Cancer Therapy: Preclinical

Clinical Cancer Research

Immunotherapy with a HER2-Targeting *Listeria* Induces HER2-Specific Immunity and Demonstrates Potential Therapeutic Effects in a Phase I Trial in Canine Osteosarcoma

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Abstract

Purpose: Recombinant *Listeria* vaccines induce tumor-specific T-cell responses that eliminate established tumors and prevent metastatic disease in murine cancer models. We used dogs with HER2/neu$^+$ appendicular osteosarcoma, a well-recognized spontaneous model for pediatric osteosarcoma, to determine whether a highly attenuated, recombinant *Listeria monocytogenes* expressing a chimeric human HER2/neu fusion protein (ADXS31-164) could safely induce HER2/neu-specific immunity and prevent metastatic disease.

Experimental Design: Eighteen dogs that underwent limb amputation or salvage surgery and adjuvant chemotherapy were enrolled in a phase I dose escalation clinical trial and received either 2×10^8, 5×10^8, 1×10^9, or 3.3×10^9 CFU of ADXS31-164 intravenously every 3 weeks for 3 administrations.

Results: Only low-grade, transient toxicities were observed. ADXS31-164 broke peripheral tolerance and induced antigen-specific IFNγ responses against the intracellular domain of HER2/neu in 15 of 18 dogs within 6 months of treatment. Furthermore, ADXS31-164 reduced the incidence of metastatic disease and significantly increased duration of survival time and 1-, 2-, and 3-year survival rates when compared with a historical control group with HER2/neu$^+$ appendicular osteosarcoma treated with amputation and chemotherapy alone.

Conclusions: These findings demonstrate that ADXS31-164 administered in the setting of minimal residual disease can induce HER2/neu-specific immunity and may reduce the incidence of metastatic disease and prolong overall survival in a clinically relevant, spontaneous, large animal model of cancer. These findings, therefore, have important translational relevance for children with osteosarcoma and adults with other HER2/neu$^+$ cancers. *Clin Cancer Res; 22(17); 4380–90. ©2016 AACR.*

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Introduction

Pediatric osteosarcoma is a highly aggressive mesenchymal bone tumor and is the second most common malignancy in adolescents (1). Treatment consists of chemotherapy, radiotherapy, and radical surgery. Despite aggressive treatment, metastatic disease is common and results in mortality rates of 30% to 40% within 5 years of diagnosis, making pediatric osteosarcoma the second highest cause of cancer-related death in children and adolescents (2). There are no effective treatments for metastatic disease and the current focus of drug development strategies is prevention of metastases rather than their treatment (3).

To evaluate novel strategies to prevent metastatic disease, we have turned to the dog, a widely recognized, clinically relevant, spontaneous large animal model that recapitulates many aspects of human osteosarcoma. These aspects include high genetic instability and histologic heterogeneity of the tumor, aggressive local disease, early metastases, and comparable treatment options (4–7). Approximately 90% to 95% of dogs with osteosarcoma have micrometastases at diagnosis and despite limb amputation and adjuvant chemotherapy, most are euthanized due to progressive metastatic disease within 1 year of diagnosis (8). These similarities make dogs with spontaneous osteosarcoma ideal to evaluate the safety and efficacy of immunotherapies administered in the setting of minimal residual disease, to prevent metastatic disease and inform human clinical trials.

HER2/neu is a tyrosine kinase receptor that belongs to the family of EGFRs (9). It is frequently overexpressed in carcinomas of the breast, prostate, pancreas, and gastrointestinal tract where it denotes an aggressive phenotype, high metastatic rate, and poor

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Translational Relevance

Pediatric osteosarcoma is a highly aggressive mesenchymal bone tumor affecting children in their first and second decades of life. Despite aggressive treatment, 30% to 40% of patients develop metastatic disease for which there are no effective treatments and die within 5 years of diagnosis. Prevention of metastatic disease is necessary to improve survival times. Here, we show in client-owned dogs with spontaneous osteosarcoma that administration of a recombinant HER2/neu-expressing *Listeria* immunotherapy following amputation and chemotherapy safely induces HER2-specific immunity, prevents metastatic disease, and prolongs overall survival. Our results hold significant translational relevance for the treatment of pediatric osteosarcoma and other HER2/neu$^+$ cancers in humans.

prognosis. HER2/neu is also expressed in 40% of pediatric and canine osteosarcoma, where it is linked to reduced response to neoadjuvant chemotherapy, high metastatic rates, and shorter overall survival times (10–13). Recent reports suggest that HER2/neu is expressed by cancer stem cells in both osteosarcoma and mammary carcinoma and that HER2/neu targeted immunotherapies may eliminate these cells and prolong overall survival (12, 14–16).

Herein, we describe a clinical trial to evaluate the safety and efficacy of ADXS31-164, a highly attenuated, recombinant *Listeria monocytogenes* (*Lm*) expressing a chimeric human HER2/neu construct (17, 18), to induce HER2/neu-specific immunity and prevent metastatic disease in dogs with appendicular osteosarcoma following amputation and chemotherapy. *Lm* is a facultative, anaerobic, intracellular bacteria that infect mononuclear cells and are potent stimulators of innate and adaptive immunity (19). Once in the phagosome, *Lm* secretes the pore-forming lysin listeriolysin O (LLO) that enables it to escape into the cytosol, thus accessing the class I antigen processing pathway (19). As such, antigens fused to a truncated form of LLO (tLLO) are also secreted and presented in the context of both MHC I and MHC II enabling priming of both CD8$^+$ and CD4$^+$ T cells, respectively (20). Attenuated, recombinant *Lm* containing fragments of either rat or human HER2/neu fused to tLLO break peripheral tolerance to HER2/neu and mediate cytotoxic T-cell–dependent tumor regression and prevention in multiple different mouse models of primary and metastatic HER2/neu$^+$ cancers (17, 18, 21, 22).

In this phase I clinical trial, we found that intravenous administration of up to 3.3×10^9 CFU ADXS31-164 was well tolerated, broke peripheral tolerance to HER2/neu, and was highly effective at preventing pulmonary metastatic disease when administered to 18 dogs with HER2/neu$^+$ appendicular osteosarcoma. Observed toxicities were low grade and transient. Overall survival was significantly prolonged in treated dogs when compared with a matched historical control group and previously published reports. These findings represent a major advance in the search for preventative therapies for metastatic osteosarcoma and have important implications for children with pediatric osteosarcoma, and humans and dogs with other HER2/neu-expressing neoplasms.

Materials and Methods

Eligibility criteria and study design

The objectives of this study were to determine the safety of ADXS31-164 and its ability to generate HER2/neu-specific immunity in dogs with spontaneous osteosarcoma following amputation and adjuvant carboplatin chemotherapy. Secondary objectives were to determine whether ADXS31-164, administered in the setting of minimal residual disease, would prevent metastatic disease and prolong overall survival.

