Polyene Phosphatidylcholine Interacting with TLR-2 Prevents the Synovial Inflammation via Inactivation of MAPK and NF- κ B Pathways

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune joint disease that causes cartilage and bone damage or even disability, seriously endangering human health. Chronic synovial inflammation has been shown to play a vital role in the disease sustainability. Therefore, down-regulation of synovial inflammation is considered to be an effective discipline for RA therapy. Polyene phosphatidylcholine (PPC) is a hepatoprotective agent, which was observed to inhibit inflammation in macrophages and prevent collagen-induced arthritis (CIA) of rats in our previous study. However, the underlying mechanism remains unclear. The present study further reported that PPC can inhibit the synovial inflammation. In lipopolysaccharide (LPS)-stimulated primary synovial fibroblasts (SFs) of mice, PPC significantly decreased pro-inflammatory cytokines production while increasing anti-inflammatory cytokines level. In this process, PPC down-regulated the expression of TLR-2 and their downstream signaling molecules such as MyD88, p-ERK1/2, p-JNK1/2, p-P38 in the MAPK pathway and p-I κ B α and NF- κ B-p65 in NF- κ B pathway. Moreover, the inhibitory effect of PPC on the above molecules and cytokines was weakened after the use of TLR-2 agonist Pam3CSK4. However, PPC lost its anti-inflammatory effect and showed an activation of MAPK and NF- κ B pathways in the TLR-2^{-/-} primary SFs after exposure to LPS. Furthermore, these results were confirmed in the SFs from the CIA mouse *ex vivo*. Collectively, this study demonstrated that PPC can alleviate synovial inflammation through TLR-2 mediated MAPK and NF- κ B pathways, which can be proposed to be a potential drug candidate for RA therapy.

Introduction

Rheumatoid arthritis (RA) is the most common autoimmune inflammatory disease in the world, affecting about 1% of the population. RA can cause loss of joint function and seriously endanger human health [1, 2]. However, its pathogenesis has not been fully elucidated due to the complex processes involving genetic factors, environmental factors and systemic immune response so far [3]. Chronic synovial inflammation is one of the characteristics of RA. There are a variety of cells in the affected articular cavity, including synovial fibroblasts (SFs) and immune cells, cooperatively contributing to joint inflammation and bone destruction [4]. Particularly, SFs represent one of the leading hyperplastic cells [5]. They produce chemokines and cytokines through the perception of dangerous molecules in the joints, to participate in the malignant inflammatory cycle [6, 7]. Furthermore, SFs can stimulate synovial vascularization through the release of proangiogenic factors, thereby promoting disease progression [8–10]. It can be concluded that the SFs act as the initiating factor of joint pathology and play a vital role in sustainability of the disease. Therefore, down-regulation of synovial inflammation is regarded to be a key discipline for the treatment of RA [11].

A large number of documents have shown that toll-like receptors (TLRs), one of the classic pattern recognition receptors in the innate immune system, are highly expressed in the SFs of RA [12, 13]. After sensitized by endogenous molecules and microbial components, TLRs activate various signal transduction pathways, contributing to the induction of various genes including inflammatory cytokines [14]. In particular, TLR-2 can promote the secretion of pro-inflammatory cytokines, and up-regulate the

production of vascular growth factor (VEGF), matrix metalloproteinases (MMPs) and adhesion molecules, thereby promoting inflammation and accelerating the development of RA diseases [12, 15, 16]. Thus, TLR-2 is recognized as a novel therapeutic target for the disease [12, 17].

MAPK inflammatory signaling pathway is a classic downstream pathway of TLR-2, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and P38. TLRs can recruit TIR-domain-containing adaptor proteins such as TIRAP, TRIF and myeloid differentiation protein 88 (MyD88), and then make proteins such as ERK1/2, JNK1/2 and P38 phosphorylated to promote production of pro-inflammatory cytokines [18]. It was reported that IL-6 and TNF- α together promote Th17 cell differentiation and IL-17 for RA angiogenesis, thereby exacerbating joint inflammation and bone destruction [10]. Therefore, several biological agents such as IL-6 and TNF- α monoclonal antibody can alleviate RA [19, 20]. In addition, IL-10 and TGF- β can inhibit the activation and cytokine production of Th cells to suppress autoreactive responses and maintain self-tolerance in immune system [21], which thereby prevents the progression of RA.

