The Inducible Nitric-oxide Synthase (iNOS)/Src Axis Mediates Toll-like Receptor 3 Tyrosine 759 Phosphorylation and Enhances Its Signal Transduction, Leading to Interferon-β Synthesis in Macrophages

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Background: TLR3 Tyr-759 phosphorylation is critical in dsRNA-mediated IFN-β production.

Results: iNOS-induced Src expression and activation amplify IFN-β production through induction of TLR3 Tyr-759 phosphorylation in macrophages.

Conclusion: Src-TLR3 interaction is important in dsRNA- and LPS-induced IFN-β production.

Significance: An essential role of the iNOS/Src/TLR3 axis is established in IFN-β production in macrophages that might have clinical applications for infectious diseases.

Double-stranded RNA (dsRNA) induces phosphorylation of Toll-like receptor 3 (TLR3) at tyrosine 759 and subsequently triggers signaling pathways to promote interferon-β (IFN-β) production. In this study, we found that dsRNA stimulation induces biphasic TLR3 Tyr-759 phosphorylation in macrophages. In addition to the immediate TLR3 Tyr-759 phosphorylation, we identified a second wave of Tyr-759 phosphorylation accompanied by an increase of both Src and ifn-β transcription in the later phase of dsRNA stimulation. Interestingly, Src phosphorylated TLR3 Tyr-759 in vitro and in vivo. However, knockdown of Src abolished the late phase of TLR3 Tyr-759 phosphorylation and decreased the nuclear accumulation of interferon regulatory factors 3 and 7 (IRF3 and -7) and IFN-β production. Reintroduction of Src restored all of these molecular changes. Notably, via down-regulation of Src, dsRNA-elicited TLR3 Tyr-759 phosphorylation, the nuclear accumulation of IRF3/IRF7, and IFN-β generation were inhibited in inducible nitric-oxide synthase (iNOS)-null macrophages. TLR3 knockdown destabilized Src and reduced the nuclear level of IRF3/IRF7 and IFN-β production in macrophages exposed to LPS (a TLR4 ligand known to induce Src and IFN-β expression). Ectopic expression of wild type TLR3, but not its 759-phenylalanine mutant, restored Src activity and ifn-β transcription. Taken together, these results suggested an essential role of the iNOS/Src/TLR3 axis in IFN-β production in macrophages.

Innate immunity is an evolutionarily conserved mechanism that controls self-nonself discrimination to protect the host from the invasion of pathogens, including viruses (1, 2). As a key player in the innate immune system, macrophages express a spectrum of pattern recognition receptors, including Toll-like receptors (TLRs)3 that recognize specific pathogen-associated molecular patterns within microbial structures (3). TLR1, -2, -4, -5, -6, and -11 are found on the cell surface, and they recognize microbial structures (1, 4). In contrast, TLR3, -7, -8, and -9 are exclusively localized in intracellular compartments like endosomes (1, 4) where they recognize pathogen-specific nucleic acids and induce type I interferon (IFN-I) production (2). It is well known that polyinosinic-polyctydilic acid (poly(I: C)) can mimic viral double-stranded RNA (dsRNA) to trigger TLR3 activation and the production of IFN-I (5).

IFN-I comprises the interferon (IFN)-α family and IFN-β and exerts a vast spectrum of biological functions. One of the well characterized functions of IFN-I is its inhibitory effect on the replication of viruses (6). This can be achieved by up-regulation of proteins such as dsRNA-dependent protein kinase, 2',5'-oligo腺ylenyl synthetase, and RNase L, which execute...

3 The abbreviations used are: TLR, Toll-like receptor; AG, aminoguanidine hemisulfate; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; Ctrl, control; FAK, focal adhesion kinase; iNOS, inducible nitric-oxide synthase; IRF, interferon-regulatory factor; PEM, peritoneal macrophage; poly(I:C), polyinosinic-polyctydilic acid; SFK, Src family kinase; SNAP, S-nitroso-N-acetylpenicillamine; 8-Br-cGMP, 8-bromo-cyclic GMP.

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their antiviral activities through different mechanisms (7). In addition, IFN-α/β regulates the homeostatic differentiation of dendritic cells, natural killer cells, B cells, T cells, and osteoclasts (7). Thus, IFN-α/β plays not only a central role in the antiviral innate immunity of mammals but also an important role in adaptive immunity (8–10).

The expression of IFN-α/β is primarily regulated by multiple transcription factors upon the activation of TLR-dependent and -independent (i.e. retinoic acid-inducible gene-1-like helicases) pathways (11, 12). In conjunction with HMG1(Y) and two other transcription factors (NF-κB and API), interferon-regulatory factors (IRFs) bind to the promoter region of the ifn-β gene and facilitate the formation of transcriptional initiation complex (13). Activation of IRF3 and IRF7 promotes ifn gene expression, and up-regulation of the expression of IFN-responsive genes is critical for the development of antiviral immunity. As a constitutively expressed protein, IRF3 is present in most cell types and shuttles between the nucleus and the cytoplasm in resting cells. In contrast, the expression of IRF7 is restricted to the lymphoid cell types and is both virus- and IFN-inducible (14–17). IRF3 and IRF7 are activated by TBK-1 and IKKε via phosphorylation on their C terminus, which leads to protein dimerization and nuclear translocation (13). In addition, phosphatidylinositol 3-kinase (PI3K)/AKT pathway-mediated IRF3 phosphorylation has been implicated in the full activation of IRF3 in TLR3-mediated signaling (18). Both IRF3 and IRF7 play essential roles in the transcriptional regulation of ifn-α/β genes (19).

