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NF-κB Is Essential for Induction of CYLD, the Negative Regulator of NF-κB

EVIDENCE FOR A NOVEL INDUCIBLE AUTOREGULATORY FEEDBACK PATHWAY*

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The transcription factor NF-κB regulates genes involved in inflammatory and immune responses, tumorigenesis, and apoptosis. In contrast to the pleiotropic stimuli that lead to its positive regulation, the known signaling mechanisms that underlie the negative regulation of NF-κB are very few. 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 by deubiquitinating tumor necrosis factor (TNF) receptor-associated factor (TRAF) 2, TRAF6, and NEMO (NF-κB essential modulator, also known as IκB kinase γ). However, how CYLD is regulated remains unknown. The present study revealed a novel autoregulatory feedback pathway through which activation of NF-κB by TNF-α and bacterium nontypeable *Haemophilus influenzae* (NTHi) induces CYLD that in turn leads to the negative regulation of NF-κB signaling. In addition, TRAF2 and TRAF6 appear to be differentially involved in NF-κB-dependent induction of CYLD by TNF-α and NTHi. These findings provide novel insights into the autoregulation of NF-κB activation.

The transcription factor NF-κB plays critical roles in regulating inflammatory and immune responses, tumorigenesis, and protection against apoptosis (1–3). Previous studies identified an inducible feedback inhibition pathway for controlling IκBα gene transcription and down-regulation of transient activation of NF-κB (4–6). Recent studies have identified the tumor suppressor CYLD[1] as a key negative regulator for NF-κB signaling by deubiquitinating tumor necrosis factor (TNF) receptor-associated factor (TRAF) 2, TRAF6, and NEMO (7–9). However, how CYLD is regulated is totally unknown. It is still unclear whether activation of NF-κB induces CYLD transcription that in turn leads to the inhibition of NF-κB especially in more delayed or persistent phase in an autoregulatory feedback manner.

To determine whether CYLD is induced during inflammation, we first sought to evaluate the effects on CYLD expression of a variety of inflammation stimuli such as proinflammatory cytokines and bacteria. Having demonstrated that CYLD is indeed induced by TNF-α, interleukin-1β (IL-1β) and nontypeable *Haemophilus influenzae* (NTHi), an important Gram-negative bacterial pathogen for respiratory infections, we next sought to determine whether activation of NF-κB is required for CYLD induction based on the fact that all of the above CYLD inducers are also potent inducers for NF-κB. Here we showed that activation of NF-κB is indeed required for CYLD induction by TNF-α, IL-1β, and NTHi and that TRAF2 and TRAF6 are differentially involved in NF-κB-dependent induction of CYLD by TNF-α and NTHi. The present study thus revealed a novel autoregulatory feedback pathway through which activation of NF-κB by TNF-α and NTHi induces CYLD that in turn leads to the inhibition of NF-κB signaling. These findings should enhance our understanding of the negative feedback regulation of NF-κB activation during inflammation.

MATERIALS AND METHODS

Reagents—MG-132 was purchased from Calbiochem. Recombinant mTNF-α, hTNF-α, and hIL-1β were purchased from R&D Systems. NTHi strain 12 was described previously (10, 11).

Cell Culture—Human cervix epithelial cell line HeLa was maintained as described (10, 11) and was used for all experiments unless otherwise indicated. All mouse embryonic fibroblast (MEF) cells were maintained as described (12–15). Wild-type (WT), IKK1$^{-/-}$, IKK2$^{-/-}$, and IKK1/2$^{-/-}$ MEFs were provided by Dr. I. Verma (12, 13); p65$^{-/-}$ and reconstituted p65$^{-/-}$ MEFs were provided by Dr. C. Y. Wang (15). WT Rat-1 cells and IKKγ (also known as NEMO (NF-κB essential modulator))-deficient cells were provided by Dr. S. Yamaoka (11, 16). Primary normal human bronchial epithelial (NHBE) cells were described previously (10–11).