Dogs with a histopathological and IHC diagnosis of HER2/neu$^+$, osteosarcoma that had undergone primary tumor removal either by amputation ($n = 17$) or limb-sparing surgery ($n = 1$) and had received 4 doses of carboplatin (target dose of 300 mg/m^2) were eligible for screening 3 weeks after their last carboplatin treatment. A thorough physical examination, complete blood count (CBC), chemistry screen (CS), and urinalysis (UA) were performed to determine general health status. Basic innate and adaptive immune functions were tested using a neutrophil oxidative burst assay and mitogen-induced lymphocyte proliferation assay, respectively. Cardiac status was evaluated by echocardiography and serum cardiac troponin I levels. Thoracic radiographs were performed to determine the presence of pulmonary metastatic disease. Only systemically healthy dogs with intact immune function, no underlying cardiac disease, and no pulmonary metastatic disease were eligible for enrollment.

A standard 3+3 phase I clinical trial design was employed, aimed at determining the MTD of ADXS31-164, administered in the setting of minimal residual disease. ADXS31-164 was administered intravenously once every 3 weeks for a total of three administrations, beginning 3 to 4 weeks after the last carboplatin treatment. Dogs were re-staged 3 weeks after their third ADXS31-164 administration and every 2 months thereafter until disease progression. Following institutional approval of a protocol amendment, dogs that were free of metastatic disease at least 5 months after receiving their third ADXS31-164 treatment were offered the option to receive additional intravenous ADXS31-164 administrations once every 4 to 6 months at a dose of 1×10^9 CFU.

Where possible, dogs that died during the course of the study underwent necropsy. The presence and location of metastatic disease were recorded and histopathology and IHC were performed to evaluate tumor subtype, grade, and HER2/neu status.

Ethics statement and regulatory approvals

This study was approved by the University of Pennsylvania's (Philadelphia, PA) Institutional Animal Care and Use Committee (Protocol number 803210) and signed owner consent was required before enrollment. The use of recombinant DNA was approved by the University of Pennsylvania's Institutional Biosafety Committee (IBC #13-016).

ADXS31-164 manufacture

The design and generation of ADXS31-164 have been reported elsewhere (18). Briefly, the *dal*, *dat*, Act-A–deleted mutant strain of *Lm* was transformed with the pADV164 plasmid carrying a chimeric human HER2/neu construct. The construct contains fragments of the 2 extracellular domains (EC1 and EC2) and one intracellular domain (IC1) of the human HER2/neu molecule that contain the majority of HLA-A2–restricted immunodominant epitopes fused to a truncated listeriolysin O (LLO). The plasmid pADV164 also contains the bacillus p60 *dal* gene and is maintained

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within the mutant *Lm* via auxotrophic complementation. There is no antibiotic resistance expression cassette. Vaccines were manufactured by Vibalogics GmbH and stored at −80°C before use.

Histopathology, staging, and IHC

Histopathological assessment of all primary and metastatic tumors was performed by a board certified veterinary pathologist (J.B. Engiles). Tumors were described as osteoblastic, chondroblastic, fibroblastic, and telangiectatic based on histologic features. All tumors were scored on the basis of mitotic index, nuclear pleomorphism, and the amount of matrix and necrosis present. Histologic scores were converted into a grade (I, II, or III) as previously described (23).

For HER2/neu staining, 5 micron sections of formalin-fixed, paraffin-embedded tissues were heated at 80°C for 20 minutes, immersed in Pro Par (clearant), and rehydrated in ethanol. Antigen retrieval was performed by boiling in sodium citrate buffer (pH ~9.0). Endogenous peroxidase was blocked using 3% hydrogen peroxide. Staining was performed with a rabbit anti-human HER2/neu antibody (Neu(c-18):sc-284, Santa Cruz Biotechnology) or a rabbit IgG isotype (Universal Negative Control serum, NC498, Biocare Medical). Bound antibody was detected using the Universal LSAB 2/HRP kit (Streptavadin-Biotin2 System; Agilent Technologies Inc.). Tissues were stained with 3,3'-diaminobenzidine solution (DAKO) and counterstained with hematoxylin. Slides were viewed using a Nikon E600 infinity corrected upright microscope. Bright field images were acquired using a Nikon Digital Sight DS-Fi1 color camera and a NIS-Element BR3.0 for image analysis. Tissue sections were evaluated and scored for HER2/neu positivity based on the percentage of HER2/neu$^+$ neoplastic cells (<10% = 1, 10%−50% = 2, >50% = 3) and HER2/neu staining intensity (weak = 1, moderate = 2, strong = 3). Of note, 10 hpf were evaluated for each tissue section. A combined HER2/neu score was obtained by multiplying the scores for HER2/neu positivity by HER2/neu staining intensity. Only dogs with greater than 10% of their tumor cells staining positive for HER2/neu were eligible for trial enrollment.

ADXS31-164 administration

Before ADXS31-164 administration, dogs received a single dose of the 5HT3 antagonist ondansetron (0.2 mg/kg) intravenously to prevent nausea and vomiting, which were reported in a phase I human clinical trial using a HPV-targeting *Listeria* construct, (24) and the H1 receptor blocker diphenhydramine (2 mg/kg) intramuscularly to prevent anaphylaxis. ADXS31-164 was administered at the following target doses; Group 1 (2×10^8 CFU), Group 2 (5×10^8 CFU), Group 3 (1×10^9 CFU), and Group 4 (3.3×10^9 CFU). The starting dose was based on that used for previous cytotoxicity studies in mice (17). ADXS31-164 was diluted in 100 mL 0.9% NaCl (Groups 1 and 2) and 200 mL 0.9% NaCl (Groups 3 and 4) and administered intravenously over 30 minutes. Temperature, pulse, respiratory rate, heart rate and rhythm, and blood pressure were monitored every hour following infusion. In cases where body temperature exceeded 103°F, dogs received intravenous fluids until their temperature fell below 103°F. Dogs were monitored hourly for signs of lethargy, nausea, or vomiting. Blood samples were drawn 24 hours and one week post vaccination, and blood cultures were performed at 24 hours post vaccination to determine the persistance of live bacteria. All dogs received a 3-day course of amoxicillin and 7-day course of S-Adenosylmethionine (SAMe) 72 hours after vaccination to eliminate any remaining ADXS31-164 and provide anti-oxidant support to the liver, respectively.

Toxicity

Toxicities occurring during the initial series of ADXS31-164 treatments (treatments 1-3) were graded according to the Veterinary Co-operative Oncology Group- Common Terminology Criteria for Adverse Events v1.1 (VCOG-CTCAE; ref. 25). To prevent bias in reporting toxicities only associated with long-term survival, toxicities occurring at subsequent ADXS31-164 administrations are not reported. Serum cardiac troponin I levels were measured and echocardiography was performed at baseline, immediately before each vaccination, 3 weeks after the last vaccination and every 2 months thereafter. Dogs surviving greater than 3 years were re-evaluated every 6 months. Parameters assessed included left ventricular fractional shortening (LVFS), left ventricular internal dimension in diastole (LVIDd), and left ventricular internal dimension in systole (LVIDs). LVIDd and LVIDs were normalized to body weight as previously described (26).

