AWARD NUMBER: W81XWH-21-1-0337

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

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REPORT DATE: August 2023

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2023	Final	01May2021-30Apr2023

4. TITLE AND SUBTITLE

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

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-21-1-0337

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

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. Our long-term 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
Unclassified	Unclassified	Unclassified	Unclassified	22	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

With accumulating evidence that estrogen signaling impacts T cell function, we proposed to test a strategy to enhance the efficacy of immune therapy using novel estrogen receptor ligands. While estrogen exposure is an established risk factor for ovarian cancer, little is known about the impact of estrogen signaling on ovarian tumor immunity and the effects of estrogen agonists or antagonists on the response to immune therapy had not been investigated. 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, 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 our goal was to determine the immunomodulatory effects of selective estrogen receptor and GPER modulators in high grade ovarian cancer models. In Aim 2 we set out to test the impact of selective estrogen receptor agonists and antagonists on the efficacy of treatment with immune checkpoint blockade. The goal of these experiments was 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 ORP Animal Care Use approval.	Mos. 1-3	Completed.
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.
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.		
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	Completed.
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		
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,	Mos. 6-18	Completed

test an association between aromatase expression and co-inhibitory receptor and ligand expression in the tumor microenvironment.		
Subtask 2. Validate findings from the TCGA data using banked tumor samples from a cohort of 30 women enrolled in a phase I/II clinical trial.	Mos. 16-22	Completed
Milestone: Determine the translational relevance of planned murine studies using patient samples		
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.	Mos. 12-18	Completed
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	Ongoing
Milestone: Select the optimal ER ligand for the treatment of ovarian cancer in combination with immune checkpoint blockade		
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	Not performed pending survival results from Major Task 5.
Milestone: Establish the contribution of immunomodulation by ER ligands to therapeutic efficacy of immune checkpoint antibodies.		
Major Task 7. Complete data analysis and draft manuscript		
Subtask 1. Compile data for presentation at national meetings	Mos. 12-24	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	Pending
Milestone: Complete a manuscript for publication		

What was accomplished under these goals?

Major activities, objectives, and significant results: Our studies demonstrated that estrogen signaling significantly modulates lymphocyte phenotype and functional status in the ovarian tumor microenvironment. While results for specific ligands were complex, there were clear differences in tumor infiltrating and tumor-associated lymphocyte subsets in response to estrogen deprivation following oophorectomy, and treatment with estrogen receptor ligands.

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. With evidence that tamoxifen can impact GPER signaling, we switched to fulvestrant as an ER antagonist.
C. Direct treatment of both murine tumor cell lines *in vitro* with estrogen receptor ligands did not significantly reduce tumor cell viability, except for G1 at high doses. G1 was also noted to increase PD-L1 surface expression in surviving BR5 and ID8 cells at high doses (**Figure 1**)

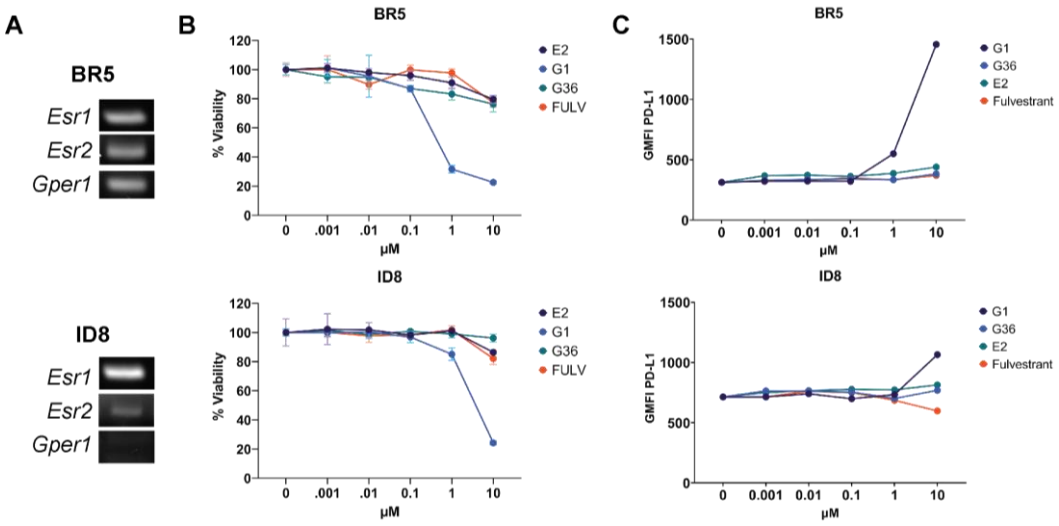

Figure 1. The impact of estrogen receptor ligands on BR5 and ID8 tumor cell viability and PD-L1 expression
A) End-point PCR analysis of *Esr1 Esr2* and *Gper1* in BR5 (top) and ID8 (bottom) cells. B) Viability as determined by MTT assay of increasing doses of ER ligands in BR5 (top) and ID8 (bottom)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

D. *In vivo*, 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 (**Figure 2**). 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. Based on these effects, day 18 was selected for comparison of T cell phenotype across experimental groups, rather than day 21 as originally planned.

