The immune environment in ovarian cancer
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Chapter 5

PD-L1 expression is associated with tumor-infiltrating lymphocytes and better prognosis in high-grade serous ovarian cancer

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*Authors contributed equally

Manuscript in preparation
Abstract

**Purpose** Tumor-infiltrating lymphocytes (TIL) are associated with favorable prognosis in high-grade serous ovarian cancer (HGSC). Nevertheless, most intraepithelial TIL upregulate PD-1, and may be suppressed by checkpoint inhibition through PD-L1 expressed in the tumor environment. We therefore analyzed whether PD-L1 and PD-1 are expressed in HGSC, how these molecules relate to immune cell infiltration and whether these molecules negatively affect the prognostic benefit of TIL.

**Methods** The expression of PD-L1 in the tumor environment was assessed by immunohistochemistry in tissue from 171 advanced stage HGSC patients. Infiltration by CD3+, CD8+, CD27+, PD-1+, CD20+, CD16+, CD14+, CD68+, CD163+, CD11c+, and LAMP3+ immune cell populations was quantified by immunohistochemistry.

**Results** PD-L1 was expressed on the tumor epithelium and on immune cells in the tumor stroma. Expression of PD-L1 was associated with better prognosis and strongly correlated with lymphoid and myeloid cell infiltration. Further, combined analysis of PD-L1 expression and infiltration by PD-1+ TIL revealed PD-L1 as a defining marker for a favorable prognosis in HGSC patients.

**Conclusions** PD-L1 expression is associated with better prognosis and might serve as a biomarker for an active immune response. Combined analysis of PD-L1 and PD-1 can be used for stratifying patients for immunotherapeutic approaches.
Introduction

High-grade serous ovarian cancer (HGSC) is a malignancy characterized by a low survival rate, largely due to diagnosis at advanced stage and a high rate of chemoresistant recurrences after initial treatment. Influx of tumor-infiltrating lymphocytes (TIL) in ovarian tumors is strongly associated with improved survival (1,2). This prognostic effect is not only dependent on the number of TIL, but also on the treatment regimen, surgical outcome, and T cell differentiation status (3). Patients treated with primary surgery (PS) and adjuvant chemotherapy benefit from a high number of infiltrating TIL, while patients treated in a neo-adjuvant chemotherapy setting (NACT) do not. Further, the prognostic benefit of TIL is largely restricted to patients in whom cytoreductive surgery results in removal of all macroscopic tumor tissue (3). Patients with TIL infiltrate and in whom the tumor cannot be completely removed surgically might therefore benefit from strategies that augment existing antitumor immunity, e.g. PD-1 checkpoint inhibition.

The PD-1 pathway is a major checkpoint in lymphocytes in which binding by its ligand PD-L1 leads to a suppression of T cell function. Checkpoint inhibition targeting the PD-1/PD-L1 axis is currently under major investigation to enhance anti-tumor immune responses. In various solid malignancies, this strategy has shown activity leading to improvement of patient survival (4,5). Interestingly, expression of PD-L1 can be found on tumor cells as well as on immune cell subsets. PD-L1 expression can be either constitutively expressed (induced by oncogenic signaling in tumor cells) (6,7) or induced upon IFN-γ expression as produced by immune cells in the tumor environment (8–10). On immune cells, expression of PD-L1 is observed mainly on tumor-infiltrating myeloid cells (TIM), such as tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC) that originate from myeloid precursors in the peripheral blood. In response to cues they receive from the tumor microenvironment these cells undergo specific differentiation. TAM can be roughly divided into two distinct polarization states: the classically activated M1 macrophages, and the alternatively activated M2 macrophages (11,12). While M1 macrophages produce IL-2 and confer anti-tumor effects, M2 macrophages are considered to be a suppressive immune population, producing IL-10 amongst other immune-suppressive cytokines. We and others have previously shown that the presence of CD163+ M2-like macrophages is associated with worse survival in ovarian tumors, at least in part by suppression of CD8+ TIL (13–15).

Nevertheless, it remains largely unknown how expression of PD-L1 correlates with TIL and TIM infiltrate in HGSC. Furthermore, since prognostic benefit from TIL is differential between patients treated with PS or NACT, differences may exist in the suppressive environment of these tumors. Therefore, we analyzed the immune infiltrate and PD-L1 expression in tumors of a large cohort of advanced stage HGSC patients treated either with PS or NACT.
Methods

Patients

Patients with advanced stage (FIGO ≥IIB) high-grade serous ovarian cancer diagnosed at the University Medical Center Groningen (UMCG) between January 2000 and December 2011 were selected for the current study. Patient data were retrieved from an institutional database into a new anonymous database, in which patient identity was protected with unique codes. According to Dutch law no approval from our institutional review board was needed. Patient characteristics are depicted in Table 1.

Tissue microarray

A tissue microarray (TMA) was constructed as described previously (3). From each TMA block 4 μm thick sections were cut and applied to APES-coated slides (Starfrost, Braunschweig, Germany). The presence of tumor in the arrayed samples was confirmed by H&E staining by a gynecologic pathologist.

Immunohistochemistry

TMA slides were stained for various immune markers by immunohistochemistry. CD8 and CD27 expression were assessed in a previous study (3). For the other markers, slides were subjected to heat-induced epitope retrieval by microwave the slides in a citrate buffer (pH 6.0), after which endogenous peroxidase was blocked in a 0.3% H₂O₂ solution. Primary antibodies were incubated overnight: mouse anti-human CD3 (DAKO; clone: F7.2.38; 1:25 in blocking buffer (1% BSA/PBS with 1% human AB serum)), rabbit polyclonal anti-human CD14 (Sino Biological; 2 μg/ml), mouse anti-human CD16 (GeneTex; clone: GTX75392; 1:50), rabbit polyclonal anti-human LAMP3 (Sino Biological, 1:25), mouse anti-human CD20 (DAKO; clone: L26; 1:200), rabbit anti-human CD11c (Abcam; clone: EP13474; 1:25). This was followed by visualization of antibody binding by use of either anti-mouse or anti-rabbit Envision (DAKO) and 3,3’-diaminobenzidine (DAB). Slides were counterstained with hematoxylin. PD-L1 and PD-1 staining was performed by use of Ventana Discovery Ultra Platform for automatic staining. Primary antibodies used for this strategy were a rabbit anti-human PD-L1 (clone E1L3N, Cell Signaling Technology) and a mouse anti-human PD-1 (clone NAT105, Abcam) antibody. Furthermore, a sequential dual staining was performed on the Ventana Discovery Ultra platform to identify CD163+ cells using a mouse anti-human CD163 antibody (clone 10D6, Leica Biosystems) with DAB chromogen, and CD68+CD163- using a mouse anti-human CD68 antibody (clone PD-M1, DAKO) with Discovery purple chromogen.
Scoring

All samples were scored by two individuals that were blinded for patient characteristics. Intratumoral lymphocytes and myeloid cells were counted manually and calculated to number of cells/mm². Stromal infiltration of myeloid populations was scored as percentage of positive staining of the total stromal region. PD-L1 expression on tumor cells as well as on immune cell infiltrates were scored as the percentage of cells with moderate to strong staining for each tumor.

Statistical analysis

Differences in cell infiltration and patient characteristics were analyzed by Spearman correlation and Kruskall-Wallis, Mann Whitney U or χ² tests. Survival analysis was performed by use of the Kaplan Meier method, with the Log rank test to detect for survival differences between groups of patients. Disease-specific survival (DSS) was defined as the time period from date of surgery or first chemotherapy treatment until death due to ovarian cancer or last follow-up. Patients were stratified on the basis of the highest tertile of immune cell infiltrate, tumoral PD-L1 expression was assessed as present or absent, and PD-L1 on stromal regions was considered positive above cut-off 10%. All p-values of <0.05 were considered significant, and all tests were performed two-sided. Analyses were performed by use of IBM SPSS 22 (SPSS inc., Chicago, USA). To profile the immune environment, heat maps were constructed by unsupervised hierarchal clustering data using complete linkage and Euclidean distance in the function ‘heatmap.2’ of the ‘gplots’ package in R. (R Core Team, a language and environment for statistical computing, 2015. R Foundation for Statistical Computing, Vienna, Austria).
Results

**PD-L1 expressed on tumor epithelium and stromal cells in HGSC and associates with improved prognosis**

We determined the expression pattern of PD-L1 in two clinically relevant cohorts of HGSC patients. The total cohort consisted of 171 advanced stage HGSC patients; patient characteristics are depicted in Table 1. This cohort has been extensively described previously (3) and consists of patients primarily treated with surgery (PS cohort, n=87) and patients treated with neo-adjuvant chemotherapy before interval surgery (NACT cohort, n=84). Analyzed tissue was obtained at the time of primary or interval surgery. Expression of PD-L1 on tumor cells was focal and present in 17.3% and 16.0% of patients in the PS and NACT cohort, respectively (Fig. 1A). Expression was limited to a small percentage of tumor cells, ranging from 1 to 20% of total tumor cells stained. Expression of PD-L1 in stromal regions of the tumor showed a pattern that reflected immune infiltrates (Fig. 1A) and was above cut-off (>10%) in 23.2% and 24.0% of patients (PS and NACT cohort; Fig. 1B). Kaplan Meier analyses revealed a significant longer disease-specific survival (DSS) for patients with any PD-L1 expression on tumor cells in the PS cohort (p=0.027, Fig. 1C). By contrast, no correlation with DSS was found for tumor epithelium-expressed PD-L1 in the NACT cohort (p=0.683, Fig. 1D). Stromal expression of PD-L1 was also significantly associated with improved survival in the PS cohort (p=0.027, Fig. 1E) and not in the NACT cohort (p=0.068, Fig. 1F).

**Expression of PD-L1 strongly associates with immune infiltration in HGSC**

Since PD-L1 can be expressed on tumor epithelium and immune cells as a result of IFN-γ produced during an ongoing immune response, we assessed whether expression of PD-L1 was associated with immune cell infiltration in HGSC. TMA slides were analyzed by immunohistochemistry for infiltration of CD3+, CD8+, CD27+, PD-1+, CD20+ TIL and CD14+, CD16+, CD11c+, LAMP3+, CD68+CD163- and CD163+ TIM cell populations (Supplementary Fig. 1A). The distribution of the immune cell populations in the tumor epithelium of both the PS and NACT cohorts is depicted in Supplementary Fig. 1B. All TIL and most TIM subsets showed a similar distribution pattern between the two cohorts, except for CD14+ and CD11c+ cells, which each were more abundantly present in tumors of patients treated by NACT (p=0.002 and p=0.012, respectively. Both tumor epithelium and stromal regions were characterized by an abundant myeloid immune infiltrate (Supplementary Fig. 1C). The most abundantly present stromal cell types in both cohorts were CD14+ and CD163+ cells, with a higher infiltrate in the PS cohort compared to the NACT cohort (p=0.002 and p=0.010, respectively). In addition, CD16+ cells were present in larger numbers in the stroma of PS patients than in the stroma of patients treated with NACT (p<0.001). As was observed for epithelial infiltration of CD11c+ cells, patients treated with NACT displayed a higher infiltration of CD11c+ into the stroma.
when compared to patients treated by PS (p<0.001).

Table 1: Clinicopathologic characteristics

<table>
<thead>
<tr>
<th></th>
<th>PS TMA cohort (N=87)</th>
<th>NACT TMA cohort (N=84)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (SD)</td>
<td>64.1 (11.3)</td>
<td>64.4 (8.6)</td>
</tr>
<tr>
<td><strong>FIGO stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIb</td>
<td>3 (3.4%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>IIC</td>
<td>5 (5.7%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>IIIa</td>
<td>2 (2.3%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>IIIb</td>
<td>6 (6.9%)</td>
<td>2 (2.4%)</td>
</tr>
<tr>
<td>IIlc</td>
<td>56 (64.4%)</td>
<td>64 (76.2%)</td>
</tr>
<tr>
<td>IV</td>
<td>15 (17.2%)</td>
<td>18 (21.4%)</td>
</tr>
<tr>
<td><strong>Surgical cytoreduction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No residual tissue</td>
<td>39 (44.8%)</td>
<td>27 (32.1%)</td>
</tr>
<tr>
<td>≤1cm residual tissue</td>
<td>15 (17.2%)</td>
<td>39 (46.4%)</td>
</tr>
<tr>
<td>&gt;1cm residual tissue</td>
<td>33 (37.9%)</td>
<td>18 (21.4%)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;59</td>
<td>27 (31.0%)</td>
<td>20 (23.8%)</td>
</tr>
<tr>
<td>≥59</td>
<td>60 (69.0%)</td>
<td>64 (76.2%)</td>
</tr>
<tr>
<td><strong>Chemotherapy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no chemotherapy</td>
<td>5 (5.7%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Platinum-based</td>
<td>82 (94.3%)</td>
<td>84 (100.0%)</td>
</tr>
</tbody>
</table>


Interestingly, patients in the PS cohort that expressed PD-L1 on tumor epithelium had significantly more intraepithelial CD3+, CD27+, PD-1+, CD16+, and CD14+ infiltrate (Fig. 2A). For PS patients that were characterized by a stromal expression of PD-L1, a similar pattern of immune cell infiltration was observed with a high epithelial infiltration of CD3+, CD8+, CD27+, PD-1+, CD16+, CD163+ and CD11c+ cells (Fig. 2B). Furthermore, stromal expression of PD-L1 was associated with increased numbers of stromal CD68+CD163+ and CD11c+ cells (Fig. 2C and 2D). Therefore, for patients treated with primary surgery, a high infiltration of TIL and TIM into the tumor epithelium is associated, in general, with PD-L1 expression on tumor cells in epithelial regions and with expression of PD-L1 on immune cells in stromal regions. By contrast, in patients that received neo-adjuvant chemotherapy prior to interval surgery, expression of PD-L1 on the tumor epithelium did not correlate to intratumoral infiltration by any immune cell population (Fig. 3A). Expression of Pd-L1 on stromal cells, on the other hand, was correlated with an increased intraepithelial infiltration of CD3+, CD8+, CD27+, PD-1+, CD20+, CD163+, CD11c+ and LAMP3+ cells, (Fig. 3B), and was associated with a higher number of stromal CD16+, CD11c+ and LAMP3+ cells (Fig. 3C and 3D).
Figure 1: PD-L1 expression in HGSC tumors. **A)** Representative images of positive and negative stained tumor and stroma for PD-L1. **B)** PD-L1 expression in percentage of total area tumor epithelium or stroma region in PS and NACT cohort. Disease-specific survival (DSS) analyses of PD-L1 expression in HGSC patients. Kaplan Meier curves depicting DSS for **C)** Tumors with or without epithelial PD-L1 expression in primary surgery cohort (PS). **D)** Tumors with or without epithelial PD-L1 expression in neo-adjuvant chemotherapy cohort. **E)** Tumors with or without stromal PD-L1 expression in PS cohort. **F)** Tumors with or without stromal PD-L1 expression in NACT cohort.
Immune infiltration in HGSC predicts PD-L1 expression

Based on these data, we wondered whether hierarchical clustering of patients based on immune cell infiltration could also predict the observed expression of PD-L1 on tumor and immune cells. Clustering patients based on immune populations revealed 3 major clusters of patients in both the PS as the NACT cohort. Different clusters were observed in PS and NACT patients, likely as a result of the different composition of the immune microenvironment. In the PS cohort, one cluster was characterized by low infiltration of all immune populations studied (cluster 1). The other two clusters both had medium to high TIL, but showed differences in the myeloid infiltrate, with cluster 2 having a high infiltration of TIM and cluster 3 a low infiltrate of these cells (Fig. 4A). In the NACT cohort, a similar cluster with low infiltrate of all cell populations could be found (cluster 1), cluster 2 was characterized by high infiltration of TIL and TIM, while cluster 3 was characterized by a high infiltration of DC subsets (LAMP3 and CD11c positive), but low to medium TIL and TIM infiltrate (Fig. 4B). Importantly, clusters characterized by a high immune infiltrate were also associated with high expression of PD-L1 on both the tumor and immune cells (Fig. 4A and 4B).

In line with the survival data observed for expression of PD-L1, survival analyses of the clusters in the PS cohort revealed that cluster 3 had a significantly longer survival as compared to cluster 1 (p=0.013), and no significant difference with cluster 2 (p=0.114) (Fig. 4C). In the NACT cohort, no differences in survival between patients within the three different clusters were found (Fig. 4D).
The immune environment in ovarian cancer

- **A**
  - PS cohort
  - High
  - Medium
  - Low
  - Cluster 1
  - Cluster 2
  - Cluster 3
  - CD3 tumor
  - CD8 tumor
  - PD-1 tumor
  - CD27 tumor
  - CD68 tumor
  - CD20 tumor
  - LAMP3 tumor
  - CD163 tumor
  - CD14 tumor
  - CD11c tumor
  - CD11c stroma
  - CD14 stroma
  - LAMP3 stroma
  - CD68 stroma
  - CD163 stroma
  - CD68 stroma
  - PD-L1 (epithelium)
  - PD-L1 (stroma)

- **B**
  - NACT cohort
  - High
  - Medium
  - Low
  - Cluster 1
  - Cluster 2
  - Cluster 3
  - CD16 tumor
  - CD14 tumor
  - CD163 tumor
  - LAMP3 tumor
  - CD14 stroma
  - CD16 stroma
  - CD68 stroma
  - CD163 stroma
  - PD-1 tumor
  - CD27 tumor
  - CD3 tumor
  - CD8 tumor
  - CD11c stroma
  - CD11c tumor
  - CD68 tumor
  - CD20 tumor
  - PD-L1 (epithelium)
  - PD-L1 (stroma)

- **C**
  - PS cohort
  - Disease-specific survival
  - Follow-up in years
  - Cluster 1
  - Cluster 2
  - Cluster 3

- **D**
  - NACT cohort
  - Disease-specific survival
  - Follow-up in years
  - Cluster 1
  - Cluster 2
  - Cluster 3

- **Table**
  - PD-L1 (epithelium)
    - PS cohort: 3.4%
    - NACT cohort: 3.4%
  - PD-L1 (stroma)
    - PS cohort: 28.6%
    - NACT cohort: 28.6%
Expression of PD-L1, infiltration by PD-1+ TIL and treatment strategy are key determinants of survival in HGSC

While the expression of PD-L1 was strongly associated with immune infiltration, not all tumors with a high PD-1+ infiltrate expressed PD-L1. Therefore, we next analyzed whether a combined analysis of PD-L1 and PD-1 expression could be used as a superior prognostic indicator in HGSC. In the PS cohort, 92.6% of the samples had infiltrating PD-1+ cells with a median infiltration of 26.79 cells/mm² tumor (IQR: 7.26-74.63). Prognostic characteristics were analyzed for differences in PD-1+ cell count in order to determine whether these factors influence intratumoral PD-1+ TIL infiltration. Age (p= 0.908), FIGO stage (p= 0.217), or surgical cytoreductive outcome (p= 0.620) did not show differences in total PD-1 count. Similar infiltration patterns were observed in the NACT cohort, with 90.4% of samples having PD-1+ cells present, with a median infiltration of 22.82 cells/mm² tumor (IQR: 7.43-73.42) and also here no differences for age (p=0.393), FIGO stage (p=0.284), or surgical cytoreduction outcome (p=0.943). Concluding that PD-1 infiltration was not dependent on any clinicopathological characteristics or treatment regimen.

For subsequent survival data analysis, patients were divided based on the group of patients with the highest infiltration of PD-1+ cells (highest tertile) vs. the group with low or no infiltration in their tumor. DSS analysis based on infiltration by PD-1+ cells revealed a significant improvement of survival (p=0.014) in the PS cohort for the group with high intratumoral infiltration compared to patients with low or no infiltration of such cells (Figure 5A). In the NACT cohort, no differences in survival were detected between the groups with either a high or a low infiltration by PD-1+ cells (p=0.916, data not shown). When the PS cohort was further categorized based on patients with or without PD-L1+ expression, a clear survival difference was observed. Indeed, patients who had a high infiltration with PD-1+ T cells and expression of PD-L1 demonstrated a significantly longer survival time compared to patients with high infiltrate of PD-1+ TIL who did not show PD-L1 expression. Survival of this group was comparable to the group with a low PD-1+ infiltrate infiltrate (Figure 5B).

In conclusion, expression of PD-L1 and infiltration by PD-1+ TIL are key determinants of survival in patients treated with primary surgery and adjuvant chemotherapy.
Figure 5: Disease-specific survival (DSS) analyses of patients with high PD-1+ cell infiltrate in HGSC patients. Kaplan Meier curves depicting DSS for A) Tumors with PD-1+ infiltrate in primary surgery cohort (PS). B) Tumors expressing PD-L1 and PD-1+ cell infiltrate.
Discussion

In the present study we demonstrate that PD-L1 can be expressed on both tumor cells and immune infiltrate in HGSC. This expression is strongly associated with increased lymphocyte and myeloid cell infiltration. In line with this observation, PD-L1 expression was associated with an improved prognosis of HGSC. Furthermore, tumor PD-L1 expression was predictive for improved prognosis in patients with high TIL infiltrate, and may therefore serve as a biomarker for an active immune response.

Checkpoint inhibition targeting PD-1 or PD-L1 has been successful in re-activating the immune response for the treatment of several solid malignancies (4,5). For ovarian cancer, success has been limited so far with blockade of PD-L1 in a phase I trial leading to an objective response in 1 out of 17 patients (5), and PD-1 blockade in a phase II trial to 2 complete responses out of 20 patients (16). For both studies, the studied population consisted of patients with platinum-resistant recurrences with heterogeneous histological and clinicopathological characteristics. Identification of biomarkers that define which patients might benefit from this treatment regimen is therefore essential to stratify patients and increase the clinical efficacy of these strategies. In melanoma, patients that responded best to this checkpoint inhibition treatment had a pre-existing immune response characterized by CD8+ (PD-1+) TIL infiltration. After treatment this immune infiltrate was extended in number and clonality of T cells (10). Unfortunately, the utility of PD-L1 as a biomarker for success of anti-PD-1 antibody treatment is limited, although in melanoma success rates are higher in patients with high levels of PD-L1 expression in their cancers (17). In a clinical trial with anti-PD-1 in ovarian cancer, PD-L1 expression on tumor cells was not correlated with success of treatment, but responding patient numbers were too small to allow a definitive analysis (16). Here, we show that PD-L1 correlates strongly with immune infiltrate and survival in HGSC, suggesting that it might serve as a biomarker for selection of patients for checkpoint blockade. Determination of cut-off for positive expression levels, as well as analysis of PD-L1 expression on tumor cells and the immune infiltrate (8) are therefore likely necessary for further evaluation of this biomarker in trials.

It has been shown previously that the vast majority of intraepithelial TIL in HGSC tumor co-express PD-1 (3,18). In confirmation of this, we found a high influx of PD-1+ TIL in these tumors to be associated with improved survival. PD-L1 expression was associated with a high influx of PD-1+ cells in the tumor, and both markers were associated with improved survival in the PS cohort. This is in line with findings in several other tumor types (19,20) and might be explained by the fact that PD-L1 upregulation on tumors can be induced by IFN-γ in the environment (9), which is likely a result of production of tumor-reactive TIL. Indeed, correlations have been found between TIL, IFN-γ and PD-L1 expression in HPV+ head and neck cancer (21). An active anti-tumor immune response may lead to an increase in IFN-γ expression...
in the tumor environment and therefore in these tumors PD-L1 expression might actually serve as a surrogate marker for a reactive immune response. To further confirm this, determination of IFN-γ expression in relation to TIL infiltrate and PD-L1 expression in these tumors will be of interest.

In further support of the finding that PD-L1 might serve as a surrogate marker for an active immune response, we found that the prognostic effect of PD-1+ TIL is only present in patients also expressing PD-L1. This subgroup therefore seems to represent the patients with a TIL population that conveys an anti-tumor response. These patients would likely benefit from therapeutic targeting of the PD-1/PD-L1 axis. The remaining group of patients who have a high influx of TIL might benefit from a combination strategy in which tumor reactivity is stimulated and PD-1/PD-L1 is blocked. With this in mind, the results of a recently started trial combining anti-CD27 with anti-PD-1 (Clinicaltrials.gov identifier: NCT02335918) will be of interest, since our recent work demonstrated CD27 and PD-1 are co-expressed on a TIL subset and a clear synergism is to be expected in those patients that do respond to anti-CD27 treatment alone (3,22). Finally, it will be meaningful to determine whether those patients that do respond to agonistic anti-CD27 treatment upregulate PD-L1 on tumor and immune cells in the tumor environment to further confirm PD-L1 as a marker for an active immune response. Furthermore, combination strategies with chemotherapy might be of interest, since carboplatin can induce PD-L1 expression in preclinical models (23), and therefore blockade of the PD-1/PD-L1 axis might increase efficacy of chemotherapy.

