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Immune-mediated eradication of cancer stem cells via polyspherex microsphere-based vaccination.

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14. ABSTRACT The prognosis for patients with metastatic breast cancer remains poor. Standard radio-and chemotherapies are effective against the majority of cells within solid tumors, but have little impact on the cancer stem cells (CSC) that perpetuate tumor growth. Many studies have implicated CSC as being critically involved in metastatic tumor spread. Thus, finding a way to specifically eradicate CSC could lead to protection from metastatic tumor progression. In this study, we attempted to use the specificity of the immune system to eradicate CSC using a vaccination technique based upon loading highly purified CSC lysates onto nanoparticles made of poly-L-lactic-co-glycolic acid (PLGA particles), then injecting these particles into tumor-bearing mice. Due to the low numbers of isolatable CSC in growing tumors, this vaccination strategy failed. However, during our vaccination strategy optimization, we identified an approach based upon incorporation of bulk tumor lysates into PLGA nanoparticles that was highly effective at eliminating metastatic breast tumors from the lungs of mice. This approach is under further investigation.					
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Introduction

The prognosis for patients with metastatic breast cancer remains poor. Standard radio- and chemotherapies are effective against the majority of cells within solid tumors, but have little impact on the cancer stem cells (CSC) that perpetuate tumor growth. Thus, such treatments selectively spare the very cell populations that give rise to recurrent tumors. CSC share many features with cells that are undergoing the epithelial to mesenchymal transition (EMT), a process that alters cellular phenotypic and functional characteristics, and facilitates metastatic dissemination. Finding a way to specifically eradicate CSC and EMT cells should therefore reduce metastatic tumor spread and diminish the ability of tumors to self-perpetuate. Because T lymphocytes can recognize subtle alterations in cell surface proteins, traffic systemically, and give rise to long-lived immunologic memory, triggering T cell recognition of CSC and EMT cells would accomplish this goal. Our objective for this project was to achieve T cell-mediated protection against metastatic breast cancer in mice by using a novel vaccination strategy to boost T cell recognition of CSC and EMT antigens. The vaccine approach was to consist of biodegradable, synthetic PLGA microspheres that are coated with CSC and EMT lysates. Vaccination with antigen-coated PLGA microspheres has been shown to induce superior levels of immunity in other model systems, versus vaccination with antigen in soluble form. We hypothesized that vaccination of tumor-bearing mice with CSC and EMT lysate coated-PLGA microspheres would lead to a robust, long-lived T cell response that would provide systemic protection against metastatic breast cancer.

Body

Original Statement of Work and Research Accomplishments

Task 1: Establish the optimal schedule for vaccine delivery and dosing.

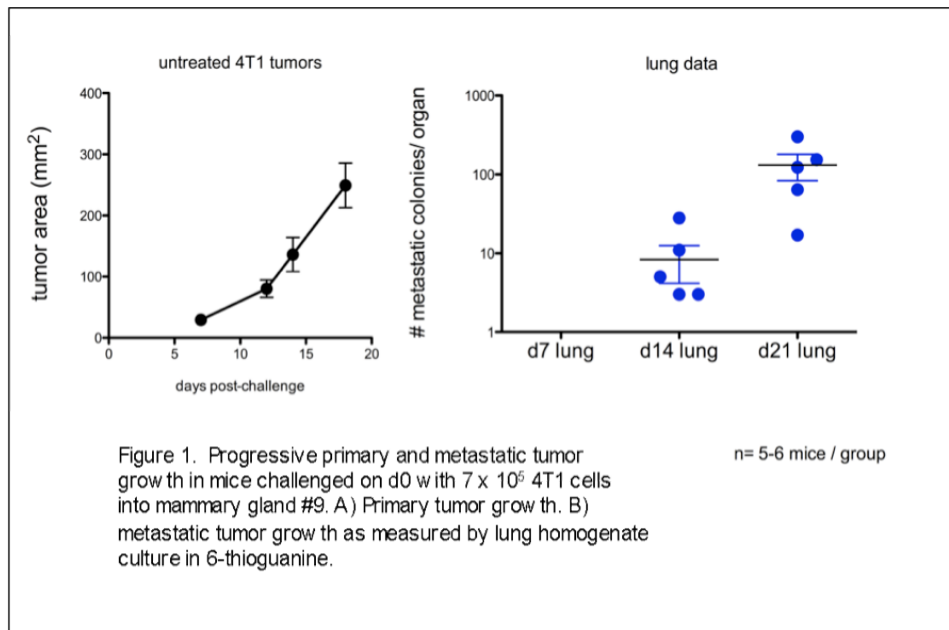
Experimental Groups:

1. 4T1 tumor challenge, no resection/ no vaccination
2. 4T1 tumor challenge, with resection and vaccination

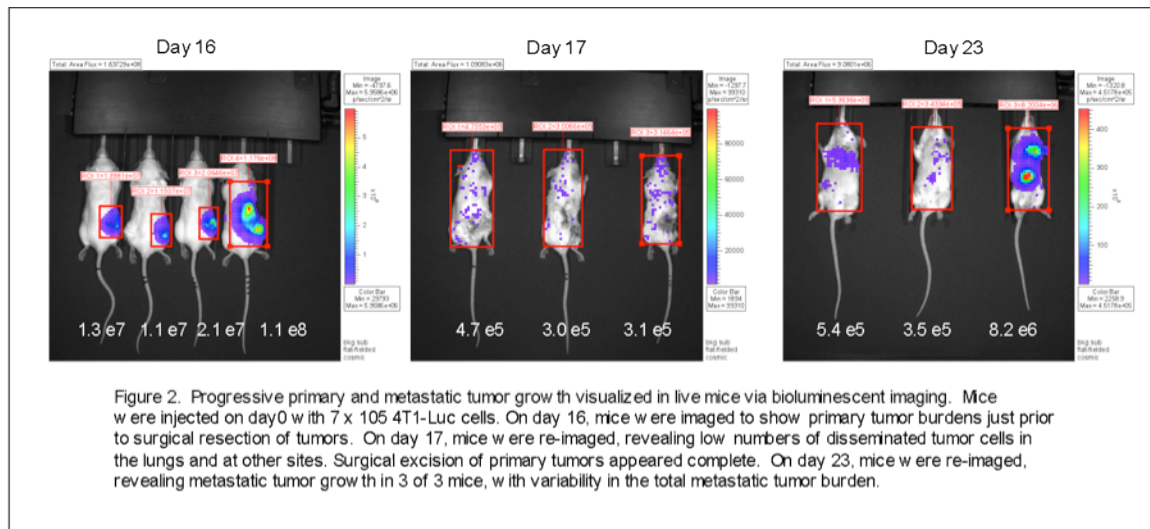
- 1a. Examine the effects of varying the microbead dose (months 1-2, 60 mice)
- 1b. Examine the effects of varying the concentration of CSC and EMT cell lysates pulsed onto microbeads (months 1-2, 60 mice)
- 1c. Examine the effects of varying the vaccination route (months 1-2, 60 mice)
- 1d. Test vaccinations using the information gained from 1a-c (months 3-4, 60 mice)

Outcomes and deliverables from this phase of the project: Identification of the optimal vaccination strategy for generating maximal effector T cell function in vaccinated mice.

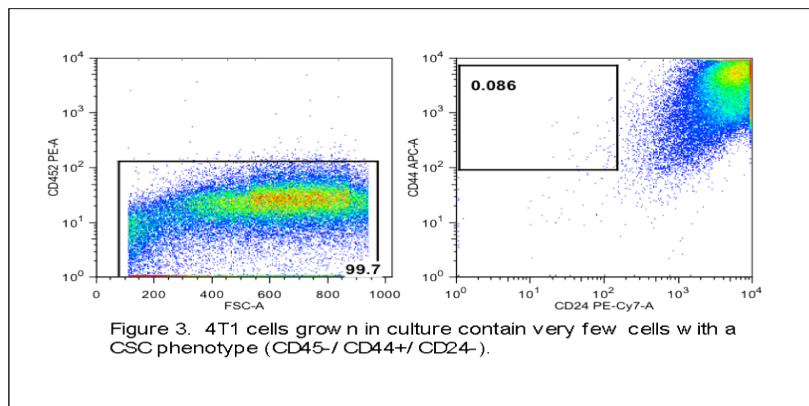
Results for Task 1. At the time this grant application was begun, we had used and reported on the 4T1 cell line for investigating host anti-tumor immunity in the absence of vaccination ¹. As we had not examined the kinetics of both primary and metastatic tumor outgrowth in this model, we began by examining these parameters in the absence of any vaccination (Figure 1).



We then performed a series of experiments in which mice were challenged with luciferase-expressing 4T1 cells (4T1-Luc) on day 0, and were imaged via IVIS bioluminescent imaging to visualize primary and metastatic tumor outgrowth in live mice. In the experiment shown, mice were imaged on day 16 post-challenge, immediately prior to surgical resection of primary tumors. Mice were re-imaged one day later, revealing low numbers of metastatic tumor cells that continued to grow out progressively over the next week (Figure 2). Mice with overly large primary tumors (those that had invaded the musculature at time of resection) were sacrificed and not used for further experimentation (Ex: mouse on right of Day 16 panel).