Src is a 60-kDa membrane-associated non-receptor tyrosine kinase. Along with its related members, including Fyn, Yes, Lck, Lyn, Fgr, Hck, and Blk, Src family kinases (SFKs) have been implicated in a spectrum of signaling pathways and cellular processes (20). Previous study revealed that Src interacts with active TLR3 on endosomes and activates PI3K/AKT, IRF3, and signal transducer and activator of transcription 1 (Stat1) in the dsRNA-treated human monocyte-derived dendritic cells (21). Unlike the highly expressed myeloid SFKs (i.e. Fgr, Hck, and Lyn), Src is barely detectable in resting macrophages, which could result in its importance being underestimated. Interestingly, our previous studies found that Src, but not its myeloid relatives, was up-regulated in macrophages stimulated with various TLR ligands (22–24). We demonstrated that LPS (a TLR4 ligand), peptideglycan (a TLR2 ligand), poly(I:C), and CpG-oligodeoxynucleotides (a TLR9 ligand) induced Src expression and subsequently promoted FAK phosphorylation, ultimately leading to the induction of macrophage migration (24). Notably, all of these events were inducible nitric-oxide synthase (iNOS)-dependent.

Mounting evidence indicates the importance of tyrosine kinase activation in dsRNA signaling (25, 26). Phosphorylation of five tyrosine residues on TLR3 (i.e. Tyr-733, Tyr-756, Tyr-759, Tyr-764, and Tyr-858) had been shown to participate in dsRNA-induced gene expression (26). Remarkably, in addition to NF-κB-mediated gene induction in dsRNA-treated cells (27), phosphorylation of TLR3 at both Tyr-759 and Tyr-858 independently mediates PI3K and TBK1 activation, leading to the phosphorylation and activation of IRF3 (18). Recently, the importance of tyrosine kinases BTK (28) and Src (29) has been implicated in dsRNA-mediated TLR3 Tyr-759 phosphorylation in different types of cells. However, whether Src is involved in TLR3 Tyr-759 phosphorylation and antiviral response in dsRNA-stimulated macrophages is still unclear. In this study, we found that dsRNA induced a biphasic (early versus late) TLR3 Tyr-759 phosphorylation in macrophages. Src could directly phosphorylate TLR3 Tyr-759 in vitro. Src-mediated late TLR3 Tyr-759 phosphorylation led to the nuclear accumulation of IRF3/IRF7 and the increment of IFN-β production. Studies conducted with wild-type (WT) and iNOS knock-out (iNOS-/-) macrophages provided further evidence regarding the concomitant induction of Src and IFN-β as well as the increased TLR3 Tyr-759 phosphorylation by a NO-mediated pathway. Remarkably, the Src-mediated TLR3 Tyr-759 phosphorylation and IFN-β generation also occurred in TLR4-engaged macrophages. Moreover, knockdown or knockout of TLR3 reduced the stability of Src- and TLR4-dependent IFN-β production. Thus, the significance of iNOS/Src/TLR3 axis in IFN-β production in macrophages is established.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies—**Aminoguanidine hemisulfate (AG), 1H-[1,2,4]oxadiazolo[4,3-α]quinazolin-1-one (ODQ), S-nitroso-N-acetylpenicillamine (SNAP), 8-bromo-cyclic guanosine monophosphate (8-Br-cGMP), poly(I:C), and LPS purified from *Escherichia coli* serotype 011:B4 were obtained from Sigma. Thioglycollate was obtained from Merck. The following antibodies were used: actin, iNOS, and FAK (Upstate); FAK Tyr(P)-861 (BIOSOURCE International); proliferating cell nuclear antigen, IRF3, and Src Tyr(P)-416 (Cell Signaling Technology); TLR3, IRF7 (Abcam, Cambridge, UK); TLR3 Tyr(P)-759 (Imgenex, San Diego, USA); Lyn, Hck, Fgr, and HRP-conjugated anti-phosphotyrosine antibody (PY20) (Santa Cruz Biotechnology, Inc.). The mouse ascites containing monoclonal anti-Src (peptide 2–17) antibody were produced by the hybridoma (CRL-2651) obtained from the American Type Culture Collection.

