Kechuangping Mixture Inhibits LPS-induced Macrophage Immune Response Through Suppressing the TLR4/MyD88/NF-κB Signal Pathway

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Research Article

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Abstract

Background: Kechuangping mixture (KCPM) based on Maxingshigan decoction has a significant clinical effect in the treatment of pediatric pneumonia, especially for asthmatic pneumonia. This study explored the effects of KCPM on lipopolysaccharide (LPS)-induced macrophage immune response and the molecular mechanism involved.

Methods: CCK-8 assays was used to detect the effect of KCPM on cell viability. The RAW 264.7 macrophages were divided into four groups: blank, LPS, KCPM, LPS+KCPM. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to examine the secretion of inflammatory factors and the expression of TLR4/MyD88/NF-κB signal pathways with or without KCPM stimulation.

Results: KCPM inhibited the differentiation of RAW264.7 macrophages induced by LPS. The secretion of pro-inflammatory cytokines IL-6 and TNF-α induced by LPS was decreased by pre-treatment with KCPM. While, treatment with KCPM advanced, the anti-inflammatory cytokines IL-10 and TGF-β increased significantly. Additionally, KCPM exhibited a potent inhibitory effect on the expression of iNOS and a promotive effect on the expression of Arg-1. Moreover, KCPM clearly suppressed the transcription level of TLR4, MyD88 and NF-κB signal pathways.

Conclusion: KCPM promoted the macrophages polarization toward to M2 phenotype, as a result, limited the macrophage immune response. TLR4/MyD88/NF-κB signal pathways may play an important role in this process.

1. Background

Macrophages colonised on the airways shortly after birth, originating from fetal monocytes[1], which are the most important innate immune cells in the lungs, and play a critical role in regulating pulmonary immune responses to inhaled pathogens and allergens. Macrophages can be activated into two phenotypes, the classically activated (M1) phenotype and the alternatively activated (M2) phenotype[2]. Under the stimulation of microbes, endotoxin, hypoxia and other inflammatory factors, M1 macrophages mainly promote Th1 immune response, which is induced by Th1 cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, or lipopolysaccharide (LPS), and generate a large number of inflammatory factors such as TNF-α, interleukin (IL)-6 and inducible nitric oxide synthetase (iNOS). M1 macrophages can effectively eliminate the pathogens in cells, promote inflammation, and induce to tissue injury. M2 macrophages are stimulated by IL-4 and IL-10, and secrete a large number of anti-inflammatory factors such as IL-10, arginase (Arg)-1 and IL-13, resulting in decreased inflammation and promoting tissue repair[3]. Thus, M1/M2 polarization balance plays an important role in the regulation of infectious immune response and affects the development of inflammation[4].

Innate immunity relies on the signals generated from membrane-bound and cytosolic pattern recognition receptors (PRRs). Toll-like receptors (TLRs) were the first PRRs to be identified and have been subsequently well characterized. TLRs are germ-line–encoded receptors that sense distinct pathogen-

associated molecular patterns (PAMPs) derived from pathogens. To date, 12 functional TLRs in mice and 10 TLRs in humans have been identified along with several TLR ligands and adaptor molecules[5]. TLR4, one of the well-characterized TLRs, senses LPS of bacteria and induces pulmonary immunity to many gram-negative pathogens, and also plays an important role in the induction of host defense against gram-positive bacterial lung infections[6]. TLR4 signalling activates transcription factors such as myeloid differentiation factor 88 (MyD88), nuclear factor kappa B (NF-κB), which are key inflammatory response mediators[7]. Activated NF-κB is released and translocated to the nucleus to participate in gene transcription, promoting macrophages to secrete IL-1, IL-12, TNF-α and other pro-inflammatory factors. IL-12 can mediate T cells polarize to Th1 cells and promote the synthesis and secretion of Th1 cytokines, which induce macrophages to M1 phenotype. Then M1 macrophages participate in the acute inflammatory response of lung tissue[8, 9]. Therefore, the TLR4/MyD88/NF-κB pathways are considered as some of the primary signaling pathways involved in pulmonary inflammatory response.

