The Tumor Suppressor Cyldromatosis (CYLD) Acts as a Negative Regulator for Toll-like Receptor 2 Signaling via Negative Cross-talk with TRAF6 and TRAF7*

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Toll-like receptor 2 (TLR2) plays an important role in host defense against bacterial pathogens. Activation of TLR2 signaling not only induces the activation of innate immunity and instructs the development of the acquired immunity but also leads to the detrimental inflammatory responses in inflammatory and infectious diseases. To avoid detrimental inflammatory responses, TLR2 signaling must be tightly regulated. In contrast to the relative known positive regulation of TLR2 signaling, its negative regulation, however, is largely unknown. In addition the distal signaling components that link TLR2 to its downstream signaling pathways have yet to be further defined. In the present study we have provided direct evidence for the negative regulation of TLR2 signaling by the tumor suppressor cyldromatosis (CYLD). We showed that activation of TLR2 signaling by TLR2 ligands including peptidoglycan (PGN), MALP-2, and Pam3CSK4 induces activation of IKKs-IκBα and MKK3/6-p38 pathways not only by TRAF6 but also by TRAF7, a recently identified TRAF family member. The activation of both pathways leads to the transcription of TNF-α, IL-1β, and IL-8 as well as CYLD. CYLD in turn leads to the inhibition of TRAF6 and TRAF7 likely via a deubiquitination-dependent mechanism. The present studies thus unveil a novel autoregulatory feedback mechanism that negatively controls TLR2-IKKs-IκBα and MKK3/6-p38-NF-κB-dependent induction of immune and inflammatory responses via negatively cross-talking with both TRAF6 and TRAF7. These findings provide novel insights into autoregulation and negative regulation of TLR signaling.

Toll-like receptors (TLRs) play critical roles in host defense against invading pathogens by recognizing microbial components and activating complex signaling networks that in turn induce distinct patterns of gene expression, which not only lead to the activation of innate immunity but also instructs the development of antigen-specific acquired immunity (1–4). However, TLRs have also been shown to be involved in the pathogenesis of autoimmune, chronic inflammatory, and infectious diseases (4). Therefore, to avoid detrimental and overactive inflammatory responses, TLR signaling must be tightly regulated. In contrast to the known positive regulation of TLR signaling, the negative regulation of TLR signaling remains largely unknown (4–6). TLRs are type I transmembrane receptors with leucine-rich repeats in the extracellular domains and cytoplasmic domains that resemble the mammalian IL-1 receptor (IL-1R) (1–3). To date, 11 members of the human TLR family have been cloned (1–3). Of these, TLR2 and TLR4 have been well studied. While TLR4 seems to be mainly involved in Gram-negative bacteria lipopolysaccharide signaling, TLR2 can respond to a variety of bacterial products, including peptidoglycan (PGN), lipoprotein, lipoteichoic acid, and lipoarabinomannan (1–3). The importance of TLR2 in host defense was further highlighted by the studies from knock-out mice showing decreased survival of TLR2-deficient mice after infection with Gram-positive Staphylococcus aureus (9). Furthermore, our recent study demonstrated that TLR2 also plays a key role in activating host immune and inflammatory response by surface lipoprotein from the Gram-negative bacterium nontypeable Haemophilus influenzae (9), a major cause of otitis media and exacerbation of chronic obstructive pulmonary diseases (10–14). Thus, it is clear that TLR2 plays a crucial role in host defense against both Gram-positive and -negative bacteria. Similar to other TLRs, the negative regulation of TLR2 signaling also remains largely unclear. Despite recent studies showing that the tumor suppressor CYLD acts as a negative regulator for TNF receptor-induced activation of NF-κB and JNK via inhibition of the tumor necrosis factors receptor-associated factor 2 (TRAF2) (15–19), its role in negatively regulating of TLR2 signaling has yet to be determined.

Although various proximal signaling components of TLR2 have been relatively well studied, the distal signaling components that link TLR2 to its downstream signaling pathways including NF-κB and MAPks remain largely unknown (1–4, 7). Among all known key signaling transducers downstream of TLR2, TRAF6 has been shown to be critically involved in activation of both NF-κB and p38 triggered by TLR family members (20–24). In addition to TRAF6, TRAF7 has been recently identified as a signaling transducer upstream of MKK3-AP1 pathway (25, 26). Its role in mediating TLR-dependent activation of both NF-κB and p38 pathways still remains unclear.

In the present study, we provided evidence for the first time that activation of TLR2 signaling by TLR2 ligands induces activation of IKKs-IκBα and MKK3/6-p38 signaling pathways not only by TRAF6 but also by TRAF7. The activation of both IKKs-IκBα and MKK3/6-p38 pathways will induce transcription of TNF-α, IL-1β, and IL-8 as well as CYLD. CYLD in turn leads to the inhibition of TRAF6 and TRAF7 likely via a deubiquitination-dependent mechanism. The present studies thus identified a novel autoregulatory feedback mechanism that negatively controls TLR2-IKKs-IκBα/MKK3/6-p38-NF-κB-dependent induction of immune and inflammatory responses via negatively cross-talking
with both TRAF6 and TRAF7. These findings provide novel insights into autoregulation and negative regulation of TLR signaling.

