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14. ABSTRACT T cells, dendritic cells (DCs), and natural killer (NK) cells are known to be involved in anti-tumor immune responses. These cell types all express a common receptor, CCR7, which binds the chemokine secondary lymphoid chemokine (SLC, also called CCL21). In Task 1, we examined the effect of SLC/CCL21 administered via a sustained delivery system in a mouse breast cancer model that mirrors the progress of human breast cancer. Utilizing this model, we found that treatment of orthotopic tumors with sustained SLC/CCL21 delivery resulted in primary tumor growth inhibition and significantly reduced spontaneous lung metastases. Examination of tumor-infiltrating leukocytes by flow cytometry revealed this treatment increased NK cells and CD8+ T cells. Our studies in Task 2 have examined the effect of surgical resection of the primary tumor in combination with sustained SLC/CCL21. Sustained SLC/CCL21 did not increase the length of survival when administered as an adjuvant immediately following tumor resection. However, sustained SLC/CCL21 delivery used as a neoadjuvant prior to tumor resection significantly increased the duration of survival. Our findings support further study of SLC/CCL21 as a therapy for breast cancer, one that may be capable of reducing residual and metastatic disease in breast cancer patients.					
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4-8
Key Research Accomplishments.....	9
Reportable Outcomes.....	10
Conclusions.....	11
References.....	11
Appendices.....	None

INTRODUCTION

For an effective antigen specific immune response to breast cancer, T cells must be activated through interaction with dendritic cells (DCs). T cells and DCs, as well as natural killer (NK) cells (which also have anti-tumor activity), express a common receptor, CCR7, which binds secondary lymphoid tissue chemokine (SLC, also known as CCL21). Expression of CCR7 allows these cells to migrate along gradients of this chemokine. In this manner SLC/CCL21 coordinates T cell co-localization with DCs, and consequently facilitates T cell activation. In murine models of cancer, recombinant SLC/CCL21 administered intratumorally has been found to induce immune-mediated tumor growth inhibition; however, repeated injections of SLC/CCL21 were required in previous studies for therapeutic efficacy. A delivery modality formulated to release pharmaceuticals over a sustained period may both ease SLC/CCL21 administration and increase its therapeutic effect, but no such methods have as yet been tested for SLC/CCL21 treatment of cancer. It is known that survival for breast cancer patients is greatly reduced when residual disease progresses following surgical resection, or when malignant cells metastasize. Initiation of a strong immune response at the primary tumor may be able to remove remaining disease following surgery, as well as eradicate any metastases. Intratumoral treatment with SLC/CCL21 has been demonstrated to increase local immune responses, but the ability of SLC/CCL21 to orchestrate immune removal of residual disease following surgical resection, or as a means to counter metastasis, has not been examined. The overall objective for this project is to further develop SLC/CCL21 as a practical immunotherapeutic for primary breast cancer through determining a highly effective delivery modality, and to determine the ability of SLC/CCL21 to direct the immune system to counter residual and metastatic disease. Our specific aims are to (1) examine a sustained release delivery modality for SLC/CCL21 in a treatment regimen of one intratumoral implantation, and (2) investigate if SLC/CCL21 given before or concurrently with surgical resection elicits an effective immune response against residual disease and metastases. These studies utilize a mouse breast cancer model to test *in vivo* immune cell manipulation by SLC/CCL21 with the goal of providing supportive data for translation of our findings into future clinical trials.

BODY

Statement of Work Task 1. Examine sustained delivery for SLC/CCL21 in a treatment regimen of one intratumoral implantation (Months 1-10).

Overview of Experimentation

Groups of BALB/c mice were injected in the mammary fat pad with CI-66, a highly metastatic adenocarcinoma cell line derived from a spontaneously arising BALB/c mammary tumor (1-2). After development of tumors, SLC/CCL21 (Peprotech, Rocky Hill, NJ) was delivered either via repeated injections in PBS or in a one-time implantation of a Hydron hydrogel polymer pellet (Interferon Sciences, New Brunswick, NJ) to allow sustained release. (Hydron is a commercially available drug delivery system that has been approved by the Federal Drug Administration for use in clinical trials of cancer treatments.) Control treatments (PBS only or PBS-Hydron) were also administered to additional groups of mice. The mice were monitored by twice weekly measurements of tumors after the initiation of therapy, and survival rates were recorded. A subset of each group of mice was used for characterization and quantification of tumor-infiltrating DCs, T cells, and NK cells. Mice that received the experimental and control treatments were also compared for differences in metastasis to the lung and bone.