Patients in our cohort were primarily treated with surgery and adjuvant chemotherapy (PS) or received neo-adjuvant chemotherapy prior to interval cytoreduction (NACT). In the PS cohort a clear benefit from TIL influx, and PD-L1 expression can be observed. In the NACT cohort, however, PD-L1 expression in these tumors as well as high TIL influx both did not confer a survival benefit. This difference with the PS cohort might be due to selection criteria for patients receiving this treatment, including widespread tumor dissemination and co-morbidities, which may have selected for differential molecular subtypes (24,25). On the other hand, neo-adjuvant chemotherapy might also directly influence immune functioning. The T cell infiltrate between these two groups did not show differences, suggesting chemotherapy might not have an effect on infiltration of TIL. However, when looking at myeloid cell populations we did find striking differences in total infiltrate between the two cohorts. The monocyte marker CD14 and the dendritic cell marker CD11c were expressed in significantly higher numbers in the NACT cohort. This higher influx might be a direct consequence of cell death as caused by the chemotherapy treatment. This, potentially immunogenic, cell death could result in higher influx of the dendritic cell population marked by CD11c. While it is tempting to speculate that these cells could support TIL activity, we did not observe any prognostic benefit from infiltration of CD11c or LAMP3 dendritic cells either alone or in combination with TIL. Nevertheless, it was recently shown that tumor-associated DCs in ovarian
cancer are suppressed to activate T cells. The cause of this might be the induction of ER stress in these cells, caused by hypoxia, nutrient starvation and oxidative stress (26). Since neo-adjuvant chemotherapy might be causative for these factors, it would be interesting to determine whether more ER stress occurs in this cohort.

By creating a suppressive environment, a higher myeloid cell infiltrate might account for the lack of survival benefit conferred by TIL in the NACT cohort. Indeed, a general higher infiltration of CD14+ cells was seen in the NACT cohort, and patients with a high infiltration of CD14+ showed a worse prognosis (not shown). One could hypothesize that chemotherapy may lead to a shift in macrophage polarization; with increased numbers of M2-like macrophages responsible for the lack of survival benefit of TIL infiltrate in NACT treated patients (27). Therefore, we analyzed expression of CD163, a marker for M2-like macrophages (28). This cell type was abundantly present in most of the tumors in both cohorts, and infiltrate was not increased in the NACT cohort. While not discussed in detail here, the suppressive function of this cell subset was observed in the PS cohort, with a high ratio of CD8+ TIL over CD163+ TIM in the tumor accounting for longer survival, confirming previous data in a different cohort (13). Again, in the NACT cohort this effect was absent, suggesting the immune infiltrate does not confer any survival effect in this cohort. To determine whether chemotherapy treatment directly affects the function of TIL infiltrate, it would be of interest to analyze tumor samples taken before and after chemotherapy treatment for a direct comparison without cohort selection bias.

In conclusion, PD-L1 expression in HGSC is highly associated with immune cell infiltrate and prognosis, and might serve as a biomarker for an active immune response. Therefore, PD-L1 could potentially be used to stratify patients for immunotherapeutic approaches.
References


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The authors would like to thank Felix Ward for technical assistance with data analysis.
Supplementary data

Supplementary Figure 1: Infiltration of tumor-infiltrating lymphocytes and myeloid cells (TIL and TIM) in HGSC tumors is dependent on primary treatment strategy. A) Representative immunohistochemistry images of all TIL and TIM markers. B) Intraepithelial infiltration of TIL and TIM in primary surgery (PS) and neo-adjuvant chemotherapy (NACT) cohort. C) Stromal infiltration of TIM in PS and NACT cohort.