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The regulation of the expression of inducible nitric oxide synthase by Src-family tyrosine kinases mediated through MyD88-independent signaling pathways of Toll-like receptor 4

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Abstract

Bacterial lipopolysaccharide (LPS) activates Toll-like receptor 4 (TLR4) leading to the expression of inflammatory gene products. Src-family tyrosine kinases (STKs) are known to be activated by LPS in monocytes/macrophages. Therefore, we determined the role of STKs in TLR4 signaling pathways and target gene expression in macrophages. The activation of NFκB, and p38 MAPK, and the expression of inducible nitric oxide synthase (iNOS) induced by LPS were not affected in macrophages deficient in three STKs (Lyn, Hck, and Fgr). These results suggest that the deletion of the three STKs among possibly nine STKs is not sufficient to abolish total activity of STKs possibly due to the functional redundancy of other STKs present in macrophages. However, two structurally unrelated pan-inhibitors of STKs, PP1 and SU6656, suppressed LPS-induced iNOS expression in MyD88-knockout as well as wild-type macrophages. The suppression of iNOS expression by the inhibitors was correlated with the downregulation of IFNβ (a MyD88-independent gene) expression and subsequent decrease in STAT1 phosphorylation. Moreover, PP1 suppressed the expression of IFNβ and iNOS induced by TRIF, a MyD88-independent adaptor of TLR4. PP1 suppressed STAT1 phosphorylation induced by LPS, but not by IFNβ suggesting that STKs are involved in the primary downstream signaling pathways of TLR4, but not the secondary signaling pathways downstream of IFNβ receptor. Together, these results demonstrate that STKs play a positive regulatory role in TLR4-mediated iNOS expression in a MyD88-independent (TRIF-dependent) manner. These results provide new insight in understanding the role of STKs in TLR4 signaling pathways and inflammatory target gene expression.
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Keywords: Lipopolysaccharide; Toll-like receptor; Src-family tyrosine kinases; Nitric oxide synthase; TRIF; IFN-beta

1. Introduction

Lipopolysaccaride (LPS), a cell wall component in the outer membrane of gram-negative bacteria, is a potent activator of the innate immune system resulting in the expression of pro-inflammatory gene products including inducible nitric oxide synthase (iNOS) in macrophages/monocytes [1,2]. Results from genetic and biochemical studies demonstrated that Toll-like receptor 4 (TLR4) is the LPS receptor and mediates LPS-induced activation of downstream signaling pathways and expression of inflammatory target genes [3,4]. TLR4 is a transmembrane receptor with an extracellular domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic Toll/interleukin-1 receptor (TIR) homology domain [5]. Broadly, the activation of TLR4 triggers the activation of two major downstream signaling pathways, MyD88-dependent and -independent [6]. MyD88,

Abbreviations: STKs, Src-family tyrosine kinases; TLR, Toll-like receptor; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; NFκB, nuclear factor κB; MyD88, myeloid differential factor 88; TRAF6, TNF receptor associated factor 6; TRIF, TIR domain-containing adapter inducing IFN-β; IFN-β, interferon β; IKK, IκB kinase; IRF-3, IFN-regulatory factor-3

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an immediate adaptor molecule, is associated with the cytoplasmic domain of TLR and triggers the activation of the downstream signaling components including IKKβ leading to the activation of NFκB. TRIF, another adaptor molecule of TLR4, initiates MyD88-independent signaling pathways resulting in the delayed activation of NFκB. TRIF also activates TBK1 and IKKε, which phosphorylate IRF3 resulting in the expression of IFNβ [7,8]. IFNβ, in turn, stimulates Janus-activated kinase (JAK) that phosphorylates and activates STAT1 leading to the expression of the secondary inducible genes such as iNOS and IP-10 [9–11].

Src-family tyrosine kinases (STKs) have been implicated to play critical roles in cellular signaling pathways in immune cells [12]. It is known that LPS can activate STKs such as Lyn, Fgr, and Hck [13,14]. LPS induces autophosphorylation of Lyn kinase in wild type, but not in TLR4-mutant (C3H/HeJ) macrophages [15]. Overexpression of a constitutively active Hck enhanced the production of TNF in response to LPS in macrophages, whereas antisense oligonucleotides to Hck interfere with LPS-induced TNF production [16]. In contrast, LPS-induced cytokine production and tumoricidal activity occurred normally in macrophages isolated from triple knockout mice (Lyn−/−, Fgr−/−, and Hck−/−) [17]. Therefore, the role of STKs in TLR4 signaling pathways and target gene expression is still not fully understood.

iNOS is overexpressed in sites of inflammation and many types of tumor tissues. What causes the overexpression of iNOS in such pathological states is not clearly understood. Elucidating the signaling pathways leading to the expression of iNOS is a key to understand why iNOS is overexpressed in such pathological states, and can provide critical information for identifying potential targets of modulation by pharmacological and dietary factors. Therefore, we investigated the role of STKs in the regulation of the signaling pathways and the expression of iNOS derived from TLR4 activation in macrophages using genetic, biochemical, and pharmacological approaches.

2. Materials and methods

2.1. Reagents

Purified lipopolysaccharide (LPS) was purchased from List Biological Lab., Inc. Herbimycin A was obtained from Sigma. PP1 (4-amino-5(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine) was purchased from BioMol (Plymouth Meeting, PA). PP3 (4-amino-7-phenylpyrazol[3,4-d]pyrimidine) and SU6656 were purchased from Calbiochem (San Diego, CA). Mouse IFNβ was purchased from CellSciences (Canton, MA). Polyclonal antibody for GAPDH were prepared and characterized as previously described [18]. Antibodies for iNOS and IκBα were purchased from Santa Cruz Biotech (Santa Cruz, CA). Monoclonal antibody for phosphotyrosine (4G10) was purchased from Upstate (Chicago, IL). Antibody for phospho-STAT1 (Y701) was from Zymed Laboratories, Inc. (South San Francisco, CA). Antibody for the active form of c-Src was obtained from Hisaaki Kawakatsu (Nippon Shinyaku Co. Ltd., Japan) [19]. Antibody for β-actin was purchased from Sigma. All other reagents were purchased from Sigma unless otherwise described.

2.2. Plasmids

The expression plasmid for a constitutively active form of TLR4 (ΔTLR4) was prepared as previously described [3]. NFκB(2x)-luciferase reporter plasmid was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). The luciferase reporter plasmid containing the promoter of inducible nitric oxide synthase was from Christopher Glass (University of California, San Diego, CA). The luciferase reporter plasmid containing mouse IFNβ promoter (−125/+55) and the expression plasmid of human TRIF were obtained from Shizuo Akira (Osaka University, Japan). Heat shock protein 70 (HSP70) β-galactosidase expression plasmid was from Robert Modlin (University of California, Los Angeles, CA). The luciferase reporter plasmid containing IP-10 promoter and the expression plasmid of mouse TRIF were obtained from Katherine A. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). The expression plasmids of a wild type of pcDNA-Lyn, a dominant-negative mutant of Lyn [pcDNA-Lyn (Y397F)] and a constitutively active form of Lyn [pcDNA-Lyn (Y508F)] were from Margaret L. Hibbs (Ludwig Institute for Cancer Research, Australia). All DNA constructs were prepared in large scale using EndoFree Plasmid Maxi kit (Qiagen, Chatsworth, CA) for transfection.

2.3. Cell culture

RAW 264.7 cells (a murine monocytic cell line, ATCC TIB-71) and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Intergen) and 100 units/ml Penicillin and 100 μg/ml Streptomycin (Invitrogen) at 37 °C in a 5% CO_2/air environment. Bone marrow-derived macrophages from the triple STKs knockout mice (Lyn−/−, Hck−/−, and Fgr−/−) were prepared as previously described [17]. Immortalized macrophages derived from MyD88 knockout or wild-type mice were established by infecting primary bone marrow cells with the J2 recombinant retrovirus at the Laboratory of Experimental Immunology, NCI-Frederick (Dr. Howard A. Young), as previously described [20].

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunobloting

SDS-PAGE and immunobloting assays were performed essentially as previously described [21,22]. The effective

exposure time and dose of the treatment were selected based on our previous studies and the published papers [23–25]. RAW264.7 cells were plated 24 h prior to the treatment for the stabilization. Cells were pretreated with various inhibitors (herbimycin A, PP1, PP3, and SU6656) or vehicle (dimethyl sulfoxide, final concentration of 0.1% (v/v)). Herbimycin A was pretreated for 30 min while PP1, PP3, and SU6656 were pretreated for 1 h. Cells were further stimulated with LPS in the absence or presence of the inhibitors. For some experiments, cells were treated with the inhibitor alone in the absence of LPS to compare the effect of the inhibitor alone on the basal level of the assays. Cell lysates for phosphotyrosine immunobloting were prepared after 30 min of LPS treatment since maximum stimulation of tyrosine phosphorylation by LPS occurred within 30–45 min of exposure, and this was reduced rapidly after 60 min [23]. Cell lysates for iNOS immunobloting were prepared after 8 h of LPS treatment since the target gene expression was observed at later time points [23,24].

Bone marrow-derived macrophages from triple knockout (TKO; Lyn−/−, Hck−/−, and Fgr−/−) or wild-type (WT) mice were stimulated with LPS (100 ng/ml) for 30 min for p38 (phosphotyrosine), IκBα, and active c-Src immunoblots, and for 12 h for iNOS immunobloting. Immortalized bone marrow-derived macrophages isolated from wild type or MyD88 knockout mice were pretreated with various concentrations of the inhibitors or vehicle (dimethyl sulfoxide) for 1 h and further stimulated with LPS (100 ng/ml) for 18 h for iNOS immunobloting.

The protein concentration of cell lysates was determined and the same amount of proteins was loaded in each lane for SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membrane. The membranes were blocked with phosphate-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk and were blotted with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL). The reactive bands were visualized with the enhanced chemiluminescence system (Amersham). The corresponding bands for Src-family tyrosine kinases, ERKs and p38 were identified using various assays including immunobloting assay and immunoprecipitation assay with specific antibody for each kinase in addition to the respective molecular sizes as described in our previous studies [23,26].

2.5. Transfection and luciferase assay

These were performed as described in our previous studies [24,27]. Briefly, RAW 264.7 cells were co-transfected with a luciferase plasmid and HSP70-β-galactosidase plasmid as an internal control using SuperFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Various expression plasmids or corresponding empty vector plasmids for signaling components were co-transfected. The total amount of transfected plasmids was equalized by supplementing with the corresponding empty vector in order to eliminate the experimental error in the transfection itself. After 24 h, cells were pretreated with various inhibitors or vehicle (dimethyl sulfoxide). Cells were further stimulated with LPS in the presence or absence of the inhibitors as described above. After 18 h, cell lysates were prepared and luciferase and β-galactosidase enzyme activities were determined using the luciferase assay system and β-galactosidase enzyme system (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity.