Real-time Quantitative Reverse Transcriptase-PCR Analysis—Real-time quantitative PCR (Q-PCR) was performed using an ABI 7700 Sequence Detection System (Applied Biosystems) as described (11). The sequences of primers and probes were as follows: human CYLD (GenBank™ accession number NM015247), 5′-ACGCCACAATCTTCATCACACT-3′ (forward primer) and 5′-AGGTCGTGGTCAAGGTTTCACT-3′ (reverse primer); TaqMan probe, 5′-6-carboxyfluorescein-AAAAAGCTGTTTCCCTTGGTACACCCCG-6-carboxytetramethylrhodamine-3′); mouse CYLD (GenBank™ accession number NM173369, 5′-CTC AGC CTA TTT AGA AAC AGA CT-3′ (forward primer) and 5′-TCT CCT GGG CCT GCA AAA T-3′ (reverse primer); rat CYLD (GenBank™ accession number XM232642), 5′-CTC AGC CTA TTT AGA AAC AGA

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[1] The abbreviations used are: CYLD, cylindromatosis; TNF, tumor necrosis factor; NTHi, nontypeable *Haemophilus influenzae*; IL, interleukin; MEF, mouse embryonic fibroblast; WT, wild-type; NHBE, normal human bronchial epithelial; siRNA, small interfering RNA; IKK, IκB kinase; NEMO, NF-κB essential modulator.

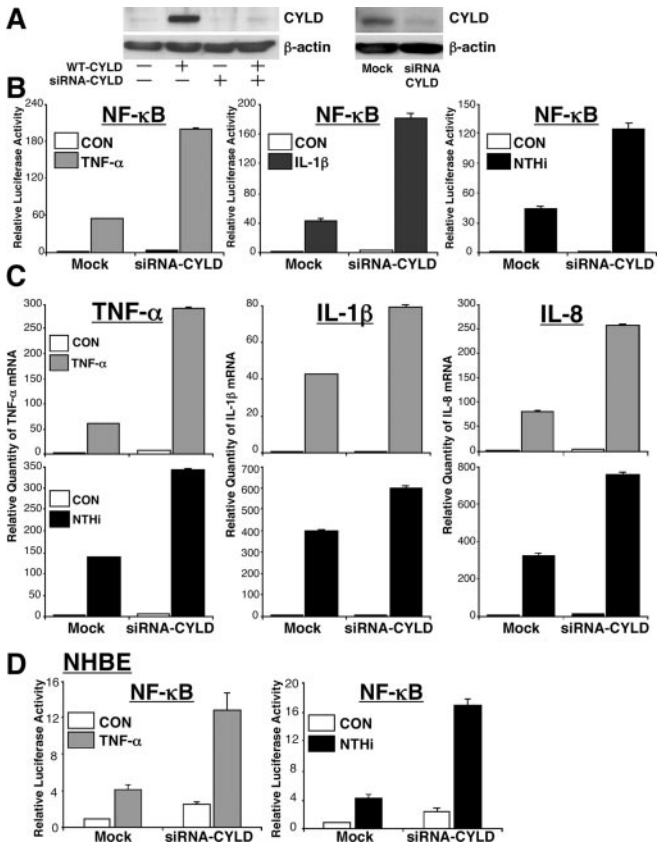

FIG. 1. **CYLD is a general negative regulator for NF-κB activation.** *A*, the exogenously expressed and the endogenous CYLD protein levels were markedly reduced by siRNA-CYLD (*left* and *right*). *B*, siRNA-CYLD enhanced activation of NF-κB by TNF-α (*left*) or IL-1β (*middle*) or NTHi (*right*). *C*, siRNA-CYLD enhanced the induction of TNF-α (*left*), IL-1β (*middle*), and IL-8 (*right*) by TNF-α (*upper*) or NTHi (*lower*) at the mRNA levels as assessed by Q-PCR. *D*, siRNA-CYLD enhanced activation of NF-κB by TNF-α (*left*) or bacterium NTHi (*right*) in primary NHBE cells. Values are the means ± S.D. ($n = 3$).