Experimental Results

SLC/CCL21 effect on mammary tumor growth and immune cell infiltration. We found that administration of SLC/CCL21-Hydron implants into CI-66 primary tumors significantly slowed their growth (**Fig. 1**). In contrast, multiple injections of SLC/CCL21 (in the absence of a sustained release system) did not reduce the tumor growth rate (**Fig. 1**).

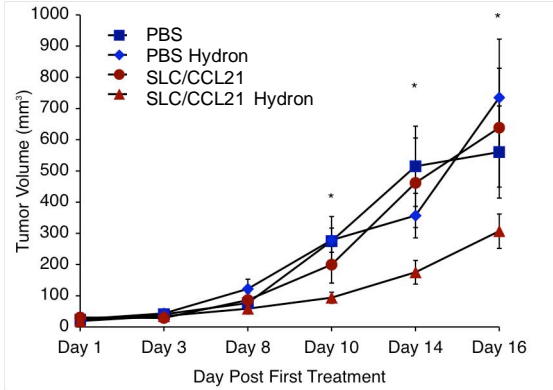


Fig. 1. CI-66 tumor growth was slowed by intratumoral SLC/CCL21-Hydron administration. BALB/c mice were injected in the fourth inguinal mammary fat pad with 1×10^5 CI-66 cells. Once tumors were palpable, the mice received 50 μ l intratumoral injections of 1 μ g SLC/CCL21 or PBS alone on days 1, 2, 3, 8, 9, and 10, or 6 μ g SLC/CCL21 in Hydron, or PBS-Hydron. Tumor growth was monitored with calipers, and volumes were calculated by the equation for a prolated sphere ($\text{width}^2 \times \text{length}/2$). By repeated measure Manova analysis, the p value for SLC/CCL21-Hydron versus PBS-Hydron = 0.01, and asterisks indicate significance. There are no significant differences when SLC/CCL21, PBS-Hydron, and PBS are compared.

Comparison of survival data from mice receiving SLC/CCL21-Hydron to that from the mice receiving the other treatments showed that that SLC/CCL21-Hydron significantly prolonged the duration of survival (e.g., median survival of CCL21-Hydron-treated mice = 46.0 days; median survival of PBS-Hydron-treated mice = 35.5 days; $p < 0.0001$). (Animals were sacrificed when tumor volumes reached 1700 mm^3 or moribund behavior (e.g., inability to obtain food and drink) was observed.) These results support our use of Hydron as a delivery system for CCL21.

In conjunction with the therapeutic effect of intratumoral SLC/CCL21-Hydron, increased numbers of infiltrating T cells and NK cells, but (surprisingly) not increased numbers of DCs, were detected in the tumor (**Fig. 2**). Both $\text{CD3}^+\text{CD8}^+\text{CD25}^-$ T cells and $\text{CD3}^+\text{CD8}^+\text{CD25}^+$ T cell populations were significantly increased (**Fig. 2**).

