

Original Article

Baicalin down regulates the expression of TLR4 and NF- κ B-p65 in colon tissue in mice with colitis induced by dextran sulfate sodium

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Abstract: Background: Baicalin is one of flavonoid extracts from *Scutellaria baicalensis*, which has several functions including anti-inflammation, anti-bacteria, antitumor and et al. However, the mechanisms of anti-inflammatory of baicalin in ulcerative colitis is not clear. Methods: Mice colitis models were established by dextran sodium sulfate, Mice administrated with baicalin (100 mg/kg) and mesalazine (100 mg/kg) twice daily by intragastric injection for 7 days after colitis induced were defined as treated group. Then the mice were sacrificed and the colon samples were collected. Toll-like receptor-2, 4, 9 were detected by immunohistochemistry. Signaling proteins such as TLR4, MyD88, and NF- κ B p65 were analyzed by western blotting. Cytokine's mRNA include TNF- α , IL-6 IL-10 and IL-13 were measured by reverse transcription polymerase chain reaction. Modified disease activity index were used to analyse the severity of the disease by assessed of diarrhea, stool (occult) blood and body weight loss of the mice. Results: Compared with control and model groups, modified disease activity index in baicalin and mesalazine treated, mice decreased gradually. Immunohistochemistry analysis showed the expression of TLR4, but not TLR2 and TLR9, in the mucosa of mice colon were decreased. Western blot analysis showed that in colitis model, the expression of NF- κ B p65 and TLR4 decreased ($P < 0.05$), while the expression of MyD88 increased significantly compared to control group, and MyD88 expression can not be repressed by baicalin ($P < 0.05$). Baicalin and mesalazine treatment suppressed the expression of TNF- α , IL-6 and IL-13 mRNA ($P < 0.05$), yet up-regulated the expression of IL-10 mRNA ($P < 0.05$), compared to the DDS and control groups. Conclusions: Baicalin administration by intragastric injection ameliorates the severity of colon inflammation. The possible mechanism of anti-inflammatory response by baicalin may involve in the blocking of the TLR4/NF- κ B-p65/IL-6 signaling pathway.

Keywords: Baicalin, ulcerative colitis, NF- κ B, TLR, MyD88

Introduction

Ulcerative colitis (UC), as well as Crohn's Disease, is one of inflammatory bowel disease (IBD) which pathogenesis is not clear. One of the potential pathogenesis may be associated with excessive immune responses against intestinal bacteria and lead to the damage of intestinal epithelial barrier via abnormal activity of some pro-inflammatory signals. Which mainly including P38/MAPK, JNK/MAPK, PI3K/Akt, and NF- κ B signaling pathways [1]. Recently, an inflammation and cancer related signal transduction pathway, MyD88/NF- κ B, raised

the attention of the researchers, Which is triggered by pattern recognition receptors (PRRs), Toll-like receptors (TLRs) via interacting with the antigen from intestinal flora. Studies have identified the immune response that induced by TLRs mediated MyD88/NF- κ B signal are exist in many conditions such as experimental cerebral ischemia [2], inflammation and carcinoma [3, 4], allergy [1, 5] and so on. UC is one of autoimmune tendency condition with a high risk of developing colorectal cancer [6] or other hematologic tumor [7]. Recent studies have showed that, TLRs/MyD88/NF- κ B signal-transducing pathway plays an important part in UC [1, 8, 9].

Traditional drug for UC such as 5-aminosalicylates (5-ASA) and corticosteroids are widely used, and some immunomodulators are used in refractory or steroid dependent UC patients. In addition, biological agents have a good effect in moderate to severe UC. Although many drugs have been used for UC treatment, the adverse effects of first line drugs will limit the use.

Baicalin (5,6-dihydroxy-4-oxygen-2-phenyl-4H-1-benzopyran-7-beta-D- glucopyranose acid, BCLN), one of flavonoid extracts from *Scutellaria baicalensis*, a well-known herbal medicine widely used in China, have several biological activities including anti-inflammatory [10, 11], antimicrobial [12], antitumor [13, 14]. Although, the anti-inflammatory mechanism of baicalin in the intestinal tract is not so clear, the decoction of *Scutellaria baicalensis*, with a large number of flavonoids, is widely used in China and East Asia [15]. Recent years, the efficacy of flavonoid extractions is widely studied [16, 17], and it was found that, the extraction of *Scutellaria* inhibit the activation of NF- κ B and then block inflammatory response [16, 18, 19], but which are seldom studied in IBD. Base on these theories mentioned above, we suppose that baicalin may inhibit the activation of NF- κ B and ultimately be benefic for the treatment of UC.

