TOLL-LIKE RECEPTOR 2 AND 4 ACTIVATE STAT1 SERINE PHOSPHORYLATION
BY DISTINCT MECHANISMS IN MACROPHAGES

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Running Title: TLR signaling induces STAT1 serine phosphorylation
SUMMARY

Engagement of Toll-like receptor (TLR) proteins activates multiple signal transduction pathways. These studies show that engagement of TLR2 and TLR4 leads to rapid phosphorylation of the transcription factor STAT1 at serine 727 (S(727) STAT1) in murine macrophages. Only TLR4 engagement induced STAT1 phosphorylation at tyrosine 701, although this response was delayed compared to S(727) STAT1 phosphorylation. Inhibition of phosphatidylinositol-3’-kinase using LY294002 blocked TLR4-induced STAT1 tyrosine phosphorylation, but this inhibitor had no effect on STAT1 serine phosphorylation. TLR-induced phosphorylation of S(727) STAT1 could be blocked by the selective p38 MAP kinase inhibitor SB203580. However, activation of p38 was not sufficient to induce S(727) STAT1 phosphorylation in macrophages. TLR2-induced activation of S(727) STAT1 phosphorylation required the adapter protein MyD88, whereas TLR4-induced activation of S(727) STAT1 phosphorylation was not solely dependent on MyD88. Lastly, TLR4-induced activation of S(727) STAT1 phosphorylation could be blocked by rotterlin, a specific inhibitor of protein kinase C δ (PKC-δ). In contrast, rotterlin had no effect on STAT1 phosphorylation induced via TLR2. Together, these data demonstrate that activation STAT1 tyrosine and serine phosphorylation are distinct consequences of TLR engagement in murine macrophages. Furthermore, p38 MAP kinase, PKC-δ, and a novel TLR2-specific signaling pathway appear to be necessary to induce S(727) STAT1 phosphorylation.
INTRODUCTION

Mammalian Toll-like receptors (TLRs) are type I transmembrane receptor that are composed of an extracellular leucine-rich repeat (LRR) domain and a highly conserved cytoplasmic Toll/IL-1R (TIR) domain (1). These receptors are expressed on a variety of cell types, including dendritic cells, macrophages, endothelial cells, lymphocytes, and epithelial cells. TLR proteins are primary signal-transducing molecules responsible for recognizing specific microbial pathogen-associated molecular patterns (PAMPs), including Gram-negative bacterial lipopolysaccharide (LPS) (2). A variety of diverse chemical structures have been identified for most of the ten known TLR proteins (reviewed in 3). The activation of TLR proteins is believed to give rise to patterns of gene expression that are necessary to initiate both innate and adaptive immunity (4, 5). TLR agonists are known to activate multiple signal transduction pathways simultaneously in target cells. These signaling events include activation of the transcription factors NF-κB and AP-1, the MAP kinases, protein kinase C isoforms, and the lipid kinase phosphatidylinositol-3’-kinase (PI-3K) (6-9).

Another early signaling event in LPS stimulated macrophages is the activation of the transcription factor STAT1 (Signal Transducer and Activator of Transcription 1). Various cytokine receptors exploit STAT proteins to transduce ligand-induced signaling. The type I interferons (IFNα/β) utilize STAT1 and STAT2 to transduce intracellular signals generated following engagement of a heterodimeric receptor complex consisting of the subunit chains, IFNAR-1 and IFNAR-2 (10, 11). IFNα/β receptor results in the cross-activation of the two receptor-associated Janus protein tyrosine kinases (Jaks), Tyk2 and Jak1 respectively. Thereby, activated Tyks and Jaks lead to the phosphorylation on Y(701) residue in STAT1 and STAT2, resulting in homodimeric (STAT1/STAT1), heterodimeric (STAT1/STAT2) or heterotrimeric (STAT1/STAT2/IRF-9) protein complexes. These multimeric complexes translocate to the nucleus where they bind to distinct DNA elements, finally leading to the activation of IFN-inducible gene expression (12, 13). Phosphorylation of Y(701) alone is sufficient to generate STAT multimers that possess DNA binding activity (15), although phosphorylation of S(727) is required for maximal transcriptional activity of STAT1 (reviewed in 14). However, the signaling mechanisms leading to STAT1 S(727) phosphorylation are not well understood. Several studies
have reported that the p38 MAP kinase is necessary for STAT1 phosphorylation at S(727), but it is likely that STAT1 is not a substrate for p38 in living cells (14).

The studies presented below show that LPS induced TLR4-dependent phosphorylation of STAT1 at both tyrosine and serine residues in murine macrophages. LPS-induced STAT1 Y(701) phosphorylation was mediated by a PI-3K-dependent mechanism, whereas STAT1 S(727) phosphorylation was PI-3K-independent. In contrast, macrophage activation via TLR2 induced phosphorylation of S(727), but not of Y(701), on STAT1. Furthermore, the adapter protein MyD88 was found to be necessary for STAT1 serine phosphorylation via TLR2, but not via TLR4. The p38 MAP kinase was necessary, but was not sufficient for TLR-dependent activation of STAT1 Ser(727) phosphorylation. Moreover, specific inhibitors of PKC-δ were found to block STAT1 Ser(727) phosphorylation induced via TLR4, but not via TLR2. These studies revealed a novel difference in the mechanism of STAT1 phosphorylation induced by engagement of TLR2 and TLR4.
EXPERIMENTAL PROCEDURES

