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MAST3 modulates the inflammatory response and proliferation of fibroblast-like synoviocytes in rheumatoid arthritis

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ABSTRACT

Via promoting synovitis, pannus growth and cartilage/bone destruction, fibroblast-like synovial cells (FLSs) play a significant role in the pathogenesis of rheumatoid arthritis (RA). In our study, rats were induced with complete freund's adjuvant (CFA) to be animal models for studying the RA pathogenesis. Microtubule-associated Serine/Threonine-protein kinase 3 (MAST3) has been documented to play a critical role in regulating the immune response of IBD (Inflammatory bowel disease) and involved in the process of cytoskeleton organization, intracellular signal transduction and peptidyl-serine phosphorylation, but its role in the progression of RA remains unknown and is warranted for investigation. So, we tried our best to investigate the mechanism and signaling pathway of MAST3 in RA progression. In the synovial tissue and FLSs of AA rats, we have found that MAST3 was significantly up-regulated than normal. Furthermore, MAST3 overexpression could promote proliferation and inflammatory response of FLSs. In the aspect of mechanism, we discovered that the expression of MAST3 might involve in NF-κB signaling pathway in RA. On the whole, our results suggested that MAST3 might promote the proliferation and inflammation of FLSs by regulating NF-κB signaling pathway.

1. Introduction

Rheumatoid arthritis (RA) is recognized as chronic synovitis, synovial membrane formation, synovial tissue proliferation and progressive destruction of articular cartilage and bone [1]. As a common chronic systemic autoimmune joint disease, RA eventually leads to joint deformity and disability [1–3]. Simultaneously, angiogenesis is essential for maintaining a chronic inflammatory state by delivering inflammatory cells to the synovitis site and providing nutrients to the synovial membrane [4]. Nevertheless, some biological agents for treatment of RA still remains generally indistinct [5]. Developing new treatments for specific pathophysiological factors will generate crucial understanding of the complex mechanisms of RA and produce better outcomes. Therefore, to explore the pathogenesis and treatment of rheumatoid arthritis is the key to the treatment of RA.

Fibroblasts-like synocytes (FLSs), one of the most significant RA effector cells, located in the juncture place of knee-joint, exhibit some characteristics of malignant cells [6–8]. As the major cell population in invasive pannus of RA synovium, migration and invasion of activated FLSs actively participate in the inflammatory processes of RA [9]. And some secretory factors produced in the pathogenesis of RA, such as pro-inflammatory cytokines TNF-α and IL-6 [10,11], matrix metalloproteinases and angiogenic factors [12]. FLSs could lead to vasospasm formation and cartilage and bone destruction by regulating the secretion of inflammatory mediators, such as TNF-α and IL-6 [13,14]. So, inhibition of inflammatory response and FLSs proliferation is an ideal target for treating RA. Activated FLSs show tumor-like behaviors, such as increased migration, decreased contact inhibition and reduced attachment- dependent growth [15]. Therefore, inhibiting inflammatory response and proliferation of the FLSs would be a potentially desired strategy for therapying RA.

As one of the Microtubule-Associated Serine/Threonine Kinase family (MAST1-4, and MAST-like), MAST3 is encoded by an open reading frame of 1309 amino acids [16]. Because MAST2 plays an important role in inflammation, MAST3 has the high sequence identity within functional domains of the MAST family members like MAST2, which could be involved in similar pathways. Consequently, it was found the highest expression of MAST3 in HEK293 cells and THP-1 monocytes in

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a panel of cell lines. MAST3 is also revealed that expressed in primary murine and human immune cells by further expression analysis (CD4 + and CD8 + T cells, CD19 + B cells). It is demonstrated that homology of the human MAST3 amino acid sequence to the mouse homologous sequence is 91% [17]. John Rioux and colleagues have demonstrated that MAST3 modulated a set of genes and had a role in the immune response via NF-κB pathway in IBD [18]. In addition, expression of the MAST3 gene set is abundant in both inflammatory and non-inflammatory tissues, which is showed by researchers [19]. MAST3 is overexpressed in inflamed tissues, but is also found ubiquitously at a much lower level [20]. Previous study has proved that MAST3 is involved in the regulation of NF-κB activation via TLR4, but does not participate in the TLR5 signaling pathway.

As it is known to us, many research studies [3,21] have been proved that cell proliferation is related with the expression of c-Myc and Cyclin D1. Although the exact cause of RA is unclear, some studies have shown that proliferation of FLSs and immune dysregulation of inflammatory cytokines are involved in inflammation and proliferation of synovial cell, which leading to joint destruction in patients with RA [22]. However, proliferation, migration and invasion of activated FLSs currently have been kept unknown on the molecular mechanisms. Up to date, there is no further research report on the function of MAST3 in RA. Elucidation of MAST3′s role in regulating protein interactions in inflammation of chronic synovial, which would help to identify the complex molecular events that lead to RA [23].

2. Materials and experimental procedures

2.1. Materials and reagents

High glucose Dulbecco's modified Eagle's medium (DMEM) was obtained from Hyclone (Logan, UT, United States). The Fetal bovine serum (FBS) was acquired from Gibco (United States). Complete Freund's Adjuvant (CFA) was purchased from Sigma Chemical (St. Louis, MO, United States). Rabbit anti- MAST3 (BS5790) was purchased from Bioworld (Shanghai, China). Rabbit anti-TNF-α (ab6671), anti-IL-6 (ab9324) were purchased from Abcam (Britain). Rabbit antip-P65 (YP0191, phospho Ser536), rabbit anti-IkB (YT2419), and rabbit anti-p-IkB (YP0151, phospho Ser32/S36) polyclonal antibody were obtained from ImmunoWay Biotechnology Company (Plano, TX, United States). anti-cMyc and rabbit anti-CyclinD1 monoclonal antibody were purchased from Cell Signaling (Danvers, MA, USA). Rabbit anti-P65 (EAP1020), anti-β-actin (EGM0345) and enzyme Linked Immunosorbent Assay (ELISA) kit were purchased from Elabscience Biotechnology Co, Ltd (Wuhan, China). Secondary antibodies were recruited from Beijing Zhong-shan Biotechnology Corporation (Beijing, China) including goat anti-rabbit and goat anti-mouse immunoglobulin (IgG) horse radish peroxidase (HRP). Cell Cycle Studies Using kits were buyed by BestBio (Shanghai, China).

2.2. Experimental animals

The experiments were approved by the Ethic Committee of Experimental Animals of the Anhui Medical University, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For the purpose of inducing RA rat models, adult female Sprague-Dawley (SD) rats (140–160 g) were administered Complete Freund's Adjuvant (CFA) 0.2 mL by paw injection at the left paw. Meanwhile, normal control rats were treatment with normal saline at the same time. All efforts were made to minimize the use of animals used and their suffering. After a single injection, the rats were sacrificed at day 25 after injection for histopathological examinations and FLSs preparation.

2.3. Histopathology

The knee joint tissues of SD rats were fixed with 4% paraformaldehyde for 24 h, and 10% EDTA (ethylenediaminetetraacetic acid) was decalcified for 30 days. After decalcification and Paraffin embedding, the knee tissue sections (5 μm) were stained with hematoxylin and eosin (HE). According to a standard procedure, HE and immunohistochemistry were performed. By an Olympus BX-51 microscope, the stained sections of HE was observed and photographed accompanied by changes, which is including vasospasm, inflammatory cell infiltration, and synovial hyperplasia. In addition, the individual score is supplemented by the total score, 0 is normal, and 3 is divided into the maximum degree and degree of participation [24].