Nuclear factor Kappa B (NF- κ B) is a crucial transcription factor that can be activated by TLRs, resulting in the production of pro-inflammatory cytokines [22]. NF- κ B-p65 is expressed in the cytoplasm and has no transcriptional activity under normal circumstances. I κ B α is an inhibitor of NF- κ B-p65, and it can combine with NF- κ B-p65 in a polymerized manner in the cytoplasm. When stimulated with LPS, phosphorylated I κ B α is immediately dissociated from the polymer and degraded NF- κ B-p65, which is previously inhibited by I κ B α , as a result, can be activated and quickly enters into the nucleus from the cytoplasm to regulate the transcription of inflammatory factors [23, 24]. Thus, inactivation of NF- κ B pathway can effectively hamper inflammation.

Polyene phosphatidylcholine (PPC) is a hepatoprotective agent, which has been widely used to treat various types of hepatopathy such as viral hepatitis, liver damage or nonalcoholic steatohepatitis [25–28]. The drug can be extracted from soy and rich in polyunsaturated fatty acids [29]. Phosphatidylcholine (PC) is the main component of PPC. In recent years, several conjugates containing PC such as tuftsin-phosphorylcholine (TPC) have been reported to improve lupus nephritis, inflammatory bowel disease (IBD) and experimental autoimmune encephalomyelitis [30–32]. In consistent to these results, our previous study showed that PPC can inhibit the inflammatory response in LPS-stimulated macrophages and ameliorate CIA in rats [33]. However, its underlying mechanism remains largely unclear. The present study further investigated the effects of PPC on SFs inflammation and explored the possible role of TLR-2 in this process. The results showed PPC interacting TLR-2 inactivates MAPK and NF- κ B pathways to alleviate synovial inflammation. Overall, our finding indicates that PPC may be a potential drug candidate for RA therapy.

Materials And Methods

Isolation and Culture of SFs

SF tissues were isolated from the knee joint cavity of SD rats, C57BL/6J and TLR-2^{-/-} mice (Shanghai Experimental Animal Center, Shanghai, China). The culture process was concisely described as follows. The SFs were dissected, and immersed in 100 mm plates with phosphate buffered saline (PBS) containing penicillin-streptomycin (100 ng/mL, Pen/Strep, GIBCO, USA). After that, the SFs were transferred into 10 mL Dulbecco's modified Eagle medium (DMEM, GIBCO, USA) with freshly-made and filtered collagenase IV (5 mg/mL, Worthington Biochemical Corporation, USA), 10% fetal bovine serum (FBS, GIBCO, USA) and 1% Pen/Strep in 50ml tubes, and then incubated in the shaking water bath at 37°C for 50 min to 1 h at a maximum shaking speed. The digested SFs were centrifuged at 1500 rpm for 5 min at room temperature, and the cell pellets were re-suspended in fresh DMEM supplemented with 10% FBS and 1% Pen/Strep. The isolated SFs were seeded into 6 well plates at a density of 110⁶/mL. When the cell confluence reaches 90% of the plate, the cells were subcultured using trypsinization, and collected for further experiments.

Immunofluorescence

The morphology and purity of prepared SFs were identified by detecting the expression of Vimentin protein (the specific marker of fibroblast cells) using immunofluorescence staining. SFs were washed with PBS three times and fixed with 200 µL of methanol per well. Then the cells were washed with PBS, and incubated for 1 h in blocking solution containing 2% BSA (Beijing Zhongshan Jinqiao Biological Technology Co, Ltd, Beijing, China). The cells were incubated with vimentin antibodies (1: 500, Abcam, USA), followed by incubation with FITC-conjugated goat anti-rabbit secondary antibody (1:1000, Abcam, USA). Finally, the cells were counterstained with DAPI and visualized by BX51 positive immunofluorescence microscopy (OLYMPUS, Japan).

Cell Viability Assay

SFs were seeded in 96-well plates at a density of 2×10³/well in 100 µL volume. After cultured in 37°C, 5% CO₂ incubator for 24 h, the cell medium was replaced with PPC at different concentrations (0, 10, 20, 30 and 50 µg/mL). At the time points of 20, 44 and 68 h, cell counting kit 8 reagent (CCK8, Beyotime Biotech, Beijing, China) was added into the medium (10 µL/well). The absorbance values of the 96-well plates were detected at 450 nm after 4 h using a microplate reader.