Many researches found that Traditional Chinese herbs can regulate the polarization, apoptosis and immune activity of macrophages, thereby reducing the inflammatory response and controlling the progress of inflammation[10]. Kechuangping mixture (KCPM) is a prescription with decades of clinical experience in our department. It has a significant clinical effect in the treatment of pediatric pneumonia. This prescription is based on Maxingshigan decoction (MXSGD) mentioned in "Treatise on Febrile Diseases", which is composed of *Mahuang*, *fried bitter almond*, *raw gypsum*, *reed root*, *Houttuynia*, *big green leaves of Polygonum*, *fried perilla seed*, *Liping seed*, *fried Raphani seed*. The whole prescription has the effect of promoting lung function, relieving cough, clearing heat, resolving phlegm and relieving asthma. Previous studies in our department have shown that its combination with ribavirin can significantly inhibit respiratory syncytial virus and reduce the dosage of KCPM and ribavirin[11], but its mechanism is not clear. MXSGD has been used to treat severe pulmonary diseases, especially for pneumonia, which can reduce the inflammatory response of patients, alleviate the disease progress, and ameliorate the prognosis[12]. Therefore, whether KCPM play a role in the treatment of pediatric pneumonia mainly through inhibiting the inflammatory response of macrophages? What is the specific mechanism of its inhibitory effection on macrophage inflammatory response? All these problems need to be solved.

In this study, RAW264.7 macrophages were stimulated with LPS in the presence or absence of KCPM. The effects of KCPM on the activation of RAW264.7 macrophage, the secretion of inflammatory factors and the modifications of TLR4/MyD88/NF-κB signal pathways were examined. Through the establishment of cell inflammation model, the effect of KCPM on macrophage immune response was studied. Aimed to provide the theoretical basis for the following-up study which will explore the effect of KCPM on macrophage immune response in pediatric pneumonia.

2. Methods

2.1. Reagents

The mice mononuclear macrophage RAW264.7 cell line was presented by Beijing Union Medical College Hospital. KCPM was a Chinese patent medicine in Beijing Friendship Hospital (the original solution contains crude drug 5.5g/ml). DMEM medium (Hyclone USA, SH30022.01), fetal bovine serum (GIBCO USA, 10270-106), lipopolysaccharide (LPS) (Sigma China, l2630), DEPC (Amresco USA, E174); The Cell Counting Kit-8 (CCK-8) (Biyuntian Biotechnology Co., Ltd., China, c0037), Trizol (Beijing all gold Biotechnology Co., Ltd., H10318, China), Reverse transcription Kit (Beijing all gold Biotechnology Co., Ltd., AQ131-01), primer synthesis (Shanghai Jierui Bioengineering Co., Ltd.), FastKing cDNA first strand synthesis Kit (Tiangen biochemical technology (Beijing) Co., Ltd., KR116-02).

2.2. Instruments

XD-101 CO_2 cell incubator (Sanyo company, Japan), SW-CJ-1FD cell super clean workbench (Antai company, Sujing group, China), RT-6000 enzyme scale instrument (Redu Life Science Co., Ltd., Shenzhen, China), Nanodrop 2000 ultra micro spectrophotometer (Thermo company, USA), IX51 biological inverted microscope (Olympus company, Germany); Stepone Plus real time PCR (Applied Biosystems, USA).

2.3. Cell culture

RAW264.7 cells were cultured in endotoxin-test DMEM with 10% fetal bovine serum (FCS) in the presence of 5% CO_2 at 37°C prior to treatment, cells were incubated overnight at 37˚C. The cells were cultured into 21-wells plate with a density of 1×10^5 cells/well, set up four groups: blank, LPS, KCPM, LPS+KCPM optimal dose group (the optimal dose was selected by CCK-8 method), each group set up two double holes, repeat once. Cells were incubated in the presence of 5% CO_2 for 24h at 37˚C. Then, after the KCPM group and LPS+KCPM group were treated with KCPM for 4h, LPS was added to the LPS group and LPS+KCPM group with the final concentration 1mg/L, equal volume PBS was added to the blank and KCPM group. The cells were cultured continuly for another 24h.

