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Unique immune characteristics and differential anti-PD-1-mediated reinvigoration potential of CD8$^+$ TILs based on *BRCA1/2* mutation status in epithelial ovarian cancers

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ABSTRACT

Background We aimed to investigate the distinct immunological characteristics of the tumor immune microenvironment in epithelial ovarian cancer (EOC) according to *BRCA1/2* mutations status and differential PD-1 expression levels.

Methods Tumor-infiltrating lymphocytes (TILs) were collected from patients with newly diagnosed advanced-stage EOC (YUHS cohort, n=117). This YUHS cohort was compared with The Cancer Genome Atlas (TCGA) data for ovarian serous cystadenocarcinoma (n=482), in terms of survival outcomes and immune-related gene profiles according to *BRCA1/2* status. We used multicolor flow cytometry to characterize the immune phenotypes and heterogeneity of TILs with or without *BRCA1/2* mutations. *In vitro* functional assays were conducted to evaluate the reinvigorating ability of CD8$^+$ TILs on anti-PD-1 treatment.

Results We found that EOC patients with *BRCA1/2* mutations (*BRCA1/2*mt) exhibited better survival outcomes and significantly higher tumor mutation burden (TMB), compared with *BRCA1/2* non-mutated (*BRCA1/2*wt) patients. Furthermore, CD8$^+$ TILs within *BRCA1/2*mt tumors displayed characteristics indicating more severe T-cell exhaustion than their *BRCA1/2*wt counterparts. Notably, the capacity for anti-PD-1-mediated reinvigoration of CD8$^+$ TILs was significantly greater in *BRCA1/2*wt tumors compared with *BRCA1/2*mt tumors. Additionally, within the *BRCA1/2*wt group, the frequency of PD-1^{high}CD8$^+$ TILs was positively correlated with the reinvigoration capacity of CD8$^+$ TILs after anti-PD-1 treatment.

Conclusion Our results highlight unique immune features of CD8$^+$ TILs in EOC and a differential response to anti-PD-1 treatment, contingent on *BRCA1/2* mutation status. These findings suggest that immune checkpoint blockade may be a promising frontline therapeutic option for selected *BRCA1/2*wt EOC patients.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The contribution of *BRCA1/2* mutations to the tumor immune microenvironment (TIME) in epithelial ovarian cancer (EOC) has not previously been well investigated. The rise of poly (adenosine diphosphate [ADP]-ribose) polymerase inhibitors has dramatically shifted the EOC treatment landscape, especially for patients with *BRCA1/2* mutations or homologous-recombination deficiency (HRD). However, at least 50% of EOC patients lack *BRCA1/2* mutations and HRD. A deeper understanding of the TIME relative to *BRCA1/2* mutation status is crucial to devise new treatment strategies to enhance survival outcomes for patients without *BRCA1/2* mutations.

WHAT THIS STUDY ADDS

⇒ Here, we highlighted the distinct immunological properties of CD8$^+$ tumor-infiltrating lymphocytes in terms of exhaustion status and ability to be reinvigorated by anti-PD-1, based on *BRCA1/2* mutation status. We found that among EOC patients without *BRCA1/2* mutations, "high PD-1^{high} expressers" could be promising candidates for PD-1 blockade therapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study provides a rationale supporting front-line immune checkpoint blockade therapy for selected patients with *BRCA1/2* non-mutated EOC.

INTRODUCTION

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy, causing the deaths of over 152,000 women worldwide each year.[1] Even with an aggressive standard of care, including cytoreductive surgery and platinum-based adjuvant chemotherapy, the majority of patients relapse within 2 years of diagnosis, with a poor 5-year overall survival (OS) rate of less than 40%.[2] Patients experience repeated recurrence and progressively worsening responses to subsequent therapies, eventually becoming refractory to all treatment modalities.[3–5] Thus, preventing recurrence is a primary challenge in ovarian

cancer treatment. In this regard, poly (adenosine diphosphate [ADP]-ribose) polymerase inhibitors (PARPis) have recently emerged as a transformative treatment for EOC, exhibiting significant clinical efficacy, particularly in EOC patients carrying breast cancer susceptibility gene (BRCA) mutations or with homologous recombination deficiency (HRD).[6 7]

Among cases of high-grade serous ovarian cancer (HGSC), approximately 20% harbor mutations in the BRCA1 or BRCA2 genes (*BRCA1/2*), either from germline or somatic sources or due to hypermethylation.[8] BRCA1/2 is related to the high-fidelity homologous recombination (HR) DNA repair pathway.[9] Therefore, ovarian cancers with these mutations (*BRCA1/2*mt) predominantly rely on less accurate DNA repair mechanisms, rendering them more susceptible to DNA-damaging agents, which contributing to better survival outcomes in these cases compared with those without these mutations.[10–12] The emergence of PARPis has further improved the progression-free survival (PFS) rates among EOC patients with *BRCA1/2* mutations.[7 13 14]

However, over 50% of EOC patients do not have *BRCA1/2* mutations or HRD.[15 16] Thus, there is an urgent need for innovative treatment strategies to enhance survival outcomes among patients without these mutations, primarily by preventing recurrence. In this context, combinational therapy with immune checkpoint blockades (ICBs)—such as anti-PD-1 and anti-PD-L1—has been tried in several studies. Notably, preliminary studies have suggested that compared with HR-proficient tumors, HRD tumors (or *BRCA1/2*mt) exhibit a unique tumor immune microenvironment (TIME), characterized by a high tumor mutation burden (TMB), significant neoantigenicity, profuse tumor-infiltrating lymphocytes (TILs), and elevated PD-L1 expression.[17–19] However, mixed clinical results have been obtained when using ICB monotherapies in patients with *BRCA1/2* mutations. The KEYNOTE-100 study investigated pembrolizumab monotherapy in patients with both BRCA and HRD statuses and found no discernible differences between responders and non-responders.[20] On the other hand, Matsuo *et al* reported a promising 67% objective response rate following nivolumab salvage therapy in a small group of *BRCA1/2*-mutated patients.[21] These findings highlight the need for more extensive studies to elucidate the nuances of the TIME with regard to *BRCA1/2* mutation status and to identify predictive biomarkers for the effectiveness of ICBs in EOC without *BRCA1/2* mutations.

In the present study, we postulated that the TIME in *BRCA1/2*mt EOC would exhibit different immunological properties compared with its non-mutated counterpart (*BRCA1/2*wt). We compared the expressions of immune-related genes using data on ovarian serous cystadenocarcinoma from The Cancer Genome Atlas (TCGA). Moreover, we examined TILs from patients with newly diagnosed advanced-stage EOC (n=117). We analyzed both the immune exhaustion phenotypes and the capacity of TILs to rejuvenate in response to anti-PD-1, with the aim of developing a method to predict responses to PD-1 blockade.