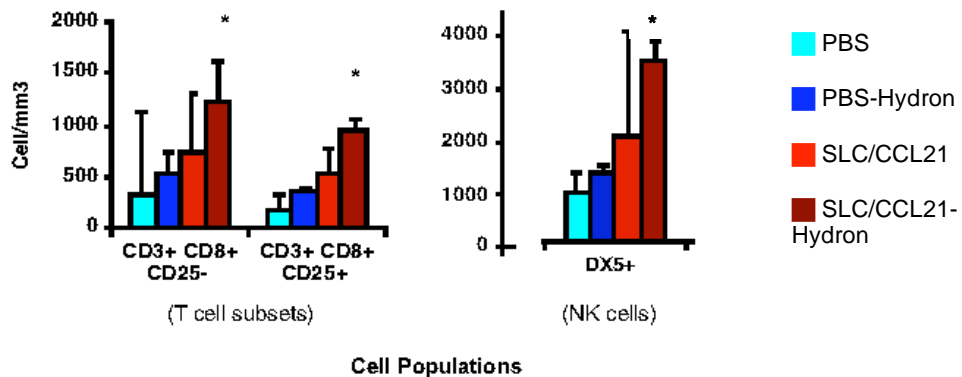


Fig. 2. Intratumoral SLC/CCL21-Hydron increased the absolute number of CD8^+ T cells and NK cells in CI-66 mouse mammary tumors. Tumors were initiated and treated as described for Figure 1. At 7 days post-treatment, tumors were resected, and infiltrating mononuclear leukocytes were isolated and assessed by flow cytometry. Data are presented as cells/tumor volume (mm^3) to normalize for minor differences in tumor sizes between groups. The DX5 monoclonal antibody recognizes CD49b, a marker expressed on a majority of NK cells in BALB/c mice. For $\text{CD8}^+\text{CD25}^-$, $\text{CD8}^+\text{CD25}^+$, and DX5^+ cells, the difference between the SLC/CCL21-Hydron group versus the PBS-Hydron or PBS group was significant (at $p < 0.05$, indicated by asterisks), and the differences between the SLC/CCL21-Hydron group versus the SLC/CCL21 group and the SLC/CCL21 group versus the PBS group were not significant ($p > 0.05$).

The CD3⁺CD8⁺CD25⁺ T cell subset predominantly consists of T cells that are cytolytically active, though it can also include rare T cells with a suppressive function (3-5). Our original plan included analyzing cytolytic function and cytokine secretion of intratumoral T cells (in comparison with lymph node and spleen T cells), but insufficient cells were obtainable from the tumors of the SLC/CCL21-treated mice to perform these additional assays. In total, our data shown in **Figures 1 and 2** suggest that SLC/CCL21-Hydron is effective at slowing orthotopic CI-66 tumor growth, and this effect is paralleled by induction of an intracellular infiltration by CD8⁺ T cells and NK cells, although not by DCs.

SLC/CCL21 effect on metastasis of mammary tumors. In our study, the number of metastases was found to be significantly lower in the lungs of SLC/CCL21-treated mice relative to PBS- and PBS-Hydron-treated mice (**Fig. 3, left panel**). Numbers of lung metastases in SLC/CCL21-Hydron-treated mice were significantly lower than in PBS-treated mice and trended lower than in PBS-Hydron-treated mice, although the p value for the comparison between the SLC/CCL21-Hydron-treated and PBS-Hydron-treated groups did not quite reach significance (p=0.058) (**Fig. 3, left panel**). Assessment of CI-66 tumor cells in the bone marrow showed that there was a trend toward fewer tumor cells in the bone of SLC/CCL21-Hydron-treated mice relative to PBS-Hydron-treated mice (p=0.16), although the difference was not significant at p<0.05 (**Fig. 3, right panel**). SLC/CCL21 was not more effective at reducing the number of tumor cells in the bone than PBS or PBS-Hydron (**Fig. 3, right panel**).

