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ARTICLE

RNF144B inhibits LPS-induced inflammatory responses via binding TBK1

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Abstract

Innate immune responses need to be precisely controlled to avoid prolonged inflammation and prevent unwanted damage to the host. Here, we report that RNF144B responded dynamically to LPS stimulation and negatively regulated LPS-induced inflammation. We found that RNF144B interacted with the scaffold/dimerization domain (SDD) of TANK binding kinase 1 (TBK1) through the in between RING (IBR) domain to inhibit its phosphorylation and K63-linked polyubiquitination, which led to TBK1 inactivation, IRF3 dephosphorylation, and IFN-β reduction. RNF144B knockdown with siRNA increased IRF3 activation and IFN-β production in response to LPS stimulation. Our study identifies that RNF144B interaction with TBK1 is sufficient to inactivate TBK1 and delineates a previously unrecognized role for RNF144B in innate immune responses.

KEYWORDS

RNF144B, TBK1, LPS, inflammatory responses

1 | INTRODUCTION

The innate immune system is the first line of defense for the host against invading microbial organisms. The recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) elicits innate immune responses.[1] However, excessive or prolonged immune responses to infection may lead to inflammation-related diseases or death.[2] TLR-mediated signaling plays essential roles during bacteria and virus infection.[3] TLR4 detects LPS of Gram-negative bacteria;[4] TLR2 recognizes lipoteichoic acid (LTA) of Gram-positive bacteria,[5] whereas TLR3, TLR7, TLR8, and TLR9 identify virus infection by viral RNA or DNA binding in the endosome.[6] Upon recognition of PAMPs, most TLRs, which localize on the cellular membrane, recruit and activate the intermediate signaling adaptor TRAF6; TLR4 and endosome bound receptors, such as TLR3, recruit TRIF to activate TANK binding kinase 1 (TBK1). Activated TBK1 subsequently phosphorylates the transcriptional factors IFN regulatory factor 3 (IRF3) and 7 (IRF7) to facilitate type I IFN production.[7] Shortage of IFN production brings about chronic infection, whereas persistent IFN production gives rise to autoimmune diseases or inflammatory diseases such as rheumatoid arthritis and sepsis.[8,9] So, precise control of IFN production is important for efficient pathogen clearance without host damage.

The kinase TBK1 is essential for IFN production, and its activation state must be tightly controlled to maintain immune homeostasis. It has been reported that TBK1 activity can be modified in various ways, including protein level regulation, phosphorylation, and ubiquitination. The E3 ligases RNF128 and Nrdp1 have been reported to

Abbreviations: CTD, C-terminal domain; DEG, differential expressed gene; HMDM, human monocyte-derived macrophage; IBR, in between RING; IRF3, regulatory factor 3; IRF7, regulatory factor 7; LTA, lipoteichoic acid; PAMPs, pathogen-associated molecular patterns; PRRs, pattern-recognition receptors; SDD, scaffold/dimerization domain; siRNA, small interfering RNA; TBK1, TANK binding kinase 1.

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activate TBK1 by promoting its K63-linked polyubiquitination.[10,11] DTX4 and TRIP, 2 E3 ligases, inhibit TBK1 by promoting its proteasome degradation.[12,13] TBK1 activity can also be negatively regulated by removing its K63-linked ubiquitin chains via a series of deubiquitinating enzymes, such as CYLD and USP2b.[14,15] Although these studies clearly demonstrated that these ligases and deubiquitinating enzymes are essential for TBK1 regulation, it is still valuable to search for potential E3 ligases, which target TBK1.

RNF144B, also known as PIR2, is an E3 ligase, which contains 2 RING domains, an in between RING (IBR) domain, and an uncharacterized, evolutionally highly conserved C-terminal domain (CTD).[16] RNF144B was previously shown to be induced by p53 and p63 to regulate the stability of $p21^{WAF1}$ or p63, processes which mediate cell growth arrest and epithelial homeostasis, respectively.[17,18] Furthermore, the N-terminal domain of RNF144B was shown to be responsible for E2 ubiquitin-conjugating enzyme binding, whereas the C-terminus was required and sufficient for p53-dependent but caspase-independent cell apoptosis induction, both of which demonstrate a role of RNF144B for switching a cell from p53-mediated growth arrest to apoptosis. RNF144B was also induced by p73 upon DNA damage, and preferentially promoted ubiquitin-mediated degradation of deltaNP73, the dominant negative isoform of p73, which led to the relief of the inhibitory effects on p73 induced apoptosis following DNA damage.[19] On the other hand, RNF144B was revealed to be a mitochondrial trans-locating E3 upon apoptotic stimulation and protected cells from unprompted Bax activation and caspase-dependent cell death by regulating the level of Bax.[20] The evidence clearly demonstrated that RNF144B is closely related with cell apoptosis and tumorigenesis and it was also reported recently that RNF144B was inducible by LPS in human macrophages. However, the function of RNF144B in LPS-induced innate immune responses has not been addressed.

In the present study, we demonstrated that RNF144B is induced by LPS in a MyD88 dependent NF-κB activation and acts as an inhibitory effector on LPS-induced IFN production by interacting with TBK1. Overexpression of RNF144B decreases TBK1 phosphorylation and IRF3 activation independent of its E3 ligase activity. Mechanistically, RNF144B associates with TBK1 scaffold/dimerization domain (SDD) and inhibits its K63 linked polyubiquitination.

2 | MATERIALS AND METHODS

2.1 | Small interfering RNA and plasmids

RNA oligonucleotides for small interfering RNA (siRNA) were synthesized (Genepharma, Shanghai, China) with the following sequences: for the nonsilencing control, 5′-UUCUCCGAACGUGUCACGU-3′, for MyD88, siRNA#1: 5′-GGCAACUGGAACAGACAAA-3′, siRNA#2: 5′-GGGCAUCACCACACUUGAU-3′, for RNF144B, 5′-CCUUCUUGCCACCUGAAAU-3′. Flag-tagged TBK1, TBK1 truncations, Myc-tagged RNF144B, and RNF144B truncations were sub-cloned into pCDNA3.1. Sequenced results confirmed that these sequences were correct.

2.2 | Cell culture

The human monocyte cell line THP-1 and 293T cells were obtained from the Cell Resource Center (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). THP-1 cells were maintained in RPMI-1640 medium, and 293T cells were maintained in DMEM. All cell cultures were supplemented with 10% FBS and penicillin or streptomycin.

