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ORIGINAL ARTICLE

Differential expression of toll-like receptor 3 and 5 in ileal pouch mucosa of ulcerative colitis patients

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Abstract
Background and aims The pathogenesis of pouchitis, major complication after restorative proctocolectomy, and ileal J pouch-anal anastomosis (IPAA) in patients with ulcerative colitis (UC) is still unclear. Changes in intraluminal bacterial colonization and correlated changes of pouch mucosa are thought to play an important role. Toll-like receptors (TLRs) as part of the innate immune system are capable of recognizing bacterial antigens. Their activation can lead to secretion of proinflammatory mediators. In this study, TLR2, 3, 4, and 5 expression profiles in the pouch mucosa of patients with UC and IPAA were analyzed and correlated with pouchitis.
Materials and methods Clinical symptoms, endoscopy, and histology were assessed in 35 patients using the Heidelberg Pouchitis Activity Score to classify patients as either having pouchitis or not. TLR mRNA expression in normal ileal mucosa and pouch mucosa was investigated by performing semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). The results of RT-PCR were associated with the pouchitis score.
Results In the analysis of all patients, TLR3 expression was decreased significantly whereas TLR5 expression was increased significantly in pouch mucosa compared to normal ileal mucosa (p-values 0.0076 and 0.016, respectively). A more detailed analysis upon dividing the patients into patients with and without pouchitis showed decreased TLR3 expression in the pouch mucosa only of patients without pouchitis (p-value=0.0067). TLR5 expression was increased in the pouch mucosa only of patients with pouchitis (p-value=0.023). No differences in TLR2 and 4 expression were found in either group.
Conclusion Differential expression of TLR3 and 5 suggests bacterial involvement in the pathogenesis of pouchitis in patients with UC.

Keywords Toll-like receptor · Pouchitis · Ulcerative colitis

Abbreviations

IPAA	Ileal J pouch-anal anastomosis
UC	Ulcerative colitis
CD	Crohn's disease
TLR	Toll-like receptor
PAMP	Pathogen-associated molecular pattern
LPS	Lipopolysaccharide
NFκB	Nuclear factor κB

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IBD	Inflammatory bowel disease
mRNA	Messenger RNA
PAS	Pouchitis Activity Score
RT-PCR	Reverse transcriptase polymerase chain reaction
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IQR	Interquartile range
DC	Dendritic cells

Introduction

Pouchitis, a mucosal inflammation of the ileal reservoir, is the most common late complication in patients with ulcerative colitis (UC) and may compromise the long-term outcome after restorative proctocolectomy with ileal J pouch-anal anastomosis (IPAA) [1, 2]. In a large study, the cumulative risk of pouchitis was estimated to be 48% at 10 years [3]. There is an increasing consensus that pouchitis can be diagnosed according to the clinical symptoms and the endoscopic and histological findings using a validated pouchitis score [4–7].

However, the pathogenesis of pouchitis remains unclear. Several possible causes have been suggested, including recurrent UC, Crohn's disease (CD), ischemia, and genetic or immune susceptibility [1, 8, 9]. Apart from this, there is some evidence that pouchitis may represent a response to antibiotics and probiotic therapy [10–15], and a possible correlation between the concentration of anaerobic bacteria and unspecific histological changes of villous atrophy and chronic inflammatory cell infiltration [1]. These findings support a causal relationship between non-physiological fecal stasis in the ileal mucosa, anaerobic bacterial overgrowth, and development of "colon-like" mucosal morphology in the pouch [1, 16]. This suggests that bacteria play some role in the pathogenesis of inflammation of the pouch.

In previous studies, it has been shown that luminal bacteria play a crucial role in the initiation of intestinal inflammation, in general [17, 18]. However, no detailed information about the exact mechanisms of interaction between luminal bacteria and the intestinal mucosa exists so far. It is possible that bacteria initiate inflammation directly by breaking through the mucosal barrier as a result of epithelial damage or via paracellular routes. Another possibility is an indirect inflammatory immune response mediated through receptors.

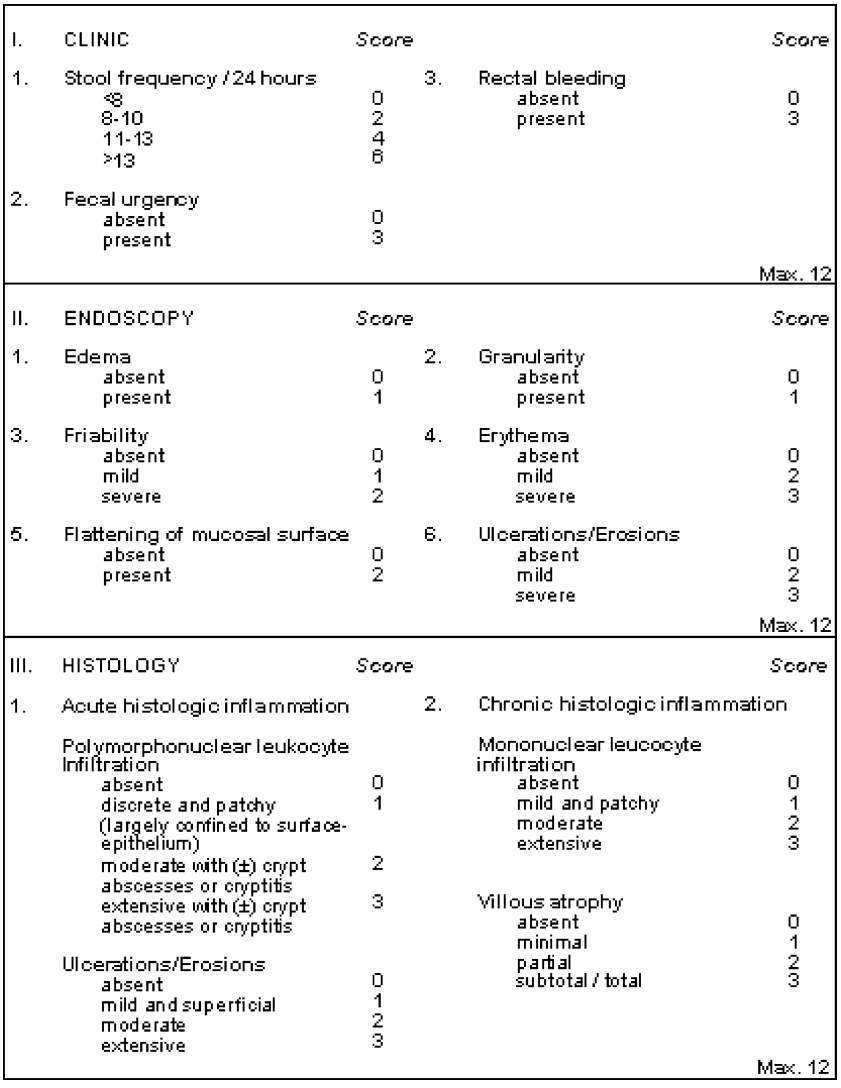
It was previously demonstrated that various intestinal epithelial cell lines constitutively express several members of a novel family of the so-called Toll-like receptors (TLRs) with a transmembrane domain that may serve as a major link between innate and adaptive mucosal immune responses [19]. To date, ten different human TLRs are known, homologues of the *Drosophila* toll protein, which appear to be pattern recognition receptors activated by pathogen-associated molecular patterns (PAMPs) such as different bacterial or viral surfaces [20–24]. It was shown in vitro that lipopolysaccharide (LPS), a component of the surface of Gram-negative bacteria, is a potential trigger for secretion of different pro-inflammatory cytokines and chemokines in intestinal epithelial cells via distinct signaling pathways through TLRs [19]. TLRs are activated by different PAMPs; the two best investigated TLRs being TLR4, mainly for LPS of Gram-negative bacteria, and TLR2, mainly for the lipoproteins of Gram-positive bacteria [25–28]. TLR3 has been recently shown to be activated by viral dsRNA and TLR5, specifically by bacterial flagellin [29–32]. TLR2, 3, 4, and 5 then rapidly activate an intracellular pathway via an adapter protein named MyD88, by which nuclear factor κB (NFκB) then regulates immunomodulatory genes and finally leads to the secretion of cytokines, co-stimulatory and antigen-presenting molecules, nitric oxide, and antimicrobial peptides or to cell death [33–36]. It is still not known whether dysregulation of TLR-mediated microbial recognition is present in infectious and inflammatory diseases. However, previous studies suggest that TLR dysregulation may be associated with increased or decreased susceptibility to intestinal infection [37–40]. Two recently published reports showed TLR dysregulation in patients with inflammatory bowel disease (IBD) or in intestinal inflammation in general [41, 42].

