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In vivo efficacy of chitosan/interleukin-12 in controlling lung metastasis in 4T1 breast cancer model

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Abstract

Cancer immunotherapy has emerged as a leading front in cancer treatment. In contrast to other treatment methods such as radiation and surgery, immunotherapy trains the patient's body to recognize and eliminate tumors, thus preventing reoccurrence of secondary tumors. IL-12 has been shown to display a potent anti-tumor immune response in mice; however, when clinical trials were conducted, it was shown to be toxic and in some cases fatal when administered systemically.[1,2] Because of this, local delivery of IL-12 is under investigation. Our lab has shown that when paired with the polymer chitosan, local, intratumoral (i.t.) injections of IL-12 are retained in the tumor microenvironment and can lead to the elimination of tumors and generate tumor specific immune cells while reducing toxicity.[3,4] In the present study, these previous findings are expanded upon and the potential for the coformulation of chitosan and IL-12 (chitosan/IL-12) to control metastasis is evaluated in a highly metastatic mammary adenocarcinoma model, 4T1. Mice treated with chitosan/IL-12 neoadjuvant to primary tumor resection showed increased survival rate when compared to mice treated with IL-12 alone. Mice treated with chitosan/IL-12 were then shown to contain fewer lung metastases. Moreover, a clinically relevant means of confirming immunity via delayed type hypersensitivity (DTH) response was observed in cured mice. Toxicology analysis also indicated that intratumoral chitosan/IL-12 induced minimal toxicity. This study demonstrated that neoadjuvant chitosan/IL-12 prior to tumor resection is safe and can generate an anti-tumor response that offers protection from metastatic disease.

Introduction

The transformation of normal cells to cancerous cells results in gene mutations that may potentially act as foreign antigen to initiate an immune response; however, tumor cells release factors that induce immunosuppression. The tumor microenvironment contains tumor-infiltrating lymphocytes which have the capabilities to launch a potent anti-tumor response; however, their activity is suppressed by factors such as transforming growth factor beta and interleukin-6 which favor the

development of immunosuppressive cell populations such as myeloid-derived suppressor cells and regulatory T cells. These cell types release factors that further suppress the immune system. As a result, the cascade of signals that lead to immune activation is inhibited and the tumor is allowed to grow and develop undetected.

Cytokine therapy has emerged as an effective means of overcoming the immunosuppressive tumor microenvironment by boosting the immune response to tumor antigens. Interleukin-12 (IL-12) has been shown to generate a potent anti-tumor response and tumor-specific immunity. Mechanisms include (i) activation and expansion of CD8+ T cells and natural killer cells, (ii) increased production of interferon-gamma and (iii) suppression of angiogenesis, (iv) enhanced trafficking of T cells, and (v) activation of dendritic cells; however, when evaluated under clinical trials, it has been shown to be toxic and sometimes fatal.[1,2,5] In a Phase 2 clinical trial involving 17 patients, systemic administration of IL-12 resulted in 12 hospitalizations and 2 deaths.[2] The failure of these studies may have resulted from the inability of systemic IL-12 delivery to achieve biologically relevant IL-12 concentrations in the tumor microenvironment at the maximum tolerated dose in humans (<500 ng/kg).[2,3] Local delivery strategies are now being investigated to maximize IL-12 delivery to the tumor microenvironment while reducing systemic toxicity.

Our lab has demonstrated that when paired with the polymer chitosan, the local delivery of IL-12 is significantly enhanced and can lead to the elimination of tumors while minimizing toxicity.[3,4] Chitosan is a natural polysaccharide derived from the chitin existing in the exoskeletons of crustaceans and insects as well as the cell walls of fungi. Chitosan is non-toxic (LD50 > 16 g/kg), biodegradable, non-immunogenic, and widely used in a number of commercial and biomedical applications.[6] Chitosan's high viscosity allows for enhanced retention of IL-12 in the tumor microenvironment and has also been shown to loosen gap junctions to enhance paracellular transport.[7]

In addition to eliminating murine tumors, our lab has shown that local chitosan/IL-12 immunotherapy can generate immune cells that can recognize and attack tumor cells; however, it is not known if these cancer-specific immune cells can prevent metastasis.[3,4] The ability of new cancer therapies to control metastasis is crucial since 9 of 10 cancer patient deaths are due to secondary tumors.[8] Immunotherapies, such as chitosan/IL-12, have the potential to provide protection from recurrence and reduce complications due to secondary tumors.

