Regulation of TRIF-mediated innate immune response by K27-linked polyubiquitination and deubiquitination

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TIR domain-containing adaptor inducing interferon-β (TRIF) is an essential adaptor protein required for innate immune responses mediated by Toll-like receptor (TLR) 3- and TLR4. Here we identify USP19 as a negative regulator of TLR3/4-mediated signaling. USP19 deficiency increases the production of type I interferons (IFN) and proinflammatory cytokines induced by poly(I:C) or LPS in vitro and in vivo. Usp19−/− mice have more serious inflammation after poly(I:C) or LPS treatment, and are more susceptible to inflammatory damages and death following Salmonella typhimurium infection. Mechanistically, USP19 interacts with TRIF and catalyzes the removal of TRIF K27-linked polyubiquitin moieties, thereby impairing the recruitment of TRIF to TLR3/4. In addition, the RING E3 ubiquitin ligase complex Cullin-3-Rbx1-KCTD10 catalyzes K27-linked polyubiquitination of TRIF at K523, and deficiency of this complex inhibits TLR3/4-mediated innate immune signaling. Our findings thus reveal TRIF K27-linked polyubiquitination and deubiquitination as a critical regulatory mechanism of TLR3/4-mediated innate immune responses.

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TLR3/4-mediated induction of downstream effector genes in primary mouse immune cells or fibroblasts. USP19-deficiency enhances TLR3/4-mediated response in vivo. We next investigated the function of USP19 in the regulation of TLR3/4-mediated innate immune and inflammatory responses in vivo. Age- and sex-matched Usp19+/+ and Usp19−/− mice were intraperitoneally injected with poly(I:C) plus
d-galactosamine or LPS. Since poly(I:C) alone is insufficient to cause inflammatory death of mice, d-galactosamine is an agent used to enlarge systemic inflammatory responses of mice injected with poly(I:C). As shown in Fig. 3a, poly(I:C)- and LPS-induced production of IFN-β, TNF, IL-6, and CXCL10 was significantly increased in the sera of Usp19−/− mice compared to Usp19+/+ mice. Hematoxylin-eosin (HE) staining analysis indicated that more serious inflammation was observed in the lungs of Usp19−/− mice after injected with poly(I:C) plus d-galactosamine or LPS (Fig. 3b). Comparing to the Usp19+/+ littermates, Usp19−/− mice were more susceptible to inflammatory death induced by poly(I:C) plus d-galactosamine or LPS (Fig. 3c). After administration of Salmonella typhimurium, the livers and spleens of Usp19+/+ mice carried less bacteria than that of the Usp19−/− littermates. These results indicated that Usp19−/− mice had increased ability to clear Salmonella typhimurium (Fig. 3d). In addition, Usp19−/− mice produced higher levels of serum TNF and IL-6 (Fig. 3e), had intensified inflammation in the small intestinal villus (Fig. 3f) and more dramatic loss of body weights (Fig. 3g), and was more susceptible to inflammatory death after administration of Salmonella typhimurium (Fig. 3h). These data suggest that USP19 attenuates TLR3/4-mediated inflammatory responses in mice.

USP19 removes K27-linked polyubiquitin moieties from TRIF. We next determined the molecular mechanisms responsible for the inhibitory effects of USP19 on TLR3/4-mediated inflammatory responses. We firstly examined the effects of USP19 on signaling mediated by the shared components of TLR3/4-triggered pathways. Reporter assays indicated that USP19 inhibited ISRE activation mediated by overexpression of TRIF but not TBK1 or IRF3 (Fig. 4a). USP19 interacted with TRIF but not TLR3, TLR4, TRAM, MyD88, or TBK1 in mammalian overexpression systems (Fig. 4b). Domain mapping experiments indicated that the TIR (aa348–580) and the C-terminal domain (aa532–712) of TRIF could independently interact with USP19. On the other hand, the C-terminal USP but not the N-terminal tandem CHORD-SGT1 (CS) domains of USP19 had the ability to interact with TRIF (Fig. 4c). Consistently, USP19 mutants lack of its USP domain also lost their abilities to inhibit poly(I:C)- and LPS-triggered IFN-β promoter activation (Fig. 4d). Endogenous USP19 was associated with TRIF in unstimulated cells and their association was increased after poly(I:C) or LPS stimulation (Fig. 4e). These results suggest that USP19 acts at the level of TRIF.

