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E3 ubiquitin ligase tripartite motif 7 positively regulates the TLR4-mediated immune response via its E3 ligase domain in macrophages

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ABSTRACT

Members of the tripartite motif (TRIM) family as E3 ubiquitin ligases have been regarded as critical regulators of innate immunity and antiviral response. However, the role of TRIM7 is still elusive. Here, we provide evidence for the importance of TRIM7 in regulation of the TLR4-mediated innate response. In detail, we find that TRIM7 is highly expressed in antigen-presenting cells like macrophages. Knockdown of TRIM7 clearly inhibits the LPS-induced production of IFN-β, TNF-α and IL-6 in macrophages. Conversely, forced expression of TRIM7 could exert an opposite effect on these pro-inflammatory cytokines. Further analysis indicates that such effect is mediated by the TLR4-associated signaling pathways including MAPKs, NF-κB and IRF3-involved pathways. Truncation of the E3 ligase domain on TRIM7 may reduce the production of pro-inflammatory cytokines, suggesting a critical role of this domain in the regulation of LPS-initiated innate response. Taken together, we report here that TRIM7 may facilitate the TLR4-mediated innate response via its E3 ligase domain in macrophages, which provides new insight into the mechanistic role of TRIM7 in innate immunity.

1. Introduction

Infection of cells by microorganisms activates the inflammatory response. The initial sensing of infection is mediated by innate pattern recognition receptors (PRRs)including Toll-like receptors(TLRs) (Kawai and Akira, 2010; O'Neill et al., 2013).Toll-like receptor 4 (TLR4) is one of the well-studied PRRs for the bacterial lipopolysaccharide (LPS) (Rosadini and Kagan, 2016; Kuzmich et al., 2017),which plays a pivotal role in the initiation and elimination of invading pathogens as aberrant activation of TLR4 has been closely associated with inflammatory and autoimmune diseases (Mukherjee et al., 2016). It has been well-documented that activation of TLR4 depends on the sequential binding of LPS-binding protein (LBP),CD14 and MD-2,consequently forming the TLR4-MD2-LPS complex and resulting in the initiation of signal transduction through the MyD88-dependent or TRIF-dependent pathways (Park et al., 2009; Peri and Piazza, 2012; Guven-Maiorov et al., 2015). MyD88-mediated signaling which occurs mainly at the plasma membrane may activate several transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). These transcription factors thereby induce the transcription and release of proinflammatory cytokines including tumor necrosis factor alpha (TNF-α), IL-1β and IL-6 (Molteni et al., 2016; Vijay, 2018; Blasius and Beutler, 2010). Unlike the MyD88-dependent signaling, activation of TRIF-dependent pathway usually occurs at the endosomal compartment initially by recruiting and phosphorylating IFN regulatory factor 3 (IRF3) (Liu et al., 2015). The Phosphorylated IRF3 then dimerizes and translocates to the nucleus to induce the transcription of IFN-β (Honda et al., 2006). The proinflammatory cytokines and type I interferons (IFNs) produced by either way could not only directly suppress the microbial infections, but also activate the adaptive immunity to eliminate the invading pathogens.

Tripartite motif proteins (TRIMs) which belong to E3 ligase family play important roles in some aspects of the immune system, including innate response, adaptive immunity and antimicrobial autophagy. There have been more than 80 TRIM genes reported in mammals (Hatakeyama, 2017). The TRIM proteins are named as their conserved RBCC domain which includes an E3 ligase domain(R) of areally

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interesting new gene (RING), one or two B-box domains (B) and a coiled-coil domain (CC) (Ebner et al., 2017; Reymond et al., 2001). Recently, TRIMs have been described to assemble signaling complexes, alter subcellular localization, mediate proteolytic degradation, or modulate the host's transcription or protein composition by coordinating the ubiquitination of target proteins (Rajsbaum et al., 2014; Versteeg et al., 2014; Esposito et al., 2017). Lines of evidence have shown that microbial pathogens might exploit the ubiquitin modification to evade the host immune system (Jiang and Chen, 2011). Recent studies also show that many TRIM proteins serve as regulators of innate immunity via PRRs such as TLR and RIG-I or via cytokines such as IFN and TNF-α during the signaling transduction (Jiang and Chen, 2011). As examples, TRIM52 overexpression could promote the activation of the NF-κB pathway, and then lead to an increased expression of TNF-α and IL-6 (Fan et al., 2017). TRIM38 could interact with TRAF6 and TRIF, and subsequently mediate K48-linked poly-ubiquitination of TRAF6 and TRIF for proteasome degradation (Zhao et al., 2012). TRIM39 might negatively regulate the NF-κB signaling in collaboration with Cactin induced by TNF-α (Suzuki et al., 2016).

TRIM7 was first identified as glycogenin interacting protein (GNIP) and has been reportedly involved in glycogen synthesis. Knockdown of Trim7 in the primary*K-Ras*^{G12D}*; p53ΔL*$^{/}$*ΔL*(*KP*) lung tumor cells could reduce cell proliferation and decrease the tumor growth in xenograft experiments. Consistently, deletion of Trim7 in an established xenograft tumor also leads to a reduced tumor growth, indicating that intervention of the MSK1/Trim7 RING domain/AP-1 co-activator 1 (RACO-1) signaling pathway may be a potential strategy for the tumor treatment (Chakraborty et al., 2015). However, the role of TRIM7 in the TLR-mediated signaling pathway has not been clarified yet.

In this study, we aim to elucidate the role of TRIM7 in the LPS-initiated inflammatory pathway. We show that TRIM7 could increase the LPS-induced production of TNF-α, IL-6 and IFN-β. Further analysis indicates that such effect is mediated by the TLR4-associated signaling pathways including MAPKs, NF-κB and IRF3-involved pathways. TRIM7 truncation analysis reveals that deletion of the E3 ligase domain may significantly attenuate the effect of TRIM7 on LPS-triggered innate response. These results indicate that TRIM7 may serve as a positive regulator of TLR4-mediated signaling via its E3 ligase domain.

2. Materials and methods

2.1. Mice and reagents

C57BL/6 mice (6–8 weeks) were obtained from Joint Ventures Sipper BK Experimental Animal Co. (Shanghai, China). All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with the approval of Medical Ethics Committee of Jinling Hospital, Nanjing, Jiangsu, China. Thioglycollate Broth, LPS (*E. coli* O111: B4) and Poly (I:C) were from Sigma-Aldrich (St. Louis, MO, USA). Phosphorothioate modified CpG-ODN (B-type CpG-ODN) and qPCR primers were synthesized by Sangon (Shanghai, China) and purified as previously described (Yao et al., 2009).RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). Anti-TRIM7 antibody was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibodies for phosphorylated IKKα/β, p65, IκBα, p38, JNK1/2, ERK1/2 and IRF3 were purchased from Cell Signaling Technology (Danvers, MA, USA). The mouse primary cells were kindly provided by Dr. Yang Yang.

2.2. Cell culture

The primary peritoneal macrophages were prepared from thioglycollate-induced mice by the intraperitoneal injection of thioglycollate broth into C57BL/6 J mice 3–4 days in advance as described previously (Chu et al., 2016). Mouse macrophages were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 1% Penicillin and Streptomycin.

2.3. Plasmid construction and transfection

The HA-tagged recombinant vectors encoding the mouse TRIM7 (GeneBank accession no. NM_001347446.1) were amplified from cDNA of mouse peritoneal macrophages and were then subcloned into the modified pcDNA3.1 vectors (Invitrogen). The vectors expressing the truncated TRIM7 (Δ82–510 amino acids) were constructed with primers: TRIM7-ΔRING sense 5′ CCGCTCGAGAGGACACGGATGGCGACT GTG 3′; TRIM7-ΔRING antisense 5′ CCCAAGCTTGGAGGCCAGATTCT CAAGTATG 3′. All constructs were confirmed by DNA sequencing. Plasmids were transiently transfected into macrophages with jetPEI reagents (Polyplus Transfection) following the manufacturer's instructions.

2.4. Real-time quantitative PCR(qPCR)

The total RNA was extracted and reversely transcribed according to the instructions and qPCR was performed with SYBR **Green** PCR kit (Takara Bio, Shiga, Japan) and LightCycler 2.0 (Roche Diagnostics, CA, USA) as described previously (Wang et al., 2016). The levels of indicated genes were determined by evaluating the threshold cycle (Ct) of the target genes after normalization against the Ct value of β-actin and calculated with the formula $2^{-\triangle\triangle Ct}$ (Sakaguchi et al., 2013). The specific primers used for qPCR assays are listed as following:

TNF-α sense 5′ GACGTGGAACTGGCAGAAGAG 3′,
TNF-α antisense 5′ TTGGTGGTTTGTGAGTGTGAG 3′;
IFN-β sense 5′ CAGCTCCAAGAAAGGACGAAC 3′,
IFN-β antisense 5′ GGCAGTGTAACTCTTCTGCAT 3′;
IL-6 sense 5′ TAGTCCTTCCTACCCCAATTTCC 3′,
IL-6 antisense 5′ TTGGTCCTTAGCCACTCCTTC 3′;
β-actin sense 5′ AGTGTGACGTTGACATCCGT 3′,
β-actin antisense 5′ GCAGCTCAGTAACAGTCCGC 3′;
TRIM7 sense 5′ACAGAAACAGAATGAGAACCTGG3′,
TRIM7 antisense 5′GCTCAGTGTGCTTTTGAACTCC 3′.

2.5. RNA interference

The mouse primary peritoneal macrophages were prepared and cultured as described above overnight followed by the change with the fresh medium before RNA interference. The cells were transfected with siRNA duplexes using the Lipofectamine®RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. The sequences of siRNAs specific for the mouse TRIM7 are as follows:

TRIM7 siRNA 734: 5′ CCAGGACUUGCCUAACCAU 3′,
TRIM7 siRNA 922: 5′ GGGCAAUGCAACUCAACAA 3′,
TRIM7 siRNA 1031: 5′ GGGAGCAGUGUCCUUCUAU 3′,
TRIM7 siRNA 1132: 5′ CCGGCACAUACUUGAGAAU 3′,
Negative control (NC) siRNA: 5′ UUCUCCGAACGUGUCACGU 3′,

These siRNAs were designed and synthesized by GenePharma Co. (Shanghai, China). After 48 h, the cells or culture supernatants were harvest for the analyses of western blot or qPCR or ELISA.

2.6. ELISA assay

TNF-α, IL-6 (R& D Systems, MN, USA) and IFN-β (PBL, NJ, USA) in the supernatants were measured with ELISA kits according to the manufacturer's instructions.