2.4. Cell viability assay

CCK-8 assays to measure the viability of cells. Briefly, the cells were cultured into 96-wells plate with a density of 5×10^3cells/well and incubated overnight in the presence of 5% CO_2 at 37˚C. Then the cells were washed with fresh 1% FCS and treated with or without KCPM (5500.0、2750.0、1375.0、687.5、343.7、171.9、85.9、42.9、21.5、10.7mg·ml^{-1}) for 24h. The cells were washed with PBS. In each well, 100 μl CCK-8 solution was added and incubated for 4h at 37˚C. The absorbance was measured at 450nm. The cell viability was calculated and represented graphically.

2.5. Cell Morphology

Morphological differentiation of RAW264.7 macrophages in each group was observed under inverted light microscope.

2.6. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The total RNA of RAW264.7 cells samples were extracted using TRIzol® according to the manufacturer's protocol. Then, RNA quality was determined by measuring the 260/280 ratio. Samples measuring >2.0 were considered to be of sufficient quality for further analysis. A total of ~1.5 μg total RNA was reverse transcribed to cDNA following the Revert Aid first-strand cDNA synthesis kit. The primer sequences are presented in Table 1.

Table 1

Reverse transcription-quantitative polymerase chain reaction primer sequences.

Peimer	Peimer sequences
Mus GAPDH-F	*CGAGAATGGGAAGCTTGTC*
Mus GAPDH-R	*TCACACCCATCACAAACATG*
Rattus IL-6-F	*CAAGAGACTTCCAGCCAGTTG*
Rattus IL-6-R	*TTTTCTGACAGTGCATCATCG*
Rattus TNF-α-F	*CTCAGCCTCTTCTCATTCCTG*
Rattus TNF-α-R	*TGATCTGAGTGTGAGGGTCTG*
Mus IL10-F	*GGGAAGACAATAACTGCACC*
Mus IL10-R	*GTCTTCAGCTTCTCACCCAG*
Mus TGF-β-F	*CCTGTCCAAACTAAGGCTCG*
Mus TGF-β-R	*CTTCCCGAATGTCTGACGT*
Mus iNOS-F	*CATCGGATTTCACTTGCAAG*
Mus iNOS-R	*TGAGCTGGTAGGTTCCTGTTG*
Mus Arg1-F	*CCCAGATGTACCAGGATTCTC*
Mus Arg1-R	*TTCCATCACCTTGCCAATC*
Mus TLR4-F	*CAGAACTTCAGTGGCTGGATT*
Mus TLR4-R	*GGAAGCTTTCTAGAGAGGCCA*
Mus MyD88-F	*TACTGAAGGAGCTGAAGTCGC*
Mus MyD88-R	*AACTCGATATCGTTGGGGC*
Rattus NF-κB-F	*CATCAAGATCAATGGCTACA*
Rattus NF-κB-R	*CACAAGTTCATGTGGATGAG*

2.7. Statistical analysis

GraphPad Prism v.8 software (GraphPad Software, Inc.) was used to analyze the mRNA expression levels data. Comparisons between the control and experimental groups were made using a one way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Effect of KCPM on cell viability

CCK-8 assays was used to detect the effect of KCPM on cell viability. As shown in Figure 1, RAW264.7 macrophages were treated with different concentrations of KCPM (5500.0、2750.0、1375.0、687.5、343.7、171.9、85.9、42.9、21.5、10.7mg·ml^{-1}), and the 85.9mg/ml concentration was the closest to 50% viability rate. Therefore, 85.9mg/ml was selected as the optimal concentration for the further study.

3.2. Effect of KCPM on LPS induced RAW264.7 macrophages activation

As shown in Figure 2, compared with the blank group, LPS promoted the differentiation of RAW264.7 macrophages, increased cell pseudopods under the light microscope, and the cell morphology changed from the bright circle in the blank group to the polygon, star or shuttle shape with antennae in the LPS group. However, RAW264.7 cells treated with KCPM (85mg/ml) advanced, when stimulated by LPS their long angle and differentiation status were relieved, and the proportion of cell pleomorphism was decreased, which indicated that KCPM could inhibit the differentiation of RAW264.7 macrophages induced by LPS.

