Tumor Suppressor Cylindromatosis Acts as a Negative Regulator for \textit{Streptococcus pneumoniae}-induced NFAT Signaling*

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To whom correspondence should be addressed: Box 672, University of Rochester Medical Center, Rochester, New York 14642 and the Cardiovascular Research Institute, University of Rochester

Here we show that \textit{S. pneumoniae} activates nuclear factor of activated T cells (NFAT) signaling pathway and the subsequent up-regulation of inflammatory mediators via a key pneumococcal virulence factor, pneumolysin. We also demonstrate that \textit{S. pneumoniae} activates NFAT transcription factor independently of Toll-like receptors 2 and 4. Moreover, \textit{S. pneumoniae} induces NFAT activation via both Ca^{2+}-calcineurin and transforming growth factor-β-activated kinase 1 (TAK1)-mitogen-activated protein kinase kinase (MKK) 3/6-p38α/β-dependent signaling pathways. Interestingly, we found for the first time that tumor suppressor cylindromatosis (CYLD) acts as a negative regulator for \textit{S. pneumoniae}-induced NFAT signaling pathway via a deubiquitination-dependent mechanism. Finally, we showed that CYLD interacts with and deubiquitiniates TAK1 to negatively regulate the activation of the downstream MKK3/6-p38α/β pathway. Our studies thus bring new insights into the molecular pathogenesis of \textit{S. pneumoniae} infections through the NFAT-dependent mechanism and further identify CYLD as a negative regulator for NFAT signaling, thereby opening up new therapeutic targets for these diseases.

In the host innate immune system, epithelial cells are situated at host/environment boundaries and thus act as the first line of host defense against various pathogens (1, 2). The principal challenge for the host is to efficiently detect the invading pathogen and mount a rapid defensive response. Epithelial cells are thus responsible for the initiation of local immune responses that may be crucial for prevention of invasive infection. Among the various invading pathogens, Gram-positive bacterium \textit{Streptococcus pneumoniae} is known as an important human pathogen that colonizes the upper respiratory tract and is a major cause of morbidity and mortality worldwide (3). \textit{S. pneumoniae} causes invasive diseases such as pneumonia, meningitis, and otitis media. Despite the importance of pneumococcal diseases, little is known about the molecular mechanisms by which \textit{S. pneumoniae}-induced inflammation is regulated, especially the negative regulatory mechanisms. Here we show that \textit{S. pneumoniae} activates NFAT signaling pathway and the subsequent up-regulation of inflammatory mediators via a key pneumococcal virulence factor, pneumolysin. We also demonstrate that \textit{S. pneumoniae} activates NFAT transcription factor independently of Toll-like receptors 2 and 4. Moreover, \textit{S. pneumoniae} induces NFAT activation via both Ca^{2+}-calcineurin and transforming growth factor-β-activated kinase 1 (TAK1)-mitogen-activated protein kinase kinase (MKK) 3/6-p38α/β-dependent signaling pathways. Interestingly, we found for the first time that tumor suppressor cylindromatosis (CYLD) acts as a negative regulator for \textit{S. pneumoniae}-induced NFAT signaling pathway via a deubiquitination-dependent mechanism. Finally, we showed that CYLD interacts with and deubiquitiniates TAK1 to negatively regulate the activation of the downstream MKK3/6-p38α/β pathway. Our studies thus bring new insights into the molecular pathogenesis of \textit{S. pneumoniae} infections through the NFAT-dependent mechanism and further identify CYLD as a negative regulator for NFAT signaling, thereby opening up new therapeutic targets for these diseases.
phosphorylated. To translocate NFAT into the nucleus, NFAT needs to be dephosphorylated. The phosphorylation status of NFAT is regulated by various kinases and phosphatases, including p38 MAPK and calcineurin, a Ca\(^{2+}\)/calmodulin-dependent serine-threonine phosphatase (10, 14). The role of NFAT and how its function is regulated in bacterial infections, however, remain largely unknown.

