Streptavidin: A Novel Immunostimulant for the Selection and Delivery of Autologous and Syngeneic Tumor Vaccines

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Abstract

Induction of antitumor immunity using autologous tumor proteins is an attractive approach to cancer therapy. However, better methods and stimulants to present these autologous proteins back to the immune system are needed. Here, we identify streptavidin as a novel carrier protein and stimulant, and test the efficacy of both syngeneic (rat) and autologous vaccines (dogs) using streptavidin in combination with reduced soluble tumor proteins. Initial syngeneic vaccine studies in the 9L rat glioma model were used to optimize vaccine dose and selectivity. Cytokine and blood analysis was used to monitor the response. Rats receiving two vaccinations of syngeneic tumor vaccine demonstrated a statistically significant (P<0.05) survival advantage compared with controls (adjuvant only). Notably, vaccination also led to remission rates of between 30% and 60% in the aggressive 9L glioma model. Antibodies to streptavidin were detected in the serum of vaccinated rats; however, antibody levels did not correlate with the response. The cytokine TNF-α was upregulated in vaccine-treated rats, whereas ICAM1 was downregulated. After engraftment, vaccinated rats maintained CD4+ and CD8+ T cells, and total lymphocyte levels closer to normal baseline than those in the controls. Twenty-five dogs treated with autologous vaccine preparations using streptavidin as a stimulant showed no adverse reactions, irrespective of additional chemotherapy and other medications. In this study, we developed a novel method for producing syngeneic and autologous vaccines using streptavidin selectivity and immunogenicity. These vaccines show efficacy in the 9L glioma rat model. Safety was also demonstrated in canine patients presenting with cancer treated with autologous vaccine. Cancer Immunol Res 2(5): 469–79. ©2014 AACR.

Introduction

Cancer immunotherapy continues to gain recognition as an oncology treatment as new and improved cancer vaccines and other immunostimulants are produced. Using a patient’s own tumor proteins to produce a vaccine is an attractive concept, and a number of autologous vaccines are undergoing preclinical evaluation (1). Other immune therapies, such as dendritic cell therapy, have moved into the clinic, but while they increase survival time in patients they do not initiate immunologic rejection or remission of a tumor (2, 3). Induction of immunologic rejection and remission remains the holy grail of cancer therapy. As more information on the complexities of immune suppression and stimulation and how to manipulate these emerges, better immune therapies will follow.

Cancer vaccines aimed at promoting an immune response to combat cancer vary greatly in design from whole tumor cell, cell and tumor lysate, tumor antigen, dendritic cell–mediated, DNA-, and bacterial-derived vaccines (4, 5). Most of these approaches use the patient’s own material either directly or for extraction or stimulation in vitro (6). These autologous vaccine types match the patient’s MHC molecules as well as tumor-associated antigens. Although this is an advantage, tumor cells or proteins alone generally have poor immunogenicity, probably because the antigens are readily tolerized and they differ little from self. Various immunostimulants are used to overcome the tolerization effect (6, 7), the most well known being Bacille Calmette–Guerin (BCG), which has been used with irradiated autologous tumor cells in several trials (6).

Several autologous cancer vaccines that showed promise in early-phase studies have failed to deliver survival benefits for patients with advanced disease, showing benefits only for early-stage disease (8, 9). Therefore, work to produce autologous vaccines, which can demonstrate efficacy in all stages of disease, needs to continue.

In this study, we identify streptavidin as a novel immunostimulant and use this characteristic for the development of both syngeneic and autologous vaccines. Syngeneic vaccines are assessed in the highly aggressive GS-9L rat glioma model (10) and autologous vaccines in a clinical setting for dogs presenting with advanced cancers.

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This work is a novel approach to cancer vaccine development harnessing the immunogenic properties of bacterial proteins together with the modification of a patient’s own tumor proteins.

Materials and Methods

Cell line
The rat glioma GS-9L (9L) cell line derived from a N-nitrosomethylurea–induced glioma (ECACC cat no. 94110705) was cultured in Basel Medium Eagle (BME) media (Life Technologies Pty Ltd.) supplemented with 10% FBS (Life Technologies) and was grown in standard conditions (37°C humidified incubator with 5% CO2). The 9L line was checked for Mycoplasma, and no additional authentication was performed.