3.3. Effect of KCPM on LPS induced inflammatory factors secretion

As shown in Figure 3, compared with the blank group, the expression of IL-6 and TNF-α in LPS group increased significantly ($P<0.01$), while the expression of IL-6 and TNF-α in LPS+KCPM group was significantly lower than that in LPS group ($P<0.01$), which proved that KCPM could significantly reduce the secretion of pro-inflammatory factors and inhibit the macrophage inflammatory response. While the expression of IL-10 and TGF-β in LPS+KCPM group was higher than that in LPS group ($P<0.01$), which meant that KCPM could significantly increase the secretion of anti-inflammatory factors.

Under the stimulation of LPS, RAW264.7 cells expressed significantly increased *iNOS* ($P<0.01$) and significantly decreased *Arg-1* ($P<0.01$) compared with blank group, suggesting that macrophage polarized to M1 phenotype. After the intervention with KCPM, in LPS+KCPM group, the expression of *iNOS* was significantly decreased ($P<0.01$), while the expression of *Arg-1* was significantly increased ($P<0.01$) compared with LPS group. It is proved that KCPM can inhibit the pro-inflammatory factors secretion and increase the anti-inflammatory factors secretion, promoting the macrophages polarization toward to M2 phenotype.

3.4. Effect of KCPM on LPS induced TLR4/MyD88/NF-κB signal pathway

As shown in Figure 4, the transcription levels of TLR4, MyD88 and NF-κB in the LPS group was increased significantly compared with that in the blank group ($P<0.01$). After the advanced intervention with KCPM, the transcription levels of TLR4, MyD88 and NF-κB in LPS+KCPM group were significantly decreased than that in LPS group ($P<0.01$). It is indicated that KCPM can inhibit the transcription levels of TLR4/MyD88/NF-κB signaling pathway induced by LPS.

4. Discussion

In this study, LPS-induced RAW264.7 macrophages were used as the TLR4-activated cell model to investigate the effect of KCPM on macrophage inflammatory mediators. To determine the optimal concentration of KCPM, CCK-8 assays were conducted. We confirmed that KCPM could inhibit M1 polarization and promote M2 polarization may through the inhibition of TLR4/MyD88/NF-κB signal pathways.

Lung macrophages are important innate immune cells, which play critical roles in lung homeostasis, host defense against pathogens, and resolution of inflammation. These diverse functions of macrophages are achieved by the plasticity of these cells, which, depending on signals present in their microenvironment, can polarize into a plethora of different phenotypes[13, 4]. In infected tissues, macrophages are first polarized to pro-inflammatory M1 phenotype to assist the host against pathogens. Subsequently, macrophages are polarized to form an anti-inflammatory response to the M2 phenotype and repair damaged tissue[15]. Recently, the regulation of macrophage polarization to regulate its immune function has been successfully stimulated. A variety of strategies have been used to modulate macrophage polarization to treat disease.

Chinese herbs are a traditional medicinal resource in China, which has a stimulating activity and regulating effect on the immune system. Macrophages may be one of the target cells for Chinese herbal medicine's anti-inflammatory effect[16, 17]. KCPM was formulated on the basis of MXSGD, which reduced Glycyrrhiza and added reed root, Houttuynia cordata, big green leaves of Polygonum, stir fried Perilla seed, Tingli seed and stir fried Radish seed, thus the effect of clearing heat and resolving phlegm was enhanced. Modern pharmacology research shows that the traditional Chinese medicine of clearing away heat has anti-inflammatory effect[18, 19]. In our study, we found that KCPM could inhibit the expression of IL-6 and TNF-α, promote the expression of IL-10 and TGF-β. Research about Xiaoer Magan Granule which formulated on the basis of MXSGD, also found that it could reduce the level of inflammatory cytokines such as IL-6, IL-8, TNF-α, and IFN-γ, so as to improve the pneumonia clinical symptoms such as cough, expectoration, fever and rale[20]. The anti-inflammatory effect of Ephedra-Glycyrrhiza showed that it can reduce the inflammatory factors TNF-α and IL-β in mice lung tissue with pneumonia[21]. More studies have found that Maxingshigan decoction can effectively reduce lung inflammation, protect immune organs and regulate cytokine balance. Its mechanism was through inhibiting the activation of TLRs signal pathways, alleviate lung injury caused by infection[22, 23]. So, we inferred that the KCPM play a role in the treatment of pneumonia through inhibiting the secretion of macrophage pro-inflammatory factors .

