

RESEARCH ARTICLE

Expression of inflammatory genes in the colon of ulcerative colitis patients varies with activity both at the mRNA and protein level

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ABSTRACT. **Background:** Ulcerative colitis (UC) is a chronic inflammatory disorder of the gastrointestinal tract involving aberrant activation of innate and adaptive immune responses. We aimed to study the expression profiles of the susceptibility gene Nod1 and selected pro- and anti- inflammatory cytokines during different stages of UC. **Methods:** 65 patients with mild, severe or remission stage of UC, and 22 normal colon mucosal biopsies from control individuals were included in the study for measuring the expression of nucleotide binding oligomerization 1(Nod1) and related pro- and anti-inflammatory cytokines using quantitative reverse transcription-PCR (qRT-PCR). mRNA expression levels were then correlated with severity of disease. In order to check their expression at the protein level, immunohistochemistry (IHC) was performed using Nod1, TNF- α , IFN- γ , IL-17, IL-23 and IL-13 antibodies. **Results:** Significant increases in Nod1 expression with simultaneous increases in pro-inflammatory cytokines TNF- α , INF- γ , IL-17 and IL-23 mRNA levels were observed in patients with mild and severe ulcerative colitis *versus* control individuals. The expression levels reverted back towards normal levels in patients during remission. However, mRNA expression of selected anti-inflammatory cytokines such as IL-11 and IL-13 were substantially lower in patients compared with control samples when measured using qRT PCR. Levels of IL-10 however, although exhibiting a decreasing trend, did not attain significance. **Conclusions:** Our results show that with simultaneous increase in Nod1 expression, expression profiling of downstream inflammatory cytokines that are activated in UC patients, displayed different patterns according to the severity of the disease. These may be potential prognostic biomarkers for diagnosing UC patients.

Key words: NOD1, qRT-PCR, immunohistochemistry, cytokine profiles, ulcerative colitis

Ulcerative colitis is a chronic inflammatory bowel disease characterized by inflammation that starts at the rectum area (proctitis), and may extend to variable distances within the colon.

It is a continuous inflammation of the intestinal lamina propria, starting from the rectum and involving the whole colonic mucosa. Of the many inflammatory genes involved, cytokines play a key role in the initiation, exacerbation and perpetuation of the disease resulting in mucosal injury. An imbalanced immune response to luminal antigens and activation of the transcription factor NF- κ B seem to be crucial in the pathogenesis of IBD [1]. In addition, NF- κ B leads to subsequent production of pro-inflammatory cytokines. The transcription factor promotes both gene expression and production of mRNA but downstream checkpoints exist (miRNA, post-translation modification, etc), that may also alter the expression of downstream cytokines to some extent. Pattern recognition receptors (PRRs) include transmembrane proteins such as Toll-like

receptors (TLRs) and C-type lectin receptors, as well as cytoplasmic proteins such as retinoic acid-inducible gene (RIG)-I-like receptors and NOD-like receptors [2-4]. Among the NOD proteins, NOD1 and NOD2 recognize components of bacterial cell walls [5]. The expression of both NOD1 and NOD2 is regulated by pro-inflammatory cytokines [6]. NOD1 senses meso-diaminopimelic acid (meso-DAP) containing peptidoglycan [7, 8]. NOD1 protein acts independently of TLR cascades, but finally synergizes with TLR to initiate innate immune response against microbes [9].

Cytokines induce a disease-specific immune response in UC and Crohn's disease (CD). In addition to the classical pro-inflammatory cytokines, such as IL-1, IL-6 and TNF- α , associated with the pathogenesis of UC [10], a complex network involving the Th2 cytokine, IL-13 has also been found to be involved. TNF- α , produced by APCs and macrophages, activates neutrophils, stimulates B cells and increases the production of IFN- γ by T-cells [11].

Accumulating evidence has implicated IFN- γ as being crucially involved in chronic inflammatory diseases, such as rheumatoid arthritis [12].

IL-17A produced by Th17 cells is a key regulator of homeostasis and epithelial barrier function [13]. It can be protective against infections, but can also turn pathological in several inflammatory diseases such as psoriasis, asthma and inflammatory bowel disease. Furthermore, IL-17 is involved in the pathogenesis of several autoimmune diseases. Observations made by Andoh *et al.*, 2008 suggest that the interactions between TNF- α and IL-17A/IL-17F potentially mobilize neutrophils, thus contributing to the pathophysiology of inflammatory bowel disease (IBD) [14].

Evidence for the importance of the IL-23 pathway in IBD has come from mouse models of IBD [15], and human IBD [16], in which IL-23 deficiency or blockade protects from disease. In particular, genetic polymorphisms in the IL23R represent one of the strongest associations in CD, and are also associated with UC [17], psoriasis [18], and ankylosing spondylitis [19].

IL-10 is a key regulatory cytokine and possesses immunosuppressive properties. It is mainly produced by regulatory T-cells and helps to down-regulate the production of pro-inflammatory cytokines [20]. It is important in maintaining intestinal homeostasis, as demonstrated by the observation that IL-10-deficient mice spontaneously develop colitis [21]. In patients with CD, a decreased IL-10 concentration in the ileum predicted a higher risk of disease recurrence [22]. IL-11 and IL-10 mediate anti-inflammatory effects and are able to downregulate LPS-induced NF- κ B activation [23, 24]. In addition, IL-11 is involved in the regeneration of small intestine crypt progenitor cells, neuronal differentiation and progression of carcinomas, including gastric and colorectal adenocarcinoma, suggesting a role in cellular proliferation and differentiation [25-27]. A multi-faceted function of IL-13 has been

suggested, with a universal clearing of inflammatory factors in the tissue being its main function [28]. A recent study in a mouse model of colitis has demonstrated that IL-13, through inhibition of the mixed type 1 and type 17 T-helper cell inflammatory responses, has a protective effect [29]. In this study we report the different expressions of selected pro- and anti-inflammatory cytokines as well as the expression of a pattern recognition receptor (PRR), the NOD1 known to be involved in the activation of signaling pathways leading to inflammatory responses during different stages of UC.

PATIENTS AND METHODS

Patients

Routine endoscopies of UC patients were carried out by the attending Gastroenterologist from the department of Gastroenterology, AIIMS, New Delhi. Biopsy specimens were mostly obtained from the lower part of the colon. The study protocol was approved by the Ethics Committee of the institute, and all the patients gave written, informed consent. Biopsy samples were immediately plunged into liquid nitrogen and stored at -80°C until processed. Sixty five UC patients were grouped into three categories of disease: severe, mild and remission. The categories were graded on the basis of inflammation following the Montreal Classification [30]. Biopsies from 22 subjects with normal colonoscopy and histology served as a normal control group. The control subjects were patients with functional dyspepsia. They had no gastrointestinal or liver diseases. The diagnosis of UC was established according to clinical guidelines and criteria based on endoscopic, radiological, and histopathological examinations. The demographic and clinical features of patients and controls are given in table 1.

Table 1
Demographic and clinical features of UC patient and controls.

	UC (n = 65)	Controls (22)
Sex (male/female)	47/18	13/9
Disease duration (yrs), mean \pm SD (range)	4.3 \pm 3.92 (0.1-17)	-
Age at diagnosis (yrs), mean \pm SD	37 \pm 12 (22-67)	35.3 \pm 15.1 (22-63)
15-40	39 (60%)	15 (68.2%)
$>$ 40	26 (40%)	7 (31.8%)
Disease stage, n (%)		
Remission	16 (24.6%)	-
Mild	24 (36.9%)	-
Severe	25 (38.5%)	-
Disease extent, n (%)		
Rectum	17 (26.2%)	-
Left colon	21 (32.3%)	-
Pancolitis	27 (41.5%)	-
Smoking history		
Yes	6 (9.2%)	4 (18.2%)
No	57 (87.7%)	17 (77.3%)
Ex	2 (3%)	1 (4.5%)
Family history of IBD y/n (%)	1/64 (1.5%)	0
Appendectomy y/n (%)	3/62 (4.6%)	0
Location of sampling	Rectosigmoid region	Rectosigmoid region

RNA extraction

Total RNA was extracted with Tri-Reagent procured from Sigma-Aldrich with an RNase-free DNase (Fermentas) treatment for 30 min as per the manufacturer's instructions. An additional cleanup step was used using a DNase treatment Kit (Promega, CA, USA). RNA concentration and purity were assessed spectrophotometrically (NanoDrop) with the A260/A280 ratio in the range 1.77-2.08. RNA samples were used immediately or stored at -80 °C for subsequent experiments.

