



# Mesenchymal Stem Cells Improve Rat Ulcerative Colitis through Activation of the TLR9/MyD88 Pathway

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## ABSTRACT

Mesenchymal stem cells (MSCs) could guide tissue-specific progenitor cells responsible for regenerating injured tissue. The objective of this animal study is to investigate the correlation between BMSCs pre-treated with IL-6 therapy and the TLR4/MyD88 signaling pathway in the context of ulcerative colitis (UC) *in vivo*. Bone marrow was collected from male Wistar rats (weighing 80-100 g, aged 8-10 weeks) and BMSCs were isolated. Before BMSC treatment, rats were treated with IL-6 for 24 h. The UC rat model was established using DSS induction and treated with BMSCs. TLR9 OE AAV, MyD88 shRNA AAV, corresponding OE NC and sh NC were packaged into slow virus plasmids and injected into the rats via tail vein. Western blot, PCR, immunohistochemistry (IHC) and immunofluorescence (IFC) were used to measure the expression of TLR9, MyD88, and IL-6. We employed the Disease Activity Index (DAI) to assess the extent of disease severity in all rats. With the treatment of BMSCs, the protein and mRNA levels of IL-6, TLR9, and MyD88 gradually decreased, with a significant decrease observed on days 7 and 14 compared to the model group. Silencing MyD88 reduced the expression of IL-6, which reversed the effect of overexpressing TLR9 on the changes in IL-6. In addition, in UC rats treated with BMSCs for 14 days, there was a decrease in DAI score and an increase in colonic length. However, activation of the TLR9/MyD88 pathway reversed the therapeutic effect of IL-6-pretreated BMSCs therapy on UC rats, with an increase in DAI score and a decrease in colonic length. Our research found that our results suggest that IL-6-pretreated mesenchymal stem cells improve UC in rats through the TLR9/MyD88 signaling pathway.

## Article Information

Received 30 May 2023

Revised 05 August 2023

Accepted 24 August 2023

Available online 17 November 2023  
(early access)

## Authors' Contribution

CS, YX and GQ conceived the study, designed and performed experiments and analysed the data. CW and QZ provided technical support, data collection and analysis. CS, YX and GQ wrote the manuscript. All authors provided final approval for submitted and published version.

## Key words

TLR9, MyD88, Ulcerative colitis, Mesenchymal stem cells

## INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (Kucharzik *et al.*, 2020) that is marked by alternating periods of remission and relapse. UC can develop from mild asymptomatic inflammation to severe inflammation in the colon, causing frequent bloody stools, colonic motility disorders, permanent fibrosis if left untreated, and tissue damage. Surgery may be necessary in severe cases (Colombel *et al.*, 2017; Dotti *et al.*, 2017). The reported incidence rates of UC are high, exceeding 0.3%, in developed countries, and there is a rapid increase in emerging industrialized nations (Porter *et al.*, 2020).

While the exact cause of UC is not fully understood, studies have suggested that UC is associated with factors such as diet, environment, genetics, and lifestyle (Carreras-Torres *et al.*, 2020; Sun *et al.*, 2023).

Mesenchymal stem cells (MSCs) are a type of cultivatable pluripotent stem cell capable of self-renewal and differentiation into various tissues under appropriate stimuli (Zhou *et al.*, 2021). When associated with damaged tissue, MSCs do not differentiate, but instead guide tissue-specific progenitor cells responsible for injury tissue regeneration (Rodríguez-Fuentes *et al.*, 2021). Furthermore, MSCs have been discovered to possess robust immunomodulatory and anti-inflammatory properties, holding significant promise for the treatment of various autoimmune and inflammatory disorders (Kim, 2021). Further, by using inflammation pretreatment, genetic modification or combination with other substances, and different tissue source cells, administration methods or dosages, the therapeutic efficacy of MSCs in inflammatory bowel disease can be enhanced (Zhang *et al.*, 2020). The toll-like receptor (TLR) family is expressed on sentinel cells of the immune system, including macrophages and dendritic cells (El-Zayat *et al.*, 2019). TLRs activate antimicrobial genes and inflammatory cytokines by

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0030-9923/2023/0001-0001 \$ 9.00/0



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triggering a series of signaling cascades, leading to the direct killing of invading pathogens (Li *et al.*, 2021). TLR9 signals through the MyD88-dependent pathway (Owen *et al.*, 2022). Latest studies have demonstrated that the TLR9/MyD88 axis in inflammatory bowel disease (Jing *et al.*, 2020; Li *et al.*, 2022). However, no studies have investigated changes in TLR9 and MyD88 after MSCs therapy and the impact of this signaling pathway on ulcerative colitis.

Our previous study has shown that IL-6 pretreated BMSCs can improve dextran sodium sulfate (DSS)-induced UC (Ding *et al.*, 2023). Based on these studies, we hypothesize that BMSCs therapy improves DSS-induced UC through modulation of the TLR4/MyD88 signaling pathway. We aim to explore the relationship between IL-6-pretreated BMSC therapy for UC and the TLR4/MyD88 signaling pathway. This research may offer novel research avenues for developing treatments for UC.

## MATERIALS AND METHODS

### *Isolation and culture of BMSCs*

Bone marrow was harvested from male Wistar rats (80-100 g, 8-10 weeks old) by dislocating the neck and flushing bone marrow from femurs and tibias. Harvested tissue was placed in a sterile culture dish and washed with culture medium, DMEM, containing 10% fetal bovine serum. The isolation and culture of BMSCs were performed as described elsewhere (Ding *et al.*, 2023). Before stem cell infusion, BMSCs were subjected to a pre-treatment with IL-6 for 24 h and then washed with PBS. The BMSCs utilized in this investigation demonstrated a purity of over 95% and were characterized as positive for CD44 and negative for CD34.

### *Animal model*

The rats in our animal study were kept under standard laboratory conditions, including housing in 12-h light-dark cycles, with free access to water and a regular diet, at a stable temperature between 18-24 degrees Celsius and humidity levels of 50-55%. All rats were randomly divided into following groups: blank group (6 rats, drinking water), ulcerative colitis (UC) group (6 rats, drinking water containing 2% DSS (Sigma-Aldrich, USA) for seven consecutive days), and BMSCs treatment group (18 rats at 0 d, 7 d, and 14 d, at 24 h post UC-induction, IL-6 pre-treated BMSCs ( $1 \times 10^6/0.4$  mL) were administered via the tail vein in a suspension of 0.5 mL of normal saline.), BMSC+TLR9 OE group (6 rats), BMSC+NC OE group (6 rats), BMSC+TLR9 OE+sh MyD88 group (6 rats), and BMSC+TLR9 OE+sh NC group (6 rats). Rats in both the model and blank groups received an equivalent amount of

normal saline via tail vein injection.

### *Adeno-associated virus (AAV) injection*

To overexpress TLR9 and suppress MyD88 in BMSCs treatment for UC rats. Before BMSCs treatment, 0.05 mL of TLR9 OE AAV (overexpressing TLR9), MyD88 shRNA AAV (knocking down MyD88), corresponding OE NC and shNC (GenePharma, Shanghai, China) were slowly injected into each group of rats via tail vein (viral titer:  $1 \times 10^7$  TU/mL). After injection, the needle was left in place for 10 min before removal, and the wound was sutured and disinfected. Control rats were injected with an equal volume of saline. The construction and packaging of slow virus plasmids were both carried out by Shanghai GenePharma Co., Ltd.

### *Assessment of disease activity index (DAI)*

The main evaluation criteria for the DAI included weight loss, bleeding, and fecal consistency. Weight loss was assessed by measuring the rats' weight on day 0 as the baseline and scored according to the following scale: 0, no weight change; 1, weight loss of 1%-5%; 2, weight loss of 6%-10%; 3, weight loss of 11%-15%; 4, weight loss >15%. The consistency of feces was graded as follows: 0, normal; 1, slightly loose; 2, loose; 3, approaching diarrhea; 4, severe diarrhea. Blood in feces was scored as follows: 0, no blood; 1, a small amount of blood; 2, bloody diarrhea; 3, bloody diarrhea with residual blood.

Firstly, the weight of rats was recorded before treatment and on days 0, 7, and 14 after treatment, and the weight change was calculated. Secondly, the fecal characteristics of rats were observed daily and graded according to the scoring criteria. The amount of blood in the feces was recorded and scored according to the scoring criteria. The sum of scores for the three measures was used to calculate the DAI score for each rat per day. The average DAI score of all rats was used as the DAI score for the treatment group. The DAI score was determined using the equation:  $DAI = (\% \text{ weight loss} + \text{fecal characteristic score} + \text{fecal blood score})/3$ . Post-euthanasia, the entire colon was removed from the cecum to the anus, and the length of the colon was measured to determine the magnitude of inflammation.

### *Immunohistochemistry (IHC)*

To perform IHC staining, tissue sections were initially exposed to Antigen Unmasking Solution (Vector Labs) at 95°C for 15 min. This was followed by sequential treatment with 3% H<sub>2</sub>O<sub>2</sub>, 0.5% Triton X-100, and Avidin/Biotin Blocking Kit. Afterward, the sections were blocked using 10% normal goat serum for one hour. The primary antibodies for total TLR9 (diluted at 1:50)

and MyD88 (diluted at 1:100) were incubated overnight at 4°C. Following this, the sections were treated with a secondary biotinylated goat anti-rabbit or anti-mouse antibody (diluted at 1/400) for 30 min. Subsequently, the sections were incubated with the Vectastain Elite ABC Kit (Vector Labs, Burlingame, CA). Finally, the IHC signals were revealed through the application of ImmPACT DAB Peroxidase Substrate.

#### Immunofluorescence (IFC)

Immunofluorescence was conducted to evaluate the expression of TLR9 and MyD88. The cells were fixed, permeabilized and then incubated with anti-TLR9 antibody (1:200, Sigma) and anti-MyD88 antibody (1:100, Sigma) overnight at 4°C followed with incubation with corresponding secondary antibody for 1 h at room temperature. DAPI was used for redyeing the nucleus. A Leica TCS-SP laser scanning confocal microscope was used to take the photomicrographs.

#### RT-qPCR

The expression of IL-6, TLR9 and MyD88 was determined using RT-qPCR. Total RNA was extracted from tissues using the Trizol reagent kit (Takara, Japan). The RNA concentration was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The mRNA was reverse transcribed into cDNA using the PrimeScript™ One Step RT-qPCR Kit (Takara Biotechnology Co., Ltd., Japan). The reaction mixture was then mixed and centrifuged, followed by incubation at 70°C for 15 min and 2 min of ice bath treatment. The amplification was performed using the SYBR GREEN master mix in an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, CA). The thermal cycling conditions were as follows: an initial activation step at 95°C for 15 min, followed by 35 cycles of denaturation at 4°C for 15 sec, annealing at 55°C for 25 sec, and extension at 70°C for 30 sec. Primer sequences were as follows:

<i>IL-6</i>	F 5'-GGCGGATCGGATGTTGTGAT-3' R 5'-GGACCCAGACAATCGGTTG-3'
<i>TLR9</i>	F 5'-ATGGTTCTCCGTCGAAGGACT-3' R 5'-GAGGCTTCAGCTCACAGGG-3'
<i>MyD88</i>	F 5'-TCATGTTCTCCATACCCTTGGT-3' R 5'-AAACTGCGAGTGGGGTCAG-3'
<i>GAPDH</i>	F 5'-AATGGATTTGGACGCATTGGT-3' R 5'-TTTGCACCTGGTACGTGTTGAT-3'

GAPDH was utilized as an internal control for this study. The levels of IL-6, TLR9, and MyD88 expression

were assessed using the 2<sup>-ΔΔCt</sup> method.

#### Western blot

Total proteins were extracted from colonic tissues and quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Then, 20 μg of the extracted proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The PVDF membranes were then incubated with a primary antibody at 4°C overnight. After being washed, the membranes were then incubated with Goat Anti-Rabbit IgG HandL secondary antibody (ab96899, 1/1000) at 37°C for 45 min. The primary antibodies included IL-6 Antibody (ab290735, 1/1000, Abcam), GAPDH Antibody (ab8245, 1/1000, Mybiosource). To serve as an internal control, GAPDH was utilized in this experiment. The protein bands were scanned utilizing the Pierce ECL Western Blotting Substrate (Pierce, Shanghai, China).

#### Statistical analysis

The measurement data were presented as mean ± standard deviation (SD). Statistical comparisons were carried out using a one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. A P-value less than 0.05 was considered statistically significant.

## RESULTS

#### *IL-6 pretreated BMSCs reduced the expression of TLR9 and MyD88*

We first treated DSS-induced UC rats with IL-6 pretreated BMSCs and euthanized the rats at days 0, 7, and 14 of treatment, and collected colonic tissues from all rats. WB and PCR were used to measure the protein and mRNA levels of TLR9, MyD88 and IL-6 in all groups. As shown in [Figure 1A](#), the protein levels of TLR9, IL-6 and MyD88 in the model group were remarkably elevated than those in the blank group ( $p < 0.05$ ). Following the administration of BMSCs, there was a gradual decrease in the protein levels of TLR9, MyD88, and IL-6. The protein levels of TLR9, MyD88 were significantly declined than those in the model group on days 7 and 14 after BMSCs treatment ( $p < 0.05$ ). The mRNA levels of TLR9, MyD88, and IL-6 showed a similar trend to protein levels ([Fig. 1B](#)).

#### *Detection of TLR9 and MyD88 expression in UC rats after BMSCs treatment*

To further investigate the expression of TLR9 and MyD88 in UC rats and their changes after BMSCs treatment, we also used IHC and IFC to measure the expression of TLR9 and MyD88 in the colonic tissues of all rats.

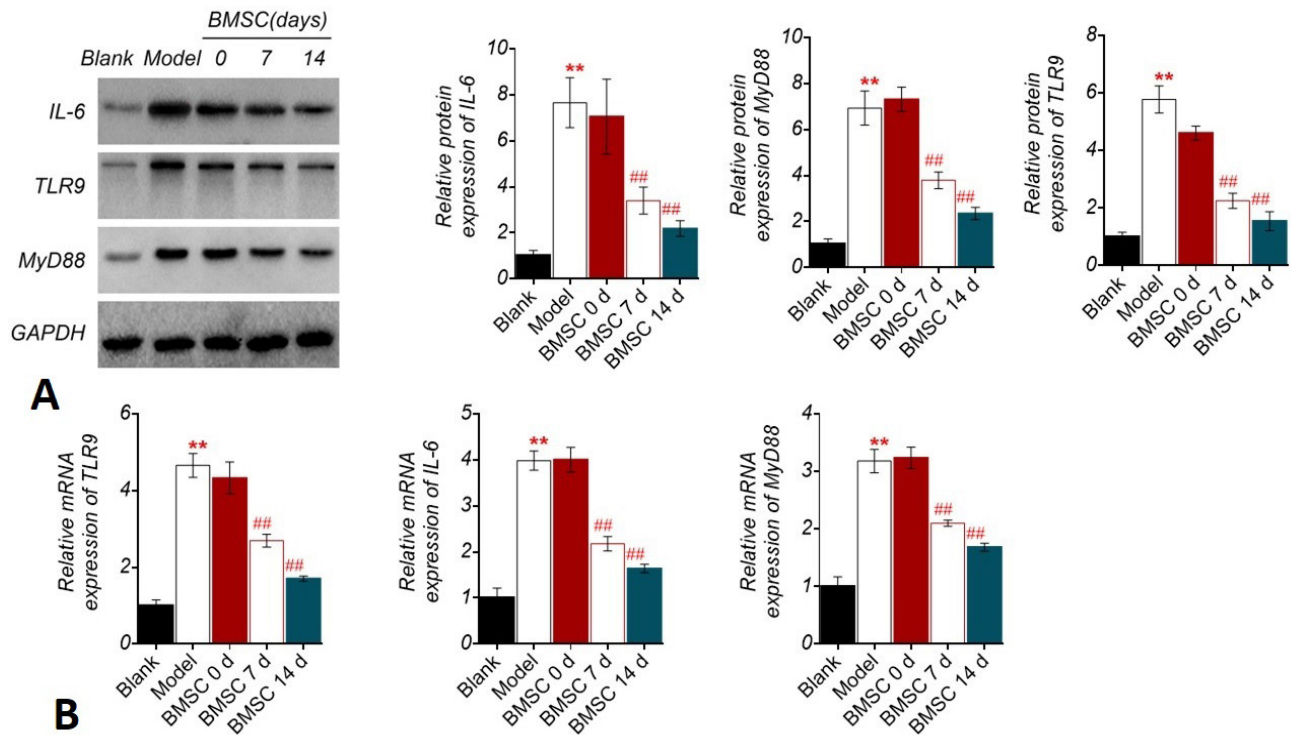


Fig. 1. IL-6 pretreated BMSCs reduced the expression of TLR9 and MyD88. The expression of IL-6, TLR9 and MyD88 were measured by WB (A) and PCR (B). The continuous data were compared using Student's t test between two groups. ANOVA followed by Tukey's post hoc test was used for comparison among three or more groups. \*\* $P < 0.05$  vs blank group. ## $P < 0.05$  vs model group.

The results of IHC showed that the expression of TLR9 and MyD88 was elevated in the colonic tissues of UC rats, and gradually decreased with BMSCs treatment (Fig. 2A). IFC also showed that the fluorescence intensity of TLR9 and MyD88 was significantly reduced after BMSCs treatment on days 7 and 14 compared to rats in model group (Fig. 2B).

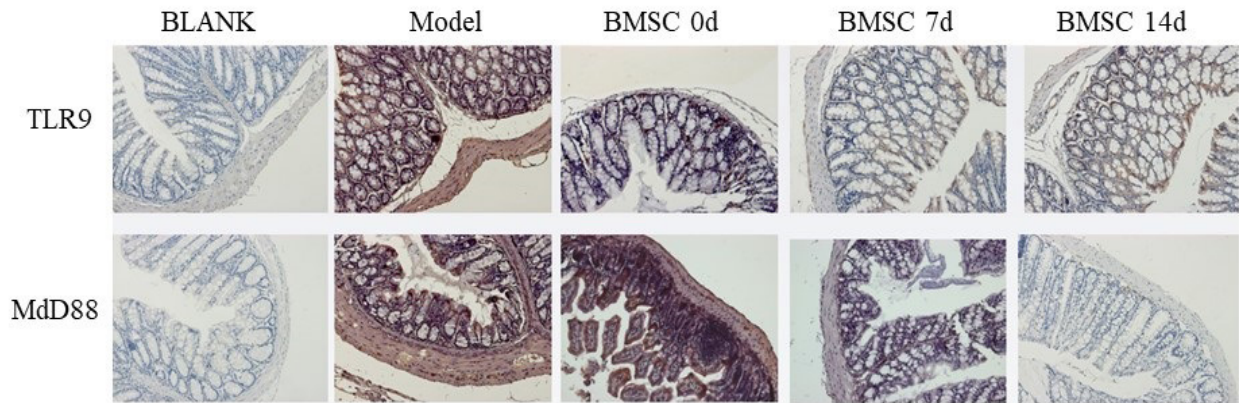
#### Activation of the TLR9/MyD88 pathway leads to an increase in the expression of IL-6

To investigate the role of TLR9/MyD88 in IL-6 pretreated BMSCs therapy for UC rats, we overexpressed TLR9 and inhibited MyD88 by using TLR9 OE AAV and MyD88 shRNA AAV before BMSCs treatment and euthanized all rats and collected colonic tissues after 14 days of BMSCs treatment. As shown in Figure 3A, after 14 days of BMSCs treatment, the levels of IL-6 in rats were significantly reduced, while in rats overexpressing TLR9, IL-6 was significantly increased ( $p < 0.05$ ), and this effect was reversed after inhibiting MyD88 expression. Compared with the TLR9 OE group, the level of IL-6 protein was significantly increased in the TLR9 OE +

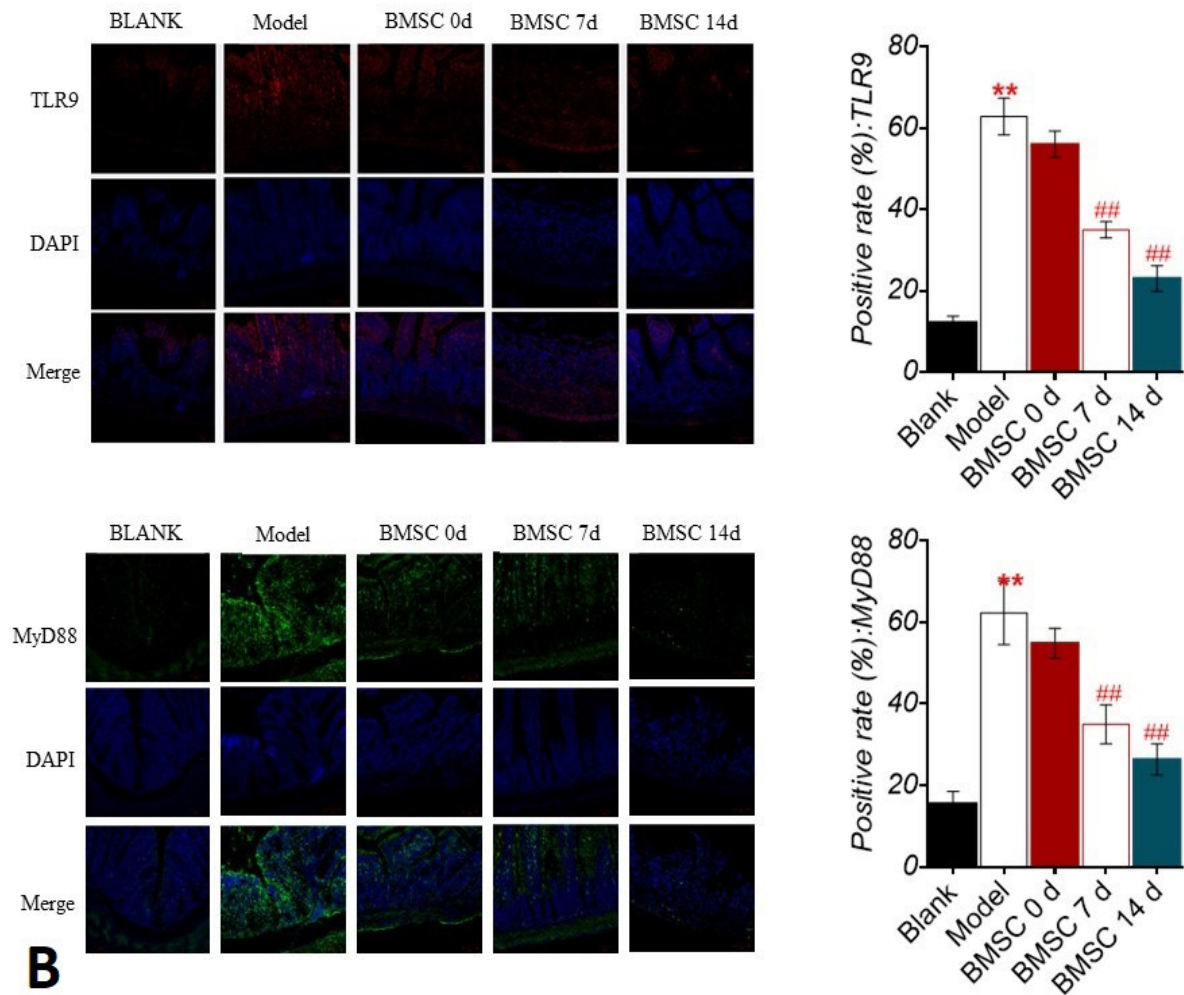
sh MyD88 group of rats. Subsequently, we used PCR to detect the mRNA levels of TLR9, MyD88, and IL-6 in the colonic tissues of rats in each group (Fig. 3B). Similarly, silencing MyD88 reduced the mRNA level of IL-6, which reversed the effect of overexpressing TLR9 on the changes in IL-6.

#### Activation of the TLR9/MyD88 pathway reversed the therapeutic effect of IL-6-pretreated BMSCs therapy on UC rats

Finally, we measured the colonic length of all rats and evaluated their DAI scores. After 14 days of IL-6 pretreated BMSC treatment, the DAI score of rats in the BMSC+TL9 OE group was significantly increased compared to the BMSC group (Fig. 4A,  $p < 0.05$ ), and this effect was reversed after silencing MyD88, with a significant decrease in the DAI score of rats in the BMSC+TL9 OE+sh MyD88 group compared to the BMSC+TL9 OE group ( $p < 0.05$ ). In addition, as shown in Figure 4B, compared with the model group, the colonic length of rats after 14 days of BMSC treatment was significantly increased ( $p < 0.05$ ). However, in rats overexpressing TLR9, the colonic length



**A**



**B**

Fig. 2. Detection of TLR9 and MyD88 expression in UC rats after BMSCs treatment by IHC and IFC. The expression of TLR9 and MyD88 were measured by IHC (A) and IFC (B). For statistical details see Figure 1.

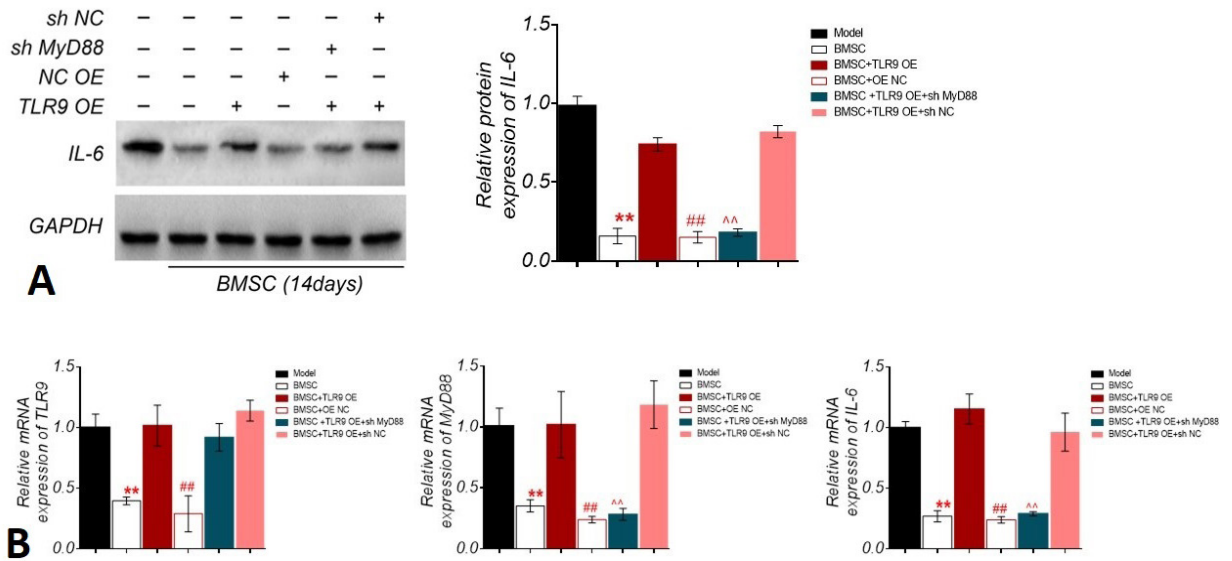


Fig. 3. Activation of the TLR9/MyD88 pathway leads to an increase in the expression of IL-6. The protein expression of IL-6 was measured by WB (A). The mRNA expression of IL-6, TLR9 and MyD88 were measured by PCR (B). For statistical details see Figure 1.

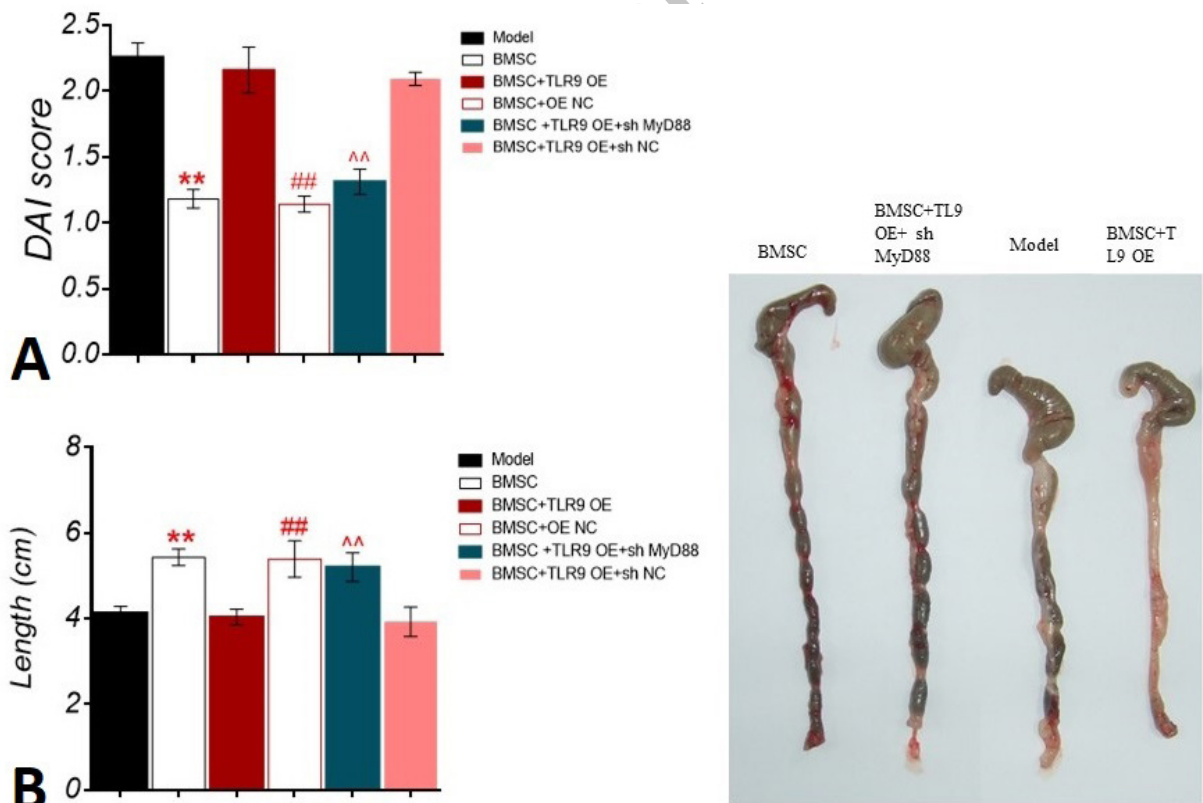


Fig. 4. Activation of the TLR9/MyD88 pathway reversed the therapeutic effect of IL-6-pretreated BMSCs therapy on UC rats. The DAI score was used to evaluate the disease severity of all rats (A). Colonic length of all rats was measured (B). For statistical details see Figure 1.

was significantly reduced ( $p < 0.05$ ), and silencing MyD88 reversed this effect. The colonic length of rats in the BMSC+TL9 OE+sh MyD88 group were remarkably elevated compared to the BMSC+TL9 OE group ( $p < 0.05$ ). These results indicated that activation of the TLR9/MyD88 pathway can reverse the therapeutic effect of IL-6-pretreated BMSCs therapy on UC rats, with an increase in DAI score and a decrease in colonic length.

