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Clostridium butyricum promotes intestinal motility by regulation of TLR2 in interstitial cells of Cajal

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Abstract. – OBJECTIVE: ***Clostridium butyricum (C. butyricum)*** **as a probiotic has been reported to have an important role in the pathogenesis of gastrointestinal diseases. However, the effects of C. butyricum on regulation of intestinal motility of ulcerative colitis (UC) remain unclear. Our study aimed to explore the cross-regulation effect of C. butyricum and toll-like receptor 2 (TLR-2) on UC.**

MATERIALS AND METHODS: Interstitial cells of Cajal (ICCs) were treated by *C. butyricum* for 2 h, the mRNA and protein levels of TLR-2, IL-6, and IL-8 were detected by RT-qPCR and Western blot. Then, TLR2-specific small interfering RNA (si-TLR2) was transfected into ICCs, and the relative expressions of IL-6 and IL-8, SCF, cell viability, ghrelin, SP, and ET were measured by RT-qPCR, Western blot, CCK-8, and ELISA. Besides, the signal pathways of NF-κB and JNK were determined by Western blot.

RESULTS: *C. butyricum* significantly increased TLR2, IL-6, and IL-8 expressions in ICCs. However, TLR2 silence alleviated *C. butyricum*-induced IL-6 and IL-8 expressions. Moreover, TLR2 silence significantly inhibited *C. butyricum*-induced cell viability in ICCs. Additionally, *C. butyricum* significantly increased SCF expression and promoted the secretion of ghrelin and SP. However, a significant reduction in the levels of SCF, ghrelin, and SP was evident in the silence of TLR2 expression. Besides, TLR2 silence reduced *C. butyricum*-activation NF-κB and JNK signal pathways in ICCs.

CONCLUSIONS: These findings revealed that *C. butyricum* promoted intestinal motility by regulation of TLR2 in ICCs, which contributed to understand the molecular mechanisms of *C. butyricum* on UC.

Key Words:
Clostridium butyricum, TLR2, Ulcerative colitis, NF-κB, JNK.

Introduction

Ulcerative colitis (UC) is a chronic disease that results in inflammation and ulceration of the colon and rectum[1]. The primary clinical features of UC are abdominal pain, diarrhea, stools mixed with blood, and intestinal motility disorders[2]. Epidemiological studies show that the incidence of UC has a significant increase around the world (about 3 million of people), and most commonly in Asian and western countries[3]. Currently, the cause of UC is still unknown, and there is no effective method to treat UC. Patients often need long-term medication to maintain life[4]. Numerous studies[5,6] mostly focus on the pathogenesis of UC, including immunity, heredity, infection, and intestinal flora. As a new direction in the pathogenesis of UC, the role of intestinal motility is being recognized and becoming a hotspot.

Emerging evidence has demonstrated that interstitial cells of Cajal (ICCs) as pacemaker cells play an important role in the regulation of intestinal motility[7]. Bernardini et al[8] reported that ICCs were actively involved in the pathogenesis of motility disturbances in UC[9]. It is well known that stem cell factor (SCF) plays a crucial role in the maintenance of ICCs phenotypes, proliferation, and differentiation[10]. Moreover, the decreased of SCF signal pathway may contribute to the loss of ICCs in gallstone disease[11]. Furthermore, gastrointestinal hormones such as ghrelin, substance P (SP), and endothelin (ET) are also important molecules that regulate intestinal motility and secretion[12,13]. Therefore, ICCs, SCF, and gastrointestinal hormones are the key regulator used to study gastrointestinal motility. However, the effects of these factors on UC remain unclear.

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Recent studies[14,15] have proven that imbalance of bacterial flora existed in human UC and experimental colitis of rat. *Clostridium butyricum* (*C. butyricum*) as a probiotic has been employed in regulating the ecological balance of intestinal bacteria[16]. Increasing evidence demonstrated that *C. butyricum* could promote *Bifidobacterium* and *Lactobacilli* proliferation, growth, and inhibit harmful bacteria and spoilage bacteria reproduction in intestinal tract[17,18]. Moreover, clinical research[19] has confirmed that *C. butyricum* combined with mesalazine in the treatment of chronic-recurrent UC was better than the use of mesalazine alone.

Based on these previous studies, we aimed to explore the effect of *C. butyricum* and toll-like receptor 2 (TLR-2) on the immune response and UC dynamics via examining relative expressions of interleukin 6 (IL-6), interleukin 8 (IL-8), SCF, and gastrointestinal hormones (ghrelin, PS, and ET). These finding will provide a new insight for treatment of UC and further guide clinical application.

Materials and Methods

Cell Culture

BALB/C mice (10-15 days old) used in this study were provided by Medical College of Qingdao University. All the experiments were approved by Animal Care and Users Committee of Medical College of Qingdao University. The mice were requested to fast for 12 h, and then were anaesthetized with chloroform and killed by cervical dislocation. The small intestine was removed (from 1 cm below the pyloric ring to the cecum) and opened along the mesenteric border. Luminal contents were washed using Krebs-Ringer bicarbonate solution (KRB, Sigma-Aldrich, St Louis, MO, USA). These tissues were pinned to the base of a Sylgard dish, and the mucosa was removed by sharp dissection.

After this, the layers of smooth muscle of the small bowel were cut into 1-2 mm^3 small pieces, and were equilibrated in nominally Ca^{2+} free solution containing, in mM: KCl 5.36, NaCl 125, NaOH 0.34, Na HCO 0.44, glucose 10, sucrose 2.9, and HEPES 11 (pH 7.4) for 30 min. Cells were then digested in an enzyme solution containing collagenase (1.3 mg/ml, Worthington Biochemical, Lakewood, NJ, USA), bovine serum albumin (BSA, 2 mg/ml, Sigma-Aldrich, St. Louis, MO, USA), trypsin inhibitor (2 mg/ml, Sigma-Aldrich), and adenosine 5'-triphosphate (ATP, magnesium salt, 0.27 mg/ml, Sigma-Aldrich) and cultured at 37°C with 5% CO_2 incubator in smooth muscle growth medium (SMGM, Clonetics, San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine SCF (5 ng/ml, Sigma-Aldrich). Animal care and experiments on animals were conducted in accordance with the guidelines issued by the Ethics Committee of Medical College of Qingdao University.

Bacterial Strains and Cell Stimulation

The *C. butyricum* used in this study was obtained from Miyarisan Pharmaceutical Co. Ltd (Tokyo, Japan). This strain was a spore-forming, gram-positive rod bacterium and produced by a butyric-acid. It was cultured in De Man Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) in an anoxic environment at 28°C [20]. Before stimulation assays, the bacteria were collected and re-suspended in antibiotic-free 1640 media (Sigma-Aldrich) at the density of 1×10^8 CFU ml-1. Cells were treated with 2 ml of 1640 media or *C. butyricum* suspensions at a designated concentration (1×10^8 CFU ml-1), and were incubated in 5% CO_2 at 37°C for 2 h.

Cell Transfection

The target small interfering RNA (siRNA) of TRL2 and the corresponding control were synthesized by GenePharma Co. (Shanghai, China). Briefly, cells were cultured in 6-well plates overnight at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Thanks for your warm work. Then, cell transfections were performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Cell Viability

Cells were seeded in 96-well plate with 5000 cells/well, and cell viability was detected by a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, Maryland, USA). In brief, after transfection for 48 h, 10 µl of CCK-8 solution was added to the culture medium, and were incubated for 1 h at 37°C in humidified 95% air and 5% CO_2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of ghrelin, SP, and ET were measured by using the specific ELISA kits (R&D Systems, Abingdon, UK) for these factors accord-

ing to the manufacturer's protocols. The optical density (OD) values were measured by a microplate reader (Molecular Devices, Silicon Valley, CA, USA) at 450 nm. The concentration of tested factors in each sample was calculated by using a standard curve according to the protocols provided by the kits.

Reverse Transcription-Quantitative PCR (RT-qPCR)

Total RNA was isolated from the cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand complementary DNAs (cDNAs) were synthesized using the PrimeScript™ RT reagent kit (TaKaRa, Dalian, China). Quantitative PCR (qPCR) was performed with 20 ng cDNA templates to measure the mRNA level using the 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The expression level of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was considered as a standard. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western Blot

We used radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) to extract the total proteins, and then the extracted proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad Laboratories) was used to separate 20 μg of total protein. The target proteins were transfected onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and then the membranes were blocked in 5% dried skim milk (Boster Biological Technology, Wuhan, China) at room temperature for 2 h. Subsequently, the membranes were incubated overnight at 4°C with specific primary antibodies for TLR2 (ab108998), SCF (ab83866), p65 (ab16502), phosphorylated (p)-p65 (ab86299), IκBα (ab32518), p-IκBα (ab92700), Jun N-terminal kinase (JNK, ab179461), p-JNK (ab76572), c-Jun (ab119944), p-c-Jun (ab81319), GAPDH (ab8245, Abcam, Cambridge, UK), interleukin 6 (IL-6, #12912, Cell signaling Technology, Inc., Danvers, MA, USA), and IL-8 (LS-C8023-100, LifeSpan BioSciences, Seattle, Washington, USA). After washing in phosphate buffered saline (PBS, Sigma-Aldrich) for three times, the membranes were incubated at room temperature for 2 h with the horse reddish peroxidase-conjugated secondary antibodies (Abcam) at a 1:5000 dilution. Protein detection was conducted by using enhanced chemiluminescence (ECL, MultiSciences Biotech, Hangzhou, China) and imaging was carried out using a BioSpectrum Gel Imaging System (HR410, UVP, USA).

Statistical Analysis

All experiments in the study were repeated in triplicate and the results of multiple experiments were presented as the mean ± standard deviation (SD). Data were analyzed by *t*-test for the significant difference by using SPSS 19.0 statistical software (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA). A p-value of < 0.05 was considered statistically significant.

Results

C. butyricum Increased TLR2, IL-6, and IL-8 Expressions in ICCs

We examined the effect of *C. butyricum* on expression levels of TLR2, IL-6, and IL-8 in ICCs by using RT-qPCR. As results showed in Figure 1A-1C, *C. butyricum* significantly increased TLR2, IL-6, and IL-8 expressions compared to control group ($p < 0.001$). Western blot results also revealed a remarkably promotion in the protein levels of TLR2, IL-6, and IL-8 by *C. butyricum* (Figure 1D). These data indicated that *C. butyricum* induced TLR2, IL-6, and IL-8 expressions in ICCs.

C. butyricum Promoted IL-6 and IL-8 Expressions by Regulation of TLR2 in ICCs

To explore whether *C. butyricum* affected IL-6 and IL-8 expression by modulation of TLR2, the specific siRNA of TLR2 (si-TLR2) was transfected into ICCs. The mRNA level of TLR2 was markedly down-regulated by si-TLR2 compared to control group ($p < 0.01$). Western blot analysis displayed a consistent result with the mRNA level in ICCs (Figure 2A). Furthermore, TLR2 inhibition significantly increased IL-6 and IL-8 expressions compared to control group ($p < 0.01$). However, after treatment by *C. butyricum*, the promotive effect was remarkably alleviated by TLR2 inhibition in ICCs ($p < 0.05$, Figure 2B-2D). Meanwhile, Western blot results showed that *C. butyricum* reduced IL-6 and IL-8 protein levels after transfection with TLR2 inhibition (Figure 2D). These results implied that *C. butyricum* induced expressions of IL-6 and IL-8 by regulation of TLR2 in ICCs

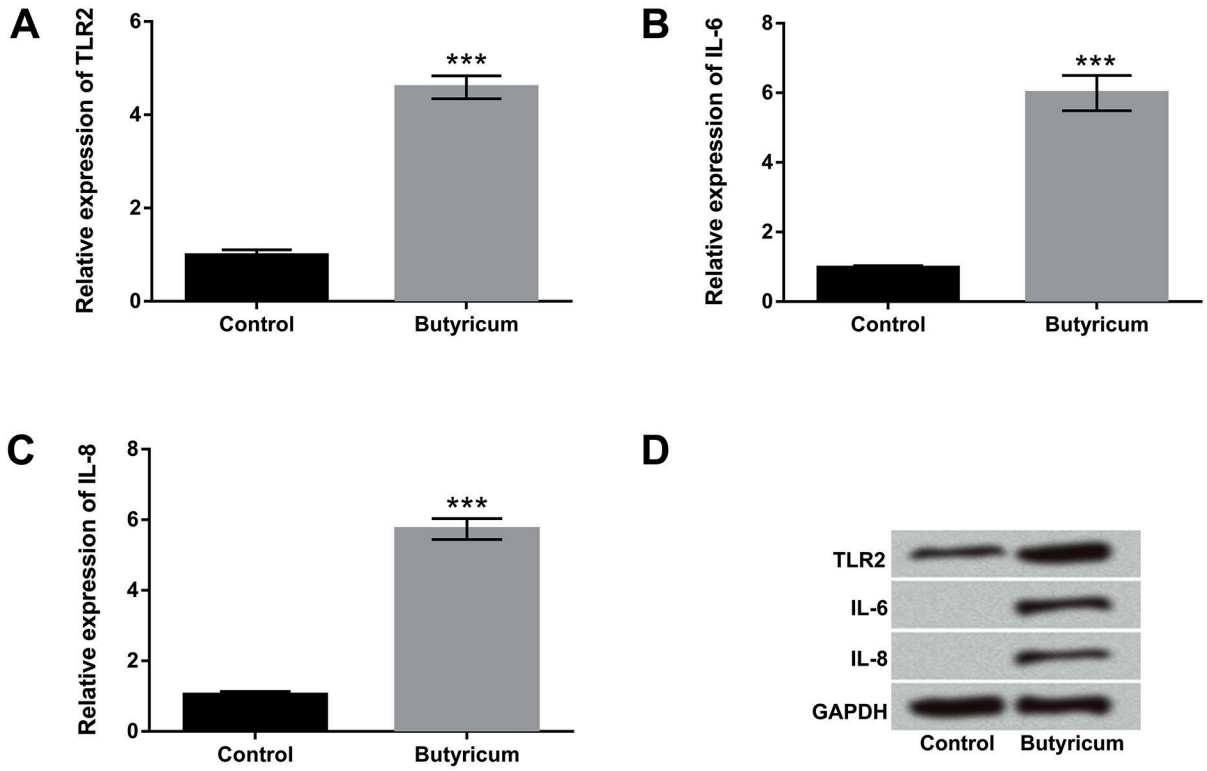

Figure 1. *C. butyricum* increased expressions of TLR2, IL-6 and IL-8 in ICCs. RNAs and proteins were extracted after a 2 h *C. butyricum* treatment, then relative mRNA expressions of (A) TLR2 (B) IL-6 and (C) IL-8 were detected by RT-qPCR; (D) relative protein levels of TLR2, IL-6 and IL-8 were examined by Western blot. *C. butyricum*: *Clostridium butyricum*; TLR2: toll-like receptor 2; IL-6: interleukin-6; IL-8: interleukin-8; RT-qPCR: reverse transcription-quantitative PCR; ***$p < 0.001$.

C. butyricum Promoted Cell Proliferation by Regulation of TLR2

Next, we investigated the effect of *C. butyricum* on cell viability in ICCs by using CCK-8 assay. As displayed in Figure 3, *C. butyricum* significantly promoted cell viability in ICCs compared to no treatment group ($p < 0.05$). However, after TLR2 knockdown via siRNA transfection, there was an evident reduction of cell viability in ICCs ($p < 0.05$). These data indicated that *C. butyricum* promoted ICCs cell proliferation by regulation of TLR2.

C. butyricum Induced Expression and Secretion of SCF, Ghrelin, and SP by Regulation of TLR2 in ICCs

Recent studies have demonstrated that SCF and gastrointestinal hormones (ghrelin, SP, and ET) are key regulators involved in gastrointestinal motility[21,22]. However, the effects of *C. butyricum* and TLR2 on SCF, ghrelin, SP, and ET in ICCs have not been investigated. As shown in Figure 4A, *C. butyricum* significantly up-regulated SCF expression compared to untreated control group ($p < 0.01$). But, the increasing effect of *C. butyricum* was alleviated in ICCs transfected with TLR2 siRNA ($p < 0.01$). Similarly, the protein level of SCF significantly increased by *C. butyricum*, but was decreased after transfection with TLR2 inhibition. Besides, *C. butyricum* induced ghrelin and SP secretion. However, TLR2 inhibition significantly reduced *C. butyricum*-induced ghrelin and SP secretion ($p < 0.05$, Figure 4C and 4D). No evident impact of *C. butyricum* and TLR2 on ET secretion was observed (Figure 4E). Taken together, these findings indicated that *C. butyricum*-induced expression and secretion of SCF, ghrelin, and SP were dependent on TLR2 in ICCs.

C. butyricum Activated NF-κB and JNK Signal Pathways by Regulation of TLR2 in ICCs

Finally, we further explored the effect of *C. butyricum* and TLR2 on NF-κB and JNK signal pathways in ICCs. As results showed in Figure 5A, *C. butyricum* increased phosphorylated p65 and IκBα expressions. However, inhibition of TLR2

decreased *C. butyricum*-induced p-p65 and p-IκBα protein levels. Furthermore, the protein levels of phosphorylated of JNK and c-Jun increased by *C. butyricum*, as well as TLR2 inhibition decreased *C. butyricum*-induced JNK and c-Jun expressions (Figure 5B). There were no significant effects of *C. butyricum* and TLR2 on p65, IκBα, JNK, and c-Jun expressions. These results provided further evidence that *C. butyricum* activated NF-κB and JNK signal pathways by regulation of TLR2 expression.

Discussion

In the present study, we demonstrated that *C. butyricum* significantly increased TLR2, IL-6, and IL-8 expressions in ICCs. However, TLR2 silence alleviated *C. butyricum*-induced IL-6 and IL-8 expressions. Moreover, *C. butyricum* promoted cell viability, up-regulated SCF expression and stimulated ghrelin and SP secretion by regulation of TLR2 in ICCs. Besides, TLR2 silence reduced *C. butyricum*-activation NF-κB and JNK signal pathways.

The intestinal tract contains a complex dynamic ecosystem, and the microflora is essential in the immune system. It is contributed to maintain gut homeostasis and intestinal function[23]. *C. butyricum* as an important symbiotic bacteria has been widely used in treating human intestinal diseases[24]. In our study, we firstly explored the effect of *C. butyricum* on the regulation of TLR2

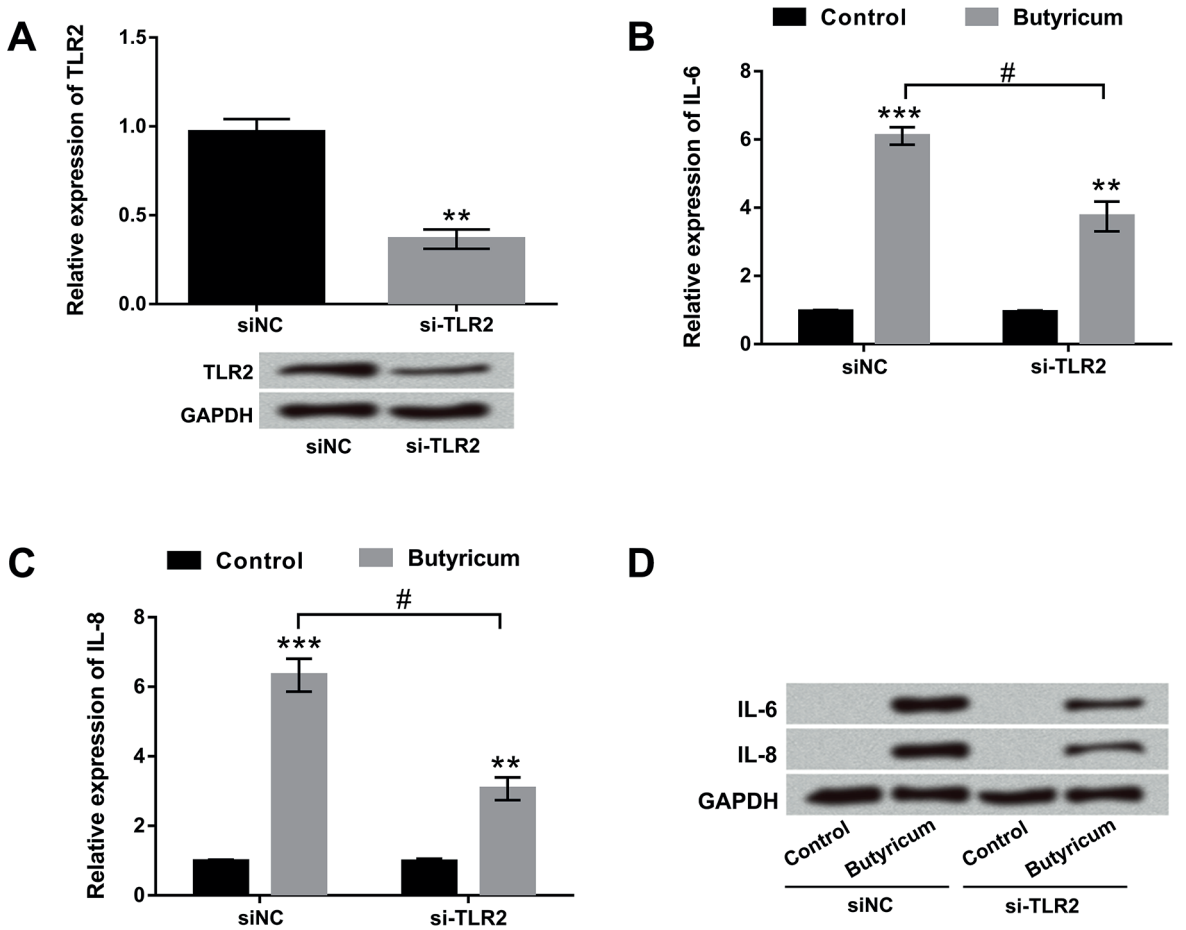

Figure 2. *C. butyricum* induced IL-6 and IL-8 expressions by regulation of TLR2. ICCs were reverse transfected with TLR2-specific siRNA for 48 h. After stimulation by *C. butyricum* for 2 h, (A) relative mRNA and protein levels of TLR2 were determined by RT-qPCR and Western blot; (B) and (C) relative IL-6 and IL-8 transcript levels were measured by RT-qPCR; (D) relative protein levels of IL-6 and IL-8 were assessed by Western blot. *C. butyricum*: *Clostridium butyricum*; IL-6: interleukin-6; IL-8: interleukin-8; TLR2: toll-like receptor 2; ICC: interstitial cells of Cajal; siRNA: small interfering RNA; RT-qPCR: reverse transcription-quantitative PCR; NC: negative control; $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the untreated control; $\#p < 0.05$ compared to siNC with *C. butyricum* stimulation.

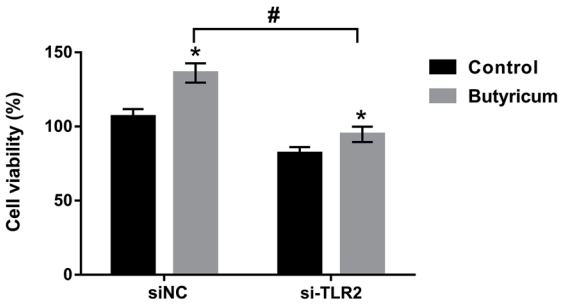

Figure 3. *C. butyricum* promoted cell proliferation by regulation of TLR2. ICCs were reverse transfected with TLR2-specific siRNA for 48 h. After stimulation by *C. butyricum* for 2 h, cell viability was analyzed by CCK-8 assay. *C. butyricum*: *Clostridium butyricum*; TLR2: toll-like receptor 2; ICC: interstitial cells of Cajal; siRNA: small interfering RNA; CCK-8: Cell Counting Kit-8; NC: negative control; *$p < 0.05$ compared to the untreated controls; #$p < 0.05$ compared to the siNC control with *C. butyricum* stimulation.

expressions in ICC. Our study found a significant induction in the level of TLR2 expression by *C. butyricum* stimulation. The result consistent with Gao et al[25] reported that *C. butyricum* was able to stimulate TLR2 production at mRNA level. Furthermore, several researches revealed that *C. butyricum* could modulate the immune response in inflammatory bowel diseases (IBD)[26,27]. Kanai et al[26] demonstrated that *C. butyricum* induced IL-10 production in IBD. Similar to these studies, our study showed that *C. butyricum* induced IL-6 and IL-8 expressions in ICCs.

It was reported that TLR2 is a pivotal molecule in host defense against gram-positive bacteria, mycoplasma, and fungus[28]. Also, TLR2 is necessary for the development of acquired immune responses to various bacterial species[29]. For example, Jeon et al[30] found that *Bifidobacterium breve* potentially produced IL-10 and IL-27 in a TLR2-dependent fashion to induce IL-10-producing in Tr1 cells. Gao et al[31] demonstrated that *C. butyricum* could increase IL-8, IL-6, and TNF-α expressions and secretions through regulation of TLR2 in HT-29 cells. Similar with these studies, our study also demonstrated that *C. butyricum* induced expressions of IL-6 and IL-8 by regulation of TLR2 in ICCs.

ICCs are specialized mesenchymal cells that play a crucial role in the regulation of gastrointestinal motility[32]. Recently, it has been verified that ICCs functioned as gastrointestinal pacemaker cells, and regulated neurotransmitters tran-

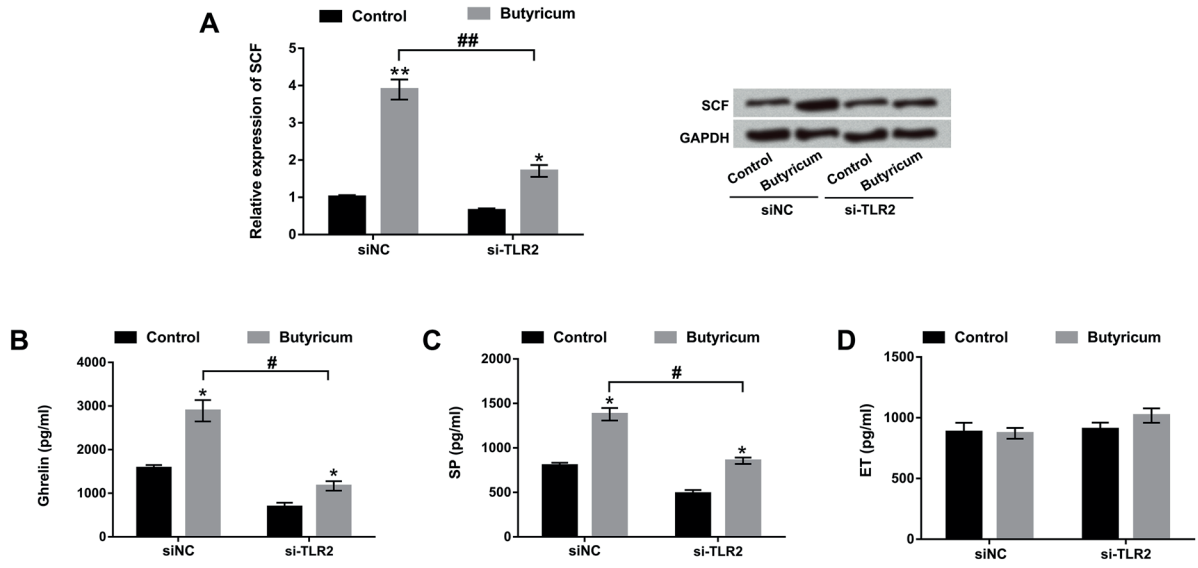

Figure 4. *C. butyricum* induced SCF expression and stimulated ghrelin and SP secretion by regulation of TLR2 in ICCs. ICCs were reverse transfected with TLR2-specific siRNA for 48 h. After stimulation by *C. butyricum* for 2 h, (A) relative mRNA and protein levels of SCF were detected by RT-qPCR and Western blot; the concentrations of (B) ghrelin (C) SP and (D) ET were examined by ELISA. *C. butyricum*: *Clostridium butyricum*; SCF: stem cell factor; SP: substance P; ET: endothelin; siRNA: small interfering RNA; RT-qPCR: reverse transcription-quantitative PCR; ELISA: enzyme-linked immunosorbent assay; *$p < 0.05$, **$p < 0.01$ compared to the untreated controls; #$p < 0.05$, ##$p < 0.01$ compared to the siNC control with *C. butyricum* stimulation.

smission[33]. Extensive experimental and clinical studies[34,35] have demonstrated that loss and/or damage of ICCs may lead to serious gastrointestinal motility disorders including in UC. Therefore, we supposed that *C. butyricum* could impact gastrointestinal motility by regulating ICCs proliferation. As expected, our study found that *C. butyricum* promoted cell viability, but TLR2 silence decreased *C. butyricum*-induced ICCs cell viability, indicating that *C. butyricum* increased ICCs proliferation by regulation of TLR2.

It has been widely accepted that SCF is the natural ligand for c-kit, and SCF/c-kit system plays an important role in the development and survival of ICCs in intestinal tract[36]. In mice mold of UC, the expression of SCF was decreased in colon tissue, indicating that the incidence of UC may be related to the regulation of the SCF/c-kit system[37]. Similar to the previous study, we found that TLR2 inhibition significantly decreased *C. butyricum*-induced SCF expression in ICCs. Further, ghrelin, SP, and ET are common polypeptide, which could promote gastrointestinal motility and involve in the pathogenesis of UC. Based on these previous studies, we also explored the effect of *C. butyricum* and TLR2 on ghrelin, SP, and ET secretion. We found that *C. butyricum* induced ghrelin and SP secretion by regulation of TLR2 in ICCs. These data indicated that *C. butyricum* could affect gastrointestinal motility by regulation of TLR2 in UC.

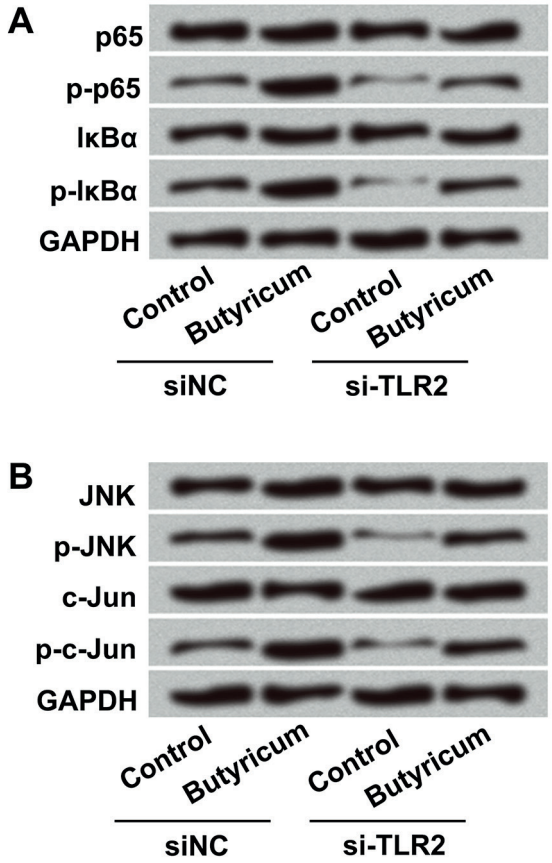

Figure 5. TLR2 inhibition reduced *C. butyricum*-activation NF-κB and JNK signal pathways in ICCs. ICCs were reverse transfected with TLR2-specific siRNA for 48 h. After stimulation by *C. butyricum* for 2 h, the main factors levels of (A) NF-κB signal pathway and (B) JNK signal pathway were examined by Western blot. TLR2: toll-like receptor 2; *C. butyricum*: *Clostridium butyricum*; NF-κB: nuclear factor-κB; JNK: Jun N-terminal kinase; ICC: interstitial cells of Cajal; siRNA: small interfering RNA.

The two principal pathways affected by *C. butyricum* are the NF-κB and JNK signal pathways and the roles of both in the pathogenesis of gastrointestinal diseases have been studied. It was reported that *C. butyricum* attenuated the phosphorylation of NF-κB, p65, and ERKs in the gastric tissues[38]. In addition, *C. butyricum* could effectively treat acute UC mice and the possible mechanism may be related to the decrease of NF-κB[39]. Furthermore, *C. butyricum* up-regulated aquqporin3 expression via JNK/p38 pathways in HT-29 cells[40]. However, the mechanisms by which *C. butyricum* exert their beneficial effects on NF-κB and JNK signal pathways functions remain unclear. In the work, we found that *C. butyricum* activated NF-κB and JNK by regulation TLR2 expression in ICCs.

Conclusions

Taken together, we demonstrated that *C. butyricum* could promote ICCs proliferation and increased the secretion of ghrelin and SP by regulation of TLR2. The findings indicated that *C. butyricum* might improve intestinal motility, and might provide a new insight for the treatment of UC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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