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ORIGINAL ARTICLE

NLRP12 reduces proliferation and inflammation of rheumatoid arthritis fibroblast-like synoviocytes by regulating the NF-κB and MAPK pathways

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ABSTRACT. Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by abnormal synovial hyperplasia and the release of inflammatory cytokines. NLRP12 is a member of the family nod-like receptor (NLR) families that are activators of inflammation. However, the role of NLRP12 in fibroblast-like synoviocytes (FLSs) is still unclear. In the present study, we have investigated the role of NLRP12 in fibroblast-like synoviocytes (FLSs). The results demonstrated that NLRP12 overexpression inhibited proliferation and promoted cell apoptosis in RA-FLSs. Moreover, NLRP12 overexpression repressed inflammation by downregulation of IL-1β, TNF-α, IL-6, IFN-γ and MCP-1 production and upregulation of IL-10 levels with knockdown of NLRP12 expression showing opposite effects. In addition, NLRP12 overexpression suppressed phosphorylation of JNK, ERK, p38 and NF-κB in RA-FLSs, whereas NLRP12 knockdown promoted phosphorylation of these proteins. In conclusion, these findings demonstrate that NLRP12 inhibits proliferation and inflammation of RA-FLSs via the regulation of the NF-κB and MAPK signaling pathways, suggesting that NLRP12 might be a potential target for RA treatment.

Key words: rheumatoid arthritis, NLRP12, proliferation, inflammation

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease featured by abnormal synovial hyperplasia and increased release of inflammatory cytokines [1-3]. The disease exhibits swelling, tenderness and destruction of synovial joints [4, 5]. RA exerts effects on people of all ages. The global incidence of RA is approximately 0.5% to 1% [6]. It is reported that genetic and environmental risk factors together contribute to the development of RA [7-10]. Currently, some drugs have been applied for RA treatment, such as antitumor necrosis factor drugs, nonsteroidal anti-inflammatory drugs, glucocorticoids and immunosuppressive agents [11, 12]. Although patients with RA have improved to some extent upon treatment, drugs have their own limitations. Therefore, it is necessary to develop effective RA therapies to meet public health need.

Fibroblast-like synoviocytes (FLSs) are involved in the pathological changes of RA [13]. Accumulating evidence has shown that RA-FLSs passively respond to the regulations from various cells, including T cells, B cells and macrophages. In addition, RA-FLSs actively regulated chronic inflammation of synovial joints, leading to cartilage and bone destruction [14]. RA-FLSs have been reported to display some tumor-like behavior, with strong proliferation ability and intracellular molecular imbalance [15, 16]. This transition behavior contributes to the progression of RA that leads to joint destruction [17]. However, the molecular mechanism underlying the activation of the proliferation and inflammation of RA-FLS remains unclear.

Nucleotide binding regions and nod-like receptor (NLR) families are reported to be activators of inflammation. NLRP12 is a recently identified member of the NLR family. NLRP12 has been shown to be a negative regulatory protein that inhibited noncanonical NF-κB activation [18]. Members of the noncanonical NF-κB family play important roles in inflammatory regulation and immune responses. Additionally, noncanonical NF-κB can induce MAPK activation [19]. It has been found that NLRP12 dramatically affects the activation of NF-κB and ERK in T cells [20]. Moreover, the role of NLRP12 in attenuating inflammatory responses has been reported. For example, a previous study has demonstrated that NLRP12-deficient mice responded to antigen immunization with hyperinflammatory T cell responses [20].

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Furthermore, NLRP12 was reported to reduce colon inflammation through maintaining microbial diversity and contributing to protective commensal bacterial growth [21]. However, the effects of NLRP12 on RA-FLSs have not been reported.

Here, we focused on investigating the role of NLRP12 in the proliferation and inflammatory response of RA-FLSs. Our results showed that NLRP12 expression was decreased in RA-FLSs, and NLRP12 inhibited proliferation and reduced inflammatory response by regulating NF-κB and MAPK signaling pathways. This study provides a new target for diagnosis and treatment of patients with RA.**Methods**

Clinical sample

Samples (n = 40) were collected from 40 patients with RA at China-Japan Union Hospital of Jilin University. Normal tissues were obtained from healthy control subjects (n = 30) who were free of other diseases, such as autoimmune disease, infectious disease and cancer. The clinical characteristics are shown in *table 1*. All individuals signed informed consent in this research. The study protocols were approved by World Medical Association Declaration of Helsinki and the Ethics Committee of China-Japan Union Hospital of Jilin University.

Isolation of RA-FLS

RA-FLSs were isolated from synovial tissue by enzymatic digestion. In brief, the synovial tissue was dissected free of fat, blood vessels and fibrous tissues and rinsed with PBS. Then, the tissue was minced and digested with Collagenase Type I (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 55minutes. After washing, the cells were cultured in DMEM containing 10% fetal calf serum, 50 IU/mL penicillin-streptomycin, 2mM L-glutamine and 10mM HEPES (all from Beyotime, Shanghai, China) at 37°C in a humidified atmosphere of 5% CO_2. Cultures were passaged every 2 or 3 days until the cell reached the logarithmic growth phase. In general, FLSs migrated out from the tissue explant and were grown into a firmly adherent cell monolayer with a confluence of 90% to 95% within 14 days. Cultured RA-FLSs were used for the following experiments between passages 3 and 8.

RA-FLSs culture and transfection

Cells were cultured with DMEM (Beyotime, Shanghai, China) supplemented with 10% fetal calf serum at 37 °C with 5% CO_2. The sequences of NLRP12 were synthesized and then inserted into pcDNA3.1 vector to generate pcDNA3.1-NLRP12 plasmid. The shRNA sequences targeting NLRP12 were inserted into pRNAH1.1 plasmid and named shNLRP12. RA-FLSs were transfected with NLRP12 overexpression (NLRP12) or negative control (NC) to achieve NLRP12 overexpression. RA-FLSs were also transfected with NLRP12 knockdown (shNLRP12) or shNLRP12 negative control (shNC) to conduct NLRP12 knockdown using Lipofectamine 3000 (Beyotime, Shanghai, China) according to the manufacturer's instructions.

Table 1
The clinical characteristics of patients with RA

Demographic and clinical characteristics of Patients with rheumatoid arthritis		
Characteristic	RA(n=40)	Control (n=30)
Age (mean±SD)	55.42±12.76	52.15±10.28
Gender: female/male	34/6	17/13
Disease duration (months): mean±SD	75.12±14.76	/
Anti-CCP>7 U/ml (%)	79.45	/
RF>20 IU/ml (%)	75.23	/
CRP>8 mg/l (%)	68.43	/
ESR>20 mm/h (%)	67.21	/
DAS28 score (%)		/
Remission (≤2.6)	13.89	/
Low (2.6–3.2)	11.92	/
Moderate (3.2–5.1)	45.31	/
High (>5.1)	28.88	/
DMARDs (%)	100	/
NSAID (%)	30	/
Prednisone (%)	46.13	/
MTX (%)	38.23	/
Leflunomide (%)	60.45	/

CCP cyclic citrullinated peptide, RF rheumatoid factor, CRP Creactive protein, ESR erythrocyte sedimentation rate,DAS disease activity score, DMARDs disease modifying anti-rheumatic drugs, NSAID non-steroidal anti-inflammatory drugs, MTX methotrexate

The cells were incubated for 24 h before performing the subsequent experiments.

Quantitative reverse transcription-PCR

Total RNA was extracted via the RNA extraction kit (Beyotime, Shanghai, China). The quantity of RNA was detected and then RNA was reverse transcribed to cDNA by SuperScript II Reverse Transcriptase (Sangon, Shanghai, China). The **quantitative reverse transcription-PCR** (qRT-PCR) was performed and the NLRP12 mRNA level was measured using SYBR Green PCR Kit (Takara, Dalian, China). Primer sequences were listed as follows: NLRP12 F: 5′-CCTCTTTGAGCCAGACGAAG-3′, NLRP12 R: 5′-GCCCAGTCCAACATCACTTT-3′. β-actin F: 5′-ATCGTGCGTGACATTAAGGAGAAG-3′, β-actin R: 5′-AGGAAGGAAGGCTGGAAGAGTGGAPDH-3′. The fold changes of mRNA were calculated by the cycle threshold (Ct) values and the 2-△△Ct method. The NLRP12 mRNA level was normalized to β-actin.

ELISA assay

Cells (5×10^4 cells/dishes) were grown in 35 mm dishes for 12 hours. After treatment for 36 hours, cells were cultured in fresh medium for 24 hours. Then, supernatant was collected and used to analyze IL-1β, TNF-α, IL-6, IL-10, IFN-γ and MCP-1 protein production by corresponding ELISA kits (Beyotime, Shanghai, China).

Western blot analysis

Protein was extracted with RIPA lysis buffer (Sangon, Shanghai, China) and resolved by SDS-PAGE. Samples were transferred onto a PVDF membrane (Millipore, Billerica, MA). The membrane was blocked in 5% skim milk at 37°C for 1 hour and incubated with antibodies against p-JNK (1:1000, CST, Shanghai, China), JNK (1:1000, CST, Shanghai, China), p-ERK (1:1000), ERK (1:1000), p-p38 (1:1000), p38 (1:1000), p-NF-κB (1:1000), NF-κB (1:1000), NLRP12(1:1000) and β-actin (1:2000) (all from Abcam, Cambridge, MA, USA) overnight at 4°C. Then, the membrane was incubated with secondary antibody (1:2000, Beyotime, Shanghai, China) at 37°C for 45minutes. Immunoreactive proteins were detected through enhanced chemiluminescence (Beyotime, Shanghai, China). Protein bands were analyzed using Image J and normalized to β-actin levels. Each experiment was carried out independently in triplicates.

Cell viability assay33333

Cells (1×10^4 cells/well) were plated in 96-well plates. Then, 10 μL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (NJJCBIO, Nanjing, China) was added to wells and incubated for 4 hours. Supernatants were removed, and dimethyl sulfoxide (DMSO) was added to terminate the reaction. The absorbance was examined through a microplate reader at 570 nm.

Cell apoptosis detection

Cells were collected and then washed with ice-cold PBS. After fixation with 70% ethanol, cells were stained with Annexin V-fluorescein-isothiocyanate (FITC) as well as propidium iodide for 25 minutes in dark. Apoptosis was detected using a FACScan flow cytometer.

Statistical analysis

Data were expressed as means ± standard deviations and analyzed using SPSS statistical software v20.0. Differences between two groups were evaluated by Student's *t*-test or Mann-Whitney *U* test. Statistical differences among groups were tested by one-way One-way analysis of variance (ANOVA) or the Kruskal-Wallis test. $P < 0.05$ was considered statistically significant.

RESULTS

NLRP12 was downregulated in RA tissues and RA-FLSs

To explore the expression of NLRP12 in RA tissues and RA-FLSs, qRT-PCR and western blot analysis were carried out. The results from qRT-PCR verified that the NLRP12 mRNA level was downregulated in RA tissues and RA-FLSs (*figure 1*A, B). Further, western blot analysis revealed that the NLRP12 protein level was decreased in RA tissues and RA-FLSs (Fig. 1C, D). The data suggested that NLRP12 may play an important role in patients with RA.

NLRP12 inhibited cell viability in RA-FLSs

We then investigated the effect of NLRP12 on cell viability of RA-FLSs. NLRP12 overexpression and knockdown were performed in RA-FLSs. Western blot analysis proved that NLRP12 protein level was increased in RA-FLS with NLRP12 overexpression, whereas NLRP12 protein level was decreased in RA-FLS with NLRP12 knockdown (*figure 2*). MTT assay demonstrated that NLRP12 overexpression repressed cell viability. In contrast, NLRP12 knockdown promoted cell viability (Fig. 2B). These findings indicated that NLRP12 repressed cell viability in RA-FLSs.

NLRP12 contributed to cell apoptosis in RA-FLSs

To examine whether NLRP12 can affect cell apoptosis in RA-FLSs, flow cytometry was conducted. Cell apoptosis was analyzed in RA-FLSs with NLRP12 overexpression or knockdown. As shown in *figure 3*, NLRP12 overexpression elevated cell apoptosis of RA-FLSs evidenced by upregulation of apoptotic cells (AnnexinV-FITC) + /PI+ and AnnexinV-FITC) + /PI-). However, NLRP12 knockdown inhibited cell apoptosis by downregulation of apoptotic cells (AnnexinV-FITC) + /PI+ and AnnexinV-FITC) + /PI-). These results implied that NLRP12 promoted cell apoptosis in RA-FLSs.

NLRP12 reduced inflammatory response in RA-FLSs

Further, we evaluated the effect of NLRP12 on inflammatory response in RA-FLSs. ELISA assays proved that NLRP12 overexpression downregulated

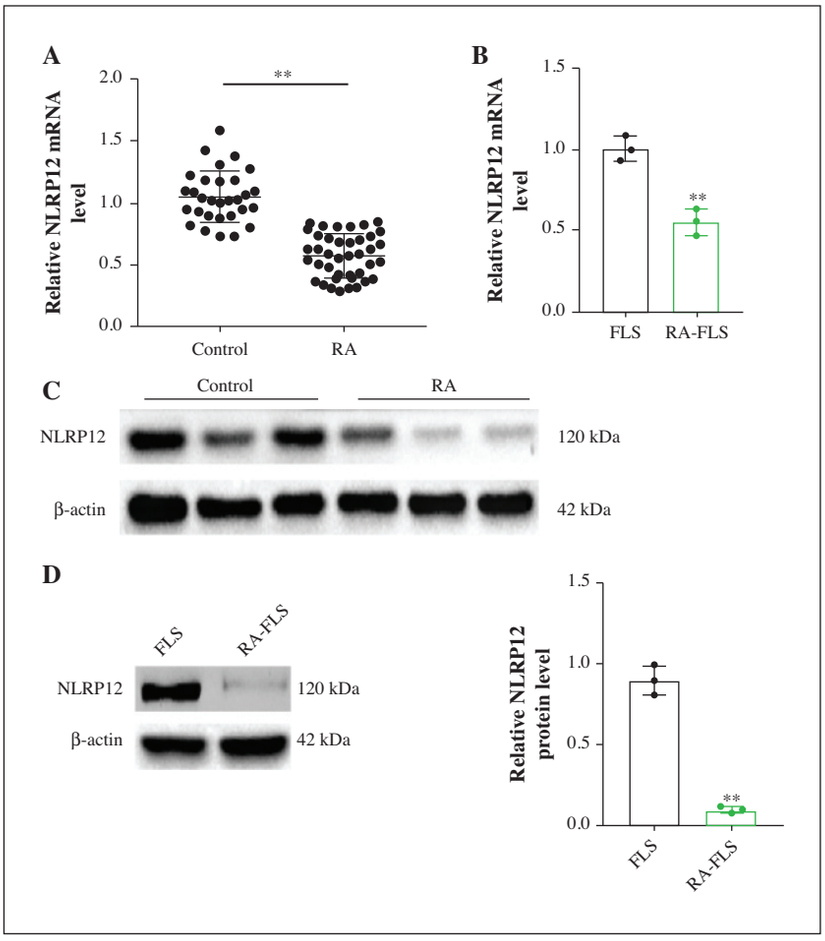

Figure 1

NLRP12 was downregulated in RA tissues and RA-FLSs. (A) NLRP12 mRNA level was measured using qRT-PCR in RA tissues. Control, n = 30. RA, n = 40. (B) NLRP12 mRNA level was examined in RA-FLSs using qRT-PCR. n = 3. (C) NLRP12 protein level was detected through western blot analysis in RA tissues. Control, n = 30. RA, n = 40. (D) NLRP12 protein level was examined by western blot analysis and the density of the bands was analyzed using Image J. n = 3. Note that a representative western blot analysis was presented in each group. **, $p < 0.01$.

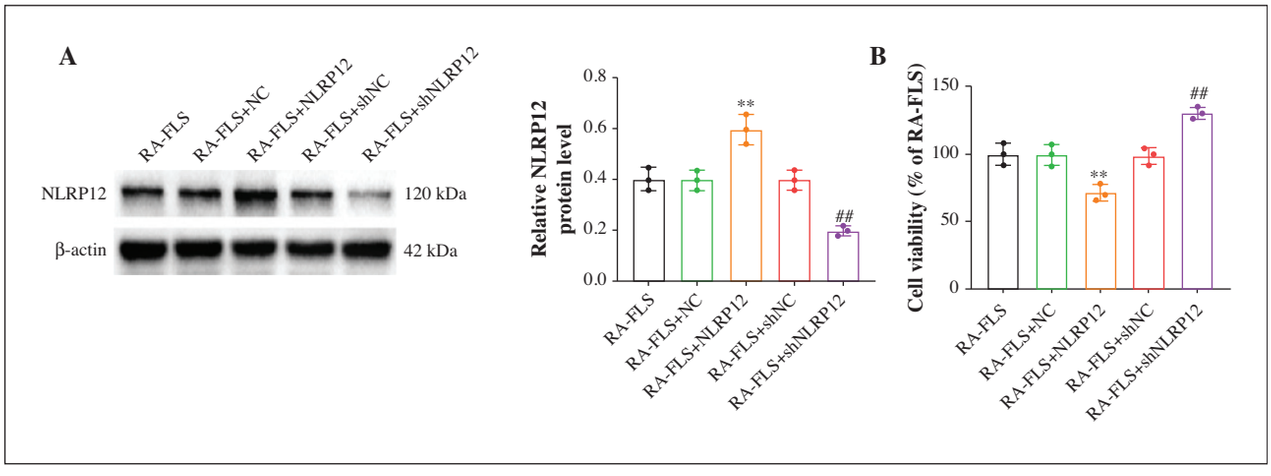

Figure 2

NLRP12 inhibited cell viability in RA-FLSs. (A) Western blot analysis was used to detect the NLRP12 protein level and the density of bands was analyzed using Image J. (B) Cell viability was measured using MTT assay. n = 3. Note that a representative western blot analysis was presented in each group. **, $p < 0.01$. ##, $p < 0.01$. * vs RA-FLS + NC. # vs RA-FLS + shNC.NC, negative control.

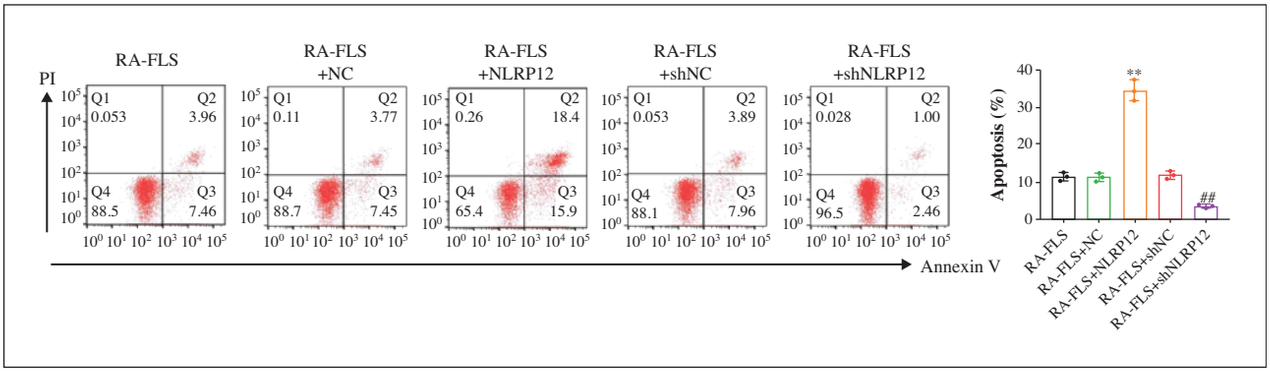

Figure 3

NLRP12 contributed to cell apoptosis in RA-FLSs. Cell apoptosis was analyzed through flow cytometry in RA-FLSs. n = 3. **, $p < 0.01$. ##, $p < 0.01$. * vs RA-FLS + NC. # vs RA-FLS + shNC. NC, negative control.

IL-1β, TNF-α, IL-6, IFN-γ and MCP-1 levels and upregulated the IL-10 level. An increase in IL-1β, TNF-α, IL-6, IFN-γ and MCP-1 levels and a decrease in the IL-10 level were found in RA-FLSs with NLRP12 knockdown (*figure 4*). The data indicated that NLRP12 decreased inflammatory response in RA-FLSs.

NLRP12 regulated NF-κB and MAPK signaling pathways

The role of NLRP12 in NF-κB and MAPK signaling pathways was also evaluated. Western blot analysis verified that NLRP12 overexpression inhibited the phosphorylation of JNK, ERK, p38 and NF-κB in RA-FLSs. Nevertheless, NLRP12 knockdown promoted phosphorylation of JNK, ERK, p38 and NF-κB (*figure 5*). These findings implied that NLRP12 was involved in the regulation of NF-κB and MAPK signaling pathways.

DISCUSSION

In the current study, we demonstrated that NLRP12 was downregulated in RA tissues and RA-FLSs, which suggests that NLRP12 may play an important role in the progression of RA. NLRP12 was then verified to suppress the proliferation and inflammation and promote cell apoptosis of RA-FLSs. In addition, NLRP12 repressed NF-κB and MAPK signaling pathways.

A previous study showed that NLRP12 deficiency promoted inflammation and proliferation and elevated

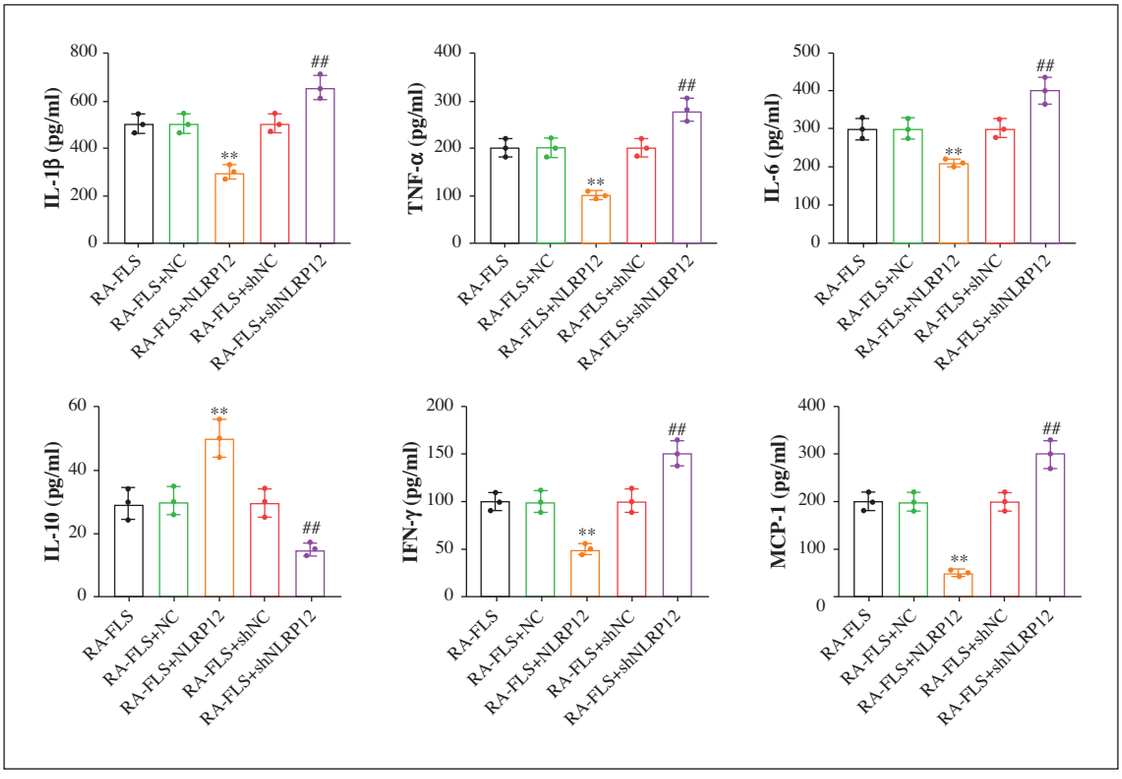

Figure 4

NLRP12 reduced inflammatory response in RA-FLSs. ELISA assays were used to measure IL-1β, TNF-α, IL-6, IL-10, IFN-γ and MCP-1 levels in RA-FLSs. n = 3. **, $p < 0.01$. ##, $p < 0.01$. * vs RA-FLS + NC. # vs RA-FLS + shNC.NC, negative control.

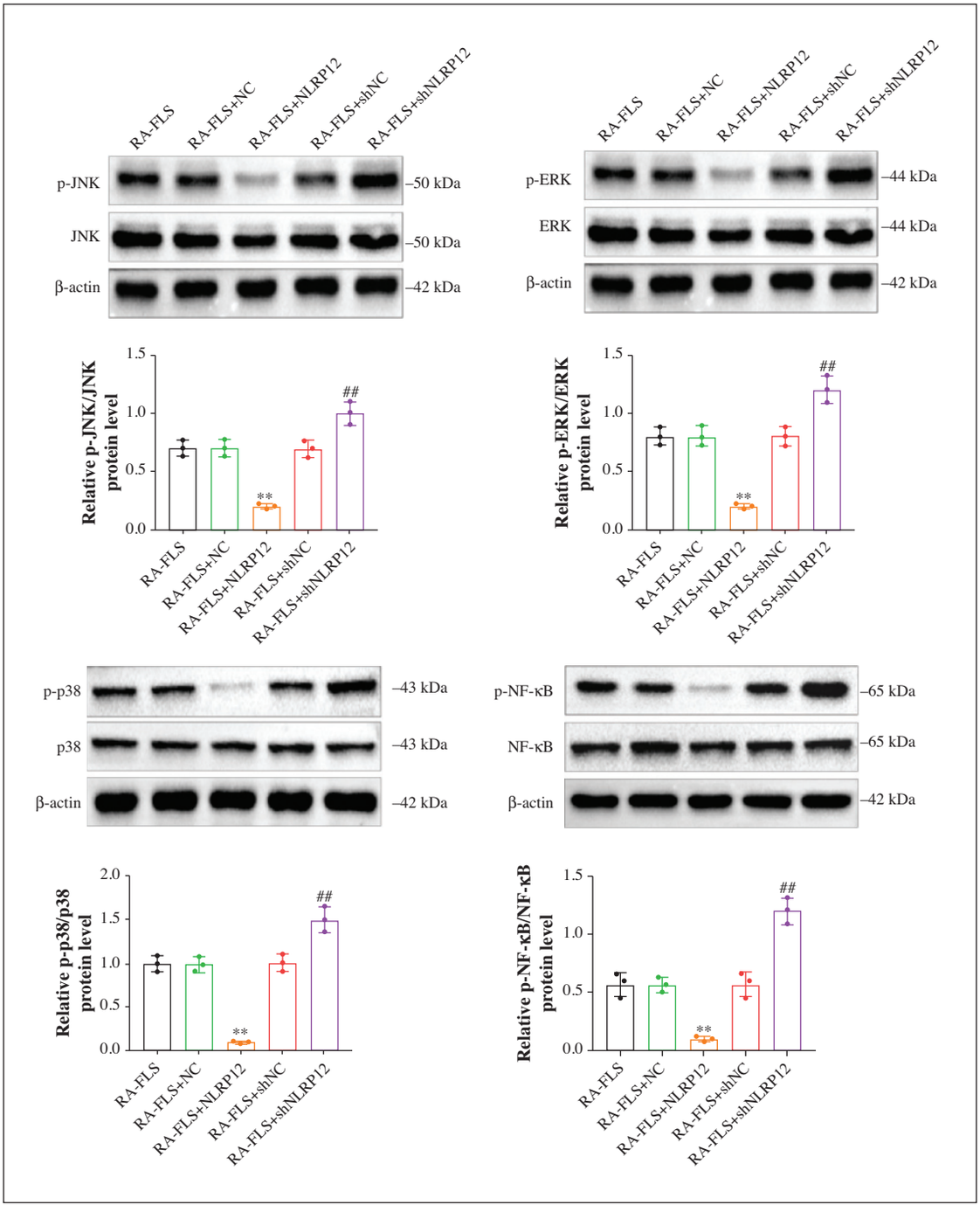

Figure 5

.**NLRP12 regulated NF-κB and MAPK signaling pathways.** The protein levels of p-JNK, JNK, p-ERK, ERK, p-p38, p38, p-NF-κB, NF-κB and β-actin were measured using western blot analysis, and the density of the bands was analyzed using Image J. n = 3. Note that a representative western blot analysis was presented in each group. **, $p < 0.01$. ##, $p < 0.01$. * vs RA-FLS + NC. # vs RA-FLS + shNC.NC, negative control.

tumor burden in hepatocellular carcinoma [22]. NLRP12, as a critical regulator, could participate in the alleviation of colon inflammation and tumorigenesis in mice [18, 23]. Moreover, auto-inflammatory disorders have been reported to be caused by mutations of NLRP12 in humans [24, 25]. In addition, NLRP12 was discovered to be expressed in human and mouse monocytic cells [26]. NLRP12 mutation in human monocytic cells exhibited hyperinflammatory in nature [27]. Interestingly, in our study, we proved that NLRP12 overexpression inhibited cell viability of RA-FLSs, whereas NLRP12 knockdown elevated cell viability. NLRP12 displayed a promoting role in cell apoptosis of RA-FLSs. Further, NLRP12 decreased IL-1β, TNF-α, IL-6, IFN-γ and MCP-1 levels and increased the IL-10 level. NLRP12 variants were reported to be related with auto-inflammatory diseases [24, 28]. These findings suggest that NLRP12 plays a suppressive role in proliferation and inflammation of RA-FLSs.

Inhibition of NF-κB and other inflammatory signaling pathways exhibited an important role in driving inflammation. Susceptibility to DSS-induced colitis was enhanced when negative regulators of NF-κB were

deleted [29-31]. NLRP12 was reported to play a protective role in intestinal inflammation through inhibiting canonical and noncanonical NF-κB [18, 23]. Accumulating evidence has revealed that NLRP12 exhibited suppressive roles in inflammation and colon tumorigenesis by inactivating NF-κB and ERK signaling in myeloid cells [23]. Furthermore, NLRP12 was negatively involved in the regulation of NF-κB and ERK in macrophages, dendritic cells, and T cells [20, 23, 32]. In our study, NLRP12 overexpression negatively regulated NF-κB signaling pathway, whereas NLRP12 knockdown promoted NF-κB signaling pathway. Additionally, NLRP12 was demonstrated to downregulate phosphorylation of JNK and p38 and inhibit MAPK signaling pathway. Consistent with these findings, Allen et al. also discovered that the phosphorylation of JNK and p38 was similar in NLRP12 deficiency and wild type exposed to Pam3Cys4 stimulation [18]. In addition, different inflammatory signaling pathways included NF-κB, MAPK, AKT and JAK/STAT signaling pathways [33]. The data implied that NLRP12 repressed proliferation and inflammation of RA-FLSs through regulating NF-κB and MAPK signaling pathways.

However, previous research has shown that NLRP12 attenuated inflammatory responses through Blimp-1 [34], suggesting that NLRP12 may be involved in regulating proliferation and inflammation of RA-FLSs by other factors. Therefore, the exact mechanism by which NLRP12 affects proliferation and inflammation of RA-FLSs remains to be fully elucidated. Besides, the role of NLRP12 in proliferation and inflammation is not evaluated in a clinical sample. The correlation between NLRP12 expression and disease activity will be further investigated. Therefore, additional experiments will be performed in the near future.

In conclusion, our results showed that NLRP12 was downregulated in RA tissues and RA-FLSs. NLRP12 inhibited the proliferation and inflammation of RA-FLSs via NF-κB and MAPK signaling pathways. These findings suggest that NLRP12 may serve as a new biomarker of RA, which provides a new therapeutic target for patients with RA.

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Competing interests

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

Ethics approval

Ethical approval was obtained from the Ethics Committee of China-Japan Union Hospital of Jilin University.

Statement of Informed Consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Availability of data and materials

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

Authors' contributions

Xin Zhang and He Nan designed the study and supervised the data collection, Jinyu Liu analyzed and interpreted the data, and Jialong Guo prepared the manuscript for publication and reviewed the draft of the manuscript. All the authors have read and approved the manuscript.

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