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TITLE: Exploiting the Immune Microenvironment Variation in Subtypes of Metastatic Prostate Cancer

PRINCIPAL INVESTIGATOR: Lauren Brady, PhD

CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Center, Seattle, WA

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Fort Detrick, Maryland 21702-5012

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6. AUTHOR(S)

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

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14. ABSTRACT

SPECIFIC AIMS and STUDY DESIGN: Aim 1: To define the immune cell types and tumor-derived cytokine effectors in specific phenotypes of mPC. This objective will validate recent findings in our group which determined differential expression of immune markers in DNPC when compared to other mPC phenotypes by applying Digital Spatial Profiling, multi-plex immunohistochemistry and digital cytometry technologies. Aim 2: To determine if tumor phenotype dictates the immune cell composition of the TME. The approach will focus on PDX models of mPC and assess whether tumor phenotype can dictate immune composition by utilizing humanized mouse models. Aim 3: Determine if therapeutics directed toward PC subtype-associated immune characteristics results in PC subtype-specific responses. The approach will utilize existing clinical therapeutics to assess efficacy in reducing tumor growth in mPC. I will determine the effect of anti-IL1R and anti-B7-H3 monoclonal antibodies (e.g. Anakinra and enoblituzumab) on tumor growth in three different PDX phenotypes of CRPC: androgen receptor positive, neuroendocrine and double-negative PCs.

15. SUBJECT TERMS
None listed.

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
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1. Introduction

Metastatic prostate cancer is currently an incurable disease state. While these cancers initially respond to therapeutics that target androgen receptor signaling, resistance is universal with progression to a state termed castration resistant prostate cancer. Notably, castration resistant prostate cancer is now recognized to comprise numerous genomic subtypes as well as cellular phenotypes that no longer rely on the androgen receptor to proliferate. Research efforts in the field of immuno-oncology have led to the development of therapeutics that effectively result in immune checkpoint blockade. Overall, with the exception of rare cases, most prostate cancers do not respond well to these treatment strategies. To date, little is known concerning the immune landscapes of castration resistant prostate cancer that may contribute to the lack of response observed to these immunotherapies. Understanding the differences in immune composition between divergent phenotypes of metastatic castration resistant prostate cancer will contribute valuable insights into improving the efficacy of targeted therapies for this patient population.

Hypothesis: The overall aim of this project is to evaluate the concept that while the vast majority of metastatic prostate cancers evade immune surveillance, they do so through distinct and varied mechanisms that associate with their underlying prostate cancer phenotype, and possibly genotype. If correct, then exploiting this concept will provide opportunities for the more precise and effective application of immune-based therapy. In this proposal we are testing the hypotheses that: (i) molecular subtypes of castration resistant prostate cancer dictate phenotype-specific microenvironments that include immune cell types and cytokines; and (ii) the immune-effectors (e.g. IL-1β; B7-H3) that associate with specific prostate cancer phenotypes can serve as selective targets for therapeutics capable of effectively activating/promoting anti-tumor immune responses.

AIM 1. Define the immune cell types and tumor-derived cytokine effectors in specific phenotypes of metastatic castration resistant prostate cancer.
AIM 2. Determine if tumor phenotype dictates the immune cell composition of the tumor microenvironment.
AIM 3. Determine if therapeutics directed toward prostate cancer subtype-associated immune characteristics results in prostate cancer subtype-specific responses.

2. Keywords

Prostate cancer, metastasis, tumor immune microenvironment, castration resistant prostate cancer