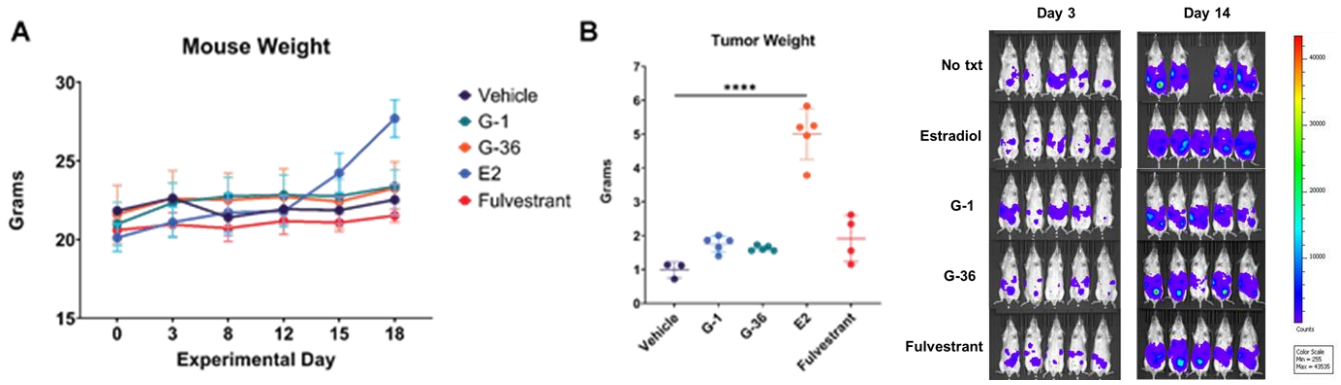

Figure 2. The impact of estrogen receptor ligands on BR5 tumor growth *in vivo*. A) Body weight of FVB mice challenged with BR5-Akt cells. B) Weight of resected tumor at 18d post-tumor challenge and illustration of tumor burden on day 14 based on luciferase flux.

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

A. 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)**

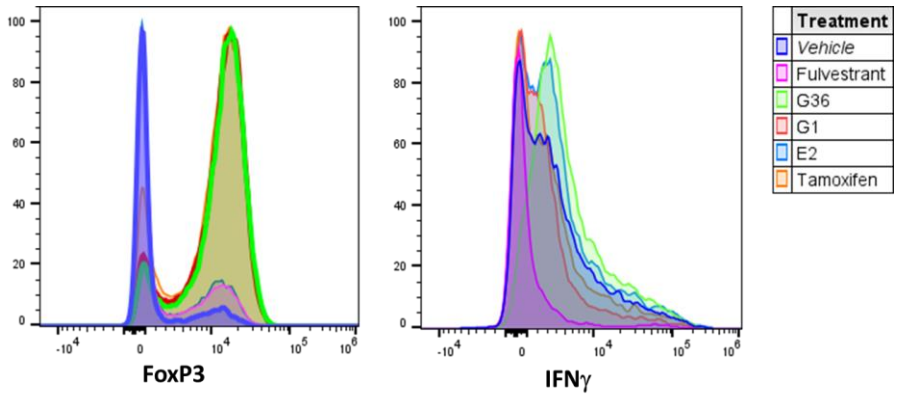

Figure 3. The impact of estrogen receptor ligands on naïve T cell viability and polarization ex vivo. A) Viability as determined by flow cytometry of increasing doses of ER ligands in naïve CD4+ (left) and CD8+ (right) T cells. B) FoxP3 (left) and IFNγ (right) staining of naïve CD4+ T cells stimulated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

B. Treatment of tumor-associated T cells *ex vivo* with ER ligands did not significantly impact effector phenotype (**Figure 4**). No difference in IFNg production was noted among treatment groups (not shown).

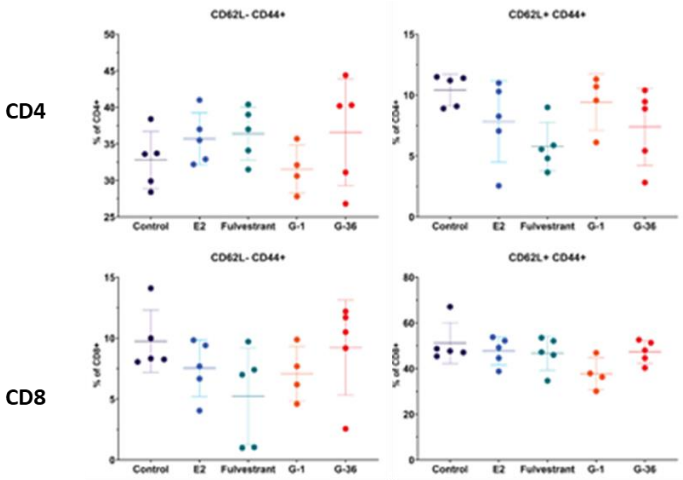

Figure 4. The impact of estrogen receptor ligands on tumor-associated T cell phenotype *ex-vivo*. The proportion of naïve and central memory CD4 (top) and CD8 (bottom) tumor-associated T cells treated *ex-vivo*.

