Midkine Promotes the Proliferation of Airway Smooth Muscle Cells Induced by LPS Through Notch2 Pathway

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Abstract

***Background**:* Airway smooth muscle cells (ASMC) can produce a variety of cytokine during inflammation, causing changes in the components of the extracellular matrix, which are related to airway remodeling. Midkine (MK) can promote the chemotaxis of various inflammatory cells and release inflammatory factors. Whether Notch and Midkine together affect the proliferation and apoptosis of airway smooth muscle cells is unclear.

Objective: To study the mechanism of Midkine on LPS-induced acute lung injury caused by airway smooth muscle cells.

***Methods**:* Airway smooth muscle cells were cultured in vitro and divided into 5 groups: control group, lipopolysaccharide group (LPS), Non-targeted siRNA group, MKsiRNA group, Notch inhibitor group (LY411575). The cell proliferation level was detected by CCK-8. The apoptosis level was detected by flow cytometry. The changes of cytokine in the Midkine/Notch2 signaling pathway were detected by Westernblot, qPCR and cellular immunofluorescence.

***Results**:* Midkine and Notch2 were highly expressed in the LPS group. MKsiRNA can effectively block the expression of Midkine induced by LPS while down-regulating the expression of Notch2. This result is the same as that of Notch inhibitor (LY411575). Exogenous Midkine promoted the proliferation of airway smooth muscle cells and reduced the rate of apoptosis in the LPS group. When the expression of Midkine was blocked, the proliferation of airway smooth muscle cells in the LPS group was significantly reduced, while apoptosis increased. Inhibiting the expression of Notch, the proliferation of airway smooth muscle cells in the LPS group decreased, and apoptosis increased.

***Conclusions**:* Midkine/Notch2 signaling pathway plays an important role in regulating airway smooth muscle cell proliferation and apoptosis in airway inflammation.

1. Background

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) refers to acute and progressive respiratory failure caused by various pathogenic factors[1]. ARDS may occur at the end-stage of various severe diseases. The occurrence and development of ALI/ARDS are mainly related to the infection of microorganisms and some non-infectious factors, such as extensive trauma, non-cardiogenic shock, poisoning, long-term cardiopulmonary resuscitation, surgery, acute pancreatitis, etc.[2].

Midkine (MK) is a new heparin-binding growth factor, which can chemotaxis a variety of inflammatory cells, release a large number of cytokines and participate in the whole inflammatory process[3,4]. At present, studies have proved that Notch receptor is one of MK receptors[5,6,7]. Studies have found that Midkine induces structural recombination of actin by binding to Notch-2 receptor, resulting in phosphorylation of STAT3 and enhancement of its activity, further promoting cell growth and differentiation regulation. Midkine activates related pathways by inducing changes in Notch-2 receptor

domain and stimulates the expression of Hes1 and NF-κB[8]. Previous studies have confirmed that Midkine is highly expressed in cystic pulmonary fibrosis[9]. Some studies have found that Midkine levels in blood of patients with acute respiratory distress syndrome increased[10] However, the related pathway mechanism of Midkine has not been fully elaborated.

Studies have found that airway smooth muscle cells can produce various inflammatory cytokines and growth factors in addition to the characteristic of contraction, and can change extracellular matrix components and further promote the process of airway remodeling [11,12]. Abnormal proliferation and hypertrophy of airway smooth muscle cells can lead to thickening of airway wall, thus causing airway remodeling [13]. Therefore, it is not clear whether MK-Notch pathway participates in the proliferation of airway smooth muscle cells. MK is likely to inhibit apoptosis and promote proliferation of airway smooth muscle cells through Notch pathway, thus promoting airway remodeling. Therefore, whether inhibition of MK expression can promote apoptosis and inhibit proliferation. In this study, the effect of Midkine on rat airway smooth muscle cells was studied by culturing rat airway smooth muscle cells in vitro.

2. Methods

2.1 Materials

Cells are derived from rat airway smooth muscle cells and purchased from Guangzhou Gennio Biological Technology Co., Ltd. All experiments were approved by the Ethics Committee.

2.2 Experimental Methods

2.2.1 Cell culture

Rat airway smooth muscle cells were incubated with DMEM (containing 10% FBS) medium in a 5% CO_2 incubator at 37 ℃, and the medium was changed regularly for serial subcultivation. The logarithmic ASMC cells were randomly selected and divided into 5 groups as follows: (a) Control group: untreated ASMC cells. (b) LPS group: ASMC cells induced by LPS were used for 72 h according to the IC50 results of LPS. (c) Non-targeted siRNA group: ASMC cells were transfected with Non-targeted siRNA and stimulated with LPS for 72 h. (d) MKsiRNA group: ASMC cells were transfected with Midkine siRNA and stimulated with LPS for 72 h. (e) LY411575 group: ASMC cells in LPS group were treated with LY411575 for 48 h according to IC50 results of Notch2 inhibitor (LY411575). The nucleotide sequence of MK siRNA is 5 '-CAAAGGCCAAAAGC CAAGAAA-3', 5 '-GAAGAGGCTCGGTACAAT-3', 5 '-CGACTGCAAATACAA GTT-3'.

2.2.2 Cell survival rate was detected by CCK8

ASMC cells were inoculated in 96-well plates at 10^5 cells/mL and 100μL per well. The following experiments were carried out 1 day after inoculation: in experiment 1, the IC50 of lipopolysaccharide (LPS) on ASMC cells were detected (Fig. 1A). In experiment 2, the IC50 of LY411575 on ASMC cells were detected (Fig. 1B). In experiment 3, the cell survival rates of control group, LPS group, Non-targeted siRNA

group and MKsiRNA group were detected (Fig. 5A). In experiment 4, the cell survival rates of control group, LPS group (0.2 mg/mL), MKsiRNA group and LY411575 group (0.03 mg/mL) were detected (Fig. 5B). In experiment 5, the cell survival rate of different concentrations of exogenous recombinant Midkine (rMK) on control ASMC was detected (Fig. 6). In experiment 6, the cell survival rate of ASMC treated with different concentrations of exogenous recombinant Midkine (rMK) for different times in LPS group was detected (Fig. 7).

2.2.3 The expression of related factors was detected by Western blot

The total proteins of ASMC cells in control group, LPS group (0.2 mg/mL), Nontargeted siRNA group, MK siRNA group and LY411575 group (0.03 mg/mL) were extracted and prepared according to the standard procedure and quantified by BCA method. Add the balanced protein to the prepared electrophoresis gel for electrophoresis, and stop the electrophoresis when the protein loading buffer runs to the bottom. The protein on the electrophoretic gel was transferred to PVDF film by wet rotation method. The film was incubated with primary antibody (Anti-Midkine, Anti-Notch2, Anti-β-actin) (Abcam) at 4℃ overnight, and then the film was incubated with Goat Anti-Rabbit IgG at room temperature for 1h on the next day. After washing, ECL luminescent reagent was used and exposed by gel imaging system (Tanon). The β-actin was used as the internal reference protein, and the ratio of the gray value of the target band to the gray value of the internal reference band was used as the relative expression amount of the protein. In experiment 1, the expression of Midkine in control group, LPS group (0.2 mg/mL), Nontargeted siRNA group and MK siRNA group was detected (Fig. 2A-B). In experiment 2, the Notch2 expression was detected in control group, LPS group (0.2 mg/mL), LY411575 group and MK siRNA group (Fig. 2C ~ D).

