A recombinant HER2/Neu expressing *Listeria monocytogenes* (*Lm*-LLO) immunotherapy delays metastatic disease and prolongs overall survival in a spontaneous canine model of osteosarcoma

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Abstract

Osteosarcoma (OSA) is an aggressive mesenchymal bone tumor that affects approximately 3000 children annually in the USA. Treatment consists of neoadjuvant chemotherapy, radiotherapy and radical surgery. Despite treatment, metastatic disease is common and results in 30-40% mortality within 5 years of diagnosis. Novel therapies that prevent metastatic disease are required to improve outcome. HER2/Neu is a tyrosine kinase receptor belonging to the EGFR family. It is expressed in ~40% of pediatric OSA and is linked to reduced response to neoadjuvant chemotherapy, high metastatic rates and short overall survival time. Recent reports indicate that HER2/Neu is expressed on OSA tumor initiating cells and that immune targeting of HER2/Neu delays metastatic disease.

Large breed dogs spontaneously develop OSA that recapitulates many aspects of pediatric OSA including histologic heterogeneity, aggressive local disease and early metastases. At diagnosis, 95% of dogs have micrometastatic disease and despite amputation and chemotherapy, the median survival time is 10 months with most dogs euthanized due to progressive metastatic disease. As in pediatric OSA, HER2/Neu is expressed in ~40% of canine appendicular OSA making pet dogs a relevant model to evaluate the effects of HER2/Neu targeted immune therapy on metastatic disease prevention.

We performed a Phase I clinical trial to evaluate the safety and efficacy of an attenuated, recombinant *Listeria monocytogenes* (*Lm*) expressing a chimeric human HER2/Neu fusion protein (ADXS31-164) to prevent metastatic disease in dogs with HER2/Neu+ appendicular OSA. *Lm* secretes a pore-forming lysin, listeriolysin O (LLO) that enables it to escape the phagosome and access the class I processing machinery of antigen-presenting cells. As such, recombinant *Listeria*, engineered to express tumor antigens fused to LLO, induce potent tumor-specific CD8 T cell responses that mediate tumor regression in murine models. Eighteen dogs with HER2/Neu+ OSA that had undergone amputation and carboplatin chemotherapy received 1 x 10^8, 5 x 10^8, 1 x 10^9 or 3 x 10^9 CFU of ADXS31-164 intravenously every 3 weeks for three administrations. ADXS31-164-associated toxicities were low grade and transient. ADXS31-164 administration broke peripheral tolerance to the highly conserved IC1 domain of HER2/Neu. At the time of writing, 12/18 dogs have not developed pulmonary metastatic disease. Vaccinated dogs showed a statistically significant increase in overall survival compared to a historical HER2/Neu+ control group (median survival in HER2/Neu+ control dogs (n=11) was 316 days (p=0.032); median survival in ADXS31-164 treated dogs has not been reached). Our results indicate that ADXS31-164 breaks peripheral tolerance to HER2/Neu and significantly delays metastatic disease in a clinically relevant, spontaneous model. This work has important implications for pediatric OSA and other human cancers that express HER2/Neu.

Figure 1. Radiographic images of primary and metastatic osteosarcoma in a human (left panels) and canine (right panels) patient. In both species, primary lesions are characterized by areas of marked proliferation and lysis in the bone metaphysis. Pulmonary metastatic disease is the principal cause of morbidity and mortality in both species.

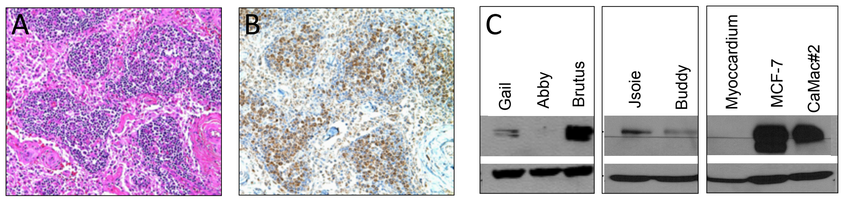

Figure 2. HER2/Neu expression in canine primary osteosarcoma. A. H&E stain of primary OSA from a dog showing nests of malignant osteoblasts and osteoid deposition. **B** Immunohistochemical evaluation of canine primary OSA showing HER2/Neu expression within malignant osteoblasts. **C.** Western blot of primary OSA samples from 5 privately owned dogs showing variable expression of HER2/Neu. MCF-7; human mammary carcinoma, CAMAC2; canine mammary carcinoma (positive controls)

