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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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. The Journal of Immunology, 2012, 188: 2567–2574.

 Toll-like receptors play a pivotal role in defense against invading pathogens through the detection of pathogen-associated molecular patterns (1–4). Recognition of pathogen-associated molecular patterns by TLRs initiates signaling pathways through the recruitment of MyD88 and Toll/IL-1R domain-containing adaptor that induces an IFN-β (TRIF) to the Toll/IL-1R domain located in the cytoplasmic tails of TLRs (1–4). Recruitment of MyD88 promotes the interaction and phosphorylation of IL-1R–associated kinases and subsequent activation of TNFR-associated factor 6 (TRAF6) and TGF-β–activated kinase 1 (TAK1), leading to the activation of NF-κB and MAPK, and the induction of proinflammatory cytokines (1–4). Recruitment of TRIF mainly promotes the activation of TRAF3/NAP1 and subsequent activation of TBK1/IKKε kinase (IKKε), leading to the phosphorylation, dimerization, and nuclear translocation of IRF3 and the induction of IFN-β (1, 2, 4). TRIF-mediated signaling also activates the NF-κB and MAPK pathways through RIP1 and TAK1 (5, 6). Although activation of TLR signaling and secretion of proinflammatory cytokines are important for the elimination of invading microorganisms, uncontrolled activation may lead to autoimmune and inflammatory diseases (7). Therefore, TLR signaling must be tightly controlled to prevent excessive inflammatory response.

Protein ubiquitination has emerged as a crucial posttranscriptional modification to provide specificity and regulate the intensity of TLR signaling (8–10). K63-linked ubiquitination of TRAF6, RIP1, and IKK-γ is essential for the activation of TLR signaling (11–13). In contrast, K48-linked ubiquitination can promote the degradation of target molecules through proteasome, therefore limiting innate immune response (14–17). Protein ubiquitination requires the sequential action of three enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3). Among those, E3 ligases bind specifically to target proteins and confer specificity of the reaction. Although several E3 ligases have been demonstrated to play an important role in the regulation of immune responses, the function of the majority of E3 ligases remains elusive and needs further investigation.

A large family of E3 ligase is the tripartite motif (TRIM) family proteins, which are composed of >70 members in humans (18). The characteristic structure of TRIM proteins is the presence of a RING (R) domain, one or two B-boxes (B), and a coiled coil (CC) domain (19, 20). TRIM proteins have been implicated in many biological processes including cell differentiation, apoptosis, transcriptional regulation, and signaling transduction (21–24). Several members of TRIM proteins are encoded within the MHC class I region, including TRIM10, 15, 26, 27, 31, 38, 39, and 40 (25). But the function of these TRIM proteins, especially in the regulation of immune response, remains largely unknown.

In this report, we identified TRIM38 (also known as RoRet) as a negative feedback regulator in TLR signaling by targeting TRAF6. Specifically, TRIM38 bound to TRAF6 and promoted
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 obtained from 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, NLC-L, 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, pentidoglycan (PNG), and polynosinic:polycytidylic acid [poly(IC)] were purchased from Invivogen (San Diego, CA). JSH-23 was purchased from Calbiochem (San Diego, CA). IFN-γ was from PeproTech (Rocky Hill, NJ). LPS, LTA, PNG, 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 100 ng/ml, respectively. Abs specific for Abs against TRAP6 and TRAF3 (sc-7221; Invitrogen), TRAF6 (sc-948), hemagglutinin (HA; sc-7392), Ub (sc-8017), JNK (sc-474), p38 (sc-353), ERK (sc-94), β-actin (sc-81178), and protein G-arose (sc-2002) were used for immunoprecipitation (IP) were from Santa Cruz Biotechnology (Santa Cruz, CA). The Abs specific to Myc (2272), Flag (F3165) was from Sigma. The Abs for Flag (F3165) 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 Flag and 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. Xueqin Cao (Second Military Medical University, Shanghai, China). Expression vectors for TRAF6, TRAF3, IKK-β, and TAK1 were gifts 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 G. M. Boss (Emory University School of Medicine). Expression vectors for HA-UB WT and mutant K48, K63, K48R, and K63R were from Dr. Hui Wu (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 (San Diego, San Diego, CA). Target sequences for transient silencing were 5'-GAG-GAUCUGCGGAAAAACAAUUU-3' (siRNA 1) and 5'-GACUAAAGGUGGAAAGAUUUU-3' (siRNA 2) for TRIM38; “scrambled” control sequences were 5'-UUCUCCGAAGUGUCAGCUU-3'.

In vivo siRNA transfection

In vivo siRNA transfection to knock down TRIM38 expression in peritoneal macrophages was performed as described previously (27). In brief, female C57BL/6J mice (4 wk old) were i.p. injected with thioglycolate to elicit peritoneal exudate cells. After 3 d, 6 nmol siRNA was incubated with Geneporter 2 Transfection Reagent according to manufacturer’s instructions and injected into mice i.p. After 48 h, the mice were stimulated with TLR agonists administered i.p.

Detection of cytokine production

After cell stimulation, the concentrations of TNF-α and IL-6 in culture supernatants or peritoneal lavage were measured by ELISA kits (R&D Systems, Minneapolis, MN).

IP and Western blot analysis

For IP, whole-cell extracts were collected 36 h after transfection and were lysed in IP buffer containing 1% (v/v) Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 50 mM EDTA, 150 mM NaCl, and a protease inhibitor “mixture” (Merck). After centrifugation for 10 min at 14,000 x g, supernatants were collected and incubated with protein G Plus-Agarose IP reagent together with 1 μg monoclonal anti-Flag or 1 μg anti-HA. After 6 h of incubation, beads were washed five times with IP buffer. Immunoprecipitates were eluted by boiling with 1% (w/v) SDS sample buffer. For Western blot analysis, immunoprecipitates or whole-cell lysates were loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as described previously (26).

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 LTA or PGN (TLR2 ligand), poly(I:C) (TLR3 ligand), and R848 (TLR7 ligand) also greatly enhanced TRIM38 protein expression. In vivo, TRIM38 expression 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-κB–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.
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 expression plasmid, and these changes were 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.
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.
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. 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). TRIM30α 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/IKKβ 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, HA-TRIM38 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 NF-κB and MAPK pathways in response to IL-1 and RANKL (38). Wang et al. (39) reported that transubiquitination induced by two TRAF6 mutines 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 conclusion 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 as 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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ubiquitin ligase is essential for RIG-I-mediated antiviral activity. 
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’-GTCACCTCTTGCCTCCATCTGC-3’ and reverse: 5’-ACATCTTCCACCCCTGTTTTTG-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).