Since USP19 is a deubiquitinating enzyme, we next determined whether it deubiquitinates TRIF. We found that overexpression of USP19 but not its enzymatic inactive mutant USP19(C607S)
removed the polyubiquitin moieties from TRIF (Fig. 5a). Conversely, polyubiquitination of TRIF was increased in Usp19−/− compared to Usp19+/+ BMDMs following poly(I:C) or LPS stimulation (Fig. 5b). Consistently, USP19 but not Usp19 (C607S) inhibited poly(I:C)- and LPS-induced activation of the IFN-β promoter in reporter assays (Supplementary Fig. 4a). Reconstitution of USP19 but not Usp19 (C607S) into Usp19−/− MLFs inhibited poly(I:C)- and LPS-induced transcription of Ifnb1 and Cxcl10 (Supplementary Fig. 4b). These data suggest that USP19 functions by deubiquitinating TRIF.

We next determined the types of ubiquitin chains of TRIF that were removed by USP19. By co-transfection of TRIF with ubiquitin mutants that contains only a single lysine residue (KO), we found that USP19 removed K27-linked polyubiquitin moieties from TRIF (Fig. 5c). By co-transfection of TRIF with ubiquitin mutants in which only one lysine reside is mutated to arginine, we found that USP19 failed to remove polyubiquitin moieties from TRIF only when K27 was mutated to arginine (Fig. 5c). These results suggest that USP19 deubiquitinates K27-linked polyubiquitin moieties from TRIF. Consistently, K27-linked polyubiquitination of TRIF was profoundly increased in USP19−/− in comparison to USP19+/+ cells following poly(I:C) or LPS stimulation (Fig. 5d). These results suggest that USP19 specifically removes K27-linked polyubiquitin moieties from TRIF following TLR3/4 activation.

Deubiquitination of TRIF impairs its recruitment to TLR3/4. We next determined how USP19-mediated deubiquitination of TRIF impairs TLR3/4 signaling. In mammalian overexpression systems, USP19 disrupted the interaction between TRIF and TLR3, but had no marked effects on the interaction between TRIF and TBK1 or TRAF3 (Fig. 6a). In similar experiments, USP19 (C607S) had no marked effects on both TRIF-TLR3 and TRIF-MLFs inhibited poly(I:C)- and LPS-induced transcription of Ifnb1 and Cxcl10 (Supplementary Fig. 4b). These data suggest that USP19 functions by deubiquitinating TRIF. However, USP19 had no marked effects on the interaction between TRIF and TBK1 (Fig. 6b). Endogenous communoprecipitation experiments indicated that more TRIF was recruited to TLR3 in USP19-deficient 293-TLR3 cells following poly(I:C) stimulation (Fig. 6c). These results suggest that USP19 impairs the association of TRIF with TLR3 after TLR3 activation in a deubiquitinating enzymatic activity dependent manner.
Similarly, we found that overexpression of USP19 but not USP19(C607S) inhibited the association between TRIF and TLR4 (Fig. 6d). In these experiments, USP19 had no marked effects on the association between TLR4 and TRIF-related adapter molecule (TRAM) (Fig. 6d), which acts as a link between TLR4 and TRIF. As expected, USP19 but not USP19(C607S) inhibited the interaction between TRAM and TRIF (Fig. 6d). Consistently, TRIF-TLR4 and TRIF-TRAM associations were markedly increased in USP19-deficient cells after LPS stimulation (Fig. 6e).

Taken together, these results suggest that USP19 inhibits the recruitment of TRIF to TLR3/4 by removing K27-linked polyubiquitin moieties from TRIF.

Cullin-3–Rbx1–KCTD10 catalyzes TRIF K27 polyubiquitination. Since USP19-mediated removal of K27-linked polyubiquitin moieties from TRIF negatively regulates TLR3/4-triggered innate immune responses, these findings further underscore the importance of USP19 in regulating TLR3/4 signaling.

**RAW TEXT END**
immune and inflammatory responses, we sought to identify the E3 ubiquitin ligase(s) that mediate K27-linked polyubiquitination of TRIF. We screened a cDNA library containing 352 individual expression clones of ubiquitin-related proteins by reporter assays. These efforts led to the identification of eight candidates which have the abilities to potentiate poly(I:C)-triggered and TRIF-mediated activation of the IFN-β promoter (Supplementary Fig. 5a, b). We then used CRISPER/Cas9 technology to knockout these candidate proteins in 293-TLR3 cells. We found that deficiency of KCTD10, HERC4, RNF13, or RNF72 but not other tested ubiquitin-related proteins markedly inhibited poly(I:C)-triggered transcription of IFNB1 gene (Fig. 7a). In mammalian overexpression systems, KCTD10 strongly mediated K27-linked polyubiquitination of TRIF, while HERC4, RNF13, and RNF72 had minimal effects (Fig. 7b). Endogenous coimmunoprecipitation experiments indicated that KCTD10 was associated with TRIF in un-stimulated cells, and the association was increased at the early time points (10 or 15 min) and then decreased to basal levels at the later time points (after 20 or 30 min) after poly(I:C) or LPS stimulation (Fig. 7c, d). Interestingly, the association of TRIF with USP19 was increased at the later time points after poly(I:C) or LPS stimulation (Fig. 7c, also see Fig. 4e). In addition, poly(I:C)- and LPS-induced K27-linked polyubiquitination of TRIF was markedly decreased in KCTD10-deficient cells compared with control cells (Fig. 7e). KCTD10 is a substrate-specific adapter in the Cullin-3-Rbx1-KCTD10 RING E3 ligase complex20,21. Consistently, KCTD10-mediated K27-linked polyubiquitination of TRIF was abolished in Cullin3- and Rbx1-deficient cells, suggesting that KCTD10-mediated polyubiquitination of TRIF is dependent on Cullin-3 and Rbx1 (Fig. 7f). qPCR experiments indicated that poly(I:C)-induced transcription of IFNB1 and CXCL10 genes was inhibited in KCTD10−/−, Cullin-3−/−, and Rbx1−/− in comparison to control cells (Supplementary Fig. 5c). Deficiency of KCTD10 also inhibited LPS- but not PGN- or R848-induced transcription of downstream genes (Supplementary Fig. 5d). Consistently, deficiency of KCTD10 inhibited poly(I:C)- and LPS-induced phosphorylation of TBK1 and p65 (Supplementary Fig. 5e). These results suggest that the Cullin-3-Rbx1-KCTD10 E3 ubiquitin ligase complex mediates TLR3/4-mediated signaling by catalyzing K27-linked polyubiquitination of TRIF.

Lys523 of TRIF is modified by K27-linked polyubiquitination. We next investigated which lysine residues in TRIF are targeted by Cullin-3-Rbx1-KCTD10 E3 ligase complex for K27-linked polyubiquitination. We carried out a systematic lysine (K) to arginine (R) mutation scanning and tested the effects of USP19 on their activation of ISRE in reporter assays. As shown in Fig. 8a, USP19 markedly inhibited ISRE activation mediated by wild-type and all of the tested mutants of TRIF, with the exception of TRIF (K523R). In these experiments, we also noticed that TRIF(K523R) had markedly reduced ability in activating ISRE in comparison to wild-type and other examined mutants (Fig. 8a). Further we investigated the functions of the TRIF(K523R) by reconstituting it into TRIF-deficient 293-TLR3 cells. We found that TRIF(K523R) partly lost the ability to mediate poly(I:C)-triggered induction of downstream IFNB1 and CXCL10 genes compared with wild-type TRIF and TRIF(K529R) (Fig. 8b). Further experiments indicated that mutation of K523 but not other examined lysine residues of TRIF to arginine abolished its K27-linked polyubiquitination (Fig. 8c). In similar experiments, K63-linked polyubiquitination of TRIF was comparable between wild-type TRIF and its K523R, K415R and K529R mutants (Fig. 8d). Moreover, KCTD10 catalyzed K27-linked polyubiquitination of wild-type TRIF and TRIF (K529R) but not TRIF(K523R) (Fig. 8e). Taken together, these results suggest that the Cullin-3-Rbx1-KCTD10 E3 ligase catalyzes K27-linked polyubiquitination of TRIF at K523.

