

Curcumin Improve Tendinopathy via Regulation TLR4 Pathway in Vitro and Vivo Study

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Research

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Abstract

Background

Tendon disorders are a common type of soft tissue disease mainly characterized by various tendon syndromes with chronic pain. The purpose of this study was to evaluate curcumin had effects to improve tendon disorders by vitro and vivo study.

Results

Curcumin shows pharmacological activities similar to that of traditional anti-inflammatory agents and favorable therapeutic effects on various chronic inflammation-related diseases. In our present study, we discussed curcumin had effects to improve tendinopathy by vitro and vivo study. In further research, the study clearly explained the mechanisms by vitro study. The results were shown that curcumin had effects to improved tendinopathy which induced by TLR4/NF- κ B(p65) pathway activating in vivo and vitro experiments. However, with TLR4 supplement, the treatment effect of curcumin has been disappear.

Conclusion

Curcumin had effects to improve tendinopathy via regulation depressing TLR4/NF- κ B(p65) pathway and suppressing p-NF- κ B(p65) nuclear volume.

Introduction

Tendon injuries and disorders are increasing with the popularity of personal computers and smart phones, the increased participation in physical exercise and sports, continual incidence of accidents and a rapidly aging population. Tendon disorders are a common type of soft tissue disease mainly characterized by various tendon syndromes with chronic pain, mostly found in athletes and aging population (Koca TT, 2017; Snedeker JG, 2017). These disorders are considered to be a degenerative disease with manifestations of tendon extracellular matrix degradation, replacement of ordered microstructure with disordered mucilage matrix, increased cell counts with decreased long spindle-shaped tendon cells and increased fibrocartilage-like cells, and increased neovascularization (Morita W, 2016; Ackermann PW, 2016; Schwellnus MP, 2018). Tendinopathy, tendon calcification and degeneration, dyskinesia and secondary arthritis bring enormous economic and social burdens. Therefore, it is of great significance to seek effective and safe medications for these diseases.

Curcumin, a polyphenol extracted from turmeric, has long been used in food. Curcumin shows pharmacological activities similar to that of traditional anti-inflammatory agents (Goel A, 2008; Zhang W, 2018) and favorable therapeutic effects on various chronic inflammation-related diseases. For instance, curcumin protects against inflammatory kidney injury in rats with diabetic nephropathy (Vanaie A, 2019), and plays a protective role in the brain by reducing inflammation and enhancing blood-brain barrier in a rat model of inflammatory response with ischemic brain injury (Pluta R, 2018). Similarly, curcumin is shown to reduce inflammatory responses by regulating NF- κ B-related signaling pathways in a rat model

of sepsis with acute lung injury (Shaikh SB, 2019). Anti-inflammatory, anti-oxidative and anti-apoptotic effects of curcumin have also been confirmed in studies on osteoarthritis (Crivelli B, 2019). However, effects of curcumin in improving tendinopathy and related mechanisms have been rarely reported to date.

On this basis, in the present study, tendinopathy models were established *in vivo* and *in vitro* to observe the changes in tendon fibrosis, apoptosis and proliferation of tendon cells treated with curcumin, and the improvement effect of curcumin on tendinopathy-induced cell apoptosis and proliferation damage as well as its relationship with inflammatory signaling pathways and the underlying mechanisms were explored via determining the expression of proteins in inflammation-related signaling pathways, providing a novel and reliable theoretical basis for the treatment of tendon disorders.

Materials And Methods

Materials

Experimental animals and establishment of animal models

Thirty CL female SD rats, 4-week old, weighting 150-180g, were purchased from Shanghai Slack Laboratory Animals Co., Ltd. These animals were anesthetized with chloral hydrate (0.3~0.5g/kg, ip) and placed on the operation table. After disinfected with the iodophor, type I collagenase (12.5 U/leg) was injected into bilateral achilles tendons along the muscle from the calcaneus to simulate tendon injury. 30 rats were randomly divided into the NC group, the Model group, the Cur-Low group, the Cur-Middle group and the Cur-High group, with 6 rats in each group. Animals in the Model group, the Cur-Low group, the Cur-Middle group and the Cur-High group were given 20 mg/kg, 40 mg/kg and 200 mg/kg curcumin daily by gavage after successful modeling, and those in the NC group were given the same volume of 0.9% saline by gavage, for 28 days. After fasting for 12hrs, tendons were removed from these rats and fixed with 4% paraformaldehyde for 24hrs. These tissues were then imbedded with paraffin and sliced into 5- μ m sections.

Isolation and identification of tendon stem cells

Newborn (1-3d) SD rats were decapitated and soaked in 75% alcohol for 5mins. Tendons were removed aseptically and washed with sterile PBS containing penicillin, streptomycin and amphotericin B for 3 times. After removing adipose tissue, necrotic tissue and red blood cells, the tendon tissue was diced, transferred into a centrifuge tube, and digested with an appropriate amount of collagenase at 37°C for 1hr, shaking once every 5mins. The digestion was terminated by adding Ham's-F12 containing 10% FBS. The diced tissue was washed 3 times and the digestion solution was collected and filtered with 70- μ m and 40- μ m cell filters. The filtrate was centrifuged at 1500rpm for 11mins, the supernatant was discarded, and an appropriate amount of D/F12medium supplemented with 10% FBS was added to re-suspend cell pallet. The cell suspension was inoculated into a polylysine-coated flask and cultured in an incubator with 5% CO₂ and saturated humidity at 37°C. Medium was changed every 2 days. Cultured cells were collected by centrifugation at 1000rpm for 5mins, washed twice with PBS, and centrifuged again to remove the

supernatant. Appropriate amount of antibody was added to the cells according to the instructions provided with the antibody, and then incubated at 37°C for 30mins away from light. After adding 400µl PBS, the cells were subjected to flow cytometry for phenotype analysis.

Establishment of cell model of tendinopathy

Three-passage tendon stem cells (TDSCs) were collected and seeded into each well of a 96-well plate at 2000 cells per well, with 6 replicate wells for each group. 100µl basal medium was added to each well and 50ng/ml TGF-β1 (Sigma, USA) was added to the TDSCs in each well to establish the cell model of tendinopathy. Control cells were incubated with PBS-DMSO.

Cell Treatment

Three-passage TDSCs were selected and divided into the NC group, the Model group, the Cur-Low group, the Cur-Middle group and the Cur-High group. Cells in the Model group, the Cur-Low group, the Cur-Middle group and the Cur-High group were treated with TNF-β1 to construct the cell model of tendinopathy, and then treated with 20mg/mL, 40mg/mL and 200mg/mL curcumin, respectively. Cells in the NC group were routinely cultured. After 48hrs of treatment, these cells were used for subsequent assays.

Cell transfection

Three-passage TDSCs were inoculated into each well of a 6-well plate before transfection. After 70-80% confluency was reached in the next day, si-NC, si-TLR4 and TLR4 (KeyGEN Biotech, Nanjing, China) were transfected into these cells with liposome 3000, respectively. After 48 hours of transfection, different treatments were performed according to the experimental protocol for further assays.

Serum collection and testing

After treated for 28 days, rats in each group were fasted for 12hrs and then anesthetized with chloral hydrate (0.3-0.5 g/kg, ip). Abdominal cavity was opened and blood was taken from the inferior vena cava. Blood samples were centrifuged at 3000g/min for 5mins and serum was taken and stored at -20°C for testing.

HE staining

Sections were routinely dewaxed, rehydrated and soaked in xylene for 5mins and with replaced xylene for extra 5mins, followed by immersion in anhydrous ethanol for 5mins, in 95% ethanol for 5mins, in 85% ethanol for 5mins, in 70% ethanol for 5mins, and washed with PBS for three times 3mins each time. The sections were incubated with nuclear staining solution (Reagent I in the kit) for 3-5mins and washed with water for about 30-60secs. Then the sections were incubated with Color separation solution I (Reagent II) for about 20secs and washed with water for 30-60secs, followed by incubation with Color separation solution II (Reagent III) for about 40secs and washing with water for about 30-60secs. The sections were then stained with plasma-cell staining solution (Reagent IV) for 2mins, and the excess dye solution was

washed with color-enhancing solution (Reagent V) twice. The sections were dried with filter paper and mounted for microscopic examination.

Masson staining

After routine processing, tissue sections were stained with hematoxylin for 5mins and washed with distilled water. Sections were differentiated with 0.1% HCl, washed with running tap water, and stained with aniline blue solution. Then these sections were counterstained with acid ponceau red staining solution for 5mins, rinsed with distilled water, and incubated with 1% phosphomolybdic acid for 50secs. After water on the slide was shaken off (instead of washing with water), the sections were stained with fast green staining solution for 5secs, washed with tap water, and dried in an electric heating air drying oven. After that, the sections were mounted with neutral gum and cover glass and observed and photographed microscopically. The percentage of collagen-positive area in rat tendon tissue in each treatment group was analyzed using the Image J software.

TUNEL staining

After routine processing, the sections were incubated with 1% Triton-100 at room temperature (RT) for 15mins, and washed with PBS for 5mins for 3 times. Sections were treated with 3% H₂O₂-methanol solution for 15mins and washed with PBS for 5mins for 3 times. For each section, 100µl prepared proteinase K was added dropwise and incubated at 37°C for 30mins, and then 100µl Streptavidin-HRP labeled working solution was added and incubated in moistened conditions at 37°C for 1hr away from light. After washing with PBS for 3 times and 5mins each time, the sections were incubated with hematoxylin staining solution for 10mins and rinsed with distilled water. If the staining was too dark, sections were differentiated with 0.1% HCl. After rinsing with tap water, the sections were incubated with aniline blue solution. Sections were immersed in 70% ethanol, 85% ethanol, 95% ethanol, and anhydrous ethanol, respectively, 5mins for each time, and in xylene for 10mins and replaced xylene for extra 10mins. After drying, sections were mounted with neutral gum and sealed with cover-slides. Tissue cells were observed under a light microscope and 3 areas high-expression areas were chosen and photographed. The number of positive apoptotic cells in tendon tissue was determined by Image J image analysis software for each group.

IHC staining

After routine processing, sections on high temperature plastic dyeing rack was placed in a dyeing box containing antigen retrieval buffer and heated to medium heat for 10mins in a microwave oven. The dyeing box was removed and immersed in running water (the slides were not taken out of the buffer and cooled). After natural cooling and washing with PBS for 3 times, 2 drops of 3% H₂O₂-methanol solution were added to each section and blocked at RT (15-25°C) for 10mins and washed with PBS for 3mins × 3 times. 1% BSA 50-100µl was added dropwise to each section and incubated at RT for 20mins. After shaking off the liquid (no washing), 50-100µl primary antibody was added dropwise and incubated overnight at 4°C. After washed with PBS for 3mins × 3 times, 50µl enhancer was added dropwise and

incubated at RT for 20mins in a wet box. Sections were washed with PBS for 3mins × 3 times, and 50µl universal mouse/rabbit polymer was added dropwise and incubated for 20mins in a humid chamber at 37°C. After washing with PBS for 3mins × 3 times, 2 drops of freshly prepared DAB solution were added to each slide and the dyeing degree was observed microscopically. Staining was immediately terminated if the dyeing was appropriate by gently rinse the slide with tap water for 15mins and then with distilled water. Sections were incubated with hematoxylin solution for 10mins and rinsed with distilled water. If the staining was too dark, sections were differentiated with 0.1% HCl. After rinsing with tap water, the sections were incubated with aniline blue solution. Sections were immersed in 70% ethanol, 85% ethanol, 95% ethanol, and anhydrous ethanol, respectively, 5mins for each time, and in xylene for 10mins and replaced xylene for extra 10mins. After drying, sections were mounted with neutral gum and sealed with cover-slides. The expression of proteins in tissue cells was observed under a light microscope, and 3 high-expression areas were selected and photographed (magnification at 400× for all photos). Expression of proteins was analyzed by Image J software for each treatment group. TLR4 (ab22048, Abcam, Cambridge, UK), NF-κB (p65) (ab1650, Abcam, Cambridge, UK) and p-NF-κB (p65) (ab86299, Abcam, Cambridge, UK) were purchased from Abcam.

RT-PCR analysis

Rat tendon tissues or cells were collected and total RNA was extracted. Concentration of total RNAs was determined and cDNAs were synthesized by reverse transcription according to the instructions from the kit. 2µl cDNA was used for RT-PCR reaction with GAPDH as an internal reference. The sequences of primers used were shown in Table 1. RT-PCR reaction conditions included pre-denaturing at 94°C for 30s, and 40 cycles of denaturing at 94°C, 5s, and annealing at 60°C. Three independent experiments were conducted. Relative expression of the target genes was calculated by the $2^{-\Delta\Delta Ct}$ method.

Table 1. The primer sequence

Gene name	Primer sequence	Size
TLR4	F: 5'- TCCAGAGCCGTTGGTGTATCTT-3'	132bp
	R: 5'- ACAATTCGACCTGCTGCCTCA-3;	
NF-κB(p65)	F: 5'- TGTGGTGGAGGACTTGCTGAGG-3'	141bp
	R: 5'- GGAGTGCTGCCTTGCTGTTCTT-3'	
GAPDH	F: 5'- AGGTTGTCTCCTGTGACTTCAA-3'	130bp
	F: 5'- CTGTTGCTGTAGCCATATTCATTG-3'	

Western blot analysis

Total protein was extracted from the tendon tissue or cells and the concentration was determined by BCA method. Certain amount of total protein was separated by 12% SDS-PAGE gel electrophoresis with voltage

of 80 V for 90mins and then with 100V until the end of run (i.e., bromophenol blue band to 0.5cm from the bottom of the separation gel). The separated proteins in the gel were transferred to PVDF membrane with constant current 280mA for 1hr, and the membrane was stained with ponceau-S for 10mins to confirm the successful transfer. The successfully transferred membrane was blocked with TBST blocking solution containing 5% skim milk powder for 1.5hrs. Then primary antibody was added and incubated at 4°C overnight. Secondary antibody was added and incubated with the membrane at 37°C for 2hrs, followed by rinsing with TBST for 10mins × 3 times and color development. Protein bands were analyzed using Quantity One software to determine the absorbance of the target protein and β-actin and calculate the relative expression level of the target protein.

