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 IkB 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 IkBa gene transcription and down-regulation of transient activation of NF-κB (4–6). Recent studies have identified the tumor suppressor CYLD7 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 hTNF-α, hTNF-β, 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 NM105247), 5'-ACGCCAACATCCTCATTCA-TCACT-3' (forward primer) and 5'-AGGTCGGTGTTCAACAGTTGATCA-CT-3' (reverse primer); TaqMan probe, 5'-6-carboxyfluorescein-AAAA-AGCGTGTTCCTTGGTACACCCCG-6-carboxytetramethylrhoda-mine-3'; mouse CYLD (GenBank™ accession number NM173369, 5'-CTC AGC AGA TTT AGA AGA AGA CT-3' (forward primer) and 5'-TCT CCT GGG CCT GCA AAA T-3' (reverse primer); rat CYLD (GenBank™ accession number XM226242, 5'-CTC AGC AGA TTT AGA AGA AGA GAG ACA AAA T-3' (reverse primer).

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7 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.
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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. 1A, left). Consistent with this result, the endogenous CYLD protein was also greatly reduced (Fig. 1A, 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. 1B, 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 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. 1D, 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,
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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 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. 3A, 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. 3B, both 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 blocking NF-κB in primary NHBE cells (Fig. 3C). 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. 2C, 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. 2D). 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 loop. 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. 3A, 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. 3B, both 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 blocking NF-κB in primary NHBE cells (Fig. 3C). 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. 2C, 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. 2D). 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.

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. 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
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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. 4A, 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 activity by CYLD knockdown (Fig. 4B). 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 activity 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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