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TITLE: Elucidating and Therapeutic Targeting of Prostate Bone Metastasis

PRINCIPAL INVESTIGATOR: Y. Alan Wang

CONTRACTING ORGANIZATION: The University of Texas MD Anderson Cancer Center

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6. AUTHOR(S)

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5e. TASK NUMBER

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas MD Anderson Cancer Center,
1515 Holcombe Blvd, Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT

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

U.S. Army Medical Research and Materiel Command
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14. ABSTRACT

Prostate cancer (PC), the most common non-cutaneous malignancy in men in the United States(1), often progresses to metastatic castration resistant prostate cancer (mCRPC) in bone. While immune checkpoint blockade (ICB) has yielded meaningful responses across many cancers, clinical trials with anti-CTLA4 or anti-PD1 have shown minimal activity in prostate cancer patients. Tumor Immune Micro Environment (TIME) has been increasingly recognized to play essential roles in regulating tumor proliferation, angiogenesis, invasion, metastasis, immune evasion, and resistance to therapeutics but TIME of bone metastases of PC is relatively poorly defined. We hypothesize that the immune suppressive TIME within the bone metastases may exert an important suppressive role on effector immune cells, including CD8 T cells and that depleting bone metastases infiltrating immune suppressive myeloid cells (ISMC) will overcome de novo resistance of ICB therapy against mCRPC. To study the TIME of metastatic PC in bone and dissect the mechanism of ICB failure, we have established syngeneic mouse models in which primary PC cell lines are established from prostate tumor cells of *CPPSML* mice and injected into C57BL/6 host through intra-femoral injection to generate bone metastasis. We first plan to perform imaging Mass Cytometry (iMC) using 31-panel antibody to comprehensibly define the TIME in this syngeneic model. The iMC results will be validated with IHC co-staining or multiplex IHC staining. Then we will combine ICB with targeted depletion of ISMCs to see if this approach enhances ICB. Specifically, we will use CXCR2 inhibitor, GSK inhibitor, CSF1R inhibitor or anti-IL6 antibody with or without ICB. Finally, emergence of novel cell populations and immune regulators after combination treatment will be subjected to detailed analysis. We will isolate those resistant cells and perform RNAseq, RPPA and cytokine array to understand resistance mechanism.
We have successfully established the syngeneic mouse model for the bone met PC using repeated enrichment of DX1 cells from bone mets after intracardiac injection. We also established protocol for iMC and obtained a 31-panel antibody for iMC, conjugated with different metal labels and validated.

15. SUBJECT TERMS

Prostate cancer, immune suppressive myeloid cells, imaging mass cytometry, immune checkpoint blockade, metastasis,

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a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Prostate cancer (PC), the most common non-cutaneous malignancy in men in the United States(1), often progresses to metastatic castration resistant prostate cancer (mCRPC) in bone. While immune checkpoint blockade (ICB) has yielded meaningful responses across many cancers, clinical trials with anti-CTLA4 or anti-PD1 has shown minimal activity in prostate cancer patients. Tumor Immune Micro Environment (TIME) has been increasingly recognized to play essential roles in regulating tumor proliferation, angiogenesis, invasion, metastasis, immune evasion, and resistance to therapeutics but TIME of bone metastases of PC is relatively poorly defined. We hypothesize that the immune suppressive TIME within the bone metastases may exert an important suppressive role on effector immune cells, including CD8 T cells and that depleting bone metastases infiltrating immune suppressive myeloid cells (ISMC) will overcome de novo resistance of ICB therapy against mCRPC. To study the TIME of metastatic PC in bone and dissect the mechanism of ICB failure, we have established syngeneic mouse models in which primary PC cell lines are established from prostate tumor cells of *CPPSML* mice and injected into C57BL/6 host through intra-femoral injection to generate bone metastasis. We first plan to perform imaging Mass Cytometry (iMC) using 31-panel antibody to comprehensibly define the TIME in this syngeneic model. The iMC results will be validated with IHC co-staining or multiplex IHC staining. Then we will combine ICB with targeted depletion of ISMCs to see if this approach enhances ICB. Specifically, we will use CXCR2 inhibitor, GSK inhibitor, CSF1R inhibitor or anti-IL6 antibody with or without ICB. Finally, for resistant cases, we will try to dissect the mechanism. Emergence of novel cell populations and immune regulators after combination treatment will be subjected to detailed analysis. We will isolate those resistant cells and perform RNAseq, RPPA and cytokine array to understand resistance mechanism.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Prostate cancer, immune suppressive myeloid cells, imaging mass cytometry, immune checkpoint blockade, metastasis,

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

1. Complete approval of all experimental procedures at the institutional level and order reagents and animals.
2. Establish iMC protocol for bone mets microenvironment study.

