**PD-L1 expression is associated with tumor-infiltrating T cells
and favorable prognosis in high-grade serous ovarian cancer**

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Abstract

Objective. As a negative regulator of T cells, Programmed Death Ligand 1 (PD-L1) is both an indicator and inhibitor of anti-tumor immune responses, which has led to confusion about its prognostic significance. We investigated the primary source of PD-L1 expression in epithelial ovarian cancer and its relationship to tumor-infiltrating lymphocytes (TIL) and associated gene products.

Methods. Tissue microarrays containing high-grade serous carcinomas (HGSC) and endometrioid, clear cell and mucinous ovarian cancers from optimally debulked patients were assessed by immunohistochemistry for expression of PD-L1 and other markers (CD68, CD3, CD8, PD-1, CD103, FoxP3 and CD25). The Cancer Genome Atlas was interrogated for associations between PD-L1 expression and immune-related transcriptional and genomic features of HGSC.

Results. PD-L1 was primarily expressed by tumor-associated CD68⁺ macrophages rather than tumor cells. PD-L1⁺ cells frequently co-localized with CD8, CD4 and PD-1⁺ TIL, CD25⁺FoxP3⁺ Tregs, and other TIL subsets. PD-L1⁺ cells were prognostically favorable in HGSC. Moreover, the presence of both PD-L1⁺ cells and CD8 TIL was associated with better prognosis than CD8 TIL alone. PD-L1 gene expression was independent of BRCA status. At the transcriptional level, PD-L1 was associated with both cytolytic (granzyme B, T-bet and IFN- γ) and suppressive (PD-1, CTLA-4, LAG3 and IDO-1) gene products.

Conclusions. PD-L1 is primarily expressed by macrophages in ovarian cancer and is strongly associated with both cytolytic and regulatory TIL subsets, resulting in a net positive association with survival. Tumors containing PD-L1⁺ macrophages appear caught in an immunological stalemate that may require multi-pronged immunotherapy to alleviate.

44 **Keywords:** PD-L1, adaptive resistance, ovarian cancer

Introduction

The programmed death 1 (PD-1) pathway has emerged as a powerful immune regulatory mechanism in cancer, prompting intensive efforts to understand its physiological regulation, prognostic significance, and therapeutic potential. PD-1 is a cell surface receptor expressed by activated CD4 and CD8 T cells and a variety of other immune cells (1). PD-1 has two known ligands, of which PD-L1 appears most relevant in the tumor microenvironment. PD-L1 can be expressed by tumor cells, tumor-associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs), dendritic cells (DCs), T cells and B cells (1). Binding of PD-L1 to PD-1 inhibits T cell receptor (TCR) signaling via engagement of SHP-1 and -2 phosphatases, resulting in reduced T-cell proliferation and cytokine production and increased susceptibility to apoptosis (for review see (2)). Disruption of the PD-L1/PD-1 interaction using antibodies to either molecule (referred to as PD-1 blockade) can restore T cell function, leading to enhanced anti-tumor immune responses (3).

PD-1 blockade represents one of the most promising advances in oncology in recent years, with impressive responses rates reported in diverse malignancies such as melanoma, non-small cell lung cancer (NSCLC), renal cancer (RCC), and Hodgkin's lymphoma (3). More modest results are being reported for other malignancies, including ovarian cancer (4-7), prompting research into the underlying determinants of response. One emerging theme is that PD-1 blockade is most effective against tumors with high mutation loads (8). In addition, response to PD-1 blockade has been linked to the presence of PD-L1⁺ cells and tumor-infiltrating lymphocytes (TIL) in the tumor microenvironment (9). These determinants may be related, as TIL can recognize somatic mutations and have been shown to correlate with the density of predicted mutant epitopes across a range of human cancers (10). Thus, a better understanding of

the factors that promote TIL and PD-L1 expression in the tumor microenvironment may enable improved clinical deployment of PD-1 blockade.

Although our understanding of the regulation of PD-L1 expression is incomplete, several factors have been identified. In particular, the canonical T cell-derived cytokine interferon-gamma (IFN- γ) can induce PD-L1 expression on a variety of cell types (11). This creates an important negative feedback loop in which activated T cells, by releasing IFN- γ , induce expression of PD-L1 on target cells, which in turn dampens T cell activation. Indeed, in a phenomenon known as adaptive resistance, TIL have been shown to induce PD-L1 expression by tumor cells, leading to diminished anti-tumor immunity (11). PD-L1 expression can also be induced by cytokines such as IL-2, IL-7, IL-15, IL-10 and IL-32 γ (12-14). In addition to cytokines, hypoxic conditions can induce PD-L1 expression via the hypoxia-inducible factor 1 alpha (HIF-1 α) pathway (15). Regulation of PD-L1 expression can also differ according to cell type; for example, IL-10 and IL-32 γ can induce PD-L1 on myeloid cells, but not tumor cells (12). Reflecting these complex regulatory mechanisms, four general categories of tumor microenvironment have been proposed based on the patterns of PD-L1 expression and TIL in melanoma: ***Type I*** - PD-L1 expression in the presence of TIL (*adaptive resistance*); ***Type II*** - lack of both PD-L1 expression and TIL (*immunological ignorance*); ***Type III*** - PD-L1 expression in the absence of TIL (*intrinsic induction*); and ***Type IV*** - lack of PD-L1 expression but presence of TIL (*immunological tolerance*) (9).

The physiology of PD-1 is similarly complex. Although it was initially described as a marker of T cell exhaustion during chronic viral infection, PD-1 is now known to be transiently upregulated as a normal feature of TCR activation. Thus, the process of T cell exhaustion involves not only PD-1 expression but the acquisition of additional exhaustion markers, such as

TIM-3 and LAG-3, combined with the sequential loss of effector functions (16). We recently reported in ovarian cancer that PD-1 is selectively expressed by TIL with an intraepithelial (as opposed to stromal) location (17). Accordingly, we found that PD-1 is co-expressed with CD103, an integrin subunit that facilitates infiltration of epithelial tissues by T cells (17). Although PD-1⁺CD103⁺ CD8 TIL were inactive when assessed directly *ex vivo*, they lacked expression of other exhaustion markers and retained the ability to produce cytokines upon *in vitro* stimulation, consistent with a quiescent rather than terminally exhausted phenotype (17). Likewise, in melanoma PD-1 demarcates tumor-reactive, functionally competent CD8 TIL (18). Thus, in the setting of cancer, PD-1 might more accurately be viewed as a marker of T cell activation than exhaustion.

In one of the first studies of PD-L1 expression in any human cancer, Hamanishi and colleagues reported in epithelial ovarian cancer (EOC) that PD-L1 had a tumor-intrinsic expression pattern, meaning it was primarily expressed by tumor cells and inversely correlated with TIL (19). Consistent with this pattern, they reported that PD-L1 was associated with poor prognosis (19). This and other reports established the paradigm of the PD-1/PD-L1 axis being immunosuppressive in cancer. However, these findings appear inconsistent with subsequent studies showing that PD-L1 is induced by IFN- γ and hence is often associated with CD8 TIL (11). Furthermore, the Hamanishi study did not distinguish between the different histological subtypes of EOC, which have distinct molecular, immunological and clinical characteristics (20). In particular, HGSC, the most common and fatal form of EOC (20), is the only subtype that harbors brisk, prognostically favorable TIL responses (21). Finally, with the recent surge of clinical interest in PD-1 blockade, a number of specific, validated anti-PD-L1 antibodies have become available for immunohistochemistry (IHC), which has prompted a major re-assessment

of PD-L1 expression in cancer (22). Using these newer antibodies and a large tumor tissue microarray (TMA) spanning the major histological subtypes of EOC, we evaluated the expression pattern and prognostic significance of PD-L1. In contrast to the above study (19), we found that PD-L1 was primarily expressed by TAMs and strongly associated with TIL in all histological subtypes of EOC. In addition, PD-L1 showed a positive association with survival in HGSC. Thus, consistent with the concept of adaptive resistance, PD-L1 expression appears to be an indicator of robust, prognostically favorable TIL responses in HGSC.

Materials and Methods

Patient cohort and tissue microarray

To evaluate PD-L1 expression across various ovarian cancer subtypes, perform prognostic studies, and determine TIL subset associations, we used a previously described tissue microarray (TMA) (21) comprising 490 EOC cases seen at the BC Cancer Agency from 1984 to 2000 (OvCaRe Ovarian Tumour Bank, Vancouver, BC, Canada). All tumor tissues were obtained at the time of primary surgery, and patients were deemed optimally debulked. In addition, we used a 17-case lung cancer TMA for anti-PD-L1 antibody validation and a 74-case HGSC TMA containing for CD68/PD-L1, CD3/CD8/PD-L1 and CD103/PD-1/PD-L1 co-expression. These latter tissues and clinical data were obtained through the BC Cancer Agency's Tumour Tissue Repository, a member of the Canadian Tissue Repository Network. All specimens and clinical data were obtained with either informed written consent or a formal waiver of consent under protocols approved by the Research Ethics Board of the BC Cancer Agency and the University of British Columbia.

Immunohistochemistry

Anti-PD-L1 rabbit monoclonal antibodies were from Spring Bioscience (clone SP142, Pleasanton, CA) and Cell Signaling Technology (clone E1L3N, Danvers, MA). Antibodies were validated using the lymphoma cell lines Karpas 1106P (PD-L1 positive control) (23) and Su-DHL-5 (PD-L1 negative control) (24) (both cell lines provided by Dr. Christian Steidl, BC Cancer Agency). Cell lines were suspended in Histogel, formalin fixed, paraffin embedded and processed for immunohistochemistry as described below for tumor tissue. All staining was performed using an Intellipath FLX autostainer (Biocare, Concord, CA) and Biocare reagents

unless otherwise specified. Specific details regarding immunohistochemical staining procedures are described in ***Supplementary Methods***. A Nuance multispectral imaging system (Perkin Elmer, Waltham, MA) was used to assess and display co-localization of markers.

TMA scoring and statistical analysis

For PD-L1, TMA cores were evaluated by two independent observers and scored as positive or negative for PD-L1 using a threshold of ≥ 1 positive cells (in one or both of duplicate cores, regardless of cell type or location). For CD8 TIL, TMAs were scanned using a Vectra automated quantitative pathology imaging system and counted using Inform advanced image analysis software (Perkin Elmer, Waltham, MA).

Statistical analyses were performed using Graphpad software. Univariate survival analysis was performed using the Kaplan-Meier method, and *P* values were determined using log-rank test. Multivariate survival analysis was performed using a Cox regression model. *P*-values < 0.05 were regarded as significant.

Gene expression and survival analysis of The Cancer Genome Atlas (TCGA) dataset

Gene expression data from The Cancer Genome Atlas (<http://cancergenome.nih.gov/>) was downloaded from cBioPortal (<http://www.cbioportal.org/>) (25). Associations between PD-L1 (*CD274*) expression and immune response genes were based on Agilent microarray z-scores in the provisional Ovarian Serous Cystadenocarcinoma dataset (n=558 cases) with manually appended clinical data. Statistical associations between *CD274* and 18 other immune response genes were tested using Pearson correlations between z-score values. Unpaired two-tailed t-tests were used to compare mean gene expression levels after stratification of cases according to below- or above-median expression of *CD274*. Bonferroni's correction was used to mitigate

multiple testing errors; P-values of < 0.0028 ($0.05/18$) were considered significant. For analysis of BRCA1/2 mutations, we used a subset of TCGA cases ($n=316$) for which exome sequencing data was available. BRCA1/2 mutation calls were obtained from cBioPortal, and BRCA1 methylation calls were kindly provided by Dr. Douglas Levine, Memorial Sloan Kettering Cancer Center. To investigate the relationship between *CD274* gene expression and mutation load, we used a subset of TCGA cases ($n=220$) for which non-synonymous single nucleotide variant counts and *CD274* gene expression values were available (courtesy of Scott Brown and Dr. Robert Holt, BC Cancer Agency's Genome Sciences Centre).

Results

PD-L1 is expressed predominantly by tumor-associated macrophages in epithelial ovarian cancer

When this study began, there was uncertainty in the field over which anti-PD-L1 antibodies most accurately detect PD-L1 by IHC. Therefore, we evaluated two recently developed anti-PD-L1 rabbit monoclonal antibodies from Spring Bioscience (clone SP142) and Cell Signaling Technology (clone E1L3N). Both antibodies demonstrated specificity and good staining intensity when used to stain formalin-fixed, paraffin embedded samples of lymphoma cell lines that were known to be either PD-L1 negative (SU-DHL-5; (24)) or PD-L1 positive (Karpas 1106P; (23)) (**Fig 1, top left and right**). In addition, both antibodies exhibited highly similar staining patterns on NSCLC specimens (**Fig 1, lower left**). Importantly, consistent with prior reports (26, 27), PD-L1 expression was largely restricted to tumor cells in NSCLC. In contrast, in HGSC both antibodies yielded a focal, patchy PD-L1 expression pattern that appeared to predominantly involve tumor-associated immune cells and only occasional patches of tumor cells (**Fig 1, lower right**). Because this was markedly distinct from the widespread and predominantly tumor cell-restricted PD-L1 expression pattern that has been previously reported for ovarian cancer (19), we performed additional studies to better define the cell types expressing PD-L1 in EOC.

Based on their morphology as well as reports from other cancer types (11), we reasoned that the majority of PD-L1⁺ immune cells observed in HGSC might be tumor associated macrophages (TAMs). To address this, HGSC tumors underwent three-color IHC with antibodies against PD-L1, the tumor-cell nuclear marker WT-1 and the myeloid/macrophage marker CD68. Three different staining patterns were observed: **1**) no PD-L1 expression (46.3%

of cases) (**Fig 2A, upper**); **2**) PD-L1 staining predominantly on CD68⁺ cells (40.5% of cases) (**Fig 2A, middle**); and **3**) PD-L1 staining on both tumor cells and CD68⁺ cells (13.2% of cases) (**Fig 2A, lower**). We did not observe any tumors containing PD-L1⁺ tumor cells without PD-L1⁺ macrophages. Staining of adjacent tumor sections with anti-cytokeratin antibody confirmed that WT-1⁺ tumor regions represented tumor epithelium (data not shown). Two-color IHC followed by image analysis confirmed the predominant expression of PD-L1 by CD68⁺ cells (**Fig 2B**). Thus, in contrast to a prior report (19), PD-L1 was expressed more frequently by TAMs than tumor cells (**Fig. 2C**). This pattern of PD-L1 expression was observed for both stromal and intraepithelial macrophages and in both primary and metastatic tumor sites (Fig. 2C).

PD-L1 is expressed at variable levels in all major subtypes of epithelial ovarian cancer

We assessed the association between PD-L1 and CD8 TIL by performing two-color immunohistochemistry on a previously described TMA containing 490 optimally debulked cases spanning the major histological subtypes of EOC (21). As described above for HGSC, PD-L1 expression was focal and patchy in all EOC subtypes. Although this staining pattern made it difficult to precisely enumerate PD-L1⁺ cells, we scored tumors as PD-L1 positive or negative using a threshold of ≥ 1 positive cells in either of two TMA cores, irrespective of cell type or intratumoral location (**Table 1**). Using this binary scoring method, 171 of 490 tumors (34.9%) were PD-L1 positive, including 112/195 (57.4%) of the HGSC subtype (**Table 1**). Among serous cases, the percentage of PD-L1 positive tumors increased with grade ($P=0.011$) but not stage of disease (**Table 1**). The proportion of PD-L1 positive tumors was lower, yet substantial, for the mucinous (8/30, 26.7%), endometrioid (30/125, 24%), and clear cell (21/129, 16.2%) subtypes (**Table 1**).

PD-L1 expression is associated with multiple TIL subsets

With the exception of the mucinous subtype, PD-L1 positive tumors contained significantly higher densities of CD8 TIL than PD-L1 negative tumors (**Fig 3A**). Moreover, 169/171 tumors that were PD-L1 positive (across all subtypes) also contained CD8 TIL (**Table 1**). However, the correlation between PD-L1 and CD8 was unidirectional, as there were numerous examples of tumors (across all subtypes) that contained CD8 TIL yet were PD-L1 negative (**Fig 3A and Table 1**). Using the classification scheme proposed by Teng and colleagues for melanoma (9), HGSC cases showed the following distribution: *Type I* PD-L1⁺/CD8⁺ (57.4%), *Type II* PD-L1⁻/CD8⁻ (5.1%), *Type III* PD-L1⁺/CD8⁻ (0%), and *Type IV* PD-L1⁻/CD8⁺ (37.4%) (**Table 1 and Supplementary Fig 1**). Thus, in HGSC the Type I pattern was most prevalent, followed by the Type IV pattern and, far less commonly, the Type II and III patterns. Notably, for the other histological subtypes of EOC, the distribution was skewed toward the Type IV (PD-L1⁻/CD8⁺) pattern, with modest representation of the Type I and II patterns, and only rare examples of the Type III pattern (**Table 1**).

The 490-case TMA described above has been evaluated for various other immune markers in prior studies (21, 28-30). Using this data, we found that PD-L1 positivity was associated with TIL expressing CD8, CD103, PD-1 and FoxP3 (**Fig 3A**). These associations were significant for the HGSC, endometrioid and clear cell subtypes; mucinous cases did not reach statistical significance likely due to small sample size. Recognizing that FoxP3 alone does not adequately define Tregs, we also investigated the association between PD-L1 and CD25⁺FoxP3⁺ TIL using existing data from the HGSC cohort (28). PD-L1 positive tumors

contained significantly more CD25⁺FoxP3⁺ Tregs than PD-L1 negative tumors (**Fig 3B**). PD-L1 expression was also associated with MHC class I ($P < 0.0001$) and II ($P = 0.0024$) in HGSC.

Multi-color IHC was used to assess the spatial relationships between PD-L1⁺ cells and TIL in the tumor microenvironment in 74 HGSC cases. We performed three-color IHC with antibodies to CD3, CD8 and PD-L1 (**Fig 3C, upper**). As before, PD-L1 expression was largely restricted to cells that morphologically resembled macrophages and tumor cells, with little to no expression on CD3⁺ or CD8⁺ TIL. PD-L1⁺ cells were frequently found in close proximity to both CD8 and CD4 TIL (the latter being visualized as CD3⁺CD8⁻ cells) with no obvious skewing toward either subset (**Fig 3C, upper**). We have previously shown that intraepithelial, prognostically favorable CD8 TIL frequently co-express PD-1 and the integrin subunit CD103 (17, 29); therefore, we also performed three-color IHC with antibodies to PD-1, CD103, and PD-L1. Although the staining patterns were complex, CD103⁺PD-1⁺ TIL were frequently present in PD-L1 positive tumor regions (**Fig 3C, lower**). Thus, PD-L1⁺ cells and TIL consistently showed a close spatial relationship in the tumor microenvironment.

