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M. STANISLAWOWSKI[1], P.M. WIERZBICKI[1], A. GOLAB[1], K. ADRYCH[2], D. KARTANOWICZ[1], J. WYPYCH[2], J. GODLEWSKI[3], M. SMOCZYNSKI[2], Z. KMIEC[1,3]

# DECREASED TOLL-LIKE RECEPTOR-5 (TLR-5) EXPRESSION IN THE MUCOSA OF ULCERATIVE COLITIS PATIENTS

[1]Department of Histology, Medical University of Gdansk, Poland; [2]Department of Hepatology and Gastroenterology, Medical University of Gdansk, Poland; [3]Department of Histology and Embryology, Medical Faculty, University of Warmia and Mazury, Olsztyn, Poland

Objective: Although there is a convincing evidence supporting an important role for microorganisms in the pathogenesis of Inflammatory Bowel Disease (IBD) which comprises ulcerative colitis (UC) and Crohn's disease (CD), the specific mechanisms involved remain unclear. Toll-like receptors (TLR) recognize various molecules of microbiota including flagellin, the principal protein of motile comensal and pathogenic bacteria implicated in the pathogenesis of IBD. Aim: To investigate the expression of the TLR-5 receptors at the mRNA and protein levels in the mucosa of UC patients. Materials and Methods: TLR-5 mRNA was quantified by the validated real-time PCR (QPCR) in mucosal biopsies of 99 UC patients and 34 control patients and TLR-5 protein was detected by immunohistochemistry (IHC) in 57 UC and 10 control patients. Results: Significantly decreased TLR-5 gene expression at mRNA and protein level was found in the mucosa of patients with moderate and severe disease activity as compared to patients with low UC activity and control. TLR-5 immunoreactivity was found in the mucosa of UC patients and normal controls in the cytoplasm of enterocytes and at their basolateral domain. However, the intensity of the IHC reaction in specimens from UC patients was substantially lower than in control samples. Conclusion: The decreased expression of TLR-5 gene and protein in the mucosa of UC patients suggests that down-regulation of TLR-5 is probably caused by the increased number of ligand molecules in the proximity of epithelial cells in the inflamed tissue.

Keywords: *TLR-5 expression, ulcerative colitis, QPCR, immunohistochemistry*

## INTRODUCTION

Toll-like receptors (TLRs) of both classical immune competent cells (*e.g.* macrophages, dendritic cells and neutrophils) as well as vascular endothelial and epithelial cells recognize various molecules classified as pathogen-associated molecular patterns (PAMPs), present on the surface of both pathogens and commensal microorganisms (1). Recent cellular and mouse studies demonstrated that activation of Toll-like receptor responses in intestinal epithelial cells by commensal bacteria plays a key role in maintaining colonic homeostasis and controlling tolerance in the gut (2, 3). It was also shown that dysregulation of intestinal immune responses to commensal/pathogenic microbiota and other environmental factors, together with a genetic predisposition, contributes to the development of chronic inflammation, which is the hallmark of Crohn's disease (CD) and ulcerative colitis (UC), collectively known as IBD (4-6). Studies of intestinal bacterial populations in IBD patients showed altered proportion of major intestinal bacterial species including motile, *i.e.* flagellated bacteria species such as *Salmonella*, *Listeria*, *Pseudomonas*, and *Escherichia* strains (7). The bacterial flagellum is basically made up by the protein flagellin which is known to be a ligand for the Toll-like receptor 5 (TLR-5) (8, 9, 10). Although the ligation of flagellin by TLR-5 induces release of proinflammatory cytokines from cultured intestinal epithelial cells (8), little is known about TLR-5 status in IBD. Therefore, the aim of this study was to characterize the expression of TLR-5 receptors in the intestinal mucosa of UC patients using real-time PCR (QPCR) and immunochistochemistry (IHC).

