

Inhaled recombinant human IL-15 in dogs with naturally occurring pulmonary metastases from osteosarcoma or melanoma: a phase 1 study of clinical activity and correlates of response

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ABSTRACT

Purpose Although recombinant human interleukin-15 (rhIL-15) has generated much excitement as an immunotherapeutic agent for cancer, activity in human clinical trials has been modest to date, in part due to the risks of toxicity with significant dose escalation. Since pulmonary metastases are a major site of distant failure in human and dog cancers, we sought to investigate inhaled rhIL-15 in dogs with naturally occurring lung metastases from osteosarcoma (OSA) or melanoma. We hypothesized a favorable benefit/risk profile given the concentrated delivery to the lungs with decreased systemic exposure.

Experimental design We performed a phase I trial of inhaled rhIL-15 in dogs with gross pulmonary metastases using a traditional 3+3 cohort design. A starting dose of 10 µg twice daily × 14 days was used based on human, non-human primate, and murine studies. Safety, dose-limiting toxicities (DLT), and maximum tolerated dose (MTD) were the primary objectives, while response rates, progression-free and overall survival (OS), and pharmacokinetic and immune correlative analyses were secondary.

Results From October 2018 to December 2020, we enrolled 21 dogs with 18 dogs reaching the 28-day response assessment to be evaluable. At dose level 5 (70 µg), we observed two DLTs, thereby establishing 50 µg twice daily × 14 days as the MTD and recommended phase 2 dose. Among 18 evaluable dogs, we observed one complete response >1 year, one partial response with resolution of multiple target lesions, and five stable disease for an overall clinical benefit rate of 39%. Plasma rhIL-15 quantitation revealed detectable and sustained rhIL-15 concentrations between 1-hour and 6 hour postnebulization. Decreased pretreatment lymphocyte counts were significantly associated with clinical benefit. Cytotoxicity assays of banked peripheral blood mononuclear cells revealed significant increases in peak cytotoxicity against canine melanoma and OSA targets that correlated with OS.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Although strategies to activate T cells are the focal point of the burgeoning immuno-oncology field, there is a growing appreciation that other immune cells, such as natural killer (NK) cells, play important roles in tumor surveillance and elimination and therefore represent attractive candidates for expanding the promise of immunotherapy. Dogs are outbred companion animals that develop spontaneous cancers in the setting of an intact immune system, allowing for the study of complex immune interactions during cancer therapies while also addressing endpoints of efficacy and toxicity.

WHAT THIS STUDY ADDS

⇒ This phase I clinical trial in dogs with gross pulmonary metastatic disease from osteosarcoma and melanoma builds on prior work in human and canine immunotherapy trials using immunostimulatory cytokine therapy to demonstrate that inhaled recombinant human interleukin-15, the prototypical NK stimulatory cytokine, induces encouraging antitumor responses using a novel, first-in-dog approach with evidence for clinical and immunologic activity.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY

⇒ These interesting findings are expected to have relevance for the design and translation of ongoing innovative immunotherapy approaches in both dogs and people.

Conclusions In this first-in-dog clinical trial of inhaled rhIL-15 in dogs with advanced metastatic disease, we observed promising clinical activity when administered as a monotherapy for only 14 days. These data have

significant clinical and biological implications for both dogs and humans with refractory lung metastases and support exploration of combinatorial therapies using inhaled rhIL-15.

BACKGROUND

Cytokine immunotherapies have been a key area of research focus in cancer immunotherapy given their ability to stimulate powerful immune responses.^{1,2} Interleukin-15 (IL-15) in particular has shown promise as an antitumor immunotherapeutic and has been used to stimulate endogenous cytotoxic natural killer (NK) and T cell responses against advanced cancers.^{3–9} However, while durable responses have been achieved in some patients, clinical trials of IL-15 monotherapy for patients with advanced cancer have frequently resulted in stable disease as the best response, and many of these trials were limited by systemic toxicity.^{10,11}

Distinct from direct intratumoral therapy, inhaled cytokine therapy offers the advantages of regional delivery of immunotherapy to the lungs, a frequent site of gross metastatic disease, while limiting systemic exposure and potential toxicity. In addition, the lungs are known to harbor a unique immune microenvironment, likely secondary to frequent pathogen exposure, and significant tissue-resident NK and CD8 +T cell immune populations have been identified in the lungs with both a memory-like and heightened cytotoxic phenotype.¹²

Cytokine immunotherapy using the inhalation route of delivery has previously been administered and demonstrated to be safe using IL-2 therapy for both dogs and humans with pulmonary metastases.^{13–15} Inhaled IL-2 has also been used in human cancer trials with response rates of approximately 15%.^{14,16,17} Yet, interest in this approach did not materialize in part because of concerns regarding the immunosuppressive effects of IL-2, including upregulation of regulatory T cells (Tregs) and induction of activation-induced cell death in cytotoxic T cells.³ Given that there are critical differences in the mechanisms of action of IL-15 and IL-2, we sought to evaluate the safety and potential clinical efficacy of inhaled IL-15 as a phase I, proof-of-concept trial in dogs with pulmonary metastases from melanoma and osteosarcoma (OSA).

Dogs are companion animals that develop spontaneous cancers in the setting of an intact immune system, which allows for the study of complex immune interactions during cancer therapies while also addressing efficacy and toxicity endpoints. Melanoma and OSA are common canine malignancies, estimated to occur in approximately 20,000–100,000 US dogs per year.^{18,19} Additionally, perhaps more so than humans, melanoma and OSA are overwhelmingly lethal diseases in dogs^{18,20,21} with median survival times of less than 1 year. Although melanoma and OSA are associated with distinct immune tumor microenvironments (TMEs) and differential response to immunotherapy in humans, there are few, if any, effective immunotherapy options in dogs. Therefore, dogs with

these cancers are ideal candidates for novel immunotherapy approaches.

Here, we report the results of a first-in-dog phase I clinical trial of inhaled recombinant human (rh) IL-15 in dogs with gross pulmonary metastases from melanoma or OSA. We observed 50 µg inhaled twice daily × 14 days to be the recommended phase 2 dose with two dose limiting toxicities (DLTs) observed in the 70 µg cohort. Among 18 evaluable patients, we observed an objective response rate of 11%, including one complete response (CR) that lasted >1 year and a clinical benefit rate of 39%. Immune correlative assays demonstrated systemic effects of therapy with evidence for delineation of responders and non-responders based on baseline absolute lymphocyte count (ALC) and correlation of ex vivo peripheral blood cytotoxicity assays with survival. Taken together, these data support ongoing evaluation of inhaled IL-15 as a strategy to improve immunotherapy outcomes in both dogs and people with gross pulmonary metastatic disease.

METHODS

Patients

Client-owned pet dogs with metastatic melanoma or OSA were enrolled in this phase I study. Eligible dogs were required to be greater than 1 year old, weigh at least 8 kg, have a VCOG-CTCAE 1.2 performance score of 0 or 1, and have one or more lung lesions consistent with metastatic disease measuring at least 1 cm on thoracic radiographs. The clinical protocol for this trial is included in the online supplemental material. Dogs were also deemed able to be successfully trained to accept nebulization with owners willing to administer inhaled rhIL-15 twice daily. All owners provided signed informed owner-consent.

We also performed two separate pilot, first-in-dog clinical trials to: (1) assess the feasibility of palliative radiation therapy (RT), allogeneic natural killer (NK) cell transfer, and subcutaneous (SQ) rhIL-15 (3 µg/kg) in dogs with locally advanced, non-metastatic melanoma and (2) the safety and preliminary assessment of response of ALT-803 in dogs with metastatic cancer.²² The clinical protocol for these trials are also included in the online supplemental material.

Study design

For the inhaled rhIL-15 trial, the primary objective was to determine the maximum tolerated dose (MTD) using a traditional 3+3 cohort design with a dose-escalation schedule.²³ Escalation was based on assessment of DLTs, defined as any grade 3 non-hematologic or grade 4 hematologic toxicity as defined by the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events (VCOG-CTCAE V.2).²⁴ The starting dose of rhIL-15 was 10 µg. Dose escalation followed a fixed dose modified Fibonacci method, where the dose is escalated 100%, 67%, 50%, 40% and then 33% of the previous cohort dose.

Study treatment

The primary investigational agent used in this trial was *Escherichia coli* derived rhIL-15 manufactured by the Biopharmaceutical Development Program of the Division of Cancer Treatment and Diagnosis/National Cancer Institute using current Good Manufacturing Practices. Patients were treated with inhaled rhIL-15 twice daily for 14 days at dose levels of 10, 20, 33, 50 or 70 µg (figure 1A). The first treatment was performed at the UC Davis VMTH to ensure that the patient and the owners were properly trained and tolerated the therapy. The nebulizer was attached to a fitted veterinary anesthesia cone, and depending on patient comfort and tolerability, the cone was placed directly over the patient muzzle (online supplemental figure 1) or placed within a plastic boot cover that was placed over the muzzle during delivery. Owners were instructed to perform treatments a minimum of 8 hours apart, and owners were requested to complete a treatment log. ALT-803, also known as N-803, was a generous gift from Altor Bioscience, now merged with NantCell/ImmunityBio. Dogs received once weekly ALT-803 for a total of four treatments.

Clinical and investigational assessments

Clinical assessments and vital signs were performed prior to the first treatment and then 1 hour, 3 hours, and 5 hours after the first treatment was completed. Blood work was performed at baseline and then at day 7, day 14, and day 28 of study. Plasma rhIL-15 concentrations were measured in a subset of patients analyzing samples collected prior to the first treatment, 5 min after starting treatment, at completion of the first inhaled treatment, and at 1, 4, and 6 hours post-treatment. Response was assessed based on the Response Evaluation Criteria for Solid Tumors in Dogs (RECIST V.1.0) by a board-certified veterinary radiologist (EGJ).²⁵ Due to the frequent rapid progression of metastatic OSA and melanoma in canine patients, 10 mm lesions were considered acceptable for enrollment rather than 20 mm. To be considered stable disease (SD), patients had to have a minimum of 28 days between imaging to meet criteria for response assessment of between <20% increase and <30% decrease in longest tumor diameter. Three-view thoracic radiographs were performed within 1 month prior to beginning therapy and were repeated on days 28, 42, 70, and 98 after initiation of inhaled rhIL-15. Patients could be restaged early if clinically indicated, and patients surviving beyond day 98 were offered imaging every 28 days.

Flow cytometry and RNA sequencing

Canine whole blood was collected at indicated time points. Plasma EDTA and whole-blood serum samples were kept on ice and stored at -80°C within 30 min of collection. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density gradient centrifugation (Lymphocyte Separation Medium, Corning Life Sciences), followed by red blood cell lysis as described previously.²⁶ Staining for flow cytometry was performed

as described previously using the following antibodies: CD45-EF450 (clone YKIX716.13, Invitrogen # 48-5450-42), CD3-FITC (clone CA17.2A12, BioRad #MCA1774F), NKp46-PE (clone 48A, kind gift of Dr. Dean Lee), and CD8-APC (clone YCATE55.9, Invitrogen #17-5080-42).⁹ Live/dead staining performed using Fixable Viability Dye 780. Cells were processed for RNA isolation using the Total RNA Purification Plus Micro Kit (Norgen Biotek, Ontario, Canada). RNAseq libraries were prepared by the University of California, Davis, Bioinformatics Core. RNA sequencing data will be made available on NCBI GEO.

Human IL-15 ELISA

rhIL-15 plasma concentrations were measured using the Human IL-15 Uncoated ELISA kit (Invitrogen #88-760) according to the manufacturer's instructions, with some modifications. In place of the kit standards, ELISA standards were generated using serial dilutions of the clinical stock rhIL-15 and ranged from 15.625 to 1000 pg/mL. Undiluted plasma (100 µL) was used per well, except for the samples from trial #21 620 (RT, allogeneic NK cells, and SQ rhIL-15), which were diluted 1:10. All samples were run in duplicate and repeated twice.

Cytotoxicity assays

Canine OSA (OSCA-78) and melanoma (M5) tumor cell lines were obtained from established immortalized stocks.²² Cytotoxicity was assessed by labeling target cells with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen #C34554) for 5 min at room temperature prior to coculturing with thawed patient PBMCs. After 18 hours of overnight effector/target coculture, cells were stained with Fixable Viability Dye 780 and analyzed by flow cytometry. Per cent cytotoxicity was calculated according to the following formula: $[\text{CFSE}^+ \text{FVD780}^+ / (\text{CFSE}^+ \text{FVD780}^+ + (\text{CFSE}^+ \text{FVD780}^-))] \times 100$.⁹ Adjusted cytotoxicity was determined by subtracting spontaneous killing of target cells when no effectors were added (0 E:T).

Multiplex analysis of cytokines

Multiplex analysis was performed using the Luminex 200 system (Luminex, Austin, Texas, USA) by Eve Technologies Corp (Calgary, Alberta). Thirteen markers were measured in the samples using the Canine Cytokine 13-Plex Discovery Assay (MilliporeSigma, Burlington, Massachusetts, USA) according to the manufacturer's protocol. The 13-plex panel consisted of GM-CSF, IFNγ, IL-2, IL-6, IL-7, IL-8/CXCL8, IL-10, IL-15, IL-18, IP-10/CXCL10, KC-like, MCP-1/CCL2, and TNFα. Assay sensitivities of these markers range from 3.2 to 21.0 pg/mL.

Statistical analysis

We used Excel (Microsoft), Prism software (GraphPad Software Inc), and SAS Enterprise Guide V.7.15 (Cary, North Carolina, USA) for graph generation and statistical analysis. Data are expressed as mean±SEM. Where appropriate, normality of distribution was confirmed using Shapiro-Wilk normality test. Differences between two groups were analyzed using the paired or unpaired

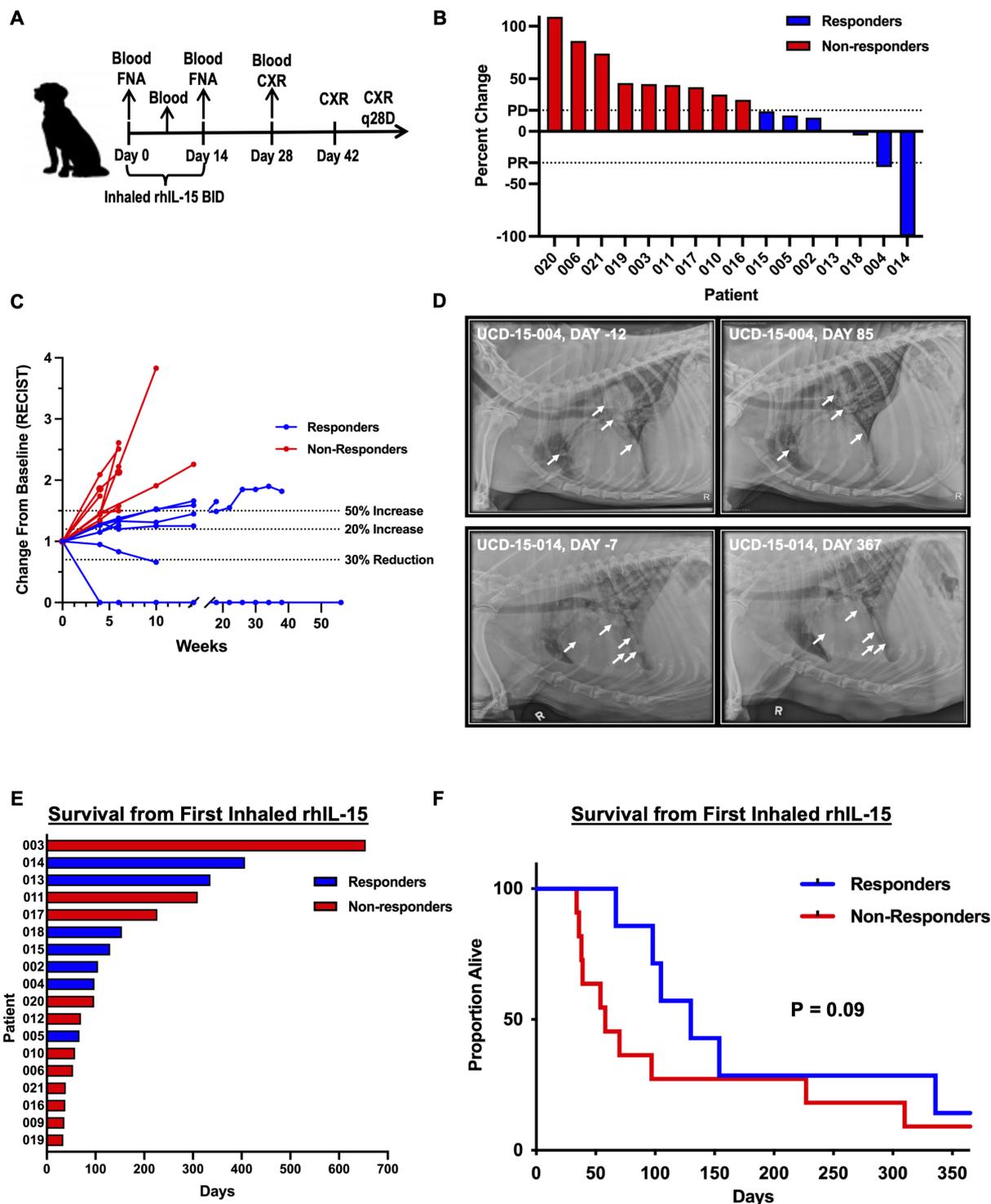


Table 1 Patient demographics and response

Patient ID	Dose (µg)	Weight (kg)	Age (years)	Sex	Disease	Breed	Best response	Response duration (days)	Survival from first IL-15 (days)
IL-15-001	10	32.2	7.6	MC	OSA	Golden retriever	NE		37
IL-15-002	10	49.0	11.1	MC	OSA	Golden retriever	SD	55	105
IL-15-003	10	47.6	8.6	FS	OSA	Labrador mix	PD		655
IL-15-004	10	28.3	11.0	FS	OSA	Golden retriever	PR	85	98
IL-15-005	10	23.4	12.3	MC	OSA	Shepherd mix	SD	46	67
IL-15-006	10	21.2	11.3	FS	OSA	Shepherd mix	PD		54
IL-15-007	20	29.5	7.5	MC	MEL	Boxer	NE		14
IL-15-008	20	21.3	13.7	FS	OSA	Greyhound	NE		21
IL-15-009	20	34.2	12.8	FS	OSA	German Shorthaired Pointer	PD		36
IL-15-010	33	25.2	10.8	FS	MEL	Golden retriever	PD		58
IL-15-011	33	27.9	12.3	MC	OSA	Border collie mix	PD		310
IL-15-012	33	26.6	9.7	MI	MEL	Irish water spaniel	PD		70
IL-15-013	50	35.4	11.1	MC	MEL	Pit bull mix	SD	147	336
IL-15-014	50	39.3	12.6	FS	MEL	Labrador	CR	350	407
IL-15-015	50	33.3	11.6	FS	MEL	Mix	SD	56	130
IL-15-016	70	46.3	8.5	MC	MEL	Mix	PD		38
IL-15-017	70	34.7	9.7	MC	MEL	Doberman	PD		227
IL-15-018	70	21.5	12.7	FS	MEL	Terrier mix	SD	42	154
IL-15-019	70	8.5	13.8	MC	MEL	Mix	PD		34
IL-15-020	70	34.5	12.7	MC	MEL	Labrador	PD		97
IL-15-021	70	24.5	3.0	MI	OSA	Belgian sheepdog	PD		39

CR, complete response; FS, female spayed; IL-15, interleukin-15; MC, male castrated; MEL, melanoma; MI, male intact; NE, not evaluable; OSA, osteosarcoma; PD, progressive disease; PR, partial response; SD, stable disease.

