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RESEARCH

The protective role of curcumin in human dental pulp stem cells stimulated by lipopolysaccharide via inhibiting NF-κB p65 phosphorylation to suppress NLRP3 inflammasome activation

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Abstract

Objectives This study aims to investigate the anti-inflammatory effect of curcumin and underlying mechanisms regarding the modulation of the nod-like receptor pyrin domain containing 3 (NLRP3) inflammasome in human dental pulp stem cells (hDPSCs).

Materials and methods The impact of curcumin on the viability of hDPSCs was evaluated. The effect of curcumin on the expression of IL-1β and NLRP3 in hDPSCs stimulated by lipopolysaccharide (LPS) was assessed. Then, LPS-primed hDPSCs were pre-treated with curcumin before ATP triggering NLRP3 inflammasome activation, and NLRP3 inflammasome-related mediators were assessed. The mechanism of curcumin inactivation of LPS plus ATP-induced inflammasome associated with NF-κB pathway was explored. The NF-κB pathway related pro-inflammatory mediators at mRNA and protein levels were evaluated. The expression of NF-κB p65 and phosphorylation p65 was visualized after curcumin or NF-κB inhibitor administrating respectively in hDPSCs with an activated NLRP3 inflammasome. Statistical analysis was performed.

Results While curcumin at the concentration of 0.5–5 μM showed no obvious impact on the viability of hDPSCs, it significantly decreased IL-1β and NLRP3 mRNA expression in LPS-induced hDPSCs in a dose-dependent manner. Curcumin significantly inhibited the LPS plus ATP-primed NLRP3 inflammasome activation in hDPSCs (NLRP3, ASC, caspase-1, and IL-1β). Curcumin evidently attenuated the LPS plus ATP-induced expression of NF-κB pathway-related pro-inflammatory mediators (IL-6, IL-8, TNF-α, and COX-2). Furthermore, curcumin effectively reduced p65 phosphorylation, which acts as an NF-κB inhibitor in hDPSCs with an activated NLRP3 inflammasome.

Conclusions Curcumin pre-treatment may exert an anti-inflammatory role via inactivation of the NLRP3 inflammasome by inhibiting NF-κB p65 phosphorylation in cultured hDPSCs.

Clinical relevance Curcumin may have therapeutic potential in pulp inflammation.

Keywords Curcumin · Human dental pulp stem cells · NLRP3 inflammasome · NF-κB · Pulp inflammation

Introduction

Preserving pulp vitality and its lifelong function are the main goals of any dental treatment. The vital pulp can reduce root fracture and improve the long-term survival rate of teeth [1, 2]. However, achievement of successful outcomes with vital pulp preservation, especially direct pulp capping to intervene in inflamed pulp due to caries, is unpredictable, with a success rate ranging from 31.8 to 91.3% [3, 4]. One of the main reasons the success rate fluctuates is that the degree and scope of pulp inflammation are hard to estimate. Existing inflammation in dental pulp tissue may lead to a degenerative pulp reaction or

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pulp necrosis, leading to vital pulp preservation failure [5]. Currently, there are no available clinical chairside diagnostic techniques and quantitative indicators for reliably calculating the degree of pulp inflammation [6]. In addition, the commercially available pulp capping agents lack obvious anti-inflammatory properties to favor a perfect predictable prognosis of direct pulp capping treatment for inflamed pulp. Thus, it is necessary to upgrade the treatment methods for the preservation of inflamed pulp. It is anticipated that more targeted molecules or drugs can be developed to decrease pulp inflammation and repair inflamed pulp.

Curcumin is a natural polyphenol that possess various health benefits including anti-inflammatory, anti-oxidant, anti-bacterial, anti-cancer, and promoting wound healing and regeneration properties [7, 8]. Curcumin, also a natural pigment, is widely used in food and as a food additive approved by the World Health Organization and the Food and Drug Administration of the USA [9]. Curcumin has been widely used as a folk medicine to eliminate infections and inflammatory conditions associated with periodontal diseases [10, 11]. Curcumin inhibits the degradation and collagenase-mediated breakdown of extracellular matrix molecule in periodontitis by downregulating matrix metalloproteinase-9 [12]. It has also been demonstrated to inactivate the nuclear transcription factor-κappa B (NF-κB) pathway and reduce the output of inflammatory cytokines, preserving periodontal tissue from damage in the treatment of gingivitis and periodontitis [13]. Recently, it was reported that phytosomal curcumin might affect the stemness of mesenchymal stem cells derived from dental pulps via up-regulating the expression of DSPP (dentin sialophosphoprotein) and VEGF (vascular endothelial growth factor) genes involved in pulp regeneration or repair [14]. Phytosomal curcumin enhances the immunoregulatory properties of human dental pulp stem cells (hDPSCs) by altering immunoregulatory-related miRNAs and CD200 expression [15]. Meanwhile, curcumin-loaded liposome decreases the secretion of the proinflammatory cytokines and restores homeostasis in hDPSCs stimulated by free resin monomer-2-hydroxyethyl methacrylate [16]. Moreover, curcumin also raises the alkaline phosphatase (ALP) activity and gene expression level to induce early osteogenic differentiation in hDPSCs [17]. The poly-ε-caprolactone/gelatin scaffold containing curcumin has been reported as a potential reparative endodontic material to have antimicrobial, antioxidant, and anti-inflammatory roles besides the induction of mineralization in hDPSCs [18]. These previous findings suggest that curcumin may have therapeutic potential in dental pulp regeneration. However, the anti-inflammatory effects of curcumin alone on inflamed pulp are still not fully understood; more investigation is necessary for its potential use to modulate pulp inflammation and promote pulp repair.

Lipopolysaccharide (LPS), a fundamental by-product of gram-negative bacteria, is known as a potent inducer of pulp inflammation [19, 20] and is also widely used for establishing in vitro pulpitis models [21]. We have previously compared the differences between *Escherichia coli* (*E.coli*) and *Porphyromonas gingivalis* LPS in the capacity to provoke inflammation in hDPSCs and demonstrated that *E. coli* LPS was a more stable and potent inducer to stimulate dental pulp stem cells inflammation [22], which is in line with previous studies [23, 24]. Accordingly, *E. coli* LPS is used as a stimulus to establish in vitro pulpitis model in this study.

The nod-like receptor pyrin domain containing 3 (NLRP3) inflammasome comprises the receptor protein NLRP3, the adaptor protein ASC, and pro-caspase-1 [25]. Upon stimulation, the NLRP3 inflammasome is activated and causes pro-caspase-1 aggregation, leading to automatic cleavage of pro-caspase-1 into the mature caspase-1. The caspase-1 protein eventually cleaves the precursor form of IL-1β (pro-IL-1β) into the active form IL-1β, mediating an inflammatory response [25, 26]. NLRP3 inflammasome has recently been suggested to play an essential role in dental pulp immune defense [27]. It was reported that the NLRP3 inflammasome components are differentially expressed in pulpitis with distinct severities [28]. The protein level of NLRP3 inflammasome-related molecules including NLRP3, caspase-1, and IL-1β in irreversible pulpitis tissues was remarkably higher than that in reversible pulpitis and normal pulp tissues, which suggests that activation of the NLRP3 inflammasome could be regarded as one of the indicators of pulp inflammation aggravation [28]. Given the proinflammatory activity of the NLRP3 inflammasome, agents designed to modulate its activation may block pulpitis progression.

Curcumin potently suppresses NLRP3 inflammasome activation in the murine macrophages triggered by LPS combined with multiple NLRP3 inflammasome activators, such as nigericin, monosodium urate crystal (MSU), and adenosine triphosphate (ATP), thus providing the potential to treat septic shock in mice [29]. Curcumin directly inhibits NLRP3 inflammasome activation in LPS-primed macrophages, attenuating IL-1β secretion and preventing high-fat-diet–induced insulin resistance and MSU–induced peritoneal inflammation in mice [30]. However, the influence of curcumin involves in regulating NLRP3 inflammasome-mediated pulp inflammation induced by LPS plus ATP has never been indicated. hDPSCs, a type of mesenchymal stem cell with self-renewal, multidirectional differentiation, and immunomodulatory functions [31], may play an essential role in pulp repair and regeneration under conditions of pulpitis [32]. Therefore, we explored the role of curcumin and its underlying mechanisms in hDPSCs.

The NF-κB pathway is a typical target for impeding pulp inflammation [33]. It has been demonstrated that NF-κB activation is involved in LPS-induced up-regulation of

NLRP3 and IL-1β in human dental pulp cells [34]. Additionally, canonical NLRP3 inflammasome activation in macrophages is induced by priming with microbial components such as LPS through the NF-kB pathway [35]. Therefore, we hypothesized that curcumin could also exert an anti-inflammatory effect via inactivation of the NLRP3 inflammasome mediated by inhibiting the NF-κB signaling pathway in hDPSCs. In this study, we set out to investigate whether curcumin pre-treatment could play an anti-inflammatory role in pulpitis provoked by LPS or plus ATP stimulation and the underlying mechanisms involved in the inhibition of NLRP3 inflammasome activation in inflamed hDPSCs.

Materials and methods

Preparation of a curcumin solution

Curcumin powder (≥ 99.5%; Supelco, USA) was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 50 mmol/L. The solution was aliquoted into Eppendorf tubes and stored at −20 °C for later use.

Cell culture

hDPSCs were isolated from pulps of freshly discarded non-carious teeth ($n = 6$), cultured in α-modified Eagle medium (α-MEM; HyClone, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% streptomycin/penicillin, and incubated in a 5% CO_2 atmosphere at 37 °C, as previously described [36]. hDPSCs were identified and characterized as described in our previous study [22]. The use of human dental tissues was approved by the Ethics Committee, and informed written consent was obtained from all participants in the study.

Cytotoxicity test

The effect of curcumin on cell viability was detected using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan). Cells at passage 3 were cultured in 96-well plates (10^4 cells per well) for 24 h and then treated with curcumin at the final concentration of 0.5, 1, 5, and 10 μM, respectively, and continually cultured for 24 h or 48 h. After treatment, the culture medium was removed, and 110 μl detection solution (serum-free culture medium: CCK-8 reagent = 10:1) was added to each well. The plates were further incubated in the dark for 2 h; then, the absorbance at 450 nm was read by a microplate reader.

qRT-PCR analysis

hDPSCs at passage 3 were seeded in six-well plates at a density of 10^5 cells per well and cultured until 90% confluent. The cells were pretreated with curcumin at various concentrations for 2 h before the addition of *E. coli* LPS (1 μg/ml, *E. coli* LPS 055: B5, Sigma, Japan) and cultured for another 3 h. To investigate whether the NLRP3 signaling pathway was involved, the NLRP3 inflammasome activator ATP (5 mM, MedChemExpress, USA) was added for the last 30 min to induce NLRP3 inflammasome activation. Cells without stimulation with LPS or ATP were used as controls. Total RNA was extracted from each culture using TRIzol reagent (Invitrogen, USA), and the isolated total RNA (1 μg per sample) was used to synthesize cDNA using a PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa Bioengineering Company, Japan). The target cDNA fragment was amplified by quantitative PCR on LightCycler® 480 real-time PCR instruments (Roche, Germany). The reaction conditions were as follows: 95 °C, 30 s; 95 °C, 5 s; and 60 °C, 30 s, for 40 cycles. The primers used in the experiment are listed in Table 1. GAPDH was used as an internal reference. The relative target gene expression level in each group was normalized to that of GAPHD and evaluated by the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

hDPSCs at passage 3 were seeded in six-well plates at a density of 10^5 cells per well and cultured until 90% confluent. The cells were pre-treated with curcumin for 2 h before 1 μg/mL *E. coli* LPS stimulation for an additional 24 h; then, 5 mM ATP was added for the last 30 min. The cell

