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Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines

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Balanced production of type I interferons and proinflammatory cytokines after engagement of Toll-like receptors (TLRs), which signal through adaptors containing a Toll–interleukin 1 receptor (TIR) domain, such as MyD88 and TRIF, has been proposed to control the pathogenesis of autoimmune disease and tumor responses to inflammation. Here we show that TRAF3, a ubiquitin ligase that interacts with both MyD88 and TRIF, regulated the production of interferon and proinflammatory cytokines in different ways. Degradative ubiquitination of TRAF3 during MyD88-dependent TLR signaling was essential for the activation of mitogen-activated protein kinases (MAPKs) and production of inflammatory cytokines. In contrast, TRIF-dependent signaling triggered noncanonical TRAF3 self-ubiquitination that activated the interferon response. Inhibition of degradative ubiquitination of TRAF3 prevented the expression of all proinflammatory cytokines without affecting the interferon response.

Balanced production of type I interferons and proinflammatory cytokines, such as tumor necrosis factor (TNF), is proposed to have a key role in the pathogenesis of autoimmune diseases[1]. Furthermore, interferon production can suppress tumors, whereas TNF and other inflammatory cytokines can promote tumor growth[2,3]. Yet the mechanisms that balance the production of type I interferon and proinflammatory cytokines are poorly understood. The main receptors able to induce both cytokine classes are Toll-like receptors (TLRs), which respond to ligands of microbial, fungal, viral and mammalian origin[4–6]. Despite the deployment of common signaling pathways, such as mitogen-activated protein kinase (MAPK) cascades and transcription factor NF-κB signaling dependent on the kinase IKK, different TLRs elicit distinct biological responses, with some being more potent inducers of proinflammatory cytokines and others mainly inducing interferons and interferon-related genes. The biochemical basis for the response specificity is poorly understood, although it has been attributed to differences in the deployment of adaptor proteins[7] and selective activation of interferon-regulatory factors (IRFs), such as IRF3, by TLRs that trigger the interferon response[8].

TLRs recruit four Toll–interleukin 1 (IL-1) receptor (TIR) domain–containing adaptors, including MyD88 (A003535), TRIF (A004068), TRAM and TIRAP, to their cytoplasmic TIR domains[9–15]. These adaptors control distinct responses classified as either MyD88 dependent or TRIF dependent[4,16]. Whereas the MyD88-dependent response mediates induction of proinflammatory cytokines, the TRIF-dependent response is critical for the induction of interferons and interferon-related genes[10,11]. How the two responses are activated differently is unknown, but published studies have highlighted a critical role for the signaling protein TRAF3 (A002309) in the induction of interferon-related genes and inhibition of inflammatory cytokines[17,18]. However, TRAF3, which is necessary for IRF3 activation, interacts with both MyD88 and TRIF. Although TRAF3 positively regulates IRF3 and the type I interferon response[18], it negatively regulates MAPK signaling by CD40 ligand and BAFF, members of the TNF family[19]. In contrast, the related protein TRAF6 positively controls MAPK signaling by TNF receptors and TLRs[20]. What makes TRAF3 function negatively in one response and positively in another is unknown. It is also unclear why MyD88, which interacts with TRAF3, does not lead to IRF3 activation after TLR4 engagement.

Using TLR4 as a prototypical TLR that elicits both MyD88- and TRIF-dependent responses, we now show that differences in the ubiquitination of TRAF3 are the key to the selective production of type I interferons versus proinflammatory cytokines. TRIF-mediated signaling triggered TRAF3 self-ubiquitination through noncanonical polyubiquitination linked to the lysine at position 63 of the ubiquitin molecule (K63-linked), which was essential for activation of IRF3 and the interferon response. In contrast, MyD88-dependent signaling through TRAF6 and the ubiquitin ligases cIAP1 and cIAP2 (called 'cIAP1/2' here) resulted in degradative ubiquitination of TRAF3, which was required for MAPK activation and induction of proinflammatory cytokines and chemokines. Elimination of cIAP1 and cIAP2 resulted in highly specific inhibition of proinflammatory genes without any effect on the anti-inflammatory and tumor-suppressive interferon response.

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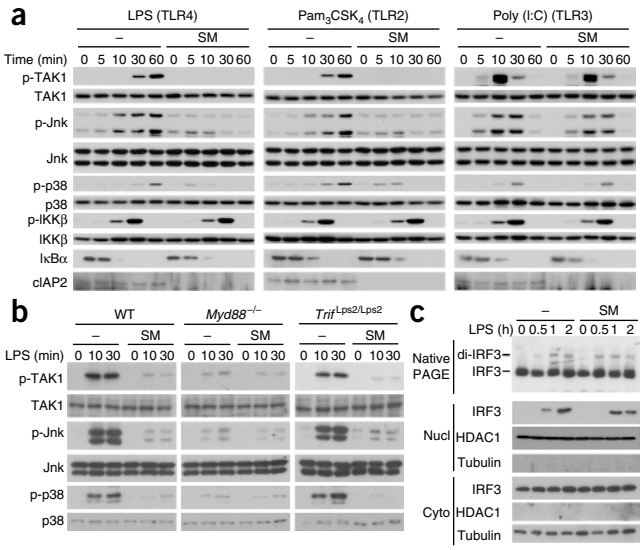

Figure 1 Role of cIAP1/2 in TLR-mediated MAPK signaling. (**a**) Immunoblot analysis of phosphorylation (p-) of the kinases TAK1, Jnk, p38 and IKKβ, as well as total IκBα (signaling activation) and cIAP2 (confirmation of SM effect), in lysates of BMDMs stimulated for various times (above lanes) with the TLR agonists LPS (100 ng/ml), Pam_3CSK_4 (1 μg/ml) or poly(I:C) (30 μg/ml) with (SM) or without (–) 4 h of pretreatment with the cIAP1/2 antagonist SM (0.1 μM). (**b**) Immunoblot analysis of protein phosphorylation in lysates of wild-type (WT), *Myd88*$^{-/-}$ and *Trif* $^{Lps2/Lps2}$ BMDMs stimulated with LPS with or without pretreatment with SM. (**c**) Immunoblot analysis of the dimerization of IRF3 (di-IRF3) in BMDMs stimulated with LPS with or without pretreatment with SM, separated by native PAGE and probed with anti-IRF3 (top). Middle and bottom, immunoblot analysis of cytosolic (Cyto) and nuclear (Nucl) extracts of the BMDMs described above. HDAC1 and tubulin serve as markers for nuclear and cytosolic fractions, respectively. Data are representative of two to four independent experiments.

