Role of TLR4 Tyrosine Phosphorylation in Signal Transduction and Endotoxin Tolerance*

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In this study, we examined whether tyrosine phosphorylation of the Toll-IL-1 resistance (TIR) domain of Toll-like receptor (TLR) 4 is required for signaling and blocked in endotoxin tolerance. Introduction of the P712H mutation responsible for lipopolysaccharide (LPS) unresponsiveness of endotoxin tolerance. Introduction of the P712H mutation, receptor (TLR) 4 is required for signaling and blocked in

tion of the Toll-IL-1 resistance (TIR) domain of Toll-like

p38 and JNK mitogen-activated protein kinases, IκB-α degradation, and activation of NF-κB and RANTES reporters. Likewise, full-length human TLR4 expressing Y674A or Y680A mutations showed suppressed capacities to mediate LPS-inducible cell activation. Signaling deficiencies of the Y674A and Y680A TLR4s correlated with altered MyD88-TLR4 interactions, increased associations with a short IRAK-1 isoform, and decreased amounts of activated IRAK-1 in complex with TLR4. Pretreatment of human embryonic kidney (HEK) 293/TLR4/MD-2 cells with protein tyrosine kinase or Src kinase inhibitors suppressed LPS-driven TLR4 tyrosine phosphorylation, p38 and NF-κB activation. TLR2 and TLR4 agonists induced TLR tyrosine phosphorylation in HEK293 cells overexpressing CD14, MD-2, and TLR4 or TLR2. Induction of endotoxin tolerance in HEK293/TLR4/MD-2 transfectants and in human monocytes markedly suppressed LPS-mediated TLR4 tyrosine phosphorylation and recruitment of Lyn kinase to TLR4, but did not affect TLR4-MD-2 interactions. Thus, our data demonstrate that TLR4 tyrosine phosphorylation is important for signaling and is impaired in endotoxin-tolerant cells, and suggest involvement of Lyn kinase in these processes.

Activation of innate immune responses is critical for the early host defense against microbial infections and for subsequent development of adaptive immunity (1–4). Toll-like receptors (TLRs)†† play a central role in these processes by sensing conserved pathogen-associated molecular patterns (PAMPs) from bacteria (TLR2, TLR4, TLR5, TLR9) (4–10) and viruses (TLR4, TLR3, TR7–9) (11–15). All mammalian TLRs share a similar structural organization, with an ectodomain containing leucine-rich repeats, a transmembrane domain, and a cytoplasmic domain with an intracellular Toll-IL-1 resistance (TIR) domain essential for signal transduction (1–4,16). TLRs sensing of certain bacterial structures (e.g. lipopolysaccharide (LPS) is initiated by a co-receptor, CD14, that acts by binding various PAMPs and presenting them to TLRs that trigger signal transduction (21–23). Several other molecules, e.g. CD11b/CD18 (Mac-1), CD36, and Dectin-1, have been also identified as co-receptors that facilitate TLR-mediated signaling (24–29). In the case of TLR4, an extracellular protein, MD-2, is necessary for TLR4 responsiveness to LPS (30–32), and is part of a tri-molecular signaling complex comprised of CD14, LPS, and TLR4 (32–34).

Recognition of PAMPs by TLRs leads to TLR oligomerization and recruitment of adapter proteins and kinases to their intracellular TIR signaling domains. These processes trigger activation of transcription factors and expression of cytokines, as well as adhesion and co-stimulatory molecules via two main signaling pathways. The “MyD88-dependent” pathway is activated by all TLRs except TLR3, engages adapter proteins MyD88 and TIR domain containing adapter protein (TIRAP) also called MyD88-like adapter (Mal) (engaged by TLR2 and TLR4), as well as the kinases IL-1R-associated kinase (IRAK)-4 and IRAK-1. This pathway results in early NF-κB and MAPK activation, production of proinflammatory cytokines, and B cell

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The abbreviations used are: TLR, Toll-like receptor; TIR, Toll-IL-1R resistance; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; DC, dendritic cells; STF, soluble tuberculosis factor; HEK, human embryonic kidney cells; PAMPs, pathogen-associated molecular patterns; TRIF, TIR domain containing adapter-inducing interferon; MAL, MyD88-like adapter; IRAK, IL-1R-associated kinase; IFN, interferon; Ab, antibody; p, phospho; hu, human; HA, hemagglutinin; HRP, horseradish peroxidase; WT, wild-type; LPS, lipopolysaccharide; IL, interleukin; ELISA, enzyme-linked immunosorbent assay.
proliferation (1–4, 16, 18, 35, 36). The “MyD88-independent” pathway is triggered by TLR4 and TLR3 only, uses the adapters TIR domain containing adapter-inducing interferonβ (TRIF) and TRIF-related adapter molecule (TRAM) (TLR4) or TRIF alone (TLR3), and kinases TRAF-associated NF-κB activator (TANK)-binding kinase (TBK)-1 and IκB kinase (IKK)-α. This pathway mediates DC maturation, activation of interferon regulatory factors (IRFs), delayed NF-κB and MAPK activation, and induction of type I interferons (IFNs) (37–52). Mice deficient for either the MyD88-dependent or MyD88-independent components are resistant to LPS, but more susceptible to bacterial infections, indicating that both pathways play a role in antimicrobial defense (36, 40).

