Puerarin alters the function of monocytes/macrophages and exhibits chondroprotection in mice

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Abstract. Recent studies have suggested that puerarin may impede osteoclastogenesis and facilitate bone regeneration, in addition to attenuating tissue inflammation. The present study investigated the therapeutic effects of puerarin on inflammatory responses and monocyte recruitment in *in vitro* and *in vivo* osteoarthritis (OA) models. Puerarin treatment increased the proliferation of OA chondrocytes, as determined by Cell Counting Kit-8 assay. In addition, the present results suggested that puerarin suppressed the interleukin-1β-induced production of inflammatory cytokines in OA chondrocytes and monocytes/macrophages, as assessed by ELISA. In a mouse model of mono-iodoacetate-induced OA, the present histological analyses suggested that administration with puerarin attenuated the inflammatory profile of OA joints and reduced cartilage destruction. Using flow cytometry, a decreased number of myeloid-derived C-C chemokine receptor 2^+/lymphocyte Ag $6C^+$ monocytes was identified in the blood of OA mice treated with puerarin compared with control OA mice. Furthermore, quantitative real-time polymerase chain reaction analysis suggested that puerarin treatment decreased C-C chemokine ligand 2 expression in arthritic tissues. Collectively, the results suggested that puerarin treatment limited the recruitment of inflammatory monocytes. In summary, the present study provided pre-clinical evidence that puerarin may serve as a potential target in the treatment of OA.

Introduction

Osteoarthritis (OA) is a common and progressive joint disease associated with aging, obesity, joint injury and heredity (1). OA may affect the quality of life of patients and result in socio-economic burden to families, communities and nations (2). Accumulating evidence suggests that chronic inflammation serves an important role in the pathogenesis and prognosis of OA (2). Of note, persistent infiltration and proinflammatory differentiation of monocytes may induce chronic inflammation in OA (3). Therefore, suppression of inflammation and prevention of articular cartilage degeneration are important factors in the treatment of OA.

Puerarin is a phytoestrogen with potential beneficial effects in attenuating neuronal injury, diabetic kidney disease and heart failure (4-7). The herbal compound modulates oxidative stress and inflammatory responses in cardiovascular diseases (8). Furthermore, it has been reported that puerarin may be beneficial in treating diabetes mellitus (5). Additionally, the protective roles of puerarin have been reported in a number of cell lineages, including endothelial progenitor cells, cardiac muscle cells and neuronal cells (9,10). Puerarin has been hypothesized to act in an arginase 2-dependent manner, a mitochondrial enzyme, via modulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-associated oxidative stress (6,11-13). In addition, puerarin alters the production of innate immune cells by regulating the differentiation of progenitor cells via estrogen receptors (14). Previous studies reported the protective effects of puerarin on the viability and function of endothelial progenitor cells derived from bone borrow and peripheral blood (10,14). Furthermore, puerarin exhibits ameliorative effects in various inflammatory disorders via inhibiting the degranulation of mast cells, reducing monocyte adhesion to endothelial cells and modifying chemotactic agent expression in cardiac fibrotic tissue (15-17). Innate immune signal transduction pathways, including nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein kinase and extracellular signal-regulated kinase signaling may be affected by treatment with puerarin (14,18,19).

It has been hypothesized that puerarin exerts potent anti-inflammatory and chondroprotective effects by inhibiting osteoclast formation, bone loss and collagen degradation (20-22); however, its regulatory effects on monocyte/macrophage function remain unclear. Therefore, the present study aimed to investigate the therapeutic effects of puerarin on inflammatory responses and monocyte recruitment within *in vitro* and *in vivo* models of OA.

Materials and methods

Reagents. Puerarin was purchased from PI & PI Biotech Inc. (Guangzhou, China). Cell Counting Kit-8 (CCK-8) reagent was