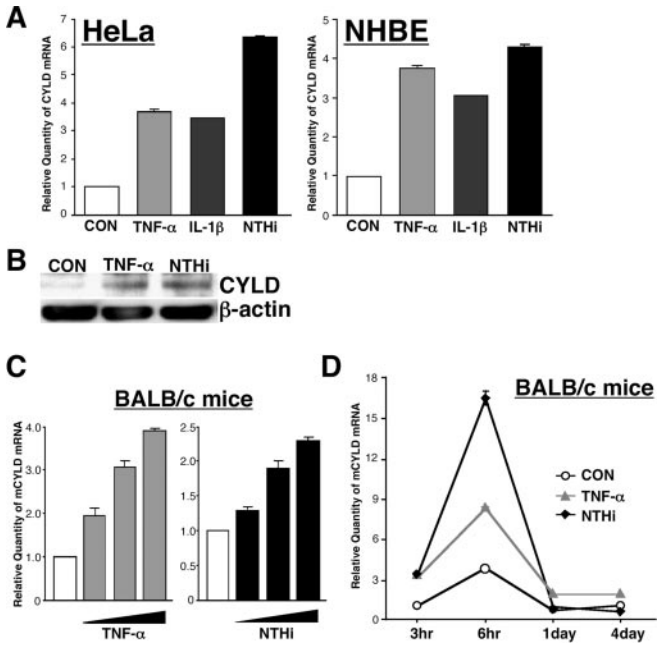

FIG. 2. **CYLD is induced by a variety of NF-κB inducers *in vitro* and *in vivo*.** *A*, CYLD expression was induced at the mRNA level by TNF-α, IL-1β, and NTHi in HeLa (*left*) and primary NHBE cells (*right*). *B*, CYLD expression was also induced at the protein level. *C* and *D*, CYLD expression was induced by TNF-α and NTHi in the lungs of BALB/c mice *in vivo* in a dose-dependent (*C*) and a time-dependent manner (*D*). Values are the means ± S.D. ($n = 3$).

AT-3′ (forward primer) and 5′-TCT CCT GGG CCT GCA AAA T-3′ (reverse primer).

Plasmids, Transfections, and Luciferase Assays—The plasmids WT-CYLD, IκBα(S32/36A), IKK2(K49A), p65, and NF-κB luciferase were described previously (7, 10, 11). All transient transfections were carried out in triplicate using TransIT-LT1 reagent (Panvera, Madison, WI). The transfected cells were treated with TNF-α, IL-1β, or NTHi for 5 h before being harvested for luciferase assay. Luciferase activity was normalized with respect to β-galactosidase activity.

RNA-mediated Interference—RNA-mediated interference for down-regulating CYLD expression was done using small interfering RNA (siRNA)-CYLD (pSUPER-CYLD) as described previously (8).

Western Blot Analysis—Western blot analysis was performed as described (8). The rabbit polyclonal antibody against CYLD, developed in the laboratory of Dr. Mosialos, was raised against amino acids 538–953 of human CYLD. Antibodies against IKK1, IKK2, and NEMO were purchased from Cell Signaling, p65 was from Santa Cruz Biotechnology, and β-actin was from Sigma.

In Vivo Study—7–8-Week-old BALB/c mice (Charles River Laboratories) were used in this study. After the trachea was surgically exposed by middle line incision in the skin, TNF-α or NTHi was directly injected into the trachea. Lung tissues were collected and then stored at −80 °C; total RNA was isolated from the frozen tissue. For inhibition study, mice were pretreated with 1 mg/kg MG-132 interperitoneally 1 h before inoculation of TNF-α or NTHi. Three mice were used for each inoculation group. The House Ear Institute Institution's Animal Care and Use Committee (IACUC) approved all of the animal protocols used in this study.

RESULTS AND DISCUSSION

CYLD Is a General Negative Regulator for NF-κB Activation—We first sought to determine whether CYLD indeed acts as a negative regulator for NF-κB activation by a variety of stress stimuli using a siRNA approach (8). We first confirmed the efficiency of CYLD-specific siRNA (siRNA-CYLD) in reducing CYLD expression in HeLa cells co-transfected with WT CYLD and siRNA-CYLD or empty vector. As expected, the CYLD protein was markedly reduced by siRNA-CYLD (Fig. 1*A*, *left*). Consistent with this result, the endogenous CYLD protein was also greatly reduced (Fig. 1*A*, *right*). We then assessed the effect of siRNA-CYLD on NF-κB activation by TNF-α, IL-1β, and bacterium NTHi (10–11). As shown in Fig. 1*B*, CYLD knockdown by siRNA-CYLD greatly enhanced NF-κB activation. To determine whether CYLD knockdown also enhances NF-κB-dependent transcription of several key inflammatory mediators, we next assessed the effect of siRNA-CYLD on TNF-α- and NTHi-induced up-regulation of TNF-α, IL-1β, and IL-8 using Q-PCR analysis. As shown in Fig. 1*C*, CYLD knockdown greatly enhanced induction of TNF-α, IL-1β, and IL-8 by TNF-α and NTHi (*upper* and *lower panels*), respectively. To further confirm whether CYLD knockdown also enhances NF-κB activation in primary epithelial cells, we then examined the effect of siRNA-CYLD on NF-κB activation in primary NHBE cells. As evidenced in Fig. 1*D*, NF-κB activation was markedly enhanced by siRNA-CYLD in NHBE cells. Similarly, activation of NF-κB induced by other known NF-κB inducers phorbol ester (phorbol 12-myristate 13-acetate) and peptidoglycan was also enhanced by siRNA-CYLD (data not shown). Taken together, these data indicate that CYLD is indeed a negative regulator for NF-κB activation induced by a variety of known NF-κB stimuli.