Rats
Female Fischer 344 rats (150–200 g; Animal Resources Centre, Perth, WA, Australia) were maintained at the Kearns Facility, Kolling Institute (Sydney, NSW, Australia) under standard conditions (12 hours light/dark cycles and free access to food and water). Rats were euthanized at ethically approved endpoints (tumor size > 13.5 cm3) and samples were collected. All procedures involving animals were carried out in accordance with the recommendations in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The protocol for this study was approved by the Royal North Shore Hospital Animal Care and Ethics Committee (protocol number 1110-018A).

9L glioma model
Rats (>13 weeks of age) were anesthetized using fluorothane inhalation and injected with 1 × 106 9L glioma cells in 100 μL of serum-free BME media subdermally in the flank. Tumors were allowed to grow to approximately 1 cm3 and were centrifuged at 12,100 g for 30 minutes at 4°C. The pellet was removed and block unbound amine groups. Tumors were removed and immediately frozen in liquid nitrogen.

Perfusions
Rats were anesthetized, the thoracic cavity was opened, and 0.2 mL of heparin (5,000 U/mL; Celsus) was injected into the left ventricle of the heart to prevent clotting. A needle was inserted via the left ventricle into the ascending aorta and the right atrium was cut. The blood was cleared with a PBS flush, and then 30 mL of biotin-SS (0.5 mg/mL; Thermo Scientific) was slowly perfused into the rat. This was followed by 20 mL of 0.05 mol/L Tris buffer to flush out unreacted biotinylation reagent and block unbound amine groups. Tumors were removed and immediately frozen in liquid nitrogen.

Vaccine dosing studies
Vaccines were prepared as described for the initial vaccine trial. Vaccine cohorts were given one to three doses of vaccine intraperitoneally and a fourth group received two doses of vaccine subcutaneously. All groups were challenged with 1 × 106 9L cells in the flank 2 weeks after the second booster and this was termed day 0. Tumors were measured by calipers three times per week and tumor volume was calculated using the equation (width2 × length)/2 = cm3.

Vaccine components experiment
This experiment was designed to evaluate the individual components of the vaccine. In preliminary work, we demonstrated that nonreduced tumor lysate had no effect on tumor growth (Supplementary Fig. S1); therefore, this group was not retested. Five treatment groups were tested: (i) reduced lysate (R-Lysate); (ii) streptavidin-only (Streptavidin; 50 μg/rat); (iii) vaccine reduced with streptavidin [Vaccine (50); 50 μg/rat]; (iv) vaccine-reduced high-dose streptavidin [Vaccine (100); 100 μg/rat]; and (v) control (FIA in PBS).

Multiple tumors were harvested and solubilized to produce a pooled tumor lysate. A 1-mL aliquot of tumor lysate was used per 3 rats. Processing involved protein precipitation, centrifugation, and drying as described above (see “Initial vaccine trial” above) with pellets resuspended in 1.2 mL of sterile PBS and mixed 1:1 with FIA for a final volume of 2.4 mL unless otherwise stated. Each rat received a 400-μL s.c. vaccination. For the R-Lysate
group, the lysate was reduced for 1 hour with 20 mmol/L Tris (2-carboxy-ethyl)-phosphin-HCl (TCEP) and then processed. For the streptavidin-only group, 300 μg of streptavidin (Genscript) was suspended in 1 mL of SDS buffer and then processed.

Vaccine (50) was prepared by reducing 2 mL of tumor lysate for 1 hour in 20 mmol/L TCEP, then, 300 μg of streptavidin (1 mg/mL) was added and incubated for 2 hours at room temperature. Each 1 mL of reduced and streptavidin-labeled lysate was then processed and resuspended in 1.2 mL (0.6 mL PBS + 0.6 mL FIA). Vaccine (100) was prepared similarly except that 300 μg of streptavidin was added to 1 mL of reduced lysate. All groups received a second vaccination 3 weeks after the first. Blood was collected from all rats 7 days after the second vaccination, at multiple time points after engraftment, and at sacrifice.

Cytokine analysis
Cytokine profiling of rat serum was performed using the rat Cytokine Bioplex (Bio-Rad) and the rat Proteome Profiler Array (R&D Systems) according to each manufacturer's instructions. Rat serum samples (100 μL) were also screened using ELISA for rat C-reactive protein (Becton Dickinson), CINC-1 (R&D Systems), ICAM1 (R&D Systems), interleukin (IL)-4 (R&D Systems), TNF-α (R&D Systems), and IFN-γ (Bender Systems) according to the manufacturer's instructions.

