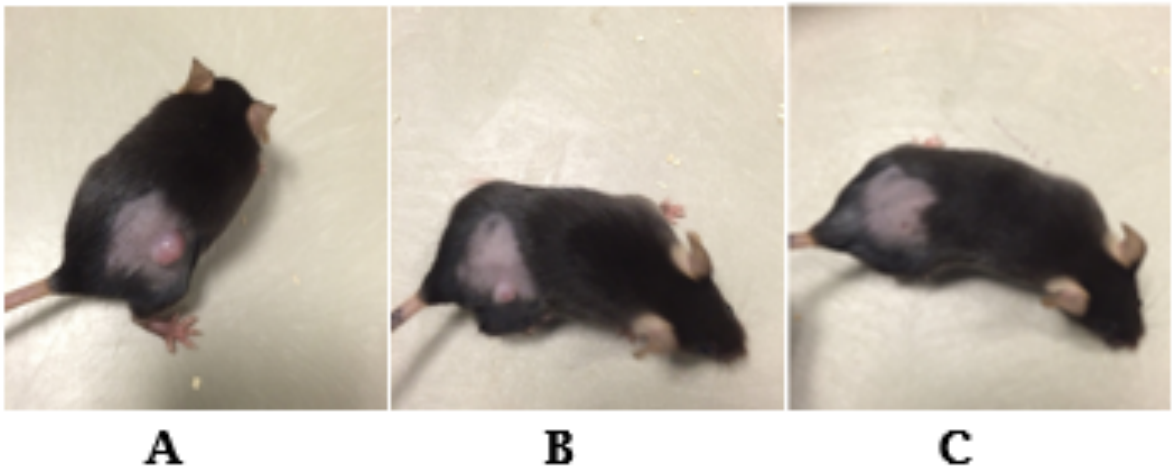

Figure 5: Selected mice from day 18. A: PBS Group B: Anti-CTLA-4 C: Anti-CTLA-4/Chitosan

From Figure 5c the sores that were mentioned previously can be faintly seen as a scab. This severity of sore was representative of the group, however, bleeding was recorded in one of the mice. Overall, the results from this study supported initial hypothesis.

3.4 Combination Therapy of Anti-CTLA-4 and IL-12 in a Novel Hydrogel

From the conclusion of the previous study, further investigation into hydrogel delivery was needed. Our lab has developed a novel controlled release hydrogel. We wanted to utilize this hydrogel to deliver anti-CTLA-4. Additionally, we wanted to initiate a combination study including IL-12 and anti-CTLA-4 delivered in the gel. In Figure 6, growth curves are shown. This study was actually repeated as the first attempt was done with mice that already had severe dermatitis. Initially, we proceeded to run the experiment in hopes that the mice would recover from their skin condition. Over the course of the experiment the mice were monitored extensively and never regained normal skin health. By day 14, the mice had scabbing from dry,

irritated skin and the control mice did not grow tumors at the same rate as we had seen before. Therefore, we decided to improve on the experiment by performing it again with healthier mice and additional groups so as to measure the effects of the gel alone.