Real-time PCR

RNAs were reverse transcribed to cDNAs using TaqMan reverse transcription reagents. Real-time PCRs were performed on the resulting cDNAs using the fast SYBR Green in ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Real-time primers specific for inflammatory genes IL-10, IL-11, IL-13, IL-17, IL-23, TNF- α , IFN- γ , *NOD1* and GAPDH (used as an endogenous control) were designed with Primer Express software (Applied Biosystems) listed in *table 2*. Primer sequences were subjected to BLAST analysis to check for any untoward homology with non-targeted sequences. They were also checked using SNP database for any known SNP near the 3' end of primers.

The amplified products of *NOD1*, IL-10, IL-11, IL-13, IL-17, IL-23, TNF- α , IFN- γ , genes were used for real time analysis. The real-time PCR were carried out with a 100 nM concentration of each forward and reverse primer in a total volume of 50 μ l. In order to check for the presence of contaminating DNA in the real-time reactions, we included reverse transcriptase-negative reactions as control. Final data were analyzed using the software available with the ABI PRISM 7500 sequence detection system. Dissociation

curves for the PCR samples were made by an additional denaturation step at 95 °C for 15s, annealing at 60 °C for 20s, and with a slow increase in temperature back to 95 °C with a ramp time of 19 min 59 s to ensure amplification of the correct genes. Relative quantification of cDNA was done using the $\Delta\Delta CT$ method following normalization with a house-keeping gene GAPDH, which was found to be stable in the present setting.

Histology

All histological experiments were performed using 4% paraformaldehyde-fixed colon biopsy tissue blocks. Colon biopsy tissues were fixed on slides and were dehydrated through an ascending ethanol series, cleared with xylene, embedded in paraffin and sectioned until crypts were clearly represented in the sectioning plane. Five μ m-thick sections were cut and mounted onto slides for hematoxylin and eosin (H&E) staining before microscopy [31].

Immunohistochemistry

Immunohistochemistry procedures for biopsy samples were carried out using a Heat-Induced Epitope Retrieval (HIER) protocol [32]. After blocking, test slides were exposed to polyclonal goat anti-*NOD1*-BIOT Fab-fragments 1:75 (R&D Systems) in 0.01M PBS (pH 7.6), or polyclonal goat anti- TNF- α , IFN- γ , IL-23 (Sigma Aldrich) and IL-17-BIOT Fab-fragments 1:75 (Santa Cruz Biotech) in 0.01 M PBS (pH 7.6) solution, while negative control sections were incubated with PBS missing specific antibodies. Test and control slides were incubated in a humid chamber at 37 °C for 2 h. After incubation, slides were rinsed twice in PBS (pH 7.6) for 4 min each time, followed by rinsing twice in Tris-HCl (pH 7.6) for another 4 min each. After rinsing, slides were covered with 70 μ L of

Table 2
PCR primers for qRT PCR.