ELISpot analysis

Cryopreserved PBMCs from each indicated time point were thawed, rested overnight at 37°C, and counted. Cells were stimulated for 5 days with 2.0 μmol/L pools of overlapping human HER2/neu peptides (11 mers overlapping by 5 amino acids) that represent the EC1, EC2, and IC1 domains of HER2/neu present in the chimeric vaccine, plus rhIL2 (Invitrogen) or left unstimulated in the presence of rhIL2. Cells were harvested, washed, and counted. IFNγ ELISpot assays were performed according to the manufacturer's protocol using a commercial canine IFNγ ELISpot assay kit (R&D Systems). Briefly, 0.1 to 3×10^5 stimulated cells were incubated with 2.5 μmol/L of EC1, EC2, or IC1 peptide pools or no peptide (to determine background counts) for 24 hours. All assays were performed in duplicates and the average number of spots is reported. Plates were developed according to the manufacturer's instructions. Spots were counted using a CTL-Immunospot analyzer (C.T.L). Samples that had background counts of 0 were assigned a count of '1' to enable fold increase following peptide stimulation to be determined.

Statistical analysis

The paired *t* test (two tailed) was used to compare continuous variables between baseline and subsequent measurements within groups. Continuous data were tested for normality before application of the t test. For within dose group comparisons, the change or percent change in variable was averaged for each dog's treatments before analysis using the paired *t* test (two tailed). This maintains the assumption that the effects of each ADXS31-164 treatment are independent of one another. The one-way ANOVA was used to compare the magnitude of change in continuous variables from baseline to 4 hours or baseline to 24 hours between dose groups. In these analyses, the dog was the repeated effect and the dose and vaccine number were fixed effects. The repeated measures ANOVA was also used to determine whether there was a correlation between magnitude of change in hematological parameters and survival outcome. A P value <0.05 was considered significant for all comparisons. Disease-free interval (DFI) was defined as the time between amputation and development of detectable metastatic disease. Overall survival (OS) was defined as

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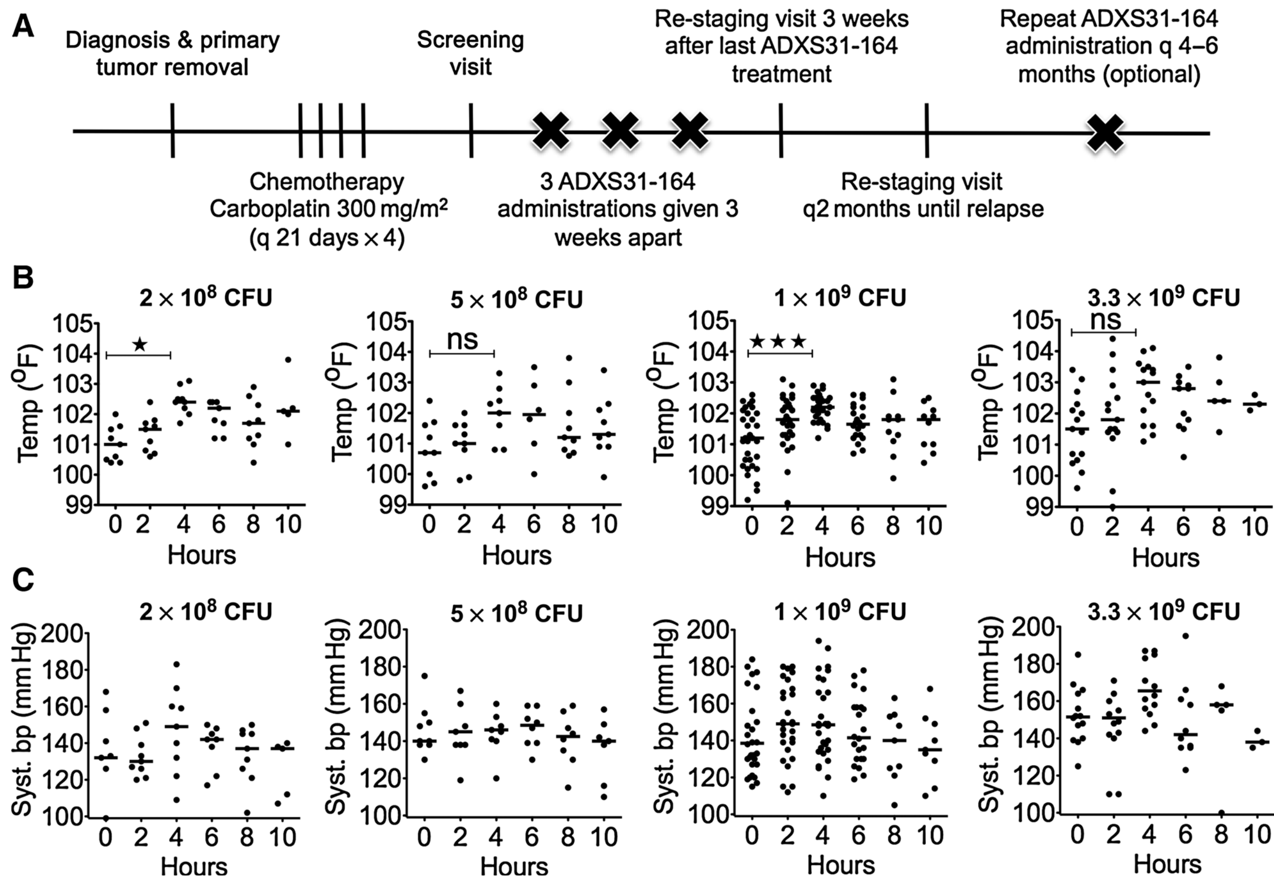

Figure 1.

Effects of ADXS31-164 on body temperature and blood pressure. Dogs enrolled were monitored for up to 10 hours after intravenous infusion of ADXS31-164. Clinical trial design (**A**); body temperature (**B**); and systolic blood pressure (**C**). Body temperature and systolic blood pressure were recorded at baseline and every 2 hours post ADXS31-164 administration. Each dot represents an individual subject at one ADXS31-164 treatment and horizontal lines represent the median values. Dogs with preexisting metastatic disease treated on a compassionate care basis are included. ★, $P < 0.05$; ★★, $P < 0.005$; ★★★, $P < 0.0005$; ns, not significant.

the time between amputation and death from any cause. OS was used instead of disease specific survival since necropsy results were not available on all dogs to confirm osteosarcoma-related death. In this way, conservative estimates of survival are reported. Data collected between July 2012 and September 2015 were used in the analysis. The Kaplan–Meier method was used to generate survival curves and determine DFI and median survival time (MST). For DFI analysis, dogs were censored if they did not have metastatic disease at the time of last follow up. For OS analysis, dogs were censored if they were alive at the last follow up. Statistical analysis was performed using SAS version 9.3 software and GraphPad Prism.

