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

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Received 14 November 2021; accepted 20 January 2022

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 disease sustainability. Therefore, downregulation 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 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 downregulated the expression of TLR-2 and their downstream signaling molecules such as MyD88, p-ERK1/2, p-JNK1/2, and p-P38 in MAPK pathway and p-IκBα and NF-κB-p65 in NF-κB pathway. Moreover, the inhibitory effect of PPC on the above molecules and cytokines was weakened after pre-treatment with TLR-2 agonist Pam3CSK4. In addition, PPC lost its anti-inflammatory effect and its suppressing capability on MAPK and NF-κB pathways in TLR-2−/− primary SFs after exposure to LPS. Collectively, this study demonstrated that PPC can alleviate synovial inflammation through TLR-2-mediated MAPK and NF-κB pathways, which can be proposed to be a potential drug candidate for RA prevention.

KEY WORDS: rheumatoid arthritis; polyene phosphatidylcholine; TLR-2; synovial fibroblasts; MAPK pathway; NF-κB pathway.
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 [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 the sustainability of the disease. Therefore, downregulation 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 being 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 upregulate 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 the production of pro-inflammatory cytokines [18]. It is 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 the immune system [21], which thereby prevents the progression of RA.

Nuclear factor Kappa B (NF-κB) is a crucial transcription factor that can be activated by TLRs, resulting in the production of pro-inflammatory cytokines [22]. NF-κB-p65 is expressed in the cytoplasm and has no transcriptional activity under normal circumstances. IκBα is an inhibitor of NF-κB-p65, and it can combine with NF-κB-p65 in a polymerized manner in the cytoplasm. When stimulated with LPS, phosphorylated IκBα is immediately dissociated from the polymer. And NF-κB-p65, as a result, can be activated and quickly enter into the nucleus from the cytoplasm to regulate the transcription of inflammatory factors [23, 24]. Thus, the inactivation of the NF-κB 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 is 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 with 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 SF inflammation and explored the possible role of TLR-2 in this process. The results showed that PPC interacting TLR-2 inactivates MAPK and NF-κB 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 preparation process of primary SFs was concisely described as follows. The SF tissues 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 50-mL 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 1 × 10^6/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 a 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 2×10^3/well in 100 μL volume. After being cultured at 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 1×10^6 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 a 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 was 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 anesthetized and subcutaneously injected with 100 μL of CII on day 1 and day 10 for
arthrits induction. Controls received an equal volume of Freund’s complete adjuvant at 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)**

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 (ΔCt) normalized by β-actin with the 2-ΔΔCt method. The primer sequences for mice can be found in our previous study [33], and the primer sequences for rat genes are listed in Table 1.

**Western Blotting Analysis**

Total proteins were extracted from SFs and quantified with a 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, IkBa, p-IkBα, NF-κB, NF-κB-p65, β-actin antibody, Abcam, UK, 1:1000 dilutions) at 4 °C for 12 h. The secondary antibodies (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 ± SD. Statistical significance was determined using a 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**

**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. But its underlying mechanism remains unclear. In view of the vital role of SF inflammation in the pathogenesis of RA, we speculated that PPC plays a protective effect through inhibiting SF inflammation. To demonstrate the hypothesis, we evaluated the effect of PPC in LPS-triggered inflammation in the primary SFs from C57BL/6J mice. The production of IL-6 and TNF-α triggered by LPS was significantly inhibited following PPC treatment \( (P < 0.001, \text{Fig. 1a}) \).
and b). On the contrary, PPC significantly increased the levels of anti-inflammatory cytokines IL-10 and TGF-β ($P < 0.001$, Fig. 1c and d). The results suggested that PPC improves LPS-induced inflammation in the SFs in vitro.

**PPC Does Not Affect the Proliferation and Migration of Primary SFs**

To exclude the possibility that PPC inhibits SF inflammation via cell cytotoxicity, this study determined the effect of PPC on the proliferation and migration of primary SFs (Supplementary Fig. 1a). As shown in Supplementary Fig. 1b, different concentrations (0, 10, 20, 30, and 50 μg/mL) of PPC did not significantly inhibit 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 Fig. 1c). Overall, these results showed that PPC does not affect the proliferation and migration of primary SFs, exhibiting a safe effect on SFs.

**PPC Inhibits LPS-Activated SFs Inflammation via TLR-2**

As a conservative pattern recognition receptor, TLR-2 plays a critical role in mediating the inflammatory response of SFs. It is reported that the TLR signaling inhibition of oxidized phospholipid relies on TLR-2 in macrophages [35]. 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 ($P < 0.001$, Fig. 2a–c). However, PPC treatment significantly inhibited TLR-2 expression activated by LPS ($P < 0.001$, Fig. 2a–c).