2.6. Real-time reverse transcription (RT)-PCR analysis of interferon-beta (IFNβ) expression

This assay was performed essentially the same as previously described [28]. RAW264.7 cells were pretreated with PP1, PP3, or vehicle for 1 h. Cells were further stimulated with LPS for 4 h. Total RNAs were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instruction. Five micrograms of total RNAs were used for cDNA synthesis with SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Quantitative Real-time PCR was performed with Light Cycler (Roche Molecular Biochemicals) using LightCycler FastStart DNA Master SYBR Green I kit. The primers used to detect mouse IFNβ are as follows: forward primer, 5′-TCCAAGAAAGGACGAACATTCG-3′; reverse primer, 5′-TGAGGACATCTCCCACGTCAA-3′. The primers for mouse β-actin (used as an internal control) are as follows: forward primer, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′; reverse primer, 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′. The following program was used: denaturation at 95 °C for 5 min and 45 cycles consisting of denaturation at 95 °C for 10 s, annealing at 56 °C for 5 s, and extension at 72 °C for 13 s. The specificity of the amplified PCR products was assessed by a melting curve analysis. The crossing-point value (Cp), which is inversely proportional to the initial template copy number, was determined by the Light Cycler Software Program provided by the manufacturer. The fold induction of IFNβ expression was calculated by $2^{\Delta\Delta Cp}$, where $\Delta\Delta Cp = \Delta Cp_{\text{treatment}} - \Delta Cp_{\text{control}}$, after normalizing for β-actin using $\Delta Cp = Cp_{\beta\text{-actin}} - Cp_{IFN\beta}$.

3. Results

3.1. A tyrosine kinase inhibitor, herbimycin A, suppresses Toll-like receptor 4 (TLR4)-induced activation of signaling pathways and expression of inducible nitric oxide synthase (iNOS) in macrophages

To determine whether tyrosine kinases are involved in TLR4-mediated signaling pathways in macrophages,

RAW264.7 cells were pretreated with a general tyrosine kinase inhibitor, herbimycin A, and further stimulated with LPS (TLR4 agonist) or a constitutively active (CA) TLR4. LPS stimulated the tyrosine phosphorylation of MAPKs (ERK-1, ERK-2, and p38) in RAW264.7 cells as compared with unstimulated cells in a dose- and time-dependent manner as shown in our previous studies [23]. In this study, herbimycin A suppressed the LPS-induced phosphorylation of ERK1, ERK2, p38, as well as Src-family tyrosine kinases (STKs) in a dose-dependent manner (Fig. 1A). Herbimycin A also inhibited the activation of NFκB induced by LPS or constitutively active TLR4 in RAW264.7 cells (Fig. 1B). Finally, LPS- or TLR4-induced expression of target genes such as inducible nitric oxide synthase (iNOS) was inhibited by herbimycin A as determined by immunobloting and promoter reporter gene assay (Fig. 1C and D). Herbimycin A at the concentrations used and the incubation time did not cause any cytotoxicity. These results demonstrate that herbimycin A inhibits both ligand-dependent and -independent activation of TLR4, and further suggest that tyrosine kinases are one of the downstream signaling components of TLR4 leading to the expression of iNOS in macrophages.

3.2. Lyn, one of the STKs, is not required for TLR4-mediated induction of signaling pathways and iNOS expression in macrophages

STKs are implicated as one of the downstream signaling components activated by LPS [12–14]. Among nine members of STKs (Lyn, Hck, Fgr, Src, Lck, Blk, Yes, Fyn, and Yrk), Lyn is one of the major STKs expressed in macrophages and known to be activated by LPS [13,15]. Therefore, we determined whether Lyn is a required signaling component for TLR4-mediated signaling pathways and the expression of target genes such as iNOS in macrophages.

For the biochemical approach, RAW264.7 cells were transfected with various expression plasmids of Lyn. A dominant-negative mutant of Lyn, which was created by the substitution of the tyrosine 397 residue with phenylalanine [29], did not suppress NFκB activation induced by LPS or TLR4 in RAW264.7 cells (data not shown). Neither