2.4. Cell culture

We obtained FLSs from the synovial tissues of AA and control rats by using the method of tissue explant cultivation [25]. The fresh synovial tissue of the rat knee joint was taken, chopped, placed in a cell culture flask, and 20% (v/v) heat-inactivated fetal bovine serum (FBS, Millipore) was added to high glucose DMEM medium (Hyclone, USA), and penicillin-streptomycin solution (Beyotime, China). All cells were cultured at 37 °C at an atmosphere of 5% CO_2 for 7 days. The adherent cells were cultured in medium after removal of the synovial pieces. We observed cell morphology, growth status and purity via the inverted microscope. The cells were at 70–80% confluences then trypsinized and separated at a 1:2 ratio. Meanwhile, vimentin's expression was measured by using the immunofluorescence cytochemistry method. When the cultured synoviocytes grew to three passages, most of item maintained a uniform population of FLSs. We principally used the FLSs of passages 3 in the next experiments.

2.5. Rat arthritis scores and paw swelling score

Animals were raised individually in transparent Beaker for an extended period. Rat arthritis score was evaluated according to the following rules: severity of redness (0-normal, 1-slightly red/purple, 2-red/purple), degree of swelling (0-normal, 1-slight swelling at the injection site, 2-swelling at the injection site and toes or ankle, 3-swelling at the injection site, toes and ankle) and variation of claw (0-normal, 1-slightly curved, 2-curved, 3-almost closed). Individual scores are additional parts of calculating the total score [26]. Then we measured secondary paw swelling by the paw volume meter. The time was respectively at the 12th, 14th, 16th, 18th, 20th, 22th and 24th days. Paw swelling score is the difference between the paw volume of the 12th, 14th, 16th, 18th, 20th, 22th and 24th days and paw volume without injecting CFA at the first day. Each group contains 6 rats.

2.6. Quantitative real-time PCR (Q-PCR)

According to the manufacture's protocol, total RNA was extracted from synovial tissues and cultured FLSs by using TRIZol (Invitrogen, USA). We quantified expressions of RNA by the Thermo Scientific NanodROP 2000 Spectrophotometer (Thermo Scientific, USA). The primers which we used were as following Table 1.

Firstly, we synthesized cDNA by using a PrimeScript RT Reagent kit (Takara, JAP). Secondly, we set the reverse transcription condition: 37 °C for 15 min, 85 °C for 5 s, and 37 °C for 10 min. Lastly, Real-time q-PCR was performed as 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C about 1 min by using Thermo Step One. The specificity of the products was estimated by melting curve analysis. Threshold cycle values were normalization of β-actin gene expression and were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primers used for real time-PCR.

Gene (Rat)	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)
MAST3	CTACACCAGCTCCCTTTCCA	CCATCCTCATCCACCACACT
TNF-α	ACTCCCAGAIAIAIAGCAIAGCAIA	CAGTTCCACATCTCGGATCA
IL-6	GAGCCCACCAGGAACGAAAGTC	TGTTGTGGGTGGTATCCTCTGTGAA
GAPDH	GGACCTCATGGCCTACATGG	TAGGGCCTCTCTGCTCAGT

2.7. Western blot

Synovial tissues and Cultured FLSs were lysed by using Radio-Immunoprecipitation Assay (RIPA) reagent containing 1% phenylmethanesulfonyl fluoride (PMSF) (Beyotime, China). We quantified the extract protein concentration by the Thermo Scientific NanodROP 2000 Spectrophotometer (Thermo Scientific, USA). Through a 10% sodium dodecyl sulfate polyacryl-amide gel electrophoresis (SDS-PAGE), equal amounts of protein were electrophoresed, and then blotted protein onto PVDF membranes (Millipore Corp, Billerica, MA, USA). After blockade with 5% milk, incubation of nitrocellulose blots with primary antibodies diluted in primary antibody dilution buffer (Beyotime, China). Primary antibodies recognition were MAST3 (1:500), TNF-a (1:500), IL-6 (1:500), P65 (1:500), p-P65 (1:500), IκB (1:1000) and p-IκB (1:500), c-Myc (1:500), CyclinD1 (1:500) and β-actin (1:1000) were used respectively.

After 12 h or 24 h, we washed the membranes for 3 times with TBS/Tween 20 (0.075%), then incubated blots with secondary antibodies (1:5000) for 1 h, such as goat anti-mouse or goat anti-rabbit horseradish peroxidase (HRP, Zhongshan Biotechnology Corporation, Beijing). Next, after the membranes were tautologically washing for 3 times with TBST (Boster, China), we analyzed expressions of protein blots by ECL-chemiluminescence kit (ECL-plus, Thermo SCIENTIFIC, USA). Compared with the control in the blot after normalized to β-actin, change in band density appears as a change which was presented as fold changes.

2.8. Cell cycle assay

Firstly, FLSs were seeded into 6-well plate for 12 h. Then the FLSs were transfected with the MAST3-RNAi or pex-MAST3. After cultured for 48 h, we trypsinized the FLSs by trypsin digestion and fixed item in 75% cold ethanol at 4 °C overnight. Then we washed FLSs by using cold PBS, incubated item with 20 μl RNase A at 37 °C for 30 min. With that, we added 400 μl propidium iodide (PI) staining buffer to each tube under certain conditions, which was at 4 °C for 30 min–1 h in the dark. Cell cycle was detected by flow cytometry (BD FACS Verse, USA), and multi-cycle data analysis software package was used to calculate the number of cells in G0/G1, S, G2/M phase.

2.9. Immunohistochemistry staining and detection of MAST3 expression

On the one hand, we fixed fresh synovial tissues with 4% paraformaldehyde for 24 h and then embedded in paraffin. Paraffin-embedded tissue joints were sliced to a thickness of 4 μm. Then partially dewaxed, dehydrated, and antigen searched in a microwave oven at 0.01 M citrate (pH = 6.0). Follow on, for the purpose of blocking endogenous peroxidase activity, each section was treated with 3% hydrogen peroxide (H_2O_2) for 5–10 min. The sections washed for three times with cold PBS, blocked by 5% bovine serum albumin for 30 min, rabbit polyclonal antibody against MAST3 (1:50, Bioworld, China) at 4 °C overnight. At day 2, the sections were incubated with horseradish peroxidase anti-mouse IgG antibody (Zhong-shan Biotechnology Corporation, Beijing) for 30 min at room temperature. Detection of MAST3 expression was used by diaminobenzidine tetrahydrochloride solution (DAB, Cowin Bioscience, China) staining for development of brown color. All sections were analysied and photographed with a fluorescence microscope.

On the other hand, the FLSs were cultured for 24 h on the cover slips of the 6 well plates and were fixed with ice-cold methyl alcohol for 15 min. And then blocked with 5% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) for 1 h. Immunofluorescence staining was performed with Rabbit anti-MAST3 (1:50) and anti-Vimentin (1:100) (Alexa Fluor 594 Conjugated) (both from Cell Signaling, USA) overnight at 4 °C. Alexa Fluor 488-Conjugated Affini Pure Goat anti-rabbit IgG (H + L) (Beijing Zhongshan Biotechnology Corporation, China) was used in the dark for 1 h at 37 °C as secondary antibody. 20 μl DAPI (Invitrogen, Carlsbad, CA, USA) were added to every hole for Nuclear staining.

2.10. Small RNAi transfection

According to the manufacturer's instructions, the FLSs were transfected with 100 nM of small interfering RNA (siRNA) by using Lipofectamine™ 2000 (Invitrogen, CA, United States). The siRNA-MAST3 (MAST3-RNAi) sens strand is 5′- GCAAGUCCACAAGCAGUAUTT-3′ and antisense strand is 5′- AUACUGCUUGUGGACUUGCTT-3′. A negative scrambled siRNA (GenePharma, Shanghai, China) was used in parallel, the forward strand is 5′-UUCUCCGAACGUGUCACGUTT-3′ and antisense strand is 5′-ACGUGACACGUUCGGAGAATT-3′. The cells were transfected with Opti-MEM at 37 °C for 6 h, then we changed to DMEM with 20% FBS. After cells were cultured for another 48 h, related indicators were detected by Q-PCR, Western blot, and flow cytometer (FCM).

2.11. Plasmid construction and transfection

Overexpression plasmid for MAST3 was generated by GenePharma (Shanghai, China). The primers which we used were as following as Table 2. The FLSs (1×10^4 cells) were cultured in 6-well plates with Opti-MEM for 6 h, then transfected with pex-MAST3 by using Lipofectamine™ 2000 (Invitrogen, United States) following as the manufacturer's manuals. The cells were cultured at 37° C for 6 h, then the Opti-MEM was changed with DMEM with 20% fetal bovine serum, and cultured for another 48 h. Further experiments are the same as Small RNAi Transfection, such as qRT-PCR, Western blot, and flow cytometer.

2.12. Statistical analysis

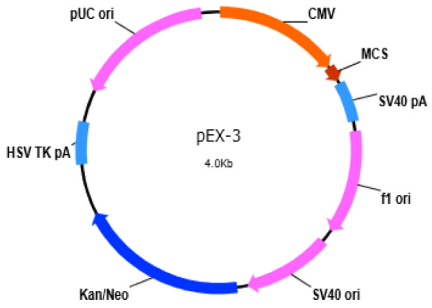
Data are expressed as mean ± standard deviation (S.D.) and analyzed by SPSS 17.0 software. Statistical significance was determined by the student's *t*-test to compare mean or one-way ANOVA with post hoc Dunnett's test. In all cases, the results were from at least three experiments, meanwhile, when $*p < 0.05$ and $**p < 0.01$, $\#p < 0.05$ and $\#\#p < 0.01$. data were considered to be statistically significant.

3. Results

3.1. The expression of MAST3 was up-regulated in AA FLSs.

We established models of AA by injection with the CFA and conducted Morphological observation on day 24. To confirm the model of

Table 2
Plasmid construction of MAST3.



GCT AGC GCT ACC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA GTC GAC GGT ACC
NheI XhoI BglII HindI EcoRI PstI SalI Kpn I

GCG GGC CCG GGA TCC ATC ACC GGT ATC GGC GCG CCA TTG ATA TCA ATG CGG CCG C
SacII SmaI BamH I AgeI AscI EcoRV NotI

AA was established successfully, we have surveyed typical photos of non-injected hind paws (Fig. 1A). We have found that MAST3 was positive for AA rats synovial tissues by hematoxylin and eosin (HE) staining and immunohistochemical analysis (Fig. 1B). To identify the variation of MAST3 expression between AA rats and normal rats, we conducted a series of experimental procedure, including western blot, qRT-PCR, immunohistochemical analysis and immunofluorescence cytochemistry assay. Compared with normal rats, the protein levels of MAST3 were significantly higher in AA rats synovial tissues and cells by western blot (Fig. 1C). As the same as western blot results of AA rats synovial tissues (Fig. 1D (a)) and cells (Fig. 1D (b)), QRT-PCR data suggested that the mRNA levels of MAST3 were higher. With a spindle shape according to FLS morphological feature, the expression of Vimentin studies (Fig. 1E) indicated that the cells were derived from synovial tissues was FLSs. And MAST3 was positive for AA rats synovial cells by immunofluorescence cytochemistry assay (Fig. 1F). The rat weight was weighed and rat arthritis score was evaluated (supplement Fig. 1G, H). AA model showed obvious swelling of the foot after non-injection, which was showed by foot swelling meter (supplement Fig. 1I). The result suggested that AA models had a variety of RA joint pathological features such as synovial hyperplasia, pannus formation and inflammatory cell infiltration, which suggested that AA rats have been successfully established.

3.2. MAST3-RNAi suppresses proliferation of FLSs.

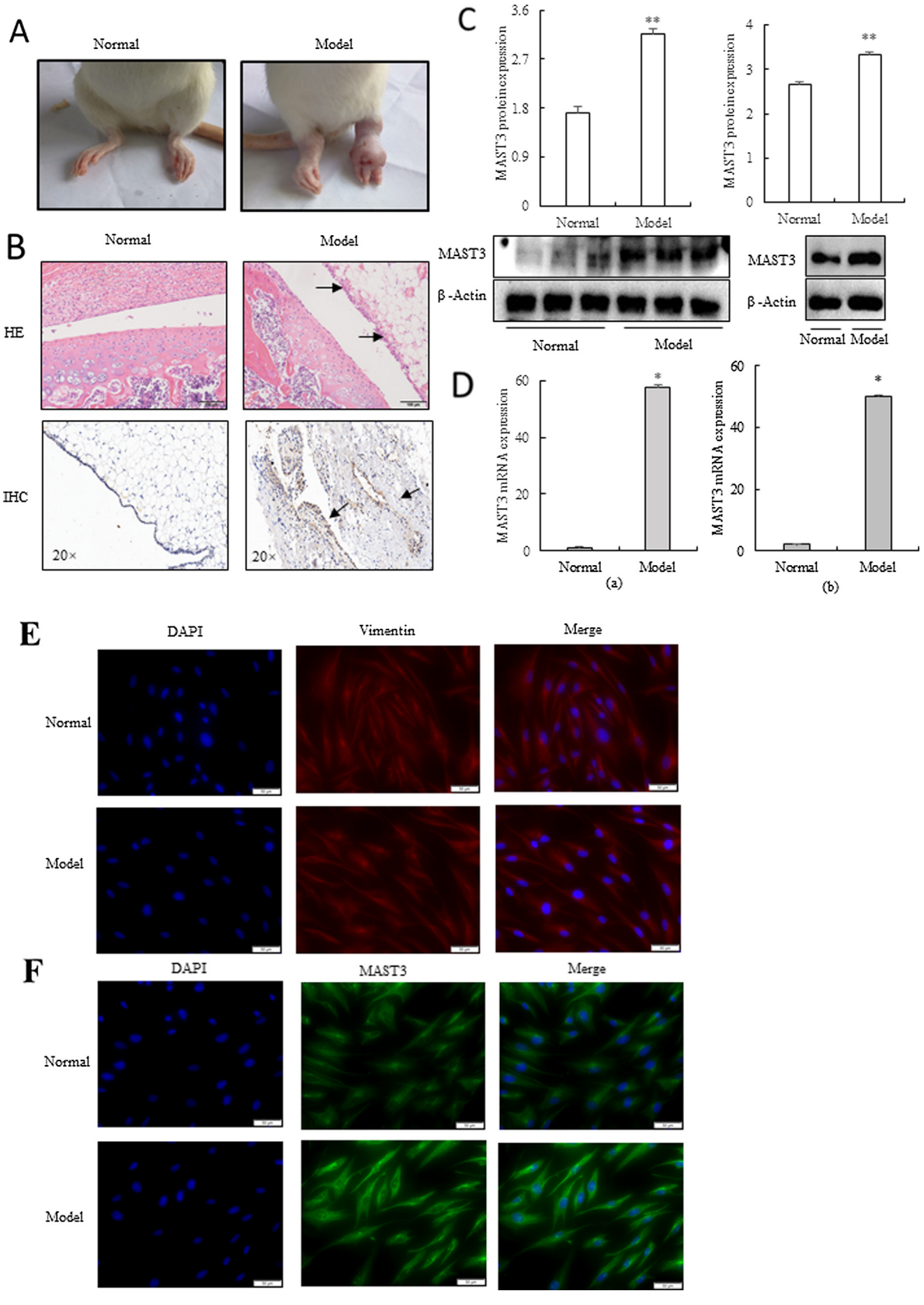
When transfected with MAST3-RNAi, western blot results revealed that the protein levels of MAST3 were significantly downregulated in cells compared to control cells (Fig. 2A). It indicated that it was successful for the transient transfection of MAST3-RNAi. In order to provide more evidence that MAST3 is involved in the proliferation of FLSs, siRNA specific for rat MAST3 was used to down-regulate gene expression in AA FLSs. 100 nM Control siRNA or MAST3-RNAi was exposed to normal and AA FLSs for 48 h, the expressions of c-Myc and Cyclin D1 protein were decreased by MAST3-RNAi in normal and AA FLSs (Fig. 2B). As same as expected, accordingly, cell cycle analysis also showed that FLSs transfected with MAST3-RNAi had different degrees of decline in S and G2/M phases of normal and AA rats (Fig. 2C).

3.3. MAST3-RNAi decreases inflammatory response of FLSs.

We transducted the MAST3-RNAi with lipidosome in AA FLSs in order to provide additional evidence to prove that MAST3 is involved in the inflammatory response of AA FLSs. Firstly, compared with normal rats, the protein levels of TNF-α and IL-6 were higher which displayed by western blot in AA rats synovial tissues (Fig. 3A). As the same as synovial tissues of AA rats, western blot results of FLSs showed the same results (Fig. 3A). Meanwhile, qRT-PCR data showed the same result as Fig. 3A. Likewise, compared with normal rats, the mRNA levels of TNF-α and IL-6 were higher in AA rats synovial tissues (Fig. 3B (a)) and cells (Fig. 3B (b)). In a forthcoming experiment, to analyze whether the effect of MAST3 knockdown is related to the inflammatory response of AA FLSs, we performed AA FLSs transfected with control siRNA or MAST3-RNAi. Serological test was performed to explore the levels of IL-6 and TNF-α in culture medium collected from the culture supernatant by ELISA kits. The results showed that the inflammatory cytokines secreted by the transfected cells were significantly lower than those in the control group (Fig. 3C). Interestingly, MAST3-RNAi induced an obvious decrease of levels of TNF-α and IL-6 protein in AA FLSs, which showed by western blot (Fig. 3D). After transient transfection of MAST3-RNAi, qRT-PCR results showed that MAST3-RNAi induced a significant decrease of levels of TNF-α and IL-6 mRNA in AA FLSs (Fig. 3E). The results were representative of at least three independent experiments. These results represent at least three independent experiments. Above results indicated that silencing of MAST3 inhibited the expression of inflammatory cytokine in FLSs.

3.4. Over expressing MAST3 increases proliferation of FLSs

Meanwhile, over expression vector with pex-MAST3 was used to upregulate MAST3 in AA FLSs. Compared with control cells, western blot results showed that MAST3 protein level was significantly upregulated in cells transfected with pex-MAST3 (Fig. 4A). It's the opposite of knocking down MAST3, the expressions of c-Myc and Cyclin D1 protein were remarkably upregulated in AA FLSs compared to normal FLSs (Fig. 4B). Correspondingly, flow cytometry analysis showed that in normal and AA rats, we used pex-MAST3 to increase S and G2/M phases significantly. Figs. 2 and 4 indicated that silencing/over-expressing MAST3 regulates proliferation of FLSs.



(caption on next page)

3.5. Over expressing MAST3 increases inflammatory response of FLSs.

Over expression vector with pex-MAST3 was used to regulate inflammatory response in AA FLSs. Serological test documented that the inflammatory cytokines secreted by the transfected cells were significantly higher than those in the control group (Fig. 5A).

Fig. 1. The expression of MAST3 was up-regulated in AA FLSs. Bioreplicon cell experiments were performed with 3 rats per group. (A) Representative paw images of normal rat and AA rat models. Morphological observations were taken on the 24th day after CFA induction, and photographs of typical non-injected hind paws were taken. (B) Hematoxylin andeosin (HE) stained sections suggested that AA models presented multiple RA joint pathological features. The results of immunohistochemistry showed that MAST3 was positive for AA rats synovial tissues compared with the normal group. (C) Western blot results showed that the protein levels of MAST3 was up-regulated in AA rats synovial tissues and FLSs compared with the normal group. (D) The results of qRT-PCR suggested that the level of MAST3 mRNA in synovial tissue (a) and FLS cells (b) of AA rats was higher than that of normal rats. β-actin served as the expression of an internal control. (E) Synovial cells of normal and model rats were identified by Vimentin in Immunofluorescence technique, which showed a spindle shape in accordance with FLSs morphological feature. Immunofluorescence cytochemistry assay also found MAST3 was positive for AA rats synovial cells. Representative views from each group are presented. The results are presented as mean ± S.D. and representative images are shown. **P < 0.01 versus normal group.

Undoubtedly, lipidosome-mediated transduction resulted in MAST3 over expression in AA FLSs. The protein levels of inflammatory cytokine (TNF-α and IL-6) of AA FLSs higher than normal by western blot (Fig. 5B). Meanwhile, after transient transfection of pex-MAST3, expressions of TNF-α and IL-6 mRNA were measured in normal and model FLSs. The qRT-PCR results showed that pex-MAST3 induced a distinct increase of TNF-α and IL-6 mRNA level in AA FLSs (Fig. 5C). Contrary to Fig. 3, over expressing MAST3 increased inflammatory cytokine expression of AA FLSs.

3.6. MAST3 might be involved in NF-κB signaling pathway in RA

On account of MAST3 is related to NF-κB signaling pathway, to investigate the mechanism of MAST3 involved in RA progression, the protein levels of p-p65 and p-IκB were detected by us in AA rats synovial tissues and cells. Compared with normal rats, western blot results showed that the protein levels of p-p65 and p-IκB were upregulated in AA rats synovial tissues and cells (supplement Fig. 6C). Similarly, the protein levels of p-IκB were also increased in AA rats synovial tissues and cells compared with normal rats (supplement Fig. 6D). We measured the protein levels of p-P65 and p-IκB in FLSs transfected with pex-MAST3 or MAST3-RNAi. Compared with control siRNA in AA FLSs, knocking down of MAST3 by siRNA significantly reduced the protein levels of p-p65 and p-IKB (Fig. 6A). On the contrary, MAST3 overexpression could improve the protein expression of p-P65 and p-IkB by western blot (Fig. 6B). These data suggested that MAST3 may be potentially involved in regulating RA progression through the course of NF-κB signaling pathway in AA FLSs.

As a classical signaling pathway, NF-κB is a key regulator of inflammatory processes and has regulatory functions for a variety of cytokine levels. NF-κB is normally stored in the cytoplasm in an inactive state that binds to inhibitor IκB (inhibitor of NF-κB). When the FLSs are transfected by MAST3, IκB kinase (IKK) activation phosphorylates IκB to form P-IκB. Then, it binds to P-P65 to promote the rapid translocation of free NF-κB to the nucleus, and induces transcription of related genes and participates in the regulation of cell proliferation. Absolutely, the Phosphorylation of IκB and P65 represents the degree of activation of the NF-κB pathway and also reflects the importance of MAST3-mediated proliferation of FLSs in our study. Critically, we showed that elevated expression of NF-κB signaling molecular proteins subunits of p65 and its inhibitor proteins of IκB-α in phosphorylation levels after transfecting pex-MAST3. However, there was no significant change in the expression of p-65 and IκB proteins. In our experiment, the results indicated that MAST3 might regulate the processes of inflammatory response and proliferation of FLSs in AA, which could be tightly related to NF-κB signaling pathway (Fig. 7).

4. Discussion

The one of topical model of experimental Rheumatoid arthritis (RA) is Adjuvant arthritis (AA), because it has analogical characteristics to RA in the matter of histology and immunology [27]. And AA is induced by injection complete Freund's adjuvant (CFA) [15,26]. AA animal models are similar to RA patient with inflammatory arthritis together with synovial inflammation and hyperplasia [28]. Fibroblast-like synoviocytes (FLSs) play a significant role in the pathogenesis of RA and have been substantiated in inflammatory arthritic diseases as key effector cells [11,29]. As is well- known to us, one of the critical features of RA is excessive proliferation of FLSs which leads to cartilage and bone destruction. In addition, lymphocyte and macrophage infiltration [30], recruitment and retention are promoted by the hyperplastic FLSs population with generating cytokines [31], chemokines [32], cell adhesion molecules [33] and extracellular matrix proteins [30]. However, up to now, there are no valid drugs to target the FLSs in RA. In fact, the treatment of RA includes only some common drugs, such as DMARDs, non-steroidal anti-inflammatory drugs (NSAIDs) and biological agents, which may cause serious side effects, including gastrointestinal lesions, cardiovascular complications and reproductive toxicity [34]. Hence, better treatments need be paid to more and more attentions and even to find an effective strategy for treating rheumatoid arthritis is a huge challenge. And it remains unresolved that the underlying mechanisms driving FLSs activation.

MAST3 belongs to the MAST kinase family, which has four other members including MAST1, MAST2, MAST4 and a mast-like protein, MASTL. The MAST3 protein is a four a-helix bundle composed of different domains, which have a protein kinase domain and a PDZ domain that mediates binding to PTEN [17]. Some researchers [20] have discovered that MAST3 play a critical role in inflammatory diseases in recent years. In particular, previous studies [19] showed that MAST3 modulated expressions of a set of genes by comparing whole-genome expression between cells with overexpressing the MAST3 gene and control cells. Most of these genes are regulated by MAST3 play a crucial part in immune functions, such as regulating NF-κB, cell adhesion and/or migration and production of proinflammatory cytokines. Significantly, it was found that expression of these genes correlated temporally with NF-κB activity. Under consideration, we first hammered at exploring the functional effects of MAST3 in FLSs of AA rats by western blot and qRT-PCR. Wonderfully, compared to normal group, the results indicated that MAST3 expression was significantly upregulated in AA synovial tissues and FLSs. In like manner, the expression of MAST3 was also respectively increased in AA synovial tissues and FLSs by immunohistochemical and immunofluorescence analysis.

In addition, TNF-α and IL-6 as proinflammatory cytokines attach importance to the progression of RA and play a significant role in the pathogenesis of RA inflammation. One of the major drivers is increased activation of NF-κB for dysregulated inflammatory response during the disease [35–37]. The transcription of various genes is controlled by NF-κB involved in apoptosis, cell-cycle progression and proliferation [38]. Aberrantly activation of NF-κB is a key player in pathogenesis of various diseases such as atherosclerosis, asthma and diabetes. Objective to better explore the functional mechanism of MAST3 in RA, we observed results of overexpression and knockdown of MAST3 in cell models. The expressions of TNF-α and IL-6 and the proliferation of FLSs by regulation of NF-κB activation is required to prevent inflammation and damage. Especially, transient transfection of MAST3-RNAi completely reduced the levels of TNF-α and IL-6 after culturing 48 h. Overexpression of MAST3 does the opposite. In our study, we showed that elevated expression of NF-κB signaling molecular proteins subunits of P65 and its inhibitor proteins of IKBα in phosphorylation levels after transfecting pex-MAST3. However, there was no significant change in the expression of P65 and IκB proteins. Meanwhile, the phosphorylation of IκB and P65 represents the degree of activation of the NF-κB pathway

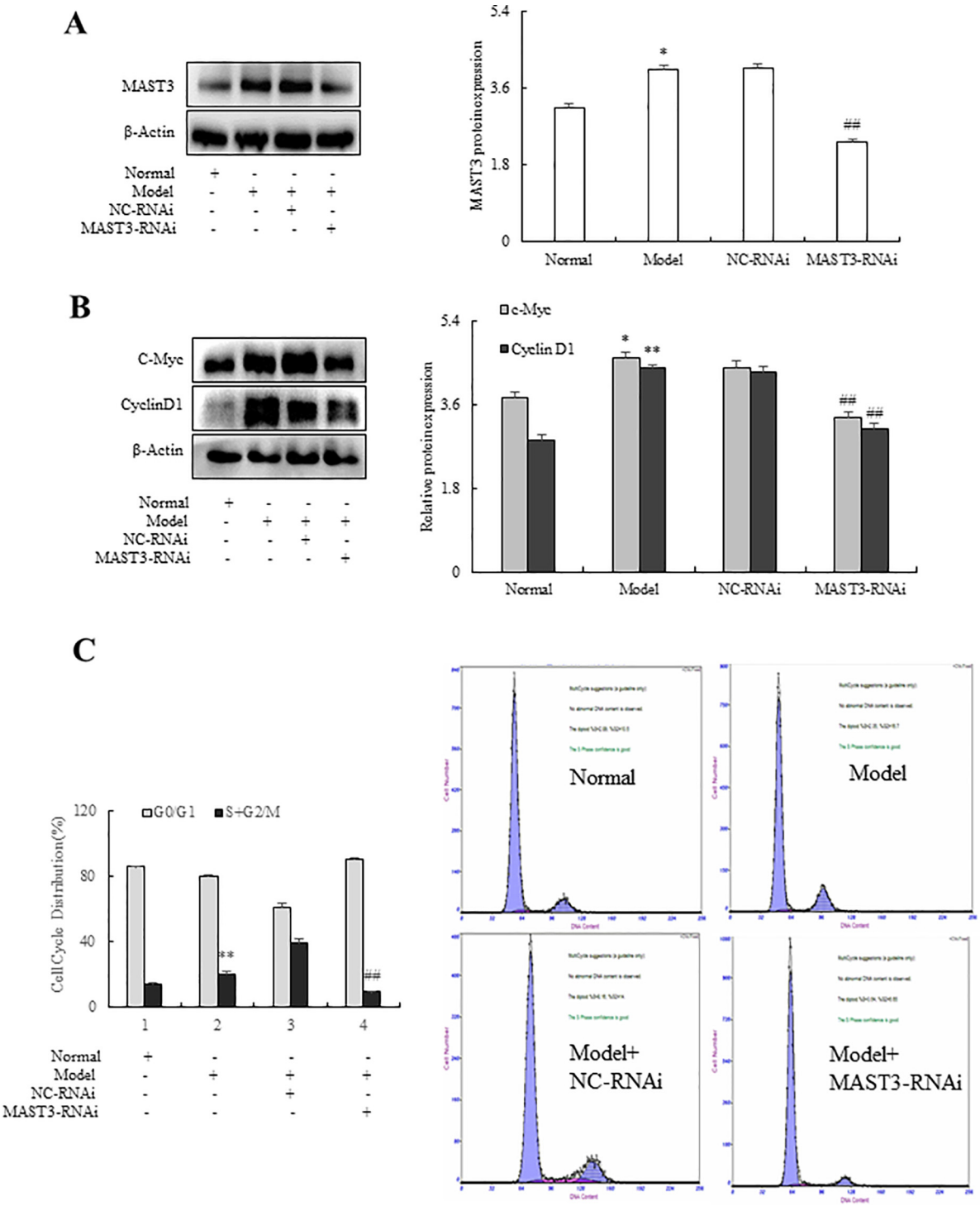


Fig. 2. MAST3-RNAi suppresses proliferation of FLSs. (A) Western blot results showed that the protein level of MAST3 was significantly decreased in cells transfected with MAST3-RNAi compared with control cells. (B) The protein levels of c-Myc and Cyclin D1 were analyzed by Western blot in FLSs with MAST3-RNAi. (C) Flow cytometry analysis showed that the G1 phase cell block was reduced in the MAST3-RNAi group compared with the model group. The results are presented as mean ± S.D. and representative images are shown. *$p < 0.05$, **$p < 0.01$ versus model group.

and also reflects the importance of MAST3-mediated proliferation of FLSs There is increasing evidence that the NF-κB signaling pathway involved in regulating local inflammation, prompting facilitating pannus formation and the arthritic joint destruction of RA [15].

Meanwhile, inhibition of MAST3 expression with MAST3-RNAi could down-regulate protooncogene c-Myc, cyclin protein Cyclin D1. It is known that Cyclin D1 and c-Myc are proved to be related with cell proliferation [39]. In our study, our results found that pex-MAST3 could upregulate expressions of c-Myc, Cyclin D1. What is more, the cell cycle was arrested in the G1 phase after silencing MAST3. Come to conclusion, overexpression MAST3 may promote RA progression potentially *via* enhancing cell proliferation regulation. In brief, pex-MAST3 significantly produced proliferation of FLSs in AA. Taken together, these data suggested that MAST3 mediated activation and proliferation of FLSs, and was closely associated with NF-κB signaling pathway in RA. To comprehensively explore the functional effects of

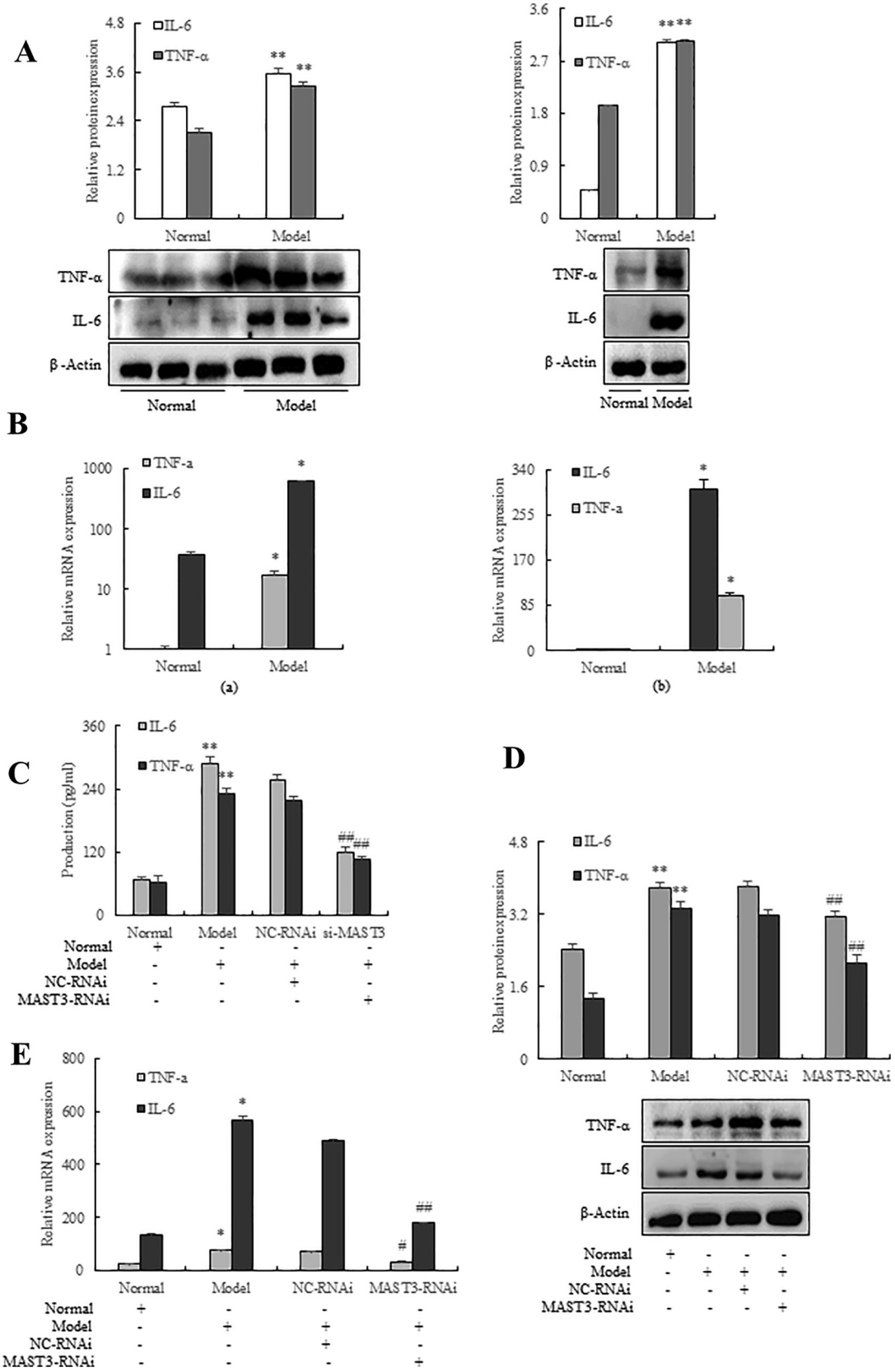


Fig. 3. MAST3-RNAi decreases inflammatory response of FLSs. (A) Compared with normal group, western blot results showed that the protein levels of TNF-α and IL-6 was higher in AA rats synovial tissues and cells. (B) Real-time quantitative PCR analysis suggested that the mRNA levels of TNF-α and IL-6 were higher in AA rats synovial tissues (a) and FLSs (b) compared with normal rats. (C) The levels of TNF-a and IL-6 in FLSs supernatants were detected by ELISA. All values were expressed as mean ± S.D. *$p < 0.05$, **$p < 0.01$ versus Normal. (D) Western blot showed that MAST3-RNAi induced a significant downregulation of the expressions of TNF-α and IL-6 protein in AA FLSs. (E) After transient transfection of MAST3-RNAi, qRT-PCR data showed that MAST3-RNAi induced a significant decrease of TNF-α and IL-6 mRNA level in AA FLSs. All values were expressed as mean ± S.D. *$p < 0.05$, **$p < 0.01$ versus Normal. #$p < 0.05$, ##$p < 0.01$ versus model group.

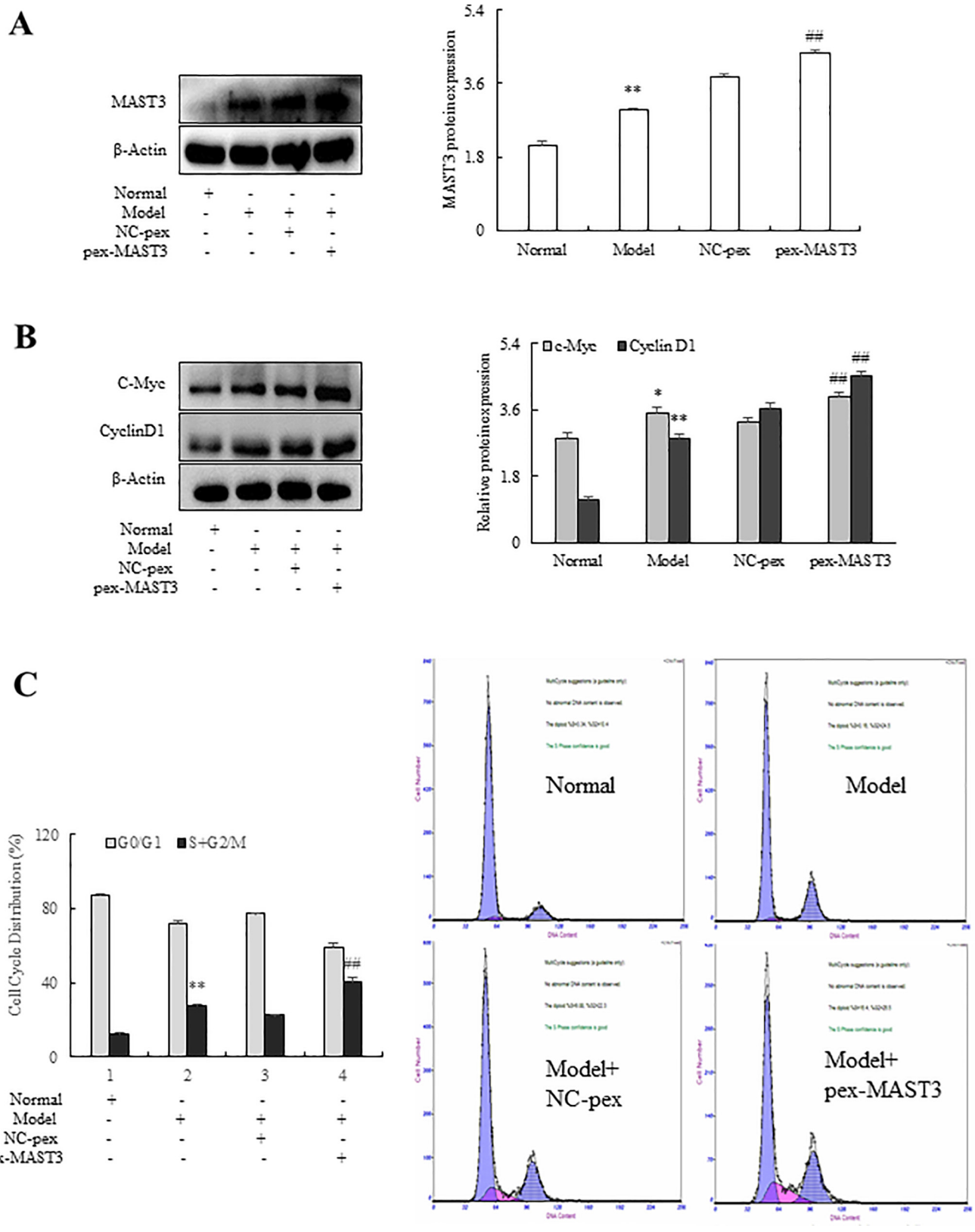


Fig. 4. Over expressing MAST3 increases proliferation of FLSs. (A) Western blot results showed that the protein level of MAST3 was significantly increased in cells transfected with pex-MAST3 compared with control cells. (B) The protein levels of c-Myc and Cyclin D1 were analyzed by Western blot in FLSs with pex-MAST3. Representative bands and images of three independent experiments. (C) Flow cytometry analysis showed that the G1 phase cell block was reduced in the pex-MAST3 group compared with the model group. The results are presented as mean ± S.D. and representative images are shown. *$p < 0.05$, **$p < 0.01$ versus model group. #$p < 0.05$, ##$p < 0.01$ versus model group.

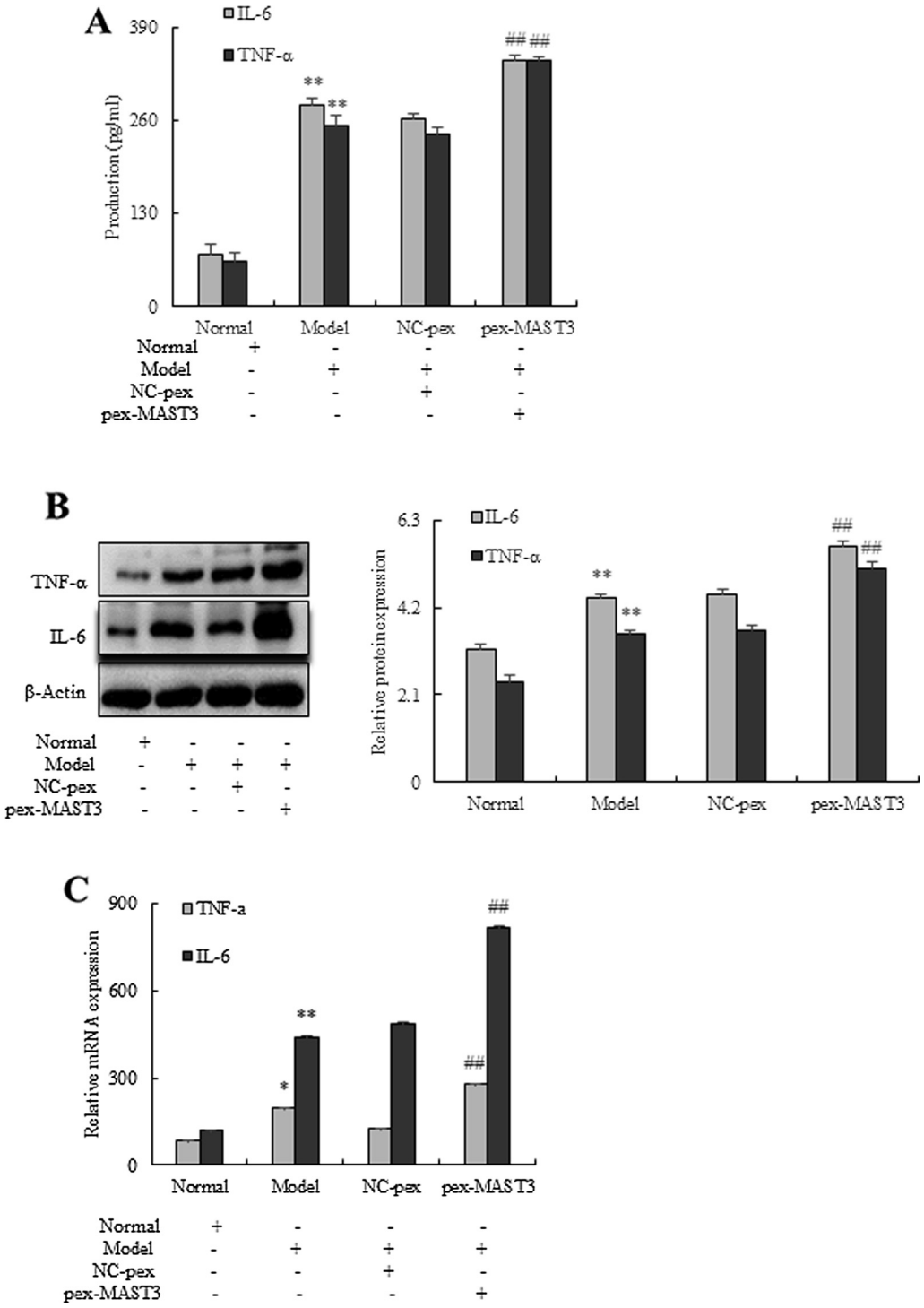


Fig. 5. Over expressing MAST3 increases inflammatory response of FLSs. (A) The levels of TNF-a and IL-6 in FLSs supernatants were detected by ELISA. (B) Western blot showed that pex-MAST3 induced a significant upregulation of TNF-α and IL-6 protein level in AA FLSs. (C) After transient transfection of pex-MAST3, the expressions of TNF-α and IL-6 mRNA were measured in synovial cells of normal and model rats. qRT-PCR data showed that pex-MAST3 induced an obvious increase of TNF-α and IL-6 mRNA level in AA FLSs. All values were expressed as mean ± S.D. *$p < 0.05$, **$p < 0.01$ versus Normal. #$p < 0.05$, ##$p < 0.01$ versus model group.

MAST3 in RA, we required to do further studies. As a result, our findings suggested that MAST3 be closely associated with activation of NF-κB signaling pathway, then could play a pivotal role during FLSs activation. This is one more capable and qualified opinion that pex-MAST3 promotes AA FLSs proliferation, indicating that the potential of MAST3 may be a potential therapeutic target for RA. Absolutely, we favourably discovered the effect of MAST3 in processes of FLSs including the inflammatory response and proliferation. It is obliged to admit the fact that our experiments still exist certain limitations. We principally executed some experiments to explore the effects of MAST3 on FLSs in vitro. We look forward to proceed further researches to study the inhibitory effect of MAST3 on inflammation both in animal and human.

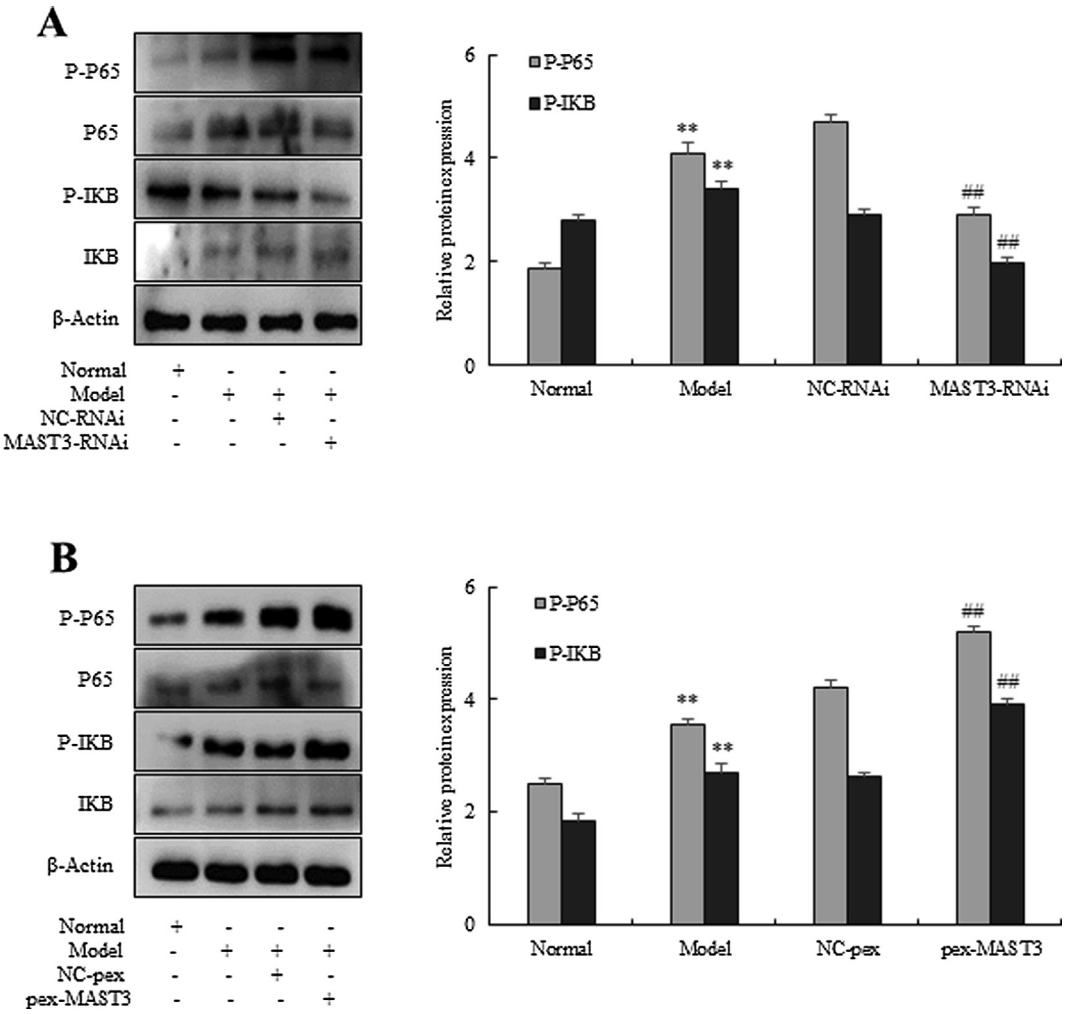


Fig. 6. Studying on NF-κB signaling pathway. (A) After transfected with MAST3-RNAi in FLSs, the protein levels of p-P65 and p-IkB were detected by Western blot. (B) The protein levels of p-P65 and p-IκB were detected by Western blot in FLSs with pex-MAST3. Each group at least contains 5 rats. All values were expressed as mean ± S.D. *p < 0.05, **p < 0.01 versus Normal. #p < 0.05, ##p < 0.01 versus model group.

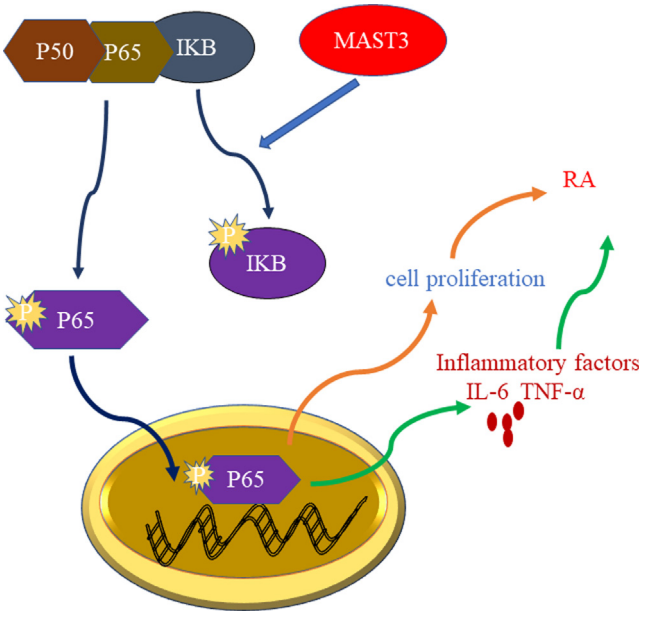


Fig. 7. Schematic model of MAST3-mediated NF-κB signaling in regulating proliferation and inflammation in FLSs.

5. Conclusion

In the experimental arthritis model, we successfully explored the effect of MAST3 on inflammatory response and proliferation of FLSs in vitro. In synovial tissues of inflamed joints as well as in FLSs, we explored that the expression of MAST3 was significantly increased. Silencing or overexpressing of MAST3 was involved in the process of proliferation and inflammatory response of FLSs. Moreover, the expressions of P-P65 and P-IκB were influenced by the expression of MAST3. These results indicated that MAST3 might regulate the processes of inflammatory response and proliferation of FLSs in AA, which could be tightly related to NF-κB signaling pathway. Come to conclusion, we believe that MAST3 potentially provide a new direction for the study of RA and even would be a new target for treating RA.

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Author contributions

All authors listed have made a direct and indirect contribution to the study, and approved it for publication full of expectation.

Declaration of Competing Interest

All authors have taken part in this study. They all declared that they have anything to disclose regarding funding from industries or conflict of interest as for the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105900.

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