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Biology of Human Tumors

Clinical Cancer Research

Clinical Use of Programmed Cell Death-1 and Its Ligand Expression as Discriminatory and Predictive Markers in Ovarian Cancer

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Abstract

Purpose: We aimed to establish whether programmed cell death-1 (PD-1) and programmed cell death ligand 1 (PD-L1) expression, in ovarian cancer tumor tissue and blood, could be used as biomarkers for discrimination of tumor histology and prognosis of ovarian cancer.

Experimental Design: Immune cells were separated from blood, ascites, and tumor tissue obtained from women with suspected ovarian cancer and studied for the differential expression of possible immune biomarkers using flow cytometry. PD-L1 expression on tumor-associated inflammatory cells was assessed by immunohistochemistry and tissue microarray. Plasma soluble PD-L1 was measured using sandwich ELISA. The relationships among immune markers were explored using hierarchical cluster analyses.

Results: Biomarkers from the discovery cohort that associated with PD-L1$^+$ cells were found. PD-L1$^+$ CD14$^+$ cells and PD-L1$^+$ CD11c$^+$ cells in the monocyte gate showed a distinct expression pattern when comparing benign tumors and epithelial ovarian cancers (EOCs)—confirmed in the validation cohort. Receiver operating characteristic curves showed PD-L1$^+$ and PD-L1$^+$ CD14$^+$ cells in the monocyte gate performed better than the well-established tumor marker CA-125 alone. Plasma soluble PD-L1 was elevated in patients with EOC compared with healthy women and patients with benign ovarian tumors. Low total PD-1$^+$ expression on lymphocytes was associated with improved survival.

Conclusions: Differential expression of immunological markers relating to the PD-1/PD-L1 pathway in blood can be used as potential diagnostic and prognostic markers in EOC. These data have implications for the development and trial of anti–PD-1/PD-L1 therapy in ovarian cancer. *Clin Cancer Res; 23(13); 3453–60. ©2016 AACR.*

Introduction

Ovarian cancer remains the most lethal gynecological cancer in the developed world. Five-year survival is typically 46% (1). Advances in therapies have improved survival, but overall burden of mortality remains high, accounting for 4.2% of deaths worldwide (2). Standard treatment includes cytoreductive debulking surgery and chemotherapy. Epithelial ovarian cancer (EOC) accounts for 90% of all ovarian cancers and is therefore the focus of this research.

Patients who have no apparent residual disease following surgery (complete cytoreduction) have improved prognosis; however, patients with EOC often present late, which contributes to poor survival (3, 4). No biomarkers currently exist to allow the accurate prediction of achieving complete cytoreduction, nor the patient's response to surgery. In women with a pelvic mass, an elevated CA-125 has sensitivity of 72% and specificity of 78% for EOC (5). Only 50% of early-stage patients have increased CA-125. Therefore, CA-125 is an unreliable biomarker, and the development of an accurate biomarker may improve diagnosis and possibly survival.

EOC is regarded as immunogenic, and immune responses may play an important role in prognosis. For example, overall survival (OS) for EOC is influenced by the presence or absence of tumor-infiltrating lymphocytes (TIL; 5-year OS; 73.9% vs. 11.9%, respectively; ref. 6). The total number of lymphocytes has also been shown to be prognostic (7), and the composition of tumor-infiltrating immune cells changes according to stage of disease (8). A meta-analysis evaluating the prognostic value of TILs in EOC has shown that intraepithelial CD3$^+$ or CD8$^+$ TILs correlate with significantly longer OS [HR, 2.24; 95% confidence interval (CI), 1.71–2.91; for TIL-negative tumors; ref. 9).

The tumor microenvironment uses several mechanisms to evade antitumor immune responses. Programmed cell death

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Translational Relevance

This study is set apart from others in this field by using liquid biopsies to detect immune molecules of interest in the context of a solid tissue cancer. We found that differential expression of blood-based immune markers can discriminate benign tumors from ovarian cancer and appear to be prognostic. Furthermore, our work shows that tissue-based immune markers are associated with survival, and soluble PD-L1 in the blood may be a surrogate marker for expression of PD-L1 in tissue.

These are significant findings as these markers may be useful as clinical tools early in a patient's management by predicting prognosis and potential response to anti–PD-1 therapy, which could be determined with a blood test. There is now an opportunity to target this pathway to achieve therapeutic response and improved survival using novel anti–PD-1 and anti–PD-L1 antibodies by the potential use of these biomarkers.

ligand 1 (PD-L1) is highly upregulated on some tumor cells as well as immune cells and has been implicated in tumor immune homeostasis (10–12). PD-L1 suppresses T-cell activation when it interacts with its receptor (PD-1) expressed on activated T cells (13). Phosphorylation of the PD-1 receptor, upon ligand engagement, causes downregulation of the antigen receptor signaling and inhibits T-cell proliferation.

High PD-L1 expression on tumor cells has been associated with poor prognosis in EOC (14). Furthermore, we have previously shown significant differences in the expression of PD-L1 on monocytes and PD-1 on T cells, obtained from blood and ascites, in patients with benign and malignant ovarian tumors (11). These studies demonstrate the potential for PD-1/PD-L1 to be used as diagnostic and prognostic markers in EOC. Targeted immunotherapies that block PD-1/PD-L1 interactions have shown great promise in the treatment of cancer and are being tested in EOC (15).

