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The iNOS/Src/FAK axis is critical in Toll-like receptor-mediated cell motility in macrophages

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ABSTRACT

The Toll-like receptors (TLRs) play a pivotal role in innate immunity for the detection of highly conserved, pathogen-expressed molecules. Previously, we demonstrated that lipopolysaccharide (LPS, TLR4 ligand)-increased macrophage motility required the participation of Src and FAK, which was inducible nitric oxide synthase (iNOS)-dependent. To investigate whether this iNOS/Src/FAK pathway is a general mechanism for macrophages to mobilize in response to engagement of TLRs other than TLR4, peptidoglycan (PGN, TLR2 ligand), polyinosinic–polycytidylic acid (polyI:C, TLR3 ligand) and CpG-oligodeoxynucleotides (CpG, TLR9 ligand) were used to treat macrophages in this study. Like LPS stimulation, simultaneous increase of cell motility and Src (but not Fgr, Hck, and Lyn) was detected in RAW264.7, peritoneal macrophages, and bone marrow-derived macrophages exposed to PGN, polyI:C and CpG. Attenuation of Src suppressed PGN-, polyI:C-, and CpG-elicited movement and the level of FAK Pi-Tyr861, which could be reversed by the reintroduction of siRNA-resistant Src. Besides, knockdown of FAK reduced the mobility of macrophages stimulated with anyone of these TLR ligands. Remarkably, PGN-, polyI:C-, and CpG-induced Src expression, FAK Pi-Tyr861, and cell mobility were inhibited in macrophages devoid of iNOS, indicating the importance of iNOS. These findings corroborate that iNOS/Src/FAK axis occupies a central role in macrophage locomotion in response to engagement of TLRs.

1. Introduction

Macrophages are critical players in innate immunity induced by invaded microorganisms or injured tissues. This is via the interaction between the conserved structures called pathogen-associated molecular patterns (PAMPs) in microorganisms or damage-associated molecular patterns in the released endogenous molecules from the damaged cells and the pattern-recognition receptors, such as Toll-like receptors (TLRs) in macrophages [1,2]. TLRs contain N-terminal extracellular leucine-rich repeats that mediate the recognition of specific pathogen components, a membrane-spanning domain that determines the cellular localization, and a C-terminal intracellular region similar to that of the IL-1 receptor known as the Toll/IL-1 receptor (TIR) domain, which is essential for downstream signaling [3]. There are 10 TLRs that have been identified in humans and 12 in

Abbreviations: AG, aminoguanidine hemisulfate; BMDMs, bone marrow-derived macrophages; FAK, focal adhesion kinase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; ODN, oligodeoxynucleotide; PAMPs, pathogen-associated molecular patterns; PEMs, peritoneal macrophages; PGN, peptidoglycan; polyI:C, polyinosinic: polycytidylic acid; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; SFKs, Src family kinases; TLRs, Toll-like receptors; WT, wild type

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mice [1]. Certain TLRs (i.e. TLR1, 2, 4, 5, 6 and 11) are found on the cell surface, while others (i.e. TLR3, 7, 8 and 9) are detected almost exclusively in intracellular compartments such as endosomes [1,4]. TLR2 recognizes peptidoglycan (PGN), a major bacterial cell wall component. TLR4 acts as a signaling receptor for lipopolysaccharide (LPS), an outer membrane component of Gram-negative bacteria [5,6]. As TLR3 senses viral double-stranded RNA [7], TLR9 detects the unmethylated CpG-oligodeoxynucleotides (CpG) that are frequently found in bacteria, but not in vertebrate DNA [8]. Subsequent to recognition of a PAMP, TLR recruits a combination of TIR-containing adaptors, including MyD88, TIRAP/Mal, TRAM and TRIF. It is well documented that TLR9 utilizes MyD88, TLR3 needs TRIF, TLR2 requires MyD88 and TIRAP, and TLR4 uses all the four aforementioned adaptors [3]. Through individually preferential adaptors, engagement of TLRs triggers downstream signaling pathways to activate NF-κB or MAP kinase and generate proinflammatory cytokines required for host defensive strategies [1,4,9].

As a nonreceptor tyrosine kinase, Src is the prototype of a family of highly conserved proteins (Fyn, Yes, Fgr, Hck, Lck, Lyn, Blk, and Yrk), which have been implicated in diverse signaling pathways and various cellular processes [10]. Though Lyn, Fgr and Hck are the predominant Src family kinases (SFKs) in macrophages [11,12], their expression is unaltered in response to LPS. By contrast, Src is barely detectable in resting macrophages, but can be greatly induced by LPS [13,14]. Considering the importance of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), the two inducible enzymes in infection and inflammation, the significance of Src in macrophage physiology is anticipated and ready to be disclosed.

Focal adhesion kinase (FAK) is an intracellular tyrosine kinase localized predominantly within focal adhesion [15,16] and participates

