Deactivating TBK1 Immunity by Dephosphorylating and IFN Production and Antiviral Innate Phosphatase PP4 Negatively Regulates Type I Xingguang Liu, Zhenzhen Zhan, Hao Cao, Xuefeng Xie, Linshan Yang, Peng Zhang, Yihan Chen, Huimin Fan, Zhongmin Liu and Xingguang Liu

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Phosphatase PP4 Negatively Regulates Type I IFN Production and Antiviral Innate Immunity by Dephosphorylating and Deactivating TBK1

Zhenzhen Zhan,*† Hao Cao,* Xuefeng Xie,* Linshan Yang,* Peng Zhang,‡ Yihan Chen,*† Huimin Fan,* Zhongmin Liu,* and Xingguang Liu‡

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The phosphorylation and activation of kinase TANK-binding kinase 1 (TBK1) plays crucial roles in the production of type I IFN mediated by TLR and retinoic acid–inducible gene I–like receptors. Type I IFN expression must be tightly regulated to prevent the development of immunopathological disorders. However, how the activated TBK1 is negatively regulated by phosphatases remains poorly understood. In this study, we identified a previously unknown role of protein phosphatase (PP4) by acting as a TBK1 phosphatase. PP4 expression was upregulated in macrophages infected with RNA virus, vesicular stomatitis virus, and Sendai virus in vitro and in vivo. Knockdown of PP4C, the catalytic subunit of PP4, significantly increased type I IFN production in macrophages and dendritic cells triggered by TLR3/4 ligands, vesicular stomatitis virus, and Sendai virus, and thus inhibited virus replication. Similar results were also found in peritoneal macrophages with PP4C silencing in vivo and i.p. infection of RNA virus. Accordingly, ectopic expression of PP4C inhibited virus-induced type I IFN production and promoted virus replication. However, overexpression of a phosphatase-dead PP4C mutant abolished the inhibitory effects of wild-type PP4C on type I IFN production. Mechanistically, PP4 directly bound TBK1 upon virus infection, then dephosphorylated TBK1 at Ser172 and inhibited TBK1 activation, and subsequently restrained IFN regulatory factor 3 activation, resulting in suppressed production of type I IFN and IFN-stimulated genes. Thus, serine/threonine phosphatase PP4 functions as a novel feedback negative regulator of RNA virus-triggered innate immunity. The Journal of Immunology, 2015, 195: 000–000.

A key feature of the antiviral innate immune response is the rapid induction and extracellular secretion of type I IFN that restricts virus replication (1, 2). Cytoplasmic RNA sensors such as retinoic acid–inducible gene 1 (RIG-I) and melanoma differentiation–associated gene 5 (MDA5), two well-known retinoic acid–inducible gene I–like receptor family proteins, can recognize viral RNA and trigger antiviral signaling pathways (3, 4). RIG-I and MDA5 contain CARD domains that recruit IFN-β promoter stimulator 1 (IPS-1), which is an adaptor molecule localized on the mitochondrial membrane (4, 5). IPS-1 associates with TNFR-associated factor (TRAF)3, leading to the phosphorylation at Ser72 and activation of TANK-binding kinase 1 (TBK1) (6, 7). The activated TBK1 phosphorylates and activates IFN regulatory factor (IRF)3 and IRF7 to induce the production of type I IFN and IFN-stimulated genes (ISGs), which is responsible for inhibition of virus replication and clearance of virus-infected cells (8). Additionally, TLR3 also can recognize viral dsRNA to trigger type I IFN production through TBK1-mediated IRF3 activation (9).

Although sufficient production of type I IFN is crucial for virus clearance, uncontrolled and excessive type I IFN expression causes pathological immune response to the host or autoimmune disorders, and thus host cells have developed distinct strategies to tightly regulate the activation of antiviral signaling and maintain the homeostasis of both the innate and adaptive immunity (10–12). As a critical kinase and central player involved in type I IFN signaling, TBK1 activation can be regulated by multiple molecules in various ways, such as phosphorylation, ubiquitination, and kinase activity modulation (13, 14). However, how the kinase activity of TBK1 is switched off by its phosphatases remains poorly understood. Until now, there are three phosphatases that have been reported to negatively regulate type I IFN production by targeting TBK1 (15–17). Src homology 2 domain–containing protein tyrosine phosphatase 2 (SHP-2) inhibits TBK1 activity through a phosphatase activity–dependent mechanism (15). The absence of SHP-1 results in the

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; HA, hemagglutinin; IPS-1, IFN-β promoter stimulator 1; IRAK1, IL-1R–associated kinase 1; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; MDA5, melanoma differentiation–associated gene 5; MOI, multiplicity of infection; ODN, oligodeoxynucleotide; poly (I:C), polyinosinic-polycytidylic acid; PP, protein phosphatase; PP4C, catalytic unit of PP4; PPMB12, PP Mg2+/Mn2+–dependent 1B; RIG-I, retinoic acid–inducible gene I; SeV, Sendai virus; SHP-2, Src homology region 2 domain–containing phosphatase 2; siRNA, small interfering RNA; TBK1, TANK-binding kinase 1; TRAF, TNFR-associated factor; VSV, vesicular stomatitis virus.

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increased level of TBK1 phosphorylation (16). However, it is unlikely that SHP-2 and SHIP-1 function as TBK1 phosphatases to directly dephosphorylate and deactivate TBK1 (15, 16). Only one serine/threonine phosphatase, protein phosphatase (PP) Mg\(^{2+}\)/Mn\(^{2+}\)-dependent 1B (PPM1B), is found to bind TBK1 and dephosphorylate TBK1 at Ser\(^{172}\), and then inhibit TBK1 activation in type I IFN signaling and antiviral innate immunity.

HBK293T cells and HeLa cells (17). However, whether other phosphatases also function in the direct regulation of phosphorylation and kinase activity of TBK1 in innate immune cells remains unknown, which needs to be further elucidated.