Results

Eighteen dogs that fulfilled the eligibility criteria were enrolled in this phase I clinical trial. Seventeen dogs underwent limb amputation and one had limb salvage surgery. All dogs received 4 doses of carboplatin (dose range from 194 mg/m^2 to 307 mg/m^2; median 282 mg/m^2) and were confirmed to be free of pulmonary metastatic disease 3 weeks after their last carboplatin dose, prior to administration of ADXS31-164. The age, breed, sex, tumor location, subtype, grade, and HER2/neu status were recorded (Supplementary Table S1). A standard 3+3 dose escalation trial design was employed where Group 1 received 2×10^8 CFU of ADXS31-164 (n = 3), Group 2: 5×10^8 CFU (n = 3), Group 3: 1×10^9 CFU (n = 9), and Group 4: 3.3×10^9 CFU (n = 3) according to the trial protocol (Fig. 1A). Six additional dogs that fulfilled the eligibility criteria were recruited to Group 3 because of grade 2 thrombocytopenia occurring in one dog from the first and one from the second group of 3 dogs treated in that dose group. Five additional dogs with HER2/neu$^+$ primary tumors that underwent limb amputation and carboplatin treatment but had pulmonary metastatic disease at screening were treated on a compassionate care basis with either 1×10^9 ($n = 3$) or 3.3×10^9 ($n = 2$) CFU ADXS31-164 (Supplementary Table S2).

Safety and toxicity

Safety was evaluated for all 23 dogs that received one or more ADXS31-164 treatments. All dogs tolerated ADXS31-164 well with only transient, low-grade toxicities observed on the day of administration (Table 1). Mild increases in body temperature occurred within 4 hours of administration in all dose groups

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Table 1. Treatment-related adverse events occurring at or within 48 hours of ADXS31-164 vaccination

			Number of dogs with treatment-related adverse events				
ADXS31-164 dose (CFU)	**Grade**	**Parameters**	**2×10^8 $n = 3$**	**5×10^8 $n = 3$**	**1×10^9 $n = 12$**	**3.3×10^9 $n = 5$**	**Total 23**
General disorders							
Pyrexia	1	103.5–104°F	1	1	1	1	4
	2	>104–105.8°F	0	0	0	3	3
Vomiting	1	<3 episodes in 24 hours					
	2	3–10 episodes in 24 hours	0	0	1	0	1
Cardiovascular abnormalities							
Arrhythmias	1	Asymptomatic	0	2[a]	1	1	4
	2	Nonurgent intervention	0	0	0	1[a]	1
Tachycardia	1	Asymptomatic	1	0	2	0	3
Hypotension			0	0	0	0	0
Hypertension	1	Systolic ≥160 mm Hg	2	3	9	5	19
Hematologic parameters							
Thrombocytopenia	1	100,000/μL to <LLN	3	2	7	1	13
	2	50,000−99,000/μL	0	0	2	0	2
Biochemical parameters (increased)							
γ-GT			0	0	1	0	1
ALKP	1	>ULN to 2.5x ULN	0	1	5	2	8
	2	>2.5 to 5x ULN	1	0	0	0	1
ALT	1	>ULN to 1.5x ULN	0	0	3	0	3
	2	>1.5 to 4x ULN	1	0	0	1	2
AST	1	>ULN to 1.5x ULN	1	1	3	2	7
	2	>1.5 to 2x ULN	0	0	2	0	2
	3	>2.0 to 10x ULN	0	0	1	0	1
Cardiac Troponin I		>0.2 μg/L	0	0	1	0	1

NOTE: To eliminate bias in recording adverse events in dogs with a favorable outcome, adverse events that occurred only during the initial 1 to 3 treatments for all 23 dogs are reported. Two dogs with preexisting metastatic disease and elevations in cTnI at baseline showed a reduction in cTnI after vaccination, although levels did not return to normal. These dogs are not included in this table. The asterisk denotes two exceptions to this with a ventricular tachycardia and grade II, accelerated idioventricular rhythmn occurring at repeat administration of ADXS31-164 in 2 dogs.

Abbreviations: LLN, lower limit of normal; ULN, upper limit of normal.

[a]Seen at repeat ADXS31-164 administration after initial series.

(Fig. 1B). There were no statistical differences in the magnitude of temperature increase between dose groups (2×10^8 and 5×10^8, $P = 0.55$; 2×10^8 and 1×10^9, $P = 0.43$; 2×10^8 and 3.3×10^9, $P = 0.47$ and data not shown). Hypotension, reported as a dose-limiting toxicity in humans receiving an HPV-E7 targeting recombinant *Lm* (24), was not observed at any dose (Fig. 1C). Transient nausea and vomiting occurred within 4 hours of treatment irrespective of dose.

Transient increases in total WBC and neutrophil counts and decreases in lymphocyte and platelet counts occurred 24 hours after ADXS31-164 administration in all dose groups (Supplementary Fig. S1A). There was no difference in the magnitude of total WBC, neutrophil, lymphocyte, monocyte, or platelet changes amongst dose groups (Supplementary Fig. S1B). However, there was a significant difference in the magnitude of WBC, neutrophil, and monocyte responses and a trend toward significance in platelet responses in dogs that survived greater than 18 months compared with dogs that died within 18 months of diagnosis (Fig. 2). Mild increases in the liver enzymes ALKP and AST occurred in approximately half of the dogs consistent with subclinical inflammation caused by the hepatotropic *Lm* (Table 1). All hematologic and biochemical changes were asymptomatic and transient. Blood cultures were performed in 19 of 23 dogs 24 hours after ADXS31-164 administration and all were negative, consistent with rapid clearance of the highly attenuated *LmddA* strain (27). Eleven of the 18 dogs and 2 dogs treated on a compassionate care basis received between 1 and 4 additional doses of ADXS31-164 following the initial course (Supplementary Table S3). Similar low-grade, transient side effects were noted as with the initial series.

Although immune targeting of HER2/neu has been reported to cause cardiotoxicity (28, 29), no significant, sustained changes in cardiac parameters were identified in any dog (Supplementary Fig. S2A–S2D). One dog in Group 3 with a mildly increased cTnI level pretreatment showed an increase in cTnI during the initial vaccination series, and again at the time of relapse. These increases were not accompanied by deteriorating cardiac function. One dog with pre-existing metastatic disease and one that developed metastatic disease after the initial ADXS31-164 series developed an accelerated idioventricular rhythm and ventricular tachycardia, respectively, within 2 hours of an additional ADXS31-164 administration. One dog was treated with membrane stabilizers, beta blockers, and corticosteroids which had little effect; the second dog was not treated. In both cases, the arrhythmias were asymptomatic and resolved completely within one week.

