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TITLE: Combining Androgen Deprivation and Immunotherapy to Prevent Progression to Castration-Resistant Prostate Cancer

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14. ABSTRACT
Recently, we found that in a PTEN-deficient mouse PCa model, castration induces an immunosuppressive state within the tumor that is concurrent with tumor recurrence. Mechanistically, this response to ADT is mediated by soluble mediators (TNF and CCL2), facilitating communication between tumor, stromal and immune cell populations within the tumor microenvironment. Based on these preliminary data, we hypothesize: Blocking myeloid suppression prevents progression to castration resistant prostate cancer. We test this hypothesis in three aims. Aim 1 examines the mechanism of paracrine TNF signaling between tumor, stromal and myeloid cell populations within the TME, in inducing immune suppression following ADT. Aim 2 tests whether blocking the transit and/or function of myeloid suppressive cell populations prevents CRPC (tumor recurrence following ADT). We also determine the role of PTEN in ADT-induced immune evasion. Aim 3 tests the hypothesis that ADT, in men with locally advanced PrCa, increases serum TNF and CCL2, as well as circulating myeloid cells, by assessing samples from an ongoing clinical study

15. SUBJECT TERMS
androgen, castration, immunotherapy, prostate, cancer, TNF, CCL2, tumor associated macrophages, CD8 T-cells

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1) Introduction

Most prostate cancer (PCa) deaths are due to castration resistant PCa (CRPC). While androgen deprivation therapy is the standard of care for patients with advanced PCa, nearly universal progression to CRPC occurs 2-3 years after ADT is initiated. Although there have been key advances in the treatment of CRPC, even the best therapies are not curative. One approach to this problem is to improve the initial treatment of advanced prostate cancers, by combining complementary therapies with ADT, to prevent progression of such advanced cancers to CRPC. Immunotherapy, typically employing T-cell 'checkpoint' inhibitors, has provided very durable remissions, verging on cure for a variety of cancer types. However, CPIs have *not* been effective in prostate cancers, perhaps because such cancers are 'cold' (lacking cytolytic CD8 T-cells). Cold tumors may be caused by infiltration of myeloid cell populations – tumor associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) – into the tumor immune cell microenvironment (TIME). In preliminary data accompanying this proposal, we demonstrate that castration of a PTEN-deficient mouse PCa model induces an immunosuppressive state within the tumor that is concurrent with the onset of tumor recurrence. The response to castration/ADT is tri-phasic: a pro-apoptotic regression phase where tumor shrinks, followed by selection for a residual population of resistant stem-like tumor cells and finally recurrent growth as CRPC. Using PCa cell lines to model the first two phases of the response to ADT, we have shown that ADT induces apoptosis, thereby enriching for an ADT-resistant stem/progenitor population that we demonstrate is an *in vitro* source of enhanced TNF production. Mechanistically, in our model system the response to ADT is driven by the soluble mediators TNF and CCL2, which facilitate communication within the TIME. Specifically, a TNF-CCL2-CCR2 paracrine loop is induced between prostate cancer cells and non-tumor cells in the microenvironment: TNF produced by tumor cells acts on myofibroblasts and TAMs to induce CCL2 production, which in turn recruits tumor-associated macrophages (TAMs) and possibly MDSCs. Analysis of public PCa data sets shows TNF and stem/progenitor marker expression are both increased in CRPC, consistent with our hypothesis that ADT drives the development of an immuno-suppressive state via a cytokine switching mechanism that triggers the TNF-CCL2-CCR2 axis in the TIME.

2) Keywords

androgen, castration, immunotherapy, prostate, cancer, TNF, CCL2, tumor associated macrophages, CD8 T-cells, myeloid-derived suppressor cells, CCR2, TNF receptors, tumor microenvironment

3) Accomplishments

What were the major goals of the project?

We proposed that TNF promotes an immunosuppressive state via CCL2, to drive castration-resistant tumor growth.

- Aim 1 determines how TNF signals in the castrate tumor microenvironment.
- Aim 2 tests our immune suppression hypothesis in three sub-aims.
- Aim 3 tests the hypothesis that ADT administered to men with locally advanced PCa increases

serum TNF and CCL2

What was accomplished under these goals?