Wound-healing Assay

SFs in the logarithmic growth phase were seeded into each well at a density of 1×10⁶ and then cultured with PPC (0, 10, 20, 30 and 50 µg/mL). A sterile 200 µL pipette tip was used to perpendicularly scratch one uniform horizontal line each well. The shed cells were washed with PBS and then added new medium to the culture plates. Photographs were taken at 0 h, 24 h and 48 h with XSZ inverted biological microscope (Chongqing, China) to evaluate the migration capacity of the cells.

Cell Treatment

In vitro experiments, the primary SFs, isolated from the knee joints of normal wild type C57BL/6J and TLR-2^{-/-} mice, were seeded in 24-well or 6-well plates, and then treated 24 h with indicated

concentrations of PPC (Sanofi Aventis, Spain), LPS (100 ng/mL, Sigma, USA), LPS plus PPC, or vehicle control, respectively. Alternatively, to study the roles of TLR-2 in inflammatory response, Pam3CSK4 (TLR-2 agonist) was added to SFs 2 h before the addition of indicated stimulus. In *ex vivo* experiments, the primary SFs were isolated from the knee joints of CIA, PPC pretreated CIA and vehicle control SD rats. The supernatants were collected to detect the cytokine production, and the cells were collected for western blot or Quantitative real-time RT-PCR analysis.

CIA model Induction

The CIA model in SD rats were established as our previous study [33]. SD rats were randomly divided into 3 groups with 10 rats in each group: CIA group; PPC treatment group (100 µg/rat) and control group without being modeled. Then 2.5 mL Bovine collagen type II (CII, 5 mg) was dissolved in 2.5 mL Freund's complete adjuvant, finally making into an emulsifiable liquid of 1 mg/mL. Rats were anaesthetized and subcutaneously injected with 100 µL of CII on day 1 and day 10 for arthritis induction. Controls received equal volume of Freund's complete adjuvant in the same time. PPC, at a dose of 100 µg/rat, was administered to CIA rats by tail vein injection on days -1 (the day before the first immunization) and 9.

Enzyme-linked Immunosorbent Assays (ELISA)

Purchased from eBioscience, USA, the levels of IL-6, TNF-α, TGF-β and IL-10 in cultured cell supernatants were determined by Enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, USA) according to the manufacturer's instructions.

Quantitative Real-time RT-PCR

RNA was isolated from SFs using TRIzol (Life Technologies, USA), and cDNA was reverse transcribed from RNA using PrimeScript™ RT Master Mix (Takara, Japan). Then cDNA was amplified using SYBR® Premix Ex Taq™ (Takara, Japan) with gene-specific primers. Quantitative PCR analyses were performed in a LightCycler® 480 II detection system (Roche Applied Science, Penzberg, Germany) under the following thermal cycler conditions: one cycle of 5 min denaturation at 95°C, and then 15 s at 95°C, 15 s at 60°C, and 15 s at 72°C for 45 cycles using the primers. All experiments were performed in triplicate and the Ct values were normalized to endogenous reference (β-actin). The relative expression of detection indexes of this manuscript was indicated by comparative cycling threshold (Ct) normalized by β-actin with the $2^{-\Delta\Delta Ct}$ method. The primer sequences for mouse can be found in our previous study [33], and the primer sequences for rat genes were listed in Table 1.

Western Blotting Analysis

Total proteins were extracted from SFs and quantified with bicinchoninic acid protein concentration assay kit (Beyotime Biotech, Beijing, China). Sample proteins were separated by electrophoresis in 10% SDS-PAGE with a Bio-Rad electrophoresis system (Hercules, CA, USA). Membranes were incubated the primary antibodies (rabbit TLR-2, MyD88, ERK1/2, p-ERK1/2, JNK1/2, p-JNK1/2, P38, IκBα, p-IκBα, NF-κB, NF-κB-p65, β-actin antibody, Abcam, UK, 1:1000 dilutions) at 4°C for 12 h. The secondary anti-bodies (anti-rabbit IgG, 1:2000 dilutions) were incubated for 2 h at room temperature. The membrane containing

antibody protein complexes was visualized with an enhanced chemiluminescence detection system on radiograph film (Bio-rad, Hercules, CA, USA). The bands were scanned and analyzed by the software Quantity ONE (Bio-rad, Hercules, CA, USA). The expressions of protein in each sample were normalized by β -actin.

