CNOT1 is involved in TTP-mediated ICAM-1 and IL-8 mRNA decay

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Received September 11, 2017; Accepted May 21, 2018

DOI: 10.3892/mmr.2018.9213

Abstract. Subunit 1 is the scaffold protein of the carbon catabolite repressor protein 4 (CCR4)-negative on TATA (NOT) complex (CNOT1). In our previous study, it was reported that tristetraprolin (TTP) could recruit subunit 7 of the CCR4-NOT complex (CNOT7) to induce the degradation of intercellular adhesion molecule-1 (ICAM-1) and interleukin-8 (IL-8) mRNA in human pulmonary microvascular endothelial cells (HPMECs). It was additionally demonstrated that TTP, CNOT7 and CNOT1 formed a complex in HPMECs. However, whether CNOT1 is involved in TTP-mediated ICAM-1 and IL-8 mRNA decay remains unclear. The present study demonstrated that CNOT1 knockdown improved ICAM-1 and IL-8 mRNA stabilization and protein expression levels. The immunofluorescence results demonstrated that CNOT1, CNOT7 and TTP are co-localized in the cytoplasm. CNOT1 silencing abolished CNOT7 and TTP coimmunoprecipitation. However, CNOT7 silencing did not influence CNOT1 and TTP coimmunoprecipitation, and TTP silencing additionally did not influence CNOT1 and CNOT7 coimmunoprecipitation. These results together with the authors' previous study, have identified that CNOT1 provides a platform for the recruitment of TTP and CNOT7, and is involved in TTP-mediated ICAM-1 and IL-8 mRNA decay.

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Abbreviations: CCR4, carbon catabolite repressor protein 4; NOT, Negative on TATA; CNOT1, subunit 1 of the CCR4-NOT complex; CNOT7, subunit 7 of the CCR4-NOT complex; TTP, tristetraprolin; ARE, AU-rich element; HPMECs, human pulmonary microvascular endothelial cells; PAN, poly (A)-specific nuclease

Key words: CNOT1, tristetraprolin, CNOT7, AU-rich element, mRNA decay, inflammatory mediator

Introduction

Tristetraprolin (TTP) is a RNA-binding protein with two zinc fingers, which are necessary for TTP to bind to the AU-rich elements (AREs) in the 3'-untraslated region (3'-UTR) of target mRNA (1), and to subsequently promote mRNA deadenylation and decay (2,3). TTP-mediated mRNA decay is a typical model of posttranscriptional regulation in inflammatory mediator expression (4). However, TTP does not have deadenylase activity and other proteins that have deadenylase activity are required to cooperate with TTP to mediate mRNA decay.

The carbon catabolite repressor protein 4 (CCR4)-negative on TATA (NOT) complex consists of multiple subunits that are involved in the regulation of gene expression at different levels (5). One of the enzymatic activities of the CCR4-NOT complex is deadenylation (6). In yeast, the CCR4 subunit provides deadenylase activity, while in other eukaryotes the deadenylase activity is provided by CCR4 and CCR4-associated factor 1 [CAF1; as known as CCR4-NOT complex subunit 7 (CNOT7) in human cells] (7,8). Schwede *et al* (9) reported that CNOT7, rather than CCR4, is necessary for the decay of an ARE-containing reporter mRNA in human cells. In humans, CNOT7 is one of the subunits of the CCR4-NOT complex and interacts with CNOT1, the scaffold protein of the CCR4-NOT complex (10). Another previous study demonstrated that CNOT1 may

directly bind to TTP (11). These results implied that CNOT1 could interact with CNOT7 and TTP. The authors' previous studies have demonstrated that CNOT1, CNOT7 and TTP were coimmunoprecipitated in human pulmonary microvascular endothelial cells (HPMECs); TTP could bind to the AREs of intercellular adhesion molecule-1 (ICAM-1) and interleukin-8 (IL-8) mRNA, and CNOT7 was involved in ICAM-1 and IL-8 regulation by TTP (12,13). On the basis of these results, together with the results of other studies (11,14), it was hypothesized that CNOT1 may provide a platform to recruit CNOT7 and TTP with ARE-bearing mRNA, and be involved in TTP-mediated ICAM-1 and IL-8 mRNA decay.

The present study in HPMECs reported that CNOT1 knockdown increased ICAM-1 and IL-8 mRNA stabilization, and protein production following stimulation with tumor necrosis factor (TNF)-α. The immunofluorescence results demonstrated that CNOT1, CNOT7 and TTP co-localized in the cytoplasm. In addition, CNOT1 silencing abolished CNOT7 and TTP coimmunoprecipitation (Co-IP). However, CNOT7 silencing did not influence CNOT1 and TTP Co-IP, and TTP silencing additionally did not influence CNOT1 and CNOT7 Co-IP. In conclusion, these results together with the results of our previous study, revealed that CNOT1 may provide a platform to recruit TTP and CNOT7, and may be involved in TTP-mediated ICAM-1 and IL-8 mRNA decay in HPMECs.

Materials and methods

Materials. The materials used in the present study were as follows: HPMECs and Endothelial Cell Medium (ECM; ScienCell Research Laboratories, Inc., San Diego, CA, USA). TNF-α (cat. no. 300-01A; PeproTech, Rocky Hill, NJ, USA). Lipofectamine 2000™ (cat. no. 11668-027), Opti-Minimum Essential Medium I reduced serum medium and TRIzol® reagent (all Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Actinomycin D (ActD; cat. no. A1410; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Immunoprecipitation kit (IP; cat. no. K286-25; BioVision, Inc., Milpitas, CA, USA). The Radioimmunoprecipitation Assay Lysis Buffer (cat. no. P0013C; Beyotime Institute of Biotechnology, Haimen, China). Protease inhibitors (cat. no. R1321) and RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Fermentas; Thermo Fisher Scientific, Inc.). Ribolock RNase Inhibitors (cat. no. E00381; Thermo Fisher Scientific, Inc.). Protein assay reagent (cat. no. KGPBCA; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). SYBR Green PCR Master Mix (cat. no. 04913850001; Roche Diagnostics GmbH, Mannheim, Germany). IL-8 ELISA kit (cat. no. 431507; BioLegend, Inc., San Diego, CA, USA). CNOT1, CNOT7 and TTP small interfering RNAs (siRNAs) and the control siRNAs (Shanghai GenePharma Co., Ltd., Shanghai, China). The following antibodies were used: Rabbit anti-TTP antibody (cat. no. ABE285; Merck KGaA), mouse anti-CNOT7 antibody (cat. no. sc-101009), mouse anti-TTP antibody (cat. no. sc374305; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-CNOT1 antibody (cat. no. 14276-1-AP; Wuhan Sanying Biotechnology, Wuhan, China), rabbit anti-ICAM-1 antibody (cat. no. 4915; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse anti-β-tubulin antibody (cat. no. M20005; Abmart, Shanghai, China), mouse anti-GAPDH antibody (cat. no. AT0002; CMCTAG, Inc., Milwaukee, WI, USA), rabbit immunoglobulin G (IgG; cat. no. A7016) and mouse IgG (cat. no. A7028; Beyotime Institute of Biotechnology).

Cell culture, treatment and transfection. HPMECs were cultured in ECM at 37˚C in an incubator supplied with 5% CO_2. Cells were subcultured to passages 5 to 8 for all experiments and activated with TNF-α (10 ng/ml) in the subsequent experiments. siRNAs were used to silence CNOT1, CNOT7 and TTP. The siRNA transfection experiment was performed as previously described (13) using Lipofectamine 2000™ and the final concentration of CNOT1 siRNA, TTP siRNA and CNOT7 siRNA was 100, 80 and 100 nM, respectively. The cells were incubated for 24 h, transfection medium was replaced with complete endothelial cell medium, and the incubation was continued for an additional 24 h prior to using the cells. The target sequences of siRNAs are as follows: CNOT1 siRNA 1 sense, 5'-CUUCACGUCGUGAAUACCUCATT-3' and antisense, 5'-UGAGGUAUUCACGACGUGAAGTT-3'; CNOT1 siRNA 2 sense, 5'-CAAUUCGCCAACUUAUCA UGCTT-3' and antisense, 5'-GCAUGAUAAGUUGGCGAA UUGTT-3', negative control siRNA sense, 5'-UUCUCCGAA CGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUU CGGAGAATT-3'. TTP siRNAs, CNOT7 siRNAs, and their control siRNAs were as previously described (12,13).

Western immunoblotting experiments. The western immunoblotting experiments were performed as previously described (13). The primary antibodies (TTP, CNOT1, CNOT7, ICAM-1, β-tubulin and GAPDH antibodies) were diluted at 1:1,000 with 0.1% PBST containing 5% bovine serum albumin (BSA; cat. no. ST023; Beyotime Institute of Biotechnology). The horseradish peroxidase-conjugated secondary antibodies [goat anti-rabbit immunoglobulin G (IgG; cat. no. A0208; Beyotime Institute of Biotechnology) and goat anti-mouse IgG (cat. no. A0216; Beyotime Institute of Biotechnology)] were diluted at 1:5,000 with 0.1% PBST containing 5% BSA. The target bands were developed using an enhanced chemiluminescent kit (cat. no. 32134; Thermo Fisher Scientific, Inc.) and exposed on film. The band images were obtained using the BenQ Scanner (5560C) and analyzed with ImageJ software 1.8.0 (National Institutes of Health, Bethesda, MD, USA; rsb.info.nih.gov/ij/).

ELISA. An IL-8 ELISA kit was employed to detect IL-8 levels in the HPMEC culture supernatant. An IL-8 standard was used to construct standard curves. Absorbance at 450 nm was read using a microplate reader and IL-8 concentrations were calculated, according to the standard curves.

RNA isolation, cDNA synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following cell transfection and activation using TNF-α (10 ng/ml), total RNA was extracted at 0, 2, 4, 8 and 12 h using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and synthesized and analyzed as previously described (13). The GAPDH, ICAM-1 and IL-8 mRNA primers were the same as those previously described (12). The $2^{-\Delta\Delta Cq}$ method was used for RNA quantification (15).

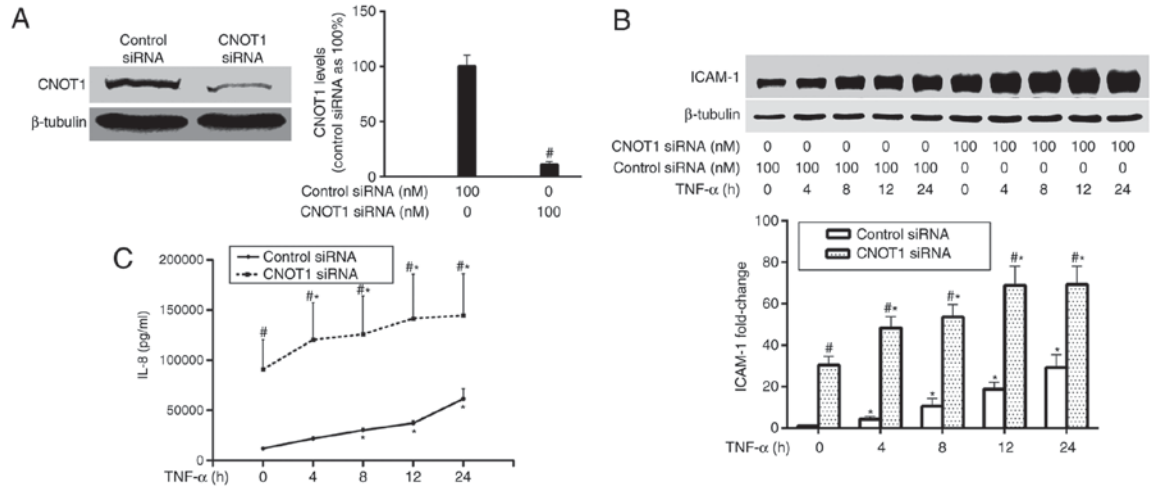

Figure 1. CNOT1 silencing increases the TNF-α-induced expression of ICAM-1 and IL-8. (A) Western immunoblotting band density results demonstrated that CNOT1 knockdown reduced CNOT1 levels when compared with the control and (B) CNOT1 knockdown increased ICAM-1 levels following TNF-α stimulation when compared with the control. (C) The ELISA results revealed a significant increase in IL-8 levels in CNOT1 knockdown HPMECs and the control following activation by TNF-α, and the IL-8 levels in CNOT1 knockdown HPMECs were significantly increased when compared with the control. The data are expressed as the mean ± standard error of the mean (n=3). #P<0.05 vs. control siRNA-transfected cells; *P<0.05 vs. cells at TNF-α 0 h. IL, interleukin; siRNA, small interfering RNA; HPMECs, human pulmonary microvascular endothelial cells; CNOT1, subunit 1 of the carbon catabolite repressor protein 4-negative on TATA complex; TNF, tumor necrosis factor; ICAM-1, intercellular adhesion molecule 1.

Analysis of mRNA stability using RT-qPCR. Cells were transfected using siRNAs for 48 h, as aforementioned, and subsequently activated using TNF-α (10 ng/ml) for 4 h at 37˚C, then ActD (5 μg/ml) was added to block transcription at 37˚C for 60, 120, 180 and 240 min. RNA was extracted at 0, 60, 120, 180 and 240 min following the addition of ActD, synthesized and analyzed as previously described (12).

Immunofluorescence experiments. HPMECs were stimulated using TNF-α (10 ng/ml) for 4 h and fixed with 4% formaldehyde at room temperature for 30 min, permeabilized with 0.2% Triton X-100 and incubated with antibodies (1:100, diluted with 0.1% PBST containing 5% BSA) specific to CNOT1, TTP and CNOT7. Following incubation with these antibodies for 2 h at room temperature, the cells were washed with PBS and incubated with Alexa Fluor 488 (cat. no. A11001) or Alexa Fluor 568 (cat. no. A11011)-conjugated secondary antibodies (both Thermo Fisher Scientific, Inc.; 1:200, diluted with 0.1% PBST containing 2% BSA). DAPI was used to stain the cell nuclei for 5 min at room temperature at a concentration of 1 μg/ml. The cells were observed using a Leica DM2500 fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany; magnification, x630).

Co-IP experiments. The Co-IP experiments were performed as previously described (12). HPMECs were transfected with CNOT1, CNOT7 or TTP siRNA for 48 h, as aforementioned, and stimulated with TNF-α (10 ng/ml) for 4 h, then ~2x10^7 cells were lysed in 500 μl non-Denaturing Lysis buffer, mixed on a rotary mixer for 30 min at 4˚C, centrifuged at 10,000 x g for 10 min at 4˚C and the cell extract was susbequently transferred to chilled fresh tubes. A total of 10 μl supernatant was collected for use as the input. Following antibody [8 μg CNOT1 antibody (cat. no. 14276-1-AP), 10 μg TTP antibody (cat. no. ABE285) and 10 μg CNOT7 antibody (cat. no. sc-101009)] and Protein A/G Sepharose beads (from the IP kit) binding for 4 h at 4˚C on a rotary mixer, the 2X SDS-PAGE loading buffer was added to the washed beads and boiled. The eluent was saved by centrifugation at 2,000 x g for 2 min at 4˚C for western immunoblotting experiments.

Statistical analysis. SPSS 19.0 was used for statistical analysis (IBM Corp., Armonk, NY, USA). The data are expressed as the mean ± standard error of the mean. The experiments were repeated three times. Student's t-test and analysis of variance with Bonferroni's post hoc test were used to analyze the differences between the groups and compare the differences between the different time points within the same treatment group, respectively. P<0.05 was considered to indicate a statistically significant difference.

Results

CNOT1 knockdown promotes the TNF-α-induced expression of ICAM-1 and IL-8 in HPMECs. In previous studies it was demonstrated that CNOT1, CNOT7 and TTP were coimmunoprecipitated, TTP was able to bind to the ARE sequences of ICAM-1 and IL-8 mRNAs, and CNOT7 was involved in ICAM-1 and IL-8 regulation by TTP (12,13). As CNOT1, CNOT7 and TTP were coimmunoprecipitated and TTP and CNOT7 were involved in the regulation of ICAM-1 and IL-8 expression, whether CNOT1 affected ICAM-1 and IL-8 expression was examined further in the present study.

Following transfection, HPMECs were stimulated using TNF-α (10 ng/ml). Total protein and the supernatant were collected at 0, 4, 8, 12 and 24 h following TNF-α stimulation. Western immunoblotting results indicated that CNOT1 siRNA (100 nM) significantly reduced CNOT1 to ~11% of the control (P<0.05; Fig. 1A). Fig. 1B demonstrated that TNF-α stimulation significantly increased ICAM-1 levels, which in CNOT1 knocked-down HPMECs were increased when compared with the control siRNA treated cells (P<0.05). In addition, the ELISA results demonstrated that CNOT1 knockdown

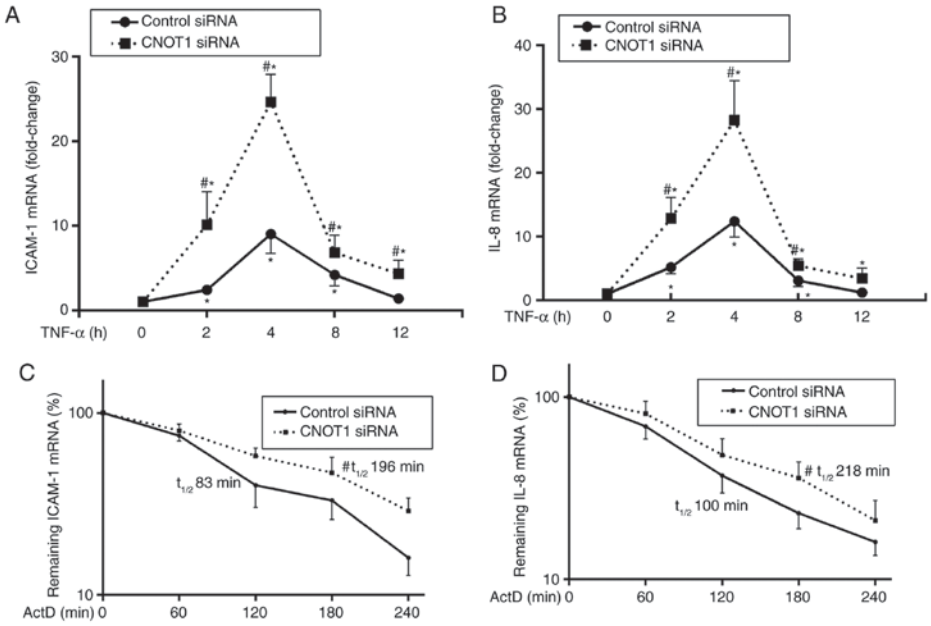

Figure 2. CNOT1 silencing improves ICAM-1 and IL-8 mRNA stability. (A) ICAM-1 and (B) IL-8 mRNA expression significantly increased in CNOT1-silenced HPMECs following TNF-α stimulation. (C) CNOT1 knockdown improved ICAM-1 and IL-8 mRNA stabilization, and the average $t_{1/2}$ of ICAM-1 mRNA was 196 and 83 min in the CNOT1-silenced HPMECs and the control, respectively. (D) The $t_{1/2}$ was 294 and 131 min for IL-8 mRNA in the CNOT1-silenced HPMECs and the control, respectively. The data are expressed as the mean ± standard error of the mean (n=3). #P<0.05 vs. control siRNA-transfected cells; *P<0.05 vs. cells at TNF-α 0 h. IL, interleukin; siRNA, small interfering RNA; HPMECs, human pulmonary microvascular endothelial cells; CNOT1, subunit 1 of the carbon catabolite repressor protein 4-negative on TATA complex; TNF, tumor necrosis factor; ICAM-1, intercellular adhesion molecule 1; $t_{1/2}$, half life; ActD, Actinomycin D.

significantly promoted IL-8 expression compared with the control group and the peak level of IL-8 was observed at 24 h following TNF-α activation (P<0.05; Fig. 1C). These results indicated that CNOT1 may be involved in the TNF-α-induced expression of ICAM-1 and IL-8 in HPMECs.

CNOT1 silencing increases ICAM-1 and IL-8 mRNA stability in HPMECs. As CNOT1 silencing increased ICAM-1 and IL-8 levels in HPMECs, whether CNOT1 effected the expression and stability of ICAM-1 and IL-8 mRNA with AREs at the 3'-UTR was investigated. Cells were transfected with CNOT1 siRNA or control siRNA for 48 h, and then activated using TNF-α (10 ng/ml). Total RNA was extracted at 0, 2, 4, 8 and 12 h and analyzed using RT-qPCR. Fig. 2A demonstrated that the ICAM-1 mRNA expression levels reached the peak level at 4 h following TNF-α activation in control HPMECs and subsequently reduced to baseline following 8 h. CNOT1-silenced HPMECs expressed ~2.7-fold more ICAM-1 mRNA when compared with the control at 4 h and the ICAM-1 mRNA levels at 12 h of TNF-α activation remained significantly elevated compared with the baseline (P<0.05). Similar results were observed for IL-8 mRNA (P<0.05; Fig. 2B). These results demonstrated that CNOT1 knockdown promoted ICAM-1 and IL-8 mRNA expression in HPMECs following TNF-α activation.

Furthermore, whether CNOT1 influenced ICAM-1 and IL-8 mRNA expression by altering mRNA stability was analyzed. CNOT1-silenced and control HPMECs were activated using TNF-α for 4 h, and ActD was added to block transcription. Total RNA was isolated at 0, 60, 120, 180 and 240 min following the addition of ActD and analyzed with qPCR. As demonstrated in Fig. 2C and D, CNOT1 knockdown significantly stabilized ICAM-1 and IL-8 mRNA when compared with the control, and the average half-life of ICAM-1 mRNA was 196 min in CNOT1 knockdown HPMECs and 83 min in the control (P<0.05). For IL-8 mRNA, the average half-life was 218 min in CNOT1 knockdown HPMECs and 100 min in the control (P<0.05). These results implied that CNOT1 reduced ICAM-1 and IL-8 mRNA levels by decreasing the stability of the two mRNAs.

CNOT7, CNOT1 and TTP co-localize in the cytoplasm of HPMECs. The results of the authors' previous studies together with the above results indicated that CNOT7, CNOT1 and TTP were coimmunoprecipitated in HPMECs, and all of them may be involved in the regulation of ICAM-1 and IL-8 expression (12,13). The cellular localization of CNOT7, CNOT1 and TTP was subsequently investigated in HPMECs by immunofluorescence experiments in the present study. Fig. 3A demonstrated that CNOT7 and CNOT1 were detected in the cytoplasm and nuclei. CNOT7 was localized in the cytoplasm with a small fraction localized in the nuclei, while CNOT1 was detected in the nuclei and a small fraction located in the cytoplasm. Fig. 3B indicated that CNOT7 and TTP were primarily localized in the cytoplasm and a small fraction of the two were located in the nuclei. As illustrated in Fig. 3C, TTP and CNOT1 were detected in the cytoplasm and nuclei. These results demonstrated that CNOT7, CNOT1 and TTP were detected in the cytoplasm and nuclei, which implied that they were co-localized in the cytoplasm and nuclei following TNF-α stimulation.

Association of CNOT1 with TTP and CNOT7. As CNOT1, CNOT7 and TTP were co-localized in the cytoplasm and were coimmunoprecipitated in HPMECs (12), together with the results published by other studies that CNOT1 could interact with CNOT7 and TTP (10,11), it was hypothesized that CNOT1

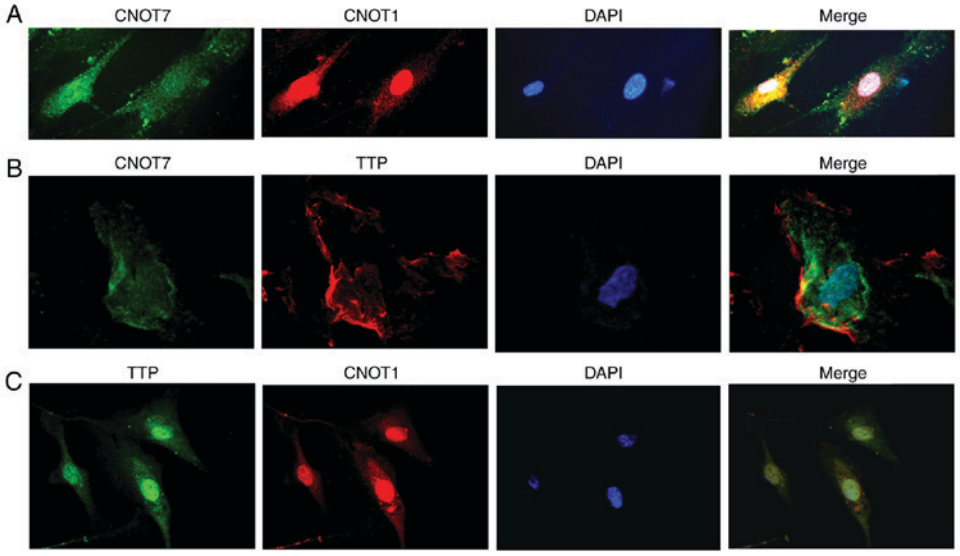

Figure 3. CNOT7, CNOT1 and TTP co-localize in the cytoplasm of HPMECs. HPMECs were activated using tumor necrosis factor-α (10 ng/ml) and DAPI was used to stain the cell nuclei. CNOT7 and CNOT1 were detected in the cytoplasm and nuclei (magnification, x630); (A) CNOT7 was primarily located in the cytoplasm and a small fraction was localized in the nuclei, while CNOT1 was primarily detected in the nuclei and a small fraction was localized in cytoplasm. (B) CNOT7 and TTP were mainly located in the cytoplasm, though a small fraction was localized in the nuclei. (C) TTP and CNOT1 were detected in the cytoplasm and nuclei. siRNA, small interfering RNA; HPMECs, human pulmonary microvascular endothelial cells; CCR4-NOT, carbon catabolite repressor protein 4-negative on TATA; CNOT1, subunit 1 of the CCR4-NOT complex; CNOT7, subunit 7 of the CCR4-NOT complex; TTP, tristetraprolin.

may serve as a platform that can recruit CNOT7 and TTP with ARE-containing mRNA, leading to mRNA decay. CNOT1, CNOT7 and TTP siRNA were used to silence the corresponding protein to investigate the effect of knocking-down one protein on the Co-IP of the other two proteins. The western immunoblotting results indicated that CNOT7 siRNA (100 nM) and TTP siRNA (80 nM) reduced the CNOT7 and TTP levels to ~10 and ~15% of the controls, respectively (Fig. 4A and B). The efficiency of CNOT1 silencing was investigated as described above. The Co-IP experiments were performed as described above and the results demonstrated that CNOT1, TTP and CNOT7 were immunoprecipitated using the CNOT1, TTP or CNOT7 antibody, whereas no CNOT1, TTP or CNOT7 band was detected when using the control antibody in the control cells (Fig. 4C-E). Notably, TTP was undetectable when using CNOT7 antibody in CNOT1-silenced cells, and CNOT7 was not detectable when using TTP antibody (Fig. 4C). However, TTP knockdown did not impact CNOT1-CNOT7 Co-IP (Fig. 4D); neither did CNOT7 knockdown affect CNOT1-TTP Co-IP (Fig. 4E). These results indicated that CNOT1, CNOT7 and TTP were coimmunoprecipitated in HPMECs, and CNOT1 could serve as a platform, which recruits CNOT7 and TTP, while TTP and CNOT7 could not directly combine.

Discussion

Inflammatory mediator expression is regulated at the transcriptional and/or posttranscriptional levels. ARE-mediated mRNA decay (AMD) is an important process during post-transcriptional regulation (16). A number of RNA-binding proteins including TTP (12,13), Human antigen R (17) and poly(A)-binding protein (18) are involved in AMD. TTP is known to serve a critical role in AMD. TTP may bind to the ARE motifs on the 3'-UTR of target mRNA and cause mRNA rapid deadenylation and decay (2,3). It was demonstrated in our previous studies that TTP bound to the AREs of ICAM-1 and IL-8 mRNA, and destabilized the two mRNAs (12,13). TTP and CNOT7 are involved in the regulation of ICAM-1 and IL-8 expression (12,13). In addition, TTP, CNOT7 and CNOT1 were coimmunoprecipitated in HPMECs (12,13). In the present study, CNOT1 silencing increased ICAM-1 and IL-8 mRNA stability. CNOT1, CNOT7 and TTP co-localized in the cytoplasm, and CNOT1 silencing abolished CNOT7 and TTP Co-IP. However, CNOT7 silencing did not influence CNOT1 and TTP Co-IP, and TTP silencing additionally did not influence CNOT1 and CNOT7 Co-IP. These results implied that CNOT1 may serve as a platform to recruit TTP and CNOT7, and be involved in TTP-mediated mRNA decay in HPMECs.

TTP has been proven to mediate ARE-bearing mRNA rapid deadenylation and degradation (2,3); however, TTP itself does not have any deadenylase activity. Previous studies have investigated the proteins that are involved in TTP-mediated mRNA decay. Yamashita *et al* (19) reported that the poly(A)-specific nuclease 2 (PAN2)-PAN3 complex and the CCR4-NOT complex serve critical roles in deadenylation. The PAN2-PAN3 complex hydrolyses poly(A) in a distributive manner (individual and not continuous), whereas the CCR4-NOT complex hydrolyses poly(A) in a processive (continuous) manner and may promote rapid deadenylation (19). Previous studies have additionally demonstrated that CCR4 is involved in the regulation of mRNAs without a dedicated destabilizing motif (19,20) and CAF1 (CNOT7 in human cells), rather than CCR4, is necessary for the degradation of ARE-containing mRNA (9). These results demonstrated that CNOT7 may serve a critical role in ARE-bearing mRNA decay and the authors' previous study confirmed this hypothesis (12). Although TTP, CNOT7 and CNOT1 were coimmunoprecipitated in HPMECs (12), the associations between TTP and CNOT7 have not been completely identified.

CNOT7 is a subunit protein of the CCR4-NOT complex, while CNOT1 is the scaffold protein of the CCR4-NOT complex (10).

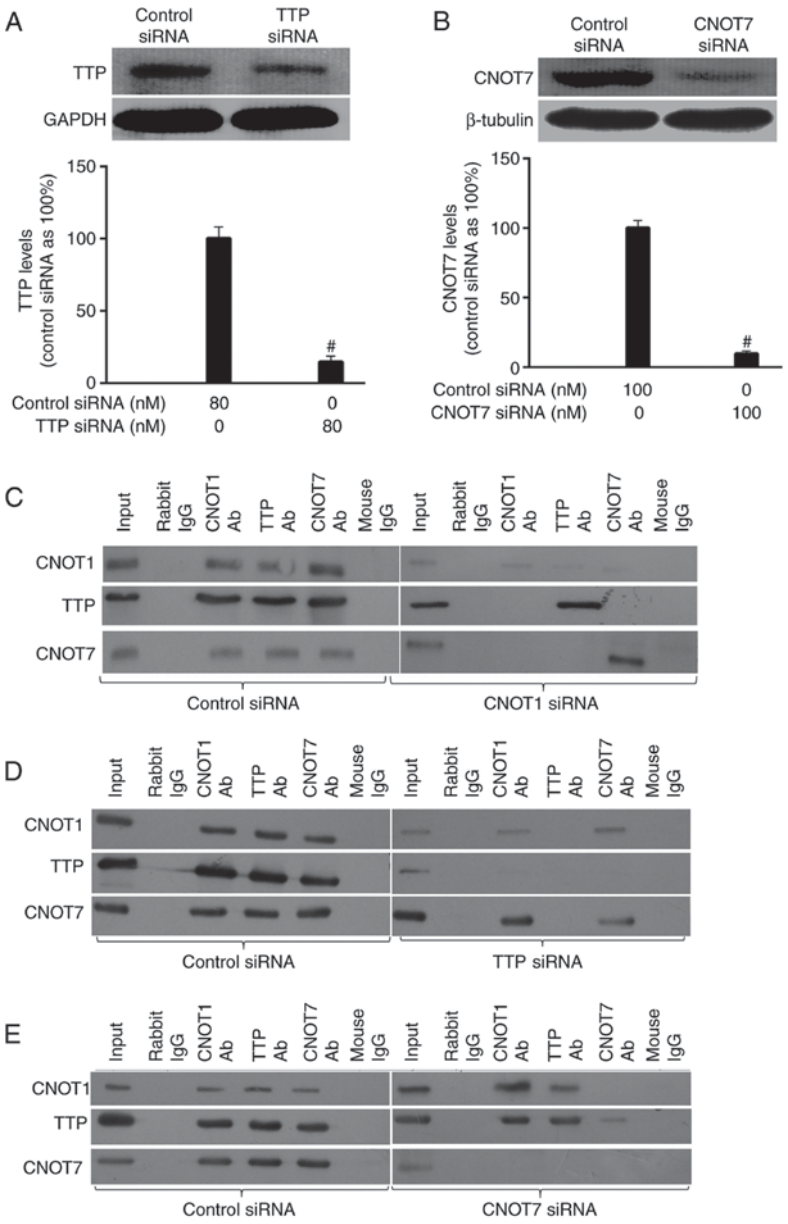

Figure 4. Association of CNOT1 with TTP and CNOT7. Western immunoblotting results indicated that CNOT7 siRNA (100 nM) and TTP siRNA (80 nM) reduced (A) TTP and (B) CNOT7 levels to 15 and ~10% of the controls, respectively. Following transfection, human pulmonary microvascular endothelial cells were activated using tumor necrosis factor-α (10 ng/ml) for 4 h, the Co-IP experiments were subsequently performed using rabbit or mouse IgG as the control. The Co-IP results demonstrated that CNOT1, TTP and CNOT7 were immunoprecipitated by the CNOT1, TTP or CNOT7 Ab, whereas no CNOT1, TTP or CNOT7 bands were detectable when using the control antibody in the control cells. (C) TTP was undetectable when using the CNOT7 antibody in CNOT1-silenced cells, neither was CNOT7 detectable when using the TTP antibody. (D) However, TTP knockdown did not impact CNOT1-CNOT7 Co-IP; nor did (E) CNOT7 knockdown affect CNOT1-TTP Co-IP. The data are expressed as the mean ± standard error of the mean (n=3). $^{\#}$P<0.05 vs. control siRNA-transfected cells. siRNA, small interfering RNA; CCR4-NOT, carbon catabolite repressor protein 4-negative on TATA; CNOT1, subunit 1 of the CCR4-NOT complex; CNOT7, subunit 7 of the CCR4-NOT complex; ICAM-1, intercellular adhesion molecule 1; Co-IP, coimmunoprecipitation; TTP, tristetraprolin; IgG, immunoglobulin G; Ab, antibody.

Fabian *et al* (11) reported that CNOT1 may directly bind to the C terminus of TTP, which provides critical evidence for investigating the model of TTP-mediated mRNA decay. As TTP and CNOT7 interact with CNOT1 (11,21), together with the results of our previous study that revealed that TTP, CNOT7 and CNOT1 were coimmunoprecipitated in HPMECs, it was hypothesized that CNOT1 may serve as a platform that recruits CNOT7 and TTP with ARE-containing mRNA, and they may collectively exert post-transcriptional control of AMD. The authors' previous studies confirmed that TTP and CNOT7 were involved in the regulation of the expression of ICAM-1 and IL-8 (12,13). Based on the above hypothesis, it was speculated that CNOT1 may also be involved in the regulation of ICAM-1 and IL-8 expression, and the results of the present study confirmed this supposition. Furthermore, in the present study it was demonstrated that CNOT1, CNOT7 and TTP co-localize in the cytoplasm, and CNOT1 silencing abolished CNOT7 and TTP Co-IP; however, CNOT7 or TTP silencing did not influence CNOT1-TTP or CNOT1-CNOT7 Co-IP, respectively. These results implied that CNOT1 may bind to TTP and CNOT7, respectively, while TTP and CNOT7 may not directly combine. These results, together with those of the aforementioned previous studies, suggest that CNOT1 may recruit CNOT7 and TTP with ARE-bearing mRNA and together may promote AMD.

In conclusion, CNOT1 may directly bind to the C terminus of TTP and recruit CNOT7 to deadenylate TTP-bound mRNA; however, the mechanism by which TTP rapidly dissociates from CNOT1 and goes on to carry target mRNA remains unclear. In addition, the upstream factors that regulate CNOT7 deadenylase activity are also not well known. Further studies are required to address these questions. A CNOT1 deficiency animal model is additionally required to investigate the effect of CNOT1 on inflammatory mediator expression and acute lung injury *in vivo*.

Acknowledgements

The authors would like to thank Mr. Jin Yang, Mr. Wen-Xue Liang, Mrs. Huan-Huan Zhang, Mrs. Ting Zhang and Mr. Shao-Lin Zhao (all from the First People's Hospital of Lianyungang City, Lianyungang, China) for their technical help.

Funding

The present study was supported by the China Postdoctoral Science Foundation (grant no. 2015M570420), the Jiangsu Provincial Health and Family Planning Commission Science Foundation (grant no. H201558), the National Natural Science Foundation of China (grant nos. 81300052 and 81670073), the Natural Science Foundation of Jiangsu Province (grant nos. BK20130402 and BK20161383) and the Lianyungang Science and Technology Bureau Foundation (grant no. SH1401).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

J-XS and RH analyzed and interpreted the data. X-CZ and C-CL conducted the experiments. J-XS, J-SL and X-ML designed the experiments. HW, YS and XS helped design the experiments and analyzed the data. J-XS wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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