TRIM30α negatively regulates TLR-mediated NF-κB activation by targeting TAB2 and TAB3 for degradation

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Toll-like receptor (TLR) signaling is pivotal to innate and adaptive immune responses and must be tightly controlled. The mechanisms of TLR signaling have been the focus of extensive studies. Here we report that the tripartite-motif protein TRIM30α, a RING protein, was induced by TLR agonists and interacted with the TAB2-TAB3-TAK1 adaptor-kinase complex involved in the activation of transcription factor NF-κB. TRIM30α promoted the degradation of TAB2 and TAB3 and inhibited NF-κB activation induced by TLR signaling. In vivo studies showed that transfected or transgenic mice overexpressing TRIM30α were more resistant to endotoxic shock. Consistent with that, in vivo ‘knockdown’ of TRIM30α mRNA by small interfering RNA impaired lipopolysaccharide-induced tolerance. Finally, expression of TRIM30α depended on NF-κB activation. Our results collectively indicate that TRIM30α negatively regulates TLR-mediated NF-κB activation by targeting degradation of TAB2 and TAB3 by a ‘feedback’ mechanism.

Another intermediary complex, TRIKA2, consists of TAK1 and two adaptor proteins: TAK1-binding protein 1 (TAB1; A002247) and TAB2 (ref. 8). TAB2 was first identified as an adaptor linking TAK1 to TRAF6. Subsequent studies showed that TAB2 contains a zinc-finger domain that binds ‘preferentially’ to polyubiquitin chains linked by K63, which is thought to be necessary for the activation of TAK1 and IKKγ. TAB2 has been reported to facilitate TRAF6 ubiquitination, thus contributing to NF-κB activation8,10, the related protein TAB3 has also been reported to function redundantly with TAB2. Inhibition of both TAB2 and TAB3 by small interfering RNA (siRNA) blocks NF-κB activation11.

TRIM30α belongs to a family of tripartite-motif (TRIM) proteins involved in the regulation of cell proliferation, differentiation, development, oncogenesis, apoptosis and antiviral responses12. Studies have shown that some TRIM family members are critical to innate immunity; TRIM5 (refs. 12–14) and TRIM25 (ref. 15), for example, have shown that some TRIM family members are critical to innate immunity; TRIM5 (refs. 12–14) and TRIM25 (ref. 15), for example, have been shown to restrict viral infection. Here we demonstrate that TRIM30α, induced by TLR ligands in an NF-κB-dependent way, interacted with the TAK1-TAB2-TAB3 complex. Through this interaction, TRIM30α ‘targeted’ TAB2 and TAB3 for degradation and prevented TRAF6 autoubiquitination, thus negatively regulating TLR signaling. We demonstrate the in vivo biological function of TRIM30α in endotoxic shock models in transfected and transgenic mice overexpressing TRIM30α. In vivo ‘knockdown’ of

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Figure 1  TRIM30α is induced by TLR agonists and is specifically expressed in lymphoid tissues. (a) Immunoblot of lysates of HEK293T cells transfected with pHA-CMV-ANKLC together with Flag-tagged TRIM30α, TRIM34 or empty vector, analyzed with polyclonal anti-TRIM30α (α-TRIM30α) or anti-Flag (α-Flag); (b) Immunoblot of lysates of BMDCs (top) or J774 cells (bottom) treated for 0–24 h with LPS (200 ng/ml or 1 μg/ml, respectively), analyzed with polyclonal anti-TRIM30α or anti-actin (loading control). (c) Immunofluorescence microscopy of BMDCs treated for 8 h with LPS, stained with polyclonal anti-TRIM30α. Original magnification, ×630. (d) Immunoblot of lysates of BMDCs treated for 12 h with PBS (Vehicle; control), poly(I:C) (25 μg/ml), LPS (200 ng/ml or CpG (6 μg/ml), with detection of endogenous TRIM30α with polyclonal anti-TRIM30α. (e) Immunoblot of mouse tissues prepared and quantified by bicinchoninic acid assay (50 μg protein loaded per sample), analyzed with polyclonal anti-TRIM30α. Arrow indicates TRIM30α. kDa, kilodaltons. Data are representative of at least three independent experiments.

