Effects of All-Trans Retinoic Acid on the Optimization of Synovial Explant Induced by Tumor Necrosis Factor Alpha

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Summary Current studies focused on the effects of all-trans-retinoic acid (ATRA) on synovial explants from rats with rheumatoid arthritis (RA) induced by lipopolysaccharides (LPS). In our study, synovial membranes were extracted aseptically from the quadriceps femoris of the knee joint of rats, and then incubated in medium containing 10% neonate bovine serum for 24 h adaptive culture. We first measured variations of correlation factors in synovium at 24, 48, 72, 96 and 120 h in control medium or in medium containing 20 ng/mL tumor necrosis factor alpha (TNF-α) (TNF-α-experiment). Then, we investigated the synovium exposed to three ATRA concentrations after 48 h incubation (ATRA-experiment). The effects of ATRA on synovitis were evaluated by observing the expression of inflammatory cytokines, angiogenic factors and the production of proteases in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and apoptosis and autophagy. In TNF-α-experiment, the secretion of nitric oxide (NO), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-9 (MMP-9) increased significantly after TNF-α stimulation without pathological damage to the synovium. Hence, we successfully obtained the synovial explants model, which had longer inflammatory response time. In the ATRA-experiment, ATRA suppressed the secretion of IL-6 and NO, downregulated the NF-κB P65 and Bcl-2, increased levels of autophagy marker protein LC3, but different doses of ATRA showed inconsistent regulatory effects on VEGF and MMP-9. In short, ATRA inhibited TNF-α induced synovitis by the regulation of inflammatory cytokines and inhibiting NF-κB signal transduction and potentially promoting autophagy, apoptosis and angiogenesis, displaying its role in alleviating synovial inflammation in patients with RA.

Key Words all-trans retinoic acid, rheumatoid arthritis, synovial explant, tumor necrosis factor alpha, immune inflammatory reaction

Rheumatoid arthritis (RA) is a common inflammatory autoimmune disease characterized by persistent, symmetrical polyarthritis involving the small joints of the hands, wrists and feet (1). The main pathological manifestations of RA are inflammatory cell infiltration, synovial proliferation and pannus formation, which gradually erode articular cartilage and destroy bone matrix. Improper treatment can lead to joint deformity and disability (2). Besides causing joint symptoms, RA is also associated with other non-joint diseases, such as cardiovascular diseases (3), respiratory diseases (4), malignant tumors (5). Concomitant diseases affect the prognosis of RA patients and bring high economic burden (6).

Though the pathogenesis of RA has not been fully elucidated, some researchers have hypothesized that environmental factors influence the microbiota of genetically susceptible hosts, which then lead to immune changes, resulting in the deposition of immune complex anti-citrullinated protein antibodies (ACP A) and rheumatoid factors (RF) in synovial capillaries, leading to tissue inflammation (7). Then the activation of CD4+ T cells, macrophages and synovial fibroblasts (FLS) secrete tumor necrosis factor alpha (TNF-α), interleukin-1beta (IL-1β), IL-6, vascular endothelial growth factor (VEGF), interferon-gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), and matrix metalloproteinases (MMPs), leading to joint destruction (8). Besides, inflammatory cytokines increase the expression of TNF-α, IL-1, IL-6, IL-8, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule 1 (ICAM-1) through the nuclear factor kappa-light-chain-enhancer of acti-
vated B cells (NF-κB) pathway (9). The high expression and abnormal activation of MMPs in RA patients leads to the imbalance between MMPs and its tissue inhibitor TIMPs (10), which directly degrade the extracellular matrix of cartilage and bone tissue. Meanwhile, new blood vessels in the synovial membrane can also form pannus with proliferating synovial and inflammatory cells, directly invading and eroding the cartilage and bone tissue of the inflammatory joint (11).

