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Research Article

Keywords: Inflammatory bowel disease, microbiota, innate immunity, Myeloid Differentiation Factor 88, MyD88 inhibitor, NOD-like receptor
Suppression of MyD88 disturbs gut microbiota and activates NLRs pathway hence fails to ameliorate DSS-induced colitis

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Abstract

**Background:** The interaction between gut microbiota and innate immunity plays an important role in the pathogenesis of inflammatory bowel disease (IBD). Myeloid differentiation factor 88 (MyD88) is the core adaptor for Toll-like receptors (TLRs) defending against microbial invasion and initiating downstream immune response during microbiota-host interaction. However, the role of MyD88 in the pathogenesis of IBD is controversial. This study aims to investigate the impact of MyD88 on intestinal inflammation and the underlying mechanism.

**Methods:** MyD88 inhibitor (TJ-M2010-5, TJ5) were used to investigate the effect of MyD88 suppression on acute DSS-induced colitis. Disease activity index, colon length, histological score and inflammatory cytokines were examined to evaluate the colitis severity. The RNA transcriptome analysis and 16S rDNA sequencing were used to detect the potential mechanism.

**Results:** In the acute DSS-colitis model, the colitis severity was not alleviated in TJ5-treated mice, though the significantly lower of MyD88 expression and NF-κB activation were exhibited compared with control mice. Meanwhile, the 16S rDNA sequencing and RNA transcriptome analysis showed a higher abundance of intestinal *Proteobacteria* and an up-regulation of NOD-like receptors (NLRs) signaling pathway.
in colitis mice after MyD88 suppression. Further blockade of the NLR signaling pathway or elimination of gut microbiota with broad-spectrum antibiotics in DSS-induced colitis mice treated with TJ5 ameliorated the disease severity that was not improved by solely MyD88 inhibition. The down-regulated NLR signaling pathway after broad-spectrum antibiotics treatment suggested that the MyD88 suppression-associated dysbiosis may stimulate intestinal inflammation via NLR signaling pathway.

**Conclusion**: Our study revealed that the suppression of MyD88 may be associated with unfavorable changes in the gut microbiota composition and lead to immune activation mediated by NLRs, which may play an important role in modulating intestinal inflammation.

**Keywords**: Inflammatory bowel disease; microbiota; innate immunity; Myeloid Differentiation Factor 88, MyD88 inhibitor; NOD-like receptor

**Running title**: MyD88 blockade fails to mitigate acute colitis severity

**Background**

Inflammatory bowel disease (IBD) is a chronic and relapsing inflammatory disorder predominantly affecting the gastrointestinal tract, including ulcerative colitis and Crohn’s disease (1). In recent years, the incidence of IBD has increased globally, which brings a rising burden on health care (2). Although it is generally accepted that
IBD is a result of microbial dysbiosis, exaggerated immune response, disrupted intestinal barrier and genetic susceptibility, the precise etiology of IBD remains elusive (1). This is the main reason that current therapeutic strategies for IBD show limited efficacy but substantial adverse effects in achieving long-term remission (3). Dysregulated interaction between intestinal microbiota and the immune system plays a pivotal role in the initiation and progression of intestinal inflammation (4).

Invading microbiota can stimulate immune responses through various approaches. Dendritic cells located in the lamina propria can directly interact with pathogens and serve as antigen presenting cells to stimulate adaptive T cells (5). Diverse metabolites from microbiota can regulate immune reaction through the metabolite-specific receptor such as TGR5 and AhR (6). Imbalance between gut commensal and pathogenic bacteria may lead to immune activation responsible for intestinal inflammation. Pattern-recognition receptors (PRRs) are the major receptors for host to recognize the invading microbiota through conserved molecular structure, termed pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) and the nucleotide oligomerization domain- (NOD-) like receptors (NLRs) are the two main families of PRRs (7). TLRs are mainly localized in the cell membrane and capable of recognizing a broad range of exogenous PAMPs, while NLRs major in defense against intracellular microbes (8). The recognition of TLRs can initiate a cascade of innate and adaptive immune responses and play a central part in the pathogenesis of IBD (8, 9).

The molecule myeloid differentiation factor 88 (MyD88) is the core adaptor of TLRs and one of major mediators to activate NF-κB pathway (10). Patients with IBD
showed enhanced activation of MyD88 signaling in intestinal epithelial cells (11). The upregulation of MyD88/NF-κB signal induces the production of multiple proinflammatory cytokines in IBD (12). The differentiation and effector function of T cells also require the MyD88. It was reported that deletion of MyD88 in CD4+ T cells resulted in defective differentiation of Th17 and decreased secretion of IL-17 in mice with colitis (13). These innate and adaptive immune responses mediated by MyD88 would promote the development of intestinal inflammation and aggravate epithelial injury in IBD patients (12). Thus, the MyD88 was regarded as a possible therapeutic target for IBD (10). However, the role of MyD88 in the development of IBD is complex and controversial. In addition to regulating immune responses, MyD88 is also important in modulating intestinal bacterial clearance, epithelial restoration, mucosal permeability and even microbial composition (14-17). Several animal experiments showed that deletion of MyD88 could not mitigate intestinal inflammation and even increased tissue susceptibility to colitis (18-20). Therefore, further research is required to investigate the effects of MyD88 on intestinal inflammation and the underlying mechanism.