In order to explore the anti-inflammatory mechanism of baicalin in UC, we built a mice colitis model by administrating dextran sulfate sodium (DSS), and explored the expression of PRR molecular (TLR2, 4, 9) in the intestinal mucosa, the expression of signal transduction proteins (TLR4, MyD88, NF- κ B p65) in mice colonic epithelial cells, and the expression of mRNA of cytokines (TNF- α , IL-6, IL-10 and IL-13 mRNA) in the colon tissue. We expected to clarify whether TLRs/MyD88/NF- κ B signaling pathway were influenced by baicalin in UC model and provide experimental basis for the use of baicalin as a new drug candidate for UC treatment.

Materials and methods

Animals

Thirty two female C57BL/6 (B6) mice, 6-7 weeks old, weighting 17-21 g, were purchased from Medical Experimental Animal Center of Guangdong province (SCXK2008-0002). This study was approved by the Ethics Committee of Guangdong Medical College.

Colitis induction

All mice were randomly divided into four groups, A: Control group, B: model group (DSS induced colitis without treatment, DSS group), C: baicalin treated group (DSS+BCLN group), and, D: mesalazine treated group (DSS+MSLZ group). For group B, C and D, mice were fed with 2.5% (w/v) DSS (36 to 50 kDa, Lot number: 160110, MP Biomedicals, USA) solution in purified water for 7 days as Wirtz's described [20].

Drug administration

One percent (w/v) baicalin (Nanjing Zelang Medical Technology Co., Ltd, China) at a dose of 100 mg/kg, or 1% (w/v) mesalazine (Ethypharm Industries Co., Ltd, France) at a dose of 100 mg/kg was administrated to mice in group C and D twice daily for another 7 days by intragastric injection. Mice in group B were fed normally from day 8 to day 14, and mice in group A were fed normally for 14 days.

Modified disease activity index (mDAI)

Body weight and hematochezia or fecal occult blood of mice in all groups was examined daily. Weight loss and hematochezia of mice were scored based on modified disease activity index (mDAI), as described previously [21]. The mDAI follow the scoring system as below: body weight: 0: < 5% weight loss; 1: 5 to 10% weight loss; 2: 10 to 15% weight loss; 3: 15 to 20% weight loss; and 4: > 20% weight loss. A fecal occult blood test (Nanjing Jiancheng Bio-engineering Institute, China, C027) were used to screen for occult blood in the stool and scored as follows: 0: no blood; 2: positive; and 4: gross blood. Grade 1 and 3 do not exist in this scale.

Samples preparation

Mice were sacrificed on day 15, colon samples were collected from mice instantly. Part of colon tissue were fixed in 10% formalin, treated with conventional dehydration, embedded in paraffin, sliced into sections, which were used for hematoxylin and eosin (H&E), immunohistochemical staining. Others parts were frozen at -80°C for RNA and protein extraction.

Immunohistochemistry (IHC) analysis

Four μ m-thick tissue sections were dewaxed, epitope retrieval was performed using citrate

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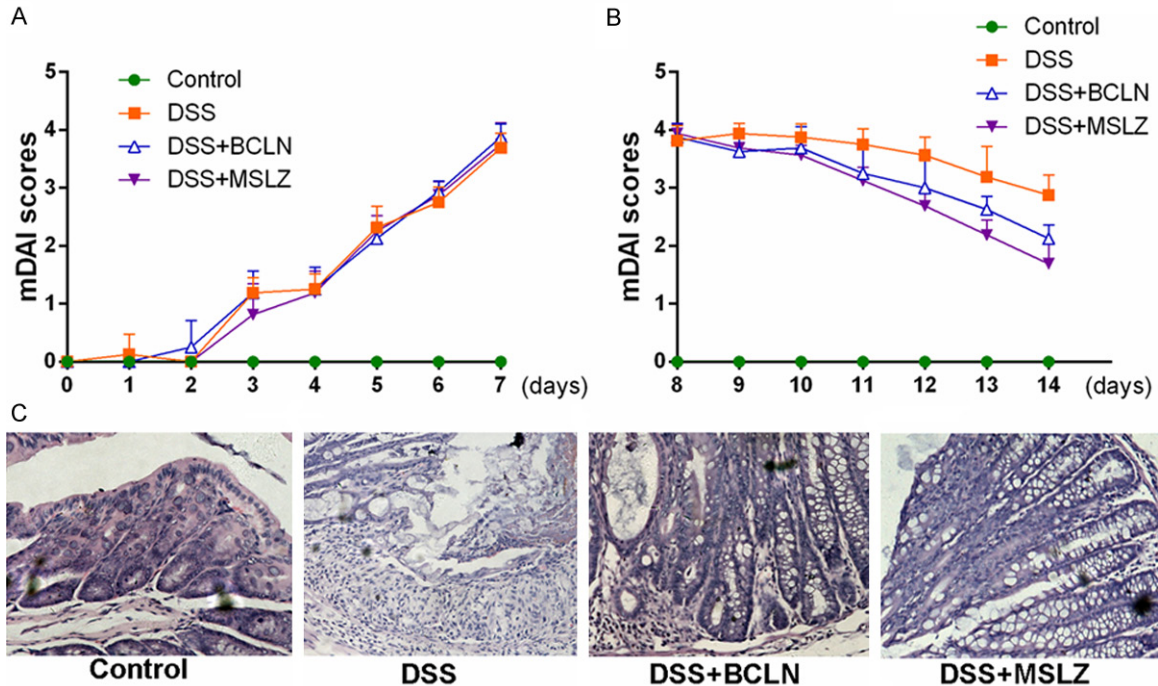