2.7. Immunoblot analysis

Total cell lysates were extracted with cell lysis buffer (Cell Signaling Technology), additional phosphatase inhibitor cocktail (Calbiochem) and 1 mM Phenylmethanesulfonyl fluoride (PMSF). Equal amounts of

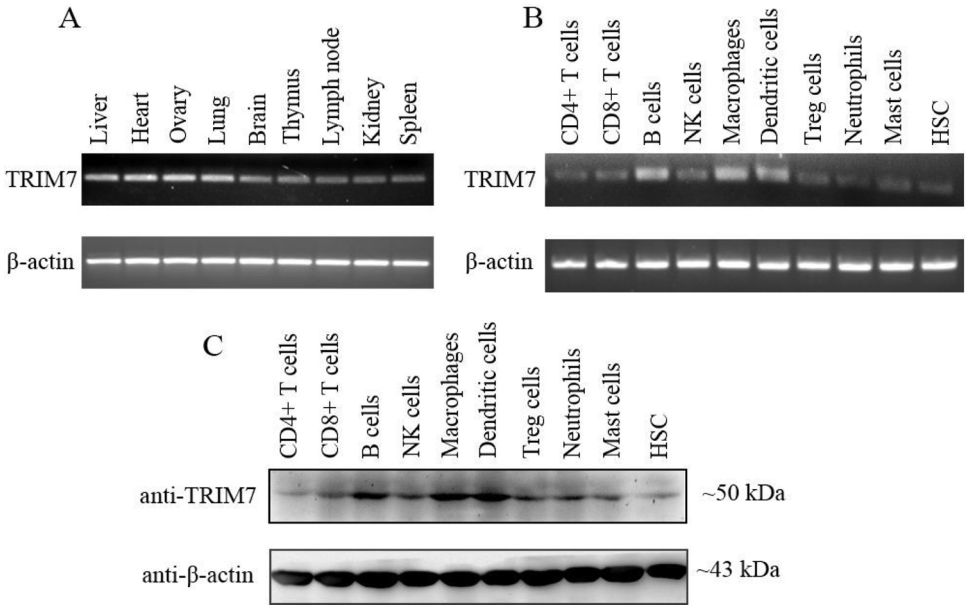

Fig. 1. Expression pattern of TRIM7 in mouse tissues and primary cells. **A.**qPCR analysis of TRIM7 transcription in different tissues. β-actin was used as control. **B and C.**qPCR and western blot analyses of TRIM7 expression in various primary cells.

proteins were prepared based on their concentration determined by the BCA assay kit (Pierce, IL, USA) for Western blot analysis with indicated antibodies as described (Wu et al., 2011).

2.8. Statistical analysis

The statistical significance of comparisons between two groups was analyzed with two-tailed Student's *t*-test. Data were presented as mean ± S.D. The *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. TRIM7 is highly expressed in antigen-presenting cells

To investigate the role of TRIM7, firstly, we checked its expression pattern in different mouse tissues and primary cells. qPCR analysis showed that TRIM7 exhibited abroad expression pattern in various mouse tissues (Fig. 1A). However, further analysis of the different primary cells revealed that TRIM7 was highly expressed merely in antigen presenting cells including the primary macrophages, B cells and dendritic cells (Fig. 1B). Consistent with qPCR results, the selective high expression of TRIM7 in antigen-presenting cells was also confirmed by western blot analysis with antibodies against TRIM7 (Fig. 1C). These results indicate that TRIM7 may play an important role in innate immunity.

3.2. TRIM7 promotes the LPS-induced production of proinflammatory cytokines and type I IFN in macrophages

Recent studies show that many TRIM proteins serve as regulators of innate immunity via PRRs such as TLR. Therefore, to determine whether TRIM7 is involved in the TLR-mediated innate immunity, we stimulated macrophages with LPS, Poly(I:C) and CpG-ODN which are the ligands for TLR4, 3, 9 respectively. qPCR and western blot analyses showed that TRIM7 expression was down-regulated immediately after LPS treatment, but restored to the initial level after 24 h stimulation (Fig. 2A-B). The tendencies of TRIM7 expression were not identical after stimulation with TLR3 and TLR9 ligands, and the change after LPS stimulation was most significant and characteristic (Fig. 2A-D). To explore the role of TRIM7 in the LPS-triggered innate response in macrophages, we constructed the pcDNA3.1 vector expressing HA-tagged full-length TRIM7 (1–510; HA-TRIM7 vector).After transfection into the primary peritoneal macrophages, the success expression of HA tagged TRIM7 was confirmed by western blot analysis (Fig. 3A-B). Overexpression of TRIM7 significantly promoted the LPS–induced expression of TNF-α, IL-6 and IFN-β at both transcriptional (Fig. 3C-E) and protein levels (Fig. 3F-H). In contrast, overexpression of TRIM7 could not enhance TLR3- or TLR9-triggered response in macrophages when the macrophages were stimulated with Poly(I:C) or CpG-ODN (Data not shown), suggesting the specific effects of TRIM7 in the TLR4-triggered response.