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obtained from MedChemExpress LLC (Monmouth Junction, NJ, USA). Fetal bovine serum (FBS) was purchased from BBI Solutions (Cardiff, UK). Dulbecco's modified Eagle's medium (DMEM)/F12 and RPMI-1640 media were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell isolation and culture. Primary human chondrocytes were isolated from cartilage tissues collected from 6 patients with OA (aged 60-63 years old; female to male ratio, 2:1) that underwent knee arthroplasty between March 2016 and January 2018 using enzyme extraction. The study was approved by the Medical Ethics Committee of Changzhou Traditional Chinese Medicine Hospital (Changzhou, China), and patients provided written informed consent. Briefly, cartilage tissue was minced into ~1 mm^3 pieces and digested in 1 mg/ml collagenase B (Roche Diagnostics, Basel, Switzerland) and 200 U/ml DNase I in a CO_2 incubator at 37˚C for 3 h. The resulting suspension was diluted with 20 ml of DMEM/F12 medium containing 10% FBS and filtered via a 70-μm cell strainer. Cells were washed with DMEM/F12/10% FBS, seeded in 6-well cell culture plates at a density of $5x10^5$ cells/well and cultured overnight at 37˚C. Additionally, a human monocytic leukemia cell line (THP-1) was obtained from the American Type Culture Collection (Manassas, VA, USA). THP-1 cells were cultured in RPMI-1640 containing 10% FBS at 37˚C and underwent differentiation with 50 ng/ml phorbol myristate acetate overnight. Then, the two cell lines were stimulated with 10 ng/ml IL-1β (Prospec-Tany TechnoGene, Ltd., East Brunswick, NJ, USA) for 12 h in the absence or presence of puerarin (25, 50 and 100 nM).

Cell proliferation assays. CCK-8 assays were performed to determine cell proliferation according to the manufacturer's protocols. Briefly, primary chondrocytes were plated at $1x10^4$ cells/well in a 96-well cell culture plate. Serial concentrations of puerarin (0, 25, 50 and 100 nM) were applied to the culture medium for 24 h at 37˚C. The working concentration selected was 50, and 50 nM puerarin was applied to the culture medium for 0, 6, 12, 24 and 48 h. CCK-8 reagent was added prior to 2 h of further incubation at 37˚C. The proliferation of cells was determined by the absorbance 450 nm as detected by a microplate reader.

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) flow cytometry assay. An Annexin V-FITC/PI detection kit (Yeasen Biotechnology Co., Ltd., Shanghai, China) was used to determine cell apoptosis according to the manufacturer's protocols. Briefly, primary chondrocytes were plated at $1x10^6$ cells/well in 6-well cell culture plates. Serial concentrations of puerarin (0, 25, 50 and 100 nM) were applied to the culture for 24 h. The cells were collected, stained with Annexin V-FITC/PI and measured using a flow cytometer. Data were analyzed using FlowJo software 7.0 (Tree Star, Inc., Ashland, OR, USA).

ELISA. Cell culture supernatants were collected and stored at -80˚C for cytokine assays. Human PGE2 (cat. no. KHL1701), IL-6 (cat. no. 88-7066-22), TNF-α (cat. no. 88-7346-22), IL-12 (cat. no. BMS2013), TGF-β1 (cat. no. BMS249-4) and IL-10 (cat. no. 88-7105-22) ELISA kits were purchased from BioSource International (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All procedures were performed according to the manufacturer's protocols.

Animal experiment. Male B6 mice (age, 8 weeks; weight, 20-22 g) were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in standard conditions of humidity and temperature (temperature, 23±2˚C; humidity, 50-80%), with free access to food and water under a 12-h light/dark cycle in the Animal Center of the Nanjing University of Chinese Medicine (Nanjing, China). The animal experimental procedures were approved by the Committee on Laboratory Animal Care of the Nanjing University of Chinese Medicine (license no. 20170621-X). To investigate the effects of puerarin following induction of OA, the mice were randomly divided into five groups, four of which were injected intra-articularly with 5 μl mono-iodoacetate (MIA; 20 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) as described previously (23). Puerarin (0, 10, 25 or 50 mg/kg) was injected intraperitoneally at a 3-day interval starting from the day prior to OA induction from day-1 to day 14. To examine the effect of puerarin on blood monocytes/macrophages migration, the mice were randomly divided into four groups, OA was induced in three groups and mice were treated with puerarin (25 or 50 mg/kg) at a 3-day interval starting, and sacrificed at day 10. The mice in the control group (NC) were not treated with MIA and OA was not induced. All mice were sacrificed under general anesthesia and their joints were collected for RNA extraction and histological analysis.

