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5, 7, 2', 4', 5'-Pentamethoxyflavanone regulates M1/M2 macrophage phenotype and protects the septic mice

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[ABSTRACT] Flavonoids have been reported to exert protective effect against many inflammatory diseases, while the underlying cellular mechanisms are still not completely known. In the present study, we explored the anti-inflammation activity of 5, 7, 2', 4', 5'-pentamethoxyflavanone (abbreviated as Pen.), a kind of polymethoxylated flavonoid, both in vitro and *in vivo* experiments. Pen. was showed no obvious toxicity in macrophages even at high dosage treatment. Our results indicated that Pen. significantly inhibited both mRNA and protein level of proinflammatory cytokines, IL-1β, IL-6, TNF-α and iNOS, which was characteristic expressed on M1 polarized macrophages. These effects of Pen. were further confirmed by diminished expression of CD11c, the M1 macrophage surface marker. Further researches showed that the mechanism was due to that Pen. downregulated the activity of p65, key transcription factor for M1 polarization. On the other hand, Pen. also enhanced M2 polarization with upregulation of anti-inflammatory factors and increase of M2 macrophage surface markers, which lead to the balance of M1 and M2 macrophages. Moreover, *in vivo* research verified that Pen. treatment alleviated LPS-induced sepsis in mice by increasing survival rate, decreasing inflammatory cytokines and improving lung tissue damage. In summary, our results suggested that Pen. modulated macrophage phenotype via suppressing p65 signal pathway to exert the anti-inflammation activity.

[KEY WORDS] Macrophage polarization; Anti-inflammation; NFκB; Sepsis; 5, 7, 2', 4', 5'-Pentamethoxyflavanone (Pen.)

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Introduction

Inflammation is a complex biologic progress that undertakes both physiological and pathological functions [1]. The acute inflammatory response can be triggered by foreign invaders and/or sterile tissue damage. Resident macrophages and mast cells initially sense the danger signal and produce a series of inflammatory factors to recruit monocytes/macrophages and neutrophils to clear pathogen and repair damaged tissue [2]. However, the continuing recruited activated effector cells and the generated inflammatory mediators are highly potent and fail to distinguish host tissue from pathogens. As a result, the dysregulated inflammation always leads to unexpected damage [3]. Given that, inflammatory responses must be tightly controlled to avoid tissue damage during related diseases [2, 4].

Sepsis is a severe systematic acute inflammatory disease which involves multiple organs failure [5]. As the main mediators of inflammation, macrophages also play essential role during progression of sepsis [5]. Initially, macrophages swallow bacteria and secret a serious of cytokines against pathogens [6]. Then, the over-activated macrophages exaggerate inflammatory response and lead to bad long-term outcomes in septic patients [5].

The activation form of macrophage was defined as classical activated macrophages, also named M1 macrophage [7]. They produce pro-inflammatory factors, including IL-1β, IL-6, TNF-α and iNOS, under the stimulation of LPS and/or IFNγ. These characters endow them with strong microbicidal and tumoricidal activity [8]. In contrast, when stimulated with IL-4/IL-13 or IL-10, macrophages undergo the alternative acti-

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vation, also named M2 macrophage [9]. M2 macrophages are considered to be involved in parasite containment, promotion of tissue remodeling, tumor progression and to show anti-inflammatory functions [10-11]. The process that macrophages differentiate to different function phenotypes is called polarization [12-14]. And in selected preclinical and clinical conditions, coexistence of cells in different activation states and unique or mixed phenotypes have been observed [7-8, 15-16]. So, targeting macrophages and modulating their phenotype is confirmed as an effective anti-inflammatory intervention.

Natural compounds have been used for combating human diseases for thousands of years. Among them, emerging evidences indicate that the flavonoids have a broad range of activities, such as antidiabetic [17], antioxidants [18]; anti-ageing [19], anti-inflammation [20], anti-viral and cancer chemoprevention [21-22]. Polymethoxylated flavone is a class of flavonoid compound containing multiple methoxyl substituents with the anti-inflammatory and immunoregulatory characteristics [23], but the underlying mechanism is still unclear.

In the present study, we evaluated the bioactivity of 5, 7, 2', 4', 5'-pentamethoxyflavanone (Pen.) in anti-inflammation. Pen. is a polymethoxylated flavone of the ingredients of *Murraya paniculata*, a traditional Chinese medicine with multiple pharmacological activities. At present, there is no information about the function of Pen. We have reported other kind of polymethoxylated flavonoid with anti-inflammatory effect in macrophages [24]. Based on our previous researches, it's necessary to deepen exploring the effect and mechanism of Pen. in inflammation. Our data suggested that Pen. obviously inhibited the M1 macrophage polarization *via* blocking translocation of p65 to nucleus, and enhanced M2 macrophage polarization simultaneously, which eventually resulting in amelioration of LPS-induced sepsis. The effect on phenotype switching of macrophage indicates that Pen. may be a novel candidate for sepsis treatment.

Materials and Methods

Mice

Female C57BL/6 mice (6–8 weeks old, 18–22 g) were obtained from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). They were maintained with free access to pellet food and water in plastic cages 21 ± 2 ºC and kept on a 12 h light/dark cycle in specific-pathogen-free (SPF) facilities. Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) 'The Detailed Rules and Regulations of Medical Animal Experiments Administration and Implementation' (Order No. 1998-55, Ministry of Public Health, China) and approved by the Laboratory Animal Ethics Committee of School of Life Sciences, Nanjing University. The animal studies were reported in compliance with the ARRIVE guidelines [25]. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

Cells

Murine Raw 264.7 cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured in DMEM (GIBCO, Grand Island, NY, Cat#11965-084) containing 10% fetal bovine serum (GIBCO, Grand Island, NY, Cat#10099-141), 100 U·mL^{-1} penicillin, and 100 mg·mL^{-1} streptomycin in 5% CO_2 at 37 ºC. THP-1 cells were purchased from Shanghai Institute of Cell Biology (Shanghai, China) and maintained in RPMI 1640 (GIBCO, Grand Island, NY, Cat#61870-044) containing 10% fetal bovine serum (GIBCO, Grand Island, NY, Cat#10099-141), 100 U·mL^{-1} penicillin, and 100 mg·mL^{-1} streptomycin in 5% CO_2 at 37 ºC.

Bone marrow-derived macrophages (BMDMs) were established as previously described, with minor modifications [24]. In brief, femurs were flushed with PBS, using a 21-ga needle. Cells were grown in RPMI 1640 containing 10% fetal bovine serum and 10 ng·mL^{-1} macrophage-colony stimulating factor (M-CSF) (Peprotech, Rock Hill, NJ, Cat# 315-02) in 5% CO_2 at 37 ºC for 7 days. Adherent macrophages were washed by PBS twist and cultured with fresh DMEM medium containing 10% fetal bovine serum.

Raw 264.7 cells and BMDMs were polarized with 20 ng·mL^{-1} LPS for 6 hours to M1 phenotype, and with 20 ng·mL^{-1} IL-4 for 6 hours to M2 phenotype. BMDM was cultured with 10 ng·mL^{-1} LPS for 3 hours followed by 5 mmol·L^{-1}ATP to induce inflammasome assemble. THP-1 was polarized with 320 nmol·L^{-1} PMA for 3 hours to obtain adherent macrophages, and then cultured with 100 ng·mL^{-1} LPS for 3 hours followed by 5 mmol·L^{-1} ATP for 1 hour to induce inflammasome activation.

Reagents

Lipopolysaccharide (LPS), 3-(4, 5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), phorbol myristate acetate (PMA) and adenosine triphosphate (ATP) were purchased from Sigma-Aldrich (St. Louis, MO). 5, 7, 2', 4', 5'-Pentamethoxyflavanone (Pen.) was isolated from *Murraya Exotica*, with the purity of 98%. Recombinant murine IL-4 and macrophage-colony stimulating factor (M-CSF) were purchased from Peprotech (Rocky Hill, NJ). FITC-anti-CD11c, IgG2a isotype control FITC were purchased from eBioscience (San Diego, CA). Antibodies against p-p65, p-IKK, p65, IKK and H3 were purchased from Cell Signaling Technology (Beverly, MA). Antibody against actin was purchased from Abmart (Shanghai, China). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

MTT assay

Cells were cultured in 96-well plates at a density of 3 × 10^5 cells/well in 0.2 mL medium and treated with various dose of Pen. for 24 hours. MTT (4 mg·mL^{-1} in PBS, 20 μL per well) was added to each well. 4 hours later, culture media was removed and 200 μL DMSO was added to dissolve the crystals. The absorption values at 570 nm were measured.

Quantitative PCR

Total RNA was extracted from cells or tissues, reverse

transcribed to cDNA and subjected to quantitative PCR, which was performed with the BioRad CFX96 ouch™ Real-Time PCR DetectionSystem (BioRad, CA,) using iQTMSYBR1Green supermix (BioRad, CA, Cat#1708880), and threshold cycle numbers were obtained using BioRad CFX manager software. The program for amplification was 1 cycle of 95 ºC for 2 min followed by 40 cycles of 95 ºC for 10 s, 60 ºC for 30 s. The primer sequences used in this study were as following: *Il-1β*, 5'-CTTCAGGCAGGCAGTATCACTC-3' (forward) and 5'-TGCAGTTGTCTAATGGGAACGT-3' (reverse); *Il-6*, 5'-ACAACCACGGCCTTCCCTAC-3' (forward) and 5'-TCTCATTTCCACGATTTCCCAG-3' (reverse); *Tnf-α*, 5'-CGAGTGACAAGCCTGTAGCCC-3' (forward) and 5'-GTCTTTGAGATCCATGCCGTTG-3' (reverse); *Inos*, 5'-CAACATCAGGTCGGCCATCACT-3' (forward) and 5'-ACCAGAGGCAGCACATCAAAGC-3' (reverse); *Arg-1* 5'-CTCCAAGCCAAAGTCCTTAGAG-3' (forward) and 5'-AGGAGCTGTCATTAGGGACATC-3' (reverse); *Il-10* 5'- GGTTGCCAAGCCTTATCGGA-3' (forward) and 5'- ACCTGCTCCACTGCCTTGCT-3' (reverse); *Fizz1*, 5'-AGGAGCTGTCATTAGGGACATC-3' (forward) and 5'-GGATGCCAACTTTGAATAGG-3' (reverse); *Ym1*, 5'-AGAAGGGAGTTTCAAACCTGGT-3' (forward) and 5'-GTCTTGCTCATGTGTGTAAGTGA-3' (reverse); *Cd163* 5'-ATGGGTGGACACAGAATGGTT-3' (forward) and 5'-CAGGAGCGTTAGTGACAGCAG-3' (reverse); *Cd206* 5'-GCAGGTGGTTTATGGGATGT-3' (forward) and 5'-GGGTTCAGGAGTGTTGTGG-3' (reverse); *β-Actin*, 5'-TGCTGTCCCTGTATGCCTCT-3' (forward) and 5'-TTTGATGTCACGCACGATTT-3' (reverse).

Cytokines assay

Cytokines, IL-1*β*, IL-6 and TNF-*α*, were measured using ELISA kits from Dakewe Biotech Co., Ltd. (Shenzhen, China, Cat# 1210122, 1210602, 1217202) according to the manufacturer's instructions. NO/Nitrite was measured by Griess reagent.

Flow cytometry

Cultured cells were harvested and washed with cold PBS twice. Collected cells were stained with FITC-CD11c for 30 min at 4 ºC in dark and analyzed by flow cytometry.

Western blot

Proteins lysed from cultured cells were separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, Cat# IBFP0785C). After blocked in 5% nonfat milk at room temperature for 1 hour, membranes were incubated with aimed primary antibodies at 4 °C overnight, and then with a horseradish-coupled secondary antibody at room temperature for 1.5 hours. Final detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK, Cat# 54-61-00).

Subcellular fractionation

Cells were separated into cytosolic and nucleus fractions using the Qproteome Nuclear Protein Kit (Qiagen, Düsseldorf, Germany, Cat# 37582) according to the procedures provided by the manufacturer.

LPS induced sepsis

C57BL/6 mice were intraperitoneally administered (i.p.) 10 mg/kg LPS to induce sepsis. Mice were randomly assigned to four experimental groups (10 mice per group) after acclimating to the housing environment for 7 days: (a) Vehicle, (b) LPS, (c) LPS with Pen. at 15 mg·kg^{-1} and (d) LPS with Pen at 15 mg·kg^{-1}. Survival rate was monitored for 72 hours, and blood samples were collected 4 hours post LPS challenge. Data collection and analysis was performed blindly, the experimenters were unaware of the group assignment and animal treatment.

Bronchoalveolar lavage fluid (BALF) collection and detection

Four hours after LPS challenge, mice were sacrificed and suffered bronchoalveolar lavage (BAL) using 0.5 mL PBS for three times. The recovery rate of the fluid was about 90%. Bronchoalveolar lavage fluid (BALF) was centrifuged at 1500 *g* for 10 min at 4 ºC [24]. Cytokines, IL-1*β*, IL-6 and TNF-*α*, in the supernatants of BALF were measured using commercial ELISA kits. Cells pellets were re-suspended and used for total cell counting with a hemacytometer.

Histological analysis

Formalin-fixed, paraffin-embedded lung tissues were sectioned at 4 μm thickness, and sections were stained with hematoxylin and erosin (HE) to assess the inflammatory cells infiltration and lung tissue damage. All sections were observed by microscope. The original amplification was 100×.

Statistical analysis

All results were expressed as mean ± SEM of three independent experiments with each experiment including triplicate sets *in vitro*. One-way ANOVA analysis and Student's *t* test were used to evaluate the differences between various experimental and control groups.

Results

Pen. inhibited M1 macrophage polarization by down-regulating the proinflammatory factors, without affecting cell viability

The chemical structural formula of Pen. was shown in Fig. 1A. We evaluated the cytotoxicity of Pen. in both rest and LPS-primed macrophages *via* MTT assay. As shown in Fig. 1B and 1C, Pen. displayed no significant effects on the proliferation of Raw 264.7 cells and BMDMs even at a higher concentration (80 μmol·L^{-1}). Then, we chose 5, 10 and 20 μmol·L^{-1} as the working concentration to assess the bioactivity of Pen.. Macrophages were stimulated with LPS to induce the classical activation. When treated with various doses of Pen., the mRNA level of proinflammatory cytokines, including *Il-1β*, *Il-6*, *Tnf-α* and *Inos*, was significantly decreased in a concentration dependent manner, both in Raw 264.7 cells and BMDMs (Fig. 1D). In line with these observations, ELISA analysis verified that the protein level of these proinflammatory factors in RAW264.7 cells was also obviously suppressed in the presence of Pen. (Figs. 2A–2D). Different from other cytokines, the secretion of IL-1*β* need activation of

signal II. So, we established inflammasome model in BMDM and THP-1 cells to study the activity of Pen.. Results in Figs. 2E and 2F displayed that LPS and ATP stimulation induced higher protein level of IL-1*β* in supernatant than LPS alone. However, Pen. shown powerful inhibition on this proinflammatory factors in a dose dependent way.

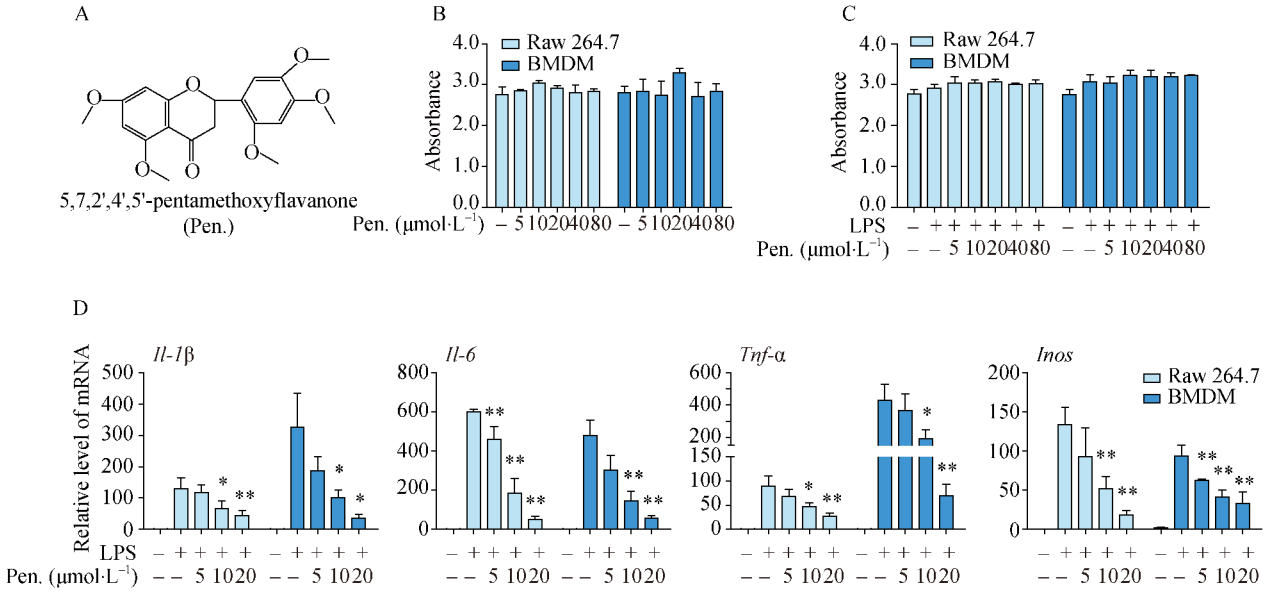

Fig. 1 Pen. inhibited LPS-induced proinflammatory cytokines in macrophage without affecting cell viability. (A) Molecule structure of Pen.. (B) Raw 264.7 cells or BMDM was incubated with various dose of Pen. for 24 h. Cell viability was measured by MTT assay. (C) Raw 264.7 cells or BMDM was incubated with various dose of Pen. in the presence of 20 ng·mL^{-1} LPS for 24 hours. Cell viability was measured by MTT assay. (D) Raw 264.7 cells or BMDM was incubated with various dose of Pen. in the presence of 20 ng·mL^{-1} LPS for 6 h. mRNA level of *Il-1*β, *Il-6*, *Tnf-α* and *Inos* was measured by Q-PCR. Data represented the mean ± SEM of three independent experiments in triplicate. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* LPS treated group

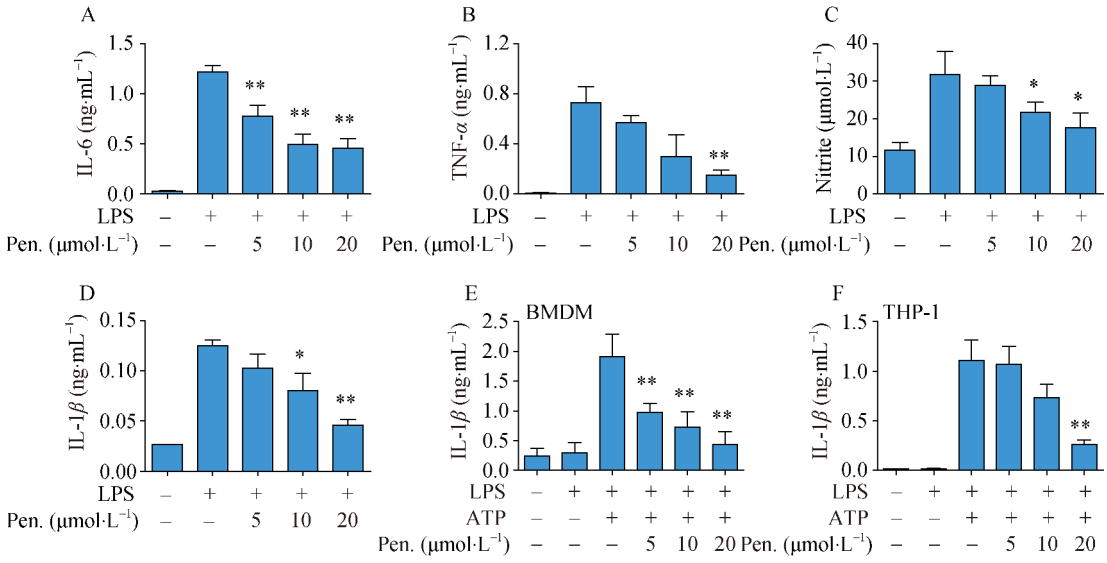

Fig. 2 Effects of Pen. on the secretion of proinflammatory factors in LPS-activated macrophages. (A-D) Raw 264.7 cells were incubated with various dose of Pen. in the presence of 20 ng·mL^{-1} LPS for 6 h. Cytokines level of IL-1*β*, IL-6 and TNF-*α* secreted was measured by ELISA. NO was by Griess reagent. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* LPS treated group. (E) BMDM was cultured with 10 ng·mL^{-1} LPS for 3 h followed by 5 mmol·L^{-1} ATP for 1 h in the presence of various does of Pen.. IL-1*β* in the supernatant was measured by ELISA. (F) THP-1 was polarized with 320 nmol·L^{-1} PMA for 3 h, and then cultured with 100 ng·mL^{-1} LPS for 3 h followed by 5 mmol·L^{-1} ATP for 1 hour in the presence of various does of Pen.. IL-1*β* in the supernatant was measured by ELISA. Data represented the mean ± SEM of three independent experiments in triplicate. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* LPS and ATP treated group

In addition, we detected the expression of CD11c, the cell surface marker of M1 macrophages, by flow cytometry. Data in Fig. 3 shown that LPS induced Raw264.7 cells polarized to M1 phenotype, with high proportion of $CD11c^+$. Pen. treatment dose-dependently decreased the percentage of $CD11c^+$ cells, compared with LPS alone treated group.

All these observations suggested that Pen. apparently repressed the M1 phenotype function property.

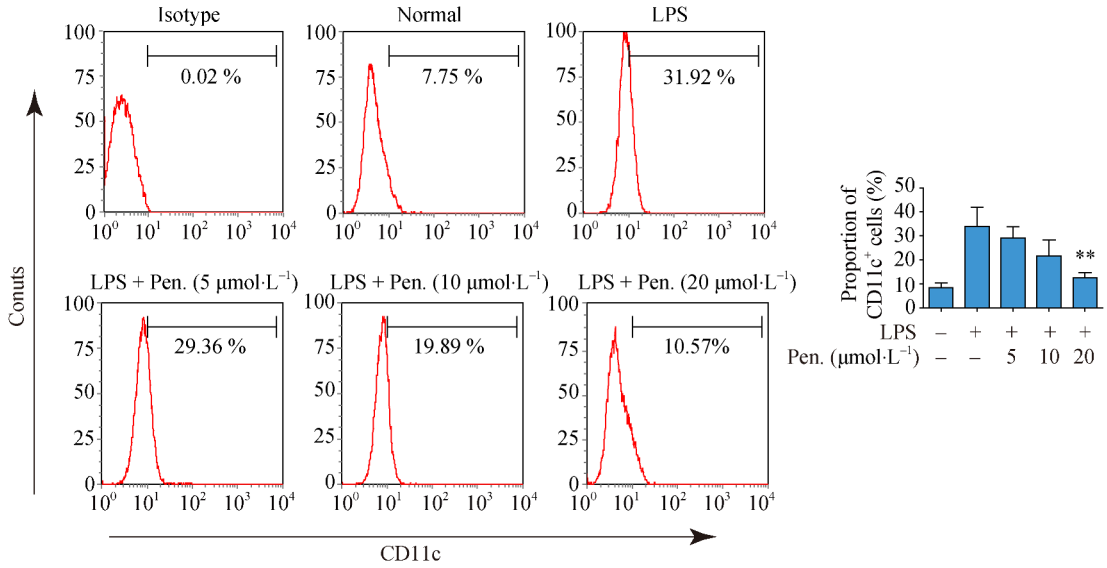

Fig. 3 Pen. blocked expression of CD11c, the surface marker of M1 macrophages. Raw 264.7 cells were incubated with various dose of Pen. in the presence of 20 ng·mL^{-1} LPS for 18 h. CD11c expression was detected by flow cytometry. The positive cells of three independent experiments were shown as a histogram of mean ± SEM. $^{**}P < 0.01$ *vs* LPS treated group

Pen. enhanced M2 macrophage polarization by up-regulating anti-inflammatory factors, including Fizz1, Ym1, Cd163, Cd206, Arg1 and Il-10

In contrast to proinflammatory phenotype, macrophages can be polarized to alternative activated macrophage (M2) under IL4/IL13 stimulation. We stimulated macrophages with murine IL-4 in the presence of a series dosage of Pen. to investigate its effect on M2 macrophage. Results in Fig. 4 shown that Pen. obviously enhanced mRNA level of M2 associated factors, as *Fizz1*, *Ym1*, *Cd163, Cd206*, *Arg1* and *Il-10,* in a dose dependent manner, both in Raw 264.7 cells and BMDMs (Figs. 4A–4F). These data indicated the promotion of Pen. on M2 polarized macrophages.

Pen. dose dependently inhibited the NF-κB signal pathway in M1 macrophages

To further elaborate underlying molecular mechanism,

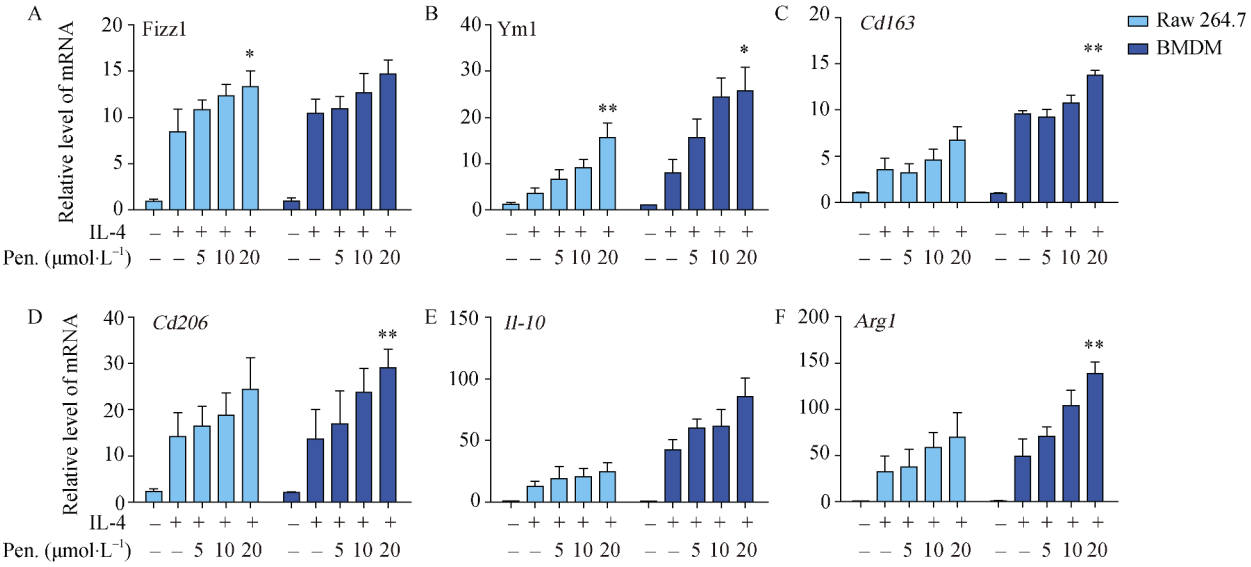

Fig. 4 Pen. enhanced M2 macrophage polarization. Raw 264.7 cells were incubated with various dose of Pen. in the presence of 20 ng·mL^{-1} IL-4 for 6 h. mRNA level of *Fizz1, Ym1, Cd163, IL4, Arg-1* and *Il-10* was measured by Q-PCR. Data represented the mean ± SEM of three independent experiments in triplicate. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* IL-4 treated group

we detected the related signal pathways of M1 macrophage. We focused on NF-κB signaling. And data showed that LPS greatly increased the protein level of p-IKK in M1 macrophage, upstream regulator of p65, but Pen. treatment inhibited p-IKK expression dose dependently, (Figs. 5A–5B). As a result, the activated form of p65, p-p65, was also obviously suppressed (Figs. 5A–5B). Since p65 need to translocate to nucleus to act as transcription factor, we detected the distribution of p65 at cytoplasm and nucleus respectively. Data in Figs. 5C and 5D displayed that Pen. downregulated the translocation of p65 from cytoplasm to nucleus under LPS stimulation. These results uncovered the underlying mechanism that Pen. executed the anti-inflammatory activity by inhibiting the activation of NF-κB signal pathway.

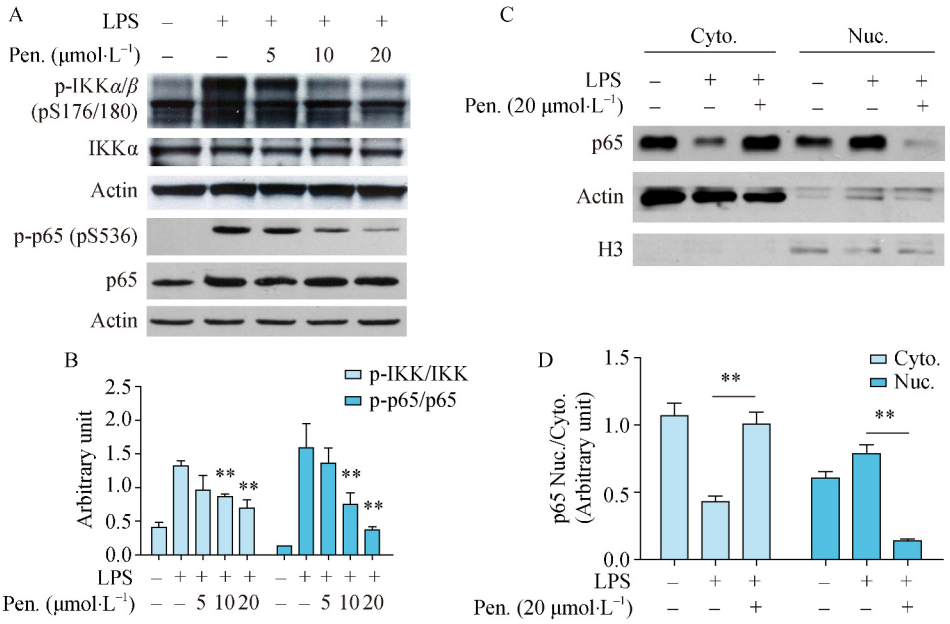

Fig. 5 Pen. downregulated the activation of p65 signal. Raw 264.7 cells were incubated with various dose of Pen. in the presence of 20 ng·mL^{-1} IL-4 for 6 h. (A) Expression of p-IKK, IKK, p-p65, p65 and Actin was measured by western blot. (B) Data represented the mean ± SEM of (A) with three independent experiments. $^{**}P < 0.01$ *vs* LPS treated group. (C) Location of p65 in cytoplasm and nucleus was measured by western blot. (D) Data represented the mean ± SEM of (C) with three independent experiments. $^{**}P < 0.01$ *vs* ??

Pen. ameliorated LPS-induced mortality, decreased proinflammatory cytokines, inhibited immunocyte infiltration and improved lung injury in mice

We detected bioactivity of Pen. *in vivo* in LPS induced septic mice. Mice was intragastric administered with various dose of Pen. when challenged with LPS. As shown in results, survival rate of 30 mg·kg^{-1} Pen.-treated group was elevated to 40%, while vehicle group was only about 10% (Fig. 6A). Proinflammatory cytokines, serum IL-6 and IL-1*β*, were obviously inhibited, which indicated the relief of system inflammatory response by Pen. treatment (Fig. 6B). We further detected the protective effects of Pen. on lung tissue. Data in Fig. 6C displayed that Pen. significantly inhibited the mRNA level of *Il-6* and *Il-1β* in lung tissue. Furthermore, IL-6 and IL-1β in BALF was also decreased in Pen.-administrated mice (Fig. 6D). Hematoxylin and eosin staining indicated that inflammatory cell infiltration and tissue damage in lung were greatly improved by Pen. treatment (Fig. 6E). These results suggested that Pen. ameliorated LPS-induced sepsis, which confirmed the anti-inflammatory activity of Pen. *in vivo*.

Discussion

Traditional Chinese medicine (TCM) is characterized by the nourishing of life and its role in many other diseases is getting more and more attention. Flavonoids are a large group of polyphenolic compounds distributed in a wide range of plants [17]. There have been many reports on their potential roles in the treatment of several kinds of disease, and their pharmaceutic potential have been evaluated in preclinical studies [21, 26-27]. Here, we studied a polymethoxylated flavonoid and confirmed its anti-inflammatory activity for the first time. Pen. was proved to ameliorate LPS-induced sepsis via modulating macrophage phenotype switching.

We first performed MTT assay on Pen.-treated rest and LPS-primed macrophages, and the results showed that Pen. did not influenced cell proliferation even at a high concentration (Figs. 1B and 1C). This helped us to rule out the cytotoxicity of Pen. in the following activity assessing experiments. Considering the important role of macrophage during inflammatory response, we detected the effect of Pen. on M1 polarized macrophages. LPS engagement activated NF-κB

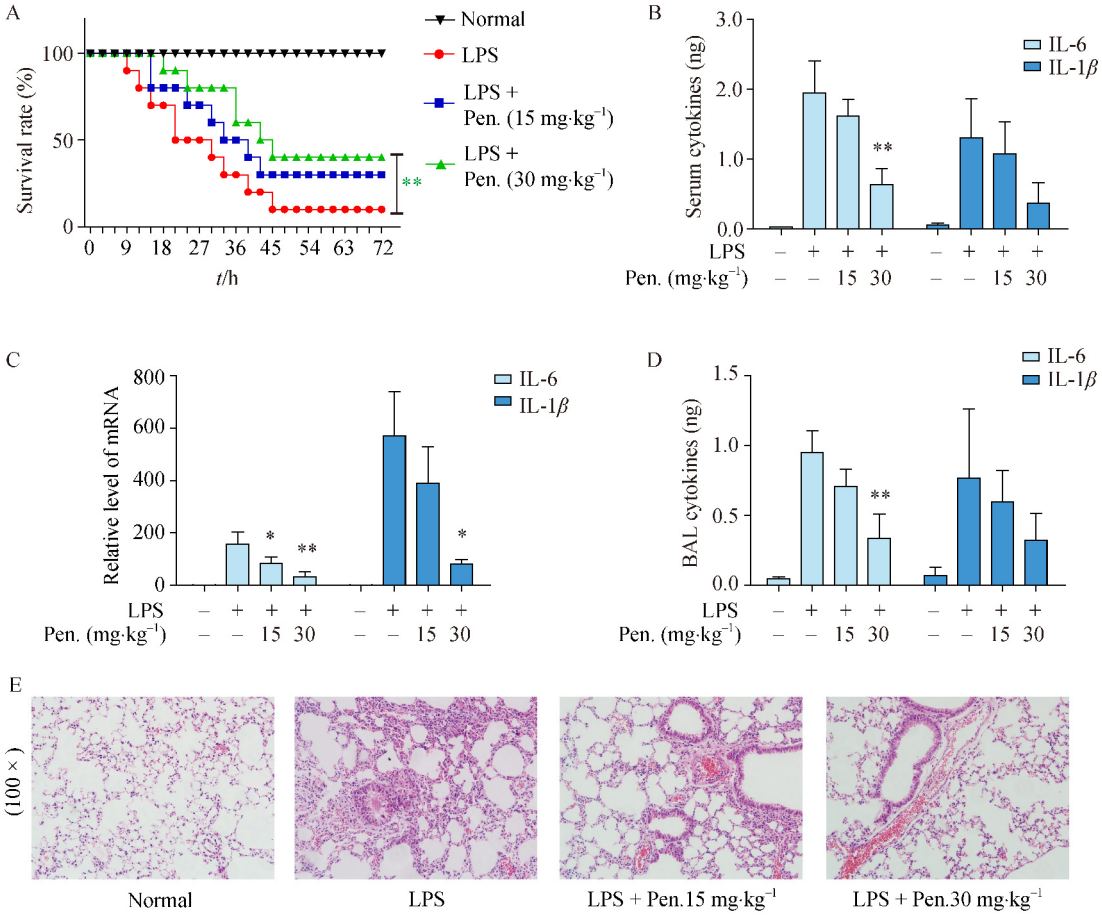

Fig. 6 Pen. protected mice from LPS-induced sepsis. Pen. (15, 30 mg·kg^{-1}) or vehicle (olive oil) was intragastric administered when LPS (10 mg·kg^{-1} i.p.) challenged. n = 10. (A) Survival rate was observed and calculated. 4 hours later, mice were scarified, serum, BALF and lung tissues were collected. (B) Cytokines of serum were measured by ELISA. (C) Cytokines in lung tissue were analyzed by Q-PCR. (D) Cytokines of BALF were tested by ELISA. (E) Hematoxylin and eosin staining of lung sections. And all sections were observed by microscope. Picture was the representative of three different views of every group. The original amplification was 100×. $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* LPS treated group

signal and elicited related proinflammatory factor gene expression. As shown in Fig. 1D, when stimulated with LPS, *Il-1β*, *Il-6*, *Tnf-α* and *Inos* expression was triggered. But Pen. treatment significantly suppressed these cytokines in a concentration dependent way, which proved the anti-inflammatory activity of this compound. In consistence with these observations, the protein level of these cytokines secreted to supernatant was also blocked (Fig. 2). However, one of these cytokines, IL-1β, needed the activation of signal II to produce the mature form [28]. IL-1β was significantly but slightly upregulated in LPS stimulated Raw 264.7 cells, which might lead to false positive results. So, we established inflammasome model on BMDM and THP-1 cells. IL-1β was enormously produced when promoted by LPS and ATP (Figs. 2E and 2F). And Pen. displayed satisfied inhibition on this cytokine, just as it did on the M1 polarized Raw 264.7 cells. Moreover, CD11c was the surface marker expressed in M1 macrophages. Treatment with Pen. apparently downregulated the proportion of CD11c positive cells. All these data suggested the anti-inflammation activity of Pen. on M1 polarized macrophages.

To further investigate the underlying molecular mechanism, we detected the related signal proteins. NF-κB p65, the master transcription factor for M1 polarization, need to be phosphorylated and translocated to nucleus to mediated target genes expression [13, 29]. And the phosphorylation of IKK, an upstream kinase, led to IκB degradation, as a result, inhibition on p65 was removed and the signal was activated [30]. Targeting these signal mediators and blocking their activity have been proved to be effective for inflammatory response. Our results shown that Pen. obviously inhibited the phosphorylation of NF-κB p65 and IKK (Fig. 5A). What's more, Pen. also broke the translocation of p65 to nucleus (Fig 5C). These observations indicated the mechanism of Pen. in anti-inflammatory activity. But there still need more experiments to be designed to further illustrate the specific molecular targets.

Moreover, the further study demonstrated that Pen. significantly promoted the ratio of M2 macrophages and in-

creased some anti-inflammatory factors, as Ym1 and IL-10 (Fig. 4). As mentioned above, Pen. had no toxic effect in macrophages, and its inhibition of M1 macrophage polarization was not achieved by the cytotoxicity. Therefore, we speculated that Pen. played an important role in regulating the balance of M1 and M2 macrophages. It can switch M1 macrophages to M2 macrophages in order to ameliorate inflammation. Such low toxic and high efficient immunomodulation is rare in other kinds of compounds, which indicates a good prospect of clinic application of Pen.

Sepsis was a sever clinical condition with high mortality rate. The excessive and unlimited inflammatory response and macrophage activation played a key role during the progression of disease [5]. Based on the effect of Pen. on M1 macrophages, we administrated LPS-induced sepsis mice with this compound and monitored the efficacy. As shown in Fig.6, Pen. treatment significantly promoted survival rate from 10% to 40% at 30 mg·kg^{-1}. Pen. inhibited proinflammatory cytokines both in serum and BAFL (Figs. 6B and 6D). This data suggested that systemic inflammation was obviously improved. Furthermore, proinflammatory cytokines in lung tissue was also suppressed (Fig. 6C). On the other hand, we proved that Pen. enhanced the M2 function property previously (Fig. 4), which contributed to anti-inflammation and tissue remodel. Combine with results in Fig. 6C, we speculated that Pen. might facilitate tissue repair other than only block tissue damage. These results confirmed the anti-inflammatory activity of Pen. *in vivo*.

In the present study, we evaluated the bioactivity of Pen. *in vitro* and in *vivo*. Our results proved the anti-inflammatory activity of Pen. on macrophage and detected underlying mechanism for the first time. Pen. significantly inhibited M1 polarized macrophages, while enhanced M2 phenotype simultaneously, without any obvious cytotoxicity. And considering the widely distribution of Pen. in plant, our study might be used in developing effective but more cheaper strategies for inflammatory diseases.

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