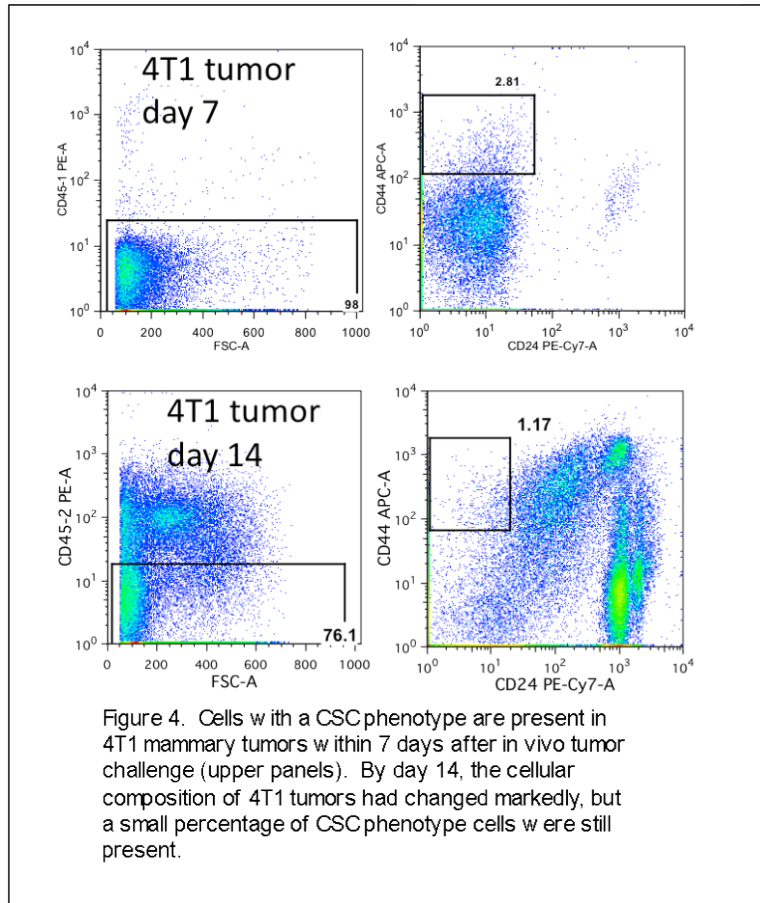


Cancer stem cells are defined in the 4T1 model system as being CD45-/ CD44+/ CD24-, so we examined our parental cell line to determine the % of cells present that had this phenotype. We found that very few cells were present in cell cultures that had this phenotype (Figure 3).

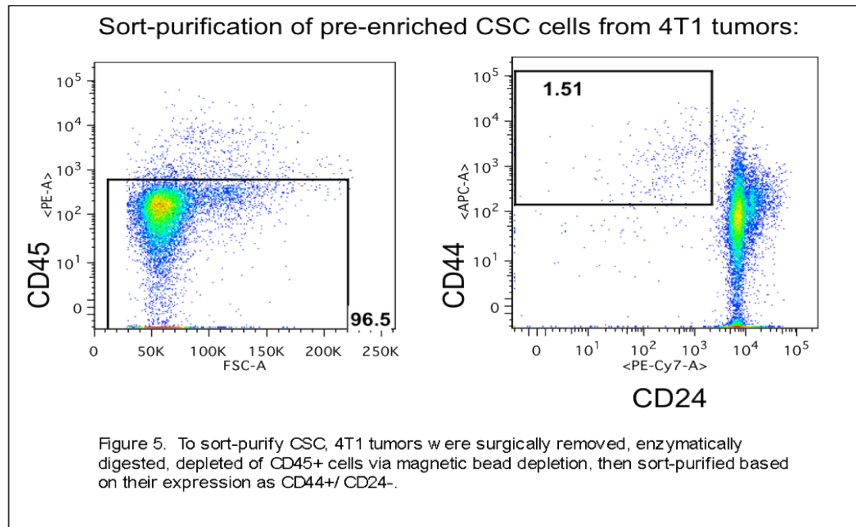


We then proceeded to examine the percentage of detectable CSC phenotype cells in growing 4T1 mammary tumors in vivo. We found that CSC phenotype cells were

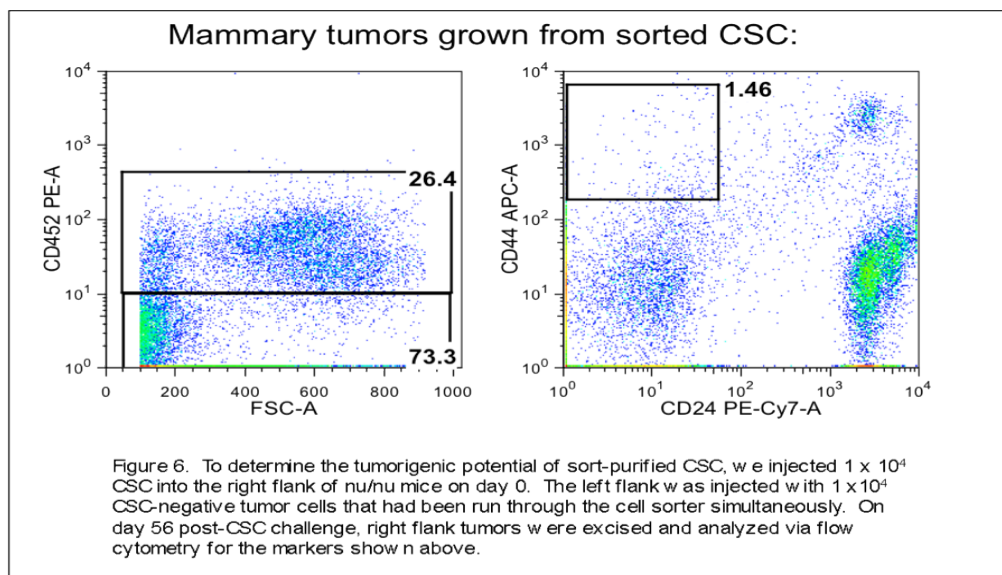
present by day 7 post-challenge, and although the cellular composition of growing tumors changed dramatically over the next week, CSC phenotype cells were still present in vivo at day 14 (Figure 4).



