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Protein Kinase D1 Is Essential for MyD88-Dependent TLR Signaling Pathway

Jeoung-Eun Park,* Young-In Kim,* and Ae-Kyung Yi2*†

Protein kinase D1 (PKD1) has been shown to be involved in certain MAPK activation and cytokine expression by several TLR ligands. However, the precise physiological role of PKD1 in individual signaling from TLRs has not been fully addressed. In this study, we provide evidence that PKD1 is being activated by TLR ligands, except the TLR3 ligand. PKD1 activation by TLR ligands is dependent on MyD88, IL-1R-associated kinase 4 and 1, but independent of TNF-α receptor-associated factor 6. PKD1-knockdown macrophages and bone marrow-derived dendritic cells revealed that PKD1 is indispensable for the MyD88-dependent ubiquitination of TNF-α receptor-associated factor 6; activation of TGF-β-activated kinase 1, MAPKs, and transcription factors; and expression of proinflammatory genes induced by TLR ligands, but is not involved in expression of type I IFNs induced by TLR ligands and TRIF-dependent genes induced by TLR3 and TLR4 ligands. These results demonstrate that PKD1 is essential for MyD88-dependent proinflammatory immune responses. *The Journal of Immunology, 2009, 182: 6316–6327.

When the body is infected by microbial pathogens, host innate defense responses are initiated by recognition of specific structural motifs, known as pathogen-associated molecular patterns, present in microbial pathogens by several groups of pattern recognition receptors, including TLRs, in host cells. The main response to pathogenic infection by TLRs is induction of proinflammatory cytokines to inhibit the growth and dissemination of invading pathogens and to promote acquired immunity to specifically remove the pathogens.

Each member of the TLR family, which is composed of at least 13 family members, has an extracellular leucine-rich repeat domain with a unique capacity for recognizing specific pathogen-associated molecular patterns, and an intracellular Toll/IL-1R (TIR) domain with a capacity for recruiting and transducing signals through adaptor(s) containing a TIR domain (1, 2). Recruitment of different combinations of TIR domain-containing adaptors to each different TLR provides a molecular basis for the complexity of differences in gene expression profiles induced by individual TLRs. MyD88, the first discovered TIR domain-containing adaptor molecule, is recruited to all TLRs, with the exception of TLR3, and is essential for production of inflammatory cytokines induced by those TLRs (2). MyD88 recruits IL-1R-associated kinase (IRAK) 4, IRAK1, and/or IRAK2 to the TLR/MyD88 signaling complex. IRAK1 or IRAK2 becomes rapidly phosphorylated by IRAK-4, leaves the receptor complex, and then associates with TNF-α receptor-associated factor (TRAF) 6. Binding of TRAF6 to IRAK1 or IRAK2 leads to the activation of TGF-β-activated kinase 1 (TAK1) that ultimately results in activation of signaling cascades, leading to the activation of MAPKs and NF-κB and subsequent expression of proinflammatory cytokines, chemokines, and oncogenes (2–4). MyD88 is also essential for the activation of IFN regulatory factor (IRF) 7 by TLR7 and TLR9 in plasmacytoid dendritic cells (DCs) and activation of TRAF3 by TLR9 in macrophages and DCs, which lead to production of type I IFNs (5, 6). MyD88 deficiency in macrophages and DCs results in lack of NF-κB activation, MAPK activation, and proinflammatory cytokine production by TLR2, TLR5, TLR7, and TLR9 (7–10). Although TLR4 ligand-mediated production of proinflammatory cytokines is completely suppressed, TLR4 ligand-mediated activation of NF-κB and MAPKs is delayed, but not inhibited, and production of IFN-β and IFN-γ-inducible protein 10 (IP-10) is not produced in MyD88-deficient cells (11, 12). In addition, TLR3 ligand-mediated TNF-α production is normal in MyD88-deficient cells (13). The presence of these MyD88-independent pathways leads to the identification of other TIR domain-containing adaptor proteins, as follows: MyD88 adaptor-like (MAL)/TIR domain-containing adaptor protein (TIRAP; functions to recruit MyD88 to TLR2 and TLR4), TIR domain-containing adaptor-inducing IFN-β (TRIF), and TRIF-related adaptor molecule (functions to recruit TRIF to TLR4) (13–19). TRIF is the TIR domain-containing adaptor for TLR3 and is required for the TLR4-induced MyD88-independent pathway (13, 16). TRIF recruits receptor-interacting protein 1 and two TRAF family members, TRAF6 and TRAF3 (20–22). Receptor-interacting protein 1 and TRAF6 are required for the TRIF-dependent NF-κB activation and proinflammatory cytokine production, whereas TRAF3 recruits TANK binding kinase 1 and IκB kinase to activate IRF3, resulting in the
production of IFN-β and chemokines. Although there is increasing information about signaling pathways of TLRs and TIR domain-containing adaptors, the complete picture of the pathways downstream of the individual TLRs and TIR domain-containing adaptors remains yet to be revealed.

