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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.

Toll-like receptors (TLRs) are widely distributed on antigen-presenting cells, especially macrophages and dendritic cells (DCs)[1–3], and function as primary sensors of invading pathogens by recognizing conserved microbial molecules (pathogen-associated molecular patterns), which is essential for eliciting the innate response and enhancing adaptive immunity to pathogens. However, excessive activation of the TLR signaling pathway contributes to the pathogenesis of autoimmune, chronic inflammatory and infectious diseases[4]. TLR signaling and function, therefore, must be under tight negative regulation to maintain immune balance.

Stimulation of TLRs initiates the signaling cascade, which leads to activation of the transcription factor NF-κB and stress-activated protein kinases[5] and then induces the expression of genes encoding immune and proinflammatory molecules[6]. Activation of NF-κB by a TLR signal depends on the activity of the inhibitor of NF-κB (IκB) kinase (IKK) complex. In turn, activation of both IKK and TAK1 (A002249), the kinase that activates IKK, requires ubiquitinated 'lysine 63' (K63) chains synthesized by TRAF6, a RING domain–containing E3 ubiquitin ligase[7,8]. After forming oligomers, TRAF6 can ubiquitinate itself with K63-linked polyubiquitin chains, a modification that contributes to its activity; this ubiquitination of TRAF6 requires the ubiquitin-conjugating protein UBC13 and the UBC-like protein UEV1A, factors identified before as 'TRIKA1' (TRAF6-regulated IKK activator 1)[7].

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 IKK[9]. TAB2 has been reported to facilitate TRAF6 ubiquitination, thus contributing to NF-κB activation[9,10]; 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 activation[11].

TRIM30α belongs to a family of tripartite-motif (TRIM) proteins involved in the regulation of cell proliferation, differentiation, development, oncogenesis, apoptosis and antiviral responses[12]. Studies 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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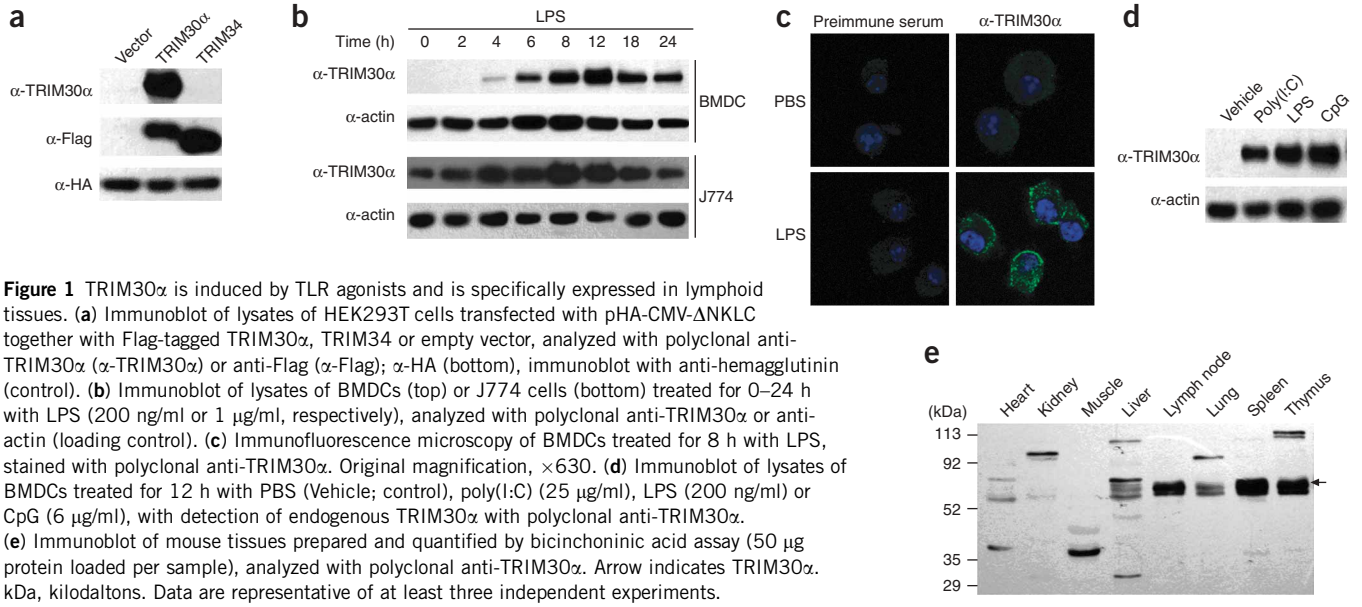

