ISSN: 2689-8616

Research Article

Advancements in Journal of Urology and Nephrology

Methylprednisolone Protects Severe Acute Pancreatitis And Pancreatitis-Associated Acute Lung Injury in Mice By Inhibiting NLRP3 Inflammasome Through NF-κB

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Submitted: 17 Mar 2022; **Accepted:** 23 Mar 2022; **Published:** 30 Mar 2022

Citation: *Chuan-jiang Liu, Chao He, Qiang Fu, Pan Liu, Peng Xia, Xu Zhang, Rui Chen, Zhen-sheng Zhai, Yu-zhu Wang, Tao Qin, Wenjing Zhou, Hong-wei Zhang. (2022). Methylprednisolone protects severe acute pancreatitis and pancreatitis-associated acute lung injury in mice by inhibiting NLRP3 inflammasome through NF-κB. Adv J Uro Nephro, 4(1), 23-31.*

Abstract

In serve acute pancreatitis (SAP), the rapid production and releasing of inflammatory cytokines can cause local and systemic excessive inflammation, especially pancreatitis-associated acute lung injury (P-ALI). Methylprednisolone (MP) is a synthetic corticosteroid with potent anti-inflammatory and antioxidant properties used as therapy for a variety of diseases. In this study, we found MP, used in the early phase of SAP, decreased the levels of IL-1β and TNF-α in serum and peritoneal lavage fluids (PLF), reduced the level of serum amylase and the expression of MPO in lung tissue, attenuated the pathological injury of the pancreas and lungs in a dose-dependent manner. The expression of NLRP3 and IL-1β in pancreas and lungs was down regulated significantly depending on the MP concentration. In vitro, MP reduced the levels of IL-1β and TNF-α by down regulating the expression of NLRP3, IL-1β and p-NF-κB in isolated peritoneal macrophages. Taken together, MP can attenuate the injury of pancreas and lungs, and the inflammatory response in SAP mice by down regulating the activation of NF-κB and the NLRP3 inflammasome.

Keywords: Severe Acute Pancreatitis, Pancreatitis-Associated Acute Lung Injury, Methylprednisolone, Nlrp3, Nf-Kb

Introduction

SAP, with approximately 15%-20% mortality, is one of the clinically acute severe illnesses that includes local pancreatic inflammation and excessive systemic inflammatory response syndrome, which can lead to multiple organ dysfunction syndrome (MODS) [1]. Epidemiological data show that the incidence of SAP has increased in recent years [2]. Pancreatitis-associated acute lung injury (P-ALI) is the most common and severe complication of SAP. P-ALI can develop into acute respiratory distress syndrome (ARDS), which is the leading cause of early death in SAP patients [3,4].

Although the pathogenesis of SAP has not been thoroughly studied, it is widely accepted that the severity of SAP is determined by the injury of the pancreas and other organs, inflammatory response, and neutrophil infiltration [5,6]. Therefore, reducing the damage of local pancreatic inflammation and excessive systemic inflammatory response may be an effective treatment of SAP.

MP is a synthetic corticosteroid with potent anti-inflammatory and antioxidant properties used as therapy for a variety of diseases. MP can alleviate spinal cord injury by suppressing post-traumatic inflammatory activity through the TNF-α-NF-κB cascade in rats [7]. A previous study suggested that intravenous MP therapy contributed to a favorable prognosis of pancreatitis systemic lupus erythematosus [8]. In this study, we investigate the protective role of MP in the pancreas and lungs of an early stage SAP mouse model was established by intraperitoneal injection of caerulein (Cae) plus lipopolysaccharide (LPS). The underlying mechanisms were explored in vivo and in vitro.

Materials and Methods

Animal Care

All animal experiments were approved by the Animal Research Ethics Committee of Henan Provincial People's Hospital, Zhengzhou, China. All operations were executed under chloral hydrate anesthesia, and all efforts were made to minimize suffering.

In vivo SAP Model

Twenty-four male C57BL/6 mice (6-8 weeks) were divided into four groups (sham group, Cae+LPS group, Cae+LPS+10 mg/kg MP group and Cae+LPS+20 mg/kg MP group) randomly (n=6). All mice were anesthetized with 10% chloral hydrate (300 mg/kg, i.p.). The SAP mouse model was established by administering an intraperitoneal injection of Cae (Sigma, America) for 12 h (50 μg/kg in 0.2 ml saline hourly) and a dose of LPS (Sigma, America) (10 mg/kg in 0.2 ml saline) after Cae. MP (Selleck, America) was administered via tail vein injection for 10 mg/kg or 20 mg/kg after the first Cae injection. All of the mice were sacrificed 12 h later. The pancreas and lungs were isolated, fixed in 4% Formalin, and embedded in paraffin according to standard practices. Blood serum and PLF were collected for the detection of IL-1β, TNF-α and amylase.

Culture and Treatment of Peritoneal Macrophages

Peritoneal macrophages were isolated and grown in DMEM medium (Gibco, America) supplemented with 10% fetal bovine serum (Gibco, America) in a humidified incubator at 37 °C with an atmosphere of 5% CO2. Peritoneal macrophages were plated in 12-well plates at a density of 1×10^6/well with medium containing 50 ng/ml LPS. The following morning, the media was replaced with different doses of MP for 3 h. Then, the cells were stimulated with ATP (5 mM) for 1 h, nigericin (10 μM) for 30 min or MSU (10 μg/ml) for 3 h. Cells and supernatants were collected for qRT-PCR, Western Blotting and enzyme-linked immunosorbent assay (ELISA).

H&E Staining

The pancreatic and lung tissues were sectioned into 5 μm sections and stained with hematoxylin and eosin after fixation. The extent of acinar cell injury and necrosis was quantified by computer-morphological examination by experienced morphologists in a blinded manner. We chose 10 microscopic fields randomly for each tissue sample.

Elisa

ELISA was performed to detect the concentrations of IL-1β and TNF-α in the serum and PLF following the manufacturer's instructions.

Amylase detection

The amylase assay kit (Jiancheng Bio, Nanjing, China) was used to measure the amylase expression levels of serum and PLF following the manufacturer's instructions.

Western Blot

Total protein was extracted by RIPA Lysis Buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), according to the manufacturer's instructions (Beyotime Bio, Wuhan, China). Protein lysates, 40 μg, from each sample were loaded and separated on a 12% SDS polyacrylamide gel. Proteins were electrophoretically transferred onto a PVDF membrane (Millipore, Bedford, MA, USA), and incubated with anti-mouse monoclonal NLRP3, NF-κB, pNF-κB and β-actin antibody at 1/1000 (CST, America) at 4°C overnight. On the following day, membranes were incubated with HRP secondary antibodies (1:5000) at 37°C for 2 hours, and signals were visualized with an electrochemiluminescence kit (Pierce, Rockford, IL, USA).

qRT-PCR

Total RNAs were isolated by using TRIzol reagent (Invitrogen, America) and then reverse-transcribed into cDNAs with PrimeScript RT Master Mix (Takara, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the SYBR Premix Ex TaqTM kit (Takara, Japan). Specific primer sequences are listed below: NLRP3 (forward) 5'- TTGAAGATGTGGACCTCAAG-3' and NLRP3 (reverse) 5'- CAATCATGAGTGTGGCTAGA-3'. IL-1β (forward) 5'-CACAGAGGATACCACTCCCAACA-3' and IL-1β (reverse) 5'- TCCACGATTTCCCAGAGAACA-3'. TNF-α (forward) 5'- CCTGTAGCCCACGTCGTAG-3' and TNF-α (reverse) 5'-CGTCATACTCCTGCTTGCTG-3'. β-actin (forward) 5'- GTACGCCAACACAGTGCTG-3' and β-actin (reverse) 5'- CGTCATACTCCTGCTTGCTG-3'. Expression of NLRP3 and IL-1β, relative to β-actin, was determined using the 2-ΔΔCT method.

Statistical Analysis

The results were shown as the mean ± SD from at least three separate experiments. Statistical analysis was performed using SPSS 19.0 software and comparisons were made using Student's t-test and one-way ANOVA. $P<0.05$ was considered statistically significant.

Results

Effect of MP on pancreatic histopathological damage, water content and levels of serum amylase in the SAP mouse model

As shown in Figure 1, MP attenuated the damage of pancreatic tissue in Cae + LPS induced SAP mice in a dose-dependent manner. H&E staining revealed standard histological examination of edema and necrosis, neutrophil infiltration, and hemorrhage in Cae + LPS induced SAP group, while these were markedly reduced in a dose-dependent manner in the Cae + LPS + MP 10 mg/kg and Cae + LPS + MP 20 mg/kg groups (Figure 1-A, B). Additionally, the water content of the Cae + LPS + MP 20 mg/kg group was significantly decreased compared with that of the Cae + LPS group (Figure 1-C). In addition, after intraperitoneal injection of 20 mg/kg MP, the levels of serum amylase were significantly reduced compared with the levels of the Cae + LPS group (Figure 1-D).

