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*Original Article*

# KLF-4 is a novel target of $^{99}$Tc-MDP mediating chondrocytes inflammation via NF-κB/iNOS/VCAM1 signaling

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**Abstract:** Osteoarthritis (OA) is a common joint disorder with varying degrees of inflammation in which the failure of chondrocytes to maintain a homeostatic balance between matrix synthesis and degradation plays pivotal role. The ideal anti-OA drug should have immunomodulatory effects while at the same time having limited or no toxicity. Yunke ($^{99}$Tc-MDP) has been widely used in China for the treatment of OA with good efficacy in suppressing inflammation. But the effect target and the underlying molecular mechanism in chondrocytes is still largely unknown. In this study, we examined the anti-inflammatory effects of YunKe in IL-1β-induced inflammatory chondrocytes HC-a cell lines and found KLF4 enhanced significantly in Yunke treated inflammatory cells. Lentivirus-mediated KLF4 knockdown or overexpression models were employed to detect the role of KLF4 in IL-1β-induced HC-a cells. Real-time RT-PCR and western blot were used to measure the expression of classic inflammation indicator MCP-1, IL-6, apoptosis regulators caspase-3, Bcl-2 and key factors of signal pathways. It was found that without KLF4, the anti-inflammation effect of Yunke will declined obviously and induced cell apoptosis, meanwhile, over-expression of KLF4 raised the anti-inflammation effect of Yunke and reduced cell apoptosis further. Our results suggest that Yunke could ease the IL-1β-induced chondrocytes inflammation and reduced cell apoptosis via targeting KLF4 partly and NF-κB/iNOS/VCAM1 pathways played important roles in this biological course.

**Keywords:** $^{99}$Tc-MDP (Yunke), osteoarthritis, chondrocytes, inflammation, Krüppel-like factor 4 (KLF4)

## Introduction

Osteoarthritis (OA) is the most common degenerative joint disease with swelling and severe pain which is characterized by chronic synovitis and progressive joint damage resulting from the degeneration of articular cartilage and the loss of cartilage matrix in affected joints in the world [1]. Cartilage is recognized as one of the main targeted structures responsible for joint diseases in OA patients. The pathological process of the disease involves changes in the survival of chondrocytes and is often associated with an inflammatory response. Chondrocytes are the only cells in articular cartilage, which play an important role in maintaining matrix integrity, pathological cascade process and tissue homeostasis [2]. Cartilage damage results from the failure of chondrocytes to maintain a homeostatic balance between matrix synthesis and degradation [3]. In this regard, chondrocytes inflammation plays a pivotal role in cartilage damage and the pathogenesis of OA [4].

Although the pathogenesis of OA has not been fully elucidated, it is generally accepted that cellular immune abnormality plays a crucial role. Increased production of proinflammatory cytokines (such as IFN-γ, IL-6 and TNF-α) and reduced levels of anti-inflammatory cytokines (such as IL-10 and factor TGF-β), which are correlated with disease activity [5, 6].

$^{99}$Tc-methylene diphosphonate ($^{99}$Tc-MDP, Yunke), a chemical compound consisting of technetium-99 conjugated with methylene diphosphonate, has been widely used in China for the treatment of OA with good efficacy. $^{99}$Tc-MDP is

effective in suppressing inflammation and bone erosion as well as reducing the production of serum TNF-α and IL-1β [7, 8]. We are interested in whether this drug exerts its therapeutic effect on OA by modulating inflammation reaction in chondrocytes and its underlining molecular mechanism.

Krüppel-like factor 4 (KLF4) is a zinc-finger transcription factor that plays a key role in cellular differentiation and proliferation during normal development and in various diseases, such as cancer. The results of recent studies have revealed that KLF4 is expressed in multiple vascular cell types, including phenotypically modulated smooth muscle cells, endothelial cells and monocytes/macrophages [9]. KLF4 is known to mitigate inflammation in several cell types. KLF4 was reported as an important role in controlling macrophage activation in 2005 [10]. Then the roles of Klf4 in mediating neuroinflammation and regulating the immunomodulatory activities of microglia were clarified [11, 12]. Besides, KLF4 mediated the inflammation in epidermal keratinocytes [13], kidney proximal tubular cells [14], astrocytes [15]. Interestingly, KLF4 is a newly identified critical target for the anti-inflammatory effect of honokiol (HNK) [16]. But the expression of KLF4 in IL-1β induced chondrocytes and if the KLF4 would be a critical target for the anti-inflammatory effect of Yunke were still unknown.