RESULTS

MAPK signaling dependent on cIAP1/2 and MyD88

The ubiquitin ligases cIAP1 and cIAP2 are redundant E3 ubiquitin ligases that direct degradative (K48-linked) ubiquitination of TRAF3 and are critical for two-stage MAPK signaling induced by the costimulatory molecule CD40, in which assembly of the receptor-associated signaling complex is followed by translocation of the multiprotein complex to the cytosol, the site at which MAPK cascades are activated[19]. Using a small-molecule mimetic of Smac (the antagonist of inhibitor of apoptosis protein), which triggers rapid cIAP1/2 degradation[21,22], we found that cIAP1 and cIAP2 were also involved in TLR signaling. In bone marrow–derived macrophages (BMDMs) and the mouse macrophage line RAW264.7, pretreatment with the Smac mimetic (SM) inhibited activation of the MAPK kinase kinase TAK1, but not of IKK, by ligation of TLR4 and TLR2 (**Fig. 1a** and **Supplementary Fig. 1**). SM had no effect on TAK1-MAPK activation by TLR3, which signals exclusively through TRIF. Congruently, TRIF-defective *Trif*$^{Lps2/Lps2}$ BMDMs showed relatively intact lipopoly-saccharide (LPS)-induced TAK1-MAPK activation through TLR4 that remained sensitive to treatment with SM, but residual TAK1-MAPK activation in *Myd88*$^{-/-}$ BMDMs was barely affected by SM (**Fig. 1b**). Neither cIAP1 nor cIAP2 was involved in TRIF-mediated signaling necessary for interferon expression, as pretreatment with SM did not prevent dimerization or nuclear translocation of IRF3 (**Fig. 1c**). The effects of SM were specific, as RAW264.7 cells in which cIAP1/2 expression was silenced by short hairpin RNA (shRNA) specific for cIAP1/2, cIAP1/2-deficient multiple myeloma cells or RAW264.7 cells incubated with a proteasome inhibitor also showed defective LPS-induced activation of TAK1-MAPK (**Supplementary Fig. 2a–c**). Furthermore, silencing of TRAF3 rendered RAW264.7 macrophages resistant to treatment with SM (**Supplementary Fig. 2d**). Hence, the E3 ligases cIAP1 and cIAP2, which trigger K48-specific degradative ubiquitination of TRAF3[19,23], are important for MyD88-dependent activation of MAPK but are dispensable for TRIF-dependent induction of interferons.

TRAF3 and cytosolic translocation of MyD88 signaling complexes

To study the formation of TLR4-associated signaling complexes, we separated BMDMs into membrane fractions (which contain plasma and endosomal membranes) and cytosolic fractions after LPS stimulation and analyzed these by immunochemistry. LPS induced rapid but transient recruitment of MyD88, TRAF6, TRAF3, IKKγ (also known as NEMO), cIAP1/2, the ubiquitin-conjugating enzyme Ubc13 and TAK1 to TLR4 and more persistent TRIF recruitment, which lasted at least 30 min (**Fig. 2a**). TLR4 was not detected in the cytosolic fraction, but immunoprecipitation with antibody to TAK1 (anti-TAK1) demonstrated the LPS-induced formation of a large cytosolic complex that persisted for at least 30 min after receptor stimulation and contained MyD88, TRAF6, IKKγ, cIAP1/2, Ubc13, the MAPK kinase MKK4 (which was not part of the receptor-associated complex) and TAK1, but not TRAF3 or TRIF (**Fig. 2a**). Pretreatment with SM stabilized the receptor-associated complex and prevented cytosolic translocation of the TAK1-associated complex (**Fig. 2a**). These results suggest that after assembly on the cytoplasmic face of TLR4, the MyD88-nucleated signaling complex, containing TRAF6, IKKγ, cIAP1/2, Ubc13 and TAK1, translocates to the cytosol, leaving behind TLR4 and TRAF3, and incorporates MKK4. Translocation of the complex required cIAP1/2 and was