TRIM30α by siRNA further confirmed its critical function in tolerance to lipopolysaccharide (LPS). Our results collectively demonstrate a feedback mechanism for the negative regulation of TLR-mediated NF-κB activation.

RESULTS

TLR agonists induce TRIM30α

To investigate how the TLR signaling pathway is regulated, we did a cDNA microarray analysis of bone marrow–derived DCs (BMDCs) with or without LPS treatment (data not shown). We found that the expression of TRIM30α mRNA was much higher after 4 h of LPS treatment. To aid in our evaluation of TRIM30α protein expression, we generated a TRIM30-specific polyclonal antibody; this antibody specifically detected a band of about 58 kilodaltons in human kidney HEK293T cells transfected with a Flag-tagged TRIM30α plasmid but not in cells transfected with the control plasmid or a plasmid expressing the control TRIM family member, TRIM34, which is 39% identical to TRIM30α in amino acid sequence (Fig. 1a).

Thus, with this specific antibody, we found TRIM30α protein increased after LPS stimulation and reach a peak at 8 or 12 h in BMDCs or in the macrophage cell line J774 (Fig. 1b). We confirmed that observation by confocal microscopy. Although it has been suggested that TRIM30α is located in both the cytoplasm and the nucleus, by analysis of a fusion protein of enhanced green fluorescent protein and TRIM30α12, we found TRIM30α only in the cytoplasm even after LPS challenge (Fig. 1c and not shown). Because LPS-triggered TLR signaling was able to induce TRIM30α, we sought to determine whether other TLR agonists have a similar function. Indeed, we found that in addition to LPS (a TLR4 ligand), CpG dinucleotide (a TLR9 ligand) and polyinosinic-polycytidylic acid (poly(I:C), a TLR3 ligand) induced TRIM30α expression in BMDCs (Fig. 1d). The tissue distribution of TRIM30α expression, assessed by immunoblot analysis, showed that organs of the immune system, such as spleen and lymph nodes, had high expression of TRIM30α (Fig. 1e), consistent with the pattern of its mRNA expression (Supplementary Fig. 1 online). These data collectively indicate that TRIM30α is induced by LPS, CpG and poly(I:C) and is specifically expressed in lymphatic tissues.

TRIM30α interacts with TAK1-TAB2-TAB3

TRIM family proteins are also known as ‘RBCC’ proteins, as they contain an ‘RBCC’ motif in their amino terminus12 consisting of a RING domain, one or two B-boxes and a coiled-coil region. This domain arrangement is reminiscent of that of TRAF6, which is critical in TLR signaling; indeed, TRAF6 also has a tripartite motif in its amino terminus composed of a RING domain, a TRAF zinc finger and a coiled-coil region (Fig. 2a). This inclusion of the RBCC motif in TRIM30α suggests that some TRIM family proteins may function as signal modulators. Because TRIM30α was located in the cytoplasm and was induced by LPS ligands, we explored whether TRIM30α could regulate TLR-mediated signaling. We first tested whether TRIM30α was able to interact with the kinases involved in TLR signaling: we tested its interaction with several kinases, including TAK1, BTK, TBK1, IRAK2 and IRAK4, but found that only TAK1 bound to TRIM30α in coinmunoprecipitation assays (Fig. 2b). We further confirmed the interaction between TRIM30α and TAK1 with Flag-tagged TRIM30α and hemagglutinin-tagged TAK1; TAK1 immunoprecipitated with TRIM30α and vice versa (Fig. 2c). We also determined that the interaction between TRIM30α and TAK1 was dependent on the RING domain, as deletion of the RING domain (AR) or substitution of the cysteine residue at position 35 of the RING domain with alanine (C35A; Fig. 2a) resulted in less interaction (Fig. 2d). We also assessed in the interaction between endogenous TRIM30α and TAK1 in J774 cells; treatment for 2 h with LPS enhanced this interaction (Fig. 2e).

