CYLD and the NEMO Zinc Finger Regulate Tumor Necrosis Factor Signaling and Early Embryogenesis*

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Background: Nuclear factor-κB (NF-κB) regulates expression of genes responsible for cell survival and immunity.

Results: Cylindromatosis (CYLD) protein activated NF-κB through interaction with other pathway proteins.

Conclusion: The non-catalytic function of CYLD is important for TNF-induced NF-κB signaling during embryogenesis.

Significance: Our findings may explain some of the developmental and immunologic findings in patients with immune deficiency disorders.

NF-κB essential modulator (NEMO) and cylindromatosis protein (CYLD) are intracellular proteins that regulate the NF-κB signaling pathway. Although mice with either CYLD deficiency or an alteration in the zinc finger domain of NEMO (K392R) are born healthy, we found that the combination of these two gene defects in double mutant (DM) mice is early embryonic lethal but can be rescued by the absence of TNF receptor 1 (TNFR1). Notably, NEMO was not recruited to the TNFR1 complex of DM cells, and consequently NF-κB induction by TNF was severely impaired and DM cells were sensitized to TNF-induced cell death. Interestingly, the TNF signaling defects can be fully rescued by reconstitution of DM cells with CYLD lacking ubiquitin hydrolase activity but not with CYLD mutated in TNF receptor-associated factor 2 (TRAF2) or NEMO binding sites. Therefore, our data demonstrate an unexpected non-catalytic function for CYLD as an adapter protein between TRAF2 and the NEMO zinc finger that is important for TNF-induced NF-κB signaling during embryogenesis.

The transcription factor nuclear factor-κB (NF-κB)† regulates expression of genes responsible for multiple biological processes including development, inflammation, and tumorigenesis (1–3). Upon TNF-α engagement at the TNFR1 receptor, multiple signaling constituents including TNFR1-associated death domain protein (TRADD), Fas-associated death domain protein (FADD), TNF receptor-associated factor 2 (TRAF2), and receptor-interacting protein (RIP) are recruited to the proximal signaling complex to activate the IkB kinase (IKK) complex (4). The IKK complex is composed of the catalytic IKKα/β kinases and a regulatory protein, NF-κB essential modulator (NEMO; also known as IKKγ). Lysine 329 in the NEMO zinc finger (ZF) domain has been reported to be a Lys-63-linked polyubiquitination site, and nearly 50% of cases of the human disease ectodermal dysplasia with immune deficiency result from hypomorphic mutations in the NEMO ZF domain. It has therefore been proposed that the ZF domain of NEMO is important for efficient NF-κB activation. However, the precise mechanism by which the NEMO ZF domain modulates NF-κB signaling is not known.

Cylindromatosis protein (CYLD) is a ubiquitin hydrolase that removes both linear and Lys-63-linked ubiquitin chains from NEMO and other proximal signaling proteins (5–8), and has also been reported to interact with the NEMO ZF domain. Genetic alterations in CYLD have been observed in patients with familial cylindromatosis and multiple myeloma, malignancies that are associated with sustained NF-κB activity (9, 10). Although these results suggest that CYLD terminates signaling events, they do not rule out the possibility of other physiologically relevant functions for CYLD. To investigate the potential role of interactions between CYLD and NEMO, we generated and characterized double mutant (DM) mice that have a mutation in NEMO at lysine 392 (K392R or KR) (11) and are deficient in CYLD (12).

Experimental Procedures

Mice—CYLD-deficient mice (12), NEMO-K392R (NEMO-KR) mice (11) and WT mice were bred in the mouse specific pathogen-free animal facility at the NIAID, National Institutes of Health. TNFR1-deficient mice were purchased from The Institute for Medical Research.
nitrocellulose membrane (Invitrogen). Western blots were developed using an enhanced chemiluminescence system according to the manufacturer’s instructions (Amersham Biosciences). For immunoprecipitation, cell lysates were normalized with respect to protein concentration, precleared with protein G-conjugated Sepharose-4B (Sigma), and then incubated with the appropriate antibodies at 4 °C for 2–16 h followed by incubation with protein G-conjugated beads for 1 h. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with the appropriate antibodies.

For Fc-TNF pulldown, MEFs were treated with Fc-TNF-α (1,000 ng/ml) for the indicated time. Cells were harvested in lysis buffer containing 1% CHAPS (Sigma) and incubated with protein G-conjugated Sepharose-4B and 2 μg of different CYLD plasmids as indicated in 100 μg of Nucleofector reagent in a sterile cuvette, and an electric pulse (program T-20) was applied. After 24 h, the cells were treated with 10 ng/ml TNF-α for 4 h, and luciferase activity in the cell lysate was measured. The transfection efficiency was measured by co-transfection with 1 μg Renilla luciferase expression vector and measuring enzymatic Renilla luciferase activity.

Results

NEMO-K392R/CYLD Double Mutant Mice Exhibit Embryonic Lethality That Is Rescued by TNFR1 Deficiency—Both CYLD-deficient mice and NEMO-KR mice are phenotypically normal and fertile (11, 12). Because the NEMO gene is on the X chromosome, we bred NEMO-KR (CYLD+/−NEMO66/kr) female mice with CYLD-KO (CYLD−/−NEMO66/kr) male mice to generate CYLD+/−/NEMO-KR mice, which were then intercrossed. Although CYLD+/−/NEMO-KR mice appeared healthy and bred normally, we were unable to generate viable double homozygous mutant mice (Fig. 1A). To investigate this further, we sacrificed CYLD+/−/NEMO-KR pregnant female mice to isolate embryos at embryonic day 12.5. Normally developing and absorbed embryos were noted in the uterus, and PCR genotyping confirmed that the absorbed embryos were invariably double mutants (Fig. 1B). Moreover, a backcross of CYLD+/−/NEMO+kr mice with CYLD+/−/NEMO+kr did not generate any viable double homozygous mutant mice. Thus, double mutation of CYLD and NEMO-KR causes embryonic lethality.

Blocking the TNF pathway by deletion of the TNF or TNFR1 gene prevents TNF-α-induced apoptosis in embryonic fetal livers (14, 15). We therefore interbred TNFR1-deficient mice with CYLD+/−/NEMO-KR mice to determine whether the developmental lethality of DM embryos could be rescued by the absence of TNFR1 signaling. Genotyping of live born mice from CYLD+/−/NEMO-KR/TNFR1−/− intercrosses showed that triple mutant animals were successfully generated when animals
**CYLD and NEMO Regulate TNF Signaling**

| Genotype          | Weaning Expectation |
|-------------------|---------------------|
| CYLD+/+NEMO-KR    | 41 (33.25)          |
| CYLD+/−NEMO-KR    | 92 (66.50)          |
| CYLD−/−NEMO-KR    | 0 (33.25)           |
| Total             | 133                 |

**Figures:**

A. Genotype Weaning Expectation table.

B. Images showing CYLD+/+NEMO-KR, CYLD+/−NEMO-KR, and CYLD−/−NEMO-KR conditions.

C. Gel electrophoresis images for CYLD, NEMO, TNFR1, and Triple conditions.

D. Gel electrophoresis images for MEF DNA and MEF Cell Lysate with size markers for WT and Mut conditions.

E. Bar graphs showing cell survival percent for WT, KO, KR, and DM cell types.

F. Bar graphs showing cell survival percent for Medium, TNF, TNF/Z, and TNF/N conditions.

G. Western blot images for WT and DM conditions with Time (6hr) and Cleaved Caspase3, PARP, and β-actin markers.
were homozygous TNFRI−/− (Fig. 1C). These data confirmed that the in utero death of CYLD-KO/NEMO-KR DM mice is mediated by TNFRI signaling and can be rescued by TNFRI deficiency.