The PP2A family, a serine/threonine phosphatase family that comprises a common domain of PP2A-like phosphatase, includes PP2A, PP4, PP5, and PP6 and is implicated in many biological processes (18). PP4 is a holoenzyme that is comprised of the catalytic subunit of PPR (PP4C) in association with PP2R regulatory subunits (19). PP4 is highly conserved during evolution and is ubiquitously expressed in many tissues. Although PP4 has been shown to be involved in multiple cellular processes, including regulation of microtubule organization, DNA damage repair, thymocyte development, and regulatory T cell functions, its role in the regulation of type I IFN signaling and antiviral immunity remains unknown (20–23). In the present study, we found that PP4, but not other members of PP2A family, most significantly inhibited RNA virus-induced IFN-β reporter activity by utilizing IFN-β luciferase reporter–based screen. Furthermore, PP4 directly interacted with TBK1 after virus infection, then dephosphorylated TBK1 at Ser\(^{172}\) and inhibited TBK1 activation, and subsequently inhibited type I IFN production and antiviral innate immune response. Our findings identify serine/threonine phosphatase PP4 as a phosphatase for TBK1 to maintain immune homeostasis during antiviral innate immunity.

Materials and Methods

Mice and reagents

C57BL/6 mice 6–8 wk old were from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University (Shanghai, China). LPS (011B:B4) and polyinosinic-polycytidylic acid (polyI:polyC) was from Sigma-Aldrich. CpG oligodeoxynucleotide (ODN) was synthesized and repurified as described previously (24). Ab specific for the PP4 catalytic subunit was from Bethyl Laboratories. Abs to TBK1 phosphorylated at Ser\(^{172}\), IRF3 phosphorylated at Ser\(^{396}\), total TBK1, IKK ε, and hemagglutinin (HA) tags were from Cell Signaling Technology. 2 FP4 INHIBITS ANTIVIRAL RESPONSE BY DEPHOSPHORYLATING TBK1

RNA interference

The sequences of small interfering RNAs (siRNAs) targeting two different sites of PP4 catalytic subunit (PPp4c) were gene 5′-TGGACTCGCC-CAGTCACTAG-3′ (no. 1) and 5′-CGCTGGTTCATCACTGT-3′ (no. 2). The control small RNA sequence was 5′-TTCTCCGAAGCTGCT-3′: siRNA duplexes were synthesized by GenePharma (Shanghai, China) and transfected into mouse peritoneal macrophages and BMDCs using INTERFE(Rn (Polyplus Transfection) according to the manufacturer’s instructions. For in vivo silencing of PP4C in peritoneal cells, siRNA duplexes were complexed with in vivo jetPEI reagent (Polyplus Transfection) at the N/P ratio of 7 according to the instructions, and then the transfection mixture was i.p. injected into mice.

RNA quantification

Total RNA was extracted with TRIzol reagent (Invitrogen). Quantitative real-time PCR analysis was performed with LightCycler (Roche) and a SYBR RT-PCR kit (Takara Bio). The primers for PP4C mRNA analysis were: 5′-CGCTGGTAAAGAGGAACCACTG-3′ (forward) and 5′-CGCCACCTACTCTGAAAGC-3′ (reverse). The primers for mouse Ifnb1, Ifnr4, human IFNB1, ISG15, and CCL15 mRNA analyses have been described (25, 26). Data were normalized to β-actin expression in each sample.

Detection of cytokines

IFN-β and IFN-α levels in the supernatants were measured by an ELISA kit (PBL Assay Science) according to the manufacturer’s instructions.

Assay of luciferase reporter gene expression

HEK293 cells were cotransfected with the indicated luciferase reporter plasmid, pRL-TK-Renilla-luciferase plasmid, and the indicated other constructs. Total amounts of plasmid DNA were equalized with empty control vector. After 24 h, luciferase activities in cell lysates were measured using Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Data were normalized for transfection efficiency by dividing firefly luciferase activity with that of Renilla luciferase.

Immunoblot and immunoprecipitation analyses

Cells were lysed with cell lysis buffer (Cell Signaling Technology) supplemented with protease inhibitor mixture. Nuclear protein was prepared with NE-PER nuclear and cytoplasmic protein extraction reagent (Thermo Scientific). Protein concentrations of the extracts were measured with BCA assay (Thermo Fisher Scientific). The immunoblot analysis and immunoprecipitation analysis were performed as described previously (25, 26).

GST pull-down assay

GST fusion proteins containing the wild-type PP4 catalytic subunit (GST-PP4C) were expressed in Escherichia coli (BL21) and purified by GST affinity chromatography (Thermo Fisher Scientific). GST-PP4C protein was incubated with recombinant TBK1 protein (Abcam) at 4˚C for 30 min and further incubated with glutathione–Sepharose 4B (Thermo Fisher Scientific) for 2 h in immunoprecipitated buffer containing Nonidet P-40. After washing three times, the precipitates were subjected to Western blot analysis as previously described (24).

In vitro phosphatase assay

Recombinant active TBK1 protein (Abcam) was preincubated with wild-type or R236L mutant GST-PP4C protein in iced water for 15 min. Then recombinant IRF3 protein and a reaction buffer containing ATP (Upstate Biotechnology) were added, followed by incubation for 20 min at 30˚C. Phosphorylation of TBK1 and IRF3 was detected by immunoblot with Ab to TBK1 phosphorylated at Ser\(^{172}\) and Ab to IRF3 phosphorylated at Ser\(^{9}\) as previously described (15).

Kinase activity assay

Endogenous TBK1 or IKKe protein was immunoprecipitated from cell lysates with anti-TBK1 or IKKe Abs. Pellets were washed three times with lysin buffer and then resuspended in kinase reaction buffer (40 mM Tris-HCl [pH 7.5], 20 mM MgCl\(_2\), 0.1 μg/μl BSA, and 0.05 mM DTT). The

Prepared and cultured as described previously (26). The plasmids were nucleofected into mouse peritoneal macrophages and RAW264.7 cells with corresponding Nucleofector kits using Amazta Nucleofector II biosystems (Lonza).

