CTLA-4 blockade synergizes therapeutically with PARP-inhibition in BRCA1-deficient ovarian cancer

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Abstract

Immune checkpoint blockade has shown significant therapeutic efficacy in melanoma and other solid tumors, but results in ovarian cancer have been limited. With evidence that tumor immunogenicity modulates the response to checkpoint blockade, and data indicating that BRCA-deficient ovarian cancers express higher levels of immune response genes, we hypothesized that BRCA(-) ovarian tumors would be vulnerable to checkpoint blockade. To test this, we used an immunocompetent BRCA1-deficient murine ovarian cancer model to compare treatment with CTLA-4 or PD-1/PD-L1 antibodies alone or combined with targeted cytotoxic therapy using a PARP-inhibitor. Correlative studies were performed in vitro using human BRCA1(-) cells. We found that CTLA-4 antibody, but not PD-1/PD-L1 blockade, synergized therapeutically with the PARP-inhibitor, resulting in immune-mediated tumor clearance and long-term survival in a majority of animals (p<0.0001). The survival benefit of this combination was T cell-mediated and dependent on increases in local IFN-gamma production in the peritoneal tumor environment. Evidence of protective immune memory was observed greater than 60 days after completion of therapy. Similar increases in the cytotoxic effect of PARP-inhibition in the presence of elevated levels of IFN-γ in human BRCA1(-) cancer cells supports the translational potential of this treatment protocol. These results demonstrate that CTLA-4 blockade combined with PARP-inhibition induces protective anti-tumor immunity and significant survival benefit in the BRCA1(-) tumor model, and support clinical testing of this regimen to improve outcomes for women with hereditary ovarian cancer.
Introduction

Recent advances in the development of immunotherapeutics have focused on T cell checkpoint blockade to promote induction and maintenance of an anti-tumor effector response (1). To date, significant therapeutic benefit has been realized with antibodies to CTLA-4 (cytotoxic T lymphocyte antigen-4, CD152) or PD-1 (programmed cell death protein-1, CD279) in melanoma and other solid tumors (2). The rationale for this approach is based on evidence that T cell activity is locally suppressed in the tumor microenvironment of many cancers, and that release of these inhibitory signals permits immunologic clearance of tumor cells (1). With phase III studies documenting long-term survival in as many as 40% of patients with advanced melanoma, current efforts are focused on identifying patients who are likely to respond and developing combination strategies to extend the benefit of checkpoint blockade to a majority of cancer patients (2).

Ovarian cancer has been identified as a rational target for immune therapy, however these tumors have been considered relatively resistant to checkpoint blockade (3,4). This is based on studies in murine models and clinical trials which showed limited response of ovarian tumors to CTLA-4 antibodies (5-7). Although two patients included in an early clinical trial of CTLA-4 blockade experienced a transient decrease in serum tumor markers, clinical disease regression has not been demonstrated (6,7). Because of the poor prognosis associated with ovarian cancer and the clear need for new treatment options, identifying strategies to enhance the efficacy of immunomodulatory regimens for the treatment of this disease remains a priority.

A recent study demonstrating that patients responding to CTLA-4 inhibition for the treatment of melanoma were more likely to have genetically heterogeneous tumors that expressed a panel of antigenic peptides, indicates that tumor immunogenicity modulates the efficacy of checkpoint blockade (8). Based on this, and other studies indicating that enhanced tumor antigenicity sensitizes cancers to checkpoint blockade therapy, combinatorial treatment regimens using cytotoxic agents together with checkpoint inhibitors have been proposed to optimize clinical outcomes (4). With evidence that a subset of ovarian cancers associated with
germline mutations in BRCA1/2 genes may be more immunogenic (9-11), we hypothesized that BRCA(-) tumors would be particularly vulnerable to checkpoint blockade.

Approximately 10-20% of ovarian cancer cases are attributed to hereditary syndromes, most commonly germline mutations in BRCA-1/2 genes that regulate double-stranded DNA repair (12,13). Targeted therapy of BRCA-deficient (BRCA(-)) cancers has been achieved using poly(ADP-ribose) polymerase (PARP)-inhibitors, which block BRCA-independent DNA repair and induce selective lethality in BRCA(-) cancer cells (14,15). Although PARP-inhibitors significantly improve progression-free survival in patients with germline BRCA mutations, to date this strategy has not demonstrated an improvement in cancer-specific mortality (16-18). With evidence that immune priming is required for successful anti-CTLA-4 therapy, we tested whether targeted cytotoxic therapy with a PARP-inhibitor would sensitize ovarian tumors to immune checkpoint blockade and optimize survival in a hereditary cancer model.

Here we demonstrate that combined treatment using a PARP-inhibitor together with CTLA-4 blockade induces long-term survival in a BRCA1-deficient ovarian tumor model. The efficacy of this regimen is mediated by the local induction of anti-tumor immunity and the production of increased levels of IFN-γ in the peritoneal tumor environment. A similar response by human BRCA1(-) cancer cells to PARP-inhibition in the presence of high levels of IFN-γ supports the translational relevance of this strategy for the treatment of women with hereditary ovarian cancer.
Materials and Methods

Ovarian cancer cell lines and murine tumor models

The BRCA1 deficient (BR5-Akt, BRCA1(-)) and sufficient (T22) epithelial ovarian cancer cell lines were generated on an FVB background as previously described (19) and were a kind gift from Sandra Orsulic. The ID8 cancer cell line was generated from C57BL/6 ovarian epithelial cells (20). Murine cell lines were maintained in DMEM (Hyclone) supplemented with 10% Fetalclone III (Hyclone), 10mM HEPES (Corning), 1 mM sodium pyruvate (Hyclone), 100 U/ml penicillin and 100 ug/ml streptomycin (Gibco) (DMEM-Complete). The human ovarian cancer cell lines were purchased from ATCC and cultured according to ATCC protocol: UWB1.289 wt (BRCA-1 deficient) was established from a papillary serous ovarian tumor from patient with a germline BRCA1 mutation within exon 11 and a deletion of the wild-type allele, and UWB1.289+BRCA1 cells were transfected to express wild-type BRCA1 using a pcDNA3 plasmid carrying wild-type BRCA1 (21).