This study set out to analyze the in vivo impacts of MyD88 inhibition by a MyD88 inhibitor on acute intestinal inflammation in terms of the crosstalk between gut microbiota and the innate immune system. Our results revealed a potential role of MyD88 in shaping the structure of gut microbiota and a cooperation mechanism of TLRs-and NLRs- mediated immunological pathways.
Methods

Mice

The wild-type C57BL/6J female mice (6–8 weeks old) were purchased from HFK Bioscience and were randomly divided into control and treatment groups. All mice were bred under conventional laboratory conditions at the animal center of Tongji Medical College. Experimental protocols were approved by the Institute Animal Care and Use Committee at the Tongji Hospital, Wuhan, China.

Regents

The MyD88 inhibitor TJ-M2010-5 (TJ5) was generated in the School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (WIPO Patent Application Number: PCT/CN2012/070811) and kindly provided by Prof. Ping Zhou. NLRs inhibitor NOD-IN-1 (NI1) was purchased (MedChemExpress, MJ, USA). Imipenem (Merck Sharp & Dohme Corp. U.S.A) and vancomycin (VANCOCIN ITALIA S.R.L) were obtained from Tongji Hospital. Antibodies used for western blot were purchased from Cell Signaling Technology (MA, USA), ABclonal technology (Wuhan, China) and Proteintech (Chicago, USA).

Induction of Colitis and Treatment

Acute colitis was induced in C57BL/6J mice by treating with 3% (wt/vol) dextran sulphate sodium (DSS, 36–50 kDa, MP Biomedicals, USA) in the drinking water for 7 days. For the MyD88 inhibition study, mice were intraperitoneally injected with
50mg/kg of TJ-M2010-5 daily from one day before the first DSS administration to the 7th day of DSS challenge. To eliminate the gut microflora, mice were given imipenem (I, 1g/L) and vancomycin (V, 1g/L) in drinking water 3 days before and during DSS administration. The NOD-IN-1 was administrated at a dose of 20 mg/kg per day via intraperitoneal injection.

The Disease Activity Index (DAI) and Histological Score

Mice were monitored daily for weight, stool consistency and rectal bleeding. The DAI was calculated under the guidance of a previously established scoring system (Supplementary Table 1) (21).

The colonic tissues were fixed in 4% formaldehyde, paraffin-embedded, then sectioned at 3-6 μm and stained with hematoxylin and eosin (H&E). Each section was evaluated by three blinded researchers through the histological score (HS) system (Supplementary Table 2) (22).

RNA Extraction and RT-PCR

Total RNA was extracted by lysing colonic tissue with Trizol reagent. Complementary DNA (cDNA) was synthesized from 0.5ug of total RNA using a reverse transcriptase kit (Vazyme, Nanjing, China), and RT-PCR was performed using SYBR Green qPCR Master Mix (Vazyme) on an ABI StepOne Real-Time PCR system (Thermo Fisher Scientific, MA, USA). All primers were synthesized by Tsingke Biological Technology (Beijing, China). (Supplementary Table 3). The relative mRNA
expression levels of target genes were analyzed by using the $2^{-\Delta\Delta CT}$ method with $\beta$-actin as the reference gene.

**Western Blotting**

Colonic protein was extracted using a protein extraction kit (Solarbio, Beijing, China). The protein lysates were run on SDS-PAGE and transferred to a PVDF membrane (Millipore, Darmstadt, Germany). The PVDF membranes were blocked with 5% non-fat milk and were incubated at 4°C overnight. Afterwards, the membranes were incubated with anti-rabbit/mouse IgG conjugated to horse-radish peroxidase (HRP) for 2 h at room temperature, then visualized by ECL assay kit (Boster Biological Technology, Wuhan, China.).