Table 1 Primers used for real-time quantitative PCR analysis

Gene	Sequence (5′–3′)
IL-1β	5′-TGCACGATGCACCTGTACGA-3′ 5′-AGGCCCAAGGCCACAGGTAT-3′
TNF-α	5′-CAGAGGGAAGAGTTCCCCAG-3′ 5′-CCTCAGCTTGAGGGTTTGCTAC-3′
IL-6	5′-GTGAGGAACAAGCCAGAGC-3′ 5′-TACATTTGCCGAAGAGCC-3′
IL-8	5′-TTTTGCCAAGGAGTGCTAAAGA-3′ 5′-AACCCTCTGCACCCAGTTTTC-3′
COX-2	5′-CTGGCGCTCAGCCATACAG-3′ 5′-ACACTCATACATACACCTCGGT-3′
NLRP3	5′-TGGCTGTAACATTCGGAGATTGTGG-3′ 5′-GCTTCTGGTTGCTGCTGAGGAC-3′
Caspase-1	5′-GGTGCTGAACAAGGAAGAGATGGAG-3′ 5′-TGCCTGAGGAGCTGCTGAGAG-3′
ASC	5′-GCTGCTGGATGCTCTGTA-3′ 5′-GGCTGGTGTGAAACTGAAG-3′
GAPDH	5′-ATGGGGAAGGTGAAGGTCG-3′ 5′-GGGGTCATTGATGGCAACAATA-3′

supernatants were collected from each group and stored at −80 °C until tested. Total protein was extracted from the cells using RIPA lysis buffer containing protease inhibitors. A BCA protein assay kit was used to determine the protein concentration. Protein lysates were then separated by 10% SDS–PAGE and electrophoretically transferred to polyvinylidene fluoride membranes. After blocked with 5% nonfat milk for 2 h at room temperature, the membranes were incubated, respectively, with anti-NLRP3, anti-caspase-1(1:1000 dilution in TBST, Cell Signaling Technology, USA), anti-IL-1β (1:500 dilution in TBST, Aibotech Biology, China), and anti-GAPDH antibodies on a shaker at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated anti-rat IgG or anti-mouse IgG secondary antibodies for 1 h at room temperature. The protein bands were visualized with a chemiluminescence imaging system using a Western blotting detection kit (ECL). Band intensities were normalized using β-GAPDH band from the same sample by densitometric analysis with ImageJ software.

Cytometric bead array

The concentrations of pro-inflammatory cytokines (IL-6 and IL-8) in the collected cell supernatants were determined using the cytometric bead array (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, IL-6 and IL8 beads were incubated with the cell supernatants and PE detection reagent at room temperature for 3 h. Then, the wash buffer was added to wash the beads three times. The beads were resuspended in buffer and detected on BD Accuri C6 flow cytometer. The data was analyzed by FCAP software.

Immunofluorescence staining of NF-κB p65 and phosphorylated p65 (p-p65)

hDPSCs at passage 3 were cultured on glass slips in 24-well plates at the density of 5×10^4 per well until 80% confluent. The culture medium was replaced with an FBS-free medium for overnight starvation. The cells were pre-treated with curcumin (1 or 5 μM) or the NF-κB inhibitor Bay 11-7082 (5 μM, Sigma, Japan) for 2 h before *E. coli* LPS (1 μg/ml) stimulation for another 3 h, and 5 mM ATP was added for the last 30 min. After treatment, the hDPSCs were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 30 min, and blocked with 1% BSA and 10% goat serum for 1 h. Then, the cells were incubated with monoclonal rabbit primary antibodies against human p65 (1:400, Cell Signaling Technology, USA) or human p-p65 (1:200, Cell Signaling Technology, USA) overnight at 4 °C. After rinsing with PBS repeatedly, a secondary antibody (goat anti-rabbit IgG, green fluorescence) was added and incubated for 2 h at room temperature. 4′,6-Diamidino-2-phenylindole (DAPI) and rhodamine rubipeptide were added, respectively, to stain the nuclei (blue) or cytoskeleton (red). The p65 and p-p65 staining was visualized using an inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics 26.0 with at least three biological replicates. The normality and homogeneity of variance for each data point were identified, and the data are represented as the mean ± standard error. All experimental data were determined by the Shapiro–Wilk test to be normally distributed. One-way analysis of variance (ANOVA) was performed followed by the post hoc Tukey test (variance homogeneity) or Dunnett's T3 tests (variance heterogeneity) to identify significant differences in the expression of mediators among the groups. Statistical significance was set at $p < 0.05$ (*).

Results

Effect of curcumin on the viability of cultured hDPSCs

The impacts of different concentrations of curcumin on the viability of cultured hDPSCs are shown in Fig. 1. The culture medium without addition of curcumin or containing 0.02% DMSO was used as blank or negative controls, respectively. After incubation for 24 h or 48 h, there were no apparent differences between the viability of hDPSCs treated with curcumin (0.5, 1, or 5 μM) and that of controls. However, cells exposed to curcumin at a high concentration of 10 μM showed evident cytotoxicity compared to the control ones ($p < 0.05$).

Curcumin decreased IL-1β and NLRP3 mRNA expression in LPS-stimulated hDPSCs

The effects of curcumin on IL-1β and NLRP3 mRNA expression in hDPSCs stimulated with LPS were determined by qRT-PCR. Cells treated with only LPS were used as the positive control and without LPS and curcumin as the negative control. LPS led to a significant increase in the mRNA expression of IL-1β ($p < 0.01$) and NLRP3 ($p < 0.05$). Curcumin at concentrations of 1 and 5 μM significantly reduced the mRNA expression of IL-1β ($p < 0.001$). The mRNA expression of NLRP3 was also obviously decreased after 5 μM curcumin treatment ($p < 0.01$). Interestingly, the inhibitory effects on IL-1β and NLRP3 expression were dose-dependent (Fig. 2).