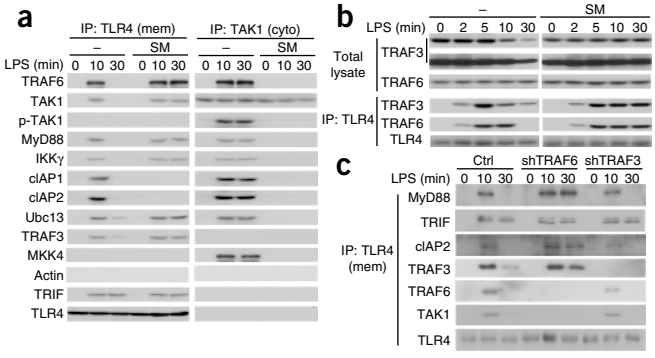

Figure 2 TLR4 engagement induces an MyD88-associated signaling complex that undergoes cIAP1/2- and TRAF6-dependent cytosolic translocation after TRAF3 degradation. (**a**) Immunoprecipitation (IP), with anti-TLR4 and anti-TAK1, of immunocomplexes from membrane (mem) and cytosolic (cyto) fractions of BMDMs stimulated with LPS with or without pretreatment with SM, followed by immunoblot analysis with antibodies to the molecules along the left margin. (**b**) Immunoprecipitation (with anti-TLR4) of immunocomplexes from lysates of RAW264.7 cells stimulated with LPS with or without pretreatment with SM, followed by immunoblot analysis of TLR4 immunocomplexes and total lysates with anti-TRAF3, anti-TRAF6 and anti-TLR4. (**c**) Immunoprecipitation of TLR4-associated proteins from membrane fractions of RAW264.7 cells transduced with lentivirus containing no insert (control; Ctrl) or shRNA specific for TRAF3 (shTRAF3) or TRAF6 (shTRAF6) and then stimulated with LPS, followed by immunoblot analysis. Data are representative of two to three independent experiments.

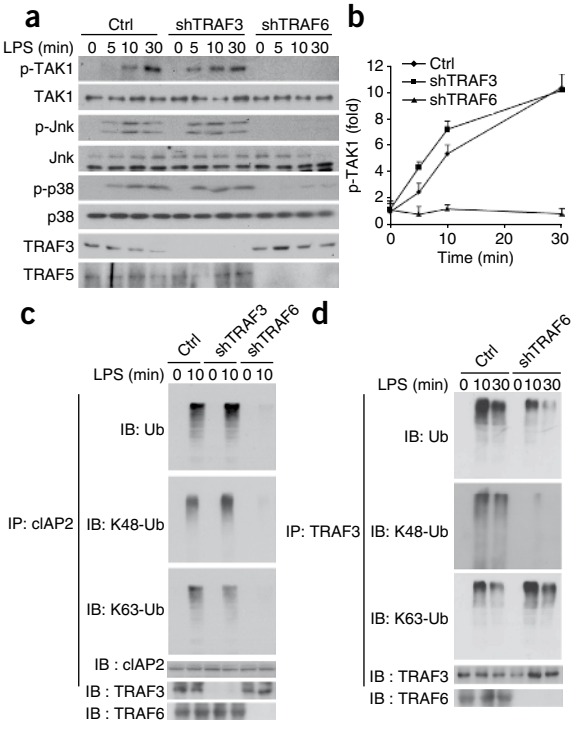

Figure 3 TRAF6 is required for LPS-induced activation of TAK1 and ubiquitination of cIAP2 and TRAF3. (**a**) Immunoblot analysis of the phosphorylation of TAK1 and the MAPKs Jnk and p38, as well as total TRAF3 and TRAF6, in LPS-stimulated control RAW264.7 cells (Ctrl) and RAW264.7 cells in which TRAF3 (shTRAF3) or TRAF6 (shTRAF6) was silenced. (**b**) TAK1 phosphorylation kinetics in LPS-stimulated control RAW264.7 cells and RAW264.7 cells in which TRAF3 or TRAF6 was silenced, assessed by densitometric analysis of experiments similar to that in **a**; results are presented relative to phosphorylation intensity at time 0. (**c**) Immunoprecipitation of cIAP2 from LPS-stimulated control RAW264.7 cells and RAW264.7 cells in which TRAF3 (shTRAF3) or TRAF6 (shTRAF6) was knocked down, followed by extensive washing and immunoblot analysis with anti-ubiquitin, anti–K48-linked ubiquitin (K48-Ub), anti–K63-linked ubiquitin (K63-Ub) or anti-cIAP2. Bottom, immunoblot analysis of TRAF3 and TRAF6 in lysates without immunoprecipitation. (**d**) Immunoprecipitation of TRAF3 from LPS-stimulated control RAW264.7 cells and RAW264.7 cells after TRAF6 knockdown, followed by immunoblot analysis as described in **c**. Data are representative of two to three independent experiments (error bars (**b**), s.d.).

therefore inhibited by pretreatment with SM, which also blocked the recruitment of MKK4 and phosphorylation of TAK1-MAPK, which occurred in the cytosol and not at the receptor (**Fig. 2a**).

Cytosolic translocation of the CD40-assembled signaling complex requires the E3 ubiquitin ligase activity of cIAP1/2 and correlates with TRAF3 degradation[19]. We examined the fate of TRAF3, which was present in the TLR4-anchored complex but was not part of the cytosolic TAK1-associated complex. Total TRAF3 protein abundance rapidly, but incompletely, decreased within 10 min of LPS stimulation, whereas the abundance of total TRAF6 remained constant (**Fig. 2b**). TRAF3 degradation was inhibited by pretreatment with SM. Similarly, TLR4-associated TRAF3 rapidly decreased at 10 min after stimulation, and this degradation was inhibited by SM (**Fig. 2b**). TLR4-associated TRAF6, however, was unchanged between 5 and 10 min after LPS addition, but after 10 min it was undetectable except in cells pretreated with SM (**Fig. 2b**). Notably, small amounts of TRAF3 remained associated with TLR4 even at 30 min after stimulation (**Fig. 2b**). This residual TRAF3 was probably engaged in MyD88-independent signaling. Silencing of TRAF6 in RAW264.7 macrophages prevented the recruitment of TAK1 to TLR4 but had no effect on the recruitment of MyD88, TRIF or cIAP2, whereas silencing of TRAF3 did not affect the recruitment of any of these proteins (**Fig. 2c**). Notably, silencing of TRAF6 slowed down disassociation of the MyD88-assembled complex from the receptor.

Unlike TRAF2, however, TRAF6 does not interact directly with cIAP1/2 (ref. 24 and data not shown). As recruitment of cIAP2 to TLR4 was dependent on MyD88 but not TRIF (**Supplementary Fig. 3a**), we examined whether MyD88 and TRIF can interact with cIAP1/2. Consistent with the genetic analysis, precipitation experiments with fusion proteins of glutathione *S*-transferase and MyD88 or TRIF showed an interaction between cIAP2 and MyD88 but not between cIAP2 and TRIF (**Supplementary Fig. 3b**). However, it remains to be determined whether this protein interaction is direct.

It has been proposed that the recruitment of MyD88 and TRIF to the TIR domain of TLR4 is sequential and mutually exclusive[25,26]. Consistent with the MyD88 dependence of the recruitment of cIAP2 to TLR4, immunoprecipitation of the membrane fraction with anti-cIAP2 resulted in the isolation of TLR4 and MyD88 but not of TRIF (**Supplementary Fig. 4a**). Furthermore, inhibition of TLR4 endocytosis with the dynamin inhibitor dynasore[27] had no effect on the recruitment of MyD88 or cIAP2 to the receptor, but blocked TRIF recruitment (**Supplementary Fig. 4b**). We conclude that TRIF and MyD88 are recruited to separate pools of receptors. Because SM inhibited the dissociation of MyD88 from the receptor without affecting TRIF recruitment, whereas dynasore inhibited TRIF recruitment without affecting MyD88 recruitment, it seems that each adaptor is recruited independently to TLR4.

TRAF6 is required for ubiquitination of cIAP2 and TRAF3

We examined the effect of silencing TRAF3 and TRAF6 on TLR4-induced signaling responses. LPS-induced TAK1-MAPK activation were barely detected in TRAF6-deficient cells, whereas depletion of TRAF3 accelerated their activation (**Fig. 3a**,**b**). However TRAF3 was required for IRF3 activation, but TRAF6 was not (**Supplementary Fig. 5**). LPS triggered polyubiquitination of cIAP2 and TRAF3 (**Fig. 3c**,**d**). Total, K48-linked and K63-linked ubiquitination of cIAP2 were TRAF6 dependent but TRAF3 independent (**Fig. 3c**). Depletion of TRAF6 diminished the total and K48-linked, but not the K63-linked, polyubiquitination of TRAF3 (**Fig. 3d**). Congruently, ablation of TRAF6 inhibited LPS-induced degradation of TRAF3 (**Fig. 3a**). Likewise, treatment with SM inhibited total, but not K63-linked, ubiquitination of TRAF3 (**Fig. 4a**), which suggests that cIAP1 and cIAP2 are responsible for K48-linked ubiquitination of TRAF3, as observed during CD40 signaling[19]. Akin to TRAF2 during CD40 signaling[23], TRAF6 may mediate TLR4-induced activation of cIAP1 and cIAP2 through their K63-linked ubiquitination and is therefore needed for TRAF3 degradation. As TRAF6 is a K63-specific E3 ligase, the K48-linked ubiquitination of cIAP2 that shows TRAF6 dependence is most probably due to self-ubiquitination by cIAP2 or cIAP1.

TLR3 also triggered K63-linked ubiquitination of TRAF3 (**Supplementary Fig. 6a**). Notably, the ratio of K63-linked to total ubiquitination of TRAF3 was higher for TLR3, which signals exclusively through TRIF. Indeed, TLR4-induced K63-linked ubiquitination of TRAF3 was TRIF dependent and MyD88 independent (**Supplementary Fig. 6b**). In contrast, SM-sensitive ubiquitination of TRAF3 was MyD88 dependent, consistent with its reliance on cIAP1 and cIAP2, which are recruited to TLR4 through MyD88.

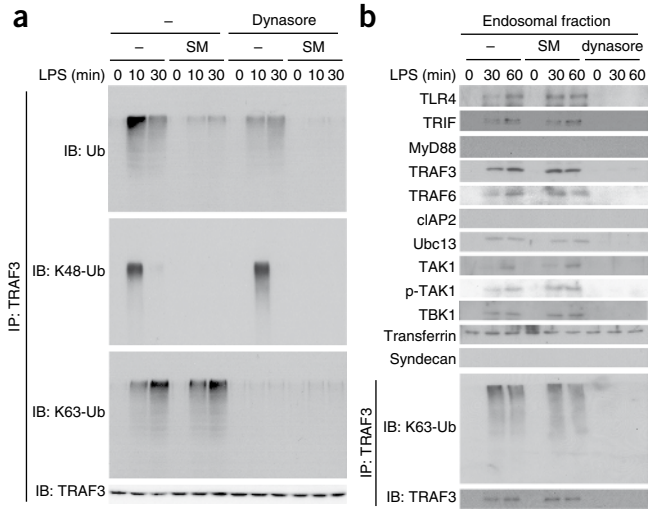

Figure 4 LPS-induced K63-linked TRAF3 self-ubiquitination depends on TLR endocytosis. (**a**) Immunoblot analysis of the ubiquitination of TRAF3 (as described in **Fig. 3c**) immunoprecipitated from RAW264.7 cells stimulated with LPS, with or without pretreatment with SM, in the presence or absence of dynasore (80 μM). (**b**) Immunoblot analysis of RAW264.7 cells stimulated with LPS, with or without pretreatment with SM, in the presence or absence of dynasore, then lysed and fractionated on a discontinuous sucrose gradient for isolation of the endosomal fraction, followed by solubilization and then immunoblot analysis of endosome-associated proteins (top). Bottom two blots, immunoblot analysis as described above of the ubiquitination of TRAF3 immunoprecipitated from the endosomal fraction. Data are representative of two to three independent experiments.