**Gut Microbiota Analysis**

The microbial composition among studied groups was compared using LEfSe analysis. Feces were collected and washed by sterile PBS, then immediately frozen at −80°C. Fecal bacterial DNA was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, GA, USA). PCR amplification of 16S rRNA genes was carried out using the paired primers for the V3-V4 region of the 16S rRNA gene (Supplementary Table 4). The total numbers of bacteria per gram of feces were determined by the DNA copies level of the V3-V4 region of the bacteria 16S rRNA gene. The demultiplexed reads were clustered into operational taxonomic units (OTUs) of more than 97% similarity using UPARSE. The Shannon, Simpson, Chao, ACE indexes were used for the alpha-
diversity analysis by using mothur (version 1.33.3, http://www.mothur.org/). The principal coordinate analysis (PCoA), heatmap analysis, and species abundance analysis were performed by the R software (version 3.2.3, R Development Core Team, 2016). The sequencing service was provided by Bioyi Biotechnology Co., Ltd. Wuhan, China.

RNA Transcriptome Analysis

Total RNA was extracted from colonic tissue by Trizol. The preparation of cDNA transcriptome library followed the TruSeqTM RNA sample preparation Kit from Illumina (CA, USA). After qualification and quantification of cDNA, RNA sequencing was performed on the NovaSeq 6000 system from illumine. Data analysis was performed on the free online platform of Majorbio Cloud Platform (www.majorbio.com). The raw data were subject to quality control, pre-processing, read mapping and quantification of gene expression level. GO enrichment and KEGG pathway analysis were performed to identify the functional processes and metabolic pathways that the differentially expressed genes were enriched.

Statistical Analysis

All data are expressed as the mean ± SEM. Data between two groups using the unpaired t-test and comparisons between multiple groups using the ANOVA analysis via the GraphPad Prism software (version 7.01, GraphPad Prism Inc., USA). A two-sided P value of less than 0.05 was considered statistically significant.
Results

MyD88 inhibition failed to ameliorate the disease severity of acute DSS-induced colitis in spite of the restricted activation of NF-κB

We first tested the effects of the MyD88 inhibitor (TJ5) on the intestinal inflammation. Western blot analysis showed that TJ5 administration could significantly restrict the expression of MyD88 in colon tissue. Compared to PBS-treated mice, TJ5-treated mice exhibited markedly reduced the level of phosphorylated NF-κB in DSS-induced colitis colon (Figure 1A-B).

Mice challenged with DSS for 7 days were observed with increased DAI, worse histological score and shortened colon than mice without DSS challenge no matter the TJ5 was administrated or not (Figure 1C-F). Moreover, the expression of proinflammatory cytokines including TNF-α, IFN-γ and IL-1β was not significantly suppressed in TJ5-treated mice (Figure 1G).

Epithelial tight junction proteins (TJs) including ZO-1, occludin and claudin-5 were reduced in DSS-treated mice compared to the normal controls. Suppression of MyD88 could partially reserve the expression of TJs after DSS administration (Figure 1H). Since MyD88 was associated with tissue repair after epithelial damage, we detected the factors involved in tissue repair including TGF-β, EGF and COX-2 within the intestinal mucosa. The results revealed that TJ5-treated mice showed considerable differences in the expression level of TGF-β, EGF and COX-2 compared to the normal controls after DSS challenge (Supplementary Figure 1).

Taken together, these data indicated that TJ5 could inhibit the My88 expression
and partially suppress the activation of NF-κB, but it could not significantly ameliorate
the disease severity of acute DSS-induced colitis measured by DAI, HS and several
proinflammatory cytokines including TNF-α, IFN-γ and IL-1β.

MyD88 inhibition disturbed the composition of intestinal microbiota in mice

We next analyzed the possible reasons for the comparable colitis severity between
MyD88-suppressed mice and their control animals after DSS challenge. In light of the
important role of MyD88 in the interaction between gut microbiota and host immunity,
we examined the effect of MyD88 suppression by TJ5 on the gut microbial ecosystem.
16S rDNA sequencing analysis of feces revealed that the diversity and abundance
indexes of bacteria including Sobs, Chao1, Shannon and Simpson were significantly
different among the H₂O + PBS, DSS + PBS, H₂O + TJ5 and DSS + TJ5 groups (Figure
2A). The PCoA analysis revealed that the composition of gut microbiota separated
significantly in the four groups (Figure 2B). At the phylum level, TJ5-treated mice
showed increased Bacteroidetes and Proteobacteria, and decreased Firmicutes in the
colon compared to the PBS-treated control. In DSS-induced colitis, the abundance of
Proteobacteria was increased in TJ5-treated mice as compared to PBS-treated mice
(Figure 2C, 2F and Supplementary Figure 2). At the class level, the dominant bacterial
community of PBS-treated mice was Clostridia. Compared with the PBS-treated mice,
TJ5-treated mice showed reduced Clostridia and increased Bacteroidia (Figure 2D).
Compared to the DSS + PBS group, the abundance of Gammaproteobacteria
significantly increased in the DSS + TJ5 group (Figure 2D and 2F). Within the
Proteobacteria phylum, Enterobacteriales were predominantly increased at order level in TJ5-treated mice (Figure 2E and 2F). Whereas, Clostridiales which belong to Firmicutes exhibited a significant decrease in TJ5-treated mice (Figure 2E). These data revealed that MyD88 suppression would lead to an unfavorable alteration in the composition of gut microbiota.

