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Fecal microbiota transplantation alleviates experimental colitis through the Toll-like receptor 4 signaling pathway

Wen X *et al*. FMT ameliorates colitis *via* TLR4

Abstract

BACKGROUND

Fecal microbiota transplantation (FMT) has shown promising therapeutic effect on mice with experimental colitis and patients with ulcerative colitis (UC). FMT modulates the Toll-like receptor 4 (TLR4) signaling pathway to treat some other diseases. However, it remains unknown whether this modulation is also involved in the treatment of UC.

AIM

To clarify the necessity of TLR4 signaling pathway in FMT on dextran sodium sulphate (DSS)-induced mice and explain the mechanism of FMT on UC, through association analysis of gut microbiota with colon transcriptome in mice.

METHODS

A mouse colitis model was constructed with wild-type (WT) and TLR4-knockout (KO) mice. Fecal microbiota was transplanted by gavage. Colon inflammation severity was measured by disease activity index (DAI) scoring and hematoxylin and eosin staining. Gut microbiota structure was analyzed through 16S rRNA sequencing. Gene expression in the mouse colon was obtained by transcriptome sequencing.

RESULTS

KO (DSS + Water) group and KO (DSS + FMT) group displayed indistinguishable body weight loss, colon length, DAI score and histology score, which represented FMT could not inhibit the disease in KO mice. In the mice treated with FMT, the relative abundance of *Akkermansia* decreased, and *Lactobacillus* became dominant. In particular, compared

with those in WT mice, the scores of DAI and colon histology decreased obviously in KO-DSS group. Microbiota structure showed significant difference between KO and WT mice. *Akkermansia* were the dominant genus in healthy KO mice. The ineffectiveness of FMT in KO mice was related to decreased abundance of *Akkermansia*. Gene Ontology enrichment analysis showed that DEGs between each group were mainly involved in cytoplasmic translation and cellular response to DNA damage stimulus. The top 9 genes correlated with *Akkermansia* included Aqp4, Clca4a, Dpm3, Fau, Mcrip1, Meis3, Nupr1 L, Pank3, and Rps13 ($|R| > 0.9$, $P < 0.01$).

CONCLUSION

FMT may ameliorate DSS-induced colitis by regulating TLR4 signaling pathway. TLR4 modulates the composition of gut microbiota and the expression of related genes to ameliorate colitis and maintain the stability of the intestinal environment. *Akkermansia* bear great therapeutic potential for colitis.

Key Words: Toll-like receptor 4; Fecal microbiota transplantation; Colitis; *Akkermansia; Lactobacillus;* Aquaporin 4; Transcriptome sequencing

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Core Tip: Recent studies have shown that fecal microbiota transplantation (FMT) had a therapeutical role on inflammatory bowel disease patients. The Toll-like receptor 4 (TLR4) signaling pathway may play a critical role in intestinal injury and repair. Here, we carry out animal experiments to explore the role of TLR4 in the dextran sodium sulphate induced mice colitis and the treatment of FMT.

INTRODUCTION

Recent studies support that inflammatory bowel disease (IBD) can be categorized as a "microbial dysbiosis diseases", because of its progression synchronizing the dysbacteriosis of gut microbiota[1]. Host physiology, such as barrier function, metabolism, immune responses and homeostasis, involves microbiome-induced cell signaling, proliferation and neurotransmitter biosynthesis[2]. In IBD patients, intestinal bacterial diversity decreases and the bacterial community structure changes[3]. In dextran sodium sulphate (DSS)-induced colitis mice, some probiotics, including *Lactbacillus* and *Bifidobacterium*, are significantly reduced[4]. New evidence indicates that IBD is not merely a consequence of chronic inflammation, but disruption of gut microbiome and destruction of intestinal epithelial barrier[5].