TRAF3 K63-linked ubiquitination depends on endocytosis

After activation, TLR4 undergoes dynamin-dependent endocytosis, which is required for TRIF-dependent interferon signaling but not for MyD88-mediated signaling[25]. As TRAF3 is a positive effector of the TRIF-dependent interferon response, we examined whether its noncanonical K63-linked ubiquitination was linked to TLR4 endocytosis. Inhibition of TLR4 endocytosis with dynasore modestly diminished total LPS-induced ubiquitination of TRAF3 but strongly inhibited K63-linked ubiquitination of TRAF3 (**Fig. 4a**). Pretreatment with SM diminished total ubiquitination of TRAF3 but had no effect on its K63-linked ubiquitination, whereas treatment with both SM and dynasore abolished ubiquitination of TRAF3 altogether (**Fig. 4a**). Treatment with dynasore alone did not block activation of MAPKs or IKK (**Supplementary Fig. 7**).

We isolated the endosomal compartment (**Supplementary Fig. 8**) at various points after TLR4 activation and analyzed its composition. LPS induced the association of TLR4, TRIF, TRAF6, TRAF3, Ubc13, TBK1 and TAK1, but not of MyD88 or cIAP2, with endosomes (**Fig. 4b**). Endosomal TRAF3 was K63 polyubiquitinated and did not undergo LPS-induced degradation. Treatment with dynasore prevented LPS-induced endocytosis of TLR4 and its associated proteins, but treatment with SM did not.

TRIF-dependent K63-linked ubiquitination of TRAF3 is associated with IRF3 activation and is akin to K63-linked ubiquitination of TRAF6, thought to be due to RING finger–mediated self-ubiquitination[28]. To determine whether K63-linked ubiquitination of TRAF3 is also RING dependent, we introduced C68A and H70A substitutions, analogous to TRAF6-inactivating substitutions[28], into the TRAF3 RING finger. We silenced TRAF3 in cells and reconstituted the cells with either wild-type TRAF3 or the RING-finger mutant of TRAF3. Both TRAF3 forms underwent LPS-induced polyubiquitination, but K63-linked polyubiquitination of the RING-finger mutant of TRAF3 was much less that of wild-type TRAF3 (**Fig. 5a**). Congruently, in cells in which TRAF3 was

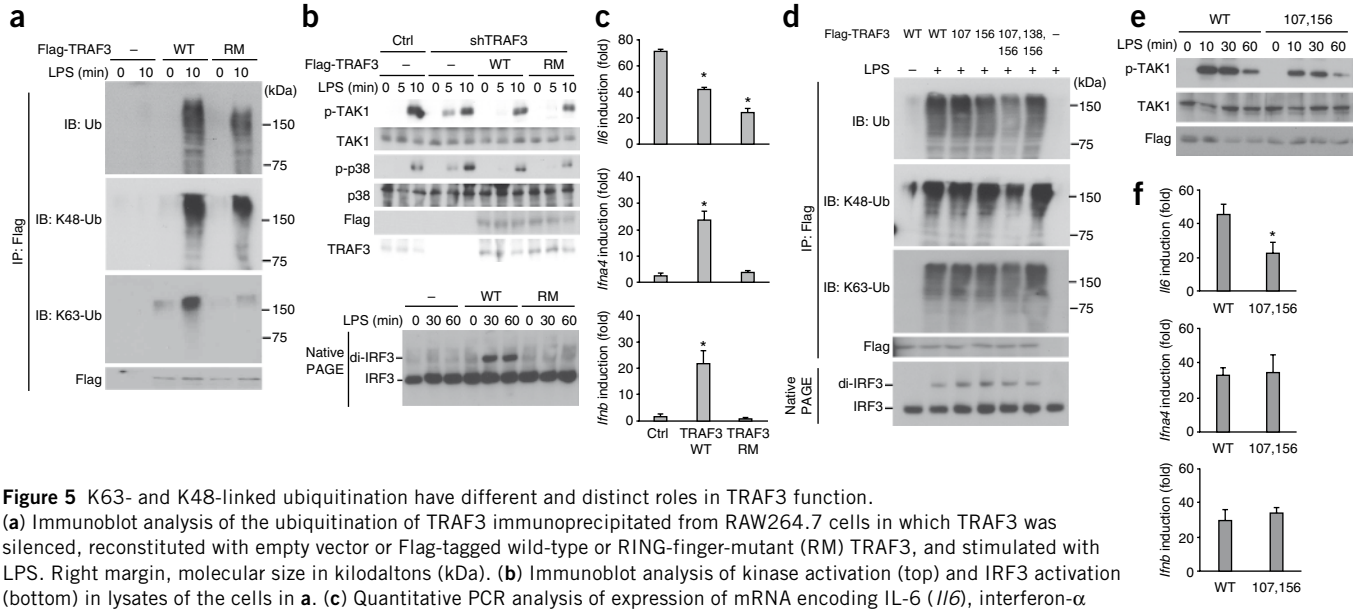

Figure 5 K63- and K48-linked ubiquitination have different and distinct roles in TRAF3 function. (**a**) Immunoblot analysis of the ubiquitination of TRAF3 immunoprecipitated from RAW264.7 cells in which TRAF3 was silenced, reconstituted with empty vector or Flag-tagged wild-type or RING-finger-mutant (RM) TRAF3, and stimulated with LPS. Right margin, molecular size in kilodaltons (kDa). (**b**) Immunoblot analysis of kinase activation (top) and IRF3 activation (bottom) in lysates of the cells in **a**. (**c**) Quantitative PCR analysis of expression of mRNA encoding IL-6 (*Il6*), interferon-α (*Ifna4*) and interferon-β (*Ifnb*) among RNA extracted from the cells described in **a**, presented relative to the expression of cyclophilin mRNA. $*P < 0.05$ (Student's *t*-test). (**d**) Immunoblot analysis of the ubiquitination of TRAF3 and activation of IRF3 in RAW264.7 cells in which TRAF3 was silenced; cells were reconstituted with empty vector (–), Flag-tagged wild-type TRAF3, or TRAF3 with the substitution(s) K107R (107), K156R (156), K107R and K156R (107, 156), or K138R and K156R (138,156), and analyzed as described in **a** before (–) and after (+) LPS stimulation. (**e**) TAK1 activation and TRAF3 expression in cells in which TRAF3 was silenced; cells were reconstituted with Flag-tagged wild-type TRAF3 or TRAF3 with the substitutions K107R and K156R, and were stimulated with LPS. (**f**) Quantitative PCR analysis of expression of mRNA encoding IL-6, interferon-α and interferon-β among RNA extracted from the cells described in **e**, presented relative to the expression of cyclophilin mRNA. $*P < 0.05$ (Student's *t*-test). Data are representative of two or more independent experiments (**a**,**b**,**d**,**e**) or two independent experiments (average and s.d. of triplicates; **c**,**f**).

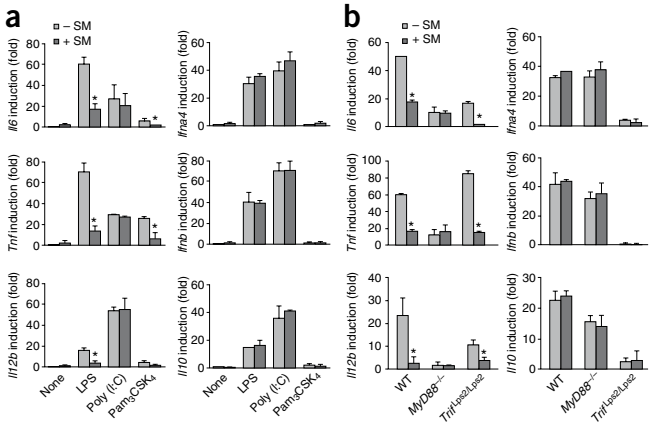

Figure 6 Differences in the regulation of TLR4-induced inflammatory cytokines and interferon-related genes. (**a**) Quantitative PCR analysis of mRNA expression in BMDMs stimulated for 2 h (*Il6*, *Tnf* and *Il12b*) or for 6 h (*Ifna4*, *Ifnb* and *Il10*) with various TLR agonists (horizontal axis) with (+SM) or without (–SM) pretreatment with SM. (**b**) Quantitative PCR analysis of mRNA expression in *MyD88*$^{-/-}$ and *Trif*$^{Lps2/Lps2}$ BMDMs stimulated with LPS for 2 h or 6 h (as described in **a**) with or without pretreatment with SM. $*P < 0.05$ (Student's *t*-test). Data are representative of two independent experiments (average and s.d. of triplicates).

silenced, reconstitution with either wild-type or RING-finger-mutant TRAF3 delayed activation of TAK1-MAPK, but only wild-type TRAF3 supported activation of IRF3 (**Fig. 5b**). Furthermore, both TRAF3 isoforms resulted in less IL-6 induction, but only wild-type TRAF3 supported the induction of type I interferon (**Fig. 5c**).

We systematically substituted lysine residues with arginine residues throughout TRAF3 to identify acceptors for K48-linked polyubiquitin chains. We combined single mutants with less ubiquitination to generate double mutants, among which TRAF3 with K107R and K156R substitutions showed the greatest, but still incomplete, decrease in LPS-induced K48-linked ubiquitination with little if any effect on K63-linked ubiquitination (**Fig. 5d**). When expressed in macrophages in which TRAF3 was silenced, TRAF3 with K107R and K156R substitutions supported LPS-induced activation of IRF3 and induction of interferon mRNA, but it led to less activation of TAK1 and lower induction of IL-6 mRNA than did wild-type TRAF3 (**Fig. 5d–f**).

SM differentially affects TLR-mediated gene induction

To determine the role of the two different modes of ubiquitination of TRAF3 in TLR signaling, we downregulated cIAP1 and cIAP2, which are responsible for degradative ubiquitination of TRAF3 (refs. 23,29,30) by treating BMDMs with SM. This treatment inhibited the induction of genes encoding inflammatory cytokines and chemokines, including *Tnf*, *Il6*, *Il12b*, *Il12a*, *Cxcl2* and *Cxcl1*, by LPS (TLR4 ligand) and Pam_3CSK_4 (TLR2 ligand) but had no effect on their induction by poly(I:C) (TLR3 ligand; **Fig. 6a** and **Supplementary Fig. 9a**). SM, however, had no effect on induction of the genes encoding interferon-α and interferon-β or interferon-related genes, including *Il10*, *Cxcl10*, *Ccl5* and *Ccl2*, in response to any TLR agonist (**Fig. 6a** and **Supplementary Fig. 9a**). We noted similar effects on cytokine gene induction in cIAP1/2-deficient RAW264.7 cells (**Supplementary Fig. 9b**) and multiple myeloma cells (**Supplementary Fig. 9c**). The cIAP1/2-dependent induction of inflammatory cytokines by TLR4 was unique to the MyD88-dependent response, as pretreatment with SM inhibited LPS-induced inflammatory cytokines and chemokines in *Trif*$^{Lps2/Lps2}$ BMDMs, which were impaired in the induction of interferon-related genes (**Fig. 6b** and **Supplementary Fig. 9b**). In contrast, induction of interferon-related genes and residual inflammatory cytokine gene expression in LPS-stimulated *Myd88*$^{-/-}$ BMDMs were not affected by pretreatment with SM. Hence, the two responses, one entailing induction of inflammatory cytokines and the other encompassing type I interferon and interferon-related genes, are separately regulated and show differences in their sensitivity to SM.

