Bcl-XL and Mcl-1 upregulation by calreticulin promotes apoptosis resistance of fibroblast-like synoviocytes via activation of PI3K/Akt and STAT3 pathways in rheumatoid arthritis

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Abstract

Objective

Fibroblast-like synoviocytes (FLS) play key roles in synovium hyperplasia and pannus formation in rheumatoid arthritis (RA). The present study was undertaken to explore the mechanisms that calreticulin (CRT) promoted anti-apoptosis of RA FLS.

Methods

The expression of CRT and anti-apoptotic proteins Bcl-XL and Mcl-1 in RA synovium were detected by immunohistochemistry. The expression of Bcl-XL and Mcl-1 in RA FLS by CRT were determined. The phosphorylation of Akt and STAT3 was detected by western blot. The effect of CRT on proliferation of RA FLS was examined by MTT assay. The ability of CRT to inhibit RA FLS apoptosis was assessed by flow cytometry.

Results

Increased expressions of CRT, Bcl-XL and Mcl-1 were detected in RA synovium compared with osteoarthritis (OA). Moreover, CRT expression correlated positively with Bcl-XL and Mcl-1 in RA, respectively. In vitro, CRT induced upregulation of Bcl-XL and Mcl-1 protein levels in RA FLS, in dose/time dependent manners. Upregulated expression of Bcl-XL and Mcl-1 induced by CRT were inhibited by PI3K/Akt or STAT3 pathways inhibitors in RA FLS, respectively. The increased phosphorylation levels of Akt and STAT3 were also detected with CRT incubation, in dose/time dependent manners. Additionally, CRT rescued apoptosis of RA FLS mediated by FasL.

Conclusion

This study showed that upregulation of Bcl-XL and Mcl-1 expression in RA FLS by CRT were PI3K/Akt and STAT3 signal pathways dependent, and promoted the anti-apoptosis of RA FLS. Therefore, this may represent a therapeutic target for the treatment of RA.

Key words

calreticulin, Bcl-XL protein, myeloid cell leukaemia sequence 1 protein, synoviocytes, rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is a chronic immune-mediated inflammatory disease characterised by hyperplasia of synovium and pannus formation, leading to joint destruction and functional disability. Several cell types, including fibroblast-like synoviocytes (FLS), macrophages, T cells, B cells, etc., and the complex interaction of many pro-inflammatory factors are involved in the pathogenesis of RA (1). Current studies imply that FLS are not only passive responders but imprinted aggressors in RA (2, 3). As the dominant components of the hyperplastic synovium and invasive pannus, FLS play a key role in RA by producing various pro-inflammatory cytokines, adhesion molecules, matrix degrading proteases as well as angiogenic factors, which contribute to the perpetuation of joint inflammation (2-5). Additionally, the stable activation of RA FLS leads to aggressive phenotype and shows tumour cell-like characteristics, such as abnormal proliferation and resistance to apoptotic stimuli (6).

Survival of FLS is a dynamic process, which reflects the balance between cell death and regeneration in RA synovium. Normal cells culminate their life span with a programmed cell death process, apoptosis, through either extrinsic or intrinsic pathways. Apoptosis is a highly regulated process that confers advantages in keeping the structural and functional homeostasis of tissues. Besides, cell proliferation can replace aging or dying cells through self-renew. Recently, studies have indicated that the relative sparing of FLS from apoptosis occurred in RA synovium (2, 3, 7), suggesting that the regulation of the apoptotic pathway may be dysfunctional.

Bcl-2 homology (BH) domains containing proteins, members of the B-cell lymphoma 2 (Bcl-2) family, regulate the intrinsic pathway of apoptosis by altering mitochondrial permeability and release of apoptotic protease activating factor 1 (apaf-1) and cytochrome C. Both B-cell lymphoma-extra large (Bcl-XL) and myeloid cell leukaemia 1 (Mcl-1) are important members of anti-apoptotic proteins of the Bcl-2 family. Bcl-XL is long form of Bcl-x, which is a dominant regulator of programmed cell death in mammalian cells, and displays cell death repressor activity (8). The C terminus of Bcl-XL contains a clear mitochondrial outer membrane (MOM)-targeting motif and shares sequence homology with those of Bax, Bak and other MOM-targeted Bcl-2 proteins (8). Bcl-XL avoids cells from apoptosis both by interacting with Bax to prevent its membrane integration and by interacting with Bid and other BH3-only proteins to sequester their pro-death activity. Mcl-1 primarily localises to MOM where it can inhibit the release of cytochrome C (9). Mcl-1 plays a major pro-survival role by sequestering Bak on the MOM and preventing Bak oligomerisation. In addition, Mcl-1 has also been shown to inhibit mitochondrial calcium signals following an apoptotic stimulus (10). Recent studies have demonstrated that both Bcl-XL and Mcl-1 are involved in RA pathogenesis (11-13).

