Cancer Therapy Vol 6, 827-840, 2008

Anti-tumor response induced by autologous cancer vaccine in canine lymphoma

Research Article

Michael Lawman[1,*], Shabnam Eidizadeh[1], Christopher Selmon[3], Curtis Kane[2], Laura Xigacos[2], Laura Kaufman[1], Neil Shaw[2], Pat Lawman[3]

[1]St. Joseph's Children's Hospital, Tampa, FL,
[2]Florida Veterinary Specialists, Tampa, FL,
[3]Morphogenesis, Inc., Oldsmar, FL

***Correspondence:** Michael Lawman, PhD, Department of Pediatrics, Division of Hematology/Oncology, St Joseph's Children's Hospital of Tampa, 3001 Martin Luther King Blvd, Tampa, FL 33607, USA; Tel: (813) 870-4504; e-mail: Michael.lawman@baycare.org

Key Words: immunotherapy, cancer vaccines, canine lymphoma, immune response, humoral and cell mediated immunity, autologous and allogeneic

Abbreviations: Ampicillin, (amp); B lymphocytes, (B cells); Bovine serum albumin(BSA); Cell mediated immunity, (CMI); Dimethylsulfoxide, (DMSO); Dulbecco's Modified Eagle's Medium, (DMEM); *Escherichia coli*, (*E. coli*); Fetal Bovine Serum, (FBS); Fluorescein isothyiocynate, (FITC); Fluorescent activated cell sorter, (FACS); Horseradish peroxidase(HRP); Immunoglobulin G, (IgG); Iscove's Modified Dulbecco's Medium, (IMDM); Luria-Bertani, (LB); N-2-Hydroxethylpiperazine-N'-2-ethanesulfonic acid, (HEPES); Neomycin sulfate, (Neo or G418); Phosphate buffered saline, (PBS); Polyacrylamide gel electrophoresis, (PAGE); Polyvinylidene Difluoride, (PVDF); Sodium dodecyl sulfate, (SDS) T lymphocytes, (T cells); Tris buffered saline, (TBS); Tris, tween buffered saline, (TTBS)

Received: 1 April 2008; electronically published: October 2008

Presented in the Theilen Tribute Symposium at UC Davis 31st May- 1st June 2008.

Summary

***ImmuneFx™* is an immunotherapy based on tumor cells transfected with the gene encoding Emm55, a *Streptococcus pyogenes* serotyping antigen. The *ImmuneFx* cancer vaccine was tested in 7 canine lymphoma patients with naturally-occurring disease. Humoral immunity was assessed by both PAGE Western blot analysis and quantitative Western dot-blot immunoassays. Cell-mediated immunity to autologous and allogeneic tumor cells was assessed by CTL assays. Clinical response was assessed by standard measurement criteria, and internal lesions were monitored by ultrasound. All dogs exhibited no adverse side-effects from the vaccine; developed an antibody response to the *ImmuneFx* priming antigen; and developed an antibody response to multiple autologous tumor cell antigens and tumor cell antigens from all other canine breeds on the trial. Furthermore, all dogs showed significant $CD8^+$-mediated cellular cytotoxicity specific to their own tumor cells but also demonstrated the presence of $CD4^+$ effector cells in the expanded lymphocyte population. The cytotoxic activity against the allogeneic target was attributable to the activity of $CD4^+$ effector cells. While all dogs showed positive clinical response to varying degrees, a therapeutically significant anti-tumor response was elicited against the slower growing indolent lymphoma. In this one dog (Golden Retriever), a pre-study abdominal ultrasound revealed mesenteric lymphadenopathy and that her spleen was coarse in echotexture and echogenicity with ill-defined nodular lesions. After 12 vaccine doses over 5 months, a post-vaccination ultrasound was unremarkable, with no abnormalities noted in the spleen and no mesenteric lymphadenopathy in evidence. Continued clinical evaluation reports reveal that no new lesions have been observed from the onset of the vaccine regimen, and all previous lesions have regressed. These results support the safety and anti-tumor response of the *ImmuneFx* cancer vaccine in dogs with naturally-occurring lymphoma and suggest that the same response would be similar in other veterinary cancers.**

I. Introduction

Canine lymphosarcoma or lymphoma is a highly aggressive hematological tumor (either B or T lymphocytes) (Wilkerson et al, 2005) and is the third most common cancer diagnosed in dogs. The annual incidence rate seems to be rising from 33 in 100,000 dogs (Teske, 1994) to 114 cases per 100,000 (Dobso et al, 2002). Although any age dog can be affected, the average age of

dogs with lymphoma is between 6-9 years. Breeds such as Boxers, German Shepherds, Golden Retrievers, Scotties and Pointers are more likely to develop this type of cancer, yet males and females are equally at risk. Chemotherapy is the current treatment of choice; however, recurrence and multi-drug resistance are common (Simon et al, 2008).

Cancer vaccines and other immunotherapies act by activating the immune system to fight tumor cells in either a specific or non-specific manner. The mechanism of action differs with different types of therapy (Quintin-Colonna et al, 1996; Dow et al, 1998; Hogge et al, 1999). *ImmuneFx* acts by providing a priming action for the immune system which initiates an immune response that is specific to the patient's tumor cells. This can be better understood when viewed from the perspective of the immune system. All cells have unique proteins or markers on their surface termed *antigens*, and many cancer cells make tumor-specific antigens (Anderson et al, 2003). Unfortunately, sometimes tumor antigens are "hidden" from the immune system and the body is unable to make an effective immune response against what it recognizes as "self". When this happens, tumors are said to escape immune surveillance. The goal of cancer immunotherapy is to educate the immune system to recognize these cancer-specific antigens and then reject any cell which bears them (Boon and Bruggen, 1996). Normally, foreign antigens are recognized by antigen-presenting cells, which then activate both arms of the immune system; humoral or antibody mediated, which is orchestrated by B cells and cell-mediated, which is orchestrated by T cells (Suto and Srivastava, 1995). Thus, the optimum strategy would be to design an immunotherapy which would make tumors appear 'foreign' so the immune system will recognize them as such, resulting in a tumor-specific immune response, which would eradicate the malignant cells. *ImmuneFx* was designed to provide the 'foreign' antigen which initiates a tumor-specific immune response.

The priming antigen, Emm55, is a serotyping protein (Lancefield, 1962) normally expressed on the surface of the bacterium, *Streptococcus pyogenes* and is highly antigenic but not rheumatogenic (Boyle et al, 1994; Hollinghead et al, 1994; Cunningham et al, 1997). To create the vaccine, the gene encoding the Emm55 antigen is introduced into the patient's autologous tumor cells in the form of DNA plasmid by *in vitro* transfection. The successfully targeted tumor cells express the Emm55 antigen both intracellularly as well as on the surface of the cells. Prior to using these targeted cells in the patient, the cells are γ irradiated to inhibit proliferation *in vivo*. Since the Emm55 antigen is expressed on the surface of the tumor cells, it acts as a signal to the recipient's immune system to activate. In this way, a strong immune response is directed to the tumor in a way that was not possible before. While many immunotherapies utilize immunopotentiators such as protein, bacterial, viral, chemical or naked DNA adjuvants which can evoke an immune response, the response is not necessarily directed toward tumor cells or subpopulations of tumor cells expressing minor antigenic variants. This point is worth emphasis. Although each tumor, regardless of its tissue of origin, probably begins by the clonal reproduction of a single cell, additional changes eventually give rise to an amazing diversity of different sub-clones and as such are antigenic variants. Once under the selective pressure of the host's immune response, low antigenic variants gain advantage over sub-clones which express fewer or more immunogenic molecules. The less immunogenic and the lower the density of the tumor-associated antigens on the cell surface, the more likely the tumor cells will fall below the threshold of immune detection and become invisible to host surveillance. Although some of the tumorogenic sub-clones may be destroyed using conventional therapies and some methods of immunotherapy, rarely are all the different sub-clones killed. It is reasonable to postulate that a cross section of tumor cells from each cancer patient would serve as a more effective vaccine because each tumor cell carries its own complete complement of tumor antigens. This means that tumor cell variants, such as radio- or chemo-resistant variants, would be specifically targeted by the immune response primed by the Emm55 antigen if they were used as the basis for the vaccine. And, since these variant cells are usually the cells that are responsible for relapses, metastases and eventual demise of the cancer patient, it is critical that these variants are specifically targeted with an effective therapy, in particular, *ImmuneFx*.

The purpose of this study was to assess the safety and tolerability of *ImmuneFx* and to test its ability to initiate a tumor-specific immune response in patients with canine lymphosarcoma. As expected, *ImmuneFx* elicited a tumor-specific immune response (both humoral and cell-mediated) in all the canine patients with lymphosarcoma and produced no toxic side effects.

