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PAP-1 ameliorates DSS-induced colitis with involvement of NLRP3 inflammasome pathway

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ABSTRACT

Background: Macrophages are a primary type of innate immune cells activated in colitis. Kv1.3 channel is one of the major potassium channels in macrophages. NLRP3 inflammasome is a downstream molecule of Kv1.3 channel. PAP-1, a specific Kv1.3 channel blocker, has been shown to have immune-regulatory effects.
Objective: To investigate the effect of PAP-1 on intestinal inflammation in DSS-induced colitis and explore its possible mechanism.
Methods: C57BL/6 mice were divided into four groups: normal control group, normal + PAP-1 injection group, DSS model group, DSS model + PAP-1 injection group. Experimental colitis was induced by 5% DSS treatment; mice were injected intraperitoneally with PAP-1 from the first day for 7 consecutive days; then all mice were sacrificed, followed by isolation of colon tissue, peritoneal macrophages and spleen macrophages. The anti-inflammatory effects of PAP-1 and the expression levels of Kv1.3, iNOS, pro-IL-1β, IL-1β and NLRP3 inflammasome were measured.
Results: PAP-1 reduced DSS-induced colonic pathological damage, DAI score, MPO activity and levels of IL-1, IL-6, TNF-a, IL-18. Compared with the DSS model group, the expression of Kv1.3, iNOS, NLRP3, ASC, caspase-1p20, pro-IL-1β and IL-1β in colon were decreased in the DSS-induced colitis mice with PAP-1 injection. PAP-1 also reduced the expression of Kv1.3, iNOS, NLRP3, caspase-1p20 and IL-1β on macrophages in colitis mice.
Conclusion: PAP-1 had protective effects on DSS-induced colitis, which might be ascribed to the regulation of NLRP3 inflammasome pathway. Therefore, we found that PAP-1 was useful as a therapeutic agent in IBD and suggested a potential important role of PAP-1 in NLRP3 inflammasome-associated diseases.

1. Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is characterized by chronic, relapsing and remitting intestinal inflammation resulting from interplay of genetic and environmental factors that trigger an abnormal immune response [1,2]. However, the precise aetiology of IBD is still not clear. Since the acute Dextran Sodium Sulfate (DSS)-induced mice colitis model is characterized by diarrhoea, bloody feces, weight loss and colon histopathological changes as seen in IBD [3], experimental animal models of colitis, which have been considered a tool to investigate the IBD molecular and cellular mechanisms, are more and more often used to develop novel anti-inflammatory drugs recently. As T and B cells are dispensable in the acute inflammatory response [4], the model is particularly useful to study the contribution of innate immune mechanisms in IBD. It is widely accepted that macrophages play an important role in the innate immune inflammatory response. Besides, macrophages have been thought to be a primary type of innate immune cells that is usually activated in DSS-induced colitis [5]. Increased levels of pro-inflammatory cytokines interleukin-1β (IL-1β) are in active IBD and correlated with the severity of inflammation [6]. And the levels of IL-1β are enhanced in colonic mucosa and peritoneal macrophages in DSS-induced colitis [7], implying that innate immune response of macrophages plays an integral role in colitis.

The maturation and secretion of IL-1β are managed by the inflammasome, especially the most studied and widely accepted Nucleotide-binding oligomerisation domain receptor, pyrindomain containing (NLRP)3inflammasome [8]. The NLRP3 inflammasome is a molecular platform that comprises NLRP3, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and caspase-1 (cysteine-requiring aspartate protease-1). In some cases, NLRP3 is united with ASC adapter protein, which in turn recruits caspase-1 that can be cleaved to its activated form (p10 and p20). Finally,

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activated caspase-1 facilitates the cleavage of pro-IL-1β and pro-IL-18 to their biologically active forms [9]. Recent studies have showed that the low levels of intracellular K^+ are the final common mechanism of NLRP3 inflammasome activation of caspase-1 [10]. It was also reported that the NLRP3 inflammasome may be an effective downstream molecule of the voltage-gated potassium channel Kv1.3 in hypertension development, and that blockage of the Kv1.3 channel can inhibit the activation of the NLRP3 inflammasome pathway and the release of IL-1β in peripheral blood T-lymphocytes of hypertensive patients [11], indicating that Kv1.3 may be a potential target, and its blockers can alleviate the severity of NLRP3-related immune inflammatory diseases.

The Kv1.3 channel plays an important role in immune cell activation as well as in maintaining a negative membrane potential through K^+ efflux [12] and controlling activation and proliferation processes of macrophages [13,14]. Macrophages, which act as antigen presenting cells modifying the cytokine milieu and the signaling intensity of T cell, can switch the immune response to inflammation or tolerance. Therefore, the inhibitory effects of specific Kv1.3-blockers on macrophages may partially account for the benefits of Kv1.3-based therapies. PAP-1, also named 5-(4-phenoxybutoxy)psoralen, has been applied effectively in animal studies [15] and proved to be a novel specific small molecule Kv1.3 channel blocker without any effects of cytotoxicor phototoxic [16]. In this study, we try to investigate whether PAP-1 can attenuate DSS-induced mice colitis and explore its possible mechanism.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (aged 6–8 weeks) were purchased from Animal Center of Anhui Province (Hefei, China). The mice were kept in the room temperature environment with 12 h dark/light cycles, and were allowed free food and water *ad libitum* for one week to be adapted to the environment before experiment. All animal experimental procedures adhered to the guidelines of the Medicine Research Ethics Committee of Anhui Medical University and the relevant regulations.

2.2. Reagents

DSS (M_r ~40,000), PAP-1and Cremophor EL were purchased from Sigma (St. Louis, MO. USA), myeloperoxidase (MPO) kit and Hemoccult assay kit were purchased from Nanjing Jiancheng Bioengineering institute(Nanjing, China). IL-1, IL-6, TNF-α and IL-18 ELISA kits were purchased from Wuhan Xinqidi Bio-Technology Co., Ltd. (Wuhan, China). Anti-Kv1.3, anti-iNOS, anti-pro-IL-1β , anti-IL-1β and anti-ASC antibodies were from BiossLtd (Shanghai, China). Anti-NLRP3 and anti-caspase-1p20 were from Adipogen (SanDiegoCA, USA).