We then began an exhaustive series of experiments in which we attempted to enrich sufficient numbers of CSC to use in our vaccination protocols. The kinetics of CSC accumulation in primary tumors was evaluated over time (not shown) to determine the optimal time at which to harvest primary tumors for CSC purification. We used a variety of pre-enrichment strategies in an attempt to increase our yield of CSC from primary tumors. These included using side-population gating, Aldefluor labeling to detect stem cells that expressed Aldehyde Dehydrogenase, depletion of CD45+ leukocytes, selection of CD44+ cells, depletion of CD24+ cells, and a number of combinations of the above (not shown). Our results indicated that CSC were very sensitive to manipulation, and that increased tumor processing was associated with decreased CSC yield. We therefore found that the best method for obtaining CSC via sort-purification from primary tumors was to negatively deplete CD45+ leukocytes, following by immunostaining for CD45, CD44, and CD24, and sort-purification based on the above-mentioned CD45-/CD44+/CD24- phenotypic profile (Figure 5).



Although these cells were phenotypically identical to CSC reported by others, we performed a set of experiments to verify their tumorigenic potential *in vivo*. Low numbers of sort-purified CSC (1×10^4 cells/mouse) were injected into the right flank of nu/nu mice (to allow tumor growth in the absence of immune-mediated rejection, as nu/nu mice lack T lymphocytes). As controls, an equal number of CSC-negative tumor cells that had been simultaneously run through the cell sorter were injected into the left flank of nu/nu mice. Tumors were observed in 3 of 5 nu/nu mice that received sorted CSC by day 60, whereas 0 of 5 mice that received non-CSC developed tumors, even after 120 days (not shown). In CSC-challenged mice that grew tumors, we excised tumors and evaluated their cellular composition. The composition was strikingly similar to tumors that arose after challenge with parental 4T1 cells grown in culture, indicating that CD44+/CD24- CSC had given rise to tumors that contained CD44+/CD24+ and CD44-/CD24+ tumor cells, as well as CD45+ leukocytes, and a small population of CD45-/CD44+/CD24- CSC (Figure 6).



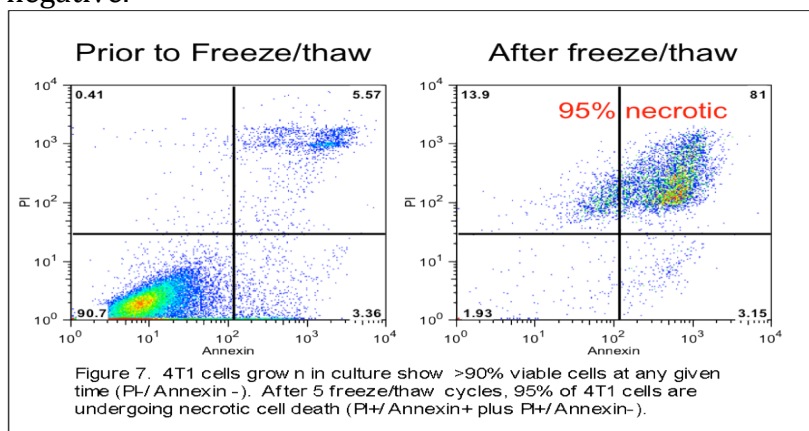
Major obstacle to completion of planned studies:

We were unable to obtain more than 85,000 sorted CSC from any given experiment, despite the fact that we were using large, day 18 4T1 tumors pooled from 15-20 individual mice per experiment. After 6 months of sort-purification, we had collected lysates from < 600,000 sorted CSC, which yielded an insufficient amount of protein to follow our optimized vaccination protocol (see below). During this time, we also attempted to differentiate CSC from 4T1 cells grown in vitro, reasoning that this might allow us to generate larger numbers of CSC than were possible to obtain through cell sorting, through the addition of various concentrations of TGF β or 5-Azacytidine (among others) into 4T1 culture medium. However, no approach that we tried resulted in a detectable population of CD45-/CD44+/CD24- CSC during in vitro culture (not shown).