II. Materials and Methods

A. Original gene cloning and vector synthesis

The parental vector, pJLA602, (kindly provided by Dr. Michael Boyle) was isolated and purified from *E. coli* DH5α (Boyle et al, 1995) using standard techniques (Sambrook et al, 1989). The *emm55* cDNA was excised using the restriction endonucleases *Bam* HI and *Eco* RI and ligated into similarly digested pcDNA3, a mammalian expression vector that in addition to features common to other such vectors, contains a *neo* gene for the selection of stably transfected cells. The ligation reactions were used to transform competent *E. coli* JM109 cells. Cultures were selected on LB agar plates supplemented with ampicillin. Purified plasmid DNA from three clones was analyzed by restriction endonuclease digestion and agarose gel electrophoresis. A single clone was selected and a restriction map was generated using *Pst* I, *Hind* III, *Nde* I, *Eco* RV, *Bam* HI, *Eco* RI and *Xba* I. The restriction analysis confirmed the successful sub-cloning of the complete *emm55* gene into pcDNA3 (pcDNA3/*emm55*).

B. Production of plasmid vector DNA

Laboratory scale production of the pcDNA3/*emm55* plasmid was carried out by allowing frozen cultures of *emm55*-transformed *E. coli* (strain, JM109) to thaw on ice for 5-10 minutes. To ensure single colonies, 50 µl of the bacterial suspension was spread onto five 100 mm diameter LB agar plates supplemented with 50 µg/ml of ampicillin which were then incubated at 37°C overnight. Following overnight growth, single colonies were harvested, placed in 5 ml of LB broth supplemented with 50 µg/ml of ampicillin and grown overnight at 37°C in a shaker incubator. A loop of culture was then streaked

onto fresh LB/amp agar plates for future reference. Small quantities of the pcDNA3/*emm55* plasmid DNA were prepared using Qiagen's Spin Miniprep Kit (Valencia, CA) according to the manufacturer's instructions/protocol. Briefly, overnight cultures were centrifuged (3,000 rpm) and the supernatant discarded. The resulting bacterial pellet were resuspended in 250 µl of the buffer provided and placed in a microcentrifuge tube. To this suspension, 250 µl of a lysis buffer (lyseblue) was added and the suspension thoroughly mixed by inverting the tube 4-6 times. Following mixing, 350 µl of a provided buffer was added and mixed and the lysed bacterial suspension was placed in a table top microcentrifuge and centrifuged for 10 minutes at 13,000 rpm. The supernatant was then harvested and applied to QIAprep spin column and centrifuged (13,000 rpm) for 10 seconds. The flow-through was discarded. The spin column was washed in the provided buffer, again discarding the flow-through. To elute the plasmid DNA, the spin column was placed on top of a fresh microcentrifuge tube and 50 µl of elution buffer was added. The tube was incubated at room temperature for 1 minute, and then centrifuged (13,000 rpm). The eluted plasmid preparations was collected and stored at -80ºC until required.

C. Analysis of plasmid DNA

Purity, concentration and yield of plasmid DNA was determined using standard spectrophotometric methods and as described (Farrell, 1993). Briefly, the concentration and purity of the DNA was estimated using a Beckman dual beam-spectrophotometer and a nucleic acid program module. The concentration of the nucleic acid is given by:

[DNA] µg/ml = A260 x Dilution x Expansion coefficient of DNA

Purity of the plasmid preparations are taken from the ratio of the OD260/280 reading. For the purposes of future trials, DNA should have an OD reading of 2.0 +/- .02 as determined by these methods. To further assess the purity of the plasmid, all plasmid preparations were subjected to agarose gel electrophoresis using Ambion's Northern Max protocol. Briefly, 1% agarose plus ethidium bromide was used in a horizontal mini-electrophoresis cell. The gel was loaded with 20 µl of sample plus an equal volume of glyoxal loading dye. The gels were run using 5 V/cm (85 V for 90 minutes).

D. Patient recruitment protocol

For either enrollment in or exclusion from this study, patients were required to meet the following criteria:

- Patients must have histologically or cytologically confirmed evidence of multi-centric lymphoma.
- Patients must not have had chemotherapy or wide field radiotherapy prior to beginning protocol therapy and no immunosuppressive therapy, including corticosteroids.
- Patients can be any canine breed, age, sex in otherwise good health.
- Patients must have staging diagnostic.
- Patients must have normal organ and marrow function.
- Patients must have an owner with the ability to understand and the willingness to sign a written informed consent document and comply with the protocol.
- Patients who have had chemotherapy, radiation therapy, or corticosteroid therapy prior to entering the study are excluded.
- Patients may not be receiving any other investigational agents.
- Patients with a history of allergic reactions attributed to compounds of similar chemical or biologic composition to *Streptococcus pyogenes* are excluded.
- Patients with a life-threatening illness unrelated to cancer are excluded.
- Patients with an additional uncontrolled or active illness; other than lymphoma are excluded.

E. Preparation of canine leukocyte conditioned medium

Canine peripheral blood mononuclear cells from normal donors were cultured at a cell concentration of $2x10^6$/ml in15 ml of RPMI containing 5% FBS and 1ug/ml phytohemagglutanin. Cell cultures were then incubated at 37^0C for 48 hours in a humidified atmosphere of 5% CO_2 in air. Following the incubation, the cell suspension was harvested, centrifuged (2,500 rpm for 15 minutes) and the supernatant collected. The cell-free supernatant was then filtered (Millipore) through a 0.22µm membrane and stored at 4^0C until required.

F. Transfection of canine lymphoma tumor cells

Patients complying with the entry criteria were invited to enter the study following the owner's informed consent. Patients were subjected to complete medical, physical, hematologic and biochemical examinations (Cell Blood Count, Serum Chemistry Panel, and Urinalysis) and diagnostic imaging (thoracic radiographs and abdominal ultrasound) to document general fitness and stage the disease to proceed with the trial.

For the production of the canine lymphoma vaccine, tumor tissue was collected via fine needle aspiration from affected lymph nodes at diagnosis and deposited into sterile tubes containing tissue culture media supplemented with fetal bovine serum. Lymphoma cells isolated from tumor samples were transfected with the pcDNA3/*emm55* plasmid by electroporation. Cells expressing the Emm55 antigen were selected in the presence of G418 and canine lymphocyte conditioned medium and expanded to the required cell concentration. Prior to use, the transfected cells were γ irradiated (10,000 rads, 30 minutes) then stored in liquid nitrogen in 2 ml DMEM supplemented with 10% DMSO and 20% FBS.

Prior to beginning the actual clinical study, vaccine was produced from tumor samples of 5 dogs with confirmed cases of lymphoma in order to test the robustness of the vaccine production procedure. The same protocol was followed to produce vaccine for these 5 dogs and the 9 dogs enrolled in the trial. Electroporation conditions and efficiencies for each dog are shown in **Table 2**. The number of tumor cells present in the tissue biopsies ranged from $1.2x10^6$ to $2.7x10^8$. In all cases, there were sufficient cells to produce vaccine. Cells expressing the Emm55 antigen were selected in the presence of G418 and canine lymphocyte conditioned medium and expanded to the required cell concentration. Seven days post-transfection, Emm55 expression was determined by immuno-fluorescence. From the time the biopsy was performed, it took an average of 14 days to produce sufficient cells for at least 4 vaccine doses.

G. *ImmuneFx* administration and clinical testing

Immediately before administration of the vaccine, heparinized blood (20 ml) was drawn for *in vitro* immunological studies and for clinical pathology (full blood count and clinical chemistry). A urine sample was obtained for urinalysis for protein and blood. The disease was restaged in accordance with current guidelines. All vaccine doses were administered intravenously at a cell concentration of $1x10^7$ in a volume of 2 ml. Patients were monitored immediately following injection by veterinary staff and overnight for side-effects compatible with an anaphylactic or other reaction to the to vaccine (vomiting, diarrhea, collapse, shock). Any reaction was treated immediately.

Table 1. Summary of patient information and timeline of protocol administration in the *ImmuneFx* canine lymphoma study. *ImmuneFx* autologous vaccine was successfully created for and administered to all dogs in the study. Trial endpoints were met for all patients.

