

ORIGINAL ARTICLE



Favorable prognostic impact of Natural Killer cells and T cells in high-grade serous ovarian carcinoma

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ABSTRACT

Introduction: The aim of the present study was to investigate the prognostic impact of intratumoral cytotoxic T cells, Natural Killer (NK) cells, neutrophils and PD-L1 expression in patients with epithelial ovarian cancer.

Methods: All patients diagnosed with high-grade serous carcinoma (HGSC) in Denmark in 2005 were included in the study. Immunohistochemical staining for PD-L1, CD8, CD66b and CD57 was performed on tumor tissue from 283 patients. Cell densities were analyzed using a digital image analysis method. The primary endpoint was overall survival (OS).

Results: The median OS for HGSC patients was 30 months. It was 45 months in patients with high level of CD57+ NK cells (≥ 10 cells/mm²) compared with 29 month in patients with low level (< 10 cells/mm²) ($p = .0310$). The median OS was 37 and 25 months in patients with high vs. low level of CD8+ T cells (cutoff 80 cells/mm²) ($p = .0008$). In multivariate analysis, high numbers of CD57+ NK cells and CD8+ T cells remained independent markers of favorable OS, adjusted hazard ratio (HR) 0.67; $p = .041$, and HR 0.72; $p = .020$, respectively. PD-L1 expression was associated with improved OS (37 months vs. 22 months, $p = .0006$), but was only borderline significant in the multivariate analysis (HR 0.77, $p = .061$). CD66b + neutrophils had no association with OS.

Conclusions: In patients with HGSC tumor-infiltrating CD57+ NK cells and CD8+ T cells had favorable prognostic impact, while PD-L1 expression had borderline favorable prognostic significance. CD66b + neutrophils had no prognostic association. These findings may influence future immunotherapy development.

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Introduction

Epithelial ovarian cancer (EOC) is a highly malignant disease. High-grade serous carcinoma (HGSC) is the predominant histological subtype (70%), and often diagnosed in an advanced stage with abdominal spread. Consequently, the curative potential is limited and the outcome is fatal in most patients [1,2].

The treatment is far from ideal, and new approaches are needed. Checkpoint inhibitor treatment has seen great success in some malignant tumors with long-lasting responses and even curative potential [3,4]. Similar results have not been reported in HGSC [5], and a better understanding of the immune system may be important for the development of effective immunological treatment of HGSC.

The presence of immune cells in malignant tumors is well known, and the possible correlation with prognosis has been thoroughly investigated, especially concerning T cells and in particular the cytotoxic T cell, which has been correlated with prognosis in EOC and other malignant tumors [6–11]. The regulation of T cell activity is the primary approach in

recent immunotherapy, either as checkpoint inhibition or T cell therapy [12,13].

Natural Killer (NK) cells are part of the innate immune system. They hold anticancer potential through tumor cell lysing without prior immunization, and they have been investigated for their therapeutic potential in some cancers [14,15]. The prognostic role of tumor-infiltrating NK cells has been investigated in several studies in different cancers pointing toward a positive prognostic impact [16], but the possible prognostic role of intratumoral NK cells in HGSC has only been addressed in one small study suggesting a negative correlation between NK cells and prognosis [17].

Neutrophils are key players in the immune system, constituting 70% of the blood leucocytes. Their functional profile is diverse and complex. Some studies suggested a tumor promotive function through the promotion of angiogenesis, chronic inflammation, metastasis, proliferation and immunosuppression while others showed anti-tumor capabilities through acute inflammation, autophagy, anti-body-dependent cellular cytotoxicity and activation of NK and T

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cells [18–21]. The majority of studies on various cancers points to an unfavorable prognostic role of tumor-infiltrating neutrophils [22,23], but there are no studies on intratumoral neutrophils and prognosis in ovarian cancer.

PD-L1 is a transmembrane protein binding to PD-1 on the T cell with an inhibitory function. PD-1/PD-L1 blockage is a cornerstone in modern immunotherapy, but the prognostic relevance of PD-L1 expression in HGSC is unclear. PD-L1 expression is correlated with a poor prognosis in many cancers [24]. A meta-analysis of 60 studies supported the negative prognostic aspect but with a high degree of heterogeneity across tumor types. In EOC, the literature is sparse with conflicting results from small studies [25–30].

The aim of the present study was to investigate the prognostic impact of intratumoral PD-L1 expression, T cells, neutrophils and NK cells in a population-based cohort of high grade serous ovarian cancer patients.

Material and methods

Patient cohort

The study cohort consisted of all women diagnosed with HGSC in Denmark in 2005 [31] with clinical characteristics registered in the DGCG (Danish Gynecologic Cancer Group) database. Neoadjuvant treatment was not introduced in 2005, making all tissue specimens from this national population-based cohort chemotherapy-naïve. In the nationwide electronic pathology database, Patobank, the surgical specimens of all EOC were identified and requested from the different pathology departments. A total of 496 patients were diagnosed with EOC in Denmark in 2005. Formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks were sent to the Department of Pathology, Vejle Hospital. Tumor tissue was from either the ovaries, the fallopian tubes or the peritoneum. Tumor tissue blocks from 412 patients were confirmed to be EOC and contained sufficient tumor tissue for the immunohistochemical stainings. Of these, 283 were confirmed as HGSC. One tumor FFPE-block was selected from each patient. Date of death, as well as clinical and pathological data was available for all patients.

The study was approved by the Regional Committee on Health Research Ethics for Southern Denmark (project ID: S-20100043) and the Danish Data Protection Agency (18/3854) and complied with the Helsinki declaration.

Immunohistochemistry

Four 3.5 µm thick slides were cut from each FFPE-block. Immunohistochemical staining for PD-L1, CD8, CD66b and CD57 was performed using a Benchmark Ultra automated instrument (Ventana Medical Systems, Roche, Tucson, AZ, USA). A positive and negative control was included in each run. For the PD-L1 analysis, SP263 (Roche Diagnostics, Basel, Switzerland) was selected based on a review of the literature concerning the validity of the most used clones for PD-L1 staining [32,33]. The SP263 clone was delivered ready to use,

with no further dilution needed. All slides were hematoxylin-stained.

In this study, we chose a CD8 antibody as a marker for the cytotoxic T cell based on its predominant expression on this cell type (clone C8/144B, 1:200, Cell Marque, Rocklin, CA, USA) [34]. We used CD57 antibody as a marker for NK cells [35] (clone NK-1, 1:800, Thermo Fischer Scientific, Waltham, MA, USA). As a marker of neutrophils, CD66b which is known to be consistently expressed on neutrophils was selected [36] (clone G10F5, 1:600, BD Biosciences Pharmingen, San Diego, CA, USA).

PD-L1 scoring

Scoring of the PD-L1 expression was performed on all 283 specimens using a conventional light microscope by two of the authors (JRH and MW) independently of each other and blinded to the data. PD-L1 expression was evaluated as the percentage of tumor cells with PD-L1 expression, as shown in Figure 1. In case of disagreement between the observers, a consensus was achieved. The interrater agreement had a Kappa coefficient of 0.6717.

CD8+, CD66b + and CD57+ cell scoring

Automated image analysis, which is a well-established method in the routine setting of pathology, was used in this study. All slides were scanned by a high-resolution scanner, NanoZoomer (Hamamatsu Photonics, Hamamatsu City, Japan), at ×400 magnification. The tumor area was manually outlined on each scanned slide by the two observers assisted by digital image analysis (Visiopharm, Hoersholm, Denmark). Stromal areas were excluded, so only the intratumoral density was analyzed. For the scoring, an app-based algorithm was developed using the Visiopharm Integrator System Software (Visiopharm, Hoersholm, Denmark), the validity of which has proven comparable to stereology [37,38]. The output was the number of stained cells/mm² tumor area. To validate the digital image analysis, these results were initially compared to manual counting on the scanned slides. Five randomly selected areas of 0.2 mm² each were evaluated by JRH in 15 test samples for each antibody. The manual evaluation results were correlated with the software analysis showing Pearson's correlation coefficients of 0.9956, 0.9964 and 0.9729 for CD8, CD66b and CD57, respectively, indicating a good linear correlation of manual counting and the software analysis.

Evaluation

PD-L1 expression was categorized as PD-L1 negative when less than 1% of tumor cells expressed PD-L1 and PD-L1 positive with at least 1% expression. This cutoff was chosen based on the relatively low expression of PD-L1 in ovarian cancer and is the same cutoff chosen by others regarding ovarian cancer and other cancers [30,39]. It was difficult to find validated and standardized cutoff values in the literature for CD8, CD66b and CD57, since there were many different

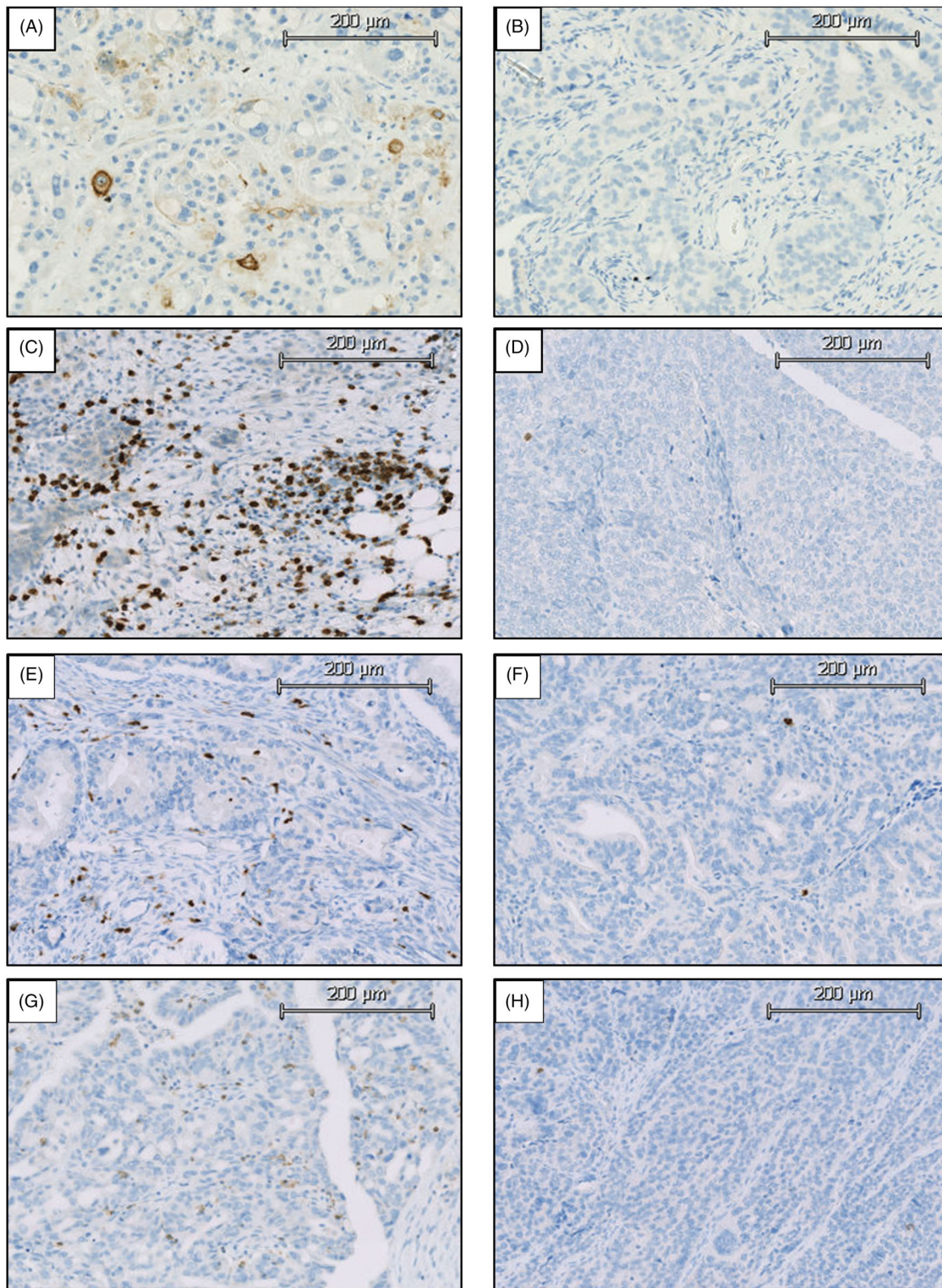


Figure 1. Intratumoral PD-L1 expression and immune cell infiltration. Representatives of the immunohistochemical stainings. (A) PDL1 expression $>1\%$. (B) PD-L1 expression $<1\%$. (C) CD8 >80 cells/mm². (D) CD8 <80 cells/mm². (E) CD66b >60 cells/mm². (F) CD66b <60 cells/mm². (G) CD57 >10 cells/mm². (H) CD57 <10 cells/mm².

cutoffs and fundamental differences in the scoring method. Most of the prior studies in ovarian cancer have counted the number of cells/field view using a light microscope. The output generated from our imaged based digital analysis was number of cells/mm² and is not directly comparable to number of cells/field view due to significant variances in the area 'field view' depending on the optical lens used, which is

often not defined in the studies. It should also be noted that earlier IHC studies, in general, have counted cells in selected parts of the pathology slide and not the whole slide. The use of automated imaged analysis is increasing due to its capability of analyzing larger tissue areas within a shorter time than manual counting, and the technology has the advantage of results being less subjective and more reproducible.

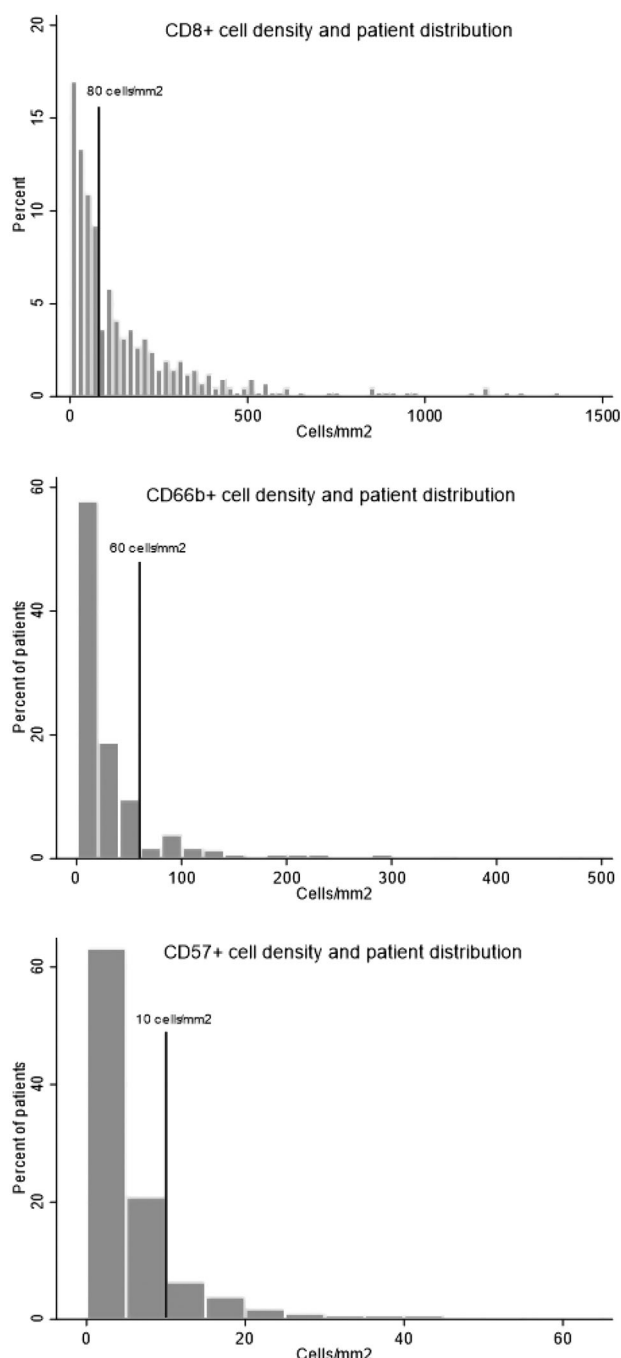


Figure 2. Patient distribution regarding CD8+ T cells, CD66b+ neutrophils and CD57+ NK cells. A similar pattern is seen in all three, i.e., a clustering of patients with cell densities less than 80, 60 and 10 cells/mm², respectively.

In order to identify a cutoff, distributional graphs of the respective cell densities were generated. All three cell types (CD8+, CD66b+ and CD57+) had the same distributional pattern with a high-frequency group of patients having a narrow interval of low cell density and a low-frequency group having a wide interval of high cell density (Figure 2). Cutoffs were set at the points where the distributional graphs optimally separated the two groups, which they all did at the same frequency of 8% (Figure 2). Separation resulted in cutoffs of 80 cells/mm², 60 cells/mm² and 10 cells/mm² for CD8+, CD66b and CD57+ cells, respectively (Figure 2). The

cutoffs categorized cell densities into high and low density as shown in Figure 1.

Statistical analysis

The primary endpoint was overall survival (OS). The Kaplan–Meier plots were used to compare survival and differences analyzed by log-rank testing. An estimation of hazard ratios (HRs) was made using a univariate Cox regression model integrating generally acknowledged prognostic variables in EOC. Markers were only included in the final multivariate Cox regression model if significant ($p < .05$) in the primary Cox regression and likelihood ratio test. Finally, the proportional hazards assumption was tested on all Cox regression models.

Interrater agreement was measured using Cohen's kappa coefficient. Linear and non-linear correlations were evaluated using Pearson's and Spearman's correlation coefficients, respectively. Chi-square test was used as a measure of association between variables. Descriptive, correlational, survival and regression analyses were performed using STATA version 15[®] (StataCorp, College Station, TX, USA).

Results

A total of 283 patients were diagnosed with HGSC in Denmark in 2005 and were included in the study. Median OS was 30 months (95% CI 25.9–35.1 months), and follow-up was 13 years.

PD-L1

PD-L1 expression $\geq 1\%$ was found in 164 (58%) patients. Only few patients had PD-L1 expression as high as 10%, and none had over 50%. PD-L1 expression was significantly associated with optimal debulking (less than 1 cm residual disease), but not with FIGO stage (Table 1).

High expression of PD-L1 was significantly correlated with favorable OS (Figure 3). The median OS was 37 months in the PD-L1 positive group vs. 22 months in the PD-L1 negative group, $p = .0008$. The multivariate analysis showed the same tendency with an HR of 0.77 but with a borderline significant p value of .061 (Table 2).

CD8+ T cells

Approximately, half of the patients had a cytotoxic T cell density in the tumor of more than 80 cells/mm². In general, the T cell was the most abundant intratumoral immune cell of the three investigated cell types. There was no significant association between stage, residual disease and cytotoxic T cell density. High CD8+ T cell density was correlated to significantly longer OS (Figure 3). The median OS was 37 months in the high-density group vs. 25 months in the low-density group, $p = .0008$. The multivariate analysis depicted in Table 2 showed that T-cell infiltration held independent significance in HGSC with an HR of 0.72 in favor of high T cell density, $p = .020$.

Table 1. Baseline patient characteristics.

	Cohort	PD-L1 ≥1%	CD8+ T-cells ≥80 cells/mm ²	CD66b+ neutrophils ≥60 cells/mm ²	CD57+ NK-cells ≥10 cells/mm ²
Median age	63	64	65	61	68
All high grade serous (N)	283	163	161	41	45
FIGO stage					
I	24 (8.5%)	15 (9%)	14 (9%)	2 (5%)	5 (11%)
II	24 (8.5%)	19 (12%)	17 (11%)	2 (5%)	6 (13%)
III	176 (62%)	98 (61%)	99 (61%)	27 (66%)	23 (51%)
IV	58 (21%)	30 (18%)	30 (19%)	10 (24%)	11 (25%)
<i>p</i> Value ^a		.157	.502	.718	.467
Surgery status					
Optimal debulking	114 (44%)	89 (55%)	86 (53%)	20 (49%)	24 (53%)
No optimal debulking	134 (52%)	69 (42%)	69 (43%)	20 (49%)	17 (38%)
Unknown	8 (3%)	5 (3%)	6 (4%)	1 (2%)	4 (9%)
<i>p</i> Value ^a		.041	.059	.987	.014

Optimal debulking is defined as less than 1 cm residual disease (according to the current definition in 2005 when the patients in the cohort were diagnosed). No optimal debulking is defined as residual disease after debulking or no debulking surgery.

^aPearson's chi-square test.

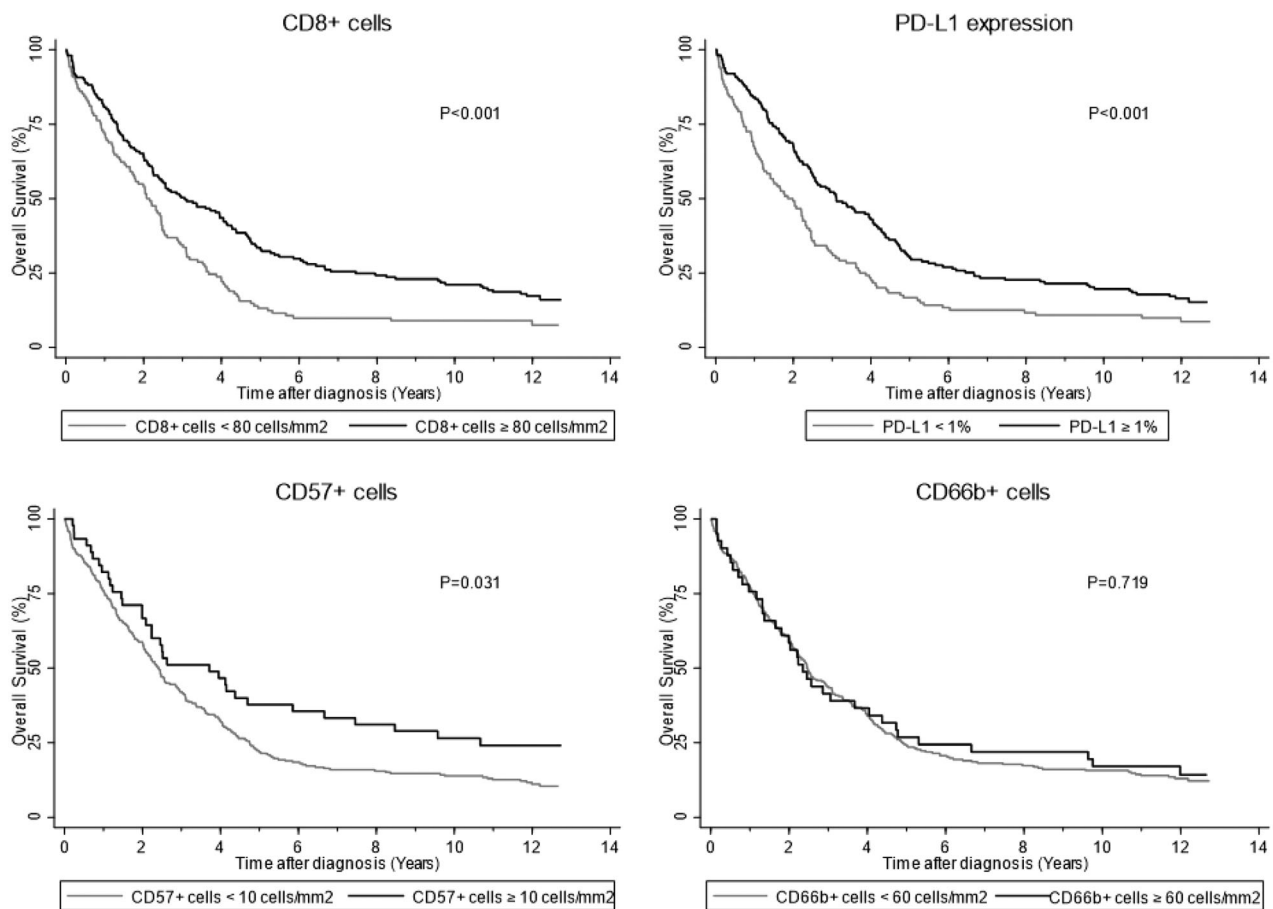


Figure 3. Favorable prognostic impact of high levels of intratumoral CD8+ T cells, CD57+ NK cells and PD-L1 expression. The Kaplan–Meier curves of overall survival in ‘high-grade serous adenocarcinomas’ ($n = 283$). *p* Values derived from log rank tests.

CD66b + neutrophils

Neutrophils were less abundant than T cells intratumorally, and less than one-fifth of the patients had a neutrophil density of ≥ 60 cells/mm² (Table 1). Necrotic areas of the tumor held a very high density of neutrophils, but they were left out of the analysis to avoid artifacts. There was no association between stage, residual disease and neutrophil density. Our analysis showed that neutrophil density did not correlate to OS in HGSC (Figure 3).

CD57+ NK cells

The density of CD57+ cells was generally very low, and the majority of patients had a CD57+ cell density of less than 10 cells/mm² (Table 1). A significant association between the achievement of radical surgery and high CD57+ cell density was found (Table 1). We observed a significant correlation between high CD57+ cell density and favorable OS in HGSC (Figure 3). In patients with high vs. low CD57+ cell density, the median OS was 45 and 29 months, respectively.

Table 2. Multivariable analysis.

High-grade serous carcinoma (n = 283)		
Prognostic factor	HR (95% CI)	p Value
Age	1.03 (1.02–1.04)	<.001
FIGO stage		
I–II	Ref.	
III–IV	3.42 (2.17–5.41)	<.001
Optimal debulking		
Yes	Ref.	
No	1.55 (1.18–2.03)	.002
PDL1		
<1%	Ref.	
≥1%	0.77 (0.59–1.02)	.061
CD8+ T-cells		
<80 cells/mm ²	Ref.	
≥80 cells/mm ²	0.72 (0.54–0.95)	.020
CD57+ NK-cells		
<10 cells/mm ²	Ref.	
≥10 cells/mm ²	0.67 (0.46–0.98)	.041

Hazard ratios (HRs) derived from multivariate Cox regression analysis of 'High-grade serous carcinomas'. Known prognostic variables and investigated markers who were significant in univariate Cox regression were integrated in the multivariate analysis.

($p = .0310$). The multivariate results depicted in Table 2 showed that CD57+ cell infiltration had independent prognostic significance with an HR of 0.67 ($p = .041$).

PD-L1 expression and immune cell density

CD57+ cell density correlated moderately with CD8+ cell density by Pearson's correlation coefficient = 0.53, $p < .001$. CD66b+ cell density did not correlate to CD57+ or CD8+ cell densities. PD-L1 expression correlated moderately with CD8+ cell density (Spearman's rho = 0.42, $p < .001$). PD-L1 expression did not correlate to CD57+ (Spearman's rho = 0.13) or CD66b+ (Spearman's rho = 0.03) cell densities. PD-L1 expression vs. no expression in the combined CD8+ and CD57+ low group ($N = 107$) correlated significantly with survival with median OS of 29.7 months vs. 19.9 months, $p = .033$. In patients with combined CD8+ and CD57+ high group ($N = 37$), PD-L1 expression had no prognostic impact.

Discussion

The present study, conducted in an unselected population-based cohort, is the first to demonstrate a positive prognostic significance of CD57+ NK cell tissue infiltration in HGSC. Furthermore, it confirms the relevance of CD8+ T cell infiltration and shows a trend for a positive impact of PD-L1 expression. CD66b+ neutrophils were not associated with a poor prognosis.

The median OS of the investigated cohort was poor (30 months) compared to OS from the literature (approximately 36 months in GOG-111 and SCOTROC trials [40,41]). Trial patients fulfill several inclusion criteria and are selected for the trials by clinicians, making them highly selected. The poorer survival of the present population-based cohort, underlines the importance of investigating cohorts without patient selection, to make results more representative, reflecting survival in a real-world setting.

The immune system interaction with the tumor is immensely complex. We focused on evaluating the two main

areas, i.e., the adaptive and the innate, of the immune system. As to the adaptive immune system, we investigated the part of the immune system prioritized in recent oncology immune research; T cells and the associated PD1/PD-L1 system. In relation to the innate immune system, we investigated the abundant immune cells; the neutrophils and NK cells. Our hypothesis was that immune cells had prognostic impact in newly diagnosed HGSC.

In this study, high cytotoxic T cell infiltration reflected a positive prognostic impact for HGSC, a result well in line with most previous research in ovarian cancer and the majority of other cancers. Goode and Sousa [11] conducted a comprehensive study on T cell infiltration in ovarian cancer, analyzing 3196 cases of HGSC. They demonstrated a significant HR of 0.75 in favor of high T-cell infiltration. Their detection method was different from that of ours, and the results are therefore not directly comparable, but they point in the same direction.

We found a relatively low PD-L1 expression in HGSC which was in line with earlier studies [26,30] and could explain why studies with PD-L1 based treatment have shown modest outcomes in EOC [42]. In lung cancer and melanoma patients, the expression of PD-L1 is generally much more pronounced and an expression of 50% in the tumor tissue is not rare [43]. PD-L1 expression on tumor cells was distinguished from PD-L1 expression on immune cells (e.g., macrophages) by the difference in appearance in the microscope. This is a limitation to the study as in cases with highly dysmorphic tumor cells, it could be challenging to distinguish the different cell types. Nevertheless, the PD-L1 expression was in general rather low compared to other tumor types. For patients with HGSC, there was a trend for positive OS impact of PD-L1 expression, though only borderline significant in the multivariate analysis. This observation is in contrast to most other cancers, where PD-L1 expression is a negative prognostic factor, but a positive prognostic impact in EOC has also been demonstrated by others [28,30]. We and others have found that CD8+ cells are a prognostically relevant player in the tumor-host interaction in HGSC, but the importance of the regulation *via* PD1/PD-L1 is unclear. T cells are regulated by many other factors, e.g., inhibition and activation by neutrophils [44]. The majority of previous studies points to an unfavorable prognostic role of neutrophils [22,23], and the lack of a negative prognostic impact of CD66b+ neutrophils in the present study might suggest a dichotomized function as described by Brandau et al. [20].

Even though generally acknowledged as part of the innate immune system, the NK cell has many facets and also contains properties of the adaptive immune system. We found it relevant to investigate NK cells due to their capability of killing tumor cells without prior immunization and antibody-dependent cellular cytotoxicity [14]. Moreover, the NK cell has received less attention from the oncology community compared to the T cell. This may be caused by preliminary, less convincing and contradictory prognostic studies on tumor-infiltrating NK cells [16,17]. In addition, early clinical trials based on either adoptive NK cell therapy or modulating NK cell activity have shown only modest effect [15]. Due to limitations with the classical NK-cell CD56 staining, we used

CD57 as marker for NK cells, although some T-cells also express CD57 [45]. The present study showed that high CD57+ cell density was associated with longer OS in univariate as well as multivariate analysis. Li et al. [17] investigated tissue from 82 patients with mixed histology operated over a period of 10 years at Kyoto University Hospital. They indicated a poor prognosis with higher CD57+ cell infiltration. The study and its cohort differ from that of ours when it comes to size and histology. Only 36 patients in the Li et al. study had serous carcinoma; information on grade was not provided [17]. The superior representativeness and size of our population-based study substantiate our results. Also, the majority of previous studies on other cancers points in the same direction as the one presented here. A meta-analysis by Hu and Wang [16] included 26 studies of which 13 showed prognostic importance. The overall result of the analysis showed a significant correlation between high CD57+ cell infiltration and favorable OS across cancer types, but not in ovarian cancer. The present study is the first to show that CD57+ cells play a favorable prognostic role in the tumor microenvironment in HGSC, supporting that NK cells have clinically relevant antitumor functions in HGSC. Antitumor functions could include both direct cancer cell killing or activation of other constituents of the immune system. Our results encourage further investigation of NK cells as a prognostic marker.

In this national population-based cohort, we were able to retrieve tissue and performed analyses on 84% of the total number of patients diagnosed with EOC in Denmark in 2005. This is a relatively high fraction of a representative cohort and is a major strength of the study. Most clinical studies include referred patients, which potentially hampers the generalizability of the results. Limitations are the age of the cohort and the retrospective nature of the study. However, despite advances in the fields of VEGF and PARP inhibitors and the addition of these new biological agents to the existing treatment, the basic treatment strategies with debulking surgery followed by platinum-based combination chemotherapy as the main part of first line treatment have not been changed and OS in EOC has not undergone major changes since 2005, making these results well applicable to patients of today. Limitations in the prognostic evaluation of tumor cell PD-L1 expression are the difficulty in differentiating between expression on tumor cells and infiltrating immune cells, which also can express PD-L1. This may have impacted results in the multivariate analysis where a drop in significance was observed when combining PD-L1 expression with CD8+ cells.

We introduced a new scoring system for evaluating immune cell infiltration based on digital imaging giving an advantage in total tumor area analysis and reproducibility. The cutoff was done consistently for all immune cells analyzed. These cutoffs should be validated.

Conclusions

In patients with HGSC, tumor-infiltrating NK cells and T cells had a favorable prognostic impact, and PD-L1 expression

had borderline positive prognostic significance. Neutrophils had no prognostic association. These findings may influence future immunotherapy development in high grade serous ovarian carcinoma.

Disclosure statement

No potential conflict of interest was reported by the authors.

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