LPS Determination

LPS levels in the sera of CIA model and control mice were detected using a chromogenic end-point TAL kit (Xiamen Bioendo Technology Co., Ltd, Xiamen, China) according to the manufacturer's protocol. The absorbance was determined at a wavelength of 545 nm using a spectrophotometer (Asuragen ClinBio128, USA). All samples for LPS measurements were performed in duplicate.

Statistical Analysis

Data were analyzed using GraphPad Prism software 8.0 and were presented as the mean $\pm$ SD. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by the post hoc or Tukey test for multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

Results

1. PPC can significantly alleviate LPS induced inflammation in SFs

The CIA model shares many similarities in the clinical manifestations and pathogenesis with RA patients [34]. We previously reported that PPC can effectively improve the condition of CIA in rats [33], providing a new option for RA intervention therapy. But its underlying mechanism remains unclear. In view of the vital role of SFs inflammation in the pathogenesis of RA, we speculated that PPC plays the protective effect on CIA through inhibiting SFs inflammation. To demonstrate the hypothesis, the present study isolated primary SFs from the rat's joints and detected the mRNA expression of cytokines. As shown in Figure 1a-c, the expression of pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α) were significantly upregulated in the SFs of CIA ($P < 0.01$). However, PPC treatment significantly reversed their elevated expression ($P < 0.01$). Furthermore, the anti-inflammatory cytokine TGF- β was significantly upregulated after PPC treatment in the SFs derived from CIA rats ($P < 0.001$, Figure 1d).

LPS is the inducer of sterile inflammation, and is increasingly recognized to involve in the RA progression [35]. We showed an elevated level of LPS in the serum of CIA group compared to that in vehicle control ($P < 0.01$, Figure 1e). However, PPC treatment significantly lowered LPS production in CIA rats ($P < 0.05$).

To further confirm the role of PPC in SFs inflammation, we evaluated the effect of PPC in LPS-triggered inflammation in the primary SFs from C57BL/6J mice (Supplementary Figure 1a). The production of IL-6 and TNF- α triggered by LPS was significantly inhibited following PPC treatment ($P < 0.001$, Figure 1f and 1g). Moreover, PPC supplementation significantly increased the levels of anti-inflammatory cytokines IL-

10 and TGF- β ($P < 0.001$, Figure 1h and 1i). Taken together, these results *ex vivo and in vitro* suggested that PPC improves the CIA through downregulating SFs inflammation.

2. PPC does not affect the proliferation and migration of primary SFs

To exclude the possibility that PPC inhibits SFs inflammation via cell cytotoxicity, this study determined the effect of PPC on the proliferation and migration of primary SFs. As shown in Supplementary Figure 1b, different concentrations (0, 10, 20, 30 and 50 $\mu\text{g/mL}$) of PPC did not significantly inhibited the normal proliferation of SFs within 72 h using CCK8 assay in comparison to vehicle control ($P = 0.05$). Moreover, there was no obvious difference in the refilling of the scratch edges after treatment with mentioned concentrations of PPC (Supplementary Figure 1c). Overall, these results showed that PPC does not affect the proliferation and migration of primary SFs, exhibiting a safe effect on SFs.

3. PPC inhibits LPS-activated SFs inflammation via TLR-2

As a conservative pattern recognition receptor, TLR-2 plays a critical role in mediating inflammatory response of SFs. It is reported that the TLR signaling inhibition of oxidized phospholipid relies on TLR-2 in macrophages [36]. We therefore examined the effect of PPC on TLR-2 expression in primary SFs. In LPS-stimulated SFs, the mRNA expression and protein level of TLR-2 were significantly upregulated ($P < 0.001$, Figure 2a and 2b). However, PPC treatment significantly inhibited TLR-2 expression activated by LPS ($P < 0.001$, Figure 2a and 2b).

