

Streptavidin: A novel immunostimulant for the selection and delivery of autologous and syngeneic tumor vaccines

Chris Weir¹, Amanda L. Hudson¹, Elizabeth Moon¹, Angus Ross², Miles Alexander²,
Lyndsay Peters³, Veronika Langova⁴, Stephen J. Clarke¹, Nick Pavlakis¹, Ross Davey¹ and
Viive M. Howell¹

¹Bill Walsh Translational Cancer Research Laboratory, Kolling Institute of Medical Research, Royal North Shore Hospital and the Sydney Medical School, University of Sydney, Sydney Australia. ²Ku-ring-gai Veterinary Hospital, Sydney, Australia, ³Northern Blood Research Laboratory, Kolling Institute of Medical Research, Royal North Shore Hospital, Sydney Australia, ⁴Small Animal Specialist Hospital, Sydney, Australia.

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Corresponding Author:

Dr Chris Weir, Senior Research Fellow

Bill Walsh Translational Cancer Research Laboratory, Kolling Institute of Medical Research,

Level 8, Kolling Building, Royal North Shore Hospital,

St Leonards 2065, NSW Australia;

(Phone) +61 2 9926 4718 (Fax) +61 2 9926 4035 (Email) chris.weir@sydney.edu.au

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ABSTRACT

Induction of antitumor immunity utilizing 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) utilising streptavidin in combination with reduced soluble tumor proteins. Initial syngeneic vaccine studies in the 9L rat glioma model were used to optimise vaccine dose and selectivity. Cytokine and blood analysis was used to monitor the response. Rats receiving two vaccinations of syngeneic tumor vaccine demonstrated a significant ($P<0.05$) survival advantage compared with controls (adjuvant only). Notably, vaccination also led to remission rates of between 30 to 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 while ICAM1 was down regulated. Post-engraftment, vaccinated rats maintained CD4⁺, CD8⁺ T cells, and total lymphocyte levels closer to normal baseline than that of the controls. Twenty five dogs treated with autologous vaccine preparations utilising 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 utilising 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.

INTRODUCTION

Cancer immunotherapy continues to gain recognition as an oncology treatment as new and improved cancer vaccines and other immune stimulants are produced. Utilizing a patient's own tumor proteins to produce a vaccine is an attractive concept and a number of autologous vaccines are undergoing pre-clinical evaluation (1). Other immune therapies such as dendritic cell therapy have moved into the clinic but whilst they increase survival time in patients they do not initiate immunological rejection or remission of a tumor (2, 3). Induction of immunological 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 utilise the patient's own material either directly or for extraction or stimulation *in vitro* (6). These autologous vaccine types match the patients MHC molecules as well as tumor-associated antigens. While 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 immune-stimulants 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 utilize 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.

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 BME media (Life Technologies Australia Pty Ltd, Mulgrave, Victoria, Australia) supplemented with 10% fetal bovine serum (Life Technologies) and 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 under standard conditions (12 hrs light/dark cycles and free access to food and water). Rats were euthanized at ethically

approved endpoints (tumor size $> 13.5\text{cm}^3$) and samples 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 anaesthetized using fluorothane inhalation and injected with 1×10^6 9L glioma cells in 100 μl serum free BME media subdermally in the flank. Tumors were allowed to grow to approximately 1cm^3 and either perfused (described below) or excised and frozen immediately for processing into vaccines.

For orthotopic brain experiments, a flank tumor of approximately 1cm^3 was harvested, cut into 1mm pieces and briefly stored at 4°C in serum free BME media. Rats were anaesthetized using fluorothane. A small hole was drilled in the left side of the rat skull into which a piece of the flank tumor was implanted, the hole sealed with bone wax (Ethicon, Somerville, New Jersey, USA), and the scalp closed. Buprenorphine (Temgesic, 0.4 mg/kg, Schering-Plough, Kenilworth, New Jersey, USA) was administered subcutaneously for pain relief. Following recovery, rats were monitored daily for signs of distress as described previously (11).

Perfusions

Rats were anaesthetized, the thoracic cavity opened and 0.2 mL heparin (5000 U/mL, Celsus Cincinnati, Ohio, USA) 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 phosphate buffered saline (PBS) flush and then 30 mL of

biotin-SS (0.5 mg/ml, Thermo Scientific, Scorsby, Victoria, Australia) was slowly perfused into the rat. This was followed by 20 mL 0.05 M TRIS buffer to flush out unreacted biotinylation reagent and block unbound amine groups. Tumors were removed and immediately frozen in liquid nitrogen.

Initial vaccine trial

Every 1 g of biotin-perfused tumor was homogenized and solubilised in 40 ml of 0.05 M Tris, 0.15 M NaCl and 1% SDS buffer (pH 7.6 TBSSDS) with complete protease inhibitor cocktail (Roche, Castle Hill, NSW, Australia) to produce a tumor lysate, which was centrifuged at 12,100 g for 30 mins at room temperature (RT). The supernatant was collected and applied to a TBSSDS-equilibrated streptavidin column (Thermo Scientific) at a ratio of 2 volumes supernatant to 1 column volume and allowed to incubate for 1 hr. Unbound proteins were then washed through with 5 column volumes of TBSSDS. The bound proteins were released with TBSSDS containing 50 mM DTT by incubating for 1 hour at RT before eluting. The eluted vaccine proteins (2 ml) were then precipitated overnight with 20 ml of acetone at -20°C, followed by centrifuging at 12,100 g for 30 mins at 4°C. The pellet was allowed to dry before being resuspended in 200 µl sterile phosphate buffered saline (PBS). Each 200 µl batch was mixed with 200 µl Freund's Incomplete Adjuvant (FIA; Sigma-Aldrich, St Louis, Missouri, USA) for use as the individual vaccine for each rat. Rats received 400 µl of vaccine (test) or FIA in PBS (controls) intraperitoneally (i.p), followed by a booster shot 3 weeks later. Rats were then challenged with 1×10^6 9L cells in the flank 2 weeks after the second booster and this was termed day zero. Tumors were measured by calipers 3 times per week and tumor volume calculated using the equation $(\text{width}^2 \times \text{length})/2 = \text{cm}^3$.

