International Immunopharmacology 75 (2019) 105741

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

MCP-induced protein 1 attenuates sepsis-induced acute lung injury by modulating macrophage polarization via the JNK/c-Myc pathway

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ARTICLE INFO

Keywords:
MCP-induced protein 1
Macrophage polarization
JNK
Sepsis
Acute lung injury

ABSTRACT

Sepsis is a potentially fatal systemic inflammatory response syndrome caused by infection. In this study, we evaluated the effects of MCP-induced protein 1 (MCPIP1), a recently discovered inflammation-related ribonuclease, on sepsis-induced acute lung injury (ALI) and investigated the underlying mechanisms. Cecal ligation puncture and lipopolysaccharide induction were performed on Sprague-Dawley rats and RAW264.7 cells, respectively, to establish sepsis-induced ALI models. The proteasome inhibitor MG132 used as an activator of MCPIP1 overexpression, and we showed that MG132 can indeed increase the expression of MCPIP1. MCPIP1 overexpression induced by MG132 alleviated sepsis-induced pathologic changes, water content and protein leakage in the lungs, and induction of systemic inflammatory mediators, and improved the 7-day mortality rate in the model rats. We also showed that MCPIP1 p showed romoted macrophage polarization from the M1 to the M2 type in sepsis-induced ALI. Furthermore, MCPIP1-enhanced M2 polarization was inhibited by an MCPIP1-targeting small interfering RNA (siMCPIP1) in RAW264.7 cells. Further mechanistic studies showed that the promotive effect of MCPIP1 on M2 polarization was related to the inhibition of c-Jun N-terminal kinase (JNK) and its downstream transcription factor c-Myc in the in vitro model. Conversely, siMCPIP1 transfection resulted in the recovery of JNK and c-Myc expression in LPS-treated cells. Taken together, these findings indicate that MCPIP1 plays a protective role in sepsis-induced ALI by modulating macrophage polarization through inhibition of the JNK/c-Myc signaling pathway. Our study presents a potentially novel therapeutic strategy for the treatment of lung injury involving the inflammatory cascade.

1. Introduction

Sepsis is a potentially fatal systemic inflammatory response syndrome caused by infection [1]. Multiple organ dysfunction may occur secondary to severe sepsis. In such a scenario, the lung is the first organ to be affected, and lung dysfunction may eventually lead to acute lung injury (ALI) or acute respiratory distress syndrome [2,3]. Although there have been rapid advancements in anti-microbial drugs and organ function support technology in recent years, the mortality rate of ALI caused by sepsis is still high. This may be associated with the complicated mechanism of sepsis-induced ALI. The main pathological changes observed in ALI secondary to sepsis include severe inflammation, infiltration of inflammatory cells, interstitial edema, impaired function of pulmonary vascular endothelial cells, impaired function of the alveolar barrier, and changes in pulmonary vascular permeability [4]. The underlying molecular mechanisms include the release of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, HMGB-1 and others, which can lead to activation of the corresponding intracellular signal transduction system and induce apoptosis, thus aggravating ALI [5,6]. Thus, investigation into the inflammatory mechanisms in sepsis-induced ALI may reveal important targets for the treatment of this condition.

As indispensable components of the innate immune system, macrophages display remarkable heterogeneity and play a pivotal role in inflammatory responses, and they can be classified according to their phenotype and the cytokines they secrete [7]. Further, macrophage polarization plays critical roles in many human inflammatory pathways, including would healing, atherosclerosis, obesity and insulin resistance, cancer, rheumatoid arthritis, and bacterial and parasitic infection [8,9]. According to their polarization, macrophages are divided into the classically activated M1 and selectively activated M2 macrophages. M1 macrophages mainly produce inflammatory mediators, including TNF-α, IL-1, IL-6, reactive oxygen species, and reactive nitrogen, M2 macrophages, which are involved in tissue repair and reducing inflammation [7,10]. It has been reported that macrophage

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https://doi.org/10.1016/j.intimp.2019.105741
Received 5 April 2019; Received in revised form 3 June 2019; Accepted 3 July 2019
Available online 16 July 2019

polarization plays an important role in ALI: M1 macrophages promote the production of free radicals and inflammatory cytokines, leading to further aggravation of ALI, while selective activation of M2 macrophages can help alleviate inflammation [11]. Thus, it would be interesting to investigate whether regulation of macrophage polarization can be used to alleviate inflammation-related injury in ALI.

Although the regulation of macrophage polarization is known to affect the inflammatory responses in sepsis, the underlying mechanisms are quite complicated. c-Jun N-terminal kinase (JNK) has been found to play important roles in the regulation of inflammation and the progression of sepsis [12,13]. Further, recent studies have reported the critical role of JNK in IL-4-induced macrophage polarization [14,15], and it was shown that JNK activation is required for glycyrrhizic acid-induced M1-related cytokine production [16]. c-Myc is a typical downstream transcription factor of the JNK pathway [17], and its inhibition was found to lead to a reduction in pro-inflammatory signaling [18]. Based on these findings, it might be interesting to investigate the possible role of the JNK/c-Myc pathway in macrophage polarization that occurs during inflammatory responses in sepsis-induced ALI.

MCP-induced protein 1 (MCPIP1) is a recently discovered inflammation-related ribonuclease that can catalyze the degradation of a series of pro-inflammatory cytokines and their messengers [19]. It was recently demonstrated that IL-4 treatment of murine macrophages induces the expression of not only STAT6 and KLF4 but also MCPIP as well as M2 markers [20]. Further, it was demonstrated mice with myeloid cell-specific knockout of MCPIP are incapable of IL-4-induced M2 polarization; this is indicative of the role of MCPIP in macrophage polarization. However, there is very little research on the role of MCPIP in inflammatory and/or immune diseases. Based on all these findings, we formulated the following hypothesis for the present study: MCPIP overexpression alleviates sepsis-induced ALI by promoting M1-to-M2 polarization via its inhibitory effects on the JNK/c-Myc pathway.