Genes	Accession No.	Forward primer/Reverse primer	Expected size of amplicon
<i>NOD1</i>	NM_006092.2	F-GAAGAGCAGGGCCACAGTGAGATG R-GCCAAGGCCTGAGGTCCACGTA	307 bp
GAPDH	NM_002046.3	F-GCTCCTCCTGTTGACAGTC R-GCAACAATATCCACTTACCGAG	180 bp
<i>NOD2</i>	NM_022162.1	F-AGCGAGACTGAGCAGACAC R-CAGGTTGCCGATCTTCACAC	197 bp
IFN γ	NM_000619.2	F- CAGCTCTGCATCGTTTGGG R-GTTCATTATCCGCTACATCTGAA	119 bp
TNF α	NM_000594.2	F-CCCCAGGGACCTCTCTCAA R-TGAGGTACAGGCCCTCTGAT	212 bp
IL17	NM_002190.2	F-CTACAACCGATCCACCTCACCTT R-CACGTTCCCATCAGCGTTGATGC	121 bp
IL23	NM_016584.2	F-GCCAGCTTCATGCCTCCCTACT R-GACTCAGGGTTGCTGCTCCATG	200 bp
IL10	NM_000572.2	F-CCGAGATGCCTTCAGCAGAGTG R-TTCTCAGCTTGGGGCATCACCTC	168 bp
IL11	NM_000641.2	F-TGCGGGGGCAGTGGGAGC R-CAGGGTGCCCAGCTCGGG	145 bp
IL13	NM_002188.2	F-GGCAGCATGGTATGGAGCATCAAC R-GCAGAATCCGCTCAGCATCCTC	120 bp

bp, base pair

a 1:250 secondary biotinylated antibody associated with a streptavidin complex (Invitrogen, UK) in 0.05 M Tris-HCl (pH 7.8) and incubated in a humid chamber at 37 °C for 45 min. After incubation, slides were rinsed three times in Tris-HCl (pH 7.6), for 4 min each and then peroxidase activity was visualized using the 3, 30-diaminobenzidine (DAB) kit (Invitrogen, UK) solution at room temperature for 10 min. After color development, slides were rinsed three times in Tris-HCl (pH 8.0) buffer solution, for 4 min each time, dehydrated in an ascending ethanol series and mounted using Hydromatrix® [33]. All imaging was carried out with an N-SIM super-resolution microscope with a simple PCI image capture system (Nikon Instruments Inc., Japan).

Statistics

Data were presented as mean \pm SEM. The probability of significant differences in real time data during the different stages of the disease *versus* control was calculated by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. All data were shown as their mean fold-change values. The relationship between two parameters was assessed by Spearman's correlation coefficient test. Gene transcription results were considered significant when $t > 1.5$ -fold change was observed at a $p < 0.05$ probability level.

RESULTS

Expression of inflammatory genes in UC

NOD1 genes were highly expressed (8-fold) in severe UC patients ($p = 0.0069$) compared to control and mild UC patients. The level reverted back to normal in the remission stage ($p = 0.0289$) (figure 1A).

Compared to the level in controls, the IFN- γ mRNA level exhibited differences among the patient groups. An upward trend was observed, particularly in patients with mild to severe disease ($p = 0.018$, $p = 0.0006$) (figure 1C). Real time analysis revealed a higher expression level of TNF- α ($p = 0.022$, $p = 0.006$) (figure 1B) in mild and severe UC samples respectively. During remission, mRNA levels of both IFN- γ ($p = 0.036$) (figure 1C) and TNF- α ($p = 0.018$) (figure 1B) attained normal values compared to the patient group in the severe stage of the disease.

Among the pro-inflammatory cytokines, higher expression of both IL-17 ($p = 0.01$ in mild UC, $p = 0.025$ in severe UC) (figure 1D), and IL-23 ($p = 0.03$ in mild UC, $p = 0.007$ in severe UC) (figure 1E) were observed in patient groups. Both IL-17 ($p = 0.008$) (figure 1D) and IL-23 ($p = 0.007$) (figure 1E) reverted back to normal in the patient group in the remission stage, confirming their transient upregulation only during the disease condition.

Among the anti-inflammatory cytokines studied here, no significant changes were observed in the levels of IL-10 and IL-13 in the different patient groups, but were present in low levels in active stages of the disease (figures 1F,H); only IL-13 was significantly higher in the patient group in remission ($p = 0.041$). IL-11 expression was low in the severe UC group ($p = 0.019$), and remained low even in patients in remission (figure 1G).

H&E staining and immunohistochemistry

General H&E stained biopsy samples shows loss of crypts, mucosal erosions, ulcers, destruction of lamina propria layer and infiltration of inflammatory cells around the mucosal layer in patient group with severe UC. Clear differences were observed in patients with severe UC compared to controls and subsequent reformation of lamina propria and crypt was seen in patients with remission stage (data not shown).

