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Quercetin as a promising intervention for rat osteoarthritis by decreasing M1-polarized macrophages via blocking the TRPV1-mediated P2X7/NLRP3 signaling pathway

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Abstract

Osteoarthritis (OA) is characterized by an imbalance between M1 and M2 polarized synovial macrophages. Quercetin has shown protective effects against OA by altering M1/M2-polarized macrophages, but the underlying mechanisms remain unclear. In this study, rat chondrocytes were treated with 10 ng/mL of IL-1β. To create M1-polarized macrophages in vitro, rat bone marrow-derived macrophages (rBMDMs) were treated with 100 ng/mL LPS. To mimic OA conditions observed in vivo, a co-culture system of chondrocytes and macrophages was established. ATP release assays, immunofluorescence assays, Fluo-4 AM staining, Transwell assays, ELISA assays, and flow cytometry were performed. Male adult Sprague–Dawley (SD) rats were used to create an OA model. Histological analyses, including H&E, and safranin O-fast green staining were performed. Our data showed a quercetin-mediated suppression of calcium ion influx and ATP release, with concurrent downregulation of TRPV1 and P2X7 in the chondrocytes treated with IL-1β. Activation of TRPV1 abolished the quercetin-mediated effects on calcium ion influx and ATP release in chondrocytes treated with IL-1β. In the co-culture system, overexpression of P2X7 in macrophages attenuated the quercetin-mediated effects on M1 polarization, migration, and inflammation. Either P2X7 or NLRP3 knockdown attenuated IL-1β-induced M1/M2 polarization, migration, and inflammation. Moreover, overexpression of TRPV1 reduced the quercetin-mediated suppressive effects on OA by promoting M1/M2-polarized macrophages in vivo. Collectively, our data showed that quercetin-induced suppression of TRPV1 leads to a delay in OA progression by shifting the macrophage polarization from M1 to M2 subtypes via modulation of the P2X7/NLRP3 pathway.

KEYWORDS
M1 macrophage polarization, NLRP3, osteoarthritis, P2X7, quercetin, TRPV1

Wenjun Li and Hebei He these authors have the same contributions.

1 | INTRODUCTION

Osteoarthritis (OA) primarily affects individuals aged 60 years and above, and is characterized by structural changes in articular cartilage, osteophyte development, and synovial inflammation known as synovitis (Hügle & Geurts, 2017; Peat et al., 2001). Chondrocytes, the cells residing in articular cartilage, are essential for preserving a delicate balance between the synthesis and degradation of extracellular matrix (Shi et al., 2019). In OA, synovitis is linked to increased chondrocyte apoptosis, ectopic bone formation, hypertrophy, and the manifestation of clinical symptoms (Sanchez-Lopez et al., 2022). The central characteristic of synovitis is an accumulation of polarized macrophages within the synovium's intimal lining. Synovial macrophages function as immune cells that undergo activation, accumulation, and polarization into M1- and M2-polarized subtypes Z (Utomo et al., 2016; Zhang et al., 2020). M1 macrophages are known for producing proinflammatory mediators such as IL-1 and TNF-α, while M2 macrophages exhibit anti-inflammatory properties that contribute to tissue repair and remodeling (Favero et al., 2019; Zhang et al., 2020). Thus, developing strategies that inhibit M1 macrophage polarization remains an important area of OA research.

Quercetin, a widely studied and abundant flavonoid compound found in various plants, exhibits diverse biological activities such as cytoprotective, antithrombotic, antioxidant, and anti-cancer effects (D'Andrea, 2015). Research has demonstrated that quercetin has the ability to inhibit iNOS and COX-2 expression, TNF-α and IL-1β secretion by M1 macrophages, and upregulate IL-10 expression in M2 macrophages (Tsai et al., 2021). Additionally, quercetin may exert its immunoregulatory effects on macrophages by inhibiting NLRP3 inflammasome activity (Luo et al., 2022). Accumulating evidence suggests that quercetin's suppressive effects on M1 macrophage polarization play a role in obesity-induced steatohepatitis (Kim et al., 2016), diabetic wound repair (Fu et al., 2020), and neutrophilic airway inflammation (Wang et al., 2023). A recent study by Hu et al. (2019) revealed that quercetin exerts chondroprotective effects in OA by suppressing inflammation, reducing chondrocyte apoptosis, and inducing synovial macrophage polarization towards the M2 subtype. However, the specific mechanisms by which quercetin influences the polarization of M1 synovial macrophages remain to be fully understood and require further investigation.

Quercetin has been shown to dose-dependently suppress a transient increase in receptor potential vanilloid 1 (TRPV1) expression in the spinal cord, and effectively alleviate paclitaxel-induced neuropathic pain (Gao et al., 2016). Additionally, quercetin was found to reduce calcium (Ca^{2+}) influx induced by histamine four receptor activation via the TRPV1 channel (Yang et al., 2021). As a non-selective cationic channel, TRPV1 is primarily found in primary nociceptive sensory neurons, and responds to various stimuli such as temperature, mechanical forces, and chemicals (Straub, 2014; Venkatachalam & Montell, 2007). TRPV1 activation has been associated with immune cell regulation (Bertin et al., 2014) and the induction of proinflammatory polarization (M1) in macrophages (Simeoli et al., 2017). When TRPV1 is activated, it triggers an influx of Ca^{2+}, which acts as a signal to elicit neuronal responses (Caterina et al., 1997). Because it is highly sensitive to extracellular Ca^{2+} (Yan et al., 2011), the purinergic 2X7 (P2X7) receptor, a member of the P2X subfamily of purinergic receptors, can be activated by elevated levels of extracellular adenosine 5′-triphosphate (ATP) (Stokes et al., 2015). Moreover, an increase in intracellular ATP concentration was found to cause a negative shift in the voltage-dependent activation of TRPV1 channels (Shimizu et al., 2022). The NLRP3 inflammasome, which comprises the recognition receptor NLRP3, adapter ASC, and the effector protein caspase 1, is an extensively studied element of the NLR family. Yue et al. (2017) found that activation of the P2X7-NLRP3-IL-1β pathway is triggered by elevated extracellular ATP levels in the hippocampus, coupled with chronic stress, resulting in the development of persistent depressive behaviors. A noteworthy finding by Kelly et al. (2015) revealed the involvement of TRPV1 in the polarization of macrophages, specifically M1 macrophages, which correlates with synovitis and OA severity. Building on these findings, it is plausible to speculate that quercetin might impact the polarization of M1 synovial macrophages in OA by modulating the P2X7-NLRP3 signaling pathway through the regulation of TRPV1-mediated Ca^{2+} influx.

To test our hypothesis, we initially investigated the impact of quercetin on Ca^{2+} influx, ATP release, as well as on TRPV1 and P2X7 expression levels in IL-1β-induced rat chondrocytes. By performing a series of experiments, we further assessed the effects of quercetin on the TRPV1-mediated P2X7/NLRP3 signaling pathway, which is involved in the polarization of M1 synovial macrophages. For this purpose, we established a co-culture system that used IL-1β-induced rat chondrocytes and macrophages. Lastly, we examined the regulatory role of TRPV1 in mediating the beneficial effects of quercetin in a rat model of OA by administering quercetin via intra-articular injection.

2 | MATERIALS AND METHODS

2.1 | Isolation and identification of rat chondrocytes

Male Sprague Dawley (SD) rat knee articular cartilage was used to isolate primary chondrocytes, which were then cultured according to a well-established method (S. N. Wang, Zhao, et al., 2015). Briefly, samples of knee articular cartilage tissue were aseptically dissected into small fragments. Next, the cartilage pieces were thoroughly rinsed five times with PBS and then digested for 1 h in 0.25% trypsin–EDTA solution. The cartilage fragments were then rinsed and subsequently incubated for 4 h in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12) containing 0.2% collagenase type II (Hyclone, Logan, UT, USA); after which, the resulting chondrocytes were released and collected by centrifugation at 1200 rpm for 5 min. Next, the collected cells were cultured in DMEM-F12 containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. An immunofluorescence assay was performed to confirm the identity of the rat chondrocytes, based on the expression of specific markers such as collagen type II alpha 1 chain (COL2A1) and chondroitin sulfate (CS). Once the cells reached 80%–90% confluence during the third passage, they were utilized for all subsequent experiments.

2.2 | In vitro cytotoxicity test

Rat chondrocytes were seeded in triplicate into 96-well plates at a density of 1×10^4 cells per well and allowed to attach for 12 h. Next, the cells were incubated with various concentrations (0, 2, 4, 8, 16, 32, and 64 μM) of quercetin (Sigma-Aldrich Corp., St Louis, MO, USA) for 24 h. Following a 2 h incubation with 10 μL of CCK-8 solution (C0038, Beyotime, Shanghai, China) at 37°C, we used a spectrophotometer to determine the optical density (OD) value of each well at a wavelength of 450 nm. Data obtained from at least three independent experiments were analyzed using GraphPad Prism 8.0 software to determine the IC_{50} (half maximal inhibitory concentration) values for each treatment group.

