Tumor Suppressor CYLD Acts as a Negative Regulator for Non-Typeable *Haemophilus influenzae*-Induced Inflammation in the Middle Ear and Lung of Mice

Jae Hyang Lim1, Hirofumi Jono1, Tomoaki Koga1, Chang-Hoon Woo2, Hajime Ishinaga3, Patricia Bourne5, Haodong Xu3, Un-Hwan Ha1, Haidong Xu1, Jian-Dong Li1,*

1 Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York, United States of America, 2 Cardiovascular Research Institute, University of Rochester Medical Center, Rochester, New York, United States of America, 3 Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, New York, United States of America

Non-typeable *Haemophilus influenzae* (NTHi) is an important human pathogen causing respiratory tract infections in both adults and children [1]. In adults, it exacerbates chronic obstructive pulmonary diseases, and in children, it causes otitis media, the most common childhood infection and the leading cause of conductive hearing loss [2–4]. Despite the need for prophylactic measures, development of a vaccine for preventing NTHi infections has been difficult and still remains a great challenge. Moreover, inappropriate antibiotic treatment contributes to the worldwide emergence of antibiotic-resistant strains of NTHi. Therefore, there is an urgent need for developing alternative therapeutic strategies for the treatment of NTHi infections based on understanding the molecular pathogenesis of NTHi infections. Like most other bacterial infections, NTHi infection is characterized by inflammation, which is mainly mediated by nuclear factor kappaB (NF-κB)-dependent production of pro-inflammatory mediators [5–8]. We have previously shown that NTHi induces Toll-like receptor (TLR) 2-dependent activation of NF-κB via an IKKβ-IκBα- and p38 mitogen-activated protein kinase (MAPK)-dependent signaling pathway [6,9,10]. However, the key signaling adaptors that link TLR2 with IKK and MAPK in mediating NTHi-induced inflammation remain unknown.

INTRODUCTION

Non-typeable *Haemophilus influenzae* (NTHi), a gram-negative bacterium, is an important human pathogen in both adults and children [1]. In adults, it exacerbates chronic obstructive pulmonary diseases, and in children, it causes otitis media, the most common childhood infection and the leading cause of conductive hearing loss [2–4]. Despite the need for prophylactic measures, development of a vaccine for preventing NTHi infections has been difficult and still remains a great challenge. Moreover, inappropriate antibiotic treatment contributes to the worldwide emergence of antibiotic-resistant strains of NTHi. Therefore, there is an urgent need for developing alternative therapeutic strategies for the treatment of NTHi infections based on understanding the molecular pathogenesis of NTHi infections. Like most other bacterial infections, NTHi infection is characterized by inflammation, which is mainly mediated by nuclear factor kappaB (NF-κB)-dependent production of pro-inflammatory mediators [5–8]. We have previously shown that NTHi induces Toll-like receptor (TLR) 2-dependent activation of NF-κB via an IKKβ-IκBα- and p38 mitogen-activated protein kinase (MAPK)-dependent signaling pathway [6,9,10]. However, the key signaling adaptors that link TLR2 with IKK and MAPK in mediating NTHi-induced inflammation remain unknown.

Although the inflammatory response triggered by bacteria is essential for eradicating bacterial pathogen, excessive inflammatory response is clearly detrimental to the host, due to severe tissue damage [11,12]. To avoid overactive and detrimental inflammatory responses in infectious disease, the bacteria-induced inflammatory response must be tightly regulated. During evolution, the host has developed a variety of strategies to prevent detrimental inflammatory response during bacterial infections. Among all strategies, bacteria-induced negative feedback regulation is thought to play a critical role in preventing overactive inflammatory response by tightly regulating the activity of the key receptor-dependent signaling adaptors specifically activated by bacterial pathogens. In the pathogenesis of chronic obstructive pulmonary diseases and otitis media, to avoid detrimental inflammatory responses in NTHi infection, TLR2 signaling must be tightly regulated. However, despite the importance of tight regulation in preventing overactive inflammatory response, the molecular mechanisms underlying the negative feedback regulation of inflammation in the pathogenesis of NTHi infection remain unknown.

CYLD was originally identified as a tumor suppressor, loss of which causes a benign human syndrome called cylindromatosis [13–15]. In vitro studies have indicated that CYLD is a member of the deubiquitinating enzyme family that specifically digests polyubiquitin chains. Transfection studies showed that CYLD deubiquitinates TRAF2 and TRAF6 and acts as a negative regulator for activation of NF-κB by tumor necrosis factor receptor (TNFR) and TLR [16–18]. Recently, we, together with others, showed that CYLD also negatively regulates activation of MAPKs, including p38 MAPK [19–22]. Moreover, the expression of CYLD is itself under the control of NF-κB [20,23], suggesting...
that CYLD is involved in a negative feedback regulation of NF-κB activation and NF-κB-dependent gene expression. Given the important role that NF-κB plays in host immune and inflammatory response in bacterial infection, it is logical to hypothesize that CYLD may act as a negative regulator for immune and inflammatory response against invading bacteria such as NTHi in vivo. However, despite recent studies demonstrating the role of CYLD in regulating T cell receptor signaling and tumor cell proliferation in vivo, the biological role of CYLD especially its negative role in inflammation in vivo still remains unknown.

In the present study, by using NTHi-induced otitis media and pneumonia model in wild type (WT) and Cyld-deficient mice, we provide in vivo evidence that NTHi induces pro-inflammatory response through TLR2-dependent MyD88-TRAF6/7-NF-κB signaling pathway, and CYLD negatively regulates NF-κB-dependent inflammatory response by NTHi via deubiquitination of TRAF6 and TRAF7. These studies may lead to development of novel therapeutic strategies for controlling overactive inflammatory response in respiratory bacterial infections.

MATERIALS AND METHODS

Mice

**Cyld**<sup>−/−</sup> mice were generated by homologous recombination as previously reported [21]. A Cyld-gene targeting construct was designed to delete exons 2 and 3 and replace them with a lacZ reporter and a neomycin resistance gene. The targeting vector was linearized and electroporated into 129S ES cells. Clones resistant to G418 were selected and screened for homologous recombinants by Southern blot analysis. Two targeted ES cell clones were microinjected into C57BL/6 blastocysts, and the resulting chimeras were mated to C57BL/6 females to generate mice heterozygous for the Cyld mutation. Homozygous knockout animals were obtained by mating of heterozygous males and females. Genotyping was performed by PCR on tail-derived genomic DNA, and absence of RNA transcript and protein expression was confirmed by RT-PCR and Western blot analysis. **Th2**<sup>−/−</sup> and **MyD88**<sup>−/−</sup> were described previously [24].

Animal experiments

For NTHi-induced otitis media model in WT and Cyld<sup>−/−</sup> mice, anaesthetized mice were transtympanically inoculated with 1 × 10<sup>7</sup> CFU of NTHi under the surgical microscope, and saline was inoculated as control. The inoculated mice were then sacrificed by intraperitoneal inoculation of 100 mg/kg sodium pentobarbital at 3, 6, 9, 24, 72, and 168 hours after inoculation of NTHi. Eardrum was inspected under the otoscope and findings were recorded. To assess the mRNA expression of inflammatory mediators, total RNA was extracted from the bulla of NTHi and saline-inoculated ear at the time points indicated above and real-time quantitative PCR (Q-PCR) was performed as previously [20]. For histological analysis, dissected temporal bones were stained with hematoxylin and eosin, and then sections were stained with hematoxylin and eosin (H&E) to visualize inflammatory response and pathological changes in the middle ear. H&E-stained middle ear sections were embedded in paraffin, and sectioned at 5-μM thickness. Sections were then stained with hematoxylin and eosin (H&E) to visualize inflammatory response and pathological changes in the middle ear.

For NTHi-induced pneumonia model in WT and Cyld<sup>−/−</sup>, Th2<sup>−/−</sup>, and MyD88<sup>−/−</sup> mice, anaesthetized mice were intratracheally inoculated with 5 × 10<sup>7</sup> CFU of NTHi, and saline was inoculated as control. The inoculated mice were then sacrificed by intraperitoneal inoculation of 100 mg/kg sodium pentobarbital at the time points indicated above. For histological analysis, dissected lung was inflated and fixed with 10% buffered formaldehyde, embedded in paraffin, and sectioned at 5-μM thickness. Sections were then stained and inspected as described above. For polymerase chain reaction (PCR) analysis, bronchoalveolar lavage (BAL) was performed by cannulating the trachea with sterilized PBS. Cells from BAL fluid were stained with Hemacolor (EM Science) after cytospin centrifugation (Thermo Electronic Co.). To assess the mRNA expression of inflammatory mediators, total RNA was extracted from the lung of NTHi and saline-inoculated lung at the time points indicated above and Q-PCR was performed as described previously [20]. All animal experiments were approved by the Institutional Animal Care and Use Committee at University of Rochester.

**Bacteria strain and culture**

Clinical isolate of NTHi wild-type strain 12 was used in *in vitro* cell culture experiments and *in vivo* animal experiments [9]. Bacteria were grown on chocolate agar at 37°C in an atmosphere of 5% CO<sub>2</sub> for overnight and inoculated in brain heart infusion broth supplemented with 3.5 μg of NAD per ml (BHI). After overnight incubation, bacteria were subcultured into 5 ml of fresh BHI and the log phase NTHi that was monitored by measurement of optical density (OD) value was washed and suspended in phosphate-buffered saline for *in vitro* cell experiments and in isotonic saline for *in vivo* animal experiments. For *in vitro* experiments, the epithelial cells were treated with NTHi at a multiplicity of infection (MOI) of 1:25 for various times as indicated. For *in vivo* animal experiments, NTHi was inoculated into the middle ear for otitis media model and the lung for pneumonia at a concentration of 1 × 10<sup>7</sup> CFU/ear and 5 × 10<sup>7</sup> CFU/lung, respectively.

**Cell culture**

Human middle ear epithelial cell line HMEEC-1, a commonly used middle ear cell line, was derived by human papilloma virus immortalization of primary human middle ear epithelial cells, and was maintained in a 1:1 mixture of Bronchial Epithelial Basal Medium (BEBM) and Dulbecco’s modified Eagle’s medium (DMEM) as described [6,9,25]. Airway epithelial cell line A549 was maintained as described [9,26]. Primary normal human bronchial epithelial cell (NHBEC) were purchased from Cambrex and were maintained as described previously in Bronchial Epithelial cell Growth Medium (BEGM) [6,23,25]. For air-liquid interface culture, NHBEC cells were cultured as described previously [27]. In brief, NHBEC cells were seeded at 2 × 10<sup>4</sup> cells/cm<sup>2</sup> onto 24-mm-diameter, 0.4 μm pore size, semi-permeable membrane inserts (Corning) in BEBM. The cultures were grown submerged for the first 7 days and then, the air-liquid interface was created by removing media from the apical compartment of the cultures. The culture media were changed every other day until the air-liquid interface was then created and were changed daily by replacing fresh media only to the basal compartment during the air-liquid interface culture. NHBEC cells were grown in air-liquid interface for 2–3 weeks before being used for the proposed experiments. All cells were cultured in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C.

**Transfection and Small Interfering RNA (siRNA)**

The plasmids TLR2, MyD88, TRAF6, and TRAF7 DN, WT CYLD, and NF-κB-luciferase reporter were described previously...
[6,9,20,23,25]. Cells were cultured on 24-well plates. After 24 hours, cells were co-transfected with or without NF-κB-luciferase reporter plasmid and various expression plasmids as indicated in the figure legends. 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 Co.) following the manufacturer’s instructions. At 40 hours after the start of transfection, cells were inoculated with NTHi for 5 hours before cell lysis for luciferase assay or mRNA analysis as described previously. RNA-mediated interference for down-regulating CYLD expression was done using siRNA-CYLD as described previously [20,23].

**Western blot (WB) analysis and immunoprecipitation (IP)**

Antibodies against TRAF6, HA and Ubiquitin were purchased from Santa Cruz Biotechnology, and CYLD was from IMGENEX.

Western blots were performed as described [20,23] and following the manufacturer’s instruction. Briefly, Western blots were performed using whole cell extracts, separated on 8–10% SDS-PAGE gels, and transferred to poly-vinylidine difluoride membranes. The membrane was blocked with 5% nonfat milk, incubated in a 1:2,000 dilution of a primary antibody, and incubated with 1:2,000 dilution of the corresponding secondary antibody.

Figure 1. **Cyld**<sup>−/−</sup> mice are hyperresponsive to NTHi-induced inflammation in the ear and lung of the mice. A, NTHi was trans-tympanically inoculated into the middle ears of WT and Cyld<sup>−/−</sup> mice and bullae were dissected from WT and Cyld<sup>−/−</sup> mice inoculated with NTHi and saline for the control for histological analysis (H&E stain, 200×). B, NTHi was intratracheally inoculated into the lungs of WT and Cyld<sup>−/−</sup> mice and lung tissues were dissected from WT and Cyld<sup>−/−</sup> mice inoculated with NTHi and saline for the control for histological analysis (H&E stain, 200×). C & D, NTHi was trans-tympanically (C) or intratracheally (D) inoculated into the middle ear (C) or lung (D), respectively, of WT and Cyld<sup>−/−</sup> mice and mRNA expression of the inflammatory mediators, IL-1β and MIP-2, was measured by Q-PCR analysis *p<0.05 compared with control inoculation in WT mice, **p<0.05 compared with NTHi inoculation in WT mice. Values are means ± S.D. (n = 3). CON, control.

doi:10.1371/journal.pone.0001032.g001
antibody. The membrane was reacted with chemiluminescence reagent ECL to visualize the blots. For immunoprecipitation, cell lysates were immunoprecipitated with 1 μg of the appropriate antibodies for overnight at 4°C and then conjugated to protein A/G-agarose beads for 2 hours at 4°C.

Statistical analysis
Data were analyzed using Student’s t-test. A value of $p<0.05$ was considered significant.

RESULTS
Cyld\(^{-/-}\) mice are hyperresponsive to NTHi-induced inflammation in the ear and lung of the mice
To determine the biological role of CYLD in regulating inflammation in vivo, Cyld-deficient mice were generated by using standard targeting technique as described previously [21]. Briefly, Cyld\(^{-/-}\) mice were generated by replacing exons 2 and 3 of Cyld gene with a neomycin resistance-lacZ cassette. Homozygous mutant was ascertained by PCR genotyping from tail tissue of mutant mice. RT-PCR analysis confirmed the absence of Cyld transcripts in Cyld\(^{-/-}\) mice, and immunoblot analysis of mouse embryonic fibroblast (MEF) using an antibody against CYLD showed Cyld deficiency. Cyld-deficient mice exhibit no overt abnormalities and have a normal lifespan.

We next investigated the negative role of CYLD in inflammatory response against NTHi infection in the middle ear and lung of Cyld\(^{-/-}\) mice. Age- and sex-matched WT and Cyld\(^{-/-}\) mice were inoculated with NTHi and inflammatory response in the middle ear and lung of infected mice was monitored up to 7 days. Histological examination of the middle ear revealed enhanced inflammatory responses including enhanced leukocyte infiltration in Cyld\(^{-/-}\) mice compared with WT mice (Fig. 1A). Consistent with this result, histological examination of the lung of NTHi-inoculated mice showed enhanced leukocyte infiltration in peribronchial and interstitial area in Cyld\(^{-/-}\) mice compared with WT mice (Fig. 1B).

NTHi-induced inflammatory response and leukocyte infiltration are mediated by pro-inflammatory mediators, including IL-1\(\beta\) and MIP-2 [5,6]. Thus we measured expression of IL-1\(\beta\) and MIP-2 at mRNA levels in the middle ear and lung from both WT and Cyld\(^{-/-}\) mice 9 hours after NTHi inoculation. Compared with WT mice, Cyld\(^{-/-}\) mice showed higher levels of IL-1\(\beta\) and MIP-2 mRNA expression in the middle ear (Fig. 1C) and in the lung (Fig. 1D). In addition, we also found significant increases in the levels of TNF-α mRNA expression in Cyld\(^{-/-}\) mice (data not shown).

Figure 2. NTHi-induced CYLD is responsible for down-regulation of inflammatory response. A & B. NTHi induced CYLD expression at both mRNA (A) and protein levels (B) in HMEEC-1 cells. C. NTHi induced CYLD expression at the mRNA level in the middle ear of WT mice. D & E. NTHi induced expression of CYLD and IL-1\(\beta\) (D) or MIP-2 (E) at the mRNA level in the lung of WT mice. *$p<0.05$ compared with CON. Values are means ± S.D. (n = 3). CON, control.

doi:10.1371/journal.pone.0001032.g002
shown). These results demonstrate that CYLD plays an important role in negatively regulating NTHi-induced inflammatory response in vivo.

**NTHi-induced CYLD is responsible for down-regulation of inflammatory response**

It is well known that a variety of genes involved in inflammatory response undergo changes in expression pattern in response to bacterial infection [28–30]. We recently found that the endogenous expression level of CYLD is relatively low in middle ear and lung under physiological condition [13], but highly inducible by bacterial pathogens [20,23], we hypothesized that CYLD is induced by NTHi and increased CYLD expression will in turn lead to inhibition of NTHi-induced inflammatory response, thereby preventing overactive inflammatory response that is detrimental to the host. We tested our hypothesis by first evaluating the effect of NTHi on CYLD expression. As shown in Fig. 2A, NTHi induced CYLD expression at the mRNA level in middle ear HMEC-1 cells. The induction of CYLD by NTHi was also confirmed at the protein level by performing Western blot analysis (Fig. 2B). Similar result was also observed in human airway A549 cells and the primary human airway epithelial cells cultured under both regular liquid culture and physiological air-liquid interface condition (Data not shown). Moreover, up-regulation of CYLD by NTHi was also observed in the middle ear of NTHi-inoculated WT mice (Fig. 2C). Marked induction of CYLD by NTHi was observed at 6–9 hours, and an even greater induction of CYLD was still observed at 3–7 days. Consistent with these findings, marked induction of CYLD by NTHi was observed in the lungs of NTHi-inoculated WT mice along with down-regulation of pro-inflammatory mediators, IL-1β and MIP-2 (Fig. 2D & E). It is interesting to note that the peak of CYLD induction was clearly preceded by the peak of the induction of IL-1β and MIP-2, thereby suggesting that NTHi-induced CYLD is responsible for down-regulation of inflammatory response.