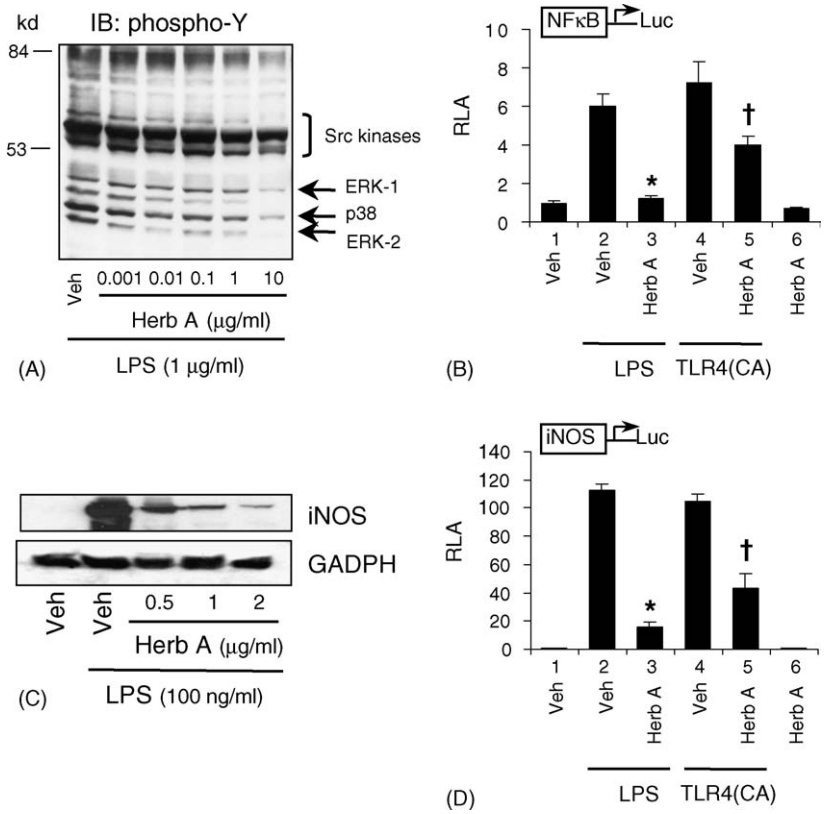

Fig. 1. A general protein tyrosine kinase inhibitor, herbimycin A suppresses the activation of mitogen-activated protein kinases (MAPKs) and NFκB, and the expression of iNOS induced by LPS or TLR4 in macrophages. (A, C) RAW264.7 cells were pretreated with herbimycin A (Herb A) for 30 min and further stimulated with LPS for 30 min (A) or for 8 h (C). Cell lysates were analyzed for phosphotyrosine (phospho-Y), iNOS, and GAPDH immunoblots. (B, D) RAW264.7 cells were transfected with a luciferase reporter plasmid containing NFκB binding site (B) or iNOS promoter (D). For LPS treatment, cells were pretreated with herbimycin A (1 μg/ml) for 30 min and further stimulated with LPS (100 ng/ml) for 6 h. For TLR4 experiment, cells co-transfected with the expression plasmid of constitutively active TLR4 or empty vector (pcDNA) were treated with herbimycin A for 18 h. Cell lysates were prepared and luciferase and β-galactosidase enzyme activities were measured as described under Section 2. Relative luciferase activity (RLA) was determined by normalization with β-galactosidase activity and presented as fold induction compared with the vehicle control. Values are mean ± S.E.M. ($n = 3$). (*) Significantly different from lane 2, $p < 0.05$. (+) Significantly different from lane 4, $p < 0.05$. The panels are representative data from more than three independent experiments. IB: immunoblot; Veh: vehicle; Herb A: herbimycin A; CA: constitutively active.

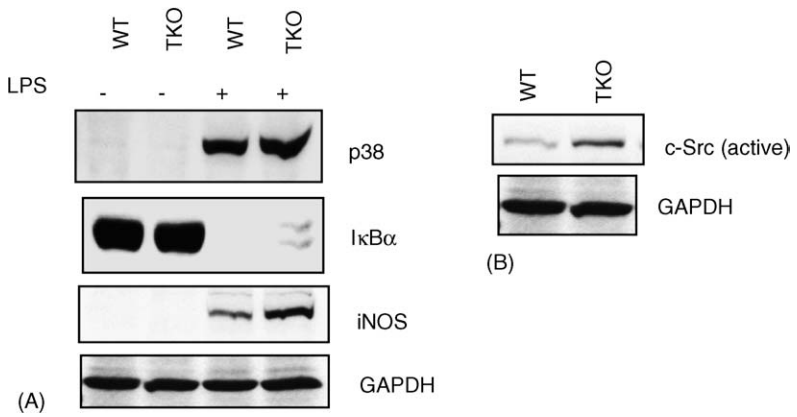

Fig. 2. The deletion of three Src kinases (Lyn, Hck, and Fgr) does not alter the activation of NFκB or p38 MAPK, and the expression of iNOS induced by LPS in macrophages. Bone marrow-derived macrophages from triple knockout (TKO; Lyn−/−, Hck−/−, and Fgr−/−) or wild-type (WT) mice were stimulated with LPS (100 ng/ml) for 30 min for p38 (phosphotyrosine), IκBα, and active c-Src immunoblots, and for 12 h for iNOS immunoblots. The panels are representative data from more than three independent experiments. IB: immunoblot.

wild-type Lyn nor constitutively active Lyn was sufficient to induce NFκB activation (data not shown).

For the genetic approach, triple STK knockout mice with deletions of Lyn, Hck, and Fgr, the three major STKs expressed in macrophages [30], were used. The phosphorylation of p38 MAPK and the degradation of IκBα (NFκB activation) induced by LPS in bone marrow-derived macrophages isolated from triple STK knockout mice (Lyn−/−, Hck−/−, and Fgr−/−) were not altered as compared with those in wild-type macrophages (Fig. 2A). The expression of iNOS induced by LPS was not diminished in macrophages derived from the triple knockout mice (Fig. 2A). Together, these results suggest that Lyn is neither sufficient nor required to activate TLR4-signaling pathways and to induce target gene expression including iNOS in macrophages.

Interestingly, the activity of c-Src, another STK, was potentiated in macrophages isolated from triple STK knockout mice (Lyn−/−, Hck−/−, and Fgr−/−) as shown by the immunobloting with a specific monoclonal antibody for an active form of c-Src (Fig. 2B). These results suggest, first, that there may be functional compensation by other STKs in triple STK knockout macrophages, and second, that therefore the knockout of three STKs cannot overcome the functional redundancy of other STKs present in the triple knockout mice.

3.3. A selective inhibitor of STKs, PP1, suppresses the expression of iNOS induced by LPS in macrophages

Due to the possibility of the functional redundancy of multiple STKs present in macrophages, the mutation or knockout of individual STK may not be an appropriate approach to investigate the role of STKs in TLR4 signaling pathways unless all STKs are deleted or inactivated. There-

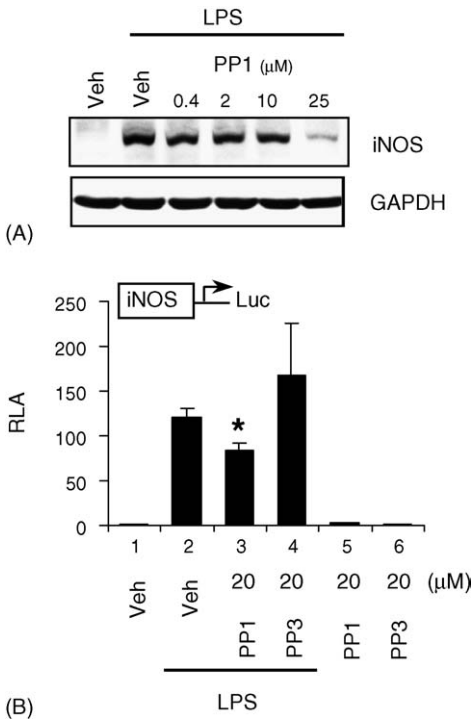

Fig. 3. An inhibitor of Src-family tyrosine kinases (PP1) suppresses LPS-induced expression of iNOS in macrophages. (A) RAW264.7 cells were pretreated with various concentrations of PP1 for 1 h and further stimulated with LPS (1 μg/ml) for 8 h. Cell lysates were analyzed for iNOS and GAPDH immunoblots. (B) RAW264.7 cells were transfected with a luciferase reporter plasmid containing iNOS promoter. After cells were pretreated with Veh, PP1, or PP3 for 1 h, cells were further treated with Veh or LPS (100 ng/ml) in the absence or presence of PP1 or PP3 for 8 h (lane 1, Veh alone; lane 2, LPS plus Veh; lane 3, LPS plus PP1; lane 4, LPS plus PP3; lane 5, PP1 alone; lane 6, PP3 alone). Cell lysates were prepared and relative luciferase activities (RLA) were determined as described in the legend of Fig. 1. Values are mean ± S.E.M. ($n = 3$). (*) Significantly different from lane 2, $p < 0.05$. The panels are representative data from more than three independent experiments. Veh: vehicle.

fore, we used a selective inhibitor of STKs, PP1 [31,32] to inhibit all STKs. PP1 suppressed the expression of iNOS induced by LPS as determined by both immunobloting and promoter reporter gene assay in RAW264.7 cells (Fig. 3). PP3, an inactive analog, did not significantly affect the expression of iNOS induced by LPS (Fig. 3B). These results suggest that STKs play a positive regulatory role in the expression of iNOS in TLR4 signaling pathways in macrophages.

3.4. A selective inhibitor of STKs, PP1, suppresses the expression of IFNβ and the subsequent phosphorylation of STAT1 induced by LPS in macrophages

The expression of iNOS induced by LPS requires the preceding de novo synthesis of IFNβ [10,33]. LPS-induced expression of IFNβ is mediated through TRIF-dependent signaling pathways of TLR4 (the primary downstream signaling pathways of TLR4) [7]. IFNβ, in turn, activates IFNα/β receptor in autocrine/paracrine manner leading to the phosphorylation of STAT1 (the secondary downstream signaling pathways of TLR4) [9,11]. The expression of iNOS induced by LPS was abolished in macrophages derived from IFNα/β receptor knockout or STAT1 knockout mice showing that the expression of iNOS is IFNβ- and STAT1-dependent [10]. Therefore, we determined whether the suppression of LPS-induced iNOS expression by PP1 is mediated through the reduction of IFNβ expression. PP1 suppressed, but PP3 (an inactive analog of PP1) did not inhibit, the endogenous IFNβ expression induced by LPS as determined by quantitative RT-PCR analysis as well as the transactivation of IFNβ promoter in RAW264.7 cells (Fig. 4A and B). PP1 also inhibited the transactivation of

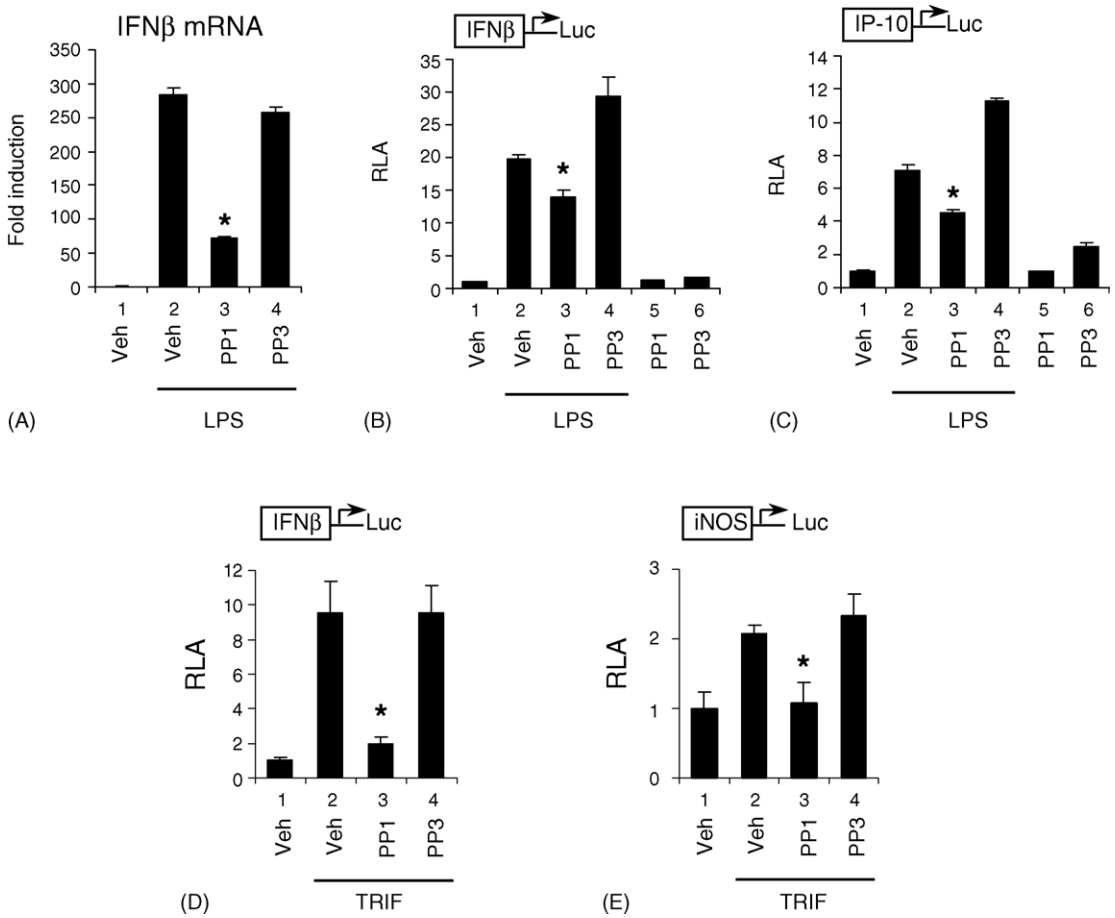

Fig. 4. An inhibitor of Src-family tyrosine kinases (PP1) suppresses the expression of IFNβ induced by LPS in macrophages. (A) RAW264.7 cells were pretreated with PP1 or PP3 (20 μM each) for 1 h and further stimulated with LPS (10 ng/ml) for 4 h. Total RNAs were extracted and the levels of IFNβ expression were determined by the quantitative real-time RT-PCR analysis. IFNβ expression was normalized with β-actin (internal control) expression and presented as fold inductions compared with the vehicle control. (B, C) RAW264.7 cells were transfected with a luciferase reporter plasmid containing IFNβ promoter (−125/+55) (B) or IP-10 promoter (C). After cells were pretreated with Veh, PP1 (20 μM), or PP3 (20 μM) for 1 h, cells were further treated with Veh or LPS (10 ng/ml) in the absence or presence of PP1 or PP3 for 8 h (lane 1, Veh alone; lane 2, LPS plus Veh; lane 3, LPS plus PP1; lane 4, LPS plus PP3; lane 5, PP1 alone; lane 6, PP3 alone). (D) RAW264.7 cells were transfected with the IFNβ promoter luciferase plasmid and the expression plasmid of mouse TRIF and further treated with PP1 or PP3 (20 μM each) for 18 h. (E) 293T cells were co-transfected with the iNOS promoter luciferase reporter plasmid and the expression plasmid of human TRIF and treated with PP1 or PP3 (20 μM each) for 18 h. Cell lysates were prepared and relative luciferase activities (RLA) were determined as described in the legend of Fig. 1. Values are mean ± S.E.M. ($n = 3$). (*) Significantly different from lane 2, $p < 0.05$. The panels are representative data from more than three independent experiments. Veh: vehicle.

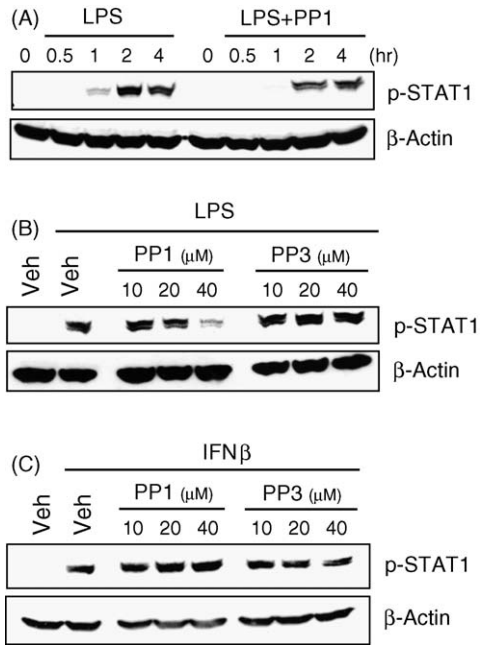

Fig. 5. An inhibitor of Src-family tyrosine kinases (PP1) suppresses LPS-, but not IFNβ-, induced phosphorylation of STAT1 in macrophages. RAW264.7 cells were pretreated with PP1 (20 μM in panel A) for 1 h. For panel A and B, cells were further stimulated with LPS (100 ng/ml) for (A) indicated time or (B) 1 h. For panel C, cells were stimulated with IFNβ (50 units/ml) for 30 min. Cell lysates were analyzed for phospho-STAT1(Y701) and β-actin immunoblots. The panels are representative data from more than two independent experiments. Veh: vehicle.

IP-10 promoter increased by LPS, which is one of the IFNβ-inducible genes [7] in RAW264.7 cells (Fig. 4C). Furthermore, PP1 inhibited the expression of IFNβ and iNOS induced by the overexpression of TRIF, an adaptor that triggers MyD88-independent signaling pathways of TLR4 (Fig. 4D and E).

PP1 suppressed the tyrosine phosphorylation of STAT1 induced by LPS in RAW264.7 cells in time- and dose-dependent manner (Fig. 5A and B). Next, we determined whether the suppression of iNOS expression by PP1 was derived from the inhibition of the upstream signaling pathways of IFNβ (the primary signaling pathways of TLR4) or the downstream signaling pathways of IFNβ (the secondary signaling pathways of TLR4). In other words, we tried to determine where STKs are located in TLR4 signaling pathways, either before IFNβ expression or after IFNβ expression. For that reason, we induced STAT phosphorylation by stimulating the cells with exogenous IFNβ without passing through the primary signaling pathways of TLR4. If PP1 inhibited IFNβ-induced STAT phosphorylation, this should mean that STKs play a role in IFNβ downstream signaling pathways. However, PP1 did not inhibit exogenous IFNβ-induced STAT phosphorylation (Fig. 5C). These results suggest that PP1 acted on the upstream signaling pathways of IFNβ (the primary signaling pathways of TLR4), but not the downstream signaling pathways of IFNβ (the secondary signaling pathways of TLR4). Therefore, our results further suggest that the inhibition of LPS-induced iNOS expression and STAT phosphorylation by STK inhibitors is due to the reduction of IFNβ production (a primary inducible gene), but not due to the direct inhibition of IFNβ downstream signaling pathways.

Together our results suggest that the suppression of LPS-induced iNOS expression by PP1 is at least partly mediated through the inhibition of IFNβ expression and the subsequent decrease in STAT1 phosphorylation. The results also suggest that STKs play a positive regulatory role in MyD88-independent (TRIF-dependent) signaling pathways of TLR4 leading to the expression of target genes such as IFNβ, iNOS, and IP-10.

3.5. Structurally different selective inhibitors of STKs, PP1 and SU6656, suppress LPS-induced iNOS expression in MyD88 knockout as well as wild-type macrophages

Finally, to determine the role of STKs in MyD88-independent pathway of TLR4, two structurally different inhibitors of STKs, PP1 [31,32] and SU6656 [25] were treated to the immortalized bone marrow-derived macrophages isolated from wild-type or MyD88 knockout mice. Consistent with the results in RAW264.7 cells, the expression of iNOS induced by LPS was suppressed by both PP1 and SU6656 in immortalized wild-type macrophages (Fig. 6). Furthermore, the suppressive effect of PP1 and SU6656 on LPS-induced iNOS expression was still observed in MyD88 knockout macrophages (Fig. 6) demonstrating that suppression of iNOS expression by PP1 and SU6656 is at least partly mediated through the inhibition of MyD88-independent pathways. These results are consistent with the results that PP1 suppressed TRIF-induced iNOS expression (Fig. 4) suggesting that STKs play a positive regulatory role in TRIF-dependent (MyD88-independent) signaling pathways of TLR4.

4. Discussion

Our results demonstrated that Src-family tyrosine kinases (STKs) positively regulate the MyD88-independent signaling pathways of Toll-like receptor 4 (TLR4) leading to the expression of MyD88-independent target genes such as inducible nitric oxide synthase (iNOS) and IFNβ in macrophages. Two structurally unrelated inhibitors of STKs, PP1 and SU6656, suppressed TLR4-mediated induction of iNOS expression. The suppression was associated with the decrease in IFNβ expression and consequent attenuation of STAT1 phosphorylation. Although it is known that type I IFNs play an important role in protecting cells from viral infections [34,35], recent evidence suggests that NO (nitric oxide) and type I IFNs (IFNα and IFNβ) are important mediators of LPS-induced endotoxic shock [36]. IFNβ-null

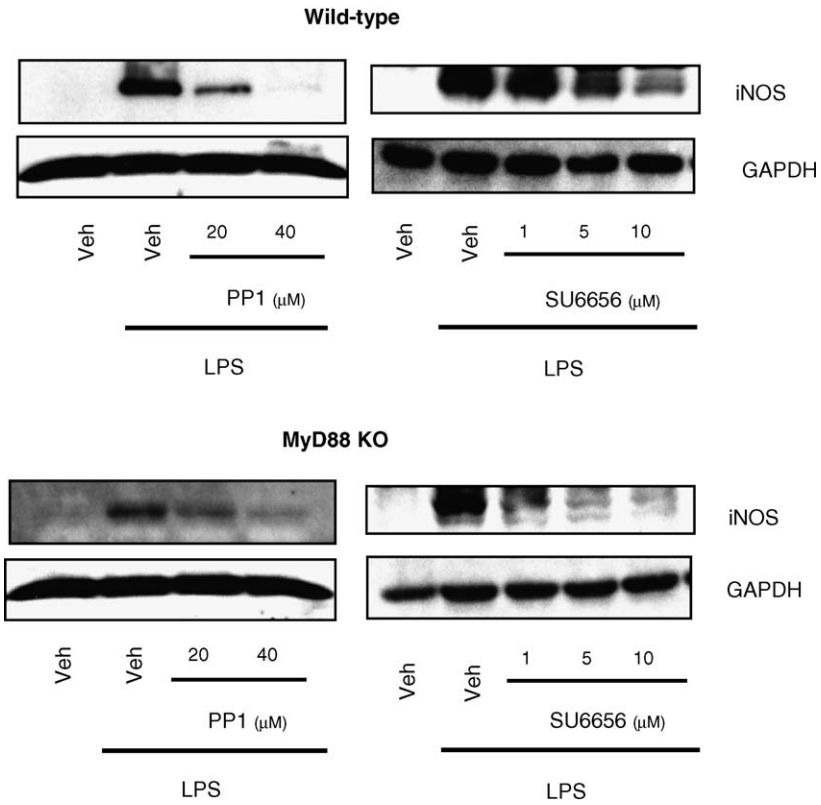

Fig. 6. Selective inhibitors of Src-family tyrosine kinases, PP1 and SU6656, suppress LPS-induced iNOS expression in MyD88 knockout as well as wild-type macrophages. Immortalized bone marrow-derived macrophages isolated from wild type or MyD88 knockout mice were pretreated with various concentrations of PP1 or SU6656 for 1 h and further stimulated with LPS (100 ng/ml) for 18 h. Cell lysates were analyzed for iNOS and GAPDH immunoblots. The panels are representative data from more than three independent experiments. Veh: vehicle; KO: knockout.