2.3 | Chondrocyte treatment

Rat chondrocytes were seeded into DMEM-F12 complete medium and allowed to culture for 2–3 days until they reached approximately 80% confluence. To evaluate the impact of quercetin on osteoarthritic chondrocytes, the cells were first treated with 10 ng/mL IL-1β (Pepro Tech, NJ, USA) for 24 h at 37°C in a 5% CO_2 atmosphere. Subsequently, the chondrocytes were exposed to both a low dose (LD: 0.3-fold IC50) and high dose (HD: 0.8-fold IC50) of quercetin for 24 h. Meanwhile, 0.1% dimethyl sulfoxide (DMSO) was added to chondrocytes from the control and IL-1β groups. Moreover, to explore the potential effect of TRPV1 on calcium ion influx and ATP release, 10 μM capsaicin (a TRPV1 activator) was added to chondrocytes in the IL-1β + quercetin groups and the cells were incubated for 24 h.

2.4 | Immunofluorescence assay

Rat chondrocytes from various groups were first washed with PBS and then fixed with 4% paraformaldehyde for 30 min. Subsequently, they were subjected to permeabilization with 0.5% Triton X-100 for 15 min and then blocked for 1 h with 5% BSA (Solaibio, Beijing, China). Next, the chondrocytes were incubated overnight at 4°C with primary antibodies against COL2A1 (ab34712, Abcam, UK), CS (ab11570, Abcam), TRPV1 (ab203103, Abcam), and ATP synthase C (ab181243, Abcam, UK), and then subsequently treated with a Goat anti-rabbit IgG (Alexa Fluor® 488) secondary antibody (Bosterbio, Wuhan, China) at a 1/200 dilution. After staining with DAPI, the samples were examined using a confocal microscope (Leica, Wetzlar, Germany).

2.5 | Intracellular Ca^{2+} measurements

Fluo-4 AM (CAS No.: 273221-67-3, MedChemExpress, NJ, USA) was used to measure intracellular Ca^{2+} concentrations according to the manufacturer's instructions. Rat chondrocytes from various groups were plated in 6-well plates and allowed to grow for 48 h; after which, they were washed three times with PBS and incubated with Fluo-4 AM working solution for 40 min. The cells were then washed again with PBS, harvested by trypsinization, and centrifuged. After resuspending the cell pellet, the cells were re-seeded into 96-well plates for analysis of intracellular calcium levels using a fluorescence spectrophotometer (SpectraMax, San Jose, CA, USA).

2.6 | Macrophage polarization and transfection

Rat bone marrow derived macrophages (rBMDMs) were obtained from the Cell Bank of Type Culture Collection at the Chinese Academy of Sciences (Shanghai, China), and subsequently cultured in DMEM (Gibco, NY, USA) supplemented with 1% penicillin/streptomycin (Gibco) and 10% FBS (Gibco) at 37°C. To induce M1-polarized macrophages, rBMDMs were treated with 100 ng/mL LPS (Sigma-Aldrich). To investigate the involvement of ATP in NLRP3 activation and macrophage polarization, cultured rBMDMs were transfected with a P2X7 overexpression vector (NM_002562.6, CDS-1788 bp, pcDNA3.1), si-P2X7 (5′- AAA GCA AAC GUA GGA AAA GAU -3′), or si-NLRP3 (5′- AAU AGA GAU UCU CGA AAG GUA -3′) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h of transfection, the macrophages were harvested for co-culture with IL-1β-induced rat chondrocytes.

2.7 | Co-culture

Macrophages from the P2X7, si-P2X7, or si-NLRP3 groups were seeded in the top chambers of a Transwell system (aperture = 0.4 μm, Corning, Tewksbury, MA, USA), while IL-1β-induced rat chondrocytes (2.2×10^5 cells per well) with or without quercetin treatment were seeded into the bottom chambers. After 24 h of incubation, the macrophages were collected for further analysis.

2.8 | Macrophage detection by flow cytometry

The activated macrophages derived from rBMDMs were phenotyped via flow cytometry. The cells were stained with Live/Dead Fixability Viability Dye, as well as antibodies against CD68 (a pan macrophage marker, ab283654, Abcam, Cambridge, UK), CD86 (an M1 marker, ab220188, Abcam), and CD163 (an M2 marker, ab182422, Abcam). The rBMDMS-activated M1 macrophages and M2 macrophages were defined as $CD68^+CD86^+$ and $CD68^+CD163^+$, respectively.

2.9 | Transwell assay

Transwell migration and invasion assays were performed using an 8.0-μm pore size insert and a BioCoatTM Matrigel® Invasion Chamber (Corning), respectively. For co-culture experiments, 1×10^5 macrophages were seeded into the upper inserts for the Transwell migration assay and 3×10^5 cells in serum-free medium were seeded for the Transwell invasion assay. After 24 h, the macrophages that migrated

Gene	Forward (5′ - 3′)	Reverse (5′ - 3′)
TRPV1	CTGACGGCAAGGATGACTAC	CAGGGCAAAGTTCTTCCAGT
P2X7	ATTACGGCACCATCAAGTGG	CGCTGATATAGCTTGTCGCT
NLRP3	TGGTGAATTCCGGCCTTACT	TGCAGAAGTCCCTCACAGAG
GAPDH	CCTCGTCTCATAGACAAGATGGT	GGGTAGAGTCATACTGGAACATG

TABLE 1 Primers for quantitative real-time PCR.

or invaded to the bottom surface were fixed with 4% paraformaldehyde, and then stained with 0.1% crystal violet. Finally, the cells were observed using a DMR inverted microscope (Leica Microsystems, Wetzlar, Germany).

2.10 | Apoptosis analysis by flow cytometry

An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) cell apoptosis kit (Invitrogen, Thermo Fisher Scientific, Inc.) was used to determine the apoptotic rates of chondrocytes and macrophages. Briefly, aliquots of cells from different experimental groups were harvested and washed twice with PBS buffer (pH = 7.4). The cells were then suspended in binding buffer and subsequently treated with 5 μL of Annexin V-FITC dissolved in 1× binding buffer for 15 min. Next, 5 μL of PI dissolved in 150 μL of 1× binding buffer was added to the cells, and the mixture was incubated in the dark for 5 min at room temperature. Ultimately, the apoptotic rate was assessed using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

2.11 | Quantitative real-time PCR

Total RNA was extracted from both chondrocytes and macrophages using TRIzol reagent. Next, complementary DNA (cDNA) was synthesized from the mRNA by using a SYBR Premix Ex TaqTM Kit (Takara, Kusatsu, Japan). Quantitative real-time PCR was performed by using 20 μL of SYBR green qPCR Master Mix on a LightCycler 480 II instrument (Roche, Basel, Switzerland). The primer sequences for the target genes are listed in Table 1, and GAPDH served as a reference gene. Relative fold-levels of gene expression were calculated using the comparative threshold cycle (Ct) technique, and specifically the $2^{-\Delta\Delta CT}$ method.

2.12 | In vivo studies

A total of 50 male SD rats (age, 5 weeks; weight range, 220–260 g) were obtained from the Guangdong Medical Experimental Animal Center (Guangzhou, China). The rats were housed in a controlled environment with a 12-h light/dark cycle and were given unrestricted access to both water and food. After anaesthesia with chlora hydrate (30 mg/kg), OA was induced in the rats by using the modified Hulth method, as previously described (Li et al., 2021). Animals in a sham surgery group ($n = 10$) did not have their meniscus resected; however, the skin was incised and then sutured. After a two-week period, the OA rats received a daily intraperitoneal injection of quercetin (100 mg/kg, Sigma-Aldrich) for period of 12 weeks. Rats in the OA + quercetin group were further divided into two subgroups: one subgroup received an intra-articular injection of lentivirus particles (concentration = 1×10^8 TU/mL) overexpressing TRPV1 twice a week for a period of 4 weeks, while the other group was administered lentivirus-control vector particles at the same concentration and schedule. The sham group received intra-articular injections of PBS. Thus, the rats were categorized into five groups: sham, OA, OA + quercetin, OA + quercetin + vector, and OA + quercetin + TRPV1 ($n = 10$ rats per group). At the end of the 4-week period following intra-articular injection of lentivirus particles, the rats were euthanized with a pentobarbital overdose. Joint cavity flush solutions were collected from rats in the different groups for use in ATP and ELISA assays, and samples of articular cartilage were collected for histological and western blot analyses. All animal experiments (including primary cell isolations, and rat model creation, et al.) were conducted in accordance with existing guidelines and approved by the Ethics Committee of Affiliated Guangdong Second Provincial General Hospital of Jinan University (No. TJGC-2021005).

2.13 | ATP assay

Cellular ATP levels were quantified using an Enhanced ATP Assay Kit (Sigma-Aldrich). Rat chondrocytes or joint cavity flush solutions from various experimental groups were lysed in ATP lysis buffer, and then centrifuged at 12,000 rpm for 10 min to collect the supernatant. The supernatant was then mixed with the ATP detection working dilution. The concentration of ATP in each sample was determined by comparing it to an ATP standard curve.

2.14 | Enzyme-linked immunosorbent assay

Supernatant samples from rat chondrocytes, macrophages, and joint cavity flush solutions were collected for analysis. Specific enzyme-linked immunosorbent assay (ELISA) kits (Proteintect, Wuhan, China) were used to assess the levels of specific inflammatory biomarkers, such as TNF-α, IL-10, IL-1β, IL-18, and IL-33.

2.15 | Histological analysis

Articular cartilage tissues obtained from rats in different groups were fixed in 10% paraformaldehyde for 24 h, and then de-calcified in 20%

EDTA for approximately 20 days. After decalcification, the cartilage tissues were dehydrated using a graded ethanol series, embedded in paraffin, and sectioned into 5.0 μm thick slices, as previously described (Tang et al., 2017). The sections were then subjected to staining with hematoxylin and eosin (H&E) and safranin O-fast green, as described in manufacturer's instructions from Sigma-Aldrich. Subsequently, the stained sections were observed and photographed under a light microscope.