METHODS

TCGA data analysis

We obtained TCGA data of serous cystadenocarcinoma patients (n=482) from the cBioPortal (Memorial Sloan Kettering Cancer Center, https://www.cbioportal.org).[22] Data were downloaded regarding *BRCA1/2* status, survival, mRNA expression Z-scores, and TMB. Updated follow-up survival data were downloaded using the R package TCGAbiolinks. An unpaired t-test or Mann-Whitney test was performed to compare gene expression according to *BRCA1/2* status. The Kaplan-Meier method and log-rank test were used to compare PFS and OS. We analyzed the relationships of *BRCA1/2* mutations with TMB and neoantigen load using the maftools R package. T-cell exhaustion (TEX) signaling pathway signatures and hallmark gene sets were retrieved from the Molecular Signatures Database (MSigDB, V.7.2)[23] and analyzed as previously reported.[24]

Study patients and lymphocyte isolation

The study population included treatment-naïve patients with ovarian, fallopian tube, and primary peritoneal carcinoma (collectively termed EOC), who underwent surgical resection between April 2018 and March 2022 at Yonsei Cancer Center (YUHS cohort, n=117). Our present analysis included only patients with stages III–IV EOC and confirmed *BRCA1/2* status. The patients' demographic and clinical information is provided in table 1. None of the patients received chemotherapy or radiotherapy before surgery.

For translational research, fresh tumor tissues were collected on the day of resection. To prepare single-cell suspensions from tumor tissues, we performed mechanical and enzymatic dissociation using the Tumor Dissociation Kit (Miltenyi Biotec: 130-095-929) according to the manufacturer's instructions. Isolated single-cell suspensions from tumors were filtered through a 100 µm pore cell strainer and cryopreserved until further use. *BRCA1/2* mutations status was determined by genetic testing using genomic DNA from peripheral blood, or by NGS analysis of formalin-fixed paraffin-embedded tumor tissue. Patients with *BRCA1/2* mutations detected in genomic or somatic DNA were defined as the *BRCA1/2* mutant group, and the remaining patients were defined as the non-mutant group. Within the *BRCA1/2* mutant group, germline *BRCA1/2* mutant (gBRCA1/2) were identified as those who detected *BRCA1/2* mutations in genomic DNA from peripheral blood. Somatic *BRCA1/2* mutants (sBRCA1/2) were defined as patients who exclusively detected *BRCA1/2* mutations in tumor tissue (not in peripheral blood).

Flow cytometry and immunostaining

Cryopreserved TILs were thawed and then stained using the LIVE/DEAD Fixable Near IR Cell Stain Kit (Life

Table 1 Baseline and disease characteristics of the study population

	BRCA1/2 non-mutated (n=78)	*BRCA1/2* mutated (n=39)	Total (n=117)	P value
Age, years				0.055
Median (range)	59.5 (40–83)	56 (36–78)	58 (36–83)	
Stage, n (%)				0.690
III	31 (39.7)	17 (43.6)	48 (41.0)	
IV	47 (60.3)	22 (56.4)	69 (59.0)	
Histology type, n (%)				0.455
Serous	73 (93.6)	39 (100)	112 (95.7)	
Others*	5 (6.4)	0 (0.0)	5 (4.3)	
Histological grade, n (%)				
G1	0 (0.0)	7 (17.9)	7 (6.0)	<0.001
G2	1 (1.3)	3 (7.7)	4 (3.4)	
G3	71 (91.0)	29 (74.4)	100 (85.5)	
Unknown	6 (7.7)	0 (0.0)	6 (5.1)	

*Other histology included three endometrioid, one clear cell, and two carcinosarcoma.

Technologies, Carlsbad, California, USA). Next, the cells were washed once and stained with fluorochrome-conjugated antibodies in the dark at 4°C for 30 min. For Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Foxp3, T cell factor-1 (TCF-1), and thymocyte selection-associated HMG BOX (TOX) staining, the cells were fixed and permeabilized using the Foxp3 staining buffer kit (eBioscience, San Diego, California, USA) following the manufacturer's instructions. Multicolor flow cytometry was performed using a BD FACSLylic (BD Biosciences, San Jose, California, USA). Data were analyzed with FlowJo software V.10.8 (BD Life Sciences) (flowjo.com). Online supplemental table S1 presents the reagents used for flow cytometry.

In vitro T-cell proliferation assay

Cryopreserved TILs were thawed and then suspended in RPMI 1640 containing 10% fetal bovine serum and incubated for 8 hours at 37°C under 5% CO_2. TILs were labeled with CellTrace Violet (CTV; Thermo Fisher Scientific). We cultured 100,000 cells in 200 µL medium in each well of a 96-well round-bottom culture plate, with stimulation using soluble anti-CD3 antibody (10 ng/mL, OKT-3; eBioscience) in the presence of 5 µg/mL anti-PD-1 (EH12.2H7) or isotype control ($mIgG_1$, MOPC-21) (all from BioLegend). After 96 hours of culture in the 5% CO_2 incubator, cells were harvested and stained with the following fluorochrome-conjugated monoclonal antibodies: anti-CD8 (RPA-T8; BioLegend), anti-CD3 (HIT3a; BioLegend), anti-CD4 (SK3; BioLegend), anti-CD14 (MϕP9), anti-CD19 (HIB19; eBioscience), and 7-aminoactinomycin D (eBioscience). $CTV^{low}CD8^+$ T cells were counted as proliferated cells, and the fold changes of $CTV^{low}CD8^+$ T cells were calculated based on isotype control.

Statistical analysis

Demographic data are presented using descriptive statistics and are summarized as median (range) or frequency (percentage). The D'Agostino & Pearson omnibus normality test was used to test for a normal distribution of continuous data. The unpaired t test or Mann-Whitney U test was used to compare the continuous variables. Categorical variables were analyzed by the Pearson χ^2 test. OS and PFS were analyzed using the Kaplan-Meier method and log-rank tests. Statistical analyses were performed using Prism software V.8 (GraphPad Software, San Diego, California, USA), SPSS software V.27 (IBM), or R statistical software V.4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). For all analyses, a $p<0.05$ represented statistical significance.