Studies have shown that the proteasome inhibitor MG132 can significantly increase the expression of MCPIP1, both at the mRNA and protein level, without altering the stability of MCPIP1 [21]. Therefore, in this study, we used MG132 to establish an overexpression model of MCPIP1 in rats. The findings of both in vivo and in vitro experiments demonstrated that MCPIP1 can attenuate sepsis-induced ALI by regulating macrophage polarization in rats and that this process may involve the JNK/c-Myc pathway.

2. Materials and methods

2.1. Reagents and antibodies

MG-132 was purchased from Calbiochem, Germany. Lipopolysaccharide (LPS) (*Escherichia coli*, serotype 0111, B4; Sigma–Aldrich, Saint Louis, MO, USA) was dissolved in phosphate-buffered saline as a stock solution and used upon thawing. Anti-Arg1 and anti-Mrc1 antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-MHCII and anti-iNOS antibodies were purchased from eBioscience Biotechnology (San Diego, CA, USA). Anti-JNK and anti-c-Myc antibodies were purchased from ProteinTech Group, CA, USA. Anti-β-actin antibody was purchased from Zsbio Commerce Store, Beijing, China.

2.2. Animals

The study protocol was approved by the Animal Care and Use Committee of the School of Medicine, Yangzhou University (Yangzhou, China) and was in accordance with the guidelines for the care and use of animals set by the Chinese government.

Adult male Sprague–Dawley rats (250–300 g) were housed in air-filtered and were given ad libitum access to food and water. Animals were housed at constant temperature (20–24 °C) and constant humidity (50–70%) with a 12/12-h light/dark cycle. The study protocol was approved by the Animal Care and Use Committee of Yangzhou University (Yangzhou, China) and was in accordance with the guidelines for the care and use of animals set by the Chinese government.

2.3. Experimental procedure and animal model of ALI

ALI was induced in the rats by cecal ligation perforation (CLP), based on a previously published CLP model of polymicrobial sepsis [22]. Briefly, after induction of anesthesia and disinfection of the surgical area, a midline incision was made through the skin. About two-thirds of the cecum was connected with 4–0 silk sutures, and an 18-gauge needle was used to ligate the ligature. A small amount of excrement was squeezed out, 3 ml of saline preheated to 37.0 °C was intraperitoneally (i.p.) administered for fluid resuscitation, and the abdomen was closed using 4–0 silk sutures in both running layers. After abdomen closure, the rats were returned to their cages and provided food and water ad libitum. The success rate of CLP-induced ALI in this study was 91.43%.

The ALI model rats were allocated into four groups of eighteen rats each, using a random number table: (1) The sham group comprised sham-operated animals that underwent only laparotomy. (2) The MG132 group comprised rats that were pretreated with MG-132 (10 mg/kg, dissolved in 0.25 mL dimethyl sulfoxide and administered i.p.) 30 min before the sham procedure (only laparotomy). (3) The CLP group comprised rats in which CLP was performed. (4) The MG132-CLP group comprised rats that were pretreated with MG-132 injection (10 mg/kg in dimethyl sulfoxide, i.p.) 30 min before the CLP procedure was performed.

For the procedures, the rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.; Merck, Darmstadt, Germany), and anesthesia was maintained by additional injections (15 mg/kg, i.p.) administered every hour under hemodynamic monitoring. The rats were placed in the supine position on a heating blanket and under a heating lamp, to ensure a body temperature of 37 °C throughout the experiment. A polyethylene catheter was placed in the femoral artery to monitor mean arterial pressure and heart rate, as well as for blood sampling. The arterial catheter was infused with physiologic saline at 0.5 mL/h. The rats were sacrificed by draining of blood from the heart 6 h after administration of physiologic saline or CLP.

2.4. Determination of water content and histologic examination

To evaluate the severity of ALI, CLP-induced pulmonary edema was assessed based on the wet-to-dry weight ratio of the lung. The right upper lobe of each lung was weighed immediately after extraction and placed in a 60 °C oven for 72 h. The dried tissue was then weighed to determine the wet-to-dry weight ratio. Samples from the inferior lobe of the right lung were fixed in 4% paraformaldehyde solution, dehydrated sequentially in 50% to 100% alcohol, and treated with xylene solution. Then, the tissues were embedded in paraffin, sectioned (thickness, 4 μm), and stained with hematoxylin and eosin. The samples were assigned an injury score for each of these four categories: alveolar and interstitial edema, microhemorrhage, inflammatory infiltration, and microatelectasis or alveolar overdistension. The injury scores were assigned as follows: 0, absent with normal appearance; 1, slight; 2, intermediate; and 3, severe [23,24]. The lung injury score was calculated by adding the individual injury scores for each category. The scoring was performed by a pathologist who was blinded to the data, who used a light microscope (×40, Olympus) to view the stained tissue samples.

2.5. Protein leakage from capillaries

Pulmonary microvascular permeability was determined using the Evans blue dye extravasation method at 6 h after CLP or the sham operation. Evans blue dye (30 mg/kg, Sigma–Aldrich) was administered intravenously at 30 min before the rats were sacrificed. Lungs were perfused to remove blood and extracted. The dye content in lung tissue was determined by spectrophotometry at an optical density of 620 nm [25].

2.6. Immunohistochemical staining

The lung tissues were embedded in Tissue-Tek (O.C.T. Compound; SAKURA Finetek, Tokyo) and frozen in liquid nitrogen for preparation of cryosections. Frozen lung tissues were cut to a thickness of 6 μm, immunostained and visualized by confocal microscopy (Fluoview FV 10i; Olympus, Tokyo). The sections were fixed in acetone for 10 min, blocked with Block Ace (Dainippon Sumitomo Pharma, Tokyo) for 10 min, and incubated with primary (rabbit polyclonal anti-MCPIP1 antibody, 1:1000; GeneTex, USA) and secondary antibodies (1:1000 dilution; Chinese Fir Golden Bridge Biotechnology) for 60–120 min.