Figure 1 TRIM30α is induced by TLR agonists and is specifically expressed in lymphoid tissues. (**a**) Immunoblot of lysates of HEK293T cells transfected with pHA-CMV-ΔNKLC together with Flag-tagged TRIM30α, TRIM34 or empty vector, analyzed with polyclonal anti-TRIM30α (α-TRIM30α) or anti-Flag (α-Flag); α-HA (bottom), immunoblot with anti-hemagglutinin (control). (**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 another 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 TRIM30α[16], 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 terminus[12] 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 TLR 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 coimmunoprecipitation 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 (ΔR) 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 pathway[8]. 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

independent of the RING domain, as this interaction was enhanced considerably when we used the C35A TRIM30α mutant (**Fig. 2f**,**h**). Finally, we did coimmunoprecipitation experiments with lysates of J774 or BMDCs after LPS challenge; this showed specific interaction between endogenous TRIM30α and TAB2 (**Fig. 2i**). Our results collectively demonstrate that TRIM30α interacts with TAK1, TAB2 and TAB3 but not with TAB1.

TRIM30α promotes the degradation of TAB2 and TAB3

When we transfected the same amount of plasmid expressing TAB2 or TRIM30α together into HEK293T cells, we detected only weak expression of TAB2 protein, whereas TAB1, which failed to bind to TRIM30α 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 affect on TAB2 expression (**Fig. 3b**). Like the weak expression of TAB2 in the presence of TRIM30α, we also found weak expression of TAB3 in cells that expressed TRIM30α (**Supplementary Fig. 2** online). These results suggest that TRIM30α may have a specific function in the degradation of TAB2 and TAB3. We further found that the accumulation of TAB2 protein was inhibited by TRIM30α in a dose-dependant way (**Fig. 3c**), yet the effect of TRIM30α was not on the amount of TAB2 mRNA (**Fig. 3d**). This result indicated that TRIM30α 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[12,17], we tested whether the C35A substitution of TRIM30α impaired the ability of TRIM30α to degrade TAB2. We found that the C35A mutant did not efficiently decrease TAB2 and TAB3 expression as did wild-type TRIM30α (**Fig. 3e** and **Supplementary Fig. 2**). We then investigated whether the down-regulation of TAB2 expression by TRIM30α was dependent on the ubiquitin-proteasome pathway. Neither lactacystin nor MG132, inhibitors of the ubiquitin-proteasome pathway, was able to block the downregulation of TAB2 by TRIM30α (**Fig. 3f**,**g**). We also noted that TRIM30α could not undergo autoubiquitination, analogous to TRAF6 autoubiquitination[18,19], an indication of E3 ligase activity; this supported the conclusion that TRIM30α may not have E3 ligase activity (data not shown). However, we did find that downregulation of TAB2 was blocked by NH_4Cl 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 NH_4Cl 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 TRIM30α localized together with TAB2 in lysosomes (**Fig. 3h**) but not in early endosomes or the Golgi apparatus (data not shown). These data suggest that TRIM30α induces TAB2 degradation through a pathway independent of ubiquitin and proteasomes but dependent on lysosomes.

To further confirm that TRIM30α targets TAB2 for degradation *in vivo*, we assessed TAB2 expression after 'knockdown' of TRIM30α by siRNA. Initially we designed three pairs of siRNA molecules specific

