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E3 Ubiquitin Ligase Tripartite Motif 38 Negatively Regulates TLR-Mediated Immune Responses by Proteasomal Degradation of TNF Receptor-Associated Factor 6 in Macrophages

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Activation of TLR signaling in the innate immune cells is critical for the elimination of invading microorganisms. However, uncontrolled activation may lead to autoimmune and inflammatory diseases. In this article, we report the identification of tripartite motif (TRIM) 38 as a negative feedback regulator in TLR signaling by targeting TNFR-associated factor 6 (TRAF6). TRIM38 was induced by TLR stimulation in an NF-κB-dependent manner in macrophages. Knockdown of TRIM38 expression by small interfering RNA resulted in augmented activation of NF-κB and MAPKs, and enhanced expression of proinflammatory cytokines, whereas overexpression of TRIM38 has an opposite effect. As an E3 ligase, TRIM38 bound to TRAF6 and promoted K48-linked polyubiquitination, which led to the proteasomal degradation of TRAF6. Consistently, knockdown of TRIM38 expression resulted in higher protein level of TRAF6 in primary macrophages. Our findings defined a novel function for TRIM38 to prevent excessive TLR-induced inflammatory responses through proteasomal degradation of TRAF6.

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Abbreviations used in this article: HA, hemagglutinin; IKK, IκB kinase; IP, immunoprecipitation; LTA, lipoteichoic acid; PGN, peptidoglycan; poly(lC), polynosinic:polycytidylic acid; siRNA, small interfering RNA; TAB, TAK1-binding protein; TAK1, TGF-β-activated kinase 1; TRAF, TNFR-associated factor; TRIF, Toll/IL-1R domain-containing adaptor that induces an IFN-β; WT, wild-type.

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K48-linked polyubiquitination, leading to the TRAF6 proteasomal degradation.

Materials and Methods

Mice and cells

C57BL/6j mice were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Medical School of Shandong University, Jinan, Shandong, China. Mouse macrophage cell line RAW264.7 and human HEK293 cells were provided by the American Type Culture Collection (Manassas, VA). HEK293-TLR2/TLR3/TLR4/TLR7/TLR8 cell lines were obtained from Invivogen (San Diego, CA). Mouse primary peritoneal macrophages were prepared as described previously (26). The cells were cultured at 37°C under 5% CO2 in DMEM supplemented with 10% FCS (Invitrogen-Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Reagents and Abs

MG132, nocodazole, NL-CL, chloroquine, LPS (Escherichia coli, 055: B5), and lipopolysaccharide (LTA) were purchased from Sigma (St. Louis, MO), and LPS was repurified as described previously (26). R848, peptidoglycan (PGN), and polyinosinic-polycytidylic acid [poly(IC)] were purchased from Invivogen (San Diego, CA). JS-39 was purchased from Calbiochem (San Diego, CA). IFN-γ was from PeproTech (Rocky Hill, NJ). LPS, TLR, PGN, poly(IC), R848, and IFN-γ were used at a final concentration of 100 ng/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml, 1 μg/ml, and 1 μg/ml, respectively. The Abs specific to TRAF6 (sc-7221), TRAF3 (sc-948), hemagglutinin (HA; sc-7392), Ub (sc-7151), TRIM38 (sc-747), p38 (sc-353), ERK (sc-94), β-actin (sc-81178), and protein G-agarose (sc-2002) were used for immunoprecipitation (IP) were from Santa Cruz Biotechnology (Santa Cruz, CA, The Abs specific to Myc (2272), phospho-JNK (9251), phospho-p38 (9215), phospho-ERK (9101), and phospho-β-actin were from Cell Signaling Technology (Beverly, MA). The Ab for FLAG (F3165) was from Sigma. The Abs for TAK1 (ab79363) and TRIM38 (ab69977) were from Abcam (Cambridge, MA). The Ab for Lys48-specific ubiquitin (05-1307) was from Millipore (Billerica, MA). Their respective HRP-conjugated secondary Abs were purchased from Santa Cruz Biotechnology.