MTT assay

Cells were cultured for 24hrs in each group and the cell proliferation ability was measured according to the instructions of the MTT cell proliferation and cytotoxicity test kit. Briefly, cells in each group were harvested and washed twice with PBS. Cells were collected by centrifugation again and 5.0×10^5 cells were re-suspended in 500μl DMEM containing 0.5% MTT and cultured extra 4hrs. Then the culture medium was carefully removed and discarded, and 100μl DMSO was added to each well. The plate was shaken at a low speed for 10mins to dissolve the crystals formed, and the absorbance of each well was measured with a microplate reader. Cell proliferation rate was calculated for each group.

Flow cytometry analysis for cell apoptosis

Cells were cultured for 24hrs in each group and cell apoptosis was determined according to the instructions of Annexin V-FITC apoptosis detection kit. Briefly, cells in each group were harvested and washed twice with PBS. Cells were collected by centrifugation again and 5.0×10^5 cells were re-suspended in 500μl 1×Binding buffer. 1μl Annexin V-FITC and 5μl PI were added, mixed well and incubated for 5mins away from light. The cells were then subjected to cytometry analysis and apoptosis was recorded.

Cell TUNEL assay

Cell sample (cell smear) was naturally dried and fixed with 4% paraformaldehyde fixative for 30mins or overnight to improve cell permeability, followed by washing with PBS for 3mins × 3 times. 1% Triton-100 was added to the cells and incubated at RT for 15mins. After washed with PBS for 5mins X 3 times, 3% H₂O₂-methanol solution was added to the sample and incubated for 15mins, followed by washing with PBS for 5mins X 3 times. 100μl Protease K was added to each sample and incubated at 37°C for 30mins, and 100μl Streptavidin-TRITC labeled working solution was added and incubated for 1hr in dark and moistened condition at 37°C for 1hr. After washing with PBS for 5mins X 3 times, 100μl DAPI working solution was added to each sample and incubated at RT for 5mins away from light, followed by washing with PBS for 5mins X 3 times, samples were observed under a fluorescence microscope and the number of apoptotic cells in each group was determined using Image J image analysis software.

Cell immunofluorescence assay

Cell sample (cell smear) was naturally dried and fixed with 4% paraformaldehyde fixative for 30mins or overnight to improve cell permeability, followed by washing with PBS for 3mins × 3 times. 2 drops of 3% H₂O₂-methanol solution was added to each slide and blocked at RT (15-25°C) for 10mins. After washing with PBS 3 times, 50-100µl of ready-to-use goat serum was added to each slide and incubated for 20mins at RT, and then 50-100µl primary antibody was added and incubated at 37°C for 2hrs in a wet box. After washing with PBS for 3 times, 50-100µl FITC-labeled secondary antibody was added and incubated at 37°C for 1hr away from light. The slide was washed with PBS for 3 times and 50-100µl DAPI dye solution was added to each sample for 5mins at RT in darkness. The slide was then mounted with anti-quenching sealant and the nucleus translocation of proteins was observed under a microscope. 3 high-expression areas were selected and photographed.

Statistical analysis

Data were analyzed using SPSS 21.0 statistical software. Data with normal distribution were presented as mean ± standard deviation (Mean ± SD). One-way ANOVA was used and the LSD-t test was applied for pairwise comparison between groups. P < 0.05 was considered statistically significant.

Results

Inflammatory factors and pathological changes of tendons in rats

Elisa results showed that the levels of serum TNF-α, IL-1β and IL-6 in the Model group were significantly increased compared with that in the NC group (p<0.001 for all comparisons; Fig.1A). Serum levels of TNF-α, IL-1β and IL-6 were significantly down-regulated after curcumin treatment (p<0.05 for all comparisons; Fig.1A) with a significant dose-effect relationship, and a significant difference between the curcumin-treated groups was noted (p<0.05 for all comparisons; Fig.1A). From Fig.1B it was seen that the structure of cells was clear in the NC group with no obvious necrosis and degeneration and no inflammatory cell infiltration in tendon interstitium; the structure of cells in the Model group was blurred with no significant necrosis and slight degeneration, and a large amount of inflammatory cell infiltration was observed in the interstitium; and after the intervention of curcumin, the cell structure was clear with no obvious necrosis and slight degeneration, and a small amount of inflammatory cell infiltration was noted in the Interstitium.

Collagen content and apoptotic cells in rats

Tendon fibrosis and tendon cell apoptosis were determined by Masson and TUNEL staining. The degree of fibrosis and the number of apoptotic cells in the Model group were significantly higher than those in the NC group (P<0.05 for all comparisons; Fig.2A&2B). After treatment with different concentrations of curcumin, the degree of fibrosis and the number of apoptotic cells in these curcumin groups were significantly lower than those in the Model group (P<0.05 for all comparisons; Fig. 2A & 2B), and significant differences were observed among the curcumin groups (P< 0.05 for all comparisons; Fig.2A & 2B).

Related genes and proteins levels in rat tendon tissue by RT-qPCR and WB

The results of RT-qPCR and WB showed that compared with the NC group, the expression levels of TLR4 and NF- κ B (p65) genes and proteins ($P < 0.001$ for all comparisons; Fig.3A&3B) as well as the ratio of p-NF- κ B (p65)/NF- κ B (p65) were significantly increased ($P < 0.001$, Fig.3B) in the Model group. After treated with curcumin, the expression levels of TLR4 and NF- κ B (p65) genes and proteins ($P < 0.001$ for all comparisons; Fig. 3A & 3B) as well as the ratio of p-NF- κ B (p65) / NF- κ B (p65) ($P < 0.001$, Fig. 3B) were significantly decreased in the curcumin-treated groups compared with those in the Model group, with significant differences among the curcumin groups ($P < 0.05$ for all comparisons; Fig.3B).

Expression of TLR4, NF- κ B (p65) and p-NF- κ B (p65) proteins in rat tendon tissue by IHC assay

IHC assay showed that the expressions of TLR4, NF- κ B (p65) and p-NF- κ B (p65) proteins in the Model group were significantly higher than those in the NC group ($P < 0.001$ for all comparisons; Fig.4A-4C). After treatment with different concentrations of curcumin, expressions of TLR4, NF- κ B (p65) and p-NF- κ B (p65) proteins were significantly reduced compared with those of the Model group ($P < 0.001$ for all comparisons; Fig.4A-4C), and the differences among the curcumin groups were statistically significant ($P < 0.05$ for all comparisons; Fig.4A-4C).

Identification of tendon stem cells (TDSCs) and cell proliferation rate of each group

The isolated TDSCs were identified by flow cytometry. The results showed that the percentages of CD44- and CD90-positive cells were $97.63 \pm 1.57\%$ and $98.06 \pm 2.03\%$, respectively, and those of CD34 and CD106-positive cells were only $0.14 \pm 0.07\%$ and $0.16 \pm 0.05\%$, respectively, indicating that TDSCs was successful purified and could be used in subsequent experiments(details in Fig.5A). The MTT assay showed that the proliferation of TDSCs in the Model group was significantly reduced compared with that of the NC group ($P < 0.001$, Fig.5B). After treated with curcumin, cell proliferation rate was significantly increased in each curcumin group compared with that of the Model group ($P < 0.05$ for all comparisons; Fig.5B), and significant differences were noted among the curcumin treatment groups ($P < 0.05$ for all comparisons; Fig.5B).

Concentration of inflammatory factors and hydroxyproline (Hyp) in supernatant of cell culture medium, number of apoptotic cells and apoptosis rate

The ELISA assay revealed that the levels of TNF- α , IL-6, IL-1 β and Hyp in the Model group were significantly higher than those in the NC group ($P < 0.001$ for all comparisons; Fig.6A). The results of flow Cytometry and TUNEL assay confirmed a significant increase in apoptosis rate and number of apoptotic cells in the Model group ($P < 0.001$ for all comparisons; Fig.6B & 6C); after intervention with curcumin, concentrations of TNF- α , IL-6, IL-1 β and Hyp in the culture supernatant were significantly decreased ($P < 0.05$ for all comparisons; Fig.6A-6B) with a significant dose-effect relationship ($P < 0.05$ for all comparisons; Fig. 6A-6B).

Expression of related genes and proteins and measurement of nucleus-translocating p-NF- κ B (p65)

RT-qPCR and WB assay showed that the expression levels of TLR4 and NF- κ B (p65) genes and proteins ($P < 0.001$ for all comparisons; Fig.7A & 7B) and the ratio of p-NF- κ B (p65)/NF- κ B (p65) ($P < 0.001$, Fig. 7B) were significantly increased in the Model group compared with those in the NC group. Meanwhile, immunofluorescence assay showed that the amount of nucleus-translocating p-NF- κ B (p65) was significantly increased ($P < 0.001$; Fig.7C). After treatment with curcumin, expression levels of TLR4 and NF- κ B (p65) genes and proteins ($P < 0.001$ for all comparisons; Fig. 7A & 7B), the ratio of p-NF- κ B (p65) / NF- κ B(p65) ($P < 0.001$, Fig. 7B) and the amount of nucleus-translocating p-NF- κ B (p65) ($P < 0.05$, Fig.7C) were significantly reduced in the curcumin treatment groups compared with those in the Model group nucleus-translocating nucleus, and significant dose-effect relationship was observed in each curcumin treatment group ($P < 0.05$ for all comparisons; Fig. 7).

Effect of TLR4 on the expression of inflammatory factors in tendon cell model

Expression levels of TNF- α , IL-1 β , IL-6 and Hyp in the Model group were significantly increased compared with those in the NC group ($P < 0.001$ for all comparisons; Fig.8). After treated with curcumin (200mg/mL) or transfected with si-TLR4, the levels of TNF- α , IL-1 β , IL-6 and Hyp were significantly decreased compared with those of the Model group ($P < 0.001$ for all comparisons; Fig.8). When TLR4 was transfected into cells, the levels of TNF- α , IL-1 β , IL-6 and Hyp in the Cur+TLR4 group were significantly increased compared with those in the Cur group ($P < 0.001$ for all comparisons; Fig.8).

Role of TLR4 in the effect of curcumin in improving the proliferation and apoptosis of tendon model cells

Compared with the NC group, the proliferation rate of the tendon stem cells was significantly reduced and the apoptotic rate and the number of apoptotic cells were significant increased in the Model group ($P < 0.001$ for all comparisons; Fig.9A-9C). After treated with curcumin (200mg/mL) or transfected with si-TLR4, cell proliferation rates were significantly increased and the apoptotic rate and the number of apoptotic cells were significantly decreased in the Cur group and the si-TLR4 group compared with those in the Model group ($P < 0.001$ for all comparisons; Fig9A-9C). When TLR4 was transfected into the cells, the proliferation rate of TDSCs was significantly decreased and the apoptotic rate and the number of apoptotic cells were significantly increased in the Cur+TLR4 group compared with that of the Cur group ($P < 0.001$ for all comparisons; Fig.9A-9C).

Role of TLR4 in the effect of curcumin in improving tendon model cells

Compared with the NC group, the expression levels of TLR4 and NF- κ B (p65) genes and proteins ($P < 0.001$ for all comparisons; Fig.10A & 10B) and the ratio of p-NF- κ B (p65) / NF- κ B (p65) ($P < 0.001$, Fig.10B) as well as the nucleus-translocating p-NF- κ B (p65) ($P < 0.001$, Fig.10C, as evidenced by cellular immunofluorescence assay) were significantly increased in the Model group. After treated with curcumin (200 mg/mL) or transfected with si-TLR4, the expression levels of TLR4 and NF- κ B (p65) genes and proteins ($P < 0.001$ for all comparisons; Fig.10A & 10B), the ratio of p-NF- κ B (p65)/NF- κ B (p65) ($P < 0.001$, Fig.10B) and the nucleus-translocating p-NF- κ B (p65) ($P < 0.001$, Fig.10C) were significantly decreased in the Cur group and the si-TLR4 group compared with the Model group. When TLR4 was transfected into

the cells, the expression levels of TLR4 and NF- κ B (p65) genes and proteins ($P < 0.001$ for all comparisons; Fig.10A & 10B) and the ratio of p-NF- κ B (p65) / NF- κ B (p65) ($P < 0.001$, Fig.10B) as well as the nucleus-translocating p-NF- κ B (p65) ($P < 0.001$, Fig.10C, as evidenced by cellular immunofluorescence assay) were significantly increased in the Cur+TLR4 group compared with those of the Cur group.

Discussion

Excessive use or age-related degeneration of tendons may induce acute and chronic tendon injuries. The incidence of chronic tendinopathy increases dramatically with age. Surveys show that 13% of the population in the 50-year-old age group develops tendinopathy, which increases to 20% in the 60-year-old age group and 31% in the 70-year-old age group (Peach MS, 2017; Jensen G, 2017). Repaired tendons are easy to fracture again due to the fact that they are scar tissues with abnormal structure and low mechanical properties, and up to 40% of patients may suffer tendon re-fracture after surgery (Han F, 2015). At the same time, repaired tendons are usually associated with problems such as adhesion and strength reduction, making the treatment of injured tendons particularly important. Traditional treatments for tendon injuries include physical therapies and medications including non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids (Beerts C, 2017; Okonta KE, 2017; Homayouni K, 2016), and certain disadvantages, such as lack of efficacy or serious side effects, still exist in these treatments.

In the late stage of tendon healing, excessive synthesis of ECM enhanced by the overexpression of TGF- β 1 may also lead to tendon adhesion, excessive fibrosis, and massive scar tissue formation. The morphology and function of cells in this scar tissue are quite different from those of normal tendon cells. The structure of the matrix surrounding these cells is usually poor with disordered arrangement. Abnormal tissue remodeling will result in increased cross-sectional area of the tendon and poor function and hardness, although the strength is slightly improved. For instance, after ruptured flexor tendon is healed, the scar tissue may entangle with the flexor tendon sheath, which seriously impairs the function of hands. Study by Riessen et al. (Riessen R, 2001) showed that TGF- β 1 directly activated the expression of COMP/TSP-5, resulting in tendon adhesion. Davies et al (Davies MR, 2016) demonstrated that the TGF- β 1 pathway plays an essential role in the pathological processes of fibrosis and apoptosis in various organs and tissues. In the present study, an animal model was established by injecting type I collagenase and a tendon cell model by using TGF- β 1 to induce primary TDSCs. The results showed that the level of fibrosis was significantly increased in the tendon tissue of the animal model and the level of Hyp, a biomarker for fibrosis, and the apoptotic rate of TDSCs were also significantly increased in the tendon cell model, indicating that the tendon animal and cell models were successfully established.