2.3. Experiment model and treatment

The mice were randomly allotted to 4 groups: (A)normal control group, (B)normal + PAP-1 injection group, (C)DSS model group, (D)DSS model + PAP-1 injection group. Experimental colitis was induced by replacing the drinking water with 5% DSS for 7 days (from day 1 to day 7). The normal + PAP-1injection group and DSS model + PAP-1 injection group were intraperitoneally injected with 3 mg/kg of PAP-1 three times daily from day 1 to day 7. The dosage of PAP-1 in vehicle (saline with25% Cremophor EL) was based on our pre-experiment and the previous research by some researchers [15].The normal control group and DSS group were intraperitoneally injected with an equal volume of vehicle daily from day 1 to day 7.

2.4. Sample collection

All mice were sacrificed on day 8, and then they were immediately intraperitoneally injected with 10 mL RPMI 1640, followed by peritoneal lavage fluids being collected after 5 min of gentle rubbing move. The peritoneal lavage fluids and spleen from the mice are ready for the next step of macrophage extraction. The distal end of the colon was excised. Some were fixed in 10%formalin for hematoxylin and eosin (H&E) staining. The remaining was straightway stored in liquid nitrogen for subsequent detection.

2.5. Isolation of peritoneal macrophages and splenic macrophages

Peritoneal macrophages from all mice were collected as in Chiang et al. [17]. Shortly, the cell pellets were suspended in RPMI-1640 complete medium after the peritoneal lavage fluids were centrifugated at 1000 rpm for 10 min. Meanwhile, spleen collected from sacrificed mice were chopped up and passed through a fine steel mesh to harvest a homogeneous cell suspension. In order to remove erythrocytes, recovered spleen cells were resuspended in lysis buffer for 5 min. Then, spleen cells were harvested and suspended in RPMI-1640 complete medium again [18,19]. Finally, Cells were cultivated in 12-well plate in RPMI 1640 complete medium containing 10% FBS and 1% penicillin–streptomycin. After 2 h in a humidified 5% CO_2 incubator at 37 °C, cells were rinsed twice with RPMI 1640.The adherent cells were used as peritoneal macrophages and splenic macrophages [20]. Finally, macrophages were collected for subsequent detection straightway stored in liquid nitrogen.

2.6. Evaluation of the severity of colitis

The mice were observed once daily for weight, the stool consistency and frequency, and the presence of blood in feces by performing fecal occult blood test. The Disease Activity Index (DAI) was calculated as previously described [21].After experiments, the mice were sacrificed, the entire colon was quickly removed for *ex vivo* study. Segments of the colon were fixed with 10% normal buffered formalin, embedded in paraffin. The paraffin-embedded colonic tissue were sectioned at 5 μm in thickness and then partially stained with H&E [21,22].

2.7. Enzyme-Linked Immunosorbent Assays (ELISAs)

Colonic tissues were weighed and homogenized in cold physiologic saline for 10% colonic tissue homogenate. Colonic homogenate supernatants were collected after centrifuged at 14,000 r/min for 15 min at 4 °C. The supernatants were used for quantified detection of MPO, IL-1, IL-6, TNF-α and IL-18 level by using ELISA kits according to the manufacturer's instructions.

2.8. RNA extraction and RT-qPCR

Total RNA was extracted from macrophages and colonic tissue using TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA). Complementary DNA (cDNA) was synthesized from total 1 μg RNA following the instructions of the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The obtained1μl cDNA from each sample was amplified using SYBR greenfor Quantitative PCR. The conditions of PCR were as follows:95 °C for 2 min, 35 cycles of 95 °C for 10 s, 60 °C for 20 s.The sequences of primers (Bio-engineering (Shanghai) Co., Ltd., China) were (forward and reverse, respectively):β-actin:5′-AGTGTGACGTTGACATCCGT-3′ and 5′-TGCTAGGAGCCAGAG CAGTA-3′;Kv1.3:5′-CCCATCGACATCTTCTCCGA-3′ and 5′-CCCTCATC CTCACGGAACTT-3′;iNOS: 5′-CCTTGTTCAGCTACGCCTTC-3′ and 5′-CTTCAGAGTCTGCCCATTGC-3′. The mRNA expression was normalized to β-actin expression.

2.9. Protein extraction and western blot analysis

The protein expression was detected by western blotting. Proteins were extracted from tissues by being homogenized with lysis buffer,

separated using SDS-PAGE and transferred to PVDF membrane (Millipore, Germany) according to standard methods. Then, the membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated with primary antibodies overnight at 4 °C. The primary antibodies were as described by antibody manufacturers;NLRP3(1:1000), ASC(1:300), Caspase-1 p20(1:1000), IL-1β(1:300), pro-IL-1β(1:300), Kv1.3(1:300), iNOS(1:300) and β-actin (1:1000). The secondary horseradish peroxidase(HRP)-linked anti-rabbit IgG was incubated with each membrane for 2 h at room temperature after washing with TBST. ECL western blotting detection system (Amersham, USA) and Image J software were used to visualize and analyze the final results.

2.10. Statistical analysis

SPSS software version 17 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A two tailed p of < 0.05 was considered to be statistically significant. Data was analyzed using one-way ANOVA and least significant difference (LSD) t-test and presented as the means ± SD.

3. Results

3.1. PAP-1treatment ameliorates DSS-induced colitis

The model of DSS-induced colitis was constructed successfully. PAP-1-treated mice were protected from DSS-induced injury on aspects of decreased body weight loss (Fig. 1a). PAP-1 also reduced the shortening of colon length in colitis mice (Fig. 1b). Moreover, the DSS model + PAP-1 injection group had a much lower DAI score and reduced MPO activity compared with the DSS model group after induction of colitis (Fig. 1c). Microscopically, histological results demonstrated important pathological changes mainly located in the mucosa and sub-mucosa, including loss of goblet cells, formation of local erosions and ulcers, and abundant infiltration of neutrophils and monocytes, as well as distortion of crypts in the colon specimens of DSS model group mice. The injection of PAP-1 improved these changes significantly (Fig. 1d). The above findings suggested PAP-1 treatment alleviated DSS-induced colitis.

In addition, compared with those of the control group, the levels of

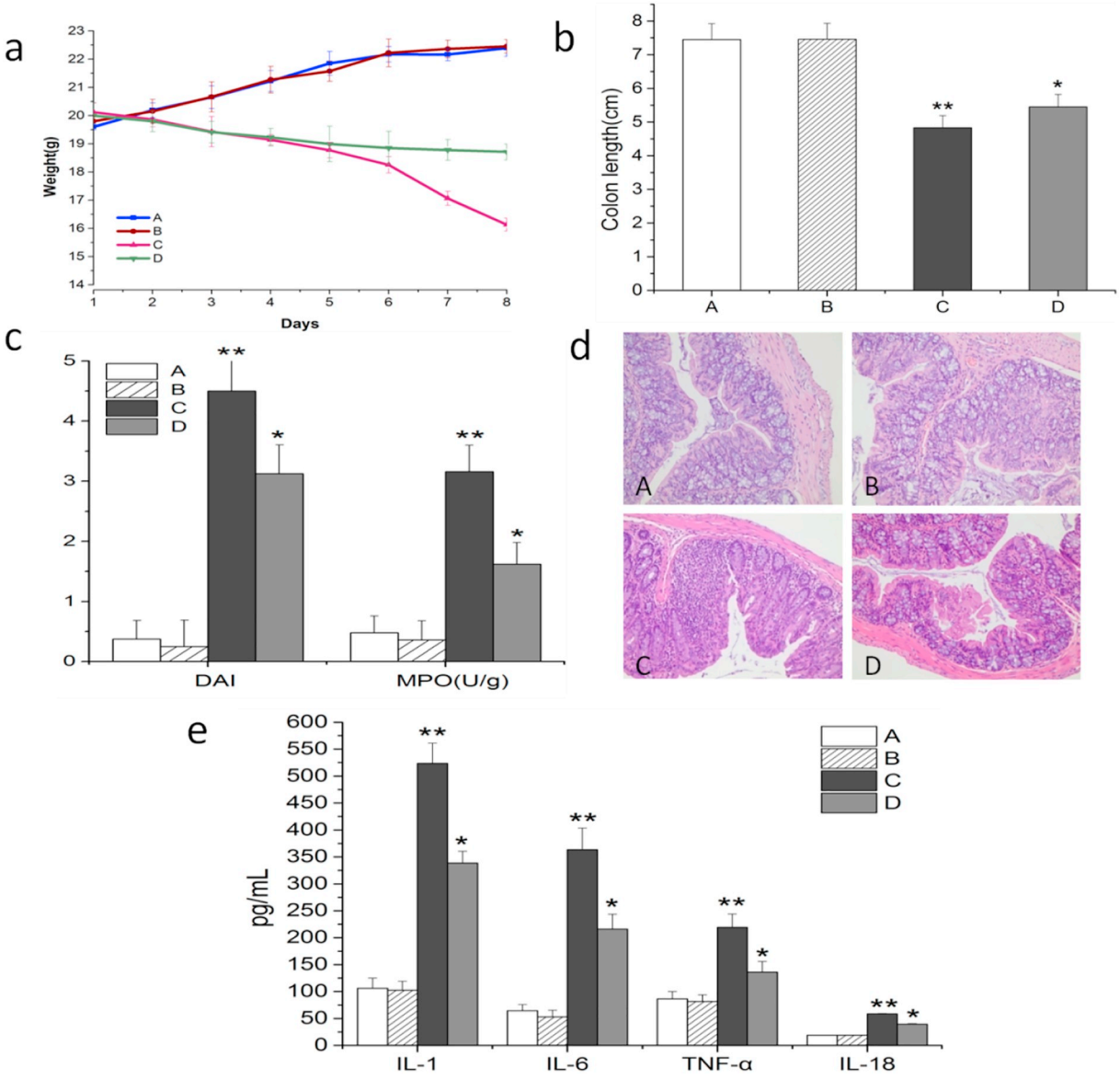

Fig. 1. Effects of PAP-1 on colitis in DSS-induced colitis mice ($n = 8$). a. The daily mean weight change in each group. b. The colon length in each group. c. The colonic DAI scoring and MPO activity in each group. d. The colonic histological findings in each group (HE × 20). e. The levels of inflammatory cytokines in each group. A: normal control group; B: normal + PAP-1injection group; C: DSS model group; D: DSS model + PAP-1 injection group. Values were shown as the means ± SD, $^{**}P < 0.05$ *vs* normal control group, $^{*}P < 0.05$ *vs* DSS model group.

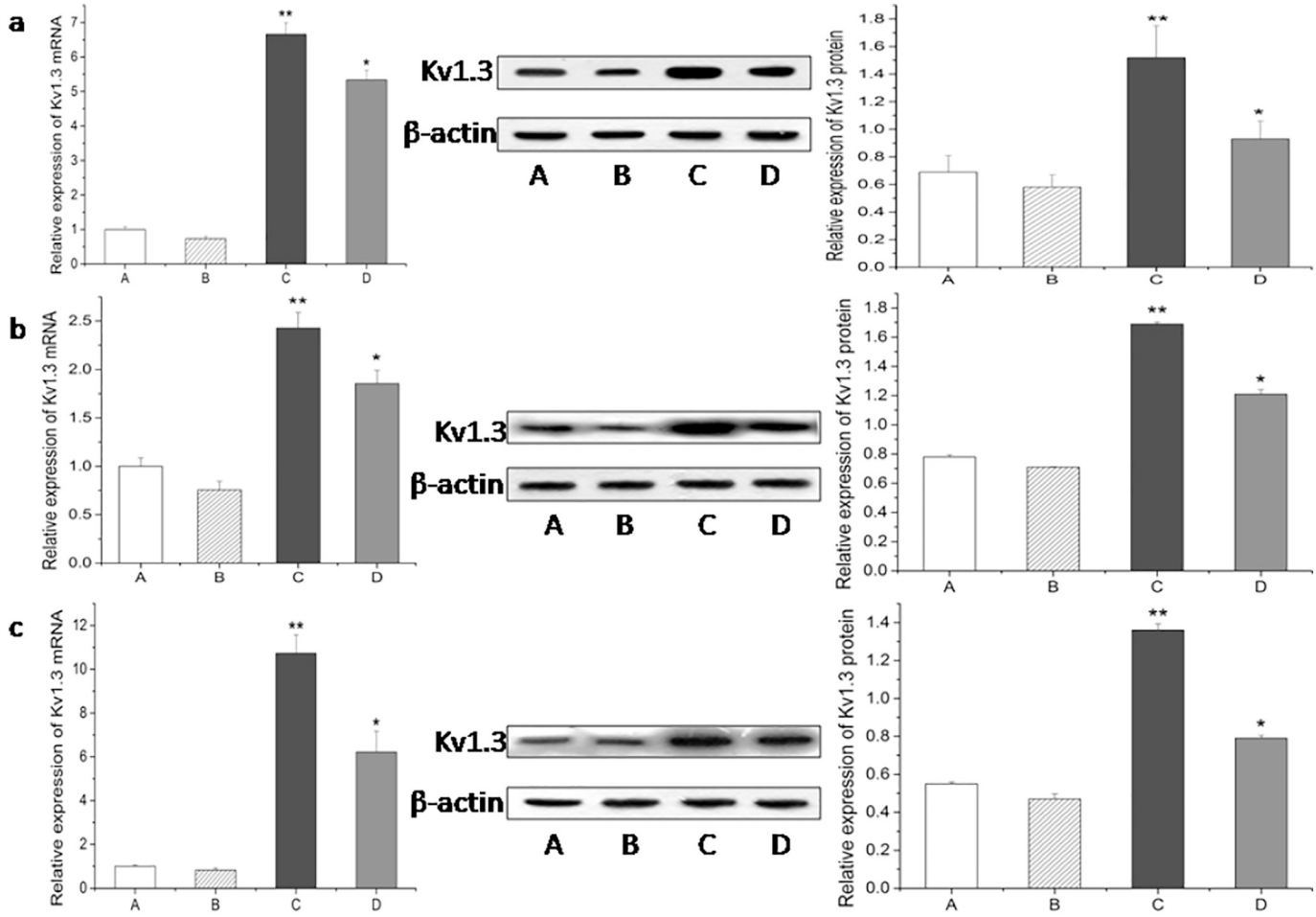

Fig. 2. Effects of PAP-1 on the expression of Kv1.3 in DSS-induced colitis mice ($n = 6$). (a) Relative Kv1.3 expression of colonic tissues was detected by RT-qPCR and western blotting. (b) Relative Kv1.3 expression of peritoneal macrophages was detected by RT-qPCR and western blotting. (c) Relative Kv1.3 expression of splenic macrophages was detected by RT-qPCR and western blotting. Data were expressed as fold changes relative to β-actin. A: normal control group; B: normal + PAP-1injection group; C: DSS model group; D: DSS model + PAP-1 injection group. Values were shown as the means ± SD, $^{**}P < 0.05$ *vs* normal control group, $^{*}P < 0.05$ *vs* DSS model group.

IL-1, IL-6, TNF-α and IL-18 in the colonic tissues were increased in DSS group and DSS + PAP-1injection group. However, DSS + PAP-1 injection group mice showed more obvious reduced level of the above pro-inflammatory cytokines compared with DSS group mice (Fig. 1e,f). The above evidence suggested that PAP-1 may ameliorate colonic damage in colitis by inhibiting the release of pro-inflammatory factors.

3.2. PAP-1 down-regulates Kv1.3 expression in DSS-induced colitis

By RT-qPCR and western blotting, we observed that Kv1.3 expression was enhanced significantly in the colon of mice treated with DSS, as compared to the normal control group. Interestingly, intraperitoneal injection of PAP-1 markedly decreased colonic Kv1.3 expression in DSS-induced colitis (Fig. 2a). Meanwhile, we found that increased Kv1.3 expression appeared on peritoneal macrophages and splenic macrophages in DSS-treated mice. Moreover, consistent with colonic Kv1.3 expression, PAP-1 treatment decreased the Kv1.3 expression of the above two types of macrophages in DSS-induced colitis mice (Fig. 2b; Fig. 2c).

3.3. PAP-1 diminishes macrophages activation

We observed that iNOS, a macrophage activation maker, was increased in colonic tissues consequent to DSS-induced injury as demonstrated by RT-qPCR and western blotting. Besides, DSS + PAP-1 injection group showed decreased iNOS expression in colonic tissues compared to DSS group (Fig. 3a). In addition, we found that increased iNOS expression appeared on peritoneal macrophages and splenic macrophages in DSS group. Moreover, PAP-1 treatment decreased iNOS expression of the above two types of macrophages in DSS-induced colitis mice (Fig. 3b and c). In sum, the above results suggested that PAP-1 diminishes macrophages activation in DSS-induced colitis.

3.4. PAP-1 inhibits NLRP3 inflammasome pathway

It has been reported that NLRP3 inflammasome plays an important role in the DSS-induced colitis [23].To investigate whether PAP-1 mediate protection from colitis through regulation of NLRP3 inflammasome, we tested the expression of NLRP3, ASC, caspase-1p20, pro-IL-1β and IL-1β in colon samples by western blotting. The results showed that PAP-1 effectively suppressed NLRP3, ASC, caspase-1p20, pro-IL-1β and IL-1β expression in the colon of DSS-treated mice (Fig. 4a). For further study, the expression of NLRP3, caspase-1p20 and IL-1β were also examined in peritoneal macrophages and splenic macrophages. Surprisingly, consistent with protein changes in the colon, PAP-1 also decreased NLRP3, caspase-1p20 and IL-1β protein expression significantly in peritoneal macrophages and splenic macrophages in DSS-induced colitis (Fig. 4b and c). It has been reported that Caspase-1 cleavage is a key step for NLRP3 inflammasome activation [9]. And caspase-1p20 is an important part of the active cleaved Caspase-1, which is required to cleave the pro-IL-1β into mature IL-1β

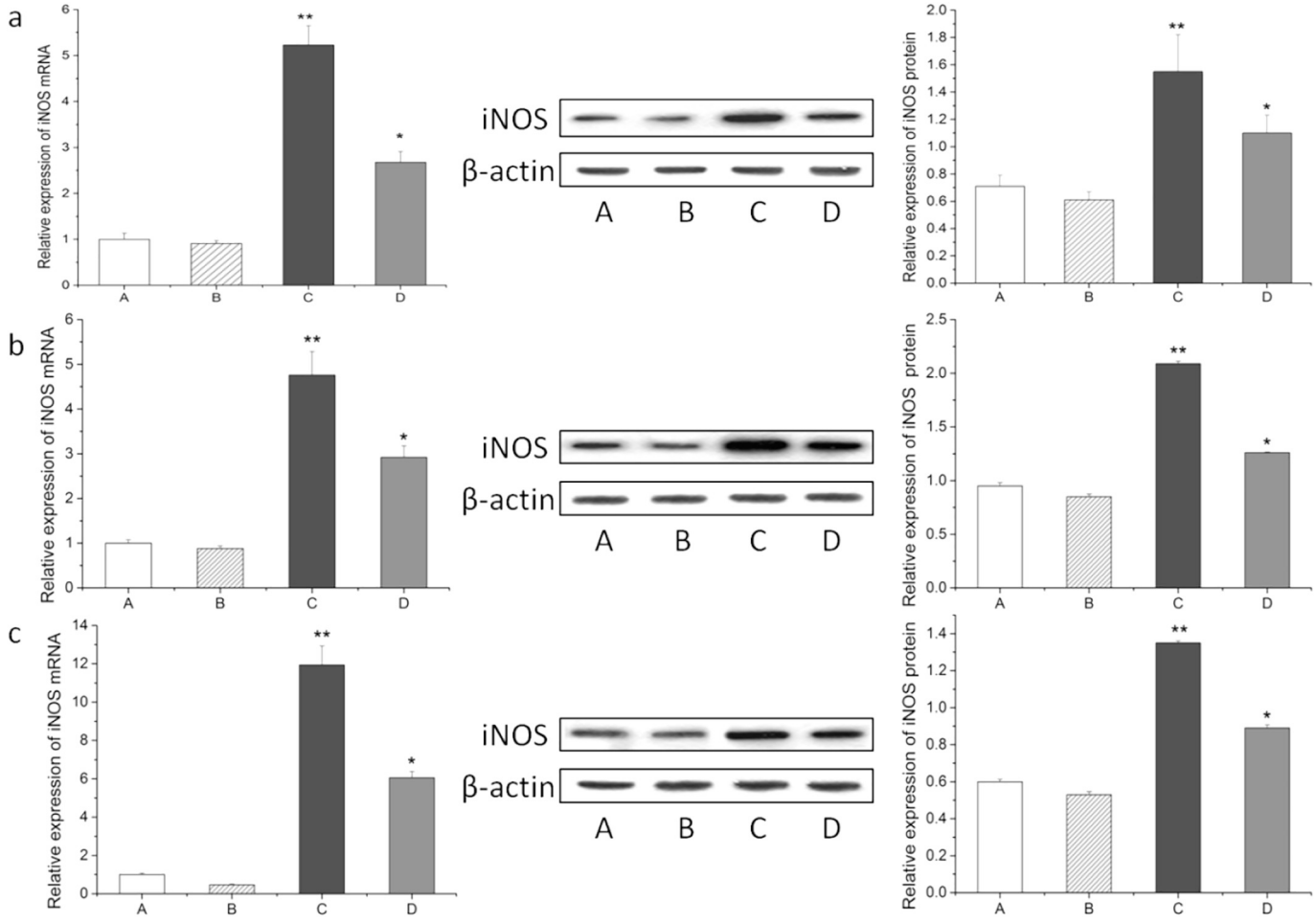

Fig. 3. Effects of PAP-1 on the expression of iNOS in DSS-induced colitis mice (n = 6). (a) Relative iNOS expression of colonic tissues was detected by RT-qPCR and western blotting. (b) Relative iNOS expression of peritoneal macrophages was detected by RT-qPCR and western blotting. (c) Relative iNOS expression of splenic macrophages was detected by RT-qPCR and western blotting. Data were expressed as fold changes relative to β-actin. A: normal control group; B: normal + PAP-1injection group; C: DSS model group; D: DSS model + PAP-1 injection group. Values were shown as the means ± SD, $^{**}P < 0.05$ *vs* normal control group, $^{*}P < 0.05$ *vs* DSS model group.

depending on NLRP3 inflammasome [24]. Therefore, PAP-1 could inhibit NLRP3 inflammasome protein expression and activation, as well as its downstream inflammatory cytokine release.

4. Discussion

IBD refers to immunologically mediated intestinal disorders characterized by recurring mucosal inflammation of the gastrointestinal tract [25].The clear aetiology and pathogenesis of IBD is still unknown. Although 5-ASA, glucocorticosteroids, immunomodulatory agents and biological agents are mainstay therapies for colitis in the clinic, many problems of serious side effects, poor-responders and lack of sustained efficacy often occur [26,27]. So it is highly significant to explore therapeutic agents with high efficiency and few side effects for patients with IBD. Classical DSS-induced acute colitis model is most commonly used for colitis studies and particularly useful to study the contribution of innate immune mechanisms in IBD [28–30]. In this study, we for the first time adopted the intraperitoneal injection of the Kv1.3 blocker PAP-1 in DSS-induced colitis mice and found that PAP-1 could alleviate the acute colitis in mice through inhibiting NLRP3 pathway. PAP-1 was always used to study T_{EM} -mediated inflammatory reaction [16]. So far, there have been few studies on the effects of PAP-1 on innate immune responses. This study showed PAP-1effectively mitigated the inflammatory responses in mice colitis, which was confirmed by the clinical symptoms and histopathological manifestation of mice. In addition, PAP-1 administration reduced the production of pro-inflammatory cytokines (IL-1, IL-6, TNF-α, IL-18 and IL-1β) and MPO activity. In the colonic mucosa of IBD patients, there were many macrophages playing vital roles in maintaining intestinal homeostasis. They could secrete many pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β), which triggered robust inflammatory reactions. And the down-regulation of these pro-inflammatory cytokines alleviated the occurrence and development of IBD [31,32]. Hence, the above results suggested that PAP-1 might be beneficial for the cure of colitis.

The innate immune system not only resisted exogenous bacteria and viruses, but also promoted inflammation [33]. Inflammasome was an important part of them and involved in the evolvement of various diseases. In particular, NLRP3 inflammasome was most widely studied [34]. Activated NLRP3 inflammasome was oligomerized by NLRP3 and ASC, which in turn recruited pro-Caspases-1 to active Caspase-1 as required to transform pro-IL-1β into mature active IL-1β. The IL-1β played a very important role in intestinal inflammation among multiple cytokines [35]. Overactivation of NLRP3 inflammasome has been shown to play a crucial role in various inflammatory diseases, such as atherosclerosis [36], Alzheimer's disease [37], and diabetes [38]. And NLRP3 inflammasome also played an important role in the pathogenesis of IBD [39,40]. Previous studies have shown that in patients and animal models, the expressions of NLRP3, ASC, and caspase-1 were evidently increased in colonic tissues of colitis; Moreover,

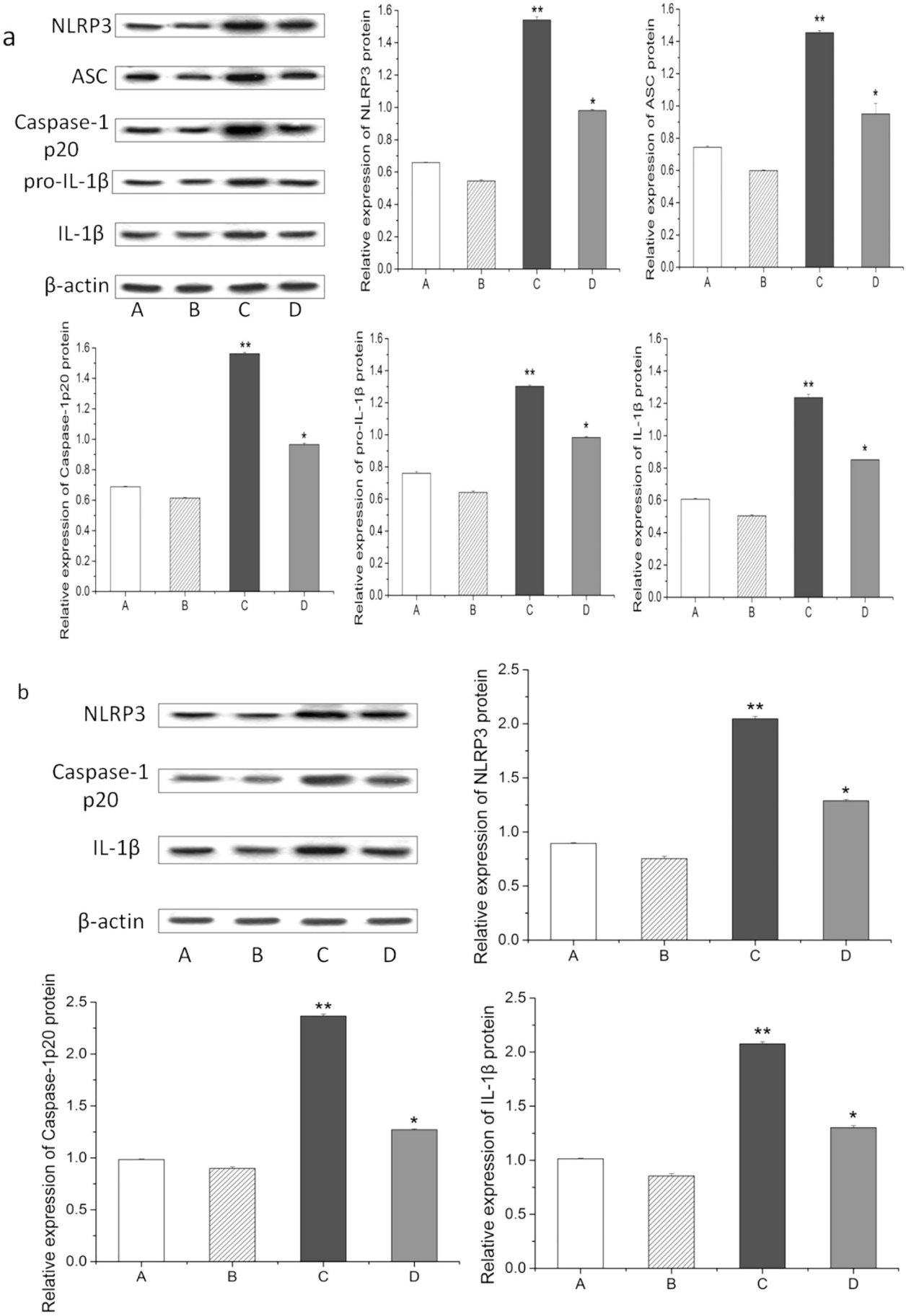

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Fig. 4. Effects of PAP-1 on NLRP3 inflammasome pathway in DSS-induced colitis mice (n = 6). (a)Relative expression of NLRP3, ASC, caspase-1p20, pro-IL-1β and IL-1β in colonic tissues were determined by western blotting. (b)Relative expression of NLRP3, caspase-1p20 and IL-1β in peritoneal macrophages were determined by western blotting. (c) Relative expression of NLRP3, caspase-1p20 and IL-1β in splenic macrophages were determined by western blotting. Data were expressed as fold changes relative to β-actin. A: normal control group; B: normal + PAP-1injection group; C: DSS model group; D: DSS model + PAP-1 injection group. Values were shown as the means ± SD, $^{**}P < 0.05$ *vs* normal control group, $^{*}P < 0.05$ *vs* DSS model group.

NLRP3, ASC, and caspase-1 knockout mice presented marked mitigation in the disease severities of colitis. Most notably, inhibition of the NLRP3 inflammasome pathway could reduce the severity of colitis [40]. Furthermore, Potassium ion was an essential mediator in NLRP3 inflammasome activation [41]. Intracellular low potassium induced by potassium ions efflux through potassium channels played an important role in the activation of NLRP3 inflammasome in turn, the suppression of potassium ions outflow inhibited the activation of NLRP3 inflammasome [42]. It has been shown that K + efflux might be a common feature required for NLRP3 inflammasome activation [10]. Previous studies also reported that the NLRP3 inflammasome might be an effective downstream molecule of Kv1.3 channel in hypertension development, and that blockage of the Kv1.3 channel could inhibit the activation of the NLRP3 inflammasome in hypertensive patients [11]. In this study, the specific Kv1.3 blocker PAP-1 showed great potential in the inhibition of the expressions of Kv1.3, NLRP3, ASC, Caspases-1p20, pro-IL-1β and IL-1β in colon with colitis, suggesting that PAP-1 could inhibit colonic NLRP3 inflammasome activation in colitis.

It is widely acknowledged that epithelial barrier disruption in IBD causes bacterial antigens invading into the mucosal layer, resulting in activation of the mucosal immune response [43]. During this process, macrophages played an important role because they could produce large amounts of inflammatory cytokines, such as IL-1, IL-6, and TNF-α, which further exacerbated the severity of colitis [44]. NLRP3 and ASC complex was mainly expressed in these primary macrophages [45], which were activated in DSS-induced mouse model of colitis [46]. The activated macrophages could secrete high levels of IL-1β, which was associated with colitis activity and its severity in patients and DSS-induced colitis mice [47,48]. A previous study pointed that Kv1.3 could be involved in macrophages apoptosis [49], macrophages migration [50], macrophages proliferation and activation [51,52]. Furthermore, it also suggested that Kv1.3 might become a novel target for the treatment of immune-related inflammatory diseases, such as asthma [53] and atherosclerosis [54]. Therefore, two types of primary macrophages isolated from each group of mice, including peritoneal macrophages and splenic macrophages, were used in this study. Similar to the findings in the colon, PAP-1 inhibited the expressions of Kv1.3, NLRP3, Caspases-1p20, IL-1β and iNOS in peritoneal macrophages and splenic macrophages, indicating that PAP-1 inhibited NLRP3 inflammasome activation in macrophages of colitis mice and reduced the activation of macrophages themselves.

In conclusion, PAP-1 could ameliorate DSS-induced colitis. The activation of NLRP3 inflammasome and macrophages played an important role in DSS-induced colonic inflammation damage. PAP-1 might

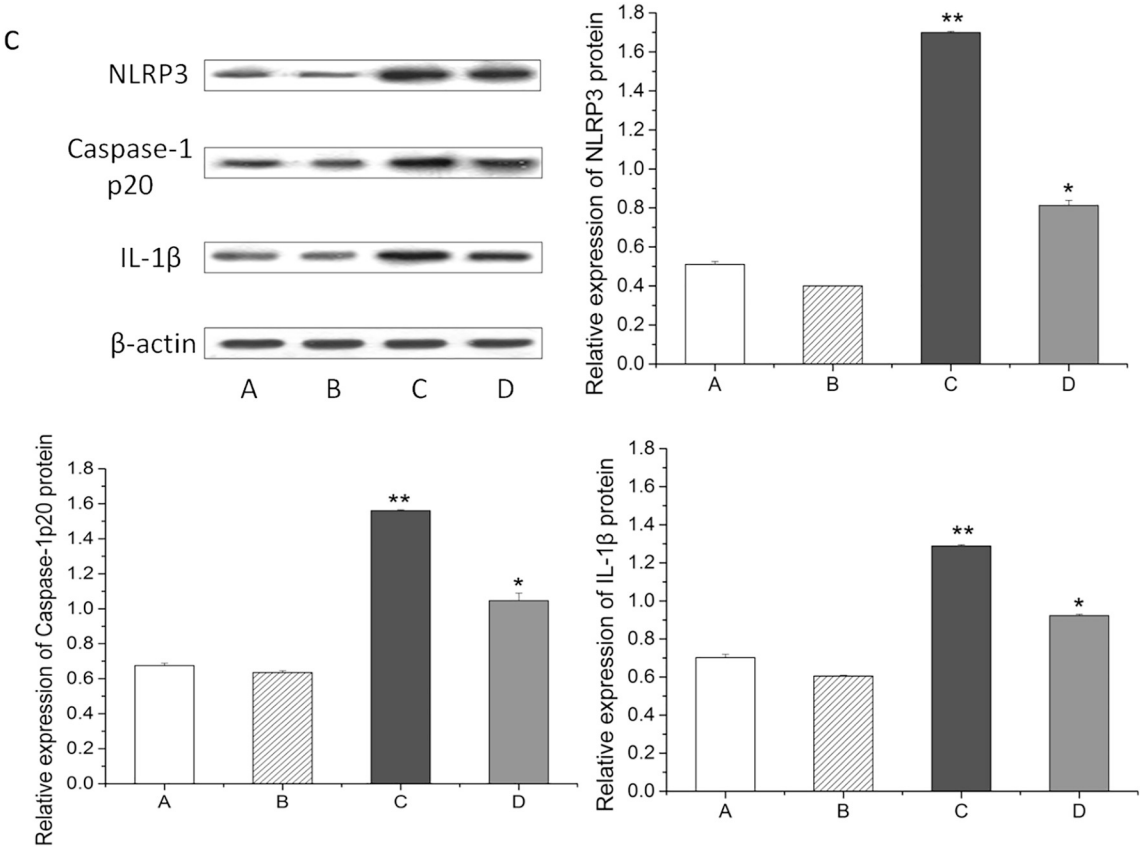

Fig. 4. (*continued*)

mitigate the severity of colitis in mice *via* inhibition of NLRP3 inflammasome pathway and macrophages activation.

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Author's contributions

YM, *CF* and SD performed the animal, cellular and molecular experiments. YM and XL analyzed the experimental data and wrote the manuscript. JH, JX and QM designed the experiments. XL and QM revised the manuscript and provided financial support.

Declaration of Competing Interest

None confliction.

Annex 1

	Means + SD	1	2	3	4	5	6
Fig. 2(Kv1.3)							
a/RT-qPCR	A:1 ± 0.08	1.13	0.91	0.99	0.92	1.04	1.04
	B:0.73 ± 0.07	0.68	0.84	0.76	0.69	0.81	0.66
	C:6.66 ± 0.33	7.14	6.89	6.52	6.17	6.66	6.61
	D:5.34 ± 0.27	5.09	5.09	5.57	5.72	5.41	5.19
a/western blot	A:0.70 ± 0.005	0.699	0.692	0.702	0.704	0.697	0.695
	B:0.58 ± 0.13	0.599	0.574	0.58	0.569	0.593	0.587
	C:1.52 ± 0.022	1.545	1.5	1.52	1.498	1.544	1.524
	D:0.93 ± 0.004	0.933	0.939	0.931	0.935	0.928	0.936
b/RT-qPCR	A:1 ± 0.09	1.01	1	1.08	1.11	0.89	0.92
	B:0.76 ± 0.09	0.71	0.67	0.85	0.88	0.68	0.77
	C:2.43 ± 0.16	2.58	2.38	2.26	2.67	2.39	2.31
	D:1.86 ± 0.13	1.8	1.99	1.99	1.74	1.68	1.96
b/western blot	A:0.80 ± 0.015	0.782	0.81	0.803	0.814	0.787	0.792
	B:0.72 ± 0.003	0.714	0.717	0.72	0.72	0.718	0.715
	C:1.69 ± 0.013	1.687	1.678	1.703	0.691	1.701	1.674
	D:1.22 ± 0.031	1.21	1.201	1.258	1.235	1.245	1.189
c/RT-qPCR	A:1 ± 0.06	0.93	0.94	1.06	0.98	1.08	1.01
	B:0.83 ± 0.09	0.92	0.84	0.95	0.76	0.77	0.74
	C:10.73 ± 0.83	10.48	11.16	11	9.51	11.96	10.41
	D:6.22 ± 0.95	6.79	7.03	5.18	5.4	5.79	7.48
c/western blot	A:0.55 ± 0.010	0.548	0.563	0.543	0.554	0.539	0.559
	B:0.48 ± 0.026	0.464	0.505	0.458	0.488	0.477	0.494
	C:1.36 ± 0.032	1.34	1.399	1.347	1.325	1.384	1.377
	D:0.79 ± 0.015	0.785	0.813	0.787	0.776	0.801	0.805
Fig. 3(iNOS)							
a/RT-qPCR	A:1 ± 0.13	1.04	0.85	0.85	1.08	1.11	1.12
	B:0.91 ± 0.06	0.97	0.94	0.82	0.86	0.99	0.9
	C:5.23 ± 0.42	4.83	5.75	4.97	4.9	5.75	5.25
	D:2.68 ± 0.23	2.39	2.58	2.51	2.96	2.82	2.86
a/western blot	A:0.71 ± 0.011	0.7	0.723	0.71	0.699	0.722	0.712
	B:0.6 ± 0.007	0.612	0.6	0.6	0.607	0.608	0.596
	C:1.55 ± 0.010	1.554	1.545	1.565	1.556	1.563	1.544
	D:1.1 ± 0.005	1.098	1.108	1.1	1.104	1.105	1.096
b/RT-qPCR	A:1 ± 0.08	0.93	1.09	0.97	1.02	0.91	1.1
	B:0.88 ± 0.06	0.8	0.9	0.85	0.95	0.92	0.84
	C:4.76 ± 0.53	5.45	4.25	5.27	4.13	4.68	4.95
	D:2.92 ± 0.26	2.81	3.11	2.99	3.29	2.84	2.55
b/western blot	A:0.95 ± 0.032	0.924	0.987	0.95	0.983	0.92	0.958
	B:0.86 ± 0.026	0.832	0.883	0.85	0.879	0.827	O.861
	C:2.08 ± 0.024	2.092	2.051	2.095	2.107	2.066	2.064
	D:1.26 ± 0.006	1.263	1.251	1.257	1.257	1.252	1.264
c/RT-qPCR	A:1 ± 0.08	1.08	1.06	0.92	0.99	0.9	1.07
	B:0.46 ± 0.05	0.46	0.49	0.37	0.48	0.45	0.5
	C:11.94 ± 0.99	13.44	12.45	12.2	12.03	10.69	11.07
	D:6.06 ± 0.33	6.15	6.03	5.66	6.64	6.07	5.86
c/western blot	A:0.61 ± 0.013	0.604	0.623	0.598	0.612	0.594	0.619
	B:0.53 ± 0.016	0.52	0.549	0.522	0.539	0.538	0.511
	C:1.35 ± 0.010	1.344	1.362	1.346	1.339	1.356	1.359
	D:0.89 ± 0.017	0.88	0.91	0.881	0.9	0.9	0.871
Fig. 4							
a/NLRP3	A:0.66 ± 0.003	0.657	0.662	0.658	0.656	0.661	0.661
	B:0.54 ± 0.009	0.538	0.554	0.54	0.547	0.55	0.534
	C:1.54 ± 0.020	1.521	1.56	1.54	1.52	1.558	1.54
	D:0.98 ± 0.007	0.977	0.977	0.989	0.985	0.984	0.973
a/ASC	A:0.74 ± 0.007	0.741	0.739	0.753	0.746	0.735	0.749
	B:0.60 ± 0.003	0.601	0.601	0.596	0.598	0.602	0.597
	C:1.45 ± 0.011	1.466	1.444	1.453	1.463	1.442	1.455
	D:0.95 ± 0.066	0.916	0.909	1.026	0.985	0.874	0.991

a/Caspase-1p20	A:0.69 ± 0.003	0.69	0.691	0.685	0.689	0.687	0.693
	B:0.61 ± 0.005	0.618	0.608	0.613	0.613	0.617	0.608
	C:1.56 ± 0.010	1.573	1.552	1.561	1.563	1.551	1.571
	D:0.97 ± 0.010	0.959	0.977	0.96	0.971	0.954	0.97
a/pro-IL-1β	A:0.76 ± 0.010	0.752	0.771	0.76	0.751	0.77	0.762
	B:0.64 ± 0.010	0.647	0.646	0.63	6.51	0.635	0.635
	C:1.30 ± 0.008	1.31	1.295	1.3	1.293	1.304	1.31
	D:0.98 ± 0.006	0.979	0.99	0.981	0.985	0.975	0.987
a/IL-1β	A:0.61 ± 0.006	0.6	0.611	0.611	0.614	0.604	0.603
	B:0.50 ± 0.007	0.512	0.498	0.501	0.409	0.496	0.507
	C:1.24 ± 0.020	1.258	1.219	1.23	1.253	1.243	1.214
	D:0.85 ± 0.001	0.849	0.852	0.85	0.85	0.85	0.848
b/NLRP3	A:0.89 ± 0.008	0.888	0.902	0.89	0.884	0.897	0.896
	B:0.75 ± 0.021	0.735	0.776	0.75	0.773	0.732	0.757
	C:2.05 ± 0.024	2.072	2.026	2.04	2.021	2.067	2.052
	D:1.29 ± 0.013	1.274	1.301	1.29	1.276	1.302	1.286
b/Caspase-1p20	A:0.98 ± 0.005	0.989	0.985	0.978	0.979	0.982	0.99
	B:0.90 ± 0.015	0.91	0.905	0.881	0.889	0.893	0.917
	C:2.37 ± 0.020	2.378	2.344	2.377	2.355	2.355	2.388
	D:1.27 ± 0.008	1.266	1.281	1.27	1.263	1.273	1.278
b/IL-1β	A:1.01 ± 0.005	1.017	1.017	1.008	1.01	1.02	1.011
	B:0.86 ± 0.022	0.88	0.837	0.85	0.833	0.862	0.875
	C:2.08 ± 0.020	2.098	2.059	2.07	2.083	2.054	2.093
	D:1.30 ± 0.019	1.321	1.283	1.3	1.301	1.281	1.319
c/NLRP3	A:0.51 ± 0.015	0.497	0.527	0.507	0.523	0.514	0.493
	B:0.40 ± 0.001	0.4	0.401	0.399	0.402	0.399	0.4
	C:1.70 ± 0.006	1.704	1.693	1.7	1.705	1.698	1.695
	D:0.81 ± 0.027	0.834	0.821	0.782	0.802	0.79	0.842
c/Caspase-1p20	A:0.68 ± 0.013	0.691	0.67	0.666	0.682	0.662	0.686
	B:0.64 ± 0.010	0.647	0.628	0.633	0.644	0.626	0.639
	C:1.56 ± 0.050	1.558	1.566	1.557	1.554	1.563	1.561
	D:1.05 ± 0.040	1.096	1.023	1.018	1.07	1.074	0.996
c/IL-1β	A:0.70 ± 0.017	0.718	0.706	0.685	0.7	0.687	0.721
	B:0.61 ± 0.005	0.605	0.61	0.601	0.6	0.609	0.606
	C:1.29 ± 0.006	1.292	1.293	1.281	1.285	1.297	1.287
	D:0.92 ± 0.008	0.929	0.914	0.924	0.914	0.93	0.921

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