Received: 26 December 2019 | Accepted: 26 June 2020

DOI: 10.1111/jvim.15852

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Autologous cancer cell vaccination, adoptive T-cell transfer, and interleukin-2 administration results in long-term survival for companion dogs with osteosarcoma

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Abstract

Background: Osteosarcoma (OSA) in dogs is an aggressive bone tumor with frequent chemotherapy failure and translational relevance for human health.

Hypothesis/Objectives: We hypothesized that dogs with OSA could be treated safely by ex vivo activated T-cells that were generated by autologous cancer vaccination and supported by interleukin-2 (IL-2) treatment with survival more than twice that reported for amputation alone.

Animals: Osteosarcoma-bearing dogs (n = 14) were enrolled in a single-arm prospective trial after complete staging before amputation. Four healthy dogs also were treated in a safety study.

Methods: Autologous cancer cell vaccinations were administered intradermally and dogs underwent leukapheresis. Mononuclear cell products were stimulated ex vivo with a T-cell-activating agent. Activated product was transfused and 5 SC IL-2 injections were administered q48h. Dogs were monitored for metastasis by thoracic radiography every 3 months.

Results: Autologous cancer cell vaccine and activated cellular therapy (ACT) products were successfully generated. Toxicity was minimal after premedicants were instituted before ACT. With premedication, all toxicities were grade I/II. Median disease-free interval for all dogs was 213 days. One dog developed cutaneous metastasis but then experienced spontaneous complete remission. Median survival time for all dogs was 415 days. Five dogs survived >730 days.

Conclusions and Clinical Importance: This immunotherapy protocol without cytotoxic chemotherapy is safe and tolerable. Compared to historical amputation reports, survival was notably prolonged in this group of patients. Additional prospective

Abbreviations: ^{18}F-FDG PET/CT, fluorine-18-flourodeoxyglucose positron emission tomography/computed tomography; ACT, activated cellular therapy; ALP, alkaline phosphatase; CARs, chimeric antigen receptor T-cells; DFI, disease-free interval; DTH, delayed-type hypersensitivity assay; HER-2, human epidermal growth factor receptor 2; IFN-γ, interferon-gamma; IL-2, interleukin-2; IL-6, interleukin-6; L-MTP-PE, liposomal muramyl tripeptide; MNC, mononuclear cell; OSA, osteosarcoma; TNF-α, tumor necrosis factor-alpha.

studies are warranted to elucidate active immunologic mechanisms and further improve disease response and survival.

KEYWORDS
canine, immunotherapy, interleukin 2, leukapheresis, osteosarcoma

1 | INTRODUCTION

Osteosarcoma (OSA) accounts for up to 85% of all primary bone tumors in dogs, and is the most prevalent bone cancer in juvenile and young adult humans.[1-4] Treatment of OSA in dogs has been predicated upon surgical removal of the tumor followed by adjuvant cytotoxic chemotherapy.[2,3] In dogs and in humans with metastatic disease, survival is short, with little improvement in longevity despite various adjuvant and neoadjuvant chemotherapy protocols developed over the past few decades.[5-7] The failure to continue to improve survival with chemotherapy has driven investigations of novel treatments.

Suggestion of immune activation by surgical site infection after limb-sparing surgeries has driven immunotherapy strategies using therapeutic vaccines, biologic vectors, adoptive transfer of T-cells, monoclonal antibodies, and modulation of immune signaling.[8-10] Nonspecific immunotherapy in the form of liposomal-encapsulated muramyl tripeptide (L-MTP-PE) extended survival compared to empty liposomes in dogs with OSA treated by amputation.[11] Xenogeneic T-cell transfer has been performed using an attenuated, immortalized human cytotoxic line, TALL-104, in dogs with OSA and free of radiographically evident metastasis after amputation and cisplatin chemotherapy.[12] Outcomes were similar to historically reported surgery and chemotherapy alone, suggesting minimal benefit to the cellular intervention. Autologous chimeric antigen receptor (CAR) T-cells specific for human epidermal growth factor receptor 2 (HER-2) have been generated to treat OSA in dogs.[13] Preliminary studies of a HER-2-targeted transgenic, attenuated *Listeria* therapeutic vaccine for OSA in dogs yielded increased disease-free intervals (DFI) and survival times compared to historical controls when administered to dogs in remission based on radiography after adjuvant chemotherapy.[14] Therapeutic studies in humans using CAR T-cells, checkpoint inhibitors, and tumor-infiltrating lymphocytes have identified the importance of cancer-associated antigen and neoantigen-specific effector T-cells for successful anticancer immunity.[15-20] Effector T-cells can reject large, bulky metastatic cancers and substantially prolong patient survival.

The treatment strategy used in our study is termed vaccine-enhanced adoptive T-cell treatment with cytokine boost. In previous studies in rodents, this strategy indicated that spontaneous micrometastases could be permanently eliminated by combining cancer cell vaccination, activated cellular therapy (ACT), and interleukin-2 (IL-2).[21,22] A founding principle of this approach is that tumors contain multiple mutations, some of which are translated into neoantigens that render the tumors immunogenic.[23-27] Studies in mice first indicated that even the least immunogenic tumors can elicit detectable immune responses after administration of attenuated autologous cancer cell vaccines and a potent immunological adjuvant.[28-30] However, the immune responses may be insufficient to eliminate cancer, even when cancer cells are combined with a very potent immune stimulus (eg, cytokines including granulocyte-monocyte colony stimulating factor or bacterial components such as *Propionibacterium acnes* or Bacillus Calmette-Guerin).[30] The specific context and chronology of antigenic presentation is likely important. Early studies in mice also indicated that, although primed T-cells removed from immune animals were unable to kill cancer cells, cytotoxic effector T-cells restimulated with antigen in vitro killed cancer cells in a major histocompatibility-restricted cancer neoantigen-specific manner.[31,32] In addition, polyclonal populations of cancer neoantigen-specific effector T-cells can be generated by stimulating cancer neoantigen-primed T-cells with antigen and recombinant human IL-2.[33,34] Those studies indicated that adoptive transfer of ex vivo-activated effector T-cells into cancer-bearing animals resulted in recipient animals being cured in a cancer neoantigen-specific manner.[33,35] This approach also was successfully applied to humans with advanced metastatic renal cell carcinoma and resistant, recurrent malignant glioma.[36,37] In both studies in humans, subsets of patients with advanced, treatment-resistant disease achieved radiologic improvement of their tumors and clinical benefit.