Alternate Approach: Developing an optimized vaccination protocol using bulk 4T1 tumor lysates incorporated into PLGA microspheres.

When the difficulties with sort-purification of CSC arose, we decided that rather than to wait to begin vaccination optimization experiments until we had sufficient numbers of sorted CSC, we would begin testing vaccination strategies with bulk tumor lysates. As shown in Figure 4, 4T1 mammary tumors contained a small percentage (1-2%) of CSC. We therefore used bulk tumor lysates to begin testing vaccination strategies while we were attempting to increase our yield of sorted CSC from primary tumors.

To prepare lysates from 4T1 tumor cells, we used a strategy of subjecting cells to multiple freeze/thaw cycles to induce necrotic cell death, as necrotic cell fragments have greater abilities to stimulate immune responses than do apoptotic cell fragments^{2,3}. Our optimized protocol included 5 rounds of freezing cells in liquid nitrogen, followed by 5 rapid thaws in a 37C water bath. To confirm that this induced necrosis, treated cells were analyzed by flow cytometry with Propidium Iodide and Annexin V (Figure 7). Necrotic cells stain positively for propidium iodide, while apoptotic cells will exclude this dye and remain propidium iodide-negative.



We tested a variety of vaccination strategies, based upon a thorough literature review. We decided to investigate two approaches. One included the use of adenoviral-encoded GM-CSF (AdGM-CSF), which is known to induce dendritic cell maturation and enhance antigen presentation ⁴. The other approach included the use of a cocktail of TLR ligands that had shown success in an HIV vaccination model ⁵. Toll-like receptors (TLRs) are expressed on cells of the innate immune system that allow for detection of pathogen-associated molecular patterns that are common to many types of pathogens, including bacteria, viruses, and intracellular parasites. TLR ligation therefore represents a “danger” signal to the immune system, and triggers innate immune responses that aid in priming T-cell mediated immunity against antigens presented in the context of these danger signals. Robust immune responses are produced in response to “prime/boost” vaccination strategies, and rapid induction of effector and memory T cell responses using PLGA microparticles had been shown with an accelerated prime/boost regimen where the boost was given 7 days after priming ⁶. We therefore decided to adopt this strategy for testing in our two approaches. For these experiments, bulk tumor lysates were either adsorbed onto the surface of commercially available Phosphorex PLGA microparticles, or were encapsulated into custom-fabricated PLGA microparticles at The University of Iowa in the laboratory of Dr. Aliasger Salem. Experiments using adsorbed tumor lysates were less successful than those using custom PLGA particles, so only results using the latter are shown.

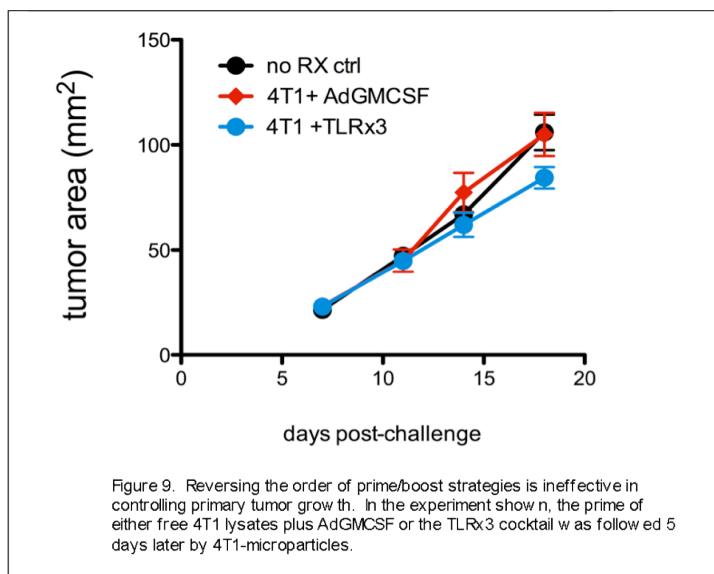
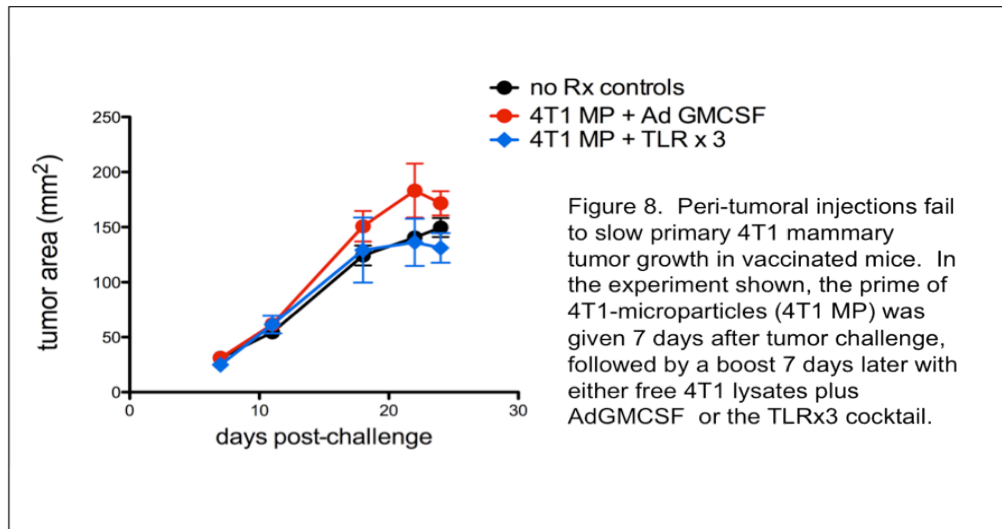
Vaccination strategies:

1. **Prime:** bulk 4T1 lysates (8 x 10⁴ cell equivalents/mouse, approximately 50ug protein) encapsulated into PLGA micropsheres, given sc on day 7
Boost: free 4T1 lysates (50ug/mouse) plus 1 x 10⁸ PFU of AdGM-CSF

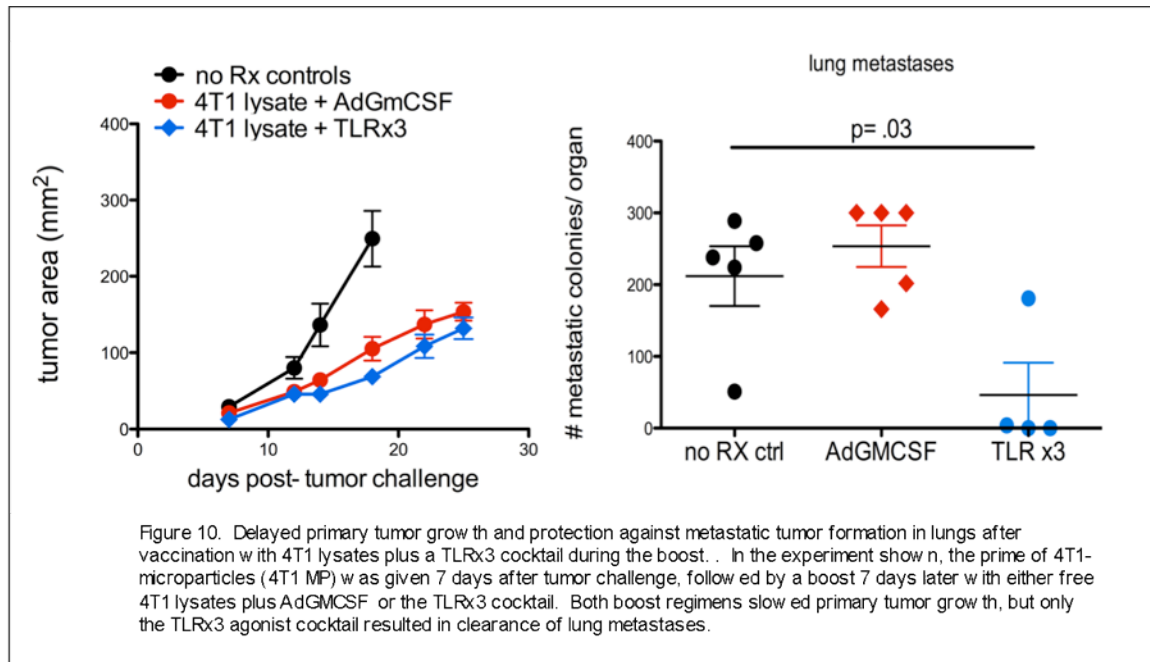
2. **Prime:** bulk 4T1 lysates (8 x 10⁴ cell equivalents/mouse, approximately 50ug protein) encapsulated into PLGA micropsheres, given sc on day 7
Boost: free 4T1 lysates (50ug/mouse) plus a TLR cocktail
MALP2 @ 0.2 ug/mouse (TLRs 2 and 6)
PolyI:C @ 50ug/mouse (TLR 3)
CpG 1826/1585 @ 4ug/mouse (TLR 9)

We tested various permutations of these two strategies that included priming on different days post-tumor challenge, altering the route by which the prime and boost injections were delivered, and altering the location of these injections (not shown). We also examined whether switching the order of prime and boost strategies was more effective. During the course of these studies, we found that peri-tumoral injection of prime and boost vaccinations was not effective in slowing

primary tumor growth with either approach (Figure 8) and that switching the order of prime and boost regimens from that stated above was also ineffective (Figure 9).



