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TITLE: Impact of Estrogen Signaling on Tumor Immunity and Response to Immune Therapy in Ovarian Cancer

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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)

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8. PERFORMING ORGANIZATION REPORT NUMBER

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U.S. Army Medical Research and Development Command
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Immune therapy has been a major breakthrough in cancer treatment, however the benefit for women with ovarian cancer has been limited. Our prior work demonstrated that modifying conditions in the tumor environment can significantly improve treatment with immune therapy in ovarian cancer models. Identifying new strategies to modify the tumor environment is expected to allow women with ovarian cancer to optimally benefit from immune therapy.

The hormone estrogen impacts immune function in both healthy people and in cancer models. Estrogen receptors on tumor cells have been targeted for ovarian cancer treatment, but the impact of estrogen on immune cells in the ovarian tumor environment is not known. We sought to test whether selective agents targeting distinct estrogen signaling pathways can enhance the effects of immune therapy in ovarian cancer.

Results to date indicate that estrogen signaling impacts both tumor cell and immune cell viability and function. Planned experiments in Year 2 will test whether these effects can enhance treatment with immune therapy in mice. Our goal is to identify a combination of estrogen signaling agents and immune checkpoint antibodies that optimally induce an immune response against ovarian cancer.

15. SUBJECT TERMS Estrogen, immunotherapy, immune checkpoint inhibition, immune checkpoint blockade, PD-1, CTLA-4, GPER

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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TABLE OF CONTENTS

1. INTRODUCTION:

Estrogen exposure is an established risk factor for ovarian cancer development. Estrogen signaling has been targeted therapeutically for women with recurrent ovarian cancer, but these regimens are directed at hormone receptors on tumor cells. Little is known, however, about the impact of estrogen signaling on tumor immunity or the response to cancer immune therapy. We sought to address this gap in knowledge by examining the impact of canonical and non-canonical estrogen signaling on T cell function and response to immune therapy in ovarian cancer models. A role for estrogen in modulating the response to immune therapy is supported by differences in treatment outcomes in men and women receiving immune checkpoint inhibitors for cancer treatment. Notably, ovarian tumors have been relatively resistant to immune checkpoint blockade (ICB), despite a strong rationale for immune therapy in this disease. Accumulating evidence that estrogen signaling impacts T cell function in cancer models supports further investigation of estrogen receptor modulators as candidate agents for combinatorial immunotherapy regimens. Immunomodulatory effects of estrogen are mediated through canonical estrogen receptors ERα and Erβ as well as non-canonical G protein-coupled estrogen receptor (GPER). With evidence that estrogen deprivation, through oophorectomy, induces an effector phenotype and IFNγ production by T cells in ovarian cancer models, we hypothesized that selective GPER antagonists would prime the ovarian immune microenvironment to enhance response to immune checkpoint inhibition. In Aim 1 we will determine the immunomodulatory effects of selective estrogen receptor and GPER modulators in high grade ovarian cancer models. In Aim 2 we will test the impact of selective estrogen receptor agonists and antagonists on the efficacy of treatment with immune checkpoint blockade. These experiments are expected to to identify novel strategies to amplify the benefit of immune therapy in women with ovarian cancer.

2. KEYWORDS:

Estrogen, immunotherapy, immune checkpoint inhibition, immune checkpoint blockade, PD-1, CTLA-4, GPER

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Research-Specific Tasks:

Report period	Target Date (Month(s) relative to start date)	**Actual Completion Date or % of Completion**
Specific Aim 1: Define the impact of canonical and non-canonical estrogen signaling on the functional capacity of tumor-associated lymphocytes.		
Major Task 1: Obtain regulatory approval for planned experiments		
Subtask 1. The experiments in this protocol are included in UNM IACUC protocol number 16-200274 renewed in 2020. On notification of funding, we will additionally apply for USAMRMC	Mos. 1-3	Completed.