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

A. At the 18d time point post tumor challenge we observed a significant increase in the proportion of naïve CD4 and CD8 T cells in the peritoneal cavity of E2-treated mice relative to vehicle treated controls. An increase in CD4 T cells was also evident in mice treated with the GPER agonist G1. A higher proportion of naïve T cells and a corresponding reduction in central memory cells were evident in E2 treated mice (**Figure 5**).

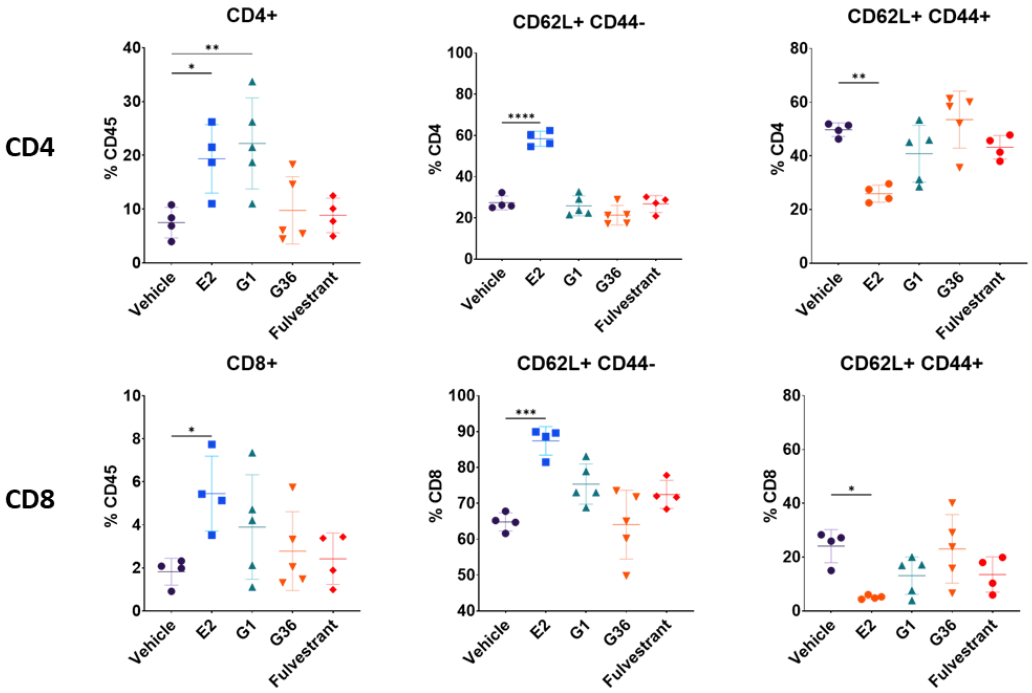

Figure 5. Effect of in vivo treatment with estrogen receptor ligands on T cell phenotype and distribution in the peritoneal cavity of tumor-bearing mice. Mice subjected to oophorectomy underwent tumor challenge on day 0, and began treatment with estrogen receptor ligands as indicated beginning on day 3. Animals were euthanized on day 18 and peritoneal T cells were collected for flow cytometric phenotyping. E2 treatment resulted in rapid tumor growth which was associated with changes in T cell phenotype in the peritoneal cavity.

B. We observed a decrease in the proportion of IFNγ^+ T cells but an increase in TNFα^+ T cells in E2-treated mice relative to vehicle treated controls (**Figure 6**).

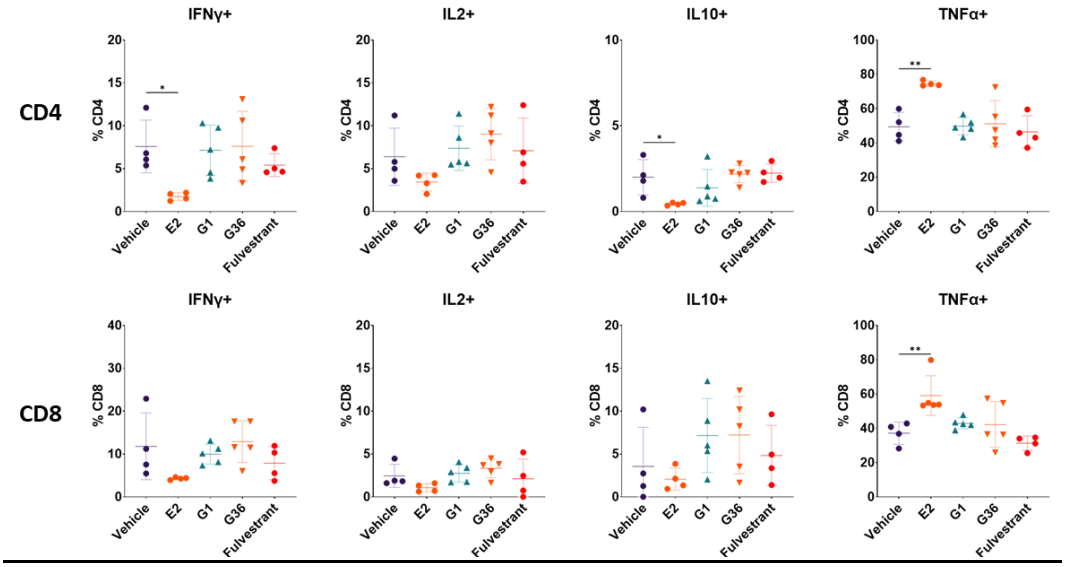

Figure 6. The impact of estrogen receptor ligands on tumor-associated T cell function *in vivo*. Cytokine production ex vivo was used as a surrogate marker for T cell functional status in the peritoneal tumor environment. On day 18, T cells were isolated from the peritoneal cavity and stimulated ex vivo. The proportion of CD4 or CD8 T cells producing each cytokine was compared by flow cytometry.

