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USP25 promotes endotoxin tolerance via suppressing K48-linked ubiquitination and degradation of TRAF3 in Kupffer cells

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ABSTRACT

The inhibition of tumor necrosis factor receptor-associated factor 3 (TRAF3) degradation induces endotoxin tolerance (ET) in macrophages. However, the mechanisms leading to TRAF3 inhibition by ET are largely unknown. Here, we found that ubiquitin-specific peptidase 25 (USP25), a deubiquitinating enzyme (DUB), interacted with TRAF3 and stabilized ET in Kupffer cells (KCs). Lentiviral knockdown of USP25 activated K48-linked ubiquitination of TRAF3 and the cytoplasmic translocation of the Myd88-associated multiprotein complex in tolerized KCs. This outcome led to a subsequent activation of Myd88-dependent c-Jun N-terminal kinase (JNK) and p38-mediated downregulation of inflammatory cytokines. The overexpression of TRAF3 attenuated the proinflammatory effects of USP25 knockdown in tolerized KCs. Thus, our findings reveal a novel mechanism of endotoxin-mediated TRAF3 degradation in KCs.

1. Introduction

Endotoxin tolerance (ET), which is a transient state in sepsis, is a mechanism that prevents proinflammatory cytokine overproduction and subsequent tissue damage (Poplutz et al., 2017). Monocytes isolated from septic patients demonstrate a diminished ability to release proinflammatory cytokines following endotoxin administration (Escoll et al., 2003; Faas et al., 2002). In reducing excessive systemic inflammation in sepsis, ET also causes immunosuppression and secondary infections that may lead to increased morbidity and mortality (Perkins et al., 2010). Therefore, understanding the mechanisms leading to ET may lead to improved treatments for sepsis.

Kupffer cells (KCs) are critical players in mediating ET. Mechanisms, including toll-like receptor 4 (TLR4) (Li et al., 2015; Nakasone et al., 2016) induction of inhibitory proteins, such as Nrf2 and Twist-2 (Li et al., 2015, 2014a), may explain some effects on ET in KCs conditions. Our previous study indicated that the degradation of tumor necrosis factor receptor-associated factor 3 (TRAF3) in KCs is inhibited during ET through suppression of K48 ubiquitin ligase activity (Li et al., 2016). TRAF3 is an E3 ubiquitin ligase that mediates TRIF- and Myd88-dependent pathways (Häcker et al., 2006; Oganesyan et al., 2006; Tseng et al., 2010). TRIF- and Myd88-dependent pathways play critical roles in the development and progression of ET (Rajaiah et al., 2015). Mitogenactivated protein kinase (p38) and c-Jun N-terminal kinase (JNK) are key signaling intermediates in the TRIF and Myd88-dependent regulation of ET (Cabal-Hierro et al., 2014; Ma et al., 2017). Conversely, deubiquitination is mediated by deubiquitinating enzymes (DUBs), which are key to cellular functions, including gene expression, cell-cycle regulation, signal transduction, and protein degradation (Komander et al., 2009; Reyes-Turcu et al., 2009). USP25, which is a member of the USP subfamily, is involved in the immune response (Zhong et al., 2012), endoplasmic-reticulum-associated protein degradation (Blount et al., 2012), and cell migration and invasion (Li et al., 2014b). TRAF3 is regulated by USP25 during TLR4-mediated signaling (Zhong et al., 2013); however, it is unknown if this mechanism occurs during ET. Here, we demonstrated that USP25 is important in regulating the protective functions of KCs in ET. USP25 deficiency in KCs potentiated LPS-induced ubiquitination of TRAF3 through a physical interaction that enhances the production of proinflammatory cytokines and impairs the production of anti-inflammatory cytokines. USP25-TRAF3 interactions specifically removed K48-linked polyubiquitin chains from TRAF3, allowing TRAF3 to induce ET. Thus, USP25 is a critical regulator of ET and acts by preventing the degradation of TRAF3.

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2. Methods

2.1. Reagents

USP25, TRAF3, JNK, P38, phospho (p)-p38, p-JNK, IL-1β, IL-10, and actin antibodies were purchased from Abcam (Cambridge, MA, UK), and antibodies for K48-linked ubiquitin were purchased from Millipore (Billerica, MA, USA). Mouse IL-1β and IL-10 enzyme-linked immunosorbent assay (ELISA) kits were from Boster Biological Technology (Wuhan, China). Ultrapure 0111: B4 LPS was from Sigma-Aldrich (St. Louis, MO, USA) and TRAF3, USP25 shRNA (m), lentiviral particles, Polybrene®, and puromycin dihydrochloride were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Adeno-associated Viral Vectors Helper-Free System was purchased from HanBio (Shanghai, China). The coding sequence (CDS, Gene ID: 498951) for USP25 and TRAF3 were cloned into pHBAAV-CMV-MCS-EGFP vectors to construct pHBAAV-CMV-USP25-EGFP that stably expressed IL-34.

2.2. Animals

Only male C57BL/6 mice that were 8 weeks of age were used for these experiments (Experimental Animal Center of Chongqing Medical University, Chongqing, China). The animals were humanely handled in accordance with the National Institutes of Health guidelines for animal research. The mice were randomly selected for inclusion into group 1: the ET group (intraperitoneal injections of 0.5 mg/kg LPS); or group 2: the non-ET (NET) group (intraperitoneal injections of sterile normal saline). Twenty-four hours after initial injections, the mice were given 15 mg/kg LPS. Each group of 10 mice was used to observe weight change and survival rate, and 5 mice were used for lentiviral infection.

2.3. Preparation of recombinant adenovirus

Adenovirus containing USP25 was purchased from Hanbio Biotechnology (Shanghai, China) and propagated in 293 cells. Infected 293-cell supernatants were subjected to cesium chloride density gradient centrifugation to purify the virus. The titers were determined using a plaque assay, and 1×10^9 plaque-forming units were intravenously administered to mice (n = 5) prior to LPS stimulation.

2.4. Isolation and culture of KCs

Mouse livers were perfused in situ with 10 ml PBS (3 ml/min) at 37 °C to remove red blood cells. The liver was then excised, and the tissue was minced before digestion with 1640 (RPMI 1640, HyClone) containing 0.1% type IV collagenase at 37 °C for 30 min. The cell suspension was centrifuged at 300 × g for 5 min at 4 °C. The cell sediment, identified with F4/80 and CD11b by flow cytometry (Fig. S), mostly were KCs (Li et al., 2014c). KCs were cultured in 6-well plates ($3–4 \times 10^5$ cells/well) in Dulbecco's Modified Eagle's medium (DMEM, HyClone, USA). KCs represented greater than 70% of the cells as identified by flow cytometry with F4/80 and CD11b. KCs were either pretreated with 10 ng/ml LPS (ET group) or DMEM medium (NET group). The cells in each group received fresh media containing LPS (100 ng/ml) 24 h later.

2.5. Lentiviral particle transduction in KCs

Lentiviral transduction of KCs with USP25 and TRAF3 shRNA (m) was performed as previously described (Li et al., 2016). In brief, the cells were cultured overnight in 12-well plates (2×10^5 cells/well) in complete media. The media were replaced with media containing 5 μg/ml Polybrene® (sc-134220) and lentiviral particles containing shRNAs for 24 h and then cultured for an additional 48 h in complete medium without Polybrene. Puromycin dihydrochloride (6 μg/L) was then added to select for stable clones.

2.6. Liver function analysis

Commercial kits and an automatic biochemical analyzer (Olympus-AU5400, Japan) provided by the Laboratory of The Second Affiliated Hospital of Chongqing Medical University were used for ALT and AST measurements.