The precise mechanisms of synoviocytes apoptosis and proliferation in RA are currently unclear. Recent studies demonstrated that phosphatidylinositol 3-kinase (PI3K)/Akt and signal transducers and activators of transcription 3 (STAT3) are both important signal pathways involved in the pathogenesis of many inflammation diseases such as RA, systemic lupus erythematosus (SLE) and type 1 diabetes (T1D) (14, 15). Fundamental studies have demonstrated critical roles of PI3K/Akt and STAT3 signal pathways in apoptosis, proliferation, cellular chemotactic responses, etc. (16, 17). Those two signal pathways have been reported to regulate the expression of anti-apoptotic Bcl-2 family molecules and proliferation of synoviocytes in RA. Cytokines such as TNF-α, IL-22 and IL-17 were able to induce the expression of Bcl-2, Bcl-XL and Mcl-1 in RA FLS partly through the PI3K/Akt and STAT3 pathways, resulting in resistance apoptosis of cells (11-13). In addition, PI3K/Akt and STAT3 signal pathways were overactive in RA FLS (18, 19).

Calreticulin (CRT), a ubiquitous and highly conserved Ca^2+ binding protein, is implicated in many biological activities including Ca^2+ homeostasis, cell adhesion, antigen presentation,

apoptosis and anti-tumour immune response process (20, 21). Recently, high expression of CRT was detected in RA patients, as well as a significant positive correlation between serum and synovial fluid levels of CRT and disease activity (22, 23). In addition, current investigations demonstrated that CRT was associated with RA progress through transducting shared epitope signal (24), promoting angiogenesis (25), as well as inhibiting T cell apoptosis (22). Meanwhile, CRT was recently identified as an upstream regulator of PI3K/Akt and STAT3 pathways (26, 27). Together, these studies suggested that CRT might be involved in the pathogenesis of RA primarily by promoting the apoptotic resistance of synovial cells. However, the roles and molecular mechanisms of CRT in the anti-apoptosis/proliferation of RA FLS remain unknown.

In the present study, we hypothesised that CRT might play an important role in the anti-apoptosis/proliferation of RA FLS through increasing the expression of anti-apoptotic proteins Bcl-XL and Mcl-1. We explored the effect of CRT on the expression of Bcl-XL and Mcl-1 in RA FLS and the potential signal pathways. The roles of CRT on anti-apoptosis/proliferation of RA FLS were further investigated.

Materials and methods

Patients and samples

Synovial tissues were obtained from RA (n=13) and osteoarthritis (OA) (n=14) patients undergoing synovectomy or total knee replacement surgery at General Hospital of Tianjin Medical University, Tianjin, China. All patients with RA fulfilled the 2010 Rheumatoid Arthritis Classification Criteria (28), and all patients with OA fulfilled the ACR 1995 criteria for OA (29). The study was approved by the Medical Ethics and Human Clinical Trial Committee (TMUhMEC 2013031) of Tianjin Medical University, and informed consent was obtained from all the patients and control subjects.

Cell culture

In vitro cell culture system with isolated FLS was employed in this study. For preparation of FLS, the synovium was minced and incubated with 4 mg/ml type I collagenase (Worthington Biochemical, Freehold, NJ, USA) in serum-free Dulbecco's modified Eagle's medium (DMEM) for 1 hour at 37°C, centrifuged, resuspended, and cultured in DMEM supplemented with 10% FBS at 37°C in 5% CO2 atmosphere. FLS between passages 3 to 8 were used for the subsequent experiments. Cells in this study were synchronised in serum starvation media (FLS media with 0.1% FBS) for 24 h before the addition of the appropriate stimuli. After 8 generations, another cultured FLS line was used to perform the subsequent experiments.

Immunohistochemistry

Sections were dewaxed, rehydrated, depleted of endogenous peroxidase activity by adding 0.3% hydrogen peroxide and blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. The sections were incubated overnight at 4°C with rabbit anti-CRT (ThermoFisher Scientific, Waltham, MA, USA) at 1:1000 dilution, or rabbit anti-Bcl-XL (Cell Signaling Technology, Beverly, CA, USA) at 1:300 dilution, or mouse anti-Mcl-1 (GeneTex, USA) at 1:1000 dilution. After washing with PBS for three times, the slides were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody (ZSGB-BIO, Beijing, China) for 1 h at room temperature, and chromogenic reaction was developed with diaminobenzidine for 5–10 min. The sections were counterstained with haematoxylin, dehydrated and mounted. The expression of CRT, Bcl-XL and Mcl-1 were observed using microscopy and the images were analysed by the BioMias 2000 image analysis and processing system.

Quantitative polymerase chain reaction (q-PCR)

Total RNA was extracted from FLS using TRIzol (Invitrogen, Carlsbad, CA, USA) and converted to cDNA using FastQuant cDNA First Strand cDNA Synthesis Kit (TianGen, Beijing, China). q-PCR amplification was performed using SYBR Green SuperReal PreMix Plus (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The primers used were as follows: Bcl-XL forward primer, 5'- CACCCCAGGGACAGCATATC-3', and reverse, 5'-GTCAGGAACCAGCGGTTGAA-3', product size 117bp; Mcl-1 forward primer, 5'-GGACAAAACGGGACTGGCTA-3' and reverse 5'-CAGCAGCACATTCCTGATGC-3', product size 101bp; β-actin forward primer, 5'- TGGCACCCAGCACAATGAA-3', and reverse, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3', product size 186bp. Relative expression levels were determined by normalisation to β-actin using the ΔΔCt method. The experiments were repeated at least three times.

