

Deubiquitinase USP14 is upregulated in Crohn's disease and inhibits the NOD2 pathway mediated inflammatory response *in vitro*

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ABSTRACT

The nucleotide binding oligomerization domain containing 2 (NOD2) protein and its ligand *N*-acetyl muramyl dipeptide (MDP) are crucially involved in Crohn's disease (CD). However, the mechanism by which NOD2 signaling is regulated in CD patients remains unclear. Ubiquitin specific protease (USP14) is a deubiquitylase that plays an important role in immunity. This study aimed to investigate the mechanism by which USP14 regulates NOD2 induced inflammatory response in CD and inflammatory bowel diseases (IBD). Our results showed that USP14 protein and mRNA levels in intestinal tissues of CD patients were significantly higher than those in healthy controls. In addition, USP14 was upregulated in IBD mouse model. While treatment with MDP, TNF- α or the Toll-like receptor 1/2 agonist Pam3CSK4 all led to significantly higher mRNA levels of TNF- α , IL-8 and IL-1 β in THP-1 cells, pretreatment with USP14 inhibitor IU1 could stimulate further upregulation of TNF- α , IL-8 and IL-1 β . In particular, MDP promoted the activation of JNK, ERK1/2 and p38 as well as NF- κ B in THP-1 cells, and IU1 significantly enhanced the MDP-induced activation of these proteins without effects on USP14 protein level. Furthermore, the JNK inhibitor sp600125, ERK1/2 inhibitor U0126 or P38 MAPK inhibitor PD169316 significantly decreased the mRNA levels of TNF- α , IL-8 and IL-1 β in THP-1 cells stimulated by both IU1 and MDP. In conclusion, our findings suggest that USP14 could inhibit MDP-induced activation of MAPK signaling and the inflammation response involved in IBD, and that USP14 is a potential therapeutic target for IBD.

Key words: inflammatory bowel disease; Crohn's disease; USP14; MAPK; NF- κ B.

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Introduction

Crohn's disease (CD) is a common type of inflammatory bowel diseases (IBD), which include a variety of chronic inflammatory disorders in the gastrointestinal tract.¹ The pathogenesis of IBD remain elusive, although recent evidence suggests that host immune response, gut microbiome and genetic and environmental factors all contribute to the development of IBD.^{2,3}

The nucleotide binding oligomerization domain containing 2 (NOD2) protein is a pattern recognition receptor that is broadly expressed intracellularly in macrophages, dendritic cells, and other cells.⁴ Upon binding to the ligands such as *N*-acetyl muramyl dipeptide (MDP), a component of bacterial peptidoglycan, NOD2 is activated and binds to receptor-interacting serine-threonine kinase 2 (RIP2), leading to TRAF6-mediated ubiquitination of NF- κ B essential modulator (NEMO) and the activation of NF- κ B pathway.⁵ Therefore, NOD2 plays an important role in inflammatory response involved in CD. Ubiquitin specific protease (USP14) is a deubiquitylase and is a member of the ubiquitin specific protease family. USP14 plays an important role in immunity. Knockdown of USP14 augmented the degradation of TNF- α in LPS-stimulated macrophage cells.⁶ USP14 inhibited RNA virus induced type I IFN signaling pathway by promoting K63-linked RIG-I deubiquitination.⁷ NF- κ B, as a key transcription factor to induce inflammation response, is an important downstream target of RIG-I activation.⁸⁻¹⁰ Therefore, we postulated that USP14 regulates NOD2 induced activation of inflammation in IBD. This study aimed to investigate the mechanism by which USP14 regulates NOD2 induced inflammatory response in CD. First, we detected USP14 expression in CD patients and mouse model of IBD by immunohistochemistry and fluorescence *in situ* hybridization analysis. Next, we treated human monocytic cell line THP-1 with NOD2 ligand MDP to establish cell inflammation model to investigate the effects of USP14 inhibitor on the production of inflammatory factors as well as the activation of NF- κ B signaling and MAPK signaling.

Materials and Methods

Clinical samples

This study was approved by Ethics Committee of Changsha Central Hospital (Approval No. 2023036) and all patients provided informed consent. Colonic mucosal tissues were obtained from surgical specimens from 12 patients with CD (6 males and 6 females, age 20 -60 years old) and 12 healthy controls (6 males and 6 females, age 20 -60 years old).

Animals

Animal experiments were approved by Animal Care and Use Committee of Changsha Central Hospital (approval no. 20200512). Acute colitis was induced by giving 4% Dextran sulfate sodium (DSS, MP Biomedicals, Santa Ana, CA, USA) orally in drinking water, tissues were collected on day 7 of the DSS treatment regimen. The disease activity index (DAI) score was recorded by assessing the body weight, stool and hematochezia of the mice on a daily basis. Histological disease scores were evaluated based on the presence of crypt loss and inflammatory cell infiltration, as described previously.¹⁰ The mice were divided into three groups: group A received saline instead of DSS as control, group B received DSS to induce IBD, and group C received DSS

plus USP14 inhibitor IU1 (MCE, Monmouth Junction, NJ, USA). Samples were fixed in 10% neutral buffer tissue fixative solution and made into histopathological sections.

