

Original Paper

Cylindromatosis (CYLD), a Deubiquitinase, Attenuates Inflammatory Signaling Pathways by Activating Toll-Like Receptor 3 in Human Mesangial Cells

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Key Words

Cyld • *Lupus nephritis* • MDA5 • Mesangial cells • RIG-I • TLR3

Abstract

Background/Aims: Cylindromatosis (CYLD), a deubiquitinase, negatively regulates nuclear factor- κ B in various cells. However, its potential roles in glomerular inflammation remain unclear. Because the activation of the Toll-like receptor 3 (TLR3)/type I interferon (IFN) pathways plays a pivotal role in chronic kidney diseases (CKD), we examined the role of CYLD in the TLR3 signaling in cultured human mesangial cells (MCs). **Methods:** We stimulated CYLD-silenced MCs with polyinosinic-polycytidylic acid (poly IC), a synthetic analogue of dsRNA, and studied representative TLR3/IFN- β pathways (i.e., TLR3/IFN- β /retinoic acid-inducible gene-1 (RIG-I)/CCL5, and TLR3/IFN- β /melanoma differentiation associated gene 5 (MDA5)/CXCL10 axes) using RT-PCR, western blotting, and ELISA. We also used immunofluorescence staining and microscopy to examine mesangial CYLD expression in biopsied specimens from patients with CKD. **Results:** CYLD silencing resulted in an increase of poly IC-induced RIG-I and MDA5 protein levels and increased CCL5 and CXCL10 mRNA and protein expression, but unexpectedly decreased mRNA expressions of RIG-I and MDA5. Interestingly, CYLD silencing did not affect IFN- β or the phosphorylated STAT1 (signal transducers and activator of transcription protein 1). CYLD was highly expressed in biopsied specimens from patients with proliferative lupus nephritis (LN). **Conclusion:** CYLD inhibits post-transcriptional regulation of RIG-I and MDA5 expression following TLR3 activation in MCs. CYLD may be involved in the pathogenesis of CKD, especially pathogenesis of LN.

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Introduction

Cylindromatosis (CYLD) was initially identified as a mutated tumor-suppressor gene in familial cylindromatosis, a genetic disorder exhibiting multiple benign skin tumors [1, 2]. CYLD encodes a ubiquitin-specific protease (deubiquitinase) which removes the linear and Lys63-linked ubiquitin chains from ubiquitinated proteins, and thereby regulating cell proliferation and inflammation [1-4]. Reportedly, CYLD negatively regulates nuclear factor- κ B (NF- κ B) during innate immune responses in various cell types [2, 4]. Further, CYLD downregulates cytoplasmic responses to viral RNAs, including antiviral responses mediated by retinoic acid-inducible gene-I (RIG-I) in 293 EBNA cells infected with Sendai virus [5, 6]. On the other hand, CYLD was found to regulate ciliogenesis in ciliated epithelial cells independently of NF- κ B, suggesting that CYLD regulates cell physiology through alternative signaling pathways [7]. However, roles of CYLD in the pathogenesis of chronic kidney diseases (CKD) have not been fully elucidated [8].

Recently, dysregulated endosomal Toll-like receptor (TLR) signaling has been reported to sustain activation of the type I interferon (IFN) system, sometimes leading to the development of systemic lupus erythematosus (SLE) [9]. This theory supports a crucial role for the innate immune system and adaptive immunity in the pathogenesis of SLE. Further, viral dsRNA can reportedly activate TLR3 signaling in renal macrophages, plasmacytoid dendritic cells and renal mesangial cells (MCs) in a murine model of lupus nephritis (LN), resulting in excess production of pro-inflammatory cytokines and type I IFNs [10]. Given the importance of induction of type I IFNs by dysregulated TLR3 activation in the pathogenesis of LN and SLE [10-12], we have previously studied the TLR3/IFN- β signaling pathways following activation by polyinosinic-polycytidylic acid (poly IC). Poly IC is a synthetic analogue of viral dsRNA, which elicits a "pseudoviral" infection in cultured human MCs [11]. Mesangial chemokine/cytokine expression via upregulation of TLR3/IFN- β signaling was found to play a pivotal role in the pathogenesis of CKD, especially in LN [13-18]. Activation of TLR3/IFN- β signaling pathways in MCs was confirmed by renal biopsy specimens obtained from patients with proliferative LN [14, 16, 17]. However, to our knowledge, expression and roles of CYLD in the inflammatory signaling pathways activated by TLR 3 in human MC have not been studied. In this study, we investigated the roles of CYLD in representative TLR3/IFN- β cascades, TLR3/IFN- β /RIG-I/CCL5 and TLR3/IFN- β /melanoma differentiation-associated gene 5 (MDA5)/CXCL10 pathways in poly IC-treated MCs [13, 14].

Materials and Methods

Reagents

Poly IC and anti-actin rabbit IgG were from Sigma (St Louis, MO, USA). Small interfering RNA (siRNA) against CYLD (SI00110082) and non-silencing negative control siRNA (1027281) were from Qiagen (Hilden, Germany). Lipofectamine RNAiMAX was from Invitrogen (Frederick, MD, USA). The illustra RNAspin kit was from GE Healthcare (Buckinghamshire, UK). M-MLV reverse transcriptase and dNTP mix were from Thermo Fisher Scientific (Asheville, NC, USA). SsoAdvanced Universal SYBR Green Supermix was from Bio-Rad (Hercules, CA, USA). Oligonucleotide primers for reverse transcription and polymerase chain reaction (RT-PCR) were synthesized by Greiner Japan (Atsugi, Japan). Precast polyacrylamide gels were from ATTO (Tokyo, Japan). Anti-CYLD rabbit IgG (8462) was from Cell Signaling Technologies (Danvers, MA, USA). Mouse IgG against anti-phosphorylated STAT1 (signal transducers and activator of transcription protein 1) (p-STAT1) (sc-136229) and rabbit anti-STAT1 (sc-592) were from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-MDA5 antibody was from Immuno-Biological Laboratories (Takasaki, Japan). Enzyme-linked immunosorbent assay (ELISA) kits for CCL5 and CXCL10 were from R&D systems (Minneapolis, MN, USA). siRNAs against IFN- β and an anti-RIG-I antibody were previously described [13].

Cells

Normal human MCs were purchased from Lonza (Basel, Switzerland) and were maintained using the culture medium for growing MCs according to the supplier's protocol [13-18]. Cells were transfected with specific siRNA against human CYLD or with a non-silencing negative control using the Lipofectamine RNAiMAX reagent. Forty-eight hours after transfection, cells were treated with 30 µg/mL poly IC and further incubated for indicated durations. Cells were subjected to quantitative reverse-transcriptase PCR (RT-PCR) or western blotting and the conditioned media were collected for ELISAs. Cell viability was not affected in these experiments.

RNA extraction, reverse transcription and quantitative RT-PCR analysis

Total RNA was extracted from cells and reverse-transcribed into single-strand cDNA using M-MLV reverse transcriptase. cDNAs for IFN-β, CCL2, CCL5, CXCL10, RIG-I, MDA5, or 18S ribosomal RNA were amplified using SsoAdvanced Universal SYBR Green Supermix. The primers used are summarized in Table 1.

Western blotting

After the incubation, cells were lysed in the reducing Laemmli sample buffer. Cell lysates were electrophoresed on a 7.5% or 12.5% polyacrylamide gel, and proteins were transferred to polyvinylidene difluoride membranes. Membranes were incubated with antibodies against CYLD (1:1000 dilution), p-STAT1 (1:5000), STAT1 (1:10000), RIG-I (1:10000), MDA5 (1:1000) or actin (1:5000 dilution), and subsequently with a horseradish peroxidase-conjugated secondary antibody. Chemiluminescence substrates were used for detection. The intensity of the bands was quantified using image J software, and normalized with actin.

CCL5 and CXCL10 ELISA

Cell-conditioned media were collected after incubation and CCL5 and CXCL10 concentrations in the media were determined using ELISA kits according to the manufacturer's protocols.