C. E2 treatment reduced the proportion of regulatory FoxP3+ CD4 T cells in the tumor microenvironment, while both G1 and G36 significantly increased FoxP3+ regulatory T cells. Notably, changes in T cell phenotype and distribution in the peritoneal cavity differed from results in splenocytes of mice treated with estrogen receptor ligands (**Figure 7**).

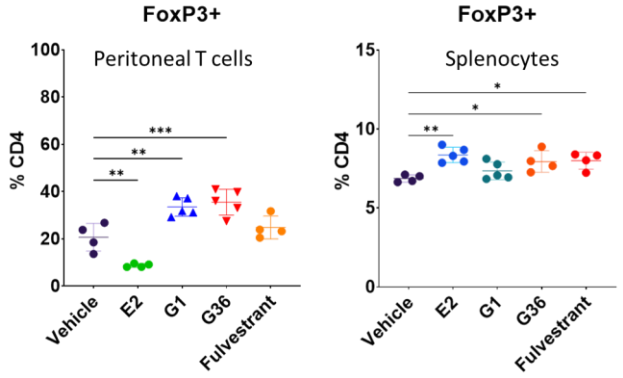

Figure 7. Estrogen receptor ligands differentially impact the proportion of regulatory T cells in the peritoneal tumor microenvironment and systemically. E2 treatment reduced the percentage of CD4+ cells expressing FoxP3, while both the GPER agonist and antagonist increased FoxP3 expression among CD4+ T cells in the peritoneal tumor microenvironment. Among splenocytes, E2, G36, and fulvestrant increased FoxP3 expression among CD4+ T cells.

Aim 1d. Test a correlation between estrogen signaling and immune checkpoint expression in ovarian tumors from patients.

A. Analysis of TCGA data:
   a. TCGA includes data from 300 women with ovarian cancer. Of those, follow-up time is documented for 299 patients. Using aromatase expression as a surrogate for estrogen status, we assessed for association between aromatase (CYP19A1) expression and expression of immune checkpoint ligands and receptors in the tumor microenvironment (CTLA4, PD1, PDL1), as well as FoxP3 expression. These analyses indicated that CTLA4, PD1, PDL1, and FoxP3 expression were strongly positively correlated ($p<0.0001$ for all correlations) in the TCGA patient samples, as expected. Interestingly, these four markers were negatively correlated with aromatase expression, but only the correlation between FoxP3 and aromatase expression was statistically significant (-0.121; $p=0.037$).
   b. When univariate Cox models were developed for progression-free survival (PFS) for each of these markers, CTLA4 expression was significantly negatively associated with PFS ($p=0.048$;

hazard ratio (HR)=0.82). In the multivariate Cox model that included all genes, the HR for high CTLA4 expression was 0.69 (p=0.059), while the HRs for the other markers ranged from 0.96-1.18.

c. When comparing expression of aromatase with expression of canonical and non-canonical estrogen receptor genes (ESR1, ESR2, and GPER1), the only significant correlation was with ESR2 (0.231, $p<0.0001$). None of these genes were significantly associated with disease outcomes in the univariate or multivariate Cox models (HRs range 0.88-1.1; $p>0.1$ for all comparisons).

d. For logistic regression analyses evaluating associations between gene expression and patient outcomes, we defined a binary PFS outcome based on progression $\leq$ 2 years (n=160) or >2 years (n=81). There were 58 patients who had no documented date of progression and were lost to follow-up prior to year 2 that were not included in this analysis. Similarly, cases with overall survival (OS) $\leq$2 years (n=59)or > 2 years (n=183) were identified. There were 57 patients who had no documented date of death and were lost to follow-up prior to year 2 who not included in this analysis. Neither univariate nor multivariate Cox Models demonstrated any significant association of 2-year PFS or 2-year OS with expression of any of the genes analyzed (CYP19A1, CTLA4, PD1, PDL1, FOXP3, ESR1, ESR2, GPER).

B. RNAseq data from 30 patients enrolled in a clinical trial of olaparib and tremelimumab for the treatment of recurrent BRCA1 or BRCA2 mutation associated ovarian cancer was assessed for any association between estrogen status and response to immune therapy.

a. RNAseq was performed on samples from 33 trial subjects. Samples from 3 patients failed quality control, so data from 30 subjects were available for analysis.

b. An association between clinical outcomes and expression of aromatase, immune checkpoint ligands and receptors, and canonical and non-canonical estrogen receptors was assessed. Subjects were divided into those who had clinical benefit from treatment (complete or partial response or stable disease as the documented best overall response), and those who did not demonstrate clinical benefit (progressive disease). In an unbiased analysis comparing gene expression in patients with or without clinical benefit, none of the target genes (CYP19A1, CTLA4, PD1, PDL1, FOXP3, ESR1, ESR2, GPER) were significantly differentially expressed (AUC $\leq$0.65 for all genes) among study subjects based on treatment outcomes.