**Animals—**C57BL/6 mice were utilized to prepare peritoneal macrophages (PEMs). C57BL/6 inducible nitric-oxide synthase knock-out (C57BL/6-NOx<sup>-/-</sup>) (iNOS<sup>-/-</sup>) (30), TLR3 knock-out (TLR3<sup>−/-</sup>) (5), and wild type (WT) mice of the same age and sex were used to assess the role of iNOS and TLR3 in poly(I:C)- or LPS-induced TLR3 Tyr-759 phosphorylation and IFN-β secretion. TRIF knock-out (TRIF<sup>−/-</sup>) (32) and WT mice were used to study the importance of TRIF in NO-mediated IFN-β production. All experiments using laboratory animals were carried out in accordance with guidelines of the China Medical University and the National Cheng Kung University.

**Cell Culture and Collection of PEMs—**Maintenance of C3H10T1/2 fibroblast-expressing vector control (Neo) and v-Src (IV5) (generously provided by Dr. Sarah J. Parsons) was described previously (33). The murine macrophage cell line, RAW264.7 (American Type Culture Collection) was cultured and propagated in RPMI 1640 medium supplemented with 10% fetal calf serum (HyClone, Logan, UT) and 2 mM L-glutamine at 37 °C in humidified atmosphere of 5% CO<sub>2</sub> and air. Preparation of PEMs was described previously (23). Briefly, they were collected by peritoneal lavage from rats (Sprague-Dawley) or mice.
given an intraperitoneal injection of 8 ml and 1 ml of thioglycollate broth, respectively, 4 days before harvest. The PEMs were washed with Ca\(^2+\) - and Mg\(^2+\) -free phosphate-buffered saline and plated in fetal calf serum-containing RPMI medium for 6 h. Then the cells were washed with medium to remove non-adherent cells. The resultant macrophage monolayer with greater than 98% purity, evaluated by morphology and phagocytosis property, was used for experiments. Bone marrow-derived macrophages were collected and differentiated as described previously (24).
Site-directed Mutagenesis—The generation of 759F-TLR3-expressing construct (pCMV/hTLR3–759F) was performed by the QuickChange Lightning site-directed mutagenesis kit (Stratagene) utilizing human TLR3-expressing plasmid pCMV/hTLR3 as a template and a pair of primers with mutation at the selected residues for a PCR. The mutated sequence was confirmed by the Sanger-dideoxy DNA sequencing method.

RNA Interference—The generation of RAW264.7 cells stably expressing nonspecific siRNA (Ctrl), src siRNA (siRNA-1), src siRNA and avian c-Src (siRNA-1/Src6 and siRNA-1/Src15) was described previously (23, 24). Similarly, RAW264.7 cells were transfected with plasmid DNA pLKO.1-mtlr3-1 (puro) (targeted sequence, 5′-CTCTTTGAACTCCTCTTCATA-3′), or both (bottom) for various durations (0, 1, 2, 6, 12, and 24 h). The amount of *ifn*-β and *nos2* transcript was analyzed by RT-PCR, and *gapdh* was utilized as an internal control for amplification efficiency. The same cells were incubated with SNAP for 0, 0.17, 1, and 2 h, and their lysates were Western immunoblotted by antibodies that recognize TLR3 Tyr(P)-759, TLR3, FAK Tyr(P)-861, FAK, Src Tyr(P)-416, and actin. *, Tyr-416-phosphorylated Src.

Preparation of Total Lysates and Nuclear Extracts—Cell lysis was carried out with modified radioimmunoprecipitation assay buffers as described previously (24), and nuclear extracts were prepared using the NE-PER kit (Pierce). Protein concentration was determined by a BCA protein assay kit (Bio-Rad).

iNOS/Src/TLR3 Axis in TLR-mediated IFN-β Production

**FIGURE 3.** **NO activates Src to phosphorylate TLR3 Tyr-759 and increase the level of *ifn*-β transcript.** A, RAW264.7-derived Ctrl, siSrc-1, and siSrc-1/ Src6 cells were stimulated with poly(I:C) (20 μg/ml; top), SNAP (100 μM; middle), or both (bottom) for various durations (0, 1, 2, 6, 12, and 24 h). The amount of *ifn*-β and *nos2* transcript was analyzed by RT-PCR, and *gapdh* was utilized as an internal control for amplification efficiency. B, the same cells were incubated with SNAP for 0, 0.17, 1, and 2 h, and their lysates were Western immunoblotted by antibodies that recognize TLR3 Tyr(P)-759, TLR3, FAK Tyr(P)-861, FAK, Src Tyr(P)-416, Src, and actin. *, Tyr-416-phosphorylated Src.

**FIGURE 4.** **Src is important in poly(I:C)-induced IFN-β secretion in macrophages.** The RAW264.7 (RAW) cell line and its derived cells Ctrl-1, Ctrl-2, siSrc-1, siSrc-1/Src6, and siSrc-1/Src15 were stimulated with or without poly(I:C) for 24 h. Their lysates (100 μg) were resolved by SDS-PAGE and immunoblotted with specific antibodies to show the induction and activation of Src in the treated cells. The concentration of IFN-β in the supernatants of these cells was determined by ELISA. **,** p < 0.001. Error bars, S.D.