Prior exposure to LPS induces a state of cell refractoriness to subsequent LPS challenge known as endotoxin tolerance, which is thought to limit excessive inflammatory responses (review in 53, 54). A subset of septic shock survivors develop a tolerant phenotype manifested by decreased monocytic responses to LPS and increased susceptibility to bacterial infections (55). The molecular mechanisms of tolerance remain poorly understood. Although LPS tolerance was suggested to be the consequence of decreased TLR4/MD2 expression (56), other studies showed unaltered TLR4 expression, but suppressed IRAK-1-MyD88 interactions and IRAK-1 activation in LPS-tolerant cells (57–61). Our previous results also demonstrated comparable TLR4 expression in normal and LPS-tolerant mouse macrophages (62) and human monocytes (63), whereas recruitment of MyD88 to TLR4 and IRAK-1 activation was blunted (63). In this report, we suggest that impaired recruitment of MyD88 to TLR4 and activation of IRAK-1 in endotoxin-tolerant cells is secondary to a decrease in TLR4 tyrosine phosphorylation. Our findings support the hypothesis that tyrosine phosphorylation of the TLR4 TIR domain is required for TLR4 signaling, and is blocked in endotoxin-tolerant cells. Tyrosine-deficient TLR4 species exhibited constitutive associations with MyD88, suppressed LPS-inducible MyD88 recruitment to TLR4, increased interactions with a short isoform of IRAK-1, and impaired activation of IRAK-1 at the receptor. Furthermore, endotoxin-tolerant cells were found to show suppressed LPS-inducible TLR4 tyrosine phosphorylation, which correlates with impaired MyD88 recruitment to TLR4 and inhibited IRAK-1 activation. Moreover, the ability of protein tyrosine kinase and Src kinase inhibitors to block TLR4 tyrosine phosphorylation and signaling, coupled with the observation of LPS-inducible recruitment of Lyn kinase to TLR4 and its impairment in LPS-tolerant cells, suggest involvement of Lyn kinase in TLR4 signaling.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cell Culture**—Polyclonal antibody (Ab) to human TLR4 (H80), Abs to MyD88, IκB-α, tubulin, β-actin, and IRAK-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phospho (p)-p38 and anti-pJNK Abs were from Promega (Madison, WI). Anti-FLAG monoclonal Ab (M2), M2-horseradish peroxidase (HRP) conjugate, and anti-hemagglutinin (HA) polyclonal Ab were obtained from Sigma, and anti-phosphotyrosine Ab PY20 was from BD Biosciences (San Jose, CA). Human HEK293T cells were maintained as described previously (64). Protein-free, phenol-water-extracted *Escherichia coli* LPS and mycobacterial soluble tuberculin factor (STF) were prepared as described (65, 66), and the synthetic TLR2 agonist, Pam3Cys S-[2,3-bis(palmitoyloxy)-2-(R)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys4-OH trihydrochloride) was purchased from EMC Microcollections GmbH (Tubingen, Germany). An HEK293 cell line stably transfected with untagged human TLR4 and FLAG-tagged human MD-2 (HEK/TLR4/MD-2) was kindly provided by Dr. Douglas Golenbock (University of Massachusetts Medical School, Worcester, MA). Protein tyrosine kinase inhibitors herbimycin A, genistein, and Src kinase inhibitors PP1 and PP2 were purchased from Calbiochem. Human monocytes were prepared by counterflow elutriation and resuspended in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA).

**Recombinant Plasmids and Transient Transfection**—pCDNA3-YFP-human (hu)TLR4, pCDNA3-huCD14, pCMV1-FLAG-huTLR2, pCMV-β-galactosidase, and pELAM-luciferase, were obtained from Dr. Douglas T. Golenbock (University of Massachusetts Medical School, Worcester, MA). pEFBOS-His/FLAG-huMD-2 was provided by Dr. Kensuke Miyake (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan), and pCDNA3-YFP-MyD88 was obtained from Dr. Katherine A. Fitzgerald (University of Massachusetts Medical School). pGL3-RANTES-luciferase reporter plasmid was kindly provided by Dr. John Hiscott (McGill University, Montreal, Canada). Expression vectors encoding HA-ΔTLR4 WT and HA-ΔTLR4 P712H have been described (67), and expression plasmids pFLAG-CMV-1 encoding WT or PGV714–716 CD4-TLR4 were kindly provided by Dr. Stephen T. Smale (Howard Hughes Medical Institute, UCLA, Los Angeles, CA). P714H, Y674A, and Y680A mutations were introduced into the TIR domain of CD4-TLR4 or YFP-TLR4 by site-directed mutagenesis, using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). HEK293T cells were cultured overnight in 150-mm TC dishes (5 × 10^6^ cells per dish) and cotransfected for 3 h with expression vectors as described in the figure legends (25 μg of total plasmid DNA per dish) using Superfect transfection reagent (Qiagen, Valencia, CA). After 48 h, cellular extracts were prepared as described (68). For real-time PCR analyses and determination of cytokine levels by ELISA, transfections were carried out in 6-well plates according to the manufacturer’s protocol.

**Isolation of RNA, Real-time PCR Analysis, and IL-8 Secretion**—Total RNA was isolated with RNeasy kits (Qiagen), followed by DNase digestion and re-purification as recommended by the manufacturer. cDNA was prepared from 1 μg of RNA using the Reverse Transcription System (Promega), and subjected to real-time PCR with gene-specific primers for human hypoxanthine phosphoribosyltransferase (HPRT), IL-8, TNF-α, and IFN-β on a MyIQ minicycler with Optical Detection Module (Bio-Rad). Real-time PCR data were processed using 2^-ΔΔCT method (69). IL-8 levels in supernatants were measured by ELISA in the Cytokine Core Laboratory (University of Maryland, Baltimore).
Co-immunoprecipitation and Immunoblotting—Cell extracts (1–3 mg of total protein) were precleared with protein G-agarose beads (20 μl per sample, Roche Applied Science, Indianapolis, IN) and incubated overnight with 1 μg of respective Ab in lysis buffer containing 20 mM HEPES (pH 7.4), 0.5% Triton X-100, 150 mM NaCl, 12.5 mM β-glycerophosphate, 50 mM NaF, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science). Thereafter, protein G-agarose beads were added (45 μl per sample) and incubation continued for 4 h. Beads were washed five times with lysis buffer, and proteins were separated by SDS-PAGE on 4–20% minigels (Invitrogen), transferred to Immobilon-P membranes (Millipore, Billerica, MA), and subjected to Western analysis as described previously (68).