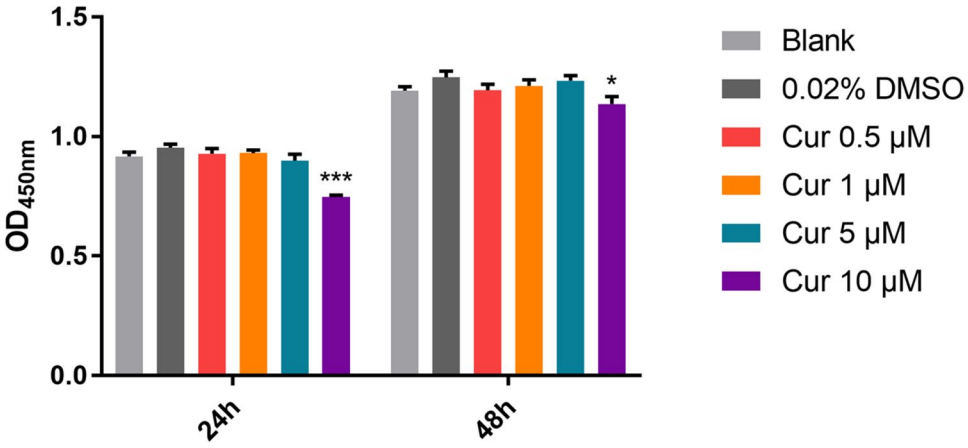

Fig. 1 Impacts of curcumin on the viability of cultured hDPSCs by the CCK-8 assay. The data are represented as the mean ± SEM ($n = 4$/each). *$p < 0.05$ and ***$p < 0.001$

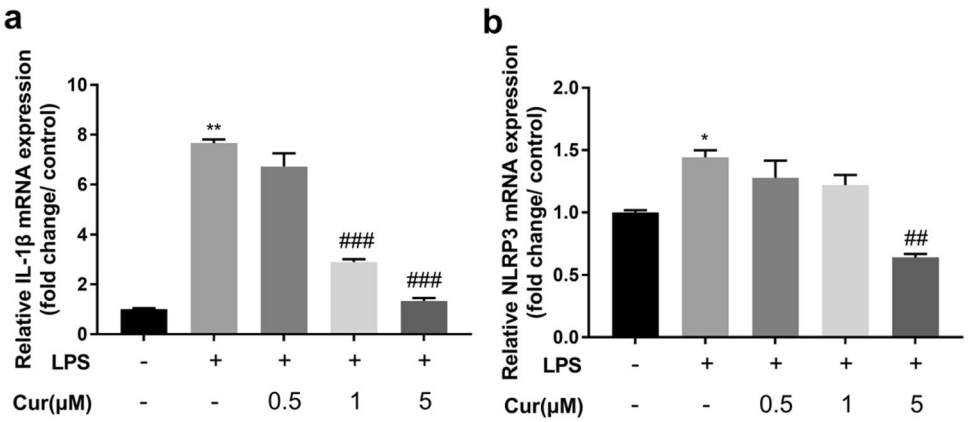

Fig. 2 Curcumin reduced the LPS-induced mRNA expression of IL-1β and NLRP3. **a** IL-1β mRNA. **b** NLRP3 mRNA. The data are presented as the mean ± SEM ($n = 3$/each). *$p < 0.05$ and **$p < 0.01$ vs. the negative control group, ##$p < 0.01$ and ###$p < 0.001$ vs. the LPS-only group

Curcumin inhibited NLRP3 inflammasome activation in LPS plus ATP-stimulated hDPSCs

To investigate the inhibitory effect of curcumin on the activation of the NLRP3 inflammasome in hDPSCs, we pretreated LPS-primed hDPSCs with curcumin followed by the addition of the NLRP3 inflammasome activator ATP. We first examined whether LPS plus ATP could activate the NLRP3 inflammasome in primary cultured hDPSCs. Our results revealed that LPS plus ATP successfully activated the NLRP3 inflammasome. The cells in the LPS plus ATP-stimulated group exhibited significantly increased mRNA expression of NLRP3, adaptor apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1, and IL-1β (Fig. 3a–d). The relative mRNA levels of these genes were higher in the LPS plus ATP treatment group than in the LPS group. Moreover, LPS treatment alone did not significantly increase the mRNA expression of ASC (Fig. 3b). ATP treatment alone did not modulate NLRP3 inflammasome component expression in hDPSCs (Fig. 3). In the curcumin treatment groups, we detected that curcumin at the concentration of 1 or 5 μM significantly downregulated the mRNA levels of NLRP3 inflammasome pathway-related mediators including NLRP3, ASC, Caspase-1, and IL-1β in hDPSCs stimulated with LPS plus ATP in a dose-dependent manner by qRT -PCR (Fig. 3a–d). These observations were further confirmed by Western blotting analysis: the combination of LPS and ATP significantly provoked higher protein levels of NLPR3, caspase-1, and IL-1β in hDPSCs. Curcumin at 1 or 5 μM considerably reduced the protein expression of NLRP3, Caspase-1, and IL-1β in hDPSCs (Fig. 3e–g).

Curcumin inhibited NF-κB-dependent inflammatory mediators in hDPSCs stimulated with LPS plus ATP

We next explored the underlying mechanism by which curcumin attenuated the activation of the NLRP3 inflammasome in hDPSCs, and whether curcumin suppressed NLRP3 inflammasome activation associated with NF-κB pathway. The expression of proinflammatory mediators associated with NF-κB pathway and pulp inflammation in NLRP3 agonist (LPS combined with ATP)-activated hDPSCs after curcumin treatment were examined by qRT-PCR and CBA. We found LPS combined with ATP significantly upregulated the expression of NF-κB pathway-related inflammatory mediator genes at mRNA level including IL-6, IL-8, COX-2, and TNF-α (Fig. 4a–d) and protein level including IL-6 and IL-8 (Fig. 4e–f). Nevertheless, administration of 1 or 5 μM

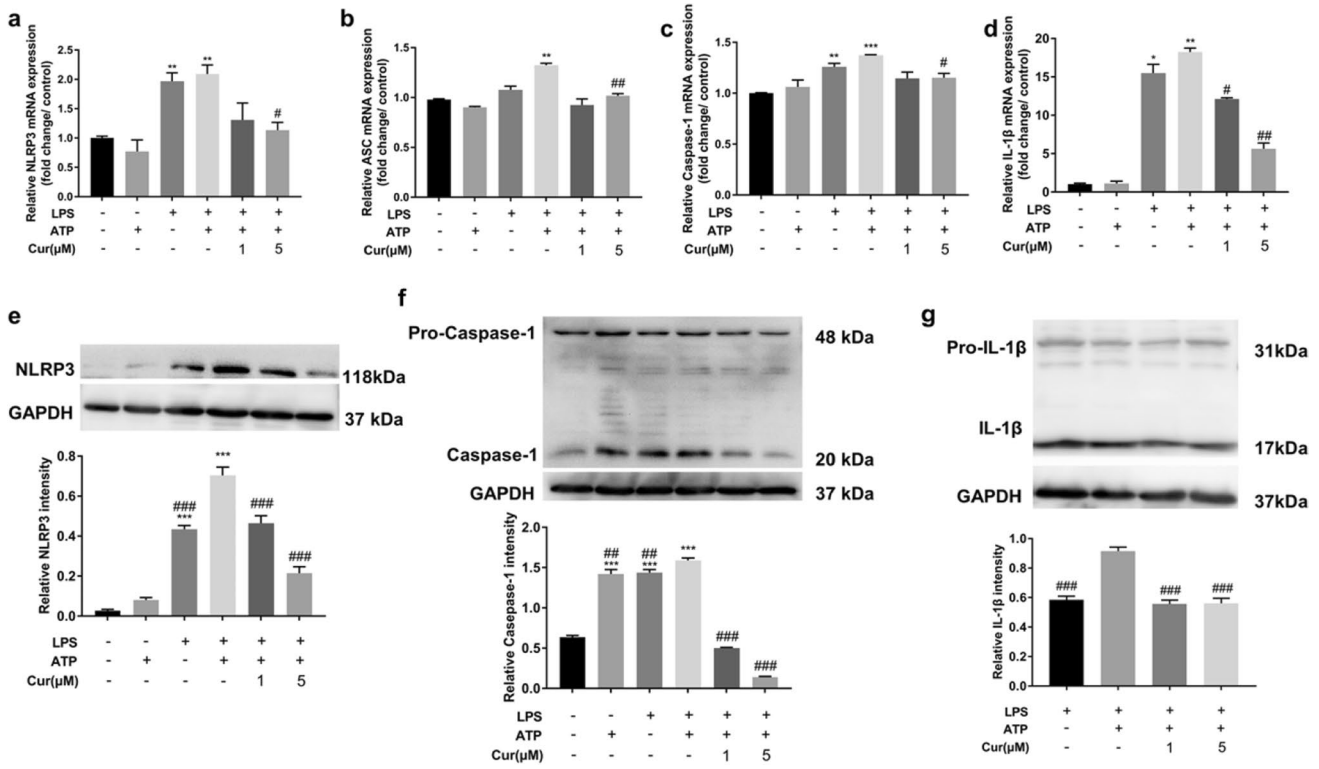

Fig. 3 Curcumin inhibited NLRP3 inflammasome activation in hDPSCs. The mRNA expression of NLRP3 inflammasome pathway-related mediators: **a** NLRP3mRNA, **b** ASC mRNA, **c** caspase-1 mRNA, and **d** IL-1β mRNA. The protein expression of NLRP3, caspase-1, and IL-1β by western blotting analysis (**e**–**g**). The right panel shows the quantitative measurements of relative intensity/GAPDH of NLRP3 (**e**), Caspase-1 (**f**) and IL-1β (**g**) using the ImageJ software. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. without LPS or curcumin-stimulated group; #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ vs. the LPS plus ATP-stimulated group

curcumin had a significant suppressive effect on the expression of proinflammatory cytokines (Fig. 4). These results indicate curcumin participants in the modulation of NF-κB pathway in NLRP3 agonist-activated hDPSCs.

Curcumin inhibited NF-κB p65 phosphorylation in hDPSCs stimulated with LPS plus ATP

To further investigate whether curcumin inhibited the activation of the NLRP3 inflammasome by regulating the NF-κB pathway in hDPSCs, LPS-primed hDPSCs were pre-treated with or without curcumin or NF-κB inhibitor Bay 11-7082 for 2 h, and the NLRP3 agonist ATP was added for the last 30 min. As shown in Fig. 5a, immunofluorescence staining showed that LPS plus ATP obviously increased the nuclear distribution of p65 in hDPSCs. Curcumin at concentration of both 1 and 5 μM evidently reduced the nuclear translocation of p65 in NLRP3 agonist (LPS plus ATP)-activated hDPSCs (Fig. 5a), which was consistent with the addition of the NF-κB pathway inhibitor Bay11-7082. The P-p65 immunofluorescence assay also confirmed that curcumin acting as Bay11-7082 attenuated the abundance of phosphorylated (P)-p65 in LPS plus ATP-stimulated hDPSCs (Fig. 5b). Furthermore, we examined the protein expression of NF-κB P-p65 in cell cultures by Western blot. Curcumin treatment effectively downregulated the expression level of NF-κB P-p65 in hDPSCs stimulated with LPS plus ATP (Fig. 5c). Taken together, the results suggest that curcumin may inhibit the NF-κB p65 phosphorylation (at least partly) to inactivate the NLRP3 inflammasome in hDPSCs.

Discussion

In this study, we first screened cytotoxicity of curcumin at a gradient concentration in cultured hDPSCs and found that curcumin at the concentration of 0.5, 1, and 5 μM exhibits no impact on hDPSCs viability. In agreement with a previous study in which curcumin has a dose-dependent effect on the viability of adult neural stem/progenitor cells [37], our results also showed that curcumin at the concentration of 1–5 μM did not affect the viability of cultured hDPSCs, whereas curcumin at the concentration of 10 μM inhibited the proliferation of hDPSCs. These results pave the way for subsequent experiments. On the other hand, it could also be inferred that curcumin cytotoxicity did not mediate the

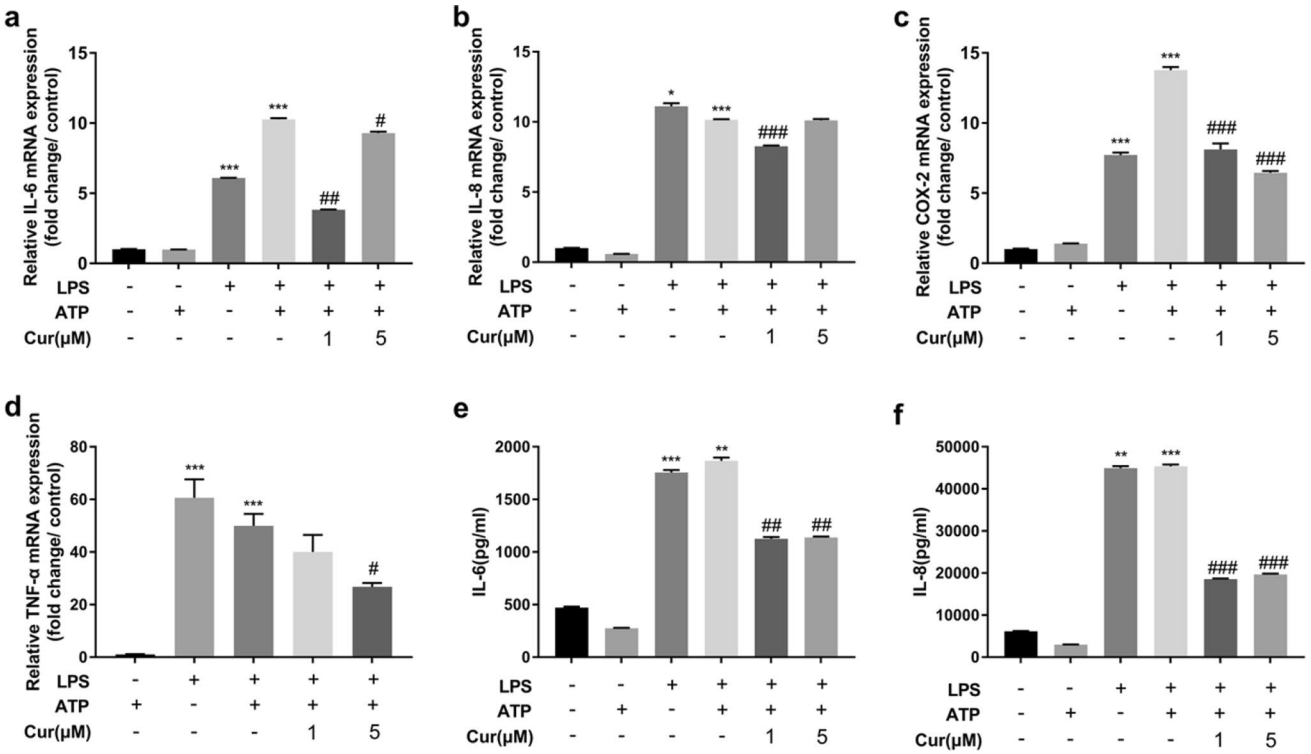

Fig. 4 Curcumin inhibited NF-κB-dependent inflammatory cytokines in hDPSCs stimulated with LPS plus ATP. At mRNA level: **a** IL-6, **b** IL-8, **c** COX-2, **d** TNF-α; at protein level: **e** IL-6 and (f) IL-8. The data are presented as the mean ± SEM ($n = 3$/each). ***$p < 0.001$ vs. without LPS or curcumin-stimulated group; #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ vs. the LPS plus ATP-stimulated group

inhibitory effect of curcumin on the expression of inflammatory mediators in hDPSCs.

LPS, a virulence factor of gram-negative bacteria released during bacterial proliferation, can provoke dental pulp inflammation, leading to dental pulp or periapical diseases [38]. LPS at a concentration of 1 μg/ml induced evident inflammatory mediator upregulation and allowed the construction of a pulpal inflammation model in vitro [21]. Consistent with previous study, an in vitro inflamed environment was constructed by stimulating hDPSCs with1 μg/ml LPS in our study. Our results showed that curcumin pre-treatment at the concentration of 1 and 5 μM had an anti-inflammatory effect on LPS-stimulated inflamed hDPSCs, acting as an inhibitor of NLRP3 inflammasome activation in hDPSCs by inhibiting the phosphorylation of the NF-κB subunit p65.

The inflammasome is composed of various proteins that participate in the host immune responses to infection and cell injury [39]. The NLRP3 protein was reported to be strongly positively expressed in dental pulp cells in an inflammatory environment, while positive staining was not apparent in healthy pulp cells [27]. The NLRP3 mRNA level was consistently upregulated in hDPSCs stimulated with LPS. Our results indicated that curcumin reduced NLPR3 expression in hDPSCs in a dose-dependent manner. Interestingly, curcumin also inhibited IL-1β expression in a dose-dependent manner and 5 μM curcumin most markedly inhibited the mRNA expression of NLRP3 and IL-1β. Consequently, we hypothesized that curcumin inhibits IL-1β expression by participating in governing the NLRP3 inflammasome in hDPSCs.

Next, we investigated whether curcumin exerts anti-inflammatory effects by suppressing the NLRP3 inflammasome activation in hDPSCs. It is well-established that the robust activation of NLRP3 inflammasome is activated undergoing a two-step signal process in macrophages: priming and activating [29]. The first signal (priming) is provided by microbial components such as TLR ligands or endogenous cytokines and leads to the upregulation of NLRP3 and pro-interleukin-1 beta (IL-1β) through the activation of NF-κB. Subsequently, the second signals lead to several molecular and cellular events such as potassium efflux, mitochondrial dysfunction, or lysosome rupture to trigger the robust activation of NLRP3 inflammasome, resulting in proteolytic cleavage of caspase-1 and the maturation of IL-1β [35]. In our study, LPS was used as the first signal to prime hDPSCs. Consistent with previous studies, the mRNA expression levels of NLRP3 inflammasome components including NLRP3, pro-IL-1β, and pro-caspase-1 were upregulated [35, 40]. ATP, a damage-related molecular pattern derived from autogenous cells undergoing death or stress,

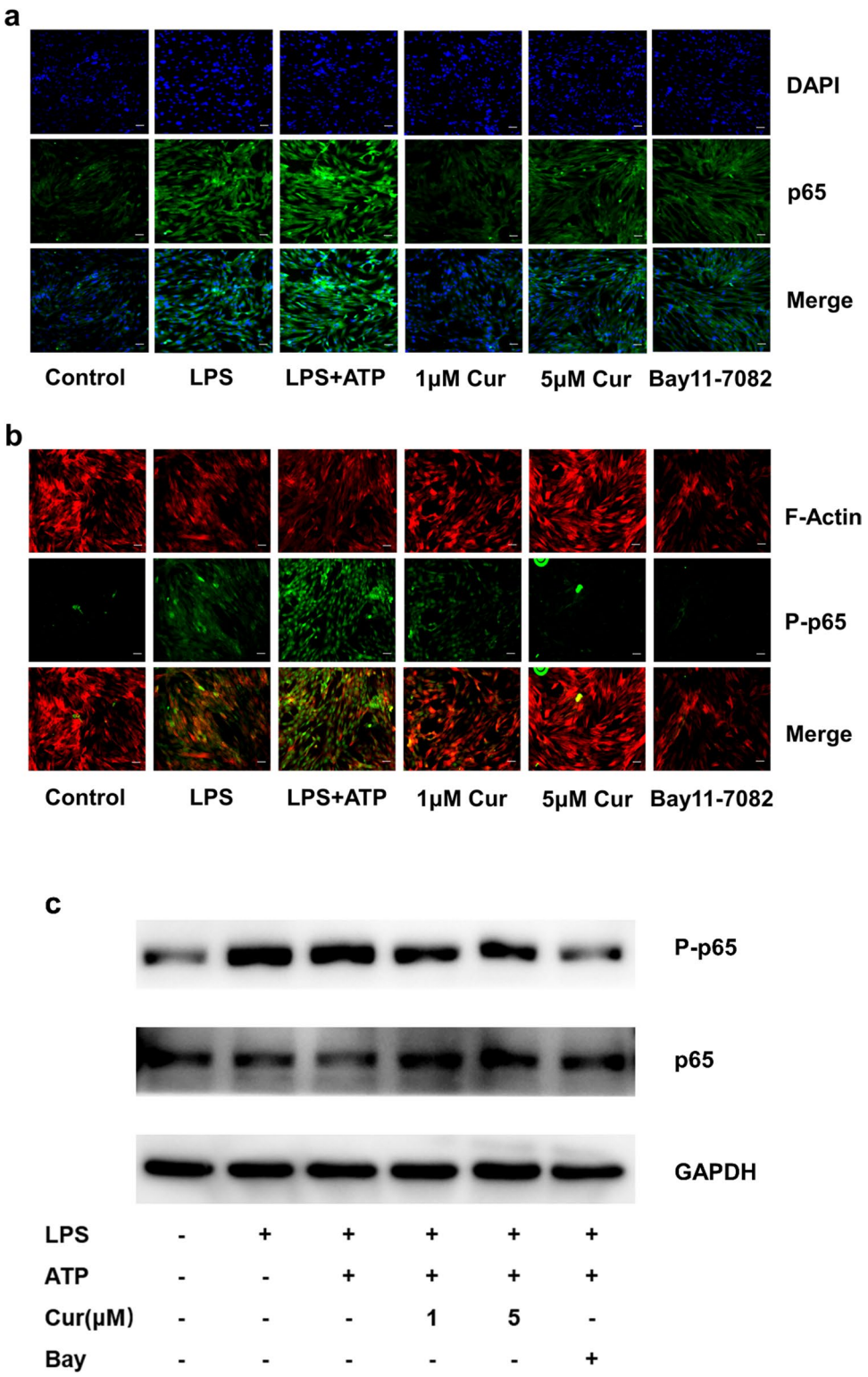

Fig. 5 Curcumin inhibited NF-κB p65 phosphorylation in hDPSCs stimulated with LPS plus ATP. The nuclear translocation of NF-κB p65 by immunofluorescence staining: Nuclei were stained blue, cytoskeletal proteins (F-actin) red, and p65 (**a**) and P-p65 (**b**) green. Scale bar = 50μm. **c** The nuclear translocation of NF-κB p65 was confirmed by western blot

is often used as a second signal to assemble and activate the NLRP3 inflammasome, which promotes the production of caspase-1, causing the cleavage of pro-IL-1β to generate mature IL-1β [41, 42]. Our findings were in line with a previous report [34], in which LPS combined with extracellular ATP (5 mM) successfully activated the NLRP3 inflammasome pathway in hDPSCs. The expression of NLRP3, caspase-1, and IL-1β was substantially increased in LPS plus ATP-treated hDPSCs; the expression levels were higher than those in the LPS alone group. In addition, ASC, one of

the NLRP3 inflammasome components, was significantly increased only in the LPS plus ATP group ($p < 0.001$), confirming that dual signaling is required to completely activate the NLRP3 inflammasome in hDPSCs.

