IRE1α Promotes Cell Apoptosis and an Inflammatory Response in Endoplasmic Reticulum Stress-Induced Rheumatoid Arthritis Fibroblast-Like Synovial Cells by Enhancing Autophagy

(IRE1α Menggalakkan Apoptosis Sel dan Tindak Balas Keradangan dalam Retikulum Endoplasma yang Disebabkan oleh Tekanan Reumatoid Artritis Fibroblas Seperti Sel Sinovial dengan Meningkatkan Autofagi)

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ABSTRACT

Endoplasmic reticulum (ER) stress can induce autophagy via the unfolded protein response (UPR), and autophagy can regulate the activation of inflammasomes. Inositol-requiring enzyme 1α (IRE1α) is a transducer of the UPR in cells with ER stress. Here, we investigated the role of IRE1α and its impact on ER stress in rheumatoid arthritis fibroblast-like synovial cells (RA-FLSs). RA-FLSs were isolated from rheumatoid arthritis (RA) patients and stimulated with thapsigargin (TG) to produce ER stress cells. ER stress-, autophagy and the expression of apoptosis-associated factors were investigated by western blotting and the qRT-PCR. Cellular ROS levels were assessed by flow cytometry. ELISAs were performed to determine the concentrations of inflammatory mediators. TG treatment promoted IRE1α, GRP78, CHOP, and ATP6 mRNA and protein expression. ROS generation was increased in TG-induced RA-FLSs; additionally, TG was found to induce cell inflammation by upregulating the expression of inflammasome markers and the concentrations of inflammatory mediators. The levels of autophagy markers, apoptosis-associated proteins, and mRNA were increased in TG-stimulated RA-FLSs. However, transfection with si-IRE1α suppressed TG-induced increases in ROS generation, inflammation levels, cell apoptosis, and autophagy in RA-FLSs. Treatment with the autophagy activator RAPA attenuated the protective effects of IRE1α silencing on TG-induced RA-FLS apoptosis and inflammatory damage. Our findings showed that in RA-FLSs, IRE1α silencing alleviated ER stress-induced inflammation and apoptosis caused by autophagy.

Keywords: Autophagy; endoplasmic reticulum stress; inositol-requiring enzyme 1α; rheumatoid arthritis fibroblast-like synovial cells; thapsigargin

ABSTRAK

Tekanan retikulum endoplasma (ER) boleh mendorong autofagi melalui tindak balas protein terungkap (UPR) dan autofagi boleh mengawal pengaktifan inflamasom. Enzim 1α yang memerlukan inositol (IRE1α) ialah transduser UPR dalam sel dengan tekanan ER. Di sini, kami mengkaji peranan IRE1α dan kesannya terhadap tekanan ER dalam sel sinovial seperti fibroblas artritis reumatoid (RA-FLSs). RA-FLS telah diasingkan daripada pesakit reumatoid artritis (RA) dan dirangsang dengan thapsigargin (TG) untuk menghasilkan sel tekanan ER. Tekanan ER, autofagi dan ekspresi faktor berkaitan apoptosis telah dikaji oleh pemedapan Western dan qRT-PCR. Tahap ROS sel dinilai oleh sitometri aliran. ELISA dilakukan untuk menentukan kepekatan mediator keradangan. Rawatan TG menggalakkan ekspresi mRNA dan protein IRE1α, GRP78, CHOP dan ATP6. Penjanaan ROS telah meningkat dalam RA-FLS yang disebabkan oleh TG; tambahan TG didapati mendorong keradangan sel dengan mengawal selia ekspresi penanda inflamasom dan kepekatan mediator keradangan. Tahap penanda autofagi, protein berkaitan apoptosis dan mRNA telah meningkat dalam RA-FLS yang dirangsang TG. Walau bagaimanapun, pemindahan dengan si-IRE1α menindas peningkatan yang disebabkan oleh TG dalam penjanaan ROS, tahap keradangan, apoptosis sel dan autofagi dalam RA-FLSs. Rawatan dengan pengaktif autofagi RAPA melemahkan kesan perlindungan pembungkaman IRE1α pada apoptosis RA-FLS yang disebabkan oleh TG dan kerosakan keradangan. Penemuan kami menunjukkan bahawa dalam RA-FLSs, pembungkaman IRE1α mengurangkan keradangan dan apoptosis yang disebabkan oleh tekanan ER yang disebabkan oleh autofagi.

Kata kunci: Autofagi; enzim 1α yang memerlukan inositol; reumatoid artritis fibroblas seperti sel sinovial; tekanan retikulum endoplasma; thapsigargin

INTRODUCTION

Rheumatoid arthritis (RA) is a long-term systemic inflammatory autoimmune disease which is characterized by joint inflammation and swelling (Sparks 2019). The clinical symptoms of RA (musculoskeletal pain, swelling, and stiffness) can seriously affect an individual's physical function and quality of life (Pirmardvand et al. 2018). Furthermore, when compared with the general population, RA patients are at higher risks for developing respiratory diseases, osteoporosis, cardiovascular diseases, and cancer (Cock & Hyrich 2018). Drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, conventional synthetic disease-modifying antirheumatic drugs (DMARDs), biological DMARDs, and targeted synthetic DMARDs are the most common treatments for RA. However, approximately 20%-30% of RA patients remain refractory to current therapies; furthermore, those therapeutic agents can sometimes produce serious gastrointestinal and cardiovascular side effects (Tang 2020). Therefore, it remains crucial to study the pathogenesis of RA and search for new therapies.

ER stress occurs when the capacity of the endoplasmic reticulum (ER) to properly fold newly synthesized proteins becomes saturated (Kabala et al. 2017; Rahmati et al. 2018). Evidence shows that hypoxia, inflammation, and amino acid deficiency may trigger the accumulation of unfolded/misfolded proteins in the ER, which is a phenomenon collectively known as the unfolded protein response (UPR) (Qi et al. 2017). The main purpose of the UPR is to maintain cell homeostasis and survival by reducing the rate of mRNA translation while increasing the production of certain proteins, such as ER chaperones. However, under conditions of long-term and high ER stress, the UPR-mediated adaptive response is not sufficient to restore normal cellular functions, and instead activates the apoptosis signalling pathway, eventually leading to programmed cell death (Hetz 2012; Kong et al. 2018). There are three ER transmembrane protein sensors: inositol-requiring enzyme 1 alpha (IRE1α), PKR-like ER kinase (PERK), and activated transcription factor 6 alpha (ATF6α). These sensors are responsible for activating UPR signaling (Moore & Hollien 2012). Under normal conditions, IRE1α, PERK, and ATF6 are maintained in an inactive form by the action glucose-regulated protein 78 kDa (GRP78/Bip). However, when cells experience ER stress, GRP78 can dissociate from the UPR proteins to induce UPR signaling (Han & Kaufman 2017; Rahmati et al. 2018). For example, IRE1α homodimerizes and triggers the unconventional splicing of X-box binding protein 1 (XBP1) messenger RNA, which leads to a frameshift mutation in XBP1. This makes it a critical transcription factor for restoring cell homeostasis (Kabala et al. 2017; Song et al. 2020). ER stress is closely related to the immune process via crosstalk that occurs between UPR and inflammatory signaling pathways (Navid & Colbert 2017). While ER stress has been considered to be a common cellular response in RA (Wang et al. 2018), our understanding of the correlation between ER stress and RA pathology remains largely incomplete. It has been proven that rheumatoid arthritis fibroblast-like synovial cells (RA-FLSs) play an important role in RA pathogenesis (Zhou et al. 2020). However, it is noteworthy that unlike other cell types, RA-FLSs show a resistance to ER stress-caused apoptosis, and the underlying mechanism for that resistance remains unknown. The most likely cause is due to increased autophagy and the unique cellular phenotype induced by autophagy (Shin et al. 2010; Yamasaki et al. 2006).