Student's t-test as appropriate for parametric data and the Mann-Whitney test or Wilcoxon signed-rank test for non-normally distributed data. For analysis of three or more groups, one-way analysis of variance tests were performed with Tukey's or Dunnett's post hoc test as appropriate. To analyze differences in fold change over time, we used a mixed effects model with a random intercept for the subjects and fixed effects for responders (Y or N). Correlations between two values were performed using Spearman correlation test. Kaplan-Meier curves and log-rank test were used to compare survival outcomes between subgroups. $P < 0.05$ was considered statistically significant unless an adjusted p value was indicated.

RESULTS

Patients and treatment

For the index trial of inhaled rhIL-15, a total of 21 dogs were enrolled including 11 dogs with melanoma and 10 dogs with OSA. Patient characteristics are summarized in [table 1](#). Twenty dogs successfully completed the 14-day course of inhaled rhIL-15. One dog was euthanized after presenting with bicavitary effusion on day 14. A

postmortem necropsy confirmed a previously undetected right atrial melanoma lesion extending through the wall of the heart into the cardiac lumen.

Dose escalation and treatment related adverse events

Treatment-related serious adverse events (SAEs) are listed in [table 2](#), and all study related AEs are provided independent of attribution (online supplemental table 1). No fevers, hypotension or other abnormalities were identified during the monitoring period following the first treatment. Several owners reported a mild increase in coughing during or immediately following inhaled treatments at home. One dog enrolled in the 10 µg dose level was allowed to enroll with a grade 3 alkaline phosphatase elevation, a grade 2 alanine transaminase (ALT) elevation, and normal aspartate transaminase (AST). At the completion of inhaled therapy, this patient presented with a new metastatic lymph node and progressive hepatopathy. These were determined to be grade 3 ALT and AST elevations. Based on progressive disease (PD), the owners withdrew the dog from the study without a hepatic ultrasound. The owners also declined necropsy at the time of euthanasia. Without confirmation of hepatic

Table 2 Treatment-related serious adverse events (AEs)

AE	Grade AE in dose cohort					Grade 3 and above # affected/%
	10 µg	20 µg	33 µg	50 µg	70 µg	
ALP, high	3, 3, 4				3	4/19.0
Dyspnea			4		4	2/9.5
Potassium, high		3			3	2/9.5
Soft tissue necrosis					3*, 3*	2/9.5
Acidosis		3				1/4.7
ALT, high	3*					1/4.7
AST, high	3*					1/4.7
Dehydration		4				1/4.7
Diarrhea		3				1/4.7
Hypoxia					4	1/4.7
Lethargy/fatigue		3				1/4.7
Lymph leakage					3	1/4.7
Peritonitis, non-septic		3				1/4.7
Total	5	6	1	0	7	19

*Attribution possibly related to treatment. All others attributed to disease progression. ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase.

metastasis, the progressive hepatopathy was attributed possibly to treatment. Therefore, this dose level was expanded to enrol three additional dogs. No other SAEs were observed at this dose level, so the study was advanced to dose level 2 (20 µg). Treatment related SAEs were not documented in any additional patients until the 70 µg cohort was reached. At this dose level, we observed clinically evident (grade 3) necrosis or abscessation of confirmed metastatic melanoma in the lymph nodes of two of six dogs. These were categorized as DLTs, and dose escalation was stopped per protocol. Accordingly, the MTD was determined to be 50 µg.

Clinical response and outcomes

Overall, three patients died or were euthanized prior to day 28 (and were thus not evaluable per protocol), leaving 18 patients evaluable for response (table 1). Among 18 evaluable dogs, 1 dog demonstrated a CR, 1 showed a partial response (PR), 5 had SD, and 11 had PD. Figure 1B depicts a waterfall plot of percent change in size of index lesions when best response was achieved as stratified by responders and non-responders. Figure 1C depicts the summary of all RECIST evaluable patient timepoints over time. Two patients (UCD-IL15-009, UCD-IL15-012) had extensive PD noted on radiographs that precluded RECIST evaluation. One patient with OSA treated at the 10 µg dose demonstrated a strong PR (figure 1D), and one patient with melanoma treated in the 50 µg cohort experienced a complete remission of diffuse metastatic pulmonary lesions (figure 1D). This CR was maintained >1 year until presentation with localizing neurologic signs of unclear etiology. The owners elected euthanasia and declined necropsy such that the cause of neurologic signs

was undetermined, although brain metastases from melanoma remained within the differential diagnosis. Notably, there was no clinical or radiographic evidence of recurrent melanoma in any other location. Additionally, there were two patients with OSA with SD (46 and 55 days), and three patients with melanoma with SD (42, 56, and 147 days). Overall, the median survival time from the time of first inhaled rhIL-15 treatment was 82.5 days (range 36–655) for OSA patients and 113.5 days (range 34–407) for melanoma patients. Consistent with our response data (figure 1B,C), we observed that patients showing clinical benefit tended to have improved overall survival compared with non-responders (figure 1E,F), although the differences in survival between responders and non-responders were not statistically significant ($p=0.09$). The large majority of patients (16 of 18 evaluable dogs) had lung only disease at the time of therapy, having previously undergone treatment of the primary tumor. Interestingly, of the two patients with their primary tumor intact, the clinical course of the primary tumor mirrored the response of the lung metastases (one CR and one PD).

Plasma rhIL-15 quantitation and cytokine induction

Plasma sampling for rhIL-15 quantitation was instituted with patient UCD-IL15-011 (33 µg cohort) as part of our correlative assays. Results of our analysis correlate with human studies examining dosing of rhIL-15,^{6–8} demonstrating a time of maximum plasma concentration (T_{max}) at approximately 4 hours (figure 2A). While rhIL-15 was numerically detectable and increased at 4 hours in all patients, concentrations were nevertheless below the limit of quantification (<20 pg/mL) at the 4-hour plasma sample in 2/2, 1/3, and 4/6 dogs in the 33, 50, and 70

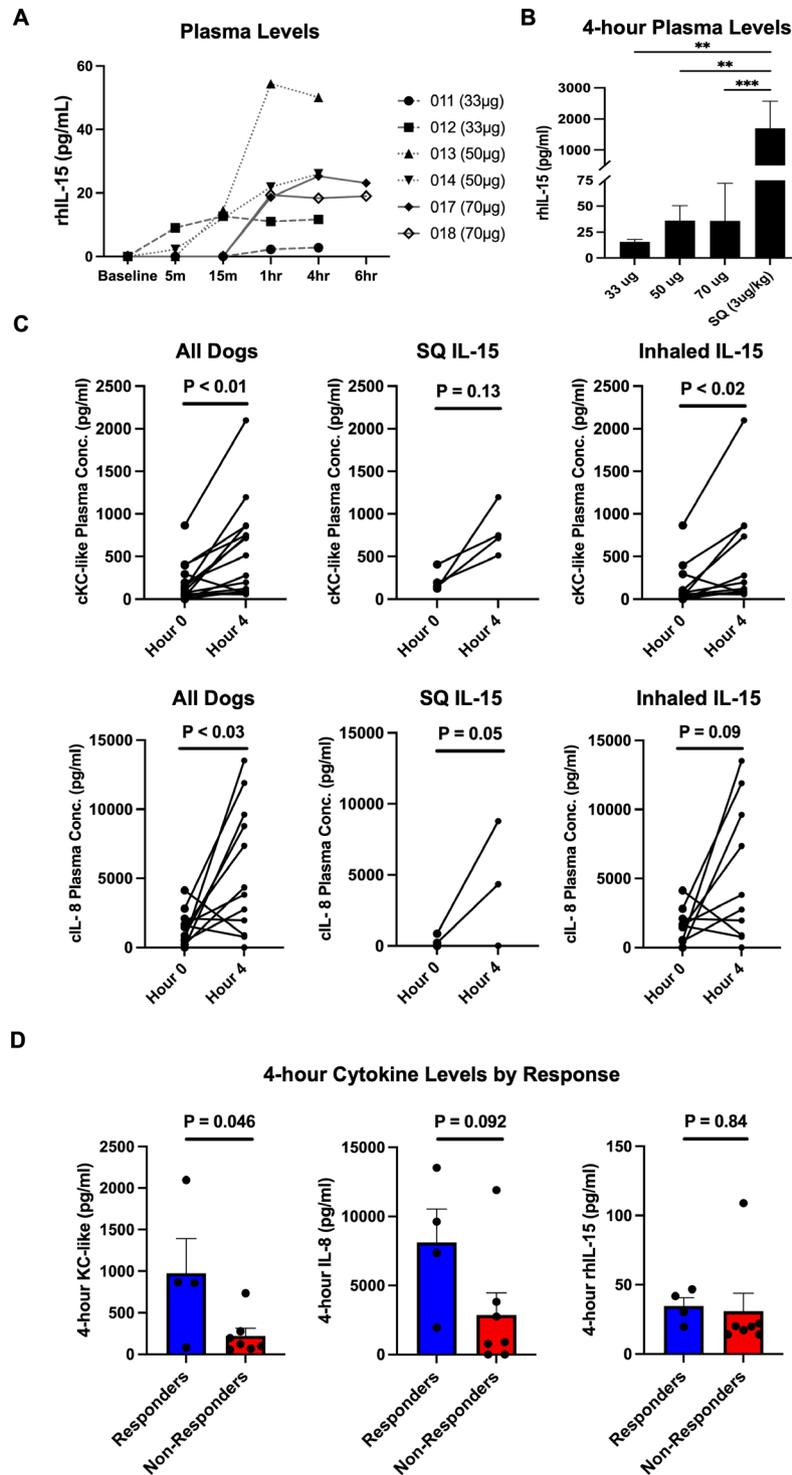


Figure 2 Plasma rhIL-15 quantitation and cytokine response. (A) Samples were collected prior to first inhaled rhIL-15 treatment and then at indicated time points for 6 hours after treatment initiation and measured by ELISA. Changes in plasma rhIL-15 concentrations were plotted over time for two patients in each of the 33–70 μ g cohorts. (B) Concentration of rhIL-15 in plasma of patients in the 33 μ g, 50 μ g, and 70 μ g inhaled rhIL-15 cohorts as well as those from a separate canine clinical trial where dogs with locally advanced melanoma were treated with palliative RT, allogeneic NK transfer, and 3 μ g /kg rhIL-15 SQ to promote NK engraftment. Bars represent plasma rhIL-15 pretreatment and at the presumed Tmax of 4 hours post-treatment for each patient. Plasma rhIL-15 was detectable in all patients but below the limit of quantitation in several patients. Individual groups were compared using one-way analysis of variance with Tukey’s multiple-comparison test. *** $P < 0.001$; ** $p < 0.01$. (C) Cytokines induced by rhIL-15, IL-8 and KC-like were quantified in the plasma of all patients (left) as well as separately within the SQ rhIL-15 trial cohort (center) and inhaled rhIL-15 cohorts (right) using a canine multiplex assay. (D) Four-hour KC-like levels (left) were significantly higher in responders compared with non-responders ($p = 0.046$), and a similar trend was noted with 4-hour IL-8 levels (middle) ($p = 0.092$). Responders and non-responders did not show differences in 4-hour rhIL-15 levels (right). rhIL-15, recombinant human interleukin-15; SQ, subcutaneous.

µg cohorts, respectively. These results are consistent with minimal systemic exposure to rhIL-15 after inhalation (figure 2B), as quantifiable plasma levels of rhIL-15 ranged from 20 to 124 pg/mL. In contrast, plasma obtained from dogs with buccal melanoma treated SQ with 3 µg/kg rhIL-15 as part of a separate clinical trial of palliative RT and allogeneic NK transfer yielded 4-hour concentrations for rhIL-15 ranging from 740 to 2954 pg/mL (figure 2C). Demographic data for these patients receiving SQ rhIL-15 are included in online supplemental table 2. Importantly, baseline plasma levels of canine IL-15 were not detected with the human IL-15 ELISA nor was rhIL-15 detectable on the canine Luminex assay. Taken together, these data indicate that systemic exposure to inhaled rhIL-15 is low, but rhIL-15 exposure appears to be maintained at relatively stable plasma levels for up to 6 hours. These data also align with previously published studies demonstrating that the majority of radio-labeled inhaled rhIL-2 remains within the lungs of dogs after inhalation,¹³ but over 24 hours, slow release into the systemic circulation occurs. Despite low systemic exposure to rhIL-15 via the inhaled route, induction of KC-like and IL-8 were noted in the plasma 4 hours after treatment in the majority of dogs after either SQ or inhaled dosing (figure 2C). Interestingly, we observed that 4-hour plasma levels for both canine KC-like and IL-8 chemokines (figure 2D) were higher in responders compared with non-responders with the differences for KC-like reaching statistical significance ($p=0.046$), while for IL-8, the differences approached but did not reach statistical significance ($p=0.09$). In contrast, 4 hours following initial inhaled IL-15 treatment, there was no difference in plasma levels of human IL-15 between responders and non-responders (figure 2D). We did not observe any correlation between absolute canine IL-8 or KC-like plasma levels and human IL-15 levels at 4 hours, nor did we observe a correlation between changes in IL-8 or KC-like levels and change in plasma IL-15 levels (online supplemental figure 3). Unlike human studies examining systemic delivery of rhIL-15, plasma concentrations of IL-6 or IFN γ 4 hours post-treatment were largely unchanged in most dogs (online supplemental figure 4).

Hematological evaluation

Baseline and induced hematological parameters have previously been shown to correlate with response and/or outcome in dogs and human patients undergoing cancer therapy.^{25,26} We, therefore, evaluated before, during, and after therapy in all evaluable dogs. As shown in figure 3A, dogs that demonstrated clinical benefit had significantly lower baseline ALC compared with patients with no clinical benefit (1040 ± 650 vs 1485 ± 268 , $p=0.03$). Moreover, as shown in figure 3B, responding patients showed a significant greater fold change increase in ALC compared with non-responding patients ($p=0.02$). ALC fold change was on average 0.32 higher in responders than in non-responders ($SE=0.12$, $p=0.02$). Given that KC-like and IL-8 chemokines are classically associated with neutrophil recruitment and migration,²⁷ we also evaluated blood

levels of neutrophils in our inhaled rhIL-15 cohort. As shown in figure 3C, absolute numbers of neutrophils were higher at baseline in non-responders compared with responders (8464 ± 6292 vs 5995 ± 1920), but this difference was not statistically significant ($p=0.33$). Similarly, as shown in figure 3D, we observed higher blood neutrophil levels at all time points among non-responders compared with responders, but these differences were not statistically significant ($p=0.16$).

We also evaluated the frequencies of circulating NK and T cells and differential gene expression of NK cell subsets from patients on the inhaled IL-15 trial using CD3-NKp46+ to identify canine NK cells (figure 3E).^{22,28} Overall, as shown in figure 3F,G, we observed no significant differences in NK, CD3+, or CD8 + cell frequencies or fold change over time. NK cell frequencies did increase by 1.69 ± 1.11 fold on day 7 (figure 3G), but this was not statistically significant. Given that IL-15 superagonist ALT-803 has been demonstrated to act as a potent immunostimulant that is capable of eliciting both rapid and long-lasting antitumor effects and has shown exciting promise in early stage human clinical trials, including with PD-1 blockade,²⁹ we also performed a first-in-dog proof-of-concept study of ALT-803 to evaluate the safety, toxicity, and preliminary data for response rates in dogs with metastatic cancer. Patient demographics for this small cohort are depicted in online supplemental figure 5A. Recognizing the limitations of analyzing a cohort of four dogs, we observed rapid progression of disease in three dogs with survival ranging from 6 to 26 days following start of ALT-803 therapy. Since these dogs did not reach the 30-day response assessment, they were deemed non-evaluable. The fourth dog demonstrated PD as the best response and survived 117 days from therapy initiation. We performed immune assessment for these patients where specimens were available (online supplemental figure 5B,C). Anecdotally, CD8 + frequencies appeared to increase more than NKp46 + NK cells in absolute numbers, while there was a 2.3 ± 1.1 fold change in NK cell frequencies at day 7, which was not statistically significant (online supplemental figure 5D).

