Phosphorylated Toll-Like Receptor 2 Interacts with Fyn and Cross-Talks with the Phosphorylation-Independent TLR2-Signaling Pathway

Robert W. Finberg, Ching Yim, Jing Yan, Lu Cheng Cao, Leisa Mandell and Evelyn A. Kurt-Jones*

University of Massachusetts Medical School, 364 Plantation St. LRB 226, Worcester, MA 01605, USA

Abstract: Following ligand stimulation, several Toll-like receptors (TLRs) are phosphorylated at tyrosine residues in their intracellular domains. However, the precise chain of events leading to tyrosine-phosphorylation-dependent TLR-mediated cytokine secretion has not been defined. We focused on elaborating the signaling pathway of tyrosine-phosphorylated TLR2. We demonstrated that two tyrosine residues in the intracellular domain of TLR2, Y616 and Y761, were important for cytokine secretion. We also showed that the src-kinase, Fyn, is constitutively associated with TLR2. TLR2-ligand stimulation increases the amount of phosphorylated Fyn and phosphorylated TLR2. The p85-PI3K complex together with PKCζ associated with TLR2 in a src-kinase dependent manner. We identified crosstalk between this complex and two components of the TLR2 tyrosine-phosphorylation-independent pathway, IRAK-1 and TRAF6. Our results demonstrate that the downstream events of ligand-stimulated TLR2, including activation of NFκB and Erk1/2 as well as cytokine secretion, are src-kinase dependent.

Keywords: CD14, Fyn, *H. pylori*, IRAK-1, TIR domain, TLR2, and TRAF6.

INTRODUCTION

The innate immune system is the host's first line of defense against microbial infections and responds to pathogens by recognizing molecules unique to pathogens known as pathogen-associated molecular patterns (PAMPs). Innate immune receptors that recognize PAMPs are termed pattern-recognition receptors (PRRs). In mammals, two major families of leucine-rich repeat-containing PRRs are present on the cell surface: CD14 and Toll-like receptors (TLRs).

TLRs signal through their intracellular domain which contains a consensus Toll-IL-1 receptor (TIR) domain [1]. Different signaling complexes have been associated with TLRs depending on the phosphorylation state of tyrosines in their TIR domain. The TLR tyrosine phosphorylation-independent signaling pathway involves the association of the TIR domain with the TIR-containing adaptor molecule, MyD88, with or without the participation of TIRAP/MAL (another TIR adaptor molecule) and the sequential binding of IRAK-1/IRAK-4 and TRAF6 to the complex. This complex in turn activates TAK1, a mitogen-activated protein kinase kinase kinase (MAPKKK), which leads to the activation of both NFκB translocation (*via* IKKs and IκBα) and MAPKs (JNK and p38 *via* MKK4/7 and MKK3/6, respectively).

The tyrosine-phosphorylation-dependent TLR signaling complex involves the association of TLRs with the p85 subunit of PI3K, MyD88, Rac1 and Btk [2-4]. In the case of TLR2, the formation of the latter complex requires the phosphorylation of its two tyrosine residues, Y616 and Y761, which presumably bind to p85 through its SH2 domains. One of the downstream products of this pathway is the phosphorylation of the p65 subunit of NFκB and the induction of p65 transactivation [4-6]. It is also known that TLR3 and TLR4 [7-9] are tyrosine-phosphorylated following ligand engagement. Src-family kinases have been implicated as the kinases responsible for the phosphorylation of TLR2 and TLR4 [9, 10]. The complete sequence of signaling events involved in this pathway, however, is poorly understood. In addition, the cooperation between the tyrosine phosphorylation-independent and the phosphorylation-dependent pathways is unknown.

This study retraced the signaling sequence that starts with ligand stimulation of TLR2. The results demonstrated that TLR2-mediated cytokine induction involves the activation of the src-family kinase Fyn, as well as activation of IRAK/TRAF6 prior to phosphorylation of the p65 subunit of NFκB. Our study of the signaling pathway of tyrosine-phosphorylated TLR2 is the first to demonstrate that the two TLR signaling pathways, the well-established tyrosine-phosphorylation-independent pathway (mediated thru IRAK-1 – TRAF6) and the tyrosine-phosphorylation-dependent pathway (mediated thru src – PI3K), act in concert with each other and that Fyn, together with p85, IRAK-1, TRAF6 and PKCζ form a unified signaling chain that transmits signals from TLR2 to downstream mediators leading to cytokine secretion.

MATERIALS & METHODS

Reagents

Stimulants were obtained from the following sources: Peptidoglycan (PGN) from ICN (Costa Mesa, CA); AraLAM (LAM) was provided by Dr. John Belisle (Fort Collins, CO); Heat-killed *Listeria monocytogenes* (HKLM), diacylated lipopeptide Pam2 and triacylated lipopeptide Pam3 were a gift from Dr. Douglas Golenbock (University of Massachusetts Medical School, Worcester, MA); *Helicobacter pylori*

*Address correspondence to this author at the University of Massachusetts Medical School, 364 Plantation St. LRB 226, Worcester, MA 01605, USA; Tel: 508-856-3531; Fax: 508-856-6176;
E-mail: evelyn.kurt-jones@umassmed.edu

1874-2262/12

(*H. pylori*) NCTC 11637 (ATCC, Manassas, VA) was provided by Dr. Jean-Marie Houghton (University of Massachusetts Medical School, Worcester, MA). IL-1β was purchased from R&D Systems; TNFα was purchased from Prepro Tech; lipopolysaccharide (LPS) from *E. coli* serotype 0111:B4 was purchased from Sigma (St. Louis, MO) and phenol extracted as previously described [11]; and Zymosan was also purchased from Sigma (St. Louis, MO) and preparations consisted of yeast cell walls (ghost cells) with an average particle diameter of 3 microns. Inhibitors were obtained from the following sources: PP1 from Biomol (Plymouth Meeting, PA) and PP2, PP3, and SU6656 were purchased from Calbiochem (San Diego, CA). Antibodies (Ab) used in this study were obtained from the following sources: Anti-FLAG M2 and isotype controls UPC10, MOPC195, MOPC21/31c (Sigma); anti-p85a (clone AB6, Upstate Biotechnology, Lake Placid, NY); anti-p85a (Z8), anti-PKCζ (H1), anti-IRAK (F4 and H273), anti-TRAF6 (H274 and D10), anti-GFP (FL and B2), anti-Fyn (15 and FYN3) and anti-c-Src (H12 and N16) (Santa Cruz Biotechnology, CA); and anti-ERK1/2 and anti-ERK1/2 pT/pY 185/187 (Invitrogen, Carlsbad, CA). ELISA kits against human IL-1β, IL-6, IL-8, MCP-1 and TNFα were from BD Pharmingen (San Diego, CA).

Cells and Cell Culture

Stably-transfected cell lines expressing TLR2 or CD14 were generated as previously described [12]. Briefly, HEK293 cells were transfected with pCDNA3-hTLR2-YFP (H2.YFP) and plasmids encoding puromycin resistance (gift of Dr. Richard Kitchens, University of Texas Southwestern Medical Center), pCDNA3-hCD14 and/or pFLAG-hTLR2 (Tularik, San Francisco, CA; FLAG-epitope tagged at the N-terminus) using Escort reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. HEK293 cells (ATCC, Rockville, MD) were grown in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA). A Chinese hamster ovary (CHO) cell line transfected with CD14 and FLAG-TLR2 (CHO-3E10-TLR2) was provided by Dr. Douglas Golenbock (University of Massachusetts Medical School, Worcester, MA). CHO-3E10-TLR2 cells were maintained in HAM F-12 culture medium (BioWhittaker) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone), 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (BioWhittaker). Human monocytes were prepared from discarded peripheral blood cells from platelet donors as previously described [13]. Briefly, mononuclear cells were isolated by Ficoll density centrifugation. T cells and NK cells were depleted by incubating the mononuclear cells with anti-CD2 and anti-CD3 monoclonal antibodies followed by goat anti-mouse coupled magnetic beads. After removal of the T cells and NK cells with a magnet, the purity of the monocyte preparation was determined by flow cytometry with phycoerythrin-conjugated antibodies specific for CD14, CD4 and CD3. Monocyte purity was routinely >90%. Monocytes were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin-streptomycin. All cell lines were cultured at 37°C in the presence of 5% CO_2 in a humidified incubator.