Gut microbiota is intimate with inflammation-related activities. Fecal microbiota transplantation (FMT) has shown high efficacy and safety in treating ulcerative colitis (UC)[6,7], owing much to its immunomodulatory and anti-inflammatory functions[8]. Our previous study has well-described that FMT can counter DSS-induced colitis in mice by increasing the relative abundance of *Lactobacillus*[9]. FMT has also shown therapeutic potential for a range of other diseases, such as hepatic disorders and metabolic syndrome[10]. Recent studies have demonstrated that Toll-like receptor 4 (TLR4) is exploited by FMT in treating many diseases, such as spleen deficiency diarrhea[11], Parkinson's disease[12,13], developmental arsenic neurotoxicity[14], fluorosis[15] and acute lung injury[16]. Previous studies have indicated that FMT intervention can inhibit the activation of the NF-κB signaling pathway[17], which is downstream of TLR4. However, there have been limited studies investigating the role of TLR4 in FMT for UC.

As a class of transmembrane proteins that recognize invading microbes and activate immune cells, toll-like receptors (TLRs) regulate gene transcription and acquired intestinal immune response[18]. In the etiology of IBD, microbes in the intestinal lumen induce abnormal immune responses, along with excessive leakage of bacterial antigens into the mucosa[19]. TLR4, an important immune activator, is highly expressed in intestinal epithelial cells and lamina propria cells of UC patients[20]. It binds to ligands to activate cytokine signaling, recruit inflammatory cells, and damage intestinal

mucosal barrier, all aggravating intestinal inflammatory lesions. More importantly, substantial evidence supports a pro-inflammatory role of the TLR4 signaling pathway in UC. Expression levels of TLR4 were positively correlated with disease activity indices (DAI), endoscopy scores and histopathological scores[21]. DSS-induced colitis deteriorates in mice with TLR4 overexpression[22,23], but maintains stable in TLR4-deficient mice[24,25]. Multiple experiments have shown that inhibiting TLR4 signaling pathway can prevent DSS-induced colitis[26,27]. While TLR4 plays a crucial role in intestinal injury and repair, its role in shaping the colonic bacterial homeostasis and microbiota-related immunity remains poorly understood.

Our previous studies have confirmed the efficacy FMT on IBD, but the mechanism has never been reported. Therefore, we explored the role of TLR4 in the mechanism through which FMT copes with DSS-induced colitis in the mice.

MATERIALS AND METHODS

Animals

Wild-type (WT) C57BL/10J mice and TLR4-knockout (KO) mice on the C57BL/10J background [female; 6 wk to 8 wk of age; weighing 18-20 g; specific pathogen-free (SPF) grade] were purchased from the Model Animal Research Center of Nanjing University. All mice were reared in an SPF condition at the experimental animal center of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University. Throughout the acclimatization and study periods, all mice were maintained in a 12 h-light/12 h-dark cycle (21 °C ± 2 °C with a relatively constant humidity of 45% ± 10%) and had access to food and water ad libitum. All mice were group-housed and reared in the standard cage, with TLR4-/- mice kept separately from C57BL/10J mice in different cages.

DSS-induced colitis

Dextran sodium sulphate (DSS) (36-50 kDa) was purchased from MP Biomedicals LLC and dissolved in distilled water. Experimental colitis was induced performed as

detailed previously with minor changes[9]. For different groups, the mice were administered with 2.5% (w/v) DSS in drinking water for 7 d. The mice in KO (DSS + FMT) group were fed with fecal microbiota from healthy WT mice from d 8 (once every 2 d) until the end of the experiment while the mice in the KO (DSS + water) group with normal saline at the same time. The mice were evaluated daily by scoring *via* the disease activity index (DAI)[28]. The DAI score was calculated by a 0-4 scale graded mentioned in our previous publication[29].

Fecal preparation and transplantation

The process of FMT were performed according to the previous description[30]. Briefly, feces from donor mice (healthy WT mice) were collected and resuspended in sterile normal saline at 0.125 g/mL, then 0.2 mL of this suspension was administered to mice once every two days by oral gavage. This process lasted 7 d.