In the present study, a cell model was established using LPS stimulation in RAW264.7 cells. LPS was demonstrated to promote the secretion of TNF-α and IL-6. The mouse macrophage RAW264.7 cell line is a type of monocyte macrophage in mice with leukemia, which is commonly used in biological experiments investigating inflammation[24]. TNF-α is the earliest endogenous mediator of an inflammatory reaction, and IL-6 is a major pro-inflammatory cytokine that serves an important role in the acute phase response of inflammation[25]. When infections or tissue injuries occur, IL-6 and TNF-α was promptly produced by macrophages and contributes to removal of infectious agents and restoration of damaged tissues through activation of immune, hematological, and acute-phase responses. The TNF-α and IL-6 influence the ability to limit pathogen infection but their over-production might result in inflammatory disorders[26–29]. Thus, the proper expression is very important for host defense. KCPM could inhibit the expression of IL-6 and TNF-α, limit the uncontrolled excessive or persistent pro-inflammatory production, as a result, preventing the further tissue damage of macrophage immune response in the pneumonia.

In our study we also found that KCPM could promote the secretion of TGF-β and IL-10. TGF-β is an evolutionarily conserved pleiotropic factor that regulates a myriad of biological processes including development, tissue regeneration, immune responses, and tumorigenesis[30]. It is necessary for lung organogenesis and homeostasis as evidenced by genetically engineered mouse models[31]. Collectively, TGF-β inhibits the development of immunopathology to self or nonharmful antigens without compromising immune responses to pathogens[32]. IL-10 is a potent anti-inflammatory cytokine that plays a crucial, and often essential, role in preventing inflammatory and autoimmune pathologies[33]. IL-10 emerged before the adaptive immune response and elicit diverse host defense mechanisms, especially from epithelial cells during an infection. It promotes innate immune responses from tissue epithelia that limit the damage caused by both viral and bacterial infections. It also facilitate tissue healing after infection /inflammation[34]. In this regard, IL-10 suppresses pro-inflammatory responses, limiting tissue disruption resulting from an inflammatory response[35]. In our research, KCPM could promote the secretion of TGF-β and IL-10, so as to limit the macrophage immune response, relief the tissue disruption, and ameliorate the prognosis of pneumonia.

The present study found that KCPM limited LPS-induced M1 macrophage polarization, inhibited the expression of iNOS, promoted the expression of Arg-1, and induced macrophage polarization to the M2 phenotype, so as to play an anti-inflammatory role. iNOS, an intracellular marker of M1 subpopulation, has been found supressed by herbs. iNOS converts l-arginine to l-citrulline and nitric oxide that interacts with reactive oxygen species to exert pro-inflammatory effects[36]. Chinese herbs has been found powerful potential in alleviating inflammatory response, they promoted the expression of Arg-1, while the expression of iNOS was decreased, induced the polarization of macrophages toward an M2 phenotype[37–40].

TLR4/MyD88/NF-κB pathways are considered to be pivotal in the inflammatory response. TLR4 can be activated by LPS, it may be the target protein of Chinese herbs, which inhibited LPS-induced inflammation in RAW264.7 cells[41]. Research about Chinese herbs found that Chinese herbs inhibited

LPS-induced TLR4 and MyD88 expressions in RAW264.7 macrophages, as a result, inhibiting the production of inflammatory mediators[42, 43]. Of the several transcriptional factors activated by inflammatory responses during bacterial infections, NF-κB plays a critical role in several signal transduction pathways. It is a key transcription factor that promotes transcription of genes encoding pro-inflammatory cytokines[44]. Many studies reports that inhibition of the NF-κB signaling pathway reduces the production of inflammatory mediators, promotes the development of M2 macrophage polarization, stimulation of the NF-κB signaling pathway can also polarize M2 macrophages into M1 macrophages[45, 46]. Hence, agents that are able to inhibit TLR4/MyD88/NF-κB transcriptional regulation and modulate the inflammatory response may have therapeutic effect. In this study we found that inhibition of the TLR4/MyD88/NF-κB signal pathway was associated with the inhibitory effects of KCPM on inflammatory mediators in LPS-stimulated RAW264.7 cells. We inferred that KCPM inhibit the macrophage immune response though the TLR4/MyD88/NF-κB signal pathway.