3. Accomplishments

Career development tasks and timeline for expected completion

	Year 1	Year 2
Subtask 1: Participate in online Master's program (University College Cork) in Clinical Trials, modules including research ethics and data management.	Completed/Awarded	
Subtask 2: Attend intermediate and advanced R-programming classes, Biomedical Research Integrity seminars and specialized workshops (grant writing, career development) and join Fred Hutch Bioinformatics interest group.	Completed	In Progress
Subtask 3: Attend a national scientific meeting, e.g. AACR, to present scientific findings and gain peer reviewed feedback.	Delayed due to COVID-19 pandemic	Anticipated
Subtask 4: Present at weekly prostate cancer research group meetings. Receive constructive criticism/critique with respect to scientific data, visual representations, and organization/delivery.	Completed	In Progress
Subtask 5: Interact at the interface of patient engagement and support by attending and speaking at support groups/patient symposiums. Organize prostate cancer	Completed (joined PNW SPORE support group as facilitator)	In Progress

specific community events e.g. Break-out exercise session at annual IPCR patient symposium.		
Subtask 6: Attend grant writing, 'grant review', presentation skills, and mentoring bootcamps offered through UW ITHS	Completed	In Progress
Subtask 7: Didactic and hands-on training in the genesis and analysis of the MISTRG humanized immune system model	Completed	
Subtask 8: Didactic and hands-on training in the histology of primary and metastatic prostate cancer	Completed	In Progress
Subtask 9: Develop research plan for independent position and deliver formal presentations to PPCR faculty for critique and feedback.		Pending

Major Project Goals

Aim 1: **Define the immune cell types and tumor-derived cytokine effectors in specific phenotypes of mCRPC.**	Year 1	Year 2
Subtask 1: CIBERSORT digital cytometry analysis of Nelson lab RNA-seq data set, validated with publicly available metastatic prostate cancer cohorts e.g. Stand Up To Cancer	Completed with assistance from Ilsa Coleman	
Subtask 2: Obtain serial FFPE sections of patient samples of interest, in addition to TMA sections for multi-plex IHC validation study.	Completed	
Subtask 3: Digital spatial profiling of full face FFPE sections and associated data analysis		Pending
Subtask 3: Multiplex IHC optimization on two panels of immune markers utilizing TMAs constructed from patient samples assessed for CIBERSORT and DSP. Consecutive analysis of validation data.	Completed	In Progress
Aim 2: Determine if tumor phenotype dictates the immune cell composition of the TME.	**Year 1**	**Year 2**
Subtask 1: Implantation of PDX tumors comprising CRPC subtypes in humanized mouse models and subsequent tumor harvesting.	Completed (see section 5)	In Progress
Subtask 2: Analysis of harvested tumors by multiplex IHC and bulk RNA-seq and cytokine array assays.	Completed	In Progress
Aim 3: Determine if therapeutics directed toward PC subtype-associated immune characteristics results in PC subtype-specific responses.	**Year 1**	**Year 2**
Subtask 1: Propagation of PDX mouse models, treatment with IL1R and B7-H3 monoclonal antibodies, tumor growth measurements, and tumor harvesting.		Pending

Subtask 2: Analysis of harvested tumors by multiplex IHC and bulk RNA-seq and cytokine array assays.		Pending
Subtask 4: Assemble data and prepare manuscripts.		Pending

What was accomplished under these goals?

Aim 1: Define the immune cell types and tumor-derived cytokine effectors in specific phenotypes of metastatic castration resistant prostate cancer.

Throughout the first year of support as part of this funded proposal, we focused on defining the immune composition of different phenotypes of metastatic castration resistant prostate cancer.

1. In collaboration with senior bioinformatician, Ilsa Coleman, we determined the immune composition of different tumors as part of both publicly available datasets, TCGA and SU2C, and our internal datasets derived from the University of Washington's (UW) rapid autopsy program **(Fig 1)**. Melanoma and Ovarian tumors had higher numbers of immune cells when compared to metastatic prostate tumors. In metastatic tumors derived from the UW rapid autopsy program substantial differences in immune cell composition was observed across different phenotypes. For example, statistically significantly higher levels of CD8 T-cells were present in the double negative phenotype when compared to the androgen receptor positive neuroendocrine negative (AR+NE-) phenotype **(Fig 1)**.

