Transcriptional modulation of pattern recognition receptors in acute colitis in mice

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ABSTRACT

Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), contribute to the development of intestinal inflammatory diseases, like inflammatory bowel disease (IBD). Supporting investigations of the underlying mechanisms of IBD, this study provides an extensive PRR expression survey together with T-cell associated factors along the murine colon during experimental colitis. 8–12 week-old C57BL/6 mice were treated with dextran sodium sulfate (DSS) to induce colitis. The mRNA expression levels of *Tlr1–9*, *Nod1*, *Nod2*, T cell subset-associated master transcription factors and cytokines were determined using qPCR. The expression of TLR2, 4, 5 and 6 was determined with immunohistochemistry. Th1 and Th17 associated responses were quantified in the mesenteric lymph nodes (mLNs) using flow cytometry. In DSS treated mice, the mRNA expression of the majority of PRRs was increased relative to healthy controls and correlated with the degree of inflammation. The exceptions were *Tlr1* and *Tlr5*, which displayed unchanged and down-regulated transcription, respectively. Furthermore, in healthy animals, there was increased transcription of *Tlr2*, *3* and *5* near the caecum as opposed the region near the rectum. Within the inflamed regions, the mRNA expression of Th1-, Th17- and regulatory T-cell associated cytokines was enhanced, while there was no change for Th2-associated cytokines. In agreement with the mRNA expression, enhanced IFNγ and IL-17 producing cells were observed in stimulated mLNs. This study provides an extensive expression survey of PRRs along the colon during the acute colitis and shows that the induced inflammation is characterized by a Th1- and IL-17 mediated cytokine response.

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1. Introduction

Over the last decade, the incidence rate of IBD and especially Crohn's disease (CD) has increased [1]. Currently, there is still no curative therapy for IBD and the treatments that do exist focus mainly on relieving symptoms and often lead to unwanted side effects [2]. Due to the need for improved medications that target more than just symptoms, there is a growing interest in targeting receptors that may modulate IBD initiation and/or progression, such as PRRs [3].

PRRs are expressed by a broad range of immune cells. They are able to recognize pathogen-associated molecular patterns (PAMPs), molecules that are unique to bacteria, fungi, parasites, and viruses; and their stimulation leads to the initiation of immune defense mechanisms. Studies using germ-free animals and observations from patients undergoing fecal-stream diversion have demonstrated that the development and maintenance of colitis are strongly associated with the presence of intestinal flora. Given the requirement of bacteria for the initiation of intestinal inflammation, PRRs may be important for the development and, possibly, the chronic nature of IBD, and therefore serve as a potential target for future treatments.

Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-containing proteins (human: NOD, mouse: Nod) of the Nod-like receptor family are two important groups of PRRs. To date, 13 TLRs are known, of which TLR1–9 are conserved in both humans and mice. TLRs can be located both extracellularly and intracellularly [4]. They are able to provide protection against a broad range of pathogens by recognizing specific PAMPs, such as bacterial cell wall components (TLR2, TLR4 and TLR5), bacterial-derived DNA (TLR9), and virus-derived RNA (TLR3, 7 and 8). TLR1 and TLR6 both pair with TLR2 to form heterodimers that recognize triacyl- and diacyl-lipopeptides from bacteria, respectively [5].

The NOD/Nod proteins are localized exclusively within the cytosol and are able to detect peptidoglycans, components on the bacterial

cell walls. The NOD2 protein encoded by *NOD2* was the first gene that was directly linked to the development of IBD, especially CD [6]. A c-terminal variant of *Nod2* results in reduced downstream NF-κB activation after stimulation [7,8]. Polymorphisms of *Tlrs* are also associated with the susceptibility of IBD development, such as the Asp299GLy polymorphism of *Tlr4*, which results in a deficient Lipopolysaccharide (LPS) response and polymorphisms of *Tlr1, 2* and *6* [9,10]. In addition to genetic associations, clinical studies have shown enhanced expression of TLRs including TLR2, TLR3, TLR4 and TLR8 in the inflamed colonic tissue of IBD patients [3,11–13].

Experimental colitis in mice has been used to help elucidate the role of PRRs in the development of IBD. Among mouse models of colitis, the DSS-induced colitis is by far the most commonly used due to its simplicity and its ability to induce a predictable inflammation in the colon [14,15]. Though, DSS colitis was first considered an ulcerative colitis model with type 2 T-helper (Th2) skewing, gene scans [16] and additional research have shown that the acute DSS model induces many genes associated with CD, including type 1 T-helper cell (Th1) and T-helper 17 (Th17) associated cytokine expression [17–20]. Studies using the DSS colitis model in TLR$^{-/-}$ mice have shown that TLRs have the capacity to influence colitis development. Both TLR4$^{-/-}$ and TLR2$^{-/-}$ mice experience a more severe colitis than control mice [21–23] while TLR9$^{-/-}$ mice are reported to have reduced symptoms [24]. Treatment of acute DSS colitis with TLR ligands have shown varying effects which appear to be highly dependent on the mode of administration. The effects include protective effects with the TLR3 ligand, poly I:C [25] and the TLR1/2 ligand, PAM$_3$Cys-SK$_4$ [23] and ambiguous effects with the TLR9 ligand, CpG DNA [24,26] and the TLR5 ligand, flagellin [27,28].

Despite the major role PRRs could play in IBD development and progression, the underlying mechanisms remain unknown. It is further complicated by the fact that PRRs, on the one hand, are known to stimulate the immune response, yet on the other hand, often have a protective role in in vivo models of colitis by maintaining the barrier function of the intestinal epithelial cell layer [21–23,25]. In order to help decipher the precise role of PRRs in colitis, we have provided an extended overview of the transcriptional modulation of *Tlr1–9, Nod1* and *Nod2* along the colon during DSS-induced colitis. We have also determined the location of expression for TLR2, 4, 5 and 6 in the colon with immunohistochemistry. Furthermore, we have simultaneously assessed the mRNA expression of cytokines and transcription factors representing different T-helper cell populations.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). All mice were used at 8–12 weeks of age and were housed under standard conditions in the animal facilities at Utrecht University. All animal experiments were approved by and were in accordance with the guidelines of the Dutch Experimental Animal Commission. The approval document is encoded with 2008.II.03.030.

2.2. Experimental colitis

Experimental colitis was induced in groups of 9 mice by adding 1.5% DSS to the drinking water of the mice for 6 days. The weight was measured every day until the end of the experiment. Mice were sacrificed on day 7 and colitis was evaluated according clinical and histological parameters. The clinical features of colitis were determined by using the data collected from the weight measurement, feces condition and the weight/length ratio of the isolated colons. The feces condition score was determined from two parameters: stool consistency (0 = normal, 1 = soft with normal form, and 2 = loss of form/diarrhea) and fecal bleeding (0 = no blood, 1 = blood observation

using Colo-rectal Test kit (Axon Lab AG, Germany), and 2 = blood observation without test).