Discussion

TRIF is an essential adapter protein for TLR3/4-mediated innate immune responses. The activity and availability of TRIF are strictly controlled by several posttranslational modifications to exert sufficient protective immunity and avoid excessive immune damage after certain viral and bacterial infection. Although several E3 ubiquitin ligases have been reported to regulate TRIF polyubiquitination, it remains unknown on how deubiquitination of TRIF is regulated and whether such a regulation modulates TRIF-mediated innate immune responses. In this study, we have identified the first deubiquitinating enzyme, USP19, which mediates deubiquitination of TRIF and regulates TRIF-mediated innate immune responses.

Several lines of evidence support a key role of USP19 in TRIF-mediated signaling. Overexpression of USP19 inhibited poly(I:C)- and LPS-induced activation of NF-κB and IRF3, whereas USP19-deficiency had the opposite effects. Deficiency of USP19 increased serum cytokine levels after administration of poly(I:C) and LPS, and promoted inflammatory death caused by administration of poly(I:C) or LPS, or infection of Salmonella typhimurium. Previously, it has been shown that USP19 negatively regulates RNA virus-triggered type I IFN induction by deubiquitinating Beclin-1 and impairing RIG-I-MAVS/VISA association in certain human cell lines22. However, our results indicated that USP19-deficiency did not affect induction of downstream effector genes after infection with both RNA and DNA viruses or stimulation with TLR2 and TLR7/8 ligands in mouse primary immune cells. It is possible that human and mouse USP19 has distinct functions.

Coimmunoprecipitation experiments indicated that USP19 was associated with TRIF, and this association was increased upon
**Fig. 4** USP19 acts at the level of TRIF. **a** Overexpression of USP19 inhibits TRIF-mediated ISRE activation. HEK293 cells were transfected with ISRE reporter and the indicated adapter expression plasmids together with a control or USP19 expression plasmid for 24 h before luciferase assays were performed. Graphs show mean ± SD; n = 3 independent samples. **P < 0.01 (unpaired t-test); N.S., not significant. **b** USP19 interacts with TRIF in overexpression systems. HEK293 cells were transfected with HA-USP19 and the indicated adapter expression plasmids. Twenty hours after transfection, co-immunoprecipitation was performed with anti-Flag or control IgG. The immunoprecipitates and lysates were analyzed by immunoblotting with anti-HA or anti-Flag. The immunoprecipitates and lysates were analyzed by immunoblotting with indicated antibodies. Data are representative of three experiments with similar results. Source data are provided as a Source Data file. Error bars represent standard deviation of the mean. **c** Domain mapping of the interaction between USP19 and TRIF. HEK293 cells were transfected with the indicated truncations before co-immunoprecipitation and immunoblotting analysis with the indicated antibodies. The schematic presentations of USP19 and TRIF truncations are shown at the top. **d** Effects of USP19 and its truncations on poly(I:C) and LPS-induced IFN-β promoter activation. The 293-TLR3 or 293-TLR4 cells were transfected with the IFN-β promoter reporter and Flag-USP19 or its truncations for 24 h, and then left untreated, treated with poly(I:C) (50 μg/ml) or LPS (100 ng/ml) respectively for 8 h before luciferase assays. Graphs show mean ± SD; n = 3 independent samples. **P < 0.01, ***P < 0.001 (one-way ANOVA followed by Dunnett’s test); N.S., not significant. **e** Endogenous USP19 is associated with TRIF. The 293-TLR3 or 293-TLR4 cells were left untreated or treated with poly(I:C) (100 μg/ml) or LPS (100 ng/ml) respectively for the indicated times. Cell lysates were immunoprecipitated with anti-TRIF or control IgG. The immunoprecipitates and lysates were analyzed by immunoblotting with indicated antibodies. Data are representative of three experiments with similar results. Source data are provided as a Source Data file. Error bars represent standard deviation of the mean.
stimulation with poly(I:C) and LPS. Overexpression of USP19 but not its inactive mutant USP19(C607S) removed K27-linked but not other types of polyubiquitin moieties from TRIF, whereas USP19-deficiency increased poly(I:C)- and LPS-induced K27-linked polyubiquitination of TRIF. Further experiments indicated that wild-type USP19 but not USP19(C607S) impaired the interaction of TRIF with TLR3 or TLR4/TRAM, whereas USP19-deficiency increased the interactions. Our results suggest that TRIF is modified by K27-linked polyubiquitination, and USP19 specifically removes K27-linked polyubiquitin moieties from TRIF following TLR3/4 activation, resulting in impairment of recruitment of TRIF to TLR3/4 and inhibition of innate immune responses.

Previously, it has been shown that USP19 is involved in many cellular processes including autophagy and immune response23, endoplasmic reticulum-associated degradation24, misfolding...
**Fig. 5** USP19 removes K27-linked polyubiquitin moieties from TRIF. a USP19 but not its C607S mutant debiquitinates TRIF. HEK293 cells were transfected with HA-TRIF and Myc-Ub together with empty vector, Flag-USP19 or Flag-USP19(C6). Twenty hours after transfection, ubiquitination assays were performed with the indicated antibodies. The detailed information of ubiquitination assays is described in the Methods. b USP19-deficiency potentiates polyubiquitination of TRIF after poly(I:C) and LPS stimulation. Usp19<sup>−/−</sup> and Usp19<sup>−/−</sup> BMDMs were left untreated or treated with poly(I:C) (100 μg/ml) or LPS (100 ng/ml) respectively for the indicated times. Cell lysates were immunoprecipitated with anti-TRIF. The immunoprecipitates and lysates were analyzed by immunoblotting with the indicated antibodies. c Overexpression of USP19 removes K27-linked polyubiquitin moieties from TRIF. HEK293 cells were transfected with Flag-TRIF, Myc-USP19, HA-ubiquitin or its mutants (KO, K-only; KR, K is mutated to R) together with a control and USP19 expression plasmid for 24 h before ubiquitination assays with the indicated antibodies. d USP19-deficiency potentiates poly(I:C)- and LPS-induced K27-linked polyubiquitination of TRIF. The 293-TLR3 cells or 293-TLR4 cells were left untreated or treated with poly(I:C) (100 μg/ml) or LPS (100 ng/ml) respectively for the indicated times before ubiquitination assays with the indicated antibodies. Data are representative of two or three experiments with similar results. Source data are provided as a Source Data file.

**Fig. 6** USP19-mediated debiquitination of TRIF impairs its function. a Overexpression of USP19 inhibits the interaction of TRIF with TLR3 but not with TBK1 or TRAF3. HEK293 cells were transfected with Flag-TLR3, Flag-TBK1, or Flag-TRAF3 and HA-TRIF together with a control or Myc-USP19 expression plasmid for 20 h. Coimmunoprecipitation was performed with anti-Flag or control IgG. The immunoprecipitates and lysates were analyzed by immunoblotting with anti-Flag or anti-IgG. The immunoprecipitates were analyzed by immunoblots with the indicated antibodies. Data are representative of three experiments with similar results. Source data are provided as a Source Data file.
associated protein secretion, cell proliferation, hypoxia, muscle, and adipogenesis. It has been reported that USP19 catalyzes the removal of various types of ubiquitin chains to regulate different physiological processes. For example, USP19 negatively regulates type I IFN signaling pathway by blocking RIG-I-VISA interaction in a Beclin-1-dependent manner, in which USP19 stabilizes Beclin-1 by removing its K11-linked polyubiquitin chains at lysine 437. USP19 removes K63-linked polyubiquitin moieties from HDAC1/2, which is crucial for regulation of HDAC1/2 activity in DNA damage repair. USP19 deconjugates K63- and K27-linked polyubiquitin chains from TAK1 after TNF or IL-1β stimulation, leading to impairment of TAK1 activity and inhibition of TNF- and IL-1β-triggered inflammatory response. USP19 deconjugates K48-linked polyubiquitin chains from HRD1, which prevents proteasomal degradation of HRD1 and ER-associated protein degradation (ERAD).

Using an expression screen approach, we found that the Cullin-3-Rbx1-KCTD10 E3 ubiquitin ligase complex was responsible for mediating K27-linked polyubiquitination of TRIF.

**Figure Legend:**

- **A:** mRNA level of IFNB1 after treatment with poly(I:C).
- **B:** Western blot analysis of HA-K27-O Flag-αHA αTRIF αTRIF β-actin.
- **C:** IP Ab: Pre poly(I:C): 0 15 30 60 90 (min) USP19 KCTD10 TRIF β-actin.
- **D:** IP Ab: Pre LPS: 0 10 20 40 60 (min) KCTD10 TRIF β-actin.
- **E:** poly(I:C): 0 60 90 Con KCTD10-KO IP: αTRIF Ub-K27 TRIF β-actin.
- **F:** HA-K27O Rbx1-KO: Cullin-3-KO: F-KCTD10: IP: αTRIF αHA αTRIF.
Deficiency of this complex impaired K27-linked polyubiquitination of TRIF as well as induction of downstream effector genes after poly(I:C) and LPS stimulation. Coimmunoprecipitation experiments indicated that the association of TRIF with KCTD10 was increased at the early time point and then decreased thereafter, whereas its association with USP19 was increased at the late time points. These results suggest that increased K27-linked polyubiquitination of TRIF by the Cullin-3-Rbx1–KCTD10 complex at the early phase of TLR3/4 stimulation promotes innate immune responses, while USP19-mediated deubiquitination of TRIF at the late phase terminates TLR3/4-triggered innate immune responses. In light of these observations, it would be interesting to determine how USP19 enzymatic activities are regulated to deconjugate distinct linkage-types of polyubiquitin moieties of various substrates in different signaling pathways.

Our experiments also identified K523 of TRIF as the target residue for K27-linked polyubiquitination by the Cullin-3–Rbx1–KCTD10 complex. Mutation of K523 of TRIF to arginine impaired its K27-linked polyubiquitination as well as its ability to mediate downstream signaling. In addition, KCTD10 failed to catalyze K27-linked polyubiquitination. Recently, structural studies suggest that R522 and K523 of TRIF are crucial for its direct catalysis. In light of these observations, it would be interesting to determine how USP19 enzymatic activities are regulated to deconjugate distinct linkage-types of polyubiquitin moieties of various substrates in different signaling pathways.

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Methods

Mice. We generated Usp19-deficient mice by standard CRISPR/Cas9-mediated gene editing strategy. Gene sequencing results showed that a thymidine was inserted into the third exon of Usp19 gene, which caused a reading-frame shift and the early translational termination of Usp19 after aa 4235. Mice were maintained in special pathogen-free facility of College of Life Sciences at Wuhan University. Eight-to-ten-week-old age, and sex-matched mice were used in all the experiments. Animals were handled according to the Guidelines of the China Animal Welfare Legislation, as approved by the Committee on Ethics in the Care and Use of Laboratory Animals of College of Life Sciences, Wuhan University.

Reagents and antibodies. Mouse monoclonal antibodies against Flag (Origene, 1:2000, F3165), HA (Origene, 1:2000, H9080), β-actin (Sigma, 1:10,000, A2228), Myc (CST, 1:1000, 5605), p-IκBα (CST, 1:1000, 9246L); rabbit antibodies against p-IRF3 (CST, 1:500, 37829), USP19 (Abcam, 1:1000, ab93159), TRIF (Abcam, 1:1000, ab108689), p65 (Santa Cruz Biotechnology, 1:1000, 71675), p-65(S536) (CST, 1:1000, 3033), TBK1 (Abcam, 1:1000, ab68076) and p-TBK1 (Abcam, 1:1000, ab109272), ubiquitin (Abcam, 1:500, ab134953), TLR3-linkage specific polyubiquitin (Abcam, 1:1000, 181537), TRIF (CST, 1:500, 6961), TRIF (R&D, 1:500, AF1478), TRAM (Abcam, 1:1000, ab69160), KCTD10 (Proteinintech, 1:1000, 27279–1-AP); poly(C) (Invivogen), LPS (Sigma), R848 (Invivogen), PGN (Invivogen), human IFN-γ (Peprotech), murine M-CSF (Peprotech), Trizol (Takara Bio), SYBR Green (Bio Rad), dual-specific luciferase assay kit (Promega), D-Gal (Bio Rad), polybrene (Millipore, TR-1003-G), type II collagenase (Worthington), DNase I (Sigma, Aldrich), d-galactosamine hydrochloride (D-Gal) (Sigma); and ELISA kits for TNF-α (Cantell strain) (Charles River Laboratories), HSV-1 (KOS strain) (China Center for Type Culture Collection, Wuhan, China) were obtained from the indicated companies.

Constitutive. Mammalian expression plasmids for Flag-, Myc- or HA-tagged USP19, KCTD10 and the other ubiquitin-related proteins, TRIF and their mutants were constructed by standard molecular biology techniques. Other plasmids used in this study were previously described.