Immunohistochemical distribution of Nod1 in the biopsy samples revealed that the protein is mainly expressed in epithelial cell on mucosal surface. During the active stage of the disease, the number of Nod1 positive cells correlated with the intensity of the inflammatory infiltrate and was higher in the areas of ulceration. A significant upregulation of Nod1 protein expression was observed in colonic epithelium of patients belonging to severe group as compared to the control group (figures 2A-C).

IFN- γ cell expression was usually stronger than TNF- α expression. It was abundantly positive in colonic epithelial cells in all the patient groups compared to control group (figures 2D-I). Signal of IFN- γ was more pronounced in lamina propria areas showing their involvement in patient group with severe disease (figures 2D-F).

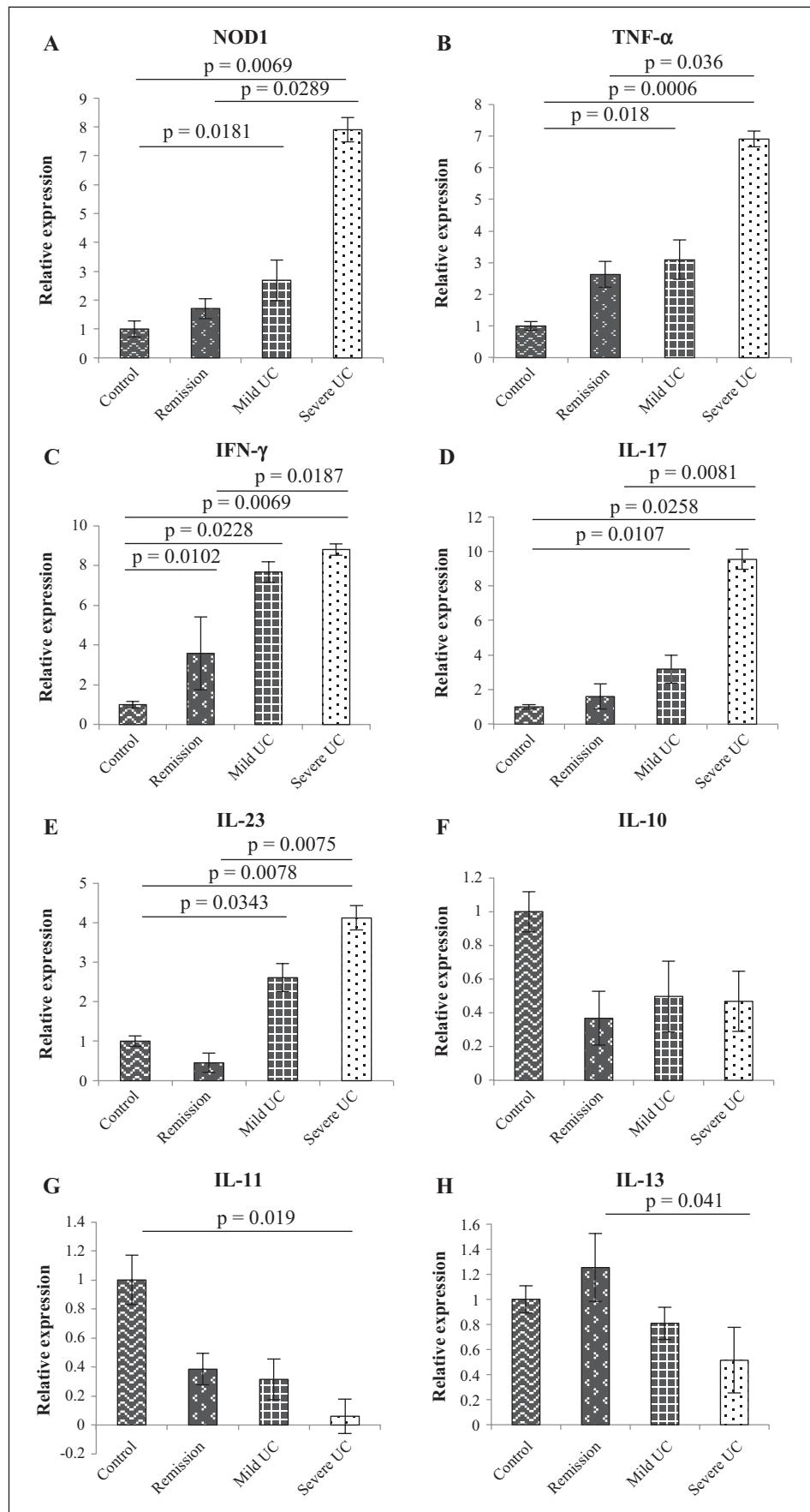
Expression of IL-17A protein was more abundant in severe stage category of patients and located mainly in the lamina propria area (figure 2N). In control group, IL-17A expression mainly restricted to the crypt region (figure 2M). IL-17A expression was more apparent in the surface epithelium as well as in the crypts during remission (figure 2O). It is likely that IL-17A expression during UC may be associated with altered immune and inflammatory responses in the intestinal mucosa.