Mice were purchased from Charles River Laboratories and maintained in a specific pathogen-free facility. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of New Mexico. 2x10^5 BRCA1(-) cells or 5x10^6 ID8 cells were injected intraperitoneally in 6-8 week old mice at in PBS (Corning). The number of tumor cells inoculated was selected such that mice reliably developed tumors and ascites within 40-60 days after tumor challenge. Mice were weighed at least twice weekly and were observed daily for ascites development. For survival studies, mice were sacrificed with any evidence of tumor morbidity or at a weight of 30g due to ascites accumulation. Alternatively, 2x10^5 BRCA(-) cells in 20 ul were surgically implanted orthotopically under the left ovarian bursa as previously described (22). At 21 days, mice were euthanized and the ovaries were removed, photographed and weighed.

Antibodies and reagents

The PARP-inhibitor (ABT-888; Veliparib) was purchased from Active Biochem and diluted in PBS from frozen stock for experiments. For in vivo experiments, the PARP-inhibitor was administered orally at indicated concentrations in a 20 ul volume using a pipette.
The CTLA-4 hybridoma (clone UC10-4F10-1), and IFN-γ hybridoma (clone R4-6A2) were purchased from ATCC. PD-1 (clone C1-G4) and PD-L1 (B7-H1, clone 10B5) hybridomas were kindly provided by Dr. Lieping Chen. Hybridomas were grown and monoclonal antibodies (mAbs) were purified as previously described (23). For in vivo experiments, 100 µg of CTLA-4, 300µg of PD-1 or PD-L1 mAbs diluted in 300 µl of PBS was injected intraperitoneally at the timepoints indicated.

Purified TNF-α neutralizing mAb (clone XT3.11) was purchased from BioXcell. Neutralizing antibodies to IFN-γ or TNF-α (0.5 mg) were injected intraperitoneally every four days beginning on day seven after tumor challenge, as previously described (24,25). Mouse recombinant TNF-α was purchased from eBioscience. Recombinant human TNF-α, recombinant mouse IFN-γ and recombinant human IFN-γ were purchased from Biolegend.

Characterization of leukocyte populations

For ex vivo analysis, T cells were collected from peritoneal washings taken from mice after CO₂ euthanization as previously described (26). Splenocytes were isolated from whole spleens removed following peritoneal washings; cells were disaggregated with the frosted portion of glass slides, passed through a 70 micron mesh screen, and centrifuged at 1500 rpm for 5 min. Red blood cells were lysed with ACK buffer, centrifuged, resuspended in DMEM-C and passed through a second 70 micron screen. Viable cells were counted by trypan blue exclusion and used for analyses.

In vitro tumor cell death assays

For in vitro analysis of PARP-inhibitor and cytokine mediated tumor cell lethality, 1x10⁴ murine tumor cells per well were plated in 24 well plates and exposed to increasing concentrations of the PARP-inhibitor alone or with IFN-γ or TNF-α with a final volume of 1 ml/well. Human ovarian cancer cell lines UWB1.289wt and UWB1.289+BRCA1 were plated at 1x10⁵ cells per well due to their slower growth rate. After 72 hours, samples were retrieved using a 1:1 mix of cellstripper (Corning) and trypsin-EDTA (Corning) to release adherent cells,
and centrifuged for 3 minutes at 1500 rpm. Cells were then washed in PBS, stained with the fixable viability dye ZombieNIR (Biolegend) for 10 minutes, and fixed for analysis. For cells stained intracellularly for active-Caspase-3, manufacturer instructions were followed (active Caspase-3 apoptosis kit, BD Biosciences).

**Tumor colony assay**

To quantitatively compare peritoneal tumor burden, an *ex vivo* tumor colony assay was adapted from Shoemaker et al (27). Briefly, peritoneal cells were plated in 10-fold serial dilutions starting at 1x10^6 cells/ml in 48 well plates (Costar). After 72 hours of incubation, colonies of growing tumor cells were distinguished from adherent non-tumor cells by circular colony formation of 10 or more cells (i.e. non-tumor cells were not observed to proliferate or form circular colonies). Colonies were counted at the dilution in which colonies were easily distinguishable and separated from other colonies. This value was multiplied by the dilution value for number of tumor cells/million cells in the peritoneal cavity.

**T cell functional assays**

For analysis of cytokine production by T cells, 5x10^6 total peritoneal cells or splenocytes were restimulated in 24 well plates with cell stimulation cocktail (1.34 uM ionomycin, 81 nM PMA, 10.6 uM brefeldin A and 2 uM monensin, eBioscience). After 5 hours cells were harvested, fixed, and permeabilized before staining for intracellular cytokine production (eBioscience FoxP3 staining buffer set).

For T cell receptor-specific restimulation of T cells, 5 x 10^6 cells were plated in 24-well plates coated with 10 ug/ml anti-CD3 antibody (no azide/low endotoxin; clone 145-2C11; BD Biosciences). After 18 hours, samples were harvested, pooled and filtered through a 0.45 micron syringe filter. Supernatant was assayed for IFN-γ (eBioscience) or TNF-α (Biolegend) by enzyme-linked immunosorbant assays (ELISA). To test whether cytokines produced by T cells contributed to a tumor cell cytotoxicity when combined with the PARP-inhibitor, 250 ul of cell-free supernatant was added to 24 well plates containing 1x10^4 murine tumor cells and the PARP-
inhibitor in a final volume of 1 ml (1:4 dilution of supernatant). After 72 hours cells were assayed for viability as described above.