Autophagy has been shown to protect cells from ER stress-triggered cell death. Interestingly, ER stress is also an effective cause of autophagy (Song et al. 2017). Autophagy, as an evolutionarily conserved cellular mechanism for maintaining cell homeostasis, is closely related to ER-induced apoptosis via common upstream signaling pathways (Song et al. 2018). A recent study showed that IRE1α was activated under conditions of ER stress and contributed to cell survival by increasing autophagy during the initial phase of hepatic steatosis (Wu et al. 2020), which suggested the existence of crosstalk between the key UPR component IRE1α and autophagy. Furthermore, autophagy plays a key role in activating and regulating inflammasomes, and participates in reducing the symptoms of inflammasome-related diseases (Liu 2019). It has also been suggested that IRE1α participates in the assembly of inflammasomes (Bronner et al. 2015). In the present study, an *in vitro* model of ER stress was established to investigate the underlying mechanisms by which IRE1α and autophagy regulate the occurrence and development of ER stress-induced inflammation in RA-FLSs.

MATERIALS AND METHODS

PATIENTS

Samples of synovial tissue were collected from 6 RA patients who underwent knee replacement surgery at Linyi People's Hospital. The patients included 2 males and 4 females; age range, 43 to 67 years (mean = 57.8 ± 9.1 years). The course of disease ranged from 6 to 30 years (mean = 14.5 ± 9.2 years). Erythrocyte sedimentation rate (ESR) ranged from 30 to 63 mm/h (mean = 40.8 ± 12.1

mm/h), and C-reactive protein (CRP) ranged from 24.3 to 62.7 mg/L (mean = 40.6 ± 14.8 mg/L). All the patients fulfilled the 2010 American College of Rheumatology/ European League Against Rheumatism Classification Criteria for Rheumatoid Arthritis (Aletaha et al. 2010). Subjects with other autoimmune diseases, infections, tumors, or other severe systemic diseases were excluded from enrollment. The enrolled RA patients suffered from severe knee joint deformities, were undergoing surgical knee replacement, and agreed to have a sample synovial membrane tissue collected during surgery. Each patient provided their written informed consent for study participation prior to enrollment. The study protocol was approved by the Ethics Committee of Linyi People's Hospital (Ethics approval number: YX200223).

ISOLATION OF RA-FLSS AND CELL CULTURE

Samples of RA synovial tissue were cut into 1 × 1 mm pieces and added to 3 mL of 0.2% type collagenase. The samples were then incubated for 1.5 h at 37 ℃ with shaking every 30 min to aid with digestion. Next, the digested tissue mixtures were centrifuged at 1000 rpm for 5 min, and the supernatants were discarded. The pelleted RA-SFSs were then cultured in Dulbecco's Modified Eagle's Medium (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum and 1% penicillin/ streptomycin mixture at 37 °C in a 5% CO_2 atmosphere with saturated humidity. The medium was replaced every 3 days. When the cells reached 80% confluence, passaging was performed using 0.25% trypsin. After completing 3 passages, the RA-FLSs were harvested for use in experiments.

CELL TREATMENT

When the cultured RA-FLSs reached 80% confluence, they were stimulated with 5 µM Thapsigargin (TG, Sigma-Aldrich, St. Louis, MO, USA) for 60 h to induce ER stress. For induction or inhibition of cellular autophagy, the cells were pretreated with either rapamycin (RAPA, Sigma-Aldrich) or 3-Methyladenine (3-MA, Sigma-Aldrich) for 1 h; after which, they were washed with fresh medium and then treated with TG.

QUANTITATIVE REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (qRT-PCR)

TRIzol reagent (Invitrogen, Waltham, MA, USA) was used to extract the total RNA from RA-FLSs, and a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine RNA concentrations. cDNA synthesis was performed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The qRT-PCR was performed by using a SYBR Green Supermix kit (Bio-Rad) on a real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). The primers used for RT-PCR were synthesized by Sangon Biotech (Shanghai, China), and the primer sequences are shown in Table 1. The threshold cycle values of target genes were normalized to GAPDH expression, and gene expression calculated using the $2^{-\Delta\Delta Ct}$ method.

TABLE 1. The primer sequences for qRT-PCR assay

Gene	Primer sequences
GAPDH	Forward: 5'-TGTTCGTCATGGGTGTGAAC-3'
	Reverse: 5'-ATGGCATGGACTGTGGTCAT-3'
GRP78	Forward: 5'-CATCACGCCGTCCTATGTCG-3'
	Reverse: 5'-CGTCAAAGACCGTGTTCTCG-3'
CHOP	Forward: 5'-GGAAACAGAGTGGTCATTCCC-3'
	Reverse: 5'-CTGCTTGAGCCGTTCATTCTC-3'
ATF6	Forward: 5'-AGCAGCACCCAAGACTCAAAC-3'
	Reverse: 5'-GCATAAGCGTTGGTACTGTCTGA-3'
IRE1α	Forward: 5'-CACAGTGACGCTTCCTGAAAC-3'
	Reverse: 5'-GCCATCATTAGGATCTGGGAGA-3'
BCL-2	Forward: 5'-TGTGTGTGGAGAGCGTCAA-3'
	Reverse: 5'-AGAGACAGCCAGGAGAAATCAA-3'
BAX	Forward: 5'-GCCGAGTCACTGAAGCGA-3'
	Reverse: 5'-CCACAAAGATGGTCACGGTC-3'
Caspase-3	Forward: 5'-CAGAACTGGACTGTGGCATTG-3'
	Reverse: 5'-GCTTGTCGGCATACTGTTTCA-3'

SMALL INTERFERING RNA (siRNA) TRANSFECTION

siRNA for IRE1α (si-IRE1α) and a negative scrambled siRNA (si-NC) were synthesized by GenePharma (Shanghai, China). Si-IRE1α or si-NC (100 nM) was transfected into RA-FLSs by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. After 48 h of transfection, the cells were harvested for use in subsequent experiments.

