Neoplasma 2022; 69(6): 1418–1424

doi:10.4149/neo_2022_220509N498

TLR2 and TLR4 in colorectal cancer: relationship to tumor necrosis and markers of systemic inflammation

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Received May 9, 2022 / Accepted October 8, 2022

In colorectal cancer (CRC), systemic inflammation is associated with poor prognosis, but the underlying mechanisms are not fully characterized. Tumor necrosis may contribute to systemic inflammation by inducing interleukin (IL)-6 signaling, and proinflammatory cytokines such as IL-6 and IL-8, and matrix metalloproteinase (MMP)-8 also are linked to adverse CRC outcomes. Because Toll-like receptors (TLRs) are important mediators of inflammatory responses, we investigated the roles of TLR2 and TLR4 in CRC-associated systemic inflammatory responses, especially tumor necrosis. In 118 patients with CRC, extensive tumor necrosis was associated with low TLR4 expression in tumor cells. Tumor cell TLR4 expression was inversely correlated with serum IL-6 and MMP-8 levels, blood total leukocyte and neutrophil counts, and serum C-reactive protein levels. Tumor cell TLR2 expression was not significantly associated with necrosis or systemic inflammation, but low expression in normal mucosa was linked to high serum MMP-8 and IL-8. These findings indicate that tumor necrosis is associated with low TLR4 expression in cancer cells and that low TLR4 expression correlates with a strong systemic inflammatory response. The low TLR2 expression in normal mucosa and its association with systemic inflammation suggest that the normal mucosa may reflect or contribute to the systemic inflammatory response.

Key words: TLR2, TLR4, tumor necrosis, systemic inflammation, colorectal cancer

Systemic inflammation predicts poor prognosis in colorectal cancer (CRC) [1–4] but the pathogenesis of cancer-related systemic inflammation is inadequately characterized. Both the innate and adaptive immune networks are known to be involved [5, 6].

Toll-like receptors (TLRs) operate in the innate immune system and are widely distributed in tissues. They provide the first line of defense against microbes and recognize dangerous molecules released from damaged tissues. Usually, ligand binding to TLRs triggers a signaling cascade leading to inflammatory response [7, 8]. Soluble forms of some TLRs, including TLR2 and TLR4, are released into the circulation from tissues and blood cells and may downregulate the innate inflammatory response [9]. Soluble TLR2 is formed by enzymatic shedding of the ectodomain, but mechanisms of the formation of soluble TLR4 are not known [10]. Both local expression and levels of soluble TLRs are usually associated with infections and inflammatory conditions.

TLR2 recognizes several bacterial, fungal, and viral proteins, including cell wall components of gram-negative and gram-positive bacteria [11]. TLR4 detects lipopolysaccharide from gram-negative bacteria [12]. Both of these TLRs recognize endogenous ligands released as a result of cell death or injury, including damage-associated molecular patterns (DAMPs) [11, 12]. TLR4 activation induces interleukin (IL)-6 and IL-8 expression, and soluble TLR2 reduces IL-8 production [13]. Both IL-6 and IL-8 are considered important cytokines in CRC progression, contributing to tumor cell growth, proliferation, migration, and angiogenesis [14].

Neutrophil activation by DAMPs leads to the degranulation of matrix metalloproteinase (MMP)-8, which has an

essential role in neutrophil infiltration and function. Accordingly, high serum MMP-8 levels have been associated with systemic inflammation and adverse outcomes in CRC [15]. The prognostic significance of TLR2 or TLR4 expression in CRC is a matter of controversy, and mechanisms mediating the prognostic effect are largely unknown. Several studies imply, however, that TLR2 and TLR4 may be involved in the progression of CRC and in the malignancy-associated systemic inflammatory response [16].

Tumor necrosis also represents an indicator of adverse prognosis in CRC [17] and has been associated with the systemic inflammatory response, including high serum IL-6 levels. How tumor necrosis induces inflammatory and prognostic effects is not clear. We hypothesize that both TLR2 and TLR4 could be involved because they recognize endogenous ligands released from damaged cells [18]. Furthermore, the inflammatory response mediated by TLR activation may induce programmed necrosis [19].

