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RESEARCH

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Effect of curcumin on the expression of NOD2 receptor and pro-inflammatory cytokines in fibroblast-like synoviocytes (FLSs) of rheumatoid arthritis (RA) patients

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Abstract

Background Previous studies has shown that nucleotide-binding and oligomerization domain-containing protein 2 (NOD2) is expressed in Fibroblast-like synoviocytes (FLSs) of rheumatoid arthritis (RA) patients which is stimulated by muramyl dipeptide (MDP) present in the joint environment and induces inflammation via the NF-κB pathway. Also, other studies have shown that curcumin inhibits proliferation, migration, invasion, and Inflammation and on the other hand increases the apoptosis of RA FLSs. In this study, we aim to evaluate the effect of curcumin, a natural anti-inflammatory micronutrient, on the expression of NOD2 and inflammatory cytokines.

Methods Synovial membranes were collected from ten patients diagnosed with RA and ten individuals with traumatic injuries scheduled for knee surgery. The FLSs were isolated and treated with 40 μM curcumin alone or in combination with 20.3 μM MDP for 24 h. mRNA was extracted, and real-time PCR was performed to quantitatively measure gene expression levels of *NOD2*, *p65*, *IL-6*, *TNF-α,* and *IL-1β*.

Results The study findings indicate that administering MDP alone can significantly increase the mRNA expression levels of *IL-6* and *IL-1β* in the trauma group and *TNF-α* in the RA group. Conversely, administering curcumin alone or in combination whit MDP can significantly reduce mRNA expression levels of *P65* and *IL-6* in FLSs of both groups. Moreover, in FLSs of RA patients, a single curcumin treatment leads to a significant reduction in *NOD2* gene expression.

Conclusion This study provides preliminary in vitro evidence of the potential benefits of curcumin as a nutritional supplement for RA patients. Despite the limitations of the study being an investigation of the FLSs of RA patients, the results demonstrate that curcumin has an anti-inflammatory effect on *NOD2* and NF-κB genes. These findings suggest that curcumin could be a promising approach to relieve symptoms of RA.

Keywords Curcumin, Fibroblast like synoviocytes, Inflammation, NOD2, Rheumatoid arthritis, Autoimmune disease

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is characterized by chronic inflammation of the synovium and destruction of bone and cartilage [1]. Although the exact cause of this disease is unknown, It has been revealed that various environmental and genetic factors, including the immune response stimulator, contribute to its development [2]. In RA disease, the innate immune system is activated, leading to the production of pro-inflammatory cytokines at high levels [3]. Fibroblast-like synoviocytes (FLSs) play a fundamental role in cartilage destruction, inflammation, and autoimmunity in the joint environment [4]. Inflammatory conditions and cell destruction in the joints can trigger the production and release of endogenous molecules, such as ATP, which can activate nucleotide-binding oligomerization domain-containing protein 2 (NOD2)-like receptors (NLRs) and result in recurrent inflammation in RA [5]. In addition, an association has been established between infectious organisms, such as Proteus and cytomegalovirus, and RA [6, 7], although the exact mechanism behind this relationship is not yet clearly understood. Molecular mimicry has been suggested as a possible mechanism involved in this association. NOD2 is connected with both infectious inflammation and sterile inflammation induced by endogenous molecules in autoimmune diseases. This protein stimulates the inflammatory response through the nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [8] and is expressed in limited cells, including hematopoietic cells, epithelial cells, Paneth cells, and FLSs [9]. In individuals with RA, the activation of the NF-κB pathway results in the development of inflammation by inducing the production of various pro-inflammatory mediators like interleukin (IL)-1β, IL-2, IL-6, IL-8, tumor necrosis factor (TNF)-α, chemokines, adhesion molecules, and matrix metalloproteinases (MMPs) [10]. p65 (also named RelA) is one of five members of the NFKB family which is involved in inflammatory responses. In the joints of patients with RA, FLSs produce an excessive amount of IL-6, which exacerbates synovitis by inducing neovascularization, inflammatory cell infiltration, and synovial cell hyperplasia [4, 11, 12]. Curcumin, a natural polyphenol and active ingredient in turmeric, has been shown to possess anti-inflammatory and anti-oxidant properties in previous studies [13–15]. It can prevent NOD2 oligomerization and inhibit NOD2 signaling-induced inflammation [16]. In the present study, we investigated the inhibitory effects of curcumin on the expression of NOD2 receptors and pro-inflammatory cytokines in isolated FLSs from both RA patients and non-arthritic individuals.

Material and methods

Patients and control subjects

The study included ten patients with RA who were scheduled to undergo knee replacement surgery and ten non-arthritic individuals who had experienced traumatic injuries and were scheduled for arthroscopy at Shariati Hospital in Tehran, Iran between 2018 and 2019. Both study groups were matched in terms of gender and age(Six women and four men, the mean age of the groups was 54 ± 10). All participants provided written consent, and the Human Research Ethics Committee of Tehran University of Medical Sciences approved the study (Ethic code: IR.TUMS.SPH.REC.1398.106). Diagnosis of RA disease was confirmed by a rheumatologist using the 2010 American College of Rheumatology (ACR) criteria [17]. Smokers in the RA group and individuals with a history of other autoimmune, rheumatologic, and cancer diseases, or who were taking glucocorticoid medications in the trauma group were excluded from the study.

Cell culture

The synovial membranes were washed sequentially with 1X phosphate-buffered saline (PBS; Gibco Invitrogen, USA), 70% ethanol, and 1X PBS solution containing 2% penicillin/streptomycin and 2% amphotericin B (Sigma-Aldrich, USA). The synovium fragments were cut into very small pieces in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, USA) with 1 mg/mL of type VIII collagenase (Sigma-Aldrich, USA) and incubated at 37 °C for 80 min. After centrifuging at 1000 g for 10 min, the resulting pellet was cultured in T25 flasks with DMEM containing 1% penicillin and streptomycin and 10% heat-inactivated fetal bovine serum (FBS; Gibco, BRL, USA).

FLS Characterization

At all stages, we continuously examined the morphology and growth phase of the cells under an inverted microscope (Nikon Eclipse TS100, Japan). For immunofluorescence staining, FLSs were washed with PBS and fixed with cold methanol. After fixation, we washed the cells again with PBS and incubated them for 1 h with the blocking buffer containing PBS/Triton-X100 and 1% bovine serum albumin (BSA). After removing the flow-through, the primary antibody (anti-fibroblast surface protein, FSP; ab11333, Abcam, UK) was added to the cells and incubated overnight at 4 °C. Then FLSs were washed with PBS and incubated with a secondary antibody (sheep anti-mouse Ig-FITC-conjugated, IbnSina, ARI2011F = 1/200), for 1 h at room temperature. Subsequently, we washed the cells

with PBS and stained their nuclei with 4′,6-diamidino-2-phenylindole dye (DAPI = 1 mg/ml). Finally, we evaluated the stained FLSs under an inverted fluorescence microscope. Flow cytometry was utilized to confirm the purity of the FLSs and the resulting data were analyzed using the FlowJo software (Tree Star, Ashland, USA). First, the cells were exposed to fluorescein isothiocyanate (FITC)-conjugated anti-human CD90 (Thy-1) (ab225), CD13 (ab227663), CD44 (ab6124), and CD68 (ab31630) (All antibodies purchased from Abcam, UK) for 60 min at 37 °C. Isotype-matched antibodies were used for 30 min on ice in the dark as a control. The cells were then analyzed with a Partec, GmbH flow cytometer (Germany). The FLSs were found to be positive for CD90, CD13, and CD44 markers, but they do not express CD68 (Fig. 1).

Analysis of cell survival by MTT assay

FLS cells were cultured in a 48-well plate and incubated overnight. The cells were then treated with 20.3 µM N.Acetyl Muramyl-L-alanyl-D-isoglutamine (MDP) (Sigma-Aldrich, USA) [18, 19] and 35, 40, 45 µM curcumin (Sigma-Aldrich, USA) [20, 21] for 12 or 24 h at 37 °C. After the respective incubation periods, 50 µL of the MTT (5 mg/ml) [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] solution (Sigma-Aldrich, USA) was introduced to each well, and the cells were incubated for 4 h. Following that, 500 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich USA) was added to dissolve the crystals in the dark, and the plate was placed on a shaker for 10 min. The absorbance of the wells was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader (BioTek-ELx800, USA).