Breed	Age (yrs)	Sex	Weight (Kg)	Disease Stage	Vaccine Produce	Weekly doses	Monthly Doses	Immune Response	
								Humoral	CMI
1. Bulldog	5	Male	28.2	3a	YES	2	0	+	+
2. Golden Retriever	11	Female	37.7	4	YES	9	12	+	+
3. Dachshund	7	Male	6.2	3a	YES	5	0	+	+
4. Vizsla	4	Male	28.4	4/5	YES	7	3	+	+
5. Greyhound	10	Female	32.2	4/5	YES	7	1	+	+
6. Welsh Corgi	10	Male	16.4	4	YES	4	0	+	+
7. Chow Chow	10	Male	20.5	4	YES	4	0	+	+

Table 2. Clinical outcome of ongoing canine study. Autologous *ImmuneFx* cancer vaccine was administered to canine lymphoma patients of various breeds. The time from biopsy to vaccine administration; vaccine administration to chemotherapy (when administered); diagnosis to outcome; diagnosis to outcome; first vaccination to outcome and the number of days survival attributable to vaccine are shown for each patient. For dogs not receiving chemotherapy, survival attributable to vaccine was calculated by subtracting the days survived beyond the outer limit of expectation (28 to 42 days) from diagnosis to outcome. For dogs receiving chemotherapy, survival attributable to vaccine was calculated by subtracting the days survived beyond the outer limit of expectation (42) from time of chemotherapy (days not shown) to outcome. The outcome for each patient is listed as NCRE (non-cancer related euthanasia); CRE cancer related euthanasia or ongoing (the patient is still being evaluated). The effect on survival is survival time attributable to vaccine plus 42 divided by the outer limit of expectation (42). The study is ongoing with respect to the Golden Retriever. Note: NAD = not able to determine.

Patient number and Breed	Biopsy to Vaccine #1 (days)	Vaccine #1 to chemo (days)	Diagnosis to outcome (days)	Vaccine #1 to outcome (days)	Survival Attributable to Vaccine (days)	Outcome	Effect on Survival
1. Bulldog	24	-	50	26	8	NCRE	NAD
2. Golden Retriever	16	-	647+	555+	Ongoing	Ongoing	Ongoing
3. Dachshund	15	-	74	39	YES	CR?E	~2X
4. Vizsla	21	0	190	168	YES	NCRE	NAD
5. Greyhound	18	52	212	126	YES	CRE	~3X
6. Welsh Corgi	33	0	90	57	YES	CRE	NAD
7. Chow Chow	26	33	86	48	YES	CRE	~2X

H. Safety and tolerability

Safety and Tolerability of *ImmuneFx* was monitored according to the following toxicity guidelines used by the National Cancer Institute:

Grade 0: *No adverse effects;*

Grade 1: *Mild symptoms*: responsive to drugs and appropriate supportive measures, for example, mild fever, nausea, diarrhea, mild respiratory complications and/or mild respiratory distress that respond clinically to treatment

Grade 2: *Moderate symptoms*: responsive to drugs and appropriate supportive measures. Moderate signs/symptoms are similar to Grade 1, except they are moderate in nature

Grade 3:*Severe symptoms*: non-responsive to drugs and persisting for more than 24 hours. These sign/symptoms are criteria for pausing the study. For example, cardiac arrhythmia, certain infections, severe immunological or anaphylactic reactions and other severe but non-life-threatening system complications

Grade 4: *Life-threatening events*: These signs/symptoms are criteria for stopping the study. For example, high fever for more than 72 hours after *ImmuneFx* delivery that is insensitive to anti-pyretics and not attributable to other causes and other extreme symptoms not attributable to other etiology

Grade 5: *Fatal event*

A summary of the patient information prior to entering the clinical protocol is shown in **Table 1**. Seven dogs were enrolled, each representing a different breed (Bulldog, Golden Retriever, Dachshund, Vizsala, Greyhound, Welsh, Corgi and Chow Chow). The dogs aged from 4 years to 11 years and there were a mixture of sexes (2 female and 5 male). All the dogs presented at the time of treatment with late stage disease (stage 3 through stage 5). Owners of the dogs were offered conventional treatment, chemo/radiation, or experimental immunotherapy treatment with *ImmuneFx*. Owners were also told that at anytime on the experimental protocol they could remove their dogs from the *ImmueFx* protocol and request that their pet be put on the Wisconsin Protocol. **Table 1** also shows that an autologous vaccine was manufactured for each dog. All the dogs received the vaccine intraveneously weekly for 4 weeks and then monthly thereafter. The two dogs that did not receive monthly doses of autologous vaccine (Dachshund and Bulldog) did not survive their disease and died just prior to receiving monthly vaccine doses. All dogs were shown to have generated both a humoral and cellular immune response to the autologous tumor cells.

I. Evaluation of *emm55* gene expression in transfected canine lymphoma cells

Expression of Emm55 was evaluated using two methods. First, transfected tumor cells were analyzed using flow cytometry. Anti-Emm55 murine monoclonals 8F-10; 25C and

15β3 used in these assays were provided by Dr. M. Boyle. Transfected canine lymphoma cells were incubated with primary antibody, at 4^0C, for 60 minutes. Following incubation with the primary antibody, cells were washed 3 times in incomplete IMDM. Primary labeled lymphoma cells were resuspended and then incubated with a FITC-labeled rabbit anti mouse IgG (heavy and light chain) at 4ºC for 30 minutes. Again the labeled cells were washed 3 times and finally resuspended in incomplete IMDM. Labeled cells were then analyzed for Emm55 antigen expression using a BD, FACSCalibur flow cytometer (BD Biosciences, CA). For the detection of intracellular antigens by either flow cytometry or immunofluorescence, cells were fixed and permeabilized using CalTag Laboratories Fix and Perm reagent. These reagents are intended for the fixation and permeabilization of cells in suspension. The protocol facilitates the intracellular localization of both unlabeled and fluorochrome-labeled antibody without interfering with the morphological scatter properties (both forward and side scatter) of the cell population and therefore allows for intracellular antigen analysis as well as cell surface antigen expression. The procedure followed was as directed by manufacturer's instructions. Briefly, transfected lymphoma cells were with the required concentration of primary antibody, mixed gently and incubated for 15 minutes at room temperature. Following incubation with the primary antibody, 100 µl of the fixative reagent was then added and the cells were incubated for a further 15 minutes at room temperature. Following this incubation the cells were washed once in PBS + 0.1% NaN_2 +5% FBS and centrifuged at 350 x G and the supernatant removed. To the pellet was added 100ul of the supplied permeabilization media in conjunction with the required concentration FITC-labeled secondary antibody. The cells were gently mixed and incubated at room temperature for a further 20-to-25 minutes. Following this incubation the cells were again washed once in PBS + 0.1% NaN_2 +5% FBS, the cell pellet was resuspended in IMDM. The cell suspension was either subjected to cyto-centrifugation and cells examined by fluorescent microscopy or by FACS analysis.

Western blot analysis, a semi-quantitative measurement, was used to evaluate expression of the Emm55 protein in tumor cells and to monitor the development of antibodies to Emm55 and tumor cell antigens produced in vaccinated animals. In this assay, $5x10^6$ total tumor cells were lysed using 1 ml of a mammalian lysis buffer (*Mammalian Cell-PE LB*, Geno-Tech, St. Louis, MO). The cell lysates were then clarified by centrifugation and the supernatant harvested and stored. The lysates were subjected to 10% SDS-Polyacrylamide gel electrophoresis at 200 V for 45 minutes. Transfer of the separated proteins from the gel to PVDF membrane was carried out using a BioRad Semi-dry Transfer System at 100 mA for 60 minutes. Following transfer the blot/PVDF membrane was washed overnight using 100 ml of TTBS with 1% non-fat dried milk. The PVDF/blot was then transferred to a glass hybridization tube and 15 ml of anti Emm55 monoclonal antibody (clone 25C3) at a dilution of 1:5,000 was added. The PVDF/blot was then incubated and rotated in a hybridization oven at 37ºC for 30 minutes. After this incubation step, the PVDF/blot was washed 3 times in 15 ml of TTBS buffer. Following the final wash, 20 ml of a 1:20,000 dilution of a sheep anti-mouse IgG (horseradish peroxidase labeled) was added and the PVDF/blot incubated for a further 30 minutes at 37ºC. The PVDF/blot was then washed 3 times in TTBS. Detection of the bound antibody was carried out using Amersham's ECL Western Blotting Analysis System in accordance with the manufacturer's instructions. Labeled bands were detected by placing the PVDF/blot on an X-ray film and exposing the film for varying times depending on the signal strength of the blot.