ADXS31-164 prolongs overall survival and reduces the development of metastatic disease

For the 18 dogs with no evidence of metastatic disease at enrollment and treated with ADXS31-164, the median DFI was 615 days and MST was 956 days (Fig. 3A and B). Overall survival rates at 1, 2, and 3 years for dogs treated with ADXS31-164 were 77.8%, 67%, and 56%, respectively. The MST of all 23 dogs (including the 5 with pre-existing metastatic disease) was 738 days with overall survival rates at 1 and 2 years of 60.9% and 52%, respectively (data not shown). In both cases, these results compare favorably with multiple studies evaluating dogs with osteosarcoma treated with amputation plus carboplatin, where reported DFI ranged from 123 to 257 days, MST ranged from

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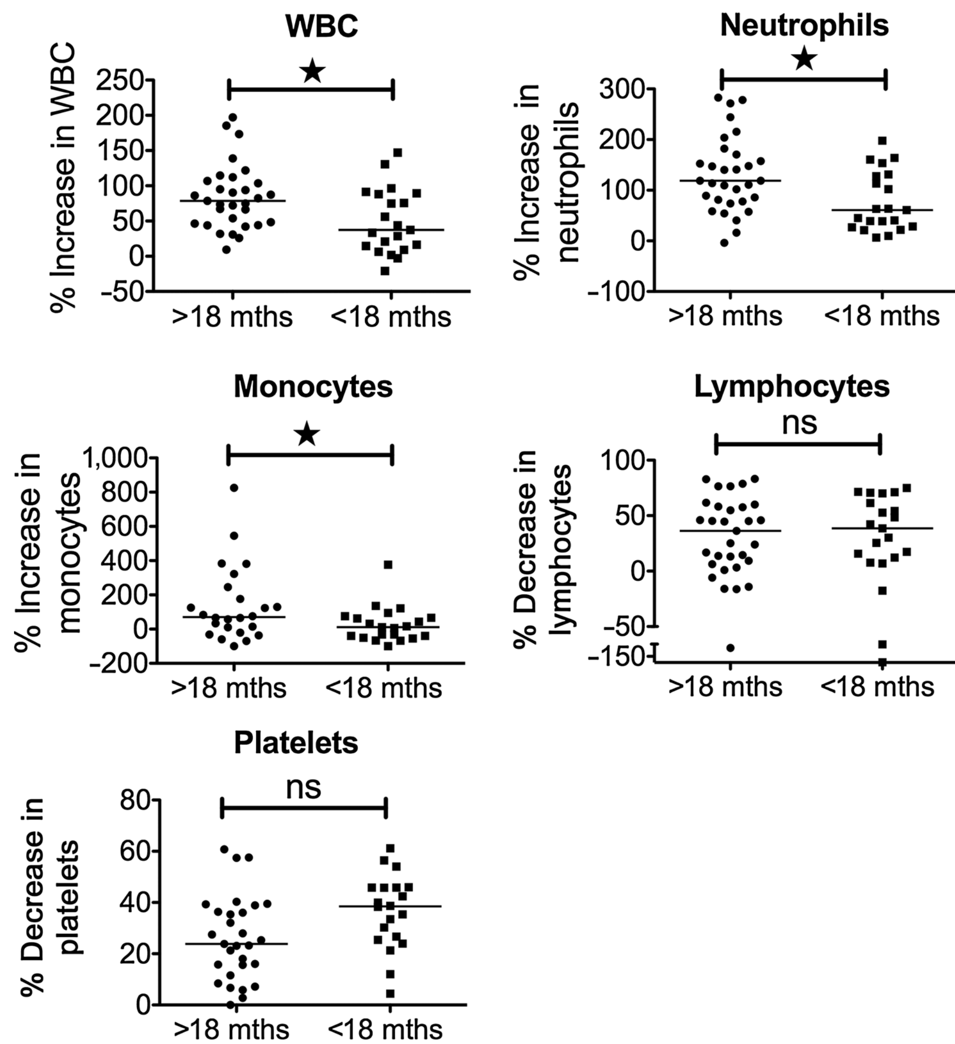

Figure 2.
Effects of ADXS31-164 on hematologic parameters at 24 hours post infusion and correlation with outcome. The percent change in WBC, neutrophils, lymphocytes, monocytes, and platelets was calculated at 24 hours post ADXS31-164 for each dog and plotted in relation to survival outcome. For survival outcome, dogs that lived longer than 18 months postdiagnosis were categorized as "Long-Term Survivors" and dogs that died within 18 months were categorized as "Short-Term Survivors." Each dot represents an individual subject at one ADXS31-164 treatment and horizontal lines represent median values. Dogs with preexisting metastatic disease treated on a compassionate care basis are included. ★, $P < 0.05$; ns, not significant.

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207 to 321 days (30–34) and overall survival rates at 1 and 2 years were 35.4% and 10% to 15%, respectively (7, 30, 32, 35).

Given that the eligibility criteria of absence of metastatic disease following chemotherapy might introduce bias in MST and overall survival rates, we retrospectively evaluated a historical control group of 18 dogs from our pathology database with HER2/neu$^+$ primary osteosarcoma that had undergone amputation and chemotherapy and were confirmed to be free of pulmonary metastatic disease following chemotherapy (Supplementary Table S4). There were no significant differences in demographics of the ADXS31-164-treated and control groups (Supplementary Table S5). Although there were 3 more dogs with grade III tumors in the control group than the ADXS31-164-treated group, the grading score previously described and used here has been shown not to correlate with outcome (23). The MST for the historical control group was 423 days, which was significantly shorter than ADXS31-164–treated dogs ($P = 0.014$, HR 0.33; 95% confidence intervals; CI, 0.136–0.802; Fig. 3B). Overall survival rates at 1, 2, and 3 years were 55%, 28%, and 22%, respectively, for the historical control group. Eight dogs were censored from the DFI analysis in the ADXS31-164 treatment group. Ten dogs in the ADXS31-164–treated group and 4 dogs in the control group were censored from the overall survival analysis. The median follow-up time for censored patients was 640 days in the ADXS31-164–treated group and 635 days in the control group. Taken together, the results from this small group of dogs demonstrate that treatment with ADXS31-164 following amputation and chemotherapy significantly prolongs overall survival.

Ten out of 18 dogs treated with ADXS31-164 relapsed, 6 with pulmonary metastases and 4 with bone metastases. Three dogs with bone metastases progressed to pulmonary metastases. One dog with a bone lesion in the sacrum was euthanized because of aspiration pneumonia and one dog with a solitary pulmonary nodule was euthanized because of nephroblastoma. Dogs that relapsed received different rescue chemo- and radiation therapies at the discretion of the primary clinician (Supplementary Table S3).

HER2/neu-specific immune responses

Immunologic responses against the human EC1, EC2, and IC1 domains of HER2/neu (sharing 89%, 93%, and 98% identity with canine HER2/neu, respectively) were evaluable in 17 dogs at baseline and occurred in 4 of 17, 5 of 17, and 1 of 17 dogs, respectively (Table 2 and Supplementary Table S3). Three weeks

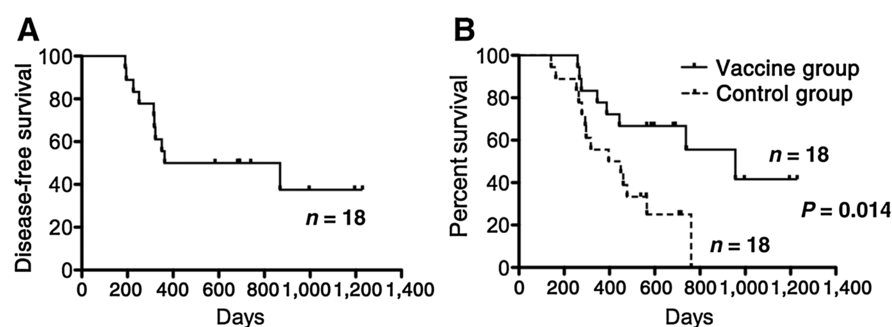

Figure 3.
Kaplan–Meier survival curves. **A,** disease-free survival for dogs without preexisting metastatic disease ($n = 18$). **B,** comparison of overall survival between ADXS31-164–treated dogs ($n = 18$) and historical controls ($n = 18$).