It has been reported that TAK1 plus TAB1 and TAB2 constitute the so-called ‘TRIak2 complex’ in the TLR signaling pathway5. We thus tested whether TRIM30α would also interact with TAB1, TAB2 and TAB3. There was indeed interaction between TRIM30α and TAB2 in cells expressing the proteins (when the expression vectors were transfected at a ratio of 1:3 (TRIM30α/TAB2); Fig. 2f); like TAB2, TAB3 immunoprecipitated together with TRIM30α, but TAB1 did not (Fig. 2g). Notably, the binding of TRIM30α to TAB2 and TAB3 was
and TAB3 but not with TAB1.

**TRIM30x promotes the degradation of TAB2 and TAB3**

When we transfected the same amount of plasmid expressing TAB2 or TRIM30x together into HEK293T cells, we detected only weak expression of TAB2 protein, whereas TAB1, which failed to bind to TRIM30x in our previous experiments (Fig. 2g), was well expressed (Fig. 3a). As a control, we also transfected plasmids expressing Trim34 and TAB2 together into cells and found, as anticipated, that Trim34 had no effect on TAB2 expression (Fig. 3b). Like the weak expression of TAB2 in the presence of TRIM30x, we also found weak expression of TAB3 in cells that expressed TRIM30x (Supplementary Fig. 2 online). These results suggest that TRIM30x may have a specific function in the degradation of TAB2 and TAB3. We further found that the accumulation of TAB2 protein was inhibited by TRIM30x in a dose-dependent way (Fig. 3c), yet the effect of TRIM30x was not on the amount of TAB2 mRNA (Fig. 3d). This result indicated that TRIM30x interfered with TAB2 expression during the post-transcriptional process.

Because RING domains have been reported to be involved in protein-protein interactions and many RING domain proteins have potential ubiquitin E3 ligase activity," we tested whether the C35A substitution of TRIM30x impaired the ability of TRIM30x to degrade TAB2. We found that the C35A mutant did not efficiently decrease TAB2 and TAB3 expression as did wild-type TRIM30x (Fig. 3e and Supplementary Fig. 2). We then investigated whether the down-regulation of TAB2 expression by TRIM30x was dependent on the ubiquitin-proteasome pathway. Neither lactacystin nor MG132, inhibitors of the ubiquitin-proteasome pathway, was able to block the down-regulation of TAB2 by TRIM30x (Fig. 3f). We also noted that TRIM30x could not undergo autoubiquitination, analogous to TRAF6 autoubiquitination, and the absence of an E3 ligase activity; this supported the conclusion that TRIM30x may not have E3 ligase activity (data not shown). However, we did find that down-regulation of TAB2 was blocked by NH2Cl and chloroquine, both inhibitors of lysosomal protein degradation (data not shown). Furthermore, accumulation of endogenous TAB2 was inhibited after LPS challenge and was restored by NH2Cl and chloroquine in J774 cells (Supplementary Fig. 3a online) and in THP-1 cells, a human monocyte cell line (Supplementary Fig. 3b). Indeed, by confocal microscopy, we found that TRIM30x localized together with TAB2 in lysosomes (Fig. 3h) but not in endosomes or the Golgi apparatus (data not shown). These data suggest that TRIM30x induces TAB2 degradation through a pathway independent of ubiquitin and proteasomes but dependent on lysosomes.

To further confirm that TRIM30x targets TAB2 for degradation in vivo, we assessed TAB2 expression after 'knockdown' of TRIM30x by siRNA. Initially we designed three pairs of siRNA molecules specific...
for TRIM30x (T1, T2 and T3) and a control siRNA, as described before20. We found that T3 most efficiently inhibited TRIM30x expression in HEK293T cells transfected with TRIM30x (T1, T2 and T3) and a control siRNA, as described above. We found an even greater difference in the knockdown of TRIM30x (T1, T2 and T3) and a control siRNA, pretreated for 12 h (Fig. 3i) compared to the untreated control (Fig. 3j). As T3 was the most effective in inhibiting ectopic and endogenous expression of TRIM30x, we used it in subsequent experiments. We next analyzed endogenous TAB2 expression after LPS stimulation of J774 cells treated with T3 or control siRNA. We found that knockdown of TRIM30x by T3 resulted in higher TAB2 expression during a secondary LPS challenge (Fig. 3k). As the formation of TRAF6 oligomers is necessary for its autoubiquitination8, we examined the possibility that TRIM30x would affect the formation of TRAF6 oligomers. We detected the formation of TRAF6 oligomers by expressing and coimmunoprecipitating two distinctly tagged TRAF6 proteins, hemagglutinin-tagged TRAF6 and Flag-tagged TRAF6; as reported before19, we found formation of TRAF6 oligomers when it was overexpressed in HEK293T cells. We found that expression of TRIM30x did not interfere with the formation of TRAF6 oligomers (Fig. 4c).