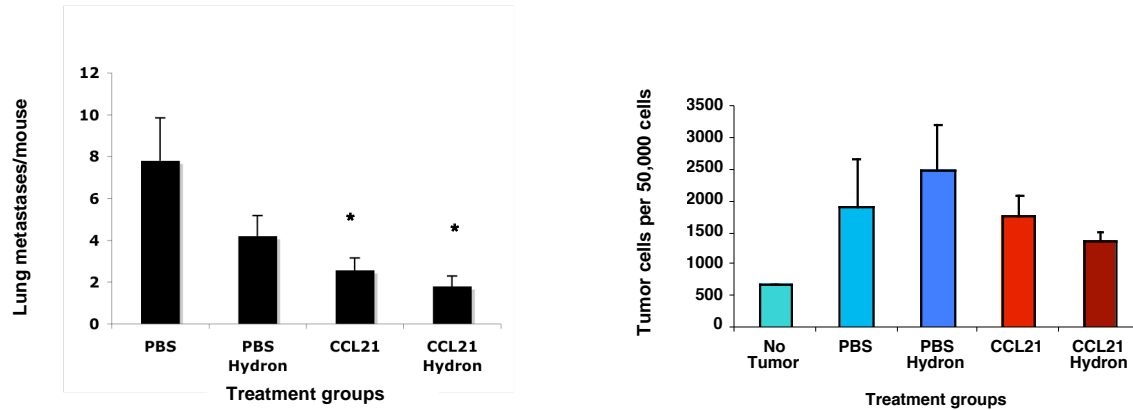


Fig. 3. Intratumoral administration of SLC/CCL21-Hydron significantly decreased the number of metastases in the lung, relative to PBS, and resulted in a trend toward a decrease in the bone marrow. BALB/c mice were injected orthotopically in the fourth inguinal mammary fat pad with 1×10^5 mammary tumor CI-66 cells. Once the tumors reached palpable size (at day 14 post tumor injection), the mice received 50 μ l intratumoral injections of 1 μ g SLC/CCL21 or PBS alone (days 1, 2, 3, 8, 9, and 10), or 6 μ g of SLC/CCL21 in Hydron or PBS-Hydron alone (day 1). At day 30 post-tumor cell injection (at the time the mice had developed 1700 mm³ diameter tumors), the mice were sacrificed and lungs and femurs were harvested. (**Left panel**) Lung metastases were enumerated. (**Right panel**) A single cell suspension was produced from bone marrow flushed from the femurs, and tumor cells in the bone marrow were enumerated.

Conclusions from Task 1. Our results from Task 1 indicate SLC/CCL21-Hydron intratumoral administration slows tumor growth significantly better than SLC/CCL21 without Hydron, PBS-Hydron, or PBS without Hydron. SLC/CCL21-Hydron lengthened significantly the duration of survival as compared to the other treatments tested. SLC/CCL21-Hydron implantation into tumors significantly increased T and NK tumor infiltration (compared to SLC/CCL21 without Hydron, PBS-Hydron, or PBS without Hydron), but not infiltration by DCs. In the T cell

infiltrates, both CD3⁺CD8⁺CD25⁺ (predominantly cytolytically active T cells) and CD3⁺CD8⁺CD25⁻ subsets were significantly increased. The numbers of lung metastases were significantly lower in the lungs of SLC/CCL21-Hydron-treated mice compared to PBS-treated mice and very nearly significantly lower for PBS-Hydron-treated mice.

Statement of Work Task 2. Investigate if SLC/CCL21 given before or concurrently with surgical resection elicits an effective immune response against residual disease and metastases (Months 11-22)

Overview

Groups of BALB/c mice were injected in the mammary fat pad with Cl-66, and tumors were allowed to develop. Because our experiments in Task 1 showed Hydron-SLC/CCL21-Hydron to elicit a greater therapeutic effect than SLC/CCL21-PBS, Hydron was used in Task 2 to provide sustained release of SLC/CCL21. The studies to accomplish Task 2 involved examining the effect of intratumoral administration of SLC/CCL21 prior to tumor resection (neoadjuvant treatment) and the effect of administration of SLC/CCL21 immediately post-surgery to the site from which the primary tumor has been resected (adjuvant treatment). Experimental animals were observed for survival, recurrence of primary tumors, and differences in metastasis. In the assessment of survival, animals were sacrificed when tumor volumes reached 1700 mm³ or the animals exhibited moribund behavior (e.g., inability to obtain food and drink).

Experimental Results

SLC/CCL21 as a neoadjuvant. Orthotopic mammary tumors were established by the injection of 1×10^5 cl-66 cells into the fourth inguinal mammary fat pad of female BALB/c mice. Once the tumors reached 60 mm³ (Day 0), PBS in Hydron or SLC/CCL21-Hydron (6 µg of CCL21/mouse) was implanted in the tumor. Four days following initial treatment, surgical resection of the tumors was carried out and the mice were monitored for survival, primary tumor recurrence, and metastases. As shown in **Figure 4**, SLC/CCL21-Hydron prolonged the survival of mice from which the primary mammary tumors had been resected significantly longer than PBS-Hydron did ($p=0.035$). A group of mice that received primary tumor resection but neither PBS-Hydron nor SLC/CCL21-Hydron survived for a length of time similar to that of the PBS-Hydron group; thus the SLC/CCL21-Hydron group survived significantly longer than the tumor resection only group ($p=0.046$) (**Fig. 5**).

Additional assessments of mice receiving SLC/CCL21 as a neoadjuvant. Mice that had received CCL21-Hydron neoadjuvant + tumor resection, PBS-Hydron neoadjuvant + tumor resection, or tumor resection only were examined for recurrence of the primary tumor or metastases to other sites. By day 70, in the CCL21-Hydron neoadjuvant group, no tumors at the primary site (i.e., the fourth mammary fat pad) or at any other sites were detectable by palpation following primary tumor resection. In contrast, about 30% of the mice in the PBS-Hydron neoadjuvant + tumor resection and tumor resection groups had palpable tumors (only very rarely located at the original tumor site). Subsets of the mice that had received CCL21-Hydron neoadjuvant + tumor resection, PBS-Hydron neoadjuvant + tumor resection, or tumor resection only were compared for metastases by euthanizing the animals and examining the lungs. Representative examples are shown in **Figure 6**. In our experiments to address Task 2, intratumoral immune cell infiltration was not examined, since we had assessed immune cell infiltration in Task 1 and infiltration results in this Task would not be expected to differ from our findings in Task 1.