To investigate a possible effect of bacterial overgrowth on pouch mucosa, this study compared the expression profile of TLR2, 3, 4, and 5 between pouch mucosa and normal ileal mucosa that is not affected by fecal stasis. To elucidate the association between TLRs and the bacterial effect on the pathogenetic mechanism of pouchitis, the expression profile of TLR2, 3, 4, and 5 messenger RNA (mRNA) expression was investigated in subgroups of patients with and without pouchitis.

Table 1 Patient characteristics

Number of patients, *N*	35	100%
Sex, male/female	20/15	57.1/42.9%
Median age at IPAA (years)	36	IQR, 27–44
Median age at diagnosis (years)	24	IQR, 16–32
Median age of the pouch	2,5	IQR, 1,2–5,8
Duration of disease (years)	8.4	IQR, 3.3–14.8
Extent of disease, pancolitis/ left-sided colitis	30/6	82.9/17.1%
CAI[a] (points)	7.5	IQR, 4–12
Pouchitis, yes/no	20/15	57.1/42.9%
Median follow-up (years)	2.2	IQR, 0.8–5.6

[a] Three patients with missing values. Quantitative parameters are presented with median value and interquartile range



I. CLINIC	Score		Score
1. Stool frequency / 24 hours		3. Rectal bleeding	
<8	0	absent	0
8-10	2	present	3
11-13	4		
>13	6		
2. Fecal urgency			
absent	0		
present	3		
			Max. 12

II. ENDOSCOPY	Score		Score
1. Edema		2. Granularity	
absent	0	absent	0
present	1	present	1
3. Friability		4. Erythema	
absent	0	absent	0
mild	1	mild	2
severe	2	severe	3
5. Flattening of mucosal surface		6. Ulcerations/Erosions	
absent	0	absent	0
present	2	mild	2
		severe	3
			Max. 12

III. HISTOLOGY	Score		Score
1. Acute histologic inflammation		2. Chronic histologic inflammation	
Polymorphonuclear leukocyte infiltration		Mononuclear leucocyte infiltration	
absent	0	absent	0
discrete and patchy (largely confined to surface-epithelium)	1	mild and patchy	1
		moderate	2
moderate with (±) crypt abscesses or cryptitis	2	extensive	3
extensive with (±) crypt abscesses or cryptitis	3	Villous atrophy	
		absent	0
		minimal	1
		partial	2
		subtotal / total	3
Ulcerations/Erosions			
absent	0		
mild and superficial	1		
moderate	2		
extensive	3		
			Max. 12

Fig. 1 The Heidelberg Pouchitis Activity Score consists of three equivalent parts (clinical, endoscopic, and histological scores) with a maximum of 36 points. Pouchitis is defined as that with a total score of ≥13 points

Materials and methods

Patients and tissues

From April 1999 to October 1999, tissue samples were obtained from a cohort of 35 UC patients undergoing pouchoscopy at the Department of Surgery, University of Heidelberg for routine follow-up after total proctocolectomy and hand-sutured ileal J pouch-anal anastomosis. Inclusion criteria were age ≥18 years and history of UC with IPAA. Exclusion criteria were IPAA for familial adenomatous polyposis and suspected CD. All of these patients were included consecutively. The patient characteristics are presented in Table 1.

Eight mucosal specimens were taken endoscopically from each patient out of the ileal afferent loop mucosa and pouch mucosa. Ileal afferent loop mucosa, located proximal to the ileoanal pouch and unaffected by fecal stasis, was referred to as normal ileal mucosa. Biopsy pliers with a diameter of 0.7 cm were used. Specimens were snap-frozen in liquid nitrogen immediately after removal and stored at −82°C. Corresponding formalin-fixed specimens were evaluated histologically.

The patients were informed of the purpose of the study and the possible risks involved in the sampling before they gave their informed consent.