2.7. Enzyme-linked immunoabsorbent assay

Bronchoalveolar lavage fluid (BALF) was collected and centrifuged at 6 h after the procedures (CLP/sham) were performed. The concentrations of TNF-α, IL-1β, IL-6 and IL-10 were measured using a commercially available enzyme-linked immunoassay (ELISA) kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

2.8. Survival curves

To observe the effect of MCPIP1 on survival, 40 more Sprague-Dawley rats were randomly divided into four experimental groups as described earlier ($n = 10$ per group). Survival was assessed every day until the endpoint of 7 days. Survival data were analyzed utilizing log-rank or χ2. A p-value of < 0.05 was considered statistically significant.

2.9. Cell culture

The mouse macrophage line RAW264.7 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in an atmosphere of 95% air and 5% CO_2. When the cells reached 80% confluence, they were seeded into 24-well or 6-well plates for further experiments.

The RAW264.7 cells were treated with 10 μg/mL LPS for 6 h to simulate sepsis model in vitro. And the cells in treatment group were treated with 1 μM MG132 for 6 h before LPS stimulation.

2.10. Short-interfering RNA

Pre-designed siRNA targeting human MCPIP1 as well as its negative control were purchased from Seville Biotechnology Co. Ltd. (Wuhan, China) Either siRNA was transfected into the RAW264.7 cells by electroporation using the Amaxa Electroporation Unit according to the manufacturer's instructions. After 24 h, the cells were harvested and protein was isolated to assess MCPIP1 knockdown. The transfected cells were left quiescent for 24 h and then pretreated with MG132 for 6 h, followed by treatment with LPS for 6 h.

2.11. Western blotting and real-time PCR

The right middle lobe and transfected RAW264.7 cells were used for protein and RNA isolation. Total protein or RNA was harvested and western blot or RT-PCR analyses were performed as described previously [24]. The RT-PCR primers are listed in Table 1. β-actin was used as an internal control.

2.12. Statistical analyses

Statistical calculations were undertaken using SPSS v19.0 (IBM, Armonk, NY, USA). Continuous data were tested for normality using the Shapiro–Wilk test, and were determined to have a normal distribution. Homogeneity of variance was tested using Bartlett's test. If Bartlett's test indicated that group comparisons had equal variance, one-way analysis of variance (ANOVA) was used. Data are the mean ± SD, Nonparametric statistical analyses (Kruskal–Wallis one-way ANOVA on mean ranks) were used for evaluation of histopathologic scores because of unequal variance. $p < 0.05$ was considered significant.

Table 1
List of primers used for RT-PCR.

MCPIP1	Forward	5′-CCCCCTGACGACCCTTTAG-3′
	Reverse	5′-GGCAGTGGTTTCTTACGAAGGA-3′
iNOS	Forward	5′-CCCTTCCGAAGTTTCTGGCAGCAGCG-3′
	Reverse	5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′
MHC II	Forward	5′-GCCTGAAGCAGCAGATGAATG-3′
	Reverse	5′-CACACTGGGGCTTGTAGGAA-3′
Arg1	Forward	5′-GTGAAGAACCCACGGTCTGT-3′
	Reverse	5′-GCCAGAGATGCTTCCAACTG-3′
Mrc1	Forward	5′-CTTCGGGCCTTTGGAATAAT-3′
	Reverse	5′-TAGAAGAGCCCTTGGGTTGA-3′
JNK	Forward	5′-CCGGACAAGCAGTTAGATGA-3′
	Reverse	5′-CACCTGTGCTAAAGGAGACG-3′
c-Myc	Forward	5′-CCCAAATCCTGTACCTCGTC-3′
	Reverse	5′-TCTTCTTCAGAGTCGCTGCT-3′
β-actin	Forward	5′-ACGTTGACATCCGTAAAGAC-3′
	Reverse	5′-GAAGGTGGACAGAGAGGC-3′

3. Results

3.1. Intraperitoneal injection of the proteasome inhibitor MG132 increases the expression of MCPIP1 in rats

Immunofluorescence images showed that the expression of MCPIP1 in rat lung tissue seems higher in the MG132-pretreated groups than in the sham and CLP groups (Fig. 1A). Proved by the results of western blotting and RT-PCR, both of them showed that pretreatment with MG132 could significantly promote the expression of the MCPIP1 protein and mRNA in rat lung tissue (Fig. 1B and C).

3.2. MCPIP1 overexpression induced by MG132 regulates the inflammatory response in sepsis rats

The ELISA results showed that the concentration of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in lung tissue was significantly lower in the MG132-pretreated groups than in the non-treated groups (CLP and sham), while the concentration of the anti-inflammatory cytokine IL-10 was significantly higher with MG132 pretreatment (Fig. 2). These findings indicate that MCPIP1 overexpression can alleviate the inflammatory reactions of lung tissue in sepsis-induced ALI rats.

3.3. MCPIP1 overexpression induced by MG132 attenuates sepsis-induced ALI and improves survival

Photomicrographs taken at 6 h after CLP revealed infiltration of inflammatory cells into the lung interstitium and alveolar spaces, thickening of alveolar walls, and intra-alveolar exudation (Fig. 3A). However, MG132 pretreatment in the CLP-MG132 group attenuated these effects of CLP. Further, semi-quantitative assessment with the lung injury score demonstrated that the degree of lung injury in the MG132-CLP group was lower than that in the CLP group (Fig. 3B). The lung wet-to-dry-weight ratio at 6 h after CLP administration was significantly higher in the CLP group than in the CLP-MG132 group (Fig. 3C). Examination of extravasation of Evans blue dye showed that CLP induced a significant increase in leakage into the lung (Fig. 3D), which was attenuated by MG132 pretreatment. Thus, pretreatment with 10 mg/kg MG132 resulted in a significant reduction in lung edema and capillary leakage.

As shown in Fig. 3E, the survival rate of rats in the sham group was 100%, and the survival rate was significantly lower in the CLP group. Furthermore, at 6 h after CLP, all the animals had developed early

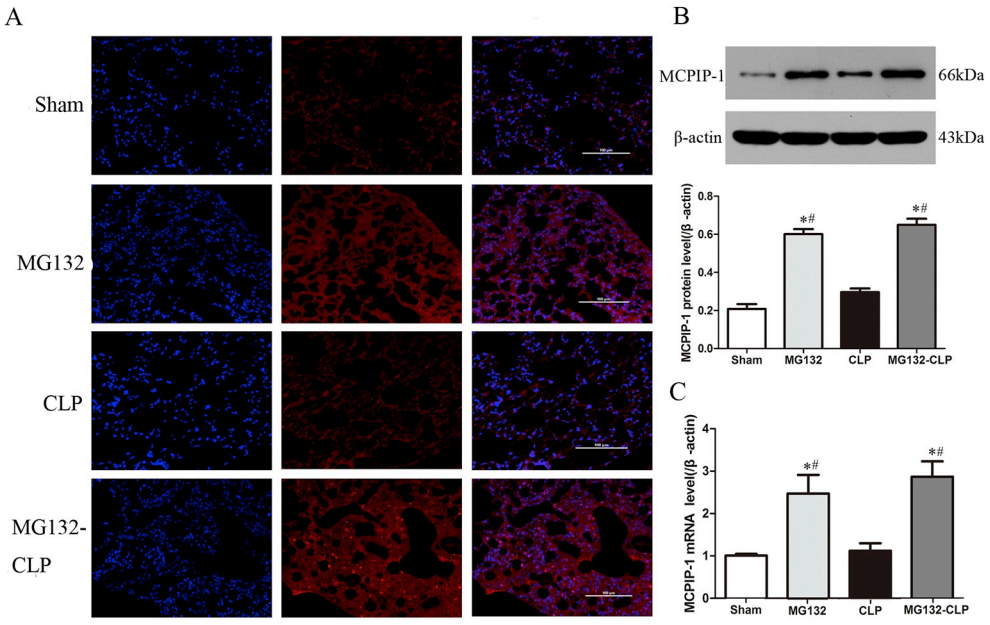

Fig. 1. Intraperitoneal injection of the proteasome inhibitor MG132 increases the expression of MCPIP1 in model rats. (A) Representative immunocytochemical images showing that MG132 induced an increase in the expression of MCPIP1 in rat lung tissue. Scale bar = 100 μm. (B) Representative western blot showing the effects of MG132 on the expression of MCPIP1 in model rats, and densitometric findings showing that intraperitoneal injection of MG132 could induce MCPIP1 expression in rats (β-actin was used as an internal control). (C) mRNA expression of MCPIP1. Data are shown as the mean ± SD ($n = 6$), one-way analysis of variance was used. $^{*}P < 0.05$ versus the sham group; $^{\#}P < 0.05$ versus the CLP group.

clinical signs of sepsis, including lethargy, piloerection, and diarrhea. The survival rate after CLP was 50% on the second day and 0% on the fourth day. In the CLP-MG132 group, however, pretreatment with MG132 improved the survival rate to 70% on the second day and to 50% on the fourth day, and by day 7, 30% of the rats survived.

3.4. MCPIP1 regulates macrophage polarization in LPS-stimulated RAW264.7 macrophages

Apart from proteasome inhibition, MG132 has also been reported to inhibit calpains. Therefore, to minimize any toxic effects of MG132, a low dose of 1 μM was used. This concentration is below the in vitro IC_{50} for calpain inhibition and far below the reported IC_{50} for calpain inhibition measured in a cell-based assay [26]. We found that the expression of MCPIP-1 was up-regulated in MG132-treated cells (Fig. 4A), consistently, the expression of the M2 macrophage markers Arg1 and Mrc1 was not remarkable in LPS-stimulated cells, but it was significantly upregulated in the cells after MG132 preconditioning (Fig. 4C, D). Conversely, M1 macrophage markers iNOS and MHCII was most obvious in the LPS-stimulated cells. However, MG132 pretreatment could effectively reduce the expression of iNOS and MHCII induced by LPS (Fig. 4E, F). These results indicate that the over-expression of MCPIP1 can enhance M2 macrophage polarization and reduce M1 polarization in LPS-stimulated RAW264.7 macrophages.

3.5. Knockdown of MCPIP1 inhibits M2 macrophage polarization in MG132-treated RAW264.7 macrophages

To further verify the correlation of MCPIP1 with macrophage polarization, we performed loss-of-function studies with siRNA targeting MCPIP1 in RAW264.7 macrophages and used western blotting and RT-PCR to determine the expression of MCPIP1 in the macrophages (Fig. 5A). The expression of MCPIP1 was not obvious in the control or MCPIP1 siRNA (siMCPIP1) cells, but it was notable after MG132 pretreatment with or without LPS stimulation. However, the siMCPIP1-transfected cells exhibited low expression of MCPIP1 even after MG132

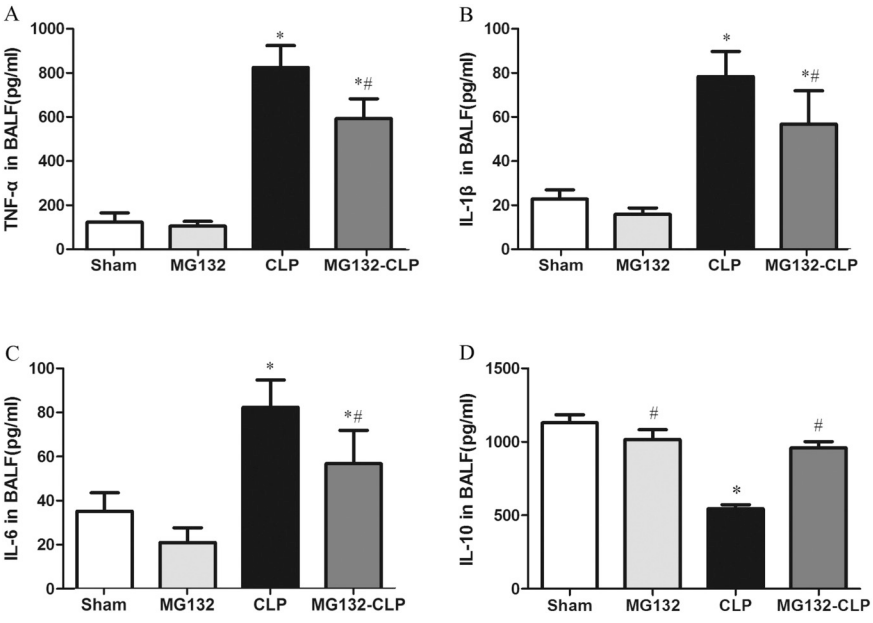

Fig. 2. MCPIP1 overexpression induced by MG132 regulates the inflammatory response in sepsis-induced ALI rats. The concentration of TNF-α (A), IL-1β (B), IL-6 (C) and IL-10 (D) in BALF, as determined by ELISA. MG132 alone did not affect the baseline values. Cecal ligation and puncture (CLP) altered these variables significantly. Pretreatment with MG132 reduced the concentration of TNF-α, IL-1β and IL-6 and increased the concentration of IL-10 in the CLP animals. Data are shown as the mean ± SD (n = 6), one-way analysis of variance was used. $^{*}P < 0.05$ versus the sham group; $^{\#}P < 0.05$ versus the CLP group.

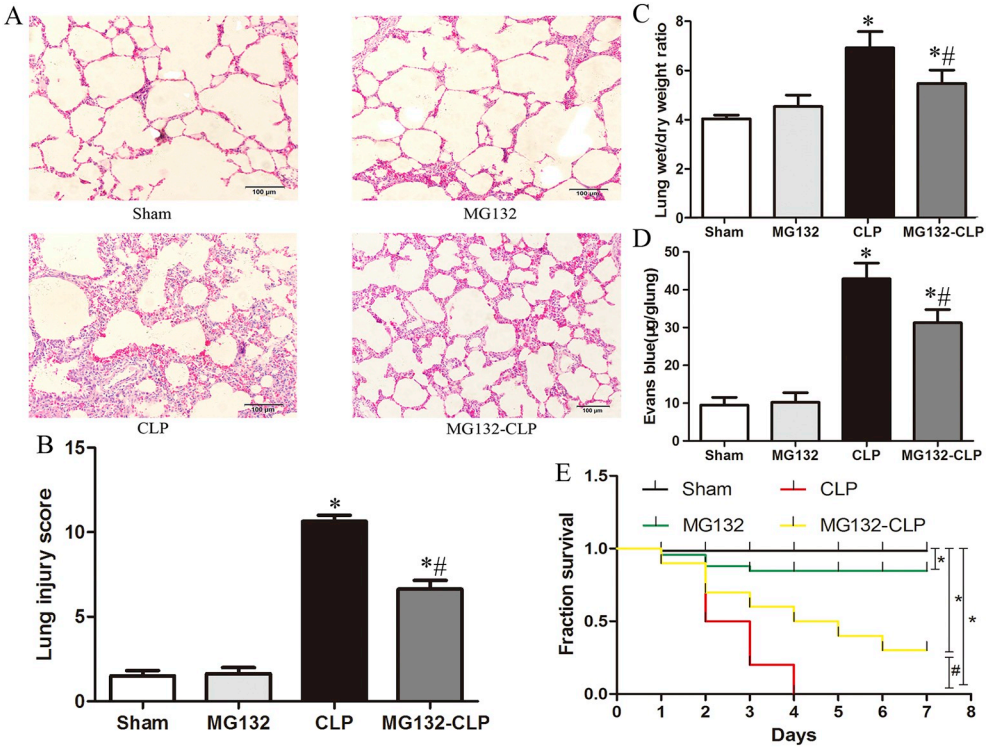

Fig. 3. MCPIP1 overexpression induced by MG132 attenuates sepsis-induced ALI and improves rats survival. (A) Representative photomicrographs of lung tissues stained with hematoxylin and eosin at 6 h after CLP administration. CLP caused infiltration of inflammatory cells into the lung interstitium and alveolar spaces, thickening of alveolar walls, and intra-alveolar exudation. Original magnification, ×10. (B) Semi-quantitative analysis of lung tissues based on the lung injury score (n = 6). (C) Lung edema determined based on the wet-to-dry lung weight ratio (n = 6), and (D) protein leakage from capillaries measured by Evans blue dye extravasation in model sepsis rats (n = 3). (E) Survival rate of mice subjected to CLP (n = 10). Data are shown as the mean ± SD, nonparametric statistical analyses were used for evaluation. *P < 0.05 versus the sham group; #P < 0.05 versus the CLP group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pretreatment (Fig. 5B, C). Furthermore, transfection of siMCPIP1 resulted in an increase in the mRNA expression of the M1 macrophage markers iNOS and MHCII in MG132-treated macrophages (Fig. 5D, E) and a reduction in the mRNA level of the M2 macrophage markers Arg1 and Mrc1 (Fig. 5F, G). These in vitro results confirm that MCPIP1 plays a critical role in the regulation of macrophage polarization.

3.6. MCPIP1 regulates LPS-induced macrophage activation via inhibition of JNK and c-Myc

In order to investigate the molecular mechanism by which MCPIP1 regulates macrophage polarization, we used western blotting analysis to determine the correlation of MCPIP1 with JNK and its downstream

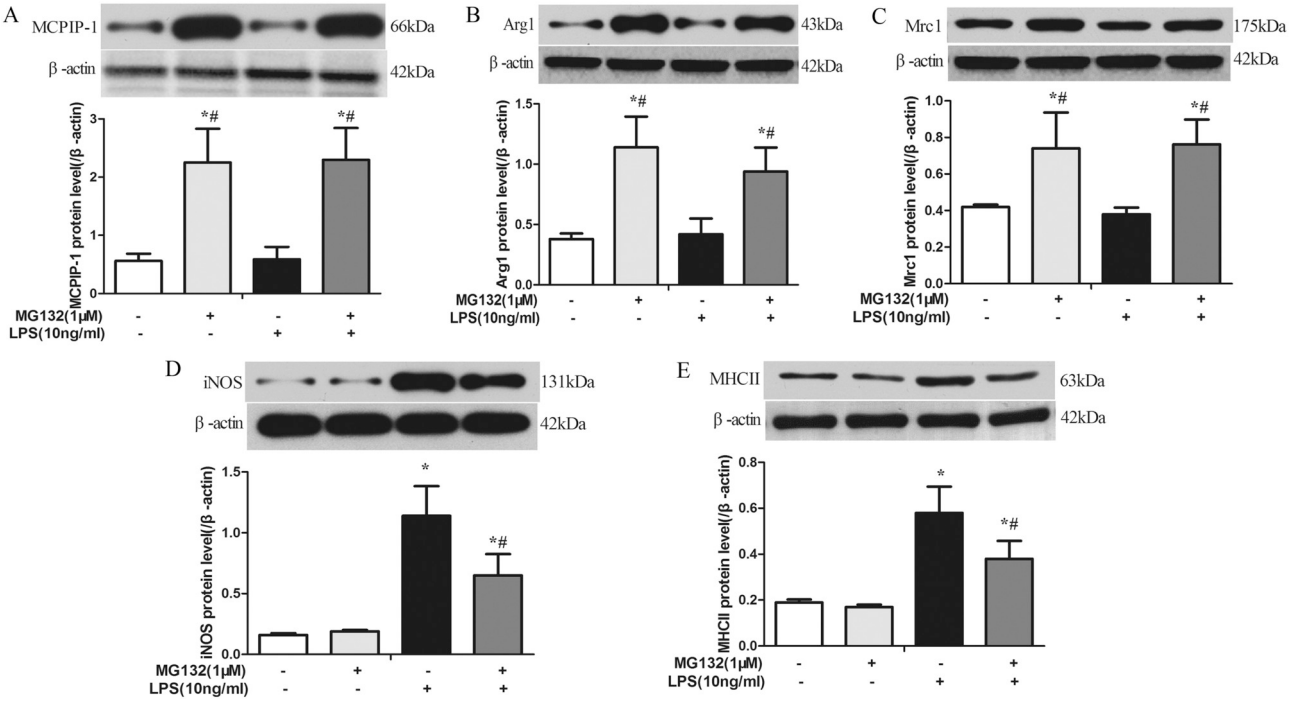

Fig. 4. MCPIP1 negatively regulates macrophage polarization in sepsis-induced ALI. (A) Representative western blot showing the effects of MCPIP1 on the expression of macrophage polarization markers in rat lung tissues, with β-actin as an internal control. The protein level of the M2 markers Arg1 (B) and Mrc1 (C) were not obvious in the CLP rats, but were upregulated by MG132 pretreatment. The protein level of the M1 markers iNOS (D) and MHCII (E) were upregulated in the CLP rats and suppressed by MG132 pretreatment. Data are shown as the mean ± SD (n = 3), one-way analysis of variance was used. *P < 0.05 versus the sham group; #P < 0.05 versus the CLP group.

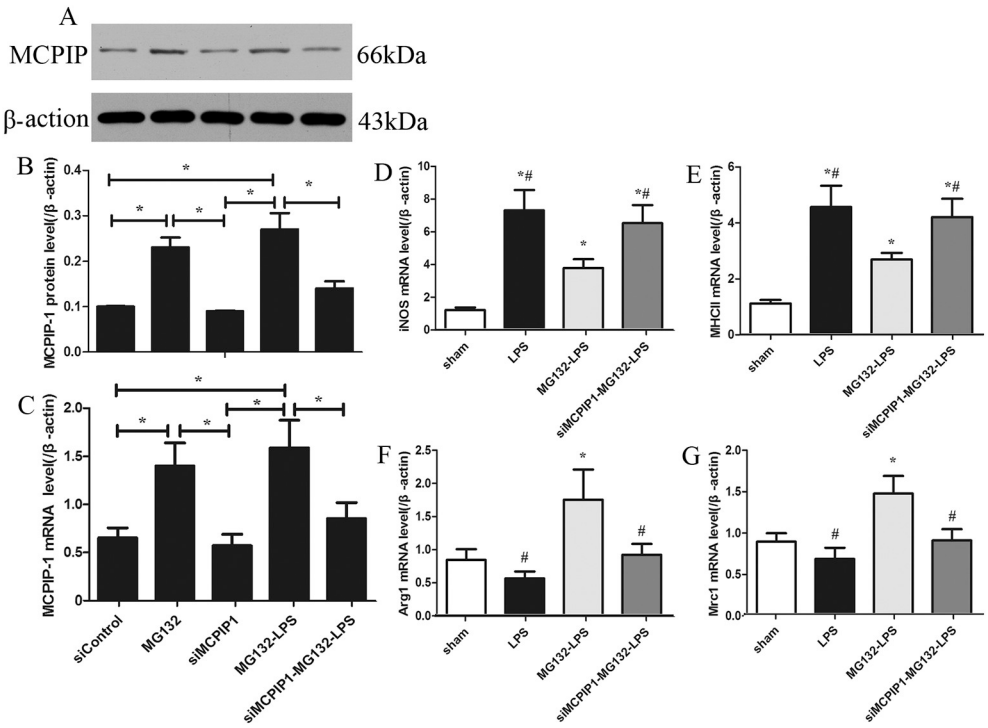

Fig. 5. Knockdown of MCPIP1 inhibits M2 macrophage polarization in MG132-treated RAW264.7 macrophages. (A) Representative western blot showing the expression of MCPIP1 in RAW264.7 line cells, with β-actin as an internal control. (B) Densitometric findings showing that the expression of MCPIP1 was upregulated by MG132 even in LPS-stimulated cells; however, MCPIP1-targeting small interfering RNA (siMCPIP1) reduced its expression even in MG132-treated cells. (C) The mRNA level of MCPIP1 in each cell group was consistent with its protein level. Data are shown as the mean ± SD (n = 3), and significant differences are indicated by * ($P < 0.05$). Knockdown of MCPIP1 by siMCPIP1 resulted in recovery of the expression of iNOS (D) and MHCII (E) even with MG132 pretreatment in LPS-stimulated cells, and reduction of the expression of Arg1 (F) and Mrc1 (G) even in MG132-treated cells. Data are shown as the mean ± SD (n = 3), one-way analysis of variance was used. *$P < 0.05$ versus the sham group; #$P < 0.05$ versus the MG132-LPS group.

transcription factor c-MycFig. 6A, C, E). We found that the expression of JNK and c-Myc was upregulated in macrophages stimulated by LPS alone. However, in MG132-pretreated cells, JNK and c-Myc expression was downregulated while MCPIP1 expression was upregulated. Further, inhibition of MCPIP1 expression by siRNA against MCPIP1 resulted in an increase in the expression of JNK and c-Myc (Fig. 6). Thus, MCPIP1 may regulate macrophage polarization by inhibiting the JNK-c-Myc signaling pathway.

4. Discussion

Through the findings of this study, we have proven our hypothesis that MCPIP1 ameliorates sepsis-induced ALI by regulating macrophage polarization via the JNK/c-Myc pathway. We used the proteasome inhibitor MG132 to induce MCPIP1 overexpression, and our results demonstrate that proteasome inhibition with a low dose of MG132 (10 mg/kg) results in a tremendous increase in the protein expression of MCPIP1 in both lung issue and RAW264.7 macrophages.

In recent years, the role of MCPIP1 in the inflammatory response has attracted much attention. For example, it has been reported that MCPIP1 negatively regulates HMGB1-mediated neuroinflammation and neuronal toxicity [27]. Further, it was revealed that MCPIP-1 maintains epithelial homeostasis by controlling the IL-8 mRNA level and plays a crucial anti-inflammatory role not only in myeloid cells but also in epithelial cells [28]. Our data demonstrate that pulmonary levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were increased markedly in rats that underwent CLP, and that MCPIP1 overexpression resulted in a decrease in the accumulation of these cytokines. Furthermore, expression of the antiinflammatory factor IL-10 was upregulated by MCPIP1 overexpression in BALF. Thus, our findings are in agreement with previous reports on the antiinflammatory role of MCPIP1, and they indicate that MCPIP1 plays a negative regulatory role in the inflammatory response in sepsis-induced ALI rats.

The role of MCPIP1 in sepsis-induced ALI has not been fully characterized, but it has been reported that MCPIP-1 can improve the survival rate in mouse models of septic shock induced by LPS [29]. In the present study, sepsis-induced ALI was confirmed by histological analysis in rats in which CLP was performed to induce ALI. In keeping with the pathogenesis of sepsis-induced ALI, CLP resulted in an increase in water content and protein leakage in the lungs. MCPIP1 overexpression induced by MG132 significantly attenuated these abnormalities, indicating the therapeutic role of MCPIP1 in sepsis-induced ALI in rats. Further, although the 7-day survival rate of rats in the MG132 group was slightly lower than that in the sham group, the overexpression of MCPIP1 ultimately resulted in an improvement in the overall survival rate of the model rats. Thus, these findings are in agreement with the study on LPS-induced septic shock. However, we found that MG132 treatment alone (without CLP) was found to decrease the survival rate of rats; this may be a result of its toxic or other side effects and its potential physiological effects in other tissues and organs [30,31]. The other physiological effects of MG132 need to be further studied.

Accumulating evidence has suggested that macrophage-mediated inflammation plays a crucial role in a number of inflammatory diseases, such as ALI and sepsis [32]. In keeping with this, the present results show that the high expression of MCPIP1, which was associated with inflammatory mediation, has a significant effect on macrophage polarization in the lung tissue of model ALI rats. MCPIP1 overexpression suppressed gene expression of M1 macrophage markers, while it enhanced the gene expression of M2 macrophage markers in CLP rats. The results of the in vivo experiments were confirmed by in vitro findings in LPS-induced RAW264.7 cells. In the in vitro model, MCPIP1 overexpression induced by MG132 significantly enhanced the expression of M2 macrophages markers in cells in which LPS was administered. However, knockdown of MCPIP1 by siRNA resulted in recovery of the expression of M1-associated markers, even with MG132 pretreatment, in LPS-stimulated cells, and reduced the expression of M2-associated markers, even in MG132-treated cells. Thus, MCPIP1 seems to be necessary for the transformation of macrophages from the M1 to the M2 type. Taken together, our results demonstrate that MCPIP1 can promote macrophage polarization from the M1 to the M2 protective phenotype both under in vitro and in vivo conditions.

We further investigated the mechanisms underlying MCPIP1-induced M2 polarization of macrophages in the in vitro model of ALI. These effects of MCPIP1 may be associated with its role in the inflammatory response, as ALI in the setting of sepsis is known to be the result of the actions of an integrated network of soluble inflammatory

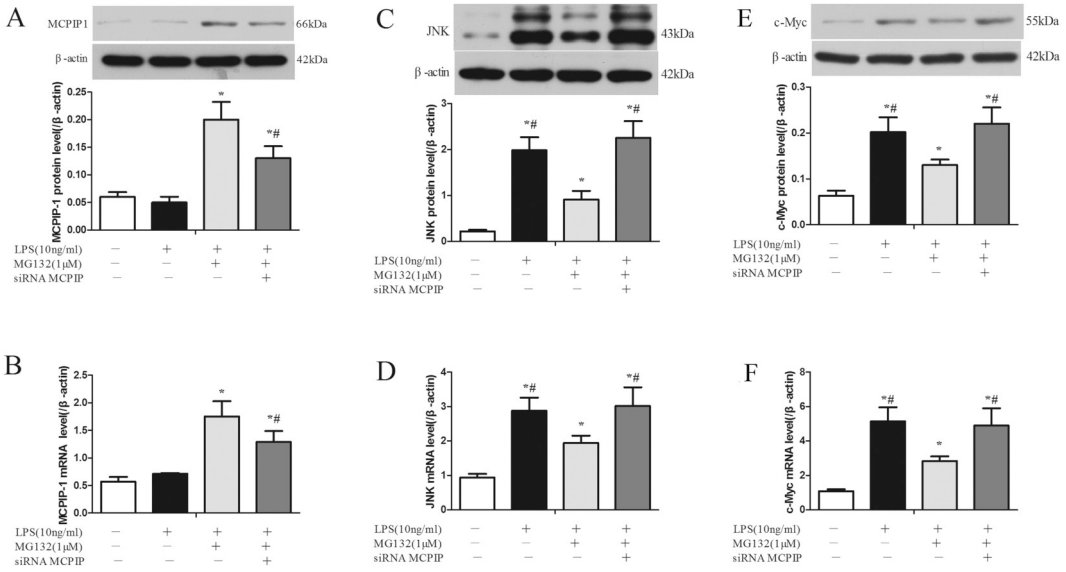

Fig. 6. MCPIP1 negatively regulates LPS-induced macrophage activation via inhibition of the JNK/c-Myc pathway. (A) Representative western blot showing the expression of MCPIP1 in RAW264.7 cells, with β-actin as an internal control. The expression of MCPIP1 protein and mRNA were activated by MG132 and suppressed by an MCPIP1-targeting small interfering RNA (siMCPIP1) even in MG132-treated cells (A, B). The expression of JNK was upregulated by LPS stimulation and suppressed by MG132 pretreatment; however, siMCPIP1 resulted in recovery of its expression even with MG132 pretreatment in LPS-treated cells(C, D). The expression of c-Myc corresponded to that of its upstream molecule JNK (E, F). Data are shown as the mean ± SD (n = 3), one-way analysis of variance was used. $*P < 0.05$ versus the sham group; $\#P < 0.05$ versus the MG132-LPS group.

mediators and various inflammatory cells [33]. JNK is known to be an important upstream regulator of the expression of inflammatory mediators induced in response to stress, cytokines and cytoskeletal reorganization [34,35]. The JNK pathway is also being increasingly recognized as a potential target of sepsis treatment. Previous studies have demonstrated that JNK inhibition by SP600125 suppressed the release of TNF-α into BALF, as well as suppressed LPS-induced increase in the DNA binding activity of NF-κB by downregulation of the phosphorylation of its inhibitor κB-α [36,37]. Consistent with the previous data, we found that MCPIP1 could inhibit LPS-induced JNK expression, as well as the expression of its downstream transcription factor c-Myc. Conversely, MCPIP1 inhibition by siRNA led to the resumption of JNK and c-Myc expression in LPS-stimulated cells. Thus, the promotion of M2 polarization by MCPIP1 may be mediated by inhibition of JNK and c-Myc pathways.

We must acknowledge the limitations of our experiments. One of the limitations was that the observation period was limited to 6 h. However, the later phase of sepsis is characterized by endotoxin tolerance, a phenomenon associated with the inability of cells to upregulate inflammatory cytokine expression and high expression of M2 macrophage markers, which result in greater susceptibility to reinfection [38,39]. Thus, the later-stage effects of MCPIP1 on endotoxin tolerance require further study. Another limitation is that our in vivo results do not exclude the possibility that MCPIP1 may have effects in other cells, such as alveolar epithelial cells and pulmonary vascular endothelial cells [28,40]. Moreover, several studies have reported that MCPIP1 can induce neuronal apoptosis in acute spinal cord injury or DNA damage in cholesterol-treated endothelial cells [30,31]. This means that MCPIP1 may carry certain risks as a therapeutic target, and therefore, further research is needed to investigate the toxic effects of MCPIP1 in sepsis-induced ALI.

With the help of a CLP-induced model of ALI in rats, we demonstrated that MCPIP1 overexpression induced by MG132 can alleviate sepsis-induced pathologic changes, increase in water content and protein leakage in the lungs, and induction of systemic inflammatory mediators, and thereby improve the 7-day mortality rate in sepsis-induced ALI. Further, the results of our molecular analysis indicate that MCPIP1 ameliorated sepsis-induced ALI by reversing macrophage polarization and that the potential mechanism of action may be (at least in part) through the inhibition of the JNK/c-Myc signaling pathway. Thus, MCPIP1 may have potential as an effective therapeutic agent for the treatment of lung injury involving the inflammatory cascade.

Declaration fo Competing Interest

None.

Disclosure

The authors declare that there are no conflicts of interest.

Financial support

This study was supported by the National Natural Science Foundation of China [grant numbers 81571936, 81401626].

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