Recently, the tumor suppressor cylindromatosis (CYLD), known as a deubiquitinase, loss of which causes a benign human syndrome called cylindromatosis, has been identified as a key negative regulator for nuclear factor-κB (NF-κB) signaling, T cell development, B cell development, and tumor cell proliferation (15–21). Most recently we have shown that CYLD is induced by Gram-negative and Gram-positive bacterial pathogens and that CYLD regulates acute lung injury in lethal *S. pneumoniae* infections (16, 18, 22, 23). The biological role of CYLD in *S. pneumoniae*-induced inflammation, however, remains largely unknown.

In the present study, we investigated the molecular mechanisms underlying *S. pneumoniae*-induced NFAT activation and the subsequent inflammatory mediator production. We show that *S. pneumoniae* PLY activates NFAT transcription factor and subsequent inflammatory mediator production. *S. pneumoniae*-induced NFAT activation is mediated by Ca\(^{2+}\)/calcineurin and transforming growth factor-β-activated kinase 1(TAK1)-mitogen-activated kinase (MKK) 3/6-p38α/β pathways independently of Toll-like receptor 2 (TLR2) and TLR4. Moreover, we show that deubiquitinating enzyme CYLD inhibits *S. pneumoniae*-induced NFAT activation and subsequent inflammatory mediator production through negative cross-talk with TAK1. Our study thus provides new insights into a novel role of NFAT signaling pathway in *S. pneumoniae*-induced inflammation and also identifies CYLD as a critical negative regulator for NFAT signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids**—Nifedipine was purchased from BIOMOL International (Plymouth Meeting, PA). SB203580 and ionomycin were purchased from Calbiochem (La Jolla, CA). Cyclosporin A (CsA) and EGTA were purchased from Sigma-Aldrich. The plasmids NFAT luciferase reporter, NF-κB luciferase reporter, p38αDN, p38βDN, MKK3DN, MKK6DN, TAK1DN, TAK1WT, siRNA-CYLD, wild-type CYLD (FLAG-tagged), deubiquitinase mutant CYLD-C/S, and CYLD-H/N were previously described (15, 16, 24, 25).

**Bacterial Strains, Culture Conditions, and Purification of PLY**—Clinical isolates of *S. pneumoniae* strains D39 (S.p.) and D39 isoegenic PLY-deficient mutant (Ply mt) were used in this study. *S. pneumoniae* was grown on chocolate agar plates and in Todd-Hewitt broth supplemented with 0.5% yeast extract at 37 °C in a humidified 5% CO\(_2\) water-jacketed incubator. After overnight incubation, *S. pneumoniae* was centrifuged at 10,000 × g for 10 min, and the supernatant was discarded. The resulting pellet of *S. pneumoniae* was suspended in phosphate-buffered saline and sonicated. Subsequently, the lysates were collected and stored at −70 °C (27). The purification of PLY was performed as described previously (28).

Cell Culture—Human lung epithelial cell line A549 and human cervix epithelial cell line HeLa cells were maintained as described previously (22). Stable cell lines HEK293-pcDNA, HEK293-TRL2, and HEK293-TRL4 were kindly provided by Dr. Douglas T. Golenbock. All stable cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 0.5 mg/ml G418, and 10 μg/ml ciprofloxacin (Cellgro, Herndon, VA) as described (15). Wild-type (WT), *Cyld*-/-, *Tlr2*-/-, and *Tlr4*-/- mouse embryonic fibroblasts (MEFs) were obtained from E13 embryos and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 units/ml, 0.1 mg/ml, respectively). WT, *Cyld*-/-, *Tlr2*-/-, and *Tlr4*-/- MEFs and mice were obtained as described previously (17, 23, 28). All cells were maintained at 37 °C in an atmosphere of 5% CO\(_2\).