All-trans retinoic acid (ATRA) also known as retinoic acid, is involved in cell proliferation, differentiation, apoptosis and exhibits anti-inflammatory, anti-tumor and immunoregulatory properties (12). Researches showed that ATRA intervention can reduce inflammation in rheumatoid arthritis (13, 14), promoting the expression of anti-inflammatory Foxp3 of regulatory T cells and inhibit pro-inflammatory Th17 cells (15). ATRA can also down-regulate the production of NO and the expression of iNOS in peripheral blood mononuclear cells of patients with RA mediated by IL-6 (16), reducing proliferation, migration and invasion of FLS in rheumatoid arthritis. Our preliminary animal experiments have also demonstrated that ATRA can alleviate joint inflammation in CIA rats, inhibiting the expression of inflammatory cytokines and proteins associated with cartilage damage, and have a potentially beneficial effect on RA (17).

Several literatures have reported that ATRA plays a therapeutic role in a variety of diseases by inducing autophagy, promoting apoptosis and inhibiting cell proliferation (18). It has also been suggested that decreased synovial proliferation is due to the imbalance between cell proliferation and programmed cell death, and the regulation of apoptosis and autophagy may be a potential treatment for RA (19). However, no study has been conducted on the effect of ATRA on RA through angiogenesis, autophagy and apoptosis. Simultaneously, it is unclear whether ATRA can regulate the expression of related factors through the NF-κB pathway in RA synovitis.

Consequently, we hypothesized that ATRA could inhibit NF-κB activation by regulating the autophagy and apoptosis of synovial cells, thereby reducing synovial proliferation and inflammatory factor secretion and alleviating RA symptoms. Current studies on RA mainly focus on animal models and cell culture. But FLS culture in vitro could not maintain the initial morphology of synovial tissue for in-situ observation of various types of cells, so the response of synovial cells may be inconsistent with that of the whole tissue. In our previous study (20), a tissue culture model of synovitis was constructed by using lipopolysaccharides (LPS) in reference to the method of Hyc. Nevertheless, due to the short duration of cytokines produced by the LPS-induced model, the duration of ATRA intervention was limited. Given that TNF-α is an effective paracrine inducer of IL-1, IL-6, IL-8 and GM-CSF (21), and also stimulates FLS expression of intercellular adhesion molecule 1 (ICAM-1) and MMPs associated with joint destruction (22), which is similar to the characteristics of synovitis in patients with RA, we used TNF-α-induced synovial explant to optimize the deficiencies in the construction of previous model, and observed the effect after ATRA intervention.

MATERIALS AND METHODS

Animals and ethic statement. Twenty Wistar female rats, aged 7–8 wk and weighing 220–240 g, were obtained from Chengdu Dossy Experimental Animals Co., Ltd. [License No. SCXK (Sichuan) 2015-030, China] and placed in a specific pathogen free (SPF)-grade lab with a 12:12 h light/dark cycle, which provided food and water ad libitum. All experiments were performed in a manner to reduce the suffering of animals based on the Guidebook for the Care and Use of Laboratory and the requirements of the National Act for the Use of Experimental Animal (China). The Ethics Committee of Sichuan University West China Medical Laboratory and the requirements of the National Act for Animal Experimentation approved this study. This research was funded by the National Natural Science Foundation of China (NSFC) [grant number 811372983].

The separation and culture of synovial tissue. Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (Sinopharm Chemical Reagent Co., Ltd., China) at 0.3 mL/100 g body weight, and then were executed with a cervical dislocation. The separated hindlimb tissues were soaked in 75% alcohol (Sinopharm Chemical Reagent Co., Ltd.) for 2 min, and then quickly removed and put into a pre-cooled phosphate-buffered saline (PBS) solution supplemented with penicillin and streptomycin (HyClone, Logan, UT, USA). Temporarily stored in an ice box. Referring to the synovial membrane separation method adopted in the previous studies (20) and made some improvement, after separating synovial membrane and patellar ligament, quadriceps femoris was separated from the femoral shaft to the tibial end by sticking to the upper end of the femur. Since the synovial membrane covered the tendon surface, the quadriceps femoris could be gently lifted to completely expose the synovial membrane connected with the tendon. The complete synovial tissue was removed to a sterile environment. A total of 40 synovium were separated from rats.