To further confirm the observation above, we knocked down the endogenous expression of TRIM7 in primary peritoneal macrophages by using several TRIM7-targeting siRNAs. qPCR analysis indicated that siRNA922 showed the highest efficiency on the suppression of the endogenous TRIM7 transcription in primary peritoneal macrophage(decreased by˜35%)as compared with other siRNAs, which was also confirmed by western blot analysis (Fig. 4A-B). Then, by using this TRIM7-specific siRNA, we checked its effects on the LPS-induced response of macrophages. Knockdown of TRIM7 significantly decreased the LPS–induced expression of TNF-α, IL-6 and IFN-β in primary peritoneal macrophages at both mRNA (Fig. 4C-E) and protein levels (Fig. 4F-H).Overall, these results suggest that TRIM7 may promote the production of proinflammatory and type I IFN cytokine in macrophages by TLR4, but not TLR3 or TLR9.

3.3. TRIM7 enhances TLR4-mediated signaling in macrophages

To investigate the molecular mechanism by which TRIM7 promotes the TLR4-triggered production of inflammatory cytokines and type I IFN, several key signaling molecules in the TLR4-mediated pathway including MAPKs, NF-κB and IRF3 (Qian and Cao, 2018) were analyzed in TRIM7-overexpressed macrophages. As shown in Fig. 5A, TRIM7 overexpression significantly promoted the LPS-induced activation of ERK1/2, p38 and JNK1/2. Conversely, silencing of TRIM7 in primary peritoneal macrophages significantly inhibited the phosphorylation of ERK1/2, p38 and JNK1/2 induced by LPS (Fig. 5B). It has been reported that LPS ligation could activate IKK complex and in turn phosphorylate IκBα. Phosphorylated IκBα could then be degraded by 26S proteasome,

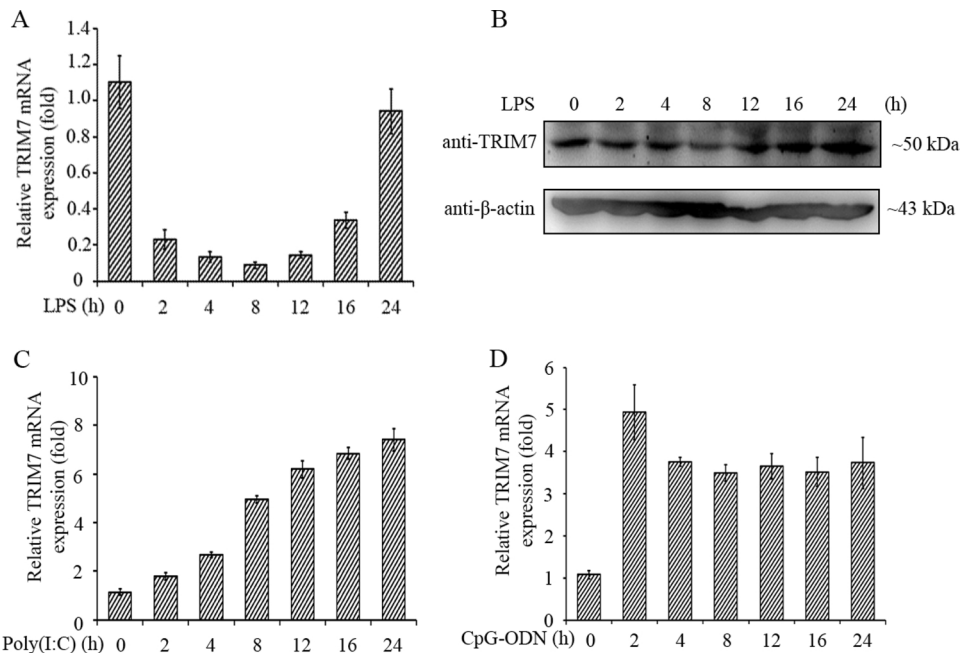

Fig. 2. TRIM 7 expression is down-regulated by LPS stimulation, but not by Poly(I:C) or CpG-ODN. **A.** Primary peritoneal macrophages were treated with 100 ng/ml LPS. The mRNA level was detected by qPCR at the time point of 2, 4, 8, 12, 16 and 24 h, respectively after LPS stimulation. β-actin was used as internal control. **B.** Western blot analysis of TRIM7 expression as described in (A). **C–D.**Primary peritoneal macrophages were treated with 10 μg/ml Poly(I:C) (**C**) or 1 μM CpG-ODN (**D**).The mRNA level was detected by qPCR at the time point of 2, 4, 8, 12, 16 and 24 h, respectively after the stimulation. Data are shown as mean ± SD of three independent experiments.

and consequently induce the nuclear transport of NF-κB proteins and initiate the downstream transcription of target genes(O'Neill et al., 2013).So, we further examined whether TRIM7 was involved in the TLR4-induced activation of IKKα/β, p65 and IκBα. Overexpression of TRIM7 in macrophages could obviously enhance LPS-stimulated phosphorylation of IKKα/β, p65 and IκBα (Fig. 5A). Conversely, TRIM7 knockdown in macrophages could suppress the LPS-induced phosphorylation of these proteins (Fig. 5B). Considering that both IRF3 and NF-κB activation contribute to the TLR4-mediated transcriptional activation of IFN-β gene (Kawai and Akira, 2010), we then checked the effect of TRIM7 on the LPS-triggered activation of IRF3. Overexpression of TRIM7 promoted the phosphorylation of IRF3 (Fig. 5A), while silencing of TRIM7 markedly suppressed the LPS-induced phosphorylation of IRF3 (Fig. 5B).These results indicate that TRIM7 may promote the production of TNF-α, IL-6 and IFN-β by the activation of MAPKs, NF-κB, and IRF3 pathways in macrophages, suggesting an important role of TRIM7 in the TLR4-mediated signaling induced by LPS.

