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**14. ABSTRACT**

Osteosarcoma is one of the most common cancers of children and adolescents and young adults (AYAs). The outcomes for with metastatic osteosarcoma are especially dismal because there are no effective options to treat this condition. Our objectives are to tilt the immune landscape of metastatic osteosarcoma from immunosuppression to immunoreactivity, and to generate pre-clinical support to translate this innovative immunotherapy for the treatment of children and AYAs with metastatic bone cancer. To achieve this, we propose to combine an oncolytic virus, which will kill tumor cells and activate the immune system in the tumor environment, with a drug that blocks the myeloid checkpoint to enhance phagocytosis and antigen presentation as well as the immune exhaustion checkpoint to enhance tumor killing by T cells and NK

**15. SUBJECT TERMS**

Osteosarcoma, metastasis, tumor immunity, oncolytic virotherapy, vesicular stomatitis virus, immune checkpoints, CD47, PD-1, PD-L1, drug development, animal

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## 1. Introduction

Osteosarcoma is one of the most common cancers of children and AYAs. In particular, the outcomes for patients with metastatic disease dismal because there are no effective options for this condition. We have recognized that the mutational profiles in osteosarcoma create fertile environments for immunotherapy, and that the immune response is an important determinant of disease progression. These discoveries are foundational for our application. In particular, immunotherapy is an appealing modality to treat metastatic cancer not only because tumors that are resistant to conventional treatment, such as radiation and chemotherapy, can be attacked with immunologic approaches, but also because this modality can reach tumors that are inaccessible for conventional treatments. For osteosarcoma, the inaccessibility of metastasis is a major factor that influences prognosis. Our recent work shows that osteosarcomas segregate into distinct groups with “immunologically silent” and “immunologically aware” tumors. Patients with immunologically aware tumors have significantly better outcomes than patients with immunologically silent tumors. However, rationally designed immunotherapies have yet to be tested in the setting of metastatic osteosarcoma.

Oncolytic viruses represent an emerging form of immunotherapy. Preclinical studies indicate that the oncolytic VSV we are employing in this project can reach - and selectively replicate in - tumor tissues, leading to their destruction and the consequent activation of anti-tumor immunity, although it is virtually certain that these agents will find their role as part of combinatorial therapies such as immune checkpoint blockade. The latter therapy has revolutionized the management of patients with advanced melanoma and lung cancer, and it represents a promising treatment approach for many other cancers. But despite its success in these areas, many cancers are unresponsive to immune checkpoint blockade. Even among patients with cancers that are approved indications for immune checkpoint blockade, many fail this therapy.

Blockade of the CD47 myeloid checkpoint is another emerging immunotherapy. Therapies that block the interaction of CD47 and SIRP$\alpha$ stimulate tumor cell phagocytosis and induce anti-tumor immune responses. CD47 blockade in being evaluated in various clinical trials in combination with other immune-activating molecules.

There are many challenges associated with the investigation of novel immunotherapies, and particularly combinatorial approaches. These difficulties are compounded in childhood cancers, and in particular in osteosarcoma which is a very rare tumor. We have thus learned about pathogenesis of this disease using conventional laboratory animal models (mice), but these models have limitations for therapy development. Studying spontaneous dog cancers as models to develop new therapies has gained favor due to the similarities in incidence and natural history of these diseases. Dogs are treated with similar protocols and dosing schedules as humans, providing a clinically realistic setting for drug development in a compressed timeline. Furthermore, canine clinical trials will almost always include animals of both sexes, allowing sex to be considered among the important biological variables. Biological samples to monitor safety, clinical toxicities, and local and systemic immune responses can be obtained from veterinary patients, so in addition to potential improvements in treatment modalities and dose schedules, veterinary trials also create opportunities to identify companion biomarkers to predict patient responses[19].

We and others have leveraged the fact that osteosarcoma is common in dogs, and although it is primarily seen in adult and aged dogs, strong similarities in the biology of the disease across dogs and humans provide opportunities to overcome challenges and develop innovative strategies for translation. Dogs as a model have their own limitations; however, we and others have identified instances where the biology allows us to utilize dogs to advance immunotherapy for this disease. A consistent conclusion from canine studies is that immunotherapies that activate innate immunity improve survival for dogs with non-metastatic appendicular osteosarcoma, an observation also seen in humans. Less work has been done in the metastatic setting, where the benefits remain unclear.

This project is designed to begin to address the potential to reach and manage metastatic osteosarcoma lesions using a combination of two novel immunotherapeutic agents.

## 2. Keywords

Osteosarcoma, metastasis, tumor immunity, oncolytic virotherapy, vesicular stomatitis virus, immune checkpoints, CD47, PD-1, PD-L1, drug development, animal models.

## 3. Accomplishments

**NOTE**: ON 4/February/2021, the PIs received an email notice from DOD describing the concern shared by CDRMP about the impacts of COVID-19 on Service Members and the public, and specifically on the challenges it posed to ongoing research. In that email, we were asked to disclose significant COVID-19-related developments that would impact performance of this project.

On 25/May/2021, Dr. Modiano (contact PI) notified Dr. Senkevitch that our laboratories had been operating at less than full capacity due to the COVID-19 responses adopted by our institutions, which were magnified by supply chain disruptions, which continue to this day, and by personnel departures.

We anticipate that a no-cost extension may be required to complete the work proposed for this project.

- **What are the major goals of the project?**

The project has three specific aims:

**Specific Aim 1: Optimize dose and schedule for combination VSV-IFNβ-NIS and P-DIC in mouse models of metastatic osteosarcoma.**

**Milestone**: Project Specific Approvals by Local IACUC and IBC (Month 0). 100% completed.

**Milestone**: Project Specific Approvals by ACURO (Months 0-2). 100% completed.

**Milestone**: Production of VSV-IFNβ- NIS for mouse studies. (Months 0-4). 100% completed.

**Milestone**: Production of P-DIC. (Months 0-4). 100% completed.

**Milestone**: Cell authentication. (Quarterly). Ongoing.

**Milestone**: Define toxicity of combination VSV-IFNβ-NIS and P-DIC (ONIx). (Months 4-8). 50% complete.

**Milestone**: Define optimal dose for combination VSV-IFNβ-NIS and P-DIC. (Months 4-8). 25% complete.

Milestone: Complete necropsies. (Months 4-12). Deferred.