Specific Aim 2: Test the impact of estrogen receptor modulation on response to immune therapy in ovarian cancer models.

Aim 2a.1. Determine the impact of estrogen receptor modulation on tumor-associated T cell function following PD1 immune checkpoint inhibition in tumor-bearing mice.

A. *In vivo* studies combining estrogen receptor ligands and PD1 immune checkpoint blockade had to be terminated by day 18 due to rapid tumor growth. Post-necropsy assessment revealed a significant increase tumor burden in mice receiving E2 treatment in combination with immune checkpoint antibody 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 8**).

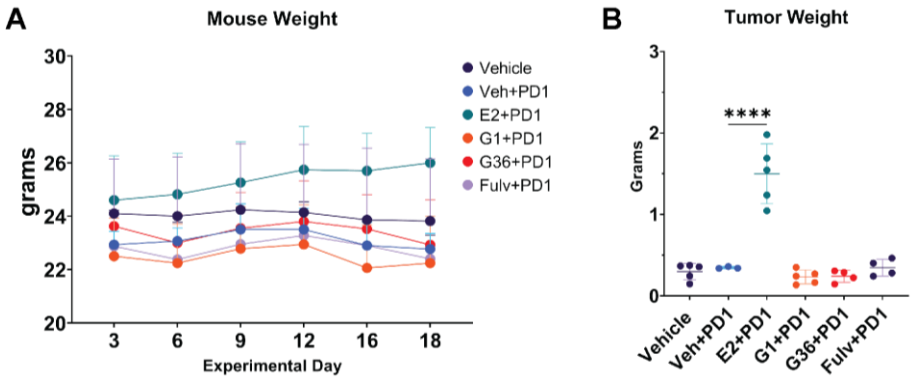

Figure 8. The impact of estrogen receptor ligands on BR5 viability following PD1 immune checkpoint inhibition *in vivo*. A) Body weight of FVB mice challenged with BR5-Akt cells. B) Weight of resected tumor at 18d post-tumor challenge

A. 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. Notably, the reduced proportion of central memory T cells seen in the E2 monotherapy group in prior experiments was not as pronounced in combination with PD1 blockade. Similarly, while no differences in effector or IFNγ-producing T cells were noted among PD1 antibody treatment groups, the marked reduction in IFNγ seen in mice treated with E2 alone was less apparent with E2 in combination with PD1 blockade. We interpret these results as evidence that effects of immune checkpoint inhibition may have overcome immunosuppressive effects of E2 in the tumor microenvironment (**Figure 9**).

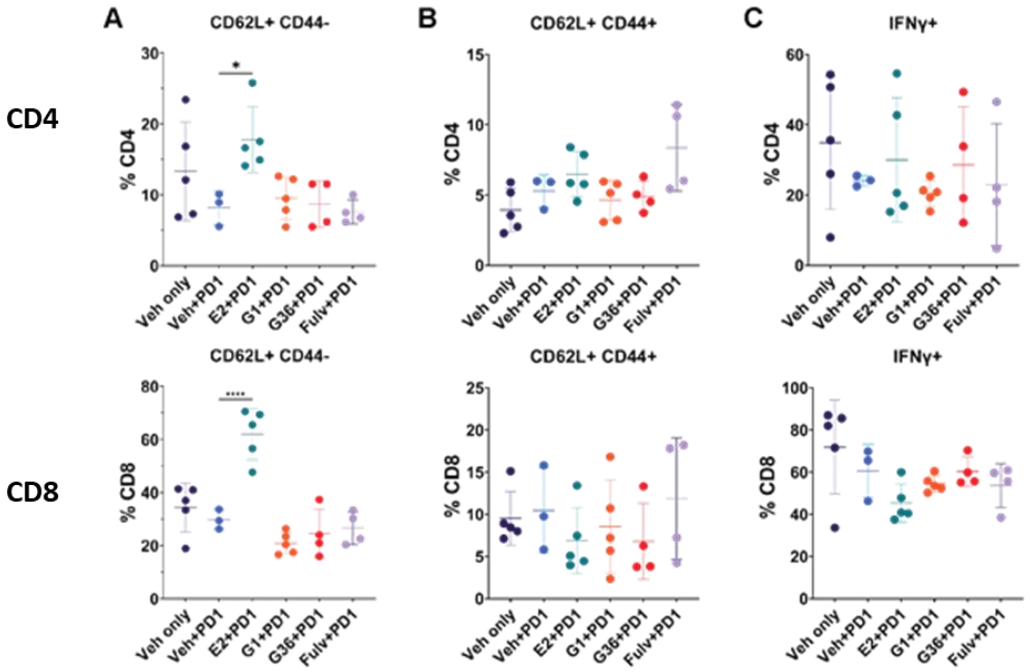

Figure 9. The impact of estrogen receptor ligands on tumor-associated T cell activation and effector function following PD1 immune checkpoint inhibition *in vivo*. The proportion of naïve (A), central memory (B), and IFNγ-producing (C) CD4 (top) and CD8 (bottom) tumor-associated T cells.