The objectives of our study were to determine safety and identify an efficacy signal of this strategy as a treatment for newly diagnosed canine patients with OSA. We hypothesized that dogs with OSA could be treated safely by ex vivo-activated T-cells that were generated by autologous cancer vaccination and supported by low-dose IL-2 treatment. We further hypothesized that dogs would survive more than twice as long as reported for amputation alone. The primary endpoint of the study was survival fraction beyond 268 days (twice that of amputation alone) in a single-stage phase II assessment according to a previously described method.[38] Treating dogs with naturally-occurring disease under these conditions allows this combination immunotherapy to be tested in patients with a natural cancer-burdened immune system and minimal disease. The outcomes obtained in such studies could serve as a blueprint for application of vaccine-enhanced ACT with ex vivo-activated effector T-cells in a wide variety of malignancies in dogs and humans.

2 | MATERIALS AND METHODS

2.1 | Trial design

We designed a single-site, single-arm, open-label prospective trial of vaccine enhanced ACT using low-dose IL-2 in client-owned dogs with OSA presented to the University of Missouri Veterinary Health Center

(MU-VHC). Client-owned dogs of any age, sex, or breed and >20 kg in body weight that were diagnosed with OSA of the appendicular skeleton by cytology (with alkaline phosphatase [ALP] confirmation) or histopathology were eligible for enrollment. Informed owner consent was obtained using an approved University of Missouri Animal Care and Use Committee protocol (#8280). Dogs needed to have adequate bone marrow function and lymphocyte counts as measured by CBC and normal organ function as assessed by serum biochemical profile (alanine aminotransferase less than 3 times the upper limit of normal and normal serum creatinine concentration). Treatment with nonsteroidal anti-inflammatory drugs or other analgesics was allowed before enrollment and during the trial. Dogs were not eligible if they had previously undergone chemotherapy, radiation therapy, or surgery, if they had evidence of soft tissue or bone metastasis, or if they had clinically relevant comorbidities that would limit their expected lifespan. All dogs were screened for evidence of pulmonary neoplasia by thoracic radiography and bone metastasis or metachronous primary lesions using Technetium-99m-methyldiphosphonate bone scintigraphy.

2.2 | Vaccine preparation

Enrolled dogs underwent therapeutic amputation. From the amputated limb, an average of 1.1 cm^3 of tissue was collected from the tumor site and shipped to ELIAS Animal Health for autologous vaccine preparation. The remaining tissue was submitted for histopathologic review by 1 of 2 pathologists (G.J. and D.Y.K.). Osteosarcoma was confirmed histologically in all cases. Cancer tissue was enzymatically digested as described previously[36] and the isolated cells were expanded if necessary to generate sufficient numbers. Isolated cells were irradiated using 2 doses of 25 Gy (50 Gy total) to halt replication while remaining viable, using an E-Beam irradiator (Sterigenics, Oakbrook, Illinois). Before vaccine preparation and after irradiation, cells were assessed for morphology to ensure viability. For each vaccination, 3×10^7 cultured cells were combined with 0.4 mg of killed *P. acnes* (Immunoregulin, Neogen Corp, Lansing Michigan) and stored frozen in autologous serum and dimethylsulfoxide until thawed and used for vaccination. The vaccine was administered intradermally once weekly over 4 sites at each administration (over superficial cervical lymph nodes bilaterally and popliteal lymph nodes bilaterally) for a total of 3 vaccination visits beginning 7 days after amputation. Vaccine was administered over the skin of the amputation site for the missing limb. After the third vaccination, aspirin (Aspirin, Time-Cap Labs Inc, Farmingdale, New York; 81 mg PO) was dispensed and was to be given 36 and 12 hours before leukapheresis to decrease platelet clumping in the apheresis tubing.

2.3 | Leukapheresis

Two weeks after the third vaccination, CBC, serum biochemistry, and urinalysis were repeated before the leukapheresis procedure. Dogs were premedicated with a combination of dexmedetomidine (Dexmedetomidine, Orion Corp, Espoo, Finland; 5-8 μg/kg IV once) and nalbuphine (Nalbuphine hydrochloride, Hospira Inc, Lake Forest, Illinois; 0.2-0.4 mg/kg IV once), induced with propofol (Diprivan, Fresenius Kabi USA LLC, Lake Zurich, Illinois; 6 mg/kg IV to effect), and maintained on isoflurane (Isoflurane, Akorn Animal Health, Inc, Lake Forest, Illinois) gas, according to hospital protocol. Vascular access was obtained using a jugular dual-lumen dialysis catheter (Dialysis catheter [8 or 10 Fr, 15 or 20 cm], Covidien Ltd, Dublin, Rep. of Ireland). While anesthetized, the dogs were kept on a heating pad to prevent hypothermia. Temperature, pulse, cardiac rate and rhythm, respiration, mucous membrane color, and analgesia were monitored at least every 15 minutes using visual observation, ECG, auscultation, respiratory monitor and pulse oximeter, or some combination of these, as appropriate. Leukapheresis was performed by processing 3 total blood volumes over approximately 4 hours using the Spectra Optia Apheresis System with a Terumo BCT Collection Set (Spectra Optia Apheresis System and Terumo BCT Collection Set, Terumo BCT, Inc, Lakewood, Colorado) using the mononuclear cell (MNC) collection protocol. Calcium gluconate (Calcium gluconate, Nova-Tech, Inc, Grand Island, Nebraska; 10% solution) was administered as necessary beginning at a dosage of 0.5 mL/kg/h; and adjustments were made after review of serum ionized calcium concentration performed using a Stat Profile Critical Care Xpress analyzer (Stat Profile Critical Care Xpress, Nova Biomedical, Waltham, Massachusetts) machine. Blood was collected for post-procedure CBC and serum biochemistry panels. Collected MNCs were shipped in packaging to maintain 2 to 8°C to ELIAS Animal Health for ex vivo expansion and stimulation with a proprietary superantigen cocktail.

2.4 | Activated product preparation

The collected MNC were separated from plasma by centrifugation. The MNC were monitored daily while being incubated for 4 to 7 days (median, 5 days) in cell culture medium (Iscove's Modification of IMDM, GE Healthcare Lifesciences, Logan, Utah), autologous serum, and a proprietary superantigen cocktail. At harvest, cells were washed 3 times (1 exception, patient 002) and resuspended in sterile phosphate buffered saline (PBS; Gibco PBS, Thermo Fisher Scientific, Waltham, Massachusetts). Experimental replicates in preclinical testing identified no remaining superantigen in the product after 3 washes. Aliquots were removed and evaluated for % viability and cell count. In all products, cell viability was ≥72% and cell counts were ≥83.5% lymphocytes or lymphoblasts assessed by morphology. For each autologous T-cell infusate, harvested cells were suspended in sterile PBS, collected into a sterile infusion bag, and stored refrigerated until used for administration within 30 hours. Dosage ranged from 9.3×10^6 per kg body weight to 4.0×10^7 per kg body weight.