J. Evaluation of humoral immune response in dogs vaccinated with *ImmuneFx*

The development of humoral responses in the dogs under study was followed by PAGE Western blot as previously described and quantitatively by a western dot blot procedure. As stated earlier, immediately before administration of the vaccine, heparinized blood (20 ml) was drawn for *in vitro* immunological studies and for clinical pathology (full blood count and clinical chemistry). The blood sample was transferred to a 50 ml tube and subjected to centrifugation (2,500 rpm) for 25 minutes. Following centrifugation, the plasma was removed and dispensed into a sterile 15 ml conical tube, the buffy coat was also carefully removed in a maximum of 5 ml volume from the cell pellet and dispensed into a 15 ml conical tube. To the cell suspension was added 10 ml of RPMI 1640 medium supplemented with FBS (5% v/v). Peripheral blood leukocytes were enriched using a one-step Ficoll gradient. Peripheral blood leukocytes were layered onto a Ficoll density gradient and centrifuged for 45 minutes at 3,000 rpm. Following the centrifugation step, the lymphocyte-enriched cell population was harvested from the layer of cells suspended at the interface of the gradient and media, these cells were washed twice and finally stored in liquid nitrogen in a storage media supplemented with 10% DMSO and 20% FBS until used in i*n vitro* cellular cytotoxicity studies.

K. Quantitative dot blot analysis for determining the humoral response of canines to vaccination

The dot blot analysis was carried out as follows: Prior to use the nitrocellulose membrane was equilibrated in TBS for 10 minutes. Following equilibration, the membrane was placed in the BioRad Dot Blot Apparatus according to manufacturer's instructions. In each well was added autologous canine lymphoma cell lysate at a predetermined protein concentration and a vacuum applied. Once the antigen was applied to the dot blot, the membranes were then blocked using 200 µl of 0.002% BSA in TBS. Following the blocking step, the membranes were washed twice with TTBS applying vacuum after each wash. Two fold dilutions were made of the canine sera ranging from 1:20-to-1:10,240 using 0.002% BSA in TBS. The dilutions were applied to the wells (2 wells/dilution) and allowed to filter through the nitrocellulose by gravity at room temperature for 30-45 minutes. After the incubation with the test serum, the membranes were washed three times with TTBS, applying the vacuum between each washing step. A 1:1,000 dilution of the HRP-labeled secondary antibody (goat-anti canine IgG) was then applied to the membrane and allowed to filter by gravity for 30-40 minutes. Again, following the incubation with the secondary antibody the membrane was washed twice with TTBS, applying vacuum after each wash. The membranes were then removed from the apparatus and incubated with 10 ml of 4-chloro-1-napthol supplemented with 20 µl of hydrogen peroxide. After spots developed, a photo of the membrane was taken and the concentration of the antibody expressed at the highest dilution to give a reaction was recorded.

L. Evaluation of the cellular immune response of canine lymphocytes to autologous tumor cell targets

The cellular immune responses to autologous tumor cells were evaluated using a non-isotopic-based CMI assay (CytoTox 96®, Promega, Madison, WI) according to manufacturer's instructions. Briefly, suspensions of Ficoll-purified canine peripheral blood lymphocytes were prepared from the test samples. The cells were resuspended in RPMI 1640 supplemented with 10% FBS, 2-mercaptoethanol, and HEPES.

The lymphocytes, at $5x10^6$ cells/well, were then incubated with stimulator cells ($2x10^5$ autologous tumor cells treated with 100 µg/ml mitomycin C for 30 minutes at 37ºC). Plates were then incubated at 37ºC in a humidified 5% CO_2 atmosphere for 6-to-7 days to expand tumor-specific effector cells. To measure cytotoxicity against tumor cell targets, effector cells were harvested following *in vitro* stimulation for 6-to-7 days and incubated with target cells at various effector-to-target ratios in a short term cytotoxicity assay (4 hours).

III. Results

A. Clinical assessment of *ImmuneFx* in dogs with lymphoma

Although clinical response was not a primary endpoint of this study, patients with measurable disease were assessed by standard criteria involving the measurement of the size of peripheral lymph nodes. Internal lesions were monitored by ultrasound. The data in **Table 2** summarizes the clinical outcome of the trial. Due to an episode of dietary indiscretion, the Bulldog developed a severe case of food poisoning and was humanely euthanatized before the end of the study. At the request of the owners, two dogs, the Vizsla and the Corgi, were administered chemotherapeutic agents alongside the vaccine from day 1. As the data show (**Figures 1-4**), these dogs made significant humoral and cell mediated responses during the time of the chemotherapy treatment. No conclusions can be drawn as to the effect that the vaccine may have had in influencing the overall length of their survival. However, the chemotherapy received by 2 other dogs was administered later, at day 52 post-vaccination for the Greyhound and day 33 post-vaccination for the Chow. By taking the outer limit of their expected survival time at diagnosis, the data is suggestive of extended survival rates between 2 and 3 times normal life expectancy. The patient still being followed, the Golden Retriever, had a slower growing indolent form of lymphoma. In fact, she had presented originally with enlarged lymph nodes 1 year prior to entering the study. At the time she received the first vaccine dose, she was diagnosed with disseminated stage 4 multi-centric indolent intermediate-size lymphoma, which was confirmed by flow cytometry. Similar to the remaining dogs in the study, she exhibited no adverse side-effects from the vaccine. Furthermore, like the other dogs on the trial, she developed a highly significant antibody response to the *ImmuneFx* priming antigen and tumor cell antigens as well as a strong cellular response to autologous tumor cells and tumor cells from all other canine breeds on the trial.

In this one dog (Golden Retriever), a pre-study abdominal ultrasound revealed mesenteric lymphadenopathy and that her spleen was coarse in echotexture and echogenicity with ill-defined nodular lesions. After 12 vaccine doses over 5months, a post-vaccination ultrasound was unremarkable, with no abnormalities noted in the spleen and no mesenteric lymphadenopathy in evidence. Continued clinical evaluation reports reveal that no new lesions have been observed from the onset of the vaccine regimen, and all previous lesions have regressed.

B. Transfection efficiency of canine lymphoma with pcDNA3/*emm55*

The tumor cells recovered from the biopsies ranged from $1.2x10^6$ to $2.7x10^8$, with an average cell count of $9.8x10^7$. The electroporation conditions and transfection efficiency of pcDNA3/*emm55* plasmid in canine lymphoma tumor cells are shown in **Table 3**. The ability to transfect and obtain expression of the targeted gene (*emm55*) was experimentally determined in these dogs prior to enrollment. The initial transfection efficiency of pcDNA3/*emm55* ranged from 9 to 20%.

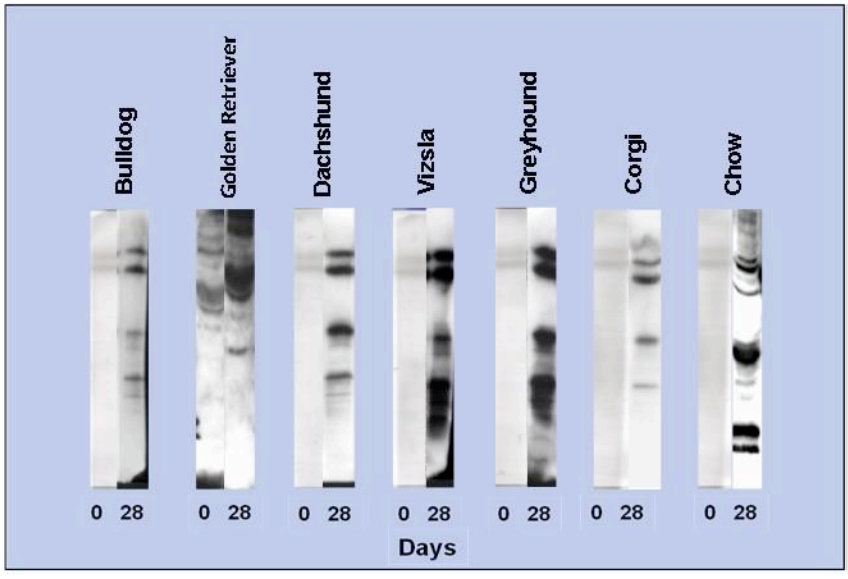

Figure 1. Humoral response to *ImmuneFx* vaccine (Emm55/autologous tumor cells). Western blot analysis of pre-immune sera (Day 0) and post-immune sera (Day 28) from patients receiving the *ImmuneFx* autologous lymphoma cancer vaccine showed that all dogs had strong reactivity to multiple tumor-specific antigens.