The protein kinase D (PKD) family is a group of three structurally related serine/threonine kinases (PKD1/protein kinase C (PKC) μ, PKD2, and PKD3/PKCα) that regulate diverse cellular and subcellular processes, such as transportation of vesicles; regulation of cell shape, motility, and adhesion; activation of MAPKs and NF-κB; and expression of various genes (23). Several studies have indicated that PKD family proteins might play a role in TLR signaling. LPS-mediated p38 activation and TNF-α secretion in microglial cells are suppressed by a PKC/PKD inhibitor (inhibits PKCα, PKCβ, and PKD3), G60976 (24). PKD1 has been shown to bind and phosphorylate human TLR5, and G60976 suppresses a TLR5 ligand flagellin-mediated p38 activation and IL-8 production in epithelial cells (25). TLR1/2 ligand palmitoyl-3-cysteine-serine-lysine-4 (Pam3CSK4) induces activation of PKDs via a PKC-independent manner, and Pam3CSK4-induced expression of heat shock protein 27 and MCP-1, as well as activation of PKDs, is inhibited by G60976, but not by broad-spectrum PKC inhibitors, in mouse bone marrow-derived mast cells (26). In addition to these studies, we recently found that PKD1, but not PKD2 and PKD3, is recruited to the TLR9/MyD88/IRAK/TRAF6 signaling complex and being activated upon the TLR9 ligand CpG DNA stimulation and required for activation of NF-κB and MAPKs, and subsequent expression of cytokines (27). In the present study, we investigated whether all TLR ligands induce activation of any PKD family protein and physiological roles of PKD1 in individual signaling from TLRs.

Materials and Methods

Mice

C57BL/6 and BALB/c mice at 4–5 wk of age were obtained from the Frederick Cancer Research and Development Center, National Cancer Institute, and were used within 3 wk. TLR9 gene-deficient (TLR9−/−) mice and MyD88−/− mice were provided by S. Akira (Osaka University, Osaka, Japan). TLR2 gene-deficient (TLR2−/−, Tlr2mice) mice, TLR4 P712H mutant (Tlr4−/−, Tlr4mice) mice, and TRIF-defective mutant (the deletion of a single guanine within codon 708 of TRIF gene, Tlr3mice) mice were purchased from The Jackson Laboratory. All animal care and housing requirements were set forth by the National Institutes of Health Committee on the Care and Use of Laboratory Animals of Institute of Laboratory Animal Resources, and all animal protocols were reviewed and approved by the University of Tennessee Animal Care and Use Committee.

Isolation of murine peritoneal macrophages, cell lines, and culture conditions

Peritoneal macrophages were isolated, as described (27). Peritoneal macrophages and RAW264.7 cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO2 humidified incubator. All culture reagents were purchased from Invitrogen and Sigma-Aldrich.

Oligodeoxynucleotides and reagents

Nucleotide-resistant phosphorothioate oligodeoxynucleotides 1826 (CpG DNA) were purchased from Coley Pharmaceutical Group. Pam3CSK4 (synthetic tripalmitoylated lipopeptide), peptidoglycan (PGN), diacylated synthetic lipidopeptide (PSL-1) flagellin, imiquimod, and poly(I:C) were purchased from Invitrogen. Ultrapure LPS (Escherichia coli 0111:B4) and muramyl dipeptide (MDP) were purchased from List Biological Laboratories and Sigma-Aldrich, respectively. Recombinant murine cytokines (IL-1β, IL-18, and IFN-γ) were purchased from R&D Systems. PMA was purchased from Calbiochem.