After sacrificing the mice, the colons were excised between the ileocaecal junction and rectum and were prepared for histological evaluation. The colon was opened longitudinally, placed on a piece of blotting paper, and fixed in 10% formalin. After fixing, the colons were rolled, paraffin-embedded, and sectioned (5 μm). Two researchers assessed general inflammatory features blindly after staining sections with hematoxylin and eosin according the assessment system described below. Individual scores were tallied for the proximal colon (characterized by bulges in the colon wall) and the distal colon (the region starting from end of proximal portion stretching to the anus). Assessments included four pathological criteria: the extent of cellular infiltration (0: no infiltration, 1: infiltration between the crypts, 2: infiltration in the submucosa, 3: infiltration in the muscularis externa, and 4: infiltration in entire tissue); cover area of cellular infiltration in the region (0: no infiltration, 1: <25%, 2: 25%–50%, 3: 50%–75%, and 4: >75%); loss of crypts (0: no damage, 1: 30% shortening of crypts, 2: 65% shorting of crypts, 3: total loss of crypts, and 4: loss of entire epithelial layer); extent of crypts loss in the region (0: no crypt loss, 1: <25%, 2: 25% – 50%, 3: 50%–75%, 4:>75%). Ly-6B + cell infiltration was considered an additional indicator for colitis and stains primarily neutrophils and macrophages. The Ly-6B staining is described in Section 2.3, "Immunohistochemical staining".

2.3. Immunohistochemical staining

For immunohistochemical staining, 5-μm-thick sections were subjected to a heat-induced epitope retrieval step. Slides were washed with 1× phosphate buffered saline (PBS) buffer and blocked with rabbit or goat serum, before an overnight incubation at 4 °C with primary antibodies against Ly-6B (MCA771GA, AbD Serotec, Dusseldorf, Germany), TLR2 (ab24192, Abcam, Cambridge, UK), TLR4 (ab47093, Abcam, Cambridge, UK), TLR5 (ab62460, Abcam, Cambridge, UK) and TLR6 (SAB1300202, Sigma, UK). For detection, biotinylated goat anti-rat (E0468, Dako, Glostrup, DK) and goat anti-rabbit (E0432, Dako, Glostrup, DK) secondary antibodies were administered followed by incubation with peroxidase-labeled streptavidin (Vectastain Elite ABC kit, PK-6200, Vector, Burlingame, CA USA). The peroxidase activity was visualized using DAB (D5637, Sigma, UK). Background staining was determined by substituting the primary antibody with the relevant isotype control, either rat (ab37261, Abcam, Cambridge, UK) or rabbit (ab27472, Abcam, Cambridge, UK) IgG antibody.

2.4. mRNA expression analysis

The colons were divided into four equal sections: proximal (P), medial 1 (M1), medial 2 (M2) and distal (D). The total RNA was isolated using the RNAeasy kit (Qiagen, Germantown, MD, USA) and, subsequently, reverse transcribed into cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Real-time PCR was performed using iQ SYBR Green supermix kit (Bio-Rad, Hercules, CA USA) with the CFX 96 Real-time system (BioRad, Hercules, CA USA) and the relative mRNA expression values were calculated using Bio-Rad CFX manager V1.6. The sequence of specific primers for *Nod1* and *Nod2*, *Tlrs*, T cell transcription factor genes, and the gene for the household protein ribosomal protein S13 (*Rps13*) are listed in Table 1. The primers for the cytokines: tumor necrosis factor-α (*Tnf*), interferon-γ (*Ifn-gamma*), interleukin-1β (*Il-1beta*), *Il-6*, monocyte chemotactic protein-1 (*Ccl-2*), *Il-4, Il-6, Il-10, Il-12p35, Il-17, Il-23p19* and transforming growth factor β (*Tgf-beta*) were purchased from SABioscience (Frederick, MD, USA). The final data for the target samples were normalized against the internal control *Rps13*.

Table 1
qPCR primer sequences for PPRs, T cell transcription factors and RPS13.

	Primer sequence 5′ → 3′	
	Forward primer	Reverse primer
Tlr1	GGTGTTAGGAGATGCTTATGGGG	GATGTTAGACAGTTCCAAACCGA
Tlr2	CCAGACACTGGGGGTAACATC	CGGATCGACTTTAGACTTTGGG
Tlr3	GGGGTCCAACTGGAGAACCT	CCGGGGAGAACTCTTTAAGTGG
Tlr4	GCCTTTCAGGGAATTAAGCTCC	AGATCAACCGATGGACGTGTAA
Tlr5	TCAGACGGCAGGATAGCCTTT	AATGGTCAAGTTAGCATACTGGG
Tlr6	GACTCTCCCACAACAGGATACG	TCAGGTTGCCAAATTCCTTACAC
Tlr7	TCTTACCCTTACCATCAACCACA	CCCCAGTAGAACAGGTACACA
Tlr8	GGCACAACTCCCTTGTGATT	CATTTGGGTGCTGTTGTTTG
Tlr9	ACTCCGACTTCGTCCACCT	GGCTCAATGGTCATGTGGCA
Nod1	GAAGGCACCCCATTGGGTT	AATCTCTGCATCTTCGGCTGA
Nod2	CCGCTTTCTACTTGGCTGTC	GTGATTTGCAGGTTGTGTGG
Tbet	GCCAGCCAAACAGAGAAGAC	AAATGTGCACCCTTCAAACC
Gata3	GCGGTACCTGTCTTTTTCGT	CACACAGGGGCTAACAGTCA
Foxp3	CACTGGGCCTTCTGGGTATGT	AGACAGGCCAGGGGATAGTT
Rorc-γt	TGCAAGACTCATCGACAAGG	AGGGGATTCAACATCAGTGC
Rps13	GTCCGAAAGCACCTTGAGAG	AGCAGAGGCTGTGGATGACT

2.5. Intracellular cytokine staining

Mice were treated with DSS or water for 6 days and on day 7 the mice were sacrificed. After sacrificing the mice, the mesenteric lymph nodes were isolated and prepared as single-cell suspensions. All reagents and antibodies used were obtained from eBioscience (San Diego, CA, USA). Briefly, 10^5 cells were transferred to 96 well round bottom plates and activated by incubating at 37 °C for 24 h in wells coated with anti-CD3. After stimulation, cells were incubated for 4 h with Brefeldin A. Before staining, Fc-receptors were blocked using CD16/Cd32 to prevent non-specific binding of antibody. The cells were stained extracellularly with antibodies for CD4. Subsequently, the cells were fixed and permeabilized using the Foxp3 intracellular staining buffer set and stained with anti-IL-17A PE and anti-IFNγ PerCP-Cy5.5. The samples were read on a BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA). Intracellular cytokine staining was analyzed using BD FACSDiva software (BD Biosciences).

2.6. Statistical analysis

Means with SEM are represented in each graph. Statistical analysis was performed using GraphPad Prism version 5.0 for windows (GraphPad Software, San Diego, CA USA). p-Values were calculated using the Mann–Whitney test. p-Values considered as significant are indicated as *** <0.001, **<0.01, and *<0.05.

3. Results

3.1. Colitis severity during DSS treatment

Experimental colitis in C57BL/6 mice was induced by adding 1.5% DSS to the drinking water for six days. As expected, weight loss and diarrhea with blood were observed only in the treated group (Fig. 1A and B). The colon length and weight ratio was significantly decreased in the DSS-treated mice as shown in Fig. 1C indicating severe inflammation.