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

B. Similar to observed effects with E2 monotherapy and E2+PD1ab, a higher tumor burden was associated with increased naïve CD4 T cells in the peritoneal cavity of mice treated with

E2+CTLA4ab relative to vehicle treated controls (**Figures 10, 11**). As with PD1 checkpoint blockade, no difference in the proportion of IFNγ-producing T cells were noted among treatment groups, however the reduction in IFNγ seen with E2 monotherapy was attenuated in CD8 T cells in response to E2 combined with CTLA4 blockade. Additionally, IL-10 production by CD4 and CD8 T cells was markedly reduced in response to all estrogen receptor ligands in combination with CTLA4 immune checkpoint inhibition (**Figure 12**). Finally, while no significant difference in immune checkpoint receptor expression was evident in the tumor microenvironment, differences were observed in both CD4 and CD8 T cells in the spleen in response to combined treatment with CTLA4 antibody and estrogen receptor ligands (**Figure 13**).

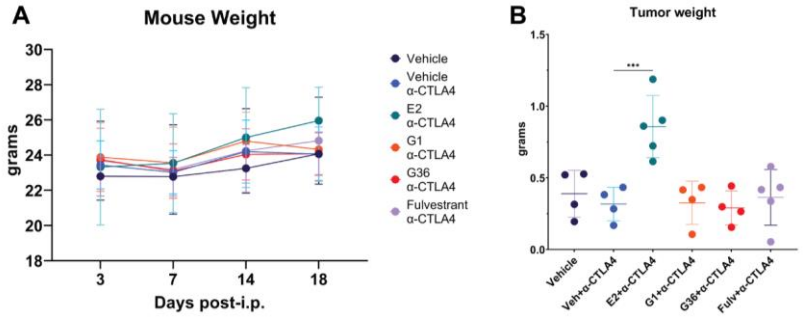

Figure 10. The impact of estrogen receptor ligands on BR5 viability following CTLA4 immune checkpoint inhibition *in vivo*. A) Body weight of FVB mice challenged with BR5-Akt cells. B) Weight of resected tumor at 18d post-tumor challenge

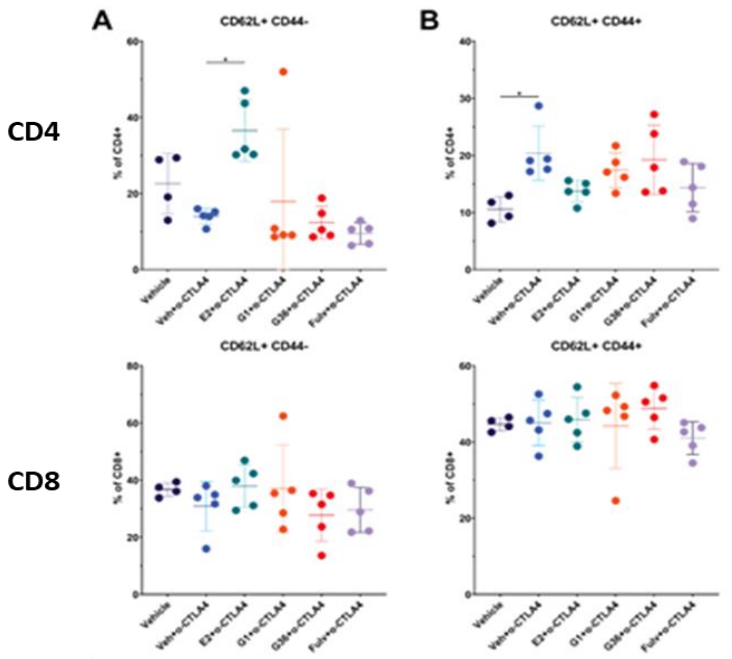

Figure 11. The impact of estrogen receptor ligands on tumor-associated T cell activation following CTLA4 immune checkpoint inhibition *in vivo*. The proportion of naïve (A), effector (B) CD4 (top) and CD8 (bottom) tumor-associated T cells.