**Measurement of IFN-β Production**—Cells were seeded onto 96-well plates at 2 × 10⁵ cells/ml and stimulated with or without poly(I:C) (20 μg/ml), SNAP (100 μM), or 8-Br-cGMP (100 μM) for various times, as indicated. IFN-β concentration in the culture supernatants was measured using an enzyme-linked immunosorbent assay (ELISA). The ELISA kit for murine IFN-β was purchased from PBL Biomedical Laboratories.

**In Vitro Kinase Reaction**—The in vitro Src kinase reaction was performed as described previously (33). Briefly, TLR3 was immunoprecipitated from lysates (1.5 mg) of RAW264.7 cells. The immunoprecipitates was determined by ELISA. **,** p < 0.001. Error bars, S.D.

**RT-PCR**—The amount of *ifn*-β, *nos2*, *src*, *tlr3*, and *gapdh* transcript was semiquantitated by RT-PCR. Sequences of primer pairs used were as follows: *ifn*-β, 5′-CATCACTATAAGCAGCTCCA-3′ (forward) and 5′-TTCAAGTTGAGAGGCCGTTGAG-3′ (reverse); *nos2*, 5′-GCATTTCACACAAACAGAAGTTGTCGTCA-3′ (forward) and 5′-TCATTGTACTCTGAGGCTGAACACA-3′ (reverse); *src*, 5′-CTGCGTGGACCTTTCTCAAGGG-3′ (forward) and 5′-GTACAGAGCAGCTTCAGGG-3′ (reverse); *tlr3*, 5′-CTCCTGAACACGGCCACCTA-3′ (forward) and 5′-GTCCACTTCAGCCAGAGAA-3′ (reverse); *gapdh*, 5′-CCATCACCATCTTCAGAG-3′ (forward) and 5′-CTCTGCTTCCACCCCTTTT-3′ (reverse). PCR products were resolved in 2% agarose gel and detected by ethidium bromide staining.
Statistical Analysis—Each experiment was performed at least three times. Unless indicated, the results were presented as means ± S.D. from a representative triplicate experiment. The significance of difference was assessed by Student’s t test. Bonferroni correction was used for controlling type I error in multiple comparisons.

RESULTS

Src Mediates TLR3 Phosphorylation at Tyr-759 in dsRNA-treated Macrophages—Previously, TLR3 Tyr-759 phosphorylation was demonstrated to be essential in dsRNA-elicited activation of PI3K and full activation of IRF3 (18). Here, a time course study with Western immunoblotting revealed two phases of TLR3 Tyr-759 phosphorylation occurring in poly(I:C)-treated macrophages. Whereas the first phase (early) appeared within 10 min (~0.17 h) and then declined, the second phase (late) gradually appeared at the 6-h time point and was sustained in PEMs exposed to dsRNA (Fig. 1A, left). Similar results were observed in RAW264.7 cells exposed to dsRNA (Fig. 1A, right).

The biphasic Tyr-759 phosphorylation of TLR3 was also detected in the immunoprecipitated TLR3 prepared from RAW264.7 cells exposed to dsRNA (Fig. 1B). Because the late TLR3 Tyr-759 phosphorylation occurred together with the enhanced Src protein expression and kinase activity (as reflected by FAK Tyr(P)-861) (23) (Fig. 1A), Src was speculated to be responsible for the late TLR3 Tyr-759 phosphorylation in dsRNA-treated cells. To test this hypothesis, we examined the level of TLR3 Tyr-759 phosphorylation at different time points (i.e., 0.17, 1, and 24 h) in dsRNA-stimulated RAW264.7-based control cells (Ctrl), Src-deficient cells (siSrc-1), and Src-deficient cells expressing avian Src (siSrc-1/Src15) (avian c-src mRNA contains sequences that were not recognized by the applied small interfering RNA) (23). As shown in Fig. 1C, the late but not the early TLR3 Tyr-759 phosphorylation was affected by Src silencing, and this effect was reversed by the ectopic expression of Src. Of note, TLR3 could associate with Src in RAW264.7 cells overexpressing Src (siSrc-1/Src6) (Fig. 2A) or fibroblasts with (IV5) or without (Neo) v-Src (Fig. 2B).