To diagnose pouchitis, the 36-point Heidelberg Pouchitis Activity Score (PAS) was used (Fig. 1). The PAS evaluates clinical, endoscopic, and histological features as the three fields of manifestation of pouchitis, with each part of the score having a maximum of 12 points. Investigations were performed independently by an experienced clinician, endoscopist, and pathologist. Within one of these parts, 5 and more points defines a

Table 2 Comparison of Toll-like-receptor expression in ileum and pouch

	Ileum (n=35)		Pouch (n=35)	
	Median net intensity (IQR)	Median TLR/GAPDH ratio (IQR)	Median net intensity (IQR)	Median TLR/GAPDH ratio (IQR)
GAPDH	48,704 (31,650–66,921)		47,611 (38,847–63,784)	
TLR2	15,896 (5,828–21,679)	0.29 (0.16–0.45)	15,961 (6,706–26,600)	0.28 (0.17–0.54)
TLR3	28,499 (13,699–38,409)	0.57 (0.29–0.8)	18,999 (8,543–30,180)	0.39 (0.2–0.52)
TLR4	2,205 (0–8,592)	0.05 (0–0.18)	7,727 (316–14,637)	0.14 (0.01–0.33)
TLR5	2,658 (0–8,325)	0.08 (0–0.19)	7,440 (2,372–14,059)	0.13 (0.07–0.28)

Net intensity values and TLR/GAPDH ratios with interquartile range of 35 pouch mucosa samples and of 35 ileal afferent loop mucosa samples are given

pouchitis. A score of 13 and more points is taken as a diagnosis of pouchitis [7].

Semiquantitative reverse transcriptase polymerase chain reaction of Toll-like receptor 2, 3, 4, and 5

Isolation of total RNA

After homogenization of the biopsy specimen, total RNA was isolated using the QIAGEN RNeasy Kit. To avoid any amplification from genomic DNA contamination, the samples were pretreated with DNAse.

cDNA synthesis

Of the total RNA, 1 μg was reverse transcribed according to the Superscript II protocol for reverse transcriptase polymerase chain reaction (RT-PCR) (GIBCO BRL) using first-strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, and 15 mM MgCl2), 0.1 M dithiothreitol, dNTP Mix, oligo(dT)primer, and 200 units of reverse transcriptase in a 20-μl volume.

PCR amplification of cDNA

PCR amplification was carried out on 3-μl aliquots of diluted cDNA (c=1 μg/μl) in a total volume of 50 μl using 2.5 units of Taq DNA Polymerase. As described previously, specific cDNA products were amplified using specific pairs of primers and conditions for TLR2, 3, 4, and 5 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene, which was used as an internal control in each sample [43]. The number of cycles was defined in the exponential phase of the reaction to guarantee valid quantification.

The primers used were previously described by Hausmann et al. [42].

PCR conditions were GAPDH (25 cycles) at 60°C, TLR2 (30 cycles) at 56°C, TLR3 (30 cycles) at 55°C, TLR4 (32 cycles) at 57°C, and TLR5 (30 cycles) at 55°C. The specimens were denatured at 94°C for 30 s and annealed at primer-specific temperatures for 30 s, which was extended at 72°C for 30 s. Negative controls were run simultaneously to exclude contamination by genomic DNA for each reaction. The reactions were performed in an Eppendorf Thermocycler (MASTER CYCLER gradient 5331). The PCR products were then separated on a 2% agarose gel containing ethidium bromide using a 100-bp DNA ladder (GIBCO BRL).

The absorbance values for each band were measured as net intensities by densitometry with a digital documentation system (Kodak 1D Image Analysis Software). For each individual sample, a ratio was calculated between TLR and the GAPDH bands (TLR ratio=TLR net intensity/GAPDH net intensity). This ratio represents an indication of the relative abundance of the two templates of the samples and therefore represents the amount of TLR mRNA expression [43, 44].

Statistical analysis

SAS software (Release 8.02, SAS Institute, Cary, NC, USA) was used for statistical analysis. Paired comparisons of TLR/GAPDH ratio values measured in pouch mucosa samples and in normal ileal mucosa samples within each individual patient and within groups were performed using Wilcoxon's signed-rank test. A p-value of less than 0.05 was considered to show statistical significance. The distributions of TLR/GAPDH ratio values in pouch mucosa samples and in normal ileal mucosa samples are presented by box-and-whisker plots. TLR/GAPDH ratio values and net intensities are reported as median and interquartile ranges (IQR, lower quartile–upper quartile). All tests were two-sided.

Results

The mRNA of the housekeeping gene GAPDH was expressed constantly in all 70 tissue samples with a median net intensity of 48,244.