2.5 | Activated product administration

Six days after leukapheresis, the activated T-cell product was harvested and shipped to the MU-VHC in a refrigerated transport

container for administration. The activated T-cell product was supplied in a 150 mL IV infusion bag. The product arrived the morning of infusion and the bag was warmed to room temperature (by touch) for at least 60 minutes and then gently agitated to ensure that T-cell adhesion or clumping was minimized and that the majority of cells were in suspension. To maintain sterility, the bag was not breached for sampling. The contents were infused over 30 minutes. Recombinant human IL-2 (IL-2, Miltenyi Biotec Inc, Auburn, California; 20 000 IU/kg SC) then was administered 5 times at 48-hour intervals, beginning the day after T-cell IV infusion. Dogs were reevaluated by examination and 3-view thoracic radiographs beginning 3 months after screening and every 3 months thereafter until metastatic disease was identified.

2.6 | Enrollment and measured outcomes

Fourteen dogs were enrolled. Protocol was followed regarding amputation and surgical sample collection, cancer cell vaccination, leukapheresis, ACT, and low-dose IL-2 injections. One screened dog was excluded because of the presence of skeletal metastasis. Disease-free interval and overall survival time (OST) were defined as time from cytological diagnosis to disease progression and death, respectively. Final median DFI and OST were calculated using Kaplan-Meier log-rank survival analysis. Dogs were censored if they were alive and disease free at study conclusion (n = 5). Dogs that died underwent necropsy if possible.

2.7 | Protocol deviations

The first dog to undergo ACT received the cells in a larger volume than the intended 150 mL (500 mL). Immediately after ACT infusion, the dog developed fever (grade II), lethargy (grade III), vomiting (grade I), and erythema (grade II). This reaction prompted suspending the study to perform a study of tolerability in normal dogs. When the study was temporarily suspended, the second dog enrolled was removed from the study and did not undergo leukapheresis or receive ACT or IL-2. Both dogs were included in the intent-to-treat analysis. Variation in the time necessary to prepare vaccine or administer components of the treatment was recorded as protocol deviations. Intradermal delayed-type hypersensitivity (DTH) tests initially were planned, but eliminated because of inadequate tumor cell material after vaccine preparation. One patient (patient 011) received only 2 vaccinations because of poor cancer cell growth ex vivo.

2.8 | Healthy dog study

After noting adverse effects of ACT in the first OSA-bearing dog to receive T-cells, 4 healthy, purpose-bred dogs (2 male and 2 female hound crosses) underwent leukapheresis, ACT, and IL-2 administration according to the method previously described. Dogs were housed at Xenometrics, LLC. Before infusion, dogs were treated with diphenhydramine (Diphenhydramine hydrochloride, APP Pharmaceuticals, LLC, Schaumburg, Illinois; 2 mg/kg IM) and maropitant citrate (Cerenia, Zoetis, Inc, Kalamazoo, Michigan; 1 mg/kg SC). Firocoxib (Previcox, Merial, Duluth, Georgia; 5 mg/kg PO q24h) was given PO 1 day before infusion and on the morning of infusion. The activated T-cells were infused IV, with most infusions finished within 30 minutes (infusion flow rates of 200 mL/h). These premedicants became standard protocol for dogs 004-015 before ACT administration.

In the healthy dogs, the T-cell product was cultured for aerobic and anaerobic microorganisms by ELIAS. For sterility testing, 1 mL from each T-cell product was added to both a thioglycollate culture tube (IDEXX BioResearch, Pathology Services, West Sacramento, California) and a trypticase soy broth culture tube (Remel culture tubes, ThermoScientific, Lenexa, Kansas). The tubes were incubated at 37°C for 14 days and evaluated daily for microbial growth. In addition, each animal's blood was cultured aerobically and anaerobically for bacterial growth approximately 24 hours before ACT and 24 hours after the start of ACT. Approximately 10 mL of whole blood was collected into a single 80 mL blood culture bottle (BACTEC, Beckton, Dickinson, Franklin Lakes, New Jersey). The bottles were stored at room temperature and shipped the same day of collection whenever possible.

Interleukin-2 (50 000 IU/kg, which was higher than the dosage used in the OSA dogs SC in alternating locations) then was administered at 48-hour intervals for 5 doses, beginning the day after ACT. Dogs were monitored for adverse reactions, and injection site locations and clinical observations were documented in the study file. Serum was collected from the healthy dogs and analyzed for the presence of a panel of cytokines, growth factors and chemokines, including IL-2, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interferon-gamma (IFN-γ). Two milliliters of blood were collected in serum separator tubes for quantitation of these cytokines before leukapheresis and at 3, 6, 12, 18, 24, 36, and 48 hours after ACT. An additional 2 mL of blood was collected in the same manner for cytokine analysis 24 hours after the final administration of IL-2. On the final day of the study (1 week after final IL-2 injection), 2 mL of blood was collected to assess resolution of cytokine production. Serum was removed from the serum separator tube and then frozen immediately at −80°C. Serum samples were thawed on ice and duplicate samples were prepared in accordance with the ProcartaPlex multiplex Immunoassay kit (ProcartaPlex Assay, ThermoScientific) instructions. Data were collected on a Luminex 200 instrument, and further analyses were performed using Microsoft Excel. Cytokine measurements were not performed in the cancer-bearing dogs.

2.9 | Treatment for cytokine release syndrome

After the reaction consistent with cytokine release syndrome in the first dog, guidelines were developed for treating this reaction in future patients. Dogs were observed for hypotension, pyrexia, vomiting, diarrhea, lethargy, and signs of acute kidney injury. If any of these were observed, the protocol allowed for 0.25 mg/kg dexamethasone SP (Dexium-SP, Bimeda, Oakbrook Terrace, Illinois) to be administered IV once

q24h for up to 3 days. No subsequent dog in the protocol required treatment for cytokine release syndrome after initiation of premedication.

2.10 | Statistical evaluation

Time to event analyses were performed using Kaplan-Meier single group survival analysis with 95% confidence intervals (CI) generated. This study was a pilot trial to identify initial signal of efficacy. Fourteen dogs was a sufficient number to determine a minimum long-term survival rate of 30% with the assumption that 5% of dogs with OSA typically are long-term survivors. Using a sample size calculation of single-stage phase II design, 3 responders surviving >268 days among 14 dogs would predict a 30% minimum response rate with $P = .05$ and power of .84.[38] Descriptive statistics were performed using SigmaPlot (SyStat Software, San Jose, California).