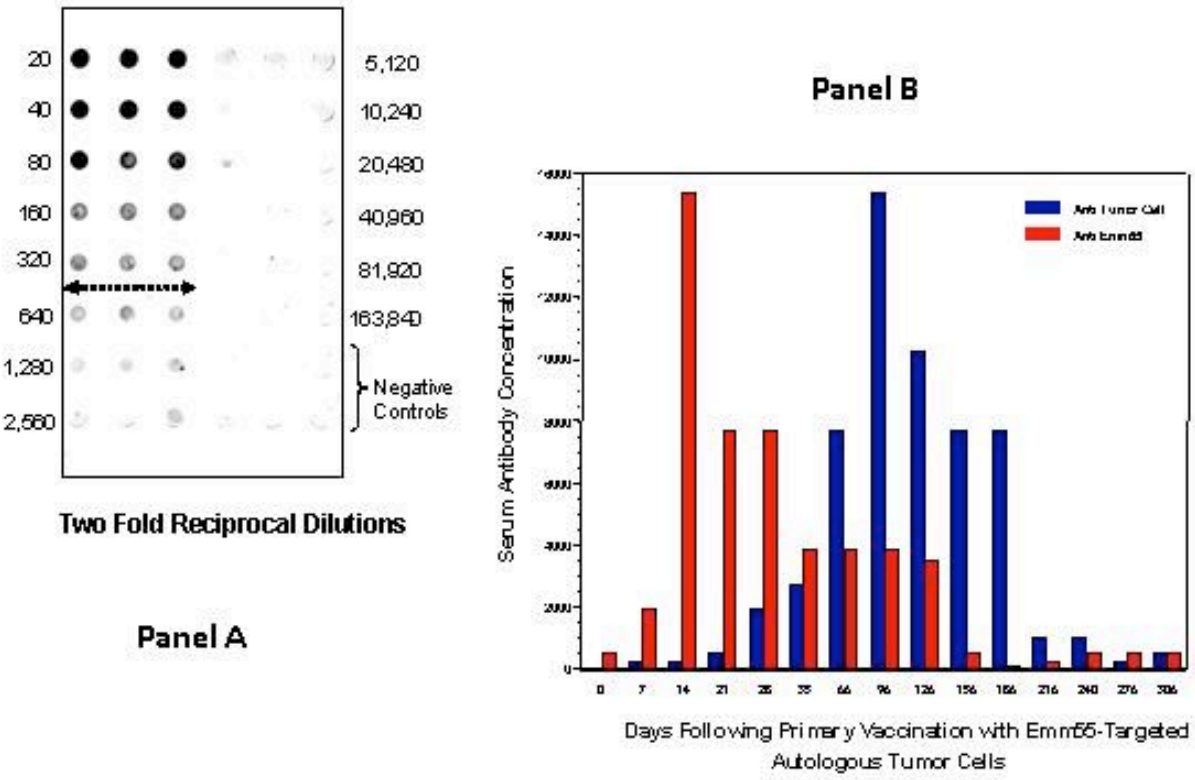

Figure 2. Humoral response induced by *ImmuneFx* vaccine (Emm55/autologous tumor cells) in Golden Retriever. Sera from the Golden Retriever was taken at weekly/monthly intervals following vaccination with *ImmuneFx* vaccine and tested for antibody to both the Emm55 priming antigen and autologous lymphoma tumor cells using Western dot blot analysis. The figure in panel A is representative of the dot blot assay. The bar graph in panel B on the right shows the antibody titers over time as determined in the dot blot assay.

Following neomycin (G418) selection, between 60 and 75% of the transfected cells expressed Emm55 as determined by flow cytometric analysis. The average time from biopsy to development of vaccine for use in the dogs was 14 days in culture. Viable vaccine cells from all the dogs under study were stored in liquid nitrogen for the development of subsequent vaccine doses.

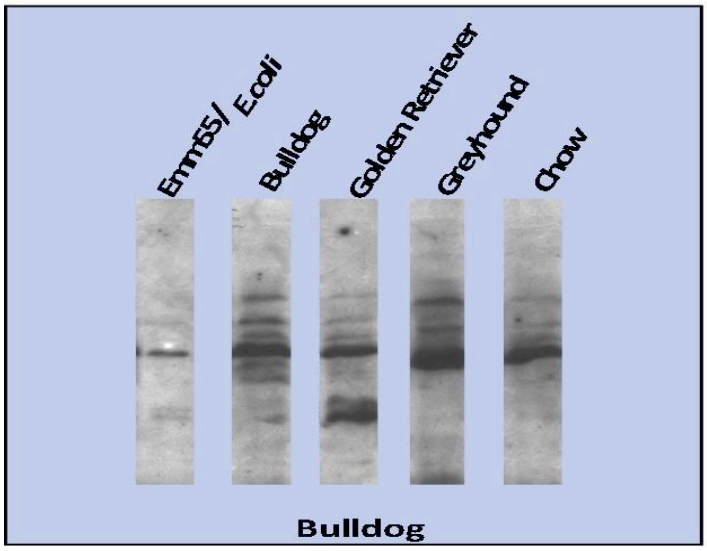

Figure 3. Cross reactivity of canine immune sera induced by *ImmuneFx* vaccine (Emm55/autologous tumor cells). Western blot analysis of immune sera from one breed, Bulldog, showing cross reactivity of the humoral response to tumor cells from several of the other breeds.

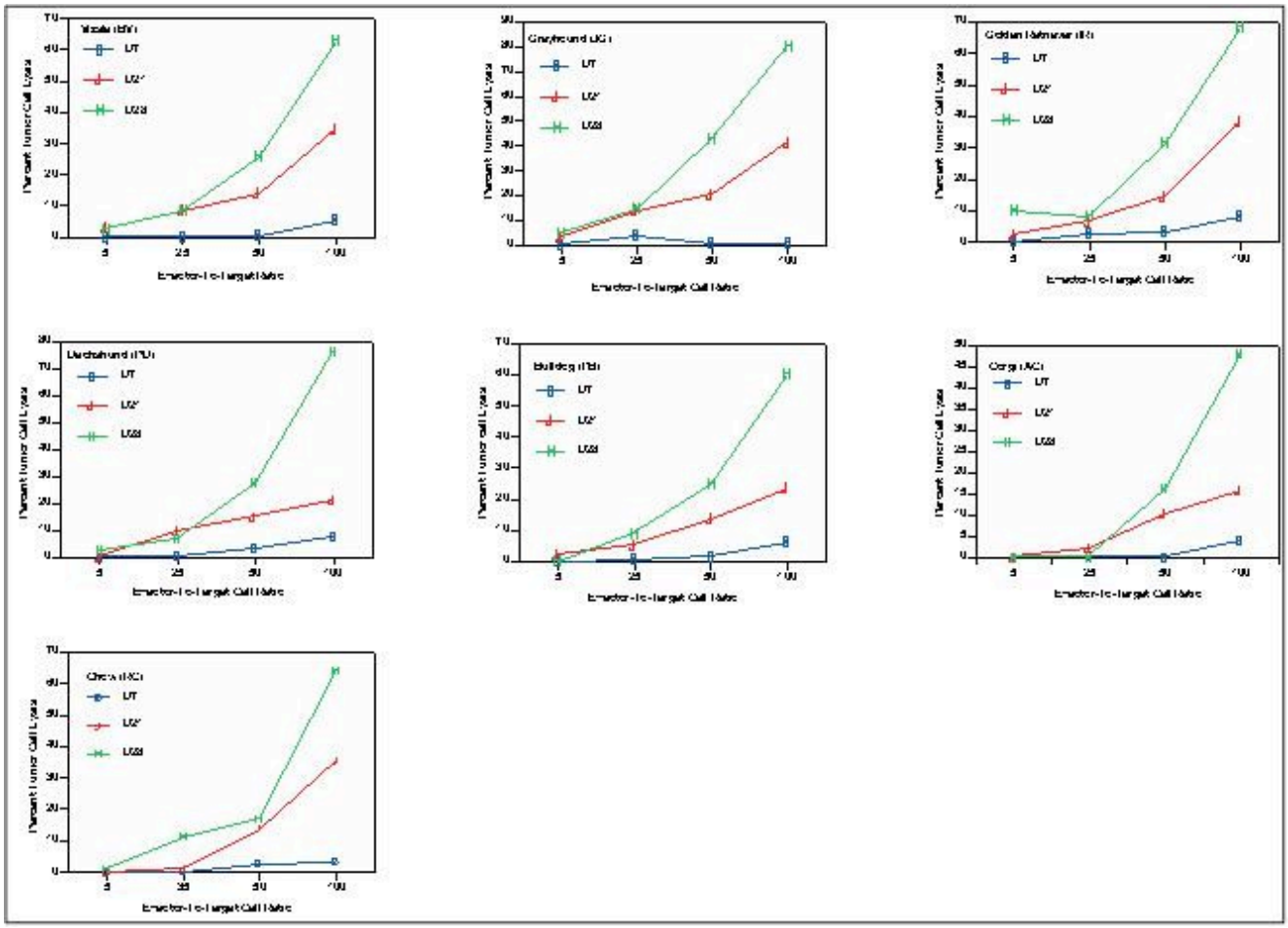

Figure 4. Cell-mediated response induced by *ImmuneFx* vaccine (Emm55/autologous tumor cells). Cell-mediated response in individual dogs at day 0, day 21 and day 28 was determined. The data indicate that patients receiving the autologous *ImmuneFx* vaccine produced a strong cellular response to their own tumor cells. This response increased over time.