**Milestone**: Data analysis; organization and conclusions. (Months 4-12). Ongoing.

**Milestone**: Quality control and quality assurance for data – manuscript preparation and submission. (Months 8-18). Ongoing.

**Specific Aim 2: Define immunological effects and mechanism of action for combination VSV IFNβ-NIS and P-DIC**

**Milestone**: Assessment of immune response induced by VSV-IFNβ-NIS and P-DIC in mice with metastatic osteosarcoma (Months 8-24). 25% completed.

**Milestone**: Data analysis; organization and conclusions (Months 8-24). Ongoing.

**Milestone**: Quality control and quality assurance for data – manuscript preparation and submission (Months 12-24). Ongoing.

**Specific Aim 3: Characterize safety, efficacy, and immunomodulatory effects of VSV-IFNβ- NIS and P-DIC in dogs with naturally occurring metastatic osteosarcoma**

**Milestone**: Production of in vivo grade VSV-IFNβ-NIS (Months 0-8) 100% completed.

**Milestone**: Production of in vivo grade P-DIC (Months 0-8) 100% completed.

**Milestone**: Identify safe dose to guide clinical development (Months 20-24) 25% complete.

**Milestone**: Assessment of immune response induced by VSV-IFNβ-NIS and P-DIC in dogs with metastatic osteosarcoma (Months 20-24) Not yet started.

**Milestone**: Data analysis; organization and conclusions. (Months 20-24) Not yet started.

**Milestone**: Quality control and quality assurance for data – manuscript preparation and submission. (Month 24) Not yet started.

**Milestone**: Planning for pediatric clinical trial planning (translation to humans) (Months 20-24) Not yet started.

- **What was accomplished under these goals?**

**Milestone**: Project Specific Approvals by Local IACUC and IBC (Month 0). 100% completed.
- University of Minnesota IACUC protocol 2004-38033A was approved on July 20, 2020
- Mayo Clinic IACUC protocol A00005309-20 was approved on September 30, 2020

**Milestone**: Project Specific Approvals by ACURO (Months 0-2). 100% completed.
- ACURO approval for Proposal Number CA190276, Award Number W81XWH-20-1-0682 was obtained on November 20, 2020.

**Milestone**: Production of VSV-IFNβ- NIS for mouse studies. (Months 0-4). 100% completed.
- Viral production was completed at Mayo Clinic and passed all quality controls. See below for experimental data using VSV-IFNβ- NIS in mouse studies.

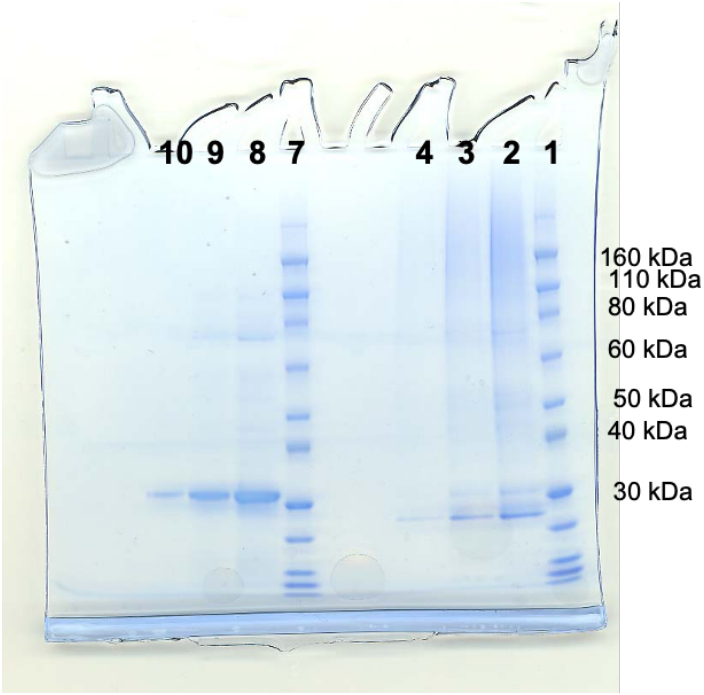
**Milestone**: Production of P-DIC. (Months 0-4). 100% completed.
- We had a pre-existing batch of P-DIC (now called Oncoimmunology accelerator, or ONIx) that was sufficient for pilot studies (see below for experimental data using ONIx in pilot mouse studies). This product had been previously tested for sterility and potency, and the data were provided in the original grant application.

However, production of ONIx for this project was delayed because of the response to the COVID-19 pandemic. The University of Minnesota maintained minimal operations from March 2020 until approx.

May of 2021, primarily focused on development of COVID-19 tests, ventilators, and other procedures that aided the national response to this public health crisis. Laboratories and facilities, including the Molecular Cell Therapy Center, where drug production for ONIx was to take place, were closed as part of the need to avoid having people in close contact, as well as to divert all physical resources (pipettes, glassware, etc.) to COVID-related research, development, diagnosis, treatment, and response operations.

In May of 2021, PIs at the University of Minnesota were directed to submit individual plans to resume work under the umbrella of a University Health Sciences-wide “Sunrise Plan.” With few exceptions, the individual plans required staff to work in staggered shifts with a maximum occupancy in labs of 50%. As part of the implementation of that plan, Dr. Vallera was able to resume synthesis of ONIx and complete the batch for pre-clinical work (in mice and dogs). The production run was completed on May 27, 2021 and vialed for use in 3-mL vials at a concentration of 9 mg/mL (total yield = 3.1g).

Purity of the product was confirmed by SDS-PAGE (Figure 1).




1: Novex MW Ladder
2: 5 µg UMN-ONix NR
3: 2.5 µg UMN-ONix NR
4: 1.25 µg UMN-ONix NR
5: Blank
6: Blank
7: Novex MW Ladder
8: 5 µg UMN-ONix R
9: 2.5 µg UMN-ONix R
10: 1.25 µg UMN-ONix R

Figure 1. Coomassie blue-stained gel showing a titration of the final ONIx peptide under non-reducing conditions (lanes 2-4) and under reducing conditions (lanes 8-10)

Sterility testing for ONIx is ongoing (sacrificial vials were sent for testing to IDEXX Laboratories). Potency testing (measured as displacement of anti-CD47 and anti-PD-L1 antibodies from human A549 and canine CLBL-1 cells is in progress in Dr. Modiano’s lab.

**Milestone**: Cell authentication. (Quarterly). Ongoing.
- The PI labs follow rigorous SOPs for cell authentication. It should be noted that since virtually no work was done in the laboratories in the period between March 2020 and May 2021, authentication of resources was not necessary.