NOD-like receptor signaling pathway was the major immune-related pathway upregulated in DSS-induced colitis mice treated with TJ5

To explore the signaling pathways possibly mediating the unmitigated colitis in DSS-fed mice after MyD88 suppression, we performed the RNA transcriptome analysis of colon tissue from the DSS + TJ5 mice versus the DSS + PBS mice. The comparative analysis revealed that 56 genes were differentially expressed between the two groups (Figure 3A). The differentially expressed genes were subjected to additional GO analysis and KEGG enrichment analysis. GO analysis showed that MyD88 suppression in acute DSS-induced colitis was related to the defense response, immune response and response to organism/external biotic stimulus (Supplementary Figure 3). The KEGG pathway analysis showed that immune-related pathways, including the IL-17, TNF and NOD-like receptor (NLR) signaling pathways were the major pathways associated with MyD88 inhibition in DSS-induced colitis (Figure 3B). Real-time PCR confirmed that only genes related to the NLR signaling pathway exhibited enhanced mRNA expression in DSS + TJ5 mice compared to the DSS+PBS mice (Figure 3C and Supplementary Figure 4-6).
Further suppression of NLR signaling pathway ameliorated the unmitigated colitis severity in TJ5 treated DSS-colitis mice

The above results demonstrated that MyD88 inhibition could not alleviate the colitis severity and was associated with the alteration of gut microbiota composition and the upregulation of NLR signaling pathway in mice with DSS challenge. To better understand the role of NLR signaling pathway in colitis under MyD88 inhibition conditions, TJ5-treated mice were intraperitoneally injected with NLRs inhibitor NI1. Western blot analysis indicated that the protein expression levels of NLRs and NLRs-induced MAPK signal were significantly upregulated in the DSS+TJ5 group compared with the DSS+PBS group. Administration of NI1 could suppress the upregulation of NOD-like receptor signal in mice with DSS-induced colitis (Figure 4A). Meanwhile, mice from the DSS+TJ5+NI1 group displayed a significantly decreased DAI, HS and colon shortening than those from the DSS+PBS group or the DSS+TJ5 group (Figure 4B-E). The mRNA expression of proinflammatory cytokines in colonic tissues, including IL-1β, IFN-γ, and TNF-α, were decreased in DSS-induced colitis mice by treating NI1 and TJ5 as compared to mice solely treated with TJ5 (Figure 4F). These data suggested that further inhibition of NLR signaling pathway in TJ5-treated DSS-colitis mice could ameliorate the colitis severity that was not improved by solely MyD88 inhibition with TJ5.

Antibiotics treatment altered colonic bacterial flora in mice

Since the important role of MyD88 in the interaction between gut microbiota and
host immunity, we next examined the combination effect of MyD88 suppression and antibiotics on the gut microbial ecosystem. Animals were given imipenem (I; 1g/L) and vancomycin (V; 1g/L) in drinking water for 3 days before and during DSS administration to eliminate the gut microflora. Total number of bacteria per gram feces was much less after antibiotics treatment (Figure 5A). 16S rDNA sequencing analysis of colonic mucosal samples revealed that the diversity and abundance indexes of microbiota, including Sobs, Chao1, Shannon and Simpson, were significantly different among the H2O + PBS, DSS + PBS, DSS + TJ5, DSS + I&V and DSS + TJ5 + I&V groups (Figure 5B). The PCoA analysis revealed that the composition of colonic microbiota separated significantly in the five groups (Figure 5C). At the phylum level of DSS-induced colitis mice, TJ5-treated and/or I&V-treated mice showed increased Proteobacteria, and decreased Bacteroidetes in the colonic microbiota composition compared to the PBS-treated mice (Figure 5D). The total number of Proteobacteria per gram feces was largest in TJ5-treated mice among the five groups (Figure 5E). Whereas, the total number of Proteobacteria per gram feces exhibited a significant decrease after antibiotics treatment (Figure 5E). These data revealed that the effect of MyD88 suppression on gut microbiota could be altered after antibiotics treatment.