3.4. TRIM7 promotes the TLR4-mediated activation through its E3 ligasedomain

The E3 ligase domain(RING) on TRIMs has been reportedly involved in the ubiquitination of key proteins in TLR signaling transduction (Ozato et al., 2008). So, to check whether this domain of TRIM7 was required to promote the TLR4-mediated production of inflammatory cytokine and type I IFN, two pcDNA3.1 vectors were constructed expressing full-length TRIM7 (1–510; TRIM7 vector) or E3 ligase domain–depleted TRIM7 (82–510; TRIM7-ΔRING vector) under the control of a CMV promoter. Transfection of the TRIM7 vector into the TRIM7-silenced macrophages could rescue the attenuated expression of inflammatory cytokines and type I IFN, which, however was not observed when the cells were transfected with the TRIM7-ΔRING vector (Fig. 6). These results indicate that the E3 ligase domain of TRIM7 is essential for its function in the TLR4-triggered innate immune response.

4. Discussion

Innate immunity plays an essential role in the control and elimination of bacterial infection through production of inflammatory cytokines (Liu et al., 2015). Production of inflammatory cytokines requires the recognition of bacterial pathogen-associated molecular patterns by TLRs receptors. Full activation of TLR4 could be significant part in the initiation of innate response and subsequent elimination of invading pathogens (O'Neill and Bowie, 2007).It has been well-documented that TLR4 could be activated through either MyD88-dependent or TRIF-dependent pathways. TLR4-deficient mice show a suppressed production of type I IFNs and proinflammatory cytokines and are highly susceptible to bacterial infection (Deng et al., 2013). So, recently, much attention has been devoted to identifying the regulators of TLR4-triggered immune response attracts. Our group previously reported that CHIP could positively regulate TLR4 signaling by recruiting and polyubiquitinating Src and atypical PKC?? (Yang et al., 2011). In this study, we provide evidence for the critical role of TRIM7 in TLR4-associated signaling pathways and the production of TLR4-induced inflammatory cytokine. Overexpression of TRIM7 results in the activation of MAPKs, NF-κB and IRF3, and leads to an increase in LPS-triggered production of type I IFN, TNF-α and IL-6. TRIM7 truncation analysis reveals that deletion of the E3 ligase domain may significantly attenuate the effect of TRIM7 on LPS-triggered innate response. These results indicate that TRIM7 may positively regulate the TLR4-mediated signaling via its E3 ligase domain. To our knowledge, this is the first report showing the biological significance of E3 ligaseon TRIM7 in the regulation of the innate inflammatory response.

TRIM7 has been reportedly involved in some important biological processes including tumor cell proliferation and glycogen metabolism (Montori-Grau et al., 2018; Zhan et al., 2015). Here, we show that TRIM7 is critical for the TLR4-associated signaling pathways and the production of TLR4-induced inflammatory cytokine. However, interestingly, LPS treatment may reduce TRIM7 expression initially in macrophages. Considering the importance of TLR4 in the host antibacterial immunity, one possibility for this result is that bacteria may modulate TRIM7 expression to escape immune surveillance and facilitate bacteria replication. Moreover, we demonstrate that TRIM7 is primarily expressed in antigen-presenting cells including macrophages, dendritic cells and B cells. Over-expression and silencing experiments above indicate that TRIM7 could facilitate the LPS-initiated TLR4 signaling by promoting the phosphorylation levels of MAPKs, NF-κB and IRF3.

TRIM7 has been functionally linked to lys63-ubiquitination-mediated protein activation. However, the role of TRIM7 in innate immunity and inflammation is still limited. As previously reported, TRIM7 as an E3 ligase could mediate K63-linked ubiquitination of the AP-1