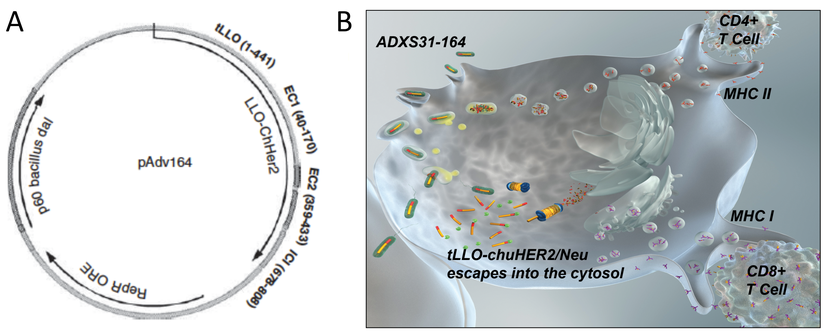

Figure 3. ADXS31-164 recombinant HER2/Neu plasmid and cartoon of exploitation of listeriolysin O (LLO) to deliver chimeric HER2/Neu into the cytosol and MHC I processing pathway. A. pAdv164 expressing a chimeric HER2/Neu fusion protein consisting of 2 extracellular domains and one intracellular domain of human HER2/Neu fused to a truncated LLO. The plasmid is maintained within the recombinant dal/dat$^-$ Act-A$^-$ listeria strain (*LmddA*) by means of auxotrophic complementation of the dal gene. **B.** Through the action of LLO, ADXS31-164 escapes from the phagosome before fusion with the lysosome. tLLO-huHER2/Neu is secreted into the cytosol and gains access to the MHC I processing pathway.

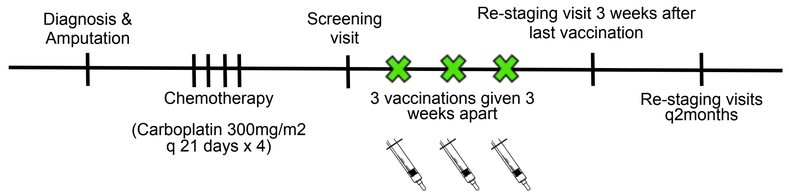

Figure 4. Schematic of this phase I, 3+3 clinical trial to evaluate the safety and efficacy of ADXS31-164 in dogs with HER2$^+$ osteosarcoma. Privately owned dogs with spontaneous, HER2+ appendicular OSA underwent standard of care amputation and follow up carboplatin chemotherapy. Three weeks after the last carboplatin dose, dogs were vaccinated with either 2x10^8, 5x10^8, 1x10^9 or 3x10^9 CFU of ADXS31-164 intravenously. Dogs were re-staged every 2 months until death to determine vaccine efficacy in preventing metastatic disease.

AGE	BREED	SEX	TUMOR LOCATION	SUBTYPE	GRADE	HER2 SCORE	DOSE	CHEMOTHERAPY	OVERALL SURVIVAL (days)
Group 1									
12.5	American Pit Bull	FS	Proximal humerus	Osteoblastic	II	2	2 x 10^8	Carboplatin x 4	738
11.5	Mixbreed	FS	Distal radius	Osteoblastic	I	5	2 x 10^8	Carboplatin x 4	267
9	Labrador	MC	Proximal humerus	Fibroblastic	II	7.5	2 x 10^8	Carboplatin x 4	911+
Group 2									
6	Mixbreed	FS	Distal tibia	Osteoblastic	I	4.5	5 x 10^8	Carboplatin x 4	880+
7	Rottweiler	MC	Distal ulnar	Osteoblastic	III	2.25	5 x 10^8	Carboplatin x 4	859+
4.5	English Bulldog	MC	Proximal humerus	Osteoblastic	II	4	5 x 10^8	Carboplatin x 4	346
Group 3									
6	OES	MC	Distal femur	Osteoblastic	II	1.5	1 x 10^9	Carboplatin x 4	678+
9	Greyhound	MC	Proximal humerus	Osteoblastic	II	5	1 x 10^9	Carboplatin x 4	444
8	Golden Retriever	MC	Distal ulnar	Fibroblastic	I	3	1 x 10^9	Carboplatin x 4	422+
2	Labrador	FS	Proximal tibia	Fibroblastic	I	4.5	1 x 10^9	Carboplatin x 4	372+
7.5	Cavalier King Charles	FS	Proximal tibia	Osteoblastic	II	7.5	1 x 10^9	Carboplatin x 4	373+
6.5	Golden Retriever	FS	Distal radius	Osteoblastic	I	4.5	1 x 10^9	Carboplatin x 4	364+
10	Greyhound	MC	Distal femur	Osteoblastic	II	2	1 x 10^9	Carboplatin x 4	276
5.5	Labrador	MC	Distal femur	Osteoblastic	I	9	1 x 10^9	Carboplatin x 4	279+
9	Golden Retriever	FS	Distal femur	Osteoblastic	I	6	1 x 10^9	Carboplatin x 4	246+
Group 4									
6.6	Great Dane	MC	Distal radius	Osteoblastic	II	7.5	3 x 10^9	Carboplatin x 4	259
7	Mixbreed	MC	Proximal humerus	Osteoblastic	II	9	3 x 10^9	Carboplatin x 4	270+
6.5	Rottweiler	FS	Proximal humerus	Osteoblastic	II	6	3 x 10^9	Carboplatin x 4	266+

