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CONTRACTING ORGANIZATION: Johns Hopkins University, Baltimore, MD

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14. ABSTRACT

Immune checkpoint blockade has been mostly ineffective for osteosarcoma. The presence of tertiary lymphoid structure (TLS) in the tumor is thought to be essential for immunotherapy to be effective. TLS are found in tumors of some but not many osteosarcoma patients, raising a question how we can promote the development of such immunostimulatory tumor environment in the remaining population of osteosarcoma patients. We hypothesize that simultaneously activating STING and lymphotoxin beta receptor (LTbR) pathways promotes the formation of intratumoral high endothelial venules (HEVs) and TLS-like lymphoid structures in osteosarcoma, thereby reprograming the immune landscape of osteosarcoma to the one that is favorable for immune checkpoint inhibition therapies. Aim 1 will determine the osteosarcoma immune landscape created by STING/LTbR agonist combination therapy. Aim 2 will determine the effect of STING/LTbR agonist combination on osteosarcoma progression and examine whether this treatment sensitizes osteosarcoma to immune checkpoint inhibition

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION

Currently, patients with chemoresistant metastatic sarcomas such as osteosarcoma and rhabdomyosarcoma have no effective treatment options. Immune checkpoint blockade has been mostly ineffective for various types of sarcomas. The presence of tertiary lymphoid structure (TLS) in the tumor is thought to be essential for immunotherapy to be effective. TLS are found in tumors of some but not many sarcoma patients, raising a question how we can promote the development of such immunostimulatory tumor environment in the remaining population of sarcoma patients. We hypothesize that simultaneously activating STING and lymphotoxin beta receptor (LTβR) pathways promotes the formation of intratumoral high endothelial venules (HEVs) and TLS-like lymphoid structures in sarcomas, thereby reprograming the immune landscape of these tumors to the one that is favorable for immune checkpoint inhibition and other cancer therapies including conventional chemotherapies. The purpose of this project is to examine the therapeutic potential of this strategy by treating sarcomas with agonists for STING and LTβR in orthotopic mouse sarcoma models.

2. KEYWORDS

Rhabdomyosarcoma, tertiary lymphoid structure (TLS), high endothelial venule (HEV), sarcoma, osteosarcoma, rhabdomyosarcoma, immune checkpoint inhibition therapy, immunotherapy, stimulator of interferon genes (STING), lymphotoxin beta receptor (LTβR), lymphocytes, B cells, T cells, germinal center

3. ACCOMPLISHMENTS

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

Specific Aims
Aim 1: To determine the osteosarcoma immune landscape created by STING/LTβR agonist combination therapy
Aim 2: To determine the effect of STING/LTβR agonist combination on osteosarcoma progression and examine whether this treatment sensitizes osteosarcoma to immune checkpoint inhibition

Task 1: Establish models of orthotopic mouse osteosarcoma and agonist treatment (months 1~3)
Task 2: Examine HEV formation in agonist-treated osteosarcoma (months 1~15)
  - Milestone 1: Demonstrate the effect of agonists on intratumoral HEV formation in osteosarcoma

Task 3: Determine immune cell profile and cytokine milieu (months 6~24)
  - Milestone 2: Demonstrate the changes in Th1/Th2 immune cell infiltration depending on the different treatments.
  - Milestone 3: Demonstrate the changes in Th1/Th2 cytokine landscape in the tumors depending on treatments.

Task 4: Examine treatment effect on tumor growth and metastasis (months 16~30)
  - Milestone 4: Demonstrate that tumor suppression depends on the tumor immune environment observed in Aim 1.

Task 5: Examine efficacy of checkpoint immunotherapy (months 19~36)
  - Milestone 5: Demonstrate that the effectiveness of immune checkpoint blockade with/without STING agonist/LTβR agonists

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

Task 1: Establish models of orthotopic mouse osteosarcoma and agonist treatment (months 1~3)

We successfully developed orthotopic sarcoma models in syngeneic mice. For this, MOS-J, M3-M-9, and 76-9 sarcoma cells were transduced with the firefly luciferase gene and subsequently implanted in the calf muscle via paratibial injection. The tumor growth was analyzed by caliper measurements as well as by IVIS bioluminescence imaging. This was an important accomplishment for the project because, it turned out, these three sarcoma models have different levels of MHC-class I expression, providing an excellent opportunity to examine the importance of T cell-dependent adaptive immunity for HEV and TLS formations in sarcomas.