Vaccine dosing studies

Vaccines were prepared as described for the initial vaccine trial. Vaccine cohorts were given 1-3 doses of vaccine i.p and a 4th group received 2 doses of vaccine subcutaneously (s.c). All groups were challenged with 1×10^6 9L cells in the flank 2 weeks after their last vaccination.

Acquired immunity experiments

Rats that survived the initial vaccine study and vaccine dosing studies (n=9) were considered in remission and assessed for acquired immunity. The surviving rats were split into 2 cohorts for rechallenge with cells or orthotopically with tumor.

Vaccine Components experiment

This experiment was designed to evaluate the individual components of the vaccine. In preliminary work we demonstrated that non-reduced tumor lysate had no effect on tumor growth (SI Figure 1) therefore this group was not retested. Five treatment groups were tested: 1: reduced lysate (R-Lysate); 2: streptavidin only (Streptavidin, 50 μ g/rat); 3: vaccine reduced with streptavidin (Vaccine (50), 50 μ g/rat); 4: vaccine reduced high dose streptavidin (Vaccine (100), 100 μ g/rat); 5: 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 (“Initial Vaccine Trial”) with pellets resuspended in 1.2 ml sterile PBS and mixed 1:1 with FIA for a final volume of 2.4 ml unless otherwise stated. Each rat received a 400 μ l s.c. vaccination. For the R-lysate group, the lysate was reduced for 1 hr with 20mM TCEP and then processed. For the streptavidin only

group, 300 μ g streptavidin (Genscript, Piscataway, New Jersey, USA) was suspended in 1 ml SDS buffer and then processed.

Vaccine (50) was prepared by reducing 2 ml of tumor lysate for 1 hr in 20 mM TCEP; then 300 μ g streptavidin (1 mg/ml) was added and incubated for 2 hrs at RT. Each 1 ml of reduced and streptavidin-labelled 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 μ g streptavidin was added to 1 ml 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, Gladesville, NSW, Australia) and the rat proteome profiler™ array (R and D systems, Minneapolis, Minnesota, USA) according to each manufacturer's instructions. Rat serum samples (100 μ l) were also screened using ELISA for rat C-Reactive protein (Becton Dickinson, San Jose, California, USA), CINC-1 (R and D systems), ICAM1 (R and D systems), IL-4 (R and D systems), TNF- α (R and D Systems), INF- γ (Bender Systems, Burlingame, California, USA) 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, Kilsyth, Victoria, Australia) then developed with BCIP/NBT (Sigma-Aldrich).

We developed an ELISA assay to measure the level of streptavidin-specific antibodies in the serum. Maxisorp ELISA plates (NUNC, Roskilde, Denmark) were coated overnight at 4°C with streptavidin (100 µl/plate, 10 µg/ml in 0.1 M NaHCO₃) (Calbiochem, La Jolla, California, USA). Plates were then blocked in 3 % BSA in PBS for 1 hr at 37°C. Rat serum samples were diluted 1:1000 in 1 % BSA in PBS and 100 µl incubated on plate at 37°C for 1 hr. The plate was then washed 4 times with 0.05% Tween-20 in PBS and incubated with a goat anti-rat-HRP antibody (1:2000 in 1% BSA in PBS; Sigma-Aldrich) for 1 hr at 37°C. Plates were washed again, TMB substrate (R and D Systems) added for 10 minutes and then the absorbance read at 450 nm.

Circulating immune cell profiling

Circulating white blood cell levels were measured by Fluorescence-activated cell sorting (FACS). 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) FITC, and CD45 PE/Cy7 (Biolegend) for 15 minutes at RT. Red cells were lysed using 10 mM Tris and ammonium chloride buffer (pH 7.4). All cells gated as CD45 positive were analyzed. Monocytes, neutrophils and lymphocytes were analysed by Forward Scatter *versus* Side Scatter. Cell subtypes were analysed as follows: CD4 T cells (CD45+/CD3+/CD4+), CD8 T cells (CD45+/CD3+/CD8+), Natural Killer (NK) cells (CD 45+/CD3-/CD161a+) and B Cells (CD45+/CD3-/CD45 RA+). Cell numbers per µl were calculated as follows:

$$\frac{\text{Cell number}}{25 \mu\text{l}} \times \frac{\text{Bead number}}{\text{Bead count}}$$

Canine vaccine production and phase I safety study

Approval was obtained from the Regeneus (Gordon, Sydney, Australia) Animal Care and Ethics Committee to undertake a safety trial of the vaccine treatment in dogs from 2 veterinary oncology practices in Sydney. 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 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 homogenised in 4 ml of TBSSDS buffer with complete protease inhibitor (Roche). The soluble fraction was collected by centrifuging at 12,100 g for 30 minutes. Each 1 ml of lysate was treated with 20 mM TCEP for 1 hr at RT then 100 µg of biotin-NHS (Thermo Scientific) was added for 2 hrs with gentle mixing. Following this 100 µg recombinant streptavidin (Genscript) was added. This was incubated for 2 hrs at RT. The biotin-streptavidin labelled lysate was then processed and the pellet resuspended in 600 µl PBS. This was split into two 0.3 ml aliquots and stored at -20°C until use. Dogs received vaccine mixed 1:1 with FIA s.c in the dorsum at the scruff of 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 check-ups with the treating veterinarians with a census date of May 31st 2013.