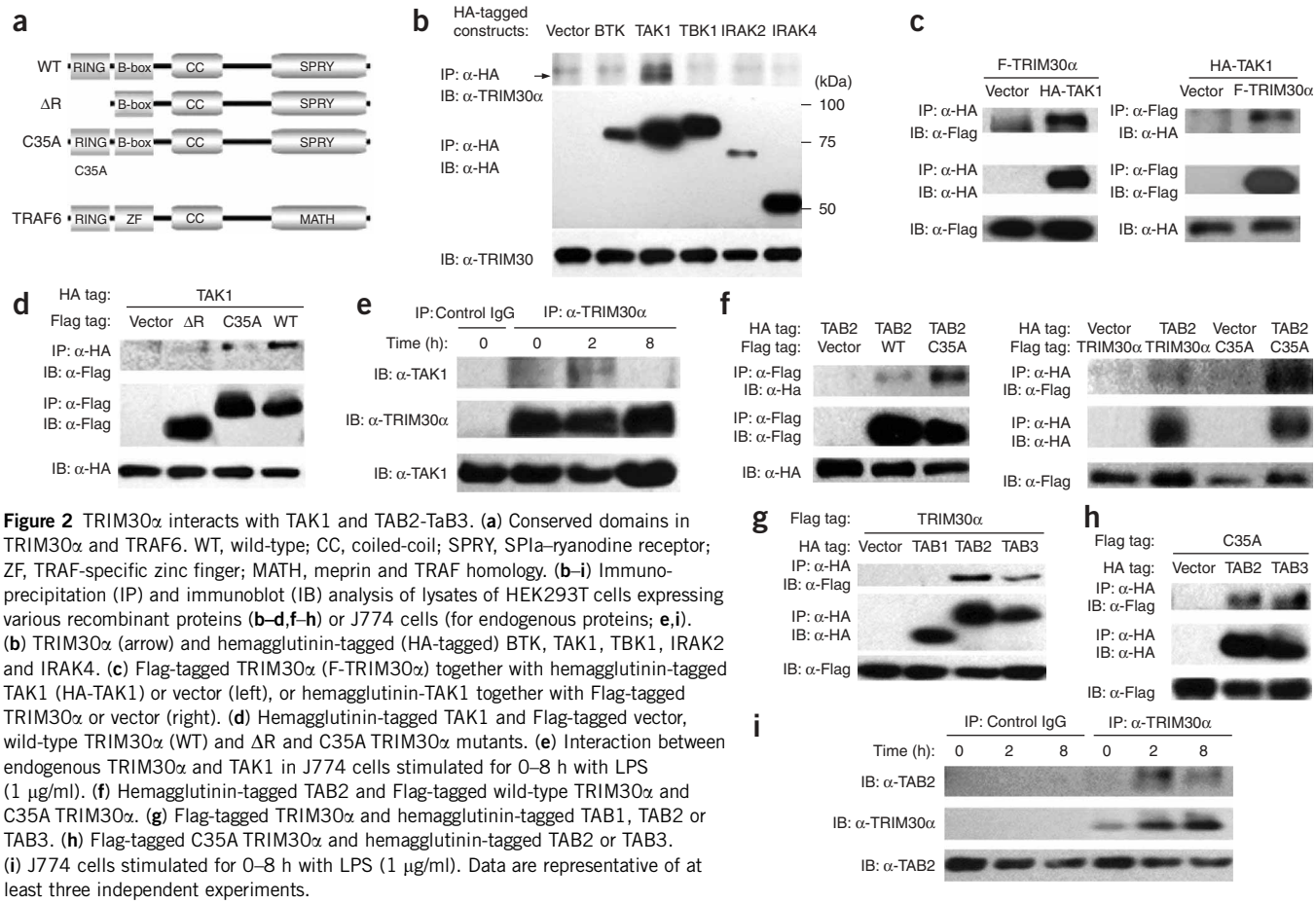

Figure 2 TRIM30α interacts with TAK1 and TAB2-TaB3. (**a**) Conserved domains in TRIM30α and TRAF6. WT, wild-type; CC, coiled-coil; SPRY, SPIa–ryanodine receptor; ZF, TRAF-specific zinc finger; MATH, meprin and TRAF homology. (**b**–**i**) Immunoprecipitation (IP) and immunoblot (IB) analysis of lysates of HEK293T cells expressing various recombinant proteins (**b**–**d**,**f**–**h**) or J774 cells (for endogenous proteins; **e**,**i**). (**b**) TRIM30α (arrow) and hemagglutinin-tagged (HA-tagged) BTK, TAK1, TBK1, IRAK2 and IRAK4. (**c**) Flag-tagged TRIM30α (F-TRIM30α) together with hemagglutinin-tagged TAK1 (HA-TAK1) or vector (left), or hemagglutinin-TAK1 together with Flag-tagged TRIM30α or vector (right). (**d**) Hemagglutinin-tagged TAK1 and Flag-tagged vector, wild-type TRIM30α (WT) and ΔR and C35A TRIM30α mutants. (**e**) Interaction between endogenous TRIM30α and TAK1 in J774 cells stimulated for 0–8 h with LPS (1 μg/ml). (**f**) Hemagglutinin-tagged TAB2 and Flag-tagged wild-type TRIM30α and C35A TRIM30α. (**g**) Flag-tagged TRIM30α and hemagglutinin-tagged TAB1, TAB2 or TAB3. (**h**) Flag-tagged C35A TRIM30α and hemagglutinin-tagged TAB2 or TAB3. (**i**) J774 cells stimulated for 0–8 h with LPS (1 μg/ml). Data are representative of at least three independent experiments.