TRIM30x prevents TRAF6 autoubiquitination

It has been documented that TAB2 facilitates TRAF6 autoubiquitination and thus contributes to NF-κB activation18. Therefore, the function of TRIM30x in targeting TAB2 has the potential to interfere with TRAF6 autoubiquitination. When we transfected Flag-tagged TRAF6 and hemagglutinin-tagged ubiquitin together into HEK293T cells, we readily detected autoubiquitination of TRAF6, as reported before18. Expression of wild-type TRIM30x together with TRAF6 resulted in less TRAF6 autoubiquitination, but expression of the C35A or AR TRIM30x mutant together with TRAF6 did not (Fig. 4a). Consistent with that result, knockdown of TRIM30x by T3 enhanced TRAF6 autoubiquitination in J774 cells during a secondary LPS challenge (Fig. 4b). As the formation of TRAF6 oligomers is necessary for its autoubiquitination8, we examined the possibility that TRIM30x would affect the formation of TRAF6 oligomers. We detected the formation of TRAF6 oligomers by expressing and coimmunoprecipitating two distinctly tagged TRAF6 proteins, hemagglutinin-tagged TRAF6 and Flag-tagged TRAF6; as reported before19, we found formation of TRAF6 oligomers when it was overexpressed in HEK293T cells. We found that expression of TRIM30x did not interfere with the formation of TRAF6 oligomers (Fig. 4c).

Phosphorylation of IkBz is a critical event after TRAF6 activation and is needed for IkBz degradation and NF-κB activation. When we knock down TRIM30x expression in J774 cells with T3, IkBz phosphorylation was enhanced after LPS challenge (Fig. 4d). During a secondary LPS challenge, after 12 h of pretreatment with LPS, we found an even greater difference in IkBz phosphorylation in cells pretreated with T3 (Fig. 4e). Hence, TRIM30x prevents TRAF6 autoubiquitination, TRAF6 oligomerization and IkBz phosphorylation. This suggests that TRIM30x prevents these downstream events by both preventing autoubiquitination through direct binding to TAB2 and blocking TAB2 interaction with TRAF6.
autoubiquitination, diminishes IkBα phosphorylation and is involved in regulating LPS-mediated signaling.

**TRIM30α inhibits TRAF6-induced NF-κB activation**

Because TRIM30α suppressed IkBα phosphorylation, we wondered if TRIM30α would also negatively regulate TLR-mediated NF-κB activation. We therefore assessed the effect of TRIM30α on NF-κB activation with luciferase reporter assays. First we noted that overexpression of TRIM30α did not result in NF-κB activation (data not shown). We then assessed the expression of TRAF6 alone or TRAF6 plus TAB2 or TAK1 plus TAB1 in HEK293T cells and found that all three conditions led to much more NF-κB reporter activity (Fig. 5a–c). When we added a plasmid expressing TRIM30α in increasing concentrations, we noted a dose-dependent inhibition of NF-κB activity induced by TRAF6 or TRAF6 plus TAB2 (Fig. 5a,b) but not of NF-κB activation induced by TAK1 plus TAB1 (Fig. 5c). Moreover, TRIM30α did not interfere with TBK1- or TRIF-induced activation of a promoter driven by an interferon-stimulated response element or the promoter of the gene encoding interferon-β (data not shown). We further assessed the effects of TRIM30α mutants on the induction of NF-κB activity by TRAF6 or TRAF6 plus TAB2. As anticipated, the ΔR and C35A TRIM30α mutants failed to block NF-κB activity (Fig. 5d,e). This is in agreement with our data showing that the ΔR and C35A mutants lost the ability to promote TAB2 degradation. These results collectively show that TRIM30α negatively regulates NF-κB activation, probably by targeting TAB2.