**Flow cytometry and analysis**

Flow cytometry was performed using a BD Biosciences LSRFortessa. For direct ex vivo staining of lymphocytes, 1-2 x 10^6 cells were resuspended in 100 ul of FACS buffer (PBS + 1% Fetalclone III) and Fc receptors were blocked for 10-15 minutes at 4 degrees C with 1 ug/sample of 2.4G2 mAb (CD16 and CD32 blockade) and 1 ug/sample of mouse IgG (ThermoPierce). Cells were fluorescently labeled with antibodies for 30 minutes at 4 degrees C, washed and resuspended in fixation buffer (2% formaldehyde in PBS), or intracellularly stained according to the manufacturer’s protocol (eBiosciences). Fluorescently labeled anti-mouse monoclonal antibodies used for flow cytometry were: IFN-γ-FITC, IFN-γ-PE, CD44-PerCP-Cy5.5, CD4-APC, CD8a-APC (Biolegend); CD62L-FITC, FoxP3-PE, TNF-α-PerCP-eFluor 710, CD45-eFluor 450, CD4-eFluor450, CD62L-eFluor 450, CD4-APC-eFluor 780 and CD8a-APC-eFluor 780 (eBioscience); active-Caspase-3-PE (BD Biosciences). Flow cytometry data was analyzed with FCS Express 4 (De Novo Software).

**Histological analysis**

Omenta were retrieved from experimental mice and fixed in 10% formalin vials (Evergreen Scientific). Tissue samples were sectioned (5 μm), stained with hematoxylin (Sigma-Aldrich) and Eosin (RICCA Chemical Company), and imaged at 20x magnification with a Nikon DS-Fi1 camera mounted on a Nikon Eclipse E400 microscope running NIS-Elements software.

**Statistical methods**

Statistical analysis was done using Excel (Microsoft) and GraphPad Prism 6 (Graphpad Software, Inc.). All data are presented as means with error bars representing the standard error of the mean (SEM). Statistical comparisons between experimental groups were analyzed by one-way and two-way analysis of variance followed by Tukey’s procedure for multiple comparisons. Pairwise comparisons of two groups were made
using the student’s t-test. Kaplan-Meier survival curves were computed and the log-rank statistic was used to evaluate differences among groups. A p value < 0.05 was taken to indicate statistical significance. In all figures, p values are denoted as the following: ****p<0.0001; *** p<0.005; ** p<0.025; *p<0.05.
Results

IFN-γ enhances the cytotoxic effect of PARP-inhibition in a BRCA1- ovarian cancer model

Early studies of CTLA-4 blockade demonstrated that in vivo treatment induces the expansion of memory CD8+ T cell populations capable of producing interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (28,29). IFN-γ and TNF-α have been shown to inhibit proliferation and promote apoptosis of tumor cells in many tumor models (30-32). As a preliminary step to evaluating combined checkpoint blockade and PARP-inhibition, we tested whether IFN-γ or TNF-α would similarly amplify the cytotoxic effect of PARP-inhibition in a BRCA1(-) cancer model in vitro.

The BR5 ovarian cancer cell lines are currently the only available BRCA-deficient murine ovarian cancer model which can be used in immunocompetent mice (19). BR5-Akt overexpress Akt, (hereafter referred to as BRCA1(-) cells), and intraperitoneal inoculation of this cell line results in the rapid accumulation of peritoneal tumors with high grade serous histology and abdominal ascites, mimicking the patterns observed in patients. We confirmed the presence of the inactivating insertion in BRCA1 in these cells with PCR (not shown), and the “BRCAness” phenotype by selective sensitivity to PARP-inhibition in vitro compared with syngeneic cell lines with wild-type BRCA function (Fig. 1A). In vivo cytotoxicity was also demonstrated in response to PARP-inhibition in this model, with a reduction in tumor burden observed in treated mice as early as Day 7 after intraperitoneal inoculation with BRCA1- tumor cells (Fig. 1B; n=5 mice per group treated with 40mg/kg/day of the PARP-inhibitor beginning on Day 3 after tumor challenge).

We next evaluated the cytotoxic efficacy of increasing concentrations of IFN-γ or TNF-α alone or combined with the PARP-inhibitor in both the BRCA1(-) and BRCAwt cell lines. When tumor cells were exposed to IFN-γ or TNF-α at concentrations approximating physiologic conditions, a minimal cytotoxic effect was observed in both models. However when IFN-γ or TNF-α was combined with the PARP-inhibitor, a significantly higher rate of cell death was induced when compared with PARP-inhibition alone. This effect was
not seen in BRCA wt tumor cells, in keeping with the selective sensitivity of BRCA1(-) cells to PARP-inhibition (Fig. 1C, 1D, 1E, 1F). Cell death in BRCA1(-) cells exposed to PARP-inhibition and TNF-α or IFN-γ was also associated with increases in activated Caspase-3, indicating that treatment induced tumor cell apoptosis (Fig. 1G, 1H), while no change in Caspase-3 was noted in BRCA wt cells (data not shown). However, as the entire population of dead/dying cells did not express active Caspase-3, the possible contribution of other cell death pathways could not be excluded. These results demonstrate an interaction between TNF-α, IFN-γ, and the PARP-inhibitor in the BRCA1(-) model, and indicate that increases in effector cytokines in the tumor environment may enhance tumor cell death following targeted therapy.