To further understand the role of TLR-2 in the anti-inflammatory action of PPC, we determined the effect of PPC on SFs isolated TLR-2^{-/-} mice. As shown in Figure 2c-2f, LPS still both stimulated the high levels of pro-inflammatory cytokines (IL-6, TNF- α) and anti-inflammatory cytokines (IL-10 and TGF- β) in the TLR-2^{-/-} SFs ($P < 0.01$). Moreover, PPC significantly upregulated the level of IL-10 in TLR-2^{-/-} SFs after exposure to LPS ($P < 0.01$, Figure 2e). However, PPC lost its capability to decrease the production of IL-6 and TNF- α , and to increase the TGF- β production in the absence of TLR-2. Collectively, these data indicated that TLR-2 is required for the anti-inflammatory role of PPC on SFs inflammation.

4. PPC downregulates NF- κ B pathway in SFs via TLR-2

MyD88/NF- κ B is the classic pathway that mediates LPS-induced inflammation in macrophages [37]. We therefore asked whether PPC can regulate the pathway in LPS-stimulated SFs. As shown in Figure 3a, LPS could increase the level of phosphorylation of I κ B α (Ser32/36), thereby eliminating its inhibitory effect on NF- κ B and promoting inflammation. However, PPC treatment inhibited the p-I κ B α (Ser32/36) and hinder NF- κ B activation (Figure 3a). Furthermore, the inhibition effect of PPC became weakened in TLR2^{-/-} SFs (Figure 3b). The nuclear translocation of NF- κ B-p65 is vital for the transcriptome of proinflammatory cytokines. We therefore examined the expression of NF- κ B-p65 in the cytoplasm and the nucleus of SFs. As shown in Figure 3c, the expression of NF- κ B-p65 was increased under LPS treatment both in cytoplasm and nucleus of wild type SFs, while the produce of NF- κ B-p65 was suppressed by PPC supplementation. However, the inhibitory effect of PPC on the expression of NF- κ B-p65 was weakened in

the absence of TLR-2 (Figure 3d). These results showed that TLR-2/ NF- κ B pathway is the downstream pathway for the anti-inflammatory effect of PPC.

5. PPC inactivates MAPK pathway in SFs via TLR-2

MAPK pathway is also a classical downstream pathway in TLR-2-triggered inflammation [38]. We next determined the proteins expression of p-P38, p-ERK1/2, ERK1/2, p-JNK1/2, JNK1/2 in LPS-stimulated SFs in the presence or absence of PPC. As shown in Figure 4a, the expression of p-P38 (Thr180/Tyr182), p-ERK1/2 (Thr202/Tyr204), p-JNK1/2 (Thr183/Tyr185) was significantly upregulated in LPS-stimulated SFs in comparison to vehicle control. Nevertheless, PPC treatment significantly down-regulated the levels of those phosphorylated proteins. Moreover, in TLR-2^{-/-} SFs, PPC could not inhibit the expression of p-P38, p-ERK1/2, p-JNK1/2 induced by LPS (Figure 4b). These results indicated that PPC inactivates MAPK pathway in SFs via TLR-2.

6. The anti-inflammatory effect of PPC in SFs relies on TLR-2 signaling

To further confirm whether TLR-2 is the pathway for PPC regulates the anti-inflammatory response, we pre-treated the SFs using Pam3CSK4 (TLR-1/2 agonist), and then stimulated the cells by PPC and/or LPS for 24 h. In the vehicle treated SFs, it was seen that PPC inhibited the phosphorylation of proteins in MAPK and NF- κ B pathways (Figure 5a). However, in TLR-2 over-expressed SFs, PPC had no obvious effect on these proteins. Furthermore, we determined the production of inflammatory cytokines in the supernatants of mentioned cell cultures. As shown in Figure 5b, PPC could not inhibit the production of IL-6 and TNF- α , conversely, decrease the TGF- β level in LPS stimulated SFs after Pam3CSK4 pretreatment ($P<0.01$). Overall, these *in vitro* results suggested that the anti-inflammatory effect of PPC in SFs relies on TLR-2 pathway.

