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TNF-α/calreticulin dual signaling induced NLRP3 inflammasome activation associated with HuR nucleocytoplasmic shuttling in rheumatoid arthritis

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Abstract

Objective The present study was undertaken to validate whether TNF-α and calreticulin (CRT) serve as dual signaling to activate nucleotide-binding oligomerization domain-, leucine-rich repeat- and pyrin domain-containing 3 (NLRP3) inflammasome in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) and HUVECs. The effect of human antigen R (HuR) in NLRP3 inflammasome activation was also explored in RA FLS.

Methods Immunofluorescence was used to determine the expression of NLRP3 and adaptor protein apoptosis associated speck-like protein containing a CARD (ASC) in RA synovial tissue and HuR location in RA FLS. Western blot and quantitative real-time PCR were employed to measure the priming effect of NLRP3 inflammasome in cells and HuR expression in synovial tissue. The concentrations of IL-1β and IL-18 were detected by enzyme linked immunosorbent assay. Immunohistochemistry was used to visualize the expression of HuR in synovial tissue. HuR knockdown in RA FLS was achieved by siRNA-mediated gene silencing.

Results Higher expression of NLRP3 and ASC in RA synovial tissue than those in osteoarthritis was detected. The staining of NLRP3, ASC and cleaved IL-1β were observed in FLS and vascular endothelial cells in RA synovium. Expression of NLRP3 and pro-IL-1β in RA FLS and HUVECs treated with TNF-α was increased. The pro-IL-18 expression was also enhanced in HUVECs, but not in RA FLS. TNF-α/CRT dual stimulation of cells gave rise to caspase-1 p20 expression and the secretion of IL-1β. The secreted IL-18 was also elevated in HUVECs but not in RA FLS. HuR expression was significantly elevated in RA synovial tissue. TNF-α initiated the nucleocytoplasmic shuttling of HuR in both FLS and HUVECs. The knockdown of HuR in FLS incubated with TNF-α led to reduced caspase-1 p20 protein expression and further resulted in decreased secretion of IL-1β in the presence of CRT.

Conclusions TNF-α/CRT dual signaling induced NLRP3 inflammasome activation, which could be suppressed by HuR knockdown presumably due to the block of HuR translocating from nucleus to cytoplasma.

Keywords NLRP3 inflammasome · Calreticulin · Human antigen R · Tumor necrosis factor-α · Rheumatoid arthritis · Fibroblast-like synoviocytes

Introduction

The nucleotide-binding oligomerization domain-, leucine-rich repeat- and pyrin domain-containing 3 (NLRP3) protein is a member of the nucleotide-binding domain and leucine-rich repeat containing (NLR) family, serving as the pattern recognition receptor (PRR) sensor of the NLRP3 inflammasome. The NLRP3 inflammasome plays a pivotal role in inflammation and autoimmunity predominantly due to its ability to induce production of the pro-inflammatory cytokines IL-1β and IL-18 and drive pyroptotic cell death [1]. Actually, NLRP3 inflammasome is a cytoplasmic protein

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complex, and the activation of it involves two stages. Before oligomerization of this complex, a priming step must occur first. The expression of NLRP3 is induced by priming at transcriptional [2] or post-translational [3] levels, which provides the first signal for NLRP3 inflammasome activation. Then the system can be ultimately activated by a large number of second signals including exogenous microbe-associated molecular patterns or endogenous damage-associated molecular patterns (DAMPs), leading to the combination of NLRP3 with the adaptor protein apoptosis associated speck-like protein containing a CARD (ASC). The whole event initiates the autocatalytic activation of caspase-1. Cleaved caspase-1 subsequently triggers the maturation of the precursors of IL-1β and IL-18 [4]. Typically, priming is accomplished in vitro using a microbial Toll-like receptor (TLR) ligand such as LPS [2, 5]. However, it has been clarified that the inflammasome is activated during the progression of sterile inflammatory diseases such as rheumatic disease, atherosclerosis and metabolic disease. Therefore, it is time to consider the endogenous factors (e.g., tumor necrosis factor) and mechanisms that mediates priming and activation if the inflammasome in these conditions.

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by invasive synovial inflammation. There are a few data on NLRP3 inflammasome expression in RA. Sidiropoulos et al. showed that expression of NLRP3, caspase-1 and IL-1β were enhanced in peripheral blood cells of patients with active RA [6, 7]. The potentially pathogenic effect of NLRP3 inflammasome in RA is also illustrated by the discovery that lower NLRP3 activity in PBMC and granulocyte of RA serves as a protective factor [8] and that single nucleotide polymorphisms (SNPs) in NLRP3 are closely related to RA susceptibility [9]. Moreover, excessive NLRP3 inflammasome activation drives arthritis pathogenesis in $A20^{myel\text{-}KO}$ mice [10].

The RNA-binding protein (RBP) human antigen R (HuR/ELAVL1) is a member of the embryonic lethal abnormal vision (ELAV)-like/Hu-protein family of RBPs [11]. It is one of the most extensively studied regulators of the post-transcriptional gene expression and plays an important role in inflammation and cancer, apoptosis and proliferating and angiogenesis [12]. HuR protein is comprised of three RNA recognition motifs (RRMs) and a flexible hinge region, predominantly recognizing the AU-rich elements (ARE) in 3′ UTR of target mRNAs by RRMs to stabilize these mRNAs and promote the translation of them. HuR is a nucleocytoplasmic shuttling protein and primarily located in the nucleus [13, 14] in rest cells. Upon various stimuli (e.g., viral infection, cytokines, heat shock, and UV irradiation), HuR translocates to the cytoplasm, which is required for its mRNA-stabilizing function [15]. The potential mechanism by which HuR achieves these effects is that HuR prevent target mRNAs from degradation by competing with other RBPs (such as tristetraprolin) and microRNAs [14, 16]. Currently, many factors have been demonstrated to be downstream targets of HuR, especially those of cytokines, oncogenes, and inflammatory factors. Therefore, increased HuR expression or aberrant nuclear/cytoplasmic distribution is always accompanied with exacerbation of inflammation and poor outcomes of cancer.

CRT is an endoplasmic reticulum (ER) resident protein responsible for maintaining Ca^{2+} homeostasis and glycoprotein folding [17]. Recently, extracellular CRT has emerged as a member of DAMPs which present at higher concentrations in the plasma and synovial fluid of RA patients, playing a pro-inflammatory role in RA [18–20]. Existing research found that extracellular CRT was increased in the joints of RA patients and inhibited FasL-mediated apoptosis of T cells [18]. Our previous study showed that serum CRT levels were closely related to RA disease activity score (DAS28) [19] and that extracellular CRT promoted angiogenesis in RA [20]. Although CRT has been implied to be involved in RA synovitis, the pathogenic roles of CRT have not been investigated thoroughly.

This study was undertaken to explore the effect of dual signals of TNF-α (a most dominant pro-inflammatory factor in RA) and extracellular CRT on NLRP3 inflammasome activation in fibroblast-like synoviocytes (FLS) and vascular endothelial cells in RA [21, 22]. We also aimed to investigate whether HuR, a critical regulator of the post-transcriptional gene expression, plays a role in NLRP3 inflammasome activation mediated by TNF-α/CRT dual signaling in RA FLS, which may provide a novel target for clinical treatment of RA.

Materials and methods

Patients and samples

Synovial membrane (SM) tissues were obtained from patients with RA ($n = 8$) and osteoarthritis (OA) ($n = 10$) during synovectomy at General Hospital, Tianjin Medical University, Tianjin, China, and Tianjin Hospital, Tianjin, China. Patients who suffered from other chronic diseases or any acute infections within 3 months were excluded from this study. All patients with RA fulfilled the American College of Rheumatology and European League Against Rheumatism (ACR/EULAR) 2010 criteria for RA [23] and all patients with OA fulfilled the ACR 1995 criteria for OA [24]. Local ethics approval for all experiments was provided by the Medical Ethics and Human Clinical Trial Committee of Tianjin Medical University (ethical approval number TMUhMEC 2013031). The informed consent was obtained from all patients.

SM samples shipped in liquid nitrogen were then frozen for immunofluorescence. SM samples shipped in sterile RPMI-1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS) were then fixed with 4% paraformaldehyde for 48 h at 4 °C for paraffin embedding and immunohistochemistry, or stored at 4 °C for FLS isolation.

Immunofluorescence analysis

The SM tissue sections (5 μm thick) were prepared using a LEICA CM 1860 cryostat. Slides were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 1 h at room temperature. Primary antibodies were added in blocking buffer (1:50 dilution) and slides were incubated overnight at 4 °C. The primary antibodies are as followed: Goat anti-human NLRP3 (ab4207, Abcam), rabbit anti-human TMS1 (ASC) (GTX102474, GeneTex), rabbit anti-human cleaved IL-1β (83186S, Cell Signaling Technology), mouse anti-human PDPN (MCA2543, AbD Serotec), mouse anti-human Endosialin (CD248, clone B1/35) (MAB2626, Millipore) and mouse anti-human CD31 (ab24590, Abcam). After washing with TBS for three times, the slides were incubated with secondary antibodies in blocking buffer (1:500 dilution). The secondary antibodies are as followed: Alexa Fluor™ 488 conjugated donkey anti-goat IgG or Alexa Fluor™ 488 conjugated donkey anti-rabbit IgG and Alexa Fluor® 594 conjugated donkey anti-mouse IgG (all from Invitrogen, Carlsbad, CA, USA). Slides were then washed in TBS and counterstained with DAPI for 10 min. Images were obtained using a 20 × or 40 × objective on a fluorescence microscopy.

For cell immunofluorescence assay, RA FLS and HUVECs were fixed with 4% paraformaldehyde for 20 min, permeated with 0.5% triton X-100 for 10 min, blocked with 5% BSA for 1 h and incubated with mouse anti-human primary antibody recognizing HuR (sc-5261, Santa Cruz Biotechnology). Alexa Fluor™ 488 conjugated donkey anti-mouse IgG was used to detect primary antibody–antigen complexes. After counterstained with DAPI, sections were observed by a fluorescence microscopy. All images were processed using Image J software to calculate mean fluorescence intensity.

Cell preparation and stimulation

For isolation of RA FLS, the synovium tissue 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) (Gibco, USA) for 1 h at 37 °C, then centrifuged, resuspended, and cultured in DMEM supplemented with 10% FBS at 37 °C in 5% CO_2 atmosphere. FLS between passage 3 to 8 were used for the subsequent experiments. Cells were synchronized in serum starvation media (DMEM containing 0.1% FBS) for 24 h before indicated stimulation.

For preparation of human umbilical vein endothelial cells (HUVECs), primary HUVECs were isolated from fresh human umbilical cords via collagenase digestion, and immediately maintained in DMEM supplemented with 10% FBS at 37 °C in an atmosphere of 5% CO_2. All HUVECs were used after no more than five passages. A total of 4×10^5 cells per condition in 2 ml DMEM containing 10% FBS were used for stimulation experiments.

For NLRP3 inflammasome priming, the cells were treated with TNF-α (0, 10, 100 ng/ml) (PEPROTECH, USA) or LPS (0, 20, 200 ng/ml) (Solarbio Life Science, Beijing, China) for 6 h. For inflammasome activation, the cell culture supernatant of TNF-α or LPS pre-treated cells was discarded, and cells were incubated with complete medium containing 10 μM Nigericin (Invitrogen) for 1 h. Cell culture supernatant was discarded again and the cells were continue to be cultured with complete medium for 36 h. Alternatively, the cell culture supernatant of TNF-α or LPS pre-treated cells was discarded, and cells were incubated with CRT (0, 1, 10, 20 μg/ml) (ab40609, Abcam) for 36 h.

Western blot analysis

Proteins were extracted from RA FLS, HUVECs or SM tissue using RIPA lysis buffer supplemented with PMSF (Solarbio Life Science) and quantified with a BCA protein concentration determination kit (Beyotime, Shanghai, China). Cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, USA) at 250 mA for 1.5 h at 4 °C. The membranes were blocked with 5% BSA (diluted with TBS containing 0.1% Tween-20) for 1 h at room temperature and incubated with primary antibodies overnight at 4 °C. The following antibodies were used: NLRP3 (AG-20B-0014-C100), caspase-1 p20 (AG-20B-0048B-C100) (both from AdipoGen Life Sciences, USA), pro-IL-1β (12242S, Cell Signaling Technology), IL-18 (PAA064Hu01, Cloud-Clone Corp, Wuhan, China), HuR (sc-5261, Santa Cruz Biotechnology) and β-actin (TA-09, ZSGB-BIO, Beijing, China). After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody (ZSGB-BIO, Beijing, China) for 1 h at room temperature. Proteins were detected using an Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).

Quantitative real-time PCR

Total cellular RNA from cells and SM tissue were extracted using TRIzol (Invitrogen) and converted to cDNA using

FastQuant cDNA First Strand cDNA Synthesis Kit (TianGen, Beijing, China). Real-time PCR amplification was performed using the platinum SYBR Green SuperReal PreMix Plus (Tiangen Biotech, Beijing, China), following the manufacturer's instructions. Results were normalized with the ACTB transcript as an internal control and then used to calculate expression levels according to the ΔΔ comparative threshold method [25]. The following primers pairs were used: GAPDH: forward, 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse, 5′-TGGTGAAGACGCCAGTGGA-3′; NLRP3: forward, 5′-AACAGCCACCTCACTTCCAG-3′ and reverse, 5′-CCAACCACAATCTCCGAATG-3′; IL-1β: forward, 5′-CCAGGGACAGGATATGGAGCA-3′ and reverse, 5′- TTCAACACGCAGGACAGGTACAG-3′; IL-18: forward, 5′-GGCCTCTATTTGAAGATATGACT-3′, and reverse, 5′-AGTATGTATAAAGATAGCCAGCCTAGAGG-3′; HuR: forward, 5′-CCGTCACCAATGTGAAAGTG-3′ and reverse, 5′-TCGCGGCTTCTTCATAGTTT-3′.

ELISA

The secretion of IL-1β and IL-18 in cell culture supernatant was quantified by Human IL-1β and IL-18 Enzyme linked immunosorbent assay (ELISA) Kit (Elabscience, Wuhan, China) according to the manufacturer's protocol. The concentrations were assessed by a microplate reader (Synergy 2) at 450 nm.

Immunohistochemistry

The paraffin embedded human SM sections were dewaxed in xylene and hydrated through ethanol to water, then depleted of endogenous peroxidase activity by adding 0.3% hydrogen peroxide. Slides were blocked with 5% BSA for 1 h at room temperature. The sections were incubated overnight at 4 °C with mouse anti-human HuR (sc-5261, Santa Cruz Biotechnology) at 1:100 dilution. After washing with PBS for three times, the slides were incubated with HRP-conjugated anti-mouse secondary antibody (ZSGB-BIO) for 1 h at room temperature. Chromogenic reaction was developed with diaminobenzidine for 5–10 min. The sections were counterstained with haematoxylin, dehydrated and mounted. The HuR expressions were observed by microscopy and processed using Image J software to calculate mean optical intensity.

siRNA-mediated gene silencing

Cells were seeded in 6-well plates the day before transfection. Adjust the cell density so that 60–80% confluency were reached at the time of transfection. After washing with PBS, cells were incubated with siRNA at 37 °C in 5% CO_2 atmosphere for 4–6 h. siRNA was prepared by mixing lipofectamine 3000-containing Opti-MEM (Invitrogen) and siRNA-containing Opti-MEM together, and this mixture was then incubated at room temperature for 30 min. Cells were generally used for assays 24 h post-transfection. Then cells were stimulated as indicated for inflammasome priming and activation experiment. Negative control siRNA (SI03650325) and HuR siRNA (SI00300139) were purchased from Qiagen. siRNA sequences were: NC siRNA (5′-UUAAGAGGCUUGCACAGUGCA-3′), HuR siRNA_1 (5′-UUCAUCGUCCUGUGUCGAACC-3′;), HuR siRNA_2 (5′-UUGGACUGAAGCCUCAAGCCG-3′).

mRNA stability measurement

Cells transfected with NC siRNA or HuR siRNA were incubated with TNF-α for 6 h and then treated with 2 μg/ml of actinomycin D (Santa Cruz Biotechnology) to stop transcription and then lysed for NLRP3 mRNA analysis at the indicated times after actinomycin D addition.

Statistical analysis

Data were presented as mean ± standard deviation (SD) and were processed with SPSS software 16.0 (SPSS Inc., USA). Student's t test was used for a comparison between two different groups. Differences among multi-group were analyzed with one-way analysis of variance (ANOVA), and Student–Newman–Keuls test was further employed for a comparison between two groups. A p value of less than 0.05 was considered statistically significant.

Results

Increased expression of NLRP3 inflammasome related proteins in rheumatoid synovium

Expression of NLRP3 (0.089 ± 0.005, 0.074 ± 0.003, $t = 2.559$, $p = 0.034$) and ASC (0.076 ± 0.006, 0.056 ± 0.006, $t = 2.406$, $p = 0.043$) protein were significantly elevated in synovial tissue of patients with RA compared with OA, respectively (Fig. 1a–d). The glycoproteins podoplanin (PDPN) and CD248 are thought to identify discrete populations of fibroblasts in the rheumatoid synovium [26]. NLRP3, ASC and cleaved IL-1β were all present in PDPN+ and CD248+ fibroblast as co-localizations of these NLRP3 inflammasome related proteins with PDPN and CD248 were observed, respectively (Fig. 2a–c). There were also staining for NLRP3, ASC and cleaved IL-1β in CD31+ vascular endothelial cells (Fig. 2a–c). Isotype controls are in online supplementary figure S1.

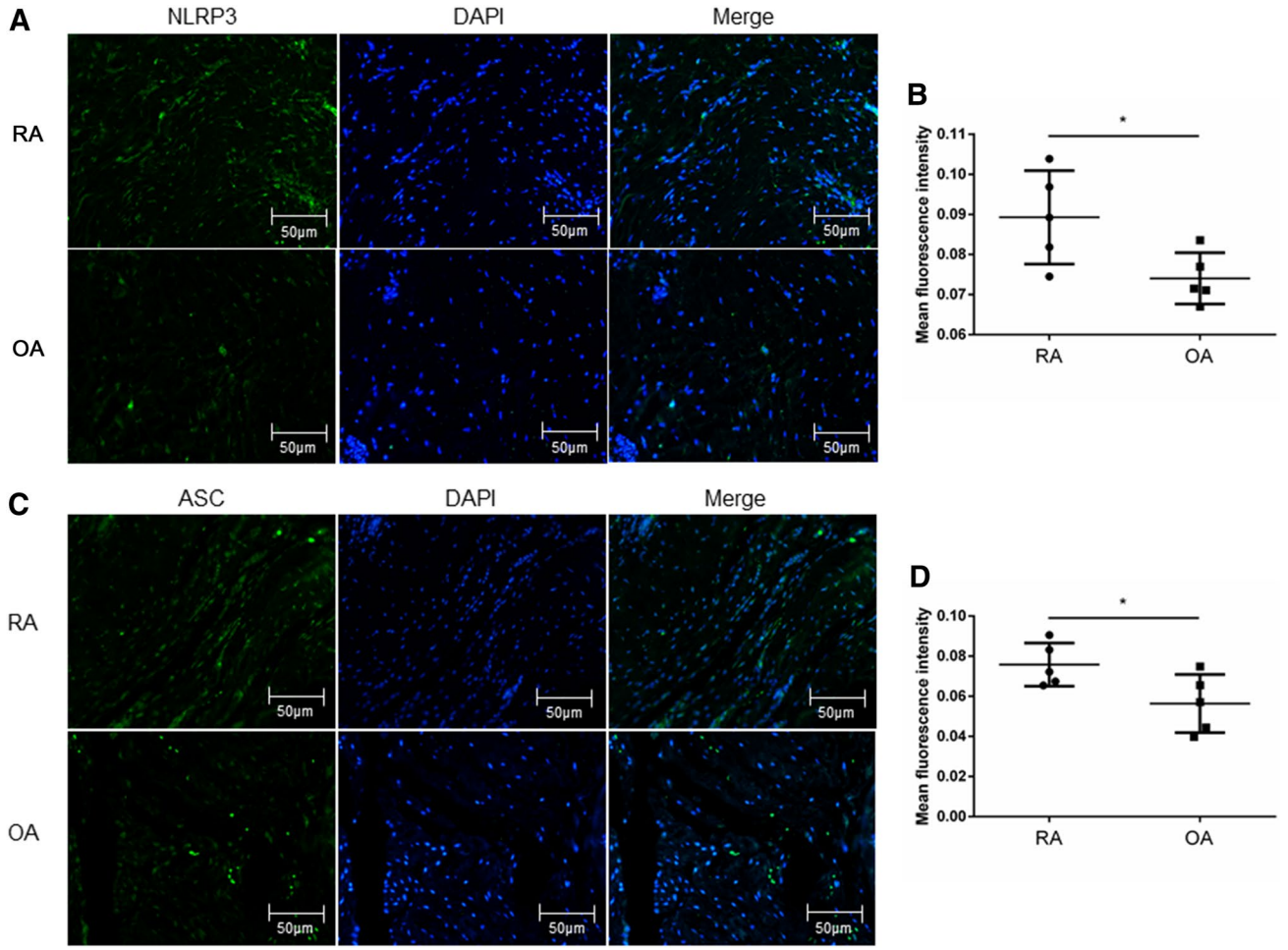

Fig. 1 Elevated NLRP3 and ASC proteins in rheumatoid synovium. Tissue sections from RA or OA synovium were stained using antibodies against NLRP3 (**a**) and ASC (**c**), and with DAPI (4′6-diamidino-2′-phenylindole) nuclear stain. Images are representatives of experiments. Sections of RA ($n=5$) or OA ($n=5$) synovium were stained with antibody against NLRP3 (**b**) and ASC (**d**), and staining was quantified using Image J, by mean fluorescence intensity (MFI) calculation. *$p<0.05$

TNF-α is a potential priming signal of the NLRP3 inflammasome in RA FLS and HUVECs

We next turned to the role of TNF-α as a potential priming signal for NLRP3 inflammasome activation in RA FLS and HUVECs. Here we showed that priming of RA FLS with TNF-α led to dose dependent NLRP3 and pro-IL-1β protein expression, yet did not affect the protein level of pro-IL-18 (Fig. 3a). In line with these data, TNF-α incubation consequently resulted in a considerable induction of NLRP3 mRNA and IL-1β mRNA expression whereas did not affect the mRNA level of IL-18 (Fig. 3c–e). Interestingly, LPS treatment had no promoting effect on NLRP3 and pro-IL-1β expression at either protein or mRNA levels (Fig. 3a, c, d). As to IL-18, although its mRNA expression could be elevated by LPS (Fig. 3e), the protein level of pro-IL-18 were remained almost unchanged. In HUVECs, TNF-α incubation induced dose dependent protein expressions of NLRP3, pro-IL-1β and pro-IL-18, which were marginally lower compared with LPS priming (Fig. 3b). As expected, priming of HUVECs with TNF-α or LPS significantly increased these three mRNA expressions with relative weaker priming effect of TNF-α (Fig. 3f–h).

CRT is an activation signal of NLRP3 inflammasome

We first studied whether TNF-α-primed RA FLS and HUVECs were capable of NLRP3 inflammasome activation. TNF-α-primed cells were stimulated with Nigericin, a canonical NLRP3 stimulus, and total NLRP3 inflammasome activity were investigated represented by caspase-1 cleavage. In RA FLS, caspase-1 p20 was increased in a dose-dependent manner in TNF-α-primed cells, whereas it was almost unchanged in LPS-primed cells (Fig. 4a). Analogous results were obtained when pro-IL-1β cleavage was studied. The concentration of secreted IL-1β in cell culture supernatant

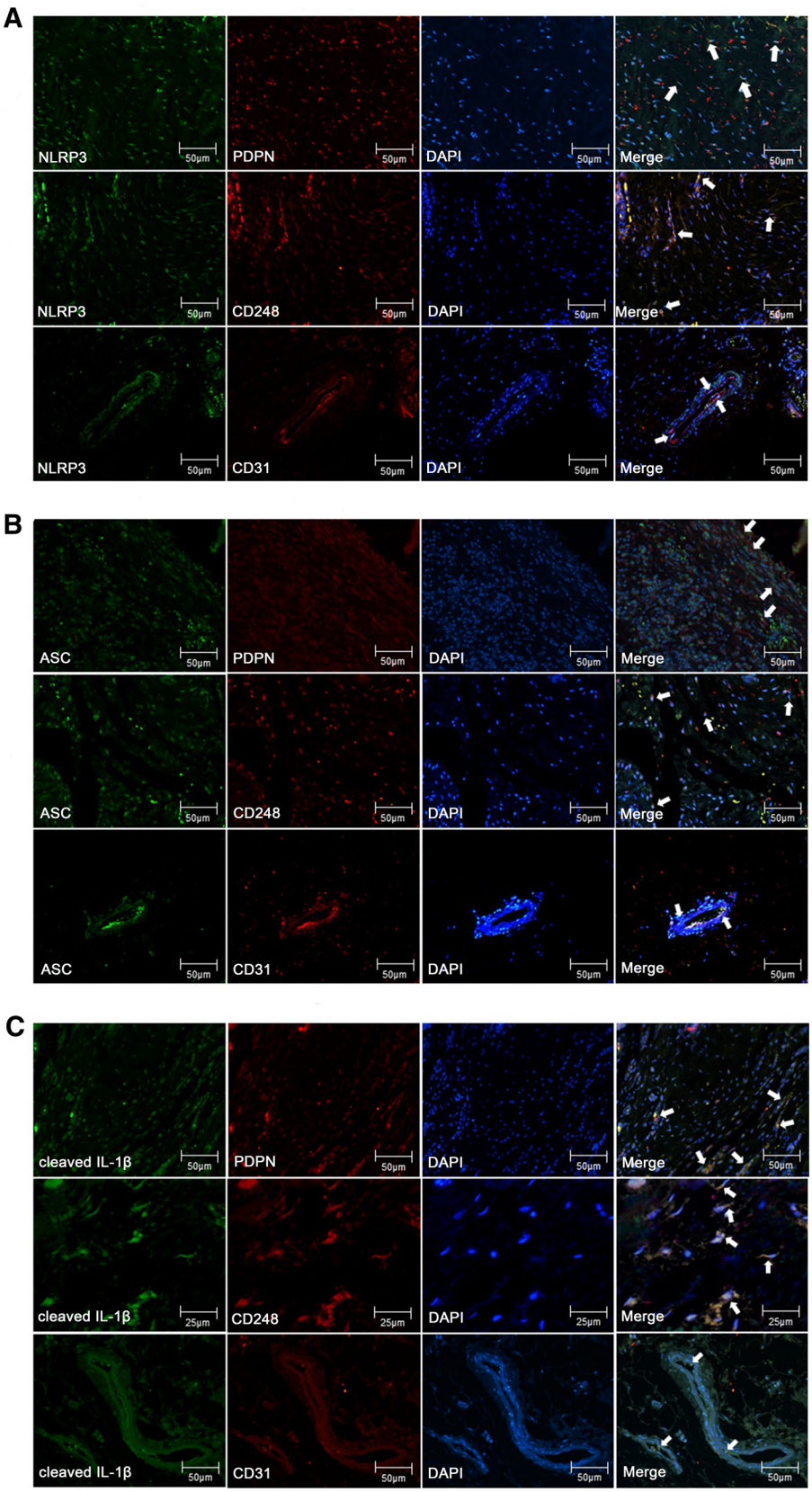

Fig. 2 Expression of NLRP3 inflammasome related proteins in rheumatoid synovium. Tissue sections from RA synovium were stained using antibodies with the specificities indicated, and the co-localizations of NLRP3 (**a**), ASC (**b**) and cleaved IL-1β (**c**) with cell markers of fibroblast and endothelial cell were observed, respectively. Arrows represent co-localizations. Images are representatives of experiments

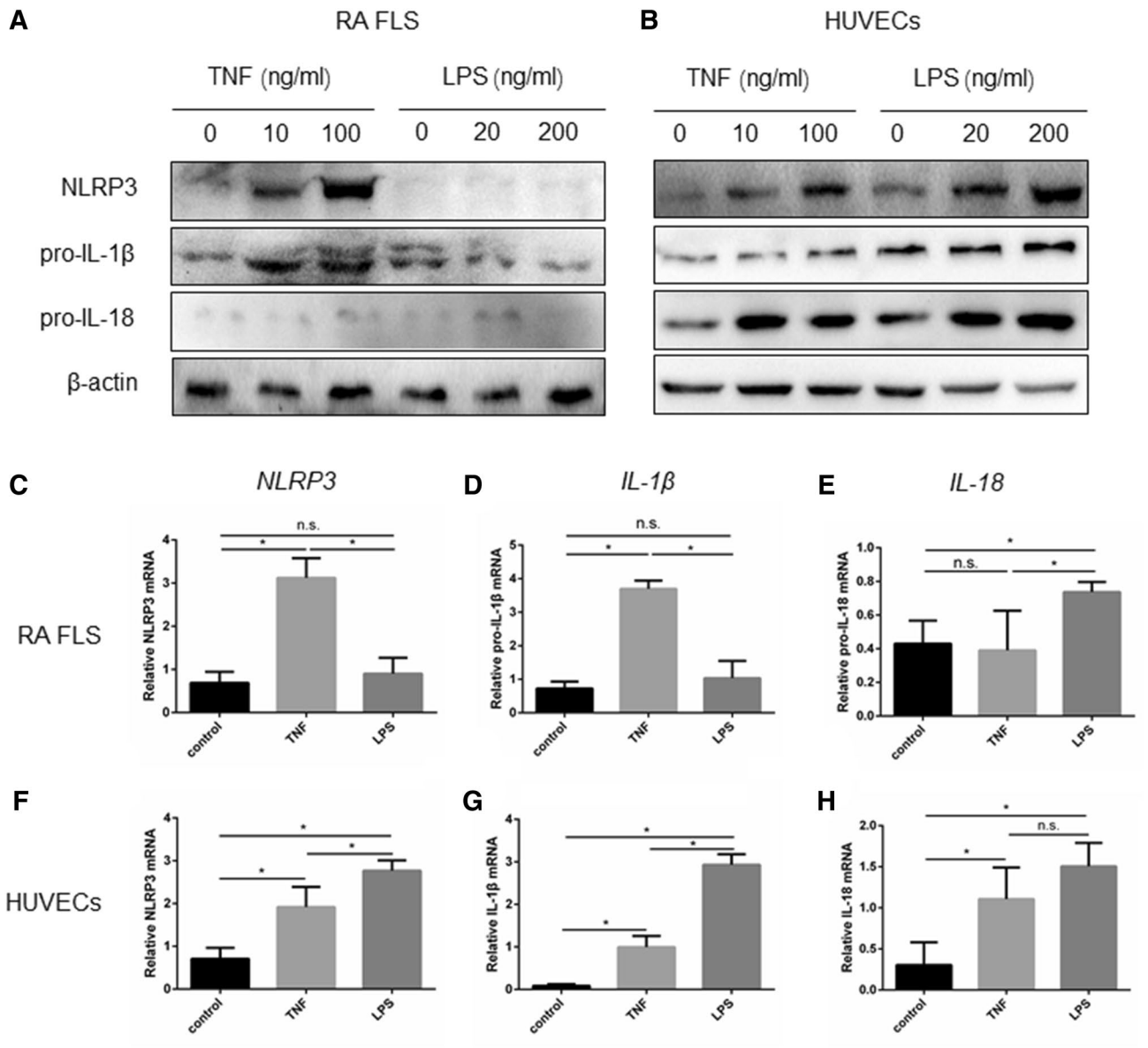

Fig. 3 Priming effect of TNF-α on NLRP3 inflammasome in RA FLS and HUVECs. RA FLS (**a**) and HUVECs (**b**) were treated with TNF-α (0, 10, 100 ng/ml) or LPS (0, 20, 200 ng/ml) for 6 h, and NLRP3, pro-IL-1β, pro-IL-18 and β-actin proteins were determined by western blot. Images are representatives of at least two independent experiments. RA FLS (**c**–**e**) and HUVECs (**f**–**h**) were left untreated or treated with TNF-α (100 ng/ml) or LPS (200 ng/ml) for 6 h. The mRNA levels of NLRP3, IL-1β and IL-18 were measured by qRT-PCR. Indicated mRNA levels are shown as relative expression normalized to GAPDH. GAPDH is loading control. Data shown are from three independent experiments. $^{*}p < 0.05$, *n.s.* not significant

was increased in TNF-α/Nigericin dual signaling stimulated cells and no significant increase was detected in LPS/Nigericin treated cells (Fig. 4c). The levels of secreted IL-18 were unchanged in both TNF-α and LPS pre-treated cells stimulated by Nigericin compared with priming signals alone, respectively (Fig. 4d). In HUVECs, both TNF-α and LPS-primed cells were capable of NLRP3 inflammasome activation by Nigericin represented by enhanced caspase-1 cleavage (Fig. 4e) and elevated IL-1β and IL-18 secretion (Fig. 4g, h).

We next investigated whether the effect of CRT on inflammasome activation. The dose-dependent increase of caspase-1 p20 in TNF-α primed RA FLS showed that CRT only activate NLRP3 inflammasome in the presence of TNF-α rather than LPS (Fig. 4b), which accompanied with increased release of IL-1β but not IL-18 (Fig. 4c, d). In HUVECs, CRT was able to activate NLRP3 inflammasome in both TNF-α and LPS-primed cells in that caspase-1 p20, secreted IL-1β and IL-18 were increased simultaneously (Fig. 4f, h).

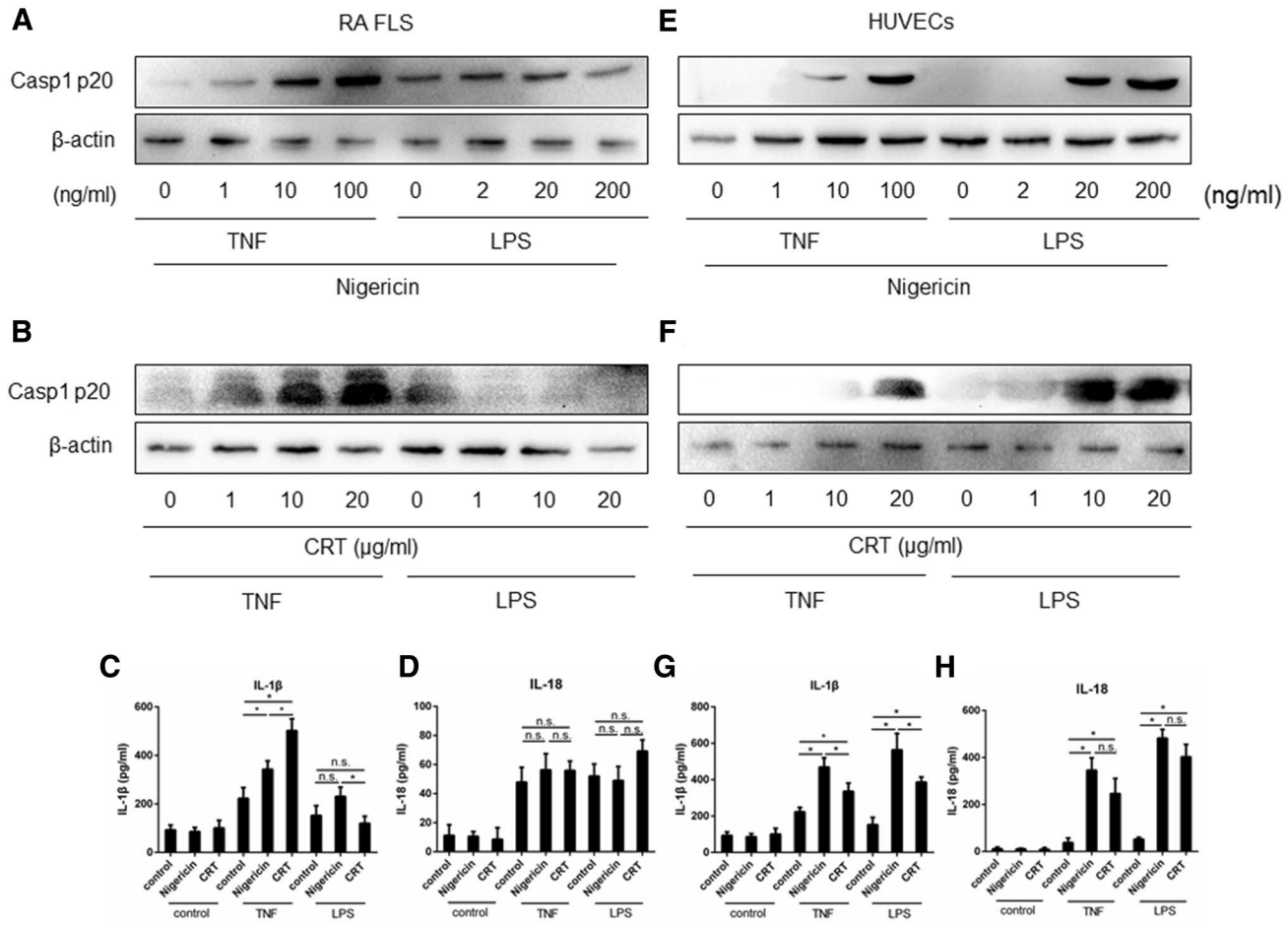

Fig. 4 CRT promoted NLRP3 inflammasome activation in RA FLS and HUVECs. TNF-α (0, 10, 100 ng/ml) or LPS (0, 20, 200 ng/ml) pre-treated cells were then stimulated with Nigericin (10 μM) for 1 h. Caspase-1 p20 and β-actin proteins in lysates of RA FLS (**a**) or HUVECs (**e**) were analyzed by western blot. Images are representatives of at least two independent experiments. TNF-α (100 ng/ml) or LPS (200 ng/ml) pre-treated cells were then stimulated with CRT (0, 1, 10, 20 μg/ml) for 36 h. Caspase-1 p20 and β-actin proteins in lysates of RA FLS (**b**) or HUVECs (**f**) were analyzed by western blot. Images are representatives of at least two independent experiments. IL-1β (**c**, **g**) and IL-18 (**d**, **h**) concentration in cell culture supernatant of RA FLS (**c**, **d**) or HUVECs (**g**, **h**) both stimulated as indicated were measured by ELISA. Data shown are from three independent experiments. $^{*}p < 0.05$, *n.s.* not significant

Together, these data revealed that CRT was a strong activation signal of NLRP3 inflammasome in TNF-α primed RA FLS, and in TNF-α or LPS-primed HUVECs. Notably, the activation of NLRP3 inflammasome in RA FLS was independent of LPS stimulation.

Elevated expression of HuR in RA synovium

To address the expression of HuR in RA, the synovial slides of patients with RA and OA were analyzed by immunohistochemistry. HuR was strongly expressed in RA synovium, which was speck-like and localized in lining layer, vascular endothelium and perivascular areas, whereas HuR only strongly expressed in vascular endothelium of OA synovium (Fig. 5a). The results of mean optical density suggested that HuR expression (0.190 ± 0.009, 0.023 ± 0.002, $p < 0.01$) was significantly increased in RA synovium compared with that in OA (Fig. 5b). Meanwhile, western blot analysis of synovial tissue displayed a considerable amount of HuR protein in RA (Fig. 5c). Similarly, mRNA level of HuR in RA was also dramatically elevated (Fig. 5d).

Effect of NLRP3 inflammasome priming signal TNF-α on HuR expression and nucleocytoplasmic distribution

To determine whether TNF-α impacted on HuR expression and nucleocytoplasmic shuttling, we next measured the protein and mRNA level of HuR in RA FLS and HUVECs treated with TNF-α. HuR protein were almost unchanged in cells with different concentrations stimulation of TNF-α or LPS (Fig. 6a). Treatment of

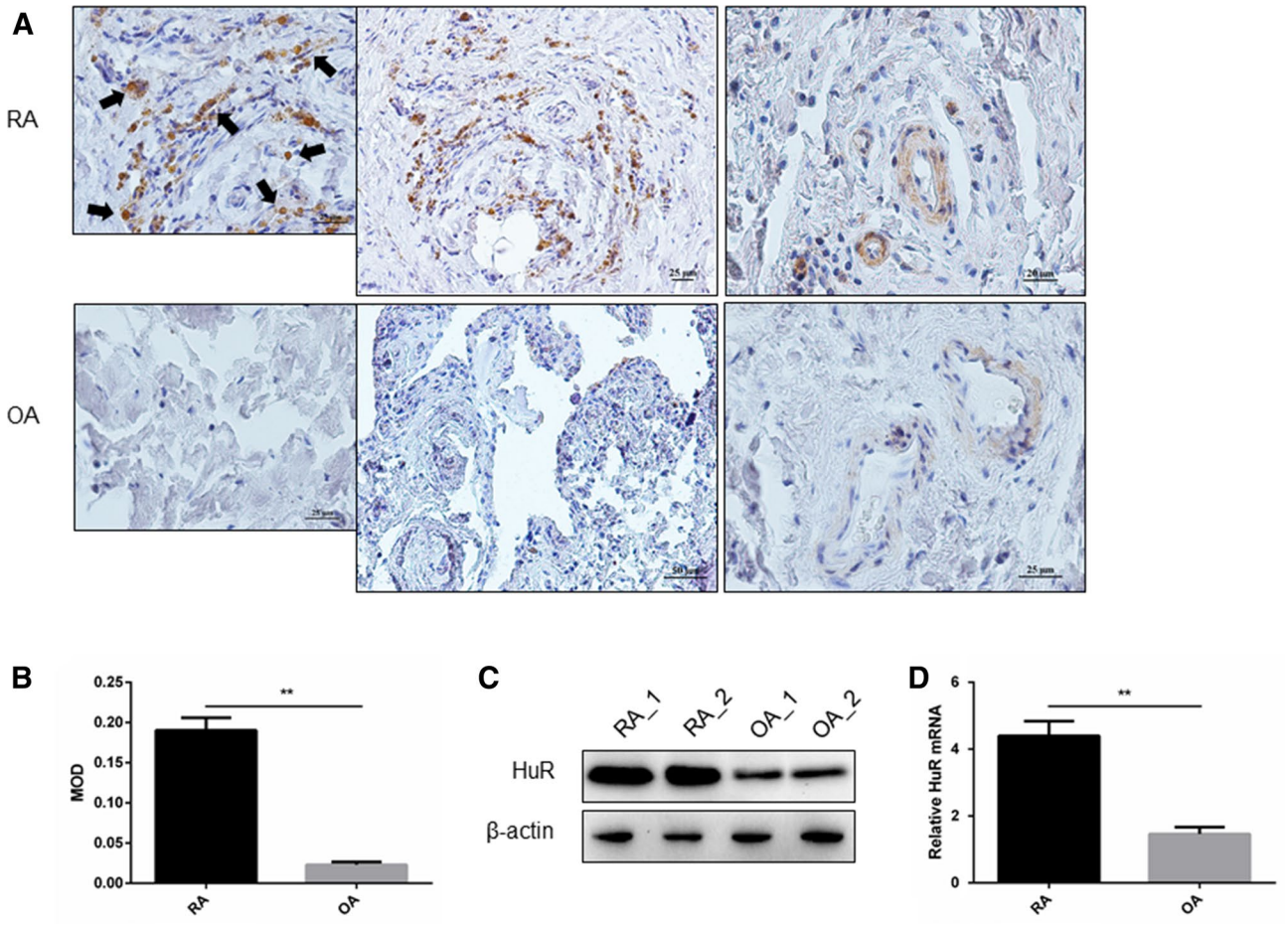

Fig. 5 Enhanced HuR expression in rheumatoid synovium. **a** The expression of HuR in RA ($n=3$) and OA ($n=3$) synovium were determined by immunohistochemistry. Middle panels show the synovial lining layer area, whereas endothelium of blood vessels in the sublining is shown in right panels (left panels were obtained by magnifying a part of the middle panels). Arrows represent speck-like HuR accumulation. Images are representatives of experiments. **b** The HuR expressions in **a** were analyzed using Image J, by mean optical intensity (MOD) calculation. $^{**}p<0.01$. **c** The expression of HuR protein in RA ($n=2$) and OA ($n=2$) synovium were also explored by western blot. **d** The mRNA expressions of HuR in RA ($n=3$) and OA ($n=3$) synovial tissue were analyzed by qRT-PCR. Indicated mRNA levels are shown as relative expression normalized to GAPDH. GAPDH is loading control. $^{**}p<0.01$

TNF-α or LPS also did not considerably influence the mRNA expression of HuR (Fig. 6b). However, TNF-α indeed affected the nucleocytoplasmic distribution of HuR. The immunofluorescence results demonstrated that HuR expression in cytoplasma turned higher than that in nucleus at approximately 4 h point of TNF-α incubation in most RA FLS (Fig. 6c, left panel). Presumably, HuR protein translocated from nucleus to cytoplasma. In HUVECs, the nucleocytoplasmic shuttling occurred at 6 h point and accomplished at 8 h point due to the fact that the HuR staining in cytoplasma was gradually stronger while that in nucleus turned weaker until annihilation at 8 h point of TNF-α incubation (Fig. 6c, right panel).

HuR knockdown suppressed TNF-α mediated NLRP3 priming and CRT induced inflammasome activation

Because the 3′UTR of NLRP3 protein had been reported to contain ARE [27], we next aimed to explore if HuR was a potent regulator of NLRP3 expression. To this end HuR expression in RA FLS was knocked down using siRNA and NLRP3 expression in response to TNF-α stimulation was assayed. Our results showed that HuR protein and mRNA expressions were efficiently reduced by siRNA transfection (Fig. 7a–c). Importantly, expression of NLRP3 was considerably decreased in cells lacking HuR expression (Fig. 7d). Because HuR is known to stabilize target mRNA, we also

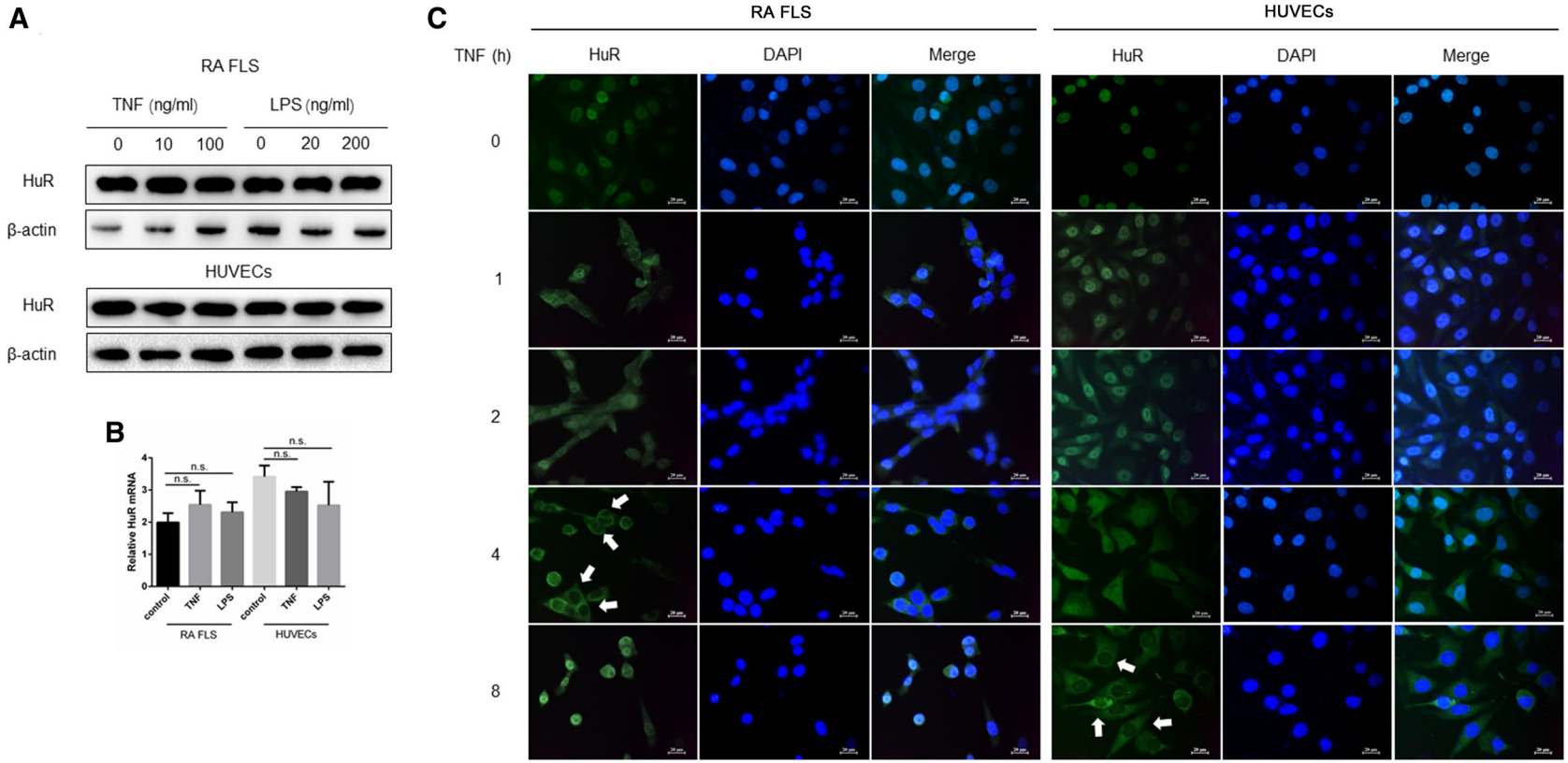

Fig. 6 Effect of TNF-α on HuR expression and distribution in RA FLS and HUVECs. **a** RA FLS and HUVECs were treated as indicated and HuR protein was investigated by western blot. Images are representatives of at least two independent experiments. **b** HuR mRNA expressions in RA FLS and HUVECs both treated with TNF-α (100 ng/ml) or LPS (200 ng/ml) were analyzed by qRT-PCR. Indicated mRNA levels are shown as relative expression normalized to GAPDH. GAPDH is loading control. Data shown are from three independent experiments. *n.s.* not significant. **c** RA FLS and HUVECs were treated with TNF-α (100 ng/ml) for different period of time as indicated. The HuR distribution in cells was stained by immunofluorescence. Arrows represent the cells that HuR staining in cytoplasma was stronger than that in nucleus

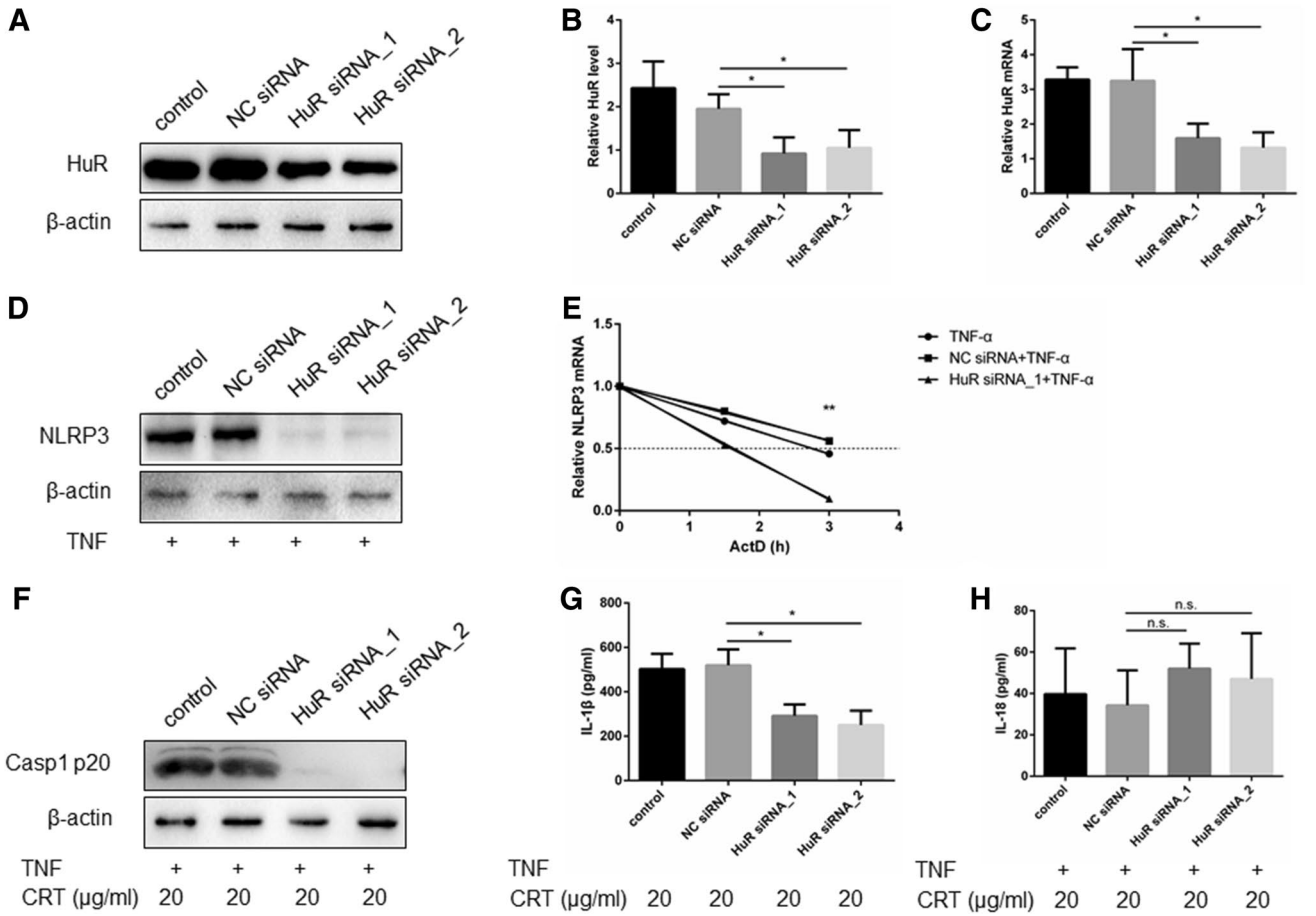

Fig. 7 HuR knockdown suppressed NLRP3 priming and inflammasome activation. RA FLS were untreated or transfected with NC siRNA or HuR siRNA_1 or HuR siRNA_2. The transfection efficiency was evaluated by HuR protein detection (**a**) and HuR mRNA analysis (**c**). **a** Images are representatives of three independent experiments. **b** Protein abundance in **a** was estimated by scanning densitometry, with normalization against the loading control β-actin. $*p<0.05$. **c** Data shown are from three independent experiments. Indicated mRNA levels are shown as relative expression normalized to GAPDH. GAPDH is loading control. $*p<0.05$. **d** Cells were transfected as indicated and treated with TNF-α (100 ng/ml) for 6 h. HuR and β-actin protein expressions were measured by western blot. Images are representatives of two independent experiments. **e** RA FLS transfected as indicated were incubated with TNF-α for 6 h and then treated with 2 μg/ml of Actinomycin D. NLRP3 mRNA was analyzed by qRT-PCR at 0 h, 1.5 h and 3 h after Actinomycin D addition and normalized to GAPDH. The relative mRNA levels at 1.5 h and 3 h point are shown as fold of that at 0 h point. Data shown are from three independent experiments. **f** RA FLS transfected as indicated were incubated with TNF-α for 6 h and then treated with CRT for 36 h. The expression of caspase-1 p20 in cell lysates was measured by western blot (**f**), and secreted IL-1β (**g**) and IL-18 (**h**) in cell culture supernatant were investigated by ELISA. **f** Images are representatives of at least two independent experiments. **g**, **h** Data shown are from three independent experiments. $**p<0.01$, $*p<0.05$, *n.s.* not significant

measured mRNA stability of NLRP3 in RA FLS. We found that NLRP3 mRNA was destabilized after transfected with HuR siRNA_1 when compared with the stability of control groups (Fig. 7e). As such, whether HuR could further influence the NLRP3 inflammasome activation mediated by CRT in RA FLS was investigated. As expected, the HuR siRNA transfected cells manifested a reduced amount of caspase-1 p20 in the presence of TNF-α and then CRT (Fig. 7f), suggesting that HuR knockdown suppressed the activity of NLRP3 inflammasome. And the reduced concentration of released IL-1β also supported this result (Fig. 7g). Nevertheless, HuR knockdown did not affect the IL-18 release in cell culture supernatant which constantly sustained a low level (Fig. 7h).

Discussion

RA is a progressive inflammatory autoimmune disease. TNF-α plays a leading role as one of the pro-inflammatory cytokines contributing to RA induced synovitis [28]. CRT is a multi-functional ER protein implicated in the pathogenesis of RA. In the present study, the effect of TNF-α/CRT dual signaling on activating NLRP3 inflammasome was explored

for the first time. And HuR, one of the RBPs, promoted NLRP3 priming and inflammasome activation by stabilizing NLRP3 mRNA. The mechanism by which HuR achieved this effect presumably owed to its translocation from nucleus to cytoplasma (Fig. 8).

In this study, expression of NLRP3 and ASC was significantly elevated in RA synovium. Our results were consistent with a previous study by Alexander et al. [29] which showed that a large amount of expression of inflammasome protein components in RA synovium and that B cells, neutrophils and macrophages were all NLRP3 inflammasome-expressing cells in RA. In our study, the production of NLRP3 inflammasome-related proteins in resident cells (FLS of both lining and sublining layers and vascular endothelial cells) of RA synovium were certified, which laid the foundation for follow-up experiments.

We next explored the effect of TNF-α on NLRP3 inflammasome priming. TNF-α proved to be a potential priming signal in RA FLS and HUVECs, leading to the production of NLRP3 and pro-IL-1β in RA FLS, and the expression of NLRP3, pro-IL-1β and pro-IL-18 in HUVECs. Our results were consistent with an analogous study recently carried out that TNF-α selectively primes the NLRP3 inflammasome in bone marrow-derived macrophages (BMMFs) in aging-associated metabolic disturbances [30]. Besides the TNF-dependent and spontaneous inflammasome activity, other sterile inflammatory diseases also show an activated NLRP3 inflammasome, which can be primed by a series of endogenous factors. For instance, products of lipid metabolism, such as sterol regulatory element binding proteins (SREBPs), can either facilitate or diminish priming of the inflammasome [31]. Oxidized LDL is shown to provide signal 1 to prime NLRP3 inflammasome in mouse models of atherosclerosis [32]. The sub-lytic membrane attack complex (MAC) of complement in inflammatory microenvironment primes inflammasome in mouse macrophages [33]. In addition, we found that RA FLS could not be primed by LPS which is a typical priming signal of NLRP3 inflammasome. One likely answer might be that the downstream signals of TLR (e.g., MyD88 and the downstream kinases IRAK1 and IRAK4) were somehow blocked in this case, given the previous demonstration that they participated in the transcriptional regulation of NLRP3 [34, 35]. Moreover, existing research had clarified that priming step involves transcriptional upregulation of pro-IL-1β, inflammasome sensor NLRP3 and pro-IL-18 by NF-kB-dependent de novo protein synthesis [1]. An additional aspect of priming is the post-translational modification of NLRP3, during which NLRP3 is deubiquitinated to a stand-by status [33]. The latter requires a short-term exposure to LPS for about 30 min. This may provide another explanation for the silencing of the effect of LPS.

In our study, CRT was identified as a potent activating signal of NLRP3 inflammasome in RA FLS and HUVECs, which provides evidence for a single DAMP being able to activate the inflammasome to produce modest levels of IL-1β and IL-18. This consequence is consistent with chronic inflammatory condition occurring during RA synovitis. It has been reported that several molecular and cellular events are involved in the triggering for NLRP3 inflammasome activation, including reactive oxygen species (ROS) [36], K^+ efflux [37], Ca^{2+} signaling [38], mitochondrial dysfunction [39], and lysosomal rupture [38]. The specific mechanism by which CRT exerted the influence remained to be elucidated.

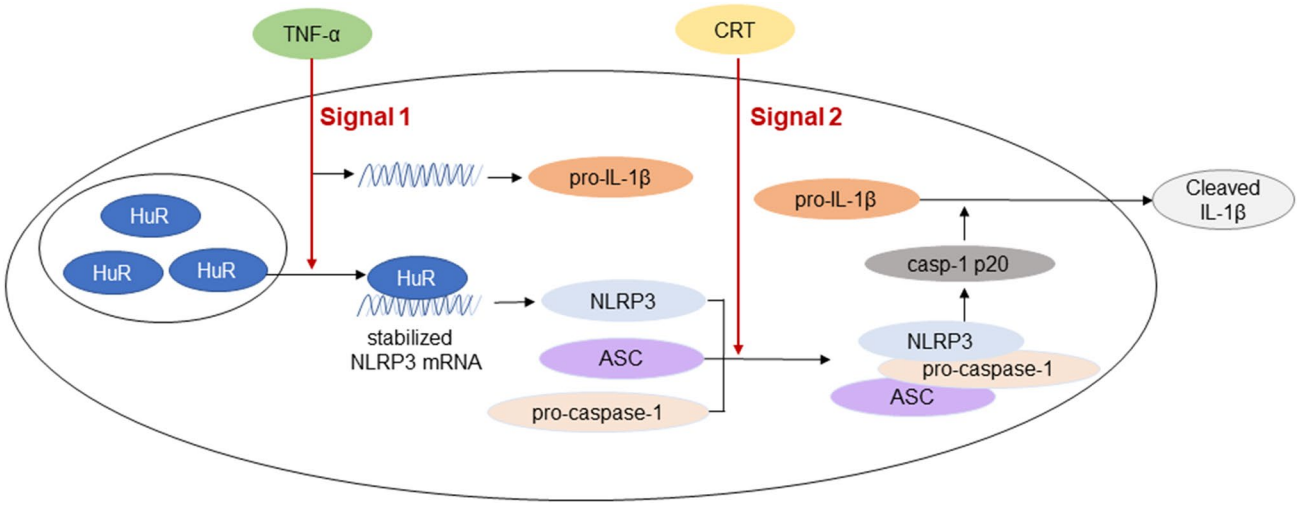

Fig. 8 TNF-α/CRT dual signaling induced NLRP3 inflammasome activation associated with HuR nucleocytoplasmic shuttling in RA FLS. TNF-α (signal 1) promoted NLRP3 expression by inducing HuR translocating from nucleus to cytoplasma to stabilize NLRP3 mRNA and facilitate NLRP3 protein expression. In response to CRT (signal 2), NLRP3 oligomer are formed, which evoked the cleavage of caspase-1 and further triggered the cleaved IL-1β secretion

In the past decade, interests have shifted towards the function of CRT in extracellular compartment, driven by discoveries such as the role of CRT on angiogenesis [20], focal adhesion of T cells [40], clearance of apoptotic cells [41] and anti-cancer immunity. Recent years, many other reports have implicated that extracellular CRT may function as a 'danger signal' for the immune system, including the effect on immunogenic cell death (ICD) and innate and adaptive immunity [18, 42–44]. For instance, CRT could regulate innate immune responses through several binding partners like complement C1q [42] and low-density lipoprotein receptor-related protein 1 (LRP-1) [43], leading particularly to phagocytic removal of target cells. CRT was also capable of inhibiting FasL-mediated apoptosis via binding to soluble FasL in synovial fluid [18] and promoting apoptosis resistance of FLS in RA [44]. Inflammasome mediated release of pro-inflammatory factors is a crucial part of innate immunity, thus our results provide another line of CRT exerting pro-inflammatory effect in autoimmune disease.

We further demonstrated that the distribution of HuR could be altered by TNF-α in FLS and HUVECs. The knockdown by HuR siRNA transfection caused the decrease of NLRP3 protein expression and reduced mRNA stability, and further led to suppressed inflammasome activity. Our results suggested that HuR stabilized the mRNA of NLRP3 and promoted NLRP3 inflammasome activation in RA FLS. Previous studies showed that phosphorylation modified the subcellular localization of HuR, which further induced its translocation from the nuclear to the cytoplasmic compartment [45, 46]. Given the fact that both p38 and MK2 are capable of phosphorylating HuR directly [45, 46] and that p38 MAPK and MK2 can be activated by TNF-α in RA FLS [47], we speculate that TNF-α led to the phosphorylation of HuR through activating p38 MAPK signaling pathway, which further affected the nucleocytoplasmic shuttling of HuR. Nevertheless, our speculation needs to be confirmed by further experiments. During detailed analysis of the human NLRP3 sequence, O'Neill et al. [48] found that human *NLRP3* could be alternatively polyadenylated, leading to transcripts containing a long or short 3′-UTR isoform. Tristetraprolin (TTP), another RBP, could bind to a main ARE of the long 3′-UTR isoform to destabilize the NLRP3 mRNA for degradation and repress NLRP3 expression, which further suppressed NLRP3 inflammasome activity. Moreover, it is extensively accepted that upon LPS stimulation, p38 signaling leads to the phosphorylation of TTP, which inhibits its ability to degrade target mRNAs [49]. Furthermore, it has been implicated that aberrant activation of MAPK p38 in the RA synovium leads to the accumulation of TTP in a phosphorylated and inactive form [48]. Therefore, it is likely that HuR competing with TTP to facilitate NLRP3 expression and inflammasome activation in inflamed RA synovium.

Together, our study indicated the TNF-α priming signal and CRT activation signal of NLRP3 inflammasome in RA FLS and HUVECs. In response to TNF-α stimulation, HuR translocated from nucleus to cytoplasma to stabilize NLRP3 mRNA and facilitate NLRP3 inflammasome activation. Future studies will investigate the molecular mechanisms involved in the effect of TNF-α and CRT and the relationship between HuR and TTP in NLRP3 inflammasome activation in RA.

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Author contributions All authors have contributed in a substantive and intellectual manner.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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