Western blot

Proteins were extracted from FLS using RIPA lysis buffer supplemented with protein phosphatase inhibitor cocktail and centrifuged for 15 minutes at 14,000 rpm, 4°C. The protein concentrations in the supernatant were measured using the BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Samples were separated in 10% SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Millipore Co., Billerica, MA, USA). Blocking was performed in 5% milk for 1 h at room temperature and membranes were incubated in primary antibodies overnight at 4°C. The primary antibodies to Bcl-XL, Mcl-1, phospho-(p)Akt, Akt, p-STAT3, STAT3 and β-Actin (all from Cell Signaling Technology, Beverly, CA, USA) were 1:1000-1:2000 diluted in 5% BSA/TBST, respectively. The membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibody (ZSGB-BIO, Beijing, China) for 1 h at room temperature and proteins were detected using a Pro-light HRP Chemiluminescent Kit (Tiangen Biotech, Beijing, China). The experiments were repeated at least three times.

MTT cell proliferation assay

RA FLS (100μl) were seeded into a 96-well plate at a density of 1×10^4 cells/well in complete DMEM medium until the cells reached 50–60% confluence. The cells were then cultured in the presence of CRT (0, 1, 10, 50 ng/

ml) for 48 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (20 μl, 5 mg/ml) were added to each well and incubated for 4 h at 37°C. Thendimethylsulphoxide (DMSO) (150 μl) were added to each wells to dissolve the formazan crystals. Absorbance was measured at 490 nm. The experiments were repeated at least three times.

Flow cytometry analysis

Cell apoptosis was determined by flow cytometry with Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, RA FLS were washed with cold PBS and resuspended in binding buffer at a concentration of 1×10^6 cells/ml. Cell solution (100μl) was incubated with 5 μl FITC Annexin V and 5 μl PI for 15 minutes at room temperature in dark. The percentage of apoptotic RA FLS were analysed by flow cytometry. The experiments were repeated at least three times.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Differences between multi-groups were analysed by one-way analysis of variance (ANOVA), and Student-Newman-Keuls test was further used for a comparison between two groups. The Spearman's rank correlation coefficient was used for correlation analyses. The percentage of apoptotic cells were analysed by χ^2 test. Values for $p<0.05$ were considered statistically significant. SPSS software 17.0 was used for statistical analysis.

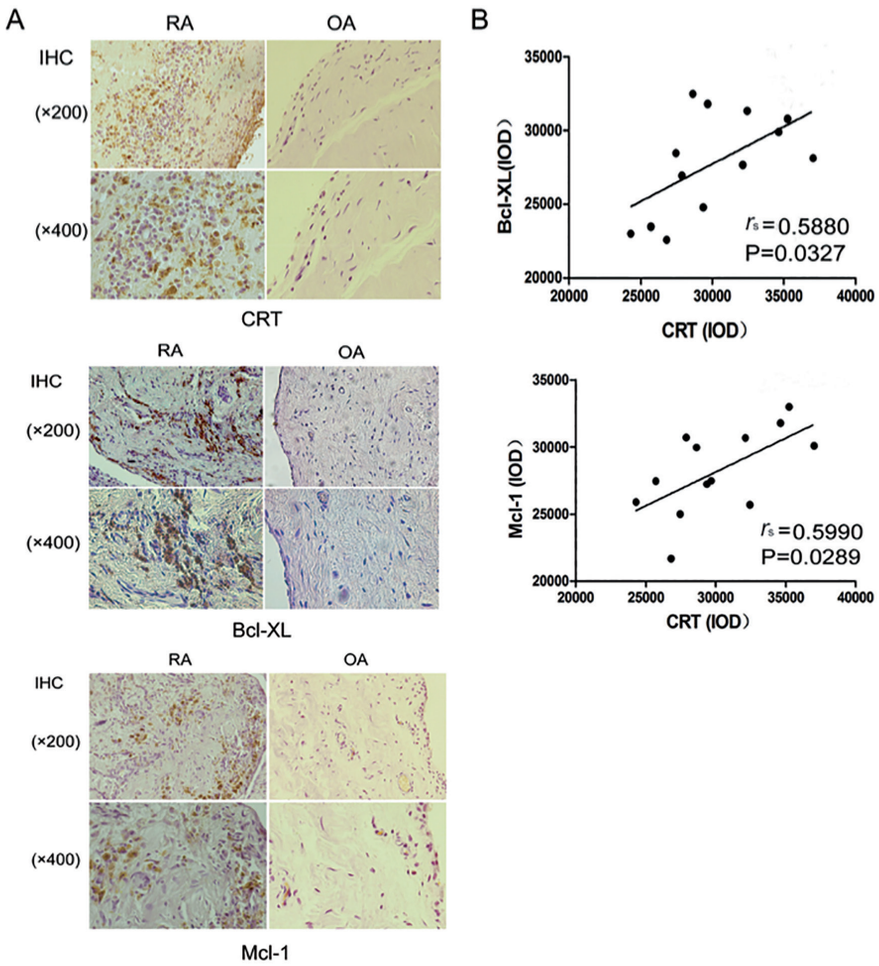

Fig. 1. Increased expression of CRT in RA synovium correlated positively with Bcl-XL and Mcl-1 expression.

