

## Original Article

# Effect of Torin 1 on suppressing inflammation in mice with dextran sodium sulfate-induced colitis

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**Abstract:** The etiology of inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC) and Crohn's disease (CD), still remains unknown. It is also has great importance to develop new therapeutic strategies for the clinical treatment of IBD. In this study, we investigated the role of Torin 1, an inhibitor of mTOR, in Dextran sulfate sodium (DSS)-induced experimental colitis. We found that Torin 1 treatment significantly attenuated body weight loss and reduced the mortality induced by DSS. In addition, Torin 1 prevented DSS-induced colonic pathological damage, inhibited the production of pro-inflammatory cytokines in colon tissues. In vitro, we found in mouse macrophage cell line RAW264.7, Torin 1 significantly suppressed the cytokines expression and inhibited the activation of mTOR and NF- $\kappa$ B signaling pathway. In conclusion, our study demonstrated that Torin 1 may exert anti-inflammatory effect via modulation of mTOR/NF- $\kappa$ B signaling, suggesting that Torin 1 might be a potential effective drug for inflammatory bowel diseases.

**Keywords:** Torin 1, DSS, colitis, mTOR, NF- $\kappa$ B

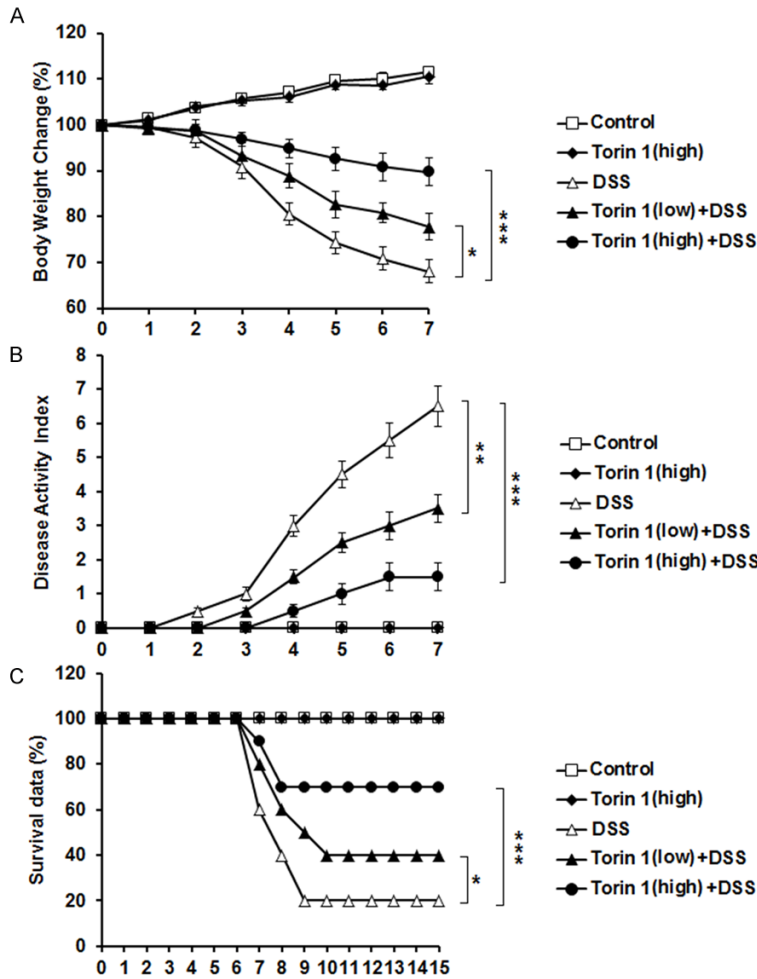
## Introduction

Millions of people worldwide are affected by inflammatory bowel disease (IBD), characterized by inflammation in the intestine and colon, as well as mucosal tissue damage [1]. It includes two main clinical forms: Crohn's disease (CD) and ulcerative colitis (UC). Although the precise etiology causing the disease still remains unknown, it has been demonstrated that the chronic mucosal inflammation in IBD is related to the hyperactivation of effector immune cells, which produce high levels of pro-inflammatory cytokines like tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6), thereby resulting in colonic tissue damage [2, 3].