Transfection Studies

FLAG-hTLR2 mutants were created by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers used to create pFLAG-hTLR2 (Y616A) were CCATGGCCT GTGGGCTATGAAAATGATGTGGG (forward primer) and CCCACATCATTTTCATAGCCCACAGGCCATGG (reverse primer). Primers used to create the Y761A mutation were GAACACCAAGACCGCCCTGGAGTGGCCCATGGACG AG (forward primer) and CTCGTCCATGGGCCACTCCA GGGCGGTCTTGGTGTTC (reverse primer). All transient transfections were done with the Polyfect transfection reagent (Qiagen, Valencia, CA).

Western Blotting and ELISA Analysis

Cells were stimulated with either heat-killed *Listeria monocytogenes* (HKLM) (10^8/ml) or *H. pylori* (10^7/ml) in Dulbecco's Modified Eagle Medium (DMEM), 10% FBS for the indicated time points (between 1-10 minutes). Cells (~$3x10^6$ HEK cells or ~$5x10^6$ peritoneal exudate cells [PECs, for pERK1/2 western]) were lysed in 1% Triton X-100, 20 mM Tris-HCl [pH 7.7], 150 mM NaCl, 1 mM sodium vanadate, 10 mg/ml leupeptin, 0.15 U/ml aprotinin, 1 mM PMSF on ice for 30 min. and centrifuged. A Bradford Assay was performed to determine protein concentration and 200 μg of protein used for the immunoprecipitations with protein G agarose. Samples were subjected to electrophoresis through a mini-SDS-PAGE gel (7.5% or 4-15% gradient) and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA or 5% milk in TBS-Tween 20 (0.1%) for 1 hour. Following two washes with TBS-Tween 20, the membrane was incubated with various antibodies for 1 hour at room temperature or overnight at 4°C. The membrane was washed twice in TBS-Tween 20 and incubated in the appropriate horse radish peroxidase (HRP)-conjugate secondary Ab, washed 2x and developed by ECL (GE Healthcare). If needed, immunoblots were stripped using stripping buffer (Chemicon, Temecula, CA) for 12 min. at room temperature, washed 3x in TBS-Tween 20/5% milk and re-immunoblotted. ELISA assays were performed on cells grown and stimulated in 24-well plates (~$2x10^5$ for CHO and HEK cells, $1x10^6$ for peripheral blood mononuclear cells (PBMCs)). ELISA assays were performed at least twice with duplicates for each sample.

Immunocomplex *In Vitro* Kinase Assay

CHO-3E10-TLR2 cells (~$3x10^6$ cells) were lysed on ice for 15 min. in lysis buffer (0.5% NP40; 150 mM NaCl; 20 mM Tris [pH 7.4]; 10 mM iodoacetimide; 1 mM sodium vanadate; 10 mg/ml leupeptin, 0.15 U/ml aprotinin; and 1 mM PMSF). Insoluble debris was removed by microcentrifugation (15,000 x *g*) for 15 min. The lysates were incubated for 2 hrs. at 4°C with mAb's previously bound to protein-G agarose beads (Boehringer Mannheim, Indianapolis, IN). The beads were then washed 2x in lysis buffer and once in kinase buffer (25 mM HEPES [pH 7.5], 1 mM $MnCl_2$ and 0.1 mM sodium vanadate). The beads were resuspended in 50 ml of kinase buffer with 20 mCi (γ-^{32}P) ATP (New England Nuclear, Boston, MA) and incubated for 15 min. at room temperature and then washed once with lysis buffer with 10 mM EDTA and once with lysis buffer alone. Samples were either boiled in 1x reducing Laemmli sample buffer and subjected directly to SDS-PAGE analysis

or eluted in elution buffer (0.5% SDS, 10 mM HEPES [pH 7.5] and 1 mM NaF) at 70°C for 3 min. and diluted 30 fold with cold lysis buffer. The eluate was subjected to re-immunoprecipitation with various mAb's or pAb's, washed 2x in lysis buffer, boiled in 1x reducing Laemmli sample buffer and subjected to electrophoresis through a 9% SDS-PAGE gel.

RESULTS

Both the CD14 Signaling Complex and the TLR2 Signaling Complex Contain Src-Kinases But Have Observable Differences in Complex Composition

Our laboratory previously demonstrated that CD14, a co-receptor with TLR2 for a wide variety of microbial ligands, is constitutively associated with src-kinases [13]. Therefore, we began by investigating the possibility that the activation of the TLR2 signaling complex is due to its association with CD14 and CD14-associated src-kinases. To address this, the kinase activities of the TLR2-associated signaling complex and the CD14-associated signaling complex were compared using the immunocomplex *in vitro* kinase method to label TLR2- and CD14- associated phosphoproteins. PP1 and PP2 inhibited the src-family kinase-mediated ^{32}P-labeling of the TLR2- and CD14-associated phosphoproteins. Both the TLR2 and the CD14 complexes contained src-kinase bands at ~60kD and the activations of both complexes were sensitive to src-kinase inhibitors (Fig. **1**). However, TLR2 was associated with a signaling complex that differed from that of CD14 in some important aspects, notably an unidentified band at ~90-95kD that was not found in the CD14 complex (Fig. **1**). We focused on the TLR2 signaling pathway to further examine the events of the tyrosine-phosphorylation-dependent TLR signaling pathway.

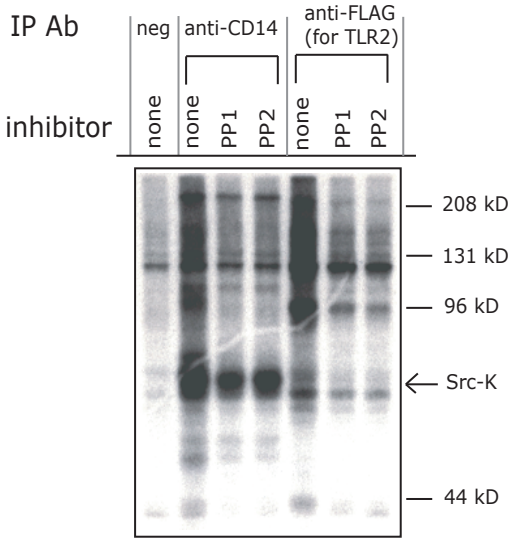

Fig. (1). ***In vitro*** **assay demonstrates that both the TLR2 and CD14 signaling complexes contain src-kinases but have notable differences in complex components.** CD14 and FLAG-TLR2 were immunoprecipitated from CHO-3E10-TLR2 cell lysates and subjected to *in vitro* kinase assays. The immunoprecipitates were left untreated or treated with two different src kinase inhibitors (5μM PP1 or 5μM PP2). The position of src kinases (Src-K) are indicated by the arrow. A representative of two independent experiments is shown.

