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ORIGINAL ARTICLE

The inflammatory microenvironment in epithelial ovarian cancer: a role for TLR4 and MyD88 and related proteins

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Abstract The tumor-associated inflammatory microenvironment may play a pivotal role in epithelial ovarian cancer (EOC) carcinogenesis and outcomes, but a detailed profile in patient-derived tumors is needed. Here, we investigated the expression of TLR4- and MyD88-associated markers in tumors from over 500 EOC patients using immunohistochemical staining. We demonstrate that high expression of TLR4 and MyD88 predicts poorer overall survival in patients with EOC; most likely, this is due to their association with serous histology and features of high tumor burden and aggressiveness, including stage, grade, and ascites at surgery. Combined TLR4 and MyD88 expression appears to serve as an independent risk factor for shortened survival time, even after covariate adjustment (both moderate HR 1.1 [95 % CI 0.7–1.8], both strong HR 2.1 [95 % CI 1.1–3.8], both weak as referent; $p = 0.027$). We reveal that in EOC tissues with elevated expression of both TLR4 and MyD88 and activated NF-κB signaling pathway, expression of hsp60, hsp70, beta 2 defensin, and HMGB1 are also enriched. In total, these results suggest that activation of TLR4/MyD88/NF-κB signaling by endogenous ligands may contribute to an inflammatory microenvironment that drives a more aggressive phenotype with poorer clinical outcome in EOC patients.

Keywords Epithelial ovarian cancer · Toll-like receptor four (TLR4) · Myeloid differentiation primary response gene eighty-eight (MyD88) · Endogenous ligands · NF-κB signaling pathway

Introduction

Epithelial ovarian cancer (EOC) remains the most lethal gynecologic malignancy and the fifth leading cause of cancer death among women in the USA [1]. Less than 40 % of women with EOC are cured [1] due to the lack of effective screening strategies and the non-specific nature of early signs and symptoms associated with this disease, resulting in advanced stage at diagnosis in most patients [2]. Although most patients with advanced disease are initially highly responsive to surgery and chemotherapy, the majority of them succumb to recurrent disease, which tends to resist to current treatments [3]. Therefore, investigations to understand the etiology and chemoresistance mechanisms of EOC are urgently needed.

The innate immune system recognizes the presence of bacterial pathogens through expression of a family of membrane receptors known as toll-like receptors (TLR) [4]. Although their expression is well-established in immune cells, TLRs are also found in myriad human cancers, including EOC [5]. Moreover, accumulating evidence reveals that toll-like receptor 4 (TLR4) exerts the ability to create an inflammatory microenvironment for EOC cells through the activation of the

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nuclear factor-kappaB (NF-κB) signaling pathway, with the help of myeloid differentiation primary response gene 88 (MyD88), a TLR signaling adapter protein [6, 7]. This hypothesis also attributes TLR4/MyD88/ NF-κB signaling pathway to one of the EOC's chemoresistance mechanisms, but it has only been studied in small sample sets of EOC tissues [8–10]. Moreover, this cascade can be initiated by binding of TLR4 with bacterial products such as lipopolysaccharide (LPS), or synthetic molecules like paclitaxel, one of the major chemotherapy agents used for the vast majority of patients with advanced stage EOC [6, 7]. In addition to these ligands, some damage-associated molecular patterns (DAMPs) released by necrotic cells are also reported to activate TLR4 signaling and serve as endogenous ligands for TLR4 [11]. However, the expression profiles of those endogenous ligands and their associations with TLR4/MyD88 and NF-κB signaling pathway in EOC remain largely unknown.

In the current study, we investigate TLR4, MyD88, and their associated inflammatory markers in the EOC microenvironment using immunohistochemical (IHC) staining of ovarian cancer tissues from a large cohort of patients, with particular interest in seven DAMPs, five key markers of NF-κB signaling pathway, and a downstream reporter of NF-κB signaling pathway.

Materials and methods

Patients and specimens

Established Institutional Review Board (IRB)-approved protocols were used to recruit patients from the Departments of Gynecologic Surgery and Medical Oncology at Mayo Clinic with pathologically-confirmed EOC diagnosed between 2000 and 2009. Patients provided written informed consent and permission for active follow-up. Five tissue microarrays (TMAs) were constructed [12] of triplicate 0.6 mm cores from a single formalin-fixed, paraffin-embedded block of tumor tissue from a total of 517 patients who had not received neo-adjuvant chemotherapy prior to surgery. Vital status was updated annually using medical records and active follow-up.