3 | RESULTS

3.1 | Recruitment

Fourteen dogs (patients 002-015) with spontaneously occurring OSA were enrolled in the clinical trial. One screened dog (patient 001) was denied trial enrollment because of confirmed skeletal metastasis on bone scan, and 3 others were denied enrollment because of pulmonary metastatic disease on initial screening. Because bone scanning was part of the research protocol, the first dog was assigned a study number, but eliminated from evaluation when the scan was positive. Dogs were enrolled from October 2015 to December 2017. All dogs were followed until development of metastasis or 2 years beyond treatment. Minimum follow-up on long-term survivors was 2 years. Two dogs were enrolled before the healthy dog study, and 12 additional dogs afterward. Dog breeds included Labrador retrievers (n = 4), Golden retrievers (n = 2), and Rottweilers (n = 2) with the remaining dogs being a boxer, German short-haired pointer, Great Dane, greyhound, and mastiff. The median age of dogs was 6.6 years (range, 4-9.6 years) with a median weight of 39 kg (range, 20-68.8 kg). Locations of tumor included distal radius (n = 7), tibia (n = 3, distal; n = 2, proximal), proximal humerus (n = 2), and distal femur (n = 1). Three patients had increased ALP activities, a known negative prognostic indicator in dogs with OSA.[39] Alkaline phosphatase activity in the remainder of the dogs was within reference intervals. All surgical biopsy specimens supported the cytologic diagnosis of OSA and were reported as either OSA or OSA with chondroblastic (n = 2), fibroblastic (n = 1), telangiectatic (n = 1), or giant cell type (n = 1) differentiation.

3.2 | Treatment administered

Fourteen dogs received autologous vaccinations. The vaccine was created using irradiated autologous cancer cells to ensure that cancer neoantigens were presented to generate an immune response specific to the patient's disease. Dogs were prescribed 3 vaccines; 1 patient (patient 011) only received 2 vaccinations because of poor cancer cell growth ex vivo. Eleven dogs underwent leukapheresis, 2 dogs developed metastasis before apheresis, and the study was suspended temporarily, causing removal of 1 dog from the study. Eleven dogs received ACT. Ten dogs received IL-2. The first dog to receive ACT did not receive IL-2 after experiencing a severe reaction (Figure 1).

3.3 | Toxicoses observed

Patient 002 completed leukapheresis and ACT, but was withdrawn from the trial after fever (grade II), lethargy (grade III), vomiting (grade I), and erythema (grade II) immediately after ACT. Initial fever was consistent with cytokine release syndrome and resolved with fluid therapy. A second fever developed the next day, and was suspected to be caused by sepsis. *Achromobacter*, a gram negative nonfermentative rod, was cultured from multiple sites in the patient, but no organisms were cultured from the initially prepared activated T-cell product. The dog developed suspected bronchopneumonia after vomiting (ie, alveolar pattern in the right middle lung lobe on thoracic radiographs). Systemic antimicrobials (enrofloxacin [Baytril, Bayer HealthCare LLC, Shawnee Mission, Kansas, 10 mg/kg IV q24h] and ampicillin sulbactam [Unasyn, Pfizer Inc, New York, New York, 22 mg/kg IV q8h]), IV fluids, and maropitant (1 mg/kg SC q24h) were initiated and the fever resolved. The dog recovered 48 hours after initiation of parenteral medications. Interleukin-2 injections were not administered and the dog was followed to monitor for development of metastasis. Cytokines were not measured in this dog. Patient 003 was removed from the trial before the leukapheresis and ACT pending safety data from normal dogs. Except for patient 002, which experienced adverse effects attributable to the intervention before the addition of premedicants, adverse effects were low grade and transient, based on Veterinary Cooperative Oncology Group-Common Terminology Criteria for Adverse Events.[40] Toxicoses are summarized in Table 1. For leukapheresis, all dogs were placed under general anesthesia for the entirety of the procedure. All dogs required calcium supplementation during the procedure above the starting dosage of 0.5 mL/kg/h. One dog (patient 004) developed second degree atrioventricular block with normal blood pressure during leukapheresis, which resolved after cessation of the cell collection. Another dog (patient 010) had persistent grade II neutropenia after leukapheresis that resolved after 6 weeks. Although most adverse effects were gastrointestinal in nature and occurred during IL-2 administration, these events are most likely attributable to ACT with delayed onset.

3.4 | Outcomes

Disease-free interval was evaluated in dogs on an intent-to-treat basis. Median DFI of dogs on an intent-to-treat basis (n = 14) was 213 days (95% CI, 170-256 days; Figure 2). Median overall survival time for all dogs was 415 days (95% CI, 79-751 days; Figure 3). Five dogs were still alive and 4 disease-free at analysis, all of which