We were able to successfully slow primary tumor growth using a strategy in which the prime/boost strategy listed above was administered sc on the flank opposite the growing mammary tumor. We believe that the reason for this was increased T cell priming in the absence of immunosuppressive cells and cytokines that are known to be present in both the tumor itself and the tumor-draining lymph node. As effector T cells traffic systemically once they exit the lymph node in which they are primed, we reasoned that priming in the absence of tumor-derived suppression might produce a more robust immune response, and this appears to be the case. In addition to slowing primary tumor outgrowth, this strategy resulted in the almost complete clearance of metastatic tumor outgrowth in the lungs of tumor-bearing mice (Figure 10).



Therefore, although our initial strategy of using sorted CSC in a PLGA vaccination protocol did not yield positive results, we succeeded in developing a vaccination protocol using bulk mammary tumor lysates encapsulated into PLGA microparticles, followed by simultaneous ligation of multiple TLRs during the boost, that was highly effective in eliminating metastatic tumor outgrowth in the lungs.

Future studies:

We will continue this avenue of investigation, by evaluating the efficacy of this approach when combined with surgical excision of primary mammary tumors. This scenario would provide a clinically relevant application of our approach and could lead to future clinical application of this strategy as PLGA microparticles and TLR agonists are already being investigated in clinical trials for cancer and other diseases.

Task 2: Evaluate the T cell responses in mice with metastatic 4T1 mammary carcinoma, following vaccination with CSC and EMT cell-lysate pulsed microbeads.

Experimental Groups:

1. 4T1 tumor challenge, no resection/ no vaccination
2. 4T1 tumor challenge, with resection and vaccination
3. 4T1 tumor challenge, with resection and vaccination (empty beads)

4. 4T1 tumor challenge, with resection/ no vaccination
5. 4T1 tumor challenge, sham resection and vaccination
6. 4T1 tumor challenge, sham resection/ no vaccination

- 2a. Characterize the T cell phenotype, cytokine production, and Foxp3 expression at days 24-30 post-tumor challenge (months 5-7, Experimental groups 1-4 only, 120 mice)
- 2b. Characterize the in vivo cytolytic activity of T cells (months 5-7, Groups 1-4 only, 80 mice)
- 2c. Perform in vivo depletions of T cells to determine effects on metastatic tumor outgrowth (months 5-8, Groups 1-4 only, 80 mice)

Outcomes and deliverables from this phase of the project: Understanding of how the proposed vaccination strategy impacts the phenotype and function of tumoricidal effector T cells in vivo.

Due to difficulties in developing an efficacious vaccination protocol, no progress was made on this Task.

Task 3: Evaluate the efficacy and safety of the vaccination strategy in mice.

Experimental Groups: same as in Task 2.

- 3a. Quantitate the numbers of lung, liver, and lymph node metastases using culture of homogenized organs in 6-thioguanine (months 8-10, Experimental Groups 1-6, 120 mice)
- 3b. Quantitate the relative tumor outgrowth in mice using bioluminescent imaging of live animals (months 8-9, Experimental Groups 1-6, 90 mice)
- 3c. Determine effects of the vaccination strategy on CSC and EMT cell percentages and phenotypes (months 8-11, Experimental Groups 1-6, 120 mice)
- 3d. Determine the extent to which systemic T cell responses protect mice from re-challenge with 4T1 tumor cells in the contralateral mammary gland (months 11-12, done in conjunction with 3b, no additional mice required)
- 3e. Determine if autoimmunity develops in vaccinated mice (months 8-12, done in conjunction with 3c and 3d, no additional mice required)

Outcomes and deliverables from this phase of the project: A determination of the safety and efficacy of this vaccination strategy in mice.

Due to difficulties in developing an efficacious vaccination protocol, no progress was made on this Task. However, no undesirable side effects were observed in mice treated with the 4T1bulk lysate/PLGA prime followed with a free 4T1 lysate/ TLRx3 boost.

Key Research Accomplishments:

1. We developed a novel vaccination strategy for metastatic breast cancer that involved priming tumor-bearing mice with bulk tumor cell lysates encapsulated into PLGA microspheres on day 7, followed by a boost on day 14 consisting of free 4T1 lysates in combination with a cocktail of 3 TLR agonists. This strategy was found to delay the growth of primary breast cancers in mice, and to almost completely eliminate the formation of metastatic tumor outgrowth in the lungs of mice.
2. We determined that tumor lysate encapsulation into custom PLGA microparticles is more effective as a vaccine component than the same tumor lysates adsorbed to the surface of commercially available PLGA microparticles.
3. We determined that it is not feasible to isolate large enough numbers of CSC from murine tumors to test their efficacy in a vaccination protocol.

Reportable Outcomes:

1. **Abstracts:** BC097268 “Immune-mediated eradication of cancer stem cells via poly(D,L-lactic acid co-glycolic acid) microsphere-based vaccination” 2011 Era of Hope Conference, Orlando, FL. Authors: Tamara Kucaba, Vijaya Joshi, Aliasger Salem, Thomas Griffith, and Lyse Norian
2. **Presentations:** Department of Pharmaceutics, The University of Iowa, May 2011. Host: Aliasger Salem, Ph.D.
3. **Research Opportunities Applied For:** Holden Comprehensive Cancer Center Breast Cancer Research Interest Group. “A novel immunotherapeutic vaccine for metastatic breast cancer based upon PLGA nanoparticle delivery of tumor lysates” November, 2011 application deadline, In preparation.