Synovial tissues were rinsed for 3 times in pre-cooled sterile PBS solution supplemented with penicillin and streptomycin and then transferred to each well of 24-well culture plates (NEST Biotechnology Co., Ltd., China). One milliliter medium containing Dulbecco’s Modified Eagle Medium (DMEM)/F12 (Chengdu Hali Biotech. Co., Ltd., China), 10% neonate bovine serum (Chengdu Hali Biotech. Co., Ltd.), penicillin (100 U/mL) and streptomycin (100 μg/mL) were added in each well, which is incubated adaptively in a 5% CO2 incubator with 100% relative humidity atmosphere (Thermo Scientific, USA) at 37°C for 24 h, and then the medium was discarded.

Establishment of synovitis explants. The difference in TNF-α-experiment was whether to add 20 ng/mL TNF-α (Peprotech) or not, including 5 culture time points (24 h, 48 h, 72 h, 96 h, 120 h). Each 4 membranes were
randomly divided into the following ten groups: Control group 1 (incubated in DMEM/F12 medium for 24 h, C1), Control group 2 (incubated in DMEM/F12 medium for 48 h, C2), Control group 3 (incubated in DMEM/F12 medium for 72 h, C3), Control group 4 (incubated in DMEM/F12 medium for 96 h, C4), Control group 5 (incubated in DMEM/F12 medium for 120 h, C5), and TNF-α-challenged group 1 (exposed to 20 ng/mL TNF-α for 24 h, TNF-α-1), TNF-α-challenged group 2 (exposed to 20 ng/mL TNF-α for 48 h, TNF-α-2), TNF-α-challenged group 3 (exposed to 20 ng/mL TNF-α for 72 h, TNF-α-3), TNF-α-challenged group 4 (exposed to 20 ng/mL TNF-α for 96 h, TNF-α-4), TNF-α-challenged group 5 (exposed to 20 ng/mL TNF-α for 120 h, TNF-α-5). To make sure the sufficient nutrients supply, the medium of each group was added DMEM/F12, 1% FBS, BCA, Model control group (incubated with DMEM/F12 with 0), 2‰ dimethyl sulphoxide (DMSO), BCA, the remaining groups were added with culture medium containing 1% FBS, BC, and all the other groups were stimulated with 20 ng/mL TNF-α for 24 h, and then the medium was changed. After 48 h of incubation, three ATRA intervention groups were added with culture medium containing ATRA, and the remaining groups were added with corresponding medium. The six groups are as follows: Blank control group (incubated with DMEM/F12 with 1% FBS, BC), Model control group (exposed to 20 ng/mL TNF-α, TC), Solvent control group [exposed to 20 ng/mL TNF-α and 2‰ dimethyl sulphoxide (DMSO), SC], ATRA-challenged group 1 (exposed to 1 µM ATRA, ATRA-1), ATRA-challenged group 2 (exposed to 10 µM ATRA, ATRA-2), ATRA-challenged group 3 (exposed to 100 µM ATRA, ATRA-3): The medium was changed every 24 h, and the incubation was stopped at 120 h and then the synovial explants were dehydrated and collected. Media was collected and stored at −20°C.

HE staining and pathological alteration. Pathological alteration in a synovial explant was evaluated by HE staining in accordance with a previous study. Briefly, the fixed tissues were dehydrated with ethanol, soaked in wax, embedded in paraffin and trimmed to 3–4 µm. After being drained, they were placed in a 60°C incubator for 1 h, and then dyed with haematoxylin (Wuhan Servicebio Biotechnology, China) for 5 min and eosin (Wuhan Servicebio Biotechnology) for 3 min. The images are then viewed under an optical microscope (Nikon Eclipse CI). According to Krenn’s pathological scoring criteria for synovitis (23), synovial cell proliferation, inflammatory cell infiltration and vascular proliferation were graded. The total score was recorded as synovitis score, 0–1 for no synovitis (grade 0), 2–3 for mild synovitis (grade 1), 4–6 for moderate synovitis (grade 2), and 7–9 for high synovitis (grade 3). HE staining was performed in the TNF-α-experiment.