Histopathology

Mice were euthanized by cervical dislocation, and their abdominal cavity were opened immediately. The colon tissue was dissected; colons were measured for colon length, and tissues were examined for gross macroscopic appearance and stool consistency. The distal colon segment was placed in 10% neutral buffered formalin for 24 h, embedded in paraffin and cut into sections 4 μm in thickness. The sections were then stained with hematoxylin and eosin (H&E). H&E-stained sections were examined for inflammation and tissue damage by an experienced pathologist in a blinded manner. Tissue histology was scored by summing the scores of the following parameters[31] according to previous research: Inflammation extent, crypt aberrant, lymphocyte infiltration and colon wall aberrant.

Fecal DNA extraction and 16S rRNA sequencing

Fecal DNA extraction and 16S rRNA sequencing were performed as previously reported[9]. The V3-V4 hypervariable region of the bacterial 16S-rRNA gene was

amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAA T-3′) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, United States)[9]. All PCR products were extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States)[32]. Purified amplicons were sequenced on the Illumina MiSeq PE300 platform (Illumina, San Diego, United States). The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 and merged by FLASH version 1.2.7. Operational taxonomic units (OTUs) with 97% similarity cutoff[33] were clustered using UPARSE version 7.1[34], and chimeric sequences were identified and removed. Bacterial alpha-diversity was determined by sampling-based OTUs analysis. Species accumulation curves analysis was performed to assess the rationality and efficiency of the sequencing depth. Principal component analysis (PCA) was implemented in R programming. Using the Wilcoxon rank-sum test, the bacterial taxonomic analysis was performed for comparison at bacterial phylum, class, order, family, genus levels between two groups. Based on the matrix of normalized relative abundance, bacteria with significantly different abundances between assigned taxa were determined by LEfSe with the Kruskal-Wallis rank-sum test ($P < 0.05$), linear discriminant analysis (LDA) was used to assess the effect size of each feature [LDA score (log10) = 3 as cut-off value].

Transcriptome analysis

Total RNA was extracted from inflammatory colonic tissue. For sequencing, a 1-cm colon tissue was sampled from the site about 2 cm from the anus, regardless of whether there was visible inflammation. The tissue samples with minimum and maximum histological scores were removed. Then, the colon samples from four randomly chosen animals in each group was used for sequencing. Methods for amplifying and sequencing followed those previously published[9,29]. Briefly, a total amount of 2 μg RNA per sample was used to sequence on the Illumina Hiseq 4000 platform. Differential expression analysis was performed using the DESeq R package (1.10.1)

according to the manufacturer's protocol. Then, to explore the potential function of the DEGs, GOseq R package[35] and KOBAS software[36] were used to test the enrichment of DEGs in Gene Ontology (GO) functional annotations[37] and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways[38].

Correlation analysis for gut microbiota and transcriptome

We used Metastats software to confirm the difference in the relative abundance of microbiota among the samples ($P \leq 0.05$). Then we used DEG-seq to carry out transcriptome difference analysis (the threshold is padj < 0.05 & |log2FC| > 1). Finally, R psych software package was used to analyze the Spearman association between the transcriptome and intestinal microflora. Those with $|R| > 0.8$ and $P < 0.05$ (strong correlation) were screened for mapping.

Statistical analysis

Differences were analyzed using *t*-test with Graphpad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, United States). Results were shown as mean ± SEM; $P < 0.05$ was considered as statistically significant.

RESULTS

FMT could not improve the acute colitis induced by DSS in TLR4-KO mice

In our previous experiment, we found that FMT is effective to treat colitis. We further explored whether this efficacy is related to TLR4 pathway. Acute DSS-induced colitis was induced in 8 animals per group using 2.5% DSS in the drinking water. Strikingly, after gavage with fecal microbiota from the WT mice, the mice in KO (DSS + water) and KO (DSS + FMT) groups displayed indistinguishable body weight loss, colon length, DAI score, histology score (Figure 1A-D). Beyond our expectation, FMT posed no effect on colonic inflammation in TLR4-KO mice. Then, we compared the expression of TLR4 gene in the intestine of WT mice before and after FMT in our previous experiments. The

transcriptome sequence data indicated that DSS increased, but FMT effectively decreased the expression of TLR4 (Figure 1E).

