# LIM mineralization protein-1 inhibits IL-1β-induced human chondrocytes injury by altering the NF-κB and MAPK/JNK pathways

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**Abstract.** Osteoarthritis (OA) is a common degenerative disease that is associated with the degradation of articular cartilage. Accumulating evidence has confirmed that LIM mineralization protein-1 (LMP-1) is an important agent of bone formation and has been shown to be osteoinductive in various types of disease. However, the underlying mechanisms of LMP-1 in the pathogenesis of OA remain unknown. The present study aimed to evaluate the role and potential mechanism of LMP-1 in IL-1β-stimulated OA chondrocytes. CHON-001 cells were transfected with pcDNA3.1-LMP-1, pcDNA3.1, negative control-small interfering (si)RNA or LMP-1 siRNA for 24 h and then induced by IL-1β for 12 h to establish an OA model *in vitro*. Cell viability, apoptosis and inflammatory cytokine (IL-6, IL-8 and TNF-α) release were assessed using MTT assay, flow cytometry and ELISA, respectively. The expression levels of LMP-1, cleaved-caspase 3, phosphorylated (p)-p65, p65, p-JNK and JNK were analyzed using reverse transcription-quantitative PCR and western blotting. Overexpression of LMP-1 notably alleviated the IL-1β-induced inflammatory response in CHON-001 cells, as shown by increased cell viability, decreased apoptosis, suppressed expression of cleaved-caspase 3 and a decreased cleaved-caspase 3/caspase 3 ratio. Moreover, IL-1β-induced secretion of IL-6, IL-8 and TNF-α in CHON-001 cells; this was reversed by pcDNA3.1-LMP-1. However, knocking down LMP-1 expression exert opposite effects on the IL-1β-induced inflammatory response in CHON-001 cells, as evidenced by the decreased cell viability, increased apoptosis, enhanced expression of cleaved-caspase 3 and cleaved-caspase 3/caspase 3 ratio and enhanced secretion of IL-6, IL-8 and TNF-α observed. The present data demonstrated that LMP-1 siRNA notably inhibited LMP-1 expression, suppressed cell viability, promoted apoptosis and enhanced cleaved-caspase 3 expression and cleaved-caspase 3/caspase 3 ratio. In addition, LMP-1 siRNA promoted the release of inflammatory factors in CHON-001 cells. It was also found that pcDNA3.1-LMP-1 inhibited p-p65 and p-JNK expression, as well as decreasing the p-p65/p65 and p-JNK/JNK ratio. Nevertheless, there was no significant difference in the mRNA expression levels of p65 and JNK between the groups. Taken together, these findings indicated that overexpression of LMP-1 alleviated IL-1β-induced chondrocytes injury by regulating the NF-κB and MAPK/JNK pathways, suggesting that LMP-1 may be a valuable therapeutic agent for OA treatment.

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*Key words:* LIM mineralization protein-1, IL-1β, chondrocytes, NF-κB and MAPK/JNK pathways

## Introduction

Osteoarthritis (OA) is a common degenerative joint disease that is caused by strain, trauma, joint deformity and other factors (1). At present, it has been reported that 240 million individuals suffer from OA globally, mostly affecting those who are middle-aged or elderly (2). The number of patients with OA will increase with the aging population, which will result in the heavy economic burden for patients and society (3). Current therapies for OA include pharmacological treatment and methods to relieve pain, whereas knee arthroplasty is the final therapeutic option if other methods fail (4,5). Nevertheless, the pathogenesis of OA is yet to be fully elucidated and it is important to discover effective therapeutic methods for patients with OA.

IL-1β, a member of the IL-1 cytokine family, is involved in articular cartilage degeneration (6). IL-1β is also a vital regulator in the pathogenesis of OA (7). IL-1β signaling has been considered a promising target to treat OA (8). Previous *in vivo* models and clinical trials have displayed contradictory results. Although a number of studies have shown that IL-1 inhibition is an effective treatment, other studies have also shown that IL-1 inhibition is not an effective analgesic/anti-inflammatory therapy, especially from previous large-scale clinical studies targeting IL-1β (9-12). Chondrocytes are the only cells found

in articular cartilage and serve a key role in maintaining matrix integrity (13). IL-1β-stimulated chondrocytes have been used to mimic articular cartilage injury *in vitro* (14). IL-1β-stimulated CHON-001 cells have been widely used as an *in vitro* model to study OA (15-18).

NF-κB, a common transcription factor, serves a key role in the regulation of cellular responses, including inflammation, the immune response and apoptosis (19-21). Under physiological conditions, NF-κB is located in the cytosol and is combined with its inhibitory protein, IκB. Once IκB is phosphorylated, the NF-κB hetero-dimer is translocated to the nucleus where it activates transcription of target genes (21,22). It has also been reported that NF-κB is crucial for OA occurrence and progression. Hu *et al* (23) observed that loganin improves cartilage degeneration and OA progression in a mouse model by suppressing the NF-κB pathway in chondrocytes. Moreover, Zhou *et al* (24) confirmed that kinsenoside relieves OA by repolarizing macrophages via inactivation of the NF-κB/MAPK pathway and protection of chondrocytes. MAPKs, a group of serine/threonine protein kinases, are involved in the control of cellular responses, such as apoptosis and the inflammatory response to cytokines (25). Lee *et al* (26) reported the protective effects of aqueous extract of *Anthriscus sylvestris* on relieving OA both *in vitro* and *in vivo* by inhibiting the MAPK and NF-κB pathways.

LIM mineralization protein-1 (LMP-1), an intracellular mediator of bone formation, has been reported to promote bone formation (27). LMP-1 has been reported to suppress TNF-α-induced intervertebral disc degeneration (28). However, to the best of our knowledge, its role in OA and chondrocytes has not been previously reported.

The present study aimed to evaluate the role of LMP-1 in relieving the inflammatory response stimulated by IL-1β during the pathogenesis of OA. It was hypothesized that after the OA cell model was established using IL-1β, LMP-1 would exhibit a protective effect against OA in IL-1β-stimulated chondrocyte inflammation and that the mechanism underlying the protective effect of LMP-1 may be associated with the NF-κB and MAPK/JNK pathways.

## Materials and methods

*Cell culture*. CHON-001 cells were purchased from the American Type Culture Collection, seeded in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (HyClone; Cytiva) and 1% penicillin-streptomycin and maintained at 37˚C with 5% $CO_2$ in an incubator. CHON-001 cells were exposed to IL-1β (0.1, 2.0, 5.0 and 10.0 ng/ml; Sigma-Aldrich; Merck KGaA) at 37˚C for 12 h (29).

*Construction of pcDNA3.1-LMP-1, pcDNA3.1, LMP-1 small interfering (si)RNA and negative control (NC) siRNA*. The LMP-1 plasmid (pcDNA3.1-LMP-1), empty plasmid (pcDNA3.1), LMP-1-siRNA (sense, 5'-GGAAUUUGCACG GACAGGCTT-3' and anti-sense, 5'-GCCUGUCCGUGCAAA UUCCTT-3') and NC siRNA (forward, 5'-UUCUCCGAACG UGUCACGUTT-3' and reverse. 5'-ACGUGACACGUUCGG AGAATT-3') were constructed by GenePharm, Inc. The full length of LMP-1 gene (accession no. AAK30567) was amplified using RT-PCR and then cloned into vector plasmid using the PCR cloning technique (30). Briefly, total RNA from CHON-001 cells was isolated using an TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The cDNA was obtained using a PrimeScript™ RT kit (Takara Bio, Inc.). The temperature protocol for the reverse transcription reaction was as following: 25˚C for 5 min, 42˚C for 60 min and 80˚C for 2 min. Subsequently, amplification of the LMP-1 gene was performed using a PCR Amplification kit (cat. no. R011; Takara Bio, Inc.) with primers LMP-1-forward, 5'-ATGGATTCCTTCAAAGTAGTGCTG-3' and reverse, 5'-TCACACATGAGAGAAGGCATGG-3'. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 30 sec.

*Cell transfection*. CHON-001 cells ($5x10^4$ cells/well) were transfected with 0.5 μg pcDNA3.1-LMP-1, 0.5 μg pcDNA3.1, 50 nM LMP-1-siRNA and 50 nM NC-siRNA at 37˚C for 24 h using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. At 24 h post-transfection, reverse transcription-quantitative (RT-q)PCR was performed to evaluate the efficiency of cell transfection.

*MTT assay*. Following transfection for 24 h and/or treatment with IL-1β for 12 h, CHON-001 cells ($2x10^3$ cells per well) were cultured into 96-well plates at 37˚C. Then, cells were treated with 10 μl MTT (5 mg/ml) solution and continuously incubated for a further 4 h. Following treatment, the solution was removed and 100 μl DMSO was added to each well to dissolve the formazan product. Finally, the optical density (OD) was measured at a wavelength of 570 nm using a multifunctional plate reader (BioTek Instruments, Inc.) following 15 min of vibration mixing, according to the manufacturer's protocol.

*Flow cytometry analysis*. Following transfection for 24 h or treatment with IL-1β for 12 h, CHON-001 cell apoptosis was measured using an Annexin V-FITC/PI apoptosis detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. In brief, CHON-001 cells ($1x10^6$) were collected though centrifugation (1,000 x g) at 4˚C for 5 min, then the cells were stained using 5 μl Annexin V-FITC and 5 μl PI for 30 min at room temperature in the dark. The number of apoptotic cells were examined using a Cell Lab Quanta™ SC flow cytometer (Beckman Coulter, Inc.) and analyzed using Kaluza analysis software (version 2.1.1.20653; Beckman Coulter, Inc.).

*Western blot analysis*. Total protein was extracted from CHON-001 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and quantified using a BCA Protein assay kit (Invitrogen; Thermo Fisher Scientific, Inc.). The protein samples (40 μg per lane) were then separated via 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). Following blocking with 5% skimmed milk in PBS-0.1% Tween-20 at room temperature for 1.5 h, the membranes were cultured with primary antibodies against GAPDH (1:1,000; cat. no. 5174; Cell Signaling

Technology, Inc.), LMP-1 (1:1,000; cat. no. ab182153; Abcam), phosphorylated (p)-p65 (1:1,000; cat. no. ab76302; Abcam), p65 (1:1,000; cat. no. ab32536; Abcam), p-JNK (1:1,000; cat. no. sc-6254; Santa Cruz Biotechnology, Inc.), JNK (1:1,000; cat. no. sc-7345; Santa Cruz Biotechnology, Inc.), caspase 3 (1:1,000; cat. no. 14220; Cell Signaling Technology, Inc.) and cleaved-caspase 3 (1:1,000; cat. no. 9654; Cell Signaling Technology, Inc.) overnight at 4˚C. After washing in TBS-Tween-20, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (both 1:2,000; cat. nos. 7074 and 7076, respectively; both Cell Signaling Technology, Inc.) at room temperature for 1.5 h. Finally, the bands were visualized using Pierce ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. ImageJ v.2.0 software (National Institutes of Health) was used to quantify the band intensity.

*ELISA*. The secretion of IL-6 (cat. no. PI330), IL-8 (cat. no. PI640) and TNF-α (cat. no. PT518) in CHON-001 cell culture supernatant (collected through centrifugation at 500 x g at 4˚C for 5 min) were quantitatively detected using ELISA kits (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Then, the OD value at 450 nm was measured on a Multiscan spectrum.

*RT-qPCR analysis*. LMP-1, p65 and JNK expression levels were measured via RT-qPCR. Isolation of RNA from CHON-001 cells was performed using an TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was obtained using a PrimeScript RT kit (Takara Bio, Inc.). The temperature protocol for the reverse transcription reaction was as following: 25˚C for 5 min, 42˚C for 60 min and 80˚C for 2 min. qPCR amplification was then performed with SYBR Premix Ex-Taq (Takara Bio, Inc.) on an ABI PRISM 7900 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 30 sec. GAPDH was used as the internal control. Primers were obtained from Sangon Biotech Co., Ltd. as follows: LMP-1-forward, 5'-TAGCCAGTGTGGGAAGGTCCTGGAAGAG-3' and reverse, 5'-CTTCTTGCACTTGGCACAGCTGGGTG-3'; p65 forward, 5'-ACAACAACCCCTTCCAAGAAGA-3' and reverse, 5'-CAGCCTGGTCCCGTGAAATA-3'; JNK forward, 5'-CACAGTCCTAAAACGATACC-3' and reverse, 5'-CCACACAGCATTTGATAGAG-3' and GAPDH forward, 5'-TCAACGACCACTTTGTCAAGCTCA-3' and reverse, 5'-GCTGGTGGTCCAGGGGTCTTACT-3'. Target gene expression was assessed using the $2^{-\Delta\Delta Cq}$ method (31).