PD-L1 expression is associated with favorable prognosis in HGSC

In univariate analysis, PD-L1 positivity was associated with disease-specific survival in HGSC (HR=0.4864; 95% confidence interval=0.3206-0.7378; $P=0.0007$) (**Fig. 4A**). PD-L1 remained a significant factor in multivariate survival analysis when age, stage, and grade were used as covariants (HR = 0.607; 95% confidence interval = 0.399–0.925; $P = 0.02$). By contrast, PD-L1 positivity showed no prognostic significance in low-grade serous, mucinous, endometrioid or clear cell cancers, similar to previous results for CD8 TIL and PD-1 TIL in this cohort (17, 21). When PD-L1 and CD8 scores were considered together in the HGSC subtype,

three distinct prognostic groups emerged. Relative to PD-L1⁻/CD8⁻ cases, PD-L1⁺/CD8⁺ cases were associated with the best prognosis (HR 0.2191, 95% confidence interval 0.06664 to 0.7207) followed by PD-L1⁻/CD8⁺ cases (HR 0.4781, 95% confidence interval 0.1750 to 1.307) (**Fig. 4B, left**). Similar trends were observed in TCGA data (Supplementary Fig. 2); however, values did not reach statistical significance. When PD-L1 and PD-1 scores were considered together, only PD-L1⁺/PD-1⁺ tumors were associated with a prognostic advantage (HR 0.4763, 95% confidence interval 0.3110 to 0.7292 compared to all other cases) (**Fig. 4B, right**).

PD-L1 expression is associated with both cytolytic and regulatory gene signatures

To further investigate the immunological features of PD-L1 positive tumors, we interrogated the HGSC dataset from TCGA (25). Despite the reported association between BRCA1 disruption and the C2/Immunoreactive subtype of HGSC (31), PD-L1 gene expression showed no association with BRCA1 mutation or methylation (P=0.8342; ANOVA). Similarly, PD-L1 gene expression showed no association with BRCA2 mutation (P=0.5856; two-tailed t test) or somatic mutation load as determined by whole exome sequencing (Pearson's $r^2=0.004$, P=0.3729). Consistent with our IHC results, analysis of TCGA gene expression data revealed an association between PD-L1 (*CD274*) and the macrophage-related gene product *CD68* (**Fig. 5A, Supplementary Table 1**). Furthermore, *PD-L1* gene expression was associated with multiple cytotoxic lymphocyte-related gene products, including granzyme B (*GZMB*), T-bet (*TBX21*), and IFN- γ (*IFNG*) (**Fig. 5B, Supplementary Table 1**). However, PD-L1 gene expression was also associated with immunosuppressive gene products, including PD-1 (*PDCDI*), CTLA-4, LAG-3, and IDO-1 (**Fig. 5C, Supplementary Table 1**). Thus, gene expression data provided further

evidence that PD-L1 positive tumors harbor a mixture of cytolytic and regulatory immune cells and associated factors.

Discussion

The PD-1/PD-L1 axis is showing great promise as a target for immune modulation in cancer, and the pattern of PD-L1 expression in relation to TIL is emerging as an important determinant of clinical response (9). We report here that PD-L1 expression is prevalent across all major histological subtypes of EOC. PD-L1 expression was far more common on TAMs than tumor cells. Indeed, PD-L1 expression by tumor cells was evident in only 13.2% of HGSC tumors, all of which also contained PD-L1⁺ macrophages. Moreover, we found a strong, positive association between PD-L1 expression and the presence of multiple TIL subsets, including CD8⁺, CD103⁺, PD-1⁺, and FoxP3⁺ TIL. Accordingly, *in silico* analysis of TCGA gene expression data revealed associations between PD-L1 and both cytolytic and regulatory gene products. In HGSC, PD-L1 positivity was associated with favorable prognosis, even when the analysis was restricted to CD8 TIL positive cases. Thus, despite being a negative regulator of T cell activation, PD-L1 was associated with robust, prognostically favorable TIL responses. Our findings are consistent with the concept of adaptive resistance, wherein activated T cells trigger negative feedback mechanisms in the tumor microenvironment, resulting in an immunological stalemate. We speculate that such responses may be re-activated when patients undergo standard treatment, resulting in improved tumor control and favorable prognosis.