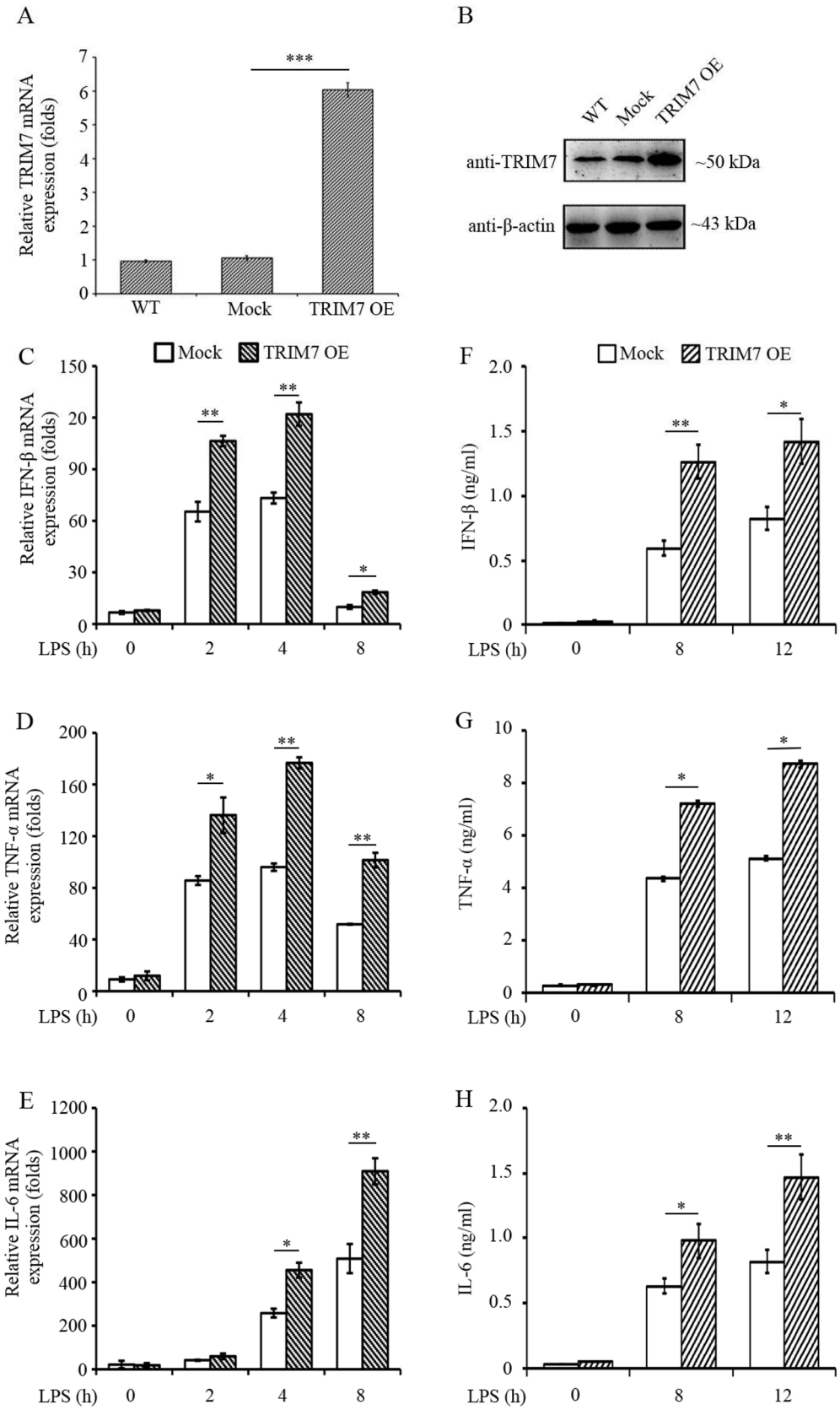

Fig. 3. TRIM7 overexpression in macrophages up-regulates the production of proinflammatory cytokines and type I IFN. **A.** Macrophages (3×10^5) were transiently transfected with plasmid expressing full-lengthTRIM7 (TRIM7 OE) or empty vector (Mock). After 24 h, the cells were harvested for qPCR analysis. **B.** Macrophages (2×10^6) were transiently transfected with plasmid expressing full-length TRIM7 (TRIM7 OE) or empty vector (Mock). After 24 h, the cells were harvested for Western blot analysis. **C–E.**As described in **A**, after 24 h transfection, the cells were stimulated with 100 ng/ml LPS. Transcriptional levels of IFN-β (**C**),TNF-α (**D**) and IL-6 (**E**)were measured by qPCR at the indicated time points after stimulation. **F–H.**IFN-β (**F**), TNF-α (**G**) and IL-6 (**H**) in the supernatants were detected by ELISA. Results were obtained in three independent experiments. Data are shown as mean ± SD. *, $P < 0.05$; **, $P < 0.01$.

coactivator RACO-1, then leading to RACO-1 protein stabilization (Chakraborty et al., 2015).Although in the present study no protein is shown to interact with TRIM7 or be modified with K63-ubiquitination, we have already prepared different truncated TRIM7 including delta RING (82–510 aa), N165 (1–165 aa), N275 (1–275 aa), C165 (166–510 aa), C275 (276–510 aa)(data not shown), which will be used to further investigate how TRIM7 functions in the TLR4-mediated innate response.