Our observations further revealed that the IL-23 expression in epithelial cell was more pronounced than IL-17A expression in colonic biopsy samples. The expression was more localized in the cells of lamina propria (figure 2K) in patient groups. In control samples, IL-23 expression was more apparent in the surface mucosal epithelial cells (figure 2J). This difference probably reflects increasing number of infiltrates in the lamina propria area during disease condition. Crypts were more involved in remission cases as the expression was quite high in these cells (figure 2L).

Interestingly, we did not observe significant change either in the mRNA or protein expression in IL-10 and IL-11 levels at any group of patients. Scattered IL-13 positive cells were seen in the lamina propria in colonic mucosa of severe category of patients (figure 2Q). Very low expression of IL-13 was identified in the crypt region in remission category of patients (figure 2R). In control group, weaker signals were observed on the epithelial layer of mucosal surface reflecting low level of expression (figure 2P).

DISCUSSION

It is known that NOD1 expression by epithelial cell lines is upregulated by IFN γ acting through the transcription factor IFN-regulatory factor 1 (IRF1) at the *CARD4* promoter [34]. Increased expression of Nod1 gene both at the

**Figure 1**

Differential mRNA Expression of A. *NOD1*, B. TNF α , C. IFN γ , D. IL-17 and E. IL-23, F. IL-10, G. IL-11 and H. IL-13 by real time analysis in the biopsy samples from control *versus* different UC patient groups- remission, mild and severe stages of the disease. The expression level for each parameter is presented as a median ratio to the expression of normal gut. p values are shown on top of the bars.