FIGURE 5. iNOS participates in Src induction and the IFN-β production in macrophages stimulated with poly(I:C). RAW264.7 cells were either pretreated with or without AG (2 mM) or ODQ (100 μM) for 30 min and then stimulated with or without poly(I:C) for 48 h (A) or simply incubated with or without SNAP (100 μM) or 8-Br-cGMP (cGMP, 100 μM) for 48 h (B). The amount of ifn-β transcript in these cells was analyzed by RT-PCR, and gapdh was utilized as an internal control for amplification efficiency. PEMs from WT and iNOS−/− mice were stimulated with or without poly(I:C) (C) or SNAP and 8-Br-cGMP (cGMP) (D) for 48 h. Equal amounts (100 μg) of their lysates were resolved by SDS-PAGE and probed with specific antibodies to indicate concurrent expression of Src and iNOS. The concentration of IFN-β in the culture supernatants was determined by ELISA. ***; p < 0.001. Error bars, S.D.
Furthermore, an in vitro kinase reaction was performed with immunoprecipitated TLR3 incubated with or without baculovirus-expressed active Src for 10 and 30 min. As shown in Fig. 2C, tyrosyl phosphorylation of TLR3 was undetected in the absence of Src. In contrast, the level of tyrosyl-phosphorylated TLR3 and TLR3 Tyr-759 phosphorylation was elevated in the presence of Src in a time-dependent manner, suggesting that Src might directly phosphorylate TLR3 on Tyr-759 in dsRNA-treated macrophages. Notably, constitutive TLR3 Tyr-759 phosphorylation was observed in IV5, as compared with that in Neo (Fig. 2D). This further corroborated the role of Src in mediating TLR3 Tyr-759 phosphorylation. Consistent with the notion that TLR3 Tyr-759 phosphorylation is responsible for dsRNA-mediated PI3K/AKT activation (18), depletion of Src not only impaired the TLR3 Tyr-759 phosphorylation but also abolished AKT activity at the late phase (i.e. 24 h) of dsRNA stimulation in RAW264.7 cells, which could be restored by the ectopic expression of Src. Consequently, dsRNA-mediated production of IFN-β was also suppressed by silencing Src, and this suppression was restored by the ectopic expression of Src (Fig. 4).

Although ectopically expressed Src restored IFN-β production in Src-deficient cells, it did not accelerate dsRNA-induced transcription of the ifn-β gene (Fig. 3A, top), suggesting that in addition to Src expression, Src activation might also be critical in this process. It has been demonstrated that NO can induce and activate Src (34). Indeed, in the presence of SNAP (a NO donor), the increase of Src Tyr-416 phosphorylation and the phosphorylation of Src substrates (i.e. FAK Tyr-861 phosphorylation and TLR3 Tyr-759 phosphorylation) was observed in siSrc-1/Src6 cells at 1 h post-treatment (Fig. 3B). Furthermore, the appearance of ifn-β transcript was accelerated in these cells stimulated with SNAP (Fig. 3A, middle) or dsRNA plus SNAP (Fig. 3A, bottom) as compared with the control. Given that the expression and activation of Src are iNOS-dependent (23, 24) and pivotal for IFN-β generation, the involvement of iNOS in IFN-β production was investigated. Because soluble guanylyl cyclase (sGC) is a main target of NO whose activation generates cGMP (a second messenger) (35), the effect of the inhibitors for iNOS and sGC on IFN-β production was determined. AG (an iNOS inhibitor) and ODQ (a sGC inhibitor) decreased dsRNA-elicited ifn-β mRNA expression and IFN-β secretion (Fig. 5A).
whereas SNAP and 8-Br-cGMP (a permeable cGMP analog) induced the generation of IFN-β (Fig. 5B). The conditioned media of PEMs derived from WT and iNOS−/− mice treated with or without dsRNA were also analyzed. As shown in Fig. 5C, dsRNA-stimulated IFN-β production was impaired in the iNOS-null PEMs. Remarkably, SNAP and 8-Br-cGMP stimulated the secretion of IFN-β in iNOS-deficient macrophages to a level comparable with that in WT macrophages (Fig. 5D). Of note, the reduced secretion of IFN-β was correlated to the diminished level of Src in dsRNA-treated PEMs devoid of iNOS (Fig. 5C). These data implied that the NO/cGMP pathway could induce IFN-β production via Src expression and activation. To further test this hypothesis, RAW264.7-derived Ctrl-1, Ctrl-2, siSrc-1, and siSrc-1/Src15 cells with ectopic expression of avian Src (siSrc-1/Src6, siSrc-1/Src15) were stimulated with SNAP, and the amount of IFN-β secreted was measured by ELISA. As shown in Fig. 6, concomitant induction of Src, FAK Tyr-861 phosphorylation, and IFN-β secretion were observed in RAW264.7, Ctrl-1, and Ctrl-2 but not in siSrc-1 cells at 24 h after SNAP stimulation. Markedly, the production of IFN-β and FAK Tyr-861 phosphorylation could be restored in siSrc-1/Src6 and siSrc-1/Src15 upon SNAP stimulation (Fig. 6). Thus, the vital role of Src in IFN-β induction elicited by dsRNA/iNOS was confirmed.