Sources of macrophages
LPS-hyporesponsive C3H/HeJ mice and normal C3H/OuJ mice were purchased from Jackson Laboratories (Bar Harbor ME). TLR2−/− and MyD88−/− mice were provided by Dr. Shuzio Akira (University of Osaka Medical School, Osaka) and have been described previously (16). These mice were backcrossed into a C57BL/6 background for 4 generations prior to use. C57BL/6 mice from Jackson Laboratories were used as controls for the TLR2-deficient mice. Primary peritoneal macrophages were prepared from these mice using thioglycollate elicitation, as previously described (17). The murine macrophage RAW264.7 cell line (American Type Culture Collection, Mananas VA, ATCC TIB-71) were cultured in LPS-free Dulbecco’s modified Eagle’s medium containing 10% (v/v) heat-inactivated fetal bovine serum, 1% L-glutamine, and 10 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Grand Island, NY) at 37 °C in air supplemented with 5 % CO2.

Plasmids and reagents
The constitutively active form of murine TLR4 was previously described (6). The dominant-negative p85 expression plasmid was kindly provided by Dr. Julian Downward (Imperial Cancer Research Fund, London), and was previously described (18). The PKC-δ dominant-negative expression plasmid was provided by Dr. Michael Simons (Dartmouth Medical School), and was previously described (19). The human IFN-β promoter luciferase reporter plasmid was provided by Dr. John Hiscott (McGill University, Montreal), and was previously described (20). The murine COX2 promoter luciferase reporter plasmid was provided by Dr. Daniel Hwang (University of California, Davis), and was also previously described (6). All plasmids were prepared using the EndoFree plasmid kit as recommended by the manufacturer (Qaigen, Valencia, CA). Highly purified protein-free E. coli K235 LPS was prepared as decribed by Hirschfeld et al. (21). The synthetic lipopeptide Pam3Cys (S-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys4-OH trihydrochloride) was from EMC Microcollections GmbH (Tubingen, Germany). LY294002 was from Sigma (St. Louis, MO). Bisindoylmaleamide, rottlerin, and SB203580 were purchased from Calbiochem (La Jolla, CA). Recombinant murine IFN-γ was purchased from R&D Systems (Minneapolis, MN) and
recombinant murine IL-1β was purchased from Peprotech (Rocky Hill, NJ). Antibodies against Akt, S(473)-phosphorylated Akt, STAT1, Y(701)-phosphorylated STAT1, and phosphorylated MAP kinase p38 were from Cell Signaling Technology (Beverly, MA). The antibody recognizing S(727)-phosphorylated STAT1 was from Upstate Biotechnology (Lake Placid, NY).

**Transfection and luciferase reporter assays**

RAW264.7 cells were plated in six-well plates (1.2 x 10^6 cells/well) and transfected with the appropriate plasmid DNA, including a β-galactosidase expression plasmid (HSP70-β-gal) as an internal control, using SuperFect transfect reagent (Qiagen) according to the manufacturer’s instruction. One day after transfection, relative luciferase activity was determined by normalization with β-galactosidase activity as previously described (6, 22). All assay were performed in triplicate, and a single representative experiment is shown. Data are expressed as mean values ± SEM.

**Western blot analysis**

Cells were harvested and washed once with PBS (pH 7.5), and then lysed for 30 min on ice in lysis buffer (150 mM NaCl, 50 mM Tris-Cl [pH 8.0], 5 mM EDTA, 1% Nonidet P-40) with protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma). Cell lysates were clarified by centrifugation at 4 °C for 10 min at 12,000 x g. Protein concentrations of the lysate were measured by Bradford method (Bio-Rad, Hercules, CA) and equal amount of total protein per lane was fractionated on 10% SDS-polyacrylamide gel using Laemmli sample buffer (23). Gels were transferred to polyvinylidene difluoride membranes. The membranes were blocked with TBS containing 0.05% Tween-20 and 5% nonfat dry milk, and then incubated with the indicated antibodies and an appropriate HRP-conjugated secondary antibody as described in elsewhere (6). Bound antibodies were visualized using the enhanced chemiluminescence system (Pierce, Rockford, IL).

**RT-PCR**

Semi-quantitative RT-PCR amplification of total RNA was performed as previously described (20). The oligonucleotide primers used for amplification of the murine IFN-β PCR product were: 5’-TCCAAGAAAGACGACATTCG-3’ and 5’-TGAGGACATCTCCCACGTCA-3’
(annealing temperature, 55 °C).
RESULTS

TLR engagement leads to phosphorylation of STAT1

Previous reports have demonstrated that TLR signaling leads to the activation of MAP kinases and the transcription factors NF-κB and AP-1 in macrophages (6, 7). Subsequent studies sought to determine whether engagement of TLR proteins also leads to the activation of the transcription factor STAT1. RAW264.7 murine macrophages were stimulated with the TLR4 agonist *E. coli* LPS, or with the synthetic lipopeptide TLR2 agonist Pam3Cys, for various times. Whole cell lysates were prepared, and lysates were then analyzed by western blotting. STAT1 activation was measured using specific antibodies that discriminate between STAT1 phosphorylated at serine 727 and tyrosine 701. As shown in Figure 1A, *E. coli* LPS was capable of inducing the phosphorylation of STAT1 at both serine and tyrosine residues. Pam3Cys was capable of inducing STAT1 S(727) phosphorylation with similar kinetics to LPS (Fig. 1B), but this TLR2 agonist was incapable of inducing STAT1 tyrosine phosphorylation (data not shown). The inability of TLR2 agonists to induce STAT1 tyrosine phosphorylation has been previously reported, and is due to the inability of TLR2 agonists to induce IFN-β secretion and subsequent engagement of the type I IFN receptor (20).

The kinetics of STAT1 serine phosphorylation were rapid and sustained, with maximal serine phosphorylation occurring in less than 30 min, whereas STAT1 tyrosine phosphorylation was both delayed and transient. Two distinct species of tyrosine-phosphorylated STAT1 were observed, corresponding to STAT1α (92 kDa) and STAT1β (84 kDa). A single species of serine-phosphorylated STAT1 was observed, corresponding to STAT1α. The STAT1β splice variant lacks the C-terminal serine phosphorylation site present in STAT1α. In the figures, this serine phosphorylated form of STAT1α will simply be referred to as P-S(727)STAT1. STAT1 tyrosine phosphorylation was first observed approximately 2 hr after LPS stimulation, as previously reported (20, and references therein), and was substantially diminished by 10 hr after LPS stimulation. This reduction in STAT1 tyrosine phosphorylation coincided with an overall increase in total STAT1 levels, and may reflect an overall increase in the total cellular content of STAT1. Alternatively, transient STAT1 tyrosine phosphorylation may reflect the action of protein tyrosine phosphatases. These data reveal that engagement of TLR2 and TLR4 leads to...
the rapid serine phosphorylation of STAT1. Moreover, the distinct kinetics of STAT1 serine and tyrosine phosphorylation suggests that these events are consequences of distinct signal transduction pathways.

To determine whether TLR4 was necessary for the activation of STAT1 serine phosphorylation, peritoneal macrophages from normal C3H/OuJ and TLR4-mutant C3H/HeJ mice were stimulated in vitro with E. coli LPS for 20 and 40 min. STAT1 serine phosphorylation was measured using western blotting, as described above. As shown in Figure 2A, STAT1 serine phosphorylation was strongly induced in the C3H/OuJ macrophages. In contrast, no induction of STAT1 S(727) phosphorylation was observed in the TLR4-mutant macrophages. Our published studies have previously shown that TLR4 was also necessary for LPS-induced STAT1 tyrosine phosphorylation (20). In parallel studies, Pam3Cys was found to induce rapid STAT1 serine phosphorylation in macrophages from normal C57BL/6 mice, but not from TLR2-/- mice (Figure 2B). Together, these findings demonstrate that TLR2 and 4 are necessary for STAT1 activation by Pam3Cys and E. coli LPS, respectively. Lastly, STAT1 serine phosphorylation could still be rapidly induced in the TLR4-mutant C3H/HeJ macrophages by the TLR2 agonist Pam3Cys, and in TLR2-deficient macrophages by the TLR4 agonist E. coli LPS (data not shown).

**PI-3K mediates TLR-induced STAT1 tyrosine phosphorylation, but not serine phosphorylation**

Previous studies have reported that serine phosphorylation of STAT1 could be mediated by PI-3K in IFN-γ-stimulated fibroblasts (24). We subsequently sought to determine whether STAT1 S(727) phosphorylation induced via TLR2 and TLR4 was also mediated by PI-3K. To test this possibility, RAW264.7 macrophages were stimulated with E. coli LPS in the presence and absence of the specific PI-3K inhibitor LY294002. As shown in Figure 3, STAT1 serine phosphorylation was not inhibited by LY294002, demonstrating that this pathway is not dependent on PI-3K. In contrast, both STAT1 tyrosine phosphorylation and phosphorylation of the PI-3K-dependent kinase Akt were inhibited by LY294002 in a dose-dependent manner in LPS-stimulated RAW264.7 cells (Fig. 3B). As shown in Figure 3C, STAT1 serine phosphorylation induced by Pam3Cys was not inhibited by LY294002, demonstrating that TLR2-dependent serine phosphorylation is not dependent on PI-3K. Thus, PI-3K mediates STAT1
tyrosine phosphorylation, activated via TLR4, but does not mediate serine phosphorylation in macrophages activated by engagement of TLR2 and TLR4.

**PI-3K does not mediate induction of IFN-β gene expression by E. coli LPS**

IFN-β has been previously shown to mediate LPS-induced STAT1 tyrosine phosphorylation in macrophages (20, and references therein). The finding that LY294002 could inhibit LPS-induced STAT1 tyrosine phosphorylation, suggested that PI-3K might be necessary for induction of IFN-β expression in LPS-stimulated macrophages. To determine whether PI-3K plays a role in LPS-induced IFN-β production, RAW264.7 cells were stimulated with E. coli LPS in the presence and absence of LY294002. Total RNA was then isolated from the cells 2 hr later, and IFN-β mRNA levels were measured using semi-quantitative RT-PCR. As shown in Figure 4A, LY294002 did not inhibit LPS-induced endogenous IFN-β mRNA expression. A second experimental approach was then used to confirm that PI-3K does not mediate the activation of IFN-β gene expression. RAW264.7 cells were co-transfected with an expression plasmid that encodes a constitutively active TLR4 mutant (TLR4-CA) and an IFN-β-luciferase reporter plasmid. In some cases, cells were also transfected with an expression plasmid encoding a dominant-negative (kinase-dead) mutant of the p85α regulatory subunit. As shown in Figure 4B, the dominant-negative p85α mutant failed to block TLR4-induced IFN-β promoter activation in the transfected macrophages. The capacity of this dominant-negative p85α mutant to block activation of a PI-3K-dependent promoter was confirmed in additional experiments using RAW264.7 cells co-transfected with an iNOS-luciferase reporter plasmid and the expression plasmid encoding the dominant-negative p85α mutant. (data not shown). Together, these findings demonstrate that PI-3K does not mediate activation of IFN-β gene expression by LPS and TLR4.

**PI-3K does not mediate STAT1 tyrosine phosphorylation induced by exogenous IFN-β**

The finding that PI-3K did not mediate the induction of IFN-β gene expression by LPS raised the alternative possibility that PI-3K might be necessary for signaling via the type I IFN receptor (IFNAR). In order to test this possibility, the capacity of LY294002 to block STAT1 tyrosine phosphorylation induced by exogenous IFN-β was evaluated. As shown in Figure 5, exogenous IFN-β rapidly induced STAT1 tyrosine phosphorylation and LY294002 had a negligible effect on IFN-β-induced STAT1 tyrosine phosphorylation. These findings demonstrate that PI-3K does
not mediate STAT1 activation via IFNAR signaling. Given the findings that PI-3K was not necessary either for LPS-induced IFN-β gene expression (Fig. 4), or IFNAR signaling (Fig. 5), these combined observations suggest that PI-3K mediates LPS-induced IFN-β secretion by macrophages (although not specifically tested in our studies). This possibility is consistent with the findings of Ohmori et al. who reported that LY294002 lowered IFN-β secretion by LPS-stimulated RAW264.7 cells, compared with controls (25).

**p38 MAP kinase mediates TLR-induced serine phosphorylation of STAT1**

Although the identity of the protein kinase that phosphorylates STAT1 at serine residues in macrophages has not been definitively established (26), activation of serine STAT1 phosphorylation has been previously shown to be dependent on the p38 MAP kinase (27, 28). To evaluate the role of p38 in TLR-induced STAT1 serine phosphorylation, RAW264.7 macrophages were stimulated with the TLR2 and TLR4 agonists Pam3Cys and *E. coli* LPS (respectively) in the presence and absence of the specific p38 inhibitor SB203580. A concentration of SB203580 was used (20 \( \mu \)M) that completely blocked TLR-dependent activation of p38 in RAW264.7 cells, without affecting viability of the cells (data not shown). Whole cell lysates were then analyzed by western blotting, using anti-phospho-p38 antibodies. As shown in Figure 6, SB203580 treatment inhibited serine STAT1 phosphorylation induced by both Pam3Cys and *E. coli* LPS. These findings demonstrated that p38 was necessary for TLR-induced serine phosphorylation of STAT1.

**p38 MAP kinase activation is not sufficient to induce STAT1 serine phosphorylation**

Because both TLR proteins and the type I IL-1 receptor activate similar signal transduction pathways, including the activation of MAP kinases (3), the capacity of exogenous IL-1β protein to activate STAT1 serine phosphorylation was also assessed. RAW264.7 macrophages were stimulated with recombinant murine IL-1β (100 ng/ml) for 10 and 20 minutes. The activation of p38 and STAT1 was measured by western blotting, as described above. As shown in Figure 7, p38 phosphorylation was rapidly induced in the macrophages following IL-1β stimulation. In contrast, no induction of STAT1 serine phosphorylation was observed in the IL-1-stimulated macrophages, demonstrating that p38 activation is not sufficient for STAT1 activation. Together with the results shown in Figure 6, these findings suggest that TLR engagement triggers the
activation of a protein serine kinase that phosphorylates STAT1 in a p38-dependent manner. Both TLR agonists and IL-1β are capable of activating p38, although only TLR agonists can activate STAT1 serine phosphorylation in macrophages. Thus, this protein serine kinase appears to distinguish the IL-1 from the TLR signaling pathways in these cells. Alternatively, IL-1β may induce STAT1 serine phosphorylation, while also activating a serine phosphatase that de-phosphorylates S(727).

**Role of PKC-δ in TLR-dependent activation of STAT1 serine phosphorylation**

Subsequent studies sought to determine the identity of the STAT1 serine kinase activated by engagement of TLR proteins. Several candidate kinases had been previously identified in macrophages, including isoforms of Protein Kinase C (PKC). To evaluate the role of PKC isoforms in TLR-induced STAT1 serine phosphorylation, RAW264.7 macrophages were stimulated with the TLR2 and TLR4 agonists Pam3Cys and *E. coli* LPS (respectively) in the presence and absence of the pan-PKC-specific inhibitor bisindoylmaleamide (Bis), or the PKC-δ-specific inhibitor rottlerin. Whole cell lysates were then analyzed by western blotting. As shown in Figure 8, both Bis and rottlerin inhibited STAT1 S(727) phosphorylation induced by LPS, but not Pam3Cys. Taken together, these data indicate that TLR2 and TLR4 engagement activates distinct STAT1 serine kinases and that the STAT1 serine kinase activated by *E. coli* LPS is a PKC family member, possibly PKC-δ.