Plasmids, generation of FLAG-tagged PKD-expressing macrophages and gene-specific knockdown macrophages, transient transfection, reporter gene assay, and RT-PCR

FLAG-tagged PKD-expressing macrophages and gene-specific knockdown macrophages were generated, as described (27). Transient transfections, reporter gene assays, and RT-PCR were done, as previously described (28). Luciferase activity was normalized using pRL-TK-luciferase activity (Renilla) in each sample. Actin or GAPDH was used as a loading control for all RT-PCR. All primers for cloning, gene-specific small hairpin interfering RNA (shRNA targeting), and RT-PCR were purchased from Integrated DNA Technologies, and sequences of primers are listed in Table I or described previously (27).

Generation of murine bone marrow-derived DCs (BMDCs) and small interfering RNA (siRNA) transfection

Mouse bone marrow cells were obtained by flushing femoral and tibial bone marrow cavities with PBS using a 10-ml syringe with a 25-gauge needle. After removing RBC, the bone marrow cells were resuspended in conditioned medium (RPMI 1640 supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), 2-ME (50 μM), 10% heat-inactivated FBS, and 20 ng/ml mouse rGM-CSF (BD Biosciences)) and plated at 2 × 105 cells/ml in a 100-mm petri dish. On days 3 and 6, medium was replaced with fresh conditioned medium. On day 8, BMDCs (5 × 105/ml) were cultured overnight in medium without antibiotics and then transfected with 100 nM nontarget siRNA (Dharmacon) or PKD1-specific siRNAs (5′-CTTGGATCATCAGGACCTTCTCCAA 3′) using lipofectamine (Invitrogen), according to the manufacturer’s protocol.

ELISA, Western blot assay, and EMSA

Concentrations of the selected cytokines in culture supernatants, levels or phosphorylation status of specific proteins in whole cell extracts, and nuclear DNA-binding activities of NF-κB and AP-1 were analyzed by ELISA, Western blot assay, and EMSA, respectively, as described previously (27, 28). Actin was used as a loading control for all Western blot assays. All recombinant murine cytokines and Abs specific for murine cytokines were purchased from BD Biosciences. Ab specific for TLR9 was purchased from IMGENEX. Ab specific for IRAK4 was purchased from ABGENT. Ab specific for IRAK1 was purchased from Upstate Biotechnology. Abs specific for TLR4, MyD88, TRAF6, PKD, IκBα, or IκBβ were purchased from Santa Cruz Biotechnology. All phospho-specific Abs were purchased from Cell Signaling Technology.

In vitro kinase assays

Each FLAG-tagged PKD protein in whole-cell lysates was immunoprecipitated with anti-FLAG Ab. The resulting immune complexes were subjected to in vitro kinase assay using Syntide-2 (Sigma-Aldrich) as a PKD substrate, as previously described (29).
**FIGURE 1.** TLR ligands induce activation of PKD1 in macrophages. A, RAW264.7 cells were stimulated with medium (med), CpG DNA (12 μg/ml), LPS (50 ng/ml), Pam3Cys (Pam3CSK4, 1 μg/ml), PGN (10 μg/ml), FSL-1 (0.1 μg/ml), flagellin (1 μg/ml), imiquimod (10 μg/ml), poly(I:C) (PIC; 50 μg/ml), MDP (10 μg/ml), or PMA (10 ng/ml) for 45 min. B, RAW264.7 cells were stimulated with poly(I:C) (PIC; 50 μg/ml) or MDP (10 μg/ml) for the indicated time period. CpG DNA (12 μg/ml) or PMA (10 ng/ml) was used as positive control. Activation status of PKDs was detected by Western blot assay using Abs specific for the phosphorylated forms of PKDs (pPKD744/748, pPKD916). Phosphorylation of p38 was detected as an indication that each stimulus was functional. C and D, RAW264.7 cells stably expressing empty vector, FLAG-tagged PKD1, FLAG-tagged PKD2, or FLAG-tagged PKD3 were stimulated as indicated for 45 min. Each PKD family protein in whole-cell lysates was immunoprecipitated with anti-FLAG Ab. Kinase activity of PKDs was analyzed by in vitro kinase assay using syntide-2 as a PKD substrate (top). Expression and phosphorylation status of each PKD were analyzed by immunoblotting with anti-FLAG and anti-phospho-PKD Abs, respectively (bottom). E, Control luciferase-knockdown (Luc shRNA) or PKD1-knockdown (PKD1 shRNA) macrophages were stimulated as indicated for 45 min. Activation status of PKDs was detected by Western blot assay.
Flow cytometric analysis

To analyze cell surface expression of CD86, cells were stained with allophycocyanin-conjugated rat anti-mouse CD86 or allophycocyanin-conjugated isotype control. CD86 expression was analyzed with BD FACS Aria II flow cytometer (BD Biosciences). All Abs were purchased from BD Biosciences.