MATERIALS AND METHODS

Reagents and Plasmids—Caffeic acid phenethyl ester (CAPE), MG-132, and SB203580 were purchased from Calbiochem (La Jolla, CA). PGN from S. aureus, Pam3CSK4 and R837 (Imiquimod) were purchased from InvivoGen (San Diego, CA). MALP-2 was purchased from ALEXIS Biochemicals (San Diego, CA). The plasmids IκBα (S32/ 36A), IKKα (K44M), IKKβ (K49A), NEMO DN, fp38(AF), fp38β2, (AF), MKK3β(A), MKK6β(A), TRAF2 DN, TRAF6, TRAF6 DN, HA-TRAF7, HA-TRAF7 DN, HA-CYLD, FLAG-CYLD, and NF-κB-luciferase reporter were described previously (10–15, 25, 27, 28).

Cell Culture—Human lung epithelial cell line A549 and human cervix epithelial cell line HeLa were maintained as described (10, 33, 34). Stable cell lines of HEK293-pcDNA, HEK293-TLR2, and HEK293-TLR4 were kindly provided by Dr. Douglas T. Golenbock (28, 29). All stable cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen), 0.5 mg/ml G418, and 10 μg/ml ciprofloxacin (Cellgro, Herndon, VA) in a 5% saturated CO2 atmosphere at 37 °C.

Transfection and Luciferase Assay—Cells were cultured on 24-well plates. After 24 h, cells were co-transfected with NF-κB-luciferase reporter plasmid and various expression plasmids as indicated in the legends to Figs. 1–5. Empty vector was used as a control and was added where necessary to ensure a constant amount of input DNA. All transient transfections were carried out in triplicate using a TransIT-LT1 reagent (Mirus, Madison, WI) following the manufacturer’s instructions. At 40 h after the start of transfection, cells were pretreated with or without chemical inhibitors including 10 μg/ml CAPE, 10 μM MG-132, and 1 μM SB203580 for 1 h. PGN (5 μg/ml), MALP-2 (10 ng/ml), Pam3CSK4 (1 μg/ml), or R837 (10 μg/ml) were then added to the cells for 5 h before cell lysis for luciferase assay. Luciferase activity was normalized with respect to β-galactosidase activity.

Real-time Quantitative Reverse Transcription-PCR (Q-PCR) Analysis—Total RNA was isolated by using TRIZol reagent (Invitrogen) by following the manufacturer’s instructions. For the reverse transcription reaction, TaqMan reverse transcription reagents (Applied Biosystems) were used. Briefly, the reverse transcription reaction was performed for 60 min at 37 °C, followed by 60 min at 42 °C by using oligo(dt) and random hexamers. PCR amplification was performed by using TaqMan universal master mix for human TNF-α, IL-1β, IL-8, and CYLD, or by using SYBR Green universal master mix for human TRAF7. In brief, reactions were performed in duplicate containing 2× universal master mix, 1 μl of template cDNA, 100 nM primers, and 100 nM probe in a final volume of 12.5 μl, and they were analyzed in a 96-well optical reaction plate (Applied Biosystems). Probes for TaqMan include a fluorescent reporter dye, 6-carboxyfluorescein, on the 5′ end and labeled with a fluorescent quencher dye, 6-carboxytetramethylrhodamine, on the 3′ end to allow direct detection of the PCR product. Reactions were amplified and quantified by using as ABI 7700 sequence detector and the manufacturer’s corresponding software (Applied Biosystems). Relative quantity of mRNAs were obtained by using the comparative Ct method (for details, see User Bulletin 2 for the Applied Biosystems PRISM 7700 sequence-detection system) and was normalized by using TaqMan predeveloped assay reagent human cyclophilin as an endogenous control (Applied Biosystems). Universal master mix and TaqMan predeveloped assay reagents (primer and probe mixture of human TNF-α, IL-1β, and IL-8) were purchased from Applied Biosystems. The primers and probe for human CYLD were as follows: 5′-ACG CCA CAA TCT TCA CAC T-3′ (forward primer) and 5′-AGG TGG TGG TCA AGG TTT CAC T-3′ (reverse primer); TaqMan probe, 5′-6-carboxyfluorescein-AAA AAG CTG TTT CCC TTG GTA CAC CCC C-6-carboxytetramethylrhodamine-3′. The primers for human TRAF7 were as follows: 5′-TGG AGT TGC GGC GGG-3′ (forward primer) and 5′-AGC GGC GCG TTG ATG T-3′ (reverse primer).

Immunofluorescence—Cells were cultured on four-chamber slides. After treatment with PGN, the cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 15 min. Fixed cells were subsequently blocked with 1.5% bovine serum albumin in PBS for 20 min and incubated with 1:250 dilution of mouse anti-p65 NF-κB antibody for 1 h (Santa Cruz Biotechnology). Primary antibody was detected with 1:200 dilution of fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology). Samples were examined and photographed by using an Axiopt microscope (Zeiss). For analyzing the co-localization of CYLD with TRAF6 and TRAF7, cells were co-transfected with the indicated combinations of TRAF6, HA-TRAF7, and FLAG-CYLD plasmids. After fixing, permeabilizing, and blocking, the cells were incubated with rabbit anti-TRAF6 antibody (Santa Cruz Biotechnology), rabbit anti-HA antibody (Santa Cruz Biotechnology), or mouse anti-FLAG antibody (Sigma). Primary antibodies were detected with fluorescein isothiocyanate-conjugated anti-mouse (Santa Cruz Biotechnology) or rhodamine-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology).

Small Interfering RNA (siRNA)—RNA-mediated interference for down-regulating CYLD expression was done using small interfering siRNA-CYLD (pSUPER-CYLD) as described previously (5, 15). For down-regulating TRAF7 expression, the siRNA-TRAF7 was purchased from Dharmacon. HEK293-TLR2 cells were cultured on 48-well plates. A final concentration of 200 nM siRNA-TRAF7 was co-transfected with NF-κB-luciferase reporter plasmid into 40–50% confluent cells using Lipofectamine 2000 (Invitrogen). At 40 h after the start of transfection, cells were treated with PGN, MALP-2, Pam3CSK4, or R837 for 5 h before being harvested for Luciferase assay. In contrast, for measuring siRNA effectiveness, cells were cultured on 12-well plates before co-transfection of siRNA-TRAF7 with HA-TRAF7, and Western blot analysis was performed. For confirming the down-regulation of the endogenous TRAF7, cells were transfected with siRNA-TRAF7, and Q-PCR was performed.

Western Blot Analysis and Immunoprecipitation—Antibodies against phospho-IκBα (Ser-32), phospho-p38 (Thr-180/Tyr-182), p38, and phospho-MKK3/6 (Ser-189/207) were purchased from Cell Signaling Technology (Beverly, MA), HA-probe (Y-11), and TRAF6 (H-274) were from Santa Cruz Biotechnology; TRAF6 (IM-536) was from IMGENEX (San Diego, CA), CYLD was from ALEXIS Biochemicals (San Diego, CA), FLAG and β-actin were from Sigma. Western blots were performed as described (11, 27) and following the manufacturer’s instructions. Briefly, Western blots were performed using whole cell extracts, separated on 6–10% SDS-PAGE gels, and transferred to polyvinylidine difluoride membranes (Pall Life Sciences, Pensacola, FL). The membrane was blocked with a solution of PBS containing 0.1% Tween 20 (PBS-T) and 5% nonfat milk. After three washes in PBS-T, the membrane was incubated in a 1:2000 dilution of a primary antibody. After another three washes in PBS-T, the membrane was incubated with 1:2000 dilution of the corresponding secondary antibody. The membrane was reacted with chemiluminescence reagent ECL (Amersham Biosciences) to visualize to blots. For immunoprecipitation, Cells were lysed in lysis buffer (20 mM Tris base, 50 mM NaCl, 50 mM sodium pyrophosphate, 30 mM NaF, 5 μM zinc chloride, 2 mM iodoacetic acid,
1% Triton X-100, pH 7.4) supplemented with “Complete” protease inhibitors (Roche Applied Science). 500 μl cell lysates (400–500 μg of total cellular protein) were precleared with protein A/G-agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. After centrifugation, supernatant was immunoprecipitated with 2 μg of the appropriate antibodies for 90 min at 4°C and then conjugated to protein A/G-agarose beads for overnight at 4°C. Immunoprecipitates were washed four times with the lysis buffer and suspended in a sample buffer (15% glycerol, 0.14 M Tris-HCl, pH 6.8, 1% SDS, 0.5 M dithiothreitol, 0.0006% bromphenol blue).