Immunohistochemistry

Fifteen markers were used in this study including TLR4, MyD88, seven DAMPs or endogenous ligands of TLR4-heat shock 60 kDa protein (hsp60), heat shock 70 kDa protein (hsp70), gp96, fibrinogen, heparan sulfate, beta 2 defensin, high-mobility group box 1 (HMGB1), five key markers of NF-κB signaling-IκB kinase β (IKKβ), NF-kappa-B inhibitor α (IκBα), phospho- IκBα, p50, phospho-p65, and a downstream reporter of NF-κB signaling-matrix metallopeptidase 9 (MMP9). IHC staining was optimized using positive and negative control tissues. Following citrate (Dako K5207) or EDTA (Chem Lab) antigen retrieval, TMAs were stained with antibodies diluted in Bkg reducing diluent (Dako S0809) followed by EnVision™/ EnVision™+detection system (Dako K4001/K4061) or Mach3 detection system (Biocare M3R531); dilution and manufacturer's information for the antibodies are listed in Supplemental Table 1. Stained TMAs were scanned by a BLISS slide scanner system (Bacus Laboratories, Inc.) using a Zeiss Axioplan 2 microscope at ×40 magnification.

TMA scoring and categorization

TMA cores found to contain inadequate tumor tissue were excluded. For cases with multiple cores successfully scored; the maximum value was used. Viewers trained by pathologist (DWV) and blinded to the clinical covariates conducted the scoring. For each marker, a subset of cores were scored by DMV and the trained viewer independently, and the agreement of the two viewers was statistically acceptable (weighted kappa range from 0.43 to 0.77, data not shown). Scoring strategies are listed in Supplemental Table 1. For phospho-p65, the percentage of positive cells in nuclei (<10 %, ≥10 %) was scored; for all other markers, the intensities of nuclear and cytoplasmic staining in the majority of cells (none, weak, moderate, strong) were scored. Some groups were combined due to small sample size, as listed in Supplemental Table 1.

Statistical analysis

Overall survival was defined as time from diagnosis to death from any cause. Kaplan-Meier curves and corresponding log-rank (Mantel-Cox) tests were used to visually compare survival across levels of expression. Cox proportional hazard regression was used to estimate hazard ratios (HRs) and 95 % confidence intervals (CIs) for association of expression values with disease outcome. Markers with unadjusted p value <0.05 were further analyzed in covariate-adjusted Cox proportional hazard models including clinical factors associated with overall survival-age at diagnosis (quartiles <53, 53–60, 61–70, >70); histology (serous, mucinous, endometrioid, clear cell, other); stage (I-II, III-IV); grade (1, 2, 3), and surgical debulking (no macroscopic disease, macroscopic disease <1 cm, macroscopic disease >1 cm) with a forward selection strategy. Correlations between expression and clinical/pathological characteristics were assessed using Pearson's chi-square test or, where sample size was < five, Fisher's exact test. Pairwise correlations between markers were analyzed with Spearman's rho test with Fisher's z transformation to calculate 95 % confidence intervals. A two-sided p value <0.05 was considered statistically significant; no adjustment for multiple testing was done. All statistical analyses were carried out using SPSS v22.

Results

Expression of DAMPs, TLR4, MyD88, key markers of the NF-κB signaling pathway and MMP9 in EOC tissues

Clinical/pathological characteristics of the study cohort are listed in Table 1. All markers are detected in a subset of EOC tissues. For the majority (99.6 %, 487 of 489) of EOC patients investigated, there is at least one of the seven DAMPs detected, while for 74.8 % (366 of 489) patients, there are at least five DAMPs detected. Moreover, expression of TLR4 is detected in more than 70 % of EOC tissues, and expression of MyD88 is observed in more than 79 % of cases. Defined as having both IKKβ and MMP9 positive in the same case [13], activation of NF-κB signaling pathway is observed in 76.0 % (393 of 466) cases. Representative images for all markers investigated are showed in Supplemental Fig. 1 and Supplemental Fig. 2, and distributions of them, including by histologic types, are summarized in Fig. 1.