ORP Animal Care Use approval.		
Subtask 2. Genomic analysis of samples from subjects enrolled in INST 1419 is approved by the Western IRB (Study#1159254, March, 2016). Data is de-identified for analysis. Apply for Department of Defense USAMRMC Office of Research Protections Human Research Protection Office approval.	Mos. 1-3	Completed. (Documentation of exempt status submitted)
Major Task 2: Characterize the effects of estrogen signaling on tumor proliferation and growth kinetics.		
Subtask 1. Test the growth kinetics and co-inhibitory receptor expression on murine ovarian cancer cells [BR5-Akt (FVB), ID8P53- (C57/B6)] treated with estrogen receptor agonists and antagonists *in vitro*.	Mos. 1-3	Completed.
Subtask 2. Evaluate tumor engraftment and growth kinetics *in vivo* in mice subjected to oophorectomy prior to tumor challenge. 5 mice per group: untreated, G1, G15, estradiol, tamoxifen	Mos. 3-12	Completed in the BR5-Akt FVB model.
Milestone: Establish a baseline measure of the impact of estrogen receptor modulators on tumor growth and disease outcomes. Status: completed two independent experiments in the BR5-Akt FVB model.		
Major Task 3. Test the effects of estrogen receptor ligands on tumor-associated lymphocytes		
Subtask 1. Perform *in vitro* studies to measure the effects of ER modulators on lymphocyte proliferation in the presence or absence of IL2. Cells will be analyzed for expression of phenotypic markers by flow cytometry. Cytokine production will also be evaluated after PMA/ionomycin stimulation. Splenocytes and IP T cells will be retrieved from tumor bearing mice on day 21 for *in vitro* studies. To obtain sufficient IP T cells, 20 mice will be used per mouse strain per replicate.	Mos. 3-9	Initial experiments completed. Confirmatory replicates projected to be completed within 3 months.
Subtask 2. Assess the impact of ER modulators after antigen-independent activation *in vitro* with CD3/CD28 antibodies. Cytokine production and surface marker expression will be measured by flow cytometry as outlined above. 20 mice per mouse strain per replicate.	Mos. 3-9	Completed.
Subtask 3. Compare the phenotype and distribution of peritoneal and splenic lymphocytes on day 21 after tumor challenge retrieved from tumor-bearing mice treated with ER agonists or antagonists.	Mos. 9-18	Completed in the BR5-Akt FVB model.
Milestones: Determine the direct effects of select ER and GPER agonists and antagonists on tumor-associated lymphocyte phenotype and function Status: Initial in vitro T cell studies completed but confirmatory experiments are still underway. In vivo studies have been completed in the BR5-Akt FVB model.		
Major Task 4. Test an association between estrogen signaling and co-inhibitory ligand/receptor expression in tumor samples from women with ovarian cancer		
Subtask 1. Using TCGA data from women with ovarian cancer, test an association between aromatase expression and co-inhibitory receptor and ligand expression in the tumor microenvironment.	Mos. 6-18	Initial interrogation of TCGA gene

Additional comparisons will evaluate whether aromatase expression is linked with published IFNg gene signatures.		sets underway.
Subtask 2. Validate findings from the TCGA data using banked tumor samples from a cohort of 49 women enrolled in a phase I/II clinical trial.	Mos. 16-22	Planned for Year 2.
Milestone: Determine the translational relevance of planned murine studies using patient samples Status: planned for Year 2 of this project.		
Specific Aim 2: Test the impact of estrogen modulation on response to immune therapy in ovarian cancer models.		
Major Task 5. Examine the impact of ER ligands on T cell phenotype and function in mice receiving immune checkpoint inhibitors		
Subtask 1. Oophorectomized mice subjected to tumor challenge will be treated with ER ligands prior to the initiation of immune checkpoint inhibition with anti-PD1, anti-PDL1 or anti-CTLA4 antibodies. Animals will be euthanized on day 21 for phenotypic and functional analysis of peritoneal and splenic lymphocytes by flow cytometry as outlined in Task 3. 3 mice per treatment group per mouse strain: untreated, ICI monotherapy (PD1ab or CTLA4ab), PD1+CTLA4ab, ICI combined with G1, G15, tamoxifen or estradiol.	Mos. 12-18	Planned for Year 2 but currently 33% complete.
Subtask 2. Perform survival studies to determine the impact of ER ligands on the therapeutic efficacy of immune checkpoint antibodies *in vivo*. 11 mice per group per mouse strain: untreated, ICI monotherapy (PD1ab or CTLA4ab), PD1+CTLA4ab, ICI combined with G1, G15, tamoxifen or estradiol	Mos. 14-22	Planned for Year 2.
Milestone: Select the optimal ER ligand for the treatment of ovarian cancer in combination with immune checkpoint blockade Status: Planned for Year 2.		
Major Task 6. Isolate the impact of ER ligand treatment on tumor associated lymphocyte function in response to immune checkpoint inhibition *in vivo*		
Subtask 1. Perform adoptive transfer experiments using T cells from mice treated with the optimal combination of an ER ligand and immune checkpoint antibody to test whether changes in T cell function contribute to treatment benefit. 10 per group of donor mice per mouse strain: untreated, optimal ICI+ER ligand combination; G1, G15. 10 per group of recipient mice – 5 will receive IP lymphocytes, 5 will receive splenocytes.	Mos. 16-24	Planned for Year 2.
Milestone: Establish the contribution of immunomodulation by ER ligands to therapeutic efficacy of immune checkpoint antibodies. Status: Planned for Year 2.		
Major Task 7. Complete data analysis and draft manuscript		
Subtask 1. Compile data for presentation at national meetings	Mos. 12-24	Initial results presented at the Annual Meeting