RESULTS

Both IKKs-1αBα and MKK3/6-p38 MAPK Signaling Pathways Are Required for TLR2-dependent Activation of NF-κB and NF-κB-dependent Transcription of Inflammatory Mediators—TLR2 plays an essential role in host defense response against bacterial pathogens by the recognition of conserved components of bacteria (1–8). Among various TLR2 ligands, PGN has been well recognized as an important ligand for TLR2-dependent activation of host immune and inflammatory responses (1–3, 7–8). Despite the known involvement of IKKs-1αBα pathway in TLR2-dependent activation of NF-κB, it is still unclear whether other signaling pathways such as p38 MAPK is also required for TLR2-dependent NF-κB activation. To fully elucidate the signaling pathways involved in TLR2-dependent NF-κB activation and NF-κB-dependent transcription of inflammatory mediators, we initially sought to confirm the involvement of IKKs-1αBα pathway in TLR2-dependent activation of NF-κB. We first assessed the effect of PGN on NF-κB-dependent promoter activity and nuclear translocation of p65 subunit of NF-κB in HEK293 cells that stably express wild-type TLR2, TLR4, or empty vector-pcDNA (hereafter called HEK293-TLR2, HEK293-TLR4, and HEK293-pcDNA, respectively). As shown in Fig. 1, A and B, PGN potently induced activation of NF-κB and p65 nuclear translocation in HEK293-TLR2 but not in HEK293-TLR4 or HEK293-pcDNA cells, suggesting PGN activates NF-κB specifically via TLR2 signaling. Consistent with this finding, PGN also potently induced the transcription of several inflammatory mediators, including TNF-α, IL-1β, and IL-8 that are known to be mainly controlled by NF-κB as assessed by real-time quantitative PCR analysis (Fig. 1C). We next examined whether IKKs-1αBα pathway is indeed involved in TLR2-dependent activation of NF-κB. Perturbing IKKs-1αBα signaling using CAPE (30) and MG-132 (31), specific inhibitors for nuclear translocation of NF-κB degradation of IκBα, respectively, or co-expressing dominant-negative mutant forms of IκBα, IKKα, IKKβ, or NEMO (also known as IKKγ) greatly inhibited PGN-induced NF-κB activation in HEK293-TLR2 cells (Fig. 1D).

Many cellular stress stimuli can simultaneously activate both NF-κB and p38 MAP kinase modules (13, 14, 21–25, 32–34). Due to this overlap, we explored the possibility that activation of p38 and MKK3/6, its known immediate upstream activators, is involved in the PGN-induced NF-κB activation. As shown in Fig. 1E, perturbing MKK3/6-p38 signaling using either SB203580, a specific inhibitor for p38 (35), or co-expressing dominant-negative mutant forms of p38α, p38β, MKK3, or MKK6, greatly inhibited PGN-induced NF-κB activation in HEK293-TLR2 cells (Fig. 1E). Interestingly, PGN-induced nuclear translocation of p65 was only blocked by CAPE and MG-132 but not by SB203580, suggesting IKKs-1αBα and MKK3/6-p38 MAPK signaling pathways are involved in TLR2-dependent activation of NF-κB via distinct mechanisms, p65 translocation-dependent and -independent manner, respectively. Consistent with the results observed using NF-κB promoter, PGN-induced transcription of TNF-α, IL-1β, and IL-8 was also inhibited by CAPE, MG-132, and SB203580 (Fig. 1G). Together, these data suggest that both IKKs-1αBα and MKK3/6-p38 MAPK signaling pathways are required for TLR2-dependent activation of NF-κB and NF-κB-dependent transcription of inflammatory mediators.

The Tumor Suppressor CYLD Negatively Regulates TLR2-dependent Activation of Both NF-κB and p38 MAPK Pathways—Due to the important role that negative signaling regulator plays in controlling host immune and defense response against bacterial infections, we next sought to identify the negative regulator for TLR2-dependent activation of both NF-κB and p38 MAPK pathways (4–6). Recent studies have identified the tumor suppressor CYLD, loss of which causes a benign human syndrome called cylindromatosis, as a key negative regulator for NF-κB signaling mainly activated by TNF-α (15–19). It is still unclear whether CYLD also acts as a negative regulator for TLR2-dependent activation of NF-κB and p38 signaling pathways. To determine whether CYLD negatively regulates TLR2 signaling, we first assessed the effect of co-expressing wild-type (WT) CYLD on PGN-induced NF-κB activation. As shown in Fig. 2A, co-expressing wild-type CYLD greatly inhibited PGN-induced NF-κB activation. We next sought to determine whether CYLD indeed acts as a negative regulator for PGN-induced NF-κB activation using a siRNA approach (8). We first confirmed the efficiency of CYLD-specific small interfering RNA (siRNA-CYLD) in reducing CYLD expression in HEK293-TLR2 cells co-transfected with siRNA-CYLD or control siRNA (5, 15). As expected, the CYLD protein was markedly reduced by siRNA-CYLD (Fig. 2B, left). Consistent with this result, the endogenous CYLD protein was also greatly reduced (Fig. 2B, middle). We then assessed the effect of siRNA-CYLD on NF-κB activation by PGN (10, 11). As shown in Fig. 2B (right), CYLD knockdown by siRNA-CYLD greatly enhanced NF-κB activation by PGN. To confirm whether CYLD indeed acts as a negative regulator for TLR2 signaling, we next assessed the effects of CYLD knockdown on NF-κB activation induced by more specific TLR2 ligands including MALP-2 and Pam3CSK4. As shown in Fig. 2C, CYLD knockdown using siRNA-CYLD enhanced NF-κB activation by both MALP-2 and Pam3CSK4, confirming that CYLD is indeed a negative regulator for TLR2 signaling. We then sought to determine whether CYLD also acts as a negative regulator for PGN-induced activation of both IKKs-1αBα and MKK3/6-p38 signaling pathways. As shown in Fig. 2D, co-expressing WT CYLD inhibited, whereas CYLD knockdown by siRNA-CYLD greatly enhanced, PGN-induced phosphorylation of IκBα, MKK3/6, and p38. Moreover, co-expressing WT CYLD also inhibited, whereas CYLD knockdown by siRNA-CYLD greatly enhanced, PGN-induced transcription of TNF-α, IL-1β, and IL-8 using Q-PCR analysis (Fig. 2E). Taken together, these data indicate that CYLD is indeed a negative regulator for TLR2-mediated NF-κB activation via negative cross-talk with IKKs-1αBα and MKK3/6-p38 signaling pathways.

