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USP19 Inhibits TNF-α– and IL-1β–Triggered NF-κB Activation by Deubiquitinating TAK1

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The dynamic regulations of ubiquitination and deubiquitination play important roles in TGF-β–activated kinase 1 (TAK1)-mediated NF-κB activation, which regulates various physiological and pathological events. We identified ubiquitin-specific protease (USP)19 as a negative regulator of TNF-α– and IL-1β–triggered NF-κB activation by deubiquitinating TAK1. Overexpression of USP19 but not its enzymatic inactive mutant inhibited TNF-α– and IL-1β–triggered NF-κB activation and transcription of downstream genes, whereas USP19 deficiency had the opposite effects. *Usp19*$^{-/-}$ mice produced higher levels of inflammatory cytokines and were more susceptible to TNF-α– and IL-1β–triggered septicemia death compared with their wild-type littermates. Mechanistically, USP19 interacted with TAK1 in a TNF-α– or IL-1β–dependent manner and specifically deconjugated K63- and K27-linked polyubiquitin chains from TAK1, leading to the impairment of TAK1 activity and the disruption of the TAK1–TAB2/3 complex. Our findings provide new insights to the complicated molecular mechanisms of the attenuation of the inflammatory response. *The Journal of Immunology*, 2019, 203: 259–268.

The transcription factor NF-κB is involved in the regulation of various cellular events, such as immune response, inflammation, cell proliferation, and death (1–3). In resting cells, NF-κB is sequestered in the cytoplasm through association with inhibitory IκB proteins. Following stimulation with exogenous infection, endogenous damage, and tissue stress, the IκB proteins are rapidly phosphorylated by IκB kinase (IKK) complex, consisting of catalytic subunits IKKα, IKKβ, and NF-κB essential modulator. Phosphorylated IκBs are ubiquitinated and subsequently degraded through 26S proteasomes, which leads to the release of NF-κB and allows its translocation to the nucleus, thereby initiating the expression of specific target genes (4, 5).

Signaling pathways triggered by the proinflammatory cytokines such as TNF-α and IL-1β have been intensively studied. Upon binding to TNF-α, TNFR1 recruits downstream adaptor TNFR-associated protein with a death domain (TRADD). TRADD further recruits cellular inhibitor of apoptosis proteins (cIAPs), TNFR-associated factor (TRAF) 2, and TRAF5, which catalyzed K63-linked polyubiquitination on receptor-interacting protein 1. The K63 ubiquitin chains preferentially recruit TGF-β–activated kinase 1 (TAK1)–binding protein (TAB) 2 and TAB3 and form a signal complex. Then, TAK1 is autophosphorylated at its theronines 184 and 187 and activates the IKK complex, leading to the phosphorylation and degradation of IκBα and the activation of NF-κB. The binding of IL-1β to IL-1R triggers recruitment of the adaptor protein MyD88 and IL-1β–associated kinase (IRAK) 1 and IRAK4. TRAF6 binds to IRAK1 and mediated K63-linked free ubiquitin chains generation with the E2 enzyme Ubc13-Uev1A, which in turn recruits TAK1–TAB2/3 complex subsequent to the activation of TAK1 and transactivation of NF-κB downstream genes (5–7).

TAK1 belongs to the mitogen-activated protein kinase kinase kinase (MAPKKK) family, which functions as a pivotal activator of distinct signals by mediating the activation of NF-κB and MAP kinases (8–10). Several reports have demonstrated that ubiquitination and deubiquitination are critical for the regulation of TAK1-mediated NF-κB activation (11, 12). For example, the E3 ubiquitin ligase tripartite motif 8 (TRIM8) positively regulates TNF-α– and IL-1β–triggered NF-κB activation by catalyzing K63-linked polyubiquitination of TAK1 (13). Itch–Cyld complex cleaves K63-linked ubiquitin chains from TAK1 and catalyzes its K48-linked ubiquitination and degradation to terminate NF-κB activation (14). Ubiquitin-specific protease (USP) 4 downregulates TNF-α–induced NF-κB activation by deubiquitinating TAK1 (15). USP18 inhibits NF-κB activation and Th17 differentiation by deubiquitinating TAK1–TAB1 complex (16, 17). However, whether additional deubiquitinating enzymes (DUBs) or E3 ligases are involved in the regulation of NF-κB activation remains unclear.

In the current study, we identified USP19 as a negative regulator of TNF-α– and IL-1β–triggered NF-κB activation. USP19 is a DUB and was initially identified as a regulator of myofibrillar protein expression in rats. USP19 has important functions in skeletal muscle atrophy, antiviral response, and stabilization of transmembrane endoplasmic reticulum–associated degradation substrates (18–21). In this study, we found that USP19 interacted with TAK1 in a TNF-α– and IL-1β–dependent manner

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; BMDM, bone marrow–derived macrophage; CS, CHORD-SGT1; DUB, deubiquitinating enzyme; HA, hemagglutinin; IKK, IκB kinase; IRAK, IL-1β–associated kinase; IRF, IFN regulatory factor; qPCR, quantitative PCR; RNAi, RNA interference; TAB, TAK1-binding protein; TAK1, TGF-β–activated kinase 1; TRADD, TNFR-associated protein with a death domain; TRAF, TNFR-associated factor; TRIM8, tripartite motif 8; USP, ubiquitin-specific protease.

and specifically cleaved K63- and K27-linked polyubiquitin chains from TAK1, which resulted in the impairment of TAK1 phosphorylation and the disruption of the TAK1–TAB2/3 complex, thereby terminating the TAK1-mediated induction of NF-κB downstream genes. Our findings uncovered an important regulatory mechanism that limits excessive inflammatory response.

Materials and Methods

Mice

Usp19$^{-/-}$ mice on the C57BL/6 background were generated by the CRISPR/Cas9 method and obtained from the Wuhan University A3 Animal Center with free access to water and regular chow. Mice were maintained in the special pathogen-free facility of College of Life Sciences at Wuhan University. 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 at Wuhan University. Eight-week-old male and female mice were randomly allocated for each experimental group, and littermates were used as controls.

Reagents, Abs, and cells

TRIzol (9109; Takara Bio); SYBR Green (172-5274; Bio-Rad Laboratories); dual-specific luciferase assay kit (E1980; Promega); polybrene (TR-1003-G; MilliporeSigma); ELISA kits for murine TNF-α, IL-1β, IL-6, and MCP1 (EK0394 and 0441; Bosterbio); recombinant human and mouse TNF-α and IL-1β (300-01A, 200-01B, 315-01A, 211-11B; PeproTech); D-galactosamine hydrochloride (G0500; Sigma-Aldrich); Abs against Flag, hemagglutinin (HA), and c-Myc (OriGene); p-IκBα (9246L, Cell Signaling Technology), p-TAK1 (Thr187) (4536, Cell Signaling Technology), β-actin (A2228; Sigma-Aldrich), and p-p65 (S536) (3033, Cell Signaling Technology); USP19 (ab93159; Abcam); p65 and TRAF6 (71675 and 8409; Santa Cruz Biotechnology); ubiquitin (ab7254; Abcam); K27-linkage specific polyubiquitin (ab181537; Abcam); K48-linkage specific polyubiquitin (ab140601; Abcam); K63-linkage specific polyubiquitin (ab179434; Abcam); and TAK1 (ab109526; Abcam) were purchased from the indicated companies. HEK293 cells were obtained from American Type Culture Collection.

Constructs

Mammalian expression plasmids for Flag- or HA-tagged USP19 and TAK1 and their mutants were constructed by standard molecular biology techniques. Other plasmids used in this study were previously described (13, 22, 23).

Generation of bone marrow–derived macrophages and bone marrow–derived dendritic cells

Bone marrow–derived macrophages (BMDMs) and bone marrow–derived dendritic cells (BMDCs) were generated as described (24, 25). Bone marrow–derived cells (1×10^7) were cultured in RPMI 1640 medium containing 10% FBS and 10% conditional medium from M-CSF-L929 or GM-CSF-L929 cells in a 100-mm dish for 5 d for the generation of BMDMs or BMDCs.