In this study, we investigate a panel of immunological markers, chosen based on previously published work in cancers including EOC, in order to identify diagnostic and prognostic markers that may facilitate clinical decision making (11, 14, 16). These markers may pave the way for a rapid test to discriminate between EOC and benign tumors.

Patients and Methods

Ethical approval was granted by West London and GTAC Research Ethics Committee (04/Q0406/79 and 10/H0707/7).

Blood, ascites, and tumor samples were collected before or during surgery and before chemotherapy (174 women enrolled 2007–2012; Supplementary Fig. S1). Blood and/or ascites were obtained from 126 women: 11 healthy volunteers, 10 borderline ovarian tumors, 71 EOCs, and 34 benign ovarian tumors. Data from all patients with ovarian pathology (EOC, benign, borderline) were used in the creation of the heatmaps, and data from healthy patients were used as a control group for the ELISA experiment. Data from benign and EOC patients were used to compile all other figures, and the characteristics of these patients are summarized in Supplementary Table S1. The discovery set included 48 benign and EOC patients and the validation set 49. Data from 30 consecutive patient samples from the discovery set have been previously published (11). All patients with EOC had primary cytoreductive surgery followed by standard chemotherapy; 72% achieved complete surgical cytoreduction. Tumor from a further 48 patients was analyzed by immunohistochemistry (IHC; 33 high-stage serous EOC and 15 benign tumors).

OS and progression-free survival (PFS) were measured as days between diagnosis and death or progression, respectively, or last known contact. Patients lost to follow-up were censored when they were last seen. Median follow-up was 20 months. CA-125 was recorded at presentation.

Isolation of mononuclear cells from peripheral blood (PBMCs) and ascites (AMCs)

Mononuclear cells were obtained from blood and ascites by gradient separation as previously published (11).

Flow cytometry

Cells isolated from blood and ascites were incubated with Fc receptor blocking agent (Miletenyi Biotec). Mononuclear cells were stained with FITC-, phycoerythrin (PE)-, and allophycocyanin (APC)-conjugated monoclonal antibodies against CD3, CD4, CD8, CD28, CD19, CD69, CD25, CD86, CD14, CD11c, and PD-1 (BD Bioscience), FOXP3 (Biolegend), CTLA-4, and HLA-DR (Abcam) specific for surface and intracellular cell markers. Matched isotype controls were used for each antibody to determine the gates (Supplementary Fig. S2). The full protocol has been previously published (11).

Construction of tissue microarrays and IHC

PD-L1 expression on tumor-associated inflammatory cells was assessed by IHC staining (Dako) sections of ovarian tumors. Tissue microarrays were constructed using 48 ovarian tumors. Each paraffin-embedded tissue block had two areas identified to represent the tumor's center and invasive margin; five cores were taken from each tumor. Cores were cut (5 μm) and then microwaved (10 minutes at 850W in pH 6.0 citrate buffer), for antigen retrieval, and stained with hematoxylin and eosin and for immunological markers using the avidin–biotin complex (ABC; Vectorlab) and Supersensitive Polymer detection (Biogenex). Total number and percentage of PD-L1–positive ($PD\text{-}L1^{+}$) tumor inflammatory cells were determined by two pathologists. The cells were identified by their morphology and location within the tumors, i.e., epithelial or stromal. When multiple sections from one tumor were assessed, the mean was calculated.

Hierarchical clustering

Hierarchical cluster analysis was applied using Euclidean distance and average linkage method. The stability of clusters was evaluated via a bootstrap resampling method implemented in *pvclust* package in R (v2.10.1), which estimated an approximately unbiased (AU) *P* value for each cluster using 1,000 times multiscale bootstrap resampling (17). Clusters were considered strongly supported by the data when the AU *P* value was >95%.

Statistical analysis

Statistical analyses were performed using R and SPSS. The Mann–Whitney *U* test was used to assess differential expression of immune markers between tissue types (significance $P < 0.05$). In boxplots, the upper and lower limits of the box represent 75th

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and 25th centiles, respectively. The horizontal line represents median. Whiskers indicate upper or lower quartile plus or minus 1.5 times the interquartile range. ROC curves assessed each biomarker's tissue discriminatory ability by determining the AUC. OS and PFS were determined using univariate log-rank and multivariable Cox proportional hazards adjusting for age, stage, grade, histology, and residual disease. Patients with EOC were allocated to groups (high or low) using the IHC score's median expression level as the cut-off. Expression of markers was a continuous variable in Cox models.

Sandwich ELISA for the detection of soluble PD-L1

Immuno Maxisorb 96-well plates (Fisher Scientific) were coated with capture antibody (rabbit anti-human PD-L1, Sino-Biological), diluted in coating buffer (0.1 mol/L Sodium carbonate, pH 9.5) to a final concentration of 1 μg/mL, overnight at 4°C, then washed with wash buffer (PBS with 0.05% Tween-20). Assay diluent (AD, 200 μL; PBS with 10% heat-inactivated FCS, at pH 7.0) was added as a blocking agent and incubated (1 hour at room temperature). Plasma samples were centrifuged at 22,000 g. Note that 100 μL of plasma samples or recombinant PD-L1 protein (Sino Biological), used to create the standard curve, were added to each well and incubated (overnight 4°C). The plate was washed before 100 μL of goat anti-human PD-L1 detection antibody (R&D Systems), 0.1 μg/mL, was added and incubated (2 hours at room temperature) and washed with wash buffer. Streptavidin–HRP complex (Biolegend) was diluted 1:1,000 in AD and added to the plate and incubated (2 hours at room temperature). The plate was washed, and 50 μL of tetramethylbenzidine substrate (BD Bioscience) was added and incubated (30 minutes at room temperature). Stop solution (50 μL 1 mol/L H_3PO_4) was added and absorbance at 450 nm measured.