Several animal models of experimental colitis have been developed to help investigate the molecular and cellular mechanisms of the disease. Dextran sulfate sodium (DSS) is commonly used to chemically induce intestinal inflammation in mice [4, 5]. Similar to human UC, DSS-induced colitis is limited to the colonic mucosa and is characterized by diarrhea, bloody feces, weight loss, colonic ulceration,

macrophages and granulocytes infiltration, as well as an increased production of inflammatory mediators, including IL-6, TNF- $\alpha$ , IL-1 $\beta$  [6, 7].

mTOR is a serine-threonine protein kinase that regulates protein synthesis, cell growth, and cell proliferation in response to growth factors and nutrients [8]. It is well studied that mTOR plays essential roles in tumorigenesis [9-12]. Furthermore, it was reported that lipopolysaccharide (LPS) stimulation of macrophages leads to activation of mTOR, and the mTOR pathway regulates the production of nitric oxide and activates STAT1-dependent transcription in macrophages in response to LPS [13-15]. A recent study showed that an mTOR inhibitor, rapamycin, leading to suppression of experimental chronic colitis through blunting leukocyte adhesion and extravasation in the gut mucosa [16]. Another study showed that treatment with everolimus (another mTOR inhibitor) ameliorating established murine colitis by affecting the number of T cells and the release of IFN- $\gamma$  in lamina propria [17]. Taken together, these studies suggest that mTOR inhibitors could be potential strategy for treatment of UC.



**Figure 1.** Torin 1 treatment attenuated body weight, DAI scores, and mortality in DSS-treated mice. A. Basal body weight changes of each group (n = 6 per group) after DSS induction of colitis (high dose: 20 mg/kg, low dose: 10 mg/kg). B. Disease progression of each group (n = 6 per group) after DSS induction of colitis. C. Survival rate was monitored until day 15 after the initiation of DSS (n = 10 per group). The data are representative of three independent experiments and presented as mean  $\pm$  S.D. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

In this study, we examined the role of Torin 1, a new mTOR inhibitor, in DSS-induced murine colitis. We found Torin 1 treatment attenuated DSS-induced body weight loss and reduced the mortality. Moreover, Torin 1 treatment prevented DSS-induced colonic pathological damage, meanwhile inhibited pro-inflammatory cytokines production in colon tissues. *In vitro*, Torin 1 significantly suppressed the cytokines expression in mouse macrophage cell line RAW264.7 cells and inhibited the activation of mTOR and NF- $\kappa$ B signaling pathway. Based on these data, we suggest that Torin 1 could ameliorate DSS-induced colitis in mice.