following treatment, IFNγ responses against the IC1 domain were detected above baseline in 5 dogs. Ten additional dogs developed delayed IFNγ responses against the IC1 domain 2, 4 or 6 months later (Table 2 and Supplementary Table S3). Three dogs developed IC1-specific IFNγ responses at the time of relapse. Two dogs developed robust responses 1 to 2 months after relapse and rescue chemotherapy. One dog developed marked IC1 responses at the 16-month recheck when ALKP increased to greater than 2 × normal, suggesting subclinical relapse. The ALKP continued to rise in this dog on repeat rechecks and the dog was diagnosed with multiple bone metastases 4 months later. Of the 8 of 15 dogs that developed IC1-specific IFNγ responses without clinical relapse before or at their 6th month recheck, 7 are still alive with survival times ranging from 682 to 1,229 days (Table 2). Of the 10 of 18 dogs that relapsed, 7 had no increase in IFNγ responses against the IC1 domain of HER2/neu 3 weeks after ADXS31-164 and 3 had less than a 5-fold increase compared with baseline (Table 2 and Supplementary Table S3). Six of eight dogs with pulmonary metastatic disease diagnosed at screening (screen failures) had no evidence of IC1-specific IFNγ responses at baseline (Supplementary Table S3).

IFNγ responses against the EC1 and EC2 domains were induced or augmented 3 weeks after the third ADXS31-164 treatment in 6 and 5 dogs, respectively. Delayed IFNγ responses against the EC1 and EC2 domains occurring between 2 and 6 months after treatment were induced or augmented above baseline in 3 and 8 dogs, respectively. IFNγ responses detected in the peripheral blood were generally not maintained following treatment. Eleven dogs received repeat injections of ADXS31-164 and of the 9 dogs evaluable 2 to 4 months later, 4 had detectable increases in IFNγ responses against all 3 HER2/neu domains (Supplementary Table S3).

Evidence of ADXS31-164–associated T-cell responses within the pulmonary parenchyma

Five dogs with metastatic pulmonary nodules at screening were treated on a compassionate care basis. One dog with multiple pulmonary metastases and two dogs with pulmonary and bone metastases received one treatment each before disease progression. Two dogs presented with a single metastatic pulmonary nodule and received a total of 4 and 5 ADXS31-164 treatments, respectively (Supplementary Tables S2 and S3). Additional pulmonary lesions developed in one dog but not in the other, although the pre-existing pulmonary nodule doubled in size every 3 weeks (Fig. 4A and B). A CT scan one week after the third treatment confirmed the absence of additional metastases and the dog underwent metastatectomy. Before surgery, indocyanine green was administered intravenously and at surgery, fluorescence under near infra-red light was seen in the pulmonary nodule and several other areas of grossly normal pulmonary tissue (Fig. 4C and D). Histopathology of the nodule revealed metastatic osteosarcoma, surrounded by a thick fibrous capsule (Fig. 4E). $CD3^+$ T cells surrounded the nodule but were not present within the neoplastic tissue (Fig 4G and H). Other areas identified by fluorescence showed focal areas of T-cell infiltrates (Fig. 4F, I, and J) that were associated with large, pleomorphic vimentin-positive spindle cells that contained prominent mitotic figures (Fig 4K and L). Although the exact nature of these pleomorphic cells is unknown, they may represent metastatic sarcoma cells being actively targeted by ADXS31-164 primed T cells, providing insight into the mechanism by which ADXS31-164 prevents metastatic disease. The dog remained free of disease for 5 months before developing widespread, aggressive, $HER2/neu^+$ metastatic disease (Supplementary Table S6).

Necropsy findings

Eight out of eighteen dogs died during the study period and 5 of these dogs underwent necropsies (Supplementary Table S6). Four dogs had multifocal metastatic osteosarcoma involving the lungs, bone, mediastinum, and kidney. One dog had nephroblastoma with a single pulmonary nodule identified on CT. The latter was not evaluated by histopathology, so its origin is unknown. All dogs presenting with metastatic disease died (OS range 153–336 days, median 233 days). All metastatic lesions evaluated were positive for HER2/neu expression suggesting loss of HER2/neu expression was not responsible for relapse.

Discussion

Osteosarcoma is considered an immunologically responsive tumor and dogs with spontaneous osteosarcoma can be used to evaluate the effectiveness of immunotherapy to prevent metastases and subsequently inform human clinical trials (3, 7, 36, 37). Previously, nonspecific immune stimuli, such as spontaneous bacterial infections following limb sparing surgery (38, 39) or liposome encapsulated muramyl tripeptides (L-MTP-PE) administered after amputation and platinum therapy have resulted in significant prolongation of metastasis-free interval (11.3 months vs. 7.6 months; control) and overall survival (14.4 months vs. 9.8 months; control) in dogs with osteosarcoma (37, 38). Consequently, L-MTP-PE (mifamurtide; MEPACT) was evaluated in, and received approval for pediatric osteosarcoma in Europe in 2009. Our results show a DFI of 615 days and MST of 956 days for dogs without pre-existing metastases at enrollment. In this phase I study, there was no placebo-controlled group; however, when compared with a matched historical control group, dogs that received ADXS31-164 showed a significant survival advantage

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Table 2. Summary of IFNγ ELISpot data obtained at baseline and at different time points following ADXS31-164