Our findings contradict a prior study in EOC in which PD-L1 expression was reported to be inversely associated with TIL and patient survival (19). Although the authors of that study did not describe which cell types stained positive for PD-L1, the staining pattern they presented

appeared to primarily involve tumor epithelium rather than TAMs. We believe the difference in staining patterns is most likely attributable to the use of different antibodies against PD-L1. Indeed, given the surge of interest in the PD-1/PD-L1 pathway, this issue has received considerable attention in the literature recently. The 27A2 antibody used in the study by Hamanishi and colleagues (19) has since been found by others to exhibit high background staining (32). Likewise, another HGSC study recently reported extensive expression of PD-L1 on tumor cells (33); however, the anti-PD-L1 antibody used in this study (clone EPR1161(2)) has since been discontinued by the manufacturer due to concerns about product quality (according to the company website). For the current study, we selected two antibodies that were specifically developed and validated for use on FFPE tissue (34, 35). In addition, we performed IHC using an automated instrument that generates highly reproducible results. The two antibodies yielded very similar staining patterns which matched the expected results for NSCLC and other control tissues. Moreover, in EOC they revealed a TAM-predominant, TIL-associated PD-L1 expression pattern that has been reported in several other cancers and fits with the known regulatory properties of PD-L1 (11, 36-39). Thus, the PD-L1 expression pattern reported here is consistent with recent literature using the latest generation of antibodies. Our study differed from that of Hamanishi and colleagues (19) in two other ways. First, we stratified our results according to the different histological subtypes of EOC, which are now recognized to have distinct genetic, molecular, clinical and immunological properties (20, 21). This stratification proved to be important, as the association between PD-L1, TIL and prognosis was seen only in the HGSC subtype; in the Hamanishi study, only 28/70 tumors were of serous histology. Second, our outcomes analysis was focused on optimally debulked cases, which generally show a stronger prognostic effect of TIL compared to suboptimally debulked cases (21). Thus, Hamanishi's study

made a seminal contribution to the field by highlighting the importance of PD-L1 as an immunosuppressive factor in EOC. Our findings refine this view by defining TAMs as the major source of PD-L1 expression in EOC and demonstrating a positive association between PD-L1, TIL and patient survival in optimally debulked HGSC.

The association between PD-L1 expression and TIL is consistent with recent findings in melanoma and a variety of epithelial cancers (11, 36, 38). Using laser capture microdissection, Taube and colleagues (11) detected IFN- γ at the interface between TIL and PD-L1⁺ cells in melanoma, providing direct evidence for an IFN- γ -mediated adaptive resistance mechanism. Accordingly, using the TCGA dataset, we found an association between IFN- γ and PD-L1 at the gene expression level (**Supplementary Table 1**). Taube and colleagues (11) also found that a significant proportion of tumors contained TIL yet were PD-L1 negative, suggesting that not all TIL responses are sufficiently vigorous to induce PD-L1 expression. As for the prognostic benefit associated with PD-L1 positivity, they speculated this might reflect the fact that many patients in their study had received some form of immunotherapy (36). However, patients in our study received only standard chemotherapy, suggesting that the immunological benefits associated with adaptive resistance extend to conventional treatments as well. To explain this, we speculate that PD-L1 expression may be a marker of arrested TIL responses that are rejuvenated by the immune stimulatory effects of chemotherapy (40). Furthermore, PD-L1 could potentially diminish the suppressive activity of PD-1⁺ Tregs (28), resulting in a net positive effect on anti-tumor immunity. Thus, given the complexity of the tumor microenvironment, there are several possible mechanisms to account for the survival benefit associated with PD-L1 expression.

Although the strong association between PD-L1 expression and TIL reported here is suggestive of an adaptive resistance mechanism, one still needs to account for why PD-L1 is

predominantly expressed by TAMs rather than tumor cells. If T-cell derived IFN- γ is indeed a major mediator of PD-L1 expression in ovarian cancer, as described in other settings (11), one could speculate that ovarian tumor cells may be less IFN- γ responsive than TAMs. Alternatively, perhaps while cleaning up tumor debris, TAMs come to present high levels of tumor antigen, making them prime targets for T cell recognition. In this scenario, expression of PD-L1 may provide a means to protect TAMs from bystander killing. Moreover, these macrophages could conceivably be present in both the stromal and intraepithelial regions of the tumor, as was observed herein by IHC. This scenario would also fit well with our previous findings that CD103⁺ (intraepithelial) CD8 tumor infiltrating lymphocytes frequently express the immune checkpoint molecule PD-1 and are essentially quiescent in the tumor microenvironment (17). Thus it is unlikely that these CD8 cells would be capable of driving widespread PD-L1 expression on tumor cells themselves.