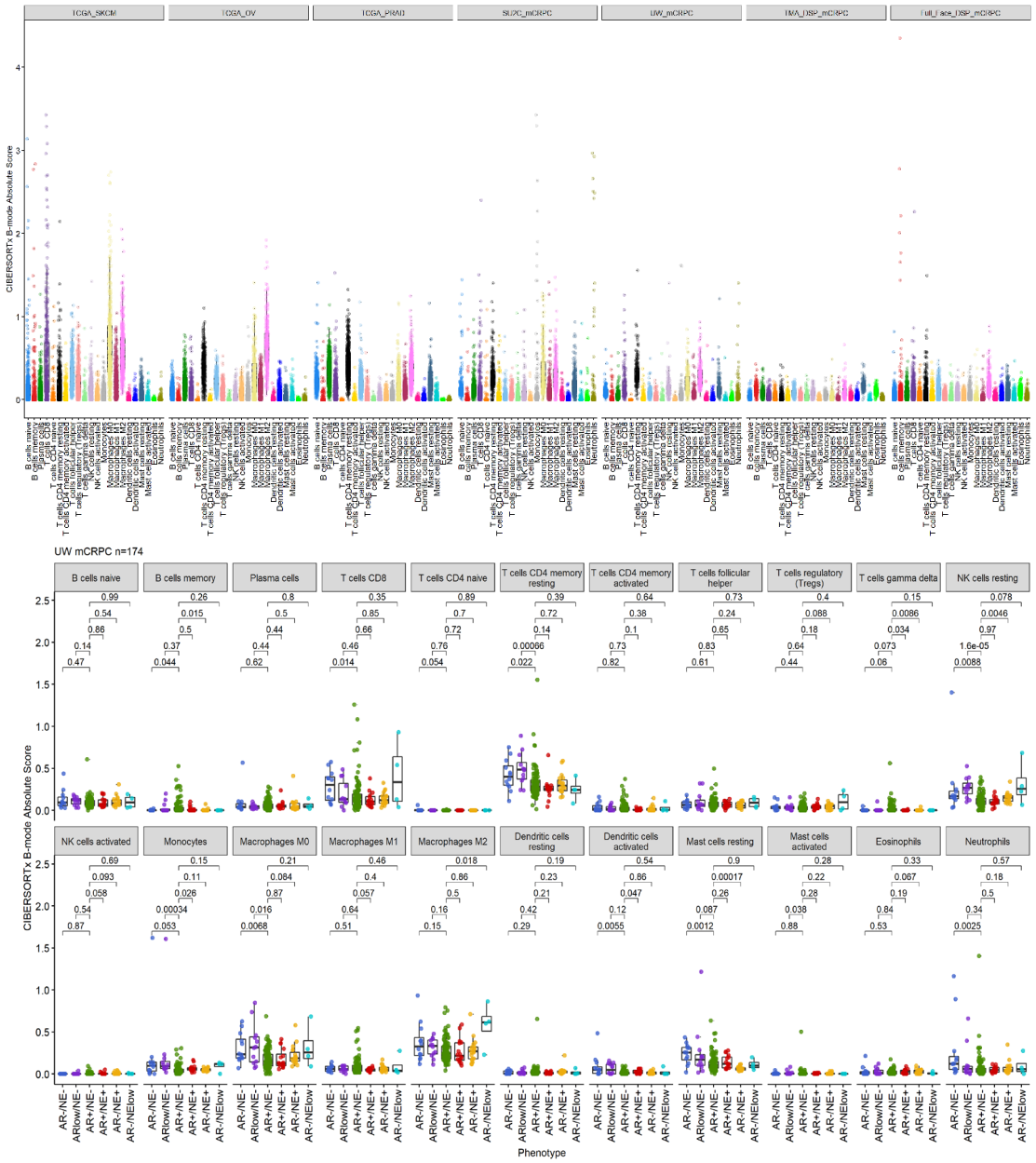

Fig 1: Top: Computational analysis, CIBERSORTx, of immune composition of tumors derived from TCGA, SU2C and University of Washington (UW) rapid autopsy program. Bottom: CIBERSORTx immune analysis of UW tumors only.