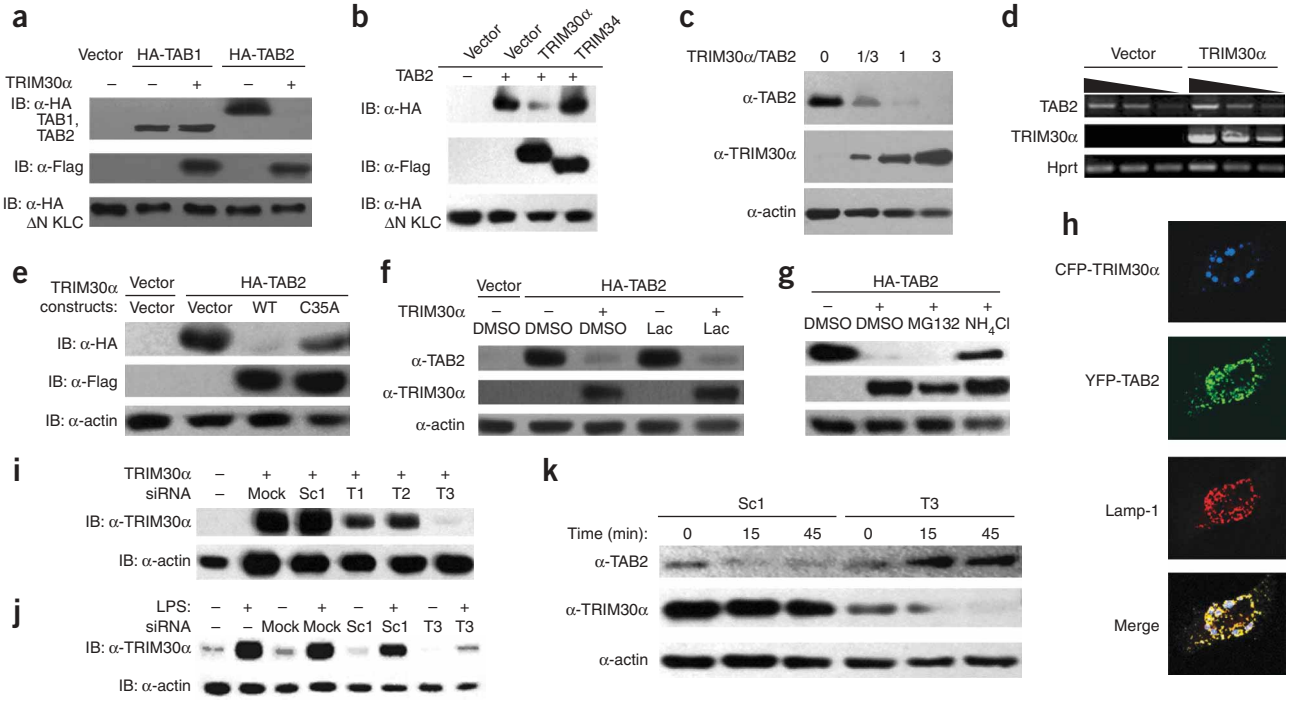

Figure 3 TRIM30α targets TAB2. (**a**,**b**) Immunoblot analysis of lysates of HEK293T cells transfected with pHA-CMV-ΔN KLC together with Flag-tagged TRIM30α or TRIM34 and hemagglutinin-tagged TAB1 or TAB2. α-HA ΔN KLC, anti-hemagglutinin detecting deletion of the amino terminus of kinesin light chain (transfection efficiency control). (**c**) Immunoblot analysis of lysates of HEK293T cells transfected with hemagglutinin-tagged TAB2 and increasing doses of TRIM30α; anti-actin (bottom), loading control. (**d**) RT-PCR analysis of mRNA from HEK293T cells coexpressing TAB2 and TRIM30α. Wedges indicate decreasing amounts of input DNA; HPRT (encoding hypoxanthine guanine phosphoribosyl transferase), loading control. (**e**) Immunoblot analysis of lysates of HEK293T cells coexpressing Flag-tagged TRIM30α and C35A TRIM30α and hemagglutinin-tagged Tab2 or control vector. (**f**,**g**) Immunoblot analysis of lysates of HEK293T cells transfected for 24 h with Flag-tagged TRIM30α and hemagglutinin-tagged TAB2 or vector control, then treated for 6 h with DMSO (negative control), 10 μM lactacystin (Lac), 20 μM MG132 or 5 mM NH_4Cl. (**h**) Immunofluorescence microscopy of HeLa cells transfected for 24 h with cyan fluorescent protein–labeled TRIM30α (CFP-TRIM30α) and yellow fluorescent protein–labeled TAB2 (YFP-TAB2), then stained with anti-LAMP-1 and indodicarbocyanine-tagged secondary antibody. Original magnification, ×630. (**i**) Immunoblot analysis of lysates of HEK293T cells transfected with TRIM30α and TRIM30α-specific siRNA (T1, T2, T3) or negative control siRNA (Sc1), or mock transfected (Mock). (**j**) Immunoblot analysis of lysates of J774 cells transfected with T3 or control siRNA, or mock transfected. (**k**) Immunoblot analysis of lysates of J774 cells transfected with T3 or control siRNA, pretreated for 12 h with LPS (200 ng/ml) and then rechallenged for 0–45 min (above lanes) with LPS. Data are representative of at least three independent experiments.

for TRIM30 (T1, T2 and T3) and a control siRNA, as described before[20]. We found that T3 most efficiently inhibited TRIM30α expression in HEK293T cells transfected with TRIM30α (**Fig. 3i**); we also noted inhibition of expression of endogenous TRIM30α with T3 in J774 macrophage cells (**Fig. 3j**). As T3 was the most effective in inhibiting ectopic and endogenous expression of TRIM30α, 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 TRIM30α by T3 resulted in higher TAB2 expression during a secondary LPS stimulation after initial pretreatment with LPS for 12 h (**Fig. 3k**); we found no substantial change in TAB2 expression during the primary stimulation (data not shown). These data collectively indicate that TAB2 is a specific target of TRIM30α *in vitro* and *in vivo*.

TRIM30α prevents TRAF6 autoubiquitination

It has been documented that TAB2 facilitates TRAF6 autoubiquitination and thus contributes to NF-κB activation[10]. Therefore, the function of TRIM30α 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 before[8,18]. Expression of wild-type TRIM30α together with TRAF6 resulted in less TRAF6 autoubiquitination, but expression of the C35A or ΔR TRIM30α mutant together with TRAF6 did not (**Fig. 4a**). Consistent with that result, 'knockdown' of TRIM30α 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 autoubiquitination[8], we examined the possibility that TRIM30α 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 before[19], we found formation of TRAF6 oligomers when it was overexpressed in HEK293T cells. We found that expression of TRIM30α did not interfere with the formation of TRAF6 oligomers (**Fig. 4c**).

Phosphorylation of IκBα is a critical event after TRAF6 activation and is needed for IκBα degradation and NF-κB activation. When we 'knocked down' TRIM30α expression in J774 cells with T3, IκBα 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 IκBα phosphorylation in cells pretreated with T3 (**Fig. 4e**). Hence, TRIM30α prevents TRAF6

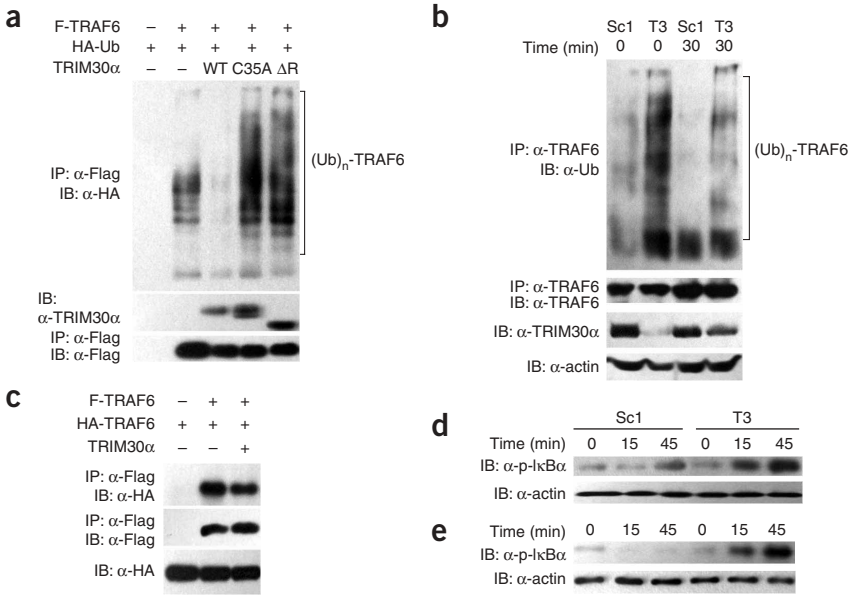

Figure 4 TRIM30α prevents TRAF6 autoubiquitination. (**a**) Immunoprecipitation of lysates of HEK293T cells expressing hemagglutinin-tagged ubiquitin (HA-Ub) and Flag-tagged TRAF6 (F-TRAF6) in the presence or absence of wild-type TRIM30α or the C35A or ΔR TRIM30α mutant, followed by immunoblot analysis of expressed proteins (below) and TRAF6 autoubiquitination (above; $(Ub)_n$-TRAF6). (**b**) Immunoprecipitation of J774 cells transfected for 24 h with T3 or control siRNA and then treated for 0 or 30 min (above lanes) with LPS, followed by immunoblot analysis as described in **a**. (**c**) Immunoprecipitation and immunoblot analysis of lysates of HEK293T cells expressing hemagglutinin- and/or Flag-tagged TRAF6 and TRIM30α. (**d**,**e**) Immunoblot analysis of phosphorylated IκBα (p-IκBα) in lysates of J774 cells transfected with T3 or control siRNA, and then treated for 0–45 min (above lanes) with LPS (**d**) or first pretreated with LPS for 12 h and then treated for 0–45 min (above lanes) with LPS (**e**). Data are representative of three experiments.

autoubiquitination, diminishes IκBα phosphorylation and is involved in regulating LPS-mediated signaling.

TRIM30α inhibits TRAF6-induced NF-κB activation

Because TRIM30α suppressed IκBα 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 activation (**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 immunoassay (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

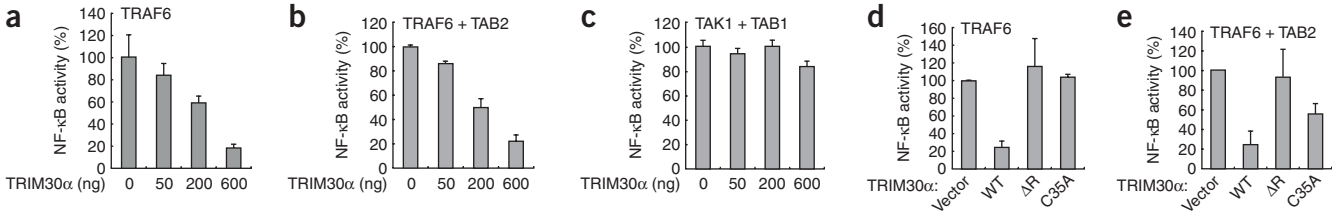

Figure 5 TRIM30α inhibits NF-κB activity. (**a**–**c**) Dual-luciferase assay of lysates of HEK293T cells transfected with an NF-κB reporter plasmid and increasing concentrations of a plasmid expressing TRIM30α, plus plasmids expressing TRAF6 (**a**); TRAF6 and TAB2 (**b**); or TAK1 and TAB1 (**c**). (**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.

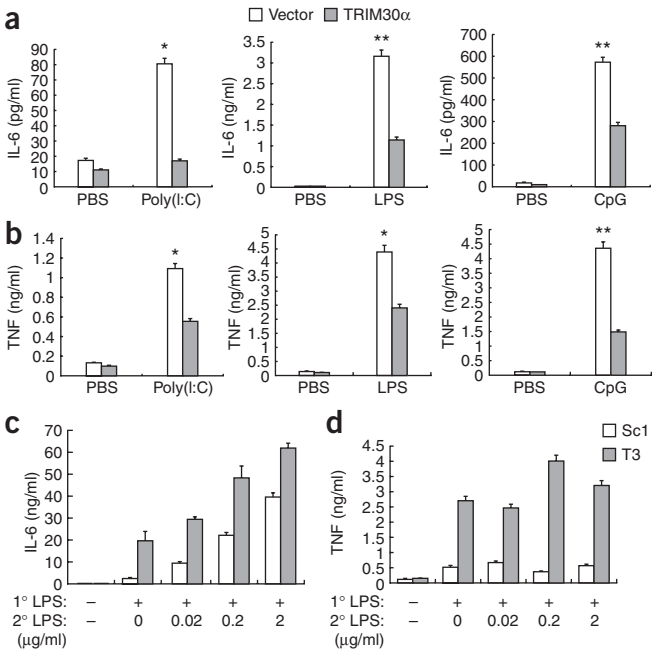

Figure 6 TRIM30α inhibits IL-6 and TNF production. (**a**,**b**) ELISA of IL-6 (**a**) or TNF (**b**) in supernatants of J774 cells transfected with TRIM30α or control vector and then stimulated with poly(I:C), LPS or CpG. (**c**,**d**) ELISA of IL-6 (**c**) or TNF (**d**) in supernatants of J774 cells 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 'silencing' of TRIM30α with T3 (**Fig. 6d**). These results further support the conclusion that TRIM30α inhibits NF-κB activation stimulated by TLR signaling.

In vivo endotoxin challenge

To assess the *in vivo* function of TRIM30α in TLR signaling, we used an endotoxin shock model. We first used polyethylenimine-mediated DNA transfection of whole mice[21]. 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 transfected by this method[21]. As anticipated, mice injected intravenously with the TRIM30α-expressing plasmid expressed more TRIM30α at 24 h after transfection (**Fig. 7a**). We then subjected the mice to endotoxin shock by intraperitoneal challenge with LPS and D-galactosamine. Mice transfected with the TRIM30α-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 TRIM30α-expressing plasmid was delayed (**Fig. 7b**). This indicated that TRIM30α was able to protect mice from endotoxin shock. To further confirm those results, we generated transgenic mice overexpressing TRIM30α. By RT-PCR and immunoblot analysis, we identified the transgenic mice by their higher expression of TRIM30α (**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 TRIM30α is an inducible protein and thus will not have an effect during primary stimulation with LPS. We then tested whether 'knockdown' of TRIM30α 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 TRIM30α by immunoblot analysis. As expected, TRIM30α 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 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 TRIM30α is involved in LPS-induced tolerance. Our data collectively indicate that TRIM30α is a negative regulator of TLR signaling.

TRIM30α expression depends on NF-κB activity

TRIM30α is induced by TLR ligands and functions as a negative regulator of NF-κB activation. We sought to determine whether TRIM30α expression is regulated in an NF-κB-dependent way.