Both TRAF6 and TRAF7 Are Required for Mediating TLR2-dependent Activation of NF-κB and p38 MAPK Pathways—We next sought to determine how CYLD negatively regulates both IKKs-1αBα and MKK3/6-p38 signaling pathways. Given the distinct features of both pathways, it is more logical that CYLD negatively regulates both pathways by inhibiting shared signaling transducers upstream of both IKKs-1αBα and MKK3/6-p38 signaling pathways rather than directly interacting with both pathways. In review of all known shared signaling transducers upstream of both IKKs-1αBα and MKK3/6-p38 pathways, the TRAFs, a family of adaptor proteins, have been shown to be critically involved in activation of both NF-κB and p38 triggered by TNF receptor and TLR family members (15–19). Among all six TRAFs identified previously, TRAF2 has been identified as a signal transducer associated with TNF receptors, whereas TRAF6 has been known to be associated
FIGURE 1. Both IKK-α/β and MKK3/6-p38 MAPK signaling pathways are required for TLR2-dependent activation of NF-κB and NF-κB-dependent transcription of inflammatory mediators. 

A, PGN induced NF-κB-dependent promoter activity in HEK293-TLR2 but not in HEK293-TLR4 cells. B, PGN induced nuclear translocation of p65 subunit of NF-κB in HEK293-TLR2 but not in HEK293-TLR4 cells. C, PGN induced TNF-α, IL-1β, and IL-8 expressions at mRNA level in HEK293-TLR2 cells, as assessed by performing Q-PCR analysis. D, CAPE, MG-132, and overexpression of dominant-negative mutant forms of IκBα, IκBβ, IKKα, and NEMO inhibited PGN-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. E, SB203580 and overexpression of dominant-negative mutant forms of p38α, p38β, MKK3, and MKK6 inhibited PGN-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. F, CAPE and MG-132 (MG) but not SB203580 (SB) blocked PGN-induced nuclear translocation of p65 subunit of NF-κB in HEK293-TLR2 cells. G, CAPE, MG-132, and SB203580 inhibited PGN-induced TNF-α, IL-1β, and IL-8 expressions at mRNA level in HEK293-TLR2 cells. Values are the means ± S.D. (n = 3). CON, control.
with TLRs (15–19). In addition, TRAF7 has been recently identified as a signaling transducer upstream of MEKK3-AP1 pathway (25, 26). Its role in mediating TLR-dependent activation of both NF-κB and p38 pathways has yet to be determined. To explore the roles of TRAFs in TLR2-dependent activation of NF-κB and the NF-κB-dependent transcription of inflammatory mediators, we first assessed the effects of perturbing

**FIGURE 2.** The tumor suppressor CYLD negatively regulates TLR2-dependent activation of both NF-κB and p38 MAPK pathways. A, overexpression of WT CYLD inhibited PGN-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. B, CYLD knockdown by siRNA-CYLD markedly reduced both exogenous and endogenous expression of CYLD and enhanced PGN-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. C, CYLD knockdown by siRNA-CYLD enhanced MALP-2- and Pam3CSK4-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. D, overexpressing WT CYLD inhibited, whereas CYLD knockdown by siRNA-CYLD enhanced, PGN-induced phosphorylation of IκBα, M KK3/6, and p38 in HEK293-TLR2 cells. E, overexpressing WT CYLD inhibited, whereas CYLD knockdown by siRNA-CYLD enhanced, PGN-induced TNF-α, IL-1β, and IL-8 expression at mRNA level in HEK293-TLR2 cells. Values are the means ± S.D. (n = 3). CON, control.
Both TRAF6 and TRAF7 are required for mediating TLR2-dependent activation of NF-κB and p38 MAPK pathways. A, overexpression of dominant-negative mutant forms of TRAF6 and TRAF7 but not TRAF2 inhibited PGN-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. B, overexpression of a dominant-negative mutant form of TRAF7 inhibited MALP-2- and Pam3CSK4-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. C, TRAF7 knockdown by siRNA-TRAF7 reduced exogenous expression of TRAF7 in HEK293-TLR2 cells. D, TRAF7 knockdown by siRNA-TRAF7 reduced endogenous expression of TRAF7 at mRNA level in HEK293-TLR2 cells. E, TRAF7 knockdown by siRNA-TRAF7 inhibited PGN-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. F, TRAF7 knockdown by siRNA-TRAF7 inhibited MALP-2- and Pam3CSK4-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. G, TRAF7 DN and siRNA-TRAF7 did not affect IKKβ-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. H, TRAF7 knockdown by siRNA-TRAF7 inhibited PGN-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. I, TRAF7 knockdown by siRNA-TRAF7 inhibited MALP-2- and Pam3CSK4-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells.
TRAF signaling on TLR2-mediated NF-κB activation. As shown in Fig. 3A, co-expression of a dominant-negative mutant of TRAF6 but not TRAF2 inhibited PGN-induced NF-κB activation. More interestingly, overexpressing a dominant-negative mutant of TRAF7 also inhibited PGN-induced NF-κB activation (Fig. 3A). Similarly, perturbing TRAF7 signaling by co-expressing a dominant-negative mutant of TRAF7 also inhibited NF-κB activation by MALP-2 and Pam3CSK4 (Fig. 3B). Since this is the first time to demonstrate the novel involvement of TRAF7 in TLR2-dependent activation of NF-κB, we next sought to confirm its novel involvement by using a siRNA approach. We first confirmed the efficiency of TRAF7-specific small interfering RNA (siRNA-TRAF7) in reducing TRAF7 expression in HEK293-TLR2 cells co-transfected with HA-TRAF7 and siRNA-TRAF7. As expected, the TRAF7 protein was markedly reduced by siRNA-TRAF7 (Fig. 3C). Consistent with this result, the endogenous TRAF7 mRNA was also greatly reduced (Fig. 3D). We then assessed the effect of siRNA-TRAF7 on TLR2-mediated NF-κB activation. TRAF7 knockdown by siRNA-TRAF7 greatly inhibited NF-κB activation not only by PGN (Fig. 3E) but also by MALP-2 and Pam3CSK4 (Fig. 3F). To rule out the nonspecific effects of perturbing TRAF7 signaling, we then assessed the effect of co-expressing a dominant-negative mutant of TRAF6 or siRNA-TRAF7 on NF-κB activation induced by overexpressing IKKβ (IKK2) that acts downstream of TRAFs. As shown in Fig. 3G, IKKβ-mediated NF-κB activation was almost unaffected. To further determine whether TRAF7 also acts downstream of other TLRs, we evaluated the effect of perturbing TRAF7 signaling on NF-κB activation induced by TLR7 ligand. As shown in Fig. 3H, co-expressing a dominant-negative mutant of TRAF7 and TRAF7 knockdown also inhibited NF-κB activation by R837, a TLR7 ligand, indicating that TRAF7, similar to TRAF6, may also act as an important signaling transducer downstream of more TLRs. We next sought to determine whether TRAF6 and TRAF7 also act as common signaling transducers in mediating PGN-induced activation of both IKKs-IκBα and MKK3/6-p38 signaling pathways. As shown in Fig. 3, I and J, co-expressing a dominant-negative mutant of either TRAF6 or TRAF7 inhibited PGN-induced phosphorylation of IκBα and p38. The involvement of TRAF7 in mediating PGN-induced activation of both IκBα and p38 was also confirmed in HEK293-TLR2 cells using siRNA-TRAF7 (Fig. 3K). Moreover, overexpressing a dominant-negative mutant of either TRAF6 or TRAF7 also inhibited PGN-induced transcription of TNF-α, IL-1β, and IL-8 as assessed using Q-PCR analysis (Fig. 3L). Collectively, these data suggest that both TRAF6 and TRAF7 are required for mediating TLR2-dependent activation of NF-κB and p38 MAPK pathways and the NF-κB-dependent transcription of inflammatory mediators.

We have shown that both TRAF6 and TRAF7 are necessary for TLR signaling. It is still unclear whether activation of TRAF6 or TRAF7 is sufficient for activating NF-κB activation nor is whether they act in a co-operative manner. As shown in Fig. 4A, overexpressing wild-type TRAF6 induced potent activation of NF-κB. In contrast, overexpressing wild-type TRAF7 alone did not induce potent NF-κB activation but
FIGURE 5. CYLD inhibits TLR2-dependent NF-κB activation via negative cross-talk with both TRAF6 and TRAF7 likely in a deubiquitination-dependent manner. A, CYLD knockdown by siRNA-CYLD enhanced wild-type TRAF6- and TRAF7-induced NF-κB-dependent promoter activity in HEK293-TLR2 cells. B, CYLD interacted with TRAF6 and TRAF7 in HEK293-TLR2 cells. Cells were co-transfected with HA-CYLD and TRAF6 or FLAG-CYLD and HA-TRAF7. Whole cell extracts were analyzed by immunoblotting (IB) with anti-TRAF6, anti-HA, or anti-FLAG antibodies either directly or after co-immunoprecipitation (IP) with control IgG, anti-TRAF6, anti-HA, or anti-FLAG antibodies. C, CYLD co-localized with TRAF6 and TRAF7 in HEK293-TLR2 cells. Cells were co-transfected with FLAG-CYLD and TRAF6 (upper) or FLAG-CYLD and HA-TRAF7 (lower), and their cellular localization was analyzed by staining with anti-FLAG (green), anti-TRAF6 (red), and anti-HA (red) antibody. D, PGN induced ubiquitination of TRAF6 and TRAF7. Cells were transfected with TRAF6 or HA-TRAF7. At
synergistically enhanced TRAF6-induced NF-κB activation. Interestingly, co-overexpressing a dominant-negative mutant of TRAF7 or siRNA-TRAF7 greatly inhibited WT TRAF6-induced NF-κB activation (Fig. 4, B and C). To further determine whether both TRAF6 and TRAF7 physically interact with each other, we performed co-immunoprecipitation experiments. Results shown in Fig. 4D suggest that TRAF6 and TRAF7 are indeed physically associated with each other in HEK293-TLR2 cells co-transfected with TRAF6 and TRAF7. Thus, our data demonstrate that TRAF7, although alone is insufficient for inducing NF-κB activation, may act as an important co-transducer for enhancing TRAF6-induced activation of NF-κB via an IKKs-IκBα and MKK3/6-p38-dependent mechanism.

