



# Study Protocol

## I. 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.

## II. 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.

## III. 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

#### IV. 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  $\geq$  10 kg
  - VCOG-CTCAE 1.1 performance score of 0 or 1
  - 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 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. Blood sample for PK will be collected, then 3mcg/kg rhIL-15 will be given SQ. Dogs will be monitored for two hours post infusion for any reactions per protocol sheet. Additional PK samples will be collected at 1 and 4 hours post-rhIL-15 injection. Dog will then be discharged.
6. One day post RT: Physical Exam. CBC, Chemistry panel, PBMCs, plasma, and PK samples 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>)

## V. 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  $7.5 \times 10^6$  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  $7.5 \times 10^6$  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  $1 \times 10^9$  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

## VI. 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

## VII. 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.

## VIII. 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, Monjazez 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.

## IX. 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
PK				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:

**Cryovial labeling example**

Sample Type  
Patient Number  
Age  
Date/Initials

## 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

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.

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.

## APPENDIX G

**NK Cell/RT Study SOP for rhIL-15 PK plasma sample collection:****Materials:**

3mL purple top (EDTA) blood tube  
1.8mL Cryo Vials (screw top)  
1000uL Pipette  
Sharpie “super permanent ink”

**Time points:**

1. Pre-rhIL-15 injection
2. 1 hour post-injection
3. 4 hours post injection
4. 18-24 hours post injection #1 and prior to injection #2

**For each time point, label 2x cryo tubes with the following information:**

Patient VMTH ID  
NK Cell  
Date  
Time point (“pre”, “1hr post”, “4hr post”, “24hr post”)  
“PK Plasma”

**Plasma collection procedure for each time point:**

1. Collect 2-3 mL blood into EDTA collection tube (2mL minimum)
2. Immediately place sample on ice and centrifuge at 2,500 x g for 10 minutes at 4°C
3. Transfer 500uL of plasma using a pipette to each of 2x 1.8mL cryo tubes on ice.
  - Be careful not to disturb the lower blood layer
  - If less than 1ml plasma available, transfer 400uL of plasma to one tube and the remaining plasma to second tube.
  - If more than 1ml plasma available, please transfer the remaining plasma into a third, labeled tube
4. Store at -80°C within 30 min of collection (plasma samples should remain on ice until transferred to -80 storage)

The storage box is located in the -80 freezer in the main lab Open the inside freezer door to the 3<sup>rd</sup> shelf down, the rack on the far right, first drawer. There is a box in that drawer labeled for “NK Cell Study PK samples”. Place the cryo tubes, in chronological order, in the next available spots.