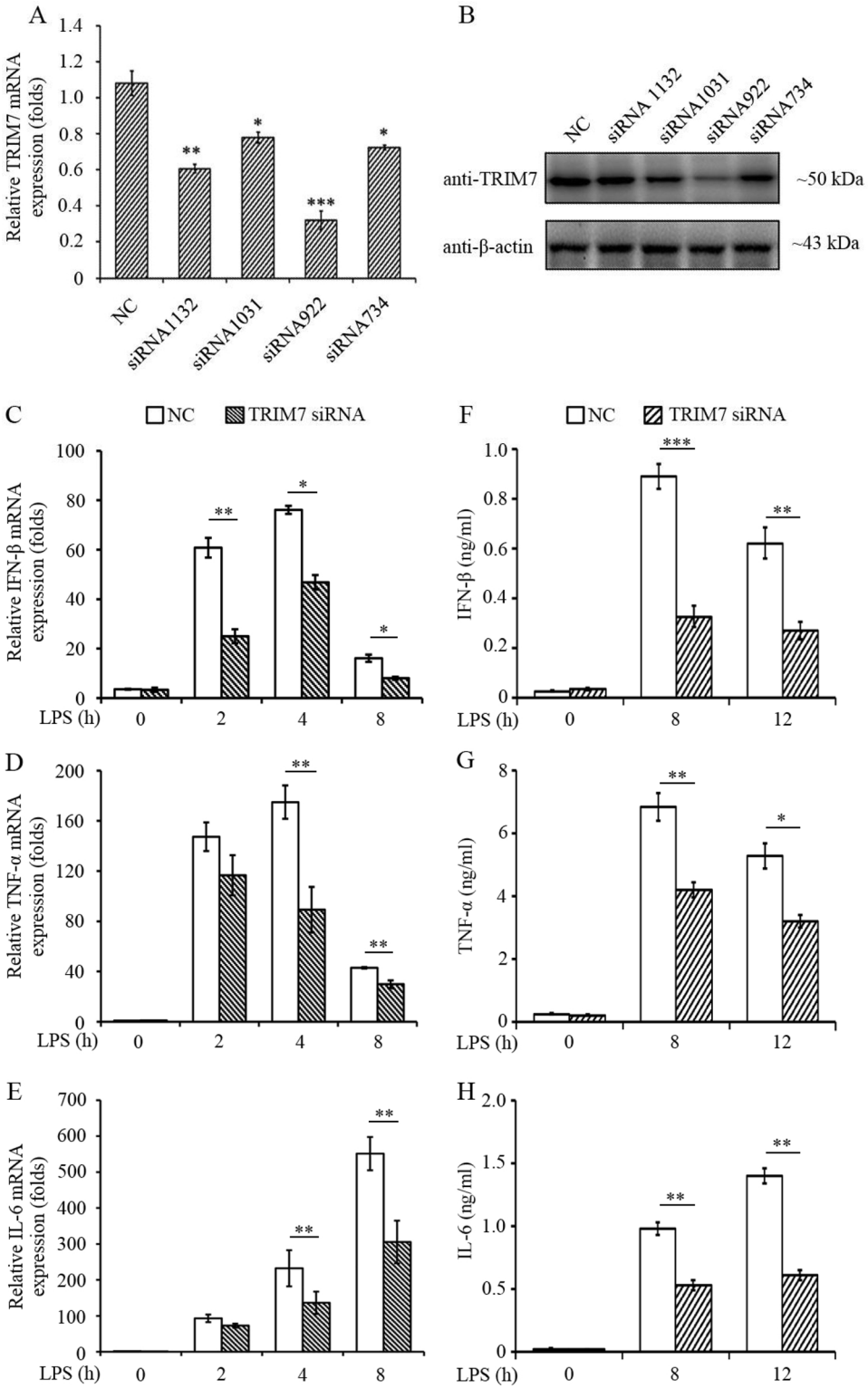

Fig. 4. Silencing of TRIM7 suppresses the production of LPS-induced proinflammatory cytokines and type I IFN in macrophages. **A–B.**Primary peritoneal Macrophages were transiently transfected with TRIM7 siRNA or negative control siRNA (NC). After 48 h, the efficiency of silencing was examined by qPCR (**A**)and western blot (**B**). **C–E.**Primary peritoneal macrophages (3×10^5) were transfected with siRNA 922 or negative control siRNA. After 48 h, the cells were stimulated with 100 ng/ml LPS. Transcriptional levels of IFN-β (**C**), TNF-α (**D**) and IL-6 (**E**) were measured by qPCR at the indicated time points after stimulation. **F–H.**IFN-β (**F**), TNF-α (**G**) and IL-6 (**H**) in the supernatants were detected by ELISA. Results were obtained in three independent experiments. Data are shown as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

In summary, here, we show that TRIM7 expression could be downregulated by LPS stimulation and TRIM7 may serve as a positive regulator for the TLR4-mediated signaling via its E3 ligase domain, which provides new insight into the mechanistic role of TRIM7 in innate immunity.

Author contributions

Mingfeng Lu and Xuhui Zhu designed the experiments. Mingfeng Lu, Xuhui Zhu, Zhizhou Yang, Wei Zhangand Qijian Ji performed the experiments. Mingfeng Lu, Xuhui Zhu and Shinan Nie analyzed the data. Zhaorui Sun, Xin Chen, Jin Zhu and Changjun Wang contributed to the preparation of reagents or materials. Mingfeng Lu and Xuhui Zhu wrote the paper.

Conflicts of interest

The authors declare no financial conflict of interest.

