AWARD NUMBER: W81XWH-21-1-0332

TITLE: Ultrasound-Guided Targeted STING Activation for Breast Cancer Immunotherapy

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REPORT DATE: July 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2022	Annual	01Jul2021-30Jun2022

4. TITLE AND SUBTITLE

Ultrasound-Guided Targeted STING Activation for Breast Cancer Immunotherapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-21-1-0332

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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Houston, TX 77030-4017

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Despite recent successes of cancer immunotherapy, only a fraction of patients with breast cancer benefit from such treatment. Therefore, there is an urgent and unmet need to develop more effective immunotherapy strategies for breast cancer patients. Purpose: Our proposal aims to develop a novel and translatable strategy using microbubble-assisted US activation (MUSIC) to enhance STING activation in antigen presenting cells (APCs) to treat highly aggressive breast cancers. Scope: We will first evaluate MUSIC's ability to activate STING in vivo and its antitumor effect against different subtypes of breast tumors. We will then determine whether MUSIC treatment can produce systemic antitumor immune responses as a monotherapy or in combination with an anti-PD-1 antibody against metastatic breast cancers. Results: We have shown that MUSIC treatment promotes the activation of cGAS-STING pathway in APCs and enhance type I interferon responses by bone-marrow derived APCs as well as tumor-associated macrophages (TAMs) from both mouse and human. We have also found that MUSIC inhibited tumor growth in syngeneic breast cancer models and its effect is dependent on host STING signaling. Significance: These results confirm our hypothesis that MUSIC can activate STING in APCs and promote antitumor immune responses against breast cancer in vivo.

15. SUBJECT TERMS
Ultrasound, breast cancer, immunotherapy, cGAS-STING, microbubble, image guidance

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	9	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

Cancer immunotherapy that targets the adaptive immune system such as immune checkpoint blockers has recently emerged as a revolutionary treatment for metastatic cancers including melanoma, non-small cell lung and renal cell carcinoma. The efficacy of cancer immunotherapy against breast cancer, however, has been limited, with only a subpopulation of patients with triple negative breast cancer appearing to benefit in the case of immune checkpoint inhibitor treatment. Increasingly, there is a realization that to improve the response rate of cancer patients to immunotherapy, stimulation of the innate immune system is also highly critical. As a result, agonists that target several innate immune regulators including the cytosolic DNA sensor cyclic GMP-AMP Synthase-Stimulator of Interferon Genes pathway (cGAS-STING) are now being investigated and produced highly promising results. However, STING agonists such as cyclic GMP-AMP (cGAMP), cannot easily penetrate into antigen presenting cells (APCs) cytosol, which is needed to activate inflammatory response and generate antitumor immunity, thus severely limiting their clinical potential. Our present proposal aims to address this major limitation of STING-targeted cancer immunotherapy by proposing an innovative microbubble-assisted ultrasound-guided platform to efficiently deliver cGAMP into APCs to prime potent antitumor T cell responses. This technology platform, which we termed Microbubble-assisted UltraSound-guided Immunotherapy of Cancer (MUSIC), comprises of a clinically approved microbubble (MB) contrast agent modified to load cGAMP and to specifically target immune cells. When applying ultrasound pulses with a clinical scanner, the microbubbles will generate small transient pores on APC's surface and release cGAMP directly into the cytosol to activate STING and down-stream pathways. We hypothesize that MUSIC represents a revolutionary way to deliver STING agonists into immune cells and provide an effective immunotherapy strategy to treat metastatic breast cancer. Furthermore, it can be combined with T cell therapies such as anti-PD-1 antibody to produce improved therapeutic effect across multiple breast cancer subtypes. In our current study, we are testing our overall hypothesis by completing the following specific aims. In Aim 1, we evaluated MUSIC treatment's ability to activate STING and inhibit breast tumor growth in vivo. In Aim 2, we will determine the antitumor effect of MUSIC against metastatic breast cancers, and study whether MUSIC can be combined with anti-PD-1 therapy to produce more potent antitumor immune response. We will also evaluate the toxicity profile of the combination treatment in preparation for clinical trials. Cancer immunotherapy has revolutionized the way we treat patients with many types of cancers and patients diagnosed with previously terminal cancers can now achieve long-term remission. Our proposed research will further advance the field and provide new ways to boost the efficacy of cancer immunotherapy in breast cancer. If successful, our proposed research can overcome a major technical hurdle that is currently facing cancer immunotherapy targeting the STING pathway and greatly advance its progress towards clinical translation.