CYLD Is Induced by a Variety of NF-κB Stimuli in Vitro and in Vivo—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 CYLD is induced by a variety of inflammation stimuli such as proinflammatory cytokines and bacteria. We thus tested our hypothesis by assessing the effects on CYLD expression of TNF-α,

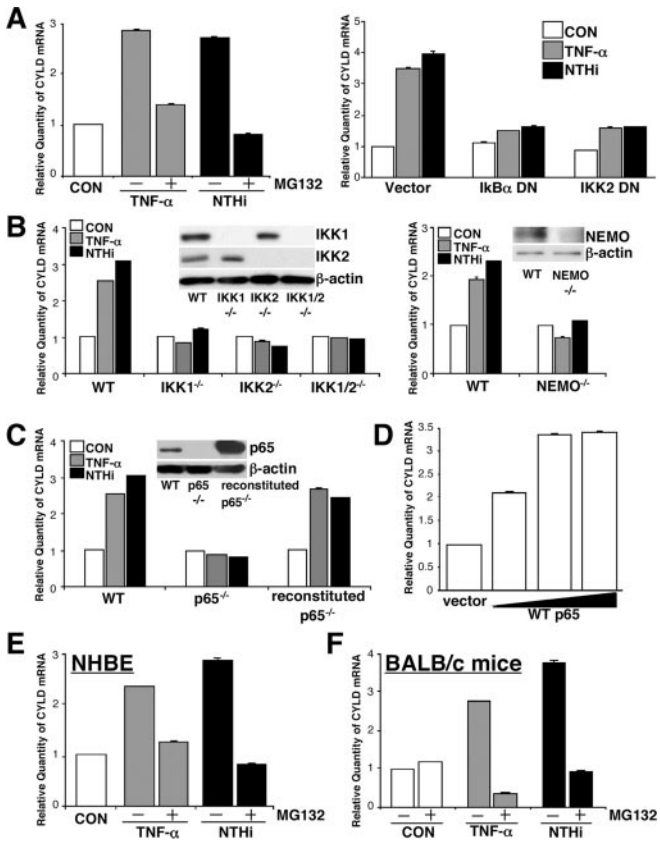

FIG. 3. **NF-κB is essential for induction of CYLD by TNF-α and NTHi.** *A*, MG-132 (*left*) and expressing a transdominant mutant of IκBα or a dominant-negative mutant of IKK2 (*right*) blocked CYLD induction. *B*, TNF-α or NTHi induced CYLD expression in WT but not in IKK1$^{-/-}$, IKK2$^{-/-}$, IKK1/2$^{-/-}$ MEFs (*left*) or NEMO-deficient cells (*right*). *C*, TNF-α or NTHi induced CYLD expression in WT or p65$^{-/-}$ MEFs reconstituted with WT p65 but not in p65$^{-/-}$ MEFs. *D*, expressing WT p65 induced CYLD expression. *E*, MG-132 blocked CYLD induction in primary NHBE cells. *F*, MG-132 blocked CYLD induction in the lungs of BALB/c mice *in vivo*. Values are the means ± S.D. (n = 3).

IL-1β, and NTHi that are known as highly potent NF-κB inducers. As shown in Fig. 2*A*, TNF-α, IL-1β, and NTHi strongly induced CYLD expression at the mRNA level in HeLa (*left*) and NHBE cells (*right*) by Q-PCR analysis. Consistent with this result, induction of CYLD by TNF-α and NTHi was also observed at the protein level (Fig. 2*B*). Similarly, CYLD induction by peptidoglycan, phorbol 12-myristate 13-acetate, and Gram-positive bacterium *Streptococcus pneumoniae* was also observed (data not shown), suggesting that induction of CYLD may be generalizable for a variety of NF-κB inducers. To further confirm whether CYLD is also induced *in vivo*, we next determined the effects of TNF-α and NTHi on CYLD expression in the lungs of the mice. As shown in Fig. 2*C*, both TNF-α and NTHi induced CYLD expression in a dose-dependent manner, respectively. The induction of CYLD became evident at 3 h, greatly up-regulated at 6 h, and returned to base-line level by 4 days after inoculation of either TNF-α or NTHi (Fig. 2*D*). Collectively, these data demonstrate that CYLD is induced by a variety of NF-κB stimuli including TNF-α and bacterium NTHi *in vitro* and *in vivo*.