Two Conserved Tyrosine Residues in TLR2 are Required for Phosphorylation-Dependent TLR2 Signaling

Two highly conserved tyrosine residues in the intracellular domain of TLR2, Y616 and Y761, have been implicated in phosphorylation-dependent TLR2 signaling [4]. To investigate the role of Y616 and Y761, HEK cells expressing CD14 (but not TLR2) were transiently transfected with TLR2 wild-type; TLR2^{Y616A}, TLR2^{Y761A} single mutants; or the TLR2$^{Y616A, Y761A}$ double mutant. The level of expression of WT-TLR2 and the TLR2 mutants was comparable (data not shown). Transfected cells were then stimulated with the TLR2/6 ligand Pam2 and the TLR2/1 ligand Pam3 and the production of the cytokine IL-8 was measured. Both the TLR2^{Y761A} mutant and the double mutant greatly reduced IL-8 production while the TLR2^{Y616A} mutant either enhanced IL-8 production (in the case of Pam2 stimulation) or had no effect (Pam3 stimulation) (Fig. **2A**).

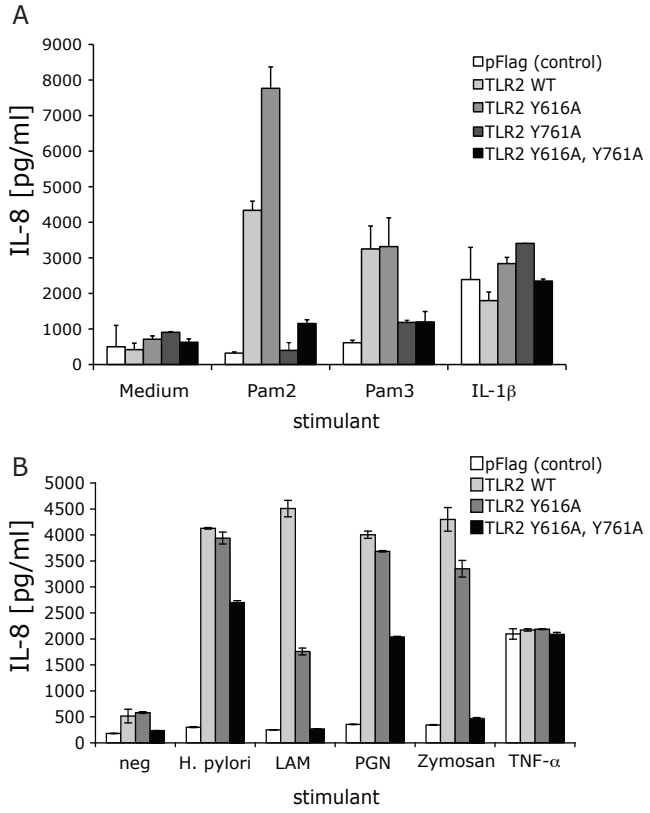

Fig. (2). TLR2 tyrosine mutants are defective in TLR2-ligand-stimulated signal transduction. (**A**) HEK-CD14 cells were transiently transfected with control plasmid (pFLAG), TLR2^{WT}, TLR2^{Y616A}, TLR2^{Y761A} or TLR2$^{Y616A, Y761A}$. After 24 hr, the cells were stimulated with Pam2, Pam3 or IL-1β (100ng/ml each) for 24 hr and supernatants analyzed by IL-8 ELISA. IL-1β was included as a negative control. (**B**) Differential sensitivity of TLR2 mutant-stimulated response to different ligands. HEK-CD14 cells were transiently transfected with control plasmid, TLR2^{WT}, TLR2^{Y616A} or TLR2$^{Y616A, Y761A}$. After 24 hr, the cells were stimulated overnight with the following: *H. pylori* (10^7/ml), araLAM (LAM, 1μg/ml), peptidoglycan (PGN, 10μg/ml), zymosan (10μg/ml) or TNFα (negative control, 100ng/ml). The supernatants were analyzed for IL-8 using an ELISA. Data shown are representative of two independent experiments each with duplicates for each sample, and are reported as means and SD for replicates.

Using a panel of TLR2 ligands (Fig. **2B**) we showed that the $TLR2^{Y616A,\ Y761A}$double mutant had a significantly reduced ability to stimulate IL-8 secretion with all of the ligands tested while the $TLR2^{Y616A}$ single mutant showed a significant reduction in IL-8 secretion when stimulated by araLAM (a TLR2/1 ligand). Both TLR2/1 and TLR2/6 ligands required both tyrosine residues, demonstrating that the main signaling pathway originates from TLR2. Thus, both tyrosine residues 616 and 761 of TLR2 are important for TLR2 signaling.

TLR2 is Constitutively Associated with the Src-Kinase Fyn

The two conserved tyrosine residues in TLR2 could be required for binding to signaling molecules such as src-kinases. To explore the role of src-proteins in TLR2 signaling, we examined if TLR2 physically associates with Fyn, a widely-expressed, membrane-bound src-kinase. The association of Fyn with TLR2 was probed by co-immunoprecipitation - western blotting in TLR2-HEK cells transfected with Fyn. The results demonstrated that TLR2 is constitutively associated with Fyn (Fig. **3A**).

TLR2 is tyrosine-phosphorylated following ligand stimulation [4, 10]. Therefore, we asked if the association of src kinases with TLR2 was affected by the phosphorylation state of TLR2. TLR2-HEK cells were ligand-stimulated, src-kinases immunoprecipitated, and tyrosine-phosphorylated proteins were co-immunoprecipitated with src-kinases and visualized by western blotting. As shown in Fig. (**3B**), a ~90kD phosphoprotein co-immunoprecipitated with src-kinases following ligand stimulation. Stripping and re-probing of the blot revealed that this tyrosine-phosphorylated band was TLR2 (Fig. **3B**, middle panel). The appearance of TLR2 as a doublet indicated that two different phosphorylation states were present in the src-kinase-associated TLR2 molecules. Both bands were visualized by anti-phosphotyrosine antibodies, and likely represent mono- and di-phosphorylated forms of TLR2. This is consistent with the fact that there are two putative tyrosine phosphorylation sites in TLR2 (it is likely that the PAGE experiment was unable to separate the two different forms of mono-phosphorylated TLR2). Following ligand-stimulation with peptidoglycan (PGN) the level of tyrosine-phosphorylation of src-kinase bound TLR2 increased (Fig. **3B**, upper panel) while the amount of TLR2 that was associated with src-kinases changed only slightly (Fig. **3B**, middle panel). The amount of src-kinases stayed constant (Fig. **3B**, lower panel).

Src-family kinases are activated by their associations with tyrosine-phosphorylated receptor proteins and the subsequent phosphorylation of a conserved tyrosine residue within their activation loops [14]. Thus, the question of whether Fyn is phosphorylated following TLR2 ligand stimulation was investigated. HEK293 cells expressing FLAG-TLR2 and CD14 (TLR2-HEK) were stimulated with PGN and the tyrosine phosphorylation of Fyn investigated by immunoprecipitation - western blotting. As shown in Fig. (**3C**, upper panel) Fyn was rapidly tyrosine-phosphorylated at Tyr59 following TLR2 activation while the amount of Fyn stayed constant (lower panel).