Statistical Analysis

One way ANOVA survival curve statistical analyses and Tukey's multiple comparison tests were performed using Graph Pad Prism® 4. A P-value < 0.05 was considered significant.

RESULTS

Initial vaccine shows efficacy with no adverse effects in rats with glioma

The initial vaccine trial comprised rats treated with 2 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 the 3 rats in the vaccine-treated group developed tumors, however 1 of these resolved over time and the tumor had disappeared by day 58 post-engraftment (Figure 1A). Two rats were considered “in remission” after surviving beyond 100 days and were kept for rechallenge in the acquired immunity experiments described below. By contrast, in control adjuvant-vaccinated rats the average tumor progression time until ethical endpoint was 35 days (Figure 1B). Overall there was a significant survival advantage in the vaccinated group compared to the adjuvant alone group ($P < 0.05$).

Immunity to glioma induced in rats with 2 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 (Figure 2A). However, significantly extended survival relative to that of the controls was observed for rats receiving 2 doses (Figure 2B, $P < 0.05$) and 3 doses (Figure 2C, $P < 0.05$).

Interferon- γ levels were elevated significantly 21 days post tumor challenge in rats receiving 1 or 2 vaccinations compared to control (SI Figure 2, $P < 0.05$). IL-4 levels were decreased significantly at day 21 in rats receiving 1 or 2 vaccinations compared to control (SI Figure 2, $P < 0.05$). There was no significant difference in vaccine efficacy of 2 doses whether administered i.p. (Figure 2B) or s.c. (Figure 2D) and all further vaccinations were given s.c.

Nine rats from this trial showed no sign of tumor 150 days post-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, Figure 1) were rechallenged with 9L glioma cells. A highly significant increase in response was observed with vaccinated rats surviving to 150 days with no sign of tumors (Figure 2E, $P < 0.01$). 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 post-engraftment whereas all control mice died of disease within 25 days of orthotopic engraftment (Figure 2F, $P < 0.001$). 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 5 - 6 bands between 50 and 75 kDa (Figure 3A). Proteomic profiling identified peptides with sequences matching streptavidin (52.8 kDa tetramer, data not shown) and this was confirmed by western blotting with anti-streptavidin (Figure 3B). This suggested that streptavidin was being inadvertently eluted from the streptavidin affinity column.

These results led us to hypothesise that streptavidin was eliciting the antibody response and stimulating the immune system. To test this, unperfused (non-biotinylated) tumor was applied

to the streptavidin column. A complex banding pattern was observed (Figure 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 non-biotinylated 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 ($P < 0.001$, Figure 4A, Table 1). This combination also induced remission in 2 of 6 rats (Figure 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 (Figure 4A, Table 1). Retesting Vaccine (50) *versus* controls and lower doses of streptavidin confirmed its survival advantage (SI Figure 3, $P < 0.05$).

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 were assayed for anti-streptavidin antibodies. Rats treated with streptavidin showed reactivity to streptavidin that remained stable until sacrifice

(Figure 4C). However, Vaccine (50)-treated rats demonstrated approximately 6 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. Further, as remission was only observed in Vaccine (50) rats, a low level antibody response may be optimal. The same sera (all groups and all timepoints) were also used for western blotting and ELISAs of unprocessed tumor lysates pooled from 6 additional rats. No reactivity was observed in any samples (data not shown). In combination, these data provide further evidence for streptavidin as a key immunogenic component in the vaccine.

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

Circulating cytokine levels were assessed at day 21 post-engraftment, and/or at endpoint (Table 1, Figure 4, SI Figure 4). The pro-inflammatory 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 (Figure 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 ($P < 0.05$, Table 1). Similarly, the pro-inflammatory interleukin 1 family cytokine IL-1 α was significantly elevated in Vaccine (50)-treated rats not in remission. ICAM1, which can be induced by pro-inflammatory cytokines, was significantly lower in the Vaccine (50) group than in controls at day 21 post-engraftment (Figure 4E, $P < 0.05$). No significant changes in cytokine levels were observed for rats treated with R-lysate or Vaccine (100).

Different cellular responses observed post-tumor engraftment between vaccination with Vaccine (50) and adjuvant only

Circulating immune cell levels were determined by multi-color FACS assays in peripheral blood collected from the control (vaccination with adjuvant) and Vaccine (50) groups (Figure 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 post tumor engraftment (Day 21). B cells for the Vaccine (50) group returned to normal while those of the control group remained significantly higher ($P < 0.05$, Figure 5); NK cells decreased significantly to below baseline for both groups ($P < 0.05$, Figure 5); and monocyte levels for the Vaccine (50) group decreased significantly below baseline and those of controls ($P < 0.05$, Figure 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 ($P < 0.05$, Figure 5). Neutrophil and lymphocyte levels remained stable with the exception of significantly lower lymphocyte levels in controls post-tumor ($P < 0.05$, Figure 5).