Streptavidin antibody and ELISA
Western blotting of tumor lysate or vaccine was performed with polyclonal rabbit anti-streptavidin antibodies (Sigma-Aldrich) and goat anti-rabbit alkaline phosphatase (Merck) and then developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT; Sigma-Aldrich).

We developed an ELISA assay to measure the level of streptavidin-specific antibodies in the serum. MaxiSorp ELISA plates (NUNC) were coated overnight at 4°C with streptavidin (100 μL/well, 10 μg/ml in 0.1 mol/L NaHCO3; Calbiochem). Plates were then blocked in 3% bovine serum albumin (BSA) in PBS for 1 hour at 37°C. Rat serum samples were diluted at 1:1,000 in 1% BSA in PBS and 100 μL/well incubated on plate at 37°C for 1 hour. The plate was then washed four times with 0.05% Tween-20 in PBS and incubated with a goat anti-rat-horseradish peroxidase antibody (1:2,000 in 1% BSA in PBS; Sigma-Aldrich) for 1 hour at 37°C. Plates were washed again, TMB substrate (R&D Systems) was added for 10 minutes, and then the absorbance was read at 450 nm.

Circulating immune cell profiling
Circulating white blood cell levels were measured by fluorescence-activated cell sorting (FACS) analysis. A sample of blood was collected into a 0.5-mL EDTA tube. For each sample, 25 μL of blood was added to a Trucount tube (BD Pharmingen) and then labeled with 20 μL rat T/B/NK cell cocktail (BD Pharmingen), 10 μL each of rat CD8a PE, CD4 (domain 1) fluorescein isothiocyanate (FITC), and CD45 PE/Cy7 (BioLegend) for 15 minutes at room temperature. Red cells were lysed using 10 mmol/L Tris and ammonium chloride buffer (pH 7.4). All cells gated as CD45+ were analyzed. Monocytes, neutrophils, and lymphocytes were analyzed by forward scatter versus side scatter. Cell subtypes were analyzed as follows: CD4 T cells (CD45+CD3+/CD4+), CD8 T cells (CD45+CD3-/CD8+), natural killer (NK) cells (CD45+CD3-/CD161a+), and B cells (CD45+/CD3-/CD45 RA+). Cell numbers per μL were calculated as follows:

\[
\text{Cell number} = \frac{25 \mu L}{\text{Bead number}} \times \text{Bead count}
\]

Canine vaccine production and phase 1 safety study
Approval was obtained from the Regenueus Animal Care and Ethics Committee to undertake a safety trial of the vaccine treatment in dogs from two veterinary oncology practices in Sydney, Australia. Following provision of vaccine information, owners gave informed written consent to include their dog in the study. Participation was irrespective of tumor type, prior cancer treatment, or age of the dog. Dogs presenting between March 2011 and March 2013 were included in this study. Tumors were either surgically removed or if inoperable a biopsy was taken, with a minimum of 100 mg required to make a vaccine. Samples were frozen (−20°C) immediately after excision. Every 100 mg of tumor was homogenized in 4 mL of TBSDS buffer with complete protease inhibitor cocktail (Roche). The soluble fraction was collected by centrifuging at 12,100 × g for 30 minutes. Each 1 mL of lysate was treated with 20 mmol/L TCEP for 1 hour at room temperature, and then 100 μg of biotin-NHS (Thermo Scientific) was added for 2 hours with gentle mixing. Following this, 100 μg of recombinant streptavidin (Genscript) was added. This was incubated for 2 hours at room temperature. The biotin–streptavidin-labeled lysate was then processed and the pellet was resuspended in 600 μL of PBS. This was split into two 0.3-mL aliquots and stored at −20°C until use. Dogs received vaccine mixed at 1:1 with FIA subcutaneously in the dorsal or the neck. This was repeated 3 weeks later. Dogs were monitored for signs of anaphylaxis for 30 minutes after vaccinations, and subsequent health was monitored by regular checkups with the treating veterinarians with a census date of May 31, 2013.

Statistical analysis
One-way ANOVA survival curve statistical analyses and Tukey multiple comparison tests were performed using GraphPad Prism 4. A P < 0.05 was considered statistically significant.