Table 1 Clinical and pathological characteristics of 517 EOC patients

	Number (%)
Age at diagnosis, years	
Mean, Median, Range	61.5, 61, 21–93
Race	
White	489 (98 %)
Non-white	8 (2 %)
Unknown	20
Histology	
Serous	370 (72 %)
Endometrioid	73 (14 %)
Clear cell	32 (6 %)
Mucinous	19 (4 %)
Others	23 (4 %)
Stage	
I–II	124 (24 %)
III–IV	393 (76 %)
Grade	
1	32 (6 %)
2	55 (10.6 %)
3	430 (83 %)
Ascites at surgery	
Yes	267 (63 %)
No	156 (37 %)
Unknown	94
Surgical debulking	
No macroscopic disease	238 (47 %)
Macroscopic disease <1 cm	219 (43 %)
Macroscopic disease >1 cm	54 (11 %)
Follow-up time, years	
Mean, Median, Range	5.1, 4.7, 0.01–14.6
Vital status	
Alive	175 (34 %)
Dead	342 (66 %)

Numbers may not add to 517 due to missing values (six for surgical debulking)

Expression of TLR4 and MyD88 associate with shortened overall survival time, while combined expression of TLR4 and MyD88 provides additional patient stratification in EOC

Associations between expression of markers investigated and overall survival time were then analyzed. Unadjusted Kaplan-Meier analysis and log-rank testing (Fig. 2a and Fig. 2b), as well as unadjusted Cox regression analysis (Table 2), suggest that elevated expression of TLR4 and MyD88 are each associated with shorter overall survival (strong vs moderate vs none/weak intensity). However, no association between other markers investigated and overall survival is detected with unadjusted Cox regression (Supplemental Table 2). With covariate adjustment for age, histology, stage, grade, and surgical debulking status, risk estimates for TLR4 and MyD88 are attenuated (e.g., HR = 1.78 for strong vs none/weak before adjusted and HR = 1.36 after adjusted for TLR4), and their associations with survival are no longer statistically significant at $p < 0.05$ (Table 2). Because in vitro studies suggest that MyD88 is required for TLR4 induced NF-κB signaling pathway activation and then survival of EOC cells, we then combined TLR4 and MyD88 expression by stratifying patients into three groups: (1) both markers none/weak ($N = 41$, used as reference); (2) both markers moderate ($N = 90$) and (3) both markers strong ($N = 27$). This revealed low, intermediate, and high risk groups of EOC patients (Fig. 2c) (HR = 1.66 for both moderate and HR = 2.74 for both strong compared to both none/weak, $p < 0.001$, Table 3). Moreover, this result remained statistically significant, albeit attenuated, after covariate adjustment (HR = 1.12 for both moderate, and HR = 2.09 for both strong compared to both none/weak, $p = 0.027$, Table 2).

Expression of TLR4 and MyD88 varies by histology and correlates with clinical/pathologic characteristics

To determine whether important clinical features may be driving the association with TLR4 and MyD88 and outcome, we compared marker distributions across these features using Pearson's chi-square and Fisher's exact testing. While expression is not associated with age at diagnosis or debulking status, EOC cases with high expression of TLR and MyD88 are enriched for serous histology (TLR4 $p < 0.001$, MyD88 $p = 0.029$, Table3). What's more, expression of TLR4 and