Using RNA sequencing, we then analyzed the differential gene expression of CD5-depleted PBMCs from a subset of inhaled rhIL-15 dogs for which adequate samples were available (figure 3H–K). As shown by multidimensional scaling plots, there were significant differences in clustering between CD5-depleted and CD5^{bright} subsets consistent with CD5 primarily representing a T cell marker, especially for the cells with strong expression (figure 3H). In contrast, we observed no significant clustering for the differential gene expression of enriched peripheral blood NK cells in the CD5 depleted subset when analyzed by inhaled rhIL-15 dose (figure 3I), cancer type (melanoma vs OSA, figure 3J), or even best response to therapy (figure 3K), reinforcing the complexities of the multiple host/cancer and genetic/epigenetic variables impacting gene signatures. We also analyzed patient's samples for quantitative differences in differential gene expression

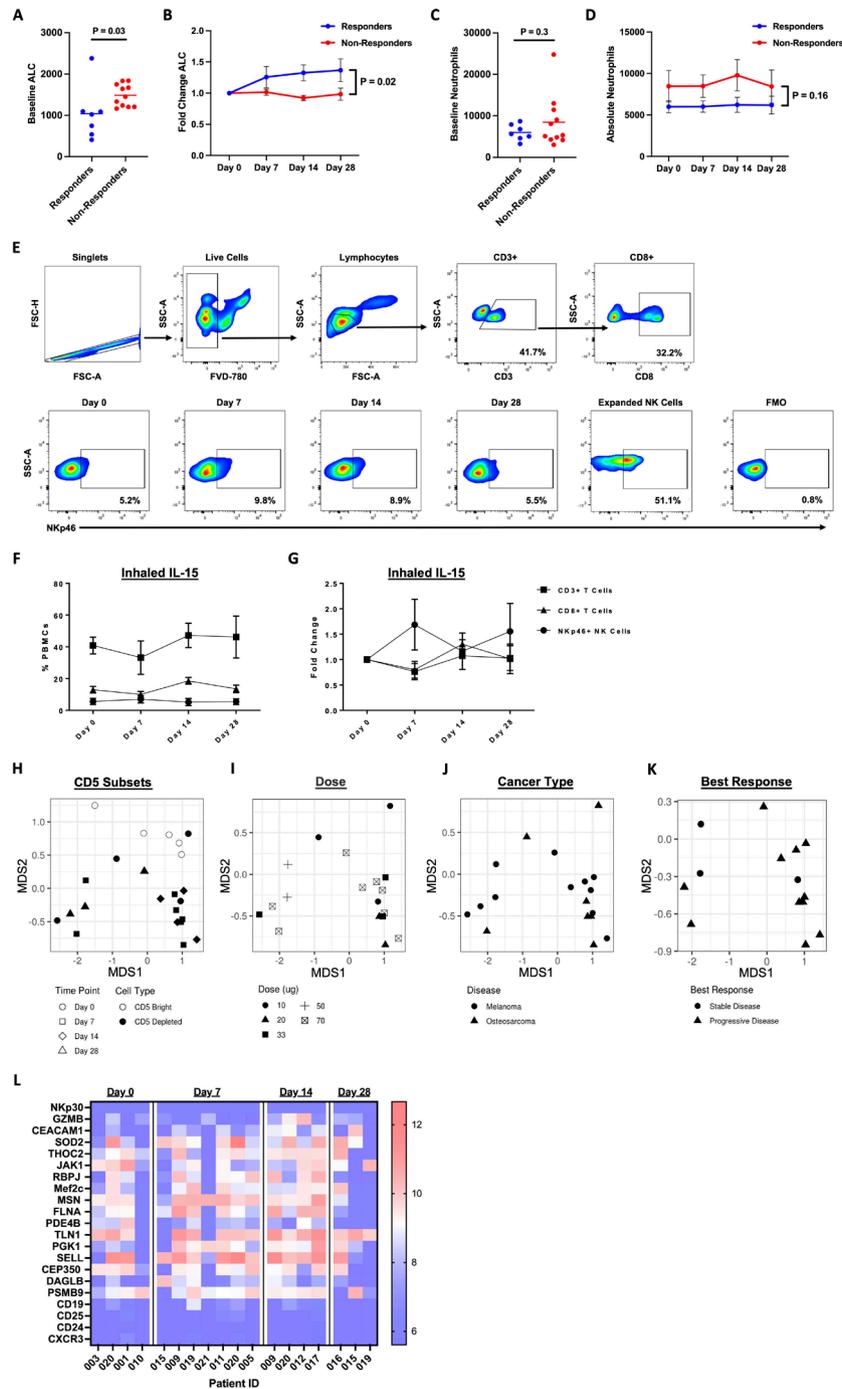


Figure 3 Baseline lymphopenia is associated with response to therapy. (A) Baseline absolute lymphocyte counts (ALCs) were determined. Day 0 ALC was lower among responders compared with non-responders ($p=0.03$ by Kruskal-Wallis Test). (B) Fold change from baseline ALC was on average 0.32 higher in responders than in non-responders ($SE=0.12$, $p=0.020$). (C) There is no difference in baseline neutrophil counts between responders and non-responders ($p=0.3$). (D) Neutrophil counts were higher in non-responders at all time points, but these differences were not statistically significant ($p=0.16$). (E) Representative flow cytometry staining is shown for PBMCs, including parent gating and CD3+, CD8+, and Nkp46 +lymphocyte subsets. Nkp46 staining is shown for one patient for all time points along with primary cultured NK cells and Fluorescence Minus One (FMO) of positive and negative controls. (F) Frequencies of CD3+, CD8 + and Nkp46 +lymphocyte subsets in the peripheral blood in patients over time. (G) Fold change of CD3+, CD8 + and Nkp46 + lymphocyte subsets were calculated compared with day 0 values as the reference. (H) Multidimensional scaling (MDS) plots based on mathematical distances of differential gene expression for CD5 depleted versus CD5^{bright} subsets using RNA sequencing on PBMC samples at indicated time points. Plot demonstrates significant differences in clustering. (I–K) MDS plot for differential gene expression of CD5 depleted PBMCs enriched for NK cells showing mathematical distances of gene profile based on inhaled IL-15 dose, cancer diagnosis, and best clinical response, respectively. (L) Heatmap of 21 genes of interest demonstrates subset of genes induced in CD5 depleted PBMCs at time points postinitiation of inhaled IL-15 compared with day 0 gene expression. NK, natural killer; PBMC, peripheral blood mononuclear cell.

at indicated time points (figure 3L). Overall, we did not observe significant changes in the gene expression of canonical NK genes in the peripheral blood of patients on trial, but we did see notable changes in genes linked to NK function and maturation. Three genes of particular interest which were upregulated in peripheral blood NK cells after initiation of therapy were: recombination signal binding protein for immunoglobulin kappa J region, which has been linked to reductions in NK cell populations in peripheral blood when deleted³⁰; myocyte enhancer factor 2C, which has been associated with profound defects in the production of B cells, T cells, NK cells and common lymphoid progenitor cells when deficient³¹; and moesin, which causes NK cells to exhibit increased cell death and impaired signaling in response to IL-15 when deficient (figure 3L).³²

Changes in PBMC cytotoxicity

Given reports that have shown that cytotoxicity of circulating NK and cytotoxic T cells also correlates with cancer outcomes,^{33–34} we sought to analyze the cytotoxicity of patient PBMCs over time against OSA and melanoma targets in vitro using cryopreserved specimens from our patients. As shown in figure 4A, we used a CFSE-labeling technique to delineate target cells from effector cells in this in vitro assay. We verified that changes in cytotoxicity over time were related to treatment rather than random variation by confirming stable cytotoxicity in PBMCs over time from healthy beagles not undergoing therapy (online supplemental figure 2). As shown in figure 4B, PBMCs post-inhaled rhIL-15 treatment demonstrated significantly increased peak cytotoxicity against both OSA and melanoma tumor lines ($p < 0.001$). As shown in figure 4C, cytotoxicity of PBMCs against OSCA-78 correlated significantly with patient survival from inhaled rhIL-15 treatment ($r = 0.693$, $p = 0.002$). Similarly, as shown in figure 4D, the change in cytotoxicity of PBMCs from baseline to peak also demonstrated a significant positive correlation with patient survival from inhaled rhIL-15 treatment ($r = 0.579$, $p = 0.02$). We did not observe a correlation of maximal cytotoxicity of PBMCs against M5 with patient survival from inhaled rhIL-15 (figure 4E) but noted a modest correlation between change in cytotoxicity of PBMCs from baseline to peak and patient survival, which was not statistically significant ($r = 0.357$, $p = 0.15$), as shown in figure 4F. Figure 5G–I represent examples of patients with SD, CR, and PD, respectively.

Analysis of plasma canine cytokines

To assess the effect of inhaled rhIL-15 on the induction of cytokines, we evaluated the absolute concentrations of canine (c) cytokines in plasma over time (figure 5A–M). There was marked variability in plasma concentrations of cGM-CSF, cINF γ , cIL-7, cIL-10, cIL-15, cIL-18, cKC-like, and cTNF α among subjects, and we did not observe any significant differences among responders and non-responders (figure 5A, B, E, G, H, I, K and M, respectively). Figure 5N–Q show the fold change of canine

cytokines over time in responders compared with non-responders. Fold change of IFN γ , IL-2, IL-6, and IL-18 over time showed no differences between responders and non-responders, although responders generally showed greater fold change of IL-2 at each time point. Figure 5R–W demonstrate canine cytokine concentrations at baseline in dogs with OSA versus melanoma. The concentrations of cGM-CSF, cIL-6, cIL-7, cIL-10, cIL-15, and cTNF α at baseline were generally higher in the dogs presenting with OSA than those with melanoma. The concentrations of cIL-10 were significantly higher in dogs with OSA compared with those with melanoma ($p = 0.004$, figure 5U). The levels of cIL-7 and cIL-15 were higher at baseline in dogs with OSA compared with those with melanoma, with the differences approaching statistical significance ($p = 0.054$ and $p = 0.076$, respectively) (figure 5T and V).

DISCUSSION

To the best of our knowledge, this is the first study to implement inhaled rhIL-15 as a novel cancer immunotherapeutic in a first-in-dog clinical trial. The primary objectives of this phase I trial were to identify treatment-related adverse events (AEs), DLTs, and to determine a MTD for follow-up therapeutic trials. Attribution of AEs can present challenges in late-stage metastatic disease, particularly in subjects who experience rapid progression with short survival times.^{18–35} Importantly, but also somewhat surprisingly, we observed no evidence of pulmonary-related SAEs or DLTs despite the inhalational route. This is highly relevant for combination therapies, especially given the recent development of canine PD-1 and PD-L1 inhibitory antibodies for clinical usage since these agents in humans have been observed to cause potentially severe pneumonitis.^{36–38} Instead, the DLT we observed in two dogs in the 70 μg cohort was abscessation or necrosis within regional lymph nodes confirmed to harbor metastatic melanoma. While infection/necrosis of lymph nodes does occasionally occur in the natural progression of metastatic cancer, we concluded that rhIL-15 therapy might be related to the occurrence of these events in our patients. We therefore determined that the MTD of inhaled rhIL-15 in cancer-bearing dogs was 50 μg twice daily. Of note, although the rationale that the highest dose that does not cause dose-limiting toxicity will result in the greatest probability of efficacy may not apply in the era of immunotherapy where clinical benefit may not be dose dependent, our impression is that a higher dose of inhaled rhIL-15 is preferable for phase 2 studies, especially given the uncertainties of using biomarkers for dose selection.

Based on prior inhaled rhIL-2 data in dogs with primary or metastatic gross disease within the lungs, we anticipated that we might see responses to inhaled rhIL-15 in dogs with metastatic OSA or melanoma. While response was not a primary endpoint, we were encouraged to see documented responses, including a PR and a durable

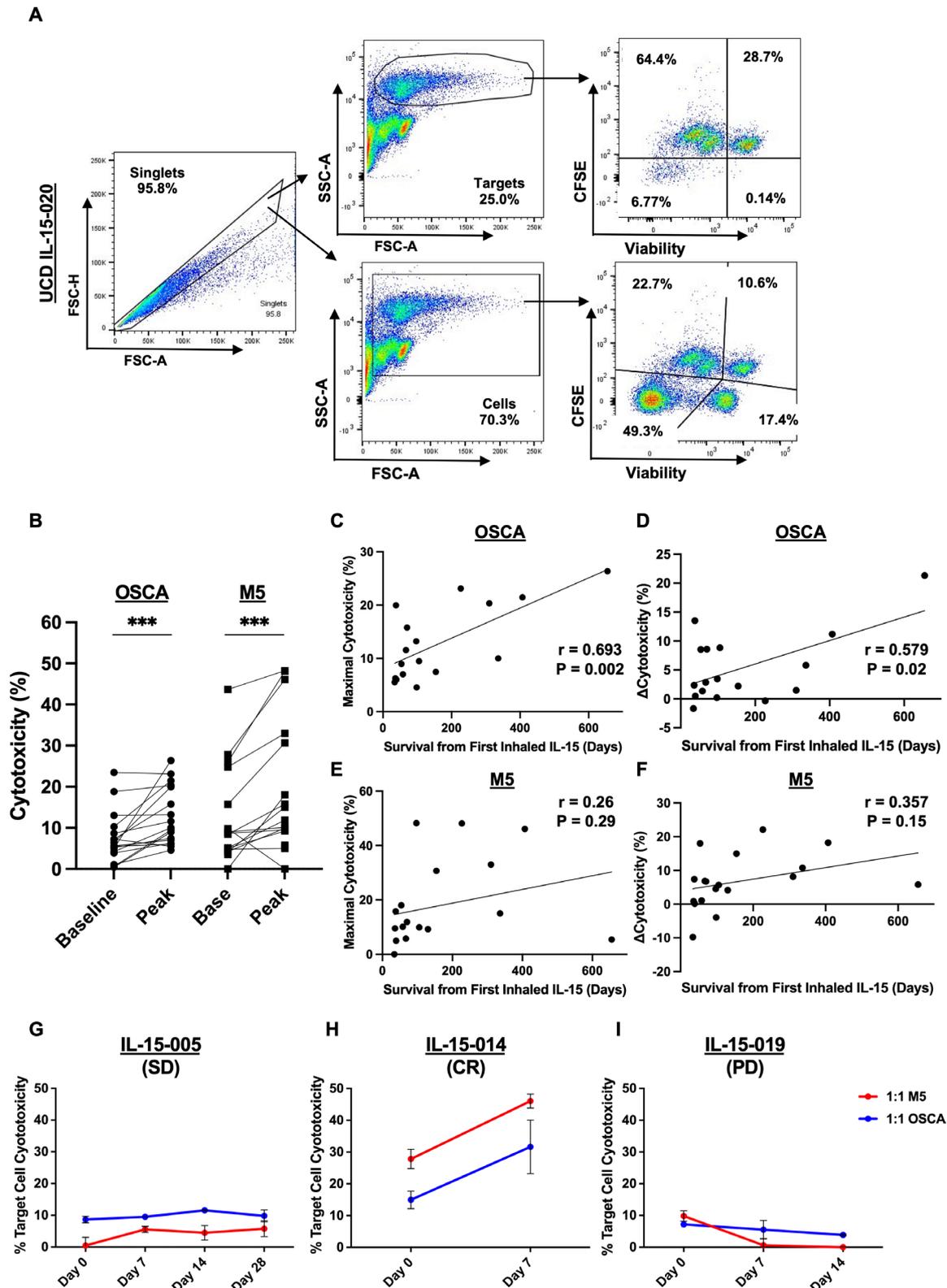


Figure 4 Cytotoxic function of patient PBMCs pretherapy and post-therapy. (A) Representative flow cytometry gating distinguishes PBMC effectors from CFSE-labeled OSA and melanoma target cells with dead cells staining positive for Viability Dye 780. (B) Cytotoxicity, calculated by flow cytometry, of PBMCs against osteosarcoma (OSCA-78) and melanoma (M5) cell lines was significantly increased postinhaled rhIL-15 treatment ($p < 0.001$). PBMCs targeting OSCA had significant correlation (Spearman correlation coefficient, ρ) of (C) maximal cytotoxicity and (D) change in cytotoxicity with survival. (E) Maximal cytotoxicity of PBMCs targeting M5 did not show significant correlation to survival. (F) Minimal correlation existed between change in cytotoxicity and survival in PBMCs targeting M5. Representative examples of changes in cytotoxicity, at 1:1 effector to M5 (red) or OSCA (blue) target ratios, in a patient with (G) stable disease (SD), (H) complete response (CR), and (I) progressive disease (PD). PBMCs, peripheral blood mononuclear cells.

