Endoplasmic reticulum stress perpetuated toll-like receptor signalling-mediated inflammation in rheumatoid arthritis via X-box-binding protein-1

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Abstract

Objective

Multiple physiological and pathological conditions interfere with the function of the endoplasmic reticulum (ER). However, much remains unknown regarding the impact of ER stress on toll-like receptors (TLRs) -induced inflammatory responses in rheumatoid arthritis (RA). The aim of this study was to reveal the effects of ER stress and its regulator, X-box-binding protein-1 (XBP-1), on the inflammatory response of RA synovial fibroblasts (RASF) to different TLRs ligands.

Methods

ER stress was induced in RASF by incubating with thapsigargin (Tg). TLR2 ligand Pam3CSK4, TLR3 ligand PolyIC, TLR4 ligand LPS were used to stimulate the cells. Effects of ER stress on TLRs-induced inflammatory mediators were determined by using RT-PCR, qPCR and ELISA analysis. Western blots analysis was used to detected the signalling pathways in this process. For gene silencing experiment, control scrambled or XBP-1 specific siRNA were transfected into RASF. T helper (Th)1/Th17 cells expansion was determined by flow cytometry analysis, and IFN-γ/IL-17A production in supernatants were collected for ELISA assay.

Results

ER stress potentiated the expression of inflammatory cytokines, MMPs and VEGF in RASF stimulated by different TLRs ligands, which was companied with enhanced the activation of NF-κB and MAPKs signalling pathways. Silencing XBP-1 in RASF could dampen TLRs signalling-simulated inflammatory response under ER stress. Moreover, blockade of XBP-1 reduced the generation of Th1 and Th17 cells mediated by RASF, and suppressed the production of IFN-γ and IL-17A.

Conclusion

Our findings suggest that ER stress and XBP-1 may function in conjunction with TLRs to drive the inflammation of RASF, and this pathway may serve as a therapeutic target for the disease.

Key word

rheumatoid arthritis, endoplasmic reticulum stress, X-box-binding protein-1, Toll-like receptor signalling

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease, which is characterised by abundant immune cell infiltration and an unusual expansion of the synovium, leading to progressive joint destruction. Although the pathogenesis of RA remains not fully understood, recently an increasing number of studies have shown that RA synovial fibroblasts (RASF) play a major role in initiating and driving RA (1-2). RASF contribute to joint inflammation and bone destruction possibly via multiple mechanisms. RASF can reduce contact inhibition, resist to apoptosis, and increase the ability to invade bone and cartilage (3). RASF, as non-immune cells, can respond to endogenous ligands of pattern recognition receptors and inflammatory cytokines. Moreover, RASF may promote immune responses by interacting with immune cells and supporting ectopic lymphoid-like structure formation in synovial tissues (4).

The endoplasmic reticulum (ER) is the cellular organelle that directs the folding, processing and trafficking of unfolded proteins, which include many key components of the immune response. An accumulation of unfolded or misfolded proteins in the ER leads to stress conditions. Oxidative, proteotoxic, metabolic stresses as well as hypoxia and impaired calcium balance are common inducers of ER stress. The resulting accumulation of misfolded proteins in the ER triggers a set of signals referred as the unfolded protein response (UPR), which aims at relieving the burden by slowing down the global translation rate and increasing the production of a selected set of proteins particular ER chaperone 78 kDa glucose-regulated protein (Grp78) (5). The coordinated UPR acts through triggering three main signalling cascades: inositol-requiring enzyme 1α (IRE1α), protein kinase R-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) pathways (6). IRE1α could generate splice variant of transcription factor X box-binding protein-1 (XBP-1), which activates the genes involved in protein degradation and controls the transcription of chaperones (7). ER stress and UPR signalling pathways have been implicated in the pathogenesis of a variety of human disease conditions ranging from neurodegenerative, pulmonary to viral infections and inflammatory diseases (8-9). In RA, studies have shown that ER stress-associated genes were highly expressed in synovium and synovial macrophages. The ER stress could facilitate the production of IL-6, IL-8 as well as IL-1β in RASF (10). Moreover, TNF-α and IL-1β promoted the expression of Grp78, which was associated with RASF angiogenesis, proliferation and survival (11).

Toll-like receptors (TLRs) are a class of proteins that play a fundamental role in the innate immune system. They recognise ''pathogen-associated molecular patterns (PAMP)'' which are structurally conserved molecules derived from microbes and are distinguishable from host molecules, and activate the innate immune responses (12). TLRs activation induce the downstream signalling via mitogen-activated protein kinase (MAPK) or transcription factors such as nuclear factor-kappa B (NF-κB) or interferon regulatory factor (IRF), resulting in the expression of inflammatory cytokines and type I interferon (13). Our previous studies have shown that RASF expressed a variety of TLRs, such as TLR2, TLR3 as well as TLR4, and these TLRs resulted in the vast production of IL-6, IL-8, MMPs and VEGF in RASF (14). Other studies have found that similar to TNF-α inhibitors, TLR2 blockade significantly inhibited the secretion of IL-6 and IL-1β from RASF (15). A recent research showed that the ligation of TLRs could transform RA myeloid cells into M1 macrophages, and that IL-6 secreted from M1 macrophages and RASF participated in Th17 cell development (16). Together these studies suggest that TLRs could be key mediators involving in promoting inflammatory response in the RA joint.