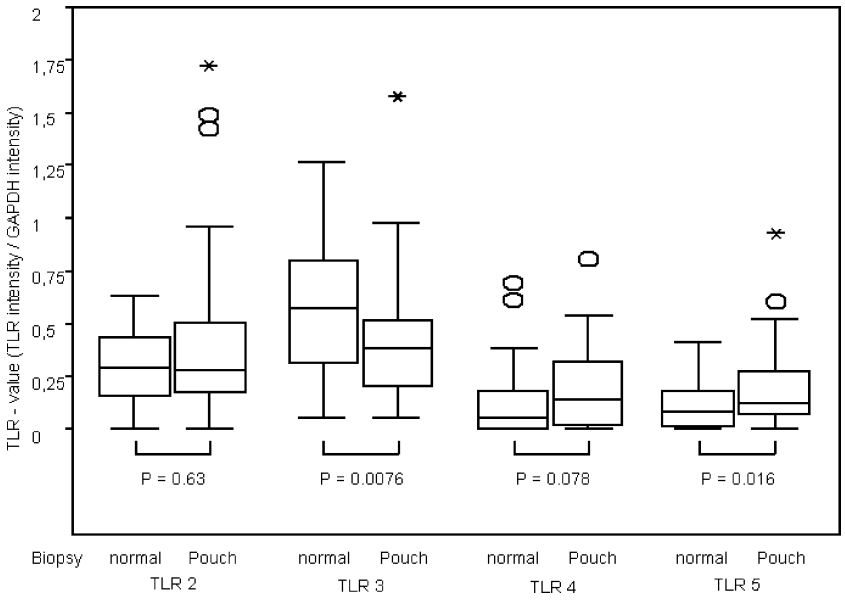


Fig. 2 A comparison of TLR2, 3, 4, and 5 expression in normal ileum and pouch mucosa shows significant differential expression of TLR3 and 5. TLR expression is presented as a *box-and-whisker* plot: minimum 25th percentile, median, 75th percentile maximum, and extreme values outside the interquartile range

TLR mRNA expression in ileal pouch mucosa compared to normal ileal mucosa

TLR2 mRNA expression in ileal pouch mucosa did not differ from the TLR2 mRNA expression in normal ileal mucosa (p=0.63). TLR3 mRNA expression in the pouch mucosa was significantly lower than the TLR3 mRNA expression in normal mucosa (p=0.0076). The TLR4 mRNA expression in pouch mucosa was nearly three times higher than in normal mucosa (p=0.078). However, this difference did not reach statistical significance. The TLR5 mRNA expression in pouch mucosa was also significantly higher than in normal mucosa (p=0.016) (Table 2; Fig. 2).

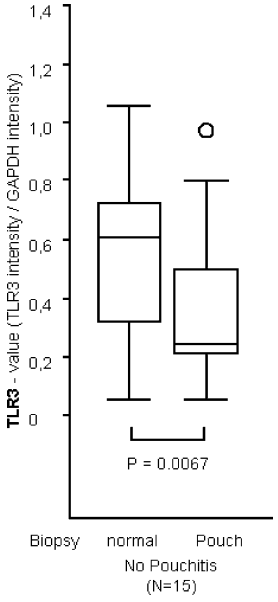


Fig. 3 TLR3 expression in normal ileum of the afferent loop compared to pouch mucosa of patients without pouchitis. TLR expression is presented as a *box-and-whisker* plot: minimum 25th percentile, median, 75th percentile maximum, and extreme values outside the interquartile range

Association between the pouchitis score and TLR mRNA expression in ileal pouch mucosa as compared to normal ileal mucosa

According to the pouchitis score, 20 of the 35 patients (57.1%) were diagnosed with pouchitis, while 15 (42.9%) did not have pouchitis.

For TLR2 and 4 mRNA, no differential expression was found between normal ileal mucosa of the afferent loop and pouch mucosa, irrespective of the pouchitis diagnosis. In patients without pouchitis, there was a statistically significant decrease in expression of TLR3 mRNA in pouch mucosa compared to normal ileal mucosa (p=0.0067) (Fig. 3) in contrast to patients with pouchitis in whom no differential expression of TLR3 was observed. In patients with pouchitis, a significantly increased expression of TLR5 mRNA in pouch mucosa was found as compared to normal ileal mucosa (p=0.023) (Fig. 4), whereas patients without pouchitis showed no differential expression of TLR5.