2.2.4 qPCR was used to detect the mRNA expression of related factors

The total RNA of ASMC cells in control group, LPS group (0.2 mg/mL), Nontargeted siRNA group, MK siRNA group and LY411575 group were extracted by TRizol method and prepared according to standard procedure. RNA purity and concentration were measured. The primers of Midkine, Notch2 and GAPDH were as follows (Midkine: FORWARD: 5 '-CAGACCCAGCGCATCATTG-3') were designed and synthesized by Bioengineering (Shanghai) Co., Ltd.; REVERSE: 5 '-TCTTGGAGGTGCAGGCTTG-3'. Notch2: FORWARD: 5 '-GGTGGTCAAGAGCCCTGTGT-3'; REVERSE: 5 '-TGGCCTGCGTCACACAGTA-3'. GAPDH: FORWARD: 5 '-CAGCCAGGAGAAATCAAACAG-3'; REVERSE: 5 '-GACTGAGTACCTGAACCGGC-3'). Reverse transcription was performed using a reverse transcription kit (Thermo Reagent Company). Real-time fluorescence quantitative PCR detection was carried out by PCR fluorescence quantitative kit (Biosharp). The reaction conditions were pre-denaturation at 95 ℃ for 5 min; the reaction was repeated at 95 ℃ for 10 sec, 60 ℃ for 30 sec (40 cycles), and extended at 60 ℃ for 30sec. The qPCR reaction system was 20 μL, including Hieff ® qPCR SYBR ® Green Master Mix 10μL, Forward Primer (10M) 0.5μL, Reverse Primer (10M) 0.5μL, template DNA 1μL and sterile ultrapure water 8 μL. GAPDH was used as internal reference, and 2 − Ct was used to represent the ratio of target gene expression in each group, and compared with the control group. In experiment 1, the expression of Midkine mRNA in control group, LPS group (0.2 mg/mL), Nontargeted

siRNA group and MK siRNA group was detected (Fig. 3A). In experiment 2, the Notch2 mRNA expression was detected in control group, LPS group (0.2 mg/mL), LY411575 group and MK siRNA group (Fig. 3B)

2.2.5 Immunofluorescence assay was used to detect the expression of related factor proteins

ASMC were inoculated in confocal culture dish at the rate of 105 cells/mL and 1mL per well, and the control group, LPS group (0.2 mg/mL), Nontargeted siRNA group, MK siRNA group and LY411575 group were prepared. Immunofluorescence staining was performed after successful preparation. PBS buffer was used for washing 3 times, and 3 min each time, 4% paraformaldehyde was used to fix cells for 15 min, and PBS buffer was used for washing 3 times, and 3 min each time. 0.5% TritonX-100 was used to penetrate at room temperature for 15 min, and PBS buffer was soaked for 3 times, and 3 min each time. A proper amount of normal goat serum was added dropwise and sealed at room temperature for 30 min. Add enough diluted primary antibody (Anti-Midkine, anti-Notch2) and put it into a wet box, put it in a 4 ℃ refrigerator, and incubate overnight. The next day, PBST buffer was used for washing 3 times, and 3 minutes each time. Diluted fluorescent second antibody (Goat Anti-Rabbit IgG) was added dropwise and incubated on a room temperature shaking for 1 h. 10 μL DAPI was added dropwise and incubated in light for 5 min, then the nuclei were stained. Soak and wash with PBST for 3 times, each time for 3 minutes. Anti-fluorescence quencher was added dropwise. Images were collected under a fluorescence microscope. In experiment 1, the expression of Midkine protein in control group, LPS group (0.2 mg/mL), Nontargeted siRNA group and MK siRNA group was detected (Fig. 4A). In experiment 2, the expression of Notch2 protein in control group, LPS group (0.2 mg/mL), Nontargeted siRNA group, MK siRNA group and LY411575 group was detected (Fig. 4B)

2.2.6 Apoptosis rate was detected by flow cytometry

ASMC was inoculated into 6-well plates at 10^5 cells/mL and 2 mL per well. After inoculation, the following experiments were carried out: in experiment 1, the effects of control group, LPS group, MK siRNA group, Non-targeted siRNA group and different concentrations of recombinant Midkine (rMK) on the apoptosis rate of ASMC cells in LPS group were detected (Fig. 8). In experiment 2, the effects of control group, LPS group, MKsiRNA group and LY411575 group on apoptosis rate were detected (Fig. 9).

2.3 Statistical analysis

The experimental data were expressed by mean ± standard deviation (±s) and processed by SPSS 20.0 software. One-way ANOVA was used to compare the differences between groups, and independent sample t test was used for intra-group comparison. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Detection of cell activity by CCK8

The effects of LPS and Notch2 inhibitor (LY411575) on the inhibition rate and cell survival rate of ASMC cells were detected by CCK-8 kit. The results showed that the IC50 of LPS was 0.2 mg/mL, and the IC50 of Notch2 inhibitor (LY411575) was at 0.03 mg/mL (Fig. 1A-B).

3.2 The expression of related factors detected by Western blot

Western blot was used to detect the expression of Midkine protein. The protein band image was analyzed by WCIF Hmage J image analysis software and standardized with β-actin. The results showed that the expression of Midkine protein in LPS group was significantly higher than that in control group (* $P < 0.05$), and the expression of Midkine protein in MK siRNA group was lower than that in LPS group (# $P < 0.05$) (Fig. 2A-B). Western blot was used to detect the expression of Notch2 protein. The protein band image was analyzed by WCIF Hmage J image analysis software and standardized with β-actin. The results showed that the expression of Notch2 protein in LPS group was higher than that in control group (* $P < 0.05$), MK siRNA group (# $P < 0.05$) and LY411575 group (# $P < 0.05$) (Fig. 2C-D).

2.3 The mRNA expression of related factors detected by qPCR analysis

The expression of Midkine and Notch2 mRNA in each group was detected by qPCR. The relative expression of Midkine mRNA in LPS group was higher than that in control group (* $P < 0.05$) and MKsiRNA group (# $P < 0.05$). (Fig. 3A). The relative expression of Notch2 mRNA in LPS group was higher than that in control group (* $P < 0.05$), MKsiRNA group (# $P < 0.05$) and LY411575 group (# $P < 0.05$). (Fig. 3B).

3.4 The expression of Midkine and Notch2 in ASMC of each group observed by immunofluorescence method

The expression of Midkine in LPS group was higher than that in control group by immunofluorescence method ($P < 0.01$). The expression of Midkine in MKsiRNA group was lower than that in LPS group ($P < 0.01$). Midkine expression in non-target siRNA group was slightly lower than that in LPS group, but the difference had no statistical significance ($P > 0.05$) (Fig. 4A). The expression of Notch2 in LPS group was higher than that in the control group ($P < 0.05$), the expression of Notch2 in LPS group was higher than that in MK siRNA group ($P < 0.05$), and the expression of Notch2 in LPS group was higher than that in LY411575 group ($P < 0.05$) (Fig. 4B).

3.5 Cell proliferation test

3.5.1 CCK8 kit was used to detect the cell survival rate of cells in control group, LPS group, Non-target siRNA group, MK siRNA group and LY411575 group.

According to the results of CCK-8 kit analysis, the survival rate in LPS group was lower than that in control group ($P < 0.05$), the survival rate in MK siRNA group was lower than that in LPS group ($P < 0.05$), and there was no significant difference in survival rate between the Non-target siRNA group and the LPS

group, showing no statistical significance (P > 0.05) after LPS intervention (Fig. 5A). The cell survival rate in LY411575 group was lower than that in LPS group (P < 0.05) (Fig. 5B).

3.5.2 CCK8 kit was used to detect the proliferation of recombinant Midkine (rMK) on normal ASMC

rMK had no significant effect on the survival rate of ASMC cells in normal group, without statistical significance (P > 0.05) (Fig. 6).

3.5.3 CCK8 kit was used to detect the proliferation of rMK with different concentrations on LPS with different concentrations groups for different times.

The proliferation ability of rMK in LPS group increased significantly with the increase of rMK concentration (P < 0.05), but there was no significant difference in cell survival rate between 48 h and 72 h treatment (P > 0.05) (Fig. 7).

3.6 Apoptosis assay

3.6.1 Apoptosis experiment of LPS group treated with different concentrations of recombinant Midkine (rMK)

The apoptosis rate of ASMC cells was detected by flow cytometry, and the flow cytometry diagram is shown in Fig. 8A. The results showed that the apoptosis rate of cells in LPS group was higher than that in control group (* P < 0.05), and the apoptosis rate of cells in MKsiRNA group was higher than that in LPS group (# P < 0.05). The apoptotic rate of cells in LPS group was significantly lower than that of 1ng/mL, 10ng/mL, 100ng/mL rMK treated groups (# P < 0.01), but there was no significant difference between rMK with different concentrations groups (P > 0.05) (Fig 8B). The apoptotic rate of cells in LPS group was significantly lower than that in LPS group (# P < 0.01) after treatment with 1ng/mL, 10ng/mL, 100ng/mL rMK.

3.6.2 Effects of LY411575 on the apoptosis in LPS group

The apoptosis rate of ASMC cells was detected by flow cytometry, and the flow cytometry diagram is shown in Fig. 9A. The results showed that the apoptosis rate of cells in MKsiRNA group was significantly higher than that in LPS group (P < 0.01), and the apoptosis rate of cells in LY411575 group was significantly higher than that in the LPS group (P < 0.01) (Fig. 9B).

4. Discussion

Acute lung injury is a clinical disease with high mortality, which brings great treatment pressure to the clinic. Although great progress has been made in diagnosis and treatment, its poor prognosis is still an urgent clinical problem to be solved. When acute lung injury occurs, apoptosis and necrosis of vascular endothelial cells and the impaired integrity of vascular wall will result in increased permeability and pulmonary edema. Lipopolysaccharide (LPS) is a component of Gram-negative bacteria cell wall because

it can induce inflammatory reaction and secretion of various cytokines, and it is close to ALI caused by clinical infection, ALI models are widely used at home and abroad[14, 15]. Therefore, this study designed to use LPS to prepare an acute lung injury model of rat airway smooth muscle cells. Some studies have found that when LPS is used to induce acute lung injury model, TNF is significantly higher than that in the control group. TNF-α can cause pulmonary vascular endothelial cell injury, change vascular permeability and cause pulmonary edema, which may promote Midkine expression through NF-κB pathway [16,17]. Midkine can promote the release of MCP-1, MIP-2 and other chemokines, and promote the aggregation of macrophages and neutrophils[18]. Midkine expression in normal organs is usually very weak, but it can be induced during oxidative stress, inflammation and tissue repair[19, 20]. In this study, LPS-induced rat airway smooth muscle cells can enhance Midkine expression in vitro, while low expression in control airway smooth muscle cells. However, the role of Midkine in LPS-induced acute lung injury caused by airway smooth muscle cells and related signaling pathways have not been fully clarified.

Initial studies found that Midkine can promote cell proliferation[21]. Midkine expression has been found in many tumors and inflammatory diseases, and its participation in inflammatory reactions and anti-apoptosis effects has been confirmed[22]. In order to study the effect of Midkine on proliferation and apoptosis of airway smooth muscle cells, this study quoted recombinant Midkine (rMK) to observe whether it has proliferation effect on airway smooth muscle cells. Previous studies have shown that after HCl aspiration and mechanical ventilation, collagen deposition and hydroxyproline deposition in lung tissue of wild-type mice lead to a decrease in lung compliance. In Midkine-/-mice, this phenomenon is significantly reduced. Inhibition of Nox1, Midkine or Notch2 can reduce the occurrence of epithelial-mesenchymal transformation, which indicates that Midkine plays an important role in airway remodeling [10,23]. Zhang et al. Also proved that MK promotes hypoxia-induced proliferation and differentiation of human lung epithelial cells [24]. In this study, MK siRNA was used to inhibit the expression of Midkine and LPS was used to induce acute lung injury. The results showed that the cell survival rate of MK siRNA group was lower than that of LPS group, which also indicated that Midkine could reduce LPS-induced acute lung injury. Interestingly, recombinant Midkine was applied to airway smooth muscle cells without LPS treatment and airway smooth muscle cells without LPS treatment. Comparing the two groups, it was found that recombinant Midkine could promote the proliferation of airway smooth muscle cells in LPS treatment group and reduce cell apoptosis, while there was no obvious change in LPS treatment group. The results of this study show that Midkine participates in the repair of inflammatory tissues, promotes cell proliferation and inhibits cell apoptosis, further causing airway remodeling, while inhibiting Midkine can inhibit cell proliferation and promote cell apoptosis.