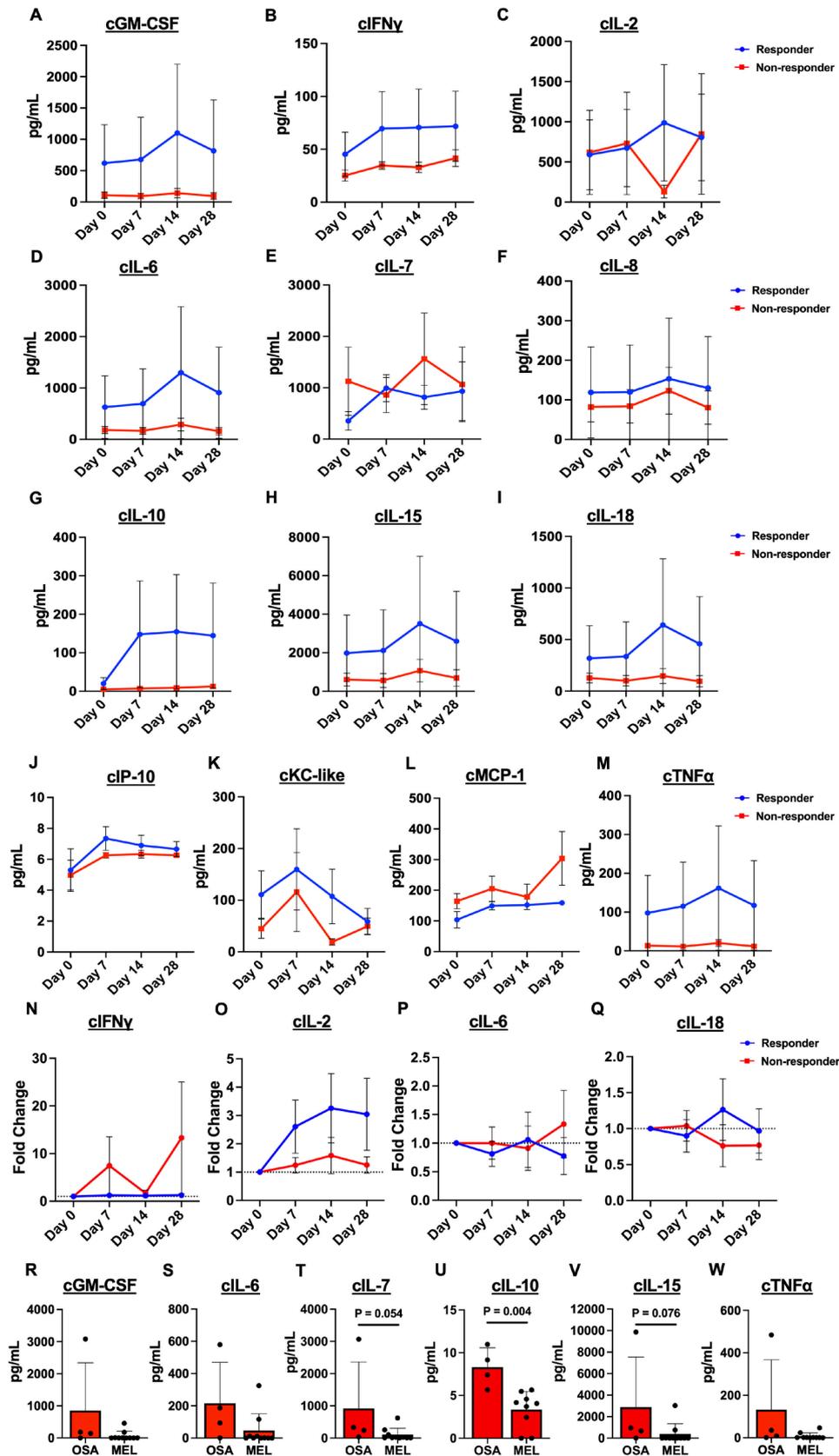


Figure 5 Plasma cytokine levels. Plasma cytokine values measured by canine Luminex assay are depicted comparing responders to non-responders over time: (A) cGM-CSF, (B) cINF γ , (C) cIL-2, (D) cIL-6, (E) cIL-7, (F) cIL-8, (G) cIL-10, (H) cIL-15, (I) cIL-18, (J) cCXCL10, (K) cKC-like, (L) cMCP-1, and (M) cTNF α . Fold change of (N) cINF γ , (O) cIL-2, (P) cIL-6, and (Q) cIL-18 levels fluctuated over time but were not significantly different between responders and non-responders. Concentrations of (R) cGM-CSF, (S) cIL-6, (T) cIL-7, (U) cIL-10, (V) cIL-15, and (W) cTNF α at baseline were higher in patients with OSA compared with those with melanoma with a significant difference observed in cIL-10 ($p=0.004$). OSA, osteosarcoma.

CR >1 year in dogs with diffuse and bulky metastatic disease. Equally important is that these responses were prolonged despite receiving a limited 2-week window of inhaled cytokine. This short treatment duration was based on the likelihood that dogs would generate neutralizing antibodies to rhIL-15, making prolonged treatment a risk for allergic responses and immune pathology. Ultimately, the documented response rate per modified RECIST criteria remains low in our study. Human clinical trials evaluating the efficacy of single-agent rhIL-15 for advanced solid tumors via SQ, intravenous bolus, or continuous infusion yielded significant induction of NK and lymphocyte populations, but no documented clinical responses were noted with monotherapy.^{6,39} While we did observe clinical responses in dogs treated via inhalation, the overall response rate of CR and PR remains relatively modest (11%), and the role for rhIL-15 may likely be most impactful in combination with other existing or novel immunotherapies. At the same time, we anticipate that studies of inhaled canine IL-15 will be highly informative given the potential for delayed responses with immunotherapy (which may convert some patients with SD at early time points to delayed responses after longer exposure). We hypothesize that a longer course of inhaled IL-15 using canine IL-15 will lead to a higher response rate and greater clinical benefit, especially since we and others have shown that canine cytokines (IL-2 and IL-15) are more potent in stimulating immune responses in canine PBMCs and canine NK cells than human cytokines.^{28,40}

The finding that patients with low ALC at baseline were more likely to have responses or SD is also intriguing. While this finding was statistically significant, conclusions based on a small phase 1 study should be interpreted with caution as additional studies are clearly needed. While it is possible that a pre-existing cytokine profile could favor or enhance the activity of inhaled rhIL-15, it is also possible that responses in patients with low lymphocyte counts could be unrelated to the activity of rhIL-15. Depletion of lymphocytes improves the effect of adoptive cell transfer by eliminating Tregs and by removing endogenous lymphocytes, thereby leading to more 'space' for adoptively transferred cells to proliferate in the setting of increased availability of homeostatic cytokines like IL-7 and IL-15.^{41–43} Thus, the decreased baseline ALC seen in responders in our cohort could be linked to less competition for exogenous cytokine among endogenous NK and cytotoxic T cells.

Distinct tumor types may also contribute to differences in cytokine profiles and response to treatment. Although melanoma and OSA are both aggressive cancers with poor prognosis, they have unique immune phenotypes in both dogs and human.^{18,19,21,40} Plasma cytokine profiles were generally higher in dogs with OSA than dogs with melanoma, although only IL-10 was significantly higher in OSA. IL-10 can promote increased cytotoxicity and IFN γ secretion in NK cells⁴⁴ but has an overall immunosuppressive effect on immune cells,

contributing to cancer cells' immune escape.⁴⁵ Concentrations of IL-7, a critical cytokine in the homeostatic expansion of T cells,^{43,46} showed a trend to be higher in OSA versus melanoma, although remained highly variable among patients. Overall, this suggests that cytokine elaboration can be related to tumor type although other immune-modifying factors such as age, sex, and body habitus are clearly also important. Similarly, immune cell populations and cytokine patterns in the blood may not represent mechanisms of response or resistance in the lung TME. For example, NK cells in healthy human lungs comprise approximately 10% of tissue-resident lymphocytes and primarily express a CD56^{dim} perforin^{high} phenotype.^{47,48} Neoplastic transformation of lung tissue recruits largely non-cytotoxic CD56^{bright} cells,⁴⁷ and intravenous infusion of IL-15 induces an expansion of CD56^{bright} cells with enhanced cytotoxic function and increased cytokine production compared with CD56^{dim}.⁴ Inhaled rhIL-15 ultimately may have many different effects in the target tissue versus PBMCs, and further studies are required to determine effects of inhaled rhIL-15 on the native lung and the TME of lung metastases, as well as any crosstalk.

In summary, our first-in-dog phase 1 clinical trial using a novel inhaled delivery route of rhIL-15 produced promising clinical activity and identified an MTD of 50 μ g twice daily. We also observed that the cytotoxicity of circulating effector cells in the blood while on therapy as well as baseline ALC appear to correlate with clinical benefit. Ultimately, these results support ongoing investigation of inhaled rhIL-15 in treating dogs with metastatic melanoma and OSA with an emphasis on combinatorial approaches.

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Contributors RBR, SLS, WJM, MSK, and RJC designed the study. RBR, DY, SJJ, LEF, RVB, EGJ, JHB, JW, LAW, KW, EES, SSW, KAS, SA-N, ATL, WC, MSK, and RJC conducted the experiments and collected the data. SLS provided statistical analysis. RBR, DY, SMC, SJJ, AMR, CD, SLS, AAG, and RJC analyzed the data. RBR, DY, SMC, AMR, and RJC wrote the manuscript. All authors provided critical review of the manuscript. RBR and RJC are responsible for the overall content as guarantors.

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Competing interests None declared.

Patient consent for publication Not applicable.

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Data availability statement Data are available on reasonable request.

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Study Protocol

STUDY TITLE	Allogeneic NK Cells and palliative radiotherapy in the treatment of canine cancer		
STUDY NICKNAME	NK Cell/RT Study		
IACUC #	21620	GRANT #	N/A
REDCap Project #	N/A	CAMPUS ACCOUNT #	V4V0003
SPONSORED PROGRAMS #	N/A	VMACS ACCOUNT #	X00V03
STUDY PHASE	Pilot Study – Proof of Concept		
STUDY DRUG or DEVICE	Allogenic NK Cells and IL-15		
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	Phone		
	Email		
SPONSOR	UCDMC – Cancer Center		
SPONSOR CONTACT	Name		
	Phone		
	Email		
INITIAL EFFECTIVE DATE	6/23/2020		
AMENDMENT VERSION #			
AMENDMENT EFFECTIVE DATE			

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Study Narrative

I. Study Location(s), Study Personnel and Contact Information

- A. Study Location: This study will be conducted at the UC Davis, VMTH
 B. Investigators: Michael Kent, Robert Canter

Role	Name	Best Contact Number	Email
CO-PI	Michael Kent	1-530-400-1693	mskent@ucdavis.edu
CO-PI	Bob Canter	215-964-0468	rjcanter@ucdavis.edu

C. Clinical Trial Coordinators

Main Line: 530-754-1954

General E-mail: vetclintrials@ucdavis.edu / oncologyclinicaltrials@ucdavis.edu

Name	Best Contact Number	Email
Emily Phenix	Office: (530) 754-1297 Cell: (530) 304-8255	eaphenix@ucdavis.edu
Jacque Young, RVT	Office: (530) 754-1355 Cell: (530) 304-8563	jacyoung@ucdavis.edu

D. Veterinary Center for Clinical Trials (VCCT)

Name	Best Contact Number	Email
Christine Munsterman Financial Analyst	Office: (530) 754-7746	cmmunsterman@ucdavis.edu
Kate Hodges Clinical Trials Coordinator Supervisor	Office: (530) 754-1953 Cell: (510) 610-5655	khhodges@ucdavis.edu

II. Overview / Rationale

Use of allogeneic NK Cells in treating cancer in dogs

Previously we have shown that intratumoral injections of autologous NK cells into osteosarcoma lesions in dogs resulted in alterations in the local tumor microenvironment and that the transferred cells persisted after injection with evidence of immune activation/ stimulation. In this previous work, we used IL-2 to stimulate NK Cell activation. To address the difficulties with the use of autologous NK Cells as well as disadvantages of using IL-2 (including the possible upregulation of Tregs), our goal is to investigate the use of systemically delivered allogeneic NK cells along with the use of subcutaneous IL-15 to activate them.

The main objectives of this study are to provide preliminary safety data on allogeneic NK cells in canine cancer and to test 1) whether the transferred NK cells will engraft into the recipient dog, 2) whether these cells will localize to tumors post-radiotherapy (which we have showed in pre-clinical models), and 3) persist as least in the short term in the tumor. This study is designed to provide preliminary data to seek funding for a larger study and to compliment the other work being doing by our group in the area of NK cell therapies.

III. Study Objectives

- A. Primary -Safety of combined Palliative RT followed by NK Cell and rhIL-15 administration
- B. Secondary – Determination of retention of Allogeneic NK Cells after adoptive transfer as well as effects on immune function systemically and in the local tumor environment.

IV. Investigational Plan

- A. Overall Study Design – Proof of concept and preliminary safety study
- B. Statistical Plan
 1. Sample Size Determination: This is a proof-of-concept study to determine initial safety and to determine if allogeneic NK cells will target to a tumor and be retained after administration. This will allow us to plan further studies.
 2. Statistical Methods: Descriptive
 3. Subject Populations for Analyses: Dogs being treated with a palliative course of RT for a solid tumor with a tumor that is accessible for repeated biopsy.
- C. Selection of Study Population
 1. Number of Subjects: 5
 2. Species: Dogs
 3. Breed: Any
 4. Initial Age: Any
 5. Weight: >10kg on day 1
 6. Sex: Female or Male, either intact or neutered/spayed
 7. Origin / Source: Client Owned Pets

8. Identification: Dogs will be identified by their given name and owner's surname, as recorded in their medical records. In addition, dogs will be identified by their unique medical record number in VMACs
 9. Previous Treatments: Normal vaccination and general health care practices are permitted. No prior chemotherapy within 2 weeks to day 0. No prior immunotherapy or radiation therapy within the last 4 weeks.
 10. Husbandry: Normal feeding and housing post procedures until dog is released to their owners. Normal feeding and housing as provided by individual dog owners post procedures.
- D. Study Schedule – See Appendix A

V. Study Implementation

- A. Inclusion Criteria
- Histologic or cytologic diagnosis of a tumor
 - Patient planning to undergo a course of radiation therapy
 - Gross tumor of at least 3cm
 - Body Weight ≥ 10 kg
 - VCOG-CTCAE 1.1 performance score of 0 or 1
 - HCT $\geq 25\%$
 - Neutrophil Count $\geq 2,000/\text{ul}$
 - Platelet Count $\geq 75,000/\text{ul}$
 - Creatinine \leq ULN; bilirubin \leq ULN; ALT \leq ULN; AST \leq ULN Any other clinically significant grade 2 or higher hematologic, biochemical abnormality.
 - Metastatic disease allowed if clinically safe for anesthesia.
- B. Exclusion Criteria
- Chemotherapy within 2 weeks of day 0
 - Immunotherapy or previous radiation therapy within 4 weeks of Day 0
 - VCOG-CTCAE 1.1 performance score of 2 or higher
 - Concurrent therapy - NSAIDS acceptable if needed for pain control and patient has been receiving for 2 weeks or more. Tramadol, Opioids, Gabapentin, Pamidronate and zoledronate also acceptable.
 - No systemic corticosteroids within two weeks of starting protocol.

C. Visit Descriptions

1. Pre-Enrollment: within 21 days of enrollment the dog must be screened for all inclusion and exclusion criteria. Also, the owner will be responsible for obtaining a CBC, Chemistry Panel, Urinalysis and thoracic radiographs. The owner is responsible for the cost of the screening visit as well. Please note that the CBC and Chemistry panel should be attempted to be done within one month of the treatment planning CT and starting radiotherapy to limit owner costs as this will be required for anesthesia planning. Canter lab is contacted with schedule.
2. Radiotherapy Treatment Planning CT: While not part of the study itself, this must be planned to allow sufficient time prior to starting radiotherapy to allow treatment planning. Contact RT staff for scheduling this and start of RT.
3. First radiation therapy: Physical Exam. PBMCs and plasma sample are collected. Prior to the first irradiation and after the dog is anesthetized, two 4mm punch biopsies will be obtained. Radiation reaction is scored along with VCOG adverse events.
4. Mid Treatment Visit: Physical Examination, CBC, Chemistry Panel, PBMCs and plasma sample are collected. Radiation reaction is scored along with VCOG adverse events.
5. Last RT Visit: Physical Exam. CBC, Chemistry panel, PBMCs and plasma sample are collected. Radiation reaction is scored along with VCOG adverse events. After the last irradiation and before the dog is recovered from anesthetized two 4mm punch biopsies will be obtained. The dog will be recovered from anesthesia. 15 minutes prior to NK Cell infusion dog will be given 2mg/kg diphenhydramine SQ or IM. NK cells will be injected IV as a slow bolus. 3mcg/kg rhIL-15 will then be given SQ. Dogs will be monitored for two hours post infusion for any reactions per protocol sheet. Dog will then be discharged.
6. One day post RT: Physical Exam. CBC, Chemistry panel, PBMCs and plasma sample are collected. Radiation reaction is scored along with VCOG adverse events. 3mg/kg rhIL-15 will then be given SQ. Dogs will be monitored for two hours post infusion for any reactions per protocol sheet. Dog will then be discharged.
7. One week post RT: Physical Exam. Radiation reaction is scored along with VCOG adverse events. CBC, Chemistry panel, PBMCs and plasma sample are collected. Dog is anesthetized. Two 4mm punch biopsies will be obtained.
8. Two weeks post RT: Exam. Radiation reaction is scored along with VCOG adverse events. CBC, Chemistry panel, PBMCs and plasma sample are collected. Dog is then finished with the study.