	Baseline			3 wks post V3			2 mth reck			4 mth reck			6–7 mth reck			8 or 10 mth reck			11–12 mth reck			16 or 18 mth reck			DFI	OS	
	EC1	EC2	IC1	EC1	EC2	IC1	EC1	EC2	IC1	EC1	EC2	IC1	EC1	EC2	IC1	EC1	EC2	IC1	EC1	EC2	IC1	EC1	EC2	IC1	(days)	(days)	
Group 1																											
Dog 1	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+++	$+^{V}$	$-^{V}$	$-^{V}$	++++	++++	++++	-	-	-	350^{B}	738	
Dog 2	-	+++	-	-	-	-	-	+++	+++		Deceased														185^{L}	267	
Dog 3	-	+++	-	-	-	-	+++	+++	++	++	+++	+++	$-^{V}$	$-^{V}$	$-^{V}$	+	++	+	$-^{V}$	$-^{V}$	$-^{V}$	$-^{V}$	$-^{V}$	$-^{V}$		1,229+	
Group 2																											
Dog 4	++	-	-	+++	+++	+++	-	-	-	-	-	-	+++	-	-	$-^{V}$	$-^{V}$	$-^{V}$	+	-	-	-	-	-		1,195+	
Dog 5	+++	++	-	+	-	-	++	++	++	-	-	-	$-^{V}$	$-^{V}$	$-^{V}$	-	-	-	-	+	+	++*	-*	+++*	869^{B}	956	
Dog 6	-	-	-	+	++	+		ND		ND	ND	ND		Deceased											318^{B}	346	
Gourp 3																											
Dog 7	-	-	-	-	-	-	-	-	-	-	-	+++	-	+++	++	$-^{V}$	$-^{V}$	$-^{V}$	-	-	-	$-^{15mth}$	$-^{15mth}$	$-^{15mth}$		996+	
Dog 8	-	-	-	-	-	-	-	-	-	$++++^{V}$	$++++^{V}$	$++++^{V}$	$+^{V}$	$-^{V}$	$-^{V}$		Deceased									322^{B}	444
Dog 9	++	-	-	+	-	-	+	-	-	-	+	+	-	-	+	$-^{V}$	$+++^{V}$	$+++^{V}$	+	-	-		NR			740+	
Dog 10	-	-	-	+	+++	+++	+	-	-	$-^{V}$	$-^{V}$	$-^{V}$	++	++	+++	$+^{V}$	$-^{V}$	$-^{V}$		NR						690+	
Dog 11	-	+	-	+	+	-	-	-	-	$-^{V}$	$+++^{V}$	$++++^{V}$		ND		-	-	+		NR						691+	
Dog 12	-	-	-	-	-	-	-	+	+	$-^{V}$	$-^{V}$	$-^{V}$	+++	+++	+	$-^{V}$	$-^{V}$	$+^{V}$		NR						682+	
Dog 13	-	-	-	+	-	+		Deceased																	251^{L}	276	
Dog 14		Failed QC		-	-	-	-	+	-	ND	ND	ND		Deceased											363^{L}	388	
Dog 15	-	-	-	-	+	-	+	+	+	-	-	++	+	+	+		ND			NR					315^{L}	564+	
Gourp 4																											
Dog 16	+++	++	++	-	+	+	ND	ND	ND		Deceased														226^{L}	259	
Dog 17	-	-	-	+	++	+		ND			ND			ND			ND			NR					190^{L}	597+	
Dog 18	-	-	-	-	-	-	-	-	-	-	-	-	$-^{V}$	$+^{V}$	$-^{V}$		NR									584+	

NOTE: − and + represent fold increase of spots above unstimulated control; - represents <2 fold increase; + represents 2-5 fold increase; ++ represents 5-10 fold increase; +++ represents 10-50 fold increase; ++++ represents >50 fold increase. In instances where there were no spots in the unstimulated control, the number 1 was assigned to allow for fold increase to be calculated. Shaded boxes indicated time of relapse. *, rising levels of ALKP, a biomarker of metastatic disease.

Abbreviations: B, bone; V, additional ADXS31-164 treatments; L, lung; ND, samples that have not yet been collected/evaluated for ELISpot and/or run at that timepoint; NR, sample time points have not yet been reached; reck, re-check evaluation.

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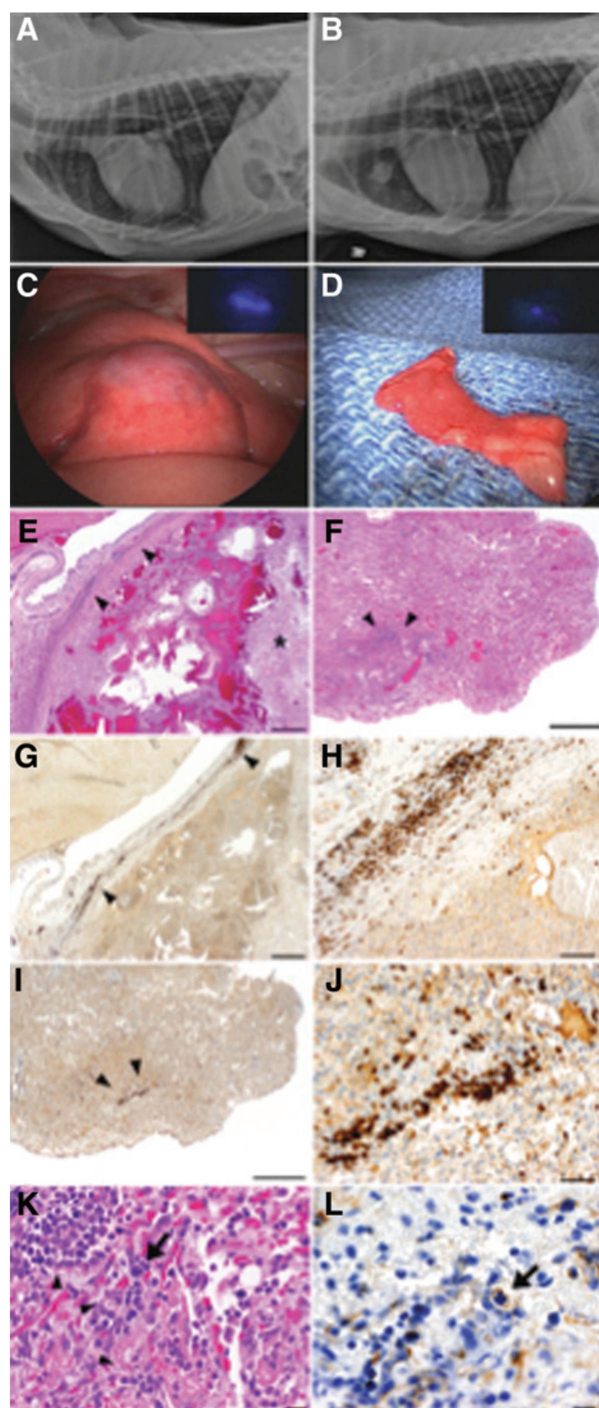

Figure 4.
Evidence of tumor-associated inflammatory T-cell infiltrates following ADXS31-164 treatment. **A** and **B,** thoracic radiographs taken 3 weeks after carboplatin and before ADXS31-164 show a single pulmonary nodule in the right cranial lung lobe (**A**) and 3 weeks after the third ADXS31-164 treatment (**B**) showing an increase in nodule size but no additional nodules. **C** and **D,** pulmonary nodule identified at thoracoscopy (**C**) that, following administration of ICG, fluoresces under near infra-red light (inset). Grossly normal pulmonary tissue removed at metastatectomy (**D**) showing fluorescence under near infra-red light (inset). **E** and **F,** histopathology shows the partially necrotic and hemorrhagic metastatic nodule (**E**) with neoplastic tissue (asterisk) separated from the pulmonary parenchyma by a thick fibrous capsule (arrowheads), and a focal basophilic inflammatory infiltrate (**F,** arrowheads) within the grossly normal pulmonary tissue that fluoresced under near infra-red light in **D** (scale bar = 500 μm). **G** and **H,** CD3 immunohistochemical stain of the same metastatic nodule (**G**: scale bar = 500 μm; **H**: scale bar = 100 μm) shows $CD3^+$ T cells concentrated in the fibrous capsule (**G,** arrowheads) but excluded from the inner neoplastic tissue (H, lower right). **I** and **J,** CD3 immunohistochemical stain of grossly normal lung tissue shown in D and F (**I,** scale bar = 500 μm; **J,** scale bar = 100 μm) shows one of several regions of focal aggregates of $CD3^+$ T cells (**I,** arrowheads; **J**). **K,** within the basophilic inflammatory focus in **F** are few pleomorphic spindle cells that are undergoing mitosis (arrow) surrounded by lymphocytes and few neutrophils (H&E, scale bar = 20 μm). **L,** vimentin IHC stain of the same focus shows lymphocytic infiltrates associated with pleomorphic vimentin-positive spindle cells that exhibit mitosis (arrow). Scale bar = 10 μm.