FMT changed the intestinal flora of TLR4-KO mice

Subsequently, we investigated whether FMT changed the composition of gut microbiota in KO (DSS + water) and KO (DSS + FMT) groups. We employed LEfSe to evaluate the bacterial taxa (at genus level) in two groups (Figure 2A). The dominating taxa in KO (DSS + FMT) group were enriched in *Lactobacillus*, which indicated that we had successfully transplanted the gut microbiota of healthy WT mice. Meanwhile, KO (DSS + FMT) group had a lower abundance of *Akkermansia*, which meant FMT could alter the relative abundance of *Akkermansia* in KO mice (Figure 2B).

TLR4 knockout alleviated colitis induced by DSS

We used TLR4-deficient mice and WT mice to testify whether TLR4 may protect mice from DSS-induced colitis. The mice in KO-DSS (n = 8) and WT-DSS (n = 7) groups were given distilled drinking water containing 2.5% DSS for 7 d (Figure 3A). Compared with WT mice, KO mice showed lower susceptibility to DSS, as manifested by their much smaller body weight loss (Figure 3B), lower DAI (Figure 3C), and longer colons (Figure 3D). Compared to WT-DSS group, mice in KO-DSS group exhibited more intact colon structure, less severe crypt damage and reduced inflammatory infiltration (Figure 3E). In summary, KO mice showed increased tolerance to DSS-induced colitis.

TLR4 deficiency influenced the diversity and composition of gut microbiota

We further investigated whether the protection against DSS-induced colitis was attributed to TLR4 knockout or microbiota re-composition. We detected the gut microbiota of WT and KO mice in the basal and DSS-treated states. We analyzed the beta-diversity of microbiota based on PCA. An evident clustering separation between OTUs revealed the different community structures between each two groups,

suggesting that these communities are distinct in terms of their compositional structure (Figure 4A and 5A).

At the phylum level, TLR4 deficiency decreased the abundance of *Bacteroidetes* and increased the abundances of *Actinobacteria* and *Verrucomicrobia* ($P < 0.05$, Figure 4B), compared to those in the WT mice. After DSS induction, a significant increase of phylum *Proteobacteria* was observed in WT-DSS group, as compared to that in KO-DSS group ($P < 0.05$, Figure 5B). *Verrucomicrobia* were the most abundant phylum among those with significant differences ($P < 0.05$). At the genus level, *Akkermansia* abundance was significantly higher in the KO mice than in the WT mice either healthy or diseased ($P < 0.05$, Figure 4C and 5C). To further investigate the potential effect of microbiota composition on DSS-induced colitis, we used the LDA of effect size (LEfSe) to detect the marked differences in the dominant bacterial communities between the two groups (Figure 4D and 5D). Specifically, *Lactobacillus* and *Peptococcus* were enriched in WT-CON group (Figure 4D), while *Escherichia_Shigella* and *Anaerotruncus* were enriched in WT-DSS group (Figure 5D). Interestingly, *Akkermansia* and *Bifidobacterium* were enriched either in healthy and diseased KO mice (Figure 4D and 5D). The collective results of our study indicate clear differences in the intestinal microbiome between WT mice and KO mice, both in healthy conditions and during illness. These findings highlight the important role of TLR4 in shaping the composition and diversity of the intestinal microbiota.