**Transfections and Luciferase Assay**—Cells were cultured on 24-well plates. After 24 h, cells were co-transfected with NFAT or NF-κB luciferase reporter plasmid and various expression plasmids as indicated in the figures or figure legends. All transfections were carried out in triplicate using TransIT- LT1 reagent (Mirus, Madison, WI) following the manufacturer’s instruction. In all co-transfections, an empty vector was used as a control. At 40 h after the transfection, cells were pretreated with or without chemical inhibitors, including EGTA, nifedipine, CsA, and SB203580 for 1 h. *S. pneumoniae* D39, Ply mt, PLY, or ionomycin 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 PCR Analysis**—Total RNA was isolated with TRizol reagent (Invitrogen) by following the manufacturer’s instruction. For the reverse transcription reaction, TaqMan reverse transcription reagents (Applied Biosystems) were used as described (22). PCR amplifications were performed by using SYBR Green Universal Master Mix for human cyclooxygenase-2 (COX-2), IL-6, mouse COX-2, and mouse IL-6. In brief, reactions were performed in duplicate containing 2× Universal Master Mix, 1 μl of template cDNA, 100 nM primers in a final volume of 12.5 μl, and they were analyzed in a 96-well optical reaction plate (Applied Biosystems). Reactions were amplified and quantified by using an ABI 7500 sequence detector and the manufacturer’s corresponding software (7000v1.3.1; Applied Biosystems). The relative quantities of mRNAs were obtained by using the comparative Ct method and were normalized with predeveloped Taqman assay reagent human cyclophilin or mouse glyceraldehyde-3-phosphate dehydrogenase as an endogenous control (Applied Biosystems). The primers for human COX-2 were as follows: forward primer, 5'-GAAATCTACCAGGCAAAATG-3'; reverse primer, 5'-TCGTGACAGGTCGCTGCCAGAATG-3'. The primers for mouse COX-2 were as follows: forward primer, 5'-CCAGCATTCCACCACAGGAAATG-3'; reverse primer, 5'-CTCCTCGTGAGCTCTGAGCTCAG-3'. The primers for mouse COX-2 were as follows: forward primer, 5'-GGCTACATCCATGAGCAGTACAGT-3'; reverse primer, 5'-ACCAGGTCTCTGTTTCCTAG-3'. The primers for human IL-6 were as follows: forward primer, 5'-GAGGATAACCACCCAAACAGAC-3'; reverse primer, 5'-AAGTGCATCATGTTTTCATACA-3'.
Western Blot Analysis and Immunoprecipitation—Antibodies against phospho-MKK3/6 (Ser-189/207), MKK3, phospho-p38 MAPK (Thr-180/Tyr-182), and p38 MAPK were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against CYLD were purchased from Imgenex Corp. (San Diego, CA). Antibodies against ubiquitin and TAK1 were purchased from Sigma. Western blot analysis was performed as described previously to measure inflammatory cell migration and cell number from the BAL fluid was counted under the microscope (18). All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Rochester.

RESULTS

S. pneumoniae PLY Acts as a Potent Inducer of NFAT and the Subsequent Inflammatory Mediators in Vitro and in Vivo—To determine whether S. pneumoniae induces NFAT activation

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay—Nuclear protein extraction was performed as described previously (30, 31). 5–7 μg of nuclear extracts was prepared and non-radioactive electrophoretic mobility shift assay was performed using an EMSA kit according to the manufacturer’s instruction (Pierce). Oligonucleotide (oligo) 5′-ACGCCAAAAGAGGAAAATTTGTTCTACACA-3′ was used as the consensus NFAT binding site-containing probe. Oligo was obtained from Integrated DNA Technologies and end-labeled with biotin-N4-CTP using terminal deoxynucleotidyl transferase (Pierce).

Calcineurin Phosphatase Assay—Calcineurin phosphatase activity was measured by using the Calcineurin Assay kit (BIOMOL) according to the manufacturer’s instruction.

Animal and Animal Experiments—C57BL/6 strain was purchased from NCI, National Institutes of Health. Cyld−/− mice were reported previously (23). For S. pneumoniae- or PLY-induced inflammation in the lungs of the mice, under anesthesia animals were intratracheally inoculated with 5 × 107 colony-forming units of S. pneumoniae D39 or PLY mt lysate or 80 ng of PLY in 50 μl of saline; 50 μl of saline was inoculated as control inoculation in all the experiments. Animals were sacrificed 6 h after inoculation with an overdose injection of sodium pentobarbital, and mRNA expressions of COX-2 and IL-6 were measured from the lung tissues of inoculated mice by Q-PCR analysis as described above. For the S. pneumoniae-induced inflammation in Cyld−/− mice, both wild-type and Cyld−/− mice were intratracheally inoculated with 1 × 107 colony-forming units of S. pneumoniae, and mRNA expressions of COX-2 and IL-6 were measured from the lung tissues of wild-type and Cyld−/− mice 6 h after inoculation. Broncho-alveolar lavage (BAL) was performed as described previously to measure inflammatory cell migration into the airway from the lungs of wild-type and Cyld−/− mice, and cell number from the BAL fluid was counted under the microscope (18). All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Rochester.
and NFAT-dependent inflammatory response, we first investigated NFAT-dependent transcriptional activity by using NFAT-dependent luciferase reporter in HEK293 cells. As shown in Fig. 1A, *S. pneumoniae* (S.p.) induced NFAT activation in a dose-dependent manner, and similar results were observed in HeLa and A549 cells (data not shown). We have shown previously that PLY is an important virulence factor in *S. pneumoniae* infections (23, 28). To determine whether PLY plays an important role in *S. pneumoniae*-induced NFAT activation, NFAT promoter assay was performed using *S. pneumoniae* wild-type D39 and isogenic mutant Ply mt. As shown in Fig. 1B, NFAT promoter activation was induced by *S. pneumoniae* D39 and PLY, but not by PLY-deficient mutant Ply mt. Moreover, PLY induced NFAT activation in a dose-dependent manner. Consistent with these results, DNA binding activity of NFAT was markedly induced by *S. pneumoniae* (Fig. 1C). Because a variety of genes involved in inflammatory response undergo changes in expression pattern in response to bacterial infection, it is of particular interest and important to determine whether *S. pneumoniae* induces NFAT expression. We performed experiments to evaluate the effect of *S. pneumoniae* on NFAT expressions. Lung epithelial cell A549 was first treated with *S. pneumoniae* lysate, and the expression of NFAT1, 2, 3, 4, and 5 at mRNA levels was then measured by performing real-time quantitative RT-PCR analysis. Interestingly, none of the NFAT isotypes was up-regulated by *S. pneumoniae* lysate (data not shown). This finding suggests that *S. pneumoniae* induces NFAT activity mainly by inducing its activation rather than up-regulating its expression (14).

To further determine whether *S. pneumoniae* also induces NFAT-dependent expression of inflammatory mediators, we investigated the effects of *S. pneumoniae* on expression of COX-2 and IL-6, which are known as NFAT-dependent genes (32, 33), by Q-PCR analysis. As shown in Fig. 1D, both COX-2 and IL-6 were markedly induced by *S. pneumoniae* and PLY in both HEK293 cells and lung epithelial cell line A549. To further determine whether *S. pneumoniae* also induces NFAT-dependent gene expression in vivo, we investigated the expression of COX-2 and IL-6 by Q-PCR analysis in the lungs of C57BL/6 mice. As shown in Fig. 1E, both COX-2 and IL-6 were up-regulated in the lungs of *S. pneumoniae*- or PLY-inoculated mice, but not in the lungs of Ply mt-inoculated mice. Collectively, these data demonstrate that *S. pneumoniae* PLY induces NFAT activation and the subsequent inflammatory mediators both in vitro and in vivo.