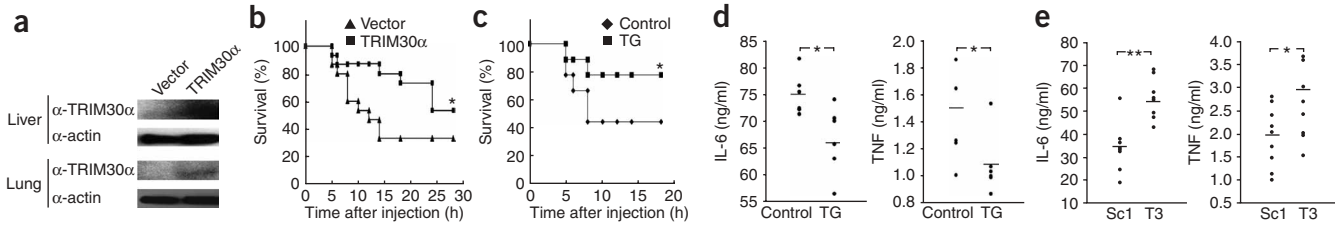

Figure 7 TRIM30α contributes to protecting mice from endotoxin shock *in vivo*. (**a**) Immunoblot analysis of TRIM30α in lysates of liver and lung tissue from mice ($n = 2$ per group) at 24 h after transfection with TRIM30α or vector plasmid. (**b**) Survival of mice ($n = 15$ per group) transfected with TRIM30α plasmid or control vector and injected intraperitoneally with LPS and D-galactosamine. (**c**) Survival of TRIM30α-transgenic mice (TG) and littermate control mice ($n = 9$ per group) injected intraperitoneally with LPS and D-galactosamine. (**d**) ELISA of TNF and IL-6 in plasma from TRIM30α-transgenic mice or control littermate mice ($n = 6$ per group) injected intraperitoneally with LPS. (**e**) ELISA of TNF and IL-6 in plasma from mice ($n = 9$ per group) transfected with T3 or control siRNA and injected intraperitoneally with LPS. *, $P < 0.05$; **, $P < 0.01$. Data are representative of at least two experiments.

Initially we found that TRIM30α induced by LPS in BMDCs was inhibited by two inhibitors of NF-κB, 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-κB. 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-κB-dependent pathway. Our data collectively demonstrate that TRIM30α is a negative regulator of the TLR-mediated NF-κB 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 established[22–24]. Studies have shown that this signaling pathway is under the precise control of many negative regulators[25]. Here we have shown that TRIM30α functions as a negative modulator of the TLR signaling pathway, by targeting TAB2 and TAB3, and contributes to the inhibition of TLR-mediated NF-κB activation. Consistent with the finding that TRIM30α expression was induced after stimulation with ligands such as LPS, we have shown that TRIM30α was critical for LPS-induced tolerance. Further studies showed that both TRIM30α-transgenic mice and mice transfected with TRIM30α were more resistant to endotoxin shock, whereas *in vivo* knockdown of TRIM30α by siRNA impaired LPS-induced tolerance, which demonstrated that TRIM30α negatively regulated LPS-mediated signaling *in vivo*.

TRIM30α belongs to the TRIM family, which has attracted attention in recent years. Some TRIM family members, such as TRIM5, TRIM25, TRIM21 (ref. 26) and TRIM32 (ref. 27), have been shown to have previously unknown functions in regulating innate immunity, which suggests that a large family of immune-modulating molecules is emerging. Indeed, our work has demonstrated the molecular and biological functions of TRIM30α; TRIM30α has been reported before as 'rpt-1'[28], a regulatory protein altered because of a deletion mutation in its mRNA sequence. Notably, an ortholog (at the sequence level) of the gene encoding TRIM30α does not seem to be present in the human genome. The reason of this is not yet clear. Given the finding that a similar pathway seems to operate in a human monocyte cell line (THP-1), we suggest that some other protein(s) in human cells would fulfill the TRIM30α function, despite the fact that such a protein may not be recognizable as a direct TRIM30α ortholog by sequence similarity.