**Milestone**: Define toxicity of combination VSV-IFNβ-NIS and P-DIC (ONIx). (Months 4-8). 50% complete.
- To confirm the safety of VSV and ONIx combination therapy, a pilot study was carried out in the well-established A20 (Balb/c) murine lymphoma model. Mice with established A20 tumors were treated with either PBS, VSV ($1x10^8$ $TCID_{50}$/mouse), ONIx (5mg/kg for 6 doses), or combination of VSV and ONIx. Mice were monitored for adverse events, including weight loss. The results show that

all treatments, including combination therapy were well tolerated, with minimal transient weight loss and resumption of weight gain in mice at approximately the same rate as the controls (Figure 2.).

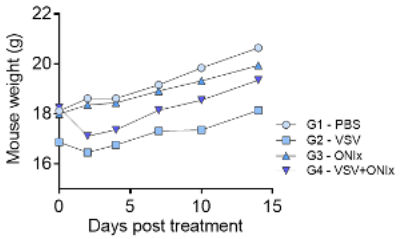



Figure 2. ONIx does not cause weight loss or impede weight gain in mice.
Mice were treated as described in the text above. ONIx treatment alone did not alter the course of weight gain in the mice. The VSV treatment group included smaller mice (see weight at Day 0) and the trend for weight gain was comparable

**Milestone**: Define optimal dose for combination VSV-IFNβ-NIS and P-DIC. (Months 4-8). 25% complete.
- The K7M2 model (and its non-metastatic counterpart, K12) are both challenging to work with. The tumors are syngeneic with Balb/c mice, where reagents are more limited, and in our hands, they grow slowly with incomplete penetrance (less than 50% tumor take in some experiments). We generated a K12 cell line incorporating chicken ovalbumin (as a target antigen for immune cells) and Gaussia luciferase using CRISPR-Cas9. However, the cells do not show significant luciferase activity, and certainly not enough for *in vivo* experiments. Our attempts to introduce genes into K7M2 cells repeatedly failed. We have since obtained a K7M2 cell line that is stably transfected with firefly luciferase from colleagues at West Virginia University, but we have not had a chance to test their growth *in vivo* due to the challenges with COVID-19. To complicate matters, the animal facilities at the University of Minnesota are experiencing a severe shortage of caretaker staff and the PIs have been asked to reduce the number and frequency of animal experiments, or not to start any new ones until further notice when staffing can be restored.

The C57Bl/6 (B6) mouse model offers numerous advantages, including a Th1 bias in their immune response (better suited for mechanistic studies of cancer immunotherapy) and greater availability of reagents. We recently learned that several syngeneic B6 osteosarcoma cell lines were developed by our colleagues at Baylor College of Medicine. One in particular (F420) has been modified with firefly luciferase and seems to be well suited for experiments *in vivo*. We are currently in process of obtaining these cells from Baylor through an MTA.

Another approach we have taken is to develop the MC17 murine sarcoma model as an alternative to K7M2. Although MC17 represents a fibrosarcoma, it is a reliable model for drug development, and it is especially recalcitrant to conventional immunotherapy (MC17 is the prototypical “cold tumor”). Yet, MC17 can respond to unconventional immunotherapies. For example, we have developed a molecule called eBAT, which consists of full-length human EGF linked to the amino terminal fragment (ATF) of human urokinase plasminogen-type activator (uPA) and to a Pseudomonas exotoxin (PE) modified to enhance its cytotoxicity and reduce its immunogenicity.

We have shown that eBAT has activity against a wide variety of sarcomas and it effectively targets sarcoma stem cells. eBAT can also kill activated endothelial cells, potentially depleting or normalizing tumor vasculature. But most importantly for this discussion, eBAT seems to remodel the immune and inflammatory tumor microenvironment (TME) by targeting urokinase receptor (uPAR)-expressing, immunosuppressive myeloid cells, potentially enhancing the immune response against tumors. We tested this premise using MC17 and a uPAR-knockout (KO) called D10. MC17 and D10 cells are highly resistant to eBAT *in vitro*, and *in vivo*, the tumors attract large numbers of myeloid cells, including $F480^{+}$ tumor associated macrophages (TAMs) (Figure 3A). These TAMs were unaffected in uPAR-KO mice that were injected with uPAR-KO D10 cells and treated with meBAT, but they were ablated when WT mice injected with WT MC17 tumors were with a mouse-specific version of eBAT called meBAT (Figure 3B). To define the impact of remodeling the MC17 sarcoma immune landscape more clearly, WT C57/Bl6 mice (CD45.1) were lethally irradiated and rescued by bone marrow reconstitution using

syngeneic WT (CD45.2) donors or uPAR-KO (CD45.2) donors. Engraftment efficiency was approx. 75-85% for both groups. This provided a control group (WT), and a group where the only hematopoietic (+/- vascular) cells would lack uPAR expression. The mice were then injected with mCherry-labeled WT MC17 cells and treated 2 cycles of eBAT or meBAT. Figure 4A shows that, despite the fact that MC17 cells were resistant to the cytotoxic effects of eBAT and meBAT, both drugs reduced the tumor burden in mice whose hematopoietic cells expressed WT uPAR. The therapeutic benefit was completely ablated in mice whose hematopoietic cells had uPAR knocked out. Consistent with what we observed in the WT and germline uPAR KO mice, eBAT and meBAT remodeled the myeloid landscape of the MC17 sarcomas (Figure 4B). The total numbers of $CD11b^+$ myeloid cells were similar in the WT and uPAR-KO bone marrow recipients. However, a significant number of $CD11b^+$ cells in the WT bone marrow recipients also stained positive for mCherry, suggesting these myeloid cells had been reprogrammed from an immunosuppressive phenotype to a pro-immune phenotype and had at least formed complexes, but more likely engulfed $mCherry^+$ MC17 tumor cells. This increased myeloid cell phagocytosis was not seen in the uPAR-KO bone marrow recipients. The pro-immune myeloid remodeling appeared to be functionally significant: we observed a reproducible increase of $CD3^+$ T-cells infiltrating the TME in the WT bone marrow recipients (Figure 4C), whereas virtually no T cells were present in the TME of the uPAR-KO bone marrow recipients, and the overall survival time (based on time to a tumor endpoint) was also improved in in the WT bone marrow recipients (Figure 4D) as compared with the uPAR-KO bone marrow recipients.