*S. pneumoniae* Activates NFAT Transcription Factor Indepedently of TLR2 or TLR4—Because of the important roles of Toll-like receptor (TLR) 2 and TLR4 in cellular responses to pneumococcal components (34–36), we investigated whether TLR2 or TLR4 is involved in activation of NFAT by *S. pneumoniae* by using a variety of HEK293 cells that stably overexpress either TLR2, TLR4, or an empty vector (HEK293-TLR2, HEK293-TLR4, and HEK293-pcDNA, respectively). Surprisingly, *S. pneumoniae* induced potent NFAT activation even in HEK293-pcDNA cells, which do not express TLR2 or TLR4 (Fig. 2A). These results are rather unexpected, because both TLR2 and TLR4 were previously shown to be important for the host defense response against *S. pneumoniae* infections. To further confirm the requirement of both TLR2 and/or TLR4 for *S. pneumoniae*-induced NFAT activation, we performed NFAT promoter assay in TLR2−/− and TLR4−/− MEFs. As shown in Fig. 2B, *S. pneumoniae* still induced potent NFAT activation in TLR2−/− or TLR4−/− MEFs. Taken together, we conclude from these data that TLR2 or TLR4 signaling pathway is not required for *S. pneumoniae*-induced NFAT activation.

*S. pneumoniae* Activates NFAT through Ca2+-calcineurin Signaling Pathway—Having demonstrated the role of PLY in inducing NFAT-dependent expression of inflammatory mediators, we next sought to investigate the signaling mechanisms underlying NFAT activation by PLY. Based on previous studies showing that PLY induces cellular Ca2+ influx in various cell types, and the important role of Ca2+ for NFAT signaling (10, 37, 38), we investigated the role of Ca2+ in *S. pneumoniae*-induced NFAT activation. As shown in Fig. 2C, a specific Ca2+ chelator, EGTA, greatly inhibited *S. pneumoniae*-induced NFAT activation. Because of the important role of the L-type Ca2+ channel for Ca2+ influx and NFAT activation (39), we next investigated the requirement of L-type Ca2+ channel by using its specific inhibitor, nifedipine. As shown in Fig. 2C, *S. pneumoniae*-induced NFAT activation was not inhibited by nifedipine. Collectively, these data demonstrate that *S. pneumoniae* induces NFAT activation via a PLY-induced Ca2+ influx, but not L-type Ca2+ channel.

![Figure 2](image-url)
NFAT is normally present in the cytoplasm in an inactive state and is highly phosphorylated. To translocate NFAT into the nucleus, NFAT needs to be dephosphorylated. A common phosphatase to achieve dephosphorylation of NFAT is calcineurin, a Ca^{2+}/calmodulin-dependent serine-threonine phosphatase (10, 14). To determine the involvement of calcineurin in *S. pneumoniae*-induced NFAT activation, we investigated the effects of CsA, a specific inhibitor for calcineurin, on NFAT activation using NFAT reporter assay and Q-PCR analysis. As shown in Fig. 2, D and E, pretreatment with CsA inhibited *S. pneumoniae*-induced NFAT activation and COX-2 expression in a dose-dependent manner. Moreover, to investigate whether *S. pneumoniae* activates calcineurin phosphatase, we measured calcineurin phosphatase activity upon *S. pneumoniae* treatment. As shown in Fig. 2F, *S. pneumoniae* induced calcineurin activity. Taken together, our data indicate that *S. pneumoniae* activates NFAT-dependent gene expression through a Ca^{2+}-calcineurin-dependent signaling pathway.