Results

**TLR ligands, except TLR3 ligand, induce activation of PKD1**

Recent studies using pharmacological inhibitor of PKC/PKD indicated that PKD family proteins may be implicated in TLR signaling (24–26). In addition, we found that PKD1 is being activated by TLR9 ligands and plays a pivotal role in TLR9-mediated innate immune cell activation (27). However, it is currently not known whether PKD1 functions in other TLR signaling pathways. Using Abs specific for phosphorylated forms of PKD family proteins, we have investigated whether TLR ligands activate PKD family member in macrophages. As shown in Fig. 1B, all TLR ligands activate any PKD family member in macrophages. However, the TLR3 ligand poly(I:C) and the NOD2 ligand MDP did not induce phosphorylation of PKDs, although they induced phosphorylation of MAPK p38 (Fig. 1, A and B). This result indicates that the TLR ligand that induces recruitment of MyD88 to its receptor activates one or more PKD family members. We further investigated whether PKD family member(s) is being activated by these TLR ligands using macrophages that express each FLAG-tagged PKD family member (27). Phosphorylation status and kinase activity of PKD family members were detected by phospho-PKD-specific Western blot analysis and in vitro kinase assay, respectively, following immunoprecipitation of each FLAG-tagged PKD family member. As shown in Fig. 1C, all ligands for TLRs that use MyD88, including CpG DNA, LPS, PGN, flagellin, and imiquimod, induced both kinase activity and phosphorylation of PKD1 in RAW264.7 cells, whereas TLR3 ligand poly(I:C), which transduces its signal through TRIF, did not. In contrast, these TLR ligands induced kinase activity and phosphorylation of neither PKD2 nor PKD3. Of note, PMA induced activation of all three PKD family members. These results indicate that PKD1 may be a common downstream target for signaling mediated through MyD88.

In addition to TLRs, IL-1R and IL-18R contain a TIR domain and transduce the signal through MyD88 (30). Therefore, we further investigated whether IL-1β and IL-18 also induce activation of PKD1. As shown in Fig. 1D, both IL-1β and IL-18 induced kinase activity and phosphorylation of PKD1, but not PKD2 or PKD3. Moreover, all TLR ligands, IL-1β, and IL-18 failed to induce phosphorylation of PKDs in PKD1-knockdown macrophages, further confirming that they only activate PKD1, not PKD2 or PKD3 (Fig. 1E). Of note, mRNA and protein levels of TLR signaling molecules, PKD2, and PKD3 in PKD1-knockdown macrophages were comparable to those in control luciferase-knockdown macrophages (27). Taken together, our results demonstrate that TLR/IL-1R/IL-18R ligands, except for the TLR3 ligand, activate PKD1, and suggest that PKD1 activation by these ligands may facilitate the signals transduced through MyD88.

**TLR ligands activate PKD1 via a MyD88-dependent pathway**

Because our results demonstrated that PKD1 is being activated by TLR ligands and cytokines that use adaptor molecule MyD88 to transduce their signals, we further investigated whether TLR ligands activate PKD1 through a MyD88-dependent manner. As shown in Fig. 2A, PAMCSK4, LPS, and CpG DNA failed to activate PKD1 in macrophages lacking their own functional receptor. In addition, PKD1 activation by TLR ligands (CpG DNA, LPS, and PGN) failed to induce PKD1 activation through a MyD88-dep
induce activation of PKD1 in both IRAK4-knockdown macrophages and IRAK1-knockdown macrophages. In contrast, PKD1 activation by these TLR ligands was substantially suppressed in TRAF6-knockdown macrophages. Expression of TLRs, MyD88, and PKD1 was normal in IRAK4-knockdown macrophages, IRAK1-knockdown macrophages, and TRAF6-knockdown macrophages (Fig. 3, A, B, E, and F) (31). Of note, PMA-mediated PKD activation in IRAK4-knockdown, IRAK1-knockdown, or TRAF6-knockdown macrophages was comparable with that in the control luciferase-knockdown macrophages. These results indicate that IRAK4 and IRAK1, but not TRAF6, are necessary for TLR ligand-mediated PKD1 activation and that TRAF6 may either be a signaling partner or a downstream signaling effector of PKD1 rather than an upstream activator of PKD1 in TLR/MyD88 signaling.