To determine the extent of inflammation at the proximal and distal ends of the colon, the colons were fixed and stained with hematoxylin and eosin and scored for disease severity. DSS-treated mice displayed extensive signs of inflammation as compared to healthy mice (Fig. 1D). The damage was predominantly found in the distal
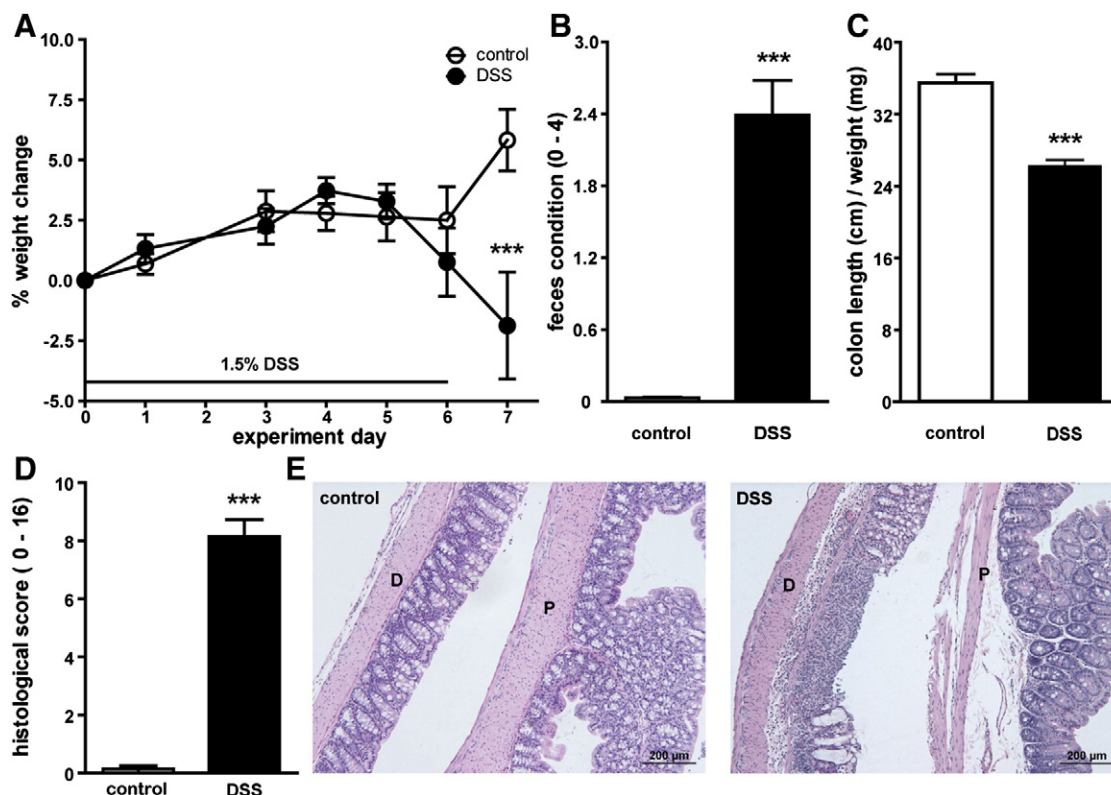

Fig. 1. DSS treatment induces colitis in mice. C57BL/6 mice have received either normal drinking water or drinking water with 1.5% DSS for 6 days. (A) Percent body weight change of mice over time. (B) Fecal condition of healthy mice (control) or DSS-treated mice (DSS) was determined by measuring diarrhea and blood in feces on day 6. (C) On day 7, the mice were sacrificed and the ratio of colon length over weight was determined in individual colons. Results are expressed as mean ± SEM for n = 9 mice per group, * p < 0.05; *** p < 0.001. (D) Histological quantification of inflammatory markers was determined within colons derived from both healthy mice and DSS-treated mice. The quantification parameters are described in experimental procedures. Results are expressed as mean ± SEM for n = 3 mice per group, *** p < 0.001. (E) H&E staining of colons from either healthy mice or DSS-treated mice. Both proximal (P) and distal (D) colon regions are shown. The photos are representative for n = 3 mice per group.
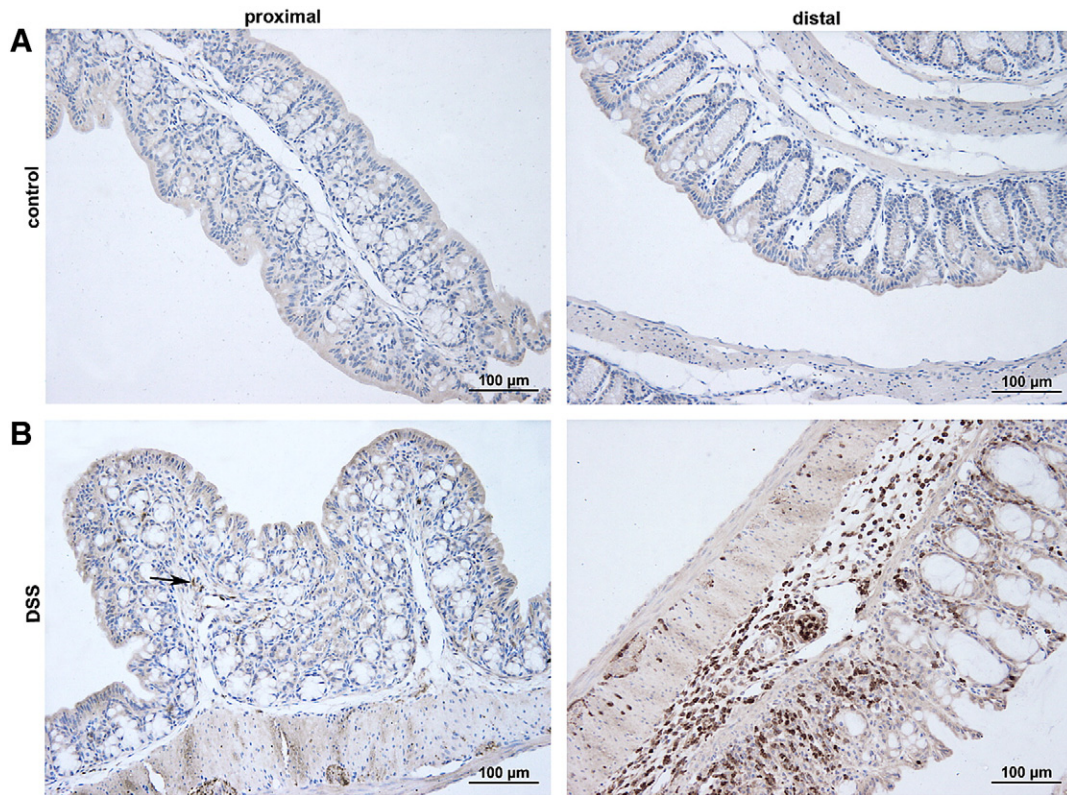
Fig. 2. Massive Ly-6B+ cell influx in the distal colon region of DSS-treated mice. The colon was divided into proximal and distal regions according to the morphological characteristics described in experimental procedures and assessed for the presence of Ly-6B+ cells, which are mainly neutrophils and inflammatory macrophages. Immunohistochemical staining was used to visualize Ly-6B+ cells at the proximal and distal colon region of healthy mice (A) and of DSS-treated mice (B). An example of a positively stained cell is indicated with the arrow. The pictures are representative of n = 3 mice per group.

region of the colon and included complete loss of crypt structure. The proximal region, in contrast, remained generally unscathed. Submucosal inflammatory cell infiltration was observed in 50–70% area of the whole colon, most prominently in distal (D) areas when compared to the proximal part of the colon (Fig. 1E). Immunohistochemistry was employed to determine the extent of infiltrated cells expressing Ly-6B, which exhibits particularly high expression on the surface of neutrophils and inflammatory macrophages. The regional tissue damage was mirrored by the immune cell influx measured by Ly-6B, which was mainly constrained to the distal regions of the colon in DSS-treated mice and not found in the control mice (Fig. 2A and B). Thus, the inflammation detected in the colon of our DSS-treated mice conformed to the generally accepted pathology of DSS-induced colitis.

3.2. mRNA expression levels of Nod1 and Nod2 are increased during DSS colitis

To determine the mRNA expression of Nod1 and Nod2 during DSS colitis, we investigated specific mRNA expression along the length of the colons isolated from both healthy mice and mice suffering from DSS-induced colitis (Fig. 3A). The mRNA transcripts of both Nod1 and Nod2 were up-regulated within the inflamed distal region of the colon. In the non-inflamed proximal region, there was no up-regulation of the mRNA encoding Nod1 and Nod2 (Fig. 3B) demonstrating that up-regulated expression correlates with the presence of gross inflammation.

3.3. mRNA encoding TLRs that recognize bacterial PAMPs are differentially modulated in DSS colitis

The main crux of this survey was to assess the mRNA expression of the conserved Tlr1–9 along the length of the colon in both healthy

mice and diseased mice. Of particular interest are the TLRs that recognize bacterial-associated PAMPs as it is known that intestinal inflammation relies on the presence of intestinal bacteria. The mRNA expression of bacterial-associated Tlrs (Tlr1, 2, 4, 5, 6, and 9) is shown in Fig. 3C. In healthy mice, the mRNA expression of these Tlrs is stabilized along the length of the colon, with exception of Tlr2 and Tlr5, which had a higher expression in the proximal colon region. In DSS-treated mice, Tlr2, 4, 6 and 9 expression levels were up-regulated in the inflamed colon region. TLR1 and TLR6 both pair with TLR2 to form functional heterodimers and discriminate triacyl- and diacyl-lipopeptide, respectively [29]. Though both Tlr2 and Tlr6 were up-regulated within the inflamed region of the colon, Tlr1 displayed no enhanced expression. Interestingly, TLR5, which recognizes flagellin, was observed to have a decreased mRNA expression within the inflamed distal region of the colon.

3.4. Colitis leads to the up-regulation of TLR transcripts that are associated with virus detection

TLR3 and TLR7/8 are known to provide protection against viral infection, recognizing double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA), respectively [30–32]. In order to determine the effects of inflammation on the expression of the virus-associated TLRs, we analyzed their mRNA expression along the length of colons obtained from both DSS-treated and healthy mice (Fig. 3D). The mRNA expression pattern of Tlr3 detected in the colon was similar in both DSS-treated mice and healthy mice; although a marginal increase was observed in the inflamed colon region of DSS-treated animals. In contrast to Tlr3, large increases of mRNA expression were seen for both Tlr7 and Tlr8 within the inflamed regions as compared to control colon samples.
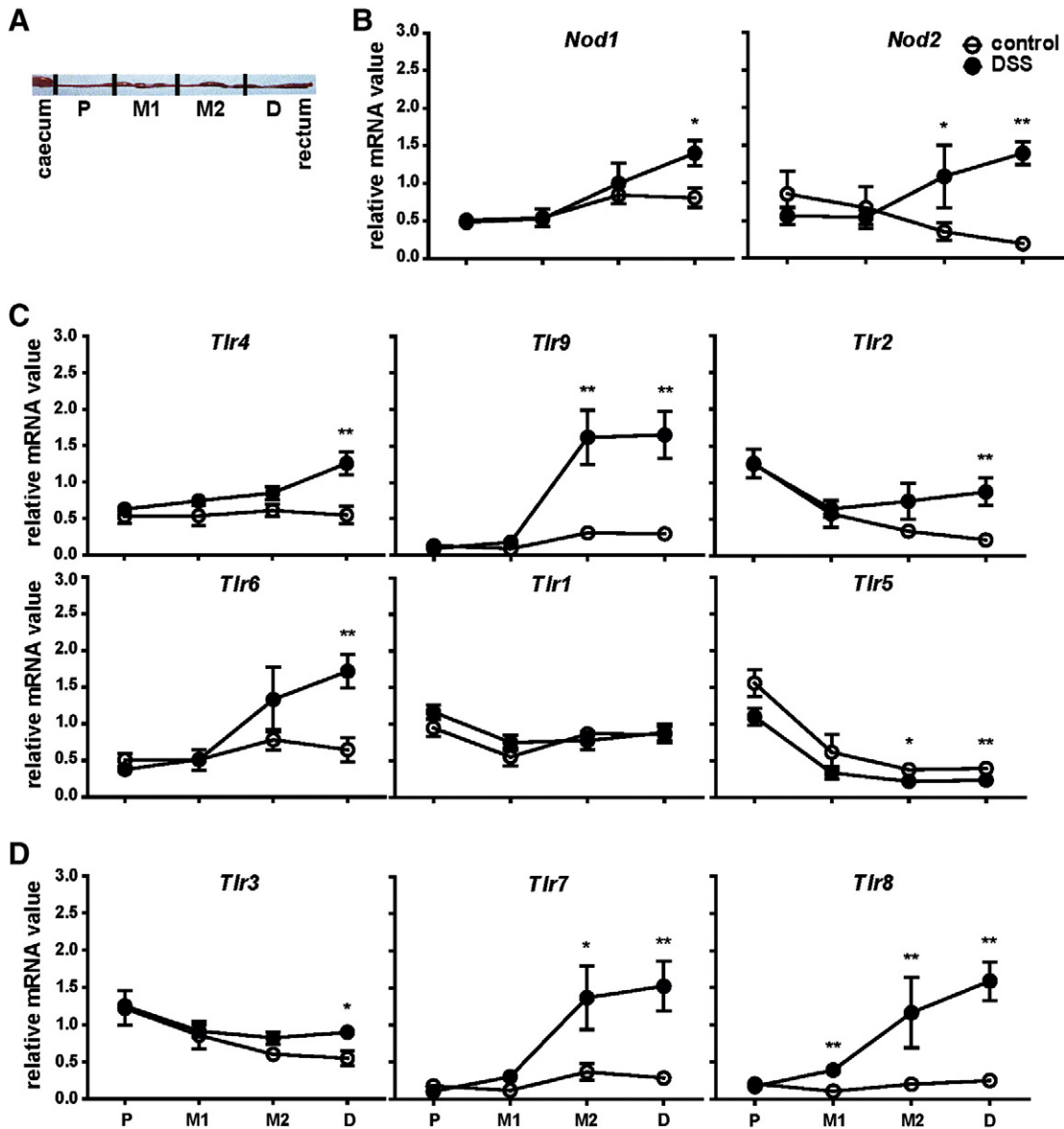
Fig. 3. The mRNA expression of PRRs is differentially modulated in the colons of DSS-treated mice. A) Photo of colon illustrating the four regions that were examined: P = proximal region, M1 = proximal middle region, M2 = distal middle region, D = distal region. mRNA expression of NOD proteins (B), bacterial-associated *Tlrs*: *Tlr4*, *Tlr9*, *Tlr2*, *Tlr6*, *Tlr1* and *Tlr5* (C) and virus-associated *Tlrs*: *Tlr3*, *Tlr7* and *Tlr8* (D) were examined in four different colon regions using qPCR. Results are expressed as mean ± SEM, n = 6 mice per group, pooled from two independent experiments, * p < 0.05; ** p < 0.01; *** p < 0.001.

3.5. Expression of TLR2, 4, 5 and 6 in the colon

We assessed the expression of several bacterial-associated TLR molecules (TLR2, 4, 5, and 6) in the colons of both healthy and DSS-treated mice using immunohistochemical staining. TLR4 positive cells were found throughout the colon in both healthy and DSS-treated mice. TLR4 was frequently found expressed in the epithelial layer found at the base of the crypts in the distal colon of both healthy and DSS-treated mice (Fig. 4A).