		of the Society of Gynecologic Oncology, March 18-22, 2022, Phoenix, AZ.
Subtask 2. Draft manuscript for submission in collaboration with members of the biostatistics core facility.	Mos. 18-24	Planned for Year 2.
Milestone: Complete a manuscript for publication Status: Initial results presented at a national meeting. Manuscript planned for Year 2.		

What was accomplished under these goals?

Specific Aim 1: Define the impact of canonical and non-canonical estrogen signaling on the functional capacity of tumor-associated lymphocytes.

Aim 1a: Characterize the effects of estrogen signaling on tumor proliferation and growth kinetics.

A. Using end-point PCR we confirmed that both BR5 and ID8 cells express *Esr1* and *Esr2*. Notably, only BR5 cells appear to express *Gper1*
B. In vitro experiments indicated that the GPER agonist G1 is toxic to both BR5 and ID8 cells at high doses
C. G1 increases PD-L1 surface expression at high doses in surviving BR5 and ID8 cells (**Figure 1**)

Figure 1

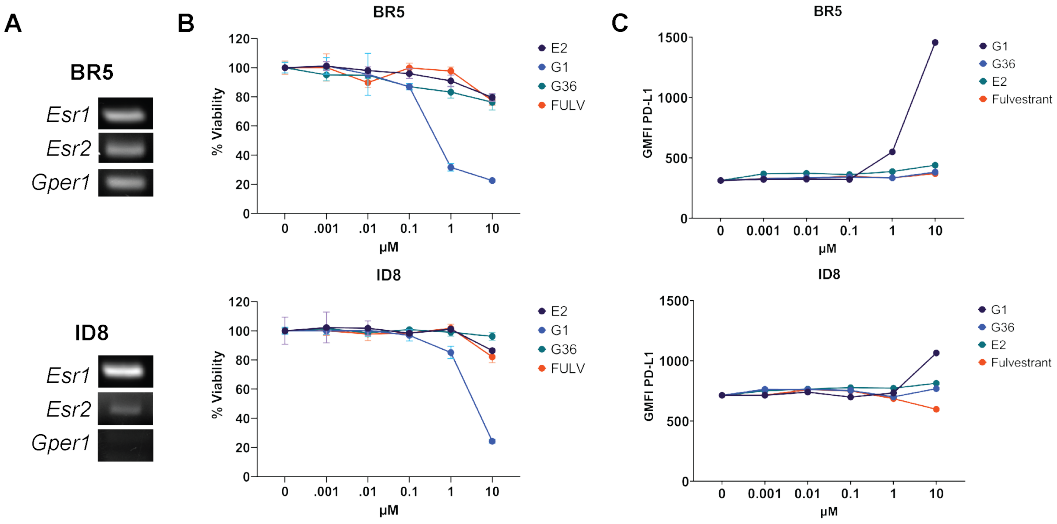

Figure 1. The impact of estrogen receptor ligands on BR5 and ID8 viability and PD-L1 expression A) End-point PCR analysis of *Esr1 Esr2* and *Gper1* in BR5 (top) and ID8 (bottom) cells. B) Viability as determined by MTT assay of increasing doses of ER ligands in BR5 (top) and ID8 (bottom) cells. C) Flow cytometry analysis of PD-L1 in BR5 (top) and ID8 (bottom) cells in response to increasing doses of ER ligands

D. Unexpectedly, treatment with the canonical estrogen ligand E2 accelerated tumor growth such that animals had to be euthanized 18 days post-tumor challenge due to ascites accumulation

(**Figure2**). Post-necropsy assessment of tumor burden confirmed a significant increase tumor burden relative to vehicle-treated controls. No significant differences were observed in mice treated with G1, G36 or fulvestrant at this time point.

Figure 2

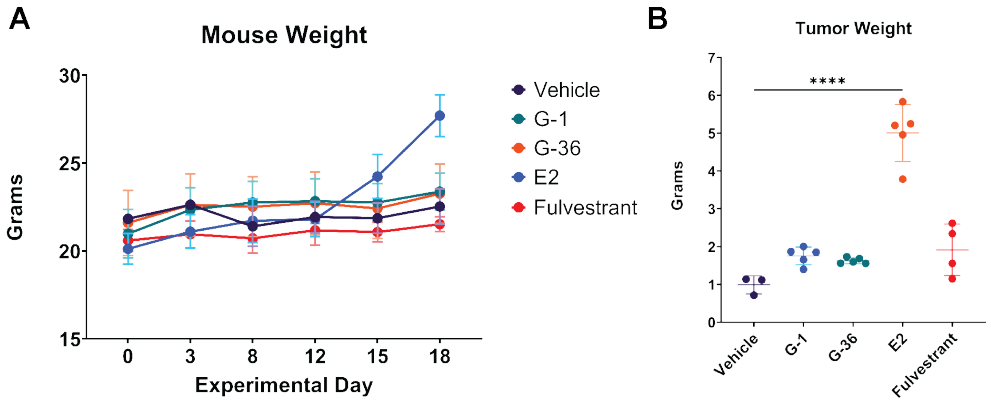

Figure 2. The impact of estrogen receptor ligands on BR5 viability *in vivo*. A) Body weight of FVB mice challenged with BR5-Akt cells. B) Weight of resected tumor at 18d post-tumor challenge

Aim 1b. Define the direct effects of estrogen receptor ligands on tumor-associated lymphocytes ex vivo.

A. Similar to experiments using tumor cells, high doses of G1 were toxic to naïve CD4 and CD8 T cells (**Figure 3**)
B. We found that ER ligands had differential impacts on T cell polarization. Tamoxifen, G1 and G36 were found to promote FoxP3 expression in iTreg conditions. Conversely, G36 and E2 were found to promote IFNγ production in Th1 conditions.

Figure 3

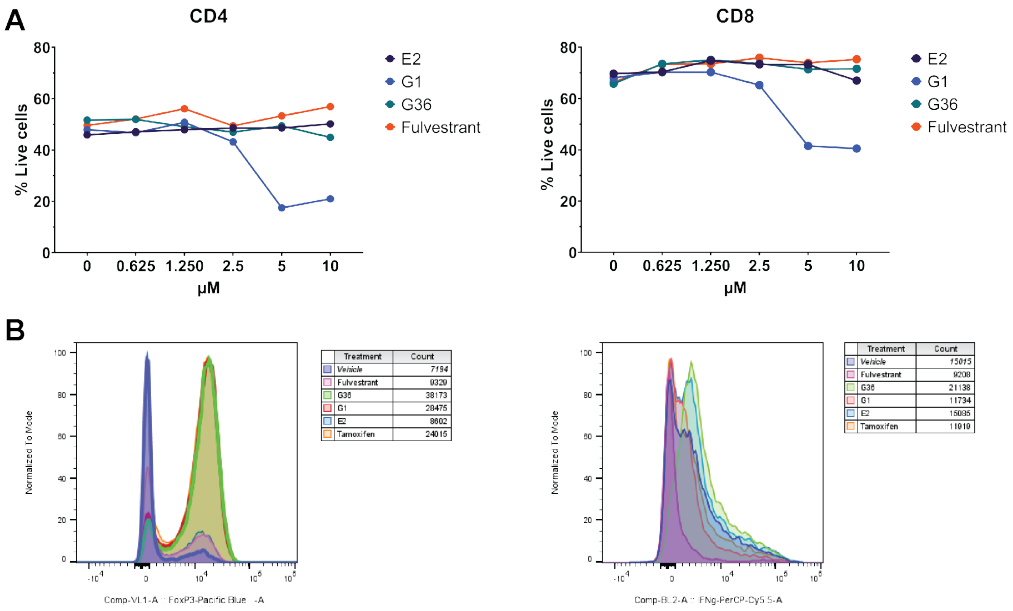

Figure 3. The impact of estrogen receptor ligands on naïve T cell viability and polarization. A) Viability as determined by flow cytometry of increasing doses of ER ligands in naïve CD4+ (left) and CD8+ (right) T cells. B) FoxP3 (left) and IFNγ (right) staining of naïve CD4+ T cells stimulated in iTreg and Th1 conditions respectively.