MyD88-dependent TLR ligands failed to induce ubiquitination of TRAF6 in PKD1-knockdown macrophages

Because our results indicate the possibility that TRAF6 may either be a signaling partner or a downstream signaling effector of PKD1 in TLR/MyD88-signaling pathway, we further investigated whether PKD1 is required for TLR ligand-mediated activation of TRAF6. Ubiquitination of TRAF6 has been shown to be required for activation of all TRAF6 downstream signaling events (3). Therefore, we detected ubiquitination of TRAF6 as an indication of TRAF6 activation. As shown in Fig. 4, CpG DNA, LPS, and PGN induced ubiquitination of TRAF6 in control cells. However, CpG DNA and PGN (TLR ligands that use only the MyD88-dependent pathway) failed to induce ubiquitination of TRAF6 in PKD1-knockdown macrophages. In contrast, LPS (which uses both MyD88-dependent and TRIF-dependent pathways)-mediated ubiquitination of TRAF6 was delayed and attenuated, but not abolished in PKD1-knockdown macrophages.
PKD1 is essential for MyD88-dependent pathway, but not TRIF-dependent pathway

Activation of TAK1 in TLR signaling is dependent on a MyD88/IRAK4/IRAK1/TRAF6 pathway as well as a TRIF/TRAF6 pathway (32–34). Activation of TAK1, in turn, leads to activation of NF-κB and MAPKs that regulate expression of proinflammatory cytokines and chemokines. To determine the precise physiological role of PKD1 in individual TLR signaling, we generated PKD1-knockdown macrophages and investigated whether PKD1 is required for the activation of TAK1 and its downstream MAPKs and NF-κB. Expression of TLRs and TLR-downstream signaling modulators in PKD1-knockdown macrophages was comparable to that in control luciferase-knockdown macrophages (27). CpG DNA-, PGN-, or IL-1β (which uses only the MyD88-dependent pathway)-mediated activation of TAK1, MAPKs (ERK, JNK, and p38), and transcriptional factors (NF-κB, AP-1, CREB, and STAT1) was ablated in PKD1-knockdown macrophages (Fig. 5, A, C, E, and G). However, LPS (which uses both MyD88-dependent and TRIF-dependent pathways)-mediated activation of TAK1, MAPKs, and transcription factors (NF-κB, AP-1, CREB, and STAT1) was delayed and attenuated, but not abolished in PKD1-knockdown macrophages (Fig. 5). In addition, activation of TRIF-dependent transcription factor IFN-α-stimulated responsive element (ISRE) by LPS was not altered in PKD1-knockdown macrophages (Fig. 5G). As expected, poly(I:C)- or IFN-γ-mediated activation of transcription factors (NF-κB, AP-1, CREB, and ISRE) was not suppressed in PKD1-knockdown macrophages (Fig. 5G). These results suggest that PKD1 is required only for the MyD88-dependent activation of TAK1, MAPKs, and transcription factors.