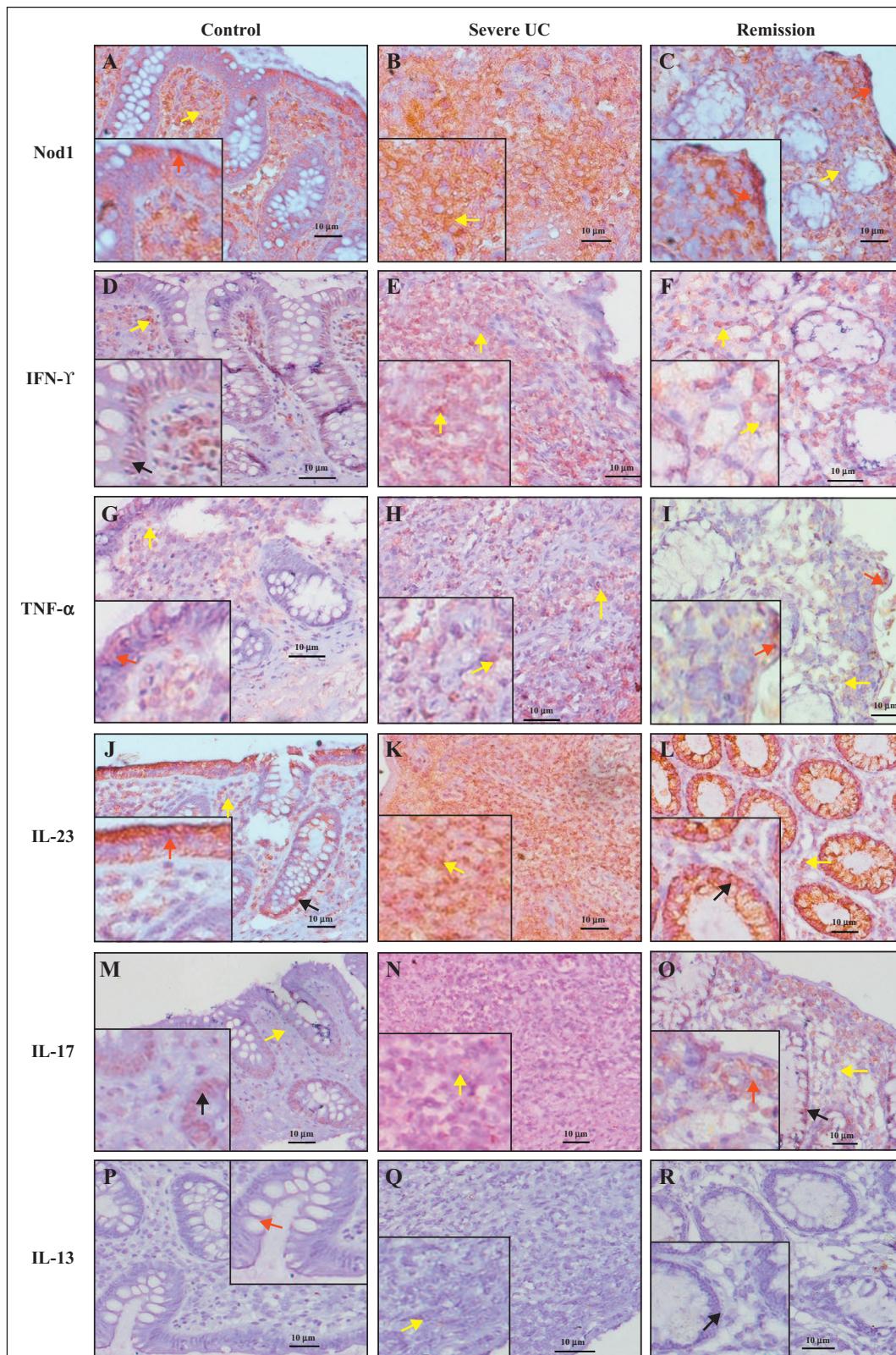


Figure 2

Protein expression of Nod1 and cytokines in biopsy samples of different groups of patients *versus* control. Distribution of positive cytokine expression in the biopsy tissues of control, severe UC and remission samples are shown by immunohistochemistry. The Nod1, Th1 cytokines (IFN- γ , TNF- α), IL-23 and Th17 expressing cytokines, IL-17 and IL-13 (anti-inflammatory cytokines) in control mucosa (Yellow arrows A, D, G, J, M) and severe UC (Yellow arrows B, E, H, K, N, Q) were predominantly in the subepithelial layer of the stroma. In remission, they were evenly distributed in the stroma (Yellow arrows C, F, L, O). Red arrows indicate expression of the cytokines in the epithelial layer. Black arrows show the expression restricted to crypt regions.

mRNA (8 fold) as well as the protein level in severe category of patients as seen in IHC, supports earlier observation by Magalhaes *et al.* [35] where Nod1 expression within

the stromal compartment was shown to be necessary for priming of effector CD4+Th2 responses and specific IgG1 antibodies.

IFN- γ was observed to be highly expressed in the mRNA level in severe category of UC patients. This supported our IHC data where the number of IFN- γ producing cells in the lamina propria was also significantly increased as compared to controls supporting earlier observation by Camoglio *et al.*, 1998 [36].