TRIM30α has a RING domain at its amino terminus that is believed to relate to ubiquitin E3 ligase activity[17]. Some members of TRIM family have been shown to regulate protein degradation. For example, TRIM25-mediated activation of RNA helicase RIG-I is dependent on the E3 ligase activity of TRIM25 (ref. 15), whereas TRIM5 targets the human immunodeficiency virus group-associated antigen polyprotein for degradation[14]. We thus initially thought that TRIM30α might degrade TAB2 by the ubiquitin-proteaseome pathway, as the RING domain was critical for TAB2 degradation. However, further experiments did not identify any E3 ligase activity for TRIM30α, and TAB2 degradation could not be blocked by MG132 or lactacystin. This suggested that TAB2 degradation was independent of the 26S proteasome pathway. We also found that the RING domain of TRIM30α facilitated the interaction between TRIM30α and TAK1, but it is not yet known whether this interaction contributes to the degradation of TAB2. However, substitution or deletion of the TRIM30α RING domain impaired the ability of TRIM30α to negatively regulate TLR-mediated NF-κB activation, which suggests that TAK1 may be involved in TAB2 degradation. As the C35A TRIM30α mutant showed enhanced interaction with TAB2, the binding of TRIM30α itself does not seem to affect TAB2 function. Another finding to be considered is that the interaction between TRIM30α and TAB2 or TRIM30α and TAK1 was enhanced after 2 h of LPS challenge and decreased at 8 h, which indicated that the interactions were triggered by TLR signaling, yet the mechanism remains undefined. In conclusion, degradation of TAB2 and TAB3 by TRIM30α was responsible for its inhibition of NF-κB 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 NH_4Cl 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 lysosome[29]. 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 lysosome[30]. 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-κB activation triggered by TLR3, TLR4 and TLR9. Therefore, the critical function of TRIM30α in the attenuation or termination of NF-κB 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-κB activation[11] 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. Miyazaki[33]). 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 tbk1 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-Flag 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 described[34]. 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[21]. The plasmid pBUDCE4.1-TRIM30α 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 D-galactosamine and were monitored for survival for the ensuing 30 h. For the endotoxicity studies, 6- to 8-week-old female TRIM30α-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% CO_2 at 37 °C in DMEM supplemented with 10% (vol/vol) FBS, penicillin (100 U/ml) and streptomycin (100 U/ml). Lipofectamine (Invitrogen) was used for transient transfection of HEK293T cells. The procedure for generating BMDCs has been described[35]. 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. 'Silencing' RNA was transfected with GenePorter2 as described[36]. J774 cells were transfected with Nucleofector (program T-20; Amaxa). For stimulation, J774 cells were treated with LPS (1 μg/ml), poly(I:C) (25 μg/ml) or CpG (6 μg/ml). For all transfection experiments, pHA-CMV-ΔN KLC (for expression) or nonspecific siRNA was used as control.

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 1.0% (vol/vol) Nonidet P40, 20 mM Tris-HCl, pH 8.0, 10% (vol/vol) glycerol, 150 mM NaCl, 0.2 mM Na_3VO_4, 1mM 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-Agrose Immunoprecipitation reagent (Santa Cruz) together with 0.5 μg monoclonal anti-Flag (M2; F3165; Sigma-Aldrich) or 0.5 μg anti-hemagglutinin (HA.11; 16B12;CO-MMS-101R; Covance). After 4 h of incubation at 4 °C, beads were washed three times with lysis buffer. Immunoprecipitates were eluted by boiling with 1% (wt/vol) SDS sample buffer. For immunoblot analysis, immunoprecipitates or whole-cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad) and hybridized to various antibodies. After three washes with 1% (vol/vol) Tween-20 in Tris-buffered saline, membranes were further incubated for 1 h at 25 °C with horseradish peroxidase–conjugated anti–mouse immunoglobulin G (HAF 007; R&D Systems) or anti–rabbit immunoglobulin G (4050-05; Southern Biotechnology Associates). After washing, peroxidase activity was detected by chemiluminescence according to the manufacturer's instructions (Pierce).

Immunofluorescence microscopy. BMDCs or J774 cells grown on glass coverslips were stimulated for 8 h with LPS (100 ng/ml). Cells were washed with PBS, fixed for 5 min in cold methanol and made permeable in 75% ethanol. After rehydration with PBS at 25 °C, coverslips were blocked and stained with antiserum to TRIM30α (dilution, 1:1,500) or preserum as a control, followed by fluorescein isothiocyanate–conjugated anti–rabbit immunoglobulin G (49076; BD). Nuclei were stained with 4,6-diamidino-2-phenylindole and fluorescent images were captured with a Leica TCS SP2 laser confocal microscope. For localization of TAB2 and TRIM30α, HeLa cells were transfected for 24 h with cyan fluorescent protein–labeled TRIM30α and yellow fluorescent protein–labeled TAB2. Cells were then stained with antibody to LAMP-1 (Ly1C6; VAM-EN001C; Stressgen), early endosome antigen 1 or *trans*-Golgi network 381 (dilution, 1:150) or control antibody, followed by indodicarbocyanine-conjugated goat anti–mouse immunoglobulin G (115-175-146; Jackson ImmunoResearch).

Analysis with siRNA. The siRNA constructs were designed as described[20]. The forward siRNA sequences targeting TRIM30 mRNA were T1 (5′-CAGCU CUCAUUGAAGAGGUTT-3′), T2 (5′-GGAGAAUGAGGAGCUGCAGTT-3′) and T3 (5′-CUGCGGUGCUCUCAUCUAUTT-3′); siRNA was chemically synthesized by Dharmacom. The negative control siRNA was from Dharmacom (D-001210-01-20).

Reporter assays. HEK293T cells were transfected with luciferase reporter plasmids combined with pNF-κB-TA-Luc for the NF-κB reporter or pISRE-Luc (Stratagene), 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 TRIM30α 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.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A002249 and A002247.

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

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