In this study, I evaluated the efficacy of neoadjuvant chitosan/IL-12 immunotherapy prior to primary tumor resection in reducing lung metastasis in the 4T1 breast cancer model. Efficacy was analyzed using survival rates and metastasis quantification. Furthermore, a DTH response was evaluated to identify a potential method for confirming tumor specific immunity. Chitosan/IL-12 related toxicity was also assessed to observe any effect on toxicity associated with IL-12.

Materials and Methods

Animals, cell line, and reagents

Female BALB/c mice, 8-12 weeks old, were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed and maintained under pathogen-free conditions in microisolator cages. Animal care was in compliance with the recommendations of *The Guide for Care and Use of Laboratory Animals* (National Research Council).

The parental 4T1 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's Modified Eagle Medium containing high glucose and 10% heat-inactivated fetal bovine serum supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (all from Hyclone, Logan, UT).

Recombinant murine IL-12 was purchased from Peprotech (Rocky Hill, NJ). Chitosan glutamate (Protosan G 213) was purchased from NovaMatrix (Sandvika, Norway).

Tumor model

Tumors were generated via subcutaneous installation of $1x10^5$ 4T1 tumor cells/100 µL PBS in the shaved left flank of each mouse. Mice were anesthetized with ketamine (75 mg/kg)/xylazine (15 mg/kg) prior to implantation. Treatments containing 50 µL of 1.5% (w/v) chitosan admixed with IL-12 (2 µg) in DPBS, IL-12 (2 µg) in DPBS, 1.5% (w/v) chitosan in DPBS, or DPBS were administered via intratumoral injections on days 6, 9 and 12 following tumor implantation. Mice were anesthetized prior to treatments.

Resection of primary tumor

To model neoadjuvant immunotherapy, surgical resection was performed following intratumoral treatments. Mice were anesthetized, and tumors were resected as described previously 15 days following tumor implantation.[9] The 4T1 model develops lung micrometastasis as soon as 5-7 days following implantation; therefore, there sufficient time has elapsed for metastasis to occur at the time of resection. Wounds were closed using wound clips. Clips were removed once wounds were healed.

Survival analysis following primary tumor resection

Following primary tumor resection, mice were kept alive until they became morbid, at which point they were euthanized. Examination of lungs confirmed death due to metastatic disease. Mice surviving >80 days were deemed cured.

Lung metastasis quantification

Metastasis quantification was performed using 6-thioguanine supplemented medium as described previously.[9] Briefly, 5 weeks following primary tumor resection, 3 to 5 mice from each group were euthanized and lungs were removed. Lungs were then minced and digested using collagenase type IV/elastase cocktail. (Worthington Biochemical, Lakewood, NJ). Samples were filtered and cultured for 14 days in medium containing 6-thioguanine (Sigma-Aldrich, St. Louis, MO). Methylene blue was then used to stain and visualize 4T1 colonies.

Evaluation of antigen specific delayed type hypersensitivity (DTH) response

A radiation dose of 3000 cGy was used to attenuate 4T1 cells. Cured mice were given intradermal injections of $1x10^5$ irradiated 4T1 cells/10 µL PBS in one ear and 10 µL PBS in the opposite ear. Prior to injection and 24 hours post injection, ear thickness was measured using a thickness gage (Mitutoyo, Aurora, IL).