![Graphs showing CYLD expression and inflammatory response](image-url)
NTHi induces inflammatory response through TLR2-MyD88-TRAF6/7-NF-κB signaling pathway

We next sought to identify the molecular target of CYLD in negatively regulating NTHi-induced inflammation. We first examined if TLR2-MyD88 and TRAFs, a family of adaptor proteins, are critically involved in mediating NTHi-induced inflammation. As shown in Fig. 3A, overexpressing dominant-negative mutants of TLR2, MyD88 or TRAF6 or TRAF7, a newly identified member of TRAF family, inhibited NF-κB activation by NTHi in human middle ear HMEEC-1 cells. Similar results were also observed in human airway epithelial A549 cells and human primary bronchial epithelial NHBE cells (Data not shown). Moreover, overexpressing dominant-negative mutants of MyD88, TRAF6 or TRAF7 also inhibited NTHi-induced expression of IL-1β and IL-8 in HMEEC-1 cells (Fig. 3B). Consistent with these in vitro findings, Tlr2<sup>−/−</sup> mice showed significant reduction in both IL-1β and MIP-2 expression, when mice were intratracheally inoculated with NTHi (Fig. 3C). Similar result was observed in MyD88<sup>−/−</sup> mice (Fig. 3D). Moreover, histological analysis of lungs of NTHi-inoculated mice revealed markedly reduced inflammatory response in Tlr2<sup>−/−</sup> mice compared with WT mice (Fig. 3E). Thus, it is evident that TLR2-dependent MyD88-TRAF6/7 signaling pathway plays a critical role in mediating NTHi-induced NF-κB-dependent inflammatory response in vitro and in vivo.

CYLD acts as a negative regulator for NTHi-induced NF-κB activation via negative cross-talk with TRAF6/7

Having demonstrated the critical role of TLR2-dependent MyD88-TRAF6/7 signaling pathway in NTHi-induced NF-κB-dependent inflammatory response, we next sought to determine if CYLD negatively regulates NTHi-induced NF-κB activation by targeting TRAF6/7. We initially evaluated the role of CYLD in activation of NF-κB and the resultant cytokine induction by NTHi by CYLD knockdown in human middle ear epithelial HMEEC-1 cells. We first confirmed the efficiency of CYLD-specific siRNA (siRNA-CYLD) in reducing CYLD expression. As expected, endogenous CYLD protein was markedly reduced by siRNA (Fig. 4A, upper panel). CYLD knockdown by using siRNA-CYLD enhanced activation of NF-κB by NTHi, whereas overexpression of WT-CYLD inhibited it (Fig. 4A, lower panel & Fig. 4B). Moreover, CYLD knockdown enhanced NTHi-induced DNA binding activity of NF-κB (Fig. 4C). Consistent with these results, siRNA-CYLD also enhanced the induction of CYLD in In Vivo Inflammation

![Figure 4. CYLD acts as a negative regulator for NTHi-induced NF-κB activation via negative cross-talk with TRAF6/7.](http://www.plosone.org/doi/abs/10.1371/journal.pone.0001032.g004)
IL-1β and IL-8 expression by NTHi in middle ear epithelial HMEEC-1 cells (Fig. 4D). Similar results were also observed in human airway epithelial A549 cells and primary airway epithelial NHBE cells (Data not shown). Our data thus suggest that CYLD indeed acts as a negative regulator for NTHi-induced NF-κB-dependent inflammatory response in middle ear and respiratory epithelial cells.

We next sought to determine how CYLD inhibits NTHi-induced NF-κB activation. We previously showed that TRAF7 cooperates with TRAF6 in mediating NF-κB activation. Since we have shown that TRAF6 and 7 are involved in NTHi-induced NF-κB activation, it is likely that CYLD may inhibit NTHi-induced NF-κB activation by targeting TRAF6 and 7. We first tested our hypothesis by evaluating if CYLD knockdown or overexpressing WT CYLD alters NF-κB activation induced by expressing WT TRAF6 and 7. As shown in Fig. 4E, TRAF7 synergistically enhanced TRAF6-induced NF-κB activation, and overexpression of WT-CYLD inhibited TRAF6 and 7-induced NF-κB activation, whereas CYLD knockdown by siRNA-CYLD enhanced NF-κB activation induced by wt-TRAF6 and 7. Our data thus suggests that CYLD inhibits NF-κB by targeting TRAF6 and 7.

CYLD inhibits TRAF6 and TRAF7 in a deubiquitination-dependent manner
Because CYLD is known as a deubiquitinating enzyme (DUB) and polyubiquitination plays an important role in TRAFs activation [16–18,20], we next determined if CYLD deubiquitinates TRAF6 and 7. We first examined whether NTHi induces polyubiquitination of TRAF6 and TRAF7. As shown in the Fig. 5A & C, NTHi induced polyubiquitination of TRAF6 and TRAF7 in a time-dependent manner, respectively. Moreover, co-expression of WT-CYLD caused a reduction in TRAF6 and TRAF7 ubiquitination, and CYLD knockdown by siRNA-CYLD markedly increased levels of ubiquitinated TRAF6 and TRAF7 in HeLa cells (Fig. 5B & D). Together, we conclude that CYLD inhibits NTHi-induced NF-κB-dependent inflammatory response by deubiquitinating TRAF6 and TRAF7.

DISCUSSION
Negative feedback regulation plays a critical role in preventing overactive and detrimental inflammatory response in a variety of human diseases including infectious diseases [31]. To fully understand the molecular mechanisms underlying the negative feedback regulation.
regulation of inflammatory response in NTHi infections including otitis media and chronic obstructive pulmonary diseases, it is essential to identify the key receptor-mediated signaling adaptors. We previously showed that TLR2 is required for NTHi-induced NF-κB-dependent inflammatory response [6,9,10]. However, the key signaling adaptors that link TLR2 with NF-κB still remain unknown. In the present study, by overexpressing dominant-negative mutants of TLR2, MyD88 and TRAF6/7 and by using Tlr2<sup>−/−</sup> and MyD88<sup>−/−</sup> mice, we demonstrated that NTHi induced NF-κB-dependent inflammatory response through TLR2-dependent MyD88-TRAF6/7 signaling pathway [Fig. 6]. This finding is of particular interest because disrupting the signaling-mediated by TRAFs, the bottleneck of the receptor-mediated signaling, should efficiently block NTHi-induced TLR2-dependent NF-κB activation and the subsequent inflammatory response. Thus, the TRAF6/7 complex appears to be an attractive target for therapeutic intervention [32–36]. Indeed, over the past decade significant effort has been put into developing therapeutic strategies to shut down TRAF6 signaling for reducing the mortality associated with septic shock [37]. Since TRAF6/7 complex is essential for transducing NTHi-initiated signal from surface TLR to the NF-κB in the nucleus, it would be thus an ideal target for negative feedback regulation in the pathogenesis of bacteria infection.

The deubiquitinating enzyme CYLD was initially identified as a tumor suppressor because loss of which causes a benign human tumor called cylindromatosis [13–15]. In vitro studies indicate that CYLD inhibits TNF-α-induced NF-κB activation by deubiquitinating TRAF2 [16–18]. Ubiquitination is previously known for its role in targeting proteins for degradation by the proteasome, but evidence of the nonproteolytic functions of ubiquitin is also rapidly accumulating. Initial evidence for the regulatory, rather than proteolytic, function of ubiquitin is provided by study of the TRAF proteins, which function as ubiquitin ligases to synthesize lysine 63 (K63)-linked polyubiquitin chains to mediate activation of downstream kinases through a proteasome-independent mecha-

![Figure 6. Schematic representation of the negative regulation of NTHi-induced inflammatory response by CYLD.](doi:10.1371/journal.pone.0001032.g006)
nism [36]. Despite the role of CYLD in inhibiting TRAF2 by deubiquitinating it, its role in deubiquitinating other TRAF family members remains unclear. The role of CYLD in regulating NTHi-induced NF-κB activation also remains unknown. Moreover, despite its known role in regulating T-cell development and tumor cell proliferation in vitro [39,40], its role in regulating inflammation in bacterial infections in vivo is still unclear. In the present study, we have shown that, by performing the inflammatory phenotype analysis of Cyld−/− mice, CYLD negatively regulates NTHi-induced inflammatory response in the middle ear and lung of Cyld−/− mice (Fig. 1), and this negative regulation results in enhanced pathological changes in the middle ear and lung as well as increased expression of pro-inflammatory mediators in vivo. Thus our data provide direct in vivo evidence for the negative regulation of CYLD in NTHi-induced inflammation via a deubiquitination-dependent inhibition of TRAF6/7 in the middle ear and lungs of mice.