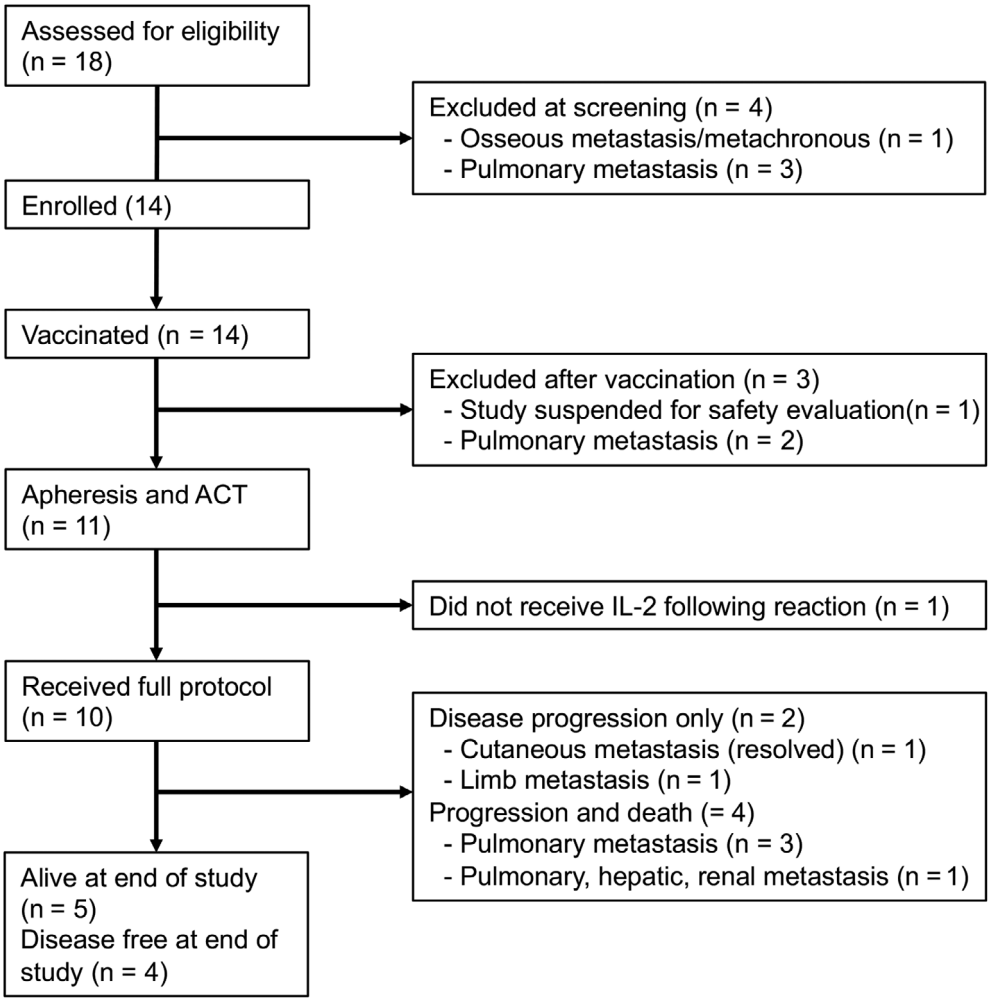

FIGURE 1 CONSORT diagram illustrating the outcomes of evaluated and enrolled dogs

TABLE 1 Toxicoses summarized by organ system. Attributions were assigned by temporal correlation to each phase of the trial. Reactions that occurred before the second dose of IL-2 were attributed to ACT. Reactions that continued after the second dose of IL-2 were attributed to IL-2

	Toxicosis						
Procedure	**Admin site**	**Gastro-intestinal**	**Bone marrow**	**Cardiac**	**Respiratory**	**Constitutional**	**Fever**
Vaccine	ISR(14) - I	V(1[b]) - I					
Leukapheresis			M(1) - II	AV(1) - II			
ACT		V(1[a]) - I D(2[a]) - I/II			C(1[a]) - I	L(1[a]) - II, W(1[a]) - II	F(1[a]) - III
IL-2		V(2) - I, N (2) - I D(3) - I/II (2)					

Notes: Letter indicate toxicosis, (n) is the number of patients, and roman numerals indicate grade of toxicosis. Example: D(3) - I/II/III means 3 patients had grade I, II, or III diarrhea.
Abbreviations: AV, AV block; C, cough; D, diarrhea; F, fever; ISR, injection site reaction; L, lethargy; M, myelosuppression; N, nausea; V, vomiting; W, weight loss.
[a]Patient treated before premedicants.
[b]Toxicosis unlikely attributable to intervention.

received the entire protocol of autologous vaccinations, leukapheresis, ACT, and low-dose IL-2 (Figure 3). One dog (patient 009) developed an OSA on a remaining limb at 902 days, confirmed by cytology, and was euthanized at 946 days. The dog that received ACT but not IL-2 was disease-free for 627 days and survived 709 days, receiving no further treatment. Another dog (patient 007) had disease progression at day 140 after amputation. A 3 cm cutaneous metastatic lesion was confirmed by cytology of fine needle aspirates with characteristic sarcoma cells that were ALP positive and the dog was removed from the study with no further thoracic radiographs performed. However, upon

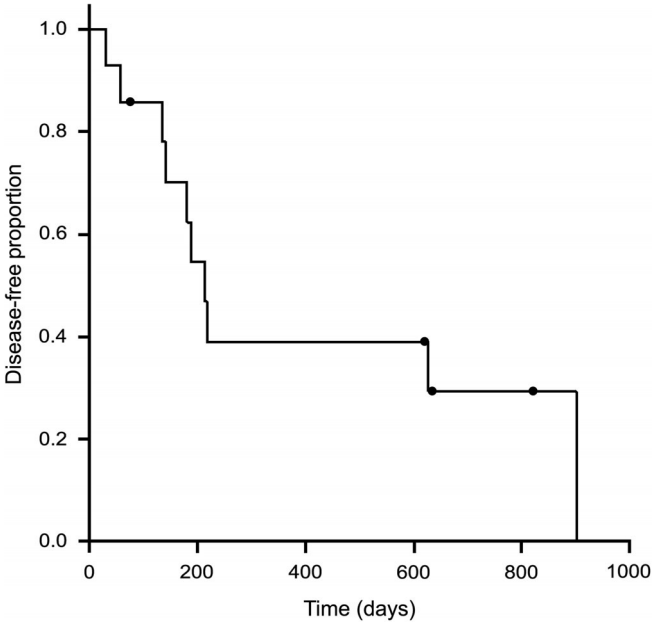

FIGURE 2 Disease-free interval (DFI) for all dogs on an intent-to-treat basis. The median DFI for all dogs (n = 14) was 213 days. Dogs that were alive at the end of the study (n = 5) were censored (dots) as well as 1 dog (003) which was euthanized at the primary veterinarian without disease progression having continued to be monitored for metastasis at the trial site

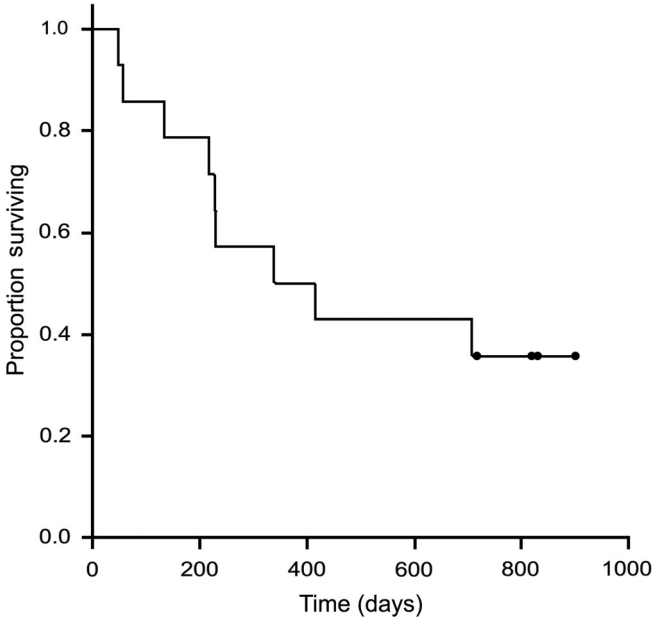

FIGURE 3 Overall survival time (OST) for all dogs on an intent-to-treat basis. The median OST for all dogs (n = 14) was 415 days. Dogs that were alive at the end of the study (n = 5) were censored (dots). The median survival of these 5 dogs at the time of analysis was 822 days

follow-up with the dog's owner, it was confirmed that the dog was still alive and the metastatic lesion had resolved. The dog returned to the MU VHC and a fluorine-18-flourodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) scan was performed. The previous metastatic mass was absent and no other hypermetabolic or metastatic lesions were noted. The dog was deemed to be in a second complete remission and has been followed subsequently without further metastasis. An image of the ^{18}F-FDG PET/CT scan is provided in Figure 4. The 3 dogs with increased ALP activity at diagnosis lived 134, 415, and 719 days after diagnosis with the last dog still alive at time of writing and disease free.

