Microbiome manipulation in the management of acute and chronic colitis

A preclinical research approach

Bin Zheng

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Microbiome manipulation in the management of acute and chronic colitis

A preclinical research approach

Beïnvloeding van het microbioom als behandel-ing voor colitis

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(met een samenvatting in het Nederlands)

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Contents

Chapter 1

GENERAL INTRODUCTION

Inflammatory Bowel disease (IBD) which includes Crohn's disease (CD) and ulcerative colitis (UC) is a chronic inflammatory disorder of the gastrointestinal tract characterized by remissions and relapses. The symptoms of IBD include amongst others rectal bleeding, diarrhea, abdominal pain and weight loss [1]. Although individuals of any age can develop IBD, the onset of the disease generally happens in early adult life and last throughout the whole life. The peak age for CD is between 20 and 30 years and for UC between 30 and 40 years. Regarding the location of inflammation, UC is generally limited to the colon, while CD can involve any part of gastrointestinal tract in a non-continuous manner [2]. IBD is a disease that has a large impact on both the social and personal life, since it dramatically reduces the quality of life and work capability of the patients. The last fifty years, both the incidence and prevalence of IBD are increasing worldwide and this increase is more than 10 fold in Western countries within this time frame [3, 4]. In addition, studies examining the epidemiology of childhood-onset IBD indicate that the incidence in young kids is also rising internationally [5].

Despites the increased demand for an improved IBD therapy, a curative therapy for this disease remains absence. The current therapy consists of antibiotics to eliminate the infections, aminosalicylate or corticosteroids to reduce the inflammation, immune suppressing drugs and biologicals against specific targets (anti-TNFα etc.). However, these drugs have either very little efficacy [6] or other limitation like not suitable for long-term use and unwanted side effects [7, 8]. Hence, a new IBD therapy is needed, which suppresses or cures IBD symptoms without serious side effects and induces long-lasting remission.

IBD pathogenesis

Although the etiology of IBD has been extensively studied during the last decades, the underlying mechanisms and the exact triggers remain unclear. The current knowledge of IBD pathogenesis is obtained from genome wide association studies, clinical research and preclinical studies using animal models. From these data it is likely that aberrant activation or impaired down regulation of innate immunity, and hyperactive adaptive immune responses against commensal enteric bacteria within genetically susceptible hosts might contribute to IBD. Moreover, the onset or reactivation might be triggered by extern environmental factors such as diet change, infections or even psychological stress.

Genetic studies highlight the importance of the host-microbe interaction [9]. Disturbance of the interaction such as defects in recognition pathways or aberrant regulation of innate and/or adaptive immunity could contribute to development of the disease. In literature, multiple variants of genes belong to pathogen recognition signalling pathway (*Nucleotide-binding oligomerization domain-containing protein 2(NOD2)* [10, 11]*, Toll like receptors (TLRs)* [12-16]*)*, the interleukin (IL)-23/Thelper cell (Th)17 pathway (*IL23R* [9, 17]) and regulatory T cell (Treg) pathway (*IL10* [18, 19]) have been reported to be associated with IBD development.

Clinical studies and animal studies confirmed the genetic data. Dendritic cells (DCs) derived from CD patients have a decreased autophagy activity, resulting in reduced bacterial cleaning and impaired antigen presenting. An increased bacterial load and incorrect immune priming can trigger the development of IBD [20]. Aberrant expression of TLRs has been found in the intestine of IBD patients as well, though the exact role of TLRs is still not fully understood [21-23]. Using animal models, TLR-mediated epithelial barrier regulation [24] and T cell modulation [25] mechanisms are described to contribute to IBD development. An inaccurate T cell response is the major player in IBD development. The classic T cell subsets, Th1- and Th2-mediated immune responses were suggested to be responsible at least in part for the extensive inflammation and tissue damage that manifests in IBD patients [26, 27]. However, there is increasing evidence that Th17 cells, which are characterized by IL17 cytokine production, are the third CD4+ T cell lineage that contributes to the pathogenesis [26, 28]. Treg cells are known to have the unique regulatory function by suppressing the activity of other T cell subsets and induce immune tolerance [29]. IBD genome wide association studies as well as animal models, point to the role of abnormal Treg cells in number as well as in function during the development of IBD which may cause the abnormal and extensive immune response towards commensal intestinal bacteria resulting in inflammation [30].

Animal models mimicking IBD

Although animal models cannot represent the human IBD disease totally, they are useful tools to investigate IBD pathogenesis. At this moment, more than 60 different animal models have been established and can be classified into chemical induced, immune cell transferred and genetic modulated models [31]. Disadvantages of immune cell transferred and genetic modulated animal models is that they are either immune-compromised or genetic modified with unknown side effects. In contrast, chemical-induced colitis models are restricted from these side effects.

Among the chemical-induced animal models, DSS is by far the most commonly used due to its simplicity and the capability to induce predictable intestinal inflammation [32, 33]. DSS-induced colitis has been considered as an erosive, self-limiting model of acute colonic inflammation that is only driven as far as we know by innate immune responses [34]. However, a recent study demonstrated that bacteria penetrated the inner mucosa layer before inflammation in the acute colitis model [35]. Triggering of host immunity by the penetrated bacteria can activate the resident innate immune cells and subsequently lead to adaptive immune responses in which antigen-specific T cells are involved. Furthermore, single DSS exposure induced acute colitis can develop into chronic colitis that is accompanied by a progressive production of Th1 and Th17 cell-associated cytokines [36]. However, chronic colitis models are probably more representative using repeated DSS exposure cycles interspersed with recovery periods since it mimics the remission-relapse course of IBD after disease onset [37].

Pathogen recognition receptors (PRRs)

The interaction between the intestinal microbiota and host immune system is a critical factor for IBD pathogenesis. The key process is the recognition of microbes by PRRs: all immune responses start with recognition of the pathogen.

TLRs are the best studied PRRs that sense a broad spectrum of invading pathogens by recognizing pathogen-associated molecular patterns (PAMPs). Up to date, 13 TLRs have been found; TLR1 - TLR10 in human, Tlr1 - Tlr9 and Tlr11 - Tlr13 in mice. Among these TLRs, TLR1 - TLR9 are conserved in both human and mice. Depending on subcellular location, TLR1-TLR9 could be divided into two groups, cell surface TLRs and intracellular TLRs. The cell surface TLRs recognize PAMPs that are mainly belonging to bacterial cell wall components or on the bacterial cell surface, such as lipopeptides and peptidoglycal (TLR1/TLR2, TLR2/TR6), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5). In contrast, the intracellular TLRs mainly recognize microbial nucleic acid including viral double-strand RNAs (TLR3), single-strand RNAs (TLR7 and TLR8) and CpG ODN (TLR9) [38]. TLR activation induces a signalling cascades that are either myeloid differentiation primary response gene 88 (MyD88) dependent or TIR-domain-containing adapter-inducing interferon- β (TRIF) dependent [38].

The MyD88-dependent pathway is required for all TLRs except TLR3, and lead to activation of NFκβ resulting in production of proinflammatory cytokines such as tumor necrosis factor (TNFα), IL1β and IL6 [38, 39]. The TRIF dependent pathway is activated by TLR3 and endocytosed TLR4 ligation, leading to the production of type I interferon (IFN) [38]

NOD-like receptors are another family of PRRs. NOD1 and NOD2 are two members of this PRR family that is exclusively located in cytosol and are capable of recognizing distinct peptidoglycan fragment [40, 41]. Activation of these receptors induces the production of proinflammatory cytokines and antimicrobial molecules that provide the protective defence for the host [42, 43].

Although the PRRs play an important role in host defense system against pathogens, aberrant activation leads to extensive inflammation and tissue damage. Hence it is important to tightly control the signaling pathway induced by these PRRs in the intestine, since they are continuously exposed to an enormous load of intestinal bacteria. A selected number of the PRRs are expressed in endosomes (TLR3, TLR7-TLR9) and the basolateral membrane (TLR5 and TLR9) of epithelial cells, where they are not exposed to the bacteria load. Despite these specific expression locations, negative regulation mechanisms provide extra control for regulated activation of the PRRs signaling pathway. Limited data are available regarding the negative regulation mechanisms. The known negative regulation mechanisms includes the production of inhibitors such as peroxisome proliferator-activated receptor γ (PPARγ), Tumor necrosis factor alpha–induced protein 3 (A20) and Toll interacting protein (Tollip); production of adaptor protein variant, which lead to dissociation of the downstream adaptor complex; production of soluble PRRs that will sequester the available ligands [44, 45]. In addition, though the inhibition mechanism is still unknown, a rapid inhibition of TLR5 signaling has been observed after prolonged flagellin-induced activation [46].

IBD and PRRs

As the host immune system - intestinal microbiota interaction is central in the pathogenesis of IBD, dysregulated PRRs signaling pathways might contribute to IBD developement.

PRR gene knockout mice provided important data regarding their contribution to intestinal homeostasis and colitis developement. TLR5 deficient mice develop spontaneous colits [47]. Depletion of TLR2 and TLR4 increases susceptibility to DSS colitis [24, 48]. Interestingly, TLR4 deficiency could both exacerbate and ameliorate $IL10^{-/-}$ mediated colitis in mice. TLR9 deficient mice increase susceptibility in DSS-induced acute colitis, but are resistance to chronic DSS colitis [49, 50]. The *NOD2* mutant was the first genetic variant that was associated with the susceptibility of IBD [11]. Mice with genetic $Nod2^{-/-}$ are more susceptible to TNBS induced colitis [51].

IBD and Probiotics

The human gut content is a unique microenvironment where a large population of microorganisms coexist in close contact with the host immune system and gut epithelial cells. The balance within the bacteria community and the interaction between the intestinal microbiota and host immunity are critical factors for IBD pathogenesis, though the underlying mechanisms are relative unclear [52]. Dysbiosis or altered intestinal microbiota is associated with IBD pathogenesis [53]. This leads to the hypothesis that remodelling of the microbiomal composition can reverse the development of IBD. Antibiotics recognized as an established treatment for IBD disease support this hypothesis [54]. In addition, CD patients achieved clinical and histological remission after fecal microbiota transplantation [55].

Probiotics, defined as “live microorganisms which when administrated in adequate amounts confer a healthy benefit for the host” have the capability to remodel the microbiota balance, which makes it a potential candidate to prevent or treat IBD. The gut-derived bacteria from the genera *Lactobacillus* and *Bifidobaterium* are the most studied probiotics. Data from human cell cultures and animal studies indicate that probiotics carry out their effects on three different manners [56]. Firstly, probiotics promote competitive exclusion of pathogenic bacteria by either directly inhibit their activity or through influence of probiotics on the composition of intestinal microbiota. Secondly, certain probiotic strains maintain and enhance the epithelial barrier function by an increased production of defensin [57] and tight junction proteins [58], and preventing apoptosis of the epithelial cells [59]. Thirdly, probiotics have the capability to modulate both the innate and adaptive intestinal immune system [60]. Since PRRs are the initial step of the immune regulation response. PRRs are suspected to be involved in the probiotic-mediated immune modulation. For example, activation of TLR2 and TLR9 was shown to be important for the cytokine induction capability of two specific probiotic strains derived from the genera *Lactobacillus* and *Bifidobaterium*

[61]. Treatment with probiotic mix VSL#3 was reported to damp TLR4-induced human DC activation [62]. Using human cell culture system, it has been show that gut-derived bacteria were able to modulate T cell polarization in peripheral blood mononuclear cell (PBMC) by inducing different T-cell subsets in a strain dependent manner [63, 64]. Moreover, two independent clinical studies, using two different *Bifidobacteria* strains, have demonstrated their immune modulating capacities by both enhancing the transforming growth factor β (TGFβ) signaling and increasing peripheral Treg cells numbers [65, 66].

Aim and outline of this thesis

This thesis aims to provide new insight in the role of PRRs during the development of IBD by using animal models for acute and chronic colitis. In addition, selected probiotic strains that can modulate the intestinal microbiome were applied to investigate the possible effects on inflammation and tissue damage in acute and chronic colitis.

In **chapter 2**, an extensive survey of the expression of Tlr1–9, Nod1 and Nod2 in both healthy and inflamed colon tissue in acute colitis is provided. In addition, the changes in PRR mRNA expressions are correlated with mRNA expression of cytokines and transcription factors representing different T-helper and regulatory T cell populations is analyzed simultaneously.

In **chapter 3**, our aim was to determine if antigen-specific T cells could be induced during acute DSS colitis and whether they could be detected after disease resolution. To induce antigen-specific T cells, the tracking antigen, ovalbumin (OVA), was administered orally during colitis initiation. Disease severity was monitored, and the antigen-reactivity of CD4+ T cells was examined. The results indicate that during acute DSS colitis T cells develop that are specific against oral antigens and that they are found systemically after colitis resolution.

In **chapter 4**, the role of TLR6 stimulation in inducing a Th1 and Th17 response is examined by using an animal model for acute colitis, human PBMC and human biopsy samples. All data indicate that stimulation of TLR6 promote Th1 and Th17 responses, which is thought to mediate the development of IBD, in particular CD [26]. The effects of *L. rhamnosus* and *B. breve* before and during acute DSS colitis are described in **chapter 5**. While *L. rhamnosus* had little effect on disease severity, *B. breve* ameliorated DSS-induced colitis, increased Treg-and Th2-associated responses and locally reduced Th17 cells. In **chapter 6**, the mRNA expression of Tlr1–9, Nod1 and Nod2 in both healthy and inflamed colon was investigated in the chronic DSS colitis model. In addition, mRNA expression of cytokines and transcription factors representing different T-helper cell populations has been described. In **chapter 7**, the therapeutic effects of *L. rhamnosus* and *B. breve* in the chronic model of DSS are presented. Different effects of both probiotic strains were observed during remission and chronic phase of colitis in mice. In **chapter 8**, a general summary and discussion of this thesis is given.

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Chapter 2

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.

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 triacy- 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, PAM3Cys-SK4 [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.

Materials and Methods

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.

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, 2 = loss of form/diarrhea) and fecal bleeding (0 = no blood, 1 = blood observation using Colo-rectal Test kit (Axon Lab AG, Germany), 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, 4: infiltration in entire tissue); cover area of cellular infiltration in the region (0: no infiltration, 1: < 25%, 2: 25%-50%, 3: 50%-75%, 4: >75%); loss of crypts (0: no damage, 1: 30% shortening of crypts, 2: 65% shorting of crypts, 3: total loss of crypts, 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 the section, "Immunohistochemical staining".

Immunohistochemical staining

For immunohistochemical staining, 5-µm-thick sections were subjected to a heat-induced epitope retrieval step. Slides were washed with 1x 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, Cambrige, UK), TLR4 (ab47093, Abcam, Cambrige, UK), TLR5 (ab62460, Abcam, Cambrige, 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 EliteABC 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 (ab 37261, Abcam, Cambrige, UK) or rabbit (ab27472, Abcam, Cambrige, UK) IgG antibody.

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 sythesis kit (BioRad, Hercules, CA USA). Real-time PCR was performed using iQ SYBR Green super mix kit (BioRad, 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*.

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 hours in wells coated with anti-CD3.

Table 1. qPCR primer sequence

	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	CACTGGGCTTCTGGGTATGT	AGACAGGCCAGGGGATAGTT
Rorc	TGCAAGACTCATCGACAAGG	AGGGGATTCAACATCAGTGC
Rps13	GTCCGAAAGCACCTTGAGAG	AGCAGAGGCTGTGGATGACT

After stimulation, cells were incubated for 4 hours 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 Biosciencse, Franklin Lakes, NJ USA). Intracellular cytokine staining was analyzed using BD FACSDiva software (BD Biosciences)

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.

Results

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

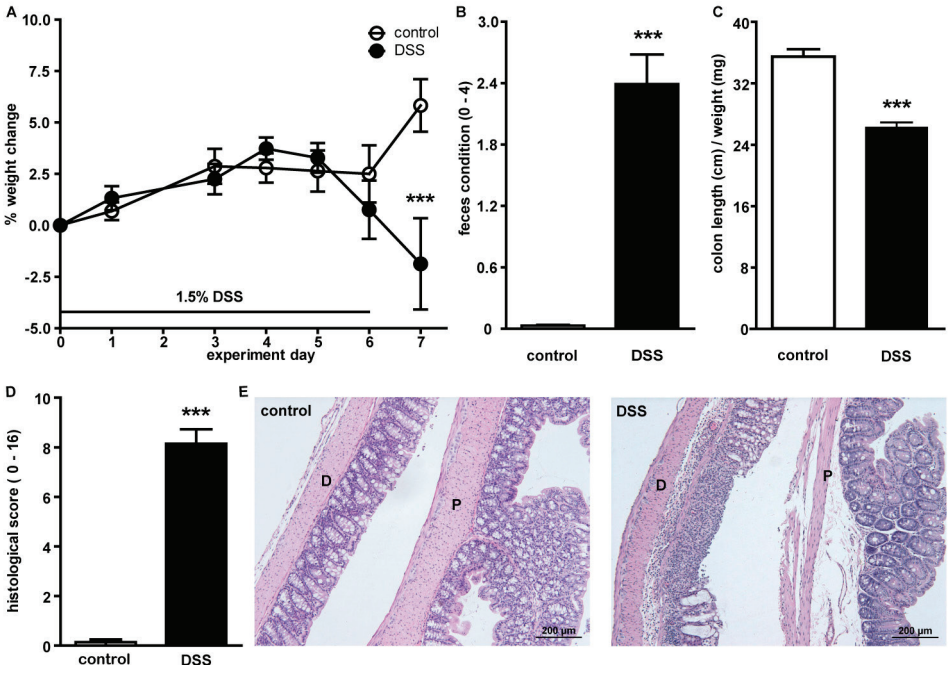

Figure 1. DSS treatment induces colitis in mice.

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 < 0.001. (D) Histological quantification of inflammatory markers was determined within colons deried 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.

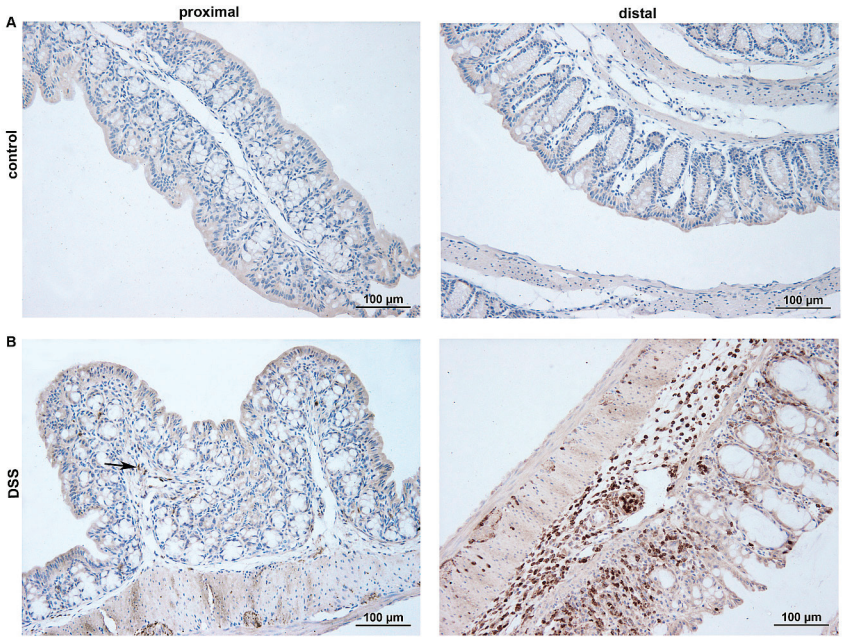

Figure 2. Massive Ly-6B+ cell influx in the distal colon region of DSS-treated mice.

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.

treated group (Fig.1A and 1B). 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 region of the colon and included complete loss of crypt structure and submucosal cell infiltration. The proximal region, in contrast, remained generally unscathed (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 2B). Thus, the inflammation detected in the colon of our DSS-treated mice conformed to the generally accepted pathology of DSS-induced colitis.

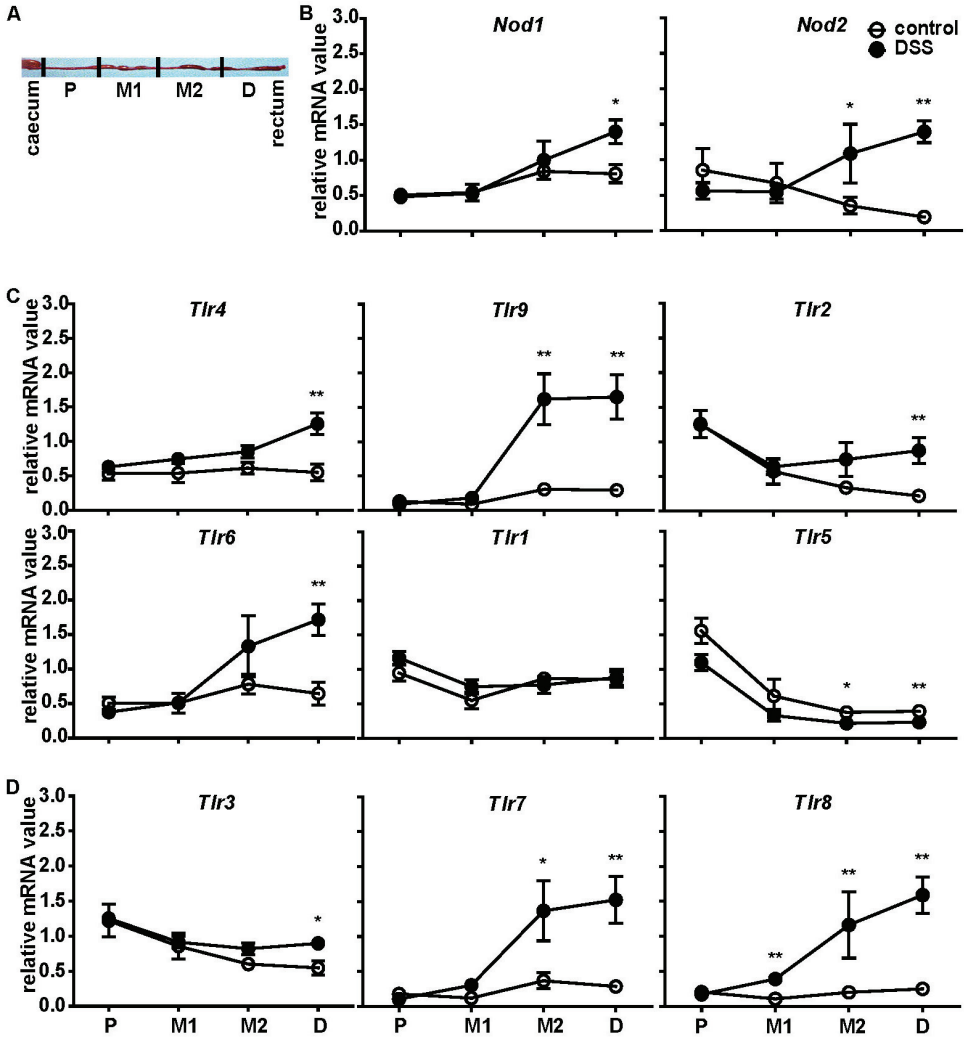

Figure 3. The mRNA expression of PRRs is differentially modulated in the colons of DSS-treated mice.

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, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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.

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.

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.

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).

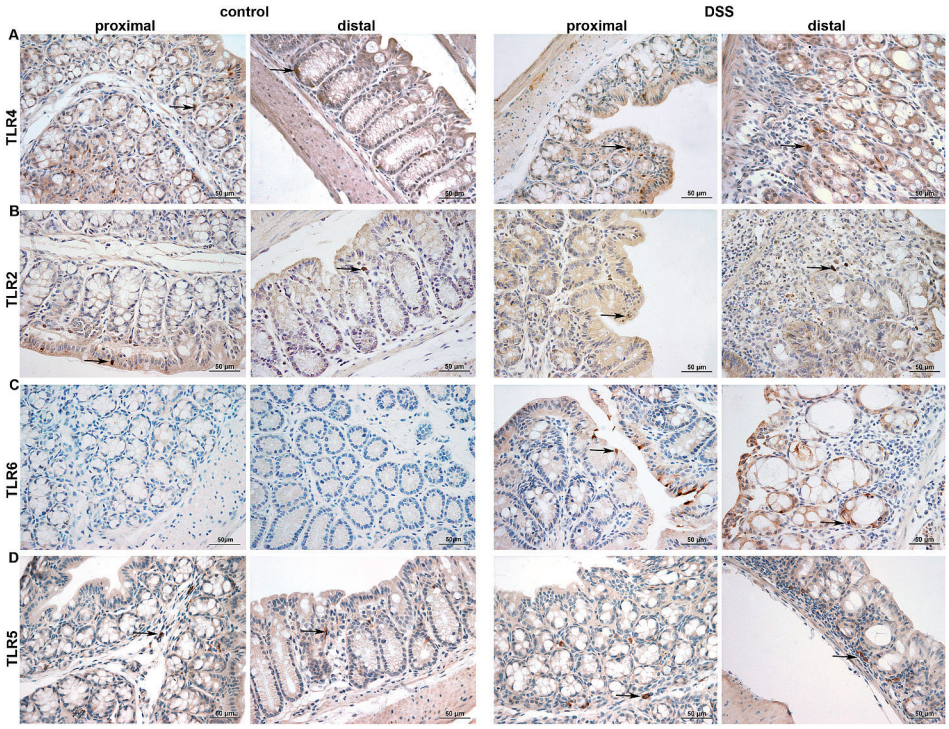

Figure 4. Expression of TLR2, 4, 5 and 6 within the colon.

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.

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.

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

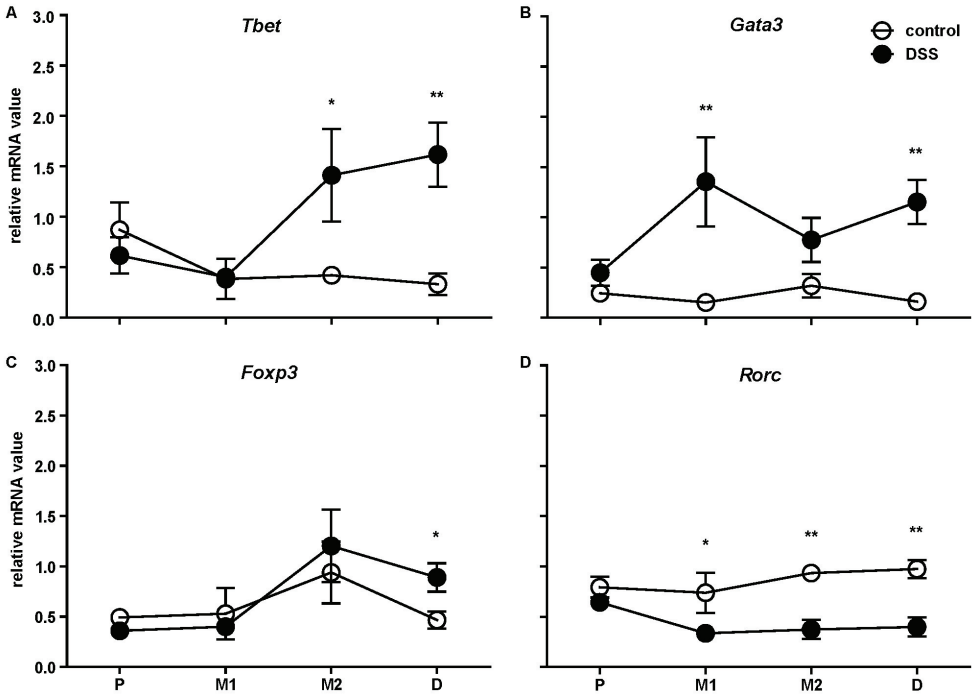

Figure 5. Colitis leads to modulations in the transcription activity of T-cell associated transcription factors.

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 ± SEM, n = 6 mice per group, pooled from two independent experiments, *P < 0.05; **P < 0.01; *** P < 0.001.

(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 5C). 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).

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

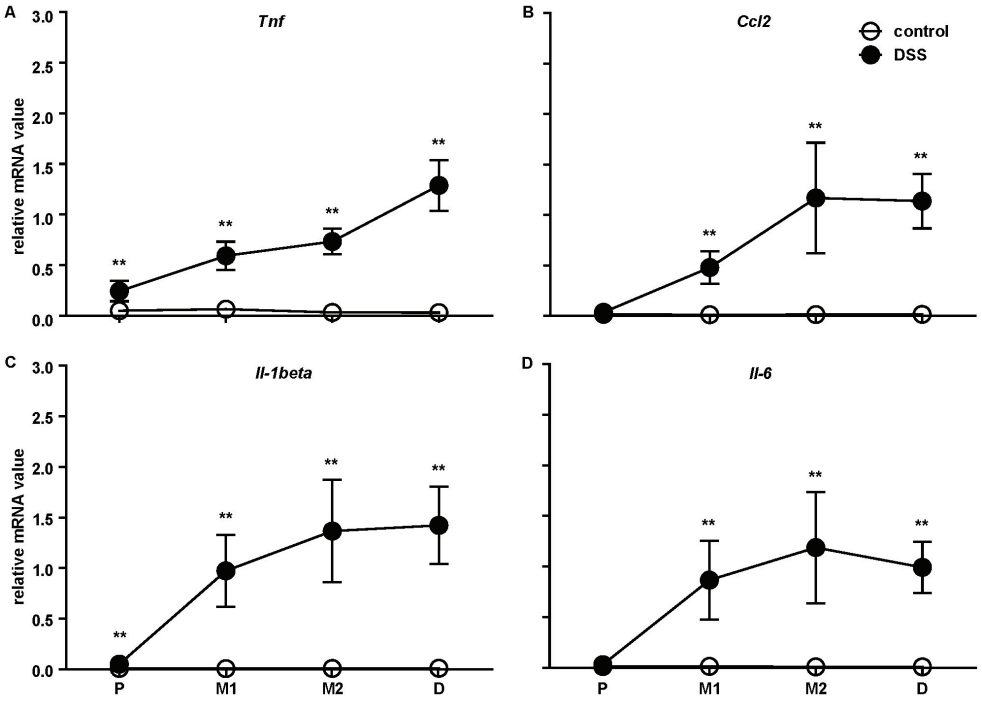

Figure 6. Modulated PRR expression is coupled with increased mRNA transcripts of pro-inflammatory cytokines in DSS-induced colitis.

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.

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-6D). 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. 7B). 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. 7C) [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).

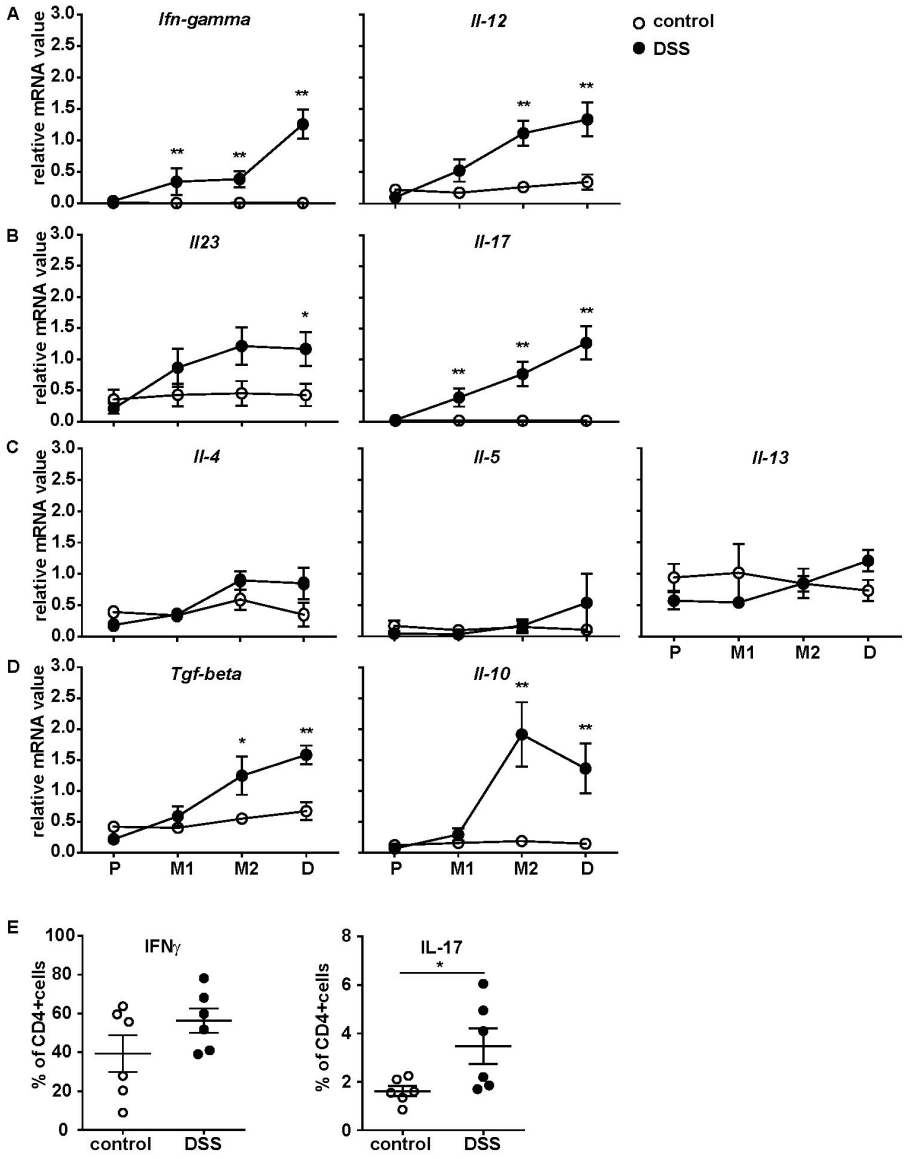

Fig. 7. Modulation of PRR expression is coupled with increased Th1/Th17 cell-associated cytokine production.

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.

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.

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.

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 colons. 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 gut-associated lymphoid tissue also leads to increased Th17 polarization (chapter 4), 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. 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 result 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 I pathway [54-56]. Dendritic cells express high levels of TLR7 and TLR9 and are producers of type I 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 I IFN in the intestinal tract of mice and reduces DSS-induced colitis [55]. Analysis of type I 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 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 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 induced 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 led 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*, which are associated with the differentiation of Th1 cells. In addition, the percentage of IFNγ+CD4+ T cells tended to be higher in mLN obtained from mice undergoing DSS colitis.

Previously, an enhanced expression of 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 colon is, at the moment, not clear. However, it has been shown that conditions, which favor Treg, naturally antagonize Th17 polarization [60-62]. 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 coworkers [59] that the IL-17 production in the colon progressively increased during a chronic DSS colitis model, suggests IL-17 expressing T cells may play a role in the chronic phase of colitis development.

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.

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Chapter 3

NEW PERSPECTIVE ON DEXTRAN SODIUM SULFATE COLITIS: ANTIGEN-SPECIFIC T CELL DEVELOPMENT DURING INTESTINAL INFLAMMATION

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Abstract

CD4+ T cell responses against oral antigens can develop in inflammatory bowel disease (IBD) patients, which may modulate disease. Dextran sodium sulfate (DSS) colitis is commonly used to study IBD, however, it is not considered the best model in which to study T cell involvement in intestinal disease. Our aim was to determine if antigen-specific T cells could be induced during DSS colitis and if they could be detected after disease resolution. To induce antigen-specific T cells, the tracking antigen, ovalbumin (OVA), was administered orally during colitis initiation. Disease severity was monitored, and the antigen-reactivity of CD4+ T cells examined using CD69 expression. While OVA-directed, CD4+ Foxp3+ regulatory T cells could be detected in the spleens of both OVA-treated control and DSS mice, OVA-reactive, CD4+ Foxp3-T cells were only found in the OVA and DSS-treated mice. These results indicate that during DSS colitis T cells develop that are specific against oral antigens, and they are found systemically after colitis resolution. This gives added depth and utility to the DSS model as well as a way to track T cells that are primed against luminal antigens.

Introduction

Inflammatory bowel disease (IBD) consists of several chronic inflammatory diseases of the gastrointestinal tract of which Crohn's Disease (CD) and Ulcerative colitis (UC) are the most prevalent. The etiology is largely unknown, but a widely recognized hallmark is abnormal T cell responses towards intestinal bacteria [1]. CD4+ T cells that are responsive to CBir1 (flagellin), oral antigens, enterobacteria and commensal flora [2–6] have been detected. The pathogenicity of these CD4+ T cells has been confirmed in severe combined immunodeficient (SCID) mice after T cell transfer [5,7], and it has been demonstrated that microbiota-specific effector T cells generated during gastrointestinal inflammation are long-lived giving them the potential to lead to chronic inflammation [8]. Furthermore, two of the most widely used drugs for IBD, tumor necrosis factor inhibitors and azathioprine, work, at least in part, via mechanisms that suppress T cell responses [9,10]

A role for T cells in IBD is further supported by genome-wide association studies, which show that T helper type 17 (Th17) cells and regulatory T cells (Tregs) are important for both UC and CD [11]. Th17 cells recruit and stimulate neutrophils via activation of local tissues using interleukin (IL)-17A and IL-17F, and Tregs regulate effector T cells through a variety of mechanisms, both cell-contact dependent and independent, to prevent autoimmunity and maintain peripheral tolerance [1]. The presence of high amounts of Th17 cells and Th17 cellderived cytokines in the inflamed colon tissue of IBD patients underscores their likely contribution to intestinal inflammation [12].

The possibility of treating IBD by interfering with the development of pathological T cells is enticing. To specifically target T cells, knowledge about their antigen-specificity would be useful as well as information about the triggers that lead to their development. To study adaptive immune responses within colitis, the T cell transfer model of colitis is preferred [13]. In this model, naïve T cells are transferred to an immune compromised host. The caveat of this model is that it relies on a genetically compromised host and an abnormal imbalance of naïve and regulatory T cells that is not found in wild type animals. This model, thus, does not give insight into the immunological processes behind the development of pathological T cells in an, otherwise, healthy animal.

The dextran sodium sulfate (DSS) model of colitis, in contrast to the T cell transfer model, is a robust model of colitis induced by administering dissolved DSS in the drinking water and is inducible in all backgrounds of mice [14]. It also responds to many drugs used to treat IBD, making it highly representative of IBD [15].

DSS is often considered a toxicity model as *in vitro* studies testing the effects of DSS on epithelial cell lines show that direct exposure causes the cell cycle arrest of epithelial cells [16]. However, these *in vitro* studies did not take into account the role of the mucus layer found in *in vivo* conditions. It is now known that DSS causes intestinal mucus to become permeable to bacteria and possibly to other luminal antigens. This would allow bacteria to come into contact with the epithelial layer below [17] and with the transepithelial dendrites of antigenseeking dendritic cells [18]. This would suggest that the DSS model, instead

of being purely a toxicity model, is also modeling mucus loss and the eventual bacterial penetration found during intestinal trauma.

The fact that acute DSS colitis can be induced without the help of T cells, using purely the innate immune system [19], has made it a poor candidate for T cell research. However, it is known that an adaptive immune response does develop, and T cells accumulate at the site of inflammation [20]. Furthermore, certain mouse strains (including C57Bl/6) develop long-term chronic inflammation characterized by substantial neutrophil infiltration that does not subside [21,22]. This suggests a possible role for T cells that do develop during the DSS-induced acute inflammation.

Very little specific research regarding T cell development in the model exists. As it has not been previously published if antigen-specific T cells develop in mice experiencing DSS colitis, our aim was to investigate if CD4+ T cells directed against oral antigens could be found after the resolution of colitis. We found that while healthy mice developed CD4+ T cells that were reactive against the tracking antigen, ovalbumin (OVA), in only the Treg (CD4+ Foxp3+) population, DSStreated mice developed reactive CD4+ T cells in both the conventional T cell population (CD4+ Foxp3-) and the Treg population. This demonstrates that potentially proinflammatory, antigen-specific CD4+ T cells do develop during DSS colitis and that they can be tracked, making the DSS model more useful for T cell research.

Materials and Methods

Ethics statement

All experiments were performed in accordance with the guidelines issued by the Dutch ethics committee for animal studies. The protocol was specifically approved by the ethics committee for animal studies of Utrecht University (DEC Number: 2008.II.06.051 and 2010. II.01.013). All efforts were made to minimize suffering.

Animals

Female C57BL/6 mice and OTII transgenic 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.

Experimental colitis

Experimental colitis was induced in mice by adding 1.5% (w/v) DSS (MP Biomedicals LLC, Illkirch, France) to the drinking water of the mice for 6 days. Mice were sacrificed on either day 7 or 14 after starting DSS, depending on the experiment. Exposure to OVA to develop OVA-directed T cells was accomplished by adding OVA (Sigma-Aldrich, St. Louis, MO USA) to the drinking water (140 µg/ml) during DSS administration.

The Disease Activity Index (DAI) of colitis used in this manuscript is an adaptation of the method introduced by Cooper et al. [22]. It was determined by combining the scores collected from the weight measurement, feces condition and the detectable presence of blood in the feces leading to a score between 0-8 for each mouse. The loss of weight was scored as follows relative to starting weight: 0 = no weight loss, 1 = 15% weight loss. The feces condition score was scored as follows: 0 = normal, 1 = soft with normal form, 2 = loss of form/diarrhea and 3 = no feces produced. Fecal blood was tested with a Colo-rectal test kit (Axon Lab AG, Stuttgart, Germany). Fecal blood was scored as follows: 0 = no blood, 1 = blood.

Histological evaluation of colon damage and immunohistochemistry

On day 7 or day 14 (1 day and 8 days after the end of the DSS cycle respectively), colons were excised between the cecum and the rectum and were prepared for histological evaluation. The colon was opened longitudinally, washed in phosphate buffered saline (PBS), placed on a piece of blotting paper, fixed in 10% formalin for 24 hours, routinely embedded in paraffin as swiss-roles and sectioned (5 µm). The damage and infiltration for samples collected on day 14 were blindly assessed after staining sections with H&E. 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) and combined for a final histological score. Assessments included four pathological criteria: the severity of cellular infiltration (0 = no infiltration, 1 = infiltration between the crypts, 2 = infiltration in the submucosa, 3 = infiltration in the muscularis externa, 4 = infiltration in entire tissue); the extent of cellular infiltration in the region (0 = no infiltration in the region, 1 = < 25%, 2 = 25%-50%, 3 = 50%-75%, 4 = >75%); percent loss of crypts (0 = no damage, 1 = 30% shortening of crypts, 2 = 65% shorting of crypts, 3 = total loss of crypts, 4 = loss of entire epithelial layer) and the extent of crypt loss (0 = no crypt loss, 1 = < 25%, 2 = 25%-50%, 3 = 50%-75%, 4 = > 75%).

For the immunohistochemical T cell staining, colons collected from mice on day 7 were used. Colon sections (5 µm) were deparafinized, dehydrated and treated for endogenous peroxidase activity by incubating with 0.03% H_2O_2 in methanol for 30 minutes. Antigen retrieval was performed by boiling the slides for 15 min in a 10mM Tris/1mM EDTA, pH 9.0 buffer. After cooling and being rinsed three times with PBS, the slides were blocked with 5% goat serum (Dakocytomation, Glustrup, Denmark) in 1% bovine serum albumin (BSA) in PBS for 20 minutes at room temperature. Afterwards, the sections were incubated overnight with anti-CD3 antibodies (Dakocytomation) in 1% BSA/PBS for 45 minutes. After thoroughly washing with PBS, the slides were incubated with biotinylated goat-antirabbit antibodies (Dakocytomation) and streptavidinhorseradish peroxidase (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA USA) for 45 minutes at room temeperature. Specific binding was detected by incubating the sections with 0.05% diaminobenzidine-tetrahydrochloride (Sigma-Aldrich, St. Louis, MO USA)/0.015% H_2O_2 /0.01 M TrisHCL, pH

7.6 for 10 minutes resulting in a brown staining product. Sections were counterstained with Mayers' haematoxylin (Merck, Darmstadt, Germany), dehydrated and mounted. Slides without primary antibody incubations were included as negative controls.
Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera. Contrast of the H&E stained sections was improved and magnification bars were added using Adobe Photoshop CS5.

Isolation of lymphoid organs and colon cells

Mesenteric lymph nodes (mLNs) and spleens were excised from mice and kept on ice in PBS. To make single cell suspensions, the organs were crushed and the slurry was filtered using 70 µm filters to collect the single cells. The spleens were further purified by destroying the red blood cells by isotonic shock. Cell counts in mLN and spleen were determined using a Coulter Counter (Beckman Coulter, Woerden, the Netherlands). Cells were kept cold until use.
Colon mononuclear cells were isolated from both control and DSS-treated mice on day 7 by incubating the colons with a solution of 0.25 M EDTA for four 15-minute cycles; adding fresh solution after each cycle. The colon tissue was manually disrupted by forcing it through a 70 µm filter. All incubations were performed at 37°C. The mononuclear cells were then isolated by using a 40/90% percoll gradient. Colon mononuclear cells were kept cold until use.

Detection of memory T cells

Single cell suspensions were first washed with ice cold 1% BSA/PBS. To prevent background staining, cells were first incubated with unlabeled anti-CD16/32 (eBioscience, San Diego, CA USA) for 15 minutes on ice. Cell samples were then stained with antibodies specific for mouse CD3, CD4, CD62L, and CD44 (eBioscience) in the dark, on ice for 30 minutes. After washing with 1% BSA/PBS, the samples were measured with a BD FACSCanto™ II (BD Biosciences, Franklin Lakes, NJ USA). Analysis of the flow cytometry data was performed using BD FACSDiva software (BD Biosciences).