RESULTS

Improved survival outcomes in BRCA1/2mt EOC patients and differences in gene expression profiles in the tumor microenvironment based on *BRCA1/2* mutation status

Within the YUHS cohort (n=117), 78 patients (66.7%) were *BRCA1/2*wt, and 39 patients (33.3%) were *BRCA1/2*mt (table 1 and figure 1A). The two groups were similar in terms of median age, FIGO stage, and histology type, but differed in histologic grade (table 1). Compared with *BRCA1/2*wt patients, *BRCA1/2*mt patients had longer PFS (HR 0.30, 95% CI 0.18 to 0.49, p<0.0001) and OS (HR 0.12, 95% CI 0.43 to 0.33, p=0.0136) (figure 1B). Among 39 patients with *BRCA1/2* mutations, 28 had germline *BRCA1* mutation (gBRCA1), 8 had germline *BRCA2* mutation (gBRCA2), and 3 had somatic *BRCA1/2* mutations (sBRCA1/2) (online supplemental figure S1A). When comparing the survival outcomes according to germline *BRCA1/2* mutation status, PFS and OS did

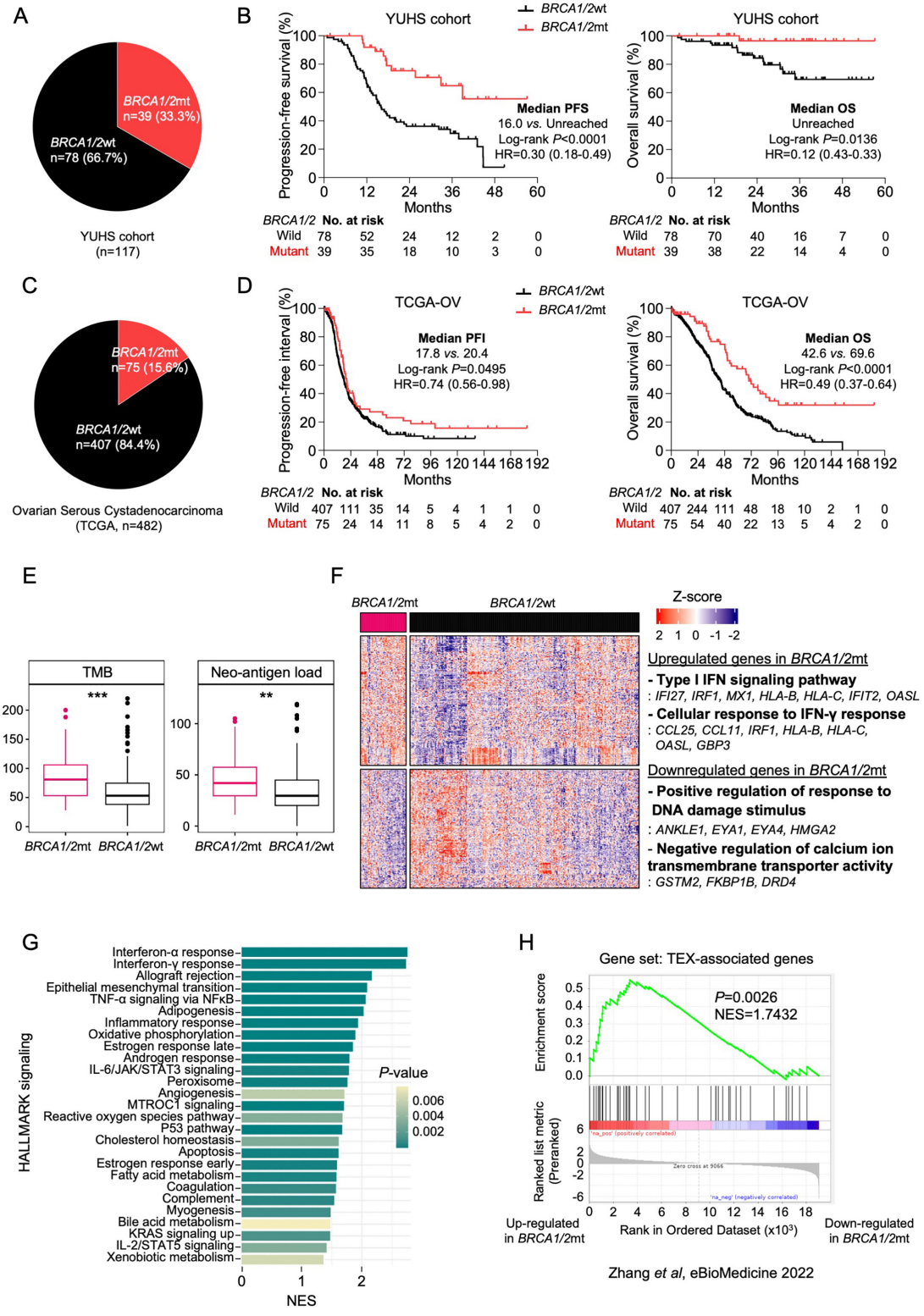

Figure 1 Differences in survival outcomes and tumor microenvironments in epithelial ovarian cancer, according to *BRCA1/2* mutations status. (A) Patient distribution according to *BRCA1/2* status in the YUHS cohort. (B) Comparison of PFS and OS between patients with *BRCA1/2* mutation (*BRCA1/2*mt) and *BRCA1/2* non-mutation (*BRCA1/2*wt) in the YUHS cohort. (C) Patient distribution according to *BRCA1/2* status in the TCGA cohort. (D) Comparison of PFI and OS between *BRCA1/2*mt and *BRCA1/2*wt patients from the TCGA data. (E) Comparison of tumor mutation burden (TMB) and neoantigen load between ovarian cancers with *BRCA1/2*mt and *BRCA1/2*wt from the TCGA data. (F) Comparison of gene expression profiles between ovarian cancers with *BRCA1/2*mt and *BRCA1/2*wt from the TCGA data. (G) Hallmark pathway enrichment analysis, and (H) GSEA of T-cell exhaustion (TEX)-associated genes, according to *BRCA1/2* status in TCGA data. *p<0.05; **p<0.01; ***p<0.001. GSEA, gene set enrichment analysis; NES, normalized enrichment score; ns, not significant; OS, overall survival; PFI, progression-free interval; PFS, progression-free survival; TCGA, The Cancer Genome Atlas.

not significantly differ between patients with germline *BRCA1*mt vs *BRCA2*mt (online supplemental figure S1B). We also further analyzed the TCGA data. Among 482 cases of ovarian serous cystadenocarcinoma in the TCGA data, 75 patients (15.5%) had *BRCA1/2* mutations (figure 1C). Similar to in the YUHS cohort, the *BRCA1/2*mt EOC patients from the TCGA data exhibited a longer progression-free interval (HR 0.74, 95% CI 0.56 to 0.98, p=0.0495) and OS (HR 0.49, 95% CI 0.37 to 0.64, p<0.0001) compared with *BRCA1/2*wt patients (figure 1D).