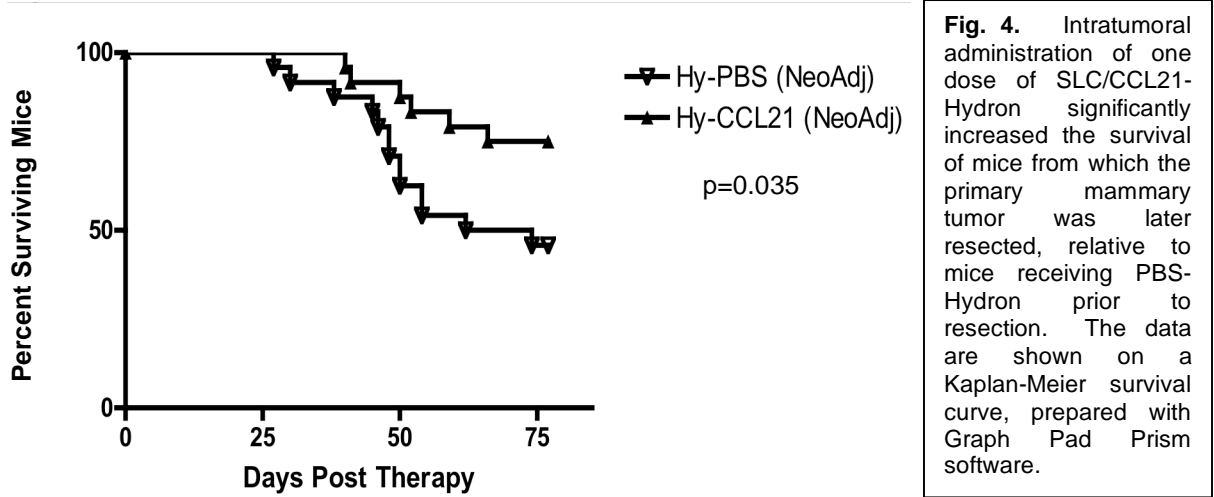


Fig. 5. Intratumoral administration of one dose of SLC/CCL21-Hydrone significantly increased the survival of mice from which the primary mammary tumor was later resected, relative to mice receiving resection alone. The data are shown on a Kaplan-Meier survival curve, prepared with Graph Pad Prism software.

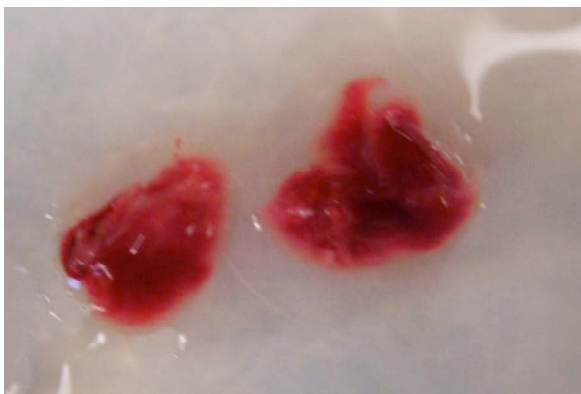
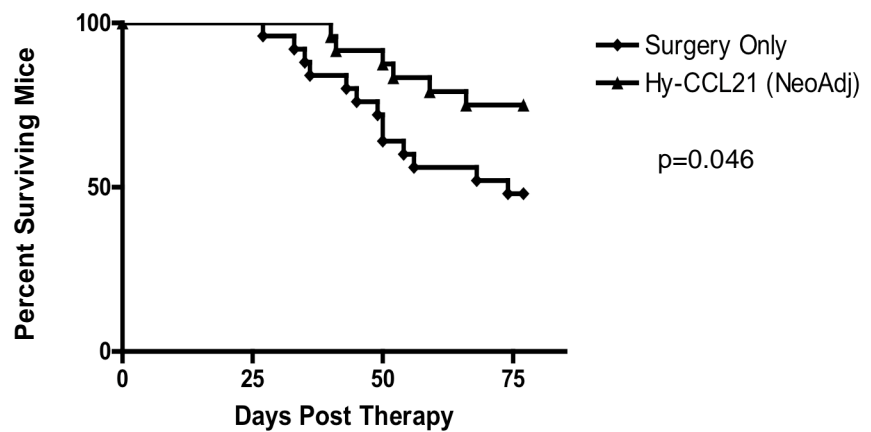