Whole blood toxicology following treatment

Blood composition and chemistry analyses were performed on mice bearing 4T1 tumors treated with chitosan/IL-12 or DPBS via intratumoral injection. A separate cohort of mice received 4 consecutive daily doses of IL-12 (2µg) via intraperitoneal injection to serve as a positive control for toxicity. Submandibular bleeding was performed to collect blood samples. Samples were analyzed for leukocytes, lymphocytes, monocytes, neutrophils, red blood cells, hemoglobin, hematocrit, albumin, alkaline phosphatase, alanine aminotransferase, amylase, total bilirubin, blood urea nitrogen, calcium, phosphorus, creatinine, glucose, sodium, potassium, total protein and globulin. Blood composition and chemistry analyses were performed on a VetScan HM5 and VetScan VS2 respectively (both from Abaxis, Union City, CA).

Results

Chitosan/IL-12 neoadjuvant to tumor resection increased survival rate

Survival analysis indicated that 63% of mice treated with chitosan/IL-12 and 20% of mice treated with IL-12 alone survived >80 days and were deemed cured (Figure 1). All mice treated with chitosan or saline died within 35 days. Lung metastases were observed in all mice that died.

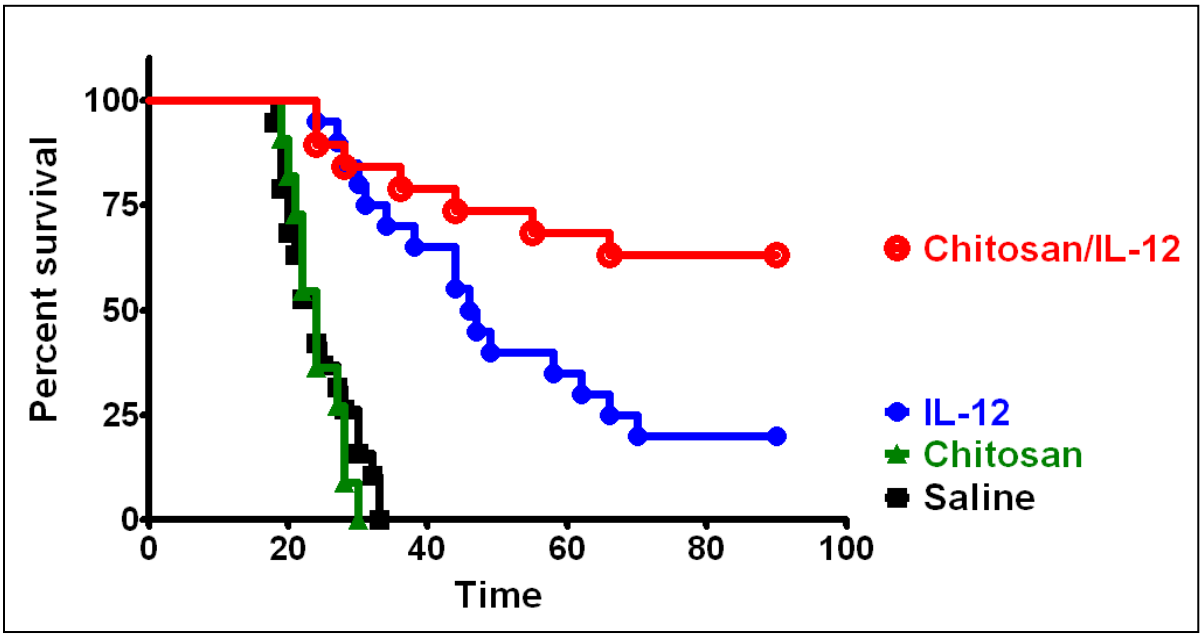

Figure 1: Survival rate of mice following primary tumor resection after three rounds of intratumoral treatment on days 6, 9 and 12 after tumor implantation. Resection was performed on day 15.