2.1. work with iMC core facility to acquire and establish necessary iMC grade antibodies and metal conjugation of antibodies.
2.2. test iMC antibodies for mouse specificity on mouse tissue slides.

3. Establish metastatic bone tumors through intra femoral injection
4. Order animals for injection, and harvest and prepare bone mets for downstream analysis.
5. iMC analysis of bone mets samples

5.1. staining and iMC data acquisition
5.2. IHC and multiplex IHC staining for further validation

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Establishment of bone metastatic model: To study the tumor immune microenvironment in bone metastatic prostate cancer, we have established syngeneic mouse model in which primary prostate cancer cell line DX1-GFP isolated from *CPPSML* mice (Lu et al., Nature 2017) was enriched for metastatic cells (DX1-BM) through repeated intracardiac injection and isolation from bone marrow. DX1-BM cells were then injected into the femur of C57BL/6 mice and resulting tumor was monitored by X ray (A), BLI (B) and histopathology C) (Fig 1). Tumors were apparent between week 2 and 3.

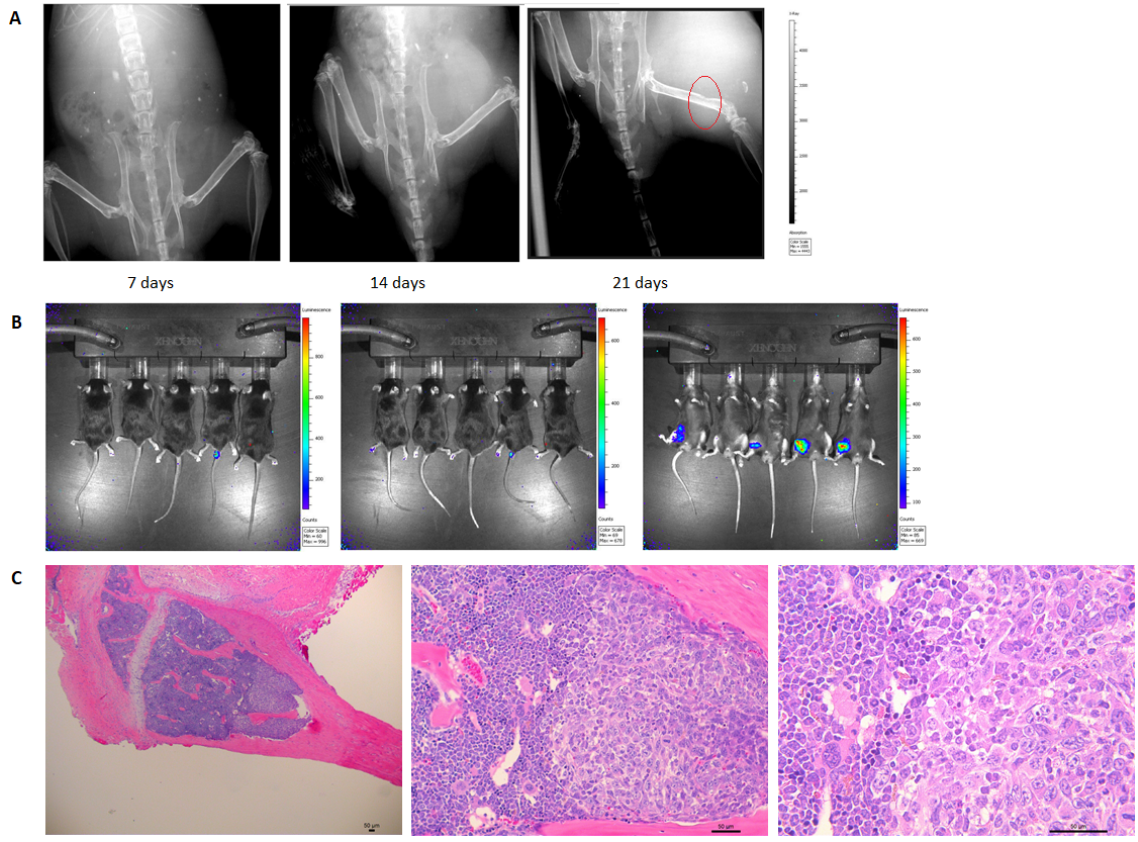

Figure 1: Establishment of bone metastatic model

Established iMC protocol: To gain a comprehensive view of bone metastatic TIME, we developed a 31-panel antibody iMC protocol in collaboration with Dr. Jared Burks who is running an iMC core facility at MD Anderson Cancer Center. To detect various cell populations in bone mets microenvironment, we have included markers for immune suppressive myeloid cells (CD11b, Gr1, S100A9, Ly6G, Arg1, Mac-2), for T-cells (CD8, CD4, CD3, FoxP3, Tbet), and tumor cells (AR, EpCAM, GFP). We further expanded the antibody panel to include other immune regulators expressed by tumor infiltrating immune cells including CTLA4, PD1, PD-L1, PD-L2, VISTA, LAG3, TIM3, TIGIT, ICOS, OX40, GITR, CD40, 4-1BB, B7-H3, B7-H4. To determine whether infiltrating T-cells are functional, we included antibodies for proliferation marker (Ki67) and cytokine production (TNFa, IL-2 and IFNg). Various lanthanide metal conjugated antibodies were obtained from iMC antibody bank at the core facility (Table 1).