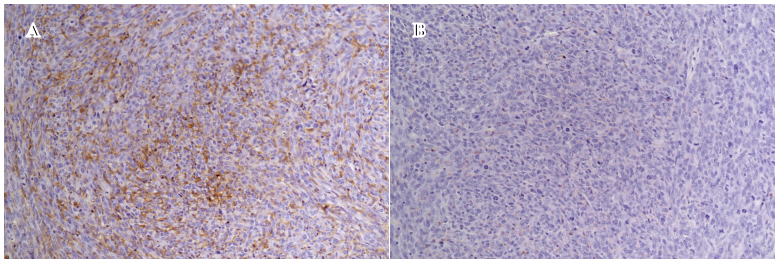



Figure 3. Depletion of $F4/80^+$ Immunosuppressive TAMs in mouse sarcoma treated with meBAT. Representative IHC to document the presence of $F4/80^+$ TAMs (brown staining) in mouse tumors. Panel A: uPAR KO mouse inoculated with D10 (uPAR KO) cells and treated with two cycles of meBAT starting on day-16. Panel B: WT mouse inoculated with WT MC17 cells and treated with two cycles of meBAT starting on day-16. Tumors were harvested at day-28 and day-30, respectively. Note the depletion of TAMs in panel (B) as compared to panel (A). Even though staining in the tumors was variable, decreased $F4/80^+$ TAM density was reproducibly observed only in WT mice harboring WT tumors treated with meBAT or eBAT. (Magnification 200x).

In the interim while the MC17 model was transferred to Mayo Clinic, Dr. Naik carried out a pilot study in the well-established A20 murine lymphoma model to confirm the safety of combination VSV and P-DIC combination therapy. This was done concurrently with the tolerability experiment described in Figure 2. The results show that mice that received both VSV and combination therapy had transient tumor remissions (including formation of scabs on the tumor site), with a significant survival benefit compared to PBS treatment (Figure 5). These data support further analysis of this combination therapy in relevant murine sarcoma models.

Milestone: Complete necropsies. (Months 4-12). Deferred.
- Dr. Modiano, Ms. Schulte, and Dr. Kim performed necropsies for all the experiments using MC17 at the University of Minnesota, but absent a reliable osteosarcoma model, we have not performed the immune experiments in our lab. Dr. Naik is developing this aspect of the project as described above and in the Milestones for Aim 2.

**Milestone**: Data analysis; organization and conclusions. (Months 4-12). Ongoing.
- The project investigators have been holding regular meetings to update the status of the project.

**Milestone**: Quality control and quality assurance for data – manuscript preparation and submission. (Months 8-18).
- QC and QA for data are performed on a continuous basis. We do not have sufficient data to prepare or submit a manuscript yet, at least partly due to the delays caused by COVID-19.

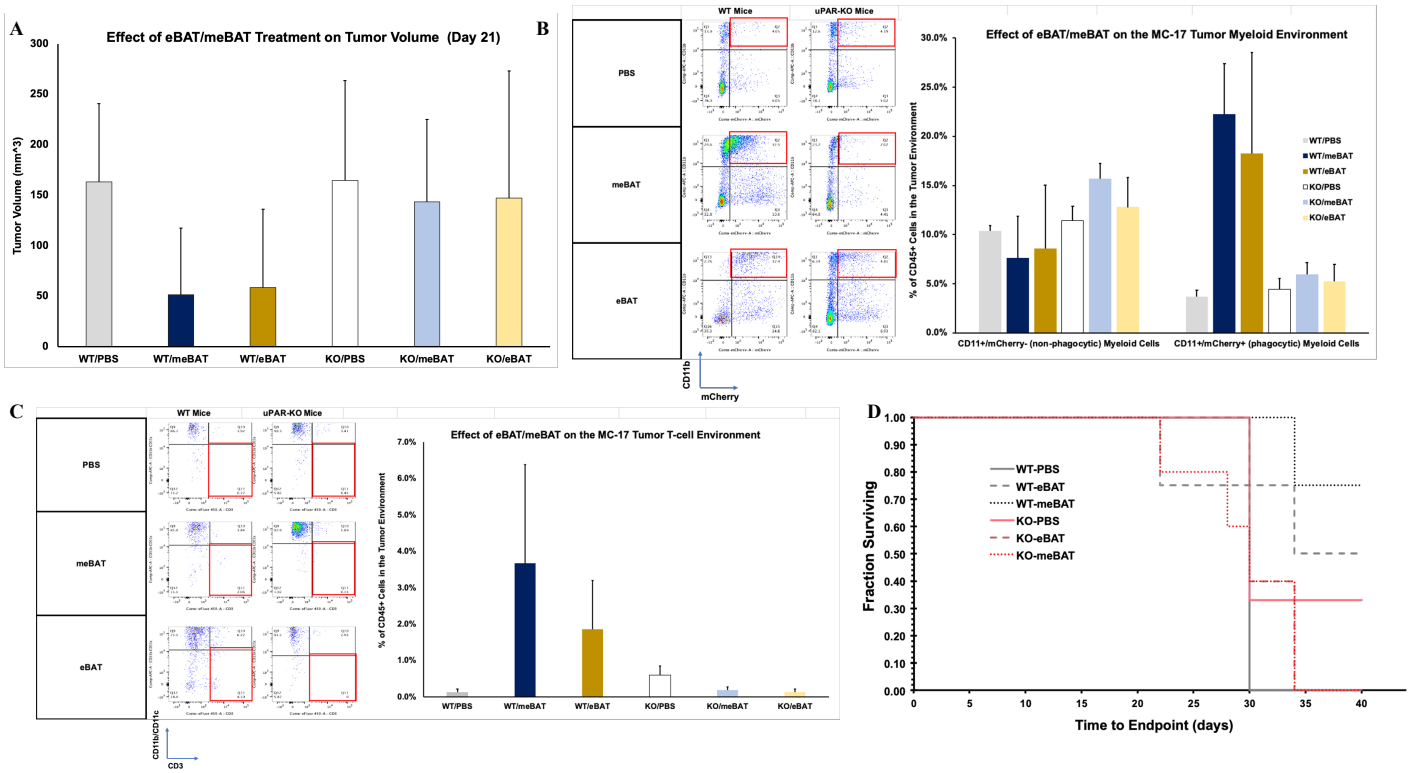
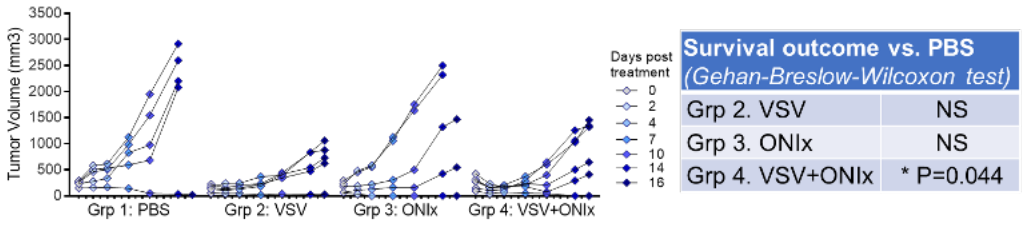