In this study, only in vitro studies were carried out, further animal experiments are still needed to verify the above results. Further research about the effect of KCPM on the protein expression of TLR4/MyD88/NF-κB signal pathways were also needed.

5. Conclusion

To sum up, this study preliminarily explored the effect of KCPM on LPS induced immune response of RAW264.7 macrophages, and found that KCPM had a certain inhibitory effect on LPS-induced RAW264.7 macrophages immune response. The anti-inflammatory mechanisms of KCPM was suppressing the release of TNF-α and IL-6, promoting the release of IL-10 and TGF-β. This effection may through inhibiting the expression of TLR4/MyD88/NF-κB signaling pathways, which provided a theoretical basis for the future study of the effect of KCPM on macrophage immune response in pediatric pneumonia.

Declarations

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributors

All of the authors had full access to the full dataset (including the statistical reports and tables) and take responsibility for the integrity of the data and the accuracy of the analysis. LS, CH conceived and

designed the study. LS, YAJ, YLJ collected the data and developed the analysis. LS interpreted the data. LS wrote the first draft of the paper. CH reviewed and approved of the final report.

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Figures

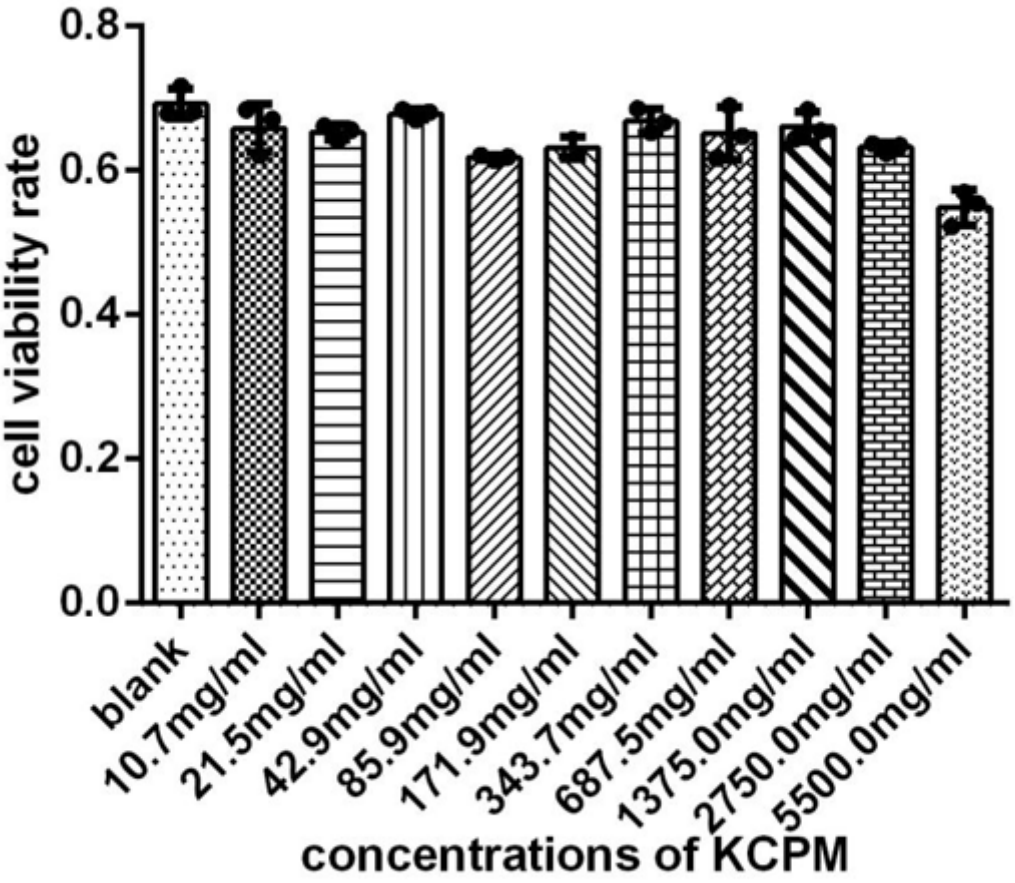
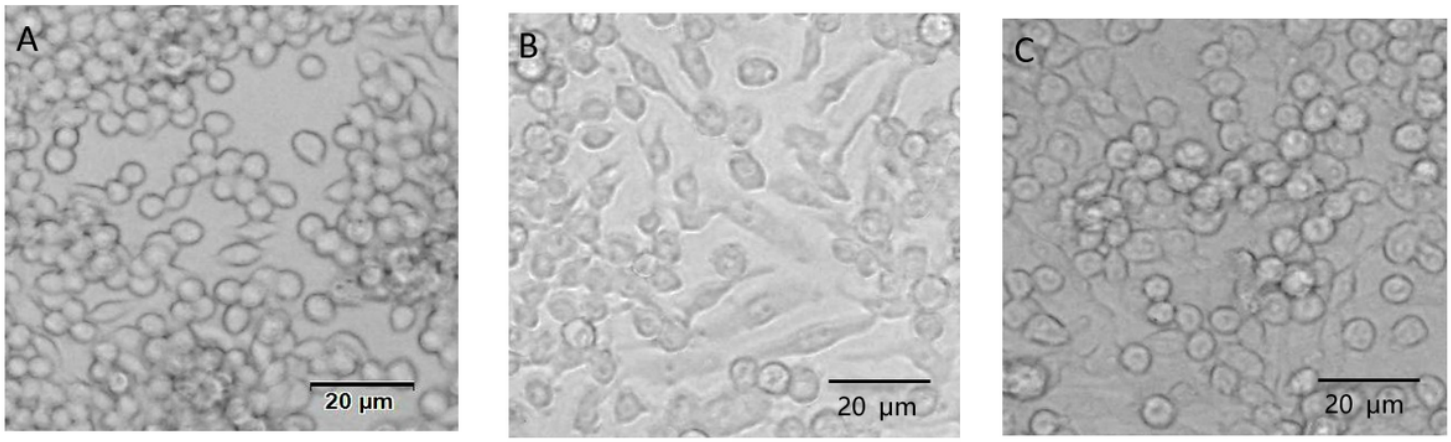