**TRIM30α inhibits the production of interleukin 6 and TNF**

As the genes encoding interleukin 6 (IL-6) and TNF are two common genes regulated by NF-κB and are used as ‘hallmarks’ of NF-κB activation, we next tested whether TRIM30α would regulate the production of those two cytokines. We stimulated J774 cells with LPS, poly(I:C) or CpG and measured the cytokines by enzyme-linked immunosorbent assay (ELISA); we found that large amounts of IL-6 and TNF were induced by those TLR ligands. However, when TRIM30α was overexpressed in the cells, induction of IL-6 and TNF was significantly suppressed relative to their induction in cells expressing vector control (Fig. 6a,b). In contrast, expression of the ΔR or C35A TRIM30α mutant did not inhibit the production of IL-6 and TNF (data not shown). To further confirm the suppression of IL-6 and TNF, we ‘silenced’ TRIM30α by siRNA in J774 cells and assessed the production of IL-6 and TNF after LPS stimulation. We found no evident difference in TNF production during the primary stimulation with LPS regardless of whether TRIM30α was ‘knocked down’ or not (data not shown). As TRIM30α is induced by LPS, we tested the possibility that TRIM30α might be involved in endotoxin tolerance. We

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**Figure 5** TRIM30α inhibits NF-κB activity. (a–c) Dual-luciferase assay of lysates of HEK293T cells transfected with an NF-κB reporter plasmid and increasing concentrations of a plasmid expressing TRIM30α, plus plasmids expressing TRAF6 (a); TRAF6 and TAB2 (b); or TAK1 and TAB1 (c). (d,e) Dual-luciferase assay of lysates of HEK293T cells transfected with an NF-κB reporter plasmid and plasmid expressing wild-type TRIM30α or the ΔR or C35A TRIM30α mutant, plus plasmids expressing TRAF6 (d) or TRAF6 and TAB2 (e). Results are presented as percent activity relative to the activity in cells not transfected with TRIM30α (set as 100%). Data (mean and s.e.m.) are representative of at least three independent experiments.
three experiments. and analyzed after 24 h. Data (mean and s.e.m.) are representative of IL-6 (Fig. 6a, b) ELISA of IL-6 (a) or TNF (b) in supernatants of J774 cells transfected with TRIM30x or control vector and then stimulated with poly(I:C), LPS or CpG. (c, d) ELISA of IL-6 (c) or TNF (d) in supernatants of J774 cells transfected for 24 h with T3 or control siRNA, then pretreated for 18 h with LPS (200 ng/ml; 1 LPS) and then restimulated with LPS (0–2 μg/ml (below bars); 2 LPS) and analyzed after 24 h. Data (mean and s.e.m.) are representative of three experiments.

pretreated J774 cells for 18 h with LPS (200 ng/ml) and evaluated IL-6 production after rechallenge with LPS; IL-6 production was enhanced in cells treated with T3 relative to that in cells treated with control siRNA (Fig. 6c). TNF production, normally low in cells receiving secondary LPS stimulation, was higher in cells after ‘snoozing’ of TRIM30x with T3 (Fig. 6d). These results further support the conclusion that TRIM30x inhibits NF-κB activation stimulated by TLR signaling.

In vivo endotoxin challenge
To assess the in vivo function of TRIM30x in TLR signaling, we used an endotoxin shock model. We first used polyethylenimine-mediated DNA transfection of whole mice. We confirmed the efficiency of DNA ‘transfection’ with this method by immunoblot analysis. We tested liver and lung, as they have been reported to be efficiently transfectected by this method. As anticipated, mice injected intravenously with the TRIM30x-expressing plasmid expressed more TRIM30x at 24 h after transfection (Fig. 7a). We then subjected the mice to endotoxin shock by intraperitoneal challenge with LPS and O-galactosamine. Mice transfected with the TRIM30x-expressing plasmid were more resistant to endotoxin shock, as determined by survival rate, than were those transfected with control vector plasmid. Furthermore, the death of mice transfected with the TRIM30x-expressing plasmid was delayed (Fig. 7b). This indicated that TRIM30x was able to protect mice from endotoxin shock. To further confirm those results, we generated transgenic mice overexpressing TRIM30x. By RT-PCR and immunoblot analysis, we identified the transgenic mice by their higher expression of TRIM30x (Supplementary Fig. 4a online). Using the same treatment protocol, we found that transgenic mice were more resistant to endotoxin shock than were their nontransgenic littermates (Fig. 7c). Consistent with that result, the LPS-driven cytokines in plasma (TNF and IL-6) were less abundant in the transgenic mice (Fig. 7d).

Because TRIM30-deficient mice are not yet available, we used in vivo siRNA delivery with polyethylenimine. We were not able to detect a substantial difference in the ability of mice injected with T3 and mice injected with control siRNA to resist primary endotoxin shock (data not shown). This may have been because endogenous TRIM30x is an inducible protein and thus will not have an effect during primary stimulation with LPS. We then tested whether ‘knockdown’ of TRIM30x would affect in vivo LPS-induced tolerance. We pretreated mice with low dose of LPS at day 5 and day 3 before LPS rechallenge and delivered T3 or control siRNA in vivo on day 1 before LPS rechallenge. At 24 h after transfection, we challenged mice with LPS (50 mg LPS per kg body weight (50 mg/kg)) and evaluated the in vivo ‘knockdown’ efficiency of TRIM30x by immunoblot analysis. As expected, TRIM30x expression was impaired in mice transfected with T3 relative to its expression in mice transfected with control siRNA (Supplementary Fig. 4b). We also found that IL-6 and TNF in plasma from T3-transfected mice were much higher after 2 h of LPS secondary challenge (Fig. 7e), which suggested that endogenous TRIM30x is involved in LPS-induced tolerance. Our data collectively indicate that TRIM30x is a negative regulator of TLR signaling.

TRIM30x expression depends on NF-κB activity
TRIM30x is induced by TLR ligands and functions as a negative regulator of NF-κB activation. We sought to determine whether TRIM30x expression is regulated in an NF-κB-dependent way.
Initially we found that TRIM30α induced by LPS in BMDCs was inhibited by two inhibitors of NF-kB, the serine protease inhibitor TPCK (N-tosyl-L-phenylalanine chloromethyl ketone) and PDTC (pyrrolidine dithiocarbamate; Supplementary Fig. 5a online). We further confirmed the TRIM30α expression profile in BMDCs isolated from mice deficient in the p50 subunit of NF-kB. As expected, p50-deficient BMDCs failed to express TRIM30α after challenge with LPS, poly(I:C) or CpG (Supplementary Fig. 5b). These data show that TRIM30α expression is controlled by an NF-kB-dependent pathway. Our data collectively demonstrate that TRIM30α is a negative regulator of the TLR-mediated NF-kB signaling pathway by targeting TAB2 through a feedback mechanism.

**DISCUSSION**

The pivotal function of TLRs in initiating early innate immune response and in directing the later adaptive immune response is well established22–24. Studies have shown that this signaling pathway is negatively regulated LPS-mediated NF-kB activation. Nevertheless, the mechanism remains undefined. In conclusion, degradation of TAB2 and TAB3 by TRIM30α was responsible for its inhibition of NF-kB activation, and interference with TRAF6 autoubiquitination by TRIM30α would be due to the degradation of TAB2 and TAB3 because TAB2 and TAB3 facilitated TRAF6 autoubiquitination. We further found that TAB2 degradation could be partially inhibited by NH4Cl or chloroquine, inhibitors of lysosomal protein degradation. It is well known that TLR9 and TLR3 are located in the endoplasmic reticulum and that TLR9 signals after translocation from the endoplasmic reticulum to CpG DNA in the lysosome29. Although TLR4 is located on the cell membrane, its internalization and modification in the lysosome have also been reported, which suggests that functional regulators of TLR signaling would be found in the lysosome30. We have shown that TAB2 localized together with TRIM30α in the lysosome, consistent with the idea that TAB2 degradation depends on the lysosomal degradation pathway. Because most TLR signaling pathways converge at a complex consisting of TRAF6, TAB2, TAB3 and TAK1 (refs. 31,32), degradation of TAB2 and TAB3 would restrict downstream TLR signaling, such as NF-kB activation triggered by TLR3, TLR4 and TLR9. Therefore, the critical function of TRIM30α in the attenuation or termination of NF-kB activation induced by many TLR ligands probably contributes to control of the amplification cascade of innate immune response. The importance of TAB2 and TAB3 downstream of TNF receptor or IL-1 receptor signaling in effecting NF-kB activation31 also suggests that targeting of TAB2 and TAB3 by TRIM30α may be a mechanism for modulating many types of immune responses.

**METHODS**

**Mouse strains and conditions.** TRIM30α-transgenic mice were generated in the laboratory of B.S.; expression of mouse gene encoding TRIM30α was under control of the enhancer of the cytomegalovirus intermediate-early gene and the promoter of the chicken gene encoding β-actin (pcAGGS; a gift from J. Miyazaki33). Nontransgenic littermates served as control for the transgenic mice. Mice deficient in p50 and control wild-type mice were gifts from J. Geng. Female C57BL/6 mice 6–8 weeks old were from the Shanghai SLAC Laboratory Animal Company. All mice were maintained in clean and comfortable animal rooms at the Shanghai SLAC Laboratory Animal Company and the animal facility of the Shanghai Laboratory Animal Center. All mice had free access to water and a standard laboratory diet (provided by the Shanghai SLAC Laboratory Animal Company).

**Constructs and reagents for cDNA.** Sequences encoding TRIM30α, TAK1, TAB1, TAB2, TAB3, irak4, irak2, btk and tk1 were amplified by PCR with cDNA from BMDCs challenged for 4 h with LPS. TRIM30α, deletion mutants and point mutants were cloned into the pCDNA3.0-HA vector. All kinases, TAK1 deletion mutants, TAB1, TAB3, and TAB2 and its mutants were cloned into the pCDNA3.1-HA vector. Expression constructs of Flag-tagged TRAF6 (gifts from C. Wang) were cloned into the pCDNA3.1-HA vector by PCR. The ubiquitin plasmid was from G. Pei. All reagents were from Sigma unless stated otherwise. Polyclonal antibody to TRIM30α (anti-TRIM30α) was generated by immunization of rabbits with TRIM30α expressed by Escherichia coli; this procedure has been described34. Anti-TAB2 (sc-20756) was from Santa Cruz; poly(I:C) and CpG were from Invitrogen; and antibodies to early endosome
antigen 1 (610456) and trans-Golgi network 38 (610898) were from BD PharMingen.

In vivo transfection and in vivo stimulation. Plasmid or siRNA was delivered into 6- to 8-week-old C57BL/6 female mice with polyethylenimine (Qbiogene) by tail vein administration as described. The plasmid pBUDCEAI-TRIM30z or control vector was mixed for 20 min at 25 °C with polyethylenimine at a nitrogen/phosphorus weight ratio of 10; for siRNA, nitrogen/phosphorus ratio was 5. For each mouse, 200 μl of the mixture containing 60 μg DNA or siRNA was injected. For in vivo overexpression assays, after 24 h of in vivo transfection, mice were injected intraperitoneally with 1,000 ng LPS and 8 mg of galactosamine and were monitored for survival for the ensuing 30 h. For the endotoxiness studies, 6- to 8-week-old female TRIM30z-transgenic or control mice were challenged with LPS (50 mg/kg, administered intraperitoneally) and, 2 h later, a sample of blood was obtained and plasma TNF and IL-6 were measured by ELISA. For LPS-induced tolerance assay, mice were pretreated with LPS at a dose of 50 mg/kg 5 d before LPS rechallenge and LPS at a dose of 20 mg/kg 3 d before LPS rechallenge and in vivo siRNA was delivered 1 d before LPS rechallenge. After 24 h, mice were challenged with LPS at a dose of 50 mg/kg. Then, 2 h later, plasma was collected and TNF and IL-6 were measured by ELISA. For analysis of in vivo knockdown efficiency, mice were killed 8 h after the LPS challenge and then lungs, spleens and lymph nodes were prepared for immunoblot analysis. Animal experiments were done with approval of the Shanghai Institutes for Biological Sciences Biological Research Ethics Committee.