2. KEYWORDS

Ultrasound, breast cancer, immunotherapy, cGAS-STING, microbubble, image guidance

3. ACCOMPLISHMENTS

What were the major goals of the project?

Major Milestone 1 Assessing the antitumor effect of MUSIC treatment in orthotopic syngeneic murine breast cancer models and characterize immune cell profiles within tumors after treatment (Proposed completion date: 7/2022, completion date: 7/2022)

Major Milestone 2: ACURO approval (Proposed completion date: 1/2022, completion date: 10/2021)

Major Milestone 3: Determination of mechanism of action of MUSIC's antitumor responses against localized breast cancer (Proposed completion date: 7/2023)

Major Milestone 4: Determined the antitumor effect of MUSIC against metastatic breast cancer and determine the extent of tumor infiltrating immune cells after different treatment regimens. (Proposed completion date: 1/2024)

Major Milestone 5: Determined the antitumor effect and systemic immunity of MUSIC treatment against metastatic breast cancers. (Proposed completion date: 1/2024)

Major Milestone 6: Determined the antitumor effect and toxicity effect of combined MUSIC and antiPD1 treatment against metastatic breast cancers. (Proposed completion date; 7/2024)

What was accomplished under these goals?

Major Milestone 1: Assessing the antitumor effect of MUSIC treatment in orthotopic syngeneic murine breast cancer models and characterize immune cell profiles within tumors after treatment (Proposed completion date: 7/2022, completion date: 7/2022)

Major activities:
- Performed experiments to test antitumor effect of MUSIC on orthotopically implanted murine breast tumors.
- Performed experiments to characterize immune cell profiles in treated tumor samples

Specific objectives:
To investigate whether MUSIC promotes produces antitumor effect against murine breast cancer model and modify the local immune landscape.

Significant results or key outcomes:
1) We have shown that MUSIC treatment promotes the activation of cGAS-STING pathway in APCs and enhance type I interferon responses by bone-marrow derived APCs as well as tumor-associated macrophages (TAMs) from both mouse and humans (**Figure 1**). For this experiment, we collected bone marrow-derived APCs and TAMs from C57BL/7 mice, and treated them with MUSIC, cGAMP or microbubbles alone. We then measured the activation of cGAS-STING and downstream effectors. We also performed assays to measure both mRNA and protein expressions of type I interferons using ELISA. Finally, we incubated the treated APCs spiked with cOVA peptide with T cells from transgenic OT-I and OT-II mice and measured the proliferation of T cells using flow cytometry. We found that MUSIC treatment significantly enhanced the phosphorylation of STING and downstream effect IRF3 in APCs. This effect was abolished when treating APCs from STING KO mice. We also noted that the increased STING phosphorylation is associated with increased production of type I IFN, which against was negated when using STING KO APCs. Interestingly, the MUSIC treated APCs were more efficient in priming both CD4 and CD8 T cells in a STING dependent manner compared to the treatment with cGAMP alone.