## MATERIALS AND METHODS

### *Patients*

In the prospective study specimens were obtained from two gastroenterological clinics of the Pomerania region of Poland from 2006 to 2008. The study was approved by local ethical committee. Informed, written consent regarding the use of tissue samples and mucosal biopsies was obtained from all patients before colonoscopy which was scheduled for follow-up or relapsing disease.

UC patients were divided into 3 groups according to clinical activity and classified according to the 'Montreal Classification'

(11). The diagnosis of UC was based on clinical, radiographic, endoscopic and histopathological findings. Clinical activity of UC was graded in a semi-quantitative manner according to the scoring system (Clinical Activity Index) described by Rachmilewitz (12). Group I included UC patients with mild (n=41), group II with moderate (n=50), and group III with high (n=8) UC activity. The control group consisted of 34 patients who underwent colonoscopy as a part of a routine surveillance for colorectal carcinoma (CRC). These patients had no CRC or IBD in family history and presented normal mucosal histology. None of the control patients was on medication at the time of investigation.

The basic characteristics of the patients is shown in *Table 1*. At the time of the study, all UC patients were treated with mesalamine or sulfasalazine, whereas UC patients with high disease activity (group III) were also treated with oral or enema corticosteroids. None of the group III patients was treated with antibiotics or immunomodulators (6-mercaptopurine, azathioprine, and/or methotrexate). Statistically significant differences between groups' age, sex, disease duration, active smoking and number of erythorcytes were not found. However, Erythrocyte Sedimentation Rate increased, and serum iron concentration decreased proportionally to the disease activity (*Table 1*).

*Mucosal biopsies and nucleic acids extraction*

The procedure for sampling mucosal biopsies and further processing has been in detail described in an accompanying paper (13). Shortly, colonic biopsy specimens were taken from the endoscopically active lesions (inflamed mucosa). One biopsy for routine histological examination was fixed in 10% formalin, whereas other specimens from the closest location obtained for RNA and immunochemical assessments were immediately placed in sterile vials, quick frozen in liquid nitrogen, and stored at -85°C. RNA was isolated using Total RNA kit (A&A Biotechnology, Gdynia, Poland) based on the phenol-chlorophorm-isoamyl alcohol and silica membrane technique (14). Isolated RNA was stored at -85°C. 2 µl of RNA was reversibly transcribed using M-MLV Transcriptase and oligo-$dT_{15}$ primer (Promega, Madison, WI, USA) in a total volume of 25 µl and stored at -25°C.

*Quantitative PCR analysis*

The expression rate of the TLR-5 gene was analyzed by Real-time PCR using iQ Cycler (Bio-Rad, Hercules, CA, USA) with Sybr®Green I as a fluorophore. TLR-5 expression ratio was determined by the comparative method (Livak's $2^{-\Delta\Delta Ct}$ equation) (15) in relation to the mean rate of two housekeeping genes ACTB and RPL32, which have constant expression in ulcerative colitis and Crohn's disease (constancy of the expression was established in preliminary experiments - data not shown). Primers' sequences were as follows: TLR-5; sense: 5'-TCAAACCCCTTCAGAGA ATCCC, antisense: 5'-TTGGAGTTGAGGCTTAGTCCCC [16], ACTB; sense: 5'-TGTGCCCATCTACGAGGGGTATGC, antisense: 5'-GGTACATGGTGGTGCCGCCAGACA [16], RPL32; sense: 5'- TGACAACAGGGTTCGTAGAAGAT, antisense: 5'-GTTCTTGGAGGAAACATTGTGAG. The components of the PCR reactions were as follows; 0.4 µl tissue cDNA (equivalent of 32 ng of total mRNA), 100 nM primers and Real-time PCR iQ SybrGreen SuperMix (Bio-Rad, Hercules, CA, USA) were mixed to obtain a final volume of 17 µl. All reactions were performed in duplicate. For TLR-5, ACTB and RPL32, the amplification profile was 30-s denaturation at 95°C followed by 30-s annealing at 55°C for TLR-5 and RPL32, and 30-s annealing at 60°C for ACTB, 1-min elongation at 72°C and 5-s fluorescence reading at 77°C for a total of 35 cycles. Amplification of the expected single products was confirmed by dynamic melt-curve and by electrophoresis on 1% agarose gels stained with ethidium bromide. Fluorescence data were automatically collected and analyzed by iCycler iQ Optical Sofware ver. 3.0a (Bio-Rad, Hercules, CA, USA).