In the present study, it is clear that CYLD acts as a critical negative regulator for NTHi-induced inflammation in the middle ear and lung. However, the expression of CYLD in the middle ear and lung is relatively low under physiological condition. Moreover, Cyld−/− mice exhibit no overt abnormalities under normal conditions [21,40]. Moreover it was recently reported that Cyld−/− mice are more susceptible to chemical-induced skin-tumorigenesis even though they appear normal under physiological condition. These interesting findings thus led us to hypothesize that CYLD, although expressed at relatively low level under physiological conditions, is up-regulated by bacteria during middle ear and lung infection, which in turn leads to the attenuation of NTHi-induced inflammation. In view of CYLD in negative regulation of bacteria-induced TLR2-dependent inflammatory response, it is interesting to note that CYLD expression itself is also regulated by bacteria-induced TLR activation [20,23]. CYLD is rapidly induced after activation of TLR2 by various bacterial pathogens including PGN, MALP-2, Pam3CSK4, and NTHi. Our finding reveals novel molecular mechanisms by which bacterial pathogens including NTHi induce CYLD expression, which in turn attenuates bacteria-induced inflammatory response. Given that prolonged CYLD expression is anticipated to suppress adequate host response to the invading pathogens, which is necessary for the adequate host defense, it is also of particular interest to investigate how CYLD expression is negatively regulated. This study should provide novel insights into how inflammation is tightly regulated and may lead to the development of novel therapeutic strategies for modulating inflammation.

ACKNOWLEDGMENTS

We are grateful to Drs. R. Bernardes and G. Courtois for kindly providing reagents.

Author Contributions

Conceived and designed the experiments: J-DL, JHL. Performed the experiments: JHL, HJ, TK, HI, HK, HH, BZ, HB, HX. Contributed reagents/materials/analysis tools: J-DL. Wrote the paper: J-DL, JHL.