2. During year 1, we collected tumors from the UW rapid autopsy program that comprise the different phenotypes of advanced disease and designed two multiplex immunohistochemistry (IHC) panels focused on immune markers (panel 1 – CD8, CD4, FOXP3, TIM-3, PD1, pancytokeratin (panCK) and DAPI and Panel 2 CD68/CD163 cocktail, CD14, CD66b, PDL1, B7H3, panCK and DAPI). We performed multiplex IHC with these panels on a tissuemicroarray (TMA) comprised of 28 patients with 2 metastatic sites per patient. By utilizing HALO software, we designed algorithms that segmented each tissue core into tumor/stroma/glass/tissue of origin which allowed an accurate enumeration of intra-tumoral immune cells, in addition to defining the stromal composition. Overall, with the exception of B7H3 which we previously described as being highly expressed across the spectrum of prostate cancer tumor cells, intra-tumor expression of immune cell markers was low **(Fig 2)**.

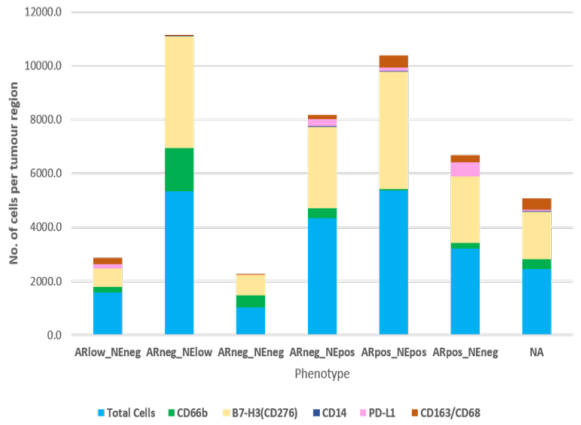

Fig 2: Number of cells per tumor region sub-grouped by castration resistant prostate cancer phenotype. Tumor cells highly express B7H3 across all phenotypes, with low numbers of immune cells

3. We next analyzed the presence of each immune cell type across the different tumor phenotypes by ANOVA. Significant differences in CD66b expression (neutrophil) were observed between phenotypes, with significantly higher numbers of neutrophils present in the double negative (AR-NE-) phenotype when compared to the other phenotypes. Significantly higher numbers of neutrophils were also observed in the androgen receptor low neuroendocrine negative (ARlowNE-) and androgen receptor negative and neuroendocrine low (AR-NElow) phenotypes **(Fig 3)**.

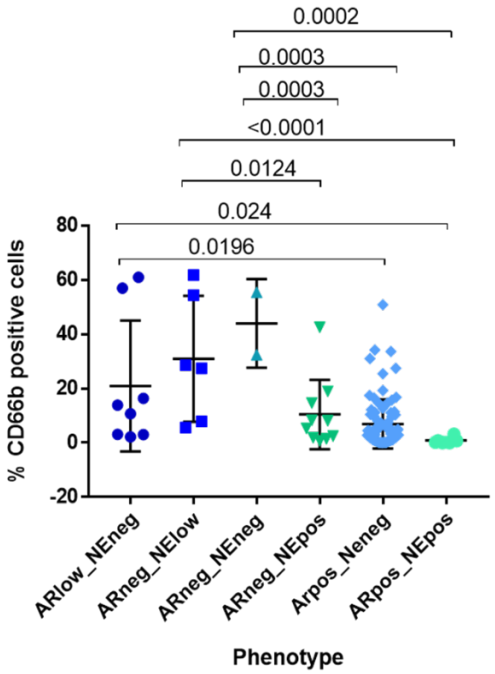

Fig 3: CD66b (neutrophil) intra-tumoral expression by phenotype. Data analyzed by 1-way ANOVA.

Aim 2: Determine if tumor phenotype dictates the immune cell composition of the tumor microenvironment.

During this period of support, I have focused on implanting patient derived xenograft (PDX) models of castration resistant prostate cancer into humanized mouse models (MISTRG). The MISTRG mice have a humanized innate immune system allowing us to observe the immune cell composition of the tumors representing the different phenotypes.