TLR2 was expressed along the length of colons in cells in the epithelial layer and lamina propria (Fig. 4B) of both healthy and DSS treated mice. In contrast to TLR2 and TLR4, the heterodimer partner of TLR2, TLR6, was detected only in the epithelial layer of colons derived from DSS-treated mice (Fig. 4C). The TLR6 positive cells were found at both the proximal and distal areas, clustered in patches. Unlike TLR2, 4 and 6, TLR5 staining was clearly found on mononuclear cells within the lamina propria as well in the epithelial cell layer (Fig. 4D) in both healthy and inflamed colons.

3.6. Colitis leads to modulations in the mRNA expression of T cell-associated master transcription factors

Histological analysis of our DSS-treated mice displayed both extensive damage and inflammatory monocyte infiltration within the distal region of the colon congruent with DSS colitis characteristics already published. However, clinical observations add limited information about the type of adaptive immune response present locally within the tissue (Th1, Th2, Treg or Th17). Next, we measured the mRNA expression of the transcription factors, T-bet (*Tbet*), GATA-3 (*Gata3*), Foxp-3 (*Foxp3*) and RORγt (*Rorc*), which are associated with Th1 cells, Th2 cells, regulatory T cells (Treg cells) and Th17 cells, respectively (Fig. 5) [20]. Similar to the *Tlr* expression patterns, the mRNA of the Th1-associated transcription factor *Tbet* was increased in the distal region (Fig. 5A). In the same region, *Gata3* and *Foxp3* were also moderately increased (Fig. 5B and C). Interestingly, the mRNA expression of *Rorc*, the transcription factor associated with Th17 polarization, was decreased in the DSS-treated mice as compared to healthy mice (Fig. 5D).
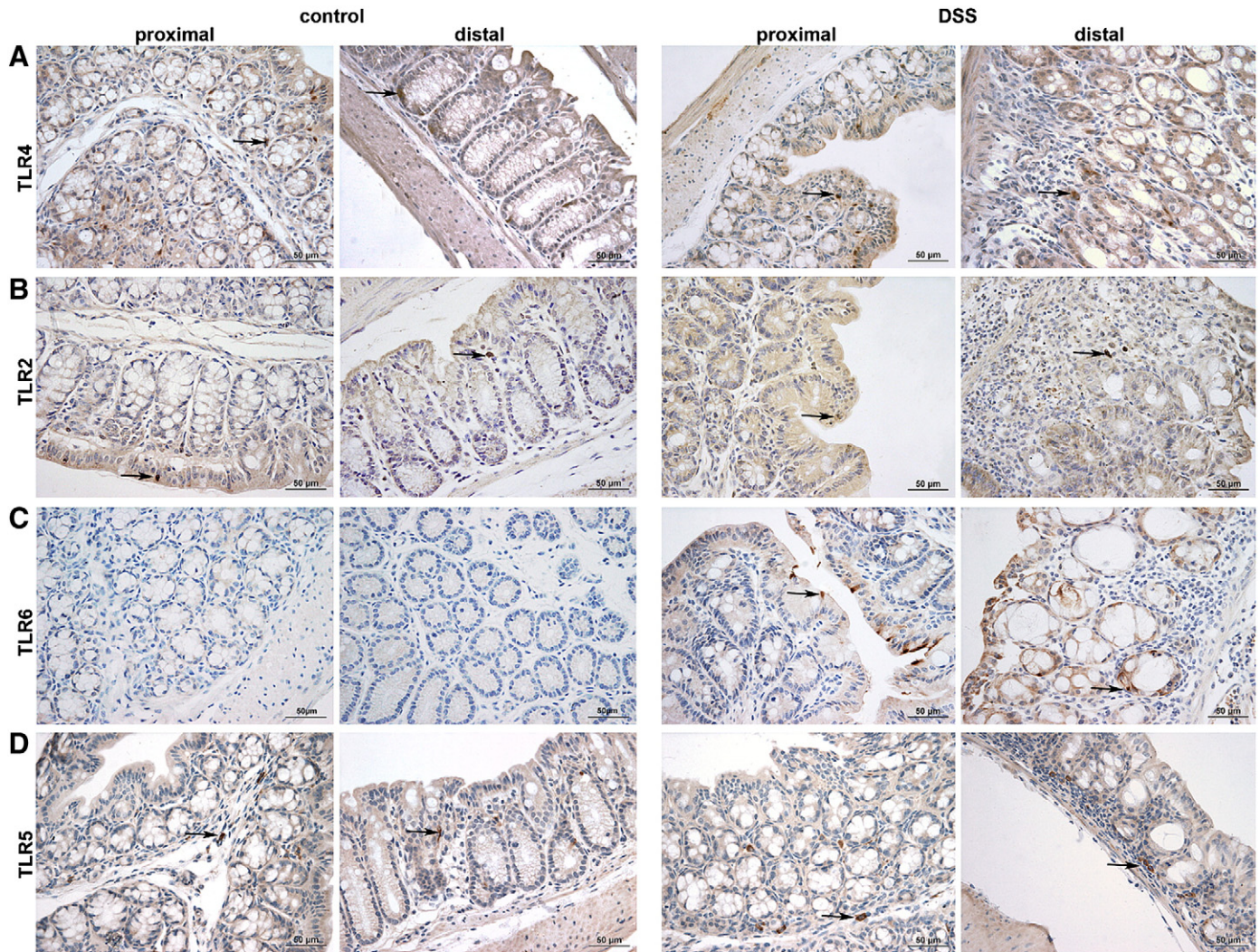
Fig. 4. Expression of TLR2, 4, 5 and 6 within the colon. The colon was divided into proximal and distal region according the morphological characteristics described in experimental procedures. Immunohistochemical staining was used to visualize TLR4 (A), TLR2 (B), TLR6 (C) and TLR5 (D) at both proximal and distal colon regions of either DSS-treated mice or healthy mice. An example of a positively stained cell is indicated with an arrow in each photo. The images are representative of n = 3 mice per group.

3.7. Colitis leads to increased transcripts of Th1/Th17 associated cytokines

Although the analyzed transcription factors are generally representative of the different T cell populations, there are also other cells that utilize these transcription factors. To provide further information about the underlying adaptive immune response, we performed a cytokine mRNA analysis, taking into account the different regions of the colon. We observed increased mRNA expression levels for various pro-inflammatory cytokines including *Tnf*, *Il-1beta*, *Ccl-2*, and *Il-6* in the inflamed distal region of the colon from DSS-treated mice (Fig. 6A–D). Furthermore, consistent with our transcription factor data, the mRNA transcripts of cytokines associated with Th1 (*Ifn-gamma* and *Il-12*) were also increased at the inflamed distal region (Fig. 7A), whereas Th2 associated cytokines (*Il-4*, *Il-5* and *Il-13*) were clearly not up-regulated (Fig. 7C). Interestingly, although the mRNA of the Th17 associated transcription factor, *Rorc*, was decreased in the DSS-treated mice, the Th17 associated cytokines (*Il-23* and *Il-17*) did show increased expression as reported in another study (Fig. 7B) [33]. This supports the concept that DSS colitis leads to Th1 and Th17 associated responses and also supports the relevance of this model for CD, which also is characterized by a Th1/Th17 response [20,33]. Finally, we also observed an increased mRNA expression of anti-inflammatory cytokines (*Tgf-beta* and *Il-10*) at the inflamed distal region, indicating a possible increase in activity or numbers of regulatory T cells (Fig. 7D).