Accomplished under Aim 1.

From our revised SOW, we proposed to pursue the following in the 1st year of the application:

Tasks	Timeline
SPECIFIC AIM 1: examines the role of the TME. We test the hypothesis that ADT-induced TNF derives from epithelial tumor cells and acts on macrophages and/or myofibroblasts in the TME.	
Major Task 1: Determine source of TNF *in vivo*. The purpose is to understand whether tumor cells or microenvironment cell populations are producing TNF in response to castration.	Months
Subtask 1: establish TNF KO/ hi-myc + luc model. Includes breeding and genotyping mice.	1-6
Subtask 2: measure tumor growth rate. Employs serial use of high frequency ultrasound imaging of tumors in the mice.	7-12

One of the over-arching goals of our research is to understand the response of prostate cancers to androgen deprivation therapy. In patients, the response is multi-phasic (Fig. 1), including a regression phase as the initial response. Almost every tumor shrinks and in patients the PSA level is reduced to a very low level. We believe there is a selection phase that promotes stem-

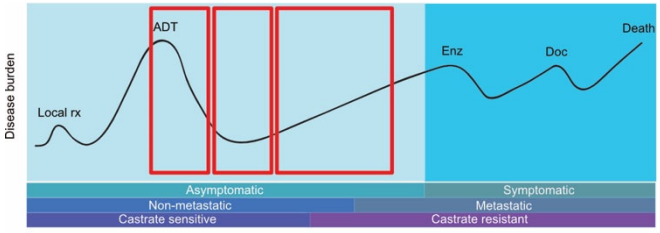

Fig. 1. Patient response androgen deprivation therapy. The scheme plots tumor burden (PSA) in lethal prostate cancer, over time. Red boxes define 3 phases of the response: regression (apoptosis of tumor cells); selection (for a CSC-enriched population) and recurrence.

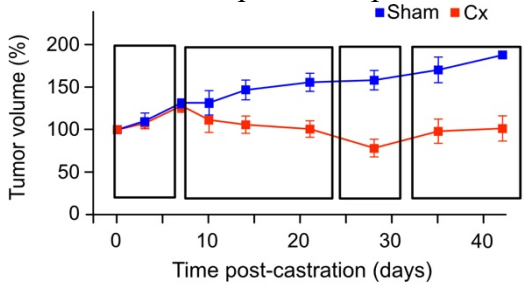

Fig. 2. Response of PBcre x PTENf/f mice to castration. The volume (determined by HFUS) of sham castrated (Sh) and castrated (CX) tumors over time. Black boxes correspond to (L to R): 'delay'; regression, selection and recurrence.

ness and then there is almost universal recurrence as castration resistant prostate cancer (CRPC). We have modeled the response to ADT in genetically engineered mouse models (GEMMs) such as the PBcre x PTEN f/f mouse and observe a similar pattern of regression, selection and recurrence (Fig. 2). In these mice there is also an initial 'delay' phase which we have investigated in separately funded studies, and shown is due to a transient compensatory up-regulation of glucocorticoid

signaling. While the model shown in Fig. 2 is an excellent one for studying human prostate cancers, it is relatively time-consuming to manipulate the genetics of the tumor cells (only) as this requires altering the mouse germline, complex breeding of the resulting mice, and waiting for tumor development. An alternate approach is to use a tumor cell lines derived from the GEMM as an implantable tumor. The cell line can be genetically manipulated much more quickly, allowing us to pursue the experiments within the time frame of the grant. While there is a PTEN-deficient prostate cancer cell line derived from the GEMM illustrated in Fig. 2, it does not grow as an allograft and therefore cannot be used (we need to use immuno-competent mice since we are investigating the role of the immune system). However, we observed very similar kinetics of prostate cancer response to CX in another GEMM – the Hi-MYC model which has a tumor-specific transgenic over-expression of c-MYC (Fig. 3 and see also Fig. 5), an oncogene that is also frequently amplified or over-expressed in human PrCa. In the case of the Hi-MYC GEMM, prostate cancer cell lines have been established, which allowed us to consider this for use as a syngeneic allograft model. Therefore, we performed a series of preliminary studies to examine the kinetics of Hi-MYC tumor growth in normal mice. We confirmed that the tumors were castration dependent and that the tumor volume (as measured by high frequency ultrasound (HFUS)) regressed, and subsequently recurred as we expected (Fig. 4). However, the recurrence rate was far too rapid to allow for us to either accurately measure or experimentally modulate the molecular events of tumor immune microenvironment. We also examined the response of other Hi-MYC cell lines they behave similarly. Thus, we believe that is likely an inherent limitation of these model. Specifically, we think that during *in vitro* establishment in culture, a tumor sub-clone(s) that has enhanced growth properties is selected for. Unfortunately, this makes it unfeasible to complete subtasks 1-3 of this first major task.

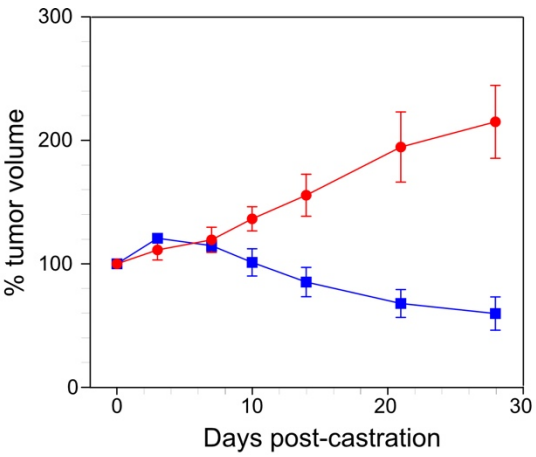

Fig. 3. The response of hi-MYC GEMM to castration. Similar to Fig. 2, the volume of prostate cancers in hi-MYC mice were determined by HFUS. Although not shown in this figure, the cancers recur at ~5 weeks post-CX.

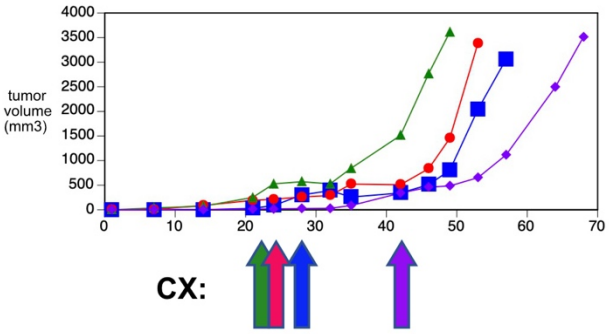

Fig. 4. The response of hi-MYC allografts to castration. Mice were implanted with hi-MYC tumors HFUS imaged to determine tumor volume and CX at the indicated time (days). Colors correspond to independent mice/ implants.

As noted below, we have elected to revise the SOW for Aim 1 to look at a related aspect of TNF's role in the response to castration, which will not require time-consuming genetic manipulation of the GEMM model.

Accomplished under Aim 2

SPECIFIC AIM 2: Aim 2 tests our immune suppression hypothesis in three sub-aims.	
Major Task 1: Determine if therapies directed against TNF and/or CCL2 signaling will prevent castrate resistant recurrence. This provides information on whether or not these soluble mediators are acting in as part of a signaling cascade and complements the insight from Aim 1.	Months
Subtask 1: Obtain PF4136309 (CCR2 antagonist). Purchase or obtain *gratis* from Pfizer.	1-3
Subtask 2: Monitor tumor volumes on required cohorts of PTEN-deficient mice. Employs serial use of high frequency ultrasound imaging of tumors in the mice.	4-16
Subtask 3: Castrate 1st mouse cohort, blind operator to treatments.	16
Subtask 4: Monitor tumor volumes. Employs serial use of high frequency ultrasound imaging of tumors in the mice.	17-24
Subtask 5: Castrate 2nd mouse cohort, blind operator to treatments. Employs serial use of high frequency ultrasound imaging of tumors in the mice.	25
Subtask 6: Monitor tumor volumes. Employs serial use of high frequency ultrasound imaging of tumors in the mice.	25-32
Subtask 7: Analyze HFUS data. Employ AMIRA software to segment tumor volumes.	32-36