Ligand-Dependent Formation of the TLR2 - Fyn - p85 Complex

An important interaction of ligand-stimulated TLR2 is the association of TLR2 with the p85 subunit of PI3K [4]. Tyrosine kinases are required to phosphorylate the tyrosines in the TLR2 intracellular domain in order for them to associate with the p85 SH2 domain(s). We hypothesized that src-kinases are required for this phosphorylation event. To investigate this, TLR2-HEK cells were stimulated with heat-killed *Listeria monocytogenes* (HKLM) with or without pre-incubation with the src-kinase inhibitor PP1, and the association of TLR2 and p85 detected by co-immunoprecipitation - western blotting. Notably, stimulation with whole bacteria was necessary to provide the robust response required to see the interaction. The results demonstrated that the ligand-activated association of TLR2

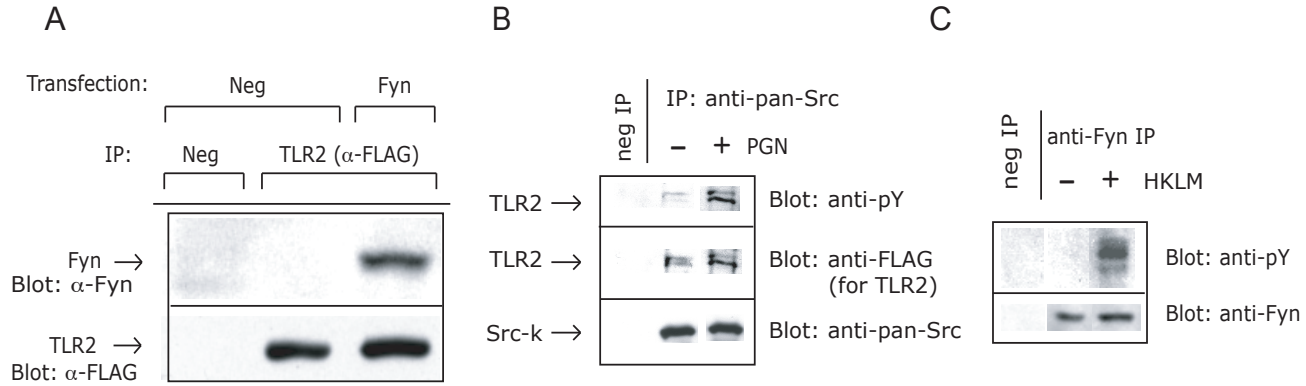

Fig. (3). Association of TLR2 with the src-kinase, Fyn. (**A**) HEK-TLR2 cells were transiently transfected with Fyn or control plasmid and then lysed after 2 days. Lysates were immunoprecipitated with anti-FLAG (for TLR2) or isotype control antibody and immunoblotted with anti-Fyn (upper panel). The blot was then stripped and re-probed with anti-FLAG (lower panel). (**B**) Ligand stimulation increased tyrosine-phosphorylation of src-kinase associated TLR2. HEK-TLR2-CD14 cells were treated with peptidoglycan (PGN, 100 mg/ml) for 6 minutes and lysed. The lysates were immunoprecipitated with anti-pan-src mAb or isotype control Ab and immunoblotted with 4G10-HRP anti-phosphotyrosine Ab (anti-pY, upper panel). The blot was then stripped and re-probed with anti-FLAG Ab (to visualize TLR2, middle panel) and then anti-pan-src Ab (lower panel). (**C**) TLR2 stimulation activated Fyn. HEK-TLR2-CD14 cells were treated with heat-killed *Listeria monocytogenes* (HKLM, 10^8/ml) for 8 minutes and lysed. The lysates were immunoprecipitated with anti-Fyn monoclonal Ab or isotype control Ab and immunoblotted with RC20-HRP anti-phosphotyrosine Ab (anti-pY, upper panel). The blot was then stripped and re-probed with anti-Fyn pAb (lower panel). All blots shown are representative of at least two independent experiments.

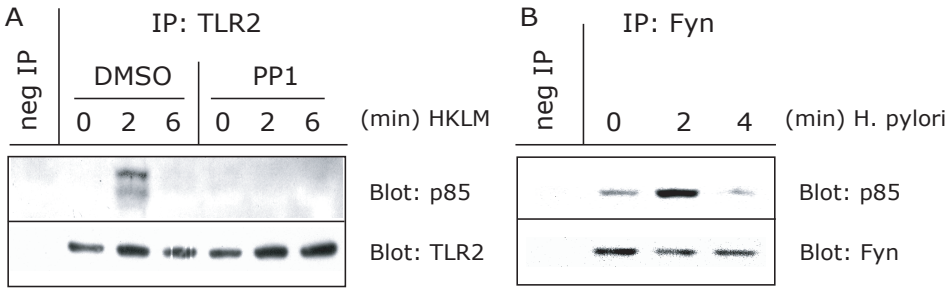

Fig. (4). TLR2 association with p85 is src-kinase dependent and the src-kinase Fyn interacts with p85. (**A**) HEK-TLR2-CD14 cells were pre-incubated with PP1 (25μM) for 1 hr and then stimulated with heat-killed *Listeria monocytogenes* (HKLM, 10^8/ml) for the indicated time points. The lysates were immunoprecipitated with anti-FLAG antibody or control antibody and immunoblotted with either anti-p85 (upper panel) or anti-FLAG (lower panel). (**B**) TLR2-stimulated association of Fyn and p85. HEK-TLR2-CD14 cells were stimulated with *H. pylori* (10^7/ml) for the indicated time points and lysed. The lysates were immunoprecipitated with anti-Fyn antibody or control antibody and immunoblotted with anti-p85 (upper panel). The blot was then stripped and re-probed with anti-Fyn (lower panel). Each blot is representative of three independent experiments.

with p85 in TLR2-HEKs occurred with comparable kinetics as reported previously (Fig. **4A**) [4]. Pre-incubation of the cells with PP1 abolished this association.

Since TLR2 associates with the src-kinase Fyn and p85, the ligand-dependence of the association of Fyn with p85 was further investigated. TLR2-HEK cells were stimulated with *Helicobacter pylori* (*H.pylori,* a TLR2 ligand). Our laboratory has previously demonstrated that *H.pylori* stimulates macrophages in a TLR2-dependent manner and stimulates TLR2-HEK cells but does not stimulate untransfected HEK cells [15]). The association of Fyn with p85 was detected by co-immunoprecipitation – western blotting. As shown in Fig. (**4B**), ligand stimulation increased the amount of p85 associated with Fyn. We found that stimulation with *H.pylori* gave a much stronger signal than heat-killed *Listeria monocytogenes* so we used this stimulus in subsequent experiments.

Novel Interactions with the TLR2-src-p85 Pathway

The TLR2-src-p85 pathway leads to the transactivation of p65-NFκB(4). PKCζ, a protein which associates with TLR2 [16] and is activated by PI3-kinase, effects p65 transactivation [17]. Thus, we investigated the TLR2-stimulated association of p85 with PKCζ using co-immunoprecipitation - western blotting. As shown in Fig. (**5A**), TLR2-ligand activation stimulated the association of p85 with PKCζ, indicating that PKCζ is involved in the TLR2-p85 pathway. This novel result established that TLR2, p85 and PKCζ lie on the same signaling pathway.