**Checkpoint blockade with CTLA-4 antibody combined with PARP-inhibition enhances T cell effector function in the peritoneal tumor environment**

In addition to increasing the frequency of T cells producing IFN-γ, CTLA-4 antibody treatment has been shown to enhance the proliferative response of effector T cells (29,33). To evaluate whether checkpoint blockade modified the phenotype or functional status of T cells in the BRCA1(-) tumor environment *in vivo*, we subjected peritoneal T cells from treated mice to flow cytometric analysis. In these experiments, BRCA1(-) tumor-bearing animals were treated with antibodies to CTLA-4 or PD-1 as monotherapy or combined with PARP inhibition (20mg/kg/day). CTLA-4 or PD-1 antibody was administered on Day 4 after tumor challenge. In addition, a second dose of PD-1 was administered on Day 11 because PD-1 signaling is thought to impact peripheral T cell exhaustion, a late event in the establishment of anti-tumor immunity. Results from these experiments demonstrated a marked increase in the proportion of CD8+ cells with an effector/memory phenotype in mice receiving CTLA-4 antibody together with the PARP-inhibitor (Fig. 2A). This effect was specifically observed among T cells in the peritoneal tumor environment, and not the spleen (Fig. 2B). In contrast, despite evidence that ovarian tumors express PD-L1 (34,35), and that tumor-infiltrating lymphocytes upregulate PD-1, blockade of the PD-1/ PD-L1 pathway did not significantly increase the proportion of effector/memory cells in this model (Fig 2A, 2B).
To determine whether changes in T cell phenotype following checkpoint blockade in combination with PARP-inhibition were associated with increases in local levels of IFN-γ or TNF-α, we evaluated peritoneal T cells retrieved on Days 7, 14, and 21 for intracellular cytokine production by flow cytometry. Cells were restimulated ex vivo for five hours with PMA and ionomycin in the presence of Golgi transport inhibitors, and then CD4+ and CD8+ T cells were analyzed for IFN-γ, and TNF-α cytokine production. In these experiments, neither monotherapy with CTLA-4 or PD-1 blockade alone nor PD-1 blockade together with PARP-inhibition significantly increased cytokine production by CD4+ or CD8+ T cells in peritoneal samples (Fig. 2C). In contrast, combination therapy with the PARP-inhibitor and CTLA-4 antibody did significantly boost both IFN-γ and TNF-α production in CD4+ and CD8+ T cells in the peritoneal tumor environment on Day 21. The percentage of polyfunctional peritoneal CD8+ T cells producing both IFN-γ and TNF-α was also significantly increased in mice receiving CTLA-4 antibody and PARP-inhibitor combined therapy. Like the increase in effector/memory cells, these changes in cytokine production were evident in the peritoneal tumor environment, but similar effects were not seen among splenic T cells (Fig. 2D). From these data, we conclude that CTLA-4 blockade, but not inhibition of PD-1 signaling, induces a Th1 effector phenotype among T cells in the peritoneal tumor environment when combined with PARP-inhibition in a BRCA1(-) ovarian cancer model.

*Increases in IFN-γ production in response to combined CTLA-4 blockade and PARP-inhibition in vivo are sufficient to enhance tumor cell cytotoxicity*

Our results demonstrate that combined therapy with the PARP-inhibitor and CTLA-4 antibody enhances IFN-γ and TNF-α production by peritoneal T cells in response to T cell receptor (TCR)-independent stimulation with PMA and ionomycin. In order to quantify production of these cytokines specifically in response to TCR engagement by peritoneal T cells, we cultured Day 21 peritoneal cells with anti-CD3 antibody for 18 hours in culture. In this manner, only T cells were stimulated to express effector cytokines, allowing direct quantification of IFN-γ and TNF-α (35). The results of this experiment demonstrated that both IFN-γ and TNF-
α levels were highest following anti-CD3 treatment of peritoneal cells from mice receiving combined treatment with the PARP-inhibitor and CTLA-4 antibody, with a greater than 20-fold increase in IFN-γ and an approximately 5-fold increase in TNF-α compared with controls (Fig. 3A, 3B).

To determine whether high cytokine levels in the peritoneal tumor environment could induce a cytotoxic effect similar to that seen using recombinant cytokines in vitro (Fig. 1), tumor cells were cultured with cell-free supernatant retrieved from the T cell stimulation experiments described above in the presence of increasing concentrations of the PARP-inhibitor. As expected, the supernatant from restimulated T cells harvested from BRCA1(-) tumor-bearing mice receiving combination therapy had the greatest effect on BRCA1(-) tumor cell death when combined with the PARP-inhibitor in vitro (Fig. 3C, 3D, black lines). Supporting a role for IFN-γ and TNF-α in this effect, cell death in response to supernatant from T cells retrieved from mice receiving combination therapy was attenuated when neutralizing antibodies to IFN-γ or TNF-α were added to cell cultures (Fig. 3C, 3D, red lines). As seen with recombinant cytokines in vitro, this effect was restricted to BRCA1(-) tumor cells; BRCAwt cells cultured under the same conditions showed no evidence of cytotoxicity (Fig. 3E). Together these data indicate that combination therapy with the PARP-inhibitor and CLTA-4 antibody promotes a strong Th1 T cell response in the peritoneal tumor environment which results in local increases in effector cytokines that are sufficient to enhance BRCA1(-) tumor clearance in vivo.