Results

Discovery of differentially expressed immune markers in PBMCs and AMCs of patients with EOC or benign ovarian tumors

To determine differential expression of immune markers between benign tumors and EOC, immune markers on PBMCs and AMCs were analyzed by flow cytometry. Heatmaps represent immune marker data obtained from 48 patients in the discovery cohort tissue microarray (Fig. 1). There were four distinct clusters of markers in PBMCs and AMCs (Supplementary Fig. S3). Percentage of monocytes PD-L1$^+$, monocytes PD-L1$^+$ CD14$^+$, and monocytes PD-L1$^+$ CD11c$^+$ showed a distinct expression pattern when comparing benign tumors and EOCs (Fig. 1A).

Validation of discovered immune markers

Some immune markers found in the monocyte gate were differentially expressed between benign tumors and EOC; percentage of PD-L1$^+$ cells, PD-L1$^+$ CD14$^+$ cells, and PD-L1$^+$ CD11c$^+$ cells

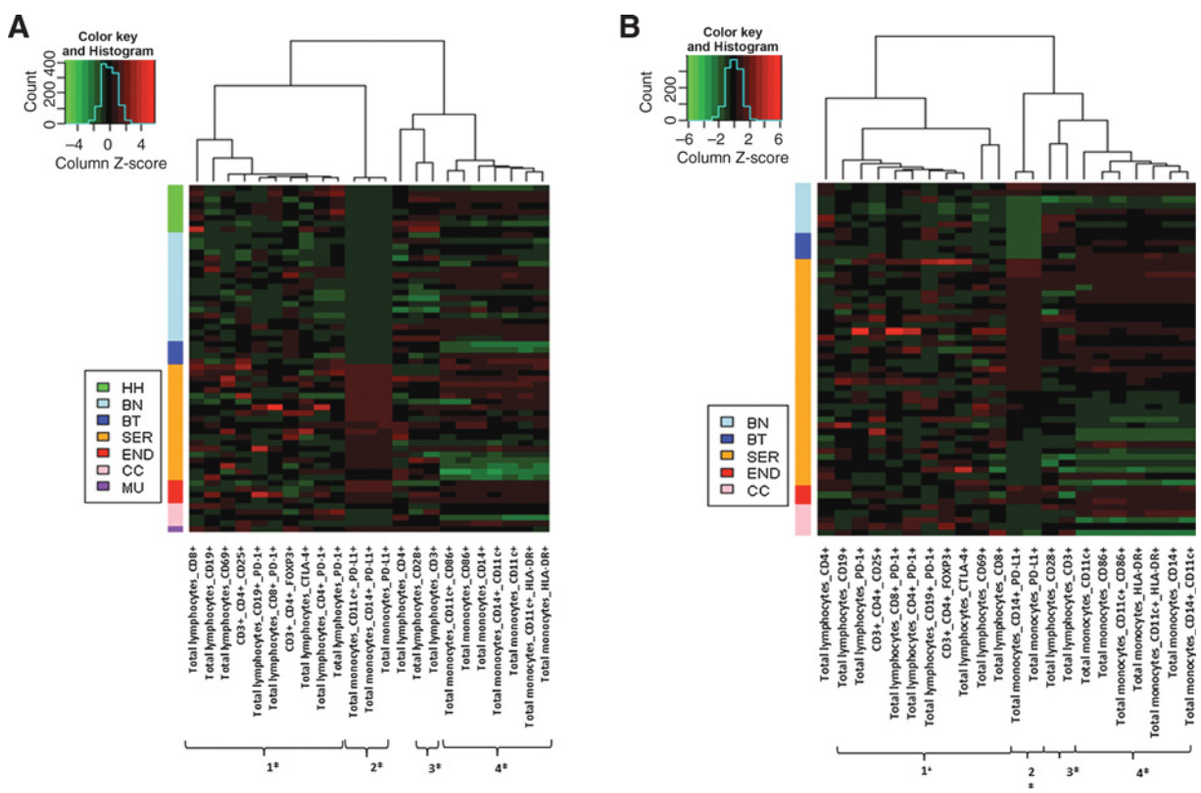

Figure 1.

Heatmaps of candidate biomarkers in blood and ascites. In PBMC samples, 23 markers were analyzed (**A**), and 22 markers were studied in ascites samples (**B**). Unsupervised hierarchical clustering of candidate biomarkers in healthy controls (HH), benign ovarian tumors (BN), borderline ovarian tumor (BT), and EOCs (SER, serous; END, endometrioid; CC, clear cell; MU, mucinous). *, cluster is well supported by data (AU $P > 95\%$); +, cluster not significant (AU P value 90%).