DISCUSSION

TLRs detect microbes, viruses and endogenous ligands to mediate the induction of genes encoding inflammatory cytokines, chemokines, interferons and interferon-related molecules[16]. In general, TLRs that recognize bacteria induce proinflammatory cytokines, chemokines and antimicrobial peptides, whereas those that detect viruses trigger the interferon response[31]. How these two responses, which depend on engagement of MyD88 and TRIF, are balanced to control autoimmunity[1] and pro-tumorigenic versus anti-tumorigenic inflammation[2] has remained unknown until now[32]. TRAF3 is uniquely required for the TRIF-dependent interferon response[17,18], but it is also a negative regulator of MAPK activation[19]. We therefore explored the basis for the different activities of TRAF3 and examined whether TRAF3 is involved in determining the balance between inflammatory cytokines and type I interferons. We found that although TRAF3 was incorporated into both MyD88- and TRIF-assembled multiprotein complexes, its signaling function was regulated in different ways by alternative ubiquitination modes. In the MyD88-assembled signaling complex, TRAF3 underwent degradative K48-linked ubiquitination dependent on TRAF6 and on cIAP1 and cIAP2, the latter being direct K48-specific TRAF3 ubiquitin ligases[19,23]. Notably, cIAP1 and cIAP2 were present only in the MyD88-assembled signaling complex but not in the TRIF-assembled signaling complex. Degradative ubiquitination of TRAF3 in the MyD88 complex precluded IRF3 activation and instead promoted cytosolic translocation of the entire signaling complex. This allowed MAPK activation and induction of inflammatory genes. In contrast, the association of TRAF3 with the cIAP1/2-devoid, endosomal TRIF signaling complex resulted in its K63-linked self-polyubiquitination, a modification that was required for IRF3 activation and induction of the interferon response. It should be noted that MyD88 and TRIF are not part of the same signaling complex and differences in their signaling potentials correlate with their ability to selectively engage cIAP1/2 and thereby dictate the nature of TRAF3 ubiquitination. Despite the absence of cIAP1/2, the TRIF-assembled signaling complex can also activate TAK1 to some extent and this may have accounted for the weak induction of inflammatory cytokines that was SM resistant, seen in MyD88-deficient cells.

The TRAF3 relatives TRAF2 and TRAF6 are E3 ubiquitin ligases that selectively catalyze K63-linked polyubiquitination of themselves[33] and other proteins, such as cIAP2 (ref. 23). Their activity depends on Ubc13, a K63-specific ubiquitin-conjugating enzyme[33] that is essential for TNF receptor- and TLR-induced activation of MAPK[34]. We have now demonstrated that as with its relatives, the TRAF3 RING finger was required for its K63-linked ubiquitination in the TRIF signaling complex, but unlike TRAF2 or TRAF6, K63-linked ubiquitination of TRAF3 was not totally dependent on Ubc13 (unpublished results), which thus explains the activation of the interferon response in Ubc13-deficient cells[34]. Notably, during MyD88 or CD40 signaling[19], TRAF3 did not undergo K63-linked self-ubiquitination and instead acted as an inhibitor of MAPK activation and inflammatory cytokine induction. This inhibitory activity did not require the RING finger of TRAF3 and was eliminated after its proteasomal degradation, which was promoted by its 'decoration' with canonical K48-linked polyubiquitin chains. The extent of K63-linked ubiquitination of TRAF3 correlated with interferon induction, being

the highest for TLR3-stimulated macrophages. In the case of TLR4, K63-linked ubiquitination of TRAF3, just like IRF3 activation, depended on receptor endocytosis and TRIF rather than MyD88.

Our results have demonstrated that MyD88-dependent MAPK signaling proceeds through a two stage mechanism, similar to that described before for CD40 and other TNF receptors[19]. This mechanism involves receptor-induced assembly of a multiprotein complex containing MyD88, TRAF6, Ubc13, IKKγ, cIAP1/2, TAK1 and TRAF3. Complex assembly resulted in TRAF6 activation, which led to K63-linked ubiquitination of cIAP1 and cIAP2 and enhancement of their activity as TRAF3 K48-specific E3 ligases[19,23]. Degradation of TRAF3 allowed translocation of the MyD88-associated signaling complex to the cytosol, where TAK1 and its subordinate MAPKs are activated. Interference with this process by SM-induced elimination of cIAP1/2 selectively inhibited the induction of inflammatory cytokines and chemokines without any deleterious effect on the interferon response, which includes induction of the anti-inflammatory cytokine IL-10. Notably, activation of IKK by TLR4, which also depends on TAK1 (ref. 35 and data not shown), was not affected by SM-induced inhibition of TAK1 phosphorylation. This suggests that unlike MAPK signaling, IKK activation depends on TAK1 but not on its protein kinase activity, an important concept that merits further investigation. Although it did not prevent NF-κB activation, interference with two-stage TLR signaling through SM-induced elimination of cIAP1/2 was sufficient for selective inhibition of the production of inflammatory cytokines and chemokines but had no deleterious effect on the interferon response. We therefore propose that SM and similar cIAP1/2 antagonists may serve as superior anti-inflammatory drugs that will not compromise antiviral immunity. This may be of importance in inflammatory diseases that respond to type I interferons[1], as well as cancer whose growth is stimulated by proinflammatory cytokines, such as TNF, but is inhibited by type I interferons[2]. Furthermore, selective inhibition of TNF and other proinflammatory cytokines without a concomitant decrease in interferon production may be useful in the treatment of autoimmune disease caused by increased TNF and decreased type I interferons[1].

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A003535, A004068 and A002309.

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

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AUTHOR CONTRIBUTIONS

P.-H.T. and M.K. planned and designed all experiments and wrote the manuscript; P.-H.T. and A.M. did most experiments; W.Z. and T.M. helped with cell cultures, TRAF3 mutants and immunoprecipitation experiments; and D.A.A.V. provided the HWA4C4 K63-specific antibody to ubiquitin.