2.7. Coimmunoprecipitation and immunoblotting

Following cross linking and sonication, lysates (200 μl) were split into two samples, 10 μl from each was saved as input for comparison with immunoprecipitates, and the remaining lysate was diluted to 250 μl with sonication buffer and immunoprecipitated overnight at 4 °C with anti-TRAF3 antibody or rabbit IgG control antibody (Abcam, ab46540) according to the manufacturer's directions. Prior to the immunoprecipitation, the antibodies were prebound for 2 h at RT to 100 μl of protein A coupled magnetic beads (Dynabeads, Invitrogen) in PBS with 5% BSA. Cell lysates were resuspended in ice-cold lysis buffer containing 0.2% Nonidet P-40 and 150-mM NaCl in 10-mM Tris (pH 7.4). Cytoplasmic fractions were prepared as previously described (Tseng et al., 2010). Ubiquitination was measured by adding N-ethylmaleimide (20 mM, NEM, Sigma) to lysates. SDS-polyacrylamide gel electrophoresis for immunoblotting was performed using a Bio-Rad Mini protein apparatus (Bio-Rad, Hercules, CA, USA) and electrotransferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked and probed with the indicated antibodies (described above).

2.8. Quantitative polymerase chain reaction (qPCR)

TRIzol (Takara, Dalian, China) was used to lyse cells and, following RNA isolation, cDNA synthesis was performed using the PrimeScript® RT reagent Kit with gDNA Eraser (Takara, Japan). Reactions were performed in a Bio-Rad CFX Connect TM Real-Time System (Bio-Rad, Hercules, CA, USA). The following qPCR primer sequences were used: mouse USP25, forward 5'-AGGTACATCAGCGTGGGAAG-3′, reverse 5'－CCTGAATGCCCTGTTTGACT-3′; mouse TRAF3, forward 5'-TCAGG AAATTTGCCTATTGAAAATTT-3′, reverse 5'-GCTTTGTCTTTCTTGTTA TCTTTTAAGTTGT-3′ and β-actin: forward 5'CATTGTGATGGACTCCG GAG-3′; reverse 5'-CTGCCGGTCCAGTAGTATA-3′. Beta-actin was used to normalize values and results are presented as the mean of duplicates.

2.9. Histological analysis

Liver tissues were fixed in 10% neutral formalin, dehydrated, and embedded in paraffin, and 5-μm-thick sections were placed on glass slides. The slides were stained with hematoxylin and eosin (H&E).

2.10. ELISA

Culture supernatants were assayed for IL-1β and IL-10 levels using mouse ELISA kits according to the manufacturer's protocol (Boster Biological Technology, Wuhan, China).

2.11. Statistical analysis

Data are represented as the mean ± SD and were analyzed using SPSS 20.0 software (SPSS, Inc., Chicago, IL). One group was compared by one-way analysis of variance (ANOVA). Two groups were compared by two-way Repeated Measures ANOVA for multiple comparisons. Differences were deemed significant if $p < 0.05$.