Notch2 is one of the receptors of Midkine, which can hydrolyze and cleave Midkine at two different sites after binding to Midkine, enabling it to enter the nucleus and play the role of transcription activator, participating in a variety of cell processes[25,26]. LY411575 is a-secretase inhibitor, which has been proved to inhibit osteoclast differentiation and bone destruction through Notch/HES1/MAPK/Akt pathway[27]. In this study, LY411575 was cited to inhibit Notch expression. Notch2, as a receptor of MK, can cause tumor invasion and metastasis by regulating epithelial-mesenchymal transformation (EMT), and may be down-regulated by its inhibitors or natural compounds, resulting in EMT reversing to

mesenchymal-to-epithelial transformation[28]. Notch2 interacts with MK in human lung epithelial cells, and MK-induced EMT is affected by mechanical stretching. Inhibition of Notch2 signal blocks EMT more effectively than targeting endogenous MK. Silencing Midkine or Notch2 can slow down the occurrence of pulmonary fibrosis[29]. Danahay et al. found that Notch2 signal mediates cytokines to induce metaplasia of human airway epithelial goblet cells to goblet cells[30]. Carrer et al. Found that antisense oligonucleotide (ASO) can down-regulate Jag1 and Notch2 on goblet cell metaplasia of allergen-induced asthma and inhibit airway remodeling[31]. The results of this study found that Midkine expression was down-regulated, and Notch2 expression was down-regulated, which was consistent with previous results. Tian et al. found that Midkine can promote the proliferation of gastric cancer tissues and gastric cancer cells through Notch signaling pathway, while recombinant Midkine can weaken cisplatin-induced apoptosis of gastric cancer cells, and application of MKsiRNA can enhance cisplatin-induced apoptosis of gastric cancer cells. The apoptosis of MKsiRNA transfected cells may be through mitochondrial-apoptosis body pathway, with down-regulation of Bcl-2 expression, up-regulation of Bax expression, reduction of mitochondrial membrane potential, release of cytochrome c, activation of caspase-3, caspase-8 and caspase-9, especially caspase-3 and caspase-9, inducing apoptosis[32,33]. This study found similar results on airway smooth muscle cells. Inhibition of Midkine expression or inhibition of Notch2 expression can inhibit the proliferation of airway smooth muscle cells and promote their apoptosis, which further indicates that Midkine and Notch2 participate in airway remodeling of lung tissue at the same time.

5. Conclusion

Taken together,the results of this study demonstrated that Midkine has a role in acute lung injury models.In addition, Midkine could promote the proliferation of airway smooth muscle cells and reduce cell apoptosis through Notch2 pathway. This discovery may provide a novel insight into the mechanism of airway smooth muscle in airway remodeling.However, There are still unsolved puzzles.This study confirmed that Midkine plays a proliferative role through Notch2 pathway in LPS-induced acute lung injury model of airway smooth muscle cells in vitro, but the downstream proteins of Midkine/Notch2 signaling pathway have not been fully elucidated, and further research is still needed. Therefore, the author hopes that lentivirus and other technologies can be used instead of siRNA technology in future experiments to further verify the effect of Midkine.

Abbreviations

COPD:Chronic Obstructive Pulmonary Disease; LPS:Lipopolysaccharide; MK:Midkine; rMK:recombinant Midkine;IC50:The Half Maximal Inhibitory Concentration;PTPζ:Protein Tyrosine Phosphatase-ζ; ALK: Anaplastic lymphona kinase; ALI: Acute lung injury; WB: Western Blot; RAS: Renin-angiotensin system; MAPK: Mitogen-activated protein kinase;Bcl-2: B-cell lymphoma 2; NADPH:Nicotinamide Adenine Dinucleotide Phosphate; EMT: Epithelial-mesenchymal transition ; TNF-α: Tumor necrosis factor alpha;

TGF-β: Transforming growth factor beta; ASO : Antisense oligonucleotide; ASMC: Airway smooth muscle cells. EMT: Epithelial-mesenchymal transition.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Competing interests

The authors state that there are no conflicts of interest to disclose

Authors' contributions

XL and QH. conceived and designed the experiments;QH and TD performed the experiments and collected the date;LL,JQ,QL,KL analyzed the data and prepared figures and tables; DS,SX,HW,XW performed part of experiments and contributed reagents/materials/analysis tools;XL supervised the work.All authors reviewed and approved the manuscript.

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Figures

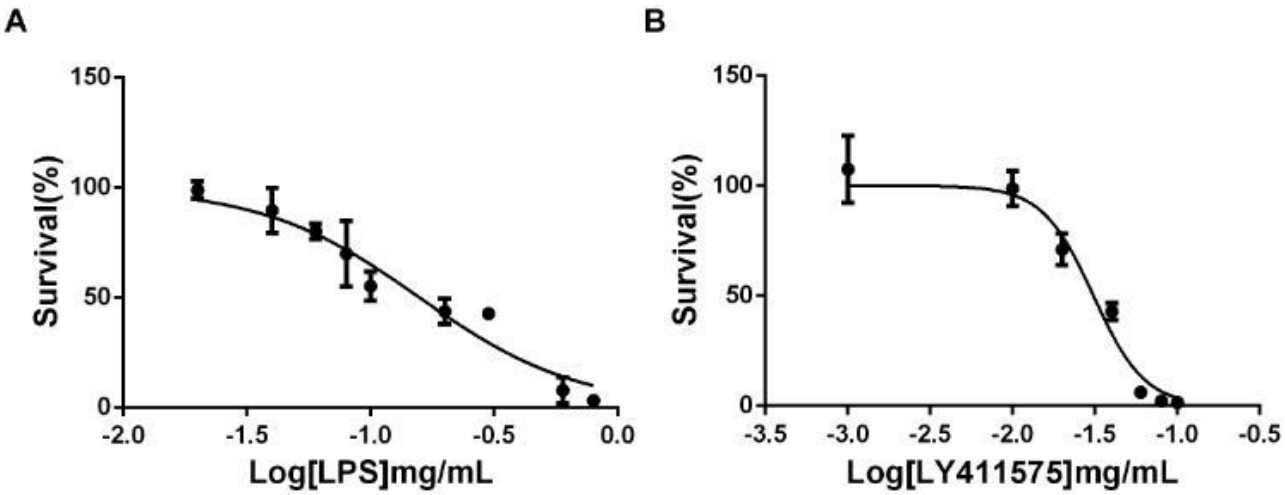

Figure 1

Half-inhibitory concentration (IC50) of LPS and Notch inhibitor on airway smooth muscle cell cells. N=6, $\bar{x}$ ±s. Fig.1 A shows the cell survival rate of LPS to ASMC. Fig. 1B shows the cell survival rate of Notch inhibitor (LY411575) against ASMC.

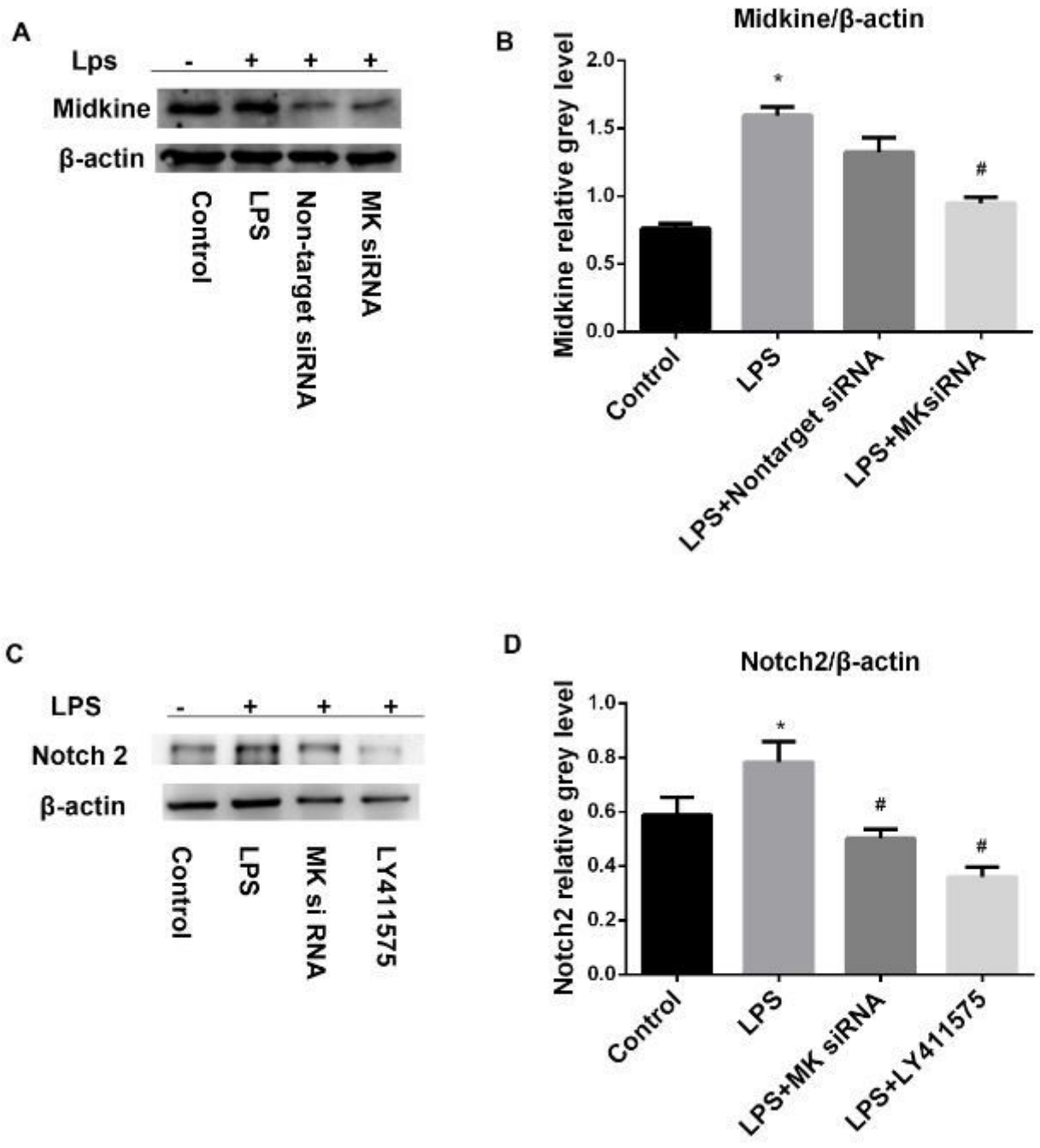

Figure 2

Expression of Midkine and Notch2 in airway smooth muscle cells of acute lung injury model rats. (A) Western blot analysis of Midkine protein bands in control group, LPS group (0.2 mg/mL), Non-targeted siRNA group and MK siRNA group. (B) The relative expression of Midkine protein in control group, LPS group (0.2 mg/mL), Non-targeted siRNA group and MK siRNA group. (C) Western blot analysis of Notch2 protein bands in control group, LPS group (0.2 mg/mL), MK siRNA group and LY411575 group. (D) The relative expression of Notch2 protein in control group, LPS group (0.2 mg/mL), MK siRNA group and LY411575 group. N=3, $\bar{x}\pm s$. * indicates that compared with the control group, * $P < 0.05$. # indicates that compared with LPS group, # $P < 0.05$.

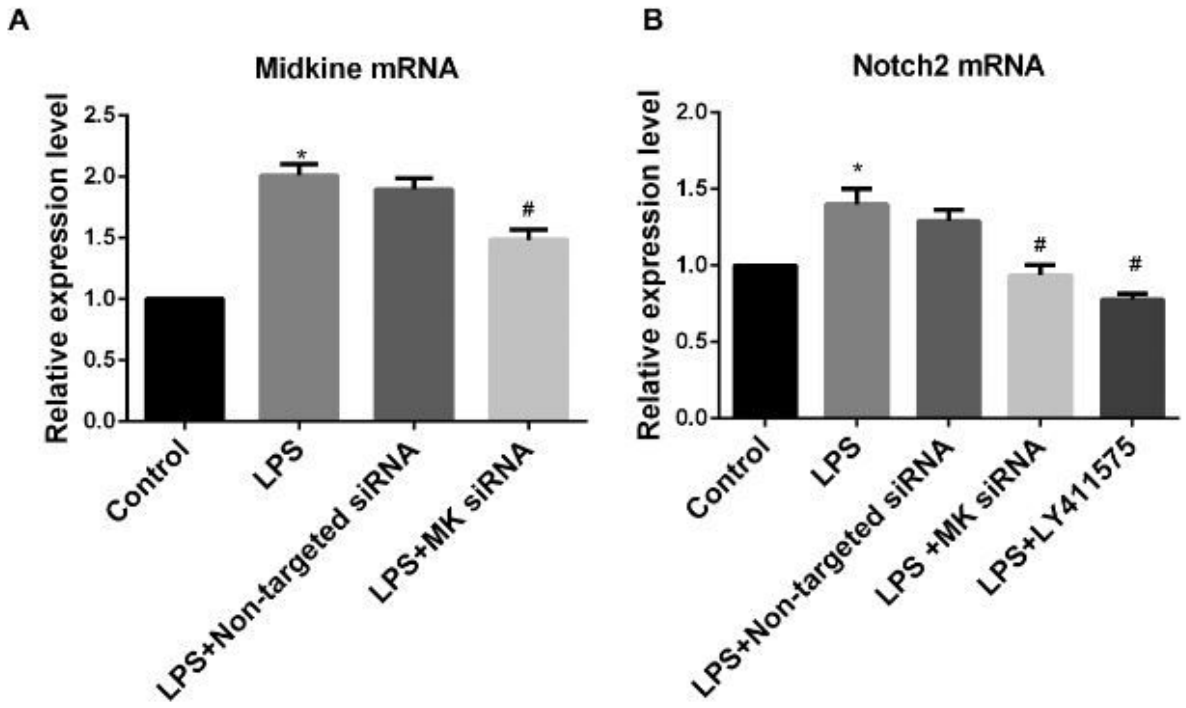

Figure 3

Expression of Midkine and Notch2 mRNA in airway smooth muscle cells of acute lung injury model rats. (A) The relative expression of Midkine mRNA in ASMC of control group, LPS group (0.2 mg/mL), Non-targeted siRNA group and MK siRNA group. (B) The relative expression of Notch2 mRNA in control group, LPS group (0.2 mg/mL), Non-targeted siRNA group, MK siRNA group and LY411575 group. N=3, $\bar{x}\pm s$. * indicates that compared with the control group, * $P < 0.05$. # indicates that compared with LPS group, # $P < 0.05$.

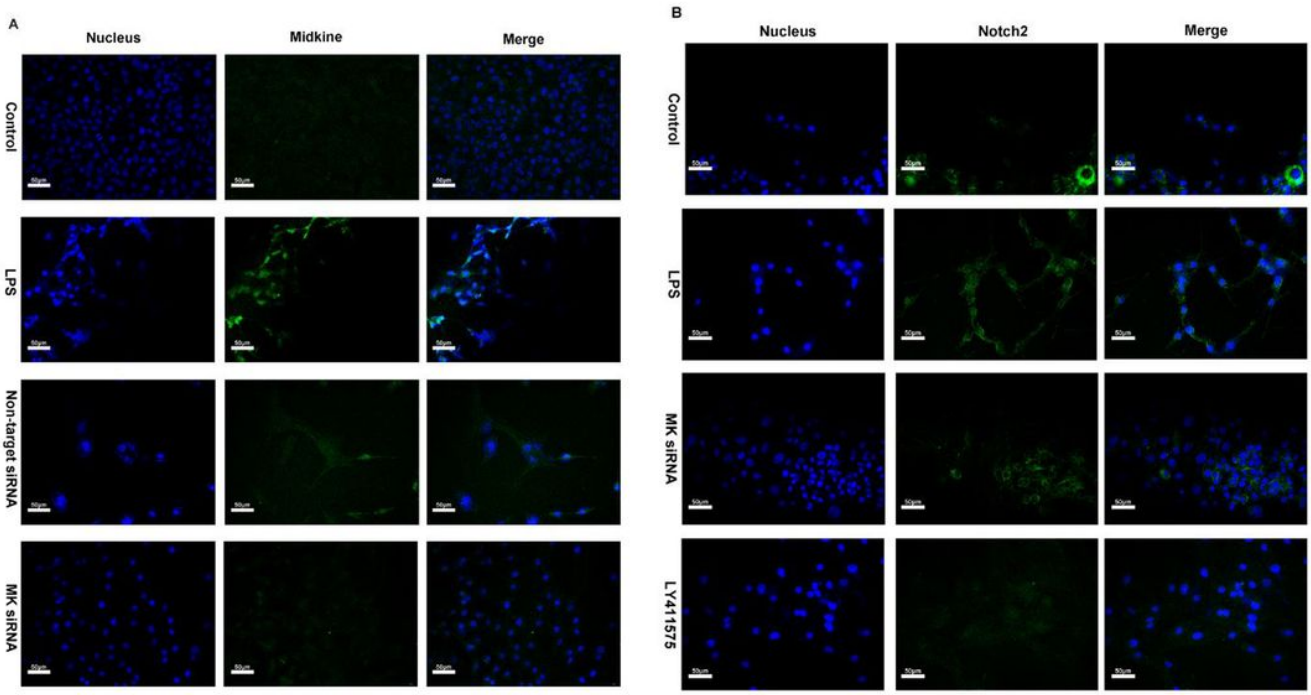

Figure 4

Cell immunofluorescence image. (A) Midkine expression in control group, LPS group, Non-target siRNA group and MKsiRNA group (× 400). (B) Notch2 expression in control group, LPS group, MKsiRNA group and LY411575 group (× 400). N=3, $\bar{x}$ ±s. The nucleus stained by DAPI and the fluorescence staining showed blue light. The expression of Midkine protein was mainly located in cytoplasm, showing green fluorescence. The expression of Notch2 protein is mainly located on the cell membrane, showing green fluorescence.

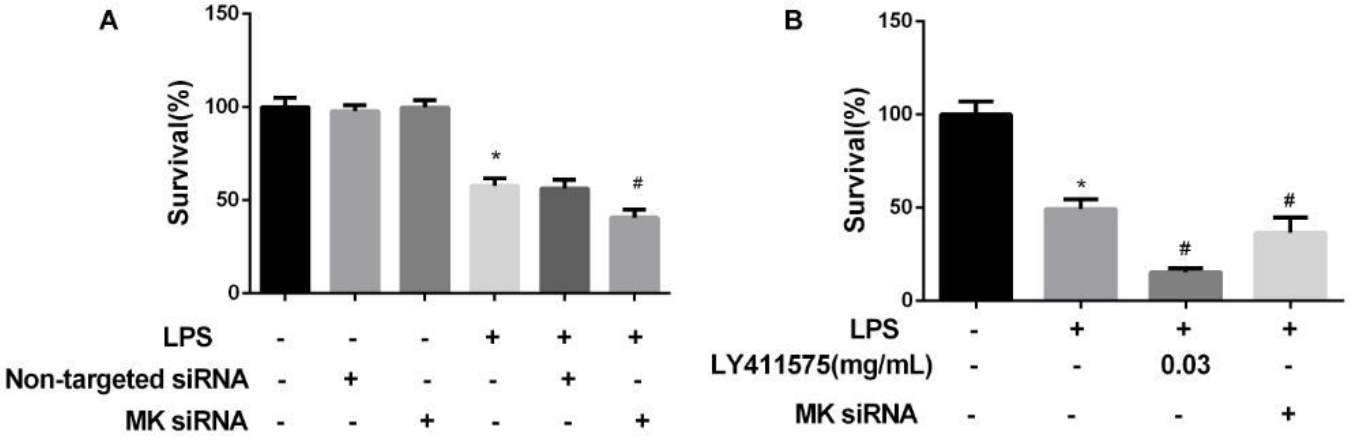

Figure 5

Cell survival rate. (A) Cell survival rate of control group, LPS group, Non-target siRNA group and MKsiRNA group. (B) The cell survival rate of control group, LPS group, MKsiRNA group and LY411575 group. N=6, $\bar{x}$±s. * indicates that compared with the control group, * P < 0.05. # indicates that compared with LPS group, # P < 0.05.

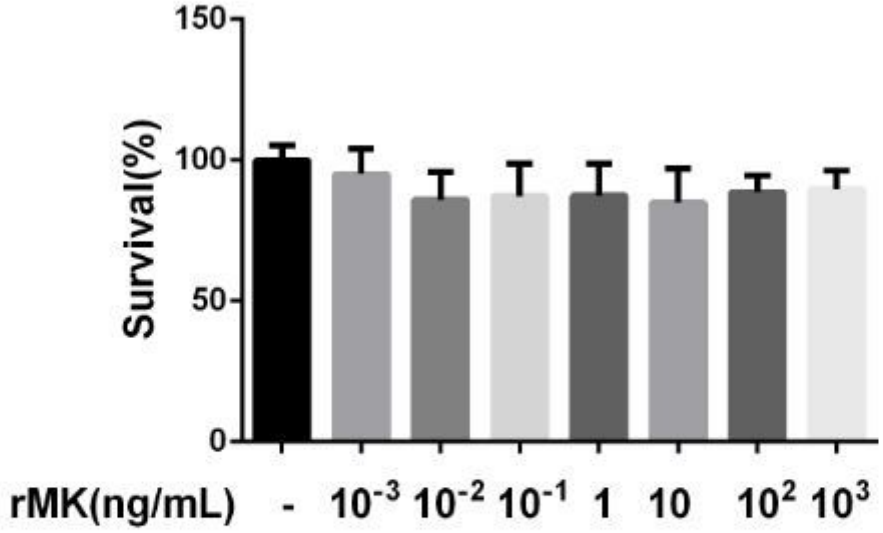

Figure 6

The survival rate of ASMC treated with rMK with different concentrations. N=6, $\bar{x}$ ±s.

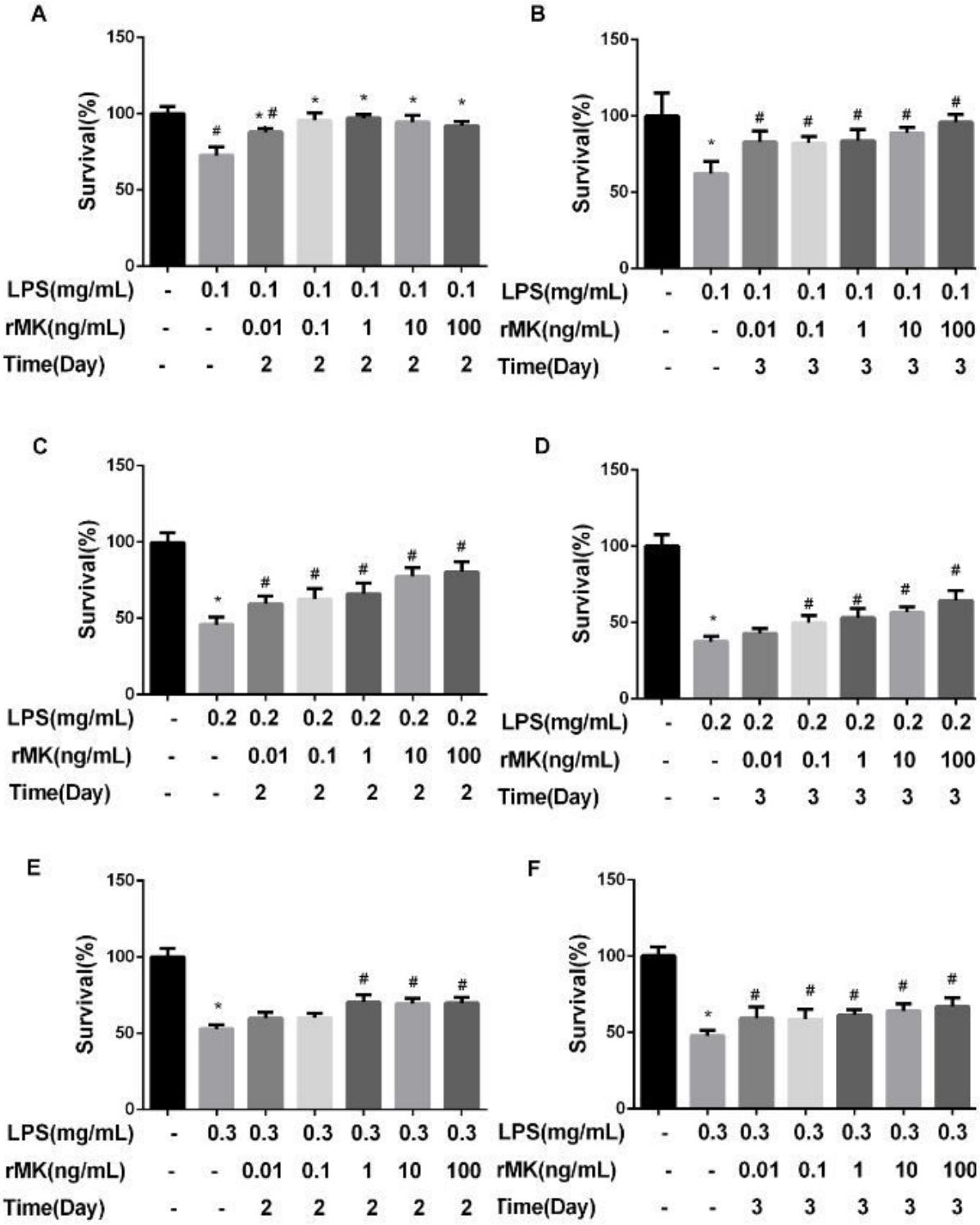

Figure 7

Proliferation experiments of LPS with different concentrations groups treated with rMK with different concentrations for different times. N=6, $\bar{x}$ ±s. A: the survival rate of ASMC treated with 0.1 mg/mL LPS and rMK with different concentrations for 2 days. B: the survival rate of ASMC treated with 0.1 mg/mL LPS and rMK with different concentrations for 3 days. C: the cell survival rate of ASMC treated with 0.2 mg/mL LPS and rMK with different concentrations for 2 days. D: the survival rate of ASMC treated with

0.2 mg/mL LPS and rMK with different concentrations for 3 days. E: the survival rate of ASMC treated with 0.3 mg/mL LPS and rMK with different concentrations for 3 days. F: the survival rate of ASMC treated with 0.3 mg/mL LPS and rMK with different concentrations for 3 days. * indicates that compared with the control group, * P < 0.05. # indicates that compared with LPS group, # P < 0.05.

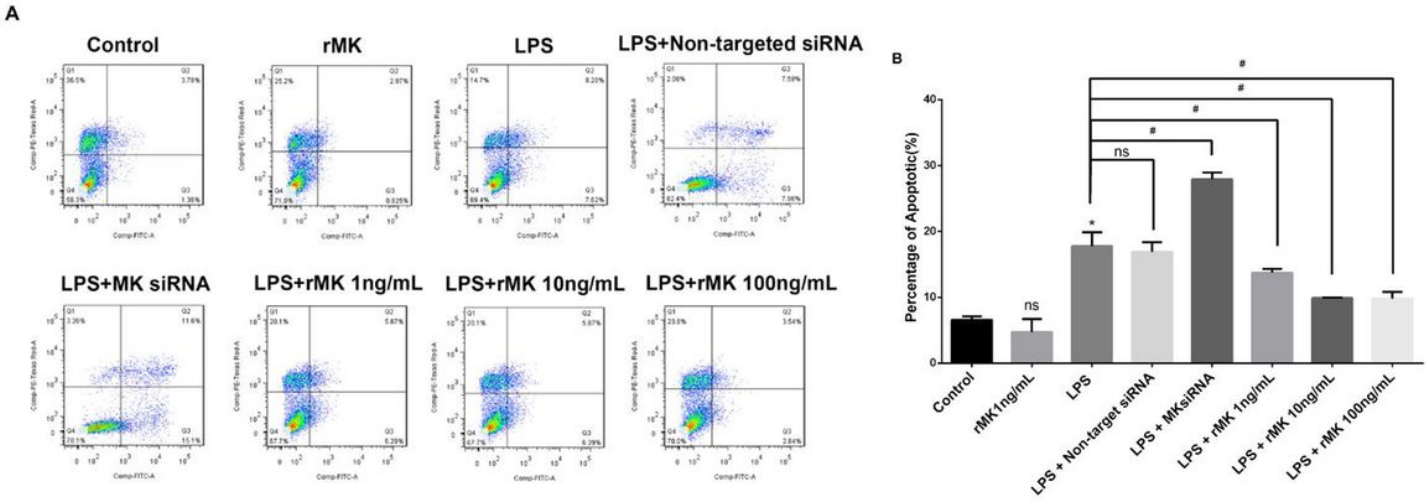

Figure 8

Effect of rMK with different concentrations on apoptosis in LPS group. A is flow cytometry and B is the apoptosis rate of control group, 1ng/mL rMK + ASMC group, LPS group, Nontargeted siRNA group, MKsiRNA group, LPS + 1ng/mL rMK, LPS + 10ng/mL rMK, LPS + 100ng/mL rMK. N=3, $\bar{x}\pm s$, * indicates that compared with the control group, * P < 0.05. # indicates that compared with LPS group, # P < 0.05.

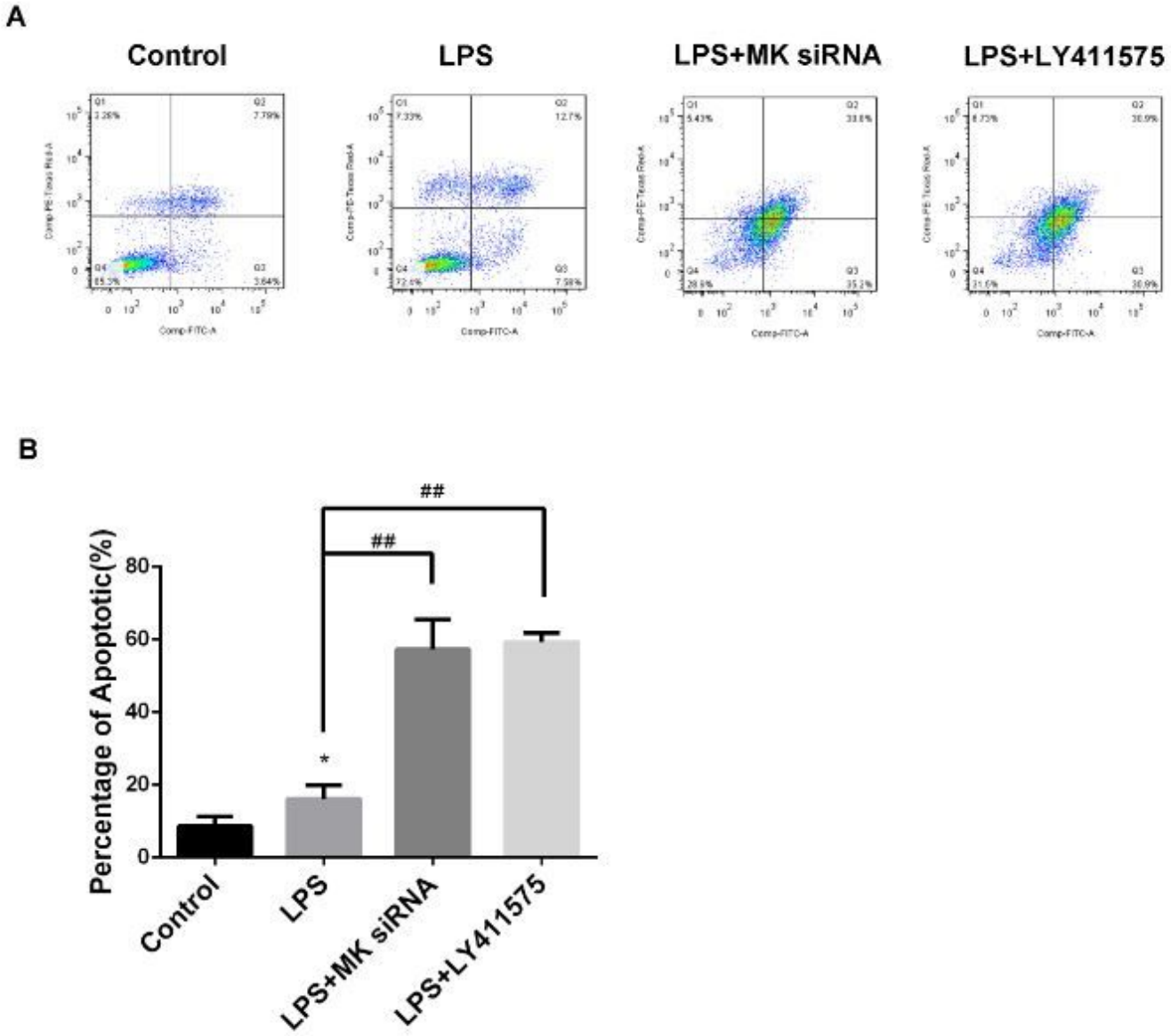

Figure 9

Effects of LY411575 on apoptosis in LPS group. A is flow cytometry and B is the apoptosis rate of control group, LPS group, MKsiRNA group and LY411575 group. N=3, $\bar{x}$ ±s, * indicates that compared with the control group, * P < 0.05. # indicates that compared with LPS group, # # P < 0.01.