Since the mRNA data suggested a Th1- and Th17-associated immune response during DSS colitis, we decided to measure the intracellular expression of IFNγ and IL-17 in CD4+ T cells isolated from mLN of both DSS-treated mice and healthy control mice using intracellular cytokine staining. After aspecific stimulation with anti-CD3, increased amounts of CD4+IL-17+ T cells were observed in DSS-treated mice as compared to healthy mice (Fig. 7E). The percentage of CD4+IFNγ+ T cells also tended to be higher in the DSS-treated mice, though the increase was not significant.

4. Discussion

To our knowledge, this is the first extensive survey of the mRNA expression of PRRs along the length of the colon from both healthy and DSS-treated mice exclusively using sensitive real-time PCR. Though current gene array studies offer some insight into the expression of PRRs, gene arrays are not nearly as sensitive as real-time PCR [34]. Furthermore, we have measured the transcription of PRRs along the length of the colon. This has allowed us to observe that the majority of PRRs modulate their expression in accordance with the level of inflammation and damage.
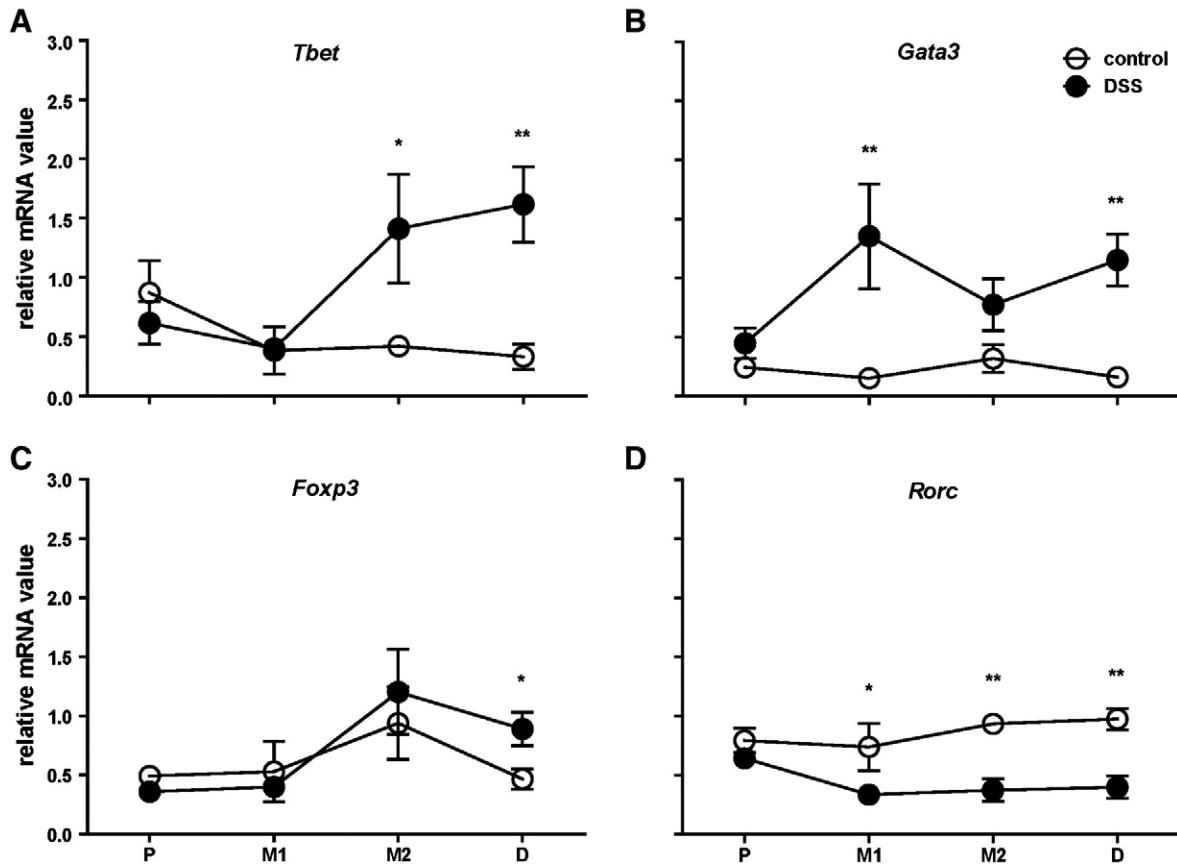
Fig. 5. Colitis leads to modulations in the transcription activity of T-cell associated transcription factors. The mRNA expression of the T cell subtype-associated master transcription factors were quantified along the colon from both DSS-treated mice and healthy mice using qPCR. *Tbet* (A), *Gata3* (B), *Foxp3* (C) and *Rorc* (D) are the master transcription factors representative for Th1, Th2, Treg and Th17 cells, respectively. Results are expressed as mean $\pm$ SEM, n = 6 mice per group, pooled from two independent experiments, * p < 0.05; ** p < 0.01; *** p < 0.001.

As we used colon tissue to isolate our RNA, the source of the mRNA transcripts for the PRRs may be a result of both increased transcription within resident immune cells (e.g. macrophages, mast cells and lamina propria resident lymphocytes) and intestinal epithelial layer cells, as well as transcriptional activity from infiltrating cells (e.g. neutrophils, macrophages and T cells) [35,36]. Our immunohistochemical staining showed that in the colon, TLR4 and 6 were expressed by cells only within the epithelial layer while TLR2 was expressed by both cells in the epithelial layer and cells within the lamina propria. TLR5, in contrast, was only expressed by mononuclear cells in the lamina propria. Interestingly, TLR6 was detected only in inflamed distal colons mirroring the TLR6 mRNA expression data. TLR6, which pairs with TLR2, recognizes diacylated lipopeptides [37] and is capable of detecting *Candida albicans* [38]. It has been shown that TLR6 stimulation can drive a Th17 response via the mucosal surface of the lungs [39]. In a separate study, we've now found that stimulation of TLR6 in the gut-associated lymphoid tissue also leads to increased Th17 polarization (unpublished results), suggesting that TLR6 plays a similar role at the mucosal surface of the intestines. Furthermore, a TLR6 polymorphism has been reported to be associated with the extent of IBD in the colon and, thus, *Tlr6*'s unique expression pattern may be indicative of strong role in initiating and/or supporting inflammation.

The fact that TLR2$^{-/-}$ and TLR4$^{-/-}$ mice treated with DSS have a more severe colitis than wild type mice, suggests that they may have a protective role. However, TLR2 and TLR4 both regulate the epithelial barrier integrity. An extensive study on TLR2 showed that deficiencies of TLR2 results in early disruption of tight junction [23]. At the same time, TLR2, as well as TLR4, are employed by a host of immune cells to stimulate immune response. Therefore, our expression results

cannot be directly interpreted. The enhanced *Tlr2* expression during colitis may both help the barrier function repair mechanism, and, on the other hand, encourage the immune response by stimulating the recruitment of neutrophils [21,51] and activating dendritic cells and macrophages [52,53]; all mechanisms to protect against bacterial infection induced by the DSS-treatment. Ultimately, results from TLR$^{-/-}$ mice should be treated with caution. Mechanisms induced in TLR$^{-/-}$ mice may differ from that in wild-types, since the knock-out mice lack TLRs from birth.

The increased expression of TLR7 and TLR9 mRNA in the colon of DSS-treated mice might suggest the activation of IFN type 1 pathway [54–56]. Dendritic cells express high levels of TLR7 and TLR9 and are producers of type 1 IFN [57]. Stimulation of TLR9 and associated IFNα production is reported to be anti-inflammatory in IBD [55,56]. In addition, the TLR7 ligand imiquimod induces also type 1 IFN in the intestinal tract of mice and reduces DSS-induced colitis [54]. Analysis of type 1 IFN mRNA expression in inflamed colon of mice in this study did not reveal an association between the enhanced expression of TLR7 or TLR9 mRNA and IFNα mRNA, since no or very limited expression of IFNα mRNA was detected (data not shown). The observed increased expression of *Tlr7* and *Tlr9* in the inflamed colon could be due to a rebound protection mechanism in DSS-colitic mice.