A: Representative immunohistochemical images of CRT, Bcl-XL and Mcl-1 in RA and OA synovium. Bcl-XL and Mcl-1 were strongly expressed in RA synovium mainly localised to synovial vascular endothelial cells, inflammatory cells, and perivascular areas in both the synovial lining and sublining layers.

B: Scatter plots demonstrating the correlations of CRT protein levels with Bcl-XL and Mcl-1 expression in RA synovium. The integrated optical density (IOD) values of immunohistochemical images were analysed. Correlations of CRT expression in RA synovium (n=13) with Bcl-XL and Mcl-1 were assessed by Spearman's rank correlation coefficient, respectively. Dots represented individual patients, and Spearman r_s and p-values were showed in each graph.

Results

Increased expression of CRT in RA synovial tissue correlates positively with Bcl-XL and Mcl-1

CRT, Bcl-XL and Mcl-1 expression in RA synovium were detected by immunohistochemistry. The results showed that CRT was highly expressed in RA synovium compared with less expression in OA. Strong staining of CRT localised primarily in largely aggregated cells such as fibroblast-like synoviocytes (FLS) and inflammatory cells in both the lining and sublining layers of RA synovium (Fig. 1A). There were less aggregated FLS and inflammatory cells, and only in a minority of synoviocytes was CRT staining positive in OA. Bcl-XL and Mcl-1, anti-apoptotic proteins, were also strongly expressed in RA synovium, located in both the lining and sublining layers. Bcl-XL was largely expressed in FLS, synovial vascular endothelial cells and perivascular areas. In addition, the expression of Mcl-1 was mainly localised in FLS, inflammatory cells and neovascular regions. According to the above results, the relationship between expression of CRT and anti-apoptotic proteins was investigated. The integrated optical density (IOD) values of immunohistochemical images were analysed by the BioMias image analytical instrument. And then, Spearman's rank correlation analyses showed significant positive correlations between expression of CRT with Bcl-XL (r_s=0.5880, p=0.0327) and Mcl-1 (r_s=0.5990, p=0.0289) in RA synovium, respectively (Fig. 1B).

CRT upregulated the expression of Bcl-XL and Mcl-1 in RA FLS

To further evaluate the role of CRT on the expression of Bcl-XL and Mcl-1, isolated RA FLS were cultured with CRT at different concentrations or du-

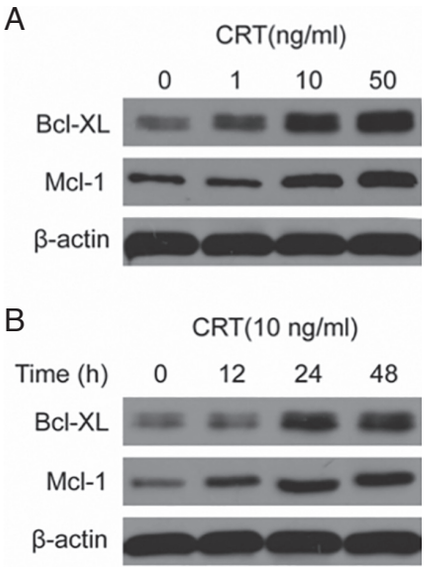

Fig. 2. CRT increased the expression of Bcl-XL and Mcl-1 in RA FLS.
A: FLS from patients with RA were cultured with various concentrations of CRT (0, 1, 10 and 50 ng/ml) for 48h, and the protein levels of Bcl-XL and Mcl-1 were determined by western blot.
B: Western blot analysis of Bcl-XL and Mcl-1 expression in FLS from OA cultured with CRT (10 ng/ml) for 0, 12, 24 and 48h, respectively. Our results showed representative data of at least three independent experiments.

rations, and the expression of Bcl-XL and Mcl-1 were determined by western blot. The results showed expression of Bcl-XL and Mcl-1 proteins increased when RA FLS were incubated with CRT, especially at the concentration of 10 and 50 ng/ml (Fig. 2A), which were comparable with the results in our previous study (23). With incubation time prolonged (0, 12, 24 and 48 h), the expression of Bcl-XL and Mcl-1 in RA FLS increased gradually in the present of CRT (10 ng/ml) stimulation, respectively (Fig. 2B). Together, our data revealed that CRT upregulated the expression of anti-apoptotic proteins Bcl-XL and Mcl-1 in RA FLS, in dose/time-dependent manners.

PI3K/Akt and STAT3 signal pathways were involved in CRT-induced Bcl-XL and Mcl-1expression upregulation in RA FLS

The mRNA levels of Bcl-XL and Mcl-1 expression were also increased with CRT (10 ng/ml) incubation (Fig. 3A). We further studied the molecular mechanisms underlying Bcl-XL and Mcl-1 upregulation by CRT in RA FLS. The upregulated mRNA levels of anti-apoptotic proteins Bcl-XL and Mcl-1 were decreased by LY294002 (PI3K inhibitor) incubation in RA FLS. Besides, after treated with STA-21 (STAT3 inhibitor), the transcription of anti-apoptotic proteins were reduced in RA FLS (Fig. 3A). Consistent with the results in mRNA levels, the increased protein levels of Bcl-XL and Mcl-1induced by CRT were also inhibited by LY294002 or STA-21, respectively (Fig. 3B).

Subsequently, the impact of CRT on the activation of PI3K/Akt and STAT3 signal pathways, represented by phosphorylation of key molecules Akt and STAT3, were explored in RA FLS. Both Akt and STAT3 phosphorylation were visibly increased treated with CRT (10 ng/ml) in a time-dependent manner in RA FLS, the activation of which occurred around 30 min, and the maximal activation was observed followed by stimulation of 120 min (Fig. 3C). In addition, CRT stimulation increased the phosphorylation levels of Akt and STAT3 in RA FLS, in a dose-dependent manner (Fig. 3D). PI3K or STAT3 inhibitor alone had no significant effect on the expression of Bcl-XL and Mcl-1 (data not shown). Together, these results demonstrated that CRT was able to activate PI3K/Akt and STAT3 signal pathways, and both PI3K/Akt and STAT3 signalling pathways were involved in CRT-induced Bcl-XL and Mcl-1 upregulation in RA FLS.

CRT inhibited FasL-mediated apoptosis of RA FLS

Having indicated that anti-apoptotic proteins were upregulated by CRT incubation, the role of CRT on the proliferation of RA FLS was studied with MTT assay. Our results revealed the proliferation of RA FLS was just slightly increased after stimulation with CRT at different concentrations (0, 1, 10 and 50 ng/ml), but was not statistically significant ($F=2.88$, $p>0.05$) (Fig. 4A). The impact of CRT on the apoptosis resistance was further investigated. RA FLS were pre-incubated with different concentrations of CRT (0, 10 and 50 ng/ml) for 48h and then cultured with FasL (100 ng/ml) for 24h, and the apoptosis of RA FLS was assessed by flow cytometry. As the results suggested, the population of apoptotic RA FLS significantly increased treated with FasL alone. However, CRT pre-incubation inhibited FasL-mediated apoptotic death of RA FLS (Fig. 4B). These results suggested that increased expression of CRT in local lesions of RA promoted hyperplasia of synovium might partly through inhibiting apoptotic death of RA FLS.

Discussion

Fibroblast-like synoviocytes (FLS) are dominant cell component mediating the pathogenesis of RA through persisting chronic inflammation. Increasing anti-apoptosis of FLS leads to synovium hyperplasia and pannus formation in RA. Our research demonstrated that the expressions of CRT, Bcl-XL and Mcl-1 were upregulated in RA synovium, and increased CRT was able to activate the PI3K/Akt and STAT3 signal pathways which consequently elevated the expression of anti-apoptotic proteins Bcl-XL and Mcl-1 in RA FLS, leading to resistance to apoptosis of FLS.

In the present study, high expression of CRT and anti-apoptotic proteins Bcl-XL and Mcl-1 was demonstrated in RA synovium, and the expression of CRT correlated positively with Bcl-XL and Mcl-1, respectively. Recent evidences indicated that CRT was able to transfer from the endoplasmic reticulum (ER) to the cytoplasm, cell surface and extracellular milieu under certain circumstance such as cell stress (20, 21, 30). Intrinsic control of apoptosis requires regulation of mitochondrial outer membrane permeabilisation by Bcl-2 family proteins, including both Bcl-XL and Mcl-1. Our previous studies illustrated that the expression of CRT was increased in RA, including serum, synovial fluid and synovial tissue, and serum levels of CRT were positively correlated with disease activity (23). The histological distribution of CRT was similar with the locations of anti-apoptotic proteins both Bcl-XL and Mcl-1 in RA synovium. Our results on Bcl-XL expression were similar to those of a previous study which showed that RA was associated with elevated expression of Bcl-XL in the synovial lining layer and inflammatory cells, particularly plasma cells

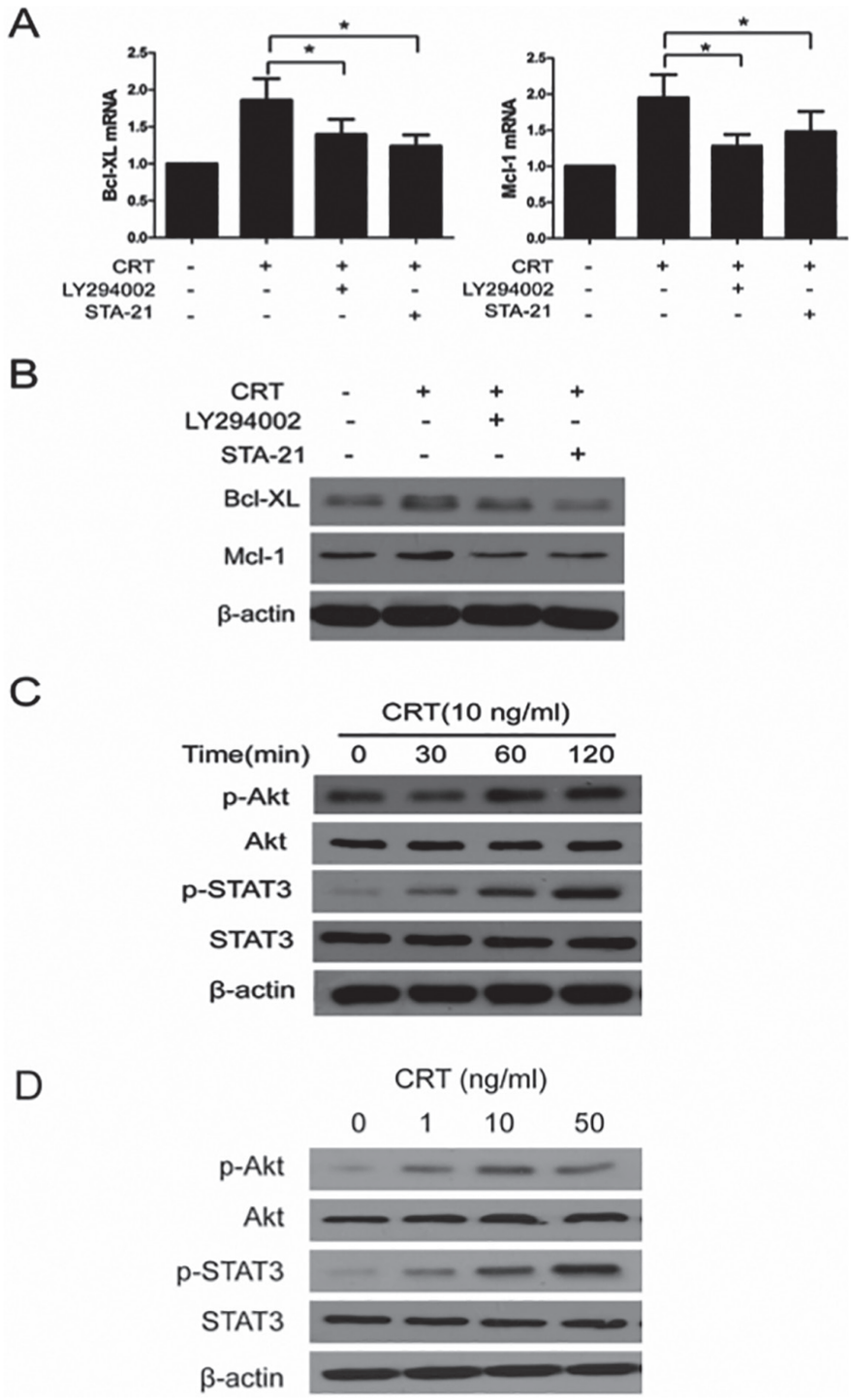

Fig. 3. PI3K/Akt and STAT3 signalling pathways were involved in upregulation of Bcl-XL and Mcl-1 expression.

A: RA FLS were incubated with or without LY294002 (PI3K inhibitor, 25μM) or STA-21(STAT3 inhibitor, 25 μM) for 1h before incubation with CRT (10 ng/ml) for 48h. The levels of Bcl-XL and Mcl-1 mRNA were determined by q-PCR.

*$p< 0.05$, *vs* CRT-treated group.

B: Western blot detected the expression of Bcl-XL and Mcl-1 in RA FLS treated with CRT (10 ng/ml) in the presence or absence of PI3K or STAT3 inhibitor.

C: Akt and STAT3 phosphorylation in RA FLS stimulated by CRT (10 ng/ml) for different time periods (0.5, 1 and 2 h) were assessed by western blot.

D: Akt and STAT3 phosphorylation in RA FLS stimulated by CRT (0, 1, 10 and 50 ng/ml) for 2 h were assessed by western blot. All western blot results shown in figures are representative data of at least three independent experiments.

(31). A prior study on Mcl-1expression in RA showed that Mcl-1 was highly expressed in the FLS of synovial lining and sublining layers of RA synovium, which supported our results in the present study (32). Our results suggest that high expression of Bcl-XL and Mcl-1 might play an important role in the apoptosis resistance of synoviocytes and inflammatory cells in RA synovial tissues, which was associated with the elevated expression of CRT.

In order to explore whether the expression of Bcl-XL and Mcl-1 was regulated by CRT, an *in vitro* RA FLS cultrure system was employed. Our data showed higher Bcl-XL and Mcl-1 expression in RA FLS after treated with CRT. CRT increased the expression of Bcl-XL and Mcl-1 in protein level of RA FLS, in dose/time-dependent manners. Previous studies revealed that proinflammatory factors like IL-22, IL-17 and TNF-α, etc. could upregulate the expression of anti-apoptosis proteins of Bcl-2 family such as Bcl-2, Bcl-XL and Mcl-1 in RA FLS (12, 13, 32). Importantly, the concentrations of CRT used in our study closely resembled the actual levels of CRT measured previously in a clinical environment of RA (22, 23, 25), suggesting that the effects of CRT observed in our study were most likely to occur *in vivo*. Heat-shock proteins, specifically HSP 72, expression inhibited apoptosis in prostate tumour cell lines, which might be mediated by the production of survival factors such as Mcl-1, Bcl-2 and Bcl-XL (33). Both HSP 72 and CRT are molecular chaperones in the endoplasmic reticulum. Our previous results indicated that increased expression of CRT in local lesions of RA synovium might play an important role in pathogenic mechanisms of RA by regulating the expression of anti-apoptosis proteins in FLS.

Our results showed that the increased expression of Bcl-XL and Mcl-1 triggered by CRT was regulated by PI3K/Akt and STAT3 signal pathways. Both the activation of Akt and STAT3 were increased in RA FLS in the presence of CRT, in a dose/time-dependent manner. Several studies have demonstrated that PI3K/Akt and STAT3 signal pathways were involved in physiological func-

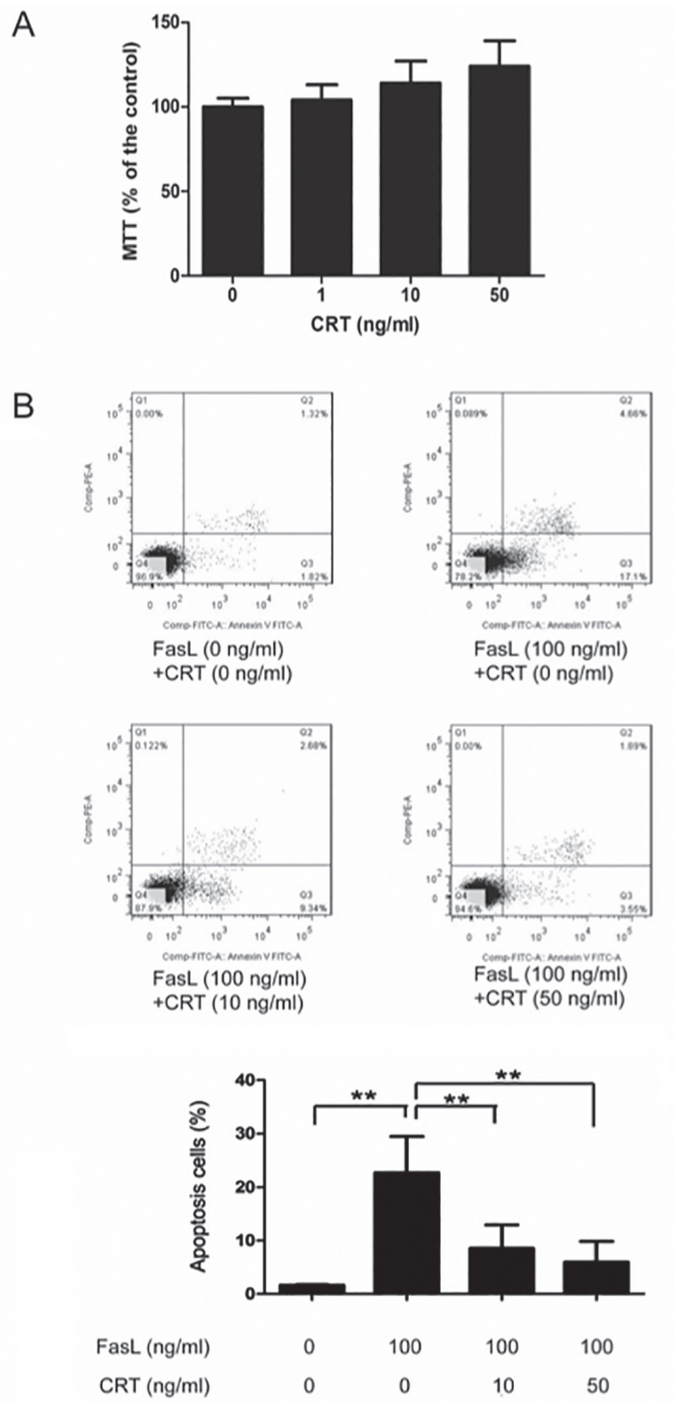

Fig. 4. CRT rescued FasL-mediated apoptosis of RA FLS.
A: The effect of CRT on the proliferation of RA FLS. RA FLS were treated with different concentrations of CRT (0, 1, 10 and 50 ng/ml) for 48h and cell proliferation was determined by MTT assay. Slightly increases in proliferation of RA FLS were observed.
B: CRT inhibited apoptosis of RA FLS. RA FLS were pre-incubated with different concentrations CRT (0, 10 and 50 ng/ml) for 48h followed by treatment with FasL (100 ng/ml) in serum-free DMEM for 24h. Apoptotic cells in RA FLS were measured by flow cytometry using Annexin V-FITC and PI staining. The percentage of apoptotic cells in FasL treatment group significantly increased compared with the control. FasL-induced apoptotic death of RA FLS was inhibited by CRT pre-incubation. $^{**}p < 0.01$.

tions of cells including apoptosis, proliferation, and autophage, etc. (34-38). The key cellular processes regulated by PI3K/Akt signal pathway include cell growth, migration and differentiation. One of the substrates of Akt is tuberous sclerosis complex (TSC)1/2, which can be phosphorylated and inhibited by Akt, leading to activation of the central regulator of cell growth, mammalian target of rapamycin complex 1(mTORC1). Mutations on the PI3K/Akt/mTORC1 axis were amongst the most prevalent in human cancers, illustrating its importance in growth factor signalling (34). Once cells are stimulated by cytokines and growth factors, STAT3 is phosphorylated by receptor-associated Janus kinases (JAK), forms homo- or heterodimers, and translocates to the cell nucleus where it act as transcription activators. Intriguingly, these two pathways are crosslinked with each other in regulating apoptosis/proliferation. Several studies have shown that expression of anti-apoptotic proteins of the Bcl-2 family were regulated by PI3K/Akt and STAT3 signal pathways (11-13). Inhibition of Mcl-1 by epigallocatechin-3-gallate was partly mediated via down-regulation of the TNF-α-induced Akt phosphorylation (11). Activation of STAT3 triggered by IL-22/IL-17 upregulated the expression of Bcl-2 which protected RA FLS against sodium nitroprussiate-induced apoptosis (12, 13). Both transcription factors STAT3 and NF-κB were downstream of PI3K/Akt signal pathway, and regulated the expression of Bcl-x gene encoding Bcl-XL (39). Besides, transcription factor STAT3 was able to regulate Mcl-1 expression (37). However, IL-35 signalling through STAT1 and inhibition of the expression of mediators of angiogenesis and inflammation in RA FLS, indicated that the physiological function of STATs might be distinct (40). These studies suggested the enhanced activation of the PI3K/Akt and STAT3 (6, 41) mediated by CRT in inflamed synovium could increase the expression of anti-apoptotic proteins, leading to apoptosis resistance of synoviocytes.

The effects of CRT on the anti-apoptosis of RA FLS were further investigated. Our results showed that CRT significantly inhibited FasL-mediated apoptotic death of RA FLS, which might be explained by the increased expression of anti-apoptosis proteins such as Bcl-XL and Mcl-1 in RA FLS mediated by CRT. Of note, the investigation whether CRT mediates the extrinsic pathway of apoptosis in RA FLS and the detailed

mechanisms is under study. Several researchers have reported that CRT could induce anti-apoptosis in RA and other diseases (22, 42). CRT had the capacity to directly bind to FasL and to inhibit FasL-mediated apoptosis of Jurkat T cells, and thus might play a role in inhibiting apoptosis of inflammatory T cells in RA (22). Our preliminary study showed that CRT inhibited tumour necrosis factor related apoptosis-inducing ligand (TRAIL)-mediated T cell apoptosis partly through competitive binding of CRT and TRAIL in RA (data not shown). A recent study showed that thrombospondin 1 (TSP1) signal through the CRT/low density lipoprotein receptor-related protein 1(LRP1) complex in fibroblasts confers resistance to anoikis (anchorage-independent survival) in fibroblasts under non-adherent experimental conditions (42). Interestingly, high expression of CRT can lead to distinct results between cell death and survival. In human colorectal cancer cells, high expression of CRT induced by chemotherapeutics drugs could increase the immunogenic cell death including apoptosis and autophagy, and the cell surface exposure and secretion of CRT were regulated by PI3K/Akt signal pathway (43). The distinction may be due to the fact that colorectal cancer and RA are two different diseases. In addition, cell physiological characteristics between human colorectal cancer cells and RA FLS are not consistent. Our results also showed no significant effect of CRT on the proliferation of RA FLS. These findings indicated that CRT might be involved in the pathogenesis of RA primarily by promoting the apoptotic resistance other than proliferation of RA FLS.

In conclusion, our data identified that increased expression of anti-apoptotic proteins Bcl-XL and Mcl-1 were positively correlated with the high expression of CRT in synovial tissues of RA. CRT could upregulate the expression of Bcl-XL and Mcl-1 in RA FLS, which was PI3K/Akt and STAT3 signal pathways dependent. Moreover, we demonstrated that CRT protected RA FLS from FasL-induced apoptotic death. Taken together, our results suggested that CRT-mediated Bcl-XL and Mcl-1 upregulation promoted anti-apoptosis of FLS through the activation of PI3K/Akt and STAT3 pathways, which might represent a novel therapeutic target for the treatment of RA.

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