Intracellular cytokine staining

Single cell suspensions of mesenteric lymph nodes and spleens were cultured in Roswell Park Memorial Institute medium (RPMI, Life Technologies, Paisley, Scotland) supplemented with 1 unit/ml penicillin, 1 µg/ml streptomycin, 50 µM β-mercaptoethanol and 5% FCS in 96 well round-bottom plates at a concentration of 105 cells per well. Intracellular cytokine staining was performed on cells after 24 hours stimulation with purified anti-CD3 (eBioscience) at 2 µg/ml in the medium. Cells were then stained with antibodies for CD4, IFNγ and IL-17A (all antibodies from eBioscience) using the Foxp3 intracellular staining kit (eBioscience). To prevent background staining, cells were first incubated with unlabeled anti-CD16/32 (eBioscience) for 15 minutes on ice as suggested in the manufacturer's protocol. Fixed samples were kept at 4°C until reading with the flow cytometer. Samples were measured

using a BD FACSCanto II flow cytometer. Analysis of the FACS data was performed using BD FACSDiva software (BD Biosciences)

Serum amyloid A measurement

Serum was collected on day 14 via heart puncture and tested for serum amyloid A (SAA). The SAA ELISA was obtained from Invitrogen (Paisley, UK) and was used according the manufacturer's directions. The results were read using the Bio-Rad (Hercules, CA USA) iMark™ microplate reader and analyzed using Microplate Manager 6.1 software (Bio-Rad).

***In vivo* evaluation of antigen presentation**

OTII mice were sacrificed and the spleens were removed and kept on ice. Single cell suspensions of the spleens were prepared. The prepared splenocytes were then stained with the vital dye, carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Europe BV, Leiden, The Netherlands). For CFSE staining, cells were suspended in 1% BSA/PBS containing 0.5 µM CFSE and then incubated for 10 minutes at 37°C in the dark. Fetal calf serum (FCS) was then added until an end concentration of 5% was reached. Cells were then washed with 1% BSA/PBS two times before intravenous transfer to mice. 10 million cells were transferred per mouse. After transfer, colitis was induced in the recipient mice by adding DSS (1.5%) to the drinking water for 6 days. On the fifth day of DSS water, mice were administered 400 µg of endotoxin-free OVA (Hyglos GmBH, Bernried, Germany) in saline via oral gavage. Three days later, on day 8, mice were sacrificed and lymphoid organs were removed. *In vivo* proliferation of transferred OTII cells was measured by flow cytometry using a BD FACSCanto II flow cytometer (BD Biosciences). Removed lymphoid organs were prepared as cell suspensions and co-stained with fluorescently labeled anti-CD4 antibodies (eBioscience). Dividing cells were visualized by looking at the dilution of the CFSE intensity of CD4+ lymphocytes. Proliferation of transferred OTII cells was quantified by looking at the percent divided cells within the total CFSE+CD4+ population.

***Ex vivo* evaluation of T cell antigen-reactivity**

Mesenteric LNs and splenocytes were isolated from mice at day 14 of the experiment. Cells were re-stimulated with 25 µg/ml OVA (Sigma-Aldrich) or anti-CD3 (2 µg/ml; eBioscience) and cultured with RPMI medium supplemented with 1 unit/ml penicillin, 1 µg/ml streptomycin, 50 µM β-mercaptoethanol, and 5% FCS in 96 well round-bottom plates at a concentration of 105 cells per well. After 48 hours, cells were harvested and stained with fluorescently labeled antibodies. To prevent background staining, cells were first incubated with unlabeled anti-CD16/32 (eBioscience) for 15 minutes on ice. Cells were first stained extracellularly with anti-CD4 and anti-CD69 and then stained intracellularly for Foxp3. All antibodies and the Foxp3 intracellular staining reagents were obtained from eBioscience. Analysis of the flow cytometry data was performed using BD FACSDiva software (BD Biosciences).

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). Where appropriate, either the unpaired or paired student's T test or 1- way ANOVA with post-hoc test (Dunnett) were applied. P-values considered as significant are < 0.05.

Results

Acute DSS-induced colitis leads to increases in CD4+ central memory T cells

To learn more about the adaptive immune response during colitis, we induced acute DSS colitis in mice. As expected, the colitis symptoms peaked at 7 days after the start of DSS (Figure 1A), and the colons were significantly shortened (Figure 1B). Immunohistochemical staining for CD3 in the colons revealed that T cells collected in the inflamed areas of the

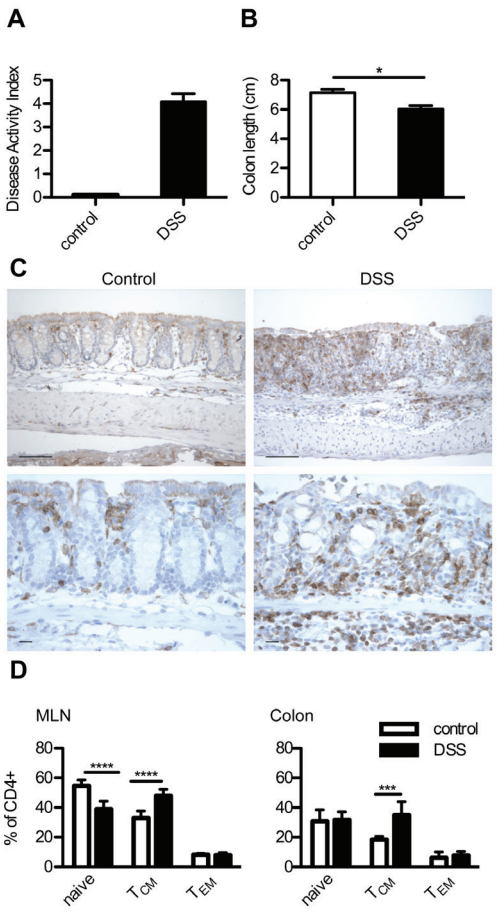

Figure 1. During colitis, T cells accumulate in the inflamed regions of the colon.

Mice were treated with DSS for 6 days and sacrificed on day 7. The mice displayed signs of colitis including (A) an increased Disease Activity Index (DAI) and (B) shortened colons. C) Immunohistochemical staining of CD3+ cells in colons obtained from both control (left panes) and DSS-treated (right panes) mice. Top panes are 200x (bar: 5 µm) and bottom panes are 400x magnification (bar: 1 µm). Increased CD3+ cells are observed in inflamed colons. D) Naïve (CD4+ CD62L+ CD44-), central memory (TCM, CD4+ CD62L+ CD44+) and effector memory (TEM, CD4+ CD62L- CD44+) T cells were measured in the mLNs and colon mononuclear cell suspensions using flow cytometry. Results are expressed as mean ± SEM, N = 4-6 mice per group.
***P < 0.001; **** P < 0.0001.

colon (Figure 1C). To characterize the activation states of the cells, flow cytometry was used to determine the relative percentages of naïve (CD4+ CD62L+ CD44-), central memory (TCM, CD4+ CD62L+ CD44+) and effector memory CD4+ T cells (TEM, CD4+ CD62L-CD44-) in colon mononuclear cells and mLN cells collected from healthy and DSS-treated mice. In the colon and the mLN, naïve CD4+ T cells were the most abundant, comprising 30%-60% of the CD4+ T cells. During inflammation, naïve T cells were significantly decreased in the mLN ($P < 0.0001$, Figure 1D). In healthy colons and mLNs, CD4+ TCM cells comprised 20%-40% of the population. During colitis, CD4+ T_{CM} cells increased significantly in both the colons ($P < 0.001$) and the mLNs ($P < 0.0001$). CD4+ T_{EM} cells, the least abundant population, comprised approximately 10% of CD4+ T cells (Figure 1D). Changes in the CD4+ T_{EM} cell population magnitude were not apparent in the colons or mLNs.

Th17 cells are detected in the spleen after resolution of DSS-induced colitis

As increased central memory T cells were formed as a result of DSS colitis, the possibility existed that pro-inflammatory T cells could be found in the lymphoid organs of DSS-treated mice after acute disease resolution. Thus, cell suspensions of isolated mLNs and spleens from control and DSS-treated mice were activated with anti-CD3. Intracellular cytokine staining for IFNγ, IL-17A, IL-4 and IL-10 was performed 24 hours later. Increased IL-10 and IL-4 producing CD4+ T cells were not observed (data not shown). However, CD4+ T cells isolated from spleens of DSS-treated mice produced more IL-17A as compared to healthy spleens after anti-CD3 stimulation ($P < 0.01$; Figure 2A and B). Although suggestively increased in both tissues, IFNγ was not significantly raised. This shows that after the resolution of acute colitis, during the chronic phase of DSS colitis, Th17 cells can be detected in the spleen.

Oral OVA is taken up from the colons from both healthy and DSS-treated mice

The development of antigen-specific T cells depends on the presence of antigen presenting cells. Oral antigen is taken up predominately by dendritic cells (DCs) in the intestinal tract and presented to T cells in the Peyer's patches and in the draining lymph nodes, such as the mLNs [23]. The efficiency of oral antigen presentation has not been investigated during DSS-induced colitis. To be certain that ingested OVA would be properly presented within healthy and inflamed intestinal tracts, CFSE-labeled OTII cells were adoptively transferred into mice two days before DSS induction. Transgenic OTII mice have CD4+ T cells with T cell receptors (TCRs) specific for an OVA epitope presented in the context of the murine MHC class II molecule, IA^b. Three days after oral exposure of OVA, both DSS-treated and healthy mice displayed expanded CD4+ T cells in the mLN (Figure 3A). The percentages of proliferated CFSE-labeled cells were significantly higher in mice given OVA than in the controls for both DSS-treated and healthy mice ($P < 0.01$ and $P < 0.05$ respectively, Figure 3B). This indicates that antigen-presenting cells in DSS-treated mice took up oral antigens in the gastrointestinal tract and efficiently presented them during inflammation in a manner similar to healthy mice.

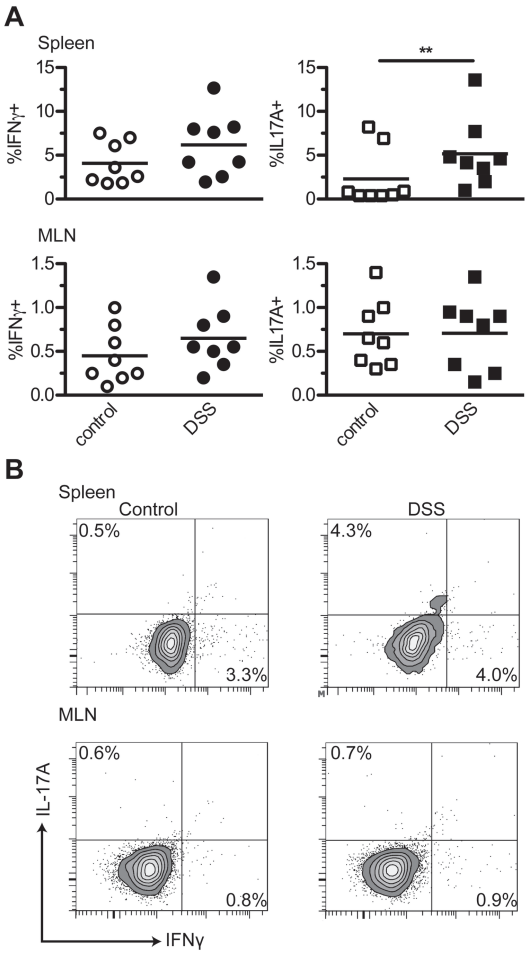

Figure 2. Th17 cells are detected in the spleen after colitis resolution.

IFNγ and IL-17A producing CD4+ T cells were detected in the spleens and mLNs, 14 days after the start of DSS using intracellular cytokine staining. A) Percentages depicted are the populations of cytokine expressing CD4+ cells within the total CD4+ population. Bars indicate the mean, N = 8 mice per group. ** $P < 0.01$. B) Representative FACS contour dot plots for spleen and mLN showing intracellular staining of IL-17A and IFNγ within the gated CD4+ T cell population. Percentages within CD4+ T cell population are shown.

To control for spontaneous proliferation of the OTII cells, CFSE positive cells were also examined in non-local lymph nodes (axillary lymph nodes), which would be less likely to come in contract with orally ingested antigen. T cell proliferation was not observed in the axillary lymph nodes (Figure 3C).

Oral OVA does not change clinical parameters of DSS-induced colitis

In IBD patients, responses to orally administered antigens are measured in the peripheral blood mononuclear cells, 1-2 weeks after the oral feeding of antigens [2]. To provide oral tracking antigens during DSS colitis, we administered OVA via the drinking water along with dissolved DSS for 6 days. The expectation was that oral OVA would not influence the clinical parameters. However, as oral tolerance induction against bystander antigens has been known to result in the amelioration of chronic inflammatory disease models in a phenomenon called bystander suppression [24, 25], we monitored the DSS colitis phenotype closely.

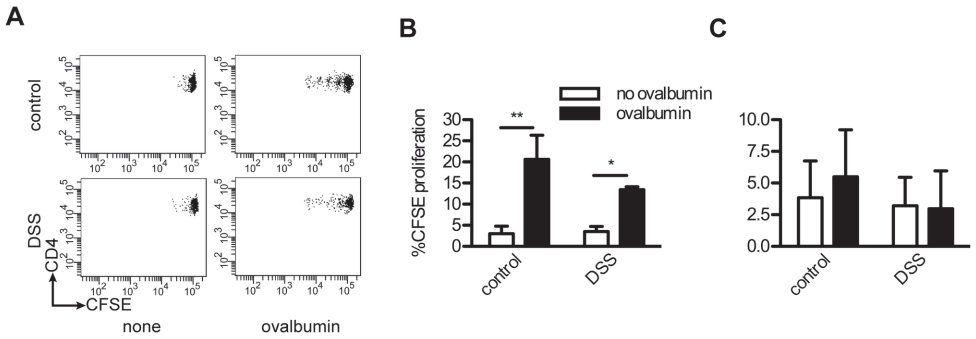

Figure 3. Oral antigens are presented in the draining lymph nodes of both healthy and DSS treated mice.

A) OVA presentation in the gastrointestinal tract was visualized by the proliferation of adoptively transferred, CFSE labeled, OTII T cells. Representative FACS dot plots displaying OTII T cell proliferation within the mLNs of both healthy and DSS-treated mice after oral gavage of saline, "no antigen" or oral gavage with OVA, "ovalbumin". The loss of CFSE intensity is an indication of dividing T cells. B) Percent proliferated OTII cells found within isolated mLNs. C) Percent proliferated OTII cells in the non-local, axillary lymph nodes. Results for (B) and (C) are expressed as mean ± SEM, N = 4 mice per group, pooled from two independent experiments. * $P < 0.05$; ** $P < 0.01$.

There was no indication that OVA treatment led to lessened or worsened colitis severity, as judged by the investigated clinical parameters (Figure 4A–G). The weight loss (Figure 4A) and DAI (Figure 4B) measured on day 6 and 13 were normal for the DSS colitis model despite OVA in the drinking water. The total amount of cells in the mLNs (Figure 4C) and spleens (Figure 4D) was similarly increased by DSS treatment in both OVA-treated and untreated animals during sacrifice on day 14. The acute phase protein, SAA, was found to be similarly increased in the day 14 sera from both DSS-treated groups compared to control groups (Figure 4E). Histological analyses of the colons revealed no obvious differences in gross damage or general cellular infiltration between the groups (Figure 4F and G). Both the OVA-treated DSS group and the untreated DSS group had colons with apparent cellular infiltrations, crypt damage and edema (Figure 4G). No differences were detected either clinically or microscopically as a result of adding OVA to the drinking water during DSS colitis.

Oral OVA does not change the CD4+ T cell subset composition

To determine if the general CD4+ T cell reactivity and subset composition had changed as a result of OVA and DSS in the drinking water at one week after disease resolution, the percentage of CD4+ T cells, the ratios of CD4+ Foxp3-/ CD4+Foxp3+ T cells and percentages of IL-17A+ and IFNγ+ T cells were determined in spleen and mLN cells after stimulation with anti-CD3. As shown in Figure 5A, administering OVA together with DSS did not change the percentage of CD4+ found in the samples as compared to DSS alone. Nor did it alter the ratios of CD4+ Foxp3- (conventional T cells)/CD4+ Foxp3+ (Tregs; Figure 5B). Moreover,

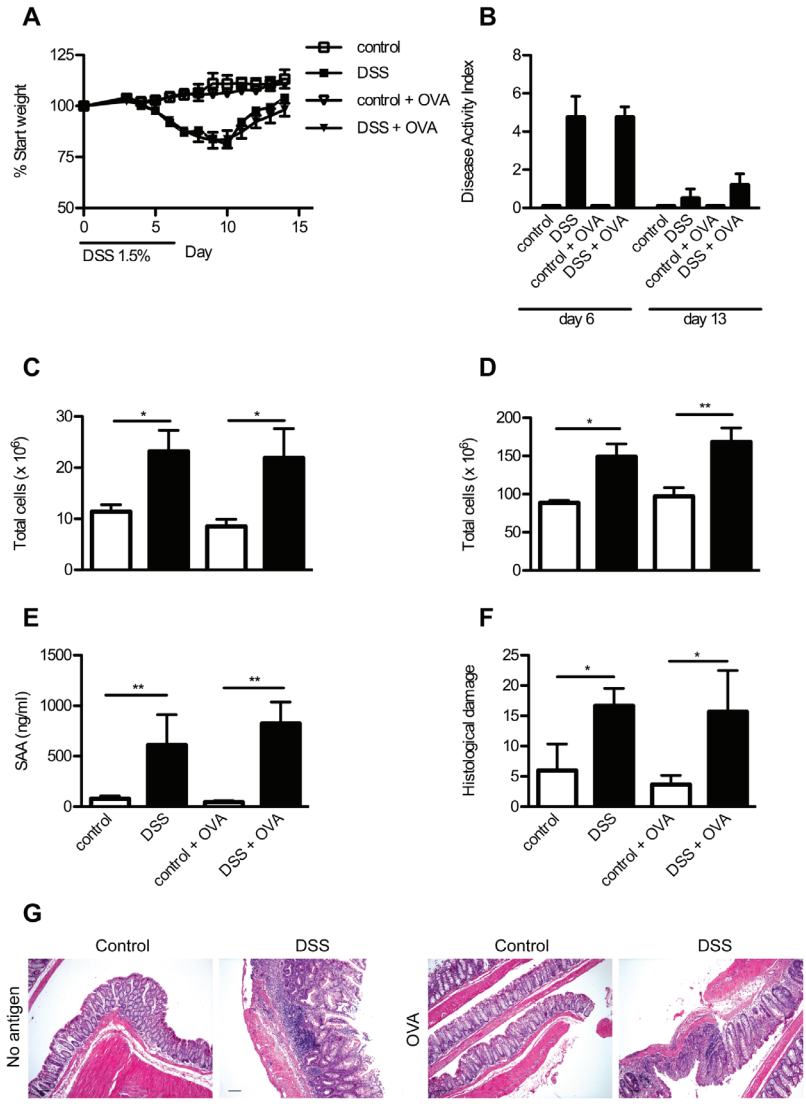

Figure 4. DSS and OVA treated mice have the same clinical phenotype as mice treated with DSS alone.

Mice were treated with both DSS and OVA for 6 days, and several clinical parameters were measured during disease progression and after sacrifice. A) Percent weight gain relative to starting weight was measured in each individual mouse for 14 days. B) DAI was calculated on day 6 and day 13 for all groups as described in the materials and methods. N = 10-15 mice before day 7, N = 5-10 after day 7, mice are pooled from two independent experiments. Mesenteric LNs C) and spleens D) were obtained from mice sacrificed on day 14. Cell suspensions were prepared and total cells counted in each organ. Samples (N = 5) were pooled from two independent experiments. E) SAA was measured in the serum from mice sacrificed on day 14. Samples (N = 5) were pooled from two independent experiments. F) Several colons collected from mice on day 14 were examined and scored for histological damage parameters as described in the materials and methods (N = 3, from a single experiment). G) Representative photos are shown for colonic sections from control, DSS-treated, OVA treated controls and OVA and DSS-treated mice. Bar is 5 µm. All graphical results are expressed as mean ± SEM in the bar graphs. * $P < 0.05$; ** $P < 0.01$.

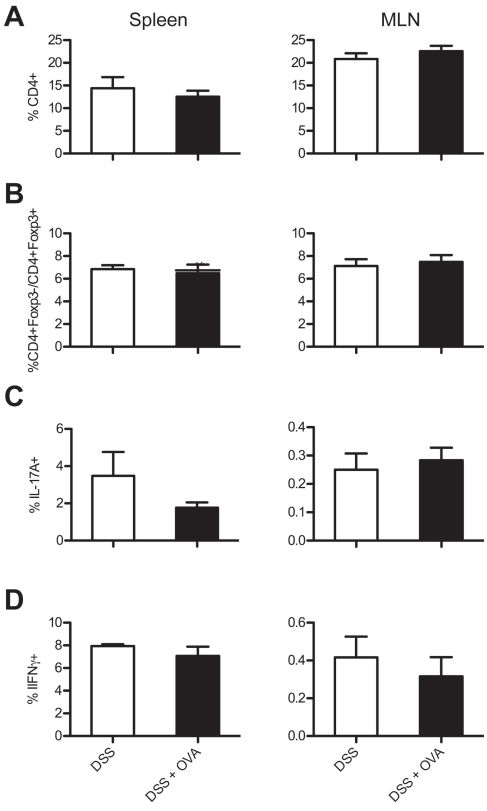

Figure 5. DSS and OVA treated mice have aspecific T cell responses similar to mice treated with DSS alone.

Mice were either treated with DSS alone or with DSS and OVA. Spleens and mLNs were removed, stimulated and examined by flow cytometry. A) Percentages of CD4+ T cells were determined within cultures after stimulation with anti-CD3 for 48 hours. N = 4-9 mice per group. B) The ratios of the percentages of Foxp3- and Foxp3+ cells (Foxp3-/Foxp3+) were determined within the CD4+ T cell population. N = 4-9 mice per group. Using intracellular cytokine staining, percentages of IL-17A+ (C) and IFNγ+ cells (D) within the CD4+ cell population were determined after stimulation. N = 3 mice per group. All graphical results are expressed as mean ± SEM in the bar graphs.

the percentages of IL-17A+ and IFNγ+ CD4+ T cells in the mLNs and spleens after ex vivo stimulation remained unchanged after the addition of OVA to the DSS model.

Increased percentages of OVA-reactive splenic CD4+ Foxp3-T cells are detected in DSS and OVA treated mice after resolution of colitis

To determine if the splenic CD4+ cells were reactive to oral antigens, CD4+ cells from the mLNs and spleens were examined one week after colitis resolution in mice that were administered OVA. Cell suspensions from mLNs and spleens were restimulated with OVA and examined for the expression of the very early activation marker CD69. As shown in Figure 6A and B, OVA-reactive T cells were found in the splenic CD4+ Foxp3+ (Treg) populations of both mice treated with DSS and OVA and the OVA-treated healthy controls. However, mice treated with DSS and OVA uniquely had significantly increased percentages of splenic, OVA-reactive CD4+ Foxp3-T cells (conventional T cells). Reactive cells were not observed in the mLNs (Figure 6C and D), and no OVA-directed cells were detected in the control mice not administered OVA in the drinking water (Figure 6A–D). Taken together, these results indicate that antigen-specific CD4+ Foxp3-T cells develop during active colitis, and that they can be found in the spleens of mice after DSS resolution. Moreover, this implies that OVA can be used as a T cell tracking antigen within DSS colitis.

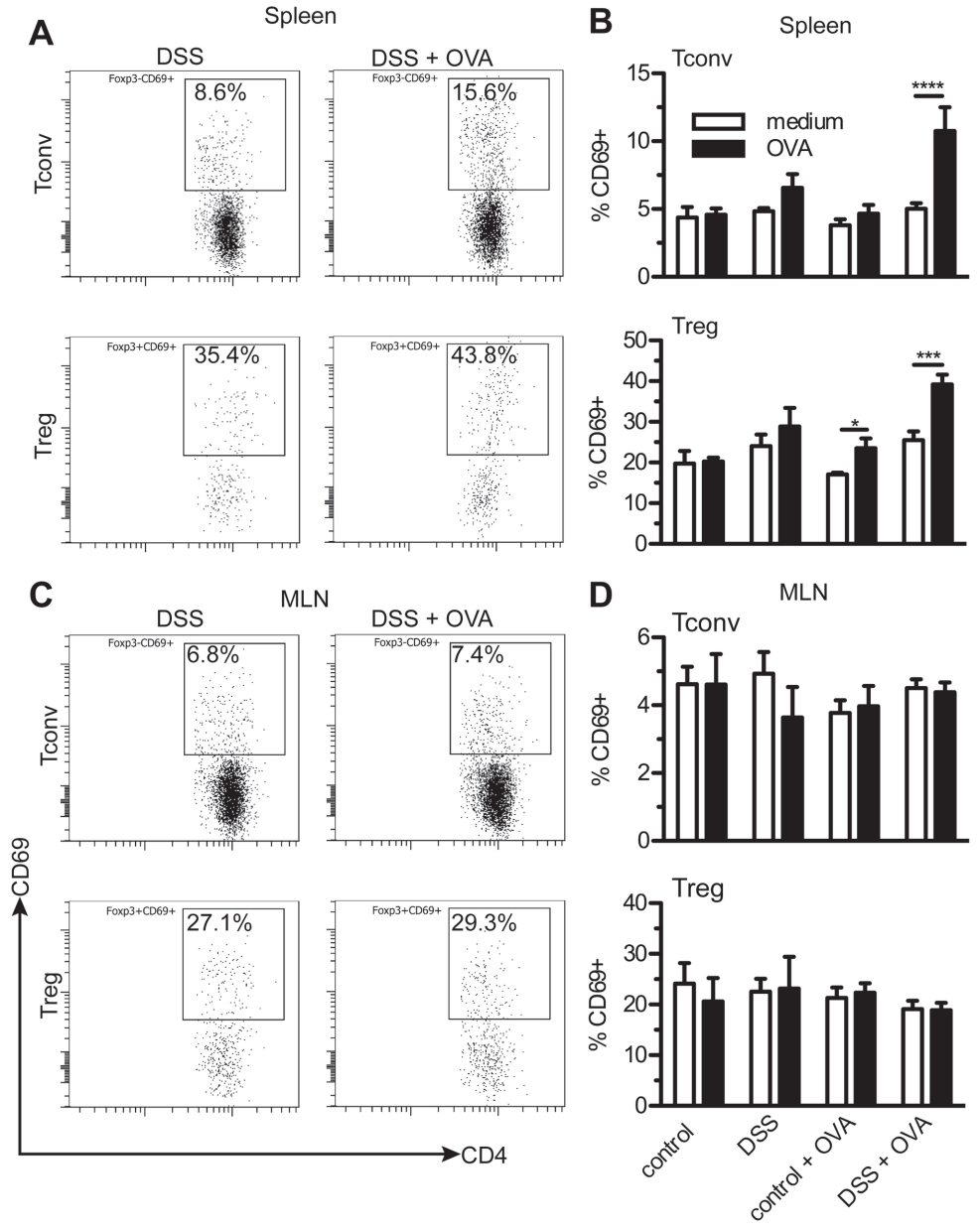

Figure 6. OVA-directed, CD4+ Foxp3-T cells are detected only in mice treated with DSS and OVA.

Both mLN and spleen cell suspensions were prepared 14 days after the start of the DSS and OVA treatment for all groups. Cells were stimulated with OVA, and CD69 expression was measured using flow cytometry. A) Representative FACS dot plots of the CD69 expression in CD4+ Foxp3- (Tconv) and CD4+ Foxp3+ (Treg) T cells in the spleen. Percentages shown are the % CD69+ cells in the specific gated Tconv or Treg populations. B) The mean percentage CD69 expression was calculated for Tconv and Treg in the spleen cells of each group. C) Representative FACS plots of the CD69 expression in Tconv and

Discussion

Research looking at the role of CD4+ T cells in IBD has shown that IBD patients develop T cells that are directed towards luminal antigens like flagellin, intestinal bacteria and oral antigens [2–6]. This information, often the result of examining patient CD4+ T cell clones, does little to explain the actual function of CD4+ T cells in promoting IBD chronicity and how they are generated. *In vivo* models of IBD can be helpful for this research. Lodes et al. justified their finding of anti-CBir1 (flagellin) antibodies and CBir1-specific CD4+ T cells in CD with an *in vivo* experiment. They found that transferring CBir1- specific CD4+ T cells to SCID mice led to the development of colitis, while CD4+ T cells with a normal repertoire of TCRs did not, indicating that antigen specificity is of importance in CD4+ T cell-mediated colitis [5]. Moreover, a study of antigen specificity of CD4+ T cells isolated from SCID mice suffering from colitis after transfer of naïve T cells showed that these cells were highly reactive to fecal extracts and are Th1 skewed [7]. These studies underscore the importance of the T cell transfer model of colitis for intestinal CD4+ T cell research. However, because they are induced in SCID mice, which lack functional T and B cells, they are not the best representatives of human IBD and do not allow the study of the immunological processes that lead to the generation of IBD-inducing T cells.

Looking for new alternatives, we chose to use the DSS model of colitis to determine if T cells reactive against luminal antigens could be developed *in vivo* in an experimental colitis model using wild type mice. The DSS-induced colitis model is advocated as a highly relevant model for IBD, being sensitive to common IBD therapeutics [15], sharing a similar gene expression as IBD [26] and displaying T cell accumulation in the inflamed colon [20,27] similar to what is found in IBD patients [28]. Furthermore, many have observed a chronic pathology that develops after the acute inflammation has passed, which includes changes in crypt morphology with lymphocytosis and a Th1/Th2 cytokine profile [14,21,22,29].

This chronic pathology could be caused by memory T cells. Memory T cells are known to function as sentinels of the immune system and often reside in the periphery [30]. During DSS-induced inflammation, tertiary lymphoid structures that are adjacent to the intestinal epithelial layer develop [31], which likely house resident memory T cells. We found increased numbers of T_{CM} cells in our colon mononuclear cell suspensions of our DSS-treated mice. T_{CM} are differentiated mainly on the expression of CD62L, an adhesion molecule that allows them to enter and stay in lymphoid tissues like colon patches. Increased T_{CM} in the colon during DSS colitis could be responsible for the chronic colitis pathology later found in mice [21]. T_{CM} are known to regain effector functions and expand when they re-encounter their cognate antigens [32], which would lead to immune cascades that re-ignite inflammation.

We found that during DSS colitis, both conventional T cells and Tregs were generated against oral antigens, while healthy mice only developed OVA-reactive Tregs. Classically, exposure to oral antigens leads to Foxp3+ Treg responses that control untoward responses to microbiota and food antigens that are induced via CD103+ DCs producing TGFβ, retinoic acid and prostaglandins [23]. However, in the DSS model of colitis, the weakening of the mucus

barrier allows the penetration of bacteria to the underlying immune cells [17]. This likely leads to the release of an abundance of proinflammatory cytokines [33], and this would allow the generation of other non-regulatory CD4+ T cell effector subsets. This concept was supported by our ability to only detect OVA-directed conventional T cells in DSS-treated mice.

We were only able to find oral antigen reactive T cells and cytokine-producing effector T cells within the spleen and not the mLN. Literature supports this observation as T cells are known to travel to the spleen after the resolution of acute inflammation [30]. Moreover, Hall et al. demonstrated that after resolution of acute DSS colitis (day 25 after the start of DSS), there is a striking increase of activated CD4+ T cells in the spleen, while the percentage of activated T cells within the mLN normalizes [34]. We cannot eliminate the possibility that examination of mLNs using a more refined technique, such as tetramer staining, may reveal the presence of OVA-reactive T cells. However, despite lack of sensitivity, it is clear that OVA directed responses are more pronounced in the spleen at the time point that we tested.

To our knowledge, we have demonstrated for the first time that oral antigen-specific T cells form during DSS colitis and that they can be found systemically after the resolution of colitis. This gives added depth and usefulness to the DSS colitis model, specifically as oral antigens can now be considered as a strategy to track T cells primed by gut luminal antigens. This new perspective on the DSS-induced colitis model will allow insight into the inflammatory processes needed to initiate the development of antigen-specific T cells during acute gastrointestinal inflammation and their role in disease chronicity.

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Chapter 4

TOLL-LIKE RECEPTOR 6 STIMULATION PROMOTES T-HELPER 1 AND 17 RESPONSES IN GASTROINTESTINAL-ASSOCIATED LYMPHOID TISSUE AND MODULATES MURINE EXPERIMENTAL COLITIS

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Abstract

T-helper 1 and 17 (Th1/Th17) responses are important in inflammatory bowel disease (IBD), and research indicates that Toll-like receptor 6 (TLR6) stimulation leads to Th17 cell development within the lung. The gastrointestinal tract, like the lung, is a mucosal surface that is exposed to bacterially derived TLR6 ligands. Thus, we looked at the effects of TLR6 stimulation on the expression of Th17-, Th1-, and regulatory T-cell-associated transcription factors; RORγt, T-bet, and Foxp3, respectively; in CD4+ T cells within gut-associated lymphoid tissue (GALT) *in vitro* and *in vivo*. Cells from GALT and spleen were stimulated with anti-CD3 and TLR ligands for TLR1/2 and TLR2/6 (Pam3CSK4 and FSL-1, respectively). FSL-1 was more effective than Pam3CSK4 at inducing Th1 and Th17 responses in the GALT while Pam3CSK4 rivaled FSL-1 in the spleen. TLR6 was further explored *in vivo* using experimental colitis. *Tlr6*-/- mice were resistant to colitis, and oral FSL-1 led to more severe colitis in wild-type mice. Similar pro-inflammatory reactions were seen in human peripheral blood mononuclear cells, and TLR6 expression was directly correlated with RORC mRNA levels in inflamed intestines of IBD patients. These results demonstrate that TLR6 supports Th1- and Th17-skewed responses in the GALT and might be an important target for the development of new medical interventions in IBD.

Introduction

Toll-like receptor 6 (TLR6) is a member of the TLR family of pattern recognition receptors.[1] Like TLR1, it forms a heterodimer with TLR2.[2] The heterodimer TLR2/6 recognizes diacylated lipopeptides whereas TLR1/2 recognizes triacylated lipopeptides.[3,4] Lipopeptides are cell wall components of Gram-positive bacteria, yeasts, and mycoplasma.

Initial studies on TLR6 have suggested that TLR2/6 stimulation on dendritic cells leads to tolerogenic dendritic cell formation and regulatory T-cell (Treg) development.[5,6] Tregs have the capacity to suppress the pro-inflammatory activities of other immune cells, and they can differentiate into two main groups based on their expression of the transcription factor, forkhead box p3 (Foxp3).[7] Foxp3- Treg, often known as regulatory type 1 T cells, are induced in the periphery and produce high amounts of interleukin (IL)-10. Foxp3+ Treg, on the other hand, may be induced or thymus-derived and use additional suppression mechanisms.

Recently, contradictory results were published that demonstrated that TLR6 could support the development of T-helper 17 (Th17) cells in lung, which were protective against fungal infection.[8] Th17 cell responses are especially important for the control of extracellular bacterial and fungal pathogens and are particularly well known for their ability to stimulate the accumulation of neutrophils.[9] Th17 cells are characterized by the master transcription factor, retinoic acid-related orphan receptor γt (RORγt) and the production of IL-17A, IL-17F, IL-22, IL-26, tumor necrosis factor α, and granulocyte macrophage colony-stimulating factor. Under pro-inflammatory conditions, with high amounts of IL-23 and IL-1β, they also produce interferon γ (IFNγ).[10]

Genome-wide association scan data indicate a role of Th17 cells in inflammatory bowel disease (IBD),[11] and Th17 cells are increased in patients with a magnified activation state.[12–14] This is in addition to the classical T-cell subset associations of the two main forms of IBD: Th1 with Crohn's disease and Th2 with ulcerative colitis.[15] Crohn's disease, in particular, appears to be mediated by Th1/Th17 responses,15 including highly inflammatory Th17 cells that produce IFNγ.[12,16]

As TLR6 is involved in Th17 induction in the lung,[8] we considered that TLR6 might be involved in modulating Th17 responses in IBD. We found that TLR6 stimulation of murine gut-associated lymphoid tissue (GALT) cells was more effective than Pam3CSK4 (TLR1/2 ligand) at inducing Th1/Th17 responses *in vitro*. Moreover, *Tlr6*-/- mice were protected against colitis while the addition of TLR6 ligands during colitis led to worsened disease and increased Th17 responses after disease resolution. TLR6 stimulation may be important in controlling Th1/Th17-mediated inflammation in IBD patients.

Results

TLR6 is expressed in the intestines of IBD patients and mice with experimental colitis

Researchers have reported a role for TLR6 in the development of Th17 cells at the lung mucosa.[8] Therefore, we investigated the expression of TLR6 mRNA and protein in inflamed intestinal tissues. Immunofluorescence staining showed that TLR6 was expressed in the intestine by both crypt epithelial and non-epithelial cells, and TLR6t cells were present in IBD intestinal biopsies (Figure 1a). TLR6 and RORC mRNA were also detected in both patient and control intestines (Figure 1b). Although the mRNA levels were not significantly changed in IBD patients, the levels of the two genes were significantly correlated (Figure 1c), suggesting increased TLR6 expression has a relationship with the Th17-associated master transcription factor RORC in the intestine.

To determine *Tlr6* mRNA expression in the murine colon, we isolated mRNA from colon at both the distal (rectum) and proximal (cecum) ends during intestinal inflammation (day

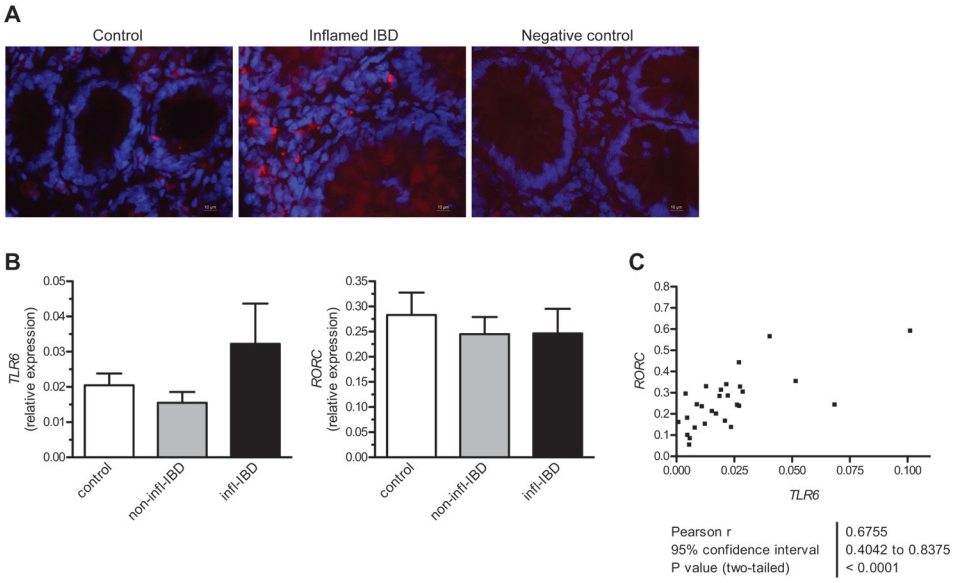


Figure 1 Toll-like receptor 6 (TLR6) is expressed during inflammatory bowel disease (IBD).

(a) Immunofluorescent staining of TLR6 in intestinal tissues obtained from a control (Control) and an IBD patient (Inflamed IBD). Negative control shows background stain without the primary antibody. Representative stains are shown for n = 3 controls and IBD patients. Red staining shows TLR6+ cells, and blue shows nucleic acids (cell nuclei). Photomicrographs were taken at x400 magnification. (b) Relative expression of RORC and TLR6 was determined for RNA obtained from colon carcinoma patients (control, n = 10), non-inflamed regions of IBD patient intestinal tissue (non-infl-IBD, n = 10) and matched inflamed regions of IBD patient intestines (infl-IBD). Data are presented as mean ± s.e.m. The measurement was performed twice independently. (c) A significant correlation was found between the mRNA levels of RORC and TLR6 in colon samples obtained from both control and IBD patient material (n = 28).

7) in the dextran sodium sulfate (DSS)-induced colitis model. In DSS colitis, the disease manifests more strongly at the distal end. Although both control and DSS-treated mice expressed *Tlr6* in the colon, during colitis *Tlr6* mRNA was significantly increased in the inflamed distal region of the colon (Figure 2a). The increased expression of TLR6 protein was confirmed by immunoblot (Figure 2b). As expected, we observed a double band of B100 kD, demonstrating that TLR6 is upregulated in the colon during colitis. To determine which immune cells express TLR6, mesenteric lymph node (MLN) cells were isolated from control and DSS-treated mice and were examined for TLR6 expression using flow cytometry. TLR6 expression was low in immune cells taken from control mice (Figure 2c). In DSS-treated mice, TLR6 expression was significantly upregulated in CD11c+ and CD3+CD4+ cells.

TLR6 stimulation increases RORγt expression in the GALT

We explored TLR1/2 and TLR2/6 stimulation in the context of murine GALT by looking at the effect of TLR ligands (TLRLs) on CD4+ T-cell responses in Peyer's patches (PPs) and MLN. Spleen was also included as a control tissue. Th17-, Th1-, and Treg-associated responses were measured by master transcription factor expression (RORγt, T-bet, and Foxp3, respectively). CD4+RORγt+ T-cell expression of IL-17A was first confirmed in MLN cells (Figure 3a). Although not all CD4+RORγt+ T cells expressed IL-17A (˷20%), the IL-17A expression was constrained to the CD4+RORγt+ T-cell population.

The expression of RORγt and Foxp3 in activated CD4+ CD69+ T cells was measured in the GALT (MLN and PP) and spleen after stimulation with anti-CD3 and TLRLs. Stimulation with FSL-1 in GALT cells led to a significant increase in RORγt+Foxp3- T cells within the

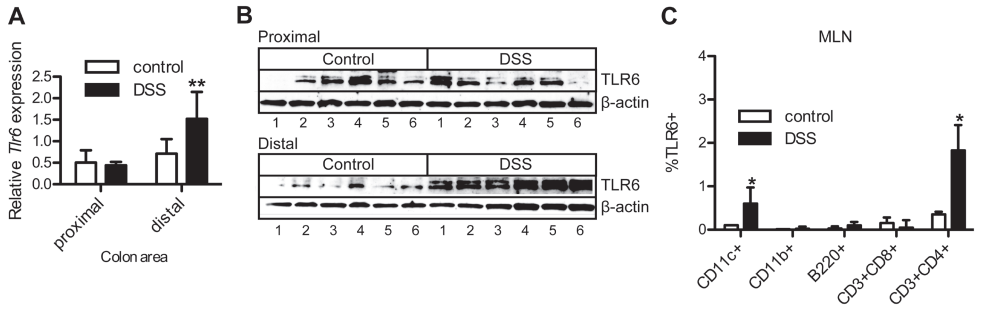


Figure 2 Toll-like receptor 6 (TLR6) expression is increased during experimental colitis.

(a) Distal and proximal regions of colon tissues isolated from control and dextran sodium sulfate (DSS)-treated mice were analyzed for Tlr6 (n = 6). Values are presented as relative to the household gene Rps13. (b) Immunoblot for TLR6 protein content in distal and proximal colon tissues of control and DSS-treated mice (n = 6). The double bands are in accordance with the staining pattern shown by the manufacturer of the TLR6 antibody. Beta-actin staining was used as a loading control. (c) mesenteric lymph node (MLN) cells from control and DSS-treated mice were stained (n = 4) with either an antibody for TLR6 or a matched isotype control. The percentage of TLR6 staining was determined for various immune cells. Matched isotype values were first subtracted. Data are presented as mean ± s.e.m. P-values considered as significant are indicated as *< 0.05, ** < 0.01.