**Figure 4**. Remodeling of the immune landscape by eBAT or meBAT treatment leads to improved outcomes in the MC17 sarcoma model. Five-week old, WT C57Bl/6 (CD45.1) mice were lethally irradiated and rescued from death by bone marrow reconstitution using WT (WT) or germline uPAR-KO (KO) C57Bl/6 (CD45.2) donors. The proportion of $CD45.2^+$ cells in the reconstituted mice was, on average, approx. 75-80%. Three weeks after bone marrow reconstitution, mice were injected subcutaneously with mCherry-labeled MC17 cells, and five days later the mice were treated with phosphate buffered saline (PBS), or with two cycles of meBAT or eBAT. The bars are color coded to assist in identification of groups: WT recipients treated with PBS are colored in gray; WT recipients treated with meBAT are colored in dark blue; WT recipients treated with eBAT are colored in gold; KO recipients treated with PBS are colored in white; WT recipients treated with meBAT are colored in light blue; and KO recipients treated with eBAT are colored in yellow. Tumor cells did not engraft in one mouse of the KO/PBS group, but this mouse was still included in the analyses of tumor size and survival. Panel (**A**) shows tumor volume in mice from the three groups at Day-21 after tumor inoculation. Panel (**B**) shows representative 2-dimensional flow cytometry dot plots of myeloid cells stained with anti-CD11b and cells expressing mCherry. The red boxes delineate putative phagocytic myeloid cells ($CD11b^+$/$mCherry^+$). Panel (**C**) shows representative 2-dimensional flow cytometry dot plots of myeloid cells stained with anti-CD11b and anti-CD11c and T cells stained with anti-CD3. The red boxes delineate T cells found in the TME. Panel (**D**) shows a Kaplan-Meier survival probability plot for all four groups. Only the mouse in the KO/PBS group and mice in the WT/meBAT and WT/eBAT groups survived longer than 34 days. The mice were sacrificed at Day-40 to maximize the probability that changes in the TME attributable to meBAT or eBAT treatment would still be detectable.




| Survival outcome vs. PBS *(Gehan-Breslow-Wilcoxon test)* | |
|---|---|
| Grp 2. VSV | NS |
| Grp 3. ONIx | NS |
| Grp 4. VSV+ONIx | * P=0.044 |

Figure 5. VSV and P-DIC combination treatment induces tumor remission in a Balb/c tumor model. Mice were injected with A20 lymphoma cells, and once visible tumors arose, they were treated with PBC (control), VSV alone, ONIx alone, or VSV + ONIx. The spider plots show tumor growth for individual mice in each group.

## Specific Aim 2: Define immunological effects and mechanism of action for combination VSV IFNβ-NIS and P-DIC

**Milestone**: Assessment of immune response induced by VSV-IFNβ-NIS and P-DIC in mice with metastatic osteosarcoma (Months 8-24). 25% completed.

- Pilot data were generated for this milestone, which were highly informative. The effect of VSV on mouse osteosarcoma, and especially on the immune landscape of osteosarcoma tumors had not been previously established. To fill this gap in knowledge, Dr. Naik's group conducted a pilot study of VSV monotherapy in the K7M2 tumor model. Figure 5 shows the study design.

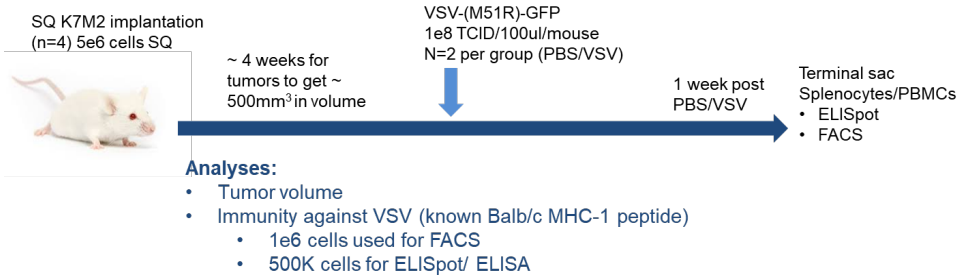



Figure 5. Study design to evaluate immune responses induced by VSV and ONIx in the K7M2 mouse osteosarcoma model.

Balb/c mice were implanted $5x10^6$ K7M2 osteosarcoma cells subcutaneously in the right flank. Tumor growth was quite slow in this model with about ~50% of mice developing tumors (N-4). Tumor bearing mice were treated with PBS or $1x10^8$ $TCID_{50}$ VSV (per mouse) given intravenously. Mice were sacrificed on study day 4 to optimize methods to measure intra-tumoral immune infiltration and antitumor immunity.

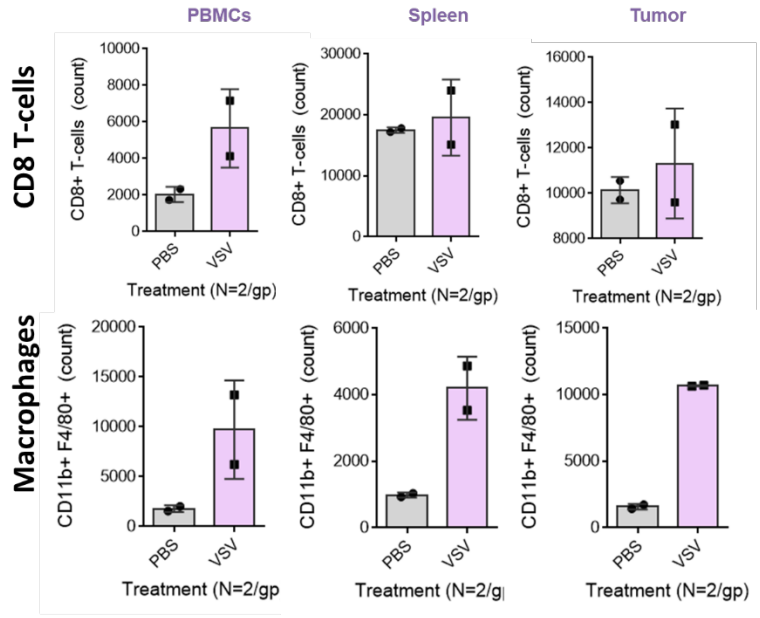



Figure 6. Presence of immune cells in blood, spleen, and tumor. PBMCs, spleens, and tumors were harvested from the mice at the time of sacrifice and the presence of CD8 T-cells and tumor-associated macrophages was established using

Preliminary assessment of CD8 T-cells and macrophages in peripheral blood mononuclear cells (PBMCs), spleen and tumor (Figure 6) show *(a)* a notable increase of CD8 T-cells in PBMCs but not in tumor and spleen; and *(b)* an increase in macrophages in PBMCs, spleen, and tumor. Additional and larger experiments will be needed to confirm these findings. Addition of checkpoint inhibition dually targeted to macrophages (CD47) and T-cells (PD-1), *i.e.*, ONIx, may potently activate antitumor responses mediated by these immune cell populations. Additional data from this experiment (ELISpot) are anticipated when a new post-doc is able to join Dr. Naik's group and add to the critical mass of personnel needed for these experiments.

To examine the potential impact that blockade of the immune exhaustion checkpoint might have on VSV therapy, T cells in PBMCs and in the spleens from mice treated with VSV were evaluated for

PD-1 expression as a marker of activation and exhaustion. Figure 7 shows the results of this experiment for CD4 and CD8 T-cells. The data show an increase in PD-1 expression on both CD4 and CD8 T-cells in blood and spleen, suggesting addition of a PD-1 inhibitor, *i.e.*, ONIx, would be a potent activator of antitumor immune responses.

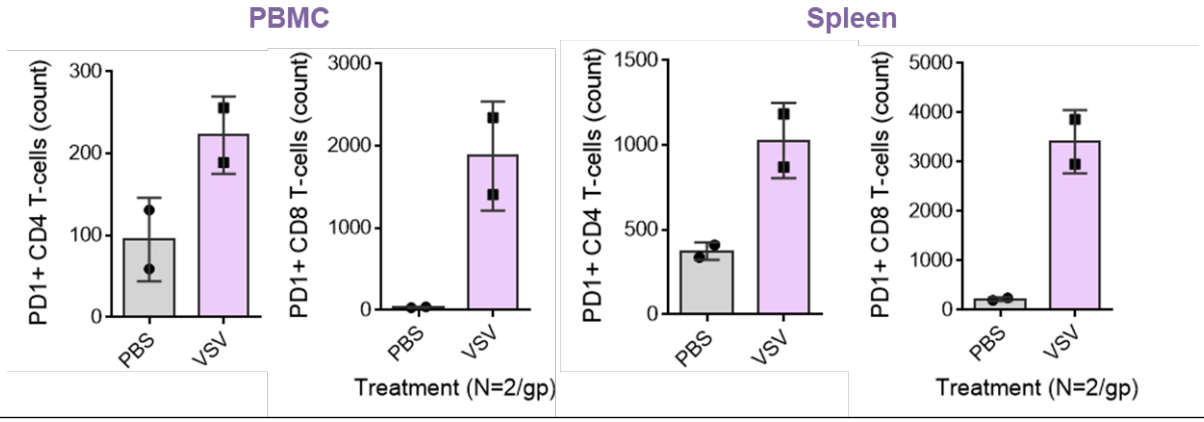



Figure 7. Increased PD-1 expression on T-cells in tumor and spleen following VSV treatment. PBMCs and spleens obtained as in Figure 4 were evaluated for CD4 T-cell activation and potential exhaustion based on expression of PD-1 using flow cytometry.

**Milestone**: Data analysis; organization and conclusions (Months 8-24). Ongoing.
- Even though the data for this aim are only emerging, they are included in the investigators have regular meetings to update the status of the project.

**Milestone**: Quality control and quality assurance for data – manuscript preparation and submission (Months 12-24). Ongoing.
QC and QA for data are performed on a continuous basis. This specific aim was only started in the latter part of year-1.

**Specific Aim 3: Characterize safety, efficacy, and immunomodulatory effects of VSV-IFNβ- NIS and P-DIC in dogs with naturally occurring metastatic osteosarcoma**

**Milestone**: Production of in vivo grade VSV-IFNβ-NIS (Months 0-8) 100% completed.
- Please see milestones for specific aim 1. Sufficient *in vivo* grade VSV-IFNβ-NIS for the canine clinical trial was produced by the Mayo Clinic investigators.

**Milestone**: Production of in vivo grade P-DIC (Months 0-8) 100% completed.
- Please see milestones for specific aim 1. This milestone was accelerated and ONIx was produced in a single batch, which should be sufficient to achieve biologically active doses so far predicted by the mouse models.

**Milestone**: Identify safe dose to guide clinical development (Months 20-24). Ongoing.
The mouse experiments shown as part of Aims 1 and 2 suggest that the dose of ONIx required to achieve a therapeutic effect is likely to be no higher than 1-2 mg/kg (or about 1/6 of the effective mouse dose).

**Milestone**: Assessment of immune response induced by VSV-IFNβ-NIS and P-DIC in dogs with metastatic osteosarcoma (Months 20-24). Not yet started.

**Milestone**: Data analysis; organization and conclusions (Months 20-24). Not yet started.

**Milestone**: Quality control and quality assurance for data – manuscript preparation and submission. (Month 24). Not yet started.

**Milestone**: Planning for pediatric clinical trial planning (translation to humans) (Months 20-24). Not yet started.

- **What opportunities for training and professional development has the project provided?**

In Dr. Modiano's lab, one of the technicians involved in the project (Ms. Taylor DePauw) was accepted into graduate school starting in the Fall of 2021. This is a great achievement for a young person who wishes to eventually contribute to the research enterprise as a principal investigator.

Dr. Makielski continues her progression towards independence, having been promoted from a post-doctoral position to a Researcher-5 position, which is in the professional academic and administrative track. Dr. Makielski is committed to developing an independent research program focused on developing more refined and accurate diagnostic methods and more effective and safer immunological therapies for osteosarcoma.