In the TCGA data, the *BRCA1/2*mt group had a significantly higher TMB and neo-antigen load, compared with the *BRCA1/2*wt group (figure 1E). *BRCA1/2*mt tumors exhibited upregulation of genes associated with the type I interferon signaling pathway (*IFI27, IRF1, MX1, HLA-B, HLA-C, IFIT2,* and *OASL*) and cellular response to interferon-γ (*CCL25, CCL11, IRF1, HLA-B, HLA-C, OASL,* and *GBP3*) (figure 1F). Gene set enrichment analysis (GSEA) of HALLMARK signaling revealed that *BRCA1/2*mt patients exhibited significant upregulation of interferon-α and γ responses, and inflammatory responses (figure 1G). *BRCA1/2*mt tumors also displayed higher enrichment scores for genes associated with T-cell exhaustion (TEX) (NES=1.74, p=0.0026) (figure 1H). Collectively, these results suggested that *BRCA1/2*mt EOC patients displayed features of highly activated immune status, but were also characterized by a more exhausted immune state, compared with *BRCA1/2*wt patients.

Tumor-infiltrating CD8⁺ T cells in *BRCA1/2*-mutated tumors exhibit features of more severe exhaustion compared with *BRCA1/2*-non-mutated tumors

The *BRCA1/2*mt tumor gene expression profiles from the TCGA data displayed both immunologically enhancing and inhibitory features (figure 1F–H); therefore, we further examined TILs from the YUHS cohort, using multicolor flow cytometry to compare the immunological characteristics according to *BRCA1/2* mutation status. Online supplemental figure S1C shows the flow cytometric gating strategies for $CD8^+$ TILs and regulatory T cells (Tregs). The percentages of $CD8^+$ TILs, Tregs, and Treg subpopulations did not significantly differ between *BRCA1/2*mt and *BRCA1/2*wt patients (online supplemental figure S1D,E). Henceforth, we prioritized $CD8^+$ TILs over Tregs and analyzed the expressions of immune checkpoint receptors (PD-1, CTLA-4, TIM-3, and TIGIT), costimulatory receptor 4-1BB (TNFRSF19, CD137), CD226 (DNAM-1), and CD39 on $CD8^+$ TILs.

We found that PD-1^+CD8^+ TILs were more prevalent among *BRCA1/2*mt patients (figure 2A). Given that the TMB and neoantigenic load were significantly higher in *BRCA1/2*mt patients than *BRCA1/2*wt patients (figure 1E), the percentages of tumor-reactive $CD8^+$ TILs (PD-1^+CD39^+ or $CD39^+CD103^+$) were significantly higher among *BRCA1/2*mt patients, compared with *BRCA1/2*wt patients (figure 2B). When analyzing transcription factors associated with T-cell exhaustion, PD-1^+CD8^+ TILs from *BRCA1/2*mut patients exhibited lower TCF-1 expression and higher TOX expression, compared with those from *BRCA1/2*wt patients (figure 2C). Moreover, PD-1^+CD8^+ TILs, representing tumor-specific $CD8^+$ TILs, exhibited higher expression levels of other immune checkpoint receptors (including CTLA-4, TIM-3, and TIGIT) in *BRCA1/2*mt patients, compared with *BRCA1/2*wt individuals (figure 2D). However, expression of the costimulatory receptor CD226 on PD-1^+CD8^+ TILs was greater among *BRCA1/2*mt patients than among *BRCA1/2*wt patients, while the expression of 4-1BB was comparable (figure 2E). In addition, when comparing germline *BRCA1*mt and *BRCA2*mt tumors, we did not find significantly different features of immune exhaustion among PD-1^+CD8^+ TILs in terms of expressions of immune checkpoint receptors and transcription factors TCF-1 and TOX (online supplemental figure S2A,B). The infiltration of tumor-reactive $CD8^+$ TILs was also comparable between *BRCA1*mt and *BRCA2*mt tumors (online supplemental figure S2C).

Anti-PD-1-mediated reinvigoration capacity of tumor-infiltrating CD8⁺ T cells is higher in *BRCA1/2* non-mutated than in *BRCA1/2*-mutated tumors

Next, we performed an *in vitro* T-cell proliferation assay to compare the reinvigoration ability of exhausted $CD8^+$ TILs after anti-PD-1 treatment. PD-1 blockade significantly enhanced the overall proliferation of $CD8^+$ TILs, irrespective of *BRCA1/2* mutation status (figure 3A–C, $CTV^{low}CD8^+$ %). However, when examining the relative fold change of $CTV^{low}CD8^+$ TILs, we found that $CD8^+$ TILs from *BRCA1/2*mt patients did not significantly reinvigorate on anti-PD-1 treatment, whereas $CD8^+$ TILs from *BRCA1/2*wt patients exhibited a significantly increased proliferative response after anti-PD-1 treatment (figure 3B,C, right graphs). More importantly, the combination of anti-PD-1 and anti-CTLA-4 treatment significantly further enhanced the effector function of $CD8^+$ TILs, only among *BRCA1/2*wt patients (figure 3D–F). There were no significant differences between *BRCA1* and g*BRCA2* mutations in capacity for anti-PD-1±anti CTLA-4-mediated functional reinvigoration (online supplemental figure S2D).

Some patients in the YUHS cohort participated in clinical trials (DUO-O, MK7339-01, ATHENA, and KGOG 3046) and received front-line ICB therapy. Some patients were unblinded for subsequent treatment; thus, we were able to classify the patients into the front-line "IO therapy group", the "non-IO therapy group", and the "unknown group" (figure 3G). In the "IO therapy group", there was no significant difference between *BRCA1/2*wt and *BRCA1/2*mt patients in terms of median age, FIGO stage, histology type and histological grade (online supplemental table S2). Among *BRCA1/2*wt patients, the IO therapy group exhibited superior PFS compared with the non-IO therapy group (figure 3H, left). On the other hand, PFS did not significantly differ among *BRCA1/2*mt