In this study, we systematically explored the effects of ER stress on the inflammatory response of RASF induced by TLRs signalling, and assessed the central role of XBP-1 in exacerbation the inflammation.

Material and methods

Tissue specimens of patients

Synovial tissue specimens which were acquired from RA patients (n=5; age, mean/range: 60.8/44-66 years; sex, female/male: 3/2) during total knee replacement surgery were used for culturing RASF. All patients fulfilled the American College of Rheumatology 1987 criteria for RA (17). Participants provided their written informed consent before participating in the study. The experiment protocols and consent forms were approved by the Institutional Medical Ethics Review Board of Peking University People's Hospital.

RASF culture and treatment

RASF were cultured as described previously (18), and cells were used at passages 4-6 for study. To induce ER stress, 5×10^4 RASF were stimulated with 1 μM thapsigargin (Tg, Sigma, St. Louis, MO, USA) for 1 h in 6-well plates. Then the cells were treated with stimuli for 4 h, as follows: 25 μg/ml polyinosinic-acid (polyIC) and 100 ng/ml lipopolysaccharide (LPS) (Sigma, St. Louis, MO, USA), 100 ng/ml Pam3CSK4 (Invitrogen, Santa Cruz, California, USA). The cells were collected for RT-PCR and qPCR assay, while the supernatants were harvested for ELISA analysis.

For gene silencing experiment, control scrambled and XBP-1 specific siRNA (Invitrogen, Santa Cruz, California, USA) were transfected into RASF according to the manufacturer's instructions, respectively. In brief, 5×10^4 RASF were planted in 6-well plates for 24 h, and then transfected with 8 μl siRNA using Transfection Reagent (Invitrogen, Santa Cruz, California, USA). After cultured overnight, the cells were collected to test gene knocking-down efficiency by RT-PCR, qPCR and western blot analysis. The cells stimulated by Tg with or without LPS for 4h were then harvested for RT-PCR and qPCR analysis, while the supernatants were collected for ELISA assay.

RT-PCR and qPCR

Total RNA of RASF was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed with the RevertAid First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's instructions. The resulting cDNA was used for RT-PCR and qPCR analyses. RT-PCR was performed to analyse the expression of XBP-1 in RASF. The primers used for detection XBP-1 were as follows: XBP-1 sense primer: 5'-CTGAAGAGGAGGCGGAAGC-3', antisense primer: 5'-AATACCGCCAGAATCCATGG-3'. The RT-PCR products were separated by gel electrophoresis on 2% agarose.

Two-step qPCR was also performed by using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Gene expression was quantified relative to the expression of the housekeeping gene GAPDH, and normalised to control by standard $2^{-\Delta\Delta CT}$ calculation.

Western blot analysis

RASF were treated with 1 μM Tg for 1 h and then incubated with 100 ng/ml LPS for another 0, 5, 15 min. The cells were harvested for western blot analysis as described previously (19). The antibodies used were as follows: anti-phospho-ERK mAb, anti-phospho-IκBα mAb, anti-phospho-JNK mAb, anti-phospho-p38 mAb, anti-p38 mAb, anti-IκBα mAb, anti-ERK mAb, anti-JNK mAb (Cell Signaling Technology, Danvers, MA, USA); anti-GAPDH mAb (Tianjin Sungene Biotech Co, Ltd, Tianjin, China).

ELISA assay

ELISA kits for measuring inflammatory cytokines in the supernatants were used as follows: IL-6, IL-8 ELISA kits (Ray Biotech, Atlanta, USA); TNF-α ELISA kit (Multi Sciences, Hangzhou, China); IL-17A and IFN-γ ELISA kits (Neobioscience Technology Co, Ltd, Beijing, China).

RASF/T cell co-culture assay

5×10^4 RASF were seeded in 6-well plates per well overnight, and then were transfected with XBP-1 siRNA as described above. After 24 h, 100 ng/ml LPS was added and incubated for another 24 h. Then RASF were washed with serum free RPMI 1640 and co-cultured with allogeneic RA patients $CD4^+$ T cells (>95% purity) at the ratio of 1:10 in the presence of anti-CD3 and anti-CD28 antibody (3 μg/ml each). After 5 days, T cells were harvested for flow cytometry analysis and the supernatants were collected for ELISA assay.

Flow cytometry analysis

RA patients' T cells were co-cultured with RASF for 5 days as described above. At the end of co-culture, the cells were treated with PMA (50 ng/ml) and ionomycin (1000 ng/ml) for 5 h in the presence of Brefeldin A (10 μg/ml). After that, T cells were stained with FITC-conjugated anti-CD4, fixed and permeabilised, followed by intracellular staining using PE-conjugated anti-IL-17A and APC-conjugated anti-IFN-γ (eBioscience, San Diego, CA, USA). Percentage of positive cells was analysed on a flow cytometer (Accuri C6, Becton Dickinson, San Diego, CA, USA).