Results
Initial vaccine shows efficacy with no adverse effects in rats with glioma
The initial vaccine trial comprised rats treated with two doses of 9L glioma vaccine or adjuvant to determine the safety and efficacy of the vaccine. The rats showed no adverse effects to the vaccination other than minor swelling at the vaccination site. Two of 3 rats in the vaccine-treated group developed tumors; however, 1 of these resolved over time and the tumor had disappeared by day 58 after engraftment (Fig. 1A). Two rats were considered “in remission” after surviving beyond 100 days and were kept for rechallenge in the acquired immunity experiments described in Materials.
and Methods. In contrast, in control adjuvant–vaccinated rats the average tumor progression time until ethical endpoint was 35 days (Fig. 1B). Overall, there was a statistically significant survival advantage in the vaccinated group compared with the adjuvant-alone group ($P < 0.05$).

**Immunity to glioma induced in rats with two vaccinations**

Given the success of the initial vaccine trial, we next determined the optimal vaccine dosage regimen. One dose did not extend survival significantly over that of control rats (Fig. 2A). However, significantly extended survival relative to that of the controls was observed for rats receiving two doses ($P < 0.05$; Fig. 2B) and three doses ($P < 0.05$; Fig. 2C).

IFN-γ levels were elevated significantly 21 days after tumor challenge in rats receiving one or two vaccinations compared with control ($P < 0.05$; Supplementary Fig. S2). IL-4 levels were decreased significantly at day 21 in rats receiving one or two vaccinations compared with control ($P < 0.05$; Supplementary Fig. S2). There was no statistically significant difference in vaccine efficacy of two doses whether administered intraperitoneally (Fig. 2B) or subcutaneously (Fig. 2D), and all further vaccinations were given subcutaneously.

Nine rats from this trial showed no sign of tumor 150 days after engraftment and were considered in remission. To ascertain whether acquired immunity had developed, 4 rats in remission (including the 2 surviving rats from the initial vaccine trial; Fig. 1) were rechallenged with 9L glioma cells. A highly statistically significant increase in response was observed with vaccinated rats surviving to 150 days with no sign of tumors ($P < 0.01$; Fig. 2E). These results suggest that rats that responded to vaccination and could fight off a tumor challenge were immune to further 9L cell challenges, possibly due to vaccine-induced immune memory.

To further assess the efficacy of the vaccine, 5 rats in remission and 6 untreated control rats had resected tumor sections engrafted orthotopically in the brain. No signs of tumor growth were observed in the vaccinated rats up to 150 days after engraftment, whereas all control rats died of disease within 25 days of orthotopic engraftment ($P < 0.001$; Fig. 2F). These results suggest that the vaccine can induce immunity across the blood–brain barrier.

**Streptavidin identified as the immunogenic agent**

Having established the optimal dosing regimen for the vaccine and its ability to induce immunity, we were interested in determining the essential immunogenic components of the vaccine. Western blot analysis of vaccine proteins probed with serum from vaccine-treated rats detected five to six bands between 50 and 75 kDa (Fig. 3A), Protemic profiling identified peptides with sequences matching streptavidin (52.8-kDa tetramer; data not shown), and this was confirmed by Western blotting with anti-streptavidin (Fig. 3B). This suggested that streptavidin was being inadvertently eluted from the streptavidin affinity column.

These results led us to hypothesize that streptavidin was eliciting the antibody response and stimulating the immune system. To test this, unperfused (nonbiotinylated) tumor was applied to the streptavidin column. A complex banding pattern was observed (Fig. 3C), suggesting that streptavidin itself was selecting the vaccine proteins. This likely occurs by binding to its RYD site via an RGD or similar sequence in the tumor proteins (12). Increasing the SDS concentration of the buffer 10-fold (to 10%) to disrupt the covalent bonds had little effect; the vaccine still yielded a complex vaccine profile even on nonbiotinylated tumors (data not shown). These findings indicate that streptavidin has a high affinity for the bound tumor proteins.

**Low-dose streptavidin in combination with reduced vaccine proteins [Vaccine (50)] showed greatest efficacy in rat glioma**

Having identified that streptavidin is the component common to all batches of vaccine, we next tested the efficacy of the vaccine with or without key steps in the production process. Rats that received the combination of reduced lysate linked with a 50-μg dose of streptavidin [Vaccine (50)] showed the greatest survival of all the groups, with an average survival of 77 days compared with 38 days for control rats ($P < 0.001$; Fig. 4A; Table 1). This combination also induced remission in 2 of 6 rats (Fig. 4B), suggesting that both reduction of proteins and streptavidin dose are important factors in vaccine production. Doubling the dose of streptavidin [Vaccine (100)] did not increase survival (Fig. 4A and Table 1). Retesting Vaccine (50) versus controls and
lower doses of streptavidin confirmed its survival advantage ($P < 0.05$; Supplementary Fig. S3).