Neoadjuvant chitosan/IL-12 reduced lung metastasis

Cell cultures from mice treated with chitosan/IL-12 displayed an average of 34.8±48.0 colonies with 2 of 5 plates containing no colonies (Figure 2). Mice treated with IL-12 alone displayed an average of 76.25±152.5 colonies and mice treated with saline displayed an average of 80±2.83 colonies. 3 of 4 samples from mice treated with IL-12 alone displayed no colonies. All samples from saline treated mice d

	Chitosan/IL-12	IL-12	Saline
average (colonies)	34.8	76.25	80

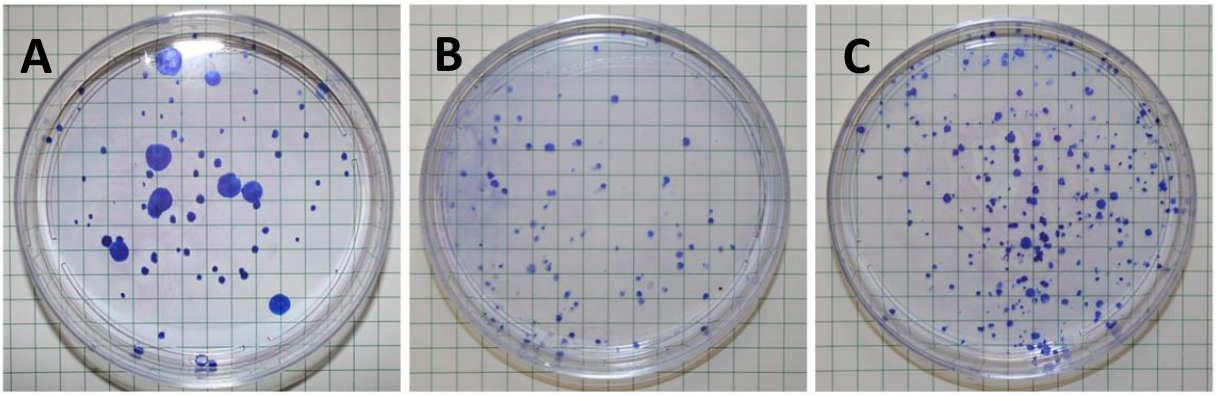

Figure 2: Culture plates containing 4T1 cells cultured from mice treated with (A) chitosan/IL-12, (B) IL-12 or (C) saline. Intratumoral treatments were administered on days 6, 9 and 12 after tumor implantation and resection was performed on day 15. Mice were euthanized 5 weeks after resection.

DTH response generated in cured mice

Ears of cured mice that were injected with irradiated 4T1 cells displayed a 37.5±10.5% increase in ear thickness 24 hours post injection (Figure 3). Increase in ear thickness was visible upon close inspection. Inflammation in the form of redness was also visible. The opposite ear which was injected with saline showed a 0.6±4.0% increase. Naïve mice showed an increase in ear thickness of 2.7±2.3% and 2.3±4.0% when injected with irradiated 4T1 cells and saline respectively. Inflammation was not present in these ears.