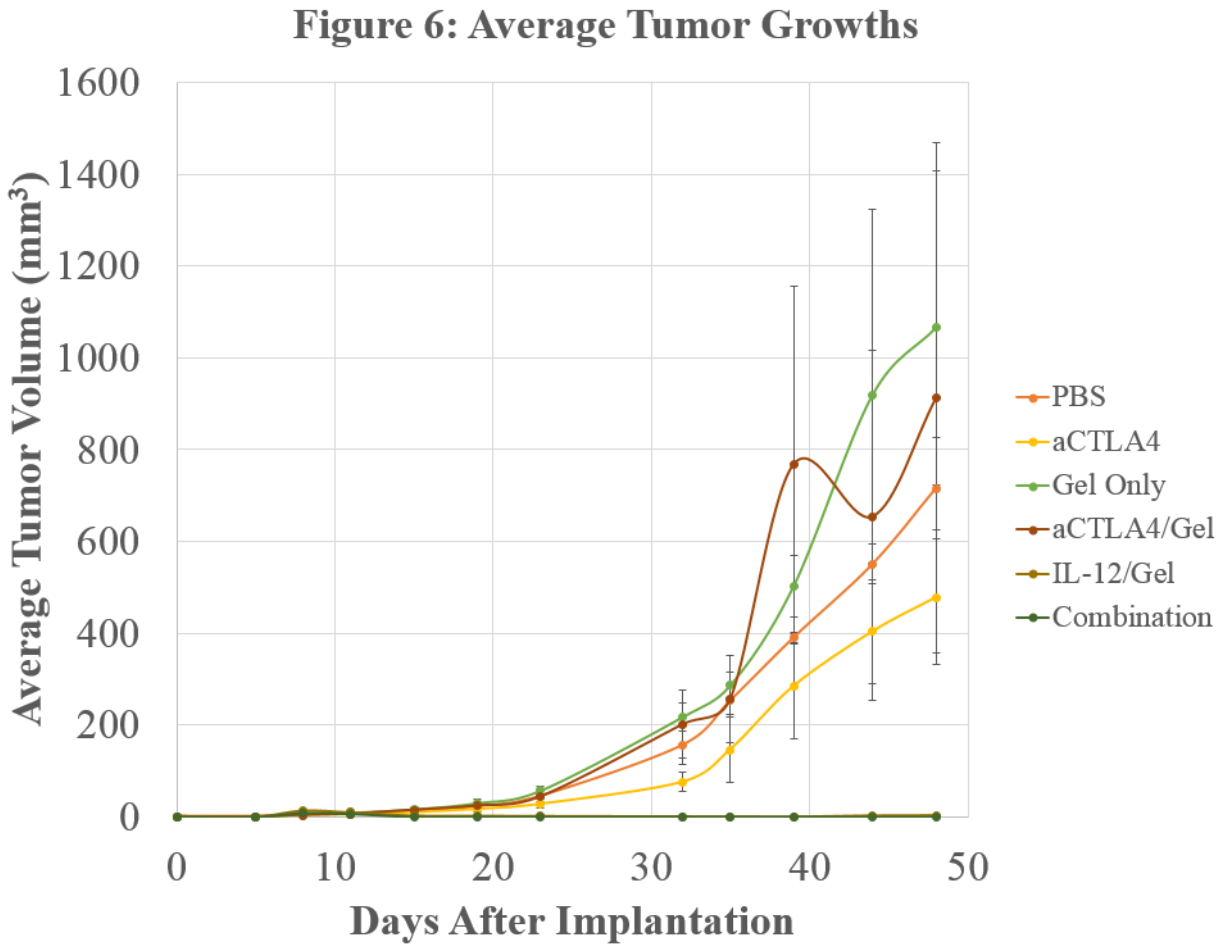

Figure 6: Growth curves from the Combination Immunotherapy study. n=5

The most notable result of this study is that after 44 days, the combination group had no detectable tumor mass. In addition, the IL-12/Gel group performed similarly but had one mouse develop and sustain a very small tumor. Average tumor volumes for the PBS, intraperitoneal anti-CTLA-4, Gel only, anti-CTLA-4/Gel were 715 mm^3, 477 mm^3, 1065 mm^3, and 911 mm^3, respectively.

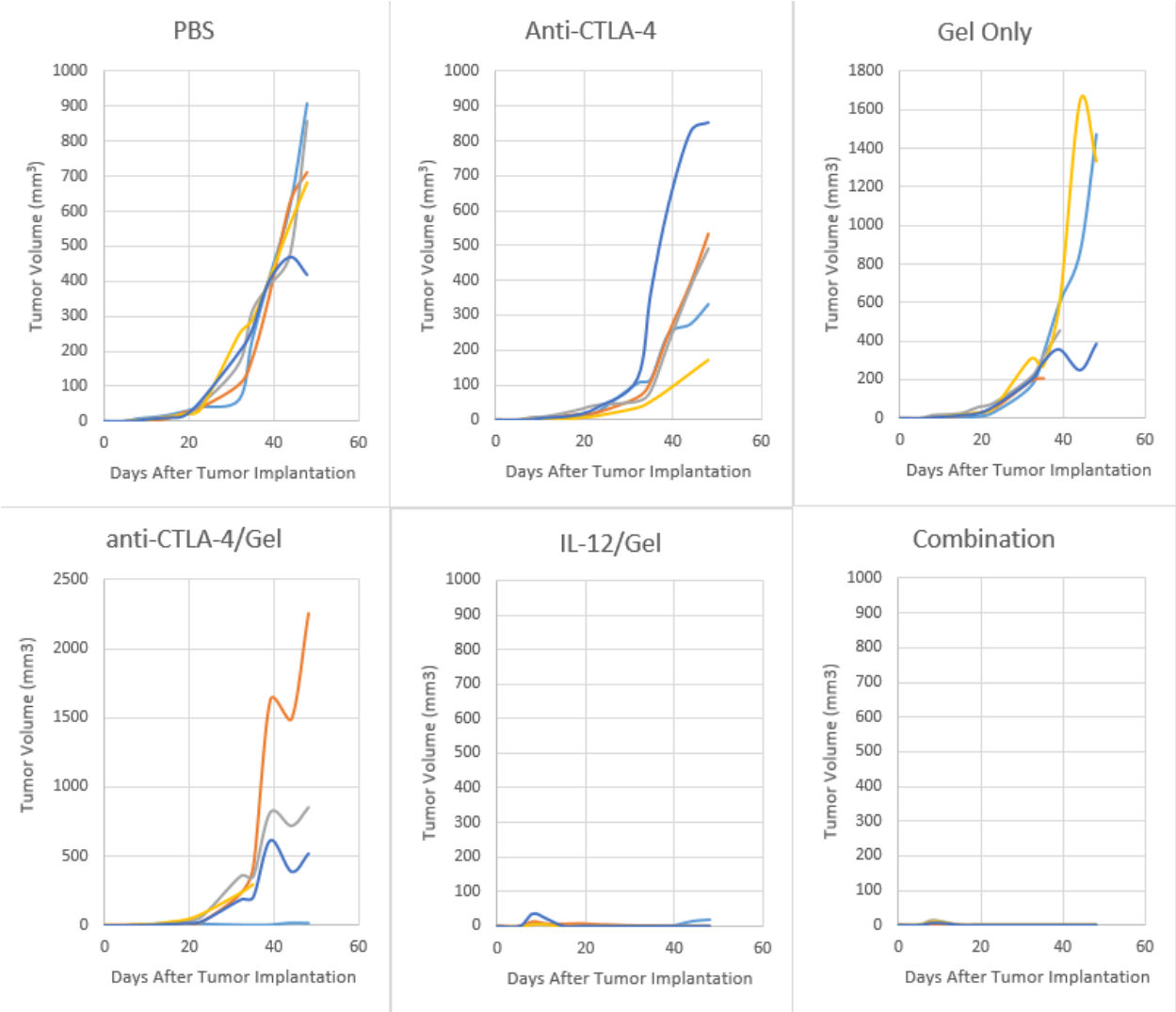

Figure 7: Individual growth curves for all treatment groups participating in the combination immunotherapy study.

Figure 7 shows the individual growth curves of the mice participating in the combination study. The group administered anti-CTLA-4 alone, intraperitoneally, developed severe dermatitis and loss most of their fur coat two weeks into the study.