Studies over recent years have confirmed that curcumin has anti-inflammatory, anticoagulant, hypolipidemic and anti-tumor effects (Bonaccorsi PM, 2019; Dai J, 2018; Li HS, 2018) and exerts its anti-inflammatory effects via inhibiting the degradation of NF- κ B inhibitor (I κ B) to block the activation of NF- κ B and release of inflammatory factors induced by cytokines (Oh SW, 2010). Curcumin may also inhibit the expression of NF- κ B, IL-1 β and IL-10 mRNAs in rats with inflammatory bowel disease (Song WB, 2010). In

vitro and in vivo assays in the present study showed that curcumin effectively improved tendon tissue fibrosis induced by tendon disorders and the induced tendon stem cell apoptosis.

The Toll-like receptor 4 (TLR4) signaling pathway plays an important role in the inflammatory response. Overexpression of TGF- β 1 may activate the TLR4/NF- κ B signaling pathway, leading to increased fibrosis and apoptosis (Kabel AM, 2019; Sun W, 2019; Jia L, 2019). It was found in the present study that the TLR4/NF- κ B signaling pathway was activated in both in vivo and in vitro models of tendinopathy, and the activity of TLR4/NF- κ B and the amount of nucleus-translocating p-NF- κ B (p65) were significantly reduced after intervention with curcumin. However, the effect of curcumin in improving tendinopathy disappeared after the cells were transfected with TLR4.

Conclusion

This result indicated that the effect of curcumin in improving tendon tissue fibrosis induced by tendinopathy as well as the induced apoptosis of tendon stem cells may be closely associated with the inactivation of TLR4/NF- κ B.

Declarations

Ethics approval and consent to participate

This study was approved by Ethics committee of the First Affiliated Hospital of Anhui Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

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Author Contributions:

Xu Bin: Designated experimental scheme and checked the manuscript; Tu Jun and Guo Ruipeng completed the whole experiment and written the manuscript.

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Availability of data and materials

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Ethics approval and consent to participate

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Consent for publication

Not applicable.

Conflict of Interest

The authors declare that they have no competing interests.

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Figures

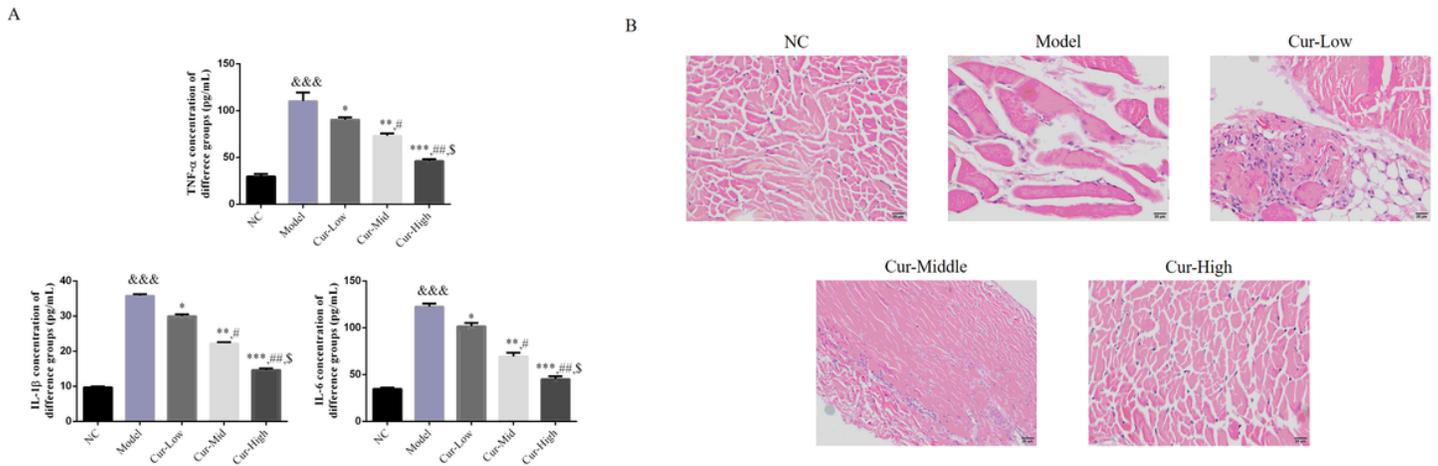


Figure 1

Inflammatory factors and pathological changes of tendons in rats NC: Normal Control group; Model: The tendinopathy Model group; Cur-Low: The tendinopathy model rats were treated with 20mg/kg curcumin; Cur-Middle: The tendinopathy model rats were treated with 40mg/kg curcumin; Cur-High: The tendinopathy model rats were treated with 200mg/kg curcumin A. Inflammatory factors of difference groups by Elisa assay &&&: $P \leq 0.001$, compared with NC group; *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group B. The pathological changes of difference groups by HE staining (400x)

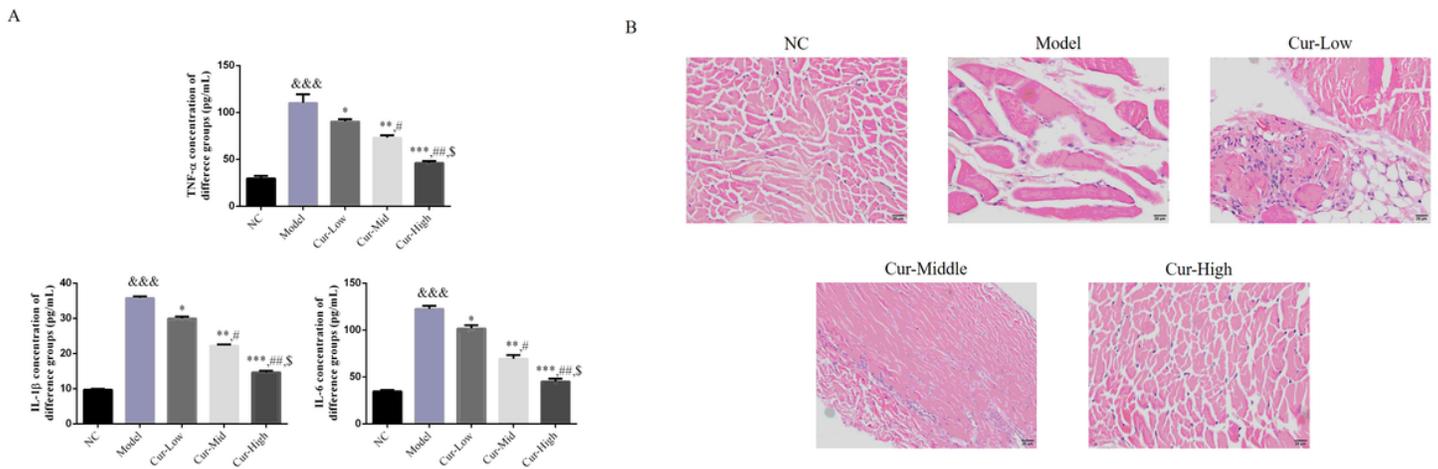


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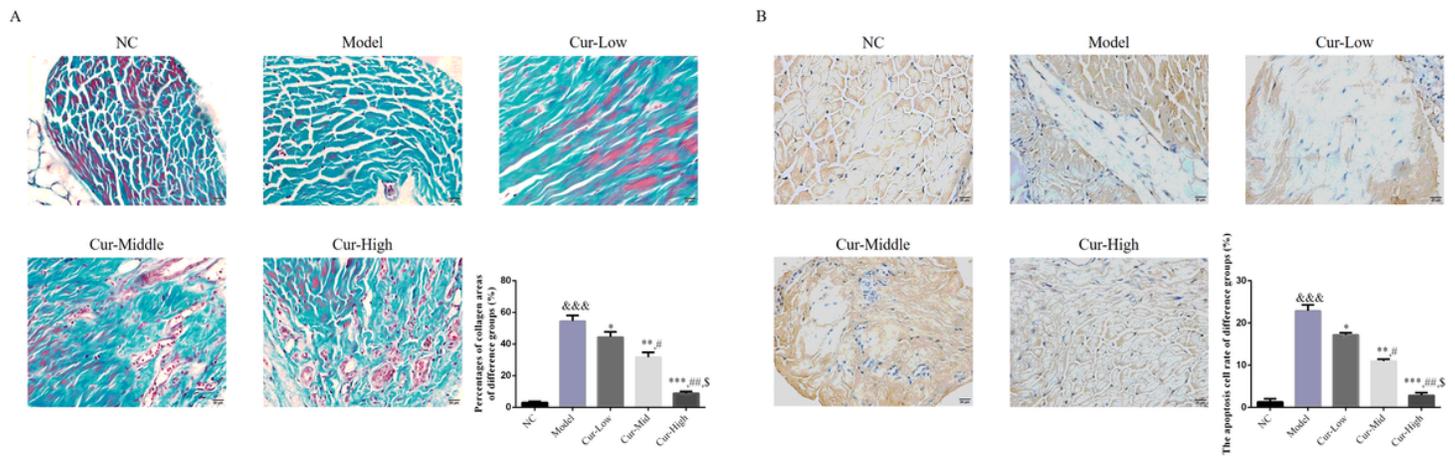


Figure 2

Collagen content and apoptotic cells in rats NC: Normal Control group; Model: The tendinopathy Model group; Cur-Low: The tendinopathy model rats were treated with 20mg/kg curcumin; Cur-Middle: The tendinopathy model rats were treated with 40mg/kg curcumin; Cur-High: The tendinopathy model rats were treated with 200mg/kg curcumin A. Percentages of collagen areas of difference groups by Masson staining (400×) &&&: $P \leq 0.001$, compared with NC group; *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group B. The apoptosis cell rate of difference groups by TUNEL assay (400×) &&&: $P \leq 0.001$, compared with NC group; *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group

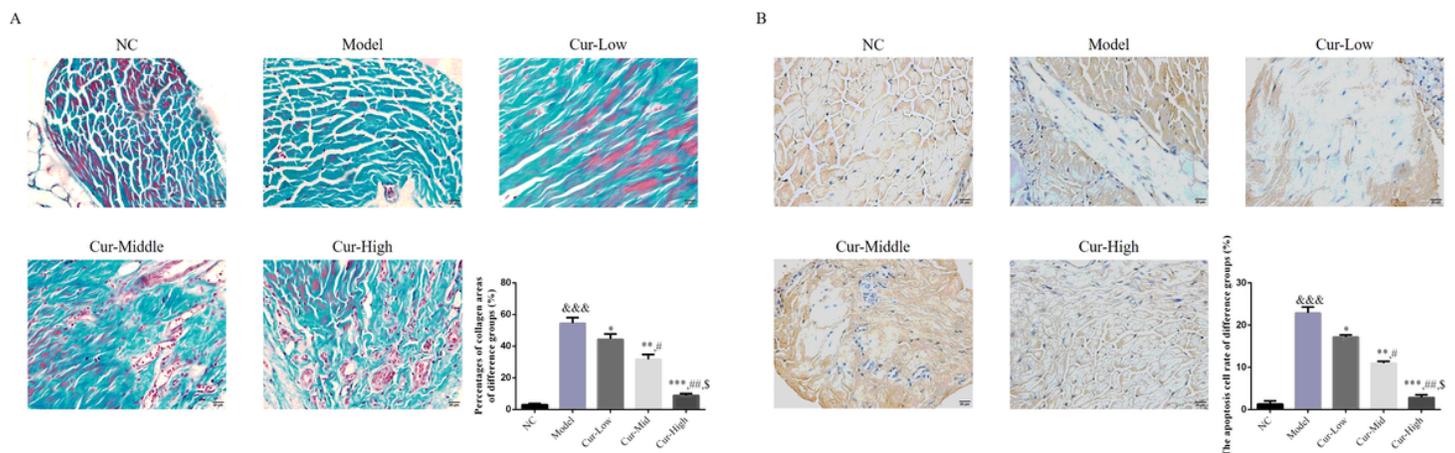
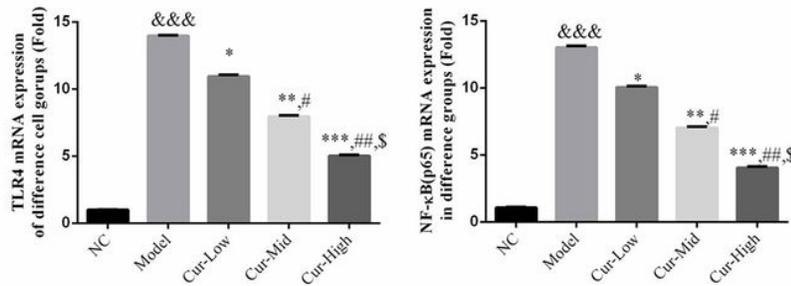


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A



B

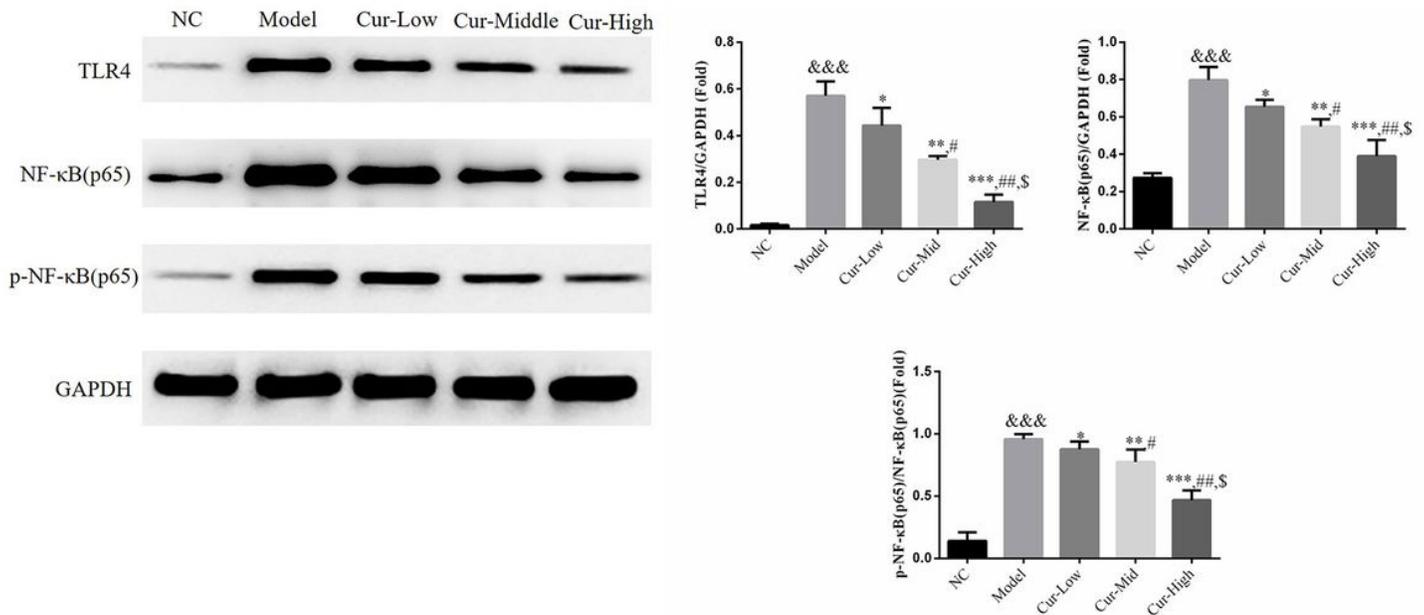


Figure 3

Related genes and proteins levels in rat tendon tissue by RT-qPCR and WB NC: Normal Control group; Model: The tendinopathy Model group; Cur-Low: The tendinopathy model rats were treated with 20mg/kg curcumin; Cur-Middle: The tendinopathy model rats were treated with 40mg/kg curcumin; Cur-High: The tendinopathy model rats were treated with 200mg/kg curcumin A. The relative genes expression of difference groups by RT-qPCR assay &&&: $P \leq 0.001$, compared with NC group; *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group B. The relative proteins expression of difference groups by WB assay &&&: $P \leq 0.001$, compared with NC group; *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group

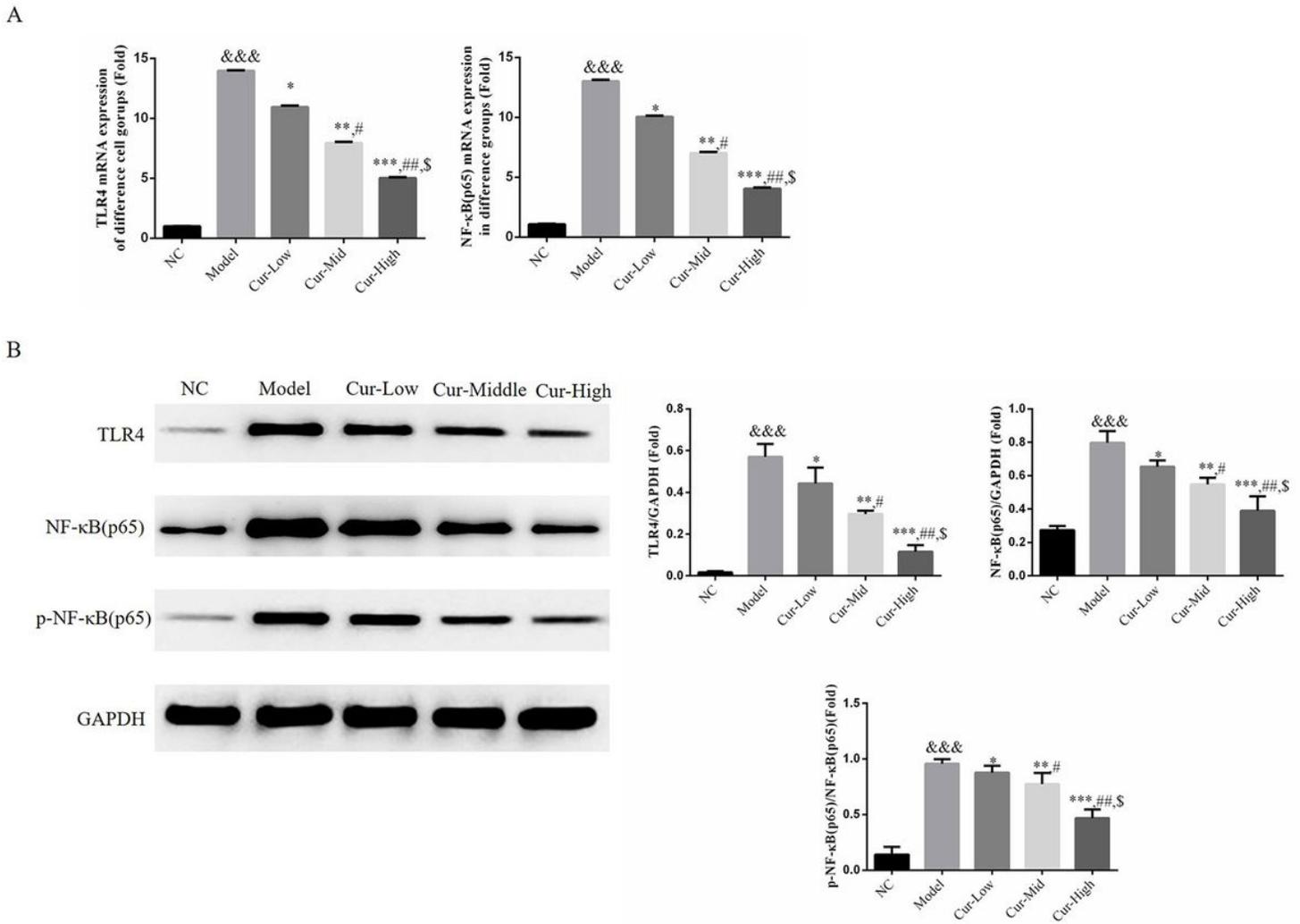


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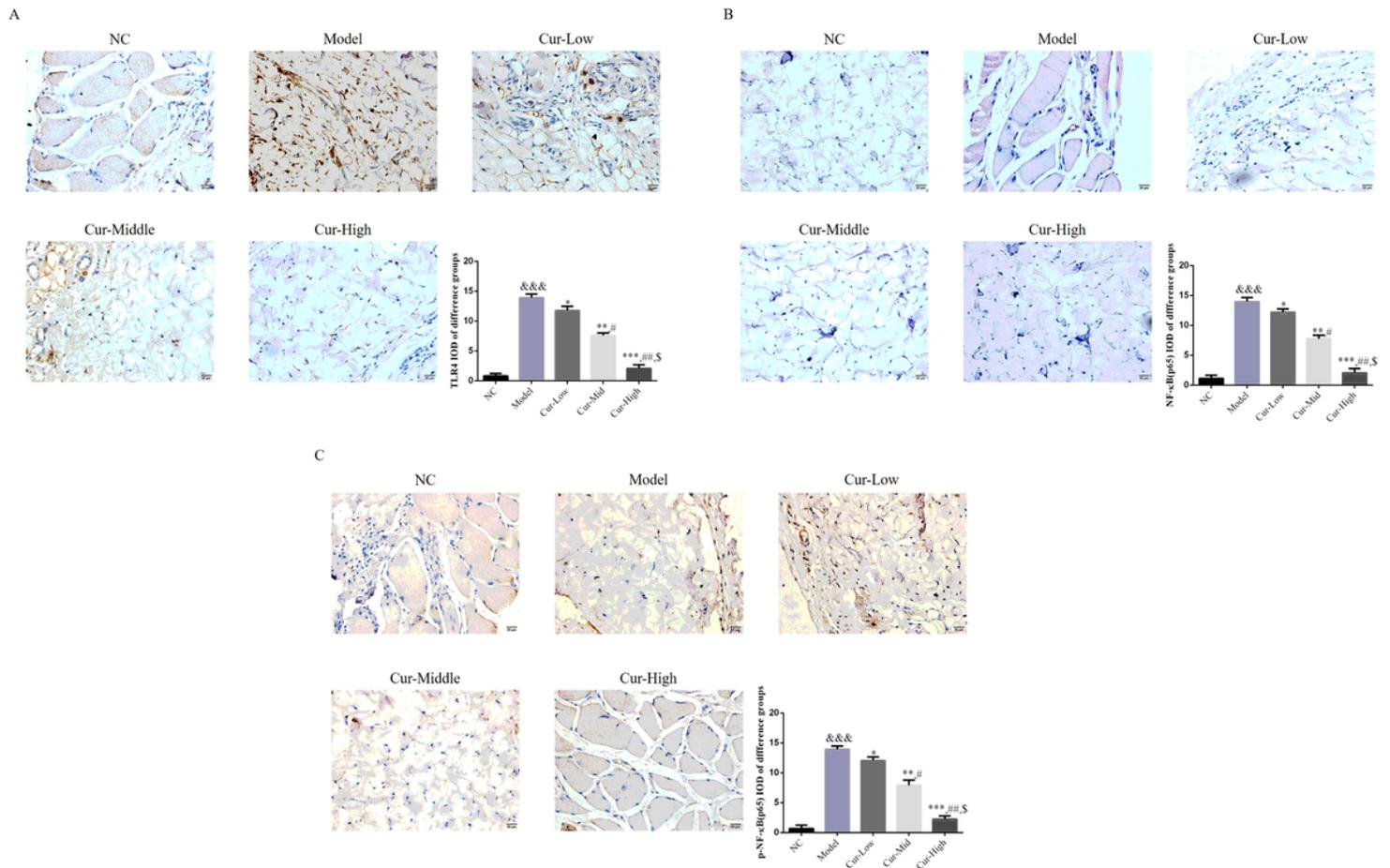


Figure 4

Expression of TLR4, NF-κB (p65) and p-NF-κB (p65) proteins in rat tendon tissue by IHC assay NC: Normal Control group; Model: The tendinopathy Model group; Cur-Low: The tendinopathy model rats were treated with 20mg/kg curcumin; Cur-Middle: The tendinopathy model rats were treated with 40mg/kg curcumin; Cur-High: The tendinopathy model rats were treated with 200mg/kg curcumin A. The TLR4 protein expression in difference groups by IHC assay (400×) &&&: $P < 0.001$, compared with NC group; *: $P < 0.05$, **: $P < 0.01$, *** $P < 0.001$, compared with Model group; # $P < 0.05$, ##: $P < 0.01$, compared with Cur-Low group; \$: $P < 0.05$, compared with Cur-Middle group B. The NF-κB(p65) protein expression in difference groups by IHC assay (400×) &&&: $P < 0.001$, compared with NC group; *: $P < 0.05$, **: $P < 0.01$, *** $P < 0.001$, compared with Model group; # $P < 0.05$, ##: $P < 0.01$, compared with Cur-Low group; \$: $P < 0.05$, compared with Cur-Middle group C. The p-NF-κB(p65) protein expression in difference groups by IHC assay (400×) &&&: $P < 0.001$, compared with NC group; *: $P < 0.05$, **: $P < 0.01$, *** $P < 0.001$, compared with Model group; # $P < 0.05$, ##: $P < 0.01$, compared with Cur-Low group; \$: $P < 0.05$, compared with Cur-Middle group

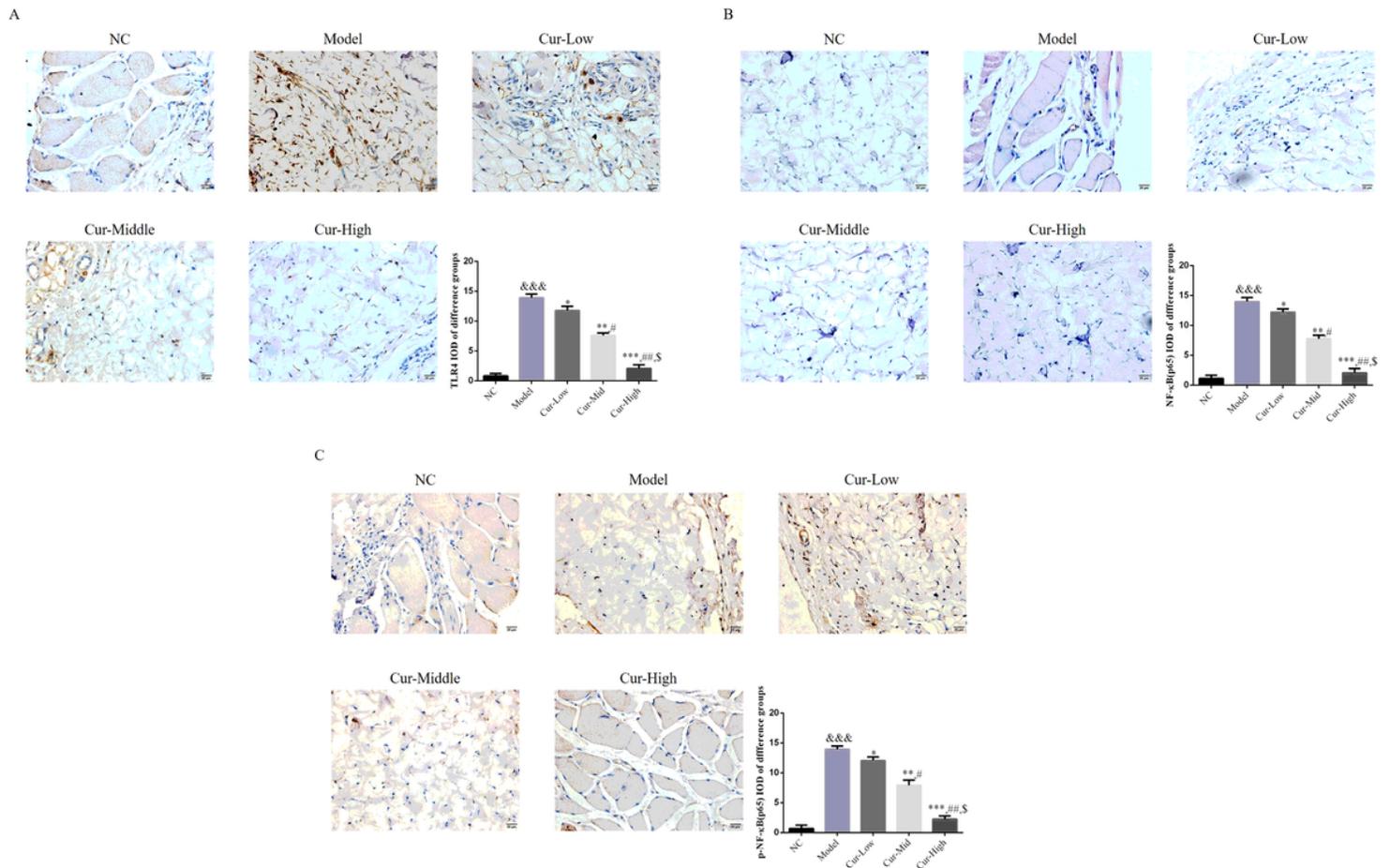
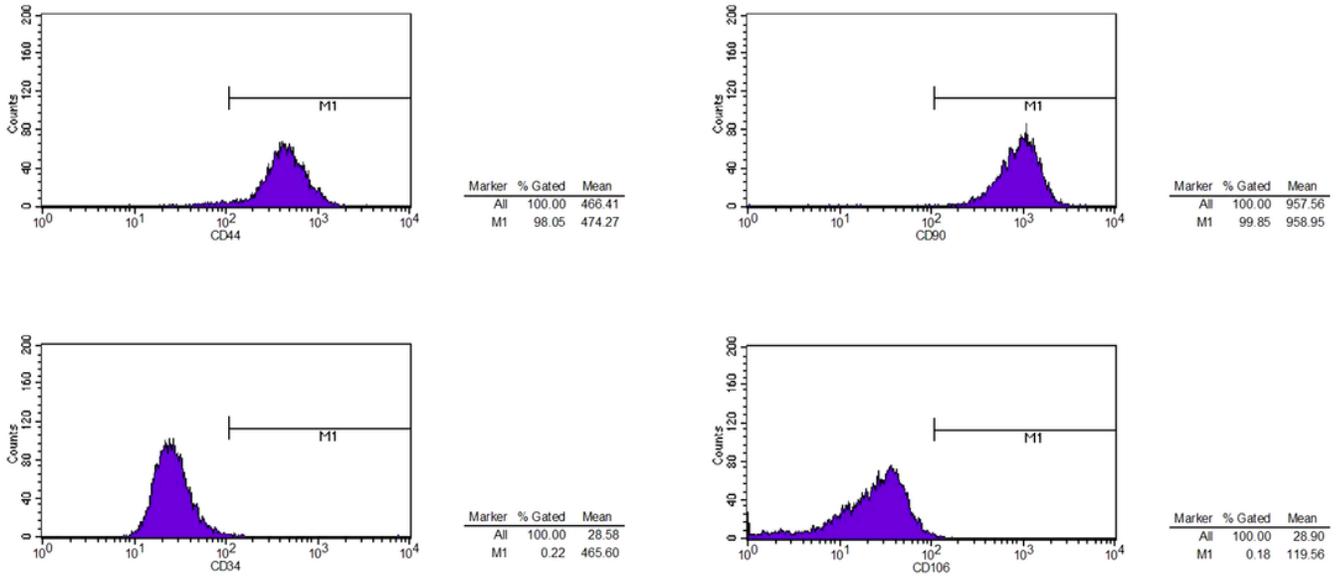


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A



B

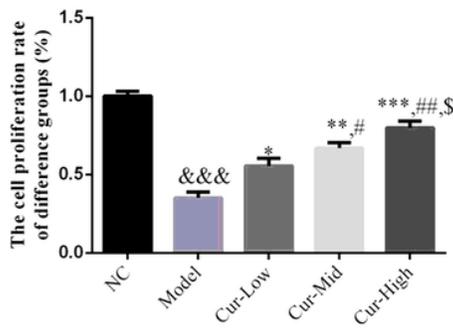
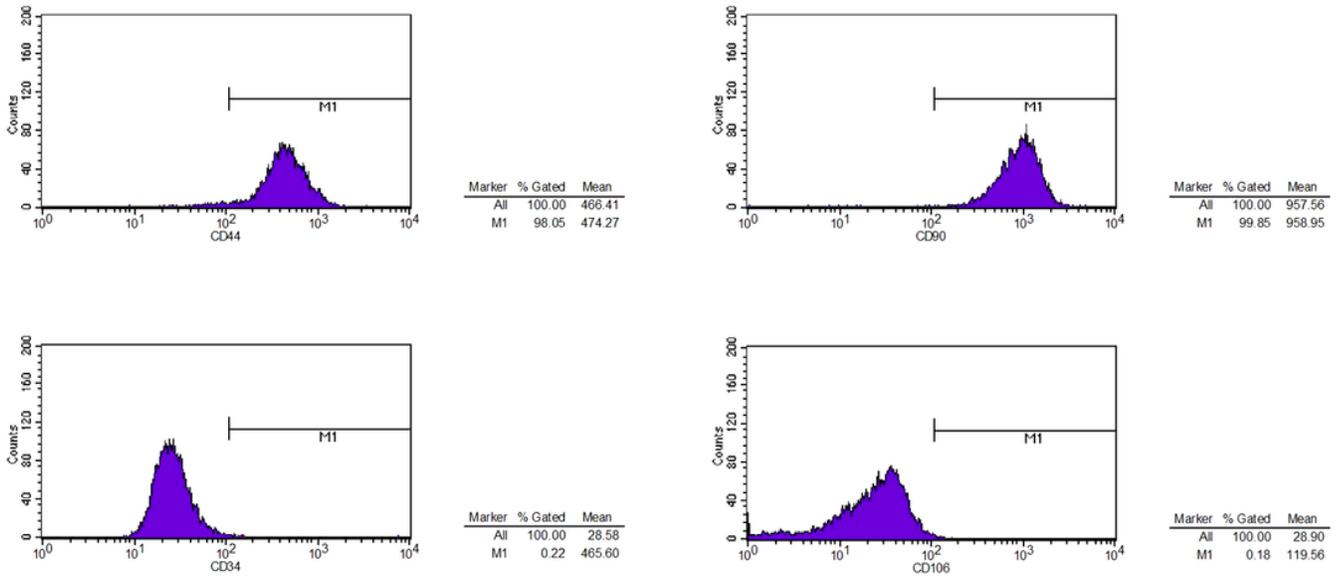


Figure 5

Identification of tendon stem cells (TDSCs) and cell proliferation rate of each group A. Identification of tendon stem cells (TDSCs) B. Cell proliferation rate of each group by MTT assay NC: Normal Control group; Model: The tendinopathy cell Model group; Cur-Low: The tendinopathy model cells were treated with 20mg/mL curcumin; Cur-Middle: The tendinopathy model cells were treated with 40mg/ mL curcumin; Cur-High: The tendinopathy model cells were treated with 200mg/ mL curcumin &&&: $P \leq 0.001$, compared with NC group; *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group

A



B

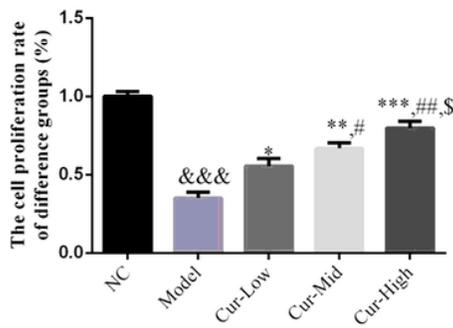


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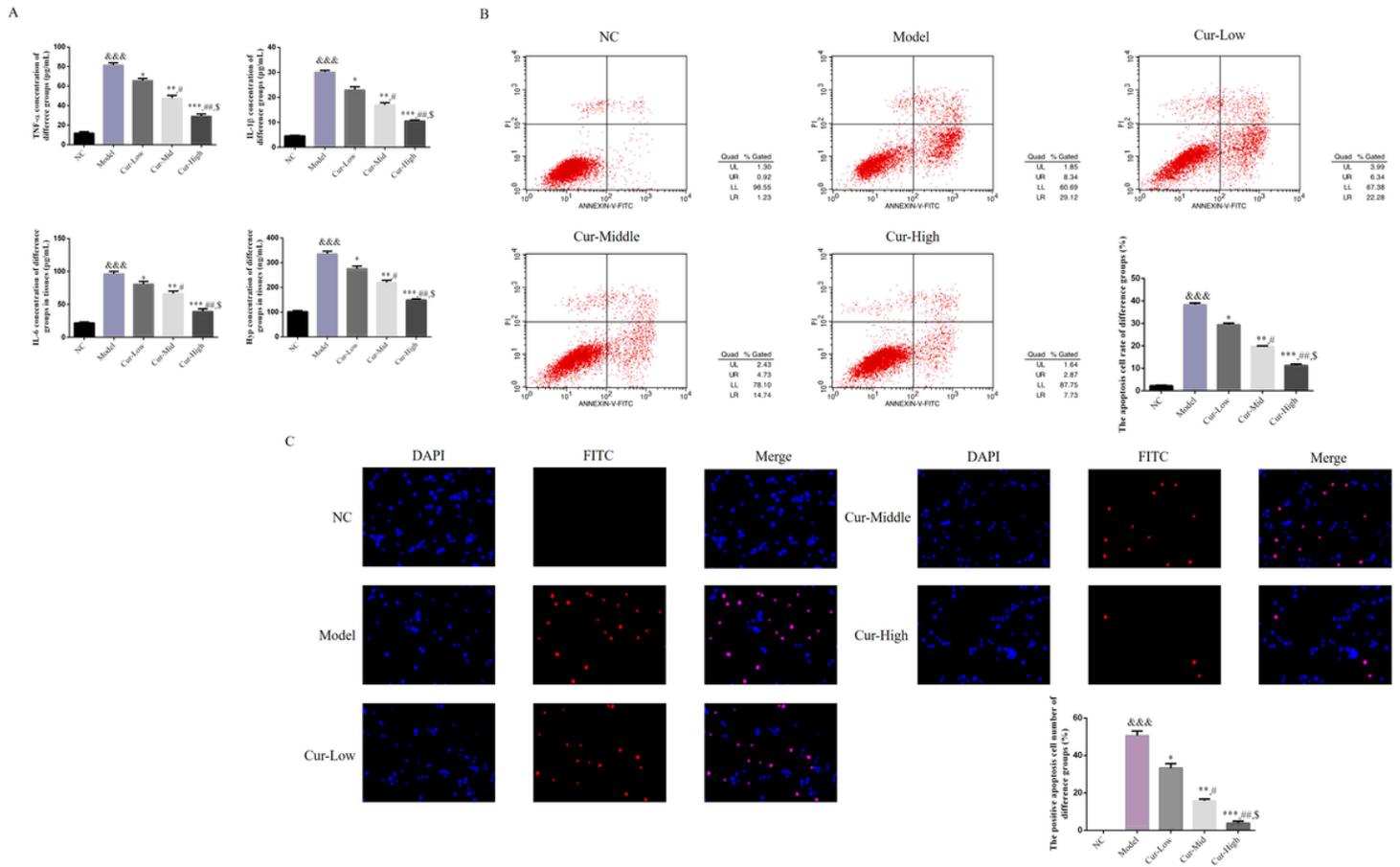


Figure 6

Concentration of inflammatory factors and hydroxyproline (Hyp) in supernatant of cell culture medium, number of apoptotic cells and apoptosis rate NC: Normal Control group; Model: The tendinopathy cell Model group; Cur-Low: The tendinopathy model cells were treated with 20mg/mL curcumin; Cur-Middle: The tendinopathy model cells were treated with 40mg/ mL curcumin; Cur-High: The tendinopathy model cells were treated with 200mg/ mL curcumin A. Inflammatory factors and hydroxyproline (Hyp) concentration of difference groups by Elisa assay &&&: $P \leq 0.001$, compared with NC group; *: $P \leq 0.05$, **: $P \leq 0.01$, *** $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group B. The apoptosis rate of difference groups &&&: $P \leq 0.001$, compared with NC group; *: $P \leq 0.05$, **: $P \leq 0.01$, *** $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group C. The apoptosis cell number of difference groups by TUNEL assay (200 \times) &&&: $P \leq 0.001$, compared with NC group; *: $P \leq 0.05$, **: $P \leq 0.01$, *** $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group

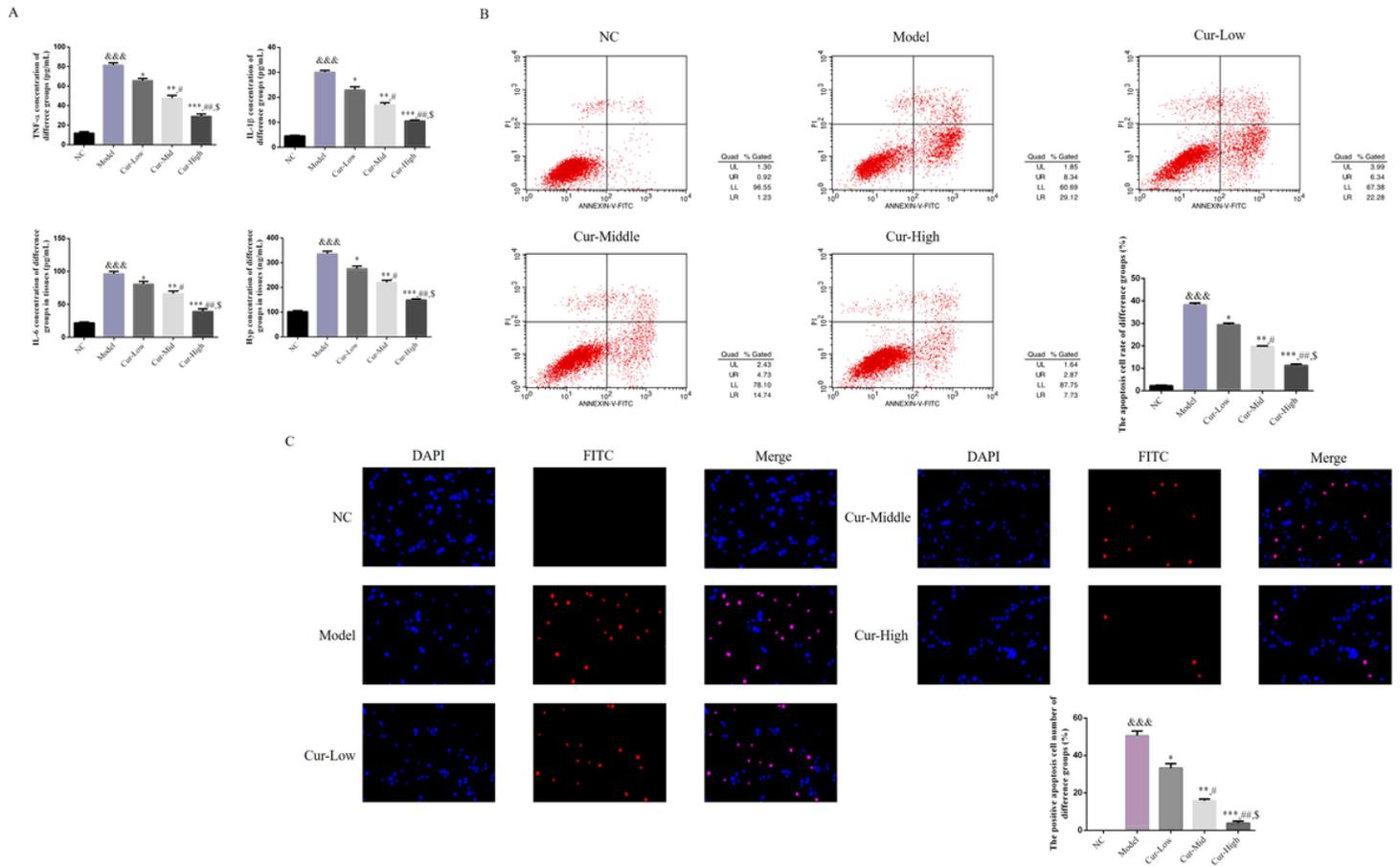
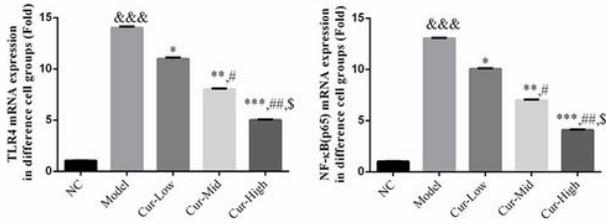


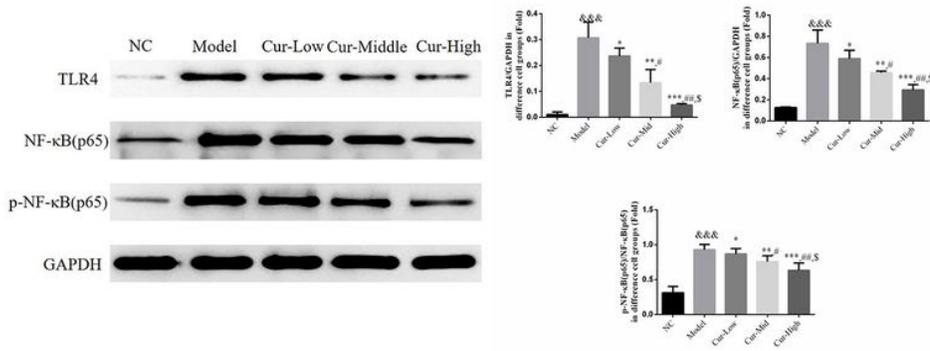
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A



B



C

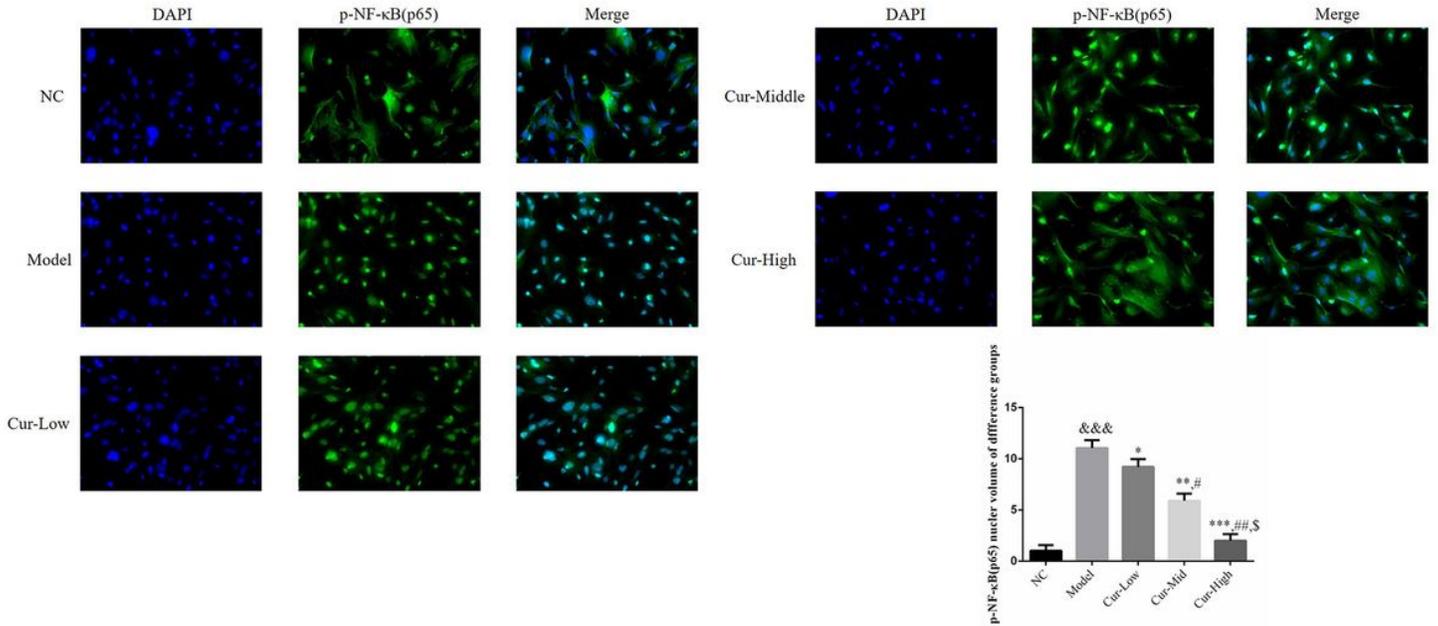
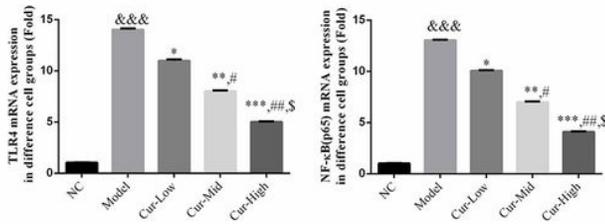


Figure 7

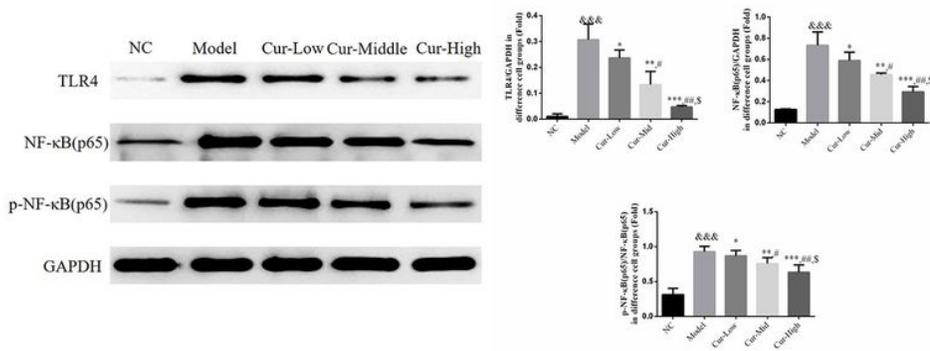
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A



B



C

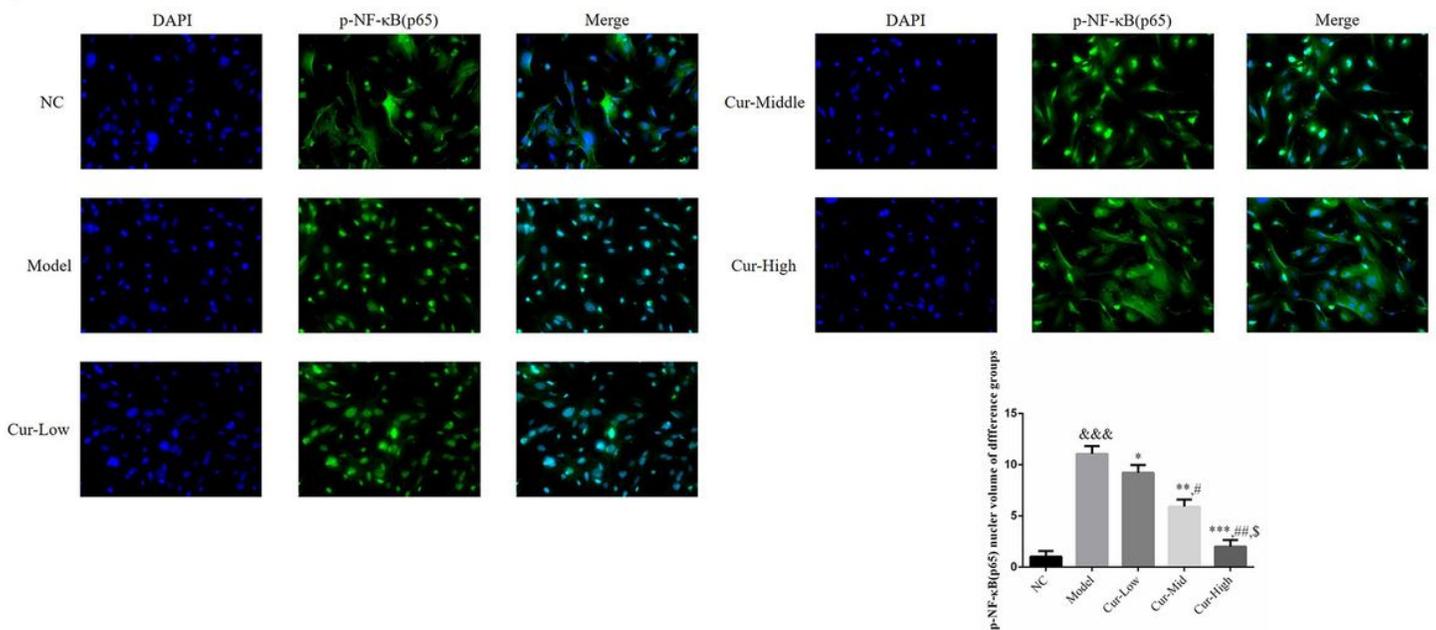


Figure 7

Relative genes and proteins levels by RT-qPCR and WB assay and measurement of nucleus-translocating p-NF-κB (p65) NC: Normal Control group; Model: The tendinopathy cell Model group; Cur-Low: The tendinopathy model cells were treated with 20mg/mL curcumin; Cur-Middle: The tendinopathy model cells were treated with 40mg/ mL curcumin; Cur-High: The tendinopathy model cells were treated with 200mg/ mL curcumin A. Relative genes expression of difference cell groups by RT-qPCR assay &&&: $P \leq 0.001$,

compared with NC group; *, $P \leq 0.05$, **, $P \leq 0.01$, *** $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group B. Relative proteins expressions of difference cell groups by WB assay &&&: $P \leq 0.001$, compared with NC group; *, $P \leq 0.05$, **, $P \leq 0.01$, *** $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group C. p-NF- κ B(p65) protein nuclear volume in difference cell groups by cellular immunofluorescence(200 \times) &&&: $P \leq 0.001$, compared with NC group; *, $P \leq 0.05$, **, $P \leq 0.01$, *** $P \leq 0.001$, compared with Model group; # $P \leq 0.05$, ##: $P \leq 0.01$, compared with Cur-Low group; \$: $P \leq 0.05$, compared with Cur-Middle group

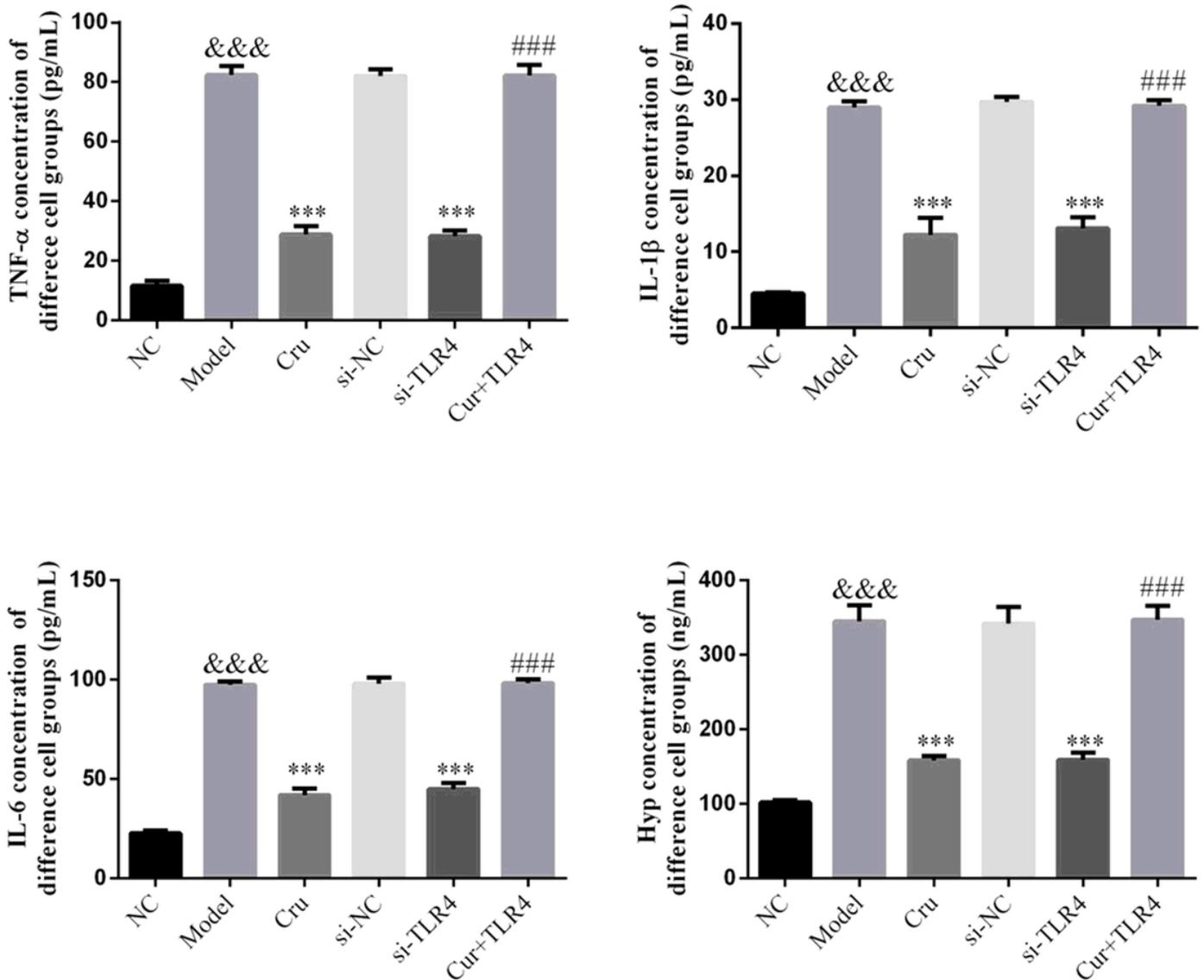


Figure 8

Effect of TLR4 on the expression of inflammatory factors in tendon cell model NC: Normal Control group; Model: The tendinopathy cell Model group; Cur: The tendinopathy model cells were treated with 200mg/

mL curcumin; si-NC: The tendinopathy model cells were transfected with empty vector; si-TLR4: The tendinopathy model cells were transfected with si-TLR4; Cur+TLR4: The tendinopathy model cells which transfected with TLR4 were treated with 200mg/ mL curcumin &&& P<0.001, compared with NC group; ***P<0.001, compared with Model group; ###: P<0.001, compared with Cur group

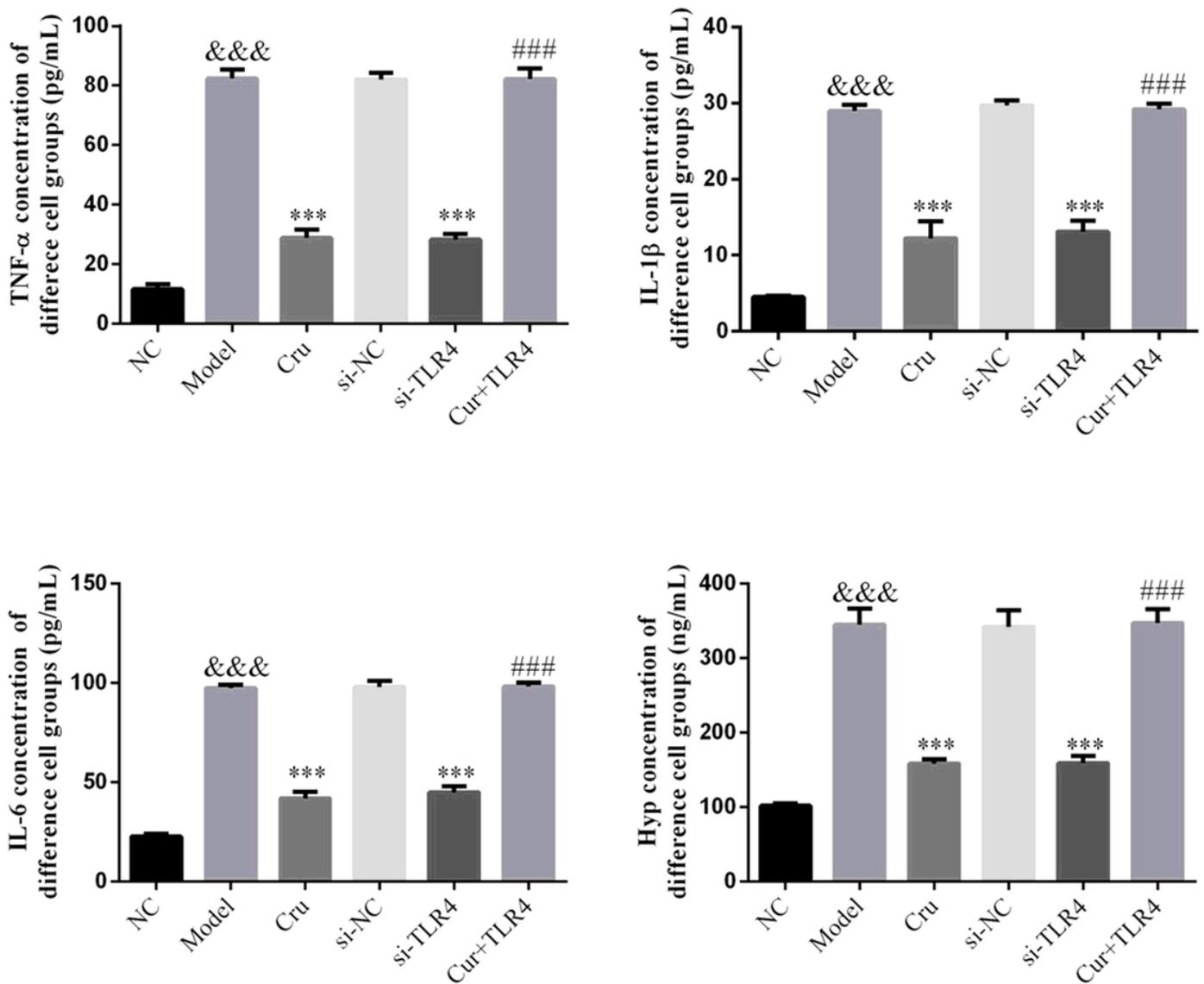


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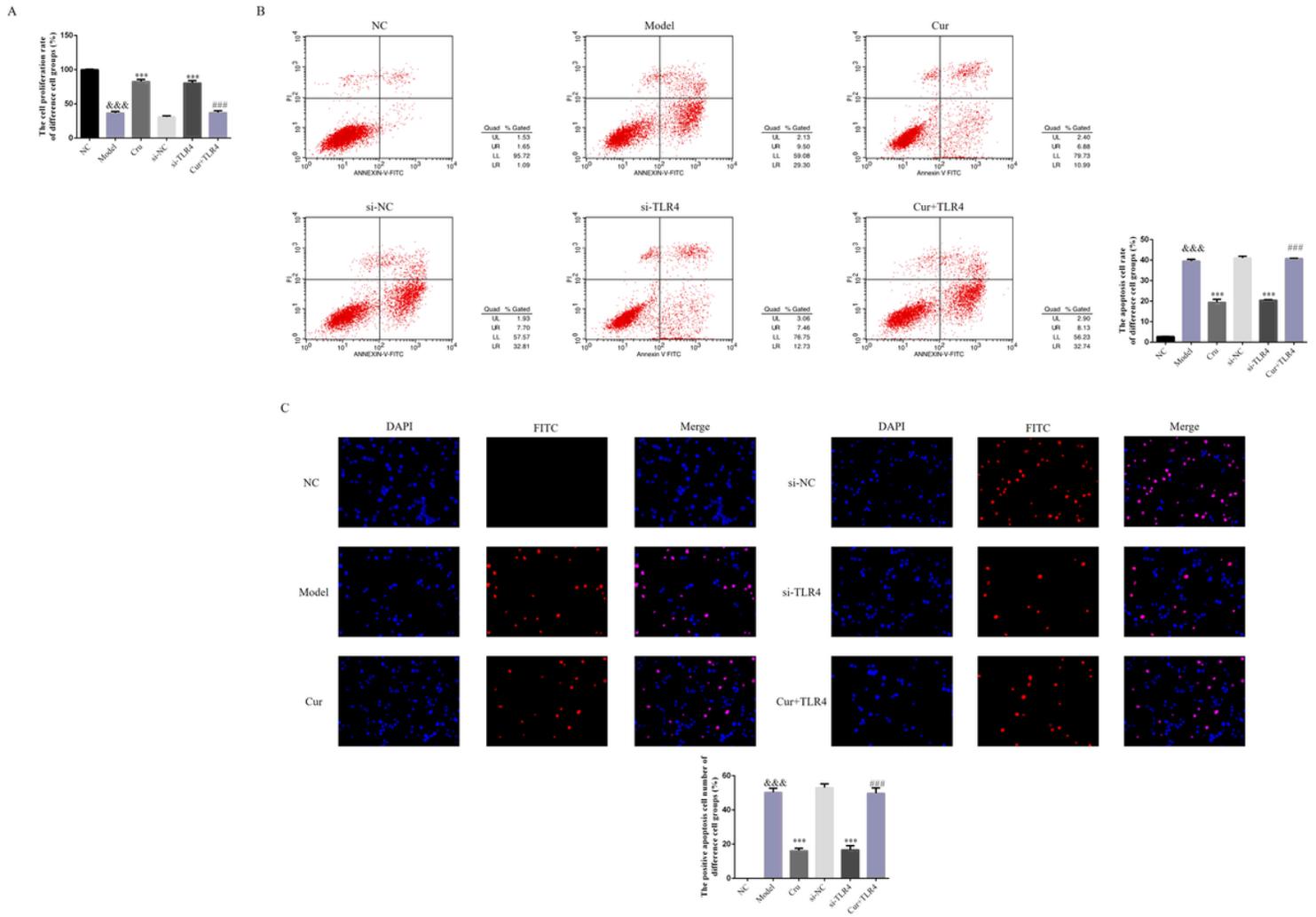


Figure 9

Role of TLR4 in the effect of curcumin in improving the proliferation and apoptosis of tendon model cells
 NC: Normal Control group; Model: The tendinopathy cell Model group; Cur: The tendinopathy model cells were treated with 200mg/ mL curcumin; si-NC: The tendinopathy model cells were transfected with empty vector; si-TLR4: The tendinopathy model cells were transfected with si-TLR4; Cur+TLR4: The tendinopathy model cells which transfected with TLR4 were treated with 200mg/ mL curcumin A. The cell proliferation rate of difference groups by MTT assay &&&: $P \leq 0.001$, compared with NC group; ***: $P \leq 0.001$, compared with Model group; ###: $P \leq 0.001$, compared with Cur group B. The apoptosis rate of difference cell groups &&&: $P \leq 0.001$, compared with NC group; ***: $P \leq 0.001$, compared with Model group; ###: $P \leq 0.001$, compared with Cur group C. The apoptosis cell number of difference cell groups by TUNEL assay (200 \times) &&&: $P \leq 0.001$, compared with NC group; ***: $P \leq 0.001$, compared with Model group; ###: $P \leq 0.001$, compared with Cur group

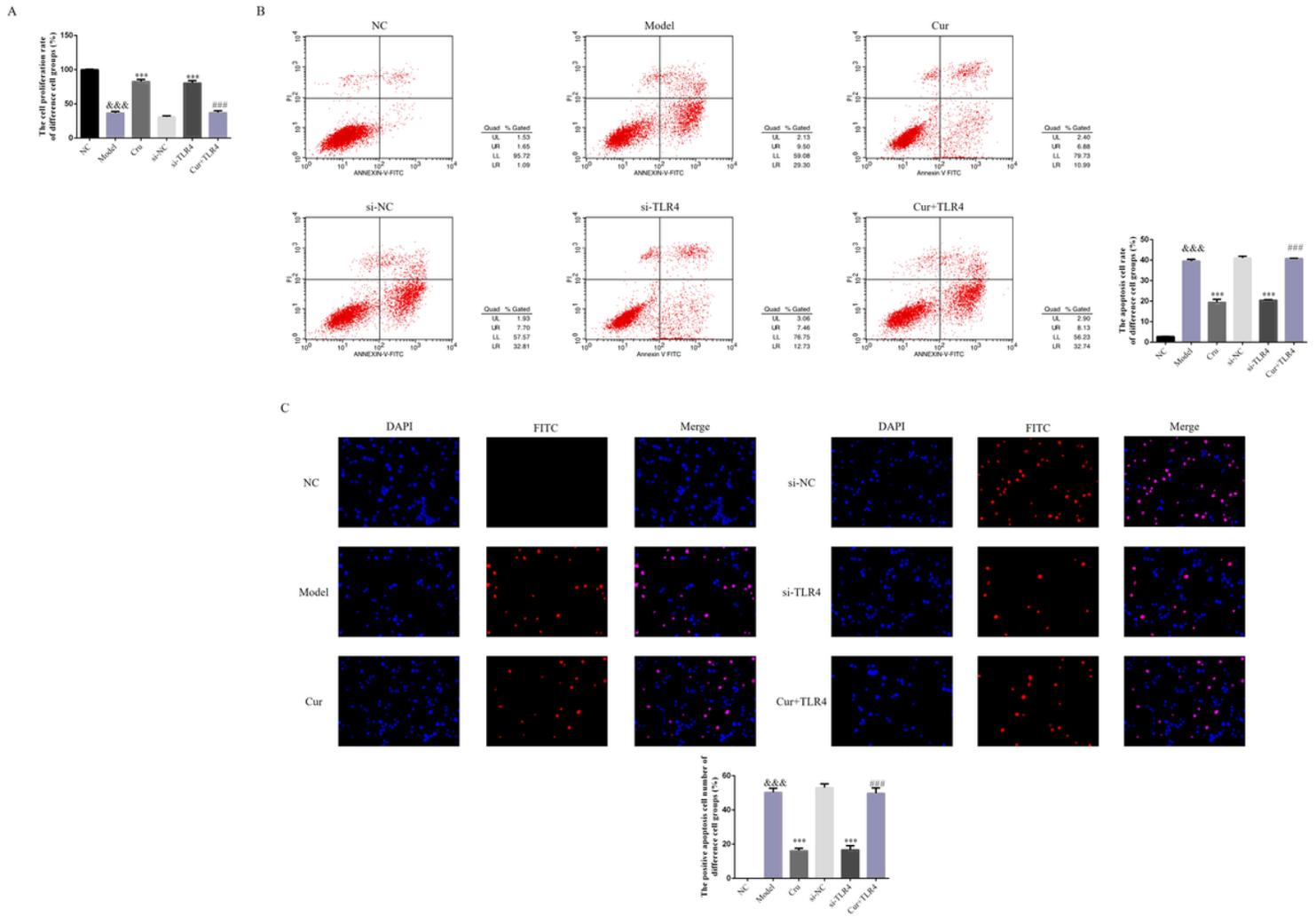
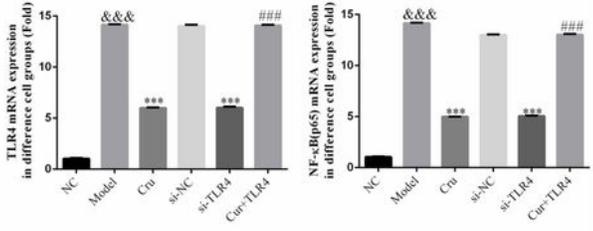