iNOS/Src Axis Is Required for the Nuclear Accumulation of IFN-β in Macrophages

iNOS/Src/TLR3 Axis in TLR-mediated IFN-β Production

FIGURE 7. The iNOS/Src axis is required for poly(I:C)-mediated nuclear accumulation of IRF3 and IRF7 in macrophages. Total lysates (100 μg) (A) or nuclear extracts (30 μg) (B) were prepared from RAW264.7-derived Ctrl, siSrc-1, and siSrc-1/Src15 cells stimulated with or without poly(I:C) or SNAP for 24 h, resolved by SDS-PAGE, and immunoblotted with specific antibodies to demonstrate that the level of TLR3 Tyr(P)-759 and IRF7 in the lysates as well as the level of nuclear IRF3 and IRF7 are Src-dependent. C, RAW264.7 cells were either pretreated with or without AG or ODQ for 30 min and then stimulated with or without poly(I:C) for 24 h or simply stimulated with SNAP or 8-Br-cGMP for 24 h. Their nuclear extracts (30 μg) were resolved by SDS-PAGE and immunoblotted with specific antibodies to examine the amount of IRF3 and IRF7. Proliferating cell nuclear antigen (PCNA) was utilized as a loading control. D, PEMs prepared from WT and iNOS−/− mice were stimulated with or without poly(I:C) or SNAP for 24 h. Their total lysates (100 μg; top) or nuclear extracts (30 μg; bottom) were resolved by SDS-PAGE and immunoblotted with specific antibodies to demonstrate that the level of nuclear IRF3 and IRF7 is correlated with the level of TLR3 Tyr(P)-759, Src, and iNOS in the lysates.
Knockdown of Src impaired TLR3 Tyr-759 phosphorylation (Fig. 7A) as well as the nuclear accumulation of IRF3/7 (Fig. 7B) in dsRNA- and SNAP-stimulated macrophages. Of note, unlike IRF3, whose expression was similar between Ctrl and siSrc-1 cells, the induction of IRF7 was reduced in siSrc-1 cells stimulated with dsRNA or SNAP, which could be reversed by the ectopic expression of Src (Fig. 7A). Thus, Src plays an important role in dsRNA- and SNAP-induced nuclear accumulation of IRF3 and IRF7. To further confirm the importance of iNOS in dsRNA-mediated nuclear accumulation of IRF3/7, RAW264.7 cells were pretreated with AG or ODQ. As shown in Fig. 7C, the nuclear accumulation of IRF3/IRF7 was abolished by AG or ODQ. In contrast, SNAP and 8-Br-cGMP increased the nuclear level of both IRF3 and IRF7 in response to dsRNA stimulation (Fig. 7D). Notably, such a phenomenon was not observed by SNAP treatment (Fig. 7D). These findings supported the importance of iNOS in TLR3-mediated nuclear accumulation of IRF3 and IRF7 for iNOS-β transcription.

TLR3 Tyr-759 Phosphorylation Also Participates in the Expression of iNOS in TLR4-engaged Macrophages—Given that LPS induces IFN-β production (38) and Src has been shown to be elevated and activated by TLR4 engagement (24), we aimed to determine whether Src-mediated TLR3 Tyr-759 phosphorylation also occurs in macrophages exposed to LPS. Intriguingly, like dsRNA-treated RAW264.7 cells, TLR3 Tyr-759 phosphorylation was observed in macrophages stimulated with LPS for 24 h (Fig. 8A). To demonstrate that this TLR3 Tyr-759 phosphorylation was also Src-dependent, RAW264.7-derived Ctrl, siSrc-1, and siSrc-1/Src15 cells were treated with dsRNA and LPS for 24 h, and the levels of TLR3 Tyr-759 phosphorylation, IRF3/7 nuclear accumulation, and the expression of iNOS were examined. As shown in Fig. 8B, the level of TLR3 Tyr-759 phosphorylation, nuclear IRF3/7, and iNOS transcript was suppressed by Src knockdown and rescued by Src reintroduction. To further verify the importance of TLR3 in eliciting iNOS-β transcription in macrophages in response to TLR4 engagement, RAW264.7-derived Ctrl and TLR3-deficient (siTLR3–1 and -2) cells were stimulated with or without dsRNA and LPS for 24 h, and the level of TLR3 Tyr-759 phosphorylation, IRF3/7 nuclear accumulation, and the expression of iNOS were examined. As shown in Fig. 9A, TLR3 knockdown impaired all of these events in macrophages exposed to either dsRNA or LPS. Interestingly, silencing TLR3 inhibited LPS-mediated Src enhancement (Fig. 9A) but not the activation of MAPK (i.e. phospho-ERK1/2 and phospho-p38 MAPK) (Fig. 9B). Concurrently, a decreased level of Src and IFN-β was also observed in TLR3-null PEMs exposed to dsRNA and LPS (Fig. 9C). Notably, unlike dsRNA treatment, there was no decrement on the level of src transcript in LPS-exposed TLR3-deficient macrophages (Fig. 9A). Further analysis of the stability of Src in RAW264.7-based control and TLR3-deficient cells exposed to LPS for 24 h revealed that the half-life of Src was greatly decreased (from >12 to <6 h) in response to TLR3 diminishment (Fig. 9D). Markedly, transient expression of human wild type (Wt) TLR3, but not phenylalanine 759 (759F) mutant, restored dsRNA- and LPS-elicited iNOS-β transcription in TLR3-deficient macrophages (Fig. 9E), further supporting the importance of Src-mediated TLR3 Tyr-759 phosphorylation in the induction of IFN-β by dsRNA or LPS.