TLR4-KO-shaped microbiota affected the transcriptome in the colon of mice

In order to further explore whether FMT can change the gene expression related to TLR4, we investigated DEGs between groups. Compared to those in the WT-DSS group, 1436 genes were differentially expressed in the KO-DSS group, and 309 genes in the KO (DSS + FMT) group. Furthermore, 193 DEGs were found between the KO-DSS group, WT-DSS group and KO (DSS + FMT) group (Figure 6A). GO enrichment analysis showed that these DEGs were mainly involved in cytoplasmic translation and cellular response to DNA damage stimulus (Figure 6B). According to the 16S rRNA sequencing

analysis, we found that *Akkermansia* dominated in the KO group. To characterize potential gene-microbe interactions, we computed gene-microbe correlations with Spearman correlation efficients (Figure 6C). The top 9 genes correlated with *Akkermansia* included Aqp4, Clca4a, Dpm^3, Fau, Mcrip1, Meis3, Nupr1 L, Pank3, and Rps13 ($|R| > 0.9$, $P < 0.01$).

DISCUSSION

Researchers have found that patients with active UC can benefit from FMT[39]. Moreover, our previous study has also verified that FMT can treat colitis in the mice. In the present study, the expression of TLR4 was upregulated by DSS, while it was downregulated after FMT. It therefore stands to reason that, by inhibiting TLR4, a protective effect from intestinal inflammation will be induced. Considering the ubiquitous involvement of TLR4 signaling pathway in the activities of the mucosa, we designed this animal study to elucidate its interaction with FMT in UC. In this study, TLR4 knockout significantly alleviated the clinical and histological manifestations of DSS-induced colitis. Notably, the increased relative abundance of the predominant *Akkermansia* species contributes to the heightened resistance against colon inflammation. Through further investigation, we have discovered that the genetic knockout of TLR4 significantly impacts the structure and composition of the gut microbiota, resulting in a shift towards an anti-inflammatory configuration. This shift plays a crucial role in promoting enhanced resistance and tolerance to colitis.

At the phylum level, DSS changed the relative abundances of *Bacteroidetes*, *Actinobacteria* and *Verrucomicrobia* in TLR4-KO mice, compared to WT mice. Possibly, the high abundance of anti-inflammatory *Akkermansia* in the gut microbiota curbs the aggravation of colitis, despite the absence of TLR4 signaling. *Akkermansia* was the dominant genus in healthy KO mice, while after the treatment of FMT, their level decreased. Compared with that in WT group, the status of colitis in KO group was not significantly attenuated by FMT, suggesting that the therapeutic effect of FMT on colitis is closely related to the TLR4 signaling pathway and *Akkermansia*.

In the gut, the expression of TLRs changes with the composition of microbiota[40], as well as the activity the intestinal epithelium, such as inflammation[41]. In the present study, we observed the difference in microbial composition between WT-DSS and KO-DSS groups. At the phylum level, KO-DSS group had a higher relative abundance of *Actinobacteria* and *Verrucomicrobia*, while WT-DSS had a higher relative abundance of *Proteobacteria*. In addition, *Verrucomicrobia* demonstrated the most significant difference at the phylum level. Lo Sasso *et al* have analyzed the composition of gut microbiota in UC patients *via* fecal microbiota whole-genome sequencing, finding increased abundance of *Proteobacteria* and decreased abundance of *Verrucomicrobia*[42]. In addition, one study characterized the mucosal microbiome of pediatric UC patients, noticing a significant decrease in the phylum *Verrucomicrobia* at the phylum level[43]. It has reported that the abundance of *Proteobacteria* increases in UC mice[44]. Moreover, the relative abundance of *Proteobacteria* in DSS-induced mice rises remarkably, compared with that in WT mice, which can be restored to normal after Lizhong therapy[45]. Consistently, this study proves that DSS can raise the abundance of *Proteobacteria* in WT mice, rather than KO mice.