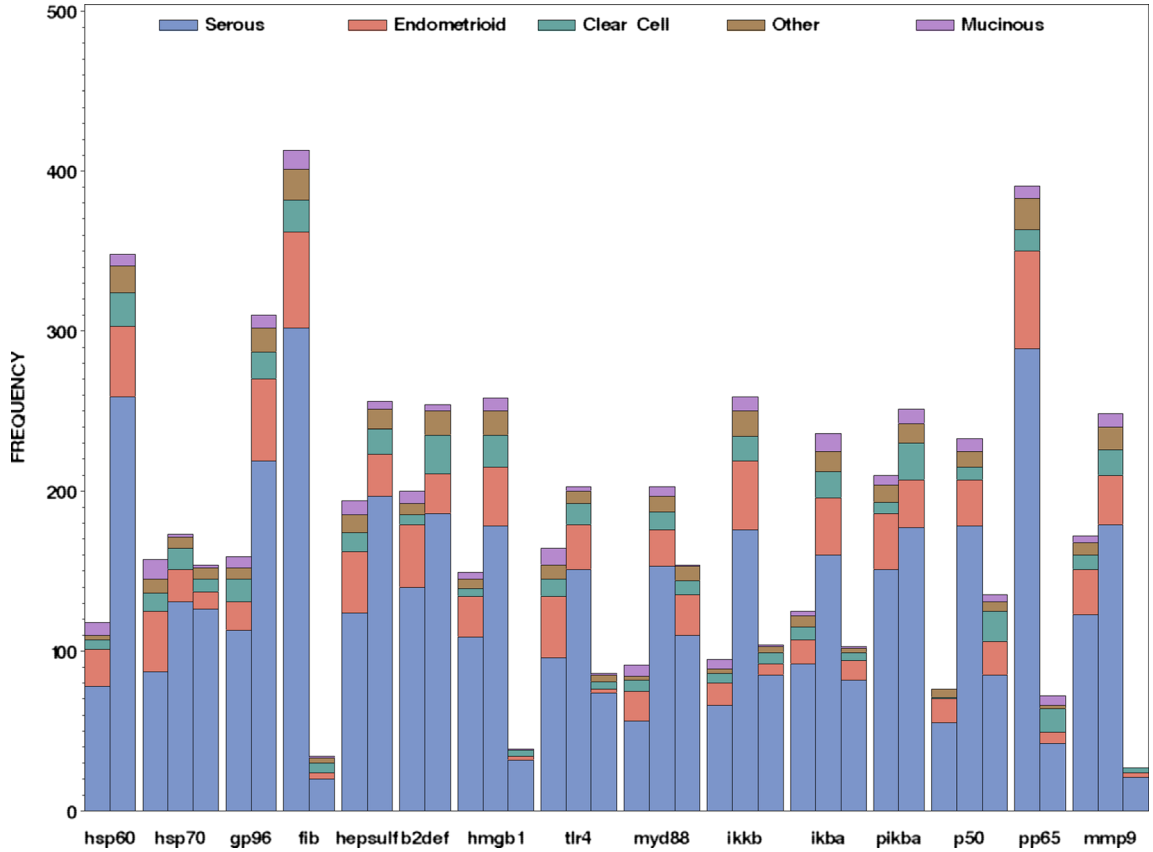

Fig. 1 Distribution of proteins investigated in EOC patients by levels of histology. The following expression levels for each protein are displayed in the figure from left to right: hsp60 none/weak and moderate/strong; hsp70 none, weak and moderate; gp96 none/weak and moderate; fibrinogen (fib) none and weak/moderate; heparan sulfate (hepsulf) none and weak/moderate; beta 2 defensin (b2def) none/weak and moderate; HMGB1 (hmgb1) none, weak and moderate; TLR4 (tlr4) none/weak, moderate and strong; MyD88 (myd88) none/weak, moderate and strong; IKKβ (ikkb) none, weak and moderate; IκBα (ikba) none, weak and moderate; phospho-IκBα (pikba) none and weak/moderate; p50 none, weak and moderate; phospho-p65 (pp65) <10 % and ≥10 %; and MMP9 (mmp9) none, weak/moderate and strong. Numbers may not add to total number of subjects overall due to missing values for some expression levels

MyD88 is also associated with features of high tumor burden and aggressiveness, including stage ($p = 0.009$ and 0.002, respectively), grade ($p < 0.001$ and $p = 0.030$, respectively) and ascites at surgery ($p = 0.039$ and 0.025, respectively, Table 3).

Several endogenous ligands of TLR4 and activation of NF-κB signaling pathway are enriched in EOC patients with combined high expression of TLR4 and MyD88

We finally evaluated expression patterns of DAMPs, TLR4, MyD88, and the NF-κB signaling pathway to gain a better understanding of their interrelationships in this study cohort. Expression of TLR4 positively correlates with hsp60, beta 2 defensin, HMGB, IKKβ, IκBα, phospho-IκBα, p50, and MMP9, while expression of MyD88 positively correlates with hsp60, hsp70, beta 2 defensin, HMGB, IKKβ, IκBα, and MMP9 (Table 4). Of note, we also assessed inter-marker correlations in ovarian tumor RNA expression data from the Cancer Genome Atlas (www.cbioportal.org) and found positive Pearson correlations with the MMP9 target gene (TLR4 = 0.46, MyD88 = 0.13). Tumors with elevated expression of combined TLR4 and MyD88 show greater expression of selected endogenous ligands of TLR4 (hsp60, hsp70, beta 2 defensin, and HMGB1) as well as NF-κB signaling pathway members (IKKβ and MMP9) (Supplemental Table 3).

