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Palmatine attenuated dextran sulfate sodium (DSS)-induced colitis via promoting mitophagy-mediated NLRP3 inflammasome inactivation

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ABSTRACT

Activation of NLRP3 inflammasomes is crucial in the pathological process of Ulcerative colitis (UC), which could be negatively regulated by PINK1/Parkin-driven mitophagy. Palmatine is a herb derived isoquinoline alkaloid with potent anti-inflammatory and anti-bacteria activities. In present study, we evaluated the effect of palmatine on dextran sulfate sodium (DSS)-induced mice colitis and examined whether its effect is exerted by promoting mitophagy-mediated NLRP3 inflammasome inactivation. The result showed that palmatine (40, 100 mg/kg) significantly prevented bodyweight loss and colonic shortening in DSS mice, and reduced the disease activity index and histopathologic score. The levels of MPO, IL-1β, TNF-α and the number of F4/80+ cells in colon of DSS mice were remarkably decreased by palmatine. Moreover, palmatine suppressed NLRP3 inflammasomes activation, but enhanced the expression of the mitophagy-related proteins involving LC3, PINK1 and Parkin in colonic tissue of DSS mice. These effects was consistent with *the* in vitro data revealing that palmatine inhibited the activation of NLRP3 inflammasomes, while promoted the expression and mitochondrial recruitment of PINK1 and Parkin in THP-1 cell differentiated macrophages. Furthermore, the effect of palmatine on THP-1 cells was neutralized by a mitophagy inhibitor Cyclosporin A (CsA) and PINK1-siRNA. In parallel, CsA significantly attenuated the therapeutic effect of palmatine in DSS mice, illustrating that the anti-colitis effect of palmatine is closely related to mitophagy. Taken together, the current results demonstrated that palmatine protected mice against DSS-induced colitis by facilitating PINK1/Parkin-driven mitophagy and thus inactivating NLRP3 inflammasomes in macrophage.

1. Introduction

Ulcerative colitis (UC), one form of inflammatory bowel diseases (IBD), is a recurrent and chronic gastrointestinal tract disease. Without efficient therapy, it would predispose sufferers to develop into colitis-associated cancer (Danese et al., 2011). Multiple factors involving gene susceptibility, imbalance of gut microbiota and disorder of mucosal immune responses are crucial pathogenic factors of UC (Maloy and Powrie, 2011). It is generally accepted that disrupted intestinal barrier results in penetration of bacterial antigens in colon, which activities mucosal immune response and then exacerbates the injury of colon (Salim and Soderholm, 2011). During this process, activated macrophages produced multiple pro-inflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor (TNF-α) and IL-18, subsequently enhancing inflammatory response and further aggravating tissue damage (Mahida, 2000). Those pro-inflammatory cytokines released by activated macrophages are closely correlate to NLRP3 inflammasome activation.

NLRP3 is the well-known member of the cytoplasmic NOD-like receptors (NLR) family, it senses the presence of pathogens through recognizing PAMPs (pathogen-associated molecular patterns) and perceives endogenous danger and stress situations via DAMPs (danger-associated molecular patterns, DAMPs) (Muruve et al., 2008; Petrilli et al., 2007). Once NLRP3 is activated, it integrates with apoptosis-associated speck-like protein (ASC) and Pro-Caspase-1 to constitute NLRP3 inflammasomes, subsequently facilitating the activation of Caspase-1. After that, the precursors of IL-1β and IL-18 are cut into mature IL-1β and IL-18, and secreted out of the cell to promote immune

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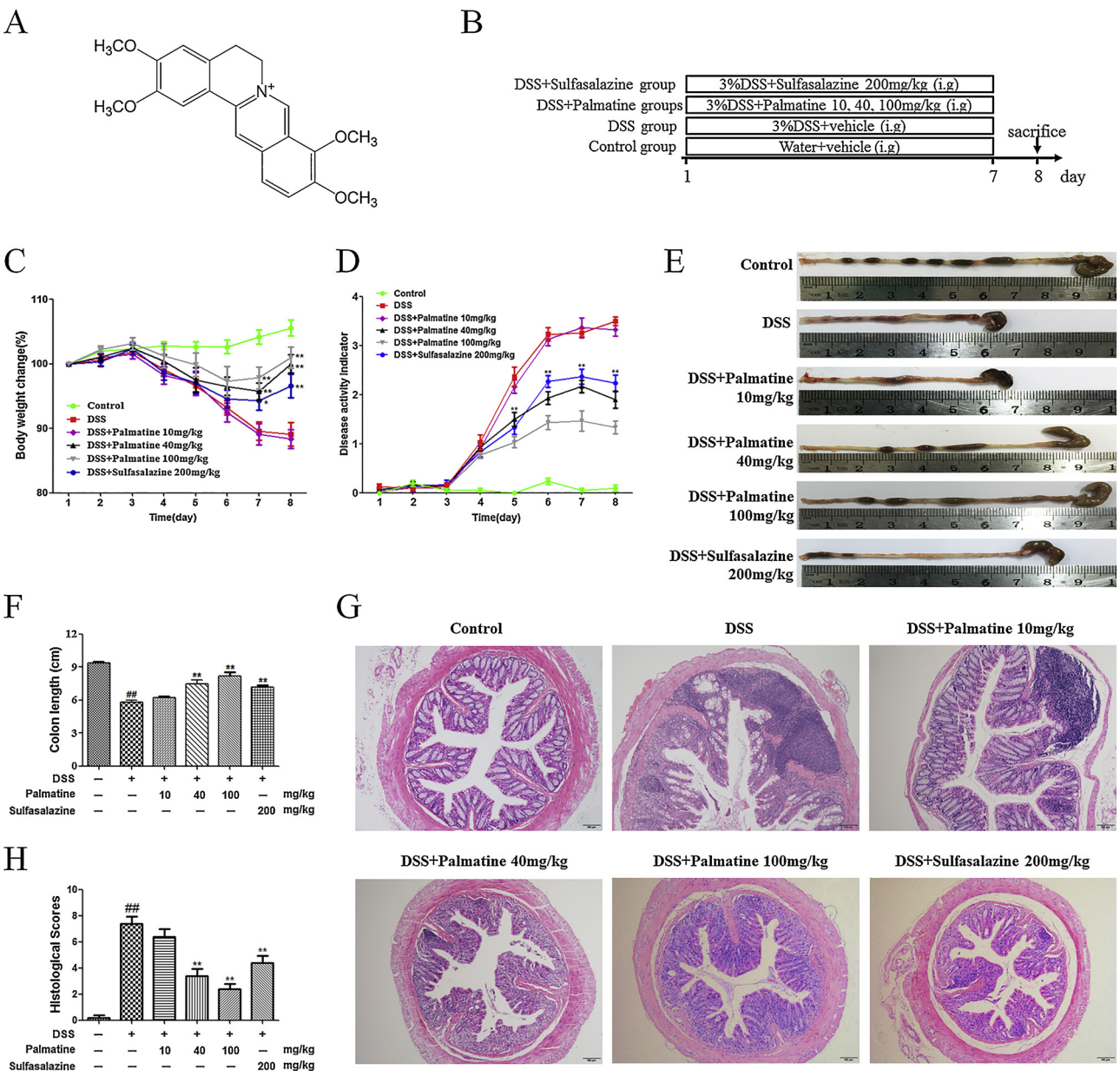