We next examined the crosstalk between the TLR2 tyrosine-phosphorylation-dependent pathway (TLR2→ c-Src/Fyn → p85 → PKCζ) and the TLR2 tyrosine-phosphorylation-independent pathway (TLR2 → MyD88 → IRAK-1/IRAK-4 → TRAF6) by examining the interactions between components of both pathways. IRAK-1 is required for the LPS-induced activation of PI3-kinase [17], LPS also induces the association of p85 with MyD88, the adaptor molecule upstream of IRAK-1 [2]. Therefore the involvement of IRAK-1 in the signaling of TLR2 to PKCζ was investigated. TLR2-HEK cells were stimulated with *H. pylori* and the PKCζ associated with IRAK-1 was detected by co-immunoprecipitation - western blotting. As shown in

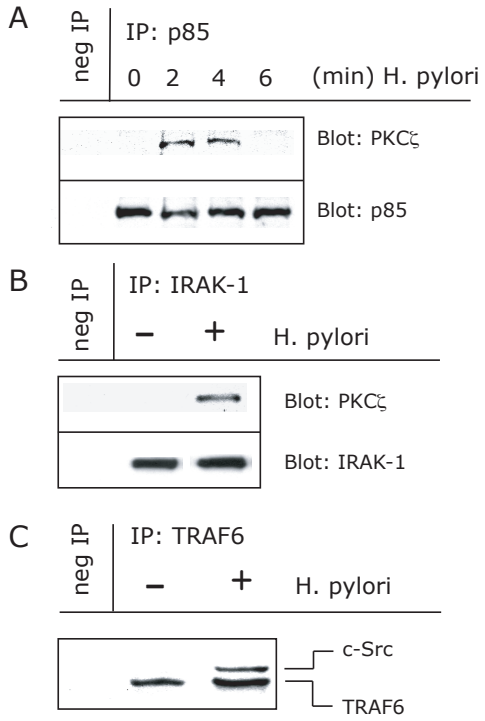

Fig. (5). TLR2-ligand stimulation induces the association of p85 and PKCζ and cross-talk with the phosphorylation-independent pathway components, IRAK-1 and TRAF6. (**A**) HEK-TLR2-YFP cells were stimulated with *H. pylori* (10^7/ml) for the indicated time points and lysed. The lysates were immunoprecipitated with anti-p85 antibody or control antibody and immunoblotted with anti-PKCζ (upper panel). The blot was then stripped and re-probed with anti-p85 (lower panel). (**B**) TLR2-stimulated association of IRAK-1 and PKCζ. HEK-TLR2-CD14 cells were stimulated with *H. pylori* (10^7/ml) for 12 minutes and lysed. The lysates were immunoprecipitated with anti-IRAK-1 antibody or control antibody and immunoblotted with anti-PKCζ (upper panel). The blot was then stripped and re-probed with anti-IRAK-1 (lower panel). (**C**) TLR2-stimulated association of TRAF6 and c-Src. HEK-TLR2-YFP cells were stimulated with *H. pylori* (10^7/ml) for 4 minutes and lysed. The lysates were immunoprecipitated with anti-TRAF6 antibody or control antibody and immunoblotted sequentially on the same blot with anti-c-Src and then anti-TRAF6 antibodies without stripping. Each blot is representative of at least two independent experiments.

Fig. (**5B**), TLR2 ligand stimulation induced the association of IRAK-1 with PKCζ. Therefore IRAK-1 was also involved in the TLR2 - PKCζ pathway.

Studies have shown that c-Src associates with TRAF6 in an overexpression system and also following IL-1 activation [18, 19]. Therefore, the TLR2-stimulated association of c-Src and TRAF6 was investigated. TLR2-HEK cells were ligand-stimulated, TRAF6 was immunoprecipitated and the associated c-Src detected by western blotting. As shown in Fig. (**5C**), TLR2 ligand activation induced the association of TRAF6 with c-Src. (A similar result has been reported by another group [20]).

Src Kinases are Required for TLR2 Activation of Downstream Signaling Events

TLR2 is known to activate mitogen-activated protein kinase ERK1/2, a critical component of the acute stress response, however, this pathway is poorly understood. We investigated the involvement of src-kinases in TLR2-mediated ERK1/2 activation. Murine peritoneal exudate cells (PEC) were pre-incubated with SU6656 [21], a specific src-kinase inhibitor, and stimulated with Pam2, a TLR2 ligand. The lysates were analyzed by western blotting with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies to detect the phosphorylation state of ERK. As shown in Fig. (**6A**), Pam2 stimulation activated the phosphorylation of ERK1/2 while SU6656 pre-incubation greatly diminished activation. Furthermore, both Pam2-stimulated and Pam3-stimulated production of the cytokine MCP-1 was reduced in the presence of the src-kinase inhibitors PP2 and SU6656 (Fig. **6B**).

Another downstream pathway activated by stimulation of TIR-containing receptors, like TLR2, is the NFκB pathway. It is thought that TLRs activate NFκB translocation *via* the tyrosine-phosphorylation-independent signaling complex (MyD88, IRAK-1/IRAK-4 and TRAF6) and that the