Discussion

Our previous study has shown that PPC downregulates the inflammatory response in macrophages and improves the condition of arthritis [33]. More recently, we reported that PPC inhibits M1 polarization in LPS-activated macrophages via reprogramming glycolipid metabolism [29]. The present study further revealed that the anti-inflammatory effect of PPC also occurs in SFs inflammation. Mechanistically, PPC could inactivate MAPK and NF- κ B pathways *via* interacting TLR-2, thereby reducing the production of proinflammatory cytokines and alleviating synovial inflammation (Figure 6).

The available drugs against RA can be classified into conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) and biological DMARDs (bDMARDs). These drugs still have several shortcomings based on Nationwide Population-Based Cohort Study [39]. Thus, it is still urgent to develop novel drugs. PC is an important part of cell membrane and organelle membrane, which has shown immunomodulator activity in recent years. For example, TPC, a chemical synthesis of a phagocytosis

peptide Tuftsin and PC, has amelioration effects in a chronic colitis murine model and murine lupus nephritis by regulation of the expression of inflammatory factors [31, 40]. It is also reported that the compound can induce the expansion of regulatory T (Treg) and regulatory B (Breg) cells to improve CIA condition [41]. In addition, VB-201, an oxidized phospholipid small molecule, is proved to constrain atherosclerosis through profoundly inhibiting TLR-2-dependent macrophage activation and phosphorylation of ERK1/2, IKK α / β , P38 and rescuing I κ B α degradation [42]. The present study showed that PPC, the derivative of PC, a well-known classic hepatinica [25–28], can mitigate synovial inflammation. In line with our finding, a previous report has reported that oral PC pretreatment alleviated the signs of experimental rheumatoid arthritis [43]. Taken together, the present study proposed that PPC may be a novel drug for RA treatment.

There is accumulating evidence shows that many endogenous TLR ligands arose at different stages of RA [12]. In the SFs of RA patients, the expression level of TLRs is closely associated with the activity score of the disease [44]. Thus, targeting TLRs is believed to be a promising therapy for RA. The present study showed that PPC prevented the synovial inflammation. Moreover, the anti-inflammatory effect was almost lost in the absence of TLR-2. In addition, pre-activation of TLR-2 using PamCSK4 also abolished PPC's anti-inflammatory capability. In line with our finding, oxidized phospholipids are reported to selectively act on TLR-2 to downregulate inflammation in macrophages [36]. Thus, these results showed that TLR-2 is required for the anti-inflammatory effect of PPC.

As the important member of TLRs, TLR-2 can recruit the toll/interleukin-1 receptor (TIR) domain containing adaptor MyD88, and activate MAPK and NF- κ B pathways, triggering downstream signaling cascades and further mediating inflammatory responses [22, 45]. On the one hand, MyD88, first, uses its death domains to recruit, orient, and activate IRAK4. IRAK1/2 can then be allowed for activation and join with MyD88 and IRAK4 to form the myddosome [46]. Formation of this signaling platform then induces TNF receptor-associated factor 6 (TRAF6), a ubiquitin protein ligase to activate MAPKs such as ERK, JNK and P38 inflammatory pathways [22] (Figure 6). The present study showed that PPC can counter the upregulated expression of key molecules in MAPK signaling pathway such as p-P38, p-ERK1/2, p-JNK1/2 induced by LPS, contributing to the anti-inflammation effect of PPC. Moreover, we provided the evidence that the inhibition of MAPK is dependent on TLR-2 signaling. On one hand, the weaken effect of PPC on the protein expression was observed in TLR-2^{-/-} SFs. On the other hand, pre-treatment of Pam3CSK4 almost completely abolishes the inhibition effect of MAPK pathway. These results together suggested that TLR-2/MAPK signaling pathway is essential for PPC's anti-inflammatory effect in SFs. Furthermore, we showed that PPC can act on TLR-2 to reduce the binding of MyD88 to TLRs and the activation of the IKKs complex, thereby reducing I κ B α phosphorylation. This in turn inhibits the nuclear translocation of NF- κ B-p65 in the cytoplasm, thereby inhibiting the transcription of related inflammatory cytokines [47].

Conclusions

The present study demonstrated that PPC interacting with TLR-2 inhibits LPS triggered synovial inflammatory response via MAPK and NF- κ B pathways, which proposes that PPC may be an effective

drug candidate for RA treatment.

