Inhibiting TAK1 Serine 412 Phosphorylation Negatively Regulates TLR Response by Phosphatase Holoenzyme PP1/GADD34

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Phosphatase Holoenzyme PP1/GADD34 Negatively Regulates TLR Response by Inhibiting TAK1 Serine 412 Phosphorylation

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The molecular mechanisms that fine tune TLRs responses need to be fully elucidated. Protein phosphatase-1 (PP1) has been shown to be important in cell death and differentiation. However, the roles of PP1 in TLR-triggered immune response remain unclear. In this study, we demonstrate that PP1 inhibits the activation of the MAPK and NF-κB pathway and the production of TNF-α, IL-6 in macrophages triggered by TLR3, TLR4, and TLR9 in a phosphatase-dependent manner. Conversely, PP1 knockdown increases TLRs-triggered signaling and proinflammatory cytokine production. Tautomycin, a specific inhibitor of PP1, aggravates LPS-induced endotoxin shock in mice. We further demonstrate that PP1 negatively regulates TLR-triggered signaling by targeting TGF-β-activated kinase 1 (TAK1) serine 412 (Ser412) phosphorylation, which is required for activation of TAK1-mediated IL-1R and TLR signaling. Mutation of TAK1 Serine 412 to alanine (S412A) significantly inhibits TLR/IL-1R–triggered NF-κB and MAPK activation and induction of proinflammatory cytokines in macrophage and murine embryonic fibroblast cells. DNA damage-inducible protein 34 (GADD34) specifies PP1 to dephosphorylate TAK1 at Ser412. GADD34 depletion abolished the interaction between TAK1 and PP1, and it relieved PP1 overexpression-induced inhibition of TLRs signaling and proinflammatory cytokine production. In addition, knockdown of GADD34 significantly promotes TLR-induced TAK1 Ser412 phosphorylation, downstream NF-κB and MAPK activation, and proinflammatory cytokine production. Therefore, PP1, as a physiologic inhibitor, together with its regulatory subunit GADD34, tightly controls TLR-induced TAK1 Ser412 phosphorylation, preventing excessive activation of TLRs and protecting the host from overwhelming inflammatory immune responses. The Journal of Immunology, 2014, 192: 000–000.

Toll-like receptors play critical roles in innate immunity and host defense. They detect invading pathogens through recognition of pathogen-associated molecular patterns (PAMPs) such as LPSs, nucleic acids, lipoproteins, and glycans derived from pathogens and initiate the activation of multiple intracellular signaling pathways (1). Among these pathways, NF-κB and MAPK pathways are most important (2). Activation of the NF-κB and MAPK pathways leads to induction of cytokines and chemokines, which shape the nature, magnitude, and duration of host proinflammatory and innate immune responses (3). Although full activation of TLR signaling is essential for host defense, dysregulation of this process contributes to many inflammatory diseases and immunopathologic disorders, such as endotoxin shock and autoimmune disease (2). It is important to elucidate the underlying molecular mechanism through which the TLR response is tightly regulated. For example, previous studies identified positive regulators of the TLR pathway, such as the, Peli1, Nrdp1, and MHC class II molecules (4–6), and negative regulators such as NLRC3, SHP-2, and MHC class I molecules (7–9); however, it remains to be explored whether there are other unidentified regulators for TLR responses.

Protein phosphorylation and dephosphorylation, mediated by a plethora of protein kinases and phosphatases, are important regulatory mechanisms in a wide variety of biological processes, such as cell development, differentiation, and transformation in higher eukaryotes. It is reported that in TLR-activated macrophage, most phosphorylation sites were on serine (84%) and threonine residues (14%), whereas tyrosine phosphorylation occurred only in 2% of the cases (10). The human genome encodes ~40 catalytic subunits of Ser/Thr phosphatases (11), which include the PP1, PP2A/PP2, PP2B/calcineurin/PP3, PP4, PP5, PP6, and PP7 of the PPP phosphatase subfamily (12). PP1 is one of the most abundant phosphatases, and it has important regulatory roles in cellular events [e.g., metabolism, gene transcription, cell polarity, vesicle trafficking and cell cycle (13)]. PP1 holoenzymes typically function as heterodimeric complexes containing one catalytic subunit and one regulatory subunit (14). Human genome encodes three PP1 catalytic subunits—PPP1Ca, PPP1CB, and PPP1Cγ (15). So far, ~180 human proteins have been identified as PP1 regulatory subunits (16). Among these regulatory subunits, PPP1r14a, PPP1r14b, PPP1r15a/GADD34 (17), PPP1r15b, PPP1r18, and KLqra1 are highly expressed in immune cells and thus might be involved in...
regulation of immune responses. The regulatory subunits may inhibit PP1 enzymatic activity, restrict its substrate specificity, or limit its subcellular localization. Studies have shown that PP1 together with GADD34 dephosphorylates IKK via adaptor protein CUEDC2, thus negatively regulating the TNF-R pathway (18). However, the roles of PP1 in TLR-mediated innate immunity have not been studied.

In TLRs and IL-1R signaling, activation of NF-κB and MAPK kinases requires a critical upstream kinase TAK1 (tumor growth factor β-activated kinase), a member of the MAP kinase kinase kinase family (19). TAK1 exists in cells as a stable complex with two structural subunits TAB1 and TAB2 (or TAB3, the TAB2 homolog). Activation of TAK1 correlates with phosphorylation of residues Thr178, Thr184, Thr187, and Ser192 in its activation loop (20, 21). In particular, phosphorylation of Thr187 is required for TAK1 kinase activation, which is increased in response to stimulation by proinflammatory cytokines and TLR ligands (20). Phosphorylation of TAK1 at sites other than those in the activation loop has also been reported in the literature (22, 23) and the PhosphoSitePlus Web site (http://www.phosphosite.org/); however, whether these phosphorylation events regulate TAK1 activation remains to be determined.

In the current study, we have demonstrated that protein phosphates PP1, together with its regulatory subunit GADD34, negatively regulate TLR-triggered inflammatory cytokine production in macrophages by specifically dephosphorylating TAK at serine 412. Mutation of TAK1 Ser412 phosphorylation induced IL-1R and TLR-mediated signaling and proinflammatory cytokine production. PP1 and GADD34 knockdown enhanced LPS-induced LPS-induced TAK1 Ser412 phosphorylation without affecting the phosphorylation of Thr187 residue, and downstream NF-κB and MAPK activation, resulting in increased proinflammatory cytokine production in macrophages. Furthermore, tautomycetin, a specific inhibitor of PP1, aggravated LPS-induced endotoxin shock in mice. These data demonstrate that PP1 inhibited TLR signaling by controlling TLR-induced TAK1 Serine 412 phosphorylation, thus serving as an important negative regulator of TLR-mediated immune responses. Our results brought new insight into the mechanism of the fine-tuning of TLR-triggered innate immune responses. In addition, our results would indicate that PP1 is a new inhibitor of the TLR signaling pathway.

**Materials and Methods**

**Abs, reagents, and plasmids**

Abs for phospho-TAK1 Thr187, TAK1 Ser412, IRAK1, IKKα/β, IκBα, IκB, ERK1/2, and p38 were purchased from Cell Signaling Technology. Ab against PP1 and GADD34 was purchased from Santa Cruz Biotechnology. TAK1 siRNA oligos were synthesized by Genepharma (Shanghai, China). siRNA duplexes were transfected into RAW264.7 cells and bone marrow–derived macrophages (BMDMs) by using INTERFERin (Polyplus) or Lipofer by using RNAiMAX (Invitrogen) according to the manufacturer’s protocol. The following sequences for siRNA oligos were used (only the sense strands were shown): human TAK1 (5'-GACUUUGACUGUAACUGGAdTdT-3'); mouse TAK1 (5'-GAGTGGACACTTCTGGCTCA-3').

**Quantitative RT-PCR**

Total RNA was extracted using TRizol (Invitrogen) according to the manufacturer’s instructions. RNA was reverse transcribed using M-MLV reverse transcriptase (Takara). Quantitative PCR was performed using SYBR Green (Takara) and the ABI 7500 real-time PCR system (Applied Biosystems). The following primers were used: mouse β-Actin-F: 5'-AAGATCGGCGTCTAGAACGC-3'; mouse β-Actin-R: 5'-CGTTGACA-TCCGTAAGAAGC-3'; mouse IL-6-F: 5'-AGTTGCCCTTCTTGGACT-3'; mouse IL-6-R: 5'-TCCAGATGTCACCAGAAAC-3'; mouse TNF-α-F: 5'-CTGGGACAGTGAATGAGCT-3'; mouse TNF-α-R: 5'-GACCCTCAGGGAAGTTCG-3'.

**In vitro phospahatase assay**

Flag-tagged TAK1 or IKKβ was transiently expressed and immunoprecipitated with M2 agarose beads as the substrates. On beads, in vitro phosphatase activity was assayed using the QuickChange Site-Directed Mutagenesis protocol (Stratagen). For TAK1, the following primers were used: 5'-CTGAGACGTTACAGTTCTGGCTCA-3'; 5'-TCCAGATGTCACCAGAAAC-3'; 5'-CTGGGACAGTGAATGAGCT-3'; 5'-GACCCTCAGGGAAGTTCG-3'.

**Statistical analysis**

Unpaired, two-tailed Student t test was used to determine the statistical differences between the data sets; p ≤ 0.05 was considered statistically significant.
significant. In the mouse endotoxin shock study, p values for differences in survival between groups were calculated by a log-rank test and analyzed for statistical significance with GraphPad Prism 4.0 software.

Results

PP1 inhibits TLR-triggered production of proinflammatory cytokines in macrophages via phosphatase activity

To explore the role of PP1 in innate immune response, we first investigated the effects of PP1 on IL-6 and TNF-α production in macrophages. Overexpression of Ppp1cc, but not Ppp1ccΔ278, a phosphatase activity-deficient Ppp1cc mutant (26), significantly suppressed LPS, CpG ODN, or poly-IC–induced production of IL-6 and TNF-α in RAW264.7 cells (Fig. 1A). Consistent with this, RNAi-based silencing of PP1 led to a substantial increase in IL-6 and TNF-α production in BMDM (Fig. 1B) and RAW264.7 cells (Supplemental Fig. 1A) stimulated by LPS, poly-IC or CpG ODN. Quantitative PCR analysis and Western blot confirmed PP1 silencing in the RAW264.7 cells and BMDM (Supplemental Fig. 1B–C). Tautomycetin is a specific inhibitor of PP1 (27, 28) and has been used to study PP1 function in animal models (29). Because the

FIGURE 1. PP1 inhibits production of TLR-triggered proinflammatory cytokines in macrophages. (A) RAW264.7 cells were transfected with empty vector (mock), Ppp1cc WT, or activity-deficient mutant (Ppp1ccΔ278) and then stimulated with LPS (100 ng/ml), poly-IC (20 μg/ml) or CpG ODN (0.3 μM) for 6 or 12 h. Secretion of IL-6 and TNF-α in the supernatant was measured with ELISA. (B) BMDM cells were transfected with scrambled or PP1-specific siRNA for 48 h and then treated as in (A). Data from (A) and (B) are plotted as means ± SE. All experiments were performed at least three times. (C, D) Sex- and age-matched mice were preinjected i.p. with 0.05-mg/kg doses of tautomycetin (TC; n = 8) or equal volume of dimethyl sulfoxide (n = 8) for 1 h before i.p. administration of LPS (10 mg/kg body weight). Serum samples were obtained 1.5 h after LPS injection and TNF-α was then measured with ELISA. (C) Data are shown as mean ± SE. (D) The survival of the LPS-challenged mice was monitored every day for 8 d. Similar results were obtained in two more independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus controls. ns, no significance.
inhibition of PP1 leads to enhanced production of proinflammatory cytokines, we wanted to know the effect of PP1 inhibition by tautomycetin in an LPS challenge–based mouse model of endotoxin shock. As shown in Fig. 1C, challenged with sublethal LPS, tautomycetin-pretreated mice produced more TNF-α than control mice did. Consequently, all pretreated mice died within 1 wk after in vivo LPS administration, whereas 62.5% of control mice survived (Fig. 1D), suggesting that inhibition of PP1 aggravates endotoxin shock in response to sublethal LPS challenge.

**PP1 inhibits TLR signaling by targeting TAK1 Ser412 phosphorylation**

Next, we examined the underlying signaling mechanism by which PP1 inhibited TLR-triggered immune responses. We first observed the effect of PP1 overexpression on TLR-induced phosphorylation of MAPKs and NF-κB, which is necessary for TLR-induced proinflammatory cytokine production in RAW264.7 cells. Compared with empty vector (mock) transfection, overexpression of Ppp1cc, but not Ppp1ccΔ278, dramatically reduced LPS-induced phosphorylation of IKKα/β and its substrate IkBα, as well as MAPks (Fig. 2A). Accordingly, in the NF-κB–based luciferase reporter assay, overexpression of MyD88, TRAF6, or TAK1/TAB1 in 293T cells enhanced reporter activity, which was impaired by co-overexpression of Ppp1cc, but not Ppp1ccΔ278 (Supplemental Fig. 2A). Furthermore, overexpression of Ppp1cc inhibited LPS-induced NF-κB reporter activity in RAW264.7 cells (Supplemental Fig. 2B). PP1 knockdown strongly enhanced LPS-induced phosphorylation of IKKα/β, IkBα, p65, ERK, JNK, and

**FIGURE 2.** PP1 inhibits MAPKs, NF-κB activation, and proinflammatory cytokine production in TLR signaling pathways by targeting TAK1 Ser412 phosphorylation. (A) RAW264.7 cells were transfected with empty vector (mock), Ppp1cc WT, or activity-deficient mutant (Ppp1ccΔ278) and then stimulated with LPS (100ng/ml) for the indicated time. Cell lysates were immunoblotted with indicated Abs. (B and C) RAW264.7 cells were transfected with scrambled siRNA or PPI-specific siRNA for 48 h and then stimulated with LPS (100 ng/ml) for the indicated time. Cell lysates were immunoblotted with indicated Abs. (D) The stable transfectants of RAW264.7 were stimulated with LPS (100 ng/ml) for the indicated time. Cell lysates were immunoprecipitated with TAK1 Ab followed by washing and on beads in vitro kinase assay by using His-MKK6 as a substrate for 30 min at 25˚C. Whole cell lysates (WCL) and assay mixtures were immunoblotted with indicated Abs. (E) RAW264.7 cells were transfected with empty vector (mock) or the indicated vectors for 36 h and then stimulated with LPS (100 ng/ml) for the indicated time. Cell lysates were immunoblotted with indicated Abs. (F) RAW264.7 cells were transfected with the indicated vectors for 36 h and then stimulated with LPS (100 ng/ml), poly-IC (20 μg/ml) or CpG ODN (0.3 μM) for 6 or 12 h. Secretion of IL-6 and TNF-α in the supernatant was measured with ELISA. Data are plotted as means ± SE. All experiments were performed at least three times. *p < 0.05, **p < 0.01, ***p < 0.001 versus controls. ns, no significance.
p38 in RAW264.7 cells (Fig. 2B) and NF-κB and AP-1 luciferase reporter activities (Supplemental Fig. 2C), demonstrating that PP1 negatively regulated TLR signaling in macrophages.

Activation of NF-κB and MAPK kinases in TLR signaling critically requires upstream kinase TAK1 (19). In the reporter assays, overexpression of TAK1 together with TAB1 activated both NF-κB and AP-1 reporter activities, which were strongly suppressed by co-overexpression of Ppp1cc. This result prompted us to investigate whether PP1 regulated LPS signaling by dephosphorylating TAK1; however, as shown in Fig. 2C, PP1 knockdown had little effect on TAK1 Thr187 phosphorylation and its upstream kinase IRAK1 phosphorylation. Although phosphorylation of Thr187 in its activation loop is required for TAK1 kinase activation. Literature and PhosphositePlus database search suggest that there are indeed multiple phosphorylation sites on TAK1. Among these sites, Ser412 phosphorylation of mouse TAK1 (mTAK1; it is Ser439 in human TAK1; Ser412 is used thereafter) caught our attention. Remarkably, Ser412 phosphorylation has been detected 92 times by mass spectrometry recorded at PhosphositePlus and analyzed in two reports (22, 23). However in these two reports, Ser412 phosphorylation was not detected in response to LPS or other NF-κB activation stimuli. To determine whether Ser412 is phosphorylated in response to TLR activation, the Flag-TAK1 protein was purified from stably overexpressed RAW264.7 cells that were treated with LPS and then analyzed by liquid chromatography coupled with tandem mass spectrometry. The result showed that the TAK1 carries phosphorylations at Ser412 (Supplemental Fig. 2D) and Thr187 (data not shown) after LPS treatment. We further used commercially available Abs specific to phosphorylated Ser412 (mTAK1; corresponding to Ser439 in human TAK1) of TAK1 to test whether Ser412 of TAK1 could be phosphorylated in LPS signaling. Interestingly, Ser412 of TAK1 was significantly phosphorylated after LPS stimulation, and PP1 knockdown enhanced and prolonged TAK1 Ser412 phosphorylation (Fig. 2C). We next examined whether PP1 can inhibit TAK1 kinase activity. The endogenous TAK1 was immunoprecipitated from Ppp1cc or its mutant Ppp1ccΔ278–stably overexpressed RAW264.7 cells, and its kinase activity was detected using MKK (K82A) purified from E.coli as TAK1 substrate. As shown in Fig. 2D, TAK1 purified from PP1 overexpressed RAW264.7 in response to LPS stimulation showed lower kinase activity toward MKK6 when compared with the TAK1 purified from GFP or Ppp1ccΔ278 overexpressed transfectants, which correlates well with the lower phosphorylation level of JNK and IκBα in cell lysates of PP1 overexpressed group. We also investigated whether PP1 negatively regulated LPS signaling by dephosphorylating TAK1 at Ser412. Overexpression of Ppp1cc diminished the LPS-induced IKKα/β, IκBα, and JNK phosphorylation in RAW264.7 cells, even when co-overexpressed with TAK1 wild type (WT). However, this diminishment was almost abolished when mutant TAK1 S412D, which mimics Ser412 phosphorylation, was co-overexpressed with Ppp1cc (Fig. 2E). Again, the suppression of TAK1-induced NF-κB and AP-1 reporter gene expression by Ppp1cc was blocked when TAK1 S412D was used (Supplemental Fig. 2E). Accordingly, co-overexpression of Ppp1cc with TAK1 WT inhibited LPS-induced IL-6 and TNF-α production; this inhibitory effect was abolished when TAK1 S412D was used (Fig. 2F). Collectively, PP1 negatively regulates MAPKs, NF-κB acti-

FIGURE 3. PP1 specifically dephosphorylates TAK1 at Ser412. (A) RAW264.7 cells were stimulated with LPS (100 ng/ml) for the indicated time. Cell lysates were immunoprecipitated (IP) with PP1 or IgG Ab, followed by immunoblotting with the indicated Abs. (B) BMDM cells were stimulated with LPS (100 ng/ml) for the indicated time. Cell lysates were subjected to immunoprecipitation with an anti-PP1 Ab. The immunoprecipitated proteins and whole cell lysates (WCLs) were detected with the indicated Abs. (C) Myc-tagged Ppp1cc and Flag-tagged TAK1 were transfected into 293T cells for 36 h. Cell lysates were used for reciprocal coimmunoprecipitation using anti-Myc or anti-Flag Abs as indicated. Flag-TRAF6 was used as a negative control. (D) Flag-tagged TAK1 alone, or with Myc-tagged Ppp1cc or Ppp1cb was transfected into 293T cells for 36 h and then subjected to immunoprecipitation by using anti-Myc Ab. Immunoprecipitated proteins and WCL were analyzed with anti-Flag or anti-Myc Abs. (E) Flag-TAK1 was expressed and immunoprecipitated from 293T cells and then incubated with the indicated amount of recombinant PP1 for 30 min at 30°C. Diphosphorylation of Ser412 and Thr187 was determined with the indicated phospho-specific Abs.
vation, and proinflammatory cytokine production in the TLR signaling pathways by dephosphorylating TAK1 Ser412.

**PP1 specifically dephosphorylates TAK1 at Ser412**

We next examined whether PP1 is physically associated with TAK1 in macrophage. As shown in Fig. 3A, PP1 interacted with TAK1 in resting RAW264.7 cells, but disassociated from TAK1 within 2.5 min of LPS treatment. Notably, the interaction between PP1 and TAK1 was gradually restored in 30 min. Similarly, the cycling of association, dissociation, and reassociation between PP1 and TAK1 also existed in primary BMDMs treated with LPS (Fig. 3B). Interestingly, the LPS-induced cycling of TAK1 and PP1 interaction inversely correlated with TAK1 Ser412 phosphorylation (Figs. 3A-3B). Simultaneously, coimmunoprecipitation experiments revealed that all three isoforms of PP1 catalytic subunit, Ppp1ca, Ppp1cb, and Ppp1cc, interacted with TAK1 (Fig. 3C). More importantly, purified PP1 decreased TAK1 Ser412 phosphorylation in a dose-dependent manner in an in vitro dephosphorylation assay, without showing effect on Thr187 phosphorylation (Fig. 3E). Consistent with this result, only high (20 nM) but not low (0.2 nM) concentrations of okadaic acid (OA) treatment enhanced TAK1 Ser412 phosphorylation in 293T cells transiently overexpressing TAK1 and TAB1 or in LPS-stimulated RAW264.7 cells (Supplemental Fig. 2G). PP1 is inhibited by OA with an IC50 of 20 nM, whereas PP2A is much more sensitive to OA, with an IC50 of 0.2 nM (30). These results indicate that PP1 acts as a physiologic TAK1 phosphatase, which specifically dephosphorylates TAK1 at Ser412.

**GADD34 mediates TAK1 Ser412 dephosphorylation by PP1**

PP1 functions as a heterodimer with one of over 180 regulatory subunits that specify its location and substrates and regulate its enzymatic activity (31). To look for the putative regulatory subunit that might specify PP1 to TAK1, we focused on several regulatory subunits of PP1 that appear to be induced after LPS treatment in macrophages. We overexpressed TAK1 together with each of the candidates to see which would coimmunoprecipitate with TAK1 and thus would mediate PP1 interaction with TAK1. Coimmunoprecipitation assay showed the strong interaction between TAK1 and GADD34. Ppp1r15b, but not others, also showed weak interaction with TAK1 (Fig. 4A). GADD34 directly interacted with TAK1, as GST-TAK1 was able to pull down MBP-GADD34 but not MBP-Ppp1cc, all of which were expressed and purified from E.coli (Fig. 4C). We next mapped the region of GADD34 that interacts with TAK1 by using various HA-tagged truncation mutants of GADD34, which were constructed according to its structure and functional domains (32). Coimmunoprecipitation revealed that the region spanning 242–540 of GADD34, which is N-terminal to the motif mediating the binding of GADD34 to PP1 (Fig. 4D), is sufficient for TAK1 binding (Fig. 4E). Consistent
with our hypothesis that GADD34 mediates PP1 interaction with TAK1 and should function as a negative regulator of TAK1 activation, siRNA-mediated knockdown of GADD34 in primary peritoneal macrophages (Fig. 5A) enhanced LPS-induced TAK1 Ser412 phosphorylation, but had no detectable effect on IRAK1 phosphorylation, which is similar to what we observed in the case of PP1 knockdown (Fig. 2C).

We then asked whether GADD34 indeed mediates the interaction between PP1 and TAK1. An Ab against TAK1 was able to coimmunoprecipitate endogenous TAK1 and PP1. This interaction was abolished when GADD34 was depleted with siRNA (Fig. 5B). More notably, PP1 overexpression inhibited LPS-induced IκBα, JNK, and p38 phosphorylation, but this effect was blocked by simultaneous knockdown of GADD34 (Fig. 5C). Accordingly, diminishment of IL-6 and TNF-α induction by PP1 overexpression in LPS-treated RAW264.7 cells was abolished when GADD34 was knocked down simultaneously (Fig. 5D).

The interaction of PP1 with TAK1 is dynamic and can be disrupted upon stimulation (Fig. 3A–B). We wondered whether the interaction between GADD34 and TAK1 is also dynamic. Coimmunoprecipitation experiments using either GADD34-specific or TAK1-specific Abs revealed that GADD34 and TAK1 exist as a complex under basal conditions, and their association is unaffected by LPS stimulation in RAW264.7 cells (Fig. 5E). Collectively, GADD34 is the regulatory subunit of PP1 to specify PP1 to dephosphorylate TAK1 Ser412.

**GADD34 negatively regulates TLR signaling and cytokine production**

We further characterized the effects of GADD34 on TLR signaling. Overexpression of GADD34 reduced LPS-induced phosphorylation in RAW264.7 cells of IKKα/β, IκBα, ERK, JNK, and p38; this was not seen with GADD34ΔC, a C-terminal truncation mutant of GADD34 that cannot form complex with PP1C (32) (Fig. 6A). Accordingly, overexpression of GADD34, but not the GADD34ΔC, reduced IL-6 and TNF-α production in RAW264.7 cells induced by LPS, poly-IC, or CpG ODN (Fig. 6B). Consistent with these results, knockdown of GADD34 in primary peritoneal macrophages (Figs. 6C–6D) strongly enhanced LPS-induced phosphorylation of IKKα/β, IκBα, ERK, JNK, and p38, and significantly increased cytokine production.

**FIGURE 5.** GADD34 mediates TAK1 Ser412 dephosphorylation by PP1. (A) Primary peritoneal macrophages were transfected with scrambled or GADD34-specific siRNA for 48 h and then stimulated with LPS for the indicated time. Cell lysates were immunoblotted with indicated Abs. (B) RAW264.7 cells were transfected with scrambled or GADD34-specific siRNA for 48 h, cells were lysed, and lysates were immunoprecipitated (IP) with IgG or TAK1 Ab followed by immunoblotting with indicated Abs. (C) RAW264.7 cells were transfected with scrambled or GADD34-specific siRNA for 24 h and then transfected with empty vector (mock) or PP1 vector as indicated. After 36 more hours, cells were treated with LPS for the indicated time. Cell lysates were immunoblotted with indicated Abs. (D) As in (C), but cells were treated with LPS for 6 or 12 h. IL-6 and TNF-α in the supernatants were measured by ELISA. Data are plotted as means ± SE. All experiments were performed at least three times. (E) RAW264.7 cells were treated with LPS for the indicated time. Cell lysates were immunoprecipitated (IP) with IgG or TAK1 or GADD34 Ab followed by immunoblotting with indicated Abs. *p < 0.05, **p < 0.01 versus controls. ns, no significance.
FIGURE 6. GADD34 negatively regulates TLR signaling and cytokine production via inhibition of TAK1 Ser412 phosphorylation. (A) RAW264.7 cells were transfected with empty vector (mock), GADD34, or a GADD34 mutant lacking the PP1 binding site (GADD34ΔC) for 36 h and then stimulated with LPS for the indicated time. Cell lysates were immunoblotted with indicated Abs. (B) As in (A), but cells were then treated with LPS (100 ng/ml), poly-IC (20 μg/ml) or CpG ODN (0.3 μM) for 6 or 12 h. Secretion of IL-6 and TNF-α in the supernatants was measured with ELISA. (C) Primary peritoneal macrophages were transfected with scrambled siRNA or GADD34-specific siRNA for 48 h and then treated with LPS (100 ng/ml) for the indicated time. Cell lysates were immunoblotted with indicated Abs. (D) As in (C), but cells were then treated with LPS, poly-IC, or CpG ODN for 6 or 12 h. Secretion of IL-6 and TNF-α in the supernatants was measured with ELISA. (E) RAW264.7 cells were transfected with the indicated vectors for 24 h and then stimulated with LPS for the indicated time. Cell lysates were immunoblotted with indicated Abs. (F)RAW264.7 cells were transfected with the indicated vectors for 24 h and then stimulated with LPS (100 ng/ml) for 6 or 12 h. Secreted IL-6 and TNF-α in the supernatants were measured with ELISA. Data from (B), (D), and (F) are plotted as means ± SE. All experiments were performed at least three times. *p < 0.05, **p < 0.01, ***p < 0.001 versus controls. ns, no significance.
To confirm that negative regulation of TLR signaling by GADD34 is mediated by TAK1 Ser412 dephosphorylation, we tested whether the TAK1 S412D mutant is insensitive to regulation by GADD34. Overexpression of GADD34 in RAW264.7 cells strongly inhibited LPS-induced phosphorylation of IKKα/β, IκBα, ERK, JNK, and p38 even when coexpressed with TAK1 WT, which alone slightly enhanced their phosphorylation (Fig. 6E). In contrast, when TAK1 S412D was used, overexpression of GADD34 did not inhibit LPS-induced phosphorylation of IKKα/β, IκBα, ERK, JNK, or p38 (Fig. 6E). In addition, overexpression of GADD34 inhibited IL-6 and TNF-α production, and this inhibition was abolished in the presence of coexpressed TAK1 S412D (Fig. 6F). Therefore, like PP1, GADD34 also negatively regulates TLR signaling through inhibiting Ser412 phosphorylation.

**TAK1 Ser412 phosphorylation is essential for TLR signaling and cytokine production**

It is well known that phosphorylation of Thr187 is required for TAK1 kinase activation and is necessary for TLR triggered signaling (20, 21). TAK1 Ser412 phosphorylation has been described in only two reports (22, 23), and the role of TAK1 Ser412 phosphorylation in TLRs triggered immune response had not been reported. We studied a number of cells and cell lines, including freshly prepared peritoneal macrophages, RAW264.7 cell line, mouse embryonic fibroblast (MEF) cell line and C6 cell line (a 293 derivative stably expressing IL-1R) with LPS and IL-1β, and detected TAK1 Ser412 phosphorylation with TAK1 phospho-Ser412 specific Ab. As seen with stimulation-dependent Thr187 phosphorylation, the treatment induced phosphorylation of Ser412 (Fig. 7A). These two phosphorylation events correlate well with each other and suggest that TAK1 Ser412 phosphorylation might be a common event in response to LPS and IL-1β stimulation in various cell types.

We next investigated the significance of phosphorylation of TAK1 Ser412 in the NF-κB and MAPK signaling pathways. LPS treatment of RAW264.7 cells resulted in TAK1 activation, which in turn led to phosphorylation of IκBα, p38, JNK, and ERK1/2 (Fig. 7B). Overexpression of TAK1 WT slightly enhanced phosphorylation of IκBα, p38, JNK, and ERK1/2. In contrast, overexpression of TAK1 Ser412 to alanine (S412A) mutant significantly inhibited phosphorylation of IκBα, p38, JNK, and ERK1/2. The inhibition by TAK1 (S412A) was comparable to the reduction induced by overexpression of TAK1 (K63W) and TAK1 (T187A), two known TAK1 kinase-dead mutants that can function as dominant-negative mutants (Fig. 7B). Knockdown of TAK1 using hTAK1-specific small RNA in C6 cells reduced IL-1β-induced phosphorylation of IκBα, p38, and JNK, which was rescued by simultaneous expression of TAK1 WT, but not T187A or S412A mutants (Supplemental Fig. 3A). Overexpression of TAK1 T187A, S412A, or K63W, but not WT in RAW264.7 cells also reduced production of IL-6 and NF-α in response to LPS, poly-IC, or CpG treatment (Fig. 7C). MEFs generated from Cre-mediated TAK1 knockout cells showed impaired phosphorylation of IκBα, JNK, p38, and ERK1/2 and production of cytokines following LPS treatment (Fig. 7D–E), which was rescued to a comparable level to WT MEFs upon overexpression of TAK1 WT, T187D, S412D, or T187D/S412D, but not T187A, S412A, or T187A/S412A (Fig. 7D–E). The T187D and S412D mutations mimic their phosphorylation, rendering them insensitive to regulation of dephosphorylation. The residual phosphorylation of these proteins in knockout MEF cells might be due to incomplete knockout of TAK1 (Supplemental Fig. 3B). Measurement of IL-6 and TNF-α mRNA induction using quantitative RT-PCR revealed similar results (Supplemental Fig. 3C). These data together suggest that TAK1 Ser412 is phosphorylated in response to TLR and IL-1R activation and required for downstream signaling and cytokine induction.

**Discussion**

TLRs have critical roles in host defense against infection by detecting conserved components of invading microbial pathogens. Although TLRs are crucial for the initiation of protective immune responses, inappropriate TLR responses can contribute to the pathogenesis of acute and chronic inflammation, as well as to systemic autoimmune diseases. Thus, exploring how TLR signaling is tightly regulated would help us to understand the immunologic balance between activation and inhibition to avoid inappropriate inflammatory responses. In this report, we demonstrate that phosphatase PP1 negatively regulated TLR-triggered innate immune responses. PP1 knockdown increased LPS-induced proinflammatory cytokine production in macrophages. Inhibition of PP1 with a small molecule inhibitor led to enhancement of TNF-α production and susceptibility to endotoxin shock in mice. Therefore, PP1 serves as a physiologic inhibitor of TLR signaling, which can prevent excessive activation of TLRs and protect the host from overwhelmed inflammatory immune responses.

TAK1 is the upstream kinase whose kinase activity is required in the IL-1R/TLR signaling pathways. Its activation leads to phosphorylation and activation of IKK–NF-κB and the three MAP kinase pathways, which lead to the production of proinflammatory cytokines (33). Phosphorylation of Thr187 is necessary for TAK1 kinase activity on its downstream substrates and cytokine induction in TLR pathway (20). TAK1 Ser412 phosphorylation was reported previously (22, 23). However, the importance of Ser412 phosphorylation in TAK1 activation, and its role in the TLR and IL-1R pathways, was unknown. In this study, we found that PP1 and its regulatory subunit GADD34 negatively regulated TLR-triggered immune response by dephosphorylating TAK1 Ser412 instead of Thr187. In fact, PP1 did not show any detectable phosphatase activity toward Thr187 in an in vitro assay using purified PP1 and preactivated TAK1. We also found that in multiple cell types, simulation of IL-1R or TLRs results in rapid phosphorylation, followed by dephosphorylation, of TAK1 Ser412. This finding suggests that TAK1 Ser412 phosphorylation is not unique to a particular cell type or a particular ligand, but instead plays a general role in the TLR and IL-1R pathways. Our data then demonstrate that Ser412 phosphorylation is as important as Thr187 phosphorylation for TAK1 kinase activation (20). Overexpression of either of these mutants had a dominant negative effect. Interestingly, although both residues are important for TAK1 kinase activation, and their phosphorylation profiles show parallel kinetics, it seems that Thr187 phosphorylation and Ser412 phosphorylation are actually independent of each other.

Thr187 is located in the TAK1 kinase activation loop; by contrast, Ser412 is near the C terminus, which is rather distant from the kinase domain. It is not clear how Ser412 phosphorylation influences TAK1 kinase activity; an understanding of this will require structural information. Notably, activation of many kinases, such as Akt, NDR, and PKC family members, requires phosphorylation of Ser/Thr residues outside of their respective activation loops for maximal activity (34). For example, full Akt activation requires phosphorylation of the hydrophobic segment at its C terminus by mTORC2 (35).

A previous report has shown that PP1/GADD34/CUEDC2 dephosphorylates IKK and regulates cytokine production in the TNF-α pathway (18). We presented several lines of evidence suggesting that the most important target of PP1 in the IL-1R and TLR signaling pathways tested in this study is TAK1.
FIGURE 7. TAK1 Ser412 phosphorylation is essential for TLR signaling and production of proinflammatory cytokines. (A) Mouse peritoneal macrophages, RAW264.7, MEF, and C6 cells, were treated by LPS (100 ng/ml for peritoneal macrophages and RAW264.7, 1 μg/ml for MEFs) or IL-1β (10 ng/ml for C6) for the indicated time.  Phosphorylation of TAK1 Thr187, Ser412, and total TAK1 were detected by immunoblotting. (B) RAW264.7 cells stably transfected with empty vector (mock), Flag-TAK1, TAK1 T187A, S412A, or K63W were treated with LPS (100 ng/ml) for the indicated time. Cell lysates were immunoblotted with indicated Abs. (C) RAW264.7 cells were treated with LPS (100 ng/ml), poly-IC (20 μg/ml), or CpG ODN (0.3 μM). IL-6 and TNF-α in the supernatants were measured with ELISA. (D) TAK1−/− MEF cells were transfected with empty vector, TAK1 WT, or the indicated mutants and were treated with LPS (1 μg/ml) for the indicated time. Cell lysates were immunoblotted with indicated Abs. (E) Cells were treated as in (D), but with longer LPS stimulation. Secretion of TNF-α in the supernatant was analyzed by ELISA. Data from (C) and (E) are plotted as means ± SE. All experiments were performed at least three times. *p < 0.05, **p < 0.01, ***p < 0.001 versus controls. ns, no significance.
1. In our in vitro phosphatase assay, PP1 did not show potent activity on IKK.
2. Using NF-κB–based reporter assays, epistatic analysis showed that PP1 functions at the level of TAK1.
3. Overexpression or knockdown of PP1 or GADD34 affected both NF-κB and MAP kinase signaling components instead of just IKK and its downstream IkBα.
4. The negative regulatory effects of PP1 and GADD34 were abolished by simultaneous expression of TAK1 S412D mutant.

The target of tautomycin inhibition also includes other proteins, such as SHP2 (36), which negatively regulates the TRIF adapter protein-dependent type I IFN and proinflammatory cyto- kine production in TLR signaling (8). It is hard to exclude the possibility that the tautomycin aggravates LPS-induced endotoxin shock in mice via inhibiting SHP2 activity. Because the IC50 values for PP1 and SHP2 enzymes is 1.6 nM and 2.9 μM, respectively (27, 36). The dominant inhibition target of tautomycin should be PP1 in the mouse model.