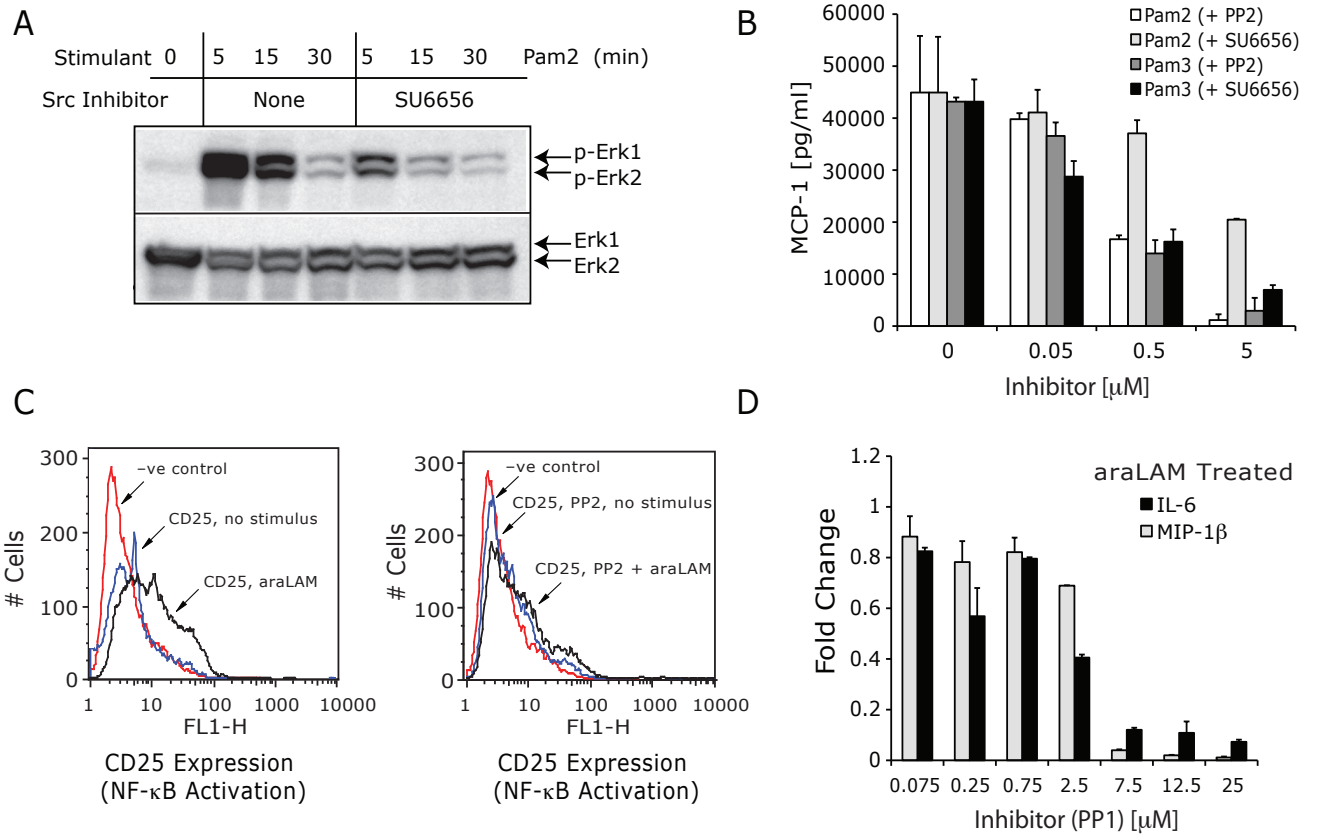

Fig. (6). Downstream signaling events of ligand-stimulated TLR2 are src-kinase dependent. (**A**) Mouse (C57BL/6 wild type) peritoneal exudate cells were stimulated with DMSO negative control (lane 1), Pam2 (100ng/ml, lanes 2-4) or Pam2 (100ng/ml) + SU6656 (2.5μM, lanes 5-7) at various time points as indicated. The lysates were blotted with anti-ERK1/2 pT/pY 185/187 (upper panel) or anti-ERK1/2 (lower panel). (**B**) Human PBMCs were pretreated with 0.05, 0.5 or 5 μM PP2 or SU6656 for 30 min and then stimulated with Pam2 or Pam3 overnight. The supernatants were analyzed for MCP-1 levels using an ELISA. (**C**) TLR2-stimulated NFκB mediated transcription was src-kinase dependent. CHO cells expressing CD14, TLR2 and an NFκB reporter gene (CD25) were incubated with DMSO control (left) or 25μM PP2 (right) for 30 minutes prior to stimulation with araLAM (1μg/ml) or media alone (no stimulus). Sixteen hours later, the cells were stained with a PE-labeled anti-CD25 Ab and NFκB-driven CD25 expression was measured by flow cytometry. Negative (-ve) control cells were stained with a labeled isotype control Ab. (**D**) Human PBMCs were pretreated with 0.075, 0.25, 0.75, 2.5, 7.5, 12.5 or 25μM PP1 or control for 1 hr and then stimulated with araLAM (1μg/ml) overnight. The supernatants were analyzed for IL-6 and MIP-1β levels by ELISA. Graphs depict cytokine levels as fold change (inhibitor treated levels/untreated levels). The data shown in A and C are representative of two independent experiments each. The data shown in B and D are the combined results of two independent experiments each with duplicates for each sample, and are reported as means and SD.

tyrosine-phosphorylation-dependent signaling complex (p85-PKCζ) activates NFκB transcriptional activation *via* p65 phosphorylation and transactivation. We investigated the role of src-kinases on the TLR2-stimulated, NFκB-dependent transcription using a reporter cell line (CHO-3E10-TLR2) that expresses CD14, TLR2 and an NFκB-driven CD25-reporter gene. Upon NFκB activation, CD25 is expressed on the cell surface, which can be detected by flow cytometry. The reporter cells were stimulated with the TLR2-ligand (araLAM) with or without the src-kinase inhibitor (PP2). Incubation with PP2 abrogated the induction of CD25 (Fig. **6C**). In addition, we investigated the effect on cytokine production in araLAM stimulated PBMCs with or without increasing concentrations of the src-kinase inhibitor PP1. The results revealed that IL-6 and MIP-1β levels were greatly reduced in the presence of the src-kinase inhibitor (Fig. **6D**).

Using a panel of stimulants (LPS, araLAM, PGN, and zymosan) and two different src-kinase inhibitors (PP1 and PP2) in PBMCs, we further confirmed that inhibiting src-kinases reduced the levels of three cytokines, IL-6, IL-8, and MIP-1β (Appendix Fig. **A1**).

DISCUSSION

Two major pattern recognition receptors, the TLRs and CD14, are responsible for mediating the host immune response. Previous studies have shown that CD14 associated with both TLR2 and src-kinases [13, 22]. A novel finding of the current study was that the signaling complexes of TLR2 and CD14 (despite containing src-family kinases as common components) have noticeable differences. To elucidate the TLR signaling pathway, our study focused on TLR2 and its tyrosine phosphorylation-dependent signaling events. This study confirmed that src-family kinases are the major tyrosine kinases responsible for TLR2 phosphorylation to initiate the tyrosine phosphorylation-dependent signaling pathway (Fig. **7**).

TLR2- and TLR4-ligands activate src-kinases. TLR2 was previously shown to directly associate with the src-kinase, c-Src [20]; our results demonstrate that another src-kinase, Fyn, is constitutively associated with TLR2 and that Fyn is phosphorylated following stimulation with a TLR2-ligand. We also demonstrate that Fyn is associated with two different forms of tyrosine-phosphorylated TLR2 and that

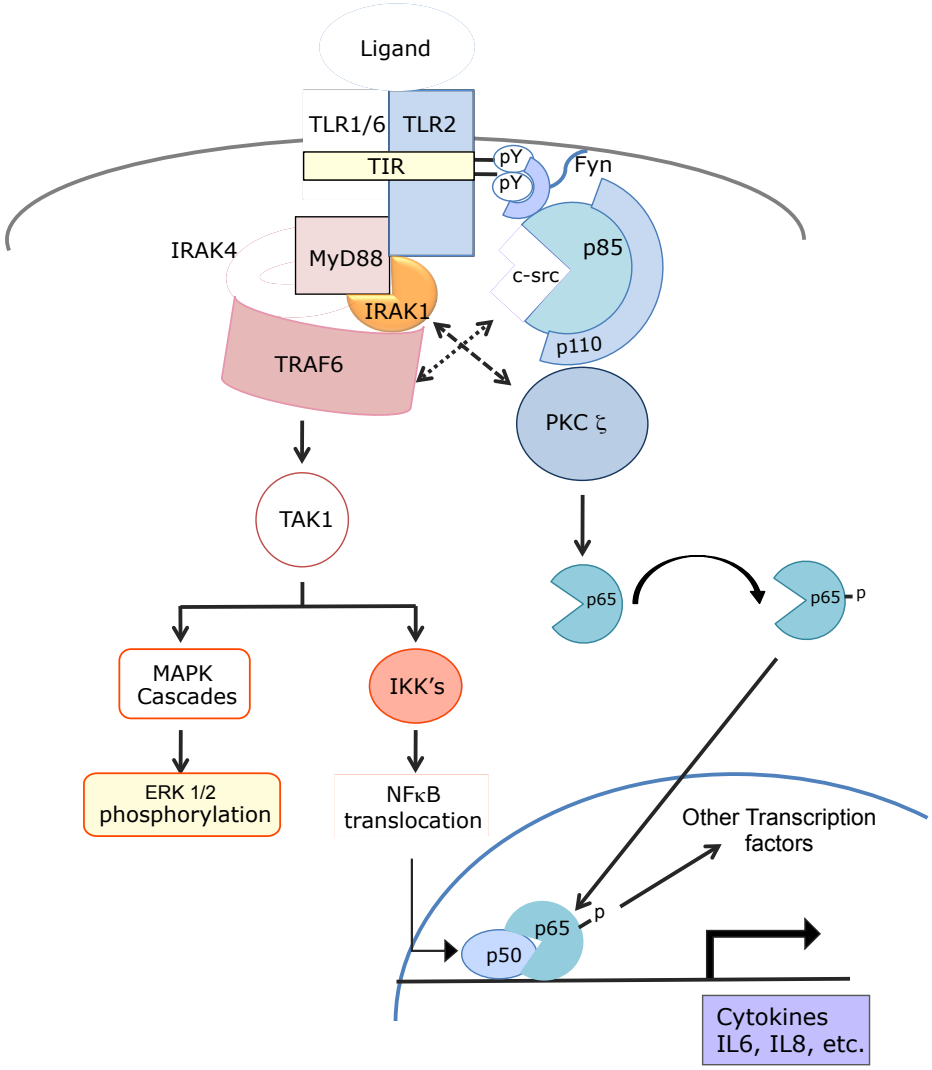

Fig. (7). Diagram representing the TLR2 ligand-stimulated tyrosine phosphorylation-independent and dependent pathways. Dotted arrows illustrate the crosstalk between the two pathways.