In the healthy colons, the expression of PRR mRNA along the colon typically followed two patterns: the same expression along the length of the colon or more expression at the proximal end near the caecum. Of all the PRRs tested, only *Tlr2*, *3*, and *5* showed a transcriptional preference for the proximal end of the healthy colon. This may be a consequence of increased exposure to the bacteria-rich caecum. Previous studies have shown a higher *Tlr5* expression in the caecum end of the colon [40] and *Tlr2* expression is induced after exposure
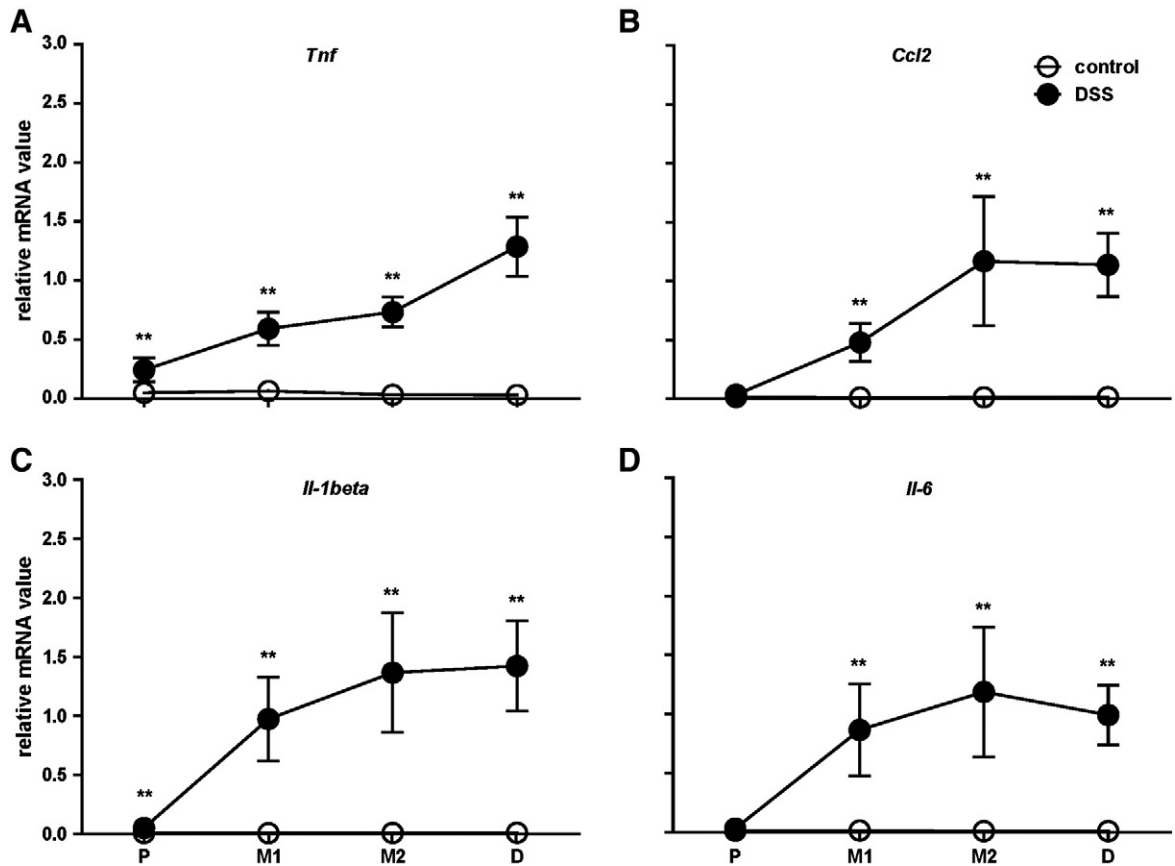
Fig. 6. Modulated PRR expression is coupled with increased mRNA transcripts of pro-inflammatory cytokines in DSS-induced colitis. The expression of pro-inflammatory cytokines *Tnf* (A), *Ccl-2* (B), *Il-1beta* (C) and *Il-6* (D) was examined in the four colon regions, illustrated previously. qPCR was used to quantify the mRNA expression level. Results are expressed as mean ± SEM, n = 6 mice per group, pooled from two independent experiments, * $p < 0.05$; ** $p < 0.01$; *** p < 0,001.

to caecal microbiota [41]. Both TLR2 and 5 detect bacterial PAMPs, however, TLR3 typically recognizes viral RNA. Though there is little known about the role of TLR3 in relation to the caecum, TLR3 is recognized as having a special role in the detection of endogenous ligands associated with cell necrosis via released RNA [42–44]. One could speculate that this mechanism could be important for intestinal immune homeostasis. Our observations of DSS-induced increased mRNA expression of TLRs, except TLR5, in the distal inflamed colon are in line with the report showing that DSS causes alterations of the inner colon mucus layer that makes it permeable to bacteria where they may trigger the epithelial cells to express higher levels of TLRs and induce an inflammatory reaction [58].

Comparing control and DSS-treated mice colon, we have found transcriptional modulation of a broad range of PRRs. Consistent with previous studies, which compare control and IBD patient colon biopsies using genome-wide microarray [45,46], we have also found increased mRNA levels for *Nod2* and *Tlr4* in the inflamed distal region of colons from DSS-treated mice as compared to non-inflamed, control colons. In the gene array, a decreased *Tlr1* expression has been found [45,46]. Although the same decreasing was not detected, in our study, *Tlr1* is the only *Tlr* remains at same expression level in both control and inflamed colons area. Furthermore, increased mRNA expression of *Tlrs* including *Tlr2* and *Tlr8* were detected in our study, which is in line with earlier reports [11,13]. A possible explanation for discrepancies between the murine data and the microarray data in humans could be the increased sensitivity of qPCR as compared to that with a typical microarray [34].

In our study, which included *Tlr1–9* and *Nod1* and *2*, only the mRNA expression of *Tlr5* displayed the unique characteristic of being down-regulated during colitis. This observation could be the

result of either the destruction of specific *Tlr5* expressing cells or an actual decrease of *Tlr5* transcription activity. The former is unlikely the case, since this would require *Tlr5* to be expressed in exclusion of other highly expressed *Tlrs*. Furthermore, no gross reductions of TLR5 staining were detected in colon sections of DSS-treated mice. Our data is in line with the study of Ortega-Cava and his colleagues, that demonstrated a decreased intestinal TLR5 expression during DSS colitis with the help of western blotting [47]. Interestingly, they demonstrated that IFNγ was able to down-regulate TLR5 expression in mice colon cells in a dose and time dependent manner [46]. Our data showed an increased expression of *Ifnγ* in the colons of mice suffering from colitis. This may be a possible explanation of why *Tlr5* reductions are observed.

Recently, UC patients were found to have lowered mucosal expression of TLR5 [48] and polymorphisms of *Tlr5* in dogs were found to be related to IBD development [40]. TLR5 is, in general, located at the basolateral surface of intact colonic mucosa [27,49], suggesting that it may have a special role during the progression of inflammation. This concept is further supported by experiments administrating the TLR5 ligand, flagellin, by rectal enema in combination with DSS colitis, which have lead to aggravated disease [27] as well as the fact that TLR5$^{-/-}$ mice suffer from spontaneous colitis [50]. These data imply that TLR5 triggering is highly important during intestinal homeostasis and the initiation of inflammation and its function will need to be addressed by future research.

