Supplementary Materials for

Combined immune checkpoint blockade as a therapeutic strategy for BRCA1-mutated breast cancer


*Corresponding author. Email: sherene.loi@petermac.org (S.L.); lindeman@wehi.edu.au (G.J.L.)

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This PDF file includes:

Fig. S1. Characterization of TILs in BRCA1- and BRCA2-mutant tumors by OPAL imaging.
Fig. S2. Output from deconstructSigs for a representative MMTV-cre/Brca1^{0/f}\p53^{+-} tumor.
Fig. S3. Effect of combination therapy with checkpoint inhibitors on mice body weight, blood count, and biochemistry.
Fig. S4. Cisplatin treatment and induction of immunogenicity markers on human TNBC BRCA1-mutated HCC1937 tumors in vitro.
Fig. S5. Characterization of lymphocyte subsets in response to immune checkpoint inhibitor therapy.
Fig. S6. Effect of combination therapy with checkpoint inhibitors on the growth of Brca1-deficient tumors in RAG1 mice.
Fig. S7. Dual checkpoint inhibitor blockade and activation of dendritic cells and polyfunctional effector T cells.
Fig. S8. Characterization of lymphocyte subsets in response to immune checkpoint inhibitor therapy.
**Fig. S1. Characterization of TILs in BRCA1- and BRCA2-mutant tumors by OPAL imaging.** Expression of CD3, CD4, CD8 and FOXP3 in TILs in the stromal and intratumoral regions (CK18+) was evaluated in BRCA1 TNBCs using OPAL serial immunostaining. Shown are two representative BRCA1-mutated TNBCs: (A) and (B). The majority of TILs are CD3+ CD4+ or CD8+ T cells, with a small number of FOXP3+ cells. The OPAL staining panel includes: tumor marker CK18 (yellow), CD3 (red), CD4 (white), CD8 (green), FOXP3 (orange), with nuclear counterstaining with DAPI (blue). Scale bar, 100 µm. (C) Representative FACS profile of a tumor from a BRCA1-mutated TNBC, showing CD3+ TILs and the expression of PD-1 on CD4+ and CD8+ T cell subsets. (D) Analysis of matched BRCA2-mutated TNBC patient stromal TIL populations for H&E, OPAL staining, stromal and intratumoral PD-L1 expression (n = 7). (E) A BRCA2-mutated TNBC. Inset shows an area of high intratumoral PD-L1 expression. The OPAL staining panel includes: tumor marker CK18 (yellow), CD3 (red), CD4 (white), CD8 (green), FOXP3 (orange), PD-L1 (cyan), DAPI (blue). Iso, Isotype-matched control antibody. Scale bars, 100 µm (left panel), 20 µm (right panel).
Fig. S2. Output from deconstructSigs for a representative MMTV-cre/Brca1<sup>fl/flp53<sup>−/−</sup> tumor. (A) The relative proportions of different mutational contexts for missense mutations. (B) Reconstruction of (A) using known mutational signatures detected in this sample. Signature 3 is known to be associated with BRCA1 deficiency. Signature 1 is age-related. Signature 21 is of unknown etiology. (C) Mutational Signature 3 for illustrative purposes (reproduced from http://cancer.sanger.ac.uk/cosmic/signatures).
Fig. S3. Effect of combination therapy with checkpoint inhibitors on mice body weight, blood count, and biochemistry. (A) Body weights of mice were determined three times a week at the same time as tumor measurements (Fig. 4A). (B) Terminal bleeds were taken on day 10, and serum creatinine, urea, activated alanine aminotransferase (ALT) and alkaline phosphatase (ALP), and blood counts were measured. Hb, hemoglobin; WBC, white blood count; UT, untreated; Cis, cisplatin.