TLR2-stimulation is necessary for the association of Fyn with p85. These findings, together with previous reports, establish that src kinases, c-Src and Fyn, contribute to the TLR2 tyrosine-phosphorylation-dependent pathway. Our observation that c-Src associates with TRAF6 following TLR2 activation, suggests that TRAF6 is also a downstream target of src-kinases, and is analogous to the recent finding that IL-1, which utilizes the same TIR-mediated pathway as that of TLR ligands, induces the formation of the c-Src-TRAF6 complex which in turn activates downstream signaling molecules *via* PI3-kinase and its target, Akt (18).

TLRs signal to downstream effectors such as NFκB by several different pathways. In particular, TLR2 leads to NFκB mediated transcription *via* two mechanisms: NFκB translocation and NFκB transactivation. The first pathway involves only serine-threonine phosphorylation events and is tyrosine phosphorylation-independent. This pathway signals through the sequence TLR2 → MyD88 → IRAK-4/IRAK-1 → TRAF6 → TAK1 → IKKα/β → IκBα degradation → NFκB translocation (Fig. **7**). The second pathway, in contrast, involves at least one tyrosine phosphorylation event and signals through the sequence TLR2-pY → p85-PI3K → PKCζ → p65 phosphorylation → NFκB transactivation (Fig. **7**). This study elaborated the crosstalk of these two pathways. In particular, this is the first study to discover the stimulation-dependent interaction between IRAK-1 and PKCζ. The experimental results could indicate that following TLR2 phosphorylation, Fyn, IRAK-1/TRAF6, p85-PI3K and PKCζ form a complex that signals downstream events (Fig. **7**).

In addition, our results suggest interactions between the signaling pathway of phosphorylated TLR2 and the downstream signaling pathway of Dectin-1 (the receptor for zymosan). The double mutant ($TLR2^{Y616A, Y761A}$) was unable to mount a cytokine response to zymosan, suggesting that phosphorylated TLR2 works together with Dectin-1 to produce the cytokine response induced by zymosan. Dectin-1 can be phosphorylated at two tyrosine residues located in its tail sequence. Phosphorylated Dectin-1 recruits the Syk kinase, which is essential for production of the cytokines IL-2 and IL-10 in DCs [23]. It is possible that Syk kinases also play a role in phosphorylated TLR2 signaling. Because a major role for Dectin-1 is to signal downstream events leading to phagocytosis [24] it will be interesting to see if phagocytosis is affected by the $TLR2^{Y616A, Y761A}$ mutations.

Our results showed that NFκB-activated transcription and Erk1/2 activation as well as cytokine secretion were all src-kinase dependent. Delude *et al*. demonstrated that NFκB translocation does not require tyrosine kinase activity [25]. Our results demonstrated that TLR2 stimulation of NFκB-dependent gene transcription was src-kinase dependent. The NFκB-dependent transcription event requires both translocation and transactivation of its p65 subunit. The p85 pathway, which activates p65 transactivation downstream, is likely to be much more sensitive to src-kinase inhibition than the IRAK-1/TRAF6-mediated pathway, which activates NFκB translocation, due to the fact that the p85 pathway involves a critical step where the phosphotyrosines of TLR2 associate with the SH2 domains of p85. The observation that src-kinase inhibition completely suppressed TLR2 association with p85 supports this hypothesis. This theory is also supported by results from the IL-1R signaling pathway, which is analogous to that of TLR2. While IL-1-activated NFκB-dependent gene transcription is inhibited by PP1, the IL-1 mediated NFκB translocation is unaffected [26].

Peptidoglycans are recognized by TLR2/6, but also by a second, separate pathway effected by intracellular receptors NOD1 and NOD2 [27]. NOD1 and NOD2 activate NFκB translocation *via* the recruitment of the serine-threonine kinase RICK/RIP2 and the polyubiquitination of RICK and NEMO/IKKγ, a precursor event to IκB degradation. NOD1- and NOD2-recruited RICK also activates the MAPK pathways *via* TAK1, a MAPKKK. The TLR2-pY → c-Src/Fyn → p85/PI3K → PKCζ pathway described here occurs independently of this NOD pathway. The NOD pathway is not known to be tyrosine kinase dependent and does not require the interaction of any phosphotyrosine- or SH2-containing signaling proteins such as c-Src, Fyn, p85 and PKCζ. We demonstrated that mutations of TLR2 tyrosines and the membrane-anchoring domain of Fyn inhibited ligand-induced cytokine responses, showing the independent significance of this pathway. While some TLR2 ligands such as *H. pylori* can signal through the NOD pathway, previous studies in our laboratory demonstrated that *H. pylori* responses in HEK cells were completely dependent on transfected TLR2 [15], showing that TLR2 is critical in the initiation of signaling responses, even if NOD1/2 contributes to the downstream responses such as cytokine secretion. The interaction of TLR2 and its signaling molecules c-Src and p85 stimulated by heat-killed bacteria were clearly demonstrated. In PBMCs, responses to bacterial peptidoglycans are presumed to occur through the synergistic responses of TLR2 and NOD1/2. The finding in this paper that *H. pylori* bacteria and *S. aureus* peptidoglycan-stimulated cytokine responses were incompletely inhibited by the TLR2 tyrosine-signaling null mutant and that the PGN - and zymosan-stimulated IL-6 secretions (compared with MIP-1β) were relatively insensitive to src-kinase inhibition did point to an additional, independent pathway in cooperation with the TLR2-mediated pathway (for some cytokines such as IL-6 and IL-8), and the NOD pathway is a possible candidate for future studies.

In conclusion, phosphorylation of two highly conserved tyrosines in the intracellular domain of TLR2 are required to initiate the phosphorylation dependent TLR2 signaling pathway. TLR2 phosphorylation could result in the formation of a TLR2-pY - src-kinase - IRAK-1/TRAF6 - p85-PI3K - PKCζ complex that in turn activates downstream effectors and leads to cytokine production. These results suggest the possibility of designing selective drugs to modulate specific components of the TLR-mediated response to microbial products.

ACKNOWLEDGEMENTS

We thank Dr. Douglas Golenbock for generously providing CD25-expressing cell lines, Dr. Jean-Marie Houghton for providing *Helicobacter*, and Dr. John Belisle (Fort Collins, CO) for providing AraLAM under NIH contract, NIAID Contract NO1-AI-75320. We thank Jayashree Paranjape for help with figures.

FUNDING

This work was supported by grants from the National Institutes of Health: RO1 A151405 (to E.K.J.) and RO1 A164349 and U54 A1057159 (to R.W.F.) and by a Juvenile Diabetes Research Fund grant (to R.W.F.).