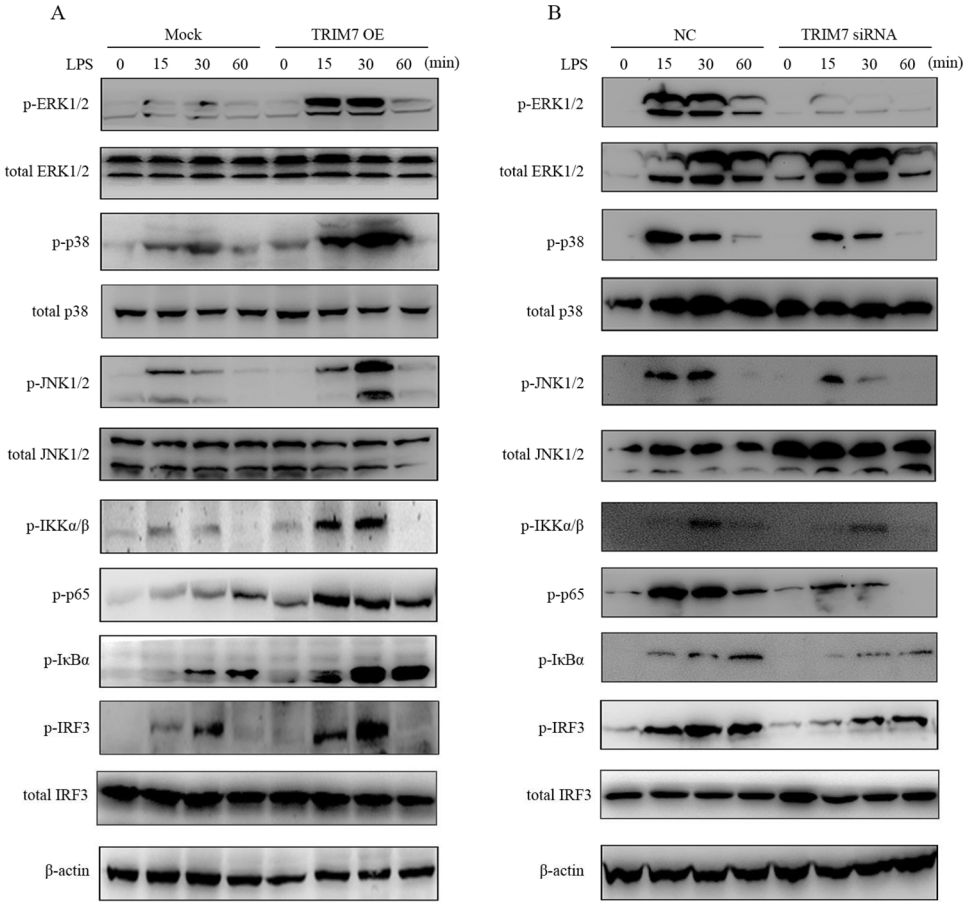

Fig. 5. TRIM7 enhances the activation of TLR4-mediated signaling pathway in macrophages. **A.** Mouse primary peritoneal macrophages (2×10^6) were transiently transfected with plasmids expressing full-length TRIM7(TRIM7 OE) or empty vector (Mock). After 24 h transfection, the cells were stimulated with 100 ng/ml LPS. Phospho-ERK1/2, p38, JNK1/2, IKKα/β, p65, IκBα and IRF3 were detected by western blot at the indicated time points after stimulation. **B.** Mouse primary peritoneal macrophages (2×10^6) were transfected with TRIM7-specific siRNA or negative control siRNA (NC). After 48 h, the cells were stimulated with 100 ng/ml LPS. Phospho-ERK1/2, p38, JNK1/2, IKKα/β, p65, IκBα and IRF3 were detected by western blot at the indicated time points after stimulation. Data are representative of three independent experiments.

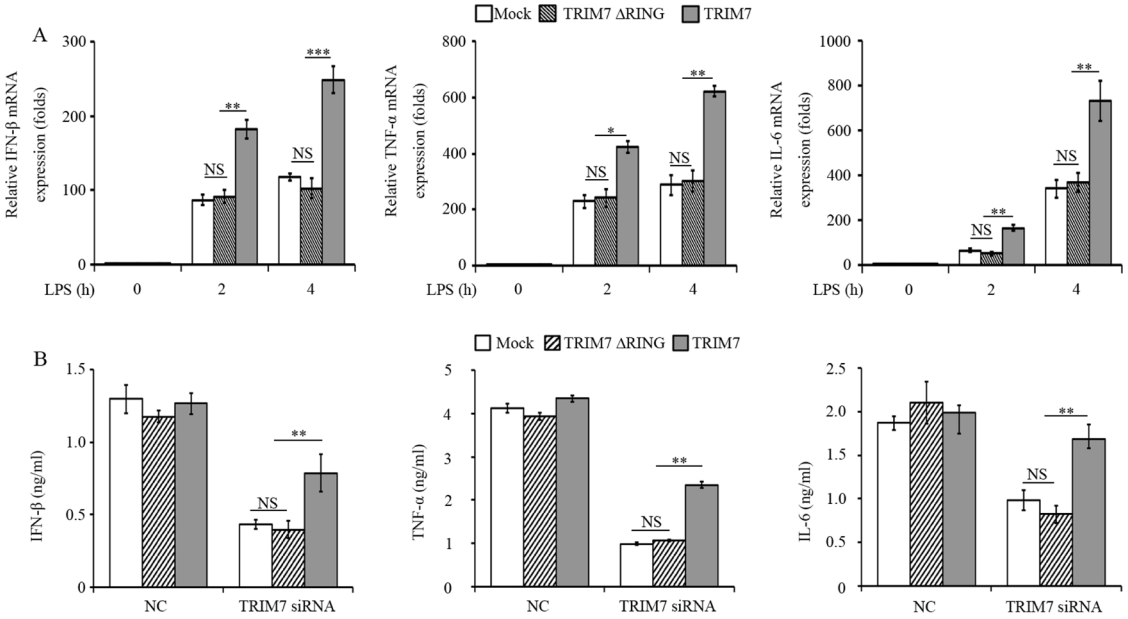

Fig. 6. TRIM7 promotes the production of the TLR4-induced inflammatory cytokine via its E3 ligase activity. **A.** Mouse peritoneal macrophages were transfected with TRIM7 siRNA for 24 h followed by further transfection with the pcDNA3.1 empty vector (Mock) or the TRIM7 ΔRING vector or the TRIM7 vector. After 24 h, the cells were then stimulated with LPS (100 ng/ml). mRNA levels of IFN-β, TNF-α and IL-6 were detected by qPCR at the indicated time points after the stimulation. **B.** Macrophages were transfected as described in (**A**), After 24 h, the cells were stimulated with LPS (100 ng/ml). The supernatants were collected after 12 h stimulation. IFN-β, TNF-α and IL-6 in the supernatants were detected using ELISA. Data are shown as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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