Previous studies have shown that DSS colitis leads to the production of Th1 and Th17 associated cytokines [16,20,59]. In line with these data, we have also found an enhanced expression of *Tbet*, and the cytokines *Ifn-γ* and *Il-12* in the inflamed colon, which are associated with the differentiation of Th1 cells. In addition, the percentage
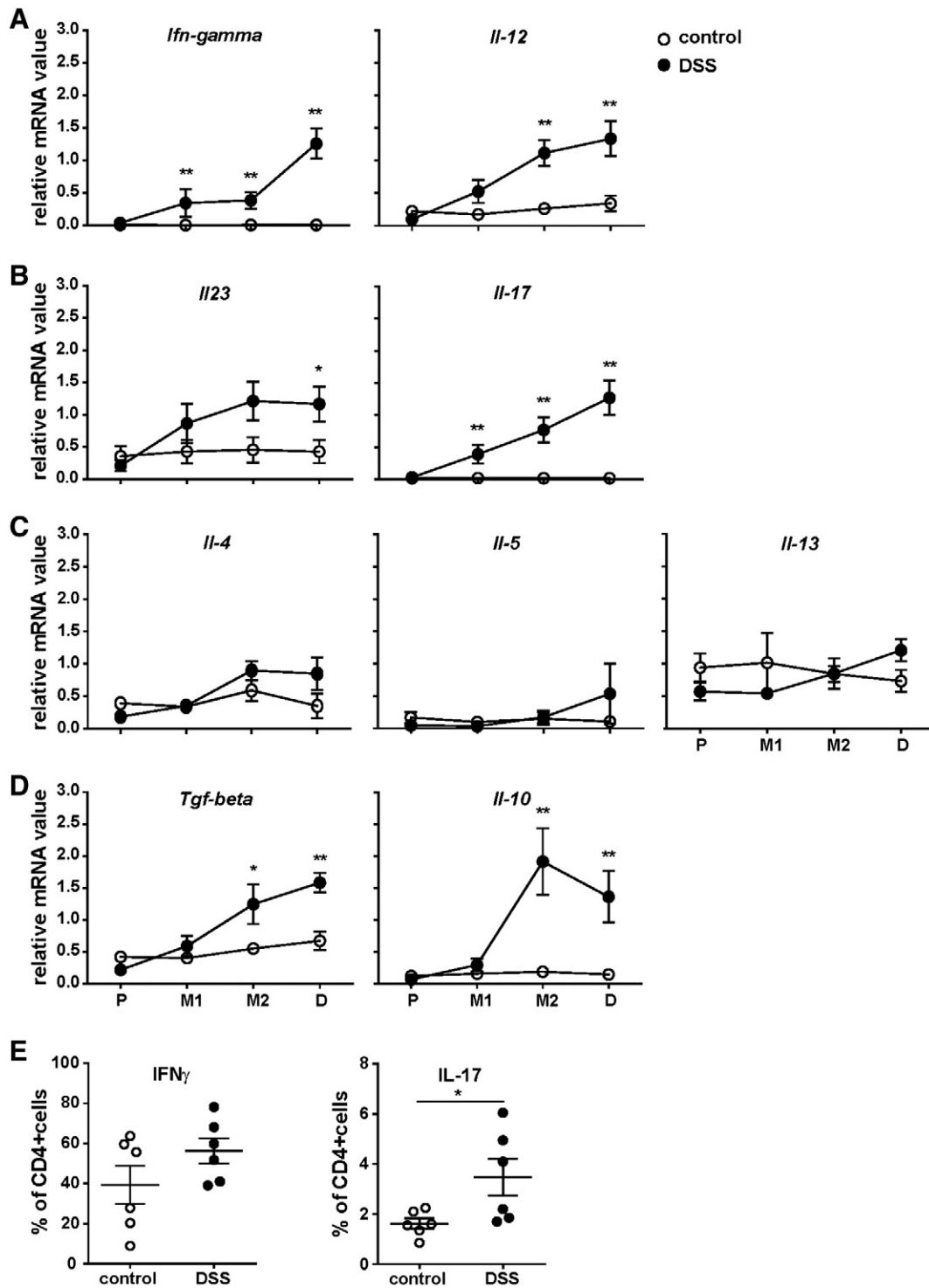
Fig. 7. Modulation of PRR expression is coupled with increased Th1/Th17 cell-associated cytokine production. The mRNA expression of T cell-associated cytokines was quantified along the colon from both DSS treated mice and healthy mice using qPCR (A–D) and the development of Th1 and Th17 associated responses was quantified in the mLN using fluorescence-activated cell sorting (FACS) analysis (E). (A) *Ifn-gamma* and *Il-12* are Th1 cell-associated cytokines. (C) *Il-4*, *Il-5* and *Il-13* are Th2 cell-associated cytokines. (B) *Il-23* and *Il-17* are Th17 cell-associated cytokines (D) *Tgf-beta* and *Il-10* are Treg cell-associated cytokines. (E) The percentage of CD4+IFN-γ+ cells (Th1 cells) and the percentage of CD4+IL-17A+ cells (Th17 cells) in cells obtained from the mLN of healthy and DSS-treated mice after CD3 stimulation. Results are expressed as mean ± SEM, n = 6 mice per group, pooled from two independent experiments, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

of IFNγ+CD4+ T cells tended to be higher in mLN obtained from mice undergoing DSS colitis.

Previously, an enhanced IL17A concentration was demonstrated in colon homogenates after 6 days DSS treatment [59]. Similarly, we also observed an increased expression of *Il-23* and *Il-17A* in the

inflamed colon, which are the inducer and effector cytokines of Th17 cells, respectively. Interestingly, this finding was not correlated with higher amounts of mRNA transcripts for the master Th17 transcription factor, *Rorc* as we had expected. The reason for the loss of *Rorc* mRNA expression in the inflamed colon is, at the moment, not

clear. However, it has been shown that conditions, which favor Treg, naturally antagonize Th17 polarization [59–62]. Based on this observation, one hypothesis could be that the transcription of *Rorc* is inhibited by factors, which stimulate *Foxp3* expression such as peroxisome proliferator-activated receptor (PPAR)-γ. PPAR-γ is a nuclear receptor protein, which can be induced after stimulation of TLR signaling pathway [63,64]. Activated PPAR-γ along with TGFβ encourages *Foxp* transcription by DNA demethylation, while inhibiting *Rorc* transcription [61,62]. Consistent with this idea, a study, which demonstrated a TRIF and MyD88 dependent *Rorc* inhibition mechanism, also showed opposing expression levels of *Foxp3* and *Rorc* [65].

We also demonstrated an increased amount of IL-17 expression CD4+ T cell in mLN from DSS-treated mice suggesting a specific development antigen-specific Th17 cells that might become relevant in a more chronic inflammation. The findings of Melgar and co-workers [59] that the IL-17 production in the colon progressively increased during a chronic DSS colitis model, suggests IL17 expressing T cells may play a role in the chronic phase of colitis development.

5. Conclusion

We provide an extensive overview of the expression of *Tlr1–9*, *Nod1* and *Nod2* in both healthy and inflamed colon tissue. Though, studies in murine models of disease can never replace direct studies within patient tissues, this work still provides valuable fundamental information regarding PRR expression in the colon. This information will help us, and others, to determine the precise role of different PRRs during the development and progression of inflammation within the colon along with providing valuable information regarding the suitability of TLRs or NOD receptors as drug targets within IBD.

Conflict of interest

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