FACS analyses showed that the levels of MHC-class I expression were M3-M-9 >> MOS-J > 76-9 (Fig. 1). There was no detectable MHC-class II expression.

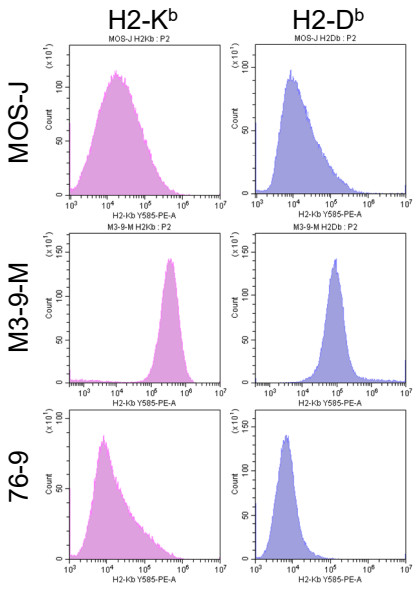

Fig. 1 MHC-class I expression comparison between sarcoma cell lines

Task 2: Examine HEV formation in agonist-treated osteosarcoma (months 1~15)

- Milestone 1: Demonstrate the effect of agonists on intratumoral HEV formation in osteosarcoma

We treated MOS-J and M3-9-M tumors with STING agonist alone, LTβR agonist alone, or STING/LTβR agonist combination and compared with untreated control. STING agonist was administered intratumorally once and LTβR was given via i.p. every 3-4 days, a total of 4 times over 10 days. These treatments rarely induced HEVs or TLS in these tumors as we previously observed in our pilot study. So, we next treated orthotopic M3-9-M tumors with 4 injections of STING agonist over 10 days, given at the same time as LTβR agonist. This treatment schedule successfully induced the formation of HEVs and TLS in M3-9-M tumors. TLS were identified as several large dense clusters of CD19$^+$ B cells developed at tumor periphery with CD3$^+$ T cells around them (Fig. 2). A number of smaller TLS were also found at the tumor periphery. These TLS were associated with HEVs detected by MECA-79 staining (Fig. 2). This result demonstrates that the simultaneous activation of STING and LTβR signaling in the tumor microenvironment by agonist combination is useful for inducing HEVs and TLS and create an immunostimulatory tumor microenvironment, suggesting a novel therapeutic strategy for sarcomas.

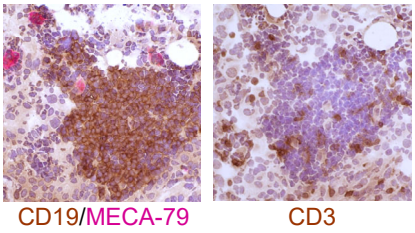

Fig. 2 Induction of TLS and HEV formation in M3-9-M tumors by STING and LTβR agonist treatment

Task 3: Determine immune cell profile and cytokine milieu (months 6~24)

- Milestone 2: Demonstrate the changes in Th1/Th2 immune cell infiltration depending on the different treatments.
- Milestone 3: Demonstrate the changes in Th1/Th2 cytokine landscape in the tumors depending on treatments.

To determine tumor infiltrating immune cell types and their gene expression including cell markers, transcription factors like Bcl6, and cytokines, we conducted a single-cell analysis of FACS-sorted CD45$^+$ cells from cell-dissociated KPC tumors from the four treatment groups (A-D) at Day 14 post treatment initiation. This study revealed that B cell population has increased in the tumors in Group C and D, corroborating with the

histological observation of TLS in these groups. Especially, the increase in Group D was substantial. We detected the significant presence of germinal center B cells in tumors of Group D, indicated as $Bcl6^{+}CD19^{+}$ B cells. Germinal center B cells were undetected in Groups A and B, supporting the idea that those TLS in Group D have functional germinal centers as a hub for B cell priming with tumor antigens. Supporting this idea, immunofluorescence staining of tumor sections demonstrated the abundant presence of $CD138^{+}IgG^{+}CD19^{+}$ B cells, i.e. antibody producing plasma cells in Group D tumors treated with STING/LTβR agonist combination. This suggests that B cells are primed, clonally expanded, and class-switched locally at TLS, again supporting the germinal center activities of the TLS. We are currently conducting B cell receptor (BCR) profiling by single-cell analysis, which will further analyze the clonality of B cells and their somatic hypermutations. These results suggest the importance of humoral immunity for the mechanism of agonist combination therapy. Therefore, we next tested the tumor-specificity of the plasma IgG from these mice. In this study, mice were treated with agonist monotherapy or combination therapy as neoadjuvant treatment followed by tumor resection surgery. The plasma was collected 2 weeks later and incubated in vitro with KPC cell suspension, and the plasma IgG and IgM binding to the cells were analyzed by FACS. We found significantly increased IgG binding to the tumor cell surface for the plasma of Group D mice, indicating that the mouse plasma contains elevated levels of tumor-specific IgG after neoadjuvant treatment with STING and LTβR agonist combination (Fig. 3).