Regrettably, Dr. Sathyanarayan left Dr. Naik's lab at the Mayo Clinic due to a COVID-19-related relocation. Dr. Naik is currently recruiting a post-doc to continue working on this project.

- **How were the results disseminated to communities of interest?**

Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

We plan to continue pre-clinical development, studies to characterize the mechanisms of action of VSV and ONIx, alone and in combination, and to initiate the canine clinical trial.

- **Other related work**

In addition to the work described here, the COVID-19-related laboratory closures gave us the opportunity to make progress on data analysis from our previously completed VSV clinical trial in dogs with naïve osteosarcoma. Most relevant for this project, we evaluated the significance of immune infiltrates to the eventual outcomes of dogs with osteosarcoma, as well as the impact that VSV had on those immune infiltrates. Biopsy samples were obtained from dogs prior to treatment, and 10 days after administration of neoadjuvant VSV (at the time of amputation and prior to chemotherapy. mRNA was extracted from the biopsies and subjected to next-generation RNA sequencing. Among the important findings, we could detect VSV mRNA in some of the post-biopsy tumor samples (but not in skin samples), indicating that the virus not only survived systemic administration, but it homed to the tumor, as expected. We also found co-regulated transcriptional programs in the tumors that indicated the samples were heterogeneous with regard to their proliferative capacity and genomic instability, as well as their immune status. Generally, osteosarcoma is an immunologically "cold" or barren tumor, but we showed previously that RNA sequencing is sufficiently sensitive to identify the presence of innate and adaptive cells (inferred from the expression of co-regulated, cell specific gene clusters). As we observed before in children, dogs that had higher adaptive immune infiltrates had better outcomes than dogs with lower adaptive immune infiltrates. But in this case, the differences were especially noticeable in dogs that received VSV, where the presence of $CD8^+$ T-cells and other adaptive immune cells was associated with a significant survival benefit as compared to dogs that had low or no detectable $CD8^+$ T cells. We believe that the VSV therapy played a role in the expanded survival of the dogs with pre-existing

immune infiltrate, since the survival of dogs that received placebo treatment was comparable to the dogs that had no pre-existing immune infiltrates (Figure 8).

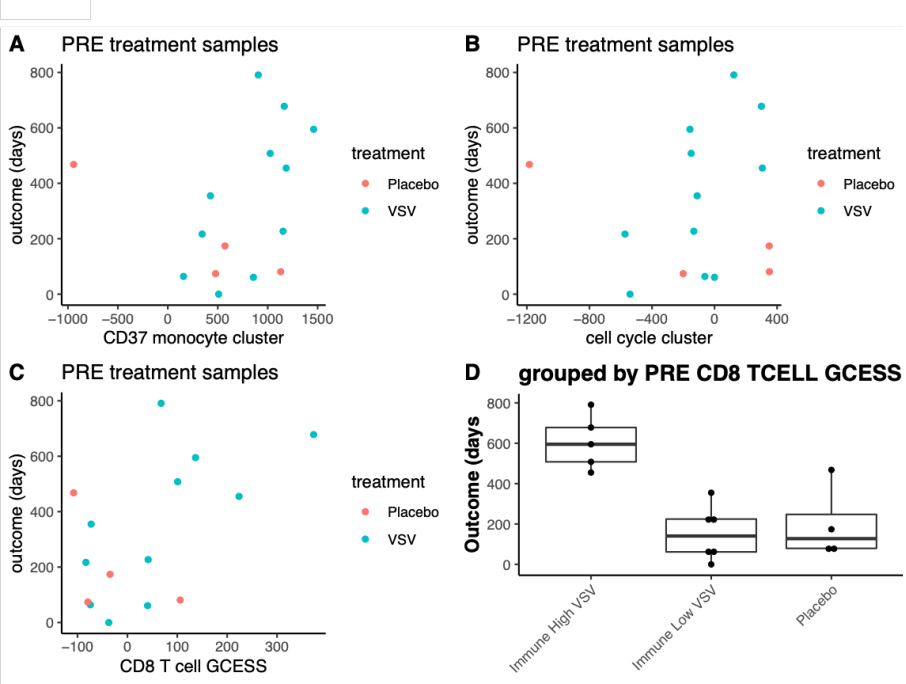



Figure 8. High immune gene cluster expression summary scores (GCESS) are associated with longer overall survival in dogs with hemangiosarcoma treated with neoadjuvant VSV followed by standard of care (the VIGOR trial). Twenty-eight (28) dogs were enrolled in the VIGOR trial evaluating safety and efficacy of neoadjuvant VSV-IFNβ in dogs with treatment naïve osteosarcoma. The study began as an open label trial to evaluate safety, as well as whether mitigation practices would be needed to control environmental shedding. Safety and absence of environmental shedding were addressed in the first 15 dogs, after which the trial was modified to a double-blinded, placebo controlled study. Thirteen additional dogs were enrolled into this second phase of the study. Pre-treatment needle core biopsies were collected from each dog prior to VSV treatment. Tumor samples were collected 10-days later at the time of definitive surgical management (amputation). Each sample was divided into sections for diagnosis (FFPE), RNAseq, and single cell disruption to establish cell lines. RNAseq was completed for 16 pre-treatment samples that passed quality control metrics (those for which the needle core biopsies were able to obtain high quality, viable tumor material). Panel (A) shows the outcome for each dog as a function of the pre-treatment CD37 (innate immune) GCESS. Panel (B) shows the outcome for each dog as a function of the pre-treatment cell cycle GCESS. Panel (C) shows the outcome for each dog as a function of the pre-treatment CD* (adaptive immune) GCESS. Panel (D) is a box and whisker plot showing the outcome for dogs with high CD8 GCESS treated with VSV, low CD8 GCESS treated with VSV, and dogs treated with placebo. The dogs with high CD8 GCESS treated with VSV survived longer than either of the other two groups (p=0.04 and p=0.06, respectively). There was no difference in survival between the dogs in the low CD8 GCESS group treated with VSV and the dogs in the group treated with placebo (p=0.55). Similar results were seen when comparing dogs where post-VSV treatment biopsy samples had high vs low CD8 GCESS.

## 4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**

Nothing to Report (yet).

- **What was the impact on other disciplines?**

Nothing to Report

- **What was the impact on technology transfer?**

Nothing to Report

- **What was the impact on society beyond science and technology?**

Nothing to Report

## 5. Changes/Problems

- **Changes in approach and reasons for change**

The K12 and K7M2 mouse osteosarcoma models have not proved to be as reliable as we expected, and so we are developing alternative models to investigate our hypotheses and address the goals of this project, including new cell lines in the C57Bl/6 background and the MC17 sarcoma model (see above).