3.5 | Necropsy findings

Nine dogs died with pulmonary metastatic disease. Necropsy was performed in 2 dogs. These dogs survived 415 and 218 days. Both were euthanized because of identified pulmonary metastases and poor quality of life. Metastatic OSA was identified in the lungs, liver, and kidneys of the first dog, and in the lungs of the second dog.

3.6 | Protocol deviations

Protocol deviations resulted primarily from longer than planned times to expand tumor cells for vaccine preparation. The median time to vaccine administration after amputation was 20 days (range, 7-34 days), compared to the intended interval of 14 days. In all but 1 case, cell material was expended on vaccine preparation and intradermal DTH testing was not possible. The first activated T-cell product was prepared in a larger than planned volume (500 mL) and cells were washed only once. Interleukin-2 injections were intended to be given every other day, but were administered around weekends and only given on weekdays. One patient (patient 011) only received 2 vaccinations because of poor cancer cell growth ex vivo.

3.7 | Healthy dog study

After suspension of the initial phase I/II clinical trial after the first 2 tumor-bearing dogs, 4 healthy dogs underwent the entire protocol, without amputation and autologous vaccination. The healthy dogs tolerated leukapheresis, ACT, and low-dose IL-2 injections well. Adverse effects were mostly gastrointestinal in nature and included infrequent emesis in 3 of 4 dogs, 1 episode of soft feces in 2 of the 4 dogs, and ptyalism in 1 dog. These time points did not correlate with the procedures and were unlikely attributable to the interventions. For all dogs, the hemoglobin, hematocrit, and red blood cell counts remained unchanged throughout the study. However, in all dogs undergoing leukapheresis (n = 4 healthy, n = 14 OSA dogs), decreases in total lymphocyte count (P = .06) and PCV (P = .2), as well as increases in serum total calcium concentration (P = .008) were identified, but were minimal. Preleukapheresis blood urea nitrogen and serum creatinine concentrations were not different from postleukapheresis results.

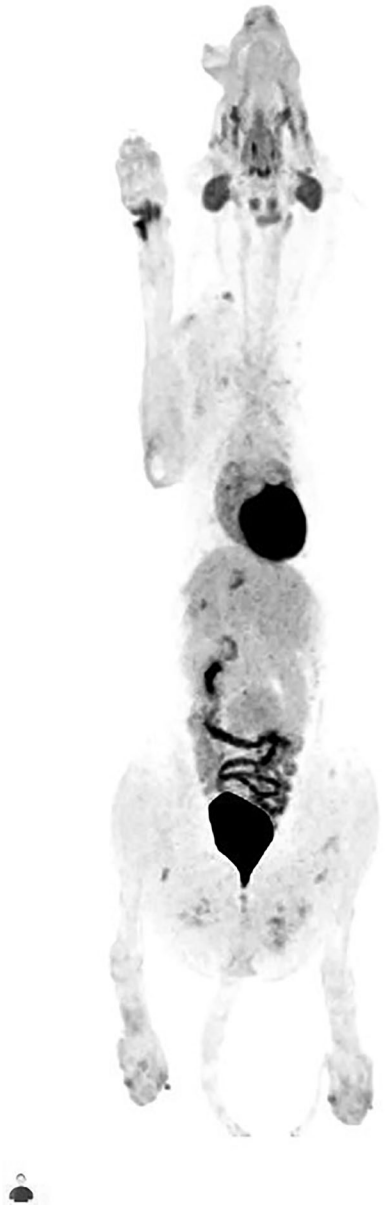

FIGURE 4 ^{18}F-FDG PET scan generated on day 363 of a dog diagnosed at day 140 with a cytology confirmed, alkaline phosphatase positive cutaneous metastatic osteosarcoma (OSA) lesion. No metastatic lesions are visible in this whole-body scan, confirming complete remission

The average leukapheresis product in healthy dogs contained over 5×10^9 viable cells (range, 2.17×10^9-1.21×10^{10}), and consisted primarily of lymphocytes and monocytes with variable contamination with granulocytes and red blood cells. The lymphocytes were primarily CD4+ and CD8+ T-cells with CD4+ T-cells predominating. The viable cell yield after superantigen stimulation typically was $>10^9$ cells and the average was 2.3×10^9 (range, 8.95×10^8-4.35×10^9). Of the cells in the activated T-cell product, >90% were lymphocytes by morphology and those lymphocytes were approximately evenly distributed between small lymphocytes and lymphoblasts. For the activated T-cell product, nearly all expressed T-cell markers and were either CD4+ or CD8+. Leukapheresis and activated T-cell product mean values are summarized in Table 2.

Serum of normal dogs contained negligible quantities of the cytokines IL-2, IL-6, TNF-α, and IFN-γ, typically elaborated by T-cells, before infusion of the activated product. Most of the pro-inflammatory cytokines increased in concentration after infusion of the activated T-cell product. Interleukin-2, IL-6, TNF-α, and IFN-γ all were increased 3 hours after infusion, with peak concentrations between 3 and 12 hours. Serum concentrations progressively decreased with time, generally reaching baseline concentrations by 24 to 36 hours after infusion (Figure 5).

4 | DISCUSSION

Limitations of our study stem primarily from the small sample size, lack of a control population, and lack of immune correlative assays. Ours was a pilot study to investigate the feasibility and possible efficacy of this immunotherapy approach after amputation for naturally occurring OSA. Furthermore, the lack of correlative immune study precludes confirming a specific immunologic mechanism of cancer cell killing beyond the clinical outcome of the cases. Live cancer cells attenuated by irradiation invariably produce the most potent anticancer immune responses when compared to other sources of cancer antigens. In vivo evidence also supports irradiation of vaccine cells when used in combination with an immunological adjuvant (bacterial or cytokine).[41-45] The adjuvant *P. acnes* is known to elicit a strong immune response, but the quantity used has not been successful as a sole treatment in the context of an autologous cancer vaccine. Interleukin-2 at the dosage used in our trial has not been associated with tumor responses in other clinical studies. The activated cell product contained a large number of highly stimulated lymphocytes, which could elicit a nonspecific anticancer immune response after infusion. It is also possible that lower than expected cell viability caused nonspecific immune stimulation leading to an antitumor response. In our pilot study, analysis of the activated cell infusate final product included sterility, cell counts, and cell viability.