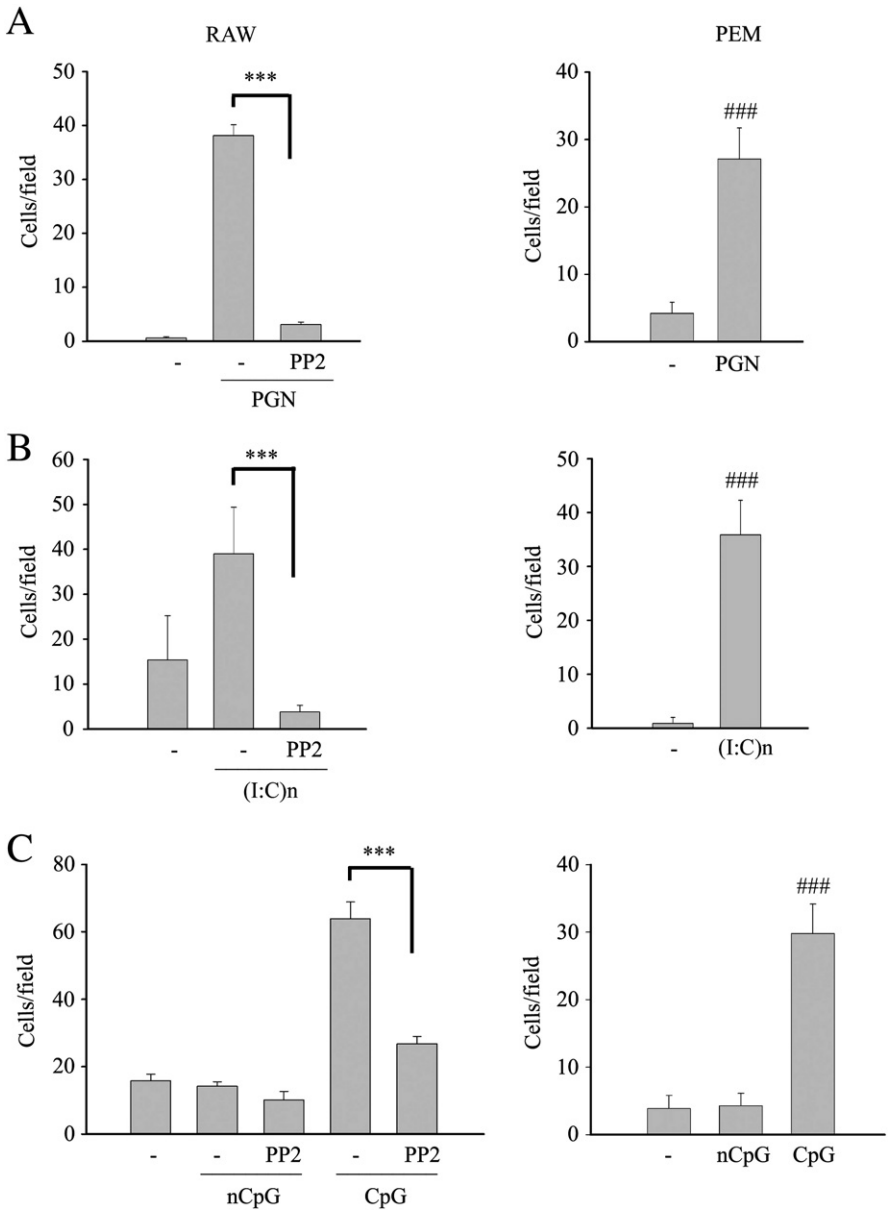

Fig. 1. The involvement of Src family kinase(s) in PGN-, polyI:C-, and CpG-stimulated macrophage migration. RAW264.7 macrophages (RAW; left) pretreated without or with PP2 (10 μM) for 1 h, and then cells were stimulated without or with (A) PGN (5 μg/ml), (B) polyI:C ((I:C)n, 20 μg/ml) and (C) nCpG or CpG (0.3 μg/ml) for 48 h. Rat PEMs (right) were stimulated with or without (A) PGN (5 μg/ml), (B) polyI:C ((I:C)n; 20 μg/ml) and (C) CpG or nCpG (0.3 μg/ml) for 48 h. The motility of each group was determined by using a modified Boyden chamber as described under "Materials and methods." $^{***}p<0.001$; $^{###}p<0.001$. Similar results were repeated three times and the representative was demonstrated.

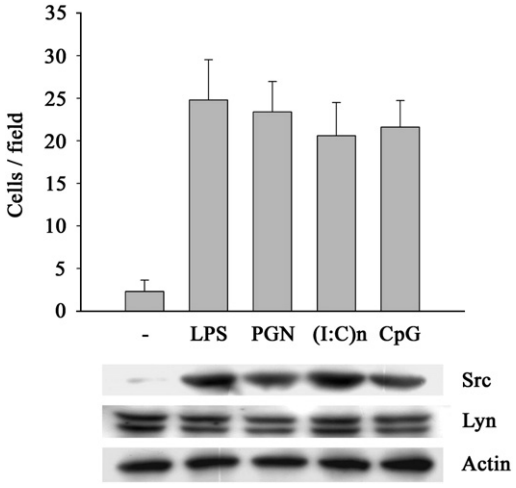

Fig. 2. Increased Src expression and motility in BMDMs exposed to LPS, PGN, polyI:C, and CpG. BMDMs were stimulated without or with LPS (100 ng/ml), PGN (5 μg/ml), polyI:C ((I:C)n; 20 μg/ml) and CpG (0.3 μg/ml) for 48 h. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. The motility of each group was determined as mentioned in Fig. 1. Similar results were repeated three times and the representative was demonstrated.

in a variety of integrin-initiated biological activities including growth, survival, cell adhesion and migration [17,18]. Deficiency of FAK results in embryonic lethality, characterized by impaired fibroblast migration [19–21]. Upon integrin stimulation, FAK becomes activated and autophosphorylated at Tyr397 that confers the binding site for Src. This facilitates Src-mediated FAK phosphorylation on several tyrosine residues including Tyr861 [22,23], whose phosphorylation promotes FAK Pi-Tyr397 [24]. It is noteworthy that FAK is initially thought to be absent or expressed at low levels in monocyte/macrophages [25,26]. However, mounting evidence reveals the presence of FAK in this cell lineage [27,28] and unveils its involvement in macrophage motility [29].

To date, study on the response of macrophages to PAMPs via TLRs has focused mainly on the generation of proinflammatory cytokines as well as the release of granular components. Few reports concerning cell migration are available. Given that Src, a downstream target of nitric oxide (NO), led to increased FAK Pi-Tyr861 and promoted cell motility in LPS-treated macrophages [14], we wondered whether the iNOS/Src/FAK axis might also contribute to macrophage mobility in response to PAMPs other than LPS. To answer this question, PGN, polyI:C and CpG known to activate TLR2, 3, and 9 respectively, were utilized to stimulate macrophages. We observed that Src was PGN-, polyI:C- and CpG-inducible and *src* siRNA inhibited TLR2-, 3-, or 9-initiated motility, which could be recovered by ectopic avian c-Src. In addition, attenuation of FAK reduced TLR ligand-induced macrophage movement. Studies conducted with wild-type (WT) and iNOS knockout ($iNOS^{-/-}$) macrophages further supported that iNOS-dependent Src induction and FAK activation was a general mechanism in macrophage motility induced by activation of a variety of TLRs.

2. Materials and methods

2.1. Reagents and antibodies

Phosphorothioate backbone-modified oligodeoxynucleotides (S-ODN) were purchased from InvivoGen (San Diego, California, USA). The sequences of the S-ODN used were 5′-TCC ATG ACG TTC CTG ACG TT-3′ (CpG DNA, ODN1826) and 5′-TCC ATG AGC TTC CTG AGC TT-3′ (nCpG DNA, ODN1826 control). Aminoguanidine hemisulfate (AG), polyI:C and *Staphylococcus aureus* PGN were obtained from Sigma (St. Louis, Missouri, USA). Thioglycollate was obtained from Merck (Darmstadt, Germany). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo-[3,4-D]pyrimidine (PP2) was purchased from Calbiochem (La Jolla, California, USA). The primary antibodies used were actin, iNOS, FAK (Upstate); FAK Pi-Tyr861 (BIOSOURCE International); Src Pi-Tyr416 (Cell Signaling Technology); Lyn, Fgr, Hck and HRP-conjugated anti-phosphotyrosine antibody (PY20) (Santa Cruz Biotechnology). The mouse ascites containing monoclonal anti-Src (peptides 2–17) were produced by the hybridoma (CRL-2651) obtained from the American Type Culture Collection.

2.2. Animals

Rats (Sprague–Dawley) were utilized to prepare peritoneal macrophages (PEMs). C57BL/6 inducible nitric oxide synthase knockout (C57BL/6-Nos™1Lau) ($iNOS^{-/-}$) and WT mice of the same age and sex were used to assess the role of iNOS in PGN-, polyI:C-, and CpG-induced Src expression in macrophages. Mice devoid of iNOS were normal in appearance, histology, growth rate and reproduction.

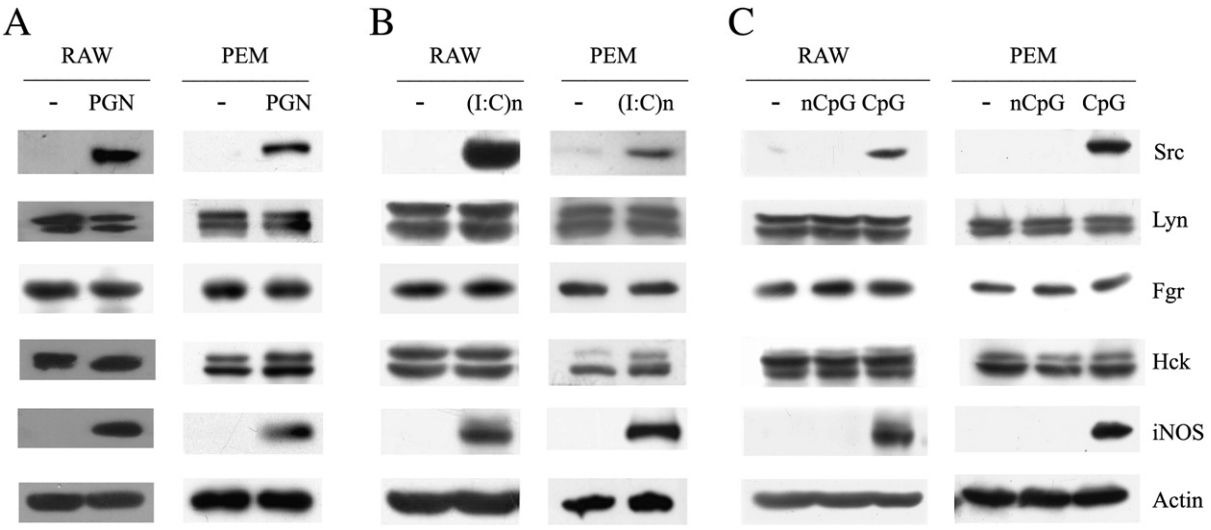

Fig. 3. PGN, polyI:C, and CpG induced the expression of Src but not its myeloid relatives in macrophages. RAW264.7 macrophages (RAW; left) and rat PEMs (right) were stimulated without or with (A) PGN (5 μg/ml), (B) polyI:C ((I:C)n; 20 μg/ml) and (C) CpG or nCpG (0.3 μg/ml) for 48 h. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. Similar results were repeated three times and the representative was demonstrated.