REACTIVE OXYGEN SPECIES (ROS) MEASUREMENT

An ROS detection kit (Beyotime) was used to measure ROS levels in the RA-FLSs. RA-FLSs were incubated in 6-well culture plates containing 10 mM 2',7'-dichlorodihydrofluorescein diacetate for 20 min at 37 °C in the dark. The cells were then washed with PBS and analyzed by flow cytometry.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The concentrations of IL-6, IL-1β, and IL-18 in the supernatants of cultured RA-FLSs were detected by using the corresponding ELISA kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocol.

WESTERN BLOTTING

The total proteins in RA-FLSs were isolated by using RIPA buffer (Beyotime, Shanghai, China) that contained protease and phosphatase inhibitors (Beyotime). The isolated proteins were then separated by gel electrophoresis. Specific antibodies against GRP78 (Cat. No: sc-13539), Caspase-1 (Cat. No: sc-398715), LC3B

(Cat. No: sc-398822), and IRE1α (Cat. No: sc-390960) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA. Antibodies against ATF6 (Cat. No: ab122897), phosphorylated IRE1α (p-IRE1α, Cat. No: ab48187), TXNIP (Cat. No: ab188865), NLRP3 (Cat. No: ab263899), Beclin-1 (Cat. No: ab210498), p62 (Cat. No: ab56416), Bax (Cat. No: ab32503), Bcl-2 (Cat. No: ab32124), and Cleaved Caspase-3 (Cat. No: ab32042) were purchased from Abcam (Cambridge, MA, USA). Antibodies against CHOP (Cat. No: AF6684) were purchased from Beyotime (Shanghai, China). The aforementioned antibodies were used for immunostaining of proteins. GAPDH (Cat. No: sc-47724, Santa Cruz Biotechnology) expression served as an internal control.

STATISTICAL ANALYSIS

Statistical results are expressed as the mean value ± SD of data obtained from at least three independent experiments. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., CA, USA) and comparisons between groups were performed using the student's t-test or ANOVA. A P-value < 0.05 was considered to be statistically significant.

RESULTS

TG PROMOTED INFLAMMATION IN RA-FLSS

To induce ER-stress, RA-FLSs were stimulated with 5 μM TG for 60 h. After treatment, the levels of GRP78, CHOP, ATF6, and IRE1α expression were up-regulated, as shown by results of western blot (Figure 1(A)) and qRT-PCR studies (P < 0.001, Figure 1(B)). Moreover,
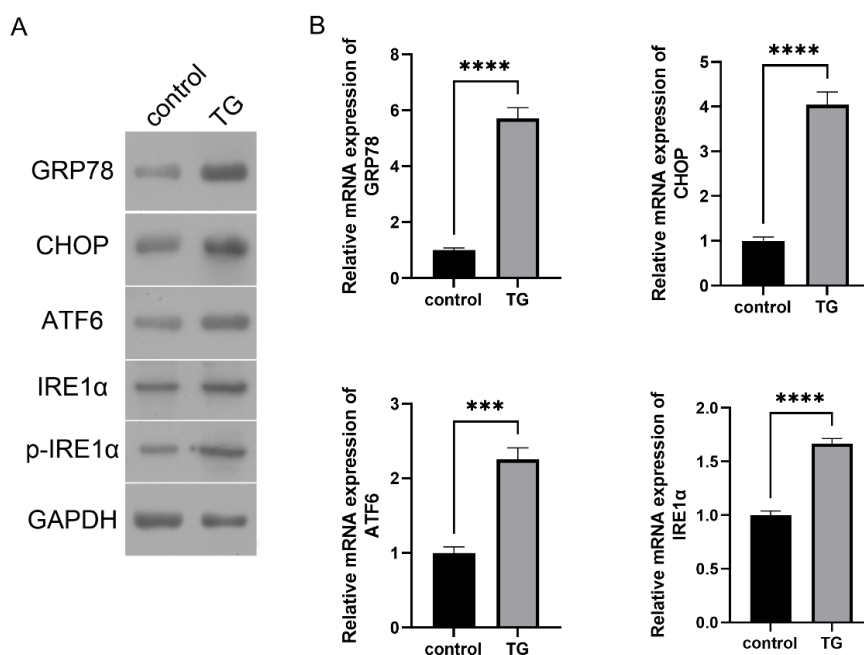

FIGURE 1. TG induced ER stress in RA-FLSs

TG was used to induce ER stress in RA-FLSs; after which, the levels of GRP78, CHOP, ATF6, and IRE1α expression were assessed by western blotting (A) and the qRT-PCR (B). Data represent the mean value ± SD, ***P < 0.001, ****P < 0.0001

treatment with TG induced increases in ROS levels in RA-FLSs, as indicted by flow cytometry (P < 0.001, Figure 2(A)). ELISA results confirmed increased levels of inflammation-related factors IL-6, IL-1β, and IL-18 in the TG-treatment group (P < 0.001, Figure 2(B)). Western blot results showed that the expression of inflammasome-associated proteins including TXNIP, NLRP3, and Caspase-1 in RA-FLSs was induced by TG (Figure 2(C)).
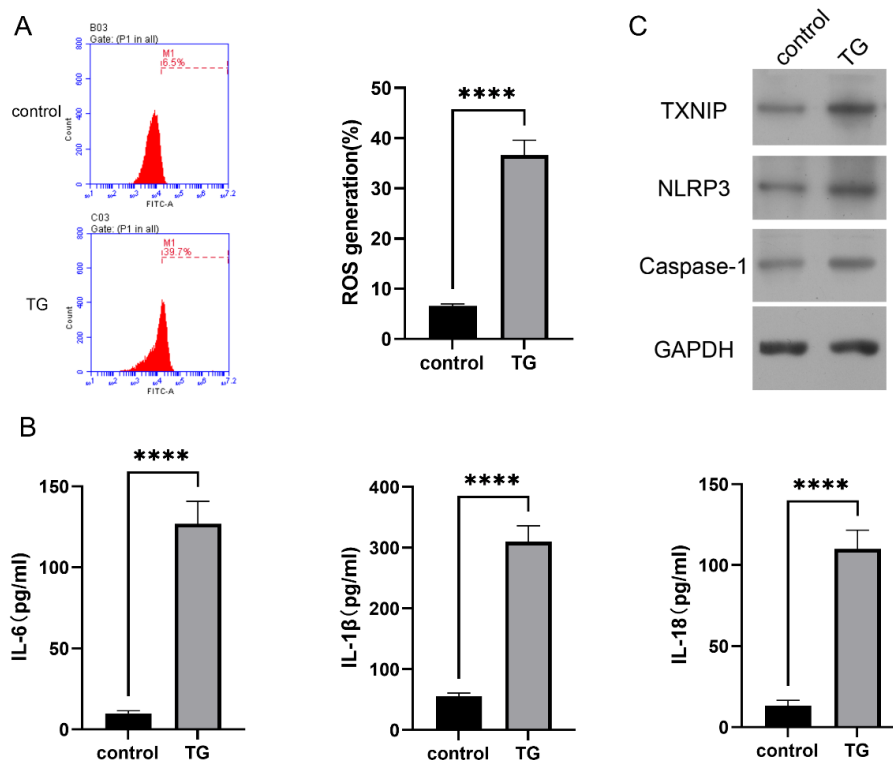

FIGURE 2. Treatment with TG induced ROS production and inflammation in RA-FLSs

(A) ROS production was detected by flow cytometry. (B) The concentrations of inflammation-related factors (IL-6, IL-1β, and IL-18) were assessed by ELISA. (C) Inflammasome protein expression including TXNIP, NLRP3, and Caspase-1 was assessed by western blotting. Data represent the mean value ± SD, ****P < 0.0001

TG PROMOTED RA-FLS APOPTOSIS BY INDUCING AUTOPHAGY

To further investigate the effect of ER on RA-FLSs, cell autophagy and apoptosis were assessed. Regarding cell autophagy, our data showed that markers of autophagosome formation and autophagy induction (LC3II/LC3I), and levels of Beclin-1 expression were increased, while p62, a marker of low autophagic activity, was down-regulated in TG-treated RA-FLSs (Figure 3(A)), indicating TG-induced cell autophagy. Western blot and qRT-PCR studies indicated that the relative expression levels of two apoptosis-related (Bax and Cleaved caspase-3) were both increased, while Bcl-2 expression was down-regulated by TG treatment in RA-FLSs (P < 0.01 or P < 0.001, Figure 3(B) and 3(C)). The above results indicated that RA-FLSs apoptosis and autophagy were significantly promoted by TG-induced ER stress.

INHIBITION OF IRE1A ALLEVIATED TG-INDUCED INFLAMMATION IN RA-FLSS BY INHIBITING AUTOPHAGY

To investigate the effect and role played by IRE1α in TG-induced ER stress, IRE1α expression in RA-FLSs was silenced by transfection with si-IRE1α. An autophagy inhibitor (3-MA) or activator (RAPA) was utilized to regulate autophagy in RA-FLSs. We found that 3-MA treatment had no effect on TG-induced GRP78, CHOP, ATF6, and IRE1α up-regulation at the mRNA and protein levels. However, inhibition of IRE1α expression significantly down-regulated GRP78, CHOP, ATF6, and IRE1α expression (P < 0.01 or P < 0.001, Figure 4(A) and 4(B)) when compared with expression of those factors in the si-NC-transfected group. In addition, when IRE1α-inhibited RA-FLSs were treated with RAPA, no significant changes occurred in expression of the above factors. The above results indicated that while inhibition
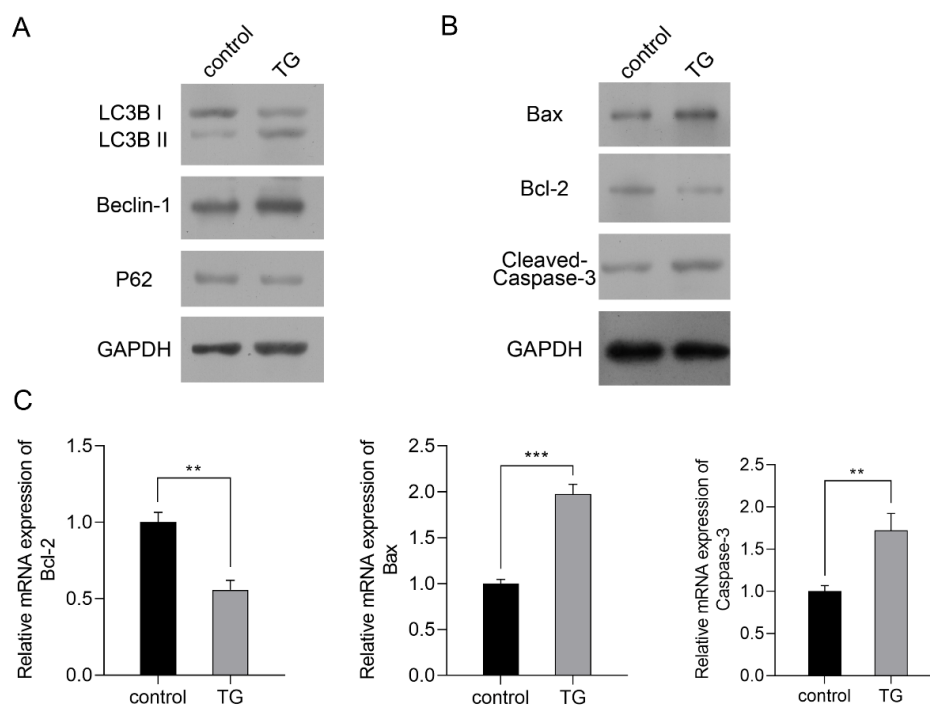
FIGURE 3. TG induced autophagy and apoptosis in RA-FLSs

TG was used to trigger ER stress in RA-FLSs; after which, (A) the levels of autophagic markers LC3B, p62, and Beclin-1 were detected by western blotting. The expression of apoptosis-related proteins (Bcl-2, Bax, and Cleaved caspase-3) in RA-FLSs was evaluated by western blotting (B) and the qRT-PCR (C). Data represent the mean value $\pm$ SD, **P < 0.01, ***P < 0.001

of IRE1α could attenuate GRP78, CHOP, ATF6, and IRE1α expression; neither an autophagy activator nor inhibitor had any effect on their expression.

However, our data showed that ROS production was reduced when autophagy was inhibited in TG-induced RA-FLSs, and the same result was observed in TG-induced RA-FLSs transfected with si-IRE1α (both P < 0.001, Figure 5(A)). However, ROS generation was increased after autophagy was activated with RAPA in IRE1α-silenced RA-FLSs (P < 0.001, Figure 5(A)). Results from cell inflammation studies showed that either inhibition of autophagy or knockdown of IRE1α could reduce the levels of TG-induced cellular inflammation by down-regulating IL-1β, IL-6, and IL-18 concentrations (all P-values < 0.001, Figure 5(B)). Moreover, further activation of autophagy in IRE1α-silenced RA-FLSs partially abrogated the protective effect of IRE1α silencing on TG-induced cell inflammation (all P-values < 0.001, Figure 5(B)). These results suggested that silencing of IRE1α had an effect similar to that of 3-MA for alleviating TG-induced ROS generation and inflammation in RA-FLSs. Furthermore, RAPA could reverse the protective effect of IRE1α silencing, which further suggested that tIRE1α silencing might reduce TG-induced ROS production and inflammation in RA-FLSs by inhibiting autophagy.

IRE1α SILENCING ALLEVIATED TG-INDUCED RA-FLS APOPTOSIS BY INHIBITING AUTOPHAGY

We also investigated the mechanism for the effect of IRE1α silencing on TG-triggered autophagy and apoptosis in RA-FLSs. Our data showed that the levels of LC3B (LC3B II/I) and Beclin-1 expression in TG-treated RA-FLSs were reduced after the cells were transfected with si-IRE1α or treated with 3-MA; however, in the si-IRE1α-transfected group, further treatment with RAPA promoted LC3B (LC3B II/I) and Beclin-1 expression. The opposite trend was found for p62 expression (Figure 6(A)). These results indicated that silencing of IRE1α had an effect similar to that of an autophagy inhibitor on alleviating TG-induced cell autophagy. Treatment with 3-MA alleviated TG-induced apoptosis by suppressing Bax and Caspase-3 protein and mRNA expression (P < 0.001 or P < 0.05) while promoting Bcl-2 expression (P < 0.001, Figure 6(B) and 6(C)). Similar trends were observed in IRE1α-silenced RA-FLSs when compared with si-NC-transfected RA-FLSs (P < 0.01); however, RAPA reversed the above effects (P < 0.001 or P < 0.05, Figure 6(B) and 6(C)). No significant change was found in Caspase-3 mRNA expression (P > 0.05, Figure 6(C)).
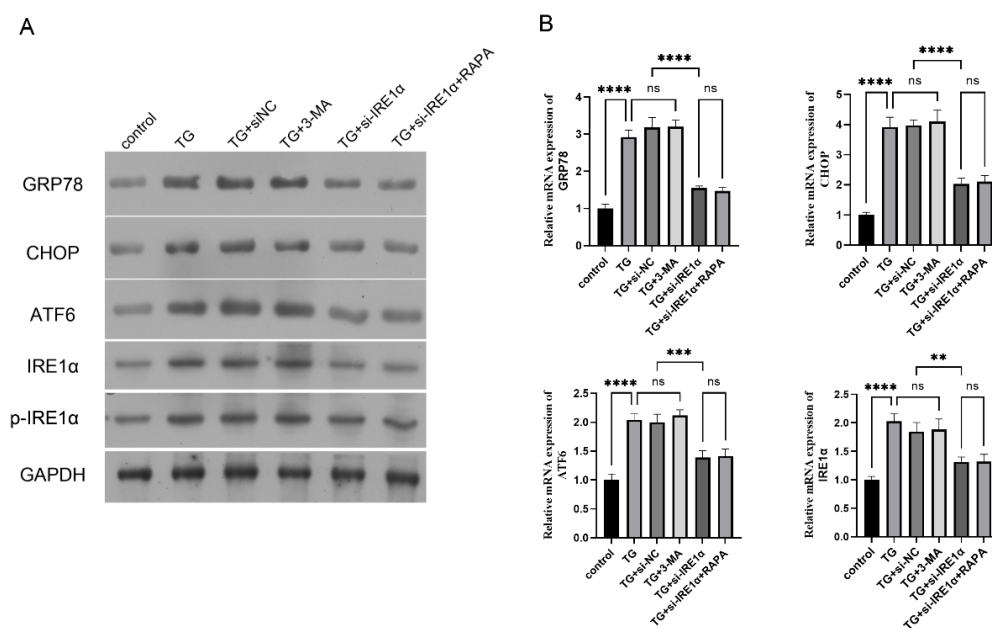
FIGURE 4. IRE1α silencing alleviated TG-induced ER stress in RA-FLSs

RA-FLSs were first treated with RAPA, 3-MA, and si-IRE1α either separately or simultaneously and then induced with TG Next, the levels of GRP78, CHOP, ATF6, IRE1α, and p-IRE1α expression were assessed by western blotting (A), and the qRT-PCR (B). Data represent the mean value ± SD, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: P > 0.05

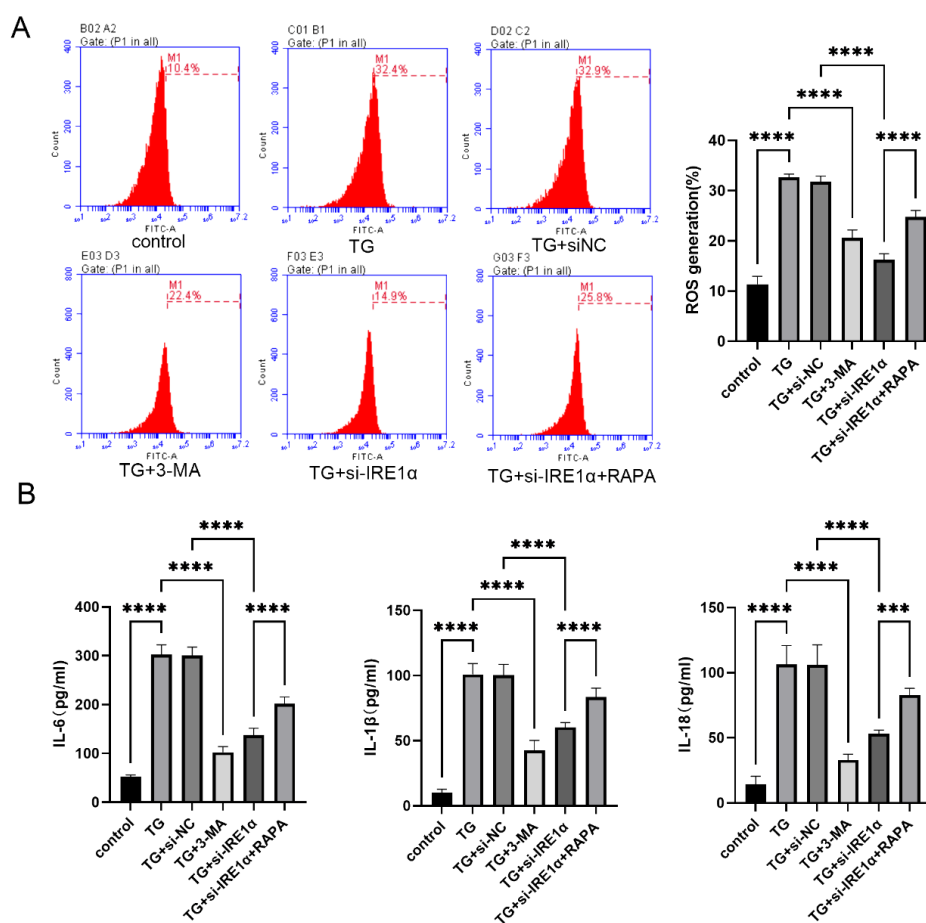FIGURE 5. IRE1α silencing alleviated ROS production and inflammation in RA-FLSs by inhibiting autophagy

RA-FLSs were first treated with RAPA, 3-MA, and si-IRE1α either separately or simultaneously and then induced with TG. (A) Flow cytometry assays were conducted to detect ROS production. (B) The concentrations of inflammation-related cytokines (IL-6, IL-1β, and IL-18) were evaluated by ELISA. Data represent the mean value ± SD, ***P < 0.001, ****P < 0.0001, ns: P > 0.05
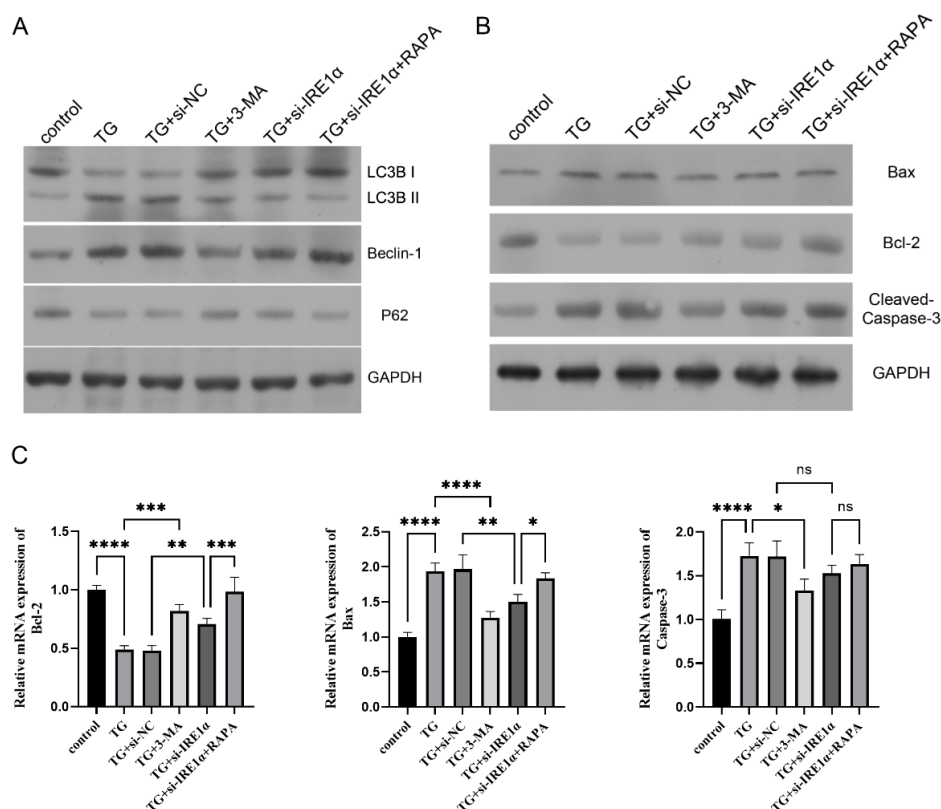
FIGURE 6. IRE1α silencing alleviated TG-induced induced autophagy and apoptosis in RA-FLSs by suppressing autophagy

RA-FLSs were first treated with RAPA, 3-MA, and si-IRE1α either separately or simultaneously and then induced with TG. (A) The expression of autophagic markers (LC3B, p62, and Beclin-1) was confirmed by western blotting. Western blotting (B) and the qRT-PCR (C) were used to detect the expression of apoptosis-associated proteins (Bcl-2, Bax, and Cleaved caspase-3) in RA-FLSs. Data represent the mean value ± SD, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: P > 0.05

DISCUSSION

Fibroblast-like synovial cells (FLSs) are aggressive and metabolically active in rheumatoid arthritis. When compared to cells found in normal synovium, RA-FLSs display an altered phenotype, as shown by a loss of contact inhibition and dependency, and thus contribute to the increased numbers of fibroblasts (Asif et al. 2017). RA-FLSs maintain their aggressive phenotype in both imprinting and epigenetics, which enables them to easily participate in the inflammatory positive feedback loop based on the synovial environment (Kabala et al. 2017). Therefore, determining the local tissue conditions that can initiate and maintain the subsequent inflammatory cycle is essential for understanding and intervening in the disease process. Previous research showed that IRE1α RNase activity was increased in the peripheral blood mononuclear cells of RA patients, suggesting that by regulating its downstream targets, IRE1α could be used to improve current diagnostic markers and increase treatment options for RA (Ahmadiany et al. 2019). In the present study, we demonstrated the potential function and mechanism of IRE1α in RA-FLSs.

The key role of UPR signal transduction is to increase protein synthesis (e.g., PERK, ATF6, and IRE1α proteins) or to activate a coordinated and regulated transcription network (e.g., ATF4, eIF2α, and XBP-1), while simultaneously maintaining cell homeostasis (Hu et al. 2019). Results from the present study indicated that the expression levels of ER stress-associated markers (GRP78, CHOP, ATF6, IRE1α, and p-IRE1α) were increased in TG-treated RA-FLSs. IRE1α is the most conserved UPR transducer. By regulating IRE1α-dependent degradation, ER stress can affect the stability of mRNAs encoded by certain genes (Maurel et al. 2014). We found that in TG-induced RA-FLSs, the over-expression of ER stress-associated genes was alleviated and homeostasis

was recovered after IRE1α expression was inhibited. Additionally, ER stress can induce inflammation and oxidative stress in a variety of disease models (Chong et al. 2017; Hong et al. 2018). Consistent with previous studies, we also found that ER stress promoted ROS production, inflammatory cytokine production, and apoptosis, which was as expected. Meanwhile, IRE1α silencing attenuated the increased ROS and inflammation levels and also the rates of apoptosis in ER stress-induced RA-FLSs. These findings clearly indicated that IRE1α silencing was involved in suppressing apoptosis and inflammation under conditions of ER stress in RA.

The removal of unfolded and misfolded proteins by autophagy is essential for cell survival. Recent studies have demonstrated the important role of autophagy in the pathogenesis and development of autoimmune diseases, including RA (Rockel & Kapoor 2017; Vomero et al. 2018), suggesting potential therapeutic targets for treating autoimmune diseases. Although IRE1α participates in regulating a variety of cellular autophagy processes (Chen et al. 2020; Hou et al. 2019), the function of IRE1α in RA-FLS autophagy remains largely unknown. Our study provides evidence for the positive regulatory role of IRE1α in RA-FLS autophagy and is supported by the observation that IRE1α knockdown decreased the levels of autophagy in ER stress-induced RA-FLSs. The same effect was observed on the expression levels of autophagy-related markers in 3-MA-treated ER-induced RA-FLSs, where 3-MA blocked the initial stage of autophagosome formation by inhibiting phosphatidylinositol 3-kinase (PI3K) (Wu et al. 2013). RAPA is a classic autophagy agonist, as it targets the mechanistic target of rapamycin (mTOR) signaling network (Gao et al. 2020). RAPA treatment can reverse the effect of IRE1α silencing on suppressing autophagy. Hence, our data showed the mechanism by which IRE1α silencing blocks the autophagic pathway in RA-FLSs.

Further investigations showed that IRE1α silencing could ameliorate ER stress via autophagy. Our results showed that 3-MA could reduce the ROS production, inflammatory response, and apoptosis induced by ER stress in RA-FLSs. In agreement with results showing that IRE1α silencing ameliorates ER stress-induced cell apoptosis and inflammation, we also found that PARA reversed the protective effect of IRE1α silencing in RA-FLSs undergoing ER stress. These data demonstrated a strong link between the processes leading to ER stress-related damage in RA-FLSs and autophagy and showed that modulation of autophagy is dependent on IRE1α expression. A previous study showed that the IRE1α-XBP1 pathway, which is activated by ER-stress, is partly involved in the induction of autophagy-dependent apoptosis in pancreatic and breast cancer cells (Zhang et al. 2019). In addition, ER stress was shown to activate the IRE1α-XBP1 signaling pathway and induce autophagy to protect endothelial cells from glucocorticoid-induced cell damage and ensure their survival and proliferation (Gao et al. 2018). In the present study, we demonstrated the cross talk that occurs between IRE1α-meditated ER stress and autophagy in RA-FLSs, suggesting that ER stress is linked to autophagy in RA, and that targeting IRE1α is a potential therapeutic strategy for treating RA. Recently, Qiu et al. (2013) proved that ubiquitination of IRE1α can regulate Toll-like receptor-induced IRE1α activation during pro-inflammatory cytokine production, and suggested IRE1α as a potential target for treating inflammatory arthritis. Our study provides a novel insight into the mechanism of IRE1α in RA treatment and supports a role for IRE1α silencing in inhibiting RA-FLS apoptosis and inflammatory responses, which at least in part might be attributable to autophagy induction.

A previous study showed that autophagy plays a dual role in cell survival. Under certain stimuli, autophagy induces apoptosis, which is accompanied by formation of numerous cytoplasmic vacuoles; under other stimuli, autophagy plays a protective role. For example, Shin et al. (2010) indicated that autophagy inhibits the ER stress response in RA fibroblasts to protect against cell death. However, Kato et al. (2014) confirmed the opposite effect of autophagy on regulating cell death pathways in RA-FLSs. In addition, Kato et al. (2014) verified that the autophagy inhibitor 3-methyladenine (3-MA) attenuated PAT-induced increases in cytoplasmic cathepsin B expression, LDH release, NLRP3 activation, cell pyroptosis, and inflammation. In our study we showed that autophagy increased ER stress-induced apoptosis in RA-FLSs.

CONCLUSION

In conclusion, our studies conducted with a TG-induced ER stress model showed that IRE1α silencing inhibited ER stress-induced autophagy, and reduced cellular ROS production, apoptosis, and inflammatory cytokine production in RA-FLSs. However, further results confirmed that an autophagy activator could reverse the protective effects of IRE1α silencing on ER stress-induced cell death in IRE1α-silenced-RA-FLSs. Therefore, our data indicate that IRE1α silencing decreases ER stress-induced apoptosis in RA-FLSs by blocking autophagy, suggesting IRE1α as a potential target for treating RA.

REFERENCES

Ahmadiany, M., Alavi-Samani, M., Hashemi, Z., Moosavi, M.A. & Rahmati, M. 2019. The increased RNAse activity of ire1α in pbmcs from patients with rheumatoid arthritis. *Advanced Pharmaceutical Bulletin* 9(3): 505-509.

Aletaha, D., Neogi, T., Silman, A.J., Funovits, J., Felson, D.T., Bingham III, C.O., Birnbaum, N.S., Burmester, G.R., Bykerk, V.P., Cohen, M.D., Combe, B., Costenbader, K.H., Dougados, M., Emery, R., Ferraccioli, G., Johanna, M.W., Hobbs, K., Huizinga, T.W.J., Kavanaugh, A., Kay, J., Kvien, T.K., Laing, T., Mease, P., Ménard, H.A., Moreland, L.W., Naden, R.L., Pincus, T., Smolen, J.S., Stanislawska-Biernat, E., Symmons, D., Tak, P.P., Upchurch, K.S., Vencovský, J., Wolfe, F. & Hawker, G. 2010. Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League against rheumatism collaborative initiative. *Annals of the Rheumatic Diseases* 69(9): 1580-1588.

Asif, A.M., Fox, D.A. & Ruth, J.H. 2017. Synovial cellular and molecular markers in rheumatoid arthritis. *Seminars in Immunopathology* 39(4): 385-393.

Bronner, D.N., Abuaita, B.H., Chen, X., Fitzgerald, K.A., Nunez, G., He, Y., Yin, X. & O'Riordan, M.X.D. 2015. Endoplasmic reticulum stress activates the inflammasome via NLRP3- and Caspase-2-driven mitochondrial damage. *Immunity* 43(3): 451-462.

Chen, H.G., Han, H.Z., Li, Y., Yu, Y.H. & Xie, K.L. 2020. Hydrogen alleviated organ injury and dysfunction in sepsis: The role of cross-talk between autophagy and endoplasmic reticulum stress. *Experimental Research International Immunopharmacology* 78: 106049.

Chong, W.C., Shastri, M.D. & Eri, R. 2017. Endoplasmic reticulum stress and oxidative stress: A vicious nexus implicated in bowel disease pathophysiology. *International Journal of Molecular Sciences* 18(4): 771.

De Cock, D. & Hyrich, K. 2018. Malignancy and rheumatoid arthritis: Epidemiology, risk factors and management. *Best Practice & Research Clinical Rheumatology* 32(6): 869-886.

Gao, G., Chen, W., Yan, M., Liu, J., Luo, H., Wang, C. & Yang, P. 2020. Rapamycin regulates the balance between cardiomyocyte apoptosis and autophagy in chronic heart failure by inhibiting mTOR signaling. *International Journal of Molecular Medicine* 45: 195-209.

Gao, Y., Zhu, H., Yang, F., Wang, Q., Feng, Y. & Zhang, C. 2018 Glucocorticoid-activated IRE1α/XBP-1s signaling: An autophagy-associated protective pathway against endotheliocyte damage. *American Journal of Physiology Cell Physiology* 315: C300-C309.

Han, J. & Kaufman, R.J. 2017. Physiological/pathological ramifications of transcription factors in the unfolded protein response. *Genes & Development* 31(14): 1417-1438.

Hetz, C. 2012. The unfolded protein response: Controlling cell fate decisions under ER stress and beyond. *Nature Reviews Molecular Cell Biology* 13: 89-102.

Hong, J., Kim, K., Park, E., Lee, J., Markofski, M.M., Marrelli, S.P. & Park, Y. 2018. Exercise ameliorates endoplasmic reticulum stress-mediated vascular dysfunction in mesenteric arteries in atherosclerosis. *Scientific Reports* 8: 7938.

Hou, Y., Fu, J., Sun, S., Jin, Y., Wang, X. & Zhang, L. 2019. BDE-209 induces autophagy and apoptosis via IRE1α/Akt/mTOR signaling pathway in human umbilical vein endothelial cells. *Environmental Pollution* 253: 429-438.

Hu, H., Wang, C., Jin, Y., Meng, Q., Liu, Q., Liu, Z., Liu, K., Liu, X. & Sun, H. 2019. Catalpol inhibits homocysteine-induced oxidation and inflammation via inhibiting Nox4/NF-κB and GRP78/PERK pathways in human aorta. *Endothelial Cells Inflammation* 42: 64-80.

Kabala, P.A., Angiolilli, C., Yeremenko, N., Grabiec, A.M., Giovannone, B., Pots, D., Radstake, T.R., Baeten, D. & Reedquist, K.A. 2017. Endoplasmic reticulum stress cooperates with Toll-like receptor ligation in driving activation of rheumatoid arthritis fibroblast-like synoviocytes. A*rthritis Research & Therapy* 19: 207.

Kato, M., Ospelt, C., Gay, R.E, Gay, S. & Klein, K. 2014. Dual role of autophagy in stress-induced cell death in rheumatoid arthritis synovial fibroblasts. *Arthritis & Rheumatology* 66(1): 40-48.

Kong, F.J., Ma, L.L., Guo, J.J., Xu, L.H., Li, Y. & Qu, S. 2018. Endoplasmic reticulum stress/autophagy pathway is involved in diabetes-induced neuronal apoptosis and cognitive decline in mice. *Clinical Science* 132(1): 111-125.

Liu, T. 2019. Regulation of inflammasome by autophagy. *Advances in Experimental Medicine and Biology* 1209: 109-123.

Maurel, M., Chevet, E., Tavernier, J. & Gerlo, S. 2014. Getting RIDD of RNA: IRE1 in cell fate regulation. *Trends in Biochemical Sciences* 39(5): 245-254.

Moore, K.A. & Hollien, J. 2012. The unfolded protein response in secretory cell function. *Annual Review of Genetics* 46: 165-183.

Navid, F. & Colbert, R.A. 2017. Causes and consequences of endoplasmic reticulum stress in rheumatic disease. *Nature Reviews Rheumatology* 13: 25-40.

Pirmardvand, C.S., Varshosaz, J. & Taymouri, S. 2018. Recent approaches for targeted drug delivery in rheumatoid arthritis diagnosis and treatment. *Artificial Cells, Nanomedicine, and Biotechnology* 46: 502-514.

Qi, L., Tsai, B. & Arvan, P. 2017. New insights into the physiological role of endoplasmic reticulum-associated degradation. *Trends in Cell Biology* 27(6): 430-440.

Qiu, Q., Zheng, Z., Chang, L., Zhao, Y.S., Tan, C., Dandekar, A., Zhang, Z., Lin, Z., Gui, M., Li, X., Zhang, T., Kong, Q., Li, H., Chen, S., Chen, A., Kaufman, R.J., Yang, W.L., Lin, H.K., Zhang, D., Perlman, H., Thorp, E., Zhang, K. & Fang, D. 2013. Toll-like receptor-mediated IRE1α activation as a therapeutic target for inflammatory arthritis. *The EMBO Journal* 32: 2477-2490.

Rahmati, M., Moosavi, M.A. & McDermott, M.F. 2018. ER stress: A therapeutic target in rheumatoid arthritis? *Trends in Pharmacological Sciences* 39(7): 610-623.

Rockel, J.S. & Kapoor, M. 2017 Autophagy: Controlling cell fate in rheumatic diseases. *Nature Reviews Rheumatology* 13(3): 193.

Shin, Y.J., Han, S.H., Kim, D.S., Lee, G.H., Yoo, W.H., Kang, Y.M., Choi, J.Y., Lee, Y.C., Park, S.J., Jeong, S.K., Kim, H.T., Chae, S.W., Jeong, H.J., Kim, H.R. & Chae, H.J. 2010. Autophagy induction and CHOP under-expression promotes survival of fibroblasts from rheumatoid arthritis patients under endoplasmic reticulum stress. *Arthritis Research & Therapy* 12: R19.

Song, S., Tan, J., Miao, Y., Li, M. & Zhang, Q. 2017. Crosstalk of autophagy and apoptosis: Involvement of the dual role of autophagy under ER stress. *Journal of Cellular Physiology* 232(11): 2977-2984.

Song, S., Tan, J., Miao, Y. & Zhang, Q. 2018. Crosstalk of ER stress-mediated autophagy and ER-phagy: Involvement of UPR and the core autophagy machinery. *Journal of Cellular Physiology* 233(5): 3867-3874.

Song, Y., Rivera, C., Mai, J. & Sun, A.W.L. 2020. Splicing HAC1/XBP1 mRNAs in cytoplasm: The non-conventional mRNA splicing reaction in the unfolded protein response OBM. *Genetics* 4(2): 11.

Sparks, J.A. 2019. Rheumatoid arthritis. *Annals of Internal Medicine* 170(1): ITC1-ITC16.

Tang, C.H. 2020 Research of pathogenesis and novel therapeutics in arthritis 2.0. *International Journal of Molecular Sciences* 21(21): 8125.

Vomero, M., Barbati, C., Colasanti, T., Perricone, C., Novelli, L., Ceccarelli, F., Spinelli, F.R., Franco, M.D., Conti, F., Valesini, G. & Alessandro, C. 2018. Autophagy and rheumatoid arthritis: Current knowledges and future perspectives. *Frontiers in Immunology* 9: 1577.

Wang, L., Dong, H., Song, G., Zhang, R., Pan, J. & Han, J. 2018. TXNDC5 synergizes with HSC70 to exacerbate the inflammatory phenotype of synovial fibroblasts in rheumatoid arthritis through NF-κB signaling. *Cellular & Molecular Immunology* 15(7): 685-696.

Wu, P., Tian, T., Zhao, J., Song, Q., Wu, X., Guo, Y., Yu, Y., Tan, S. & Xia, H. 2020. IRE1α-JNK pathway-mediated autophagy promotes cell survival in response to endoplasmic reticulum stress during the initial phase of hepatic steatosis. *Life Sciences* 264: 118668.

Wu, Y., Wang, X., Guo, H., Zhang, B., Zhang, X.B., Shi, Z.J. & Yu, L. 2013. Synthesis and screening of 3-MA derivatives for autophagy inhibitors. *Autophagy* 9(4): 595-603.

Yamasaki, S., Yagishita, N., Tsuchimochi, K., Kato, Y., Ssaki, T., Amano, T., Beppu, M., Aoki, H., Nakamura, H., Nishioka, K. & Nakajima, T. 2006. Resistance to endoplasmic reticulum stress is an acquired cellular characteristic of rheumatoid synovial cells. *International Journal of Molecular Medicine* 18(1): 113-117.

Zhang, X., Hu, P., Ding, S.Y., Sun, T., Liu, L., Han, S., DeLeo, A.B., Sadagopan, A., Guo, W. & Wang, H. 2019. Induction of autophagy-dependent apoptosis in cancer cells through activation of ER stress: An uncovered anti-cancer mechanism by anti-alcoholism drug disulfiram. *American Journal of Cancer Research* 9(6): 1266-1281.

Zhou, W., Shen, Q., Wang, H., Yang, J., Zhangm, C., Deng, Z., Wu, K., Zhou, Y., Zheng, J., Zhang, Y. & Shen, W. 2020. Knockdown of YAP/TAZ inhibits the migration and invasion of fibroblast synovial cells in rheumatoid arthritis by regulating autophagy. *Journal of Immunology Research* 2020: 9510594.

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