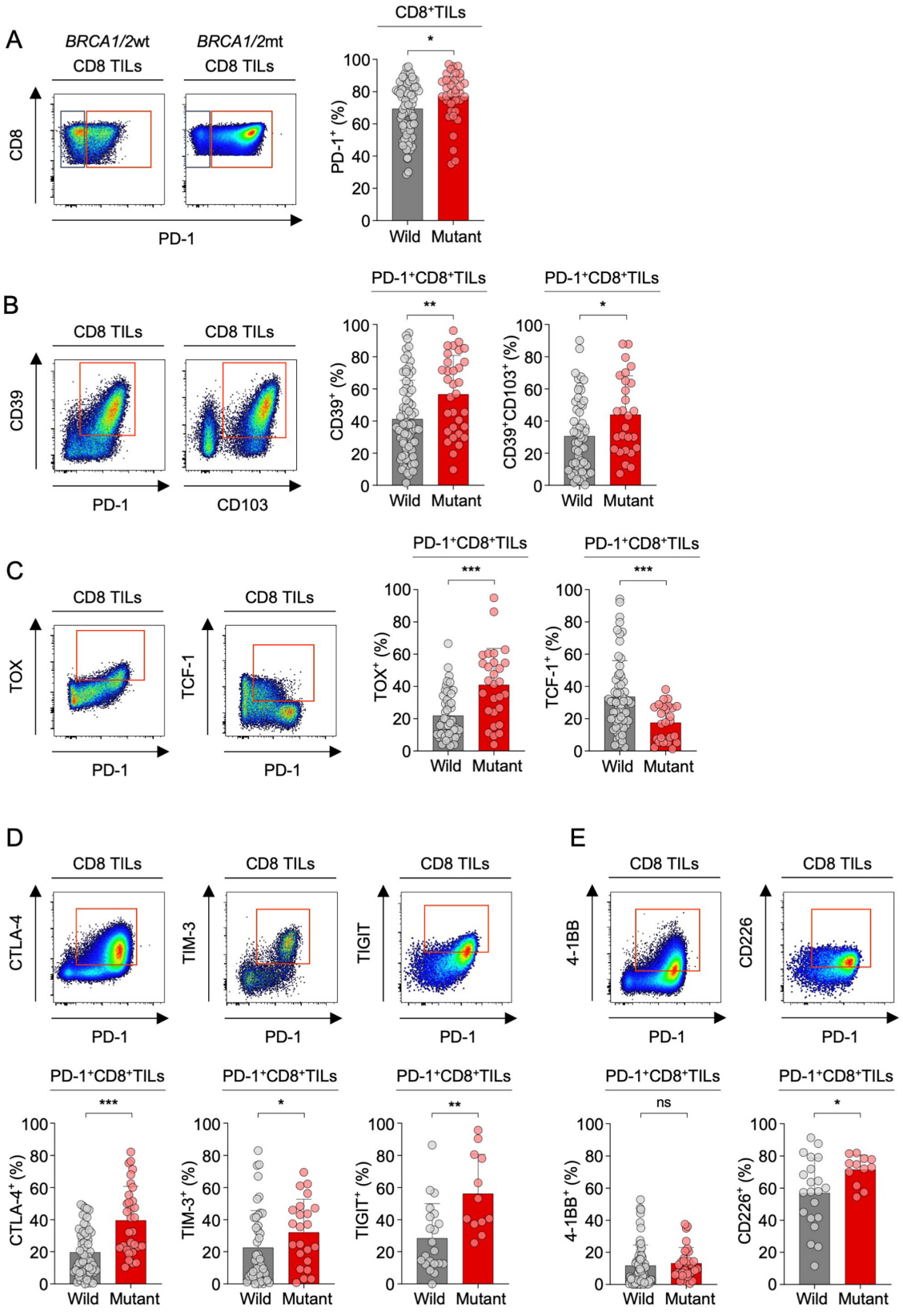

Figure 2 Comparison of tumor-infiltrating CD8⁺ T cells according to *BRCA1/2* mutations status in advanced epithelial ovarian cancer. (A) Representative dot plots and comparison of the frequencies of PD-1⁺ cells among CD8⁺ TILs between patients with *BRCA1/2* mutation (*BRCA1/2*mt) and *BRCA1/2* non-mutation (*BRCA1/2*wt). (B, C) Representative dot-plots and comparison of the frequencies of CD39⁺, CD39⁺CD103⁺ (B), TCF-1⁺, and TOX⁺ cells (C) among PD-1⁺CD8⁺ TILs between patients with *BRCA1/2*mt and *BRCA1/2*wt. (D, E) Representative dot plots and the frequencies of CTLA-4⁺, TIM-3⁺, and TIGIT⁺ cells (D) and 4-1BB⁺, and CD226⁺ cells (E) among CD8⁺ TILs between patients with *BRCA1/2*mt and *BRCA1/2*wt. ns, not significant; *p<0.05; **p<0.01; ***p<0.001. TILs, tumor-infiltrating lymphocytes.