*S. pneumoniae* Activates NFAT through Ca^{2+} Influx-TAK1-MKK3/6-p38α/β Pathway—Because we recently have demonstrated the important role of p38 MAPK in *S. pneumoniae* infections (23, 40), we investigated the involvement of MKK3/6-p38 MAPK pathway in *S. pneumoniae*-induced NFAT activation. We first examined whether *S. pneumoniae* induces activation of MKK3/6 and p38 MAPK. As shown in Fig. 3A, *S. pneumoniae*-induced activation of MKK3/6 and p38 MAPK in epithelial cells. We next investigated the requirement of MKK3/6-p38 MAPK in NFAT activation. As shown in Fig. 3B, a specific inhibitor for p38 MAPK, SB203580 (SB), inhibited NFAT activation. We further confirmed its involvement by overexpressing the dominant negative (DN) mutant form of p38α or p38β. As expected, overexpression of both p38α DN and p38β DN inhibited NFAT activation. We next sought to determine which upstream molecules transduce signals to p38 MAPK in mediating NFAT activation. Among the numerous molecules, MKK3, MKK6, and TAK1 have been shown to play important roles in p38 MAPK activation (25). As shown in Fig. 3C, overexpressing MKK3 DN, MKK6 DN, and TAK1 DN inhibited NFAT activation by *S. pneumoniae*. Because of the important role of Ca^{2+} influx in *S. pneumoniae*-induced NFAT activation, we next investigated whether Ca^{2+} influx induces p38 MAPK activation. As shown in Fig. 3D, the Ca^{2+} influx-TAK1-MKK3/6-p38 MAPK pathway in *S. pneumoniae*-induced NFAT activation. We first examined whether *S. pneumoniae* induces activation of MKK3/6 and p38 MAPK. As shown in Fig. 3A, *S. pneumoniae* induced activation of MKK3/6 and p38 MAPK in epithelial cells. We next investigated the requirement of MKK3/6-p38 MAPK in NFAT activation. As shown in Fig. 3B, a specific inhibitor for p38 MAPK, SB203580 (SB), inhibited NFAT activation. We further confirmed its involvement by overexpressing the dominant negative (DN) mutant form of p38α or p38β. 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Negative Regulation of NFAT Signaling by CYLD

![Diagram](image)

**FIGURE 4.** *S. pneumoniae*-induced NFAT signaling is negatively regulated by CYLD. A, *S. pneumoniae*-induced NFAT activation was enhanced by CYLD knockdown using siRNA in HEK293 cells (left panel), whereas *S. pneumoniae*-induced NFAT activation was inhibited by wild-type CYLD overexpression (right panel). B, *S. pneumoniae*-induced COX-2 mRNA expression was enhanced by siRNA-CYLD (left panel), whereas *S. pneumoniae*-induced COX-2 mRNA expression was inhibited by wild-type CYLD overexpression (right panel). C, NFAT activation by *S. pneumoniae* is much greater in Cyld−/− MEF than Cyld+/+ MEF. D, *S. pneumoniae* induced COX-2 and IL-6 mRNA expression to a greater extent in Cyld−/− MEF than in Cyld+/+ MEF. E, both COX-2 and IL-6 mRNA expression was enhanced in Cyld−/− mouse lung compared with Cyld+/+ mouse lung inoculated with *S. pneumoniae* for 6 h. F, *S. pneumoniae*-induced inflammatory cell migration into airway was enhanced in Cyld−/− mice. Values are the mean ± S.D. (n = 3 for A–F). S.p., *S. pneumoniae*; Con, control.

largely unknown. Moreover, based on our recent study showing that CYLD is greatly induced by bacterial pathogens, including *S. pneumoniae* (16–18, 22, 23), we hypothesized that CYLD may play an important role in *S. pneumoniae*-induced NFAT activation. We first investigated whether CYLD regulates NFAT signaling in human epithelial cells by using siRNA and overexpression of WT CYLD. Interestingly, CYLD knockdown by siRNA-CYLD potently enhanced *S. pneumoniae*-induced NFAT activation, whereas overexpression of WT CYLD inhibited (Fig. 4A). Likewise, expression of NFAT-regulated gene COX-2 was enhanced by siRNA-CYLD at the mRNA level, whereas overexpression of wild-type CYLD inhibited it (Fig. 4B). To further investigate the role of CYLD, we took advantage of available Cyld−/− MEFs. Consistent with the results obtained by using siRNA-CYLD, *S. pneumoniae*-induced NFAT activation was much greater in Cyld−/− MEFs than in Cyld+/+ MEFs (Fig. 4C). We next determined whether CYLD indeed acts as a negative regulator for *S. pneumoniae*-induced NFAT-dependent gene expression. Fig. 4D shows that *S. pneumoniae* induced COX-2 mRNA expression in Cyld−/− MEFs to a greater extent than in Cyld+/+ MEFs. Similar results were observed in *S. pneumoniae*-induced IL-6 mRNA expression. The in vivo relevance of these data was further confirmed by using the lungs of Cyld−/− mice. As shown in Fig. 4E, expression of both COX-2 and IL-6 was further enhanced in the lungs of Cyld−/− mice inoculated with *S. pneumoniae* compared with Cyld+/+ mice. Moreover, *S. pneumoniae*-induced inflammatory cell migration into the airway was also enhanced in Cyld−/− mice (Fig. 4F). Together, our data indicate that CYLD negatively regulates *S. pneumoniae*-induced NFAT activation and subsequent NFAT-regulated inflammatory mediators in vitro and in vivo.