**TNF-α Induces Apoptosis in DM MEFs**—To further study the consequences of CYLD deficiency combined with NEMO-K392R, we generated four different types of MEF: WT, CYLD-KO, NEMO-KR, and DM. These were confirmed by PCR genotyping of MEF DNA and Western blot analysis of MEF protein extracts (Fig. 1D). Treatment with human TNF-α induced time- and dose-dependent cell death in the DM MEFs but not in the other three types of MEF (Fig. 1E). To determine of the nature of the cell death induced in these cells by TNF-α, we assessed the viability of WT and DM MEFs after a 16-h treatment with TNF-α, alone or in combination with z-VAD (a pan-caspase inhibitor that blocks apoptosis) or necrostatin (an inhibitor of necrosis that inhibits the loss of mitochondrial membrane potential following TNF-α treatment). WT MEFs were relatively resistant to TNF-α-mediated cytotoxicity under all conditions, whereas DM MEFs showed increased viability in the presence of z-VAD but not necrostatin (Fig. 1F). We also measured TNF-α-induced caspase activity in the MEFs. In the presence of cycloheximide, activation of caspase-3 and PARP cleavage was detectable in WT cells at 6 h. Moreover, caspase activity was enhanced in DM MEFs (Fig. 1G), leading to increased apoptosis of these cells. Collectively, these findings indicate that TNF-α induced apoptosis, but not necrosis, in DM MEFs.

**NF-κB Activity and JNK Activation Are Impaired in DM MEFs**—The sensitivity of DM MEFs to TNF-α-induced apoptosis suggested a defect in NF-κB signaling. Because translocation of NF-κB to the nucleus results from phosphorylation and degradation of IκBα, we measured phosphorylation of IκBα in WT, CYLD-KO, NEMO-KR, and DM MEFs after stimulation by TNF-α. Phosphorylation of IκBα could be detected in wild-type cells as early as 5 min after TNF-α stimulation (Fig. 2A). However, minimal phosphorylation of IκBα was detected in extracts of DM MEFs, even after a 10-min stimulation with TNF-α. Phosphorylation of the NEMO-associated kinases IKK α/β was readily detectable in WT cells 10 min after TNF-α stimulation, whereas phosphorylation of IKK α/β was minimally detectable in extracts of DM MEFs, even after a 20-min stimulation with TNF-α. We next investigated IKK complex integrity by immunoprecipitation of whole cell extracts with anti-NEMO antibody. As shown in Fig. 2B, anti-NEMO antibody successfully pulled down Iκκβ. NEMO immunoprecipitates were used to enhance detection of phosphorylated IKK. However, no phosphorylation of IKK α/β following TNF-α stimulation could be detected in cell extracts from DM MEFs. These data suggested that the double mutation of CYLD and NEMO-KR does not disrupt IKK complex integrity, but affects IKK α/β activation in response to TNF-α.

Because activation of MAPKs by TNF is known to play a role in the regulation of cytokine gene expression, we treated MEFs with TNF-α and assessed the phosphorylation status of key signaling molecules by Western blotting. The phosphorylation status of Erk was comparable among the four different MEFs; however, activation of JNK was impaired in DM MEFs when compared with the other three types of MEF (Fig. 2C). These data suggest that, in addition to NF-κB signaling, JNK activation in response to TNF-α treatment is impaired in DM MEFs and genes that require the cooperation of NF-κB and JNK will be particularly affected.

We next examined expression of the chemokine KC (CXCL1), a well characterized NF-κB-dependent target gene that is normally up-regulated in response to TNF-α. As shown in Fig. 2D, KC expression increased following TNF-α stimulation in WT, CYLD-KO, and NEMO-KR cells, but was undetectable in DM MEFs. Although wild-type MEFs showed dose-dependent production of KC following TNF-α treatment, KC expression remained undetectable in DM MEFs at all concentrations tested (Fig. 2E). The failure of DM cells to produce KC following TNF-α stimulation is consistent with an impaired NF-κB signaling function.

**Ubiquitinated RIP and CYLD Are Recruited to the TNFRI Complex**—Treatment with TNF-α induces conjugation of RIP, a TNFR1-binding protein, with linear and Lys-63-linked polyubiquitin chains. We therefore examined RIP ubiquitination in WT, CYLD-KO, NEMO-KR, and DM MEFs using antibodies specific for the type of ubiquitin linkage (linear, Lys-63-linked, or Lys-48-linked). Both linear and Lys-63-linked polyubiquitination of RIP was enhanced in CYLD-KO and DM MEFs treated with TNF-α (Fig. 3A), suggesting that CYLD modulates both types of ubiquitin linkage to RIP. To determine whether ubiquitinated RIP is present in the TNFR1 signalosome, proteins from lysates of unstimulated cells or cells stimulated with Fc-TNF-α were pulled down by protein G beads and subjected to Western blot analysis with antibody to RIP. Ubiquitinated RIP was recruited to TNFRI in WT, CYLD-KO, NEMO-KR, and DM MEFs following treatment with TNF-α, with significant enhancement of recruitment in DM MEFs (Fig. 3B). Interestingly, CYLD was also detected in this complex, and there was no difference in CYLD recruitment between WT and NEMO-KR MEFs. CYLD has been shown to interact with the NEMO proline-rich domain (16), and these results suggest that the NEMO K392R mutation does not affect recruitment of CYLD into the TNFR1 complex.

Recruitment of RIP by TNFRI might induce cell death by enhancing formation of death complex II associated with

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**FIGURE 1.** CYLD and NEMO lysine 392 are required for embryonic development. A, genotyping results from CYLD−/−/NEMO-KR heterozygous intercross. B, gross appearance of CYLD+/−/NEMO-KR, CYLD+/−/NEMO-KR, and CYLD−/−/NEMO-KR embryos at day 12.5. C, PCR genotyping of genomic tail DNA of WT, CYLD-KO, NEMO-KR, TNFR1-KO, and CYLD-KO/NEMO-KR/TNFRI1-KO mice. Mut, mutant. D, PCR genotyping of genomic DNA (left panel) and Western blot (WB) analysis of CYLD and NEMO protein expression (right panel) in WT, CYLD-KO (KO), NEMO-KR (KR), and DM MEFs. E, TNF-induced cell death. Cell viability was measured after treatment of WT, CYLD-KO, NEMO-KR MEFs with 10 ng/ml TNF-α (left panel) or the indicated TNF-α concentrations (right panel) for 16 h. Viability is expressed relative to that of untreated cells (100%). m/m, mutant/mutant; NS, non-specific. F, WT and DM MEFs were treated with TNF-α for 16 h alone or in combination with z-VAD (TNF/2) or necrostatin (TNF/M). Data are means ± S.D. G, cleavage of caspase-3 and PARP following treatment with TNF-α. WT and DM MEFs were treated with the indicated reagents for 6 h followed by Western blotting of cytoplasmic extracts with antibodies specific for caspase-3 and PARP. The same membrane was reprobed with anti-β-actin antibody as a control to show equal protein loading. T, TNF-α; C, cycloheximide; T/C, TNF-α/cycloheximide; T/C/Z, TNF-α/cycloheximide/z-VAD.
FADD and downstream caspases (17, 18). By immunoprecipitation with anti-FADD and blotting with anti-RIP antibody, we found that more ubiquitinated RIP was associated with FADD-RIP death complex in DM MEFs than in WT MEFs (Fig. 3C). These results suggest that enhanced RIP ubiquitination in TNF-α-stimulated DM MEFs promotes the formation of death complex II and augments cell death.