Discussion

In the current study, we investigated the expression of TLR4- and MyD88-associated markers in over 500 EOC tumors using immunohistochemical staining. We reveal that high expression of TLR4 and MyD88 is associated with poorer overall survival in univariate models but attenuate after adjustment for clinical factors, most likely due to their association with serous histology and features of high tumor burden and aggressiveness, such as tumor stage, grade, and ascites at surgery. We also demonstrate, for the first time, in such a large cohort of EOC tissues that combined TLR4 and MyD88 expression serves as an independent risk factor for shortened

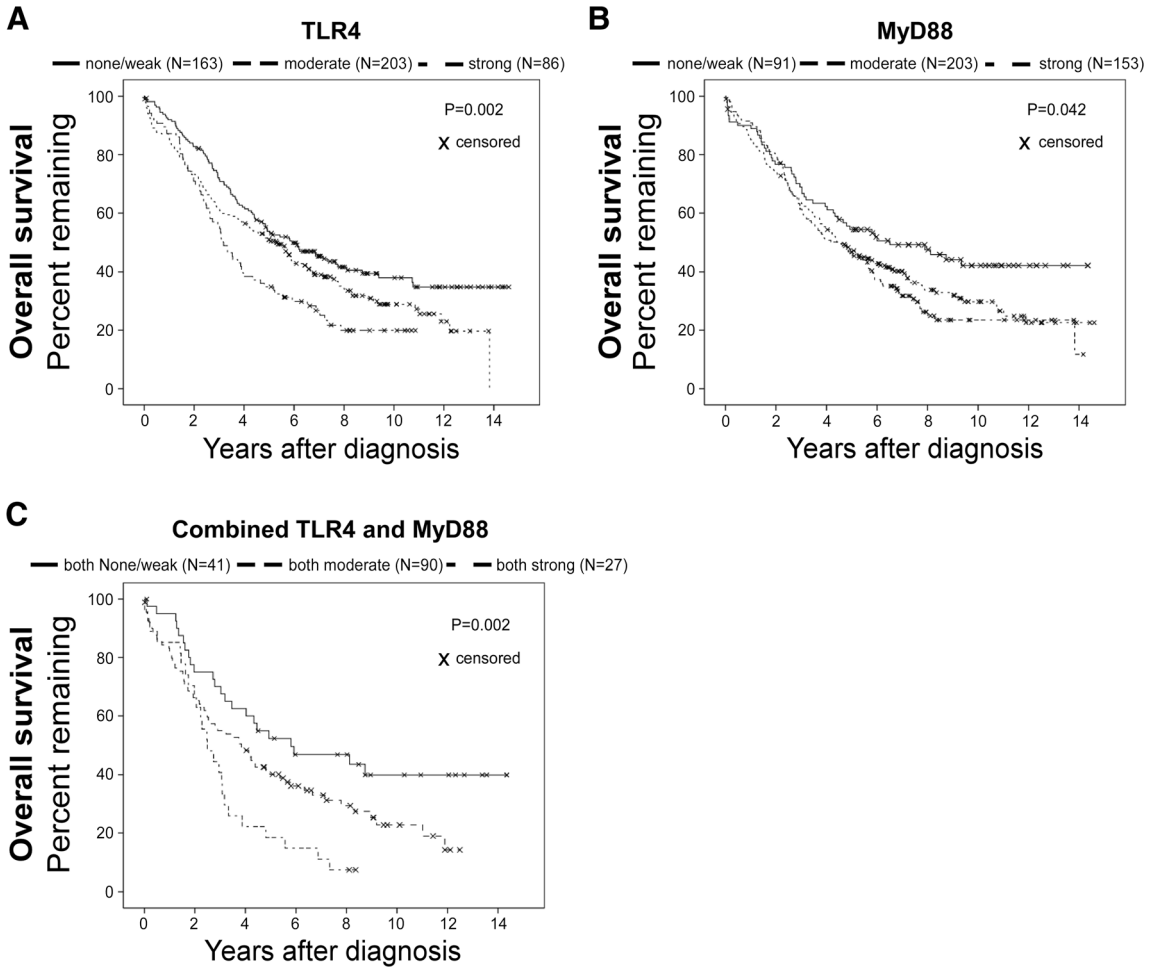

Fig. 2 Kaplan-Meier curves obtained from univariate analyses (log-rank) of EOC patients with TLR4 MyD88 and combined TLR4 and MyD88 expression. **a** Strong TLR4 expression versus moderate versus none/weak TLR4 expression for overall survival rates of EOC patients (N = 452). **b** Strong MyD88 expression versus moderate versus none/weak MyD88 expression for overall survival rates of EOC patients (N = 447). **c** Combined both strong expression versus both moderate versus both none/weak expression of TLR4 and MyD88 for overall survival rates of EOC patients (N = 158)