In order to obtain functional evidence of a role for PKC-δ in LPS-induced activation of STAT1, experiments were performed to determine whether a dominant-negative PKC-δ mutant could affect the trans-activation function of STAT1 in LPS-stimulated macrophages. RAW264.7 macrophages were transiently co-transfected with a luciferase reporter plasmid under the control of the STAT1-dependent IFN-β promoter, with and without an expression plasmid encoding a dominant-negative (kinase-dead) PKC-δ mutant (19). As shown in Figure 9A, *E. coli* LPS was a potent activator of the IFN-β promoter in macrophages, and over-expression of the PKC-δ dominant-negative mutant in these cells resulted in a 36% average reduction in promoter activity. This is consistent with an inhibition of STAT1 serine phosphorylation, which would be expected to reduce (but not abolish) the trans-activation function of STAT1. The specificity of this PKC-δ dominant-negative mutant was confirmed by the finding that this mutant did not affect activation...
of the NF-κB-dependent COX2 promoter by LPS (Fig. 9B). Together, these data provide further evidence of a role for PKC-δ in STAT1-dependent promoter activation by LPS.

**Role of MyD88 in TLR-dependent activation of STAT1 serine phosphorylation**

The findings reported above demonstrated that STAT1 serine phosphorylation could be induced by engagement of TLR2 and TLR4, but not by IL-1β. The adapter proteins MyD88 and TIRAP have been shown to mediate signal transduction via TLR2 and TLR4 (29, 30). In addition, a novel adapter protein, termed TRIF, may mediate signaling via TLR3 and TLR4, but not TLR2 (31). To assess the role of MyD88 in TLR-induced STAT1 serine phosphorylation, peritoneal macrophages were obtained from wild type (C57BL/6) and MyD88−/− mice. Cells were stimulated with *E. coli* LPS or Pam3Cys for various times, as indicated. As shown in Figure 10, both LPS and Pam3Cys induced rapid serine phosphorylation of STAT1 in wild type macrophages. In MyD88-deficient macrophages, however, LPS also induced rapid STAT1 S(727) phosphorylation, whereas Pam3Cys did not. This demonstrates that activation of STAT1 serine phosphorylation via TLR2 is MyD88-dependent. Additional signaling components, such as TRIF, might provide an alternate pathway leading to STAT1 serine phosphorylation via TLR4. Together, these findings suggest a model in which STAT1 serine phosphorylation arises from two distinct signaling pathways. One pathway is TLR-specific and leads to the activation of a protein serine kinase, and the other pathway (via either MyD88 or TRIF) leads to the activation of p38 (Fig. 11). Neither pathway alone is sufficient to induce STAT1 S(727) phosphorylation.
DISCUSSION

The objective of these studies was to characterize a novel signal transduction pathway initiated by engagement of TLR proteins. Many previous reports have documented the activation of MAP kinases and the transcription factors NF-κB and AP-1 by various members of the TLR family (6, 7). Studies using genetically modified mice have demonstrated that activation of these signaling pathway is dependent on a variety of adapter proteins, such as MyD88, TIRAP, and TRIF. MyD88 and TIRAP together appear to mediate signaling via TLR2 and TLR4 (29, 30), whereas TRIF also participates in signaling via TLR3 and TLR4 (31). In the case of TLR4 signaling, TRIF may provide an alternate signaling pathway that can activate MAP kinases in a MyD88-independent manner (32). We previously reported that TLR4-dependent signaling could activate cellular responses that are not activated by engagement of TLR2, and were not dependent on MyD88 (20). Specifically, these responses include the induction of IFN-β gene expression, activation of STAT1 tyrosine phosphorylation, and the induction of several STAT1-dependent genes (e.g., iNOS, IP-10, MCP-5). The current studies sought to characterize an additional response induced by TLR engagement, namely STAT1 serine phosphorylation, and to identify the factors necessary for this phosphorylation.

These studies revealed that STAT1 serine phosphorylation was rapidly induced following engagement of TLR2 and TLR4, as well as TLR9 (data not shown). These responses were dependent on TLR signaling as shown by the unresponsiveness of macrophages from TLR2−/−, or TLR4-mutant (C3H/HeJ), mice to the TLR agonists Pam3Cys and E. coli LPS, respectively. In contrast to STAT1 serine phosphorylation, STAT1 tyrosine phosphorylation was induced more slowly by E. coli LPS, and not at all by Pam3Cys. In the case of LPS-induced STAT1 tyrosine phosphorylation, this response was previously shown to be downstream of TLR4-induced IFN-β production, IFNAR engagement, and Jak/Tyk kinase activation (20). The inability of Pam3Cys to induce STAT1 tyrosine phosphorylation was due to the inability of this TLR2 agonist to induce IFN-β production. Although a role for PI-3K in STAT1 serine phosphorylation induced by E. coli LPS or Pam3Cys could not be demonstrated, the PI-3K inhibitor LY294002 blocked STAT1 tyrosine phosphorylation induced by E. coli LPS. Additional experiments revealed that PI-3K was not necessary for LPS-induced IFN-β expression, or for IFNAR signaling in response to
exogenous IFN-β. Therefore, these data support the possibility that PI-3K mediates IFN-β secretion by LPS-activated macrophages, a possibility previously suggested by Ohmori et al. (25). Two published studies have reported that LPS-induced STAT1 serine phosphorylation was dependent on the p38 MAP kinase (27, 28). These studies were confirmed and extended by showing that the p38 inhibitor SB203580 could also block Pam3Cys-induced STAT1 serine phosphorylation. Together with the finding that p38 activation by exogenous IL-1β protein could not activate STAT1 serine phosphorylation in macrophages, our findings demonstrate that p38 is necessary, but not sufficient, for TLR-induced STAT1 activation.