Antibiotics treatment impeded the colitis progression in MyD88-suppressed DSS-colitis mice

We hypothesized that the MyD88 inhibition-associated increase of Proteobacteria in gut might upregulate NLR signaling pathway and lead to unamended colitis severity
after MyD88 inhibition. In our study, TJ5-treated mice were orally given broad-spectrum antibiotics with a combination of imipenem and vancomycin to eliminate gut bacteria. Compared to PBS or TJ5-treated DSS-colitis mice, the antibiotics plus TJ5-treated mice exhibited significantly lower DAI, histological scores and less colon shortening after DSS administration (Figure 6A-C). Pathological findings and proinflammatory cytokine levels revealed an ameliorated colonic inflammation in TJ5-treated DSS-induced colitis mice after antibiotics treatment (Figure 6D-E). By using broad-spectrum antibiotics to restrict the gut bacteria, the expression level of NLRs and activated MAPK proteins significantly decreased in TJ5-treated mice with DSS-colitis (Figure 6F). The data suggested that increase of gut *Proteobacteria* in MyD88-suppressed mice may exaggerate intestinal inflammation by stimulating NLRs-mediated inflammatory response.

Discussion

Suppression of the overactive immune response has been regarded as the important therapeutic strategy for IBD. Our study suggested that MyD88 inhibition by TJ5 could not improve the colitis severity in an acute DSS-induced colitis model, though the activation of proinflammatory NF-κB pathway was partially restricted. The results implied that the unfavorable changes in the gut microbiota and the upregulation of NLR signaling pathway after MyD88 suppression may account for the sustained DSS associated colonic inflammation and intestinal mucosal injury after MyD88 inhibition (Figure 7).
The MyD88-mediated innate immune response was suggested to be an important promoter of intestinal inflammation and could be a potential therapeutic target for IBD. In a mouse model of azoxymethane (AOM)/DSS–induced colitis-associated cancer, MyD88 inhibitor administration significantly alleviated AOM/DSS–induced colitis with less body weight loss and lower mortality (23). In the IL10−/− mouse model of chronic intestinal inflammation, MyD88 deletion could protect IL10−/− mice from spontaneous commensal-dependent colitis (24). Interestingly, our study demonstrated that blocking the MyD88-mediated immunological pathway alone could not ameliorate the acute DSS-induced colitis severity in terms of body weight loss, DAI, histological change and several proinflammatory cytokine levels including TNF-α, IFN-γ and IL-1β, although the activation of NF-κB was significantly inhibited. This result was similar with the observations in several other recent studies. Araki and Rakoff-Nahoum found that MyD88 deficient mice exhibited increased susceptibility to DSS with severe mucosal injury, rectal bleeding and even higher mortality (18, 19). Mice with intestinal epithelial-specific blockade of MyD88 even developed spontaneous inflammation in the small intestine at 36 weeks (25). These contradictory results of MyD88 blockade in the above murine models of intestinal inflammation might be attributed to the complex role of MyD88 in intestinal mucosal barrier.

The adaptor MyD88 could mediate the activation of NF-κB signaling pathway and was classically defined as a booster of mucosal inflammatory responses (10). Besides, MyD88 was important in regulating the epithelial repair and mucosal permeability (17, 19). Our results indicated that MyD88 suppression could restrict the activation of NF-
κB but could not reduce the level of several proinflammatory cytokines including IL-1β, IFN-γ, and TNF-α. The mRNA expression level of tissue repair factors including TGF-β, EGF and COX-2 within the intestinal mucosa was not significantly depressed after MyD88 suppression. And the TJs could be partially reserved by MyD88 inhibition after DSS administration. These results suggested that MyD88 suppression in experimental colitis could limit the activation of NF-κB and would not weaken the tissue reconstruction ability and epithelial TJs production. We further dug into the possible explanation why MyD88 inhibition could not improve the acute DSS-induced colitis severity.

MyD88 plays a key role in the innate immune defense against pathogens. It was reported that autosomal recessive MyD88 deficiency in children was associated with increased susceptibility to pyogenic bacterial infection (26). Production of antibacterial agents such as antimicrobial peptides and sIgA also required MyD88 (25). As the key adaptor of TLRs, MyD88 may participate in TLRs-mediated shaping and regulating of gut microbiota (27, 28). Given that MyD88 was involved in multiple antibacterial defense pathways, the hypo-expression of MyD88 may result in the accumulation of pathogens in gut and disrupt structure of gut microbiota. Our findings revealed that MyD88 suppression mice had unfavorable alterations in gut microbial composition with increased abundance of Proteobacteria. Proteobacteria was a minor phylum in human gut and consisted of several known human pathogens, such as Escherichia, Shigella, Salmonella and Yersinia which could activate the host immune defense and trigger a proinflammatory state (29, 30). Patients with IBD was often found
with the increase of *Proteobacteria* in gut (31-33). Thus, MyD88 suppression may lead to dysbiosis and stimulate intestinal inflammation by activating other immunological pathways.

The RNA transcriptome analysis in our study showed that NLR signaling pathway was the major upregulated immunological pathway associated MyD88 suppression in DSS-induced colitis. Further blockade of the NLRs signaling pathway in MyD88-suppressed mice could ameliorate the colitis severity that was not improved by MyD88 inhibition. Besides, elimination of the intestinal *Proteobacteria* in MyD88-suppressed mice by broad-spectrum antibiotics could also alleviate the colitis severity, together with the reduced expression of NLRs and NLRs-mediated MAPK signaling pathway.

It was acknowledged that both NLRs and TLRs were the important PRRs families, which worked collaboratively to recognize PAMPs and induced immune defense against invading pathogens in intestinal epithelia (34). The NLRs were localized in the cytoplasm and have been shown a key role in the defense against intracellular microbiota. PAMPs derived from pathogens could activate NLRs, leading to downstream proinflammatory signals transduction and cytokines production (8). It implied that MyD88 inhibition and MyD88 hypo-expression-related dysbiosis would induce activation of the NLR signaling pathway in acute DSS-induced colitis, which may be the reason why MyD88 inhibition failed to mitigate colitis severity evaluated by DAI, HS and several proinflammatory cytokines including IL-1β, IFN-γ, and TNF-α.
Conclusion

In conclusion, this study indicated that blockade of MyD88-mediated immunological pathway was associated with unfavorable changes in gut microbiota and would induce activation of the NLRs-mediated immune responses. The cooperation of PRRs is important for modulating intestinal inflammation.

Abbreviations:

IBD: inflammatory bowel disease; PRRs: pattern-recognition receptors; PAMPs: pathogen-associated molecular patterns; TLRs: Toll-like receptors; NOD: nucleotide oligomerization domain; NLRs: nucleotide oligomerization domain like receptors; MyD88: myeloid differentiation factor 88; TJ5: TJ-M2010-5; NI1: NOD-IN-1; DSS: dextran sulphate sodium; I&V: imipenem plus vancomycin; DAI: disease activity index; OTUs: operational taxonomic units; PCoA: principal coordinate analysis, AOM: azoxymethane.

Declarations:

Ethics approval and consent to participate

All experimental involving animals were conducted according to the ethical policies and procedures were approved by the Institute Animal Care and Use Committee at the Tongji Hospital, Wuhan, China (Approval no. TJH-201901004).

Consent for publication
Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and the sequencing data in this study are available in the NCBI online repository (https://www.ncbi.nlm.nih.gov/, accession number: PRJNA904645 and PRJNA 904649)

Competing interests

The authors declare no competing financial interests.

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Author’s contributions

YC, ZY, JX, JH, JHL, LC, FX performed experiments; LX, SX studied the properties of the MyD88 inhibitor TJ-M2010-5; YC, ZY, LC, JH, JHL, LX, SX, analyzed data; YC, ZY, JHL, FX, JZL wrote the manuscript; JZL, US, DT, FX supervised parts of the project; FX, US, DT designed the study and obtained funding for the project. All authors read and approved the manuscript.
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Supplementary Information

Additional file 1: Supplementary Table 1. Scoring criteria for disease activity index (DAI). Supplementary Table 2. Histological grading of colitis. Supplementary Table 3. Primers used for RT-PCR. Supplementary Table 4. Primers for the V3-V4 region of the 16S rRNA gene. Supplementary Figure 1. MyD88 inhibition failed to interfere with the intestinal epithelial reconstruction ability. Relative mRNA expression levels of TGF-β1, EGF, and COX-2 in colon tissue of control and TJ5-treated mice by RT-PCR analysis. Data were represented as mean ± SEM, *p<0.05, ns: not significant. TJ5: TJ-M2010-5. Supplementary Figure 2. Significant changes in functional profiles were suggested based on phylogenetic investigations of communities through LEfSe analyses (LDA-score >2.5). The dominating taxa in DSS+TJ5 group were enriched in Proteobacteria at the phylum level. Supplementary Figure 3. The differentially expressed genes of DSS+TJ5 group compare with DSS+PBS group were analyzed by GO. Supplementary Figure 4. The differentially expressed genes (DSS+PBS group vs. DSS+TJ5 group) associated with NOD-like receptor signaling pathway were identified by RT-PCR. Data were represented as mean ± SEM, *p<0.05, ns: not significant. TJ5: TJ-M2010-5. Supplementary Figure 5. No significant difference was detected in the key genes involved in the MyD88-independent signaling pathway by RT-PCR. Supplementary Figure 6. NLRP3 signaling activation was suppressed by
TJ5 administration determined by western blot analysis. Data were represented as mean ± SEM, *p<0.05.