Major Task 3: assess hi-MYC model. The purpose is to determine if castration induced immune suppression enhances recurrence in a second model of prostate cancer.	Months
Subtask 1: Monitor tumor volumes on required cohorts of hi-MYC mice. This is to ensure we have a cohort of biologically similar tumors for the experiments.	1-6
Subtask 2: Castrate hi MYC mice & follow by monitoring HFUS. Determines the kinetics of tumor recurrence.	7-12
Subtask 3: Harvest tumor, analyze by FACS, IHC and RNAseq similar to the approaches employed in the PTEN-deficient model.	12-18

We completed the entirety of both Major Task 1 and Major Task 3 in Aim 2 during the first funding year. Specifically, we had shown in the preliminary data section of the grant application that CCL2 levels are increased at the point in time when there tumor recurrence is occurring in the PTEN-deficient GEMM. Now, we have demonstrated that CCR2 inhibitors were able to inhibit the

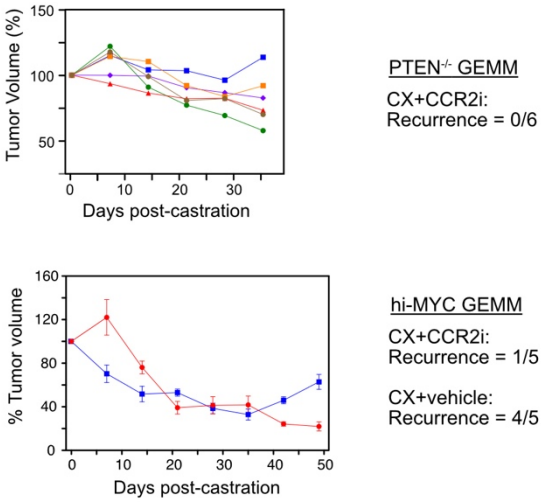

Fig. 5. CCR2 signaling blockade prevent recurrence of prostate cancers following castration. The indicated GEMM were castrated and then treated with a CCR2 inhibitor or vehicle as indicated. Tumors were HFUS imaged to determine tumor volume. Recurrence is defined as two consecutive measurements that are 10% above the nadir.

recurrence phase of the response to castration in both the PTEN-loss and the MYC-gain mouse models. This data (summarized in Fig. 5) will constitute a significant portion of manuscript #2, as mentioned below. The implications of this data are significant. They provide strong support for the concept that there is an immunological mechanism that is at least partially responsible for the castration-resistant recurrence that we have observed in our kinetic analysis of the response to ADT in two distinct prostate cancers models. It is also important to note that the GEMM models are driven by two of the most frequently altered genes seen in human cancers. Second, the importance of CCL2-CCR2 signaling also implies the immunological mechanism leading to recurrence falls into the category of 'myeloid suppression.' This is consistent with the preliminary data in our original proposal that indicated that CD8 T-cell levels *decrease*, while CD68 macrophages (tumor-associated macrophages; TAMs) *increase*. To determine if these observations in the murine models were relevant to human prostate cancer, we probed public data bases (TCGA for primary prostate cancer (PPC); c-Bioportal for CRPC) and as seen in Fig. 6, found that the same pattern was observed in human CRPC as in recurrent GEMMs.

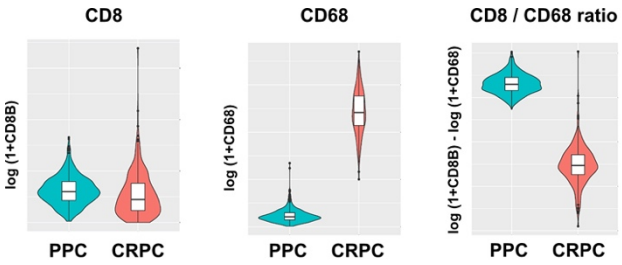

Fig. 6. Increased TAMs and decreased CD* T-cells are seen in CRPC. We examined RNAseq data from the public data bases listed in the text and determined the RNA counts corresponding to the indicated genes for the patients samples. n=495 PPC and n=270 CRPC cases.