Immunohistochemical staining

Paraffin-embedded colonic or ileal sections of patients and mice were cut at a 5 μ m thickness. The sections were processed as described previously.¹⁰ Briefly, the sections were boiled in 10 mM sodium citrate buffer for antigen retrieval. After blocking endogenous peroxidase with 3% hydrogen peroxidase, sections were blocked for 30 min with 3% bovine serum albumin, and then incubated with primary antibodies for USP14 or TNF- α (Abcam, Cambridge, UK) (1:200 dilution) overnight at 4°C. Phosphate buffered saline was used instead of primary antibodies as negative control. After incubation with horseradish peroxidase labeled secondary antibodies (Abcam) (1:500 dilution) at room temperature for 1 h, the sections were visualized with DAB, and counterstained with hematoxylin. Three sections were selected for each sample and 5 random fields of view per section were evaluated under optical microscope (XSP-C204, CIC, 40x magnification). Immunohistochemical staining was assessed using ImageJ software (ImageJ, Bethesda, MD, USA).

Fluorescence *in situ* hybridization (FISH)

The sections were analyzed by FISH as previously described.¹¹ Briefly, the sections were incubated with USP14 probe 5'-CCATTGGAGGTTTCATCTGTATTCAATTCT-3' (500 nM in hybridization buffer) at 40°C overnight. After washing, the sections were incubated with Imaging oligo (DIG) at 42°C for 3 h. Next, the sections were washed and incubated with anti-DIG-horseradish peroxidase (Jackson Immuno Research Laboratory Inc., West Grove, PA, USA) for 10 min at room temperature. The sections were then washed and incubated with FITC-Tyramide for 5 min in the dark. Finally, the sections were stained with DAPI (0.1 μ g/mL) at room temperature for 10 min and observed under fluorescence microscope (Olympus, Tokyo, Japan; 40x magnification).

Cell culture and treatment

Human monocytic cell line THP-1 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/ml streptomycin at 37°C. Cells were treated with 10 μ g/mL MDP for 24 h to induce inflammation.¹² Control cells were treated with 0.1% (v/v) solvent dimethyl sulfoxide (DMSO) as vehicle control.

Western blot analysis

Cells were lysed in lysis buffer containing protease and phosphatase inhibitors on ice, and supernatants were collected after centrifugation at 12,000 rpm for 5 min at 4°C. Next, protein samples were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane. The membrane was blocked with 3% skim milk solution in phosphate buffered saline containing 0.1% (v/v) Tween-20 (PBST) for 1 h at room temperature, and incubated with primary antibodies for USP14, Phospho-p44/42 MAPK (ERK1/2), Phospho-SAPK/JNK, Phospho-NF- κ B p65, Phospho-P38 MAPK and GAPDH (all from Cell Signaling, Danvers, MA, USA; 1:500 dilution) overnight at 4°C. The membrane was washed and then incubated with horseradish peroxidase-conjugated secondary antibodies (Beyotime Biotechnology, Haimen, China; 1:2000 dilution) at room temperature for 1 h, and the bands were detected by enhanced chemiluminescence and analyzed with Image Pro software.

RT-PCR

Total RNA was extracted from cells using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized for real-time PCR using RevertAid 1st cDNA Synth kit (Thermo Fisher Scientific). PCR primers were synthesized by Sangon (Shanghai, China) with the following sequences: TNF- α forward AAGCCTGTAGCCCA TGTTGT and reverse AGTCG-GTCACCCTTCTCCA; IL-8 forward 5'-TTGGCAGCCTTCCT-GATTT-3' and reverse 5'-TCAAAAACCTTCTCCACAACCC-3'; IL-1 β forward TGAAGTAAAGCTCTCCACCT and reverse ACTGGGCAGACTCAAATTCCA. The amplification parameters were as follows: 95°C for 10 min, 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as internal control.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0, and all values were presented as means \pm SD. Student's *t*-test were used to compare differences between groups, and $p < 0.05$ was considered significant.

Results

Upregulation of USP14 in intestinal tissues of CD patients

To determine whether USP14 is implicated in CD, we per-

formed immunohistochemistry staining for USP14. USP14 was mainly localized to the cytoplasm. Optical density analysis showed that the staining intensity of USP14 in intestinal tissues of CD patients was significantly higher compared with those of healthy controls (Figure 1 A-C).

To confirm the upregulation of USP14 in intestinal tissues of CD patients, we performed FISH to detect USP14 mRNA in the cytoplasm. We found that USP14 mRNA was very weakly stained in the cytoplasm of intestinal tissues of healthy controls but was strongly stained in the cytoplasm of intestinal tissues of CD patients (Figure 2 A,B). Quantitative analysis of staining signal showed that both percentage of positively stained cells and the density of positively stained cells were significantly higher in intestinal tissues of CD patients compared with those of healthy controls (Figure 2 C,D). Collectively, these data indicated that USP14 was upregulated in intestinal tissues of CD patients.

Upregulation of USP14 in intestinal tissues of IBD mice

Next, we established DSS induced IBD mouse model to evaluate the expression of USP14 during IBD. DSS treated mice had significantly higher disease activity index score (DAI) and higher body weight loss rate than control mice (Figure 3 A,B). Histological analysis revealed that the intestinal tissue of control mice was normal, but DSS treated mice developed serious colitis (Figure 3 C,D). Moreover, USP14 inhibitor IU1 worsened the signs of colitis (Figure 3E), accompanied by increased DAI score, body weight loss and histological score (Figure 3 B,C). Subsequently, we performed immunohistochemistry staining for USP14 and TNF- α in three groups of mice. The expression of