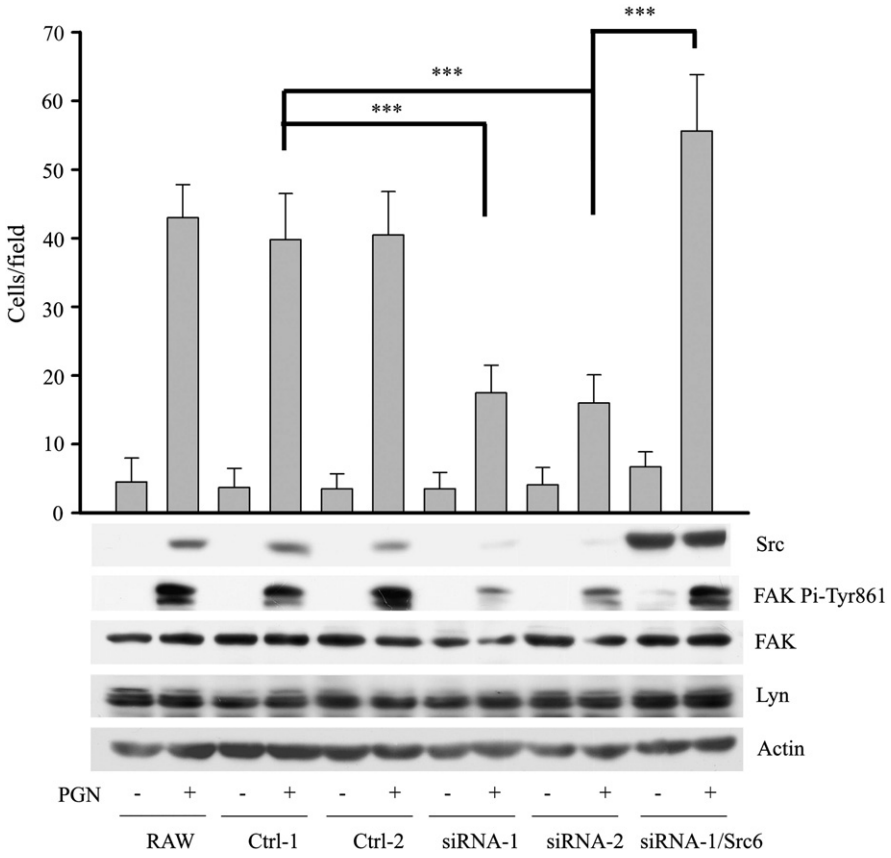

Fig. 4. Ecotopic Src restored cell migration in PGN-stimulated Src-attenuated macrophages. RAW264.7 (RAW) and its derived control cells (Ctrl-1, -2), Src-attenuated cells (siRNA-1, -2), and Src-attenuated cells harboring a plasmid encoding avian Src (siRNA-1/Src6) were stimulated without or with PGN (5 μg/ml) for 48 h. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. The motility of each group was determined as mentioned in Fig. 1. ***$p<0.001$. Similar results were repeated three times and the representative was demonstrated.

But their PEMs failed to generate NO when stimulated with LPS [30]. All experiments using laboratory animals were done in accordance with China Medical University guidelines.

2.3. Cell culture

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_2 and air. Peritoneal macrophages (PEMs) were collected by peritoneal lavage from rats (Sprague–Dawley) or wild-type and iNOS-null 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 overnight. Then the cells were washed with medium to remove nonadherent cells. And according to morphological and phagocytic criteria, the resultant macrophage monolayer was >98% pure and ready for experimentation. Bone marrow-derived macrophages (BMDMs) were collected and differentiated as described [31].

2.4. Generation of RAW264.7 macrophages expressing src siRNA

To generate cells expressing *src* siRNA (siRNA) or the non-specific siRNA (Ctrl), RAW264.7 macrophages were transfected with plasmid DNA pLKO.1-m*src* (puro) (targeted sequence: 5′-CGAGCCGCCAA-TATCCTAGTA-3′; [13]) or the negative control pSilencer plasmid (which contains sequences not present in mouse genome and provided by Ambion Inc.) by the Lipofectamine Plus method (Invitrogen) followed by puromycin or hygromycin selection. To generate Src-attenuated cells expressing ectopic Src, RAW264.7 cells expressing *src* siRNA were cotransfected with pBabe (hygro) DNA and a plasmid DNA encoding avian c-Src [32] followed by hygromycin selection.

2.5. Pretreatment with PP2

RAW264.7 cells were cultured with PP2 (10 μM) for 30 min prior to the addition of PGN, polyI:C, and CpG. These PGN-, polyI:C-, and CpG-stimulated or non-stimulated cells were then harvested and their migratory ability was determined.

2.6. Lysate preparation and immunoblot analysis

Lysis of the cells was carried out with modified radioimmune precipitation assay buffers as described before [33], and protein concentration was determined by protein assay kit (Bio-Rad). Methods for immunoblotting analysis have been described [33]. The cells lysates were resolved in an 8% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and probed with respective antibodies followed by horseradish peroxidase-conjugated protein A or horseradish peroxidase-conjugated secondary antibodies and detected by the Enhanced Chemiluminescence method (Amersham Biosciences).

2.7. Migration

The migration of macrophages exposed to PGN, polyI:C, or CpG was determined by using a modified Boyden chamber as described

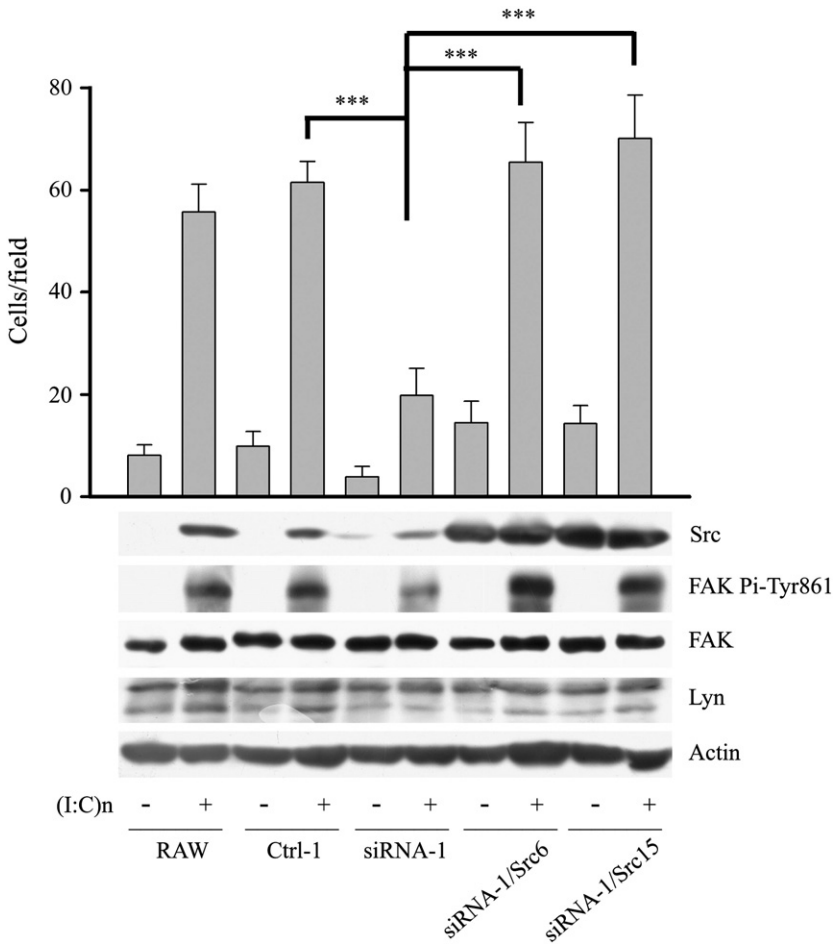

Fig. 5. Ecotopic Src restored mobility in polyI:C-exposed Src-attenuated macrophages. RAW264.7 (RAW), Ctrl-1, siRNA-1, siRNA-1/Src6 and siRNA-1/Src15 cells, as described above, were stimulated without or with polyI:C ((I:C)n, 20 μg/ml) for 48 h. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. The motility of each group was determined as mentioned in Fig. 1. ***$p<0.001$. Similar results were repeated three times and the representative was demonstrated.

before [14]. Briefly, cells without or with 48-h PGN, polyI:C, or CpG exposure were added to the upper wells (48-multiwell Boyden microchambers) at 2×10^4 cells per well. The migrated cells will traverse a polycarbonate filter (8 μm) from the upper chamber to the lower chamber, which contains 10% fetal calf serum as a chemoattractant. After 5 h at 37 °C in 5% CO_2, non-migratory cells on the upper membrane surface were removed with a cotton swab, and the cells that traversed and spread on the lower membrane surface were fixed with methanol and stained with Giemsa stain (Modified solution, Sigma). By utilizing a microscope with a 40× objective, the number of migratory cells per membrane was enumerated. Four random fields in each filter were examined. Each experiment was performed in triplicate, and migration was expressed as the mean ± S.D. of total cells counted per field. In addition, the motility of RAW264.7 and its derived Ctrl, *lyn* siRNA-1 and -2, *fak* siRNA-1 and -2 in response to LPS, PGN, poly I:C, and CpG was monitored using a Leica AS MDW system equipped with a Coolsnap HQ camera (Roper Scientific). Video images were collected at intervals of 20 min for 3 h. The positions of nuclei were tracked to quantify cell motility and analyzed with Metamorph Software (Universal Imaging Corp.).

2.8. RT-PCR

The amount of *src* and *gapdh* transcript was semi-quantitated by RT-PCR as previously described [14].

2.9. 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. Bonferronic correction was used for controlling type I error in multiple comparisons.

3. Results

3.1. PGN, polyI:C and CpG enhance cell motility of macrophages

Previously, we have demonstrated that iNOS-dependent Src induction and FAK activation are involved in TLR4-mediated macrophage migration [14]. In this study, we were interested to know whether a similar mechanism could also be applied to TLR2-, TLR3- and TLR9-elicited macrophage movement. To address this issue, PGN, polyI:C, and CpG were utilized to individually activate TLR2, TLR3 and TLR9 in macrophages. And nCpG was used as a negative control for CpG [34]. Compared to non-stimulated and nCpG-stimulated RAW264.7 macrophages, significantly increased migration was detected in cells treated with PGN (Fig. 1A, left), polyI:C (Fig. 1B, left) or CpG (Fig. 1C, left). To confirm that TLR2-, TLR3- and TLR9-evoked macrophage mobilizations are a physiological event, thioglycollate-elicited rat PEMs were harvested and their migratory potential in response to these agonists was assessed. Indeed, PEMs exposed to PGN (Fig. 1A, right), polyI:C

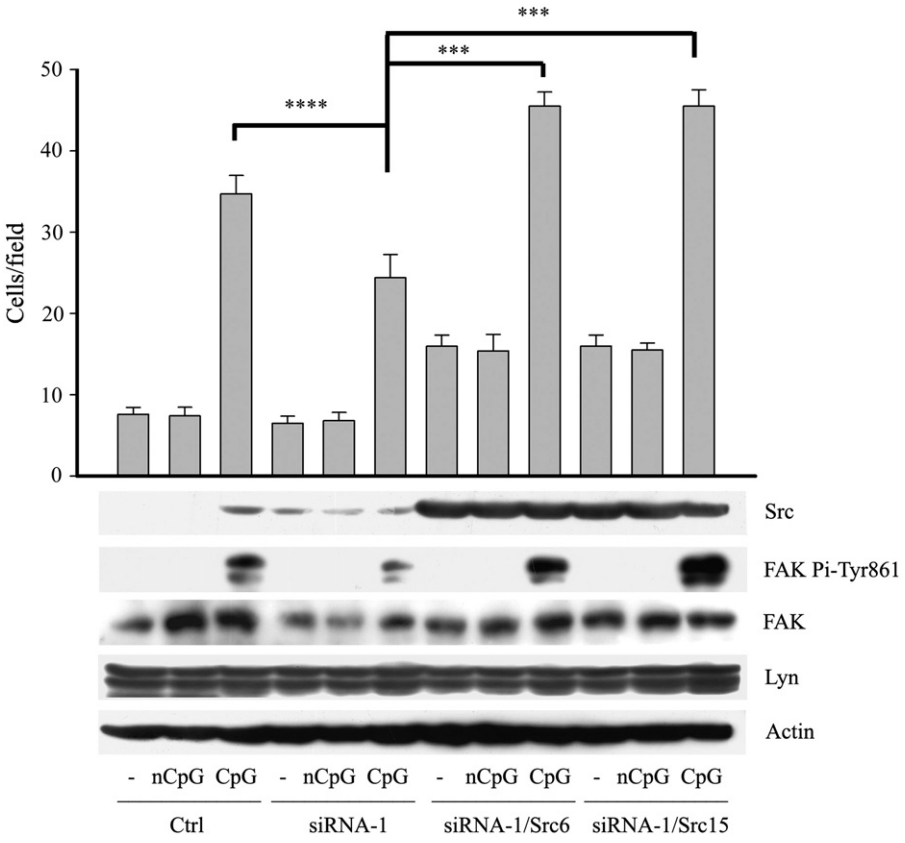

Fig. 6. Ecotopic Src restored motility in CpG-stimulated Src-attenuated macrophages. RAW264.7-derived control Ctrl, siRNA-1, siRNA-1/Src6 and siRNA-1/Src15 cells were stimulated without or with CpG or nCpG (0.3 μg/ml) for 48 h. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. The motility of each group was determined as mentioned in Fig. 1. ***$p<0.001$. Similar results were repeated three times and the representative was demonstrated.

(Fig. 1B, right) or CpG (Fig. 1C, right) exhibited an increased migratory potential as compared to those without treatment or treated with nCpG. Similar results were observed in BMDMs stimulated with LPS and the other three TLR agonists (Fig. 2). Intriguingly, this PGN- (Fig. 1A, left), polyI:C- (Fig. 1B, left), or CpG-elicited macrophage motility (Fig. 1C, left) was sensitive to PP2 (an SFK inhibitor), implicating the role of SFKs in this process.