In particular, we found that the abundance of *Akkermansia* increased in the KO-DSS group, but then dropped notably after FMT, indicating its role in the effect of FMT on UC. As reported before, the abundance of *Akkermansia* decreases in UC patients[46], but it is unclear whether this is a cause or consequence of UC. *Akkermansia* can protect intestinal barrier function and reduce the production of inflammatory cytokines[47]. On the other hand, *Akkermansia* can increase the production of short-chain fatty acids and anti-oxidant enzymes, indicating that *Akkermansia* may proliferate to alleviate colitis[48]. According to our experiment, the relative abundance of *Akkermansia* was negatively correlated with the severity of colitis in our animal models. *Akkermansia* bear great therapeutic potential for colitis. Studies on human and mice have revealed that the injection of beneficial bacteria, such as *Lactobacillus*, *Akkermansia* and *Bifidobacterium*, can alleviate the inflammation in UC patients[49-51]. In a systematic review of 3 studies, the abundance of *Akkermansia* decreases in all UC patients[52]; a high abundance of

Akkermansia could modulate host metabolism to prevent seizures[53]. In other studies, several *Akkermansia* species demonstrate abilities of modulating immune responses and protecting barrier function[54].

Despite the widely recognized beneficial properties of *Akkermansia* as a potential probiotic, it is crucial to take into account the potential occurrence of adverse effects. It was observed that colorectal cancer patients had a higher abundance of *Akkermansia_muciniphila*[3]. A prior study demonstrated that the genetic deletion of TLR4 exacerbated the severity of colon inflammation and thereby result in the decrease of the abundance of *Akkermansia*[55]. This conflicting conclusion may be explained by various factors, such as the different mouse species and the different experiment models used. When the equilibrium of the gut microbiota is disturbed, beneficial microbes have the potential to shift towards virulent species, leading to adverse effects on the host. Studies have suggested a potential link between *Akkermansia* and TLR4 signaling. A study demonstrated that the administration of anthocyanins extracted from *Lycium ruthenicum* (ACs) increases the abundance of *Akkermansia*, thus inhibiting the LPS/NF-κB/TLR4 pathway to improve intestinal function[56]. It has also been observed that inhibition of TLR4 signal pathway can increase the abundance of *Akkermansia*[57]. *Akkermansia* has been reported to promote the integrity of the intestinal barrier and regulate immune homeostasis, potentially by interacting with TLR4[58,59]. In the present study, the composition and structure of gut microbiota presented significant difference between the KO-DSS mice and the WT-DSS mice. Based on the above results, we advocate that *Akkermansia* can increase resistance to acute colitis in TLR4-KO mice. However, we need more in-depth investigations to determine if *Akkermansia* negatively associated with TLR4 are a potential target of FMT in treating UC.

TLR4 has been found to be differentially expressed in patients with early and advanced UC, indicating a close correlation between TLR4 and UC[60]. Inhibition of TLR4 was observed to significantly decrease the expression of cell cycle regulatory genes. Furthermore, TLR4 signaling in colonic epithelial cells was found to promote the recruitment of inflammatory cells through miR-155-mediated post-transcriptional

regulation[61]. Our results demonstrate that FMT down-regulated the expression of genes related to TLR4/MLCK signal pathway in WT mice, highlighting the importance of TLR4 in the effectiveness of FMT. The functional analysis revealed that most DEGs were enriched in cytoplasmic translation and cellular response to DNA damage stimulus. The top 9 DEGs strongly related to *Akkermansia* are primarily associated with cell cycle regulation, transcriptional control, apoptosis, stress responses and inflammatory responses. Their functions align with the main processes identified in the GO analysis, indicating their involvement in crucial biological pathways. These functions highlight its potential role in modulating various cellular activities. Aquaporin 4 (AQP4), a water channel protein that facilitates transmembrane water movement, has the strongest correlation[62]. AQPs are widely distributed in mammals' secretory and absorptive epithelial cells and responsible for transport and trafficking processes. In colonic inflammation, AQP4 is abundantly expressed in the basolateral membrane of colonic epithelial cells in humans and mice. As reported, the permeability of cell membranes is positively correlated with AQP4 expression[63]. AQP4 overexpression facilitates the entry of water into cytes, thereby contributing to cytotoxic edema[64-66]. AQP4 deficiency alleviates experimental colitis in the mice[67]. Although we did not use the same mouse knockout model in the present study, the effect of AQP4 on colonic inflammation is consistent with that of TLR4. Activating the HMGB1/TLR4/NF-kB pathway can increase the expression of AQP4[68,69]. Furthermore, LPS, a potent TLR4 agonist, significantly increases the mRNA level of AQP4 expression through TLR4 signaling in the cortex and astrocytes[63]. We may speculate that TLR4 deficiency could protect against colitis through increasing the abundance of *Akkermansia* and reducing the expression of AQP4. As shown by previous results, FMT can relieve colitis in WT mice. However, in this study, FMT did not exert effect on colonic inflammation in TLR4-KO mice. It is intriguing to detect that the abundance of *Akkermansia*, which had dominated in TLR4-KO mice, significantly decreased after FMT. This may be related to the decreased relative abundance of *Akkermansia*. While the DEGs mentioned above may have roles in immune regulation, inflammation, or cellular

