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13. ABSTRACT (Maximum 200 Words) To generate anti-tumor immune responses, T cells must interact with mature dendritic cells (DCs) presenting tumor-derived peptides. Natural killer (NK) cells, DCs, and T cells express a common receptor, CCR7, which binds the chemokine SLC/CCL21. SLC/CCL21 is normally expressed in the lymphoid organs and coordinates the interactions between DCs and T cells, thus initiating T cell responses. 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 progression of the human disease. Utilizing this model, we found that treatment of orthotopic tumors with sustained SLC/CCL21 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. Current studies in Task 2 are examining if the resection of the primary tumor in combination with sustained SLC/CCL21 given before or concurrent with resection will potentiate the therapeutic effects observed in Task 1. 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, and therefore a promising treatment for breast cancer patients.				
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	8
References.....	8
Appendices.....	none

SUBJECT: ANNUAL SUMMARY REPORT**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 Cl-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. 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. Our studies analyzed SLC/CCL21 effects on tumor growth, intratumoral leukocyte infiltration, and metastasis. 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).

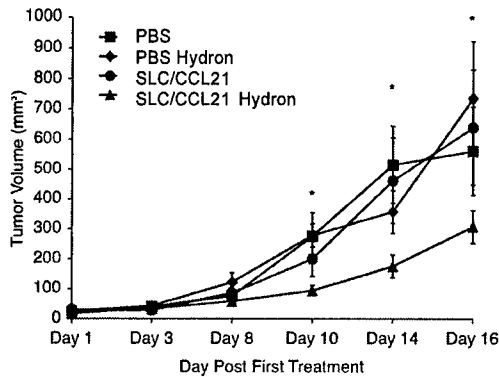


Fig. 1. CI-66 tumor growth was slowed by intratumoral SLC/CCL21-Hydron administration. BALB/c mice were injected in the fourth inguinal mammary fat pad with 1×10^5 CI-66 cells. Once tumors were palpable, the mice received 50 μ l intratumoral injections of 1 μ g SLC/CCL21 or PBS alone on days 1, 2, 3, 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.

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).

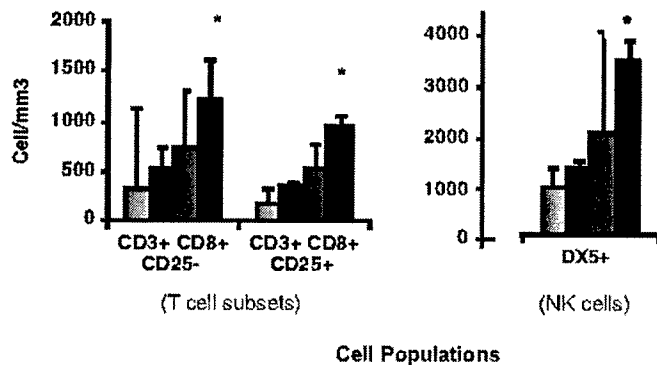


Fig. 2. Intratumoral SLC/CCL21-Hydron increased the absolute number of CD8^+ T cells and NK cells in CI-66 mouse mammary tumors. Tumors were initiated and treated as described for Figure 1. At 7 days post-treatment, tumors were resected, and infiltrating mononuclear leukocytes were isolated from the tumors. The percentages of cells in specific subsets within the tumors were assessed by flow cytometry, using a BD FACS Vantage and Attractors software (BD Immunocytometry Systems, San Jose, CA). 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 each T cell subset and for NK cells, treatments are indicated as follows (*left to right*): PBS, SLC/CCL21, PBS-Hydron, and SLC/CCL21-Hydron. 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**).

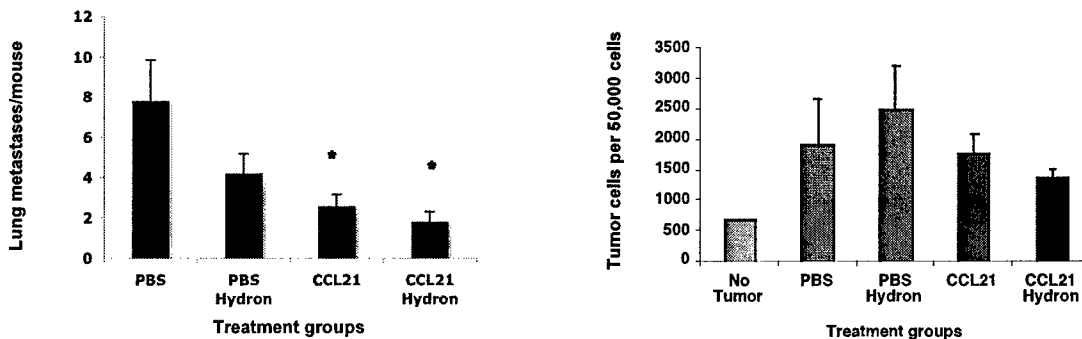


Fig. 3. Intratumoral administration of SLC/CCL21-Hydron significantly decreased the number of metastases in the lung, relative to PBS, and resulted in a trend toward a decrease in the bone marrow. BALB/c mice were injected orthotopically in the fourth inguinal mammary fat pad with 1×10^5 mammary tumor 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.

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)

As scheduled, our studies for Task 2 are currently underway. The above findings demonstrate that administration of SLC/CCL21 via the delivery of a single sustained release Hydron pellet

has better therapeutic efficacy than multiple injections of SLC/CCL21 in PBS. Hence, current studies to accomplish Task 2 involve the comparison of intratumoral administration of SLC/CCL21-Hydron prior to tumor resection to administration of SLC/CCL21-Hydron immediately post-surgery to the site from which the primary tumor has been resected. Experimental animals will be observed for increased survival, recurrence of primary tumors, and differences in metastasis. The number of treated and control mice with relapse of the primary tumor, as well as the number of metastases per mice, will be ascertained.

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 and increase intratumoral infiltration by CD8⁺ T cells and NK cells.
- SLC/CCL21-Hydron 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-Hydron-treated mice relative to PBS-Hydron-treated mice, and to be significantly lower in SLC/CCL21-treated mice relative to PBS-Hydron-treated mice. There was also a trend toward fewer CI-66 tumor cells in the bone of SLC/CCL21-Hydron-treated mice relative to PBS-Hydron-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-Hydron.

REPORTABLE OUTCOMES

- This project allowed the principal investigator, Heth Turnquist, to acquire data contributing toward the completion of his Ph.D. degree. (Accomplishment of all degree requirements is anticipated by June 30, 2005.)
- Data and expertise gained by the 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.
- The project has provided translational data relevant to a potential treatment modality for breast cancer that will be reported at the Era of Hope Meeting this summer. (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. Submitted for presentation in the P45 Vaccines and Immunotherapies Poster Session at the Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting, June 2005.)
- The project has provided preliminary data included in a proposal submitted 02/01/05 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.)

CONCLUSIONS

Overall, taking both effect on primary tumor growth rate and metastasis into account, SLC/CCL21-Hydrion appears superior to SLC/CCL21 protein delivered without Hydrion as a potential breast cancer therapy. Studies are currently underway to determine the therapeutic effect of administering SLC/CCL21 preceding or concurrent with surgical resection against residual primary tumor and metastases.

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