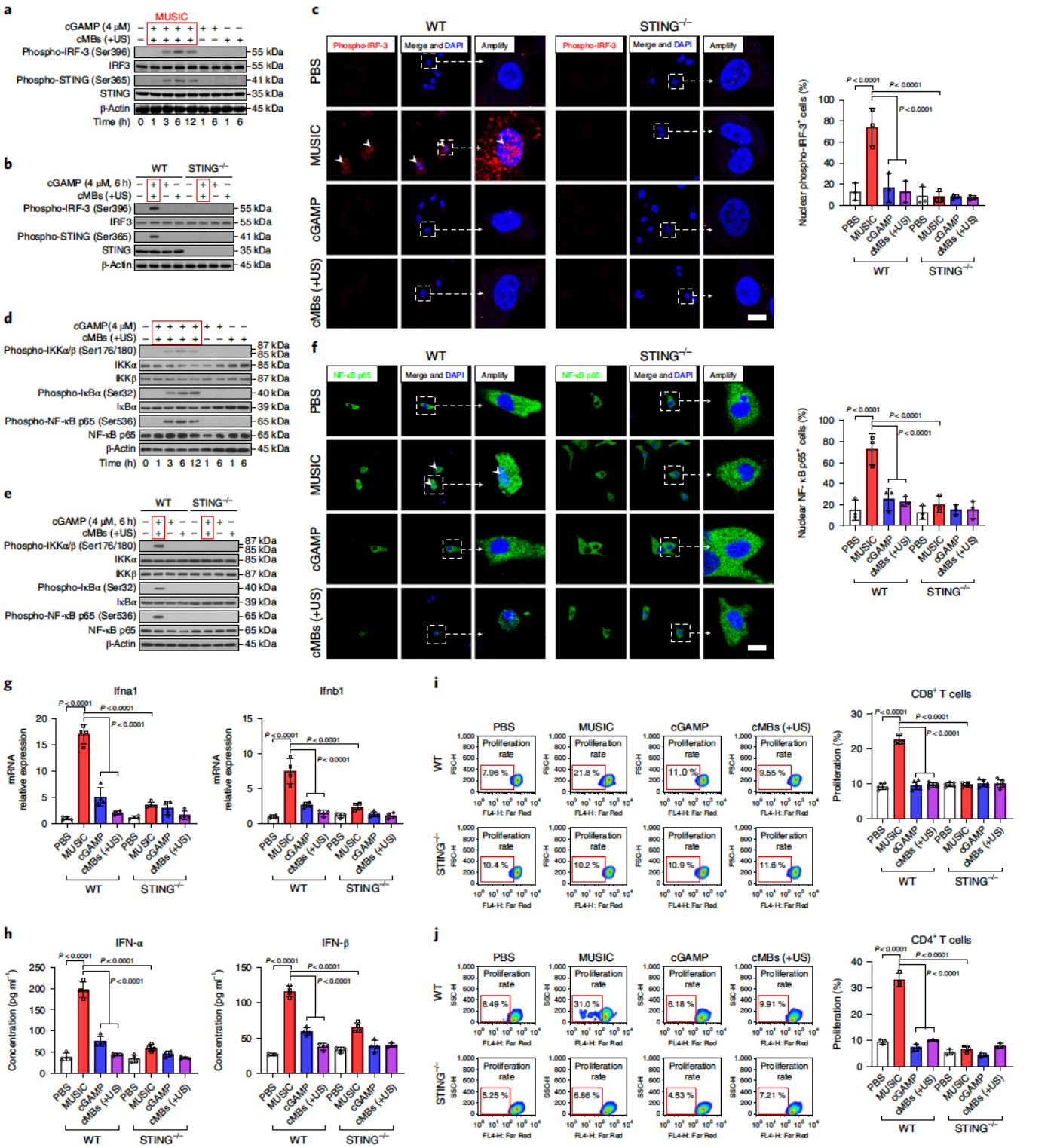

Figure 1: Sonoporation of APCs targeted by (MUSIC) induces activation of STING-IRF signaling in vitro. a,b, BMDMs from C57BL/6J mice were treated with MUSIC, cGAMP or control MBs (cMBs) (+US), respectively. After treatment, the cells were cultured for the indicated periods, followed by western blotting of proteins in the STING-IRF3 pathway. a, The strongest STING-IRF3 activation was induced 6 h after MUSIC treatment. b, STING knockout abrogated MUSIC induced STING-IRF3 activation. c, Confocal fluorescence microscopy images of nuclear translocation of activated IRF3 (phosphorylated IRF3 or pIRF3) in BMDMs at 6 h after treatment. d,e, Western blotting of NF-κB pathway signaling was performed using the same cells treated in a and b. The red box in a,b,d,e refers specifically to MUSIC treatment. f, Confocal fluorescence microscopy images of the nuclear translocation of activated NF-κB (p65) in BMDM. c,f, Scale bar, 20 μm. Right panels indicate quantification of nuclear fluorescent-positive cells done by randomly measuring 500 cells in each group. g, ifna1 and ifnb1 mRNA expression levels in mouse BMDMs treated as above were determined via real-time PCR. h, IFN-α and IFN-β cytokine released in cell culture supernatants from above were measured by ELISA. g,h, n = 4 repeats in each

group. i,j, CD8+ T cells from OT-I mice and CD4+ T cells from OT-II mice were stained with Far Red. T cell proliferation was measured by flow cytometry after co-culture with MUSIC-activated BMDMs with pulsed OVA peptides for 72 h, respectively. Right panels indicate the quantification of proliferated cells as gated. i, n = 6; j, n = 3. All data are representative from at least three biologically independent experiments. c,f–j, data are presented as mean ± s.d.; statistical significance was calculated by one-way ANOVA with Tukey's multiple comparisons test.