Aim 1c. Determine the impact of estrogen receptor modulators on T cell function in the tumor microenvironment.

A. At the 18 day time point post-tumor challenge we observed a significant increase in naïve CD4 and CD8 T cells in the peritoneal cavity of E2-treated mice relative to vehicle treated controls. Additionally, we observed a decrease in IFNγ^{+} CD4 T cells in E2-treated mice relative to vehicle treated controls (**Figure 4**).

Figure 4

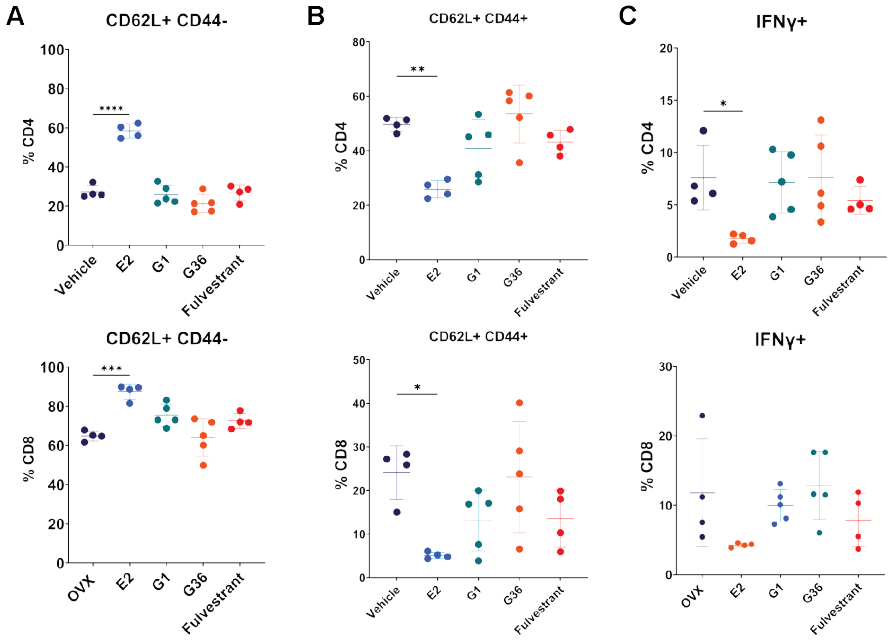

Figure 4. The impact of estrogen receptor ligands on tumor-associated T cell activation and effector function *in vivo*. The proportion of naïve (A), effector (B) and IFNγ-producing (C) CD4 (top) and CD8 (bottom) tumor-associated T cells.

Aim 2a. Determine the impact of estrogen receptor modulation on tumor-associated T cell function following CTLA4 or PD1 immune checkpoint inhibition in tumor-bearing mice.

A. In initial studies combining estrogen receptor ligands and PD1 immune checkpoint blockade, mice treated with E2+PD1ab reached our humane end-point due to ascites accumulation by day 18. Post-necropsy assessment revealed a significant increase tumor burden relative to vehicle-treated controls. No significant differences in tumor burden were observed in mice treated with G-1, G-36 or fulvestrant combined with PD1 blockade at this time point (**Figure 5**).

Figure 5

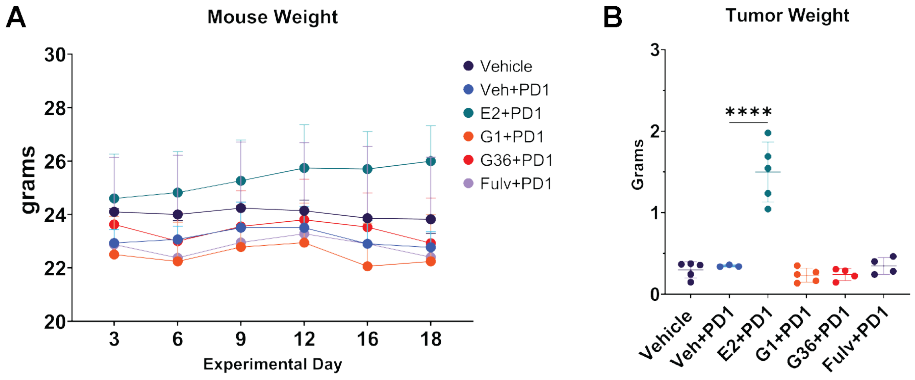

Figure 5. The impact of estrogen receptor ligands on BR5 viability following PD1 immune checkpoint inhibition *in vivo*. A) Body weight of FVB mice challenged with BR5-Akt cells. B) Weight of resected tumor at 18d post-tumor challenge

