

## **Evaluation of Combined Photodynamic Therapy (PDT) and Dendritic Cell Based Immunotherapy for Enhancement of Anti-Tumor Immunity**

### **Hypothesis**

The combined use of Photodynamic Therapy (PDT), to induce preferential necrotic or apoptotic cell death and bone marrow-derived “immature” DCs administered at the site of tumor cell death will cause apropos DC activation, maturation, and trafficking that would lead to augmented anti-tumor immunity.

### **Background**

The treatment of most cancers is still inadequate, despite steady progress. Our laboratory studies breast cancer. The number of patients diagnosed and deaths due to advanced breast cancer has increased in the past few years, reaching a level of 211,240 cases and close to 41,000 deaths for 2005. Most patients who receive even the most advanced cancer therapies available today have a relapse of the disease due to failure of destroying all the tumor cells. We aim to manipulate and use the body’s own immune system to destroy tumor cells and prevent re-growth of the tumor.

Our body’s immune response to tumors is ineffective in destroying tumor cells despite being able to recognize and respond to the tumors. Dendritic cells are critical components of the human immune system that exist as two phenotypes: “mature” and “immature.” Immature DCs have the ability to engulf antigens from dead cells or cellular debris, process them, and present them to the effector components of the immune system. This includes the ability to “cross-prime” the immune system for MHC class I responses that would normally be restricted to MHC class II. Mature DCs reside in the secondary lymphoid tissue, following a series of events including activation, maturation, and trafficking, and are important in stimulation of immune responses.

PDT involves the use of light to activate a non-toxic drug to induce cell death. Apoptosis and necrosis are the two types of cellular death, and these can be elicited by using different doses of light during PDT. During PDT, photosensitizers are given to the patient via oral gavage (PO), IV, or intra-tumoral injection. Subsequent exposure to light, in conjunction with the photosensitizers, causes cell death in the exposed region. This is effective in targeting local areas, but is not helpful in destroying metastatic tumor cells.

Our proposed cancer therapy involves a combination of PDT and dendritic cell, DC, based immunotherapy which we have termed “Immunophototherapy,” or IPT. PDT, by eliciting apoptosis or necrosis of the tumor cells, creates a collection of tumor-associated antigens (TAAs), in a small area full of dead and dying cellular debris that can activate DCs. This provides a site for antigen uptake by DCs. Bone-marrow derived DCs grown *ex vivo* injected at the site of tumor cell death would hypothetically cause 1) increased DC activation, 2) improved DC trafficking, and 3) an enhanced anti-tumor response.

## Preliminary Data

In our preliminary experiments, we conducted studies of DC trafficking and the biodistribution of the photosensitizing drug 5-aminolevulinic acid (5-ALA). We defined the kinetics of 5-ALA accumulation and its conversion to the photosensitive agent Pp IX in tumor masses. We used 0.5 cm x 0.5 cm tumor masses to evaluate this (Figure 1 & 2).

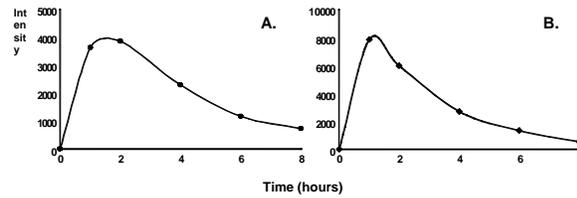
Tumor masses sized 1.0 cm x 1.0 cm were also analyzed in previous experiments. The concentration of Pp IX in the tumor tissue increased to a maximum value after 1-2 hours of drug administration. Significant decrease in concentration occurred after that time period and returned to a resting value in between 12-24 hours. Tumor size had no effect on this trend. PO and IV methods both showed similar kinetics in the distribution of 5-ALA. Although IV shows a higher maximum [Pp IX], the animal models seemed to tolerate the PO administration better than the IV probably due to the acidic 5-ALA.

This experiment showed a decrease in 5-ALA accumulation and conversion to Pp IX in the tumor periphery (Figure 2). This did not appear to be due to central necrosis and was found to be true despite tumor size. This can possibly be attributed to tumor-vasculature abnormalities and the increased

hydrostatic pressure found in the interstitial space of tumor tissue [1-5]. Also, injection of 5-ALA directly into the tumor tissue showed a more uniform distribution of the drug (Figure 2). This method proved practical and was tolerated by the animal models. The differences in dosage for each method, 5 mg for IT and

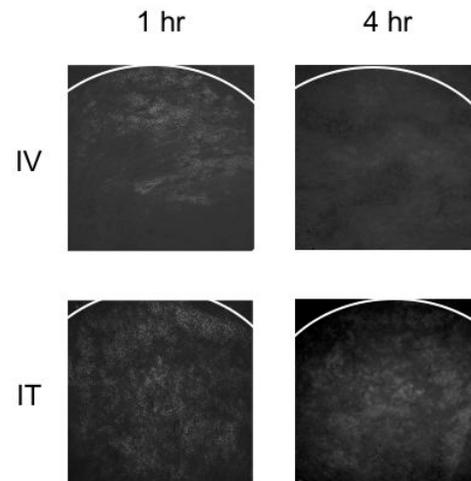
≈15 mg for PO or IV, do not appear to be a problem since the increased distribution appears to be accompanied by a slower decay rate of Pp IX fluorescence (Figure 2).

We used a light flux of 25 J/cm<sup>2</sup> that brought about a mixture of necrotic and apoptotic cell death. However, the necrosis occurred closer to the center of the cell mass and we interpreted this to be primarily a result of tumor growth as it is also seen in untreated tumors. Using a light flux of 25 J/cm<sup>2</sup>, we evaluated the ability of rat bone marrow-derived DCs, generated *in vitro* and labeled (CFSE), to travel from PDT site to drainage lymph nodes. Administration of 5 x 10<sup>4</sup> CFSE labeled DCs into the tumor mass treated

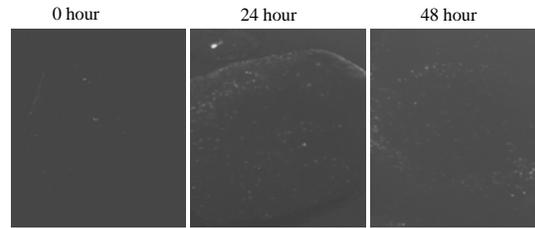


**Figure 1.** Tumor Pp IX fluorescence. 5-ALA was administered PO, by oral gavage, Panel A, or IV, by tail vein injection, Panel B, (100mg/kg in normal saline pH3.5) to animals with pre-established tumors. The data in these panels depict that obtained from animals with tumors measuring approximately 0.5 cm x 0.5 cm. Animals were euthanized at the designated time points, with immediate tumor harvest and freezing in OCT media for subsequent analysis by two photon imaging and low light fluorescence in frozen sections cut with a maximal 8 μm thickness. Individual data points represent mean of data obtained from three animals per time point.

**Figure 2.** Tumor Pp IX fluorescence. 5-ALA was administered to female rats (≤ 150g) with pre-established tumors, IV, by tail vein injection (100mg/kg pH3.5) or, IT, by direct intratumoral injection using a 28g needle and insulin syringe (5 mg in a volume of 100ul). The panels above depict low light fluorescent images covering ≈ 60% of the tumor diameter, original magnification 40x. White line overlays demarcate the approximate border of the tumor masses.

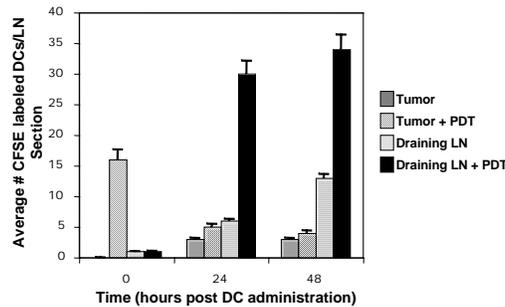


with PDT resulted in a higher number of labeled DCs that were successfully activated and trafficked to the lymph nodes (Figure 3a & 3b) than in control animals that were given 5-ALA but no light treatment. This experiment showed that cell death from PDT can increase DC activation and trafficking. Thus, I/we have all of the elements in place to formally test our hypothesis that IPT enhances anti-tumor immunity and survival when compared against PDT alone.



**Figure 3b.** CFSE labeled DC Trafficking to Draining Lymph Nodes, Post-PDT. Representative images from draining lymph nodes harvested at the designated time following adoptive transfer of  $5 \times 10^4$  CFSE labeled rat bone marrow-derived DCs into the tumor masses, 15 minutes after PDT treatment. Only fluorescent signals with appropriate cellular morphology were included, typical signal artifact is noted in upper left of the 24 hour image. Original magnification 40x.

**Figure 3a.** Trafficking of CFSE Labeled DCs. Female Rats with established tumors received 5 mg 5-ALA, IT, one hour before PDT at 25 J/cm<sup>2</sup>. Within fifteen minutes  $5 \times 10^4$  CFSE labeled and washed syngeneic BM-derived DCs were injected in a volume of 100 ul of normal saline into the tumor mass at the site of PDT. Rats were euthanized at the designated time points with tumor and draining lymph nodes harvested, frozen in OCT media, sectioned, and imaged by low light fluorescence. Controls, animals without PDT, were euthanized and had tissue harvested in an identical time frame to the experimental animals. This data represents one of two experiments that yielded similar results.