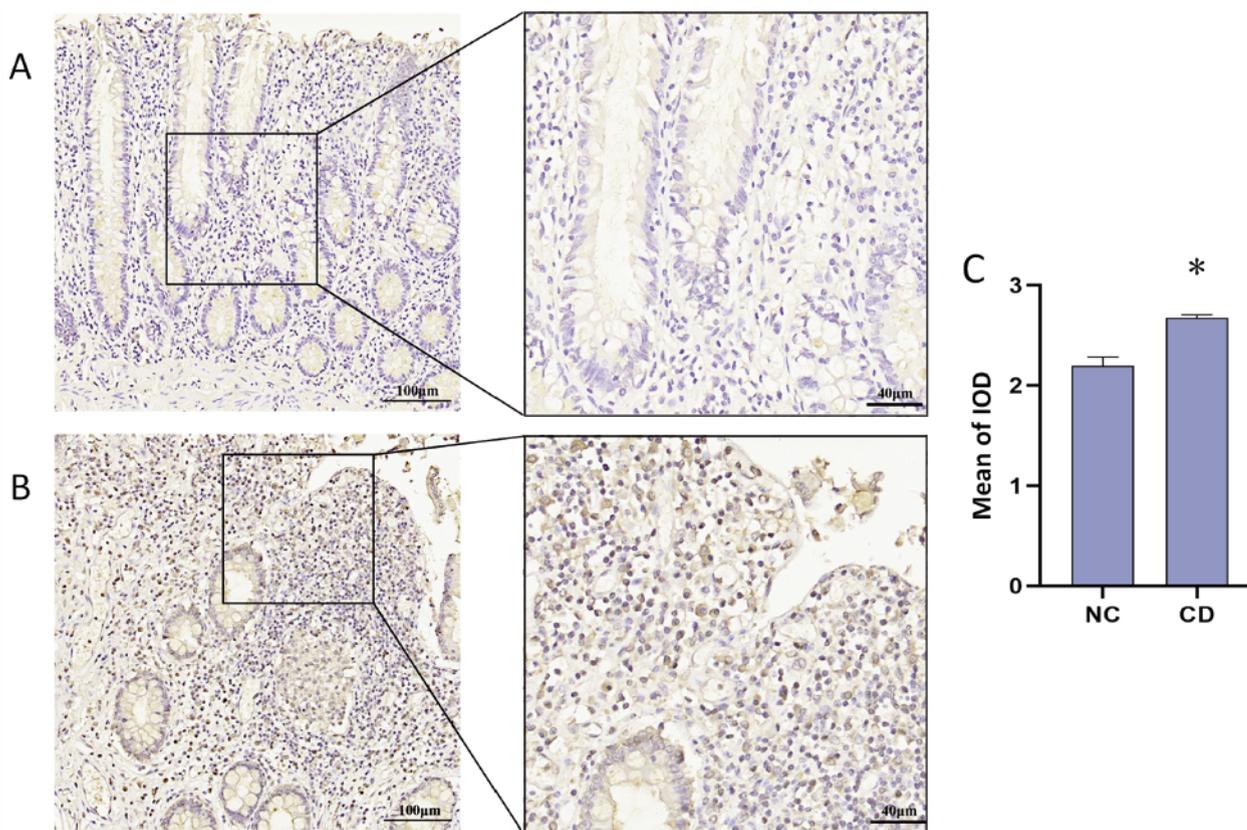


Figure 1. Immunohistochemistry analysis of USP14 in the mucosa of CD patients and healthy controls. **A)** Representative staining of USP14 in intestinal sections from 12 healthy controls. **B)** Representative staining of USP14 in intestinal sections from 12 CD patients. **C)** Staining intensity was quantified by image J software and illustrated as IOD charts (n=5 fields in each section). * $p < 0.05$ compared to healthy control (NC).

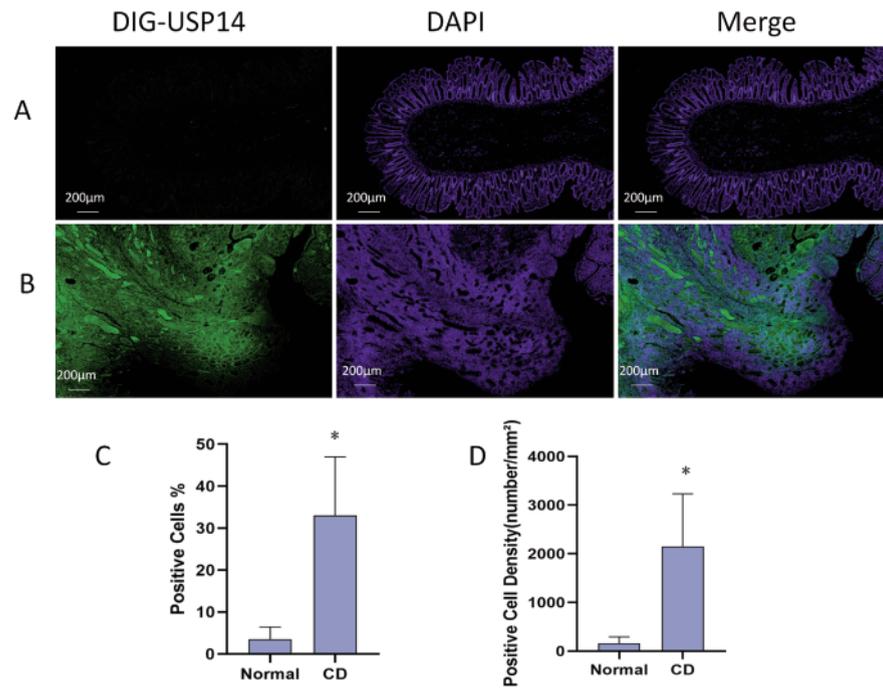


Figure 2. FISH analysis of USP14 in the mucosa of CD patients and healthy controls. **A)** Representative FISH of USP14 mRNA in intestinal sections from healthy controls. **B)** Representative FISH of USP14 mRNA in intestinal sections from CD patients; DIG-USP14 indicated positive cells stained with green with DIG-USP14 probe; DAPI indicated all cells stained with DAPI in the nuclei. **C)** Percentage of positive stained cells. **D)** Density of positive stained cells. * $p < 0.05$ compared to healthy control (normal).