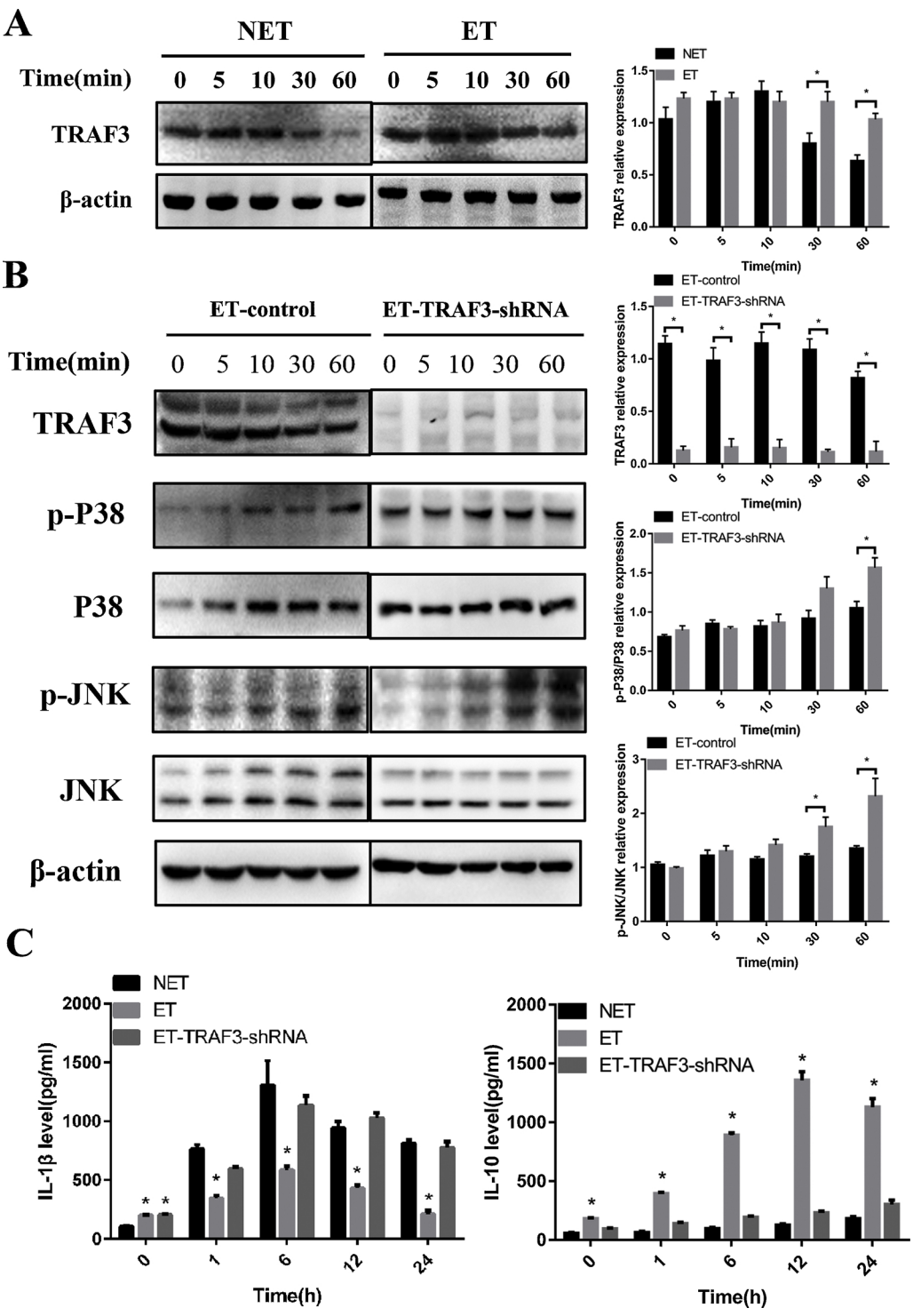

Fig. 1. The effect of TRAF3 on ET in KCs. (A) The degradation of TRAF3 in ET conditions was lower than that in NET conditions at 30 min and 60 min (0 m in. 1.03 ± 0.11, 1.23 ± 0.05; 5 min: 1.20 ± 0.10, 1.23 ± 0.05; 10 m in. 1.30 ± 0.10, 1.20 ± 0.10; 30 min: 0.80 ± 0.10, 1.20 ± 0.10; 60 min: 0.63 ± 0.05, 1.03 ± 0.05; n = 5 mice/time point/group). (B) TRAF3 deficiency promoted an inflammatory response by activation of JNK and P38 in ET conditions (p-P38/P38: 0 m in. 0.68 ± 0.02, 0.76 ± 0.05; 5 min: 0.85 ± 0.05, 0.78 ± 0.02; 10 m in. 0.81 ± 0.07, 0.86 ± 0.10; 30 min: 0.91 ± 0.10, 1.30 ± 0.15; 60 min: 1.05 ± 0.08, 1.56 ± 0.12; p-JNK/JNK: 0 m in. 1.05 ± 0.05, 0.98 ± 0.28, 5 min: 1.21 ± 0.10, 1.30 ± 0.10; 10 m in. 1.15 ± 0.05, 1.41 ± 0.10; 30 min: 1.20 ± 0.05, 1.75 ± 0.18; 60 min: 1.35 ± 0.05, 2.31 ± 0.33; n = 5 mice/time point/group). (C) TRAF3 deficiency promoted the release of IL-1β and inhibited the release of IL-10 in the supernatant of KCs in ET conditions (IL-1β: 0 h: 109.00 ± 3.60, 202.00 ± 3.60, 205.66 ± 7.02; 1 h: 763.66 ± 37.87, 347.33 ± 23.79, 599.33 ± 13.31; 6 h: 1307.66 ± 208.63, 587.33 ± 30.92, 1137.34 ± 79.60; 12 h: 942.33 ± 56.69, 432.01 ± 26.62, 1029.32 ± 282.02; 24 h: 816.02 ± 26.85, 213.06 ± 27.83, 775.33 ± 52.53; IL-10: 0 h: 56.01 ± 7.54, 180.03 ± 9.84, 94.33 ± 8.50; 1 h: 62.12 ± 11.53, 394.32 ± 11.50, 138.33 ± 12.09; 6 h: 95.66 ± 11.23, 890.34 ± 20.79, 193.01 ± 11.13; 12 h: 125.66 ± 12.50, 1356.67 ± 73.44, 230.18 ± 15.13; 24 h: 181.02 ± 18.52, 1129.56 ± 72.00, 302.02 ± 37.01; n = 5 mice/time point/group). Results are displayed as the mean ± SD. *P < 0.05.

3. Results

3.1. ET requires TRAF3

KCs isolated from C57BL/6 mice were cultured with LPS under different experimental conditions (NET:100 ng/ml; ET:10 ng/ml + 100 ng/ml) and analyzed by Western blot using TRAF3 antibody. As shown in Fig. 1A, the expression of TRAF3 in the NET group declined in a time-dependent manner; however, the expression of TRAF3 in the ET group remained elevated. As previous studies demonstrated, the concentration of IL-1β in supernatants in the ET group were lower than those in the NET group. Similarly, here, we found that the levels of IL-10 in the ET group were significantly higher than those in the NET group (Fig. 1C).

To examine the role of TRAF3 in ET, TRAF3 expression was knocked down in KCs undergoing ET. JNK and P38, which are important proteins in Myd88-dependent signaling, showed greater phosphorylation in the TRAF3-shRNA group than that in the control group. We also measured the concentration of IL-1β and IL-10 in the supernatants. The concentration of IL-1β rapidly increased in the TRAF3-shRNA group, while IL-10 was not detected, indicating that TRAF3 was essential for ET (Fig. 1C).

3.2. USP25 suppressed the K48-linked polyubiquitination profile of TRAF3

Ubiquitination is the primary mechanism by which TRAF3 degradation is regulated. To verify whether USP25, which is one of the deubiquitinating enzymes (DUBs), could interact with TRAF3 in KCs, we measured the binding of USP25 to TRAF3 by coimmunoprecipitation. As expected, USP25 interacted with TRAF3 when KCs were stimulated with LPS (Fig. 2A). KCs forced to overexpress USP25 were cultured under NET conditions, and the expression of TRAF3 and K48ub-TRAF3 was measured. Interestingly, K48ub-TRAF3 expression was slightly increased, and TRAF3 expression remained stable after stimulation with LPS (Fig. 2B). Due to TRAF3 expression, JNK and P38 were phosphorylated. IL-1β levels in the supernatants were not as high as those in the control group (Fig. 2D). However, the concentration of IL-10 in the supernatants was significantly increased (Fig. 2D). Together, these data suggest that USP25 could interact with and stabilize TRAF3, thereby contributing to ET in KCs.

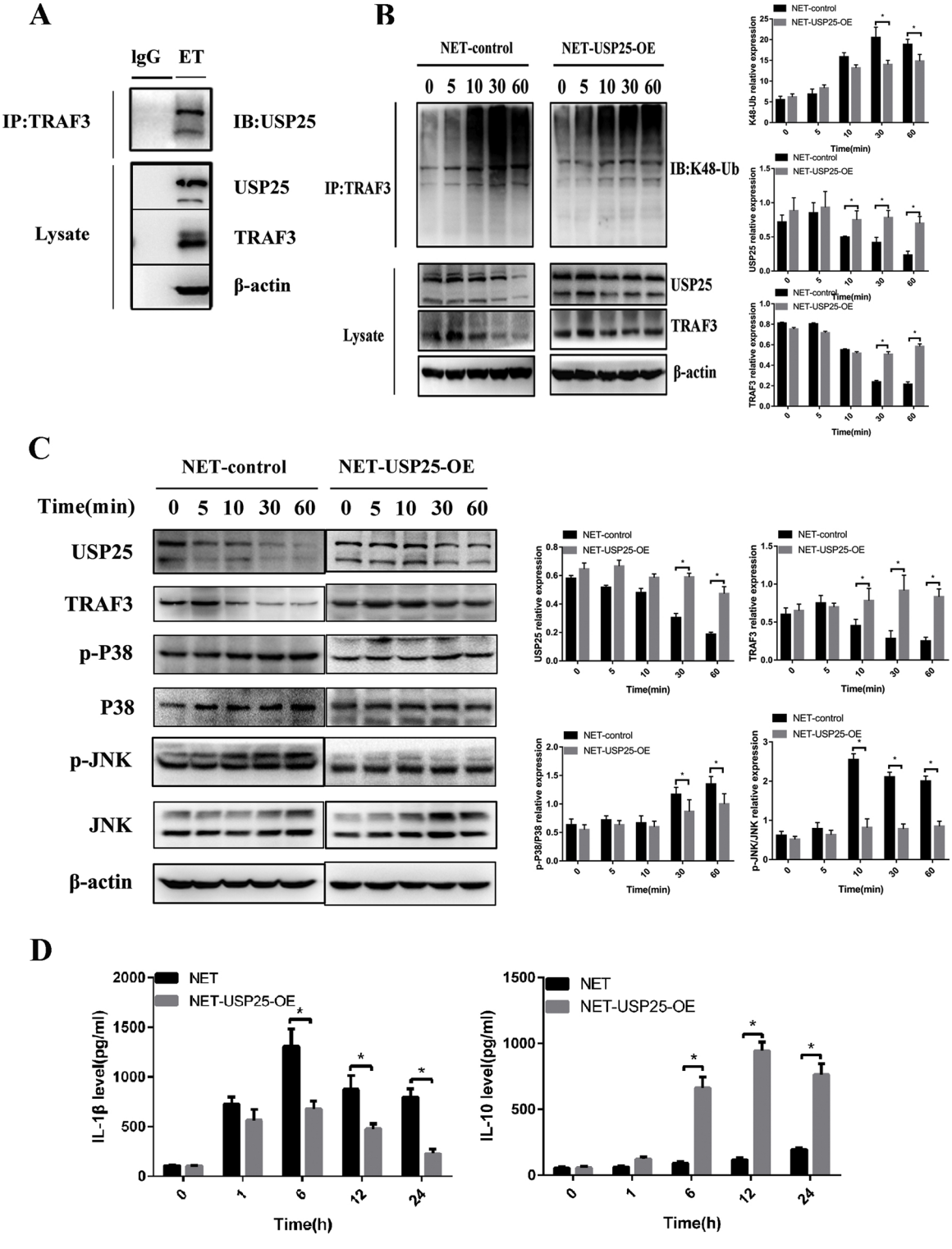

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Fig. 2. K48-linked polyubiquitination of TRAF3 is mediated by USP25 in KCs. (A) Interaction of USP25 and TRAF3 was detected by immunoprecipitation (IP). (B) Overexpression of USP25 reduced the K48-linked polyubiquitination of TRAF3 in KCs in NET conditions (K48-Ub: 0 m in. 5.5 ± 0.86, 6.16 ± 0.76; 5 min: 6.83 ± 1.25, 8.33 ± 0.76; 10 m in. 15.83 ± 1.04, 13.16 ± 0.76; 30 min: 20.50 ± 2.50, 14.00 ± 1.00; 60 min: 18.83 ± 1.25, 14.83 ± 1.60; USP25: 0 m in. 0.71 ± 0.10, 0.88 ± 0.18; 5 min: 0.85 ± 0.15, 0.93 ± 0.23; 10 m in. 0.49 ± 0.01, 0.75 ± 0.13; 30 min: 0.41 ± 0.07, 0.78 ± 0.10; 60 min: 0.23 ± 0.05, 0.70 ± 0.10; TRAF3: 0 m in. 0.81 ± 0.01, 0.75 ± 0.01; 5 min: 0.80 ± 0.01, 0.71 ± 0.01; 10 m in. 0.55 ± 0.01, 0.51 ± 0.01; 30 min: 0.23 ± 0.01, 0.50 ± 0.02; 60 min: 0.21 ± 0.02, 0.58 ± 0.02; n = 5 mice/time point/ group). (C) USP25 overexpression reduced the inflammatory response by inhibiting JNK and P38 activation in KCs in NET conditions (USP25: 0 m in. 0.58 ± 0.02, 0.64 ± 0.04; 5 min: 0.51 ± 0.01, 0.66 ± 0.04; 10 m in. 0.48 ± 0.03, 0.58 ± 0.02; 30 min: 0.30 ± 0.03, 0.59 ± 0.02; 60 min: 0.18 ± 0.01, 0.47 ± 0.04; TRAF3: 0 m in. 0.60 ± 0.08, 0.65 ± 0.08; 5 min: 0.75 ± 0.10, 0.70 ± 0.05; 10 m in. 0.45 ± 0.08, 0.78 ± 0.16; 30 min: 0.28 ± 0.10, 0.91 ± 0.20; 60 min: 0.25 ± 0.05, 0.83 ± 0.10; p-P38/P38: 0 m in. 0.63 ± 0.10, 0.55 ± 0.08; 5 min: 0.71 ± 0.07, 0.63 ± 0.07; 10 m in. 0.66 ± 0.12, 0.60 ± 0.10; 30 min: 1.16 ± 0.12, 0.86 ± 0.20; 60 min: 1.35 ± 0.13, 1.00 ± 0.18; p-JNK/JNK: 0 m in. 0.61 ± 0.10, 0.51 ± 0.07, 5 min: 0.78 ± 0.16, 0.63 ± 0.11; 10 m in. 2.55 ± 0.15, 0.81 ± 0.22; 30 min: 2.10 ± 0.12, 0.78 ± 0.12; 60 min: 2.00 ± 0.13, 0.85 ± 0.13; n = 5 mice/time point/ group). (D) USP25 overexpression inhibited the release of IL-1β and promoted the release of IL-10 in the supernatant of KCs in NET conditions (IL-1β: 0 h: 106.00 ± 9.53, 102.66 ± 8.50; 1 h: 728.33 ± 70.54, 562.66 ± 108.24; 6 h: 1306.66 ± 175.96, 677.00 ± 78.17; 12 h: 874.33 ± 140.01, 477.66 ± 49.08; 24 h: 795.66 ± 85.27, 227.00 ± 45.29; IL-10: 0 h: 54.66 ± 11.23, 57.33 ± 11.01; 1 h: 63.66 ± 6.65, 123.66 ± 16.25; 6 h: 92.00 ± 11.35, 662.33 ± 82.03; 12 h: 117.33 ± 13.50, 944.66 ± 65.37; 24 h: 195.33 ± 13.05, 763.66 ± 83.54; n = 5 mice/time point/ group). Results are displayed as the mean ± SD. *P < 0.05.

3.3. USP25 is required for KC endotoxin tolerance

USP25 alleviates K48ub-TRAF3 in NET, but whether USP25 was essential for ET is still unknown. To assess the role of USP25 in ET, USP25 expression was silenced through USP25-shRNA infection in KC cells that were then stimulated under ET-inducing conditions. The expression of K48ub-TRAF3 in USP25-shRNA KCs was increased compared to that in the control group (Fig. 3A). Due to the increase in K48ub-TRAF3, TRAF3 expression in cells was rapidly degraded (Fig. 3B). Furthermore, phosphorylation of JNK and P38 increased similarly to those levels observed in NET conditions (Fig. 3B). The concentration of IL-10 in supernatants was slightly increased but not to control levels, and IL-1β in supernatants was significantly elevated (Fig. 3C). Taken together, these data suggest that without USP25, KCs lost the ability to tolerate endotoxin and secreted a large amount of proinflammatory cytokines.

3.4. USP25 ablation attenuates the extent of endotoxin tolerance in vivo

Previously, we observed that USP25 suppressed the degradation of TRAF3 in KCs, which contributes to ET. To examine the role of USP25 in ET in vivo, mice were infected with Ad-USP25 and then subjected to ET. After a challenge with a high dose of LPS for 24 h, both Ad-USP25-infected and control mice lost weight, but the effect was greater in Ad-USP25 mice (Fig. 4A). Surprisingly, the survival rate was lower in the ET model where USP25 was silenced, especially at 24 h (Fig. 4B). Next, we compared the extent of liver damage between the two groups. H&E staining of liver tissues revealed severe swelling of the hepatocytes and a mass of inflammatory cell infiltrate in the liver tissues of the Ad-USP25 group, while hepatocyte edema was significantly relieved, and there were fewer inflammatory cells in the control group (Fig. 4C). The serum AST and ALT levels of the control group were much lower than those of the Ad-USP25 group, which indicated that USP25 promoted ET induction in vivo (Fig. 4D).

Next, we measured the level of K48ub-TRAF3 and associated proteins in KCs of Ad-USP25 mice. As expected, without inhibition by USP25, K48ub-TRAF3 was expressed at high levels in the KCs (Fig. 4E). In parallel, TRAF3 in the KCs of Ad-USP25 mice was rapidly degraded when treated with LPS. In addition, Ad-USP25 mice produced significantly higher levels of p-JNK and p-P38 in their KCs compared to control mice (Fig. 4F). Together, these data suggest that USP25 is essential for tolerance to LPS-induced septic shock in mice.

Overexpression of TRAF3 enhances USP25-shRNA KC ET in vitro

We next investigated whether TRAF3 was the primary target of USP25 in vitro. Overexpression of TRAF3 in KCs isolated from Ad-USP25 mice effectively controlled the rising phosphorylation levels of JNK and P38, especially at 60 min (Fig. 5A). Additionally, the level of IL-1β in the supernatant was significantly reduced in the Ad-USP25-TRAF3-OE group compared with that in the USP25-shRNA group (Fig. 5B). In contrast, the content of IL-10 in the supernatant was significantly increased in the Ad-USP25-TRAF3-OE group compared with that in the USP25-shRNA group, especially at 12 h and 24 h (Fig. 5C). These results suggest that the overexpression of TRAF3 was sufficient to compensate for the absence of USP25, thereby restoring ET.

4. Discussion

Sepsis is primarily caused by the destruction of the intestinal barrier and the release of large amounts of endotoxin from gram-negative bacilli. As important immune cells in the liver, KCs play important roles in the development of endotoxin tolerance. A better understanding of the mechanism of ET will provide new ideas and important treatment methods for the clinical treatment of sepsis. In this study, the role of USP25 in the formation of ET in KCs was investigated in the classic model of KCs stimulated with LPS.

The increase in anti-inflammatory cytokines (IL-10) and the decrease in inflammatory cytokines (IL-1β) are important mechanisms for ET (Zanoni et al., 2011; Husebye et al., 2006). Various cytokines, including IL-8, IL-10, IL-1β, and TNF-α, which are produced by monocytes and macrophages, respond to NF-κB stimulation in the nucleus. Our previous studies indicated that JNK and P38 MAPKs, which are the upstream regulators of NF-κB, play key roles in LPS-induced ET in KCs (Liu et al., 2008a, b). In the present study, we found that USP25 inhibited the ubiquitination and degradation of TRAF3, thereby inhibiting the activation of JNK and P38. Due to the stability of TRAF3, KCs can secrete enough of the anti-inflammatory cytokine, IL-10, to induce endotoxin tolerance (Fig. 6). To further verify the role of TRAF3, we cultured TRAF3-shRNA KCs with LPS to study ET in vitro. As expected, TRAF3-shRNA KCs failed to secrete sufficient IL-10, but they produced a large amount of IL-1β, thereby resulting in an inflammatory response. Similar studies reported that autoreactive TRAF3$^{-/-}$ B cells increased in number and displayed enhanced TLR-mediated expression, which contributed to autoimmune manifestations (Xie et al., 2011, 2007). Thus, regulation of TRAF3 expression may be the critical path for controlling inflammatory responses to TLR stimulation.

The results shown here reveal that USP25 physically interacted with TRAF3 and was decreased in KCs not undergoing ET but maintained stable levels in KCs subjected to ET stimulation. This finding suggests that USP25 expression is associated with TRAF3 expression in KCs. In cells infected with RNA or DNA viruses, USP25 protects TRAF3 and TRAF6 from virus-induced proteasome-dependent or independent degradation, respectively (Lin et al., 2015). Under these conditions, TRAF3 degradation occurs through K48-linked polyubiquitination, which results in translocation of the Myd88-associated multiprotein complex to the cytoplasm (Hildebrand et al., 2011). Similarly, following TLR4 induction, the decrease of USP25 can increase the ubiquitination and degradation of TRAF3 (Zhong et al., 2013). The upregulation of USP25 attenuates lipopolysaccharide-induced inflammatory

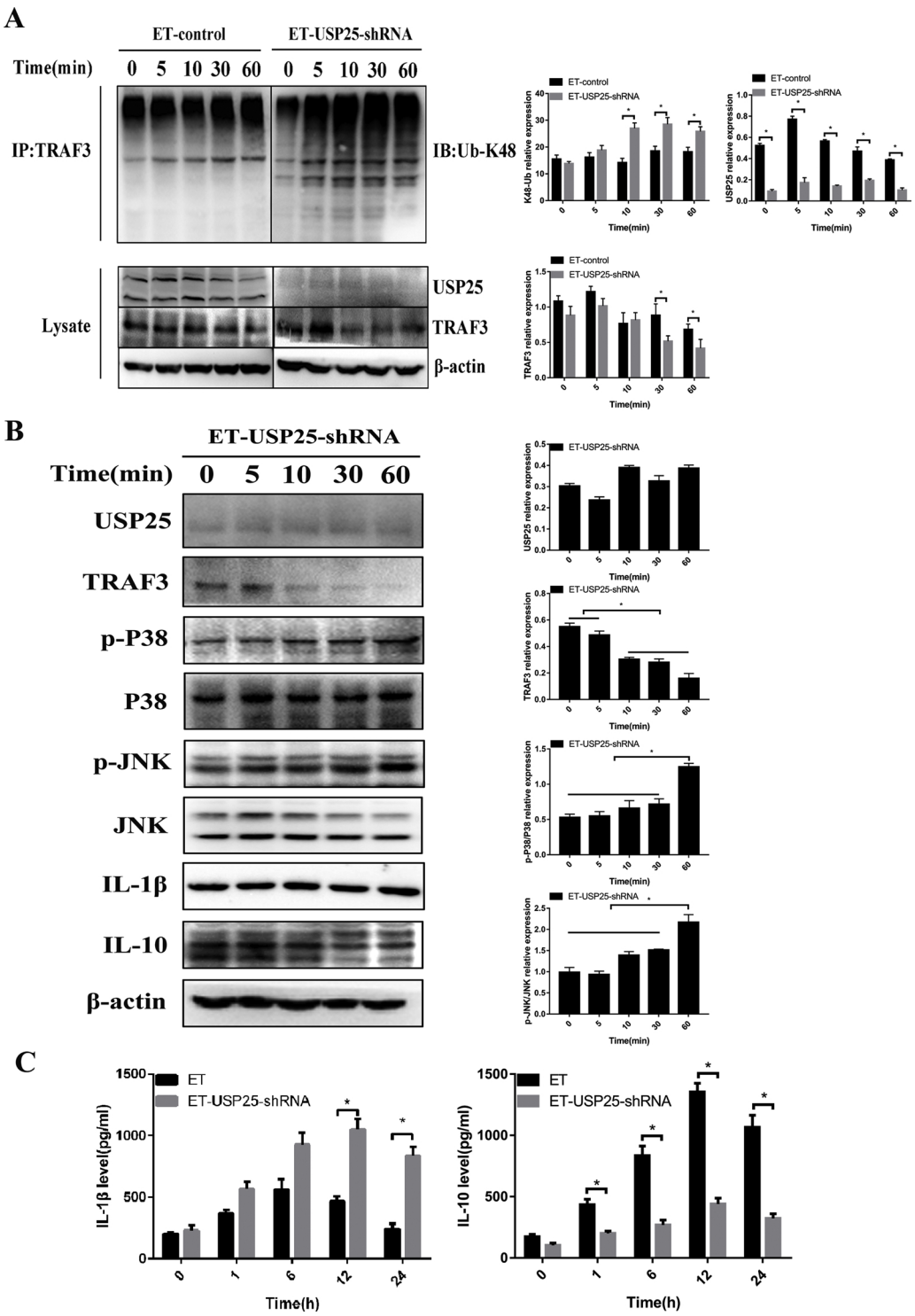


Fig. 3. USP25 knockdown caused KCs to lose endotoxin tolerance. (A) USP25 knockdown promoted the K48-linked polyubiquitination of TRAF3 in KCs in ET conditions (K48-Ub: 0 m in. 15.50 ± 1.50, 13.83 ± 0.76; 5 min: 16.16 ± 1.75, 18.83 ± 1.75; 10 m in. 14.16 ± 1.60, 27.01 ± 2.00; 30 min: 18.50 ± 1.80, 28.51 ± 2.50; 60 min: 18.16 ± 1.75, 25.83 ± 1.75; USP25: 0 m in. 0.52 ± 0.02, 0.09 ± 0.01; 5 min: 0.77 ± 0.03, 0.17 ± 0.04; 10 m in. 0.56 ± 0.01, 014 ± 0.01; 30 min: 0.47 ± 0.04, 0.19 ± 0.01; 60 min: 0.38 ± 0.01, 0.10 ± 0.02; TRAF3: 0 m in. 1.08 ± 0.07, 0.88 ± 0.12; 5 min: 1.21 ± 0.07, 1.01 ± 0.10; 10 m in. 0.76 ± 0.15, 0.81 ± 0.10; 30 min: 0.88 ± 0.16, 0.51 ± 0.07; 60 min: 0.68 ± 0.07, 0.41 ± 0.12; n = 5 mice/time point/ group). (B) USP25 knockdown promoted an inflammatory response by JNK and P38 activation in KCs in ET conditions (USP25: 0 m in. 0.30 ± 0.01; 5 min: 0.23 ± 0.01; 10 m in. 0.39 ± 0.01; 30 min: 0.32 ± 0.02; 60 min: 0.38 ± 0.01; TRAF3: 0 m in. 0.55 ± 0.02; 5 min: 0.48 ± 0.03; 10 m in. 0.30 ± 0.01; 30 min: 0.28 ± 0.02; 60 min: 0.16 ± 0.03; p-P38/P38: 0 m in. 0.53 ± 0.04; 5 min: 0.55 ± 0.06; 10 m in. 0.66 ± 0.10; 30 min: 0.71 ± 0.07; 60 min: 1.24 ± 0.05; p-JNK/JNK: 0 m in. 0.98 ± 0.11; 5 min: 0.93 ± 0.07; 10 m in. 1.39 ± 0.08; 30 min: 1.51 ± 0.02; 60 min: 2.17 ± 0.18; n = 5 mice/time point/ group). (C) USP25 knockdown promoted the release of IL-1β and inhibited the release of IL-10 in the supernatant of KCs in ET conditions (IL-1β: 0 h: 200.33 ± 15.14, 230.32 ± 41.88; 1 h: 367.10 ± 30.34, 569.02 ± 56,67; 6 h: 563.03 ± 84.30, 925.35 ± 98.00; 12 h: 466.01 ± 40.58, 1045. 03 ± 87.50; 24 h: 238.34 ± 44.24, 836.12 ± 73.74; IL-10: 0 h: 173.34 ± 19.55, 103.42 ± 18.00; 1 h: 434.00 ± 45.50, 200.66 ± 19.29; 6 h: 834.66 ± 78.58, 269.02 ± 39.94; 12 h: 1354.66 ± 71.51, 440.00 ± 47.94; 24 h: 1065.00 ± 99.53, 323.34 ± 36.69; n = 5 mice/ time point/ group). Results are displayed as the mean ± SD. *P < 0.05.