**Circulating streptavidin antibodies developed following vaccination**

Blood was collected 7 days following the second vaccination, at days 14, 28, 35, and 40 after engraftment, and at sacrifice; the serum was assayed for anti-streptavidin antibodies. Rats treated with streptavidin showed reactivity to streptavidin (Fig. 4C). However, Vaccine (50)–treated rats demonstrated approximately six times lower reactivity to streptavidin than the streptavidin-only and Vaccine (100) groups. These results suggest that the level of streptavidin antibody response is modulated by both the streptavidin dose and the reduced tumor proteins in the vaccine. Furthermore, as remission was only observed in Vaccine (50) rats, a low-level antibody response may be optimal.

**Vaccine (50) and streptavidin-alone induced responses in circulating cytokines**

Circulating cytokine levels were assessed at day 21 after engraftment, and/or at endpoint (Table 1; Fig. 4; Supplementary Fig. S4). The proinflammatory cytokine TNF-α demonstrated the most notable changes with levels at day 21 at least 3-fold higher in the Vaccine (50)–treated group than other groups (Fig. 4D). Levels remained significantly higher in rats from this group in remission (survival > 100 days; $P < 0.05$; Table 1), whereas the rats not in remission had significantly lower endpoint levels ($P < 0.05$; Table 1). IFN-γ and the chemokine MIP-3A showed opposite profiles to TNF-α at endpoint with significantly lower levels in rats in remission and higher levels in the remaining Vaccine (50) group.
Similarly, the proinflammatory IL-1 family cytokine IL-1α was significantly elevated in Vaccine (50)-treated rats not in remission. ICAM1, which can be induced by proinflammatory cytokines, was significantly lower in the Vaccine (50) group than in controls at day 21 after engraftment ($P < 0.05$; Fig. 4E). No significant changes in cytokine levels were observed for rats treated with R-Lysate or Vaccine (100).

![Figure 3. Streptavidin identified in the vaccine protein profiles. A, Western blot analysis of vaccine probed with vaccinated rat serum. B, Western blot analysis of vaccine probed with anti-streptavidin. C, silver-stained PAGE separation of a representative rat vaccine using streptavidin selection. Std, protein standards (Precision Plus; Bio-Rad Laboratories Pty Ltd.).](image)

![Figure 4. Vaccine (50) prolongs survival and induces remission, production of streptavidin (Strept) antibodies, and TNF-α. Parameters for four different vaccine types and adjuvant controls ($n = 5$) were compared: R-Lysate ($n = 6$); Vaccine (50) ($n = 6$); Vaccine (100) ($n = 3$); Strept ($n = 6$). A, mean survival times. B, survival curves plotted as percentage surviving over time. C, serum streptavidin antibody levels where day 0 is the day of engraftment. D, serum TNF-α levels at day 21 after engraftment. E, serum ICAM1 levels at day 21 after engraftment. $^*$, $P < 0.05$; $^*$*, $P < 0.01$; $^*$*, $P < 0.001$; NS, not statistically significant. $P$ values determined by one-way ANOVA except for A, which used the Tukey multiple comparison test.](image)
Different cellular responses observed after tumor engraftment between vaccination with Vaccine (50) and adjuvant only

Circulating immune cell levels were determined by multicolor FACS assays in peripheral blood collected from the control (vaccination with adjuvant) and Vaccine (50) groups (Fig. 5).

Following vaccination (Pre-tumor), increases in B cells and monocytes were observed in both Vaccine (50) rats and adjuvant-treated controls, whereas increases in NK cells were observed only in the controls. The levels of B cells and NK cells decreased after tumor engraftment (day 21). B cells for the Vaccine (50) group returned to normal, whereas those of the control group remained significantly higher (P < 0.05; Fig. 5); NK cells decreased significantly to below baseline for both groups (P < 0.05; Fig. 5); and monocyte levels for the Vaccine (50) group decreased significantly below baseline and those of controls (P < 0.05; Fig. 5). CD4 T-cell levels increased significantly above the baseline following vaccination (Pre-tumor) in the Vaccine (50) rats (P < 0.05). Post-tumor, both CD4 and CD8 T-cell levels in the Vaccine (50) group were maintained at normal baseline levels and significantly higher than those in the control group (P < 0.05; Fig. 5). Neutrophil and lymphocyte levels remained stable, with the exception of significantly lower lymphocyte levels in controls post-tumor (P < 0.05; Fig. 5).