**FIGURE 2.** Defective NF-κB signaling in double mutant MEFs. A, WT, CYLD-KO (KO), NEMO-KR (KR), and DM MEFs were treated with 10 ng/ml TNF-α for the indicated times followed by Western blotting of cytoplasmic extracts with antibody specific for phospho-IκBα (P-IκBα) or phospho-IκKa/β (P-IκKa/β). The membrane was reprobed with antibodies against IKKβ and GAPDH as controls. B, impaired phosphorylation of IKKα/β in the NEMO complex of DM MEFs. Lysates were prepared from MEFs following treatment with 10 ng/ml TNF-α. The NEMO protein complex was analyzed by immunoprecipitation with an anti-NEMO antibody followed by immunoblotting with antibodies specific for phospho-IKKα/β, IKKβ, and NEMO. C, WT, CYLD-KO, NEMO-KR, and DM MEFs were treated with 10 ng/ml TNF-α for the indicated times followed by Western blotting of cytoplasmic extracts with antibody specific for phospho-JNK (P-JNK) or phospho-ERK (P-ERK). The membrane was reprobed with antibodies against JNK and ERK as controls. D, impaired TNF-induced KC production in DM MEFs. MEFs were stimulated with 10 ng/ml TNF-α for 20 h, and the KC concentration in culture supernatant was determined by ELISA. E, KC expression in WT and DM MEFs following a 20-h treatment with increasing concentrations of TNF-α. Data are means ± S.D.
We next examined the fate of RIP in WT, CYLD-KO (KO), NEMO-KR (KR), and DM MEFs following TNF-α stimulation. In DM MEFs, RIP protein levels started to decrease after 1 h of TNF-α treatment and were almost completely undetectable after 2 h (Fig. 3D). In contrast, there was no reduction in RIP protein levels in WT, CYLD-KO, or NEMO-KR MEFs, even at 8 h. The loss of RIP in DM cells was TNF-α dose-dependent and could be prevented by the proteasome inhibitor MG-132 (Fig. 3E), indicating that the reduction in RIP levels is due to protein degradation. The degradation of RIP in TNF-α-stimulated DM cells is reminiscent of findings in NEMO-deficient cells, in which RIP levels decrease rapidly following stimulation with TNF-α (19). These results indicate that that stimulation-dependent interaction between NEMO and RIP is important for RIP stability.

**Endogenous NEMO from DM Cells Fails to Migrate onto the TNFR1 Complex**—Given our findings of severely impaired NF-κB activity in DM MEFs following TNF treatment, we tested for the presence of NEMO at the TNFR1 complex in TNF-α-stimulated DM and WT MEFs. NEMO from WT MEFs was recruited to TNFR1 as early as 5 min and increased at 15 min after treatment. In contrast, no NEMO was detected in the TNFR1 complex of DM MEFs although NEMO protein levels were similar to those of WT MEFs (Fig. 4A). In addition, in contrast to WT
MEFs, we did not detect IKKβ in the TNFR1 signaling platform of DM MEFs. These findings indicate that the impaired NF-κB activity in DM MEFs results from a failure to recruit NEMO to the TNFR1, most likely because of defective RIP-NEMO interaction.