Cell grouping

To investigate the effects of treatment on RA and non-arthritic FLSs, the cells were divided into four groups and subjected to different conditions: vehicle only, 20.3 µM of MDP, 40 µM of curcumin, or a combination of both MDP (20.3 µM) and curcumin (40 µM). The flasks were then incubated in a CO2 incubator at 37 °C for 24 h. Following this, the FLSs were trypsinized, washed, and collected by centrifugation for subsequent molecular evaluations.

Quantitative real-time PCR

To extract total RNA from the collected FLSs, the SinaPure-RNA kit (SinaClonBioScience, Iran) was used and cDNA was synthesized according to the manufacturer's protocols using the RT-ROSET kit (RojeTechnologies, Iran). For gene expression analysis, the StepOne real-time PCR System (Applied Biosystems, USA) was employed with the master mix high ROX (Takara, Japan). The genes analyzed were *NOD2, P65, IL-6, TNF-α, IL-1β,* and glyceraldehyde-3-phosphate dehydrogenase(*GAPDH*), and the relative mRNA expression levels of the genes were normalized to *GAPDH.* The fold induction or repression was measured using the comparative C_t ($2^{-\Delta\Delta Ct}$) method [22]. The primers used for this analysis are listed in Table 1.

Statistical analysis

The statistical analysis of the data was conducted using IBM SPSS software version 22 (SPSS Inc., Chicago, IL, USA). The normality of variants distribution was checked using both the Kolmogorov–Smirnov, and Shapiro–Wilk tests. For the comparison of gene expression levels between two groups (RA and non-arthritis groups), either the independent t-test or the Mann–Whitney U test was employed. Mean comparison analysis among multiple paired groups was conducted using the Friedman test. The Wilcoxon signed-rank test was performed to compare untreated and treatment groups. A P value less than 0.05 was considered statistically significant. The data were expressed as the mean ± standard error of the mean (SEM), and the plots were generated using GraphPad Prism software version 9 (GraphPad Software, La Jolla California USA).

Results

Identification of FLSs

The morphology of FLSs, characterized by a normal spindle-shaped appearance, was confirmed as originating from fibroblasts based on observations using an inverted microscope (Fig. 1A). The confirmation of this finding was further supported by ICC (Immunocytochemistry), which demonstrated the homogeneity of the FLS population after the third passage (Fig. 1B). Flow cytometry analysis showed high expression levels of specific FLS CD markers such as CD90 (94.67% ± 3.7%), CD44 (99.12% ± 2.21%), and CD13 (97.14% ± 2.06%) (Fig. 1C). Moreover, the cells

(See figure on next page.)

Fig. 1 **A** Morphology of fibroblast-like synoviocytes (FLSs) under an inverted microscope (40X). The scale bar represents 50 µm. **B** Immunocytochemistry staining analysis of fibroblast-like synoviocytes (FLSs). Stained cells with a specific antibody against fibroblast surface protein (FSP) (a), and 4′,6-diamidino-2-phenylindole (DAPI) dye (b), match shapes a & b (c) to confirm the fibroblastic origin of the cells. The scale bar represents 50 µm.**C.** Confirmation of fibroblast-like synoviocytes (FLSs) identity by flow cytometry analysis of CD90 (a), CD44 (b), CD13 (c), and CD68 (d) expression. Unstained was used as a control of test (e)

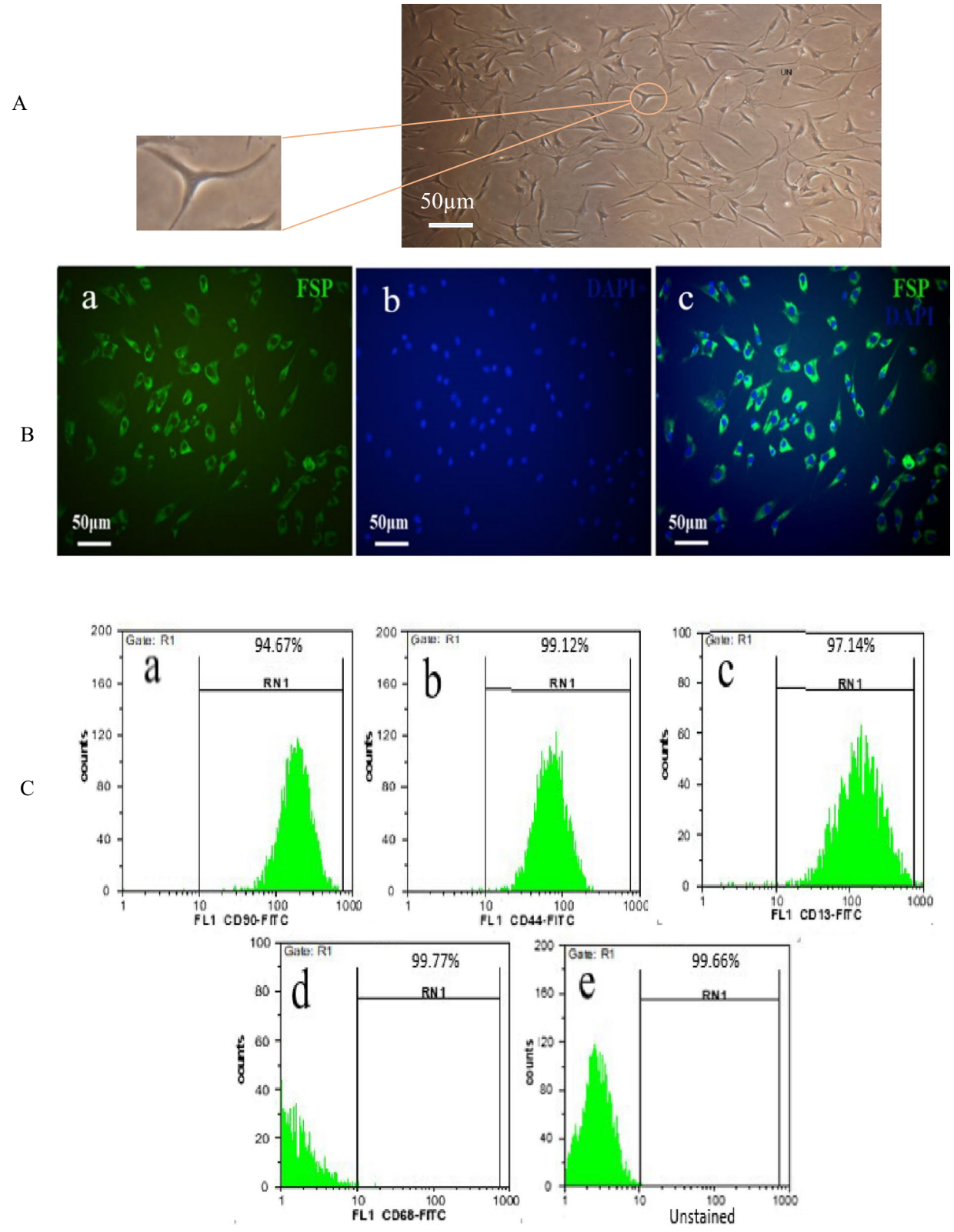

Fig. 1 (See legend on previous page.)

Table 1 Primer sequences of the studied genes in the real-time PCR

Gene name	Primer sequences (5′-3′)
NOD2	F: 5′-CACCGTCTGGAATAAGGGTACT-3′ R: 5′- TTCATACTGGCTGACGAAACC-3′
P65	F: 5′-ATGTGGAGATCATTGAGCAGC-3′ R: 5′-CCTGGTCCTGTGTAGCCATT-3′
GAPDH	F: 5′-GAGTCAACGGATTTGGTCGT-3′ R: 5′- GACAAGCTTCCCGTTCTCAG-3′
IL6	F: 5′-AATCATCACTGGTCTTTTGGAG-3′ R: 5′-GGTTATTGCATCTAGATTCTTTGC-3′
IL1B	F: 5′-ATGGCTTATTACAGTGGCAATGAG3′ R: 5′- GTAGTGGTGGTCGGAGATTCG-3′
TNFA	F: 5′-CCTGCCCCAATCCCTTTATT-3′ R: 5′- CCCTAAGCCCCCAATTCTCT-3′

were found to be negative for the macrophage marker CD68 (99.77% ± 4.01%) (Fig. 1C). These results provide conclusive evidence that the isolated cells were indeed FLSs. Consequently, cells from the third to the sixth passage were utilized for subsequent experiments.

Cytotoxic effects of curcumin and MDP on FLSs

We investigated the cytotoxic effects of curcumin and MDP on FLSs by assessing the impact of various concentrations of curcumin (35, 40, and 45 µM) and a single concentration of MDP (20.3 µM) on FLSs for 12 and 24 h using the MTT colorimetric assay. The results presented in Fig. 4 indicate that a high dose of curcumin (40 and 45 µM) reduced cell viability after 24 h. However, MDP treatment and the combination of curcumin and MDP did not exhibit any toxic effects except for the combination of curcumin at 45 µM and MDP at 20.3 µM (Additional file 1: Fig. S1).

Curcumin treatment decreases NOD2 gene expression in RA-FLSs

There were no significant differences observed in NOD2 mRNA expression between RA-FLSs and non-arthritic individuals. Similarly, no significant changes were observed in NOD2 mRNA expression upon MDP treatment of FLSs in both RA and trauma groups. However, the expression of NOD2 mRNA was significantly reduced upon curcumin treatment in RA-FLSs only. The combination treatment of FLSs did not significantly alter *NOD2* gene expression in either the RA or trauma groups (Fig. 2).

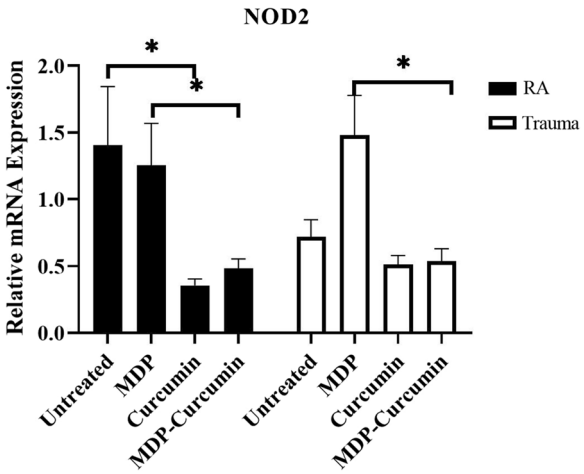

Fig. 2 The effect of muramyl dipeptide (MDP) and curcumin treatment alone and in combination on the nucleotide-binding oligomerization domain-containing protein 2*(NOD2)* gene expression in fibroblast-like synoviocytes (FLSs) of RA and trauma groups. Data are presented as Mean ± SEM (*$P < 0.05$; **$P < 0.01$)

Curcumin treatment suppresses p65 gene expression in FLSs

No significant differences were found in the mRNA expression level of *p65* between RA and healthy non-arthritic individuals. Furthermore, MDP treatment did not affect the expression of *p65* in the FLSs of both groups. However, treatment with curcumin alone or in combination with MDP significantly reduced the

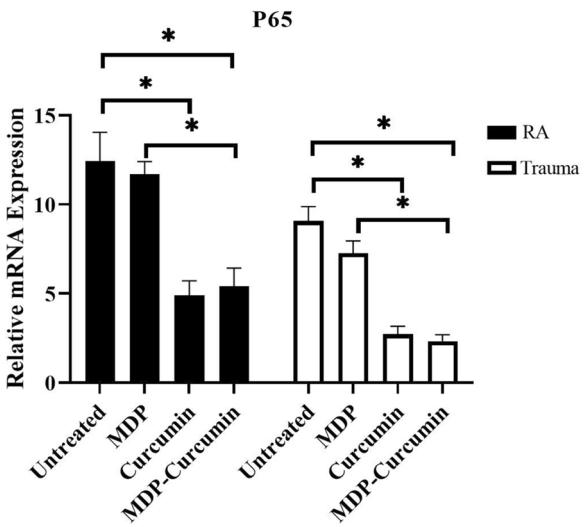

Fig. 3 The effect of muramyl dipeptide (MDP) and curcumin treatment alone and in combination on *p65* gene expression in fibroblast-like synoviocytes (FLSs) of RA and trauma groups. Data are presented as Mean ± SEM (*$P < 0.05$; **$P < 0.01$)

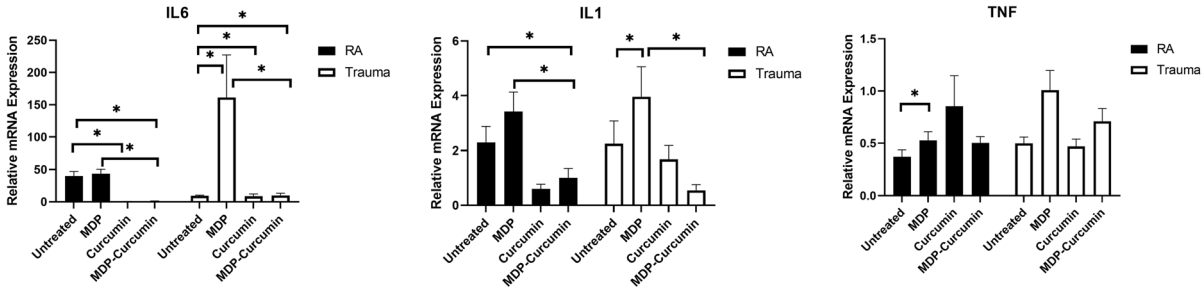

Fig. 4 The effect of muramyl dipeptide (MDP) and curcumin treatments alone and in combination on the expression of inflammatory cytokines genes including *IL-6, IL-1β* and *TNF-α genes* in fibroblast-like synoviocytes (FLSs) of RA versus trauma groups. Data are presented as Mean ± SEM (*$P < 0.05$; **$P < 0.01$)

relative mRNA expression of *p65* in both the RA and trauma groups (Fig. 3).

Curcumin treatment downregulates IL-6 gene expression in FLSs

The expression levels of *IL-6, IL-1β,* and *TNF-α* genes were comparable between RA FLSs and the trauma group ($P > 0.05$).

Treatment of FLS with MDP resulted in a significant upregulation of IL-6 and IL-1β mRNA expression in the trauma group, but did not cause any significant changes in the RA group. On the other hand, treatment with curcumin alone and in combination significantly downregulated the expression of *IL-6* gene in the FLSs of both RA and trauma groups; however, no significant changes were observed in the expression of IL-1β gene (Fig. 4).

In RA-FLSs treated with MDP, the expression of *TNF-α* gene was significantly increased, whereas MDP treatment had no significant effect on TNF-α expression in the trauma group. Neither curcumin nor its combination treatment could significantly change the expression of *TNF-α* gene in FLSs of both RA and trauma groups (Fig. 4).

Discussion

In the joint environment of RA patients, FLSs play a direct role in the development of inflammation and the destruction of bone and cartilage [4]. Considering the chronic inflammation in RA and the side effects of disease-modifying antirheumatic drugs (DMARDs), it is important to find non-toxic multifunctional substances with appropriate anti-inflammatory effects [23, 24]. Curcumin, a polyphenol and the active ingredient in turmeric, has demonstrated anti-inflammatory [25] and antioxidant properties in previous studies [14, 26]. Amalraj et al. showed that curcumin (250 mg and 500 mg) significantly reduced CRP, ESR, VAS, SJC, TJC, rheumatoid factor (RF) values, and DAS28 in active RA patients compared to placebo; Side effects have not been observed [27]. Curcumin inhibits inflammation by reducing the production of prostaglandins in FLS cells [20], inhibiting FLS hyperplasia [21], reducing osteoclast activity, and in turn, reducing local osteoporosis [28]. Zihan Xu et al. showed that Curcumin has an anti-RA effect in vivo and in vitro by inhibiting the expression of pro-inflammatory factors TNF-a, IL-6, and IL-17 and inhibiting the activation of PI3K/AKT signaling pathway [29]. Previous studies have shown that curcumin can suppress *NOD2* mRNA expression (16) and inhibit cytoplasmic p65 translocation [20]. Moreover, it has been revealed that curcumin can effectively inhibit NF-κB activation and induce ERK1/2 dephosphorylation [21]. Increased expression of NOD-2 upon MDP treatment induces the expression of IL-6 [30]. Curcumin has been shown to effectively blocks the release of IL-6 in both MH7A cells and RA-FLSs [21] and can decrease the levels of TNF-α and IL-1β proteins in the joint ankle [20]. Woźniak et al. showed that RA in vitro model, curcumin (at 10 μM) reduced the survival of synovial sarcoma SW982 cells as well as MMP1 gene expression and TNF-α protein but had no effect on the expression of IL-6 [31]. In this study, we used curcumin to inhibit the NOD2 receptor and NF-κB pathway. We found that treatment of FLSs with curcumin significantly reduced *NOD2, P65,* and *IL-6* relative mRNA expression in the RA group, and *P65* and *IL-6* expression in the trauma group. Additionally, the combined treatment of FLSs with curcumin and MDP significantly reduced *P65* and *IL-6* expression in both the RA and trauma groups.

Ospelt et al. reported that NOD-2 is expressed by RA FLSs at invasion sites in articular cartilage. Upregulation of NOD-2 by Toll-like receptor (TLR) and NOD2 ligands increases the expression of the *IL-6* gene [30]. Our study found that the expression of *NOD2,*

p65, and IL-6 genes in FLSs was higher in the RA group compared to the trauma group; but this difference was not statistically significant. In addition, there were no significant changes in the baseline expression of *IL-1β* and *TNF-α* between the two groups. We observed that RA patients who participated in our study were in the end-stage of the disease and had received immunosuppressive drugs, which may have contributed to the lack of significant differences at the baseline level. NOD2 binding to MDP leads to the activation of the NF-κB pathway, which induces the expression of inflammatory cytokines such as IL-6, TNF-α, and IL-1β, but studies have shown that IL-6 is the main product of RA FLSs [32]. IL-6 plays a critical role in the pathogenesis of RA by enhancing synovitis, inducing the infiltration of inflammatory cells into the synovium, neovascularization, and synovial fibroblast hyperplasia [11]. Our results show that MDP significantly increased *IL-6* and *IL-1β* relative mRNA expression in the trauma group and *TNF-α* relative mRNA expression in the RA group, but not in other genes (33). In conclusion, curcumin has been found to inhibit the NF-κB pathway and the inflammatory cytokine *IL-6* in FLSs, indicating its potential as a medical supplement in the treatment of RA patients. However, further studies with larger sample size are needed to confirm these findings, particularly with regards to investigating the inhibitory effect of curcumin on NOD2 receptor at the protein level and to assess cytokine secretion in the supernatant or at least intracellular protein expression which was not possible in this study due to limitations.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42358-023-00308-0.

Additional file 1. **Figure S1**. The effects of different concentrations of curcumin (35, 40, and 45 μM), muramyl dipeptide (MDP) (20.3 μM) treatment alone (a) and in combination (b) on the viability of fibroblast-like synoviocytes (FLSs). Cytotoxic effects were evaluated with MTT assay after 12 and 24 hours. Data are presented as Mean ± SEM.

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Author contributions
FAP, MNT, ASV, LA, MA, SP, and FM: Acquisition of data, drafting the article, analysis and interpretation of data, and final approval of the article. AASY, EF, AJ, and MM: The conception and design of the study, revising the article critically, interpretation of data, and final approval of the article. All authors read and approved the final manuscript.

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Availability of data and materials
The data generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study was conducted following the guidelines of the Declaration of Helsinki and received approval from the Ethics Committee of Tehran University of Medical Sciences (Approval No: IR.TUMS.SPH.REC.1398.106). The written informed consent was signed by all participants before enrolling in the study.

Consent for publication
Not applicable.

Competing interests
The authors report there are no competing interests to declare.

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