Reporter Assays—Expression vectors pELAM-luciferase (NF-κB reporter assays) and pGL3-RANTES-luciferase (RANTES reporter assays) were added (0.3 μg/well each) to corresponding transfection mixtures, pCMV-β-galactosidase reporter (0.2 μg/well) was co-transfected for normalization (NF-κB reporter assays), and total plasmid DNA amount was adjusted to 1.5 μg/well with pCDNA3. Following transfections, cells were recovered for 24 h, lysed in a passive lysis buffer (Promega) and firefly luciferase versus β-galactosidase activities were measured using Reporter Assay Systems (Promega) and β-galactosidase assay kit (Tropix, Galacto-Light System) on a Berthold LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). For RANTES reporter assays, luciferase activity was normalized to total protein concentrations measured in cell extracts.

Statistical Analysis—Statistical analysis was performed using the GraphPad Prism 4 program for Windows. Statistical differences among experimental groups were evaluated by the Student’s t test with the level of significance set at p < 0.05. Values are expressed as mean ± S.D.

RESULTS

The P712H Mutation in the TIR Domain of Murine HA-ΔTLR4 Abolishes TLR4 Signaling and Tyrosine Phosphorylation—Positional cloning of TLR4 identified a P712H mutation within the TIR domain as the molecular basis for the LPS-hyporesponsive phenotype of C3H/HeJ mice (4, 5). However, the molecular mechanisms responsible for the signaling deficiency of the mutant P712H TLR4 still remain obscure. In this study, we hypothesized that the P712H mutation alters the secondary structure of the TIR domain, resulting in the failure of TLR4 to undergo agonist-induced post-translational modifications that may be necessary for initiation of downstream signaling. Because an earlier report correlated LPS-induced TLR4 tyrosine phosphorylation with TLR4 signaling (70), we initially sought to determine whether signaling incompetence of P712H TLR4 variants also correlates with changes in TLR4 tyrosine phosphorylation. To this end, we used an expression vector encoding murine TLR4 that has a deletion of a large part of the ectodomain (ΔTLR4), rendering it constitutively active, as evidenced by activation of NF-κB, COX-2, and TLR4-MyD88 association (67). Overexpression of wild-type (WT) HA-ΔTLR4 in HEK293T cells markedly impaired tyrosine phosphorylation. Because the P712H mutation within the TIR domain as the molecular basis for the LPS-hyporesponsive phenotype of C3H/HeJ mice (4, 5). However, the molecular mechanisms responsible for the signaling deficiency of the mutant P712H TLR4 still remain obscure. In this study, we hypothesized that the P712H mutation alters the secondary structure of the TIR domain, resulting in the failure of TLR4 to undergo agonist-induced post-translational modifications that may be necessary for initiation of downstream signaling. Because an earlier report correlated LPS-induced TLR4 tyrosine phosphorylation with TLR4 signaling (70), we initially sought to determine whether signaling incompetence of P712H TLR4 variants also correlates with changes in TLR4 tyrosine phosphorylation. To this end, we used an expression vector encoding murine TLR4 that has a deletion of a large part of the ectodomain (ΔTLR4), rendering it constitutively active, as evidenced by activation of NF-κB, COX-2, and TLR4-MyD88 association (67). Overexpression of wild-type (WT) HA-ΔTLR4 in HEK293T cells markedly impaired tyrosine phosphorylation. Because an earlier report correlated LPS-induced TLR4 tyrosine phosphorylation with TLR4 signaling (70), we initially sought to determine whether signaling incompetence of P712H TLR4 variants also correlates with changes in TLR4 tyrosine phosphorylation. To this end, we used an expression vector encoding murine TLR4 that has a deletion of a large part of the ectodomain (ΔTLR4), rendering it constitutively active, as evidenced by activation of NF-κB, COX-2, and TLR4-MyD88 association (67). Overexpression of wild-type (WT) HA-ΔTLR4 in HEK293T cells markedly impaired tyrosine phosphorylation.

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FIGURE 1. The P712H mutation in the TIR domain of mouse HA-ΔTLR4 abolishes TLR4 signaling and tyrosine phosphorylation. HEK293T cells were transiently transfected with expression vectors encoding HA-tagged ΔTLR4 WT, P712H mutant, or a control vector pCDNA3. After 48 h, total RNA was isolated to examine IL-8 mRNA levels by real-time PCR (A); cell-free supernatants were collected to determine IL-8 production (B), and cell extracts prepared for Western analysis of IRAK-1, IkB-α, p38 phosphorylation, and tubulin expression (C). TLR4 species were also immunoprecipitated with α-HA Ab and subjected to immunoblotting with α-HA Ab (total TLR4 expression) and α-phosphotyrosine Ab (TLR4 phosphorylation) (D). Results of a representative experiment (n = 3) are shown.
renders human TLR4 functionally incompetent. Human TLR4 precludes TLR4 tyrosine phosphorylation and that the PGV714–716AAA mutation in the TIR domain of human TLR4 was observed (Fig. 2). The results obtained for the P712H HA-TLR4 expression of WT and PGV714–716AAA mutant. Similar to these results, we observed that overexpression of the PGV714–716AAA mutation (71) that corresponds to the homologous P712H mutation in murine TLR4 (4, 5, 71) and blocks activation of human TLR4. In doing so, we made use of the human P714A mutation (71) of the TIR domain of human CD4-TLR4 fusion protein on cell activation and TLR4 tyrosine phosphorylation.

The Effect of the PGV714–716AAA Mutation in the TIR Domain of Human CD4-TLR4 Fusion Protein on Cell Activation and TLR4 Tyrosine Phosphorylation—We next sought to extend the linkage between signaling competence of mouse TLR4 and its ability to undergo tyrosine phosphorylation to human TLR4. In doing so, we made use of the human P714A mutation (71) that corresponds to the homologous P712H mutation in murine TLR4 (4, 5, 71) and blocks activation of NF-κB, AP-1, and C/EBP-dependent reporter plasmids elicited by constitutively active WT CD4-TLR4 (71). Extending these results, we observed that overexpression of the PGV714–716AAA CD4-TLR4 (designated P714A) in HEK293T cells failed to activate IL-8 mRNA expression and secretion (Fig. 2, A and B), IRAK-1 and p38 MAPK phosphorylation, and IκB-α degradation (Fig. 2C), in contrast to robust responses elicited by WT CD4-TLR4. As shown in Fig. 2D, Western analysis of immunoprecipitated CD4-TLR4 proteins showed comparable expression of WT and PGV714–716AAA mutant. Similar to the results obtained for the P712H HA-ΔTLR4, the PGV714–716AAA CD4-TLR4 failed to undergo tyrosine phosphorylation, whereas tyrosine phosphorylation of WT CD4-TLR4 was observed (Fig. 2D). Taken together, these data indicate that the PGV714–716AAA mutation in the TIR domain of human TLR4 precludes TLR4 tyrosine phosphorylation and renders human TLR4 functionally incompetent.

CD4-TLR4 point mutants, Y674A and Y680A, were overexpressed in HEK293T cells co-transfected with NF-κB or RANTES reporters, and reporter induction was measured compared with a control (transfection with pCDNA3). The Y674A mutation completely abrogated the ability of CD4-TLR4 to activate NF-κB and RANTES reporters, showing signaling deficiency comparable to that of the P714H CD4-TLR4, whereas the Y680A substitution had a less pronounced effect (Fig. 3A). Next, we examined the effect of tyrosine deficiency in the TIR domain of CD4-TLR4 on activation of endogenous signaling intermediates, e.g., JNK and p38 MAPK phosphorylation and IκB-α degradation. Under conditions of comparable CD4-TLR4 protein expression, strong p38 and JNK phosphorylation and IκB-α degradation was elicited by WT CD4-TLR4, while CD4-TLR4 mutants deficient for 674Y or 680Y yielded little or no detectable MAPK phosphorylation and IκB-α degradation (Fig. 3B).

To demonstrate functional significance of tyrosine deficiencies for LPS-mediated signaling, the Y674A and Y680A point mutations were introduced into the TIR domain of full-length YFP-TLR4. As a reliable means to compare the extent of inhibition, the P714H point mutation (homologous to the murine P712H mutation) was also created in the TIR domain of YFP-TLR4. WT, P714H mutant, or tyrosine-deficient (Y674A, Y680A) species of YFP-TLR4 were overexpressed in HEK293T cells (together with CD14 and MD-2 to
enable LPS responsiveness), and LPS-induced NF-κB reporter activation, IkB-α degradation and phosphorylation of JNK and p38 MAPK were examined. Stimulation with LPS strongly induced NF-κB reporter activation (Fig. 3C), phosphorylation of p38, and led to degradation of IkB-α (Fig. 3D) in cells overexpressing WT YFP-TLR4. In contrast, the presence of the P714H and Y674A mutations led to complete inhibition of TLR4 to elicit these LPS-mediated responses, demonstrating that the agonist-mediated signal-transducing functions of TLR4 are also severely compromised in the Y674A tyrosine-deficient mutant. Similar to its lower inhibitory effect on the ability of constitutively active CD4-TLR4 to induce cellular activation, the Y680A YFP-TLR4 mutation also showed weaker suppression of LPS-induced activation of NF-κB reporter (Fig. 3C), and p38/JNK phosphorylation (data not shown). Together, these results show the importance of individual tyrosine residues in the TIR domain for enabling TLR4 signal-transducing capacities.

**Mutations of Y674 and Y680 Tyrosine Residues in the TIR Domain of TLR4 Alter TLR4-MyD88 Interactions, Impair Activation of IRAK-1 at the Receptor and Result in Interaction with a Short Form of IRAK-1**—In the next series of experiments, we sought to elucidate molecular mechanisms responsible for signaling incompetence of tyrosine-deficient TLR4. To this end, three distinct experimental approaches were employed to study the effect of the Y674A and Y680A mutations on TLR4 interactions with MyD88 and IRAK-1. First, we examined the effect of these mutations on the ability of constitutively active CD4-TLR4 to interact with co-expressed YFP-MyD88 and endogenous IRAK-1. Because overexpressed MyD88 is known to activate IRAK-1 constitutively (43–46), and would be expected to compromise the ability of IRAK-1 to interact with TLR4, co-transfection with YFP-MyD88 was omitted in the case of IRAK-1-TLR4 interaction studies. In agreement with previously reported data (43–46), concomitant expression of constitutively active WT CD4-TLR4 and YFP-MyD88 in HEK293T cells resulted in ligand-independent TLR4-MyD88 associations (Fig. 4A). Surprisingly, the Y674A and Y680A CD4-TLR4s were also able to interact with YFP-MyD88, and the degree of interaction correlated with their relative signaling deficiency (Fig. 4A). Specifically, WT CD4-TLR4 and the Y680A mutant with the weakest signaling deficiency showed the lowest associations, whereas non-signaling Y674A CD4-TLR4 exhibited the strongest interaction with MyD88 (Fig. 4A). Consistent with the ability of MyD88 to associate with TLR4 via their TIR domains and with IRAK-1 via death domains (72, 73), we observed higher amounts of unmodified endogenous IRAK-1 in complex with the Y674A mutant compared with that associated with WT CD-TLR4 (Fig. 4B). Of great interest, tyrosine-deficient CD4-TLR4 variants, but not WT CD4-TLR4, interacted with a short form of IRAK-1 (Fig. 4B) that showed
electrophoretic mobility similar to that reported for the IRAK-1c isoform, a negative regulator of TLR signaling (79).