Histology. Joints were fixed with 10% neutral buffered formalin, decalcified in 10% EDTA for 1 month at room temperature and subsequently embedded in paraffin. Sections (5-μm) were stained with H&E (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) to detect inflammation or with Safranin O/Fast green (Beijing Solarbio Science & Technology Co., Ltd.) to detect cartilage alterations. For hematoxylin and eosin staining, the sections were stained with hematoxylin at 25˚C for 15 min. Subsequently, the sections were washed with purified water, dehydrated in ethyl alcohol series and stained with eosin solution for 5 min at 25˚C. The samples were washed and dehydrated in ethyl alcohol, and the sections were visualized using a light microscope (DMI4000B; Leica Microsystems GmbH, Wetzlar, Germany; magnification, x200). For Safranin O/Fast green staining, the sections were washed twice for 10 min in Xylene at 25˚C, rehydrated in ascending ethyl alcohol series and stained at 25˚C for 12 h in a 1% Safranin O solution. Subsequently, the sections were washed in purified water twice for 10 min, dehydrated in ascending ethyl alcohol series, and stained at 25˚C for 10 sec in Fast Green solution. Following rinsing and removal of the Fast Green solution, the samples were imaged using a light microscope (DMI4000B; Leica Microsystems GmbH; magnification, x200).

Flow cytometric assay of blood monocytes. In total, 500 μl blood was collected from the angular vein of mice using EDTA-Na_2 anticoagulant tubes. Following dilution with an equivalent volume of PBS, the blood samples were

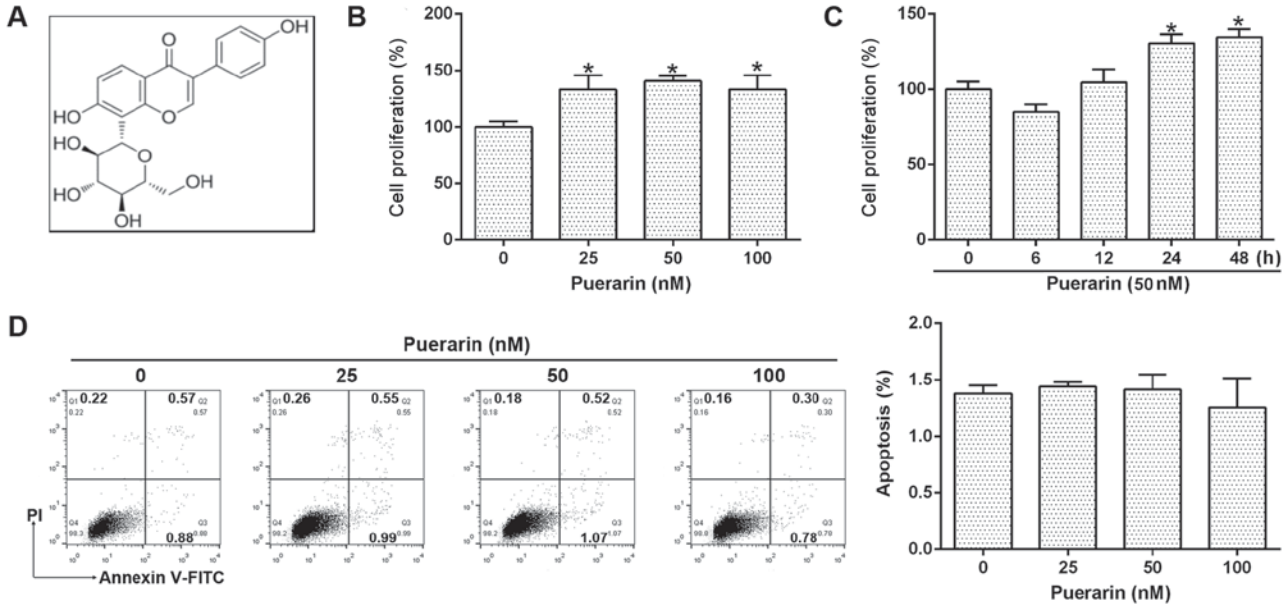

Figure 1. Effects of puerarin on the proliferation of chondrocytes. (A) Chemical composition of puerarin. (B) Effect of puerarin (0, 25, 50 or 100 nM) on the proliferation of primary OA chondrocytes 24 h following culture. (C) 50 nM puerarin was used to determine the effects of puerarin treatment over time on primary OA chondrocytes. (D) Effects of puerarin on the apoptosis of chondrocytes. Experiments were performed at least two times. Data are presented as the mean ± standard deviation. *P<0.05 vs. 0 nM. FITC, fluorescein isothiocyanate; OA, osteoarthritis.