MyD88 is essential for gene expression induced by TLR2, TLR5, TLR7, TLR8, and TLR9, whereas TRIF is essential for gene expression by TLR3 (1). However, TLR4-mediated gene expression is dependent on MyD88 and/or TRIF (12, 16, 35). The TLR4 ligand LPS-mediated expression of proinflammatory cytokines requires both MyD88 and TRIF, whereas LPS-mediated expression of IFN-β, IP-10, MCP-1, and CCL5 requires only TRIF, and either MyD88 or TRIF is sufficient for CD86 expression induced by LPS. To further determine whether PKD1 is involved only in MyD88-dependent signaling pathways, we examined the expression of various cytokines and chemokines by TLR ligands in PKD1-knockdown macrophages and PKD1-knockdown BMDCs. Expression of TLRs, TLR signaling downstream molecules, PKC family proteins, PKD2, and PKD3 was normal in PKD1-knockdown macrophages and PKD1-knockdown BMDCs (Fig. 7, A and B) (27). As shown in Figs. 6, A and B, and 7, C and D, expression of cytokines TNF-α, IL-6, IL-10, and IL-12p40 at both mRNA and protein levels in response to CpG DNA, LPS, or PGN was completely inhibited in PKD1-knockdown macrophages and PKD1-knockdown BMDCs. In addition, a TLR9 ligand CpG DNA failed to induce expression of IP-10, MCP-1, CCL5, and CD86 in PKD1-knockdown macrophages and PKD1-knockdown BMDCs (Fig. 6, C and D, and Fig. 7, D and E). However, CpG DNA-mediated IFN-β expression that is dependent on TRAF3, not TRAF6, was not suppressed in PKD1-knockdown BMDCs (Fig. 7D). LPS-mediated expression of IFN-β, IP-10, MCP-1, CCL5, and CD86 was not altered in PKD1-knockdown macrophages and PKD1-knockdown BMDCs compared with those in the control cells (Fig. 6, C and D, and Fig. 7, D and E). Moreover, expression levels of cytokines (TNF-α, IL-6, IL-10, IL-12p40, and IFN-β), chemokines (IP-10, MCP-1, and CCL5), and surface molecule CD86 induced in response to TLR3 ligand poly(I:C) were comparable in both PKD1-knockdown cells and control cells (Fig. 6 and Fig. 7, C–E). These results demonstrate that PKD1 is essential for the MyD88-dependent, but not TRIF-dependent, signaling pathways in macrophages and DCs.

We further investigated whether PKD1 is also critical in TLR ligand-mediated MyD88-dependent activation of signaling modulators and expression of cytokines and chemokines in vivo. C57BL/6 mice were stimulated with CpG DNA, LPS, or poly(I:C) in the presence or absence of PKC/PKD inhibitor Gö6976 for indicated time periods. Activation of MAPKs, degradation of IκB (as an indication of NF-κB activation), and expression of the selected cytokines and chemokines in spleen cells were analyzed. Systemic production of the selected cytokines (TNF-α, IL-6, IL-10, and IL-12) in serum was also detected. As demonstrated in Fig. 8A, systemic administration of Gö6976 effectively inhibited CpG DNA- and LPS-mediated PKD1 activation in vivo. CpG DNA failed to induce activation of JNK, p38, and ERK; degradation of IκBα and IκBβ; and expression of proinflammatory cytokines and chemokines. These results suggest that PKD1 is essential for the MyD88-dependent, but not TRIF-dependent, signaling pathways in macrophages and DCs.
PKD1 is required for MyD88-dependent activation of TAK1, MAPKs, and transcription factors. A–F, Control luciferase-knockdown (Luc shRNA) or PKD1-knockdown (PKD1 shRNA) macrophages were stimulated as indicated for 45 min (A, C, and E), 1 h (E, for NF-κB), or 4 h (E, for AP-1), or stimulated with LPS for indicated time periods (B, D, and F). Phosphorylation of TAK1, JNK, p38, ERK, CREB, and STAT1 was detected by Western blot analysis. DNA-binding activities of transcription factor, NF-κB, or AP-1, in equal amounts of nuclear extracts (3 μg/lane), were analyzed by EMSA (gel shift), and degradation of IκBα and IκBβ in cytosolic extracts was detected by Western blot analysis (WB). G, Cells were transiently transfected with AP-1-β-galactosidase, or pRL-TK-luciferase plus NF-κB-luciferase, CREB-luciferase, or ISRE-luciferase reporter genes and then stimulated as indicated for 12 h. Luciferase (NF-κB, CREB, or ISRE) or β-galactosidase (AP-1) activities in cell extracts were analyzed. Data represent the mean relative luciferase unit (RLU) ± SD of triplicates.
chemokines (TNF-α, IL-6, IL-10, IL-12, IP-10, MCP-1, and CCL5) in mice pretreated with Go6976 (Fig. 8). However, CpG DNA-mediated IFN-β expression was not suppressed in mice pretreated with Go6976 (Fig. 8B). In comparison, LPS-mediated activation of MAPKs and degradation of IκBα and IκBβ were partially (but to a substantial degree) inhibited in mice pretreated with Go6976 (Fig. 8A). LPS-mediated expression of proinflammatory cytokines (TNF-α, IL-6, IL-10, IL-12) was completely ablated in mice pretreated with Go6976 (Fig. 8, B and C). In contrast, LPS-mediated expression of IFN-β, IP-10, MCP-1, and CCL5 was not suppressed in mice pretreated with Go6976 compared with that in the control mice (Fig. 8B). Moreover, levels of MAPK activation, IκBα and IκBβ degradation, and cytokine (TNF-α, IL-6, IL-10, IL-12p40, and IFN-β) and chemokine (IP-10, MCP-1, and CCL5) expression induced in response to TLR3 ligand poly(I:C) were comparable in both control mice and mice pretreated with Go6976 (Fig. 8). Collectively, these results indicate that PKD1 is essential for the expression of proinflammatory cytokines and chemokines in vitro and in vivo.

**Discussion**

In this study, we identified PKD1 as one of the critical proximal signaling molecules in the TLR signal transduction pathway and determined the physiological role of PKD1 in the signal transduction pathways used by TLR family members. Among the three PKD family proteins, only PKD1 is activated by all TLRs that recruit MyD88 and is required for MyD88-dependent activation of MAPKs and transcription factors and subsequent expression of proinflammatory cytokine and chemokine genes by the TLR family members regardless of involvement of MAL/TIRAP. In contrast, PKD1 is not activated by TLR3 and is not necessary for signaling via the TRIF-dependent pathway of TLR3 and TLR4.

Using Abs specific for the phosphorylated forms of PKD family proteins and pharmacological inhibitor Go6976 (inhibits PKCα, PKCβI, and PKD), several recent studies have suggested that members of PKD family proteins may be implicated in activation of MAPKs and production of cytokines and chemokines in response to ligands for TLR1/2, TLR4, TLR5, and TLR9 (24–27). However, to date, it is not known whether all TLR ligands induce
activation of one or more PKD family members, and which PKD family member(s) plays a physiological role in individual signaling from TLRs. Our experiments showed that with exception of TLR3 ligand, all TLR ligands tested induced increased phosphorylation and kinase activity of PKD1, but not PKD2 and PKD3. In addition, we found that PKD1 was activated in macrophages in response to IL-1β and IL-18, cytokines that use MyD88 for transducing signals from their receptors. These indicate the possibility that PKD1 might be activated through and functioning in the MyD88-dependent pathway. Indeed, MyD88−/− macrophages showed defects in PKD1 activation in response to TLR ligands, IL-1β, and IL-18. In contrast, activation of PKD1 by TLR ligands was normal in TRIF-defective mutant (Trif−/−) macrophages. These results demonstrate that MyD88, but not TRIF, is necessary for TLR-mediated PKD1 activation.

Previous studies have shown that IRAK family proteins interact with MyD88 and TRAF6 and play a critical role in MyD88-dependent pathway. Indeed, MyD88−/− macrophages showed defects in PKD1 activation in response to TLR ligands, IL-1β, and IL-18. In contrast, activation of PKD1 by TLR ligands was normal in TRIF-defective mutant (Trif−/−) macrophages. These results demonstrate that MyD88, but not TRIF, is necessary for TLR-mediated PKD1 activation.