D. Criteria to Remove Subjects from Study Post-Enrollment

- If deemed clinically indicated by the attending clinician
- If requested by the owner

- Progression of disease is not a specific cause for removal
- E. Biological Sample Collection, Processing, & Storage (See Appendix C)
1. Blood: CBC and Chemistry panels will be turned into the VMTH Central Lab receiving using the study research form. PBMC and Plasma Samples will be given as one – two 5ml Lavender top tube to the Canter Lab for processing.
 2. Urine: N/A
 3. Other: Tissue from Biopsies – See Appendix C
- F. Imaging – Not applicable for this study except as part of screening assessment.
- G. Data Quality Assurance
- This study will be conducted in accordance with 21 CFR 511.1 and under the principles of Good Clinical Practices as defined in VICH GL9.
(<http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM052417.pdf>)

VI. Investigational Product, Drug, or Device

- A. Description
- Endogenous NK Cells**
- These cells will be grown in the Canter lab at the Institute for Regenerative Cures using similar techniques as previously described (Reference 3). GMP techniques will be used to ensure sterility of the NK cells, and cell preparations will be tested to ensure >90% viability. NK cell preparations will also be confirmed as endotoxin and mycoplasma negative prior to intravenous injection.
- Recombinant Human Interleukin-15 (IL-15)**
- E. Coli product is described by the NCI Drug Dictionary as, A recombinant agent that is chemically identical or similar to the endogenous cytokine interleukin-15 (IL-15) with immunomodulating activity. IL-15, secreted by mononuclear phagocytes (and some other cell types) following viral infection, regulates T and natural killer cell activation and proliferation. This cytokine induces activation of transcription activators STAT3, STAT5, and STAT6 via JAK kinase signal transduction pathways in mast cells, T cells, and dendritic epidermal T cells. IL-15 and interleukin-2 (IL-2) are structurally similar and share many biological activities; both may bind to common hematopoietin receptor subunits, negatively regulating each other's activity. CD8+ memory T cell number has been shown to be regulated by a balance between IL-15 and IL-2.

SEE SECTION D AND E of AP FOR FULL SOPS ON DRUG PREPARATION

- B. Treatment Regimen – The objective of this pilot trial is to determine preliminary safety and data regarding NK cell homing to tumor and changes in the immune response. Dogs will be treated with a course of palliative radiotherapy per established clinical practice. They will then receive one dose of allogeneic NK cells dosed at 7.5×10^6 NK cells/kg IV (with 5 ng/mL rhIL-15 in solution of 0.9% NaCl) after the final dose of radiation is delivered followed by two subcutaneous injections of rhIL-15 (3mcg/kg) on the final day of radiotherapy and 20-30 hours post finishing radiotherapy.
- C. Method of Assigning Subjects to Treatment Groups – Single Arm Study
- D. Preparation and Administration of Investigational Product – See SOPs
Dogs will be premedicated with diphenhydramine at 2mg/kg IM or SQ 15 minutes before NK infusion. NK Cells will be injected intravenously on the last RT visit after recovery from anesthesia. Dose of allogeneic canine NK cells is 7.5×10^6 NK cells/kg extrapolating from our prior first-in-dog NK trial. Note, this dose is well below the MTD in human clinical trials where doses of NK cells reach 1×10^9 cells/ kg. NK cells will be resuspended in a 60 mL syringe of sterile 0.9% sodium chloride. GMP techniques will be used to ensure sterility of the NK cells, and cell preparations will be tested to ensure >90% viability. NK cell preparations will also be confirmed as endotoxin and mycoplasma negative prior to infusion. Cells will be injected as a slow bolus through an IV catheter and using a closed chemotherapy system. Dogs will then be given 3mcg/kg rhIL-15 SQ directly after NK cell infusion and then 20-30 hrs post infusion.
- E. Blinding of Study Intervention: Study is unblinded
- F. Prior and Concomitant Therapy: Prior and Concomitant Therapy: Bisphosphonates allowable. NSAIDS allowed if patient requires for pain control and has been on for greater than 2 weeks. No other concurrent therapy allowed. Two-week washout from chemotherapy. Four-week washout from prior radiation therapy and/or immunotherapy. All medications will be recorded in the study CRF.
- G. Storage and Handling of Investigational Product
Allogeneic NK cells/rhIL-15 (See Appendix D for full SOP):
1. Manufacturer: Grown in Canter Lab
 2. Storage Instructions: Will be stored at 4C after delivery on day on infusion and until use.
 3. Handling Instructions: Protect from light. Maintain sterility.
 4. Dispensation Instructions:
 5. Return or Disposition Instructions: Return to Canter Lab or dispose of in chemotherapy waste.
- Rh IL-15 for SQ injection (see Appendix E for full SOP):
1. Manufacturer: NIH

2. Storage Instructions:
3. Handling Instructions:
4. Dispensation Instructions:
Clinical trials will be responsible for thawing and mixing the IL-15 and pulling up into 3mL syringes total dose will be 3mcg/kg.
5. Disposition Instructions: Return to Canter lab or dispose of any extra in chemotherapy waste

VII. Investigational Requirements

A. Informed Consent

All owners must read and sign the Owner Informed Consent Document ([Appendix F](#)) prior to enrollment of their pet into the study. A copy of the signed consent form will be provided to the owner, one copy will be scanned into the patient medical record in VMACS on the visit for Day 0 and the original signed copy will be kept in the patient study binder.

B. Adverse Events / Safety Assessment

1. Definition of an Adverse Event: Any grade 1 or higher toxicity identified by VCOG or VRTOG criteria.
2. Method of Evaluating and Recording Adverse Events: Adverse events will be recorded and documented in the binder CRF forms using criteria laid out in references 1 & 2.
3. Required Adjustments if Adverse Events Occur
 - a) *Reporting Requirements for Serious Adverse Events: In the event of a serious adverse event, the Principal investigator will be notified.*
 - b) *Unblinding Procedures: N/A*
 - c) *Stopping Rules: if at any time the protocol is not being followed, it will be reported to the IACUC. Any grade 3 or 4 toxicities, except for in the event of transient neutropenia/thrombocytopenia or moist desquamation post RT.*

- B. System of Data Capture: Paper CRF records in a study binder that will be kept with the clinical trials coordinator.

- C. Confidentiality of Data
Identity of patients and their owners will be kept confidential in any presentations or publication of the data generated in this study.
- D. Retention of Records
 1. Location of Records During the Study: Study binders will be kept in the radiation oncology department.
 2. Individual(s) with Access to Records During the Study: PI, Co-Investigators, Study Coordinators, Attending Clinical Trials Doctor
 3. Location of Records After Study Completion: Once all patients have completed the study, the binders will be transferred to the Principal Investigator.
 4. Individual(s) with Access to Records After Study Completion: PI, Co-Investigators, Study Coordinators, Attending Clinical Trials Doctor

VIII. Study Budgeting / Billing

- A. Costs Covered by Study - Once determined eligible and the pet is enrolled, the study will cover the cost of appointment fees, blood sampling, sedation, tumor biopsies, and study related NK infusion, IL-15, PPE, and procedures. Study will also provide a \$2,000 incentive for enrolling in the study to be distributed at end of the study and for use at the UC Davis Veterinary Medical Teaching Hospital.
- B. Costs Not Covered by Study - Owners are responsible for eligibility screening which may include initial or recheck office examination, bloodwork including CBC/Chemistry panel/UA, thoracic radiographs, and confirmed diagnosis of a tumor. The costs associated with radiation therapy are not covered by the study.
- C. Costs Covered for Adverse Events - If the pet experiences adverse event(s) as a result of taking part in this study, and is in need of medical treatment, the study sponsors will offer to pay for medical treatment for injury/side effects up to \$2000. The study can ONLY pay for costs of therapy incurred at the UC Davis Veterinary Medical Teaching Hospital. Costs associated with treatment beyond \$2000 will be at the expense of the owner. This does not cover costs associated with expected radiation therapy side effects.

IX. References

1. Veterinary cooperative oncology group - common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1. *Veterinary and comparative oncology*, (Jul 20, 2011).
2. Ladue T, Klein MK; Veterinary Radiation Therapy Oncology Group. Toxicity criteria of the veterinary radiation therapy oncology group. *Vet Radiol Ultrasound*. 2001 Sep-Oct;42(5):475-6. PubMed PMID: 11678573.

3. Canter RJ, Gossenbacher SK, Foltz JA, Sturgill IR, Park JS, Luna JI, Kent MS, Culp WTN, Chen M, Modiano JF, Monjazeb AM, Lee DA, Murphy WJ. Radiotherapy enhances natural killer cell cytotoxicity and localization in pre-clinical canine sarcomas and first-in-dog clinical trial. *J Immunother Cancer*. 2017 Dec 19;5(1):98. doi: 10.1186/s40425-017-0305-7. PubMed PMID: 29254507; PubMed Central PMCID: PMC5735903.

X. Appendices

A. Appendix A

Procedure	Pre-Enroll Day -21 to 0	Visit RT#1	Visit RT Mid-Treatment	Visit RT Last Day	Visit RT One Day Post	Visit 1 Week Post	Visit 2 Weeks Post
Physical Exam	X	X	X	X	X	X	X
CBC	X		X	X	X	X	X
Chemistry	X		X	X	X	X	X
Urinalysis	X						
PBMCs/plasma IL-15		X	X	X	X	X	X
Notify Canter Lab with Schedule	X						
Thoracic Radiographs	X						
Radiotherapy		X	X	X			
NK Cell IV Infusion				X			
rhIL-15 SQ Injection				X	X		
Treatment Planning CT	X						
Tumor Biopsy		X		X		X	
Adverse Event Assessment		X	X	X	X	X	x

- B. Appendix B: Case Report Forms
 - 1. Adverse Events
 - 2. Concomitant medication form
 - 3. Enrollment Form
 - 4. Inclusion/Exclusion Form
 - 5. Owner Consent Form
 - 6. Road Map
 - 7. Clinical Visit Forms
 - 8. Blood Processing
 - 9. Tumor Processing

Appendix C

Blood and Tissue Processing

Collection of Tissue Samples (at appropriate time points per protocol):

1. Number and feasibility of core biopsies of tumor tissue to be determined by treating clinicians.
2. Allocation of tumor tissue (in order of priority):
 - a. 2 cores/ punch biopsies into RPMI media at 4C for immediate analysis by flow cytometry.
 - b. If additional tissue, place into RNA later and frozen at -20C for PCR.

Collection of Blood Samples (at appropriate time points per protocol):**For Research Evaluation:**

1. 10 mLs blood in purple top tube/ tubes. Tube to be kept in 4C in refrigerator until pickup. Please call Canter/Murphy lab for pickup within 1 hour of procurement.
2. Phone Numbers to call for pick-up in order:
 - a. Sean Judge- 408-806-4945,
 - b. Logan Vick- 916-601-7629 ,
 - c. Bob Canter-215.964.0468 Email: rjcanter@ucdavis.edu

Cryovial labeling example

Sample Type
Patient Number
Age
Date/Initials

N.B.: If Canter/Murphy lab personnel coming to pick up specimens same day, please place specimens into 4C (refrigerator) in Eppendorf or other tubes. Canter/Murphy lab personnel will transport specimens for further processing as noted.

Appendix D

SOP for NK Cell/IL-15 preparation and administration

Dogs will be premedicated with diphenhydramine at 2 mg/kg IM or SQ 15 min prior to injection

1. Call/Text Clinical Trials Coordinators TBD prior to leaving with NK cells
(Address is one Garrod Drive, Davis CA, Center for Companion Animal Health)

NK cells will be injected intravenously on the last day of radiotherapy of protocol following completion of RT and recovery of the dog from anesthesia.

2. NK cells will be in T75 flasks labeled with study number in incubator.
3. Pool all the NK cells together into 50 ml conical vial. Spin at 1200 RPM for 5 min.
4. Dump supernatant. Then resuspend cells using RF10c.
5. Count the NK cells by hemocytometer to get estimate of viability. (need >90% viability). Then okay to count by Coulter as long as viability is accounted for. Use separate count sheet.
6. Spin the NK cells at 1200 RPM for 5 min. Dump supernatant.
7. Resuspend the NK cells in 60 ml of sterile 0.9% sodium Chloride
8. Add rhIL15 at 5 ng/mL.
9. NK cell preparations need to be confirmed as endotoxin and mycoplasma negative prior to adoptive transfer using kits.
10. Bring the syringe labeled on ice to the UC Davis School of Veterinary Medicine CCAH. Cover in foil as it is light sensitive. See address above. Let trials coordinator know approximate time of arrival.
11. Cells will be injected by slow IV bolus through a cephalic or saphenous vein catheter.

Call Dr. Canter with any questions: cell # 215-964-0468

Appendix E
SOP for preparation of rhIL-15 for subcutaneous injection
Pending check on available concentrations of drug.

APPENDIX F

Informed Consent document – Please note document will be available in PDF format in Box folder for printing.

I. Study Objectives

- A. Primary – To determine the maximum tolerated dose (MTD) for inhaled rhIL-15 in dogs with lung metastases.
- B. Secondary – Measure Immune correlates (tumor aspirates and systemic PBMCs), response rate, response duration, median survival time.

II. Investigational Plan

- A. Overall Study Design –Phase I Trial; Dose Escalation
- B. Statistical Plan

Sample Size Determination: Up to 18 dogs will be enrolled in this phase 1 study. Dose escalation will follow a fixed dose modified Fibonacci method, where the dose is escalated 100, 67, 50, 40 and then 33% of the previous dose as the cohorts increase. Since an MTD of IH IL-15 has not been established in dogs, we will escalate until dose-limiting toxicities (DLTs) are observed. However, we will not escalate past dose level 6 even if MTD is not reached. A DLT will be defined as = grade 3 toxicity in any category (except hematologic) according to the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events.

Statistical Methods: In this Phase I trial, the MTD will be defined as the highest dose level at which no more than 1/6 of the subjects develops a DLT. Three patients will be enrolled per dose level, with escalation to the next dose level if no DLT is observed. If one DLT is observed, the dose level will be expanded to a total of 6 patients, and escalation will occur if no more than one DLT is observed among the 6 patients. If 2 or more patients in any cohort experience DLT, this will be defined as the maximally administered dose, and the phase I study will be concluded or dose reduced to the previous dose level (then defined as the MTD). Dose level escalation will be determined based on DLTs observed during therapy, but DLTs will be monitored after the cessation of treatment, and dose de-escalation may occur if significant late DLTs are observed.

Subject Populations for Analyses:

C. Selection of Study Population

Number of Subjects: up to 21

Species: Canine

Breed: No Specification

Initial Age: At least 1-year-old on Day 0

Weight: At least 10kg on Day 0

Sex: Male or Female, Intact or Neutered

Origin / Source: Client-Owned Pets

Dogs participating in this study will be privately owned. Dogs will be identified by their given name and owner's surname, as recorded in their medical records. In addition, dogs will be identified by both their assigned by their unique medical record number in VMACs, and by a study number that will consist of UCD-followed by a 4-digit sequential number starting with the number (10) e.g. UCD-IL15-101, UCD-IL15-102, UCD-IL15-103, etc.

Additionally, Dogs will be identified in Red Cap Precinct 01 Penn Medicine with a unique ID starting with 40 (for Davis Site) followed by the corresponding above three digit number 101, 102, 103, etc. Example 40101, 40102

In the event the study opens up to additional sites, the study coordinator will assign the site a two digit code. The site will establish their subjects as listed above using their given university two or three digit initials, followed by the two digit code. (e.g. UW-IL15-201, CSU-IL15-301).

Previous Treatments: Normal vaccination and general health care practices are permitted. No prior chemotherapy within 2 weeks to day 0. No prior immunotherapy or radiation therapy within the last 4 weeks.

Husbandry: Normal feeding and housing post procedures until dog is released to their owners. Normal feeding and housing as provided by individual dog owners post procedures.

D. Study Schedule – Appendix A

III. Study Implementation

A. Inclusion Criteria:

- Dogs > 1 year

- Radiographs performed within 14 days of enrollment (Day 3) consistent with metastatic disease from cytologic or histologic confirmed osteosarcoma or melanoma.
 - Adequate local control of Primary tumor (Surgery or Radiation)
 - Body Weight \geq 10 kg
 - VCOG-CTCAE 1.1 performance score of 0 or 1
 - CBC and Chemistry performed within 14 days of enrollment (Day 3) showing adequate organ function: HCT \geq 25%, Neutrophil Count \geq 2,000/ul, Platelet Count \geq 75,000/ul, Creatinine \leq ULN; bilirubin \leq ULN; ALT \leq ULN; AST \leq ULN
 - Urinalysis performed within 14 days of enrollment (Day 3)
 - **One or more lung lesion measuring at least 1cm on radiographs**
- B. Exclusion Criteria
- Dogs unable to undergo sedation for chest films
 - Owner unwilling to administer inhaled IL-15 twice daily and/or dog unable to tolerate twice daily nebulization
 - Chemotherapy within 2 weeks of day 0
 - Immunotherapy or radiation therapy within 4 weeks of Day 0
 - VCOG-CTCAE 1.1 performance score of 2 or higher
 - Concurrent therapy (NSAIDs acceptable if needed for pain control and patient has been receiving for 2 weeks or more). Pamidronate or zoledronate also acceptable
- C. Visit Descriptions
- **Pre-Enrollment Screening (Day -7):** Dogs will be evaluated to determine eligibility. If the dog appears to meet preliminary inclusion criteria, a formal appointment/consultation (trial service) will occur. At which time, the owners will be given detailed information on the trial, including nebulization. If they are interested, the owner will review the Informed Consent Form with trial staff and sign the document. Owners will be trained in nebulization treatments (including a demonstration with the patient) and will be sent home with saline and a nebulizer to desensitize the dog. Clients will be responsible for the cost of the physical examination and the screening CBC, blood chemistry, and urinalysis, as well as any other screening tests deemed appropriate by the attending clinician. Screening CBC/CHEM/UA performed by outside reference laboratories are acceptable if they are performed within 14 days (Day -14) of enrollment (Day 3). A copy of the histologic or cytologic diagnosis of osteosarcoma or melanoma will be recorded in the study binder and in patient chart. These tests can be done prior to or after desensitization. If the dog is eligible and is successfully desensitized, then the patient will either be scheduled for an optional FNA on Day 0 with a subsequent enrollment on Day 3 or will forgo the FNA and proceed to enrollment on Day 3. Patients requiring longer than 7 days for desensitization are still eligible, however, eligibility/screening criteria will need to adhere to study requirements and therefore may need to be repeated (at the cost of the owner) to confirm eligibility.
 - **Day 0 (optional):** Quality of Life Form (QOL), Blood sample will be obtained for CBC, PBMCs and Serum; FNA of pulmonary lesion (see appendix), 3-view thoracic

radiographs (If pre-enrollment rads not done within 14 days of Day 3), primary tumor measurement (melanoma only). Owner sent home with nebulizer and drug.