**Combined treatment with a PARP-inhibitor and CTLA-4 antibody promotes IFN–γ-mediated tumor rejection in a BRCA1(-) tumor model**

With evidence that IFN-γ enhances the cytotoxic effect of PARP-inhibition in vitro, and that PARP-inhibition combined with CTLA-4 blockade increased IFN-γ production and an effector phenotype among peritoneal T cells in vivo, we evaluated the impact of this regimen on survival in the BRCA1(-) tumor model. To do this, animals inoculated with BRCA1(-) cancer cells were treated with checkpoint blockade monotherapy, or combined treatment with the PARP-inhibitor (40mg/kg/day), and survival was measured from the day of tumor inoculation on October 16, 2017. © 2015 American Association for Cancer Research.
challenge until animals reached a weight of 30g due to ascites accumulation (Supplemental Fig. 1). Results from these experiments demonstrated limited benefit from CTLA-4 monotherapy, in keeping with prior reports (4,5), however treatment with the CTLA-4 antibody together with PARP-inhibition resulted in a synergistic therapeutic effect with long-term tumor-free survival in a majority of animals (Fig. 4A). As early as Day 21, all control mice and animals receiving monotherapy had bulky solid tumors in the abdominal cavity, however among mice receiving combination therapy, only one mouse had visible tumor and two additional mice had evidence of microscopic omental implants (Fig. 4B). When experimental animals receiving combined CTLA-4 blockade and the PARP-inhibitor were examined by necropsy on Day 90, no gross tumor was evident in surviving mice. A similar effect was observed when tumors were inoculated orthotopically under the ovarian bursa and solid tumor growth was examined on Day 21 (Supplemental Fig 2). In contrast with CTLA-4, no survival benefit was observed when PARP- inhibition was combined with PD-1 or PD-L1 blockade (Fig. 4C). This is in agreement with prior data (36) and with our T cell functional analyses indicating PD-1/PD-L1 blockade in combination with PARP inhibition had limited effects on T cell activation and cytokine induction (Fig. 2). Animals inoculated with BRCAwt tumors showed no significant survival benefit was observed following treatment with the PARP-inhibitor alone or in combination with CTLA-4 antibody, which was associated with a lack any effect on peritoneal T cell populations in these mice (Supplemental Fig. 3).

With evidence that combined therapy with the PARP-inhibitor and CTLA-4 mAb enhances IFN-γ and TNF-α production by local T cells in BRCA1(-) tumor-bearing mice, we next tested whether the therapeutic efficacy of PARP inhibition together with CTLA-4 blockade was dependent on elevations in effector cytokines in vivo. To do this, mice were treated with combination therapy using the PARP-inhibitor (20mg/kg/day) and CTLA-4 antibody together with neutralizing antibodies to IFN-γ, TNF-α, or both, and monitored for survival. This experiment demonstrated that neutralization of TNF-α had a minimal effect on outcomes, however IFN-γ was required for the survival effect of combined treatment in vivo (Fig. 4D). Thus, CTLA-4 checkpoint blockade
and targeted cytotoxicity with PARP-inhibition promoted immune-mediated rejection of high grade BRCA1(-) tumors, resulting in long-term tumor free survival.

**The survival benefit of combined treatment with the PARP-inhibitor and CTLA-4 antibody is due to lasting effects on peritoneal T cells**

One of the most remarkable effects of checkpoint blockade has been the lasting therapeutic effects exhibited among patients who respond to treatment (4). Using sarcoma tumor models, Gaubin et al have demonstrated that CTLA-4 blockade can induce protective immunologic memory (37). With evidence that tumor clearance following combined CTLA-4 blockade and PARP-inhibition is dependent on local changes in T cell phenotype and cytokine production, and that it can produce a lasting treatment benefit, we evaluated peritoneal T cells from long-term survivors for evidence of a memory response. To do this, peritoneal and splenic T cells were harvested from mice surviving past Day 90 after treatment with CTLA-4 antibody and the PARP-inhibitor and analyzed for cytokine production. Following *ex vivo* stimulation with PMA and ionomycin, very high levels of IFN-γ production were observed in both peritoneal and splenic CD4+ and CD8+ T cells from combination treated mice (Fig. 5A, 5B). Thus, although initial changes in T cell phenotype and functional status were specifically seen in the peritoneal tumor environment, treated animals did develop evidence of a systemic memory response, which was associated with long-term survival following combined exposure to the CTLA-4 antibody and the PARP-inhibitor.

To confirm that the extended therapeutic benefit of combined treatment with the PARP-inhibitor and CTLA-4 antibody was T cell-mediated, adoptive transfer experiments were performed. To do this, 2x10^5 CD8+ splenocytes retrieved from mice surviving greater than 90 days following combined treatment were transferred to recipient animals, followed by intraperitoneal tumor challenge after 12 hours. In this experiment, a majority of mice receiving CD8+ cells pooled from three long-term survivors were themselves protected from intraperitoneal tumor challenge: all experimental animals survived longer than the control group, and 3/5 had no evidence of tumor at Day 90 (Fig. 5C). We interpret these results as confirmation that combined therapy using...
CTLA-4 blockade and PARP-inhibition induced a population of effector T cells which increased local levels of IFN-γ and promoted the establishment of protective immunologic memory.