Fig. 1. Palmatine alleviated DSS-induced experimental colitis in mice. (A) Chemical structure of palmatine. (B) Experimental design for palmatine treatment on DSS-induced colitis in mice. Mice were treated with 3% DSS in drinking water for 7 days to induce colitis. Sulfasalazine and palmatine were administrated for 7 days during DSS treatment via oral gavage. Mice were sacrificed at day 8. (C) Body weight changes and (D) Disease activity index (DAI) evaluations during the disease process. (E and F) Macroscopic photographs and length of the colons. (G) Representative H&E staining images of colon tissue (scale bar, 100 μm). (H) Histological scores of colons. The data are presented as the means ± SD (n = 5–8) and statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison test. Significant differences were indicated as ##P < 0.01 vs. Control group, *P < 0.05 vs. DSS group, **P < 0.01 vs. DSS group.

response (Jin and Flavell, 2010). However, without properly regulation, those inflammatory cytokines will cause hyperinflammation and trigger IBD. Emerging evidence had suggested that activation of NLRP3 inflammasomes is intimately associated with colitis (Bauer et al., 2010).

Conversely, NLRP3 inflammasomes can be negatively regulated by mitophagy, a process of degrading the damaged or dysfunctional mitochondria. Mitophagy reduces the release of damaged or dysfunctional mitochondrial contents including mtDNA and mtROS (Kim et al., 2016). To initiate mitophagy, the ubiquitin kinase PINK1 is recruited to the mitochondrial outer membrane to mediate the ubiquitin phosphorylation. Subsequently, PINK1 recruits the E3 ubiquitin ligase Parkin to establish ubiquitin chains and assemble autophagy receptors, which result in the commencement of mitophagy and then reduce the production of mtROS which is required to activate NLRP3 inflammasomes (Geisler et al., 2010; Lazarou et al., 2015; Martinon, 2010). Therefore, promoting mitophagy is a feasible method to inactivate NLRP3 inflammasomes and terminate hyper-inflammation. This strategy may provide a novel sight for treating ulcerative colitis.

Palmatine is an isoquinoline alkaloid isolated from the traditional herb *Fibraurea Recisa Pierre*. Palmatine holds wildly pharmacological effects, including anti-inflammation and anti-bacteria, and had been clinically used to treat pelvic inflammation, abdominal pain, enteritis and chronic endometritis (Qiao et al., 2015; Yan et al., 2017). However, whether palmatine had protective effects on colitis remains unclear. Therefore, in the present study, we aimed to evaluate the effects of palmtine on DSS-induced mice colitis and clarified the underlying mechanisms.

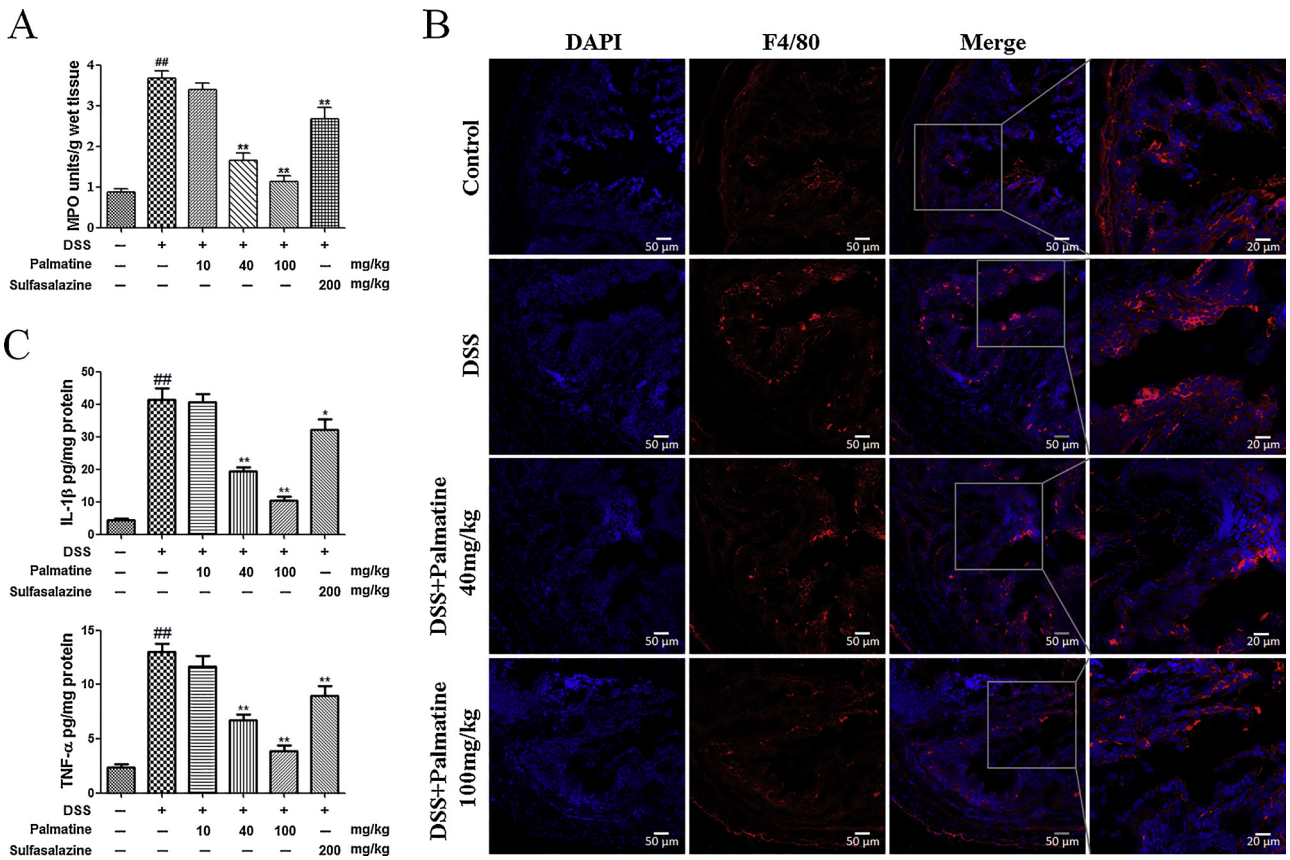

Fig. 2. Palmatine attenuated inflammatory response in DSS mice. (A) Myeloperoxidase (MPO) activity in the colon tissues was determined. (B) The level of IL-1β and TNF-α in colonic homogenate were detected by ELISA kit. (C) Sections of colon tissues were immunostained with DAPI (blue) and F4/80-FITC (red), and were observed using a confocal laser-scanning microscope (Scale bar, 50 μm). The data were presented as the means ± SD (n = 8) and statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison test. ##P < 0.01 vs. Control group, *P < 0.05 vs. DSS group, **P < 0.01 vs. DSS group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Animals

Male BALB/C mice (22–24 g) were provided from the animal center of Guangzhou University of Chinese Medicine (Guangzhou, China). Experimental protocols were strictly following the ethical regulation of the Committee for Animal Care and Use at Guangzhou University of Chinese Medicine and the Guide for the Care and Use of Laboratory Animals. Animals were housed at a temperature of 23 ± 2 °C and humidity of 55 ± 10% specific pathogen-free environment with a 12 h light/dark cycle and given free access to standard laboratory diet and water.

2.2. Cells culture and treatment

Human THP-1 cells were obtained from Central South University (Hunan, China) and were cultured at 37 °C in 1640 culture medium (Gibco, NY, USA) with 10% fetal bovine serum (10099141, Gibco) and 1% penicillin streptomycin (Gibco) in a 5% (v/v) CO_2 atmosphere.

2.3. Drugs, reagents and antibodies

Palmatine was provided by Xi'an Realin Biotechnology. Sulfasalazine was obtained from Fuda Pharmaceutical Co., Ltd (Shanghai, China). Dextran sulfate sodium (DSS, MW; 36–50 kDa) was purchased from MP Biomedicals (Irvine, CA, USA). Cyclosporin A was obtained from Meilun biotechnology Co., Ltd (Dalian, China). Adenosine 5′-triphosphate disodium salt hydrate (ATP, A7699), 4′, 6-Diamidino-2-phenylindole dihydrochloride (DAPI, D8417), phorbol myristate acetate (PMA, P1585) and lipolysaccharide (LPS, L2880) were purchased from Sigma-Aldrich. Mouse TNF-α (4281407), IL-1β (4336726) and Human IL-1β (4338671) Elisa kits were purchased from invitrogen (Carlsbad, CA, USA). F4/80 (sc-377009) and ASC (sc-271054) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Caspase-1 (ab1872), SQSTM1/P62 (ab56416), PINK1 (ab75487), Parkin (ab15954) and LC3 (ab192890) were purchased from Abcam Technology Inc (MA, USA). TOM20 (42406), NLRP3 (15101), β-actin (3700), IL-1β (12242), Cleaved-Caspase-1 (4199), Cleaved-IL-1β (83186), Alexa Fluor 488 anti-mouse IgG (4409), Alexa Fluor 555 anti-rabbit IgG (4412), HRP-linked anti-rabbit IgG (7074) and HRP-linked anti-mouse IgG (7076) were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.4. Establishment of DSS-induced colitis

After acclimatizing for at least one week, mice were randomly divided into 6 groups (n = 8): Control group, DSS group, palmatine-treatment (10, 40, 100 mg/kg) groups and Sulfasalazine (200 mg/kg) group. As previously described (Qu et al., 2017), experimental colitis was induced in mice through receiving 3% DSS dissolved in drinking water for consecutive 7 days, while the control mice were given the same volume of distilled water. In addition, mice in each group were administrated with vehicle, palmatine (10, 40, 100 mg/kg) or Sulfasalazine (200 mg/kg) as positive reference once per day respectively. The bodyweight and the presence of gross blood as well as stool consistency were recorded daily from day 1 to day 8. On day 8, all animals in this experiment were sacrificed and colon tissues were collected for analysis

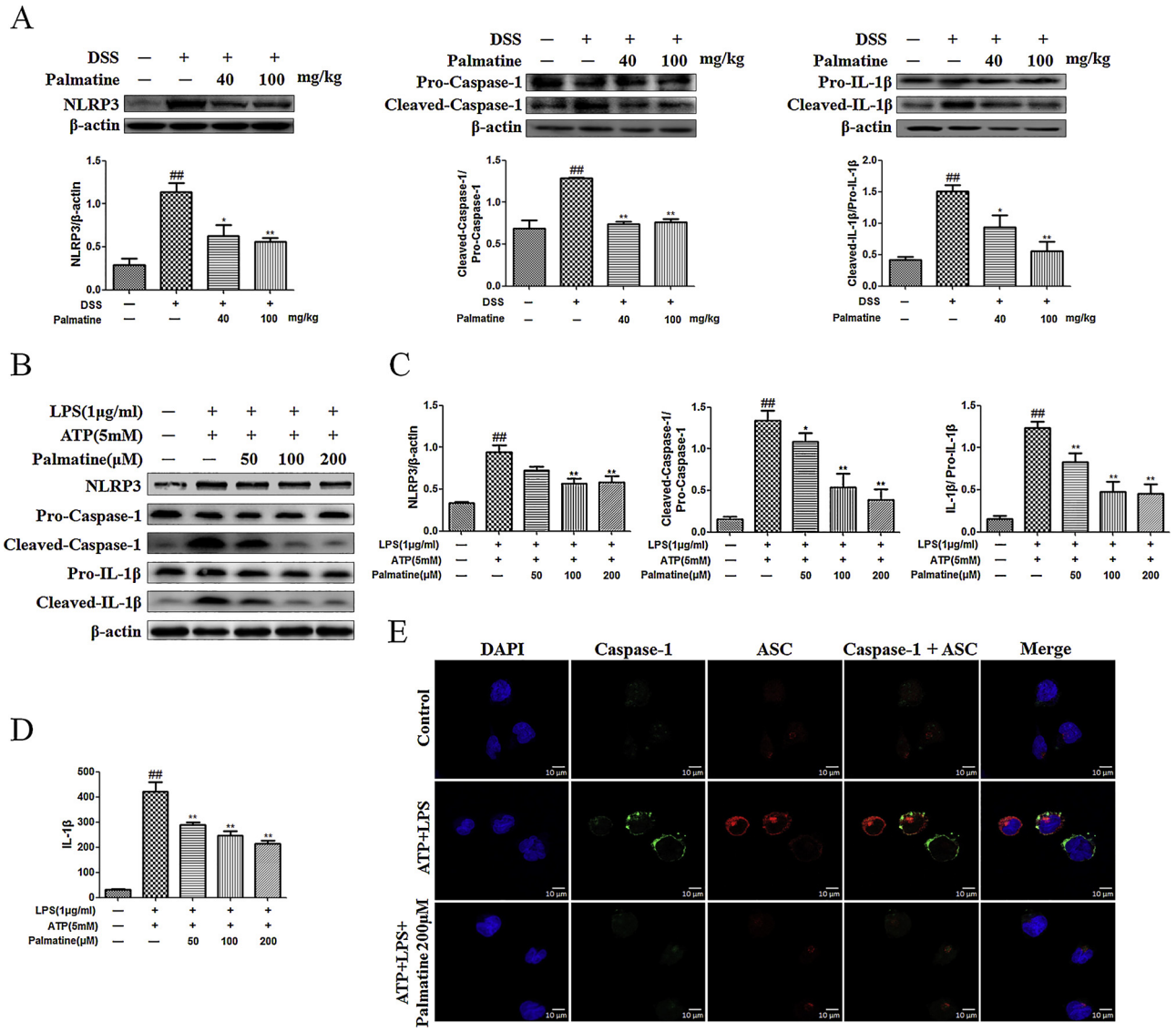

Fig. 3. Palmatine inhibited NLRP3 inflammasomes activiation in DSS mice and THP-1 differentiated macrophages (THP-Ms). (A) Protein levels of NLRP3, pro-Caspase-1, Cleaved-Caspase-1, pro-IL-1β and Cleaved-IL-1β in the colon of mice were analyzed by western blotting. THP-1 Cells were pre-treat with 300 nM PMA for 24 h to differentiate into macrophages (THP-Ms). After stimulated with 1 μg/ml LPS for 3 h and 5 μM ATP for 1 h, cells were treated with palmatine for another 3 h. (B and C) The relative protein expressions of NLRP3, Pro-Caspase-1, Cleaved-Caspase-1, Pro-IL-1β and Cleaved-IL-1β in THP-Ms. The data are representative of three experiments. (D) IL-1β in supernate was measured by ELISA kit, n = 6. (E) Cells were immunostained with DAPI (blue), Caspase-1 (green) and ASC (red), and were observed using a confocal laser-scanning microscope. The data are presented as the means ± SD and statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison test. ##P < 0.01 vs. Control group, **P < 0.01 vs. LPS + ATP group or DSS group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

after measurement of the length.

2.5. Clinical score and histological analysis

The disease activity index (DAI) was calculated by assessing loss of body weight, presence of gross bleeding and stool consistency according to the well-established methods (Alex et al., 2009). Partial colon was fixed in 10% formalin, embedded in paraffin and cut into 4 μm thicknesses. And the sections were stained with hematoxylin and eosin. Histological score was evaluated based on the scoring systems described previously (Qu et al., 2017) as follows: 0, no signs of leukocyte infiltration and inflammation; 1, few leukocyte infltration and focal inflammation; 2, moderate leukocyte infltration; 3, high leukocyte infltration and mild crypt epithelium disruption, moderate expansion of the mucosa, high vascular density and focal loss of crypts; 4, severe diffuse inflammation, massive loss of goblet cell and serious crypt epithelium disruption.

2.6. Myeloperoxidase (MPO) assay and Elisa assay

The colons were homogenized and the supernates were partially isolated to determine the protein concentration of the colon using the BCA protein assay kit (CWBIO, Beijing, China). Remaining supernates were used to measure the levels of Myeloperoxidas (MPO) and cytokines according to manufacturer's instructions of MPO assay kit (jiancheng, Nanjing, China), and mouse TNF-α and IL-1β Elisa kits.

2.7. Western blotting analysis

Total proteins were extracted from colon or cells in lysis buffer (Beyotime, Jiangsu, China) containing phosphatase inhibitor cocktail (CWBIO) and protease inhibitor (Beyotime), and the mitochondrial

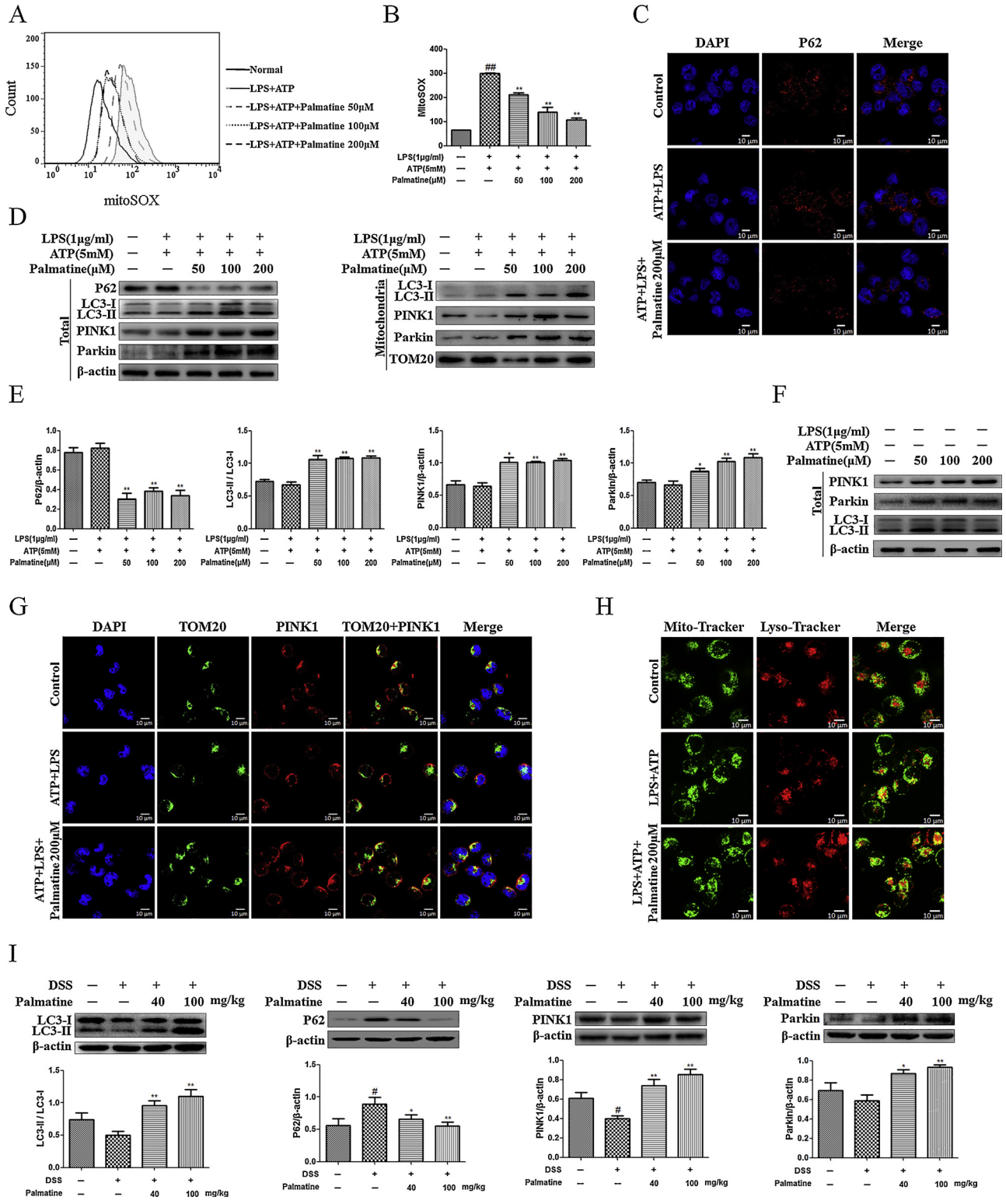

Fig. 4. Palmatine promotes PINK1 and Parkin-mediated mitophagy in THP-Ms and DSS mice. (A and B) Level of MitoSOX was detected using flow cytometry, n = 6. (C) Intracellular distribution of p62 in THP-Ms. Cells were immunostained with DAPI (blue) and P62 (red), and were observed using a confocal laser-scanning microscope (scale bars, 10 μm). (D) The protein levels of LC3, PINK1 and Parkin in THP-Ms and mitochondria were detected by western blotting. (E) Quantitative results of LC3, PINK1 and Parkin in THP-Ms (F) Representive western blotting image of PINK1 and Parkin expression in THP-Ms. THP-Ms were treated with palmtine for 3 h and the protein levels were assessed by western blotting. (G) Intracellular distribution of PINK1 and mitochondria (Tom20). Cells were immunostained with DAPI (blue), TOM20 (green) and PINK1 (red), and were observed using a confocal laser-scanning microscope (scale bars, 10 μm). (H) Mitochondria and lysosome were respectively signed the mitotracker and lysotracker, and then were observed using a confocal laser-scanning microscope (scale bars, 10 μm). (I) Protein levels of Mitophagy-related proteins including LC3, PINK1 and Parkin in DSS-treated mice were determined using western blotting. The data are presented as the means ± SD and statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison test. Significant differences were indicated as #P < 0.05 vs. Control group, ##P < 0.01 vs. Control group, *P < 0.05 vs. LPS + ATP group (or DSS group), **P < 0.01 vs. LPS + ATP group (or DSS group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

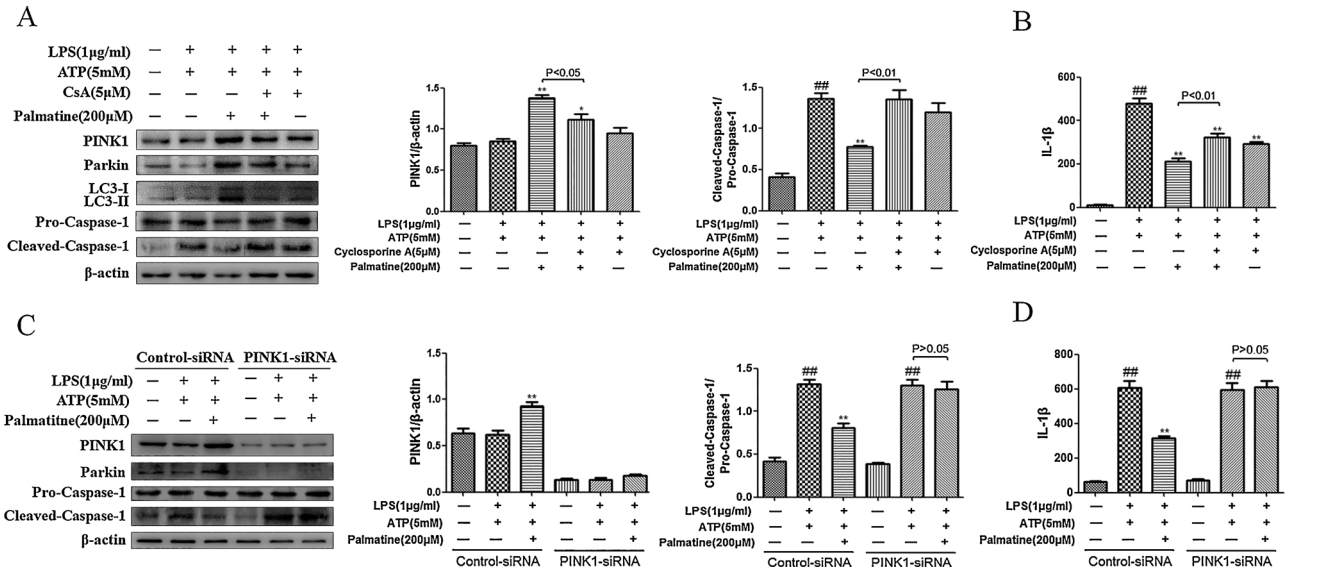

Fig. 5. Palmatine inhibited the activation of NLRP3 inflammasomes through promoting PINK1/Parkin-mediated mitophagy in THP-Ms. THP-Ms were pretreated with 1 μg/ml LPS for 3 h, and stimulated with 5 μM ATP for 1 h and subsequently treated with palmatine and Cyclosporine A for 3 h. (A) The expression levels of PINK1, Parkin, LC3, Pro-Caspase-1 and Cleaved-Caspase-1 in THP-Ms analyzed by western blotting. (B) IL-1β in supernate were measured, n = 6. (C and D) Cells were cultured without penicillin streptomycin. PINK1-siRNA and control-siRNA were transfected into THP-Ms for 54 h. After stimulated with 1 μg/ml LPS for 3 h and 5 μM ATP for 1 h, THP-Ms treated with PMT for 3 h. (C) Protein levels of PINK1, Parkin, LC3, Pro-Caspase-1 and Cleaved-Caspase-1 in the colon of mice were determined and the data of PINK1, Pro-Caspase-1 and Cleaved-Caspase-1 are representative of three experiments. (D) IL-1β in supernate were measured, n = 6. The data are presented as the means ± SD and statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison test. ##P < 0.01 vs. Control group, **P < 0.01 vs. LPS + ATP group.

proteins were extracted from cells according to the manufacturer's instructions of Cell Mitochondria Isolation Kit (Beyotime). Protein concentration was determined using the BCA protein assay kit (CWBIO). Ten micrograms of total and mitochondrial protein from cells or 50 μg total protein from colon tissue were separated by SDS-PAGE and then transferred onto 0.22 μm polyvinylidene fluoride (PVDF) membrance (Millipore, MA, USA). After blocking with 5% non-fat powdered milk (BBI Life Sciences, Shanghai, China) for 2 h at room temperature, Membranes were incubated with primary antibody overnight at 4 °C (Caspase-1 and IL-1β antibodies were used for animal sample, Cleaved-Caspase-1 and Cleaved-IL-1β antibodies were used for cell sample). Subsequently, membranes incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit Immunoglobulin G (IgG) for 1 h at room temperature. The protein was visualized by enhanced chemiluminescent HRP substrate (Millipore) via chemoluminescence and was quantified using Image J software (NIH).

2.8. Mitochondrial ROS measurement

Cells were incubated with 5 μM MitoSOX (M36008, Invitrogen) reagent working solution at 37 °C for 10 min. After washed gently three times with warm phosphate-buffered saline (PBS), cells were collected to analysis via Flow cytometry.

2.9. Imaging the combination of mitochondria and lysosome

Cells planted in Laser confocal plate (Nest, Jiangsu, China) were incubated with 150 nM Mito-Tracker Green (Beyotime) and 75 nM Lyso-Tracker Red (Beyotime) at 37 °C for 1 h. After removed the culture medium and washed, cells were photographed by laser scanning confocal microscopy.

2.10. Immunofluorescence assay

Cells planted in Laser confocal plate (Nest) or 6 μm freezing section of colon tissue was fixed by acetone, permeabilized by Triton-X-100 (Beyotime) for 10 min (F4/80 without this step), blocked with 5% goat serum (CWBIO) for 30 min and finally incubated with prime antibody overnight at 4 °C. After washed by PBST, samples were incubated with Alexa Fluor 555 or 488 antibody IgG at 37 °C for 1 h and subsequently incubated DAPI (10 μg/ml) for 5 min. Images were required by laser scanning confocal microscopy.

2.11. RNA interference

Cells were cultured in the absence of penicillin streptomycin. After premixing with Lipofectamine 3000 (L3000-008, Invitrogen), PINK1-siRNA and control-siRNA (Ribobio, Guangzhou, China) were transfected into Cells for 54 h.

2.12. Statistical analysis

Data are shown as Mean ± SD. Statistical analyses were performed using One-way ANOVA with Dunnett's Multiple Comparison test to analyze the significance among multiple groups and using unpaired Student's *t*-test to analyze the significance between two groups. P value < 0.05 was considered as significance.

3. Result

3.1. Palmatine alleviated the symptoms of DSS-induced colitis in mice

The chemical structure of palmatine is shown in Fig. 1A. To evaluate the protection of palmatine on experimental UC, we established the mice model via feeding with 3% DSS solution for consecutive 7 days (Fig. 1B). Compared with control group, mice in DSS group significantly lost their bodyweights (Fig. 1C) and had higher DAI scores(Fig. 1D) which is a vital parameter reflecting the severity of colitis (Cooper et al., 1993). Moreover, DSS mice had shorter colons than control animals (Fig. 1C–F). These results indicated that DSS induced colonic edema, presence of gross bleeding and alterations in stool consistency. However, mice in palmatine (40, 100 mg/kg) and sulfasalazine (as a

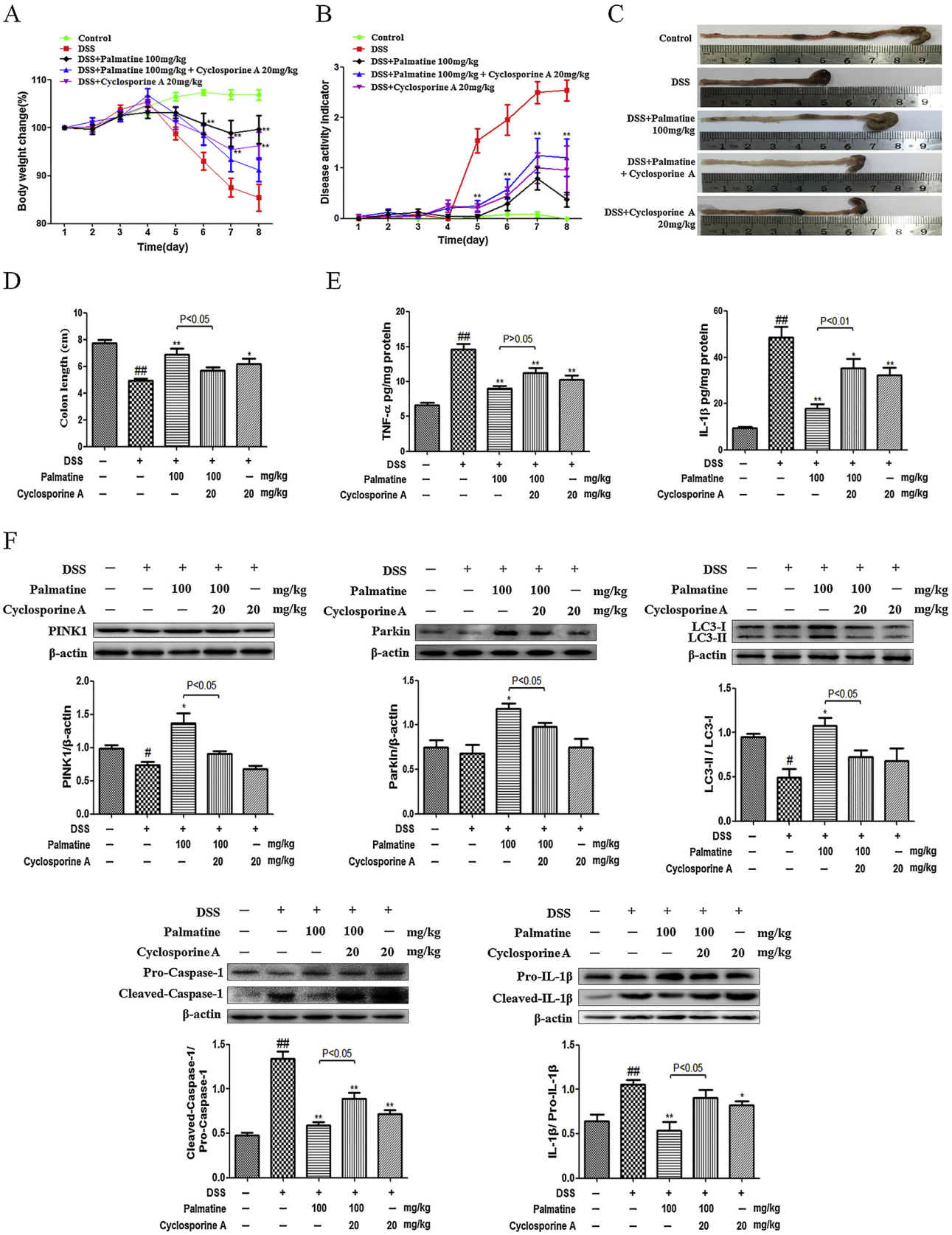

Fig. 6. Effect of Palmatine on NLRP3 inflammasomes was weakened after inhibiting mitophagy in colitis mice. Mice were received 3% DSS in drinking water for continuous 7 days. In addition, Palmatine was orally administered daily (100 mg/kg) and Cyclosporin A was intraperitoneal injected daily (20 mg/kg). (A) Body weight changes and (B) Disease activity index (DAI) during the disease process. (C and D) Macroscopic photographs and length of the colons. (E) The level of IL-1β and TNF-α in colonic homogenate were detected by ELISA kit. (F) Protein Level of PINK1, Parkin, LC3, Pro-Caspase-1, Cleaved-Caspase-1, Pro-IL-1β and Cleaved-IL-1β in colitis mice were detected by western blotting. The data are presented as the means ± SD (n = 8) and statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison test. Significant differences were indicated as ##P < 0.01 vs. Control group, *P < 0.05 vs. DSS group, **P < 0.01 vs. DSS group.

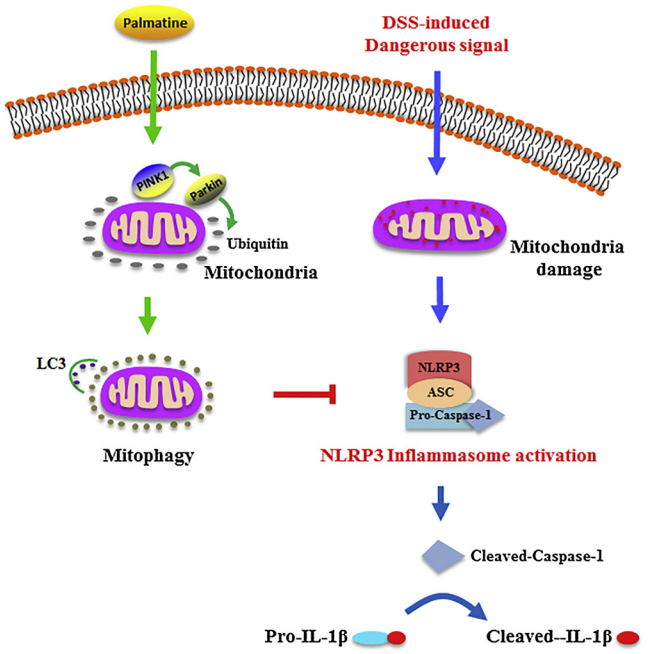

Fig. 7. Mechanism against ulcerative colitis of Palmatine. DSS-induced dangerous signal stimulates the mitochondria and leads to mitochondrial damage, which triggers the NLRP3 inflammasomes activation and finally induces the secretion of IL-1β. Palmatine promotes PINK1/Parkin-mediated mitophagy to inhibit the activation of NLRP3 inflammasomes, resulting in the decrease of IL-1β and thus alleviate inflammatory response.

positive control) groups gradually restored their bodyweights and colon length, and their DAI scores decreased. In addition, histopathological analysis revealed that DSS elicited colonic inflammation including loss of epithelial crypts, disruption of mucosal barrier and infiltration of inflammatory cells, which directly leading to higher histological score (Fig. 1G and H). By contrast, both palmatine (40, 100 mg/kg) and sulfasalazine administration improved the pathological changes and decreased the histological scores of DSS mice.

3.2. Palmatine attenuated colonic inflammation in DSS mice

Neutrophil and macrophage infiltrations are recognized as evaluative parameters for severity degree of colitis (Hall et al., 2013; Lu et al., 2014). As shown in Fig. 2A, palmatine (40 and 100 mg/kg) remarkably suppressed colonic myeloperoxidase (MPO, an indicator of neutrophil activation) activity in DSS mice, and evidently reduced the percentage of $F4/80^+$ (a marker of activated macrophage) cells (Fig. 2B) in mice colon. Meanwhile, palmatine dramatically reversed the elevated IL-1β and TNF-α in colon tissues of DSS mice (Fig. 2C).

3.3. Palmatine suppressed NLRP3 inflammasomes activation in DSS mice and THP-1 differentiated macrophages (THP-Ms)

It is well known that NLRP3 inflammasomes are of importance in the DSS-induced mice colitis (Bauer et al., 2010). We firstly detected the protein levels of NLRP3 inflammasome related proteins in colon by western blotting analysis. As shown in Fig. 3A, palmatine significantly inhibited DSS-increased protein level of NLRP3, cleaved-caspase-1 and cleaved-IL-1β in colon tissues. Moreover, we investigated whether palmatine inhibit NLRP3 inflammasome activation in PMA-primed THP-Ms. As reported (Guo et al., 2014), LPS plus ATP induced the expression of NLRP3, cleaved-caspase-1 and cleaved-IL-1β in THP-Ms, and promoted the secretion of IL-1β in supernate (Fig. 3B–D). However, palmatine treatments suppressed the generation of NLRP3, cleaved-caspase-1 and cleaved-IL-1β in THP-Ms as well as the secretion of IL-1β in supernate in a dose-dependent manner. ASC-assembled large protein complex plays an vital role in Caspase-1 activation (Elliott and Sutterwala, 2015). Next, we investigated this complex in THP-Ms via using Immunofluorescence. As shown in Fig. 3E, LPS plus ATP promoted ASC speck generation, while palmatine disrupted ASC speck.

3.4. Palmatine promoteed PINK1 and Parkin-driven mitophagy in THP-Ms and DSS mice

During the process of NLRP3 inflammasomes activation, mitophagy can reduce the accumulation of mtROS and mtDNA and thus negatively regulate NLRP3 inflammasomes (Kepp et al., 2011). In the process of mitophagy, PINK1 and Parkin are recruited into mitochondrial outer membrane to mediate mitochondrial ubiquitination. Subsequently, ubiquitinated mitochondria binds to autophagy receptor LC3-II in autophagosome. LC3-II, representing autophagosome formation, is converted from LC3-I via degrading the selective autophagy substrates P62 (Ivankovic et al., 2016; Johansen and Lamark, 2011; McLeland et al., 2011; Shaid et al., 2013). To determine whether palmatine inhibited NLRP3 inflammasome activation by promoting mitophagy, we examined the effect of palmatine on mitophagy in THP-Ms, which NLRP3 inflammasomes were activated by LPS plus ATP. Firstly, mitoSOX was used to analyze the mitochondrial ROS in THP-Ms by using flow cytometry and the result showed that LPS plus ATP significantly enhanced the generation of mitochondrial ROS in THP-Ms (Fig. 4A and B), while palmatine remarkably decreased the level of mitochondrial ROS in a dose-dependent manner. Secondly, the mitophagy-related proteins including P62, LC3, PINK1 and Parkin were determined by using immunofluorescence and western blotting. As shown in Fig. 4C–F, palmatine decreased P62 protein level and obviously promoted conversion of LC3-I into LC3-II in cytoplasm of THP-Ms either in the presence of LPS plus ATP or not. Meanwhile, the expression of LC3-II in mitochondrial was also promoted after palmatine treatment, which suggested that palmatine facilitated the combination of autophagosome and mitochondria. In addition, palmatine elevated the expressions of PINK1 and Parkin, and promoted the mitochondrial recruitment of PINK1 and Parkin in THP-Ms (Fig. 4D and E). The immunofluorescence results in Fig. 4G was consistent with western blotting results.

To identify mitophagy in THP-Ms, we used mitotracker and lysotracker to label mitochondria and lysosome. As shown in Fig. 4H, palmatine facilitated the combination of mitochondria and lysosome in THP-Ms. Furthermore, we determined the in vivo effect of palmatine on mitophagy in DSS mice. The results revealed that DSS mice had higher level of p62 and lower level of PINK1, although the reductions of LC3-II and Parkin did not reach statistical significance (Fig. 4I). However, palmatine (40, 100 mg/kg) decreased the level of P62 and obviously promoted the conversion of LC3-I into LC3-II and the expression of PINK1 and Parkin in mice colon.

3.5. Mitophagy inhibitor Cyclosporin A (CsA) and PINK1 siRNA neutrolized the effect of palmatine on NLRP3 inflammasomes activation in THP-Ms and DSS mice

To further validate the effect of palmatine on NLRP3 inflammasomes via activating PINK1/Parkin signaling pathway, we used mitophagy inhibitor (CsA) (Mauro-Lizcano et al., 2015) and PINK1 siRNA to block or silence the relative pathway. The results showed that the effects of palmatine on inhibiting activated caspase-1 and secretion of IL-1β were weakened under CsA intervention (Fig. 5A and B). As shown in Fig. 5C and D, when PINK1 expression was silenced by transfecting PINK1-siRNA on THP-Ms, the effects of palmatine on cleaved-caspase-1 and the secretion of IL-1β were blocked.

Consistently, in vivo results in Fig. 6A–F showed that CsA blocked the protective effect of palmatine on mice bodyweight, DAI and colon length. Although the expression of NLRP3 inflammasomes-related protein were also weakened in colitis mice received CsA in comparision

with DSS group (Fig. 7).

4. Discussion

UC is a chronic inflammatory disease with the highest incidence and prevalence rates in developed countries (Ordas et al., 2012). Immunosuppressants like azathioprine or methotrexate are the mainly clinical drug used to control the symptoms of UC, but most of them have severe side effects, such as steroid dependence and serious infections, and subsequently limited its application for treating UC (Liu et al., 2013; Renna et al., 2014). Therefore, efforts searching for efficient and safe novel drugs never stopped. Palmatine, an isoquinoline alkaloid from *Fibraurea Recisa Pierre*, has anti-inflammatory and antimicrobial bioactivities (Yan et al., 2017). In current study, we evaluated the effects of palmatine on anti-UC and elucidated the underlying mechanism for the first time.

DAI, bodyweight loss, colonic length and histological scores are the main parameters used to determine the degree of UC (Goyal et al., 2014). In this study, palmatine evidently improved the clinical symptoms of colitis mice by preventing the loss of bodyweights, extending the lengths of colon, down-regulating the infiltration of inflammatory cells and inhibiting the accumulation of MPO and secretion of inflammatory cytokines including TNF-α and IL-1β. Moreover, it is known that inappropriate response of macrophages causes sustaining inflammation and subsequently induce IBD (Mahida, 2000). But, palmatine markedly suppressed DSS-induced macrophage activation in colon. Those results indicated that palmatine possesses a therapeutic potential for UC.

Mitochondrial ROS is the pivotal trigger of NLRP3 inflammasomes and can be cleared via mitophagy mediated by PINK1/Parkin pathway (Hu et al., 2016; Martinon, 2010). In this study, LPS plus ATP activated NLRP3 inflammasomes and induced excessive mitochondrial ROS production, while palmatine significantly decreased mitochondria ROS level in THP-Ms. Moreover, palmatine also promoted conversion of LC3-I into LC3-II in both whole cells and mitochondria, which demonstrated that palmatine facilitated the combination of autophagosome with mitochondria and modulated mitophagy. Meanwhile, palmatine potentiated the expression of PINK1 and Parkin as well as the mitochondrial recruitment of PINK1 and Parkin. Those results suggest that palmatine inhibits the activation of NLRP3 inflammation possibly by promoting PINK1/Parkin-mediated mitophagy.

To further validate the underlying mechanism of palmatine, we used CsA to inhibit mitophagy and PINK1-siRNA to silence the expression of PINK1. CsA is an immunosuppressive agent used to suppress immune response. Besides, it is also an inhibitor of cyclophilin D, a mitochondrial matrix protein that regulates mitochondiral permeability transition and therefore modulates mitophagy (Mauro-Lizcano et al., 2015; Soriano-Izquierdo et al., 2004). Our results showed that CsA blocked the conversion of LC3 and the expression of PINK1 and Parkin. In addition, the effect of palmatine on NLRP3 inflammasomes was attenuated under CsA intervention either in vitro or in vivo. Similar outcome was observed after silencing the expression of PINK1, which demonstrated that palmatine inhibited the activation of NLRP3 inflammasomes via promoting PINK1/Parkin-mediated mitophagy.

Previous study has revealed that the LD50 of palmatine in mice acute toxicity assay was 1.534 g/kg, which is 15 folds of the highest dose used in this study. Moreover, a 90-day administration of Palmatine (521 mg/kg) did not cause mortality or any symptoms of poisoning in rats (Yi et al., 2013). Herein, the currently used doses of Palmatine can be considered as safe.

5. Conclusions

The above results clearly demonstrated that palmatine possessed a protective effect against DSS-induced colitis, which mechanism possibly mediated via enhancing PINK1/Parkin-driven mitophagy and thus inactivating NLRP3 inflammasomes in macrophages. Overall, our results suggest that palmatine may be a promising pharmaceutical candidate to treat ulcerative colitis.

Conflict of interests

The authors have declared no competing interests on this work.

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