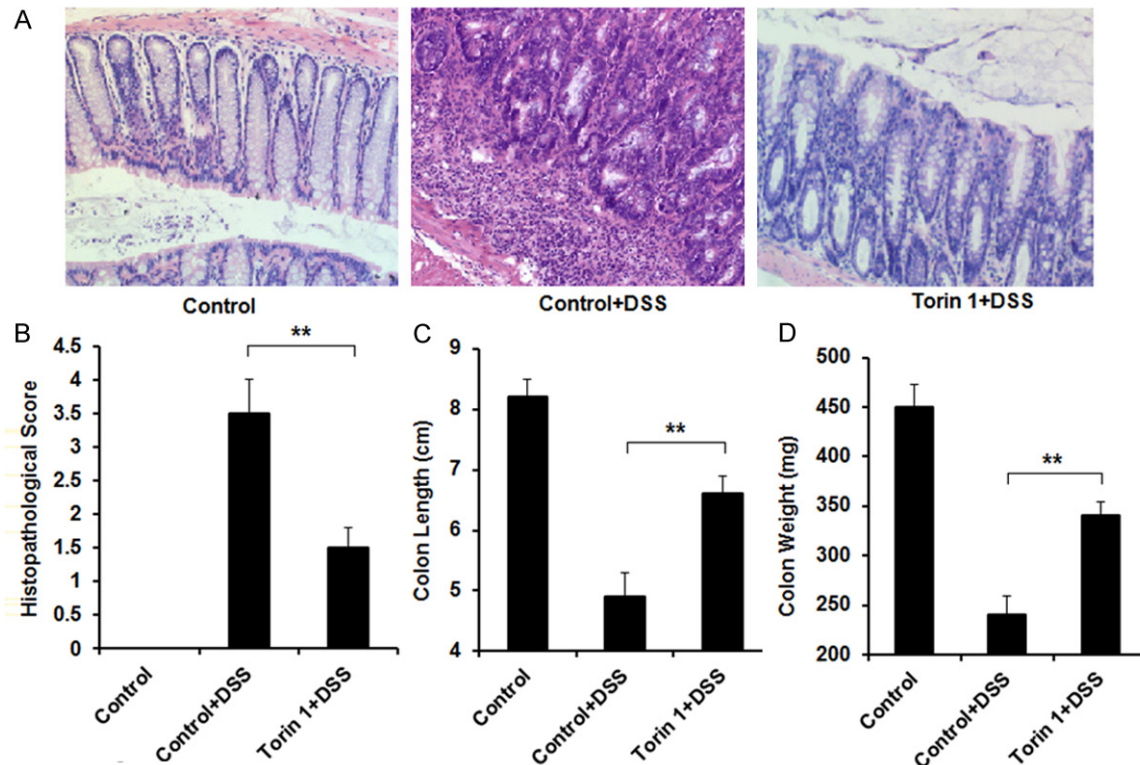
## Material and methods

### Mice and cells

Wild-type (WT) C57BL/6 (B6) mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All mice used were male, weighing 18-22 g, 8-12 weeks old and were kept at room temperature with a light-dark cycle of 12 h each day. The experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals approved by the Animal Ethics Committee of the Scientific Investigation Board of Weifang People's Hospital. Mouse macrophage cell line RAW-264.7 was obtained from American Type Culture Collection (Manassas, VA). The cells were cultured at 37°C under 5% CO<sub>2</sub> in DMEM supplemented with 10% FBS (Invitrogen-Life Technologies), 100 U/ml penicillin, and 100 mg/ml streptomycin.

### DSS-induced colitis and design of Torin 1 treatment

Acute colitis was induced by administration of DSS in drinking water. The mice were received either drinking regular water (control) or DSS (4% wt/vol, 5000 kDa; Wako Pure Chemical Industries, Osaka, Japan) drinking water (model) for 6 days, subsequently, mice were sacrificed at day 6 to separate the colons for tissue experiments or switched to regular drinking water until 15 days for survival data monitoring. The animals were given free access to water containing DSS for 6 days. The mice were randomly assigned to control, Torin 1 (20 mg/kg)-treated, DSS-treated, Torin 1 (10 mg/kg) +DSS-treated and Torin 1 (20 mg/kg) +DSS-treated groups. *In vivo*, Torin-1 (Selleckchem, Houston, TX) as a suspension in 20% N-methyl-2-pyrrolidone/40% PEG400/40% water [18], or



**Figure 2.** Torin 1 treatment reduced inflammation and colon damage in DSS-treated mice. A. The mice were sacrificed at day 6 after DSS treated, and colons were excised. Serial sections of colon tissues were stained with hematoxylin and eosin (H&E) (original magnification  $\times 100$ ). B. Histopathological score. C. Colon length. D. Colon weight. Dose of Torin 1: 20 mg/kg. The data are representative of three independent experiments ( $n = 6$  per group) and presented as mean  $\pm$  S.D.  $**P < 0.01$ .

vehicle was delivered intragastrically once per day for 6 days from the first day, respectively. *In vitro*, Torin1 was stored as a solid in the dark and dissolved at  $50^{\circ}\text{C}$  in DMSO (Sigma-Aldrich) immediately before use, and RAW264.7 cells were treated with or without LPS (100 ng/ml), together with Torin1 (10 ng/ml or 5 ng/ml) or equivalent DMSO vehicle control.