Figure 1. Protective effects of baicalin and mesalazine in mice with DSS induced colitis. A. Modified disease activity index after DSS administration during colitis induction experiment. B. Modified disease activity index of colitis during drugs intervention. C. Histological presence of colon mucosa of all 4 groups (H&E-staining).

buffer (pH=6), and then the activity of endogenous peroxidase were blocked by incubated with H_2O_2 solution for 10 minutes at room temperature. After epitope retrieval, tissue sections were incubated with rabbit purified anti-mouse TLR2 (BioLegend), rabbit anti-mouse-TLR4 (Abcam) and rabbit anti-mouse-TLR9 antibody (Abcam) respectively for 30 minutes at room temperature, washed and incubated with secondary peroxidase anti-rabbit IgG at 1:500 dilution for another 30 minutes at room temperature. After that, DAB reagents were used for chromogenic reaction. Counterstaining was performed with hematoxylin, dehydrated, cleaned in xylene, and mounted on coated slides. The results of IHC were analyzed by Image-Pro Plus (IPP 6.0 Media Cybernetics, US) software.

Western blot (WB) analysis

Colon tissues were lysed with lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 1 mM PMSF, 10 nM phosphatase inhibitor microcystin). Protein were separated on 10% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 2% BSA in TBST (0.05% Tween 20 in Tris-buffered saline) for 1

hour at room temperature, the membranes were separately incubated with anti-mouse CD282 (TLR2) (BioLegend), anti-MyD88 (Biovision), anti-NF κ Bp65(ARP) and anti- β -actin (Santa Cruz, US) antibodies at a 1:1000 dilution in TBST for 1 hour at room temperature. Then membranes were incubated with HRP-conjugated secondary antibodies (Santa Cruz, US) at a 1:10,000 dilution for 1 hr at room temperature before visualizing by using ECL detection reagents.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

The RNA in colon tissue was extracted by using Trizol reagent (Life Technologies, Copenhagen, Denmark) and chloroform following the manufacturer's instructions in the RT-PCR kit. To analyze the expression of TNF- α , IL-6, IL-10 and IL-13 mRNA, the oligonucleotide primers (Sangon, China) designed for TNF- α were 5'-AGC-ACAGAAAGCATGATCCG-3' (forward) and 5'-CT-GATGAGAGGGA GGCCATT-3' (reverse), for IL-13 were 5'-TCTTGCTTGCCTTGG TGGTCTCGC-3' (forward) and 5'-GATGGCATTGCAATTGGAGATGTTG-3 (reverse), for IL-6 were 5'-GAGGATACCACTCC-CAACAGACC-3' (forward) and 5'-AAGTCATCAT-CGTTGTCATACA-3' (reverse), for IL-10 were 5'-

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Table 1. mDAI score of mice in each group during the period of modeling

Groups	mDAI score							Sum	F	P	
	day 1	day 2	day 3	day 4	day 5	day 6	day 7				
Control (n=8)	\bar{x}	0.000	0.000	0.000	0.000	0.000	0.000	0.000	—	0.000	
	s	0.000	0.000	0.000	0.000	0.000	0.000	0.000			
DSS (n=8)	\bar{x}	0.125	0.000	1.188	1.250	2.313	2.750	3.688	1.616	204.465	0.000
	s	0.354	0.000	0.259	0.267	0.372	0.267	0.259			
DSS+BCLN (n=8)	\bar{x}	0.000	0.250	1.188	1.250	2.125	2.938	3.875	1.661	171.428	0.000
	s	0.000	0.463	0.372	0.378	0.231	0.177	0.231			
DSS+MSLZ (n=8)	\bar{x}	0.000	0.000	0.813	1.188	2.250	2.875	3.750	1.554	187.204	0.000
	s	0.000	0.000	0.530	0.372	0.267	0.231	0.378			
Sum	\bar{x}	0.031	0.063	0.797	0.922	1.672	2.141	2.828	1.208	556.751*	0.000*
	s	0.177	0.246	0.594	0.611	1.013	1.271	1.678			
F		1.000	2.333	20.615	34.350	151.667	418.333	432.616	439.921*	(F=62.994, P=0.000)#	
P		0.407	0.096	0.000	0.000	0.000	0.000	0.000	0.000*		

*F statistic and P value of main effect; #F statistic and P value of crossover effect.

Table 2. mDAI score of mice in each group during the period of treatment

Groups	mDAI score							Sum	F	P	
	day 8	day 9	day 10	day 11	day 12	day 13	day 14				
Control (n=8)	\bar{x}	0.000	0.000	0.000	0.000	0.000	0.000	0.000	—	0.000	
	s	0.000	0.000	0.000	0.000	0.000	0.000	0.000			
DSS (n=8)	\bar{x}	3.813	3.938	3.875	3.750	3.563	3.188	2.875	3.571	17.398	0.000
	s	0.259	0.177	0.231	0.267	0.320	0.530	0.354			
DSS+BCLN (n=8)	\bar{x}	3.875	3.625	3.688	3.250	3.000	2.625	2.125	3.170	25.516	0.000
	s	0.231	0.354	0.372	0.463	0.535	0.231	0.231			
DSS+MSLZ (n=8)	\bar{x}	3.938	3.438	3.188	2.688	2.063	1.625	1.250	2.598	102.365	0.000
	s	0.177	0.563	0.530	0.259	0.496	0.443	0.535			
Sum	\bar{x}	2.906	2.750	2.688	2.422	2.156	1.859	1.563	2.335	108.323*	0.000*
	s	1.715	1.656	1.630	1.498	1.428	1.278	1.134			
F		791.961	506.613	495.227	275.456	176.235	154.933	149.695	1004.906*	(F=16.823, P=0.000)#	
P		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000*		

*F statistic and P value of main effect; #F statistic and P value of crossover effect.

ATGCTGCCTGCTCTTACT GACTG-3' (forward) and 5'-CCCAAGTAACCCTTAAAGTCCTGC-3' (reverse). The oligonucleotide primers for β -actin used as a house-keeping gene were 5'-ATC-ACTATTGGCAACGAGCG-3' (forward) and 5'-TC-AGCAATGCCTGGGT ACAT-3' (reverse). Image-Pro Plus 6.0 (IPP, Media Cybernetics, US) was used to analyze relative concentration values of mRNA expression.

Statistical analysis

All the data were expressed as mean \pm standard deviation. SPSS 19.0 software was used

for one-way ANOVA and variance analyses of repeated measurement data analysis. A P-value of less than 0.05 was considered significant.

Result

Protective effects of baicalin in colitis induced by DSS

mDAI scores increased in colitis model group (group B, C, D) significantly (**Figure 1A, 1B; Tables 1, 2**) and baicalin and mesalazine treatment markedly suppressed the mDAI scores (**Figure 1C**). In addition, baicalin and mesala-

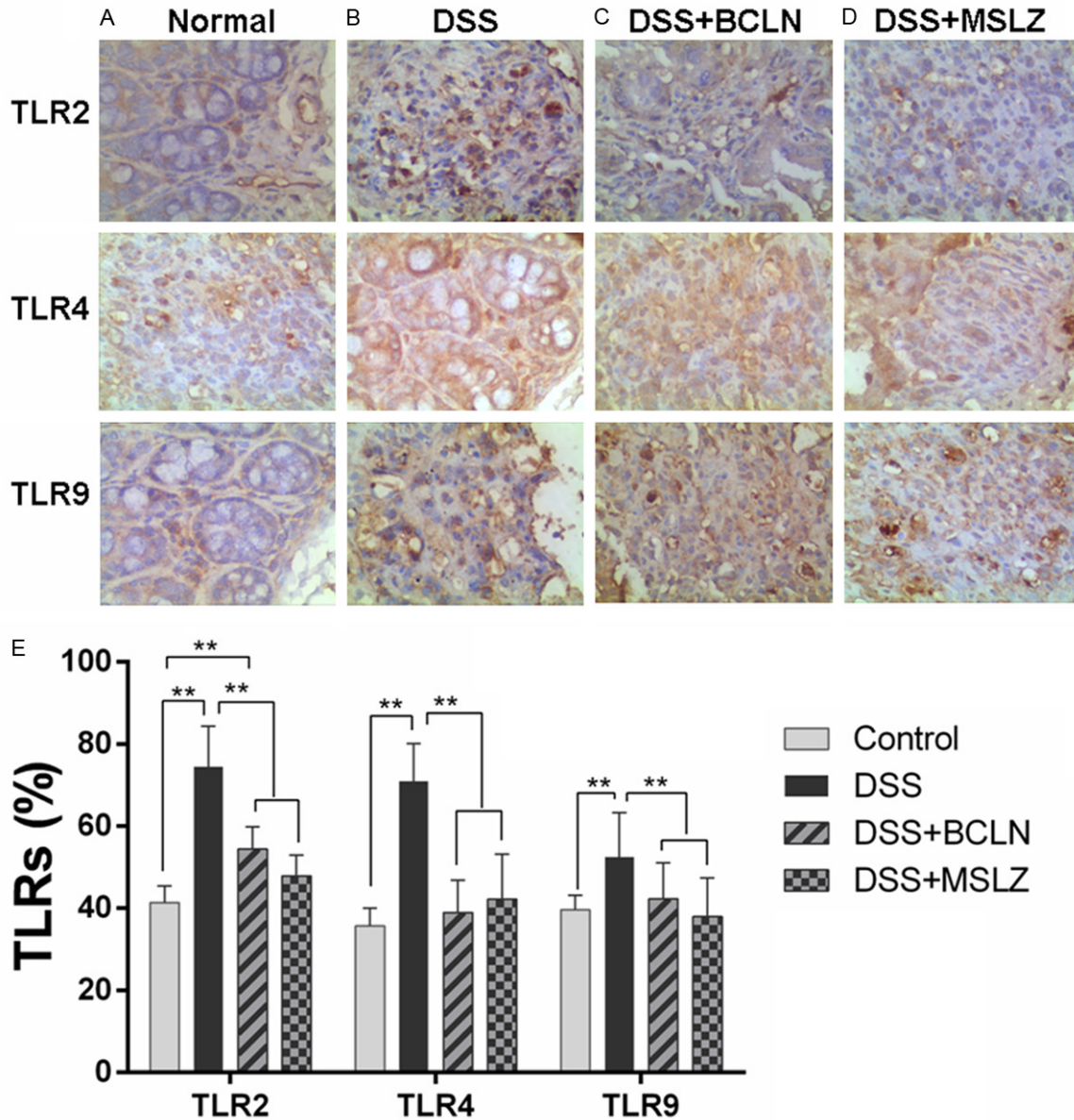


Figure 2. TLR2, 4, 9 expression on colon mucosa. A-D. The immunohistochemical analysis of TLR2, 4, 9 expressed on colon tissue; E. The calculation of TLR2, 4, 9 positive cells was performed by IPP software. Values are shown as mean \pm SEM (* $P < 0.05$, ** $P < 0.01$).

zine dramatically improved the inflammatory lesions in colon tissue by the histologic analysis (Figure 2B-D).

Baicalin treatment suppressed TLR4 expression in colon mucosa

Results of IHC in colon tissues (Figure 2A) showed the expression of TLR2, 4, 9 was increased in mice with colitis (Figure 2B-D) and The majority of the TLR2 and TLR9 positive cells were lamina propria mononuclear cells and the expression of TLR2, TLR9 were located on cytomembrance (Figure 2A). The expression

of TLR4 decreased dramatically after baicalin and mesalazine treatment. The expression of TLR9 decreased by mesalazine treatment. However, the expression of TLR2 remained unchanged after both drugs treatment.

Baicalin and mesalazine treatment decreased NF- κ B p65 expression in colon tissue

WB analysis revealed that NF- κ B p65 and MyD88 expression in colon tissue decreased by baicalin or mesalazine treatment. Whereas the decreased expression of MyD88 in baicalin and mesalazine treated group did not reach statistical levels ($P > 0.05$, Figure 3).