Next, we sought to examine the effect of tyrosine mutations in the TLR4 TIR domain on agonist-inducible TLR4–MyD88-IRAK-1 signalosome assembly. For this purpose, we used co-immunoprecipitation to study LPS-mediated recruitment of endogenous MyD88 and IRAK-1 to full-length WT, Y674A, or Y680A YFP-TLR4 variants expressed in HEK293T cells, along with CD14 and MD-2. Y674A and Y680A YFP-TLR4s co-expressed in HEK293T cells along with CD14 and MD-2. Y680A YFP-TLR4 exhibited the ability to interact with a short form of IRAK-1, which corresponds by electrophoretic mobility to a negative regulator of TLR signaling, IRAK-1c. Taken together, these data indicate that tyrosine deficiency in the TIR domain of TLR4 results in constitutive interactions with endogenous MyD88 and a short isoform of IRAK-1 (most likely representing IRAK-1c), impairs LPS-inducible recruitment of MyD88 to TLR4, and suppresses phosphorylation of IRAK-1 at the receptor.

**TLR2 and TLR4 Agonists Induce Tyrosine Phosphorylation of Respective TLRs**—Given the correlation between signal-transducing capacities of constitutively active HA–TLR4 and CD4–TLR4 proteins and their ability to undergo tyrosine phosphorylation, it was important to show agonist-induced tyrosine phosphorylation of unmodified, full-length TLR4.

To this end, HEK293T cells were transiently transfected with pCDNA3-huTLR4, along with pCDNA3-huCD14 and pEFBOS-HA-huMD-2 expression vectors to impart LPS sensitivity. In addition to transiently transfected cells, we also used an HEK293 cell line stably expressing untagged human TLR4 and FLAG-MD-2 to study TLR4 tyrosine phosphorylation. Because TLR2 has been shown to undergo tyrosine phosphorylation upon stimulation with heat-killed *Staphylococcus aureus* (74), HEK293T cells were also transiently transfected with pCMV-1-FLAG-TLR2, along with pCDNA3-huCD14 and pEFBOS-HA-huMD-2, and stimulated with two different TLR2 agonists, mycobacterial STF, and synthetic Pam3Cys. Thereafter, FLAG-TLR2 and untagged TLR4 were immunoprecipitated from cell extracts with anti-FLAG and anti-TLR4 Abs, respectively, and analyzed by immunoblotting for tyrosine phosphorylation of respective TLRs and for total TLR expression. Extending previously published data (74), both STF and Pam3Cys induced tyrosine phosphorylation of TLR2 in a time-dependent manner, with a response peaking within 5 min and declining by 15 min (Fig. 5, A and B). TLR4 also exhibited tyrosine phosphorylation within 1–15 min following LPS stimulation in HEK293/TLR4/CD14/MD-2 transient and stable transfectants (Figs. 5C and 7B). These data show that agonist stimulation induced tyrosine phosphorylation of TLR2 and TLR4 in HEK293 cells overexpressing the respective TLRs.

**FIGURE 4.** The effect of the Y674A and Y680A mutations on constitutive (CD4–TLR4) and LPS-inducible (full-length YFP-TLR4) interactions of TLR4 with MyD88 and IRAK-1 species. WT, Y674A, and Y680A variants of CD4–TLR4 were overexpressed in HEK293 cells with (A) or without (B) YFP-MyD88. CD4–TLR4 species were immunoprecipitated with α-FLAG-agarose, and TLR4 interactions with YFP-MyD88 (A) or IRAK-1 (B) were examined using α-GFP or α-IRAK-1 Abs. Total CD4–TLR4 expression was measured by immunoblot analysis. YFP–MyD88 interactions of TLR4 with MyD88 and IRAK-1 species. For this purpose, we used co-immunoprecipitation to study LPS-mediated recruitment of endogenous MyD88 and IRAK-1 to full-length WT, Y674A, or Y680A YFP-TLR4 variants expressed in HEK293T cells along with CD14 and MD-2. LPS stimulation was carried out as indicated (C) or for 5 min (D). YFP–TLR4s or endogenous MyD88 were immunoprecipitated, and TLR4, endogenous MyD88 and IRAK-1 expression was analyzed by immunoblotting with α-GFP, α-MyD88, and α-IRAK-1 Abs, respectively. Results of a representative experiment (n = 4) are shown.
Protein Tyrosine Kinase and Src Kinase Inhibitors Suppress LPS-inducible TLR4 Tyrosine Phosphorylation and Signaling—Next, we sought to gain an initial insight into which kinases could be involved in TLR4 tyrosine phosphorylation and signaling. To this end, we used protein tyrosine kinase inhibitors, genistein, and herbimycin A, as well as Src kinase inhibitors, PP1 and PP2, to examine whether inhibition of these kinases compromises LPS-mediated responses and/or TLR4 tyrosine phosphorylation. Based on the literature, pretreatment of cells with protein tyrosine kinase and Src kinase inhibitors before LPS stimulation was necessary because simultaneous addition of these inhibitors with LPS often failed to mediate a suppressive effect (70). For this reason, the effects of inhibitor pretreatment on LPS-inducible responses in HEK293/TLR4/MD-2 stably transfectants were examined. LPS stimulation resulted in marked phosphorylation of p38 evident in Me2SO (vehicle control)-pretreated cells by 15 min (Fig. 6A), and strongly induced activation of the NF-κB reporter (Fig. 6B). LPS-mediated p38 phosphorylation was abolished by prior exposure of cells to protein tyrosine kinase inhibitors herbimycin A, genistein, and Src kinase inhibitor PP1. Herbimycin A and Src kinase inhibitors PP1 and PP2 also exerted a strong inhibitory effect on LPS-induced NF-κB reporter activation. Furthermore, herbimycin A, genistein, and PP1 also completely abrogated LPS-inducible TLR4 tyrosine phosphorylation (Fig. 6A), suggesting an important role of Src kinases in LPS-mediated TLR4 tyrosine phosphorylation and signaling.

Induction of Endotoxin Tolerance Correlates with Suppressed LPS-inducible Tyrosine Phosphorylation of TLR4 and Recruitment of Lyn Kinase to TLR4 but Does Not Affect TLR4-MD-2 Interactions—Our previous studies showed that induction of endotoxin tolerance does not change total protein levels of TLR4 and MD-2, but suppresses MyD88 recruitment to TLR4 and IRAK-1 activation (63). In view of the crucial significance of TLR4 tyrosine phosphorylation for signal transduction (Figs. 1–4 and Refs. 70, 71), we next sought to determine whether LPS tolerance alters agonist-induced tyrosine phosphorylation of TLR4. First, we assessed the effect of endotoxin tolerance on tyrosine phosphorylation status of TLR4 in stably transected HEK293/TLR4/MD-2 cells. To detect these modifications, we assessed both the proportion of tyrosine-phosphorylated species of TLR4 in total immunoprecipitated TLR4 proteins, and, reciprocally, the proportion of TLR4 species among total phosphorylated proteins immunoprecipitated with anti-phosphotyrosine Ab PY20. Shown are data of one out of three experiments.
which was detected within 1–5 min in medium-pretreated cells (Fig. 7, B and C). Tolerant cells showed reduced LPS-mediated IL-8 mRNA expression (Fig. 7A) and no detectable tyrosine phosphorylation of TLR4 (Fig. 7, B and C). Total TLR4 levels were not affected by tolerance induction, confirming our previous findings (62, 63) and the results reported by others (57, 59).

In our previous study, we showed that endotoxin tolerance does not modulate TLR4 and MD-2 protein levels (63, 64). Because of the importance of relative stoichiometry of expressed MD-2 molecules in enabling optimal LPS responsiveness to TLR4 (75), the ability of MD-2 to associate with TLR4 was next examined in medium-pretreated and LPS-tolerized HEK293T/TLR4/MD-2 stable transfectants. Co-immunoprecipitation analysis revealed comparable amounts of MD-2 associated with TLR4 in control and LPS-tolerant cells regardless of whether or not cells were stimulated with LPS (Fig. 7B, third panel). These data indicate that endotoxin tolerance does not alter the ability of MD-2 to interact with TLR4.

To confirm our results obtained in the overexpression system, we examined the effect of endotoxin tolerance on tyrosine phosphorylation of endogenous TLR4 in human monocytes. LPS pretreatment of monocytes significantly inhibited LPS-mediated IκB-α degradation, p38 phosphorylation, and TNF-α gene expression compared with responses observed in medium-pretreated cells (Fig. 8, A and B), demonstrating LPS tolerance induction. LPS-tolerant human monocytes showed suppressed LPS-inducible up-regulation of both MyD88-dependent (e.g. TNF-α) and MyD88-independent (e.g. IFN-β) genes (Fig. 8B), extending our previous findings obtained in mouse macrophages (76). Fig. 8C shows that LPS failed to elicit tyrosine phosphorylation of endogenous TLR4 in endotoxin-tolerant human monocytes, in contrast to the strong response observed in control cells.

Our data using protein tyrosine kinase and Src kinase inhibitors suggest the role for Src kinases in mediating TLR4 tyrosine phosphorylation and signal transduction. To elucidate further whether Src kinases play a role in TLR4 phosphorylation, we studied whether one of the Src kinase family members, Lyn, is recruited to TLR4 upon LPS stimulation and how induction of LPS tolerance may affect this process. For this purpose, FLAG-tagged Lyn was overexpressed in HEK293/TLR4/MD-2 stable transfectants, cells were pretreated with medium or tolerized with LPS, and LPS-inducible recruitment of Lyn to TLR4 was examined by co-immunoprecipitation. LPS stimulation of medium-pretreated cells led to a strong recruitment of FLAG-Lyn to TLR4 within 1–5 min, with a maximal response reached by 15 min (Fig. 9). In contrast, very little Lyn was found in association with TLR4 in HEK293/TLR4/MD-2 cells rendered endotoxin-tolerant (Fig. 9). Taken together, these data show that induction of endotoxin tolerance results in suppression of LPS-inducible tyrosine phosphorylation of TLR4 and recruitment of Lyn to TLR4, as well as the failure of LPS to induce MyD88-dependent and MyD88-independent signaling, but does not affect TLR4-MD-2 interactions.

**DISCUSSION**

Tyrosine phosphorylation regulates signal transducing functions of many receptors, including TLR2 and TLR3 (71, 74, 77). Chen *et al.* (70) reported that LPS induces tyrosine phosphorylation of endogenous TLR4 in human monocytes, and Ronni *et al.* (71) showed abolishment of activation of NF-κB, AP-1, and C/EBP-dependent reporter plasmids by CD4-TLR4 fusion proteins with several tyrosine-alanine substitutions in their TIR domain. However, the molecular mechanisms by which alterations in tyrosine phosphorylation of the TIR domain of TLR4 affect signaling have not been elucidated. It is also not known whether the endotoxin-tolerant phenotype is secondary to...
interference with post-translational modifications of TLR4. In this report, our data support the hypothesis that agonist-induced engagement of TLRs leads to post-translational modifications within the TIR domain that may play an important role in creating docking platforms for the recruitment of downstream signal-competent adapter proteins (e.g. MyD88) and kinases (e.g. IRAK-4 and IRAK-1). Our results also suggest that one mechanism by which endotoxin tolerance inhibits recruitment of MyD88 to TLR4 and IRAK-1 activation could operate by altering TLR4 tyrosine phosphorylation, creating “signal-incompetent” TLR4 docking platforms. This is, in turn, translated into preferential association between hypophosphorylated TLR4 and a short isoform of IRAK-1 (possibly IRAK-1c), and might result in recruitment of other negative regulators of TLR4 signaling. Finally, the ability of protein tyrosine kinase and Src kinase inhibitors to block TLR4 tyrosine phosphorylation and signal transduction, coupled with the demonstration of LPS-mediated recruitment of Lyn to TLR4 and its inhibition in LPS-tolerant cells, suggest the involvement of Lyn in TLR4 tyrosine phosphorylation, signaling, and tolerance.