3.2. PGN, polyI:C, and CpG induce Src expression in macrophages

To pinpoint the SFK(s) responsible for PGN-, polyI:C-, and CpG-triggered migration, the expression of Src and its myeloid relatives (i.e. Lyn, Fgr and Hck) in RAW264.7 cells without or with stimulation of each ligand was examined. While the expression of the myeloid SFKs was almost unaltered, significant Src induction was observed in RAW264.7 cells exposed to PGN (Fig. 3A), polyI:C (Fig. 3B) or CpG (Fig. 3C). To exclude the possibility that PGN-, polyI:C-, and CpG-elevated Src expression was an in vitro artifact that only occurred in RAW264.7, PEMs and BMDMs were utilized to answer this question. As shown in Fig. 3, while the expression of Lyn, Fgr, and Hck was unchanged, Src induction was detected in PEMs after a 48-h exposure of PGN, polyI:C, and CpG. Similar findings were also obtained in BMDMs (Fig. 2). These results confirmed that PGN-, polyI:C-, and CpG-mediated Src enhancement was a *bona-fide* physiological event occurred in macrophages.

3.3. Src-mediated FAK Pi-Tyr861 in PGN-, polyI:C-, and CpG-stimulated macrophages

Following exposure to various TLR ligands, the simultaneous increase of Src expression and macrophage migration suggested the importance of Src in this process. To prove this, non-specific and *src*-specific siRNA-bearing plasmids were introduced into RAW264.7 macrophages to obtain the corresponding control (Ctrl-1 and -2) and siRNA (siRNA-1 and -2) cells, respectively. Significant Src induction was observed in parental and control cells, but not in *src* siRNA-expressing RAW264.7 cells following stimulation with PGN (Fig. 4), polyI:C (Fig. 5), or CpG (Fig. 6). Notably, like parental and control cells, the expression of Lyn was almost unaltered in Src-attenuated cells in the presence or absence of PGN (Fig. 4), polyI:C (Fig. 5), and CpG (Fig. 6). Analysis of cell motility by transwell assay revealed that PGN-, polyI:C-, and CpG-induced cell mobility was significantly suppressed in Src-attenuated RAW264.7. To verify these results, we further generated Src-attenuated cells expressing avian Src (siRNA-1/Src6, siRNA-1/Src15) [14] and demonstrated that reduced PGN-, polyI:C-, and CpG-elicited macrophage mobilization could be restored by ectopic Src (Figs. 4, 5 and 6). Since FAK is a substrate of Src and regulates macrophage motility [29], therefore, its activity (as reflected by FAK Pi-Tyr861) in these cells treated with PGN, polyI:C, or CpG was examined. Markedly, PGN (Fig. 4), polyI:C (Fig. 5), or CpG (Fig. 6) increased FAK Pi-Tyr861 in RAW264.7, which was greatly suppressed by Src deprivation and was restored by Src reintroduction.

3.4. Attenuation of Lyn does not suppress LPS-, PGN-, polyI:C-, and CpG-mediated macrophage migration

Given the almost unaltered expression of myeloid SFKs in macrophages exposed to various TLR ligands, their involvement in mobility was attempted. Among the three Src relatives, we chose Lyn to address this issue. Nonspecific and two different *lyn*-specific siRNA-bearing plasmids were introduced into RAW264.7 macrophages to obtain the corresponding control (Ctrl) and siRNA (*lyn* siRNA-1 and -2) cells,

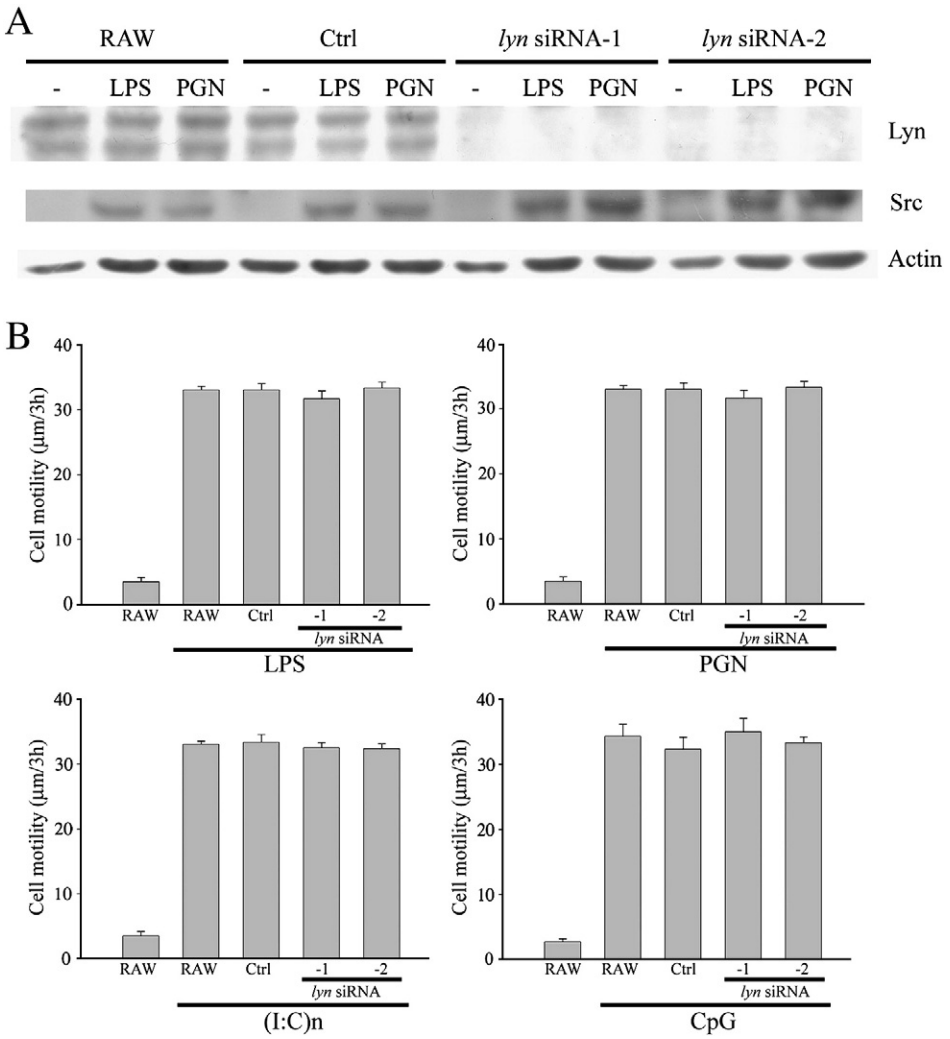

Fig. 7. Attenuation of Lyn does not impair LPS-, PGN-, polyI:C-, and CpG-induced macrophage migration. (A) The generation of Lyn-attenuated RAW264.7 (RAW) cells were performed exactly as the generation of Src knockdown cells except that plasmid DNA pLKO.1-mlyn-1 (puro) (targeted sequence: 5′-CGCGAGAGTCATCGAAGATAA-3′) and plasmid DNA pLKO.1-mlyn-2 (puro) (targeted sequence: 5′-GCCAAGGTCAACACCTTAGAA-3′) were transfected to generate *lyn* siRNA-1 cells and *lyn* siRNA-2 cells, respectively. RAW264.7 and its derived control cells (Ctrl) and Lyn-attenuated cells (*lyn* siRNA-1 and -2) were stimulated without or with either LPS (100 ng/ml) or PGN (5 μg/ml) for 48 h. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. (B) RAW264.7 and its derived cells as described above were stimulated without or with LPS, PGN, polyI:C, and CpG for 48 h. Movement of these cells were monitored by time-lapse video microscopy and tracked for 20-min intervals over a span of 3 h. Motility was calculated as velocity (μm/3 h) using image processing software as described under "Materials and methods." Data from these fields of cells (>20 cells/field) were collected and calculated in each cell line, and the results were means ± S.D. of a representative experiment.

respectively. Despite that the expression of Lyn was reduced in siRNA cells, still like parental and Ctrl cells, their Src expression was greatly augmented in response to LPS, PGN, polyI:C, and CpG (Fig. 7A and data not shown). Increased motility was also observed in Lyn-attenuated macrophages exposed to all these ligands (Fig. 7B). These findings suggested that Lyn did not contribute to TLR-mediated macrophage movement.