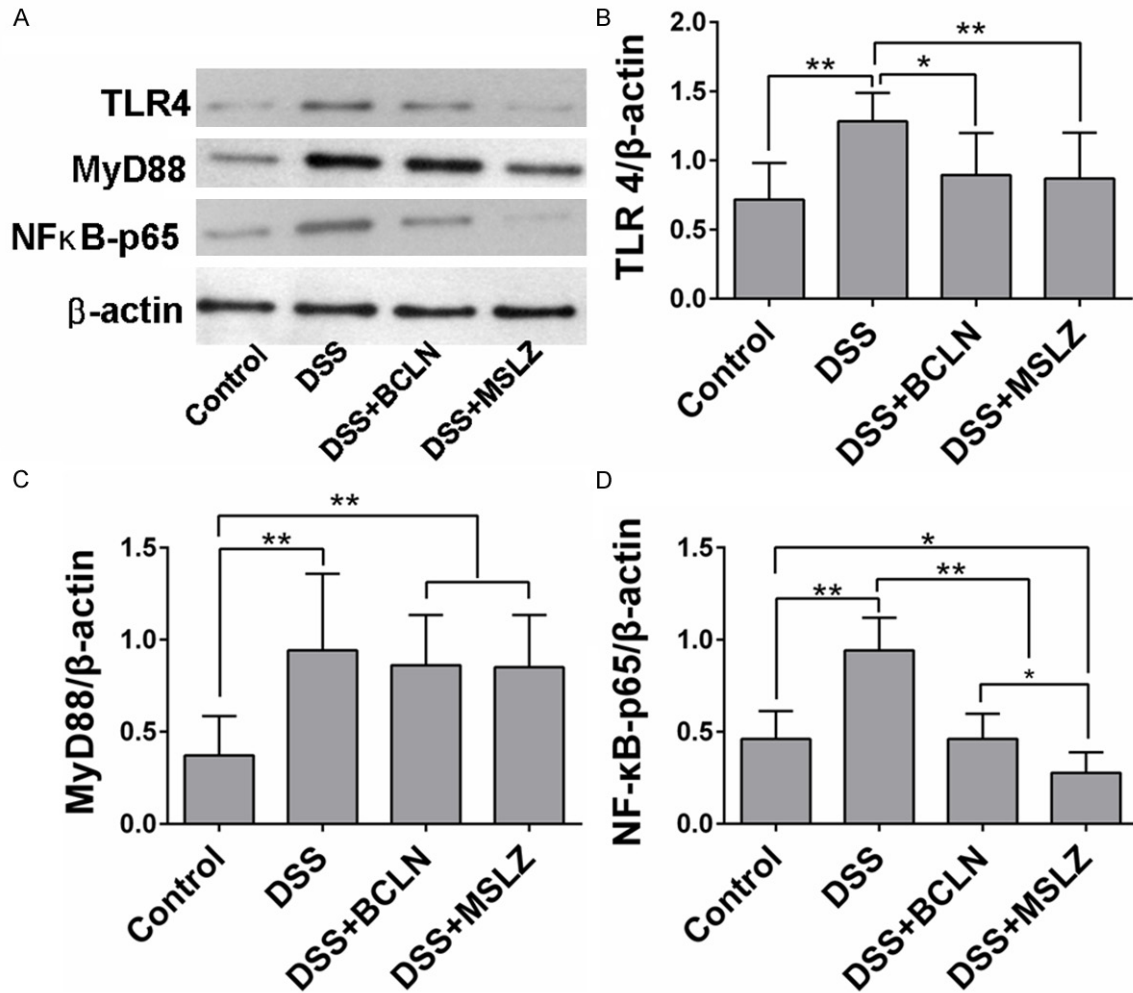


Figure 3. TLR4, MyD88, NF-κB p65 Expression in colon tissue. A. Western blot analysis of TLR4, MyD88, NF-κB p65 and β-actin. B-D. Relative concentration of TLR4, MyD88, NF-κB p65 were analyzed by IPP software. Values are shown as mean ± SEM (* $P < 0.05$, ** $P < 0.01$).

Baicalin and mesalazine treatment increased IL-10 mRNA but decreased TNF-α, IL-6 and IL-13 mRNA expressions

The mRNA of cytokines in mice colon tissues were detected by RT-PCR analysis. DSS substantially increased the TNF-α, IL-6, IL-10 and IL-13 mRNA expressed in colon tissues. On the contrary, baicalin and mesalazine treatment suppressed TNF-α, IL-6 and IL-13 mRNA expressions while elevated IL-10 mRNA expression ($P < 0.05$, **Figure 4**).

Discussion

NF-κB is a critical signaling molecule in inflammatory process, which facilitates the expression and secretion of pro-inflammatory cyto-

kines, and then lead to a series of inflammatory responses and mucosal damage. It have been identified that, inhibiting the activation of NF-κB by blocking the MyD88 signal (an upstream signal molecular of NF-κB signal pathway) will reduce the release of proinflammatory cytokines, alleviate the inflammatory response and achieve a therapeutic effect [4]. Previous studies have demonstrated the important role of TLRs/MyD88/NF-κB signaling pathway in different conditions [2, 22-24].

Our current study show that, mice induced by DSS, developed a series of inflammatory responses in the intestinal mucosa, the expression of PRRs such as TLR2, TLR4 and TLR9 were significantly increased, the expression of signal-transducing protein assayed by WB,

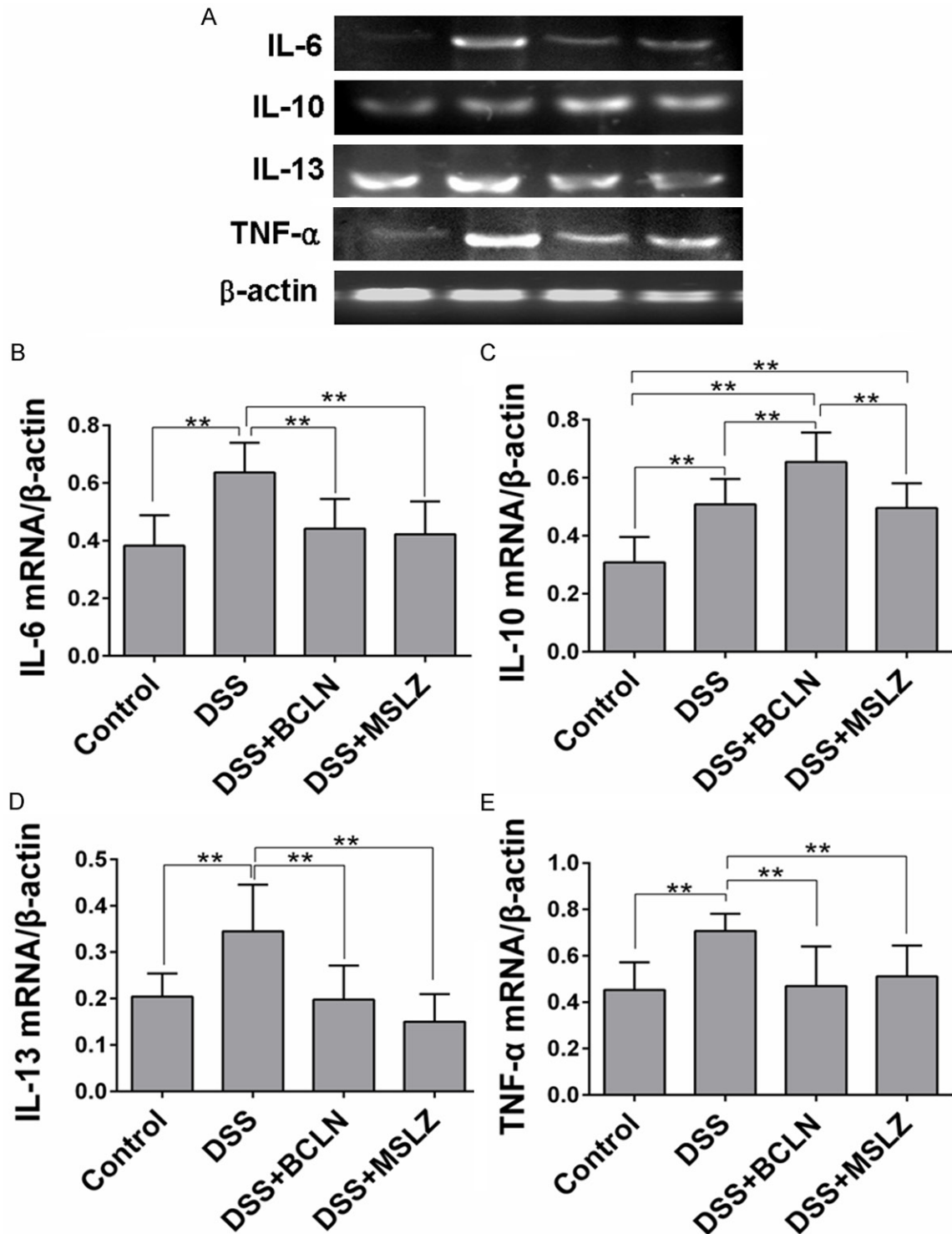


Figure 4. TNF- α , IL-6, IL-10 and IL-13 mRNA expression in colon tissue. A. TNF- α , IL-6, IL-10, IL-13 and mRNA expression in colon tissue were detected by RT-PCR analysis. B-E. Relative concentration of IL-6, IL-10, IL-13 and TNF- α mRNA were analyzed by IPP software. Values are shown as mean \pm SEM (* P < 0.05, ** P < 0.01).