- **Day 3 (Enrollment):** Physical examination, (QOL), Start nebulization with IL-15 (first dose followed by 6 hours monitoring, in hospital). Sampling catheter and serial blood sampling for PK data. **If Subject did not complete a Day 0 visit then the following will also be performed:** 3 view thoracic radiographs (if not performed within the previous 14 days), PBMC, Serum, CBC/Chem 2.
- **Day 10:** Physical examination, (QOL), CBC/CHEM/PBMCs/Serum;
- **Day 17:** Physical examination, (QOL), Blood sample CBC/CHEM/PBMCs/Serum; Ultrasound guided fine needle aspirate of a single pulmonary metastatic lesion (see attached protocol)
 - Owner returns nebulizer and unused drug to center
- Day 31:** Physical examination, (QOL), Blood sample for CBC, CHEM, PBMCs/Serum; Thoracic radiographs.
- **Day 45:** Physical examination, (QOL), Thoracic Radiographs
- **Day 73:** Physical examination, (QOL), Thoracic Radiographs
- **Day 101:** Physical examination, (QOL), Thoracic Radiographs; end-of-study
- **Long Term Follow-up:** Following the Day 101 visit, patients will return every 28 days for monitoring of disease progression. A Physical examination, (QOL), Thoracic Radiographs will be performed at these visits.

D. Criteria to Remove Subjects from Study Post-Enrollment

- If deemed clinically indicated by the attending clinician
- If requested by the owner
- **Progression of disease is not a specific cause for removal**

E. Biological Sample Collection, Processing, & Storage

Blood: PBMCs and Serum will be collected on Days 0, 10, 17, 31; CBC/Chemistry 2 performed within 14 days of day 0, and on days 10, 17, and 31.

See Appendix for PBMC/Serum Protocol and storage.

Urine: screening urinalysis only

Fine needle aspirates of metastatic pulmonary nodules for RNA analyses: Day 0 and 17 if not contraindicated in the opinion of the attending clinician.

See Appendix for FNA Protocol, and handling.

F. Imaging:

Three-view thoracic radiographs consistent with metastatic disease should be performed within one month of Day -7 documenting metastatic lung lesions for eligibility. Radiographs will be repeated on Days 0, 31, 45, 73, and 101

G. Data Quality Assurance

This study will be conducted in accordance with 21 CFR 511.1

(<https://www.gpo.gov/fdsys/pkg/CFR-2010-title21-vol6/pdf/CFR-2010-title21-vol6-sec511-1.pdf>) and under the principles of Good Clinical Practices as defined in VICH GL9.

(<http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforceme>

[nt/GuidanceforIndustry/UCM052417.pdf](https://www.bmj.com/nt/GuidanceforIndustry/UCM052417.pdf)

IV. Investigational Product, Drug, or Device

A. Description: Recombinant Human Interleukin-15 (IL-15)-E. Coli product is described by the NCI Drug Dictionary as,

- A recombinant agent that is chemically identical or similar to the endogenous cytokine interleukin-15 (IL-15) with immunomodulating activity. IL-15, secreted by mononuclear phagocytes (and some other cell types) following viral infection, regulates T and natural killer cell activation and proliferation. This cytokine induces activation of transcription activators STAT3, STAT5, and STAT6 via JAK kinase signal transduction pathways in mast cells, T cells, and dendritic epidermal T cells. IL-15 and interleukin-2 (IL-2) are structurally similar and share many biological activities; both may bind to common hematopoietin receptor subunits, negatively regulating each other's activity. CD8+ memory T cell number has been shown to be regulated by a balance between IL-15 and IL-2.
- SEE SECTION D or APPENDIX FOR FULL SOP DRUG PREPARATION/CALCULATION

B. Treatment Regimen:

The objective of this clinical trial is to determine the maximally tolerated dose (MTD) of Inhaled IL-15 to client owned animals that present with lung metastasis from melanoma or osteosarcoma.

C. Method of Assigning Subjects to Treatment Groups:

Standard 3 + 3 phase I cohort design. Dose escalation rules based on three (3) dog cohorts will be used to define a well-tolerated dose. Escalation will be based on assessment of a DLT, defined as any grade 3 non-hematologic or grade 4 hematologic toxicity. There will be a one week observation period between cohort escalations. The first two dogs in any cohort can be enrolled within one week of one another, but the 3rd dog or any other subsequent dog in the cohort must have a one-week waiting period after the previous dog enrolled.

1. If DLT is seen in 0/3 dogs, the dose will be escalated.
2. If DLT is seen in 1/3 dogs, an additional (up to) five dogs will be enrolled at that prescribed dose. If no DLT are seen with the additional five dogs (DLT 1/6), escalation may continue to the next higher dose
3. If DLT is seen in 2 or more dogs (2/2, 2/3, 2/4, 2/5 or 2/6) dogs within a group, the MTD will be defined as the dose administered in the cohort below.

The starting dose of IL-15 in the first cohort will be 10ug

D. Preparation and Administration of Investigational Product:

SEE APPENDIX FOR FULL SOP:

rhIL-15 Stock = 510ug/mL in solution of 25mM Sodium Phosphate, 500mM Sodium Chloride, pH 7.4. 1mL volume per vial. Stored at -80C. The IL-15 will be thawed per dosing cohort (See chart).

Volume chart for making up 90 or 100mL of IL-15 final working concentrations with 0.01% CSA and 0.9% Saline.

<i>DOSE LEVEL</i>	<i>Dose Escalation</i>	<i>Actual Dose</i>	Actual IL15 Conc.	Saline Vol. to Discard	1% CSA to Add	Stock 1L-15 (510µg/mL) to Add	Vials of IL-15 to Thaw
1	---	10ug	3.333 µg/mL	1.7 mL	1.0 mL	0.65 mL	1
2	100%	20ug	6.666 µg/mL	2.3 mL	1.0 mL	1.31 mL	2
3	67%	33ug	11.0 µg/mL	12.8 mL	0.9 mL	1.94 mL	2
4	50%	50ug	16.667 µg/mL	13.8 mL	0.9 mL	2.94 mL	3
5	40%	70ug	23.333 µg/mL	5.6 mL	1.0 mL	4.58 mL	5
6	33%	93ug	31.0 µg/mL	16.4 mL	0.9 mL	5.47 mL	6

Dispensing:

Clinical trials will be responsible for thawing and mixing the IL-15 and pulling up into 3mL syringes. The pharmacy will create a label: The script will be printed by the pharmacy and include:

- ***Refrigerate*** place 3mL of prepared drug into the nebulizer well. Administer twice a day over 10- 15 minutes until vapor is no longer seen, as demonstrated for owner, for 14 days. IL-15 will need to be packaged on ice for transport with care to avoid freeze/thaw.

- E. Blinding of Study Intervention:
All dogs will receive the drug. Study is unblinded.
- F. Prior and Concomitant Therapy:
Bisphosphonates allowable. NSAIDS allowed if patient requires for pain control and has been on for greater than 2 weeks. Nother concurrent therapy allowed. Two week washout from chemotherapy. Four week washout from radiation therapy and/or immunotherapy. All medications will be recorded in the study CRF.
- G. Storage and Handling of Investigational Product
Manufacturer: Biological Resources Branch; National Cancer Institute **Storage Instructions:** Store at $\leq -70^{\circ}\text{C}$
Handling Instructions: Once prepared, IL-15 may stored at 4c for up to 14 days **Dispensation Instructions:** Study medication for the nebulizer will be dispensed in 3ml syringes, for a 14 day supply. Owner will be sent home with nebulizer, tubing, nosecone and/or hood, and Personal Protective equipment including, chemo gown, disposable N95 mask, eye protection, and gloves. Owner will be instructed to use eye protection and instructions to perform in an well ventilated area. Label will be printed to include *Refrigerate* place 3mL of prepared drug into the nebulizer well. Adminster twice a day am and pm (8-12 hours apart) over 10-15 minutes as demonstrated (until no vapor is seen). *See Owner handout*.
Return or Disposition Instructions: On Study Day 17, the owner will be instructed to return the nebulizer, hose, safety equipment, and any unused drug, and all empty syringes for proper disposal. In the event the subject is not able to return for future visits, the owner will be given a FEDEX label with instructions for returning the machine.

V. Investigational Requirements

- A. Informed Consent
All owners must read and sign the Owner Informed Consent Document ([Appendix B](#)) prior to enrollment of their pet into the study. A copy of the signed consent form will be provided to the owner, one copy will be scanned in to the patient medical record in VMACS on the visit for Day 0 and the original signed copy will be kept in the patient study binder.
- B. Adverse Events / Safety Assessment
Definition of an Adverse Event: Any grade 1 or higher toxicity identified by VCOG criteria.
Method of Evaluating and Recording Adverse Events: Adverse events will be recorded and documented in the binder CRF forms.
Required Adjustments if Adverse Events Occur
- *Reporting Requirements for Serious Adverse Events: In the event of a serious adverse event, the Principal*

investigator will be notified. If 2 or more serious adverse events at a grade 3 toxicity in any category (except hematologic) are identified in a cohort, the cohort will be closed.

- *Unblinding Procedures: N/A*
- *Stopping Rules if at anytime the protocol is not being followed, it will be reported to the IACUC. Any grade 3 or 4 toxicities, except for in the event for transient neutropenia/thrombocytopenia. The study may be halted if a MTD is seen within any of the cohorts.*

C. System of Data Capture:

Paper CRF records in a study binder will be kept in Oncology.

D. Confidentiality of Data

Identity of patients and their owners will be kept confidential in any presentations or publication of the data generated in this study.

E. Retention of Records

Location of Records During the Study: Study binders will be kept in the office of CCAH 171 while the study continues to have active patients.

Individual(s) with Access to Records During the Study: PI, Co-Investigators, Study Coordinators, Attending Clinical Trials Doctor

Location of Records After Study Completion: Once all patients have completed the study, the binders will be transferred to the Principal Investigator.

VI. Appendices

A. Appendix A

<i>*Pr e - Enr oll me nt Sali ne for ow ner to des ens titize</i>	Procedure	Pre-Enroll Day -7	Patient Desensitized	Day 0	Day 3	Day 10	Day 17	Day 31	Day 45	Day 73	Day 101	Long Term Follow-up[^]
	Physical Examination	X		X	X	X	X	X	X	X	X	X
	Quality of Life			X	X	X	X	X	X	X	X	X
	CBC	X		X		X	X	X				
	CHEMISTRY	X				X	X	X				
	Urinalysis	X										
	PBMC			X		X	X	X				
	Thoracic Radiographs			X				X	X	X	X	X
	Ultrasound Guided Asp of Lung Met			X			X					
	Inhaled IL-15 (*saline)	*X	*X		X	X ¹						
	PK collection				X							

1 Owner is administering at home prior to appointment.

[^] Long Term Follow-up Visits will occur every 28 days after the Day 28 visit

Ver 2.0 04Aug2020

IACUC# 20179

IACUC - Protocol Review

9/7/18, 10:46 AM

Staff Reviewer: Nicole Dias**Committee Reviewer 1:** not assigned**Committee Reviewer 2:** not assigned**Reviewer:** Robert Rebhun - *review completed 07-25-2018*

# Questions: 3	University of California, Davis PROTOCOL FOR ANIMAL CARE & USE	Protocol: 20668
# Questions Sent: 0		Expires:
# Responses: 0		

1. Contacts

[Ask a Question \(section 1\)](#)
[Review Questions/Responses](#)

Primary Investigator		Alternate Contact	
Name:	Robert B Rebhun *	Name:	Robert J Canter
E-mail:	rbrehhun@ucdavis.edu	E-mail:	rjcanter@ucdavis.edu
Department:	VM: Surgical & Radiological Science	Department:	MED: Surgery, Div of Surgical Oncology
Telephone:	530-754-5028	Telephone:	916-734-7044
After Hours:	530-219-5960	After Hours:	215-964-0468

**Primary contact for sick animals*

2. Title

[Ask a Question \(section 2\)](#)
[Review Questions/Responses](#)

ALT-803 Immunotherapy for Treatment of Lung Metastases

Replacement Protocol: No**3. Protocol Type**

[Ask a Question \(section 3\)](#)
[Review Questions/Responses](#)

Research
VMTH Clinical Trial

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[DocUpload-01-86084-\(CTRB Consent form\)_ClinicalTrial.docx](#)

4. Species

[Ask a Question \(section 4\)](#)
[Review Questions/Responses](#)

Common Names	Total Number for Study	Name of Source of the Animals
dog - <i>client-owned dogs</i>	14	VMTH Oncology Service Population

USDA: No**Detrimental Species:** No**5. Brief Summary of Procedures**

[Ask a Question \(section 5\)](#)
[Review Questions/Responses](#)

We propose a phase 1 clinical study to assess the safety and efficacy (secondary endpoint) of ALT-803, an IL-15 superagonist. In brief, dogs with measurable (on radiographs) lung metastases who have exhausted standard treatment options or whose owners have declined such therapy will have up to 20 mLs of blood drawn by venipuncture on days 0,

7, 10, 17, 24, 31, 45, 73, and 101. Dogs will receive once weekly ALT-803 for a total of four treatments. An ultrasound guided fine needle aspirate of one pulmonary mass will be performed on day 0 (prior to starting ALT-803) and again on day 31 of the study if deemed safe to do so based on ultrasound appearance and location. Animals will be monitored for any side effects from treatment, and then followed by a chest x-ray at days 31, 45, 73, 101. After day 101, chest x-rays will be performed every 2-3 months as or as clinically recommended. The trial will formally end at the 101 day radiographs.

6. Animal Location(s)

[Ask a Question \(section 6\)](#)
[Review Questions/Responses](#)

Study Area/Laboratory:

Building - Room	AAALAC
None/Animals Will Not Leave Animal Facility	-

Overnight Housing (vivarium):

Vivarium(s)
VMTH - Small Animal Clinic

Animals will be maintained by:

Vivarium

7. Special Husbandry Requirements:

[Ask a Question \(section 7\)](#)
[Review Questions/Responses](#)

There are no special requirements.

8. Hazardous Materials:

[Ask a Question \(section 8\)](#)
[Review Questions/Responses](#)

No

9. Special Procedures and/or Activities:

[Ask a Question \(section 9\)](#)
[Review Questions/Responses](#)

None - No Special Procedures and/or Activities

10. Funding Source(s):

[Ask a Question \(section 10\)](#)
[Review Questions/Responses](#)

National Cancer Institute

11. Veterinary Care:

[Ask a Question \(section 11\)](#)
[Review Questions/Responses](#)

VMTH Small Animal Service

12. Objectives and Significance:

[Ask a Question \(section 12\)](#)
[Review Questions/Responses](#)

Objectives:

To determine the maximum tolerated dose (MTD) for ALT-803 in dogs with lung metastases. Response to therapy and peripheral immune correlates are secondary endpoints.

Significance:

If successful, this trial will provide important evidence in a large animal, outbred model of spontaneous metastasis that ALT-803 is a safe and potentially effective treatment for dogs with lung metastasis. Dogs with lung metastases have a dismal prognosis and response rates to standard chemotherapy are less than 20-30% for most tumor types. Therefore, these patients are ideal candidates for novel therapies. Future work (Aim 3) related to this grant will build upon these findings by combining this therapy with standard chemotherapy for dogs with osteosarcoma.

13. The 3 R's - Refinement, Replacement, and Reduction:
[Ask a Question \(section 13\)](#)
a) Database Search for Alternatives:
[Review Questions/Responses](#)

1) Does this project involve USDA covered species? **No**

b) Refinement:

Through the use of proper handling, we will minimize any potential pain and distress in the animals

c) Has this study been previously conducted?