CRISPR-Cas9 knockout. Genome engineering was performed utilizing the CRISPR-Cas9 system. Double-stranded oligonucleotides corresponding to the target sequences were cloned into the lentivirus vector and co-transfected with packaging plasmids into HEK293 cells. Two days after transfection, the viruses were harvested and used to infect the indicated cells. The infected cells were selected with puromycin (1 μg/ml) for at least 5 days. The following sequences were targeted for human Usp19 cDNA: 5′-GACAGAGAGTGAGGACACCC-3′; mouse Usp19 cDNA: 5′-GAGTCCTGGCCGCGCCCTCCT-3′, human KCTD10 cDNA: 5′-GAGTCCTGGCCGCGCCCTCCT-3′, mouse KCTD10 cDNA: 5′-GACGTCCACTCCTGGAGACG-3′, mouse KCTD10 cDNA: 5′-CAGGGTCCTCGGTTGTGCGCT-3′, human Usp19 cDNA: 5′-GACGTGTTATTCTTTACGGC-3′.

Preparation of BMDMs and BMDCs. The bone marrow cells were isolated from tibia and femur. For preparation of BMDMs, the bone marrow cells were cultured in RPMI 1640 medium which contains 10% FBS and murine M-CSF (10 ng/ml) for 5 days. For preparation of BMDCs, the bone marrow cells were cultured in RPMI1640 medium which contains 10% FBS and GM-CSF conditional medium for 9 days.

Preparation of MLFs. The lungs of mice (4–6 week-old) were minced, which were then digested with type II collagenase (10 μg/ml) and DNase I (20 μg/ml) in calcium and magnesium free HBSS at 37 °C for 3 h with shaking. Cell suspensions were sequentially filtered through 100 μm and 40 μm cell strainers, which were then centrifuged at 1500 rpm for 4 min. The pelleted cells were then cultured in 100 mm dishes in DMEM/Ham’s F-12 (1:1 v/v) medium containing 10% FBS, 15 mM HEPES, 2 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. One hour later, the adherent fibroblasts were washed with HBSS and then cultured in the above medium.

Administration of poly(I:C) and LPS. Age- and sex-matched Usp19−/− and Usp19+/+ mice were grouped for the experiments. The mice were injected intra-peritoneally with poly(I:C) (2 μg/g body weight) and D-galactosamine (1 μg/g body weight), or with LPS (10 μg/g body weight). The survival status of the injected mice was monitored at half an hour interval.
Fig. 8 K523 of TRIF is modified by K27-linked polyubiquitination. a Effects of USP19 on ISRE activation induced by wild-type (WT) and mutant TRIF. HEK293 cells were transfected with ISRE reporter and WT or mutant TRIF expression plasmids together with a control or USP19 expression plasmid for 20 h before reporter assays were performed. Graphs show mean ± SD; n = 3 independent samples in a, b. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA followed by Dunnett's test). N.S., not significant. b Effects of TRIF and its mutants on poly(I:C)-induced transcription of downstream genes. TRIF-deficient 293-TLR3 cells reconstituted with TRIF or its mutants were treated with poly(I:C) or left untreated for the indicated times before qPCR analysis. Graphs show mean ± SD; n = 3 independent samples in a, b. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA followed by Turkey's test). N.S., not significant. c Effects of mutation of various lysine residues of TRIF on its K27-linked polyubiquitination. Flag-tagged TRIF or its mutants were individually transfected into HEK293 cells along with HA-ubiquitin (K27O). Twenty hours after transfection, ubiquitination assays were performed with the indicated antibodies. d Effects of mutation of various lysine residues of TRIF on its K63-linked polyubiquitination. Flag-tagged TRIF or its mutants were transfected into HEK293 cells together with HA-ubiquitin (K63O). The experiments were performed similarly as c. e Effects of KCTD10 on K27-linked polyubiquitination of WT and mutant TRIF. The experiments were performed similarly as c. Data are representative of two or three experiments with similar results. Source data are provided as a Source Data file. Error bars represent standard deviation of the mean.
Supplementary Figs. 6

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Author contributions
X.W., C.L., and H.B.S. conceived and designed the study; X.W., C.L., T.X. and X.Z. performed the experiments; H.B.S., X.W., Q.Y. and C.L. analyzed the data. H.B.S., X.W., Q.Y. and C.L. wrote the manuscript.

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