Table 2 Overall survival of EOC patients and selected proteins

	Number	Unadjusted		Covariate-adjusted	
		HR (95 % CI)	p value	HR (95 % CI)	p value
TLR4			*0.002*		0.178
None/weak	163	1 (reference)		1 (reference)	
Moderate	203	1.31 (1.01–1.71)		1.15 (0.88–1.50)	
Strong	86	1.78 (1.30–2.43)		1.36 (0.98–1.88)	
MyD88			*0.042*		0.411
None/weak	91	1 (reference)		1 (reference)	
Moderate	203	1.40 (1.01–1.94)		0.96 (0.68–1.35)	
Strong	153	1.54 (1.10–2.16)		1.14 (0.80–1.63)	
Combined TLR4 and MyD88			*0.002*		*0.027*
Both none/weak	41	1 (reference)		1 (reference)	
Both moderate	90	1.66 (1.03–2.68)		1.12 (0.68–1.84)	
Both strong	27	2.74 (1.54–4.88)		2.09 (1.13–3.87)	

Univariate and multivariate analysis performed using Cox proportional hazard models. *HR* hazard ratio, *CI* confidential interval; covariate adjustments include age at diagnosis (quartiles, <53, 53–60, 61–70, and >70); stage (I–II, III–IV); histology (serous, mucinous, endometrioid, clear cell, other); tumor grade (1, 2, 3) and surgical debulking (no macroscopic disease, macroscopic disease <1 cm, and macroscopic disease >1 cm); italicized p-values are less than 0.05.

Table 3 Associations of TLR4 and MyD88 with clinical and pathological parameters, N (%)

Parameters	Expression of TLR4			*p* value	Expression of MyD88			*p* value
	Negative/low	Moderate	High		Negative/low	Moderate	High	
Age at diagnosis, years				0.503				0.112
<53	58 (35.4)	52 (25.6)	22 (25.6)		24 (26.6)	54 (26.4)	52 (33.8)	
53–61	33 (20.1)	50 (24.6)	21 (24.4)		23 (25.3)	50 (24.6)	31 (20.1)	
61–70	41 (25.0)	57 (28.1)	22 (25.6)		32 (35.2)	48 (23.6)	39 (25.3)	
>70	32 (19.5)	44 (21.7)	21 (24.4)		12 (13.2)	51 (25.1)	32 (20.8)	
Histology				*<0.001*				*0.029*
Serous	96 (58.5)	151 (74.4)	74 (86.0)		56 (61.5)	153 (75.4)	110 (71.4)	
Endometrioid	38 (23.2)	28 (13.8)	2 (2.3)		19 (20.9)	23 (11.3)	25 (16.2)	
Clear cell	11 (6.7)	13 (6.4)	5 (5.8)		7 (7.7)	11 (5.4)	9 (5.8)	
Mucinous	10 (6.1)	3 (1.5)	1 (1.2)		7 (7.7)	6 (3.0)	1 (0.6)	
Other	9 (5.5)	8 (3.9)	4 (4.7)		2 (2.2)	10 (4.9)	9 (5.8)	
Stage				*0.009*				*0.002*
I–II	52 (31.7)	39 (19.2)	16 (18.6)		34 (37.4)	41 (20.2)	30 (19.5)	
III–IV	112 (68.3)	164 (80.8)	70 (81.4)		57 (62.6)	162 (79.8)	124 (80.5)	
Grade				*<0.001*				*0.030*
1	19 (11.6)	6 (3.0)	0 (0.0)		11 (12.1)	8 (3.9)	6 (3.9)	
2	21 (12.8)	21 (10.3)	4 (4.7)		10 (11.0)	23 (11.3)	12 (7.8)	
3	124 (75.6)	176 (86.4)	82 (95.3)		70 (76.9)	172 (84.7)	136 (88.3)	
Ascites at surgery				*0.039*				*0.025*
No	58 (44.3)	61 (35.9)	20 (26.7)		37 (49.3)	54 (31.2)	45 (36.3)	
Yes	73 (55.7)	109 (64.1)	55 (73.3)		38 (50.7)	119 (68.8)	79 (63.7)	
Surgical debulking				0.088				0.068
No macroscopic disease	84 (51.9)	93 (46.5)	29 (34.1)		49 (54.4)	86 (42.8)	69 (45.4)	
Macroscopic disease <1 cm	65 (40.1)	87 (43.5)	43 (50.6)		38 (42.2)	87 (43.3)	68 (44.7)	
Macroscopic disease >1 cm	13 (8.0)	20 (10.0)	13 (15.3)		3 (3.3)	28 (13.9)	15 (9.9)	

All but three comparisons were made using chi-square tests

For histology and grade, we applied Fisher's exact test due to sample size less than in some groups; italicized *p*-values are less than 0.05.

overall survival time, providing additional stratification into low, intermediate, and high risk patient groups. Finally, we demonstrate that in EOC tissues with elevated expression of both TLR4 and MyD88, expression of several endogenous ligands of TLR4 (namely, hsp60, hsp70, beta 2 defensin, and HMGB1) and NF-κB pathway members (namely, IKKβ and MMP9) are enriched.