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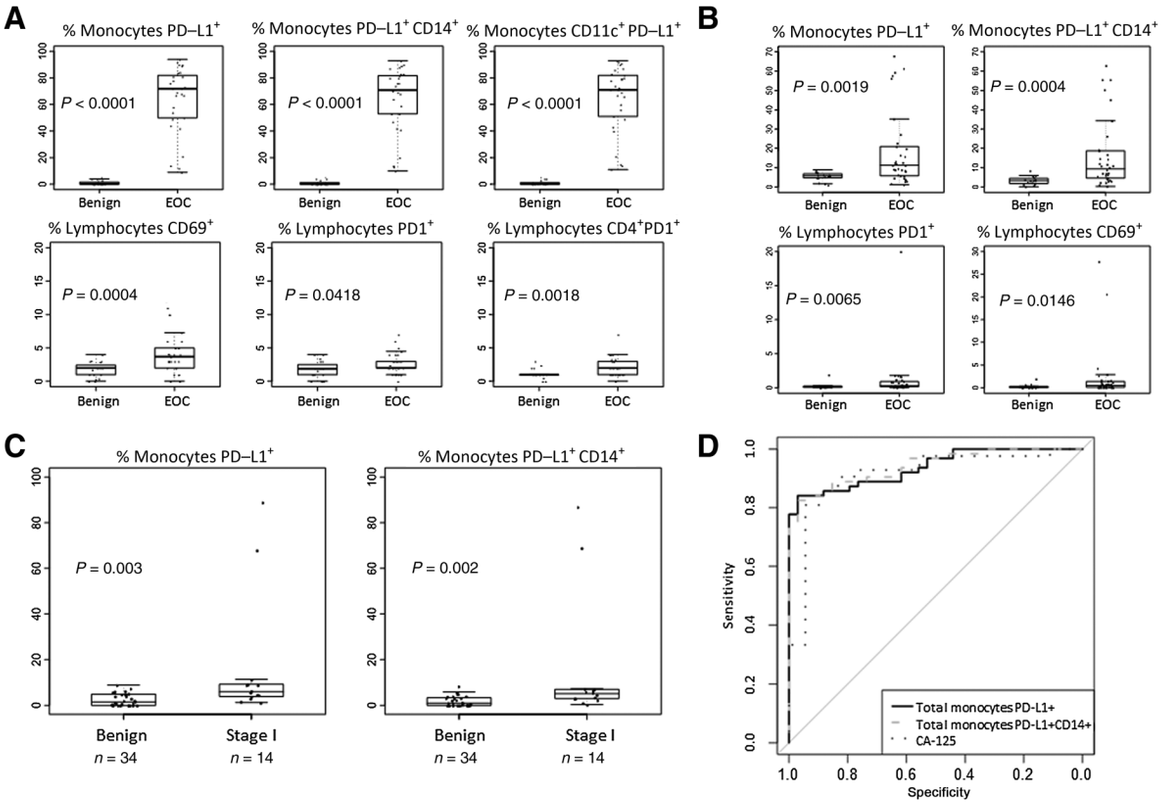

Figure 2.

Differential expression of immune markers on PBMCs in patients with benign tumors and EOCs in the discovery and validation cohorts. **A,** The percentage of monocytes and lymphocytes (and their subsets) in PBMCs from patients with benign tumors or EOC that expressed PD-1, PD-L1, and CD69. **B,** Differential expression of PBMC markers between benign tumors and EOC in the validation set. Analysis was repeated after excluding data >4 SD from the mean, to ensure statistical significance was not driven by extreme outliers, and all results remained significant ($P = 0.0019$, % monocytes PD-L1$^+$/$P = 0.0004$, % monocytes PD-L1$^+$CD14$^+$, $P = 0.009$, % lymphocytes PD-1$^+$/$P = 0.026$, % lymphocytes PD-1$^+$). **C,** The percentage of monocytes in PBMCs that were PD-L1$^+$ or PD-L1$^+$ CD14$^+$ in the validation cohort was significantly different in patients with benign ovarian tumors and stage I EOC. Analysis was repeated after excluding data >4 SD from the mean to ensure statistical significance was not driven by extreme outliers. Both results remained significant ($P = 0.005$, % monocytes PD-L1+/$P = 0.005$, % monocytes PD-L1$^+$ CD14$^+$). Statistical analysis was performed using the Mann–Whitney *U* test (**A–C**). **D,** ROC curve (combined cohorts $n = 63$) showing the sensitivity and specificity of PD-L1$^+$ and PD-L1$^+$ CD14$^+$ cells, compared with CA-125, at discriminating EOC from benign tumors; AUC (95% CI): PD-L1$^+$ = 0.94 (0.89–0.98), PD-L1$^+$CD14$^+$ cells = 0.94 (0.90–0.98), CA-125 = 0.91 (0.83–1.00).

(Fig. 2A). Markers found in the lymphocyte gate also differed between the groups; percentage of CD69$^+$ cells, CD4$^+$ PD-1$^+$ cells, and PD-1$^+$ cells (Fig. 2A). These results were consistent with the data obtained from the clustering analysis. To exclude the confounding effect of different histologic subtypes and grade, we further confined the analysis to high-grade serous (HGS) and benign tumors. Differential expression remained significant in five of the originally discovered six immune markers [monocytes (PD-L1$^+$, PD-L1$^+$ CD14$^+$, PD-L1$^+$ CD11c$^+$) and lymphocytes (CD69$^+$ and CD4$^+$ PD-1$^+$)], and marginal significance was observed in lymphocytes PD-1$^+$ (Supplementary Fig. S4A). Similar results were obtained for ascites (Supplementary Fig. S5).