The vaccine extends survival of dogs with advanced cancer

Twenty-five family pet dogs of a variety of breeds presenting with nine different types of advanced cancer were treated with vaccines prepared from each dog's own tumor using the canine vaccine production method as part of a phase I clinical trial. This method of vaccine production incorporated biotin to optimize linking of tumor proteins to streptavidin and had been previously validated in rat experiments (Supplementary Fig. S5). Vaccine protein profiles (Supplementary Fig. S6) were similar to those for the rat glioma model (Fig. 3). The tumor types and survival data for this trial are shown in Fig. 6. As a reference for the natural history of the cancers in these animals, expected survival times were taken from either individual oncology reports or published literature (13) and were based on surgery alone or standard of care for the tumor type.

Of the 25 dogs included in this study, 11 had some burden of disease at vaccination (Fig. 6). This included inoperable tumor, relapse following chemotherapy, metastases, or residual tumor remaining after surgery. Sixty-three percent of these (7 of 11) survived longer than would otherwise be expected on the basis of tumor grade, histology, and/or oncology report.

Twenty-one dogs received two vaccinations, and 71% (15 of 21) of these exceeded their expected survival time by 2 weeks to 22 months at the census date. Of the other 6 of these 21 dogs, 4 are alive but have not exceeded their expected survival; 1 died of other causes before the expected survival time and 1 died at the expected time.

No cases of anaphylaxis occurred, and the only side effect recorded was a subcutaneous nodule at the vaccination site, which resolved over time. Some of the dogs enrolled in this safety trial were also receiving or had received other therapies; thus, the additional impact of the vaccine therapy on their disease natural history cannot be truly determined. These results demonstrate the safety and possible efficacy of the vaccine in a clinical setting. The fast turnaround time from vaccine in a clinical setting. The fast turnaround time from tumor resection or biopsy also means minimal lag time to treatment, an important consideration in the clinical setting.

Discussion

In this study, we demonstrated that vaccines developed using our production method can stimulate the immune system to recognize and either slow tumor growth or induce tumor rejection. Prophylactic syngeneic vaccination of the 9L glioma rat model doubled survival in 100% of rats and led to remission in 33% of the vaccinated animals. Rechallenging rats in remission demonstrated 100% tumor rejection. Autologous vaccination in the clinical setting of dogs with advanced cancer demonstrated safety as well as "real-world" applicability of the rapid production method with initial evidence of efficacy.

Table 1. Circulating cytokine response at endpoint relative to control vaccinated rats

<table>
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<th>Group</th>
<th>N</th>
<th>Mean ± SD</th>
<th>Survival</th>
<th>Cytokine response relative to control</th>
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<td>38.4 ± 3.0</td>
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<td>Increased</td>
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<tr>
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<td>Vaccine (50)</td>
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<tr>
<td>Vaccine (100)</td>
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<td>43.5 ± 6.1</td>
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</table>

NOTE: The Vaccine (50) group was further divided into two groups: those surviving >100 days, considered in remission; and the remainder. Cytokines measured that did not show statistically significant differences in any groups: IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-17, IL-18, EPO, G-CSF, GRO-KC, MIP-1A, and Rantes.

Abbreviations: M-CSF, macrophage colony-stimulating factor; N, number in each cohort; NSD, no statistically significant differences.
A key component of the vaccine is the protein streptavidin. We discovered that streptavidin is effective at selecting tumor proteins and stimulating the immune system. The binding of streptavidin to proteins is through its RYDS sequence, which mimics the RGD cell adhesion domain of fibronectin (14). Over 60 integral membrane proteins contain an RGD sequence and could potentially bind streptavidin (12, 15). Many of these proteins, such as integrins, VEGFA, angiopoietin, osteopontin, and fibronectin, have been shown to have a role in cancer development (16, 17).