No

d) Replacement (Species Rationale):

Dogs are the target species for this therapy. Dogs will also serve as a spontaneous tumor model for future human studies.

e) Reduction (Animal Numbers Justification):

This trial will utilize an accelerated phase 1 study design. We plan to use a 40% dose escalation between single-patient cohorts in the accelerated phase which reverts to a standard 3+3 cohort design when one dose-limiting toxicity (DLT) or two moderate toxicities (excluding minor dermatologic toxicities at injection site) are observed during any cycle. We will start at 30 ug/kg and proceed to 42 ug/kg, 60 ug/kg, 82 ug/kg, 115 ug/kg, and 160 ug/kg. We estimate that up to 14 patients will be enrolled. This is based on the conversion to a 3 + 3 Study design, where up to 5 dogs could be enrolled at the first dose for which a dose limiting toxicity is observed. Assuming a patient fallout rate of 20% (2 dogs), we arrived at an estimate of 14 dogs. This study design is intended to minimize the number of dogs used in the study, while also minimizing the number of dogs exposed to potentially subclinical dosing of ALT-803.

Since an MTD of ALT-803 has not been established in dogs, we will escalate until dose-limiting toxicities (DLTs) are observed. However, we will not escalate past dose level 6 even if MTD is not reached. A DLT will be defined as = grade 3 toxicity in any category (except hematologic) according to the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events.

In this Phase I trial, the MTD will be defined as the highest dose level at which no more than 1/6 of the subjects develops a DLT.

Dose level escalation will be determined based on DLTs observed during therapy, but DLTs will be monitored after the cessation of treatment, and dose de-escalation may occur if significant late DLTs are observed. Necropsy will be strongly encouraged on all subjects, and careful histologic evaluation will be performed to determine cause of death from cancer progression versus immuno-pathology. The primary objective of this study is to determine the MTD of ALT-803. Up to 14 client-owned clinical dogs with OSA or melanoma pulmonary metastases will be enrolled in this sub-aim. In addition to toxicity, secondary outcome measures of efficacy will be evaluated including response rate, response duration, and median survival time.

14 dogs in this arm should also allow us to evaluate immune correlates.

f) Study Groups and Numbers Table:

Group	Species	Number of Animals	Procedures/Treatments
Dogs with lung metastases	dog - <i>client-owned dogs</i>	14	

14. Procedure Details:
[Ask a Question \(section 14\)](#)

a) Describe the use of animals in your project[Review Questions/Responses](#)

All owners will sign the CTRB approved consent form prior to enrollment. Up to 14 client-owned dogs with pulmonary metastasis greater than 2cm, for which all other reasonable treatment options have been exhausted or declined, will be eligible for entry into the trial. Prior to entry into the trial, the dog will undergo a physical exam and routine hematologic evaluation with a CBC and serum chemistry panel as well as a urinalysis. 2-6 mls of venous blood will be collected from the jugular vein or other peripheral vessel as is standard for clinical patients in the VMTH.

The UCD blood collection policy will be followed. (i.e. 1% of animals body weight can be collected in 24 hours every 14 days) for any samples listed above. Patients less than 10kg will be excluded.

Other analgesics as needed which as assessed by the attending clinician including NSAIDs, tramadol, gabapentin or opioids are allowed but not part of the protocol.

Appropriate sedation protocols for chest x-rays (and fine needle aspirates) may be done on an individual patient basis based upon the recommendations made by a board certified veterinary anesthesiologist from the VMTH Clinical Anesthesia Service, however, this will most commonly be performed with dexmedetomidine and butorphenol. Monitoring under sedation will be standard and as is done for all VMTH clinical patients and at a minimum will include heart rate and respiratory rate monitoring, mucous membrane color, CRT as well as assessing for appropriate depth of sedation. Recovery from sedation will take place under supervision of a VMTH clinical veterinarian or licensed veterinary technician. All compounds given to the animals are pharmaceutical grade.

Prior to therapy, an ultrasound-guided fine needle aspirate of a single pulmonary metastatic lesion will be obtained under sedation when possible and deemed safe to do so.

Day 0- Blood sample obtained for evaluation of peripheral blood mononuclear cells (PBMCs), Fine needle aspirate of pulmonary lesion, chest x-rays Day 3- ALT-803 Day 7- Blood sample for PBMCs, CBC/CHEM Day 10- Blood sample for PBMCs, CBC/CHEM, ALT-803 Day 17 - Blood sample for PBMCs, CBC/CHEM, ALT-803 Day 24 - Blood sample for PBMCs, CBC/CHEM, ALT-803 Day 31- Blood sample obtained for evaluation of peripheral blood mononuclear cells (PBMCs), Fine needle aspirate of pulmonary lesion, chest x-rays Day 42 - Blood sample for PBMCs, CBC/CHEM, chest x-rays Day 70 - Blood sample for PBMCs, CBC/CHEM, chest x-rays Day 98 - Blood sample for PBMCs, CBC/CHEM, chest x-rays

Serial measurements of blood and serum will be obtained weekly to assess for hematologic (WBC, hemoglobin, platelet count, and sub-populations) and biochemical toxicity (serum Na, K, Cl, glucose, alkaline phosphatase, ALT, AST, bilirubin, BUN, creatinine, albumin, and globulin). Blood will be obtained for PBMC evaluation

ALT-803 treatments will be given subcutaneously and all injections will be performed here at the VMTH in order to monitor for any complications. The owners will be informed to look for clinical signs such as dyspnea, tachypnea, hyperemia, anaphylaxis, depression, etc. and advised to call or come in immediately if any clinical signs should be displayed. Blood pressure, heart rate, and temperature will be monitored periodically for 6-8 hours to assess for vasomotor/hemodynamic toxicity on days of injection.

In this Phase I trial, the MTD will be defined as the highest dose level at which no more than 1/6 of the subjects develops a DLT.

This trial will utilize an accelerated phase 1 study design. We plan to use a 40% dose escalation between single-patient cohorts in the accelerated phase which reverts to a standard 3+3 cohort design when one dose-limiting toxicity (DLT) or two moderate toxicities (excluding minor dermatologic toxicities at injection site) are observed during any cycle. We will start at 30 ug/kg and proceed to 42 ug/kg, 60 ug/kg, 82 ug/kg, 115 ug/kg, and 160 ug/kg. We estimate that up to 14 patients will be enrolled. This is based on the conversion to a 3 + 3 Study design, where up to 5 dogs could be enrolled at the first dose for which a dose limiting toxicity is observed. Assuming a patient fallout rate of 20% (2 dogs), we arrived at an estimate of 14 dogs. This study design is intended to minimize the number of dogs used in the study, while also minimizing the number of dogs exposed to potentially subclinical dosing of ALT-803.

Dose level escalation will be determined based on DLTs observed during therapy, but DLTs will be monitored after the cessation of treatment, and dose de-escalation may occur if significant late DLTs are observed. Necropsy will be strongly encouraged on all subjects, and careful histologic evaluation will be performed to determine cause of death from cancer progression versus immuno-pathology.

We do not plan to escalate past dose level 6 even if MTD is not reached. A DLT will be defined as = grade 3 toxicity in any category (except hematologic) according to the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events (Veterinary Cooperative Oncology Group, 2011).

Previous toxicity studies have been performed in mice and non-human primates, both of which tolerated weekly 100 ug/kg subcutaneous dosing of ALT-803. Human clinical trials are currently underway and toxicity has been found to be acceptable in dosing ranging from 6-30 ug/kg.

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b) All Drugs and Compounds to be Administered to the Animals (except for euthanasia) - anesthetics, analgesics, neuromuscular blocking agents, antibiotics and/or experimental compounds:

Will drugs be used in this study? **Yes**

Species	Drug	Dose	Route	When and how often will it be given?
dog	Atipamazole	0.2-0.4 mg/kg	Intramuscular (IM)	For reversal of dexmedetomidine sedation. Will be administered IM as same volume as dexmedetomidine was used
dog	ALT-803	up to 160 ug/kg	Subcutaneous (SC)	Once weekly for four weeks
dog	butorphanol	0.2 mg/kg	Intravenous (IV)	If needed prior to imaging or aspirates
dog	dexamethasone	0.2 mg/kg	Intravenous (IV)	if needed for anaphylactic reaction
dog	dexmedetomidine	125-375 mg/m squared	Intravenous (IV)	If needed prior to imaging or aspirates
dog	epinephrine	0.01 mg/kg	Intravenous (IV)	once, only in extreme cases. Can be repeated if no improvement.
dog	Diphenhydramine	2-4 mg/kg	Intramuscular (IM)	if needed for anaphylactic reaction

15. Adverse Effects:

[Ask a Question \(section 15\)](#)
[Review Questions/Responses](#)

a. Describe all significant adverse effects that may be encountered during the study.

Due to treatment: anaphylaxis during administration, pulmonary edema, vomiting, diarrhea, anorexia, infection, fever, death. Due to aspirates: bleeding, pneumothorax, respiratory distress, death

b. Describe frequency for monitoring the well-being of animals on the study and criteria for terminating/modifying the procedures(s) if adverse effects are observed.

Serial measurements of blood and serum will be obtained weekly to assess for hematologic (WBC, hemoglobin, platelet count, and sub-populations) and biochemical toxicity (serum Na, K, Cl, glucose, alkaline phosphatase, ALT, AST, bilirubin, BUN, creatinine, albumin, and globulin). In this Phase I trial, the MTD will be defined as the highest dose level at which no more than 1/6 of the subjects develops a DLT. Three patients will be enrolled per dose level, with escalation to the next dose level if no DLT is observed. If one DLT is observed, the dose level will be expanded to a total of 6 patients, and escalation will occur if no more than one DLT is observed among the 6 patients. If 2 or more patients in any cohort experience DLT, this will be defined as the maximally administered dose, and the phase I study will be concluded or dose reduced to the previous dose level (then defined as the MTD). Dose level escalation will be determined based on DLTs observed during therapy, but DLTs will be monitored after the cessation of treatment, and dose de-escalation may occur if significant late DLTs are observed. Necropsy will be strongly encouraged on all subjects, and careful histologic evaluation will be performed to determine cause of death from cancer progression versus immuno-pathology. If an owner elects euthanasia, then it will be allowed. After discharge, the owner will have a number to call if there are any perceived side effects. The patients will be evaluated once weekly per protocol and on an as needed basis by the oncology service.

Adverse event forms are generated for all oncology trials based on VCOG adverse events criteria. Owners will be informed to monitor for signs of fever, lethargy, decreased appetite, respiratory changes, etc. Side effects of inhaled IL-2 have not been seen previously.

c. How will the signs listed above be ameliorated or alleviated?

Serial measurements of blood and serum will be obtained weekly to assess for hematologic (WBC, hemoglobin, platelet count, and sub-populations) and biochemical toxicity (serum Na, K, Cl, glucose, alkaline phosphatase, ALT, AST, bilirubin, BUN, creatinine, albumin, and globulin). In this Phase I trial, the MTD will be defined as the highest dose level at which no more than 1/6 of the subjects develops a DLT. If one DLT is observed, the dose level will be expanded to a total of 6 patients, and escalation will occur if no more than one DLT is observed among the 6 patients. If 2 or more patients in any cohort experience DLT, this will be defined as the maximally administered dose, and the phase I study will be concluded or dose reduced to the previous dose level (then defined as the MTD). Dose level escalation will be determined based on DLTs observed during therapy, but DLTs will be monitored after the cessation of treatment, and dose de-escalation may occur if significant late DLTs are observed. Necropsy will be strongly encouraged on all subjects, and careful histologic evaluation will be performed to determine cause of death from cancer progression versus immuno-pathology.

d. List the criteria to be used to determine when euthanasia is to be performed or the animal will be removed from the study.

If quality of life is considered compromised by the owner, then euthanasia will be performed if requested by the owner. Otherwise, all clinically available palliative care modalities will be used. If the attending clinician feels that the dog's quality of life is compromised while on this trial they can be removed from the trial without the owner's consent. Alternative palliative measures or humane euthanasia will also be offered based on the clinical situation as appropriate. Other study endpoints may include withdrawal by the owner, progression of disease, decreased quality of life, or adverse event profile.

16. Euthanasia:

[Ask a Question \(section 16\)](#)
[Review Questions/Responses](#)

Species	Method	Drug	Dose	Route	Justification for Physical Methods
dog	Overdose	Pentobarbital	100 mg/kg	Intravenous (IV)	N/A

17. Disposition:

[Ask a Question \(section 17\)](#)
[Review Questions/Responses](#)

We do not anticipate that any of these animals will be euthanized as a direct result of therapy. There may be progression of disease (despite therapy) that warrants euthanasia. When the trial period is concluded, patients will remain under the care of veterinarians within the clinical oncology service, and owners will be given further treatment options including palliative care options. All live animals will go home with their owners.

18. Roster:

[Ask a Question \(section 18\)](#)
[Review Questions/Responses](#)

Name	Title/Degree	E-mail	OccHealth Participation		ACU 101 Training	Rodent Survival Surgery Course	Qualifications
			Completion	Renewal			
Burton, Jenna H.	Asst Clin Professor	jburton@ucdavis.edu	04-20-2016	04-20-2019	10-04-2016	Not Found	Dr. Burton is a board certified medical oncologist with extensive clinical trials experience and 8 years experience treating dogs with cancer.
Canter, Robert J.	Associate Professor	rjcanter@ucdavis.edu	09-18-2015	09-16-2018	11-28-2017	10-07-2013	Will be overseeing NK

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							cell expansion and preparation. Will not be involved in handling animals
Guerrero, Teri A.	Clinical Trials Coord	tguerrero@ucdavis.edu	04-20-2017	04-20-2020	01-12-2016	Not Found	Ms. Guerrero has been working with dogs in a veterinary setting since 1996 and she has an RLAT certification. She has been employed by the veterinary school at UC Davis since 2007 and serves as the clinical trials coordinator, a position that involves patient care, client communication, and SOP development and completion.
Kent, Michael S.	Professor	mskent@ucdavis.edu	04-07-2016	03-31-2019	11-11-2016	Not Found	A board certified veterinarian with over 20 years experience working with dogs.
O'Daniel, Franklin	Staff Research Assoc II	fodaniel@ucdavis.edu	07-12-2017	07-12-2020	05-19-2017	Not Found	12 years animal handling experience including dogs. RVT and CVPM. Efficient in catheter placements, animal handling, venipuncture, anesthesia, drug calculations in dogs.
Rebhun, Robert B.	Associate Professor	rbrebhun@ucdavis.edu	07-24-2018	07-24-2021	04-07-2017	04-29-2013	10 years experience treating dogs as a practicing veterinarian. Completion of residency and board certified in medical oncology. Handling of chemotherapeutic agents was part of the oncology training.
Skorupski, Katherine A.	Assoc Prof	kskorups@ucdavis.edu	04-27-2018	04-27-2020	01-09-2018	Not Found	14 years

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	of Clin						experience treating dogs as a practicing veterinarian and board certified in Veterinary Medical Oncology.
Willcox, Jennifer	ASST PROF OF CLIN-FY	jwillcox@ucdavis.edu	11-07-2017	11-07-2019	08-01-2018	Not Found	A board certified veterinary oncologist with over 6 years experience treating dogs
Withers, Sita	GSR-FULL FEE REM	sswithers@ucdavis.edu	09-20-2016	09-06-2019	05-02-2017	08-12-2014	Board certified oncologist with over 4 years treating dogs with cancer.

Assurances for the Humane Care and Use of Vertebrate Animals:

I have read and agree to abide by the **UC Davis Policy and Procedure Manual section 290-30** ([Animal Care and Use](#)). This project will be conducted in accordance with the **ILAR Guide for the Care and Use of Laboratory Animals**, and the **UC Davis Animal Welfare Assurance** on file with the US Public Health Service. I will abide by all Federal, State, and local laws and regulations dealing with the use of animals in research.

The activities proposed in this application do not unnecessarily duplicate previous experiments [AWA 2.31(d)(1)(iii)].

I will advise the IACUC in writing of any proposed significant changes in the procedures and wait for IACUC approval prior to implementing the change. I will also advise the IACUC of any changes in personnel involved in this project.

I have read and agree with the above statement.



Supplemental Figure 1. Administration of inhaled rhIL-15. Representative image showing mechanics of administration for inhaled rhIL-15 using a nebulizer attached to a fitted veterinary anesthesia cone placed over the patient's muzzle. Owners were instructed to perform treatments twice daily, a minimum of 8 hours apart, and a treatment log was sent home to be completed by all owners. Treatments lasted approximately 10 minutes each.