In addition to IFN- γ , several other factors can induce PD-L1 expression. Hypoxia has been shown to induce PD-L1 expression via HIF-1 α (15), and TAMs are frequently found in hypoxic tumor regions. In addition, our finding that PD-L1 positive tumors have higher densities of Tregs raises the possibility that these cells might induce PD-L1 expression through secretion of IL-10, which preferentially affects myeloid cells over tumor cells (12). As for tumor-intrinsic factors, high mutation load has been proposed to promote PD-L1 expression in some cancers (9); however, we found no evidence for this in the TCGA dataset for HGSC. Furthermore, PD-L1 gene expression was not associated with BRCA1 or BRCA2 impairment, both of which are important for homologous DNA repair. Finally, PD-L1 positivity has been associated with EGFR mutations in lung cancer, activation of the phosphatidylinositol-3-OH kinase pathway in glioma,

and a variety of signaling pathways in melanoma (9). The extent to which these various factors regulate PD-L1 expression by TAMs and tumor cells in EOC awaits further investigation.

Our findings may help inform the effective deployment of PD-1 blockade in EOC. Studies in other tumor sites have found correlations between clinical response and expression of PD-L1 by tumor cells (36) or immune infiltrates (38) or the combination of PD-L1⁺ cells and CD8 TIL (39). In our analysis of optimally debulked EOC patients, the cases that best matched these criteria were those with a PD-L1⁺/CD8⁺ (Type I) pattern, which constituted 57.4% of HGSC cases and 16-27% of the other histological subtypes (**Table 1**). If one narrows this definition by focusing on PD-1⁺ TIL, 32.8% of HGSC cases showed a PD-L1⁺/PD-1⁺ pattern. Although clinical trials of PD-1 blockade in EOC are still at an early stage, objective response rates have so far ranged from 6-17% (4-7). This suggests that additional factors may need to be taken into consideration to more accurately predict responses. For example, we found in HGSC that PD-L1 was associated with other negative regulators, including Tregs, LAG-3, CTLA-4, and IDO-1 (**Supplementary Table 1**). It is possible that PD-1 blockade, by enhancing TIL activity, might exacerbate these other suppressive mechanisms, thereby dampening clinical responses. Notably, however, Herbst and colleagues found a trend toward increased response to PD-L1 blockade in tumors that expressed PD-L1 in combination with a second negative regulator (38). If a similar phenomenon applies to EOC, then the most heavily suppressed tumors might respond best to PD-1 blockade. Moreover, efficacy might be augmented by targeting these other pathways through combination approaches.

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JRW, KM and DK conceived and carried out experiments. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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Figure Legends

Figure 1

Immunohistochemical detection of PD-L1. Representative images of single-color IHC using anti-PD-L1 antibody SP142 are shown for lymphoma cell lines known to be either PD-L1 negative (SU-DHL-5; *upper left*) or PD-L1 positive (Karpas 1106P; *upper right*). The lower panels show examples of staining in non-small cell lung cancer (*lower left*) and HGSC (*lower right*).

Figure 2

PD-L1 is expressed predominantly by CD68⁺ TAMs in HGSC. **A** HGSC specimens were subjected to three-color IHC with antibodies against PD-L1 (brown), CD68 (red) and WT-1 (blue). Representative examples of the three major PD-L1 expression patterns are shown. **B** Representative images of HGSC after two-color IHC with antibodies against PD-L1 and CD68. The same field is shown as a bright field image (*upper*) with PD-L1 (blue) and CD68 (brown), or as a dark field image (*lower*) with pseudo-coloring with Nuance software to reveal double positive cells (yellow). **C** Graphical representation of the percentage of tumors from a prospective HGSC TMA (N=74) with the indicated patterns of PD-L1 expression.

Figure 3

Association between PD-L1 expression and TIL in EOC. **A** A 490-case EOC TMA was analyzed by dual-color IHC with antibodies to PD-L1 and CD8 and by single color IHC with antibodies to PD-1 (17), CD103 (29) and FOXP3 (28). Cores were scored as PD-L1 negative (open circles) or PD-L1 positive (closed circles), and the absolute number of cells that were positive for each indicated marker (regardless of location) is shown. Statistical comparisons

between indicated groups were performed using the Mann-Whitney test. **B** HGSC cases (N=195) were stained with antibodies against CD25 and FoxP3 (as previously described (28)), and the absolute number of CD25⁺FoxP3⁺ Treg cells (regardless of location) is shown for PD-L1 negative (open circles) and PD-L1 positive (closed circles) cases. **C** Three-color IHC with spectral separation (Nuance Multispectral Imaging platform) was used to assess the co-localization of the indicated TIL subsets with PD-L1⁺ cells in a 74-case HGSC TMA. *Upper panel.* Both CD4 (red) and CD8 (purple) TIL were commonly found in regions containing PD-L1⁺ cells. *Lower panel.* CD103⁺ (red) and CD103⁺PD-1⁺ TIL (purple) were commonly found in regions containing PD-L1⁺ cells.