REFERENCES

1. Foxwell AR, KylJM, Cripis AW (1998) Nontyphoidal Haemophilus influenzae: pathogenesis and prevention. Microbiol Mol Biol Rev 62: 294–308.
2. Rao VK, Krasan GP, Hendrixson DR, Davich S, St Greme JW 3rd (1999) Molecular determinants of the pathogenesis of disease due to nontypable Haemophilus influenzae. FEMS Microbiol Rev 23: 99–129.
3. Sethi S, Murphy TF (2000) Chronic obstructive pulmonary disease. N Engl J Med 343: 1969–1970.
4. Murphy TF (2000) Haemophilus influenzae in chronic bronchitis. Semin Respir Infect 15: 41–51.
5. Murphy TF (2006) The role of bacteria in airway inflammation in exacerbations of chronic obstructive pulmonary disease. Curr Opin Infect Dis 19: 225–230.
6. Watanabe T, Jono H, Han J, Lim DJ, Li JD (2004) Synergistic activation of NF-kappaB by nontyphoidal Haemophilus influenzae and tumor necrosis factor alpha. Proc Natl Acad Sci USA 101: 3563–3566.
7. Hayden MS, Ghosh S (2004) Signaling to NF-kappaB. Genes Dev 18: 2195–2224.
8. Ghosh S, Karin M (2002) Missing pieces in the NF-kappaB puzzle. Cell 109: Suppl: S81–96.
9. Shuto T, Xu H, Wang R, Han J, Li JD (2003) Activation of NF-kappaB by nontyphoidal Haemophilus influenzae is mediated by toll-like receptor 2-TAK1-dependent NIK-IKK alpha/beta-I kappa B alpha and M KK3/6/p38 MAP kinase signaling pathways in epithelial cells. Proc Natl Acad Sci USA 90: 8774–8779.
10. Imamoto A, Desbois-Mouthon C, Han J, Kai H, Cato AC, et al. (2002) Inhibition of p38 MAPK by glucocorticoids via induction of MAPK phosphatase-1 enhances nontyphoidal Haemophilus influenzae-induced expression of toll-like receptor 2. J Biol Chem 277: 4744–4750.
11. Ulevitch RJ, Tobias PS (1995) Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu Rev Immunol 13: 437–457.
12. Kurt-Jones EA, Chan M, Zhou S, Wang J, Reed G, et al. (2004) Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. Proc Natl Acad Sci USA 101: 1315–1320.
13. Biggell GR, Warren W, Seal S, Takahashi M, Rapley E, et al. (2000) Identification of the familial cylindromatous tumour-suppressor gene. Nat Genet 25: 169–163.
14. Leonard N, Chaggar R, Jones C, Takahashi M, Nikitopoulou A, et al. (2001) Loss of heterozygosity at cylindromatosis gene locus, CYLD, in sporadic skin adenomas. J Clin Pathol 54: 689–692.
15. Poblete Gutierrez P, Eggemann T, Heller D, Juergt FK, Beermann T, et al. (2002) Phenotype diversity in familial cylindromatosis: a framework mutation in the tumor suppressor gene CYLD underlies different tumors of skin appendages. J Invest Dermatol 119: 527–531.
16. Tromponki E, Hatzivasilou E, Tzichrintz T, Farmer H, Ashworth A, et al. (2003) CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature 424: 793–796.
17. Brummelkamp TR, Nijman SM, Dirac AM, Bernardi R (2003) Loss of the cylindromatous tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature 424: 797–801.
18. Kovalenko A, Chable-Besina C, Cantaugade C, Israel A, Wallach D, et al. (2003) The tumor suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. Nature 424: 801–805.
19. Reiley WW, Jin W, Lee AJ, Wright A, Wu X, et al. (2007) Deubiquitinating enzyme CYLD negatively regulates the ubiquitin-dependent kinase TAK1 and prevents abnormal T cell responses. J Exp Med 204: 1475–1483.
20. Jono H, Lim JH, Chen LF, Xu H, Trompouki E, et al. (2004) Negative regulation of JNK signaling by the tumor suppressor CYLD. J Biol Chem 279: 51611–51617.
21. Yoshida H, Jono H, Kai H, Li JD (2005) The tumor suppressor cylindromatosis (CYLD) acts as a negative regulator for toll-like receptor 2 signaling via negative cross-talk with TRAF6 AND TRAF7. J Biol Chem 280: 11411–11412.
22. Zhang J, Stirling B, Temmerman ST, Ma CA, Fuss IJ, et al. (2006) Impaired regulation of NF-kappaB and increased susceptibility to colitis-associated tumorigenesis in CYLD-deficient mice. J Clin Invest 116: 3042–3049.
23. Reiley WW, Jin W, Lee AJ, Wright A, Wu X, et al. (2007) Deubiquitinating enzyme CYLD negatively regulates the ubiquitin-dependent kinase TAK1 and prevents abnormal T cell responses. J Exp Med 204: 1475–1483.
24. Takeuchi O, Hoshino K, Akira S (2000) Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. J Immunol 165: 5392–5396.
25. Kwon SM, Wang B, Ruxter D, Lim JH, Koga T, et al. (2006) Synergistic activation of NF-kappaB by nontyphoidal H. influenzae and S. pneumoniae is mediated by CACK1, IKBlaa-KappaBalpha, and p38 MAPK. Biochem Biophys Res Commun 351: 368–375.
26. Sakai A, Han J, Cato AC, Akira S, Li JD (2004) Glucocorticoids synergize with IL-1beta to induce TLR2 expression via MAP Kinase Phosphatase-1-dependent mechanism. J Biol Chem 38171–38174.
27. Kim S, Schein AJ, Nadell JA (2005) E-cadherin promotes EGF-R-mediated cell differentiation and MUC1/CAG mucin expression in cultured human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 289: L1049–1060.
28. Dunne A, O’Neill LA (2003) The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. Sci STKE 2003: re3.
29. Akira S, Takada K (2004) Toll-like receptor signalling. Nat Rev Immunol 4: 499–511.
30. Beutler B (2004) Inferences, questions and possibilities in Toll-like receptor signalling. Nature 430: 257–263.
31. Liew FY, Xu D, Brint EK, O’Neill LA (2005) Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol 5: 446–458.
32. Deng L, Wang C, Spencer E, Yang L, Braun A, You J, et al. (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell 103: 351–361.
33. Inoue J, Ishida T, Tsukamoto N, Kobayashi N, Naito A, et al. (2000) Tumor necrosis factor receptor-associated factor (TRAF) family: adapter proteins that mediate cytokine signaling. Exp Cell Res 254: 14–24.
34. Weissman AM (2001) Themes and variations on ubiquitylation. Nat Rev Mol Cell Biol 2: 169–178.
35. Ben-Neriah Y (2002) Regulatory functions of ubiquitination in the immune system. Nat Immunol 3: 20–26.
36. Habelhah H, Takahashi S, Kadoya T, Watanabe T, et al. (2004) Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-kappaB. EMBO J 23: 322–332.
37. Fukushima T, Matsuzawa S, Kress CL, Bruey JM, Krajewska M, et al. (2007) Ubiquitin-conjugating enzyme Ubc13 is a critical component of TNF receptor-associated factor (TRAF)-mediated inflammatory responses. Proc Natl Acad Sci USA 104: 6371–6376.
38. Chen ZJ (2005) Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol 7: 758–765.
39. Reiley WW, Zhang M, Jin W, Losiewicz M, Donohue KB, et al. (2006) Regulation of T cell development by the deubiquitinating enzyme CYLD. Nat Immunol 7: 411–417.
40. Massoumi R, Chmielarska K, Hennecke K, Pfeifer A, Fassler R (2006) Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. Cell 125: 665–677.