Fig. 6. SLC/CCL21-Hydrone neoadjuvant reduced lung metastases. Mammary tumors were established by the injection of 1×10^5 cl-66 cells into the fourth inguinal mammary fat pad of female BALB/c mice. Once the tumors reached 60 mm^3 (Day 0), PBS in Hydrone or SLC/CCL21-Hydrone ($6 \mu\text{g}$ of CCL21/mouse) was implanted in the tumors of some of the mice. Primary tumors were resected at four days following initial treatment, and subsets of the mice were monitored for metastases at day 70. Representative examples of lungs are shown from a mouse in the SLC/CCL21-Hydrone neoadjuvant group (*left panel*) and one in the tumor resection only group (*right panel*). The lungs shown on the right are very pale and rigid due to heavy infiltration with metastases.

SLC/CCL21 as an adjuvant. In addition to testing SLC/CCL21-Hydron as a neoadjuvant, we also tested it as an adjuvant to surgical resection. In this case, SLC/CCL21-Hydron treatment was performed immediately following resection of the primary tumor. We demonstrated that the duration of the survival of the SLC/CCL21-Hydron adjuvant group was clearly no better than that of the surgery only group ($p=0.5256$). Our assessment is that the investigation of SLC/CCL21-Hydron in the adjuvant setting should not be further pursued in this preclinical breast cancer model. Because there was no therapeutic effect observed from the use of the SLC/CCL21 in the adjuvant setting, for the SLC/CCL21-Hydron adjuvant-treated mice we did not examine immune infiltration of the surgical site, lymph nodes, or spleen, nor did we assess cytotoxic activity of cells from the nodes or spleen.

Conclusions from Task 2. SLC/CCL21-Hydron was not effective as an adjuvant to surgery in our study, likely because the number of remaining tumor cells after tumor resection was insufficient to provoke a strong immune response when SLC/CCL21 attracted immune cells to the surgical site. In contrast, SLC/CCL21-Hydron was a highly effective neoadjuvant to surgery, prolonging the duration of survival significantly and reducing the incidence of metastases in comparison to control (PBS-Hydron neoadjuvant) and to tumor resection alone. These results suggest that SLC/CCL21 neoadjuvant treatment might be of great clinical benefit to breast cancer patients.

Statement of Work Task 3. Final data analysis and manuscript writing (Months 22-24)

Overview

The results obtained in our studies are described in two manuscripts, one that has already been submitted, and one that is in preparation. It is anticipated that the manuscript in preparation will be submitted within this month.

Results

The manuscripts containing data from this project are as follows:

Turnquist, H. R., A. E. Ashour, X. Lin, N. M. Burns, X. Wang, A. Tuli, M. A. Hollingsworth, R. K. Singh, J. E. Talmadge, and J. C. Solheim. Intratumoral CCL21 administration: similarities and disparities in effects on mammary and pancreatic tumors. *Clinical Cancer Res.*, submitted.

Ashour, A. E., X. Lin, H. R. Turnquist, N. M. Burns, X. Wang, A. Tuli, R. K. Singh, J. E. Talmadge, and J. C. Solheim. Efficacy of CCL21 implantations used in conjunction with surgical resection in a mouse mammary tumor model. In preparation.

KEY RESEARCH ACCOMPLISHMENTS

- SLC/CCL21 delivered intratumorally into CI-66 mammary tumors by a sustained release system (Hydron) was found to significantly slow growth of the treated primary tumors, prolong survival, and increase intratumoral infiltration by CD8⁺ T cells and NK cells. The T cell CD3⁺CD8⁺CD25⁺ and CD3⁺CD8⁺CD25⁻ subsets were both significantly increased, and the CD3⁺CD8⁺CD25⁺ cells have been previously demonstrated to be predominantly cytolytically active T cells.