Four of the immune markers from lymphocyte and monocyte subsets were chosen for validation [lymphocytes (PD-1$^+$ and CD69$^+$) and monocytes (PD-L1$^+$ and PD-L1$^+$CD14$^+$)]. Flow cytometry analysis was performed on blood obtained from a validation cohort of patients to confirm the previous results. Validation was only carried out in blood as this is more clinically usable as a diagnostic test than ascites. When the analysis was repeated in the validation cohort, all four immune markers continued to discriminate (Fig. 2B; Supplementary Fig. S4B).

Clinically, it is important to distinguish between benign tumors and early-stage EOC. To investigate differential expression between these tumors, PBMC expression of PD-L1 was analyzed by flow cytometry. Stage I EOC had significantly elevated total PD-L1$^+$ monocytes and PD-L1$^+$ CD14$^+$ cells in the monocyte gate compared with benign tumors (Fig. 2C). To assess the robustness of these markers at discriminating benign tumors and EOC, a ROC curve was created comparing total PD-L1$^+$ monocytes and PD-L1$^+$ CD14$^+$ cells in the monocyte gate to the well-established CA-125 biomarker (Fig. 2D). Both PD-L1$^+$ and PD-L1$^+$ CD14$^+$ cells in the monocyte gate were better predictors than CA-125 alone; PD-L1$^+$ (AUC 0.94), PD-L1$^+$ CD14$^+$ cells (AUC 0.94), and CA-125 (AUC 0.91). PD-1 and CD69 expressions on lymphocytes were not discriminatory (data not shown). To establish whether a combination of markers would be able to improve the discrimination of EOC from benign tumors, we grouped PD-L1$^+$ monocytes and PD1$^+$ lymphocytes in the ROC analysis, which showed a

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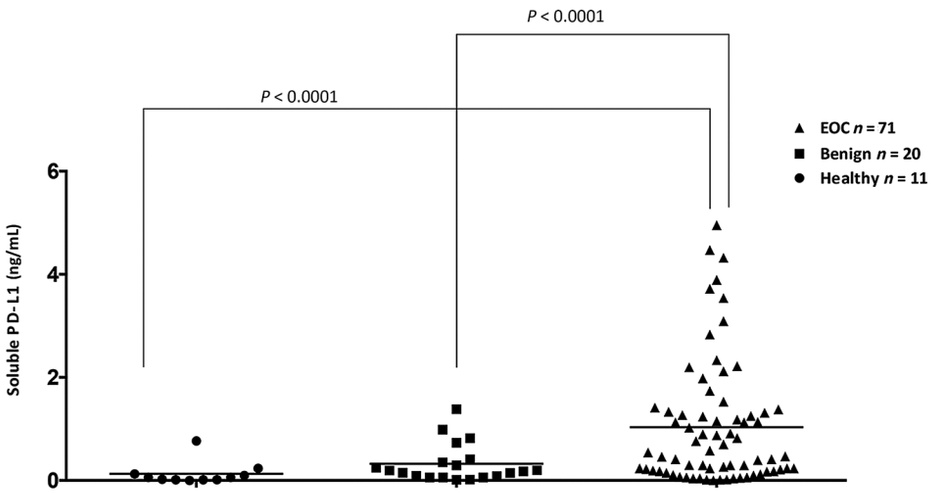

Figure 3.
Expression of sPD-L1 in plasma from healthy females and women with benign and EOC tumors. The sPD-L1 was detected using ELISA. Bars indicate the mean expression. Statistical analysis was performed using the Mann–Whitney *U* test.

comparable AUC of 0.93 as shown in Supplementary Fig. S6A. Further ROC analysis of the HGS sub-group revealed an AUC of 0.98 when using PD-L1$^+$ monocytes alone and an AUC of 0.97 when combining PD-L1$^+$ monocytes and PD1$^+$ lymphocytes (Supplementary Fig. S6B).

Investigation of soluble PD-L1 in plasma by ELISA

ELISA was used to determine soluble PD-L1 (sPD-L1) concentrations as this method is much quicker than flow cytometry and could potentially be developed into a test using sPD-L1 as a surrogate marker of tumor cell and immune cell surface expression of PD-L1. Soluble PD-L1 concentration in the plasma of healthy women, patients with benign tumors, and EOC was measured. Figure 3 shows that there were significantly higher levels of sPD-L1 in the plasma of patients with EOC compared with healthy women and patients with benign tumors. There was no significant difference between sPD-L1 levels in patients with benign tumors and healthy individuals.