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

APPENDIX

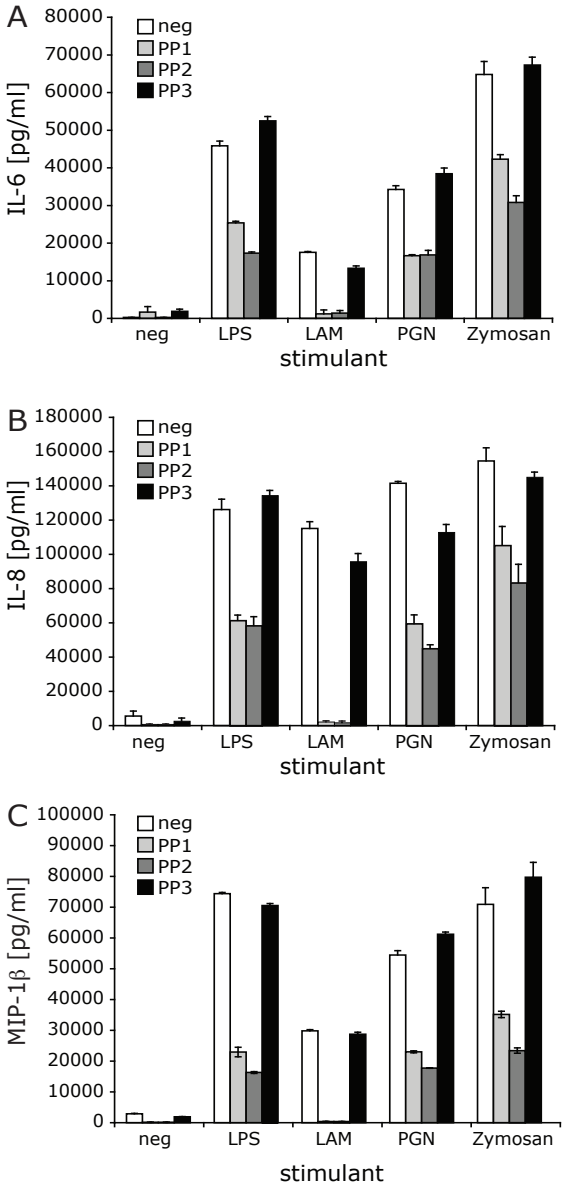

Fig. (A1). Src-kinase inhibitors decrease ligand-stimulated cytokine secretion. Human PBMCs were pretreated with 25 μM PP1, 25 μM PP2, 25 μM PP3 or control for 1 hr and then stimulated with phenol-extracted LPS (100ng/ml), araLAM (1μg/ml), peptidoglycan (PGN, 10μg/ml), zymosan (10μg/ml) or media control (neg) overnight. The supernatants were analyzed with IL-6 (upper panel), IL-8 (middle panel) and MIP-1β (lower panel) ELISA. Data shown are the combined results of two independent experiments performed with duplicates for each sample, and are reported as means and SD.

REFERENCES

[1] Martin MU, Wesche H. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. Biochim Biophys Acta 2002; 1592(3): 265-80.

[2] Ojaniemi M, Glumoff V, Harju K, Liljeroos M, Vuori K, Hallman M. Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. Eur J Immunol 2003; 33(3): 597-605.

[3] Liljeroos M, Vuolteenaho R, Morath S, Hartung T, Hallman M, Ojaniemi M. Bruton's tyrosine kinase together with PI 3-kinase are part of Toll-like receptor 2 multiprotein complex and mediate LTA induced Toll-like receptor 2 responses in macrophages. Cell Signal 2007; 19(3): 625-33.

[4] Arbibe L, Mira JP, Teusch N, *et al*. Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. Nat Immunol 2000; 1(6): 533-40.

[5] Teusch N, Lombardo E, Eddleston J, Knaus UG. The low molecular weight GTPase RhoA and atypical protein kinase Czeta are required for TLR2-mediated gene transcription. J Immunol 2004; 173(1): 507-14.

[6] Anrather J, Csizmadia V, Soares MP, Winkler H. Regulation of NF-kappaB RelA phosphorylation and transcriptional activity by p21(ras) and protein kinase Czeta in primary endothelial cells. J Biol Chem 1999; 274(19): 13594-603.

[7] Sarkar SN, Peters KL, Elco CP, Sakamoto S, Pal S, Sen GC. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. Nat Struct Mol Biol 2004; 11(11): 1060-7.

[8] Chen LY, Zuraw BL, Zhao M, Liu FT, Huang S, Pan ZK. Involvement of protein tyrosine kinase in Toll-like receptor 4-mediated NF-kappa B activation in human peripheral blood monocytes. Am J Physiol Lung Cell Mol Physiol 2003; 284(4): L607-13.

[9] Medvedev AE, Piao W, Shoenfelt J, *et al*. Role of TLR4 tyrosine phosphorylation in signal transduction and endotoxin tolerance. J Biol Chem 2007; 282(22): 16042-53.

[10] Chun J, Prince A. Activation of Ca2+-dependent signaling by TLR2. J Immunol 2006; 177(2): 1330-7.

[11] Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J Immunol 2000; 165(2): 618-22.

[12] Kurt-Jones EA, Mandell L, Whitney C, *et al*. Role of toll-like receptor 2 (TLR2) in neutrophil activation: GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils. Blood 2002; 100(5): 1860-8.

[13] Solomon KR, Kurt-Jones EA, Saladino RA, *et al*. Heterotrimeric G proteins physically associated with the lipopolysaccharide receptor CD14 modulate both *in vivo* and *in vitro* responses to lipopolysaccharide. J Clin Invest 1998; 102(11): 2019-27.

[14] Boggon TJ, Eck MJ. Structure and regulation of Src family kinases. Oncogene 2004; 23(48): 7918-27.

[15] Mandell L, Moran AP, Cocchiarella A, *et al*. Intact gram-negative Helicobacter pylori, Helicobacter felis, and Helicobacter hepaticus bacteria activate innate immunity *via* toll-like receptor 2 but not toll-like receptor 4. Infect Immun 2004; 72(11): 6446-54.

[16] Yang CS, Lee JS, Song CH, *et al*. Protein kinase C zeta plays an essential role for Mycobacterium tuberculosis-induced extracellular signal-regulated kinase 1/2 activation in monocytes/macrophages *via* Toll-like receptor 2. Cell Microbiol 2007; 9(2): 382-96.

[17] Leitges M, Sanz L, Martin P, *et al*. Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway. Mol Cell 2001; 8(4): 771-80.

[18] Funakoshi-Tago M, Tago K, Sonoda Y, Tominaga S, Kasahara T. TRAF6 and C-SRC induce synergistic AP-1 activation *via* PI3-kinase-AKT-JNK pathway. Eur J Biochem 2003; 270(6): 1257-68.

[19] Nakamura I, Kadono Y, Takayanagi H, *et al*. IL-1 regulates cytoskeletal organization in osteoclasts *via* TNF receptor-associated factor 6/c-Src complex. J Immunol 2002; 168(10): 5103-9.

[20] Lee IT, Wang SW, Lee CW, *et al*. Lipoteichoic acid induces HO-1 expression *via* the TLR2/MyD88/c-Src/NADPH oxidase pathway and Nrf2 in human tracheal smooth muscle cells. J Immunol 2008; 181(7): 5098-110.

[21] Blake RA, Broome MA, Liu X, *et al*. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. Mol Cell Biol 2000; 20(23): 9018-27.

[22] Yang RB, Mark MR, Gray A, *et al*. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature 1998; 395(6699): 284-8.

[23] Rogers NC, Slack EC, Edwards AD, *et al*. Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. Immunity 2005; 22(4): 507-17.

[24] Underhill DM. Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. Immunol Rev 2007; 219: 75-87.

[25] Delude RL, Fenton MJ, Savedra R Jr, *et al*. CD14-mediated translocation of nuclear factor-kappa B induced by lipopolysaccharide does not require tyrosine kinase activity. J Biol Chem 1994; 269(35): 22253-60.

[26] Nasuhara Y, Adcock IM, Catley M, Barnes PJ, Newton R. Differential IkappaB kinase activation and IkappaBalpha degradation by interleukin-1beta and tumor necrosis factor-alpha in human U937 monocytic cells. Evidence for additional regulatory steps in kappaB-dependent transcription. J Biol Chem 1999; 274(28): 19965-72.

[27] Shaw MH, Reimer T, Kim YG, Nunez G. NOD-like receptors (NLRs): bona fide intracellular microbial sensors. Curr Opin Immunol 2008; 20(4): 377-82.

Received: January 16, 2012 Revised: April 2, 2012 Accepted: April 3, 2012