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Kinase activity assay

Endogenous TBK1 or IKKe protein was immunoprecipitated from cell lysates with anti-TBK1 or IKKe Abs. Pellets were washed three times with lysis buffer and then resuspended in kinase reaction buffer (40 mM Tris-HCl [pH 7.5], 20 mM MgCl\(_2\), 0.1 μg/μl BSA, and 0.05 mM DTT). The
kinase activity of TBK1 or IKKε was assayed with a kinase enzyme system and an ADP-Glo kinase assay kit, according to the manufacturer’s instructions (Promega).

**In vitro and in vivo infection of vesicular stomatitis virus and Sendai virus**

Vesicular stomatitis virus (VSV; Indiana strain) was propagated and amplified by infection of a monolayer of Vero cells. Mouse peritoneal macrophages, BMDCs, and RAW264.7 cells were in vitro infected with VSV (multiplicity of infection [MOI] of 10) or Sendai virus (SeV; MOI of 1) for the indicated time. VSV RNA levels in the supernatant and cells as well as viral titers were quantified as described previously (27, 28). For in vivo virus infection, age- and sex-matched mice were i.p. infected with VSV (5 x 10^2 PFU/g) or SeV (1 x 10^3 PFU/g) for the indicated times. The peritoneal lavages were harvested, and then peritoneal fluids were used for viral titer analysis or concentrated using a Amicon ultra filter (Millipore) for ELISA of type I IFN; meanwhile, macrophages were sorted using MoFlo XDP (Beckman Coulter) from peritoneal exudate cells that were stained with F4/80 and CD11b Abs. The sorted peritoneal macrophages were used for the analysis of type I IFN mRNA and VSV RNA levels as well as for immunoblot and immunoprecipitation analysis (25).

**Statistical analysis**

The statistical significance of comparisons between two groups was determined with a two-tailed Student t test, whereas comparisons among multiple groups were assessed by ANOVA. A p value of < 0.05 was considered statistically significant.

**Results**

**IFN-β luciferase screen identifies PP4 as a regulator of anti-virus RNA innate immunity**

To systematically uncover phosphatases of the PP2A family that are involved in the regulation of innate immunity against RNA virus, we performed an IFN-β luciferase reporter–based screen in various phosphatase-overexpressed HEK293 cells infected with SeV. Luciferase activity assay showed that overexpression of PP4C, the catalytic subunit of PP4, most significantly inhibited SeV-induced IFN-β reporter activity, as compared with other PP2A family members (Fig. 1A). Furthermore, PP4C overexpression also markedly suppressed gene transcription of SeV-induced IFNβ1 and ISGs, including ISG15 and CCL5 in HEK293 cells (Fig. 1B–D). Additionally, VSV or SeV in vitro infection upregulated the mRNA and protein expression of PP4C in a time-dependent manner in macrophages (Supplemental Fig. 1A, 1B). The mRNA and protein expression levels of PP4C were also markedly increased in peritoneal macrophages harvested from mice i.p. infected with VSV or SeV for the indicated time (Supplemental Fig. 1C, 1D). Collectively, these results suggest that PP4 may be a negative feedback regulator of RNA virus-induced innate immune response.

**PP4 inhibits type I IFN and inflammatory cytokine production triggered by RNA virus and TLR in macrophages and BMDCs**

To further identify whether PP4 could affect RNA virus-triggered innate immune response in macrophages and BMDCs, we first investigated the role of PP4 in type I IFN production upon RNA virus challenge. siRNA (siRNA no. 1) specifically targeting PP4 catalytic subunit Pp4c could significantly knock down PP4C expression (Fig. 2A). Silencing of PP4C significantly increased IFN-β and IFN-α protein production, as well as IFN-β and IFN-α mRNA levels in macrophages upon infection with VSV or SeV (Fig. 2B, 2C). The same phenomenon was also observed in PP4C-silenced BMDCs infected with VSV or SeV (Fig. 2D). To further confirm the inhibitory role of PP4 on RNA virus-induced type I IFN production, we used another siRNA (siRNA no. 2) targeting another site of the Pp4c gene. Consistently, PP4C knockdown by this siRNA (siRNA no. 2) also significantly increased IFN-β and IFN-α production triggered by VSV or SeV in macrophages (Supplemental Fig. 2A, 2B). The transfection of these two siRNAs specific for PP4C did not affect the protein expression of other phosphatases such as PPM1B, SHP-2, and SHIP-1 (Supplemental Fig. 2C, D) further indicating that PP4C knockdown by these two different siRNAs is specific. Additionally, the production of type I IFN induced by LPS or poly(I:C) was also markedly increased in PP4C-silenced macrophages than that in control macrophages, suggesting that PP4 also inhibits TLR3- and TLR4-induced type I IFN production in macrophages (Fig. 2E).

Furthermore, we observed the effects of PP4C overexpression on RNA virus-induced type I IFN production in macrophages. Overexpression of PP4C significantly suppressed the production of IFN-β and IFN-α protein, as well as the expression of IFN-β and IFN-α mRNA in macrophages infected with VSV or SeV (Fig. 3A, Supplemental Fig. 3A). Considering that PP4 is a cytosolic protein phosphatase, we further explored whether the phosphatase activity of PP4C was responsible for its inhibitory role in RNA virus-triggered type I IFN production. It has been reported that the replacement of arginine 236 with leucine in PP4C (PP4C-R236L) results in the loss of its phosphatase activity, which is still capable of binding substrate proteins (21). The production of type I IFN induced by VSV or SeV was not inhibited in macrophages transfected with the PP4C-R236L mutant construct (Fig. 3A, Supplemental Fig. 3A). Similar results were also found in RAW264.7 cells overexpressed with wild-type or R236L mutant PP4C (Fig. 3B, Supplemental Fig. 3B). These data indicate that PP4 suppresses type I IFN production in macrophages and macrophages upon RNA virus infection, which depends on the phosphatase activity of PP4.

The effects of PP4 on inflammatory cytokine production triggered by TLR and RNA virus were also investigated. PP4C knockdown significantly increased the production of TNF-α and IL-6 in macrophages infected with VSV or SeV (Supplemental Fig. 3C). Similar results were also found in PP4C-silencing
macrophages stimulated with LPS, poly(I:C), or CpG ODN (Supplemental Fig. 3D). PP4C overexpression suppressed TNF-α and IL-6 production triggered by VSV, SeV, LPS, poly(I:C), or CpG ODN in macrophages (Supplemental Fig. 3E, 3F). These data suggest that PP4 also inhibits TLR- and RIG-I–triggered inflammatory cytokine production in macrophages.

**PP4 promotes VSV replication through impairment of type I IFN production**

To further investigate the biological significance of PP4 in RNA virus-induced immune response, we went on to investigate the effects of PP4 on VSV replication and the role of IFN-β in these effects. By measuring VSV titers in the culture supernatants of the infected macrophages, we found that PP4C silencing could significantly inhibit VSV replication, whereas anti–IFN-β-neutralizing Ab could reverse the suppression of VSV replication mediated by PP4C silencing, indicating that the increase of type I IFN production mediated by PP4C silencing inhibits VSV replication (Fig. 4A). Furthermore, overexpression of wild-type PP4C markedly promoted VSV replication, whereas overexpression of the PP4C-R236L mutant or addition of recombinant murine IFN-β could reverse the enhanced VSV replication mediated by wild-type PP4C overexpression (Fig. 4A). Correspondingly, the intracellular and supernatant VSV RNA levels were more significantly decreased in PP4C-silencing macrophages, and the increased VSV RNA levels were observed in wild-type PP4C-overexpressing macrophages but not in PP4C-R236L mutant–overexpressing macrophages (Fig. 4B, 4C). These data suggest that PP4 suppresses the production of type I IFN, thus promoting RNA virus replication.

**PP4 suppresses innate immunity against RNA virus infection in vivo**

We further demonstrated the in vivo biological effect of PP4 on virus infection by knocking down PP4 in mice. The siRNA duplexes complexed with in vivo jetPEI reagent were i.p. injected into mice and could significantly reduce the endogenous protein level of PP4C in macrophages from the peritoneal cavity (Fig. 5A). Mice injected with siRNA/jetPEI complex were i.p. infected with VSV or SeV. Silencing of PP4C significantly increased virus-induced IFN-β and IFN-α production in peritoneal fluids, as well as IFN-β and IFN-α4 mRNA levels in peritoneal macrophages (Fig. 5B, 5C). PP4C silencing also markedly inhibited VSV replication in peritoneal fluids and decreased the intracellular VSV RNA levels in peritoneal macrophages (Fig. 5D). These in vivo data, which are in line with the observation in vitro, further

![Figure 2](http://www.jimmunol.org/)

**Figure 2.** Silencing of PP4 increases type I IFN production triggered by RNA virus and TLR3/4 ligands in macrophages and BMDCs. (A) Mouse macrophages were transfected with control siRNA (Ctrl) or PP4C siRNA (siRNA). Forty-eight hours later, the efficiency of silencing was detected by quantitative PCR and Western blot. (B-D) Mouse macrophages and BMDCs were transfected with PP4C siRNA or control siRNA as in (A). After 48 h, cells were infected with VSV (MOI of 10) or SeV (MOI of 1), then IFN-β and IFN-α levels in the supernatants after virus infection for 24 h were measured by ELISA (B and D), and the mRNA expression of IFN-β and IFN-α4 after virus infection for 18 h were detected by quantitative PCR (C). (E) Mouse macrophages were transfected as in (A). After 48 h, cells were stimulated with LPS or poly(I:C) for 6 h, then IFN-β and IFN-α levels in the supernatants were measured by ELISA. Data are representative of three independent experiments with similar results (A, right) or are shown as mean ± SEM of three independent experiments using five mice per experiment (total 15 mice for three independent experiments) (A, left, and B–E). **p < 0.01.

![Figure 3](http://www.jimmunol.org/)

**Figure 3.** Overexpression of PP4 inhibits RNA virus-induced production of type I IFN in a phosphatase-dependent manner. (A and B) Mouse macrophages (A) and RAW264.7 cells (B) were transiently transfected with wild-type PP4C (PP4C) or PP4C-R236L mutant (PP4C mut) plasmid. After 48 h, cells were infected with VSV (MOI of 10) or SeV (MOI of 1), then IFN-β and IFN-α levels in the supernatants after virus infection for 24 h were measured by ELISA (A, upper, and B), and the mRNA expression of IFN-β and IFN-α4 after virus infection for 18 h were detected by quantitative PCR (A, lower). Data are shown as mean ± SEM of three independent experiments using 5 mice per experiment (total 15 mice for three independent experiments) (A), and three independent experiments (B). **p < 0.01.
PP4C inhibited the activation of IFN-β, RIG-I, MDA5, IPS-1, and TBK1 but not IKK

Supernatant VSV RNA replicates were measured by quantitative PCR (B). RNAs from equal volume of cellular supernatants were extracted and supernatant VSV RNA replicates were measured by quantitative PCR (C). Data are shown as mean ± SEM of three independent experiments using 5 mice per experiment (total 15 mice for three independent experiments). **p < 0.01. N.D., not detected.