Immune markers and association with survival

The four validated immune markers (% lymphocytes PD-1$^+$, % lymphocytes CD69$^+$, % monocytes PD-L1$^+$, and % monocytes PD-L1$^+$CD14$^+$) were analyzed in PBMCs of patients with EOC for the combined patient cohort ($n = 63$). Kaplan–Meier survival curves and multivariable Cox models determined whether the immune markers were associated with PFS and OS. Patients with EOC were allocated to groups (high or low) using the PBMC immune marker's median expression level as the cut-off. Percentage of lymphocytes that were PD-1$^+$ was associated with PFS (Cox $P = 0.001$; adjusted HR 1.25, 95% CI, 1.09–1.43; Fig. 4A; Supplementary Table S2). Low

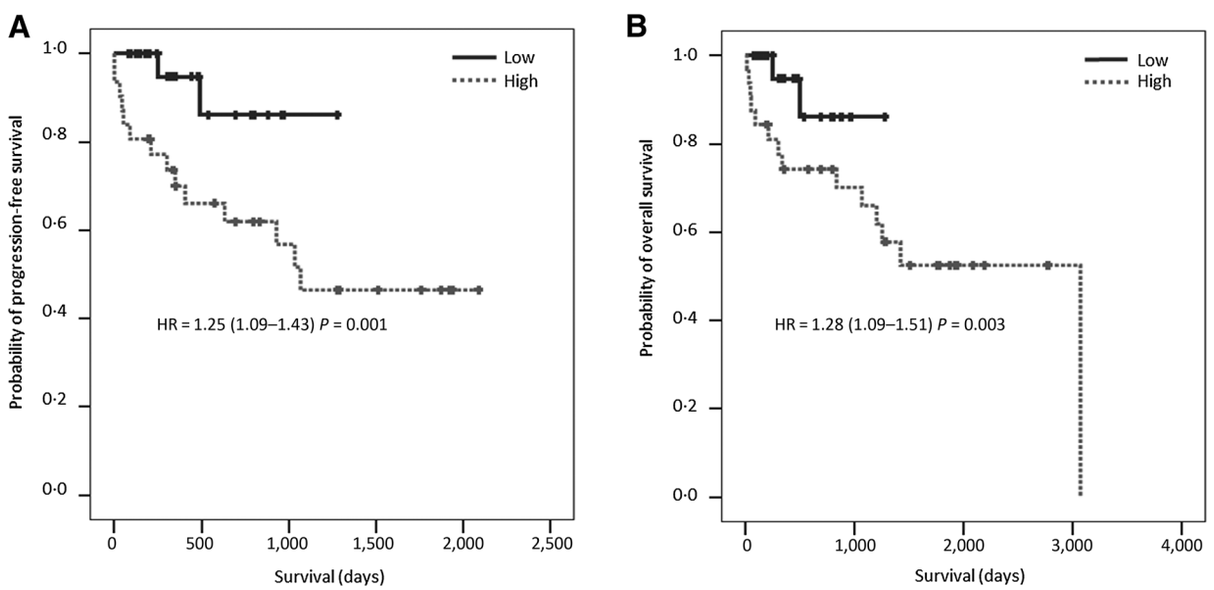

Figure 4.
Kaplan–Meier plots of PFS (**A**) and OS (**B**) according to expression of PD-1 in lymphocytes. Expression of PD-1 on lymphocytes in blood obtained by flow cytometry ($n = 63$). Log-rank $P = 0.023$ (**A**) and $P = 0.095$ (**B**). The HR was estimated by multivariable Cox models adjusted for age, stage, grade, histology, and residual disease.

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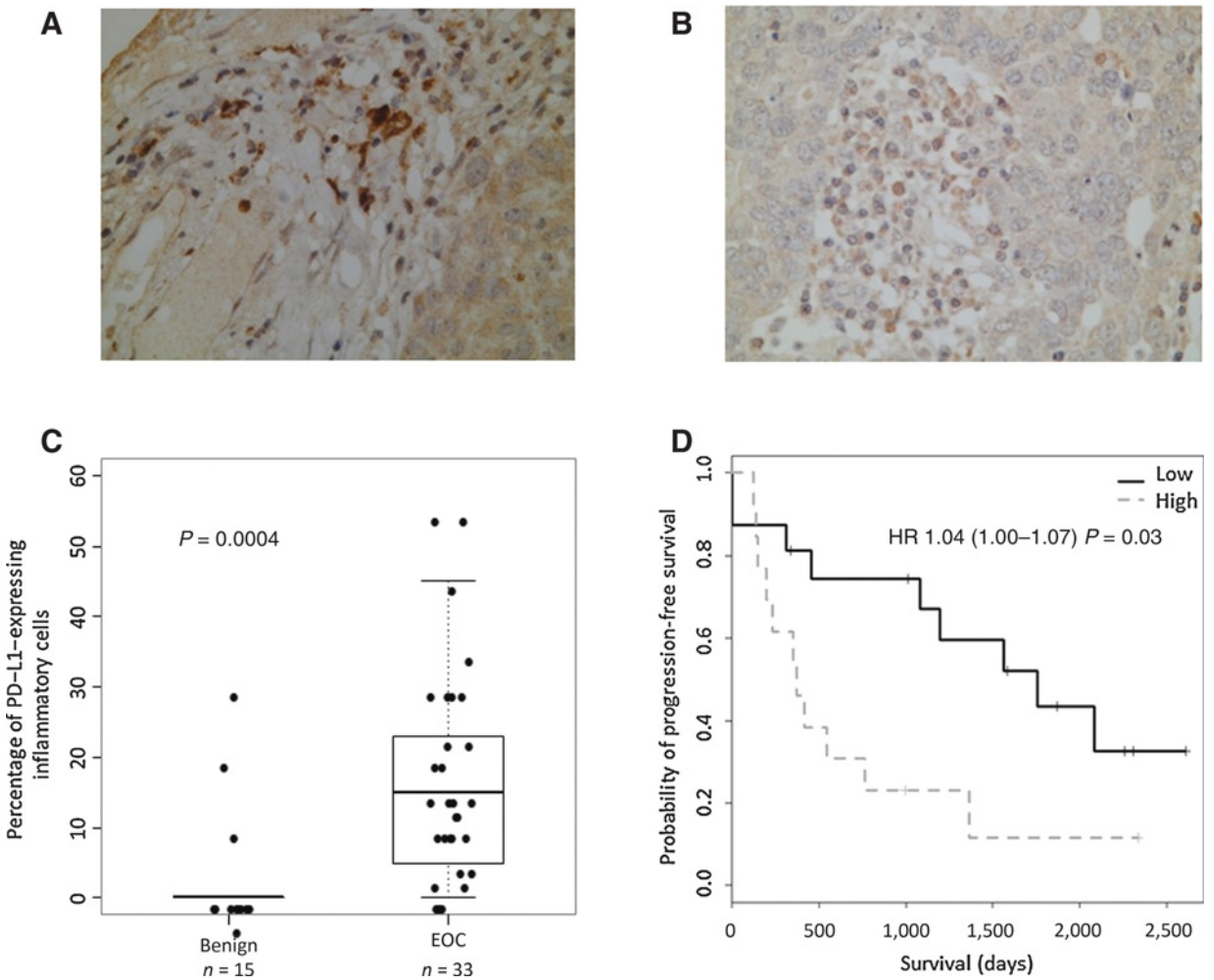