The tumor-associated inflammatory microenvironment has been recognized as a hallmark of nearly all solid malignancies [14, 15]. In EOC, the progression of tumor often correlates with tumor-associated inflammatory microenvironment [16, 17]. TLR activation by pathogen-associated molecular patterns such as LPS causes secretion of numerous cytokines and chemokines, which results in an inflammatory microenvironment largely due to the activation of the NF-κB signaling pathway [18]. Zhou et al. initially reported that TLR4 was expressed in EOC tissues and cell lines [19]. In vitro studies then revealed that LPS could induce proliferation in ovarian cancer cells expressing both TLR4 and its adapter protein-MyD88, but did not induce proliferation in cells expressing only TLR4 [6]. LPS ligation to TLR4 results in production of chemokines and cytokines that lead to chemoresistance to paclitaxel by activing the NF-κB pathway in MyD88-positive but not MyD88-negative cells [6]. Moreover, paclitaxel binding to TLR4 can also activate NF-κB pathway and induce chemoresistance only in MyD88-positive cells [7, 20], indicating that co-expression of TLR4 and MyD88 in EOC cells are essential for the activation of TLR4/MyD88/NF-κB signaling pathway.

A few prior investigations demonstrated that high expression of either TLR4 or MyD88 in EOC tissues is associated with poor survival rates [8, 9, 21]. However, these studies failed to examine the prognostic importance of combined expression of TLR4 and MyD88, as suggested by in vitro studies. In agreement with those studies, our study reveals that elevated expression of TLR4 and MyD88 associates with poor overall survival rates in a large cohort of EOC patients with unadjusted analyses, but we further show that HRs of TLR4 and MyD88 are attenuated, after covariate adjustment with

Table 4 Correlation between expression of TLR4 and MyD88 and other proteins in EOC patients ($N = 517$), Spearman's rho

	Role	Correlation with TLR4 (95 % CI)	Correlation with MyD88 (95 % CI)
hsp60	DAMP	0.13 (0.03–0.22)	0.36 (0.27–0.44)
hsp70	DAMP	0.08 (−0.02–0.17)	0.16 (0.07–0.25)
gp96	DAMP	0.06 (−0.04–0.15)	−0.01 (−0.10–0.09)
Fibrinogen	DAMP	−0.06 (−0.05–0.04)	0.15 (0.05–0.24)
Heparan sulfate	DAMP	0.15 (0.05–0.24)	0.10 (0.00–0.19)
Beta 2 defensin	DAMP	0.17 (0.07–0.25)	0.14 (0.04–0.23)
HMGB1	DAMP	0.13 (0.03–0.22)	0.19 (0.10–0.28)
TLR4	Toll-like receptor 4	1	0.04 (−0.05–0.13)
MyD88	TLR signaling adapter protein	0.04 (−0.05–0.13)	1
IKKβ	Activing kinase of NF-κB	0.37 (0.28–0.44)	0.19 (0.10–0.28)
IκBα	Inhibitor of NF-κB	0.26 (0.17–0.34)	0.12 (0.03–0.21)
Phospho-IκBα	Phosphorylated inhibitor of NF-κB	0.18 (0.09–0.27)	0.06 (−0.03–0.16)
p50	NF-κB transcription factor	0.16 (0.06–0.25)	−0.04 (−0.13–0.06)
Phospho-p65	NF-κB transcription factor	−0.02 (−0.11–0.07)	−0.01 (−0.10–0.08)
MMP9	Target gene of NF-κB	0.12 (0.02–0.21)	0.12 (0.02–0.21)

CI confidential interval, *hsp60* heat shock 60 kDa protein, *hsp70* heat shock 70 kDa protein, *HMGB1* high-mobility group box 1, *TLR4* toll-like receptors 4, *MyD88* myeloid differentiation primary response gene 88, *IKKβ* IκB kinase β, *IκBα* NF-kappa-B inhibitor α, *MMP9* matrix metallopeptidase 9, *DAMP* damage-associated molecular pattern

clinical factors consisting of age, histology, stage, grade, and surgical debulking status. As demonstrated by other investigations [9, 10], our study then shows EOC cases with high expression of TLR4 and MyD88 are enriched for serous histology, while expression of TLR4 and MyD88 are associated with features of high tumor burden and aggressiveness, including stage, grade, and ascites at surgery. This is consistent with our observation of attenuated HRs and reduced significance for TLR4 and MyD88 upon covariate adjustment, and it suggests that expression of these proteins does not independently predict outcome.