**CYLD Negatively Regulates S. pneumoniae-induced NFAT Signaling via Deubiquitination of TAK1**—Having identified CYLD as a negative regulator for *S. pneumoniae*-induced NFAT signaling, we next sought to determine how CYLD negatively regulates NFAT signaling pathway. Because CYLD has been identified as a deubiquitinating enzyme, we investigated the requirement of CYLD deubiquitinating activity in NFAT signaling. As shown in Fig. 5A, deubiquitination-deficient CYLD mutants (CYLD C/S and CYLD H/N) exhibited no inhibitory effect on *S. pneumoniae*-induced NFAT transcriptional activity, whereas NFAT activation was inhibited by overexpression of wild-type CYLD. Expression of COX-2 was also inhibited by overexpressing wild-type CYLD but not by deubiquitination-deficient mutants (CYLD C/S and CYLD H/N) as assessed by Q-PCR analysis (Fig. 5B). Similar results were observed in Cyld−/− MEFs (data not shown). Thus, our data demonstrate that CYLD inhibits NFAT signaling pathway in a deubiquitination activity-dependent manner. We next sought to determine the target molecule of CYLD. We first investigated whether CYLD inhibits *S. pneumoniae*-induced NFAT activation by targeting calcineurin. As shown in Fig. 5C, knockdown of CYLD by siRNA exhibited no inhibitory effect on calcineurin activity. Because recent reports demonstrated that TAK1 polyubiquitination is involved in its activation and TAK1 interacts with and is deubiquitinated by CYLD to prevent the excessive activation of T cells (41, 42), we sought to determine whether CYLD acts as a negative regulator for *S. pneumoniae*-induced NFAT signaling through deubiquitinating TAK1. We first determined whether *S. pneumoniae* induces TAK1 polyubiquitination. As shown in Fig. 5D, *S. pneumoniae* induced TAK1 polyubiquitination in a time-dependent manner. We
next evaluated whether overexpressing CYLD WT inhibits TAK1 polyubiquitination. As shown in Fig. 5E, the overexpression of CYLD WT inhibited S. pneumoniae-induced TAK1 polyubiquitination. Moreover, the interaction between CYLD and TAK1 was identified by performing co-immunoprecipitation experiments (Fig. 5F). Finally, to confirm whether CYLD also negatively regulates S. pneumoniae-induced MKK3/6-p38 MAPK activation was performed. As shown in Fig. 5G, S. pneumoniae-induced phosphorylation of both MKK3/6 and p38α/β was enhanced in Cyld−/− MEFs compared with Cyld+/+ MEFs. Taken together, our data demonstrate that S. pneumoniae induces TAK1 polyubiquitination and CYLD interacts with TAK1 and deubiquitinates TAK1, thus acting as a negative regulator for S. pneumoniae-induced NFAT signaling and the subsequent induction of NFAT-dependent inflammatory mediators.

DISCUSSION

In the present study, we provide direct evidence that S. pneumoniae PLY activates NFAT transcription factor and induces subsequent up-regulation of inflammatory mediators such as COX-2 and IL-6 in vitro and in vivo. S. pneumoniae-induced NFAT activation is mediated by Ca2+-calcineurin pathway and TAK1-MKK3/6-p38α/β pathway independently of both TLR2 and TLR4. Interestingly, the deubiquitinating enzyme CYLD negatively regulates S. pneumoniae-induced NFAT signaling and subsequent expression of inflammatory mediators. We have also shown that CYLD interacts with and deubiquitinates TAK1 and in turn negatively regulates activation of its downstream signaling molecules, including MKK3/6 and p38α/β. These data thus reveal a novel role of CYLD in negatively regulating NFAT signaling pathway (Fig. 6).

An important finding in this study is the direct evidence that S. pneumoniae potently induces NFAT transcriptional activity and subsequent expression of inflammatory mediators. Our findings may have important implications for host defense and innate immune response to S. pneumoniae infections. Despite a number of studies demonstrating the involvement of S. pneumoniae PLY in NF-κB-dependent inflammation at relatively later stages of infections, the role of PLY in inducing NFAT-dependent inflammation remains totally unknown. In the present study, we provided evidence for the first time to demonstrate NFAT activation and the resultant induction of pro-inflammatory mediators by S. pneumoniae PLY. Previous study showed that S. pneumoniae induced delayed activation of NF-κB in mouse lung compared with lipopolysaccharide (43). In line with
this finding, our recent study demonstrates that NF-κB activation by S. pneumoniae PLY does not appear to play a crucial role in the pathogenesis of S. pneumoniae infections at the early stage (23). Thus, PLY-induced NFAT activation and the subsequent up-regulation of pro-inflammatory mediators, such as COX-2 and IL-6, may play a critical role in the pathogenesis of S. pneumoniae infections at a relatively early stage. This notion is further supported by our data showing that S. pneumoniae PLY induces NFAT activation independently of both TLR2 and TLR4. It is now known that lung epithelial cells express relatively low levels of certain TLRs under physiological conditions and these TLRs are up-regulated during the course of infections. Thus TLR2- and TLR4-independent activation of NFAT by S. pneumoniae PLY may play a crucial role in initiating host inflammatory/immune responses at early stages of infections when the pathogens first encounter the host surface epithelial cells.

Another interesting finding in our study is that PLY plays a key role in S. pneumoniae-induced NFAT activation via a TLR-independent mechanism. Although PLY induces NF-κB activation in a TLR4-dependent manner (34), TLR4 is not required for NFAT activation by PLY. Thus, a question that has yet to be answered is how S. pneumoniae PLY regulates NFAT activation. One possibility is that PLY induces Ca\(^{2+}\) influx into the cells through its pore-forming action (37, 38). PLY has been known as a direct inducer of Ca\(^{2+}\) influx into the cells. The resultant Ca\(^{2+}\) influx in turn leads to activation of Ca\(^{2+}\)/calmodulin-calcineurin signaling and subsequent NFAT activation. Indeed, our data (Fig. 2C) showed S. pneumoniae-induced NFAT activation was inhibited by EGTA, a known Ca\(^{2+}\) chelator. Future studies will focus on elucidating how exactly Ca\(^{2+}\) influx is involved in PLY-dependent NFAT activation via a TAK1-dependent manner.

Of particular interest in our study is that CYLD negatively regulates S. pneumoniae-induced NFAT activation. A growing body of evidence suggests that the deubiquitinating enzyme CYLD may act as not only a negative regulator for NF-κB but also a negative regulator for many other cell signaling events (44). The present study provides convincing evidence for the negative regulation of NFAT by CYLD, thereby providing novel insights into our understanding of how CYLD delicately and tightly regulates inflammation via negative cross-talk with multiple key inflammation regulatory pathways. Moreover, although we previously reported that CYLD negatively regulates MKK3-p38 MAPK signaling pathway, it is still unknown how CYLD negatively regulates MKK3-p38 MAPK pathway. In the present study, we identified TAK1 as the target of CYLD in inhibitions of MKK3-p38 MAPK pathway. Our finding is in line with recent reports that demonstrated that TAK1 polyubiquitination is involved in its activation and TAK1 interacts with and is deubiquitinated by CYLD to prevent the excessive activation of T cells (41, 42). In summary, the present study identifies CYLD as a negative regulator for NFAT by deubiquitinating TAK1 and provides novel and interesting insights into the molecular pathogenesis of S. pneumoniae infection.

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