Table 1. Signalment of trial dogs, OSA location, subtype and grade, HER2 expression score (by IHC) and overall survival times. For HER2 score, 10 hpf were evaluated for stain intensity (mild=1, moderate = 2, severe =3) and distribution (<10% osteoblasts = 1, 10-50% = 2 and >50% = 3). The product of intensity and distribution scores was recorded as the overall score. Carboplatin was administered i.v. at 300mg/m^2 once q21 days for 4 doses. "+" indicates dogs that are still alive.

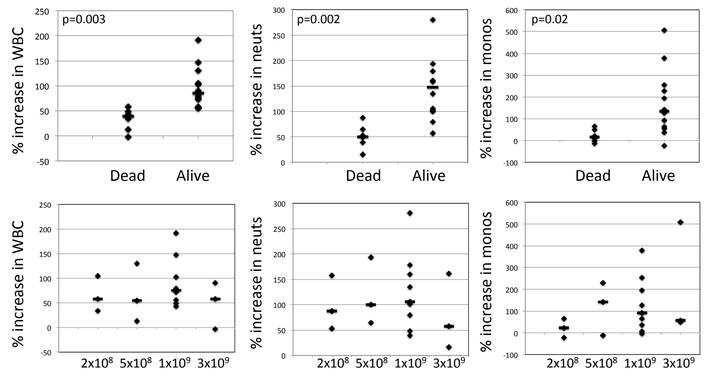

Figure 5. ADXS31-164 induced Increases in WBC, neutrophil and monocyte counts correlate with survival. WBC, neutrophil and monocyte counts were measured at baseline and 24 hours after vaccination. The percent increase was calculated following each vaccination and averaged for each dog**. Upper panel:** Results displayed according to survival. **Lower panel:** Results displayed according to ADXS31-164 dose. Horizontal bars represent median values of the group.

Number of dogs with Treatment Related Adverse Events						
ADXS31-164 dose		2x10^8	5x10^8	1x10^9	3x10^9	Total
Number of dogs recruited		3	3	9	3	18
General Disorders						
Pyrexia (T>103)		2	1	5	2	10
Fatigue		1	0	7	2	10
Nausea	Grade 1	1	2	9	2	14
	Grade 2	1				1
Vomiting	Grade 1	1	2	8	2	13
	Grade 2	2				2
Cardiovascular abnormalities						
Arrhythmias		0	1	1	1	3
Tachycardia		0	0	1	1	2
Hypotension		0	0	0	0	0
Hematological parameters						
Thrombocytopenia	Grade 1	2	2	5	1	10
	Grade 2			1	1	2
Biochemical parameters (increased)						
γ-GT		0	2	0	0	2
ALKP	Grade 1	0	1	4	1	6
	Grade 3	1				1
ALT	Grade 1	1	1	1	0	3
	Grade 2				1	1
	Grade 3	1				1
AST	Grade 1	1	1	4	1	7
	Grade 2			1		1
BUN		0	0	0	0	0
CREA		0	0	0	0	0
Cardiac Troponin I		0	0	1	0	1

Table 2. Treatment related adverse events. All reported clinical events were transient and occurred on the day of ADXS31-164 infusion. Increases in biochemical values occurred within 48 hours of vaccination and were low grade. Biochemical abnormalities resolved within one week following vaccination.

