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TITLE: Elucidating and Therapeutic Targeting of Prostate Bone Metastasis

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13. SUPPLEMENTARY NOTES

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. Our iMC test using a 31-panel antibody has not been successful, likely due to the low specificity of antibodies to mouse tissue. We will now expand our approach to examine the bone mets tumor microenvironment using spatial transcriptomics.

15. SUBJECT TERMS
Prostate cancer, immune suppressive myeloid cells, imaging mass cytometry, immune checkpoint blockade, metastasis,

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1. INTRODUCTION:

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:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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?

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). Tumors were apparent between week 2 and 3.

Using iMC to elucidate bone mets microenvironment. To gain a comprehensive view of bone metastatic TIME, we developed a 31-panel antibody iMC protocol which 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), in addition to other immune regulators expressed by tumor infiltrating immune cells (CTLA4, PD1, PD-L1, PD-L2, VISTA, LAG3, TIM3, TIGIT, ICOS, OX40, GITR, CD40, 4-1BB, B7-H3, B7-H4) as well as proliferation marker (Ki67) and cytokine production (TNFa, IL-2 and IFNg). We have tested this panel of antibody by iMC on bone mets sections, and we found only a few antibodies were positive (i.e. Ki67, Arg1, CD11b). Most antibodies have given no signal on image analysis even these antibodies recommended for mouse tissue staining.

Triple combination therapy using anti-PD1, anti-CTLA4 and SX682 on bone mets animal model.

We have injected DX1-BM cells into the femur of 48 C57 bl/6 mice and animals were then divided into five experimental group. We performed histopathological analysis of bone mets and consistent with the expected result, untreated bone mets have low levels of lymphocytes infiltration. Importantly, there were more lymphocytes infiltration in PD1 and CTLA4 treatment group and in PD1+CTLA4+SX682 treatment. Interestingly, tumor mass in bone appears to be smaller in triple treatment group only.

Treatment	Total mice	Tumor Outside	**Tumor in Cavity**	Infiltrate PMNs	Infiltrate Lymphocyte	Infiltrate Macrophage+ Monocytes
Untreated	12	11 (92%)	**8 (67%)**	3 (25%)	0	0
SX682	9	9 (100)	**3 (33%)**	0	0	0
SX682+PD1	5	3 (60%)	**3 (60%)**	1(33%)	0	1 (33%)
PD1+CTLA4	7	4 (57%)	**1 (15%)**	3 (43%)	3 (43%)	2 (28%)
SX682+PD1+CTLA4	15	9 (60%)	**7 (47%)**	6 (40%)	5 (33%)	5 (33%)
Total	48	36(75%)	**22(46%)**	13(27%)		

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

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. This fellow has been transferred to another lab at MD Anderson Cancer Center once PI has moved to the new institution.

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 have collected Bone mets tumor samples post treatment and in light of antibody deficiency for iMC analysis, we will use the control the four treatment group samples for spatial transcriptomics by the 10X visium platform and the newly acquired Nanostring CosMx. The results of these analyses will inform potential response and resistance biomarkers for better design of immunotherapy trials against castration resistant prostate cancer.

4. IMPACT:

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

Nothing to Report

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 PI has moved to Indiana University since July 1, 2022 and will continue to pursue immunotherapeutic approach targeting castration resistance prostate cancer through innovation.

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

In addition to the lack of suitable antibody for the iMC analysis, we have also encountered issue with imaging bone metastasis using MRI. We have developed additional DX1 cells line expressing novel imaging marker (Aka-Luc) and results were not promising. We have also encountered issues with access to laboratory equipment which the department has placed some restriction and the lack of access to samples generated in the current study.

Changes that had a significant impact on expenditures

Nothing to Report

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

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:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to Report

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

Nothing to Report

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Y. Alan Wang
Project Role:	PI
Researcher Identifier:	awbs://orcid.org/0000-0001-8272-2450
Nearest person month worked:	8
Contribution to Project:	Directed the project
Name:	Surendra P Chaurasiya
Project Role:	Postdoctoral Fellow
Researcher Identifier:	
Nearest person month worked:	8
Contribution to Project:	Surendra has worked on all aspect of the project
Name:	
Project Role:	
Researcher Identifier:	
Nearest person month worked:	
Contribution to 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?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: