Atorvastatin reduces calcification in valve interstitial cells *via* the NF-κB signalling pathway by promoting Atg5-mediated autophagy

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ABSTRACT

Aortic valve calcification (AVC) is a common cardiovascular disease and a risk factor for sudden death. However, the potential mechanisms and effective therapeutic drugs need to be explored. Atorvastatin is a statin that can effectively prevent cardiovascular events by lowering cholesterol levels. However, whether atorvastatin can inhibit AVC by reducing low-density lipoprotein (LDL) and its possible mechanism of action require further exploration. In the current study, we constructed an *in vitro* AVC model by inducing calcification of the valve interstitial cells. We found that atorvastatin significantly inhibited osteogenic differentiation, reduced the deposition of calcium nodules in valve interstitial cells, and enhanced autophagy in calcified valve interstitial cells, manifested by increased expression levels of the autophagy proteins Atg5 and LC3B-II/I and the formation of smooth autophagic flow. Atorvastatin inhibited the NF-κB signalling pathway and the expression of inflammatory factors mediated by NF-κB in calcified valve interstitial cells. The activation of the NF-κB signalling pathway led to the reversal of atorvastatin's effect on enhancing autophagy and alleviating valve interstitial cell calcification. In conclusion, atorvastatin inhibited the NF-κB signalling pathway by upregulating autophagy, thereby alleviating valve interstitial cell calcification, which was conducive to improving AVC.

Key words: aortic valve calcification; atorvastatin; autophagy; NF-κB.

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Introduction

Aortic valve calcification (AVC) is a degenerative disease characterized by ectopic mineralization and fibrosis of the aortic valve, which can lead to serious consequences such as stenosis, heart failure, and even sudden death.[1] The characteristics of AVC are progressive thickening and hardening of the valve, resulting in orifice obstruction.[2] With the development of an ageing population, the prevalence and number of patients with AVC are constantly increasing. Currently, surgery is still an effective treatment method against AVC. However, there are certain risks associated with surgery, especially in elderly patients with multiple underlying diseases and poor body function, which may cause other adverse reactions. Although no available drugs are capable of effectively preventing the progression of AVC clinically, emerging studies indicate the potential therapeutic targets and drug delivery system in treating calcific aortic valve.[3] Therefore, it is urgent to elucidate the pathogenesis of AVC, and find safe and effective drugs.

The pathological characteristics of AVC are similar to those of atherosclerosis, including inflammatory cell infiltration, lipoprotein deposition and calcified nodule formation.[3] Low-density lipoprotein (LDL) can trigger the migration of inflammatory cells in the aortic valve, forming a plaque-like lesion composed of LDL and inflammatory cells, leading to the generation of reactive oxygen species and LDL oxidation. This continuous inflammatory process promotes osteogenic differentiation of valve interstitial cells.[4] Valve interstitial cells are the main cell type in aortic valves, and their osteogenic differentiation is the core cellular mechanism of AVC.[5] Therefore, reducing the content of LDL is considered an effective method to improve AVC. Statins are inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase, which can effectively prevent primary and secondary cardiovascular events by lowering plasma cholesterol, improving endothelial function, and reducing inflammatory status.[6] In a follow-up study of patients with aortic stenosis, atorvastatin was found to reduce the concentration of LDL-C by 53%.[7] However, whether atorvastatin can inhibit AVC by reducing LDL and its possible mechanism of action still need further exploration.

Bioinformatics analysis of potential genes in AVC revealed that the nuclear factor-κB (NF-κB) signalling pathway was closely related to AVC.[8] NF-κB is one of the main mediators inducing inflammatory responses. The silencing of the NF-κB gene has been proven to prevent the nuclear translocation of the osteogenic-related gene RUNX2, thereby preventing cell calcification,[9,10] which indicates that NF-κB may be an effective target for treating AVC. In addition, autophagy-related 5 (Atg5)-mediated autophagy reduces the inflammatory response by inhibiting the NF-κB signalling pathway.[11] Autophagy is a key process that removes or degrades ageing cells and proteins, maintaining intracellular homeostasis. A recent study showed that inhibiting intracellular autophagy levels could promote osteogenic differentiation of valve interstitial cells.[12] Although there are currently few relevant reports, this discovery provides new insights into the pathological mechanism of AVC. However, the role of NF-κB and autophagy in the pathogenesis of AVC has not yet been elucidated.

Interestingly, atorvastatin not only alleviated radiation therapy-induced intestinal damage by activating autophagy and antioxidant effects[13] but also improved doxorubicin-induced cardiomyopathy by regulating the autophagy-lysosome pathway.[14] In addition, it can induce autophagy by inhibiting the NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome activity and regulating NF-κB signalling to suppress TNF-α-induced degradation of the rat nucleus pulposus cell matrix.[15] Therefore, we wondered whether atorvastatin could improve AVC by regulating autophagy. Against this background, it is clear that atorvastatin could affect autophagy levels, and NF-κB is a key pathway in the pathogenesis of AVC. In the present study, we aimed to investigate the effect of atorvastatin in improving AVC *in vitro* and whether the regulatory effect of atorvastatin on autophagy is involved in its underlying mechanism.

Materials and Methods

Cell culture

Immortalized human valve interstitial cells were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in complete medium containing 10% FBS (Excell Bio, Hong Kong, China), 90% high glucose DMEM (Gibco, Rockville, MD, USA) and 1% penicillin G-streptomycin double antibody (MACKLIN, Shanghai, China) at 37°C in a 5% CO_2 atmosphere. For the OM group, the valve interstitial cells were cultured in osteoblastic medium after the third generation for 14 consecutive days according to published study,[16] with the medium changed every 2-3 days. The formula for the osteogenic culture medium was as follows: 1% FBS+50 mg/mL ascorbic acid + 100 nmol/L dexamethasone + 10 mmol/L β-glycerol phosphate + DMEM. For the OM+L-ATO group, the valve interstitial cells were treated with osteoblastic medium and 1 μmol/L atorvastatin. For the OM+M-ATO group, the valve interstitial cells were treated with osteoblastic medium and 5 μmol/L atorvastatin. For the OM+H-ATO group, the valve interstitial cells were treated with osteoblastic medium and 10 μmol/L atorvastatin. For the OM+ATO+si-Atg5 group, the valve interstitial cells were treated with osteoblastic medium and 5 μmol/L atorvastatin and transfected with si-Atg5 plasmid. For the OM+ATO+RANK group, the valve interstitial cells were treated with osteoblastic medium and 5 μmol/L atorvastatin and RANK.

Western blot

Western blot was performed to detect the expression levels of osteogenic differentiation, autophagy and NF-κB signalling pathway marker proteins. Briefly, total protein was extracted using RIPA lysis buffer (Beyotime, Shanghai, China), and the BCA method was used to detect the protein concentration. The loading volume was determined with 20 μg as the loading amount. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was prepared to separate proteins, which were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Next, the membrane was blocked with 5% skim milk powder (BD, Franklin Lakes, NJ, USA) at room temperature for 2 h and incubated with P21, ALP, RUNX2, Atg5, LC3, p-IκB, IκB, p-P65, P65 and glyceraldheyde 3-phosphate dehydrogenase (GAPDH) antibodies (Abcam, Cambridge, UK; dilution 1:200) overnight at 4°C. The second day after washing the membrane, the corresponding secondary antibody (Abcam; dilution 1:1000) was incubated at room temperature for 1 h. A chemiluminescence imaging system (Tanon, Shanghai, China) was used for development, and the results were analysed using ImageJ software for greyscale analysis.

Alizarin red staining

Alizarin red staining was performed to observe the deposition of calcium nodules. The 2nd to 5th generation valve interstitial cells with exponential growth were inoculated into a 12-well plate at a density of 5×10^4 cells per well. After treatment with osteogenic medium or atorvastatin administration, cells were fixed with 4%

paraformaldehyde (Boster, Wuhan, China) for 15 min. Then, 750 µL/well of alizarin red dye solution (0.2%) was added and incubated at room temperature for 30 min after washing with double distilled water 3 times. After dyeing, 500 µL/well 10% cetylpyridine chloride solution was added and incubated at room temperature for 30 min. Finally, the OD value was detected using an RT-6000 microplate reader (Rayto, Shenzhen, China) at a wavelength of 562 nm.

Ad-mCherry-GFP-LC3 adenovirus transfection

Ad-mCherry GFP-LC3 adenovirus (Hanbio, Shanghai, China) transfection was used to detect autophagic flow in valve interstitial cells. mCherry is a red fluorescent protein that is co-labelled with GFP for LC3. During the binding process between autophagosomes and lysosomes, the green fluorescence of GFP is quenched in an acidic lysosomal environment, and cells only emit mCherry red fluorescence. In short, valve interstitial cells were inoculated into a 24-well plate at a density of 1×10^4 cells per well. After administration, 2 µL ad-mCherry-GFP-LC3 adenovirus (20 MOI) was added and incubated in an incubator for 24 h. Then, after washing with D-Hanks, the cells were switched to complete culture medium and incubated for 24 h. The results were observed, the measurement of green, yellow, and red fluorescence was conducted under a Zeiss Axio Imager A2 fluorescence microscope (Zeiss, Oberkochen, Germany).

Si-RNA transfection

Valve interstitial cells were inoculated at a density of approximately 2×10^5 to 7×10^5 cells per well into a 6-well plate for cultivation. Meanwhile, 125 µL DMEM culture medium without antibiotics and serum, 100 pmoL si-Atg5 (Shanghai Sangon Co., Ltd., Shanghai, China), and 4 µL Lipo8000™ transfection reagent (Shanghai Sangon Co., Ltd.) were added to a clean EP tube, mixed and incubated at room temperature for 6 h. According to the dosage of 125 µL Lipo8000™ transfection reagent-siRNA per hole, the mixture was uniformly added to the entire well and cultured for 2 days.

Real-time PCR analysis

The total RNA of cells cultured in a six-well plate was extracted using TRIzol reagent (Vazyme, Nanjing, China), and purified RNA was obtained after chloroform phase separation, isopropanol precipitation, and 75% ethanol washing. Then, a reverse transcription system was prepared that reacted at 37°C for 15 min and 85°C for 5 s to obtain cDNA. Next, a real-time quantitative PCR system was prepared, and the reaction was performed using CFX96 Touch 1855195 Real-time Fluorescence Quantitative PCR Instrument (Bio-Rad, Hercules, CA, USA) according to the following procedure: predenaturation at 95°C for 10 min, denaturation at 95°C for 15 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and cycle count of 40; melting curve: 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. The primers were as follows: Atg5 (forward: CTTGCATCAAGTTCAGCTCTTC; reverse: TATGTGCGTATCCCAAAACTGG); GAPDH (forward: GGTCTCCTCTGACTTCAACA; reverse: GTGAGGGTCTCTCTCTTCCT). The results were calculated by $2^{-\Delta\Delta Ct}$ and corrected with GAPDH as the internal reference gene.

ELISA

ELISA was performed to detect the expression levels of inflammatory factors in cells. After treatment, the supernatant of each group of cells was collected. According to the manufacturer's instructions (Beyotime), standard curves were created, and the expression levels of interleukin-1 beta (IL-1β), TNF-α and TGF-β were detected using an RT-6000 microplate reader (Rayto).

Statistical analysis

The experimental data are represented as the mean ± SEM, and all data were processed and statistically analysed using GraphPad Prism 8 (La Jolla, CA, USA). The comparison between the sample mean of the two groups was performed by *t*-test, and the comparison between the sample mean of multiple groups was performed by ANOVA. A p-value <0.05 indicates that the difference is statistically significant.

Results

Atorvastatin improved valve interstitial cell calcification

To explore the effect of atorvastatin on AVC, valve interstitial cells were treated with calcified medium to induce osteogenic differentiation. Western blot assay for ageing-related proteins and osteogenic-related proteins showed that compared to those in the control (CON) group, the protein expression levels of P21, ALP, and RUNX2 in OM group cells were all elevated (Figure 1 A,B; $p<0.01$, $p<0.01$, and $p<0.001$). Meanwhile, alizarin red staining showed that the formation of calcium nodules in OM group cells was significantly increased, and calcium salt deposition was worsened (Figure 1C). These results indicated that valve interstitial cells were successfully induced into osteoid differentiation. On this basis, calcified valve interstitial cells were then treated with 1, 5, and 10 µmol/L atorvastatin to evaluate the effect of atorvastatin. As the concentration of atorvastatin increased, the protein expression levels of P21, ALP, and RUNX2 gradually decreased, and the formation of calcium nodules was reduced (Figure 1 D-F; $p<0.01$ or $p<0.001$). These findings suggested that atorvastatin prevented osteogenic differentiation of valve interstitial cells.

Atorvastatin elevated autophagy levels in calcified valve interstitial cells.

We next investigated whether atorvastatin regulated autophagy in AVC to relieve calcification. The expression of the autophagy marker proteins Atg5 and LC3 and the formation of autophagic flow were detected to evaluate autophagy levels. Western blot assay showed that compared to the CON group, the expression of Atg5 and the ratio of LC3B-II/I was significantly decreased in the OM group while increased in the atorvastatin-treated groups (Figure 2 A,B; $p<0.01$ or $p<0.001$). At the same time, ad-mCherry-GFP-LC3 adenovirus was transfected into valve interstitial cells to observe autophagy flux under a fluorescence microscope. Compared to the CON group, the OM group showed diffuse yellow fluorescence. Compared to the OM group, the atorvastatin-treated groups showed an increase in yellow spots and the ratio of yellow/red fluorescence as the atorvastatin concentration increased (Figure 2 C,D; $p<0.001$). These results indicated that atorvastatin upregulated autophagy levels, which were decreased in calcified valve interstitial cells.

Atorvastatin inhibited valve interstitial cell calcification by enhancing autophagy.

Then, we constructed a si-Atg5 plasmid, which was transfected into valve interstitial cells to knock down the expression of Atg5, to confirm that atorvastatin prevented valve interstitial cell calcification by regulating autophagy. Real-time PCR analysis showed that the expression of Atg5 was successfully downregulated (Figure 3A; $p<0.001$). We first examined the change in osteogenic protein expression. Compared with the OM+ATO group, knocking down Atg5 notably increased the protein expression levels of P21,

ALP, and RUNX2 (Figure 3 B,C; $p<0.05$). Meanwhile, the formation of calcium nodules was significantly increased in the OM+ATO+si-Atg5 group (Figure 3D). These results suggested that inhibiting Atg5-mediated autophagy reversed the alleviating effect of atorvastatin on valve interstitial cell calcification.

Atorvastatin inhibited the NF-κB signalling pathway by promoting autophagy.

To discuss the deeper mechanism of atorvastatin in reducing valve interstitial cell calcification by enhancing autophagy, we next investigated the effect of inhibiting autophagy on the NF-κB signalling pathway. Western blot assay showed that compared to the CON group, the expression levels of p-IκB and p-P65 were significantly elevated, and the ratios of p-IκB/IκB and p-P65/P65 were both increased (Figure 4 A,B; $p<0.05$). This result suggested that the NF-κB signalling pathway was activated in calcified valve interstitial cells. Compared to the OM+ATO group, the ratios of p-IκB/IκB and p-P65/P65 were both decreased, indicating that atorvastatin inhibited the NF-κB signalling pathway. Furthermore, after Atg5 was knocked down, the ratios were actually increased. Given that NF-κB is the classic pathway mediating the inflammatory response, we then detected the expression of inflammatory factors. ELISA showed that the contents of IL-1β, TNF-α and TGF-β were obviously decreased in the OM+ATO group compared to the CON group which were increased in the OM+ATO+si-Atg5 group (Figure 4 C-E, $p<0.01$). These results indicated that the NF-κB signalling pathway inhibited by atorvastatin was activated after downregulating autophagy.

Activating the NF-κB signalling pathway inhibited the enhancing effect of atorvastatin on autophagy

As shown above, atorvastatin inhibited the NF-κB signalling pathway in calcified valve interstitial cells by elevating autophagy levels. Therefore, the NF-κB activator RANK was then used to further demonstrate the relationship between the NF-κB signalling pathway and autophagy. As revealed by Western blot analysis, compared to the OM+ATO group, the expression of Atg5 and the ratio of LC3B-II/I were significantly decreased in the OM+ATO+RANK group (Figure 5 A,B; $p<0.05$). In addition, ad-mCherry-GFP-LC3 transfection showed a decrease in yellow spots and in the yellow-red fluorescence ratio in the OM+ATO+RANK group (Figure 5 C,D; $p<0.001$). Collectively, the level of autophagy enhanced by ATO was decreased by activating the NF-κB signalling pathway.

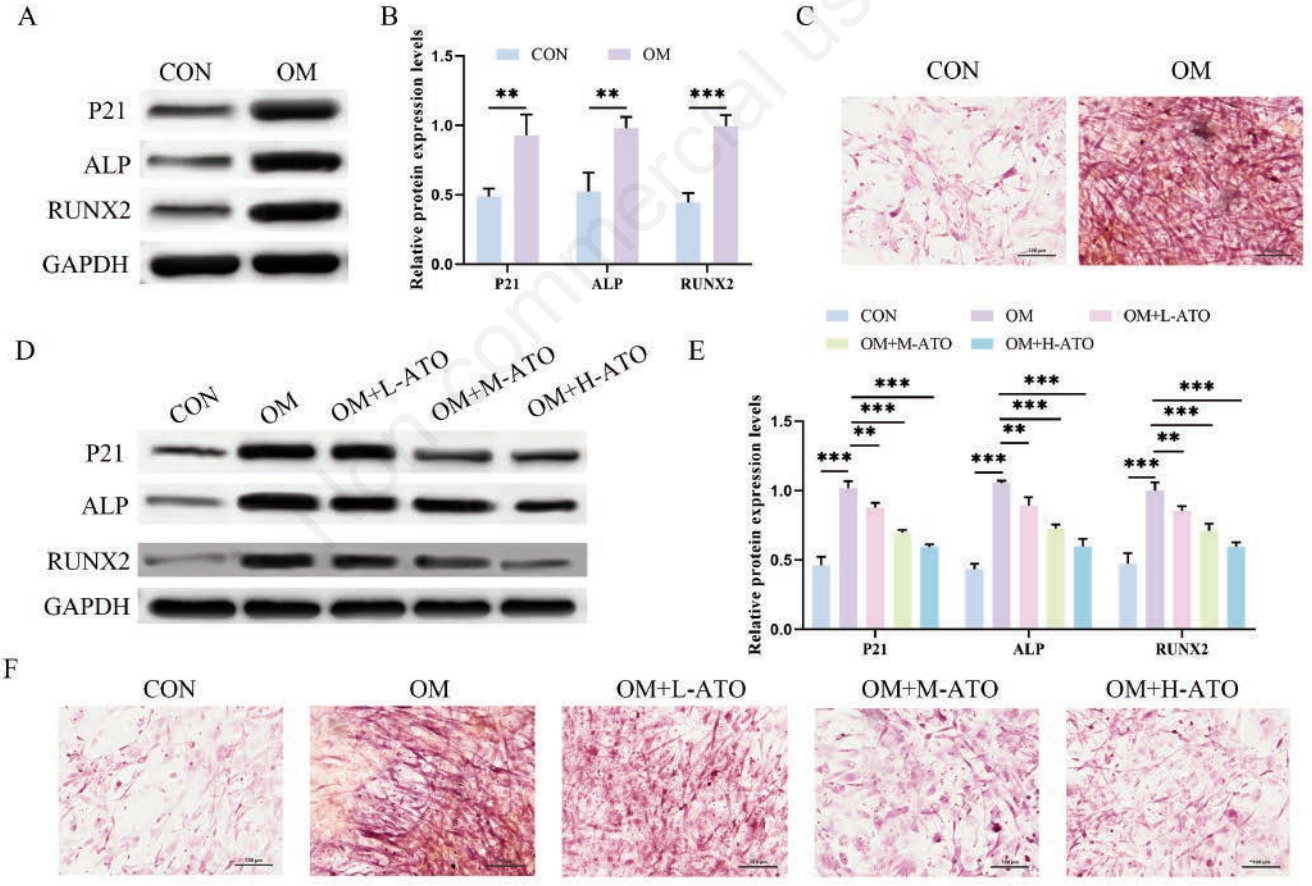

Figure 1. Atorvastatin improved valve interstitial cell calcification. **A**) Western blot analysis for the expression levels of the cell calcification marker proteins. GAPDH was used as a reference. **B**) Quantitative analysis of the expression levels of the cell calcification marker proteins; $^{**}p<0.01$, $^{***}p<0.001$. **C**) Alizarin red staining for the detection of calcium nodule deposition; scale bar: 100 μm. **D**) Western blot analysis examining the effect of atorvastatin treatment at different concentrations on the expression levels of calcification marker proteins. **E**) Quantitative analysis of the expression levels of the cell calcification marker proteins; $^{**}p<0.01$, $^{***}p<0.001$. **F**) Alizarin red staining to examine the effect of atorvastatin treatment at different concentrations on calcium nodule deposition; scale bar, 100 μm; n=3.

Activating the NF-κB signalling pathway reversed the ameliorative effect of atorvastatin on valve interstitial cell calcification

To document the role of the NF-κB signalling pathway in atorvastatin alleviating valve interstitial cell calcification, the effects of RANK on the expression levels of osteogenic differentiation proteins and calcium nodule deposition were observed. As expected, compared to the OM+ATO group, the expression of P21, ALP and RUNX2 in the OM+ATO+RANK group was markedly elevated (Figure 6 A,B; $p<0.001$). Moreover, RANK treatment induced a significant increase in calcium nodule formation (Figure 6C). These results demonstrated that RANK abolished the inhibitory effect of atorvastatin on osteogenic differentiation and calcium deposition in valve interstitial cells. Taken together, atorvastatin reduced valve interstitial cell calcification by inhibiting the NF-κB signalling pathway.

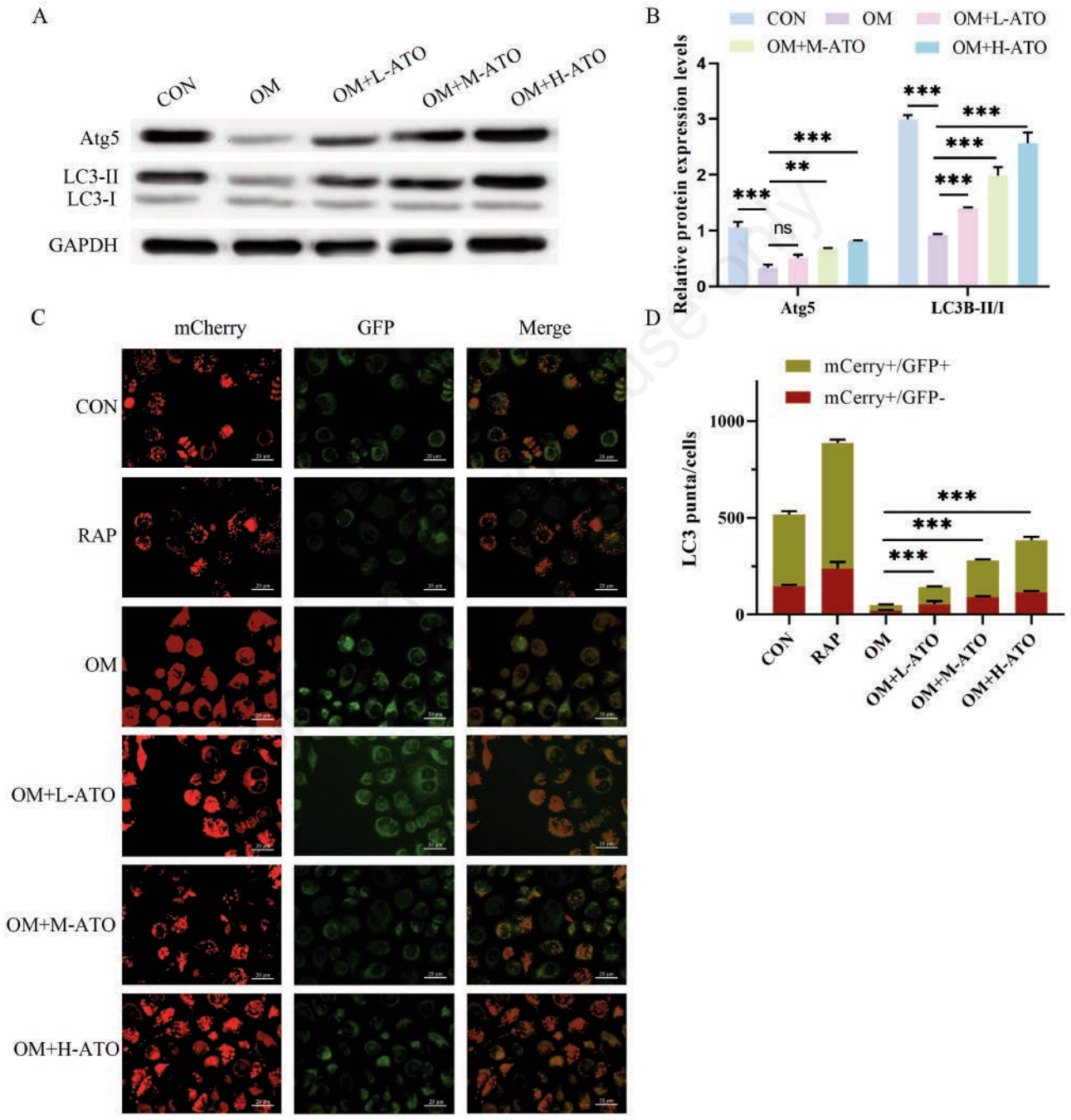

Figure 2. The effect of atorvastatin on autophagy levels in calcified valve interstitial cells. **A**) Western blot analysis for the expression levels of the autophagy marker proteins Atg5 and LC3. **B**) Quantitative analysis of the expression levels of Atg5 and LC3-II/LC3-I; $^{**}p<0.01$, $^{***}p<0.001$. **C**) Ad-mCherry GFP-LC3 adenovirus transfection to detect changes in autophagic flow. Red fluorescence represents mCherry, green fluorescence represents GFP. **D**) Statistical results of the yellow dots in merged graphic and free red dots; $^{***}p<0.001$, n=3.

Discussion

As a classic statin drug, atorvastatin has the characteristics of fast response, strong lipid-lowering effect, and long action time. It can increase the uptake and metabolism of LDL by increasing the number of LDL-receptors on the surface of liver cells.[17] In addition to its lipid-lowering effect, atorvastatin can also inhibit the proliferation and migration of vascular smooth muscle and alter blood viscosity and coagulation function. In recent years, it has been widely studied in heart and cardiovascular diseases, such as coronary heart disease,[18] myocardial infarction,[19] and heart failure.[20] Moreover, a clinical study suggested that atorvastatin treatment halts the progression of calcified aortic stenosis in patients with asymptomatic calcified aortic stenosis.[21] Therefore, in the current study, we investigated the function of atorvastatin in valve interstitial cell calcification *in vitro* and identified the regulation of autophagy and the NF-κB signalling pathway as its mechanism.

The osteogenic transformation of valve interstitial cells is believed to be the foundation for the development of AVC.[22] In our

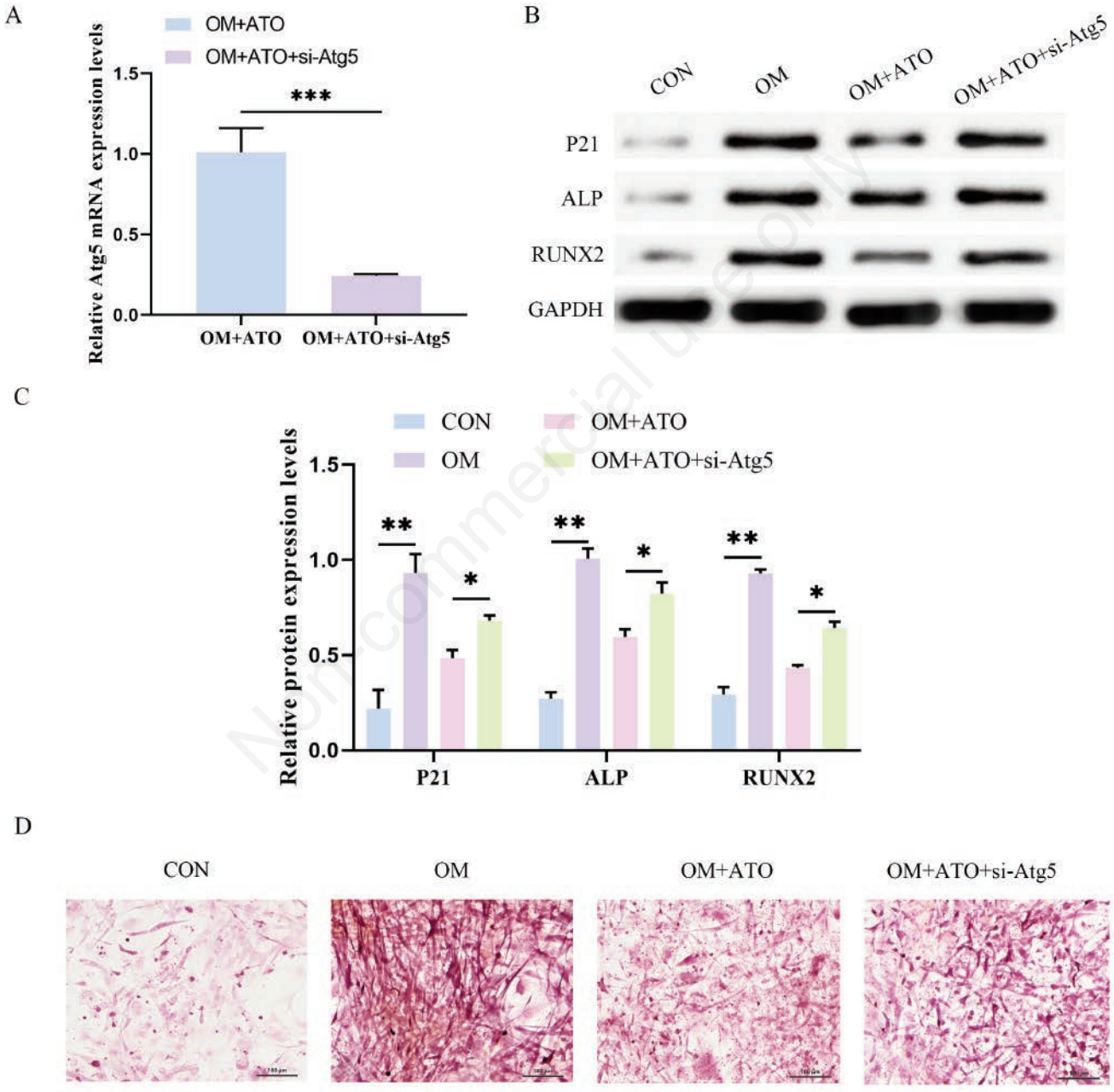

Figure 3. The effect of inhibiting autophagy on atorvastatin improving valve interstitial cell calcification. **A**) Real-time PCR assay to verify the successful knockdown of Atg5; ***p<0.001. **B**) Western blot analysis to detect the effect of knocking down Atg5 on atorvastatin-mediated inhibition of calcification marker protein expression. **C**) Quantitative analysis of the expression levels of the cell calcification marker proteins; *p<0.05, **p<0.01. **D**) Alizarin red staining to examine the effect of knocking down Atg5 on atorvastatin alleviating calcium nodule deposition; scale bar, 100 μm; n=3.

study, to induce calcification of valve interstitial cells, we cultured them in osteoblastic medium for 14 days, and ultimately, the valve interstitial cells were successfully induced to undergo osteogenic differentiation, which was specifically manifested by a significant increase in the expression levels of cell aging and osteogenic differentiation marker proteins. In addition, we found that atorvastatin inhibited the expression of RUNX2. RUNX2 is highly expressed in adult calcified aortic valves and is considered a necessary protein for osteogenic differentiation and calcification of aortic valves.[23] This suggested that atorvastatin might have the ability to reduce calcification in valve interstitial cells. The reduction in calcium nodule deposition by atorvastatin has been confirmed. Calcium nodule deposition is the main manifestation of calcification, which can cause valve thickening, stiffness, and loss of elasticity, leading to serious heart damage.[24] Taken together, the inhibitory effect of atorvastatin on valve interstitial cell calcification indicated its potential in the treatment of AVC.

Autophagy is a conserved process in which autophagy-related genes regulate the degradation of damaged organelles and macromolecules.[25] A study found that stabilizing the expression of autophagy-related mRNA and regulating autophagy flow are beneficial for delaying calcified aortic valve disease.[26] This indicates that autophagy plays an important role in AVC. In our study, atorvastatin was found to elevate the expression of autophagy-related proteins and the formation of autophagic flow in calcified valve interstitial cells. Atg5 is a classic autophagy marker protein. Thus, we observed the effect of inhibiting autophagy on atorvastatin-reducing valve interstitial cell calcification by constructing an Atg5 knockdown plasmid. The results showed that after knocking down Atg5, the expression of osteogenic proteins and calcium nodule deposition reduced by atorvastatin both increased. In conclusion, atorvastatin inhibits valve interstitial cell calcification by enhancing autophagy. It has been confirmed that the NF-κB pathway is activated in the early stages of AVC.[27] The progression of calcific aortic valve stenosis is closely associated with inflammation, playing a key role in various stages of the disease, including cell damage, transformation, and calcification processes. Also, the accumulation of lipoproteins in the aortic valve plays a central role

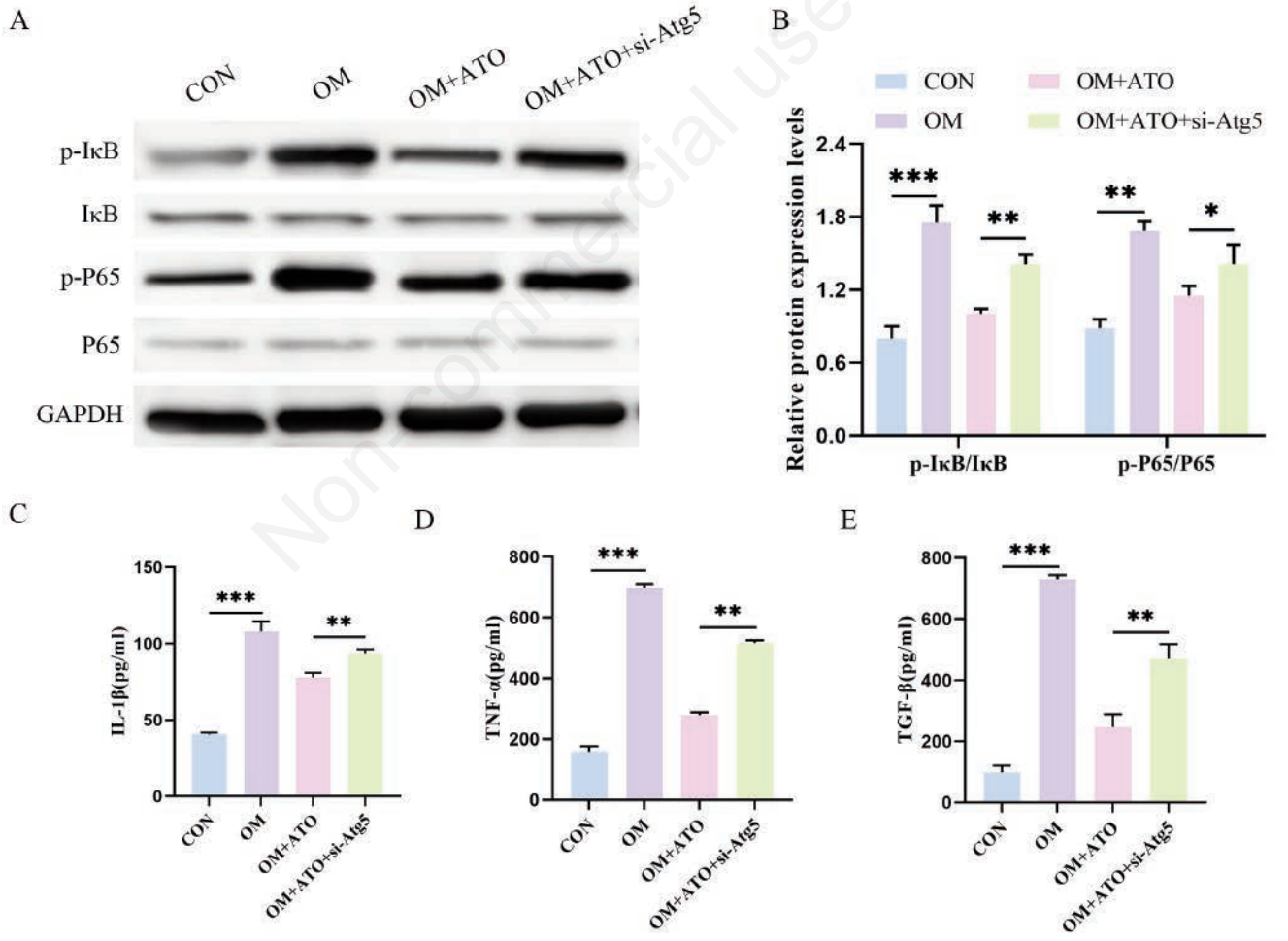

Figure 4. The effect of inhibiting autophagy on atorvastatin-mediated inhibition of the NF-κB signalling pathway. **A**) Western blot analysis to detect the expression levels of NF-κB signalling pathway marker proteins. **B**) Quantitative analysis of the ratio of p-P65/P65 and p-IκB/IκB; *p<0.05, **p<0.01, ***p<0.001. **C-E**) ELISA for the content of inflammatory factors, IL-1β, TNF-α and TGF-β; **p<0.01, ***p<0.001; n=3.

in driving inflammation, preceding the pathological mineralization seen in calcific aortic valve stenosis. This lipid retention induces a chronic low-grade inflammatory response, potentially triggering an osteogenic pathway within the aortic valves.[28] Activated NF-κB translocates into the nucleus and regulates the expression of downstream target including IL-1β and IL-6, thereby participating in various biological processes, such as the inflammatory response.[29] We found that atorvastatin inhibited the activation of the NF-κB signalling pathway in calcified valve interstitial cells, and this effect was reversed after knocking down Atg5. This indicated that atorvastatin inhibited the NF-κB signalling pathway by enhancing autophagy. In addition, we used the NF-κB activator RANK for validation. As expected, the activation of NF-κB resulted in a decrease in autophagy levels enhanced by atorvastatin. This confirmed the interaction between autophagy and NF-κB. Moreover, we validated the effect of the NF-κB signalling pathway on valve interstitial cell calcification. After RANK treatment, the expression levels of osteogenic proteins increased, which is consistent with the research results of others.[30] Meanwhile, RANK also led to an increase in calcium nodule deposition, reversing the relieving

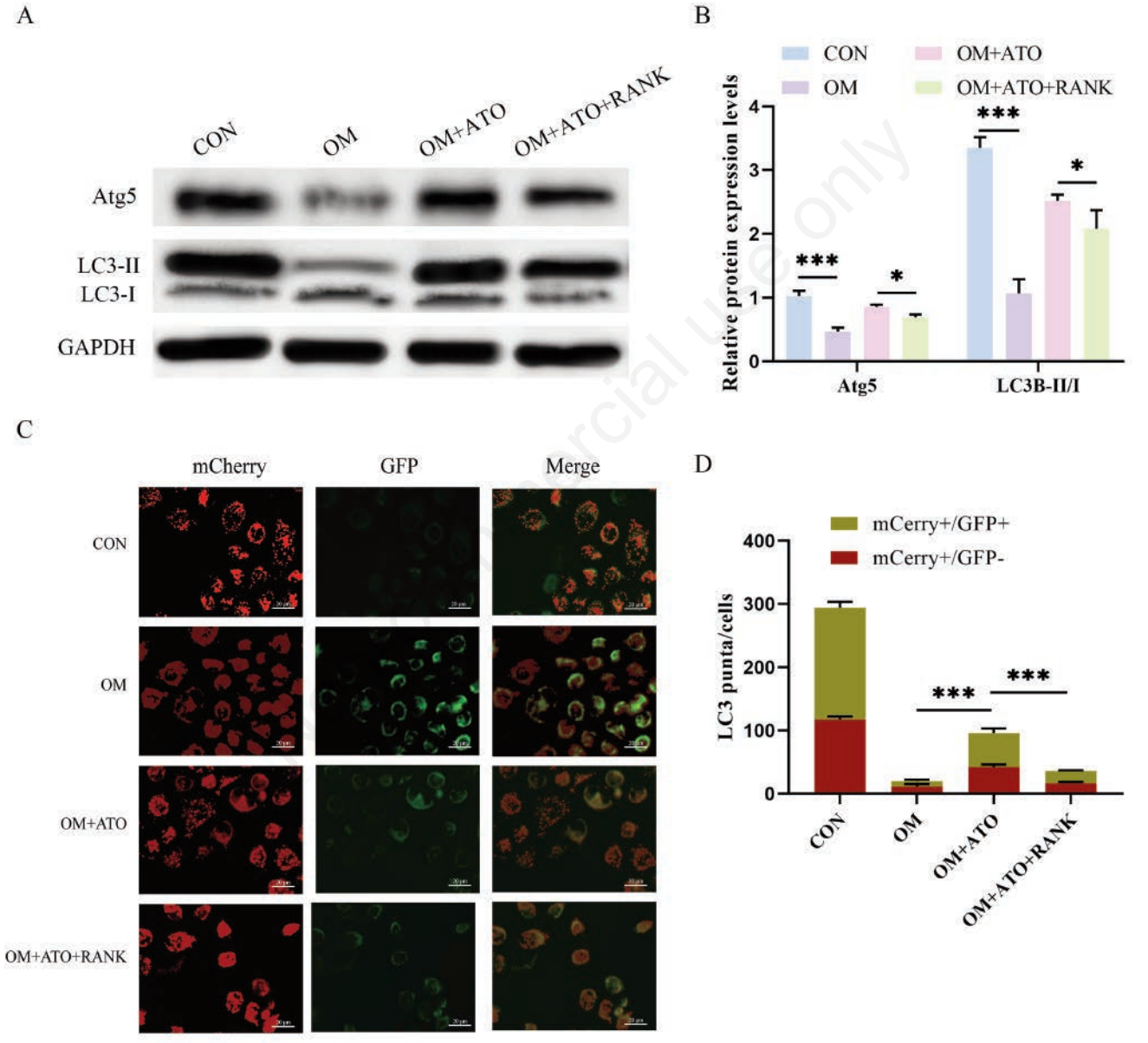

Figure 5. The effect of activating the NF-κB signalling pathway on atorvastatin-enhanced autophagy levels in calcified valve interstitial cells; n=3. **A**)Western blot analysis of the effect of RANK treatment on the elevation of the expression levels of the autophagy marker proteins Atg5 and LC3 by atorvastatin. **B**) Quantitative analysis of the expression levels of Atg5 and LC3-II/LC3-I; *p<0.05, ***p<0.001. **C**) Ad-mCherry GFP-LC3 adenovirus transfection to detect changes in autophagic flow; red fluorescence represents mCherry, green fluorescence represents GFP. **D**) Statistical results of the yellow dots in merged graphic and free red dots; ***p<0.001; n=3.

effect of atorvastatin on valve interstitial cell calcification. Therefore, inhibiting the NF-κB signalling pathway may be one of the mechanisms of atorvastatin in the treatment of AVC.

In summary, we found that atorvastatin can prevent osteogenic differentiation of valve interstitial cells. To date, there are no safe and effective drugs in clinical practice that can be used to prevent and slow AVC, but statins and angiotensin-converting enzyme (ACE) inhibitors have always been highly anticipated. Our findings suggest that atorvastatin can not only enhance the autophagy level of calcified valve interstitial cells but also inhibit the activation of the NF-κB signalling pathway. In light of these findings, the future clinical application of atorvastatin in the management of AVC holds great promise. These novel mechanisms of action provide a strong foundation for exploring the efficacy of atorvastatin in preventing and slowing the progression of AVC, offering hope for improved treatment strategies and outcomes in the field of cardiovascular medicine.

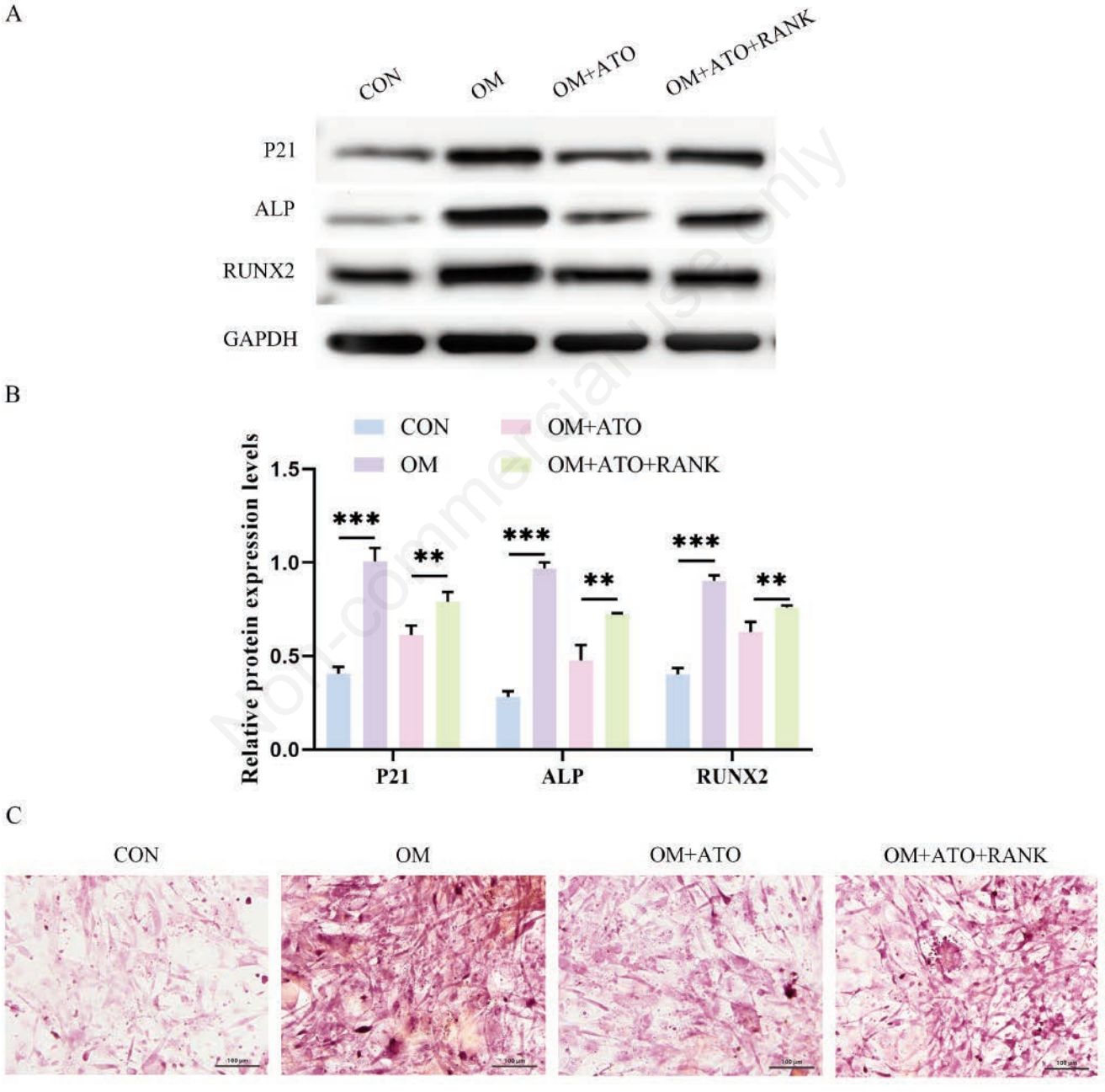

Figure 6. The effect of activating the NF-κB signalling pathway on atorvastatin improving valve interstitial cell calcification. **A**) Western blot analysis to detect the effect of RANK treatment on atorvastatin-mediated inhibition of calcification marker protein expression. **B**) Quantitative analysis of the expression levels of the cell calcification marker proteins; **p<0.01, ***p<0.001. **C**) Alizarin red staining to examine the effect of RANK treatment on atorvastatin alleviating calcium nodule deposition; scale bar, 100 μm; n=3.