Tagged Atibodies	target	label	clone	specificities	Source
Arginase-1 164Dy	Arginase-1	164Dy	D4E3M	Hu, Ms, Rb	DVS-Fluidigm
B220 176Yb	B220, CD45R	176Yb	RA3-6B2	Hu, Ms	BioLegend
b-catenin(active) 165Ho	b-catenin, active (non-phospho)	165Ho	D13A1	Hu, Ms, Rb, Mk	DVS-Fluidigm
Caspase 3 (cleaved) 172Yb	Caspase 3, cleaved	172Yb	5A1E	Hu, Ms, Rb, Mk	DVS-Fluidigm
CD117(Ms) 166Er (MDA)	CD117, c-kit	166Er	ACK2	Ms	Tonbo
CD11b 148Nd (MDA)	CD11b	148Nd	M1/70	Hu, Ms	Tonbo
CD152(Ms) 163Dy (MDA)	CD152, CTLA-4	163Dy	UC10-4F10-11	Ms	Tonbo
CD272(Ms) 153Eu	CD272, BTLA	153Eu	6A6	Ms	Tonbo
CD274(Ms) 145Nd	CD274, PD-L1, B7-H1	145Nd	10F.9G2	Ms	Tonbo
CD275(Ms) 173Yb	CD275, B7-H2	173Yb	HK5.3	Ms	Tonbo
CD279(Ms) 150Nd	CD279, PD-1	150Nd	RMP1-14	Ms	Tonbo
CD3(Ms) 151Eu	CD3	151Eu	17A2	Ms	Tonbo
CD33 145Nd (IMC)	CD33	145Nd	Polyclonal	Cross	DVS-Fluidigm
CD4(Ms) 156Gd	CD4	156Gd	GK1.5	Ms	Tonbo
CD40(Ms) 161Dy (FGK45)	CD40	161Dy	FGK45	Ms	Tonbo
CD44 111Cd	CD44	111Cd	IM7	Hu, Ms	Tonbo
CD45(Ms) 152Sm	CD45	152Sm	30-F11	Ms	Tonbo
CD8a(Ms) 160Gd	CD8a	160Gd	53-6.7	Ms	Tonbo
EpCAM 144Nd	EpCAM, CD326	144Nd	BLR077G	Hu, Ms	BETHYL
F4/80 171Yb	F4/80	171Yb	D2S9R	Ms	CST
IL-10(Ms) 158Gd (MDA)	IL-10	158Gd	JES5-2A5	Ms	Tonbo
Ki67 168Er (IMC)	Ki67	168Er	B56	Hu, Ms, Rt, Pg	DVS-Fluidigm
Ly-6G 146Nd	Ly-6G	146Nd	1A8	Ms	BioLegend
NK1.1 170Er (MDA)	NK1.1	170Er	PK136	Ms	BioLegend
p-Histone H3 176Yb (IMC)	p-Histone H3	176Yb	HTA28	Cross	DVS-Fluidigm
SMA 141Pr (IMC)	SMA	141Pr	1A4	Hu, Ms, Rb, Rt, Pg, Sh	DVS-Fluidigm
STAT3 160Gd	STAT3	160Gd	BLR098G	Hu, Ms	BETHYL
Ter119 162Dy (MDA)	TER-119	162Dy	TER-119	Ms	Tonbo
TIM-3(Ms) 162DY (MDA)	TIM-3, CD366	162Dy	RMT3-23	Ms	Tonbo
TIM-4(Ms) 147Sm	TIM-4	147Sm	RMT4-54	Ms	Tonbo
Vimentin 143Nd (D21H3)	Vimentin	143Nd	D21H3	Hu, Ms, Rb, Mk	DVS-Fluidigm