blocked using the purified anti-mouse CD16/32 antibody (dilution, 1:100; clone, 93; cat. no. 101301; BioLegend, Inc., San Diego, CA, USA) for 15 min on ice and subsequently stained with fluorescein isothiocyanate-labeled cluster of differentiation 11b (CD11b; dilution 1:200; clone, M1/70; cat. no. 11-0112-82; eBioscience; Thermo Fisher Scientific, Inc.), phyllochlorin-labeled CD115 (dilution, 1:100; clone, AFS98; cat. no. 25-1152-80; eBioscience; Thermo Fisher Scientific, Inc.), phycoerythrobilin-labeled lymphocyte Ag 6G (Ly6G; dilution, 1:100; clone, 1A8-Ly6g; cat. no. 17-9668-80; eBioscience; Thermo Fisher Scientific, Inc.), phycoerythrin-cyanin 7-labeled Ly6C (dilution, 1:100; clone, HK1.4; cat. no. 12-5932-80; eBioscience; Thermo Fisher Scientific, Inc.) and allophycocyanin-labeled C-C chemokine receptor 2 (CCR2; dilution, 1:100; clone, SA203G11; cat. no. 150621; BioLegend, Inc.) antibodies for 40 min on ice. Subsequently, red blood cells (RBCs) were removed using the RBC lysis buffer (BioLegend, Inc.). Following a centrifugation at 500 x g for 10 min at 4˚C, the remaining cell pellets were washed three times with PBS and resuspended in 300 μl fluorescent-activated cell sorting (FACS) buffer (PBS/0.5% FBS/0.06% NaN_3) for assays. Finally, data were acquired using a BD FACS Aria II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using the FlowJo software 7.0 (Tree Star, Inc., Ashland, OR, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from mouse knee cartilage and synovium using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. RT of RNA was conducted using a Moloney Murine Leukemia Virus reverse transcriptase PCR Kit (Vazyme, Piscataway, NJ, USA) according to the manufacturer's protocol. qPCR was performed using a SYBR® Green Kit (Vazyme) with the following primers: C-C chemokine ligand 2 (CCL2), forward 5'-AGG TGTCCCAAAGAAGCTGTA-3', reverse, 5'-ATGTCTGGA CCCATTCCTTCT-3'; CCL5, forward 5'-ATATGGCTCGGA CACCACTC-3', reverse, 5'-GTGACAAACACGACTGCA AGA-3'; and GADPH, forward 5'-CATGGCCTTCCGTGT TCCTA-3' and reverse, 5'-GCGGCACGTCAGATCCA-3'.

The thermocycling conditions were as follows: 30 cycles of 95˚C for 5 min, 95˚C for 30 sec, 63˚C for 30 sec and 72˚C for 30 sec. The mRNA expression levels were normalized to GADPH using the $2^{-\Delta\Delta Cq}$ quantification method as previously described (24).

Statistical analysis. All statistical analyses were performed using GraphPad Prism v5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). All experiments were repeated at least three times. The data are presented as the mean ± standard deviation. Significant differences between groups were determined using the Kruskal-Wallis test followed by Tukey's multiple comparisons test. P<0.05 was considered to represent a statistically significant difference.

Results

Effects of puerarin on the cell proliferation of chondrocytes. To investigate the effects of puerarin (Fig. 1A) on human chondrocytes, the proliferation of chondrocytes was determined following treatment with various concentrations (0, 25, 50 or 100 nM) of puerarin for 24 h. As presented in Fig. 1B, 25, 50 or 100 nM puerarin significantly increased the proliferation of cells compared with the control; thus, 50 nM puerarin was selected to determine the effects of puerarin in the further analyses. Compared with baseline proliferation prior to treatment, 50 nM puerarin induced a marked decrease in chondrocyte proliferation at 6 h, but significantly promoted cell proliferation at 24 and 48 h (Fig. 1C). These results