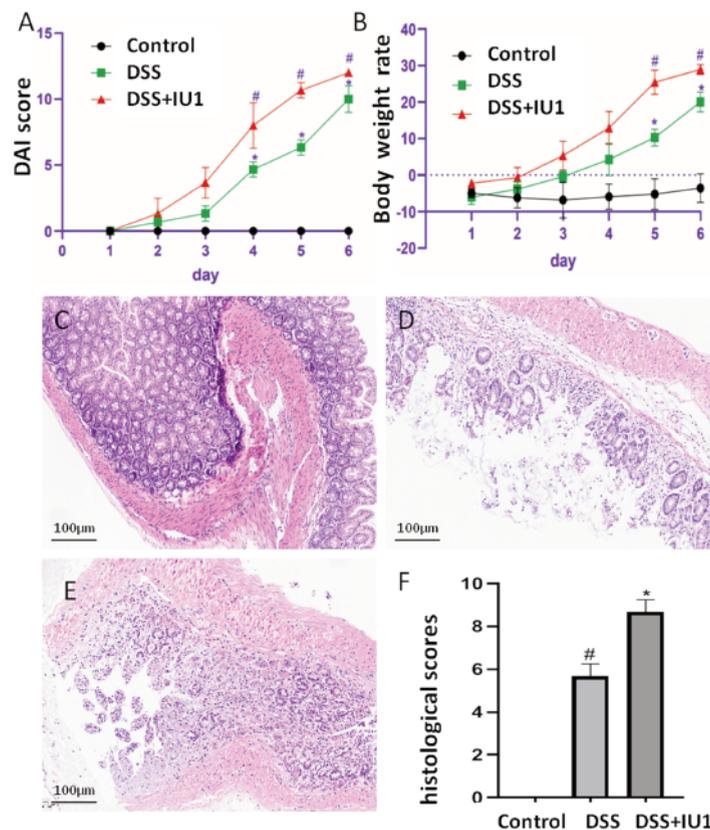


Figure 3. USP14 inhibitor alleviated DSS-induced colitis in mice. **A)** Disease activity index (DAI) score. **B)** Body weight loss rate. **C)** Hematoxylin and Eosin (H&E) staining of colon sections in control group. **D)** HE staining of colon sections in DSS group. **E)** H&E staining of colon sections in DSS+IU1 group. **F)** Histological scores of colon sections. Values were expressed as means \pm SD (n=6). * $p < 0.05$ vs DSS group; # $p < 0.05$ vs control group.

USP14 in DSS model group was significantly higher than that of vehicle control group, but the expression of USP14 in DSS+IU1 group was significantly lower than that of DSS model (Figure 4 A-D). These data indicated that USP14 was upregulated in mouse colitis model. Furthermore, the expression of TNF- α was significantly higher in DSS model group than in vehicle control group, and was even higher in DSS+IU1 group than in DSS group (Figure 5 A-D). These data indicated that the inhibition of USP14 promoted inflammation in mouse colitis model.

Inhibition of USP14 promoted inflammation in THP-1 cells

To confirm that the inhibition of USP14 promotes inflammation, we used THP-1 cell line as an *in vitro* model. MDP is known as the ligand of NOD2 to stimulate inflammation. Thus, we treated THP-1 cells with MDP to induce inflammation. The results showed that MDP treatment led to significantly higher mRNA levels of TNF- α , IL-8 and IL-1 β in THP-1 cells. After cells were pre-treated with USP14 inhibitor IU1, MDP could stimulate further

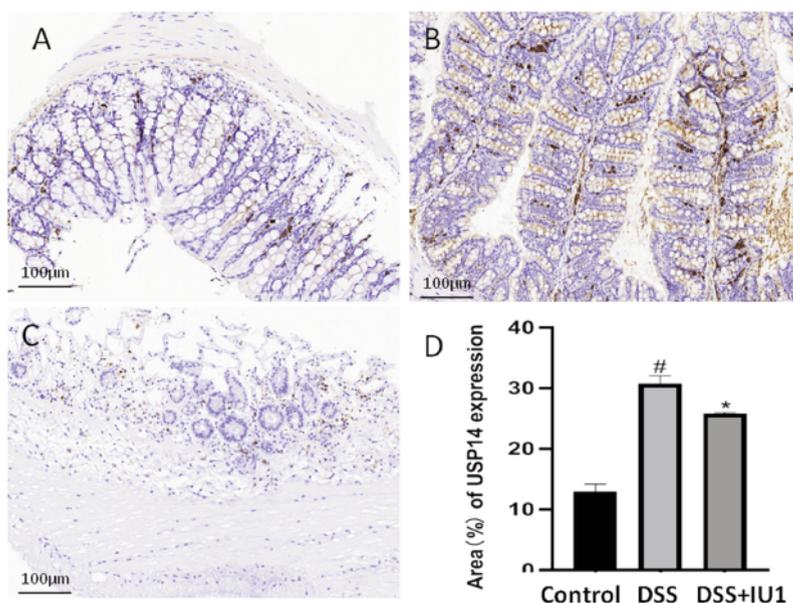


Figure 4. USP14 was upregulated in DSS-induced colitis. **A)** Immunohistochemical staining of USP14 in colon sections in control group. **B)** Immunohistochemical staining of USP14 in colon sections in DSS group. **C)** Immunohistochemical staining of USP14 in colon sections in DSS+IU1 group. **D)** Quantitation of immunohistochemical staining of USP14. Values were expressed as means \pm SD (n=6). * p <0.05 vs DSS group; # p <0.05 vs control group.