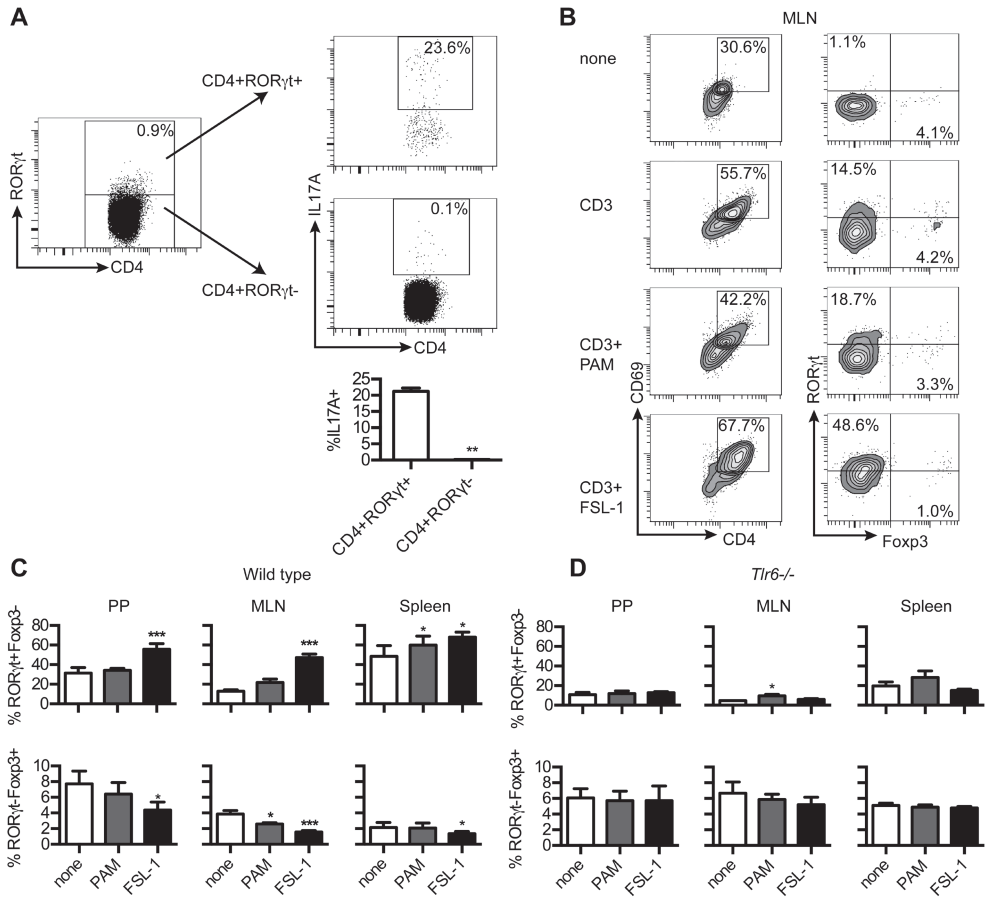


Figure 3 FSL-1 stimulation of murine gut-associated lymphoid tissue (GALT) cells increases RORγt expression and lowers Foxp3 expression.

(a) Cells were isolated from mesenteric lymph node (MLN) and stimulated with PMA/ionomycin (n = 6) and the numbers of IL-17A+ cells in the CD4+RORγt+ and CD4+RORγt- T cells population determined. Representative plots are shown for a single mouse. (b) Left column: percentages CD4+CD69+ T cells from total MLNcells after the indicated stimulation. Right column: RORγt and Foxp3 staining of theCD4+CD69+ cells in the left column. Plots are representative of the data presented in part c. (c) Cells isolated from the indicated lymphoid tissues from wild-type mice were stimulated with anti-CD3 and TLRLs. Percentages of RORγt+Foxp3- and RORγt-Foxp3+ T cells were determined from the gated CD4+CD69+ T-cell population (n = 4, pooled from two independent experiments). (d) Cells isolated from lymphoid tissues of Tlr6-/- mice were stimulated and analyzed as in part C (n = 3). Data are presented as mean ± s.e.m. *P*-values considered as significant are indicated as * < 0.05, **< 0.01 and *** < 0.001.

CD4+CD69+ population that was not observed in the other conditions (Figure 3b and c). Parallel reductions in the percentage of RORγt-Foxp3+ T cells in the FSL-1-treated GALT cells were also noted. The effect of FSL-1 was also observed in spleen cells (Figure 3c) and human peripheral blood mononuclear cells (PBMCs) (Supplementary Figure 1A online). Pam3CSK4 had little effect on GALT cells except lowering RORγt-Foxp3+ T cells in the MLN. However,

Pam3CSK4 increased the percentage of RORγt+Foxp3- T cells in the spleen (Figure 3c). Loss of TLR6 expression eliminated all FSL-1-mediated transcription factor changes (Figure 3d). Its effect on the response caused by PAM3CSK4, in contrast, was limited.

Unlike RORγt, expression of the Th1 transcription factor, T-bet, was not stimulated by the addition of TLRLs during the stimulation of MLN cells with anti-CD3. It also remained unchanged in CD4+ T cells in *Tlr6-/-* mice (Figure 4a–d).

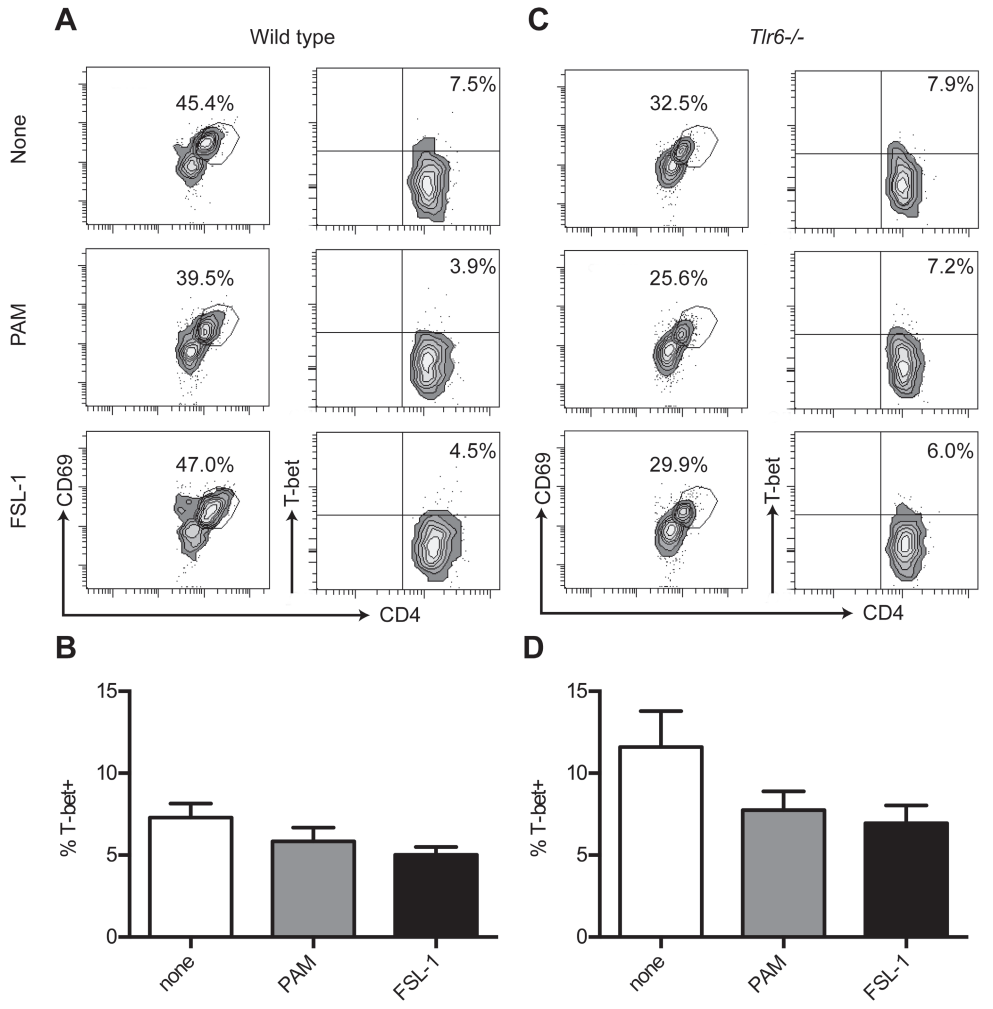


Figure 4 TLRL stimulation of murine mesenteric lymph node (MLN) cells does not affect T-bet expression.

Wild-type (a and b) and Tlr6-/- (c and d) MLNs were stimulated with TLRLs and anti-CD3. T-bet was measured after 48 h of stimulation. None, anti-CD3 alone; PAM, Pam3CSK4 and anti-CD3; and FSL-1, FSL-1 and anti-CD3. (a and c) Left column: percentages CD4+CD69+ T cells from total MLN cells after the indicated stimulation. Right column: T-bet and CD4 staining of the CD4+CD69+ cells in the left column. T-bet plots are representative of the data (n = 5) presented in parts b and d. Data are presented as mean ± s.e.m.

T-cell stimulation combined with FSL-1 increases both IFNγ and IL-17A secretion in the GALT

To further investigate influences on T-cell polarization during stimulation with anti-CD3 and TLRLs, we measured IFNγ, IL-17A, IL-10, IL-4, IL-2, IL-6, and transforming growth factor β (TGFβ) in the supernatants after 48 h of culture. IL-4 was hardly detectable in all of the cultures (data not shown). In GALT cultures, the addition of FSL-1 during anti-CD3 stimulation led to significant increases in the production of IFNγ and IL-17A that were not seen after stimulation with Pam3CSK4 (Figure 5). In PP cells, Pam3CSK4 and FSL-1 both induced a significant increase in IL-10, a trend also seen in MLN. FSL-1 significantly increased the levels of IL-2 in the MLN and PP, while Pam3CSK4 had only the same response in the MLN. Interleukin-6 was increased by both TLRLs in the GALT, while TGFβ levels remained the same in all conditions.

The spleen cells, in general, produced far higher amounts of cytokines than the GALT cells when stimulated with anti-CD3 and TLRLs. All cytokines, except TGFβ, appeared to increase. Significant changes were measured for IFNγ after FSL-1 treatment and IL-6 after Pam3CSK4 (Figure 5). Within the spleen and human PBMCs (Supplementary Figure 1B), FSL-1 and Pam3CSK4 performed almost identically with both TLRLs causing significant and not quite significant increases in the cytokines tested.

FSL-1 leads to increased Th1 and Th17 cells in GALT long-term cultures

The previous results were after short 48-h stimulations. Early changes in transcription factor expression and cytokines may not be representative of fully differentiated and expanded effector T cells. Therefore, MLN and spleen cells were stimulated with anti-CD3 in combination with the TLRLs and expanded for 7 days in culture. Restimulation with PMA/ionomycin revealed that both TLRLs led to significantly increased amounts of CD4+IL-17A+ T cells (Th17) within MLN cell suspensions (Figure 6a). However, FSL-1 uniquely led to magnified percentages of CD4+IFNγ+ T cells (Th1) in MLN cell cultures. Neither TLRL induced CD4+IL-10+ cells. In the spleen cell cultures, both TLRLs caused similar increases in CD4+IFNγ+ T cells with ˜80% of the CD4+ T cells expressing IFNγ. Though the percentage of Th17 cells appeared to increase in FSL-1-treated spleen cultures, only Pam3CSK4 treatment led to significant increases. Pam3CSK4 treatment also increased the percentage of CD4+IL-10+ T cells, while FSL-1 decreased them.

It has been reported that CD4+ T cells double positive for both IFNγ and IL-17A are particularly potent autoimmune Th17 cells that arise when Th17 are exposed to high IL-23 and IL-1β.[10] Therefore, we examined if the populations of CD4+IFNγ+IL-17A+T cells were also changed in the cultures. These rare cells increased significantly in the FSL-1-treated MLN cultures and were not affected by Pam3CSK4 treatment (Figure 6b). In expanded spleen cultures, Pam3CSK4, but not FSL-1 treatment, significantly increased the CD4+IFNγ+ IL-17A+T-cell population.

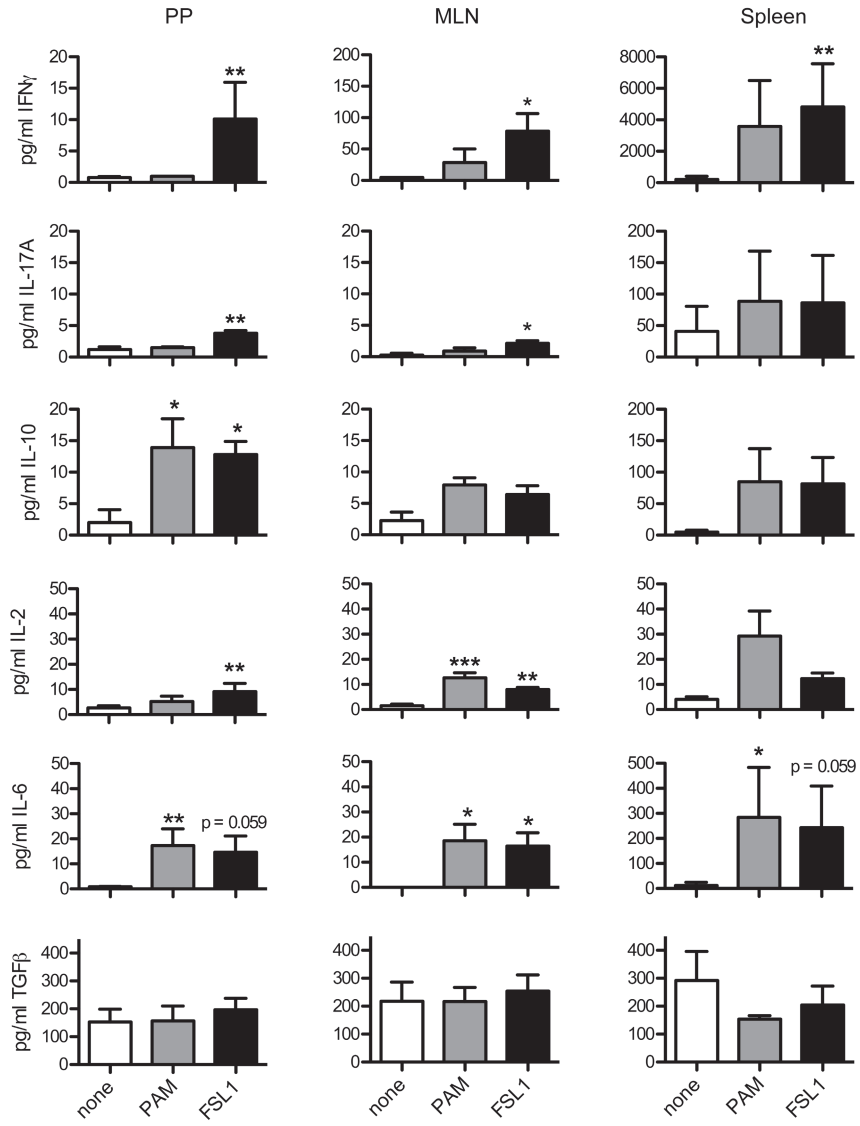


Figure 5 T-cell stimulation with anti-CD3 and FSL-1 leads to increased IFNγ, IL-17A secretion in gut-associated lymphoid tissue (GALT).

Cells isolated from murine Peyer's patches (PPs), mesenteric lymph nodes (MLNs), and spleens were stimulated with anti-CD3 and TLRLs (n = 4). After 48 h, supernatants were isolated and analyzed for cytokine content. The cytokines measured and the units are given in the y-axis. Data are presented as the Mean ± s.e.m. *P*-values considered as significant are indicated as * < 0.05, ** < 0.01, and *** < 0.001.

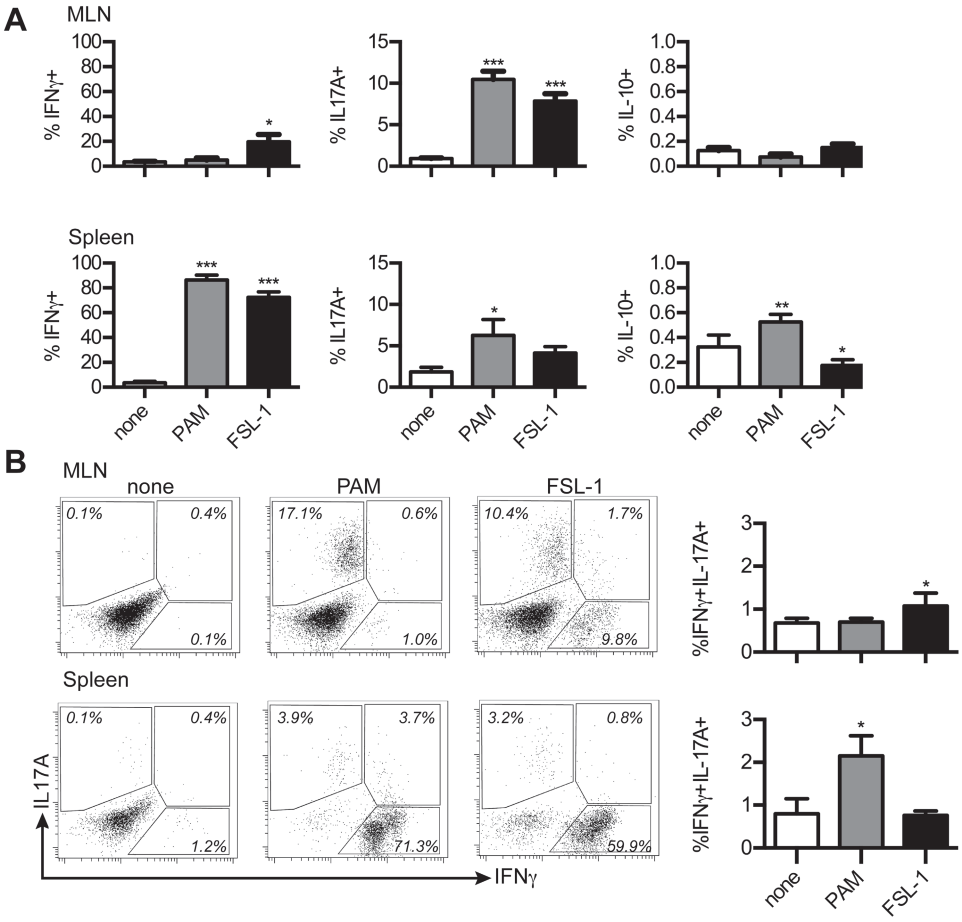
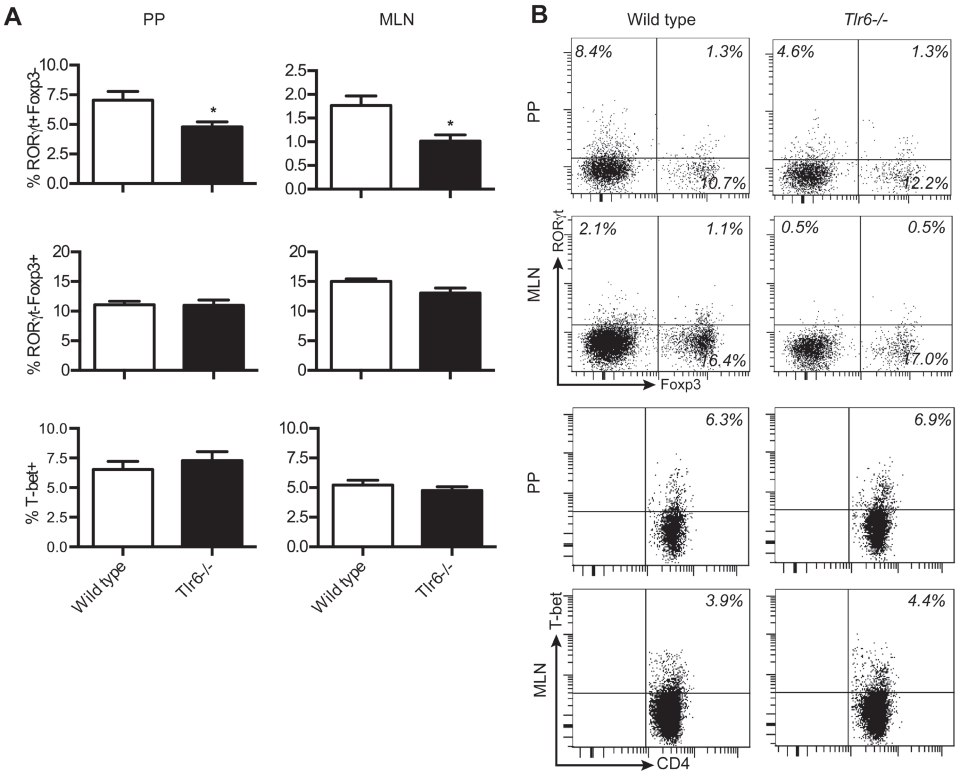


Figure 6 FSL-1 leads to increased Th1 and Th17 cells in extended mesenteric lymph node (MLN) cultures.

Cells isolated from the MLNs or spleens (n = 4) were stimulated with anti-CD3 and TLRLs for 7 days followed by PMA/ionomycin stimulation. (a) Percentages of cytokine-positive CD4+ cells are indicated. (b) Percentages of CD4+IFNγ+IL-17A+ cells are shown in the column figure on the right. Representative FACS plots for a single mouse are also shown. Data in column graphs are presented as the mean + s.e.m. *P*-values considered as significant are indicated as * < 0.05, ** < 0.01, and *** < 0.001. **Figure 7 *Tlr6-/-* mice have lower amounts of CD4+RORγt+ cells.** (a) Gut-associated lymphoid tissue (GALT) cells were isolated from both *Tlr6-/-* and wild-type mice (n = 6).RORγt, Foxp3, and T-bet expression were analyzed within the CD4+ T-cell population. (b) Representative plots of a single mouse are shown for the transcription factor staining. Data are given as mean + s.e.m. *P*-values considered as significant are indicated as * < 0.05. PPs, Peyer's patches. **Figure 7 *Tlr6-/-* mice have lower amounts of CD4+RORγt+ cells.** (a) Gut-associated lymphoid tissue (GALT) cells were isolated from both *Tlr6-/-* and wild-type mice (n = 6).RORγt, Foxp3, and T-bet expression were analyzed within the CD4+ T-cell population. (b) Representative plots of a single mouse are shown for the transcription factor staining. Data are given as mean + s.e.m. *P*-values considered as significant are indicated as * < 0.05. PPs, Peyer's patches.

Tlr6-/- mice have lowered Th1 and Th17 responses in the GALT and are protected against colitis

Tlr6-/- mice have less Th17 cells in the lung.8 The possibility existed that lowered Th17 numbers would be found in the GALT as well. To determine if this was the case, T-cell subsets were examined in the MLN and PPs of Tlr6-/- mice. In both tissues, significant reductions of CD4+RORγt+ Foxp3- T cells were observed as compared with wild-type controls while the rest remained unchanged (Figure 7a and b). Owing to the changes in the Th17 populations,



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we speculated that Tlr6-/- mice could be protected against DSS-induced colitis. Tlr6-/- mice were less susceptible to colitis than wild types, as judged by the disease activity index (Figure 8a). This difference was also observed in the colon length (Figure 8b). Both *Tlr6*-/- mice and wild-type mice had shortened colons after DSS exposure; however, the colons of the DSS-treated wild type mice were significantly shorter than the colons of the DSS-treated *Tlr6*-/- mice.

An investigation of the transcription factor expression and cytokine production in GALT CD4+ cells revealed that during colitis, CD4+RORγt+ Foxp3- T cells remained significantly reduced in *Tlr6*-/- mice compared with wild types (Figure 8c and d). Significant and almost significant reductions of CD4+T-bet+ cells were also observed in the MLN and PP of *Tlr6*-/- mice. Lowered CD4+RORγt-Foxp3+ populations were also observed in the MLN, but not in the PP.

Oral FSL-1 increases experimental colitis severity and residual Th17-associated responses

Because TLR6 expression was increased in the colon during DSS colitis, we speculated that in vivo treatment with the TLR6 ligand, FSL-1, during colitis would lead to changes in the disease progression and associated Th1 and Th17 responses. FSL-1, Pam3CSK4, or saline in combination with ovalbumin (OVA) were orally administered on day 4, 5, and 6 after the start of DSS water to both healthy and DSS-treated mice (Figure 9a). Due to the risk that oral TLRL treatment might lead to extremely severe disease, a mild form of DSS colitis was induced. Analysis of the weight scores showed that only FSL-1-treated mice experienced a significantly sharper decrease in weight on day 7 as compared with the DSS controls at these conditions (Figure 9a).

To observe changes in the T-cell compartments after inflammation, mice were sacrificed on day 14, and cytokine expression in CD4+ cells was examined in the MLN and spleen. FSL-1- and DSS-treated mice had significantly increased amounts of CD4+IL-17A+cells in the MLN relative to the mice treated with just DSS (Figure 9b). Expression of IFNγ was low in the MLN, and no appreciable numbers of CD4+IL-17A-IFNγ+ and CD4+IL-17A+IFNγ+ were noted, preventing analysis (data not shown). Although cytokines were measured in the spleen, there were no differences measured between the groups (data not shown).

Further analysis of the T-cell response using anti-CD3 stimulation and transcription factor staining revealed that FSL-1- and DSS-treated animals had approximately four times as many potential Th17 cells within the total CD4+ T cells as compared with the untreated DSS controls in the spleen while Treg populations remained unchanged (Figure 9c). In the MLNs, similar changes were observed, however, they were not quite significant. Similar responses were also observed for antigen-specific CD4+ T-cell responses directed against the tracking antigen OVA, which was administered simultaneously with the TLRLs (Supplementary Figure 2).

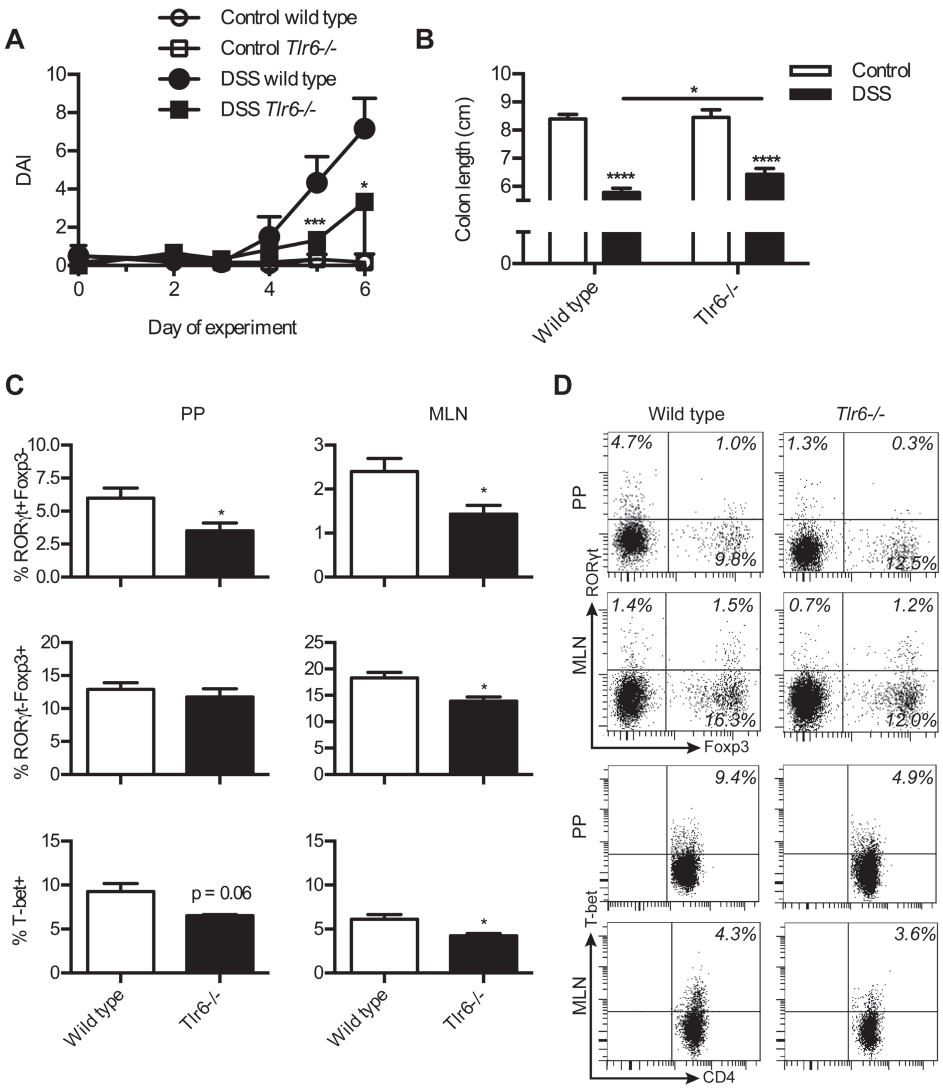

Figure 8 Tlr6-/-mice have reduced Th1 and Th17 responses and are protected from colitis.

(a) Disease activity index (DAI) is shown for healthy controls and dextran sodium sulfate (DSS)-treated wild-type and Tlr6-/- mice (n = 6). (b) In the same mice, colon lengths were measured at sacrifice (day 7 after start of DSS). (c) Transcription factor staining is shown for CD4+ T cells from the gut-associated lymphoid tissue (GALT) for the mice in the above experiment treated with DSS. (d) Representative FACS plots are shown for the data presented in c. Data are given as mean ± s.e.m. *P*-values considered as significant are indicated as * < 0.05, ** < 0.01, *** < 0.001, and **** < 0.0001. MLN, mesenteric lymph node; PPs, Peyer's patches.

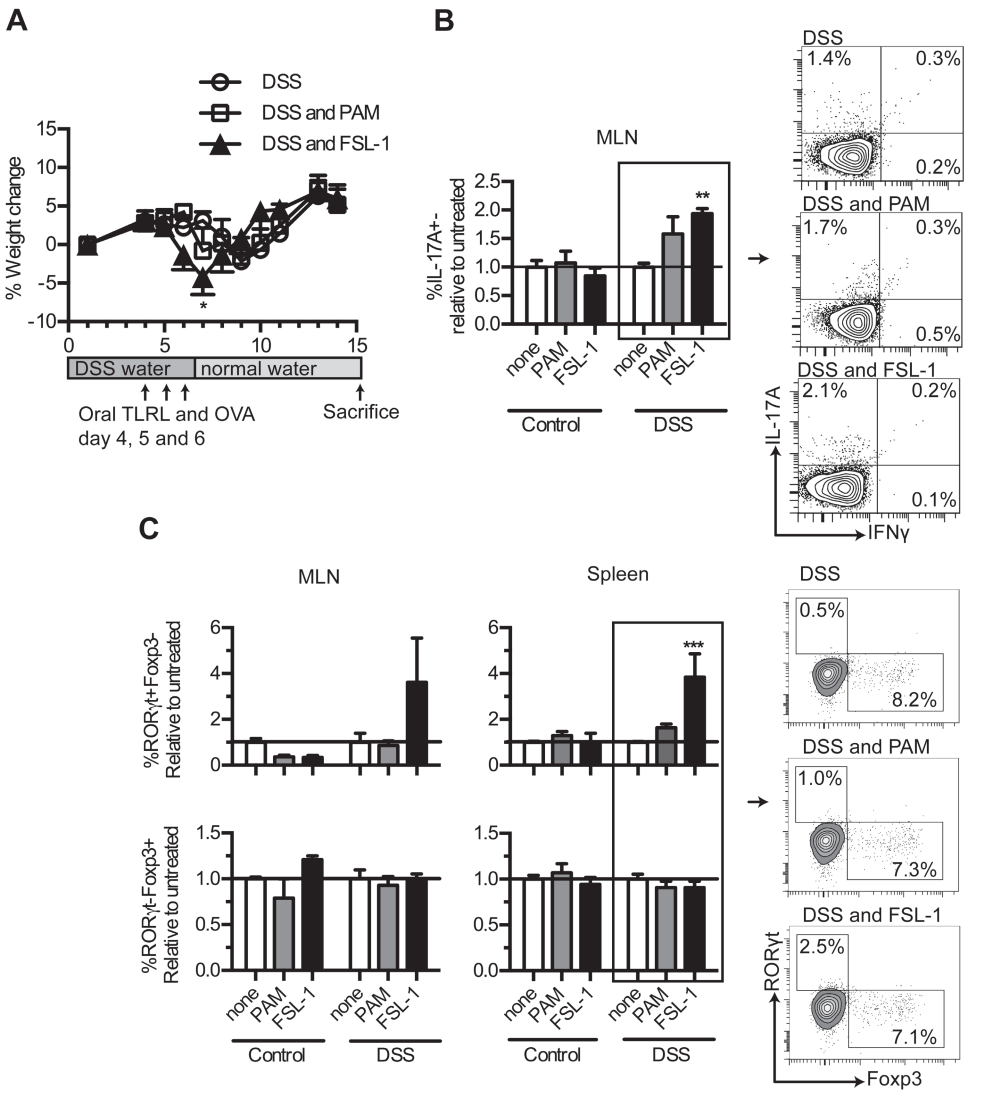

Figure 9 Oral FSL-1 worsens colitis and increases the Th17 response.

Mice were administered regular water or dextran sodium sulfate (DSS) water for 6 days. On day 4, 5, and 6, mice were given oral TLRLs combined with the tracking antigen ovalbumin (OVA). One week after stopping DSS, the mice were sacrificed (n = 5). (a) Percent weight gain of the mice over time relative to starting weight. Data are shown as the mean of each day +/- s.e.m. The experimental design is shown under the weight graph. (b) Intracellular cytokine staining on CD4-gated mesenteric lymph node (MLN) cells isolated from the *in vivo* experiment. Data are presented as relative values of the mean percentage staining found in the untreated group (the group not receiving TLRL treatment). FACS plots on the right show representative staining of the DSS samples. (c) MLN and spleen cell cultures were analyzed for percentages of RORγt+Foxp3- (Th17) and RORγt-Foxp3+ (Treg) T-cell populations in CD4+ T cells after 48 h of anti-CD3 stimulation. Data are presented in the same manner as b. Representative FACS plots on the right show examples of spleen data. Data are shown as the mean ± s.e.m. *P*-values considered as significant are indicated as * < 0.05, ** < 0.01, *** < 0.001, and **** < 0.0001.

Discussion

The role of TLR6 at the mucosal interface is not well understood. Studies by DePaolo et al.[5] demonstrate that TLR2/6 stimulation of dendritic cells, both *in vitro* and *in vivo*, is associated with the formation of suppressive immune responses. Recently, the same group also found lowered regulatory responses and enhanced Th1 and Th17 responses in TLR6-/- mice[6] during Yersinia enterocolitica infection, supporting their previous findings. In contrast, research on the lung mucosa found conflicting data. Protective Th17 responses were impaired in TLR6-/- mice during Saccharopolyspora rectivirgula and Aspergillus fumigatus infections of the lung.[8,17] Moreover, the administration of macrophage-activating lipopeptide-2 (another TLR2/6 ligand) to rat lung caused the influx of immune cells.[18] There are also conflicting reports about the effects of TLR2/6 stimulation on human Treg. Some studies show that TLR2/6 ligands suppress Treg function, like TLR1/2 ligands, while others show that it is ineffective.[19,20]

Our results show that there is a relationship between TLR6 stimulation in the GALT, and the induction of Th1/Th17 responses, which is not shared by TLR1/2. Even though Pam3CSK4 stimulation leads to responses in the GALT, they are relatively milder than those induced by FSL-1 and do not support Th1. In contrast, Pam3CSK4 appears superior to FSL-1 at inducing Th1/Th17 in the spleen. Deeper investigation of TLR6 *in vivo* further supported the *in vitro* findings for TLR6; TLR6 stimulation worsens colitis development and is involved with Th1/Th17 induction. Furthermore, TLR6 expression is increased in the distal colon during colitis, where inflammation is the highest,[21] and TLR6 is expressed in the inflamed intestines of IBD patients where it also correlates with RORC expression. Taken together, this indicates that TLR6 is an interesting player in intestinal inflammation.

Performing re-stimulations with anti-CD3 and TLR2/6 or TLR1/2 ligands showed that FSL-1, but not Pam3CSK4, induced RORγt and reduced Foxp3 in GALT cell suspensions after 48 h of culture. As CD4+ T cells do express TLR6, this effect could be mediated by direct signaling, or it could be mediated by cytokine release from other immune cells. Our results suggest that the latter could be a possibility. Th17 cells require expression of the transcription factor, RORγt.[22] Th17 differentiating conditions (anti-CD3 with low IL-2, IL-4, IFNγ, and high IL-6 and TGFβ) increase *Rorc* mRNA after 25 h.[23] In our FSL-1-treated cultures, IL-4 and IL-2 were both at low concentrations; TGFβ was present, and both IFNγ and IL-6 were increased. IL-6 induces STAT3 (signal transducer and activator of transcription 3), which is needed for *Rorc* transcription.[24] STAT3 also induces the loss of FOXP3 in human natural Tregs,[25] which was also observed. Despite high concentrations of IFNγ (which is not necessarily detrimental to Th17 induction[26]), the environment was, at a cytokine level, conducive to Th17 development and detrimental to Treg.

Despite the lack of changes in T-bet expression in GALT cells at 48 h of culture, large amounts of IFNγ were produced as well as other pro-inflammatory cytokines. As 48 h is too short of a period for naïve T cells to be stimulated, differentiate, and produce their own cytokines; the source must be other cells found in our cell suspensions. Existing memory Th1 cells are

likely candidates, and it is known that they produce IFNγ and IL-10 when costimulated with TLR2 ligands.[27] Other options are memory CD8+ T cells,[28] innate lymphoid cells,[29] dendritic cells,[30] and macrophages.[31]

To further determine the long-term effects on T-cell differentiation, extended cultures were performed with the TLRLs. In MLNs, Pam3CSK4 induced CD4+IL-17A+ cells just as well as FSL-1 but, unlike FSL-1, did not induce CD4+IFNγ+ cells. Looking at the cytokines produced during the first 48 h shows that MLN stimulated with anti-CD3 and Pam3CSK4 produced significantly higher levels of IL-6. As IL-6 combined with TGFβ is needed to differentiate Th17 cells, the development of Th17 is to be expected. The response is not, however, entirely the same as that with FSL-1, which also induced IFNγ/IL-17A double-positive cells. These cells are indicative of a highly proinflammatory cytokine milieu and promote intestinal inflammation found in IBD.[12,16] The main difference between the two stimulations was the presence of IFNγ in the FSL-1 cultures. The presence of IFNγ supports Th1 development[32] and also promotes Th17 responses.[26] This could be the main reason that FSL-1 leads to Th1/Th17 responses in the GALT.

We observed clear differences between Pam3CSK4 and FSL-1 in the GALT. This does not exclude an important role for TLR1/2 in intestinal inflammation, but does suggest that it is different than that of TLR2/6. Our results and studies by DePaolo et al.[6] indicate that TLR1/2 is responsible for intestinal Th17 responses. Still, TLR1/2 stimulation is more potent in the spleen than GALT, inducing both Th1 and Th17. This tissue specific response is an important consideration for future work on TLRLs and may offer some explanation to conflicting data in previous publications.

To investigate TLR6 in intestinal inflammation, DSS colitis was induced in *Tlr6*-/- mice. Our experiments showed that these mice were partially protected from the disease. Interestingly, *Tlr6*-/- mice have reduced numbers of Th17 cells as compared with the wild-type mice, and they remain low even during colitis. During colitis, these mice also had fewer CD4+T-bet+ cells and reduced Treg. Although the loss of Treg is puzzling, the reductions in Th1/Th17 may have influenced the severity of disease.

To learn more about the fate of CD4+ T cells primed during DSS-induced colitis, FSL-1 or Pam3CSK4 were orally administered along with the tracking antigen OVA during the induction of colitis in wild-type animals. Significantly increased Th17 responses in the FSL-1- and DSS-treated mice were found in the MLNs and spleen using several different assays. This is particularly striking as these responses were measured 7 days after DSS treatment was stopped. In contrast, residual Th1 responses were not measured. DSS colitis-associated T-cell responses within the spleen after disease resolution are not unusual. It has been observed that after DSS

colitis heals, there is a striking increase of CD4+ T cells in the spleen, while the percentage of T cells within the MLN normalizes,[33] which may also explain why the percentages of cytokine-expressing T cells was so low in our intracellular cytokine staining assay.

Our results point to TLR6 being an important driver of Th1 and Th17 responses in the GALT. This is supported by both *in vitro* and *in vivo* evidence. Moreover, the fact that RORC and TLR6 mRNA levels are correlated in human intestine and that stimulations of human PBMCs mimic the murine results, suggests a similar relationship between TLR6 and proinflammatory T-cell polarization in humans. As the reduction of Th1/Th17 responses in IBD is a prime goal for therapy, TLR6 could be a potential target for future IBD treatments.

Methods

Animals. Female C57BL/6 mice for the oral TLRL study and for the *in vitro* experiments were purchased from Charles River Laboratories (Maastricht, The Netherlands). The mice were used at 8–12 weeks of age and were housed under standard conditions in the animal facilities at Utrecht University. Breeding pairs of *Tlr6*-/- mice were obtained from Shizuo Akira (Osaka University). Male and female *Tlr6*-/- mice used both *in vivo* and *in vitro* were bred and kept in standard conditions at the Central Animal Facility of the Radboud University Nijmegen Medical Centre. Sex and aged-matched C57BL/6 controls used in the *Tlr6*-/- *in vivo* experiment were bred in the same locale as the *Tlr6*-/- mice.

Experimental colitis. Experimental colitis was induced in all mice by adding 1.5% (w/v) DSS (MP Biomedicals LLC, Illkirch, France) to the drinking water of the mice for 6 days, starting at day 1 of the experiment. Mice were sacrificed on either day 7 or 14 after starting DSS, depending on the experiment. Individual mice were considered a single experimental unit.
Oral gavages for TLRLs and OVA administration were performed on day 4, 5, and 6 to coincide with the developing intestinal inflammation. Each gavage contained 400 µg endotoxin-free OVA (Hyglos GmBH, Bernried am Starnberger See, Germany) and 80 µg of either Pam3CSK4 or FSL-1 (both from EMC microcollections, Tuebingen, Germany) in 500 ml saline solution. Both FSL-1 and Pam3CSK4 are synthetic lipopeptides. Control animals were administered saline only.
The disease activity index of colitis was determined by combining the scores collected from the weight measurement, rectal condition, feces condition, and the detectable presence of blood in the feces. The disease activity index was between 0 and 11 for each mouse. The loss of weight was scored as follows relative to starting weight: 0, 0–2.5% weight loss; 1, 2.5 – 5% weight loss; 2, 5–10% weight loss; 3, 10–15% weight loss; and 4, >15% weight loss. The feces condition score was scored as follows: 0, normal; 1, soft with normal form; 2, loss of form/ diarrhea; and 3, no feces produced. The rectal condition score was determined as follows: 0, normal; 1, irritation present; 2, mucus and/or swelling; and 3, visible blood. Fecal blood was tested with a Colo-rectal test kit (Axon Lab AG, Stuttgart, Germany). Fecal blood was scored as follows: 0, no blood; 1, blood.

Messenger RNA expression analysis. The human intestinal tissue samples in this study were obtained from surgical resection specimens and include pairs of macroscopically inflamed and normal-appearing (non-inflamed) mucosa from patients with Crohn's disease (*n* = 5) and ulcerative colitis (*n* = 5), both clinically and histologically confirmed, with normal tissue from patients with a colorectal carcinoma, at least 10 cm from the tumor, as controls. Details on the tissue specimens and patient characteristics are described in a previous study as well as the methods of total RNA isolation.[34]

For the murine colon samples, the colons were divided into two equal sections: proximal and distal. The total RNA was isolated using the RNAeasy kit (Qiagen, Germantown, MD) and, subsequently, reverse transcribed into cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA).

Real-time PCR was performed using iQ SYBR Green super mix kit (BioRad) with the CFX 96 Real-time system (BioRad). Messenger RNA for ribosomal protein S13 (RPS13) was used as the internal control in mice, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as the internal control in the human samples. The primers used for the human real-time PCR were obtained from SA Biosciences (Frederick, MD). The primer sequences used for the mouse studies were as follows: *Tlr6* fwd: 5' GACTCTCCCACAACAGGATACG 3', *Tlr6* rev: 5' TCAGGTTGCCAAATTCCTTACAC 3', Rps13 fwd: 5' GTCCGAAAGCACCTTGAGAG 3', Rps13 rev: 5' AGCAGAGGCTGTGGATGACT 3'. The final data for the target samples were normalized against the internal control GAPDH in the human samples and Rps13 in the mouse samples. The relative mRNA expression values were calculated using Bio-Rad CFX manager V1.6.

Immunofluorescence. Cryostat sections (5 mm) of the intestinal tissues of CD patients (*n*=5), UC patients (*n*=5) and normal tissue from patients with a colorectal carcinoma (*n*=5) were cut, fixed in ice cold acetone for 10 min, blocked with 5% goat serum in 1% BSA/PBS (bovine serum albumin/phosphate-buffered saline) and afterwards incubated with rabbit-anti-TLR6 (1:125, Novus Biologicals, Cambridge, UK, NBP1-54336) in 1% BSA/PBS overnight at 4 °C. After subsequent washings with PBS, the antibodies were visualized by incubation with goat-anti-rabbit-Alexa568 (1:400, Invitrogen, Breda, The Netherlands) in 1% BSA/PBS for 1 h at room temperature. Counterstaining was done with Hoechst 33,342 (Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 5 min at room temperature and slides were sealed with Prolong gold anti-fade reagent (Invitrogen). Photomicrographs were taken at room temperature with an Olympus BX-60 microscope with a x40/0.75na objective lens equipped with a Leica DFC425 C camera and controlled with LAS software 4.0.

***Ex vivo* T-cell stimulation and PBMC culture**. PPs, MLN, and splenocytes were isolated from mice. Cells were re-stimulated with either 25 $\mu g ml^{-1}$ endotoxin-free OVA (Hyglos GmBH) or 2 $\mu g ml^{-1}$ anti-CD3 (eBioscience, San Diego, CA) with or without Pam3CSK4 or FSL-1 (4 $\mu g ml^{-1}$; EMC microcollections) and cultured with RPMI medium (Roswell Park Memorial Institute

medium, Life Technologies, Paisley, Scotland) supplemented with 1 unit per ml penicillin, 1 μgml^{-1}streptomycin, 50 mM b-mercaptoethanol, and 5% FCS in 96-well round-bottom plates at a concentration of 105 cells per well. During the long stimulation, cells were incubated for 7 days before restimulation with PMA/ionomycin (Sigma). Otherwise, cells were incubated for 48 h, after which, supernatants were collected; cells were harvested and stained with fluorescently labeled antibodies.