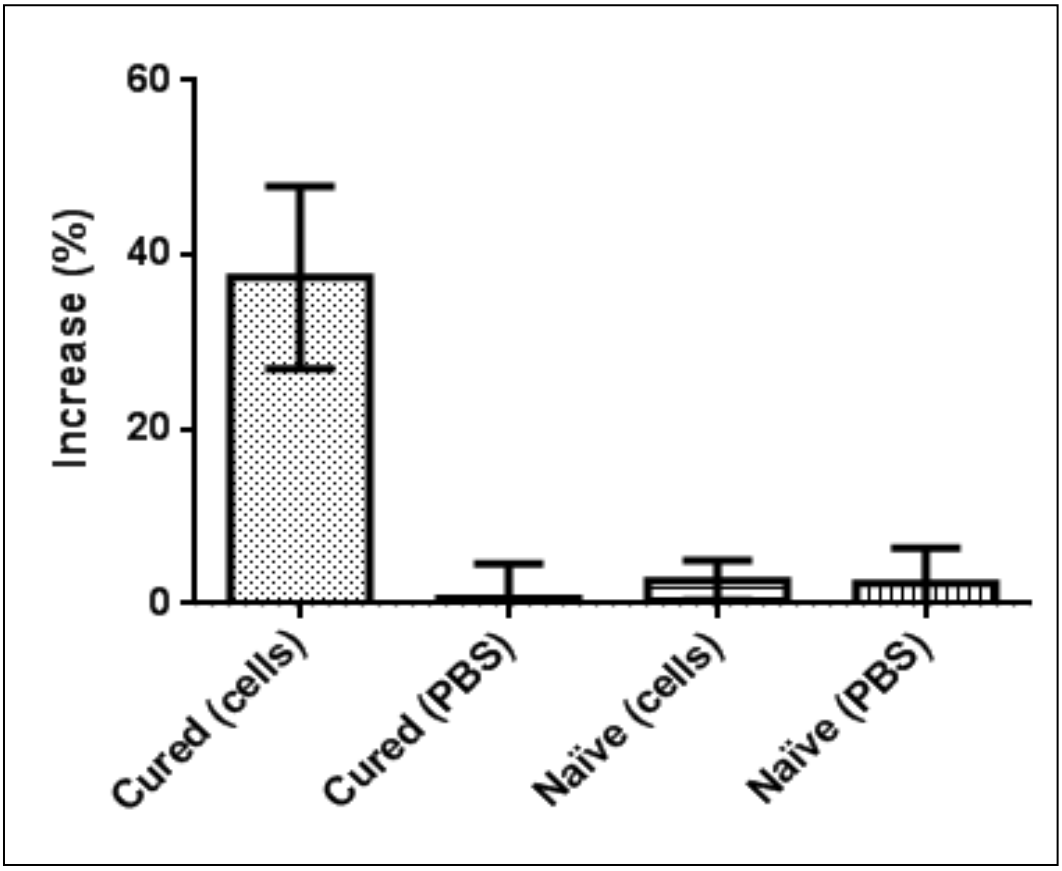

Figure 3: Increase in ear thickness 24 hours following intradermal injection with $1x10^5$ irradiated 4T1 cells in one ear and saline in the other of cured and naïve mice.

Intratumoral chitosan/IL-12 induced minor toxicity

Mice treated with chitosan/IL-12 did not show adverse response in ALT levels. Mean ALT levels in mice treated were PBS were 38±12.7 U/L and mice treated with chitosan/IL-12 were 43±7.5 U/L (Figure 4). Mice that were administered 4 consecutive IP doses of IL-12 showed a spike in ALT levels to 249±13.2 U/L. Leukopenia was seen in both mice treated with chitosan/IL-12 and consecutive IP doses of IL-12. Total leukocyte levels decreased from $7.34\pm1.3x10^9$ cells/L (PBS) to $4.59\pm2.5x10^9$ cells/L (chitosan/IL-12) and $1.42\pm0.2x10^9$ cells/L (IP lL-12). Lymphocyte levels decreased from $4.89\pm1.2x10^9$ cells/L (PBS) to $2.98\pm2.0x10^9$ cells/L (chitosan/IL-12) and $0.86\pm0.1x10^9$ cells/L (IP lL-12). Neutrophil levels decreased from $2.11\pm0.6x10^9$ cells/L (PBS) to $1.34\pm0.5x10^9$ cells/L (chitosan/IL-12) and $0.45\pm0.1x10^9$ cells/L (IP lL-12). There were no significant differences in red blood cells, hemoglobin, hematocrit, albumin, alkaline phosphatase, alanine aminotransferase (ALT), amylase, total bilirubin, blood urea nitrogen, calcium, phosphorus, creatinine, glucose, sodium, potassium, total protein or globulin.