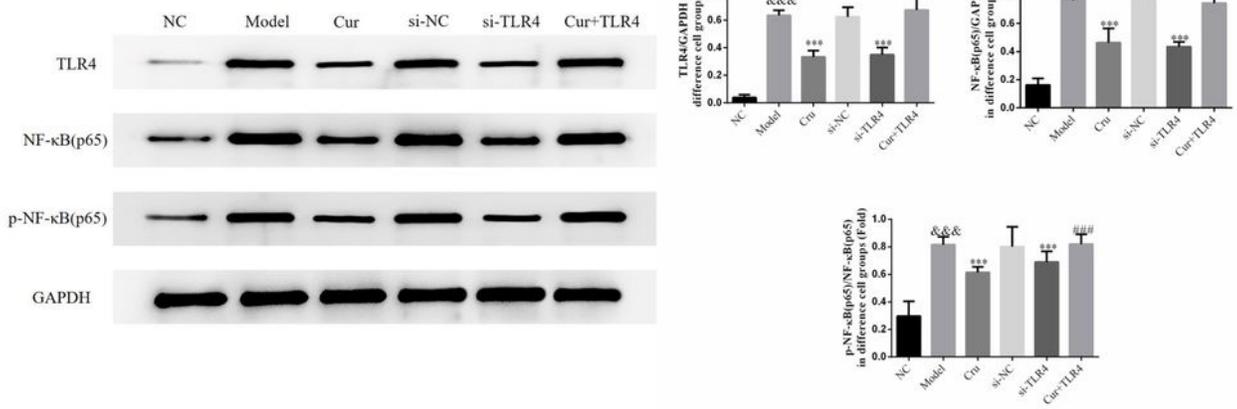
Figure 9

Role of TLR4 in the effect of curcumin in improving the proliferation and apoptosis of tendon model cells
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A



B



C

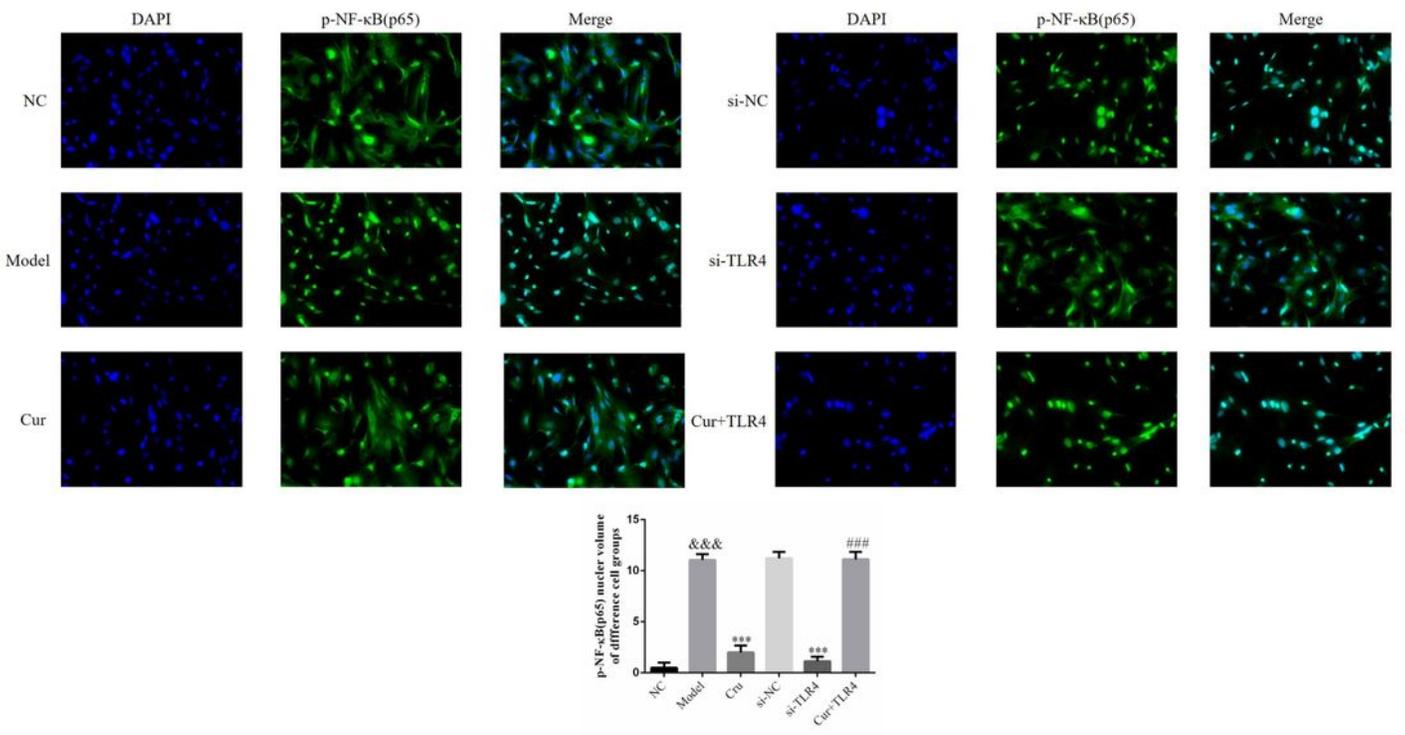
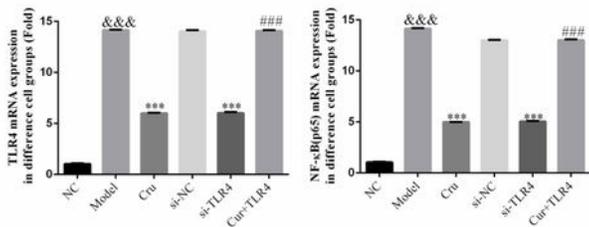


Figure 10

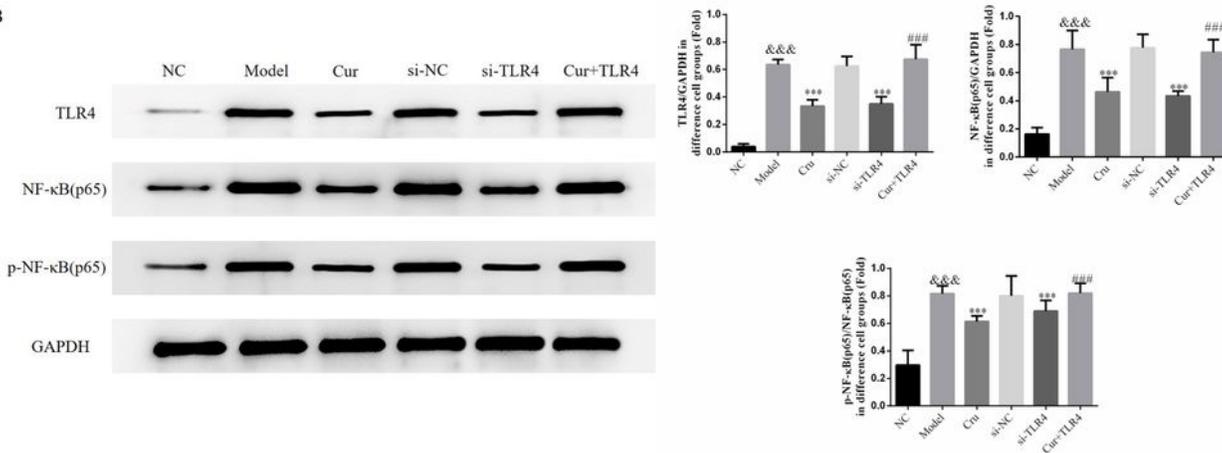
Role of TLR4 in the effect of curcumin in improving tendon model cells NC: Normal Control group; Model: The tendinopathy cell Model group; Cur: The tendinopathy model cells were treated with 200mg/ mL curcumin; si-NC: The tendinopathy model cells were transfected with empty vector; si-TLR4: The tendinopathy model cells were transfected with si-TLR4; Cur+TLR4: The tendinopathy model cells which transfected with TLR4 were treated with 200mg/ mL curcumin A. Relative genes expression of difference

cell groups by RT-qPCR assay &&&: $P \leq 0.001$, compared with NC group; $***P \leq 0.001$, compared with Model group; $###P \leq 0.001$, compared with Cur group B. Relative proteins expressions of difference cell groups by WB assay &&&: $P \leq 0.001$, compared with NC group; $***P \leq 0.001$, compared with Model group; $###P \leq 0.001$, compared with Cur group C. p-NF- κ B(p65) protein nuclear volume in difference cell groups by cellular immunofluorescence(200 \times) &&&: $P \leq 0.001$, compared with NC group; $***P \leq 0.001$, compared with Model group; $###P \leq 0.001$, compared with Cur group

A



B



C

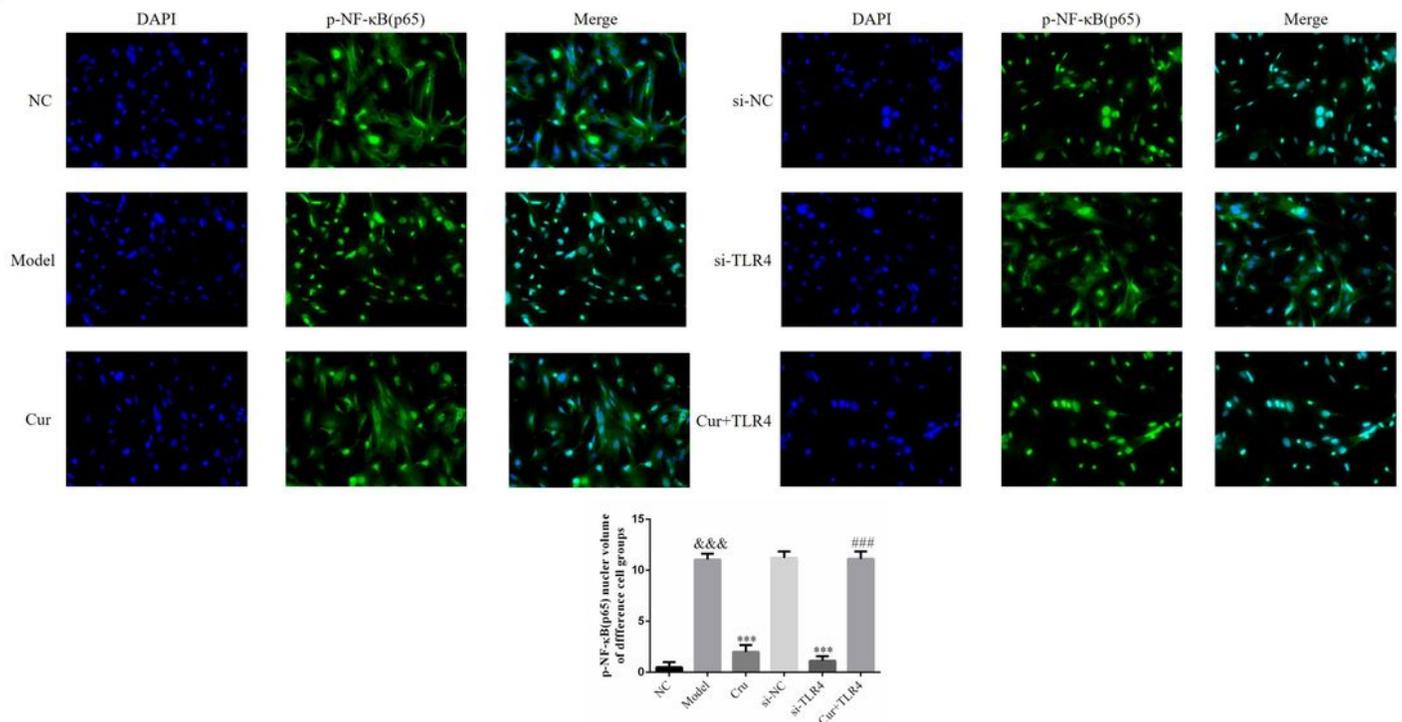


Figure 10

Role of TLR4 in the effect of curcumin in improving tendon model cells NC: Normal Control group; Model: The tendinopathy cell Model group; Cur: The tendinopathy model cells were treated with 200mg/ mL curcumin; si-NC: The tendinopathy model cells were transfected with empty vector; si-TLR4: The tendinopathy model cells were transfected with si-TLR4; Cur+TLR4: The tendinopathy model cells which transfected with TLR4 were treated with 200mg/ mL curcumin A. Relative genes expression of difference cell groups by RT-qPCR assay &&&: $P \leq 0.001$, compared with NC group; $***P \leq 0.001$, compared with Model group; $###P \leq 0.001$, compared with Cur group B. Relative proteins expressions of difference cell groups by WB assay &&&: $P \leq 0.001$, compared with NC group; $***P \leq 0.001$, compared with Model group; $###P \leq 0.001$, compared with Cur group C. p-NF- κ B(p65) protein nuclear volume in difference cell groups by cellular immunofluorescence(200 \times) &&&: $P \leq 0.001$, compared with NC group; $***P \leq 0.001$, compared with Model group; $###P \leq 0.001$, compared with Cur group

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