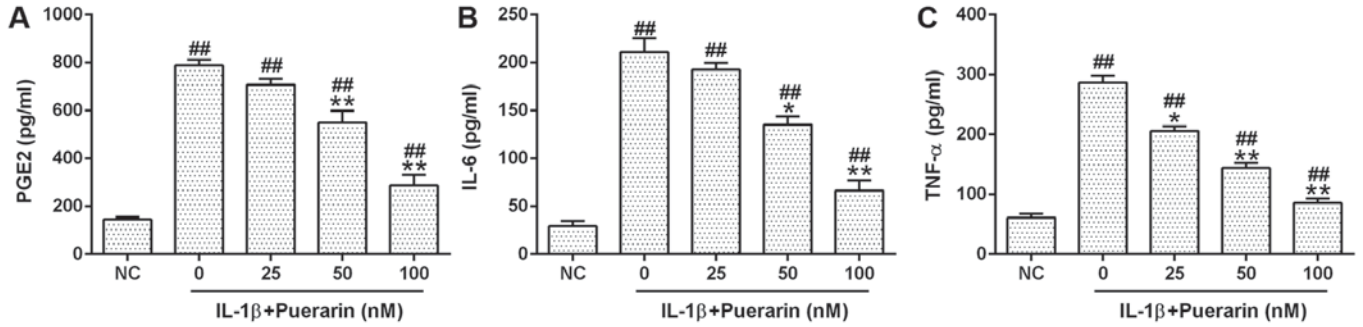

Figure 2. Effects of puerarin on the inflammatory response of chondrocytes following IL-1β stimulation. Primary osteoarthritis chondrocytes were stimulated with 10 ng/ml IL-1β in the presence of 0, 25, 50 or 100 nM puerarin. Following culture for 48 h, the levels of (A) PGE2, (B) IL-6 and (C) TNF-α in the culture supernatant were determined by ELISA. Experiments were performed at least two times. Data are presented as the mean ± standard deviation. $^{\#\#}$P<0.01 vs. NC. *P<0.05, **P<0.01 vs. 0 nM. IL, interleukin; NC, negative control; PGE2, prostaglandin E2; TNF-α, tumor necrosis factor α.

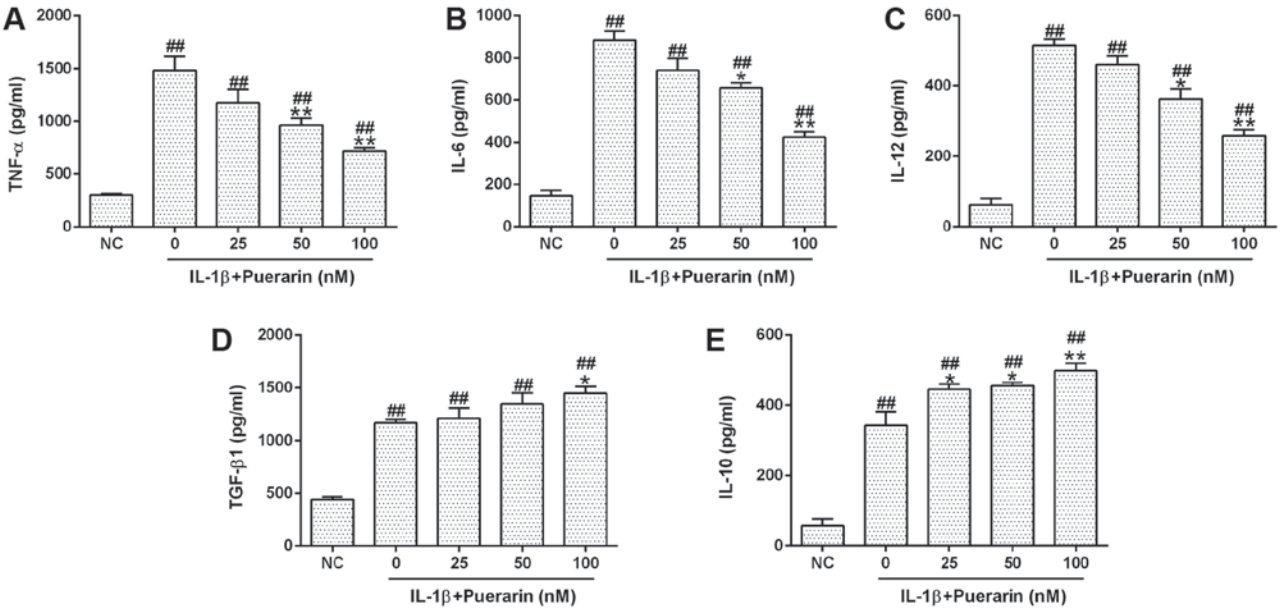

Figure 3. Puerarin treatment reduces the release of inflammatory mediators from monocytes/macrophages following IL-1β stimulation. THP-1 cells were differentiated using 50 nM phorbol myristate acetate, and then stimulated with 10 ng/ml IL-1β in the presence of 0, 25, 50 or 100 nM puerarin. Following culture for 48 h, the levels of (A) TNF-α, (B) IL-6, (C) IL-12, (D) TGF-β1 and (E) IL-10 in the culture supernatant were determined by ELISA. Experiments were performed at least two times. Data are presented as the mean ± standard deviation. $^{\#\#}$P<0.01 vs. NC. *P<0.05, **P<0.01 vs. 0 nM. IL, interleukin; NC, negative control; TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor α.

were supported by Annexin V-FITC/PI flow cytometry; no significant alterations in the apoptosis of cells were observed following puerarin treatment (Fig. 1D).

Puerarin inhibits the production of IL-1β-induced inflammatory cytokines. The effects of puerarin on IL-1β-induced inflammatory responses were subsequently investigated in OA chondrocytes. The levels of TNF-α, IL-6 and IL-12 expression were measured via an ELISA (Fig. 2A-C). It was revealed that puerarin significantly reduced the IL-1β-induced upregulation of PGE2, IL-6 and TNF-α expression levels in a dose-dependent manner.

The effects of puerarin on the function of human monocytes/macrophages were subsequently investigated. THP-1 macrophages were treated with puerarin for 24 h, followed by stimulation with IL-1β. The expression levels of TNF-α, IL-6 and IL-12 were measured by ELISA. Puerarin treatment significantly downregulated IL-1β-induced expression of TNF-α, IL-6 and IL-12 in THP-1 macrophages (Fig. 3A-C); however, the levels of anti-inflammatory cytokine expression, including TGF-β1 and IL-10, were increased in IL-1β-treated cells following puerarin exposure (Fig. 3D and E), suggesting that puerarin treatment altered the function of monocytes/macrophages.

Effects of puerarin on degenerative cartilage destruction. To evaluate the effects of puerarin on OA, a mouse model of OA was generated using a method of intra-articular injection of MIA as previously described (23). Treatment with puerarin was initiated the day prior to MIA injection, and repeated at 3-day intervals at a range of concentrations (0, 10, 25 or 50 mg/kg; Fig. 4A); a normal control (NC) group did not receive MIA. Following puerarin treatment, the degree of cartilage destruction and synovitis score were determined