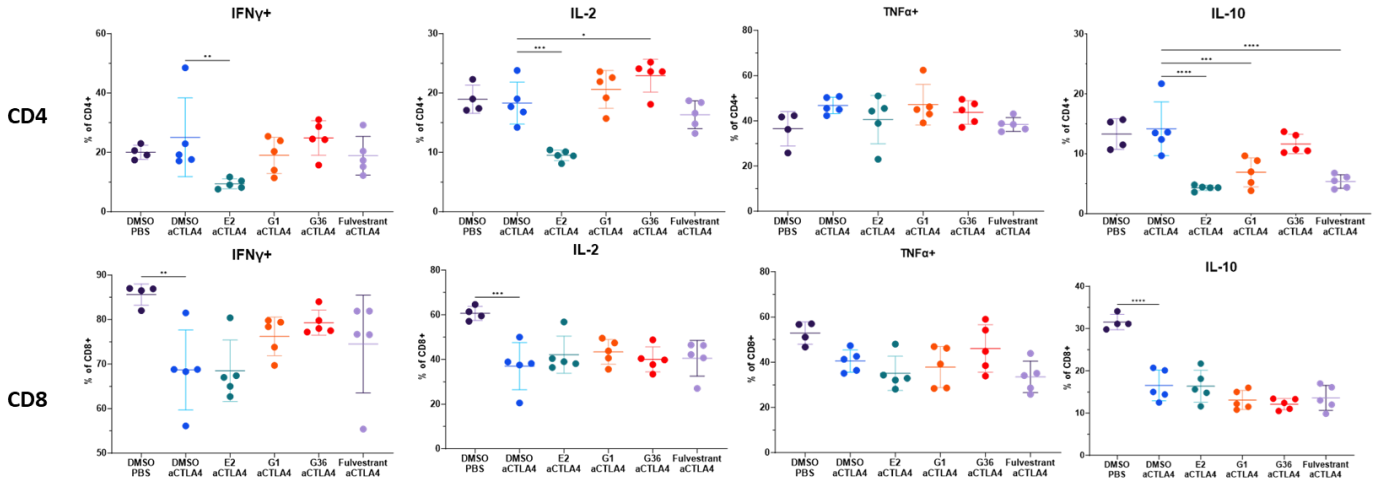

Figure 12. Estrogen receptor ligands in combination with CTLA4 immune checkpoint inhibition modulate cytokine production by tumor-associated T cells. Reduced IFNg production among CD4+ T cells was seen with E2 combined with CTLA4 immune checkpoint blockade, similar to the effects of E2 monotherapy, but these effects were attenuated in CD8+ T cells. Notably, estrogen ligand treatment reduced IL10 in combination with CTLA4 blockade, which may potentiate the immunostimulatory effects of immune checkpoint inhibition, particularly since IL-10 levels are high in the ovarian tumor microenvironment.

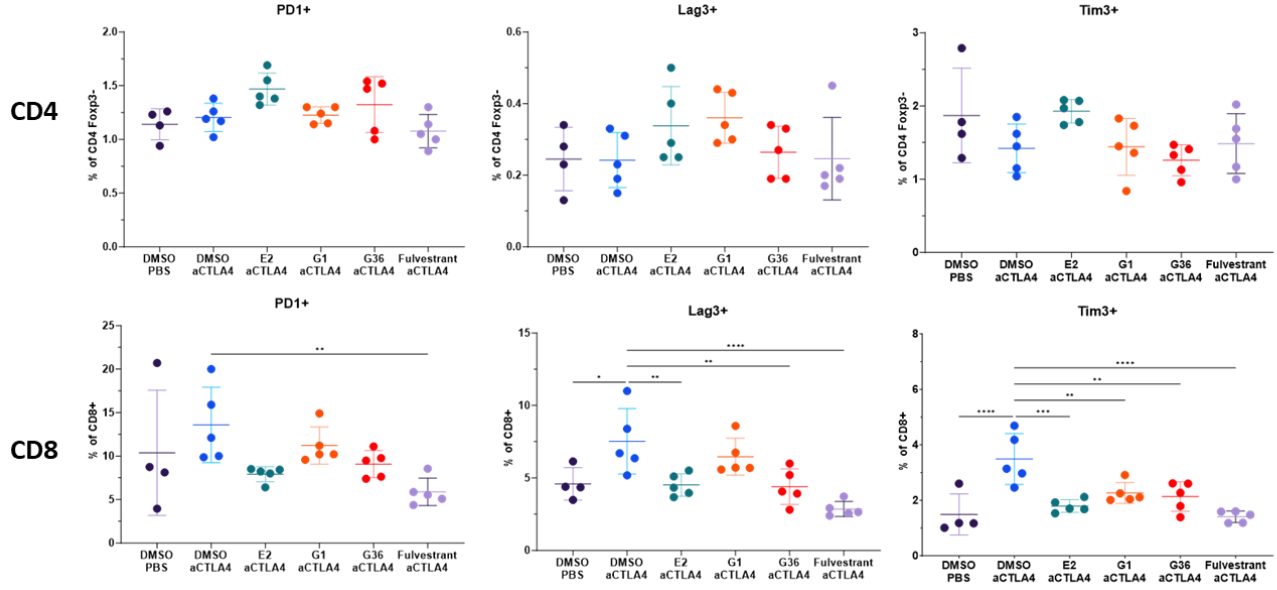

Figure 13. Combined treatment with estrogen receptor ligands and CTLA4 immune checkpoint antibody modulates immune checkpoint receptor expression in splenocytes. Although no significant difference in checkpoint receptor expression was demonstrated in the tumor microenvironment, the addition of estrogen receptor ligands modulated checkpoint expression in combination with CTLA4 antibody therapy, particularly on CD8 T cells.