The toll-like receptors (TLRs)/NF-κB pathway appears to be involved in the activation of the NLRP3 inflammasome in LPS-primed human dental pulp fibroblasts. An NLRP3 inflammasome expression reduction was observed when LPS-primed hDPCs were pre-treated with these pathway inhibitors [34]. Since curcumin can exert anti-inflammatory effect via NF-κB in other diseases model, and hence we focused on the impact of curcumin on LPS-induced priming for NLRP3 inflammasome activation in hDPSCs. Therefore, hDPSCs were pre-treated with curcumin for 2 h, then primed with LPS, and finally activated by ATP. Our findings showed that curcumin pretreatment (1 or 5 μM) significantly decreased the expression levels of NLRP3 inflammasome components including NLRP3, ASC, and caspase-1 and the terminal effector IL-1β in hDPSCs stimulated with LPS plus ATP. Previous studies have delved into the inhibitory effect of curcumin on the activation of the NLRP3 inflammasome in inflammatory diseases. Gong et al. found that curcumin dramatically decreased the production of IL-1β in macrophages triggered with various NLRP3 inflammasome activators and reduced tissue damage to improve survival in a mouse model of septic shock [29]. Curcumin was also reported to alleviate colitis in model mice via inactivation of the NLRP3 inflammasome [43]. Together, these results suggest that curcumin inhibits NLRP3 inflammasome activation in hDPSCs and may be a prominent pulp capping agent for pulp inflammation therapy.

hDPSCs play an essential role in pulp immunity by pattern recognition receptors (PPR), such as TLRs, recognizing and binding pathogen-related pattern molecules, such as LPS. Signal transduction via TLRs mediated by different adaptor proteins can promote downstream signaling via NF-κB pathway leading to the expression of downstream inflammatory mediators [44, 45]. The inflammatory response mediated by the NF-κB pathway is involved in dental pulp immunity, and the downstream pro-inflammatory mediators of this pathway, such as IL-6, IL-8, TNF-α, and COX-2, are upregulated in pulpitis [46]. In agreement with previous study, our results showed that the expression of IL-6, IL-8, TNF-α, and COX-2 was significantly upregulated in hDPSCs stimulated with LPS plus ATP and that curcumin effectively inhibited LPS plus ATP-induced proinflammatory cytokine gene expression and protein production in hDPSCs. It has been demonstrated that the NF-κB pathway is the first signaling pathway involved in NLRP3 inflammasome activation in macrophages [47]. Based on our findings, we conclude that the activation of the NLRP3 inflammasome in hDPSCs also involves NF-κB pathway, and the inhibitory effect of curcumin on NLRP3 inflammasome activation in hDPSCs is related to NF-kB. Furthermore, an NF-κB inhibitor (Bay 11-7082) markedly attenuated the upregulation of p65 phosphorylation in hDPSCs with activation of the NLRP3 inflammasome. Similarly, curcumin (1 or 5 μM) also exerted an inhibitory effect on p65 nuclear translocation as an NF-κB inhibitor. Similar regulatory mechanisms have been confirmed in macrophages. Yin et al. found that curcumin inhibited the activation of the NLRP3 inflammasome by inhibiting the NF-κB signaling pathway in macrophages [30]. Therefore, it is reasonable to speculate that curcumin may regulate NLRP3 inflammasome activation at least partly by inhibiting the phosphorylation of NF-κB p65 in hDPSCs.

In the present study, an inflammatory model of dental pulp stem cells was constructed using LPS to detect the anti-inflammatory activity of curcumin and its related mechanisms. Our findings exhibit a novel linkage between curcumin's protective effect and NF-κB/NLRP3 inflammasome mediated-inflammatory signaling pathway in hDPSCs, which expand the understanding of curcumin's anti-inflammatory activities and outline potential molecular mechanisms that explain curcumin potential protective pulpal effects. The present study provides a beneficial experimental basis for using curcumin as a potential drug used in repairing inflamed pulp. Nevertheless, curcumin has poor solubility, high metabolic rate, and low bioavailability, which greatly restricts its potential health function. Besides, the local application of curcumin is more effective than its oral administration in promoting wound healing [48]. Therefore, additional studies about a suitable vehicle for local use of curcumin via animal models of pulpitis are essential in the future.

Conclusions

Curcumin pre-treatment may exert an anti-inflammatory role through impeding the phosphorylation of NF-κB p65 to inhibit NLRP3 inflammasome activation in cultured hDPSCs, suggesting the potential of curcumin for inflamed pulp repairing or healing in vital pulp therapy.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions Chunhua Lan: investigation, data processing, and manuscript writing. Yueming Qian: methodology, project administration. Yumin Wang: reviewed and edited the manuscript. Yuemin Chen: investigation, resources. Chensheng Lin: investigation and resources. Yanding Zhang: technical support. Xiaojing Huang: conceptualization, funding acquisition, and reviewed and edited the manuscript.

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Declarations

Ethics approval The use of human dental tissues was approved by the Ethics Committee of Fujian Medical University (approval No. 201652), and informed written consents were obtained from all participants.

Competing interests The authors declare no competing interests.

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