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Regulatory mechanism of mesalazine on TLR4/MyD88-dependent pathway in mouse ulcerative colitis model

Y. LI[1,2], Q. LIU[2], J.-H. TANG[3], J.-J. WEN[1], J.-Q. ZHU[1]

[1]Department of Thyroid Surgery, West China Hospital, Sichuan University, Chengdu, China
[2]Department of General Surgery, Guizhou Provincial People's Hospital, Guiyang, China
[3]Department of Gastroenterology, Xuzhou TCM Hospital Affiliated to Nanjing University of Chinese Medicine, Xuzhou, China

Abstract. – OBJECTIVE: The aim of this study was to investigate the regulatory mechanism of mesalazine (MSLZ) on microRNA-21, microRNA-31 and Toll-like receptor 4/myeloid differentiation primary response 88 (TLR4/MyD88)-dependent pathway in 2,4,6-trinitrobenzene sulfonic acid (TNBS)/ethanol-induced ulcerative colitis (UC) model in mice.

MATERIALS AND METHODS: The UC model was constructed by coloclysis of TNBS/ethanol in mice. 60 male mice were randomly assigned into control group, model group, MSLZ group and Azathioprine (AZA) group, with 15 mice in each. Corresponding drug or saline was i.g. injected in mice for consecutive 14 days. Pathological lesions in colon tissues were observed by hematoxylin and eosin (HE) staining under the microscope. The expression levels of microRNA-21 and microRNA-31 in mouse colon tissues were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The mRNA and protein levels of relative genes in TLR4/MyD88-dependent pathway in mouse colon tissues were detected by qRT-PCR and Western blot, respectively.

RESULTS: A mouse UC model was successfully constructed based on scores of DAI, colonic damage and pathological lesions under the microscope. MSLZ markedly improved clinical symptoms and mucosal healing. Meanwhile, the protective effect of MSLZ was similar or even stronger than that of AZA. The expression levels of microRNA-21 and microRNA-31 in mouse colon tissues in the model group were significantly higher than those of the control group ($p<0.01$). Compared with the model group, both MSLZ and AZA treatment could remarkably inhibit the expressions of microRNA-21 and microRNA-31 ($p<0.01$). The mRNA and protein levels of relative genes in TLR4/MyD88-dependent pathway in mouse colon tissues were markedly upregulated in the model group when compared with those of the control group. The inhibitory effect of MSLZ on the expressions of upstream factors in TLR4/MyD88-dependent pathway (including TLR4, MyD88, TRAF-6 and NF-κB) was slightly stronger than AZA, which was weaker in inhibiting downstream factors (including TNF-α and IL-1β). However, no significant difference in the inhibition of TLR4/MyD88-dependent pathway was found between MSLZ and AZA ($p>0.05$).

CONCLUSIONS: In the TNBS/ethanol-induced UC mouse model, MSLZ could inhibit the expressions of microRNA-21 and microRNA-31 in colon tissues. Furthermore, MSLZ also inhibited the release of inflammatory factors by inhibiting the TLR4/MyD88-dependent pathway in UC mice.

Key Words:
Mesalazine, Ulcerative colitis (UC), MicroRNA-21, MicroRNA-31, TLR4/MyD88-dependent pathway.

Introduction

Ulcerative colitis (UC) is a type of inflammatory bowel disease (IBD), which is closely associated with autoimmune abnormalities[1]. Currently, aminosalicylic acid, steroid hormones and immunosuppressive drugs are the most applied treatments for UC[2,3]. However, the therapeutic efficiency of these drugs is not satisfactory. Moreover, long-term usage can even lead to severe adverse events. As a novel 5-aminosalicylic acid controlled-release formulation, mesalazine (MSLA) is the preferred drug for UC treatment. It is especially suitable for acute attacks of UC patients who cannot tolerate sulfasalazine. The specific pharmacological function of MSLA has not been completely clarified. Some studies showed that MSLA mainly acts on the intestinal inflammatory mucosa,

Corresponding Author: Jingqiang Zhu, MD; e-mail: zhujingqiang@scu.edu.cn

thus inhibiting the synthesis of prostaglandins and leukotrienes. MSLA scavenges oxygen free radicals, inhibits fatty acids in intestinal mucosa and reduces intestinal permeability. Meanwhile, it exerts a significant anti-inflammatory effect on intestinal wall inflammation. Clemett Markham[4] have indicated that MSLA is particularly effective for inflammatory intestinal wall connective tissues. However, the regulatory effect of MSLA on the immune system is rarely reported. It is suggested that MSLA downregulates nuclear factor-kappa B (NF-κB) expression in colon tissues, thereafter reducing the production of inflammatory cytokines in the colon[5]. Furthermore, other cytokines or pathways may also be involved in the anti-inflammatory regulation of MSLA[6,7]. At present, the role of Toll-like receptor 4/myeloid differentiation primary response 88 (TLR4/MyD88)-dependent signaling pathway has been well concerned in the pathogenesis of UC[8]. Previous researches have suggested that microRNA-21 and microRNA-31 are closely related to the TLR4/MyD88-dependent signaling pathway, which also participates in the regulation of intestinal inflammation[9,10]. In the present work, we first established the 2,4,6-trinitrobenzene sulfonic acid (TNBS)/ethanol-induced UC model in mice. We aimed to detect the anti-inflammatory effect of MSLZ on UC, and to elucidate whether microRNA-21, microRNA-31 and the TLR4/MyD88-dependent pathway were involved in this process. Our work might provide a theoretical basis for the treatment of UC by MSLZ.

Materials and Methods

Animals and Drugs

60 male specific-pathogen-free (SPF) C57BL/6 mice weighing 200 ± 20 g were provided by the animal center of Sichuan University and routinely fed. Mesalazine (MSLZ slow release granules; Trade name: Aidisha) was used for 7 days intra-gastric administration. The equivalent daily dose of MSLZ for mice was converted according to the surface area ratio of experimental animals to human bodies [mouse dosage (g/kg)=human daily oral dose (g)×0.0026/0.02 kg]. Azathioprine tablets (AZA) were obtained from the Shanghai Pharmaceutical Group Co., Ltd. Xinyi Pharmaceutical General Factory (lot No. 008953) (Shanghai, China). Mouse activity, hair color and stool were daily observed. This study was approved by the Animal Ethics Committee of Sichuan University Animal Center.

Reagents and Instruments

5% TNBS (Sigma-Aldrich, St. Louis, MO, USA); 10% chloral hydrate (Shanghai BioMed Lab, Co., Ltd, Shanghai, China); Absolute ethanol (Hangzhou Longshan Chemical Co., Ltd, Hangzhou, China); Central venous catheter (Shanghai Jingnian Medical Instruments Co., Ltd, Shanghai, China); Primers of microRNA-21 and microRNA-31 (Shanghai BioMed Lab, Co., Ltd, Shanghai, China); Primers of Toll-like receptor 4 (TLR4), myeloid differentiation primary response 88 (MyD88), TNF receptor associated factor 6 (TRAF-6), nuclear factor-kappa B (NF-κB), tumor necrosis factor-α (TNF-α) and interleukin 1 beta (IL-1β) (TaKaRa, Otsu, Shiga, Japan); Antibodies of β-actin and NF-κB (Cell Signaling Technology, Danvers, MA, USA); Antibodies of TLR4, MyD88, TRAF-6, TNF-α and IL-1β (Abcam, Cambridge, MA, USA); Anti-TNF-α (R&D Systems, Minneapolis, MN, USA); Secondary antibodies, electrophoresis apparatus and electrophoresis chamber (Bio-Rad, Hercules, CA, USA); RNA extraction kit and reverse transcription kit (TaKaRa, Otsu, Shiga, Japan); Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Construction of UC Model in Mice

Mice were adaptively fed for one week and fasted for 24 h. On the other day, mice were anesthetized with i.p. injection of 10% chloral hydrate at a dose of 3 mL/kg. The deep venous catheter lubricated with paraffin oil was slowly inserted into the colon about 8 cm from the anus. Coloclysis of 0.02 mL/kg 5% TNBS+0.25 mL 50% ethanol was performed in mice of the model group, MSLZ group and AZA group. However, mice in the control group received colocysis of isodose saline. At the end of colocysis, 0.3 mL of air was pushed and the anus of mice was blocked with cotton swabs. Mouse abdomen was gently rubbed for 1 minute and inverted for 5 minutes. Subsequently, mice were given to normal diet. The successful construction of the UC model in mice was evaluated by the following criteria: within 2 days after model construction, mice showed mental fatigue, wilting, laziness and decreased eating. Mice preferred to lay down and became pale. Their hair was messy without brightness. Mice experienced diarrhea and increased stool frequency. The perianal fur was sticky, and the stool was septic and bloody.

Drug Treatment and Observational Indexes

Mice in the control group and model group were i.g. administrated with 0.1 mL/kg saline. AZA was fully ground and diluted in saline for the preparation of the suspension. Mice in the AZA group were i.g. administrated with 60 mg/kg AZA. On the third day after UC model construction, saline or AZA was administrated in mice, once a day for 14 consecutive days. General characteristics of mouse spirit, diet, hair, stool frequency and property were regularly recorded. The occult blood test was performed, and Disease Activity Index (DAI) was scored as previously[6]. 24 hours after the final administration, 11 mice were randomly selected in each group sacrificed. The colon tissues that were 8 cm away from the anus were harvested. The remaining 4 mice in each group were utilized for high-throughput sequencing. Colonic inflammation evaluation methods included: 1. DAI was scored according to the percentage reduction in body mass, stool property and occult blood test of mice 14 days after drug treatment based on the standard procedure proposed by Gonzalez-Navajas et al[8]; 2. Colonic damage was scored based on the standard procedure proposed by Claridge et al[9]; 3. Colonic damage under the microscope was evaluated based on hematoxylin and eosin (HE) staining of colon tissues and the standard procedure proposed by Van der Goten et al[10].

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in colon tissues was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed according to the instructions of the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The RNA concentration was detected using a spectrometer. QRT-PCR was then performed in strict accordance with SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). The specific qRT-PCR procedure was: pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s. The relative gene expression was calculated by the $2^{-\Delta Ct}$ method. Primers used were as follows: β-actin: forward: 5'-GGAGATTACTGCCCTGGCTCCTA-3'; β-actin: reverse: 5'-GACTCATCGTACTCCTGCTTGCTG-3'; TLR4: forward: 5'-CTCACAACTTCAGTGGCTGGATTTA-3'; TLR4: reverse: 5'-GTCTCCACAGCCACCAGATTCTC-3'; MyD88: forward: 5'-TATACCAACCCTTGCACCAAGTC-3'; MyD88: reverse: 5'-TCAGGCTCCAAGTCAGCTCATC-3'; TRAF-6: forward: 5'-TTTGGCGTCGGAGACACTTG-3'; TRAF-6: reverse: 5'-TCGCTTGAAGACTGGCTGGA-3'; NF-κB: forward: 5'-CATGCGTTTCCGTTACAAGTG-3'; NF-κB: reverse: 5'-GTGCGTCTTAGTGGTATCTGTGCT-3'; TNF-α: forward: 5'-TCAGTTCCATGGCCCAGAC-3'; TNF-α: reverse: 5'-GTTGTCTTTGAGATCCATGCCATT-3'; IL-1β: forward: 5'-CCCTGAACTCAACTGTGAAATAGCA-3'; IL-1β: reverse: 5'-CCCAAGTCAAGGGCTTGGAA-3'.

Western Blotting

Colon tissues were lysed to harvest total protein, followed by determination of total protein concentration. An equal amount of protein sample was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with skimmed milk, the membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. On the other day, the membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibody at room temperature for 2-3 h. Finally, immunoreactive bands were captured by the Tanon detection system using enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Data were expressed as mean ± standard deviation ($\bar{x}\pm s$). One-way ANOVA was used to compare the difference among groups, followed by Post-Hoc Test. $p<0.05$ was considered statistically significant.

Results

Inflammatory Response in UC Mice

Compared with the control group, the intestinal wall of mice in the model group adhered more to the surrounding tissues. The ulcer was observed in the hickened intestine with hyperemia and peripheral mucosa edema. Under high magnification, colonic mucosal edema was observed. The infiltration of abundant lympho-

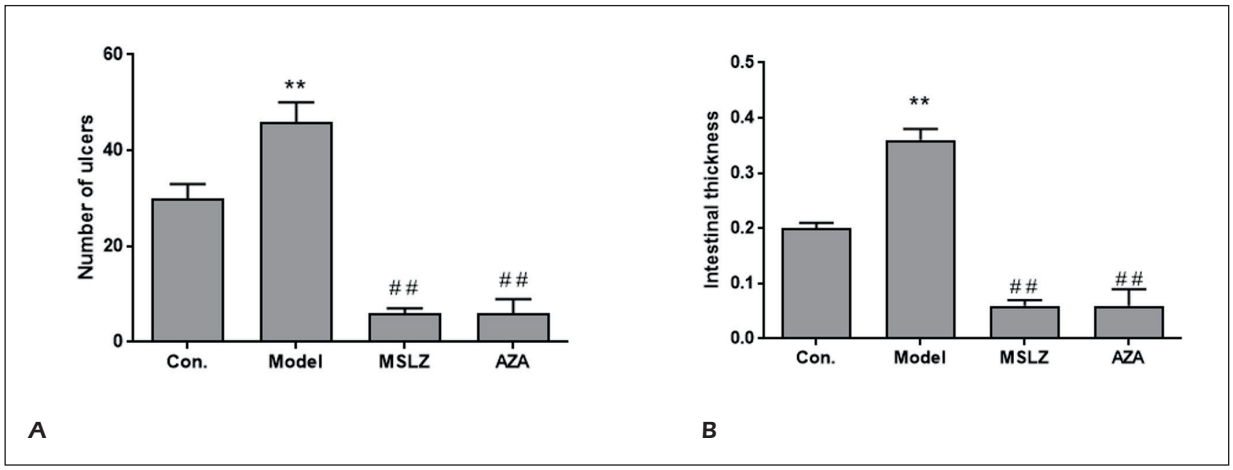

Figure 1. Inflammatory response in UC mice. ***A,*** Pathological lesions in distal colonic tissues of mice. ***B,*** HE staining of distal colonic tissues of mice (magnification 200×).

cytes, plasma cells and neutrophils in mucosa and the muscular layer were marked. Crypt abscess, ulcer formation, necrotic layer of the ulcer surface and granulation tissue were found in UC mice of the model group. However, the adhesion between the intestinal wall and the surrounding tissues was light or absent. The ulcer was rare, and the crypt structure was intact. Meanwhile, inflammatory cells such as neutrophils and lymphocytes were less infiltrated after treatment of AZA or MSLZ. Among them, UC mice in the MSLZ and AZA groups exhibited significantly less adhesion to the intestinal wall and surrounding tissues. No thickening of the intestinal tube was found. In addition, the intestinal wall was slightly thickened without ulcer formation, accompanied by mild mucosal congestion and edema (Figure 1A, 1B).

Expression Levels of MicroRNA-21 and MicroRNA-31 in Mouse Colon Tissue

QRT-PCR results indicated that the expressions of microRNA-21 and microRNA-31 in colon tissues of mice in the model group were markedly increased than those of the control group ($p<0.01$). After treatment of MSLZ or AZA, both microRNA-21 and microRNA-31 expressions were markedly inhibited when compared with the model group ($p<0.05$, $p<0.01$, Figure 2).

The mRNA Levels of Relative Genes in TLR4/MyD88-Dependent Pathway in Mouse Colon Tissues

Subsequently, we detected the mRNA levels of relative genes in the TLR4/MyD88-dependent pathway in mouse colon tissues. Significantly higher mRNA levels of TLR4, MyD88, TRAF-6,

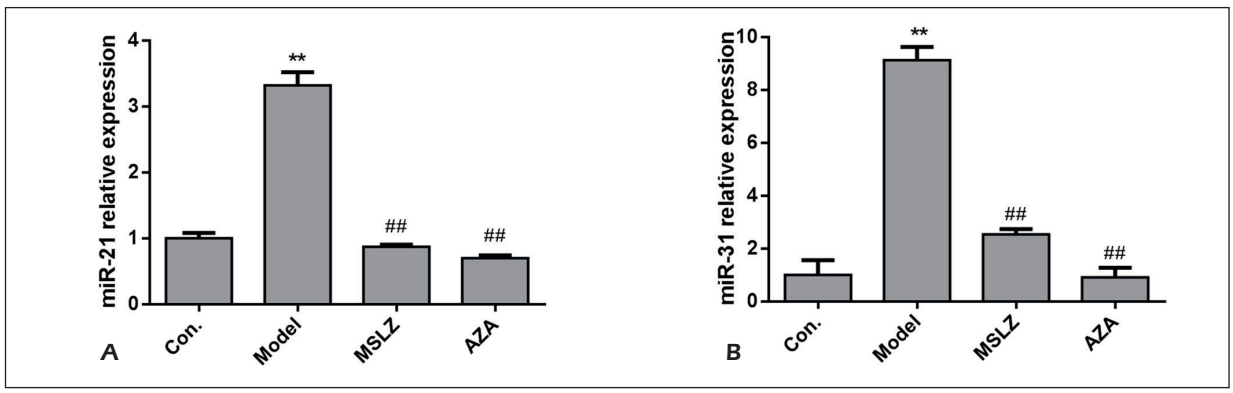

Figure 2. Expression levels of microRNA-21 and microRNA-31 in mouse colon tissues. ***A,*** Expression level of microRNA-21 in colonic tissues. ***B,*** Expression level of microRNA-31 in colonic tissues. $^{**}p<0.01$ compared with the control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with the model group.

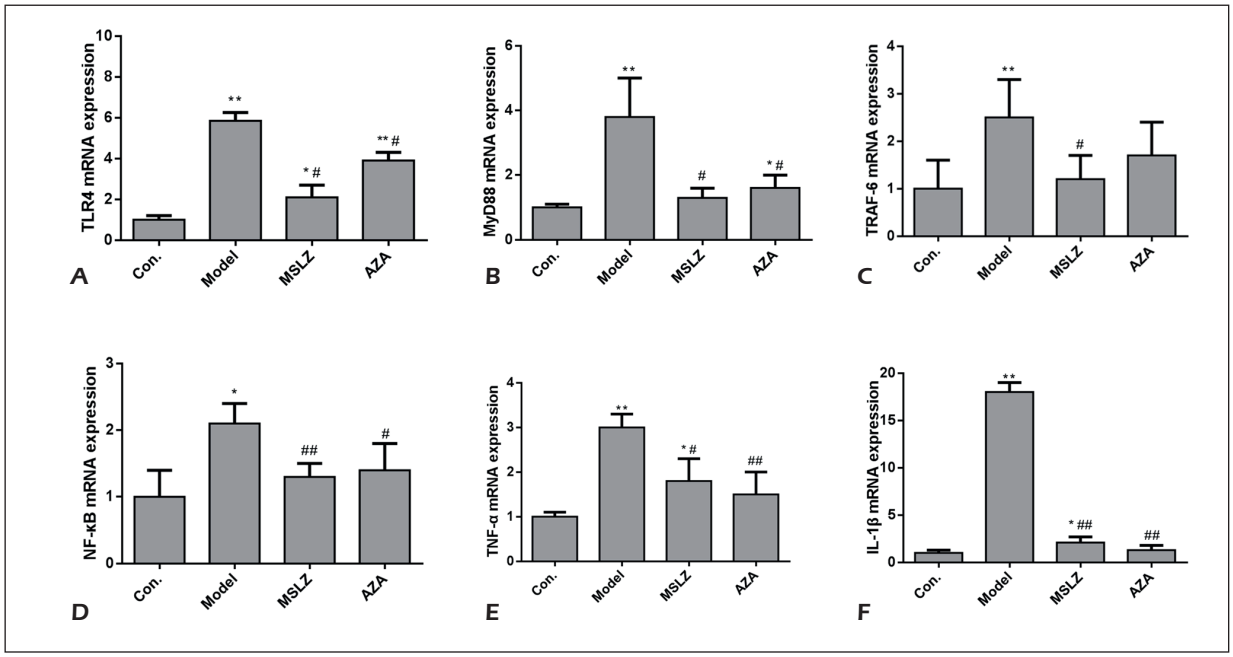

Figure 3. The mRNA levels of relative genes in TLR4/MyD88-dependent pathway in mouse colon tissues. ***A-F,*** The mRNA levels of TLR4, MyD88, TRAF-6, NF-κB, TNF-α and IL-1β ($\bar{x}\pm s$, n = 11).

NF-κB, TNF-α and IL-1β were found in the model group than those of the control group ($p<0.05$). MSLZ treatment showed remarkably stronger inhibitory effects on mRNA levels when compared with those of the AZA group. Although MSLZ and AZA treatment could both inhibit the mRNA levels of IL-1β and TNF-α, the inhibitory effect of MSLZ was markedly weaker than AZA. However, no significant difference in the inhibition of RNA levels of IL-1β and TNF-α was found between MSLZ and AZA ($p>0.05$) (Figure 3).

Protein Levels of Relative Genes in TLR4/MyD88-Dependent Pathway in Mouse Colon Tissues

Furthermore, we accessed the protein levels of TLR4, MyD88, TRAF-6, NF-κB, TNF-α and IL-1β in each group by Western blot. By comparison with the control group, the protein expression levels of the above gene were significantly higher in UC mice of the model group ($p<0.01$). MSLZ treatment could markedly inhibit their expressions than those of the model group ($p<0.01$). Consistent with the mRNA levels, the inhibitory effect of MSLZ on the protein levels of upstream factors in TLR4/MyD88-dependent signaling pathway (including TLR4, MyD88, TRAF-6 and NF-κB) was slightly stronger than AZA, which was significantly weaker in inhibiting downstream factors (TNF-α and IL-1β). However, no significant difference in the inhibition of TLR4/MyD88-dependent pathway was observed between MSLZ and AZA ($p>0.05$) (Figure 4). Quantification of protein expressions was shown in Figure 5.

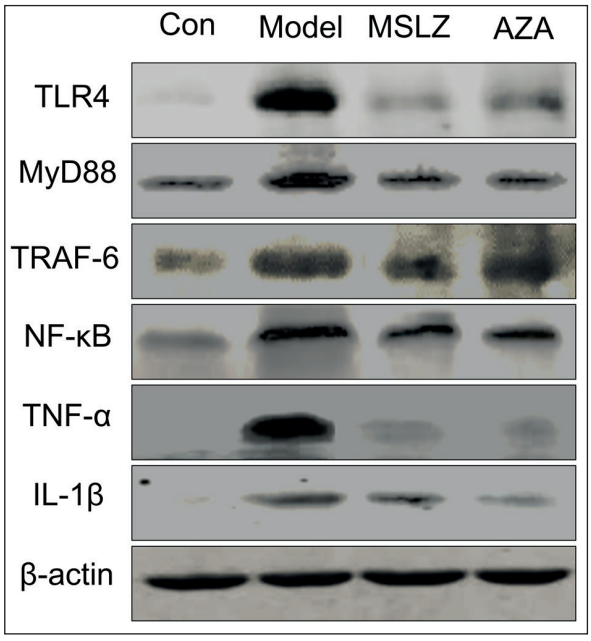

Figure 4. Protein levels of relative genes in TLR4/MyD88-dependent pathway in mouse colon tissues. ***A-F,*** The protein levels of TLR4, MyD88, TRAF-6, NF-κB, TNF-α and IL-1β ($\bar{x}\pm s$, n = 11).

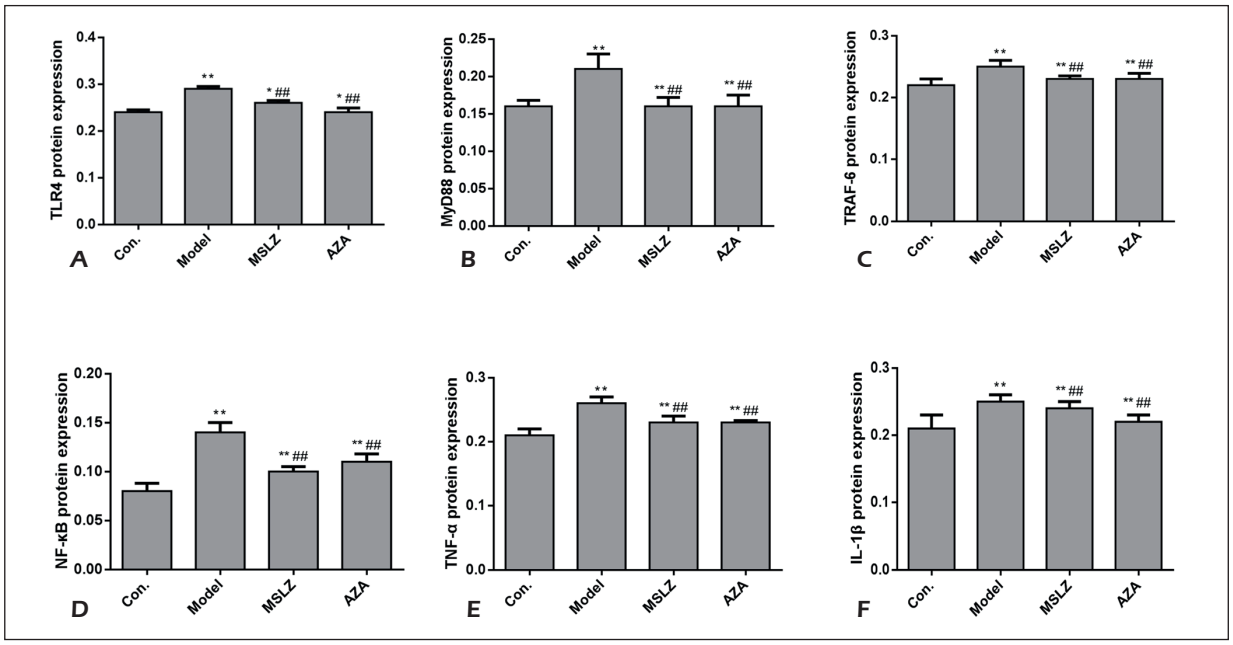

Figure 5. Quantification of protein levels of relative genes in TLR4/MyD88-dependent pathway in mouse colon tissues.

Discussion

TNBS/ethanol-induced UC model in mice is an excellent model for researches on UC. The inflammatory response in UC is closely related to immune abnormalities caused by TNBS[11]. In this study, the general characteristics of mice were regularly observed after UC model construction. We found that 2 days after UC model construction, changes in spirit, diet, hair, stool frequency and property were found in mice of the model group. The occult blood test of mice in the model group was positive. Meanwhile, DAI was about 7-13 scores in the model group, which was markedly higher than that of the control group. After animal procedures, the general situation of colon tissues was observed. Adhesion of lesions to surrounding tissues was observed in UC mice of the model group. Scores of HE and colonic damage in the model group were markedly increased ($p<0.05$). Pathological changes in mouse colon tissues of MSLZ and AZA group were significantly alleviated. These results indicated that MSLZ exerted a certain effect on the improvement of clinical symptoms and mucosal healing in UC mice, which was comparable to or stronger than AZA. MicroRNA is a non-coding endogenous RNA consisting of 19-22 nucleotides. It acts on the 3' untranslated region (3'UTR), ultimately inhibiting transcription or degrading the expression of target mRNAs[12]. Due to the diversity of miRNA binding patterns, microRNAs are important in the pathogenesis of UC[13]. Studies[14-16] have shown that microRNA-21 and microRNA-31 are mainly used as protective factors to inhibit the development of intestinal inflammation. However, in this study, we found that the expressions of microRNA-21 and microRNA-31 in UC mice were markedly increased compared with mice in the control group. MSLZ and AZA administration could significantly inhibit the expression levels of microRNA-21 and microRNA-31 in UC mice. Meanwhile, we also investigated the changes in the TLR4/MyD88-dependent pathway in UC-induced inflammation. TLR4 recognizes pathogen-associated molecular patterns or damage-associated molecular patterns, which activates relative signal kinases through intermediate-linker proteins. The subsequent activation of transcription factors allows transcriptional expressions of inflammatory effector molecules. This may finally complete innate immune responses or activate acquired immune responses and inflammation. The TLR4 pathway is primarily divided into MyD88-dependent and MyD88-non-dependent signaling pathways based on the need for adaptor protein MyD88[17]. In MyD88-dependent pathway, TLR

recruits and activates downstream IRAK4, IRAK1, ARAK2 and TRAF-6, eventually regulating MAPK, IRF5 and NF-κB. As a result, terminal inflammatory factors IL-1β and TNF-α are released[18]. In the present work, we detected the expression levels of TRL4, MyD88, TRAF-6 and NF-κB, to access the changes of the TLR4/MyD88 pathway in UC mice. Besides, we also determined the levels of IL-1β and TNF-α to elucidate the regulatory effect of TLR4/MyD88 pathway on UC-induced inflammatory response. Western blot and qRT-PCR results demonstrated that both mRNA and protein levels of TRL4, MyD88, TRAF-6 and NF-κB in mice of the model group were significantly higher than those of controls. This suggested the specific role of the TLR4/MyD88-dependent pathway in UC. Moreover, MSLZ treatment downregulated the expressions of these genes in a dose-dependent manner, especially in protein levels. Compared with positive control AZA group, MSLZ treatment inhibited the expressions of upstream factors in TLR4/MyD88-dependent pathway. However, no significant differences were found ($p>0.05$). Both MSLZ and AZA could achieve anti-inflammatory effects by inhibiting the TLR4/MyD88-dependent pathway in UC mice. The inhibition of the TLR4/MyD88-dependent pathway is one of the mechanisms of anti-inflammatory function in MSLZ. Furthermore, both the mRNA and protein levels of terminal inflammatory factors IL-1β and TNF-α were remarkably increased in the model group than those of the control group. Both MSLZ and AZA treatment could inhibit their expression levels in UC mice. AZA showed better efficacy than that of MSLZ; however, no significant difference was observed ($p>0.05$). MSLZ presented better inhibitory effect on the upstream factors in TLR4/MyD88-dependent pathway than that of AZA. However, its role in regulating downstream factors was worse than AZA. The complexity of drug regulation in signaling pathways may affect the ultimate anti-inflammatory outcome of drugs.

Conclusions

We detected that in TNBS/ethanol-induced UC mouse model, MSLZ could inhibit the expressions of microRNA-21 and microRNA-31 in mouse colon tissues. Furthermore, MSLZ inhibited the release of inflammatory factors by inhibiting the TLR4/MyD88-dependent pathway in UC mice.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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