3.5. Attenuation of FAK reduces LPS-, PGN-, polyI:C-, and CpG-induced macrophage migration

To ascertain that FAK is critical in macrophage mobilization, non-specific and two different *fak*-specific siRNA-bearing plasmids were introduced into RAW264.7 to obtain the corresponding control (Ctrl) and siRNA (*fak* siRNA-1 and -2) cells, respectively. As shown in Fig. 8, attenuation of FAK impaired macrophage mobility in response to LPS, PGN, polyI:C, and CpG. These results confirmed that Src/FAK axis played a pivotal role in various TLR ligand-elicited macrophage mobilization.

3.6. iNOS is required for PGN-, polyI:C-, and CpG-induced macrophage motility

Given that iNOS participated in LPS-mediated macrophage migration [14], therefore, we analyzed the migratory potential of PGN-, polyI:C- and CpG-treated PEMs derived from WT and iNOS$^{-/-}$ mice. Compared to WT PEMs, reduced migration evoked by PGN (Fig. 9A), polyI:C (Fig. 9B), and CpG (Fig. 9C) was observed in iNOS-null PEMs. These data indicated the involvement of iNOS in macrophage movement initiated by these TLR ligands.

3.7. PGN-, polyI:C- and CpG-elicited Src induction and FAK activation is iNOS-dependent

By modulating Src/FAK pathway, iNOS is involved in LPS-mediated macrophage mobility [14]. To study whether a similar mechanism can also be utilized by macrophages exposed to PGN, polyI:C, or CpG, cell lysates prepared from WT and iNOS$^{-/-}$ PEMs treated without or with the respective ligand were collected and analyzed. Western blot analysis revealed that concurrent with the absence of iNOS,

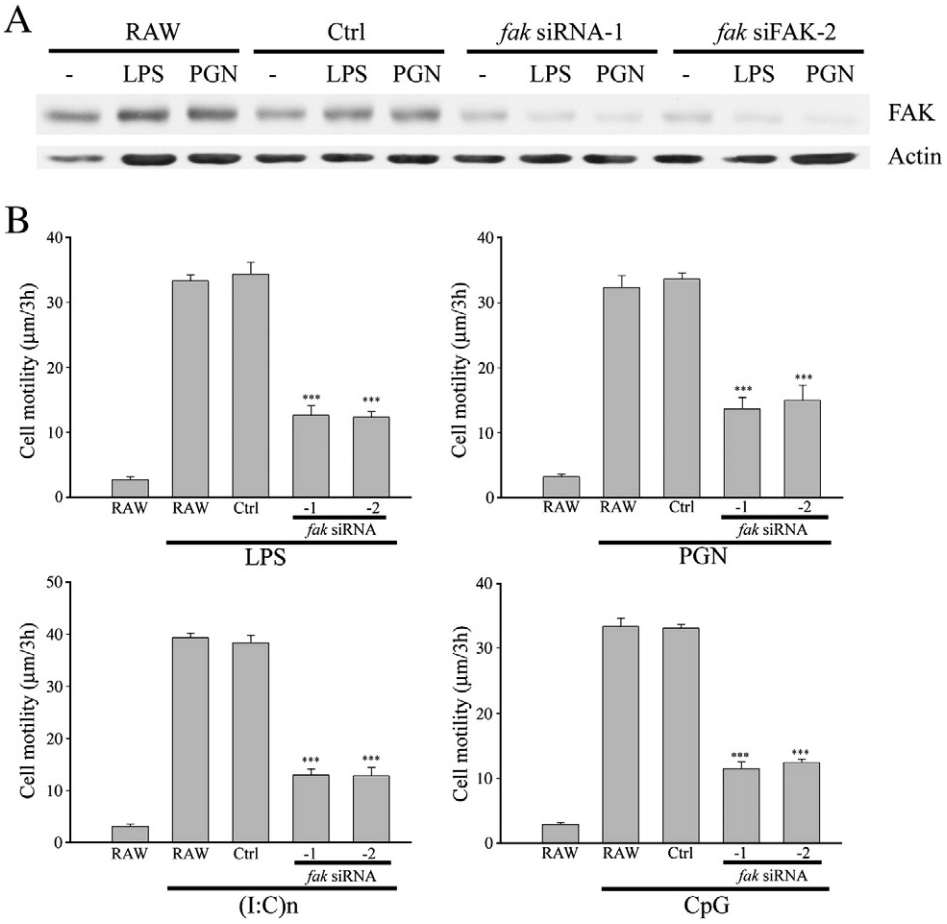

Fig. 8. FAK knockdown decreases LPS-, PGN-, polyI:C-, and CpG-induced macrophage migration. (A) Plasmid DNA pLKO.1-mfak-1 (puro) (targeted sequence: 5′-GCCTTAACAATGCGT-CAGTTT-3′) and pLKO.1-mfak-2 (puro) (targeted sequence: 5′-CCTGGCATCTTTGATATTATA-3′) were used to generate *fak* siRNA-1 and *fak* siRNA-2 (FAK-attenuated cells), respectively. RAW264.7 (RAW) and its derived control cells (Ctrl) and FAK-attenuated cells (*fak* siRNA-1 and -2) were stimulated without or with either LPS (100 ng/ml) or PGN (5 μg/ml) for 48 h. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. (B) RAW264.7 and its derived cells as described above were stimulated without or with LPS, PGN, polyI:C, and CpG for 48 h. The motility of these cells was measured exactly as described in Fig. 7. ***$p<0.001$. The results were means ± S.D. of a representative experiment.

dramatically reduced expression of Src (but not its myeloid-specific relatives) was detected in PGN- (Fig. 10, left), polyI:C- (Fig. 10, middle), and CpG- (Fig. 10, right) stimulated iNOS$^{-/-}$ PEMs. Besides, iNOS deficiency not only suppressed Src activation (as reflected by Src Pi-Tyr416), but also ablated PGN-, polyI:C-, and CpG-elicited FAK Pi-Tyr861 (Fig. 10). Consistently, aminoguanidine hemisulfate (AG, an iNOS inhibitor) treatment led to the decrement of protein and RNA transcript of Src evoked by TLR ligands (Fig. 11). These results unveiled that by virtue of Src induction and FAK activation, iNOS was crucial in PGN-, polyI:C-, and CpG-induced macrophage locomotion.

4. Discussion

Src was important in LPS-mediated macrophage motility [14]. To investigate whether Src was also critical in macrophage movement in response to other PAMPs, PGN, polyI:C, and CpG were chosen to treat macrophages in this study. Here, we observed that PGN, polyI:C, and CpG increased cell migration in RAW264.7 cells, PEMs (Fig. 1), and BMDMs (Fig. 2). The greatly reduced motility by PP2 implicated the involvement of SFKs in PGN-, polyI:C-, and CpG-triggered movement. Like LPS treatment, the inducible characteristic of Src could be detected in RAW264.7, PEMs (Fig. 3), and BMDMs (Fig. 2) exposed to these TLR agonists. Notably, Src attenuation led to reduced PGN- (Fig. 4), polyI:C- (Fig. 5), and CpG- (Fig. 6) evoked motility in RAW264.7, and ectopically expressed avian Src rescued this defect (Figs. 4, 5 and 6). These findings substantiated that Src was involved in macrophage mobilization induced by PGN, polyI:C, and CpG.

For a long period of time, FAK was thought to regulate focal adhesion turnover and mobility in fibroblasts [17,18,35]. Its involvement in macrophage migration was vague. Recently, Owen et al. have generated myeloid-specific conditional FAK knockout mice and demonstrated that PEMs derived from these mice displayed significant migration defects, which was coincident with suppressed adhesion turnover and formation of stable lamellipodia necessary for directional locomotion [29]. Meanwhile, FAK deficiency also led to decreased infiltration of monocytes into sites of inflammation in vivo. Intriguingly, sustained FAK activation that perturbed cytoskeletal disassembly was reported to be responsible for oxidized LDL-induced macrophage trapping in atheroinflammatory lesions [36]. As the role of FAK in macrophage mobilization was fully disclosed, we demonstrated that Src could mediate FAK Pi-Tyr861, an indicator of FAK activation [24], to contribute to enhanced movement in PGN-, polyI:C-, and CpG-treated RAW264.7 (Figs. 4, 5, and 6). In agreement with this notion, reduced migration was detected in FAK-attenuated RAW264.7 exposed to various TLR ligands (Fig. 8).

Due to its low basal expression in macrophages, the physiological significance of Src is concealed and underestimated. In contrast, the three myeloid Src relatives (i.e. Fgr, Hck, and Lyn) with high protein expression are spotlighted. As the expression of Src was inducible by LPS [14,37] as well as other TLR ligands (Figs. 2 and 3), Src was confirmed to

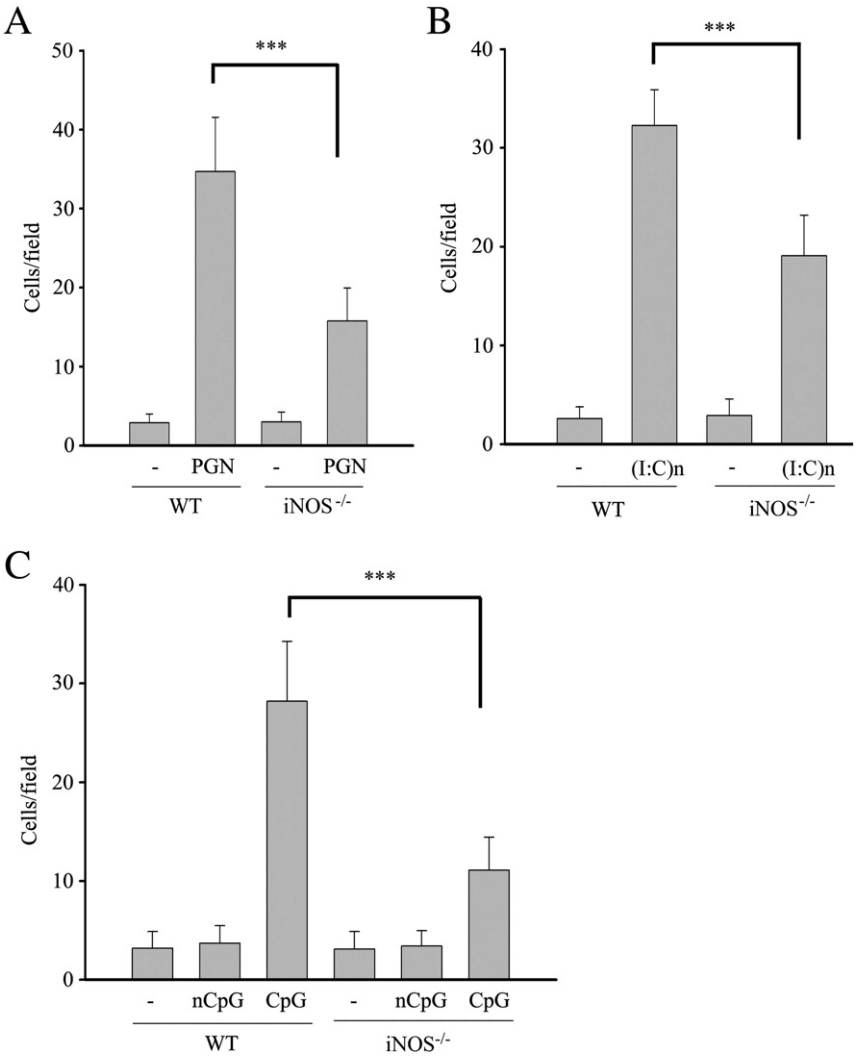

Fig. 9. iNOS is required for PGN-, polyI:C- and CpG-induced macrophage migration. PEMs from wild type (WT) and iNOS$^{-/-}$ mice were stimulated without or with (A) PGN (5 μg/ml), (B) polyI:C ((I:C)n, 20 μg/ml), and (C) nCpG or CpG (0.3 μg/ml) for 48 h. Then, the motility of PEMs (2×10^4 cells) harvested from each group was determined by a modified Boyden chamber as described under "Materials and methods." ***, $p<0.001$. Similar results were repeated three times and the representative was demonstrated.

participate in macrophage migration elicited by activation of various TLRs. However, the role of myeloid SFKs in this process was unclear. Here, with Lyn-attenuated RAW264.7, we presented evidence to exclude its participation in macrophage migration elicited by various TLR ligands (Fig. 7).

A migration-stimulatory role of NO has been reported in diverse cell types. For instance, migratory potential of primary aortic smooth muscle cells was augmented by NO [38]. Also, NO was critical for a switch from stationary to locomoting phenotype in epithelial cells [39]. Furthermore, NO played a permissive role in endothelial cell migration induced by substance P [40], endothelin [41], and vascular endothelial growth factor [42]. Recently, we reported that LPS-mediated Src induction led to increased FAK activation and cell migration in macrophages, and which was iNOS-dependent [14]. Herein, studies conducted with WT and iNOS$^{-/-}$ macrophages provided further definitive evidence regarding the role of iNOS in the concomitant induction of Src, activation of FAK (Fig. 10) and cell movement (Fig. 9) in macrophages exposed to PGN, polyI:C, and CpG. Of note, iNOS inhibitor treatment led to the decrement of protein and RNA transcript of *src* induced by TLR ligands (Fig. 11). It is well established that soluble guanylyl cyclase (sGC) is a main target of NO, and activation of sGC can convert GTP to cGMP, an intracellular second messenger. Given (1) LPS-mediated Src induction could be impaired by ODQ (an inhibitor of sGC), (2) 8-bromo-cGMP (8-Br-cGMP, a cGMP analogue) could induce Src expression, and (3) the expression of Src in iNOS-null PEMs could be restored by SNAP (a NO donor) and 8-Br-cGMP to a level comparable to that detected in normal PEMs [14], we concluded that NO/cGMP pathway could contribute to Src induction by TLR ligands.

Due to residual Src expression and activity were still detected in iNOS-null macrophages exposed to various TLR ligands (Fig. 10), therefore iNOS/NO should not be the only signaling pathway leading to Src induction. Besides, in addition to SFKs, PP2 was also reported to inhibit other protein kinases including Abl [43] involved in LPS-mediated macrophage activation [44]. Because PP2 exerted more inhibitory effect than iNOS absence did, thereby, in addition to Src, other PP2-sensitive tyrosine kinase(s) might also be involved in TLR-mediated cell motility.

In contrast to the large repertoire of rearranged receptors utilized by the acquired system, the innate immunity recognizes microorganisms via a limited number of germline-encoded pattern-recognition receptors including TLRs [4]. Irrespective of their localization and utilization of different TIR-containing adaptors, engagement of TLRs led to activation of NF-κB and augmented expression of iNOS, COX-2, and proinflammatory cytokines [6]. As PGN, polyI:C, and CpG were used as stimulants in this study, we actually analyzed TLRs located on plasma membrane (i.e. TLR2) vs. endosome (i.e. TLR3 and 9). Besides, the signaling pathways of the TLRs studied could be divided into MyD88-dependent (i.e. TLR2 and 9) and -independent (i.e. TLR3).