NF-κB Is Essential for Induction of CYLD by TNF-α and NTHi—On the basis of evidence that NF-κB controls expression of many genes involved in inflammatory response (1–3) and CYLD, a key negative regulator for NF-κB (7–9), is induced by a variety of NF-κB inducers including TNF-α or NTHi, we next sought to determine whether NF-κB is also required for induction of CYLD in an inducible autoregulatory feedback

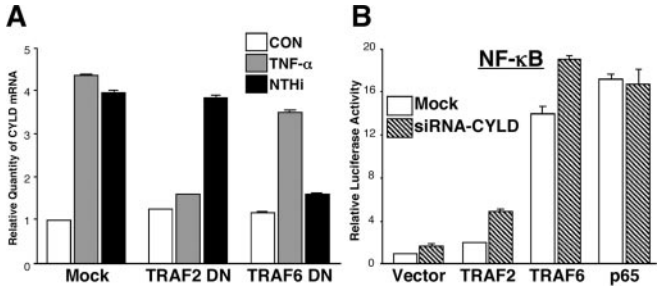

FIG. 4. **TRAF2 and TRAF6 are differentially involved in NF-κB-dependent induction of CYLD by TNF-α and NTHi.** *A*, CYLD induction by TNF-α or NTHi was differentially inhibited by expressing dominant-negative mutant of TRAF2 or TRAF6. *B*, activation of NF-κB by expressing WT TRAF2 or TRAF6, but not by expressing p65, was enhanced by siRNA-CYLD. Values are the means ± S.D. (n = 3).

manner. We first assessed the effects of blocking NF-κB signaling on CYLD induction by TNF-α and NTHi using various approaches. As shown in Fig. 3*A*, CYLD induction by either TNF-α and NTHi was greatly inhibited by blocking IKK2-IκBα signaling using MG-132 (*left*) and expressing a transdominant mutant of IκBα or a dominant-negative mutant of IKK2 (*right*) in HeLa cells (10–11), suggesting the involvement of IKK2-IκBα signaling in CYLD induction. To confirm the requirement of IKK complex in CYLD induction, we next investigated CYLD induction in MEFs derived from WT and IKK1$^{-/-}$ and IKK2$^{-/-}$ mice. As shown in Fig. 3*B*, both TNF-α and NTHi induced CYLD expression in WT but not in IKK1$^{-/-}$, IKK2$^{-/-}$, and double knockout IKK1/2$^{-/-}$ MEFs (*left*) (12–14). Likewise, TNF-α and NTHi induced CYLD expression in WT but not in NF-κB essential modulator (NEMO) or IKKγ-deficient cells (*right*) (16), thereby confirming the requirement of IKK complex signaling in CYLD induction. To determine whether NF-κB is required for CYLD induction, we then assessed the effects of TNF-α and NTHi on CYLD expression in WT and p65$^{-/-}$ MEFs (15). Fig. 3*C* shows that both TNF-α and NTHi induced CYLD expression in WT but not in p65$^{-/-}$ MEFs. When p65$^{-/-}$ MEFs were reconstituted with WT p65 expression plasmid, CYLD induction became evident in reconstituted p65$^{-/-}$ MEFs in response to both TNF-α and NTHi, indicating NF-κB is required for CYLD induction by TNF-α and NTHi. To further determine whether direct activation of NF-κB induces CYLD, we transfected HeLa cells with WT p65 expression plasmid. Interestingly, overexpression of WT p65 induced CYLD expression in a dose-dependent manner (Fig. 3*D*). Thus, it is clear that activation of NF-κB is indeed required for inducing CYLD expression. Similar to HeLa cells, induction of CYLD by TNF-α and NTHi was also inhibited by perturbing IκBα signaling using MG-132 in primary NHBE cells (Fig. 3*E*). Moreover, CYLD induction was also abolished by MG-132 in the lung of BALB/c mice, thus confirming the involvement of NF-κB signaling in CYLD induction *in vivo* (Fig. 3*F*). Taken together, our data suggest that IKK1/2/NEMO-IκBα-dependent activation of NF-κB is essential for CYLD induction by TNF-α and NTHi *in vitro* and *in vivo*, thereby providing evidence for an inducible autoregulatory feedback loop.