● SLC/CCL21-Hydrone and SLC/CCL21 treatments of primary CI-66 tumors were observed to reduce the number of lung metastases significantly relative to PBS treatment. The number of lung metastases was discovered to trend lower in SLC/CCL21-Hydrone-treated mice relative to PBS-Hydrone-treated mice, and to be significantly lower in SLC/CCL21-treated mice relative to PBS-Hydrone-treated mice. There was also a trend toward fewer CI-66 tumor cells in the bone of SLC/CCL21-Hydrone-treated mice relative to PBS-Hydrone-treated mice, although the difference was not significant at $p < 0.05$. SLC/CCL21 injection of primary tumors did not decrease metastasis to the bone, compared to either PBS or PBS-Hydrone.

● SLC/CCL21-Hydrone was found to be ineffective as a surgery adjuvant in our study; however, SLC/CCL21-Hydrone was demonstrated to be a very effective surgery neoadjuvant. SLC/CCL21 lengthen the duration of survival significantly, relative both to PBS-Hydrone neoadjuvant) and to tumor resection alone. These results suggest that SLC/CCL21-Hydrone neoadjuvant treatment may be of considerable benefit in the clinical treatment of human breast cancer.

REPORTABLE OUTCOMES

● This project allowed the original principal investigator, Heth Turnquist, to acquire data contributing toward the completion of his Ph.D. degree.

● Data and expertise gained by the original principal investigator, Heth Turnquist, during the course of this project contributed to his obtaining a post-doctoral position in immunotherapy research at the University of Pittsburgh, beginning July 1, 2005.

● This project has allowed the current principal investigator, Abdelkader Ashour, to acquire data contributing toward the completion of his Ph.D. degree (anticipated to be awarded by July-August 2006).

● Data and expertise gained by the current principal investigator, Abdelkader Ashour, during the course of this project have contributed to his applications for positions following his graduation this summer.

● The project has provided translational data relevant to a potential treatment modality for breast cancer that was reported at the Era of Hope Meeting in 2005. (Turnquist, H. R., R. K. Singh, J. E. Talmadge, and J. C. Solheim. SLC/CCL21 treatment of primary mammary tumors inhibits their growth and reduces spontaneous lung metastases. Presented in the Vaccines and Immunotherapies Poster Session at the Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting, June 2005.)

● The project provided preliminary data included in a proposal submitted by the fellowship mentor, Dr. Joyce Solheim, to the National Cancer Institute as part of a Program Project Grant application.

(Title of Program: Dendritic Cell Manipulation and Breast Cancer Therapy

Title of Project 5: Dendritic Cell Chemotaxis in Neoadjuvant Breast Cancer Therapy

Program Leader: James Talmadge, Ph.D.

Project 5 Leader: Joyce Solheim, Ph.D.

Other Project Leaders: Dmitry Gabrilovich, M.D., Ph.D.; Kenneth Cowan, M.D., Ph.D.; James Talmadge, Ph.D.; Rakesh Singh, Ph.D.)

● One manuscript has been submitted, and another is in preparation, that include data derived from this project:

Turnquist, H. R., A. E. Ashour, X. Lin, N. M. Burns, X. Wang, A. Tuli, M. A. Hollingsworth, R. K. Singh, J. E. Talmadge, and J. C. Solheim. Intratumoral CCL21 administration: similarities and disparities in effects on mammary and pancreatic tumors. *Clinical Cancer Res.*, submitted.

Ashour, A. E., X. Lin, H. R. Turnquist, N. M. Burns, X. Wang, A. Tuli, R. K. Singh, J. E. Talmadge, and J. C. Solheim. Efficacy of CCL21 as a neoadjuvant in a mouse mammary tumor model. In preparation.

CONCLUSIONS

Overall, taking the effect on primary tumor growth rate, survival, metastasis, and immune cell infiltration into account, SLC/CCL21-Hydron is superior to SLC/CCL21 protein delivered without Hydron as a potential breast cancer therapy (Task 1). Furthermore, we have assessed the therapeutic effect of administering SLC/CCL21-Hydron as a neoadjuvant prior to tumor resection or as an adjuvant immediately following tumor resection (in terms of survival, recurrence of the primary tumor, and metastases) (Task 2). These latter studies have shown that SLC/CCL21-Hydron is not therapeutic as an adjuvant, but does show significant therapeutic benefit in this mouse mammary tumor model as a neoadjuvant. These results support the testing of SLC/CCL21-Hydron in clinical trials in the neoadjuvant setting.

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