with 1-, 2-, and 3-year survival rates of of 77.8%, 67%, and 56% (vaccine group) versus 55%, 28%, and 22% (control group), respectively. Although ADXS31-164 dose did not appear to affect survival, favorable survival outcomes could be predicted from the magnitude of the inflammatory response 24 hours after ADXS31-164. Strong innate immunity induced by *Lm* contributes to its therapeutic potency by promoting cell-mediated immunity (24). Furthermore, high levels of IL12 induced by *Lm* early in infection (40) can upregulate FAS expression on metastatic osteosarcoma cells (41) leading to apoptosis when they encounter constitutively expressed FASL in the lung (42). These mechanisms may contribute to the reduced incidence of metastatic disease and prolonged overall survival observed following ADXS31-164 treatment.

Our results in dogs parallel those in mouse models and show that ADXS31-164 can safely break peripheral tolerance and induce HER2/neu-specific T-cell responses, without significant on-target, off-tumor side effects. We hypothesize that induction of HER2/neu-specific T cells is responsible for elimination of $HER2/neu^+$ metastatic cells and long-term protection from relapse. Consistent with this hypothesis, recent data suggest that, as in mammary carcinoma, HER2/neu is expressed on osteosarcoma tumor-initiating cells (TIC) and that HER2 targeting may effectively prevent metastatic disease (12, 14, 15, 43). In our study, analysis of HER2/neu-specific IFNγ responses in the peripheral blood revealed 'early responders,' identified within 3 weeks of the last vaccination and 'late responders,' who were identified 2 to 6 months later. There was no evidence of a dose-dependent effect of ADXS31-164 on induction or magnitude of HER2/neu-specific immunity, although dogs that did develop IC1-specific IFNγ responses without clinical relapse before or at their sixth month recheck tended to have prolonged overall survival. Several dogs that did not generate immune responses immediately post treatment, did so at the time of clinical relapse raising the possibility that resurgence of $HER2/neu^+$ tumor cells leads to expansion of HER2/neu-specific T cells. However, failure to identify IC1-specific IFNγ responses in 6 of 8 dogs with metastatic disease at baseline (screen failures) suggests that relapse alone is insufficient to break tolerance to HER2/neu and that ADXS31-164 priming is required for HER2/neu-specific T-cell expansion in response to relapse. Although the design of this study does not allow us to determine whether the survival benefit afforded by ADXS31-164 is HER2/neu dependent, controlled experiments using this approach in mice reveal that the antitumor effects and prolonged survival are HER2/neu dependent and not merely associated with a Coley's toxin effect (17). A randomized

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Listeria only controlled study will need to be performed to confirm a HER2/neu-dependent mechanism of action in the dog.

We hypothesize that the delayed HER2/neu-specific T-cell responses seen in our long-term survivors were associated with memory T-cell expansion, following ADXS31-164 priming, in response to subclinical disease and that these responses effectively prevent metastatic disease. Our hypothesis is further supported by the timing of HER2/neu-specific T-cell expansion, which in 5 dogs occurred approximately 8 months post diagnosis, when many dogs will develop metastatic disease and by the findings of focal pulmonary T-cell infiltrates in one dog following ADXS31-164 and metastatectomy. Previous murine studies using this recombinant *Lm* technology have found that T-cell responses induced against the IC1 domain of HER2/neu provide the longest overall survival times when compared with responses against the extracellular domains (22). This is likely associated with the resistance of the IC1 domain to accumulate mutations and escape the immune response, given its importance in intracellular signaling and sustained oncogenesis.

On-target, off-tumor side effects including cardiotoxicity have been associated with the administration of large numbers of HER2/neu-specific T cells (28) or the combination of HER2/neu targeting antibodies such as trastuzumab with anthracyclines (29). Here, we show that administration of up to 3.3×10^9 CFU ADXS31-164 is safe and despite inducing HER2/neu-specific immunity, does not lead to immediate or delayed cardiotoxicity. The MTD was not reached in this study and further escalation was not pursued as HER2/neu-specific immunologic responses were induced independently of dose. Adverse events occurred only at the time of ADXS31-164 administration and were mild and transient. Comparable hematologic changes and adverse events were reported in dogs with metastatic pulmonary osteosarcoma receiving liposome DNA complexes encoding IL2 (44). Furthermore, a similar adverse event profile, including mild elevations in hepatic enzymes, was reported following administration of an HPV targeting recombinant *Lm* to human patients with late-stage cervical cancer (45), emphasizing the predictive value of the canine model in preclinical safety studies.

To conclude, our study suggests that ADXS31-164 immune therapy prevents pulmonary metastatic disease and prolongs survival in dogs with HER2$^+$ appendicular osteosarcoma through its ability to (i) induce potent innate inflammatory responses that may sensitize metastatic osteosarcoma cells to FAS-mediated apoptosis and (ii) prime HER2/neu-specific T-cell responses that prevent or eliminate micrometastatic pulmonary disease. Furthermore, it is possible that as with human immunotherapy, antigen-specific T cells may be excluded from the metastatic tumor microenvironment. This further emphasizes the relevance of the dog with spontaneous metastatic disease for evaluating combination therapies that enable T-cell penetration, survival, and effector function within the tumor microenvironment. Prolonged overall survival in response to LE-MTPs in canine osteosarcoma paved the way for approval of MEPACT, exemplifying the value of the canine model in identifying effective therapies for pediatric osteosarcoma (3). Our results demonstrate safety and improved survival times in dogs with osteosarcoma and likely hold significant translational relevance for the treatment of pediatric osteosarcoma and other HER2/neu$^+$ cancers in humans.

Disclosure of Potential Conflicts of Interest

N.J. Mason reports receiving speakers bureau honoraria and commercial research grants from, and is an employee of and consultant/advisory board member for Aratana Therapeutics, and has ownership interest (including patents) in Advaxis Inc. Y. Paterson has ownership interest (including patents) in and is a consultant/advisory board member for Advaxis Inc. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.J. Mason, J.S. Gnanandarajah, J.B. Engiles, D. Laughlin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.J. Mason, J.S. Gnanandarajah, F. Gray, A. Gaurnier-Hausser, A. Wallecha, M. Huebner, Y. Paterson
Writing, review, and/or revision of the manuscript: N.J. Mason, J.S. Gnanandarajah, Y. Paterson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.J. Mason, J.S. Gnanandarajah, J.B. Engiles
Study supervision: N.J. Mason, J.B. Engiles, Y. Paterson
Other (lead pathologist on this study and contributed to the pathology grading scheme, acquisition and organization of data [pathology scoring and image acquisition] writing and supervision of the junior pathologist and medical residents involved in pathology): J.B. Engiles
Other (including histochemical and immunohistochemical analysis and grading of primary and metastatic lesions in study and control groups): F. Gray

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