Cell culture, transfection and stimulation. J774, HEK293T and HeLa cells were maintained in humidified 5% CO2 at 37 °C in DMEM supplemented with 10% (vol/vol) FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Lipofectamine (Invitrogen) was used for transient transfection of HEK293T cells. The procedure for generating BMDCs has been described. BMDCs were stimulated for various times with LPS (200 ng/ml) or for 8 h with LPS (200 ng/ml), poly(I:C) (25 μg/ml) or CpG (6 μg/ml); PBS served as stimulation control. Cells were then collected and prepared for immunoblot analysis. Immunofluorescence microscopy. Immunofluorescence microscopy. Immunofluorescence microscopy. Immunofluorescence microscopy.

Immunoprecipitation and immunoblot analysis. Cultures of HEK293T cells in six-well plates were transfected with various combinations of plasmids with Lipofectamine as specified by the manufacturer (Invitrogen). For immunoprecipitation, cells were collected 36 h after transfection and were lysed in lysis buffer containing 1%, (vol/vol) Nonident P40, 20 mM Tris–HCl, pH 8, 10%, (vol/vol) glycerol, 150 mM NaCl, 0.2 mM Na3VO4, 1 mM NaF, 0.1 mM sodium pyrophosphate and a protease inhibitor cocktail (Roche). For detection of the autoubiquitination of endogenous TRAF6, 5 mM N-ethylmaleimide was included as well. After centrifugation for 20 min at 14,000 g, supernatants were collected and incubated with protein A/G Plus-Agarose Immunoprecipitation Control, followed by fluorescein isothiocyanate–conjugated goat–anti–mouse immunoglobulin G (115-175-146; Jackson ImmunoResearch).

Analysis with siRNA. The siRNA constructs were designed as described. The forward siRNA sequences targeting TRIM30 mRNA were T1 (5′-CAGCU CUAUUGAGAAGGGTTU-3′), T2 (5′-GGGAUAGAGGACGUGCATTT-3′) and T3 (5′-CUGCGGGUCUCAUUTT-3′); siRNA was chemically synthesized by Dharmaco. The negative control siRNA was from Dharmaco (D-002120-01-20).

Reporter assays. HEK293T cells were transfected with luciferase reporter plasmids combined with pNF-kB-TA-Luc for the NF-kB reporter or pISRE- Luc (Strатегène), and pRL-TK (Clontech). Then, 24 h after transfection, cells were lysed and reporter activity was analyzed with the Dual-Luciferase Reporter Assay system (Promega).

ELISA. For overexpression, J774 cells were transfected for 24 h with TRIM30z or vector. Then, cells were stimulated for 24 h with LPS (1 μg/ml), poly(I:C) (25 μg/ml) or CpG (6 μg/ml); for siRNA assays, after 24 h of siRNA transfection, J774 cells were treated for 24 h with LPS (1 μg/ml). For the LPS tolerance assay, cells were pretreated for 18 h with LPS (200 ng/ml), then collected and washed twice with DMEM, and then challenged with various concentrations of LPS. Supernatants were collected and their concentration of IL-6 or TNF was determined with a mouse-specific ELISA kit (R&D Systems), followed by analysis with a SpectraMax M5 (Molecular Devices).

Reverse transcription and quantitative real-time PCR. Total RNA was extracted from cultured cells with TRIzol (Invitrogen) according to the manufacturer’s instructions. Oligo(dT) priming and Superscript III reverse transcriptase (Invitrogen) were used for reverse transcription of purified RNA. All gene transcripts were quantified by quantitative PCR with Brilliant SYBR Green QPCR Master Mix and a Light Cycler apparatus (Stratagene). Primers for PCR and gene clones are in Supplementary Table 1 online.

Statistics. One-way analysis of variance was used for multiple-group comparisons, followed by the Bonferroni procedure for comparison of means. Student’s t-test was used for the comparison of two independent groups. For all tests, a P value of less than 0.05 was considered statistically significant.


Note: Supplementary information is available on the Nature Immunology website.

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