CYLD Inhibits TLR2-dependent NF-κB Activation via Negative Cross-talk with Both TRAF6 and TRAF7 Likely in a Deubiquitination-dependent Manner—Having shown that both TRAF6 and TRAF7 are required for mediating TLR2-dependent activation of NF-κB and p38 MAPK pathways, still unknown is whether CYLD inhibits TLR2-dependent activation of IKKs-IκBα and MKK3/6-p38 pathways via negative cross-talk with TRAF6 and TRAF7. To test the hypothesis that CYLD does inhibit NF-κB activation via TRAF6 and TRAF7, we assessed the effect of CYLD knock-down on TRAF6 or TRAF7-induced NF-κB activation. As shown in Fig. 5A, CYLD knockdown enhanced NF-κB activation induced by WT TRAF6 and TRAF7, respectively, although TRAF7 induced NF-κB activation to a much lesser extent. These data suggest that CYLD inhibits NF-κB activation either by directly acting on or by acting downstream of TRAF6 and TRAF7. To determine whether CYLD directly interacts with TRAF6 and TRAF7, we then performed co-immunoprecipitation experiments. Results shown in Fig. 5B suggest that CYLD is indeed physically associated with either TRAF6 or TRAF7 in HEK293-TLR2 cells co-transfected with CYLD and TRAF6 or TRAF7. Next we determined the subcellular localization of ectopically expressed CYLD and TRAF6 or TRAF7. CYLD co-localized with TRAF6 or TRAF7 in HEK293-TLR2 cells (Fig. 5C). Thus, these observations indicate that CYLD inhibits TLR2-dependent NF-κB activation by interacting with TRAF6 and TRAF7.

On the basis of evidence that CYLD has been known as a deubiquitinating enzyme and polyubiquitination plays an important role in TRAF activation (15–24), we next sought to explore whether CYLD deubiquinates TRAF6 and TRAF7. We first examined whether PGN induces polyubiquitination of TRAF6 or TRAF7. As shown in Fig. 5D, PGN induced polyubiquitination of TRAF6 and TRAF7 in a time-dependent manner, in agreement with the notion that TRAF6 or TRAF7 overexpression leads to NF-κB activation. Moreover, co-expression of wild-type CYLD caused a reduction in TRAF6 and TRAF7 ubiquitination, and CYLD knockdown by siRNA CYLD markedly increased levels of ubiquitinated TRAF6 or TRAF7 (Fig. 5E). Although our data do not preclude the possibility that CYLD inhibits TRAF6 and TRAF7 by other post-translational modification manner, these results did provide supportive evidence for our model that CYLD inhibits TLR2-dependent activation of IKKs-IκBα and p38 likely by deubiquitinating TRAF6 and TRAF7.

CYLD Is Induced by TLR2 Ligands—Because a variety of genes involved in inflammatory response undergo changes in expression pattern after initiation of inflammation (1–3) and the endogenous expression of CYLD is relatively low in epithelial cells, we hypothesized that activation of TLR2 signaling by PGN induces CYLD, which in turn leads
to the inhibition of NF-κB by negatively cross-talking with TRAF6 and TRAF7. We thus tested our hypothesis by assessing the effect of PGN on CYLD expression in a variety of epithelial cell lines including HEK293-TLR2, A549, and HeLa cells. As shown in Fig. 6A, PGN induced CYLD expression at the mRNA level in all cell lines tested by Q-PCR analysis. Likewise, MALP-2 and Pam3CSK4 also induced CYLD expression in HEK293-TLR2 cells (Fig. 6B). Consistent with the results obtained using Q-PCR analysis, induction of CYLD by PGN was also observed at the protein level (Fig. 6C). Moreover, CYLD induction by PGN was inhibited by perturbing the TLR2-TRAF6/7-dependent IKKs-IκBα-NF-κB and p38 signaling pathways using CAPE, MG-132, SB203580, and co-expressing dominant-negative mutant of TRAF6 or TRAF7 (Fig. 6D). Taken together, our data suggest that activation of TLR2 signaling by TLR2 ligands induces the expression of CYLD, which in turn leads to the inhibition of IKKs-IκBα- and MKK3/6-p38-dependent activation of NF-κB via negative cross-talk with TRAF6 and TRAF7 likely in a deubiquitination-dependent manner (Fig. 7).