2.16 | Western blot analysis

The total proteins were extracted from rat chondrocytes, macrophages, and articular cartilage tissues by lysing the cells or tissues in radioimmunoprecipitation buffer (RIPA) supplemented with protease inhibitors. The protein concentration in each extract was quantified using a BCA Protein Assay Kit (Beyotime). Next, a 30 μg sample of protein from each extract was separated by 10% SDS-PAGE and the protein bands were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% non-fat milk, the membranes were incubated overnight at 4°C with primary antibodies against TRPV1 (ab203103, Abcam), P2X7 (ab93354, Abcam), iNOS (ab178945, Abcam), Arg1 (ab315110, Abcam), NLRP3 (ab263899, Abcam), ASC (ab309497, Abcam), cleaved caspase-1 (ab207802, Abcam), pro-caspase 1 (ab179515, Abcam), and GAPDH (ab8245, Abcam). Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Millipore, Burlington, MA, USA) at room temperature for 2 h. The immunostained protein bands were visualized using an enhanced chemiluminescence kit (Beyotime).

2.17 | Statistical analysis

All statistical analyses and data normalization were carried out using GraphPad Prism 8.0 software (GraphPad Software, Inc.). Quantitative data are expressed as a mean value ± standard deviation (SD) as determined by results from three independent experiments. Student's *t*-test was used for comparisons between two groups and one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test or Tukey's post hoc test was used to analyze statistical differences among multiple independent groups. A p-value <0.05 was considered to indicate statistical significance.

3 | RESULTS

3.1 | Assessment of quercetin's cytotoxic effects on isolated rat chondrocytes

To examine the potential effects of quercetin on OA progression, we collected rat chondrocytes from the knee articular cartilage of SD rats. Immunofluorescence assays showed strong positive staining for chondrogenic differentiation-related genes, namely COL2A1 and CS (Figure 1a), indicating the successful isolation of rat chondrocytes. Next, the chondrocytes were incubated with different concentrations of quercetin (0, 2, 4, 8, 16, 32, and 64 μM). Subsequent CCK-8 assays revealed a dose-dependent reduction in chondrocyte viability following quercetin treatment. The calculated IC50 value for quercetin in chondrocytes was 10.11 μM (Figure 1b). Based on the observation that quercetin concentrations of 2–8 μM were not cytotoxic to chondrocytes, we selected quercetin at 0.3-fold IC50 (3.03 μM) and 0.8-fold IC50 (8 μM) concentrations for further investigation.

3.2 | Quercetin-mediated suppression of calcium ion influx and ATP release in IL-1β-induced rat chondrocytes, with concurrent TRPV1 downregulation

Next, we examined the effects of quercetin on calcium ion influx and ATP release in IL-1β-induced chondrocytes. Rat chondrocytes were initially treated with 10 ng/mL IL-1β for 24 h and then exposed to 3 μM (LD) and 8 μM (HD) quercetin for an additional 24 h. Quantitative real-time PCR (Figure 1c), western blot (Figure 1d and Data S1), and immunofluorescence assays (Figure 1e) showed that quercetin treatment significantly decreased TRPV1 (a calcium ion channel gene) expression in IL-1β-induced rat chondrocytes. Moreover, higher doses of quercetin had more pronounced effects on TRPV1 downregulation. Furthermore, we also evaluated intracellular Ca^{2+} levels by using immunofluorescence staining for Ca^{2+} (Figure 1f) and Fluo-4 AM staining (Figure 1g). Those results showed a clear reduction in cytosolic-free Ca^{2+} levels in IL-1β-induced rat chondrocytes that had been treated with quercetin, with higher doses of quercetin having more pronounced suppressive effects. Similarly, the significant increases in ATP levels (Figure 1h) and immunofluorescence staining of ATP synthase C (Figure 1i) observed after IL-1β stimulation of rat chondrocytes were reversed following treatment with high doses of quercetin. Moreover, quercetin mitigated the increased apoptosis observed in IL-1β-induced rat chondrocytes, with the effects being more evident at higher doses of quercetin (Figure 1j).

3.3 | Activation of TRPV1 reversed the quercetin-mediated effects on calcium ion influx and ATP release in IL-1β-induced rat chondrocytes

To explore the role of TRPV1 in the inhibitory effects of quercetin on calcium ion influx and ATP release in IL-1β-induced rat chondrocytes, we treated IL-1β-induced rat chondrocytes with quercetin, followed by an additional treatment with 10 μM capsaicin (a TRPV1 activator) for 24 h. An evaluation of TRPV1 mRNA and protein expression did not reveal significant differences between the IL-1β + quercetin and IL-1β + quercetin + capsaicin groups, as demonstrated by quantitative real-time PCR (Figure 2a), western blot analysis (Figure 2b), and immunofluorescence assays (Figure 2c). Furthermore, P2X7 expression on chondrocytes was not changed by

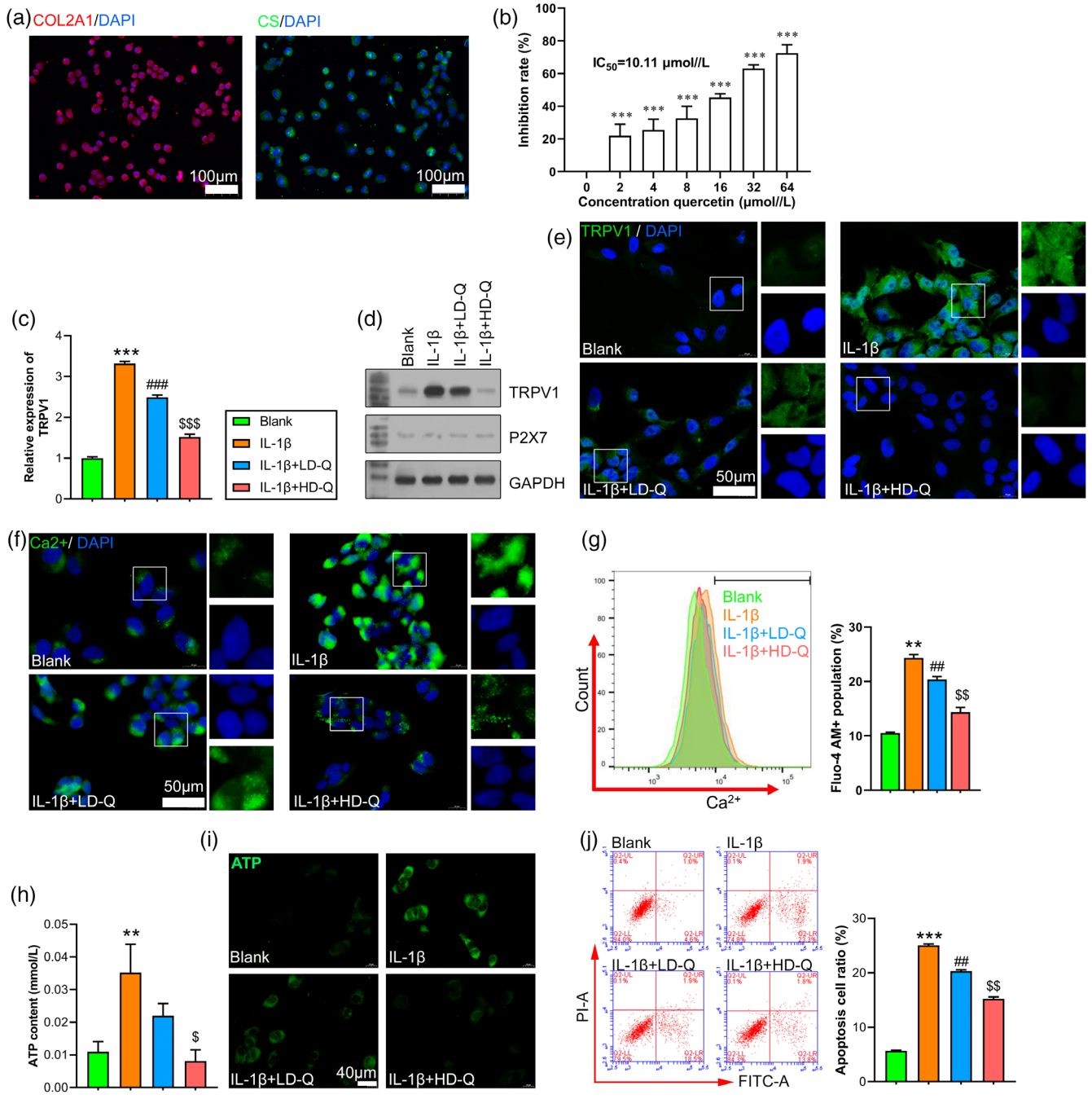


FIGURE 1 Quercetin-mediated suppression of calcium ion influx and ATP release in IL-1β-induced rat chondrocytes, with concurrent TRPV1 downregulation. (a) Immunofluorescence staining of collagen type II (COL2A1) and chondroitin sulfate (CS) markers in the rat chondrocytes. (b) Chondrocytes were treated with quercetin (0, 2, 4, 8, 16, 32, and 64 μM) for 24 h and cell viability was assessed by a CCK-8 assay. The inhibition rate of quercetin in chondrocytes was analyzed by GraphPad Prism 8.0 and the IC_{50} was 10.11 μM. Rat chondrocytes were initially treated with 10 ng/mL IL-1β for 24 h. Subsequently, the cells were exposed to 3 μM (LD) and 8 μM (HD) quercetin (Q) for an additional 24 h. The expression levels of TRPV1 mRNA (c) and protein (d) were determined in rat chondrocytes from different groups. Immunofluorescence staining of TRPV1 (e) and Fluo-4 AM (f) was shown in rat chondrocytes from different groups. (g) The intracellular Ca^{2+} concentration was measured using Fluo-4 AM staining. ATP assay was performed using the enhanced ATP assay kit (h) and immunofluorescence staining of ATP synthase C (i). (j) Cell apoptosis was determined by flow cytometry assay. All data are repeated three times and represented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with blank; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with IL-1β; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, compared with IL-1β + LD-Q. HD-Q, high concentration of quercetin; LD-Q, Low concentration of quercetin.