- **Actual or anticipated problems or delays and actions or plans to resolve them**

As we described above, the COVID-19 pandemic and the responses to the pandemic caused significant disruptions to the project. From the departure of a key person (post-doc) at the Mayo site to lab closures that lasted more than a year, to persistent problems sourcing reagents and materials and a very strong and direct request from the U of M administration to consider reducing animal activities due to staffing problems.

Unfortunately, the future course of the pandemic is unpredictable. We are planning to continue the project under the assumption that conditions will normalize, and we will be able to replace people who left the labs, procure the necessary resources (labware, reagents, etc.), and proceed with the planned experiments. We anticipate that we will request a no cost extension at the end of year-2, but we will wait to assess our remaining budget and the progress we are able to achieve this year before we decide if we will file a formal request.

- **Changes that had a significant impact on expenditures**

The COVID-19 pandemic and the resultant requirement for remote work meant that personnel effort (salary) had to be dedicated to “soft” tasks, including data analysis (see for example the data presented in Figures 3, 4, and 8 of this report), and instead of “hard” tasks (experiments in the lab). It is possible that this could create an imbalance in the budget categories at the end of the project. If re-budgeting were necessary, we will contact our program officer to discuss.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

None

- **Significant changes in use or care of human subjects**

None

- **Significant changes in use or care of vertebrate animals.**

None

- **Significant changes in use of biohazards and/or select agents**

None

## 6. Products

Nothing to Report

## 7. Participants & Other Collaborating Organizations

**What individuals have worked on the project?**

| | |
|---|---|
| Name: | *Jaime Modiano* |
| Project Role: | *PI* |
| Researcher Identifier (e.g. ORCID ID): | 0000-0001-6398-7648 |
| Nearest person month worked: | *1* |
| Contribution to Project: | *Dr. Modiano is the contact PI. He has overseen the work and contributed to the experiments conducted at the University of Minnesota, as well as to the analysis of all project data.* |
| Funding Support: | *Institutional (endowed Chair)* |

| | |
|---|---|
| Name: | *Shruthi Naik* |
| Project Role: | *Co-PI* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 6 |
| Contribution to Project: | *Dr. Naik is the Mayo site PI. She has contributed to all the experiments performed at the Mayo Clinic, including supervision of personnel (post-doc) and data analysis.* |
| Funding Support: | |

| | |
|---|---|
| Name: | *Kelly Makieslki* |
| Project Role: | *Postdoctoral research associate* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 6 |
| Contribution to Project: | *Dr. Makielski contributed to the experiments performed at the University of Minnesota (in vitro validation of ONIx, MC17 model experiments, and analysis of the VIGOR trial).* |
| Funding Support: | |

| | |
|---|---|
| Name: | *Lauren Mills* |
| Project Role: | *Research Associate* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 1 |
| Contribution to Project: | *Dr. Mills has been involved in data storage and data management, especially for sequencing data* |
| Funding Support: | |

| | |
|---|---|
| Name: | *Andy Sicheneder* |
| Project Role: | *Technician* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 1 |
| Contribution to Project: | *Mr. Sicheneder was responsible for production of ONIx* |
| Funding Support: | |

| | |
|---|---|
| Name: | *Mitzi Lewellen* |
| Project Role: | *Technician* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 1 |

| | |
|---|---|
| Contribution to Project: | *Ms. Lewellen is involved with managing inventories and the animal colonies in the Modiano lab. For this project, she has kept experimental inventories and managed the mouse colonies for the experiments shown in Figures 3 and 4* |
| Funding Support: | *The Ford Foundation (Complete only if the funding support is provided from other than this award).* |

| | |
|---|---|
| Name: | *Ashley Schulte* |
| Project Role: | *Technician* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 2 |
| Contribution to Project: | *Ms. Graef was responsible for design and oversight of the experiments shown in Figures 3 and 4. She also generated the genome-edited mouse osteosarcoma cell lines with assistance from the GESR at the University of Minnesota. She has contributed to QC testing and potency assays for ONIx and has been the lab manager and safety officer for the Modiano lab throughout the project.* |
| Funding Support: | *Karen Wyckoff Rein in Sarcoma Foundation; Morris Animal Foundation* |

| | |
|---|---|
| Name: | *Taylor DePauw* |
| Project Role: | *Technician* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 2 |
| Contribution to Project: | *Ms. DePauw was responsible for leading the ONIx validation studies (in vitro). She also was responsible for all purchasing activity and equipment maintenance in the Modiano lab.* |
| Funding Support: | |

| | |
|---|---|
| Name: | *Amber Winter* |
| Project Role: | *Certified Veterinary Technician* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 1 |

| | |
|---|---|
| Contribution to Project: | *Ms. Winter worked with Dr. Modiano and Ms. Stuebner to complete the documentation for IACUC and ACURO approval. She will be the lead nurse for the canine clinical trial.* |
| Funding Support: | |

| | |
|---|---|
| Name: | *Aishwariya Sathyanarayan* |
| Project Role: | *Postdoctoral research associate* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 2 |
| Contribution to Project: | *Dr. Aishwariya initiated the mouse experiments at Mayo Clinic before COVID-related events forced her relocation from Dr. Naik's lab.* |
| Funding Support: | |

| | |
|---|---|
| Name: | *Aaron Sarver* |
| Project Role: | *Assistant Professor* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 1 |
| Contribution to Project: | *Dr. Sarver was responsible for analysis of the RNAseq data shown in Figure 8.* |
| Funding Support: | *NIH grant R50 CA211249* |

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Several grants held by the PI at the start of this project have ended (DOD IDEA Award, K01 mentored transition to independence award where the PI was a mentor, T32 support for a postdoc, two fixed cost contracts from Boston Scientific, a contract from Vyriad, and grants from the AKC Canine Health Foundation and Morris Animal Foundation).

New grants awarded to the PI since the start of this project include a grant from Venn Foundation intended to pursue commercialization of inventions in the PI's lab and a competitive renewal from the AKC Canine Health Foundation.

The PI's effort on this grant has not changed.

- **What other organizations were involved as partners?**

The only two organizations involved, University of Minnesota and Mayo Clinic, were listed in the original application.

## 8. Special Reporting Requirements

None

## 9. Appendices

N/A