1. We chose to begin these experiments with 2 x PDX cell lines – 35CS, androgen receptor positive neuroendocrine negative prostate cancer (AR+NE-), and 176, androgen receptor low, neuroendocrine negative prostate cancer (ARlowNE-). PDX cells were implanted into both humanized MISTRG mice (case) and non-humanized MISTRG mice (control) and the mice were monitored daily with tumor measurements taken 3x weekly. There was no significant difference in tumor growth between the humanized and non-humanized mice over time (**Fig 4**), however, some humanized mice were euthanized at earlier time-points due to the onset of anemia.

After euthanasia, tumors were harvested and dissected and pieces of tumor were formalin fixed paraffin embedded (FFPE), snap-frozen in liquid nitrogen, or embedded in OCT for downstream applications. The histology of each tumor was determined by IHC staining for know PC markers (androgen receptor, prostate specific antigen, synaptophysin, chromogranin) and the morphology was confirmed by our collaborating pathologist Dr. Martine Roudier. We confirmed that the phenotype and expression of prostate related markers did not change after growth in the MISTRG mice model and phenotypes previously assigned by bulk RNA-seq remained consistent (**Fig 5**).

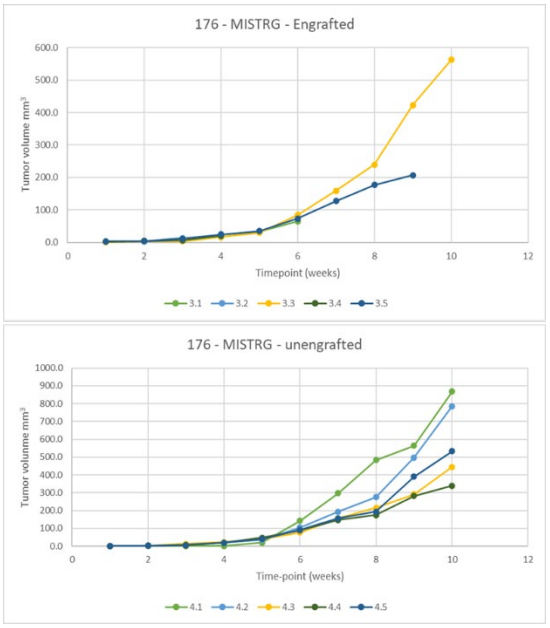

Fig 4: Representative tumor growth over time in humanized versus non-humanized MISTRG mice (176).

3. Multiplex IHC was performed on the FFPE tumors harvested from the mice, examining expression of CD3 (T-cells), CD66b (neutrophils), CD56 (natural killer cells), CD63/CD168 combined (macrophages) and panCK (epithelial cell marker). Human immune cell infiltration was confirmed to be present in all tumors excised from humanized mice, and no immune cells were present in the tumors excised from non-humanized mice, as expected. Human immune cells were predominantly present in the stroma surrounding the tumor or in intra-tumoral stroma. Immune cells were present in very low numbers within the tumor itself. Macrophages were the most abundant immune cell type in both phenotypes (**Fig 6**).

4. Immune cell composition was compared between the different phenotypes of advanced prostate cancer (176 *vs.* 35CS). Although not statistically significant, there was an increased number of both T-cells and macrophages in the 176 tumors compared to the 35CS suggesting possible differences between the two phenotypes that need to be confirmed in additional experiments (**Fig 7**).

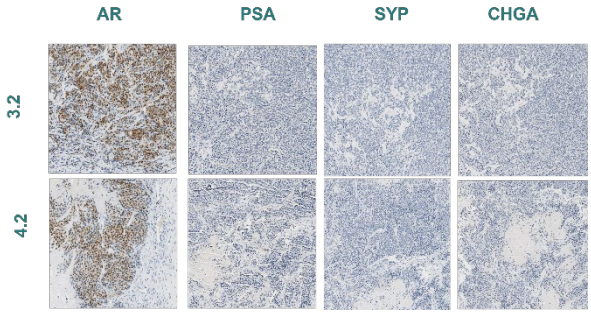

Fig 5: Representative images of IHC expression of prostate cancer specific markers in 176 tumors (ARlowNE-).