Human PBMCs were cultured in RPMI medium supplemented with 1 unit per ml penicillin, 1 μgml^{-1}streptomycin, 1mM pyruvate, 50 μgml^{-1} gentamicin, and 2.5% FCS in 96-well round-bottom plates at a concentration of 105 cells per well. Similarly to the murine experiments, the cells were incubated for 48 h with anti-CD3 (1 μgml^{-1}; eBioscience) with or without TLRLs (Pam3CSK4 or FSL-1, 4 μgml^{-1}; EMC microcollections). Upon completion of the incubation, the supernatants were collected and the cells harvested for FACS staining.

Flow cytometry. Intracellular cytokine staining was performed on either fresh or cultured cells after 5 h stimulation with PMA/Ionomycin (Sigma) and Brefeldin A (eBioscience). Cells were then stained with antibodies for CD4, IFNγ, IL-17A, IL-10, and IL-4 (all antibodies from eBioscience) using the Foxp3 intracellular staining kit (eBioscience). To prevent background staining, cells were first incubated with unlabeled anti-CD16/32 (eBioscience) for 15 min on ice as suggested in the manufacturer's protocol. Fixed samples were kept at 4 °C until reading with the flow cytometer.

Transcription factor expression was measured in murine cells or PBMCs by performing a surface stain with anti-CD4 and anti-CD69 and then staining intracellularly for Foxp3, T-bet, and RORγt.

All antibodies and the Foxp3 intracellular staining reagents were obtained from eBioscience. Before the surface stain and again during the intracellular stain, samples were blocked with unlabeled anti-human/mouse CD16/32 (eBioscience) for 15 min on ice. Fixed samples were kept at 4 °C until reading with the flow cytometer. All samples were read on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and analysis of the flow cytometry data was performed using BD FACSDiva software (BD Biosciences).

Cytokine measurements. Isolated supernatants were analyzed for cytokine production by using either the human or mouse BD Cytometric Bead Array Th1/Th2/Th17 kits (BD Biosciences) and the mouse/human TGFβ Ready-Set-Go! ELISA from eBioscience. Kits were used according to manufacturer's protocol. The bead array samples were read using a FACSCanto II flow cytometer (BD Biosciences). Data analysis was performed using the FCAP Array Analysis software and FCS Filter Software (Soft Flow Hungary Ltd., Pecs, Hungary).

Immunoblot. Intestinal tissues were weighed (100 mg ml^{-1}) and homogenized in radioimmunoprecipitation assay buffer containing protease inhibitors and EDTA (Thermo Fisher Scientific, Rockford, IL) using a Precellys 24 tissue homogenizer (Bertin Technologies,

Montigny-le-Bretonneux, France) for four times 10 s at 6,000 rpm with a minimum 5 min cooling period on ice in between. Afterwards, samples were centrifuged for 15 min at 14,000 rpm and the supernatant was transferred to a clean tube. Homogenates were separated on 4–20% (w/v) SDS gels (BioRad) and blotted to nitrocellulose membranes (Millipore, Billerica, MA). Membranes were blocked for 2 h with 5% milk proteins in PBS/0.1% Tween-20 and subsequently incubated with rabbit-anti-mouse TLR6 (Sigma, SAB1300203) in PBS/2% milk/0.1% Tween-20 overnight at 4 °C. After incubation, membranes were washed three times with PBS/2% milk/0.1% Tween-20, incubated with goat-anti-rabbit-horse radish peroxidase (Dako, Heverlee, The Netherlands) in PBS/2% milk/0.1% Tween-20, treated with commercial ECL reagents (Amersham Biosciences, Roosendaal, The Netherlands) and finally exposed to photographic film. Afterwards, blots were stripped with stripping buffer (Thermo Fisher Scientific) and reprobed with rabbit-anti-b-actin (Cell Signaling Technology, Danvers, MA). The TLR6 staining appears as double bands and this is in accordance with the staining pattern depicted by the manufacturer.

Statistical analysis. Means with s.e.m. are represented in each graph. Statistical analysis was performed using GraphPad Prism version 6.0 for windows (GraphPad Software, San Diego, CA). Correlation significance was determined using the Pearson product–moment correlation coefficient. For comparisons between two groups, T-test or Mann–Whitney test were used depending on the distribution of the data. Comparisons between three or more groups were performed with either a one-way analysis of variance or repeated measures analysis of variance followed by the Bonferroni post test where appropriate. In cases of repeated, nonparametric data, the Friedman test followed by Dunn's multiple comparison post test was used. In the situation of two independent parameters, a two-way analysis of variance was applied (either regular or repeated measures) with the Bonferroni post test.

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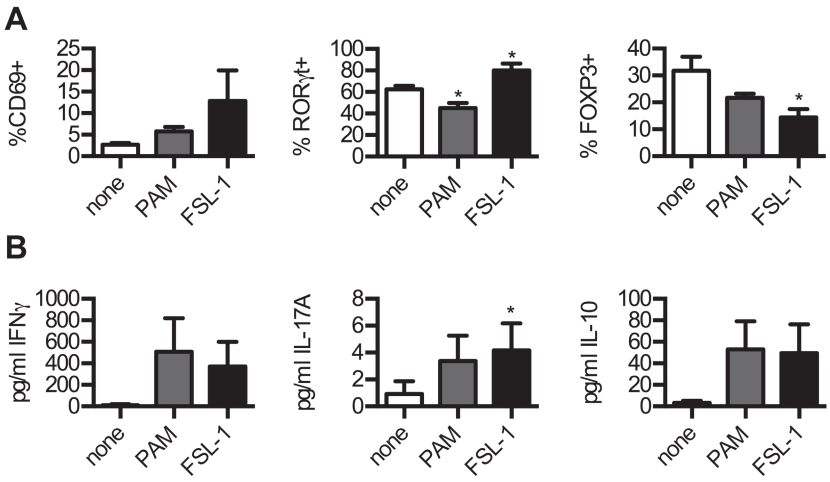
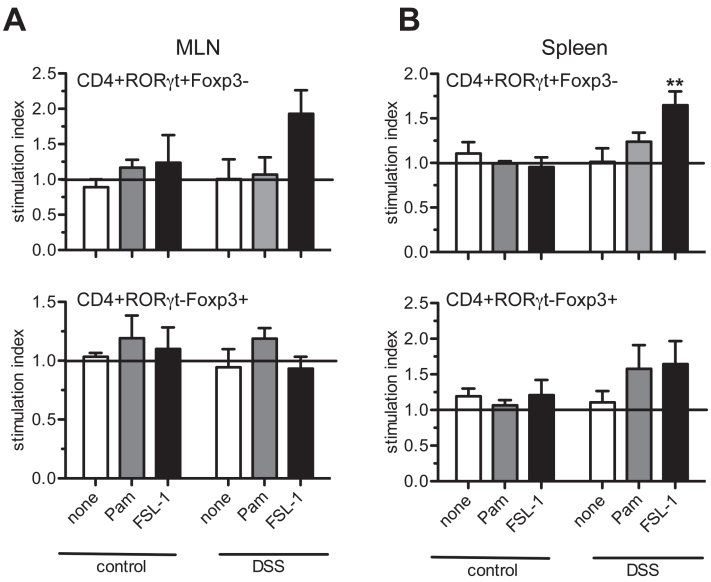
Supplementary figures and Legends:



Supplementary figure 1: Stimulation of human PBMC with FSL-1 leads to increased CD4+RORγt+ cells and decreased CD4+FOXP3+.

A) Stimulated and TLRL treated human PBMC from n = 3 donors were analyzed for transcription factor expression in gated CD69+CD4+ T cells (left graph) after 48 hours of stimulation. Data is representative of two experiments. RORγt and FOXP3 expression in CD4+CD69+ cells is shown in the middle and right graphs, respectively. B) Within the same stimulations described in part A, supernatants were isolated and analyzed for cytokines. The cytokines measured and the units are given on the y-axis. Data is presented as the mean ± s.e.m. . P-values considered as significant are indicated as * < 0.05.



Supplementary figure 2: Oral FSL-1 leads to antigen-specific responses in the Th17 compartment after intestinal disease resolution.

Cells isolated from the MLN (A) and the spleen (B) of *in vivo* treated mice (n = 5, pooled from three independent experiments) were re-stimulated with OVA. Activation was detected by measuring CD69 expression in T cells from either the CD4+RORγt+Foxp3- or CD4+RORγt-Foxp3+ populations. Data is represented as a stimulation index (the percentage CD69+ in the OVA stimulations was divided by background values found in the unstimulated wells for individual mice). Data is given as the mean ± s.e.m. P-values considered as significant are indicated as ** < 0.01.

Chapter 5

BIFIDOBACTERIUM BREVE ATTENUATES MURINE DEXTRAN SODIUM SULFATE-INDUCED COLITIS AND INCREASES REGULATORY T CELL RESPONSES

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Abstract

While some probiotics have shown beneficial effects on preventing or treating colitis development, others have shown no effects. In this study, we have assessed the immunomodulating effects of two probiotic strains, *Lactobacillus rhamnosus (L. rhamnosus)* and *Bifidobacterium breve (B. breve)* on T cell polarization *in vitro,* using human peripheral blood mononuclear cells (PBMC), and *in vivo*, using murine dextran sodium sulfate (DSS) colitis model. With respect to the latter, the mRNA expression of T cell subset-associated transcription factors and cytokines in the colon was measured and the T helper type (Th) 17 and regulatory T cell (Treg) subsets were determined in the Peyer's patches. Both *L. rhamnosus* and *B. breve* incubations *in vitro* reduced Th17 and increased Th2 cell subsets in human PBMCs. In addition, *B. breve* incubation was also able to reduce Th1 and increase Treg cell subsets in contrast to *L. rhamnosus*. *In vivo* intervention with *B. breve*, but not *L. rhamnosus*, significantly attenuated the severity of DSS-induced colitis. In DSS-treated C57BL/6 mice, intervention with *B. breve* increased the expression of mRNA encoding for Th2- and Treg-associated cytokines in the distal colon. In addition, intervention with *B. breve* led to increases of Treg and decreases of Th17 cell subsets in Peyer's patches of DSS-treated mice. *B. breve* modulates T cell polarization towards Th2 and Treg cell-associated responses *in vitro* and *in vivo*. *In vivo B. breve* intervention ameliorates DSS-induced colitis symptoms and this protective effect may mediated by its effects on the T-cell composition.

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease that affects the gastrointestinal tract and consists of two major forms, Crohn's disease (CD) and ulcerative colitis (UC). Although the exact mechanisms of IBD development still remain to be elucidated, a feature that is common to IBD pathogenesis is a dysregulated effector T cell response to the commensal microflora [1,2]. T cells are important components of the adaptive immune system. Upon activation, T cells expand and differentiate into various effector CD4+ T cells such as Th1, Th2, Th17 cells, and Treg cells. The differentiation of these T cell subsets is induced by the specific transcription factors T-bet [3], GATA3 [4], RORgt [5] and Foxp3 [6,7], respectively.

Until recently, the classical T cell subsets (Th1 and Th2) have been considered the major players during the development of IBD. However, there is an increasing body of evidence showing the importance of the Th17 pathway in IBD [2]. Th17 cells are characterized by RORgt expression and IL17 production [5,8], and increased Th17 cells have been found in IBD patients [9,10]. Although the development of Th17 cells is independent of the Th1 and Th2 program, it shares the same requirement for TGFb with Treg cells [11]. Treg cells have a unique regulatory function by suppressing the activity of other T cell subsets (Th1, Th2 and Th17 cells) and, thereby, helping control autoimmunity [12]. In contrast to Th17 cells, decreased amounts of Treg cells have been found in the peripheral blood of IBD patients as compared to normal controls [13,14]. In addition, increased apoptosis of Treg cells was found in the inflamed mucosa of IBD patients compared to non-inflamed control colons [15]. Murine models of IBD have further illustrated the protective effects of Treg cells during colitis. Immunodeficient mice that are adoptively transferred with Treg-depleted naïve CD4+ T cells develop spontaneous colitis; in contrast, mice transferred naïve CD4+ T cells combined with Treg cells do not develop colitis [16,17]. Additionally, Mice lacking interleukin (IL)-10, an important anti-inflammatory cytokine needed for both the induction of Treg cells and their effector function, spontaneously develop colitis [18].

In the last decade, products supplemented with live bacteria, called probiotics, have become increasingly popular [19]. The use of probiotics has been proposed to be beneficial for human health and there is increased interest for their use in IBD. This is due to the beneficial effect of probiotic treatment in other intestinal diseases such as traveler's diarrhea and antibiotic-associated diarrhea [20]. However, the working mechanisms of probiotics still need to be elucidated. Gut-derived bacteria from the genera *Lactobacillus* and *Bifidobaterium* are the most studied probiotics. Diverse effects of the probiotics have been demonstrated using human cell culture systems and animal models and one of the most important effects is their ability to modulate immune responses [21]. Studies using human peripheral blood mononuclear cells (PBMC) have demonstrated the abilities of gut-derived bacteria to modulate T cell polarization by inducing different T-cell subsets including Treg cells in a strain dependent manner [22,23]. Moreover, two independent clinical studies using two

different *Bifidobacteria* strains have demonstrated their immune modulating capacities by both enhancing the TGFb signaling and increasing peripheral Treg cells numbers [24,25].
Recently, Plantinga *et al* assessed the cytokine production of PBMC stimulated with two probiotic strains, *L. rhamnosus* and *B. breve*. Exposure to either bacterial strain led to increased IL-10 levels. In addition, exposure to *B. breve* led to a reduction of IFNγ production, a Th1-associated cytokine, as compared to the *L. rhamnosus* [26]. In this study, we further investigated the same probiotic strains by examining their effects on CD4+ T cell differentiation both *in vitro* and *in vivo*. We demonstrated that both strains had the ability to shift CD4+ T cell polarization in stimulated PBMCs away from Th17 cell development towards Th2 differentiation. In addition, *B. breve* induced the development of Treg cells while decreasing the development of Th1 cells. Administering these bacterial strains in the DSS-induced colitis model showed that while *L. rhamnosus* had little effect on disease severity, *B. breve* ameliorated DSS-induced colitis, increased Treg- and Th2-associated responses and locally reduced CD4+RORgt+Foxp3- T cells while simultaneously increasing CD4+ RORgt-Foxp3+ T cells.

Materials and methods

Human peripheral blood mononuclear cell stimulations

Human PBMCs were isolated from buffy coats, which were obtained from the Sanquin blood bank (Utrecht, the Netherlands). The cell fraction containing PBMCs was obtained by density centrifugation of 1:3 diluted buffy coats on Ficoll-Paque^{TM}PLUS (GE Healthcare, Eindhoven, the Netherlands). Subsequently, the obtained cells were washed with phosphate buffered saline (PBS; Lonza Verviers SPRL, Verviers, Belgium) and the erythrocytes were lysed using sterile lysis buffer (0.15M NH_4Cl, 0.01 M $KHCO_3$ and 0.1mM EDTA, pH 7.4). After lysis, the remaining cells (PBMCs) were washed again with PBS supplemented with 2% heat-inactivated Fetal Calf Serum (FCS; Lonza Verviers SPRL, Verviers, Belgium) and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza Verviers SPRL, Verviers, Belgium) supplemented with 2.5% FCS, 1% penicillin/streptomycin, 1 mM pyruvate and 50 mg/ml gentamicin.
A total of 10^5 PBMCs were incubated either with anti-CD3 (Sanquin, Amsterdam, the Netherlands) alone (at a final concentration of 1:10.000) or in combination with *L. rhamnosus* or *B. breve.* Both *L. rhamnosus* (NutRes 1 formerly known as NumRes 1) and *B. breve* (NutRes 204 formerly known as NumRes 204) were provided by Danone Research BV (Wageningen, the Netherlands) as live bacteria in a 20% glycerol stock. The bacteria:PBMC ratio was 20:1 and incubated in 96-well plates (Greiner bio-one, Stonehouse, UK) at 37°C for 48 hours or 7 days.

Experimental colitis and administration of probiotics

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.

Experimental colitis was induced by adding 1.5% DSS to the drinking water for 5 days. 10^9/ dose of *L. rhamnosus* or *B. breve* probiotics were administrated by oral gavage every two days, starting 9 days prior to the DSS treatment and continued to the end of the experiment. Colitis development was monitored by measuring the weight and the fecal condition. The fecal condition was measured on day 0, 3 and 5. On day 6, the mice were sacrificed and the colons and Peyer's patches were isolated for further analysis.

The severity of the colitis was determined by calculating the body weight change, feces condition and the colon length. The body weight change was determined by calculating the percentage of weight change relative to the starting weight before DSS treatment on day 0. The fecal condition score was determined using two parameters: stool consistency (0 = normal, 1 = soft with normal form, 2 = loss of form/diarrhea) and fecal bleeding (0 = no blood, 1 = blood observation using Colo-rectal Test kit (Axon Lab AG, Germany), 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. 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, 4: infiltration in entire tissue); cover area of cellular infiltration in the region (0: no infiltration, 1: < 25%, 2: 25%-50%, 3: 50%-75%, 4: >75%); loss of crypts (0: no damage, 1: 30% shortening of crypts, 2: 65% shorting of crypts, 3: total loss of crypts, 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%).

Ethics statement

All experiments were performed in accordance with the guidelines issued by the Dutch ethics committee for animal studies. The protocol was specifically approved by the ethics committee for animal studies of Utrecht University (DEC approval number 2009.II.06.046). All efforts were made to minimize suffering.

Immunohistochemical staining

A subset of the mice from each group was examined using immunohistochemistry. After sacrificing the mice, the colons were opened longitudinally and half of each colon was fixed in 10% formalin, rolled, paraffin-embedded, and sectioned (5mm). The sections

were subjected to a heat-induced epitope retrieval step. Slides were washed with PBS and blocked with rabbit or goat serum before an overnight incubation (4°C) with primary antibodies against Ly-6B (AbD Serotec, Dusseldorf, Germany), RORgt (eBioscience San Diego, CA USA) or Foxp3 (eBioscience San Diego, CA USA). For detection, biotinylated goat anti-rat (Dako, Glostrup, DK) secondary antibodies were administered followed by incubation with peroxidase-labeled streptavidin (Vectastain EliteABC kit, Vector, Burlingame, CA USA). The peroxidase activity was visualized using the substrate, DAB (Sigma, Gillingham, UK). The cell nuclei were visualized by a short incubation with Mayer's hematoxylin (Klinipath, Duiven, the Netherlands). Background staining was determined by substituting the primary antibody with a rat IgG isotype control (Abcam, Cambrige, UK).
The number of Foxp3+ cells was quantified by counting positive cells within the lamina propria area excluding the induced and tertiary lymphoid follicle regions. The density of RORgt+ cell was determined as follows: RORgt+ cells were counted in colonic patches and quantified as a function of 0.01mm^2 colonic patch area.

MPO measurement

A subset of the mice from each group was used to determine the MPO concentration in the colon. After sacrificing the mice, the colons were opened longitudinally and half of each colon was transferred into RIPA buffer (Thermo Scientific, Rockford, IL USA) and homogenized using a Precellys®24-Dual homogenizer (Precellys, Villeurbanne, France). The homogenates were centrifuged at 14000 rpm for 10 minutes at 4 °C and the MPO concentration in the supernatant was measured using an ELISA kit according to the manufacturer's protocol (Hycult biotech, Uden, the Netherlands).

Real-time PCR

A subset of the mice from each group was used to determine the mRNA expression of a selection of genes in the colon. After sacrificing the mice, Total RNA of 1 cm distal colon pieces 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 quantification was performed using the iQ SYBR Green super mix kit (BioRad, Hercules, CA USA) with the CFX 96 Real-time system (BioRad, Hercules, CA USA) and the RNA expression was determined using built-in detection system of CFX 96 Real-time system (BioRad, Hercules, CA USA). The RNA expression value and normalized gene expression ($\Delta\Delta C_T$) value was calculated using the built-in gene expression analysis module in CFX Manager™ software version 1.6. The sequence of specific primers for 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: *interferon gamma (Ifnγ), Il12p35, Il4, Il5, Il13, Il23p19, Il17, Il6, Tgfβ* and *Il10* were purchased from SABioscience (Frederick, MD USA). The final data for the target samples were normalized against the internal control *Rps13*.

Intracellular staining for cytokines and transcription factors

The isolated human PBMCs were incubated for 48 hour or 7 days as described. The PBMCs, which were incubated for 48 hours, were stained first extracellularly with antibodies for CD4 and CD69, followed by intracellular staining for GATA3, RORgt, FOXP3 and T-bet. The Fluorescent Minus One (FMO) control of each marker was determined by taking out the indicated marker antibody during the staining of control human PBMCs. In addition, the possible background of each marker antibody within CD4 cells was determined by substituting the indicated antibody with an appropriate isotype antibody with a matching fluorescent label.

The PBMCs, which were incubated for 7 days, were provided with fresh culture medium for 24 hours and then subsequently stimulated with PMA (50ng/ml) and ionomycin (750ng/ml) in the presence of Brefeldin A (eBioscience, San Diego, CA USA) for 4 hours. After stimulation, PBMCs were first stained extracellularly with anti-CD4, followed by intracellular staining for IL-4, IL-17, IL-10 and IFNg. The FMO controls and isotype controls of these marker antibodies were also assessed as described in the previous paragraph.

Peyer's patches isolated from the mice of experimental colitis study were prepared as single-cell suspensions by passing through a 0.75µm cell strainer. Cells were first stained extracellularly with antibodies for CD4, followed by intracellular staining for Foxp3 and RORgt. The FMO controls and isotype controls of these marker antibodies were examined in mLN cells obtained from non-treated mice. All antibodies and intracellular staining buffers were obtained from eBioscience (San Diego, CA USA). All samples were read on a BD FACSCanto II (BD Biosciencse, Franklin Lakes, NJ USA) and the data were analyzed using BD FACSDiva software (BD Biosciences). The (activated) T cells were determined by gating on CD4+ (CD69+) cells. Subsequently, the different T cells subsets were defined on the found T cell subset associated transcription factors and the specific cytokine producing T cells were found by gating on appropriated cytokines.

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 either the 2-way ANOVA followed by Bonferroni post-tests or a Mann-Whitney test. P-values considered as significant are indicated as *** < 0.001, ** < 0.01, and * < 0.05.

Results

L. rhamnosus and *B. breve* reduce Th17 differentiation in PBMCs

To assess the immunomodulatory capacity of the bacterial strains, PBMCs were stimulated with a combination of anti-CD3 together with *L. rhamnosus* or *B. breve*, and the different T cells subsets were analyzed using flow cytometry. Differences were found in the T cell subtype composition within the activated CD4+CD69+ T cells (Figure S1 and Figure 1A - D). Both strains significantly increased Th2 (CD4+CD69+GATA3+ Tbet-; Figure 1A) and decreased Th17 (CD4+CD69+RORγ+FOXP3-; Figure 1B) cell subsets. Incubation with *B. breve*, but not *L. rhamnosus,* led to a significantly increased Treg (CD4+CD69+RORγ-FOXP3+; Figure 1C) and decreased Th1 cell (CD4+CD69+ GATA3+Tbet-; Figure 1D) subsets. To further confirm the changes in T cell subsets, the IL-4, IL-17, IL-10 and IFNγ producing CD4+ T cells within total PBMCs were analyzed after 7 days of stimulation (Figure S2 and Figure 1E - H). Cytokine expression of CD4+ T cells stimulated with anti-CD3 and the bacteria mirrored the results seen with the transcription factors. Both *L. rhamnosus* and *B. breve* significantly increased the population of CD4+IL-4+ T cells and decreased the population of CD4+IL17+ T cells (Figure 1E and F). No changes were observed in the CD4+IL-10+ and CD4+IFNγ+ T cell populations for both bacteria (Figure 1G and H). The results of FMO controls and isotype controls indicate that the staining antibodies were working sufficiently and that we used proper gate-settings (Figure S1 and S2).
These data indicate that *L. rhamnosus* as well as *B. breve* are able to limit the differentiation of CD4+ T cells *in vitro* towards Th17 cells. Additionally, *B. breve* induced *de novo* Treg induction and reduced Th1 cells.

Intervention with *B. breve*, but not L. rhamnosus, ameliorates DSS-induced colitis

To study the effect of the L. rhamnosus and B. breve strains in vivo, the murine DSS-induced colitis model was used. Mice received L. rhamnosus or B. breve 9 days prior to colitis induction and the bacterial administration was continued until the end of the experiment. Control mice receiving bacteria did not display any clinical changes (data not shown). DSS treatment increased feces condition score, histology score and mildly reduced body weight and the colon length. Intervention with B. breve, but not L. rhamnosus led to improvement of feces condition and to a significant reduction of DSS-induced colon shortening, colon epithelial damage and cellular infiltration as compared to mice with DSS treatment alone (Figure 2A-D).

In order to visualize changes in infiltrating inflammatory cells in the colon after DSS treatment, immunohistochemistry was employed to determine the number of cells expressing Ly-6B, which is expressed on the surface of neutrophils and inflammatory macrophages [27]. DSS treatment significantly enhanced the infiltration of Ly-6B+ cells. Mice treated with DSS and B. breve intervention tended to have reduced amounts of Ly-6B+ cells in the colon (Figure

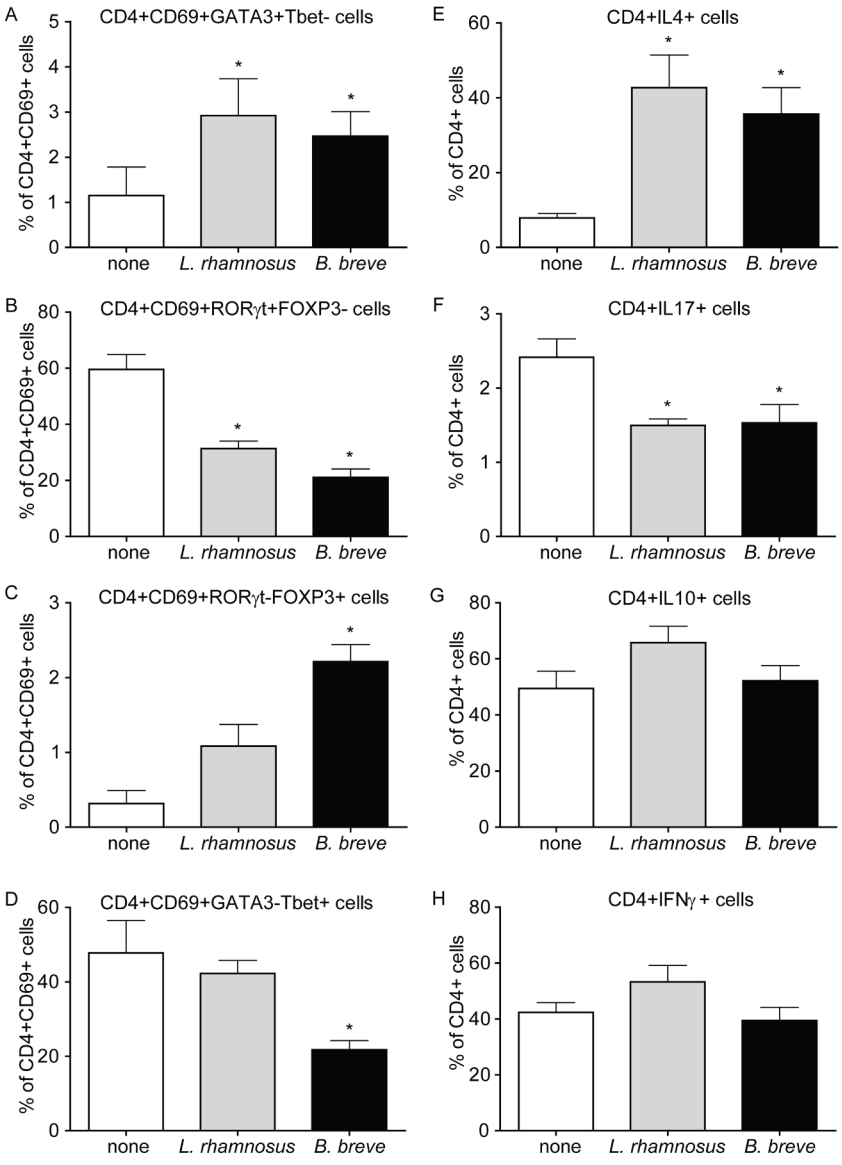
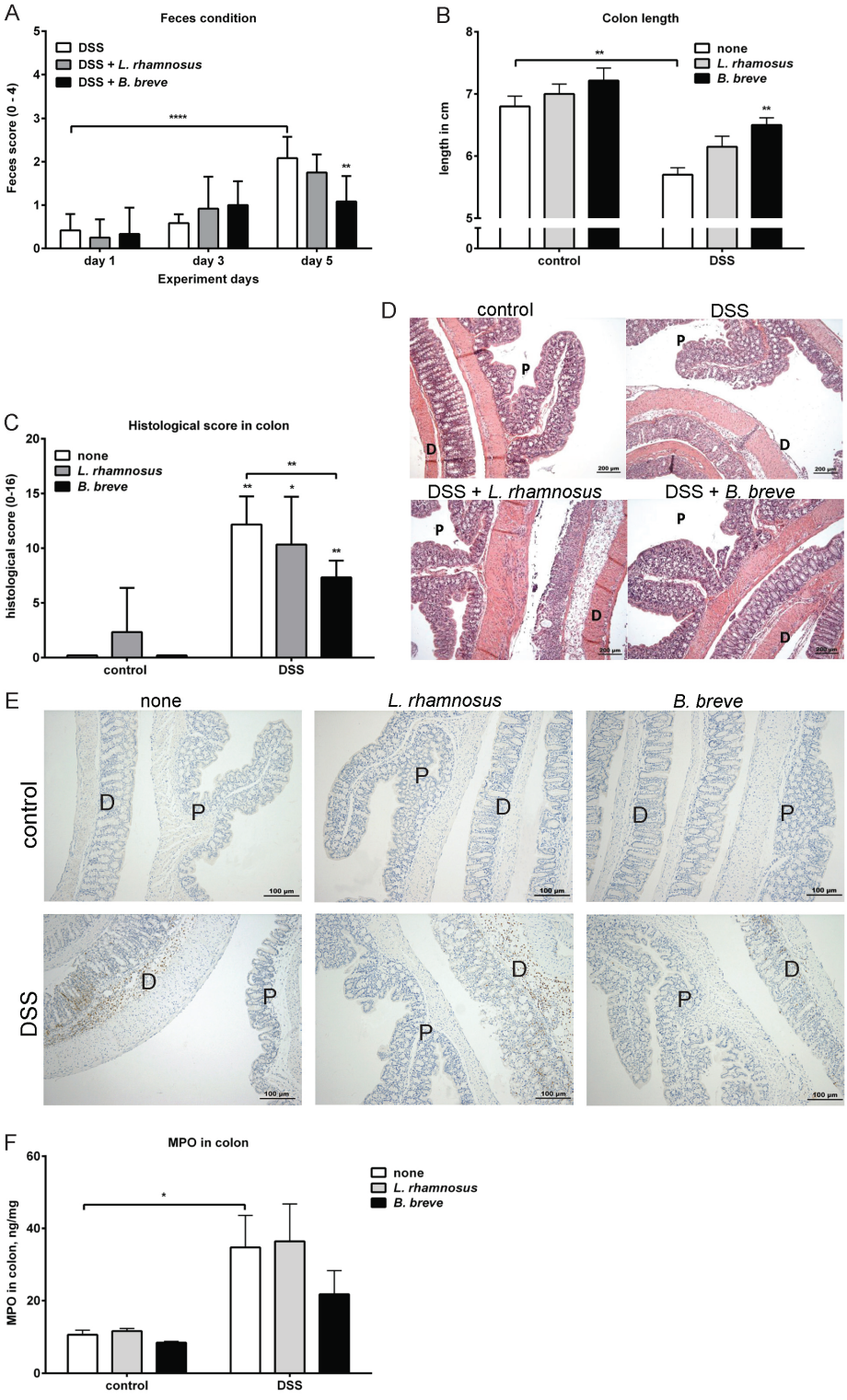


Figure 1. *L. rhamnosus* and *B. breve* alter T cell differentiation in human PBMCs

PBMCs were stimulated with anti-CD3 alone (white bars), with a combination of anti-CD3 and *L. rhamnosus* (grey bar) or a combination of anti-CD3 and *B. breve* (black bar) for 48 hours or 7 days. A-D) The percentages of Th2 (GATA3+Tbet-), Th17 (RORγ+FOXP3-), Treg (RORγ-FOXP3+) or Th1 (GATA-Tbet+) cells within the activated T cells (CD4+CD69+) in the PBMCs were determined after 48 hours of incubation. Percentages within activated CD4+CD69+ T cell population are shown. E-H) The percentages of cytokines (IL10, IL17, IL4 or IFNγ) producing CD4+ T cells in the PBMCs were determined after 7 days of incubation. Percentages within CD4+ T cell population are shown. The Results are expressed as mean ± SEM, n = 3, * $p<0.05$.


A
Feces condition
DSS
DSS + L. rhamnosus
DSS + B. breve
Feces score (0 - 4)
****
**
day 1
day 3
day 5
Experiment days
B
Colon length
none
L. rhamnosus
B. breve
length in cm
**
**
control
DSS
C
Histological score in colon
none
L. rhamnosus
B. breve
histological score (0-16)
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control
DSS
D
control
DSS
DSS + L. rhamnosus
DSS + B. breve
P
D
200 µm
E
none
L. rhamnosus
B. breve
control
DSS
D
P
100 µm
F
MPO in colon
none
L. rhamnosus
B. breve
MPO in colon, ng/mg
*
control
DSS

Figure 2. B. *breve*, but not L. *rhamnosus*, ameliorates DSS-induced colitis

C57BL/6 mice with or without probiotics treatment received either normal drinking water or drinking water with DSS for 5 days. A) The fecal condition was calculated on day 0, day 3 and day 5 after DSS treatment. On day 6, the mice were sacrificed and B) the colon length of each mouse was measured. Results are expressed as mean ± SEM, n = 6 mice per group, pooled from two independent experiments. Colons were collected and examined for histological score as described in materials and methods. C) The histological scoring graph and D) representative H&E staining photos are shown. Results are expressed as mean + SEM, n = 3 mice per group, pooled from two independent experiments. E) The presence of Ly-6B+ cells was visualized in the proximal (p) and distal (d) colons using immunohistochemistry. The pictures are representative of 3 separate mice per group obtained from two experiments. F) The concentration of MPO was measured in colon homogenates of each group. Results are expressed as mean ± SEM, n = 4 mice per group, pooled from two independent experiments. * $p < 0.05$; ** $p < 0.01$

2E). Consistent with the Ly-6B staining, quantification of MPOconcentration (an indicator for neutrophil influx) in the colon showed that DSS treatment significantly increased the MPO concentration in colon of colitis mice.
Intervention with *B. breve* reduced the MPO expression by approximately 35%, although no significant different was determined (Figure 2F). These data indicate that *B. breve* intervention leads to improvements in the outcome of DSS-induced colitis in mice.

B. breve intervention enhances the mRNA expression of Th2- and Treg-associated cytokines in distal colon

As both *L. rhamnosus* and *B. breve* were able to alter T cell differentiation *in vitro*, we investigated if *L. rhamnosus* and *B. breve* induced similar changes *in vivo* during colitis. DSS-induced colitis, on its own, significantly increased the mRNA expression of *Ifnγ, Il6, Il17* and *Tgfβ* as compared to controls. *L. rhamnosus* intervention did not modulate the transcription of cytokines in healthy control mice nor DSS-treated mice, except for a significant increase of *Il5* in DSS-treated mice (data not shown).
B. breve administration in healthy control mice, on the other hand, significantly increased mRNA transcription of Th2- (*Il4*, *Il5* and *Il13*) and Treg- (*Il10* and *Tgfβ*) associated cytokines as well as *Il23* in the colon. In contrast, Th1- (*Ifnγ* and *Il12)* associated cytokines (Figure 3A) were unaffected. *B. breve* intervention of DSS-treated mice induced a similar mRNA cytokine expression pattern in the colon as healthy control mice with *B. breve intervention*. However, the expression was more pronounced and significantly increased *Il6* and *Il17* mRNA expression levels were observed (Figure 3B). These results demonstrate that *B. breve* intervention alters mRNA expression patterns in the colon and increased the mRNA expression of *Il6* and *Il17,* and Th2 and Treg-associated cytokines.

B. breve intervention leads to increased numbers of Foxp3+ cells in the colon and altered Treg and Th17 cell populations in the Peyer's patches during colitis

As intervention with B. breve led to significant changes in cytokine transcription that were indicative for skewing in the T cell response towards a Th2 and Treg response combined

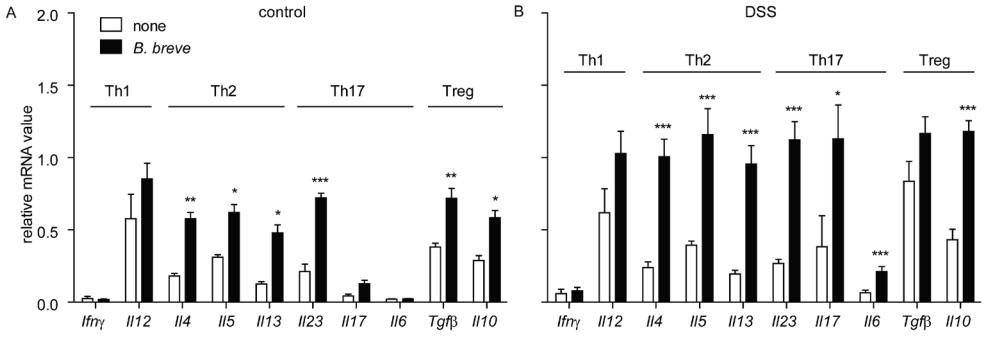

Figure 3. *B. breve* intervention changes mRNA expression of Th2-, Th17- and Treg- associated cytokines in the colon.

The mRNA expression of Th1- (*Ifnγ* and *Il12*), Th2- (*Il4, Il5* and *I13*), Th17- (*Il23* and *Il17*) and Treg- (*Tgfβ* and *Il10*) associated cytokines was quantified in the distal colons of both A) healthy and B) DSS-treated mice with or without *B. breve* intervention. Results are expressed as mean ± SEM, n = 5 mice per group, pooled from two independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

with a Th17 response, we assessed the mRNA expression of Th17-, Th2- and Treg-associated transcription factors; Rorc, Gata3 and Foxp3, respectively, in the colon. Significantly increased Gata3 and Foxp3 mRNA expression levels were detected in both healthy and DSS-treated mice receiving *B. breve*, while no different was detected for *Rorc* expression in both healthy and DSS-treated mice receiving *B. breve* (Figure 4A).

To determine whether increases in the regulatory T cell response caused by *B. breve* intervention were also reflected by an increased number of Foxp3+ cells in the colon, we visualized and quantified colon Foxp3+ cells using immunohistochemistry. Indeed, increased numbers of Foxp3+ cells were found in the colon of DSS-treated mice with *B. breve* intervention (Figure 4B). It has been shown that the conditions, which favor Treg development, naturally antagonize Th17 polarization [28].

Since Th17 cells express the transcription factor RORγt [5], we also examined the numbers of RORγt+ cells in the colon using immunohistochemistry. RORγt+ cells were found primarily in the lymphoid follicles and we analyzed the number of these cells per 0.01 mm^2 colonic patch. When analyze the effect of *B. breve* intervention on the number of RORγt+ cells in colonic patches, taking the *B. breve* intervention and exposure to water or DSS together, a trend of decreasing the amount of RORγt+ cells was observed in mice with *B. breve* (Two way ANOVA: $F_{1,8}$=4,29 p=0.07, Figure 4 C).

Although only a trend in reducing the number of RORγt+ cells by *B. breve* was observed in the colonic patches, analysis of CD4+ T cells within GALT, namely the Peyers patches of the small intestine, using flow cytometry revealed that *B. breve* intervention significantly decreased the Th17 (CD4+RORγt+Foxp3-) cell subset in Peyer's patches and significantly increased the Treg (CD4+RORγt-Foxp3+) cell subset (Figure 4D and Figure S3).

These results indicate that *B. breve* intervention is capable of increasing the Treg cell population and decreasing Th17 cells in the GALT during colitis.

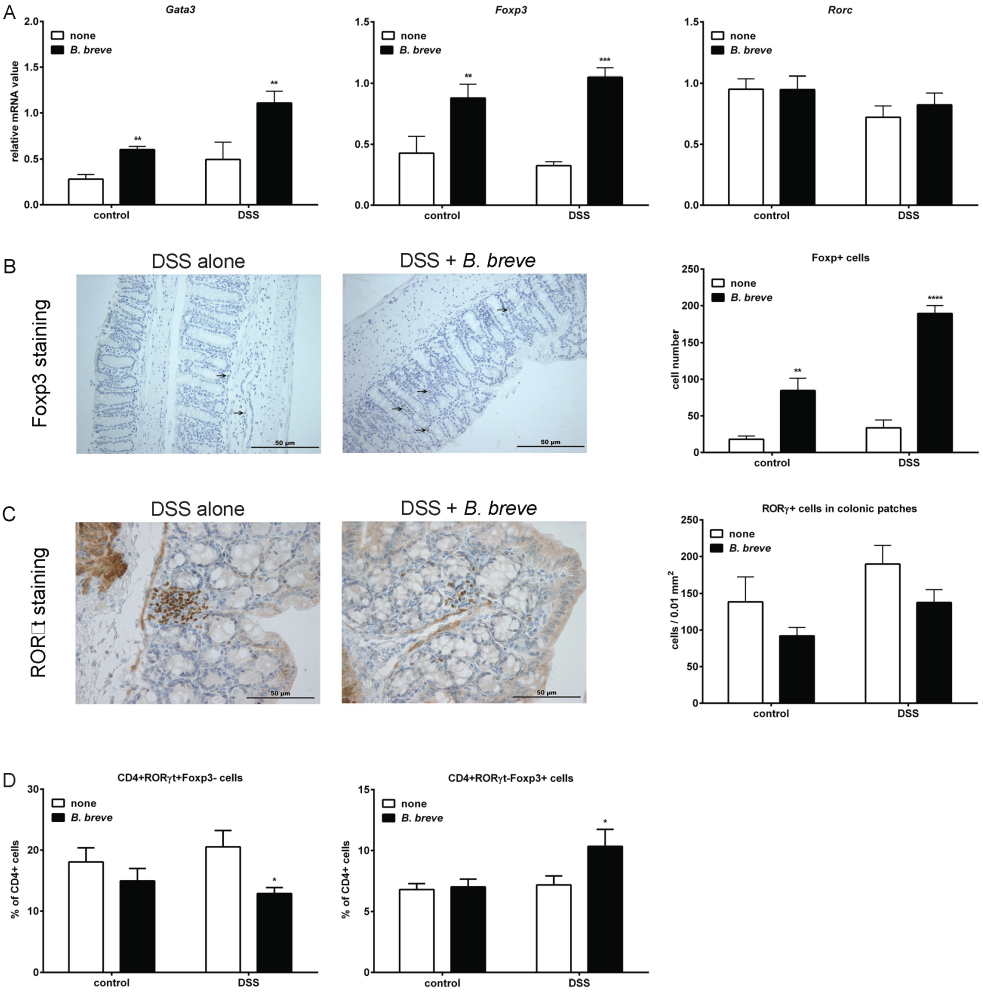

Figure 4. *B. breve* intervention leads to increased numbers of Foxp3+ cells in the colon and Peyer's patches.

A) The mRNA expression of Th2- (*Gata3)*, Th17- (*Rorc)* and Treg- (*Foxp3*) associated transcription factors was quantified in the distal colons of both healthy and DSS-treated mice with or without *B.breve* intervention. Results are expressed as mean + SEM, n = 5 mice per group, pooled from two independent experiments. B) Foxp3+ cells cells were visualized in the colon of DSS-treated mice with or without *B. breve* intervention using immunohistochemistry. The number of Foxp3+ cells was determined as described in the materials & methods and shown in the graph, The pictures are representative of n = 3 mice per group obtained from two independent experiment. C) RORγt+ cells were visualized in the colon of DSS-treated mice with or without *B. breve* intervention using immunohistochemistry. The number of RORγt+ cells was determined as described in the materials & methods and depicted in the graph. D) The percentage of Th17 cells (CD4+RORγt+Foxp3-) and Treg cells (CD4+RORγt-Foxp3+) was determined in the Peyer's patches obtained from both healthy and DSS-treated mice with or without *B. breve* intervention. Percentages within CD4+ T cell population are shown. Results are expressed as mean ± SEM, n = 6 mice per group, pooled from two independent experiments. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$.