Cell lines and retroviral gene transfer

The transduction of USP19–RNA interference (RNAi) plasmid to HEK293 cells was performed by retroviral-mediated gene transfer. Briefly, HEK293 cells plated on 100-mm dishes were transfected with the indicated retroviral plasmids (10 μg) together with the pGag-pol (10 μg) and the pVSV-G (3 μg) plasmids. After 2 d of transfection, the viruses were harvested and used to infect new prepared HEK293 cells in the presence of polybrene (8 μg/ml). The infected cells were selected with puromycin (1 μg/ml) for at least 6 d before experiments.

Transfection and reporter assays

The cells were transfected by standard calcium phosphate precipitation. To normalize for the transfection efficiency, pRL-TK (*Renilla* luciferase) reporter plasmid (0.01 μg) was used as a based control. Twenty hours after transfection, cells were treated or left untreated with the indicated stimuli before luciferase assays were performed using a dual-specific luciferase assay kit. Firefly luciferase activities were normalized based on *Renilla* luciferase activities.

Coimmunoprecipitation and immunoblot analysis

Cells were lysed in 1 ml NP-40 lysis buffer (20 mM Tris-HCl (pH 7.4–7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF). The lysate were centrifuged at 12,000 rpm × *g* for 10 min at 4°C. For each immunoprecipitation, the supernatant was incubated with 0.5 μg Ab and 35 μl 50% slurry of GammaBind G Plus-Sepharose (Amersham Biosciences) at 4°C for 2 h. The beads were washed for three times with 1 ml lysis buffer containing 500 mM NaCl. The bound proteins were separated by SDS-PAGE, followed by immunoblotting analysis with the indicated Abs.

Detection of ubiquitin-modified proteins

The experiments were performed as previously described (26, 27). Briefly, the cells were lysed in lysis buffer containing 1% SDS and denatured by heating at 95°C for 10 min. After centrifugation, the supernatants were diluted with NP-40 lysis buffer until the concentration of SDS was decreased to 0.1%, followed by coimmunoprecipitation with the indicated Abs. Ubiquitin-modified proteins were detected by immunoblots with the indicated Abs.

RNAi experiments

Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper-Retro RNAi plasmid (Oligoengine). In this study, the following sequences of human *USP19* mRNA were targeted: number 1: 5′-GAGCAAACCAGGAGAGCAA-3′; number 2: 5′-GGGCTGTGGAGAAGGATAA-3′; number 3: 5′-GATGCTGCTTTCACAGATA-3′; and number 4: 5′-GCGCTTCTCCTTTCGTAGT-3′.

Real-time PCR

Total RNA was isolated from cells using TRIzol reagent. After reverse transcription with oligo (dT) primer using a RevertAid First Strand cDNA Synthesis Kit (Fermentas), the samples were subjected to quantitative PCR (qPCR) analysis to measure mRNA expression levels of the tested genes. Data shown are the relative abundance of the indicated mRNAs normalized to that of *GAPDH or Gapdh*. qPCR was performed using the following primers: human *GAPDH*, forward 5′-GACAAGCTTCCCGTTCTCAG-3′, reverse 5′-GAGTCAACGGATTTGGTCGT-3′; human *TNFa*, forward 5′-GCCGCATCGCCGTCTCCTAC-3′, reverse 5′-CCTCAGCCCCCTCTGGGGTC-3′; human *MCP1*, forward 5′-AGAATCACCAGCAGCAAGTGTCC-3′, reverse 5′-TCCTGAACCCACTTCTGCTTGG-3′; murine *Gapdh*, forward 5′-ACGGCCGCATCTTCTTGTGCA-3′, reverse 5′-ACGGCCAAATCCGTTCACACC-3′; murine *Tnfa*, forward 5′-GGTGATCGGTCCCCAAAGGGATGA-3′, reverse 5′-TGGTTTGCTACGACGTGGGCT-3′; murine *Il1b*, forward 5′-AAAGCCTCGTGCTGTCGGACC-3′, reverse 5′-CAGGGTGGGTGTGCCGTCTT-3′; murine *Cxcl10*, forward 5′-ATCATCCCTGCGAGCCTATCCT-3′, reverse 5′-GACCTTTTTTGGCTAAACGCTTTC-3′; murine *Gbp1*, forward 5′-AGATGCCCACAGAAACCCTCCA-3′, reverse 5′-AAGGCATCTCGCTTGGCTACCA-3′; and murine *Cxcl1*, forward 5′-TCCAGAGCTTGAAGGTGTTGCC-3′, reverse 5′-AACCAAGGGAGCTTCAGGGTCA-3′.

TNF-α and IL-1β injection

Age- and sex-matched *Usp19*$^{+/+}$ and *Usp19*$^{-/-}$ mice were injected i.p. with murine TNF-α or IL-1β (150 μg/kg) plus D-galactosamine (1 mg/g) per mouse. The survival of the injected mice was monitored daily.

Statistical analysis

Unpaired Student *t* test was used for statistical analysis with Microsoft Excel and GraphPad Prism Software. For the mouse survival study, Kaplan–Meier survival curves were generated and analyzed by the log-rank test; a p value <0.05 was considered significant.

Results

USP19 negatively regulates TNF-α– and IL-1β–triggered NF-κB activation

To identify the ubiquitin-related enzymes involved in the regulation of NF-κB activation, we screened ∼350 independent cDNA expression plasmids encoding ubiquitin-related enzymes by NF-κB luciferase reporter assays and found that human USP19 inhibited TNF-α– and IL-1β–induced activation of NF-κB (Fig. 1A). To further confirm these results, we generated Flag-tagged human

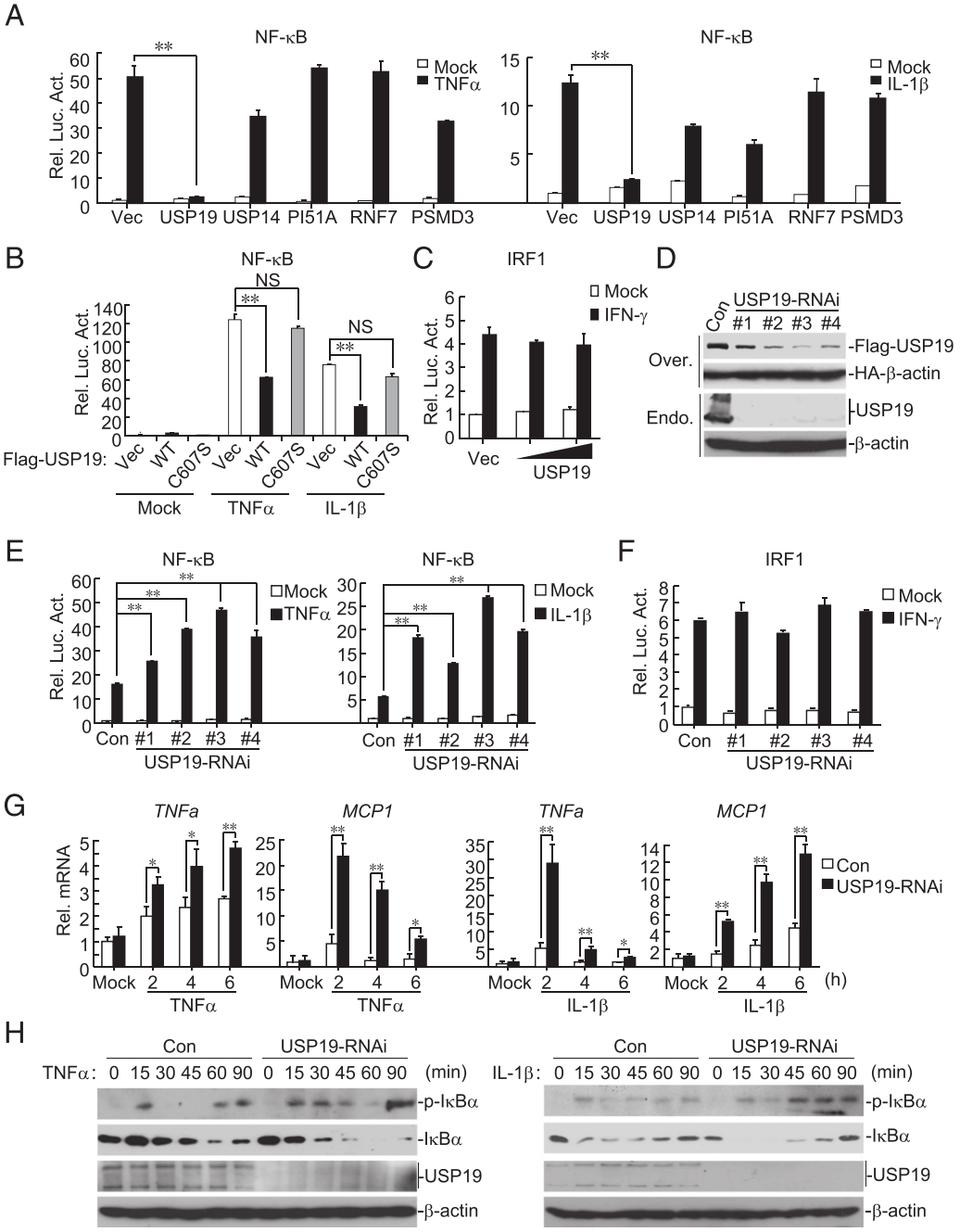

FIGURE 1. USP19 negatively regulates TNF-α– and IL-1β–triggered NF-κB activation. (**A**) Identification of USP19 by cloning expression screens. cDNA expression plasmids (50 ng) encoded the ubiquitin-related enzymes, which were individually transfected into 293 cells (1×10^4) together with the NF-κB luciferase reporter plasmids (20 ng) for 24 h and then left untreated or treated with TNF-α or IL-1β (10 ng/ml) for 10 h before luciferase assays. (**B**) Effects of USP19 on TNF-α– and IL-1β–triggered NF-κB activation. HEK293 cells (1×10^5) were transfected with NF-κB reporter (10 ng) and Flag-USP19 or Flag-USP19 (C607S) plasmids for 24 h and then left untreated or treated with TNF-α or IL-1β (10 ng/ml) for 10 h before luciferase assays. (**C**) Effects of USP19 on IFN-γ–triggered IRF1 activation. HEK293 cells (1×10^5) were transfected with IRF1 promoter reporter (100 ng) and increased amounts of Flag-USP19 plasmid for 24 h and then left untreated or treated with IFN-γ (10 ng/ml) for 10 h before luciferase assays. (**D**) Effects of USP19–RNAi on expression of USP19. In the upper panels, HEK293 cells (4×10^5) were transfected with Flag-USP19 and HA–β-actin (0.1 μg each) and the indicated USP19–RNAi plasmids (1 μg each) for 24 h before immunoblotting analysis. In the lower panels, HEK293 cells (4×10^5) were transfected with USP19–RNAi plasmids (2 μg each) for 48 h before immunoblotting analysis with the indicated Abs. (**E**) Effects of USP19 knockdown on TNF-α– and IL-1β–induced NF-κB activation. HEK293 cells (1×10^5) were transfected with NF-κB reporter plasmid (10 ng) and the indicated USP19–RNAi plasmids (0.5 μg each) for 36 h and then left untreated or treated with TNF-α or IL-1β (10 ng/ml) for 10 h before luciferase assays. (**F**) Effects of USP19 knockdown on IFN-γ–triggered IRF1 activation. HEK293 cells (1×10^5) were transfected with IRF1 promoter reporter (100 ng) and the indicated USP19–RNAi plasmids (0.5 μg each) for 36 h and then left untreated or treated with IFN-γ (10 ng/ml) for 10 h before luciferase assays. (**G**) Effects of USP19 knockdown on TNF-α– and IL-1β–induced transcription of *TNFa* and *MCP1*. HEK293 cells (4×10^5) stably transduced with control or USP19–RNAi plasmids were left untreated or treated with TNF-α or IL-1β (10 ng/ml) for the indicated times before qPCR analysis. (**H**) Effects of USP19 knockdown on TNF-α– and IL-1β–induced IκBα phosphorylation and degradation. HEK293 cells (4×10^5) stably transduced with control or USP19–RNAi plasmids were left untreated or treated with TNF-α or IL-1β (10 ng/ml) for the indicated times before immunoblotting analysis. Graphs show mean ± SD, $n = 3$. $^*p < 0.05$, $^{**}p < 0.01$.

USP19 expression plasmid and its enzymatic inactive mutant USP19 (C607S). In reporter assays, overexpression of Flag-USP19 but not USP19 (C607S) significantly inhibited TNF-α– and IL-1β–induced NF-κB activation (Fig. 1B) but had no marked effects on IFN-γ–triggered IFN regulatory factor (IRF) 1 promoter activation (Fig. 1C). These results suggest that overexpression of USP19 specifically inhibits TNF-α– and IL-1β–induced NF-κB activation.

We next determined whether endogenous USP19 is a negative regulator of NF-κB activation. We constructed four pSuper-USP19–RNAi plasmids (no. 1–no. 4), which markedly inhibited the expression of transfected or endogenous USP19 in HEK293 cells (Fig. 1D). In reporter assays, knockdown of USP19 significantly potentiated TNF-α– and IL-1β–induced NF-κB activation but not IFN-γ–triggered IRF1 promoter activation (Fig. 1E, 1F) (we selected pSuper-USP19–RNAi-no. 3 construct for additional experiments). Consistently, qPCR experiments indicated that knockdown of USP19 significantly potentiated TNF-α– and IL-1β–induced transcription of NF-κB downstream genes, such as *TNFa* and *MCP1* (Fig. 1G). In addition, knockdown of USP19 markedly promoted TNF-α– and IL-1β–induced phosphorylation and degradation of IκBα (Fig. 1H), which are hallmarks of NF-κB activation. These results suggest that USP19 is a negative regulator of TNF-α– and IL-1β–triggered NF-κB activation pathways.

To further elucidate the physiological roles of Usp19 in the regulation of NF-κB activation, we generated Usp19-deficient mice by standard CRISPR/Cas9-mediated gene editing strategy (Supplemental Fig. 1A). 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 at 42 aa (Supplemental Fig. 1B). Immunoblot analysis confirmed that Usp19 was completely lost in $Usp19^{-/-}$ cells (Supplemental Fig. 1C). The $Usp19^{-/-}$ mice were born in a sub-Mendelian ratio and exhibited normal growth and development compared with the wild-type mice (Supplemental Fig. 1D).

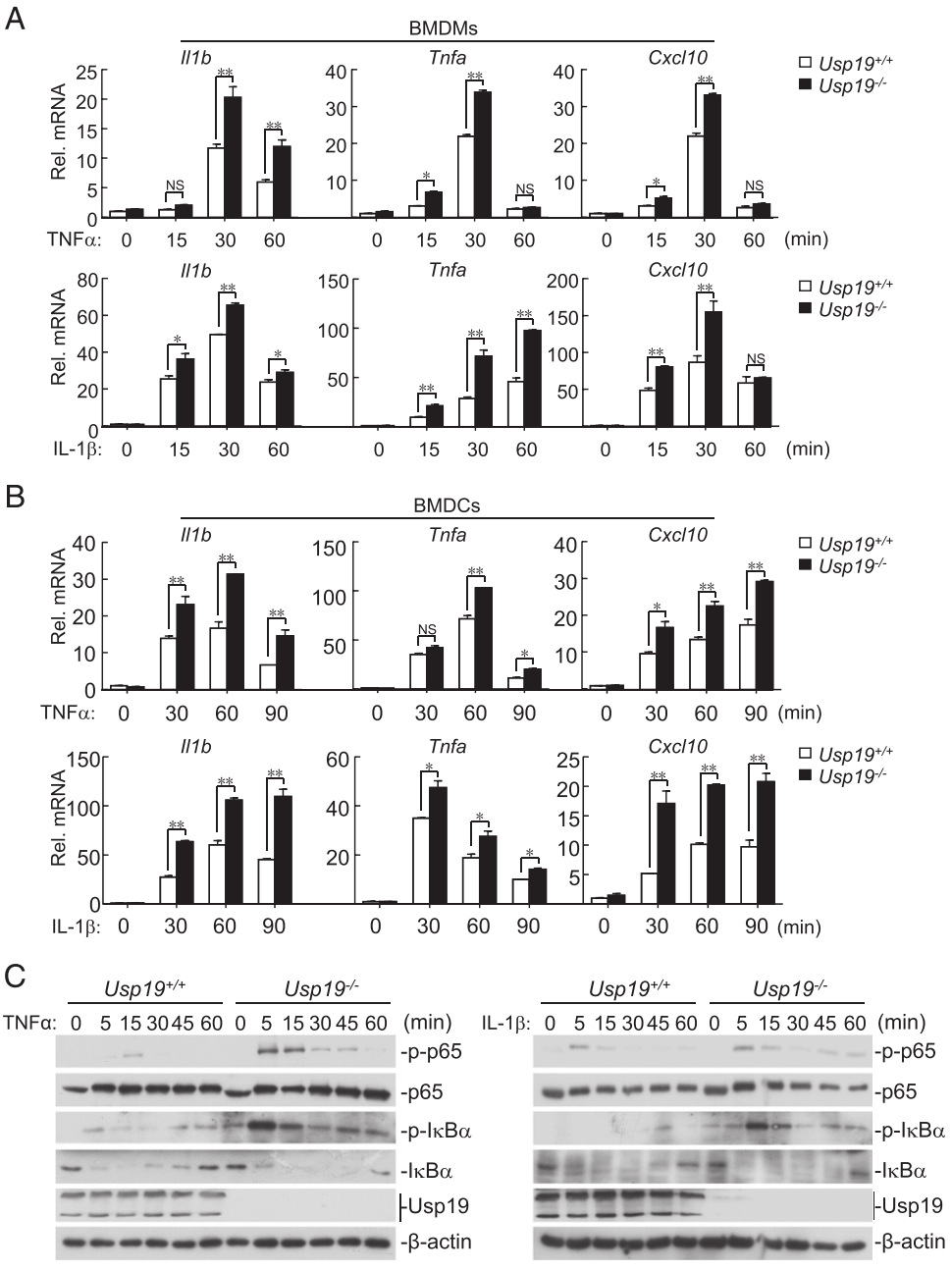

FIGURE 2. USP19-deficiency potentiates TNF-α– and IL-1β–induced NF-κB activation. (**A**) Effects of USP19-deficiency on TNF-α– and IL-1β–induced transcription of inflammatory genes in BMDMs. $Usp19^{+/+}$ and $Usp19^{-/-}$ BMDMs (2×10^6) were left untreated or treated with murine TNF-α and IL-1β (50 ng/ml) for the indicated times before qPCR analysis. (**B**) Effects of USP19-deficiency on TNF-α– and IL-1β–induced transcription of inflammatory genes in BMDCs. The experiments were performed as described in (A) except the BMDCs were used. (**C**) Effects of USP19-deficiency on TNF-α– and IL-1β–induced phosphorylation of IκBα and p65. The $Usp19^{+/+}$ and $Usp19^{-/-}$ BMDMs (2×10^6) were left untreated or treated with TNF-α or IL-1β (50 ng/ml) for the indicated times before immunoblotting analysis. Graphs show mean ± SD, $n = 3$. $^*p < 0.05$, $^{**}p < 0.01$.

The numbers and compositions of various immune cells in the thymus, spleen, and peripheral lymph nodes were comparable in wild-type and *Usp19*$^{-/-}$ mice (Supplemental Fig. 1E, 1F), indicating that Usp19 is dispensable for maturation and development of the immune cells.

We next examined TNF-α– and IL-1β–triggered NF-κB activation in *Usp19*$^{+/+}$ and *Usp19*$^{-/-}$ cells. qPCR experiments showed that TNF-α– and IL-1β–induced transcription of NF-κB downstream genes, such as *Il1b*, *Tnfa*, and *Cxcl10*, were significantly increased in Usp19-deficient BMDMs in comparison with wild-type cells (Fig. 2A). Furthermore, USP19-deficiency also increased the transcription of *Il1b*, *Tnfa*, and *Cxcl10* genes induced by TNF-α and IL-1β in BMDCs (Fig. 2B). However, IFN-γ–triggered transcription of *Cxcl1* and *Gbp1* genes were fully comparable between *Usp19*$^{+/+}$ and *Usp19*$^{-/-}$ cells (Supplemental Fig. 1G). In addition, TNF-α– and IL-1β–induced phosphorylation of p65 and IκBα were markedly increased in *Usp19*$^{-/-}$ compared with wild-type cells (Fig. 2C). Consistently, TNF-α–induced phosphorylation of ERK, JNK, and p38 were significantly enhanced in *Usp19*$^{-/-}$ in comparison with wild-type BMDMs (Supplemental Fig. 1H). These results suggest that USP19 plays a critical role in terminating TNF-α– and IL-1β–triggered NF-κB activation.

Usp19$^{-/-}$ *mice are more susceptible to TNF-α– and IL-1β–induced death*

To gain insight into the functions of USP19 in the regulation of NF-κB signaling in vivo, we monitored TNF-α– and IL-1β–induced expression of inflammatory cytokines as well as septicemia death of wild-type and Usp19-deficient mice. As shown in Fig. 3A, TNF-α–triggered expression of IL-1β, IL-6, and MCP1 was significantly increased in the sera from *Usp19*$^{-/-}$ mice compared with their wild-type counterparts. Similarly, IL-1β–triggered production of TNF-α, IL-6, and MCP1 were increased in *Usp19*$^{-/-}$ mice. H&E staining analysis indicated that more serious inflammation occurred in the lungs of *Usp19*$^{-/-}$ mice than in the lungs of wild-type mice after TNF-α or IL-1β stimulation (Fig. 3B). Consistently, Usp19-deficient mice exhibited an early death and were more susceptible to TNF-α– and IL-1β–induced septicemia death in comparison with their wild-type counterparts (Fig. 3C, 3D). These results suggest that USP19 negatively regulates TNF-α– and IL-1β–triggered inflammatory response in vivo.

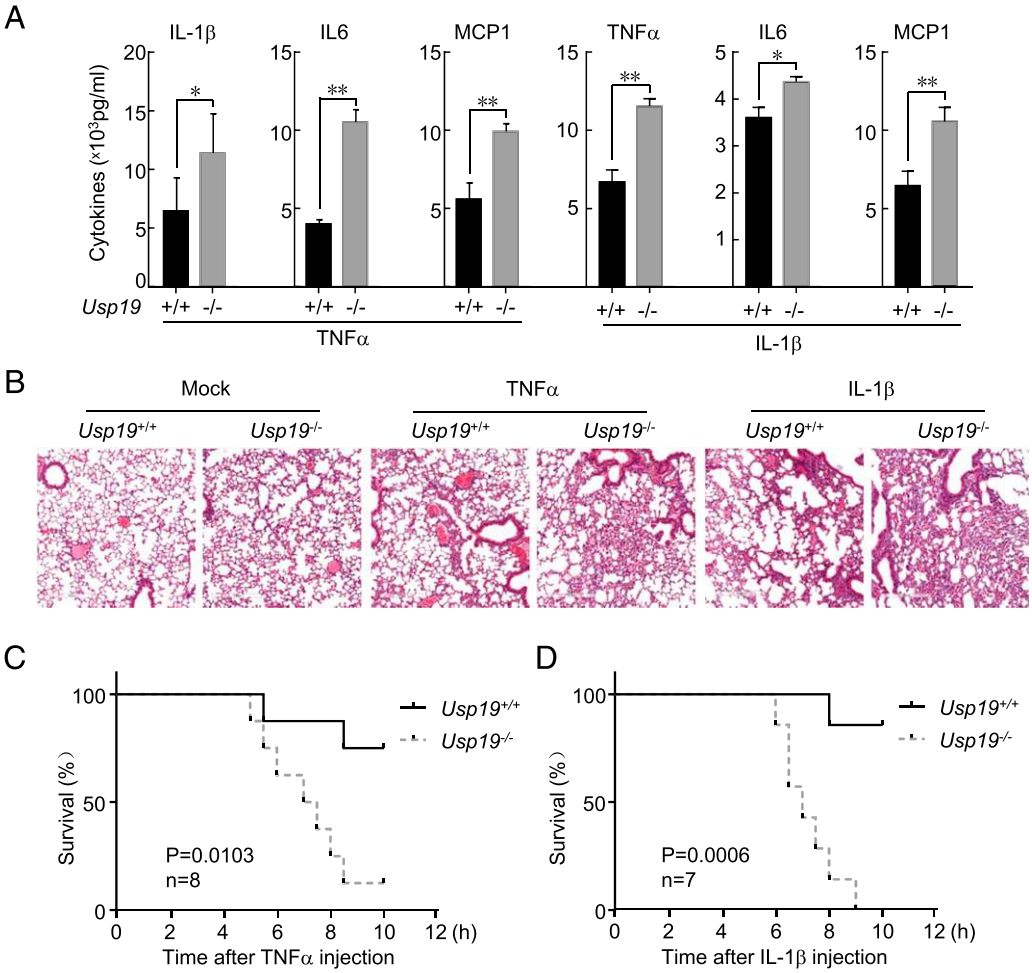

FIGURE 3. *Usp19*$^{-/-}$ mice are more susceptible to TNF-α– and IL-1β–induced septicemia death. (**A**) Effects of USP19-deficiency on TNF-α– and IL-1β–induced serum cytokine levels. Sex- and age-matched *Usp19*$^{+/+}$ and *Usp19*$^{-/-}$ mice (n = 3, 8 wk old) were injected i.p. with murine TNF-α and IL-1β (150 μg/kg) for 3 h, followed by the measurement of the indicated cytokines in the serum by ELISA. (**B**) Effects of USP19-deficiency on TNF-α– and IL-1β–induced inflammatory damage of the lungs. Sex- and age-matched *Usp19*$^{+/+}$ and *Usp19*$^{-/-}$ mice were injected with TNF-α and IL-1β, and the lungs of mice were analyzed by histology with H&E staining. (**C**) Effects of USP19-deficiency on TNF-α–induced septicemia death. Sex- and age-matched *Usp19*$^{+/+}$ and *Usp19*$^{-/-}$ mice were injected with murine TNF-α (150 μg/kg) plus D-galactosamine (1 mg/g) per mouse. The survival curve was generated by Kaplan–Meier methods followed by log-rank test analysis. (**D**) Effects of USP19-deficiency on IL-1β–induced septicemia death. The experiments were performed as described in (C) except IL-1β was used. Graphs show mean ± SD, n = 3. *$p < 0.05$, **$p < 0.01$.

USP19 regulates TNF-α– and IL-1β–triggered NF-κB activation at TAK1 level

We next determined the target(s) of USP19 in the NF-κB signaling pathways. In reporter assays, knockdown of USP19 markedly potentiated NF-κB activation mediated by TAK1–TAB1 as well as their upstream molecules, including TRAF6, MyD88, receptor-interacting protein 1, and TRADD, but had no marked effects on NF-κB activation mediated by IKKβ or p65 (Fig. 4A). In transient transfection and coimmunoprecipitation experiments, USP19 specifically interacted with TAK1 and TRAF6 but not TAB1, TAB2, TAB3, and other tested molecules (Fig. 4B, 4C). Endogenous coimmunoprecipitation experiments indicated that USP19 was associated with TAK1 in a TNF-α– or IL-1β–dependent manner (Fig. 4D), indicating that USP19 regulates NF-κB activation at the level of TAK1 following TNF-α and IL-1β stimulation.

We next mapped the domains of USP19 and TAK1 responsible for their associations. As shown in Fig. 5A, 5B, the N-terminal truncates containing the tandem CHORD-SGT1 (CS) motifs interacted with TAK1. Interestingly, the USP19 truncates lacking either the CS motifs or the peptidase domain failed to inhibit TNF-α–triggered NF-κB activation in reporter assays (Fig. 5C), indicating that the association with TAK1 and the inhibition of NF-κB activation depended on the N-terminal CS motifs and C-terminal peptidase domain, respectively. In parallel experiments, we found that the N-terminal kinase domain of TAK1 was sufficient for its association with USP19 (Fig. 5D, 5E). These data demonstrate that USP19 interacts with TAK1 kinase domain through its N-terminal CS domain, which is required for USP19-mediated downregulation of NF-κB activation.

USP19 hydrolyzes K27- and K63-linked polyubiquitin chains from TAK1

Because USP19 is a TAK1-associating DUB and its deubiquitinating enzymatic activity is required for downregulation of NF-κB activation, we investigated whether USP19 hydrolyzed polyubiquitin chains from TAK1. As expected, overexpression of USP19 but not USP19 (C607S) catalyzed deubiquitination of TAK1 (Fig. 6A). In contrast, overexpression of USP19 did not affect the polyubiquitination of TRAF2, TRAF3, or TRAF6 (Supplemental Fig. 2A, 2B). We next examined the ubiquitination status of TAK1 in the absence of USP19 and found that TNF-α–induced polyubiquitination of TAK1 was substantially increased in *Usp19*$^{-/-}$ compared with *Usp19*$^{+/+}$ BMDMs (Fig. 6B).

We next determined the types of ubiquitin chains of TAK1 that are removed by USP19. Using ubiquitin mutants that contain only one lysine residue, we found that USP19 removed K63- as well as K27-linked polyubiquitin chains from TAK1 (Fig. 6C). Consistently, TNF-α–triggered K27- and K63- but not K48-linked polyubiquitination of TAK1 were markedly enhanced in Usp19-deficient cells (Fig. 6D). These results suggest that USP19 specifically removes K27- and K63-linked polyubiquitin chains from TAK1.

TRAF6 and TRIM8 have been reported to catalyze K63-linked polyubiquitination and activation of TAK1 after TNF-α or IL-1β stimulation (13, 28). Interestingly, we found that TRAF6 but not TRIM8 promoted K27-linked polyubiquitination of TAK1 (Supplemental Fig. 3A, 3B), and TRAF6-deficiency markedly inhibited K27- and K63-linked polyubiquitination of TAK1 after IL-1β stimulation (Supplemental Fig. 3C, 3D). These data suggest that TRAF6 is a major E3 ubiquitin ligase that mediates K27- as well as K63-linked ubiquitin chains to TAK1 after TNF-α stimulation.

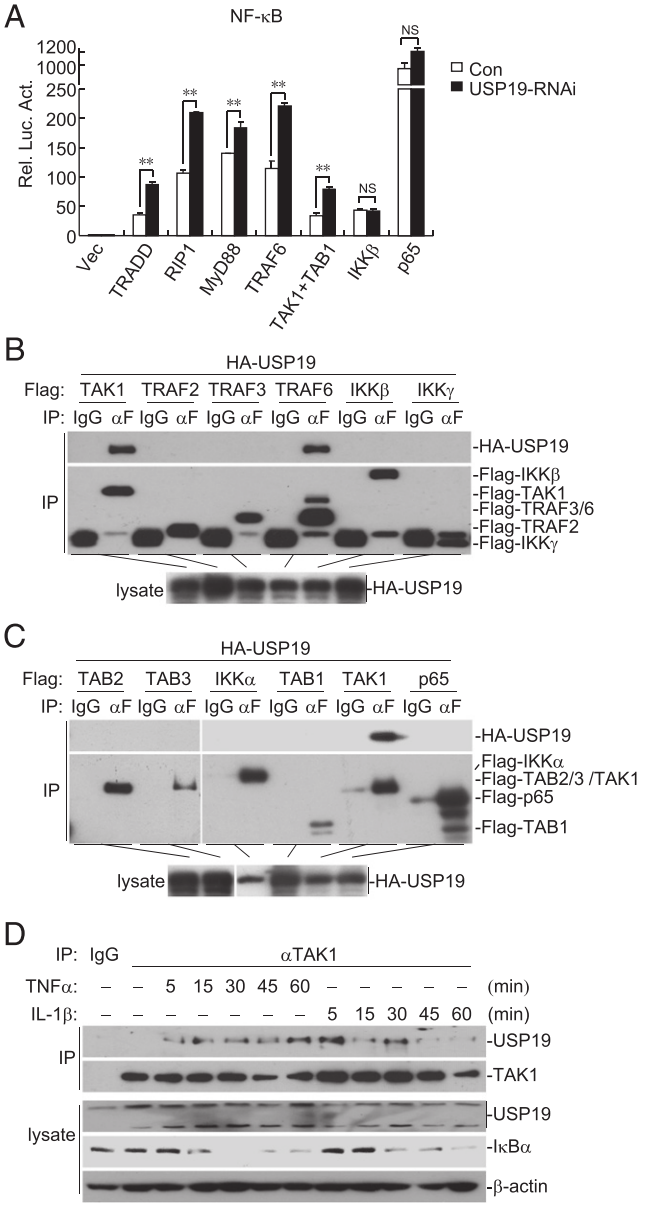

FIGURE 4. USP19 regulates TNF-α– and IL-1β–triggered NF-κB activation at TAK1 level. (**A**) Knockdown of USP19 potentiates TAK1–TAB1– but not IKKβ- and p65-mediated NF-κB activation. HEK293 cells (1×10^5) were transfected with control or USP19–RNAi plasmids (0.5 μg) along with NF-κB reporter (10 ng). Thirty-six hours later, the cells were further transfected with the indicated plasmids (0.2 μg) for 24 h before luciferase assays. Graphs show mean ± SD, $n = 3$. $^{**}p < 0.01$. (**B** and **C**) USP19 interacts with TAK1. HEK293 cells (2×10^6) were transfected with the indicated plasmids for 24 h before coimmunoprecipitation and immunoblotting analysis. (**D**) Endogenous association of USP19 with TAK1. HEK293 cells (1×10^7) were left untreated or treated with TNF-α or IL-1β (25 ng/ml) for the indicated times. Cells were lysed, and the lysates were immunoprecipitated with anti-TAK1 or control IgG. The immunoblotting analysis was performed with the indicated Abs.

USP19 inhibits TAK1 activation and TAK1/TAB complex formation

It has been implicated that K63-linked polyubiquitination of TAK1 promotes the aggregation of TAK1/TAB complex, leading to auto- or paraphosphorylation of TAK1 (29). Expectedly, TNF-α– or IL-1β–induced phosphorylation of TAK1 was markedly potentiated by USP19-deficiency (Fig. 7A). Although the functions of K27-linked polyubiquitination remain largely unknown, it has

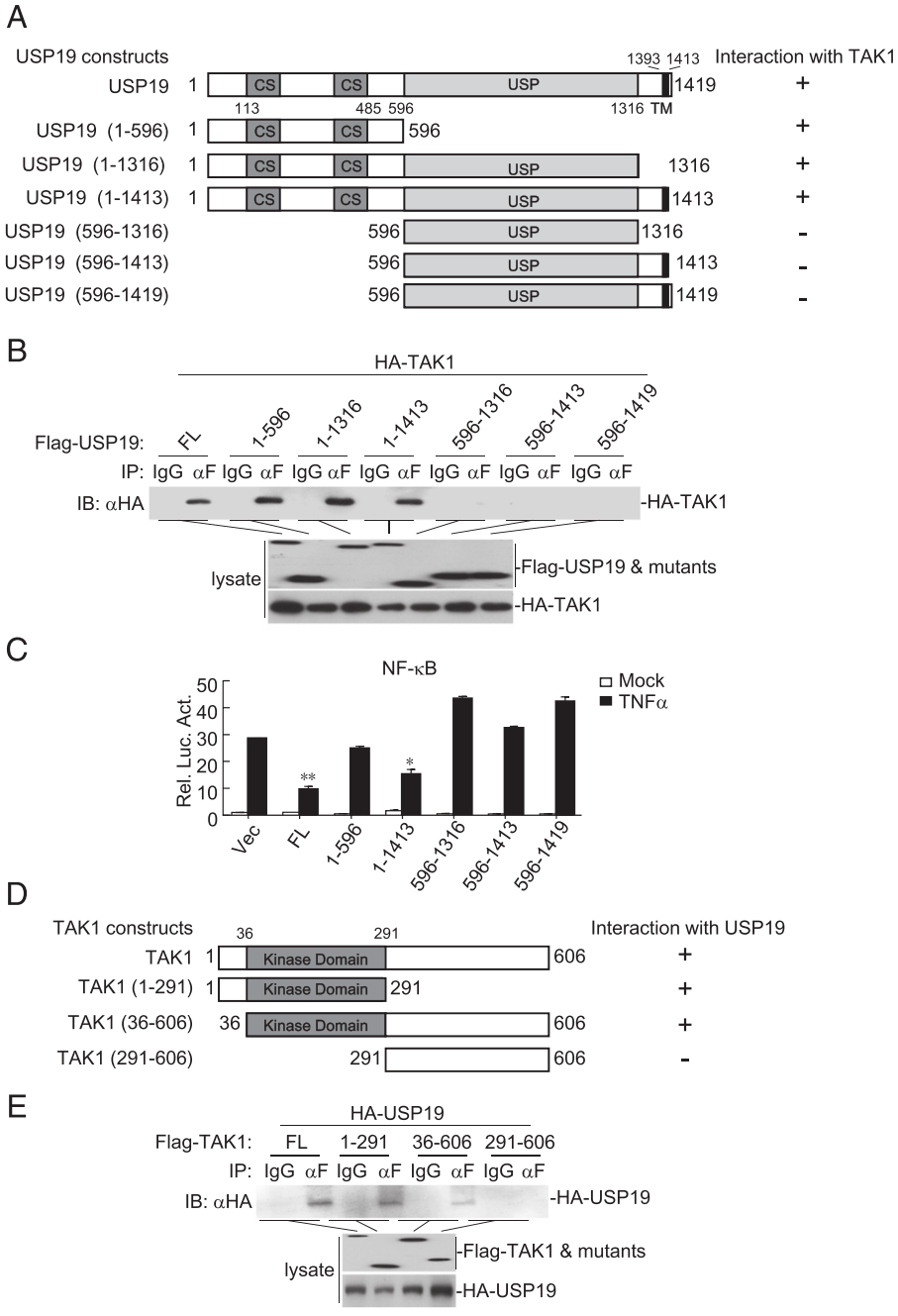

FIGURE 5. Domain mapping of USP19–TAK1 interaction. (**A**) A schematic presentation of USP19 truncations and their abilities to interact with TAK1. (**B**) The interaction between USP19 truncations and full-length TAK1. HEK293 cells (2×10^6) were transfected with the indicated plasmids for 24 h, followed by coimmunoprecipitation and immunoblotting analysis. (**C**) Effects of USP19 and its truncations on TNF-α–induced NF-κB activation. HEK293 cells (1×10^5) were transfected with NF-κB reporter (10 ng) and Flag-USP19 or its truncations for 24 h and then left untreated or treated with TNF-α (10 ng/ml) for 10 h before luciferase assays. Graphs show mean ± SD, $n = 3$. $^*p < 0.05$, $^{**}p < 0.01$. (**D**) A schematic presentation of TAK1 truncations and their abilities to interact with USP19. (**E**) Interactions between TAK1 truncations and full-length USP19. HEK293 cells (2×10^6) were transfected with the indicated plasmids for 24 h, followed by coimmunoprecipitation and immunoblotting analysis.

been reported that K27-linked polyubiquitination of MITA/STING mediates the recruitment of TBK1 after virus infection (30). We thus speculated that K27-linked polyubiquitination of TAK1 might be responsible for the recruitment of the TAB proteins. Overexpression and coimmunoprecipitation assays showed that USP19 but not USP19 (C607S) inhibited TAK1–TAB2 interaction, whereas USP19 did not affect TAK1–TRAF6 and TAK1–IKKβ interactions (Fig. 7B, Supplemental Fig. 4A–D). In addition, TAK1–TAB2 and TAK1–TAB3 associations were markedly increased in *Usp19*$^{-/-}$ compared with *Usp19*$^{+/+}$ BMDMs after TNF-α stimulation (Fig. 7C). Furthermore, in vitro binding assays demonstrated that TAK1 modified with K27- but not K63-linked ubiquitin chains exhibited enhanced binding with TAB2 and TAB3, which was substantially impaired by USP19 (Fig. 7D). These results suggest that USP19 inhibits TAK1–TAB2/3 complex formation and phosphorylation of TAK1 by removing K27- and K63-linked ubiquitin chains from TAK1.

Discussion

NF-κB is a critical transcription factor and regulates a wide spectrum of pathological and physiological events, and its activation is tightly regulated by complicated mechanisms to avoid inflammatory damage. TAK1 is a serine/threonine kinase required for TNF-α– and IL-1β–triggered NF-κB activation. The activation of TAK1 depends on the aggregation of TAK1/TABs complexes that induce auto- or paraphosphorylation of TAK1. Several studies have suggested essential roles of E3 ubiquitin ligase-mediated synthesis of free ubiquitin chains as well as K63-linked polyubiquitination of TAK1 for the activation of TAK1. In this study, we have identified USP19 as a negative regulator of TAK1 after TNF-α or IL-1β stimulation. Through unbiased NF-κB luciferase reporter screens, we identified USP19 as a potent inhibitor for TNF-α– and IL-1β–induced activation of NF-κB. We further found that overexpression of wild-type USP19 but not its enzymatic inactive mutant USP19 (C607S) inhibited TNF-α– and

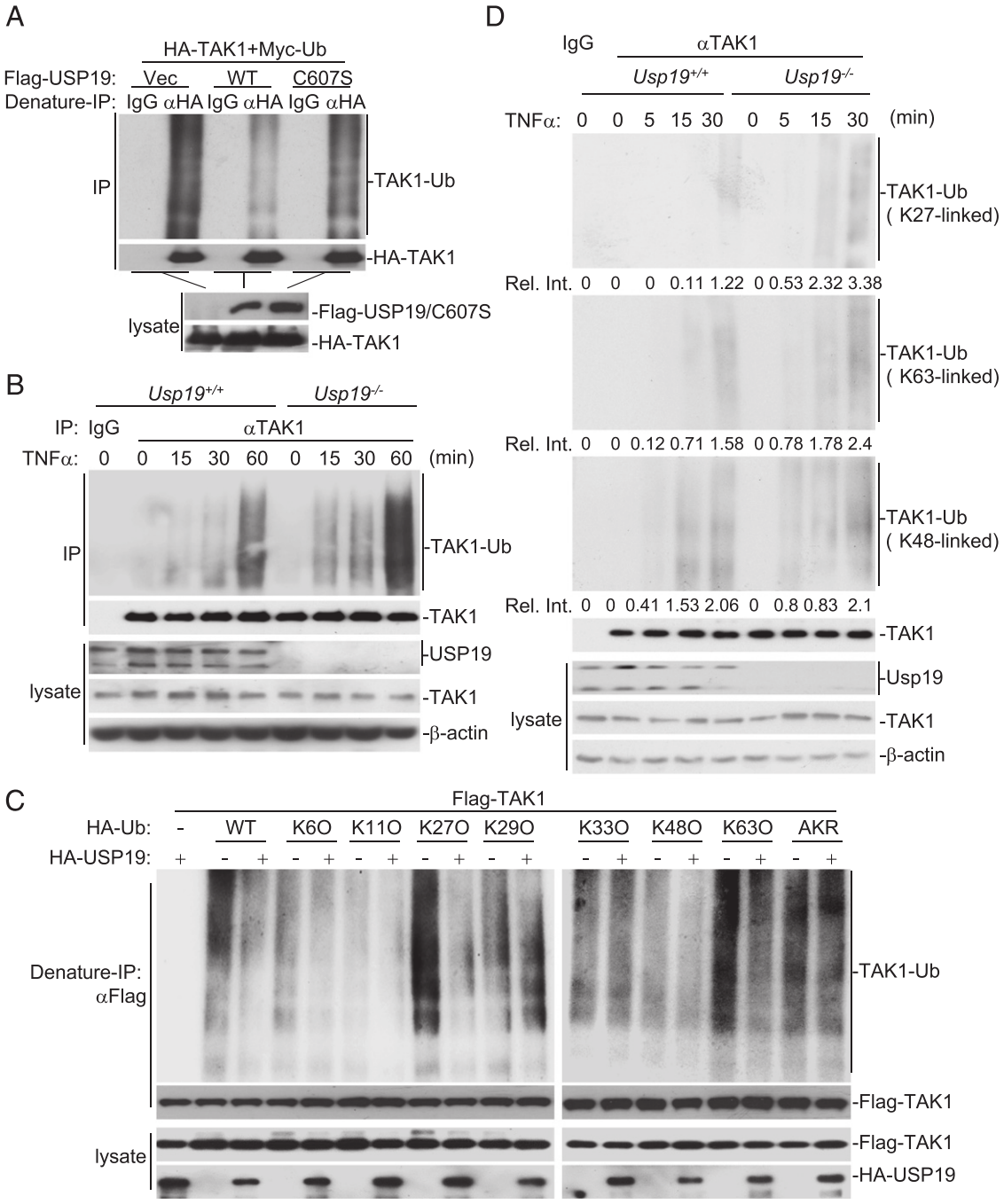

FIGURE 6. USP19 hydrolyzes K27- and K63-linked polyubiquitin chains from TAK1. (**A**) USP19 but not USP19 (C607S) deconjugates ubiquitin chains from TAK1. HEK293 cells (2×10^6) were transfected with the indicated plasmids for 24 h and then followed by denatured coimmunoprecipitation and immunoblotting analysis. (**B**) USP19-deficiency potentiates TAK1 polyubiquitination after TNF-α stimulation. *Usp19*$^{+/+}$ or *Usp19*$^{-/-}$ BMDMs (5×10^7) were treated with TNF-α (50 ng/ml) for the indicated times. The cell lysates were immunoprecipitated with anti-TAK1, and immunoblotting analysis was performed with the indicated Abs. (**C**) Overexpression of USP19 deconjugates K27- and K63-linked polyubiquitin chains from TAK1. The 293 cells (2×10^6) were transfected with Flag-TAK1 (5 μg) and USP19 (1 μg) together with HA-Ub or its mutants (1 μg each) for 24 h and then followed by denatured coimmunoprecipitation and immunoblotting analysis. Ub-AKR, all lysine residues of ubiquitin, were mutated to arginine. (**D**) USP19-deficiency potentiates TNF-α–induced K27- and K63-linked polyubiquitination of TAK1. *Usp19*$^{+/+}$ and *Usp19*$^{-/-}$ BMDMs (5×10^7) were left untreated or treated with murine TNF-α (50 ng/ml) for the indicated times, followed by coimmunoprecipitation and immunoblotting analysis. The relative intensity (Rel. Int.) of ubiquitinated TAK1 were quantified using ImageJ and normalized by immunoprecipitated TAK1.

IL-1β–triggered NF-κB activation and transcription of the inflammatory genes, whereas USP19-deficiency had the opposite effects. In addition, USP19-deficiency in mice resulted in elevated inflammatory cytokine levels in the sera after TNF-α or IL-1β challenge and increased susceptibility to TNF-α– and IL-1β–induced septicemia death. These results demonstrate that USP19 attenuates TNF-α– and IL-1β–triggered inflammatory response in vivo.