processes that could intersect with TLR4 signaling, their specific relationships with TLR4 are not extensively characterized. Notwithstanding, further studies are needed to answer whether FMT also targets *Akkermansia* to regulate the expression of related DEGs in countering colon inflammation.

In this study, we assessed the microbial diversity and composition in DSS-induced mice. The bacteria inhabited in the mucosa may play major roles in the development of IBD. So, it is necessary to explore the function of microbiota in mucosal tissues in future study. However, animal studies have certain limitations in evaluating the mechanism of TLR4. Therefore, clinical studies should be designed to unveil the interplay among TLR4, gut microbiota and UC.

CONCLUSION

TLR4 modulates the composition of gut microbiota and regulates the expression of microbiome-related genes to ameliorate colitis and maintain the stability of the intestinal environment. For the first time, we find that FMT may ameliorate DSS-induced colitis by regulating TLR4 signaling pathway. Our findings will make the treatment of patients more targeted and is worthy of clinical trials in the future.

ARTICLE HIGHLIGHTS

Research background

It is well known that microbiota dysbiosis contributes to the occurrence of inflammatory bowel disease (IBD). Fecal microbiota transplantation (FMT) has shown promising therapeutic effects in both clinical and basic studies of ulcerative colitis (UC). Substantial evidence supports a negative pro-inflammatory role of Toll-like receptor 4 (TLR4) signaling pathway in IBD. However, it remains unknown whether this modulation is also involved in the treatment of FMT on UC.

Research motivation

FMT treats other diseases by regulating the TLR4 signaling pathway. Previous studies have shown that the expression of TLR4 in the intestinal mucosa of patients with effective FMT was higher while patients with poor FMT was lower. We speculate the TLR4 signaling pathway may be involved in the therapeutic mechanism of FMT on IBD.

Research objectives

To clarify the necessity of TLR4 signaling pathway in FMT on regulating gut microbiota in dextran sodium sulphate (DSS)-induced colitis.

Research methods

Experimental colitis was constructed in wild-type (WT) and TLR4-knockout (KO) mice and fecal microbiota was transplanted by gavage. Colon inflammation severity in mouse model was measured by disease activity index (DAI) score and hematoxylin and eosin (H&E) staining. Gut microbiota alteration was analyzed through 16S rRNA sequencing. The difference of gene expression in mouse colon was obtained by transcriptome sequencing of colon tissue.

Research results

In KO mice treated with FMT or water, these two groups displayed indistinguishable body weight loss, colon length, DAI score and histology score, which represented FMT could hardly alter the progress of the disease in KO mice. Next, compared with the WT mice, the scores of DAI and colon histology decreased obviously in KO-DSS group. KO mice experienced enhanced resistibility to DSS-induced colitis. There was a significant difference in the microbiota structure between KO and WT mice. *Akkermansia* was the dominant genus in healthy KO mice. But unexpected, After the treatment of FMT, the relative abundance of *Akkermansia* decreased, while the level of *Lactobacillus* in the intestine of mice prevailed. The ineffectiveness in KO mice after FMT was related to the decrease of *Akkermansia*. GO enrichment analysis showed that DEGs between each

group were mainly involved in cytoplasmic translation and cellular response to DNA damage stimulus. Finally, we list the top 9 genes related to *Akkermansia*.