Conclusion:

The use of PLGA microparticles can provide an effective way to break immune tolerance to tumor-derived antigens. Delivery of tumor lysates encapsulated in PLGA lysates, followed by a rapid boost containing a cocktail of three TLR agonists can provide robust protection against metastatic lung cancer in a pre-clinical murine model of metastatic breast cancer in mice.

The use of CSC lysates as an immunogenic vaccine component should NOT be attempted via sort-purification of CSC from growing mammary tumors. It may be possible to develop a differentiation strategy for breast cancer CSC in vitro, but we were unable to find such a method. Thus, due to our inability to obtain sufficient numbers of CSC, we were unable to determine whether vaccination with purified CSC lysates would provide enhanced protection relative to the bulk tumor lysate/ PLGA delivery strategy we developed during the course of this year.

Additional studies on the bulk tumor lysate?PLGA vaccination strategy will be required to determine the safety of this protocol, as well as to determine the magnitude and duration of any anti-tumor T cell responses generated by this approach. To have clinical translatability, we will have to show that a protective T cell response is generated in mice that receive this vaccination after resection of primary tumors, and that the vaccination results in long-term protection from tumor re-challenge. As both PLGA microparticles and TLR agonists are being used in clinical trials as single agents, we predict that this strategy should prove to be safe and well-tolerated in patients, making it a feasible future therapeutic approach for women with metastatic breast cancer. Our initial results indicate that this approach warrants further investigation.

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Appendix A.

Era of Hope Abstract:

BC097268 “Immune-mediated eradication of cancer stem cells via poly(D,L-lactic acid co-glycolic acid) microsphere-based vaccination”

Tamara Kucaba, Vijaya Joshi, Aliasger Salem, Thomas Griffith, and Lyse Norian

The prognosis for patients with metastatic breast cancer remains poor. Standard radio- and chemotherapies are effective against the majority of cells within solid tumors, but have little impact on the cancer stem cells (CSC) that perpetuate tumor growth. Thus, such treatments selectively spare the very cell populations that give rise to recurrent tumors. CSC share many features with cells that are undergoing the epithelial to mesenchymal transition (EMT), a process that alters cellular phenotypic and functional characteristics, and facilitates metastatic dissemination. Finding a way to specifically eradicate CSC and EMT cells should therefore reduce metastatic tumor spread and diminish the ability of tumors to self-perpetuate. Because T lymphocytes can recognize subtle alterations in cell surface proteins, traffic systemically, and give rise to long-lived immunologic memory, triggering T cell recognition of CSC and EMT cells would accomplish this goal.

Hypothesis: Following resection of primary mammary tumors, vaccination with CSC and EMT cell lysates coated onto poly(D,L-lactic acid co-glycolic acid) (PLGA) microbeads will activate circulating T lymphocytes to specifically recognize and eradicate these cells, leading to systemic protection from metastatic breast cancer.

Objectives: Develop a novel vaccine strategy to achieve immune-mediated protection against metastatic breast cancer based upon targeted elimination of CSC and EMT cells.

Methods: 4T1 is a mammary carcinoma cell line that spontaneously metastasizes following transplantation into murine mammary glands. We are using both parental 4T1 cells and a 4T1-luciferase derivative that stably expresses firefly luciferase and permits in vivo quantitation of tumor growth via bioluminescent imaging. CSC/ EMT cells are defined as $CD45^{neg}/ CD44^{+}/ CD24^{neg}$ and can be sort-purified from primary 4T1 tumors by using this profile. CSC/EMT lysates are then encapsulated within PLGA microspheres, which leads to enhanced immunogenicity of the CSC/EMT-derived antigens, and used to immunize mice with established 4T1 tumors. We then monitored metastatic tumor outgrowth and T cell activation, to determine if CSC/EMT immunization could induce protective antitumor immunity and reduce metastatic tumor outgrowth.

Preliminary results: We have successfully identified $CD45^{neg}/ CD44^{+}/ CD24^{neg}$ CSC/EMT in 4T1 primary tumors, metastatic lungs, and livers. The number of CSC/EMT increases over time in all three sites. Following resection of primary 4T1 tumors, CSC/EMT can be isolated and used to immunize mice. In the absence of immunization, the endogenous immune response is insufficient to control metastatic tumor outgrowth. Our evaluations of protective antitumor immunity and T cell responses to metastatic tumor growth are ongoing.

Significance: This is a novel approach for generating systemic protection against metastatic breast cancer, a disease that is currently incurable. If proven to be safe and efficacious in mice, this vaccination strategy could be rapidly and easily translated into clinical application as an adjunct therapy for breast cancer patients.