The vaccine extends survival of dogs with advanced cancer

Twenty five family pet dogs of a variety of breeds presenting with 9 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 (SI Figure 5). Vaccine protein profiles (SI Figure 6) were similar to those for the rat glioma model (Figure 3). The tumor types and survival data for this trial are shown in Figure 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 (Figure 6). This included inoperable tumor, relapse following chemotherapy, metastases or residual tumor remaining after surgery. Sixty three percent of these (7/11) survived longer than would otherwise be expected based on tumor grade, histology, and/or oncology report.

Twenty one dogs received 2 vaccinations and 71% (15/21) 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 prior to 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 turn-

around-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 recognise 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.

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). There are over 60 integral membrane proteins that contain an RGD sequence and could potentially bind streptavidin (12, 15). Many of these proteins such as integrins, VEGF-A, 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. While the final precipitation step has been used before as a potent way to present antigens with low

immunogenicity (18), utilizing 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 disulphide 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 predominantly 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, by 21 days post 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 post 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 cancer patients are reported to be indicative of

poorer prognosis and higher tumor grade (30). Melanoma and colorectal cancer patients with a higher level of tumor-infiltrating lymphocyte (CTL) also have a better prognosis (31, 30).

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

Cytokine analysis also identified differences in the levels of IL-4 and INF- 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 significant increase in INF- γ , which is a critical immune system component of the antitumor response (35). INF- γ together with lymphocytes not only provide protection against tumor development but also assist 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.

While 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 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 if using in an adjunct setting.

In conclusion we have developed a unique vaccine process for making autologous or syngeneic tumor vaccines with evidence of both slowed tumor growth and remission. The use of streptavidin as an immune stimulant 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 randomised clinical studies aimed at proving efficacy are warranted.

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FIGURES

Figure 1. Initial vaccine shows efficacy in a rat glioma model. Rats were administered vaccine (n = 3) or adjuvant (n = 5) at days -35 and -14 and then challenged with 1×10^6 9L tumor cells in the flank at day zero **A.** Tumor growth plotted as volume over time. Black horizontal line indicates the tumor size cut off for ethical euthanasia (13.5 cm^3). **B.** Survival curves for the same rats plotted as percentage rats surviving over time.

Figure 2. A regimen of 2 vaccinations shows greatest efficacy and remission in rat glioma. Survival curves are plotted as percentage survival over time for different vaccine strategies *versus* controls. **A.** A single i.p. vaccine dose (n = 8) *versus* controls (n = 8); **B.** 2 i.p. vaccinations (n = 9) *versus* controls (n = 8); **C.** 3 i.p. vaccinations (n = 9) *versus* controls (n = 8); **D.** 2 s.c. vaccinations (n = 8) *versus* controls administered 2 s.c doses of adjuvant (n = 6); **E.** Rats in remission rechallenged with tumor cells (n = 4 of which 2 were from initial vaccine study, Figure 1) *versus* controls (n = 10); **F.** Rats in remission rechallenged with tumor sections implanted in the brain (n = 5) *versus* controls (n = 6). 2A and 2B share the same control group (2 doses of adjuvant); 2C and 2E share the same control groups (unvaccinated). There are no differences in survival between the control groups (2A-2E). NS: Not significant.

Figure 3. Streptavidin identified in the vaccine protein profiles. **A.** Western blot of vaccine probed with vaccinated rat serum; **B.** Western blot 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, Gladesville, NSW Australia). kD: kiloDaltons

Figure 4. Vaccine (50) prolongs survival and induces remission, production of streptavidin antibodies, and TNF- α . Parameters for 4 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 post-engraftment; **E.** Serum ICAM1 levels at day 21 post-engraftment. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$, NS: not significant. P values determined by one way ANOVA except for Fig 4A which used Tukey's multiple comparison.

Figure 5: Changes in immune cell profiles are induced in rats treated with Vaccine (50) relative to adjuvant treated controls. Whole blood was analysed by FACS at 3 different timepoints: prior to the first vaccination (baseline: horizontal bar; n = 12); 7 days following vaccination and prior to engraftment (Pre tumor; n = 6 per group); and 21 days post engraftment (Post (Day 21); n = 6 per group). Control: black bars; Vaccine (50): white bars; *: $P < 0.05$

Figure 6: A Phase I canine clinical trial of the autologous vaccine demonstrates safety and efficacy (n = 25). Individual cases (y axis) are described (diagnosis and disease burden at time of vaccination) and plotted against survival time in months (x axis). The expected survival times were taken from patient notes or the British Small Animal Vet Association Manual of Canine and Feline Oncology (13). ** dogs received carboplatin with vaccine; ^,^^,^^^ - vaccination given 3,4, 8 months (respectively) after resection; BAC: Broncoalvelolar carcinoma; G: grade; HG: high grade; S: stage; COPD: Chemotherapy of Mitozantrone, Doxorubicin, Vincristine and Cyclophosphamide; CHOP: Chemotherapy of Cyclophosphamide, Doxorubicin, Vincristine and Prednisone.

Table 1. Circulating cytokine response at endpoint relative to control vaccinated rats. The Vaccine (50) group was further divided into 2 groups, those surviving > 100 days, considered in remission; and the remainder.

<i>Group</i>	<i>N</i>	<i>Survival</i> <i>Mean ± SD</i>	<i>Cytokine response relative to control</i> <i>P < 0.05</i>	
			<i>Increased</i>	<i>Decreased</i>
Control	5	38.4 ± 3.0	---	---
R-Lysate	6	43.0 ± 2.7	NSD	NSD
Streptavidin	6	51.3 ± 11.3	IL-1β, IL-13, MIP-3A, VEGF	M-CSF
Vaccine (50)	6	76.8 ± 18.6		
	(4)	<i>Died < 77 days</i>	IL-1α, IFN-γ, MIP-3A	TNF-α
	(2)	<i>Survived > 100 days</i>	TNF-α	IFN-γ, MIP-3A
Vaccine (100)	3	43.5 ± 6.1	NSD	NSD

SD: Standard deviation; N: number in each cohort; NSD: No significant differences. Cytokines measured that did not show 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, Rantes.

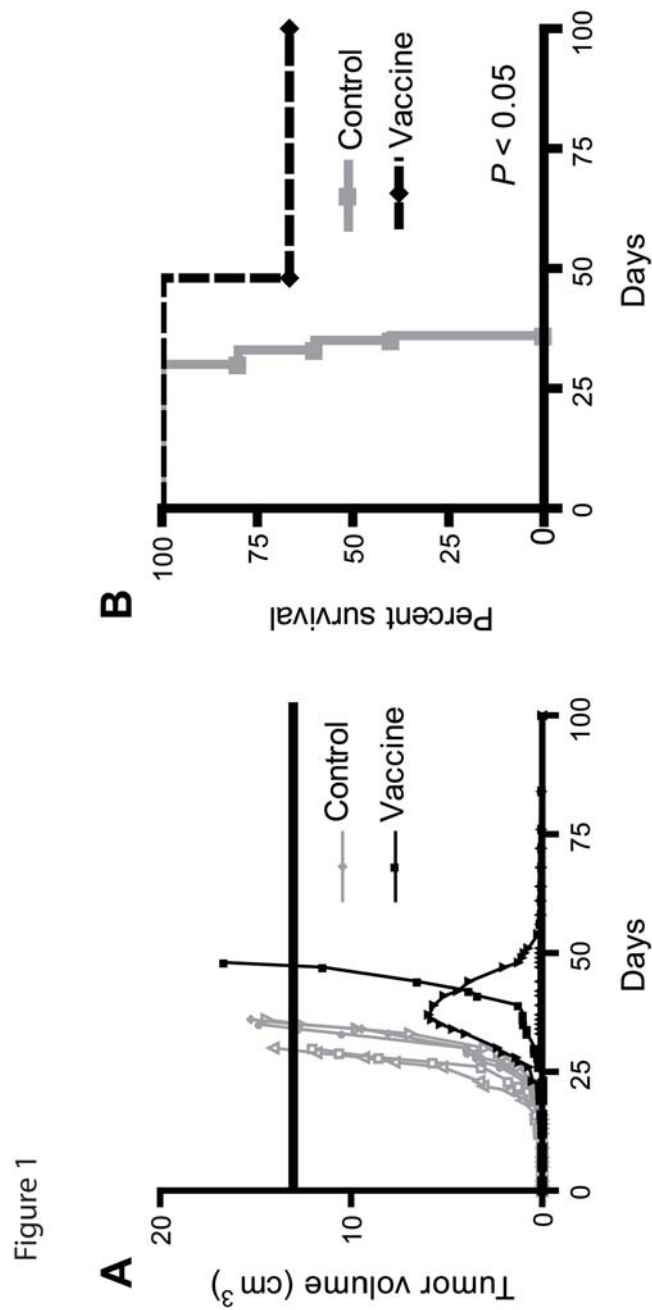
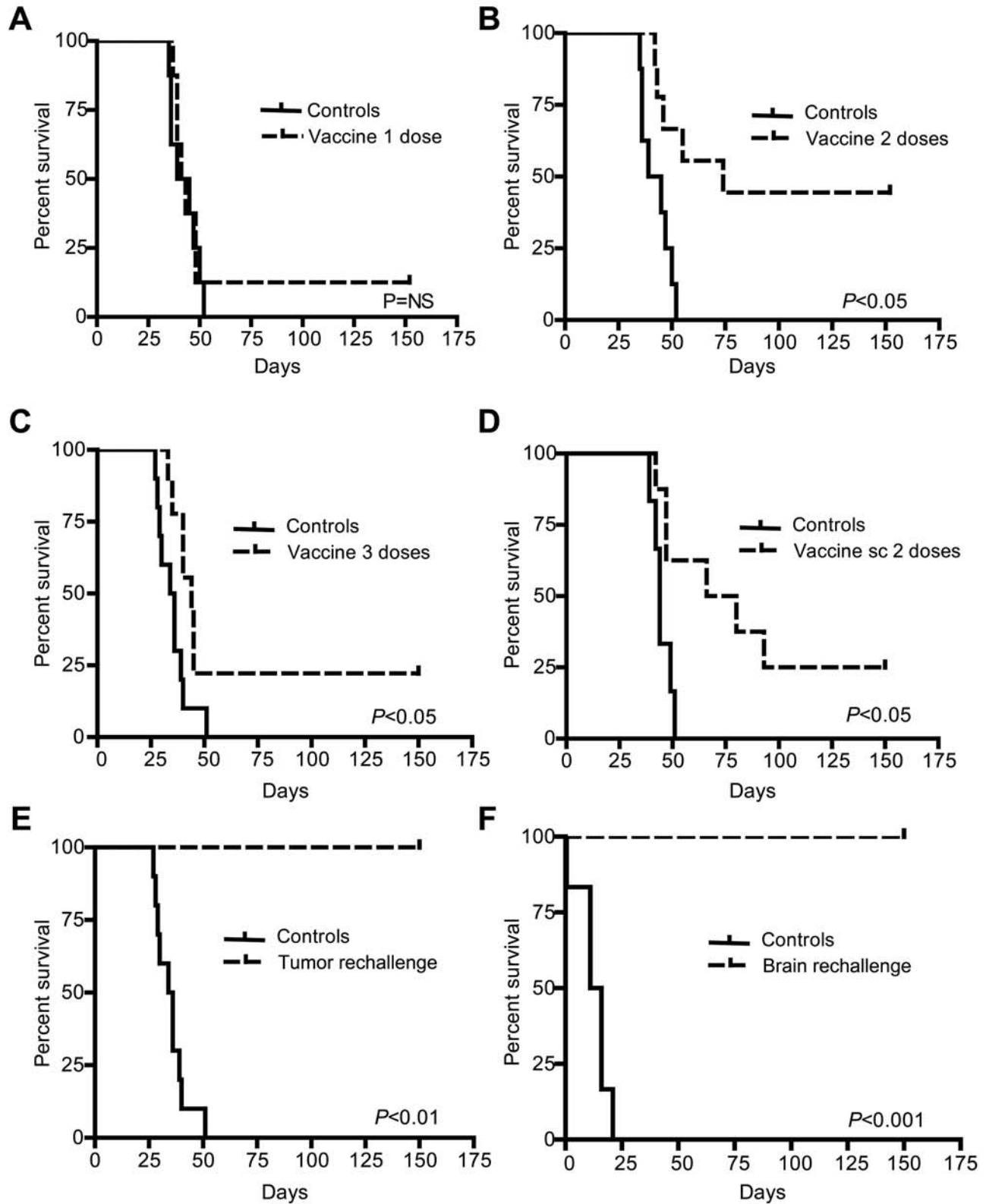