Our studies suggest that USP19 negatively regulates TNF-α– and IL-1β–triggered NF-κB activation by deubiquitinating TAK1 (Fig. 7E). USP19 interacted with TAK1 in a TNF-α– and IL-1β–dependent manner. Overexpression of USP19 caused deubiquitination of TAK1 and inhibited TAK1-mediated NF-κB activation in a DUB activity-dependent manner. In contrast, USP19 had a minimal effect on IKKβ- or p65-mediated NF-κB activation,

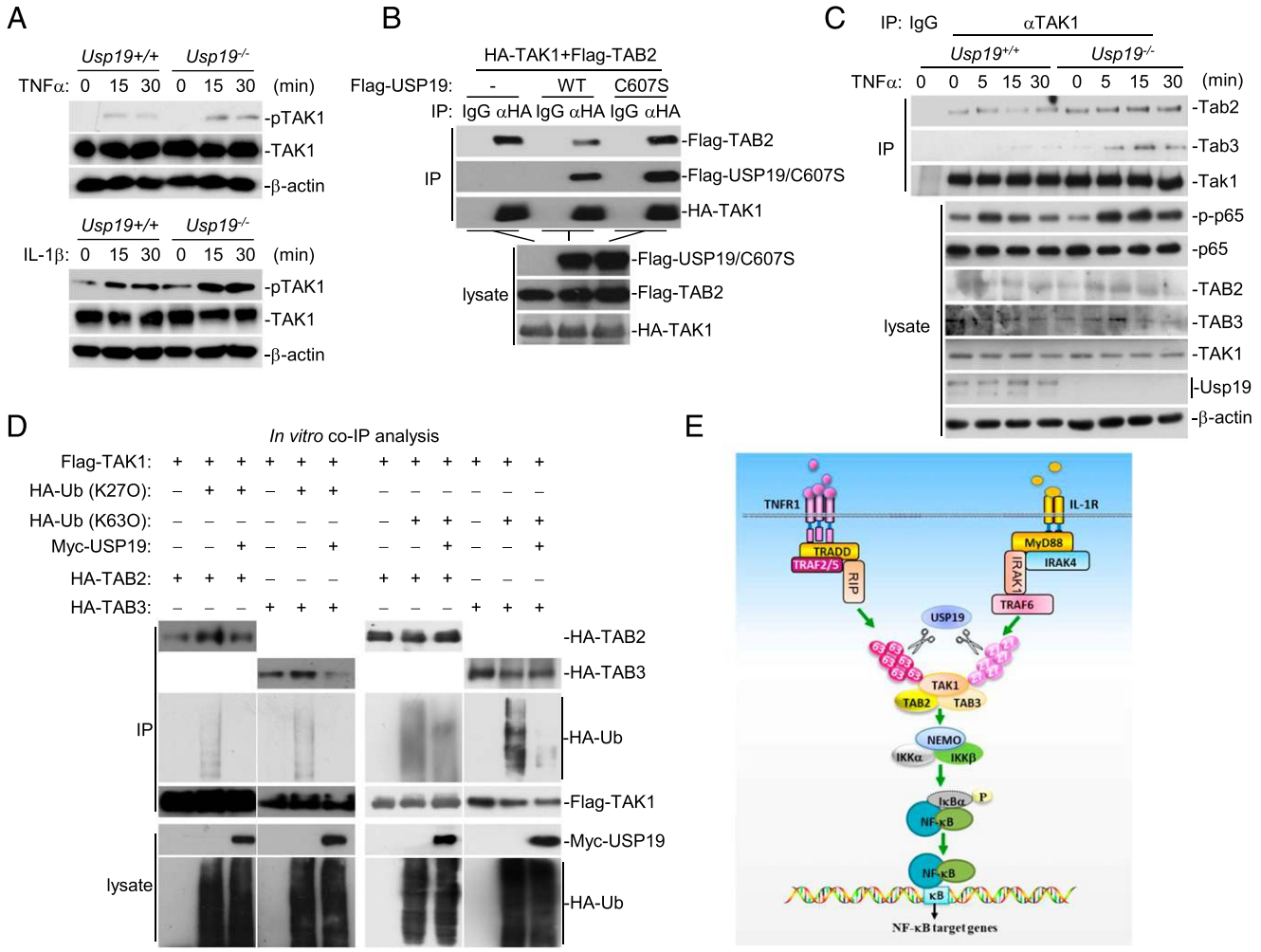

FIGURE 7. USP19 inhibits phosphorylation and complex assembly of TAK1. (**A**) Effects of USP19-deficiency on TNF-α– and IL-1β–induced Tak1 phosphorylation. The *Usp19*$^{+/+}$ and *Usp19*$^{-/-}$ BMDMs (4×10^5) were left untreated or treated with TNF-α or IL-1β (25 ng/ml) for the indicated times before immunoblotting analysis. (**B**) Overexpression of USP19 but not USP19 (C607S) inhibits TAK–TAB2 interaction. HEK293 cells (2×10^6) were transfected with the indicated plasmids for 24 h and followed by coimmunoprecipitation and immunoblotting analysis. (**C**) USP19-deficiency potentiates assembly of Tak1 complex. The *Usp19*$^{+/+}$ and *Usp19*$^{-/-}$ BMDMs (1×10^7) were left untreated or treated with TNF-α for the indicated times and followed by coimmunoprecipitation and immunoblotting analysis. (**D**) Effects of K27-linked polyubiquitination of TAK1 on its recruitment of TAB2 and TAB3. HEK293 cells were transfected with Flag-TAK1 and HA-K27O or HA-K63O ubiquitin in the presence or absence of USP19 for 24 h. The cells were lysed and immunoprecipitated with anti-Flag for 2 h at 4°C; the beads were then washed and eluted with FLAG peptide, followed by incubation with cell lysis in which HA-TAB2 or TAB3 were expressed. The mixtures were further immunoprecipitated with anti-Flag for 2 h, followed by immunoblotting analysis. (**E**) A working model on the involvement of USP19 in TAK1-mediated signaling. USP19 interacted with TAK1 in a TNF-α– or IL-1β–dependent manner and specifically deconjugated K63- and K27-linked polyubiquitin chains from TAK1, leading to the impairment of TAK1 activity and the disruption of the TAK1–TAB2/3 complex.

suggesting that USP19 regulates NF-κB activation at the level of TAK1. In addition, USP19 preferentially deconjugated K27- and K63-linked polyubiquitin chains from TAK1, thereby attenuating TNF-α– and IL-1β–induced phosphorylation of TAK1 and the subsequent transcription of proinflammatory cytokines.

Previous studies have characterized TRAF6 and TRIM8 as essential E3s catalyzing K63-linked polyubiquitination of TAK1 and promoting NF-κB activation. In this study, we found that TRAF6 mediated K27-linked polyubiquitination of TAK1. Such a modification promoted recruitment of TAB2 and TAB3 to TAK1 to form the TAK1/TABs complex, which is a prerequisite for the aggregation of TAK1/TABs complex. USP19 removed K27-linked polyubiquitin chains from TAK1, thereby inhibiting TAK1–TAB2 or TAK1–TAB3 associations. In addition, USP19 also deconjugated K63-linked polyubiquitin chains from and inhibited phosphorylation of TAK1 after TNF-α stimulation, which might be a result of impaired TAK1/TABs complexes. Thus, it is likely that USP19 regulates TAK1 activation through a step-wise manner (i.e., inhibiting the interaction between TAK1 and TABs and TAK1/TABs complex aggregation that are dependent on K27-linked or K63-linked polyubiquitination of TAK1, respectively).

USP4 and USP18 inhibit TNF-α– and TCR-triggered NF-κB activation by deubiquitinating TAK1 or TAK1–TAB1 complex, respectively (15, 17). qPCR analysis indicated that USP18 was expressed at higher levels in Th0, Th1, and Th17 cells than in inducible regulatory T cells, Th2, BMDM, and BMDC. Interestingly, USP19 was abundantly expressed in inducible regulatory T cells, Th2, BMDM, and BMDC but not in other tested cells (Supplemental Fig. 4E). These results suggest that USP18 and USP19 deubiquitinate TAK1 in a cell type–specific manner. USP18 interacts with TAK1 in unstimulated T cells and disassociates from TAK1 complexes after TCR engagement, whereas USP4–TAK1 association is induced by TNF-α stimulation. Our identification of USP19 as a negative regulator of TAK1 in response to TNF-α

and IL-1β has provided new insights into the complicated regulatory mechanisms for NF-κB–mediated inflammatory responses.

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Disclosures

The authors have no financial conflicts of interest.

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