*Statistical analysis*. All experiments were repeated three times. Statistical analysis was performed using SPSS 20.0 (IBM Corp.). Data are expressed as the mean ± SD from three independent experiments. Differences between two groups were assessed using unpaired Student's t-test; differences between multiple groups were analyzed by one-way ANOVA followed by post hoc Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*IL-1β induces inflammatory damage and significantly decreases LMP-1 expression in chondrocytes*. Previous reports have shown that IL-1β is involved in the pathogenesis of OA by causing a cascade of inflammatory and destructive reactions (7,8). In the present study, various concentrations of IL-1β (0.1, 2.0, 5.0 and 10.0 ng/ml) were applied to CHON-001 cells to establish an OA model *in vitro*. Following 12 h stimulation, cell viability and apoptosis were assessed. The data demonstrated that 5 and 10 ng/ml IL-1β notably decreased CHON-001 cell viability compared with the control group (Fig. 1A). Therefore, 10 ng/ml IL-1β was selected for subsequent analysis. It was also found that 10 ng/ml IL-1β significantly promoted CHON-001 apoptosis (Fig. 1B and C), enhanced cleaved-caspase 3 protein expression (Fig. 1D) and increased the ratio of cleaved-caspase 3/caspase 3 (Fig. 1E). Moreover, the secretion of TNF-α, IL-6 and IL-8 was increased by 10 ng/ml IL-1β (Fig. 1F-H), whereas IL-1β significantly decreased LMP-1 mRNA (Fig. 1I) and protein expression (Fig. 1J) in CHON-001 cells compared with the control group. These findings revealed that IL-1β induced CHON-001 cellular injury in OA.

*LMP-1 alleviates IL-1β-induced CHON-001 changes in cell viability and apoptosis*. To evaluate whether LMP-1 was associated with chondrocyte inflammation, CHON-001 cells were transfected with pcDNA3.1-LMP-1 or pcDNA3.1 for 24 h and stimulated with 10 ng/ml IL-1β for 12 h. Then, the expression of LMP-1 was measured using RT-qPCR and western blotting. LMP-1 expression was higher in the pcDNA3.1-LMP-1 group compared with that in the pcDNA3.1 group (Fig. 2A and B). LMP-1 enhanced cell viability, inhibited apoptosis and decreased secretion of inflammatory factors (TNF-α, IL-6 and IL-8) in CHON-001 cells (Fig. S1). Furthermore, LMP-1 was downregulated in IL-1β-induced CHON-001 cells compared with the control group. However, this inhibitory effect was reversed by pcDNA3.1-LMP-1 (Fig. 2C and D).

To investigate the role of LMP-1 in IL-1β-induced CHON-001 cells, the effect of pcDNA3.1-LMP-1 on cell viability and apoptosis was assessed. IL-1β inhibited cell viability (Fig. 2E), increased the number of apoptotic cells (Fig. 2F and G) and enhanced cleaved-caspase 3 protein expression (Fig. 2H) and the cleaved-caspase 3/caspase 3 ratio (Fig. 2I). However, these effects were reversed by pcDNA3.1-LMP-1. Thus, the data demonstrated that upregulation of LMP-1 alleviated IL-1β-induced CHON-001 apoptosis and inhibition of cell viability.

*LMP-1 relieves the IL-1β-stimulated inflammatory response in CHON-001 cells*. The effect of LMP-1 on the secretion of inflammatory factors, including TNF-α, IL-6 and IL-8, was assessed. Levels of TNF-α, IL-6 and IL-8 were significantly increased in IL-1β-stimulated CHON-001 cells, whereas pcDNA3.1-LMP-1 significantly decreased inflammatory factor release in IL-1β-treated CHON-001 cells (Fig. 3A-C). These data suggested that LMP-1 alleviated the IL-1β-induced inflammatory response in CHON-001 cells.

*LMP-1-siRNA aggravates IL-1β-induced CHON-001 apoptosis and decreased cell viability*. To determine the

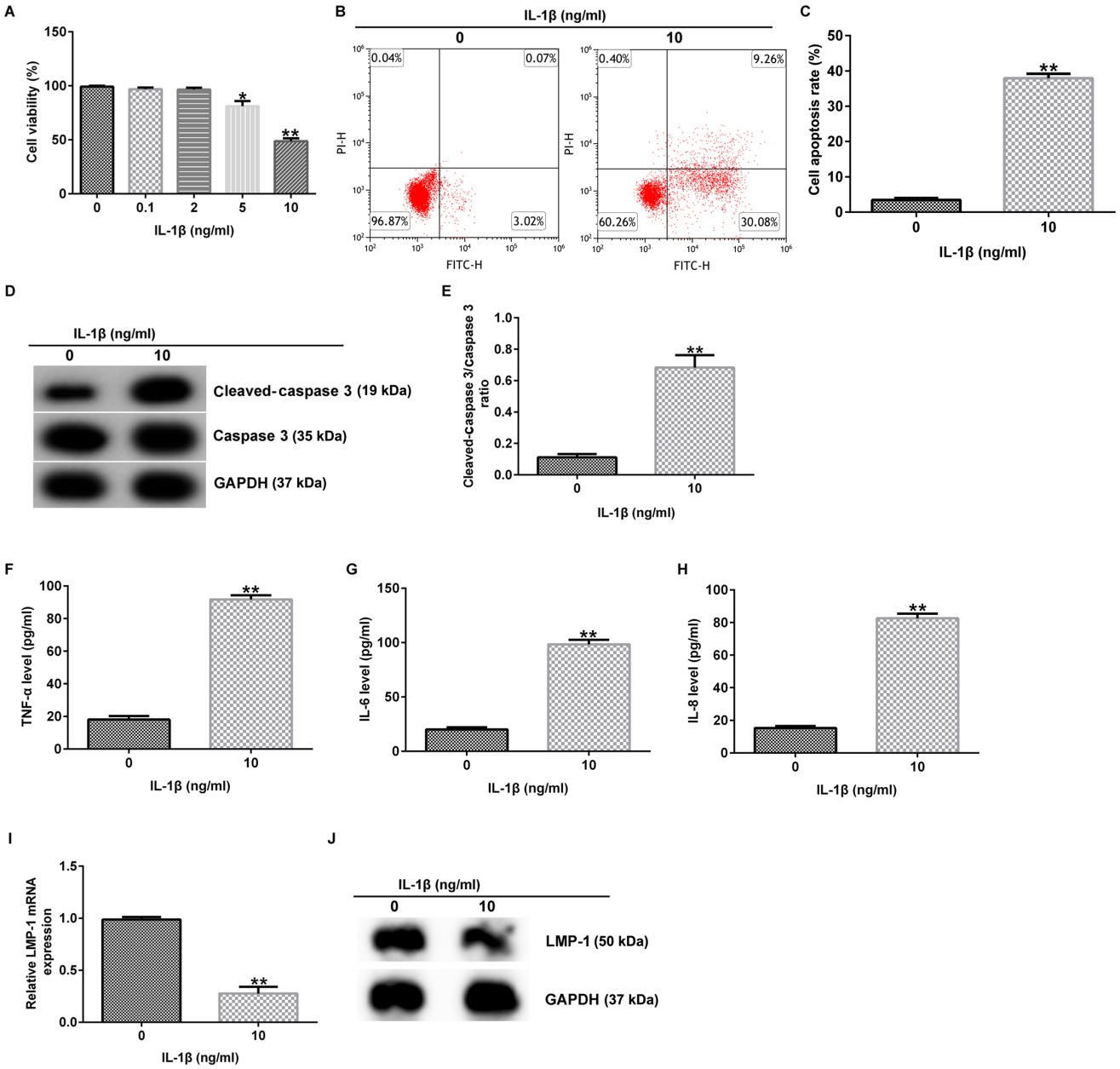


Figure 1. Effects of IL-1β on inflammatory injury in CHON-001 cells. CHON-001 cells were induced with different concentration of IL-1β (0.1, 2.0, 5.0 and 10.0 ng/ml) for 12 h. (A) Cell viability was evaluated via MTT assay. (B) Cell apoptosis was assessed via flow cytometry. (C) Quantification of the number of apoptotic cells. (D) Western blot analysis of cleaved-caspase 3 and caspase 3. (E) Ratio of cleaved-caspase 3/caspase 3. The levels of (F) TNF-α, (G) IL-6 and (H) IL-8 were assessed using ELISA. (I) Reverse transcription-quantitative PCR analysis and (J) western blotting of LMP-1 mRNA and protein expression. $^{*}$P<0.05, $^{**}$P<0.01 vs. 0. LMP-1, LIM mineralization protein-1.

protective effects of LMP-1 on IL-1β-stimulated CHON-001 cells, CHON-001 cells were transfected with NC-siRNA or LMP-1 siRNA for 24 h and stimulated with 10 ng/ml IL-1β for 12 h. RT-qPCR and western blotting revealed that LMP-1 siRNA significantly decreased LMP-1 expression (Fig. 4A and B). Compared with the NC-siRNA transfection group, LMP-1-siRNA significantly decreased cell viability, enhanced cell apoptosis and increased secretion of inflammatory factors (TNF-α, IL-6 and IL-8) in CHON-001 cells (Fig. S2). In addition, LMP-1 was downregulated in IL-1β-induced CHON-001 cells compared with the control group. LMP-1 siRNA significantly decreased LMP-1 mRNA and protein expression (Fig. 4C and D). The results demonstrated that IL-1β stimulation significantly inhibited cell viability (Fig. 4E), increased the number of apoptotic cells (Fig. 4F and G) and enhanced cleaved-caspase 3 protein expression (Fig. 4H) and the cleaved-caspase 3/caspase 3 ratio (Fig. 4I). These effects were further enhanced in the IL-1β+LMP-1 siRNA group compared with the IL-1β+NC-siRNA group. Collectively, these data suggested that LMP-1-siRNA aggravated IL-1β-induced CHON-001 apoptosis and decreased cell viability.

*LMP-1-siRNA aggravates the IL-1β-induced inflammatory response in CHON-001 cells*. The effect of LMP-1 on inflammatory factor secretion in CHON-001 cells was then evaluated. The data revealed that the levels of TNF-α, IL-6 and IL-8 were significantly enhanced in IL-1β-treated CHON-001 cells and LMP-1 siRNA further promoted TNF-α (Fig. 5A),

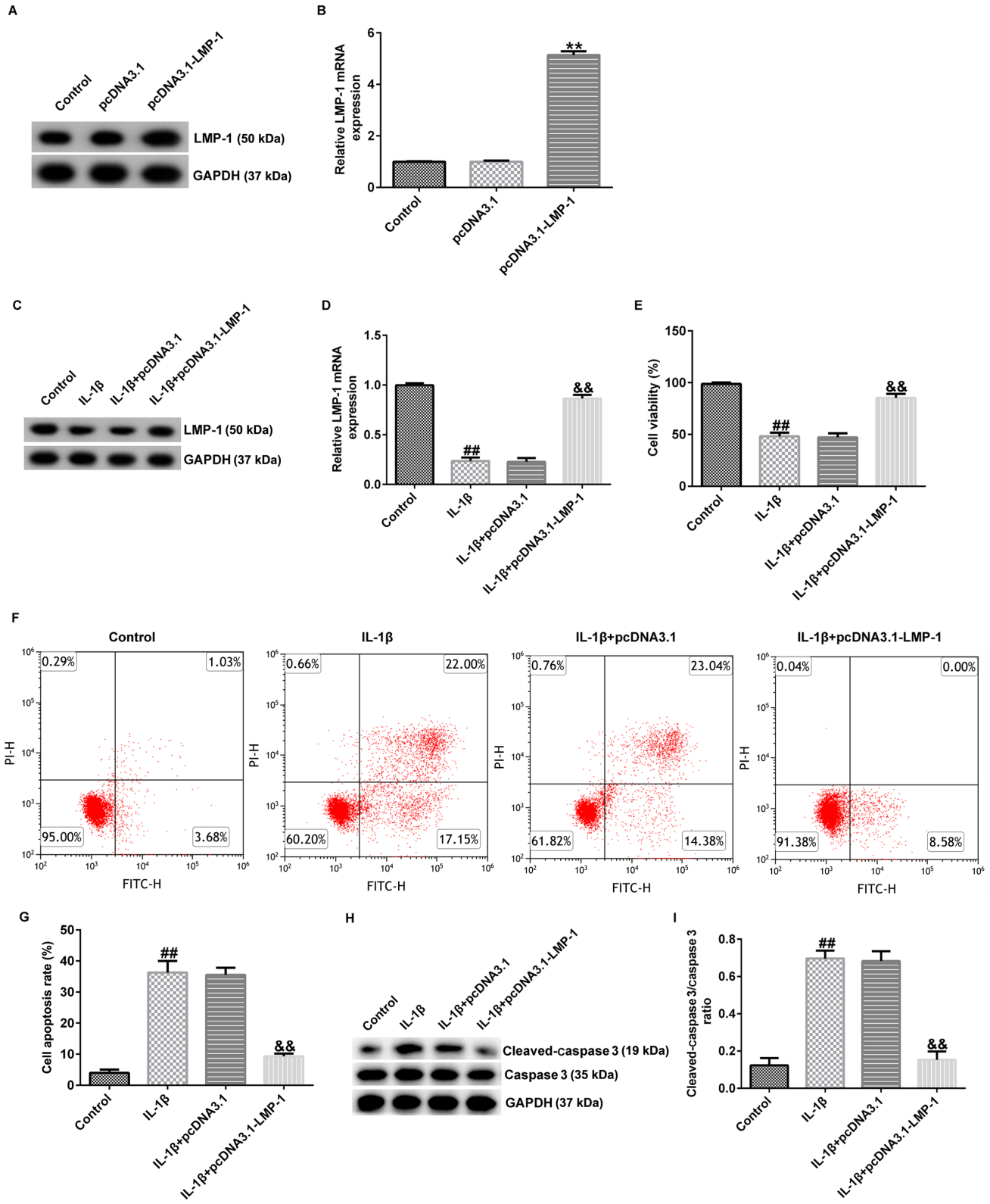


Figure 2. Effects of pcDNA3.1-LMP-1 on IL-1β-induced changes in cell viability and apoptosis. CHON-001 cells were transfected with pcDNA3.1-LMP-1 or pcDNA3.1 for 24 h and induced with 10 ng/ml IL-1β for 12 h. Expression levels of LMP-1 in pcDNA3.1-LMP-1 and pcDNA3.1 groups was determined via (A) western blotting and (B) RT-qPCR. Expression levels of LMP-1 in IL-1β+pcDNA3.1-LMP-1 and IL-1β+pcDNA3.1 groups were determined via (C) western blotting and (D) RT-qPCR. (E) MTT assay was used to assess cell viability. (F) Flow cytometry was performed to determine the number of apoptotic cells. (G) Quantification of the number of apoptotic cells. (H) Expression of cleaved-caspase 3 was evaluated using western blot analysis. (I) Ratio of cleaved-caspase 3/caspase 3. $^{**}$P<0.01 vs. pcDNA3.1; $^{\#\#}$P<0.01 vs. control; $^{\&\&}$P<0.01 vs. IL-1β + pcDNA3.1. LMP-1, LIM mineralization protein-1; RT-q, reverse transcription-quantitative.

IL-6 (Fig. 5B) and IL-8 (Fig. 5C) release in IL-1β-induced CHON-001 cells. In summary, these data suggested that LMP-1-siRNA aggravated IL-1β-induced inflammatory response in CHON-001 cells.

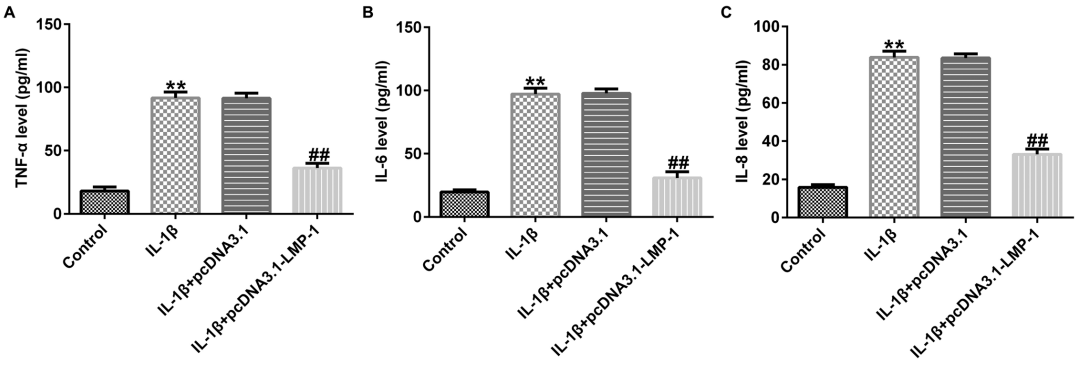


Figure 3. Effect of pcDNA3.1-LMP-1 on IL-1β-induced inflammatory cytokine secretion. CHON-001 cells were transfected with pcDNA3.1-LMP-1 or pcDNA3.1 for 24 h and stimulated with 10 ng/ml IL-1β for 12 h. The secretion of (A) TNF-α, (B) IL-6 and (C) IL-8 was assessed using ELISA. $^{**}$P<0.01 vs. control; $^{\#\#}$P<0.01 vs. IL-1β + pcDNA3.1. LMP-1, LIM mineralization protein-1.

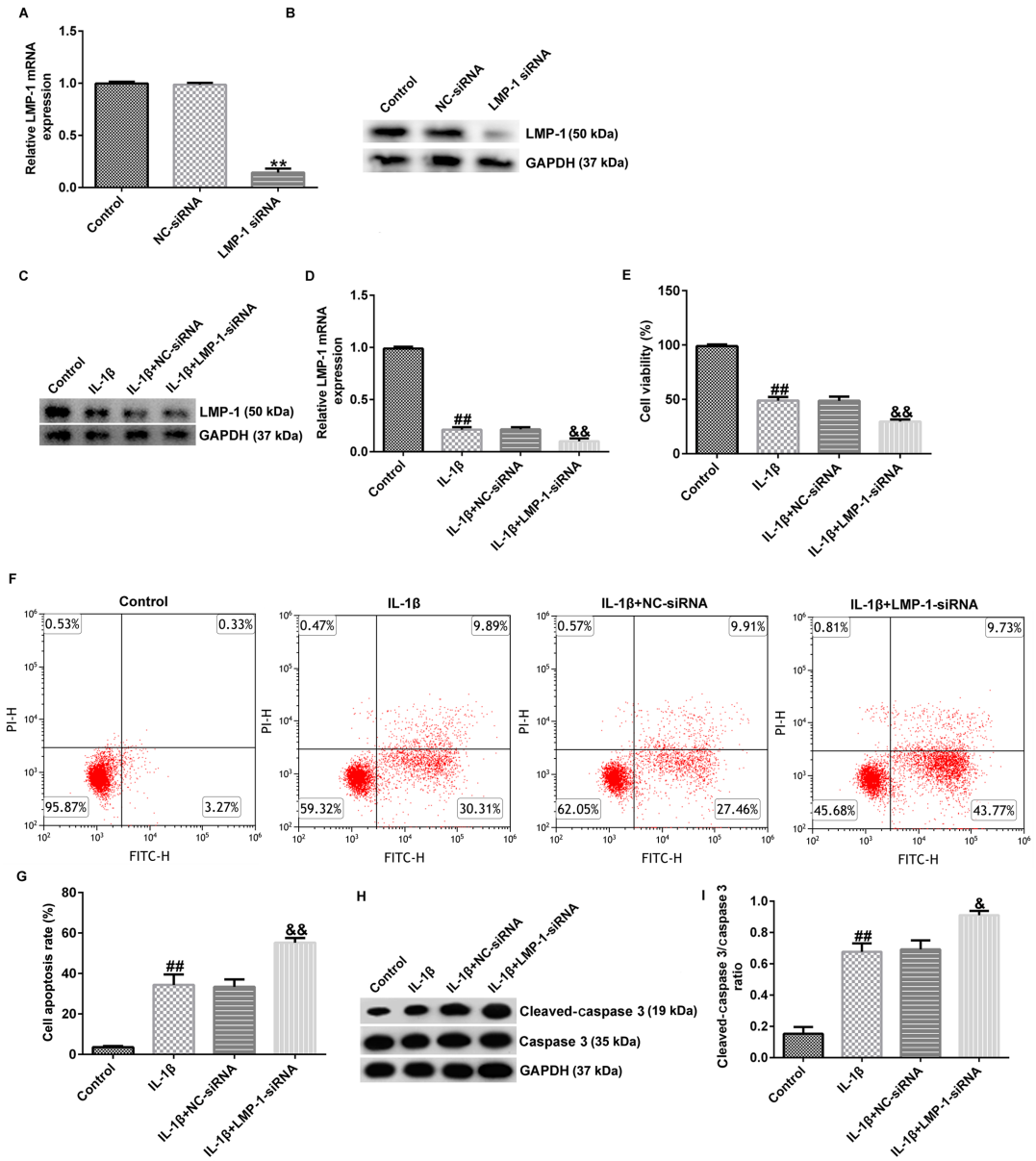


Figure 4. Effect of LMP-1-siRNA on IL-1β-induced apoptosis and decreased cell viability. CHON-001 cells were transfected with LMP-1 siRNA or NC-siRNA for 24 h and induced with 10 ng/ml IL-1β for 12 h. Expression levels of LMP-1 in LMP-1-siRNA and NC-siRNA groups was assessed using (A) RT-qPCR and western blotting (B). (C) Western blotting and (D) RT-qPCR results of LMP-1 expression in IL-1β+NC-siRNA and IL-1β+LMP-1-siRNA groups. (E) Cell viability was determined via MTT assay. (F) Cell apoptosis was evaluated using flow cytometry. (G) Quantification of the number of apoptotic cells. (H) Western blot analysis of cleaved-caspase 3 expression. (I) Ratio of cleaved-caspase 3/caspase 3. $^{**}$P<0.01 vs. NC-siRNA; $^{\#\#}$P<0.01 vs. control; $^{\&}$P<0.05 and $^{\&\&}$P<0.01 vs. IL-1β+NC-siRNA. LMP-1, LIM mineralization protein-1; RT-q, reverse transcription-quantitative; si, small interfering; NC, negative control.

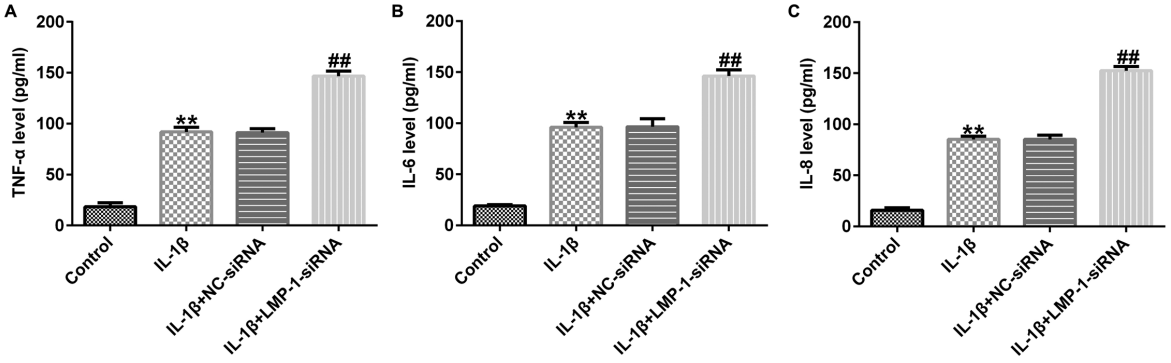


Figure 5. Effect of LMP-1-siRNA on IL-1β-induced inflammatory cytokine secretion. CHON-001 cells were transfected with LMP-1 siRNA or NC-siRNA for 24 h and stimulated with 10 ng/ml IL-1β for 12 h. The release of (A) TNF-α, (B) IL-6 and (C) IL-8 was assessed using ELISA. **P<0.01 vs. control; ##P<0.01 vs. IL-1β+NC-siRNA. LMP-1, LIM mineralization protein-1; si, small interfering; NC, negative control.

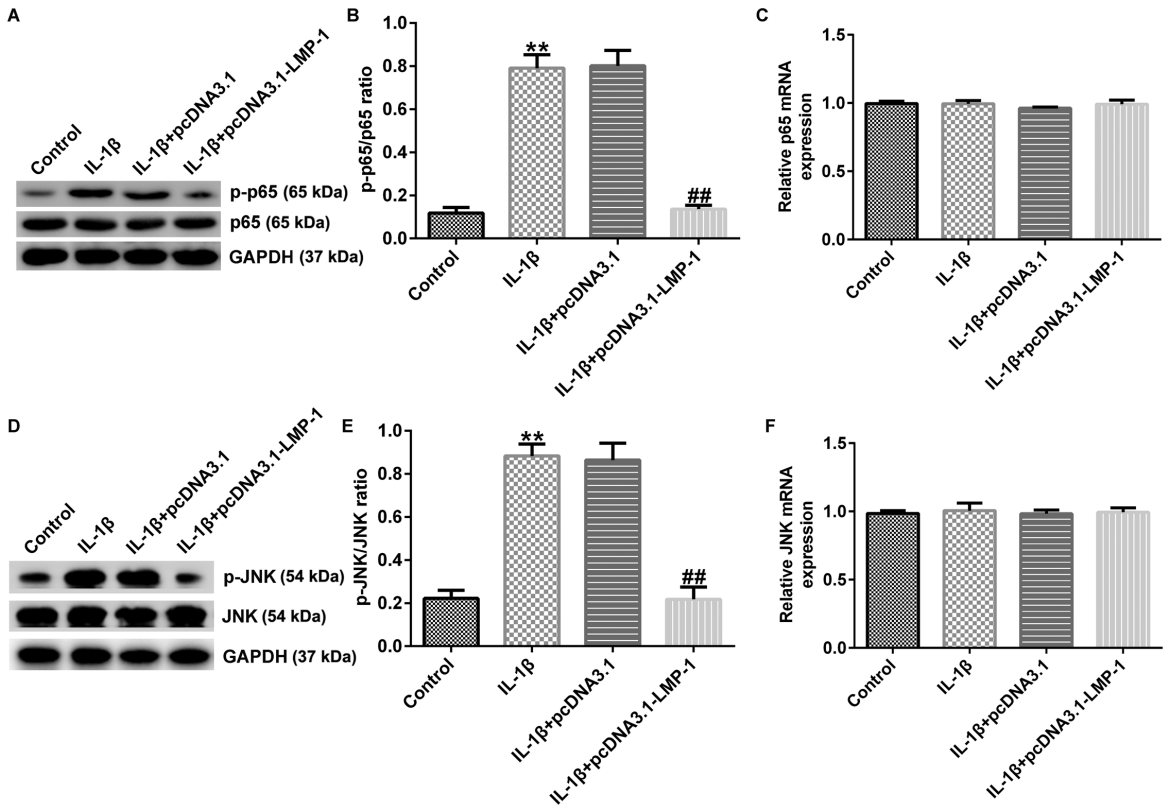


Figure 6. Effect of pcDNA3.1-LMP-1 on the NF-κB and MAPK/JNK pathways. CHON-001 cells were transfected with pcDNA3.1-LMP-1 or pcDNA3.1 for 24 h and stimulated with 10 ng/ml IL-1β for 12 h. (A) Expression levels of p-p65 and p65 were assessed via western blot analysis. (B) p-p65/p65 ratio was semi-quantified. (C) RT-qPCR analysis of p65 mRNA expression. (D) Expression levels of p-JNK and JNK were assessed via western blot analysis. (E) p-JNK/JNK ratio was semi-quantified. (F) RT-qPCR analysis of JNK mRNA expression. **P<0.01 vs. control; ##P<0.01 vs. IL-1β+NC-siRNA. LMP-1, LIM mineralization protein-1; RT-q, reverse transcription-quantitative; si, small interfering; NC, negative control; p-, phosphorylated.

*LIM-1 inhibits IL-1β-induced chondrocyte injury by regulating the NF-κB and MAPK/JNK pathways.* Previous reports have confirmed that NF-κB regulates the inflammatory response in numerous types of disease (19,21,22). Thus, the role of LMP-1 in NF-κB and MAPK/JNK pathways was assessed. CHON-001 cells were transfected with pcDNA3.1-LMP-1 or pcDNA3.1 for 24 h and stimulated with 10 ng/ml IL-1β for 12 h. Then, the expression levels of p-p65, p65, p-JNK and JNK were determined via western blotting and RT-qPCR analysis. IL-1β induced upregulation of p-p65 and increased the ratio of p-p65/p65 (Fig. 6A and B); these effects were significantly decreased in the IL-1β+pcDNA3.1-LMP-1 group. The mRNA expression levels of p65 presented no significant changes between the different groups (Fig. 6C). Consistently, IL-1β increased p-JNK protein expression and enhanced the p-JNK/JNK ratio (Fig. 6D and E); these effects were inhibited by pcDNA3.1-LMP-1. However, there were no significant differences in JNK (Fig. 6F) mRNA expression levels between groups. Taken together, these data indicated that LMP-1 inhibited IL-1β-induced human chondrocyte injury by regulating the JNK/NF-κB signaling pathway.

## Discussion

OA is a prevalent inflammatory disease, characterized by joint dysfunction, cartilage degeneration and articular cartilage breakdown (1,2). OA is a global public health problem and a cause of disability worldwide (32). Previous reports have revealed that OA is caused by numerous factors, including strain, trauma and joint deformity (1,33) and it is estimated that ~3 million new cases of OA occur every year. With the increasing age of the population, the incidence of OA has risen year by year (34). At present, there are no satisfactory therapies for chronic joint disease. Therefore, the present study aimed to identify an effective agent for OA treatment.

Previous studies have reported that IL-1β is associated with OA by regulating chondrocyte apoptosis and the inflammatory response (14,35). In the present study, CHON-001 cells were treated with different concentration of IL-1β (0.1, 2.0, 5.0 and 10.0 ng/ml) for 12 h to establish an OA model *in vitro*. It was found that the viability of CHON-001 cells was significantly decreased following treatment with 5 or 10 ng/ml IL-1β, which was consistent with a previous report (36). Thus, 10 ng/ml IL-1β induced CHON-001 cells were used as an *in vitro* OA model for subsequent experiments. CHON-001 is a fibroblast immortalized cell line established by hTERT transduction and therefore lacks certain important characteristics of primary chondrocytes. For example, primary chondrocytes are quiescent cells that rarely divide under physiological conditions, but CHON-001 cells are proliferative cells (37). CHON-001 cells, collected from an embryo (age, 18 weeks), are distinct from chondrocytes in adults (38). This was a limitation of the present study as the majority of patients with OA are elderly (39).

LMP-1, a regulator of bone formation, is found in fetal calvarial cells (27,40). Moreover, previous reports have shown that LMP-1 is involved in numerous types of disease, including tumor (41), dental caries (42), fracture repair (43) and intervertebral disc degeneration (28). Nevertheless, the mechanism by which LMP-1 protects chondrocytes from IL-1β-stimulated damage in CHON-001 cells remains unknown. Hence, the present study investigated the underlying mechanism of LMP-1 in IL-1β-induced CHON-001 cells. The results demonstrated that LMP-1 expression was significantly decreased in response to IL-1β treatment. The protective effect of LMP-1 against IL-1β-induced CHON-001 cell injury was also investigated. The present data indicated that LMP-1 expression was higher in the pcDNA3.1-LMP-1 group compared with that in the pcDNA3.1 group. LMP-1 was downregulated in IL-1β-induced CHON-001 cells compared with the control group. However, this inhibitory effect was reversed by pcDNA3.1-LMP-1. Furthermore, functional assays revealed that overexpression of LMP-1 alleviated IL-1β-induced CHON-001 cellular injury, as shown by increased cell viability, suppressed apoptosis and decreased cleaved-protein caspase 3 expression levels and cleaved-caspase 3/caspase 3 ratio.

Caspase 3, a member of cysteine-aspartic protease family, is involved in cellular components and apoptosis (44). Chung *et al* (45) reported the association between tacrolimus-induced apoptosis and caspase 3 and 12 in Jurkat cells. Consistent with earlier results (29), the present study observed that IL-1β increased cleaved-caspase 3 protein expression and promoted the cleaved-caspase 3/caspase 3 ratio. Overexpression of LMP-1 decreased cleaved-caspase 3 protein expression and the cleaved-caspase 3/caspase 3 ratio in IL-1β-induced CHON-001 cells. Due to caspase 3 cleavage, it was expected that levels of caspase 3 in cells exposed to IL-1β would be decreased but this was not the case and further research is needed.

Inflammatory factors serve a vital role in OA development, which results in joint tissue degradation (46). Chunlei *et al* (46) observed that microRNA-138-5p silencing protects chondrocytes (ATDC5 and CHON-001 cells) from IL-1β-induced inflammation by upregulating SOX9. Wang *et al* (47) reported that acupuncture can downregulate the expression of IL-1β and TNF-α in osteoarthritic chondrocytes. Yao *et al* (48) also demonstrated that Shenmai injection protects knee articular cartilage from IL-1β-induced human chondrocyte damage in OA rabbits. In addition, a previous study suggested that leonurine inhibits IL-1β-induced inflammation in a murine OA model (49). LMP-1 also exhibits an anti-inflammatory role in lipopolysaccharide-induced pre-osteoclasts (50). In the present study, the release of inflammatory cytokines (including IL-6, IL-8 and TNF-α) in IL-1β-induced CHON-001 cells was measured. IL-1β notably promoted the release of IL-6, IL-8 and TNF-α compared with the control group. Therefore, suppression of chondrocyte apoptosis and the inflammatory response may be beneficial for OA treatment. In the present study, overexpression of LMP-1 notably decreased the secretion of IL-6, IL-8 and TNF-α in IL-1β-stimulated chondrocytes. These findings demonstrated that LMP-1 alleviated the IL-1β-induced inflammatory response in chondrocytes cells.

To assess the effect of LMP-1 downregulation in IL-1β-stimulated OA chondrocytes, LMP-1 expression was knocked down in CHON-001 cells using LMP-1 siRNA. Functional analysis of LMP-1 siRNA was performed to verify whether it regulated the effects of IL-1β. The results revealed that knockdown of LMP-1 further aggravated IL-1β-induced chondrocyte injury, as evidenced by inhibited CHON-001 cell viability, increased apoptosis and enhanced IL-6, IL-8 and TNF-α release.

Previously, accumulating evidence confirmed that activated NF-κB molecules may cause articular joint damage, resulting in the progression of OA (51,52). Saito and Tanaka (51) revealed that inhibiting the Notch and NF-κB pathway prevents the progression of OA, whilst Lei *et al* (52) observed that ubiquitin specific peptidase 19 suppresses TNF-α and IL-1β-induced NF-κB activation by deubiquitinating mitogen-activated protein kinase kinase kinase 7. Furthermore, LMP-1 has been shown to promote bone formation and the association between LMP-1 and the JNK signaling pathway was confirmed in a previous study (50). The present study demonstrated that LMP-1 suppressed phosphorylation of p65 and JNK, and thus relieved the inflammatory response. However, there were no significant differences in p65 and JNK mRNA expression levels between the groups. These data suggested that LMP-1 relieved OA development by altering the NF-κB and MAPK/JNK pathways.

In conclusion, the present study identified that LMP-1 relieved IL-1β-induced inflammatory injury in CHON-001 cells via regulating the NF-κB and MAPK/JNK pathways, suggesting that targeting LMP-1 may alleviate cartilage damage in patients

with OA. Thus, upregulation of LMP-1 may be a promising therapeutic target for OA clinical treatment. However, the present study was a preliminary *in vitro* investigation of the role of LMP-1 in an *in vitro* model of OA. To confirm the role of LMP-1 in OA, more research is needed. For example, whether NF-κB and MAPK/JNK pathways are directly involved in the role of LMP-1 in IL-1β induced CHON-001 cells still needs to be analyzed, including the use of NF-κB and MAPK/JNK pathway inhibitors and agonists. Extracellular matrix degradation in response to IL-1β treatment also needs further investigation. The role of LMP-1 in other chondrocyte cell lines or primary chondrocytes should also be studied. Additional *in vivo* experiments are required to verify the precise mechanism of LMP-1 in OA progression in the future. Also, since OA can be caused by factors other than chondrocyte injury, investigation of the role of LMP-1 in synovial tissue or subchondral bone, as well as chondrocytes using a rodent OA model, should be performed. Moreover, the association between expression levels of LMP-1 and clinical characteristics of patients with OA needs to be clarified in the future.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

DO contributed to the study design, data collection, statistical analysis, data interpretation and manuscript preparation. CH contributed to data collection, statistical analysis and manuscript preparation. SL, CT, HT and YY contributed to data collection and statistical analysis. DO and CH performed the experiments. DO and CH confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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