4. Discussion

4.1 Demonstration of Efficacy

The efficacy of anti-CTLA-4 has been shown to be dependent on whether or not tumor invading myeloid cells are present. The effects of these myeloid cells are that FcγR crosslinking occurs which results in the cytolytic degradation of regulatory T cells that express CTLA-4. Since pancreatic adenocarcinoma is known to have abundant myeloid cell invading activity, it is possible that the Panc02 cell line would respond promisingly to CTLA-4 blockade[9]. The results from this quick pilot study confirmed the antitumor activity associated with anti-CTLA-4. The bi-weekly injections allowed for anti-CTLA-4 to maintain the blockade over a longer duration and to replenish antibody concentrations after clearance of the first dose. The lower average tumor size confirms that anti-CTLA-4 has potential in restricting tumor size in the Panc02 cell line. The current standard of Ipilimumab treatment for patients is by systemic route, however many patients experience autoimmune side effects like dermatitis[4]. It is possible that if the cause of these adverse side effects are the reduction of regulatory T cells, a local and controlled release delivery method could potentially limit these side effects.

4.2 Anti-CTLA-4 Dosing Study

Results from the dosing study were surprising in that the highest dosage group, 120 μg, had the highest tumor volumes when compared to the other anti-CTLA-4 treatment groups. From previous studies, local delivery at 60 μg was delivered peritumorally. This reaffirmed the decision that the 60 μg dose would be optimal moving forward with future experiments. It was unexpected that the highest dosage group, 120 μg of anti-CTLA-4, failed to restrain tumor growth and ranked last among treatment groups for tumor size. The initial hypothesis was that the higher dose would have a greater and more intense effect intratumorally. A similar

phenomena was observed in Sandin *et al.,* where treatment frequency and dosage was analyzed in their study. Their findings included an increase in the levels of regulatory T cells found in the serum of the mice treated with higher doses of CTLA-4 blockade. A possible explanation in this finding was a suppression assay performed by Sandin *et al.* finding that regulatory T cells have the potential to down regulate the proliferation of responder cells and thus lower immune response in the tumor environment[5]. In Sandin *et al.*, where treatments were given peritumorally, our study wanted to investigate the enhanced effects of direct intratumoral delivery[5]. We hypothesized that due to pancreatic tumors having a thick fibrotic stroma, local injections near the tumor may not have the same effect as drug permeability at the stroma by administering the treatments directly into tumors[10].

4.3 Co-Formulation of Anti-CTLA-4 with Chitosan

This study returned promising results as during the treatment scheduling, the mice treated with the co-formulated gel showed almost no tumor growth. Two mice eliminated their tumors during the treatment regime however once the treatments were terminated, a tumor in one of the mouse began to grow rapidly. The administered chitosan hydrogel was noted and physically detected in the flanks of the mice up to 6 days following the treatment. This indicates that the chitosan was able to maintain a sustained delivery of anti-CTLA-4 for an extended period of time. However, sores and ulcers did develop right at the tumor sites for the groups treated with the hydrogel. It is possible that the intensity of the immune response to the anti-CTLA-4 administration could have caused the ulceration. We hypothesize that the prolonged sustained delivery correlates to an increase in antitumor activity. When compared to a study done by Sandin *et al.*, tumor growth showed to only be restrained in groups treated peritumorally with anti-CTLA-4 against Panc02 tumors even with increased treatment days while when delivered in

a chitosan hydrogel intratumorally, tumor growth would cease in mice and in some cases completely regress as long as the treatments were delivered[5]. A study performed by Fransen *et al.* showed that the delivery of anti-CTLA-4 in a slow release Montanide emulsion subcutaneously only restrained tumor growth in MC-38 tumors expressing ovalbumin[6]. While the tumor is different from the Panc02 cell line used in this study, we are still able to demonstrate that intratumoral delivery is slightly more effective in that the average tumor volumes for the study performed by Fransen *et al*. were just short of 200 mm^3 on day 35 while our study showed that in the same time frame, average tumor volumes were 109 mm^3 with two mice completely lacking tumors. One of the major critiques we had for this experiment was that there was no group to test the effects of the chitosan hydrogel on tumor growth. Chitosan has been shown to have some biological activity therefore, the study needed to examine the effects of a chitosan hydrogel on pancreatic tumor growth[11]. Also of note, in Sandin *et al.,* their findings show that additional treatment frequency does not influence the growth of the tumor[5]. Our findings in this study contradict that claim. Mice treated with the co-formulation of anti-CTLA-4 and chitosan had no detectable tumor mass in 4 of the 5 mice, however after administration of the last dose on day 19, tumors began to grow. This slow release of anti-CTLA-4 in the tumor may cause a constant immune response that may be more effective than a single dose that may be cleared from the system in as little as twenty four hours.