Fig S4. Cisplatin treatment and induction of immunogenicity markers on human TNBC BRCA1-mutated HCC1937 tumors in vitro. FACS analysis of HLA-ABC (MHC-I), HLA-DR (MHC-II), PD-L1, CD80, CD86, calreticulin, and MICA/B expression (mean fluorescence index; MFI) on the HCC1937 cell line after 72 hours of treatment with vehicle, cisplatin (2 μM) alone, IFNγ (5 ng/mL) alone or the combination of cisplatin treatment and IFNγ stimulation. Experiment was performed in triplicate, and data are presented as mean ± s.e.m. P values represent one-way ANOVA and post-hoc Tukey’s tests. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Fig. S5. Characterization of lymphocyte subsets in response to immune checkpoint inhibitor therapy. (A) Quantification of the proportion of tumor-infiltrating T cells that are CD8+ (left panel) or FOXP3+ (right panel) from mice receiving the indicated treatments. *p < 0.05, **p < 0.001, ***p < 0.0001, ****p < 0.00001. (B) Representative images showing immunostaining for CD8 within tumors from mice receiving the indicated treatment (n = 3 tumors per group). Scale bars, 100 μm. (C) Stromal TILs at the experimental endpoints across three treatment arms. UT, untreated; Cis, cisplatin.
Fig. S6. Effect of combination therapy with checkpoint inhibitors on the growth of Brca1-deficient tumors in RAG1 mice. MMTV-cre/Brca1<sup>−/−</sup>/p53<sup>−/+</sup> tumor cells were injected into the mammary fat pads of Rag1/J mice. Three weeks after transplantation, mice were randomized to one of five treatment arms: (1) vehicle (PBS), (2) cisplatin, (3) cisplatin and anti-PD-1, (4) cisplatin and anti-CTLA4, or (5) cisplatin, anti-CTLA4, and anti-PD-1. Mice received cisplatin treatment on day 1 and anti-CTLA4 and anti-PD-1 treatment on day 2, 5, and 8. (A) Tumor growth curve and (B) Kaplan-Meier survival curve. In (A), the arrow depicts cisplatin treatment on day 1. Data represent mean ± s.e.m. (n = 11 mice per treatment arm).
Fig. S7. Dual checkpoint inhibitor blockade and activation of dendritic cells and polyfunctional effector T cells. (A) Plots of MHC II vs. CD11c gated on CD45^+ TCRβ^- tumor-infiltrating cells 10 days after treatment with cisplatin alone or cisplatin and combination anti-PD-1/anti-CTLA-4 blockade. Regions show the proportion of CD11c^+/MHC II^+ dendritic cells. (B) Quantification of the proportion of tumor-infiltrating dendritic cells. (C) Histograms of CD80 expression gated on CD11c^+/MHC II^+ tumor-infiltrating dendritic cells 10 days after treatment with cisplatin alone or cisplatin and combination anti-PD-1/anti-CTLA-4 blockade (solid lines) versus untreated mice (gray shaded). Percentage CD80^+ and the mean fluorescence intensity (MFI) are shown. (D) Dot plots of IFNγ vs. TNF expression gated on tumor-infiltrating CD45^+CD4^+TCRβ^+ cells from mice in the indicated groups. (E) Quantification of the proportion of CD4^+ T cells expressing IFNγ, TNF, or IFNγ plus TNF. Plots are representative of 3–4 mice per experiment. Mean ± s.e.m. are shown with one-way ANOVA and Tukey’s post-test.*p < 0.05, **p < 0.001, ***p < 0.0005, ****p < 0.0001. UT, untreated; Cis, cisplatin.
Fig. S8. Characterization of lymphocyte subsets in response to immune checkpoint inhibitor therapy. 
(A) Representative image of a mouse injected with Evans Blue dye at the tumor site, showing lymphatic drainage to the axillary node. (B) Expression of PD-1 versus CD8 on TCRβ+ T cells from the draining lymph node, with proportions shown for PD-1− and PD-1+ CD4+ T cells. (C) Quantification of PD-1+ CD4+ T cells in draining lymph nodes of mice receiving the treatments indicated. (D) Expression of PD-1 versus CD8 on splenic TCRβ+ T cells, with proportions shown for PD-1− and PD-1+ CD8+ T cells (top panels) and PD-1− CD4+ T cells (bottom panels). (E) Quantification of splenic PD-1+ CD8+ T cells and PD-1− CD4+ T cells in mice receiving the indicated treatments. (F) Quantification of CTLA-4− splenic CD4+, CD8+, or FOXP3−CD4+ T cells from the indicated groups. Flow cytometric analysis is representative of 2 experiments with n = 5 mice per group. Lymph nodes and spleens were harvested from mice 14 days after treatment with cisplatin alone or cisplatin and checkpoint inhibitor therapy. Mean ± s.e.m. are shown with one-way ANOVA and Tukey’s post-test. *p < 0.05, **p < 0.001, ***p < 0.0001. UT, untreated; Cis, cisplatin.