Accomplished under Aim 3

In Aim 3, we have been collecting and storing samples for future batch-wise analysis.

Other achievements

As an alternative to the studies we could not pursue in Aim 1, we will use our imaging expertise to investigate the role of TNF in both the initial regression phase following ADT and to determine if these events are dependent on disruption of vessels/ endothelium in the tumor. We will measure vessel morphology by anti-CD31 staining and we measure blood flow, tissue perfusion and tissue oxygenation status and confirmed that these events are consistent with TNF initiating vessel damage and hypoxia which contributes to regression by necroptosis and apoptosis of the tumor cell population. In the revised SOW, we will designate these experiments as revised Aim 1. We will use a part of year 2 to complete these experiments and submit this publication (manuscript #1).

We also used timed blockade of TNF signaling to demonstrate that both the regression and recurrence phases of prostate cancer response to castration require TNF signaling. Again, the main measure was to determine tumor volume using HFUS. This data will be part of a second publication which is in the early stages of preparation. We will use a part of year 2 to also complete the preparation and submission of this publication (manuscript #2).

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?

As noted we will use a portion of the next reporting period to prepare and publish two manuscripts describe under 'other achievements'. We will also complete the remainder of Aims 1 and 2.

4) Impact

What was the impact on the development of the principal disciplines of the project?

We demonstrated that TNF signaling is required for both the regression and recurrence phases of the response to castration. We also demonstrating that CCL2 signaling is required for the recurrence phase only – it has no role in regression. Moreover, blockade of late TNF signaling phenocopies the blockade of CCL2 signaling, consistent with a molecular mechanism in which TNF acts via NFkB to promote CCL2 expression (a well-described gene regulatory event). We have previously demonstrated that following the initial regression response to castration there is an increase in the stem-like cell character of the tumor cells which likely contributes to high NFkB levels.

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

As noted above the model system for Aim 1 was not tenable to use KO mice to test the role of TNF, and therefore we instead examined the role of TNF in castration induced vascular events using the TNF blocking reagent.

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

Nothing to report beyond that summarized in the prior paragraph.

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

Nothing to report.

6) Products

Nothing to report.

Websites or other internet sites
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications and or licenses
Nothing to report

7) Participants and other collaborating organizations

What individuals worked on the project?

John Krolewski, MD PhD. PI. 2.4 Calendar Months.

- Dr. Krolewski lead the project and analysis of the data for manuscript #2.

Kent Nastiuk, PhD. HRI Scientist (promoted to Assistant Member and changed role to co-I), 3.6 Calendar Months

- Dr. Nastiuk helped to supervise the post-doctoral fellow in the lab, managed the imaging experiments and assisted with the analysis of the data. He is leading the writing for manuscript #1.

Kevin Eng, PhD. Biostatistician, 0.96 Calendar Months

- Dr. Eng assisted with statistical analysis of the regression and recurrence kinetics.

Aerken Maolake, PhD. Post-doc. 6 Calendar Months.
- Dr. Maolake carried out the bench work on the project.

Gurkamal Chatta, MD. Co-investigator. 0.24 Calendar Months
- Dr. Chatta assisted with Aim 3.

Bo Xu, MD, PhD. Co-investigator. 0.24 Calendar Months
- Dr. Xu assisted with Aim 3.

Has there been a change in the active other support of the PI or senior/key personnel since the last reporting period?

Only for Dr. Eng –

	Level of support
DOD award W81XWH-19-1-0378 (PI: Krolewski)	8.0%
NIH award R01 CA247362-01A1 (PI: Knudsen)	5.0%
NIH award R01 CA247771 (PI: Lovell/Abrams)	1.9%

What other organizations were involved as partners?
Nothing to report

8) Special reporting requirements
Nothing to report

9) Appendices

None