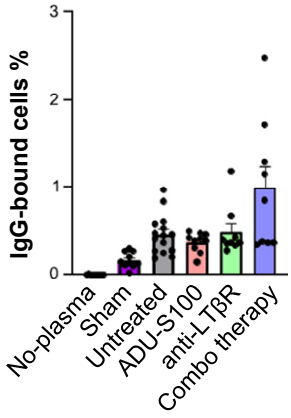

Fig. 3 Agonist combination-treated mice have an elevated level of tumor-binding IgG in plasma The binding of mouse plasma IgG to the tumor cell surface was examined by incubating plasma with KPC cells *in vitro* followed by FACS using goat anti-mouse IgG antibody. Mouse plasma was diluted 10× in PBS for this assay.

Task 4: Examine treatment effect on tumor growth and metastasis (months 16~30)

- Milestone 4: Demonstrate that tumor suppression depends on the tumor immune environment observed in Aim 1.

The effects of agonist treatments on the growth of primary tumors were investigated. We found that the LTβR monotherapy had little/no effects and the STING monotherapy (a single dose at 2 μg/tumor) had only moderate effects on the growth of primary tumors of KPC tumors and Py230 tumor at this dose (Fig. 4). In comparison, the combination therapy reduced KPC tumor burden by more than 50% and significantly delayed Py230 tumor growth (Fig. 4). However, these effects started days before TLS formation and are therefore independent of TLS. To investigate the contribution of TLS to the development of anti-tumor immunity and tumor control, we surgically resected the subcutaneous KPC tumors after mono- or combination therapy on Day 14 and reinoculated KPC tumor cells subcutaneously 2-3 weeks later to mimic tumor recurrence (Fig. 5A). Remarkably, most mice that received the neoadjuvant therapy of combined agonists prior to the tumor resection showed excellent long-term survival (Fig. 5B). The reinoculated tumor cells were initially taken by the host mice as indicated by the growth of small bumps at the injection sites for several days, but these small tumors regressed to disappear completely later, except for a few cases, demonstrating that these animals became resistant to KPC tumors. Mice that received neoadjuvant LTβR monotherapy also acquired tumor resistance, but of much lesser strength (Fig. 5B). The neoadjuvant STING monotherapy had little survival benefit, and the reinoculated tumors grew rapidly in most mice similarly to the untreated control mice. Histological examinations of the reinoculation sites 8 days later revealed extensive infiltration of $CD3^{+}$ T cells and neutrophils into the tumor interior or periphery of the mice that received neoadjuvant combination therapy.

The immune cell types responsible for this therapeutic effect were determined. We depleted $CD8^{+}$ T cells, NK cells, or neutrophils from the mice 1 day before the tumor reinoculation. The mice lost tumor resistance after $CD8^{+}$ T cell depletion. The NK cell depletion also decreased the ability of the mice to reject tumor reinoculation,

but neutrophil depletion had no effect. These results demonstrate the importance of cell-mediated cytotoxicity for the mechanism of action of the agonist combination therapy.

The evidence for the importance of humoral immunity was obtained from a serum transfer experiment. Mouse serum was collected from the donor mice that underwent neoadjuvant agonist mono- or combination therapy and tumor resection surgery. The serum was transferred to tumor-bearing recipient mice, and the tumor growth was monitored. The serum transfer from Group D mice, but not Group A mice, inhibited tumor growth of the recipient mice, indicating that the serum of Group D mice contains strong anti-tumor immunity developed in the donor mice after the neoadjuvant treatment (Fig. 6).