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

Although the current COVID-19 pandemic prevented travel to national or international scientific conferences for the first year of this award, I attend weekly Prostate Cancer Program seminar series meetings and weekly lab meetings. At both venues I have been given the opportunity to present our findings on this project, gaining positive feedback and critical evaluation of the data from experts in the field. Further, I have completed training in animal handling (as evident from the above results) and relevant courses such a R-programming that will aid in the successful continuation of this project and further my career goals.

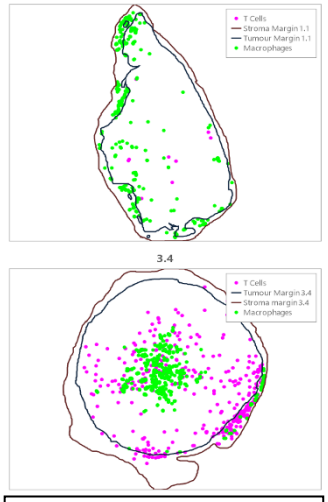

Fig 6: Representative tumors, stained with by multiplex IHC. Green dots indicate macrophages, pink dots indicate T-cells. Top 35 CS, bottom 176.

How were the results disseminated to communities of interest?

In 2021, I became facilitator of the Pacific Northwest SPORE prostate cancer patient advocate committee. Participation in these bi-monthly meetings provides critical insight from patients into our research direction, and ensures the data is disseminated in patient centered language to patients living with prostate cancer.

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

I will continue to follow the original SOW as part of this project. We will continue to pursue animal experiments to replicate *in vivo* the tumor immune microenvironment. In particular, we will focus on experiments that examine the immune composition of both the neuroendocrine and double negative phenotypes of prostate cancer to better understand the full spectrum of immune interactions in a castration resistant setting. Further, as outlined in the SOW we will investigate the effects of different therapeutic strategies in mitigating prostate cancer growth *in vivo*.

4. Impact

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

To date, our findings from this study have provided valuable insight into the immune composition of different phenotypes of metastatic castration resistant prostate cancer and have highlighted the divergent immune populations present in some phenotypes such as double negative prostate cancer. Our observations have described the low numbers of immune cells that are present within the tumor (intra-tumor), noting an increased presence of immune cells in the surrounding stromal tissue. Further, our preliminary studies in humanized mice have established the ability of the immune cells circulating in the bloodstream to enter the prostate tumor and have highlighted differences in immune cell composition between phenotypes, although this did not meet statistical significance. This enhanced understanding of the immune composition of advanced prostate cancers may aid in the future development of immunotherapies.

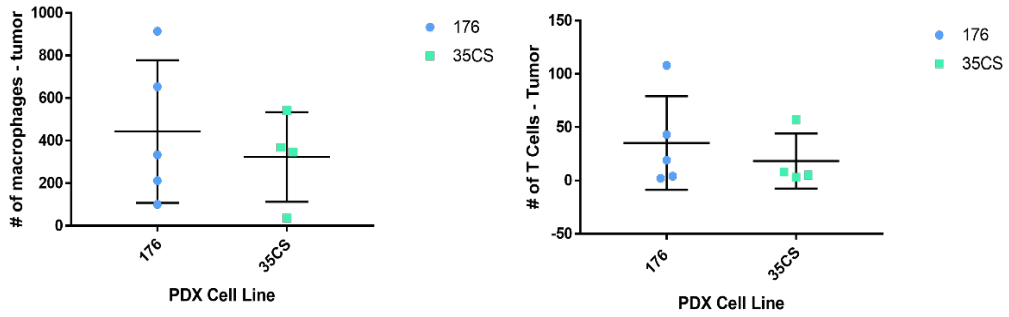

Fig 7: Comparison of intra-tumoral T-cells and macrophages between 176 and 35CS tumors grown in MISTRG humanized mice. No statistically significant differences were observed.

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

No changes to the research plan at present.

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

Some project delays associated with the COVID-19 pandemic occurred. The animal facilities operated on a limited schedule with reductions in staff including administrative staff for developing and approving animal studies. Further, supply chain issues persist with some delays in the receipt of key chemicals/reagents and plasticware.