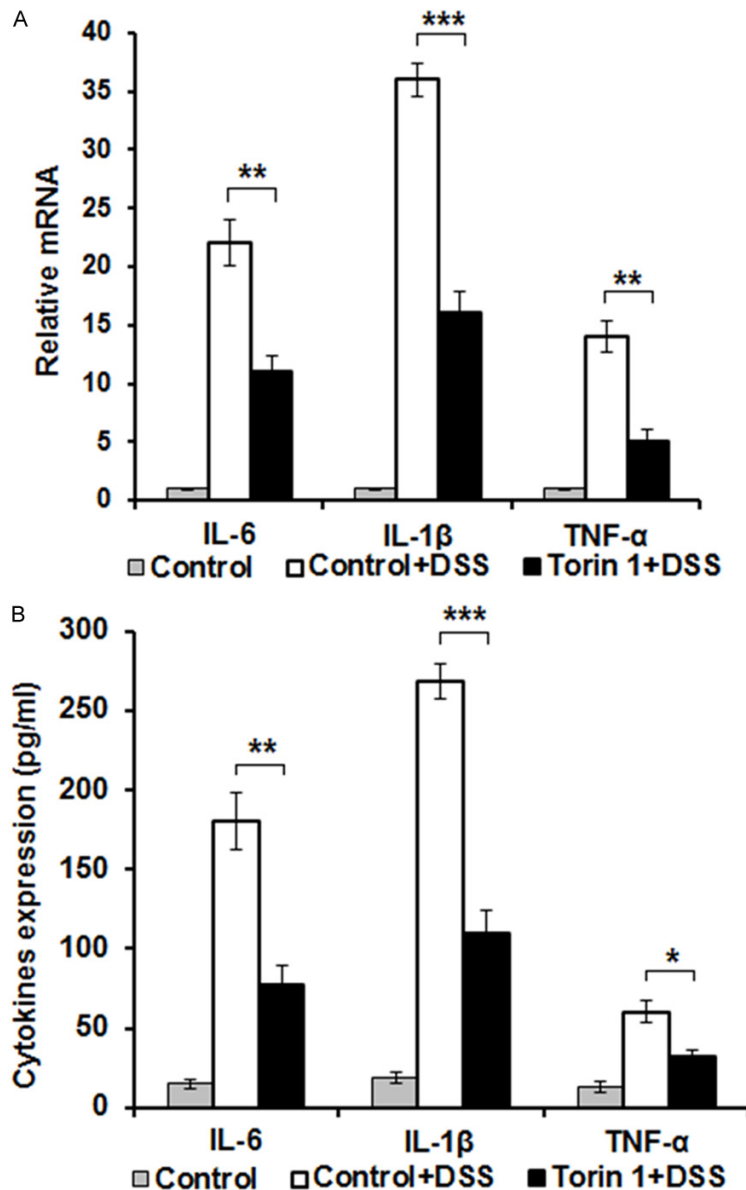
#### Evaluation of DSS-induced colitis

Mice were examined daily to determine their clinical Disease Activity Index (DAI), which was based on the degree of body weight loss, stool consistency, and fecal blood (ranging from 0 to 12). Briefly, DAI was scored as follows: weight loss (no change = 0;  $< 5\%$  = 1; 6-10% = 2; 11-20% = 3;  $> 20\%$  = 4), stool (normal = 0; soft, well-formed = 1; soft without pellets = 2; diarrhea = 4), and blood (no blood = 0; visible blood in rectum = 1; gross bleeding in rectum = 2; visible blood on fur = 4). For histological analysis, the distal colonic specimens were fixed in

10% buffered formalin and embedded in paraffin. Sections were stained with H&E, and pathological scores, ranging from 0 to 6 (combining inflammatory cell infiltration score and tissue damage score), were determined as follows: inflammatory cell infiltration in the lamina propria (occasional inflammatory cells = 0; increased inflammatory cells = 1; confluence of inflammatory cells extending to the submucosa = 2; transmural extension = 3) and tissue damage (no mucosal damage = 0; lymphoepithelial lesions = 1; surface mucosal erosion = 2; extensive mucosal damage and extension into deeper structures of the bowel wall = 3).