To further investigate the hypothesis that co-expression of TLR4 and MyD88 is essential for the activation of TLR4/MyD88/NF-κB signaling pathway which results in poor prognosis in EOC patients, our study then reveals, for the first time, in such a large cohort of EOC tissues, that combined expression of TLR4 and MyD88 possess the ability to stratify EOC patients into low, intermediate, and high risk groups according to combined TLR4 and MyD88 expression intensity. Most important, this result remains statistically significant at $p < 0.05$ after covariate adjustment, suggesting that co-expression of TLR4 and MyD88 serves as an independent risk factor that predicts shortened overall survival time in EOC patients.

It has been reported that in cancer cells, certain DAMPs, or endogenous TLR4 ligands released by cell death or cellular stress, could potentially promote cancer progression [11, 18, 22, 23], but the expression profile of endogenous ligands in EOC and their association with TLR4/MyD88/NF-κB signaling pathway have never been investigated. Here, we showed that seven DAMPs, known endogenous ligands of TLR4 are detected in a subset of EOC tissues. Furthermore, there is at least one of the seven DAMPs detected in the vast majority (99.6 %) of EOC patients investigated; for 74.8 % patients, there are more than five DAMPs detected. The lack of variation in DAMPs among the EOC tissues studied may have resulted in a reduction in statistical power and may explain why no DAMP independently associates with survival in our study cohort. Similarly, activation of NF-κB signaling pathway, defined as both IKKβ (activating kinase of NF-κB) and MMP9 (target gene of NF-κB) being positive in the same case, is observed in 76.0 % of our study cases, which may have reduced power to detect an association. However, previous studies reported that expression of p50, p65, and MMP9 in EOC tissues were adverse prognostic factor for overall survival [24–26]. In fact, our study also targeted p65 as an important NF-κB transcription factor, but cases with high intensity of p65 dominated the study cohort so dramatically (96 %, 434 of 454) that we excluded this marker from analyses. While previous studies might be biased by their relatively limited sample size (less than 100 cases), this lack of variability may impair our ability to properly analyze the status of the NF-κB signaling pathway in this study, despite the large cohort of 517 EOC samples.

Until now, the correlation between biogenesis and release of DAMPs, or endogenous TLR4 ligands and the status of TLR4/MyD88/ NF-κB signaling pathway in EOC cells remained largely unknown [11]. In particular, our findings that selective TLR4-activating DAMPs (hsp60, hsp70, beta 2

defensin, and HMGB1) are enriched in patients with high expression of TLR4 and MyD88, and that the NF-κB signaling pathway is activated (defined as high expression of IKKβ and MMP9), suggests that production and enrichment of several endogenous ligands in EOC cells may result from activation of NF-κB signaling pathway and correlate with TLR4 and MyD88 status. However, this hypothesis may warrant further in vitro investigations.

Although our study has the advantage of large sample size over prior reports, it nonetheless does not allow for histotype-specific analyses other than for serous histology (72 %, 370 of 517). Thus, further consortium-based studies to explore the prognostic importance of TLR4 and MyD88 in histologic subtypes of EOC are needed.

Conclusion

To summarize, we demonstrate that high expression of TLR4 and MyD88 predicts poorer overall survival in patients with EOC, most likely due to their association with serous histology and features of high tumor burden and aggressiveness, including stage, grade, and ascites at surgery. Combined TLR4 and MyD88 expression serves as an independent risk factor for shortened survival time. In EOC tissues with elevated expression of both TLR4 and MyD88 and activated NF-κB signaling pathway, expression of hsp60, hsp70, beta 2 defensin, and HMGB1 is also enriched. In total, these results suggest that activation of TLR4/MyD88/NF-κB signaling by endogenous ligands may contribute to an inflammatory microenvironment that promotes an aggressive phenotype and results in poor survival of EOC patients.

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Compliance with ethical standards We declare that all experiments were performed in accordance with the current law of USA. The study was approved by the Institutional Review Board (IRB) of Mayo Clinic in Rochester. Patients provided written informed consent and permission for active follow-up.

Conflicts of interest The authors declare that they have no conflict of interest

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