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 Fig. 2d–g, 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 ($P < 0.01$). However, PPC significantly upregulated the level of IL-10 in TLR-2−/− SFs after exposure to LPS ($P < 0.01$, Fig. 2f). 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 SF inflammation.

**PPC Downregulates NF-κB Pathway in SFs via TLR-2**

MyD88/NF-κB is the classic pathway that mediates LPS-induced inflammation in macrophages [36]. We therefore asked whether PPC can regulate the

![Fig. 1](image1) PPC significantly alleviates LPS-induced inflammation in mice SFs. Primary SFs isolated from C57BL/6J mice were stimulated with LPS in the presence or absence of PPC for 24 h. The supernatants were collected to analyze the cytokine level. a IL-6, b TNF-α, c IL-10, and d TGF-β. The data were presented as mean ± SD. Comparisons among multiple groups were analyzed using one-way ANOVA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. 

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**Fig. 1** PPC significantly alleviates LPS-induced inflammation in mice SFs. Primary SFs isolated from C57BL/6J mice were stimulated with LPS in the presence or absence of PPC for 24 h. The supernatants were collected to analyze the cytokine level. a IL-6, b TNF-α, c IL-10, and d TGF-β. The data were presented as mean ± SD. Comparisons among multiple groups were analyzed using one-way ANOVA. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. 

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pathway in LPS-stimulated SFs. As shown in Fig. 3a and Supplementary Fig. 2a, LPS could increase the level of phosphorylation of IkBα (Ser32/36), thereby eliminating its inhibitory effect on NF-κB and promoting inflammation. However, PPC treatment inhibited the p-IkBα (Ser32/36) and hindered NF-κB activation (Fig. 3a and Supplementary Fig. 2a). Furthermore, the inhibition effect of PPC became weakened in TLR2−/− SFs (Fig. 3b and Supplementary Fig. 2c). The nuclear translocation of NF-κB-p65 is vital for the transcriptome of pro-inflammatory cytokines. We therefore examined the expression of NF-κB-p65 in the cytoplasm and the nucleus of SFs. As shown in Fig. 3c and Supplementary Fig. 2b, the expression of NF-κB-p65 was increased under LPS treatment both in cytoplasm and nucleus of wild-type SFs, while the produce of NF-κB-p65 was suppressed by PPC supplementation. However, the inhibitory effect of PPC on the expression of NF-κB-p65 was weakened in the absence of TLR-2 (Fig. 3d and Supplementary Fig. 2d). These results showed that TLR-2/NF-κB pathway is the downstream pathway for the anti-inflammatory effect of PPC.
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MAPK pathway is also a classical downstream pathway in TLR-2-triggered inflammation [37]. We next determined the proteins expression of p-P38, p-ERK1/2, ERK1/2, p-JNK1/2, and JNK1/2 in LPS-stimulated SFs in the presence or absence of PPC. As shown in Fig. 4a and Supplementary Fig. 3a, the expression of p-P38 (Thr180/Tyr182), p-ERK1/2 (Thr202/Tyr204), and p-JNK1/2 (Thr183/Tyr185) was significantly upregulated in LPS-stimulated SFs in comparison to vehicle control. Nevertheless, PPC treatment significantly downregulated the levels of those phosphorylated proteins. Moreover, in TLR-2−/− SFs, PPC could not inhibit the expression of p-P38, p-ERK1/2, and p-JNK1/2 induced by LPS (Fig. 4b and Supplementary Fig. 3b). These results indicated that PPC inactivates the MAPK pathway in SFs via TLR-2.

The Anti-Inflammatory Effect of PPC in SFs Relies on TLR-2 Signaling

To further confirm whether TLR-2 is the pathway for PPC that regulates the anti-inflammatory response, we pretreated 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-κB pathways (Fig. 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 Fig. 5b–e, PPC could not inhibit the production of IL-6 and TNF-α, conversely, decreasing the IL-10 and TGF-β production 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 the 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 the anti-inflammatory effect of PPC in SF inflammation. Mechanistically, PPC could inactivate MAPK and NF-κB pathways via interacting TLR-2, thereby reducing the production of pro-inflammatory cytokines and alleviating synovial inflammation (Fig. 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 the Nationwide Population-Based Cohort Study [38]. Thus, it is still urgent to develop novel drugs. PC is an important part of the cell membrane and organelle membrane, which has shown immunomodulatory activity in recent years. Historically, the protective effect of a phosphatidylcholine-enriched diet has been demonstrated in animal models of lipopolysaccharide-induced experimental neuroinflammation and 2,4,6-trinitrobenzenesulfonic acid–induced mucosal inflammation [39, 40]. What’s more, as a crucial factor of the membrane architecture, exogenous PC is shown to increase tolerance to ischemia and hypoxia, and inhibit leukocyte accumulation and inflammation in the hindlimb reperfusion model [41]. TPC, a chemical synthesis of a phagocytosis peptide Tuftsin and PC, has amelioration effects in mice models of chronic colitis and lupus nephritis by regulation of the expression of inflammatory factors [31, 42]. Moreover, the compound is reported to induce the expansion of regulatory T (Treg) and regulatory B (Breg) cells to improve CIA condition [43]. 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, IKKα/β, and P38 and rescuing IκBα degradation [44]. The present study showed that PPC, the derivative of PC, a well-known classic hepatoprotective medicine [25–28], can mitigate synovial inflammation.