## DISCUSSION

Despite the availability of many effective drugs and treatments for UC, there are still challenges and limitations in its treatment. Therefore, researchers are actively seeking new treatment methods and drugs to improve treatment outcomes and alleviate patient suffering, highlighting the need for new treatment strategies and targets (Yeshi *et al.*, 2020; Tripathi and Feuerstein, 2019). Now, we demonstrated that IL-6-pretreated BMSCs improve the development of DSS-induced UC in rats by modulating the TLR4/MyD88 signaling pathway.

The potential of MSCs in the treatment of diverse diseases, both immune and non-immune, has been widely acknowledged (Markov *et al.*, 2021). The pathological changes of UC include inflammation, ulcers and repair of the intestinal mucosa. As stem cells that can differentiate into multiple cell types, MSCs can promote intestinal mucosal repair and regeneration, and enhance intestinal mucosal barrier function (Hosseini-Asl *et al.*, 2020). Sala *et al.* (2015) and Song *et al.* (2017) have both demonstrated that MSC therapy can improve colitis symptoms, increase weight and decrease inflammatory cytokine levels. Moreover, evidence suggests that using an inflammatory stimulus to prime MSCs before transplantation may aid their survival in inflamed areas, and enhance their capacity to regulate local immune responses, thereby empowering them with stronger abilities to manage the tissue microenvironment (Barrachina *et al.*, 2020). Based on this, we used IL-6-pretreated BMSCs to treat DSS-induced UC rats, and the results showed that this type of treatment had a significant therapeutic effect. Therapy utilizing BMSCs has been shown to reverse the pathological changes induced by UC, leading to improvements in colon length and a reduction in DAI scores.

TLR9 not only leads to activation of the innate immune system, but also plays a critical role in the subsequent development of the immune response (Pollak *et al.*, 2022). TLRs can induce the secretion of type I interferons (IFNs), such as IFN- $\alpha$  and IFN- $\beta$ , to respond to viral DNA or RNA (Fitzgerald and Kagan 2020). Moreover, TLRs also trigger the activation of inflammatory cytokines, including IL-1 $\beta$ , IL-6, and TNF, which play crucial roles

as mediators of the inflammatory response and can induce acute inflammatory reactions against pathogens (Akhtar *et al.*, 2020). After TIR domain stimulation, it recruits the adapter protein MyD88 and initiates downstream signaling cascades, leading to the production of cytokines as a characteristic of pro-inflammatory responses (Zheng *et al.*, 2019). To investigate the role of the TLR9/MyD88 signaling pathway in BMSCs therapy for UC, we used WB, PCR, IHC, and IF to detect the expression of TLR9 and MyD88 in UC rats. The results showed that the expression of TLR9 and MyD88 in UC rats gradually decreased after treatment with BMSCs. Jing *et al.* (2020) suggested that upregulation of TLR9-induced MyD88 exacerbates stretch-induced inflammation and injury in a cellular injury model (Li *et al.*, 2022). Li *et al.* (2022) research also found that TLR9 and Myd88 expression was significantly increased in TNBS-induced colitis rats, and inhibiting the TLR9/Myd88/NF- $\kappa$ B signaling pathway can alleviate TNBS-induced colitis symptoms (Jing *et al.* 2020). These studies all suggested that TLR9/Myd88 played an important role in inflammation-induced damage and disease. In our research, we also confirmed that activating the TLR9/Myd88 signaling pathway could reverse the therapeutic effect of BMSCs treatment for UC, while downregulating MyD88 could improve the therapeutic effect.

### Limitation

Our research also has some limitations. Whether TLR9 is regulated by other genes is not clear. We also did not perform *in vitro* experiments to confirm the results of apoptosis. All these need further studies to illustrate.

## CONCLUSION

In summary, our results suggest that IL-6-pretreated mesenchymal stem cells improve DSS-induced UC in rats through the activation of TLR9/MyD88 signaling pathway. The results might provide a new integrated treatment approach for UC in clinical practice.

## ACKNOWLEDGMENT

We would like to acknowledge everyone for their helpful contributions to this paper.

### Funding

No funding support.

### Ethics statement and IRB approval

All animal experiments were performed according to the guidelines of the animal ethical organization and

obtained the permission of The Ehu Branch of Xishan People's Hospital.

#### Statement of conflict of interest

The authors have declared no conflict of interest.

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