Although sample size was small, our results are potentially generalizable because of the 36% 2-year survival rate. A pilot study of dogs with OSA receiving carboplatin chemotherapy followed by a HER-2-targeted, attenuated *Listeria* vaccine has a reported 52% 2-year survival rate.[14] A critical difference between that study and our study is that the previous dogs had to survive without relapse after a course of carboplatin chemotherapy, approximately 70 days, before receiving immunotherapy. Our dogs were preselected only for lack of gross metastatic disease at amputation. Disease-free survival for over 2 months after amputation could preselect dogs with a less aggressive disease phenotype. A nonspecific immunotherapy approach using L-MTP-PE has been reported as a single-agent immunotherapy after amputation, and resulted in a median survival of 222 days for 14 dogs treated with the agent, as compared to 77 days for dogs treated with empty liposomes.[11] Four dogs in that study remained alive 1 year and 2 alive at

TABLE 2 Mean values of leukapheresis and activated T-cell products of normal, healthy laboratory dogs. All percentages are based on % of total cells in population. Note the increase in proportion of CD4+ and CD8+ cells with the majority carrying the CD25+ activation marker

	Total cells (billions)[a]	Viability (%)[a]	Lymphocyte (%)[a]	Lymphoblast (%)[a]	Monocytes (%)[a]	Total T-cells (%)[b]	CD4 (%)[b]	CD8 (%)[b]	CD25 (%)[b]
Leukapheresis	5.4	89	52	<1	31	54	43	11	<5
Activated T-cell	2.3	80	39	56	5	87	57	31	>95

[a]Cell analyses before freezing sample: based on morphologic characterization (cytospin and Wright stain).
[b]Cells analyses after thawing frozen samples: gated on total live/nondoublet cells.

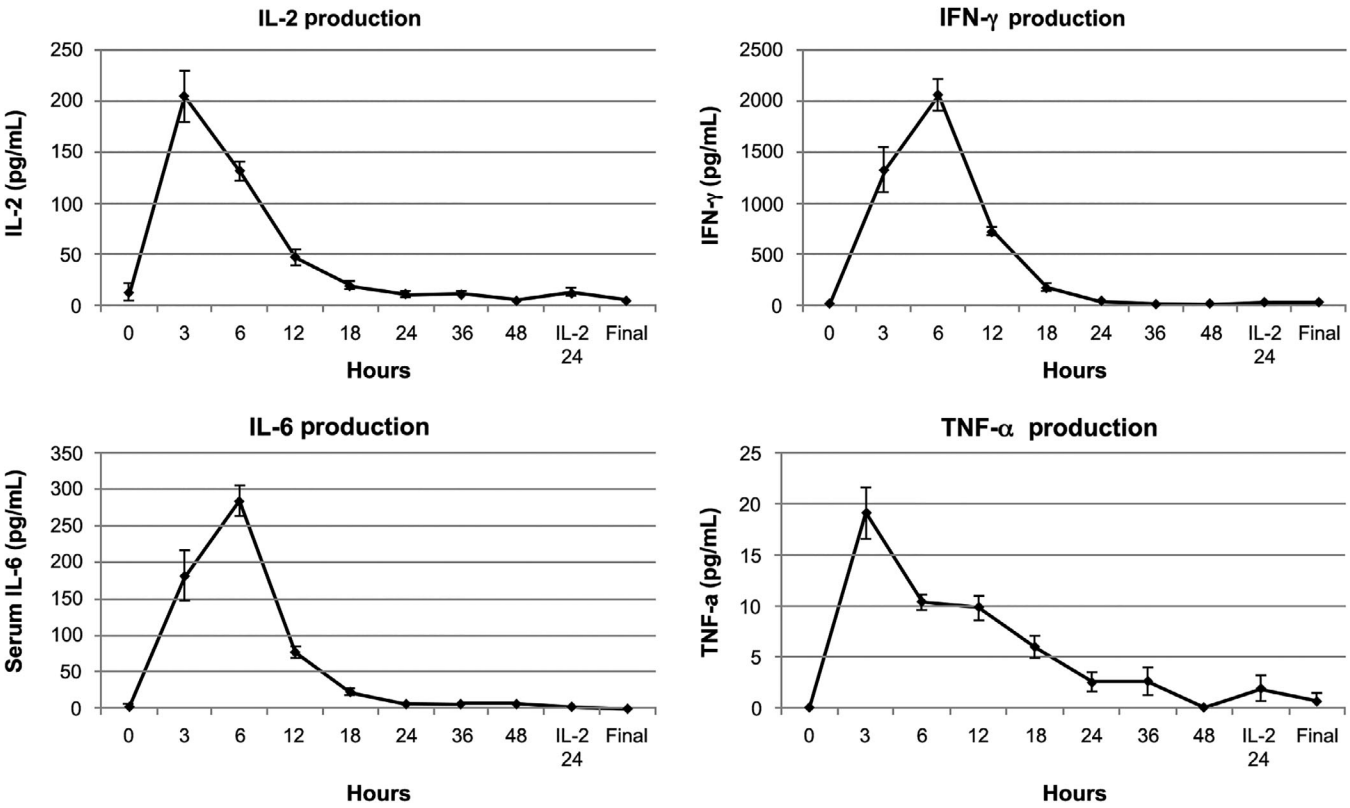

FIGURE 5 Cytokine levels in the serum of 4 laboratory dogs after activated cellular therapy (ACT). IL-2, IL-6, TNF-α, and IFN-γ were elevated above baseline for 12 hours after the infusion of the activated T-cells. Presented are cytokine levels by time point ±SEM. Minimum limits of detection are less than approximately 10 pg/mL. TNF-α lower limit of detection is approximately 6 pg/mL

2 years after amputation. The short average survival of the empty liposome-treated dogs, and only 2 surviving over 1 year is consistent with other reported results of short survival after amputation alone. Two dogs in our study died as a result of metastatic disease within the first 2 months, and 7 dogs survived >1 year (5 dogs >2 years). A long-term survival proportion this high has not been reported previously with immunotherapy alone after amputation.