Discussion

While the incidence rate of IBD has increased [29], 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 [30]. In the last decade, probiotics, defined as "live microorganisms that when administrated in adequate amounts, confer a health benefit on the host", have been proposed as potential candidates for IBD treatment. The increased interest in the immunomodulatory properties of specific probiotic strains stems from the success of using probiotics to treat a varied number of intestinal diseases [20]. Since a dysregulated T cell response is a common feature in IBD [31], we assessed the capability of two probiotic strains, *L. rhamnosus* NutRes 1 and *B. breve* NutRes 204 to modulate the development of different T cell subsets *in vitro*, using PBMCs isolated from healthy volunteers. In addition, the effect of these specific bacterial strains on the experimental colitis and the development of different T cell subset *in vivo* have been assessed. We hypothesize that these specific gut-derived bacterial strains could have protective effects on experimental colitis via their capability to modulate the development of different T cell subsets.

Our results were generally consistent with the results from previous study by Plantinga *et al* concerning the same bacteria [26]. In our study, significantly decreased CD4+CD69+RORγt+FOXP3 and CD4+IL-17+ T cell subsets were observed in PBMCs stimulated with both bacteria, however, only *B. breve* stimulation led to a reduction of the Th1 cell subset. In addition, we found *B. breve* stimulation significantly increased the FOXP3+ Treg cell subset, which is often associated with anti-inflammatory effects [12], suggesting an anti-inflammatory property of this bacterial strain.

The increased Th2 cell subset found in PBMCs stimulated with *B. breve* may contribute to the decreased Th1 cell subset due to the mutual antagonizing effects of Th1 and Th2 cells on each other [32]. Increased Th2 cells and CD4+IL4+ T cell subsets were also observed in PBMCs stimulated with *L. rhamnosus,* but no change in the Th1 cell subset was seen suggesting that *L. rhamnosus* may have a different T cell modulating mechanism.

The capability of *B. breve* to alter T cell differentiation by inducing Treg cell and reducing Th17 cell development *in vitro* indicates that using this specific bacterial strain *in vivo* may have a protective function in IBD. Murine colitis models are a useful tool to examine the clinical efficacy and possible working mechanism of probiotics in the development of IBD. A multitude of *Bifidobacteria* strains have shown protective effect in colitis models. For example, a mixture of probiotics including *Bifidobacterium longum* induces Treg cell expansion and prevents trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice [33]. In addition, prior administration of a probiotic mixture, including four *Bifidobacteria* strains, to DSS-treated mice also demonstrated protective effects [34,35].

Here, we tested the effect of *B. breve* administration in a DSS-induced colitis model. For a long time, the DSS-induced acute colitis model was regarded by some as an erosive, self-limiting model of colonic injury and inflammation. A previous study has demonstrated that T cells are not necessary in DSS-induced colitis [36]. However, recent studies show that

bacteria penetrated the mucosa layer before inflammation in acute colitis model [37] and microflora is necessary during the development of DSS colitis [38]. Penetration of bacteria in the mucosal layer will lead to the activation of resident innate immune cells that in turn can lead to an adaptive immune response where T cells are involved. Indeed, we have recently demonstrated that antigen-specific T cells develop during the acute stage of DSS-induced colitis [39]. In addition, it has been shown that transient Treg depletion leads to increased severity of DSS colitis [40]. We hypothesized that the induction of Treg cells in the intestinal mucosa by intervention with *B. breve* could induce protective effect during DSS-induced colitis. The DSS colitis model is, thus, a valuable model to investigate the rise of T cell associated responses during intestinal inflammation mimicking early IBD. Altogether, T cells can affect the development of acute colitis, although the specific mechanism still needs to be investigated.

In this study, our data show that intervention with *B. breve* is beneficial in DSS-induced colitis by improving the weight loss, fecal condition, colon histology score which includes epithelial damage and cellular infiltration and colon shortening. DSS-induced colitis is often associated with increased MPO activity, which is indicative for an increased number of infiltrating neutrophils [41]. In line with this finding, increased numbers of LyB6+ cells and increased MPO levels were found in the colons of DSS-treated mice. DSS-induced enhancement of MPO expression was decreased by 35% due to *B. breve* intervention, although this did not reach significance. Intervention with *L. rhamnosus* did not affect the DSS-induced colitis, which is similar to results found in experiments using *Lactobacillus rhamnosus GG* performed by Mileti *et al* [42]. A possible explanation could be that *L. rhamnosus* is less able to modify the T cell composition as compared to *B. breve*. it has been postulated that it is essential to target both Th1 and Th17 cells for treatment for CD, the major form of IBD [43]. The fact that *L. rhamnosus* is not as protective for DSS-induced colitis as *B. breve* could be explained by data from the *in vitro* experiments that show exposure to *B. breve* reduced both Th1 and Th17 cell subsets, whereas exposure to *L. rhamnosus* only reduced the Th17 cell subset. In addition, *B. breve* increased the expression of Treg cell–associated cytokines and transcription factors *in vivo*, while *L. rhamnosus* did not induce any of these changes.

Analysis of mRNA expression in the colon showed increased expression of Th2 (*Il4, Il5 and Il13*)- and Treg (*Il10*)-associated cytokines in both healthy and DSS-treated mice with *B. breve* intervention. An increased Th2 response often results in a decreased Th1 response due to the mutual antagonizing effects of Th1 and Th2 cells on each other [32]. Treg cells are able to repress the activity of other T cell subsets to induce an anti-inflammatory effect [12]. There are two major regulatory T cell populations, namely Foxp3+ Treg and IL10-producing type 1 regulatory T (Tr1) cells that are know to maintain intestinal homeostasis [44]. Therefore, it can be concluded that besides Foxp3+ Treg cells, Tr1 cells could also be involved. Interestingly, the increased *Il10* expression in the colon is in line with recent findings, which demonstrated an increased number of IL10 producing Tr1 cells in the colon after *B. breve* intervention [35].

Next to the increased Th2- and Treg-associated cytokines, we also observed an increased mRNA expression of Th17 associated cytokines including the effector cytokine IL17 in DSS treated mice with *B. breve* intervention as compared to DSS treatment alone. It should be noted that Th17 cells are not the only source of IL17 production as it was demonstrated that also innate lymphoid cells can produce IL17 upon activation by IL23 derived from macrophages and dendritic cells [45][46]. RORγt is the master transcription factor of Th17 cells [5], but is also expressed in IL17-producing innate lymphoid cells (ILC) [47]. We did not observe an effect of either DSS or treatment with *B.* breve on the expression of *Rorc* in the colon. The increased expression of *Il17* in the colon observed after *B. breve* intervention might be the result of IL23 mediated activation of resident ILC that are mainly found in the lamina propria in close proximity of epithelial cells. However, another possibility is that another, RORγt independent, IL17 producing source is present in the colon, such as B cells [48]. Although IL17 is often thought to promote the development of IBD [49], a recent study has demonstrated a protective function of IL17 in intestinal inflammation [50]. The exact role of IL17 during the IBD development still needs to be elucidated in additional studies.

Treg cells are associated with anti-inflammatory and tolerance inducing mechanisms [12,51]. Although it is not totally clear how Treg cells effect the development of IBD, lack of Treg cells are often found in IBD patients [14,15]. Animal models of IBD have further demonstrated the importance of Treg cells during the development of colitis [17,33]. Foxp3 expression is associated with Treg cell development [6,7] and the anti-inflammatory properties of Foxp3+ Treg cells have been demonstrated by a number of studies in both mice and humans [10,52]. In this study, *B. breve* stimulation induced Treg cell differentiation *in vitro* and *in vivo.* Moreover, *B. breve* intervention ameliorated DSS-induced colitis symptoms and increased Foxp3+ T cells in Peyer's patches. Recent studies have demonstrated that the home of Peyer's patches, the small intestine, is involved in DSS colitis [53,54]. Peyer's patches, like other lymphoid organs, contain dendritic cells that taken up antigens and present them to T cells, leading to T cell activation and differentiation. The increased Foxp3+ T cells in Peyer's patches indicate T cell differentiation that favors anti-inflammatory response. The resulting activated and expanded T cells have the potential to travel to the colon and induce immune regulation [55,56,57]. In line with this hypothesis, an increased amount of Foxp3+ cells was found in the colon of mice with *B. breve* intervention. These data suggest that the protective effects of *B. breve* on DSS colitis might lie with its capability to induce Treg cells.

In conclusion, *B. breve* NutRes 204 stimulation *in vitro* leads to T cell skewing toward Treg cells in human PBMCs. Additionally; intervention with *B. breve* NutRes 204 ameliorated DSS-induced colitis symptoms *in vivo* with an increased amount of Treg cells and a reduced amount of Th17 cells in the GALT. This suggests that patients suffering from IBD could potentially benefit from *B. breve* intervention.

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Supplementary figures and legends:

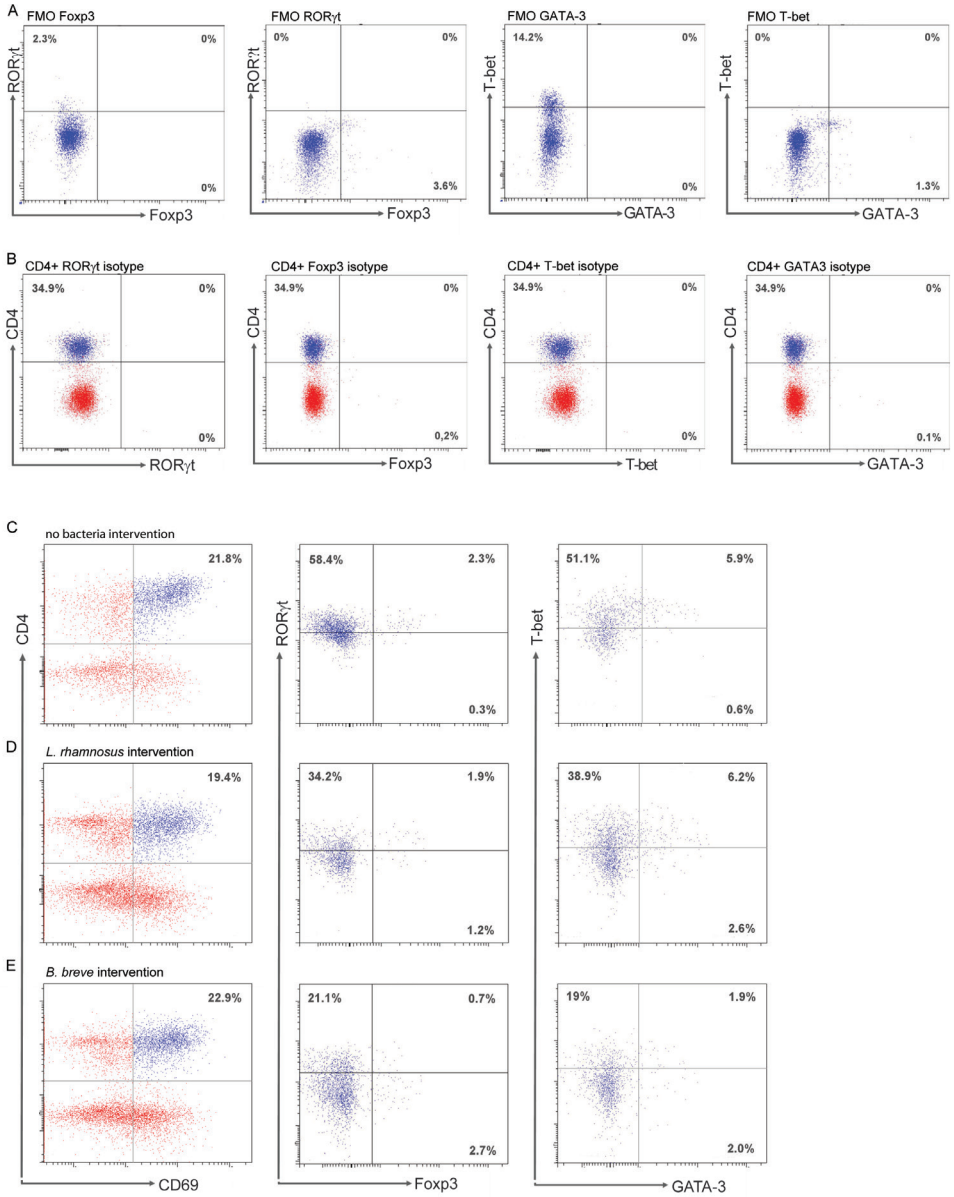

Figure S1. FACS dot plots of T cell composition in human PBMCs with or without bacteria intervention

FACS dot plots of A) fluorescence minus one (FMO) controls and B) isotype controls of FOXP3, RORγ, GATA3 and Tbet staining antibodies within CD4+ T cells are shown. Representative FACS dot plots of Th2 (GATA3+Tbet-) and Th1 (GATA-Tbet+), Th17 (RORγ+FOXP3-) and Treg (RORγ-FOXP3+) cells in the PBMCs after 48 hours incubation with either C) anti-CD3 stimulation alone, D) a combination of anti-CD3 and *L. rhamnosus,* or E) a combination of anti-CD3 and *B. breve* are illustrated. The percentage of activated CD4+CD69+ T cells is calculated within total live cells and the percentage of Th2, Th1, Th17 and Treg cells are shown within activated CD4+CD69+ T cell population.

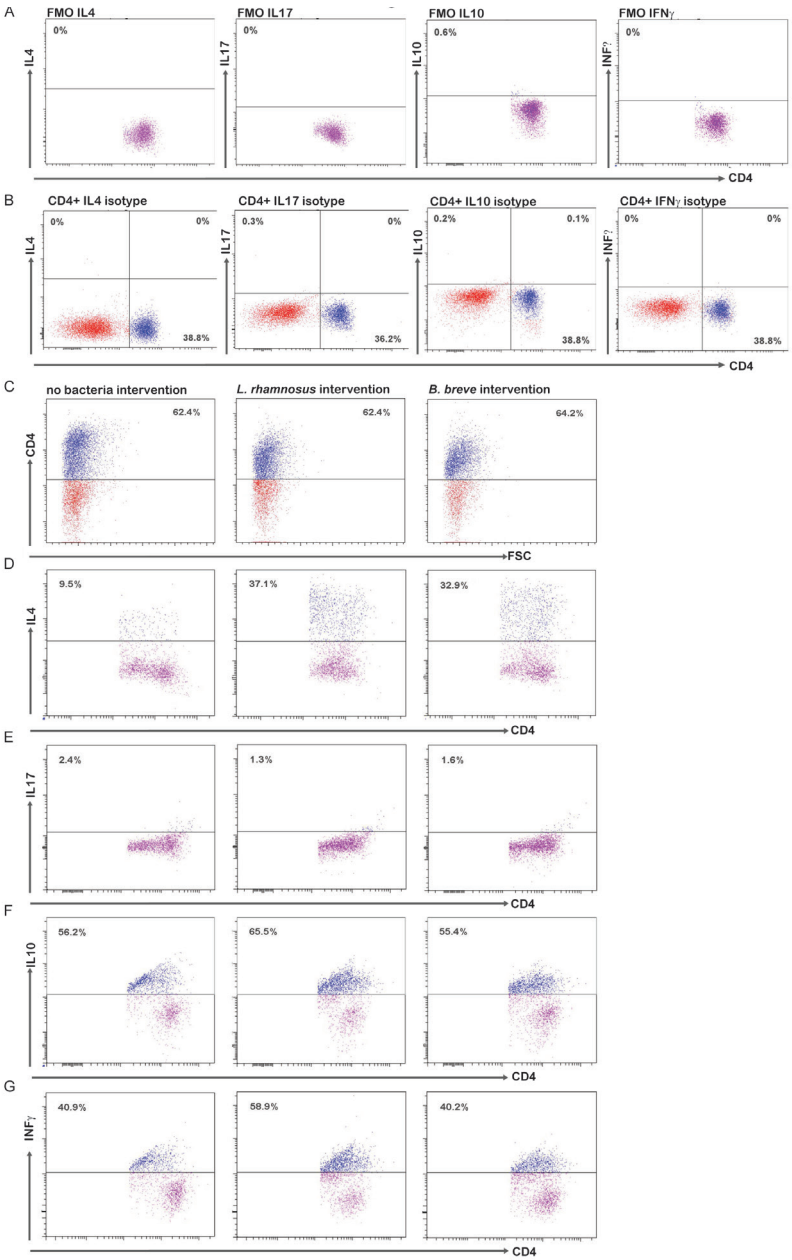

Figure S2. FACS dot plots of T cell – associated cytokine producing T cells in human PBMCs with or without bacteria intervention

A) FACS dot plots of A) fluorescence minus one (FMO) controls and B) isotype controls of IL4, IL17, IL10 and IFNγ staining antibodies within CD4+ T cells are shown. Representative FACS dot plot of CD4+ T cells in the PBMCs after 7 days stimulation with anti-CD3 alone, or a combination of anti-CD3 with either *L. rhamnosus* or *B. breve* are shown in C). Gated on the CD4+ T cells, the percentages of D) IL4+, E) IL17+, F) IL10+ and G) IFNγ+ CD4+ T cells were determined. The percentage of CD4+ T cells is calculated within total live cells and the percentages of IL4+, IL17+, IL10+ and IFNγ+ CD4+ T cells are presented within CD4+ T cell population.

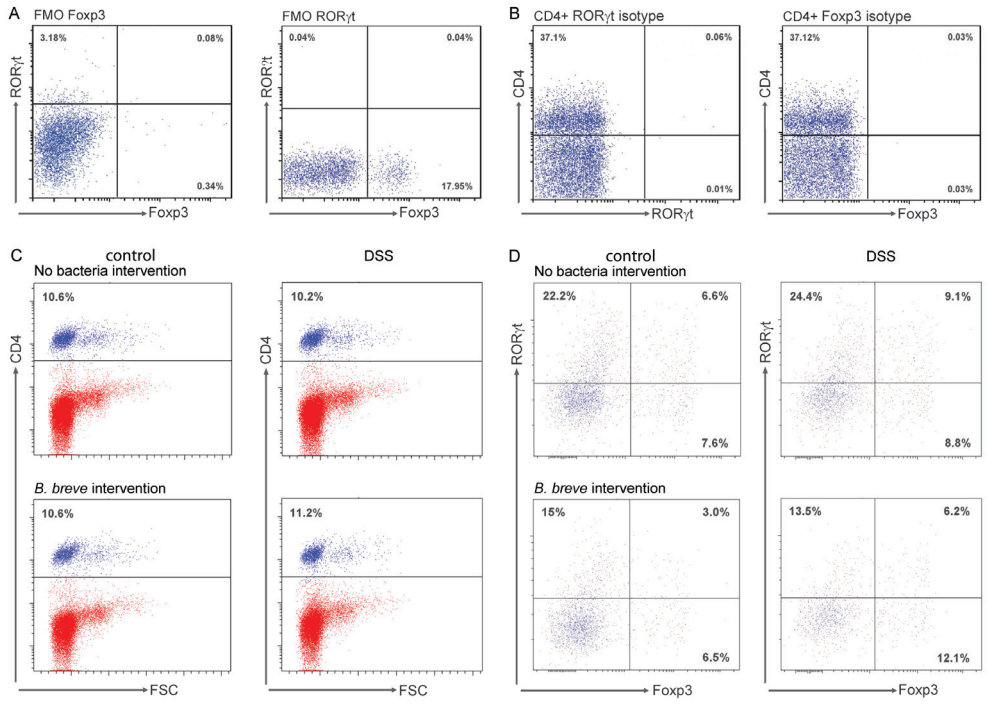

Figure S3. FACS dot plots of Treg cells and Th17 cells in the mice with or without *B. breve* intervention

A) FACS dot plots of A) fluorescence minus one (FMO) controls and B) isotype controls of Foxp3 and RORγt staining antibodies within CD4+ T cells are shown. Representative FACS dot plots of CD4+ cells in the Peyer's patches obtained from both healthy and DSS-treated mice, with or without *B. breve* intervention, are shown in C). Gated on CD4+ T cells, the percentages of D) Th17 (CD4+RORγt+Foxp3) and Treg (CD4+RORγt-Foxp3+) cells were determined. The percentage of CD4+ T cells is calculated within total live cells and the percentages of Th17 and Treg cells are determined within CD4+ T cell population.

Chapter 6

TRANSCRIPTIONAL MODULATION OF PATTERN RECOGNITION RECEPTORS IN CHRONIC COLITIS IN MICE IS ACCOMPANIED WITH TH1 AND TH17 RESPONSE

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This article is being submitted

Abstract

Pattern recognition receptors (PRRs) may contribute to inflammatory bowel diseases (IBD) development due to their microbial-sensing ability and the unique microenvironment in the inflamed gut. In this study, the PRR mRNA expression profile together with T cell-associated factors in the colon was examined using a chronic colitis mice model. 8-12 week old C57BL/6 mice were exposed to multiple dextran sodium sulfate (DSS) treatments interspersed with a rest period to mimic the course of chronic colitis. The clinical features and histological data were collected. The mRNA expressions of colonic PRRs, T cell-associated components were measured. Finally, the colons were scored for Foxp3+ cells. During chronic colitis, the histological data, but not the clinical manifestations demonstrated characteristic inflammatory symptoms in the distal colon. In contrast to acute colitis, the expression of all *Toll-like receptors (Tlrs)*, except *Tlr5* and *Tlr9*, was unaffected after repeated DSS treatments. The expression of *Nod1* was decreased, while *Nod2* increased. After third DSS treatment, only the expressions of *Tlr3* and *Tlr4* were significantly enhanced. Unlike other PRRs, decreased *Tlr5* and increased *Tlr9* mRNA expression persisted during the chronic colitis period. As the colitis progress, only the mRNA expression of *Ifnγ* and *Il17* staid increased during chronic colitis, while the acute colitis-associated increase of *Il23,* and *Il10* and *Il12* was abolished. Finally, increased histological score of Foxp3+ cell in colon was found during the chronic colitis period. This study provides an expression pattern of PRRs during chronic colitis that is accompanied by a Th1- and Th17 cell-mediated immune response.

Introduction

Inflammatory bowel disease (IBD) is a chronic gastrointestinal disorder and is characterized by a relapse-remitting course that is caused by recurrent intestinal inflammation [1]. The two major forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC), often have an onset during early adulthood and significantly affect the quality of the life [2]. The established high prevalence of IBD population in US and Europa indicate the requirement for an efficient treatment for IBD [2]. However, the existing treatments are mainly focus on relieving of symptoms and are often accompanied with unwanted side effects [3]. To elucidate the disease pathogenesis and develop more efficient treatment, there is growing interest for targeting pattern recognition receptors (PRRs). The unique microenvironment in the gut, where abundant microorganisms co-exist and the microbial-sensing ability of PRRs suggest that PRRs could contribute to both maintaining and breakdown of the intestinal homeostasis [4,5]. The development of gut dysbiosis and imbalances in host-microbiome interaction has been demonstrated to contribute to the extent, severity and chronicity of intestinal inflammation [2]. Furthermore, an aberrant immune response towards gut bacteria has been suggested to be the major contributor in the inflammatory response of IBD [6].

Toll-Like receptors (TLRs) are possibly the best studied PRRs that sense a broad spectrum of invading pathogens by recognize pathogen-associated molecular patterns (PAMPs), unique molecules on these pathogens [7]. Up to date, 13 TLRs have been discovered, from which TLR1-9 are conserved in both human and mouse. Based on the location, these TLRs can be divided into two groups; receptors expressed on the cell surface (TLR1, TLR2, TLR6, TLR4 and TLR5) and intracellular receptors (TLR3, TLR7, TLR8 and TLR9) [7]. To recognize the specific ligand, the TLRs form functional mono-dimers or a functional complex with other component such as MD-2 (for TLR4), except TLR1, TLR2 and TLR6 [7]. Both TLR1 and TLR6 form functional hetero-dimer complex with TLR2 to recognize triacyl- or diacyl-lipopeptides from bacteria, respectively [8]. Upon activation by specific ligand derived from bacteria, fungi, parasites or virus, they will induce innate immune response and strength adaptive immune response to provide protection against pathogens [9]. However, unregulated activation of TLRs could lead to extensive and chronic inflammation, which results in inflammatory disease such as IBD [4,10]. In agreement with this hypothesis, increased expression of TLRs such as TLR2, TLR3 and TLR4, are found in the colon of IBD patient [11, 12]. In addition, an association was found between the polymorphism of TLRs and the susceptibility of IBD development [13-15].

Nucleotide-binding oligomerization domain-containing protein (human: NOD; mice Nod) is another PRR family. Two member of this PRR group, NOD1 and NOD2 are located exclusively intracellular and able to detect peptidoglycan, a cell wall component on bacteria [16]. There has been great interest in studying the role of NOD2 and its related receptor NOD1 in IBD development, because NOD2 encoding gene *NOD2* is the first gene that has been directly associated with CD and confer great risk for the development of IBD [17,18]. The CD risk variant *NOD2* gene causes 'loss of function' resulting in reduced autophagy induction which

result in reduced bactria killing and impaired antigen presenting, which could be the trigger to the development of IBD [19].

Experimental animal models have been used to study the pathology of the IBD and clarify the underlying mechanisms [20]. Among these animal models, DSS-induced colitis in mice is most commonly used model to investigate IBD-like colitis due to its simplicity and the ability to induce predictable intestinal inflammation [21,22]. While single DSS exposure induces colitis modeling acute injury and repair mechanism, repeated DSS exposure cycles interspersed with recovery period mimic the chronic nature of IBD [23,24].

In our previous study, we have illustrated the colonic expression pattern of PRRs and the T-helper (Th) cell response in mice using a DSS-induced acute colitis model. To extent our knowledge of the expression of these PRRs and activated immune response during chronic colitis, the mRNA expression of these PRRs in the colon during chronic colitis was determined after repeated DSS exposure. In addition, the T cell development during the chronic colitis was monitored by measuring the mRNA expression of T cell-associated master transcription factors and cytokines.

Materials and Methods

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.

Experimental colitis

Chronic colitis was induced in groups of 6 mice by administration of 3 cycles 1.5% DSS to the drinking water of the mice for 6 days with a rest period of 10 days. Colitis development was monitored by measuring the bodyweight and scoring the feces condition during the experiment and measuring the colon length/weight ration after sacrificing the mice on the end of each DSS treatment cycle (day 7, day 23 and day 39). The feces condition assessment was started from experimental day 5 until the end of the experiment (day 39). The feces condition score was determined from two parameters: stool consistency (0 = normal, 1 = soft with normal form, 2 = loss of form/diarrhea) and fecal bleeding (0 = no blood, 1 = blood observation using Colo-rectal Test kit (Axon Lab AG, Germany), 2 = blood observation without test).

Histological evaluation of colon damage and immunohistochemical staining

After sacrificing the mice on the end of each DSS treatment cycle (day 7, day 23 and day 39), colons (n = 3) were taken out for histological evaluation and immunohistochemical staining. The colon was opened longitudinally; half of each colon was washed in the phosphate buffered saline (PBS) and placed on a piece of blotting paper. After fixing in 10% formalin for 24 hours, colons were paraffin-embedded as swiss-roles and sectioned (5μm). Two researchers assessed general inflammatory features blindly after staining sections with hematoxylin and eosin according the assessment system described before [25]. Briefly, the histological 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, 4: infiltration in entire tissue); cover area of cellular infiltration in the region (0: no infiltration, 1: < 25%, 2: 25%-50%, 3: 50%-75%, 4: >75%); loss of crypts (0: no damage, 1: 30% shortening of crypts, 2: 65% shorting of crypts, 3: total loss of crypts, 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%).

Immunohistochemistry was employed to determine the Ly-6B.2+ cells (neutrophils & some activated macrophages). The sections were subjected to a heat-induced epitope retrieval step. Slides were washed with PBS and blocked with rabbit or goat serum before an overnight incubation (4°C) with primary antibodies against Ly-6B.2 (AbD Serotec, Dusseldorf, Germany). For detection, biotinylated goat anti-rat (Dako, Glostrup, DK) secondary antibodies were administered followed by incubation with peroxidase-labeled streptavidin (Vectastain EliteABC kit, Vector, Burlingame, CA USA). The peroxidase activity was visualized using the substrate, DAB (Sigma, Gillingham, UK). The cell nuclei were visualized by a short incubation with Mayer's hematoxylin (Klinipath, Duiven, the Netherlands). Background staining was determined by substituting the primary antibody with a rat IgG isotype control (Abcam, Cambrige, UK). The neutrophil infiltration score was determined using the following 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, 4: infiltration in entire tissue); cover area of cellular infiltration in the region (0: no infiltration, 1: < 25%, 2: 25%-50%, 3: 50%-75%, 4: >75%);

Assessment of Myeloperoxidase concentration in the colon tissue

After sacrificing the mice, colons (n = 3) were taken out for myeloperoxidase (MPO) concentration assessment, marker for neutrophils. The colon was opened longitudinally; half of each colon was separated into proximal colon (characterized by bulges in the colon wall) and distal colon (the region starting from end of proximal portion stretching to the anus). Subsequently, the colons pieces were transferred into RIPA buffer (Thermo Scientific, Rockford, IL USA) and homogenized using a Precellys®24-Dual homogenizer (Precellys, Villeurbanne, France). The homogenates were centrifuged at 14000 rpm for 10 minutes

at 4 °C and the MPO concentration in the supernatant was measured using an ELISA kit according to the manufacturer's protocol (Hycult biotech, Uden, the Netherlands).

mRNA expression analysis

After sacrificing the mice, colons (n = 6) were taken out for gene expression analysis. The colon was opened longitudinally and the proximal colon was distinct from the distal colon by its specific structure (bulges in the colon wall). The total RNA was isolated using the RNAeasy kit (Qiagen, Germantown, MD USA) and, subsequently, reverse transcribed into cDNA using the iScript cDNA sythesis kit (BioRad, Hercules, CA USA). Real-time PCR was performed using iQ SYBR Green super mix kit (BioRad, 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 identical to the primer used in our acute colitis model [25]. The primers for the cytokines: tumor necrosis factor-α (*Tnf),* interleukin-1β (*Il-1beta), Il-6,* monocyte chemotactic protein-1 (*Ccl-2),* interferon-γ (*Ifn-gamma), Il-12p35, Il-4, Il-5, Il-13, Il-23p19, Il-17, Il-10* 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*.

Statistical analysis

Means with SEM are represented in each graph. Statistical analysis was performed using GraphPad Prism version 6.0 for windows (GraphPad Software, San Diego, CA USA). P-values were calculated using either the two-way ANOVA followed by Bonferroni post-tests or a Mann-Whitney test. P-values considered as significant are indicated as *** <0.001, ** < 0.01, and * < 0.05. [a] p<0.05 cycle 2 and cycle 3 control mice compare to cycle 1 control mice. [b] p<0.05 DSS-treated mice compare to control mice after each DSS treatment cycle. [c] p<0.05 cycle 2 and cycle 3 DSS-treated mice compare to cycle 1 DSS-treated mice [d] p<0.05 cycle 3 DSS-treated mice compare to cycle 2 DSS-treated mice.

Results

Repeated DSS treatment induces features of chronic colitis

In a previous study, we have induced an acute colitis by adding 1.5% DSS into drinking water of mice for 6 days and found decreased bodyweight and increased feces score [25]. In the current study, we assessed the bodyweight and fecal phenotype changes of mice undergoing repeated DSS treatments. Acute colitis was accompanied with a decreased bodyweight and increased feces score. After repeated DSS treatments, there were no longer inductions of major bodyweight loss as observed after the first DSS treatment cycle (Figure 1A). During

and after all three cycles of DSS treatment increased feces scores were found (Figure 1B). After sacrificing the mice, the colon length and weight were measured.

There was a decreased colon length/weight ratio after each DSS treatment cycle as compare to healthy mice: this ratio further decreased as the DSS treatment cycle progressed (Figure 1C).

To determine the extent of the inflammation, colonic histological score including tissue damage and cellular infiltration was accessed. The tissue damage and cellular infiltrations were observed in the colon of DSS-treated mice, predominantly in the distal part, after each DSS treatment (Figure 2). The MPO levels and Ly-6B.2 expressing cells in colon were analyzed to determine the infiltration of neutrophil cells. MPO is abundantly expressed in neutrophil granulocytes [26] and is frequently used as marker of neutrophil infiltration, while Ly-6B.2 expression is particularly high on the surface of neutrophils and some inflammatory macrophages [27].

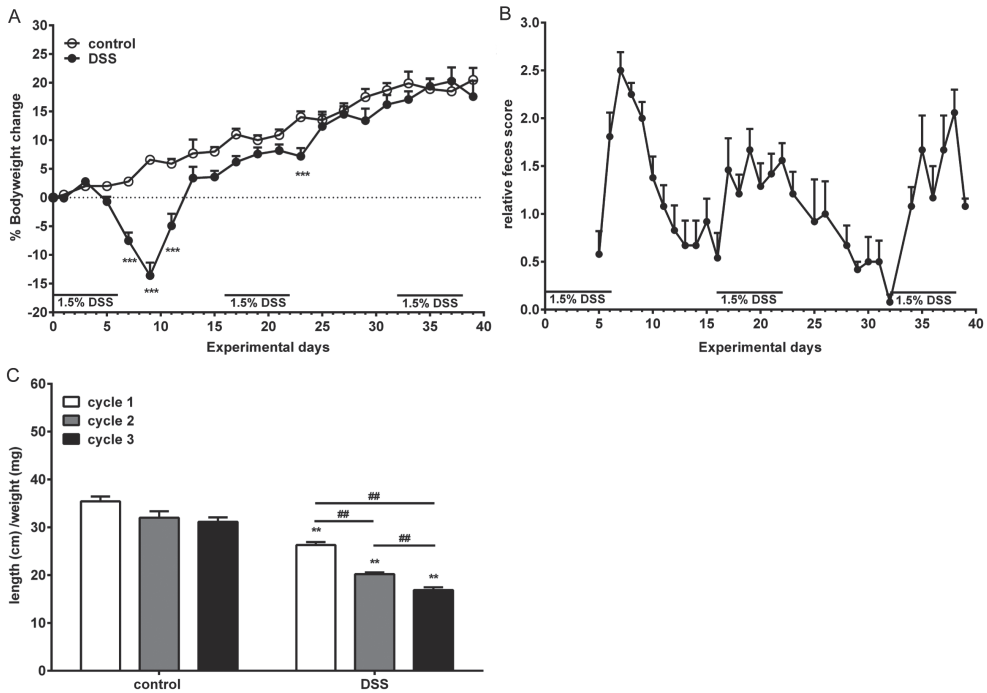

Figure 1. An increased feces score was found after each DSS treatment, while bodyweight loss was only found after acute DSS treatment

A) The bodyweight changes of control and DSS-treated mice during repeated DSS treatment are illustrated in the figure. B) The increased fecal scores of DSS-treated mice as compared to control mice after the first DSS treatment until end of the experiment are shown (n = 18 during cycle 1 DSS treatment, n = 12 during cycle 2 DSS-treatment and n = 6 cycle 3 DSS treatment). C) The colon length (cm) /weight (mg) ratio was determined (n = 6 per group). All results are expressed as ± SEM, * indicates significant different between control and DSS-treated group. # Indicates significant different between the DSS-treatment cycles ##, ** P < 0.01, *** P < 0.001.

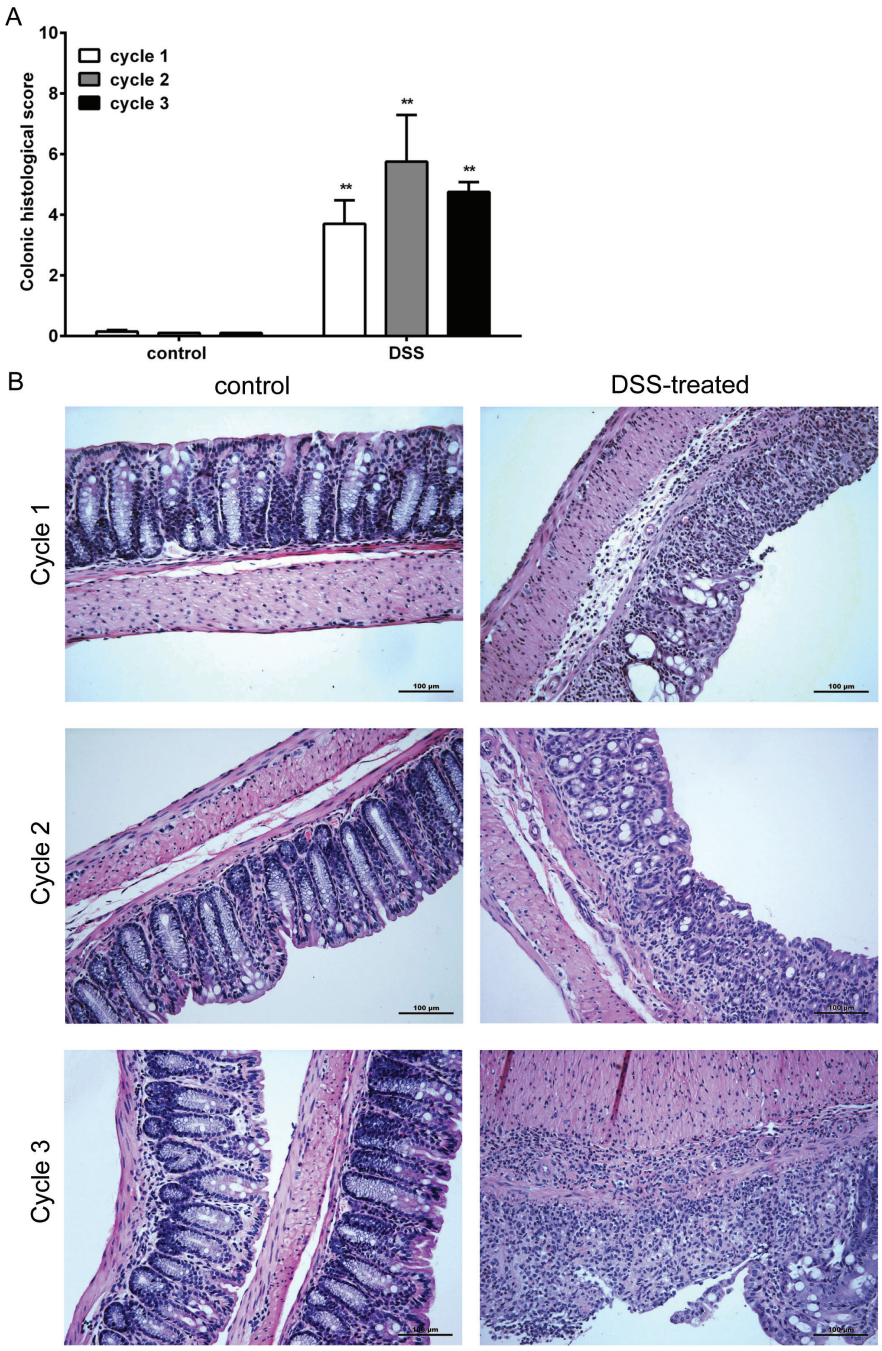

Figure 2. DSS treatment induces damage and cellular infiltration in the colon

Colons were collected for histological assessment as described in material and methods. A) Colonic histological score and B) representative histology staining photos of the colons derived from control or DSS-treated mice after each DSS treatment cycle are shown. Results are expressed as ± SEM (n = 3 per group), ** indicates significant different between control and DSS-treated group P<0.01.

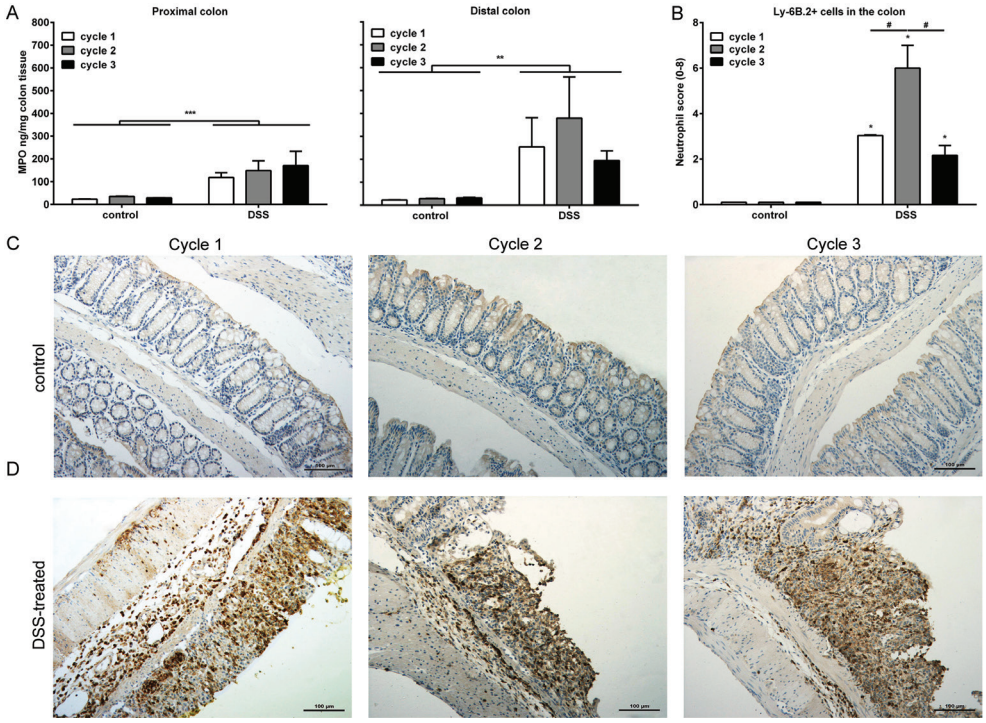

Figure 3. DSS treatment induces neutrophil influx in the colon

Colons were collected for A) MPO concentration measurement in the proximal and distal colon and B) immunohistochemical assessment of Ly-6b+ cells. Representative histochemical staining picture of Ly-6b+ cells in the colons of C) control and D) DSS-treated mice during each DSS treatment cycle are shown. All results are expressed as ± SEM n = 3, * indicates the significant different between control and DSS-treated group. # Indicates the significant different between the DSS-treatment cycles $^{\#,*}$ $P < 0.05$, $^{\#\#,**}$ $P < 0.01$, *** $P < 0.001$.

An increased expression of MPO was found in both proximal and distal part of the colon isolated from DSS-treated mice as compare to healthy control mice after each DSS treatment cycle (Figure 3A). This result indicates an increased amount of neutrophils in the colon of DSS-treated mice. The increased score of Ly-6B.2 expressing cell in the colon of DSS-treated mice supports this finding (Figure 3B-D). In addition, a significant increase of neutrophil score was found after second DSS treatment as compared with first and third DSS treatment cycle (Figure 3 B-D).

Taken together, these results indicate that repeated DSS treatments result in chronic colitis accompanied by profound neutrophil infiltration.

mRNA expression of both *Nod 1* and *Nod 2* was increased during acute colitis, while different mRNA expression patterns were found during the chronic phase

To investigate the mRNA expression of Nod1 and Nod2 during the DSS induced chronic colitis, the specific mRNA expression in both proximal and distal part of the colon isolated

from either healthy or DSS-treated mice was determined (Figure 4). The DSS induced transcriptional modulation was mainly found in the distal part of the colon (Figure 4B). In the proximal part of the colon, there was no significant change of mRNA expression between healthy and DSS-treated mice, except a decreased Nod1 mRNA expression after second DSS treatment (Figure 4A). In the distal part of the colon, there were increased mRNA expressions of both Nod1 and Nod2 during acute colitis, although the expression of Nod1 did not reach significance (Figure 4B). During chronic colitis, the mRNA expression of Nod2 was significantly increased after second DSS treatment and an enhanced trend was observed after third DSS treatment (Figure 4B). In contrast, there was a significantly reduced mRNA expression of *Nod1* during after the second DSS treatment (Figure 4B)

Tlr5 mRNA expression is the only extracellular *Tlr* that is structurally modified after every DSS treatments

To further investigate the mRNA expression of PRRs during the DSS induced chronic colitis, we have accessed the mRNA expression of TLRs located on the cell surface (*Tlr1, Tlr2, Tlr6, Tlr4 and Tlr5*) in both proximal and distal part of the colon isolated from either healthy or DSS-treated mice (Figure 5). The DSS treatment induced modulatory effects on the transcription of bacterial recognizing TLRs were mainly found in the distal part of colon (Figure 5B). In the proximal part of colon, there was no significant change of the mRNA

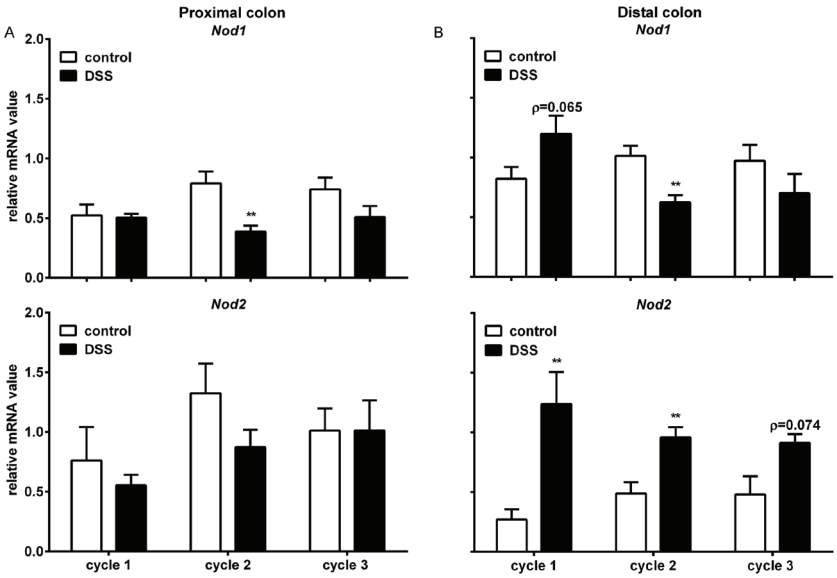

Figure 4. Acute DSS treatment increases both the *Nod 1* and *Nod 2* expression, while repeated DSS treatment induces different effect between Nod1 and Nod2 expression.