2.3 | Reagents

LPS (*Escherichia coli*, serotype 055:B5) and anti-Flag (F3165), anti-Myc (M4439), and anti-actin (A2228) were from Sigma (St. Louis, MO, USA). Malp2, poly (I:C), flagellin, R-848, and CpG DNA were obtained from Enzo Life Sciences (Farmingdale, NY, USA). Antibody to TBK1, phosphorylated TBK1, IRF3, and phosphorylated IRF3 were from Cell Signaling (Beverly, MA, USA); and anti-HA (sc-7392) was purchased from Santa Cruz (Dallas, TX, USA).

2.4 | Transfection and reporter assay

Lipofectamine 2000 and lipofectamine LTX with PLUS reagent (Invitrogen, Carlsbad, CA, USA) were used to transfect 293T cells with plasmids encoding the NF-κB luciferase reporter or pRL-TK renilla luciferase. Luciferase activity was assessed with a dual luciferase assay kit (Promega, Madison, WI, USA) and a Luminoskan Ascent luminometer (Thermo Scientific, Waltham, MA, USA). All the experiments for luciferase reporter assay were performed in 24-well dishes.

2.5 | Real-time PCR

Real-time PCR was performed on an Applied Biosystems Step Two Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and results were calculated by the comparative cycling threshold quantization method or standard curve method.[21] Real-time PCR Master Mix (Toyobo, Osaka, Japan) was used to detect and quantify the expression of the target gene. The gene encoding GAPDH was used as an internal control. The primers (F, forward; R, reverse) are listed in Table 1.

2.6 | Immunoprecipitation and immunoblot analysis

For immunoprecipitation experiments, whole-cell extracts were prepared after transfection and were incubated overnight with the appropriate antibodies, together with Protein A/G beads (Santa Cruz). Beads were then washed 3 times with lysis buffer, and immunoprecipitates were eluted with SDS loading buffer and resolved by PAGE.[22] The proteins were transferred to a polyvinylidene difluoride membrane and were further incubated with the appropriate antibodies.

2.7 | Ubiquitination assays

For analysis of the ubiquitination of TBK1 in HEK293 cells, cells were transfected with Flag-TBK1, HA-ubiquitin-WT, or HA-ubiquitin

TABLE 1 Primer sets used for quantitative PCR assay

Name of genes	Forward primer (5′–3′)	Backward primer (3′-5′)
hGAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
h*RNF144B*	CTGGTAGGCTCCACTATCTCG	GGGCAAGTGATGGGAGACC
h*IFNB1*	CAACAAGTGTCTCCTCCAAAT	TCTCCTCAGGGATGTCAAAG

mutants and Myc-RNF144B. Then, whole-cell extracts were immunoprecipitated with anti-Flag and analyzed by immunoblot with anti-HA.

2.8 | Statistical analysis

Data (combined from 3 or more independent experiments) were analyzed with Prism 6 (GraphPad, San Diego, CA, USA). One-way ANOVA and Student's *t*-test were used for comparing groups. *P* values of <0.05 were considered significant.

3 | RESULTS

3.1 | LPS-induced RNF144B expression is dependent on MyD88-mediated NF-κB activation

With the aim to find novel E3 in LPS-TLR4 signaling regulation, we searched the public microarray data in GEO (https://www.ncbi.nlm.nih.gov/gds/) database for differential expressed gene (DEG) analysis. By thoroughly screening, we located 2 datasets of GSE16193 and GSE40885. For GSE16193, the microarrays were performed on THP-1 cells, and the cells were treated with or without LPS.[23] For GSE40885, 7 healthy humans were instilled with sterile saline into a lung segment, whereas LPS into the contralateral lung. Bilateral bronchoalveolar lavage was performed and whole-genome transcriptional profiling was done on purified alveolar macrophages.[24] So by DEG analysis in both datasets, we found consistent DEGs in both cell line and primary macrophage on LPS stimulation for further analysis. We found 6 E3s differentially expressed after LPS stimulation in both datasets, with 5 up-regulated (RNF144B, PELI1, SOCS3, TNFAIP3, and BIRC3) and 1 down-regulated (RNF166) (Fig. 1A). PELI1 negatively regulates type I IFN induction and antiviral immunity in the central neural system.[25] SOCS3 negatively regulates IL-6 signaling in vivo.[26] TNFAIP3 negative regulates IRF3 signaling by physically interacting with TBK1.[27] BIRC3 transcription is induced by LPS, and it positively regulates TNFα-mediated NF-κB activation.[28] The down-regulated RNF166 potentiates RNA virus-induced IFN beta via increasing the ubiquitination of TRAF3 and TRAF6.[29] Moreover, significant up-regulation of RNF144B mRNA was found in the whole blood samples of patients with sepsis (GSE26378) (Fig. 1B), indicating the regulatory role of RNF144B in IFN production induced by LPS.

We measured the kinetics of *RNF144B* transcription as well as the expression of genes encoding inflammatory cytokines, such as TNFα, upon stimulation with LPS in THP-1 human monocytic cells. Consistent with a previously published study,[30] *TNFA* transcription was rapidly increased and reached the plateau at 3 h, and then dropped to the basal level at about 12 h after LPS stimulation (Fig. 1C). The increase of *RNF144B* transcript was concomitant with the rapid up-regulation of *TNFA* mRNA during the first 3 h. TNF expression returned to near basal level at 6 h; *RNF144B* then began to decline and reached minimum expression, which indicated that low expression of *TNFA* might not be sufficient for RNF144B induction upon LPS challenge. To further validate induced RNF144B expression by LPS stimulation, human monocyte-derived macrophage (HMDM) was used to measure RNF144B upon LPS treatment for 6 h (Fig. 1D), and RNF144B expression was dramatically induced in HMDM by LPS, which was consistent with a previous report.[31] In order to further explore the underlying mechanism of RNF144B induction by LPS, we used different ligands of TLR2, TLR3, TLR5, TLR7/8, and TLR9, including LTA, poly (I:C), flagellin, R848 and CpG oligodeoxynucleotides (ODNs) to challenge THP-1 cells and measured *RNF144B* expression level at 6 h. LTA, flagellin, R848, and CpG ODNs were sufficient for RNF144B induction, whereas poly (I:C) was not (Fig. 1E). Given that all TLRs except TLR3 signal via MyD88,[32] we suspected that RNF144B might generally respond to TLR signaling via MyD88-NF-κB activity. To test that hypothesis, firstly we designed 2 siRNAs specific for MyD88, and transfected them to THP-1 cells. The knockdown efficiency for both siRNAs were validated by Western blot (Fig. 1F), and RNF144B expression was measured upon LPS stimulation. The results showed that MyD88 knockdown remarkably reduced RNF144B expression induced by LPS (Fig. 1G). Then, we treated THP-1 cells with different concentrations of BAY-11-7085, a specific inhibitor of NF-κB (Fig. 1H), and found that BAY 11–7085 inhibited LPS-induced *RNF144B* expression in a dose-dependent manner. Collectively, our results demonstrated that RNF144B is induced by TLR ligands dependent on MyD88-NF-κB signaling.

3.2 | RNF144B negatively regulates IRF3 activation and IFN-β production

To explore the function of RNF144B in the innate antibacteria response, we designed siRNA specific for human RNF144B (hRNF144B siRNA) and transfected it into THP-1 cells. Endogenous RNF144B mRNA expression was attenuated after transfection with RNF144B siRNA at different times of LPS stimulation (Fig. 2A). Expression of *IFNB1* (which encodes IFN-β) was increased in THP-1 cells transfected with RNF144B siRNA at different time-courses, as compared to cells transfected with control siRNA (Fig. 2B) without affecting that of *TNFA* (Fig. 2C), which demonstrated that RNF144B might specifically interfere with LPS induced *IFNB1* expression.

To confirm the function of RNF144B in LPS-IFN-β signaling, we transfected the siRNA into THP-1 cells, and treat the cells with LPS at different times (0, 15, 30, 60, and 120 min) to detect the

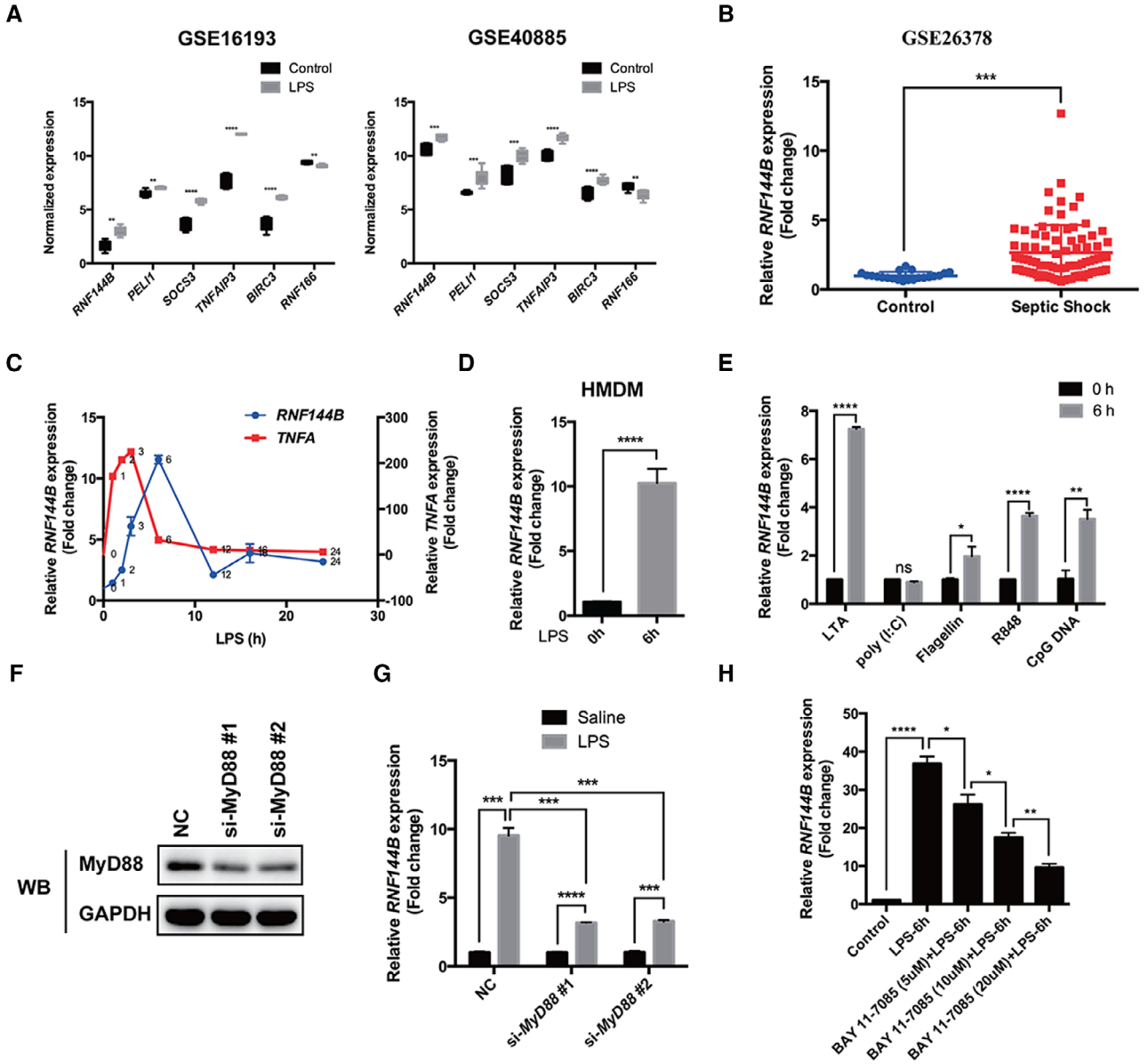

FIGURE 1 **LPS-induced RNF144B expression is dependent on MyD88-mediated NF-κB activation.** (**A**) Consistent differentially expressed E3 ligases in both GSE16193 and GSE40885. (**B**) *RNF144B* up-regulation in septic shock samples was observed in GSE26378. (**C**) *RNF144B* expressional dynamics upon LPS treatment were revealed in THP-1 cells by QPCR analysis. (**D**) *RNF144B* expression in human monocyte-derived macrophage (HMDM) induced by LPS for 6 h. (**E**) *RNF144B* expression changes were determined by LTA, poly (I:C), flagellin, R848, and CpG ODNs treatment for 6 h in THP-1 cells. (**F**) MyD88 siRNAs were transfected to THP-1 cells for 48 h, and the knockdown efficiency was confirmed by Western blot. (**G**) *RNF144B* expression was detected by QPCR analysis in THP-1 cells transfected with siRNAs of MyD88 and treated with LPS. (**H**) BAY 11–7085 inhibition of LPS-induced *RNF144B* expression were confirmed by QPCR in THP-1 cells. $^{****}P < 0.001$, $^{***}P < 0.005$, $^{**}P < 0.01$, and $^{*}P < 0.05$ (2-tailed Student's *t*-test)

active state for TBK1 and IRF3 (Fig. 2D). The protein level of phosphorylated IRF3 was quantified by ImageJ software (Fig. 2D), and it is obvious that RNF144B knockdown could enhance the phosphorylation of both TBK1 and IRF3, which are the essential for IFN-β production. To further demonstrate RNF144B influence on the activation of TBK1 mediated IRF3 activation, we cotransfected plasmids for *IFNB*-promoter driven luciferase and RNF144B overexpression in THP-1 cells, and the results showed that RNF144B overexpression dramatically attenuated *IFNB*-promoter driven luciferase activity induced by LPS (Fig. 2E). In summary, the data suggested that RNF144B has a negative effect on LPS-induced TBK1 and IRF3 activation and therefore attenuates IFN-β expression.

3.3 | RNF144B targets TBK1

It is well known that when the LPS is recognized by membrane TLR4, TLR4 dimerizes. The TLR4 dimers recruit adaptors of TRIAP and MyD88 for IRAK signaling complex docking, which further summons

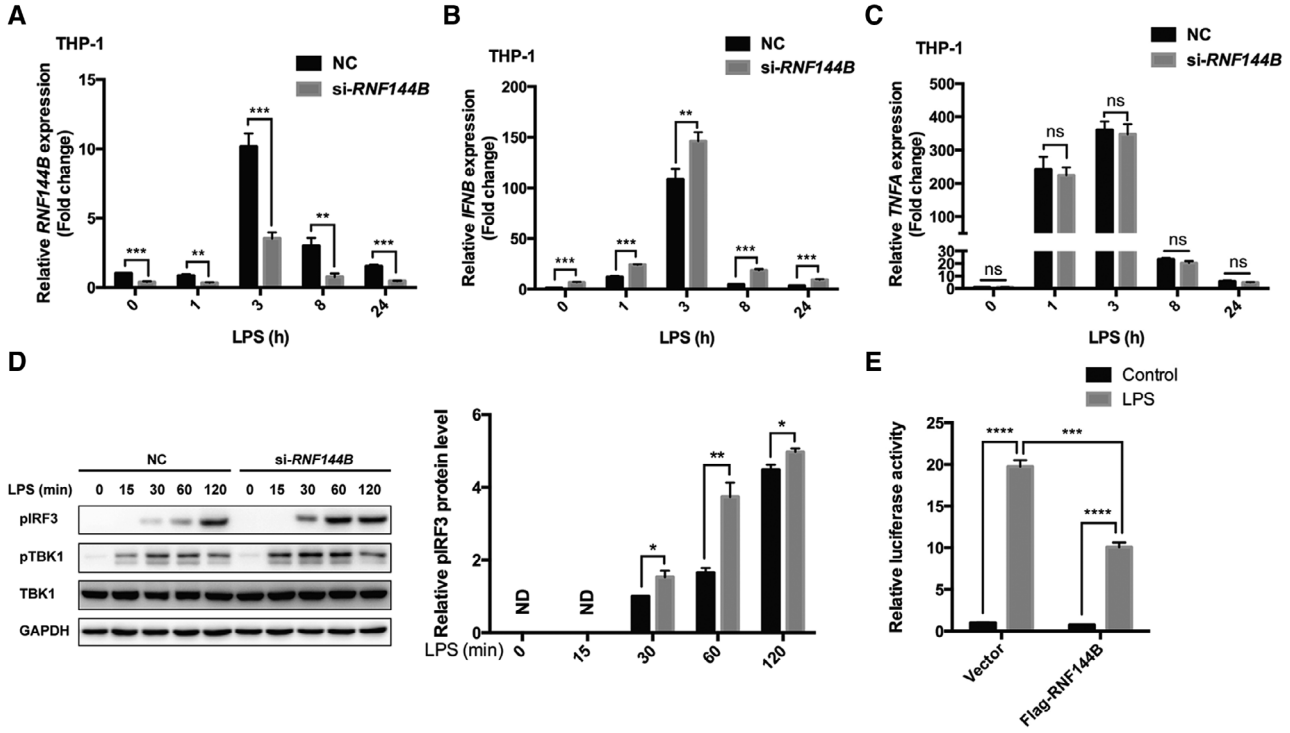

FIGURE 2 **RNF144B negatively regulates IRF3 activation and IFN-β production.** (**A**) siRNA-mediated RNF144B knockdown efficiency was determined by QPCR analysis in THP-1 cells. (**B**) *IFNB* mRNA expression was detected by QPCR analysis with RNF144B knockdown upon LPS treatment at different time points. (**C**) *TNFA* mRNA expression was detected by QPCR analysis with RNF144B knockdown upon LPS treatment at different time points. (**D**) Phosphorylation levels of IRF3 and TBK1 were detected by Western blot with RNF144B knockdown upon LPS treatment at different time points, and the protein level for phosphorylated IRF3 was quantified by ImageJ software. (**E**) *IFNB*-promoter driven luciferase plasmid was cotransfected with RNF144B over-expression plasmid in THP-1 cells and treated with LPS, and luciferase activity was detected by a dual luciferase assay kit. The indicated antibodies were used. $^{****}P < 0.001$, $^{***}P < 0.005$, $^{**}P < 0.01$, and $^{*}P < 0.05$ (2-tailed Student's *t*-test)

TRAF6, activating TAK1 and NF-κB. On the other hand, a fraction of membrane bound TLR4 is endocytosed to endosome for signaling transduction, upon which TRAM and TRIF are exploited for TRAF3 and TBK1 recruitment, which induces IFN-β expression and production. In order to decipher the potential targets of RNF144B in the IFN-β production-signaling pathway and exclude its involvement in regulating MyD88-TRAF6 signaling pathway, we constructed plasmids for Myc tag-labeled RNF144B and Flag tag-labeled TRIF, TRAF3, TBK1, and TRAF6. To test the interaction between RNF144B and these candidate targets genes, we cotransfected RNF144B along with TRIF, TRAF3, TBK1, and TRAF6 individually and the cell lysate was immunoprecipitated with Flag antibody (Fig. 3A). RNF144B coimmunoprecipitated with TRIF, TRAF3, and TBK1 except TRAF6, which demonstrated that RNF144B might form a complex with these signaling transduction molecules. TBK1 is functional downstream of both TRIF and TRAF3, so we speculated that RNF144B was most likely to target TBK1. By cotransfection of RNF144B and TRIF or TBK1 respectively in 293T cell, we found that RNF144B could both inhibit TRIF and TBK1-mediated IFN-β promoter-driven luciferase reporter activity and *IFNB1* mRNA expression (Figs. 3B and 3C). Meanwhile, by Western blotting analysis and quantification for phosphorylated IRF3 protein by ImageJ (Fig. 3D), we also detected that RNF144B could dramatically reduce TRIF and TBK1-induced IRF3 activation, indicating that TBK1 is a target of RNF144B. It is known that TBK1 and RNF144B are both structurally composed of several domains. To reveal the interaction between TBK1 and RNF144B in detail, we constructed a series of truncations of both TBK1 and RNF144B for coimmunoprecipitation analysis. The results showed that SDD of TBK1 was essential, and the IBR domain of RNF144B was indispensable for the interaction (Figs. 3E and 3F).

3.4 | RNF144B negative regulation of IFN-β production is independent of its E3 ligase activity

RNF144B is also known as "in between ring finger domains containing protein 2″ (IBRDC2) because it contains an IBR domain between 2 different RING domains. It is speculated that the cysteine of 206 (C206) residing in RING2 is responsible for E3 ligase activity; however, there is no relevant publication to verify this. In order to confirm whether the inhibitory function of RNF144B is dependent on its E3 ligase activity, we constructed various mutations and truncations of RNF144B, which may lack E3 ligase activity, including RING1 deletion, RING2 deletion, C206A, and C206A/C211A/C213/A/C215A. Mutation of IBR deletion that abrogates its ability for TBK1 binding was used as a positive control for not affecting TBK1 or IRF3 activation. We cotransfected these plasmids with IFN-β promoter-driven luciferase reporter and found

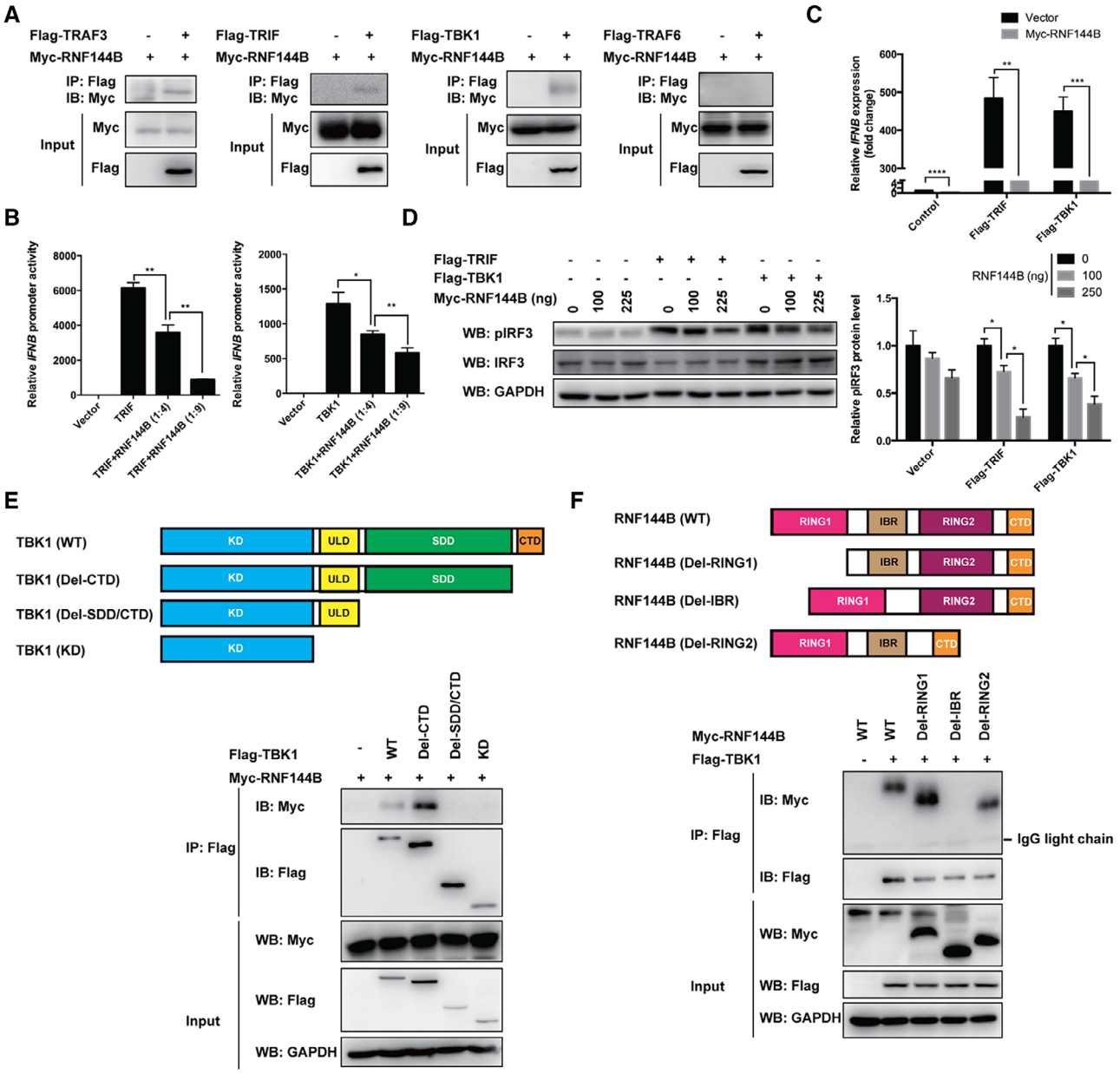

FIGURE 3 **RNF144B targets TBK1.** (**A**) Myc-RNF144B was cotransfected with Flag-tagged TRAF3, TRIF, TBK1, and TRAF6 individually, the interactions were confirmed by coimmunoprecipitation and Western blot analysis. (**B**) Luciferase activity of IFN-β promoter reporter in HEK293 cells transfected with expression plasmids for TRIF and TBK1 along with Myc-RNF144B for 24 h. (**C**) *IFNB* mRNA expression level was determined in HEK293 cells transfected with plasmids for TRIF and TBK1 along with Myc-RNF144B. (**D**) Phosphorylated IRF3 protein level was determined in HEK293 cells transfected with Flag-tagged TRIF and TBK1 along with Myc-RNF144B, and quantified by ImageJ software. (**E**) Immunoprecipitation and immunoblot analysis of Flag-tagged TBK1-WT, TBK1-del-CTD, TBK1-del-SDD/CTD, or TBK1-KD with Myc-RNF144B. (**F**) Immunoprecipitation and immunoblot analysis of Myc-tagged RNF144B-WT, RNF144B-Del-RING1, RNF144B-Del-IBR, or RNF144B-Del-RING2 with Flag-TBK1. $^{****}P < 0.001$, $^{***}P < 0.005$, $^{**}P < 0.01$, and $^{*}P < 0.05$ (2-tailed Student's *t*-test)

that all of these mutations could inhibit TBK1 induced IFN-β promoter activity except IBR deletion (Fig. 4A). Further, we cotransfected these mutations with TRIF in 293T cells to check whether RNF144B mutations could also inhibit TRIF-induced TBK1 and IRF3 phosphorylation. Consistent with the luciferase reporter assay, these mutations except IBR deletion had comparable inhibitory effects on TBK1 and IRF3 activation with wild type RNF144B (Fig. 4B). Collectively, these results demonstrated that RNF144B E3 ligase activity is not essential for its inhibitory function on TBK1 and IRF3 activation.

3.5 | RNF144B inhibits TBK1 phosphorylation and K63-linked polyubiquitination

RNF144B interaction with TBK1 is sufficient for its inhibition of TBK1-induced IRF3 activation. It is well known that TBK1 activation is dependent on its phosphorylation and K63-linked polyubiquitination states. Since E3 ligase activity of RNF144B does not promote TBK1 degradation, we hypothesized that RNF144B interaction with TBK1 might decrease its phosphorylation and K63-linked

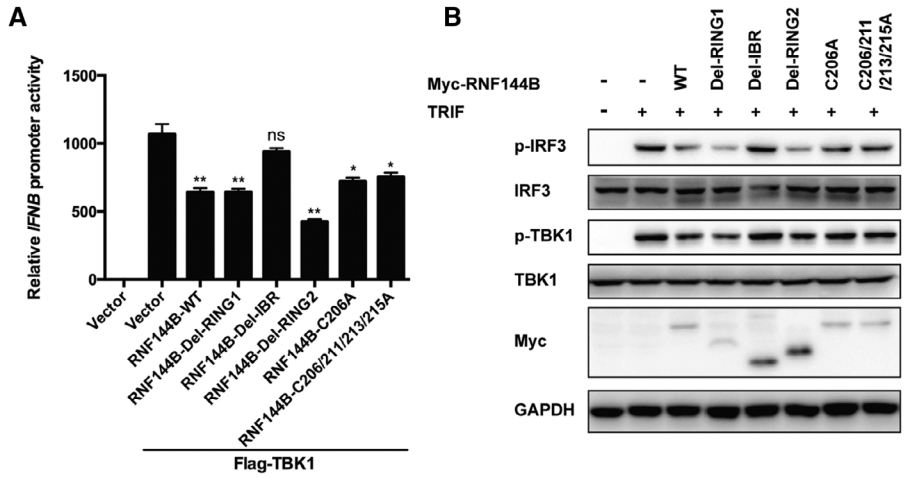

FIGURE 4 **RNF144B negative regulation of IFN-β production is independent of its E3 ligase activity.** (**A**) Luciferase activity of IFN-β promoter reporter in HEK293 cells transfected with expression plasmids of Myc-tagged RNF144B-WT, RNF144B-Del-RING1, RNF144B-Del-IBR, RNF144B-Del-RING2, RNF144B-C206A, or RNF144B-C206/211/213/215A along with Flag-TBK1 for 24 h. (**B**) Immunoblot analysis of phosphorylated IRF3 and TBK1 in HEK293 cells transfected with Flag-TRIF together with Myc-tagged RNF144B-WT, RNF144B-Del-RING1, RNF144B-Del-IBR, RNF144B-Del-RING2, RNF144B-C206A, or RNF144B-C206/211/213/215A. ****$P < 0.001$, ***$P < 0.005$, **$P < 0.01$, and *$P < 0.05$ (2-tailed Student's *t*-test)

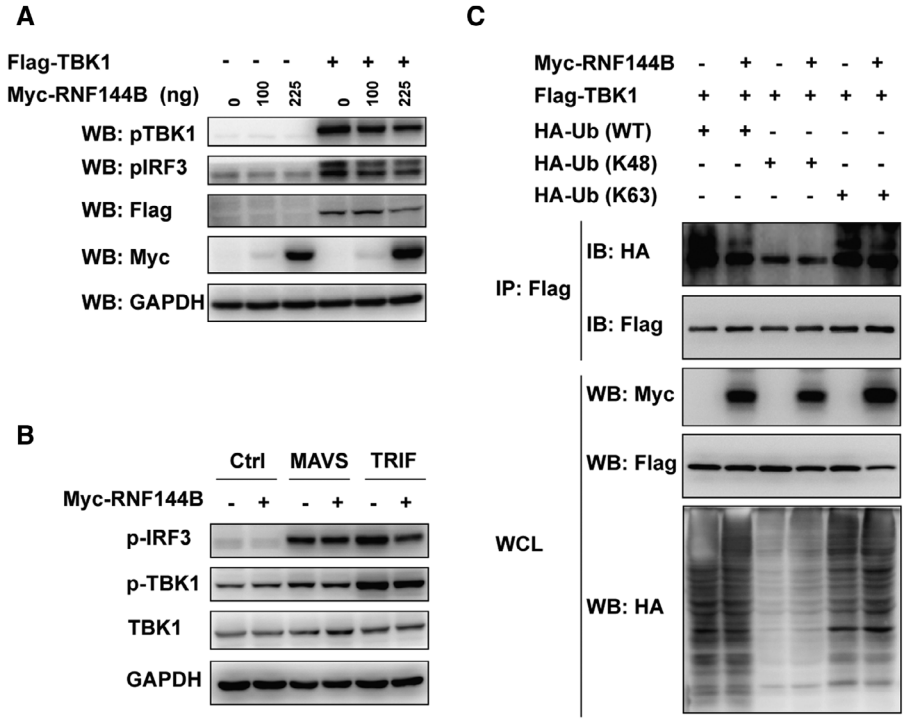

FIGURE 5 **RNF144B inhibits TBK1 phosphorylation and K63-linked polyubiquitination.** (**A**) Immunoblot for phosphorylated TBK1 and IRF3 in HEK293 cells transfected with Flag-TBK1 and different doses of Myc-RNF144B. (**B**) Immunoblot for phosphorylated TBK1 and IRF3 in HEK293 cells transfected with Myc-RNF144B together with Flag-tagged MAVS and TRIF. (**C**) Coimmunoprecipitation analysis of TBK1 ubiquitination in HEK293 cells cotransfected with Flag-TBK1, Myc-RNF144B, or control vector and HA-ubiquitin-WT, HA-ubiquitin-K48, or HA-ubiquitin-K63 plasmids

polyubiquitination. Western blot analysis of harvested cells showed that RNF144B inhibited TBK1 phosphorylation in a dose-dependent manner (Fig. 5A). To prove that RNF144B specifically inhibits LPS induced IRF3 activation, RNF144B was cotransfected with MAVS or TRIF, and protein level of phosphorylated TBK1 and IRF3 were detected (Fig. 5B). The results showed that RNF144B specifically inhibited TRIF mediated TBK1/IRF3 activation without affecting that of MAVS. Further, to determine whether TBK1 ubiquitination state was affected by RNF144B, HA-tagged ubiquitin (WT, K48, and K63) were cotransfected with RNF144B and TBK1, and the ubiquitination level

of TBK1 was detected (Fig. 5C). The results illustrated that K63 linked polyubiquitination of TBK1 was dramatically decreased by RNF144B, leaving K48 linked polyubiquitination of TBK1 unaffected. These results indicated that the binding of RNF144B with TBK1 is sufficient to inhibit its phosphorylation and K63-linked polyubiquitination.

4 | DISCUSSION

Excessive IFN-β production can cause unwanted inflammatory damage to the host. TBK1 is an essential signaling transducer for TLR-mediated production of type I IFN. Precise regulation for TBK1 activity is pivotal for maintaining immunologic homeostasis. Our present study identified RNF144B, a RING domain-containing E3 ligase, as a regulator that limits TLR4-mediated inflammatory responses through TBK1. Our findings that RNF144B was up-regulated in patients with sepsis indicated that RNF144B might have a role in the pathogenesis of certain infectious diseases. Moreover, we found that the expression of *RNF144B*, but not *RNF144A* (data not shown), dynamically responded to LPS treatment of bacterial infection. In addition to LPS, other TLR ligands, including LTA (a ligand of TLR2), flagellin (a ligand of TLR5), R848 (a ligand of TLR7 and TLR8), and CpG ODNs (a ligand of TLR9) also induced *RNF144B* expression to certain degrees; poly (I:C) (a ligand of TLR3) did not. When THP-1 cells were transfected with siRNAs targeting MyD88 or treated with BAY-11-7085, *RNF144B* induction by LPS dramatically decreased. The data demonstrated that RNF144B might generally respond to inflammatory signaling via all TLR-MyD88 except TLR3.

Here, we identified that the ubiquitin E3 ligase RNF144B suppresses LPS-mediated inflammatory responses. Our results in THP-1 cells showed that RNF144B knockdown increased LPS-induced TBK1 and IRF3 phosphorylation and increased the expression level of IFN-β. Overexpression of RNF144B restrained TRIF and TBK1-mediated IFN-β promoter activity and expression. RNF144B mutations were constructed to cover each possible active site for its E3 ligase activity, including RING1 deletion (Del-RING1), RING2 deletion (Del-RING2), 206 cysteine to alanine (C206A), and cysteines of 206, 211, 213, 215 to alanine (C206A/C211A/C213A/C215A). Like wild type RNF144B, these mutations of RNF144B have comparable inhibitory effects on TBK1-induced IFN-β promoter activity and TRIF mediated TBK1 and IRF3 phosphorylation, showing that E3 ligase activity of RNF144B is not necessary for the inhibition of LPS-induced *IFNB* expression.

TBK1 is composed of 4 characterized domains, including kinase domain (KD), ubiquitin-like domain, SDD, and CTD.[33] Coimmunoprecipitation analysis for interaction mapping showed that the IBR domain of RNF144B interacts with the SDD of TBK1. It has been structurally reported that SDD is important for TBK1 modification and function.[34] The structure reveals that SDD together with KD and ULD form an extensive network of interaction, which contributes to a dimeric assembly of TBK1. It is also reported that TBK1 undergoes K63-linked polyubiquitination at Lys 401 in SDD, and this modification requires an intact dimer and in turn is required for TBK1 activation. Double mutation of Lys401 and Lys30 of KD (K30A/K401A) abrogates TBK1 phosphorylation and fails to elicit *IFNB* mRNA expression. In addition to acting as a mediator of dimer formation and a site of polyubiquitination, the SDD is also required for proper substrate phosphorylation in cells; the conserved surface surrounding Glu580 is required for IRF3 phosphorylation and reporter activation. The SDD is not required for efficient IRF3 phosphorylation in vitro, and so this surface may be a site of interaction with substrate-recruiting adaptors such as TRIF. Since RNF144B inhibits TBK1 phosphorylation and K63-linked polyubiquitination by interacting with SDD of TBK1, there are several possible underlying mechanisms. First, the interaction between RNF144B and TBK1 may conceal the sites for TBK1 dimer formation. Secondly, the interaction may occur in the proximity of K401 or hide K401 for K63-linked polyubiquitination. Moreover, the interaction may directly affect the ability of TBK1 to phosphorylate IRF3 by blocking the interaction of TBK1 and TRIF. The identification of RNF144B as a negative regulator of IFN-β will not only have important implications for understanding of the immunologic homeostasis but also offers a potential target for the therapy of autoimmune diseases or inflammatory diseases.

AUTHORSHIP

Z.Z., B.W., and X.L. designed the research. Z.Z. generated the figures and tables and performed Western blot, coimmunoprecipitation and quantitative PCR assay. L.Y.Z. performed bioinformatics analysis. X.X.Z., L.Z., C.C., and Q.G. cultured and transfected the cells. R.W., X.Q.Y., and Y.H.Z. constructed the plasmids and performed the luciferase reporter assay. Z.Z. and X.L. interpreted the data and wrote the manuscript. All authors have read and approved the final manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

1. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell*. 2010;140:805-820.
2. Tracey KJ. Reflex control of immunity. *Nat Rev Immunol*. 2009;9:418-428.

3. Perry AK, Chen G, Zheng D, Tang H, Cheng G. The host type I interferon response to viral and bacterial infections. *Cell Res*. 2005;15:407-422.
4. Miller SI, Ernst RK, Bader MW. LPS, TLR4 and infectious disease diversity. *Nat Rev Microbiol*. 2005;3:36-46.
5. Mitchell JA, Paul-Clark MJ, Clarke GW, Mcmaster SK, Cartwright N. Critical role of toll-like receptors and nucleotide oligomerisation domain in the regulation of health and disease. *J Endocrinol*. 2007;193:323-330.
6. Wu J, Chen ZJ. Innate immune sensing and signaling of cytosolic nucleic acids. *Annu Rev Immunol*. 2014;32:461-488.
7. Takeuchi O, Akira S. Innate immunity to virus infection. *Immunol Rev*. 2009;227:75-86.
8. Beutler B. Inferences, questions and possibilities in Toll-like receptor signalling. *Nature*. 2004;430:257-263.
9. Crow MK. Type I interferon in organ-targeted autoimmune and inflammatory diseases. *Arthritis Res Ther*. 2010;12(Suppl 1):S5.
10. Song G, Liu B, Li Z, et al. E3 ubiquitin ligase RNF128 promotes innate antiviral immunity through K63-linked ubiquitination of TBK1. *Nat Immunol*. 2016;17:1342-1351.
11. Wang C, Chen T, Zhang J, et al. The E3 ubiquitin ligase Nrdp1 'preferentially' promotes TLR-mediated production of type I interferon. *Nat Immunol*. 2009;10:744-752.
12. Cui J, Li Y, Zhu L, et al. NLRP4 negatively regulates type I interferon signaling by targeting the kinase TBK1 for degradation via the ubiquitin ligase DTX4. *Nat Immunol*. 2012;13:387-395.
13. Zhang M, Wang L, Zhao X, et al. TRAF-interacting protein (TRIP) negatively regulates IFN-beta production and antiviral response by promoting proteasomal degradation of TANK-binding kinase 1. *J Exp Med*. 2012;209:1703-1711.
14. Friedman CS, O'donnell MA, Legarda-Addison D, et al. The tumour suppressor CYLD is a negative regulator of RIG-I-mediated antiviral response. *EMBO Rep*. 2008;9:930-936.
15. Zhang L, Zhao X, Zhang M, Zhao W, Gao C. Ubiquitin-specific protease 2b negatively regulates IFN-beta production and antiviral activity by targeting TANK-binding kinase 1. *J Immunol*. 2014;193:2230-2237.
16. Huang J, Xu LG, Liu T, Zhai Z, Shu HB. The p53-inducible E3 ubiquitin ligase p53RFP induces p53-dependent apoptosis. *FEBS Lett*. 2006;580:940-947.
17. Ng CC, Arakawa H, Fukuda S, Kondoh H, Nakamura Y. p53RFP, a p53-inducible RING-finger protein, regulates the stability of p21WAF1. *Oncogene*. 2003;22:4449-4458.
18. Conforti F, Yang AL, Piro MC, et al. PIR2/Rnf144B regulates epithelial homeostasis by mediating degradation of p21WAF1 and p63. *Oncogene*. 2013;32:4758-4765.
19. Sayan BS, Yang AL, Conforti F, et al. Differential control of TAp73 and DeltaNp73 protein stability by the ring finger ubiquitin ligase PIR2. *Proc Natl Acad Sci USA*. 2010;107:12877-12882.
20. Benard G, Neutzner A, Peng G, et al. IBRDC2, an IBR-type E3 ubiquitin ligase, is a regulatory factor for Bax and apoptosis activation. *EMBO J*. 2010;29:1458-1471.
21. O'donoghue SI, Gavin AC, Gehlenborg N, et al. Visualizing biological data-now and in the future. *Nat Methods*. 2010;7:S2-4.
22. Bindea G, Mlecnik B, Hackl H, et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics*. 2009;25:1091-1093.
23. Chhikara M, Wang S, Kern SJ, et al. Carbon monoxide blocks lipopolysaccharide-induced gene expression by interfering with proximal TLR4 to NF-kappaB signal transduction in human monocytes. *PLoS One*. 2009;4:e8139.
24. Reynier F, De Vos AF, Hoogerwerf JJ, et al. Gene expression profiles in alveolar macrophages induced by lipopolysaccharide in humans. *Mol Med*. 2012;18:1303-1311.
25. Xiao Y, Jin J, Zou Q, Hu H, Cheng X, Sun SC. Peli1 negatively regulates type I interferon induction and antiviral immunity in the CNS. *Cell Biosci*. 2015;5:34.
26. Croker BA, Krebs DL, Zhang JG, et al. SOCS3 negatively regulates IL-6 signaling in vivo. *Nat Immunol*. 2003;4:540-545.
27. Saitoh T, Yamamoto M, Miyagishi M, et al. A20 is a negative regulator of IFN regulatory factor 3 signaling. *J Immunol*. 2005;174:1507-1512.
28. Mahoney DJ, Cheung HH, Mrad RL, et al. Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. *Proc Natl Acad Sci USA*. 2008;105:11778-11783.
29. Chen HW, Yang YK, Xu H, Yang WW, Zhai ZH, Chen DY. Ring finger protein 166 potentiates RNA virus-induced interferon-beta production via enhancing the ubiquitination of TRAF3 and TRAF6. *Sci Rep*. 2015;5:14770.
30. Deforge LE, Remick DG. Kinetics of TNF, IL-6, and IL-8 gene expression in LPS-stimulated human whole blood. *Biochem Biophys Res Commun*. 1991;174:18-24.
31. Ariffin JK, Kapetanovic R, Schaale K, et al. The E3 ubiquitin ligase RNF144B is LPS-inducible in human, but not mouse, macrophages and promotes inducible IL-1beta expression. *J Leukoc Biol*. 2016;100:155-161.
32. Janssens S, Beyaert R. A universal role for MyD88 in TLR/IL-1R-mediated signaling. *Trends Biochem Sci*. 2002;27:474-482.
33. De Jonge HJ, Woolthuis CM, Vos AZ, et al. Gene expression profiling in the leukemic stem cell-enriched CD34+ fraction identifies target genes that predict prognosis in normal karyotype AML. *Leukemia*. 2011;25:1825-1833.
34. Tu D, Zhu Z, Zhou AY, et al. Structure and ubiquitination-dependent activation of TANK-binding kinase 1. *Cell Rep*. 2013;3:747-758.

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