In this study, we focused on the KLF4 expression, its mediation of inflammation in IL-1β treated chondrocytes and we found KLF4 is a important target for the anti-inflammatory effect of Yunke in chondrocytes.

## Materials and methods

### *Cell culture and reagents*

Human chondrocytes cell line HC-a was purchased from Scien Cell Research Laboratories (USA, Cat No 4650). HC-a cells were cultured at a density of $10^5$ cells/ml in DMEM with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. Interleukin-1β (IL-1β) is a prominent pro-inflammatory cytokine which was purchased from Genscript (Nanjing, China) and Yunke was obtained from. HC-a were divided into four groups: HC-a without any treatments (Con), HC-a treated with 10 ng/ml IL-1β for 48 h (IL-1β), HC-a treated with 50 μmol/L Yunke for 48 h (99Tc-MDP), HC-a treated with 10 ng/ml IL-1β for 48 h then treated with 50 μmol/L Yunke for another 48 h (IL-1β+99Tc-MDP). The proteins were subjected to western blot analysis to measure their levels and primary antibodies against KLF4, MCP-1, caspase-3, VCAM-1 (1:500, Abcam), P65, iNOS (1:1000, Abcam), IL-6 and Cox-2 (1:1000; Santa Cruz, CA, USA), pNF-κB (Ser536) (1:1000, Cell signaling), Bcl-2 (1:1000, Immunoway, Suzhou, China) were used. The images were captured using the Genesnap software from Syngene (Syngene, Cambridge, UK). The blots were stripped and re-probed with Anti β-actin (1:10000; Sigma Aldrich, St. Louis, MO, USA) antibodies and the protein levels were normalized to β-actin.

### *Plasmids construction and lentivirus infection*

In order to explore the inflammation mediation of KLF4 in HC-a cells, the gain-of and Loss-of-function cell models were established using lentivirus infection system. The KLF4 specific shRNA sequence (F: CCGGTGTCAAGCAGGTGCCCCGAATTCAAGAGATTCGGGGCACCTGCTTGACTTTTTTG, R: AATTCAAAAAAGTCAAGCAGGTGCCCCGAATCTCTTGAATTCGGGGCACCTGCTTGACA) was inserted into pLKD.CMV.G&PR.U6.shRNA to generate recombinant plasmid pLKD.CMV.G&PR.U6.shRNA-KLF4. Human KLF4 was obtained from cDNA library by PCR amplification, the PCR primers were: F: CAGGTCGACTCTAGAGGATCC*GCCACC*TACTCCGTCGGTGGACCGCT, R: CTCACCATGGTGGCGGAATTC*CG*AATTTTTACGGAGAAGTACACATTCCG then the PCR product was cloned into pLOV.UbiC.EGFP to generate recombinant plasmid pLOV.UbiC.EGFP-KLF4. Consequently, the lentivirus package was performed by Auragene technology (Changsha, Hunan, China). HC-a were infected with Lv-KLF4-shRNA and Lv-KLF4 at the MOI of 20 respectively to get the loss-of and gain-of-function cell models for KLF4 cellular functional experiments.

### *Real-time RT-PCR*

Total RNA was extracted from cells with Trizol reagent (Invitrogen) following the manufacturer's instructions. The relative expression level of KLF4, MCP-1, IL-6, Cox-2 mRNA was detected by real-time RT-PCR using the standard SYBR Green RT-PCR Kit (Takara, Otsu, Japan) following the manufacturer's instructions. The