Supplemental Table 1. All Serious Adverse Events

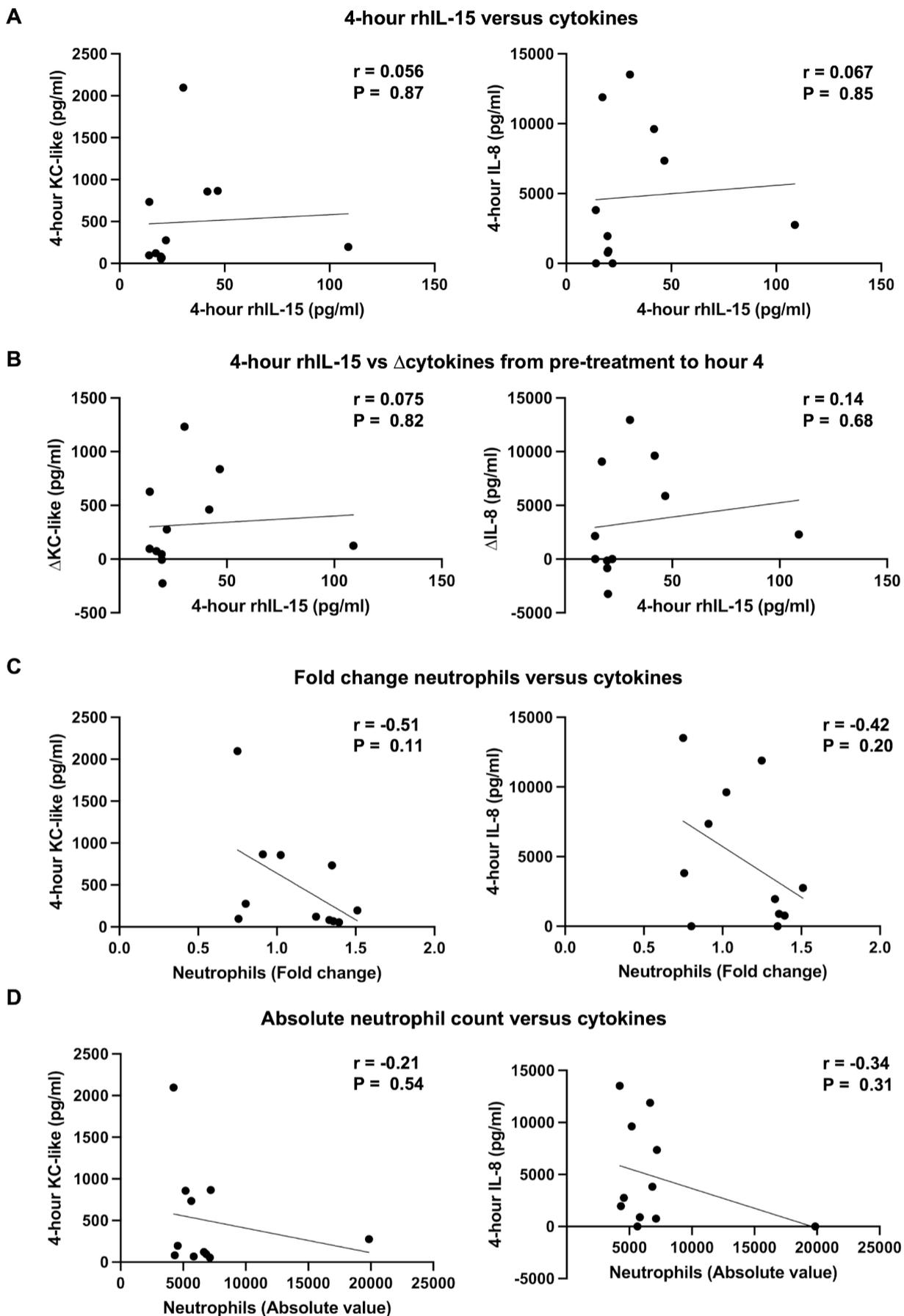
	Grade					Total
	1	2	3	4	5	
Blood/ Bone Marrow						
Anemia	8					8
Bands / left-shift	4		1			5
Basophilia	3					3
Decreased Hemoglobin	5					5
Decreased MCH	1					1
Decreased MCHC	2					2
Decreased RBC	2					2
Increased RDW	3					3
Leukocytosis	5					5
Leukopenia	2					2
Lymphopenia	5	1				6
Monocytosis	3					3
Neutrophilia	3					3
nRBCs	2					2
Reticulocytosis	4	1				5
Thrombocytosis	6					6
Unclassified cells	1					1
Cardiac General						
Sinus tachycardia	1					1
Systemic hypertension	1					1
Constitutional						
Fever	3	1				4
Hyperthermia	1					1
Lethargy / fatigue	1		1			2
Weight loss	2					2
Ear and Labrynth Disorders						
External Ear Inflammation	1					1
Gastrointestinal						
Altered Appetite	2					2
Anorexia	1	1				2
Metabolic/Laboratory						
Acidosis	1		1			2
Albumin, low	14					14
ALP, high	10	5	5	1		21
ALT, high	5	2	1			8
ALT, low	3					3
Anion gap, high	11					11
AST, high	3		1			4
AST, low	1					1
Bicarbonate, low	11	1				12
Bilirubin, high	1					1
BUN, high	4					4
BUN, low	5					5
Calcium, high	2					2
Calcium, low	7					7
Chloride, high	1					1
Chloride, low	3					3
Cholesterol, high	1					1
Creatine phosphokinase (CPK), high	10					10
Creatinine, high	1	2				3
Creatinine, low	5					5
GGT, high	3	1				4
Globulins, high	8					8
Glucose, high	4					4
Glucose, low	2					2
Magnesium, high	1					1
Magnesium, low	3					3
Phosphorus, high	3					3
Phosphorus, low	4					4
Potassium, high	11	1	2			14
Sodium, high	1					1
Sodium, low	3					3
Total protein, high	4					4
Total protein, low	2					2
Musculoskeletal / Soft Tissue						
Extremity (gait/ambulation lameness)	1	1				2
Hypertrophic Osteopathy	1	2				3
Jaw pain		1				1
Soft tissue necrosis			2			2
Neurology						
Facial nerve disorder	2					2
Seizure like episode		1				1
Ocular						
Mucoid discharge	1					1
Pulmonary / Respiratory						
Cough						0
Dyspnea		1			2	3
Tachypnea		1				1
Renal / GU						
Acute kidney injury	1	1				2
Hematuria		1				1
Proteinuria	1	1				2
Neoplasms						
Neoplasms, benign, malignant, unspecified		1				1
Vascular						
Lymph leakage	1	1	1	1		3
Totals	229	28	14	1	2	274

Supplemental Table 2. Patient demographics and clinical outcomes from pilot trial of palliative radiotherapy, allogeneic natural killer cell adoptive transfer, and subcutaneous rhIL-15 for unresectable canine melanoma.

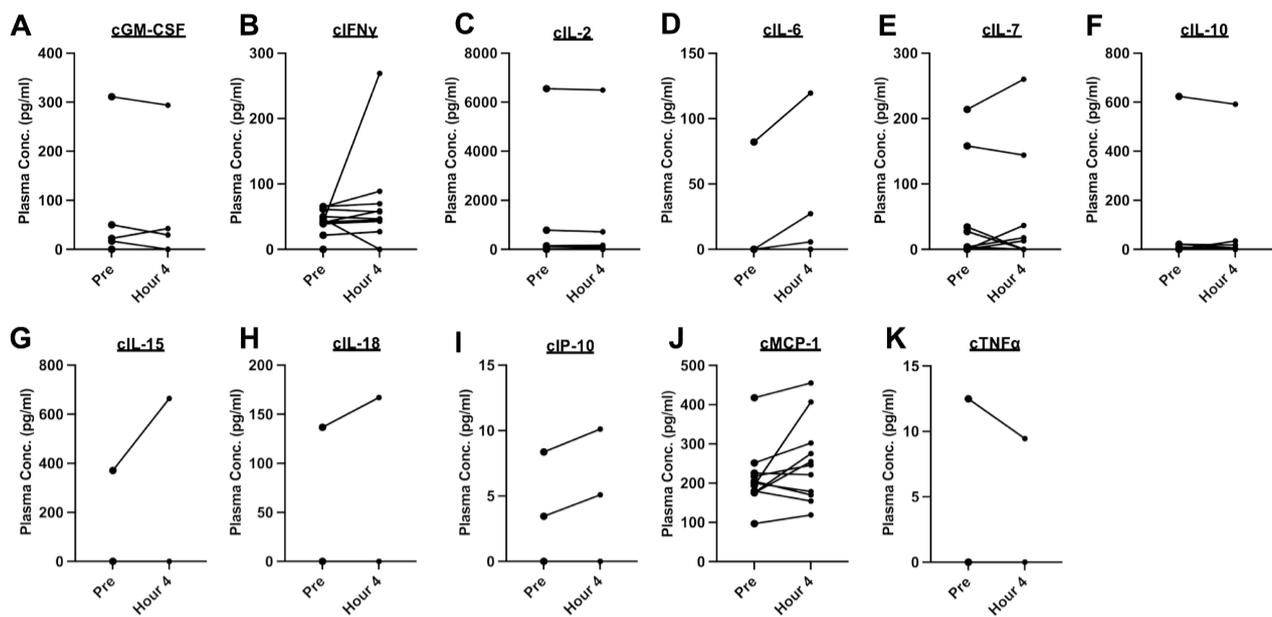
Patient ID	Dose (ug)	Weight (kg)	Age (years)	Sex [†]	Disease	Breed	VCOG-CAE Dose limiting toxicity
1	3	34.7	9.5	F	Melanoma	Labrador	none [‡]
2	3	39.0	11.3	M	Melanoma	Golden Retriever	none
3	3	10.0	13.9	M	Melanoma	Pug	none
4	3	32.0	7.1	F	Melanoma	Airedale	none
5	3	25.0	12.3	F	Melanoma	Standard Poodle	none

[†]Includes 1 castrated male and 3 spayed females

[‡]patient experienced grade 2 fevers requiring antipyretics and outpatient observation x1 week
Evaluation for response limited by concomitant administration of palliative radiotherapy



Supplemental Figure 3. Lack of correlation among plasma cytokine levels at 4 hours and blood neutrophil counts. Given rapid increases in 4-hour KC-like and IL-8, we analyzed plasma values for these cytokines. **(A)** There were no correlations of 4-hour recombinant human IL-15 (rhIL-15) with KC-like nor IL-8. **(B)** The correlations between rhIL-15 and the change in cytokine levels from pre-treatment to hour was not significant. **(C)** The correlations between 4-hour KC-like and IL-8 with fold change of neutrophils showed a negative trend, although not statistically significant. **(D)** No correlations were seen between absolute neutrophil counts and 4-hour cytokine levels.



Supplemental Figure 4. Plasma cytokine responses 4-hours post-inhalation of rhIL-15. Individual cytokines (A-K) were quantified by canine Luminex assay in the plasma pre- and 4-hours post-initial treatment.

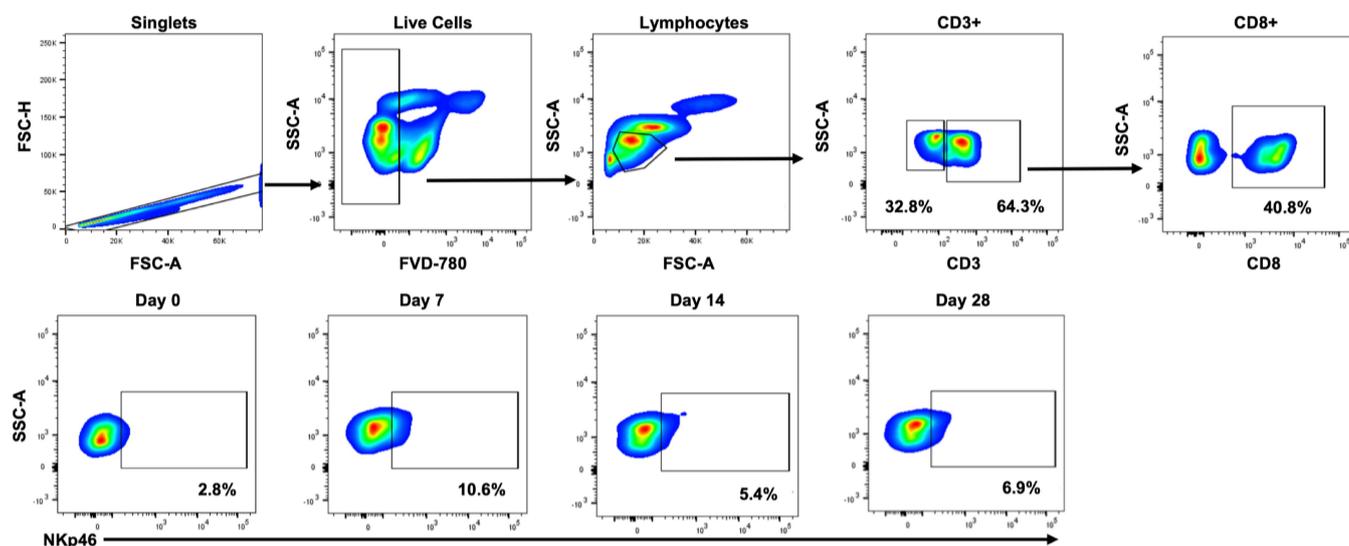
A

Patient demographics and clinical outcomes

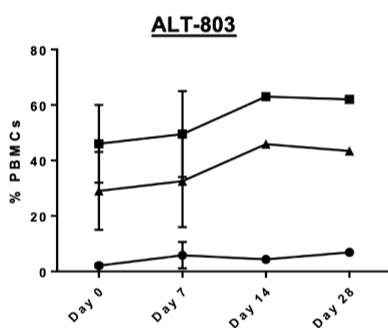
Patient ID	Dose (ug/kg)	# Doses	Weight (kg)	Age (years)	Sex [†]	Disease	Breed	VCOG-CAE Dose limiting toxicity	RECIST Response	Survival from first ALT803 treatment
803-001	30	1	44.3	8	F	Osteosarcoma	Rottweiler	NE	NE	6
803-002	30	1	50.7	6	F	Osteosarcoma	Leonberger	none	NE	19
803-003	30	2	16.9	11	M	Melanoma	Cocker Spaniel/X	none	PD	117
803-004	30	1	19.6	10	M	Carcinoma	Doberman	none	NE	26

[†]Includes 2 spayed females and 2 castrated males

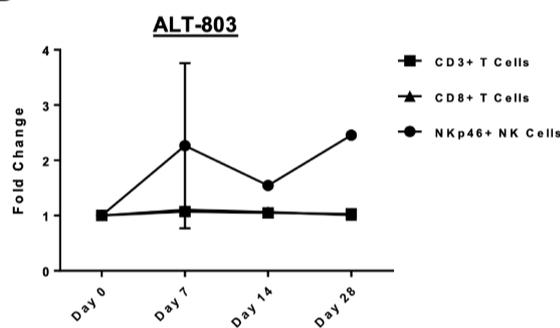
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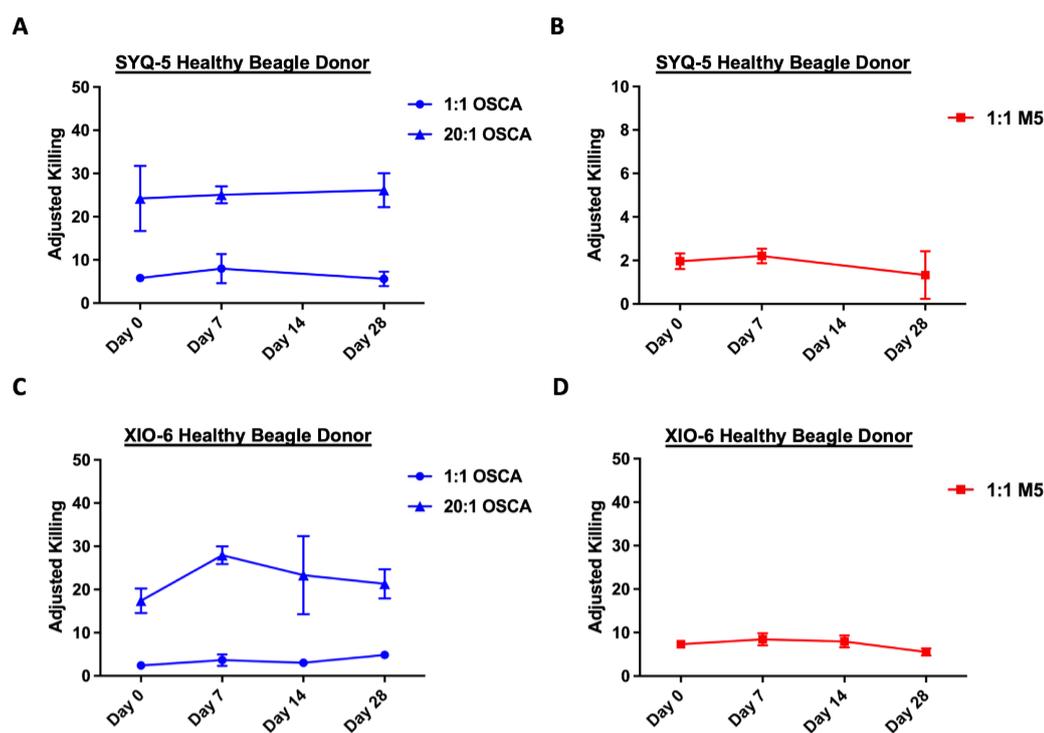
C



D



Supplemental Figure 5. Subcutaneous Superagonist ALT-803 pilot trial for metastatic canine cancer. (A) Patient demographics and clinical outcomes for the 4 patients treated with ALT-803 pilot trial. (B) Representative flow cytometry of PBMCs from patient 3 shows gating strategy for identification of lymphocyte population including CD3, CD8, and NKp46+. NKp46+ staining over time shows a peak of approximately 4-fold increase at day 7 with subsequent time points decreasing closer to baseline. (C) Frequencies of key lymphocyte populations (CD3, CD8, and NKp46) are shown graphically across indicated time point of the pilot trial, limited samples were available at days 14 and 28 because of patient dropout, most likely due to progression of disease. (D) Fold change of T cell and NK cell populations at indicated time points with increase in NKp46+ NK cells at day 7 in the context significant variability.



Supplemental Figure 2. Stability of PBMC cytotoxicity in healthy, untreated beagles over time. Beagle PBMC cytotoxicity against the canine (A) OSA target line (OSCA-78) cell line at 1:1 and 20:1 effector-to-target ratios and (B) melanoma (M5) cell line at 1:1 effector-to-target ratio showed no significant differences over multiple time points from the same donor (SYQ-5) in a schedule similar to inhaled IL-15 clinical trial. Donor beagle (XIO-6) PBMC cytotoxicity against the (C) OSCA-78 at 1:1 and 20:1 effector-to-target and (D) M5 cell lines at a 1:1 effector-to-target ratio. These experiments were repeated 3 times total.