Because the type I IL-1 receptor signals via MyD88 (33, 34), our data also suggests that MyD88 signaling is not sufficient for activation of STAT1 serine phosphorylation and that this response is mediated via a novel TLR-associated signaling pathway. The role of MyD88 in TLR-induced STAT1 serine phosphorylation was assessed directly using macrophages from MyD88-/- mice. These studies revealed that MyD88 was necessary for TLR2-dependent activation of STAT1, but not for TLR4-dependent STAT1 activation. One likely explanation for this difference comes from the potential for the TLR4-specific adapter protein TRIF to activate p38, a kinase that is necessary for STAT1 serine phosphorylation. TRIF may activate MAP kinases in a MyD88-independent manner (35), thus providing a means to activate p38 in LPS-stimulated MyD88-/- macrophages. Because TRIF does not mediate TLR2 activation by Pam3Cys, the TLR2 signaling pathway is solely dependent on MyD88 for activation of p38. This possibility is consistent with our finding that Pam3Cys failed to activate STAT1 serine phosphorylation in the MyD88-/- macrophages. Although the existence of a MyD88-independent pathway leading to MAP kinase activation via TLR4 has been previously demonstrated, the specific adapter protein that mediates this pathway has not been definitively identified. Whether this adapter protein is TRIF, or another novel factor, remains to be determined.

Our studies also attempted to shed light on the identity of the STAT1 serine kinase activated by TLR engagement in macrophages. Earlier reports have shown that p38 kinase itself only weakly phosphorylates STAT1 in vitro (28). More recently, additional kinases, including PKC-δ, PI-3K, and Ca/calmodulin-dependent kinase II (24, 36, 37), have been shown to directly or indirectly mediate STAT1 serine phosphorylation in response to IFN signaling. Two pharmacological
inhibitors of PKC were used to demonstrate a role for PKC isoforms in STAT1 serine phosphorylation induced via TLR4. Furthermore, the finding that rottlerin, a specific inhibitor of PKC-δ, could block LPS-induced STAT1 serine phosphorylation suggests a role for this particular PKC isoform. Consistent with this conclusion is the finding that a dominant-negative PKC-δ mutant partially blocking activation of a STAT1-dependent promoter by LPS. Unexpectedly, STAT1 serine phosphorylation in macrophages activated using Pam3Cys was not blocked by either rottlerin, or by the pan-PKC inhibitor bisindoylmaleamide. Thus, PKC isoforms do not appear to play a role in STAT1 serine phosphorylation induced via TLR2. The identity of this additional serine kinase, and a reason for the existence of two distinct p38-dependent pathways leading to STAT1 S(727) phosphorylation, remain to be determined.

In summary, these findings demonstrate that distinct signal transduction pathways regulate TLR-dependent STAT1 serine and tyrosine phosphorylation in macrophages. This conclusion is consistent with previous studies performed using fibroblasts (24, 38) and macrophages (28). Tyrosine phosphorylation of STAT1 is indirectly mediated by the production of endogenous type I IFN, particularly IFN-β, in LPS-stimulated macrophages (20). We have extended these earlier studies by showing that PI-3K activation is necessary for STAT1 tyrosine phosphorylation in LPS-stimulated macrophages. Because PI-3K does not appear to be necessary for the induction of IFN-β gene expression, or signaling via the IFNAR, PI-3K is likely to mediate the translation of IFN-β mRNA and/or the secretion of newly synthesized IFN-β protein. These possibilities are consistent with the findings of Ohmori et al. who suggested that PI-3K mediates IFN-β secretion by activated macrophages (25). In contrast, STAT1 serine phosphorylation was clearly independent of PI-3K in macrophages. Moreover, TLR-induced STAT1 serine phosphorylation was found to be dependent on both p38 and an additional STAT1 serine kinase, as discussed above. Thus, the mechanisms that regulate the DNA-binding and trans-activation functions of STAT1 in macrophages, via phosphorylation of tyrosine and serine residues (respectively), are highly complex and differ somewhat from similar mechanisms that have been previously described in fibroblasts. The delineation of macrophage-specific mechanisms that regulate STAT1 serine phosphorylation will be the subject of future studies.
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FIGURE LEGENDS

Figure 1. Kinetics of TLR-dependent STAT1 phosphorylation in RAW264.7 macrophages
(A) RAW264.7 murine macrophages (3 x 10^6 cells) were plated in 6-well plates 1 day prior to the experiment. Cells were stimulated with E. coli LPS (100 ng/ml) for the indicated times. The cell lysates were fractionated by SDS-PAGE, and analyzed by western blotting using an antibody against P-S(727) and P-Y(701) STAT1. Membranes were then stripped and re-probed using an antibody against total STAT1, and against β-actin as control for equal loading. In parallel dishes, RAW264.7 cells were stimulated with IFN-γ (200 U/ml, 4 hours) as a positive control for phosphorylation of STAT1. (B) RAW264.7 macrophages were cultured as described above, and then stimulated with the synthetic lipopeptide Pam3Cys (1 µg/ml) for the indicated times. The cell lysates were fractionated by SDS-PAGE, and analyzed by western blotting using an antibody against the P-S(727) STAT1, or against total STAT1, as indicated.