any of the treatments (Figure 2b and Data S1). However, immunofluorescence staining for Ca^{2+} (Figure 2d) and Fluo-4 AM staining (Figure 2e) indicated that capsaicin reversed the decreases in cytosolic-free Ca^{2+} levels in IL-1β-induced rat chondrocytes induced by quercetin treatment. Moreover, the reductions in ATP levels (Figure 2f) and the presence of apoptotic cells (Figure 2g) observed

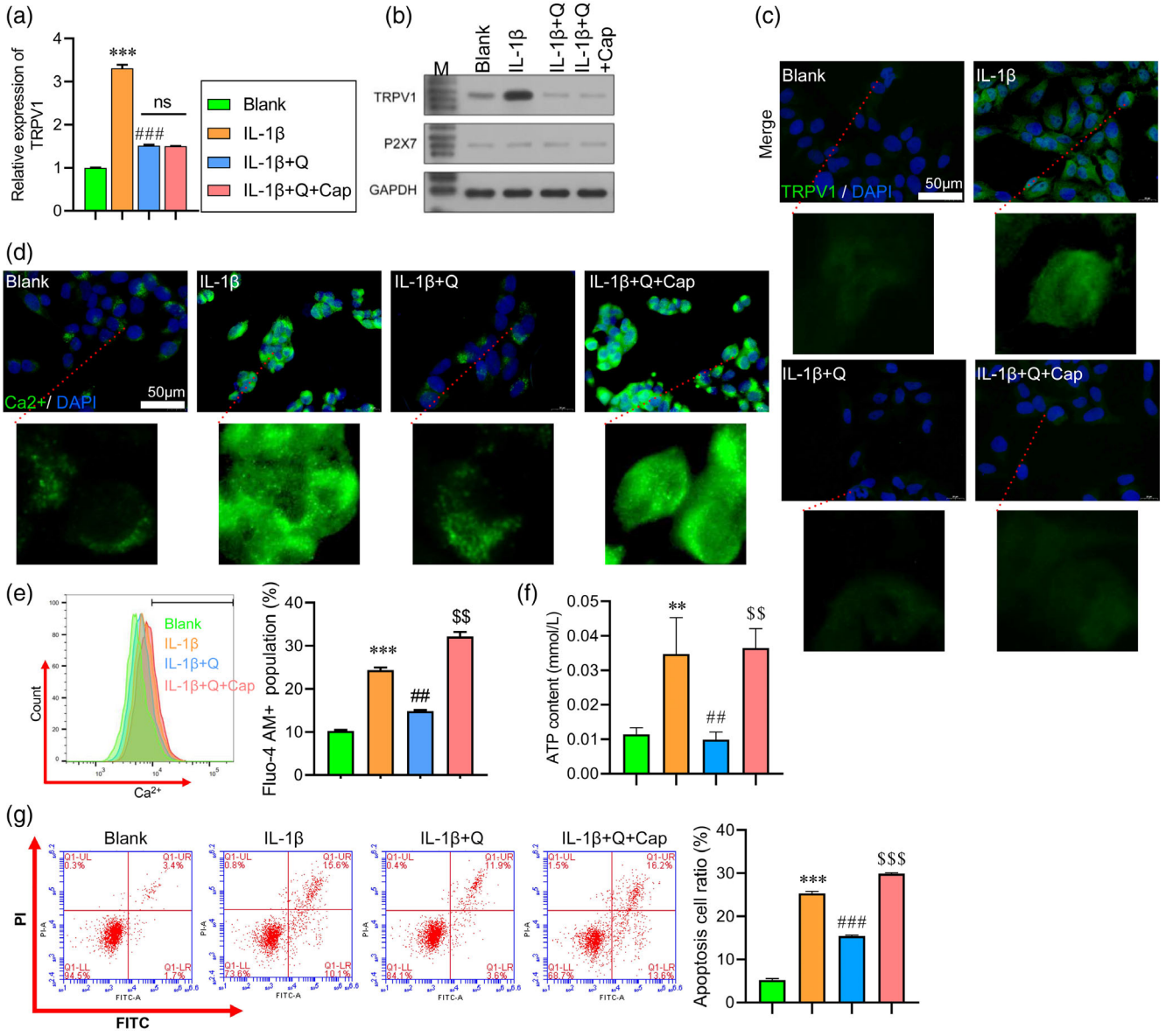


FIGURE 2 Activation of TRPV1 reversed the effects of quercetin on calcium ion influx and ATP release in IL-1β-induced rat chondrocytes. IL-1β-induced rat chondrocytes were treated with low concentration of quercetin (3 μM), and then additionally treated with 10 μM TRPV1 activator, capsaicin, for 24 h. The expression levels of TRPV1 mRNA (a) and protein (b) were determined in rat chondrocytes from different groups. Immunofluorescence staining of TRPV1 (c) and Fluo-4 AM (d) was shown in rat chondrocytes from different groups. (e) The intracellular Ca^{2+} concentration was measured using Fluo-4 AM staining. (f) ATP assay was performed using the enhanced ATP assay kit. (g) Cell apoptosis was determined by flow cytometry assay. All data are repeated three times and represented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with blank; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with IL-1β; $^{\$\$}p < 0.01$, compared with IL-1β + Q. Cap, capsaicin; Q, quercetin.

in IL-1β-induced rat chondrocytes after quercetin treatment were both restored after TRPV1 activation.

3.4 | Overexpression of P2X7 counteracted the quercetin-mediated effects on M1 polarization, migration, and inflammation in macrophages from the co-culture system

To investigate the relationship between the P2X7 receptor and macrophage M1 polarization in the co-culture system, macrophages were transfected with a P2X7 overexpression plasmid and then co-cultured with IL-1β-induced rat chondrocytes, with or without quercetin treatment. We first confirmed the successful overexpression of P2X7 in macrophages by quantitative real-time PCR (Figure 3a) and western blot analysis (Figure 3b and Data S1). As shown in Figure 4c, P2X7 overexpression led to an increase in TNF-α, levels and a decrease in IL-10 levels (Figure 3c). Subsequently, we evaluated the proportions of $CD68^{+}CD86^{+}$ (M1) and $CD68^{+}CD163^{+}$ (M2) macrophages and observed a significant reduction in the IL-1β + quercetin group when compared with the IL-1β group. Interestingly, this reduction was markedly reversed after P2X7 overexpression in macrophages within

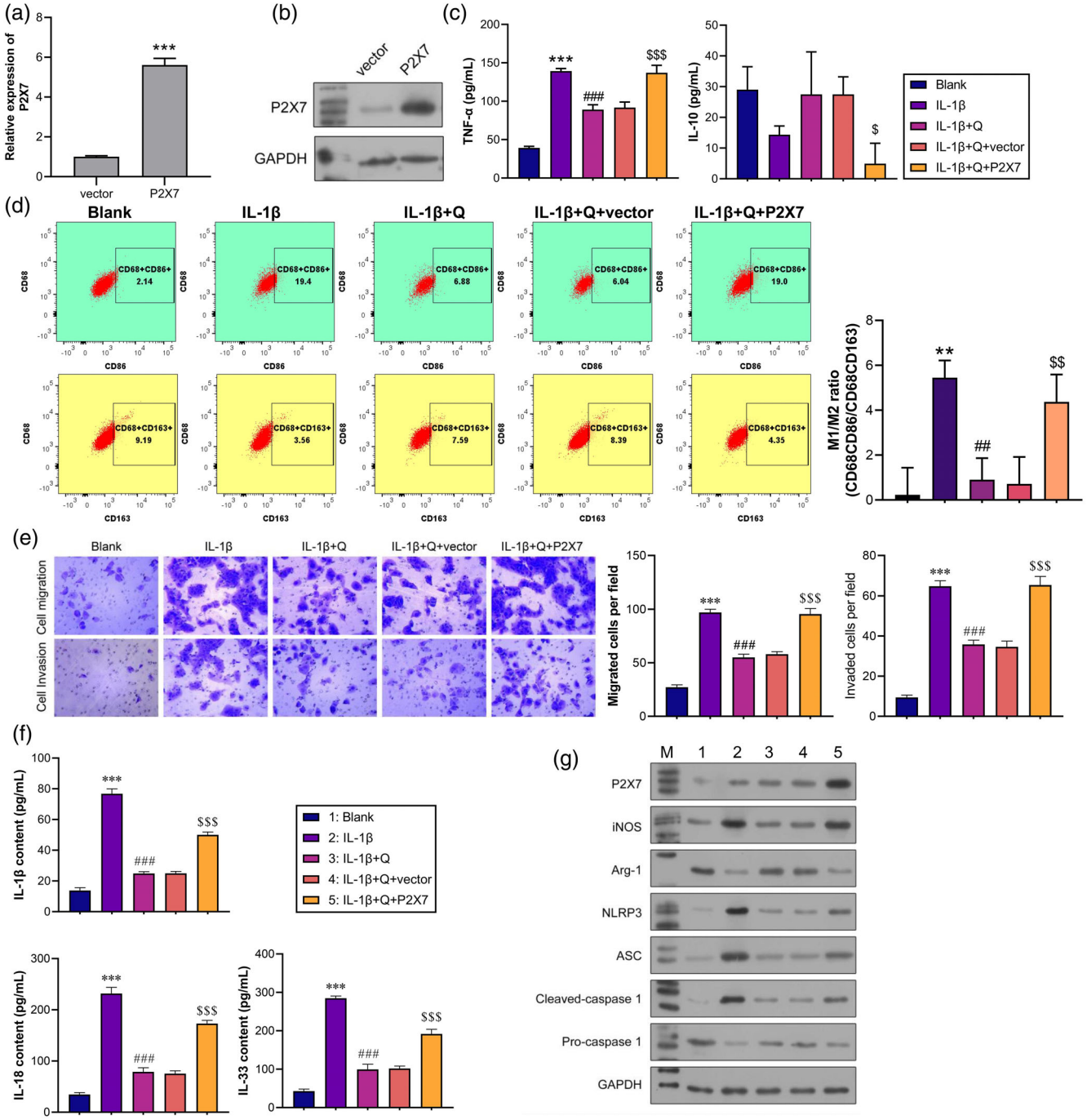


FIGURE 3 Overexpression of P2X7 counteracted the effects of quercetin on M1 polarization, migration, and inflammation in macrophages from the co-culture system. The macrophages were co-cultured with 10 ng/mL IL-1 β or quercetin (3 μM) preprocessed chondrocytes. The expression levels of P2X7 mRNA (a) and protein (b) were determined in macrophages after transfection with P2X7 overexpression plasmid or vector. $^{***}p < 0.001$, compared with vector. (c) ELISA assay was utilized to determine the concentration of TNF-α, and IL-10 in the supernatants of macrophage from the different co-culture systems. (d) Representative scheme of the different macrophage phenotypes according to different distribution for CD68, CD86 and CD163 markers (left panel). Quantitative analysis of CD86 (M1 marker) and CD163 (M2 marker) expression in $CD68^+$ cells percentage among different groups (right panel). (e) Transwell assay was used to analyze cell migration and invasion in macrophages from the different co-culture systems. 200 × magnification. (f) ELISA assay was utilized to determine the concentration of IL-1β, IL-18, and IL-33 in the supernatants of macrophage from the different co-culture systems. (g) The protein levels of P2X7, iNOS (M1 marker), Arg1 (M2 marker), NLRP3, ASC, cleaved caspase 1, and pro-caspase 1 in the macrophages were detected in macrophage from the different co-culture systems. All data are repeated three times and represented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with blank; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with IL-1β; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, compared with IL-1β + vector. Q, quercetin.

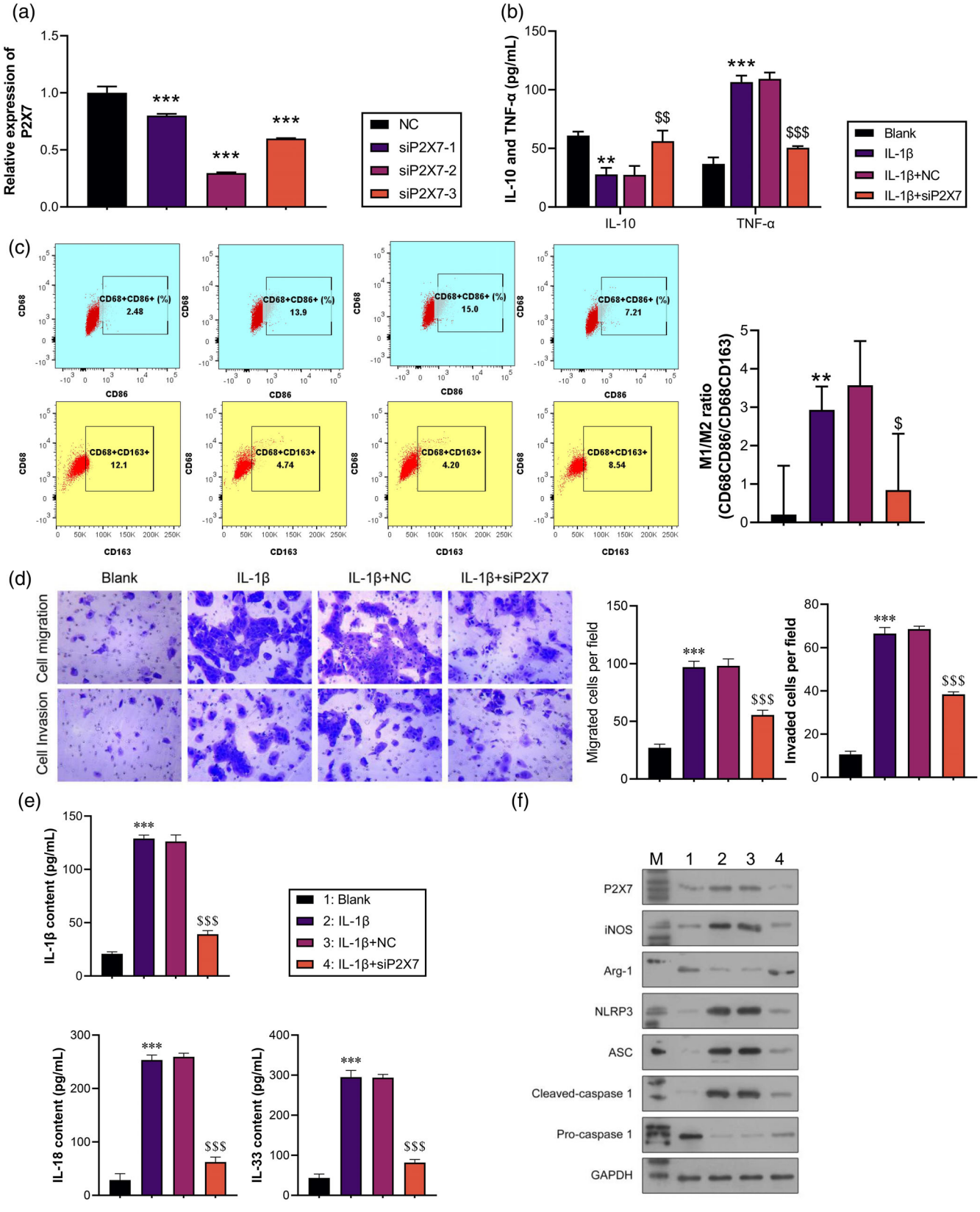


FIGURE 4 Legend on next page.

the co-culture system (Figure 3d). Transwell assays revealed that the decrease in the numbers of migrated and invaded cells induced by quercetin was reversed upon P2X7 overexpression in the macrophages (Figure 3e). ELISA results further demonstrated that P2X7 overexpression counteracted the effects of quercetin on the levels of pro-inflammatory cytokines (IL-1β, IL-18, and IL-33) in the macrophage supernatants from the co-culture system (Figure 3f). Additionally, at the molecular level, P2X7 overexpression attenuated the effects of quercetin on P2X7, iNOS (M1 marker), Arg1 (M2 marker), NLRP3, ASC, cleaved caspase 1, and pro-caspase 1 in the macrophages within the co-culture system (Figure 3g and Data S1).

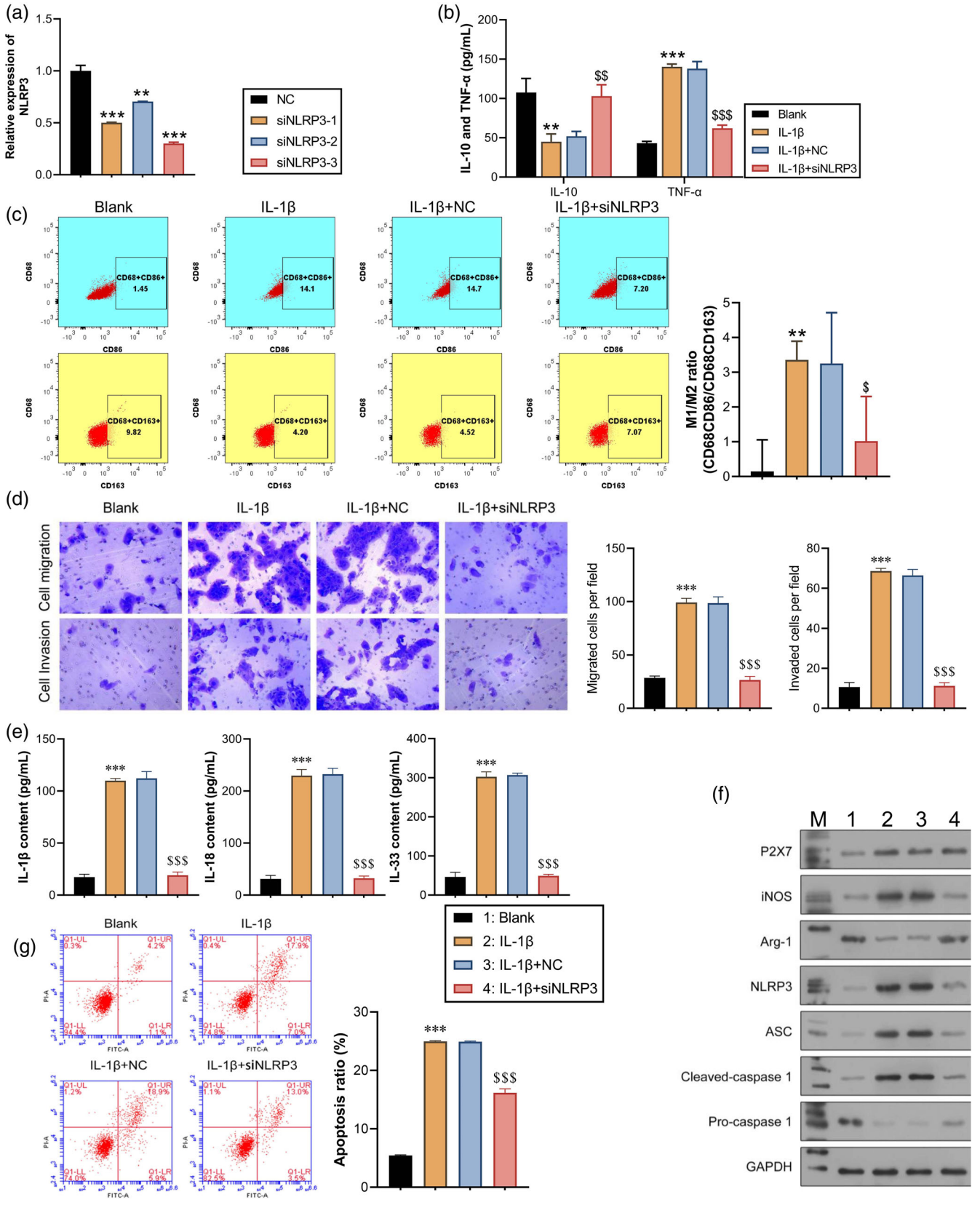
3.5 | Knockdown of P2X7 attenuated IL-1β-induced M1 polarization, migration, and inflammation in macrophages from the co-culture system

We investigated the effects of P2X7 knockdown on M1 polarization, migration, and inflammation in macrophages within the co-culture system. To achieve this, three different siRNAs targeting P2X7 were transfected into the macrophages. Among these siRNAs, si-P2X7-2 demonstrated the most potent suppressive effects on P2X7 expression in macrophages (Figure 4a), prompting its selection for further analysis. ELISA results showed that knockdown of P2X7 suppressed TNF-α expression and increased IL-10 expression (Figure 4b). The distribution of macrophage phenotypes was assessed using specific markers. Notably, knockdown of P2X7 reversed the elevated proportion of $CD68^+CD86^+$ (M1)/$CD68^+CD163^+$ (M2) macrophages in the presence of IL-1β-induced rat chondrocytes within the co-culture system (Figure 4c). Additionally, Transwell assays revealed a significant decrease in the migration and invasion of macrophages following P2X7 knockdown when co-cultured with IL-1β-induced rat chondrocytes (Figure 4d). P2X7 knockdown in the co-culture system resulted in a reversal of the increased levels of pro-inflammatory cytokines (IL-1β, IL-18, and IL-33) observed in macrophages (Figure 4e). Furthermore, P2X7 knockdown mitigated the effects of IL-1β treatment on the expression of P2X7, iNOS (M1 marker), Arg1 (M2 marker), NLRP3, ASC, cleaved caspase-1, and pro-caspase 1 in macrophages from the co-culture system (Figure 4f and Data S1). These findings highlight the role of P2X7 in regulating M1 polarization, migration, and inflammation in the co-culture system with IL-1β-induced rat chondrocytes.

3.6 | Knockdown of NLRP3 inhibited IL-1β-induced M1 polarization migration, and inflammation in macrophages from the co-culture system

Subsequently, loss-of-function assays were carried out in macrophages from the co-culture system to explore the potential correlation between NLRP3 and M1 macrophage polarization. Different siRNAs targeting NLRP3 (siNLRP3-1, siNLRP3-2, and siNLRP3-3) were used to silence NLRP3 expression. Among them, siNLRP3-3 exhibited the strongest suppressive effects on NLRP3 expression and was selected for further analysis (Figure 5a). According to Figure 6b, it can be inferred that NLRP3 knockdown reversed the suppressive effect of IL-1β on IL-10 and the enhancing effect of TNF-α (Figure 5b). Macrophages were transfected with siNLRP3-3 and then co-cultured with IL-1β-induced rat chondrocytes for subsequent experiments. The results showed a significant increase in the proportion of $CD68^+CD86^+$ (M1)/$CD68^+CD163^+$ (M2) macrophages among macrophages co-cultured with IL-1β-induced rat chondrocytes when compared to macrophages co-cultured with blank rat chondrocytes. However, this increase was remarkably reduced after NLRP3 knockdown (Figure 5c). Similar to P2X7 knockdown, NLRP3 knockdown significantly reduced the migration and invasion abilities of macrophages co-cultured with IL-1β-induced rat chondrocytes (Figure 5d). Furthermore, the elevated levels of pro-inflammatory cytokines (IL-1β, IL-18, and IL-33) in macrophages in the co-culture system were also reversed by NLRP3 knockdown (Figure 5e). Flow cytometry analyses indicated that the elevated apoptotic rates of macrophages from the co-culture system were significantly reduced after NLRP3 knockdown (Figure 5f and Data S1). Western blot analyses revealed that NLRP3 knockdown significantly affected the levels of iNOS (M1 marker), Arg1 (M2 marker), NLRP3, ASC, cleaved caspase 1, and pro-caspase 1 protein expression, but did not alter the expression of P2X7 in macrophages co-cultured with IL-1β-induced rat chondrocytes (Figure 5g).

FIGURE 4 Knockdown of P2X7 attenuated IL-1β-induced M1 polarization, migration, and inflammation in macrophages from the co-culture system. The macrophages were co-cultured with 10 ng/mL IL-1 β preprocessed chondrocytes. (a) The expression level of P2X7 mRNA was determined in macrophages after transfection with siP2X7-1, si-P2X7-2 or siP2X7-3, respectively. $^{***}p < 0.001$, compared with NC. Macrophages were transfected with si-P2X7-2 and co-cultured with IL-1β-induced rat chondrocytes. (b) ELISA was used to measure the level of IL-10 and TNF-α. (c) Representative scheme of the different macrophage phenotypes according to different distribution for CD68, CD86 and CD163 markers (left panel). Quantitative analysis of CD86 (M1 marker) and CD163 (M2 marker) expression in $CD68^+$ cells percentage among different groups (right panel). (d) Transwell assay was used to analyze cell migration and invasion in macrophages from the different co-culture systems. 200 × magnification. (e) ELISA assay was utilized to determine the concentration of IL-1β, IL-18, and IL-33 in the supernatants of macrophage from the different co-culture systems. (f) The protein levels of P2X7, iNOS (M1 marker), Arg1 (M2 marker), NLRP3, ASC, cleaved caspase 1, and pro-caspase 1 in the macrophages were detected in macrophage from the different co-culture systems. All data are repeated three times and represented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with blank; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, compared with IL-1β + NC. NC, negative control; Q, quercetin.



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3.7 | Exploring the crossroads of TRPV1 overexpression and quercetin in OA: Impact on in vivo M1 macrophage polarization and therapeutic potential

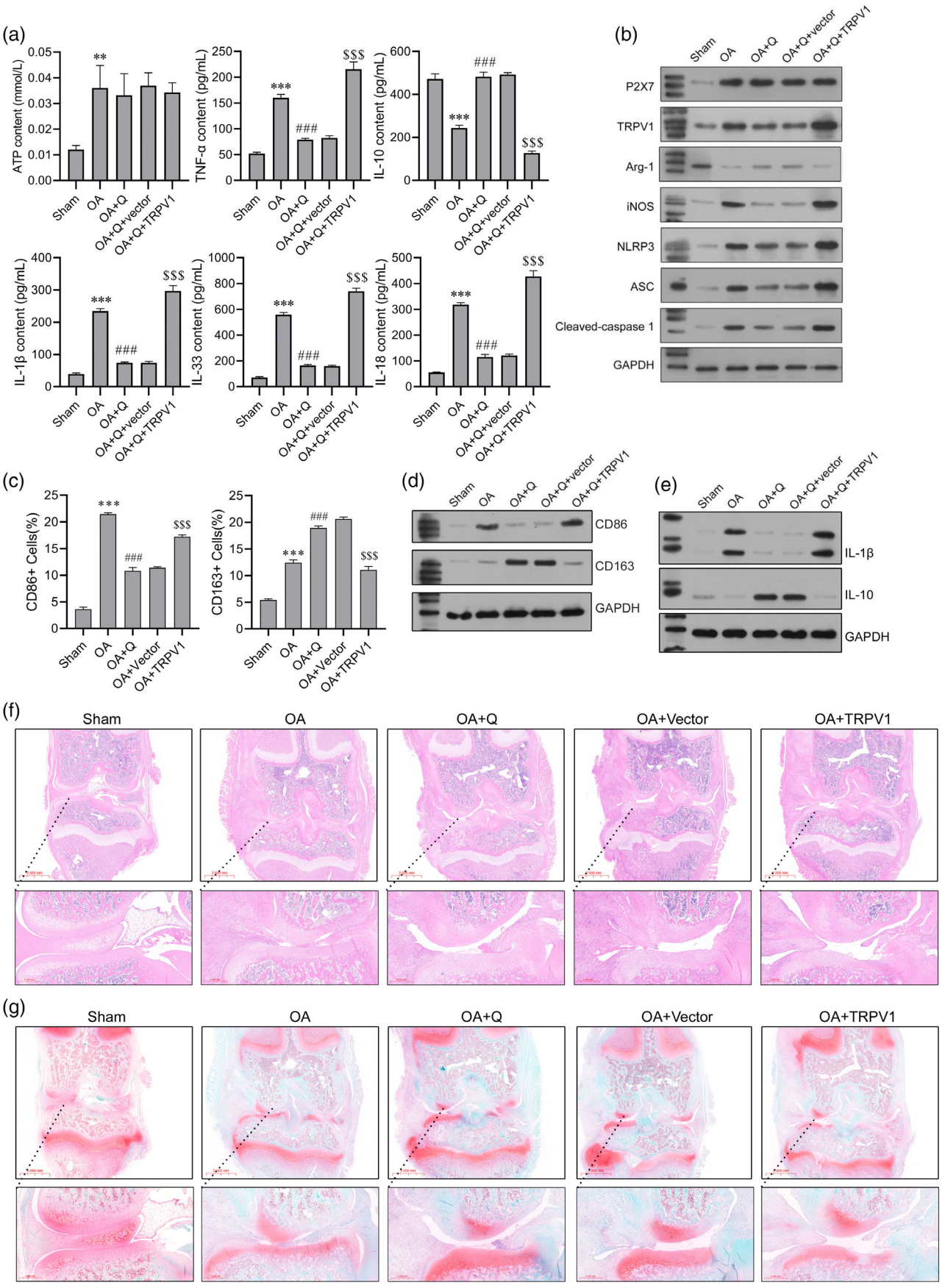
A rat OA model was induced via ACL surgery, and used to investigate the role of TRPV1 in M1 synovial macrophages in OA. Subsequently, the OA rats received intraperitoneal injections of quercetin, followed by intra-articular administration of lentivirus particles overexpressing TRPV1. Interestingly, no significant difference was observed in the regulation of ATP content in joint cavity flush solutions derived from the OA rats following quercetin treatment or TRPV1 overexpression (Figure 6a). However, TRPV1 overexpression reversed quercetin's regulation of anti-inflammatory (IL-10) and pro-inflammatory (TNF-α, IL-1β, IL-18, IL-33) cytokines in joint cavity flush solutions from the OA rats, as evidenced by ELISA results (Figure 6a). Furthermore, western blot analyses revealed that TRPV1 overexpression markedly reversed the effects of quercetin on P2X7, iNOS, Arg1, NLRP3, ASC, cleaved caspase 1, and pro-caspase 1 in articular cartilage tissues derived from the OA rats (Figure 6b and Data S1). To demonstrate the involvement of macrophages in vivo, we washed the joint cavity and subchondral bone of OA rats with PBS to obtain free cells, and then detected the molecular markers (CD86, CD163) of M1 or M2 macrophages by conducting flow cytometry assays. Those results showed that the numbers of CD86 and CD163 positive cells were both significantly increased in the OA group when compared with the Sham group, while quercetin decreased the ratio of $CD86^+$ cells and increased that of $CD163^+$ cells (Figure 6c). Furthermore, overexpression of TRPV1 partially reversed the effect of quercetin on the ratio of CD86 and CD163 positive cells. In addition, we collected the proliferative synovial tissue and detected the molecular markers (CD86, CD163, IL-1β and IL-10) of M1 or M2 macrophages by western blotting. Those results showed that quercetin inhibited CD86 expression and promoted CD163 expression, while overexpression of TRPV1 induced the opposite effects in synovial tissue (Figure 6d and Data S1). Meanwhile, the levels of IL-1β were consistent with those of CD86, and the levels of IL-10 were consistent with those of CD163 (Figure 6e and Data S1). Histopathological assessments were performed on harvested articular cartilage tissues. H&E staining revealed that the articular cartilage tissues in the sham group exhibited a normal meniscus morphology, while those in the OA model group displayed cartilage defects and joint space narrowing (Figure 6f). Safranin O-fast green staining showed that the sham group had abundant cartilage and cartilage matrix. Quercetin treatment mitigated severe cartilage and chondrocyte loss in the model group, but that effect was reversed by TRPV1 overexpression (Figure 6g). Collectively, these findings indicate that intra-articular administration of lentivirus particles resulting in TRPV1 overexpression promotes M1 synovial macrophage polarization and contributes to OA development in vivo.

4 | DISCUSSION

Numerous clinical surveys and extensive empirical evidence strongly support the significant involvement of M1/M2 macrophages in the progression of OA by contributing to the development of osteophytes and synovitis (Guo et al., 2022; Raoof et al., 2021; Woo et al., 2020). Consequently, therapeutic interventions aimed at reducing the abundance of M1 macrophages or facilitating their transition towards the M2 phenotype have emerged as promising strategies for mitigating OA progression. Hu et al. (2019) recently demonstrated that quercetin exerts protective effects against OA by simultaneously inhibiting inflammation, preventing chondrocyte apoptosis, and promoting the polarization of macrophages towards an M2 phenotype. Our study investigated the specific mechanisms underlying quercetin-induced M2 polarization. Remarkably, our observations revealed noteworthy increases in TRPV1 and P2X7 expression that were accompanied by increased Ca^{2+} influx and ATP release in IL-1β-induced rat chondrocytes. Treatment with quercetin effectively reversed those increases in RPV1 and P2X7 expression. These initial findings suggest the potential importance of TRPV1 and P2X7 in the mechanisms by which quercetin protects against OA.

TRPV1, a member of the TRPV channel family (TRPV1-6), is known for its significant role in inflammation (Bujak et al., 2019). A study conducted by Kelly et al. (2015) revealed the involvement of TRPV1 in the polarization of macrophages, specifically M1 macrophages, which correlates with synovitis and OA severity. Additionally, Zhai et al. (2020) highlighted the role of intracellular Ca^{2+} concentrations, modulated by TRPV1 ligand-gated ion channels, in determining cell fate. Sharing a similar structure with TRPV1, TRPV4 is upregulated in OA articular cartilage and induces chondrocyte apoptosis via Ca^{2+}

FIGURE 5 Knockdown of NLRP3 inhibited IL-1β-induced M1 polarization migration, and inflammation in macrophages from the co-culture system. The macrophages were co-cultured with 10 ng/mL IL-1 β preprocessed chondrocytes. (a) The expression level of NLRP3 mRNA was determined in macrophages after transfection with siNLRP3-1, si-NLRP3-2 or siNLRP3-3, respectively. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with NC. Macrophages were transfected with siNLRP3-3 and co-cultured with IL-1β-induced rat chondrocytes. (b) IL-10 and TNF-α was detected by using ELISA methods. (c) Representative scheme of the different macrophage phenotypes according to different distribution for CD68, CD86 and CD163 markers (left panel). Quantitative analysis of CD86 (M1 marker) and CD163 (M2 marker) expression in $CD68^+$ cells percentage among different groups (right panel). (d) Transwell assay was used to analyze cell migration and invasion in macrophages from the different co-culture systems. 200 × magnification. (e) ELISA assay was utilized to determine the concentration of IL-1β, IL-18, and IL-33 in the supernatants of macrophage from the different co-culture systems. (f) The protein levels of P2X7, iNOS (M1 marker), Arg1 (M2 marker), NLRP3, ASC, cleaved caspase 1, and pro-caspase 1 in the macrophages were detected in macrophage from the different co-culture systems. (g) Cell apoptosis was determined by flow cytometry assay. All data are repeated three times and represented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with blank; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, compared with IL-1β + NC. NC, negative control; Q, quercetin.