TLR4 and NF- κ B p65 were significantly increased, and the clinical parameters such as

weight loss, hematochezia or fecal occult blood were increased accordingly. In addition, the

mDAI were significantly increased. The result of histological assessment improved significantly.

After baicalin and mesalazine treatment, the symptoms of weight loss and fecal occult blood of mice were significantly ameliorated. The histological change of the intestinal mucosa were close to normal, expression of NF- κ B p65, TLR4 and expression of TNF- α , IL-6 and IL-13 mRNA were significantly elevated, accompany with an increasing levels of IL-10 mRNA. These results indicated that, TLR4/NF- κ B signal was activated in the colon mucosa of colitis induced by DSS. Baicalin inhibited the signal of NF- κ B and then suppress the production of proinflammatory cytokines such as TNF- α , IL-6 and IL-13.

In order to compare the protective effects of baicalin with traditional drug used for UC, we selected a first line drug, mesalazine, as a control drug, which was well known for anti-inflammatory effect with a mechanism of blocking NF- κ B signaling in UC patients [25]. In our study, baicalin showed a similar effect of anti-inflammatory presenting by immunological parameters in DSS+BCLN group which is closed to DSS+MSLZ group.

Considering the imbalance of pro-inflammatory and anti-inflammatory cytokines in extracellular under inflammatory condition, RT-PCR was performed to analyze the mRNA of intracellular cytokines in colon tissues. Our data suggested baicalin were able to modulate the mRNA expression of pro-inflammatory (TNF- α , IL-6 and IL-13) and anti-inflammatory (IL-10) cytokines in colon tissues, while mesalazine can only modulate the mRNA expression of pro-inflammatory (TNF- α , IL-6 and IL-13). It is well known that NF- κ B or MyD88 is a crucial factor involved in the regulation of inflammation [26], therefore, we focused on the relationship between baicalin and the activity of NF- κ B or MyD88. Whether baicalin induces anti-inflammatory cytokines via MyD88 is not yet known.

Interestingly, one of the signal-transduction proteins, MyD88, increased in DSS+BCLN and DSS+MSLZ group in our study. This change is not consistent with NF- κ B p65 and TLR4 (**Figure 4B-D**). To our knowledge, signals through TLRs 1/2/6, 5, 7-9 are mediated exclusively by MyD88 and led to activation of NF- κ B or AP-1 [27]. Our data might suggest that, baicalin might exert regulatory function via MyD88 signal, including increasing the expression of IL-10 mRNA, which needs further study.

TLRs mainly expressed on antigen-presenting cells which are important in the modulation of host immune responses. To date, 10 and 12 functional TLRs have been identified in humans and mice, respectively [28]. TLRs recognize distinct pathogen-associated molecular patterns derived from bacteria, viruses, fungi, and parasites [29]. TLR2 recognizes lipopeptides from bacteria, while TLR4 recognizes lipopolysaccharide (LPS), and TLR9 recognizes ssRNA and CpG DNA. Research indicates that TLR2 may direct tolerogenic responses, induce an increase in anti-inflammatory cytokines, such as IL-10 production [30].

The role of mediating protective effects of baicalin via TLRs signals in DSS-induced colitis was also investigated in this study. We found that, baicalin down regulated the expression of TLR4, but not TLR2 and TLR9. The level of IL-10 mRNA were elevated, which indicated baicalin might up regulated IL-10 through intervening TLR2 or TLR9 signals or other pathway. It's reported that, IL-10 was induced via TLR/MyD88/NF- κ B signaling pathway in some regulatory lymphocytes such as regulatory B cells. Whether baicalin increased IL-10 mRNA via TLR4/MyD88 pathway needs further investigate.

Our study has demonstrated the efficacy of baicalin in UC via relieving the clinical symptoms, reducing the mDAI and histological scores, decreasing the expression of TLR4 and NF- κ B p65 and also the expression of TNF- α , IL-6 and IL-13 mRNA which are mediated by NF- κ B signal, but increasing the expression of IL-10 mRNA. All these results indicate the inflammatory response may be alleviated by baicalin via blocking TLR4/NF- κ B signal transduction pathway. Therefore, TLR4/NF- κ B signaling pathway is expected to become a new target for the treatment of UC. Baicalin might contribute to the development of new pharmaceutical products for UC treatment.

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