Sequences, plasmid constructs, and transfection

Flag-tagged TRIM38 (NM_006355) expression plasmid was purchased from Origene (Rockville, MD). HA-tagged TRIM38 wild-type (WT) and domain deletion plasmids C64, C130, and N273 were constructed using PCR-generated fragments. The TRIM38 C16A mutation was generated using the KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan). All constructs were confirmed by DNA sequencing. RIP1 expression plasmid was provided by Dr. William Kaiser (Emory University). MyD88 and TRIF plasmids were gifts from Dr. Xuetao Cao (Second Military Medical University, Shanghai, China). Expression vectors for TRAF6, TRAF3, IKK-β and -α were from Dr. Michael Karin (University of California at San Diego, San Diego, CA). Expression vectors for TAK1-binding protein 1 (TAB1) and TAB2 were provided by Dr. Danying Chen (Peking University, Beijing, China). p65 expression vector was provided by Dr. Jeremy M. Boss (Emory University School of Medicine). Expression vectors for HA-UB WT and mutant K48, K63, K48R, and K63R were from Dr. Hui Xiao (Institut Pasteur of Shanghai, CAS, Shanghai, China). The PathDetect NF-κB and GAS reporting vectors were purchased from Stratagene. For transient transfection of plasmids into RAW264.7 cells, jetPEI reagents were used (Polyplus-transfection). For stable selection of cell lines overexpressing TRIM38, transfected RAW264.7 macrophages were selected with G418 (600 ng/ml) and were pooled for further experiments. For transient silencing, duplexes of small interfering RNA (siRNA) were transfected into cells with the Geneporter 2 Transfection Reagent (GTS, Minneapolis, MN).

Assay of luciferase activity

Luciferase activity was measured with the Dual-Luciferase Reporter Assay system according to the manufacturer’s instructions (Promega) as described previously (28). Data were normalized for transfection efficiency by division of firefly luciferase activity with that of Renilla luciferase.

Ubiquitination assays

For analysis of ubiquitination of endogenous TRAF6, whole-cell extracts were immunoprecipitated with anti-TRAF6 and analyzed by Western blotting with anti-UB (K48) Ab. For analysis of the ubiquitination of overexpressed TRAF6, HEK293 cells were transfected with Flag-TRAF6, HA-UB (WT), or HA-UB mutants and HA-TRIM38 WT or HA-TRIM38 mutants; then whole-cell extracts were immunoprecipitated with anti-Flag and analyzed by immunoblot with anti-ubiquitin Ab.

Statistical analysis

All data are presented as mean ± SD of three or four experiments. Statistical significance was determined with the two-tailed Student t test, with a p value <0.05 considered statistically significant.

Results

TRIM38 protein expression is induced by TLR stimulation

TRIM38 is a member of the TRIM protein family located in the MHC class I region, but its biological function, especially in the immune response, remains unknown. To explore the possible functions of TRIM38, we first measured tissue expression of murine TRIM38 by Western blot analysis. TRIM38 protein was strongly expressed in lymphoid organs such as thymus, spleen, and small intestine (Supplemental Fig. 1).

Next, TRIM38 protein expression was examined in macrophages stimulated with various TLR ligands by Western blotting. Upon stimulation with LPS (TLR4 ligand), a significant increase in expression of TRIM38 protein was detected in thioglycolate-elicited mouse primary peritoneal macrophages (Fig. 1A). Induced expression of TRIM38 protein in peritoneal macrophages was detected after stimulation with LPS for 4 h and reached the peak level after stimulation with LPS for 16–24 h (Fig. 1A). Expression of TRIM38 protein was also upregulated 4 h after LPS treatment and reached the peak level 24 h after LPS stimulation in RAW264.7 macrophages (Fig. 1A). Similarly, stimulation with LPS or PGN (TLR2 ligand), poly(I:C) (TLR3 ligand), and R848 (TLR7 ligand) also greatly enhanced TRIM38 protein expression 12 h after stimulation in peritoneal macrophages and RAW264.7 macrophages (Fig. 1B). A significant increase in expression of TRIM38 mRNA was also detected in RAW264.7 macrophages upon stimulation with LPS, poly(I:C), and PGN (Supplemental Fig. 2). LPS-induced TRIM38 protein expression was greatly inhibited by JSH-23, a NF-κB activation inhibitor (Fig. 1C). Simi-
larly, LPS-induced TRIM38 protein expression was greatly decreased by p65 siRNA transfection (Fig. 1D). Taken together, these data indicate that TRIM38 expression is restricted to the immune system and can be induced by TLR stimulation in macrophages in an NF-kB–dependent manner.

**TRIM38 inhibits TLR-induced production of proinflammatory cytokines**

To investigate the roles of TRIM38 in TLR signaling, we transfected two TRIM38-specific siRNAs into peritoneal primary macrophages. The production of TLR-induced cytokines including TNF-α and IL-6 was measured by ELISA. The expression of TRIM38 protein was greatly decreased as measured by Western blotting with transfection of TRIM38-specific siRNAs 1 and 2 (Fig. 2A). Induced expression of TNF-α and IL-6 was detected after LPS, poly(I:C), and LTA stimulation in control siRNA-transfected macrophages (Fig. 2B). In macrophages transfected with TRIM38 siRNA, LPS, poly(I:C), and LTA-induced TNF-α and expression were further enhanced (Fig. 2B). Importantly, TRIM38 siRNA 2, which has a higher efficiency to knockdown TRIM38 protein expression, has a greater potential to increase the TLR-induced cytokine production than TRIM38 siRNA 1 (Fig. 2B). Therefore, TRIM38 siRNA 2 was used in the following experiments.

To further confirm the function of TRIM38 on TLR-induced production of proinflammatory cytokines, we constructed RAW264.7 stable cell line with TRIM38 overexpression by transfecting FLAG-TRIM38 expression plasmid. Overexpression of TRIM38 was confirmed by Western blotting with both TRIM38 and FLAG Abs (Fig. 2C). TRIM38 overexpression cells and control cells were stimulated with LPS, poly(I:C), and LTA; then TNF-α and IL-6 secretion were measured by ELISA. LPS, poly(I:C), and LTA-induced expression of TNF-α and IL-6 were significantly inhibited by TRIM38 overexpression compared with cells transfected with control vector (Fig. 2D).

Because TRIM38-deficient mice are not available, in vivo TRIM38 siRNA i.p. transfection was performed to knock down TRIM38 expression in thioglycolate-elicited peritoneal exudate cells with Geneporter 2 Transfection Reagent. TRIM38 siRNA i.p. transfection suppressed TRIM38 expression in i.p. cells (Fig. 2E); at the same time, LPS-induced TNF-α and IL-6 production in peritoneal lavage was significantly increased (Fig. 2F). Collectively, these data indicate that TRIM38 is a negative regulator for TLR-induced expression of proinflammatory cytokines in macrophages.

**FIGURE 1.** TRIM38 is induced by TLR agonists. (A and B) Western blot analysis of TRIM38 expression in mouse peritoneal macrophages (MΦ) or RAW264.7 cells stimulated with various TLR agonists. (C) Western blot analysis of TRIM38 expression in mouse peritoneal macrophages pretreated with 30 μM JSH-23 for 40 min, then stimulated with LPS for 8 h. (D) Western blot analysis of TRIM38 expression in mouse peritoneal macrophages transfected with control siRNA or p65-specific siRNA, and then stimulated for 12 h with LPS. Similar results were obtained in three independent experiments.

**FIGURE 2.** TRIM38 negatively regulates TLR-induced production of proinflammatory cytokines. (A) Western blot analysis of TRIM38 expression in mouse peritoneal macrophages transfected with control siRNA or Trim38 siRNA 1 and siRNA 2 for 36 h. (B) ELISA of TNF-α and IL-6 in the supernatants of peritoneal macrophages as in (A) stimulated with LPS, poly(I:C), or LTA for 12 h. Data are shown as mean ± SD (n = 3) of one representative experiment. *p < 0.05, **p < 0.01. (C) Western blot analysis of TRIM38 expression in RAW264.7 cells stably transfected with Flag-TRIM38 expression plasmid or control empty vector (Ctrl). (D) ELISA of TNF-α and IL-6 in the supernatants of RAW264.7 cells as in (C) stimulated with LPS, poly(I:C), or LTA for 12 h. Data are shown as mean ± SD (n = 3) of one representative experiment. *p < 0.01. (E) Western blot analysis of TRIM38 expression in thioglycolate-elicited peritoneal macrophages transfected i.p. with TRIM38 siRNA for 48 h. (F) ELISA of TNF-α and IL-6 in the peritoneal lavage of mice as treated in (E) after i.p. administration with PBS or LPS for 1 h. Data are shown as mean ± SD (n = 5; **p < 0.01). Similar results were obtained in three independent experiments.
TRIM38 negatively regulates TLR-induced NF-κB activation

Production of proinflammatory cytokines upon TLR stimulation depends mainly on the NF-κB activation. To determine the function of TRIM38 on NF-κB activation, first we transfected NF-κB–luciferase reporter into RAW264.7 cells together with TRIM38 expression plasmid or control vector; then we stimulated cells with LPS and poly(I:C), and measured luciferase activity. LPS- and poly(I:C)-induced NF-κB activation was greatly decreased by transfection of TRIM38 expression plasmid compared with control vector-transfected cells (Fig. 3A). In contrast, IFN-γ–induced activation of inducible NO synthase promoter and GAS luciferase was not affected by TRIM38 expression (Fig. 3B), which requires transcription factor STAT1 for activation, suggesting that TRIM38 specifically inhibits TLR-induced activation of NF-κB.

Next, NF-κB–luciferase reporter and TRIM38 expression or control vector were transfected into HEK293 cells stably expressing TLR2, TLR3, TLR4, TLR7, and TLR8, and the luciferase activity was measured after simulating the cells with corresponding TLR agonists. In all cases, TLR-mediated NF-κB activation was significantly decreased by TRIM38 transfection (Fig. 3C). Taken together, these reporter assays indicate that TRIM38 negatively regulates TLR-induced activation of NF-κB.

To further confirm the inhibitory effect of TRIM38 on TLR-induced NF-κB activation, we stimulated stable RAW264.7 cells with TRIM38 overexpression with LPS for 0, 20, or 40 min. The activation of NF-κB was detected with IκB phosphor-specific Ab. As shown in Fig. 3D, TRIM38 expression significantly attenuated LPS-induced IκB phosphorylation. At the same time, LPS-induced phosphorylation of JNK, p38, and ERK was also greatly attenuated. The differential phosphorylation was not caused by protein degradation because JNK, p38, and ERK protein levels were not affected by TRIM38 overexpression (Fig. 3D). These results suggest that TRIM38 expression is related to the significant decrease of LPS-induced IκB, JNK, p38, and ERK phosphorylation.

Finally, TRIM38 siRNA or control siRNA were transfected into primary peritoneal macrophages to confirm the inhibitory effect of TRIM38 on TLR-induced NF-κB and MAPK signaling pathway under physiological condition. Macrophages were stimulated with LPS for 0, 15, 30, and 60 min after 48 h of siRNA transfection. Transfection of TRIM38 siRNA greatly enhanced LPS-induced IκB, JNK, p38, and ERK phosphorylation (Fig. 3E). Similarly, these changes were not caused by protein degradation because JNK, p38, and ERK protein levels were not affected by TRIM38 siRNA transfection (Fig. 3E). All together, these data suggest TRIM38 negatively regulates TLR-induced NF-κB and MAPK activation.

TRIM38 targets TRAF6

TLRs activate NF-κB through a complex cascade composed of various kinases and adaptors. To determine the molecular order and molecular targets of TRIM38 in TLR-induced NF-κB activation, we examined the effects of TRIM38 overexpression on NF-κB activation mediated by various molecules in reporter assays. Expression of MyD88, TRIF, TRAF6, and TAK1 plus TAB1 strongly induced NF-κB activation, but such activation was inhibited by the cotransfection of TRIM38 in a dose-dependent manner (Fig. 4A). In contrast, IKK-β and TAB2-induced NF-κB activation was not affected by TRIM38 (Fig. 4A), indicating TRIM38 targets proteins upstream of IKK-β to inhibit NF-κB activation.

**FIGURE 3.** TRIM38 negatively regulates TLR-induced NF-κB activation. (A and B) RAW264.7 cells were transiently transfected with NF-κB–luc, inducible NO synthase–luc, or GAS reporter plasmid together with TRIM38 expression plasmid or control plasmid, and luciferase activity was analyzed after treatment with LPS, poly(I:C), or IFN-γ for 6 h. (C) 293-TLR4/3/2/7/8 cells were transfected with NF-κB–luc reporter plasmid and TRIM38 expression plasmid or control plasmid, analyzed luciferase activity after treatment with LPS, poly(I:C), LTA, R848 for 6 h, respectively. Data are shown as mean ± SD (n = 6) of one representative experiment. **p < 0.01. (D) Western blot analysis of phosphorylated–IκB-α, phosphorylated-JNK, phosphorylated-p38, phosphorylated-ERK, and total JNK, p38, ERK in RAW264.7 cells as in Fig. 2C stimulated with LPS. Similar results were obtained in three independent experiments. (E) Western blot analysis of phosphorylated–IκB-α, phosphorylated-JNK, phosphorylated-p38, phosphorylated-ERK, and total JNK, p38, ERK in mouse peritoneal macrophages transfected with control siRNA (Ctrl) or TRIM38 siRNA (siRNA) and stimulated with LPS. Similar results were obtained in three independent experiments.
To identify TRIM38 targets in the TLR-induced NF-κB and MAPK signaling, we investigated the function of TRIM38 on the degradation of any of the signaling molecules. Various expression plasmids for TRAF6, TRAF3, RIP1, TAK1, TAB1, IKK-β, and p65 were cotransfected into HEK293 cells with TRIM38 expression plasmids, and the expression levels of the signaling molecules were analyzed by Western blotting 24 h after transfection. TRIM38 promoted degradation of TRAF6 in a dose-dependent manner (Fig. 4B, Supplemental Fig. 3). TRIM38 expression was silenced by TRIM38 siRNA transfection in peritoneal macrophages to confirm the function of TRIM38 on TRAF6 degradation under physiological condition. Transfection of TRIM38 siRNA increased TRAF6 protein level in both unstimulated and LPS-stimulated conditions compared with control siRNA transfection (Fig. 4C). As a control, TRAF3 and TAK1 protein levels were not impaired by TRIM38 siRNA transfection (Fig. 4C). All together, these data indicate that TRIM38 targets TRAF6 for degradation to inhibit TLR-induced activation of NF-κB and MAPK.

**TRIM38 interacts with TRAF6 through the PRY/SPRY domain**

To study the molecular mechanisms of TRIM38 on the degradation of TRAF6, we investigated the interaction between these proteins. HA-TRIM38 and FLAG-TRAF6 were cotransfected into HEK293 cells; 24 h after transfection, IP experiments were performed with HA or FLAG Abs. FLAG-TRAF6 was coprecipitated with HA-TRIM38 and vice versa (Fig. 5A). Endogenous interaction was also confirmed in macrophages stimulated with LPS for various times, followed by IP assays with TRIM38 or TRAF6 Ab and Western blotting with TRAF6 or TRIM38 Ab, respectively. As shown in Fig. 5B, TRAF6 was coprecipitated with TRIM38 and vice versa before and after LPS stimulation. As a control, the interaction could not be detected with normal IgG.

TRIM38, a member of the TRIM family proteins, contains a RING-finger domain, B-box domain, and C-terminal PRY/SPRY domain (Supplemental Fig. 3). To search for the domains of TRIM38 that are responsible for the interaction with TRAF6, we constructed various deletion mutants (Supplemental Fig. 3). TRAF6 was coprecipitated with TRIM38 WT, C64, C130, but not with N273, indicating the C-terminal PRY/SPRY domain is required for the interaction with TRAF6 (Fig. 5C, 5D). Consistently, the TRIM38 mutant N273 lost the ability to inhibit MyD88-induced NF-κB activation compared with WT TRIM38 (Supplemental Fig. 4). All together, these data indicate TRIM38 interacts with TRAF6 through the PRY/SPRY domain.

**FIGURE 4.** TRIM38 targets TRAF6. (A) HEK293 cells were transfected with MyD88, TRIF, TAK1 plus TAB1, TRAF6, IKK-β, or TAB2, together with NF-κB–luc reporter plasmid and increasing amount of TRIM38 plasmid, and analyzed luciferase activity. Data are shown as mean ± SD (n = 6) of one representative experiment. **p < 0.01. (B) Western blot analysis of Flag-TRAF6 in HEK293 cells transfected with Flag-TRAF6 together with increasing concentration of HA-TRIM38 expression plasmid. (C) Western blot analysis of the expression of TRAF6, TRAF3, and TAK1 in peritoneal macrophages transfected with control siRNA (Ctrl) or TRIM38 siRNA (siRNA) and then stimulated with LPS. Similar results were obtained in three independent experiments.

**FIGURE 5.** TRIM38 binds to TRAF6 through the PRY/SPRY domain. (A) Lysates from HEK293 cells transiently cotransfected with Flag-TRAF6 and HA-TRIM38 expression plasmids were subjected to IP with anti-HA or anti-FLAG Ab followed by Western blot analysis with anti-FLAG or anti-HA Ab, respectively. (B) Lysates from RAW264.7 cells stimulated with LPS for indicated time periods were subjected to IP with anti-TRAF6 or anti-TRIM38 Ab followed by Western blot analysis with anti-TRAF6 or anti-TRIM38 Ab, respectively. Proteins in whole-cell lysate were used as positive control (Input). (C and D) Lysates from HEK293 cells transiently transfected with Flag-TRAF6 and HA-TRIM38 WT, TRIM38 mutants C64, C130, or N273 were subjected to IP with anti-HA or anti-FLAG Ab followed by Western blot analysis with anti-FLAG or anti-HA Ab, respectively.
TRIM38 promotes K48-linked ubiquitination and proteasomal degradation of TRAF6

The presence of RING-finger domain indicates TRIM38 may function as an E3 ligase. Therefore, the ability of TRIM38-induced polyubiquitination of TRAF6 was investigated. Expression vector for TRAF6 and HA-ubiquitin were transfected into HEK293 cells together with TRIM38. TRIM38-mediated polyubiquitination of TRAF6 was readily detected (Fig. 6A). In contrast, the TRIM38 point mutation (C16A) with substitution of the cysteine residue at position 16 within the RING domain with alanine lost the ability to promote polyubiquitination of TRAF6 (Fig. 6A), indicating the RING-finger domain is required for the TRIM38-mediated ubiquitination of TRAF6. Similarly, the TRIM38 mutant (C64) with the deletion of the RING-finger domain lost the ability to mediate polyubiquitination of TRAF6 (data not shown). Ubiquitin mutant vectors K48 and K63, which contain arginine substitutions of all of its lysine residues except the one at positions 48 and 63, respectively, were used in the transfection assays to study the forms of TRIM38-mediated TRAF6 polyubiquitination. Surprisingly, TRIM38-mediated TRAF6 polyubiquitination could be detected in the presence of both K63 and K48 plasmid (Fig. 6B). Similarly, TRIM38-mediated TRAF6 polyubiquitination could be detected with the transfection of K63R and K48R plasmid, another two pairs of ubiquitin mutants that contain a single lysine-to-arginine mutation at positions 63 and 48, respectively (Fig. 6B), indicating TRIM38 mediates both K63-linked and K48-linked ubiquitination of TRAF6.

To confirm TRIM38-mediated, K48-linked polyubiquitination of TRAF6 under physiological conditions, we silenced TRIM38 expression by TRIM38 siRNA transfection in peritoneal macrophages. TRAF6 ubiquitination was detected by K48-specific Ab with TRAF6 immunoprecipitates from peritoneal macrophages. TRAF6 ubiquitination was detected by K48-specific Ab with TRAF6 immunoprecipitates from peritoneal macrophages.

K48-linked polyubiquitination of TRAF6 was induced by LPS stimulation. Transfection of TRIM38 siRNA greatly decreased LPS-induced K48-linked polyubiquitination of TRAF6 compared with control siRNA transfection (Fig. 6C).

K48-linked protein ubiquitination leads to the degradation of the corresponding protein by 26S proteasome. Consistently, TRIM38-induced degradation of TRAF6 protein could be reversed by proteasome inhibitor MG-132 (Fig. 6D), but not by lysosome inhibitors NH4Cl and chloroquine. Compared with WT TRIM38, TRIM38 mutants C64 and C16A, which lost the ability to mediate the ubiquitination of TRAF6, could not promote the degradation of TRAF6 (Fig. 6E). Accordingly, the TRIM38 mutant C16A and C64 lost the ability to inhibit MyD88-induced NF-κB activation compared with WT TRIM38 (Supplemental Fig. 4). Taken together, these data demonstrate that TRIM38 mediates K48-linked ubiquitination and degradation of TRAF6 to limit TLR signaling and production of proinflammatory cytokines.

Discussion

Accumulating data have confirmed that TRIM proteins play an essential role in the regulation of innate immune response (21–24). For example, TRIM25 was found to ubiquitinate the CARD domain of RIG-I through K63-linked polyubiquitin chains, which is required for the activation of downstream signaling during virus infection (29). TRIM56 was demonstrated to be required for dsDNA virus-triggered signaling through K63-linked polyubiquitination of STING at lysine 150 (30). TRIM30a was found to be a negative regulator for TLR-induced NF-κB signaling by targeting TAB2 and TAB3 for degradation through the lysosomal degradation pathway (31). TRIM27 (RFP) was found to negatively regulate antiviral and inflammatory responses by targeting IKKα/IKKβ and IKKα/TBK1 to inhibit NF-κB and IRF3 activation (32).

FIGURE 6. TRIM38 promotes K48-linked ubiquitination and proteasomal degradation of TRAF6. (A) Lysates from HEK293 cells transiently cotransfected with Flag-TRAF6, HATRIM38 WT, or TRIM38C16A and HA-Ub plasmids were subjected to IP with anti-FLAG Ab followed by Western blot analysis with anti-ubiquitin Ab. (B) Lysates from HEK293 cells transiently cotransfected with Flag-TRAF6, HA-TRIM38, or vector control and HA-Ub (WT), HA-Ub (K48), HA-Ub (K63), HA-Ub (K48R), or HA-Ub (K63R) plasmids were subjected to IP with anti-FLAG Ab followed by Western blot analysis with anti-ubiquitin Ab. (C) Lysates from mouse peritoneal macrophages transfected with control siRNA (Ctrl) or TRIM38 siRNA (siRNA) and stimulated with LPS for indicated time periods were subjected to IP with anti-TRAF6 Ab followed by Western blot analysis with anti-ubiquitin K48 Ab. (D) Western blot analysis of Flag-TRAF6 expression in HEK293 cells cotransfected with Flag-TRAF6 and HA-TRIM38 or vector control and then treated with MG132, NH4Cl, or chloroquine for 4 h. (E) Western blot analysis of Flag-TRAF6 expression in HEK293 cells cotransfected with Flag-TRAF6 and HA-TRIM38 WT, C16A, C64, or vector control plasmids. Similar results were obtained in three independent experiments.
TRIM5 was shown to bind to the TAK1 complex (TAK1, TAB2, and TAB3) and E2 ubiquitin conjugation enzymes UBC13 and UEV1A, which results in the synthesis of unattached K63-linked ubiquitin chains and the subsequent activation of TAK1 and expression of NF-κB- and AP-1–dependent genes (33). In this study, we identified TRIM38 as another TRIM protein with a role in the regulation of innate immune response. TRIM38 is encoded within the MHC class I region, but its biological function, especially in the regulation of immune response, remains largely unknown. For the first time, to our knowledge, we provide data to demonstrate that TRIM38 is a negative feedback regulator in TLR signaling. As an essential molecule in the TLR-induced NF-κB and MAPK signaling, TRAF6 has been demonstrated to be tightly regulated. Activation of TRAF6 requires K63-linked polyubiquitination (11, 13). Therefore, removing the K63-linked ubiquitin chains from TRAF6 represents a novel pathway to terminate TLR-induced signaling. For example, A20 and CYLD were demonstrated to be required for the termination of TLR-induced signaling by directly removing K63-linked ubiquitin chains from TRAF6 (34, 35). In this study, we found that TRIM38 could induce K48-linked ubiquitination and subsequent degradation of TRAF6 through proteasome. Thus, TRIM38-mediated, K48-linked ubiquitination and degradation of TRAF6 represents another novel pathway to terminate TLR-induced signaling.

Matsuda et al. (36) identified TRIM38 (RoRet) as a positive regulator for NF-κB activation in a large-scale screen. But we provide data to demonstrate TRIM38 as a negative regulator in TLR-induced NF-κB activation through siRNA knockdown experiments in vivo and in vitro, overexpression experiments, and reporter assays. The reason for this discrepancy is not clear. We found TRIM38 could induce K63-linked polyubiquitination of TRAF6. These data may explain the results observed by Matsuda et al. because K63-linked ubiquitination of TRAF6 is required for TLR signaling and NF-κB activation (11, 13). However, several reports have demonstrated that TRAF6 could activate downstream signaling independent of its autoubiquitination. TRAF6, as an E3 ligase, could promote the synthesis of unanchored polyubiquitin chains, which activate the IKK complex (37). Lysine-deficient TRAF6 has been shown to activate the NF-κB and MAPK pathways in response to IL-1 and RANKL (38). Wang et al. (39) reported that transubiquitination induced by two TRAF6 muteins is insufficient for NF-κB activation. Therefore, TRIM38 may use unknown mechanisms other than autoubiquitination to activate NF-κB. At the same time, these reports support our conclusions that TRIM38 is a negative regulator for NF-κB activation through K48-linked ubiquitination and proteasomal degradation of TRAF6. Human TRIM5α has been demonstrated to have dual roles in controlling NF-κB activation by targeting TAB2 degradation and catalyzing the synthesis of unattached K63-linked ubiquitin chains, respectively (33, 40). Thus, TRIM38 may also target different molecules to exert its dual roles in the activation of NF-κB.

In summary, we demonstrated TRIM38 was a TLR-inducible gene and identified TRIM38 as a negative regulator for TLR-induced inflammatory responses through promoting the proteasomal degradation of TRAF6. Given the importance of TRAF6 in the regulation of innate immune response during bacterial and viral infection, our study has identified a very important function for TRIM38 to limit excessive inflammatory immune response through targeting TRAF6 for proteasomal degradation.

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Disclosures
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References
20. Tarita, L., A. Bergamaschi, J. M. Luna, A. David, P. D. Uchil, F. Margottin-


E3 ubiquitin ligase TRIM38 negatively regulates TLR-mediated immune responses by proteasomal degradation of TRAF6 in macrophages

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Supplementary Figure S1-S4
Figure S1. TRIM38 is specifically expressed in lymphoid tissues. Western blot analysis of TRIM38 expression in different mouse tissues. Similar results were obtained in three independent experiments.

Figure S2. TRIM38 mRNA is induced upon TLR stimulation. RT-PCR analysis of TRIM38 mRNA expression in RAW264.7 cells stimulated with various TLR agonists for indicated time periods with the following primers: forward: 5’-GTCACTTCTTGCTCCATCTGC-3’ and reverse: 5’-ACATCTTCCACCCTGTTTTTG-3’. Similar results were obtained in three independent experiments.
Figure S3. TRIM38 promotes the degradation of TRAF6. Western blot analysis of the lysates from HEK293 cells transfected with various tagged-molecules with Myc-tagged TRIM38 for 24 h. (A) Myc-TAK1, Myc-RIP1 and Myc-TRAF3. (B) HA-IKKβ and HA-TAB1. (C) Flag-TRAF6 and Flag-p65. Similar results were obtained in three independent experiments.

Figure S4. Schematic diagram of TRIM38 WT and mutant constructs and their effect on MyD88-induced NF-κB activation. (A) TRIM38 Wild-type (WT) contains a RING domain, a B-box and a c-terminal PRY/SPRY domain. C64 lacks the RING domain. C130 lacks the RING domain and B-box. N273 lacks c-terminal PRY/SPRY domain. In mutant C16A, the cysteine residue at position 16 within the RING domain is substituted with alanine. (B) HEK293 cells were transfected with NF-κB reporter plasmid and MyD88 expression plasmid together with TRIM38 WT and mutants. 24 h later, luciferase activity was measured. Data are shown as mean±SD (n=6) of one typical experiment. (**, p<0.01).