Several lines of evidence presented in this report suggest that post-translational modifications regulate the signal-transducing functions of TLR4. First, introduction of the P712H mutation into the TIR domain of a constitutively active mouse ΔTLR4 abrogated receptor tyrosine phosphorylation and blocked its ability to elicit IRAK-1 phosphorylation and activation of p38 and NF-κB (Fig. 1). Second, expression of the PGV714→716AAA mutation (homologous to the P712H mouse mutation) in another constitutively active TLR4 construct, human CD4-TLR4, rendered it signal-incompetent and incapable of undergoing tyrosine phosphorylation (Fig. 2). Both the P712H ΔTLR4 and P714A CD4-TLR4 mutants failed to activate IRAK-1 (Figs. 1 and 2), and the mutation of P714 blocked activation of NF-κB and RANTES reporters. Furthermore, the P714H TLR4 mutants were incapable of eliciting constitutive (CD4-TLR4) and LPS-inducible (YFP-TLR4) phosphorylation of p38 and JNK, degradation of IkB-α, and NF-κB and RANTES reporter activation (Figs. 3 and 4). These results demonstrate functional significance of TLR4 tyrosine phosphorylation for activation of both MyD88-dependent and MyD88-independent signaling cascades and indicate that the conserved proline is necessary for this to occur. Third, mutation of two tyrosine residues within the TIR domain of TLR4 led to profound suppression of TLR4-mediated phosphorylation of JNK and p38 MAPK, degradation of IkB-α, and activation of NF-κB and RANTES reporters (Fig. 3). Finally, we demonstrate that TLR2 and TLR4 agonists induce tyrosine phosphorylation
of respective TLRs overexpressed in HEK293T cells, as well as endogenous TLR4 in human monocytes (Figs. 5, 7, 8). Our findings indicate that TLR4 tyrosine phosphorylation is an important post-translational modification necessary for LPS signaling.

To the best of our knowledge, this report demonstrates for the first time that inhibition of LPS-mediated MyD88-dependent and MyD88-independent signaling cascades in endotoxin-tolerant HEK293/TLR4/MD-2 cells and human monocytes correlates with the inability of TLR4 to undergo tyrosine phosphorylation. It is noteworthy that inhibited tyrosine phosphorylation of the P712H ΔTLR4 mutant (Fig. 1D) correlates with its failure to induce p38 phosphorylation, 1κB-α degradation (Fig. 1, C and D) and NF-κB reporter activation (67). It is also intriguing that the mouse P712H mutation and a functionally homologous P714A substitution both abolished the capacity of constitutively active mouse ΔTLR4 and human CD4-TLR4 to activate IRAK-1 (Figs. 1 and 2C). These data, coupled with blocked TLR4 tyrosine phosphorylation (Figs. 7 and 8), impaired recruitment of MyD88 to TLR4, and suppressed IRAK-1 activation observed in endotoxin-tolerant human monocytes (63) suggest the existence of a “no-signaling,” non-phosphorylated conformation of TLR4. Because proper TLR4 complex assembly with adapter proteins is important for recruitment and activation of IRAKs (1–3, 72, 73, 78), it was of importance to delineate whether deficient tyrosine phosphorylation of TLR4 alters recruitment of downstream adapter proteins and kinases. Co-immunoprecipitation studies revealed that tyrosine-deficient CD4–TLR4 species were not compromised in their abilities to associate with co-expressed YFP-MyD88, and, in contrast to WT CD4–TLR4, interacted with a short isoform of IRAK-1, most likely representing IRAK-1c (Fig. 4, A and B). Because the use of constitutively active CD4–TLR4 and YFP-MyD88 could yield artificial results, our next approach was to employ full-length YFP-TLR4 that are incapable of autoactivation but mediate signal transduction in conjunction with CD14 and MD-2 when activated by LPS (34). This report shows that the tyrosine-deficient Y674A YFP-TLR4 mutant, but not WT YFP-TLR4, constitutively associated with endogenous MyD88 (Fig. 4C). In contrast to potent LPS-inducible recruitment of endogenous MyD88 to WT YFP-TLR4 with the peak response at 5 min, MyD88 amounts associated with the Y674A mutant remained unchanged and were significantly lower (Fig. 4C). This is reminiscent of the failure of LPS to mediate recruitment of MyD88 to TLR4 observed in endotoxin-tolerant human monocytes (63). It is noteworthy that although the Y674A and Y680A CD4 TLR4 species were not compromised in their capacities to interact with unmodified IRAK-1, LPS-driven IRAK-1 activation at the mutant Y674A YFP-TLR4 was severely inhibited, as judged by suppressed association of activated, phosphorylated IRAK-1 with tyrosine-deficient YFP TLR4 (Fig. 4D). It is interesting to note that our recent studies demonstrated that truncated, kinase-deficient mutants of IRAK-4 that expressed mutations identified in a patient hypersensitive to LPS and suffering from recurrent infections also exhibit increased association with MyD88 and inability to interact with activated IRAK-1 (68).