Figure 4

Prognostic significance of PD-L1 in EOC. **A** The 490-case TMA was stained with anti-PD-L1 antibody, and disease-specific survival for PD-L1 positive and negative cases was assessed by Kaplan-Meier analysis. **B.** The additional prognostic value of CD8 TIL (*left*) and PD-1⁺ TIL (*right*) in the context of PD-L1 positivity was assessed using a cutoff of >1 CD8⁺ or PD-1⁺ cell per core (regardless of location). Kaplan-Meier analysis was used to compare outcomes for patients with tumors that were positive for one, both, or neither marker. The log-rank test was used to compare groups, and P values of less than 0.05 were considered significant.

Figure 5

Association of PD-L1 (CD274) with other immune-related gene products in HGSC. TCGA gene expression data was used to stratify HGSC cases as *PD-L1/CD274* positive (above median expression) or negative (below median expression). Statistical associations between *PD-L1/CD274* and the indicated immune response genes were assessed as described in Methods. P-

601 values of < 0.0028 were considered significant. **A.** CD68, a marker of TAMs. **B.** Gene products
602 associated with cytolytic T cell responses. **C.** Gene products associated with immune
603 suppression.

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Table 1. PD-L1 expression in ovarian cancer

Histologic subtype	Number of cases	Number of PD-L1 ⁺ cases (%)	Classification based on CD8 infiltration and PD-L1			
			Type I - (PD-L1 ⁺ CD8 ⁺)	Type II - (PD-L1 ⁻ CD8 ⁻)	Type III - (PD-L1 ⁺ CD8 ⁻)	Type IV - (PD-L1 ⁻ CD8 ⁺)
High Grade Serous	195	112 (57.4)	112 (57.4)	10 (5.1)	0 (0)	73 (37.4)
<i>Grade</i>						
2	56	28 (50)	28 (50)	4 (7.1)	0 (0)	24 (42.9)
3	139	84 (60.4)	84 (60.4)	6 (4.3)	0 (0)	49 (35.3)
<i>Stage</i>						
I	47	27 (57.4)	27 (57.4)	2 (7.4)	0 (0)	18 (38.3)
II	84	50 (59.5)	50 (59.5)	5 (6.0)	0 (0)	29 (34.5)
III	64	35 (54.7)	35 (54.7)	3 (4.7)	0 (0)	26 (40.6)
Low Grade Serous	11	0 (0)	0 (0)	1 (9.1)	0 (0)	10 (90.9)
<i>Stage</i>						
I	1	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
II	6	0 (0)	0 (0)	0 (0)	0 (0)	6 (100)
III	4	0 (0)	0 (0)	0 (0)	0 (0)	4 (100)
Mucinous	30	8 (26.7)	8 (26.7)	5 (16.7)	0 (0)	17 (56.7)
<i>Grade</i>						
1	11	3 (27.3)	3 (27.3)	2 (18.2)	0 (0)	6 (54.5)
2	17	4 (23.5)	4 (23.5)	3 (17.6)	0 (0)	10 (58.8)
3	2	1 (50)	1 (50)	0	0 (0)	1 (50)
<i>Stage</i>						
I	17	4 (23.5)	5 (29.4)	2 (11.8)	0 (0)	10 (58.8)
II	12	2 (16.7)	2 (16.7)	3 (25.0)	0 (0)	7 (58.3)
III	1	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
Endometrioid	125	30 (24)	28 (22.4)	18 (14.4)	2 (1.6)	77 (61.6)
<i>Grade</i>						
1	82	20 (24.4)	19 (23.2)	12 (14.6)	1 (1.2)	50 (60.1)
2	35	8 (22.9)	7 (20.0)	5 (14.3)	1 (2.9)	22 (62.9)
3	8	2 (25)	2 (25)	1 (12.5)	0 (0)	5 (62.5)
<i>Stage</i>						
I	69	21 (30.4)	20 (30.0)	9 (13.0)	1 (1.4)	39 (56.5)
II	50	8 (16)	7 (14.0)	7 (14.0)	1 (2.0)	35 (70.0)
III	6	1 (16.7)	1 (16.7)	2 (33.3)	0 (0)	3 (50.0)
Clear cell	129	21 (16.2)	21 (16.2)	39 (30.2)	0 (0)	69 (53.5)
<i>Grade</i>						
1	-	-	-			
2	-	-	-			
3	129	21 (16.3)	21 (16.3)	39 (30.2)	0 (0)	69 (53.5)
<i>Stage</i>						
I	67	8 (11.9)	8 (11.9)	19 (28.4)	0 (0)	40 (59.7)
II	54	13 (24.1)	13 (24.1)	17 (31.5)	0 (0)	24 (44.4)
III	8	0 (0)	0 (0)	3 (37.5)	0 (0)	5 (62.5)

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B

C

A

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