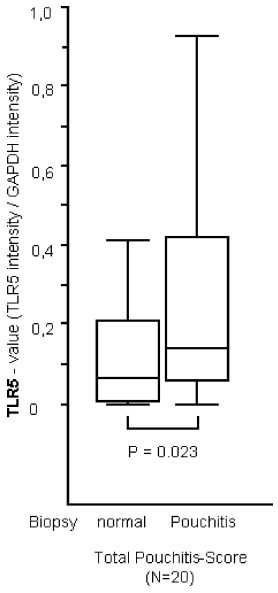


Fig. 4 TLR5 expression in normal ileum of the afferent loop compared to pouch mucosa of patients with pouchitis. TLR expression is presented as a *box-and-whisker* plot: minimum 25th percentile, median, 75th percentile maximum, and extreme values outside the interquartile range

Discussion

In this study, TLR2, 3, 4, and 5 expression profiles of ileal pouch mucosa were compared with the normal ileal mucosa of the afferent ileal loop, located proximal to the pouch and not affected by fecal stasis. Furthermore, we investigated the expression profile of TLR2, 3, 4, and 5 in subgroups of patients with pouchitis and in patients without pouchitis by comparing normal ileal mucosa with pouch mucosa to get an impression of the role of bacteria in the pathogenesis of pouchitis in UC patients. The diagnosis of pouchitis was based on the histological, endoscopic, and clinical signs of inflammation according to a validated pouchitis score, the Heidelberg PAS [6, 7].

To our knowledge, there have been no reports concerning TLR expression in ileal pouch mucosa to date.

Our main result is the statistically significant differential expression of TLR3 and TLR5 in ileal pouch mucosa as compared to normal ileal mucosa. It was surprising that no significant differential expression of TLR2 and TLR4 could be found between normal ileal mucosa and pouch mucosa.

TLR2 is activated mainly by Gram-positive bacterial cell walls, such as lipoproteins [36] or the *Staphylococcus aureus* peptidoglycan [27]. In the intestine, TLR2 was shown to remain unchanged [41] when comparing TLR expression in IBD with normal controls; this is in contrast to the results of another study, in which TLR2 upregulation was found in intestinal inflammation [42]. The latter study showed a heterogenous pattern of TLR2 and 4 distribution, with accumulation subepithelially, preferentially detectable close to the crypts. In UC patients, TLR2 and 4 were mainly restricted to the mucosa close to the epithelial layer [42]. Cario and Podolsky [41] found out that TLR2 expression was barely detectable in primary intestinal epithelial cells. According to our data, there were no differences in TLR2 expression between normal mucosa and inflamed or non-inflamed pouch mucosa.

TLR4, the most frequently investigated member of the TLR family, plays a major role in LPS signal transduction as part of the LPS receptor signaling complex [45].

Associations between TLR4 dysregulation and the intestine were found in several previous studies, one of which reported that the absence of TLR4 explains endotoxin hyporesponsiveness in human intestinal epithelium [41]. This response was also shown in humans carrying the missense mutation Asp299Gly affecting the extracellular domain of the TLR4 receptor and who have an increased risk of Gram-negative bacterial infections and bacteremia [46, 47]. In another study, decreased expression of TLR4 and MD2 was shown to correlate with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial LPS [38], yet others reported that protective roles of mast cells against enterobacterial infection are mediated by TLR4 [40]. Concerning IBD, upregulated TLR4 expression was shown in the intestinal mucosa of patients with both CD and UC [41, 42]. TLR4 expression was mostly present on basolateral surfaces of intestinal epithelial cells in colon mucosa of UC patients [41].

In our study, TLR4 expression is increased threefold in the pouch mucosa as compared to normal ileal mucosa, particularly in histologically acutely inflamed pouch mucosa. This difference, however, did not reach statistical significance.