Statistical analysis

SPSS 19.0 (SPSS, Chicago, Illinois, USA) was used for statistical analysis. Differences between groups were assessed by Wilcoxon signed-rank test, and considered statistically significant when p was <0.05.

Results

TLRs agonists stimulated RASF to produce more pro-inflammatory cytokines under ER stress

To evaluate the inflammatory response of RASF under ER stress and TLRs signalling, we first analysed the effect of Tg, a widely used ER stress inducer, on the expression of ER stress specific markers Grp78 and XBP-1. As shown in Figure 1A-B, the mRNA and protein expression of Grp78 was obviously up-regulated in RASF after stimulation with Tg for 5 h and 24 h, respectively. In addition, Tg treatment transformed XBP-1 from unspliced form to spliced form, while ligation of TLR4 with LPS did not appear to affect the Tg-triggered XBP-1 splicing in RASF (Fig. 1C). Taken together, these results suggested that Tg treatment could induce a sustained and maximal ER stress response in RASF, but LPS stimulation did not

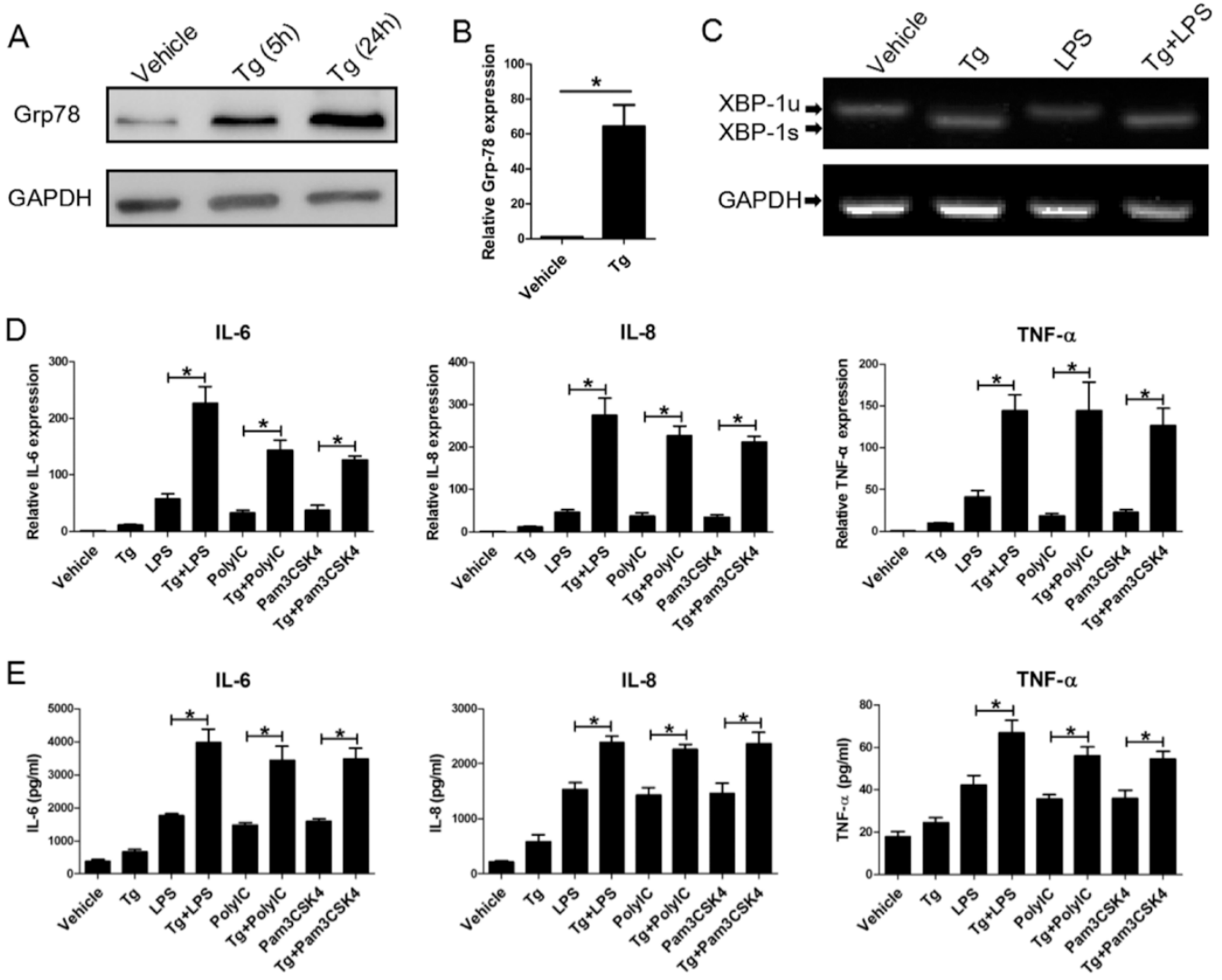

Fig. 1. TLRs agonists stimulated RASF to produce more pro-inflammatory cytokines under ER stress.
A-**B**: ER stress increased the expression of Grp78 in RASF. RASF were treated with Tg for 5 h and 24 h, and were subjected to western blot analysis (**A**) and qPCR assay (**B**).
C: Induction of XBP-1s by ER stress. RASF were pretreated with Tg for 1 h, then stimulated with LPS for another 4 h and were subjected to RT-PCR analysis.
D-**E**: Enhanced IL-6, IL-8 and TNF-α production in RASF by ER stress and different TLRs agonists. RASF were treated with Tg for 1 h and were stimulated with LPS, PolyIC and Pam3CSK4 for another 4 h, and then the cells were harvested for qPCR analysis (D) and cell culture supernatants were collected for ELISA analysis (**E**). Differences between various groups were evaluated by Wilcoxon signed-rank test, *p<0.05.
The results were presented as mean+SEM of five independent experiments.

affect the ER stress-associated genes expression.