Figure 5.

IHC tissue staining of PD-L1 on tumor-associated inflammatory cells. **A,** Moderate score staining of PD-L1$^+$ inflammatory cells in EOC. **B,** Low score staining of PD-L1$^+$ inflammatory cells in benign ovarian tumor tissue. **C,** Percentage of PD-L1$^+$ inflammatory cells in benign and EOC tissue; statistical analysis was performed using the Mann–Whitney *U* test. **D,** Kaplan–Meier survival curve showing significantly shorter PFS in women with EOC who have above median (high) levels of PD-L1$^+$ inflammatory cells in tumor tissue (log-rank $P = 0.024$). Cox model adjusted for age, stage, grade, histology, and residual disease.

percentage of lymphocytes PD-1$^+$ was associated with improved OS (Cox $P = 0.003$; adjusted HR 1.28, 95% CI, 1.09–1.51; Fig. 4B; Supplementary Table S2). The other immune markers did not correlate with survival (data not shown).

Validation of the significant immune markers by IHC

IHC was performed as a further validation of PD-L1 as a diagnostic marker in EOC. Consistent with the data obtained by flow cytometry, the results show a higher percentage of PD-L1$^+$ tumor–associated inflammatory cells in EOC when compared with benign tumor tissue (Fig. 5A–C). PD-L1 expression on tumor-associated inflammatory cells was analyzed for prognostic value. Patients were dichotomized, into high and low PD-L1 IHC expression score, using the median. A continuous Cox proportional hazards analysis showed, despite low sample numbers, that high percentage of PD-L1$^+$ tumor–associated inflammatory cells was associated with an increased risk of disease progression (Fig. 5D). No association was found with OS.

Discussion

Current diagnostic models do not enable clinicians to reliably distinguish benign tumors from early-stage EOC. Therefore, there remains a clinical need for a robust diagnostic test for ovarian masses to ensure rapid recourse to the correct treatment for patients that require the most urgent intervention. Currently, clinical indices dictate whether patients with EOC receive primary surgery or upfront chemotherapy, rather than tumor biology or a prognostic biomarker. The development of an accurate test to determine treatment and so improve outcome would be a significant step toward personalization of care in ovarian cancer. Immunological biomarkers have useful prognostic potential in a variety of cancers (16, 18) and can predict the response to novel immunotherapies such as anti–PD-L1 and anti–PD-1-blocking antibodies (19).

PD-L1 expression on tumor has been correlated previously to prognosis (11, 14, 20). To our knowledge, this is the first study investigating expression of immunological markers in the blood

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of women with ovarian masses. Our initial heatmap cluster analysis (Fig. 1) identified strong clusters separating serous and endometrioid carcinomas from benign and borderline tumors—the clustering for mucinous and clear-cell tumors was less well defined. The numbers of mucinous and clear-cell patients precluded our ability to analyze these as sub-groups and therefore we combined them for all analyses as EOC. We would expect the cluster analysis to be more clear-cut if we had focused on the serous and endometrioid subtypes, given their strong clustering in the heatmaps. However, we argue that it is important to include all EOC histology types together as one group in the analysis to reflect patient populations in the clinic, especially as all EOCs are clinically managed the same way with surgical cytoreduction and chemotherapy irrespective of histology.

We have shown differential PBMC expression of immune markers in patients with EOC and benign tumors in separate discovery and validation cohorts (Fig. 2A and B). This is clinically valuable because PBMCs are easily obtained via relatively non-invasive blood tests. Metastasis of EOC is generally not thought to be hematogenous; therefore, the discovery of immunological signatures in the blood is interesting and may represent a generalized systemic effect on the immune system with the upregulation of PD-L1 and PD-1 representing an immunoregulatory response in EOC.