Figure 1

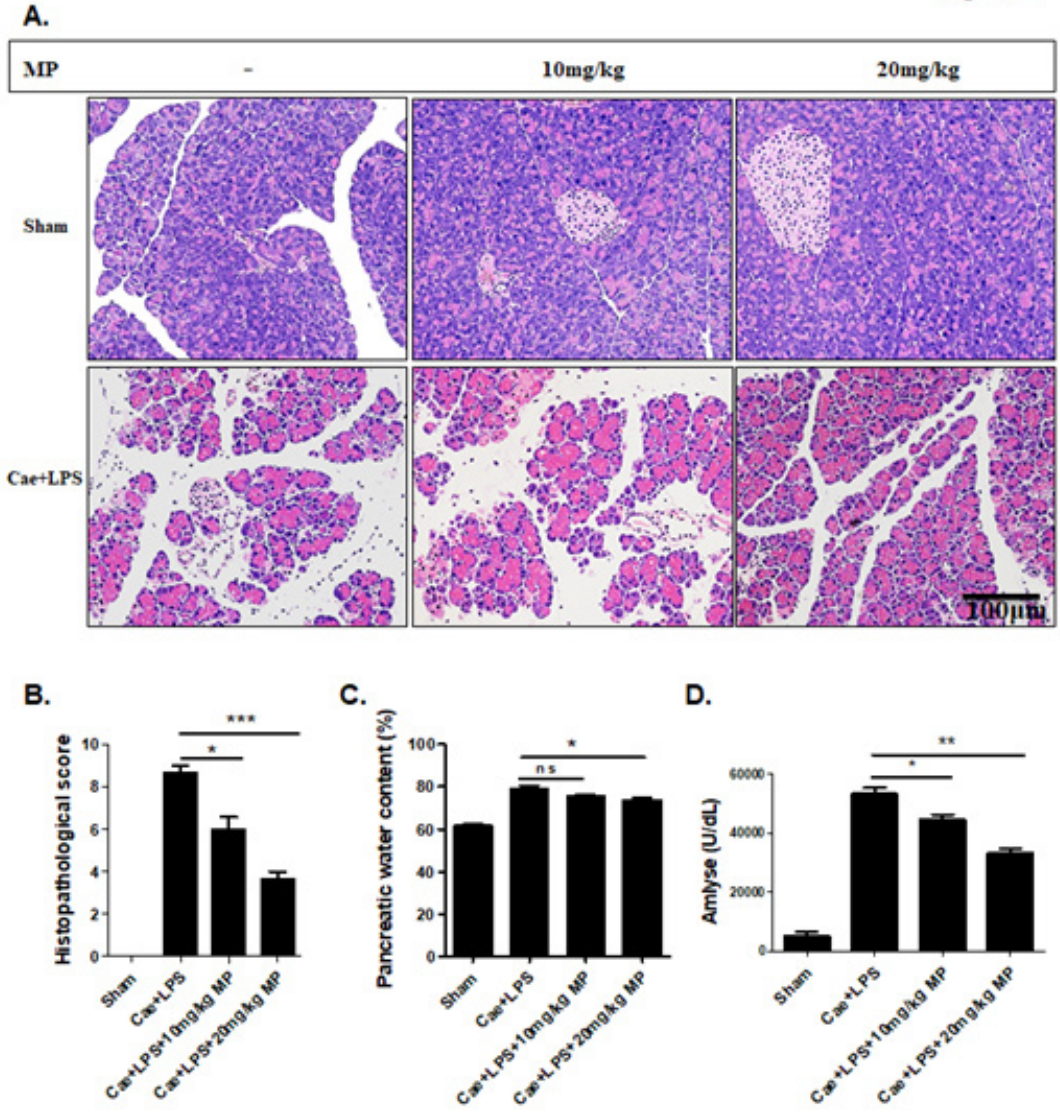

Figure 1: Effect of MP on the pancreatic histopathological damage and water content and levels of serum amylase in mice SAP model. (A) Typical HE staining of pancreatic tissue. (B) Histopathological score of pancreatic tissue. (C) Pancreatic water content percentage. (D) Levels of serum amylase were shown. nsP>0.05, *P<0.05, **P<0.01, ***P<0.001 vs Cae+LPS group.

Effect of MP on histopathological lung damage and MPO levels in the SAP mouse model

As shown in Figure 2, MP also alleviated lung injury in SAP mice induced by caerulein and LPS in a dose-dependent manner. Substantial neutrophil infiltration and congestion of alveolar septum capillaries were evident in the lungs of Cae + LPS mice. These histological features of lung injury in the MP groups were significantly reduced in a dose-dependent manner (Figure 2-A). The expression of MPO is considered a quantitative marker of neutrophil infiltration. We detected the expression of MPO in lung tissue by immunofluorescence. The results showed that the expression of MPO in the Cae + LPS + MP 10 mg/kg and the Cae + LPS + MP 20 mg/kg groups was markedly reduced compared to that in the Cae + LPS group (Figure 2-B).

Figure 2

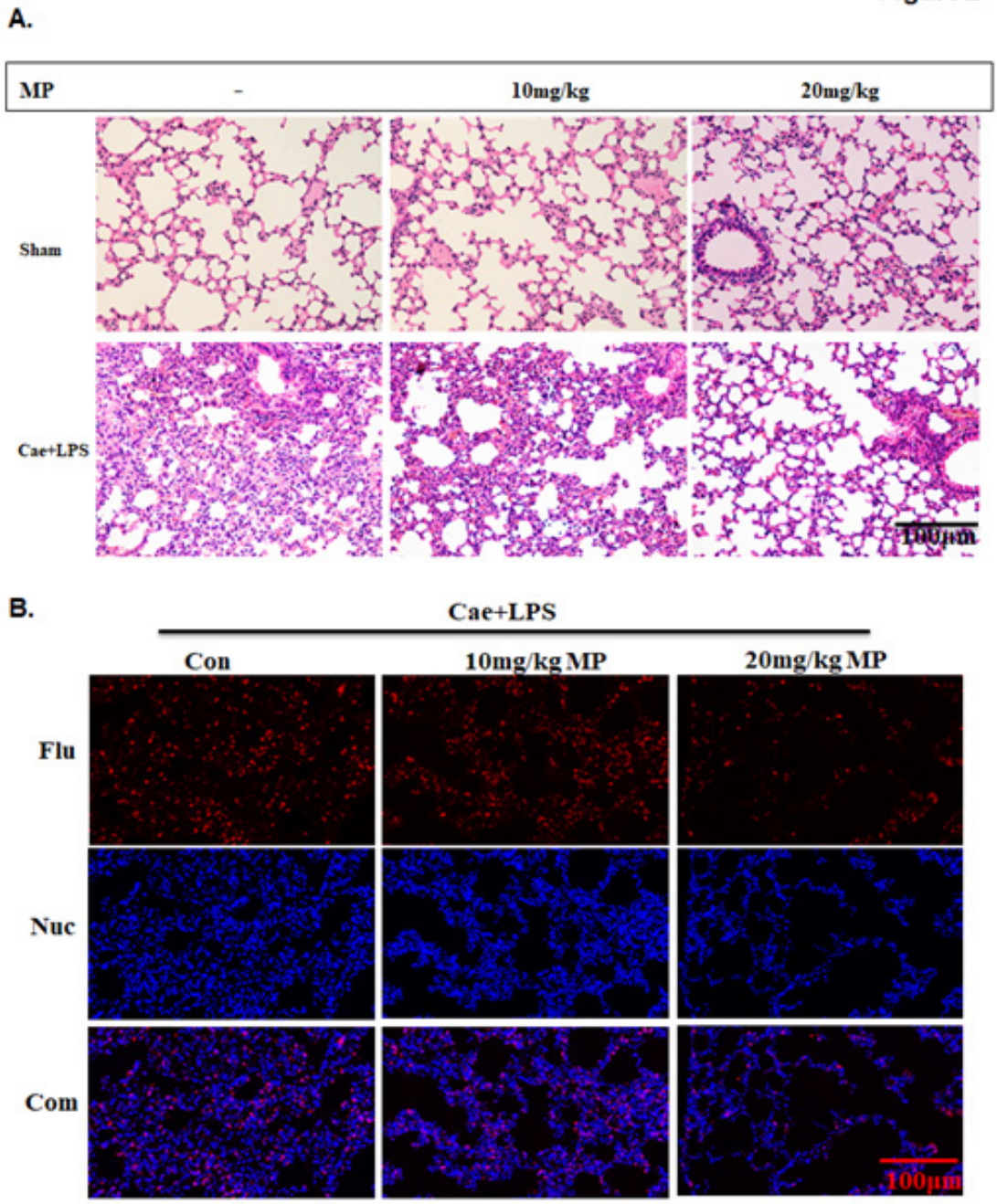

Figure 2: Effect of MP on the lung histopathological damage and MPO level in mice SAP model. (A) Typical HE staining of lung tissue. (B) Typical immunofluorescence of lung tissue.

MP Treatment Reduced the Levels of IL-1β and TNF-α in Serum and PLF

The levels of IL-1β and TNF-α in serum and PLF were used as markers of systemic inflammatory responses to SAP. The serum levels of cytokines such as IL-1β and TNF-α were detected by ELISA. As shown in Figure 3, the levels of IL-1β and TNF-α in serum were significantly lower in groups treated with 10 mg/kg and 20 mg/kg MP compared to the levels in the Cae + LPS group in a dose-dependent manner (Figure 3-A, B). Similar results were also obtained in PLF after SAP was induced (Figure 3-C, D).

Figure 3

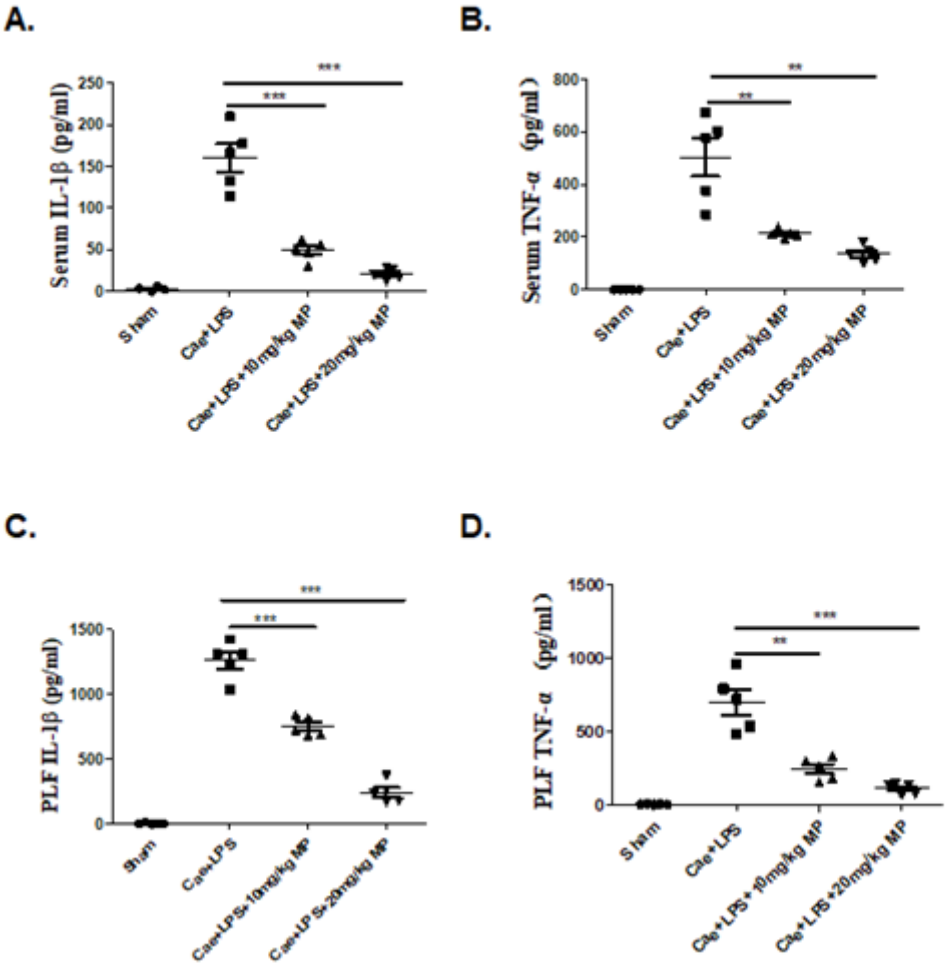

Figure 3: MP treatment could reduce the levels of IL-1β and TNF-α in serum and PLF. (A) ELISA-detected protein levels of Serum IL-1β. (B) ELISA-detected protein levels of Serum TNF-α. (C) ELISA-detected protein levels of PLF IL-1β. (D) ELISA-detected protein levels of PLF TNF-α. **P<0.01, ***P<0.001 vs Cae+LPS group.

Effect of MP on the expression levels of NLRP3, IL-1β and TNF-α in the pancreas and lungs in vivo

The NLRP3 inflammasome, IL-1β and TNF-α play an important role in SAP. Therefore, we detected the relative mRNA expression levels of NLRP3, IL-1β and TNF-α in pancreas and lungs by qRT-PCR and the protein levels of NLRP3 by Western Blot. As shown in Figure 4, MP treatment significantly reduced the mRNA expression levels of NLRP3, IL-1β and TNF-α in pancreatic tissue in a dose-dependent manner (Figure 4-A). Similar results were obtained in lung tissue (Figure 4-B). NLRP3 protein expression levels in pancreatic and lung tissue were significantly higher in the SAP mouse model while increasing doses of MP decreased NLRP3 levels (Figure 4-C).

Figure 4

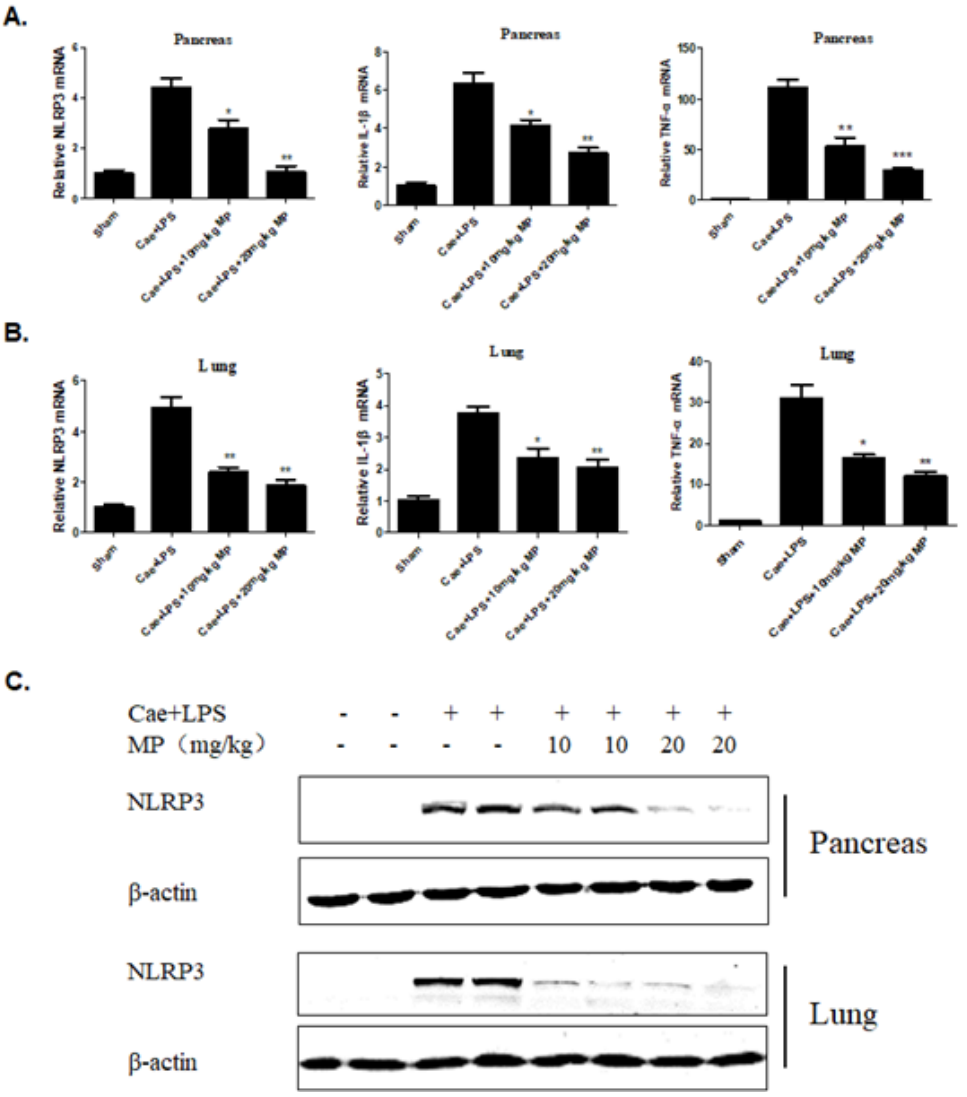

Figure 4: Effect of MP on the expression levels of NLRP3, IL-1β and TNF-α. (A) The mRNA expression levels of NLRP3, IL-1β and TNF-α of pancreas were detected by qRT-PCR. (B) The mRNA expression levels of NLRP3, IL-1β and TNF-α of lungs were detected by qRT-PCR. (C) The protein expression of NLRP3 of pancreas and lungs were detected by Western Blot. *P<0.05, **P<0.01, ***P<0.001 vs Cae+LPS group.

Effect of MP on the expression of IL-1β, TNF-α, NLRP3, NF-κB, p-NF-κB on isolated peritoneal macrophages in vitro

Experiments were carried out in vitro to confirm the protective role of MP in the NLRP3 inflammasome. Peritoneal macrophages were isolated and induced with caerulein combined with LPS. MP treatment significantly reduced the expression levels of IL-1β in culture supernatants induced with ATP, Nigericin and MSU, respectively (Figure 5-A). In addition, IL-1β levels in culture supernatants decreased in a dose-dependent manner when induced with ATP (Figure 5-B). TNF-α showed a similar trend (Figure 5-C). qRT-PCR was used to detect the mRNA expression of NLRP3, IL-1β and TNF-α in peritoneal macrophages. The results indicated that MP reduced the expression of NLRP3, IL-1β and TNF-α in a dose-dependent manner (Figure 5-D, E, F). Furthermore, the protein levels of NLRP3, NF-κB and p-NF-κB were detected by Western Blot. MP treatment reduced NLRP3 and p-NF-κB levels in a dose-dependent manner (Figure 5-G).

Figure 5

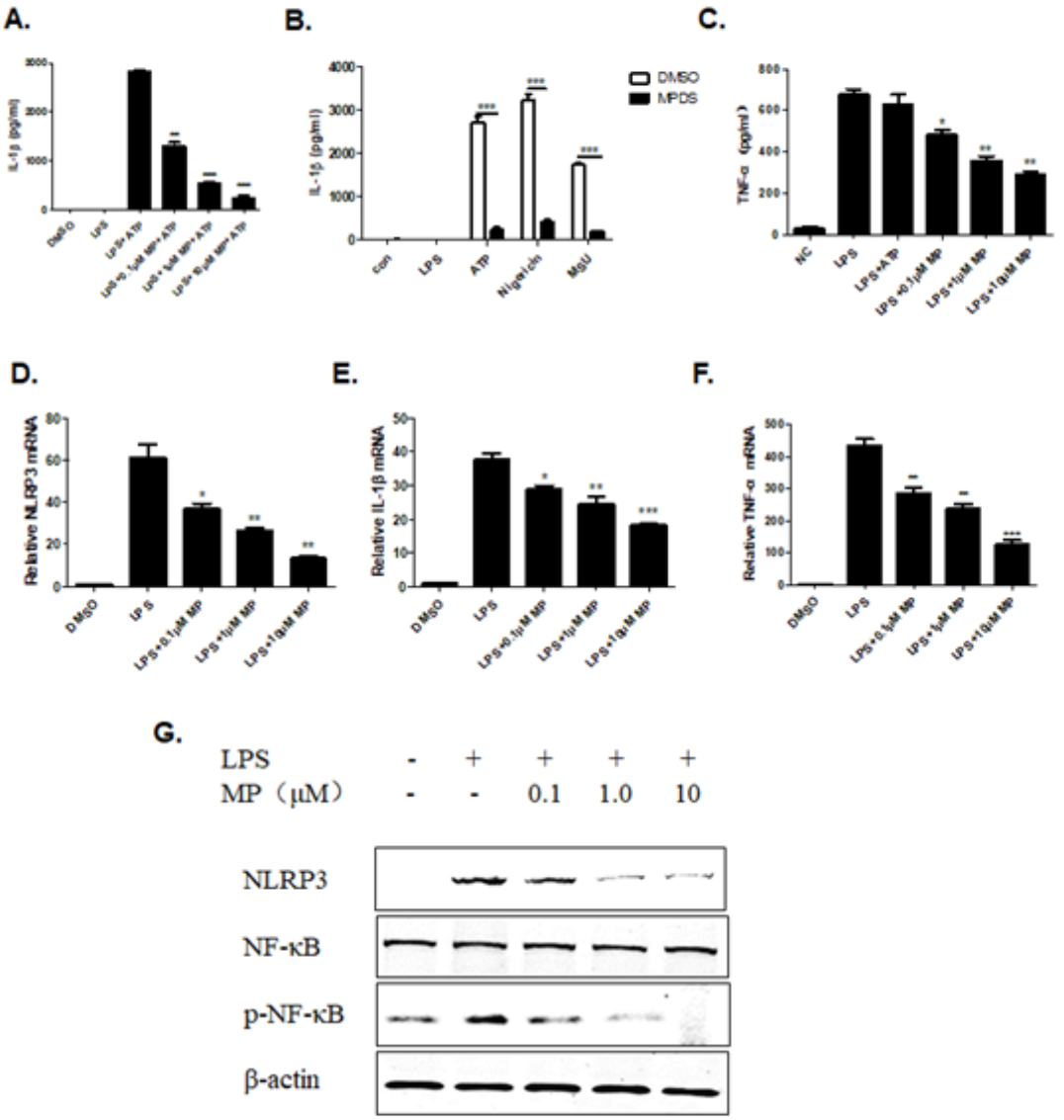

Figure 5: The effect of MP on the expression of IL-1β, TNF-α, NLRP3, NF-κB, p-NF-κB on isolated peritoneal macrophages in vitro. (A) ELISA-detected protein levels of IL-1β in culture supernatants of LPS-primed and different dose of MP and left stimulated with ATP. (B) ELISA-detected protein levels of IL-1β in culture supernatants of LPS-primed and 10μM and left stimulated with ATP, nigericin or MSU. (C) ELISA-detected protein levels of TNF-α in culture supernatants of LPS-primed and different dose of MP. (D) (E) (F) mRNA expression levels of NLRP3, IL-1β and TNF-α of peritoneal macrophages LPS-primed and different dose of MP were detected by qRT-PCR. (G) The protein level of NLRP3, NF-κB, p-NF-κB of peritoneal macrophages LPS-primed and different dose of MP were detected by Western Blot. *P<0.05, **P<0.01, ***P<0.001 vs Cae+LPS group.

Discussion

The rapid production and releasing of large numbers of inflammatory cytokines can cause local and systemic excessive inflammation in SAP. Our results demonstrated that IL-1β and TNF-α significantly increased in the SAP mouse model, which was induced by Cae + LPS and was consistent with other reports. The levels of IL-1β and TNF-α in serum and PLF were used as markers of systemic inflammatory responses to SAP. Blocking these inflammatory cytokines moderately and at the appropriate time may be an effective treatment for SAP [9,10]. The relationship between acute pancreatitis and MP is still a controversy. Most case reports reveal that steroid pulse therapy may cause acute pancreatitis [11]. MP has a powerful role in anti-inflammatory and immunosuppressive activity by inhibiting immune responses and inflammatory mediators. MP, used in the early phase, can attenuate the inflammatory responses and standard pancreatic histological examination after

induction of SAP in this mouse model. The early use of MP can improve pancreatic injury and systemic inflammation in a dose-dependent manner.

In addition, MP treatment alleviated lung injury in SAP mice. Neutrophil infiltration and congestion of alveolar septum capillaries were significantly reduced. Pancreatitis-associated acute lung injury (P-ALI) is the most common and severe complication of SAP and can develop into acute respiratory distress syndrome (ARDS), which is the leading cause of early death in SAP patients [12]. MPO is synthesized in the bone marrow and stored in azurophilic granules. Then, granulocytes enter the circulation and MPO is secreted in specific tissues when neutrophils, monocytes, and macrophages are induced by external stimuli. The increasing expression of MPO in the lungs is an important marker for the accumulation of neutrophils causing lung injury [13]. Our results confirmed that MP significantly reduces the activity of MPO in a dose-dependent manner. MP treatment can reduce neutrophil infiltration and lung injury after SAP induction in mice.

IL-1β is closely related to SAP, and its increase is positively correlated with the severity of the disease. The pathological pancreatic damage, degree of inflammation and acute pancreatitis associated acute lung injury (P-ALI) were significantly reduced after blocking IL-1β expression in SAP [14]. The maturation of IL-1β depends on activation of the NLRP3 inflammasome, which mainly consists of three parts: a leucine-rich domain, its own multimer of nucleic acids binding to the oligomerization structure domain and a variable amino acid domain [15,16].

The expression of the NLRP3 inflammasome and its components is extremely low when cells are in a quiescent condition. The activity of the NLRP3 inflammasome can be divided into two stages induced by exogenous or endogenous stimuli. In the pre-activation phase, cells recognize TNF-α and other signals in the cytoplasm, which stimulate the activation of NF-κB and IκB complexes. Then, NF-κB p50, p65 subunits are released into the cell nucleus, which can activate NLRP3 and IL-1β. The transcription and translation of precursors and IL-18 precursor-related genes release Nlrp3, pro-IL-1β, and pro-IL-18 into the cytoplasm [17]. In the second stage, the classical view is that NLRP3 binds to pro-caspase-1 through ASC under the action of active oxygen or K+ lowering conditions, it activates caspase-1, and then it cleaves IL-1β and IL-18 precursors to form mature IL-1β and IL-18 [18].

The NLRP3 inflammasome is closely related to various diseases, such as chronic inflammation, senility, auto-inflammatory syndrome, metabolic diseases, atherosclerosis lead by excessive release of inflammatory factors, diabetes, obesity, and rheumatoid arthritis [19-21]. The NLRP3 inflammasome plays an important role in the progress of AP [22]. Many studies have shown that negatively regulating the expression of the NLRP3 inflammasome can reduce the degree of inflammation in pancreatitis. Indomethacin and dexmedetomidine, which are commonly used in clinical practice, can reduce the damage and severity of pancreatic acinar cells in AP by inhibiting the expression of NLRP3, ASC, and IL-1β genes in the pancreas [23,24]. Additionally, studies have shown that hydrogen saline and sulforaphane can reduce NLRP3 inflammatory body synthesis, reduce cytokine production, and reduce pancreatic acinar cell destruction and inflammation by inhibiting NF-κB activity [25,26].

In the present study, we report that MP treatment in the early phase can reduce NLRP3 and IL-1β expression in pancreas and lungs and suppress the inflammatory cascade in vivo. Furthermore, in vitro experiments showed that MP repressed NLRP3, IL-1β and TNF-α expression in a dose-dependent manner through inhibiting NF-κB activity.

Primarily, our study indicated that MP could reduce inflammation response and mitigate the injury of both pancreatic and lung tissue in the SAP mouse model by inhibiting NF-κB activity and NLRP3 inflammasome activity. MP can be a potential treatment in the early phase of SAP.

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