TNF- α is highly expressed in UC and correlates to the grade of inflammation as observed by Olsen *et al.*, 2007 [37]. We observed higher staining with anti-TNF antibody during severe stage of the disease. It has been observed earlier that TNF- α is mainly produced by macrophages and most of the macrophages get localized in the lamina propria during the active phase of IBD, they are known to be recruited from the circulating monocyte population simultaneously with the onset of inflammation [38]. We demonstrated an increased level in TNF- α mRNA expression in inflamed mucosa in UC by RT-PCR and confirmed the same by IHC. Substantial decrease in the TNF- α positive cells during remission, suggests that upon treatment of patient, the histological parameters improved. This supports earlier observation by Olsen *et al.* where Infliximab (IFX), an anti-TNF-alpha antibody, has been shown to be effective in the treatment of UC by improving the histological parameters [39].

Upregulated expression of IL17 transcripts has been reported in biopsy specimens from both UC and CD [40]. Our observation supports the theory that increase in IL17A level leads to the induction of many pro inflammatory factors including TNF- α suggesting an important role of IL17 in localizing and amplifying inflammation [41]. In accordance with the biological properties of IL-17A, our Real time data and IHC results suggest that IL-17A derived from activated T cells could be involved in induction and persistence of mucosal inflammatory responses in UC patients.

IL-23 is found to play a critical role in the maintenance of immune response by controlling T cell memory function and by influencing the proliferation and survival of IL-17-producing Th17 cells [42]. Our results demonstrating higher expression of IL-23 in biopsy samples of severe category of UC patients corroborates earlier finding indicating that IL-23 is highly expressed in inflamed mucosa of IBD [43]. It was suggested that therapy directed against IL-23p19 may have a positive role in treatment of IBD [44].

Anti-inflammatory cytokines are known to inhibit both antigen presentation and subsequent release of pro-inflammatory cytokines thereby attenuating mucosal inflammation. The decreased levels of both the mRNA and the protein of the Th2 cytokine, IL-13, observed by us in the colonic biopsy samples, supported earlier finding by Vainer *et al.*, 2000 [28]. Severe UC is associated with decreased colonic IL-13, suggesting either that IL-13 levels are diminished as a result of exacerbations of UC, or that exacerbations follow active downregulation of IL-13. It has been observed earlier that the anti-inflammatory activity of IL-13 is partially reduced in patients with active IBD [45]. The expression of IL-13 (figure 1H) during remission was higher than in controls, indicating the protective function exerted by IL-13 during remission. Some studies however, showed high expression of IL-13 in a mouse model of UC, and correlated with the damage [46],

whereas a study conducted on UC patients [28] revealed decreased expression of IL-13 according to the severity of the UC. Our results supported the latter observation, and so the role of IL-13 remains controversial.

The ability of IL-10 to regulate intestinal inflammation, predominantly through the suppression of the activation and effector function of T cells, monocytes, and macrophages, has been well described [47-49]. IL-10 has been implicated as a key protein mediator that prevent excess TNF- α , and IFN- γ production in intracellular infections [50]. This is consistent with the report that IL-10 antagonizes the effect of IFN- γ on epithelial cell permeability [51]. We observed low expression of IL-10, with a simultaneous increase in the expression of TNF- α and IFN- γ , indicating the loss of function of IL-10 during active stages of the disease. This supports the observations made earlier by Leon *et al.*, 2009 [52], where a decrease in IL-10 mRNA levels in the affected areas of UC was observed. A reduction in expression levels of IL-11 during the severe stage of the disease supports the finding of Klein *et al.*, 2002 [53], where altered expression of IL11 was found to be involved in the genetic predisposition to UC.

Our results show a clear distinction in the expression profile of each cytokine, both at mRNA level and the protein level during different stages of the disease. Our observation of altered expression of the cytokines during severe stage of the disease and subsequent return to normal values during the remission stage of the disease was quite interesting. It suggests that the local immune responses require a tight control, the outcome of which is, in most cases, the induction of tolerance. Altogether, these data suggest that Nod1 and IFN- γ , TNF- α , IL-17 and IL-23 contribute to the inflammation found in UC patients, by stimulating T and B cells in the lamina propria. However, it is not yet clear what the implications of B cell stimulation are, and if its prevention would help to resolve the inflammation. The simultaneous higher expression of Nod1, IFN- γ , TNF- α , IL-17 and IL-23 during the onset of disease suggests their use as potential biomarkers. However, further study is required to clarify how this aspect of homeostasis is regulated.

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