#### RNA analysis

Tissues and RAW264.7 cells were collected in cell lysis buffer. Total RNA was extracted with TRIzol reagent according to the manufacturer's instructions (Invitrogen). A LightCycler (ABI PRISM 7000; Applied Biosciences) and a SYBR RT-PCR kit (Takara Biotechnology) were used



**Figure 3.** Torin 1 treatment reduced pro-inflammatory cytokines expression in DSS-treated colon. A. The mice were sacrificed at day 6 after DSS treated. The mRNA level of IL-6, IL-1 $\beta$  and TNF- $\alpha$  in distal part of colons were analyzed by qPCR. B. The protein level of IL-6, IL-1 $\beta$  and TNF- $\alpha$  in distal part of colons were analyzed by ELISA. Dose of Torin 1: 20 mg/kg. The data are representative of three independent experiments and presented as mean  $\pm$  S.D. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.

for qPCR analysis. qPCR were performed using the following primer sequences: IL-6, Forward 5'-ACAACACGGCCTTCCCTAC-3', Reverse 5'-CA-TTCCACGATTTCCAGAG-3'; IL-1 $\beta$ , Forward 5'-AC-CTTCCAGGATGAGGACATGA-3', Reverse 5'-AA-CGTACACACCAGCAGGTGA-3'; TNF- $\alpha$ , Forward 5'-GCCACCACGCTCTTCTGTCT-3', Reverse 5'-TG-AGGGTCTGGGCCATAGAAC-3'; GAPDH (internal control), Forward 5'-ACCACAGTCCATGCCATCA-C-3', Reverse 5'-TCCACCACCCTGTTGCTGTA-3'.

#### Western blot analysis

The protein in cell lysis was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by transference to a PVDF membrane (Millipore). The membrane was then blocked with 2.5% nonfat dry milk for 1 h. The antibodies specific for S6K, p-S6K, p65, phospho-p65, IKK $\beta$ , phospho-IKK $\beta$  (Cell Signaling Technology Inc, Beverly, USA) and  $\beta$ -Actin (Santa Cruz Biotechnology, Santa Cruz, USA) were added and incubated overnight at 4°C. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), the target protein was visualized by enhanced chemiluminescence (Thermo Fisher Scientific).

#### Statistical analysis

All data are presented as mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined with the two-tailed student's  $t$  test to compare two groups. One-way ANOVA was performed to compare three or more groups. If the ANOVA analysis was significant, the Newman-Keuls test was applied for comparison between each two groups. The survival curves were plotted according to the Kaplan-Meier method and compared using the

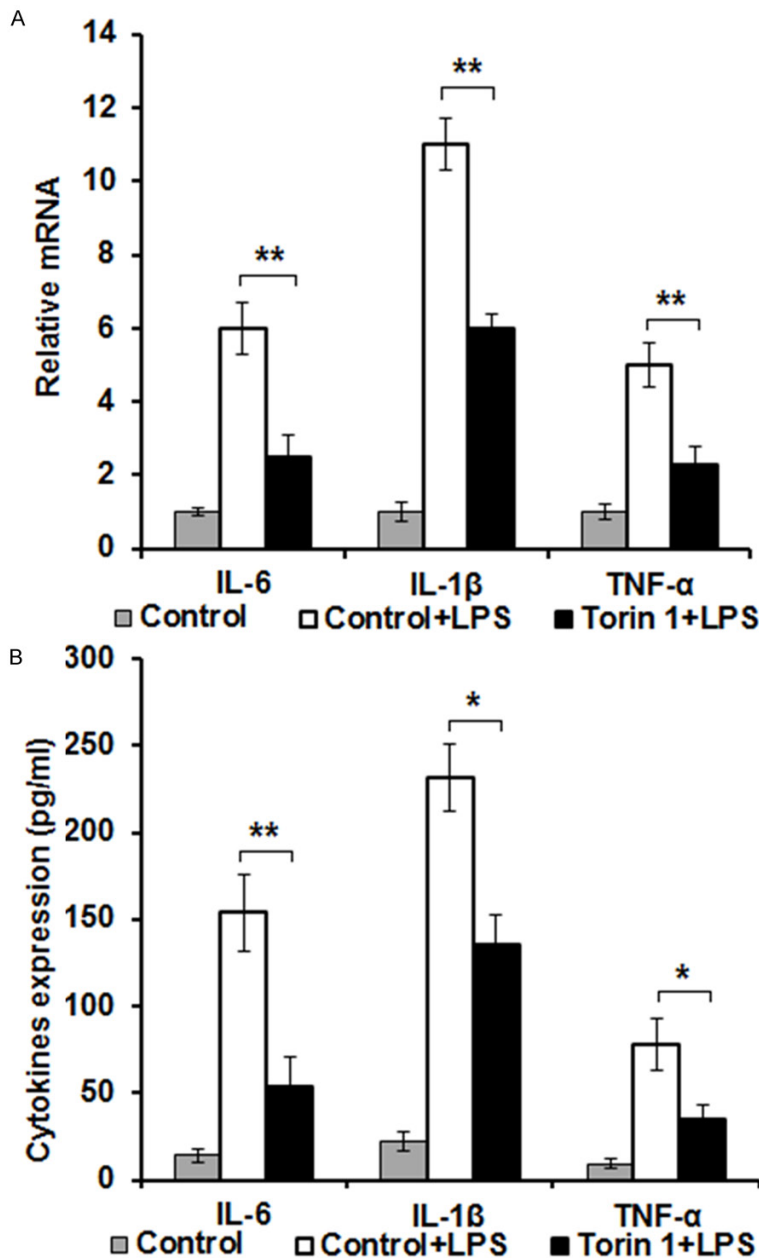
log-rank test. A  $p$  value of less than 0.05 considered statistically significant.