Figure 1

Effect of KCPM on cell viability detected by CCK-8 assays



A. Blank group, B. LPS group, C. LPS+KCPM group

Figure 2

Effect of KCPM on RAW264.7 cell morphology

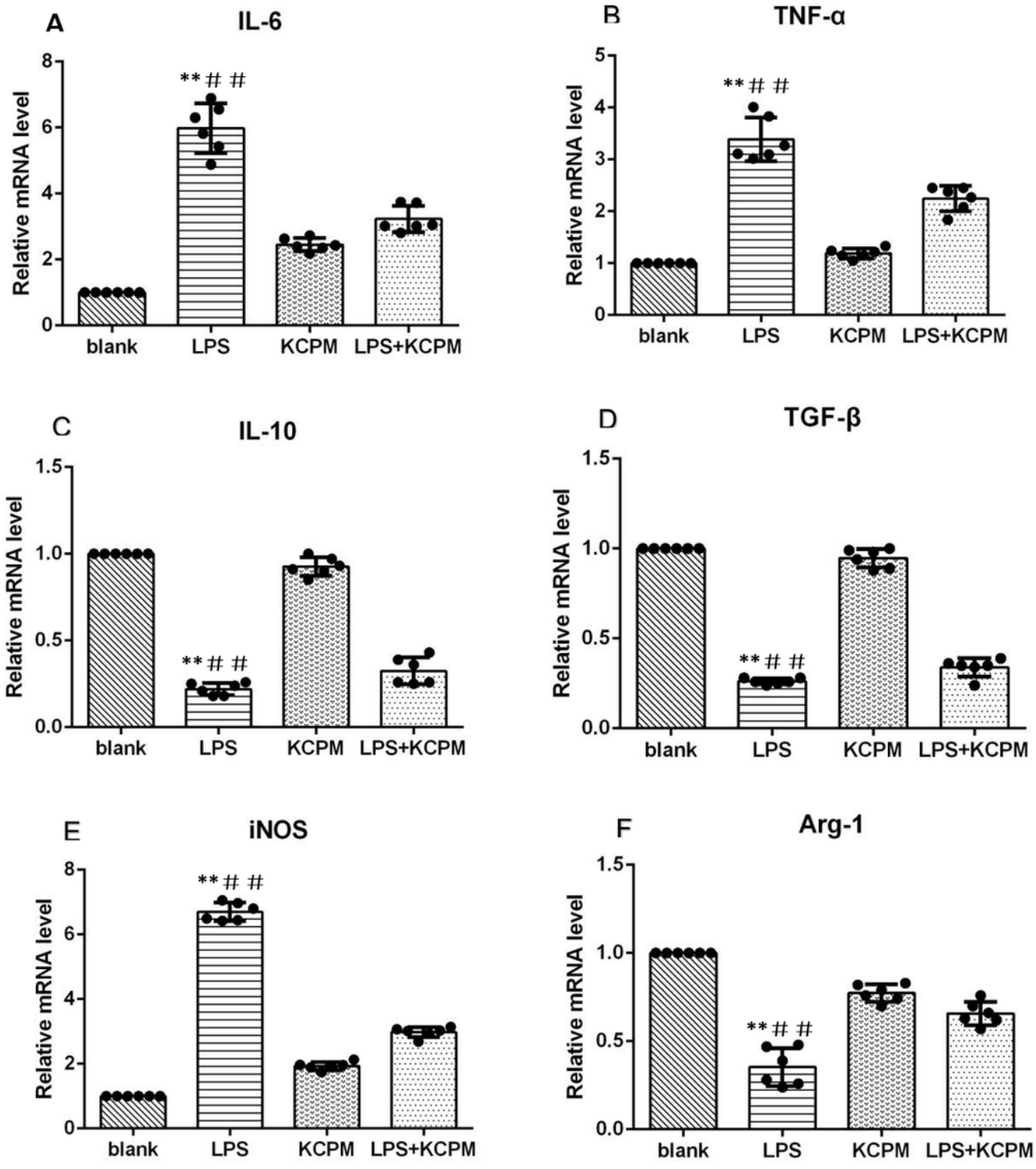


Figure 3

Effect of KCPM on LPS induced inflammatory factors secretion **P<0.01 vs. Blank##P<0.01 vs. LPS+KCPM

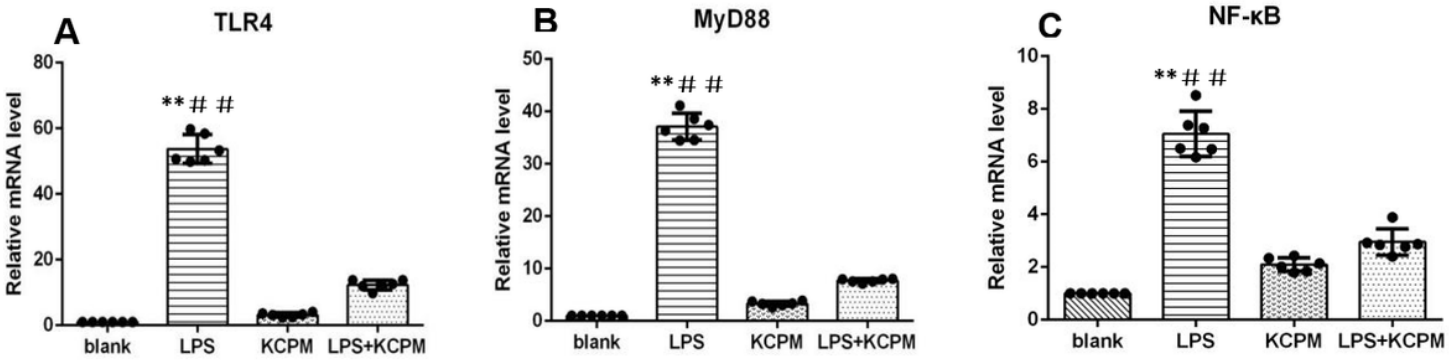


Figure 4

Effect of KCPM on LPS induced TLR4/MyD88/NF-κB singnal pathway **P<0.01 vs. Blank##P<0.01 vs. LPS+KCPM