**IFN-γ enhances cytotoxicity in human BRCA1(-) tumor cells exposed to a PARP-inhibitor in vitro**

To test the translational potential of this treatment protocol, we examined the effect of PARP-inhibition with increasing doses of recombinant IFN-γ or TNF-α on human ovarian cancer cells *in vitro*. For these studies we used the BRCA1(-) UWB1.289 cell line and UWB1.289 transfected to express wild-type BRCA1 (BRCA1*wt*), as previously described (21). The results demonstrated that IFN-γ, but not TNF-α, increased cytotoxicity in BRCA1(-) cells treated with the PARP-inhibitor *in vitro* (Fig. 6A, 6B). As in the murine experiments, this effect was not observed in BRCA1*wt* cells (Fig. 6C, 6D). These data suggest that PARP-inhibition in combination with CTLA-4 blockade may have similar therapeutic benefit in patients with BRCA1(-) ovarian cancers and support further study of this combination.
Discussion

Here we demonstrate that CTLA-4 antibody combined with targeted therapy using a PARP-inhibitor promotes long-term survival in a BRCA1(-) ovarian cancer model, and that this effect is mediated by local increases in IFN-γ production by T cells in the tumor environment. Combination therapy rapidly increases T cell recruitment, activation, and cytokine production in the peritoneal cavity, and this is followed by the induction of lasting systemic effector/memory T cell immunity. Our finding of a similar trend in human BRCA1(-) cells treated with the PARP-inhibitor in the presence of elevated levels of IFN-γ, and evidence that ascites T cells from women with ovarian cancer express CTLA-4, support the translational potential of this treatment strategy.

An important finding from these experiments is the effect of combined treatment on the peritoneal tumor environment. Prior studies using checkpoint inhibition antibodies have demonstrated a discrepancy between receptor occupancy among peripheral T cells and tumor-infiltrating T cells (36). As checkpoint inhibition is a locally mediated effect, any therapeutic benefit of checkpoint blockade requires engagement of lymphocytes in the tumor microenvironment (4). Our results demonstrate that effector/memory T cells were specifically increased in the peritoneal cavity in response to combination therapy, indicating that this regimen can effectively modulate T cells in the ovarian tumor environment. Because the ovarian tumor environment is considered tolerogenic (3), these changes indicate a significant reversal in the local immune conditions that is associated with long-term survival benefit. Furthermore, we expect that the localized increase in IFN-γ following combined treatment may prove clinically advantageous by limiting systemic toxicity (38).

It is notable that despite expression of PD-1 on ascites T cells and PD-L1 on ovarian tumor cells, inhibition of this pathway had no significant impact on survival in the BRCA1(-) model. This finding was unexpected in light of a recent report describing PD-1 pathway blockade as a strategy to enhance the efficacy of tumor vaccines in an ovarian cancer model (33). Despite differences in the dose and schedule of PD-1 antibody administration in this study, the effect of PD-1 blockade on T cell function was similar to our results, with
approximately 2% of CD8+ T cells from treated animals producing IFN-γ, which in both cases was not significantly different than controls. We interpret the selective efficacy of CTLA-4 blockade we observed as evidence that activation of new lymphocyte clones, rather than reversal of T cell exhaustion, was responsible for immune-mediated tumor clearance and long-term survival in the BRCA1(-) model (1,36,39).

Strengths of this study include our use of an immunocompetent model which reliably develops high grade serous tumors that replace the omentum and implant on serosal surfaces of the bowel and peritoneal cavity, mimicking the most common pattern found in patients. The fact that parallel results were demonstrated with BRCA1(-) human tumor cells supports further testing of this combination for the treatment of clinical disease. In addition, the long-term survival we observed has not been demonstrated previously in other studies using this model (40). Although our study is focused on ovarian cancer, we expect that our results can be extended to other BRCA(-) cancers such as breast, prostate, or pancreatic tumors that are vulnerable to the targeted effect of PARP-inhibition. We are also currently investigating strategies to extend similar benefits to patients with sporadic ovarian cancer, using metronomic chemotherapy to sensitize tumors to checkpoint inhibition (41).

Finally, these results document a novel mechanism for BRCA1(-) tumor cell death driven by an interaction between the PARP-inhibitor and IFN-γ in vitro. As a highly conserved, ubiquitous molecule required for post-translational protein modification, PARP is involved in many cellular processes in addition to DNA repair, including roles in apoptotic cell death and metabolic pathways (42-45). The observation that only a portion of tumor cell death in vitro was due to apoptosis, as shown by Caspase-3 cleavage, indicates that additional cytotoxic pathways are engaged in response to IFN-γ and the PARP-inhibitor. Accumulating reports describe a role for PARP-inhibitors in the prevention of catalytic function and PARP trapping, and it is postulated that different forms of cell death may be induced by specific mechanisms of PARP-inhibition (46-48). Therefore, it will be important to evaluate specific PARP-inhibitors in combination with immune checkpoint blockade to better parse the mechanisms of cell death and to optimize treatment protocols for patients.
Based on these data, we propose a two-phase model to describe distinct roles for the PARP-inhibitor in promoting tumor regression when combined with CTLA-4 blockade. In Phase 1, PARP-inhibition directly induces tumor cell damage, which primes and diversifies an anti-tumor T cell response, a process which is amplified by CTLA-4 blockade (49). In Phase 2, local T cells activated in the presence of CTLA-4 blockade produce increased levels of IFN-γ above a threshold required to enhance the cytotoxic efficacy of PARP-inhibition, resulting in additional therapeutic benefit through cell-intrinsic pathways. This model indicates that the therapeutic benefit of PARP-inhibition can be significantly amplified by inclusion in immunotherapeutic protocols.