FIGURE 5. PP4 inhibits VSV replication in macrophages. (A) Mouse macrophages were transfected with control siRNA (Ctrl) or PP4C siRNA (siRNA) (left) and wild-type PP4C (WT) or PP4C-R236L mutant (Mut) plasmid (right). Forty-eight hours later, cells were infected with VSV at an MOI of 10 for 1 h and washed, then fresh medium or fresh medium containing anti–IFN-β-neutralizing Ab (100 neutralizing units/ml) (left) or recombinant mouse IFN-β (100 U/ml) (right) were added as indicated. After 72 h, 50% tissue culture-infective dose (TCID₅₀) of virus in supernatants was measured. (B and C) Mouse macrophages were treated as in (A), and intracellular VSV RNA replicates were detected by quantitative PCR (B). Data are shown as mean ± SEM of three independent experiments using 5 mice per experiment (total 15 mice for three independent experiments). *p < 0.05. N.D., not detected.

FIGURE 4. PP4 inhibits VSV replication in macrophages. (A) Mouse macrophages were transfected with control siRNA (Ctrl) or PP4C siRNA (siRNA) (left) and wild-type PP4C (WT) or PP4C-R236L mutant (Mut) plasmid (right). Forty-eight hours later, cells were infected with VSV at an MOI of 10 for 1 h and washed, then fresh medium or fresh medium containing anti–IFN-β-neutralizing Ab (100 neutralizing units/ml) (left) or recombinant mouse IFN-β (100 U/ml) (right) were added as indicated. After 72 h, 50% tissue culture-infective dose (TCID₅₀) of virus in supernatants was measured. (B and C) Mouse macrophages were treated as in (A), and intracellular VSV RNA replicates were detected by quantitative PCR (B). RNAs from equal volume of cellular supernatants were extracted and supernatant VSV RNA replicates were measured by quantitative PCR (C). Data are shown as mean ± SEM of three independent experiments using 5 mice per experiment (total 15 mice for three independent experiments). **p < 0.01. N.D., not detected.

Next, we examined the underlying signaling mechanism by which PP4 inhibited RNA virus-triggered innate immune responses. We found that VSV infection-induced phosphorylations of TBK1 (Ser172) and IRF3 (Ser396) were markedly enhanced in macrophages (Fig. 6A). Accordingly, PP4C overexpression inhibited the phosphorylation of TBK1 and IRF3 in macrophages after VSV infection (Fig. 6B). Furthermore, knockdown of PP4C resulted in more VSV-induced nuclear translocation of IRF3, whereas overexpression of PP4C induced less IRF3 translocation into nucleus (Fig. 6C, 6D), suggesting that PP4 inhibits the activation of the TBK1–IRF3 signaling pathway upon RNA virus infection.

To investigate how PP4 functions in the inhibition of type I IFN signaling activation, we performed reporter assays by cotransfecting plasmids expressing PP4C and IFN-β reporter with RIG-I, MDA5, IPS-1, TBK1, IKKe, or IRF3 constructs. Overexpression of PP4C inhibited the activation of IFN-β reporter induced by RIG-I, MDA5, IPS-1, and TBK1 but not IKKe or IRF3 (Fig. 6E), suggesting that PP4 may act on TBK1 to suppress RNA virus-induced activation of type I IFN signaling. Furthermore, overexpression of PP4C dose-dependently inhibited TBK1-mediated IFN-β reporter activity (Fig. 6F). However, overexpression of phosphatase activity inactive PP4C-R236L mutant could not suppress TBK1-mediated IFN-β reporter activity as wild-type PP4C (Fig. 6G). Additionally, wild-type PP4C but not PP4C-R236L mutant also inhibited the activity of IRF3 reporter induced by TBK1 (Fig. 6H). These results indicate that PP4 negatively regulates the activation of RNA virus-triggered type I IFN pathway by functioning at TBK1.

PP4 directly interacts with TBK1

Next we investigated whether PP4 could interact with TBK1 to negative regulate RIG-I pathway activation. Immuno precipitation assays with specific PP4C or TBK1 Ab showed that PP4 could interact with TBK1 in macrophages after VSV infection (Fig. 7A, 7B). The interaction between PP4C and TBK1 was also found in peritoneal macrophages from mice i.p. infected with VSV (Fig. 7C). Furthermore, GST pull-down assay using GST-tagged PP4C protein showed that TBK1 could be pulled down by GST-tagged PP4C, suggesting that these two proteins can interact directly (Fig. 7D). However, PP4C did not bind IKKe, another kinase in RIG-I signaling, and IRF3 in immunoprecipitation assays, indicating that PP4 specifically interacts with TBK1 (Supplemental Fig. 4A). TBK1 contains the N-terminal kinase domain, the ubiquitin-like domain, and two coiled-coil domains at the C

PP44 inhibits the activation of type I IFN signaling upon RNA virus infection

Next, we examined the underlying signaling mechanism by which PP4 inhibited RNA virus-triggered innate immune responses. We found that VSV infection-induced phosphorylations of TBK1 (Ser172) and IRF3 (Ser396) were markedly enhanced in macrophages with PP4C silencing compared with nonsilencing macrophages (Fig. 6A). Accordingly, PP4C overexpression inhibited the phosphorylation of TBK1 and IRF3 in macrophages after VSV infection (Fig. 6B). Furthermore, knockdown of PP4C resulted in more VSV-induced nuclear translocation of IRF3, whereas overexpression of PP4C induced less IRF3 translocation into nucleus (Fig. 6C, 6D), suggesting that PP4 inhibits the activation of the TBK1–IRF3 signaling pathway upon RNA virus infection.

To investigate how PP4 functions in the inhibition of type I IFN signaling activation, we performed reporter assays by cotransfecting plasmids expressing PP4C and IFN-β reporter with RIG-I, MDA5, IPS-1, TBK1, IKKe, or IRF3 constructs. Overexpression of PP4C inhibited the activation of IFN-β reporter induced by RIG-I, MDA5, IPS-1, and TBK1 but not IKKe or IRF3 (Fig. 6E), suggesting that PP4 may act on TBK1 to suppress RNA virus-induced activation of type I IFN signaling. Furthermore, overexpression of PP4C dose-dependently inhibited TBK1-mediated IFN-β reporter activity (Fig. 6F). However, overexpression of phosphatase activity inactive PP4C-R236L mutant could not suppress TBK1-mediated IFN-β reporter activity as wild-type PP4C (Fig. 6G). Additionally, wild-type PP4C but not PP4C-R236L mutant also inhibited the activity of IRF3 reporter induced by TBK1 (Fig. 6H). These results indicate that PP4 negatively regulates the activation of RNA virus-triggered type I IFN pathway by functioning at TBK1.
PP4 inhibits antiviral response by dephosphorylating TBK1

**FIGURE 6.** PP4 suppresses the activation of IRF3 pathway induced by VSV infection in macrophages. (A and B) RAW264.7 cells were transfected with PP4C siRNA or control siRNA (A), and wild-type PP4C (PP4C) or mock plasmid (B). Forty-eight hours later, cells were infected with VSV for the indicated time. Phospho-TBK1 (S172) and phospho-IRF3 (S396) in lysates were detected by Western blot. (C and D) RAW264.7 cells were transfected and infected as in (A) and (B), and total IRF3 among nuclear proteins in macrophages was detected by Western blot. Numbers below lanes and graphs indicate densitometry of the protein presented relative to β-actin or lamin A/C expression in that same lane. (E) HEK293 cells were cotransfected with or without RIG-I-, MDA5-, IPS-1-, TBK1, IKKε-, or IRF3-expressing plasmid, IFN-β luciferase reporter plasmid, together with 100 ng wild-type PP4C-expressing plasmid. After 24 h, IFN-β luciferase activity was measured and normalized by Renilla luciferase activity. (F) Luciferase assay of IFN-β activation in HEK293 cells cotransfected with or without TBK1-expressing plasmid, IFN-β luciferase reporter plasmid, together with the indicated amount wild-type PP4C plasmid. (G and H) Luciferase assay of IFN-β (G) or IRF3 (H) activation in HEK293 cells cotransfected with or without TBK1-expressing plasmid, IFN-β reporter plasmid (G), or IRF3 reporter plasmids (80 ng Gal4 luciferase reporter plasmid, 20 ng Gal4-IRF3-expressing plasmid) (H), together with 100 ng wild-type PP4C (WT) or PP4C-R236L mutant (Mut) plasmid. Data are representative of three independent experiments with similar results (A–D), or are shown as mean ± SEM of three independent experiments (E–H). *p < 0.01, **p < 0.001.

**FIGURE 7.** PP4 directly binds TBK1. (A and B) Mouse macrophages were infected with VSV for the indicated time. Equal amounts of cellular lysates were immunoprecipitated with PP4C or TBK1 Ab and then detected with TBK1 and PP4C Abs. (C) GST pull-down assays were performed with GST-tagged PP4C and purified TBK1 protein. (D) Flag-tagged PP4C plasmid together with various HA-tagged TBK1 truncates were transfected into HEK293 cells. After 24 h, cell extracts were immunoprecipitated with anti-HA Ab and then immunoblotted with anti-Flag and anti-HA Abs. CC, coiled-coil domain; KD, kinase domain; ULD, ubiquitin-like domain; WT, wild-type. Similar results were obtained in three independent experiments using three mice per experiment (total nine mice for three independent experiments) (A–C) or in three independent experiments (D and E).
phages infected with VSV was markedly higher than that of control macrophages, whereas the kinase activity of IKKe protein was not affected in PP4C-silenced macrophages upon VSV infection (Fig. 8B, 8C). Additionally, wild-type PP4C overexpression markedly inhibited TBK1 phosphorylation at Ser172 and IRF3 phosphorylation in macrophages upon SeV infection, but PP4C-R236L mutant overexpression did not (Fig. 8D, 8E). Furthermore, the phosphorylation of TBK1 at Ser172 was markedly enhanced in macrophages with PP4C silencing in vivo as compared with non-silencing macrophages from mice i.p. infected with VSV (Fig. 8F). Considering that K48-linked polyubiquitination of TBK1 promotes TBK1 degradation and K63-linked polyubiquitination enhances TBK1 activation (24, 32, 33), we investigated whether PP4 affected TBK1 polyubiquitination upon virus infection. However, we failed to find that PP4 promoted or inhibited TBK1 polyubiquitination (Fig. 8G), which indicates that PP4 terminates TBK1 activation by dephosphorylating TBK1 but not by affecting TBK1 ubiquitination.

We also found that PP4C silencing enhanced K63-linked polyubiquitination of TRAF6 in macrophages infected with VSV, and PP4C overexpression significantly inhibited TRAF6-driven NF-kB reporter activity (Supplemental Fig. 4C, 4D), which is in line with the previous study that PP4 can inhibit TRAF6 ubiquitination and NF-kB activation induced by TRLR4 (30). Additionally, the knockdown of PP4C did not affect LPS-induced phosphorylation of IRAK1 in macrophages (Supplemental Fig. 4E), suggesting that PP4 inhibits TLR- and RIG-I–induced NF-kB activation by targeting TRAF6 but does not affect IRAK1 activation.

Collectively, our results demonstrate that PP4 can directly bind TBK1, then dephosphorylate TBK1 at Ser172, thus inhibiting TBK1 kinase activation and downstream IRF3 activation, resulting in the impaired type I IFN production.