The concentration of nitric oxide (NO) measured by nitrate reductase. NO concentration was evaluated using Nitric Oxide Assay Kit (Nanjing KeyGen Biotech Co., Ltd., China) according to the manufacturer’s protocols. The optical density (OD) of each sample was measured by a microplate reader (BIO-RAD, China). The concentration of NO is calculated by the manufacturer’s manual. The chemical method was used in both the experiments.

Cytokines, VEGF and MMP-9 by enzyme-linked immunosorbent assay (ELISA). IL-6, IL-1β, IFN-γ, IL-17A, VEGF and MMP-9 in the culture medium were measured using ELISA (Beijing 4A Biotech Co., Ltd., China) according to the manufacturer’s protocol. The optical density (OD) of each sample was measured by a microplate reader (BIO-RAD) and expressed as picograms per milliliter. ELISA was used in both the experiments.

Expression of NF-κB P65, IκBα using immunohistochemistry. Effects of ATRA on P65 and IκBα expression in NF-κB pathway were detected using PV two-step immunohistochemical kit (Wuhan Servicebio Biological Technology Co., Ltd., China) according to the operating instruction. The mean optical density (MOD) was calculated using Olympus CellSence image analysis software. Such immunohistochemical analysis was used in the ATRA-experiment only.

Expression of Beclin-1, LC3, p62 and Bcl-2 using immunofluorescence technique. Effects of ATRA on Beclin-1, LC3, p62 and Bcl-2 expression were detected using immunofluorescence kit (Wuhan Servicebio Biological Technology Co., Ltd.) according to the manufacturer’s protocol. The Caseviewer 3.2.1 was used to browse the digital slices and take photos. The mean optical density (MOD) was calculated by Image J 1.8.0. Such immunofluorescence analysis was used in the ATRA-experiment alone.
Effects of ATRA on Synovitis in RA

Statistical analyses. IBM SPSS Statistics 22 software was used for the statistical analysis, and values were expressed as mean±standard deviation (SD). One-way analysis of variance (ANOVA) and Least-Significant Difference (LSD) test were used to compare the mean between different groups. Histological score was determined by non-parametric Kruskal-Wallis analysis of variance and repeated measurement data were compared by repeated measure ANOVA. Statistical significance was set at α=0.05 and was acknowledged with a probability of <5%.

RESULTS

Effect of TNF-α on synovial explant weight

We first compared the weight of rat synovial explants in the control groups and the TNF-α-challenged groups at different time points. Referring to the model design method of Hyč et al. (24), the previous study of our laboratory (20) detailed the synovial membrane extraction process, but the method of opening the knee joint from popliteal fossa is more likely to damage the synovial membrane and cause certain mechanical stimulation. Therefore, in this study, we improved the synovial membrane extraction method again, and separated the quadriceps femoris from the upper femur to the tibial end by sticking closely to the femoral shaft, thus completely exposing the synovial membrane connected with the tendon. Meanwhile, because LPS may induce apoptosis and the duration of inflammatory factors is short (25), 20 ng/mL TNF-α was used to stimulate synovial membrane, and adaptive culture was performed in medium containing high concentration of serum in this study.

Forty dissected rat synovial explants were weighed after incubation. The mean weight of all synovial explants was 17.08 (16.27, 17.88) mg. There was no significant distinction in synovial mass among groups (Fig. 1), indicating no significant effect in the establishment of synovial explants on our present study.

Effect of TNF-α on the pathological alteration of synovium

In the TNF-α-experiment, HE staining was used to evaluate the pathological changes of synovial explants. The synovial membrane can be divided into two layers under the light microscope, and the synovial cells are of various forms, mainly composed of macrophages and fibroblasts, which are difficult to distinguish. Synovial cells in the surface layer are directly connected with loose fibrous connective tissue or adipose tissue in the synovial sublining layer without basement membrane. In the synovial sublining layer, there are a large number of fat cells of different sizes, which are vacuolated. The cytoplasm is lightly stained, and the cytoplasm and nucleus are on the periphery of the cell, surrounding the central lipid droplet. Cord-like collagen fibers and dispersed, ring-shaped vascular endothelial cells were

Fig. 2. Pathological alteration of synovial explants in TNF-α-experiment by HE staining (100×). A: 24 h control group; B: 24 h TNF-α challenged group; C: 48 h control group; D: 48 h TNF-α challenged group; E: 72 h control group; F: 72 h TNF-α challenged group; G: 96 h control group; H: 96 h TNF-α challenged group; I: 120 h control group; J: 120 h TNF-α challenged group.

Fig. 3. Synovitis pathology scores in TNF-α-experiment. Data were presented as mean±SD; n=4 in each group. The statistical analysis was according to non-parametric Kruskal-Wallis analysis of variance at p<0.05. *p<0.05 vs. control.
Synovitis occurred to some extent in all groups, including inflammatory cell infiltration, synovial cell proliferation and synovial matrix activation. Inflammatory cells were mainly lymphocytes and plasma cells, but Kruskal-Wallis analysis of variance showed no statistically significant difference in pathological scores between the TNF-α-challenged groups and the control groups (Fig. 3). These observations suggested that TNF-α at a concentration of 20 ng/mL had no detrimental effect on synovial explants.

**Effect of TNF-α and ATRA on the NO expression**

In the TNF-α-experiment, through repeated measure variance analysis of two factors, there was no interaction between culture mode and time, indicating that the effect of time factor is not different from that of TNF-α stimulus. In addition, the concentration of NO in the TNF-α stimulus groups were significantly higher than that in the control groups (p<0.001) (Fig. 4A), and the effect of time factor on the expression of NO was statistically significant (p<0.05), suggesting that there were significant differences in the concentration of NO at different culture times.

In the ATRA-experiment, the production of NO significantly increased in TC and SC in comparison with the BC (p<0.01). Analogously, no differences were observed between TC and SC. In contrast, intervention in the low, medium and high dose of ATRA induced a considerable reduction in NO expression compared with TC and SC (p<0.05), but there was no significant discrepancy among the three ATRA dose groups (Fig. 4B).

**Effect of TNF-α and ATRA on cytokines production**

In the TNF-α-experiment, through repeated measure variance analysis of two factors, there was an interaction between the time factor of IL-6 concentration and the culture method, while there was no such interaction in IL-1β, IFN-γ and IL-17A production. The concentrations of IL-6, IL-1β and IL-17A showed statistically significant elevating and decreasing trends with the duration of culture (p<0.05). Besides, after TNF-α treatment, a significant increase of IL-6 and IFN-γ levels were noticed in comparison with the controls (p<0.05) (Fig. 5). These observations suggested that TNF-α intervention may cause imbalance in the cytokine network.

In the ATRA-experiment, IL-6 concentrations of TC and SC were significantly higher than BC (p<0.001) after 20 ng/mL TNF-α stimulation for 120 h, indicating the occurrence of inflammatory response. For IL-6, we observed that the expression level of the three ATRA dose groups was significantly lower than that of TC and SC (p<0.001), showing a dose-dependent relation-
ship, and the IL-6 concentration of ATRA-3 was lower than that of ATRA-1 ($p<0.01$). Similarly, all ATRA concentration did produce a significant decrease in IL-1β production in comparison with TC ($p<0.01$). Concerning IFN-γ, despite no noticeable reduction both in ATRA-1 and ATRA-2 compared to TC, we noticed that 100 ATRA treatment reduced IFN-γ expression opposed to TC. Conversely, no significant decrease in IFN-γ production when treated with increasing concentrations of ATRA (Fig. 6).
Effect of TNF-α and ATRA on factors expression associated with angiogenesis

In the TNF-α-experiment, we noticed that there was an interaction between the time factor of both the concentration of VEGF and MMP-9 and the culture method, and the expression of VEGF and MMP-9 had a significant increase in TNF-α group compared with control group at each time point ($p<0.05$). Moreover, with the prolongation of time, the increase of VEGF in the TNF-α group was greater than that in the control group ($p<0.05$) (Fig. 7A). In addition, MMP-9 levels remained low in the control group, while MMP-9 levels rose sharply in the TNF-α control group (Fig. 7B). These observations suggested that TNF-α stimulus show a possible increase in angiogenesis.

In the ATRA-experiment, the expressions of VEGF and MMP-9 were manifested to be significantly higher in TC than BC ($p<0.01$), and no difference between TC and SC was observed. For VEGF, there was no significant increase in ATRA-1 as compared with TC, consistent with no increase in ATRA-3 as compared with TC. However, such difference was observed between ATRA-2 and TC ($p<0.05$). Conversely, treatment in ATRA-1 significantly suppressed MMP-9 expression in comparison with TC ($p<0.05$), but no difference was observed in ATRA-2 and ATRA-3 (Fig. 7C).

Fig. 10. Immunofluorescence staining of Beclin-1, LC3, p62 and Bcl-2 in ATRA-experiment. Images represent random selections (400× magnification) of stained sections.

Effect of ATRA on NF-κB pathway

In the ATRA-experiment, the production of P65 were reported to be significantly increased in TC and SC as compared with those in BC ($p<0.01$), and no discrepancy between TC and SC was noticed. After ATRA treatment, we observed that P65 expression in ATRA-1, ATRA-2 and ATRA-3 were lower than that in both TC and SC ($p<0.05$), but no difference among three ATRA doses (Fig. 7D). Regrading IκBα, only TNF-α stimulus reduced IκBα expression as opposed to BC, but there was no statistical difference among groups.

The immunohistochemical analysis showed that synovial cells of synovial lining were stained brown, indicating a positive reaction in the synovial lining. Moreover, the scattered synovial cells in the synovial sublining layer and around the blood vessels also showed brown-yellow positive expression (Fig. 8).

Effect of ATRA on autophagy associated marker protein

In the ATRA-experiment, the expression of LC3 were significantly decreased in TC and SC in comparison with BC ($p<0.05$), while Bcl-2 inversely increased in TC and SC as compared to BC ($p<0.01$) and no such statistic result in LC3 and Bcl-2 between TC and SC was observed. After ATRA treatment, we noticed that LC3 expression in ATRA-1 was added than both TC and SC ($p<0.05$), but Bcl-2 expression in ATRA-2 was obviously less than
both TC and SC (p<0.01). However, for Beclin-1 and p62, there was no statistical difference between each group (Fig. 9).

The immunofluorescence staining showed Beclin-1 was mainly located in vascular and cytoplasm and nucleus of synovial cells, while LC3 in the cytoplasm of fat cells and also had weak expression in the nuclei and blood vessels of fat cells. Moreover, Bcl-2 is mainly expressed in blood vessels, synovial cells and fat cells and p62 presented a very light red fluorescent signal in the blood vessels (Fig. 10).

DISCUSSION

In the TNF-α-experiment, we improved both the model extraction method and the culture method to conquer the problem that the duration of inflammatory factors was short, which limited the response time of subsequent ATRA intervention studies. The results of HE staining indicated synovitis in all groups, but there was no statistical difference in pathological score of synovitis, indicating that TNF-α stimulation had no detrimental effect on synovitis explants. Moreover, neonatal bovine serum was added to the culture medium to avoid the cytokine reduction and tissue degradation in the Hyc study (24). Pathological features of synovitis to a certain extent were observed at different time points, while no obvious central necrosis was observed, suggesting that sufficient nutrients could meet the needs of in vitro culture of synovial membrane and prolong the synovial time. With the prolongation of incubation, synovitis also appeared in the control group, which may be related to the stimulation of synovitis caused by 1% serum containing endotoxin added to the medium. For further study, we also appraised TNF-α effects on synovial explants and noticed variation in inflammatory related factors by corresponding assay kit.

IL-6 is one of the pro-inflammatory cytokines most associated with the pathogenesis of RA, and is abundant in synovial fluid and serum of patients with RA (26). ELASA results showed that TNF-α can induce synovial membrane to produce a large amount of IL-6, reaching a high point after 24 h of incubation and then gradually decreasing, which was similar to the results of previous studies (20). It is possible that mechanical stimulation in the anatomical process causes IL-6 to surge and tends to stabilize over time. However, IL-1β, IFN-γ and IL-17A levels were all at the low limit detected by the standard curve, and may not accurately reflect their dynamic changes.

IL-6-mediated inflammatory processes can increase VEGF expression (26), and hypoxia and stimulation of pro-inflammatory cytokines such as TNF-α, IL-1 and TGF-β can also induce the release of VEGF in the synovial membrane of RA (27, 28). Studies have proved that VEGF expression level is correlated with RA angiogenesis, severity of joint inflammation and occurrence of complications (29, 30). Meanwhile, MMPs is a key enzyme leading to articular cartilage degradation and bone destruction, and Stojanovic et al. also found that the presence of TNF-α G-308A allele was associated with increased MMP-9 activity in synovial fluid in early time of RA patients (31). ELASA results indicated that TNF-α stimulus can produce a great many VEGF and MMP-9 in every time point. However, the increase of VEGF produced by TNF-α stimulation is larger, suggesting that synovial tissue incubated in vitro may have potential angiogenesis with the extension of culture time, and TNF-α stimulation may cause early morphological changes of blood vessels.

Based on the above results, after 24 h of adaptive incubation, the secretion of IL-6, VEGF and MMP-9 increased significantly in the synovitis tissues of the knee joint of rats treated with TNF-α, showing inflammatory changes similar to those of RA synovitis. It can be considered that the establishment of the model of synovitis tissues in vitro was successful, and the duration of inflammatory response was prolonged, which provided sufficient duration for the follow-up intervention. Therefore, this model will be used to investigate the effect and mechanism of ATRA intervention on synovitis.

ATRA concentration ranging from 1 μM to 100 μM was employed in our study, which was higher than the usual dose used in previous studies (32, 33). Considering when the synovial membrane is studied as a whole, the cell components are more complex as compared to cell culture in vitro. Moreover, due to the large number of cells and the fat-soluble ATRA, it is possible that some of the ATRA may be stored in fat cells, resulting in a dose that is lower than the intended intervention dose. For that, we raised the dose of the intervention in the following experiments, added ATRA at 48 h after TNF-α stimulation, and collected synovium and medium at 120 h to detect relevant indicators.

Recent evidences suggested that ATRA intervention can inhibit the release of inflammatory cytokines in RA (34, 35), thereby improving the clinical symptoms and tissue structure of RA (36). In our study, we found that the inhibitory effect of ATRA on IL-6 was related to the dose of ATRA, with 100 μM of ATRA showing a stronger inhibitory effect than 1 μM of ATRA, similar to Kirchmeyer et al. (33), which showed that ATRA could reduce the expression of IL-6 in synovial fibroblasts induced by IL-1β in healthy rats. Likewise, dysregulation of IL-6 expression is considered to be an important part of the pathogenesis of RA (37).

Both VEGF and MMPs are promoters of angiogenesis, and they work together to increase synovial inflammation in RA, leading to pannus so as to erodes the cartilage and bone matrix. Our study suggested that the concentration of VEGF in synovial medium do not decrease after ATRA treatment, and even 10 μM ATRA significantly increased the level of VEGF while 1 μM ATRA enhanced the expression of MMP-9, differing from previous studies. Our earlier studies demonstrated that after ATRA interfered with HFb-RA, VEGF expression was up-regulated, and ATRA significantly increased the level of VEGF in CIA rats, which was consistent with the results of this study. However, other studies have confirmed the antiangiogenic effect of ATRA (38, 39),
reminding us that angiogenesis is a complex phenomenon and the role of ATRA in regulating angiogenesis is controversial. Hence, the role of ATRA in promoting/inhibiting angiogenesis in RA synovitis and whether ATRA can regulate the expression of MMP-9 in RA disease remains to be further investigated.

Some reports on RA patients confirmed increased endogenous NO synthesis (40), suggesting that overproduction of NO may have important significance in the pathogenesis of RA. In the TNF-α experiment, we observed that TNF-α stimulation could induce the expression of NO. But after adding ATRA, the production of NO was inhibited without dose dependence, similar to the study conducted by Algerian (16).

The NF-κB pathway plays an important role in RA (41), and TNF-α and LPS further induce NF-κB activation (42). Other researches have demonstrated that inhibition of NF-κB activation restrict the production of cytokines, chemokines, and MMPs, significantly improving chronic inflammation (43). Our findings also confirmed the cellular localization of NF-κB P65 and IκBa. TNF-α stimulation increased the expression of P65 in the synovial membrane, while ATRA down-regulated the production of P65, consistent with previous studies (44), but ATRA did not show a regulatory effect on IκBa, which is a super inhibitor of NF-κB.

Recent studies have reported the pathogenesis of RA may be related to abnormal apoptosis and autophagy (45), and the resistance of FLS to TNF-α induction of apoptosis has been considered as one of the important factors in RA (46). Immunofluorescence results showed that TNF-α induced high expression of anti-apoptotic modulating factor Bcl-2 in synovial membrane and down-regulated autophagy related protein LC3, indicating that TNF-α could induce synovial apoptotic resistance and possibly inhibit autophagy. However, TNF-α induced synovial autophagy was lower than that reported by Xu et al. (47). In our ATRA experiment, the expression of LC3 in the synovial membrane induced by TNF-α was up-regulated by 1 μM ATRA and the Bcl-2 was down-regulated by 10 μM ATRA, suggesting that ATRA may promote the level of autophagy in the synovial membrane and promote apoptosis, which is similar to the autophagy and apoptosis induced by ATRA in other tissues (18). However, different doses of ATRA did not show consistent regulation of autophagy, and Beclin-1 and p62 did not change significantly after ATRA intervention, indicating that signal transduction pathways regulating autophagy were complicated. Both the level of autophagy in the synovial membrane induced by TNF-α, which may be related to the different stages of RA and the two-way regulation of autophagy (48), and the specific mechanism by which ATRA participates in the treatment of RA by regulating autophagy and apoptosis remain to be verified in cellular and animal studies.

In conclusion, the effect of ATRA on inflammatory cytokines, angiogenic factors, NF-κB pathway and apoptosis and autophagy related proteases of RA investigated based on the successfully established TNF-α stimulated synovial explants. To simplify our results, ATRA inhibited the secretion of IL-6 and NO level, downregulated the NF-κB P65 and antiapoptotic proteins expression of Bcl-2, increased levels of autophagy marker protein LC3, but different doses of ATRA showed different regulatory effects on the expression of VEGF and MMP-9 in synovial medium, suggesting that ATRA may alleviate RA synovitis by inhibiting NF-κB signal transduction and potentially promoting autophagy, apoptosis and angiogenesis. Further studies are conducted to clarify the specific effect of ARTA on RA.

Authorship
Research conception and design: Cai Q and Li Y; experiments: Cai Q, Li Y, and Lyu F; statistical analysis of the data: Zhou M, Lu K, and Tang X; interpretation of the data: Ren D, Bao Q, and Wang D; writing of the manuscript: Cai Q, and Li Y.

Disclosure of state of COI
No conflicts of interest to be declared.

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