Figure 2



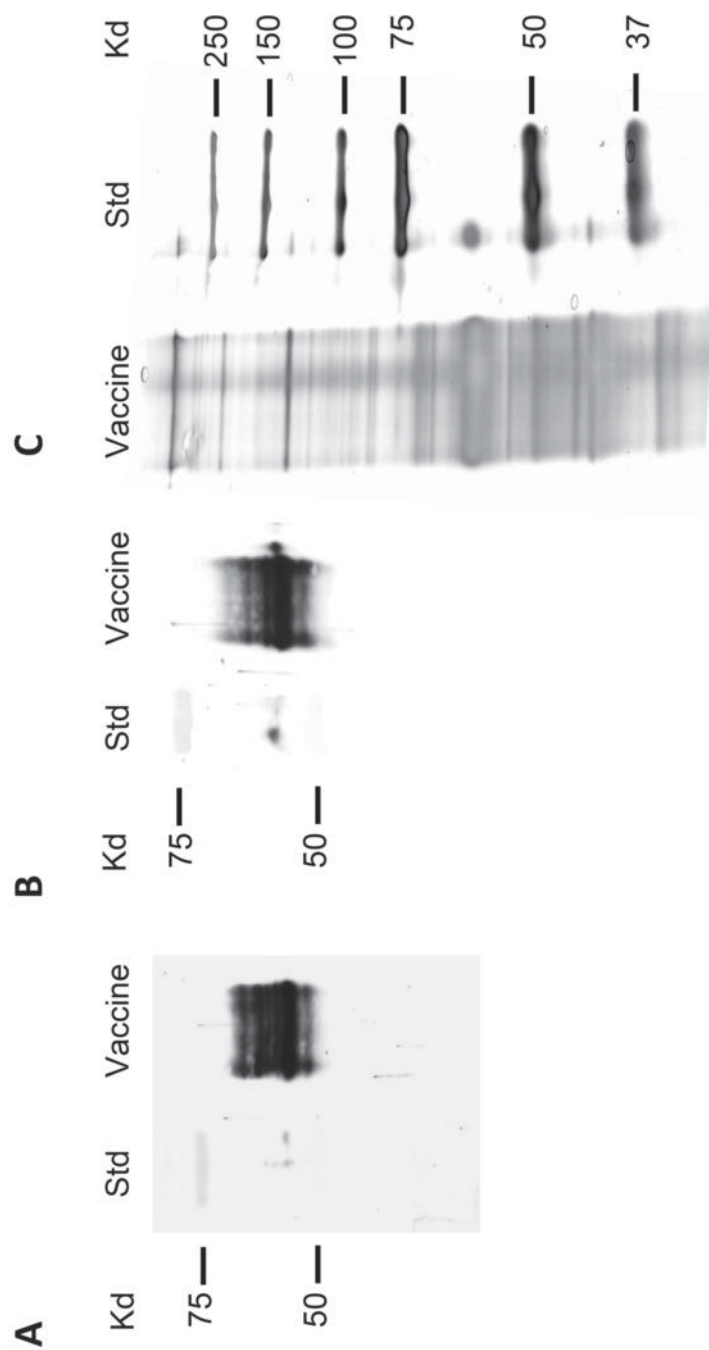
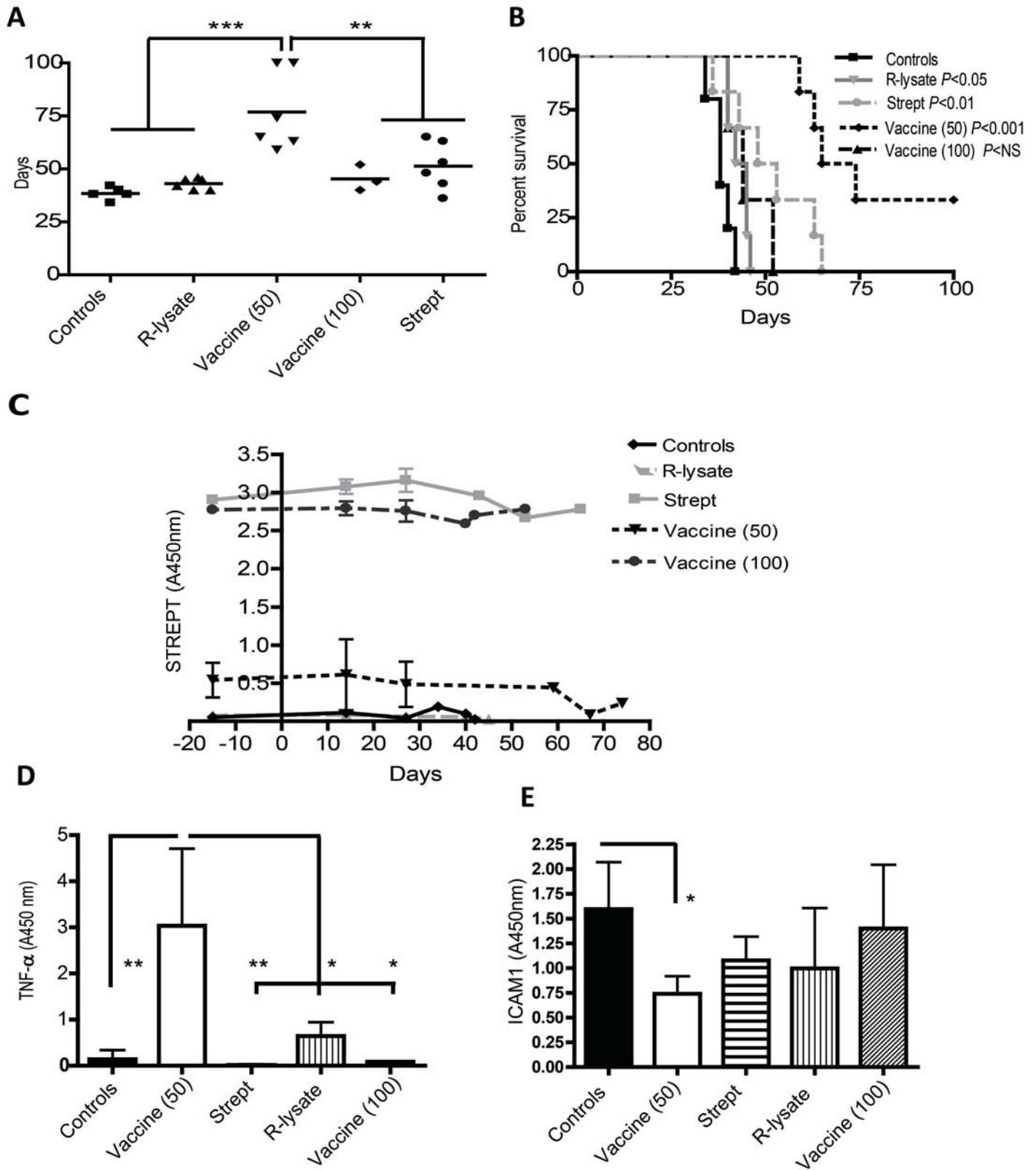