Research conclusions

FMT may ameliorate DSS-induced colitis by regulating TLR4 signaling pathway.

Research perspectives

This study provides new insights into the underlying mechanisms of FMT as a treatment for UC, which greatly helps to optimizes FMT treatment in the future.

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Figure Legends

Figure 1 Fecal microbiota transplantation did not alleviate the acute colitis induced by dextran sodium sulphate in Toll-like receptor 4-knockout mice. A: Body weight of mice during the course of colitis; B: The bar chart represents the disease activity index score of mice at day 14; C: Representative images of colons from mice (left) and statistical analysis of colon length (right); D: Representative hematoxylin and eosin staining of colon tissues, original magnification 100 ×, and histological scores (right); E: Relative quantification of transcription level of Toll-like receptor 4 in groups. Values of $^{a}P < 0.05$. DSS: Dextran sodium sulphate; FMT: Fecal microbiota transplantation; KO: Knockout; DAI: Disease activity index; TLR4: Toll-like receptor 4.

Figure 2 Fecal microbiota transplantation changed the gut microbiota of Toll-like receptor 4 knockout mice. A: The LEfSe analysis in two groups with a linear discriminant analysis score > 3.0; B: The relative abundance of *Akkermansia* in Toll-like receptor 4 knockout mice. Values of $^{a}P < 0.05$, $^{b}P < 0.01$. DSS: Dextran sodium sulphate; FMT: Fecal microbiota transplantation; LDA: Linear discriminant analysis.

Figure 3 Toll-like receptor 4 knockout alleviated dextran sodium sulphate-induced inflammation in the colon. A: Scheme of the animal experimental design; B: The change in the body weight of mice from days 0 to 7 during the disease course (knockout-dextran sodium sulphate: n = 8; wild type-dextran sodium sulphate: n = 7); C: The bar chart represents the disease activity index score at day 7; D: Representative colons (left) and statistical analysis (right) of colonic length; E: Representative hematoxylin and eosin staining of colon tissues (left), original magnification 100 ×, and histological scores (right). Values of $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ are considered as statistically significant. KO-DSS: Knockout-dextran sodium sulphate; WT-DSS: Wild type-dextran sodium sulphate; FMT: Fecal microbiota transplantation.

Figure 4 Diversities and compositions of gut microbiota in knockdown-control and wild type-control groups. A: β-diversity evaluated using the weighted UniFrac-based PCA (knockdown-control: n = 7; wild type-control: n = 7); B and C: Bar graphs showing the relative abundances of different bacteria at the levels of phylum and genus; D: The LEfSe analysis in groups with an linear discriminant analysis score > 3.0 between two group. KO-CON: Knockdown-control; WT-CON: Wild type control; LDA: Linear discriminant analysis.

Figure 5 Diversities and compositions of gut microbiota in knockout-dextran sodium sulphate and wild type-dextran sodium sulphate group. A: Multiple sample principal component analysis (knockout-dextran sodium sulphate: n = 5; wild type-dextran sodium sulphate: n = 7); B: and C: Bar graphs showing the relative abundances of different bacteria at the levels of phylum and genus; D: The LEfSe analysis in groups with a linear discriminant analysis score > 3.0. KO-DSS: Knockout-dextran sodium sulphate; WT-DSS: Wild type-dextran sodium sulphate; LDA: Linear discriminant analysis.

Figure 6 Colonic transcriptome profile and gene-microbe correlation. A: Venn diagram illustrates genes regulated by fecal microbiota transplantation and Toll-like receptor 4-knockout; B: The top 20 Gene Ontology terms enriched in these 193 differentially expressed genes (DEGs); C: Network visualizing 193 DEGs associated with *Akkermansia* ($|R| > 0.8$, $P < 0.05$). FMT: Fecal microbiota transplantation; KO: Knockout; WT: Wild type; CON: Control; DSS: Dextran Sodium Sulphate; DEGs: Differentially expressed genes.

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