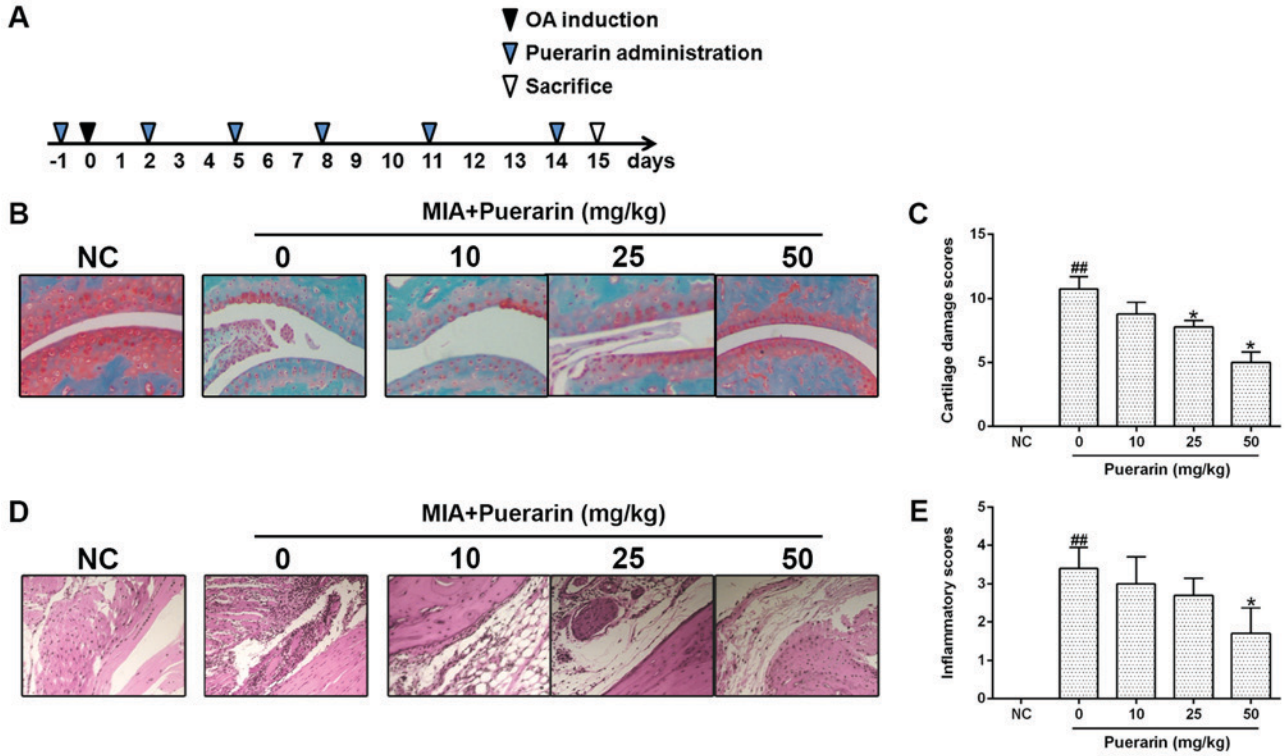

Figure 4. Puerarin treatment ameliorates the symptoms of OA in a mouse model. Mice were assigned to five groups; one group served as the control and did not receive MIA. The remaining mice were intraperitoneally administered with 0, 10, 25 or 50 mg/kg puerarin, with an interval of 3 days between injections (beginning the day prior to MIA injection). The MIA injection was performed at day 0 and mice were sacrificed 15 days later. (A) Timetable of the animal experiment. (B) Representative images of Safranin O/Fast green staining from each group (magnification, x200). (C) Cartilage damage scores. (D) Representative images of H&E staining from each group (magnification, x200). (E) Synovitis scores. N=6-8 animals/group. Data are presented as the mean ± standard deviation. $^{\#\#}$P<0.01 vs. NC. *P<0.05 vs. 0 nM. MIA, mono-iodoacetate; NC, negative control; OA, osteoarthritis.

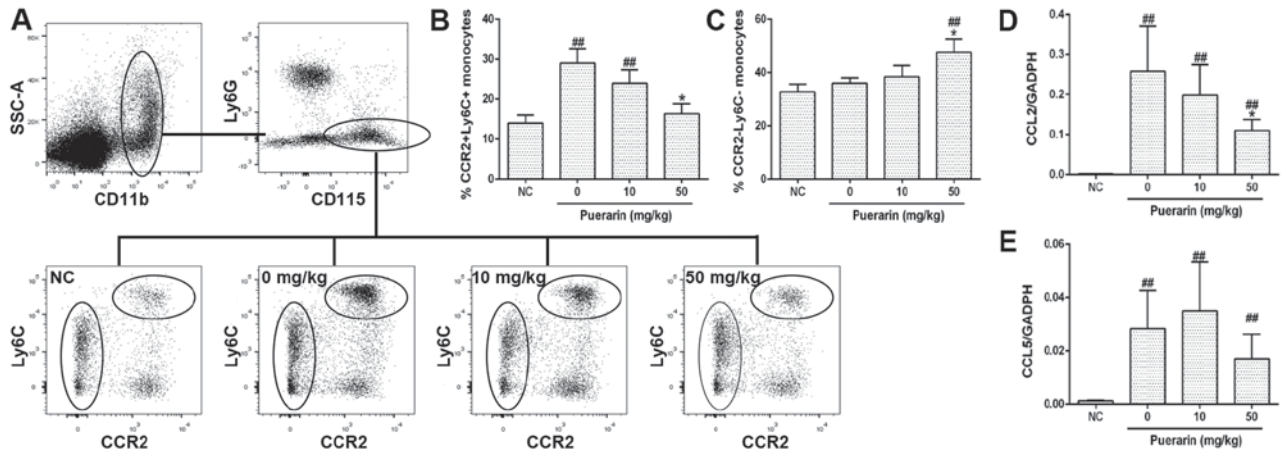

Figure 5. Effects of puerarin on blood monocytes/macrophages *in vivo*. Blood was collected on day 10 post-OA induction, stained with CD11b, CD115, Ly6G, Ly6C and CCR2 antibodies and assayed by fluorescent-activated cell sorting. (A) Gating strategy of monocytes and representative images. (B) Ratio of $CCR2^{+}/Ly6C^{+}$ cells in the $CD11b^{+}/CD115^{+}/Ly6G^{-}$ cells. (C) Ratio of $CCR2^{-}/Ly6C^{-}$ cells in the $CD11b^{+}/CD115^{+}/Ly6G^{-}$ cells. mRNA was extracted from the knee joints of mice with OA on day 14, and the levels of (D) CCL2 and (E) CCL5 expression were determined using reverse transcription-quantitative polymerase chain reaction. N=6-8 animals/group. Data are presented as the mean ± standard deviation. $^{\#\#}$P<0.01 vs. NC. *P<0.05 vs. 0 nM. CCL, C-C chemokine ligand; CCR, C-C chemokine receptor; CD, cluster of differentiation; Ly, lymphocyte Ag; NC, negative control; OA, osteoarthritis.

by Safranin O and H&E staining, respectively. MIA treatment induced a significant increase in cartilage damage compared with the NC group, which reduced in a dose-dependent manner following puerarin treatment (Fig. 4B and C). Similarly, the severity of synovitis was significantly increased following MIA treatment compared with the NC group, but was reduced in MIA-injected mice following treatment with puerarin, suggesting that 50 mg/kg of puerarin attenuated the inflammatory responses (Fig. 4D and E). Collectively, the results indicate that puerarin may ameliorate cartilage damage and synovitis-associated pathological alterations in a mouse model of MIA-induced OA.

