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Toll-like receptor (TLR) 7 and TLR8 expression on CD133+ cells in colorectal cancer points to a specific role for inflammation-induced TLRs in tumourigenesis and tumour progression ☆

Martin Grimm [a], *Mia Kim* [b], *Andreas Rosenwald* [c], *Uwe Heemann* [d], *Christoph-Thomas Germer* [b], *Ana Maria Waaga-Gasser* [a,e,*], *Martin Gasser* [b,e]

[a] *Department of Surgery I, Molecular Oncology and Immunology, University of Wuerzburg, 97080 Wuerzburg, Germany*
[b] *Department of Surgery I, University of Wuerzburg, Wuerzburg, Germany*
[c] *Institute of Pathology, University of Wuerzburg, Wuerzburg, Germany*
[d] *Department of Nephrology, University of Munich, Klinikum rechts der Isar, Munich, Germany*

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ABSTRACT

Toll-like receptor (TLR) stimulation results in activation of NF-κB, a key modulator in driving inflammation to cancer and mitogen-activated protein kinases that have been shown to recruit mitotic and cyclooxygenase-2 (COX-2) induced pathways in carcinogenesis. Here we asked whether different TLR, COX-2 and stem cell marker expression profiles in colorectal cancer (CRC) provide further evidence for this hypothesis from a clinical perspective. We analysed gene and protein expression of TLR7-TLR10, COX-2 and CD133 as a marker for colon-initiating cells in CRC patients ($n = 65$). Gene analysis demonstrated significantly upregulated TLR7–TLR10 and COX-2 expression in CRC tumour tissues. Analysis of isolated tumour cells from primary tumours showed co-expression of TLR7 and TLR8 with CD133 and gave evidence for a subpopulation of colon cancer-initiating cells. In multivariate analyses TLR8 expression was found to be an independent prognostic factor. Persistent TLR-specific activation of NF-κB in CRC and particularly in tumour-initiating cells may thus sustain further tumour growth and progression through perpetuated signalling known from inflammatory and tissue repair mechanisms with consecutive self-renewal in pluripotent tumour cells. Activation through self-ligands or viral RNA fragments may putatively maintain this inflammatory process, suggesting a key role in cancer progression.

1. Introduction

Toll-like receptors (TLR) are a family of transmembrane receptors that seem to play an important role in maintaining tissue homeostasis by regulating the inflammatory and tissue repair response to injury. Today clinical as well as experimental data clearly indicate that chronic infection and inflammation are important environmental factors and modulators leading to tumourigenesis and tumour progression.[1–3] Epidemiological studies have shown that approximately 15% of human deaths from cancer are associated with chronic viral or bacterial infection. Moreover, patients not suffering from underlying

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* *Corresponding author:* Address: Department of Surgery I, Molecular Oncology and Immunology, University of Wuerzburg, Oberduerrbacher Str. 6, 97080 Wuerzburg, Germany. Tel.: +49 931 201 31715; fax: +49 931 201 31729.
E-mail address: waaga-gasser@chirurgie.uni-wuerzburg.de (A.M. Waaga-Gasser).
[e] Both authors contributed equally to this work.
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infectious disease but from chronic inflammation, e.g. in ulcerative colitis, have a 10-fold higher likelihood of developing colorectal cancer over time.[4] This strongly suggests a relationship between long-standing infections or chronic inflammations in specific tissues or organs and tumourigenesis. In clinically well-characterised cohorts of colorectal cancer (CRC) patients, information on TLR-signalling as a potential underlying mechanism triggering inflammation, tissue remodelling and tumourigenesis remains scarce. In addition, the role of TLR-signalling during the progression of already established CRC is unclear.

During cancer progression an inflammation-like microenvironment in the tumour may augment primary as well as metastatic tumour growth. In fact, proliferating tumour cells can sustain DNA damage and mutagenesis and continue to proliferate in a microenvironment rich in growth factors secreted by inflammatory as well as cancer cells.[2] The molecular mechanisms that connect chronic inflammation and infection with cancer suggest that activation of NF-κB by the classical inhibitor-of-NF-κB kinase-(IKK)β-dependent pathway is a crucial mediator of inflammation-induced tumour growth and progression, as well as an important modulator of tumour surveillance.[1,2]

Recent studies indicate that TLR signalling contributes to the growth of tumours in numerous organs and thus may represent a general principle of tumourigenesis.[4,5] TLRs share a common signalling pathway via the adaptor molecule, myeloid differentiation primary-response protein 88 (MyD88), leading to the activation of NF-κB and MAPKs which are responsible for activation of mitotic and survival pathways.[6] During cancer progression cyclooxygenase-2 (COX-2) is linked to all essential features of carcinogenesis and may play an important role together with TLR expression.[7–10] In general, the TLR family is a family of receptors involved in microbial recognition by the innate immune system, which is thought to promote tumour growth through inflammation-dependent mechanisms.[1,11]

There is also increasing evidence that a population of CD133+ colon cancer-initiating cells is responsible for tumour formation and maintenance in colorectal cancer.[12,13] The aim of this study was therefore to investigate the expression profiles of TLRs in colon cancer cells with regard to their potential role in the initiation and progression of CRC. Moreover, we studied TLR expression in CD133+ CRC stem cells that may result in the activation of NF-κB and enhance their resistance to apoptosis. Therefore, TLR signalling in such cells may play an important role in maintaining themselves as well as re-establishing tumour heterogeneity, providing a promising molecular target of new cancer therapies.[5,14–18]

2. Materials and methods

2.1. Patients

Sixty-five patients with histologically proven colorectal cancer undergoing curative surgical resection in our Department between 01/2001 and 06/2004 were included in the study. The International Union against Cancer (UICC) classification was used for determination of the tumour stage. Tumours were evaluated for location, stage and differentiation grade. Data concerning age, gender, level of wall infiltration and lymph node metastasis were collected in a database and regular medical visits of the patients after curative therapy were performed at intervals according to the governmental guidelines for tumour patients. None of the patients had undergone neoadjuvant treatment, with any type of preoperative antineoplastic protocol (neither chemotherapy nor radiochemotherapy). Patients with rectal cancer (stages T3 NX and/or TX N+ and higher) having undergone neoadjuvant treatment were excluded from analysis. Only patients who had R0 resections were included in our study. Tumour tissue samples as well as normal colon tissues from the patients were collected with informed consent obtained before surgical resection, frozen instantly in liquid nitrogen and stored at −80 °C until analysed. With respect to the fact that colorectal cancer is a complex composition of an in parts necrotising neoplasia interacting with resident stromal components and cells and infiltrating inflammatory cells we used to take comparable areas of solid growth distant from the gut lumen. Normal colon tissues from healthy individuals (n = 10) as well as patients with colitis (data not shown) served as controls. Clinical characteristics of the study population are summarised in Table 1.

2.2. Immunohistochemical and immunofluorescent staining

For immunohistochemical analysis unconjugated TLR7 and TLR9 antibodies (Ab) were purchased from IMGENEX (San Diego, USA) and unconjugated TLR8 Ab (each at a 1:200 dilution) was provided by ProSci (CA, USA). Unconjugated cyclooxygenase (COX)-2 and TLR10 (each at a 1:100 dilution) Abs were provided by Santa Cruz (CA, USA), CD133 (1:10 dilution) Ab by Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Isotype control Abs were purchased from eBioscience (San Diego, USA). Secondary Abs used for immunofluorescence double staining of TLR7 and TLR8 were: Cy3-conjugated AffiniPure Donkey anti-rabbit IgG at a 1:200 dilution (Jackson ImmunoResearch Laboratories Inc., Suffolk, England); secondary Ab for CD133 was a FITC-conjugated AffiniPure Donkey anti-mouse IgG at a 1:200 dilution (Jackson ImmunoResearch).

The staining for TLR7, TLR8, TLR9, TLR10 and COX-2 was performed on serial cryostat sections of the 65 snap-frozen colorectal tumour specimens from early stage (UICC I/II) and late stage patients (UICC III/IV) with neighbouring normal colon tissue and, additionally, in 10 normal colon specimens. All tumours stained positive for cytokeratin-20 (CK-20) (Dako, Hamburg, Germany) and negative for cytokeratin-7 (CK-7) (Dako), a pattern characteristic of colonic adenocarcinoma.[19]

First we assessed H.E. sections from each tumour tissue to differentiate between tumour cell areas, stromal areas and infiltrating immune cells. We then stained for TLRs and COX-2 in additional serial sections. The analysis of immunohistochemical single staining for TLR7, TLR8, TLR9, TLR10, COX-2 and CD133 was performed in tissues from normal colon and colorectal cancer affected tissues. The sequential immunofluorescence double staining (co-expression) was analysed for TLR7 and TLR8 with CD133 expression.

Serial cryostat sections (5 μm) were mounted on glass slides and fixed in acetone for 10 min and then dried for 5 min. Subsequently, the slides were incubated with the pri-

Table 1 – Clinicopathological characteristics of the patients.

Characteristics	Patients ($n = 65$)	TLR7		TLR8	
		Low	High	Low	High
Age (y)					
⩽64	45 (69%)	14 (31%)	31 (69%)	11 (24%)	34 (76%)
⩾65	20 (31%)	9 (45%)	11 (55%)	3 (15%)	17 (85%)
Gender					
Male	37 (57%)	11 (30%)	26 (70%)	8 (22%)	29 (78%)
Female	28 (43%)	12 (43%)	16 (57%)	6 (21%)	22 (79%)
Primary tumour					
Colon	26 (40%)	13 (50%)	13 (50%)	9 (35%)	17 (65%)
Rectum	39 (60%)	10 (26%)	29 (74%)	5 (13%)	34 (87%)
Histological classification					
G1	12 (18%)	2 (17%)	10 (83%)	2 (17%)	10 (83%)
G2	31 (48%)	13 (42%)	18 (58%)	9 (29%)	22 (71%)
G3/4	22 (34%)	8 (36%)	14 (64%)	3 (14%)	19 (86%)
Depth of invasion					
pT1	14 (22%)	5 (36%)	9 (64%)	5 (36%)	9 (64%)
pT2	23 (35%)	10 (43%)	13 (57%)	3 (13%)	20 (87%)
pT3	17 (26%)	6 (35%)	11 (65%)	3 (18%)	14 (82%)
pT4	11 (17%)	2 (18%)	9 (82%)	3 (27%)	8 (73%)
Lymph nodes metastasis					
pN0	34 (52%)	16 (47%)	18 (53%)	9 (26%)	25 (74%)
pN1-3	31 (48%)	7 (23%)	24 (77%)	5 (16%)	26 (84%)
UICC stage					
UICC I	15 (23%)	11 (73%)	4 (27%)	2 (13%)	13 (87%)
UICC II	19 (29%)	7 (37%)	12 (63%)	7 (37%)	12 (63%)
UICC III	22 (34%)	5 (23%)	17 (77%)	5 (23%)	17 (77%)
UICC IV	9 (14%)	0 (0%)	9 (100%)	0 (0%)	9 (100%)
Mean OS (m)	32 m	41 ($n = 23$)	27 ($n = 42$)	39 ($n = 14$)	29 ($n = 51$)
Median OS (m)	31 m	49 ($n = 23$)	22 ($n = 42$)	51 ($n = 14$)	28 ($n = 51$)

y, years; G, grading; UICC, International Union against Cancer; R, residual tumour; OS, overall survival; m, months.

mary antibody or control antibody diluted in TBS plus 0.5% bovine serum albumine (BSA) overnight at 4 °C in a humidified chamber and with secondary FITC-conjugated (fluoresceinisothiocyanat) antibody for 30 min at room temperature in a humidified chamber. The slides were subsequently incubated with the second primary antibody diluted in TBS plus 0.5% BSA overnight at 4 °C in a humidified chamber followed by incubation with secondary Cy3-conjugated antibody for 30 min at room temperature in a humidified chamber. Slides were counterstained with DAPI (4′,6-diamidino-2-phenylindoldihydrochlorid) (Sigma–Aldrich, Steinheim, Germany) and covered with polyvinyl-alcohol mounting medium (DABCO) (Sigma–Aldrich) and analysed using a Zeiss camera (Jena, Germany). The photographed images using the Metamorph software package (Visitron Systems, Puchheim, Germany) were imported into the Microsoft Office Picture Manager.

For immunohistochemistry, the pretreatment fixation of the slides was the same as described for immunofluorescence. After incubation with the primary antibody, we used a horseradish peroxidase (HRP)-conjugated AffiniPure Donkey anti-mouse or a Donkey anti-rabbit or a Donkey anti-goat IgG at a 1:200 dilution (Jackson ImmunoResearch). Slides were subsequently incubated for 5 min in DAB (3,3′-diaminobenzidine) (Biogenex, San Ramon, USA), counterstained with Haemalaun and mounted with Glycergel (Dako).

The quantification of each immunoenzymatic staining of tumour cells in six individual magnified fields (×400 magnifications) for each staining sample was scored by cell counting. The magnified fields were representative for the tumour section. The results of the staining were expressed in percentages positivity (number of positive cells within 100 counted tumour cells, %). All values were expressed as mean ± SEM. Sections were evaluated by two independent, blinded investigators, separately and in case of discrepancies both evaluated the slide simultaneously and reached an agreement in their final assessment.

2.3. *Real-time quantitative reverse transcription-PCR (RT-qPCR) analysis*

To analyse gene expression of TLR7, TLR8, TLR9, TLR10 and COX-2 by RT-qPCR, we extracted total cellular RNA using the RNeasy Minikit from Qiagen (Hilden, Germany). RNA extraction was performed according to the manufacturer's instructions. To obtain a reproducible and high quality amplification for all genes, we used primer sets for TLR7, TLR8, TLR9, TLR10 and COX-2 developed by Qiagen. Matched human colon cDNA (normal colonic mucosa) was purchased from Pharmingen (Heidelberg, Germany) as control. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was

used for relative quantification and cDNA quality control. All PCR reactions were carried out with a DNA Engine Opticon 2 System (MJ Research, Biozym, Oldendorf, Germany). The relative quantification value, fold difference, is expressed as $2^{-\Delta\Delta Ct}$.

2.4. Flow cytometric analysis (FACS) of TLR7, TLR8 and CD133+ co-expression

Cells derived from patients' normal colon tissue and colorectal cancer tissue were analysed on a flow cytometer (Beckman Coulter, Krefeld, Germany) with an appropriate software package (Coulter, Epics XL-MCL, System II). In each sample we analysed a constant cluster of tumour cells. TLR7 Ab was purchased from IMGENEX (San Diego, USA) and TLR8 Ab was provided by ProSci (CA, USA). CD133-PE Ab, FITC-conjugated anti-rabbit secondary Ab and isotype control Abs were purchased from Beckman Coulter (Krefeld, Germany).

The total suspension of cells (5×10^6) was pelleted and resuspended in PBS. For intracellular staining we used the Intraprep-Kit (Beckman Coulter, Krefeld). Cells were incubated with CD133-PE conjugated or IgG-PE conjugated control Ab for 20 min. After washing, cells were incubated with second primary Ab for TLR7 and TLR8, or IgG-FITC-conjugated control Ab for 20 min followed by subsequent incubation of FITC-conjugated secondary Ab for 20 min after a washing step. The cells were analysed by FACS, equipped with the phycoerythrin emission signal detector FL2 (585 nm, red) and with the FITC emission signal detector FL1 (488 nm, green).

2.5. Statistical analysis

Statistical analysis was performed with MedCalc Software (Mariakerke, Belgium). All values were expressed as mean (SEM). Survival time was determined as the time from tumour resection to tumour-specific death and as the time from tumour resection to time of obvious recurrence. The overall survival time in association with TLR7 and TLR8 expression was estimated by Kaplan–Meier method (mean cut-off value for either high or low expressors was set at 22% for TLR7 and 34% for TLR8). In all cases, the Kolmogorov–Smirnov test was applied to test for a normal distribution. To analyse differences in the overall/tumour related survival amongst patients after successful (R0) curative surgical resection for CRC, patients were divided into two subgroups (dichotomous variables). Univariate analysis of significance for TLR7 and TLR8 expression was performed and differences in survival curves was evaluated by a log-rank test. Multivariate analysis by using the Cox Proportional Hazards Model was performed on all parameters that were found to be significant on univariate factors.

Data were analysed using the Student's *t*-test when two groups were compared. A *p*-value of less than 0.05 was accepted as statistically significant.

3. Results

3.1. TLR and COX-2 expression is associated with tumour progression

Between 30% and 40% of normal colon tissues expressed TLR7, TLR8, TLR9 and TLR10 at a baseline level of less than 10% positively stained epithelial cells. For COX-2 this was found only in 20% of normal colon tissues. Compared to control tissues, expression of TLR7, TLR8, TLR9and TLR10 in early stage tumours was found in an increased number of patients (TLR7 $n = 26/32$, 81.3%; TLR8 $n = 27/32$, 84.4%; TLR9 $n = 24/32$, 75%; TLR10 $n = 20/32$, 62.5%). COX-2 expression, alike the expression of TLR7-10 was expressed in an increased number of cases at early stages of the disease ($n = 25/32$, 78.1%). Interestingly, the overall number of tumours with elevated expression of TLRs and COX-2 further increased in late stages of the disease amongst all markers when compared between early and late stage tumours (TLR7 $n = 29/33$, 87.9%; TLR8 $n = 30/33$, 90.9%; TLR9 $n = 28/33$, 84.8%; TLR10 $n = 29/33$, 87.9%; COX-2 $n = 32/33$, 97%). Expression of TLRs was strongly associated with cancer cells but was rarely detected in stromal-tumour-infiltrating leucocytes at all tumour stages without any differences.

Furthermore, we analysed positivity of all counted cells according to the tumour stage in comparison with expression in normal colon tissues in serial sections (H.E. and immunostaining). TLR7, TLR8, TLR9, TLR10 and COX-2 expression were clearly upregulated in cancer progression ($p < 0.0001$ for each TLR compared to normal controls; UICC I/II compared to UICC III/IV: $p < 0.0001$ for TLR7 and TLR8; $p = 0.0008$ for TLR9 and TLR10; $p = 0.0007$ for COX-2) (Fig. 1A).

Fig. 1B demonstrates representative examples of an increased expression of TLR7 and TLR8 in tumour tissues of a stage II and stage IV CRC compared to normal tissue.

3.2. Analysis of TLR7, TLR8, TLR9, TLR10 and COX-2 gene expression

qRT-PCR was performed for analysis of stage-dependent differences in gene expression of TLR7, TLR8, TLR9, TLR10 and COX-2 in human colorectal carcinoma. Compared with normal tissue, gene expression of TLR7, TLR8, TLR9, TLR10 and COX-2 was significantly upregulated in both early stage (UICC I/II: TLR7 12.9, TLR8 11.3, TLR9 2.5, TLR10 1.5 and COX-2 8.2 times fold difference) and late stage colorectal cancer (UICC III/IV: TLR7 13.2, TLR8 11.8, TLR9 6.5, TLR10 2.2, COX-2 9.5 times fold difference) (for each TLR compared with normal controls $p < 0.0001$) (Fig. 2). These results demonstrated concordance with an elevated TLR and COX-2 expression in the tumour cells, as observed by serial immuno-histo chemistry. Other analysed TLRs did not show significant expression (data not shown).

For further investigation of the adoptive role of TLR7 and TLR8 in potential colon cancer-initiating cells we analysed CD133+ expression in eight out of 65 patients ($n = 8/65$).

3.3. Flow cytometric analysis of TLR7 and TLR8 expression

We next examined disintegrated tumour cells derived from CD133+ tumours ($n = 8/65$) from operated patients, that were shown by serial sectioning immunohistochemical analysis and additional H.E. staining to express a stem cell phenotype CD133. Compared with colon cells from normal colon tissues expression of TLR7, TLR8 and CD133 was increased. This was observed in the early as well as late stage tumours (normal

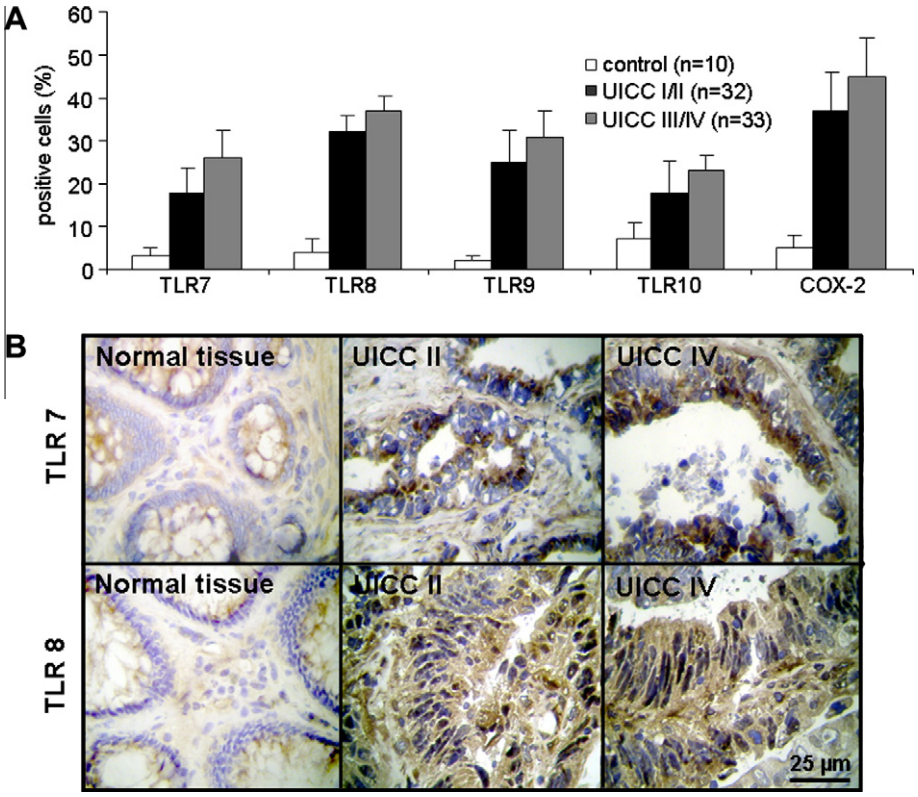

Fig. 1 – Immunohistochemical expression profiles as percentage of positive cells for TLR7, TLR8, TLR9, TLR10 and cyclooxygenase-2 (COX-2) in normal colon tissue and colorectal cancer differentiated between early and late stage tumours (UICC I/II versus III/IV) (A). Representative immunostaining for the two most relevant Toll-like receptors (TLRs), TLR7 and TLR8 (B). Analysis refers to percentages positivity of all counted cells according to normal tissue, early stage tumours (UICC I/II) and late stage tumours (UICC III/IV), respectively (A). Cytoplasmic staining pattern, brown. Original magnification: ×400 (B).

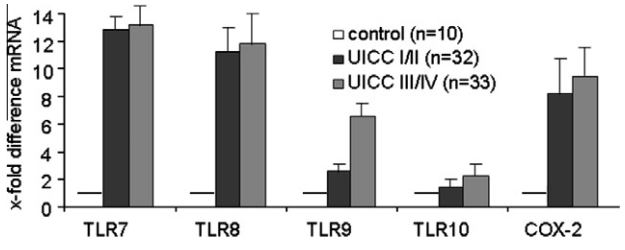

Fig. 2 – Comparison of TLR7, TLR8, TLR9, TLR10 and COX-2 gene expression in colorectal cancer using qRT-PCR. Relative quantification value, fold difference, is expressed as $2^{-\Delta\Delta Ct}$. Normal tissue is considered as onefold and is standardised to dotted line.

colon cells: TLR7: 3.5%; TLR8: 49.5%; CD133: 1.6%, data not shown; cells from UICC II tumour: TLR7: 37.9%; TLR8: 97.0%; CD133: 12.9%; cells from UICC IV tumour: TLR7: 48.4%; TLR8: 98.4%; CD133: 29.2%; $p < 0.0001$) (Fig. 3A and B, each representative of $n = 4$ tumours of early and late stage tumours). Next we performed co-expression experiments of TLR7 and TLR8 with CD133. As shown in the representative experiment Fig. 3C, TLR7 and TLR8 were co-expressed with CD133 in the tumour cells (CD133/TLR7: 22.2%; 85.7% out of all CD133+ cells; CD133/TLR8: 22.4%; 100% out of all CD133+ cells). We thus conclude that TLR7 and TLR8 are also expressed by tumour cells corresponding to undifferentiated CD133+ colon cancer-initiating cells. TLR signalling may therefore play a biological role in potential colon cancer-initiating cells.

3.4. *Co-expression of TLR7 and TLR8 with CD133*

To further assess co-expression of TLR7 and TLR8 with CD133 on tumour cells we performed immunofluorescent double staining of CD133 with TLR7 and TLR8 plus an additional serial-sectioning H.E-.staining in the tumour tissues. As shown in Fig. 4, TLR7 and TLR8 were co-expressed with CD133 (Fig. 4, representative example of $n = 8$ tumours).

3.5. *Prognostic value of TLR7 and TLR8*

To analyse differences in tumour-related survival dependent on the TLR7 and TLR8 expression we divided the patients in two subgroups as described above (dichotomous variables). The survival in the high expressing TLR7 ($p = 0.001$, hazard ratio (HR) = 3.8814, 95% (confidence interval) CI = 1.9390–7.7695) and TLR8 ($p = 0.018$, HR = 3.2202, 95% CI = 1.5274–6.7889) subgroups was significantly poorer than that of the low expressing TLR7 and TLR8 subgroups (Fig. 5A and B). Multivariate analysis using the Cox Proportional Hazards Model show ad-

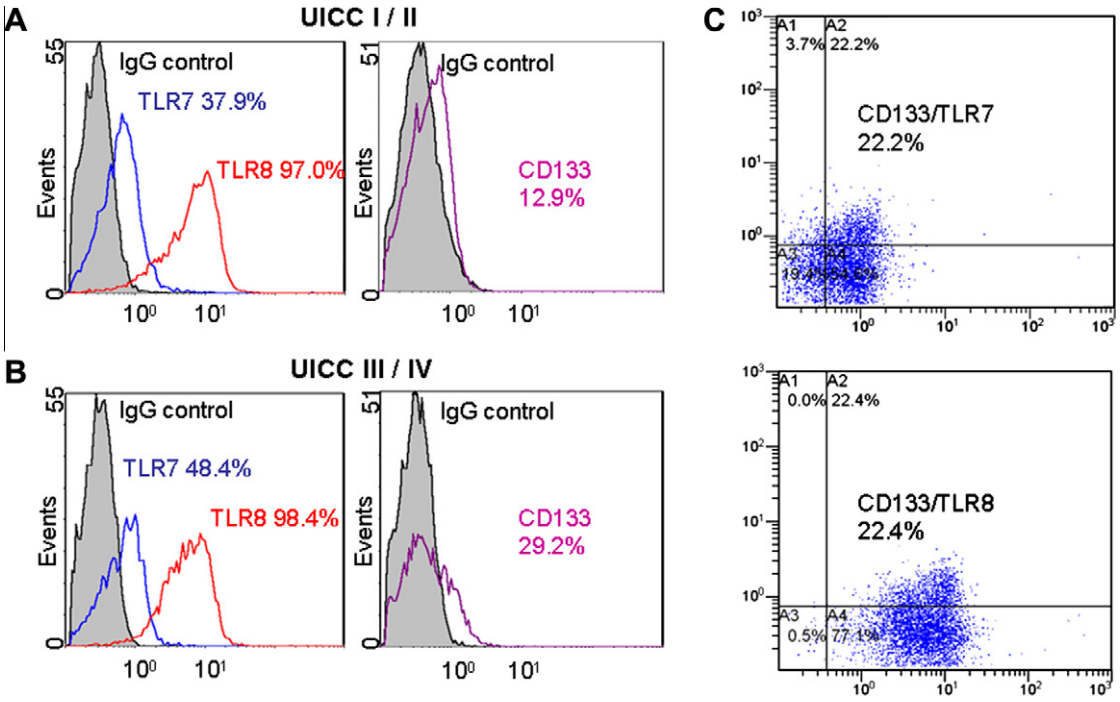

Fig. 3 – Assessment of TLR7, TLR8 with CD133 by flow cytometric analysis (FACS) analysis. FACS analysis of single stained TLR7 (blue line), TLR8 (red line) and CD133 (purple line) expressing tumour cells derived from early stage tumours (UICC I/II) (A) and late stage tumours (UICC III/IV) (B) (IgG control black line). (C) Representative example of TLR7 and TLR8 co-expression analysis with CD133 in separated colorectal tumour cells (co-expression of CD133+ with TLR7: 85.7% out of all CD133+ cells; co-expression of CD133+ with TLR8: 100% out of all CD133+ cells).

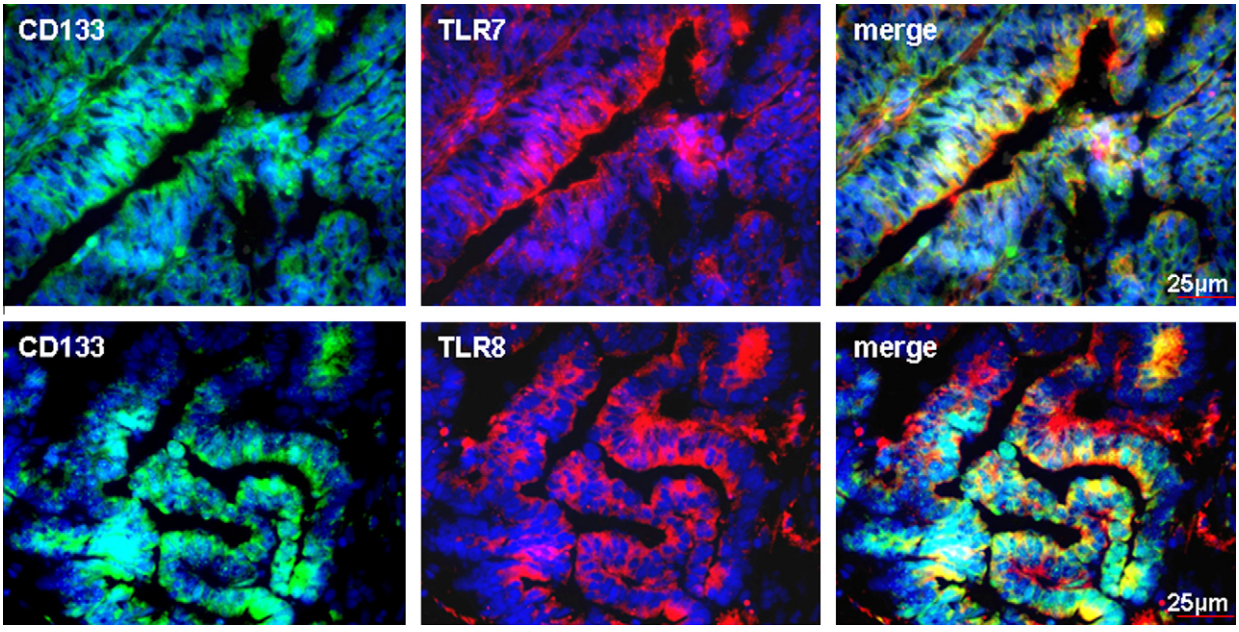

Fig. 4 – Representative images of co-expression analysis of the most relevant TLRs, TLR7 and TLR8, with CD133 by immunofluorescent double staining. Representative examples demonstrate TLR7 and TLR8 expression in CD133+ colorectal tumour specimens. FITC green fluoreszeinisothiocyanat, Cy3 red and DAPI 4′,6-diamidino-2-phenylindoldihydrochlorid blue. Calibration bar represents 25 µm.

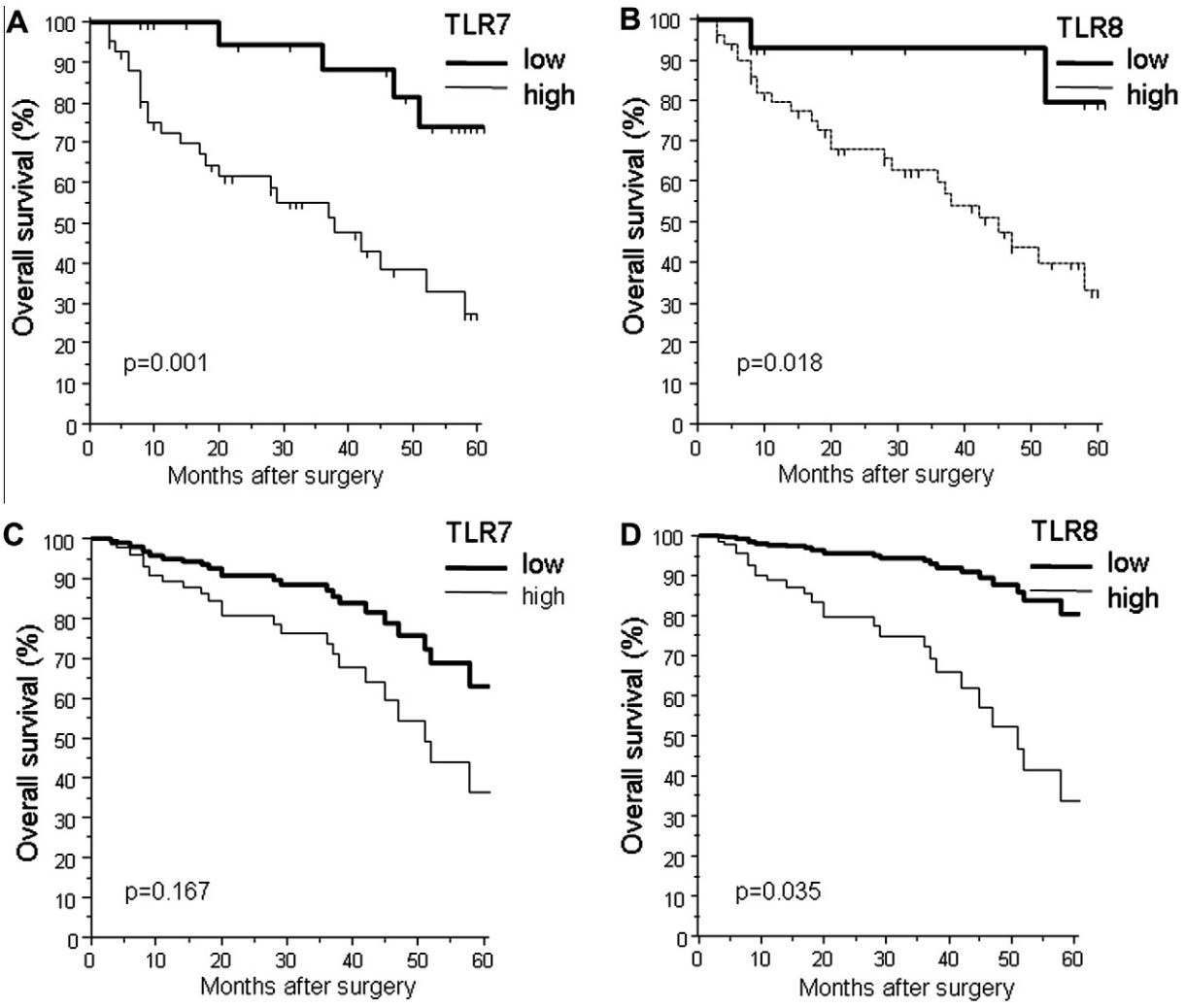

Fig. 5 – Overall survival curves by Kaplan–Meier method and Cox-regression analysis in CRC patients for TLR7 (A) and for TLR8 (B). Survival time of the censored data is indicated by short vertical lines. Multivariate analysis show survival curves for TLR7 (C) and TLR8 (D). CRC, colorectal cancer.

Table 2 – Multivariate analysis of prognostic factors of the patients (n = 65).

Variable	Unfavourable factor	Hazard ratio (HR)	95% confidence interval (CI) of HR	P Value
Depth of invasion	pT3/4	2.091	1.2527–3.4907	0.005
LN	Positive	5.468	1.9827–15.0826	0.001
TLR7 expression	High	2.195	0.7234–6.6602	0.167
TLR8 expression	High	4.997	1.1231–22.2328	0.035

LN, lymph nodes metastasis; TLR, Toll-like receptor.

vanced pT stage, lymph node metastasis and TLR8 but not TLR7 expression as independent prognostic factors (Table 2, Fig. 5C and D).

4. Discussion

Since TLR signalling may have implications for the development or progression of cancer, we studied the TLR expression in tumour cells of colorectal cancer (CRC) operated at our centre in a consecutive series. Here, we describe the new finding of highly upregulated TLR7 and TLR8 gene and protein expression in CRC which was strongly related to cancer cells but rarely detected in stromal-tumour-infiltrating leucocytes. Therefore, TLR expression of tumour-infiltrating leucocytes in the inflammatory stromal compartment seems not to be important for tumour immunity.

Several studies in the literature focused on the effects of different TLRs in the context of tumour development and progression. For example, TLR1, TLR2, TLR6, TLR7, TLR9 and TLR10 mediated proliferation and survival of multiple myeloma cells.[20] In ovarian cancer, TLR4 expression was associated with tumour progression and chemoresistance. Paclitaxel was identified as a ligand for TLR4 and the activation of the adapter protein MyD88 was linked to reduction of apoptosis in cancer cells.[4] Expression profiles of TLR1-6 and TLR9 have been described in murine and human cancer cell lines of the colon, lung, prostate, breast and melanoma. It was stated, that many of the same signalling elements are involved in tumourigenesis and tumour growth, suggesting that TLRs may support tumour growth.[15] In gastric cancer cells and precursor lesions of this tumour from patients suffering from *Helicobacter pylori* infection, TLR4, TLR5 and TLR9 were upregulated, which may have induced further cancer-promoting factors.[21] Moreover, it was suggested that TLR signalling is involved in malignant transformation of benign prostatic glands by pathogens in the urinary system.[22] Our results indicate that TLR7 and TLR8 expression are associated with tumour progression in CRC and reduced tumour-specific survival amongst patients with high TLR7 and TLR8 expression on cancer cells compared to low TLR7 and TLR8 subgroups.

Only a small subset of CD133+ tumour cells has been described to represent tumour stem cells within a solid tumour such as CRC; from our results we conclude that TLR7 and TLR8 are expressed on terminally differentiated cells as well as on pluripotent colon cancer-initiating cells.[23] Inflammation responses through the TLR signalling followed by activation of NF-κB may potentially enable tumour -initiating cells to maintain themselves as well as re-establish tumour heterogeneity by enhancing their resistance to apoptosis.[18]

Furthermore, COX-2 is linked to all essential features of carcinogenesis and may play an important role in combination with TLR expression in tumour progression. Increased invasion and angiogenesis of gastric cancer cells is mediated by COX-2 after TLR2 and TLR9 activation, leading to inflammation and tumour progression.[9] Moreover, increased COX-2 expression in human carcinomas support the suggestion, that the tumour-microenvironment contains common features of chronic inflammatory processes in parallel to all the essential features of carcinogenesis such as mutagenesis, mitogenesis, angiogenesis, reduced apoptosis, metastasis

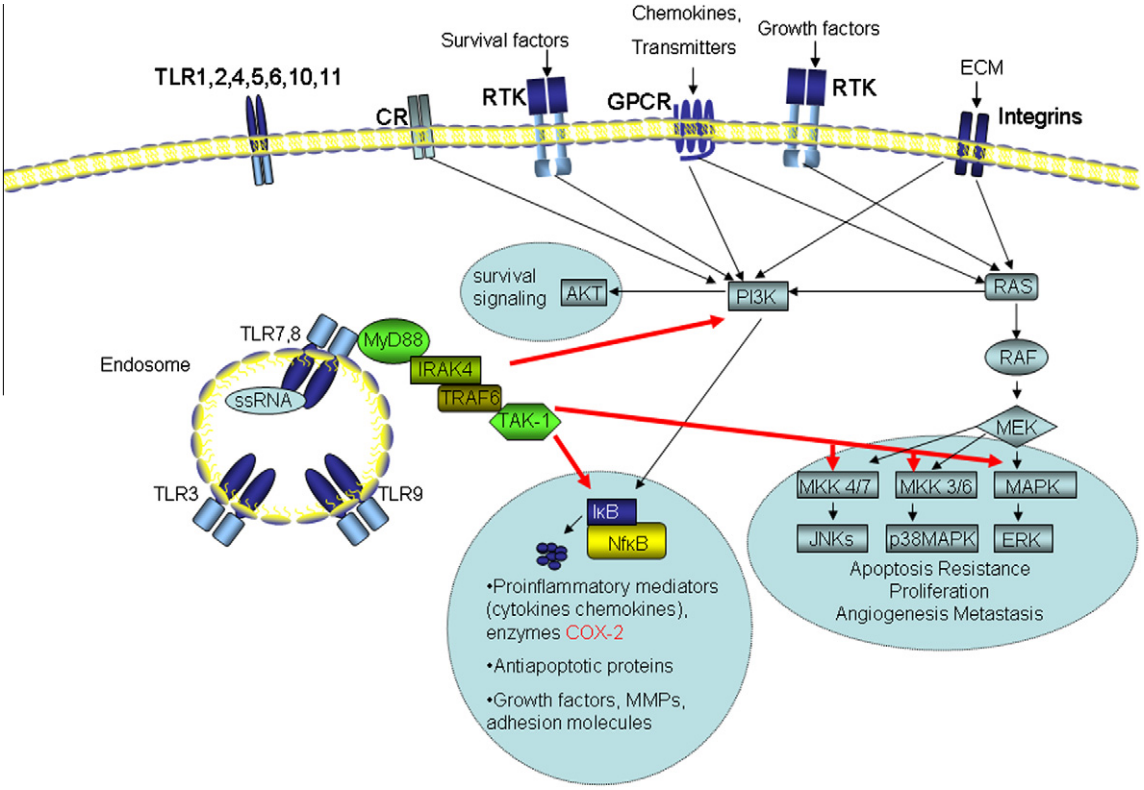

Fig. 6 – Blocking of TLR signalling might be a potential mechanism to reduce COX-2, MAPK, NF-κB and PI3K mediated apoptosis resistance, chemoresistance and tumour survival. Black arrows and red arrows (TLR signalling) show stimulatory modification. TLR, Toll-like receptor; CR, cytokine receptor; RTK, receptor tyrosine kinase; GPCR, G-protein coupled receptor; ECM, extracellular matrix; MEK, MAPK/Erk kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MKK, MAP kinase kinases; PI3K, phosphoinositide 3-kinase; JNK, Jun N-terminal kinase; MyD88, myeloid differentiation primary-response protein 88; IRAK, interleukin receptor-associated kinase; TRAF, TNF receptor-associated factor; TAK, TGF β-activated kinase; IκB, inhibitor of NF-κB; Nfκ B, nuclear factor κ B; TNF, tumour necrosis factor; COX-2, cyclooxygenase-2; ssRNA, single-stranded RNA.

and immunosuppression. All these events are linked to COX-2-driven prostaglandin E2 (PGE2) biosynthesis.[9] Recently, it was demonstrated that TLR8 signalling strongly promotes inflammatory lipid mediator biosynthesis PGE2 through the COX-2 pathway. These data provide novel insights into the innate immune response to viral infections and raise the possibility that the immune response to single-stranded RNA (ssRNA) viruses via the TLR8 pathway may implicate the lipid mediators of inflammation.[10] Interestingly, modified oligonucleotides derived from enterovirus RNA (Coxsackie B) mediated pericarditis by TLR7 and TLR8 signalling.[23] Indeed, we demonstrated increased COX-2 gene and protein expression in combination with TLR positivity in tumour tissues from CRC patients and, thus, our data support the hypothesis of inflammation-induced carcinogenesis by COX-2 associated with TLR7, TLR8, TLR9 and TLR10 upregulation in CRC.

In conclusion, chronic activation of TLRs expressed by tumour cells from CRC and pluripotent CD133+ colon cancer-initiating cells may sustain inflammation responses, mediate resistance to apoptosis and promote further tumour progression. Therefore, targeting of TLR signalling might be a potential mechanism to abrogate this inflammation-mediated effect in tumour progression (Fig. 6).

5. Ethics

The present study has been approved by the local Ethic Committees.

Conflict of interest statement

None declared.

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