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training"

activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The project provided training and professional development for one postdoctoral fellow in the field of tumor immune microenvironment. Specifically, the project provided opportunity to learn the advanced technique of imaging mass cytometry (iMC) and obtain in-depth knowledge of tumor immune microenvironment of bone metastatic prostate cancer.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

1. iMC analysis of bone mets samples
1.1. staining and iMC data acquisition
1.2. IHC and multiplex IHC staining for further validation
2. early intervention trial:
Establish DX1 femur injection into 150 mice, followed by IVIS imaging to determine starting point of treatment. Ten arms trial will be conducted to determine the best response, which are followed by weekly IVIS imaging as follows
(1) CXCR2i +ICB trial; (2) Anti-IL6+ICB trial; (3) GSK2636771 + ICB trial; (4) CSF1Ri (JNJ-40346527)+ICB trial; (5) CXCR2i+CSF1Ri+ ICB. As for control arms of the trials, we will perform (6) ICB alone trial; (7) CXCR2i trial; (8) anti-IL6 trial; (9) GSK alone trial; (10) CSF1Ri alone trial.
3. Late intervention trial:
Same procedure as above, but trial will begin when IVIS imaging showing strong intensity of tumor burden.
4. survival analysis of early and late intervention trials.
5. immunohistochemistry validation of bone mets samples from task 2 and task 3 to determine immune suppressive cell depletion
6. the best response from above combination will be tested for CD8 dependency through CD8 antibody depletion. DX1 cells will be further implanted in cured mice from above to determine immune memory.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*

- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

iMC staining, data acquisition and analysis of bone met samples were delayed due to unforeseen technical reasons. A collaborating pathologist has retired recently and it took a few months for her to become a consultant to continue work with us. It took longer than anticipated to obtain 31-panel antibody, conjugate them with various metal conjugates and validate for specificity and sensitivity on mouse tissues. Also, Hyperion Mass Imaging cytometry at MD Anderson is heavily used and scheduling could not be obtained within this reporting period. Currently all the antibodies have been obtained, conjugated and validated. iMC instrument has been booked for analysis of bone met samples in next few weeks.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each*

one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other);

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	Y. Alan Wang
Project Role:	PI
Researcher Identifier:	awbs://orcid.org/0000-0001-8272-2450
Nearest person month worked:	6
Contribution to Project:	Directed the project
Name:	Surendra P Chaurasiya
Project Role:	Postdoctoral Fellow
Researcher Identifier:	
Nearest person month worked:	12
Contribution to Project:	Surendra has worked on all aspect of the project
Name:	David Eisenbarth
Project Role:	Postdoctoral Fellow
Researcher Identifier:	
Nearest person month worked:	2
Contribution to Project:	David has worked on all aspect of the project

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A*

duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*