2) To assess the effect of MUSIC-mediated STING activation on tumor growth, mice were treated with MUSIC, cGAMP, cMBs (+US) or non-targeted (MB shell modified with non-specific IgG instead of anti-CD11b) cGAMP-nanocomplex conjugated MBs (ncMBs) (+US) and monitored over time. We observed that MUSIC given every other day for three treatments led to the most substantial tumor growth inhibition and survival benefit (Fig. 4a–d). As expected, no antitumor effects were observed in STING−/− tumor-bearing mice treated with MUSIC (Fig. 2). Notably, these MUSIC-treated tumor-free mice were resistant to EO771 tumor cell rechallenge, suggesting that MUSIC treatment produced antitumor memory responses (Fig. 2). When we collected T cells from spleens of MUSIC-treated tumor-bearing mice and rechallenged them with the EO771 tumor cells in vitro, we observed a robust IFN-γ response, thus confirming that the local MUSIC treatment generated systemic immune memory in vivo (Fig. 2).
We then analyzed treated tumor tissue samples by flow cytometry and noted a moderate increase in the populations of CD44highCD62Llow effector memory and CD44highCD62Lhigh central memory cells after MUSIC treatment.

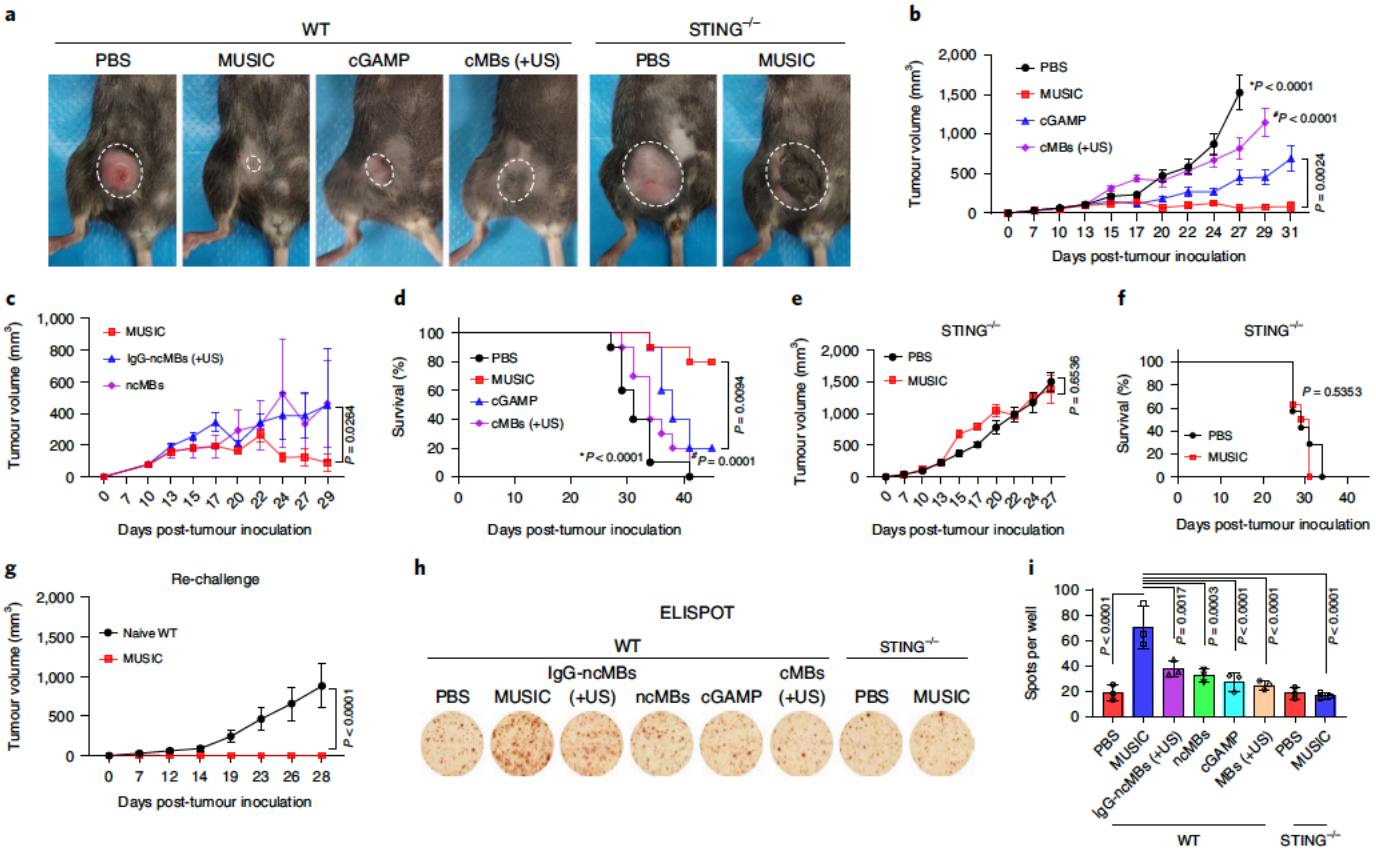

Figure. 2. MUSIC activates STING-mediated antitumor immunity. a–g, WT and STING−/− mice were inoculated with EO771 breast tumors, and treated with MUSIC, cGAMP or cMBs (+US). a, Representative photographs of mice at 24 days post-tumor inoculation. b,c,e, Tumor volumes were monitored and analyzed over the indicated periods. d,f, Survival curves for the mice in the different treatment groups. g, The six living tumor-free mice from the above MUSIC-treated group (b,d) were rechallenged with EO771 cells. Tumor volumes were measured over the following 28 days. n = 10 for all WT mice, n = 7 (PBS) or 8 (MUSIC) for STING−/− mice (a,b,d–f), n = 6 for ncMBs and n = 7 for both MUSIC and IgG-ncMBs (+US) (c). a–g, n means biologically independent animals. h,i, Splenic T cells from mice treated 18 days post-tumor inoculation were assessed by ELISPOT to further verify immune memory enhancement upon MUSIC treatment. n = 3 biologically independent samples.

