Immunity 25, 919–928, December 2006 ©2006 Elsevier Inc. DOI 10.1016/j.immuni.2006.10.014

SHP-2 Phosphatase Negatively Regulates the TRIF Adaptor Protein-Dependent Type I Interferon and Proinflammatory Cytokine Production

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Summary

The Toll-like receptor 3 (TLR3) and TLR4-signaling pathway that involves the adaptor protein TRIF activates type I interferon (IFN) and proinflammatory cytokine expression. Little is known about how TRIF pathway-dependent gene expression is regulated. SH2-containing protein tyrosine phosphatase 2 (SHP-2) is a widely expressed cytoplasmic tyrosine phosphatase. Here we demonstrate that SHP-2 negatively regulated TLR4- and TLR3-activated IFN-β production. SHP-2 inhibited TLR3-activated but not TLR2-, TLR7-, and TLR9-activated proinflammatory cytokine IL-6 and TNF-α production. SHP-2 inhibited poly(I:C)-induced cytokine production by a phosphatase activity-independent mechanism. C-terminal domain of SHP-2 directly bound TANK binding kinase (TBK1) by interacting with the kinase domain of TBK1. SHP-2 deficiency increased TBK1-activated IFN-β and TNF-α expression. TBK1 knockdown inhibited poly(I:C)-induced IL-6 production in SHP-2-deficient cells. SHP-2 also inhibited poly(I:C)-induced activation of MAP kinase pathways. These results demonstrate that SHP-2 specifically negatively regulate TRIF-mediated gene expression in TLR signaling, partially through inhibiting TBK1-activated signal transduction.

Introduction

Toll-like receptors (TLRs) are the main receptors by which immune cells recognize microbial conserved components such as lipopolysaccharide (LPS), dsRNA, and bacterial DNA. The signal transduction mechanisms of TLRs have been intensively investigated (Takeda et al., 2003; Barton and Medzhitov, 2003). Most of the members of TLR family, except TLR3, activate immune cells through the cascade that involves myeloid factor 88 (MyD88), IL-1 receptor-associated kinase 1 (IRAK1), tumor necrosis factor receptor-associated factor 6 (TRAF6), and TGF-β-activated kinase 1 (TAK1) proteins and downstream MAP kinases (MAPKs) and the transcription factor NF-κB to induce proinflammatory cytokine production (Takeda et al., 2003; Barton and Medzhitov, 2003). TRIF was initially identified as a critical MyD88-independent adaptor protein in TLR3 and TLR4 signal transduction (Hoebe et al., 2003a; Yamamoto et al., 2003). Upon TLR3 or TLR4 activation, TRIF associates with TANK-binding kinase (TBK1) and activates downstream interferon-regulating factor 3 (IRF3), mediating TLR3- and TLR4-activated type I interferon production (Oshiumi et al., 2003; Fitzgerald et al., 2003). TRIF also activates MAPKs and NF-κB and contributes to proinflammatory cytokine production in TLR3 and TLR4 signaling (Hoebe et al., 2003a; Yamamoto et al., 2003). Thus, the TRIF-TBK1 pathway is essential for TLR3- and TLR4-activated inflammatory cytokine production, type I interferon-dependent gene expression, and antigen-presenting cell maturation (Hoebe et al., 2003a, 2003b; Yamamoto et al., 2003). In addition, recent studies suggested that the TRIF-TBK1 pathway is also involved in tumor vascularization by mediating the production of angiogenesis-modulating factors (Korherr et al., 2006).

Although the full activation of macrophages is necessary for elimination of invading pathogens, inappropriate production of IFN-β and proinflammatory cytokines might promote the development of immunopathological conditions such as endotoxin shock and autoimmune responses (Karaghiosoff et al., 2003; Liew et al., 2005). More than ten negative regulators have been shown to prevent macrophages from production of excessive proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 by targeting MyD88-dependent pathway upon TLR activation. However, little is known about how TRIF pathway-activated production of type I IFN and inflammatory cytokine is negatively regulated. Given the important roles of TRIF-dependent pathway in a variety of physiological or pathological processes, it will be important to investigate how TRIF-dependent pathway is regulated in TLR signaling (Liew et al., 2005).

The Src homology 2 domain-containing protein tyrosine phosphatase 2 (SHP-2) is an evolutionarily conserved protein tyrosine phosphatase, containing two SH2 domains at the N terminus, a central catalytic domain, and a C-terminal tail (Matthews et al., 1992; Neel et al., 2003). SHP-2 positively regulates the signaling pathways of cytokines and growth factors, such as insulin, EGF, PDGF, FGF, IL-1, and IL-6 (Kharitonenkov et al., 1997; Bennett et al., 1994; Qu, 2000; You et al., 2001). In contrast to the positive role in the mitogenic pathways, SHP-2 negatively regulates the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling pathway initiated by IFN-α and IFN-γ (You et al., 1999). SHP-2 may also have an inhibitory role in activation of T and B lymphocytes (Lee et al., 1998; Feng, 1999; Sedy et al., 2005). In addition, dominantly

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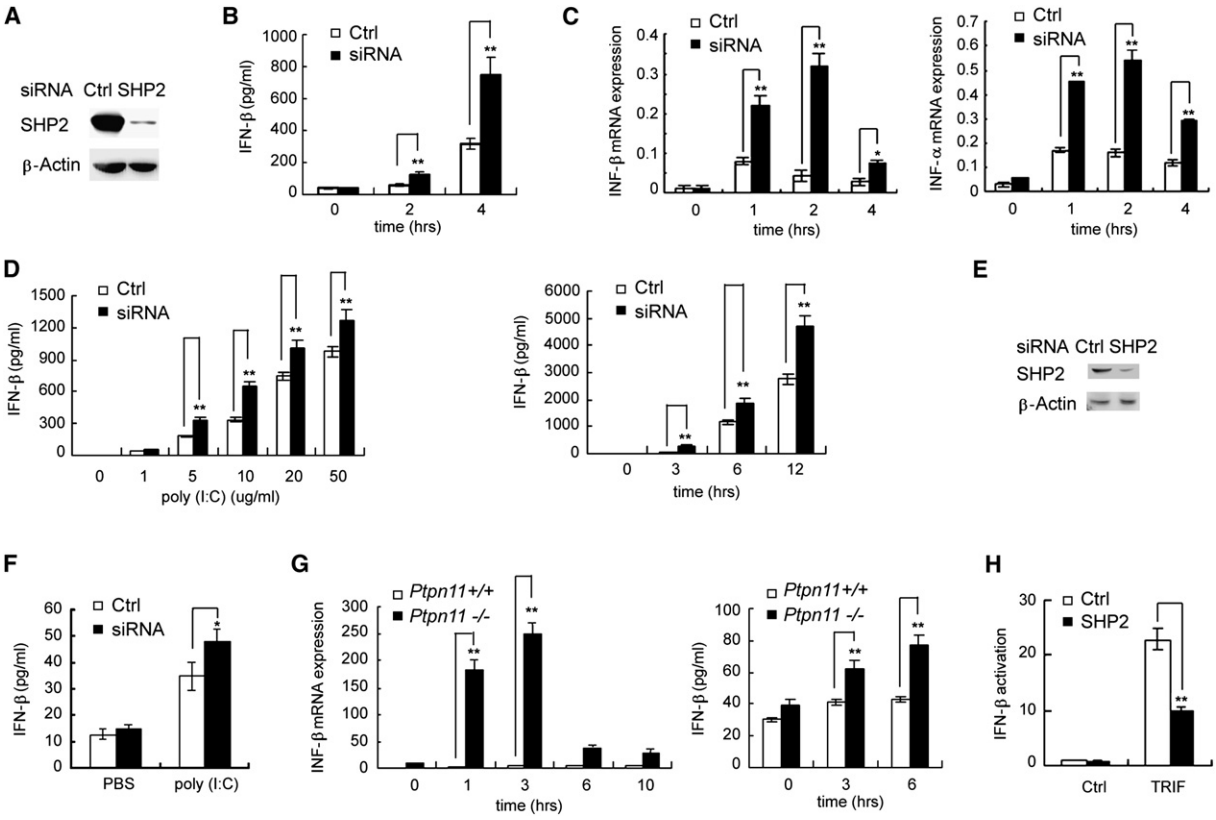

Figure 1. SHP-2 Negatively Regulates TRIF-Dependent Type I Interferon Production in TLR4 and TLR3 Signal Transduction in Macrophages

(A) Mouse peritoneal macrophages were transfected with control small RNA (Ctrl) or SHP-2 siRNA. After 36 hr, SHP-2 expression in the cells was detected by immunoblot. Similar results were obtained in three independent experiments.

(B) 1.5×10^5 mouse peritoneal macrophages were transfected with control small RNA (Ctrl) or SHP-2 siRNA (siRNA) and then treated with 100 ng/ml LPS for indicated time period. IFN-β in the supernatants was measured by ELISA. Data are shown as mean ± SE of three independent experiments (**$p < 0.01$).

(C) Mouse peritoneal macrophages were treated as described in (B). mRNA expression of IFN-4α (IFN-α) and IFN-β were analyzed. Data are shown as mean ± SD (n = 3; **$p < 0.01$). Similar results were obtained in three independent experiments.

(D) Mouse peritoneal macrophages were treated as in (B), but stimulated with poly(I:C) for 12 hr or indicated time period. IFN-β production was measured by ELISA. Data are shown as mean ± SE of three independent experiments (**$p < 0.01$).

(E) Female C57BL/6J mice (4 weeks old) were intraperitoneally injected with thioglycolate to elicit peritoneal macrophages. After 3 days, intraperitoneal siRNA transfection was performed. After 48 hr, intraperitoneal cells were collected, and SHP-2 expression in the cells was detected by immunoblot. Similar results were obtained in three independent experiments.

(F) Female C57BL/6J mice (4 weeks old) were treated as in (E), but after 48 hr, the mice were treated with PBS or 10 μg/ml poly(I:C) intraperitoneal administration. 1 hr later, peritoneal lavage was collected and IFN-β production in the lavage was detected by ELISA. Data are shown as mean ± SD (n = 5; *$p < 0.05$). Similar results were obtained in three independent experiments.

(G) *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF were stimulated with 10 μg/ml poly(I:C) for indicated time period, and IFN-β mRNA expression in the cells and IFN-β production in the supernatants was measured. Data are shown as mean ± SE of three independent experiments (**$p < 0.01$).

(H) HEK 293 cells were transfected with 100 ng TRIF expressing plasmid, 100 IFN-β luciferase reporter plasmid, 10 ng pTK-*Renilla*-luciferase, together with 100 ng SHP-2 plasmid or empty control plasmid. After 24 hr of culture, luciferase activity was measured and shown as mean ± SD (n = 3; **$p < 0.01$). Similar results were obtained in three independent experiments.

activating mutations in human *PTPN11,* encoding mutant SHP-2, is related with Noonan Syndrome (Tartaglia et al., 2001). In the present study, we demonstrate that SHP-2 inhibits TRIF-dependent type I interferon (IFN-α/β) and proinflammatory cytokine production, identifying SHP-2 as a negative regulator of TRIF-dependent TLR4 and TLR3 signal transduction.

Results

SHP-2 Negatively Regulates TRIF-Dependent Type I Interferon Production in TLR4 and TLR3 Signaling

To investigate whether SHP-2 plays a role in TRIF- and TBK1-dependent pathway, we initially examined the effects of SHP-2 expression on TLR3 and TLR4 agonists poly(I:C)- and LPS-induced type I interferon production in macrophages. Previous studies showed that synthesized small-interfering RNA could markedly suppress targeted gene expression in mouse primary peritoneal cells both in vitro and in vivo (Okazawa et al., 2005; Nakamichi et al., 2005). Thus, synthesized interfering RNA targeting mouse SHP-2, SHP-2 siRNA, was used to suppress endogenous SHP-2 expression. SHP-2 siRNA transfection resulted in more than 70% decrease in SHP-2 expression in mouse peritoneal macrophages (Figure 1A). As shown in Figure 1B, SHP-2 knockdown significantly increased LPS-induced IFN-β production in mouse peritoneal macrophages. The effect of SHP-2

knockdown on LPS-induced IFN-β expression was also analyzed by quantitative RT-PCR. SHP-2 siRNA treatment significantly increased LPS-induced IFN-β mRNA expression (Figure 1C), demonstrating that SHP-2 inhibits LPS-induced IFN-β expression at both mRNA and protein levels in macrophages. Similarly, SHP-2 siRNA treatment significantly increased LPS-induced IFN-4α mRNA expression (Figure 1C). Pretreatment with cycloheximide (CHX), an inhibitor of protein synthesis, could not diminish SHP-2 knockdown-mediated increase in LPS-induced IFN-β mRNA expression (see Figure S1 in the Supplemental Data available online). The effect of SHP-2 knockdown on poly(I:C)-induced IFN-β production was detected both in in vitro culture system and in vivo. As shown in Figure 1D, SHP-2 siRNA treatment significantly increased poly(I:C)-induced IFN-β production in cultured primary macrophages. Furthermore, SHP-2 siRNA intraperitoneal transfection suppressed SHP-2 expression in intraperitoneal cells (Figure 1E) and significantly increased poly(I:C)-induced IFN-β production in peritoneal lavage (Figure 1F). To confirm the negative regulatory role of SHP-2 in TLR3-mediated IFN-β production, the effects of SHP-2 deficiency on poly(I:C)-induced IFN-β mRNA expression and IFN-β production in mouse embryonic fibroblast (MEF) were observed. As shown in Figure 1G, poly(I:C)-induced IFN-β mRNA expression and IFN-β production were significantly increased in SHP-2-deficient (*Ptpn11*$^{-/-}$) MEF compared with that in *Ptpn11*$^{+/+}$ MEF. Because type I interferon production in TLR3 and TLR4 signaling is TRIF dependent, these results indicated that SHP-2 is a negative regulator of TRIF pathway both in vitro and in vivo. Supporting this conclusion, SHP-2 overexpression inhibited TRIF-induced IFN-β luciferase reporter gene expression in 293 cells (Figure 1H).

SHP-2 Inhibits TLR3- but Not TLR2-, TLR7-, or TLR9-Mediated Proinflammatory Cytokine Production

TRIF activates both type I interferon and proinflammatory cytokine expression in TLR signaling. Thus, we also observed the effect of SHP-2 deficiency on TRIF-activated TNF-α reporter gene expression in MEF. As shown in Figure 2A, SHP-2 deficiency significantly increased TRIF-activated TNF-α reporter gene expression in MEF, suggesting that SHP-2 also inhibited TRIF-mediated proinflammatory cytokine production. To investigate whether SHP-2 inhibited TRIF-dependent proinflammatory cytokine production, the effects of SHP-2 knockdown on TLR3-mediated IL-6 and TNF-α production was detected. As shown in Figure 2B, SHP-2 siRNA treatment significantly increased poly(I:C)-induced TNF-α and IL-6 production in primary peritoneal macrophages. Consistently, SHP-2 siRNA treatment significantly increased poly(I:C)-induced TNF-α mRNA expression in the cells (Figure 2C). To investigate whether SHP-2 specifically inhibited TRIF-dependent proinflammatory cytokine production, primary peritoneal macrophages were stimulated with LTA, R837, and CpG ODN, the agonists of TLR2, TLR7, and TLR9, respectively. SHP-2 siRNA treatment did not increase LTA-, R837-, and CpG ODN-induced TNF-α and IL-6 production in primary peritoneal macrophages (Figure 2D). We also detected poly(I:C)-, LTA-, and R837-induced IL-6 production in *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF. SHP-2 deficiency significantly increased poly(I:C)-induced IL-6 mRNA expression (Figure 2E) and IL-6 production (Figure 2F) in MEF. In contrast, SHP-2 deficiency did not increase LTA- and R837-induced IL-6 production in MEF (Figure 2F). These results provide further evidence that SHP-2 negatively regulates TRIF-dependent signal transduction.

SHP-2 Negatively Regulates TRIF-Dependent Activation of IRF3 and MAPK Pathways

IRF3 is the key transcription factor that is activated in TRIF-dependent pathway and mediates the expression of type I interferon in TLR3 and TLR4 signal transduction. We next observed the effect of SHP-2 overexpression on LPS-induced IFR3 activation by detecting IRF3 luciferase reporter gene expression. RAW264.7 cells were transfected with pcDNA-SHP-2W plasmid encoding wild-type SHP-2, or pcDNA empty control plasmid, together with Gal4-IRF3-expressing plasmid plus Gal4 luciferase reporter plasmid. As shown in Figure 3A, LPS-induced IRF3 activation was significantly inhibited by SHP-2 overexpression in a dose-dependent manner. To confirm the regulatory role of SHP-2 in TLR-activated IRF3 activation, *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF were transfected with Gal4-IRF3-expressing plasmid plus Gal4 luciferase reporter plasmid, and then stimulated with poly(I:C). SHP-2 deficiency significantly enhanced both baseline and poly(I:C)-induced IRF3 activation (Figure 3B). Consistently, SHP-2 deficiency significantly enhanced LPS- and poly(I:C)-induced IFN-β luciferase reporter gene expression (Figure 3C). These results demonstrated that endogenous SHP-2 negatively regulates IRF3 activation in TLR3 and TLR4 signaling. STAT1 activation enhances TLR4-mediated IFN-β production in macrophages. However, SHP-2 siRNA inhibited LPS-induced phosphorylation of STAT1 (Figure S2).

TRIF-dependent activation of MAPK pathways are required for cytokine production in TLR3 signaling, and therefore we investigated whether the activation of MAPK pathways in TLR3 signaling was affected by SHP-2 expression. SHP-2 deficiency enhanced poly(I:C)-induced ERK1 and ERK2, JNK1 and JNK2, and p38 MAPK activation in MEF, suggesting that SHP-2 negatively regulates TRIF-dependent activation of MAPK pathways (Figure 3D). Interestingly, SHP-2 deficiency also enhanced poly(I:C)-induced IRAK1 phosphorylation (Figure S3).

SHP-2 Inhibits TBK1-Activated Gene Expression

TBK1 functions downstream of TRIF and is essential for TRIF-induced IRF3 activation and IFN-α/β expression (Fitzgerald et al., 2003). To investigate whether SHP-2 inhibited TBK1-activated signal transduction, *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF were transfected with TBK1-expressing construct plus IFN-β reporter plasmid. As anticipated, TBK1-activated IFN-β reporter gene expression was significantly increased in *Ptpn11*$^{-/-}$ MEF compared with that in *Ptpn11*$^{+/+}$ cells (Figure 4A).

To investigate whether TBK1 was involved in the increase of poly(I:C)-induced proinflammatory cytokine production in SHP-2-deficient MEF, the effect of SHP-2 deficiency on TBK1-activated TNF-α reporter gene expression in MEF was also investigated. SHP-2 deficiency significantly increased TBK1-activated TNF-α reporter gene expression in MEF (Figure 4A). Furthermore, TBK1 expression in MEF was suppressed with small

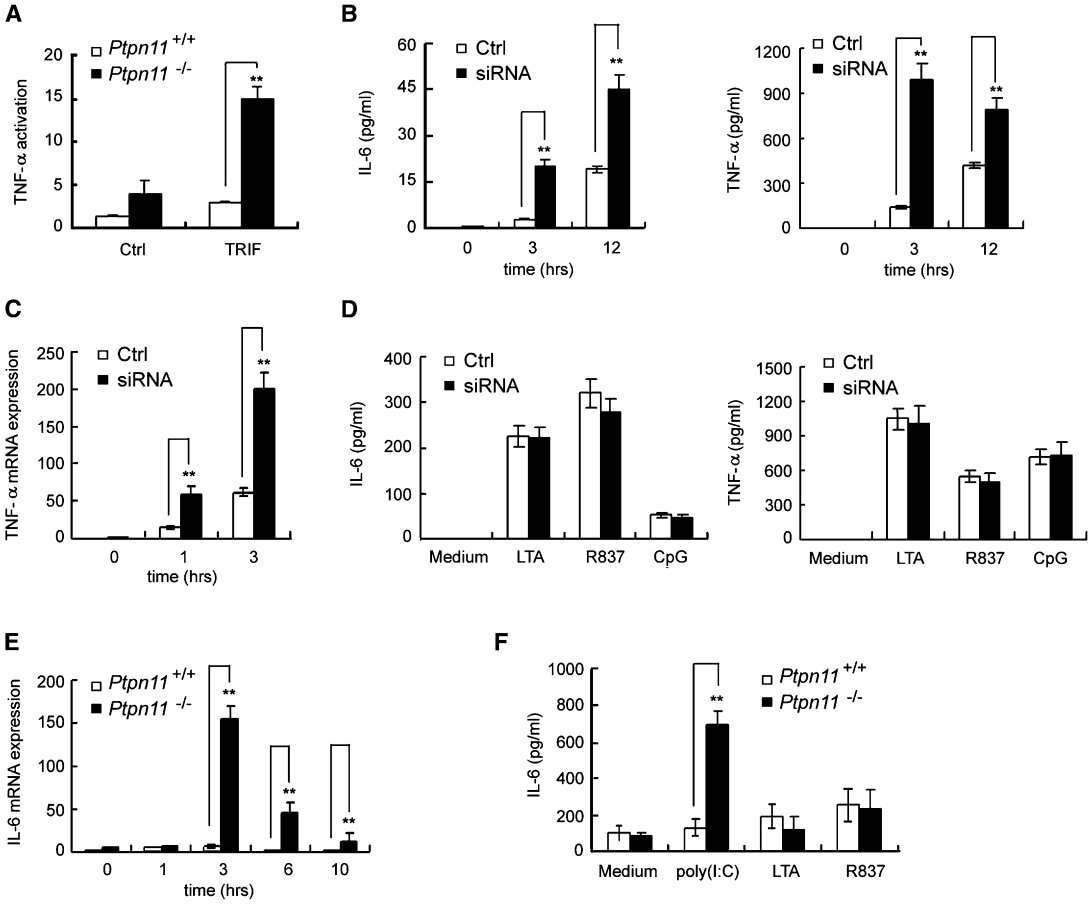

Figure 2. SHP-2 Negatively Regulates TRIF-Dependent Proinflammatory Cytokine Production

(A) *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF were transfected with 40 ng TNF-α luciferase reporter plasmid and 10 ng pTK-*Renilla*-luciferase, together with 100 ng TRIF-expressing plasmid. Total amounts of plasmid DNA were equalized with pcDNA empty control vector. After 24 hr of culture, luciferase activity was measured and normalized by *Renilla* luciferase activity. Data are shown as mean ± SD (n = 3) of one typical experiment (**p < 0.01). Similar results were obtained in three independent experiments.

(B) Mouse peritoneal macrophages were transfected with control small RNA (Ctrl) or SHP-2 siRNA (siRNA). After 36 hr, the cells were stimulated with 10 μg/ml poly(I:C) for indicated time period. Concentrations of IL-6 and TNF-α in the supernatants were measured by ELISA. Data are shown as mean ± SE of three independent experiments (**p < 0.01).

(C) Mouse peritoneal macrophages were treated as described in (B), TNF-α mRNA expression in the cells was measured by real-time RT-PCR. Data are shown as mean ± SE of three independent experiments (**p < 0.01).

(D) Mouse peritoneal macrophages were transfected with control small RNA (Ctrl) or SHP-2 siRNA (siRNA). After 36 hr, the cells were stimulated with 10 μg/ml LTA, 10 μg/ml R837, or 1 μM CpG ODN for 12 hr. Concentrations of IL-6 and TNF-α in the supernatants were measured by ELISA. Data are shown as mean ± SE of three independent experiments (**p < 0.01).

(E) *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF were stimulated with 10 μg/ml poly(I:C) for indicated time period, IL-6 mRNA expression in the cells was assayed by real-time RT-PCR. Data are shown as mean ± SE of three independent experiments (**p < 0.01).

(F) *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF were stimulated with 10 μg/ml poly(I:C), 10 μg/ml LTA, or 10 μg/ml R837 for 10 hr, IL-6 production was measured by ELISA. Data are shown as mean ± SE of three independent experiments (**p < 0.01).

interfering RNA specific to TBK1 (Figure 4B). Although TBK1 knockdown did not affect IL-6 production in *Ptpn11*$^{+/+}$ MEF, it significantly inhibited poly(I:C)-induced IL-6 production in *Ptpn11*$^{-/-}$ MEF (Figure 4C). These results suggested that TBK1-mediated signal transduction is involved in poly(I:C)-induced proinflammatory cytokine production in *Ptpn11*$^{-/-}$ MEF.

SHP-2 Inhibits TRIF-Dependent Cytokine Production in Tyrosine Phosphatase Activity-Independent Manner

It has been well established that mutation of amino acid 463 from Cys to Ser disrupted the tyrosine phosphatase activity of SHP-2 (Yin et al., 1997). To investigate whether SHP-2 inhibited TRIF-activated cytokine production through its phosphatase activity, RAW264.7 macrophages stably transfected with SHP-2W or SHP-2CS plasmids were stimulated with poly(I:C), and then TNF-α, IL-6, and IFN-β production was detected. Both SHP-2W and SHP-2CS inhibited poly(I:C)-induced TNF-α, IL-6, and IFN-β production (Figure 5), suggesting that SHP-2 inhibits poly(I:C)-induced TNF-α, IL-6, and IFN-β production in a phosphatase activity-independent manner.

SHP-2 Interacts with TBK1 and Inhibits TBK1-Activated IFN-β Expression

To understand the mechanisms by which SHP-2 negatively regulates TBK1-activated gene expression, we

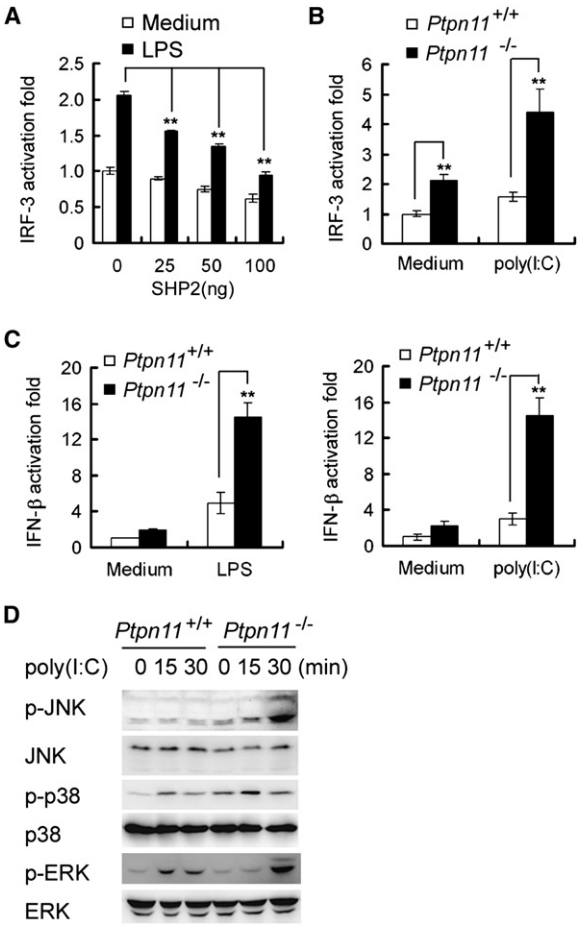

Figure 3. SHP-2 Negatively Regulates TRIF-Dependent Activation of IRF3 and MAPK Pathways

(A) RAW264.7 macrophages were transfected with 100 ng IRF3 luciferase reporter plasmids (80 ng Gal4 luciferase reporter plasmid, 20 ng Gal4-IRF3 expressing plasmid), 100 ng pTK-*Renilla*-luciferase, and indicated dose of SHP-2. Total amounts of plasmid DNA were equalized with pcDNA empty control vector. After 24 hr of culture, the cells were stimulated with 100 ng/ml LPS for 6 hr. Luciferase activity was measured and normalized by *Renilla* luciferase activity. Data are shown as mean ± SD (n = 3) of one typical experiment (**p < 0.01). Similar results were obtained in three independent experiments.

(B) *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF were transfected with 100 ng IRF3 luciferase reporter plasmids (80 ng Gal4 luciferase reporter plasmid, 20 ng Gal4-IRF3 expressing plasmid), 10 ng pTK-*Renilla*-luciferase. After 24 hr of culture, the cells were stimulated with 10 μg/ml poly(I:C) for 6 hr. Luciferase activity was measured and normalized by *Renilla* luciferase activity. Data are shown as mean ± SD (n = 3) of one typical experiment (**p < 0.01). Similar results were obtained in three independent experiments.

(C) *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF were transfected with 40 ng IFN-β luciferase reporter construct and 10 ng pTK-*Renilla*-luciferase plasmid. After 24 hr of culture, the cells were stimulated with 10 μg/ml LPS or 10 μg/ml poly(I:C) for 4 hr. Luciferase activity was measured and normalized by *Renilla* luciferase activity. Data are shown as mean ± SD (n = 6) of one typical experiment (**p < 0.01). Similar results were obtained in three independent experiments.

(D) *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF were treated with 10 μg/ml poly(I:C) for the indicated time. Phospho-ERK, phospho-38, phospho-JNK, and total ERK, p38, and JNK were detected by immunoblot. Similar results were obtained in three independent experiments.

investigated whether endogenous SHP-2 interacted with TBK1 in TLR signal transduction. After poly(I:C) treatment, RAW264.7 macrophages were lysed and SHP-2 was precipitated with specific SHP-2 antibody, and TBK1 was coprecipitated with SHP-2 after poly(I:C) treatment (Figure 6A). Reverse immunoprecipitation experiments were also carried out with TBK1-specific antibody, and SHP-2 was coprecipitated (Figure 6A), suggesting that endogenous SHP-2 was recruited to TBK1 upon TLR3 activation.

TBK1 was considered an essential intermediator downstream of TRIF in TLR3 signaling. Interestingly, mutation of K38A in the N-terminal domain of TBK1 blocked the binding of TBK1 to TRIF, suggesting that the kinase activity of TBK1 was required for the association between TBK1 and TRIF (Sato et al., 2003). We constructed mutant TBK1 K38A plasmid and coexpressed TBK1 K38A and SHP-2 in 293 cells. The K39A mutation did not significantly affect the association between TBK1 and SHP-2 (Figure S4), suggesting that the association between TBK1 and SHP-2 was not dependent on the formation of TRIF-TBK1 complex.

To determine whether SHP-2 could directly bind TBK1, purified active recombinant TBK1 was incubated with *E. coli*-expressed GST-tagged SHP-2 amino acids 230–545, and then pull-down assay was performed with glutathione-agarose. Blotting with TBK1-specific antibody revealed that TBK1 could be pulled down with GST-tagged SHP-2(230-545) (Figure 6B). More importantly, incubation with recombinant SHP-2(230-545) inhibited TBK1-mediated phosphorylation of myelin basic protein (MBP) (Figure 6C), demonstrating that SHP-2 could directly bind TBK1 and inhibit phosphorylation of substrate by TBK1. We constructed the truncated SHP-2 mutants (Figure S5) and investigated whether C-terminal domain of SHP-2 interacted with TBK1 in HEK293 cells. As shown in Figure 6D, C-terminal domain (SHP-2C273) rather than N-terminal SH2 domain of SHP-2 (SHP-2N233) was associated with TBK1. Tyrosine residues 546 and 584 in C-terminal domain of SHP-2 were reported to be required for the interaction between SHP-2 and several molecules (Vogel and Ullrich, 1996). However, truncated SHP-2C273-538 could still coprecipitate TBK1 (Figure S6), suggesting that these tyrosine residues were not required for the interaction between SHP-2 and TBK1. Consistently, SHP-2C273, SHP-2C273-538, and mutant SHP-2C273CS significantly inhibited TBK1-activated IFN-β expression (Figure 6E).

To determine how SHP-2 bound to TBK1, truncated TBK1 mutants were constructed (Figure S7) and coexpressed with SHP-2 in 293 cells. As shown in Figure 6F, SHP-2 could be coprecipitated with N-terminal domain of TBK1 (TBK1N242). In contrast, C-terminal domain of TBK1 (TBK1C243) failed to precipitate SHP-2. Similarly, truncated C-terminal domain TBK1C243-392 could not precipitate SHP-2 (Figure S8). These results demonstrated that the binding between SHP-2 with TBK1N242 domain was specific, suggesting that SHP-2 could negatively regulate TBK1-mediated signal transduction by binding to kinase domain of TBK1.

Discussion

In the present study, we demonstrate that SHP-2 negatively regulates TLR4- and TLR3-mediated IFN-4α/β expression in macrophages. We used SHP-2 siRNA to

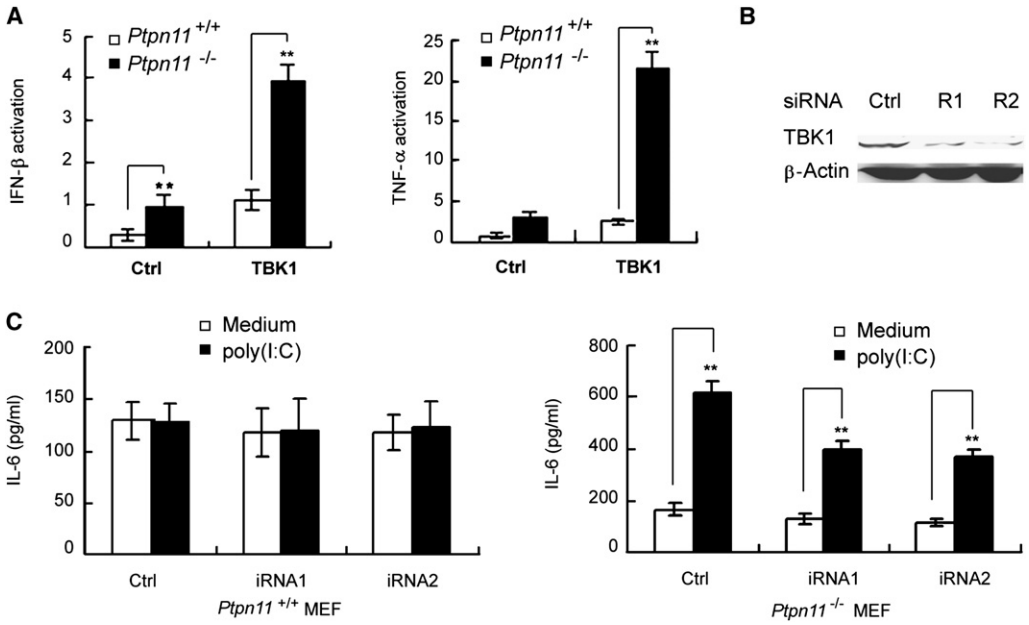

Figure 4. SHP-2 Inhibits TBK1-Activated Gene Expression

(A) *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF were transfected with 40 ng IFN-β or TNF-α luciferase reporter plasmid and 10 ng pTK-*Renilla*-luciferase, together with 100 ng TBK1-expressing plasmid. Total amounts of plasmid DNA were equalized with empty control vector. After 24 hr of culture, luciferase activity was measured and normalized by *Renilla* luciferase activity. Data are shown as mean ± SD (n = 6) of one typical experiment (**p < 0.01). Similar results were obtained in three independent experiments.

(B) MEF were transfected with control small RNA (Ctrl) or TBK1-specific siRNA (TBK1 iRNA1 or TBK1 iRNA2). After 36 hr, TBK1 and β-actin expression in the cells was detected by immunoblot. Similar results were obtained in three independent experiments.

(C) *Ptpn11*$^{+/+}$ and *Ptpn11*$^{-/-}$ MEF were transfected with control small RNA (Ctrl) or TBK1-specific siRNA (TBK1 iRNA1 or TBK1 iRNA2). After 36 hr, the cells were stimulated with poly(I:C) for 6 hr. Concentration of IL-6 in the supernatants was measured by ELISA. Data are shown as mean ± SE of three independent experiments (**p < 0.01).

suppress endogenous SHP-2 expression in primary mouse peritoneal macrophages. Correlating with the suppression of endogenous SHP-2 expression, LPS- and poly(I:C)-induced IFN-4α/β expression was increased. The negative regulatory role of SHP-2 in poly(I:C)-induced type I interferon was confirmed in *Ptpn11*$^{-/-}$ MEF.

LPS-induced IFN-α/β expression is TRIF dependent. However, SHP-2 functions in a variety of cytokine signal transduction pathways, some of which might affect LPS-induced IFN-α/β expression. In particular, the activation of STAT1 was reported to play a role in LPS-induced IFN-β production (Toshchakov et al., 2002). Thus, the effect of SHP-2 expression on LPS-induced activation of STAT1 was investigated. SHP-2 siRNA inhibited LPS-induced phosphorylation of STAT1, ruling out the possibility that SHP-2 siRNA increases LPS-induced IFN-β production through STAT1 pathway. In addition, we also used CHX to inhibit LPS-induced protein synthesis. In the presence of CHX, SHP-2 knockdown could still significantly increase LPS-induced IFN-β mRNA expression, demonstrating that the regulation of LPS-induced IFN-β mRNA expression by SHP2 is independent on synthesis of novel protein and providing additional evidence that SHP-2 directly regulates TLR4 signaling.

TBK1 is essential for TRIF-induced IRF3 activation and IFN-α/β expression. TBK1 siRNA or kinase-inactive TBK1 blocked TRIF-induced IRF3 activation (Fitzgerald et al., 2003). Immunoprecipitation experiments revealed

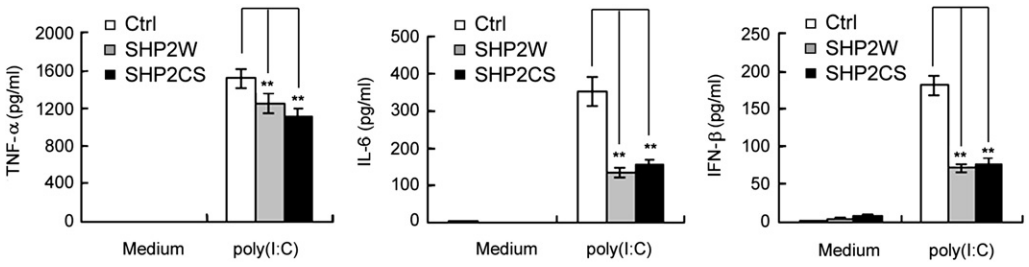

Figure 5. SHP-2 Inhibits TLR3-Activated Cytokine Production in Phosphatase Activity-Independent Manner

RAW264.7 macrophages stably transfected with SHP-2W, SHP-2CS plasmid, or empty control vector were stimulated with poly(I:C), then TNF-α, IL-6, and IFN-β in the supernatants were measured by ELISA. Data are shown as mean ± SE of three independent experiments (**p < 0.01).

that poly(I:C) treatment induced SHP-2 to associate with TBK1. IFN-β and TNF-α reporter gene expression assay demonstrated that SHP-2 deficiency significantly increased TRIF- and TBK1-activated gene expression. These results suggested that SHP-2 inhibits poly(I:C)-induced proinflammatory cytokine and type I IFN expression at least partially through TRIF- and TBK1-dependent pathway.

SHP-2 directly bound to TBK1 and inhibited TBK1-mediated phosphorylation of MBP. SHP-2 functions as a tyrosine phosphatase. However, tyrosine phosphorylation of recombinant active TBK1 could not be detected with phospho-tyrosine antibody (data not shown). In addition, mutation of C463S, which disrupted the tyrosine phosphatase activity of SHP-2, did not reverse the inhibition of poly(I:C)-induced IFN-β production and TBK1-activated IFN-β reporter gene expression by SHP-2. Thus, it appeared that SHP-2 inhibited the activity of TBK1 through a tyrosine phosphatase activity-independent mechanism. Instead, SHP-2 might prevent TBK1-mediated downstream substrate phosphorylation by binding to kinase domain of TBK1.

TBK1 phosphorylates IKK-β and has been implicated in NF-κB activation. However, poly(I:C)- and LPS-induced NF-κB activation and IL-6 production are normal in TBK1-deficient cells (Hemmi et al., 2004). These results suggested that TBK1 might not participate in TLR-activated NF-κB activity and proinflammatory cytokine production. Alternatively, TBK1 might be redundant for TLR-activated NF-κB activation. Another explanation for the inconsistency might be that some negative regulators of TBK1 exist and limit the role of TBK1 in proinflammatory cytokine production in TLR signaling. This was consistent with our observation that TBK1 induced significant TNF-α reporter gene expression in *Ptpn11*$^{-/-}$ cells but only marginal TNF-α reporter gene expression in *Ptpn11*$^{+/+}$ MEF, and was further supported by the results that TBK1 knockdown did not affect poly(I:C)-induced IL-6 production in *Ptpn11*$^{+/+}$ MEF but significantly decreased poly(I:C)-induced IL-6 production in *Ptpn11*$^{-/-}$ cells.

Presently, there is no report that TBK1 could activate MAPK pathways. Thus, there might be some molecules that contributed to the increased poly(I:C)-induced proinflammatory cytokine production in TBK1-independent manner in SHP-2-deficient cells. IRAK1 plays an important role in LPS-induced ERK1, ERK2, p38, JNK1, and JNK2 activation. SHP-2 deficiency significantly enhanced poly(I:C)-induced phosphorylation of IRAK1. However, it was reported that MAPKs were activated through IRAK1-independent pathway in poly(I:C)-induced TLR3 signal transduction (Jiang et al., 2003). Our future study will focus on how SHP-2 inhibits poly(I:C)-induced phosphorylation of IRAK1 and whether IRAK1 plays a role in poly(I:C)-induced MAPKs activation and downstream proinflammatory cytokine production in SHP-2-deficient cells.

Active control of the magnitude of immune response to microbial pathogens and endogenous TLR ligands by restricting TLR signaling is essential for protecting the host from the injury induced by excessive activation. Recent studies have identified multiple negative regulators functioning at different levels of TLR4 signaling, most of which target MyD88-IRAK-TRAF6 signal cascade (Liew et al., 2005; Kobayashi et al., 2002; Wald et al., 2003; Brint et al., 2004; Boone et al., 2004). A previous study showed that A20 also negatively regulates IRF3 activation in TLR3 signal transduction (Saitoh et al., 2005). However, it remains unknown whether these molecules also affect TRIF-dependent TLR4 signaling. To our knowledge, our results provide the first evidence that TRIF-dependent TLR4 and TLR3 signal transduction might be strictly controlled by SHP-2.

In conclusion, by identifying SHP-2 as a critical negative regulator of TRIF-dependent pathway, the present study provides insight into the mechanism of TRIF-dependent signal transduction. SHP-2 specifically inhibits MyD88-independent proinflammatory cytokine and type I interferon production in TLR3 and TLR4 signal transduction. Upon TLR3 activation, SHP-2 is recruited and directly binds to kinase domain of TBK1, resulting in the suppression of TBK1-mediated substrate phosphorylation and cytokine production. SHP-2 also inhibits TLR3-activated IRAK1 and MAPK pathways. These results demonstrate that SHP-2 negatively regulates TRIF-dependent cytokine production at least partially through inhibiting TBK1-mediated signal transduction.

Experimental Procedures

Mice and Reagents

C57BL/6J mice were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). All animal experiments were undertaken in accordance with the National Institute 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. Poly(I:C) and LPS (0111:B4) was purchased from Sigma (St. Louis, MO), and LPS was repurified as described (Hirschfeld et al., 2000). Antibodies specific to SHP-2, Myc-tag, TBK1, IRAK1, p38, ERK, JNK, and horseradish peroxidase-coupled secondary antibodies were obtained from Santa Cruz (Santa Cruz, CA). Phospho-p38, phospho-ERK, and phospho-JNK antibodies were obtained from Cell Signaling Technology (Beverly, MA). Purified recombinant active His6-tagged TBK1 protein, expressed by baculovirus in Sf21 cells, and GST-tagged SHP-2 (amino acid 330–545) protein, expressed in *E. coli*, were purchased from Upstate Biotechnology (Lake Placid, NY).

Cell Culture

Female C57BL/6J mice (5–6 weeks old) were used for the preparation of primary mouse macrophages, and thioglycolate-elicited mouse peritoneal macrophages were prepared as described (Karaghiosoff et al., 2003). The cells were cultured in endotoxin-free DMEM with 10% FCS (PAA Laboratories, Pasching, Austria). After 1 hr, nonadherent cells were removed. On the next day, the cells were transfected with Geneporter 2 Transfection Reagent (GTS, San Diego, CA) according to manufacturer's instructions. Wild-type (*Ptpn11*$^{+/+}$) and SHP-2-deficient (*Ptpn11*$^{-/-}$) mouse embryonic fibroblast (MEF) were established and cultured as described previously (You et al., 2001). Mouse macrophage cell line RAW 264.7 and human HEK 293 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described (An et al., 2005).

Plasmid Constructs and Transfection

Plasmids containing wild-type SHP-2 (pcDNA3-SHP-2W) and tyrosine phosphatase inactive mutant SHP-2 (pcDNA3-SHP-2CS) were constructed as described previously (Yin et al., 1997). Mouse cDNAs encoding TRIF and TBK1 were amplified from mRNA of RAW264.7 cells by RT-PCR, cloned into pcDNA3.1 expression vector, and sequenced (Invitrogen, Carlsbad, CA). Myc-tagged SHP-2 and TBK1 constructs were generated by PCR. IRF3 reporter plasmids were kind gifts from Dr. T. Fujita (Tokyo Metropolitan Institute of Medical Science) (Shinobu et al., 2002). Mouse DNAs for IFN-β and

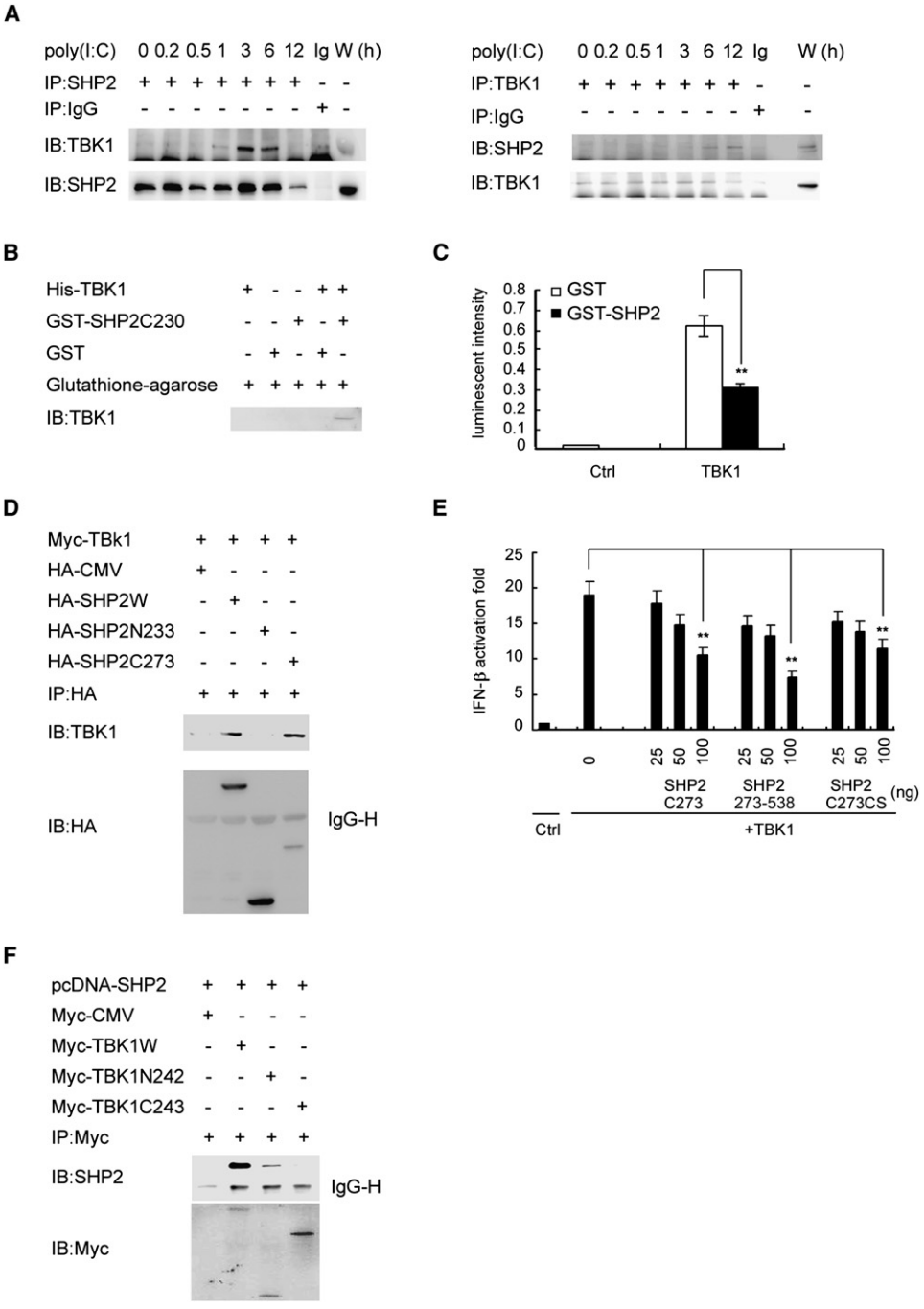

Figure 6. SHP-2 Interacts with TBK1 and Inhibits TBK1-Activated IFN-β Expression

(A) RAW264.7 cells were treated with 10 μg/ml poly(I:C) for indicated time period. Equal amount of cell lysates were immunoprecipitated (IP) with SHP-2 or TBK1-specific antibody. Precipitated proteins were blotted (IB) with SHP-2 and TBK1-specific antibody. Protein in whole-cell lysate (W) was used as positive control. Similar results were obtained in three independent experiments.

(B) Recombinant active TBK1 was incubated with GST-tagged recombinant SHP-2(230-545) or GST control recombinant protein. GST pull-down assay was performed with glutathione-agarose and blotted with TBK1-specific antibody. Similar results were obtained in three independent experiments.

(C) MBP was incubated with recombinant active TBK1, and MBP phosphorylation was detected as described in Experimental Procedures. Data are shown as mean ± SD (n = 3) of one typical experiment (**$p < 0.01$). Similar results were obtained in three independent experiments.

(D) TBK1 construct together with HA-tagged SHP-2, SHP-2N233, or SHP-2C273 plasmids were transfected into HEK293 cells. After 24 hr, SHP-2 truncates were immunoprecipitated (IP) with HA-specific antibody. Precipitated proteins were detected by immunoblot (IB). Similar results were obtained in three independent experiments.

(E) HEK293 cells were transfected with 40 ng IFN-β luciferase reporter plasmid, 10 ng pTK-*Renilla*-luciferase, 100 ng TBK1 expressing plasmid, together with indicated amount of SHP-2-expressing plasmid. Total amounts of plasmid DNA were equalized with empty control vector. After 24 hr of culture, luciferase activity was measured and normalized by *Renilla* luciferase activity. Data are shown as mean ± SD (n = 6) of one typical experiment (**$p < 0.01$). Similar results were obtained in three independent experiments.

TNF-α promoter were amplified from RAW264.7 cells by PCR and cloned in pGL3 plasmid (Promega) to construct IFN-β and TNF-α luciferase reporter plasmids. The primers used are TNF-α-F 5′ccatctg tgaaacccaataaacctc3′, TNF-α-R 5′gggagatgtggcgccttgg3′, IFN-β-F 5′agcttgaataaaatgctagctagaagctgttagaa3′, and IFN-β-B 5′caagatg aggcaaagcttcaaaggctgcagtgagaat3′. All constructs were confirmed by DNA sequencing. For stable transfection, RAW264.7 macrophages were seeded into dishes and transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The cells were selected with 300 ng/ml G418 and pooled for further experiments.

RNA Interfering

SHP-2 siRNA, small interfering RNA targeting SHP-2, was described previously (Kontaridis et al., 2004). TBK1 siRNA, small interfering RNA targeting TBK1, were ctgtgaaagtgtatgagaa (TBK1 iRNA1) and cacatgacggcgcataaga (TBK1 iRNA2). The control siRNA sequence was 5′aatcagtcacgttaatggtcg3′. For 1 × 10^6 cells, 0.4 nmol SHP-2 siRNA was mixed with 15 μl Geneporter 2 Transfection Reagent and transfected into the cells. After 6 hr, the supernatant was removed and fresh medium was added. The cells were cultured for another 36 hr before further experiments.

In Vivo siRNA Transfection

Female C57BL/6J mice (4 weeks old) were intraperitoneally injected with thioglycolate to elicit peritoneal macrophages. After 3 days, 6 nmol siRNA was incubated with Geneporter 2 Transfection Reagent according to manufacturer's instruction and then intraperitoneally injected. After 48 hr, the mice were treated with intraperitoneal administration of TLR agonist.

Detection of IFN-β, TNF-α, and IL-6 Production

2 × 10^5 cells were seeded into 24-well plates and incubated overnight. The cells were stimulated with 100 ng/ml LPS or 10 μg/ml poly(I:C) for indicated time periods. The concentrations of IFN-β, TNF-α, and IL-6 in culture supernatants were measured by ELISA Kits (R&D Systems, Minneapolis, MN).

RNA Quantification

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quantitative real-time RT-PCR analysis was performed by LightCycler (Roche) and SYBR RT-PCR kit (Takara, Dalian, China). The primers used for mHRPT, IFN-4α, and IFN-β analysis were described previously (Takaoka et al., 2005; Karaghiosoff et al., 2003). TNF-α and IL-6 primers were obtained from Biosource. Data were normalized by the level of HPRT expression in each sample.

Immunoprecipitation and Immunoblot

Cells were lysed with M-PER Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail. Protein concentrations of the extracts were measured with BCA assay (Pierce). 50 μg of protein was either used for immunoprecipitation, or loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as described previously (An et al., 2005).

Assay of Luciferase Reporter Gene Expression

RAW264.7 macrophages, HEK 293 cells, or mouse embryonic fibroblasts were cotransfected with the mixture of indicated luciferase reporter plasmid, pRL-TK-*Renilla*-luciferase plasmid, and indicated amounts of SHP-2, TRIF, or TBK1 construct. Total amounts of plasmid DNA were equalized via empty control vector. After 24 hr, the cells were left untreated or treated with TLR agonists. Luciferase activities were measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Data are normalized for transfection efficiency by dividing *Firefly* luciferase activity with that of *Renilla* luciferase.

Pull-Down Assay

Purified recombinant active TBK1 was incubated with GST-tagged SHP-2 (amino acid 330–545) at 4°C for 30 min and incubated with glutathione-agarose at 4°C for another 2 hr in IP buffer containing NP40. After washing three times, the pellet of agarose was analyzed by immunoblot.

TBK1 Kinase Activity Assay

TBK1 kinase activity was assayed with Chemiluminescent MBP Assay Kit (Upstate Biotechnology). 100 μg of recombinant GST or GST-SHP-2 (amino acid 330–545) in assay buffer was added to MBP-coated well containing Magnesium/ATP buffer. Then, 100 ng of recombinant TBK1 in assay buffer was added to appropriate wells. After incubation for 20 min, the wells were washed with washing buffer and blocked with blocking buffer. HRP-conjugated anti-phospho-MBP (phosphorylated threonine) in blocking buffer was added to the wells. After washing with PBS and washing buffer, LumiGLO Chemiluminescent substrate was added. Luminescent intensity was assayed with luminometer according to manufacturer's instructions.

Statistical Analysis

Statistical significance was determined by Student's t test, with a value of $p < 0.05$ considered to be statistically significant.

Supplemental Data

Eight Supplemental Figures and can be found with this article online at http://www.immunity.com/cgi/content/full/25/6/919/DC1/.

Acknowledgments

We thank X.W. Ma and S.Q. Li for technical assistance and Y.-W. He at Duke University for critically reading this manuscript. Supported by grants from the National Natural Science Foundation of China (30490240, 30200145, 30121002), Tsinghua-Yue-Yuen Medical Sciences Fund (202400.005-02), and the National Key Basic Research Program of China (2001CB510002).

Received: May 22, 2006
Revised: September 25, 2006
Accepted: October 17, 2006
Published online: December 7, 2006

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