Figure 2. Induction of STAT1 serine phosphorylation in wild type and TLR-deficient macrophages
(A) Peritoneal macrophages were isolated from C3H/OuJ control and TLR4-mutant C3H/HeJ mice as described in ‘Experimental Procedures’. Three days later, macrophages (3 x 10^6 cells in 60 mm dishes) were stimulated with E. coli LPS (100 ng/ml) for various times, as indicated. Cell lysates were prepared, fractionated by SDS-PAGE, and then analyzed by western blotting using an antibody against P-S(727) STAT1. Membranes were then stripped and re-probed using an antibody against total STAT1 as control for equal loading. RAW264.7 cells were also stimulated with IFN-γ (200 U/ml, 4 hours) as a positive control for phosphorylation of STAT1 on S(727).
(B) Peritoneal macrophages from wild type C57BL/6 and TLR2^-/- mice were prepared, as described in ‘Experimental Procedures’. After 3 days in culture (3 x 10^6 cells in 60 mm dishes), cells were stimulated with Pam3Cys (1 µg/ml) for the indicated times. STAT1 S(727) phosphorylation was then measured by western blotting. Total STAT1 levels were measured as a control for equal loading. Lysates prepared from IFN-γ-treated RAW264.7 cells were used as a positive control for STAT1 serine phosphorylation.

Figure 3. PI-3K mediates TLR-dependent STAT1 tyrosine, but not serine, phosphorylation
RAW264.7 macrophages (3 x 10^6 cells) were plated in 60 mm dishes 1 day prior to the experiment. Cells were pretreated with various concentrations LY294002 for 2 hours, and then stimulated with *E. coli* LPS (100 ng/ml) for 2 hours to measure STAT1 Y(701) phosphorylation (A), or for 20 min to detect STAT1 Ser(727) phosphorylation (B). To verify that LY294002 was capable of suppressing PI-3K activity, Akt S(473) phosphorylation was also measured from the same cell lysates used to detect STAT1 S(727) phosphorylation in (B). Total STAT1 levels were measured as a control for equal loading. RAW264.7 cells were also treated with IFN-γ (200 U/ml) for 4 hours as a positive control of the phosphorylation of STAT1. (C) RAW264.7 macrophages were stimulated with Pam3Cys (1 µg/ml) for 20 min in the presence of different concentrations of LY294002, as described above. STAT1 S(727) phosphorylation was measured by western blotting. Akt S(473) phosphorylation was also measured by western blotting using the same cell lysates to confirm that LY294002 blocked PI-3K activity. Lysates prepared from RAW264.7 cells were treated with IFN-γ (200 U/ml) for 4 hours were used as a positive control, and total STAT1 levels were used as a control for equal loading.

**Figure 4. PI-3K does not mediate TLR4-induced IFN-β gene expression**

(A) RAW 264.7 macrophages were pretreated 30 minutes with either DMSO (0.1 % final concentration) or LY294002 (10 µM), and then stimulated 3 hours with *E. coli* LPS (100 ng/ml). Cells were then harvested and total RNA was purified from the cell lysates. Four µg of total RNA was reverse transcribed and 200 ng of cDNA was amplified using semi-quantitative PCR to detect IFN-β, or β-actin sequences, as previously described (20). (B) RAW264.7 cells were transiently co-transfected with a luciferase reporter plasmid under the control of the IFN-β reporter plasmid (IFN-β-luc, 1 µg), the constitutively-active TLR4 expression plasmid (TLR4-CA, 1 µg), and a dominant negative p85(p85-DN, 2 µg), in the indicated combinations. The HSP70-β-gal reporter construct was included as the internal control, and the empty vector was added as necessary to bring the total amount of plasmid DNA up to 4 µg for each transfection. Transfections were performed in triplicate, and a single representative experiment is shown. Data are reported as mean values ± SEM (n=3).

**Figure 5. PI-3K does not mediate STAT1 serine phosphorylation induced by exogenous IFN-β**
RAW264.7 macrophages (3 x 10^6 cells) were plated in 60 mm dishes 1 day prior to the experiment. Cells were then stimulated with recombinant murine IFN-β (100 U/ml) for the indicated times. The cell lysates were fractionated by SDS-PAGE, and analyzed by western blotting using an antibody against P-S(727) STAT1. Membranes were then stripped and re-probed using an antibody against total STAT1 as control for equal loading. RAW264.7 cells were also treated with IFN-γ (200 U/ml) for 4 hours as a positive control of the phosphorylation of STAT1. (B) Cells were cultured as described above, pretreated with various concentrations LY294002 for 2 hours, and then stimulated with recombinant murine IFN-β (100 U/ml) for 20 min to measure STAT1 S(727) phosphorylation. Total STAT1 levels were measured as a control for equal loading. Lysates prepared from RAW264.7 cells were treated with IFN-γ (200 U/ml) for 4 hours were used as a positive control, and total STAT1 levels were used as a control for equal loading.

**Figure 6. TLR-induced STAT1 serine phosphorylation requires p38 MAP kinase**

Peritoneal macrophages (2 x 10^6 cells) from normal C3H/HeOuJ mice were prepared as described in ‘Experimental Procedures’ and cultured in 6-well plates 3 days prior to the experiment. Cells were pre-treated with the selective p38 MAP kinase inhibitor SB203580 (20 µM) for 1 hr, as indicated. The cells were then stimulated with E. coli LPS (100 ng/ml) (A) or Pam3Cys (1 µg/ml) (B), as indicated. Cell lysates were prepared 20 min later, and western blotting was used to measure STAT1 S(727) phosphorylation. Total STAT1 levels were also measured as a control for equal loading. Lysates prepared from IFN-γ-treated RAW264.7 cells were used as a positive control for STAT1 activation. Pam, Pam3Cys; SB, SB203580.