However, vaccination with streptavidin alone did not induce remission, and we combined this with soluble tumor proteins reduced under denaturing conditions to prevent refolding. Although the final precipitation step has been used before as a potent way to present antigens with low immunogenicity (18), using soluble proteins sets our process apart, as most other vaccines are derived from ethanol fixed or irradiated whole cells (19–21). These soluble proteins are then reduced with TCEP, which permanently breaks disulfide bonds and provides a stable environment for proteins (22, 23).

This combination [streptavidin plus reduced soluble proteins; Vaccine (50)] was crucial for tumor remission and rejection in the 9L glioma model. Other studies have successfully used different therapies to slow tumor growth (24, 25), but to our knowledge, this is the first report of inducing complete remission in rats using vaccines derived from solid tumors rather than cells in this aggressive model. Of note, while the 9L glioma model has been reported to be immunogenic (10), we observed 100% engraftment with no spontaneous remissions, in keeping with other reports using this cell line (24, 26–28).

Our results suggest that the vaccine modulates the immune response from a predominant antibody response (to streptavidin) to a cell-mediated response (requiring the addition of the reduced tumor lysate). B cells were increased in both the vaccine and control groups following vaccination,
demonstrating that the adjuvant FIA stimulates an increase in peripheral B cells as described previously (29). However, 21 days after engraftment, B-cell counts of vaccine-treated rats had returned to normal levels, indicating again a switch to a cell-mediated response to the tumor. In vaccine-treated rats, both CD4 and CD8 T-cell levels were maintained at normal baseline levels after tumor engraftment, whereas they decreased significantly in controls over the same time period. These observations suggest that the vaccine may aid and extend survival in part by maintaining production of CD4 and CD8 T cells during cancer progression. In support of the importance of maintaining normal lymphocyte levels, low lymphocyte levels in patients with cancer are reported to be indicative of poorer prognosis and higher tumor grade (30). Patients with melanoma and colorectal cancer with a higher level of tumor-infiltrating lymphocytes also have a better prognosis (30, 31).

The key cytokine response observed was the upregulation of TNF-α, which is known to have an antitumor effect and causes cancer cell apoptosis (32). Although streptavidin-only vaccinated rats showed increased survival, they did not show the corresponding upregulation of TNF-α as in vaccine-treated rats. ICAM1, a cytokine implicated in tumor growth and metastasis (33), was also downregulated only in vaccine-treated rats.

Cytokine analysis also identified differences in the levels of IL-4 and IFN-γ between vaccine-treated and control rats. IL-4, which has been shown to modulate tumor progression and metastasis (34), was decreased in vaccine-treated rats. Vaccine-treated rats also showed a statistically significant increase in IFN-γ, which is a critical immune system component of the antitumor response (35). IFN-γ together with lymphocytes not only provides protection against tumor development but also assists in sculpting the immunogenic phenotype of tumors for presentation as a "cancer immunoediting" process (36, 37). Taken together, the cytokine results suggest that the vaccine initiates a specific and effective immune antitumor response.

Although rat models are useful for the initial evaluation of vaccine preparations, dogs provide a clinical presentation and scenario to match that of humans in terms of presentation and time to progression. The dogs were assessed as a phase I safety trial with no adverse reactions observed when the vaccine was administered alone or in combination with a variety of other medications. These results confirm the safety of the autologous vaccine protocol.

This study also provided initial evidence for the efficacy of the vaccine in this clinical setting with canine patients presenting with varying degrees of disease (operable to metastatic) and tumor types. Dogs with residual or metastatic disease...
often survived longer than expected, indicating that vaccination can slow tumor growth. The ability to produce an autologous vaccine with a lag time of only a few days between surgery and treatment highlights its applicability to clinical situations. Furthermore, fresh tumor samples once frozen can be stored indefinitely until the vaccine is required for use in an adjunct setting.

In conclusion, we have developed a unique vaccine process for making autologous or syngeneic tumor vaccines, with evidence of both slowing tumor growth and remission. The use of streptavidin as an immunostimulant with reduced tumor proteins is effective, safe, and well tolerated in rodents and canine patients. The versatility of using streptavidin has yet to be fully studied, but it provides a novel platform for the development of improved cancer vaccines. Further randomized clinical studies aimed at proving efficacy are warranted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Patent Declaration
The use of streptavidin, biotin, and the methods used to make vaccines described in this article are subject to Provisional Patent Application No. 2012905667.

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Cancer Immunology Research

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