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ONLINE METHODS

Mice and cells. *Myd88*$^{-/-}$ mice and *Trif*$^{Lps2/Lps2}$ mice[11,36] were from S. Akira and B. Beutler, respectively. Control C57BL/6 mice were from the Jackson Laboratory. All mice were housed in a specific pathogen–free facility according to guidelines of the University of California San Diego and National Institutes of Health, and mouse protocols were approved by the Institutional Animal Care Committee of the University of California San Diego. Bone marrow was collected from femurs and tibia of mice (8–10 weeks of age) and was used to prepare BMDMs[17] that were cultured in DMEM supplemented with macrophage colony-stimulating factor (10 ng/ml) in addition to 10% (vol/vol) FBS. KMS-28BM (wild-type) multiple myeloma cells and KMS-28PE multiple myeloma cells (doubly deficient cIAP1 and cIAP2) were a gift from R. Fonseca[37]. RAW264.7 cells were cultured as described[38].

Subcellular fractionation. Subcellular fractions were prepared as described[39,40]. Cells were resuspended for 20 min on ice in a buffer containing 250 mM sucrose, 20 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride and 20 μg/ml of aprotinin and were disrupted with a Dounce homogenizer (15 strokes). After removal of nuclei by centrifugation at 1,000*g* for 10 min at 4 °C, supernatants were centrifuged at 10,000*g* for 1 h at 4 °C and the cytosolic fraction was collected. Pellets containing cellular membranes were resuspended in 10 mM Tris, pH 7.4, 150 mM NaCl, and 0.2% (vol/vol) Nonidet P-40. The nuclear fraction was made soluble in a nuclear lysis buffer containing 1% (vol/vol) Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 20 μg/ml of aprotinin and was centrifuged at 15,000*g* for 30 min at 4 °C, and the supernatant (nuclear extract) was collected. The endosomal fraction was isolated as described[41]. Cell pellets were resuspended in five volumes of a hypo-osmotic buffer (15 mM KCl, 1.5 mM magnesium acetate, 1 mM dithiothreitol and 10 mM HEPES, pH 7.5) and were homogenized with a Dounce homogenizer (20 strokes). Then, 0.1 volume of hyperosmotic buffer (700 mM KCl, 40 mM magnesium acetate, 1 mM dithiothreitol and 10 mM HEPES, pH 7.5) was added and the mixture was centrifuged for 5 min at 800*g*. The supernatant was collected and was treated for 3 min at 37 °C with 1 μg/ml of trypsin. Proteolysis was stopped with soybean trypsin inhibitor (1.5 μg/ml) and the mixture was centrifuged for 20 min at 145,000*g*. The membrane pellet was resuspended in 1 ml homogenization buffer (0.25 M sucrose, 1 mM EDTA and 10 mM Tris, pH 8.0) and was centrifuged for 2 h at 100,000*g* through a discontinuous sucrose gradient[42]. Fractions (1 ml each) were collected from the bottom of the tube. Subcellular fractions were analyzed by immunoblot with antibodies (described below) to the markers syndecan (membrane), α-tubulin (cytosol), HDAC1 (nuclear), and transferrin and EEA1 (endosome). The transferring- and EEA1-containing fractions were pooled.

Immunoblot analysis and immunoprecipitation. Total cell lysates were prepared in ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% (vol/vol) Triton X-100, 1% (wt/vol) deoxycholate, 1 mM phenylmethylsulfonyl fluoride and 20 μg/ml of aprotinin, and proteins were immunoprecipitated overnight at 4 °C. For analysis of protein ubiquitination, 20 mM *N*-ethylmaleimide (Sigma) was added to the lysis buffer. For complex coimmunoprecipitation, antibodies (**Supplementary Methods**) and cell lysates were incubated in 10 mM Tris, pH 7.4, 150 mM NaCl and 0.2% (vol/vol) Nonidet P-40.

IRF3-dimerization assay. This assay was done as described[43]. Cells were lysed in a buffer containing 50 mM Tris, pH 8.0, 1% (vol/vol) Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 20 μg/ml of aprotinin, supplemented with native PAGE sample buffer (125 mM Tris, pH 6.8, and 30% (vol/vol) glycerol). Samples were separated by native PAGE and analyzed by immunoblot.

Generation of shRNA constructs, lentiviral packaging and transduction. Lentiviral vectors encoding shRNA were constructed and packaged as described[19]. For this, 293T cells were transfected with pLSLPw-shRNA constructs along with packaging plasmids (pVSVG (Clontech) and pLV-CMV-delta 8.2 (I. Verma)) with Lipofectamine 2000 (Invitrogen). Virus-containing supernatants were collected at 48–96 h after transfection and were used to infect cells in the presence of polybrene (5 mg/ml; Sigma). After 24 h, virus-containing medium was replaced with selection medium containing puromycin (5 mg/ml; EMD). After cell growth was stable, cells were used for experiments. The oligonucleotide sequences used for shRNA expression were as follows: m-TRAF3, 5′-GCAAGAGAGAGATTCTGGC-3′; m-TRAF6, 5′-CGTCCTTTCCAGAAGTGCC-3′; m-cIAP1, 5′-GGAGTAGTTCAATGTCAT-3′; and m-cIAP2, 5′-GCACCATGCCTTTGAGCTT-3′.

Quantitative PCR analysis. Total cellular RNA from 1×10^5 cells was isolated with TRIzol (Invitrogen) and was used to synthesize first-strand cDNA with iScript cDNA synthesis kit (Bio-Rad). The amount of mRNA was measured by quantitative real-time PCR[17] (primer sequences, **Supplementary Table 1**).

Statistical analysis. Differences between averages were analyzed by Student's *t*-test. *P* values of less than 0.05 were considered significant.

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