In summary, these data show that long-term survival can be achieved in a BRCA1(-) ovarian tumor model using a PARP-inhibitor combined with CTLA-4 checkpoint blockade. The fact that both PARP-inhibitors and CTLA-4 antibodies have been well-tolerated as monotherapy in women with ovarian cancer, together with our in vitro data using human BRCA1(-) cancer cells, support the rapid translation of this treatment protocol for clinical testing. In addition, our results add to prior work suggesting that BRCA mutation status can be used to identify candidates for immunotherapeutic protocols, improving our ability to identify a treatment effect for select patients (50). Finally, we anticipate that further evaluation of PARP-inhibitors in combination with specific immune checkpoint pathways will uncover optimal pairings that will expand the clinical utility of this strategy for the treatment of patients with other BRCA(-) cancers, or with tumors deficient in alternate DNA-repair pathways.
References


Figure legends

**Figure 1.** Th1-type cytokines enhance the cytotoxic effect of PARP-inhibition in BRCA1- ovarian cancer cells. 

**(A)** BRCA1(-) (BR5-Akt) and BRCAwt (T22, ID8) cell lines were cultured in 24 well plates at a starting concentration of $1 \times 10^4$ cells/well with or without the PARP-inhibitor (PARPi) at the indicated dose, and analyzed at 72 hours for cell viability by flow cytometry. **(B)** Peritoneal cells were harvested by PBS wash on Day 7 from BRCA1(-) tumor bearing mice treated with the PARPi (40 mg/kg/day; days 3-6) and analyzed by flow cytometry for the percent of CD45(-) tumor cells (left). In addition, total ascites cells were plated at serial dilutions for 72 hr and the number of tumor colonies among total ascites cells was counted as an estimate of viable tumor burden (right). (Left p=0.0271, Right p=0.0032 student’s t-test). **(C and D)** BRCA1(-) cells were cultured in 24 well plates for 72 hours in the presence of 0, 0.5, 1, or 2 ug/ml of PARPi and either IFN-$\gamma$ (C, Upper panel, PARPi dose effect p<0.0001; IFN-g dose effect p<0.0001; interaction p<0.0001) or TNF-$\alpha$ (D, Upper panel, PARPi dose effect p<0.0001; TNF-a dose effect p<0.0001, interaction p <0.0001) at the concentrations indicated. Cells were then stained with a fixable cell viability dye and analyzed by flow cytometry for the percentage of dead cells. Upper dose response curves demonstrate the interaction dose effect between cytokine concentration and PARP-inhibitor treatment. Lower bar graphs illustrate the dose effect independent of interaction. **(E and F)** BRCAwt (T22) cells were assayed for viability as in C and D and show no treatment effect with increasing concentrations of cytokines or the PARP-inhibitor. **(G and H)** The fixable viability dye allowed for analysis of intracellular staining for active Caspase-3. BRCA(-) cells treated with PARPi and IFN-$\gamma$ (G) or TNF-$\alpha$ (H) were intracellularly stained and cells positive for active Caspase-3 are presented as a percentage of total cells (PARPi dose effect p<0.0001; IFN-g dose effect p<0.0001; interaction p<0.0001). Assays were repeated a minimum of three times. ****p<0.0001; ***p<0.005; **p<0.025; *p<0.05 by ANOVA and Tukey’s procedure for multiple comparisons.
Figure 2. Checkpoint blockade with CTLA-4 antibody combined with PARP-inhibition enhances T cell effector function in the peritoneal tumor environment. Using the protocol depicted in Supplemental Fig. 1, BRCA1(-) tumor bearing mice were sacrificed on day 21 for analysis (n=5/group). (A) peritoneal and (B) splenic CD4+ and CD8+ T cells were analyzed for CD44 and CD62L expression and percentage of CD62Llow/CD44hi cells was gated to determine the percentage of effector/memory CD4+ and CD8+ T cells of total CD4+ or CD8+ T cells, respectively. (C) peritoneal cells and (D) splenocytes were restimulated in 24 well plates with PMA and inonomycin for 5 hours in the presence of Golgi transport inhibitors. CD4+ and CD8+ T cells were then analyzed by flow cytometry for intracellular cytokine expression. The percentage of T cells from treated animals producing IFN-γ (top panel), TNF-α (middle panel) or both (lower panel) were compared with untreated controls. Data are representative of two experiments**p<0.025; *p<0.05 by ANOVA and Tukey’s procedure for multiple comparisons.

Figure 3. Increases in IFN-γ production in response to combined CTLA-4 blockade and PARP-inhibition in vivo are sufficient to enhance tumor cell cytotoxicity. Mice were treated as in Supplemental Fig. 1 and euthanized on Day 21 followed by retrieval of peritoneal cells. (n=5/group). 5x10^6 peritoneal cells were restimulated ex vivo with 10 ug/ml anti-CD3 for 18 hours. Cell-free supernatants were harvested, pooled by treatment group, and levels of (A) IFN-γ and (B) TNF-α were determined by ELISA. (C-E) Supernatants were added to BRCA(-) cells at a 4x dilution with DMEM-C in the presence of PARPi at indicated concentrations and cultured 72 hrs in the absence (black lines) or presence (red lines) of IFN-γ and TNF-α neutralizing mAbs (10 ug/ml). (C) Cells were analyzed for viability by flow cytometry and values shown are the percentage of dead cells with or without neutralizing antibody treatment. (D) Overlay of data in (C). (E) The same as (D) using BRCAwt cells (T22). ****p<0.0001; ***p<0.005; **p<0.025; *p<0.05 by ANOVA and Tukey’s procedure for multiple comparisons.
**Figure 4.** Combined treatment with a PARP-inhibitor and CTLA-4 antibody promotes IFN-γ mediated tumor rejection in a BRCA1(-) tumor model. (A) Mice were treated as depicted in Supplemental Fig. 1 (40 mg/kg/day PARPi) and survival was compared with untreated controls. (n = 10/group). (B) On Day 21, the peritoneal cavity was exposed for evaluation of macroscopic solid tumor burden, and histologic sections of omentum were examined for microscopic tumor implants. Representative samples are shown. (C) Combination therapy with PARP-inhibition (40mg/kg/day) and antibody blockade of CTLA-4, PD-1 or PD-L1 as in Supplemental Fig. 1 (n=5 to 10 per group). (D) Survival of mice treated with PARPi (20 mg/kg/day) and CTLA-4 mAb combination therapy and neutralizing antibodies to TNF-α, IFN-γ or both every 4 days beginning on Day 7. (n = 5-15/group); Survival comparisons are Kaplan-Meier curves. Differences among groups were determined with the Log Rank (Mantel-Cox) test, *** p<0.005; *p<0.05. Each experiment was repeated at least twice, with representative data shown.