Reference


Figure 1. MyD88 inhibition restricted NF-κB activation but failed to alleviate the acute colitis severity in mice. (A and B) Western blotting analysis of MyD88 protein expression and phosphorylation levels of NF-κB in colon tissue. (C) Disease activity index change, (D) colon length, (E) representative H&E staining of colon sections and (F) histological score of control and TJ5-treated mice administrated with water or 3% DSS for 7 days. (G) Relative mRNA expression levels of IL-1β, IFN-γ and TNF-α in colon tissue by RT-PCR. (H) Western blotting analysis of ZO-1, Claudin-5 and Occludin expression in colon tissue obtained from control and TJ5-treated mice administrated with water or 3% DSS for 7 days. Data were represented as mean ± SEM, *p<0.05, ns: not significant. TJ5: TJ-M2010-5.

Figure 2. Effects of MyD88 inhibition on the gut microbiota of mice. (A) Rarefaction plots based on the Sobs, Chao1, Shannon and Simpson indexes. (B) Principal coordinate analysis (PCoA) plot of the intestinal mucosa-associated
microbiota with Bray-Curtis distance. Average relative abundances of taxa at the (C) phylum level, (D) class level and (E) order level. (F) Relative abundances of indicated bacterial taxa in feces. Data were represented as mean ± SEM, *p<0.05, ns: not significant. TJ5: TJ-M2010-5.

Figure 3. MyD88 inhibition induced the upregulation of NOD-like receptor signaling pathway in mice with DSS-induced colitis. (A) RNA-sequencing was performed with isolated colon tissue from mice with DSS-induced colitis treated with PBS or TJ5. The heat map indicated the differentially expressed genes by the clustering analysis. (B) The differentially expressed genes (DSS+PBS group vs. DSS+TJ5 group) were analyzed by KEGG enrichment analysis. (C) The differentially expressed genes (DSS+PBS group vs. DSS+TJ5 group) associated with the NOD-like receptor signaling pathway were identified by RT-PCR. Data were represented as mean ± SEM, *p<0.05, ns: not significant. TJ5: TJ-M2010-5.

Figure 4. Further blockade of NOD-like receptor signaling pathway attenuated the unmitigated DSS-colitis severity in TJ5 solo treated mice. (A) Colon samples were collected after 7 days of DSS administration. Protein extracted from colon tissue was analyzed by Western blot for MyD88, NOD1/2, ERK, phospho-ERK, p38 MAPK and phospho-p38 MAPK. (B) Disease activity index change, (C) colon length and (D) histological score of mice in different groups. (E) Representative H&E staining of colon sections. (F) Relative mRNA expression levels of IL-1β, IFN-γ and TNF-α in colon
Figure 5. Alteration of colonic bacterial flora by broad spectrum Antibiotics in mice

Animals were given imipenem (I; 1g/L) and vancomycin (V; 1g/L) in drinking water for 3 days before and during DSS administration. (A) Total number of bacteria per gram feces were quantified using RT-PCR. (B) Rarefaction plots based on the Sobs, Ace, Chao1, Shannon and Simpson indexes. (C) Principal coordinate analysis (PCoA) plot of the intestinal mucosa-associated microbiota with Bray-Curtis distance. (D) Average relative abundances of taxa at the phylum level. (E) Total number of Proteobacteria per gram feces in different groups.

Figure 6. Antibiotics treatment dampened the colitis progression in TJ5-treated mice. (A) Disease activity index change, (B) colon length and (C) histological score of mice. (D) Representative H&E staining of colon sections was obtained from mice in each group. (E) Relative mRNA expression levels of IL-1β, IFN-γ and TNF-α in colon tissue by RT-PCR. (F) Protein extracted from colon tissue was analyzed by Western blot for MyD88, NOD1/2, RIPK2, ERK, phospho-ERK, p38 MAPK and phospho-p38 MAPK. Data were represented as mean ± SEM, *p<0.05. I&V, imipenem and vancomycin.

Figure 7. Schematic diagram depicting gut dysbiosis and stimulation of NOD-like
receptor signaling pathway after MyD88 suppression in intestinal inflammation.

In normal conditions, the extracellular and intracellular PPRs (TLRs and NLRs) sense intestinal microbiota through PAMPs and exert anti-bacterial effects. In the state of intestinal inflammation, though MyD88 suppression can restrict the MyD88-mediated activation of NF-κB, it also leads to unfavorable changes in the composition of gut microbiota and induces the upregulation of NOD-like receptor signaling pathway. Eliminating gut microbiota with antibiotics or blocking NOD-like receptor signaling transduction with NLRs inhibitor in combination with MyD88 suppression can ameliorate the inflammatory response. PPRs: pattern recognition receptors, TLRs: Toll-like receptors, NLRs: NOD-like receptors, PAMPs: pathogen-associated molecular patterns.
MyD88 inhibition restricted NF-κB activation but failed to alleviate the acute colitis severity in mice. (A and B) Western blotting analysis of MyD88 protein expression and phosphorylation levels of NF-κB in colon tissue. (C) Disease activity index change, (D) colon length, (E) representative H&E staining of colon.
sections and (F) histological score of control and TJ5-treated mice administrated with water or 3% DSS for 7 days. (G) Relative mRNA expression levels of IL-1β, IFN-γ and TNF-α in colon tissue by RT-PCR. (H) Western blotting analysis of ZO-1, Claudin-5 and Occludin expression in colon tissue obtained from control and TJ5-treated mice administrated with water or 3% DSS for 7 days. Data were represented as mean ± 18 SEM, *p<0.05, ns: not significant. TJ5: TJ-M2010-5.

**Figure 2**
Effects of MyD88 inhibition on the gut microbiota of mice. (A) Rarefaction plots based on the Sobs, Chao1, Shannon and Simpson indexes. (B) Principal coordinate analysis (PCoA) plot of the intestinal mucosa-associated microbiota with Bray-Curtis distance. Average relative abundances of taxa at the (C) phylum level, (D) class level and (E) order level. (F) Relative abundances of indicated bacterial taxa in feces. Data were represented as mean ± SEM, *p<0.05, ns: not significant. TJ5: TJ-M2010-5.

Figure 3

MyD88 inhibition induced the upregulation of NOD-like receptor signaling pathway in mice with DSS-induced colitis. (A) RNA-sequencing was performed with isolated colon tissue from mice with DSS-induced colitis treated with PBS or TJ5. The heat map indicated the differentially expressed genes by the clustering analysis. (B) The differentially expressed genes (DSS+PBS group vs. DSS+TJ5 group) were analyzed by KEGG enrichment analysis. (C) The differentially expressed genes (DSS+PBS group vs.
DSS+TJ5 group) associated with the NOD-like receptor signaling pathway were identified by RT-PCR. Data were represented as mean ± SEM, *p<0.05, ns: not significant. TJ5: TJ-M2010-5.

**Figure 4**

Further blockade of NOD-like receptor signaling pathway attenuated the unmitigated DSS-colitis severity in TJ5 solo treated mice. (A) Colon samples were collected after 7 days of DSS administration. Protein
extracted from colon tissue was analyzed by Western blot for MyD88, NOD1/2, ERK, phospho-ERK, p38 MAPK and phospho-p38 MAPK. (B) Disease activity index change, (C) colon length and (D) histological score of mice in different groups. (E) Representative H&E staining of colon sections. (F) Relative mRNA expression levels of IL-1β, IFN-γ and TNF-α in colon issue by RT-PCR. Data were represented as mean ± SEM, *p<0.05. NI1: NOD-IN-1.

Figure 5

Alteration of colonic bacterial flora by broad spectrum Antibiotics in mice Animals were given imipenem (I; 1g/L) and vancomycin (V; 1g/L) in drinking water for 3 days before and during DSS administration. (A) Total number of bacteria per gram feces were quantified using RT-PCR. (B) Rarefaction plots based on the Sobs, Ace, Chao1, Shannon and Simpson indexes. (C) Principal coordinate analysis (PCoA) plot of the intestinal mucosa-associated microbiota with Bray-Curtis distance. (D) Average relative abundances of taxa at the phylum level. (E) Total number of Proteobacteria per gram feces in different groups.
Antibiotics treatment dampened the colitis progression in TJ5-treated mice. (A) Disease activity index change, (B) colon length and (C) histological score of mice. (D) Representative H&E staining of colon sections was obtained from mice in each group. (E) Relative mRNA expression levels of IL-1β, IFN-γ and TNF-α in colon tissue by RT-PCR. (F) Protein extracted from colon tissue was analyzed by Western blot.
for MyD88, NOD1/2, RIPK2, ERK, phospho-ERK, p38 MAPK and phospho-p38 MAPK. Data were represented as mean ± SEM, *p<0.05. I&V, imipenem and vancomycin.

**Figure 7**

Schematic diagram depicting gut dysbiosis and stimulation of NOD-like receptor signaling pathway after MyD88 suppression in intestinal inflammation. In normal conditions, the extracellular and intracellular PPRs (TLRs and NLRs) sense intestinal microbiota through PAMPs and exert anti-bacterial effects. In the state of intestinal inflammation, though MyD88 suppression can restrict the MyD88-mediated activation of NF-κB, it also leads to unfavorable changes in the composition of gut microbiota and induces the upregulation of NOD-like receptor signaling pathway. Eliminating gut microbiota with antibiotics or blocking NOD-like receptor signaling transduction with NLRs inhibitor in combination with MyD88 suppression can ameliorate the inflammatory response. PPRs: pattern recognition receptors, TLRs: Toll-like receptors, NLRs: NOD-like receptors, PAMPs: pathogen-associated molecular patterns.

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