B. Similar to observed effects with E2 monotherapy, a significant increase in naïve CD4 and CD8 T cells was evident in the peritoneal cavity of mice treated with E2+PD1ab relative to vehicle treated controls. No differences in effector or IFNγ-producing T cells were noted.

Figure 6

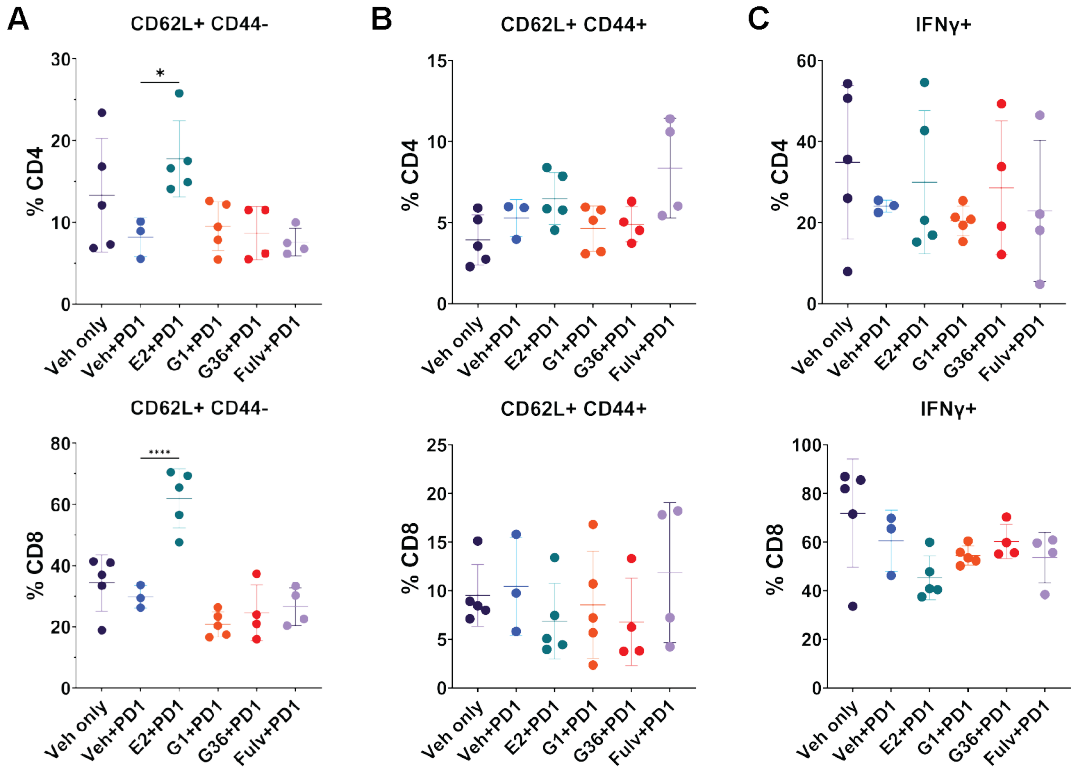

Figure 6. The impact of estrogen receptor ligands on tumor-associated T cell activation and effector function following PD1 immune checkpoint inhibition *in vivo*. The proportion of naïve (A), effector (B) and IFNγ-producing (C) CD4 (top) and CD8 (bottom) tumor-associated T cells.

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

This project has provided training and mentoring opportunities for a postdoctoral fellow and two clinical gynecological oncology fellows in training. Initial results from experiments outlined in Aim 1 were presented at the Annual Meeting of the Society of Gynecologic Oncology in Phoenix, AZ, March 18-22, 2022 by Devin Jones, MD, gynecologic oncology fellow. Trainee co-authors included Daniel Falcon, PhD, post-doctoral fellow, and Marina Miller, MD, senior clinical fellow. This project provided additional research opportunities for an undergraduate student, and mentoring opportunities for the post-doctoral fellow, through the Undergraduate Pipeline Network (UPN) summer research experience program. This undergraduate student presented her results at the UPN program's competitive symposium. Finally, initial results from these studies were also presented during a seminar series at the UNM Comprehensive Cancer Center.

how were the results were disseminated to communities of interest.