responses and inhibits IL-17-triggered signaling (Zhong et al., 2012; Ding et al., 2017). Consistent with these observations, we found decreased levels of TRAF3 K48-linked ubiquitination along with the production of the anti-inflammatory cytokine IL-10, indicating that degradation of TRAF3 is suppressed during ET. This outcome is further supported by the in vivo knockdown of USP25, which resulted in decreased survival following ET along with significant differences in weight loss and liver damage at 24 h. Our results demonstrated that USP25 knockdown significantly attenuated the extent of ET both in vitro and in vivo, suggesting that USP25 is a critical regulator of ET.

The mechanism of the USP25-TRAF3 interaction is not clear; thus, Ad-USP25 KCs were used to explore the interaction in this study. The results demonstrated that TRAF3 overexpression attenuated MAPK signaling activation and rescued USP25 deficiency. However, USP25 expression did not significantly affect survival rates. USP25, which is a member of the USP subfamily, participates in multiple biological processes, including cell growth (Xu et al., 2017) and endoplasmic reticulum (ER) stress (Jung et al., 2015). Thus, USP25 deficiency may influence other cellular functions leading to different effects on survival (Migdalska et al., 2012). The overexpression of TRAF3 alone was not

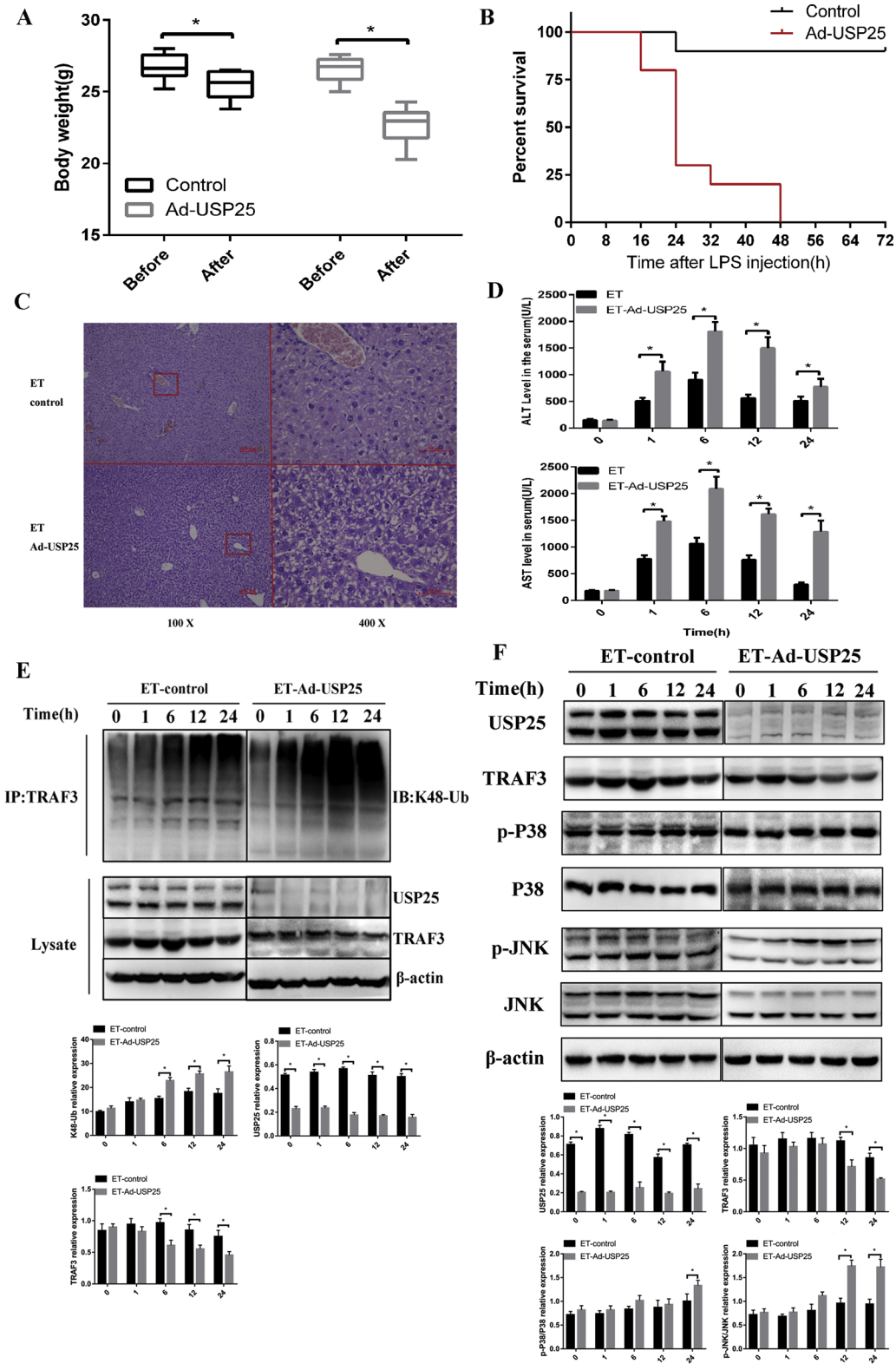
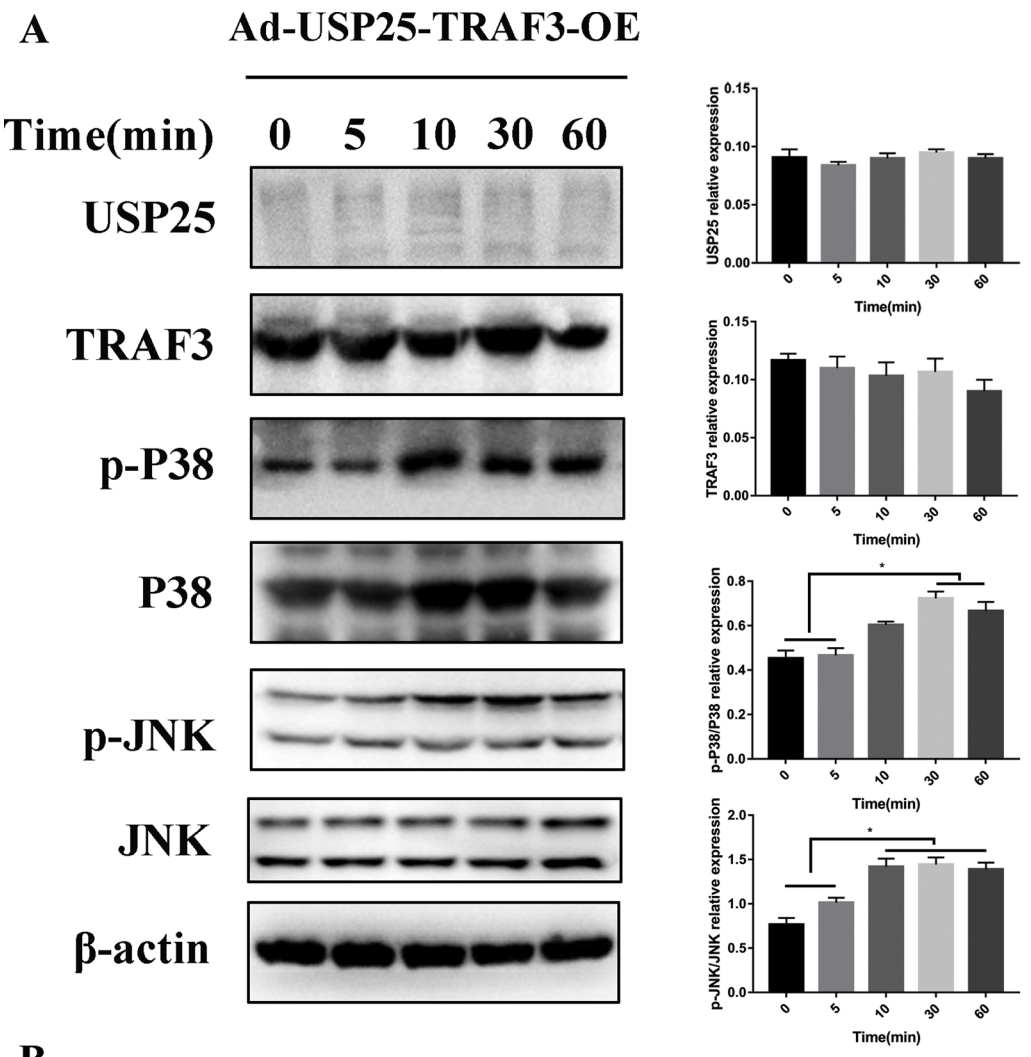


Fig. 4. The effects of USP25 ablation on the induction of ET in vivo. (A) Weight loss was greater in the USP25 knockdown group than in the control group at 24 h after LPS rechallenge (Control:26.76 ± 0.90, 25.44 ± 0.97; Ad-USP25: 26.58 ± 0.87, 22.71 ± 1.26; n = 10 mice/ group). (B) The survival rate declined significantly in the USP25 knockdown group compared to that in the control group under ET conditions (n = 10 mice/ group). (C) The severity of liver damage in the USP25 knockdown group was significantly higher than that in the control group at 24 h after the LPS challenge, as detected by H&E staining (×100; ×400) (n = 5 mice/ group). (D) The impairment of liver function, as measured by serum ALT and AST levels, in the USP25 knockdown group was significantly greater than that in the control group at 1, 6, 12 and 24 h after the LPS challenge (ALT: 0 h: 146.60 ± 21.93, 140.00 ± 18.11; 1 h: 508.60 ± 56.50, 1059.60 ± 183.89; 6 h: 907.80 ± 131.36, 1812.40 ± 173.39; 12 h: 562.20 ± 66.63, 1497.00 ± 206.84; 24 h: 508.80 ± 79.12, 774.00 ± 148.67; AST: 0 h: 177.40 ± 18.00, 181.40 ± 15.33; 1 h: 776.00 ± 67.30, 1481.40 ± 96.24; 6 h: 1062.6 ± 107.62, 2089.40 ± 227.02; 12 h: 758.60 ± 85.33, 1614.60 ± 102.48; 24 h: 299.80 ± 35.82, 1288.60 ± 205.71; n = 5 mice/time point/ group). (E) USP25 knockdown promoted the K48-linked polyubiquitination of TRAF3 in KCs in vivo (K48-Ub: 0 h: 9.83 ± 0.61, 11.26 ± 1.00; 1 h: 13.90 ± 1.76, 14.66 ± 0.80; 6 h: 15.30 ± 1.05, 22.83 ± 1.33; 12 h: 18.16 ± 1.47, 25.56 ± 1.32; 24 h: 17.40 ± 2.00, 26.43 ± 2.51; USP25: 0 h: 0.51 ± 0.01, 0.23 ± 0.02; 1 h: 0.53 ± 0.02, 0.23 ± 0.01; 6 h: 0.56 ± 0.01, 0.17 ± 0.02; 12 h: 0.51 ± 0.03, 0.17 ± 0.01; 24 h: 0.50 ± 0.02, 0.15 ± 0.02; TRAF3: 0 h: 0.84 ± 0.10, 0.90 ± 0.05; 1 h: 0.94 ± 0.09, 0.83 ± 0.07; 6 h: 0.96 ± 0.06, 0.61 ± 0.08; 12 h: 0.85 ± 0.08, 0.55 ± 0.06; 24 h: 0.75 ± 0.09, 0.45 ± 0.065; n = 5 mice/time point/ group). (F) USP25 knockdown promoted an inflammatory response by JNK and P38 activation in KCs in vitro (USP25: 0 h: 0.71 ± 0.02, 0.20 ± 0.01; 1 h: 0.87 ± 0.03, 0.20 ± 0.01; 6 h: 0.81 ± 0.02, 0.25 ± 0.06; 12 h: 0.57 ± 0.04, 0.19 ± 0.01; 24 h: 0.70 ± 0.02, 0.24 ± 0.04; TRAF3: 0 h: 1.05 ± 0.12, 0.92 ± 0.12; 1 h: 1.14 ± 0.10, 1.03 ± 0.07; 6 h: 1.15 ± 0.09, 1.07 ± 0.09; 12 h: 1.11 ± 0.06, 0.71 ± 0.10; 24 h: 0.85 ± 0.07, 0.51 ± 0.02; p-P38/P38: 0 h: 0.71 ± 0.07, 0.81 ± 0.09; 1 h: 0.73 ± 0.06, 0.81 ± 0.08; 6 h: 0.83 ± 0.06, 1.01 ± 0.10; 12 h: 0.87 ± 0.15, 0.93 ± 0.11; 24 h: 1.00 ± 0.15, 1.33 ± 0.10; p-JNK/JNK: 0 h: 0.71 ± 0.09, 0.76 ± 0.07; 1 h: 0.68 ± 0.04, 0.77 ± 0.09; 6 h: 0.80 ± 0.13, 1.11 ± 0.08; 12 h: 0.96 ± 0.10, 1.74 ± 0.12; 24 h: 0.94 ± 0.10, 1.72 ± 0.16; n = 5 mice/time point/ group). Results are displayed as the mean ± SD. *P < 0.05.