4.4 Combination Therapy of Anti-CTLA-4 and IL-12 in a Novel Hydrogel

The results of the combination study show that the combination of anti-CTLA-4, IL-12, and a hydrogel provided complete response in all mice treated. Five out of five mice were completely cured of any palpable tumor mass in the combinatory group even after treatments were terminated. These are slightly better results than with the IL-12/Gel group in which all but one

mouse completely responded. Our lab has demonstrated similar results in mice using IL-12 and a chitosan based hydrogel[7]. Figure 7 shows the individual growth curves of the IL-12/Gel group and it is notable that the one mouse that developed a tumor maintained a relatively consistent mass and behaves benign. In addition to the tumor growing at a slow rate, it is relatively small. Our results also show that the gel group performed similar to the control, however the average tumor volume in the Gel Only group was greater than that of the control treated with PBS. Furthermore, the anti-CTLA-4/gel treated group had very large tumors. This may provide some support about the dosing of anti-CTLA-4. The immune response that was induced in the anti-CTLA-4/gel group could have been so great that regulatory T cells could have been recruited to the site and suppressed the immune response[5]. Nonetheless, as far as efficacy goes, this study does demonstrate that IL-12 is more effective in treated pancreatic tumors as it showed to much less toxic and had a complete response rate of 80%. However, local delivery of anti-CTLA-4 confirmed its toxicity and ability to induce adverse autoimmune related side effects when delivered systemically. All five mice in the intraperitoneal injection group had varying levels of dermatitis and displayed ill behaviors while most of the anti-CTLA-4 groups treated intratumorally sustained minor ulceration at the tumor site.

4.5 Future Direction

Following the conclusion of the combination study, rechallenge studies will be performed to see if any cured mice have retained immunity against Panc02. Our lab has previously shown mice treated and cured with IL-12 and chitosan maintain immunity against the rechallenged cell lines[7]. We hypothesize that with the addition of IL-12 in the hydrogel treatments, some of these mice will have immunity when rechallenged on the left flank. Additionally, serum analysis should be performed in order to investigate anti-CTLA-4 blood concentration of dose levels of anti-CTLA-

4 when delivered in a hydrogel vehicle. Additionally, it has already been shown that patients receiving anti-CTLA-4 intravenously show increased regulatory T cell concentrations[12]. Therefore, seeing the effect of regulatory T cell levels in response to treatment with a hydrogel and having a prolonged immune response should be investigated in order to determine more appropriate doses for these delivery vehicles.

5. Conclusions

In conclusion, anti-CTLA-4 is a promising drug belonging to a class of therapeutics that has potential to revolutionize cancer treatments. The toxicity of anti-CTLA-4 is quite evident by the murine models we have used and more work should be done in order to reduce the toxicity levels such as delivery method. In this study, we have shown that a lower dose of anti-CTLA-4 is more effective in restraining tumor growth than when compared to a systemic delivery method. Not only is the antitumor activity treated in mice equal and perhaps more effective in the locally delivered groups when compared to the systemic group, but the mice treated locally also showed much milder side effects as a result of the treatment. Ulceration was the most common side effect in the mice treated locally. When compared to IL-12, anti-CTLA-4 performs much worse when delivered in a hydrogel based delivery vehicle. As the results show, the IL-12 group was much more effective in eradicating tumors whereas, anti-CTLA-4 in hydrogel could only restrain growth for a short period. Additionally, tumors that are more difficult to access would be able to be delivered with image guided and stereotactic injections. For tumors that are readily accessible, treatment is very favorable as the treatment can be made and injected bedside. The future of anti-CTLA-4 therapy to treat tumors may be dependent on how well the toxicity and side effects of the drug can be managed.

6. Acknowledgments

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