**Discussion**

Viral infection initiates a series of signaling cascades that lead to the production of type I IFN, finally inducing ISGs to eliminate viruses (34). The phosphorylation and activation of TBK1 is essential for IRF3 activation and type I IFN production downstream of RIG-I and MDA-5 (11, 12). Regulation of TBK1 activity is a key step in antiviral innate immunity, because excessive activation of type I IFN signaling results in immune disorder (13, 14). However, the mechanisms underlying deactivation of TBK1 remain elusive. It is widely thought that phosphatases play major roles in the control or termination of protein kinase activity (18). However, the phosphatases responsible for dephosphorylation and activity control of TBK1 are not well defined. In this study, we identify PP4 as a novel TBK1 phosphatase to dephosphorylate TBK1 at Ser172 and inhibit TBK1 activation, which suppresses the production of type I IFN induced by RNA virus and TLR and restrains the innate immune response against RNA virus in vitro and in vivo.

Although the roles of phosphatases, including tyrosine phosphatases and serine/threonine phosphatases, in the regulation of innate immune response draw more attention (35), only three phosphatases, SHP-2, SHIP-1, and PPM1B, have been found to control the activation of type I IFN signaling by acting on TBK1 until now (15–17). SHP-2 does not directly dephosphorylate TBK1 but suppresses TBK1 activity through a tyrosine phosphatase activity–independent mechanism to inhibit TRIF-dependent type I IFN and proinflammatory cytokine production (15). SHIP-1 promotes TBK1 dissociation from endosomally located TRLR3 signaling complexes and hence inhibits TBK1 phosphorylation, whereas SHIP-1 is not found to bind and dephosphorylate TBK1 (16). Thus, these studies indicate that SHP-2 and SHIP-1 inhibit TBK1 activation through other different mechanisms but not the direct dephosphorylation of TBK1. However, only PPM1B, a member of P2PC family, can interact with TBK1 and dephosphorylate TBK1 at Ser172 in HEK293T cells and HeLa cells (17). In the present study, we provide the convincing evidence that PP4 functions as a novel TBK1 phosphatase to dephosphorylate TBK1 at Ser172 and restrain the activation of TBK1 and downstream type I IFN signaling in macrophages and dendritic cells. We further explored the relationship between PP4 and these above phosphatases in regulation of TBK1 and found that PP4 did not bind PPM1B, SHP-2, or SHIP-1 upon VSV infection in macrophages (data not shown). Furthermore, overexpression of PP4 and PPM1B together more markedly suppressed transcription of Slf-induced IFNB1 gene than that did PP4 or PPM1B overexpression alone in HEK293 cells (data not shown). These results indicate that PP4 does not associate with other phosphatases implicated in regulation of TBK1 to form a complex, and PP4 may cooperate with PPM1B to dephosphorylate and deactivate TBK1 upon RNA virus infection. The interrelationship of the phosphatases in the regulation of TBK1 activation needs further investigation.
Phosphatase PP2A, which also belongs to the PP2A family as PP4, has been found to interact with IRF3, then dephosphorylate and deactivate IRF3, leading to limited type I IFN production upon SeV infection (36). In the present study, we failed to detect the interaction between PP4 and IRF3; moreover, IRF3-induced IFN-β reporter activity is not inhibited by coexpressed PP4, suggesting that PP4 cannot dephosphorylate and inactivate IRF3 as does PP2A. Thus, we propose that PP4 may collaborate with PP2A to control the activation of virus-induced type I IFN signaling through targeting different components of RIG-I signaling.

A previous study has shown that PP4 is a negative feedback regulator of TLR4-induced NF-κB activation, although the data on the effects of PP4 on the production of inflammatory cytokines and type I IFN induced by LPS are not provided (30). PP4 interacts with TRAF6 and inhibits its ubiquitination in RAW264.7 cells upon TLR4 activation through an unknown mechanism, thus suppressing TRAF6 activation and the downstream NF-κB activation (30). We also found that PP4 binds TRAF6 and PP4 inhibits K63-linked polyubiquitination of TRAF6 in macrophages infected with VSV. Moreover, PP4 overexpression also significantly inhibits TRAF6-driven NF-κB reporter activity. Because TRAF6 is a critical adapter protein involved in TLR- or RIG-I–triggered proinflammatory cytokine production (1, 7, 11), together with our data that PP4 suppresses inflammatory cytokine production induced by TLR or RNA virus, our data demonstrate that PP4 also can bind TRAF6 and hence inhibit the ubiquitination and activation of TRAF6, resulting in the impaired inflammatory cytokine production triggered by TLR- or RIG-I. It is possible that PP4 may recruit other proteins to suppress TRAF6 ubiquitination, but the exact mechanism remains unclear and needs to be further explored. Although IRAK1 is also a crucial kinase protein involved in TLR-induced proinflammatory cytokine production (1), we found that PP4 does not bind IRAK1 and affect IRAK1 phosphorylation in LPS-activated macrophages, suggesting that PP4 inhibits TRAF6-triggered NF-κB activation and proinflammatory cytokine production by targeting TRAF6 but not IRAK1. Considering that TRAF6 does not play a major role in RIG-I–triggered IRF3 activation and type I IFN production, whereas TRAF6 is primarily involved in RIG-I–triggered NF-κB activation and proinflammatory cytokine production (7, 11), we propose that PP4 primarily targets TRAF6 but not TRAF6 and hence inhibits TRAF1-driven type I IFN production upon virus infection.

Viruses have evolved elaborate strategies, such as disruption of host recognition and impairment of type I IFN signaling, to evade and subvert the innate immune responses (37, 38). Because TBK1 functions as a key kinase to initiate type I IFN signaling upon viral infection, viruses are more likely to evolve strategies to counteract innate immunity by targeting TBK1. For example, Borna disease virus protein P, povirus protein N1L, and HCV protease NS2 can directly interact with TBK1 and inhibit its kinase activity, although the mechanisms for suppression of TBK1 kinase activity remain unclear (39–41). Alternatively, viruses also can upregulate the expression levels or inhibitory functions of some negative regulatory proteins, which suppresses the activation of type I IFN signaling to impair and evade the antiviral innate immunity (37, 38). It has been reported that PP4 expression level is increased in HIV-infected monocyte-derived dendritic cells (Gene Expression Omnibus dataset GSE22589) and pandemic H1N1 influenza KYU136-infected primary lung bronchial epithelial cells (Gene Expression Omnibus dataset GSE48466) (42, 43). Taken together with our results that the expression level of PP4 is upregulated after RNA virus infection and PP4 inhibited type I IFN production, it is reasonable that some viruses may use PP4 to restrain and escape antiviral immunity, whereas which ones needs to be further investigated.

In conclusion, our data provide evidence that PP4 is a novel phosphatase known to dephosphorylate and deactivate TBK1. PP4 negatively regulates innate immunity against RNA virus to protect the host from excessive immune responses. Better understanding of the deactivation process of TBK1 will benefit the design of immunotherapy strategies for type I IFN dysregulation diseases.

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Disclosures

The authors have no financial conflicts of interest.

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Supplementary data

Phosphatase PP4 negatively regulates type I interferon production and antiviral innate immunity by dephosphorylating and deactivating TBK1

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Supplemental Fig. 1. Virus infection *in vitro* and *in vivo* up-regulates PP4C expression in macrophages. (A and B) Mouse macrophages were infected with VSV (10 MOI) or SeV (1 MOI) for the indicated times, then the mRNA and protein expression levels of PP4C were detected by quantitative PCR and western blot respectively. (C and D) Mice were intraperitoneally infected with VSV or SeV for the indicated time. The mRNA and protein expression levels of PP4C in macrophages separated from peritoneal lavages were detected by quantitative PCR and western blot respectively. Numbers below lanes and graphs indicate densitometry of the protein.
presented relative to β-actin expression in that same lane. Data are representative or shown as mean ± SEM of three independent experiments using 3 mice per experiment (total 9 mice for three independent experiments) (A, B), or are representative or shown as mean ± SEM of three independent experiments using 4 mice per group of one experiment (total 36 mice for 3 groups and three independent experiments) (C, D).
Supplemental Fig. 2. Silencing of PP4 increases RNA virus-triggered type I IFN production in macrophages. (A) Mouse macrophages were transfected with control siRNA (Ctrl) or PP4C siRNA #2 (siRNA #2). Forty-eight hours later, the efficiency of silencing was detected by quantitative PCR and western blot. (B) Mouse macrophages were transfected with PP4C siRNA or control siRNA as in (A). After 48 h, cells were infected with VSV (10 MOI) or SeV (1 MOI) for 24 h, then IFN-β and IFN-α levels in the supernatants were measured by ELISA. (C) Mouse macrophages were transfected with control siRNA (Ctrl) or two different siRNA specific for PP4C (siRNA #1 and #2). After 48 h, the protein expression levels of PP4C, PPM1B, SHP-2 and SHIP-1 were detected by western blot. Data are representative of three independent experiments with similar results (A right, C), or are shown as mean ± SEM of three independent experiments using 5 mice per experiment (total 15 mice for three independent experiments) (A left, B). **, P < 0.01.
Supplemental Fig. 3. PP4 inhibits the inflammatory cytokine production in macrophages triggered by TLR and RNA virus. (A, B) Mouse peritoneal...
macrophages (A) and RAW264.7 cells (B) were transiently transfected with flag-tagged wild type PP4C (PP4C) or PP4C-R236L mutant (PP4C mut) plasmid. After 48 h, the protein expression of wild type and mutant PP4C was detected by western blot. (C and D) Mouse macrophages were transfected with PP4C siRNA (siRNA #1) or control siRNA (Ctrl). After 48 h, cells were infected with VSV (10 MOI) or SeV (1 MOI) for 24 h (C) or stimulated with LPS, poly(I:C) or CpG ODN for 6 h (D), then TNF-α and IL-6 levels in the supernatants were measured by ELISA. (E and F) Mouse macrophages were transiently transfected with wild type PP4C (PP4C) or PP4C-R236L mutant (PP4C mut) plasmid. After 48 h, cells were treated as in (C and D), then TNF-α and IL-6 levels in the supernatants were measured by ELISA. Data are representative of three independent experiments with similar results (A, B), or are shown as mean ± SEM of three independent experiments using 5 mice per experiment (total 15 mice for three independent experiments) (C-F). **, P < 0.01.
**Supplemental Fig. 4.** PP4 binds TRAF6 upon VSV infection or LPS stimulation, and inhibits VSV-induced TRAF6 ubiquitination and TRAF6-dependent NF-κB activation but does not affect LPS-induced IRAK1 phosphorylation. (A) Mouse macrophages were infected with VSV for the indicated time. Equal amount cell lysates were immunoprecipitated with PP4C Ab and then detected with TRAF6, IKKε, IRF3 and PP4C Abs respectively. (B) Equal amount cell lysates of macrophages stimulated with LPS for the indicated time were immunoprecipitated with PP4C Ab and then detected with TRAF6, IRAK1 and PP4C Abs respectively. TRAF6 and IRAK1 in whole cell lysates (WCL) of macrophages were detected by western blot. (C) Mouse macrophages were transfected with PP4C siRNA or control siRNA. After 48 h, cells were infected with VSV for the indicated time. Equal amount cell lysates were immunoprecipitated with TRAF6 Ab and then detected with K63 ubiquitination Ab
and TRAF6 Ab. Ubiquitin in whole cell lysates (WCL) of macrophages was detected by western blot. (D) Luciferase assay of NF-κB activation in HEK293 cells co-transfected with or without TRAF6 expressing plasmid, NF-κB luciferase reporter plasmid, together with the indicated amount wild type PP4C plasmid. (E) Mouse macrophages were transfected with PP4C siRNA or control siRNA. After 48 h, cells were stimulated with LPS for the indicated time. Phospho-IRAK1 in lysates was detected by western blot. Data are representative of three independent experiments using 3 mice per experiment (total 9 mice for three independent experiments) (A-C, E), or are shown as mean ± SEM of three independent experiments (D). **, $P < 0.01$. 