Declarations

DISCLAIMER

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: WP, RXT, KYZ. Performed the experiments: ZXX, WTH, DXX. Analyzed the data: YH, ZYY, FFS, XYL, YHY. Contributed reagents/materials/analysis tools: WP, KYZ, RXT. Wrote the manuscript: ZXX, DXX, WP. All Authors have read and approved the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Competing Interests. The authors declare that they have no conflict of interest.

Consent to Participate. Not applicable.

Consent to Publication. Not applicable.

Ethics statement. All animal care and experiments were carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health (China), and approved by the Ethics Committee of Xuzhou Medical University (Xuzhou, China, SYXK (Su) 2015-0009). Each mouse was euthanized by an intraperitoneal injection of sodium pentobarbital (100 mg/kg).

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Figures

Fig. 1

Figure 1

PPC significantly alleviates LPS induced inflammation in SFs. The relative mRNA expression of pro-inflammatory cytokines IL-1 β (a), IL-6 (b) and TNF- α (c), and the anti-inflammatory cytokine TGF- β (d) in the SFs cells of CIA SD rats. (e) The level of LPS in the serum in CIA SD rats. Cytokine concentrations of IL-6 (f) TNF- α (g), IL-10 (h) and TGF- α (i) in the culture supernatants of in vitro cultured SFs isolated from normal C57BL/6J mice. The data are presented as mean $\pm$ SD. Comparisons among multiple groups were analyzed using One-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 2

Figure 2

PPC inhibits LPS-activated SFs inflammation via TLR-2. Primary SFs isolated from wild type (C57BL/6J) mice were stimulated with LPS with or without PPC for 24 h. The relative mRNA expression of TLR-2 was examined by real-time RT-PCR (a) and the protein level of TLR-2 in the SFs of wild type mice was determined by Western blot (b). Primary SFs isolated from TLR-2^{-/-} mice were stimulated with LPS with or without PPC for 24 h. The production of IL-6 (c), TNF-α (d), IL-10 (e) and TGF-α (f) of SFs isolated from TLR-2^{-/-} mice in the supernatants were determined by ELISA. The data are shown as mean ± SD. Comparisons among multiple groups were analyzed using One-way ANOVA. * P < 0.05, ** P < 0.01, *** P < 0.001.

Fig. 3

Figure 3

PPC downregulates NF-κB pathway in SFs via TLR-2. The protein levels of MyD88, p-IκBα, IκBα, β-actin on NF-κB pathway in wild-type SFs (a) and TLR-2^{-/-} SFs (b) were determined by Western blot. (c) Western blot analyses of NF-κB-p65 expression in the cytoplasm and nucleus of C57BL/6J (wild-type) SFs. (d) Western blot analyses of NF-κB-p65 expression in the cytoplasm and nucleus of TLR-2^{-/-} SFs.

Fig. 4

Figure 4

PPC inactivates MAPK pathway in SFs via TLR-2. (a) Western blot examined the protein levels of MyD88, p-P38, ERK1/2, p-ERK1/2, JNK1/2 and p-JNK1/2 in the primary SFs isolated C57BL/6J (wild-type) mice. (b) Western blot examined the protein levels of MyD88, p-P38, ERK1/2, p-ERK1/2, JNK1/2 and p-JNK1/2 in the primary SFs isolated from TLR-2^{-/-} mice.

Fig. 5

Figure 5

The anti-inflammatory effect of PPC in SFs relies on TLR-2 signalling in vitro. Primary SFs isolated from C57BL/6J mice were pre-stimulated 2 h with or without the presence of Pam3CSK4, and then inoculated with Vehicle, PPC, LPS or PPC+LPS for 24 h. The cells and supernatants were collected for further analysis. (a) The phosphorylation levels of MAPK pathway-related proteins in the absence or presence of agonists were measured by western blot. And the concentrations of IL-6 (b), TNF-α (c), TGF-α (d) with different treatments in the supernatants using ELISA. The data are presented as mean ± SD. Comparisons among multiple groups were done using One-way ANOVA. **P < 0.01, ***P < 0.001.

Fig. 6

Figure 6

Schematic diagram of the regulatory mechanism of PPC on LPS-induced inflammatory response in SFs. Schematic showed how PPC interacts with TLR-2 to attenuate the activation of NF-κB and MAPK signaling pathways, thereby decreasing the expression of downstream inflammatory cytokines.

Supplementary Files

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