It has been reported that TLRs signalling activation or ER stress status could induce RASF to produce pro-inflammatory cytokines (20-21), we next tested the effect of ER stress on pro-inflammatory cytokines production in RASF induced by different TLRs agonists, including TLR2 agonist Pam3CSK4, TLR3 agonist polyIC, and TLR4 agonist LPS. We found that Tg had a negligible effect on the mRNA expressions and supernatant levels of IL-6, IL-8 and TNF-α but markedly enhanced TLRs agonists-stimulated IL-6, IL-8 and TNF-α expression (Fig. 1D-E). Our data indicated that TLRs agonists combined with Tg could aggravate inflammatory response in RASF.

ER stress synergised with TLRs agoniststo promote higher expression of MMPs and VEGFin RASF

Angiogenesis and cellular invasion play an important role in the progression of synovitis. VEGF is the most potent angiogenic factor produced by RASF, which is essential in bone destroy and exacerbating joint damage (22). In addition, RASF could invade the extracellular matrix and secrete MMPs into synovial fluid, which are crucial for the degradation of basement membranes, migration and invasion of RASF (23). In order to further observe the effect of TLRs activation combined with ER stress on RASF, we detected the mRNA expressions of MMP1, MMP3, MMP9 and VEGF in RASF when pretreated with Tg for 1h and/or incubated with TLRs agonists for another 4 h. The results showed that the mRNA expressions of MMP1, MMP3, MMP9 and VEGF were all significantly up-reg-

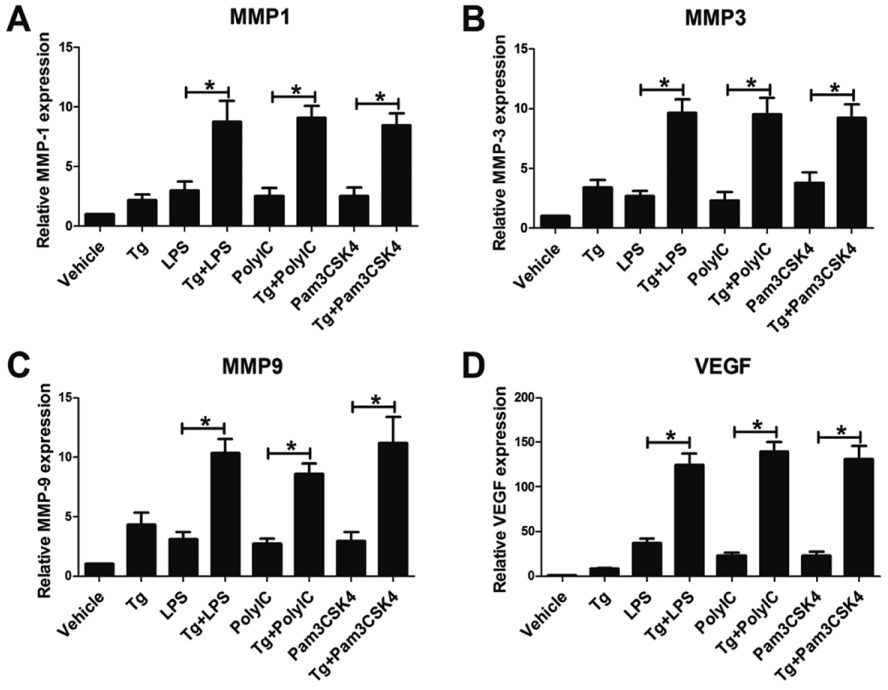

Fig. 2. ER stress synergised with TLRs agonists to promote higher expression of MMPs and VEGF in RASF. Augmented production of MMPs and VEGF in RASF by ER stress and TLRs agonists. RASF were pretreated with Tg for 1 h and were stimulated with LPS, PolyIC as well as Pam3CSK4 for another 4 h. Then the cells were harvested for qPCR analysis of MMP-1 (**A**), MMP-3 (**B**), MMP-9 (**C**) and VEGF (**D**) expression. Differences between various groups were evaluated by Wilcoxon signed-rank test, *$p<0.05$. The results were presented as mean+SEM of five independent experiments.

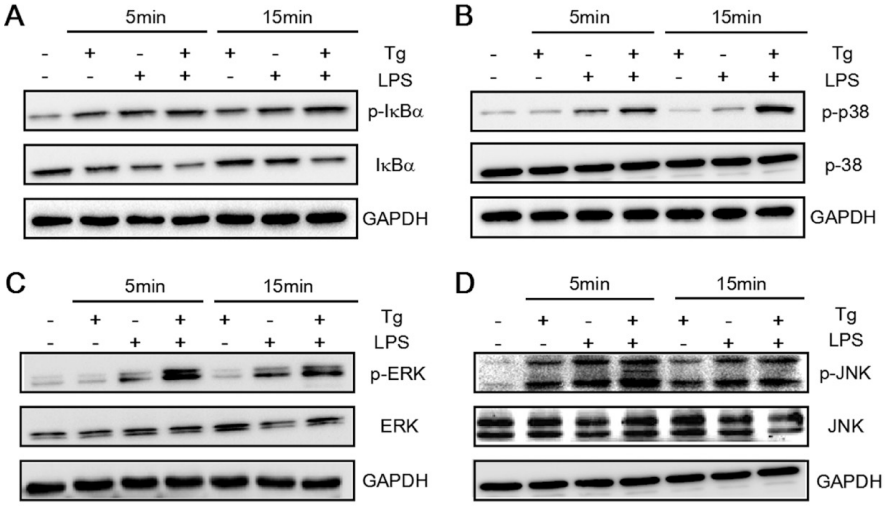

Fig. 3. ER stress promoted the activation of NF-κB and MAPKs signalling pathways induced by LPS, which might initiate the inflammatory response in RASF. RASF were stimulated with Tg for 1 h and the cells were incubated with LPS for 0, 5, 15 min, respectively. Then the cells were harvested and lysed for western blot analysis of p-IkBα (**A**), p-p38 (**B**), p-ERK (**C**), and p-JNK (**D**).
GAPDH was used as the loading control. The results were representative of at least three experiments.

ulated in RASF when stimulated with Tg combined with LPS, Pam3CSK4 as well as PolyIC compared to that only stimulated with Tg or TLRs agonists. Our results further demonstrated that Tg synergised with TLRs activation could potentiate the inflammatory effects of RASF.

ER stress promoted the activation of NF-κB and MAPKs signalling pathways induced by LPS, which might initiate the inflammatory response in RASF

Numerous findings have revealed that NF-κB and MAPKs signalling pathways are involved in inflammatory responses (24-25). To investigate whether TLRs agonists-induced inflammatory responses in RASF under ER stress are associated with NF-κB and MAPKs signalling pathways, we stimulated RASF with Tg for 1 h and incubated the cells with LPS for 0, 5, 15 min, and then detected the downstream signalling pathways response. Our results showed that after LPS stimulation, the NF-κB pathway and the MAPK pathway, including the ERK pathway, the p38 pathway, as well as the JNK pathway became activated, which could be seen from 5mins until 15 mins after stimulation. Moreover, Tg treatment could enhance LPS-induced the activation of NF-κB and MAPK signalling pathway in RASF (Fig. 4A-D). These data implied that ER stress might promote the inflammatory response in RASF through further activating the NF-κB and MAPK signalling pathways induced by LPS.

XBP-1 contributed to ER stress-augmented inflammatory response in RASF

XBP-1 plays a crucial role in ER stress-mediated inflammatory response, and it can be a link between innate immunity and ER stress (26). In order to investigate the potential role of XBP-1 involvement in ER stress-amplified inflammatory response, we silenced XBP-1 by siRNA in RASF. The knockdown efficiency of XBP-1 was evaluated with RT-PCR, qPCR and western blot analysis (Fig. 4A-B). We found when XBP-1 was blocked, the mRNA expressions and supernatant levels of IL-6, IL-8, MMPs and VEGF in RASF stimulated by Tg combined with LPS were significantly decreased (Fig. 5C-H). Our results suggested that XBP-1 contributed to the ER stress-augmented inflammatory cytokine production in RASF under TLRs ligation.

Th1 and Th17 cell expansion was significantly dampened when co-cultured with XBP-1-silenced TLR ligand-activated RASF

Our previous studies found that activated RASF could promote Th17 and Tfh-like cells expansion via IL-6 (27-29), suggesting that RASF could induce inflammatory responses via affecting T

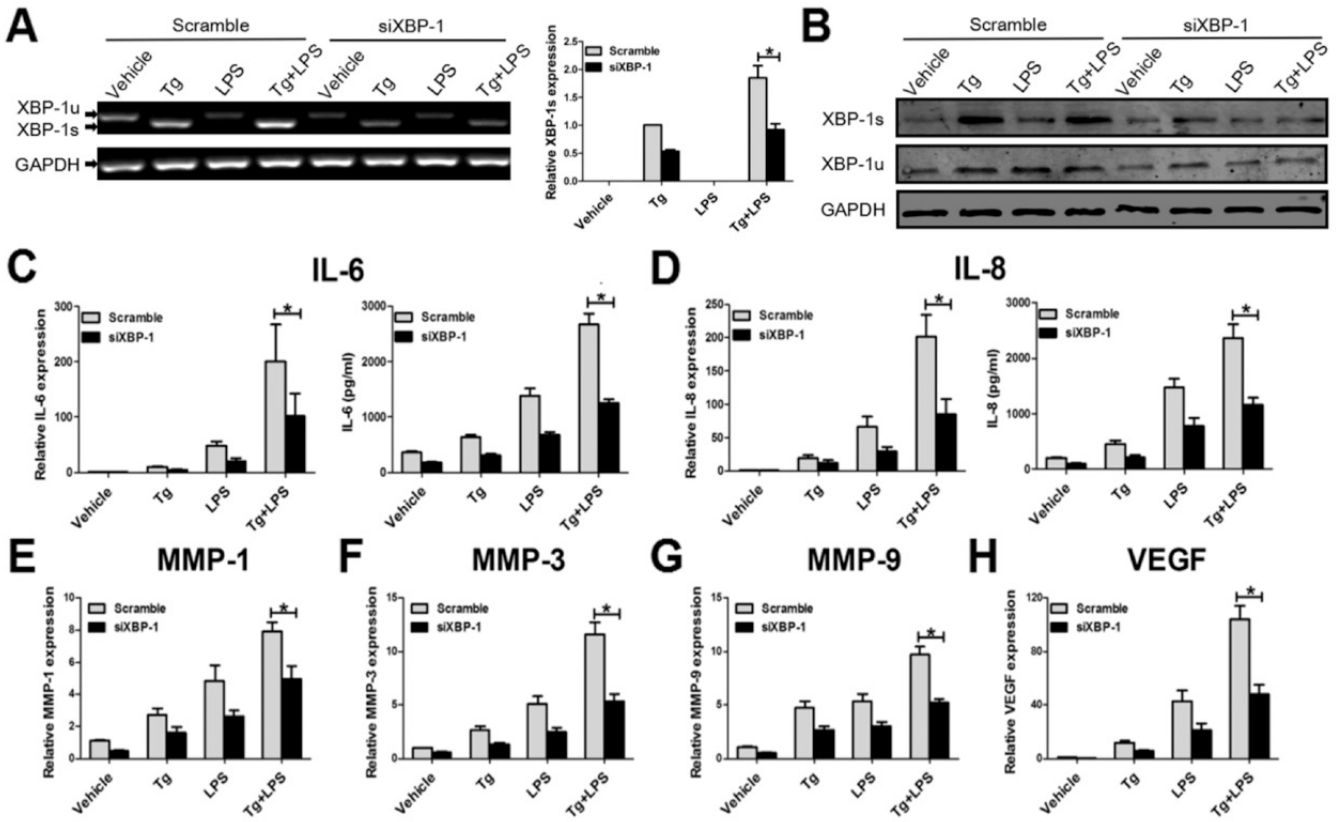

Fig. 4. XBP-1 contributed to ER stress-augmented inflammatory response in RASF. Silencing XBP-1 abolished ER stress-augmented inflammatory cytokine production induced by LPS. RASF were transfected with scramble or XBP-1 siRNA. After 24 h, cells were stimulated with LPS for 4 h. XBP-1 knockdown efficiency was confirmed by using RT-PCR, qPCR and western blot analysis (**A**-**B**). The levels of IL-6 (**C**) and IL-8 (**D**) were assayed with qPCR and ELISA analysis. Accordingly, qPCR analysis was also performed for detecting MMP-1 (**E**), MMP3 (**F**), MMP9 (**G**) and VEGF (**H**) expression. Differences between various groups were evaluated by Wilcoxon signed-rank test, *$p<0.05$. The results were presented as mean+SEM of five independent experiments.

cells. Based on aforementioned results that inflammatory cytokines production was down-regulated in XBP-1-silenced RASF, we were curious of the effect of XBP-1-silenced RASF on inflammatory T cells such as Th1 and Th17 cells. After transfected with XBP-1 siRNA or control scramble, RASF were stimulated with LPS, and then co-cultured with $CD4^+$ T cells from RA patients. The results showed that the frequencies of Th1 and Th17 cells and supernatant levels of IFN-γ and IL-17A were significantly decreased in T cells co-cultured with XBP-1-silenced RASF than with untransfected RASF after LPS stimulation (Fig. 5A-B). These results suggested that XBP-1 silencing in RASF may decrease the expansion of inflammatory Th1 and Th17 cells, which can ameliorate RA inflammatory conditions.

Discussion

In the present study, we showed that ER stress could enhance the production of inflammatory cytokines, MMPs and VEGF in RASF stimulated by different TLRs ligands, which may be associated with the activation of the NF-κB and MAPKs pathways. Additionally, XBP-1 played a pivotal part in this process, and silencing XBP-1 in RASF could dampen the inflammatory response stimulated by TLRs signalling under ER stress and reduced the generation of Th1 and Th17 cells mediated by RASF.

One of the most significant underlying factors in RA pathogenesis is infection. In RA inflamed joints present a lot of peptidoglycan, bacterial, viral DNA and viral RNA. These PAMPs can activate the corresponding TLRs generally expressed in RA synovium, inducing the production of inflammatory mediators. Endogenous heat shock protein, fibrinogen, hyaluronan and double stranded RNA from apoptotic cells, which are regarded as danger-associated molecular patterns (DAMP), have been found to exist in RA joints and also could provoke TLRs signalling to induce inflammation (30). ER stress plays a crucial role in both physiological and pathological immune responses, and it occurs when the amount of newly synthesised proteins in the ER exceeds the organelle's capacity to ensure their proper folding. In this study, we showed that ER stress synergised with TLRs signalling could induce more robust inflammatory mediator production. In RA inflamed synovium, ER stress is a common cellular response to many of the conditions RASF encounters, our results suggest that during bacterial or viral infection, RASF become more active to produce inflammatory mediators, thus exacerbating RA severity.

It is well documented that TLRs signalling promote cytokines production by activating downstream transcription factors, including NF-κB, MAPK pathway and IRFs, during innate immune responses to pathogens (31). NF-κB is a transcription factor regulating gene

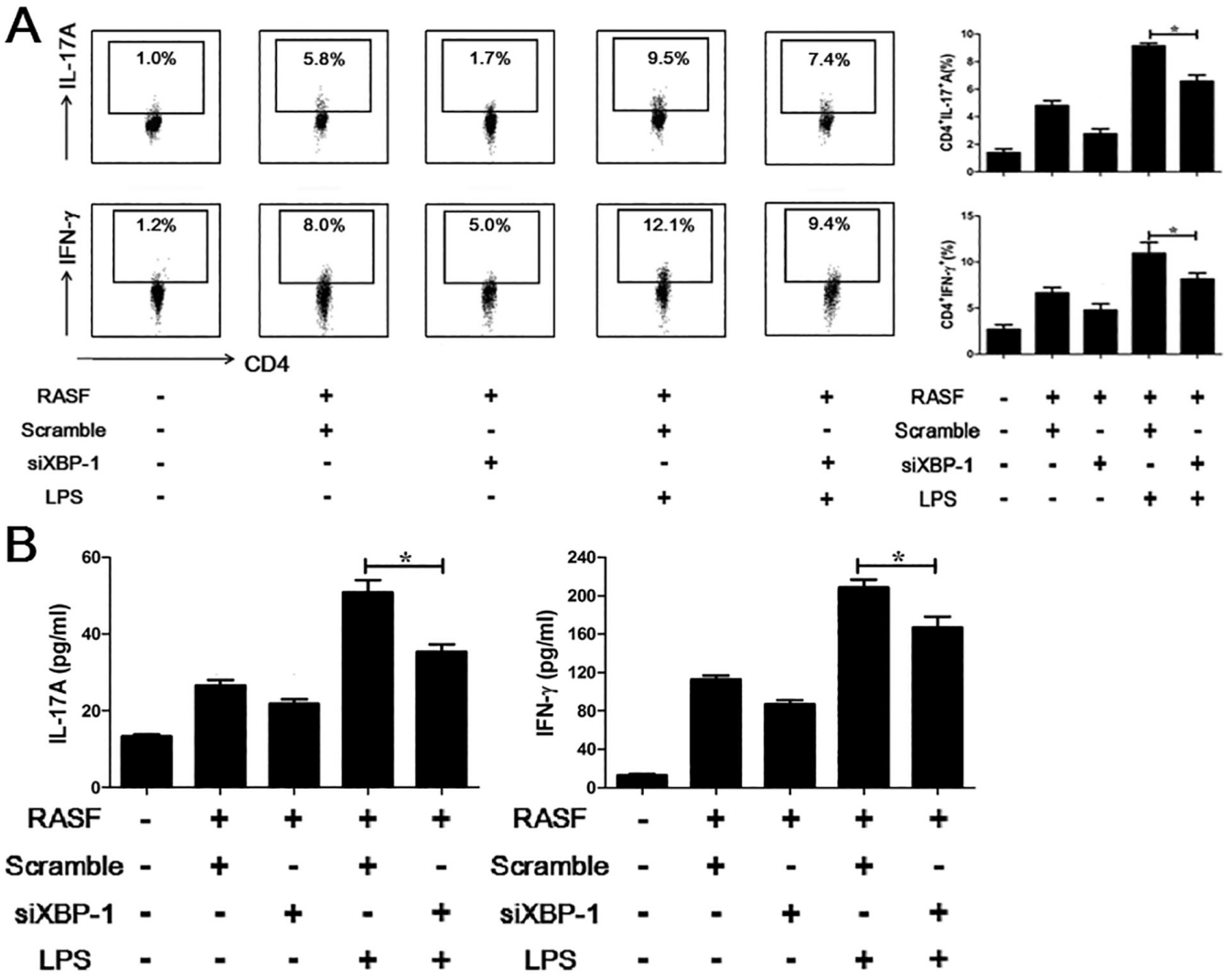

Fig. 5. Th1 and Th17 cell expansion was significantly dampened when co-cultured with XBP-1-silenced TLR ligand-activated RASF. XBP-1 knockdown dampened the expansion of inflammatory Th1 and Th17 cells mediated by RASF. RASF were transfected with siRNA XBP-1 for 24 h, and then were stimulated with LPS for another 24 h. After that, the cells were co-cultured with allogeneic T cells isolated from RA patients in the presence of anti-CD3 and anti-CD28 antibody for 5 days, and the T cells were harvested for flow cytometry analysis of the frequencies of Th1 and Th17 cells (**A**). RASF and T cell co-culture was performed as described above, and the cell culture supernatants were collected for ELISA analysis of IFN-γ and IL-17A levels (**B**). Differences between various groups were evaluated by Wilcoxon signed-rank test, *$p<0.05$.
The results were presented as mean+SEM of five independent experiments.

expression, which controls multiple cellular functions, such as inflammation, stress-induced responses as well as survival, and aberrant NF-κB pathway regulation is involved in many diseases (32). MAPKs pathway is a group of serine/threonine protein kinases and the members of the major MAPKs cascade components p38, ERK and JNK, which mediated a wide range of cellular processes, including cell proliferation, differentiation, apoptosis and immune response (33). In this study, we found that the synergistic effect of ER stress on LPS induced the generation of inflammatory mediators was controlled by NF-κB and MAPKs signalling pathways. This data confirmed previous researches showing that ER stress could induce the NF-κB pathway activation through the repression of the PERK-eIF2α-mediated translation.

XBP-1 is a major regulator of the ER stress response, which is induced through the activation of the IRE1 stress sensor. It is known that XBP-1 could induce the expression of genes involved in restoring protein folding or degrading unfolded proteins. Earlier studies have suggested that XBP-1 played an essential role in controlling TLRs signalling. Active XBP-1 could induce the expression of UPR target genes, stimulate the production of IL-6 and TNF-α by enhancing TLRs signalling, and promote the differentiation of B lymphocytes and dendritic cells (34). In macrophages, both TLR4 and TLR2 were able to specifically activate IRE1α to promote IL-6 expression in spite of the absence of an ER stress response (35). In addition, the XBP-1 deletion in macrophages could reduce the production of TNF-α and IL-1β by LPS (36). These data suggest that ER stress-related proteins affect pro-inflammatory cytokine production through modulating TLRs signalling. In this study, we found

that knocking-down XBP-1 expression in RASF could abrogate the synergy between ER stress and TLRs signalling, further elucidating the potential bridging role of XBP-1in ER stress and innate immunity. Similar to our findings, another study has shown that XBP-1 as a critical role for TLR-dependent IRE1 activation in the production of IL-6 and TNF-α in RA patients, and anti-TNF biologics therapies could be altered this pathway (21).

Th1 cells are considered to be the conventional pathogenic cells in RA, because RA is thought to be a Th1-biased disease with Th1/Th2 imbalance. Recently, attention has increasingly focused on the role of Th17 cells, a subset that produces IL-17A, IL-17F, IL-21, IL-22 and TNF-α (37). IL-17A, which synergises with TNF-α to promote the activation of fibroblasts and chondrocytes, is currently being targeted in clinical trials (38). In RA, synovial fibroblasts and T cells form a costimulatory circuit to perpetuate the inflammation. RASF could inhibit the apoptotic process of T cells and elicit their spontaneous proliferation; in turn, the increased inflammatory T cells induced more robust RASF activation (39). Lately, XBP-1 was identified as a crucial gene in the development of T cells. In mice, XBP-1 could directly enhance Th17 differentiation under hypoxic conditions, and the generation of Th17 cells was markedly reduced in the absence of XBP-1(40). In the current study, we have demonstrated that knocking-down the expression of XBP-1 inhibited RASF-mediated expansion of inflammatory Th1 and Th17 cells, which could ameliorate RA inflammatory states. Our study not only further supports the pro-inflammatory role for IRE1α-XBP-1 signalling axis, but also it demonstrates the effect of XBP-1 in RASF-mediated augment of inflammatory Th1 and Th17 cells responses.

In short, our results suggested that ER stress and XBP-1 act in conjunction with TLRs signalling to drive the inflammation in RA. This promotes us to understand the role of RASF in the pathogenesis of RA, and further indicates that XBP-1 may be a new therapeutic target for the chronic inflammatory disease.

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