The findings presented herein directly demonstrate increased associations of tyrosine-deficient TLR4 variants with a short isoform of IRAK-1 whose electrophoretic mobility corresponds to IRAK-1c (Fig. 4, B and D). IRAK-1c acts as a negative regulator of TLR4 signaling, as evidenced by suppression of IL-1 and LPS-mediated activation of NF-κB and production of TNF-α and IL-6 (79). That IRAK-1c shows impaired interactions with IRAK-4, fails to be phosphorylated by IRAK-4 (79), and exhibits increased associations with tyrosine-deficient TLR4 (Fig. 4) may explain compromised activation of IRAK-1 at the Y674A TLR4 mutant. It is tempting to speculate that preferential accumulation of IRAK-1c within hypophosphorylated TIR domains of tolerized TLR4 may account for impaired IRAK-1 phosphorylation and activation widely observed in tolerance (54, 63). In addition, we cannot exclude a possibility of preferential recruitment of another negative regulator of TLR signaling, an alternatively-spliced, short form of MyD88 (MyD88s), to tyrosine-deficient (e.g. Y674A) or tolerized, hypophosphorylated TLR4 variants. While not deficient in its interactions with IL-1R and IRAK-1, MyD88s fails to interact with IRAK-4 because of its lack of the intermediate domain critical for MyD88-IRAK-4 associations, leading to the failure of IRAK-1 phosphorylation and activation upon assembly of IL-1R/MyD88s/IRAK-1 complexes (80, 81). Finally, defective tyrosine phosphorylation within the TLR4 TIR domain could alter recruitment and/or activation of IRAK-4. Studies are in progress to address the exact molecular mechanism by which tyrosine deficiencies in the TLR4 TIR domain impair LPS-inducible MyD88 recruitment and IRAK-1 activation at the receptor complex.

It remains to be determined whether inhibition of TLR4 tyrosine phosphorylation occurs due to modulation of expression levels and/or activities of tyrosine kinases or phosphatases. Several tyrosine kinases have been implicated in TLR4-mediated signaling, including Bruton’s tyrosine kinase (82, 83), as well as p56-h, p58-hck, and p59c-fgr (84, 85). However, a possible role for tyrosine kinases for TLR4 signaling was questioned by Delude et al. (86), since inhibition of LPS-induced tyrosine kinase activity with genistein failed to inhibit LPS-mediated NF-κB translocation. Nevertheless, a recently published report on involvement of c-Src tyrosine kinase in TLR3 tyrosine phosphorylation and signaling (87) further reinforces the importance of Src kinases in TLR phosphorylation and activation. In this report, we demonstrate that protein tyrosine kinase and Src kinase inhibitors blocked LPS-mediated TLR4 tyrosine phosphorylation, activation of p38 MAPK and induction of NF-κB reporter activity (Fig. 6). Furthermore, LPS stimulation of HEK293/TLR4/MD-2 stable transfectants led to recruitment of overexpressed FLAG-Lyn to TLR4, and induction of endotoxin tolerance significantly inhibited Lyn–TLR4 association (Fig. 9). Although further experiments using Lyn gene silencing techniques and cells obtained from Lyn−/− mice are necessary to unambiguously demonstrate whether Lyn is responsible for TLR4 phosphorylation, our data presented herein support the hypothesis that Src tyrosine kinases are involved in LPS-induced TLR4 phosphorylation and its modulation in endotoxin tolerance. In addition to altered expression and/or activities of protein kinases, changes in expression and enzymatic activity of phosphatases may also contribute to sup-
pressed TLR4 tyrosine phosphorylation in tolerant cells. In this regard, Ropert et al. (88) reported that induction of tolerance to LPS coincided with increased phosphatase activity, and treatment of tolerant cells with okadaic acid, an inhibitor of phosphatase activity, restored TLR4-mediated signaling, reversing the tolerant phenotype. Nimah et al. (89) demonstrated increased expression of the dual specificity phosphatase, MAP kinase phosphatase-1 (MKP-1), during tolerance that regulates TNF-α expression by inhibiting p38 activity. In addition, MKP-1−/− macrophages exhibited a significantly reduced capacity to become tolerant to LPS (89). Because the cytosolic phosphatase SHIP has been shown to be required for the development of an LPS refractory state (6), these data further support a role for altered phosphatase activity in tolerance.

Overexpression experiments and studies in knock-out mice have unambiguously shown that MD-2 is indispensable for conferring LPS sensitivity to TLR4 (30–34). Therefore, it was of importance to elucidate whether endotoxin tolerance affects TLR4-MD-2 interactions that may be a consequence of altered TLR4 tyrosine phosphorylation. However, we observed similar amounts of MD-2 associated with TLR4, regardless of LPS stimulation or endotoxin tolerance induction, indicating that suppressed TLR4-mediated signaling in tolerized cells is not due to an altered TLR4-MD-2 association. Given the role of MD-2 in LPS binding and presentation to TLR4 (33, 34), our data are also consistent with the finding that similar amounts of labeled LPS bind to control and endotoxin-tolerant cells (41).

In conclusion, our data demonstrate that tyrosine phosphorylation of TLR4 is required for its ability to signal. We also show a correlation between abolished TLR4 tyrosine phosphorylation and suppressed LPS-mediated p38 MAPK phosphorylation, IκB-α degradation, and expression of MyD88-dependent and MyD88-independent cytokines. Similar suppression of TLR4 signaling could be recapitulated in P712H murine and P714H human TLR4 mutants that also show deficient tyrosine phosphorylation, and, conversely, tyrosine deficiency in the TIR domain of TLR4 is associated with its signaling incompetence. These results suggest an important role for the loss of LPS-induced TLR4 tyrosine phosphorylation in tolerance that is manifested at the molecular level by impaired recruitment of MyD88 to TLR4 and IRAK-1 activation. However, association of an extracellular accessory protein, MD-2, with TLR4 was found to be unaltered in LPS-tolerized cells. Suppressed LPS-inducible recruitment of endogenous MyD88 and increased association of IRAK-1c isoform with tyrosine-deficient TLR4 suggest that changes in TLR4 tyrosine phosphorylation interfere with its ability to provide proper signaling platforms for recruitment of signaling-competent intracellular adapter proteins and kinases, resulting in diminished TLR signaling in tolerized cells.

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