TRAF2 and TRAF6 Are Differentially Involved in NF-κB-dependent Induction of CYLD by TNF-α and NTHi—Having demonstrated the requirement of the IKK1/2/NEMO-IκBα-NF-κB signaling pathway in CYLD induction in an inducible autoregulatory feedback manner, still unknown are the upstream signaling components that mediate CYLD induction by TNF-α and NTHi CYLD. In review of the known signaling components mediating NF-κB activation upstream of NEMO, TNF receptor-associated factor 2 (TRAF2) and TRAF6 have been shown to act as important adaptor molecules involved in mediating

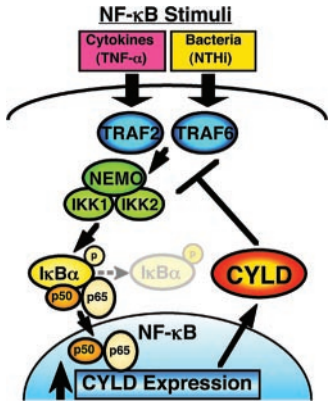

FIG. 5. **Schematic representation of the autoregulatory feedback loop through which activation of NF-κB by TNF-α and bacterium NTHi induce CYLD, which in turn leads to the inhibition of NF-κB signaling.**

TNF-α and bacteria-induced signaling leading to NF-κB activation, respectively (17, 18). Recently, interesting studies indicate that CYLD negatively regulates NF-κB activation by deubiquitinating TRAF2 and TRAF6 (7–9). We therefore investigated whether TRAF2 and TRAF6 are also involved in mediating CYLD induction. As shown in Fig. 4*A*, overexpressing a dominant-negative mutant of TRAF2 abrogated the induction of CYLD by TNF-α but not by NTHi. In contrast, transfecting the cells with a dominant-negative mutant TRAF6 inhibited the induction of CYLD by NTHi but not by TNF-α. These data suggest that TRAF2 and TRAF6 are differentially involved in CYLD induction by TNF-α and NTHi. To confirm whether inhibition of NF-κB activity by CYLD occurs through perturbing TRAF2- and TRAF6-mediated signaling, we assessed the effect of siRNA-CYLD on NF-κB activation induced by expressing WT TRAF2 and TRAF6. As expected, activation of NF-κB by expressing WT TRAF2 and TRAF6 was enhanced by CYLD knockdown (Fig. 4*B*). In contrast, activation of NF-κB by expressing WT p65 was unaffected by siRNA-CYLD. Thus, these data indicate that the inhibition of NF-κB by CYLD indeed occurs through perturbing TRAF2- and TRAF6-mediated signaling.

In summary, our findings revealed a novel autoregulatory feedback loop through which activation of NF-κB by cytokine TNF-α and bacterium NTHi induces CYLD, which in turn leads to the inhibition of NF-κB signaling (Fig. 5). In addition, TRAF2 and TRAF6 appear to be differentially involved in NF-κB-dependent induction of CYLD by TNF-α and NTHi. Moreover, the inhibition of NF-κB by CYLD occurs through perturbing TRAF2- and TRAF6-mediated signaling. Previous studies identified NF-κB-dependent transcriptional induction of its own inhibitor IκBα as an important mechanism to ensure the transient nature of NF-κB induction. It remains unclear whether the autoregulatory feedback control of NF-κB activation also occurs at the level upstream of IκBα. The present studies thus identified an autoregulatory feedback mechanism that negatively controls the more upstream signaling pathway leading to NF-κB activation. In contrast to the role that NF-κB-dependent induction of IκBα plays in controlling the transient nature of NF-κB induction, the NF-κB-dependent induction of CYLD may play a more important role in controlling the delayed activation of NF-κB induction. Thus, the involvement of the NF-κB-dependent induction of both IκBα and CYLD may be essential for ensuring the tight control of NF-κB activation in the transient and the delayed or persistent phases (19–21). It should also be noted that genomic sequence analysis revealed NF-κB sites within the putative CYLD promoter region, thereby providing further supportive information for the requirement of NF-κB in CYLD induction. Future studies will focus on cloning and identifying the regulatory region of CYLD gene that contains the functional κB site(s). In addition, the involvement of other signaling pathways in CYLD induction should also be explored as our data did not preclude the involvement of other signaling components.

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