Colons were collected for the mRNA expression assessment of PRR. The mRNA expressions of *Nod 1* and *Nod 2* in the A) proximal – and B) distal part of the colon are shown. All results are expressed as ± SEM, n = 6.

** $P < 0.001$

expressions, though a tendency of an increased *Tlr1* expression after third DSS treatment decreased mRNA expression of *Tlr5* after first DSS treatment and *Tlr6* after second DSS treatment was observed (Figure 5A).

In the distal part of colon, the acute colitis was accompanied with significant increased mRNA expressions of Tlr2, Tlr6 and Tlr4. The mRNA expression of Tlr1 was unchanged during acute colitis, whereas a significant reduced expression of Tlr5 was observed (Figure 5B). During chronic colitis, Tlr1 mRNA expression remained unchanged. Interestingly, the significant increased expression of Tlr2 and Tlr6 mRNA during the acute colitis was profoundly reduced after repeated DSS treatment, though Tlr2 tended to be increased after the second DSS treatment. In addition, a significant increased Tlr4 mRNA expression was found after third DSS treatment, but not during second DSS treatment. Tlr5 is the only extracellular TLR that was modulated in a similar way after each DSS treatment: the mRNA expression of *Tlr5* was significantly reduced after each DSS treatment (Figure 5B).

TLR9 mRNA expression is the only intracellular Tlr that is structurally modified after every DSS treatment

Intracellular TLRs comprise TLR3, TLR7 and TLR8 that recognize single- and double-strand virus RNA (dsRNA) and TLR9 that recognize CpG-rich DNA derived from virus and bacteria [28-30].

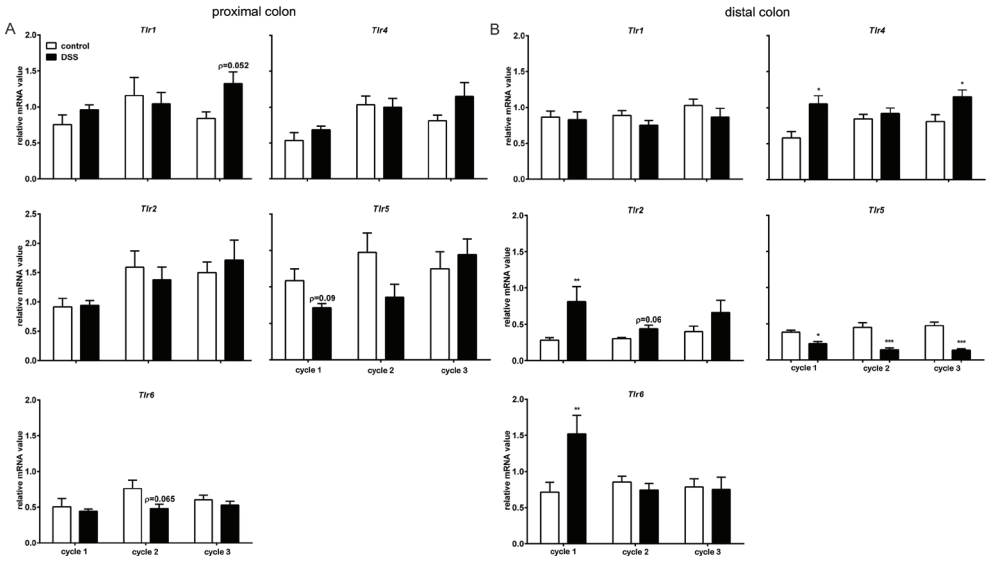

Figure 5. The mRNA expression of bacterial PAMP recognizing *Tlrs* is differently modulated by repeated DSS treatment

Colons were collected for mRNA expression assessment of PRR. The mRNA expressions of *Tlr 1*, *Tlr2*, *Tlr6, Tlr4, Tlr5,* and *Tlr9* in the A) proximal- and B) distal part of the colon are shown. All results are expressed as ± SEM, n = 6. * P < 0.05, ** P < 0.01, *** P < 0.001.

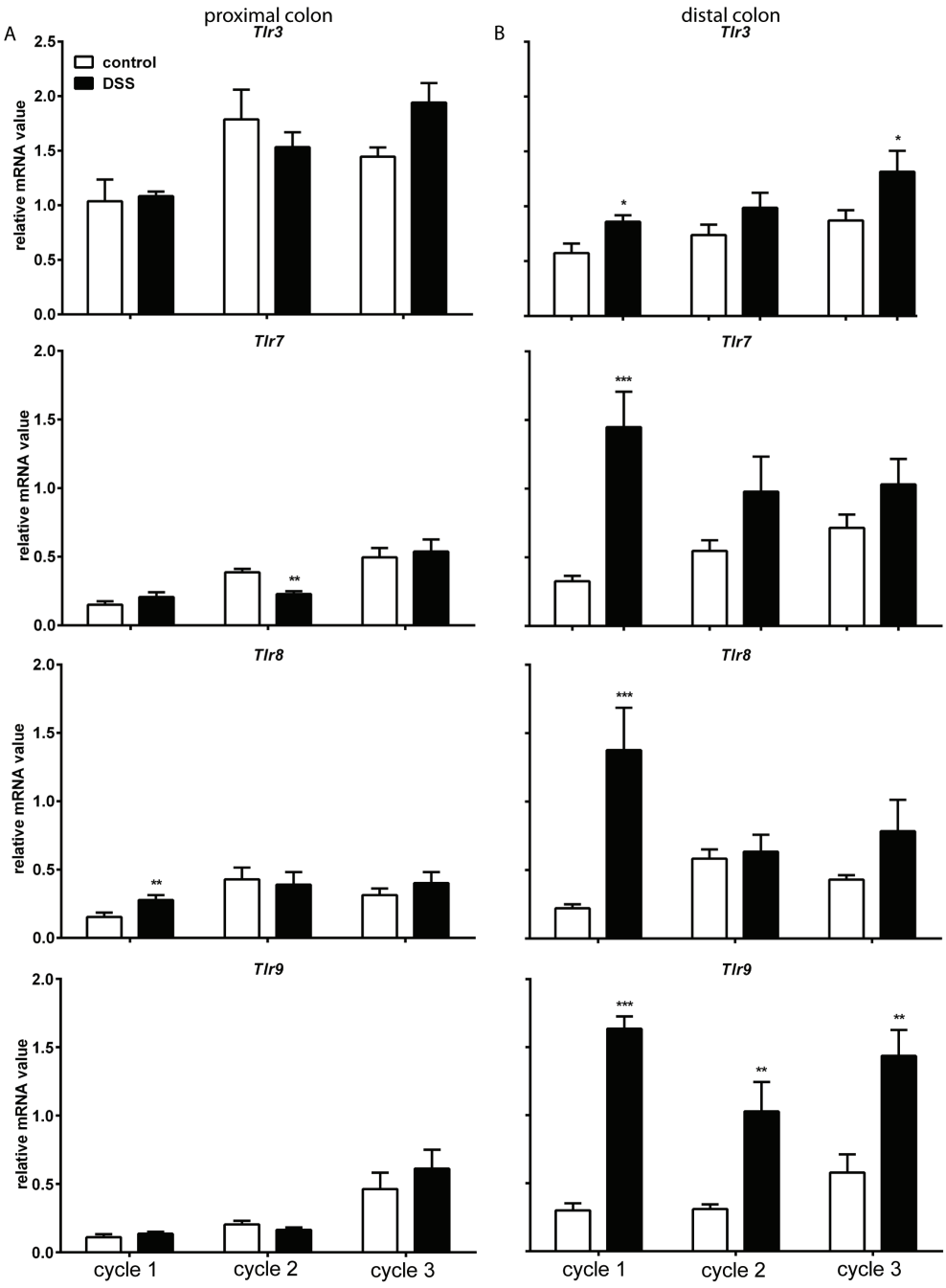

Figure 6. Repeated DSS treatment increases mRNA expression of viral-associated *Tlrs* in the distal colon.

Colons were collected for mRNA expression assessment of PRR. The mRNA expressions of *Tlr3*, *Tlr7* and *Tlr8* in both A) proximal- and B) distal part of the colon are shown. All results are expressed as ± SEM, n = 6. * P < 0.05, ** P < 0.01.

To determine the mRNA expression of this subfamily of TLR during the DSS-induced chronic colitis, the specific mRNA expression was examined in both proximal and distal part of the colon after each DSS treatment cycle (Figure 6). In the proximal part of the colon, only Tlr8 showed a significantly increased mRNA expression during acute colitis. A significantly decreased mRNA expression of Tlr7 was observed during chronic colitis after the second DSS-treatment (Figure 6A). In the distal part of the colon, the mRNA expression of all intracellular Tlrs was significantly increased during acute colitis and returned to basal expression levels after chronic DSS treatment, except for Tlr3 and Tlr9 During chronic colitis, *Tlr3* mRNA expression was significantly increased after the third DSS, while the mRNA of *Tlr9* was elevated after each DSS treatment (Figure 6B).

mRNA expression of pro-inflammatory cytokines is significantly increased during acute and chronic colitis

In a previous study, we have demonstrated that acute colitis is accompanied with increased mRNA expression of pro-inflammatory cytokines [25]. To investigate whether the increase also persists during the chronic colitis, mRNA expression of pro-inflammatory cytokines was explored (Table 1). A significantly increased mRNA expression of *Tnfα, Ccl2, Il1β* and *Il6* was observed in both the proximal and distal part of the colon during acute colitis.

In addition, a similar increased mRNA expression of these cytokines was observed during chronic colitis, although the expression of *Ccl2* in the proximal part of the colon did not reach significance after the second DSS treatment.

Chronic colitis is accompanied with an increased mRNA expression of Th1 cell-associated transcription factors and cytokines

Next, we investigate the mRNA expression of Th cell-associated transcription factors and cytokines during the DSS-induced chronic colitis (Table 2-5). To accesses the contribution of the Th1 cell response, mRNA expression of *Tbet, Ifnγ* and *Il12* was explored (Table 2). In the proximal part of the colon, there was no significant difference of *Tbet* mRNA expression between healthy and DSS-treated mice, whereas an increased mRNA expression of *Ifnγ* was observed in DSS-treated mice during acute colitis as well as chronic colitis, though the expression after third DSS treatment did not reach significance. In contrast to *Ifnγ*, there was a significant decrease in *Il12* mRNA expression after second DSS. In the distal part of the colon, increased mRNA expression of Th1 cell transcription factor *Tbet* as well as Th1 cell- associated cytokines *Ifnγ* and *Il12* was found during both acute and chronic DSS colitis, though the mRNA expression of *Il12* after third DSS treatment did not reach significance.

Pro-inflammatory cytokines				
Tnfα	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.06 ± 0.02	0.42 ± 0.1**	0.03 ± 0.01	1.01 ± 0.16***
Cycle 2	0.16 ± 0.05	0.68 ± 0.16*	0.08 ± 0.03	0.83 ± 0.08***
Cycle 3	0.16 ± 0.03	0.87 ± 0.28**	0.25 ± 0.14	1.15 ± 0.15***
Ccl2	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.01 ± 0	0.26 ± 0.08**	0.02 ± 0	1.15 ± 0.25***
Cycle 2	0.13 ± 0.03	0.25 ± 0.09	0.14 ± 0.05	0.72 ± 0.18*
Cycle 3	0.14 ± 0.04	0.62 ± 0.33*	0.17 ± 0.07	1.17 ± 0.1***
Il6	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.01 ± 0.01	0.45 ± 0.2**	0.01 ± 0	1.09 ± 0.37**
Cycle 2	0.08 ± 0.01	0.28 ± 0.04**	0.07 ± 0.01	0.78 ± 0.23**
Cycle 3	0.04 ± 0.01	0.73 ± 0.36*	0.03 ± 0	1.02 ± 0.22**
Il1β	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.01 ± 0	0.51 ± 0.18**	0.01 ± 0	1.40 ± 0.43 **
Cycle 2	0.01 ± 0	0.15 ± 0.02**	0.01 ± 0	0.68 ± 0.21*
Cycle 3	0.02 ± 0	0.57 ± 0.26*	0.02 ± 0	1.15 ± 0.17**

Table 1. DSS treatment increased mRNA expression of pro-inflammatory cytokines

Colons were collected for examination of the cytokines mRNA expression level. The mRNA expressions of pro-inflammatory cytokines *Tnfα*, *Ccl2, Il6* and *Il1β* in the proximal- and distal part of the colon are shown. All results are expressed as ± SEM, n = 6. * Indicates the significant different between control and DSS-treated mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Th1-associated transcription factor and cytokines				
Tbet	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.63 ± 0.22	0.51 ± 0.12	0.38 ± 0.08	1.52 ± 0.24**
Cycle 2	1.45 ± 0.31	1.76 ± 0.29	0.48 ± 0.09	1.65 ± 0.39**
Cycle 3	1.16 ± 0.22	1.20 ± 0.03	0.40 ± 0.11	1.27 ± 0.11**
Ifnγ	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.01 ± 0	0.19 ± 0.11*	0.01 ± 0	0.82 ± 0.13*
Cycle 2	0.05 ± 0.01	0.31 ± 0.11*	0.03 ± 0.01	0.67 ± 0.18***
Cycle 3	0.04 ± 0.02	0.43 ± 0.16$^{p=0.058}$	0.05 ± 0.04	0.92 ± 0.12***
Il12	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.20 ± 0.07	0.31 ± 0.09	0.30 ± 0.09	1.23 ± 0.18***
Cycle 2	0.52 ± 0.05	0.31 ± 0.05*	0.36 ± 0.03	0.89 ± 0.18*
Cycle 3	0.88 ± 0.18	0.64 ± 0.18	0.74 ± 0.13	1.03 ± 0.11

Table 2. DSS treatment increased the transcripts associated with Th1 response Colons were collected for examination of the mRNA expression level of Th1 transcription factors and associated cytokines. The mRNA expressions of *Tbet*, *Ifnγ* and *Il12* in the proximal- and distal part of the colon are shown. All results are expressed as ± SEM, n = 6. * indicates the significant different between control and DSS-treated mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

mRNA encoding Th2 cell-associated cytokines play a less important role during chronic colitis

To examine the involvement of the Th2 cell response, mRNA expression of *Gata3, Il4, Il5* and *Il13* was determined (Table 3). There was a significant increased mRNA expression of Th2 cell transcription factor *Gata3* in both the proximal and the distal part of colon during acute as well as chronic colitis. Interestingly, there was no significant increase in mRNA expression of the Th2 cell-associated cytokines, except *Il4* expression in the distal colon during acute

Th2-associated transcription factor and cytokines				
Gata3	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.20 ± 0.03	0.90 ± 0.28*	0.24 ± 0.05	0.96 ± 0.17 **
Cycle 2	0.35 ± 0.05	1.66 ± 0.27***	0.24 ± 0.06	1.06 ± 0.24*
Cycle 3	0.21 ± 0.03	1.51 ± 0.3***	0.12 ± 0.02	1.23 ± 0.51**
Il4	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.37 ± 0.03	0.27 ± 0.06	0.47 ± 0.08	0.87 ± 0.13*
Cycle 2	0.90 ± 0.2	1.02 ± 0.30	0.68 ± 0.1	0.49 ± 0.13
Cycle 3	1.29 ± 0.22	0.63 ± 0.08*	0.81 ± 0.14	0.57 ± 0.04
Il5	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.13 ± 0.06	0.04 ± 0.02	0.13 ± 0.07	0.13 ± 0.08
Cycle 2	0.77 ± 0.23	0.57 ± 0.23	0.59 ± 0.11	0.35 ± 0.13
Cycle 3	0.93 ± 0.12	0.58 ± 0.12$^{p=0.082}$	0.76 ± 0.13	0.31 ± 0.05*
Il13	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.98 ± 0.29	0.51 ± 0.1	0.79 ± 0.12	1.03 ± 0.13
Cycle 2	0.96 ± 0.21	1.11 ± 0.46	0.66 ± 0.16	0.71 ± 0.16
Cycle 3	1.21 ± 0.19	1.27 ± 0.28	0.82 ± 0.2	0.87 ± 0.14

Table 3. While increased Th2 transcription factor was found after each DSS treatment cycle, only *Il4* was increased after acute DSS treatment.

Colons were collected for examination of the mRNA expression level of Th2 transcription factors and associated cytokines. The mRNA expressions of *Gata3*, *Il4*, *Il5* and *Il13* in the proximal- and distal part of the colon are shown. All results are expressed as ± SEM, n = 6. * Indicates the significant different between control and DSS-treated mice. * P < 0.05, ** P < 0.01, *** P < 0.001

colitis. A significant decreased mRNA expression of *Il4* in the proximal part of colon and *Il5* in the distal part of colon was observed during chronic colitis after the third DSS treatment.

Chronic colitis was associated with increased transcript of *Il17*

In contrast to Th1 and Th2 cell transcription factors, the transcription factor of Th17 cells, *Rorc* was significantly reduced in the distal part of colon during acute colitis. In addition, the expression remained low during the chronic colitis as compared to the expression in

Th17-associated transcription factor and cytokines				
Rorc	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.77 ± 0.14	0.49 ± 0.02	0.95 ± 0.05	0.39 ± 0.08***
Cycle 2	1.13 ± 0.28	1.03 ± 0,23	0.94 ± 0.12	0.62 ± 0.1$^{p=0.089}$
Cycle 3	0.92 ± 0.11	0.98 ± 0.16	0.93 ± 0.10	0.63 ± 0.08*
Il23	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.39 ± 0.17	0.54 ± 0.19	0.44 ± 0.19	1.19 ± 0.23**
Cycle 2	0.73 ± 0.16	0.61 ± 0.10	0.84 ± 0.07	0.70 ± 0.09
Cycle 3	0.64 ± 0.13	0.96 ± 0.24	0.85 ± 0.14	0.85 ± 0.05
Il17	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.02 ± 0	0.21 ± 0.08	0.02 ± 0	1.02 ± 0.19***
Cycle 2	0.05 ± 0.02	0.28 ± 0.07	0.04 ± 0.02	0.55 ± 0.2*
Cycle 3	0.06 ± 0.03	0.85 ± 0.48	0.05 ± 0.03	1.00 ± 0.18***

Table 4. Increased mRNA expression of *Il17*, but decreased of Th17 associated transcription factors were found after each DSS treatment cycle, *Il23* was only increased after acute DSS treatment

Colons were collected for examination of the mRNA expression level of Th17 transcription factor and associated cytokines. The mRNA expression of Rorc, *Il23* and *Il17* in the proximal- and distal part of the colon are shown. All results are expressed as ± SEM, n = 6. * Indicates the significant different between control and DSS-treated mice. * P < 0.05, ** P < 0.01, *** P < 0.001

the colon of healthy mice, although it did not reach the significance after the second DSS treatment (Table 4). There was significant sustainable increase in *Il17* mRNA expressions in both the parts of the colon during chronic colitis. In addition, in the distal part of colon, a significant increased *Il23* mRNA expression was observed only during the acute colitis.

Chronic colitis increased the mRNA expression of *Il10* and enhances the number of Foxp3+ cells in the colon

To determine the contribution of Treg cell-mediated responses, mRNA expression of *Foxp3*, *Tgfβ* and *Il10* was explored (Table 5).

Although there was no change of the Foxp3 mRNA expression between healthy and DSS-treated mice, a significant increased *Tgfβ* mRNA expression was found in the distal part of colon during acute colitis, which returned to basal levels after three cycles of DSS treatment. Furthermore, there was a significant increased mRNA expression of Il10 in the distal colon

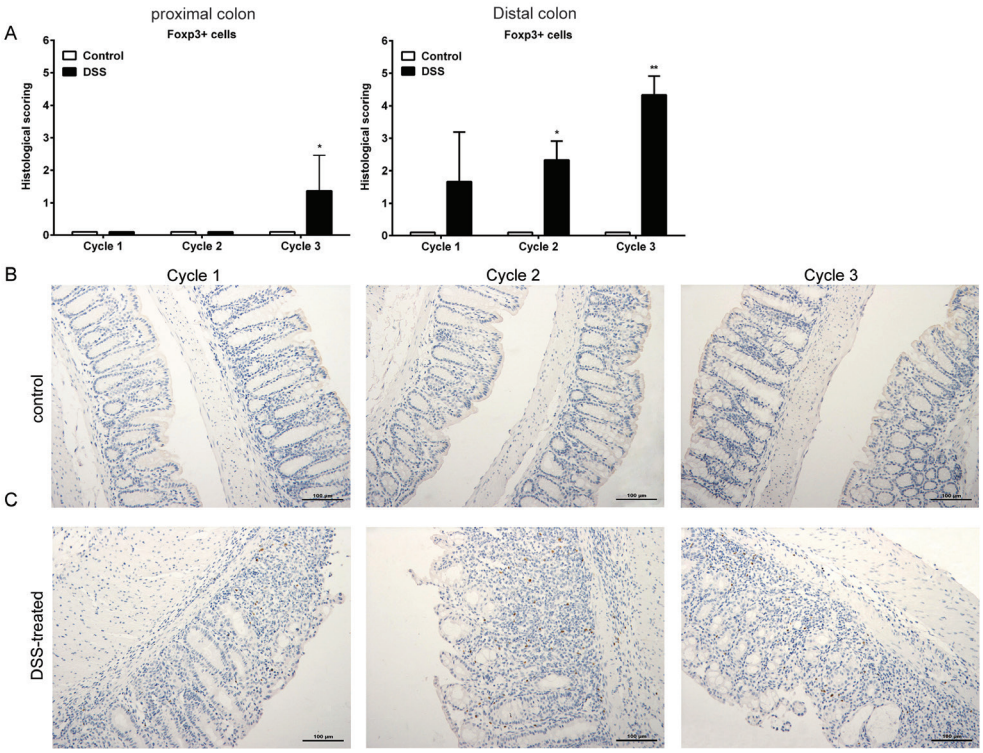

Figure 7. Increased histological score of Foxp3+ cells after each DSS treatment cycle

Colons were collected for A) immunohistochemical assessment of Foxp3+ cells. Representative histochemical staining pictures of Foxp3+ cells in the colon of B) control and C) DSS-treated mice during each DSS cycle are shown. All results are expressed as ± SEM, n = 3. * Indicates the significant different between control and DSS-treated mice: * $P < 0.05$, ** $P < 0.01$

during both acute and chronic colitis, though the expression after the third DSS treatment cycle did not reach significance. To further analysis the contribution of Treg cells during the chronic colitis, Foxp3+ cells in the colon were examined using histochemistry. There was an increased score of Foxp3+ cell in the colon during chronic colitis, but not in acute colitis (Figure 7A-C).

Treg-associated transcription factor and cytokines				
Foxp3	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.51 ± 0.15	0.38 ± 0.3	0.70 ± 0.14	0.83 ± 0.07
Cycle 2	1.07 ± 0.1	0.90 ± 0.14	0.80 ± 0.07	0.97 ± 0.09
Cycle 3	1.24 ± 0.35	0.87 ± 0.28	0.72 ± 0.1	1.03 ± 0.11$^{p=0.082}$
Tgfβ	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.41 ± 0.05	0.41 ± 0.08	0.61 ± 0.09	1.42 ± 0.19***
Cycle 2	0.50 ± 0.05	0.34 ± 0.04	0.57 ± 0.07	0.80 ± 0.13
Cycle 3	0.68 ± 0.06	0.54 ± 0.09	0.75 ± 0.09	0.84 ± 0.22
Il10	Proximal colon		Distal colon	
	Control mice	DSS-treated mice	Control mice	DSS-treated mice
Cycle 1	0.14 ± 0.04	0.18 ± 0.04	0.17 ± 0.05	0.78 ± 0.10**
Cycle 2	0.49 ± 0.06	0.45 ± 0.07	0.53 ± 0.10	1.01 ± 0.16*
Cycle 3	0.58 ± 0.09	0.54 ± 0.13	0.50 ± 0.13	0.90 ± 0.14$^{p=0.076}$

Table 5. Increased *Il10* mRNA expression was found after each DSS treatment cycle, while *Tgfβ* was only increased after acute DSS treatment

Colons were collected for examination of the mRNA expression level of Treg transcription factor and associated cytokines. The mRNA expression of Foxp3, *Tgfβ* and *Il10* in the proximal- and distal part of the colon are shown. All results are expressed as ± SEM, n = 6. * Indicates the significant different between control and DSS-treated mice. * P < 0.05, ** P < 0.01, *** P < 0.001

Discussion

In our previous study, modulated mRNA expression of colonic PRRs, pro-inflammatory cytokines, T cell-associated transcription factors and cytokines was found during acute colitis after single exposure to DSS [25]. Here, the mRNA expression of these specific genes during chronic colitis was examined in mice exposed to repeated DSS treatment interspersed with a rest period.

The DSS-induced colitis model is most widely used experiment model for studying IBD development because of its simplicity and predictable intestinal inflammation [31]. Both acute and chronic DSS-induced colitis murine models were well described in the literature [23,24]. The utilization value of this experimental colitis model for IBD development investigation is highlighted by studies that shown the DSS-induced colitis model responds to standard IBD therapeutic agents [21,32]. As compared to single DSS exposure-induced acute colitis, multiple DSS exposure induced chronic relapse colitis in mice shows a higher similarity to IBD that is characterized by remission-relapse course after disease onset [1]. The single DSS treatment induced acute colitis was characterized by clinical symptoms like decreased bodyweight and the development of bloody diarrhea. Furthermore, colon analysis showed decreased colon length/weight ratio, increased colonic tissue damage and increased number of colonic neutrophils [25]. In the chronic phase of colitis, the clinical manifestations do not always reflect the severity of intestinal inflammation [21,31]. In our multiple DSS treatment induced colitis model, the DSS induced bodyweight decrease was barely visible during the second treatment and was totally absent after the third DSS treatment. Bloody diarrhea was observed after each DSS treatment and recovered gradually after stopping the DSS treatment. However, the data, which shown faster responses of bloody diarrhea development to the repeated DSS treatments, suggest that these mice were more susceptible to reintroduced DSS treatment.

In contrast to clinical manifestations, histological analysis of colon tissue after repeated DSS treatment demonstrated characteristic inflammatory symptoms such as crypt depletion, cellular infiltration and increased number of neutrophil cells. The latter phenotype is also supported by increased MPO concentration determination. It is difficult to define whether the colonic structure change is due to incidental DSS treatment or as result of chronic inflammation process. Chronic colonic change consist phenotypes such as crypt architecture disarray, mononuclear leucocytes infiltration, transmural inflammation [31]. Our histological finding of these features and the gradually decreased colon length/weight ratio after repeated DSS treatment suggests that our colitis model may be presented as a semi-chronic relapsing colitis model that could provide valuable knowledge to help clarify the development of IBD.

During the single DSS exposure-induced acute colitis, the colonic mRNA encoding PRRs such as *Nod1*, *Nod2* and all *Tlrs*, except *Tlr1* and *Tlr5* were elevated in the inflamed colon region [25]. Activation of TLRs could induce immune responses including production of pro-inflammatory cytokines and activation of Th1 response that are important for host defense

against invading pathogens [10]. Transient activation of TLRs such as TLR4, TLR2/TLR6 in inflammatory hyporesponsive germ mice restores inflammatory responses [33]. However, aberrant and prolonged action of TLRs may leads to more tissue damage and inflammatory responses [34,35] that may result in chronic inflammatory disease like IBD [36]. Consistent with this hypothesis, increased expression of TLRs including TLR2, TLR3 and TLR4 are found in colon biopsies derived from IBD patient [11,12].

Accordingly, the extent and duration of the induction of these PRRs need to be tightly controlled. Indeed, in this study, the expression of majority of the PRRs, which was enhanced in the inflamed colon region during the acute colitis, including *Tlr2, Tlr3, Tlr4, Tlr6, Tlr7* and *Tlr8* was not observed after repeated DSS treatments during the chronic colitis. The expression of *Nod1* was even decreased after the second DSS treatment. Possible explanations of the unchanged or decreased expression of these receptors could be either that these PRRs are not involved in DSS-induced chronic colitis or these receptors were temporary insensitive/ negatively regulated after acute colitis to avoid continue activation of the inflammatory response. As TLRs signaling may be involved in many levels of inflammatory process [37,38], the latter possibility is more likely to be the case. Although the exact regulation mechanisms involved here still need to be elucidated, the importance of temporary reduced expression or silencing of these receptors was recognized and negative regulation mechanisms at multiple signaling level were reported [39,40]. Interestingly, NOD2 is one of the negative regulators of TLR-mediated signaling. Activation of NOD2 decreases TLR2-induced IL12 production of $CD11b^{+}$ cells [41]. Increased *Nod2* expression after second and third DSS exposure was observed in this study, although the expression after third DSS exposure did not reach the significance. These increased expressions could be the result of enhanced expressions of *Tnfα* and *Ifnγ,* since stimulation of human intestinal epithelial cells with TNFα or IFNγ triggers the up-regulation of NOD2 and increases MDP responsiveness [42]. The increased *Nod2* in this study may also be involved in the TLR suppression during the chronic colitis. However, the increased *Il12* expression after second DSS exposure is not in consists with this hypothesis. Keep in mind that we just take snap-shut during the DSS-induced chronic colitis, one possible explanation could be that the Nod2 mediated suppression activity did not take place yet, it will take place at later time point.

During the single DSS exposure induced acute colitis, *Tlr5* mRNA displayed a unique expression pattern of being down regulated [25]. This down-regulation was also observed after multiple DSS exposure during chronic colitis. It has been reported that TLR5 is exclusively located on the basolateral, but not on apical surface of intact intestinal epithelial [43, 44]. This allows intestinal epithelial to provide a polarized TLR5 response resulting in rapid clearance of invading pathogens that have passed the epithelial barrier. DSS treatment will lead to breakdown of the intestinal epithelial barrier and consequently increased bacteria translocation [45]. As result, TLR5 located at the basolateral surface of the intestinal epithelial cells will be exposed to abundant bacteria derived ligand, such as the TLR5 ligand flagellin. A recent study shown that prolonged exposure to flagellin will lead to flagellin-

induced tolerance accompanied by internalization of a part of the TLR5 [46]. These data provide a possible explanation of the reduced expression of *Tlr5* observed in the inflamed colon after chronic DSS treatment. In addition, we found an upregulation of the expression of *Ifnγ* in the distal colon of DSS-treated mice. Ifnγ has been show to down-regulate Tlr5 expression in mice colon cells as well in a dose and time dependent manner [47].This Ifnγ-induced feedback mechanism could be another regulation mechanism that is responsible for the observed *Tlr5* down-regulation in chronic DSS colitis. Taken together, the DSS-induced loss of *Tlr5* expression, which mirrors the observed TLR5 reduction in UC patients [48], could result in reduced clearance of infiltrated gut bacteria. This will subsequently lead to a chronic intestinal inflammation. This hypothesis is confirmed by the observation that TLR5$^{-/-}$ mice develop spontaneous colitis [49] and the finding of a strong anti-flagellin antibody response in IBD patients [50, 51].

It has been reported that TLR9 response contributes to the extension of chronic intestinal inflammation [52]. In line with this study, the mRNA expression of *Tlr9* is the only PRRs that shown an increased expression after each DSS treatment during the chronic colitis. Activation of TLR9 will induced TLR downstream MyD-88 dependent pathway, which leads to translocation of NF-κβ into the nucleus that results in expression of pro-inflammatory cytokines such as *Tnfα, Ccl2, Il1β* and *Il6* [53,54]. In addition, a recent study has shown the importance of Tlr9 signaling for the Th1 immune response in mice gut-associated lymphoid tissue (GALT) after an experimental oral infection [55] In line with these data, enhanced expression of these pro-inflammatory cytokines and Th1 associated cytokine *Ifnγ* were observed in inflamed colon of mice chronically exposed to DSS.

The DSS colitis model was first considered as an ulcerative colitis model with type 2 T-helper (Th2) cell skewing; however, increased number of studies demonstrated that many induced genes are associated with CD, including Th1 and Th17 cell-associated cytokine expression [56-58]. In this study, we also did not observe increased gene expression of any Th2 cell-associated cytokines during the acute colitis, except *Il4*. However, this increase was no longer observed during chronic colitis indicating that Th2 cell-mediated response is probably not involved in DSS induced colitis. It is unknown why the mRNA expression of Th2 cell-associated transcription factor *Gata3* was increased during both acute and chronic colitis, while the expression of majority of the associated cytokines remains unchanged. One possible explanation is derived from the Th1/Th2 cell paradigm, Th1 cell-associated transcription factor *Tbet* could distribute *Gata3* away from Th2 cell genes and induce Th1 cell differentiation [59,60]. Increased Tbet expression after DSS exposure is in line with this hypothesis.

IL10 is known as immune suppressive cytokine produced by Treg cells. Varied polymorphisms of IL10 gene are associated with early onset of UC [61]. Mice with deficient IL10 develop spontaneous colitis. In this study, increased *Il10* mRNA expression was found after single DSS exposure and second DSS exposure. However, this increased expression was not found after third DSS exposure. Despite the mRNA expression of Treg cell-associated transcription

factor Foxp3 did not shown any significant increase, histological examination of Foxp3+ cells shown increased histological score. Indicating increased amount of Foxp3+ cells in the colon after DSS treatment. Though in the mouse Foxp3+ cells are regarded as Treg cell, other Foxp3+ T cells in the intestinal tract and lymphoid organs have been described. Rubino and co-workers have found a subset of Foxp3 expressing CD8+ cytotoxic T cells that are involved in early Th17-mediated immune response to enteric pathogen in mice [62]. Furthermore, IL17- and IFNγ secreting Foxp3+ T cells have been demonstrated in peripheral lymph nodes and the spleen [63]. Taken together it could be hypothesized that the high number of Foxp3+ cells in the colon of mice undergoing chronic colitis do not represent an increased number of Treg cells, but pro-inflammatory cytotoxic of helper T cell subpopulations.

In contrast to Th2 and Treg cell-associated cytokines, increased Th1 and Th17 cell-associated cytokines and *Il10* have been shown during single DSS exposure induced acute colitis [25]. However, as the colitis development progresses, the increases of Th1 cell-associated cytokine *Il12* and Th17 cell-associated cytokine *Il23* were not enhanced after third and second DSS exposure, respectively. Analyzing the gene expression of *Ifnγ* and *Il17,* persistent increase of these gene expressions was observed during the chronic colitis. These data are in line with the finding of Melgar et al, who also shown increased expression of these gene after single DSS exposure induced chronic colitis [64]. As for human studies, similar data have also been demonstrated. IL-17A is detected in the sera of IBD patient and both IFN-γ and IL-17A producing T cells are found [65]. These results indicate that both IL-17A and IFN-γ could be possible effectors in IBD development and therefore could be potential target of IBD treatment.

In summary, we have shown the expression pattern of PRR in murine colon in a model for chronic induced by repeated cycles of DSS treatment. In addition to our previous studies in acute colitis, this report provides valuable fundamental information about PRR expression in chronic colitis. In addition, the modulation of the mRNA expression of T cell-associated cytokines was examined during the chronic colitis. Regarding PRR, the mRNA expression of *Tlr5* and *Tlr9* was modulated during both acute and chronic colitis. As for T cell-associated cytokines, the mRNA expression of *Ifnγ* and *Il17* was substantially increased during the chronic colitis mimicking the human situation in IBD. Taken together, this study might contribute to create information regarding the suitability of TLRs and NOD and related cytokines as potential targets of IBD treatment.

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Chapter 7

SPECIFIC PROBIOTIC DIETARY SUPPLEMENTATION LEADS TO DIFFERENT EFFECTS DURING REMISSION AND RELAPSE IN MURINE CHRONIC COLITIS

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Abstract

Although interest in using probiotics to prevent and treat intestinal diseases is increasing, the effects of specific probiotic strains still remain unclear. Here, we assess the therapeutic effects of two probiotic strains, *Lactobacillus rhamnosus* NutRes 1 *(L. rhamnosus)* and *Bifidobacterium breve* NutRes 204 *(B. breve)* on a dextran sodium sulfate (DSS)-induced chronic murine colitis model. The chronic colitis was induced by two DSS treatment cycles with a rest period of 10 days (the remission phase). The probiotic supplementation was started during the remission phase, after the first DSS treatment cycle, and continued until end of the experiment. In addition to clinical observations made during the experiment, cellular infiltration was measured along with mRNA expression of pro-inflammatory cytokines, T cell-associated cytokines, and Toll-like receptors (TLR) in the inflamed colon after second DSS treatment cycle. *L. rhamnosus*, but not *B. breve*, rapidly and effectively improved the DSS-induced bloody diarrhea during the remission phase. However, a contradictory effect by both probiotic strains on the fecal condition was found after re-induction of colitis. The worsening of the fecal condition was accompanied by a reduced number of neutrophils and increased expression of *Ifnγ* in the colons of DSS-treated mice. Furthermore, an increased expression of *Tlr2*, *Tlr6* and pro-inflammatory markers including *Ccl2*, *Il1β*, *Tnfα* and *Il6* was found in DSS-treated mice with *L. rhamnosus* supplementation. These results indicate that therapeutic administration of specific probiotics might be beneficial during the remission phase of colitis. However, caution should be taken as specific probiotic treatments reduce neutrophil influx, which may be the reason of exacerbation of chronic colitis.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of inflammatory bowel disease (IBD), which is a chronic inflammatory disorder of gastrointestinal tract. The disease course is characterized by series of relapse separated by remissions of varied durations; only a minority of the IBD patient undergoes a chronic, continuous disease course [1]. Although the etiology remains largely unknown, the generally accepted hypothesis is that abnormal and hyperactive immune responses against components of the microflora in a genetic susceptible hosts leads to chronic intestinal inflammation [2,3]. Recently, a new hypothesis has emerged that suggests that the underlying problem of IBD is a failure of the innate immune response towards pathogens that penetrate the mucosal barrier, which leads to d impaired neutrophil infiltration and reduced bacterial clearance [4].

The innate immune system is comprised of cells and soluble factors that defend the host against infections in a non-specific manner. Invading microbes are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). TLRs can discriminate between microbe-associated molecular patterns by recognizing microbial cell wall components (TLR1, TLR2, TLR4, TLR5, and TLR6), bacterial-derived DNA (TLR9), or virus-derived RNA (TLR3, 7 and 8) [5]. TLR2 is different from other TLRs since it needs to form heterodimers with TLR1 or TLR6 to recognize triacyl and diacyl lipopeptides respectively [6, 7]. Activation of TLRs leads to the recruitment of phagocytic leukocytes, predominantly neutrophils, to provide the first cellular immune defense against infections [8]. To prevent local damage and spread of potential pathogens, an effective clearance of invading microbes by phagocytic cells is needed.

The new hypothesis postulates that failure of microbial clearance mechanisms by diminished neutrophil chemotaxis and function results in persistent activation of immune system and drives subsequent T-cell mediated chronic inflammatory responses [9]. The observations of disrupted neutrophil accumulation in CD patients [10] and consequent impaired bacteria clearance [11] support this idea.

Probiotics are defined as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" [12]. In the recent years, the use of probiotics to treat intestinal diseases has received the attention of an increased number of researchers. Accumulated data indicate probiotics restore the intestinal microbial balance and modulate the immune response [13]. In addition, there is reasonable evidence supporting the applicability of specific probiotics in intestinal disease like IBD [13, 14]. This was also demonstrated in our previous study using a preventive approach in a mouse model of colitis. *B. breve*, but not *L. rhamnosus*, administration was found to have a beneficial effect on DSS-induced acute colitis [15].

The aim of the current study was to investigate the applicability of therapeutic administration of the same specific bacterial strains in a chronic DSS-induced colitis model in mice. We found that therapeutic supplementation with *L. rhamnosus*, but not *B. breve*, increased and accelerated the improvement of the fecal condition during the remission phase.

However, a contradictory effect on the fecal condition was observed after reintroduction of DSS colitis. Administration of *L. rhamnosus,* led to exacerbation of chronic DSS-induced bloody diarrhea. Mice receiving *B. breve* showed a similar effect, although the change was not significant. Histochemical analysis of the colons revealed a reduced number of neutrophils and MPO expression accompanied by an increased mRNA expression of the Th1-associated cytokine *Ifnγ*. In addition, administration of *L. rhamnosus* further increased the mRNA expression of pro-inflammatory markers such as *Ccl2*, *Il1β*, *Tnfα* and *Il6,* and TLRs like *Tlr2* and *Tlr6* in the colon of DSS-treated mice. These data show that *L. rhamnosus* supplementation induces a beneficial effect during the remission phase, but exacerbates inflammation during reintroduction of colitis. The increased severity, reduced neutrophils, increased pro-inflammatory and Th1 associated cytokines are reminiscent to changes found in IBD patients resulting from limited bacterial clearance, and suggest that timing is critical for probiotic treatment of IBD.

Materials and methods

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. The animal experiments were approved by and were in accordance with the guidelines of the Dutch Experimental Animal Commission. The approval document is encoded with 2009.II.06.046.

Experimental colitis and administration of probiotics

Chronic colitis was induced by administration of two cycles of 1.5% DSS separated by a 10-day rest period. 1.5% DSS was added into the drinking water during each DSS treatment cycle for 5 days. The specific bacterial supplementation was started after the first DSS treatment cycle on day 6. 10^9/dose of *L. rhamnosus* NutRes 1 or *B. breve* NutRes 204 bateria [15] were administrated by oral gavage every two days, starting the first day after the first DSS treatment cycle and continued to the end of the experiment (Figure 1A). Colitis development was monitored by measuring the bodyweight and scoring of the fecal condition. The fecal condition assessments started at the end of the first DSS treatment cycle (day 5) and were continued to the end of the experiment (day 20).

The fecal condition score was determined from two parameters: stool consistency (0 = normal, 1 = soft with normal form, 2 = loss of form/diarrhea) and fecal bleeding (0 = no blood, 2 = blood observation using Colo-rectal Test kit (Axon Lab AG, Germany), 4 = blood observation without test). The demonstrated bodyweight changes during the first DSS treatment cycle plus the remission phase (day 0 – day 15) and the second DSS treatment cycle (day 16 - day 21) were normalized against the starting weight on the beginning of each

DSS treatment cycle, on day 0 and day 15, respectively. The demonstrated fecal condition scores during the first DSS treatment cycle plus the remission phase (day 0 – day 15) and the second DSS treatment cycle (day 16 - day 20) were normalized against the score on day 5 or day 15, respectively.

Histological evaluation of colon damage and immunohistochemical staining

After sacrificing the mice at day 21, colons (n = 6) were collected for immunohistochemical staining and histological evaluation. The colon was opened longitudinally, washed in the phosphate buffered saline (PBS) and placed on a piece of blotting paper. The half of each colon was fixed in 10% formalin for 24 hours and embedded in paraffin-embedded as swiss-roles and sectioned (5μm).

Two researchers assessed general inflammatory features blindly after staining sections with hematoxylin and eosin according the assessment system described before [16]. Briefly, the histological 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, 4: infiltration in entire tissue); the area of cellular infiltration in the region (0: no infiltration, 1: < 25%, 2: 25%-50%, 3: 50%-75%, 4: >75%); loss of crypts (0: no damage, 1: 30% shortening of crypts, 2: 65% shorting of crypts, 3: total loss of crypts, 4: loss of entire epithelial layer); and the area of crypts loss in the region (0: no crypt loss, 1: < 25%, 2: 25% - 50%, 3: 50%-75%, 4: > 75%).

Immunohistochemistry was employed to determine Ly-6B.2+ cells (neutrophils & some activated macrophages). The sections were subjected to a heat-induced epitope retrieval step. Slides were washed with PBS and blocked with rabbit or goat serum before an overnight incubation (4°C) with primary antibodies against Ly-6B.2 (AbD Serotec, Dusseldorf, Germany). For detection, biotinylated goat anti-rat (Dako, Glostrup, DK) secondary antibodies were administered followed by incubation with peroxidase-labeled streptavidin (Vectastain EliteABC kit, Vector, Burlingame, CA USA). The peroxidase activity was visualized using the substrate DAB (Sigma, Gillingham, UK). The cell nuclei were visualized by a short incubation with Mayer's hematoxylin (Klinipath, Duiven, the Netherlands). Background staining was determined by substituting the primary antibody with a rat IgG isotype control (Abcam, Cambrige, UK). The number of Ly-6B.2+ cells was quantified by counting positive cells within the mucosal region throughout the whole colon.

Assessment of Myeloperoxidase concentration in the colon tissue

After sacrificing the mice, colons (n = 6) were collected for myeloperoxidase (MPO) concentration assessment, which is a marker for neutrophils. The colon was opened longitudinally and half of each colon was transferred into RIPA buffer (Thermo Scientific, Rockford, IL USA) and homogenized using a Precellys®24-Dual homogenizer (Precellys, Villeurbanne, France). The homogenates were centrifuged at 14000 rpm for 10 minutes

at 4 °C and the MPO concentration in the supernatant was measured using an ELISA kit according to the manufacturer's protocol (Hycult biotech, Uden, the Netherlands).