**Figure 7. Activation of p38 MAP kinase is not sufficient to induce STAT1 serine phosphorylation**

Peritoneal macrophages (2 x 10^6 cells) from normal C3H/HeOuJ mice were prepared as described in ‘Experimental Procedures’ and cultured in 6-well plates 3 days prior to the experiment. The cells were then stimulated with exogenous recombinant murine IL-1β (100 ng/ml) for various times, as indicated. Cell lysates were prepared and western blotting was used to measure p38 and STAT1 S(727) phosphorylation. Total STAT1 levels were also measured as
a control for equal loading. Lysates prepared from IFN-γ-treated RAW264.7 cells were used as a positive control for STAT1 activation.

**Figure 8. STAT1 serine phosphorylation induced via TLR4, but not via TLR2, is blocked by inhibitors of PKC-δ**

RAW264.7 macrophages (3 x 10^6 cells) were plated in 60 mm dishes 1 day prior to the experiment. Cells were pre-treated with the pan-PKC-specific inhibitor bisindoylmaleamide (20 µM) (A) or the PKC-δ-specific inhibitor rottlerin (20 µM) (B) for 1 hr, as indicated. The cells were then stimulated with *E. coli* LPS (100 ng/ml) or Pam3Cys (1 µg/ml), as indicated. Cell lysates were prepared 20 min later, and western blotting was used to measure STAT1 S(727) phosphorylation. Total STAT1 levels were also measured as a control for equal loading. Pam, Pam3Cys; Bis, bisindoylmaleamide; Rot, rottlerin.

**Figure 9. A PKC-δ dominant-negative mutant can partially block TLR4-induced activation of a STAT1-dependent promoter**

RAW264.7 cells were transiently co-transfected with a luciferase reporter plasmid under the control of the IFN-β reporter plasmid or murine COX2 reporter construct (2 µg) and either a dominant negative PKC-δ mutant expression plasmid (PKC-δ (DN), 2 µg), or empty vector (2 µg), in the indicated combinations. The HSP70-β-gal reporter construct (0.5 µg) was included as the internal control. Transfected cells were cultivated for a day and then stimulated with *E. coli* LPS (50 ng/ml) for 5 hr, as indicated. Transfections were performed in triplicate, and a single representative experiment is shown. Data are reported as mean values ± SEM (n=3).

**Figure 10. MyD88 is necessary for TLR2-induced, but not TLR4-induced, STAT1 serine phosphorylation**

Peritoneal macrophages from the MyD88^{+/+} (C57BL/6) and MyD88^{-/-} mice were prepared as described in ‘Experimental Procedures’ and cultured in 6-well plates 3 days prior to the experiment. The cells were then stimulated with LPS (100 ng/ml) (A) or with Pam3Cys (1 µg/ml) (B) for various times, as indicated. Western blotting was used to measure STAT1 S(727) phosphorylation. Total STAT1 levels were also measured as a control for equal loading. Lysates
prepared from IFN-γ-treated RAW264.7 cells were used as a positive control for STAT1 activation.

**Figure 11. TLR-specific and p38-dependent activation of STAT1 Ser(727) phosphorylation**

Shown is a model of signal transduction leading to STAT1 serine phosphorylation indicating the putative requirement of both the p38 MAP kinase, PKC-δ, and a novel TLR2-specific pathway that leads to the activation of a non-PKC STAT1 serine protein kinase. Our data also support the possibility that either MyD88, or TIRAP, can mediate TLR-specific activation of p38.
Abbreviations used: IFN-β, interferon β; GAS, IFN-γ-activated sequence; IRAK, IL-1 receptor-associated kinase; IRF, interferon regulatory factor; ISRE, IFN-stimulated response element; PAMPs, pathogen-associated molecular patterns; Pam₃Cys, S-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys4-OH trihydrochloride; PDK1/2, Phosphoinositide-dependent protein kinase1/2; PI-3K, Phosphoinositide 3-kinase; Bis, bisindoylmaleamide; Rot, rottlerin; LPS, lipopolysaccharide; LRR, leucine-rich repeat; Mal, MyD88-adapter-like; STAT, signal transducers and activators of transcription; TIR, Toll-interleukin 1 receptor; TIRAP, TIR domain-containing adapter protein; TLR, toll-like receptor; TRIF, TIR domain-containing adapter inducing IFN-β.

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Fig. 1
Fig. 2

A

LPS (min) 0 20 40 0 20 40
IFNγ
C3H/OuJ C3H/HeJ RAW264.7

B

Pam3Cys (min) 0 20 40 0 20 40
IFNγ
TLR2+/+ TLR2−/− RAW264.7
Fig. 3
A

![Image](Unstimulated-LPS.jpg)

IFNβ
β Actin

Ly294002 - + - +

B

![Image](IFNβ-Luc.jpg)

Fold induction

TLR4-CA - + +
p85 (DN) - - +

Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Fig. 9
Fig. 10
TRIF

TLR4

P-Ser (727)

IL-1R

TLR2

MyD88

???

TRIF

MyD88/TIRAP

???

MyD88/TIRAP

non-PKC STAT1
serine kinase

p38

PKC-δ

P-Ser (727) → STAT1

Fig. 11
Toll-like receptor 2 and 4 activate STAT1 serine phosphorylation by distinct mechanisms in macrophages

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