![Fig. 4 PPC inactivates MAPK pathway in SFs via TLR-2.](chart)

- **a** The protein levels of MyD88, p-P38, ERK1/2, p-ERK1/2, JNK1/2, and p-JNK1/2 in the SFs isolated wild-type mice.
- **b** The protein levels of MyD88, p-P38, ERK1/2, p-ERK1/2, JNK1/2, and p-JNK1/2 in the SFs isolated from TLR-2−/− mice.
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In line with our finding, a previous report has reported that oral PC pretreatment alleviates the signs of CIA [45]. It’s worth noting that PPC has only a limited therapeutic effect in CIA mice (data not shown). Thus, these studies including ours proposed that PPC may be a novel drug for RA prevention.

In our previous study, the beneficial effects of PPC on CIA rats were observed [33]. The present study further showed that PPC can effectively ameliorate the synovial inflammation in ex vivo SFs isolated from the joints of these CIA rats post-PPC pretreatment (Supplementary Fig. 4a–d), which gave a clue to investigate the protective role of PPC in CIA rats.

**Fig. 5** The anti-inflammatory effect of PPC in SFs relies on TLR-2 signaling. Primary SFs isolated from C57BL/6J mice were prestimulated for 2 h with or without Pam3CSK4, followed by inoculation 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. The concentrations of IL-6 (b), TNF-α (c), IL-10 (d), and TGF-β (e) in the supernatants. The data were presented as mean ± SD. Comparisons among multiple groups were done using one-way ANOVA. **P < 0.01; ***P < 0.001.
mechanism of PPC on CIA in the view of synovial inflammation. There is accumulating evidence showing that many endogenous TLR ligands arise 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 [46]. In line with this, we observed an elevated level of LPS in the sera of CIA rats ($P < 0.01$, Supplementary Fig. 4e). It is well recognized that LPS acts as the inducer of sterile inflammation, which involves in the RA progression [47]. Thus, targeting TLR-associated inflammation 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, preactivation of TLR-2 using Pam3CSK4 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 [35]. 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-κB pathways, triggering downstream signaling cascades and further mediating inflammatory responses [22, 48]. MyD88, first, uses its death domains to recruit,
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orient, and activate IRAK4. IRAK1/2 can then be allowed for activation and join with MyD88 and IRAK4 to form the myddosome [49]. 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] (Fig. 6). The present study showed that PPC can counter the upregulated expression of key molecules in the MAPK signaling pathway such as p-P38, p-ERK1/2, and 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 weakened effect of PPC on the protein expression was observed in TLR-2−/− SFs. On the other hand, pretreatment of Pam3CSK4 almost completely abolishes the inhibition effect of the MAPK pathway. These results together suggested that the 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 IκBα phosphorylation. This in turn inhibits the nuclear translocation of NF-κB-p65 in the cytoplasm, thereby inhibiting the transcription of related inflammatory cytokines [50]. We also showed that PPC can significantly inhibit the nuclear translocation of NF-κB-p65 in LPS-stimulated primary SFs. Correspondingly, PC is reported to inhibit TNF-α induced pro-inflammatory response in human Caco-2 cells via inhibition of NF-κB [51].

CONCLUSIONS

The present study demonstrated that PPC interacting with TLR-2 inhibits LPS-triggered synovial inflammatory response via MAPK and NF-κB pathways, which proposes that PPC may be an effective drug candidate for RA prevention.

DISCLAIMER

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

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1007/s10753-022-01633-0.

AUTHOR CONTRIBUTION

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

FUNDING

This work was supported by grants from the Starting Foundation for Talents of Xuzhou Medical College (No. D2015004), the Natural Science Foundation of the Jiangsu Higher Education Institutions (No. 15KJB310025), the Jiangsu Planned Projects for Post-doctoral Research Funds (No. 1501061A), the Natural Science Foundation of Jiangsu Province (No. BK20201459), and the Priority Academic Program Development of Jiangsu Higher Education Institutions. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DECLARATIONS

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).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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