Real-time PCR

After sacrificing the mice, colons (n = 6) were taken out for gene expression analysis. The colon was opened longitudinally and the proximal colon was distinct from the distal colon by its specific structure (bulges in the colon wall). The total RNA of the distal colon pieces 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 quantification was performed using the iQ SYBR Green super mix kit (BioRad, 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 sequences of specific primers for *Tlr1, Tlr2, Tlr6* and the gene for the household protein *ribosomal protein S13* (*Rps13*) are listed in Table 1. The primers for the cytokines: *monocyte chemotactic protein-1* (*Ccl-2), interleukin-1β* (*Il-1β), tumor necrosis factor-α* (*Tnfα), Il6, Il4, interferon gamma (Ifnγ), Il17* and *Il10* were purchased from SABioscience (Frederick, MD USA). The final data for the target samples were normalized against the internal control *Rps13*.

Statistical analysis

Means with SEM are represented in each graph. Statistical analysis was performed using GraphPad Prism version 6.02 for windows (GraphPad Software, San Diego, CA USA). P-values were calculated using either the two-way ANOVA followed by Bonferroni post-tests or, where needed, a Mann-Whitney test. A "*" indicates a significant difference between the control and DSS treated groups, while a "#" indicates a significant difference between groups with or without bacteria supplementation. The P-values considered significant are indicated as *** or ### $P < 0.001$, ** or ## $P < 0.01$, and * or # $P < 0.05$.

	Primer Sequence 5'-->3'	
	Forward primer	**Reverse primer**
Tlr1	GGTGTTAGGAGATGCTTATGGGG	GATGTTAGACAGTTCCAAACCGA
Tlr2	CCAGACACTGGGGGTAACATC	CGGATCGACTTTAGACTTTGGG
Tlr6	GACTCTCCCACAACAGGATACG	TCAGGTTGCCAAATTCCTTACAC
Rps13	GTCCGAAAGCACCTTGAGAG	AGCAGAGGCTGTGGATGACT

Table 1. qPCR primer sequences

Results

Therapeutic supplementation with *L. rhamnosus*, but not *B. breve*, accelerates the improvement of fecal condition during the remission phase, but exacerbates bloody diarrhea during a DSS-induced relapse

In a previous study, we showed that, in a preventive setting, supplementation with *B. breve* ameliorates the development of acute colitis in mice [15]. To study whether these specific bacterial strains, *L. Rhamnosus* and *B. breve*, have similar effects in a chronic model of colitis, a therapeutic approach with these probiotics was employed in a murine chronic colitis model with two repeated DSS treatment cycles (Figure 1A).

In healthy mice, supplementation with either *B. breve* or *L. rhamnosus* did not induce any clinical change as compared to mice without bacterial supplementation (data not

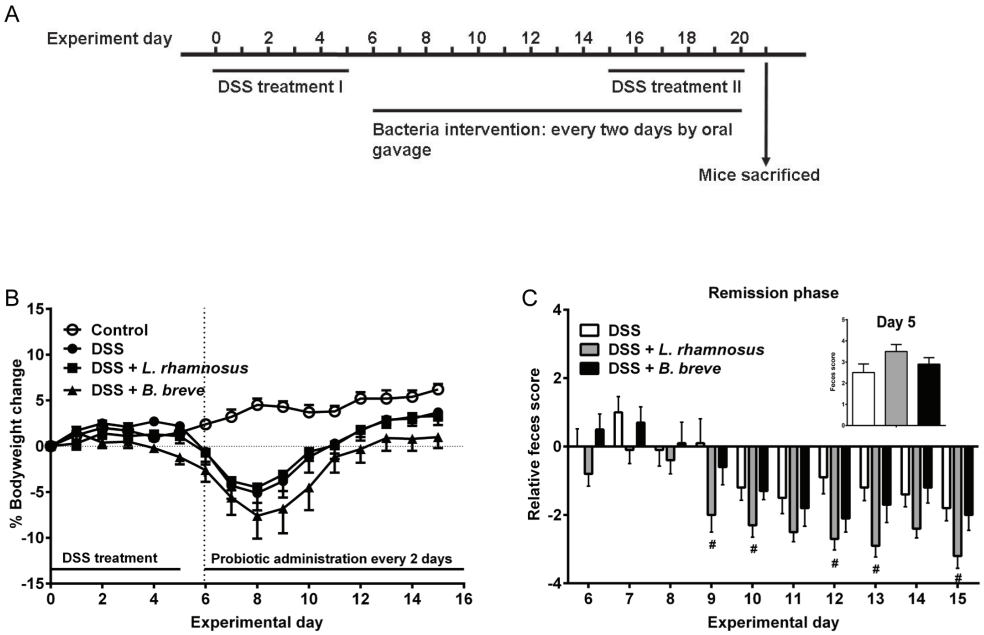

Figure 1. Supplementation with *L. rhamnosus* accelerates the improvement of fecal condition during remission phase after DSS-induced colitis in mice.

A) The study setup of the *in vivo* experiment is illustrated. Briefly, mice were treated with two cycles of 1.5% DSS separated by a 10-day rest period. 1.5% DSS was added into the drinking water during each DSS treatment cycle for 5 days. The specific bacterial supplementation was started at day 6. A 10^9/dose of *L. rhamnosus* or *B. breve* bacteria was administrated by oral gavage every two days until day 20. The mice were sacrificed at day 21. B) The bodyweight changes of the control and DSS-treated mice with or without bacterial supplementation, during the first DSS treatment cycle and the remission phase are shown. C) The fecal condition score change of DSS-treated mice during the remission phase is calculated by normalizing the results with the score on end of 1st DSS treatment cycle, day 5. The inserted graph represents the feces score on day 5 (n = 12 per group). All results are expressed as mean ± SEM. A "*" indicates the significant differences between the groups with or without DSS treatment, while a "#" indicates the significant differences between the groups with or without bacterial supplementation. * or # $P < 0.05$.

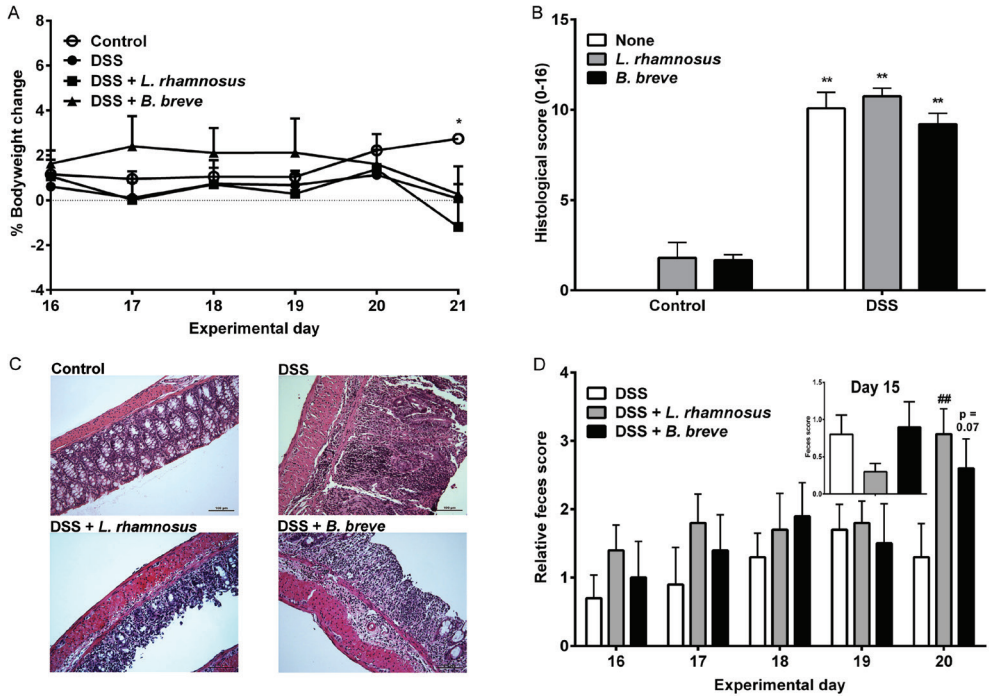

Figure 2. Supplementation with *L. rhamnosus* exacerbates DSS-induced bloody diarrhea during the second DSS treatment.

A) The bodyweight changes of the control mice and DSS-treated mice with or without specific bacterial supplementation during the second DSS treatment cycle were calculated by normalizing the results with the starting weight of 2nd DSS treatment cycle on day 15, and the results are shown in the graph. After sacrificing the mice on day 21, the colons were evaluated for tissue damage and cellular infiltration. The results are shown in the graph B) and representative images of colon of control mice and treated mice are displayed in C). The graph D) illustrates the fecal condition score changes of DSS-treated mice during the second DSS treatment cycle. The scores are calculated by normalizing the results with the score on the starting day of the second DSS treatment (day 15). The inserted graph represents the absolute feces score on day 15 (n = 12 per group). All results are expressed as mean ± SEM, The "#" indicates significant differences between the groups with or without bacteria supplementation. ## P < 0.01.

shown). The first DSS treatment cycle led to decreased bodyweight (peaked at day 8) and gradually returned to baseline during the remission phase. Therapeutic supplementation of the probiotics, starting after the first DSS cycle at day 6, had no effect on bodyweight change pattern in the remission phase of mice suffering from DSS-induced acute colitis (figure 1B). Fecal scores after the first DSS treatment had a maximal score on day 7, due to the appearance of bloody diarrhea, and returned to baseline during the remission phase. Supplementation with *L. rhamnosus,* but not *B. breve*, accelerated the restoration of fecal scores in DSS-treated mice and significantly reduced the occurrence of DSS-induced bloody diarrhea on day 9, 10, 12, 13 and 15 of the remission phase (Figure 1C).

The data of bodyweight change and the fecal score during the second cycle of DSS treatment were calculated relative to the data of starting date of second DSS treatment cycle on day 15. No significant differences in bodyweight were found between control and DSS-treated mice with or without probiotic supplementation except day 21. On day 21, a significant DSS-induced decrease in bodyweight was observed (Figure 2A). Analysis of the tissue damage and cell infiltration in the colon indicated extensive cellular infiltration and crypt loss in mice with repeated DSS treatments. Supplementation with either *B. breve* or *L. rhamnosus* had no discernible effect as demonstrated in the graph and by the representative histology photos (Figure 2B, C).

Assessment of the feces score, however, revealed that DSS-treated mice supplemented with L. rhamnosus had significantly more pronounced bloody diarrhea than DSS-treated mice without probiotics. Supplementation with *B. breve* tended to have a similar effect although this did not reach statistical significance ($p = 0.07$). (Figure 2 D)

Supplementation with L. rhamnosus and B. breve inhibits neutrophil infiltration in the colons of mice undergoing DSS-induced relapse

As neutrophils will be recruited to the targeted site upon infection or tissue damage [17, 18], we examined both MPO expression and the amount of neutrophils in the colon. MPO is abundantly expressed in neutrophil granulocytes [19] and is frequently used as marker of neutrophil infiltration, while Ly-6B.2 expression is particularly high on the surface of neutrophils and some inflammatory macrophages [20]. Repeated treatment cycles of DSS, indeed, led to an increased MPO concentration and number of Ly-6B.2+ cells in the colon (Figure 3A-C). Supplementation with the either *L. rhamnosus* or *B. breve* significantly reduced the enhanced MPO levels and the number of Ly-6B.2+ cells in the colon as shown in the graph and representative histology photos (Figure 3A-C).

Supplementation with L. rhamnosus further increases pro-inflammatory markers and Th1-associated Ifnγ expression in the colons of DSS-treated mice

To further investigate the effect of specific bacterial supplementation on the immune response in the colon, the expression of pro-inflammatory markers and T cell-associated cytokines was assessed. Figure 4 represents distal colon mRNA expression of *Ccl2*, *Il1β*, *Tnfα* and *Il6* and T cell associated cytokines *Il10* (Treg cell), *Il17* (Th17 cell), *Il4* (Th2) and *Ifnγ* (Th1) in both healthy and DSS-treated mice with or without bacterial supplementation. In healthy mice, *Il10* mRNA expression was significantly increased after *L. rhamnosus* supplementation (Figure 4A). Repeated DSS treatment significantly increased the mRNA expression of all examined cytokines in the colon of all treated mice regardless of probiotic supplementation as compared to healthy mice, except for *Il4* (Figure 4B).

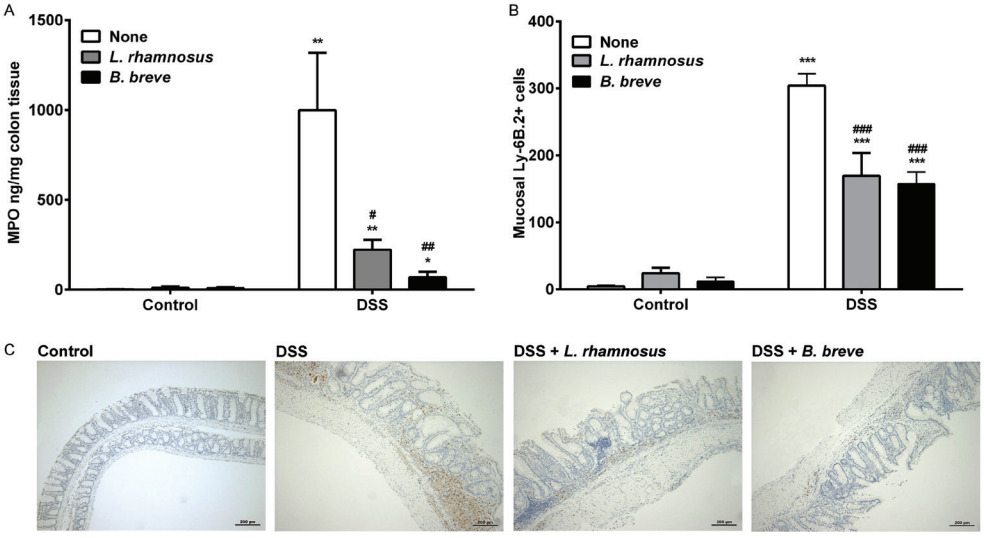

Figure 3. Specific bacterial supplementation reduces the number of neutrophils in the colons of mice undergoing an DSS-induced relapse.

After sacrificing the mice on day 21, colons were collected for A) the MPO concentration measurements and B) immunohistochemical quantification of neutrophils (n = 6 per group). C) Representative images of neutrophil staining in the colon of control mice and DSS-treated mice with or without specific bacterial supplementation are shown. All results are expressed as mean ± SEM. A * indicates the significant differences between the control and DSS-treated groups, while a # indicates the significant differences between the groups with or without specific bacterial supplementation.

*** or ### P < 0.001, ** or ## P < 0.01, * or # P < 0.05.

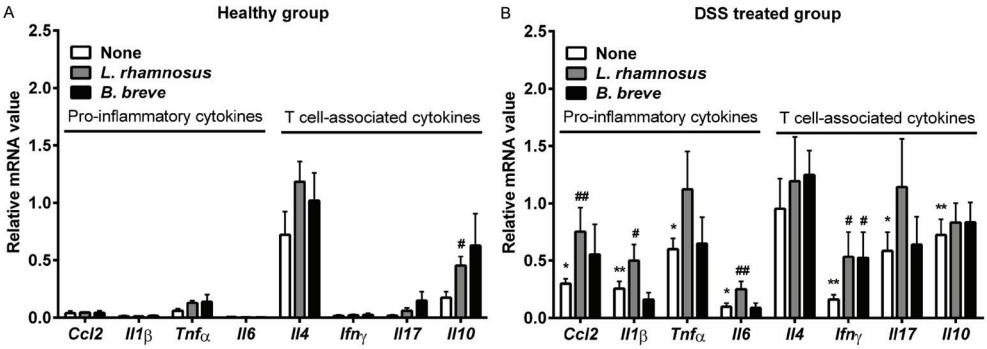

Figure 4. Supplementation with *L. rhamnosus* further increases the expression of pro-inflammatory cytokines and *Ifnγ* in mice undergoing a DSS-induced relapse.

After sacrificing the mice on day 21, colons were collected, and the mRNA expression of pro-inflammatory cytokines (*Ccl2*, *Il1β*, *Il6* and *Tnfα*) and T cell–associated cytokines (*Il4, Ifnγ, Il17* and *Il10*) was quantified in the distal colons of both A) healthy and B) DSS-treated mice with or without specific bacterial supplementation (n = 6 per group). All results are expressed as mean ± SEM, * indicates the significant different between the control and DSS-treated group, while # indicates the significant different between the group with or without specific bacterial supplementation. * P < 0.05, ** P < 0.01, ## P < 0.01, # P < 0.05.

Il4 expression was not affected by repeated DSS treatments or bacterial supplementation. Therapeutic supplementation of DSS-treated mice with *L. rhamnosus*, in contrast to *B. breve*, led to a significant increase in the expression of the pro-inflammatory markers *Ccl2, Il1ß,* and *Il6.* In addition, an increased expression of the Th1-assiociated cytokine *Ifnγ* was found in DSS-treated mice with both *L. rhamnosus* and *B. breve* supplementation as compared to mice with DSS treatment alone (Figure 4B).

Supplementation with L. rhamnosus increases mRNA expression of Tlr2 and Tlr6 in the colons of mice with repeated DSS treatment.

To investigate whether a change in colonic expression of TLRs correlates with the observed phenotype in mice suffering from colitis, we determined mRNA expression of *Tlr1, Tlr4, Tlr5* and *Tlr9.* No change in TLR expression was observed between diseased and healthy mice (data not shown). Messenger RNA expression of *Tlr2* and *Tlr6* was higher in colonic tissue of repeated DSS-treated mice when compared to healthy mice, but this did not reach statistical significance. TLR1 and TLR6 both pair with TLR2 to form functional heterodimers [6]. Although no significant differences for *Tlr1* expression were found between the different mice groups, *Tlr2* and *Tlr6* expressions were significantly increased in DSS-treated mice with *L. rhamnosus* supplementation as compared to healthy mice with the same bacterial supplementation (Figure 5 A – C).

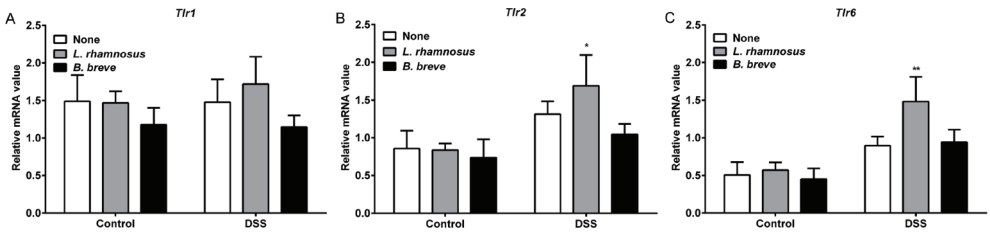

Figure 5. Supplementation with *L. rhamnosus* in mice suffering from DSS-induced chronic colitis increases the colonic mRNA expression of *Tlr2* and *Tlr6.*

After sacrificing the mice on day 21, colon tissues were collected and the mRNA expression of A) *Toll-like receptor (Tlr)* 1, B) *Tlr2* and C) *Tlr6* was quantified in the distal colon of both healthy and DSS-treated mice with or without specific bacterial supplementation (n = 6 per group). All results are expressed as mean ± SEM, * indicates the significant differences between the control and DSS-treated groups. ** $P < 0.01$, * $P < 0.05$.

Discussion

There is growing recognition of the importance of the intestinal microflora for the modulation of the host immune system. Consequently, interest in targeting microflora in order to restore immune homeostasis in intestinal disorders like IBD is also increasing. Evidence has shown that the administration of probiotics to patients suffering from IBD can manipulate the

microflora composition and induce beneficial effects [13, 21]. In patients suffering from UC, the clinical efficacy of probiotics was demonstrated by administration of VSL#3 that resulted in a remission in patients with active UC [22, 23]. In addition, treatment with *L. rhamnosus GG* was effective in maintaining remission in patients suffering from UC [24]. In the current study, supplementation with *L. rhamnosus*, but not *B. breve,* was found to accelerate and increase the improvement of the fecal condition (inhibition of DSS-induced bloody diarrhea) in mice with colitis during the remission phase. This result indicates that our *L. rhamnosus* strain has the potential to provide beneficial effects by speeding recovery and supporting the remission phase of the disease.

However, this beneficial effect was not observed when the second cycle of DSS treatment started. In contrast, *L. rhamnosus* supplementation significantly increased the bloody diarrhea after repeated DSS treatments. Supplementation with *B. breve* tended to have a similar effect, although it did not reach the statistical significance. Further investigations demonstrated that this effect seen in probiotic supplemented mice was not mirrored by worsened histological scores, represented by DSS-induced epithelial damage and total cell infiltration, but was instead accompanied by an unexpected loss of neutrophils in the colon tissue. Neutrophil infiltration is usually considered a hallmark of inflammation, and increased amounts of neutrophils are usually associated with tissue damage and severe inflammation in diseases like IBD [17, 25]. On the other hand, an alternative hypothesis for the pathogenesis of Crohn's disease suggests that diminished neutrophil recruitment and consequent impaired bacterial clearance could contribute to chronic intestinal inflammation [4]. Support for this hypothesis, in the form of reduced neutrophil accumulation and impaired clearance of bacteria from the tissue has been found in CD patients [10, 11]. In addition, a high similarity is found between patients with chronic granulomatous disease (CGD) and CD with as many as 55% of CGD patients meeting the criteria for CD. CGD is an inherited disorder that is characterized by a reduced ability of neutrophils to form reactive oxygen compounds used to kill ingested pathogens. This finding indicates that neutrophil mediated clearance actively contributes to immune homeostasis in the colon [26]. Consistent with this hypothesis, we found probiotic supplementation decreased colonic neutrophil influx and was accompanied with exacerbation of disease phenotype after the second DSS treatment. Furthermore, it is postulated that, due to the inadequate clearance of bacteria, activation of macrophages and possibly dendritic cells leads to pro-inflammatory cytokine production that, in turn, results in lymphocyte recruitment and polarization to a Th1 response [4]. In line with this hypothesis, supplementation with *L. rhamnosus* further induced the mRNA expression of pro-inflammatory markers such as *Ccl2*, *Il1β* and *Il6* in the colon of DSS-treated mice. In addition, supplementation with both *L. rhamnosus* and *B. breve* increased the mRNA expression of the Th1-associated cytokine (*Ifnβ)* in the colons of mice suffering from an induced relapse.

Besides the reduced infiltration of neutrophils, DSS-treated mice with *L. rhamnosus* supplementation induced an increased mRNA expression of *Tlr2* and *Tlr6* in the colon.

Studies investigating the effect of activated TLR2/TLR6 receptors demonstrated contradictory results. On the one hand, *in vitro,* TLR2/TLR6 stimulation induces the tolerogenic DC formation and a consequent type-1 regulatory T cell response [27]. On the other hand, our recent study has shown that TLR6 deficient mice were protected from DSS-induced colitis and that treatment with the TLR2/6 ligand, FSL-1, led to more severe colitis [28]. In addition, long-term stimulation of 7 days with TLR2/6 ligand induces Th1 and Th17 cells in mLN cells. Taken together, it could be hypothesized that supplementation with *L. rhamnonus* worsens chronic colitis by reducing neutrophil recruitment and consequently inhibiting bacterial clearance. In addition, as lipoteichoic acid of *L. rhamnosus* could activate TLR2/6 and triggers downstream signaling pathway [29], the enhanced colonic TLR2/6 expression in *L. rhamnosus* supplemented mice undergo second DSS treatment might lead to an inappropriate adaptive Th1/Th17 immune response and, as result, cause more severe and chronic colitis upon a second DSS treatment cycle.

The effects of the two probiotic strains examined in this report have been previously investigated using a preventive approach in a mouse model of colitis. While we found a preventive effect of *B. breve* and no effect of *L. rhamnosus* [15], in our current study we found a beneficial effect of *L. rhamnosus,* but not *B. breve,* during the remission phase and both probiotics worsened the fecal condition during the second DSS cycle. Although we are unable to exactly explain the differences found in these studies, one should note that the probiotics were administered when the mice were still healthy in previous preventive study. In the current study, the probiotic administration was started after the colitis had already been induced. DSS-induced colitis leads to structural change in colon including epithelial damage, a leaky gut as well as changes in immune components such as PRRs, T cells and cytokines [16]. These data suggest that the same probiotic strains may carry out different effects in an intestinal environment that is recovering from a prior inflammation.

In conclusion, we found that supplementation with *L. rhamnosus*, but not *B. breve*, affects the progression of DSS-induced colitis, and it is dependent on the status of the inflammation. During the remission phase, *L. rhamnosus* supplementation enhanced the recovery of the colitis-associated fecal condition. However, bacterial supplementation during the second cycle of DSS, mimicking a relapse, exacerbated the DSS-induced bloody diarrhea. Both *L. rhamnosus* and *B. breve* supplementation reduced the number of neutrophils in the colon of the mice suffering from DSS-induced colitis, accompanied by increased expression of Th1-associated cytokine, *Ifnγ*. In addition, *L. rhamnosus* supplementation further induced the expression of *Tlr2, Tlr6* and pro-inflammatory cytokines in colon of mice suffering from DSS-induced colitis. These data provide evidence illustrating the importance of innate immunity during the IBD development and the role of neutrophils for the elimination the resident pathogens during the inflammation.

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Chapter 8

SUMMARY AND GENERAL DISCUSSION

The prevalence and incidence of inflammatory bowel disease (IBD) are increasing rapidly, while a curative therapy is still not available. The existing therapies don't meet the medical need of the IBD patients. Increased number of studies has been conducted to investigate the pathogenesis of IBD and to identify possible targets for new treatments during the last decennia. Nowadays, although the pathogenesis of IBD remains unclear, an altered microbial balance (dysbiosis) and dysregulated immune responses against host microbiota, which leads to disturbed intestinal homeostasis, are accepted common features of IBD.
In the human gut, an enormous number of diverse microorganisms coexists without triggering the host immune defending system [1]. Since the separation between the host immune system and these microorganisms is only one single layer of epithelial cells, it is tempting to speculate that the intestinal microbiome might contribute to IBD pathogenesis as well. Studies analyzing the intestinal microbiome have revealed a range of alternations in the microbiome of IBD patients [2, 3]. Specific alterations in microbiota composition such as increased numbers of bacteria belonging to the genera *Desulfovibrio* and *Bilophila* [4, 5] and reduced numbers of bacteria belonging to *Lactobacilli* genus [2] and *Bifidobacterium* genus [6] have been identified in IBD. This microbial dysbiosis associated with reduced numbers of beneficial bacteria triggers new research: possible preventive and/or therapeutic capability of beneficial bacteria, also named probiotics, for management of IBD has been speculated.
A key component of the intestinal immune response is the pattern recognition receptor (PRR); these receptors recognize the microbes and initiate the activation of immune responses [7]. Aberrant activation or/and impaired function of the PRRs are associated with the development of IBD [7-11]. Genome-wide associated studies illustrated diverse IBD risk gene variants of PRRs including *NOD2* [12], *TLR1, TLR2* and *TLR6* [13], *TLR4* [14], *TLR5* [15] and *TLR9* [16]. In addition, clinical studies have demonstrated aberrant expression of PRRs in intestinal tissues of IBD patients, such as increased TLR2, TLR3 and TLR4 [8, 9] expression and decreased TLR5 [17] expression in intestinal epithelia cells (IECs) and mucosa, respectively.

The inflammation in a murine model of acute colitis associated with the expression of PRRs

Despite the suggested major immune modulating effects that PRRs might have in the development of IBD, the mechanisms of action of these receptors during the development of IBD remain unclear. Animal models are used to help elucidating the working mechanisms of the PRRs during the development of IBD [18]. In **chapter 2**, an extensive mRNA expression profile of PRRs including *Tlr1-9* and *Nod1* and *Nod2* is provided along the whole colon during the dextran sodium sulfate (DSS)–induced experimental acute colitis. An overall increased expression of PRRs, except *Tlr1* and *Tlr5*, was observed in the inflamed colon. *Tlr1* remained at the same expression level, while the expression of *Tlr5* was reduced. The increased expression of the *Tlrs* could be the result of sudden exposure to an enormous amount of TLR ligands derived from the gut microorganisms due to DSS-induced damaging of the epithelial layer and consequently increased microbial translocation. Additional interesting questions

are: do these alterations in mRNA expression reflect what happens during the development of IBD and what would be the consequence of the changes in mRNA expression of PPRs for the development of colitis?

Among the investigated PRRs, the TLR1 - TLR9 are conserved in both human and mice. Analysis of colonic biopsies derived from IBD patients demonstrated increased mRNA expression of different TLRs including TLR2, TLR4 and TLR9 [14] and TLR3 [8] in IECs and intestinal mucosa. In line with these findings, an increased expression of these *Tlrs* was also observed in our murine model. These data imply that the modified *Tlr* expressions found in mice suffering from acute colitis mirror at least some aspects of human IBD.

The NOD-like receptors are other family of PRR. The *NOD2* mutation was the first genetic link for the susceptibility of IBD [12]. Recently studies demonstrated that IBD-associated *NOD2*-mutation leads to loss of function of NOD2 and resulting in insufficient bacterial clearance, reduced induction of autophagy and defective antigen presentation [19-21]. Although these studies did not investigate whether restoring NOD2 function could improve the bacteria clearance and ameliorate the development of IBD, loss of function of NOD2 is linked to the development of IBD. Recently, the heat shock protein 70 that regulates anti-inflammatory molecules was shown to be able to restore the expression and activity of Crohn-associated NOD2 mutants in human cell lines [22]. Future studies to investigate the impact of this NOD2-expression induction system for the development of IBD will be extremely interesting. In our study, we observed an increased expression of *Nod2* in the inflamed colon in our murine model for acute colitis. Consistently, an increased NOD2 expression was also found in canine IBD [23]. However, to our knowledge no human studies are performed that demonstrate a similar response of NOD2 signaling. The increased expression of *Nod2* could be the natural response of exposure to enormous number of bacteria that entered the intestinal mucosal after DSS-induced epithelial injury. Take together; the modified expressions of *Nod*-like receptors found in our murine acute colitis model seem not to reflex what is observed in the inflamed intestine of IBD in human IBD. It is desirable to examine possible changes in NOD2 expression in human intestinal tissue in relation to IBD, in addition to studies regarding NOD2 mutations.

Activation of the PRRs is known to induce innate immune responses and help to coordinate the adaptive immune response [24-26]. The latter is achieved by antigen presentation, promoting the production of T cell-inducing cytokines or by function as co-stimulator receptors that direct affect the T cell response [24, 27]. In **chapter 2,** we demonstrated that the increased mRNA expression of PRRs is accompanied with increased expression of proinflammatory cytokines and Th1-, TH17- and Treg cell-associated cytokines. Activation of the PRRs induces a cascade of signal pathways that lead to activation of NF-κβ, which promote the production of the proinflammatory cytokines including tumor necrosis factor α (Tnfα), interleukin (IL)6 and IL1β [24]. In addition, PRR-induced phosphorylation of p38 mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinase (ERK) can lead to production of Treg cell-related cytokine (IL10) and TH17 cell-inducing cytokine

(IL23), respectively. TLR4 ligands activate the TRIF dependent pathway that results in production of Th1 cell-associated cytokines IL12 and type I IFNs [28, 29]. These data indicate that the increased expression of PRRs may lead to induction of an excessive proinflammatory immune responses including innate- and T cell-mediated adaptive immune system in the intestinal tract that result in a disturbed immune homeostasis.

PRRs, especially TLRs, meet many of the criteria for being a potential therapeutic target in IBD. These criteria include overexpression during disease; genetic differences correlating with risk factors for the disease. In addition, unique knockout animals used in disease models indicate the relevance of these receptors as well. So it is tempting to speculate that TLR targeting therapeutics could induce beneficial effects for IBD. Potential TLR targeting therapeutics can be (small) molecules, for example the TLR2 antagonist, OPN-305; NI-0101, an anti-TLR4 monoclonal antibody that interferes with dimerization of TLR4; or the TLR9 agonist, DIMS0150, that have been developed to inhibit immune signaling in disease-associated chronic inflammation as seen in IBD [29]. OPN-305 has not been tested in IBD yet. Recently, sustained clinical remission has been reported in IBD by local application of DIMS0150 [30, 31]. In addition, a phase I study with NI-0101 was started in 2014, however no results are available yet [32].

Another class of potential candidates targeting TLRs in IBD is probiotics. Probiotics are defined by the World Health Organization to be live microorganisms which, when administered in adequate amounts, confer a health benefit on the host [33]. Studies have shown that probiotics carry out strain-specific immune modulatory effects in a TLR dependent manner [34, 35]. Recently, several reviews summarized the clinical studies investigating the effects of lactic acid bacteria such as *Lactobacteria* and *Bifidobacteria* in the treatment of IBD [36-38]. In all randomized clinical intervention trials about 70% of the studies found beneficial effects of different bacterial strains such as maintenance of remission, clinical improvement and decreased intestinal inflammation. Although no clear role of targeting TLRs has been studied in these trials, a large number of *in vitro*- and *in vivo* studies demonstrated the contribution of TLR signaling to the probiotic-mediated immune modulatory effects [34, 35, 39, 40].

Since probiotic application for diseases like IBD attracts more and more attention, one should be conscious that the immune modulation property of a specific probiotic is highly strain dependent [41-43]. Different probiotic strains may target different TLRs or affect TLRs in different ways [43, 44]. Several studies have been performed to find the effector molecules of probiotics underlying the beneficial effects in chronic inflammation. A number of bacterial surface-associated components including lipoteichoic acid, peptidoglycan, and (glycol-) proteins have been described to directly affect the signaling pathway that underlie the immune modulatory capability [41, 42, 45]. Different composition of the cell surface components may lead to activation of different TLRs and induce different immune responses via the TLRs. For example, mutants of *Lactobacillus* strains that produce alterative surface component lipoteichoic acid were shown to induce less TLR2-dependent proinflammatory

cytokine production, but lead to higher anti-inflammatory cytokine expression resulting in an enhanced protective effect in a murine colitis model [46, 47].

T cell responses in the murine model for acute colitis

Abnormal T cell responses are recognized as a major pathogenic factor in IBD [48-50]. Long-lived microbiota-specific T cells are induced after intestinal infection indicating the contribution of disturbed T cell-mediated responses to the chronic nature of IBD [51]. Interfering with the development of these pathogenic T cells may provide a potential therapeutic treatment for IBD. To this end, an *in vivo* model that facilitates the investigation to the antigen-specificity and the induction of the development of these T cells will be very useful.

We have developed a murine colitis model that allows tracking of antigen-specific T cells. In this model, antigen-specific T cell populations including Th and Treg cells were generated against oral introduced specific antigen that was given together with DSS. In **chapter 3**, we show the increased amount of antigen-specific central memory T (T_{cm}) cells in the colon as early as 7 days after initiating of DSS-induced colitis in mice. These T_{cm} cells might be responsible for the chronic nature of DSS-induced colitis in mice as reported by us (chapter 6) and others [52].

For a long time, DSS-induced acute colitis was thought not to interfere with the adaptive immune system. This hypothesis was based on the finding that mice lacking functional CD4+ T cells develop colitis after DSS treatment [53]. However, a recent study shows a DSS-induced bacterial penetration in the inner mucosal cell layer that is normally devoid of bacteria before inflammation was detectable [54]. The DSS-induced modulation of mucus permeability will lead to an immune response to luminal antigen including those from microorganisms. Our study provided for the first time the direct evidence and presence of antigen-specific T cell responses during DSS-induced acute colitis.

Our study has a potential added value to the classic DSS colitis model, since it is now possible to track antigen-specific T cells that are developed against oral antigens. This new capability of the DSS colitis model makes it an even better tool for research into new T cell-targeting therapeutic compounds for IBD. The development of antigen-specific T cells can now be easily followed in DSS treated mice supplemented with compounds such as agonists or antagonists targeting PRRs or specific bacterial strains with implication potential.

The role of TLR6 in acute intestinal inflammation

TLRs are shown to promote the development of T cells and T cell-mediated immune responses [28, 55]. The contribution of specific TLR stimulation for the development of T cells needs to be further clarified, if TLRs are considered as potential targets for new treatments for IBD.

In **chapter 4**, the effects of TLR6 activation on T cell responses within the murine gastrointestinal-associated lymphoid tissues (GALTs) and the consequence for experimental colitis are extensively studied. First, an upregulation in the expression of mRNA encoding

for TLR6 was found in colonic biopsies of IBD patients as well as in colonic tissues obtained from an experimental colitis study. Stimulation of murine GALT cells with the Tlr6 ligand diacylated lipopeptide (FSL-1) resulted in increased expression of Th17 master transcription factor, RORγt, and IFNγ and IL-17A. In addition, FSL-1 induced the differentiation and expansion of Th1 and Th17 cell within the GALT. Since Th1 and Th17 responses seem to act synergistically in the pathogenesis of IBD [48, 56], FSL-1-induced Th1 and Th17 responses could lead to development of colitis. This hypothesis is supported by demonstrating that TLR6-/- mice show reduced polarization or expansion of Th1 and Th17, and that these mice are protected against DSS-induced colitis. Furthermore, oral administration of FSL-1 increases residual Th17-associated responses and increases the severity of experimental colitis. In addition, a correlated mRNA expression between *RORC* and *TLR6* was found in intestinal tissues obtained from patients suffering from active IBD. Collectively, these data suggest that TLR6 activation might be responsible at least in part for T cell polarization in IBD and for this reason TLR6 might be an interesting target candidate for new therapeutic avenues in the management of IBD.

As reduction of pro-inflammatory T cell response in IBD is the prime goal for therapy, blocking or reducing the expression or/and function of TLR6 should be one of the properties of TLR6 targeting compounds. An example is OPN305, a TLR2-specific monoclonal antibody developed by Opsona Therapeutics. It is able to block TLR2/6 and inhibits TLR2/6 mediated pro-inflammatory cytokine production. Although its applicability for IBD treatment still need to be tested, positive results have been seen in several other inflammatory and autoimmune disease models [57]. Recently, a combined structure- and ligand-based virtual screening approach has been presented for design of small molecule TLR2 antagonist [58]. This approach leading to identification of numbers of new antagonistic compounds gives a promising future for development of new antagonists for TLR2/TLR6, potential candidates for the treatment of IBD.

Other possible candidates to target TLR6 are specific bacterial strains. These bacterial strains can be used either as single strain or mixtures of multiple strains. It is tempting to assume that a mixture of beneficial bacteria always act in additive or synergistic ways, which may amplify the beneficial effect. However, we need to take caution when choosing and making such combinations. The possibility exists that one probiotic strain acts antagonistic against other probiotic strains [59]. Clinical studies using probiotic mixture did not always show more pronounced beneficial effects as compare to the single component probiotic strain. As matter of fact, several clinical studies have found even reduced beneficial effects of probiotic mixture [60, 61].

It is not clear which probiotics modulate the TLR6 function, however, diverse probiotic strains have been reported to induce Treg-mediated anti-inflammatory response via TLR2 [39, 62, 63], the TLR that forms heterodimers with TLR6 [64]. Among these probiotics *Lactobacilli* and *Bifidobacteria* have been studies extensively [23]. Recently, van Bergehenegouwen *et al* demonstrated that *Bifidobacteria strains,* but not *Lactobacilli* strains can induce TLR2/6

activity in HEK TLR2/6 transfected cells. Moreover, these *Bifidobacteria* strains are shown to stimulate the production of cytokines including IL10, IL6, and TNFα by DCs in a TLR2 (and possible TLR6)-dependent manner [65]. Clinical studies investigating *Bifidobacteria* have shown that different *Bifidobacteria* strains are able to induce Foxp3 T cell-mediated anti-inflammatory effects [66, 67]. It should be mentioned when using probiotics as TLR-targeting compounds that single probiotics may affect multiple TLRs. For example, an *in vitro* study using peripheral blood mononuclear cells (PBMCs) obtained from healthy individuals demonstrates that stimulation with *Bifidobacteria breve* induces TNFα and IL1β production in a TLR9-dependent manner, while reduces this cytokine production via TLR2-dependent signaling pathway [34].

The effects of beneficial microbes in the murine model for acute colitis

Probiotic *Lactobacilli* and *Bifidobacteria* can induce dendritic cell (DC) cytokine production via PRRs. Plantinga and co-workers have shown that these probiotic are able to modulate TLR-induced cytokine production by PBMCs on a strain dependent manner [68].

In **chapter 5**, the modulation capabilities of the probiotic strains *Bifidobacterium breve* NutRes 204 (*B. breve*) and *Lactobacillus rhamnosus* NutRes 1 (*L. rhamnosus*) on T cell polarization have been studied *in vitro* using PBMCs isolated from healthy volunteers. In addition, the DSS-induced acute colitis model was used to investigate whether these T cell-modulating capabilities of these beneficial bacterial strains are effective in the development of colitis.

Both *B. breve* and *L. rhamnosus* stimulation increase the percentage Th2 cells and decrease Th17 cells within the PBMCs. However, stimulation with *B. breve*, but not *L. rhamnosus*, increases the Treg cell population and decreases the percentage Th1 cells within the PBMCs. The strain-specific capacity of these probiotics to promote the T cell polarization has been reported previously with focusing on the Treg cell induction [69, 70]. Although the probiotic mechanism of action was not investigated, one potential mechanism might be mediated through recognition by the PRRs. This hypothesis is confirmed by results showing that the inhibition of TLR2, TLR4 or TLR9 lead to modulated cytokine production induced by different *Bifidobacteria* and *Lactobacilli* strains of human PBMCs and DCs [34, 65]. Activation of TLRs is known to induce the maturation of DC and consequently production of T cell-promoting cytokines that are necessary for differentiation and proliferation of distinct T cell subset [55]. In addition, recent evidence indicates that TLR signaling can promote the proliferation and differentiation of T cells also directly [27]. These data suggest that the probiotic-induced T cell polarization might be the result of indirect stimulation of innate immune cells like DCs, but also through direct activation of TLR expressed on the T cells.

The *in vivo* results mirror the data obtain *in vitro*. Supplementation with *B. breve* leads to increased Foxp3 mRNA expression and the number of Foxp3+ cells in the colon of both healthy and mice suffering from acute colitis. In addition, analyses in T cell population in Peyer's patches of colitic mice demonstrate a *B. breve*-induced increased amount of Treg

cells and a decreased amount of Th17 cells. Accordingly, ameliorated disease symptoms were found in DSS treated mice with *B. breve* supplementation, but not with *L. rhamnosus*. These data suggest that *B. breve* induces Foxp3 Treg-mediated anti-inflammatory response that lead to the reduction of the colitis symptoms. However, detail characterization of the T cell population in the *in vivo* will be necessary. The antigen-specific T cell tracking model described in **chapter 3** may be a useful tool in this case. Administration of tracking antigen together with the probiotic strains allows us to investigate the responding T cells in an antigen-specific way.

The beneficial effect of the probiotic strain *B. breve* is observed in the acute model; however acute colitis model might not be fully representative for the chronic nature of the IBD. The next logical step is testing these probiotics in a chronic colitis model to obtain more translational results regarding the effects of the examined *Bifidobacteria* and *Lactobacilli* strains in IBD.

The expression of PRRs is associated with inflammation in a murine model for chronic colitis

IBD is an intestinal inflammatory disease with a chronic nature; a chronic colitis model induced by repeated DSS exposure interspersed with recovery period can provide useful knowledge regarding the progression of the disease.

To extent our knowledge of the PRRs expression during chronic colitis, the colonic mRNA expression of PRRs during the DSS-induced chronic colitis is demonstrated in **chapter 6**. In addition, the T cell responses during the chronic colitis were monitored by measuring the mRNA expression of T cell-associated master transcription factors and cytokines. A major finding in this study regarding the PRR expression is that the mRNA expression of all *Tlrs*, except *Tlr1, Tlr5* and *Tlr9*, returned to basal level during the chronic colitis. This is in contrast to overall increased mRNA *PPR* expression found in acute colitis. During chronic colitis *Tlr1* mRNA expression was not affected in the inflamed colon. The *Tlr2* and, *Tlr3* and *Tlr4* expressions were somewhat increased after the second and third cycle DSS, respectively, but to a lesser extent when compared to acute colitis. Only the decreased *Tlr5* and increased *Tlr9* mRNA expression in colon persisted during the chronic colitis period. This modulation of TLR mRNA expression is accompanied by increased proinflammatory cytokines and Th1 and Th17 cell responses in the chronically inflamed colon. An interesting question arising from these data is: is there any association between modulated *Tlr* expression and increased proinflammatory immune response?

TLR5 recognize bacterial-derived flagellin and induces a cascade of signal transduction resulting in activation of NFκβ [71], which leads to production of proinflammatory cytokines [72]. To avoid overacting of the immune response, TLR5 is found to be expressed only on the basolateral surface of the intestinal epithelial cells and thus will not be exposed to the gut bacteria under normal conditions. Only when the invading pathogen has breached the epithelial barrier, TLR5 located on the basolateral surface will be activated to induce

rapid immune responses to promote the bacteria clearance [71, 73]. Loss of TLR5 results in severe intestinal inflammation as TLR5$^{-/-}$ mice develop spontaneous colitis [74]. The finding of abundant anti-flagellin antibodies in IBD patient suggests that the clearance of flagellin bearing bacteria is insufficient [75, 76]. In addition, a reduced TLR5 expression was found in the colon tissue derived from UC patients [17]. This decreased TLR5 expression can be the result of flagellin-induced tolerance, a phenomenon characterized by unresponsiveness to repeated flagellin stimulation and internalization of part of the TLR5 after prolonged flagellin exposure [77]. In line with these data, a reduced *Tlr5* expression level was also observed in our experimental chronic colitis model. Take together; these data suggest that reduced TLR5 expression possibly as result of flagellin-induced tolerance during IBD-associated chronic colitis can lead to insufficient bacteria clearance and ultimately development of chronic intestinal inflammation.