*Table 1.* Basic characteristics of UC and control patients.

| | **Activity of Ulcerative Colitis (CAI)** | | | **Control** |
|---|---|---|---|---|
| | Mild<br>Group I | Moderate<br>Group II | Severe<br>Group III | |
| Activity of UC<br>mean ± SD | 4 < CAI < 8<br>6.1±0.6 | 8<CAI≤12<br>9.5±1.1 | CAI>12<br>15.1±3.4 | NA |
| Number of patients (n, *%*) | 41 (*40.4*) | 50 (*50.5*) | 8 (*8,1*) | 34 |
| Sex (M/F) | 16/25 | 25/25 | 6/2 | 19/15 |
| Age<br>(mean ± SD, range, y) | 48,6±2,7<br>(22-86) | 46,3±2,0<br>(23-81) | 48,8±5,6<br>(28-74) | 54,6±2,6<br>(24-78) |
| Disease duration (mean<br>± SD, range, y) | 7,4±0,9<br>(1-30) | 7,8±0,8<br>(1-26) | 13,2±3,8<br>(5-31) | NA |
| Active smokers [a] | 27 | 31 | 6 | |
| Location | | | | |
| E1 - Proctitis | 29 | 41 | 4 | |
| E2 - Left-sided | 11 | 7 | 3 | |
| E3 -Pancolitis | 1 | 2 | 1 | |
| Laboratory findings | | | | |
| Iron in serum (normal<br>range *50 - 175*µg/dl) | 93,2±17,4 | 77,6±14,6 | 50,5±35,5* | 87,7±19,7 |
| Erythrocytes<br>($10^6$/µl) | 4,8±0,1 | 4,6±0,1 | 4,3±0,3 | 4,6±0,2 |
| ESR<br>(mm/h) | 16,5±3,0* | 18,2±2,5** | 39,4±8,2*** | 7,5±1,2 |

*, **, *** - p<0.05, 0.005, 0.001 *vs*. control; ESR- Erythrocyte Sedimentation Rate; [a] – at the time of investigation

*Immunostaining of the TLR-5 protein*

Colon biopsies that were stored at -85°C were used to obtain 12 µm-thick cryostat sections. Sections were preincubated for 30 min. at 21°C in 0,3% $H_2O_2$/methanol to block the activity of endogenous peroxidases. After washing with PBS sections were incubated with 2% rabbit serum for 1 h and incubated for 1 h at 21°C with goat primary polyclonal anti-TLR-5 antibodies (AB 1654, final dilution 1:250, Abcam Inc., Cambridge, MA, USA). Then, sections were incubated with rabbit, anti-goat biotinylated polyclonal IgG (Vectastain ABC Kit, PK-4005, Vector Labs., Burlingame, CA, USA) for 1 h followed by treating with avidin-biotinylated horseradish peroxidase reagent for 30 min. at room temperature (Vectastain ABC Kit, PK-4005, Vector Labs). Reaction products were visualized by incubating the sections with 3,3'-diaminobenzidine (DAB, SK-4100, Vector Labs.) and counterstaining with hematoxylin. After dehydration in ethanol sections were mounted in DPX and observed in Nikon Eclipse 800 microscope. As a negative control, the primary antibody was replaced with nonimmune serum in a similar dilution. The expression of TLR-5 protein was graded for both the extent and intensity of immunopositivity as described by Sinicrope *et al.* (17) and was evaluated by two independent observers (M.S. and Z.K.). The immunohistochemical analysis was performed in a blinded manner with respect to the clinical information.

*Statistical analyses*

All statistical analyses were done using Statistica ver. 7.0 software (StatSoft Inc., Tulsa, OK, USA). Statistical differences between groups were analyzed with non-parametric methods (Kruskal-Wallis test and Mann-Whitney U test for unpaired data). Spearman's test was used to estimate correlations between mRNA values and clinical-pathological variables. To assess relationships between more than two independent groups Pearson's multiple regression test was used. For all statistical tests, the level of statistical significance was set at $p<0.05$.

## RESULTS

*Levels of TLR-5 mRNA in mucosal biopsies of UC patients*

TLR-5 gene expression was significantly decreased in mucosal biopsies of UC patients with moderate (group II; p=0.004) and high (group III; p=0.00003) disease activity as compared with normal controls and group I patients (*Fig. 1*). Although 59% of moderate and 93% of severe UC cases presented decreased level of TLR-5 mRNA (as median values), there was no statistical association between groups ($\chi^2$= 6.94, p=0,13).

*Presence of TLR-5 protein in mucosal biopsies*

Due to the shortage of biological specimens, immunohistochemical assessment for TLR-5 in mucosal biopsies was performed on 10 control group and 57 UC patients (28, 23 and 6 cases from groups I, II and III, respectively).

In the mucosa of control healthy patients pronounced TLR-5 immunoreactivity was observed in the cytoplasm of epithelial cells and at their basolateral domains (*Fig. 2A*, *B*). The intensity of reaction was strong or very strong in the majority of crypts, however, in some crypts the cytoplasmic reaction was weaker (*Table 2*). Additionally, in the stromal non-epithelial tissue weak positive reaction was sometimes seen in the endothelium of small vessels.

In UC mucosa TLR-5 immunoreactivity was statistically much weaker or absent (*Table 2*) in the cytoplasm of epithelial cells, however, it was retained at their basolateral domains (*Fig 2C*, *D*). The diminished TLR-5 immunoreactivity of epithelial cells in UC mucosa was observed in all UC biopsies independent of disease activity. Although we found the lowest mucosal TLR-5 immunoreactivity in patients with high activity of UC (group III), there was no correlation between intensity of TLR-5 staining and increasing activity of UC.

## DISCUSSION

Initiation and perpetuation of the inflammatory intestinal responses in IBD may result from an exaggerated host defense reaction of the intestinal epithelium to endogenous bacterial flora (21). Intestinal epithelial cell lines constitutively express several functional Toll-like receptors (TLRs) which recognize a variety of distinct microbial components and not only play a key role in microbial recognition in innate immunity but also participate in the activation of adaptive immune responses. The dysregulation of innate and adaptive intestinal immune responses to bacterial microbiota is believed to be the hallmark of IBD pathogenesis, however, the expression of TLRs in ulcerative colitis and Crohn's disease was evaluated only by few authors (18-20). Because