#### Results

##### *Torin 1 treatment attenuated body weight, DAI scores, and mortality in DSS-treated mice*

To determine the effect of Torin 1 in experimental ulcerative colitis, mice were administered 4% DSS for 6 days to induce acute colitis. The





**Figure 4.** Torin 1 treatment decreased pro-inflammatory cytokines production in LPS-stimulated RAW264.7 cells. A. RAW264.7 cells were stimulated with LPS (100 ng/ml) together with Torin 1 (10 ng/mL) for 4 hours. The mRNA level of IL-6, IL-1 $\beta$  and TNF- $\alpha$  were analyzed by qPCR. B. The protein level of IL-6, IL-1 $\beta$  and TNF- $\alpha$  were analyzed by qPCR. The data are representative of three independent experiments and presented as mean  $\pm$  S.D. \*P < 0.05, \*\*P < 0.01.

consumption of water and food was measured throughout the experiment, and there were no significant differences between the groups (data not shown). We observed that DSS-treated mice exhibited profound body weight loss, whereas Torin 1 administration significantly attenuated the loss of body weight in a dose dependent manner (Figure 1A). We next

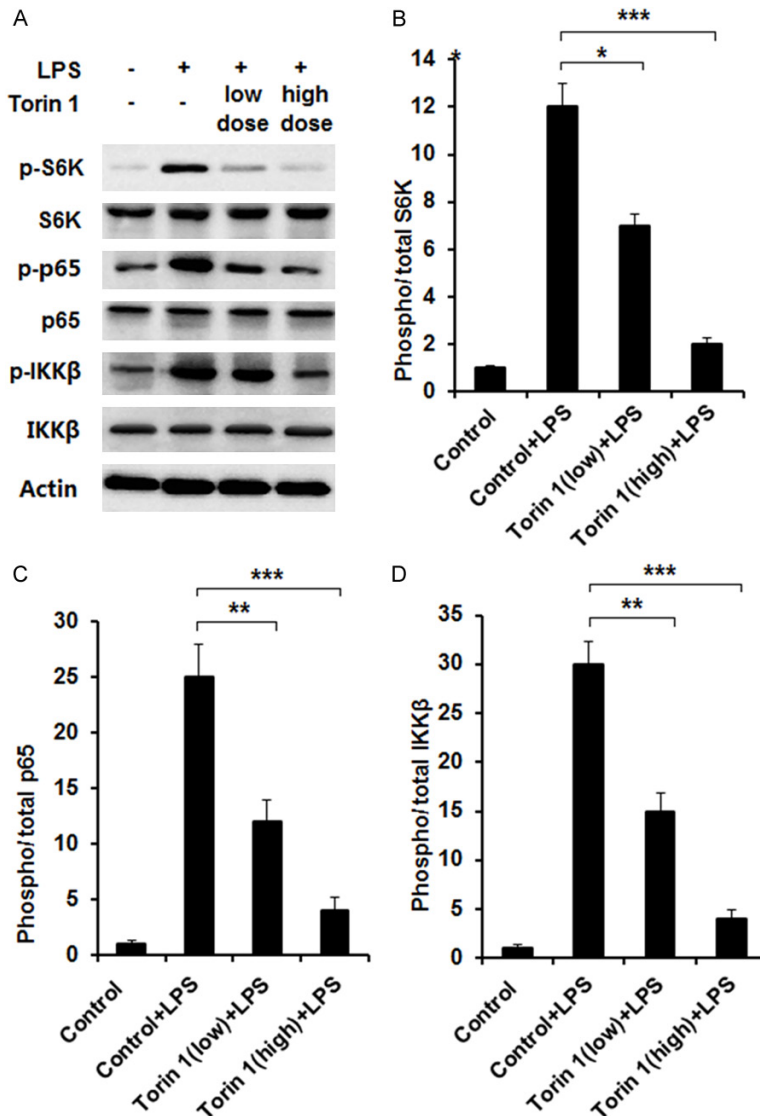
examined the clinical disease activity index (DAI), which reflected the clinical manifestation of the disease, and we found the treatment with Torin 1 significantly reduced the clinical scores of the DSS-induced colitis in mice (Figure 1B). Importantly, Torin 1 administration decreased the mortality of mice after DSS treated in a dose dependent manner (Figure 1C).