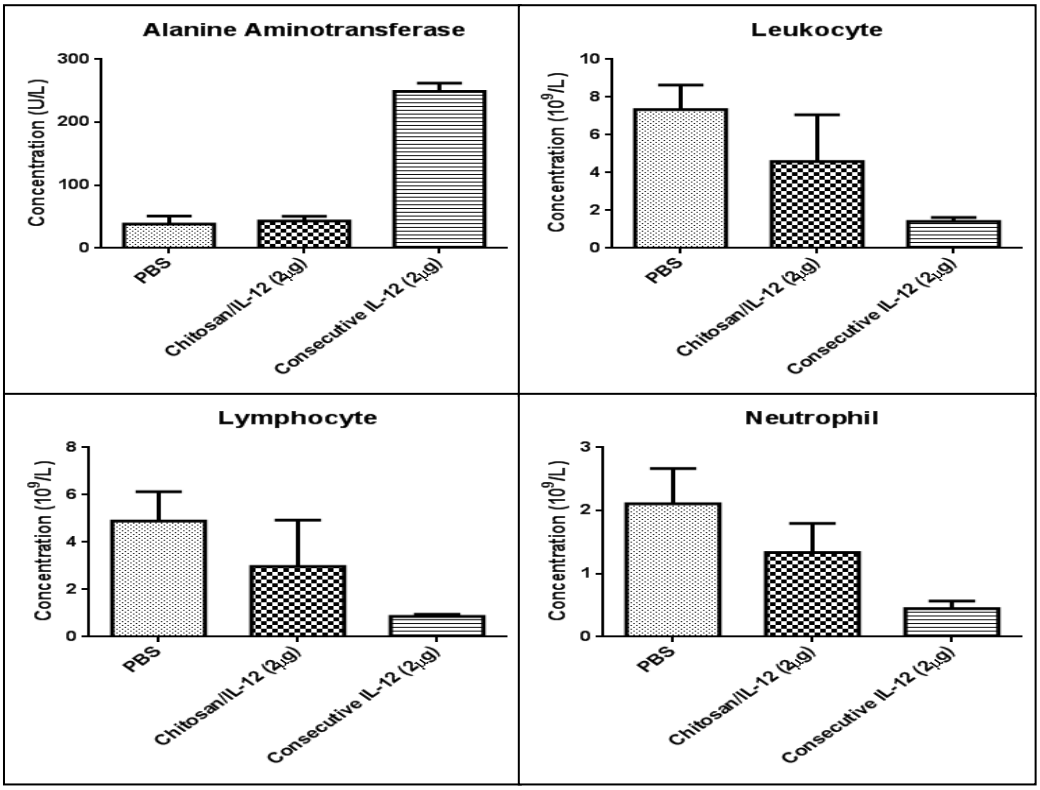

Figure 4: ALT, leukocyte, lymphocyte and neutrophil levels 24 hours after treatment with saline, chitosan/IL-12 or 4 consecutive intraperitoneal injections of IL-12.

Discussion

This study demonstrated that mice treated with intratumoral chitosan/IL-12 both increased survival rate and reduced the extent of metastasis. Results from previous studies showed that chitosan/IL-12 elicited a tumor-specific immune response that protected mice from rechallenge at the primary site; however, the efficacy of preventing metastasis relies on the ability of the immune system to launch a response within an appropriate window of time before metastatic tumor burden becomes too great. As presented in the results, metastasis was reduced when treated with only three rounds of treatment in a time span of 12 days.

Methods presented in this study also demonstrated a clinically relevant model in which the primary tumor was excised following immunotherapeutic treatment. This falls in line with the standard practice of surgically removing the tumor in breast cancer patients. A DTH response was also shown to be a viable method for detecting immunity against a specific tumor. As seen by the widely popular Mantoux test for tuberculosis, DTH analysis is an effective and efficient method for determining exposure and immunity to a specific antigen. Together, these two studies indicate that chitosan/IL-12 based immunotherapy is a highly translatable method for implementation in the clinic.

Toxicity associated with systemic IL-12 administration was also addressed. Liver function was assessed using ALT levels and results indicate normal function when compared to positive and negative controls. Leukopenia was observed 24 hours after chitosan/IL-12 treatment; however, the extent of leukopenia was considerably less than that of mice treated with consecutive IP IL-12. Leukopenia can be tolerated, but additional studies carried out at 48 hours and 72 hours post treatment will determine the duration of leukopenia.

As a whole, the research conducted in this study further validated the promise of chitosan/IL-12 as a means of controlling tumor recurrence and metastasis. Chitosan/IL-12 was shown to (i) increase survival rate following primary tumor resection, (ii) reduce the extent of lung metastasis, (iii) confirm

immunity that can be detected via DTH response, and (iv) alleviate concerns regarding IL-12 mediated toxicities. With this in mind, chitosan/IL-12 based immunotherapy may finally allow IL-12 to fulfill its potential in treating cancer effectively.

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