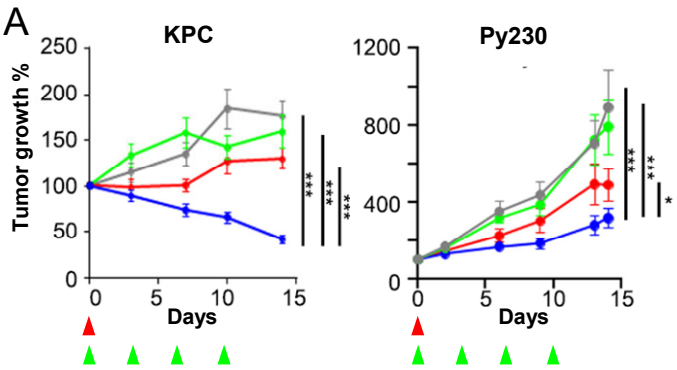

Fig. 4 Effect of agonists on subcutaneous KPC and orthotopic Py230 tumor growth Tumor growth was monitored for 14 days from the treatment start. *P<0.05, ***P<0.001

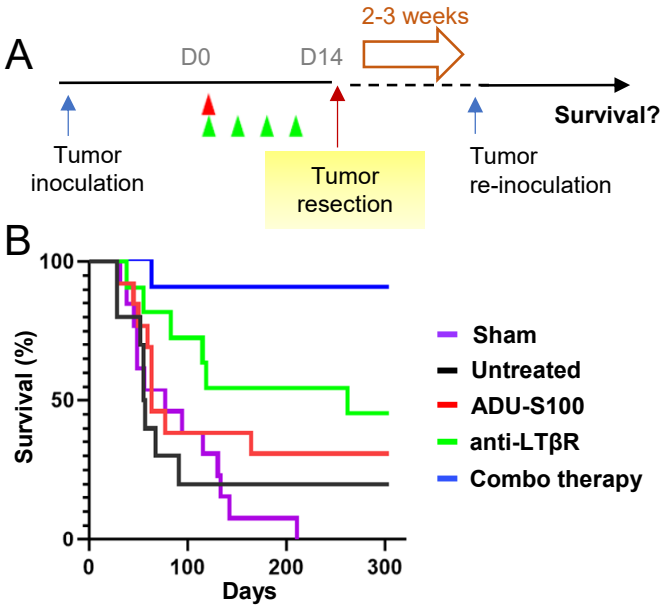

Fig. 5 Neoadjuvant STING/LTβR agonist combination protects mice from 2nd tumor challenge **A.** KPC tumor-bearing mice were treated with neoadjuvant agonist mono- or combination therapy and underwent tumor resection surgery on Day 14. KPC cells were re-inoculated 2-3 weeks later to mimic tumor recurrence. **B.** Long-term survival for 300 days of neoadjuvant/surgery-treated mice. A group of control mice didn't receive the 1st tumor inoculation but underwent sham surgery (Sham). They then received tumor inoculation 2 weeks later together with other groups. N = 10~13. Untreated vs. anti-LTβR p=0.072; Untreated vs. Combo therapy, p=0.001; anti-LTβR vs. Combo therapy, p=0.041 using Log-rank (Mantel-Cox) test.

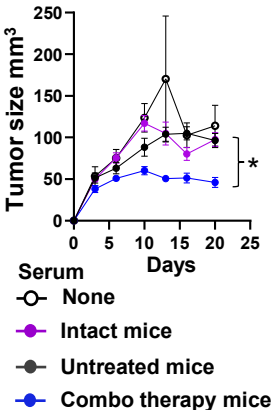

Fig. 6 Serum transfer from STING/LTβR agonist-treated mice inhibits tumor growth Treatment-naïve mice received no serum, serum from intact mice (no tumor), serum from untreated tumor-resected mice, or serum from STING/LTβR-treated tumor-resected mice. The growth of KPC tumor implants in these serum-recipient mice was monitored for 20 days. *P < 0.0001

Therapeutic effects of agonists were also investigated in the M3-9-M sarcoma model. STING monotherapy was as effective as STING/LTβR combination therapy for a short time when given 4 times. However, in a long-term observation, the combination therapy showed much more prolonged tumor suppression (Fig. 7). In comparison, STING monotherapy was only moderately effective against 76-9 sarcoma in short-term observation whereas STING/LTβR agonist combination was highly effective. These results demonstrated the benefit of STING and LTβR agonist combination in treating sarcomas.