Figure 3

Figure 4



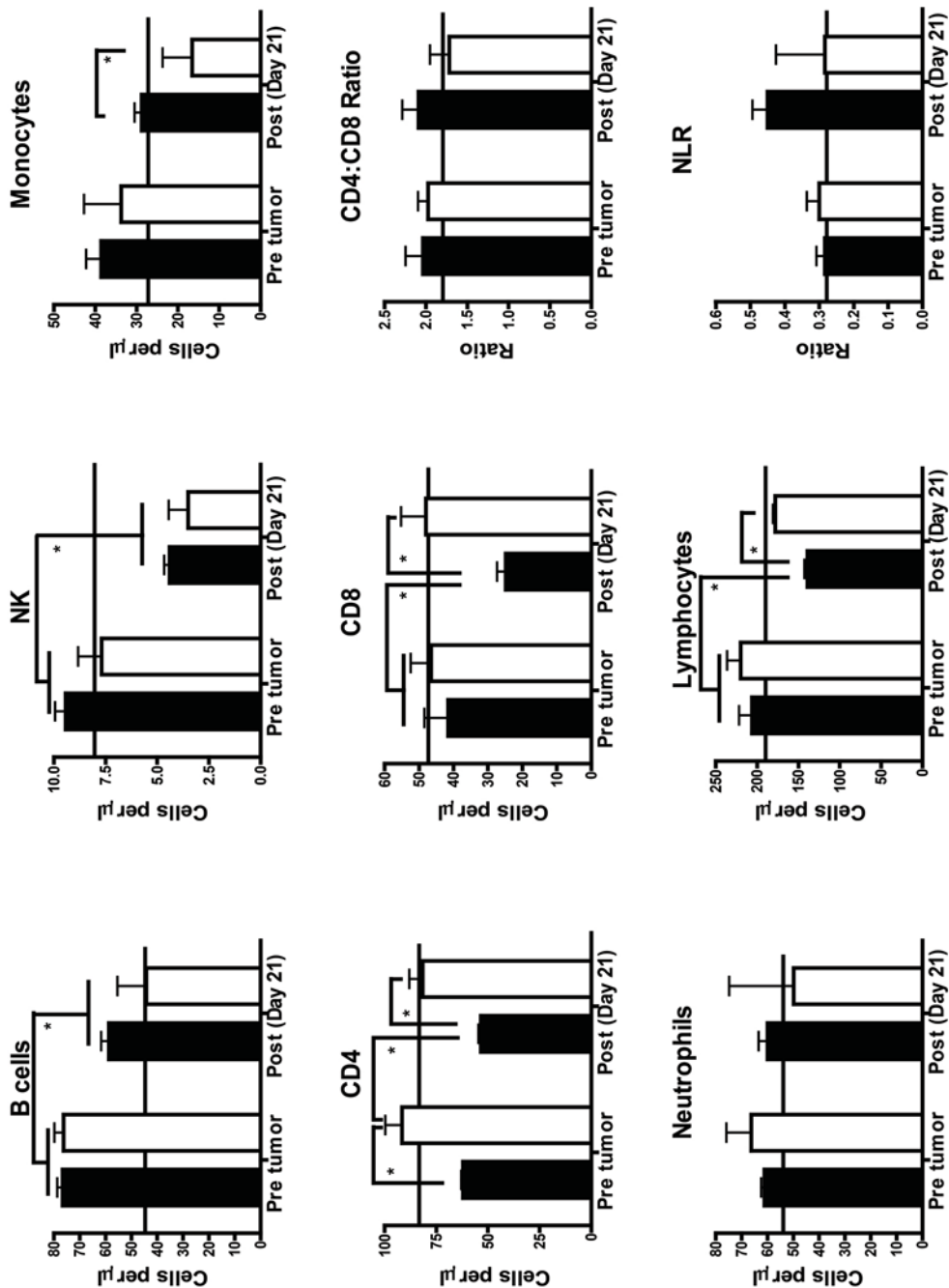


Figure 5

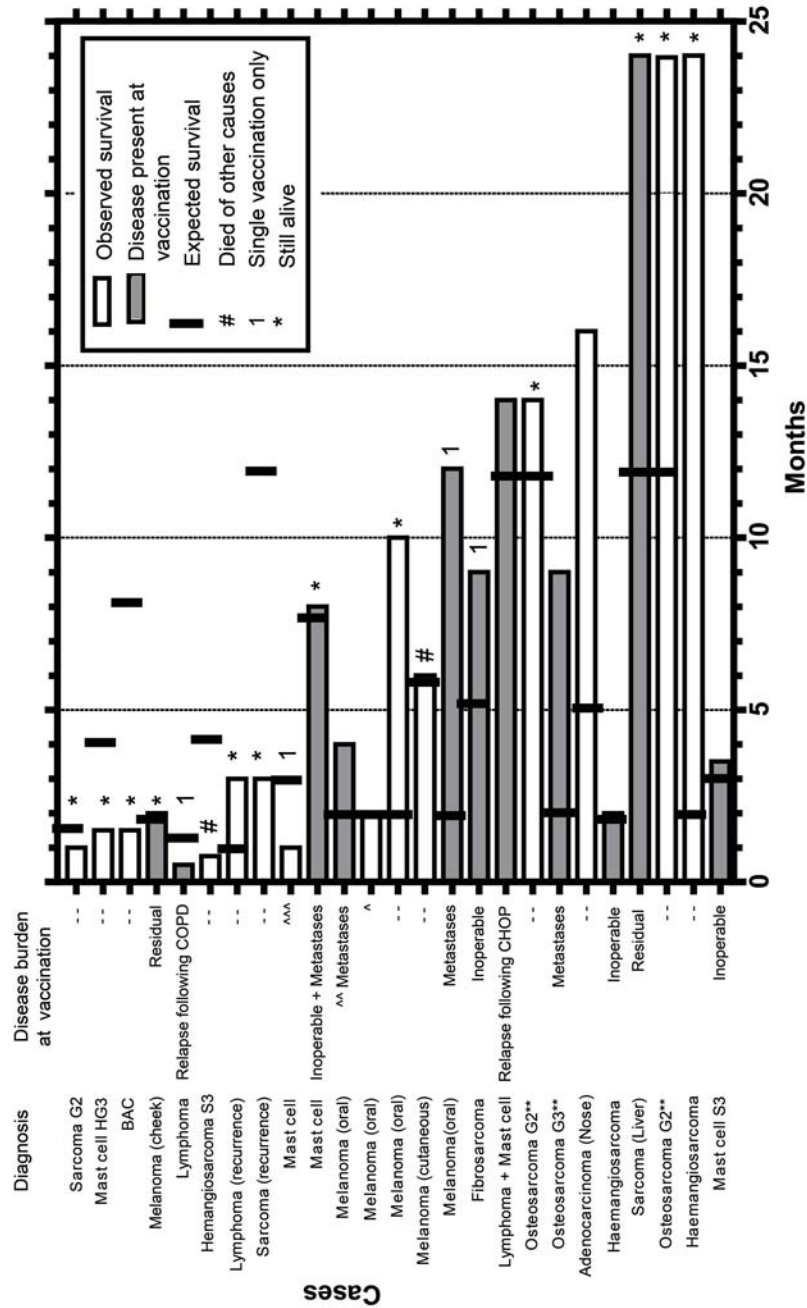


Figure 6

Cancer Immunology Research

Streptavidin: A novel immunostimulant for the selection and delivery of autologous and syngeneic tumor vaccines

Chris J Weir, Amanda Hudson, Elizabeth Moon, et al.

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