TRIF Domain-containing Adaptor-inducing IFN-β (TRIF) Is Not Required for NO-mediated IFN-β Production—TRIF is the only known TIR-containing adaptor associated with TLR3. Upon ligand (i.e. dsRNA) binding to the TLR3/TRIF, NF-κB and IRF3 are activated to turn on iNOS-β gene expression. To determine whether TRIF is required for iNOS/Src-dependent IFN-β generation, we analyzed the production of IFN-β from SNAP-treated PEMs and bone marrow-derived macrophages from TRIF-null mice. As shown in Fig. 10, the levels of SNAP-induced Src, TLR3 Tyr-759 phosphorylation, and IFN-β production were similar between WT and TRIF-null cells. Thus, this NO/Src-mediated TLR3 Tyr-759 phosphorylation and IFN-β production could be mediated via a TRIF-independent pathway.
TLR3 Is Required for v-Src-mediated Transformation—Given that TLR3 could stabilize Src, we wondered about its importance in Src-mediated cell transformation. As shown in Fig. 11A, whereas the level of Src protein and ifn-β transcript was reduced in TLR3-deficient v-Src-transformed IV5 cells, constitutive activation of Janus kinase 2 (Jak2) (as indicated by Jak2 auto-phosphorylation at Tyr-1007; i.e. Tyr(P)-1007 (39)) and Stat3 (as reflected by Stat3 Tyr(P)-705) that are important in v-Src-mediated transformation (40–45) was also inhibited (Fig. 11A). Concurrently, the ability of these cells to grow in soft agar was significantly decreased (Fig. 11B). Thus, the participation of TLR3 in v-Src-mediated cell transformation was corroborated.

DISCUSSION

Although compelling evidence indicates the importance of TLR3 Tyr-759 phosphorylation in dsRNA-triggered antiviral...
signaling (18, 26, 28), the essential role of iNOS and Src in dsRNA-mediated TLR3 Tyr-759 phosphorylation and IFN-β production has not been investigated in the past. In this study, we demonstrated that dsRNA-stimulation induced a biphasic (early versus late) TLR3 Tyr-759 phosphorylation in macrophages. The late TLR3 Tyr-759 phosphorylation was intimately correlated with the expression and activation of Src in dsRNA-treated PEMs and RAW264.7, suggesting that Src might be the kinase responsible for TLR3 Tyr-759 phosphorylation and the following IFN-β production. Indeed, dsRNA-induced TLR3 Tyr-759 phosphorylation and IFN-β production were impaired and rescued by knockdown and reintroduction of Src, respectively. Moreover, active Src could phosphorylate TLR3 in an in vitro kinase reaction and in v-Src-transformed IV5 cells, which led to constitutive expression of iIfn-β. Consistent with the fact that the enhancement and activation of Src could be mediated by NO, we further demonstrated that dsRNA-elicited iIfn-β transcription and IFN-β production was regulated by the iNOS/Src axis. Interestingly, activation of ectopically expressed Src accelerated the induction of iIfn-β transcript, further supporting this hypothesis.

The myeloid SFKs, Lyn, Fgr, and Hck, were previously shown to be dispensable for the LPS/TLR4 signaling in macrophages (46). In our study, TLR3 Tyr-759 phosphorylation was not affected by depletion of any of the three myeloid SFKs (data not shown). Moreover, Lyn knockdown did not reduce dsRNA-elicited iIfn-β transcription and IFN-β secretion (data not shown). It is also worth noting that FAK, a downstream target of Src, was involved in TLR3-mediated macrophage migration (47) but not in dsRNA-ignited IFN-β production in RAW264.7 cells (data not shown). Interestingly, the implication of EGF receptor-recruited Src in TLR3 Tyr-759 phosphorylation has been shown in constitutive Src-expressing cells (29), supporting the importance of Src in TLR3-induced signaling as we reported in this study. Recently, the role of BTK has been implicated in TLR3 Tyr-759 phosphorylation and initiating antiviral responses in dsRNA-treated macrophages (28). Given that BTK was activated within 30 min after dsRNA stimulation, BTK might be the kinase responsible for the early TLR3 Tyr-759 phosphorylation in macrophages. However, our data indicated that the early TLR3 Tyr-759 phosphorylation did not immediately elicit obvious iIfn-β transcription.