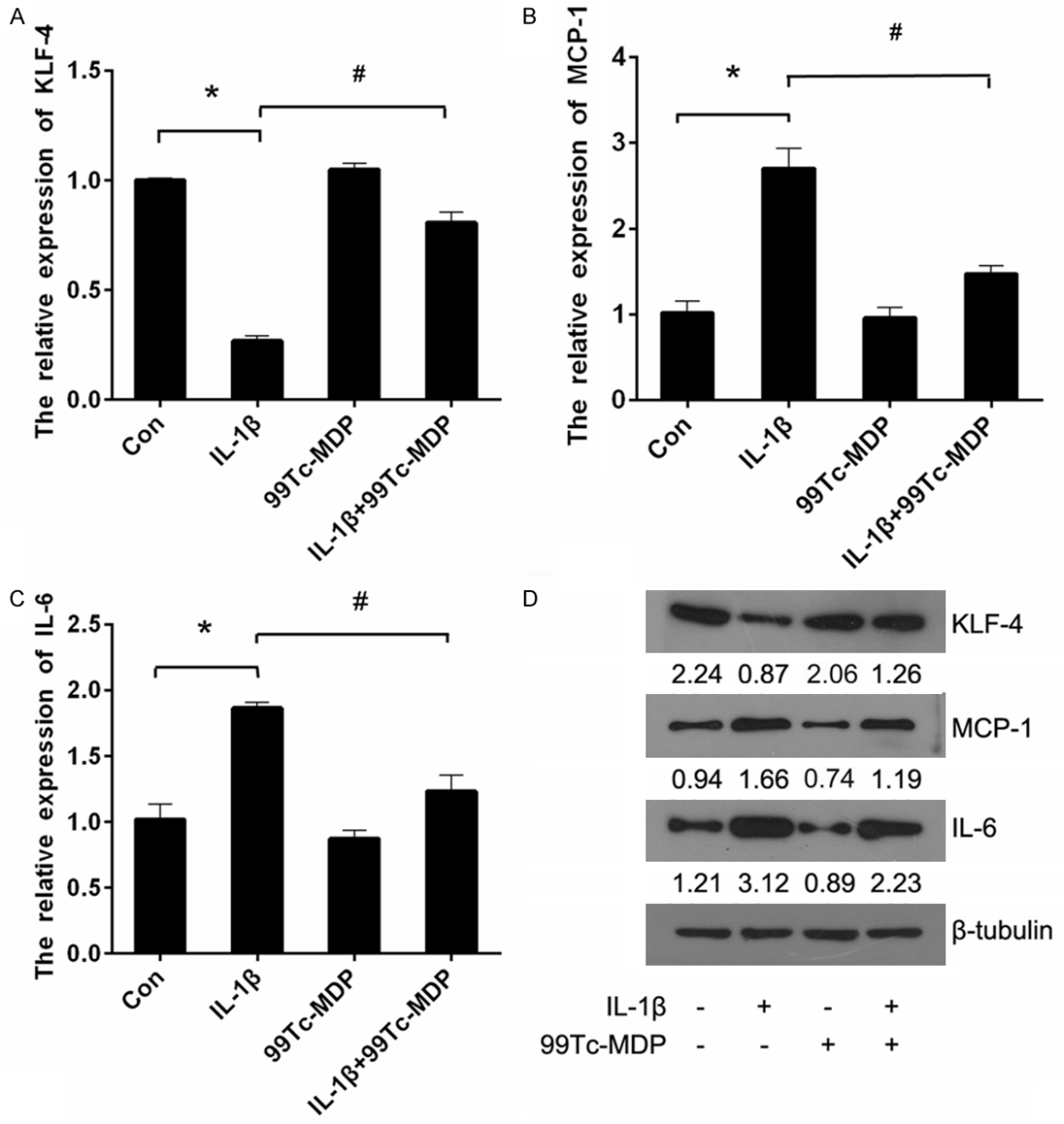


**Figure 1.** Yunke reduced the IL-1β induced chondrocytes inflammation. HC-a cells were divided into four groups: HC-a without any treatments (Con), HC-a treated with 10 ng/ml IL-1β for 48 h (IL-1β), treated with 50 μmol/L Yunke for 48 h (99Tc-MDP), treated with 10 ng/ml IL-1β for 48 h then treated with 50 μmol/L Yunke for another 48 h (IL-1β+99Tc-MDP). Relative mRNA levels and protein expression of KLF-4, MCP-1 and IL-6 in HC-a cells from different groups were detected using Real-time RT-PCR (A-C) and western blot (D). *P<0.05 VS. Con; #P<0.05 VS. IL-1β alone.

specific primer pairs are as follows: KLF4 F: CCCACATTAATGAGGCAGC and R: AGTCGCTTC-ATGTGGGAGAG; MCP-1 F: ACTTCACCAATAGG-AAGATCTCAGT and R: TGAAGATCACAGCTTCT-TTGG; IL-6 F: CCACACAGACAGCCACTCACC and R: CTACATTTGCCGAAGAGCCCT; Cox-2 F: ATCA-TAAGCAGGGCCAGCT and R: AAGGCGCAGTTTA-CGCTGTC; β-actin as an internal control, F: AGGGGCCGGACTCGTCATACT and R: GGCGGCA-CCACCATGTACCCT. The relative expression of target genes was quantified using the GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA) and 2-ΔΔCt method [17]. The values are expressed as the means ± SD from three separate experiments. Statistical analysis was carried out using SPSS 16.0 software. The difference between two groups was analyzed by the Student's t-test. A value of $P<0.05$ was considered to indicate a statistically significant result.

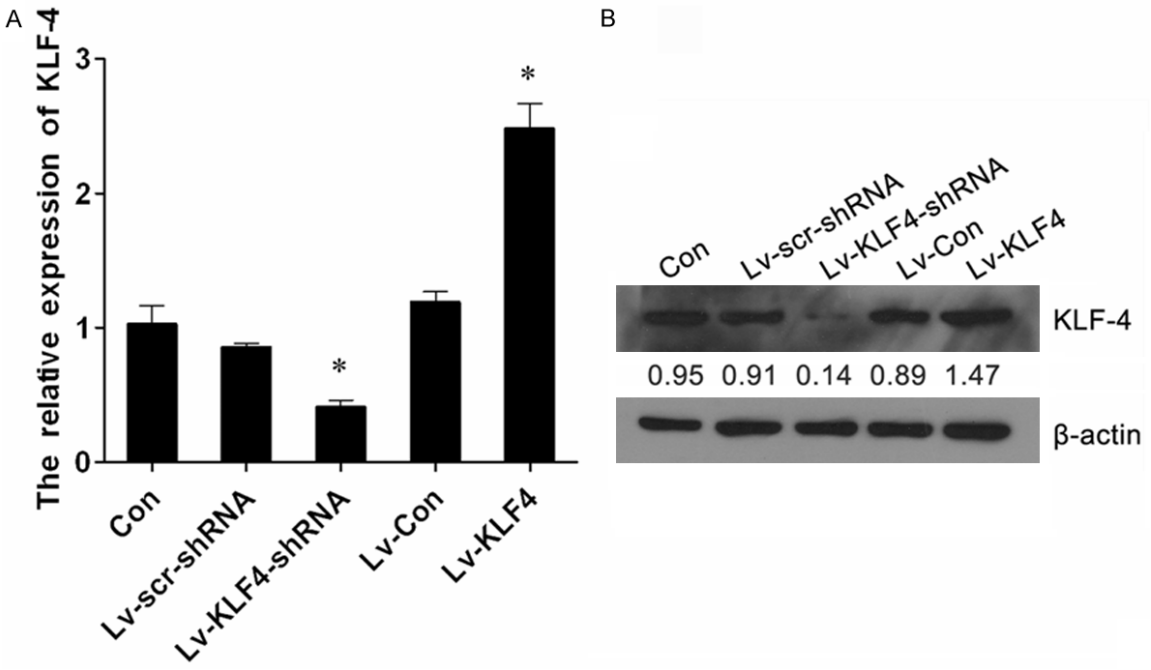


**Figure 2.** KLF-4 expression altered significantly in lentivirus infected HC-a functional model cells. Real-time RT-PCR and Western blot were performed to determine the mRNA level and protein expression of KLF-4 after infection with Lv-KLF4-shRNA and Lv-KLF4. The mRNA level of KLF-4 in gain and loss-of-function HC-a cells (A). The protein expression of KLF-4 in gain and loss-of-function HC-a cells (B). Con: cell without infection. Lv-scr-shRNA: cells were infectd with non-specific shRNA lentivirus. Lv-con: cells infected with over-expression vector. Lv-KLF4: cells infected with KLF4 over-expression lentivirus. *P<0.05 VS. Con.

*Western blotting*

Cells were solubilized in cold RIPA lysis buffer. After that, proteins were separated with 10% SDS-PAGE, and then transferred to a PVDF membrane. Membranes were blocked in 10% nonfat dried milk in PBST for 3 h and then incubated overnight at 4°C with specific primary antibodies with β-actin as a control. After incubation with the corresponding secondary antibody, immune complexes were detected using an ECL kit (Auragene Biotech, Changsha, China).

*Apoptosis analysis*

Flow cytometer was used to determine the cell apoptosis with the Annexin V-FITC Apoptosis Detection Kit. Cells were harvested and washed with cold PBS twice. After that, $10^6$ cells were resuspended in 200 μl binding buffer added with 10 μl Annexin-V-FITC and 5 μl PI-PE, and incubated in the dark for 30 min. Then, 300 μl binding buffer was added followed by flow cytometer assay. Statistical analysis was carried out using SPSS 16.0 software. The difference between two groups was analyzed by the Student's t-test. A value of $P<0.05$ was considered to indicate a statistically significant result.

## Results

*Yunke could reduce the IL-1β induced chondrocytes inflammation*

IL-1β (interleukin-1 beta) was a pro-inflammatory cyto-chemokines which could trigger inflammatory pathways in chondrocytes [11]. When the HC-a cells were treated with 10 ng/ml IL-1β for 48 h, the relative mRNA level of KLF4 was significantly reduced compared with the control group. Meanwhile, the mRNA level of inflammatory indicator protein MCP-1 and IL-6 was enhanced dramatically. While when the HC-a cells were treated with 50 μmol/L Yunke (99Tc-MDP) for 48 h, the relative mRNA level of KLF4, MCP-1 had little alteration but the IL-6 reduced significantly. It's worth noting that when the cells were treated with 10 ng/ml IL-1β for 48 h then treated with 50 μmol/L Yunke for another 48 h (IL-1β+99Tc-MDP), the relative mRNA level of KLF4 was significantly increased compared with the IL-1β group while the relative mRNA level of MCP-1 and IL-6 was

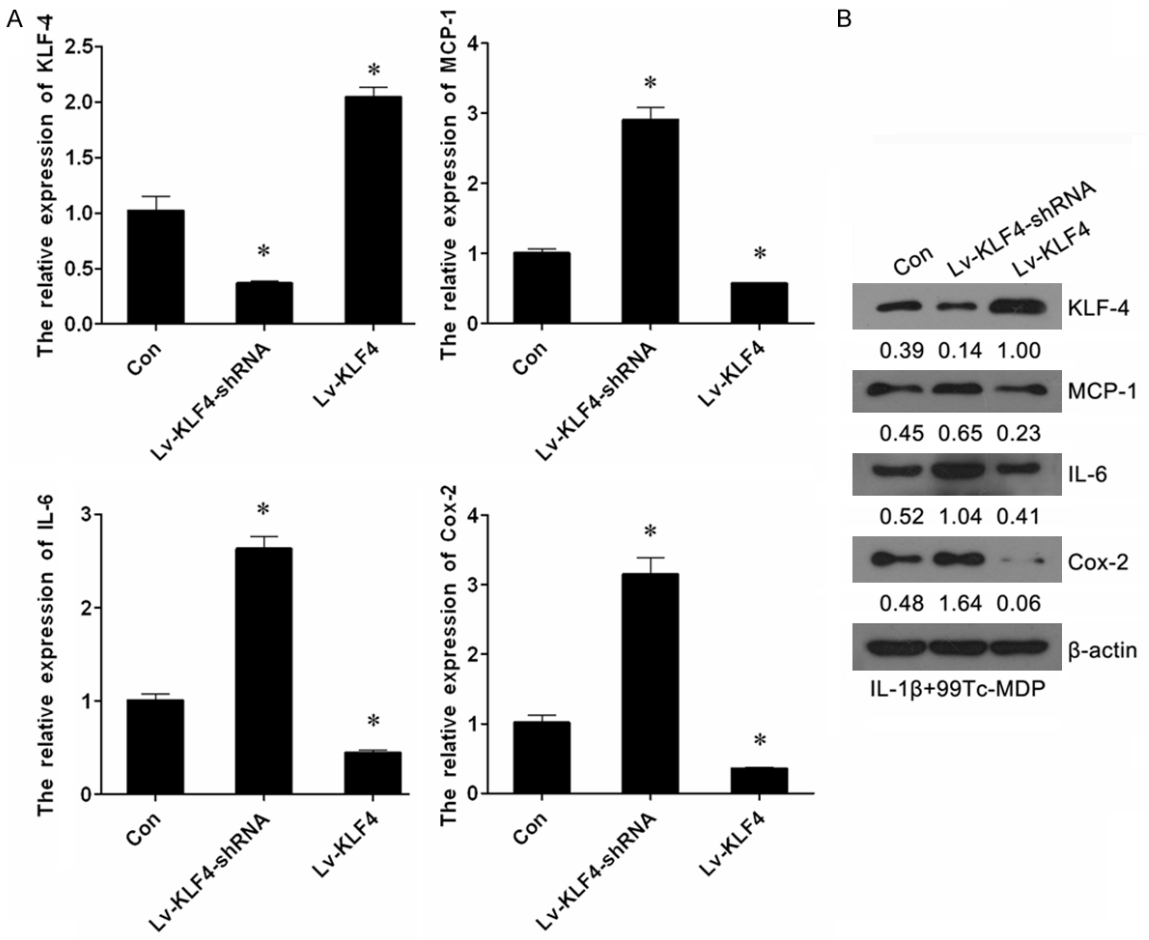


**Figure 3.** Yunke mediated inflammatory protein expression by targeting KLF-4. Parental cells (Con), KLF4 silenced cells (Lv-KLF4-shRNA) and KLF4 over-expressed cells (Lv-KLF4) were all treated with IL-1β for 48 h then with Yunke for another 48 h (IL-1β+99Tc-MDP). Relative mRNA levels and protein expression of KLF-4, MCP-1, IL-6 and Cox-2 in cells from different groups were detected using Real-time RT-PCR (A) and western blot (B). *P<0.05 VS. Con.

decreased comparing with IL-1β group (**Figure 1A-C**). The same similar results were obtained in protein level experiments (**Figure 1D**). It was found that IL-1β could inhibited KLF4 expression and enhanced inflammatory indicator protein. Yunke could not influence the KLF4 expression significantly in normal cells but could restore the KLF4 expression in IL-1β-induced KLF4 reduction effectively. These results suggest that Yunke would not affect the expression of KLF4 in normal cells but may play a role in IL-1β induced inflammation by mediating KLF4.

*Construction of lentivirus infected cell models*

In order to explore the importance of KLF4 played in anti-inflammatory effect of Yunke, gain-of and loss-of-function cell models were constructed in HC-a cells using lentivirus infection. Obviously, when the HC-a cells was infected with the Lv-KLF4-shRNA, the expression of KLF4 reduced remarkably at both mRNA and protein level. On the other hand, when the cells were infected with the Lv-KLF4, the expression of KLF4 enhanced remarkably at both mRNA and protein level compared with parental HC-a cells (**Figure 2**). The results demonstrated that the KLF4 model cells generated successfully.

*Yunke mediated inflammatory protein expression by targeting KLF4*

In order to verify if KLF4 is a direct target gene of the anti-inflammation effect of Yunke, the KLF4 gain-of and loss-of-function cell models were treated with 10 ng/ml IL-1β then with 50 μmol/L Yunke both for 48 h (IL-1β+99Tc-MDP). Then the key inflammatory indicate molecular IL-6, MCP-1, Cox-2 was measured in both mRNA

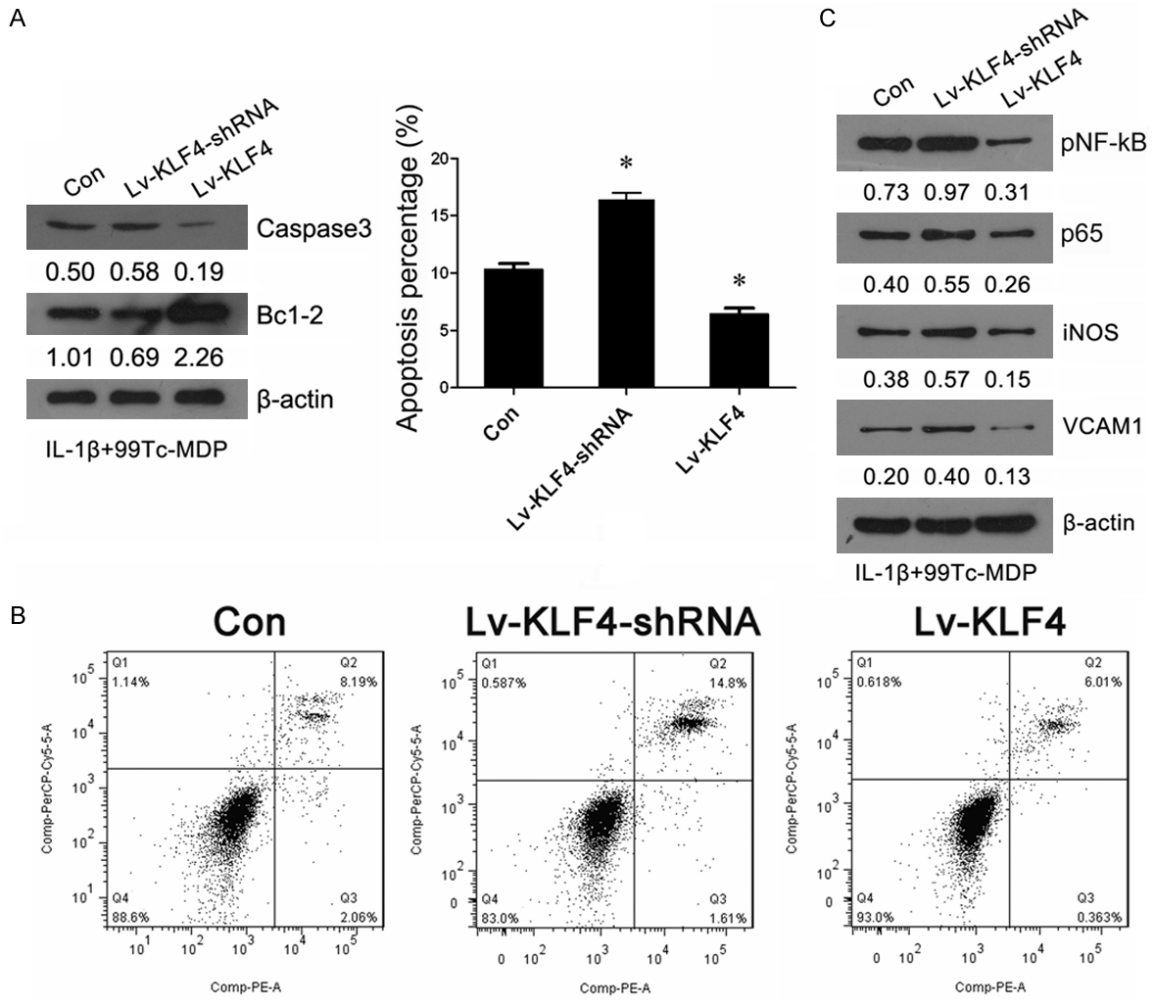


**Figure 4.** Yunke eased inflammation induced apoptosis by targeting KLF-4 via NF-κB/eNOS/VCAM1 pathways. Parental cells (Con), KLF4 silenced cells (Lv-KLF4-shRNA) and KLF4 over-expressed cells (Lv-KLF4) were all treated with IL-1β for 48 h then with Yunke for another 48 h (IL-1β+99Tc-MDP). Protein expression of apoptosis effector caspase-3 and anti-apoptosis factor Bcl-2 was detected by western blot (A). Flow cytometer apoptosis assay show the apoptosis rate of different cell groups, $^{*}$P<0.05 VS. Con, (B). Protein expression of important molecular of NF-κB/eNOS/VCAM1 pathways was detected by western blot (C).

and protein expression levels. In KLF4 loss-of-function cell models (Lv-KLF4-shRNA), Yunke could not reduce but increase the mRNA and protein expression levels of the inflammatory indicate molecular comparing with the normal HC-a cells (Con). Otherwise, in KLF4 gain-of-function cell models (Lv-KLF4), Yunke could further reduce the mRNA and protein expression levels of the inflammatory indicate molecular comparing with the normal HC-a cells (Con) (**Figure 3**). From the results, we concluded that in IL-1β induced inflammatory HC-a cells, the exertion of the anti-inflammatory effect of Yunke should via KLF4 to some extent.

*Yunke eased inflammation induced apoptosis by targeting KLF4*

Serious inflammation trigged chondrocytes apoptosis. While if Yunke plays a important role in easing inflammation-induced cell apoptosis is unknown. We generated western blot and flow cytometer apoptosis to evaluate the apoptosis status of HC-a model cells. In KLF4 loss-of-function cell models (Lv-KLF4-shRNA), Yunke could not reduce but increase the apoptosis molecular caspase-3 a little comparing with the normal HC-a cells treated with IL-1β and 99Tc-MDP (Con). While in KLF4 gain-of-function cell models (Lv-KLF4), Yunke further reduced

caspase-3 expression significantly comparing with the normal HC-a cells treated with IL-1β and 99Tc-MDP (Con). The anti-apoptosis protein Bcl-2 drew an opposite conclusion (**Figure 4A**). The same result was shown in flow cytometer apoptosis assay, when KLF4 was knock down, the anti-apoptosis effect of Yunke got weaker but when KLF4 was over-expressed, it got even much stronger (**Figure 4B**). These results suggest that Yunke eased inflammation induced apoptosis by targeting KLF4 a least partly.