In the present study, we investigated the roles of TLR2 and TLR4 in systemic inflammatory responses associated with tumor necrosis in CRC. TLR2 and TLR4 activation has been reported to induce a systemic inflammatory reaction that includes the induction of white blood cells and cytokine response [20]. Accordingly, we investigated whether features of tumor necrosis and systemic inflammation, including blood leukocyte counts and serum IL-6, IL-8, and MMP-8, are associated with serum TLR2 and TLR4 or with TLR2 and TLR4 expression patterns in carcinoma cells and in the normal intestinal epithelium.

Materials and methods

Patients. The study was based on an earlier described case series. Briefly, we used data from 149 patients newly diagnosed with CRC who underwent surgery at Oulu University Hospital between April 2006 and January 2010 and had signed informed consent to participate. The Regional Ethics Committee of North Ostrobothnia Hospital District approved both the original study design and the follow-up study (58/2005, 184/2009, 60/2012).

Clinical details and follow-up information were obtained from clinical records, and Statistics Finland provided the data on the time and cause of death. No further information for this study was obtained from the patients or from the registries. For preoperative CRC staging, we used data also collected earlier from whole-body computed tomography scans and magnetic resonance imaging scans for local staging of rectal cancer. Patients with rT3 or rT4 rectal cancer received preoperative neoadjuvant irradiation or chemoradiation therapy (n=31) and were excluded so a total of 118 patients were included in the current analyses. The TNM-6 classification system was used for staging. Patient and tumor characteristics are presented in Table 1.

Tumor histopathology. We used the World Health Organization 2010 classification to grade the differentiation [21]. The area percentage of tumor necrosis was visually estimated by manual inspection of all available tumor slides [22]. For grading necrosis, we used a three-grade scale: NG0 denoted rare areas of necrosis, NG1 denoted frequent small areas of necrosis, and NG2 denoted broad areas of necrosis [23].

Immunohistochemistry. TLR2 and TLR4 tissue expression was assessed by immunohistochemistry as previously described in detail [24, 25]. Briefly, we assessed staining intensity and the percentage of positive cells separately in the invasive front and bulk of the tumor, normal mucosa, and lymph node metastases, when present. We used a four-point scale (0–3) for staining intensity and expressed the extent of staining as the percentage of positively stained cells (0–100%). The histoscore (0–300) for the tissue samples was defined as the intensity score multiplied by the percentage of positive cells. In this work, we combined the histoscores of the tumor bulk and front by calculating the means of both values to represent the whole tumor. TLR expression was

Table 1. Characteristics of patients and the colorectal carcinomas.

	N (%)
Sex	
Male	56 (47.5)
Female	62 (52.5)
Age in years, median [min–max]	69 [36–89]
Other morbidities	
No	27 (22.9)
Yes	91 (77.1)
Type of operation	
Radical[1]	94 (80.3)
Palliative[2]	23 (19.7)
Tumor location	
Proximal colon	46 (39.0)
Distal colon	39 (33.1)
Rectum	32 (27.1)
Multiple tumors	1 (0.8)
Stage	
I	18 (15.3)
II	48 (40.7)
III	30 (25.4)
IV	22 (18.6)
Grade	
I	17 (14.4)
II	86 (72.9)
III	14 (11.9)
Data missing	1 (0.8)
Lymph node metastasis	
No	72 (61.0)
Yes	46 (39.0)
Distant metastasis	
No	96 (81.4)
Yes	22 (18.6)
Tumor necrosis	
Grade 0	68 (57.6)
Grade 1	31 (26.3)
Grade 2	17 (14.4)
Missing	2 (1.7)

Notes: [1]in one case, distant metastasis were operated radically in a second procedure; [2]in two cases, metastases were treated non-operatively (both patients were alive at the 5-year follow-up).

considered low with a histoscore ≤200 and high with scores >200.

Assessment of serum TLR2, TLR4, MMP-8, and C-reactive protein, blood leukocyte quantification, and modified Glasgow prognostic score. All serum and blood variables, including serum C-reactive protein (CRP) (mg/l) and blood leukocyte counts and differential (all 10^9/l), were determined from preoperative blood samples as described previously [2]. The modified Glasgow prognostic score (mGPS) was evaluated as score 0 for patients with normal CRP and albumin values, score 1 for patients with only elevated CRP (>10 mg/l), and score 2 for patients with both elevated CRP (>10 mg/l) and hypoalbuminemia (<35 g/l [2]). Serum concentrations of TLR2 (pg/ml) and TLR4 (ng/ml) were determined with enzyme-linked immunosorbent assays [25] and serum MMP-8 (ng/ml) by time-resolved immunofluorometric assay [23].

Assessment of IL-6 and IL-8. IL-6 and IL-8 levels were derived from our previous work with the Bio-Plex Pro Human pre-manufactured 27-Plex Cytokine Panel (Bio-Rad, Hercules, CA, USA), done according to the manufacturer's instructions, as described in detail [2]. Because IL-6 has been strongly associated with tumor necrosis, and both IL-6 and IL-8 are critical in CRC progression, we focused our analyses on these two cytokines for clarity of interpretation and to limit multiple hypothesis testing.

Statistical analyses. Summary measurements are presented as medians with 25^{th}–75^{th} percentiles or means with standard deviations (SD). We analyzed histoscore data using the Kruskal-Wallis test and categorical data by the χ^2 or Fisher's exact test. Spearman's correlation coefficients were calculated. Two-tailed p-values are presented, and the alpha level was set at 0.05. Our analyses were exploratory rather than confirmatory, so we did not apply a strict adjustment for multiple comparisons [26]. Analyses were performed using SPSS for Windows (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

Results

Tumor necrosis and TLR2 and TLR4 expression. Our previous analyses of the current case series showed that TLR2 is upregulated and TLR4 downregulated in CRC and that low expression of TLR4 is associated with an adverse prognosis [24, 25]. In the analyses for the present study, necrosis was seen in 48/116 cases (41.4%; Table 1). In two cases, necrosis data were missing. Tumor TLR2 expression was not associated with the extent (%) of tumor necrosis (Spearman correlation –0.025; p=0.795). Tumor TLR4 expression was negatively correlated with the extent (%) of tumor necrosis (Spearman correlation –0.190; p=0.041 for TLR4 histoscore and –0.188; p=0.045 for intensity of TLR4 staining in tumor front; Table 3). Extensive necrosis (graded as 2 on a three-point scale of 0, 1, and 2) was more frequent when TLR4 histoscore was low (23.6% vs. 6.7% with a high TLR4 histoscore; p=0.019). The mean TLR4 histoscore was 218 (SD 65) in tumors with grade 0 necrosis, 215 (SD 56) in grade 1, and 175 (SD 65) in grade 2 tumors (p=0.033; Figure 1).

TLR2 and TLR4 expression in cancer cells and the systemic inflammatory response. The associations between TLR2 and TLR4 expression in the carcinoma epithelium and markers of systemic inflammation (serum CRP, mGPS, blood leukocyte counts and differential, and serum IL-6, IL-8, and MMP-8) are presented in Table 2 (histoscore) and Table 3 (intensity), and scatterplots of the significant correlations of systemic inflammatory markers and TLR4 histoscore are available as Supplementary Figures. Tumor TLR2 immunoreaction was not significantly correlated with any of these markers. For TLR4 expression in tumor cells, we identified negative correlations with leukocyte and neutrophil counts, as well as with serum MMP-8, IL-6, and CRP concentrations.

TLR2 and TLR4 expression in epithelial cells of normal colorectal mucosa and systemic inflammation. A low TLR2 expression in normal colorectal mucosa was associated with high serum MMP-8 and IL-8 concentrations (Tables 2

Table 2. Correlations (Spearman) of TLR2 and TLR4 histoscores in carcinoma epithelium or normal mucosa with serum markers of systemic inflammation and blood white cell counts.

	Serum CRP	mGPS	Leukocyte	Neutrophil	Eosinophil	Basophil	Monocyte	Lymphocyte	Serum MMP-8	Serum IL-6	Serum IL-8
TLR2 tumor histoscore	–0.018	–0.060	–0.125	–0.102	–0.070	–0.149	–0.061	–0.022	0.012	–0.106	–0.012
(p-value)	(0.850)	(0.522)	(0.183)	(0.277)	(0.456)	(0.112)	(0.520)	(0.818)	(0.896)	(0.259)	(0.899)
TLR2 normal mucosa histoscore (p-value)	0.007	–0.027	–0.139	0.030	–0.125	0.036	0.014	–0.089	–0.225	–0.151	–0.249
	(0.941)	(0.777)	(0.142)	(0.753)	(0.186)	(0.707)	(0.882)	(0.349)	(0.017)	(0.108)	(0.008)
Serum TLR2	–0.112	–0.050	–0.143	–0.136	–0.202	0.068	–0.080	0.012	–0.100	–0.233	–0.134
(p-value)	(0.234)	(0.598)	(0.128)	(0.150)	(0.031)	(0.472)	(0.398)	(0.902)	(0.288)	(0.012)	(0.153)
TLR4 tumor histoscore	–0.193	–0.170	–0.223	–0.240	–0.177	0.058	–0.120	–0.038	–0.188	–0.255	–0.163
(p-value)	(0.038)	(0.070)	(0.016)	(0.010)	(0.058)	(0.540)	(0.202)	(0.688)	(0.044)	(0.006)	(0.080)
TLR4 normal mucosa histoscore (p-value)	0.127	0.094	0.025	0.123	–0.132	–0.090	–0.101	–0.024	0.008	0.025	0.031
	(0.179)	(0.320)	(0.795)	(0.191)	(0.163)	(0.340)	(0.287)	(0.798)	(0.933)	(0.787)	(0.744)
Serum TLR4	–0.073	–0.100	0.055	0.017	–0.005	–0.103	–0.115	0.074	0.091	–0.018	–0.004
(p-value)	(0.435)	(0.284)	(0.561)	(0.860)	(0.958)	(0.275)	(0.219)	(0.433)	(0.335)	(0.852)	(0.966)

Abbreviations: CRP-C-reactive protein; mGPS-modified Glasgow prognostic score; MMP-8-serum matrix metalloproteinase 8; IL-6-interleukin 6; IL-8-interleukin 8

and 3). Because the right and left sides of the colon differ biologically, including in the luminal microbiome [27], we compared normal mucosa TLR2 expression in different parts of the large intestine. The TLR2 histoscore was lower in the distal colon (median 98, range 0–250) than in the proximal colon (150, range 0–250; p=0.048, Kruskal-Wallis) or the rectum (139, range 40–300; p 0 =0.049).

This regional variation could obscure possible associations of TLR2 expression with markers of systemic inflammation, so we assessed these associations separately for each anatomic segment of the large intestine. TLR2 expression in the proximal colon showed a trend for negative correlation with MMP-8 (–0.276; p=0.077) and a significant negative correlation with IL-8 (–0.331; p=0.030). In the rectum, TLR2 in normal mucosa negatively correlated with IL-6 (–0.403, p=0.024) and IL-8 (–0.460, p=0.009), but did not correlate with either in the distal colon. TLR4 expression in normal colorectal mucosa did not differ between the proximal and distal colon and rectum (data not shown) and showed no correlations with systemic inflammation markers or serum cytokine levels (Table 2).

Serum TLR2 levels showed a negative correlation with blood eosinophil count and serum IL-6 levels, but serum TLR4 was not significantly associated with systemic inflammation markers (Table 2).

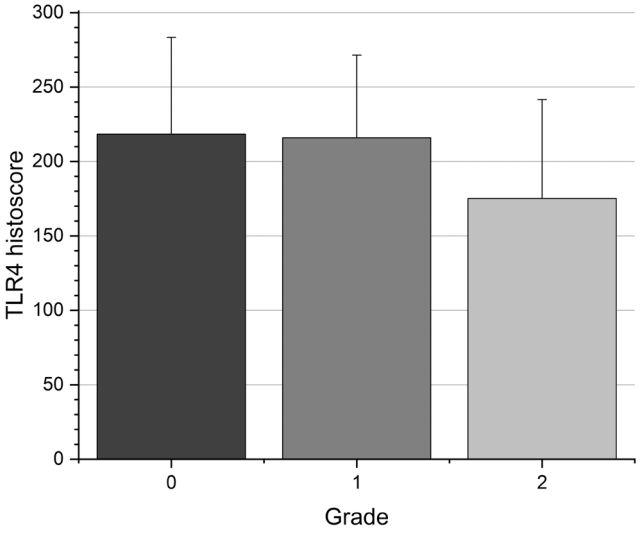

Figure 1. Relationship of TLR4 histoscore and the extent of necrosis in colorectal carcinoma. Bar chart shows mean values of TLR4 histoscores in each necrosis grade, error bars show standard deviations (SD) (p=0.033, Kruskal-Wallis).

Discussion

An activated systemic inflammatory response is associated with unfavorable CRC prognosis [1–4, 28]. Although factors driving such inflammation and the mechanisms underlying the prognostic effect are not clear [6], evidence supports a role in tumor necrosis [17, 29–31]. Because TLR2 and TLR4 mediate innate responses to both the endogenous ligands associated with necrosis and microbiological agents present in tumor tissue [8], in the current work we evaluated their associations with necrosis and the systemic inflammatory response. Tumor necrosis was linked to local downregulation of TLR4 in cancer cells, and low TLR4 in these cells was associated with the activation of systemic inflammation. TLR2 expression in carcinoma cells showed no relation to necrosis or systemic inflammation, whereas low TLR2 expression in the normal intestinal epithelium was associated with systemic inflammation. These findings suggest that TLR4 downregulation in tumor cells along with tumoral necrosis is eventually involved in the manifestation of systemic effects of tumoral necrosis in CRC. Moreover, TLR2 responses in normal mucosa may reflect or contribute to the systemic inflammatory response.

The association of tumor necrosis with TLR4 downregulation in tumor cells is a novel finding, but the mechanisms underlying the link are not obvious. Endogenous TLR ligands, including DAMPs, are released from necrotic tumor cells, and activation of TLR signaling by ligands usually leads to their increased expression [32]; thus, our findings are somewhat unexpected. The results could indicate that TLR2 and TLR4 expression levels are predominantly regulated by ligands unrelated to necrosis, possibly including the CRC-associated microbiome [33]. Alternatively, necrosis

Table 3. Correlations (Spearman) of intensity of TLR2 and TLR4 staining in carcinoma epithelium or normal mucosa with necrosis, serum markers of systemic inflammation and blood white cell counts.

	Necrosis	CRP	mGPS	Leukocytes	Neutrophils	Lymphocyte	Serum MMP-8	Serum IL-6	Serum IL-8
TLR2 intensity tumor	–0.001	0.016	–0.015	–0.102	–0.067	0.007	0.015	–0.059	0.027
(p-value)	(0.989)	(0.867)	(0.874)	(0.272)	(0.476)	(0.943)	(0.870)	(0.527)	(0.772)
TLR2 intensity normal mucosa	0.062	0.007	–0.020	–0.112	0.081	–0.139	–0.175	–0.101	–0.195
(p-value)	(0.517)	(0.938)	(0.835)	(0.234)	(0.396)	(0.141)	(0.063)	(0.285)	(0.037)
TLR4 intensity tumor	–0.170	–0.181	–0.149	–0.214	–0.241	–0.024	–0.178	–0.243	–0.150
(p-value)	(0.067)	(0.052)	(0.111)	(0.021)	(0.009)	(0.798)	(0.056)	(0.008)	(0.106)
TLR4 intensity normal mucosa	0.182	0.081	0.046	0.040	0.088	0.044	0.068	0.038	0.071
(p-value)	(0.053)	(0.391)	(0.631)	(0.667)	(0.351)	(0.641)	(0.474)	(0.688)	(0.449)

Abbreviations: CRP-C-reactive protein; mGPS-modified Glasgow prognostic score; MMP-8-serum matrix metalloproteinase 8; IL-6-interleukin 6; IL-8-interleukin 8

could be linked to some unidentified signaling pathways suppressing TLR4 expression. In carcinomas, necrosis may represent a manifestation of hypoxia [30], and some experimental evidence indicates that hypoxia suppresses TLR-mediated responses [34, 35]. However, how TLR2 and TLR4 are regulated in hypoxic conditions is unclear. Gaining a further understanding of the relationship of necrosis with tumor cell TLR4 response in CRC will require mechanistic studies.

We found an inverse association between TLR4 immunoreaction in the tumor and markers of systemic inflammation, including serum IL-6 and MMP-8, total leukocyte and neutrophil counts, and serum CRP. Although low local TLR4 expression, which also was associated with tumor necrosis in this study, might contribute to downregulating the local inflammatory response, we speculate that this downregulation might not effectively suppress the systemic proinflammatory influence of DAMPs released into the circulation from the necrotic cells [36]. We have not quantified concentrations of circulating DAMPs in our case series, but we have detected associations among tumor necrosis, circulating keratin fragments originating from necrotic tumor cells, and systemic inflammation [29]. Such findings support the concept that with necrosis, proinflammatory DAMPs are released from the tumor directly into the circulation, potentially inducing inflammation at the systemic level.

We have previously presented evidence of an association between elevated TLR2 expression in normal colorectal mucosa and higher serum TLR2 [25], suggesting a link between the normal mucosa and the systemic inflammatory milieu. Here, we found that high TLR2 expression in normal colorectal mucosa was associated with low serum MMP-8 and IL-8 concentrations, further suggesting that high intestinal TLR2 expression either reflects or contributes to the inhibition of the systemic inflammatory response. Any mechanisms remain speculative but could be related to the regulation of TLR2 expression by the luminal flora [37], with TLR2 involved in epithelial permeability [38]. Both tumor-related factors and co-existent abnormalities in seemingly normal intestinal mucosa might contribute to systemic inflammation. If findings in experimental studies support these predictions, they could open new possibilities for therapeutic targets within the systemic inflammatory response.

The limitations of our study include relatively small sample size, constraining the statistical power. Many of the correlations were at a rather low level (<0.30 or –0.30). Furthermore, considering multiple hypothesis testing [39], the observations need to be confirmed by additional studies. Nonetheless, our conclusions are supported by the detection of similar correlations for multiple markers of systemic inflammation. Tissue protein expression analyses were based on immunohistochemistry, which was analyzed semiquantitatively [40], but two investigators independently evaluated the staining, facilitating reproducibility. Furthermore, we recently reported that TLR2 and TLR4 expression evaluated with immunohistochemistry shows a good correlation with mRNA findings using *in situ* hybridization, an orthogonal method [41]. Finally, the use of tissue microarrays for tissue-based expression analyses may have decreased the detection of intratumoral variation; however, both the number and the size of cores were higher than is typical.

In conclusion, tumor necrosis in CRC is associated with low TLR4 expression in carcinoma epithelium, and low TLR4 expression is associated with systemic inflammation, as evidenced by high circulating levels of leukocytes, neutrophils, and proinflammatory cytokines. Tumoral TLR2 expression was not correlated with necrosis from systemic inflammation. In contrast, low expression of TLR2 in normal mucosa was linked to indicators of systemic inflammation, supporting the concept that the normal colon mucosa may contribute to the regulation of systemic inflammation.

Acknowledgments: We thank Erja Tomperi and Riitta Vuento for preparing the immunohistochemical stains and Anne Tuomisto and Markus J. Mäkinen for their valuable comments.

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https://doi.org/10.4149/neo_2022_220509N498

TLR2 and TLR4 in colorectal cancer: relationship to tumor necrosis and markers of systemic inflammation

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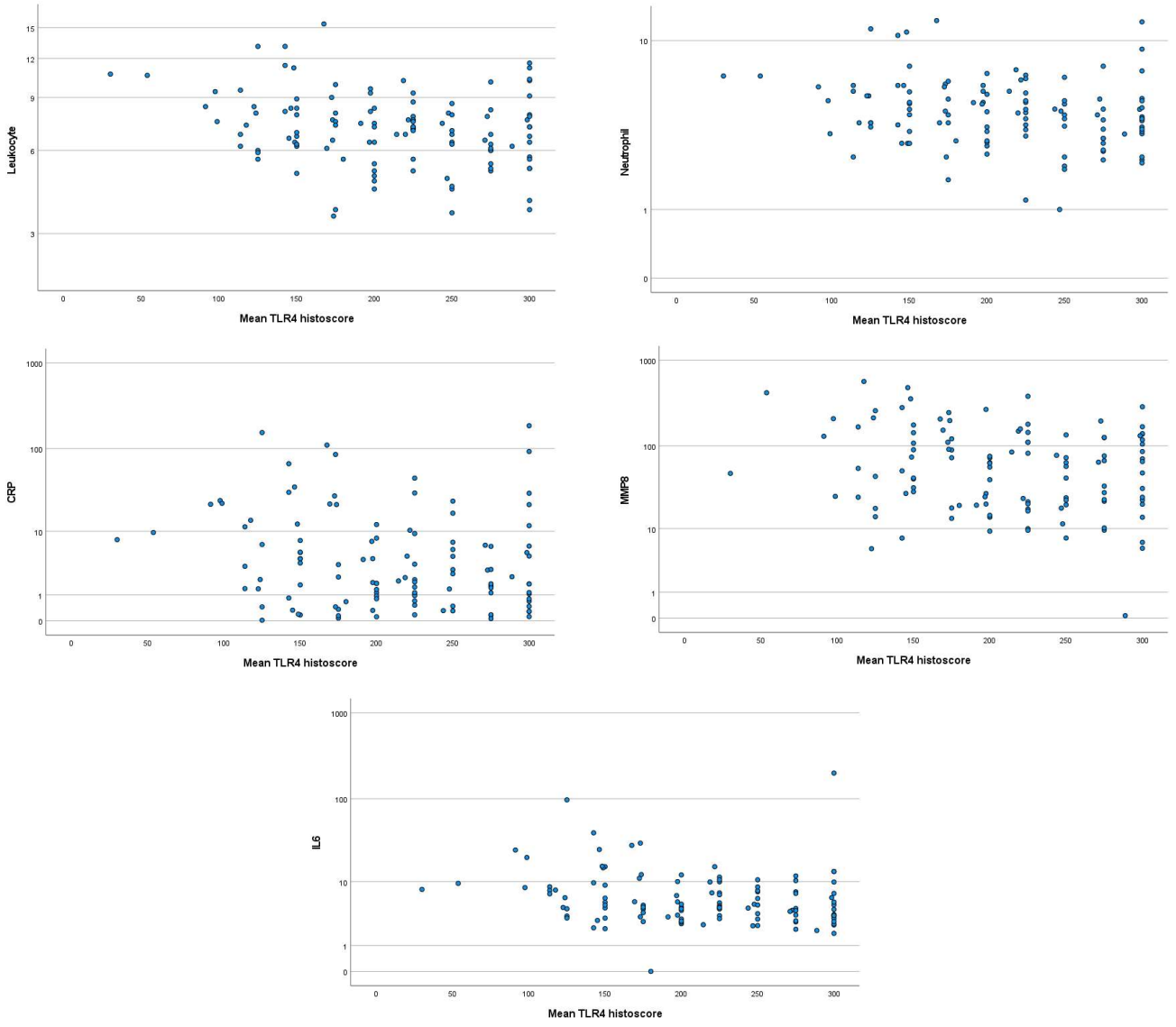
Supplementary Information



Supplementary Figures. Scatterplots showing relationship of TLR4 histoscore in carcinoma epithelium and markers of systemic inflammation including blood total leukocyte count, serum neutrophilic leukocytes count, serum CRP (C-reactive protein), serum MPP8 (matrix metalloproteinase 8), serum IL6 (interleukin 6). All shown correlations were statistically significant (p≤0.05; Spearman correlation; see Table 2).