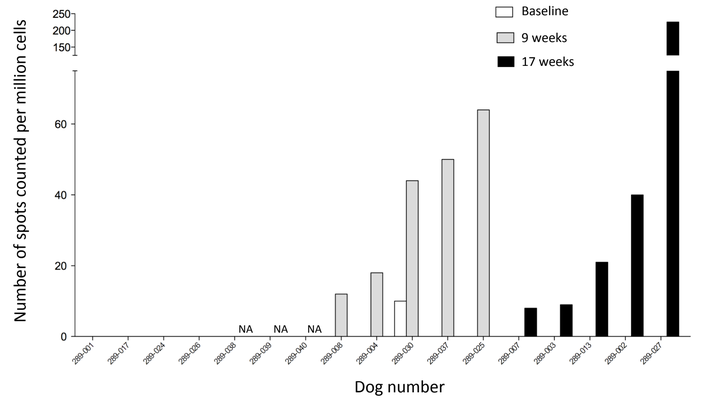

Figure 6. ADXS31-164 breaks tolerance to HER2/Neu. PBMCs were collected at baseline, 3 weeks after the 3rd vaccine (9 weeks) and 2 months later (17 weeks) and analyzed by IFN-γ ELISpot for responses to the highly conserved IC1 domain of HER2/Neu. Results divided dogs into early responders, late responders and apparent non-responders. NA indicates that the 17 week sample for these dogs has not yet been drawn.

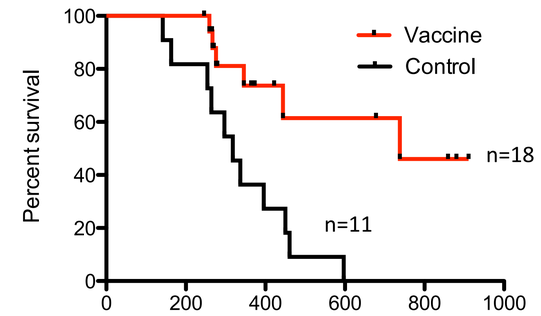

Figure 7. ADXS31-164 delays/prevents metastatic disease and prolongs overall survival in dogs with spontaneous HER2+ osteosarcoma. Kaplan-Meier survival curve of vaccinated dogs compared with a historical control group. The control group consisted of dogs with HER2+ appendicular OSA, treated with amputation and follow up chemotherapy but who did not receive ADXS31-164. p=0.002

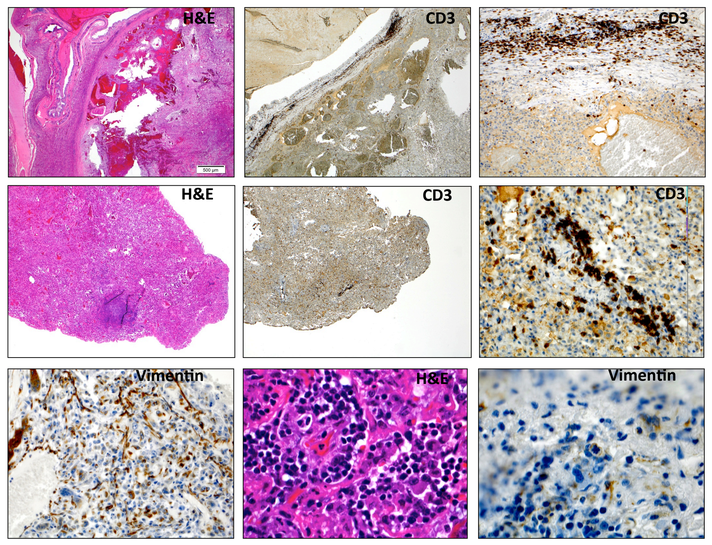

Figure 8. Histopathology and IHC of a metastatic pulmonary nodule following treatment with ADXS31-164. One week after receiving the third ADXS31-164 vaccine, a dog with pre-existing metastatic disease underwent metastatectomy. **Upper panel:** A marked CD3+ T cell infiltrate is present surrounding the fibrous capsule of the metastatic lesion. **Middle panel:** Separate focal areas of CD3+ T cell infiltrate occur within other parts of the lung, without apparent cause. **Lower panel:** Vimentin staining of pulmonary nodule (left panel) and area of focal pneumonia (middle and right panels). Occasional large, vimentin positive cells with mitotic figures are seen, surrounded by the lymphocytic infiltrate.

Summary and Conclusions

1. ADXS31-164 prevents metastatic disease and prolongs overall survival in dogs with spontaneous HER2+ OSA when administered in the setting of minimal residual disease
2. ADXS31-164 breaks peripheral tolerance to the highly conserved IC1 domain of HER2/Neu
3. The magnitude of increase in leucocytes within 24 hours of ADXS31-164 administration correlates with survival, suggesting that outcome depends in part upon the ability of the dog's immune system to respond to the vaccine
4. Administration of up to 3 x 10^9 CFU ADXS31-164 to dogs with spontaneous OSA is safe and causes only transient, low grade side effects at the time of administration
5. Prevention of pulmonary metastatic disease maybe in part associated with CD3$^+$ T cell mediated elimination of microscopic metastatic disease in the lung