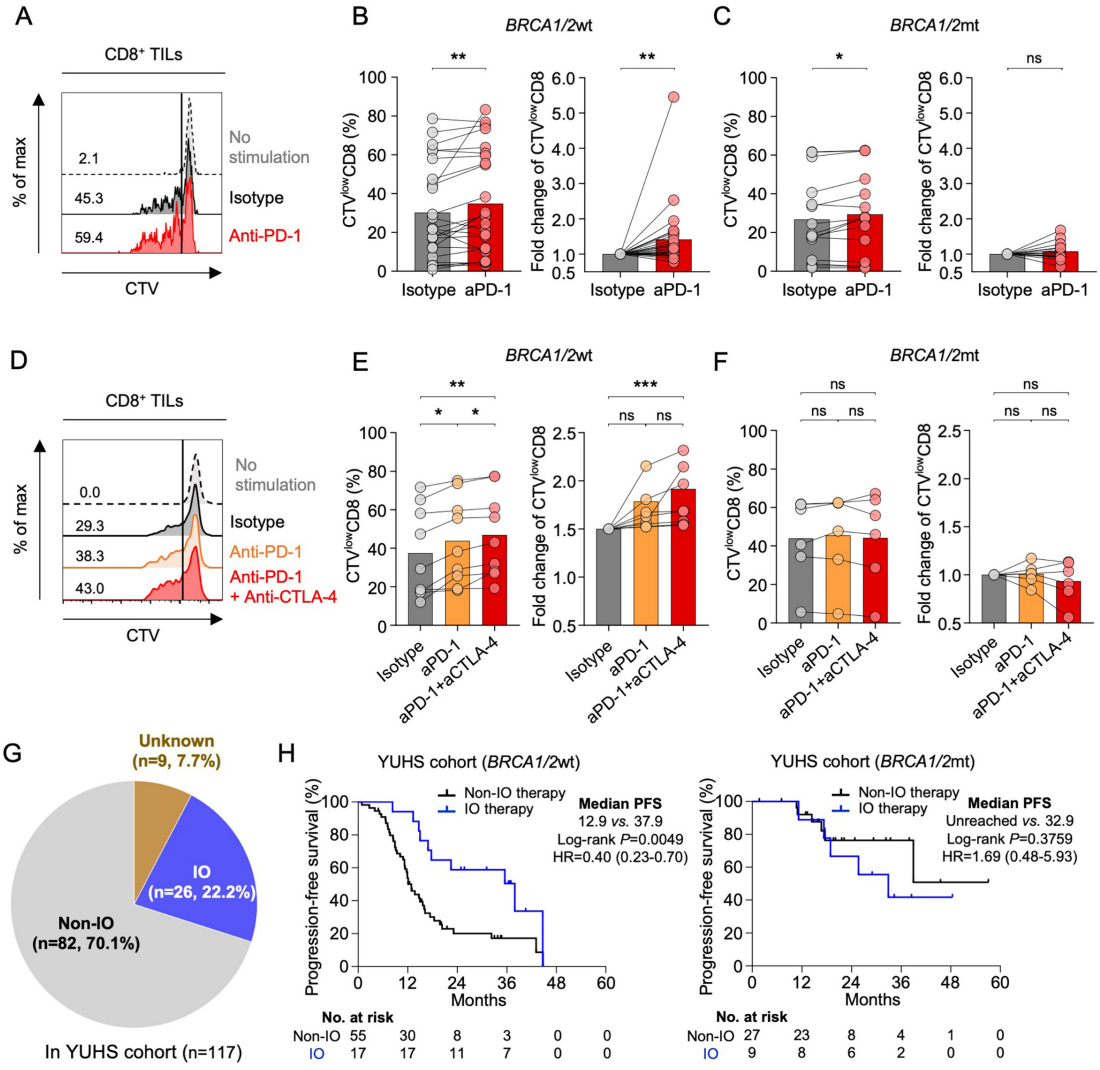

Figure 3 Immune checkpoint inhibitor-induced reinvigoration of tumor-infiltrating CD8$^+$ T cells is significantly greater in *BRCA1/2*wt than in *BRCA1/2*mt patients. CTV-labeled TILs of EOC patients were treated with anti-PD-1 ± anti-CTLA-4 or isotype control in the presence of anti-CD3 stimulation for 96 hours. Proliferative capacity was measured as the percentage of proliferated CTV^{low}CD8$^+$ TILs and the fold changes. (A, D) Representative data. (B, C) The anti-PD-1 induced proliferative capacity was analyzed in *BRCA1/2*wt (B) and *BRCA1/2*mt (C) patients. (E, F) The anti-PD-1+ anti-CTLA-4-induced proliferative capacity was analyzed in *BRCA1/2*wt (E) and *BRCA1/2*mt (F) patients. (G) Patient distribution with or without front-line immune checkpoint blockade (ICB) therapy in the YUHS cohort. (H) Comparison of PFS between patients treated with or without ICB among *BRCA1/2*wt and *BRCA1/2*mt patients. *p<0.05; **p<0.01; ***p<0.001. IO therapy, Immuno-oncology therapy; ns, not significant; PFS, progression-free survival; TILs, tumor-infiltrating lymphocytes.

patients (HR 1.69, 95% CI 0.48 to 5.93, p=0.3759, figure 3H, right).

Among *BRCA1/2*wt patients, high PD-1^{high} expressers possess exhausted but tumor-reactive CD8$^+$ TILs with high reinvigoration capacity following ICB

We subdivided *BRCA1/2*wt patients into "low responders" and "high responders" to anti-PD-1, based on the fold change of CTV^{low}CD8$^+$ TILs (median value=1.13) (figure 4A). Comparative analysis of CD8$^+$ TILs indicated that compared with "low responders, the "high responders" had higher frequencies of PD-1$^+$CD8$^+$ TILs, and of PD-1^{high}CD8$^+$ TILs (figure 4B). Notably, the frequency of PD-1^{high}CD8$^+$ TILs was positively correlated with anti-PD-1-induced reinvigoration ability (figure 4C).

Finally, we aimed to identify a subset of *BRCA1/2*wt patients in whom CD8$^+$ TILs could be efficiently reinvigorated on ICB. ROC curves were used to determine an optimal cut-off level for PD-1^{high} to discriminate "high responders" (cut-off value=22.4%) (figure 4D). We categorized the *BRCA1/2*wt patients based on the percentage of PD-1^{high} cells among CD8$^+$ TILs (figure 4E). Patients with a high percentage of PD-1^{high} cells (>22.4%) were defined as "high PD-1^{high} expressers" while others were termed "low PD-1^{high} expressers" (figure 4E,F). These two groups exhibited similar age, FIGO stage, histological type, and grade (online supplemental table S3). Notably, compared with low PD-1^{high} expressers, the high PD-1^{high} expressers exhibited significantly greater percentages of TOX$^+$ cells