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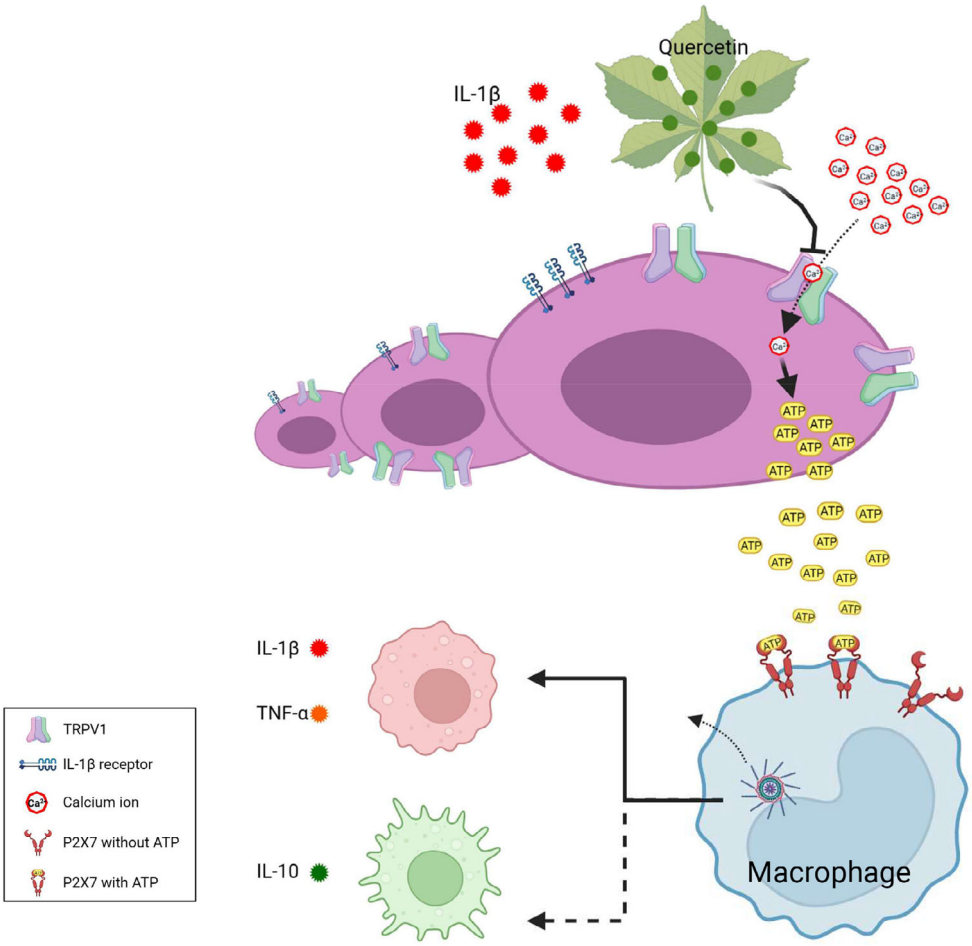

FIGURE 7 Schematic diagram showing the proposed molecular mechanisms of quercetin in alleviating OA by inhibiting M1 synovial macrophage polarization via blocking TRPV1-mediated P2X7/NLRP3 signaling pathway.

influx, leading to increased levels of cleaved caspases (Xu et al., 2019). Wei et al. (2018) suggested that an upregulated level of TRPV5 initiates extrinsic chondrocyte apoptosis via Ca^{2+} influx in OA. Chondrocytes, including those in the TRP family (TRPV1-TRPV6), respond to mechanical loading via their mechanosensitive ion channels and receptors (Barrett-Jolley et al., 2010; Gavenis et al., 2009; Somogyi et al., 2015). In line with these reports, our in vitro study supported those conclusions by showing that TRPV1 activation reversed the effects of quercetin on Ca^{2+} influx, ATP release, and apoptosis in IL-1β-induced rat chondrocytes. Therefore, quercetin's suppression of TRPV1 likely led to decreased rates of Ca^{2+} influx and apoptosis in those chondrocytes. However, another research team found that TRPV1 alleviated osteoarthritis by inhibiting M1 macrophage polarization (Lv et al., 2021) or protecting chondrocytes from ferroptosis (Lv et al., 2022). Those investigators focused on TRPV1 expression in macrophages, while our study investigated TRPV1 expression in chondrocytes, which may be the reason for the inconsistent conclusion. Fortunately, their study and our present study both found that TRPV1 expression was activated in OA. We further showed that overexpression of TRPV1 in a rat OA model negated the suppressive effects of quercetin on cartilage damage, osteophyte formation, synovitis, and M1 synovial macrophage polarization, as evidenced by an increase in the number of iNOS-positive cells. This suggests that quercetin reduces M1 macrophage polarization by downregulating TRPV1. Previous studies reported that quercetin enhanced M2 polarization in both macrophages and microglia (Tsai et al., 2021; Wang et al., 2023).

FIGURE 6 Overexpression of TRPV1 counteracted the attenuating effects of quercetin on OA by promoting M1 macrophage polarization in vivo. Rats were classified into five groups, including sham, OA, OA + quercetin, OA + quercetin + vector and OA + quercetin + TRPV1 group ($n = 10$ each group). (a) ATP assay was performed in joint cavity flush solutions using the enhanced ATP assay kit. ELISA assay was utilized to determine the concentration of TNF-α, IL-10, IL-1β, IL-18, and IL-33 in joint cavity flush solutions. (b) The protein levels of P2X7, iNOS, Arg1, NLRP3, ASC, cleaved caspase 1, and pro-caspase 1 in articular cartilage tissues were measured by western blot analysis. (c) The joint cavity and subchondral bone was washed with PBS to obtain free cells, subsequently, the molecular markers (CD86, CD163) of M1 or M2 macrophages were measured through flow cytometry assay. (d, e) The protein levels of CD86, CD163, IL-β and IL-10 in synovial tissue were measured by western blot analysis. Histological analysis was performed in articular cartilage tissues via hematoxylin and eosin (H&E) (c), safranin O-fast green (d). All data are repeated three times and represented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with sham; $^{\#\#\#}p < 0.001$, compared with OA; $^{\$\$\$}p < 0.001$, compared with OA + vector. Q, quercetin.

Our in vivo data provides new insights into the role of TRPV1 in macrophages, and thereby enhances our comprehension of the therapeutic potential associated with targeting TRPV1 in the context of OA.

We further established a co-culture system consisting of chondrocytes and macrophages to replicate physiological conditions in vivo and explore the impact of TRPV1-mediated Ca^{2+} influx on M1/M2 macrophage polarization under conditions of quercetin stimulation in the context of OA. Our results demonstrated that knockdown of either P2X7 or NLRP3 attenuated IL-1β-induced M1 polarization, migration, and inflammation. Conversely, P2X7 overexpression reversed the suppressive effects of quercetin on macrophages in the co-culture system. These findings suggest that both P2X7 and NLRP3 expression become upregulated in response to TRPV1-mediated Ca^{2+} influx (Yan et al., 2011). The P2X7 receptor, functioning as a non-selective cationic channel, becomes activated in response to elevated ATP levels (Surprenant et al., 1996). However, our results showed that the level of P2X7 expression in in vitro cells did not change, while in vivo, it was increased in the OA group. We speculate that this may be related to the increased infiltration of macrophages into the joints of OA rats. The level of P2X7 expression is closely related to the inflammatory environment (Di Virgilio et al., 2017). Another crucial element, NLRP3, comprises microparticles, ATP, cholesterol, and microbial toxins. It serves as a vital sensor of tissue damage, and thereby initiates sterile inflammation (Zhong et al., 2016). Upon interaction with the adapter protein apoptosis-associated speck-like protein (ASC), NLRP3 facilitates the recruitment of pro-caspase-1, leading to assembly of the NLRP3 inflammasome within the cytosol (Martinon et al., 2009). Ren et al. (2021) demonstrated that co-activation of macrophage-localized P2X7 receptors along with lipopolysaccharide-sensitive toll-like receptor 4 (TLR4) is necessary to initiate NLRP3 formation. This, in turn, activates caspase-1, leading to the degradation of pro-interleukin-1β (pro-IL-1β) and subsequent release of IL-1β. Remarkably, knockdown of P2X7 effectively mitigated the impact of IL-1β treatment on the expression of iNOS (M1 marker), Arg1 (M2 marker), and NLRP3 in macrophages within the co-culture system. Furthermore, activation of the P2X7-NLRP3-IL-1β pathway has also been observed in the hippocampus and is associated with chronic stress and persistent depressive behaviors (Yue et al., 2017). Additionally, involvement of the P2X7/NLRP3/caspase-1 pathways in activating the NLRP3 inflammasome has been linked to neuroinflammation seen in Alzheimer's disease (AD) (Thawkar & Kaur, 2019). Notably, inhibition of P2X7 has demonstrated encouraging results in reducing NLRP3 inflammasome activation and IL-1β production in bone marrow-derived macrophages, suggesting its potential as a promising therapeutic approach for conditions such as acute lung injury (Wang, Xie, et al., 2015). Based on these findings, we concluded that TRPV1 activation triggers an influx of Ca^{2+}, leading to increased ATP release in chondrocytes. The elevated extracellular ATP levels subsequently activate the P2X7 receptor on macrophages, which promotes their recruitment and polarization towards the M1 phenotype, and induces NLRP3 activation. This process plays a crucial role in exacerbating synovial macrophage M1/M2 polarization, and is implicated in the progression of OA.

5 | CONCLUSION

In summary, our study revealed a novel mechanism by which quercetin alleviates OA by inhibiting M1 synovial macrophage polarization. This effect is achieved by blocking the TRPV1-mediated P2X7/NLRP3 signaling pathway (Figure 7). Our findings not only highlight the impact of quercetin on synovial macrophages but also provide evidence for molecular signaling involving TRPV1, P2X7, and NLRP3 between synovial macrophages and chondrocytes during exercise therapy.

AUTHOR CONTRIBUTIONS

Wenjun Li: Conceptualization; data curation; investigation; software; writing – original draft. **Hebei He:** Conceptualization; data curation; methodology; software; writing – original draft. **Min Du:** Data curation; methodology; software. **Mu Gao:** Data curation; methodology. **Qijie Sun:** Data curation; methodology; software. **Yeyang Wang:** Data curation; methodology; software. **Hanyu Lu:** Data curation; methodology; software. **Shuanji Ou:** Data curation; methodology; software. **Changliang Xia:** Data curation; methodology; resources. **Changpeng Xu:** Conceptualization; data curation; investigation; methodology; project administration; resources; software; writing – review and editing. **Qi Zhao:** Conceptualization; data curation; methodology; project administration; resources; software; validation; writing – review and editing. **Hongtao Sun:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; writing – review and editing.

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DATA AVAILABILITY STATEMENT

All data are available from the published article.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interest regarding this research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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