**DISCUSSION**

TLR2 plays an essential role in host defense response against bacterial pathogens by the recognition of a variety of microbial ligands (1–8, 10–14, 17). Among various TLR2 ligands, PGN, MALP-2, and Pam3CSK4 have been well recognized as important ligands for TLR2-dependent activation of host immune and inflammatory responses. Activation of TLR2 signaling has been shown to induce IKKs-IκBα-dependent activation of NF-κB, which in turn leads to the NF-κB-dependent transcription of a variety of inflammatory mediators including TNF-α, IL-1β, and IL-8. In contrast to the relatively known positive signaling pathways involved in TLR2-NF-κB-dependent induction of immune and inflammatory responses, the negative signaling pathways involved in TLR2-dependent inflammatory and immune responses are largely unknown. In the present study we have provided evidence for the negative regulation of TLR2 signaling by the recently identified tumor suppressor CYLD. We showed for the first time that activation of TLR2 signaling induces activation of IKKs-IκBα and MKK3/6-p38 signaling pathways not only by TRAF6, an important signaling adaptor known to be involved for TLR2 signaling, but also by TRAF7, a recently identified TRAF family member (20–26). The activation of both IKKs-IκBα and MKK3/6-p38 pathways will lead to the transcription of TNF-α, IL-1β, and IL-8 as well as CYLD. CYLD in turn leads to the inhibition of TRAF6 and TRAF7 likely via a deubiquitination-dependent mechanism. The present studies thus unveil an autoregulatory feedback mechanism that negatively controls IKKs-IκBα/MKK3/6-p38-NF-κB-dependent induction of immune and inflammatory responses via negatively cross-talking with both TRAF6 and TRAF7. These findings provide novel insights into autoregulation and negative regulation of TLR signaling.

Of particular interest in the present studies is the direct evidence for the inhibition of TLR2-dependent activation of NF-κB and p38 signaling by tumor suppressor CYLD via negative cross-talk with TRAF6 and TRAF7. In contrast to the relatively known positive signaling pathways triggered by TLR2, the negative regulators of TLR2 signaling, especially the inducible negative regulators during microbial infections (4–6) remain largely unknown. Our results demonstrate that TLR2 ligands induce expression of CYLD during bacterial infections. CYLD then inhibits TLR2-dependent activation of NF-κB and p38, which in turn leads to the subsequent inhibition of the transcription of not only inflammatory mediators but also the CYLD itself. Thus, the involvement of NF-κB-dependent induction of CYLD, together with the IκBα induction, may be essential for ensuring the tight control of both transient and persistent NF-κB activation. The CYLD-dependent autoregulatory feedback loop may represent an important mechanism for host to self-limit serious tissue damage due to detrimental inflammatory response during microbial infections. Moreover, our finding that CYLD inhibits not only NF-κB but also MAPK p38 signaling via a TRAF6 and TRAF7-dependent mechanism may bring novel insights into our understanding of the molecular targets of CYLD, a known deubiquitinating enzyme. Previous studies have demonstrated that CYLD negatively regulates TNF-α-induced NF-κB activation via deubiquitinating TRAF2. In addition there is also recent report showing that CYLD also negatively regulates TNF-α-induced JNK activation. In the present study we showed that CYLD negatively regulates TLR2-dependent activation of not only NF-κB but also p38 by negatively cross-talking with TRAF6 and TRAF7 likely in a deubiquitination-dependent manner. Given that polyubiquitination has been suggested to play a more important role in activation of a variety of signaling pathways, it is likely that CYLD may negatively regulate more signaling pathways other than just NF-κB, JNK, and p38 pathways.

Another important finding in our studies is the novel involvement of TRAF7 in mediating TLR2-dependent activation of both NF-κB and p38 signaling (25, 26). Among all known Traf family members, TRAF6 has been known as a key signal transducer of Toll-like receptor family members (20–24). It remains unclear whether other Traf family member also mediates TLR signaling. The present studies showed for the first that TRAF7 signaling is also required for transducing TLR2 signaling. Moreover, we also showed that TRAF7, although alone, is insufficient for potently inducing NF-κB, it indeed synergizes with TRAF6 to mediate TLR2-dependent NF-κB activation likely by the
physical interaction with TRAF6. This finding may bring novel insights into the functional co-operation of TRAF family members in mediating TLR signaling. Future studies will focus on further verifying the role of CYLD in inhibiting TLR2 signaling in vivo using CYLD knock-out mouse. In addition the role of TRAF7 in TLR2 signaling in vivo should also be explored by using TRAF7 knock-out mice.

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