Overexpression of Deubiquitinating Enzyme-inactive CYLD in DM MEFs Restores TNF-α-induced NF-κB Signaling—CYLD is a deubiquitinase that negatively regulates NF-κB and JNK signaling by removing ubiquitin chains from NEMO and TRAF2. Using a protein fragment complementation assay, CYLD was shown to physically interact with NEMO and TRAF2 independently of its enzymatic function. We have shown that CYLD is recruited into the TNFR1 complex in a ligand- and time-dependent manner that is unaffected by the presence of the NEMO K392R mutation (Fig. 3C). However, in the absence of CYLD, NEMO K392R cannot be recruited into the TNFR1 complex and NF-κB activity is markedly impaired in response to TNF. We investigated the functionality of CYLD interactions with NEMO and TRAF2 using an NF-κB reporter readout and CYLD overexpression (Fig. 4B and C). Transfection of WT CYLD into DM cells rescued TNF-α-induced NF-κB luciferase activity (Fig. 4C, right panel). Interestingly, overexpression of a deubiquitinating enzyme-inactive CYLD mutant (H870N) showed greatly increased NF-κB luciferase activity when compared with WT CYLD and was fully capable of rescuing IκBα degradation in DM cells in response to TNF-α treatment (Fig. 4C, left panel). These data suggest a previously unappreciated role for CYLD in TNF-induced NF-κB activation. Using point and deletion mutants of CYLD, we determined that the CYLD H870N mutant harboring a mutation in the TRAF2 binding domain (S461A) or deletion of the NEMO binding domain (CAP3) abolished NF-κB activity when overexpressed in DM MEFs (Fig. 4B). The loss of both NEMO-CYLD-TRAF2 and NEMO-RIP interactions in DM cells leads to complete abrogation of NF-κB activity and cell death. Furthermore, this defect is predicted to be specific to TNF/TRA2 as IκBα degradation and resynthesis are normal in DM MEFs treated with IL-1β or LPS (Fig. 4D and E). These data support the hypothesis that CYLD functions as an adaptor between NEMO and TRAF2 at the TNFR1 signaling platform.
In this study, we show that mice that harbor a NEMO K392R mutation and are deficient in CYLD experience early embryonic lethality. Further genetic deletion of TNFR1 rescues embryonic development, demonstrating that the inability of DM cells to signal properly in response to TNF results in attenuated NF-κB activity and cell death. In double mutant cells, we found both linear and Lys-63-linked polyubiquitin chains on RIP in the TNFR1 complex. However, in DM cells, this complex was devoid of NEMO K392R, suggesting that interaction between the NEMO UBAN (ubiquitin binding in ABIN and NEMO) domain and polyubiquitinated RIP is also affected. Although the precise mechanism awaits further investigation, alterations in the NEMO zinc finger have been reported to affect the binding affinity of the NEMO UBAN domain to Lys-63 polyubiquitin (20). Both linear and Lys-63-linked polyubiquitination of RIP was enhanced in DM MEFs, and the altered ubiquitin chain topology bound to RIP may further reduce the capacity of the TNFR1 complex to recruit NEMO K392R via its UBAN domain.

In the context of the findings presented here, it is interesting to note that CYLD is only required for TNF signaling when the zinc finger of NEMO is mutated (K392R). NF-κB activity induced by TNF is well preserved in NEMO K392R MEFs and can be rescued in DM MEFs with overexpression of the NEMO K392R, suggesting that interaction between the NEMO UBAN (ubiquitin binding in ABIN and NEMO) domain and polyubiquitinated RIP is also affected. Although the precise mechanism awaits further investigation, alterations in the NEMO zinc finger have been reported to affect the binding affinity of the NEMO UBAN domain to Lys-63 polyubiquitin (20). Both linear and Lys-63-linked polyubiquitination of RIP was enhanced in DM MEFs, and the altered ubiquitin chain topology bound to RIP may further reduce the capacity of the TNFR1 complex to recruit NEMO K392R via its UBAN domain.

In closing, it is important to note that the zinc finger motif is stabilized through a combination of cysteine and histidine residues that are not affected by the NEMO K392R mutation. Missense mutations that disrupt the NEMO zinc finger structure cause ectodermal dysplasia with immune deficiency, although the precise mechanism by which they affect NF-κB signal strength and physiological outcome is not fully understood (21–24). Our findings suggest that such mutations affect the binding affinity of NEMO for CYLD and may explain some of the developmental and immunologic findings in patients with ectodermal dysplasia with immune deficiency.

Author Contributions—Y. Z. designed the project, collected and analyzed data, and wrote the manuscript. C. A. M. collected and analyzed data and revised the manuscript. L. W. collected and analyzed data. K. I. and E. M. O. provided reagents. J. D. A. provided reagents and revised the manuscript. D. W. B. collected and analyzed data and revised the manuscript. A. J. designed the project, analyzed data, and wrote the manuscript.

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