*Torin 1 treatment reduced inflammation and colon damage in DSS-treated mice*

We also performed the histological examination to validate the clinical data. As shown in Figure 2A, DSS treatment induced significant massive inflammatory infiltrates and disruption of mucosal structures, but the administration of Torin 1 markedly reduced the infiltration and the mucosal damage. Histological scoring showed that treatment with Torin 1 significantly reduced the overall score relative to that of the control colitis mice (Figure 2B). We also observed that after DSS-induced injury, the colon length of Torin 1 treated mice was longer than that of control mice, as well as colon weight (Figure 2C and 2D).

*Torin 1 treatment reduced pro-inflammatory cytokines expression in DSS-treated colon*

DSS-induced colitis is an inflammatory disease mediated by many pro-inflammatory cytokines [19]. To explore the effect of Torin 1 on the production of pro-inflammatory cytokines at the site of DSS-induced inflammation, after DSS challenge, expression levels of pro-inflammatory cytokines in colons of control or Torin 1-treated mice were detected. By using qPCR and ELISA, We found that pro-inflammatory cytokines, such as IL-6, IL-1 $\beta$  and TNF- $\alpha$



**Figure 5.** Torin 1 inhibited the activation of mTOR and NF- $\kappa$ B in macrophages. (A). Raw264.7 cells were stimulated with LPS (100 ng/ml) together with Torin 1 (high dose: 10 ng/mL, low dose: 5 ng/ml) for 4 hours. The expression of p-S6K, S6K, p-p65, p65, p-IKK $\beta$ , IKK $\beta$  and Actin were examined by western blot. (B-D). Quantification of protein level of p-S6K (B), p-p65 (C), p-IKK $\beta$  (D) in (A). The data are representative of three independent experiments and presented as mean  $\pm$  S.D. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

were markedly increased in control mice after DSS treatment. However, Torin 1 treated mice produced significantly less of these cytokines relative to control mice (Figure 3A and 3B).

*Torin 1 treatment decreased pro-inflammatory cytokines production in LPS-stimulated RAW264.7 cells*

During intestinal mucosa damage, LPS secreted by the intestinal flora will stimulate infiltrated of macrophages to activate the production

of inflammatory factors, leading to the colonic tissue damage [20, 21]. We examined the effect of Torin 1 on LPS stimulated pro-inflammatory cytokines production in mouse macrophage cell line RAW264.7, consistent with the data of cytokines expression in tissues, both the mRNA and protein level of pro-inflammatory cytokines such as IL-6, IL-1 $\beta$ , TNF- $\alpha$ , were decreased in Torin 1 treated group compared with control group after LPS stimulated (Figure 4A and 4B).