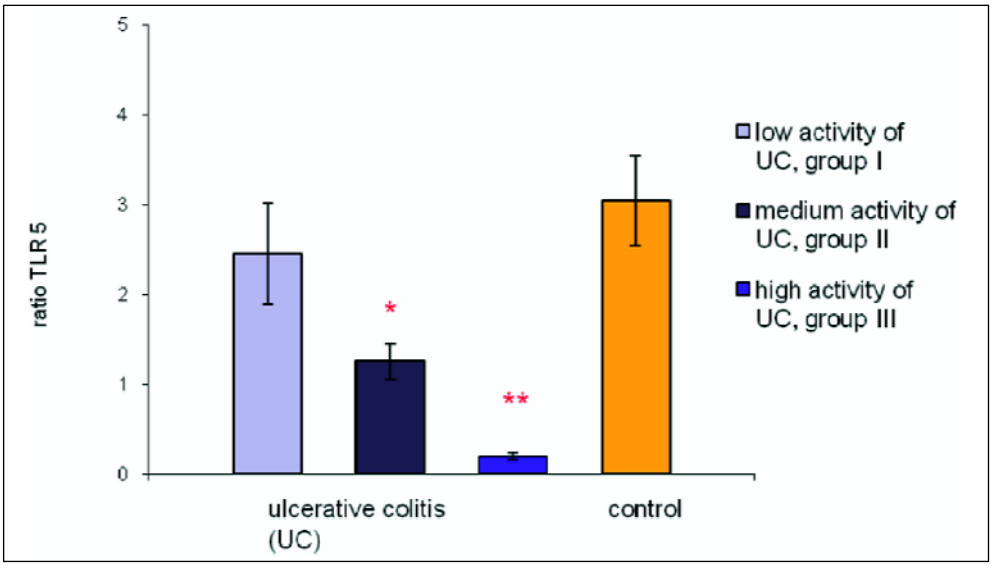


*Fig. 1.* TLR-5 gene expression in the mucosal biopsies of ulcerative colitis patients.
*,**- significantly different as compared with control, p<0,05 and p<0,001, respectively.

flagellated bacteria are important components of both nonpathogenic and pathogenic microbiota, *e.g. Salmonella*, we decided to explore the expression of the TLR-5 receptor which binds flagellin (10), the major structural protein of bacterial flagella, in active ulcerative colitis. Surprisingly, we found decreased both levels of TLR-5 mRNA and TLR-5 protein immunoreactivity in the inflamed mucosa as compared to normal controls. Our data differ from immunofluorescent study of Cario and Podolsky who reported similar intensity of TLR-5 protein immunoreactivity in the intestinal surface epithelium of control and UC patients (18). However, the analysis of their data suggests that TLR-5 immunoreaction was lower in UC mucosa since considerably longer fluorescence exposition time was used in order to get similar intensity of fluorescent staining. They also did not found TLR-5 receptors in either crypt epithelium or cell populations within the underlying lamina propria (18) contrary to our data and other studies (8). Other authors demonstrated that human mucosal TLR-5 receptors are functionally active since the infection of model intestinal epithelial cells with *Salmonella* led to an active transport of flagellin to the subepithelial compartment and subsequent binding to basolaterally expressed TLR-5, resulting in the secretion of proinflammatory cytokine IL-8 and phosphorylation and nuclear translocation of the transcription factor NF-κB in intestinal epithelial cells (8, 19). The high level of constitutive basolateral expression of TLR-5 receptors in healthy colon mucosa observed in our study and by other authors (8) suggests that under normal conditions tight junctions between enterocytes prevent luminal flagellin from evoking any significant inflammatory response. Logically, in the setting of an injured intestinal epithelial barrier TLR-5 receptors will be challenged, resulting in the up-regulation of proinflammatory response genes. However, the results of our study suggest that inflammatory damage to the colonic mucosa may result in a decreased TLR-5 expression, probably as a result