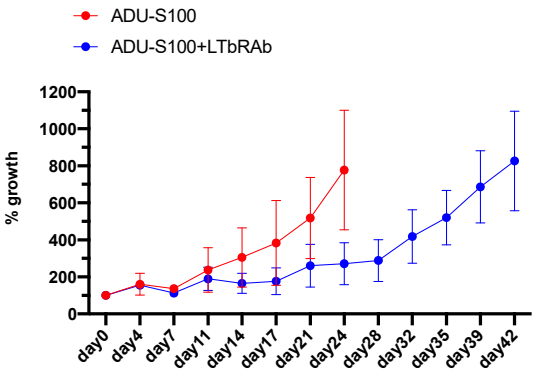

Fig. 7 Suppression of M3-9-M sarcoma growth by agonist treatment

Task 5: Examine efficacy of checkpoint immunotherapy (months 19~36)

- Milestone 5: Demonstrate that the effectiveness of immune checkpoint blockade with/without STING agonist/LTβR agonists

We found PD-L1 expression levels also vary between these sarcoma cell lines. The PD-L1 expression levels were M3-M-9 >> 76-9 > MOS-J (Fig. 8). This is crucial information for the success of Aim 2 as the PD-L1 expression level in tumor cells is one of the key factors to significantly affect anti-PD-1/PD-L1 immune checkpoint inhibition therapies. Interestingly, the treatment of KPC tumor cells in culture with STING agonist ADU-S100 increased expression of both PD-L1 and MHC-class I (Fig. 9), suggesting that the agonist combination therapy will potentiate immune checkpoint inhibition therapy. It is interesting to see whether this happens also in sarcoma cells.

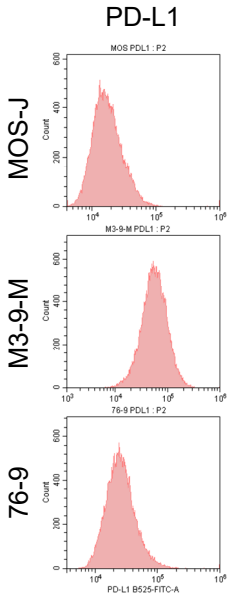

Fig.8 PD-L1 expression comparison between sarcoma cell lines

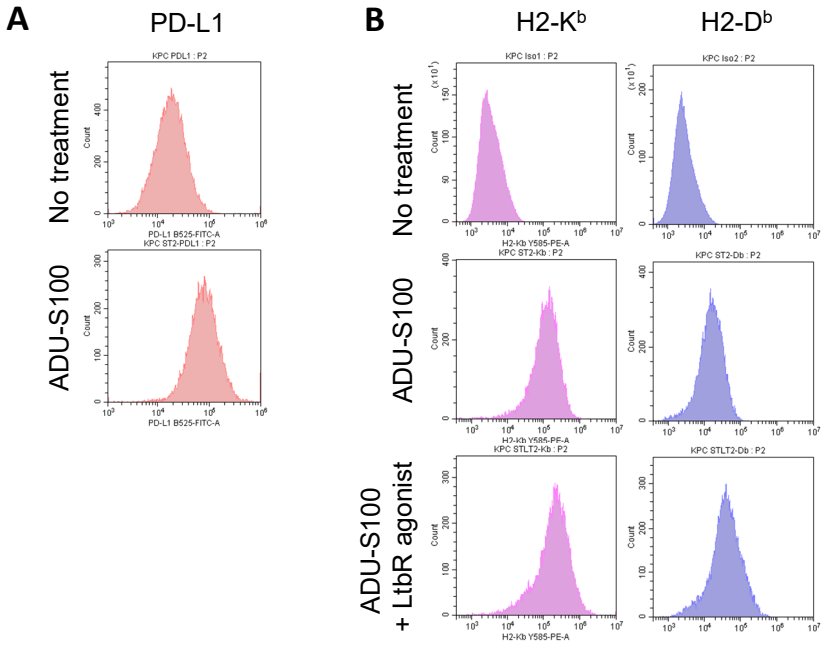

Fig. 9 PD-L1 and MHC-class I expression in KPC cells before and after STING activation by the agonist ADU-S100

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

This grant provides a training opportunity for a postdoctoral fellow. Training activities include one-on-one discussion with the mentor and group discussion with the team at the weekly lab meetings and individual progress reports. Professional development activities include gaining knowledge and skills in the area of onco-immunology and biomedical sciences as well as various scientific techniques.