## Research Plan & Procedures

I will conduct the following experiments with the assistance of Dr. Nelson's research technician. These experiments will make use of the skills that I have established to date.

In my initial set of experiments, I will harvest bone marrow and generate immature DCs. This process involves harvesting leg bones and flushing out the bone marrow. The bone marrow is then cultured for 8 to 10 days in the presence of rat GM-CSF and IL-4 in conditions previously established in Dr. Nelson's laboratory. The generation of DCs will determine the time for inoculation of animals with the tumor cells in order to have appropriate sized tumors when the DCs are ready. The DCs will be evaluated for their cell surface phenotype by staining with antibodies and flow cytometry.

I will inject four groups of 10 animals (rat models) with tumor cells and allow them to establish tumors within the animals. 5-ALA will be given to all the animals, via the PO or intratumoral (IT) routes in separate experiments. One group will receive no other treatments and should yield the most rapid tumor growth. One group will receive  $5 \times 10^4$  bone marrow derived DCs administered into the tumor. Two groups of 10 animals will be treated with PDT two hours after 5-ALA administration and one of these groups will have injection of  $5 \times 10^4$  bone marrow derived DCs into the site of PDT immediately after PDT. Animals will be followed and tumors measured no less frequently than every other day for a period that is likely to be in excess of 45 days. Animals that remain without tumor will be maintained in the vivarium with frequent evaluation not less than 120 days. This time frame means that several experiments will be initiated over the

course of the summer, but that if successful, will continue into the fall. These experiments will allow us to compare PDT to IPT and the 5-ALA group will act as the positive control for tumor growth as 5-ALA has not in the past altered tumor growth. We do not believe that DCs without PDT will result in anti-tumor activity, but the second cohort will be the control for this. These experiments will be conducted at least in duplicate for both PO and IT. We predict that PDT treated animals will have some tumor regression followed by regrowth and that IPT will either significantly delay that regrowth or cure the animals.

In future experiments that will be conducted during the course of the academic year, I will be evaluating immune responses to IPT. This will entail a similar experimental design except that animals will undergo saphenous vein phlebotomy at 7, 14, and 21 days after treatment. Serum will be collected and used for staining of the 13762 tumor cells and flow cytometry will be assessed using fluorophore labeled anti-rat secondary antibody. We will also analyze the proliferative response and cytokine release of treated animals. In separate experiments, animals will have draining lymph nodes harvested at 10-14 days post treatment for *in vitro* cellular immune assays. We will perform standard proliferation assays [6, 7] five replicates per condition with the lymphocytes stimulated by Mitomycin C treated 13762 tumor cells. <sup>3</sup>H thymidine incorporation counted by liquid scintillation will be used to assess proliferation [6,7]. ELISA kits for rat cytokines will be used to assess secreted cytokines (Tumor Necrosis Factor TNF- $\alpha$ , IFN- $\gamma$  and IL-4) in supernatant of proliferation assays.

All of these procedures will be conducted under Dr. Nelson's IACUC approved protocol #2003-2473-0. All animals will be euthanized according to proper protocol either for harvest of tissues or when animals experience any signs of distress or tumors reach the preassigned maximal volume, 10,000 mm<sup>3</sup> (approximately 3 cm in diameter).

### **Responsibilities**

I will be involved in all aspects of this project.

1. Growth and maintenance of the 13762 tumor cell line for establishing tumor in the rats. Growth of bone-marrow derived DCs *in vitro*.
2. Injection of rats with tumor; monitoring the rats.
3. Administration of 5-ALA via PO and IT methods and treat with PDT.
4. Administration of *in vitro* generated, bone marrow derived and labeled DCs.
5. Lymph node harvesting.
6. Analysis: flow cytometry for DCs and antibody responses, proliferation assays, ELISAs, etc.

### **Timeline**

Experiment #1:

Week 1-8: The animals will arrive and be allowed to rest for one week. Six animals will be euthanized to provide bone marrow for generation of DCs. I will then infect the animals with the tumor and allow the tumor to grow. I will treat the animals with the 5-ALA and PDT and observe the animals for any changes in tumor volume. This experiment will be conducted at least in duplicate for each route of 5-ALA administration

(4 experiments total). As noted above, these experiments are lengthy and I will be performing experiments with different routes of administration of 5-ALA in parallel. If results are consistent between replicate experiments, I will proceed to the second set of experiments evaluating the immune responses. Other members of the laboratory will be evaluating the effect of different light flux in similar experiments.

Week 8-16: The duplicate experiments will be conducted or completed and, if time allows, experiments to evaluate the immune responses will be initiated/conducted.

I intend on continuing my work on this project through the next school year in order to complete the set of experiments for this light flux.

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