mice are resistant to LPS-induced shock [37]. Consistently, mice deficient in type I IFN receptors have higher survival rate as compared to wild-type mice when infected by gram-positive bacterium, *Listeria monocytogenes* [38]. Therefore, our results suggest that STKs may play important roles in regulating innate immune responses and host defense against bacterial infection in macrophages.

Generally, TLR4 signaling is composed of MyD88-dependent and -independent (TRIF-dependent) pathways. It is now recognized that the majority (more than 70%) of LPS-induced genes are regulated through TRIF pathways [39]. The representative target genes dependent on TRIF signaling pathways of TLR4 include IFNβ and IFN-inducible genes such as iNOS and IP-10 [7,11,33]. Our results demonstrated that the specific inhibitor of STKs, PP1, suppressed the expression of TRIF-dependent genes, IFNβ, iNOS, and IP-10. The suppression of iNOS expression by the inhibitors of STKs, PP1 and SU6656, was still observed in the absence of MyD88. Moreover, PP1 suppressed TRIF-induced expression of IFNβ and iNOS. Together, our results suggest that STKs play a positive regulatory role in TRIF-dependent signaling pathways of TLR4.

There was a difference in the potency of PP1 in inhibiting the synthesis of IFNβ mRNA (Fig. 4A) and the transactivation of IFNβ promoter (Fig. 4B). The level of IFNβ mRNA was dramatically decreased by PP1, whereas, the inhibition of LPS-induced transactivation of IFNβ promoter by PP1 was less (Fig. 4B). These results suggest a possibility that STKs may be involved at the post-transcriptional level to regulate IFNβ expression in addition to the regulation of the upstream signaling pathways modulating the transactivation of the target gene promoter.

IFNβ expressed upon the activation of TLR4 in turn activates the IFNα/β receptor and the downstream signaling pathways, including JAK kinases, resulting in the phosphorylation of STAT1 [10,33]. The iNOS promoter contains a STAT1 binding motif and a dominant-negative mutant of STAT1 inhibits cytokine-induced iNOS promoter reporter expression [11]. In addition, LPS did not induce iNOS expression in IFNα/β receptor-knockout or STAT1-knockout macrophages [10]. Therefore, it is known that LPS-induced iNOS expression is dependent on the preceding synthesis of IFNβ and the consequent STAT1 phosphorylation in macrophages. Our results showed that PP1 suppressed LPS-induced IFNβ expression and STAT1 phosphorylation. This suppression is well correlated with the inhibition of LPS-induced iNOS expression by PP1. However, these results cannot tell us where STKs are located in TLR4 signaling pathways, either the upstream signaling pathways of IFNβ (the primary signaling pathways of

TLR4) or the downstream signaling pathways of IFNβ (the secondary signaling pathways of TLR4). If PP1 inhibits IFNβ-induced STAT phosphorylation, this should mean that STKs are involved in the downstream signaling pathways of IFNβ. Our results demonstrated that PP1 did not inhibit exogenous IFNβ-induced STAT phosphorylation. Therefore, the results suggest that STKs play a role in the primary signaling pathways of TLR4, but not the secondary signaling pathways (the downstream signaling pathways of IFNβ). This is consistent with the observation that PP1 lacks the inhibitory activity against JAK tyrosine kinases through which IFNβ induces STAT1 phosphorylation [11]. Therefore, the results suggest that STKs are involved in the regulation of the primary downstream signaling pathways of TLR4 leading to the expression of IFNβ, but not the secondary signaling pathways that are downstream of the IFNβ receptor. The signaling components that directly interact with STKs in the signaling pathways of TLR4 remain to be determined.

Our results obtained from biochemical and genetic approaches demonstrated that Lyn kinase is neither required nor sufficient to mediate TLR4 signal for the expression of iNOS in macrophages. The deletion of three STKs (Hck, Fgr, and Lyn) is not sufficient to abolish the activity of STKs completely in macrophages possibly due to the functional redundancy of other STKs present in macrophages. Indeed, the level of active c-Src, another STK, was enhanced in triple knockout macrophages showing the compensatory enhancement of other STKs activities. Although it remains to be determined which member(s) of STKs play the regulatory roles in TLR4 signaling pathways, it is possible that any individual STK can fulfill the requirement of STK activity because of the functional redundancy.

TLR4 recognizes LPS from gram-negative bacteria and plays a critical role in inducing innate immune and inflammatory responses [5,6]. Although the identity of the signaling molecules in TLR4 downstream signaling pathways that interact with STKs remains to be determined, our results demonstrate that LPS-induced expression of iNOS and IFNβ is regulated by STKs through the regulation of the TRIF-dependent signaling pathways of TLR4. These results provide new insight into the role of STKs in TLR4 signaling pathways and inflammatory target gene expression in macrophages.

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