In addition to TLR5, interleukin (IL)-1-converting enzyme protease activation factor (IPAF) was also show to recognize intracellular flagellin and leading to secretion of the cytokine IL-1β by macrophages. The potent effects of IL-1β are damped by secretory IL-1 receptor antagonist (sIL-1Ra), which is induced dependent on TLR5/flagellin interaction [78]. These data suggest in addition to insufficient bacteria clearance, decreased *Tlr 5* expression in DSS-induced chronic colitis models as well as in IBD might also lead to increased IL-1β / sIL-1Ra ratio and consequent development of excessive inflammatory symptoms in the intestinal tract.

TLR9 has been reported to generate innate immune responses against virulent bacterial pathogens in lungs [79]. The intestinal tract surface, comparable to that of lungs, is a mucosal layer separated from bacteria by just one single epithelial layer. After disruption of this physical barrier by DSS treatment, it is possible that increased *Tlr9* expression lead to amplified Tlr9 signaling which resulting in exacerbate inflammatory responses such as production of proinflammatory cytokines by innate immune cells. In addition, TLR9 was shown to be required for Th1-mediate adaptive immune responses in GALT [80]. These data suggest that the proinflammatory response observed in DSS-induced chronic colitis might be mediated via the TLR9 signaling pathway. In support of this hypothesis, studies have demonstrated that TLR9 signaling contribute to the persistence of chronic intestinal inflammation [81, 82]. However, more recently, contradictory results have been presented by other researchers who demonstrate that administration of TLR9 ligand bacterial CpG motif exert a protective effect in murine experimental colitis [83, 84]. These results may be explained by the finding that stimulation of TLR9 in different compartments can lead to diverse effect [85]. Using the NFκB activation assay, it has been shown that activation of basolateral located TLR9 of epithelial cells promotes NFκB activation, and thus results in a proinflammatory response. While TLR9 signaling through the apical epithelial surfaces effectively prevents NFκB activation, indicating a tolerance mechanism towards the commensal bacteria. The latter mode of action of epithelial TLR9 is supported by recent clinical trials where sustained clinical remission has been reported in IBD by local and

topical application of the TLR9 ligand, DIMS0150 through rectal administration [30, 31]. In conclusion, we hypothesize that commensal bacterial triggering of TLR9 on the apical side of the epithelial cells might be a protective mechanism to limit the DSS-induced chronic inflammation. Further research is needed to examine at cellular level where the DSS chronic colitis-induced upregulation of TLR9 is found, since TLR9 is also expressed by numerous immune cells such as monocytes, DCs and B cells.

Based on these data, TLR5 seems to be a good targeting candidate for the development of new therapies. It is tempting to assume that TLR5 agonists, stimulating the TLR5 signaling, can treat the development of IBD. However, using the flagellin as a TLR5 agonist in murine colitis models has shown contradictive outcomes [86, 87]. So, further clarification of the action mechanisms of TLR5 and investigating its effect in different colitis models are desirable. On the other hand, TLR9 seem to be less suitable targeting candidate as it might have a double-edge sword function in inflammation. Finally, if using these TLRs as a potential targeting candidate, we should keep in mind that the outcome can be affected by location of action and duration of the exposure of TLR ligands.

The effects of beneficial bacteria strains in the murine model for chronic colitis model

In previous paragraph, a beneficial effect of *B. breve* in DSS-induced acute colitis model in mice has been presented when administrated prior to colitis induction. In **chapter 7**, the effects of the same probiotic strains have been examined in the DSS-induced chronic colitis model. Here the effects of both bacterial strains were investigated during the resolution phase of acute colitis as well as on the induction of chronic colitis by a second cycle of DSS in the drinking water. During the remission phase, therapeutic supplementation with *L. rhamnosus*, but not *B. breve*, increased and accelerated the improvement of the fecal condition indicating a strain specific remission-inducing effect.

However, an opposite effect on the disease activity was observed after reintroduction of DSS to induce chronic colitis. Supplementation with both *L. rhamnosus* and *B. breve* exacerbate the development of colitis. This phenomenon is accompanied by a reduced number of neutrophils assessed by decreased expression of myeloperoxidase (MPO), a neutrophil-specific enzyme, in the inflamed colon. Analysis of colonic mRNA has revealed an increased mRNA expression of the Th1 cell-associated cytokine *Ifnγ* in mice supplementation with both *L. rhamnosus* and *B. breve*. In addition, administration of *L. rhamnosus* further increased the mRNA expression of pro-inflammatory markers and *Tlr2* and *Tlr6* in the colon of DSS-treated mice. Although the exact reason why the colitis deteriorates is unclear, the increased severity, reduced neutrophils, increased pro-inflammatory and Th1 cell-associated cytokines are reminiscent of changes found in IBD patients resulting from limited bacterial clearance [88]. Our results indicate the important of protective and disease limiting immune response in the development of chronic colitis.

This study demonstrates without going into detailed mechanisms that the effect of probiotics after induction of colitis during the remission phase and during exacerbation of colitis might

be different between strains. One lesson that could be learned from this study is that timing is also essential for the probiotic treatment of IBD. In addition, one should also be careful to use probiotics during the remission phase of the disease. Although the majority of the clinically tested probiotics exhibits their beneficial effect by elongate the remission phase [36], we need to remember that it is unpredictable when exacerbation will occur due to the environmental factors such as diet, infections and stress [89].

Concluding remark

IBD is a chronic inflammatory disease in the gastrointestinal tract caused by a disturbed balance between the host immune system and intestinal microbiome. As result, aberrant immune responses are developed against host microbiota. Restoring the balance by either modulating host immunity or interfering with the microbiota composition may provide novel therapies for IBD. Specific beneficial bacterial strains can be potential candidates since it can both modulate the host immune system and interfere with the intestinal microbiota composition and its metabolism. A number of probiotic mixtures and single strain probiotics have already been in use in clinical research to test their efficacy in IBD [36, 37, 90]. The majority of these clinical studies shown the potential beneficial effects of probiotics on the maintenance of IBD remission, and limited randomized controlled clinical trials are available showing remission induction or decrease in colonic inflammation. Despite the success story of some of these probiotic strains in IBD, the working mechanisms of most probiotics remain unknown. One possible important part of the mechanism of action of probiotics is the role of their interaction with host cells via PRRs. PRRs are expressed by a broad cell lines including intestinal epithelial cells, innate immunes and lymphocytes [24]. Upon activation by probiotic, PRRs induce to a cascade of signaling pathway, that finally might contribute the maintaining or recover of the intestinal homeostasis [91].

In this thesis, we focused on the examination of the potential of the two probiotic strains, *B. breve* and *L. rhamnosus*, as possible new therapeutics for IBD. The beneficial immune modulatory capacity of these probiotic strains was shown *in vitro* using human PBMCs (chapter 5 and 65). These *in vitro* results show that both probiotic strains seem to skew the adaptive immune system towards an anti-inflammatory/regulatory response as they both induce Treg cells. DSS-induced acute and chronic murine colitis models were used to investigate the effect of the *B. breve* and *L. rhamnosus in vivo*. Supplementation of *B. breve,* but not *L. rhamnosus*, prior colitis induction ameliorates the acute colitis symptoms and increases Treg response in the colon and GALT. Supplementation of *B. breve* and *L. rhamnosus* after acute colitis induction, shown that *L. rhamnosus,* but not *B. breve,* accelerated the remission phase. Notably, supplementation with both *B. breve* and *L. rhamnosus* worsen the disease activity after reintroduction of colitis. These data suggest that the effects of *B. breve* and *L. rhamnosus* are strain specific and that timing is also essential factor for probiotic treatment of IBD.

Although the potential beneficial effects of both *B. breve* and *L. rhamnosus* were demonstrated in our *in vivo* model, the direct involvements of TLRs were not investigated yet. Follow up studies examine the contribution of TLRs to these probiotic strains-mediated immune modulation would be interesting, since different *in vitro* studies have shown indispensible role of TLRs for the immune modulatory capability of these probiotic strains [34, 65, 68]. Although we did not investigate the direct involvement of TLRs in these probiotic studies, we did observed an increased TLR2 and TLR6 expression in the colon of DSS treated mice supplemented with *L. rhamnosus* during the chronic colitis. TLR2/6 signaling was shown to promote Th1 and Th17 responses and modulate the DSS-induced colitis. In addition, we have found that the expression of *Tlr6* and *Tlr2* mRNA was highly significantly correlated with the mRNA expression of pro-inflammatory cytokine *Tnfα* and *Ifnγ* and *Il17* (Figure 1). Although a significantly correlation was also observed between these cytokines and *Tlr1*, it seems the contribution of TLR1/2 seems to be less pronounced as compare to TLR6/2 according to the Pearson r-values (Figure 1).

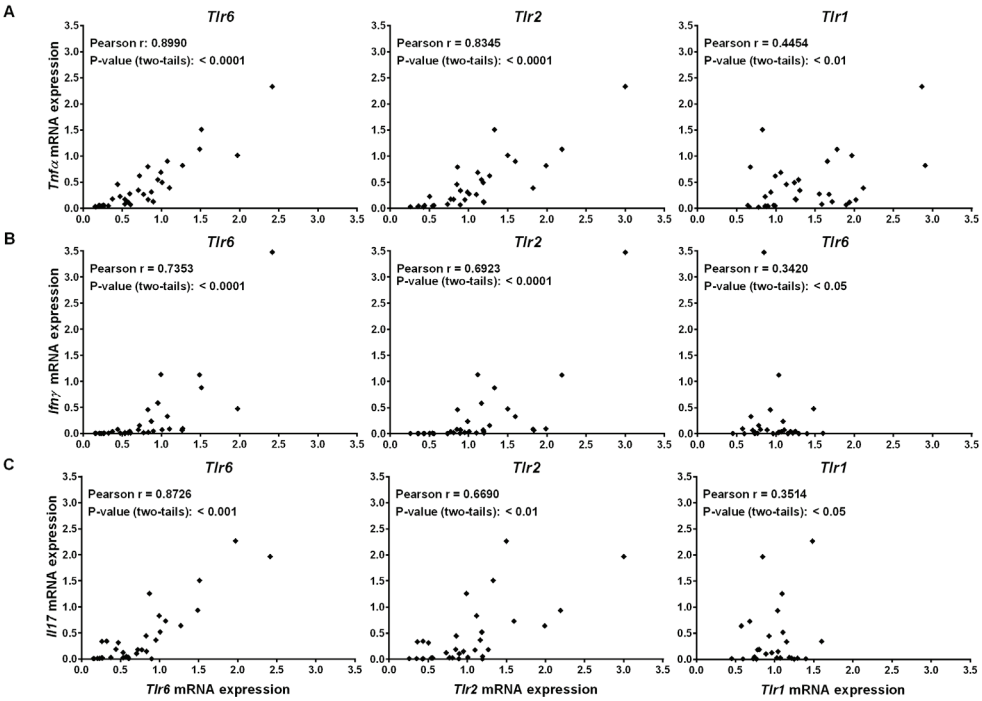

Figure 1. The mRNA expression of proinflammatory cytokines in the mouse colon is correlated with the mRNA expression of *Tlrs*.

Significant correlation was found between the mRNA level of A) *Tnfα*, B) *Ifnγ* and C) *Il17* and *Tlr6*, *Tlr2* and *Tlr1*.

Finally, despite the positive outcomes in *in vivo* models, it is still too early to make the conclusion that these specific probiotics are suitable as therapeutics for the treatment of IBD. Additional experiments, using the antigen-specific T cell tracking colitis model to characterize the involving antigen-specific T cell responses or other murine models for IBD, are required to extent our knowledge on *B. breve and L. rhamnosus* as treatment for IBD. In addition, the use of specific TLR blocking ligands or *Tlr* mutant mice will help us to determine the possible mode of action of these probiotics.

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NEDERLANDSE SAMENVATTING

Chronische inflammatoire darmziekten (inflammatory bowel diseases (Engels: IBD)) zijn een groep van chronische ontstekingsziekten die alle delen van het maagdarmkanaal kan aantasten. De twee meeste bekende zijn de ziekte van Crohn (CD) en colitis ulcerosa (UC). IBD kenmerkt zich door een onvoorspelbare wisseling tussen actieve fasen en remissies nadat de ziekte eenmaal is ontwikkeld. Als eenmaal de ziekte is vastgesteld, resulteert deze aandoening in een levenslange strijd met vaak onverklaarbare symptomen. De meeste voorkomende klachten van IBD zijn frequente buikpijnen, diarree, bloed- en slijmverlies bij de ontlasting, maar ook gewichtsverlies en algehele malaise. De kwaliteit van leven van de IBD patiënt zal dramatisch achteruit gaan. Bovendien heeft deze ziekte ook een enorme sociaaleconomische invloed op het leven van de patiënt. De ziekte openbaart zich vaak al bij jong volwassenen, die nog volop in het werkende leven staan; vaak zelfs aan het begin van hun carrière.

Uit recent onderzoek blijkt dat zowel de incidentie als de ernst van IBD sterk zijn gestegen in de afgelopen jaren. De bestaande behandelingsmethodes zijn allemaal symptoom bestrijdende therapieën. De medicijnen of behandelingsmethoden hebben grote consequenties voor de patiënten (zoals bijvoorbeeld chirurgie), zijn gelimiteerd tot beperkte patiëntengroepen (zoals de anti-tumornecrosefactor antilichaam behandeling) of gaan gepaard met ernstige bijwerkingen (zoals behandeling met prednison). Meer onderzoek naar nieuwe en effectievere behandelingsmethoden voor IBD is zeer gewenst; liefst met zo min mogelijk bijwerkingen.

Hoewel de etiologie van de ontwikkeling van IBD nog steeds onduidelijk is, duiden toenemende wetenschappelijke data erop dat de samenstelling van darmmicrobiota (het microbioom) een rol zou kunnen spelen. Het microbioom kan grote invloed hebben op bijvoorbeeld het intestinale/mucosale immuunsysteem en daardoor een belangrijke rol spelen in de pathogenese van IBD. Het lichaam van een IBD patiënt vertoont een verstoorde immuunreactie op bepaalde darmbacteriën waardoor een chronisch ontsteking in de darm zich zou kunnen ontwikkelen. Een veranderde samenstelling van het microbioom is ondertussen regelmatig beschreven. Sommige bacteriën die behoren tot de geslachten Desulfovibrio en Bilophila nemen in aantal toe terwijl andere bacteriën die behoren tot de geslachten Lactobacilli en Bifidobacterium vaak juist afnemen in aantal. Dit fenomeen zie je bij veel IBD patiënten. Er wordt gespeculeerd dat het induceren van een “gezond” microbioom zou kunnen helpen bij het herstel van sommige IBD patiënten. Deze hypothese wordt ondersteund door het succes van fecale-microbiotatransplantatie (FMT): het brengen van een gezond microbioom in IBD patiënten.

Probiotica zijn gedefinieerd door Wereldgezondheidsorganisatie en de Voedsel- en Landbouworganisatie in 2002 als “Levende micro-organismen, die wanneer in voldoende hoeveelheden toegediend, een gunstig effect hebben op de gastheer”. Tegenwoordig is het gebruik van probiotica in algemene voedingsmiddelen zoals yoghurt en melk steeds gebruikelijker geworden. Daarnaast krijgen probiotica als mogelijke behandeling voor

o.a. IBD steeds meer aandacht in het wetenschappelijk onderzoek. De theorie is dat het therapeutisch toedienen van bepaalde microbiota (probiotica) de verstoorde balans tussen het microbioom en het immuunsysteem van de gastheer kan herstellen. In vitro experimenten laten inderdaad zien dat verschillende probiotica immunologische reacties van bijvoorbeeld witte bloedcellen van zowel de mens als de muis kunnen moduleren. Deze immuunmodulatoire effecten kunnen pro- of anti-inflammatoir zijn, afhankelijk van de probiotische stam. In de darm zijn zogenaamde pattern recognition receptors (PRR) van groot belang voor het herkennen van microbiota en het initiëren van een immunologische reactie, zo ook voor de probiotica. De belangrijkste bacterie-herkennende eiwitten zijn Toll like receptoren (TLR) en Nucleotide-binding oligomerization domain receptoren (NOD). PRRs kunnen zich bevinden op een groot aantal verschillende celtypen zoals macrofagen, dendritische cellen, T cellen en epitheelcellen. Activatie van PRRs resulteert in verschillende immuunreacties afhankelijk van het PRRs type; een belangrijk fenomeen noodzakelijk om de gastheer te beschermen tegen pathogenen (virussen, bacteriën, parasieten). De familie van PRRs is zeer uitgebreid; wellicht worden daardoor wel vrijwel alle bacteriën herkend. Op dit moment zijn er 13 verschillende TLRs beschreven, waarvan TLR1 - TLR9 zowel in de mens als in de muis gevonden zijn. Zogenaamde 'genome-wide-association' studies hebben aangetoond dat verstoorde PRRs expressie en functie zijn geassocieerd met de ontwikkeling en ernst van IBD.

Het doel van dit proefschrift is meer kennis te verschaffen over de expressie van verschillende PRRs tijdens verstoorde immuunreacties betrokken bij zowel acute als chronische colitis in de muis. Verder zijn de immuunmodulerende effecten van de probiotische stammen Lactobacillus Rahmnosus (L. rhamnosus) en Bifidobacterium breve (B. breve) zowel in vitro op humane witte bloedcellen als wel in vivo in bovengenoemde muizenmodellen voor IBD onderzocht.

Diermodellen kunnen gebruikt worden om de rol van PRRs in IBD te onderzoeken. In hoofdstuk 2 is het mRNA-expressie profiel van de PRRs beschreven. De gehele lengte van de dikke darm van muizen die een acute colitis ondergaan is hiervoor intensief bestudeerd. Voor de inductie van acute colitis werd dextran sodium sulfate (DSS) toegevoegd aan het drinkwater. Acute ontsteking zorgde voor een verhoging van de expressie van alle Tlrs, behalve Tlr1 en Tlr5. De expressie van Tlr5 was verlaagd en van Tlr1 onveranderd. Deze data komen redelijk overeen met wat er is gevonden in darmweefsel van IBD patiënten; ook daar is een verhoogde expressie van TLRs waaronder TLR2, TLR3, TLR4 en TLR9 aangetoond.
Het exacte mechanisme dat leidt tot de verhoogde expressie van deze PRRs is nog niet voldoende onderzocht. Een aannemelijke verklaring voor de verhoogde Tlr expressie is dat door darmepitheelbeschadiging veel TRL liganden van darmbacteriën de aangeboren immuuncellen in de darmwand stimuleren om zodoende een sterke afweerreactie in gang te zetten. De aangeboren immuuncellen zijn door hun antigeen-presenterende capaciteit van

belang voor de ontwikkeling van een antigeen-specifiek afweerreactie. Een verhoogde gen expressie van de T helper1 (Th1) en Th17 cel-geassocieerde eiwitten (transcriptiefactoren en cytokinen) in de ontstoken muizendarm werd ook aangetoond. Th1 en Th17 cellen zijn belangrijke lymfocyten van het specifieke/adaptieve immuunsysteem, die de gastheer op een antigeen-specifieke manier beschermen tegen o.a. een infectie. Een hoge en onnodige activiteit van Th1 en Th17 cellen wordt ook gevonden in de darm van IBD patiënten. Mogelijk zijn deze T cellen essentieel voor de (chronische)ontsteking. Bovendien is in IBD patiënten aangetoond dat zogenaamde regulatoire T (Treg) cellen onvoldoende actief zijn in de regulatie/demping van afweerreacties op darmbacteriën.
In de literatuur wordt de bijdrage van de T cellen voor het ontwikkelen van IBD zeer vaak benadrukt. Om antigeen-specifieke T cellen beter te kunnen bestuderen na induceren van colitis, is het DSS colitis muizenmodel aangepast door gebruik te maken van een zogenaamd 'tracking' antigeen (bystander antigen). In hoofdstuk 3 hebben we aangetoond dat na inductie van acute colitis antigeen-specifiek T cellen aangemaakt worden in de darm. Deze T cellen zouden verantwoordelijk kunnen zijn voor de DSS geïnduceerde chronische colitis in de muis, zoals gerapporteerd in hoofdstuk 6. Aanpassing van het DSS muizenmodel met een tracking antigeen zorgt voor de beschikbaarheid van een mogelijk beter model voor IBD onderzoek om de rol van antigeen-specifieke T cellen te bestuderen.

In het hoofdstuk 4 is de rol van TLR6 op de ontwikkeling van IBD onderzocht middels in vitro en in vivo methoden. Allereerst hebben we een verhoogde expressie van TLR6 en Th17-geassocieerde eiwitten aangetoond in darmbiopten van IBD patiënten als ook in de ontstoken darm van het muizenmodel. In vitro stimulatie van uit de muizendarm geïsoleerde en humane lymfocyten met een TLR6 ligand leidt tot Th cel differentiatie richting Th1 en Th17 cellen en in vivo werden vergelijkbare resultaten gevonden na orale toediening van TLR6 ligand in de muis. Bovendien waren muizen zonder TLR6 resistent tegen DSS geïnduceerde colitis en vertoonde muizen na orale toediening van een TLR6 ligand een verergering van de DSS colitis. Deze data laten zien dat activatie van TLR6 een belangrijke rol speelt bij Th1 en Th17 responsen bij colitis en dat TLR6 mogelijk een interessant aangrijpingspunt is voor de behandeling van IBD.

Onze data als ook van andere (humane) studies illustreren de verandering van de PRRs expressie tijdens darmontsteking en laten zien dat de Th cellen belangrijke effectorcellen zijn in IBD. Dus lijken TLRs interessante aangrijpingspunten voor de behandeling van IBD te zijn. Mogelijke kandidaten hiervoor zijn specifieke bacteriën (probiotica). Een groot aantal in vitro en in vivo studies hebben aangetoond dat TLRs een belangrijke rol spelen in de werking van probiotica. Specifieke probiotische stammen kunnen verschillende TLRs beïnvloeden.

De mogelijke preventieve effecten van de bacteriële stammen L. rhamnosus en B. breve op de door DSS geïnduceerde acute colitis in muizen zijn onderzocht en beschreven in

hoofdstuk 5. In vitro testen laten zien dat stimulatie van humane witte bloedcellen met beide soorten bacteriën kan leiden tot een verhoging van het percentage Th2 cellen en verlaging van het percentage Th17 cellen. Een verlaagd percentage van Th1 cellen en een toename in Treg cellen werd alleen gemeten na stimulatie met B. breve. Dit verschil in effect kan een mogelijke verklaring zijn voor de uitkomst van de in vivo testen. De muizen kregen L. rhamnosus of B. breve oraal toegediend voor de DSS-behandeling en het toedienen van de bacteriën hield aan tot het einde van het experiment. De muizen die B. breve kregen, maar niet L. rhamnosus, laten een verminderde acute colitis zien. Uit de literatuur is bekend dat voor de behandeling van CD het belangrijk is om beide Th1- en Th17-celgemedieerde immuunreacties te verminderen. De in vivo resultaten die laten zien dat behandeling met B. breve Th1 en Th17 cellen verlaagt in plaats van alleen Th17 door de L. rhamnosus stimulatie komen overeen met de in vitro resultaten. Bovendien nam het aantal Treg cellen toe in de darmen van muizen behandeld met B. breve. Deze studie laat zien dat B. breve de potentie heeft om bescherming te bieden tegen het ontwikkelen van colitis. Dit beschermende effect is waarschijnlijk het resultaat van verandering in verschillende T cel populaties zoals het verhogen van het percentage Treg cellen en verlagen van het percentage van Th1 en Th17 cellen in de darm. Vervolgstudies zijn noodzakelijk om de effecten van de B. breve op T cellen in de darm en lymfoide organen in meer detail te begrijpen. Het DSS muizenmodel met een tracking antigeen beschreven in hoofdstuk 3 kan een interessante aanpak zijn.

Voorgaande hoofdstukken beschrijven resultaten in het veelgebruikte DSS-geïnduceerde acute colitismodel in de muis. Aangezien IBD een chronische aandoening is, zijn de immuunreacties en met name de Tlr expressie in een chronische colitis muizenmodel onderzocht en gepresenteerd in hoofdstuk 6. De chronische colitis werd geïnduceerd door herhaalde DSS-behandelingen met een rustperiode ertussen. Het idee is dat door herhaling van externe stimuli zoals DSS-behandelingen chronische colitis kan worden nagebootst, terwijl de rustperioden waarin de muizen schoon drinkwater kregen, de herstel- c.q. remissiefasen zouden kunnen representeren. In tegenstelling tot acute colitis, werd er in het chronisch colitis model geen verhoogde expressie van de genen coderend voor de meeste PRRs aangetoond, behalve voor Tlr5 en Tlr9. De expressie van het gen coderend voor Tlr5 blijft verlaagd, terwijl die voor Tlr9 verhoogd is tijdens de chronische colitis. De modulatie van Tlr5 en Tlr9 expressie in de chronisch fase van de colitis is geassocieerd met een sterke Th1 en Th17 reactie in de ontstoken dikke darm.
De verlaagde expressie van Tlr5 in ons chronische colitis model in de muis is interessant, omdat dit overeenkomt met de menselijke situatie tijdens IBD. TLR5 herkent bacterieel flagellin wat resulteert in de productie van inflammatoire eiwitten door epitheel- en immuuncellen. In de gezonde situatie komt TLR5 alleen tot expressie aan de basolaterale kant van epitheelcellen en komen zodoende niet in aanraking met darmbacteriën. Alleen wanneer een binnendringend pathogeen de epitheliale barrière gepasseerd heeft, zal TLR5 gestimuleerd worden om een afweerreactie te induceren tegen de binnendringer. In muizen

die geen TLR5 hebben ontwikkelt zich spontaan colitis en in IBD patiënten worden hoge waarden van anti-flagellin antilichamen gevonden en is de expressie van TLR5 sterk verlaagd in de ontstoken darm. Al deze data bij elkaar genomen suggereert dat klaring van bacteriën die flagellin tot expressie brengen, in IBD patiënten niet voldoende is waardoor er een overdreven immuunreactie ontstaat leidend tot chronisch ontsteking.
Studies hebben laten zien dat door TLR9 activatie de productie van pro-inflammatoire cytokinen start. Tijdens een virusinfectie in de longen is dit proces bijvoorbeeld essentieel. TLR9 is onmisbaar voor een Th1 celgemedieerde immuunreactie in het lymfoide systeem in de darm. De verhoogde expressie van de pro-inflammatoire cytokinen en Th1 celgeassocieerde eiwitten die we hebben gemeten in het chronisch colitis model komen overeen met deze data. Naast de pro-inflammatoire effecten van TLR9 zijn er ook studies die aangetoond hebben dat TLR9 een anti-inflammatoire rol kan hebben. Een beschermend effect van de behandeling met een TLR9 ligand is namelijk ook aangetoond in een colitis model in de muis. De exacte rol van TLR9 in IBD moet verder onderzocht worden om vast te stellen of dit PRR een interessant aangrijpingspunt is voor behandeling.

In het hoofdstuk 7 staan de resultaten beschreven over de effecten van bacteriële stammen L. Rahmnosus en B. breve in het chronische colitis muizenmodel. In deze studie werden de probiotische bacteriën toegediend na de eerste DSS behandeling tot het einde van het experiment. Verschillende effecten zijn gevonden tijdens de herstel- c.q. remissiefase en de tweede inductie van de colitis (de tweede DSS behandeling). Tijdens de remissiefase, zorgde L. rhamnosus, maar niet B. breve, voor een versnellend herstelproces van de acute colitis. Terwijl het toedienen van beide bacteriële stammen tijdens herhaalde inductie van de colitis leidde tot verergeren van de darmontsteking. Deze verergering ging gepaard met een sterke vermindering van de hoeveelheid neutrofielen en een toename in Th1 cellen en ontstekingsmediatoren in de ontstoken darm. L. rhamnosus zorgde bovendien voor een duidelijke stijging van de expressie Tlr2 & Tlr6 in de ontstoken darm. Deze resultaten suggereren dat TLR2/6 mogelijk een rol speelt bij de door L. rhamnosus geïnduceerde verergering van de darmontsteking. Hoewel de oorzaak van de toename in ontsteking in de dikke darm na behandeling met L. rhamnosus en B. breve nog niet duidelijk is, is een mogelijke verklaring dat er onvolledige klaring is van de binnengedrongen darmbacteriën dat vervolgens kan leiden tot sterke activatie van het immuunsysteem in de dikke darm resulterend in een heftigere ontsteking.

IBD is een chronisch inflammatoire ziekte in het maagdarmkanaal veroorzaakt door een verstoorde balans van het immuunsysteem van de gastheer op intestinale bacteriën. Het moduleren van de verstoorde immuunreactie en/of het instellen van een gezond darm microbioom kunnen een nieuwe aanpak zijn voor de behandeling van IBD. De onderzoeksresultaten beschreven in dit proefschrift laat een veranderd expressieprofiel van de PRRs zien tijdens de acute en chronische colitis. Deze veranderde PRR expressie gaat

gepaard met sterke Th1 en Th17 celgemedieerde immuunreacties leidend tot ontsteking. Het blokkeren of stimuleren van specifieke PRRs met behulp van farmaceutische moleculen kan een denkbare methode zijn om de ontsteking te voorkomen of te herstellen. Een andere mogelijkheid is het moduleren van de samenstelling van de darmbacteriën door bijvoorbeeld de behandeling met specifieke probiotische stammen.

Ondanks de successen van behandeling van IBD met specifieke probiotische stammen in klinische studies, is er nog maar weinig bekend over het werkingsmechanisme van probiotica. In dit proefschrift worden de effecten van de bacteriële stammen, L. rhamnosus en B. breve onderzocht ter voorkoming en behandeling van colitis in de muis. De resultaten laten zien dat de effecten van deze bacteriën sterk stam-afhankelijk zijn. De ene bacterie kan gunstige effectten hebben bij het voorkomen van de ontsteking, terwijl de andere stam voor een beter herstel zorgt. Daarnaast, is het tijdstip van toedienen van de probiotische bacteriën ook van belang voor het uiteindelijke effect. Een gunstig effect tijdens de herstelfase kan snel omkeren in verergering van de ontsteking als deze bacteriën tijdens het opvlammen van de ziekte worden gebruikt. Meer vervolgonderzoek is noodzakelijk om het exacte werkingsmechanisme van specifieke probiotische stammen en de mogelijke rol van PRR daarin te onderzoeken om zo een veilige en nieuwe behandelingsstrategie voor IBD te ontwikkelen.

CHINESE SAMENVATTING

中文总结

炎症性肠病 (inflammatory bowel disease,英文简称 IBD) 是一组慢性肠道疾病的统称，它可引发包括大肠以及小肠在内的肠道炎症，临床表现为频繁腹痛，腹泻，血便以及其他不适症状。两种最常见的IBD形式，是克罗恩病 (Crohn’s disease, CD) 和溃疡性结肠炎 (Ulcerative colitis, UC) 。虽然IBD对患者没有直接生命威胁，一些致命并发症如中毒性巨结肠、肠穿孔也少有发生，但由于IBD患者大多数是中青年人，做为社会最大的工作群体，这种疾病带来的不适症状给患者的日常生活带来的诸多不便将伴随患者一生。因此，这种疾病对社会和谐、稳定发展也会产生一定的影响。据统计IBD患者发生结肠癌的几率远远高于正常人，特别是幼年发病的结肠炎患者，癌变率是正常人的162倍。

最近的研究显示，近年来IBD的患病率有了大幅度的上升，相应对于治疗IBD的药物的需求也在日渐增长。但到目前为止，还没有能够彻底治愈IBD的医疗方法。现有的医疗方法主要是减轻患者所表现的症状。但这些医疗方法都有这样或那样的缺陷，比如外科切除手术会给患者造成终身不可逆转的伤害，而其他一些药物治疗或只对少数的患者有效或常伴有一些有害的副作用。因此为了寻找更有效同时又没有副作用的医疗方法，有关IBD更多更详细的研究是非常有必要的。

目前有关IBD的病因各有分说，而比较主流的说法是IBD的形成和肠道内所有细菌组成的变化有很大的关系。众所周知，在每个人的肠道内都寄生了大量的细菌。这些细菌帮助宿主分解吃下的食物有助于消化吸收，并且和宿主本身的免疫系统保持着微妙的平衡。越来越多的研究结果显示IBD患者的肠道细菌组成和健康的正常人有很大的不同。这种差异可能破坏了肠道细菌和人体免疫系统之间的微妙平衡,从而导致发病。一种新的IBD治疗法Fecale microbiota-transplantatie (FMT) 其治疗原理就是用健康人士体内的肠道细菌来取代IBD患者体内的细菌，从而恢复肠道细菌和人体免疫系统之间平衡的成功案列也从侧面证实了这种说法的可信性。近年来，添加了益生菌的食品，如酸奶等饮料变得越来越普遍。而使用益生菌作为一种针对IBD的治疗方法的可能性也吸引了越来越多科研中心的注意力。益生菌治疗可以干扰微生物群之间的平衡和恢复免疫系统的功能，从而使两者之间达到微妙的平衡，不少细胞实验以及动物实验得出的研究结果也证实了这一理论。但也有研究结果表明益生菌治疗所带来的效果取决于益生菌的种类，并不是每种益生菌都有治疗效果，某些益生菌在使用后不但没有任何治疗效果反而会使病情加重，而且每种益生菌所带来的正面效果也各不相同。

本文的中心课题是通过小白鼠模型来进一步展示IBD病发所带来的免疫

系统的变化， 特别是有关模式识别受体(Pattern recognition receptors, PRRs)的激活程度和T细胞相关的免疫反应。实验通过给小白鼠分别喂食短双歧杆菌 (*Bifidobacterium Breve, B.breve*)或鼠李糖乳杆菌(*Lactobacillus Rhamnosus, L.rhamnosus*)来研究这两种不同菌种对免疫系统起到的调节作用，从而测试这两种菌种对IBD的发病情况的影响。

为了能使大家更好的理解本文所述说的免疫系统的变化，首先向大家简单介绍一下相关免疫系统的组成部分，例如PRRs,以及与 T细胞 （白细胞的一种）相关的免疫反应。PRRs包括Toll样受体 （Toll-like receptors，TLRs）及NOD样受体 (Nod-like receptors, NLRs) 可以识别微生物特定分子就是所谓的病原相关分子模式 (Pathogen-associated molecular patterns, PAMPs)，包括细菌的碳水化合物(如脂多糖和甘露糖)；革兰氏阳性菌的肽聚糖、脂磷壁酸及真菌多糖。 PRRs分布在不同细胞的表面，包括各种免疫细胞如巨噬细胞，树突状细胞以及上皮细胞。其功能为识别病原微生物进而激活相关的免疫系统来帮助宿主抵抗外来的感染，其中也包括激活辅助T细胞(T helper cells, Th cells)相关的免疫反应。本文着重描述了Th1, Th2，Th17 以及调节T (regulatory T, Treg) 细胞群在病发后所发生的变化。Th1和Th2是两组传统的Th辅助细胞群。Th1辅助细胞的主要作用为对抗细胞内细菌及原虫的免疫反应, 其主要的执行的细胞因子是伽马干扰素 (IFNγ) 。而TH2辅助细胞的主要作用为对抗细胞外多细胞寄生虫的免疫反应，其主要的执行的细胞因子是IL-4,IL-5 & IL-13。这两种Th细胞又能互相压制彼此的信号传导路径。TH17是近几年才发现的Th輔助细胞, 其主要的执行的细胞因子是白介素17（IL17）, 主要作用为对抗细胞外细菌及微菌的免疫反应。 近年来的研究结果显示，过度激活Th17以及Th1辅助细胞相关的信号可能导致IBD的演变。Treg细胞正如名字所指，能够调节及压制其他辅助T细胞相关的信号传导，从而避免过度激活其他Th辅助细胞的信号。

本文分为两部分。 第一部分（第二章到第五章）使用的是急性结肠炎（acute colitis）小白鼠模型,通过给实验鼠喂食葡聚糖硫酸钠 (Dextran sulfate sodium,DSS)来诱发colitis。这部分研究成果描述的是病发初期免疫系统的改变。第二部分（第六章和第七章）使用的是慢性结肠炎 (Chronic colitis)的小白鼠模型。通过间接性给实验鼠喂食DSS 来诱发colitis。而在每次DSS喂食之间会有10天的间隔期，来模仿IBD患者的恢复期。这一部分所展示的是病发中后期间免疫系统的改变。

在本文第二章里，我们通过对比健康实验鼠和肠炎实验鼠肠道内PRRs的基因表达率和蛋白分布，展示了病发后PRRs的改变。 大部分的PRRs, 包括

Tlr2-Tlr4,Tlr6-Tlr9, 以及Nod1和Nod2 都处于过度激活的状态。只有Tlr1和病发前相比没有改变，而Tlr5 则处于远比病发前更低的激活状态。这些数据相当符合之前文献所发表有关PRRs在IBD患者肠道内的变化。在从IBD患者肠道所提取的细胞组织中也发现TLR2，TLR3，TLR4以及TLR9处于过度激活的状态。虽然在这里并没有详细研究导致这些 PRRs 过度激活的确切机理，但PRRs 的过度激活很可能是因为受到大量肠道细菌的刺激的缘故。因为在促进肠道炎症生成的过程中，肠道黏膜组织和上皮组织将不可避免的受到破坏，从而导致大量肠道的细菌得到释放，来刺激宿主本身的上皮细胞和免疫细胞等等。而布满在这些细胞膜上的PRRs能识别不同的细菌，进而激活一系列的免疫反应。辅助T细胞的激活就是其中一项很重要的免疫反应。 我们的数据显示，在病体实验鼠肠道内Th1和Th17相关基因的激活程度要远远高于同时期成长的健康实验鼠。Th1 和 Th17相关免疫反应在正常情况下能保护宿主抵抗病菌的感染。但在有炎症的情况下，过度激活这两组Th细胞将使炎症情况更加恶化，导致更多的细胞组织受到损害。近年来IBD临床实验所得到的结果也支持这一说法。另外，虽然我们发现在病体实验鼠肠道内Treg相关基因的激活度和健康实验鼠相比有所提高。但这显然并不足以压制所激活的免疫反应，从而达到消除炎症的目的。

一直以来，T 细胞对 IBD 发展所起到的作用被反复的强调。在本文第三章里，研究结果显示在病体实验鼠的肠道内找到了抗原特异性T细胞。这些T细胞很可能发展成记忆T细胞 （memory T cell）从而诱发chronic colitis。通过给老鼠喂食特异性抗原,我们改进了现有的老鼠模型。使其能更好的适用于研究抗原特异性T细胞在IBD中所起到的作用。

在本文第四章里，我们进一步研究了特定PRRs、TLR6的信号传导路径,及对IBD的发展所能产生的作用。首先，我们确认了TLR6以及Th17细胞相关基因确实能在IBD患者肠道切片组织里检测到。其次在体外细胞实验中，经TLR6配体刺激无论是人体细胞还是从实验鼠肠道分离出来的淋巴细胞都有向Th1和Th17细胞发展的趋势。同样的结果也在喂食了TLR6配体的实验鼠体内检测到，而且这些实验鼠的病况相对没有被喂食TLR6配体的实验鼠来说更加严重。最后我们对比了正常实验鼠和缺乏TLR6基因的实验鼠在acute colitis模型中的反应。实验数据显示缺乏TLR6基因的实验鼠在比正常的实验鼠更加能抵抗colitis的诱发。 这些数据表明，激活的TLR6信号传导路径能促进Th1和Th17免疫反应，进而诱发colitis。基于TLR6对诱发colitis的重要作用，如何调节TLR6信号传导路径使达到治疗IBD的目地将会是一个很值得研究的课题。

在本文第五章里，通过体外细胞实验以及colitis老鼠模型实验，我们测试了*B. breve*以及*L. rhamnosus*有没有可能对colitis起到预防作用。体外细胞试验结果显示，这两种细菌均能促进Th2细胞的增加和Th17细胞的减少。另外，*B. breve*的刺激更能降低Th1 细胞的数量，以及增加Treg细胞的数量。老鼠实验结果显示， 在这两种有益菌中只有*B. breve*对colitis有一定的保护作用，进一步证明了益生菌治疗所带来的效果取决于益生菌的种类。

所有以上本文使用的都是acute colitis小白鼠模型。鉴于IBD是一种慢性病，我们也使用了chronic colitis小白鼠模型用于观察免疫系统在病发中后期的变化。在本文第六章中，我们又一次对健康实验鼠和诱发了chronic colitis的病鼠肠道内的PRRs的基因表达率进行了对比。相对于使用acute colitis老鼠模型实验，这次我们主要关注的是PRRs在病发中后期的改变。在整个实验过程中只有Tlr1的基因表达率一直保持不变。其他大部分PRRs的基因表达率与病发初期相比，在经过初期的突增后又慢慢恢复到基础水平，除了Tlr5和Tlr9。Tlr5的基因表达率在病发后一直低于正常的水平，与此相反，TLR9的基因表达率一直保持着一种增长的状态。在这些Tlrs的基因表达率做出调整的同时，在病发中后期一直伴随着强烈的Th1和TH17相关的免疫反应。虽然确切调控Tlr5和 Tlr9基因表达率的机制尚不清楚，但TLR5的调控很可能是免疫系统自我调节机制所导致的结果。之前的研究结果显示长时间的刺激上皮细胞表面的TLR5蛋白质，能导致表面Tlr5蛋白质的数量的减少。这个研究成果很符合我们所测试到的TLR5基因表达率的变动。TLR9在很久以前就被证明在激活后可诱发一系列免疫反应来抵抗感染。前不久更有研究显示它对于在肠道附近淋巴系统中一个由Th1主导的免疫反应有着不可缺少的作用。

在本文第七章，我们测试了*L. rhamnosus*和*B. breve* 对已病发的实验鼠的效果。实验结果显示益生菌在病情缓解期和病情复发后有着完全不同的效果。在缓解期，使用*L. rahmnosus*能加快恢复的速度。但在病情复发后，使用*L. rhamnosus*和*B. breve*却导致了病情的恶化。虽然具体原因尚不清楚，但一个可能的解释是因为这些益生菌干扰免疫系统去彻底清除从肠道涌进的细菌，从而持续激活免疫系统， 最终导致炎症的恶化。而在实验中发现的大量减少的中性粒细胞和增强的促炎性细胞因子以及Th1细胞群比较符合这一假设。

正如本文开始所说，IBD是一种并不致命，但却能给个人以及这个社会带来很大麻烦的慢性病。本论文通过动物实验进一步增进了我们对IBD病发时免疫系统的了解，特别是肠道PRRs, 以及T细胞相关的免疫反应。为寻找新的更

有效的IBD治疗方法，提供了新的可能性，比如针对单个特定PRR，就像TLR6。而在动物模型中测试*L. rhamnosus*以及*B. breve*_的实验结果显示，益生菌的使用也可能是新的IBD治疗方法。但在使用益生菌时，需要注意几点。一是每种益生菌的效果取决于益生菌的种类；二是在时间不同的情况下，同一种益生菌所能发挥的效果也不尽相同。

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BIOGRAPHY

Bin Zheng was born on the 28th of November, 1981, in Wen Zhou, China. He grew up in Wen Zhou, where he also attended the primary school. In November 1994, he moved to the Netherlands to join family in the north of the Netherland (Friesland). He went to the secondary school (Stellingwerf College) in Oosterwolde, where he learned Dutch and followed the VWO education.

After he graduated in 2002, he moved to Amsterdam and studied Biomedical Science at the University of Amsterdam. He finished his Bachelor and Master study in 2005 and 2007, respectively. For his Master study, he did two extensive internships. The first internship was performed in Sanquin under the supervision of Dr. Maarten Rits. During this internship, different isoforms of the protein Trim5 were produced. In addition, the interactions between these isoforms and their relation with Cyclophilin A were investigated. The second internship was performed in the Netherlands Cancer Institute, Amsterdam under the supervision of Dr. Linda Smit. During this internship, deubiquitinating enzymes that are involved in Notch1 signalling were identification. The Notch signalling is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life.

In 2008, he started his PhD project within the framework of the Dutch Top Institute Pharm (TIP) project D1-101 “Exploitation of Toll-like receptors in Drug Discovery”, and under the supervision of Dr. Liz Morgan, Dr. Aletta Kraneveld, Prof. Dr. Gert Folkerts and Prof Dr. Johan Garssen at the division of Pharmacology, department of Pharmaceutical Science, Faculty of Science of the Utrecht University. During his PhD project, he has explored the intestinal expression of the pattern recognition receptors including Toll-like receptors in both acute and chronic murine colitis models. In addition, the immune responses after induction of the colitis such as T cell polarization were also examined in these murine models. Finally, in cooperation with Danone Nutricia Research group, the effects of two different probiotic strains in both murine colitis models were explored. The collected data and results during this PhD project form the basis for this thesis.

LIST OF PUBLICATION

Specific probiotic dietary supplementation leads to different effects during remission and relapse in murine chronic colitis
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Bifidobacterium breve **attenuates murine dextran sodium sulfate-induced colitis and increases regulatory T cell responses**
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ABSTRACTS

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