Effects of puerarin on blood monocytes/macrophages in vivo. The effects of puerarin on monocyte recruitment were investigated by measuring the number of monocytes in the blood and the levels of CCL2 and CCL5 expression in synovial tissues 10 days post-OA induction. All mice in the experimental groups underwent MIA-injection to induce OA on day 0; the mice in the control group were not treated with MIA. It was revealed that the number of $CD11b^+/Ly6C^-$ cells, characterized by low expression levels of CCR2 (25), was notably increased in OA mice following treatment with 50 mg/kg puerarin. Conversely, puerarin treatment significantly reduced the number of $CD11b^+/Ly6C^+$ cells, the numbers of which were increased significantly following MIA injection compared with the NC group (Fig. 5A-C). Of note, although MIA injection induced an increase in the expression levels of CCL2 and CCL5 (Fig. 5D and E), 50 mg/kg puerarin-treated mice demonstrated significantly reduced expression levels of CCL2 mRNA, suggesting that puerarin reduced the infiltration of monocytes in the mouse model of OA (Fig. 5D). Collectively, the results indicate that puerarin may suppress proinflammatory monocyte recruitment during OA.

Discussion

Puerarin is a versatile compound extracted from the root of *Pueraria* (*Radix puerariae*) (26). The therapeutic potential of puerarin has been reported *in vivo* and *in vitro*; for instance, puerarin protects retinal pericytes from apoptosis in a rat model with retinal pericyte loss (13). The molecular mechanisms underlying this effect may be associated with the inhibition of NADPH oxidase-associated reactive oxygen species (ROS) pathways and the suppression of NF-κB activation. The present study revealed that puerarin treatment suppressed the production of numerous proinflammatory cytokines from activated human chondrocytes, which may be associated with the inhibition of the ROS and NF-κB pathways. Additionally, puerarin ameliorates nerve injury-induced depression and pain following spared nerve injury in an arginase 2-dependent manner (27). Furthermore, puerarin attenuates mechanical and chemical nerve injuries in experimental models of cerebral ischemia, diabetic complication and cardiac diseases (6,8,13). These findings indicated the antioxidative, anti-inflammatory and antiapoptotic activities of puerarin *in vivo* and *in vitro*. In the present study, the potential of puerarin in the treatment of OA, and the anti-inflammatory effects of puerarin on *in vitro* and *in vivo* models of OA were investigated. Human chondrocytes exhibited increased cell proliferation following treatment with puerarin without a significant alteration in apoptosis, suggesting chondroprotective activity *in vitro*. Future experiments should aim to verify these findings and investigate the underling mechanisms using western blotting and lactate dehydrogenase release assays. Additionally, high concentrations of puerarin were used to demonstrate the effects of puerarin *in vitro*; however, further study is required to determine suitable doses and routes of administration for use in humans.

OA is a common joint disease with persistent, low-degree inflammation in the synovium (3). Puerarin has been reported to exhibit anti-inflammatory effects in various mouse models of inflammatory disease, including ischemia/reperfusion, atopic dermatitis, mastitis and collagen antibody-induced arthritis (11,16,28,29). Puerarin treatment inhibits chronic inflammation induced by lipopolysaccharide or pro-inflammatory mediators, such as IL-1β, in various types of cells (9,19,30). The present study revealed that puerarin not only inhibited inflammatory responses in IL-1β-stimulated OA chondrocytes, but may have also altered the function of monocytes/macrophages *in vitro*. A recent study identified the role of blood-derived monocytes and the CCL2/CCR2 axis in the establishment of chronic inflammation (25); however, the CCL5/CCR5 axis may be responsible for the aggravation of OA (25,31). In the present study, puerarin was administrated intraperitoneally to mice, and may therefore affect monocytes in the blood prior to migrating to joints.

In conclusion, it was revealed that puerarin protected human chondrocytes and reduced the production of inflammatory mediators. *In vivo*, puerarin ameliorated the progression of MIA-induced OA in mice and altered blood monocyte development. Further investigation is required to determine the specific mechanisms by which puerarin interacts with blood monocytes and whether puerarin alters monocyte infiltration.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LP, ZX and JP performed the animal experiments, and collected and analyzed data. LP, BW, YG and YQ contributed to the conception and design of the present study, and interpreted the data. LP and YQ drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of Changzhou Traditional Chinese Medicine Hospital (Changzhou, China), and patients provided written informed consent.

Patient consent for publications

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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