Previous studies have shown that IRAK family proteins interact with MyD88 and TRAF6 and play a critical role in MyD88-dependent ATM ubiquitination and downstream signal transduction (3, 36–39). IRAK4 has shown to induce phosphorylation of IRAK1, which reveals hidden three TRAF6-binding motifs present in C-terminal region of IRAK1, and phosphorylated IRAK1 is dissociated from MyD88 and binds to TRAF6 (39). Upon CpG DNA (or LPS) stimulation, PKD1 physically interacts with TLR9 (or TLR4), MyD88, IRAK4, IRAK1, and TRAF6, but not with TRAF3, in macrophages (data not shown) (27). However, PKD1 failed to interact with MyD88 and TRAF6 in either IRAK4-defective macrophages or IRAK1-defective macrophages (data not shown). In addition, our results showed that PKD1 activation by TLR ligands is dependent on both IRAK4 and IRAK1, whereas it is independent of TRAF6. Instead, PKD1 is appeared to be required for MyD88-dependent ubiquitination of TRAF6. Although the precise mechanism by which IRAK family proteins interact and activate PKD1 upon TLR ligand stimulation has yet to be revealed, our findings indicate that recruitment of PKD1 to the TLR/MyD88 receptor complex is indirect via its binding to IRAK4 and IRAK1, and that PKD1 might play a critical role in linking
IRAK family proteins to TRAF6. Recently using HEK293T cells and overexpression system, Bowie and colleagues (3) demonstrated that IRAK2 is involved in TRAF6 ubiquitination and NF-κB activation via a mechanism dependent on MyD88 and MAL/TIRAP, but independent of TRIF, and plays a role in TLR3-, TLR4-, and TLR8-mediated cytokine production. In addition, Akira and colleagues (4) reported that IRAK2 is dispensable for activation of the initial TLR signaling cascades and early-phase gene expression, but contributes to the sustained (late-phase) NF-κB activation using IRAK2-knockdown RAW264.7 cells (Y. I. Kim, J. E. Park, and A. K. Yi manuscript in preparation). In our hand, the levels of CpG DNA-mediated PKD1 activation, as well as activation of all three MAPKs, in IRAK2-knockdown RAW264.7 cells were comparable to those in the control RAW264.7 cells, indicating that IRAK2 is not involved in the, at least, initial phase of TLR-mediated PKD1 activation in macrophages. It is possible that IRAK2 may function as a PKD1-downstream signaling effector that connects PKD1 to TRAF6. However, physical interaction between PKD1 and IRAK2 was not detected in RAW264.7 cells within 45 min after CpG DNA stimulation, indicating that IRAK2 may be not essential for the, at least, early phase of TRAF6 ubiquitination in macrophages. Different findings between Bowie’s group and our laboratory may be due to different experimental systems used. However, we cannot rule out the possibilities that IRAK2 may be involved in the PKD1-independent late-phase TRAF6 ubiquitination induced by LPS in macrophages and/or IRAK2 may be a factor that substitutes both IRAK1 and PKD1 in the late-phase TLR responses. These possibilities are warranted to be investigated in future studies.

Similar to those observed in MyD88−/− cells (11, 16), activation of TAK1, MAPKs, and NF-κB by TLR2 ligand, TLR9 ligand, or IL-1 was completely suppressed in PKD1-knockdown macrophages. In addition, TLR4 ligand-mediated activation of TAK1, MAPKs, and NF-κB, which can be mediated through either MyD88-dependent or TRIF-dependent pathways, was delayed and attenuated in PKD1-knockdown macrophages. Furthermore, activation of TRIF-dependent transcription factor ISRE by LPS and activation of NF-κB, AP-1, CREB, and ISRE by TLR3 ligand were not altered in PKD1-knockdown macrophages. Because ubiquitination of TRAF6 is necessary for activation of TAK1, which leads to activation of NF-κB and MAPK pathways in both MyD88-dependent and TRIF-dependent pathways (32–34), and PKD1 is required for MyD88-dependent ubiquitination of TRAF6, effects of PKD1 on TLR ligand-mediated activation of MAPKs and NF-κB might be indirect due to its contribution on ubiquitination of TRAF6 and subsequent TAK1 activation. Taken together, these results demonstrated that PKD1 is essential for MyD88-dependent, but not TRIF-dependent, activation of TAK1 and its downstream signaling pathways, irrespective of involvement of MAL/TIRAP.

Production of proinflammatory cytokines, chemokines, and type I IFNs, which are critical for host defense against invading pathogens, by individual TLRs is differently regulated by signaling whole spleen cell lysates were prepared. Phosphorylation of PKD1, JNK, p38, and ERK and presence of IκBα and IκBβ in whole spleen cell lysates were detected by Western blot analysis (A). mRNA levels of the indicated cytokines and chemokines in spleen were analyzed by RT-PCR (B). Levels of the indicated cytokines in serum were analyzed by cytokine-specific ELISAs (C). Data represent the mean concentration (pg/ml) ± SD of triplicates.

**FIGURE 8.** Effect of pharmacological PKC/PKD inhibitor Go6976 on TLR ligand-mediated cytokine and chemokine expression. C57BL/6 mice were injected i.p. with DMSO or Go6976 (2.5 mg/kg body weight) at 4 and 1 h before the TLR ligand stimulation. DMSO- or Go6976-pretreated mice were injected i.p. with PBS, CpG DNA (30 μg/mouse), LPS (2 μg/mouse), or poly(I:C) (150 μg/mouse). Two hours later, mice were bled to obtain serum and then euthanized. Spleen was isolated. Total spleen RNA and...
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PKD1 activation by TLR9 involves ubiquitination of TRAF6. Ubiquitinated TRAF6 leads to the activation of IRAK1, which in turn activates signaling cascades that lead to activation of MAPKs and NF-κB, and subsequent production of proinflammatory cytokines and chemokines.

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