In summary, we have shown that PP1 is a feedback-negative regulator of TLR signaling by targeting TAK1 at Ser412 residue. The phosphorylation of TAK1 at Ser412 kinase is required for full activation of TAK1 and downstream signaling in the IL-1R and TLRs pathways. PP1, together with its regulatory subunit GADD34, associates with TAK1 in resting cells, disassociates from TAK1 upon ligand stimulation, and quickly associates with TAK1 again. The activation of TAK1 and downstream signaling in the IL-1R and TLRs is required for full activation of TAK1 in a signaling complex containing TAK1-binding protein TAB1 and TAB2. J. Biol. Chem. 280: 7357–7368.


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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


In the original Fig.7B, a blot of p-JNK was used to represent p-ERK1/2 by mistake. In the original Fig.7D, the two right panels for ERK1/2 were inadvertently duplicated.

Fig. 7 appears below with corrected panels (B) and (D). This change does not affect the conclusions or interpretations of findings presented in our article in any way.

The figure legend was correct as published and is shown below for reference.

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Figure S1. Silencing of PP1 promotes TLR-induced proinflammatory cytokine secretion in macrophages

(A) RAW264.7 cells were transfected with scrambled or PP1-specific siRNA for 48 hours and then stimulated with LPS (100ng/ml), poly (I:C) (20μg/ml) or CpG ODN (0.3μM) for 6 or 12 hours. Secretion of IL-6 and TNF-α in the supernatant was measured by ELISA. Data are plotted as means ± SE; *p < 0.05, **p < 0.01, ***p < 0.001, versus controls. All experiments were performed at least three times.

(B) RAW264.7 cells were transfected with scrambled or PP1-specific siRNA for 48 hours; the efficiency of silencing was detected by quantitative PCR and confirmed by Western blot.

(C) BMDM cells were transfected with 60 nM of scrambled or 40 nM/60 nM of PP1-specific siRNA for 48 hours and cell lysates were used for immunoblotting by using anti-PP1 to determine optimal concentration of PP1 siRNA for its efficient knockdown.
Figure S2. PP1 specifically dephosphorylates TAK1 at Ser412 and inhibits MAPKs and NF-κB activation in TLR signaling pathways

(A) 293T cells were transfected with a NF-κB luciferase reporter and the indicated vectors and 24 hours later luciferase activities were then measured.

(B) RAW264.7 cells were transfected with a NF-κB or AP-1-based luciferase reporter and empty vector (mock) or Ppp1cc vector for 24 hours and then treated with LPS (100ng/ml). 24 hours later, luciferase activities were then measured.

(C) RAW264.7 cells were transfected with scrambled or PP1-specific siRNA. After 48 hours, cells were transfected with NF-κB- or AP-1-based luciferase reporters and stimulated with LPS (100 ng/ml) for 12 hours. Luciferase activities were then measured.

(D) Tandem mass spectra of tryptic phosphopeptides pS412IQDLTVTGTEPGQVSSR identified phosphorylation sites at S412. b- and y-ion designations are shown. Mass spectrometry analysis of Flag-TAK1 protein purified from Flag-TAK1 expressing RAW264.7 stable cells with LPS stimulation for 5 min.

(E) As in (A), NF-κB- or AP-1-based luciferase reporters were determined for the indicated genes. Data from (A) - (E) are plotted as means ± SE; *p < 0.05, **p < 0.01, ***p < 0.001, versus controls. ns, no significance. All experiments were performed at least three times.

(F) Flag-TAK1 or Flag-IKKβ was expressed and immunoprecipitated from 293T cells and then incubated with the indicated amount of recombinant PP1 at 30°C for 30min. Dephosphorylation of TAK1 or IKKβ was determined with the indicated phospho-specific antibodies, respectively.

(G) 293T cells were transfected with empty vector (mock) or TAK1 with TAB1 for 36 hours, followed by treatment of Okadaic Acid (OA) at 0.2 nM or 20 nM for 3 more hours. RAW264.7 cells were pretreated with OA or DMSO for 3 hours and stimulated by LPS for 5 minutes. Cell lysates were subjected to immunoblotting with the indicated antibodies.
Figure S3. TAK1 Serine 412 phosphorylation is essential for IL-1β-induced IKK and MAP kinase activation

(A) C6 cells, a 293 derivative stably expressing IL-1R, were transfected with scrambled siRNA, or human TAK1-specific siRNA for 24 hours and re-transfected with an empty vector (mock), siRNA-resistant Flag-TAK1 WT or its mutants T187A and S412A. After 36 more hours, cells were treated with IL-1β (10 ng/ml) for the indicated time. Whole cell lysates were used for immunoblotting with the indicated antibodies.

(B) Lysates from MEFs used in Figure 7D were subjected to immunoblotting to confirm the efficiency of Cre-mediated TAK1 knockout.

(C) As in Figure 7E, but cells were stimulated with LPS for 6 or 24 hours. mRNAs were then extracted and used to determine induction of IL-6 and TNF-α by using quantitative real-time PCR (Q-RT-PCR). Data are plotted as means ± SE; *p < 0.05, **p < 0.01, ***p < 0.001, versus controls. All experiments were performed at least three times.
Figure S4. Model outlining the importance of TAK1 Ser412 phosphorylation and its tight regulation by PP1/GADD34 in innate immune responses.