A comparison of the TLR mRNA expression profiles of normal ileal mucosa and pouch mucosa showed differences in TLR3 and 5 expression. TLR3 mRNA expression was decreased in pouch mucosa compared to normal mucosa, whereas TLR5 mRNA expression was increased correspondingly. One explanation for the differential expression of TLRs in normal ileum compared to the pouch might be the concentration, the ratio, and the pattern of bacterial colonization in a pouch. It was shown that the concentration of anaerobic and aerobic bacteria is increased in the pouch in comparison to the ileal afferent loop due to fecal stasis. Moreover, the portion of anaerobic bacteria in relation to aerobic bacteria is markedly increased in the pouch compared to that in normal ileum (anaerobic/aerobic ratio 100:1 in the pouch compared to 4:1 in normal ileum) [2]. Another aspect is that bacterial surface antigen flagellin, a virulence factor of different flagellated Gram-negative bacteria, was shown to specifically activate TLR5, initiating an inflammatory cascade [30, 31]. Therefore, it can be

speculated that the increased TLR5 expression in the pouch mucosa is due to the increased concentration or ratio of flagellin-containing bacteria.

The association between the total pouchitis score and TLR expression shows that patients with no pouchitis have significantly decreased TLR3 mRNA expression in the pouch mucosa as compared to that in the normal mucosa, whereas no differences were found in TLR2, 4, and 5 expression. Patients with pouchitis showed increased TLR5 mRNA expression in pouch mucosa compared to that in normal mucosa, whereas TLR2, 3, and 4 were not expressed differentially.

As described above, the signaling pathways via different TLRs activated by several bacterial and viral characteristics lead to inflammatory responses. TLR5 has been shown to specifically mediate the response to flagellin, a component of bacterial flagella [31]. It was recently shown that bacterial flagellin is a dominant antigene in Crohn's disease [49].

Our observation that UC patients with pouchitis have increased TLR5 expression in ileal pouch mucosa indicates that the underlying pathogenetic mechanism in pouchitis might be an infection with flagellin-expressing bacteria.

The significance of this finding is that flagellin is a bacterial virulence factor of various predominantly Gram-negative bacteria, accounting for bacterial motility, helping bacteria in attaching to epithelial cells, and promoting bacterial invasion.

Although flagellin is secreted by commensal as well as pathogenic bacteria, flagellin induces inflammatory responses only if it comes in contact with the basolateral membrane, where TLR5 is located. This might be the reason why only the flagellin of pathogenic bacteria causes inflammation by crossing intestinal epithelia [30]. However, this inflammatory mechanism might also be activated not only by invasive pathogens but also by commensal bacteria passing the epithelial barrier due to epithelial barrier dysfunction, which is another mechanism suggested to be involved in the pathogenesis of pouchitis [50].

In contrast to this, the decreased expression of TLR3 in normal pouch mucosa compared to that in normal ileal mucosa indicates a downregulation of TLR3 with subsequent suppression of the inflammatory cascade. In patients with Crohn's disease, a decreased expression of TLR3 in the mucosa compared to controls was shown, irrespective of the localization and site of inflammation. No differences, however, were found in colonic mucosal specimens of UC patients. TLR3 was here mostly expressed on basolateral surfaces of intestinal epithelial cells, as well as in subepithelial blood vessels and muscle cells of the intestinal submucosa [41].

dsRNA has been recently shown to stimulate TLR3, a molecular pattern expressed by many viruses during infection. The activation of TLR3 can lead to induction of interferon-α and interferon-β, cytokines important both for antiviral responses and the production of different proinflammatory cytokines and dendritic cells (DC) maturation [29]. In addition, previous studies found that TLR3 can mediate cell-specific responses to LPS and seems to be specifically expressed by DCs [48]. Therefore, the relative increase in TLR3 in the pouch mucosa of patients with pouchitis might be a result of immigrated DCs. On the other hand, the relative increase of TLR3 could also be the response to a viral infection in pouchitis.

Futher investigations are needed to identify a possible correlation between bacterial flagellin and increased TLR5 expression in the pouch mucosa. Comparing the expression profiles of TLR3 and 5 between pouch mucosa and normal ileal mucosa and the investigation of the association between TLRs and pouchitis revealed a differential expression of TLR3 and 5, which could either be the result of or lead to adaptive reactions of the pouch mucosa due to fecal stasis and bacterial overgrowth. This might initiate or perpetuate chronic inflammation in the presence of commensal bacteria [51].

Therefore, our results indicate a possible bacterial influence on the pathogenesis of pouchitis in patients with UC mediated by TLR3 and 5. These data altogether provide new insight into the pathogenetic mechanisms of pouchitis and offer new perspectives in the therapy for pouchitis as well.

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