- **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?**

In the next reporting period, we will examine whether STING/LTβR agonist combination is effective against MOS-J osteosarcoma and produces TLS in these tumors when dosing of STING agonist is increased 4 times as we did for M3-9-M and 76-9 sarcomas. We will then conduct single-cell analyses and immunofluorescence analyses of these sarcoma tumors similar to the studies we did with the KPC tumor model. We will also conduct studies in K7M2 osteosarcoma in Balb/c mice to compare with other sarcoma models. The studies of immune cell depletion, serum transfer, and plasma-tumor cell binding will be conducted using these sarcoma tumor models to investigate the mechanism of action of the agonist combination therapy.

4. IMPACT

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

Our approach uses an agonistic antibody to activate LTβR. There have been many examples of monoclonal antibody therapies that were successfully adapted to clinical use. STING agonists are already in clinical trials, and despite some setbacks, some cGAMP-based STING agonists are promising for clinical use. Our method can induce TLS that morphologically and functionally resemble TLS observed in human cancer. Our evidence suggests the presence of germinal center activities in the agonist-treated tumors to prime, expand, and differentiate tumor-reactive B cells to $CD138^{+}$ antibody-producing plasma cells—signs of functional TLS. The ability to induce TLS in mouse tumors by drugs in a simple method has given us an unprecedented opportunity to investigate TLS. With this method as a study tool, we are now able to interrogate the precise contributions of

TLS to tumor immunity and determine the benefit of therapeutically promoting TLS formation in sarcomas. Various preclinical tumor models can be adapted to this method, and the information resulting from such studies is expected to be clinically translatable. This tool will also enable us to explore the mechanism of TLS formation and function at cellular and molecular levels. We can closely monitor the TLS formation in time-course analyses to observe the sequence of the key events—for example, to ask a question which immune cell types are recruited first to initiate the lymphoid aggregate, or how early lymphocyte aggregates become functional TLS. When combined with other systems, such as transgenic or gene knockout mice, the drug-induced TLS will be extremely useful to determine the significance of certain cell types or effector molecules involved in TLS formation and/or function. Such questions are not possible to fully address in clinical studies.

The study of plasma IgG binding to tumor cell surface showed that the plasma IgG-bound KPC cells significantly increased for the agonist combination group. This is an important observation because cancer patients with high number of IgG-bound tumor cells demonstrate significant responses to immune checkpoint inhibition and prolonged progression free survival.

- **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

Nothing to report

6. PRODUCTS

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

Name:	Masanobu Komatsu, Ph.D.
Project Role:	PI
Research Identifier:	0000-0001-7548-137X
Person months:	2.4
Contribution:	Brings expertise in tumor biology, immunology, and vascular biology. Serves as the principal investigator of this project. Oversees all aspects of proposed project, including the planning, execution, and evaluation of all proposed experiments.
Funding support:	Additional support from NIH R01 R01CA251192, Johns Hopkins University institutional funds

Name:	Yasuhiro Kikuchi, M.D. Ph.D.
Project Role:	Postdoctoral fellow
Research Identifier:	None
Person months:	6

Contribution: Conducts various in vivo analyses to determine the effect of SITNG and LTβR agonists on the tumor immune environment using mouse sarcoma models. Work with other postdocs to investigate the effect of these agonists on tumor progression, lung metastasis, and the efficacy of immune checkpoint inhibition treatment in the mouse models.
Funding support: Johns Hopkins All Children's institutional funds

Name: Maxwell Duah, M.D. Ph.D.
Project Role: Postdoctoral fellow
Research Identifier: None
Person months: 12
Contribution: Works with Dr. Kikuchi in various tasks of proposed experiments. These include immunofluorescence of tissue sections, image data analysis, *in vivo* experiments with mouse sarcoma models including the study of tumor progression, lung metastasis, and the efficacy of immune checkpoint inhibition.
Funding support: N/A

Name: Maxwell Duah, M.D. Ph.D.
Project Role: Postdoctoral fellow
Research Identifier: None
Person months: 6
Contribution: Works with Dr. Kikuchi in various tasks of proposed experiments. These include immunofluorescence of tissue sections, image data analysis, *in vivo* experiments with mouse sarcoma models including the study of tumor progression, lung metastasis, and the efficacy of immune checkpoint inhibition.
Funding support: Additional support from NIH R01 R01CA251192, Johns Hopkins All Children's institutional funds

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

Nothing to report

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

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. APPENDICES

None