Full induction of the nos2 gene (encoding iNOS) (48, 49) by LPS or pathogens requires IFN-I production, and the activation of the Jak/Stat pathway to deploy NF-κB and ISGF3 (a Stat1-Stat2-IRF9 tricomplex) (50) to its promoter region. Binding of NF-κB to the nos2 promoter is required to recruit and probably activate the kinase TFIIH, which remains to be chromatin-associated, even after NF-κB departs (51). Upon IFN-I stimulation, ISGF3 along with RNA polymerase II are recruited, and then TFIIH mediates the phosphorylation of the C-terminal tail of polymerase II. Because the NF-κB signal can precede the IFN-I signal by up to 24 h, these two processes do not need to be carried out simultaneously and display memory (51, 52). Given that IFN-β might mediate ISGF3 activation via type 1 IFN receptors (53, 54) and that the timing of the induction of nos2 and iIfn-β was simultaneously affected by Src in our kinetic study, the iNOS/Src/TLR3 Tyr-759 phosphorylation-dependent IFN-β generation found in this study might play a crucial role in establishing a positive feedback loop between iNOS and IFN-β to launch and amplify antiviral responses in macrophages. Although TRIF is essential for initial ligand-activated TLR3- and TLR4-mediated IFN-β production (32), our data indicated that it was not required in this NO-Src-TLR3 Tyr-759 phosphorylation-IFN-β pathway. Consistently, TRIF deficiency did not affect the interaction between Src and TLR3 (29).

Our time course study revealed concurrent expression of IRF7 and iNOS in the late phase when IRF3 was already activated and accumulated in the nucleus in dsRNA-treated PEMs. This is consistent with previous findings that IRF3 is the key transcription factor for early phase IFN-β production, whereas IRF7 is induced by IFN-I and plays the major role for robust production of IFN-I in response to TLR activation or viral infection (13, 16, 19, 54–56). Given that Src affects the induction of...
iNOS/Src/TLR3 Axis in TLR-mediated IFN-β Production

FIGURE 11. TLR3 participates in v-Src-mediated cell transformation. A, equal amounts of lysates prepared from IV5 and IV5 infected with lentivirus expressing luciferase siRNA (Ctrl), tlr3 siRNA1 (siTLR3–1), or tlr3 siRNA2 (siTLR3–2) were resolved by SDS-PAGE and immunoblotted by specific antibodies to show the expression of TLR3 and Src as well as the activation status of JAK2 and Stat3 (top). The amount of ifn-β, src, and tlr3 transcript in these cells was analyzed by RT-PCR, and gapdh transcript was utilized as an internal control for amplification efficiency (bottom). B, for colony formation in soft agar, 3 × 10³ cells from each cell line were applied in the top agar at the beginning, and the number of colonies (>8 cells) formed after a 10-day incubation was counted and plotted. *, p < 0.05, compared with Ctrl cells. Similar results were obtained at least twice. Error bars, S.E.

iNOS and IRF7 as well as the nuclear accumulation of both IRF3 and IRF7, Src might induce ifn-β transcription via modulation of the TBK1- and/or IKKe-IRF3 pathway in addition to the TLR3 Tyr-759 phosphorylation-P13K/AKT-IRF3 pathway (18). In fact, Src has been shown to participate in the activation of TBK1 in the E3 ubiquitin ligase CHIP-facilitated TLR4 signaling pathway (57).

In agreement with the production of IFN-β (58, 59) and the activation of the iNOS/Src axis (24) in macrophages exposed to LPS, co-emergence of Src and TLR3 Tyr-759 phosphorylation was also observed in LPS-stimulated macrophages. Remarkably, LPS-induced TLR3 Tyr-759 phosphorylation, the nuclear accumulation of IRF3/7, and the elevated level of ifn-β transcript were also Src-dependent. Given that the initial MAPK activation was not affected by TLR3 knockdown, the cross-talk between TLR4 and TLR3 could be Src-dependent and occurred in the TLR3-containing endosomes. It is conceivable that ligand-bound TLR4 induces iNOS and Src expression, which leads to TLR3-Src complex formation and the following TLR3 Tyr-759 phosphorylation. In this study, we found that without TLR3, the LPS-induced Src had a shorter half-life and could not activate IRF3 and IRF7 for ifn-β transcription. In contrast, transient expression of human TLR3 in the TLR3 knockdown macrophages restored Src expression and the following ifn-β transcription. These findings further corroborated the critical role of TLR3 in LPS-induced Src expression. Markedly, Src induction, but not ifn-β transcription, was restored in dsRNA- and LPS-treated macrophages expressing 759F-TLR3, indicating that TLR3 Tyr-759 phosphorylation was not required for Src stabilization but played an essential role in IFN-β generation.

Constitutive activation of Src resulted in TLR3 Tyr-759 phosphorylation and ifn-β transcription in v-Src-transformed cells. Given that type I IFN possesses antiproliferative activity in cancer cells, including v-Src transformed cells (31), one might speculate that TLR3 plays a negative role in cell proliferation in cancer cells. However, our studies revealed that TLR3 stabilized Src and contributed to Src-mediated anchorage-independent growth. Based on this finding, TLR3 might be a potential target for anti-cancer treatment.

In conclusion, we demonstrate that 1) Src is the tyrosine kinase responsible for TLR3 Tyr-759 phosphorylation, 2) the stability of Src is in turn regulated by TLR3, and 3) the iNOS/Src/TLR3 Tyr-759 phosphorylation axis is the common pathway for IFN-β production in macrophages exposed to dsRNA and LPS. Considering the importance of IFN-1 in antiviral and immunomodulatory effects, our findings might have implications in clinical management of infectious diseases.

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