**Figure 5.** Combined treatment with the PARP-inhibitor and CTLA-4 antibody induces protective immunity. Peritoneal cells (A) and splenocytes (B) were retrieved on Days 7, 14, and 21 from combination therapy treated mice, and from long term survivors on Day 90, and restimulated ex vivo with PMA and ionomycin for analysis by flow cytometry for intracellular IFN-γ production by CD4+ and CD8+ T cells. ** p<0.025; *p<0.05, Tukey’s multiple comparisons test. (C) Adoptive transfer of CD8+ T cells from long-term survivors protects recipients from tumor development. CD8+ T cells were isolated by MACS negative selection from long-term combination therapy survivors, pooled, and 2x10⁵ cells were adoptively transferred to recipient mice 12 hours prior to challenge with 2x10⁵ BRCA(-) tumor cells. Mice were monitored for survival. (n = 5/group) log-rank (Mantel-Cox) test.
**Figure 6.** IFN-γ enhances cytotoxicity in human BRCA1(-) tumor cells exposed to a PARP-inhibitor in vitro. BRCA1(-) UWB1.289 cells or UWB1.289 cells transfected with competent BRCA1 were cultured with titrated doses of the PARP-inhibitor in the presence of recombinant human IFN-γ or TNF-α as indicated. After 72 hours cells were analyzed for viability by flow cytometry. (A) BRCA1(-) UWB1.289 cells exposed to IFN-γ and PARP-inhibition (Upper panel, PARPi dose effect p<0.0001; IFN-γ dose effect p<0.0001; interaction p=0.3872 based on 2-way ANOVA; Lower panel ** p<0.025; *p<0.05, Tukey’s multiple comparisons test). (B) BRCA1(-) UWB1.289 cells exposed to TNF-α and PARP-inhibition (Upper panel, TNF-α dose effect p=0.0499; interaction p=0.9655 by 2-way ANOVA; Lower panel, *p<0.05, Tukey’s multiple comparisons test). (C and D) BRCA1-transfected UWB1.289 cells exposed to IFN-γ or TNF-α and PARP-inhibition (no statistically significant dose effect for IFN-γ or TNF-α).
Figure 2.

A  Peritoneal effector/memory T cells

B  Splenic effector/memory T cells

C  Peritoneal T cells

D  Splenic T cells

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CD4

CD8

CD4

CD8

PD-1 mAb

PARPi

CTLA-4 mAb

PARPi

PD-1 mAb

CTLA-4 mAb

PARPi

PD-1 mAb

CTLA-4 mAb
Figure 3.

A  IFN-γ

B  TNF-α

C  control

PARPi

CTLA-4 mAb

PARPi + CTLA-4 mAb

D  BRCA(-) cells

E  BRCA(+) cells

% dead cells

PARPi (ug/ml)

% dead cells

PARPi (ug/ml)

Control

PARPi

CTLA-4 mAb

PARPi + CTLA-4 mAb

Control + TNF/IFN mAbs

PARPi + TNF/IFN mAbs

CTLA-4 mAb + TNF/IFN mAbs

PARPi + CTLA-4 mAb + TNF/IFN mAbs
Figure 4.

A  CTLA-4 blockade and PARP-inhibition

B  Day21

C  Checkpoint blockade + PARP-inhibitor combination therapy

D  Combination therapy with cytokine neutralization
Figure 5.

A  Peritoneal T cells

B  Splenic T cells

C  Adoptive transfer of CD8+ T cells from long term survivors

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Adoptive transfer of CD8+ T cells from long term survivors
Figure 6.

A  Human BRCA-deficient

B  Human BRCA-deficient

C  Human BRCA-sufficient

D  Human BRCA-sufficient

% dead cells

IFN-γ (ng/ml) 0 1.25 2.5 5 10 20

TNF-α (ng/ml) 0 1.25 2.5 5 10 20

% dead cells

IFN-γ (ng/ml) 7/13 - 16/14: UWB1.289 wt cells: PARPi + IFN-γ synergy: % ZombieNIR+

PARP-i (20)
PARP-i (10)
PARP-i (5)
PARP-i (0)

TNF-α (ng/ml) 7/13 - 16/14: UWB1.289 wt cells: PARPi + TNF-α synergy: % ZombieNIR+

PARP-i (20)
PARP-i (10)
PARP-i (5)
PARP-i (0)

% dead cells

IFN-γ (ng/ml) 7/13 - 16/14: UWB1.289+BRCA1 cells: PARPi + IFN-γ synergy: % ZombieNIR+

PARP-i (20)
PARP-i (10)
PARP-i (5)
PARP-i (0)

TNF-α (ng/ml) 7/13 - 16/14: UWB1.289+BRCA1 cells: PARPi + TNF-α synergy: % ZombieNIR+

PARP-i (20)
PARP-i (10)
PARP-i (5)
PARP-i (0)

Human BRCA-deficient

Human BRCA-sufficient

UWB1.289 (BRCA-deficient)

UWB1.289 (BRCA-sufficient)
CTLA-4 blockade synergizes therapeutically with PARP-inhibition in BRCA1-deficient ovarian cancer

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