Table 3. Electroporation conditions and transfection efficiency of pcDNA3/*emm55* in 14 canine lymphoma tumor samples. Tumor cells in biopsies ranged from 1.2 x 10^6 to 2.7 x 10^8, with an average cell count of 9.8 x 10^7. The percentage of transfected cells expressing Emm55 post G418 selection was determined by flow cytometric analysis. The average vaccine production time was 14 days from biopsy. The numbered dogs were trial participants.

Canine Breed Tumor Cell Original	Age	Sex	Biopsy Type	Electroporation Time (mSec)	Electroporation Volts (Kv)	Transfection (%)	Expression Post G4 18 Selection (%)	Biopsy to Vaccination (days)
Labrador	NK	M	Aspirate	0.40	0.348	10-15	>60	ND
Mix Breed	10	M	Aspirate	0.40	0.342	8-12	>50	ND
Mix Breed	10	M	Aspirate	0.40	0.350	11-14	>70	ND
Retriever	12	M	Aspirate	0.40	0.196	9-12	>55	ND
Shih Tzu	9	F	Aspirate	0.40	0.380	10-14	>65	ND
Doberman	NK	M	Aspirate	0.40	0.380	10-16	>65	ND
Irish Wolf Hound	7	M	Tissue	0.58	0.530	10-14	>60	23
1. Bulldog	6	M	Aspirate	0.40	0.352	10-14	>75	24
2. Golden Retriever	12	F	Tissue	0.58	0.530	15-18	>75	16
3. Dachshund	7	M	Aspirate	0.58	0.530	9-15	>60	15
4. Vizsla	4	M	Tissue	0.58	0.530	15-18	>50	21
5. Greyhound	10	F	Tissue	0.60	0.520	15-20	>75	18
6. Welsh Corgi	11	M	Aspirate	0.56	0.520	10-13	>65	33>
7. Chow Chow	12	M	Tissue	0.58	0.520	9-13	>55	26

NK = Not Known: M = Male: F = Female: Aspirate = Needle Aspirate: Tissue = Tru-cut Biopsy: Biopsy to Vaccination = Vaccine Production plus Patient Scheduling: ND = Not Done.

C. The development of humoral immunity in dogs vaccinated with pcDNA3/*emm55*-transfected autologous lymphoma cells

Pre-immune sera and immune sera 28 days following the initial vaccination were compared by standard PAGE Western blot analysis. **Figure 1** shows the result of a Western blot analysis of all 7 dogs on the study. In all dogs, pre-immune sera showed some reactivity to autologous tumor cell lysates. The reactivity of the pre-immune sera was primarily with high molecular weight proteins. The sera from day 28 showed significant reactivity with multiple tumor cell antigens of varying molecular weights. While there were antigens common to all the dogs that reacted with autologous sera, in all cases, the post vaccination sera also reacted with cellular antigens that appeared specific for each breed. Pre-vaccine serum taken from the Golden Retriever exhibited the most reactivity with autologous tumor cell lysate. This finding is not surprising as this dog had been diagnosed with disseminated stage 4 multi-centric indolent form of the disease one year prior to going on the *ImmuneFx* study.

Sera from all the dogs were tested by a Western dot blot assay for the development of a humoral response to both the Emm55 priming antigen and to the non-transfected autologous tumor cell population. The results of one of the studies are shown in **Figure 2**. Using the modified Western dot blot assay, the development of the antibody response to the vaccine was determined for both the Emm55 priming antigen and for tumor cell antigens. The data suggest that the response in the dogs to the priming antigen was significant and became apparent early in the vaccine protocol, with peak antibody titers occurring by day 14. Significant anti-tumor cell antibody was only detected from day 21, rising to a peak response 96 days following initial vaccination. In this patient, antibody to both the priming antigen and to tumor cell antigens was still being detected 19 months following the primary vaccination. The other vaccinated dogs show similar antibody response kinetics (data not shown). In all the patients followed, antibody responses to Emm55 and tumor antigens were detected in sera until the dogs died.

Using PAGE Western blot, the cross reactivity of the individual sera was tested across all the breeds represented in the study. **Figure 3** exhibits the cross reactivity of the Bulldog, Golden Retriever, Greyhound and Chow sera using the Bulldog tumor cell lysate. In all cases, the immune sera from the dogs were able to detect tumor cell antigens in lysates derived from the Bulldog lymphoma tumor cells. As with the data shown in **Figure 1**, each sera had different reactivity and intensity with protein bands on the PAGE gel. **Table 4** is a representation of the cross reactivity of the sera from the vaccinated dogs with the tumor cell lysate from other breeds. In all cases, cross reactivity was highly significant between patients and therefore between the breeds under study.

D. The development of cellular immunity in dogs vaccinated with pcDNA3/*emm55*-transfected autologous lymphoma cells

Lymphocytes isolated from peripheral blood samples taken from the canine patients were tested for the ability to develop an *in vitro* secondary cellular response to autologous tumor cell targets as an indicator of *ImmueFx* induction of cellular immunity to their cancer. **Figure 4** exhibits the secondary CMI response in all the dogs under study as measured in a non-isotopic-based short-term cellular cytotoxicity assay. These data suggest that all the dogs developed a cell mediated immune response to their lymphoma and that this cellular response was time dependent with the highest *in vitro* induced cellular cytotoxicity occurring at day 28. Immunofluorescent studies have shown that the majority of the cells in the lymphocyte effector cell population were $CD8^+$ cells (70%-85%), with the remaining phenotype in the effector population being $CD4^+$ cells (15%-30%). The cross reactivity of the cellular response in which significant cytotoxicity was observed against allogeneic target cells, as shown in **Table 5**, may have been the result of lymphokine-induced $CD4^+$ effector cells present in the lymphocyte cultures during the time of *in vitro* restimulation.

The ability of the dogs that received chemotherapy treatment either early or late in the study to develop significant cellular responses (as well as a humoral response) has significant implications for the future of this cancer vaccine as an adjunctive therapy.

In summary, the data collected from the dogs in this study, indicate that *ImmuneFx* did not harm any of the dogs to which it was administered and that all dogs produced a measurable humoral and cellular immune response against both autologous and allogeneic lymphoma cells. Unlike other anti-cancer treatments such as chemo- or radiation therapy, which uniformly induce adverse side-effects resulting in an aftermath of devastating toxicity such as anorexia, vomiting, diarrhea, sepsis and sometimes even death, the *ImmuneFx* cancer vaccine exhibited no toxicity or other adverse side. The results presented in this manuscript, support the safety and activity of the *ImmuneFx* cancer vaccine in dogs with naturally occurring lymphoma.

IV. Discussion

Canine lymphoma represents a heterogeneous group of highly aggressive tumors often resistant to therapy (Ponce et al, 2003), and is one of the most commonly diagnosed canine malignancies (MacEwen, 1980). Since the various forms of this disease have common histories and share many of the clinical signs, classification of these malignancies is difficult. Mostly, their classification is based upon their anatomical location and is often described as multicentric, alimentary, mediastinal, and cutaneous forms (Kopla and Aizenberg, 1999; Ettinger, 2003). B-cell lymphomas are more common than T-cell lymphomas in dogs (Teske et al, 1994; Fournel- Fleury et al, 1997). In a study reported by Guija de Arespacochaga and colleagues in 2007, they showed that prevalence

Table 4. Cross reactive humoral response induced by *ImmuneFx* vaccine (Emm55/autologous tumor cells). Representative values based on Western blot and densitometry analysis shows the degree of cross reactivity between immune sera from vaccinated dogs with the tumor cells from other breeds. Autologous reactions are boxed.

	Canine ImmuneSera					
Target Cell Origin	Vizsla	Greyhound	G. Retriever	Dachshund	Bulldog	Chow
Vizsla	**4+**	3+	4+	3+	3+	3+
Greyhound	3+	**4+**	3+	4+	3+	3+
G. Retriever	3+	3+	**4+**	3+	4+	3+
Dachshund	3+	4+	3+	**4+**	4+	3+
Bulldog	4+	3+	3+	3+	**4+**	4+
Chow	3+	3+	3+	4+	3+	**4+**

Table 5. Cross reactive cellular immune response induced by *ImmuneFx* vaccine (Emm55/autologous tumor cells). Canine lymphocytes were used at an effector-to-target cell ratio of 100:1 in a short-term non-radioisotopic CMI assay. Boxed in yellow are the autologous CMI responses. The cellular response induced by autologous *ImmuneFx* cancer vaccine in patients to their own tumors was significantly higher than that observed against heterologous targets, i.e. dogs of other breeds. The only combination which showed no measurable reaction was Dachshund and Chow.