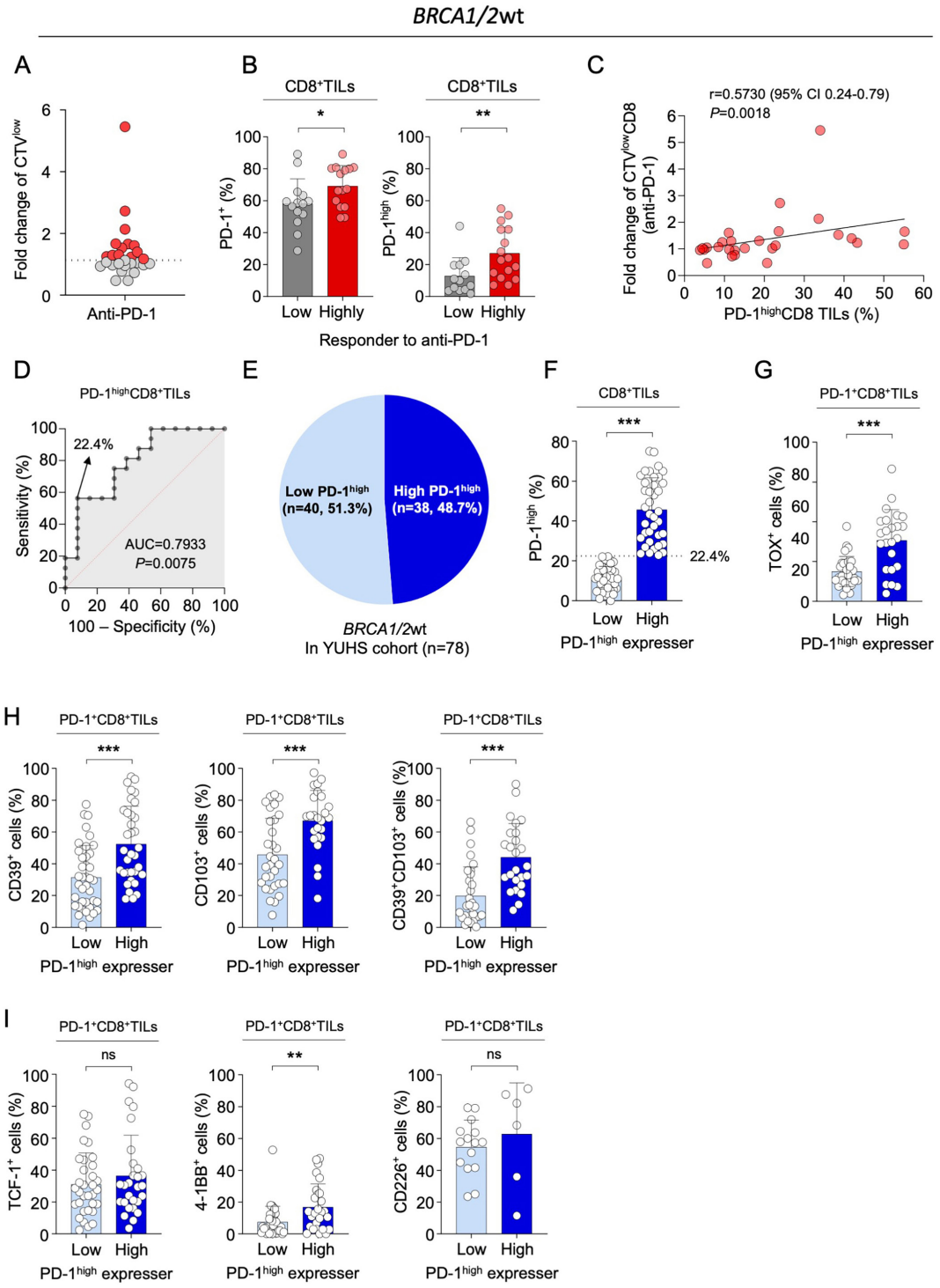

Figure 4 Among *BRCA1/2*wt, high PD-1^{high} expressers are promising candidates for immune checkpoint blockade therapy in advanced stage ovarian cancer. (A) TIL samples were grouped as "low responding TILs" (n=14) and "highly responding TILs" (n=15), based on the proliferative responses (median fold changes of CTV^{low}CD8$^+$ = 1.13). (B) Expression of PD-1 on CD8$^+$ TILs was analyzed in the "highly responding TILs" and "low responding TILs" groups. (C) The percentage of PD-1^{high} among CD8$^+$ TILs was analyzed for a correlation with the relative fold changes of CTV^{low}CD8$^+$ (anti-PD-1) using Pearson's correlation coefficients (r). (D) Performance power of PD-1^{high}CD8$^+$ TILs for predicting responses to anti-PD-1. (E) Distribution of low and high PD-1^{high} expressers, divided using the optimal cut-off value of PD-1^{high}CD8$^+$ TILs in the *BRCA1/2*wt YUHS cohort. (F) Percentages of PD-1^{high} cells among CD8$^+$ TILs in the "low PD-1^{high} expresser" and "high PD-1^{high} expresser" groups. (G) Percentages of TOX+cells among PD-1$^+$CD8$^+$ TILs in the "low PD-1^{high} expresser" and "high PD-1^{high} expresser" groups. (H) Percentages of CD39$^+$, CD103$^+$, and CD39$^+$CD103$^+$ cells among PD-1$^+$CD8$^+$ TILs in the "low PD-1^{high} expresser" and "high PD-1^{high} expresser" groups. (I) Percentages of TCF-1$^+$, 4-1BB$^+$, and CD226$^+$ cells among PD-1$^+$CD8$^+$ TILs in the "low PD-1^{high} expresser" and "high PD-1^{high} expresser" groups. *p<0.05; **p<0.01; ***p<0.001. ns, not significant; TILs, tumor-infiltrating lymphocytes.

among PD-1^{+}CD8^{+} TILs (figure 4G). PD-1^{+}CD39^{+} or PD-1^{+}CD39^{+}CD103^{+} tumor-reactive CD8^{+} TILs were also more abundant in high PD-1^{high} expressers (figure 4H). PD-1^{+}CD8^{+} TILs exhibited significantly higher 4-1BB expression in high PD-1^{high} expressers, whereas their expressions of TCF-1 and CD226 were similar between the two patient groups (figure 4I). Taken together, our results indicated that anti-PD-1-mediated reinvigoration capacity was more potent in EOC patients having a higher frequency of PD-1^{high} CD8^{+} TILs.

DISCUSSION

In the present study, we first identified unique immunological characteristics of CD8^{+} TILs in EOC patients, based on PD-1 levels and *BRCA1/2* mutation status, as outlined in online supplemental figure S3. Specifically, we found that patients with *BRCA1/2* mutations exhibited better survival outcomes compared with those without *BRCA1/2* mutation. Furthermore, *BRCA1/2*-mutated cases had significantly higher TMB and neo-antigen load compared with their *BRCA1/2* wild-type counterparts. Notably, we also identified unique gene expression profiles and tumor microenvironmental changes in *BRCA1/2*mt EOC patients, including a marked increase in the expression of genes associated with interferon response and inflammatory response, compared with in *BRCA1/2*wt tumors. These tumors also displayed significantly enriched expression of genes linked to T-cell exhaustion. Importantly, we demonstrated that the reinvigoration of CD8^{+} TILs after anti-PD-1 treatment was greater in *BRCA1/2*-non-mutated than in mutated cases. Additionally, within the *BRCA1/2*-non-mutated group, the frequency of PD-1^{high}CD8^{+} TILs positively correlated with their reinvigoration capacity following anti-PD-1 treatment.

Although PARPi maintenance therapy improves survival outcomes for patients with EOC, new treatment strategies are urgently needed for the patients with *BRCA1/2*-non-mutated EOC, because the benefits of PARPi are limited among these patients. There have been ongoing investigations into combination therapies, including with antiangiogenic agents and/or ICBs, particularly for *BRCA1/2*wt or HR proficient EOC patients. Early phase clinical trials have shown promising efficacy of ICBs in patients with *BRCA1/2*wt EOC.[25 26] Moreover, a recent phase II trial (OPEB-01) of triple combinational maintenance therapy (olaparib+bevacizumab+pembrolizumab) in second-line *BRCA1/2* non-mutated EOC patients showed a promising ORR of 68.2%, with manageable toxicity.[27] Several ongoing phase III trials—namely, DUO-O (NCT03737643), KEYLYNK-001 (NCT03740165), FIRST (NCT03602859), and ATHENA (NCT03522246)—are exploring combination therapy with ICBs in a frontline setting. Notably, the DUO-O trial has reported promising interim results with the addition of durvalumab (anti-PD-L1) and olaparib to standard front-line chemotherapy in patients with advanced *BRCA1/2*wt EOC. Given the recent treatment strategy for ovarian cancer, our current study is important in that it provides a rationale for combinational ICB therapy in patients with *BRCA1/2*wt. Notably, the present study showed that cytotoxic CD8^{+} TILs of *BRCA1/2*wt were significantly more reinvigorated by anti-PD-1, compared with those of *BRCA1/2*mt. Additionally, although this was a retrospective analysis, the survival outcomes of the IO therapy group were better than those of the Non-IO therapy group, only among *BRCA1/2*wt patients (figure 3H). Furthermore, in *BRCA1/2*wt EOC patients, ICB-mediated reinvigoration capacity was more potent among patients having a higher frequency of tumor-reactive PD-1^{high} CD8^{+} TILs. These results are summarized in online supplemental figure S3.