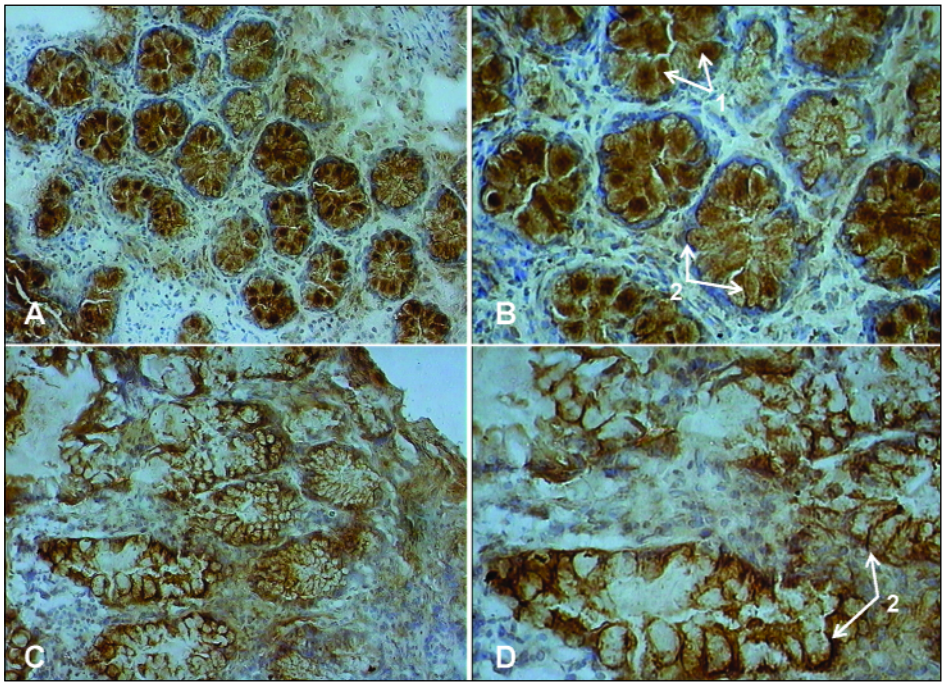


*Fig. 2.* TLR-5 protein immunoreactivity in the mucosa of UC and control patients.
A and B: control healthy patient (x200 and x400, respectively). C and D: UC patient with high disease activity, lower intensity of IHC reaction as compared to control (x200 and x400, respectively). TLR-5 immunoreactivity present in the cytoplasm of enterocytes, 1, and at their basolateral domain, 2.

*Table 2.* TLR-5 protein detection by immunohistochemistry.

| **Index of staining pattern** [a] | **0-2** Negative/ Weak n (%) | **3-4** Moderate n (%) | **5-7** Strong n (%) | **8-9** Very strong n (%) | $\chi^2$ | **p** |
|---|---|---|---|---|---|---|
| | No (%) | | | | *versus* control | |
| Control (n=10) | 0 | 1 (*10*) | 5 (*50*) | 4 (*40*) | | |
| mild UC, group I (n=28) | 1 (*4*) | 9 (*32*) | 15 (*54*) | 3 (*10*) | 2.37 | 0.12 |
| moderate UC, group II (n=23) | 4 (*19*) | 10 (*43*) | 7 (*30*) | 2 (*8*) | 7.72 | 0.007* |
| severe UC, group III (n=6) | 2 (*33*) | 3 (*50*) | 1 (*17*) | 0 | 8.60 | 0.003** |

[a] according to Sinicrope *et al.* [17], see Methods; *, ** - p<0.05, 0.005

of down-regulation caused by flagellin abundance in the damaged epithelium past the mucosal barrier.

Although serologic expression cloning has identified flagellins of intestinal microbiota as immunodominant antigens in experimental colitis in mice and in Crohn's disease patients (19, 20) the expression of TLR-5 in CD mucosa was studied only by Cario and Podolsky who found no alterations between control and CD mucosa (18). However, it has been recently found that in Crohn's disease antibodies directed against an immunodominant peptide of flagellin recognize TLR-5 as a self-antigen and are functionally active suggesting the presence of an autoimmune process as a part of IBD pathomechanism (21).

In summary, we found decreased expression of TLR-5 in the mucosa of UC patients with moderate and severe disease activity. This may represent receptor down-regulation to decrease the antigenic stimulation of inflammatory and immune pathways activated by ligand binding.

Conflict of interests: None declared.

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Author's address: Prof. Zbigniew Kmiec, Department of Histology, Medical University of Gdansk, Debinki 1 Str., 80210 Gdansk, Poland; Phone:+48583491437; Fax: +48583491419; E-mail: zkmiec@amg.gda.pl