A therapeutic benefit was noted in our dogs. Although we acknowledge the small sample size, ours is the first example of prolonged survival in OSA-bearing dogs that received only amputation and autologous cellular treatment. The proportion of dogs that appeared to benefit is similar to a previous study of L-MTP-PE. Other immunotherapy protocols, whether xenogeneic vaccines or cytotoxic T-cell infusions, all were administered to dogs that had received chemotherapy and were in clinical remission at the time of the immunotherapy intervention.[12,14,46] No dog treated in our study received chemotherapy at any point during the protocol. The largest case series of dogs with OSA treated by amputation alone reported a median survival time (MST) of 134 days.[47] Most reports of dogs that received amputation or surgical resection of primary OSA followed by adjuvant cisplatin or carboplatin chemotherapy found MST that ranged from 207 to 479 days and 2-year survival rates that ranged from 16 to 22%.[6,48-50] The finding of dogs with OSA reaching a MST of 415 days without adjuvant chemotherapy is unprecedented in veterinary clinical literature. Additionally, resolution of a cytologically documented metastatic lesion is notable because no cytotoxic or targeted chemotherapy drug has shown reliable clinical benefit for metastatic OSA in dogs.[51-53] The positive outcome of our study likely represents the

benefits of a multistaged immunotherapy combination of autologous vaccination, ACT, and low-dose IL-2 in a minimal-disease, early stage setting. Of the 10 dogs that received the entire planned protocol, 5 were long-term survivors. Although it is possible that 7 dogs with atypically less aggressive OSA were enrolled in the trial, no systematic enrollment criterion likely selected for such a possibility. Development of a metastatic lesion at 140 days postamputation, and then resolution of that lesion, supports the possibility of an immunologic mechanism. Metastatic lesions have been reported to resolve with liposomal IL-2 inhalation.[54] Other causes of spontaneous remissions of metastatic cancer are not well documented. These results, along with those seen in rodent models, suggest that testing this approach in earlier stage cancers in humans is warranted under appropriate circumstances.

Dog 002's constitutional and gastrointestinal signs after ACT are consistent with adverse effects noted in a previous study in dogs with lymphoma in which ACT resulted in adverse events varying from grade I/II (diarrhea, vomiting) to grade III (nausea).[55] Similarly, ACT treatment with TALL-104 in dogs with OSA produced peri-transfusion toxicities; 26% of dogs in 1 study had vomiting and diarrhea, which were decreased by premedication with antihistamines.[12] Adverse events occurred primarily in dog 002 after receiving activated T-cells without premedication. The activated product that dog 002 received was delivered in a larger volume and the cells were only washed once after harvest from media. Some component carryover, including superantigen, may have contributed to the dog's reaction. It is also possible the dog experienced a transfusion reaction to its autologous cells after ex vivo manipulation, particularly if viability had decreased substantially during shipping. Infrequent gastrointestinal upset was noted in healthy dogs during the tolerability study, but all dogs remained otherwise healthy for the study duration. After premedicants were instituted in the cancer-bearing dogs, no further severe adverse events were noted. Transient, low-grade gastrointestinal upset, primarily diarrhea, was noted in the immediate period after ACT. Although these effects occurred during the period of low-dose IL-2 injections, attribution was made to IL-2, but the effects may have been associated with ACT, because the effects did not continue over the course of IL-2 administration. All dogs had low-grade injection site reactions at each vaccination, suggesting reaction to the adjuvant, although DTH may have contributed.

Leukapheresis previously has been described in dogs with lymphoproliferative diseases.[56-58] Adverse effects in treated dogs mainly were associated with high dose chemotherapy or total body irradiation, and not with the leukapheresis procedure itself. In the 2 largest studies (n = 24 and n = 15), all but 1 dog in each report tolerated the procedure.[57,58] All dogs in our safety and clinical studies tolerated leukapheresis. Calcium supplementation with 10% calcium gluconate was necessary in all dogs undergoing leukapheresis. Supplementation was initiated at 0.5 mL/kg/h, but was increased in every case, up to 2.5 mL/kg/h for 1 dog. No apparent clinical abnormalities were noted from transient decreases or increases in serum ionized calcium concentration. Second degree atrioventricular block was noted in 1 dog, but it resolved after cell collection was complete and anticoagulant infusion was stopped. It was hypothesized that jugular catheter placement, anticoagulant-associated hypocalcemia immediately after the catheter in the returned blood, or both contributed to the atrioventricular block.

The study of healthy dogs allowed for evaluation of some of the immunologic mechanisms underlying ACT. The elaboration of pro-inflammatory cytokines IL-2, IL-6, TNF-α, and IFN-γ is consistent with production of these cytokines by activated T-cells causing transient inflammation that may be observed after ACT. The fact that concentrations decreased to baseline after 24 to 36 hours could be interpreted to mean that the T-cells left the blood-stream and entered tissue by that time, stopped producing cytokines over time and became memory cells, or were eliminated by the body. It is possible the cytokines may have been present in the infusate, having been elaborated by cells after being washed, suspended in sterile PBS and added into the infusion bag. Alternatively, carry-over of superantigen could explain elaboration of these cytokines, but the amount of superantigen in early, experimental batches of activated cell products was very small. Superantigen concentrations were not measured in the clinical trial batches. These factors should studied in cancer-bearing dogs in the future.

5 | CONCLUSION

With premedication, ACT was well tolerated with only low grade and transient toxicoses. These results indicate that autologous cancer vaccination, leukapheresis, and ACT of ex vivo-activated autologous T-cells and low-dose IL-2 treatment can be tolerated well in healthy and cancer-bearing dogs. Furthermore, in the micrometastatic, minimal disease setting, this immunotherapy combination can result in prolonged survival in a naturally occurring disease model. Our results support further randomized, prospective investigations of this protocol compared to dogs treated with amputation and adjuvant chemotherapy. The success of this combination immunotherapy approach warrants translation to clinical trials in OSA-bearing children in a relapse-resistant setting as well as in various other human tumors in a stage 3 setting.

ACKNOWLEDGMENTS

Abstracts were presented at the 2017 American College of Veterinary Internal Medicine (ACVIM) Forum, National Harbor, Maryland; 2018 ACVIM Forum, Seattle, Washington; 2018 Veterinary Cancer Society Annual Meeting, Louisville, Kentucky; and Paws for a Cure Research Symposium, Boston, Massachusetts, November 12, 2018. The authors thank Maren Fleer for her help during apheresis procedures at the MU-VHC, other contributory services at the MU-VHC, support faculty and staff from our Oncology service, and all of the dogs and their families for participating in the study. We thank Barry Skikne, MD, for helpful conversations.

CONFLICT OF INTEREST DECLARATION

ELIAS Animal Health holds proprietary rights to ELIAS Cancer Immunotherapy. TA Wahaus is the company's Chief Executive Officer. GW

Wood is the company's Chief Scientific Officer. N Reyes is the company's Chief Medical Officer. FL Sonderegger was employed by ELIAS Animal Health. TA Wahaus, GW Wood, and N Reyes hold stock in the company. CJ Henry and JN Bryan are on the scientific advisory board for ELIAS Animal Health.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the University of Missouri Animal Care and Use Committee protocol (#8280).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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How to cite this article: Flesner BK, Wood GW, Gayheart-Walsten P, et al. Autologous cancer cell vaccination, adoptive T-cell transfer, and interleukin-2 administration results in long-term survival for companion dogs with osteosarcoma. *J Vet Intern Med*. 2020;34:2056–2067. https://doi.org/10.1111/jvim.15852