Initial results from experiments outlined in Aim 1 were presented at the Annual Meeting of the Society of Gynecologic Oncology in Phoenix, AZ, March 18-22, 2022. This conference had over 2000 attendees, including gynecologic oncologists, medical oncologists, radiation oncologists, translational scientists, patient advocates, andfellows, residents, and students.

What do you plan to do during the neext reporting period to accompish the goals?

We will continue to follow the experimental plan outlined in the approved Statement of Work and Project Narrative. As a part of Specific Aim 1b, confirmatory *ex vivo* experiments testing the impact of ER ligands on the phenotype and function of tumor-associated T cells are underway. We will continue in vivo studies described in Aim 2 testing the impact of ER ligands on T cell phenotype and function in mice receiving anti-CTLA4 and the combination of anti-CTLA4 and anti-PD1. Results from these experiments will be used to prioritize testing in Aims 2b-c in Year 2.

4. **IMPACT:**

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

We presented preliminary results from experiments testing the effects of drugs that modify estrogen signaling on ovarian cancer cells in a dish and on tumor growth in mice at an international meeting of the Society of Gynecologic Oncology. These are the first studies of a new class of estrogen receptor modulators that selectively bind the G-protein coupled estrogen receptor (GPER) in ovarian cancer. Our data show differences in tumor growth and immune cell infiltration into tumors in mice treated with different estrogen receptor ligands. Our data support further study of the effect of these compounds in combination with immune therapy.

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.

These results may support additional studies of the impact of estrogen receptor ligands in other tumor types.

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

- Mice treated with E2 rapidly developed tumor requiring a modification of our experimental protocol to comply with humane experimental endpoints outlined in our application. We'd originally planned to examine tumor burden and T cell phenotype on Day 21 to allow comparisons with prior studies but we had to instead euthanize mice on Day 18 based on tumor growth in the experimental groups receiving E2 monotherapy or E2 in combination with PD1 antibody. We do not expect this modification to significantly impact interpretation of results but we plan repeat experiments in groups not treated with E2 to compare Day 18 results with Day 21 results.

None encountered.

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

Not applicable.

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.

Jones D, Falcon D, Miller M, Goff C, Kinjyo I, Prossnitz E and Adams S, "A pilot study of the impact of estrogen signaling on tumor immunity in a syngeneic model of ovarian cancer", presented at the 2022 Society of Gynocologic Oncology Annual Meeting, Phoenix, AZ, March 18-21, 2022.

- **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:	Sarah Adams, MD
Project Role:	Principal Investigator
Researcher Identifier:	
Nearest person month worked:	2
Contribution to Project:	Principal Investigator
Funding Support:	Department of Defense Pilot Award
Name:	Daniel Falcon, PhD
Project Role:	Post Doctoral Fellow
Researcher Identifier:	
Nearest person month worked:	8
Contribution to Project:	Oversight and conduct of experiments, mentoring for students and lab members, interpretation of data, presentation of results
Funding Support:	ASERT Fellowship, Department of Defense Pilot Award
Name:	Eric Prossnitz, PhD
Project Role:	Collaborator
Researcher Identifier:	
Nearest person month worked:	1
Contribution to Project:	Expertise in estrogen and GPER signaling in cancer, experimental design, interpretation of results
Funding Support:	No salary support on this grant
Name:	Katherine Morris, MD
Project Role:	Collaborator
Researcher Identifier:	
Nearest person month worked:	1
Contribution to Project:	Expertise in sex differences in cancer outcomes, interpretation of results
Funding Support:	No salary support on this grant
Name:	Marina Miller, MD
Project Role:	Clinical Fellow
Researcher Identifier:	
Nearest person month worked:	1
Contribution to Project:	Project development, presentation of results
Funding Support:	ACGME Fellowship

Name:	Devin Jones, MD
Project Role:	Clinical Fellow
Researcher Identifier:	
Nearest person month worked:	8
Contribution to Project:	Project development, presentation of results
Funding Support:	ACGME Fellowship
Name:	Chelsea Gregory
Project Role:	Research Technician III
Researcher Identifier:	N/A
Nearest person month worked:	3
Contribution to Project:	Technical support for ongoing experiments
Funding Support:	Department of Defense Pilot Award

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: