

## Original Article

# Luteolin regulates CLP-induced sepsis mice by inhibiting PPAR- $\gamma$ /STAT/MyD88 pathway

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**Abstract:** To investigate the effects of luteolin on cecal ligation and puncture (CLP)-induced sepsis in mice. Sepsis model was established in clean grade BALB/c inbred male mice by CLP. Toxic effects of luteolin on mouse peritoneal macrophage was tested by MTT assay. The phagocytosis by macrophage was detected by fluorescent microspheres combined with flow cytometry. The level of cytokines including TNF- $\alpha$ , IL-10, IL-1b and IL-6 in mice of different groups were detected by enzyme linked immunosorbent assays (ELISA). The level of TLR4, PPAR- $\gamma$ , STAT and MyD88 protein were detected by Western blot. Results showed that luteolin inhibited the phagocytosis of macrophages, downregulated the expression of MyD88 and TLR4 in mouse peritoneal macrophages, regulated the expression of cytokines including TNF- $\alpha$ , IL-10, IL-1b and IL-6, and reduced the level of PPAR- $\gamma$  and STAT protein, which indicated that luteolin plays a role to against sepsis by inhibit PPAR- $\gamma$ /STAT/MyD88 pathway.

**Keywords:** Luteolin, sepsis, immunity, cytokine, PPAR- $\gamma$ /STAT/MyD88 pathway

## Introduction

Sepsis is a syndrome of the systemic inflammatory response caused by infection, which causes poor organ function or insufficient blood flow [1]. Patients with severe sepsis generally present symptoms in organ failures or tissue hypoperfusion [2-4].

The development of sepsis-induced organ failure is mediated by different pathways in different organs, and MyD88 and TLR4 are usually the key factors responsible for the signal transduction [5, 6]. During the development of kidney damage induced by sepsis, the pathogens was firstly recognized by Toll-like receptors (TLRs) including TLR4, which is usually produced by sentinel cells such as macrophages to initiate inflammatory response [6, 7]. Part of the signal transduction initiated by TLR4 can be mediated by an adapter protein of TLR-Myeloid Differentiation Factor 88 (MyD88) through the binding of MyD88 to TIR domain, which lead to the recruitment of signaling proteins and the expression of the inflammatory response related factors such as tubular necrosis factor (TNF)- $\alpha$ , interleukin-6 (IL-6) and interleukin-1b

(IL-1b) [8, 9]. PPAR- $\gamma$  and IL-10 inhibited the expression of STAT-1-dependent MyD88, which in turn increased the survival rate of animal with sepsis [10]. Epidemiological studies showed that severe sepsis presented an increasing trend in high mortality rate [11, 12]. To decrease the mortality rate, different methods have been developed for treatment, however, these methods have been proved inefficient in improving the survival rate of the patients with severe sepsis [13, 14]. So, it is important to discover new drugs to increase the survival rate of severe sepsis.

Luteolin is a kind of common flavonoid that exists in many vegetables, herbs and fruits, such as celery, thyme, and honeysuckle [15]. Plants containing luteolin have been widely applied in traditional Chinese medicine to treat with various diseases including inflammatory diseases, cancer and hypertension. Relative study has proved the activities of luteolin on anti-inflammatory, antiallergic, antioxidative, and antitumorigenic reactions [15]. It can reduce the pulmonary inflammatory response to inhibit MAPK and NF $\kappa$ B pathways in mice with acute lung injury [16]. In addition, luteolin can

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inhibit polymorphonuclear neutrophils, which is a critical event that lead to host tissue injury and organ damage after trauma through MEK/ERK and PI3K/Akt pathways [17-19]. Moreover, luteolin was capable of down regulating TLR4, which could inhibit the TLR4 initiated inflammatory response [20]. Considering the important functions of luteolin, especially its anti-inflammatory activity and its interaction with inflammatory factors, it's reasonable to hypothesize that luteolin plays a role in the development of sepsis. In this study, we investigated the effects of luteolin on sepsis in mice through the establishment sepsis model by CLP and explored its possible mechanism. We found that luteolin performed in decreasing the expression of macrophages MyD88 and TLR4, regulating the expression of cytokines, and reducing the level of PPAR- $\gamma$  and STAT protein, which proved that luteolin plays a role against sepsis by inhibiting PPAR- $\gamma$ /STAT/MyD88 pathway.

### Materials and methods

#### *Experimental animals and grouping*

Clean grade BALB/c inbred male mice were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China). All mice were 8-10 weeks old with weight of  $20 \pm 2$  g. The mice were randomly divided into 4 groups: control group (Ctrl); sham-operated group (sham); CLP group (group with mice treated with cecal ligation and puncture (CLP) and 300  $\mu$ L of PBS which was used to treat the mice 2 day, and 1 h before the surgery); luteolin treatment group (group with mice treated with CLP and 300  $\mu$ L luteolin (400 mg/L) (WEIKANG ZHIHUA Co. Ltd, Sichuan, China) which was used to treat the mice 2 day, and 1 h before the surgery). Each group contained 10 mice.

#### *Sepsis induced by CLP in mice*

Mice were fasted for 24 h before surgery, and were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.4 mL/100 g). The mice were fixed on the operating table, and were disinfected by 5% iodophor, followed by deiodination with 75% alcohol. Then CLP was used to induce sepsis: a 2-cm-long incision was made along the abdomen middle line. The cecum was ligated by surgical thread at the position 1-2 cm from the end (mesangial blood vessels should be avoided), and then the liga-

tion end was punctured. The intestine was then placed back, and peritoneum and skin were sutured. All the operations are the same for the mice in sham group except that the mice in this group are not treated with cecal ligation and puncture

#### *Preparation of mouse peritoneal macrophage suspension*

BALB/c mice were sacrificed and intraperitoneally injected with 5 mL of RPMI-1640 complete medium (Gibco, USA) containing 100 mL/L of fetal bovine serum (FBS, Gibco, USA), 2 mmol/L of L-glutamine (Sigama, USA), 5  $\mu$ mol/L of  $\beta$ -dimercaptoethanol (Sigama, USA),  $1 \times 10^5$  U/L of penicillin (Roche, Shanghai, China) and 100 mg/L of streptomycin (Roche, Shanghai, China). Fingers were used to lightly massage the mouse abdomen for 5 to 10 min. The medium was extracted and the cells were counted to adjust to density to  $1 \times 10^8$  cells/L.

#### *MTT assay for drug toxicity*

The peritoneal macrophage suspension was inoculated into 96-well cell culture plate (Costar 3422, Corning Inc., NY, USA) with 200  $\mu$ L per well. After incubation for 12 h, the non-adherent cells were removed. 200  $\mu$ L of RPMI-1640 basal medium (Gibco, USA) was added into each well. The experiment includes three groups: blank group, the control group and luteolin treatment group, 6 repeat wells for each group. After incubation for 48 h, 50  $\mu$ L of MTT (5 mg/mL) (Sigma, USA) was added into each well and incubated for 4 h at 37°C. 100  $\mu$ L of supernatant was then removed and 100  $\mu$ L of DMSO was added. The absorbance at 490 nm was measured by a microplate reader. Cell relative viability = [(A test group-A blank group)/(A control group-A blank group)]  $\times$  100%.

#### *Detection of macrophage phagocytosis by florescent microspheres combined with flow cytometry*

The peritoneal macrophage suspension was inoculated into 24-well cell culture plate (Costar 3422, Corning Inc., NY, USA) with 500  $\mu$ L per well. After incubation for 12 h, the non-adherent cells were removed. 500  $\mu$ L of RPMI-1640 basal medium (Gibco, USA) was added into each well. The experiment include four groups: control group (cell suspension), luteolin treat-

**Table 1.** The survival rate of mouse macrophages after treatment with different concentrations of luteolin

Group	A <sub>490</sub> nm	%Survival rate
Blank	0.11 $\pm$ 0.01	-
Control	0.42 $\pm$ 0.02	100.00
5 $\mu$ mol/L	0.38 $\pm$ 0.01*	87.10
10 $\mu$ mol/L	0.35 $\pm$ 0.03**	77.42
20 $\mu$ mol/L	0.34 $\pm$ 0.02**	74.19

All experiments were repeated six times. Data are means  $\pm$  SD. \*represented significant differences ( $p < 0.05$ ) compared with control group, \*\*represented significant differences ( $p < 0.01$ ) compared with control group.

ment group (cell suspension + 10 or 20  $\mu$ mol/L of luteolin), bacterial lipopolysaccharide (LPS) stimulation group (cell suspension + 10 ng/mL of LPS), LPS + luteolin group (cell suspension + 10 ng/mL of LPS + 10 or 20  $\mu$ mol/L of luteolin), three repeat wells were included in each group. After incubation for 4 hours, LPS was added and incubated for 24 h. Yellow-Green Beads with a diameter of 1  $\mu$ m were added at a final concentration of  $1 \times 10^{10}$  cells/L, and kept for 30 min. Then the supernatant was removed. The cells were detected by flow cytometry in FL1 channel (Becton Dickinson, USA).

#### Detection of cytokines

Blood samples were collected and plasma was separated to determine the tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-10, IL-1b, and IL-6. The level of serum TNF- $\alpha$ , IL-10, IL-1b and IL-6 in mice of different groups were detected by enzyme linked immunosorbent assays (ELISA) in duplicate in the same run to avoid interassay variability. All cytokines were assessed with a sandwich ELISA based on a monoclonalmonoclonal antibody pair and a biotin-streptavidin amplification system (Siemens Medical Solutions Diagnostics, Los Angeles, CA), following the manufacturers protocol.

#### Western blot

The total protein of macrophages was extracted by conventional method and BCA method was used to check the protein concentration. The protein was denatured at 95°C for 5 min, and 40  $\mu$ g of protein was loaded for 12% SDS-PAGE gel running. After that the protein was transferred to polyvinylidene difluoride filter

(PVDF) membrane (Millipore, USA), and 5% skim milk was used to block the membrane for 2 h. The primary antibody of anti-MyD88, anti-TLR4, anti-p-STAT-1, anti-PPAR- $\gamma$ , anti- $\beta$ -actin (Santa Cruz Bio-technology, Santa Cruz, CA, USA) were added and incubated overnight at 4°C. After washing, the secondary antibody was added and incubated at 37°C for 2 h. Finally, ECL solution was added for exposure at darkroom. Bands can 5.0 software (Glyko, Inc., Novato, CA, USA) was used to analyze the data.

#### Statistical analysis

All data were expressed as mean  $\pm$  SD. Statistical analysis was performed using SPSS software (SPSS, Chicago, Illinois, USA). Comparisons between two groups were performed using Student's t-test.  $P$  value of  $< 0.05$  meant statistically significant;  $p$  value of  $< 0.01$  meant significantly different.

## Results

#### Toxic effects of luteolin on peritoneal macrophages

The results showed that the survival rate of mouse macrophages after treatment with different concentrations of luteolin (5  $\mu$ mol/L, 5  $\mu$ mol/L and 20  $\mu$ mol/L) for 48 h were all above 74% (**Table 1**) all of which were lower than the Median Lethal Dose, which indicated that the concentration of luteolin we used would not affect our experimental results.

#### Effects of luteolin on phagocytosis by macrophages

Mean Fluorescence Intensity (MFI) was used to indicate the phagocytosis by macrophages. As shown in **Figure 1**, the MFI value of control group was  $95.72 \pm 3.42$ , which was significantly lower than that of LPS stimulation group ( $106.99 \pm 5.23$ ) ( $p < 0.01$ ). The MFI values of the cells after 10  $\mu$ mol/L luteolin and 20  $\mu$ mol/L luteolin treatment were decreased to  $87.48 \pm 2.24$  and  $84.16 \pm 3.16$ , respectively, which were both significantly lower than that of control group ( $p < 0.01$ ). The MFI values of luteolin (10  $\mu$ mol/L) + LPS group and luteolin (20  $\mu$ mol/L) + LPS group were  $98.05 \pm 3.94$  and  $96.03 \pm 2.15$ , respectively, which were both significantly lower than that of LPS stimulation group ( $p < 0.01$ ).

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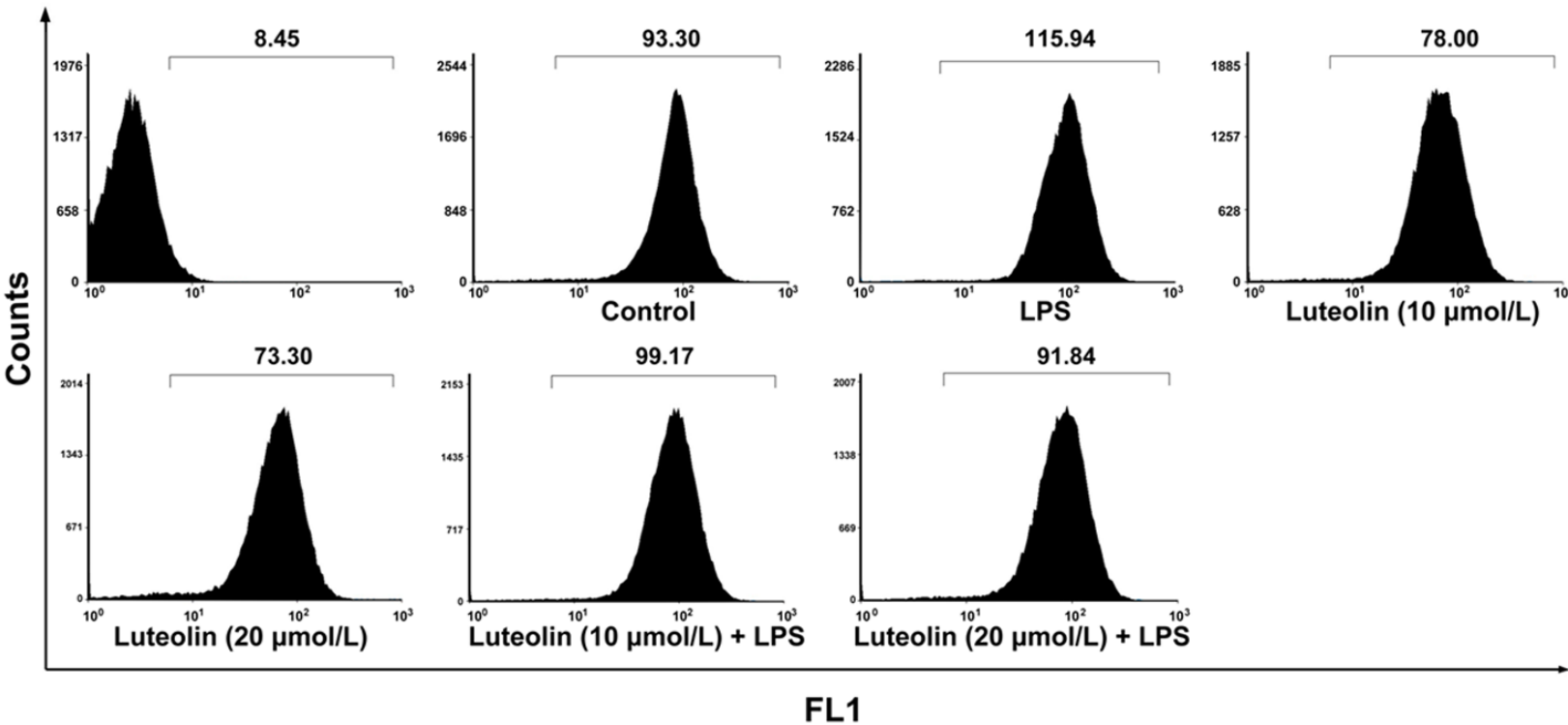
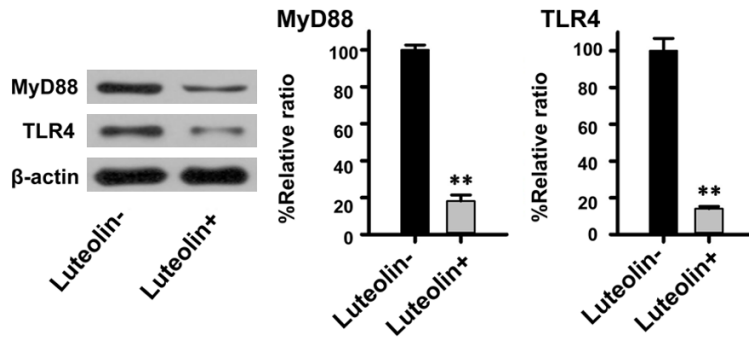


Figure 1. The MFI values of the cells in each group.



**Figure 2.** Expression of MyD88 and TLR4 protein in mouse peritoneal macrophages of different groups. \*\*,  $p < 0.01$  compared with the untreated peritoneal macrophages.

*Luteolin downregulated the expression of MyD88 and TLR4 in mouse peritoneal macrophages*

The mouse peritoneal macrophages were treated with luteolin (see the method of MTT assay), and then the total protein was extracted from cells to detect the level of macrophages MyD88 and TLR4 protein. The level of MyD88 and TLR4 protein were both significantly decreased after luteolin treatment compared with those in the untreated peritoneal macrophages ( $p < 0.01$ ) (Figure 2).

*Luteolin downregulated the expression of cytokine in mice with sepsis*

The level of cytokines (TNF- $\alpha$ , IL-10, IL-1b and IL-6) in the serum of the mice in sham group, CLP group and luteolin treatment group were detected by ELISA. The level of TNF- $\alpha$ , IL-10, IL-1b and IL-6 were all increased after CLP (Figure 3). After luteolin treatment, the level of TNF- $\alpha$ , IL-1b and IL-6 were decreased while the level of IL-10 was increased compared with those in sepsis group ( $p < 0.01$ ) (Figure 3). The application of luteolin combined with PPAR- $\gamma$  antagonist GW9662 made the decrease of TNF- $\alpha$ , IL-1b and IL-6 and increase of IL-10 more significant than the treatment with luteolin alone (Figure 3).

*Luteolin inhibited PPAR- $\gamma$ /STAT/MyD88 pathway*

The total protein was extracted from peritoneal macrophages of the mice in CLP group, CLP + luteolin group and CLP + luteolin + GW9662 group. The expression level of PPAR- $\gamma$ , STAT and

MyD88 protein were detected by western blot. We found that the expression level of PPAR- $\gamma$ , STAT and MyD88 were decreased significantly after luteolin treatment compared with those in CLP group ( $p < 0.01$ ) (Figure 4), and the application of luteolin combined with PPAR- $\gamma$  antagonist GW9662 made the decrease more significant than the treatment with luteolin alone (Figure 4).

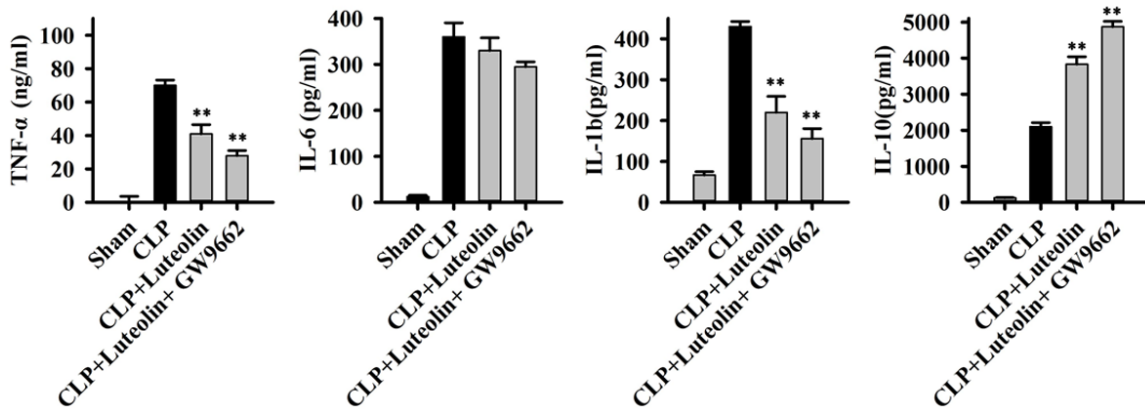
**Discussion**

Numerous studies have shown that luteolin, which is widely used in traditional Chinese medicine, can be used to treat various human diseases including Alzheimer's disease, liver disease and cancer by taking advantage of its biological activities such as anti-inflammatory, antiallergic and antioxidative functions [21-23]. In our study, we tested the effects of luteolin on sepsis through the establishment of sepsis mice model by CLP. Before the experiment, we tested the toxic effects of luteolin on peritoneal macrophages by MTT assay, we found that the survival rate of mouse macrophages after treatment with different concentrations of luteolin (5  $\mu\text{mol/L}$ , 5  $\mu\text{mol/L}$  and 20  $\mu\text{mol/L}$ ) for 48 h were all above 74% (Table 1), which would not affect our experiments.

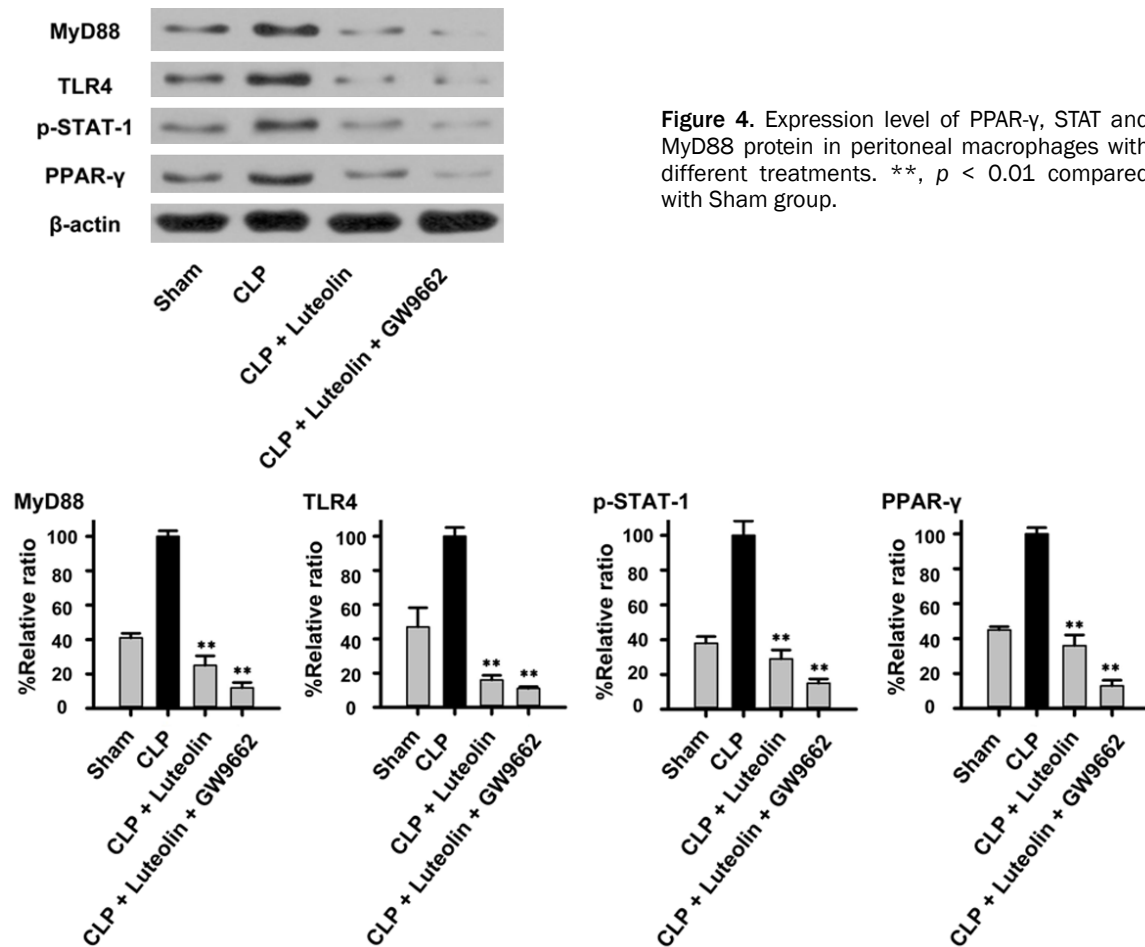
Phagocytosis, which is the primary method used to remove free microorganisms, is coupled with the inflammatory response [24]. Previous studies have shown that low concentrations of LPS can stimulate phagocytosis in mouse [25]. Consistent results were got in this study. LPS stimulation significantly increased the MFI value (the indicator of phagocytosis by macrophages) compared with that in control group ( $p < 0.01$ ) (Figure 1), while luteolin treatment significantly decreased the MFI value of the cells with and without LPS stimulation group ( $p < 0.01$ ) (Figure 1). Our data suggested that luteolin treatment can decrease the phagocytosis by macrophages under both basal and stimulated conditions, which in turn inhibit the inflammatory response.

MyD88 and TLR4 are two key factors responsible for the signal transduction involved in the organ damage induced by sepsis. The signal





**Figure 3.** Expression of NF- $\alpha$ , IL-10, IL-1b and IL-6 in cells of different groups. \*\*,  $p < 0.01$  compared with Sham group.



**Figure 4.** Expression level of PPAR- $\gamma$ , STAT and MyD88 protein in peritoneal macrophages with different treatments. \*\*,  $p < 0.01$  compared with Sham group.

transduction was initiated by TLR4 and mediated partially by MyD88 to induce the expression of inflammatory response related factors such as TNF- $\alpha$ , IL-6 and IL-1b [8, 9], while this signal transduction can be inhibited by IL-10 through the inhibitory roles of IL-10 on STAT-1-

dependent MyD88 expression [10]. In our study, the level of MyD88 and TLR4 protein were both significantly decreased after luteolin treatment compared with those in the untreated peritoneal macrophages ( $p < 0.01$ ) (Figure 2). In addition, the level of TNF- $\alpha$ , IL-1b and IL-6

were decreased after luteolin treatment, while the level of IL-10 was increased compared with that of the untreated group ( $p < 0.01$ ) (**Figure 3**). Our data suggested that luteolin treatment can inhibit signal transduction involved in the development of organ damage induced by sepsis possibly by inhibiting TLR4/MyD88 signal transduction.

Our data have shown that luteolin can upregulate the expression of TNF- $\alpha$ , IL-1b and IL-6 and downregulate the expression of IL-10 (**Figure 3**). We also found that the treatment combined luteolin with PPAR- $\gamma$  antagonist GW9662 played a better role in the regulation of the expression of TNF- $\alpha$ , IL-1b, IL-6 and IL-10 to (**Figure 3**), indicating that the roles of luteolin was related to the inhibition of PPAR- $\gamma$ . Previous studies also have shown the pivotal roles of PPAR- $\gamma$  in MyD88/TLR4 [10]. Therefore, we detected the expression of PPAR- $\gamma$  and PPAR- $\gamma$  pathway related factors-STAT and MyD88. PPAR- $\gamma$  was proved to be able to impair STAT-1 activation, which in turn reduced the STAT-1-dependent MyD88 expression [10]. In our study we found that the expression level STAT and MyD88 were both decreased significantly by luteolin treatment compared with the group without treatment ( $p < 0.01$ ) (**Figure 4**), and the application of luteolin combined with PPAR- $\gamma$  antagonist GW9662 made the decrease more significant than the treatment with luteolin alone (**Figure 4**), indicating that luteolin can inhibit the STAT-1-dependent MyD88 expression. To our surprise, the expression level of PPAR- $\gamma$  was also significantly decreased by luteolin treatment. The possible explanation is that luteolin can activate PPAR- $\gamma$  but also can downregulate its expression level, indicating the complex regulatory network of luteolin induced PPAR- $\gamma$ /STAT/MyD88 pathway.

### Conclusion

In this study, luteolin was proved to be able to activate macrophage immunity without significantly reducing the survival rate of macrophages. Our findings suggested that luteolin can decrease the expression of macrophages MyD88 and TLR4, regulate the expression of cytokines, and reduce the level of PPAR- $\gamma$  and STAT protein, which provides an evidence that luteolin plays a role to against sepsis by inhibiting PPAR- $\gamma$ /STAT/MyD88 pathway.

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### Disclosure of conflict of interest

None.

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