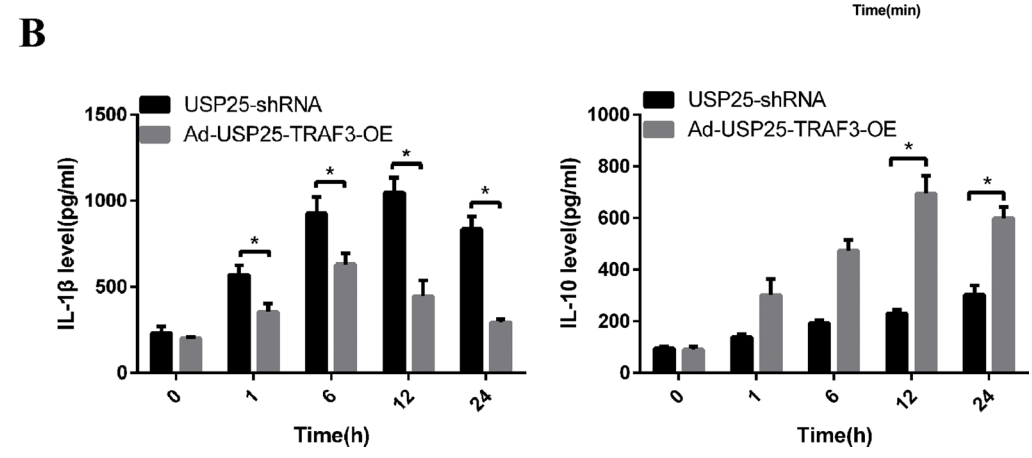


Fig. 5. Overexpression of TRAF3 rescued the effects of USP25 deficiency on ET in KCs in vitro. (A) Overexpression of TRAF3 can alleviate the positive effects of USP25 knockdown on proinflammatory factors, JNK and P38, in KCs in ET conditions (USP25: 0 m in. 0.08 ± 0.00; 5 min: 0.09 ± 0.00; 10 m in. 0.09 ± 0.00; 30 min: 0.09 ± 0.00; 60 min: 0.09 ± 0.00; TRAF3: 0 m in. 0.11 ± 0.00; 5 min: 0.11 ± 0.01; 10 m in. 0.10 ± 0.01; 30 min: 0.10 ± 0.01; 60 min: 0.09 ± 0.01; p-P38/P38: 0 m in. 0.45 ± 0.03; 5 min: 0.46 ± 0.03; 10 m in. 0.60 ± 0.01; 30 min: 0.72 ± 0.03; 60 min: 0.66 ± 0.04; p-JNK/JNK: 0 m in. 0.76 ± 0.07; 5 min: 1.01 ± 0.05; 10 m in. 1.42 ± 0.09; 30 min: 1.44 ± 0.08; 60 min: 1.39 ± 0.07; n = 5 mice/time point/ group). (B, C) Overexpression of TRAF3 can reduce the stimulatory effect of USP25 knockdown on IL-1β release and increase the inhibitory effect of USP25 knockdown on IL-10 release in supernatants of KCs (IL-1β: 0 h: 230.33 ± 41.88, 199.64 ± 9.74; 1 h: 569.00 ± 56.66, 354.18 ± 46.31; 6 h: 925.33 ± 98.00, 628.93 ± 64.39; 12 h: 1045.00 ± 87.50, 446.10 ± 91.37; 24 h:836.00 ± 73.74, 292.57 ± 16.68; IL-10: 0 h: 94.33 ± 8.50, 90.55 ± 11.37; 1 h: 138.33 ± 12.09, 301.15 ± 62.58; 6 h: 193.00 ± 11.13, 472.87 ± 41.50; 12 h: 230.01 ± 15.13, 694.59 ± 68.83; 24 h: 302.02 ± 37.00, 599.13 ± 43.14; n = 5 mice/time point/ group). Results are displayed as the mean ± SD. *P < 0.05.

enough to rescue the impact of USP25 knockdown in mice. These results suggest that other mechanisms are involved in the regulation of inflammation during ET. Moreover, mice infected with two viruses in succession, whose virulence may affect the immunity of the mice, lose the ability to tolerate endotoxins. Therefore, gene knockout mice could more accurately verify the effect of USP25 and TRAF3 on ET in vivo.

Although this study found that USP25 plays an important role in ET in mouse KCs, we did not collect data from human clinical cases of ET, which may be related to improvements in living standards and medical treatments. Therefore, we will collect relevant clinical data from patients with sepsis to verify the USP25-TRAF3 model. At the same time, potential drugs will be sought that can regulate the expression of USP25 and the ubiquitination level of TRAF3 to induce ET and improve the prognosis of sepsis.

5. Conclusion

Based on our findings, we propose a working model for the role of USP25 in KC ET. In this model, TLR4 recruits USP25 and TRAF3 to form a signaling complex and maintains the cellular abundance of TRAF3 by inhibiting its degradation and the subsequent attenuation of JNK and P38 MAPK activation, resulting in the production of anti-inflammatory

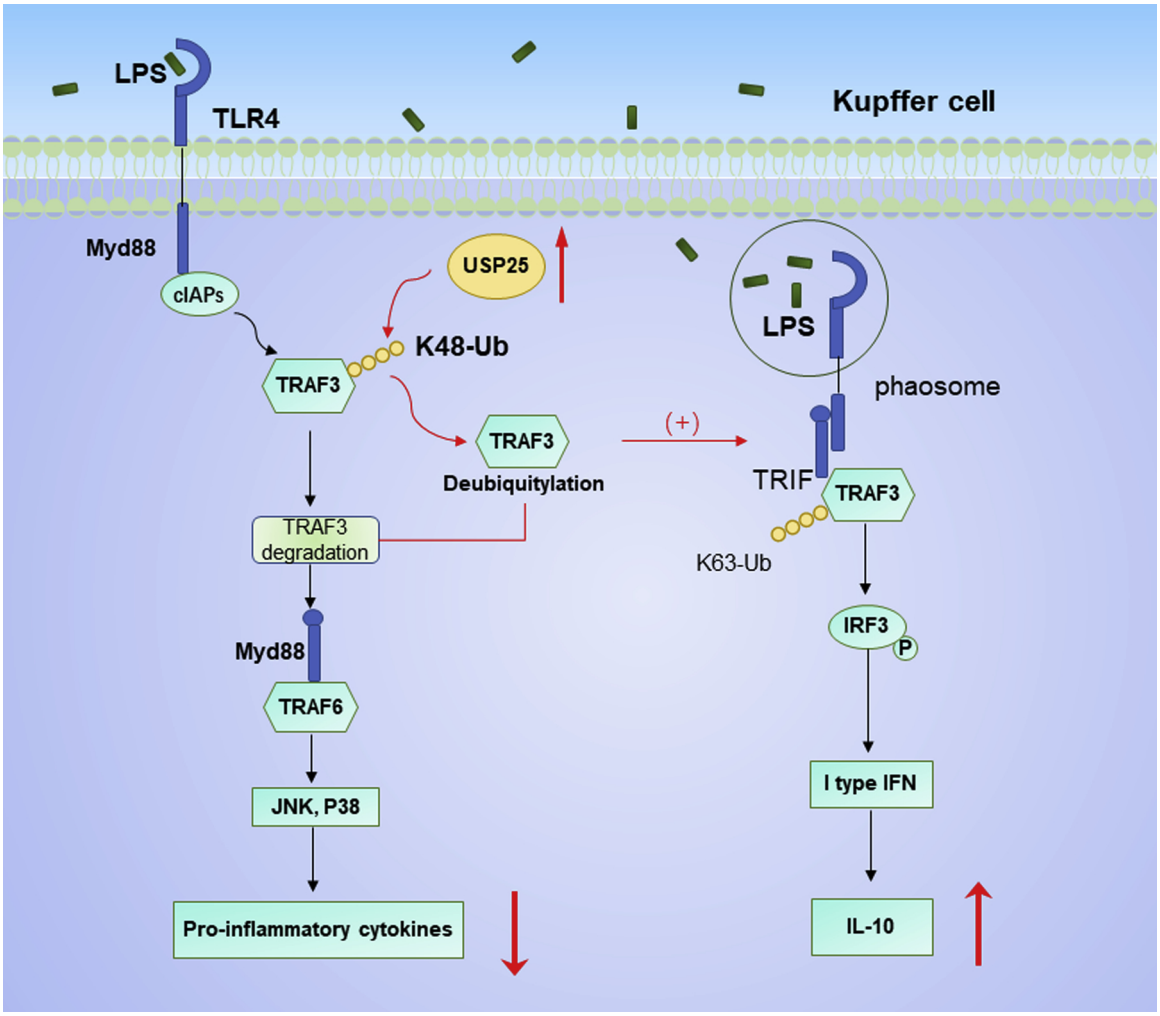


Fig. 6. The underlying mechanism of the effects of USP25 in ET. USP25 inhibits the K48-linked ubiquitination degradation of TRAF3, thereby reducing the phosphorylation levels of JNK and P38. It reduces the secretion of IL-1β, increases the secretion of IL-10 and induces endotoxin tolerance in KCs.

cytokines. This model may, in the future, provide new therapeutic targets for the treatment of immune disorders.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2018.12.017.

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