	Canine Lymphocytes					
Target Cell Origin	Vizsla	Greyhound	G. Retriever	Dachshund	Bulldog	Chow
Vizsla	**68%**	9%	27%	18%	10%	18%
Greyhound	22%	**82%**	26%	21%	19%	21%
G.Retriever	22%	31%	**60%**	25%	23%	28%
Dachshund	24%	21%	27%	**71%**	6%	15%
Bulldog	19%	9%	22%	8%	**54%**	11%
Chow	10%	6%	16%	0%	5%	**61%**

differs significantly from region to region. In their study (Guija de Arespacochaga et al, 2007), the B-cell form of the disease represented 51.2% of lymphomas, while the T-cell form accounted for only 29.3%. In similar studies by other investigators (Greenlee et al, 1990; Ferrer et al, 1993; Teske et al, 1994; Fournel-Fleury et al, 1997), it was determined that the incidence for canine T-cell lymphomas ranged from 19% in the USA to 39.7% in Europe. When dogs are diagnosed with lymphoma and do not receive an intervention therapy, they succumb to the disease within a 4 to 6 week period. Furthermore, T cell lymphomas are more aggressive and have a poorer prognosis than B cell lymphomas (Ponce et al, 2003). As stated by other investigators (Ettinger, 2003) lymphoma is a systemic disease and that to date treatment by systemic chemotherapy has been the more successful modality. Many chemotherapy protocols have been developed for the treatment of canine lymphoma. Overall, despite receiving a chemo protocol, dogs will suffer loss of remission within 6 to 9 months following treatment. Subsequent treatment will induce remission again, however, the duration of remission declines with each cycle of treatment (Berger, 2005). As with all cancers both animal and human, many factors have to be considered in selecting a protocol. These factors range form the clinical status of the patient, presentation of the disease to the previous protocol used and previous response of the patient to the protocol. Berger (2005) sets out recommendations for the selection of a protocol.

The protocols discussed in the Berger article are the induction protocols such as the COP protocol (combination protocol with Cyclophosphamide, Vincristine and Prednisone), the COAP which is the same as COP with the addition of Cytosine Arabinoside, and the Wisconsin Madison-19 (WM-19) protocol; maintenance protocols such as the LMP protocol (Methotrexate, Chlorambucil and Prednisone) and the rescue protocols, DMAC protocol (Actinomycin-D, Cytosine Arabinoside, Dexamethasone and Melphalan), the CHOP protocol (Doxorubicin,Vincristine, Cyclophosphamide, Prednisone, and Sulfatrim), Lomustine protocol (Lomustine, Prednisone), and the Protocol ADIC (Doxorubicin, and Dacarbazine). Also used as a single agent is L- asparaginase. Studies on the efficacy or toxicity using L-asparginase in combination with CHOP protocol have been reported (MacDonald et al, 2005). No difference in response rates or first remission duration was reported in those dogs receiving the CHOP protocol alone or those having the CHOP with L-asparginase. The results of the MacDonald study (MacDonald et al, 2005) suggested that the exclusion of L-asparginase from a chemotherapy protocol would not seriously affect the outcome of treatment. The authors also suggest reserving the use of L-asparginase for treating dogs, having failed to respond to induction therapy and were in clinical relapse.

In combination with chemotherapy, other drugs have also been used in the treatment of natural occurring cancers including canine lymphoma and dogs with relapsed lymphoma. Two nanopeptides of thrombospondin-1 have been shown to be active in slowing tumor growth in mice. Both of these peptides have been used in an open label trial in dogs with lymphoma in an assessment of safety, pharmokinetics, anti-tumor activity and in measuring the changes, if any, in circulating endothelial cells (Rusk et al, 2006a, b). Their data suggested that these two peptides should be further studied for their cooperative effect when used in combination chemotherapy. Monoclonal antibodies have been used as adjuvants in combination with chemotherapy (Jeglum, 1996). The monoclonal antibody, Mab 231, has been shown to have some specificity against canine lymphomas and has been reported to be able to prolong the remission stage in dogs undergoing chemotherapy protocols.

The successful outcome from any treatment for cancer is partly due to early detection of the disease. Early detection is influenced by the nature of the disease, how it presents (external versus internal) and the specific and non-specific symptoms induced by the type of malignancy. It is because of the uncertainty and delay in clinical diagnosis that when lymphoma is detected the dogs have already reached advanced stage of disease. Because diagnosis usually occurs late in the development of lymphoma, the dogs on this study had advanced stage disease with a life expectancy of between 4 to 6 weeks.

The 7 dogs on the current study were both male (5) and female (2) of various breeds; Bulldog, Vizsla, Greyhound, Golden Retriever, Dachshund, Corgi and Chow, and their ages ranged from 4 to 11 years (**Table 1**). Autologous vaccine was produced for each dog from biopsies taken from regional lymph nodes. Animals were vaccinated approximately 2 to 4 weeks from the time of biopsy. The intravenous dose, $1x10^7$ irradiated cells, was the same for all dogs. Each dog received weekly vaccine inoculations for a total of 4 weeks and in most cases went on to receive monthly doses (**Table 1**).

The data collected from all the patients indicate that *ImmuneFx* easily met the two endpoints of this pre-clinical study which were to: 1) do no harm, and 2) produce a measurable tumor-specific humoral and cellular immune response (**Figures 1-4 and Tables 4 and 5**). With the exception of an allergic reaction in one dog, which was readily treatable with Benadryl, there were no other signs of adverse reactions in any of the animals. Laboratory results have shown that all dogs on the trial not only developed a highly significant antibody response to the *ImmuneFx* priming antigen but also to cellular antigens expressed on their tumor cells. Furthermore, the immune sera from all the dogs tested recognized the tumor cells from other breeds of dog. In addition, a strong cell-mediated response to autologous tumor cells was measured. Cellular cytotoxicity was also measured against allogeneic target cells indicating that the dogs developed both $CD8^+$ T cells and lymphokine-activated $CD4^+$ effector cells. As shown in other studies, prior treatment of some of the dogs with a chemotherapeutic protocol did not influence the ability of the patient to generate an immune response. All dogs in this study, whether on or not on a chemotherapeutic protocol, generated an immune response to their own tumor cells as well as to allogeneic tumors cells. This may have important significance for undertaking further studies in which the *ImmuneFx* cancer

vaccine will be incorporated into chemotherapy protocols. In fact, others have noted that combining cancer vaccines with chemotherapy can achieve a favorable tumor reduction and thereby enhance the magnitude and duration of the immune response generated by the vaccine (Choudhury et al, 2006).

Unlike other anti-cancer treatments such as chemo- or radiation therapy, which uniformly induce adverse side-effects resulting in an aftermath of devastating toxicity such as anorexia, vomiting, diarrhea, sepsis and sometimes even death, the *ImmuneFx* cancer vaccine exhibited no toxicity in any of the canine lymphoma patients in the present study. The lack of side-effects and a strong and focused immune response to the malignancy has far reaching implications for the use of *ImmuneFx* in both the veterinary field as well as in treating human cancers.

Emm55 has been expressed on the surface of a number of different tumor cell isolated from both human and animal malignancies (melanoma, kidney carcinoma, leukemia, primitive neuroectodermal tumors, cancer stem cells, neuroblastoma and prostate cancer cells) therefore, as an immunotherapy it would be expected to work in a similar manner generating a strong tumor-specific immune response resulting in the regression of those malignancies.

The *ImmuneFx* vaccine can be used as an autologous cell vaccine, as in the present study. It can also be formulated as an allogeneic vaccine, such as the cancer vaccine currently in clinical trials for human prostate cancer (Thraves and Sutton, 2002). The third possibility is to use *ImmuneFx* as a DNA vaccine. The use of plasmid DNA directly injected *in vivo* has been shown to be effective. Plasmid DNA is less expensive to produce than cellular vaccines and is simple to purify in large quantities. Therefore, formulation of a DNA vaccine is simple and validation is straightforward. Immunization with DNA has been shown to be safe (Wang et al, 1998) and effective (Edgeworth et al, 2002). Studies in dogs with malignant melanoma have shown that *in vivo* transfection of established tumors with a bacterial superantigen (staphylococcus enterotoxin B) gene and either the gene for GM-CSF or IL-2 induced local and systemic anti-tumor immunity (Dow et al, 1998). In fact, the first therapeutic cancer vaccine approved in the US for any species is a DNA vaccine for melanoma in dogs (Bergman et al, 2003). This vaccine is a DNA plasmid encoding the human tyrosinase gene (Houghton and Vijayasaradhi, 2001) which is delivered in combination with DMRIE/DOPE (Vical) via the Biojector 2000 delivery device.