The impact of *BRCA1/2* mutations on the TIME in EOC has not been thoroughly investigated, and the correlation between *BRCA1/2* mutational status and the efficacy of anti-PD-1/L1 treatments remains unclear. A recent study reported the differential effects of *BRCA1* and *BRCA2* mutations on the response to ICB in mouse and human tumors.[28] In the current study, we compared the survival outcomes according to germline *BRCA1/2* mutation status and found that PFS and OS did not significantly differ between patients with *BRCA1*mt versus *BRCA2*mt (online supplemental figure S1B). Moreover, *BRCA1*mt and *BRCA2*mt tumors did not exhibit significantly different features of immune exhaustion among PD-1^{+}CD8^{+} TILs, and the infiltration of tumor-reactive CD8^{+} TILs was also comparable between *BRCA1*mt and *BRCA2*mt tumors (online supplemental figure S2A–C). Furthermore, *BRCA1* and *BRCA2* mutations did not significantly differ in capacity for anti-PD-1-mediated functional reinvigoration, although there remains a need for validation in a larger sample size (online supplemental figure S2D).

Several studies have reported that *BRCA1/2* mutational status is correlated with immune cell infiltration in patients with ovarian or breast cancer. For example, a recent study demonstrated that T-cell-specific BRCA1 knockout (TBKO) mice exhibited fewer total CD8^{+} cells, as well as impaired antitumor immunity because their memory tumor-infiltrating T-cell populations are reduced, more exhausted, and less cytotoxic.[29] In addition, patients with germline *BRCA1/2*-mutated recurrent ovarian cancer reportedly have fewer circulating myeloid-derived suppressor cells but higher CD8^{+} T cells in the peripheral blood.[30] In the multicenter observational, prospective cohort study of the Ovarian Tumor Tissue Analysis Consortium, CD8 TILs were evaluated from 5577 ovarian tumor sample and showed the extent of CD8 TILs differed by *BRCA* mutation status. Among HGSCs, tumors with *BRCA1* mutations had the highest CD8 TIL counts compared with non-mutated and *BRCA2* mutated tumors. Intriguingly, the survival benefit associated with CD8 TILs was found only in *BRCA1*-mutated and non-mutated tumors, not in *BRCA2*-mutated tumors.[31] Regarding the TIME, some studies have included IHC analyses demonstrating increased expressions of PD-1 and PD-L1 and increased CD3^{+} and CD8^{+} TILs, in *BRCA1/2*-mutated tumors.[17 32–34] On the contrary, there was a study reported

no significant relationship between *BRCA1/2* status and TIL density.[35]

More recently, Launonen *et al* generated spatial proteomic data using highly multiplex immunofluorescence to elucidate how *BRCA1/2* mutations shape the cellular phenotypes and spatial interactions.[36] They identified a distinct tumor microenvironment and divergent tumor–immune interactions in the *BRCA1/2*mt as compared with in HR proficient tumors. Further, consistent with our current study, they observed higher PD-1 expression on $CD8^+$ T cells in the *BRCA1/2*mt cases. Although they suggested potential roles of PD-1/L1 targeted immunotherapy, they did not perform any functional assay to measure the efficacy of PD-1 blockade according to *BRCA1/2* mutations status. Compared with these previous findings, our investigation is significant and distinct from the prior study in that we have demonstrated—using clinical samples from EOC patients—that $CD8^+$ TILs within *BRCA1/2*mt tumors displayed characteristics indicating more severe T-cell exhaustion, compared with their *BRCA1/2*wt counterparts. Furthermore, unlike the previous paper, our study elucidates the direct functional differences of $CD8^+$ TILs according to BRCA1-deficient status. Specifically, we measured the anti-PD-1-induced reinvigoration capacity of $CD8^+$ TILs and found that $CD8^+$ TILs within *BRCA1/2*mt tumors were not significantly reinvigorated by anti-PD-1 treatment, compared with $CD8^+$ TILs within *BRCA1/2*wt tumors. Additionally, in terms of the heterogeneity within TILs, we provide the first demonstration that $CD8^+$ TILs within *BRCA1/2*mt tumors exhibited features of terminally exhausted T cells, which do not respond to anti-PD-1 therapy and are difficult to reinvigorate with restored proliferative capacity.

In the current study, it is intriguing to note that the survival outcomes of the TCGA and YUHS cohorts are quite different (figure 1B,D). Although both TCGA (ovarian serous cystadenocarcinoma) and YUHS cohorts exclusively comprised patients with EOC, there were notable differences in the data collection periods: data were collected from the TCGA cohort before 2011, whereas the YUHS cohort enrolled patients between April 2018 and March 2022. Between these two time periods, there were dynamic changes in the treatment strategies for EOC, especially due to the introduction of PARP inhibitor therapy. Consequently, patients in the TCGA cohort did not receive PARP inhibitors, whereas a significant proportion of *BRCA1/2mt* patients in the YUHS cohort underwent maintenance therapy with PARP inhibitors (olaparib or niraparib). Therefore, it would be possible that *BRCA1/2*mt EOC patients showed better survival outcomes than *BRCA1/2*wt patients in the YUHS cohort, particularly given PARP inhibitors have shown a substantial benefit for *BRCA1/2*mt patients in several phase 3 pivotal clinical trials.[6 7 37]

The present study has several limitations, including concerns about selection bias, and the absence of HRD results. In fact, we did not select patient populations based on specific criteria; rather, we aimed to include patients in this study who were representative of those encountered in actual clinical practice. As this study is not a confirmative clinical study but a translational study that generates hypotheses, we acknowledge that some selection bias may be accepted. HRD status is important as *BRCA1/2* mutations in EOC. Among *BRCA1/2*wt EOC patients, HRD-positive patients may be included. More definitive immunological comparisons according to HRD status would be needed, but unfortunately, HRD tests could not be performed in this study. Nevertheless, this study is significant in that it demonstrated an immunological difference according to the presence or absence of *BRCA1/2* mutation. We acknowledge the need for further research regarding HRD.

In conclusion, our present findings suggest that "high $PD\text{-}1^{high}$ expressers" among patients with *BRCA1/2* non-mutated EOC may be ideal candidates for front-line PD-1 blockade therapy. We demonstrated that highly exhausted $PD\text{-}1^+CD8^+$ TILs were more prominent in *BRCA1/2*mt than *BRCA1/2*wt, and that the reinvigoration ability of $CD8^+$ TILs on anti-PD-1 treatment was significant only in *BRCA1/2*wt, rather than in *BRCA1/2*mt cases. Additionally, we found that the reinvigoration capability of $CD8^+$ TILs after anti-PD-1 treatment was positively correlated with the frequency of $PD\text{-}1^{high}CD8^+$ TILs in *BRCA1/2*wt patients. These findings highlight the potential of ICB therapy for specific *BRCA1/2*wt patients identified through predictive biomarkers. Clinical studies focusing on optimal ICB treatment strategies for this subgroup of EOC patients are warranted.

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