Major Milestone 2: ACURO approval

Major activities: Obtained ACURO approval of animal protocol.

Specific objectives: Obtain ACURO approval of animal protocol.

Significant results or key outcomes: Animal protocol approved by ACURO.

What opportunities for training and professional development has the project provided?
Nothing to Report

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?
For the next reporting period we aim to complete Major Milestone 3, by finishing evaluating the immune cell population profiling of tumor tissue treated with MUSIC or controls. We also plan to conduct experiments to complete milestone 4 and 5, which include evaluating the extent of T cell infiltration into breast tumors following MUSIC treatment; evaluating the antitumor effect of combined MUSIC + anti-PD1 antibodies against metastatic breast cancer; and evaluate the toxicity profiles of this combo treatment.

4. IMPACT
Nothing to report

5. CHANGES/PROBLEMS
Actual or anticipated problems or delays and actions or plans to resolve them
No problem or delayed encountered.

6. PRODUCTS
Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

Name:	Wen Jiang
Project Role:	Principal Investigator
Research Identifier:	
Nearest person month worked:	1
Contribution to Project:	Dr. Jiang supervised the research project and evaluated the data
Funding Support:	N/A

Name:	Xuefeng Li
Project Role:	Postdoctoral Fellow
Research Identifier:	
Nearest person month worked:	6
Contribution to Project:	Dr. Li performed experiments related to in vitro and in vivo characterization MUSIC in breast cancer models
Funding Support:	N/A

Name:	Yifan Wang
Project Role:	Postdoctoral Fellow
Research Identifier:	
Nearest person month worked:	6
Contribution to Project:	Dr. Wang performed experiments related to in vivo MUSIC characterization and immune assays
Funding Support:	N/A

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
Nothing to Report

9. **APPENDICES**
Nothing to report