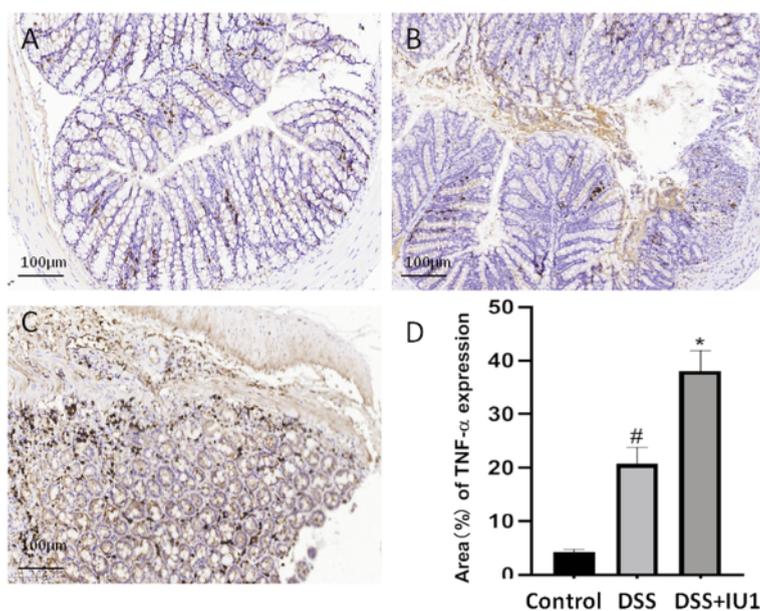


Figure 5. Expression of TNF- α in DSS-induced colitis. **A)** Immunohistochemical staining of TNF- α in colon sections in control group. **B)** Immunohistochemical staining of TNF- α in colon sections in DSS group. **C)** Immunohistochemical staining of TNF- α in colon sections in DSS+IU1 group. **D)** Quantitation of immunohistochemical staining of TNF- α . Values were expressed as means \pm SD (n=6). * p <0.05 vs DSS group; # p <0.05 vs control group.

upregulation of TNF- α , IL-8 and IL-1 β . However, without MDP stimulation IU1 could not upregulate TNF- α , IL-8 and IL-1 β (Figure 6A). Similarly, TNF- α treatment led to significantly higher mRNA levels of TNF- α , IL-8 and IL-1 β in THP-1 cells. After cells were pretreated with USP14 inhibitor IU1, TNF- α could stimulate further upregulation of TNF- α , IL-8 and IL-1 β . However, without TNF- α stimulation IU1 could not upregulate TNF- α , IL-8 and IL-1 β (Figure 6B). Toll-like receptor 1/2 (TLR1/2) agonist Pam3CSK4 treatment led to significantly higher mRNA levels of TNF- α , IL-8 and IL-1 β in THP-1 cells. After cells were pretreated with USP14 inhibitor IU1, Pam3CSK4 could stimulate further upregulation of TNF- α , IL-8 and IL-1 β . However, without Pam3CSK4 stimulation IU1 could not upregulate TNF- α , IL-8 and IL-1 β (Figure 6C). Taken together, these data suggested that USP14 could inhibit inflammation induced by various stimuli.

MAPK signaling contributed to the MDP-induced inflammation in THP-1 cells

Next, we focused on MDP-induced inflammation, and detected MAPK signaling pathways in THP-1 cells treated by MDP. MDP promoted the phosphorylation (activation) of JNK, ERK1/2 and p38 and the activation of NF- κ B in a dose dependent manner but had no effects on USP14 protein level (Figure 7A). In addition, MDP promoted the phosphorylation (activation) of JNK, ERK1/2 and p38 and the activation of NF- κ B in a time dependent manner but had no effects on USP14 protein level (Figure 7B). These results suggested that MDP promotes the activation of MAPK signaling.

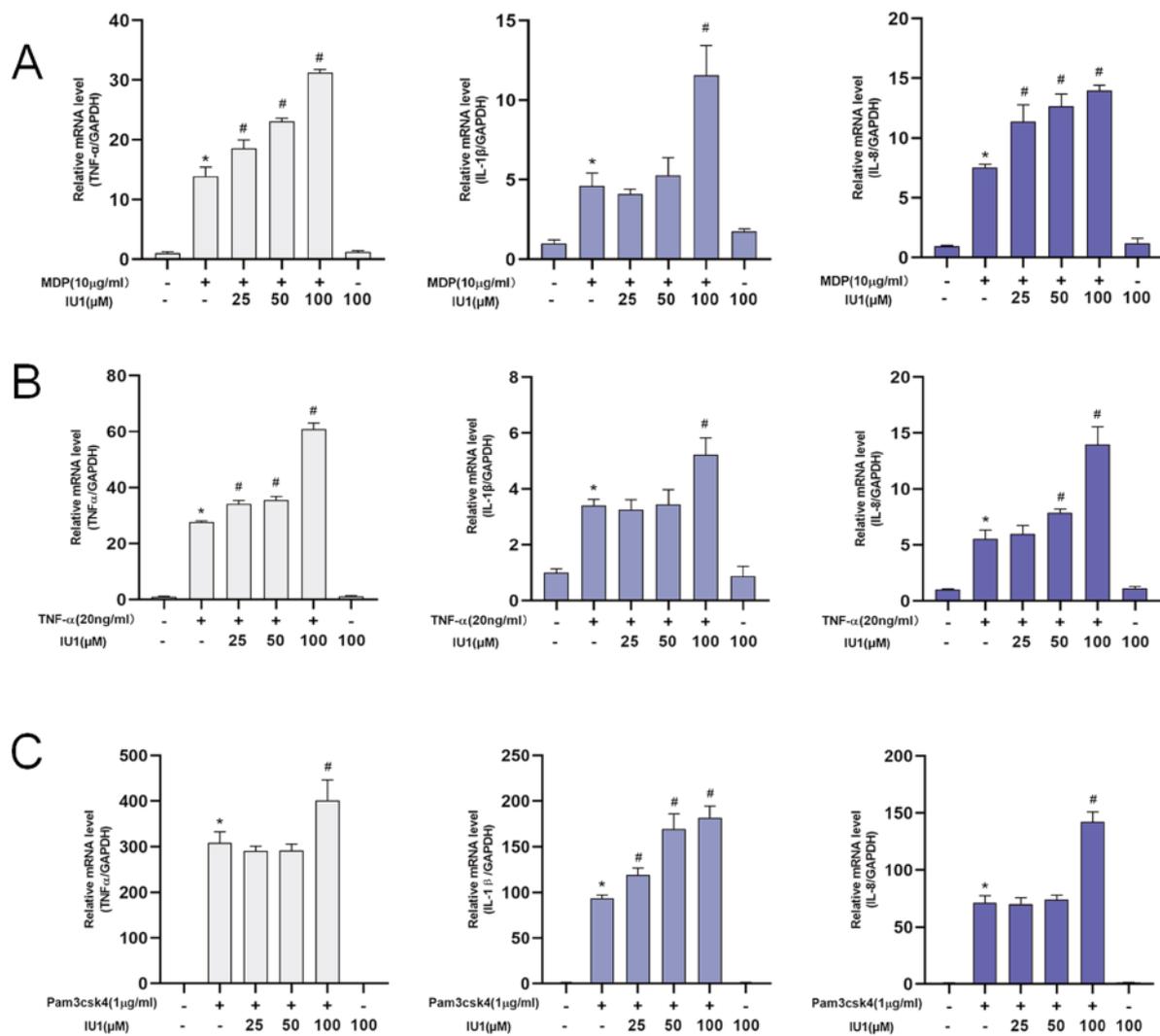


Figure 6. Inhibition of USP14 promoted inflammation in THP-1 cells. **A)** RT-PCR analysis of TNF- α , IL-1 β and IL8 mRNA levels in THP-1 cells treated with MDP or/and USP14 inhibitor IU1. **B)** RT-PCR analysis of TNF- α , IL-1 β and IL8 mRNA levels in THP-1 cells treated with TNF- α or/and USP14 inhibitor IU1. **C)** RT-PCR analysis of TNF- α , IL-1 β and IL8 mRNA levels in THP-1 cells treated with Pam3CSK4 or/and USP14 inhibitor IU1. * p <0.05 vs untreated cells; # p <0.05 vs cells treated with MDP, TNF- α or Pam3csk4 alone. Three independent experiments were performed and data represented mean \pm SD.

Inhibition of USP14 enhanced the MDP-induced activation of MAPK signaling in THP-1 cells

To understand how inhibition of USP14 promoted inflammation in THP-1 cells, we detected the effects of USP14 inhibitor IU1 on the activation of MAPK signaling. The results showed that IU1 significantly enhanced MDP-induced phosphorylation (activation) of JNK, ERK1/2 and p38 and the activation of NF- κ B compared to cells treated with MDP only but had no effects on USP14 protein level (Figure 8A).

Next, we employed JNK inhibitor sp600125, ERK1/2 inhibitor U0126 or P38 MAPK inhibitor PD169316 to pretreat THP-1 cells and then treated them with MDP or/and USP inhibitor IU1. We found that sp600125, U0126 and PD169316 significantly decreased mRNA levels of TNF- α , IL-8 and IL-1 β in THP-1 cells stimulated by both IU1 and MDP (Figure 8B). Collectively, these data suggested that USP14 could inhibit MDP-induced activation of MAPK signaling and inflammation response.

Discussion

In this study, we demonstrate that USP14 was upregulated in intestinal tissues of CD patients at both protein and mRNA levels based on immunohistochemistry and FISH analysis. Furthermore, we used NOD2 ligand MDP to stimulate inflammation in THP-1 cells and demonstrated that USP14 inhibitor IU1 could enhance MDP stimulated inflammation in THP-1 cells.

Inflammatory factors play important role in mediating manifestations of IBD.^{13,14} To reveal the mechanism by which USP14 inhibits MDP stimulated inflammation, we focused on MAPK and NF- κ B signaling pathways because recent studies suggest that USP14 may regulate MAPK and NF- κ B pathways to promote cancer and inflammation in preeclampsia.¹⁵ Our results showed that USP14 inhibitor IU1 significantly enhanced MDP-induced activation of all three branches of MAPK pathways including JNK, ERK1/2 and p38. IU1 also enhanced MDP-induced activation of

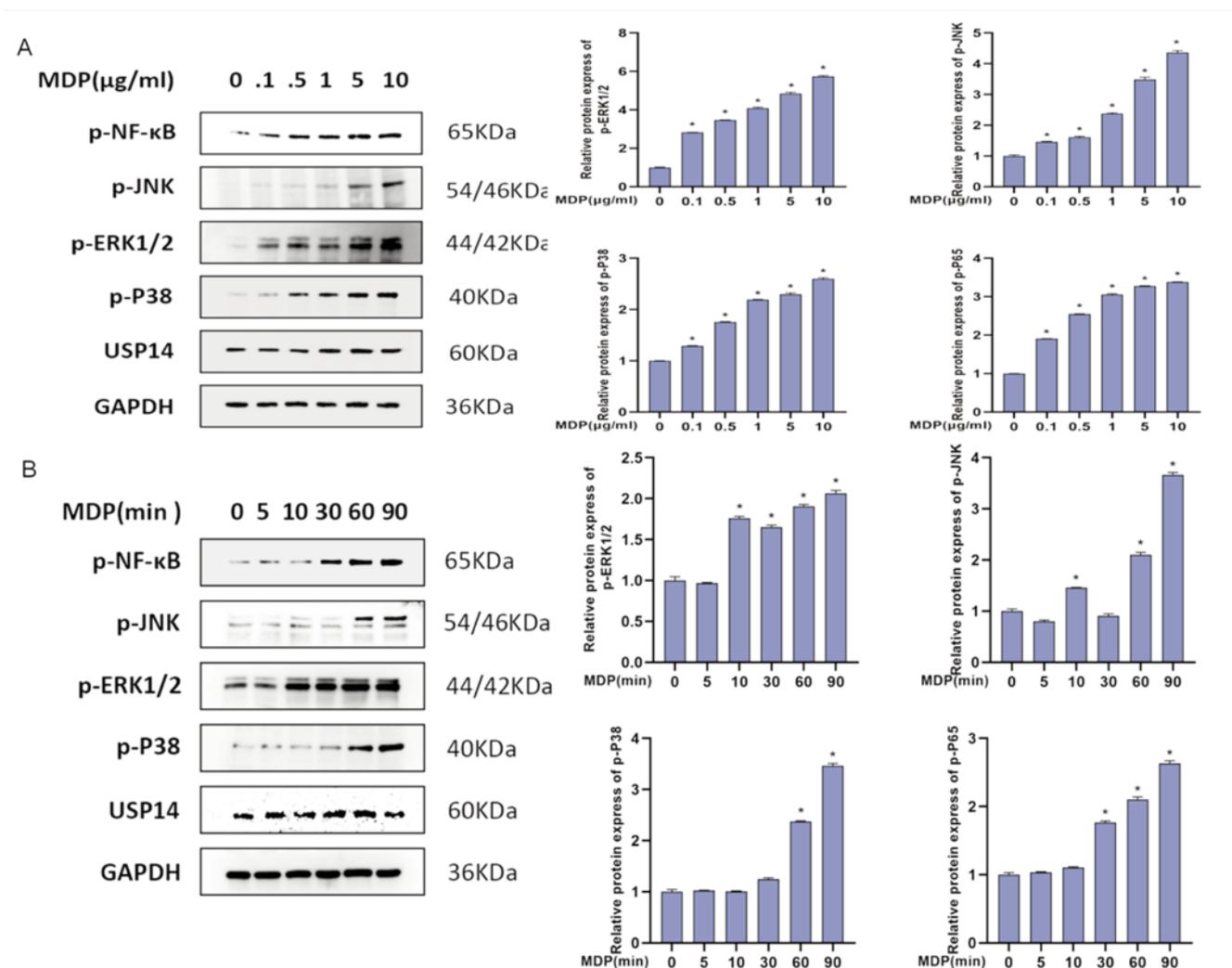


Figure 7. MAPK signaling contributed to MDP-induced inflammation in THP-1 cells. **A)** Western blot analysis of the phosphorylation of ERK1/2, JNK, p38, and NF- κ B levels in THP-1 cells treated with different concentrations of NOD2 ligand MDP. **B)** Western blot analysis of the phosphorylation of ERK1/2, JNK, p38, NF- κ B and USP14 levels in THP-1 cells treated with NOD2 ligand MDP at different time points. * $p < 0.05$ vs untreated cells. Three independent experiments were performed and data represented mean \pm SD.

NF-κB signaling, but had no effects on USP14 expression itself.

To confirm that USP14 regulates MAPK signaling to inhibit inflammation in THP-1 cells, we used inhibitors of all three branches of MAPK pathways to pretreat THP-1 cells. The results showed that MAPK inhibitors could inhibit the production of inflammatory factors TNF-α, IL-8 and IL-1β in THP-1 cells stimulated by MDP and USP14 inhibitor IU1. Collectively, these findings suggest that USP14 may inhibit MAPK signaling to downregulate the production of inflammatory factors.

Several previous studies including some from our group have shown the crosstalk of MAPK signaling and endoplasmic reticulum (ER) stress in IBD. 1⁶⁻¹⁸ It is known that ER stress leads to the

misfolding and abnormal accumulation of proteins, while ubiquitin ligases and deubiquitinases play opposite role in the maintenance of protein stability and diverse diseases such as cancer and IBD.¹⁹⁻²² Moreover, a recent study showed that USP31 played a role in regulating NF-κB signaling pathway.²³ Therefore, it would be interesting to investigate the role of USP14 in ER stress induced inflammation in IBD. Our next plan is to identify the substrates of USP14 directly involved in inflammation response in IBD.

In conclusion, our findings suggest that USP14 could inhibit MDP-induced activation of MAPK signaling and inflammation response involved in IBD and USP14 is a potential therapeutic target for IBD.

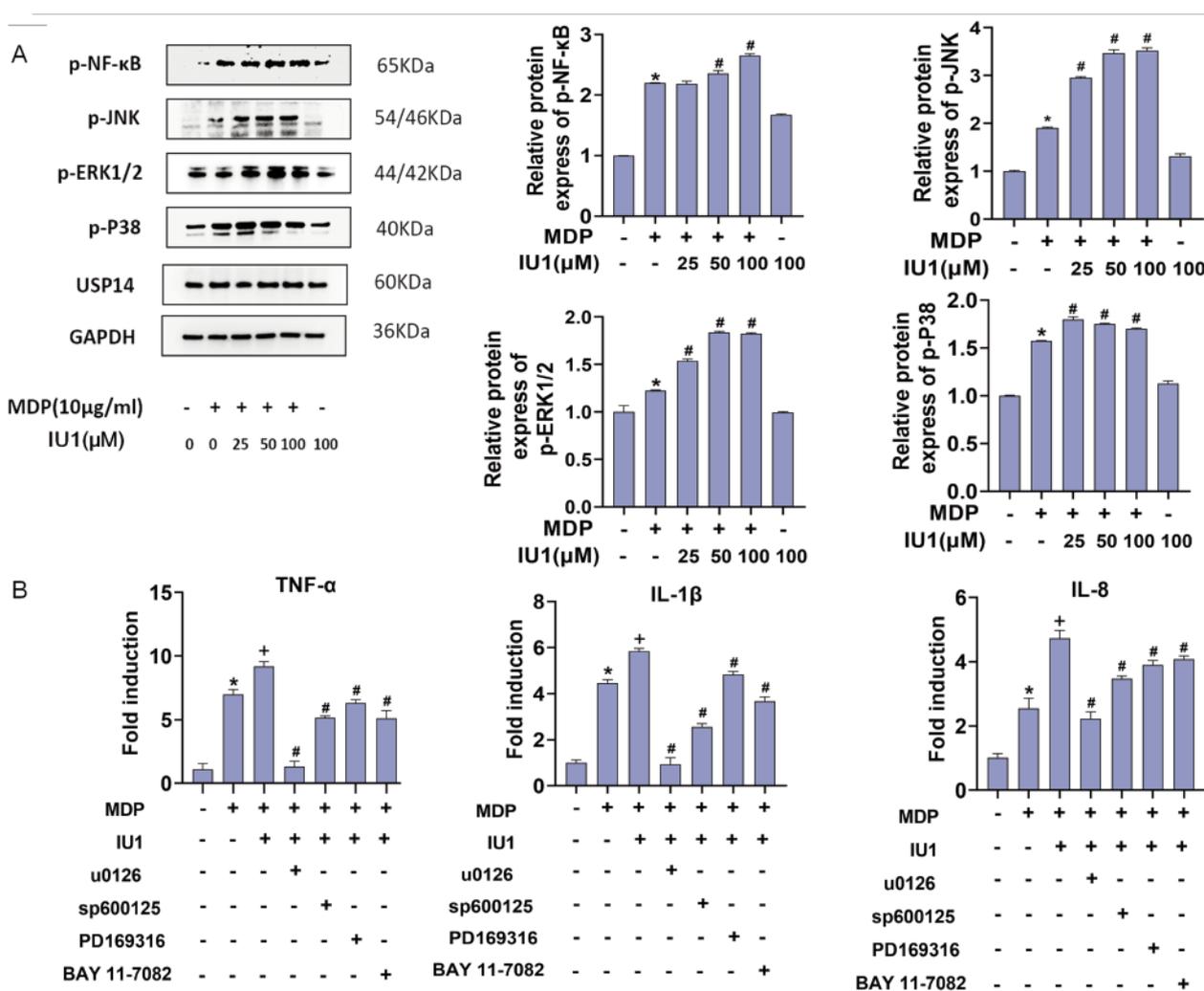


Figure 8. USP14 inhibitor enhanced MDP-induced activation of MAPK signaling in THP-1 cells. A) Western blot analysis of the phosphorylation of ERK1/2, JNK, p38, NF-κB levels in THP-1 cells treated with NOD2 ligand MDP or/and different concentrations of USP14 inhibitor IU1. B) RT-PCR analysis of TNF-α, IL-8 and IL-1β mRNA levels in THP-1 cells treated with 10 μg/mL NOD2 ligand MDP or/and 100 μM USP14 inhibitor IU1 or/and 10 μM u0126 (ERK1/2 inhibitor), 10 μM PD169316 (p38 inhibitor), 10 μM sp600125 (JNK inhibitor), 10 μM BAY 11-7082 (NF-κB p65 inhibitor). **p*<0.05 vs untreated cells; #*p*<0.05 vs cells treated with MDP and IU1. Three independent experiments were performed and data represented mean ± SD.

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