Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Regulatory effect of zinc finger protein A20 on rheumatoid arthritis through NLRP3/ Caspase-1 signaling axis mediating pyroptosis of HFLS- RA cells

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ARTICLE INFO

Original paper

Article history:
Received: May 07, 2023
Accepted: August 21, 2023
Published: August 31, 2023

Keywords:

Rheumatoid arthritis, NLRP3/ Caspase-1 signaling, zinc finger protein A20, HFLS- RA cells

ABSTRACT

As a chronic inflammatory autoimmune disease, rheumatoid arthritis (RA) causes significant destruction to joints and cartilage. So far, from RA patients, the synovial cells and subsynovial tissues reflected the positive expression of IL-18, IL-1β, Caspase-1 and NLRP3, with the synovial tissues of those patients also expressing the zinc finger protein A20 at a significantly lower level compared with osteoarthritis (OA) ones. Thus, the inhibition of the NLRP3/caspase-1 signaling pathway can effectively down-regulate the expression of IL-1β, but when NLRP3 inflammasomes are activated, they can also shear GSDMD and induce pyroptosis. These suggest that the Gasdermin family of proteins, downstream of the NLRP3 inflammasome, could be involved in pyroptosis. Previous studies have shown that A20 contributes largely as an anti-inflammatory factor in many inflammatory diseases, but it remains unclear whether zinc finger protein A20, as an inhibitor of NLRP3 inflammasomes, can play a protective role against RA by inhibiting NLRP3 inflammasome-mediated pyroptosis. Therefore, this study aimed to verify the effects of zinc finger protein A20 on NLRP3/ Caspase-1-mediated pyroptosis in rheumatoid arthritis synovial fibroblasts (HFLS-RA) cells through cell experiments and clinical bidirectional verification, aim to understand the regulatory mechanism of A20 on RA. The results of clinical trials showed that NLRP3, Caspase-1, IL-1β and IL-18 were positively scattered in RA synovial cells and subsynovial tissue. The expression level of the zinc finger protein A20 in RA synovial tissues was significantly lower than that in OA synovial tissue and was negative, while zinc finger protein A20 was strongly positive in OA synovial tissue. In addition, HFLS-RA cells with siRNA-interfering zinc finger protein A20 were constructed at the cellular level, with the results also confirming that zinc finger protein A20 can play a protective role against RA by inhibiting NLRP3 inflammasome-mediated pyroptosis. In conclusion, this study is of great significance for understanding the role of the NLRP3-caspase-1-IL-1β/ pyroptosis signaling pathway in the occurrence and development of RA. It is expected that the results will provide a theoretical basis for the immune regulation of innate immunity in the occurrence and development of RA, while providing a new therapeutic target for the clinical treatment of RA.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.8.27
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Introduction

As a chronic inflammatory autoimmune disease, rheumatoid arthritis (RA) is characterized by joint invasion as its main clinical manifestation and joint synovitis as its main feature (1). It has been reported that around 1-2% of the world population suffers from RA (2), with a significantly higher proportion of female patients compared with males (3). As noted, this condition mainly manifests itself as synovial inflammation, the destruction of joint structures as well as an abnormal proliferation and invasion of synovial fibroblasts (HFLS-RA) (4). These different processes subsequently lead to the production of local inflammatory factors (IL-18, IL-1β, IL-6, etc.) that not only degrade the extracellular matrix and cartilage proteins but also form pus, thereby further destroying the joints and cartilage. Thus, it is not surprising that as many as 60% of patients eventually present disabilities within 5-10 years (5). One important component of innate immunity is the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing (NLRP3) inflammasome (6-8). It can be activated by various endogenous danger signals, such as cellular perforin (9), extracellular ATP (10), reactive oxygen species (ROS) (11, 12), as well as other exogenous factors, leading to its inappropriate activation or mutations that are closely related to RA (13-16), inflammatory bowel disease (17-19), gout (20-22) as well as other inflammatory autoimmune diseases. Clinical studies have confirmed that in RA patients, IL-18, IL-1β, Caspase-1 and NLRP3 were positively expressed in synovial coated cells and subsynovial tissues, especially in the cytoplasm of synovial lined cells, macrophages and a few inflammatory cells within the synovial interstitium. Since Caspase-1 largely mediates IL-1β and IL-18 production, inhibition of the NLRP3/caspase-1 signaling pathway can therefore effectively down-regulate its expression. Additionally, the Gasdermin family of proteins, as the downstream of the NLRP3 inflammasome, could be involved in pyroptosis through the executive protein gasdermin D (GSDMD). This process is attributed to the cleavage of the GSDMD (23, 24) and excessive inflammatory reactions which can subsequently cause irrever-

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sible damage to the body. Therefore, even though during pyroptosis each part of the NLRP3/caspase-1/IL-1β/IL-18 pathway is involved in the pathogenesis of RA, yet the exact regulatory mechanism remains unclear.

Zinc finger protein A20 also referred to as TNF-α-induced protein 3 (TNFAIP3), represents an important molecule that is required for activating the terminal signal of NF-κB, with the process mediated by innate immune receptors such as TNF receptor (TNFR), TLRs and NOD2 (25-27). The zinc finger A20 gene, with a cDNA sequence of 4440bp as well as an open frame of 2370 bp, is found on chromosome 6q23.3, and it encodes a 90 kD protein made up of 790 amino acids (28). This protein is not only an endogenous regulator of inflammatory responses, but it also protects tissues/cells (29). Indeed, a recent study (30) showed that a specific ubiquitin-binding domain (ZnF4 and ZnF7) in A20 enabled the protein to exert its anti-inflammatory activity (31). Ubiquitin is an important modification of proteins and through the above domain, A20 can interfere with intracellular signaling pathways. For instance, it can induce ubiquitination of TNF-related acting protein (RIP) Lys48 to inhibit the activity of NF-κB, thereby reducing the production of cytokines and inflammatory mediators as well as preventing the inflammation-related pathological changes of the body from being too strong to maintain immune homeostasis. In addition, Averil Ma et al. (32) found that A20 could interact with the IL-1β precursor complex, and by inhibiting increased ubiquitination of receptor-interacting protein kinase-3-dependent, it could prevent the secretion and processing of the IL-1β precursor. At the same time, the activity of the RIPK3-dependent NLPR3 inflammasome was inhibited. In A20-absent macrophages, ubiquitination of IL-1β precursors is significantly increased depending on RIPk3 expression, and LPS alone could result in spontaneous NLRP3 inflammasome activity, hence suggesting that A20 is an important role in inhibiting NLRP3 inflammasome associated disease. In this context, Wang et al. (33) found that, for RA patients, there was a significant downregulation of A20 expression in peripheral blood mononuclear cells (PBMCs) in comparison with the control group. There was also a negative correlation between the expression level, the anti-CCP, the RA score as well as the level of C-reactive proteins. Studies have further shown the likelihood of spontaneous multi-joint involvement, similar to RA in humans, in mice where the RA susceptibility gene A20/TNFAIP3 (A20myel-KO) had been deleted. Elsby LM et al. (34) detected A20 in synovial tissues of both RA and osteoarthritis (OA) patients, with the expression being down-regulated in comparison with that of OA patients. In RA patients, expression of A20 mainly occurs in the cytoplasm of synovial lining cells, lymphocytes, fibroblasts and mast cells. In fact, the authors' previous study found a significantly lower level of A20 expression in the synovium of RA patients compared with OA. It is still unclear whether zinc finger protein A20, as an inhibitor of NLRP3 inflammasomes, can play a certain protective role against RA by inhibiting NLRP3 inflammasome-mediated pyroptosis.

At present, there are only a few reports on the role of pyroptosis in the immune regulation of RA, with no relevant studies at home and abroad on the effects and mechanism of A20 on the immune regulation of NLRP3-caspase-1-IL-1β/ pyroptosis signaling pathway in RA. Based on the above scientific hypotheses, this study conducted two-way validation experiments at the clinical and cellular levels respectively. Through immunohistochemical staining, the level of expression and significance of pyroptosis-related proteins in synovial tissue started. In addition, at the cellular level, the siRNA-interfered zinc finger protein A20 plasmid was constructed, and the gene expressions of NLRP3 and Caspase-1 were detected by RT-qPCR. NLRP3 and caspase-1 expression levels were measured by WB; The supernatant IL-1β and IL-18 were detected by Elisa. Pyrosis was detected by transmission electron microscopy. Clinical and cellular experimental data are expected to provide an important scientific basis for new target therapy of RA.

Materials and Methods

Materials

The following materials were used for the experiments: HFLS-RA cells (American Type Culture Collection (ATCC) (Manassas, VA, USA)), fetal bovine serum (Sigma, St. Louis, MO, USA), MagZol Reagent (Magen, JJ150300), DL 2000 DNA marker (TaKaRa, Tokyo, Japan, 3427B (Ax2)), Hieff TMqPCR SYBR® Green Master Mix (LoW Rox Plus,11202ES08), Primer, HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China, R223-01), anhydrous ethanol, chloroform (Guangzhou Chemical reagent, Guangzhou, China) and agarose (BIOWEST, Nuaillé, France).

Clinical data collection

The purpose of this study was to obtain clinical data on RA patients from the authors' hospital under the supervision of the hospital's Ethics Committee. Informed consent was obtained from all participants, and their rights, including information on their age, gender and results of laboratory examinations (e.g., C-reactive protein, rheumatoid factor, etc.) were fully respected. The experimental group consisted of 25 RA patients, while for the control group, 25 OA patients were selected.

The following exclusion criteria were also applied for the selection process: patients with malignant tumors; those with heart and kidney failure, severe liver diseases and other serious systemic diseases, tuberculosis, diabetes, gout, hyperthyroidism, Cushing's syndrome and other endocrine and metabolic diseases; patients with significant recent changes in body mass as a result of infection and other autoimmune diseases except RA; those undergoing preoperative radiotherapy, chemotherapy and immunotherapy; patients who did not meet the diagnostic criteria for RA.

Cell culture of MH7A cells

HFLS-RA-MH7A cells (MH7A) were cultured in DMEM to which streptomycin (100 μg/mL), penicillin G (100 U/mL) and 10% fetal bovine serum (FBS) had been added. They were then incubated under 5% CO_2 at 37°C in a humidified cell-culture incubator.

Hematoxylin and eosin (H&E) staining

After being removed, synovial tissues were fixed using 4% neutral formaldehyde. They were subsequently embedded in paraffin and cut into 5-μm sections prior to staining with Hematoxylin and eosin.

Immunohistochemical staining

Synovial tissues embedded in paraffin were sliced into 4-μm thick sections and then stained with immunohistochemical staining (En Vision method). Detailedly, the sections were stored at 4°C with the primary antibody in 5% BSA overnight before being stained. This was followed by incubation with the appropriate secondary antibody to observe pathological changes. For this set of experiments, the expression level of the following proteins was assessed: A20, IL-18, IL-1β, caspase-1 and NLRP3.

Quantitative Real-time polymerase chain reaction (qPCR)

The TRIzol method was used for extracting RNA from HFLS-RA cells prior to quantification with a NanoDrop One Microvolume UV-Vis spectrophotometer (Thermo, Waltham, MA, USA) as previously described. For selected target genes, qPCR was then used to assess the mRNA levels, with Hprt1 acting as the standard. Table 1 shows the primer sequences for this experiment. Additional inquiries regarding the primers used for the qPCR can be addressed to the corresponding author.

RNA interference assay

NC-targeting siRNAs with sequences UUCUCCGAACGUGUCACGUTT (Sense) and ACGUGACACGUUCGGAGAATT (Antisense), as well as A20-targeting ones with sequences GAGAGUGUUUGUAGUUCAUGG (Sense) and AUGAACUACAAACACUCUCUG (Antisense), were synthesized by GenePharma (Shanghai, China) for knocking down the human *A20* gene. After adding 1×10^6 RA-FLS cells to each well of 6-well culture plates, the cells were cultured for 12 hours prior to transfection with 100 pmol of siNLRP3 or siNC in DMEM. In this case, the process was achieved using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. Six hours after the transfection, fresh DMEM containing 10% fetal bovine serum was added to replace the existing medium, and this was followed by cell incubation for 36 hours, and eventually preparing cell lysates and culture supernatants.

Western blotting

Proteins that were extracted from HFLS-RA cells and liver with lysis buffer were separated by electrophoresis as described previously prior to their transfer onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). Membranes were then blocked for 1 h using non-fat milk before overnight incubation at 4°C with different primary antibodies (IL-1β and IL-18). This was followed by a 1-h incubation at room temperature with the corresponding secondary antibodies for 1 h. The ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA) was eventually used to visualize protein bands.

IL-1β and IL-18 release assay

A QuantiCyto IL-18 ELISA kit (Neobioscience, Shenzhen, China) was used alongside a CytoTox96 IL-1β kit (Promega, Madison, WI, USA), as required by the manufacturer, to measure IL-18 and IL-1β, with absorbance readings taken at 450 nm. Each experiment was performed in triplicate.

Transmission Electron Microscopy (TEM)

The two groups of RA-FLS cells, namely the NC (normal control) and the A20-silent groups, were added to 35-mm culture dishes where they were allowed to reach 80% confluency before being trypsinized. The cells were then centrifuged for 10 min at 1000 rpm, with the resulting pellet immediately fixed through a 2-h incubation in PBS containing 2.5% glutaraldehyde (vol/vol). The cell blocks were then rinsed with 0.1 M phosphoric acid buffer six times, each for 30 minutes. After being dehydrated, the cells were also embedded in Embed 812 resin. They were subsequently cut into thin sections to observe the morphology of pyroptotic cells using electron microscopy (Japan Electron Optics Laboratory Co., Ltd., JEM-1400). In this case, ImageJ software (RADIUS ALL 2.2 (Build 21230)) was used to observe the target structure in each TEM photograph.

Statistical analysis

For all data, at least three replicated measurements were taken and presented as mean ± SEM. One-way ANOVA was then performed with GraphPad Prism 8 (Graph-Pad, San Diego, CA, USA) to compare differences in the results between multiple groups. Results were considered to be statistically significant for *P*-values < 0.05 were considered as.

Results

NLRP3 was positively expressed in RA patients

Recent studies illustrated that NLRP3/Caspase-1 is a classic inflammatory signaling pathway. In this study, In the patients with RA, NLRP3 is actively expressed in synovial pericytes as well as in the subsynovial tissue, particularly in synovial lining cells, macrophages and a few inflammatory cells within the synovial interstitium. (Figure 1). Consistently, Caspase-1 was also shown to be positively expressed in synovial membranes in the presence of NLRP3, and this protein was positively expressed in the same cells and tissues as NLRP3. Furthermore, NLRP3 is activated and the cell membrane is punctured, leading to the extracellular release of many inflammatory factors, including IL-1b and IL-18. Therefore, it is not surprising that IL-1β and IL-18 are also positively expressed in the aforementioned cells like NLRP3 and Caspase-1 (Figure 1).

Table 1. Primer sequences.

Gene	Product	Sequence
β-actin	186 bp	F: TGGCACCCAGCACAATGAA
		R: CTAAGTCATAGTCCGCCTAGAAGCA
Caspase-1	142 bp	F: ATGGGCTCTGTTTTTATTGGAAG
		R: CTCTTTCAGTGGTGGGCAT
NLRP3	216 bp	F: GCCGAAGTGGGGTTCAGAT
		R: CTCACACTCTCACCCAGACG

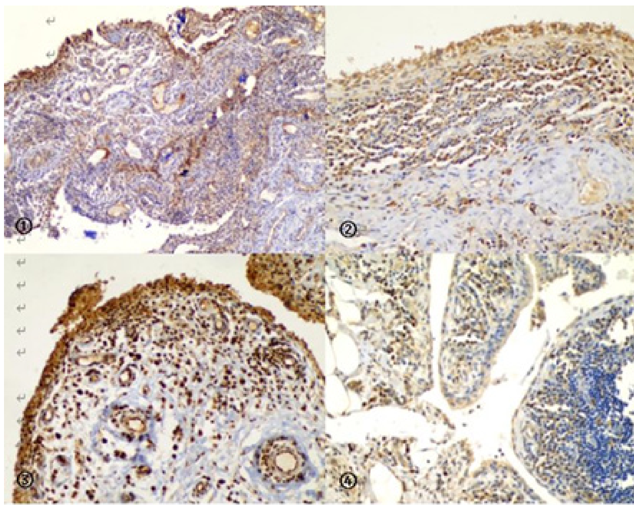

Figure 1. The expression of NLRP3 in RA synovial coated cells and subsynovial tissues. (1) NLRP3 was scattered in RA patients' synovium-coated cells and subsynovium tissues, ×100 times, En Vision method; (2) Caspase-1 was also positively scattered in similar cells and tissues, ×200 times, En Vision method; (3), along with IL-1β, ×200 times, En Vision method; (4) and IL-18, ×200 times, En Vision method.

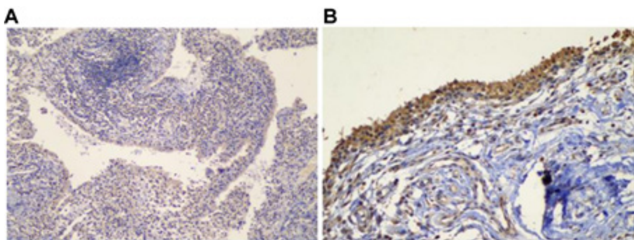

Figure 2. The expression of the zinc finger protein A20 in RA synovial coated cells and subsynovial tissues. (A) A20 was negative in the synovium-coated cells and subsynovium tissues of RA patients, ×100, En Vision method; (B) but strongly expressed in those of OA patients, ×200 times, by En Vision method.

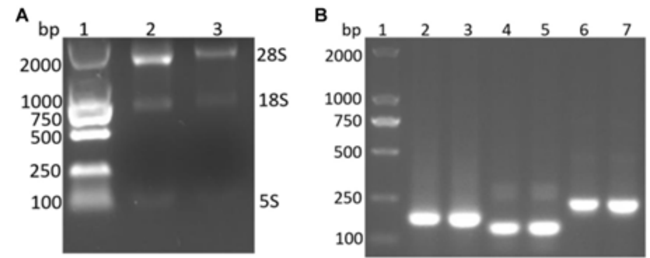

Figure 3. The mRNA expression of NLRP3 (left) and Caspase-1 (right) in MH7A cells. (A) 1. DL2000; 2. HFLS-RA-si-NC; 3. HFLS-RA-si-A20; (B) 1. DL2000; 2. HFLS-RA; 3. HFLS-RA-si-NC; 4. HFLS-RA-GAPDH positive control; 5. HFLS-RA-TNFAIP3-si-1-2059; 6. HFLS-RA-si-TNFAIP3-2-2251; 7. HFLS-RA-TNFAIP3- si-3-1830; 28S band is clearly visible, the ratio with 18S band is about 2:1, mRNA smear is visible, 5s band is wea.

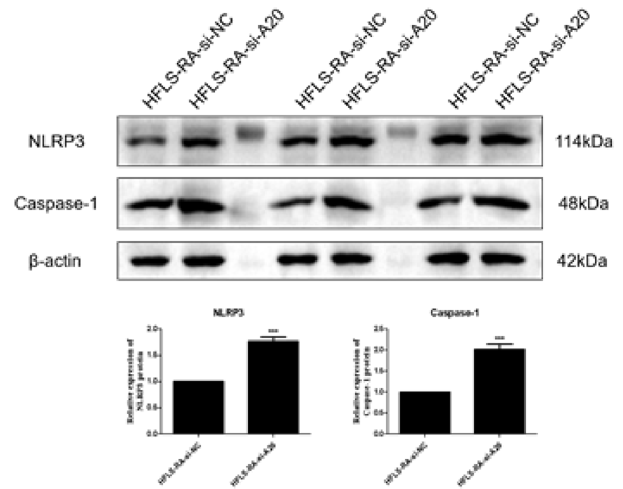

Figure 4. NLRP3 (left) and Caspase-1 (right) expression in MH7A cells. MH7A cells were treated with SiNC and SiA20 plasmid vectors. Western blot assay for IL-18 and IL-1β in MH7A cells. Statistical significance: ***P<0.001, compared with the control group. Performed with One-way ANOVA test (n=3).

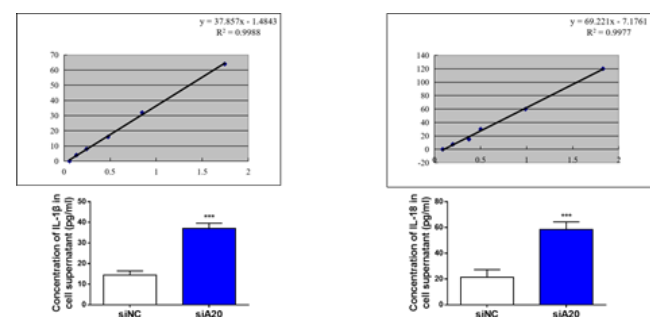

Figure 5. Expression of IL-1β (left) and IL-1β (right) in A20-deleted MH7A cells. MH7A cells were treated with SiNC and SiA20 plasmid vectors. Elisa assay for cell culture supernatant IL-1β and IL-18. Statistical significance: ***P<0.001, compared with the control. Performed with One-way ANOVA (n=3).

Zinc finger protein A20 was positively expressed in RA patients

Figure 2 shows the level of the zinc finger protein A20 (A20) in the synovium as observed after immunohistochemical staining. In RA patients, as it was the case for the above-mentioned proteins, the zinc finger protein A20 was also positively expressed in the synovial coated cells and subsynovial tissues, especially in the cytoplasm of synovial lined cells, macrophages and a few inflammatory cells in the synovial interstitium.

Deleting A20 increased the level of NLRP3 and Caspase-1 mRNA in MH7A cells

After observing a dramatic increase in the level of A20 in RA patients, MH7A cells were cultured and used to generate A20 knockout cell lines through genetic manipulation. The relationship between A20, NLRP3 and Caspase-1 was then explored, with Figure 3 showing a significant increase in NLRP3 and Caspase-1 expression after A20 deletion.

A20 deletion increased the release of NLRP3 and Caspase-1 in MH7A cells

For MH7A cells treated with si-A20, the conditional medium will be updated to determine how the deletion of A20 influences NLRP3 and Caspase-1 expression. Figure 4 highlights a significant increase in the release of NLRP3 and Caspase-1 after deleting A20.

A20 deletion increased the release of IL-18 and IL-1β

Considering that IL-18 and IL-1β are downstream targets of NLRP3 and Caspase-1, the effects of deleting A20 on the release of the two inflammatory factors were assessed based on the observed increased release of NLRP3

and Caspase1. Figure 5 shows a significant increase in the release of IL-1β and IL-1β after deleting A20.

Detection of RA-FLS cell focal death using transmission electron microscopy

As can be seen from Figure 6, the cells of the NC group had a normal morphology, with an intact cell membrane structure, a few protruding microvilli visible on the cell surface, an obvious nucleolus, a large and round nucleus as well as a uniform distribution of the chromatin in the nucleus. Intracytoplasmic organelles were also abundant and evenly distributed, with their structures being clear and normal. However, the morphology of cell structures was altered in the gene-silencing group as some of the cells were swollen and ruptured. The integrity of the cell membrane was also lost, and a large area of edema was present in the cell. In addition, a blurred depression of the nucleus's local nuclear membrane was noted, while an increase in heterochromatin pyknosis was accompanied by their uneven distribution as well as their aggregation around the nuclear membrane.

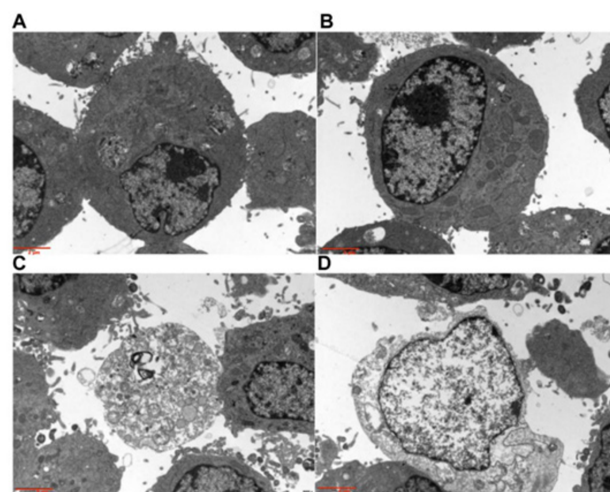

Figure 6. Ultrastructural features of RA-FLS cells, (A&B) show the cells of the NC group, while (C&D) shows the cells of the silent group treated with the detection of A20 (bar indicates 2 μm).

Discussion

RA diseases often involve inflammation, with the NLRP3-Caspase-1 pathway being key for the inflammatory response. For RA patients, an increase in the NLRP3-Caspase-1-IL-1b-IL-18 pathway was noted (Figure 1) alongside the observed increase in A20. The production of is mainly mediated by Caspase-1 largely mediates IL-1β production, hence inhibiting the NLRP3/caspase-1 signaling pathway can effectively reduce the expression of IL-1β. Furthermore, as indicated in Figure 2, there was a significantly lower level of zinc finger protein A20 expression in the synovial tissue of RA, thereby showing negative expression, while the expression level of zinc finger protein A20 in the synovial tissue of OA was strongly positive. It is known that the expression of A20 mainly occurs in the cytoplasm of synovium-coated cells, lymphocytes, fibroblasts and mast cells of the knee, hence further indicating that zinc finger protein A20 may play a protective role against RA as an NLRP3 inflammasome inhibitor by inhibiting NLRP3 inflammasome mediated pyroptosis. MH7A cells were then cultured for observing the expression pattern of A20, NLRP3, Caspase1 and IL18. Overall, the results of the clinical trials indicated that the above proteins were positively scattered positive in RA synovial cells and subsynovial tissue.

At the same time, an A20-deficient MH7A cell line was generated by genetic manipulation. In the normal cell line, deleting A20 aggravated NLRP3 and Caspase-1 overaccumulation. The zinc finger protein A20 was constructed at the cellular level, and the results also confirmed that, as an inhibitor of NLRP3 inflammasome, it could play a protective role against RA by inhibiting NLRP3 inflammasome-mediated pyroptosis. However, the reasons for this change are still unclear. In subsequent follow-up studies, the focus will be on the relationship between A20 and the NLRP3 pathway, as well as a focus on correlation analysis to further explore the trends. On the other hand, the NLRP3 pathway is closely related to pyroptosis. In this study, only the changes in the NLRP3 pathway in RA disease, without an in-depth analysis of the downstream mechanism that causes the RA diseases, were considered. The focus was subsequently on the effects of A20 deletion on pyroptosis and the NLRP3-Caspase-1- IL-1β pathway.

At present, there are only a few reports on the role of pyroptosis in the immune regulation of RA, with no similar reports at home and abroad on the effects and mechanism of A20 on the immune regulation of NLRP3-caspase-1-IL-1β/ pyroptosis signaling pathway in RA. Therefore, we postulated that zinc finger protein A20, being an inhibitor of NLRP3 inflammasome, can protect RA by inhibiting NLRP3 inflammasome-mediated pyrogenesis. Based on the above scientific hypotheses, this study conducted two-way validation experiments at the clinical and cellular levels respectively. Immunohistochemical staining was used to observe the expression level and significance of pyroptosis-related proteins in synovial tissue. In addition, at the cellular level, the siRNA-interfered zinc finger protein A20 plasmid was constructed, while the gene expressions of Caspase-1 NLRP3 were detected by RT-qPCR. NLRP3 and caspase-1 expression levels were measured by WB; The supernatant IL-1β and IL-18 were detected by Elisa. Pyroptosis was detected by transmission electron microscopy (TEM). Clinical and cellular experimental data are expected to provide an important scientific basis for new target therapy of RA.

Overall, this study provides further proof of the critical role of the NLRP3-caspase-1-IL-1β/pyroptosis signaling pathway in the development and progression of RA. Not to be overlooked, the presence of zinc finger protein A20, an inhibitor of NLRP3, provides a strong theoretical basis for the involvement of innate immunity with a view to providing new immune modulation for the occurrence and development of RA. In conclusion, this study is expected to provide a new theoretical basis for the involvement of innate immunity in the development and progression of RA and a new therapeutic target for clinical treatment.

Conflict of interests

The authors declared no conflict of interest.

Funding

This work was supported by the Tianjin enterprise post-doctoral merit funding project (TJQYBSH2018021).

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