*Yunke mediated chondrocytes inflammation and inflammatory apoptosis by KLF4 via NF-κB/iNOS/VCAM1 pathways*

To characterize the molecular mechanism underlying the role of KLF4 in Yunke mediated anti-inflammation and apoptosis, the protein levels of NF-κB, P65, iNOS, and VCAM-1 were detected in KLF4 knock down and over-expressed cell models which were co-treated with IL-1β and 99Tc-MDP using western blot analysis. The results showed that in IL-1β-induced inflammatory HC-a cells, knock down of KLF4 significantly increased pNF-κB, P65, iNOS and VCAM1 expression, the anti-inflammation effect of Yunke was eased. On the contrary, enhanced KLF4 significantly reduced pNF-κB, P65, iNOS and VCAM1 expression, the anti-inflammation effect of Yunke was further reinforced. These results demonstrated that Yunke cured chondrocytes inflammation and apoptosis targeting KLF4 via inhibiting NF-κB/iNOS/VCAM1 signaling.

## Discussion

Chondrocyte survival or apoptosis and inflammation are important in the pathogenesis of OA. IL-1β is a pro-inflammatory cytokine in OA pathogenesis, which is widely used on chondrocytes to establish an OA model [18, 19]. IL-1β can also induce chondrocyte apoptosis to establish an experimental apoptosis model [20]. In our research, we treated HC-a cells with IL-1β to generate OA cell models, and then the OA cell models were treated with Yunke to clarify its anti-inflammatory effect. Because of the anti-inflammatory effect of Yunke, although the KLF4 in normal cells did not changed, but the endogenous inflammatory molecular MCP-1 and IL-6 reduced a little but not obviously.

Yunke has been widely used in China for the treatment of OA with good efficacy, but the underlying molecular mechanism is unknown. KLF4 is a mediator of pro-inflammatory signaling in macrophages is via regulating M1/M2 macrophage polarization [10, 21] and KLF4 is a newly identified critical target for the anti-inflammatory effect of honokiol [16]. These means the role of KLF4 in inflammatory-mediation exists in cell-type-specificity. In our study, the IL-1β treatment reduced the KLF4 expression significantly, but Yunke restored the IL-1β-induced reduction of endogenous KLF4 obviously. These results suggest KLF4 may play a key role in anti-inflammatory of Yunke. The lentivirus-mediated knockdown or overexpression of KLF4 in IL-1β treated HC-a cells, and then treated with Yunke; our results demonstrated that without KLF4, the anti-inflammatory and anti-apoptosis effect of Yunke reduced a lot, while when KLF4 was over-expressed, the conclusion was opposite. Thus, we concluded that KLF4 is an anti-inflammation regulator in chondrocytes and is a critical target of Yunke at least in a sense.

IL-1β has been reported to activate some intracellular key factors on the signal pathway, such as NF-κB [22]. When NF-κB is activated by IL-1β, gene expression of TNFa, iNOS and COX-2 obviously increase [22, 23]. Notably, OA progress was aggravated because of abundant factors, such as TNFa, iNOS and COX-2 induced by activated NF-κB. Thus, we detected the relative protein expression of pNF-κB, P65, iNOS and Cox-2, and found when KLF4 was knockdown, IL-1β induced chondrocytes inflammation could not be eased effectively by Yunke comparing to normal cells, and the expression of pNF-κB, P65 increased, as same as NF-κB induced iNOS and COX-2. While, when KLF4 was over-expressed, IL-1β induced chondrocytes inflammation eased more effectively by Yunke comparing to normal cells, and the expression of pNF-κB, P65 reduced, as same as NF-κB induced iNOS and COX-2. These results were in coincidence with the previous studies. KLF4 could attenuates the inflammation-related induction of the VCAM1 gene by inhibiting the binding of P65 to the VCAM1 promoter via the association between the KLF4 and P65 [9]. In our research, the VCAM1 expression also be regulated by KLF4, but further research is needed to clarify if VCAM1 is co-regulated by KLF4 and P65 in chondrocytes inflammation.

When NF-κB is activated, P65 will translocate from cytoplasm to nucleus [24], and IL-1β induced NF-κB activation not only trigged cell inflammation but also cell apoptosis [25]. In our study, KLF4 regulates the IL-1β induced chondrocytes apoptosis and KLF4 is a novel target of anti-apoptosis which is trigged by inflammation. But if the anti-apoptosis effect of KLF4 in chondrocytes is mediated by NF-κB pathway also need further investigation.

## Disclosure of conflict of interest

None.

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