Our studies in MISTRG humanized mice were limited in duration due to the risk of anemia within this mouse model. The mice are prone to the development of anemia due to the humanized macrophages recognizing endogenous mouse blood cells as foreign pathogens. The onset of anemia shortens the lifespan of the mice currently precluding the ability for long-term studies. We are currently evaluating different options to improve these outcomes, and this may cause some delays in projected MISTRG experiments.

Changes that had a significant impact on expenditures

The COVID-19 pandemic has caused some delays with animal work and consumables as outlined above.

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
Publications, conference papers, and presentations.
Nothing to report.

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:	Lauren Brady, PhD
Project Role:	Principal Investigator
Nearest person month worked:	12
Contribution to Project:	Dr. Brady oversee's all aspects of the conduct of the research scope of work and documents and reports results.
Funding Support:	See attached Other Support document.

8. Special Reporting Requirements

Collaborative Awards: Not applicable

Quad Charts: Not applicable

9. Appendices

Nothing to report.

PREVIOUS, CURRENT AND PENDING SUPPORT – BRADY, LAUREN M.

PREVIOUS SUPPORT

None.

CURRENT SUPPORT

Title: Exploiting the Immune Microenvironment Variation in Subtypes of Metastatic Prostate Cancer
Grant #: W81XWH-21-1-0028
Time commitments: 100%
Supporting agency: DOD/USAMRAA
Name and address of the funding agency's procuring Contracting/Grants Officer:
Joshua McKean
1077 Patchel Street
Fort Detrick, MD 21702-5024
Performance Period: 04/01/2021 - 03/31/2023
Level of funding:
Brief description of the project's goals:
In this proposal I will test the hypotheses that: (i) molecular subtypes of castrate resistant prostate cancer (CRPC) comprise phenotype-specific tumor-promoting microenvironments that include immune cell types and cytokines; and (ii) the immune-effectors (e.g. IL-1β; B7-H3) that associate with specific PC phenotypes can serve as selective targets for effectively activating/promoting anti-tumor immune responses.
Overlap with proposed research: None
List of specific aims:
Aim 1: To define the immune cell types and tumor-derived cytokine effectors in specific phenotypes of mPC. This objective will validate recent findings in our group which determined differential expression of immune markers in DNPC when compared to other mPC phenotypes by applying Digital Spatial Profiling, multi-plex immunohistochemistry and digital cytometry technologies.
Aim 2: To determine if tumor phenotype dictates the immune cell composition of the TME. The approach will focus on PDX models of mPC and assess whether tumor phenotype can dictate immune composition by utilizing humanized mouse models.
Aim 3: Determine if therapeutics directed toward PC subtype-associated immune characteristics results in PC subtype-specific responses. The approach will utilize existing clinical
therapeutics to assess efficacy in reducing tumor growth in mPC.

PENDING SUPPORT

Title: Determining tangible strategies for addressing health disparities and inequities in prostate cancer clinical trials research
Grant #: 2022YI3647
Time commitments: 100%
Supporting agency: Prostate Cancer Foundation
Name and address of the funding agency's procuring Contracting/Grants Officer:
Howard R. Soule, PhD
applications@pcf.org
Performance Period: 04/01/2023 - 03/31/2026

Level of funding:
Brief description of the project's goals:
I aim to address persistent health disparities present in PCa clinical trials research by engaging with patients with PCa and patient advocates to work towards designing patient-centered, equitable interventions. In this proposal I will test the hypothesis that incorporating patient voices in clinical trial design will aid in reducing disparities in PCa clinical trials research and contribute to improving outcomes for underrepresented populations.
Overlap with proposed research: None
List of specific aims:
Aim 1: Determine patient opinions and insights into improving equity and accessibility in PCa clinical trials research.
Aim 2: Define similarities and differences between patient and health practitioner perspectives with respect to improving prostate cancer clinical trial enrollment.
Aim 3: Engage key stakeholders in the Delphi method to determine guidelines for designing equitable future PCa clinical trials.