*Torin 1 inhibited the activation of mTOR and NF- $\kappa$ B in macrophages*

It was reported that LPS stimulation of macrophages leads to activation of mTOR, and mTOR plays a key role in DSS-induced murine colitis [13-17]. In light of previous reports describing Akt-mTOR-dependent regulation of the transcription factor NF- $\kappa$ B [22], we examined the effect of present mTOR inhibitor, Torin 1, on the inhibition of mTOR and NF- $\kappa$ B in macrophages. We found LPS stimulation of macrophages leads to the phosphorylation and activation of S6K (a downstream effector of mTOR), however Torin 1 significantly inhibited the phosphorylation of S6K in a dose manner (Figure 5A and 5B). Furthermore, we

observed that treatment of Torin 1 inhibited LPS-induced phosphorylation of NF- $\kappa$ B/p65, and Ikk $\beta$  (Figure 5A, 5C and 5D). These results indicated that Torin 1 inhibited the activation of mTOR and NF- $\kappa$ B in LPS-stimulated macrophages, leading to the amelioration of DSS-induced colitis in mice.

## Discussion

Ulcerative colitis (UC) is one of the two major forms of inflammatory bowel diseases (IBDs).

Its etiology and pathogenesis is complex. UC is generally thought to be caused by the interaction between environmental factors, heredity, immune system, infection and mentality [1, 2]. Currently, there are various evolving therapeutic options for UC. Immunosuppressive drug such as TNF- $\alpha$  antibody [23], azathioprine (AZA) and methotrexate (MTX) [24] have been adopted to control the symptoms. However, these immunosuppressants have their limitations in efficacy and safety [25, 26]. Therefore, novel strategies are urgently required.

Oral administration of dextran sulphate sodium (DSS) in mice induces colitis that resembles human UC and the resulting pathological conditions correspond very well to human [6]. Previous studies showed that compounds and inhibitors are sufficient to suppress the development of DSS-induced experimental colitis [27-29]. Similar results were obtained by us. We found Torin 1, a potent inhibitor of mTOR, could significantly attenuate the loss of body, reduce the clinical score of colon, and decrease the mortality induced by DSS in mice. Furthermore, we also found that treatment of Torin 1 markedly reduced DSS-induced infiltration of immune cells and the mucosal damage.

The infiltrated immune cells such as macrophages and neutrophils are major producers of inflammatory mediators, such as TNF- $\alpha$ , IL-6, IL-1 $\beta$  that contribute to pathogenesis [30]. In the present study, we found Torin 1 treatment significantly reduced the production of these pro-inflammatory cytokines in colon tissues, therefore attenuated the development of DSS-induced colitis in mice. During intestinal mucosa damage, LPS secreted by the intestinal flora will stimulate infiltrated macrophages to activate the production of inflammatory factors [20, 21]. Therefore, we examined the effect of Torin 1 on LPS-induced cytokines expression in macrophages cell line RAW264.7, and we observed both the mRNA and protein level of these cytokines are all decreased after Torin 1 treated. These findings suggested that Torin 1 could inhibit the cytokines expression in infiltrated macrophages, therefore inhibit the progression of DSS-induced colitis.

Hu *et al.* reported that inhibition of mTOR by AZD8055 attenuates DSS-induced colitis through suppressing T Cell proliferation and balancing TH1/TH17/Treg profile [31]. Previous

reports showed that the well-known mTOR inhibitor, Rapamycin could decrease leukocyte migration in vivo and effectively reduce DSS-induced chronic colitis [16]. These findings suggested that mTOR signaling plays crucial roles in DSS-induced experimental colitis. Similar with their results, our findings showed that another potent mTOR inhibitor, Torin 1, mainly through inhibition of mTOR and NF- $\kappa$ B signal pathways to attenuate the development of colitis and the expression of pro-inflammatory cytokines. These finding indicated that mTOR inhibitors could ameliorate DSS-induced colitis through different mechanisms and multiple signal pathways, and suggested mTOR inhibitors such as Torin 1 as potential effective drugs for inflammatory bowel diseases.

In conclusion, our current study provided the evidence that Torin 1 decreased the production of pro-inflammatory cytokines through inhibition of mTOR and NF- $\kappa$ B signal pathways, therefore ameliorates DSS-induced murine colitis. This study enriched the function of mTOR inhibitors in the treatment of ulcerative colitis, and suggested Torin 1 as potential drugs for clinical treatment of inflammatory bowel diseases.

## Disclosure of conflict of interest

None.

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