The difficulty in diagnosing EOC lies in differentiating stage I EOC from benign or borderline tumors. Our data suggest that PD-L1 expression on monocytes and $CD14^+$ cells in blood is significantly upregulated in stage I EOC compared with benign tumors (Fig. 2C) which may be a useful clinical tool in women who present with a pelvic mass. Stage I tumors are more likely to be clear-cell, mucinous, or low-grade serous. In the comparison between stage I EOC and benign tumors, endometrioid, HGS, and mucinous subtypes accounted for 64%, 21%, and 15% of the tumors, respectively. Therefore, the differential expression of total $PD\text{-}L1^+$ monocytes and $PD\text{-}L1^+$ $CD14^+$ mainly reflect the difference between stage I endometrioid and benign tumors. Due to small sample size in the mucinous and clear-cell subtypes, we were unable to perform the analysis in these two subtypes. Further investigation of these markers in different EOC subtypes and grades with adequate sample size would be warranted.

Although advances have been made using predictive mathematical models and ultrasound, to distinguish ovarian tumor types, sensitivity can still be as low as 50% with specificity of 84% (21). The positive predictive value of a single CA-125 measurement for the early detection of EOC is 57% (22). Sensitivity and specificity for the combination of the ultrasound algorithm and CA-125 in specialist centers can have an AUC of 0.94 (0.93–0.95) for discriminating all-stage EOC from benign tumors (23). CA-125 in our cohort had a similar AUC of 0.91 (0.83–1.00), the wider AUC range likely due to smaller numbers in our cohort. We have shown that PD-L1 expression on circulating monocytes is comparable to CA-125 with an AUC of 0.94 (0.89–0.98) for all cancer stages (Fig. 2D). This marker therefore has the potential to serve as a useful tool for early-stage EOC diagnosis. It appears from our results that little, if anything, is gained from combining multiple markers, and $PD\text{-}L1^+$ monocytes perform well as a single marker (Fig. 2D; Supplementary Fig. S6A and S6B). Subanalysis of only HGS patients shows that $PD\text{-}L1^+$ monocytes can be used as a biomarker to discriminate HGS from benign tumors (AUC 0.98; Supplementary Fig. S6B). However, the clinical dilemma is not the ability to discriminate HGS from benign disease, it is the ability to identify early-stage EOC and therefore the analyses in Supplementary Fig. S2D and Supplementary Fig. S6A, which includes all histologies, would be more applicable to clinical practice.

Soluble PD-L1 in plasma of patients with EOC was found to be significantly higher than in patients with benign tumors or healthy individuals (Fig. 3). sPD-L1 has been shown to be elevated in the serum of patients with other cancers and was found to be associated with poor prognosis (24, 25). It has also been shown that sPD-L1 is produced and released by activated mature dendritic cells and suggested that immune and tumor cells could be the source of sPD-L1 (26). Measuring sPD-L1 may be a surrogate marker for surface PD-L1, which could be readily tested in the clinic with a blood test. Further work is required to explore whether these immunological markers in blood are raised in other conditions of inflammation, chronic infection, and autoimmunity to establish their efficacy as diagnostic biomarkers and also to monitor response to therapy or recurrence of disease.

Notably in our study, a low percentage of lymphocytes expressing PD-1 was associated with favorable survival outcome (Supplementary Fig. S4A and S4B). This is a significant finding as it may be a useful prognostic tool, early in a patient's management. PD-1 expression may also be of relevance in determining which patients may benefit from anti–PD-1/PD-L1 therapy. The median follow-up in this study was 20 months. This relatively short period is a limitation in this prospective study. However, given that the median time to first recurrence is 18 to 24 months, we can be confident in our estimation of PFS. Accurate determination of OS will require a longer follow-up.

IHC analysis of tumor tissue has shown that tumor-associated inflammatory cells had different PD-L1 expression in benign tumors and EOC (Fig. 5A–C). Furthermore, the PD-L1 expression on tumor-associated inflammatory cells appears to be significantly associated with prognosis, specifically shorter PFS (Fig. 5D). Previous studies have reported correlation with prognosis and PD-L1 expression on tumor cells across different histologic subtypes of EOC (14, 20). Our data are based on PD-L1 expression on tumor-associated inflammatory cells, rather than the tumor cells themselves. This may have more biologic significance from an immunological standpoint as a recent study, based on tissue samples of patients treated with therapeutic anti–PD-L1 antibody, showed that response correlated to the presence of PD-L1–positive tumor-associated macrophages (19). However, we recognize that our findings are not in keeping with a recent study which shows longer PFS with $PD\text{-}L1^+$ tumor–associated macrophages in HGS ovarian cancer (27). Therefore, these findings should be further investigated in a larger study.

We have shown that increased expression in monocytes of $PD\text{-}L1^+$ and $PD\text{-}L1^+$ $CD14^+$ cells and expression in lymphocytes of $PD\text{-}1^+$ and $CD69^+$ cells are associated with EOC and that expression of PD-1 on lymphocytes correlates with prognosis. These markers may therefore be useful as diagnostic tools in this disease. Furthermore, given the importance of immunoregulation through PD-1 and PD-L1, this suggests that expression of PD-1 and PD-L1 on monocytes and lymphocytes may provide important information on how and when blocking antibodies to these molecules may work.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by the other authors.

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