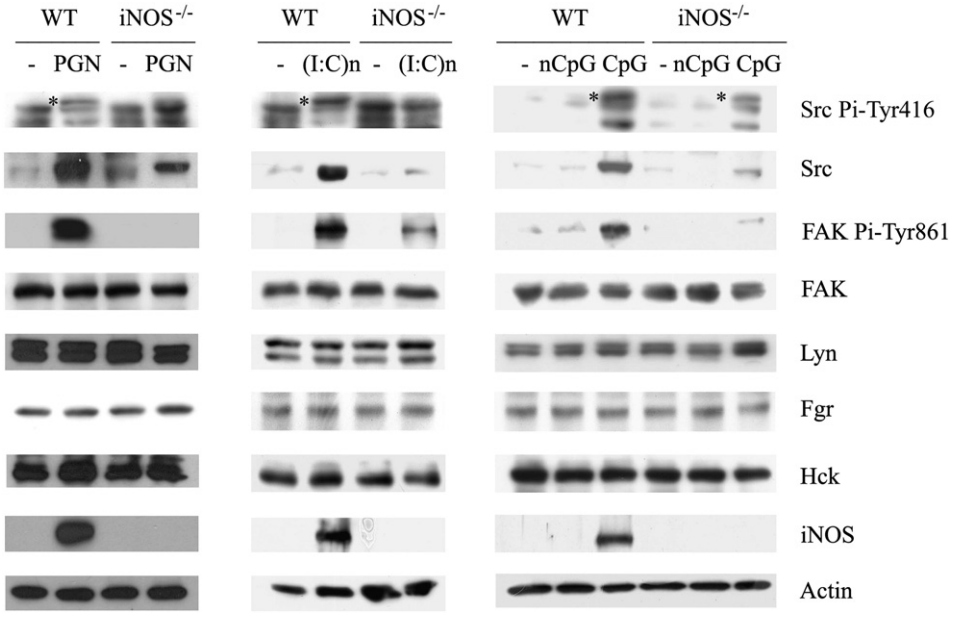

Fig. 10. iNOS was required for PGN-, polyI:C-, and CpG-evoked Src expression and FAK activation. PEMs from wild type (WT) and iNOS$^{-/-}$ mice were stimulated without or with PGN (5 μg/ml) (left), polyI:C ((I:C)n, 20 μg/ml) (middle), and nCpG or CpG (0.3 μg/ml) (right) for 48 h. Equal amounts of their lysates (30 μg) were resolved by SDS-PAGE and probed with antibodies as indicated. Asterisks marked the position of Src Pi-Tyr416. Similar results were repeated three times and the representative was demonstrated.

Intriguingly, engagement of all these three TLRs, like that of TLR4 [14], led to Src upregulation and FAK activation that were iNOS-dependent. Given that Src increased the activity of NF-κB [45,46], thereby a loop of signal amplification can be established. With what we have learned from studies conducted in RAW264.7 macrophages as well as normal vs. iNOS$^{-/-}$ PEMs, a simple model illustrating the responsible mechanism for TLR ligand-triggered, iNOS-dependent macrophage migration was proposed in Fig. 12. Given that mobilization of activated macrophages from the infection area to the regional lymph node where they present antigens to naïve CD4^{+} T cells is an indispensable step in activating the adaptive immunity [4], we speculate that the iNOS → Src → FAK signaling pathway observed in this report is likely to be a general mechanism utilized by TLR-engaged macrophages critical in host defenses.

In conclusion, we demonstrate that PGN, polyI:C, and CpG can elicit macrophage migration via their respective TLR and iNOS. Src is a critical player herein since its upregulation and activation mediated by NO results in augmented FAK activity and cell migration. Considering that the accumulation of macrophages contributes to the progression of diseases such as cancer and chronic inflammatory diseases, our data highlight the potential of iNOS, Src, and FAK to be therapeutic targets. By suppressing their activity, we might control infection, inflammation, and the maintenance of tissue homeostasis.

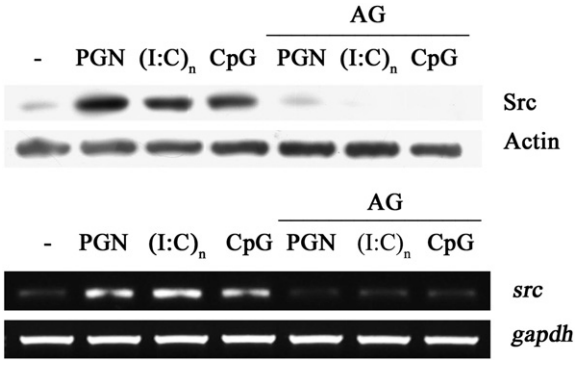

Fig. 11. iNOS inhibitor reduces both protein and RNA transcript of *src* in macrophages exposed to PGN, polyI:C, and CpG. RAW264.7 cells were pretreated without or with AG (2 mM) for 30 min, and then stimulated with PGN, polyI:C, and CpG for 48 h. Equal amounts of lysates from each sample were resolved by SDS-PAGE and probed with antibodies as indicated (upper panel). The amount of *src* transcript was analyzed by RT-PCR. *gapdh* was utilized as an internal control for amplification efficiency (bottom panel).

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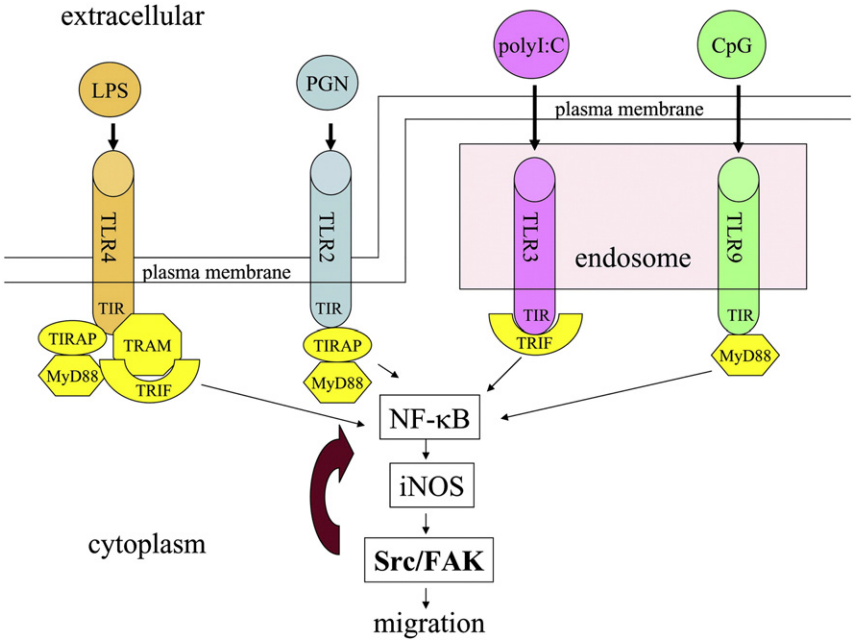

Fig. 12. Signal transduction pathways of diverse TLR-mediated macrophage migration. As LPS activates TLR4, PGN, polyI:C and CpG triggers the activation of TLR2, 3 and 9, respectively, which induces the expression of iNOS via activation of NF-κB. NO, the second messenger generated by iNOS, upregulates the expression and activity of Src, leading to FAK activation (as reflected by its Pi-Tyr861) and cell movement. Notably, Src increases the activity of NF-κB, thus a loop of signal amplification for macrophage movement can be constructed. This model highlights a general mechanism utilized in macrophages when their TLRs are engaged.

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