Impact: Consistent with our hypothesis, these immunomodulatory effects of estrogen signaling modulated T cell phenotype and functional status as monotherapy and in combination with immune checkpoint antibodies in ovarian cancer models. One outcome of our work is the demonstration that the estrogen status of cancer models may substantially impact interpretation of results. In ovarian cancer, most pre-clinical studies of cancer therapeutics are conducted in female mice with intact ovarian function, despite the fact that most women with ovarian cancer undergo oophorectomy. With evidence that estrogen signaling significantly impacts tumor immunity and lymphocyte distribution and function in the tumor microenvironment, preclinical studies of immunotherapeutic regimens may need to use mice subjected to oophorectomy to better replicate conditions in the ovarian tumor microenvironment. We expect that defining the impact of estrogen signaling pathways on

tumor immunity will pave the way for a new generation of combinatorial interventions tailored for the unique immunobiology of ovarian cancer. Based on our experience testing estrogen receptor ligands in this pilot project, we plan to shift our focus to early events in tumor development, and the opportunity to selectively modulate conditions in the tumor microenvironment to restrict tumor outgrowth in the setting of pathogenic BRCA mutations.

Challenges and goals not met: Through the course of this project, it became clear that estrogen ligands have complex effects in the tumor microenvironment. This may be due in part to overlapping effects and different mechanisms of action of available estrogen receptor modulators such as tamoxifen and fulvestrant. Depletion of ER versus blockade of estrogen signaling had differing effects on leukocyte phenotype and distribution. Although we proposed to use tamoxifen as an estrogen receptor antagonist, effects on GPER made interpretation of results challenging, so we switched to fulvestrant. This had its own challenges since fulvestrant down-regulates estrogen receptor expression, rather than blocking signaling. We also observed unexpected cytotoxic effects of GPER agonist in both BR5 and ID8 ovarian cancer cells, despite the fact that only BR5 expressed GPER. Surprisingly, G1 also increased PDL1 expression in both cell lines at high doses.

In vivo, interpretation of estrogen-mediated effects was complicated by markedly accelerated tumor growth in response to E2 in vivo. We suspect that the pronounced differences in T cell subsets in E2 treated mice may reflect the effect of a markedly increased tumor burden rather than specific effects of E2 in the tumor microenvironment, particularly since ex vivo studies did not show similar differences in phenotype. These effects were attenuated in animals treated with a combination of E2 and immune checkpoint antibody. We interpret these results as evidence of a complex interaction governed by conditions in the tumor microenvironment, including tumor-intrinsic effects, indirect immunomodulatory effects resulting from tumor progression, and direct immunomodulatory effects mediated by estrogen signaling in lymphocytes. A role for tumor-intrinsic and indirect effects is supported by differences in the observed effects on peritoneal T cells and systemic lymphocytes in the spleen. Because we did not see clear differences in T cell phenotype among treatment groups, we have not yet performed the planned adoptive transfer experiments. We will wait for results of survival studies to fine-tune plans to isolate the effects of estrogen receptor ligands on lymphocytes, particularly regarding planned timepoints for T cell harvest. In addition, based on the results of this pilot project, we plan to focus on earlier timepoints in tumor development for upcoming studies, and will restrict treatment to estradiol versus estrogen to simplify interpretation of results. Secondary experiments will investigate the impact of selective estrogen receptor and GPER modulators once primary endpoints are established using estradiol.

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. Both Drs. Miller and Jones will include their contributions to this project as part of their subspecialty thesis for gynecologic oncology certification.

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 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, and fellows, residents and students.

In addition, initial results from these studies were included in presentations about strategies to adapt immune therapy for ovarian cancer presented at the Forbeck Forum to Advance Treatment for Ovarian Cancer in March, 2022; at the University of Kansas Cancer Center in November, 2022; at the University of Maryland Department of OB/GYN Grand Rounds in February 2023; and at the University of Colorado Reproductive Sciences Seminar Series, March, 2023.

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

Nothing to report

4. **IMPACT:**

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

We presented initial results from experiments testing the effects of drugs that modify estrogen signaling on ovarian cancer cells 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. These results support further study of the effect of these compounds in combination with immune therapy.

After the end of the project period for this DOD grant, we received a new grant to fund additional studies testing whether estradiol modulates STING signaling in the setting of BRCA mutations in ovarian cancer models. This proposal built on our experience testing estrogen receptor ligands in syngeneic ovarian cancer models. We anticipate that narrowing our focus to a specific mechanism (STING activation) and limiting our studies to estradiol (present/absent) will provide a foundation for subsequent work investigating differential effects of non-canonical GPER manipulation as a potential adjuvant for immunotherapeutic regimens. We additionally submitted a proposal for an expanded project based on these studies for consideration for a Teal Expansion Award.

What was the 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?

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Nothing to report.

Changes in approach and reasons for change

Nothing to report.

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

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.

Adams S, "Immune therapy for ovarian cancer" Invited speaker, Ovarian Cancer Committee, NRG Semi-annual Meeting, July 2022, Chicago, IL

Adams S, "Context matters: optimizing treatment for ovarian cancer"
-Invited speaker and participant, Forbeck Forum to Advance Treatment for Ovarian Cancer, Monterey CA, March, 2022;
-Invited speaker, University of Kansas Department of OB/GYN Grand Rounds, November 18, 2022;
-Invited speaker, University of Maryland Department of OB/GYN Grand Rounds, February 17, 2023
- Invited speaker, University of Colorado Reproductive Sciences Seminar, March 7, 2023

- **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:	Devin Jones, 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:	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: