

ORIGINAL ARTICLE

Expression of toll-like receptors in ovarian cancer

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Summary

Purpose: Ovarian cancer continues to be the most lethal gynecologic malignancy, with a complex tumor microenvironment (TME). We investigated the immunohistochemical (IHC) expression of toll like receptors (TLRs) 4,5,7 and 9 together with CD68 and CD 163 as markers for tumor-associated macrophages (TAM) in relation to clinicopathological data.

Methods: Data from 102 patients with serous ovarian cancer treated between 2006 and 2011 was retrospectively reviewed. A TLR IHC score was developed and CD68, CD163 density scores were calculated as the mean number of positive cells from three 0.5 mm² areas.

Results: Advanced-stage disease (FIGO IIIC-IV) was present in 65.7% of cases. A TLR4 score above median was associated with peritoneal carcinomatosis (odds ratio/OR) 3.02, $p=0.019$) or ascites (OR 2.5, $p=0.041$). In FIGO stage IIIC-

IV patients with a platinum-free interval (PFI) >12 months had, in comparison with patients with PFI ≤12 months, a higher CD68 density score (191.9 ± 95.2 vs. 152.7 ± 69.4 , $p=0.066$) and a lower CD163 density score (106.7 ± 73.3 vs. 154.5 ± 73.9 , $p=0.011$). In early-stage ovarian cancer patients, TLR9 positivity was associated with a higher overall survival than in patients with absent expression (110.2 vs. 22 months, $p<0.001$), while advanced-stage patients with TLR7 positivity had a lower overall survival than patients with negative TLR7 (38.3 vs. 66.2 months, $p=0.01$).

Conclusions: Our data shows that TLRs and TAM are important prognostic markers and future studies are needed to better comprehend the immune response in ovarian cancer.

Key words: ovarian cancer, prognosis, toll like receptors, tumor associated macrophages, tumor microenvironment

Introduction

Ovarian cancer (OC) continues to be the fifth cause of cancer-related death in western countries [1]. One of the key prognostic factors is the FIGO stage, with a good 5-year survival outcome for women diagnosed with early-stage disease. On the contrary, women with stage III-IV disease have a poor survival and represent approximately 80% of the cases at diagnosis, given the nonspecific abdominal symptoms [2]. Beside initial stage at presentation, survival is closely related to the

outcome of surgical debulking, where the goal should be complete resection [3,4]. Another accepted therapeutic strategy is neoadjuvant chemotherapy followed by surgery in cases that cannot be completely debulked at presentation [5,6]. Despite aggressive initial treatment, relapse occurs in more than 70% of the cases who will finally develop chemoresistant disease and will pass away [7]. Currently there are two molecularly targeted treatments, i.e. poly(ADPribose) polymerase inhibi-

tors and anti-VEGF antibodies that have brought a lot of promise, but still they are usually limited to delaying cancer progression [8].

One of the reasons why we couldn't make a giant leap in overall survival is linked to the incomplete understanding of chemoresistance mechanisms, which affect a large proportion of patients. Recent reports highlight the complex interactions between tumor cells and the tumor microenvironment that can play a major role in influencing the therapeutic response [9,10]. A large variety of inflammatory cells has been described within this microenvironment, where the most important representatives are lymphocytes, tumor-associated macrophages (TAM) and dendritic cells [11]. Toll-like receptors (TLRs) are a family of molecules that are connected with the activation of the innate immune response [12,13]. TLRs also have a role in the modulation of cellular proliferation and survival via activation of subsequent signaling pathways [14]. Their expression has been documented in both normal and neoplastic ovarian tissue [15]. Expression of TLRs 4-5 in ovarian cancer has been linked to angiogenesis, a reduction of the innate antitumor response favoring tumor progression [16]. Current evidence underlines that TLRs can have a bidirectional effect on the immune system, promoting either tumor progression or regression. Dendritic cells and macrophages (M1-type) also express TLRs. These two cell types can initiate and activate the immune response and the recognition and clearance of tumor cells by natural killer cells and cytotoxic T lymphocytes [14,17]. The infiltration of the tumor microenvironment by M2-type (TAMs) macrophages promote tissue repair and remodeling, favoring tumor survival and growth [18]. In our study we decided to search whether a connection existed between the expression of TLRs 4,5,7,9 and CD68, CD163 as selected markers for characterizing macrophages M1/M2 polarization, in relation to various clinical markers and survival data.

Methods

Patients from our Institute treated between 2006-2011 were included in this retrospective study if they fulfilled the selection criteria. Key requirements were pathologically confirmed serous ovarian cancer, primary debulking surgery with maximum residual tumor of 10 mm, adjuvant platinum-based chemotherapy, adequate follow-up and suitable tissue available for immunohistochemical staining. Patients with neoadjuvant treatment, suboptimal surgery, incomplete files or diagnosis of a second malignancy were excluded. This study was approved by the Institutional Ethics Committee and all patients signed written informed consent.

In order to evaluate the expression of immunohistochemical markers, tissue microarrays (TMAs) were constructed by microdissection of the paraffin blocks, in areas selected by a pathologist who previously reviewed the original slides. The original paraffin blocks contained tissue that was fixed in 10% formalin and routinely processed. Sections from TMAs were cut at 4µm. The immunohistochemical stains were performed using the BOND-III Fully Automated IHC (Leica Biosystems, Germany) by using a protocol comprising the following steps: dewaxing, epitope retrieval (heat-induced by using the Epitope Retrieval Solution 1 (pH=6, Leica Biosystems, Germany), and visualization performed using the Bond Polymer Refine Detection (Leica Biosystems, Germany). The following antibodies were used: TLR 4 (clone 76B357.1, Abcam, UK, at a dilution of 1:100), TLR 5 (clone 19D759.2, Abcam, UK, at a dilution of 1:100), TLR 7 (clone EPR2088(2), Abcam, UK, at a dilution of 1:100), TLR 9 (clone 26C593.2, Abcam, UK, at a dilution of 1:100), CD68 (clone 514H12, Leica Biosystems, Germany, at a dilution of 1:100), CD163 (polyclonal AB87099, Abcam, UK, at a dilution of 1:100). Next, the slides were washed with distilled water, dehydrated, immersed in xylene and coverslipped.

TLR expression was evaluated by a senior pathologist through a semi-quantitative method, where the final score (range 0-300) was the product of staining intensity (range 0-3, corresponding to absent, weak, moderate or strong, respectively) multiplied by the percent of positive cells (range 0-100). CD68 and CD163 density scores represent the average number of positive cells on 0.5 mm² evaluated at 40× magnification on a monitor from three randomly selected areas.

Table 1. Clinical characteristics of the study group

Characteristics	n	%
FIGO stage		
I	11	10.8
II	6	5.9
III	76	74.5
IV	9	8.8
Histological grade		
Low grade (G1-G2)	54	52.9
High grade (G3)	48	47.1
Carcinomatosis at diagnosis	75	73.5
Ascites at diagnosis (>500 ml)	72	70.6
Primary surgery		
Complete debulking (R=0 mm)	68	66.7
Optimal dubulking (R<10 mm)	34	33.3
Secondary surgery		
Complete debulking (R=0 mm)	12	11.8
Optimal dubulking (R<10 mm)	4	3.9
Suboptimal dubulking (R>10 mm)	2	2.0
Relapse	79	77.5
Death	72	70.6

Statistics

Patient data was retrieved from the electronic database and from the clinical observation files. Survival events were obtained through the Regional Cancer Registry. Data analysis was performed using IBM SPSS Statistics v22 and GraphPad Prism v7. All p values displayed were two-sided, and the threshold for statistical significance was 0.05.

Results

A total number of 102 patients were selected for this study. During upfront surgery, 68 (66.7%) patients achieved complete debulking (residual tumor of 0 mm) and 34 (33.3%) had optimal debulking (residual tumor <10 mm). Median age was 57.5 years (range 43.8), and advanced-stage disease (defined as FIGO stage IIIC-IV) was present in 67 (65.7%) cases at diagnosis. Almost half (n=48, 47.1%) of patients had a high grade serous cancer. Out of the 79 (77.5%) patients that relapsed, 18 (17.7%) underwent secondary debulking surgery and subsequent chemotherapy. Death occurred in

72 (70.6%) patients during follow-up. The median follow-up (reversed Kaplan-Meier method) was 112.5 months. Detailed patient characteristics are presented in Table 1.

Overall, TLR 4,5,7,9 positivity (TLR score >0) was found in 78.4, 34.3, 15.7 and 93.1% of the cases, respectively (Figure 1). Mean CD68 and CD163 density score was 165 positive cells/0.5 mm² (SD 86.1) and 129.3 positive cells/0.5 mm² (SD 79), respectively (Figure 2). Detailed expression of IHC markers is presented in Figure 3.

TLR 4,5,7,9 scores were not normally distributed, as assessed by visual inspection of their histograms and of Normal Q-Q Plots. CD68 and CD163 density scores were normally distributed, as assessed by visual inspection. CD68 distribution had a skewness of 0.314 (standard error = 0.239) and kurtosis of -0.150 (standard error = 0.474) and CD163 distribution had a skewness of 0.311 (standard error = 0.239) and kurtosis of -0.231 (standard error = 0.474).

Among 27 patients with no evidence of carcinomatosis at diagnosis, 8 had a TLR4 score above

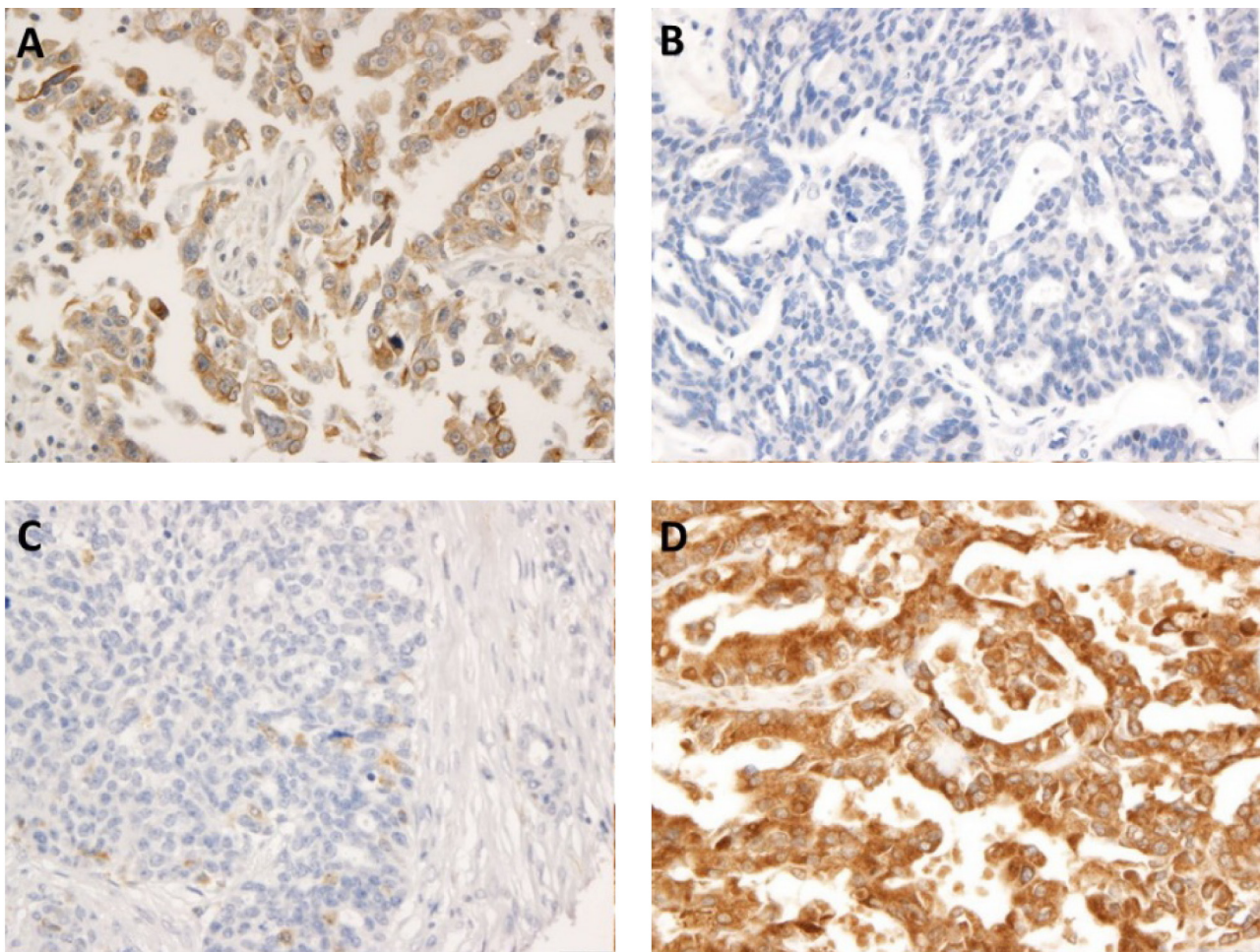


Figure 1. TLRs immunohistochemical expression. **A:** TLR4, moderate intensity; **B:** TLR5, absence of staining; **C:** TLR7, weak intensity; **D:** TLR9, strong intensity (original magnification×40).

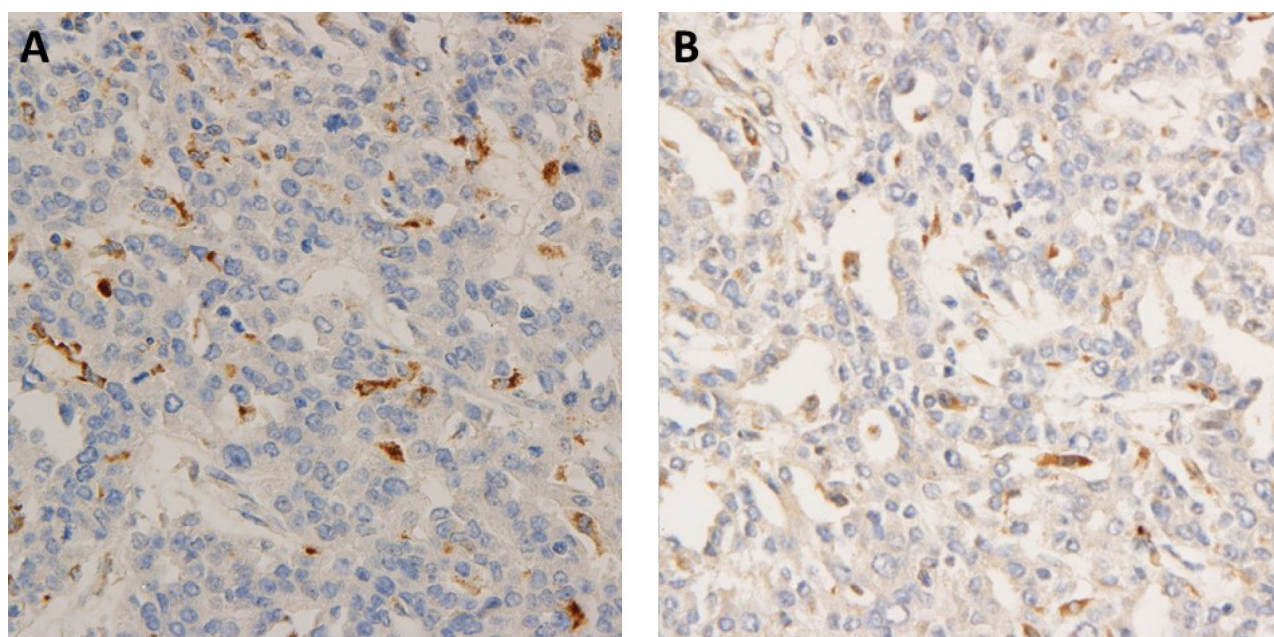


Figure 2. CD68 and CD163 immunohistochemical expression. **A:** CD68 expression on macrophages; **B:** CD163 expression on macrophages. Both pictures were realized on the same TMA block (original magnification×40).

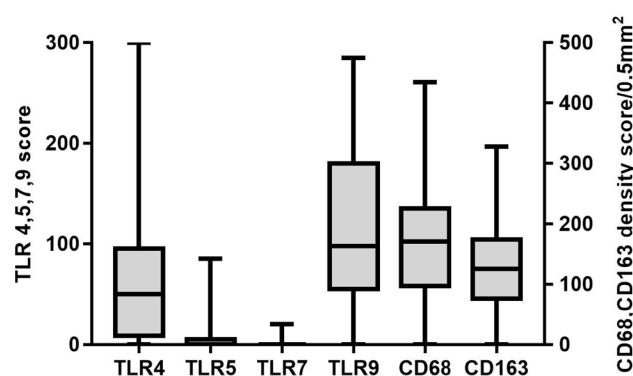


Figure 3. Box and whiskers graph of IHC markers.

median whereas 42 of the 75 patients with carcinomatosis at diagnosis had a TLR4 score above median, with an OR of 3.02 (95% CI, 1.17 to 7.76, $p=0.019$). Mann-Whitney U test was run to determine if there were differences in TLR4 score between cases with or without peritoneal carcinomatosis. Median TLR 4 score was statistically significantly higher in cases with peritoneal carcinomatosis (60) than in cases without peritoneal carcinomatosis (30), $p=0.047$.

Similarly, 10 out of the 30 patients without ascites at diagnosis had a TLR4 score above median whereas 40 out of the 72 patients with ascites at diagnosis had a TLR4 score above median, with an OR of 2.5 (95% CI, 1.02 to 6.08, $p=0.041$).

An independent-samples t-test was performed to determine if there were differences in CD68 and CD163 density scores between low and high grade ovarian cancer. The CD68 density score was higher

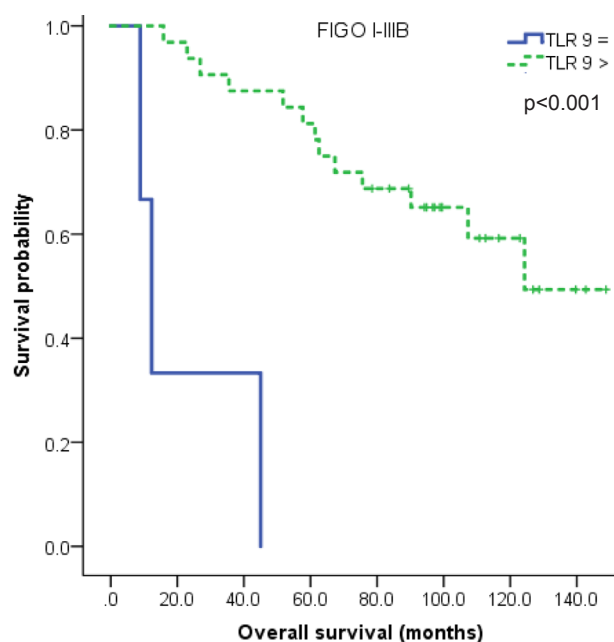


Figure 4. Kaplan-Meier overall survival based on TLR9 positivity.

in high grade samples (197.5 ± 86.7) than in low grade samples (128.5 ± 70.1), with a statistically significant difference of 68.9 (95% CI, 37.7 to 100.2), $p<0.0001$, although there was no significant difference in CD163 density score between high and low grade ovarian cancer (133.1 ± 86.0 vs. 125.1 ± 71).

An independent-samples t-test was also performed to determine if there were differences in CD68 and CD163 density scores according to the platinum-free interval that was defined as the period of time since the last date of platinum-based

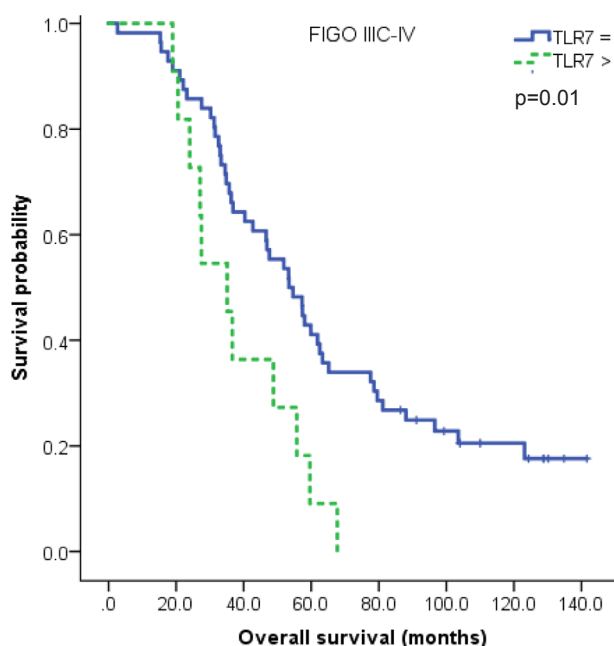


Figure 5. Kaplan-Meier overall survival based on TLR7 positivity.

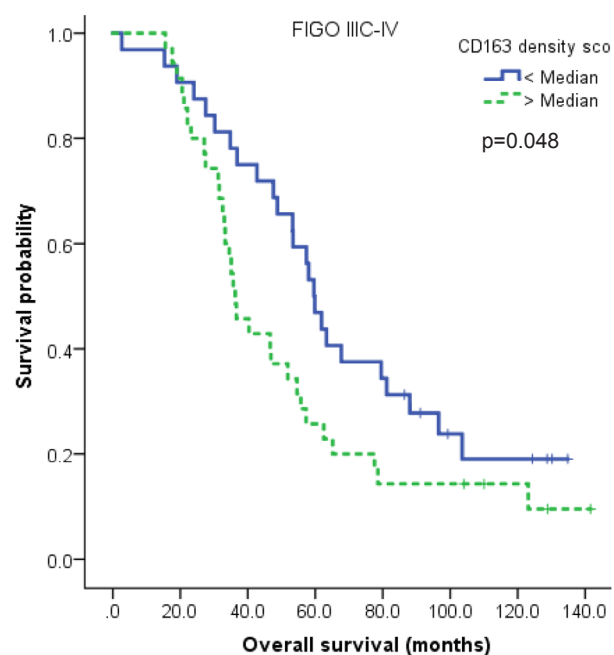


Figure 6. Kaplan-Meier overall survival based on CD163 density score.

chemotherapy until relapse or last follow-up. In the subset of patients with advanced ovarian cancer (FIGO IIIC-IV) CD68 density score was higher in patients with PFI >12 months (191.9 ± 95.2) than in patients with PFI ≤ 12 months (152.7 ± 69.4), with a difference of 39.2 (95% CI, -2.7 to 81.1), that approached statistical significance ($p=0.066$). Analyzing CD163 density score in the same subset of patients, this was higher in patients with PFI ≤ 12 months (154.5 ± 73.9) than in patients with PFI >12 months (106.7 ± 73.3), a statistically sig-

nificant difference of 47.8 (95% CI, 11.5 to 84.0), $p=0.011$.

Positivity of TLR and CD68/163 density scores was also investigated to see whether it influences survival. In early-stage (FIGO I-IIIB) ovarian cancer, patients with TLR9 positivity had a higher overall survival (mean 110.2, 95% CI 93.6 to 126.8 months) than patients with negative TLR9 (mean 22, 95% CI 0.1 to 44.6 months) that was statistically significant (log rank $p<0.001$; Figure 4).

In advanced-stage (FIGO IIIC-IV) ovarian cancer, patients with TLR7 positivity had a lower overall survival (mean 38.3, 95% CI 28.3 to 48.3 months) than patients with negative TLR7 (mean 66.2, 95% CI 55 to 77.4 months) that was statistically significant (log rank $p=0.01$; Figure 5). Also in advanced-stage, patients with a CD163 density score above median (125.83) had a lower overall survival (mean 52.7, 95% CI 40.3 to 65.2 months) than patients with a density score below median (mean 69.5, 95% CI 55.7 to 83.3 months) that was statistically significant (log rank $p=0.048$; Figure 6).

Discussion

Ovarian cancer continues to carry a significant burden of treatment associated morbidity and high mortality rates, especially in advanced-stage disease where most patients are expected to relapse and succumb to their disease [16]. Future improvements in ovarian cancer can be expected by a combination of maximizing the efficacy of surgical techniques and a better understanding of the complex tumor microenvironment through harnessing the immune's system antitumor response [17,20,21].

Our results showed that a TLR4 score above median was associated with negative clinical features at presentation such as peritoneal carcinomatosis (OR 3.02, 95% CI, 1.17 to 7.76, $p=0.019$) or the presence of ascites (OR 2.5, 95% CI, 1.02 to 6.08, $p=0.041$). It has been previously reported that TLR4 is associated with poor overall survival, probably due to its association with advanced-stage, high grade disease and presence of ascites. This can be partially explained by the activation of the TLR4/MyD88/NF- κ B pathway that can contribute to an inflammatory microenvironment harboring an aggressive phenotype [22,23].

Currently, macrophages have been separated into distinct cell populations with a wide range of functions, according to their polarization status imposed by interleukins, interferons, or TLRs stimulation [24,25]. Currently, two clusters have been described, M1 and M2. The M1 type is characterized by a pro-inflammatory status, Th1 immune

signaling and tumor suppressor functions, while the M2 type is linked to tissue repair, Th2 immune signaling and tumor promotion [26]. In this context, CD68 was used as a pan-macrophage marker expressed by M1 and M2 while CD163 expression is usually limited to M2, polarized macrophages [27].

Analyzing the CD68 and CD 163 density score in high and low grade ovarian cancer we observed that even though there was no significant change in the CD163 density score (133.1 ± 86.0 vs. 125.1 ± 71 , $p > 0.05$) there was a marked difference in the CD68 density score (197.5 ± 86.7 vs 128.5 ± 70.1 , $p < 0.0001$), suggesting that high grade ovarian cancer is associated with an increase in the M1 macrophage population, confirming similar reports from the literature, that showed differences in M1/M2 TAM expression patterns in low and high grade ovarian cancer [28].

We could also demonstrate in the subset of advanced-stage disease (FIGO IIIC-IV) that patients with a PFI > 12 months had, in comparison with patients with a PFI ≤ 12 months, a higher CD68 density score (191.9 ± 95.2 vs. 152.7 ± 69.4 , $p = 0.066$) and a lower CD163 density score (106.7 ± 73.3 vs. 154.5 ± 73.9 , $p = 0.011$), indicative for a M1 macrophage polarization that favors a longer PFI, with previous results pointing into the same direction [29]. Moreover, in advanced-stage disease (FIGO IIIC-IV), a CD163 density score above median was associated with a lower overall survival (52.7 vs 69.5 months, $p = 0.048$), indicative that M2 macrophage polarization is associated with a worse clinical outcome, reinforcing previous reports [30].

Our results also showed that in early-stage ovarian cancer patients (FIGO I-IIIB) TLR9 positivity was a marker of good prognosis, being associated with a higher overall survival than in patients with absent expression (110.2 vs. 22 months, $p < 0.001$), probably by enhancing the innate immunity. There have been several *in vivo*

studies where TLR9 agonists used either as monotherapy [31] or in association with immunomodulators [32] showed encouraging results regarding tumor regression. A recent report from two ongoing metastatic melanoma phase I studies showed that intratumoral injection of the investigational TLR9 agonists (CMP-001 and SD-101) together with checkpoint blockade therapy led to objective responses with minimal side effects, showing proof of concept in human subjects [33].

In advanced-stage (FIGO IIIC-IV) ovarian cancer, patients with TLR7 positivity had a lower overall survival than patients with negative TLR7 (38.3 vs. 66.2 months, $p = 0.01$). Poor clinical outcomes have been reported for non-small cell lung cancer patients with high TLR7 expression who had a lower PS and a smaller response rate to neoadjuvant chemotherapy [34].

Overall, in the light of the current evidence, successful results in ovarian cancer should not depend only on maximizing surgical debulking but on a combination of standard treatment and a personalized approach directed towards harnessing the capabilities of the immune system. Even though our results have been based on limited retrospective data, it is important to see that TLRs and TAM are important players of the TME and future in-depth approaches aiming to explore the complexity of tumor mechanisms involved in tumor progression and treatment resistance are needed.

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Conflict of interests

The authors declare no conflict of interests.

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