In summary the salient features of *ImmuneFx* vaccine are:

- No toxic side effects
- The induction of a strong autologous and cross reactive humoral response
- The induction of a strong autologous and cross reactive cellular response
- That it is highly specific and effective

Acknowledgements

We would like to thank Blake Singletary, Angela Rippa, Ankit Ghandi and Monica Castillo for excellent technical assistance in the completion of this study, Special thanks to Dr. Michael Boyle for providing the pJLA602 parental vector and the murine monoclonal antibodies to Emm55. The authors would also like to acknowledge the financial support of the Pediatric Cancer Foundation, Morphogenesis, Inc. and the St Joseph's Children's Hospital of Tampa.

References

Anderson M, Ruhwald M, Thorn M, Pedersen A, Matthiassen S, Buus S **(2003)** Tumor-associated antigens identified by mRNA-expression profiling as tumor rejection epitopes. **J Immune Based Ther Vaccines** 10, 1186-1195.

Bergman P, McKnight J, Novosad A, Charney S, Farrelly J, Craft D **(2003)** Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. **Clinical Cancer Research** 9, 1284-1290.

Boon T, Bruggen V **(1996)** Human tumor antigens recognized by T lymphocytes. **J Exp Med** 183, 725-729.

Boyle M, Hawlitzky J, Raeder R, and Podbielski R **(1994)** Analysis of genes encoding two unique type IIa immunoglobulin G-binding proteins expressed by a single Group A streptococcal isolate. **Infect Immun** 62, 1336.

Boyle M, Weyer-Heynermann J, Raeder R, Podblielski A **(1995)** Characterization of a gene coding for a type II° bacterial IgG-binding protein. **Mol Immunol** 32, 669-678.

Choudhury A, Mosolits S, Kokhaei P, Hansson L, Palma M, and Mellstedt H **(2006)** Clinical results of vaccine therapy for cancer: learning from history for improving the future. 95, 147-202.

Cunningham M, Antone S, Smart M. **(1997)** Molecular analysis of human cardiac myosin-cross-reactive B and T-cell epitopes of the Group A streptococcal M5 protein. **Infect Immun** 65, 3913-3923.

Dobson JM, Samuel S, Milstein H, Rogers K, Wood JL (**2002**) Canine neoplasia in the UK: estimates of incidence rates from a population of insured dogs. **J Small Anim Pract** 43, 240-246.

Dow S, Elmslie R, Willson A, Roche L, Gorman C, Potter T **(1998)** In vivo tumor transfection with superantigen plus cytokine genes induces tumor regression and prolongs survival in dogs with malignant melanoma. **J Clin Invest** 101, 2406-2414.

Edgeworth R, San J, Rosenzweig J, Nguyen N, Boyer J, Ugen K **(2002)** Vaccine development against HIV-1: current perspectives and future directions. **Immunol Res** 25, 53-74.

Ettinger S **(2003)** Principles of treatment for canine lymphoma. **Clin Tech Small Anim Pract** 92-97.

Farrell R **(1993)** RNA isolation strategies, chapter 5 in RNA Methodologies: A laboratory guide for isolation and characterization Published by **Academic Press** pp 79-82.

Ferrer L, Fondevila D, Rabanal R, Tarres J, Ramis A **(1993)** Immunohistochemical detection of CD3 antigen(pant marker)in canine lymphomas. **J Vet Diag Invest**. 5, 616-620.

Fournel-Fleury C, Magnol J, Bricaire P, Marchal T, Chabanne L, Delverdier A, Bryon P, Felman P **(1997)** Cytohistological and immunological classification of canine malignant lymphomas: comparison with human non-Hodgkin's lymphomas. **J Comp Pathol** 117, 35-59.

Greenlee P, Filippa D, Quimby F, Patnaik A, Calvano S, Matus R, Kimmel M, Hurvitz A, Lieberman P **(1990)** Lymphomas in dogs. **Cancer** 66, 480-490.

Guija de Arespacochaga A, Schwendenwein I, Weissenbock H **(2007)** Retrospective study of 82 cases of canine lymphoma in Austria based on the working formulation and immunotyping. **J Comp Path** 136 186-192.

Hege K, Jooss K, Pardoll D **(2006)** GM-CSF gene-modified cancer cell immunotherapies: of mice and men. **Int Rev Immunol** 25, 321-352.

Hogge G, Burkholder J, Culp J, Albertini M, Dubielzig R, Keller E **(1998)** Development of human granulocyte-macrophage colony-stimulating factor-transfected tumor cell vaccines for the treatment of spontaneous canine cancer. **Hum Gene Ther** 9, 1851-1861.

Hogge G, Burkholder J, Culp J, Albertini M, Dubielzig R, Yang N, MacEwen EG **(1999)** Preclinical development of human granulocyte-macrophage colony-stimulating factor-transfected melanoma cell vaccine using established canine cell lines and normal dogs. **Cancer Gene Ther** 6, 26-36.

Hollinghead S, Arnold J, Readdy T **(1994)** Molecular evolution of a multigene family in Group A streptococci. **Mol Biol Evol** 11, 208-219.

Houghton A, Vijayasaradhi S **(2001)** Patent No. 6,168,946 United States of America.

Lancefield, R **(1962)** Current knowledge of type-specific M antigens of group A streptococci. **J Immunol** 89, 307-313.

MacDonald V, Thamm D, Kurtzman I, Turek M, Vail D **(2005)** Does L-asparginase influence efficacy or toxicity when added to a standard CHOP protocol for dogs with lymphoma? **Vet Intern Med** 19, 732-736.

Mitchell M **(2001)** Principles of biologic therapy. In C. Haskell (Ed.), Cancer treatment (p. 86). WB Saunders Company.

Ponce F, Magnol J, Marchal T, Chabanne L, Ledieu D, Bonnefont C, Feman P, Fournel-Fleury C **(2003)** High grade canine T-cell lymphoma/leukemia with plasmacytoid morphology: a clinical study of nine cases. **J Vet Diagn. Invest** 15, 330-337.

Ponce F, Magnol J, Ledieu D, Marchal T, Turinelli V, Chalvet-Monfray K, Fournel-Fleury C **(2004)** Prognostic significance of morphological subtypes in canine malignant lymphoma chemotherapy. **Veterinary Journal** 167,158-166.

Quintin-Colonna F, Devauchell P, Fradelizi D, Mourot B, Faure T, Kourilsky P **(1996)** Gene therapy of spontaneous canine melanoma and feline fibrosarcoma by intratumoral administration of histoincompatible cells expressing human interleukin-2. **Gene Ther** 3, 1104-1112.

Rusk A, Cozzi E, Stebbins M, Vail D, Graham J, Valli V, Henkin J, Sharpee R, Khanna C **(2005a)** Cooperative activity of cytotoxic chemotherapy with ant-angiogenic thrombospondin-1 peptides, ABT-256 in pet dogs with relapsed lymphoma. **Clin Cancer Res** 12, 7456-7464.

Rusk A, McKeegan E, Haviv F, Majest S, Henkin J, Khanna C **(2005b)** Preclinical antiogenic thrombospondin-1 peptide mimetics, ABT-526 and ABT-510 in companion dogs with natural occurring cancers. **Clin. Cancer Res**.12, 7444-7455.

Teske E (**1994**) Canine malignant lymphoma: a review and comparison with human non-Hodgkin's lymphoma. **Vet Quart** 15, 209-219.

Thraves P, Sutton, A **(2002)** Patent No. 7,303,911. United States of America Patent and Trademark Office.

Sambrook J, Fritsch E, Maniatis T **(1989)** Molecular Cloning: A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press , pp 1-153.

Sandler A, Chihara H, Kobayashi G, Zhu X, Miller M, Scott D **(2003)** CpG oligonucleotides enhance the tumor antigen-specific immune response of a granulocyte macrophage colony-stimulating factor-based vaccine strategy in neuroblastoma. **Cancer Res** 63, 394-399.

Simon D, Moreno SN, Hirschberger J, Moritz A, Kohn B, Neumann S, Jurina K, Scharvogel S, Schwedes C, Reinacher M, Beyerbach M, Nolte I **(2008)** Efficacy of a continuous multiagent chemotherapeutic protocol versus a short-term single –agent protocol in dogs with lymphoma **J Amer Vet Med Assoc** 232, 879-885.

Suto R, Srivastava P **(1995)** A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. **Science** 269, 1585-1588.

Wang R, Doolan D, Hedstrom R, Coonan K, Charoenvit Y, **(1998)** Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. **Science** 282, 476-480.

Wilkerson M, Dolce K, Koopman T, Shuman W, Chun R, Garrett L **(2005)** Lineage differentiation of canine lymlphoma/leukemias and aberrant expression of CD molecules. **Vet Immunol and Immunopath** 106, 179-196.