Immunofluorescence staining of CYLD

Renal specimens were biopsied during routine diagnostic procedure in clinical practice. For this pilot study, we choose snap-frozen sections stored at in good condition. Specimens stained for CYLD were from patients with Class IV (A) LN (defined according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification for LN) (n=2), Class II LN (n=2), diffuse proliferative (severe form) IgA nephropathy (IgAN) (n=2), focal segmental mild proliferative (mild form) IgAN (n=2), and minimal-change nephrotic syndrome (MCNS) (as a non-inflammatory control, n=2). Since MCNS usually had no inflammation in the kidney, we used biopsy specimens of this disease as non-inflammatory control. OCT-embedded specimens were cut into 5-µm-thick sections using a cryostat, briefly fixed in cold acetone, and air-dried. Sections were transferred to slides; slides were then washed in phosphate-buffered saline (PBS) immediately before immunohistochemistry. The anti-CYLD rabbit IgG was added at a dilution of 1:50. After incubation for 40 min at 4 °C and several washes in PBS, slides were incubated sequentially with an anti-rabbit IgG conjugated with Alexa Fluor 488 at 1:50 for 30 min at 37 °C.

Table 1. Oligonucleotide primers used for quantitative real-time PCR

cDNA	Primers
IFN-β	F: 5'- CCTGTGGCAATTGAATGGGAGGC -3' R: 5'- CCAGGCACAGTGACTGTACTCCTT -3'
CCL2	F: 5'- AAAGTGAAGCTCGCACTCTCGC -3' R: 5'- ATTCTTGGGTTGTTGAGTGAGT -3'
CCL5	F: 5'- CTACTCGGGAGGCTAAGGCAGGAA -3' R: 5'- GAGGGGTTGAGACGGCGGAAGC -3'
CXCL10	F: 5'- ACCTCCAGTCTCAGCACCATG -3' R: 5'- TGGGAGGATGGCAGTGGAAG -3'
RIG-I	F: 5'- GTGCAAAGCCTTGGCATGT -3' R: 5'- TGGCTTGGGATGTGGTCTACTC -3'
MDA5	F: 5'-GTTGAAAAGGCTGGCTGAAAAC -3' R: 5'-TCGATAACTCCTGAACCACTG -3'
18S rRNA	F: 5'-ACTCAACACGGGAAACCTCA-3' R: 5'-AACCAGACAAATCGCTCCAC-3'

Statistical Analyses

Values are expressed as means \pm SD. Statistical significance was evaluated using the Student *t*-test, and $p < 0.05$ was considered significant.

Results

siRNA against CYLD increased the poly IC-induced expression of CCL5 and CXCL10 in cultured normal human MCs

Transfection of normal human MCs with siRNA against CYLD markedly enhanced CCL5 and CXCL10 expressions induced by poly IC (Fig. 1A and 1B). However, induction of neither IFN- β nor CCL2 by poly IC was affected by CYLD siRNA treatment. CYLD protein was detected in untreated cells (Fig. 2B), and CYLD protein levels were not altered after poly IC treatment (data not shown). Anti-CYLD siRNA treatment abolished CYLD protein expression as confirmed by western blotting (Fig. 2B).

Effect of CYLD silencing on RIG-I and MDA5 expression in poly IC-treated MCs

siRNA against CYLD decreased RIG-I and MDA5 mRNA expression in poly IC-treated MCs (Fig. 2A). On the contrary, RIG-I and MDA5 protein expression, which was induced by poly IC treatment, was increased by CYLD siRNA treatment (Fig. 2B, upper column). STAT1 phosphorylation was not affected by CYLD siRNA treatment (Fig. 2B, lower column).

CYLD immunoreactivity in biopsied specimens

Significant positive staining for CYLD was observed in the mesangial area in the biopsy specimen of only proliferative LN: no expression was detected in IgAN specimens even though both showed similar mesangial proliferation. No glomerular CYLD expression was detected in samples from patients with MCNS. Representative results are shown in Fig. 3.

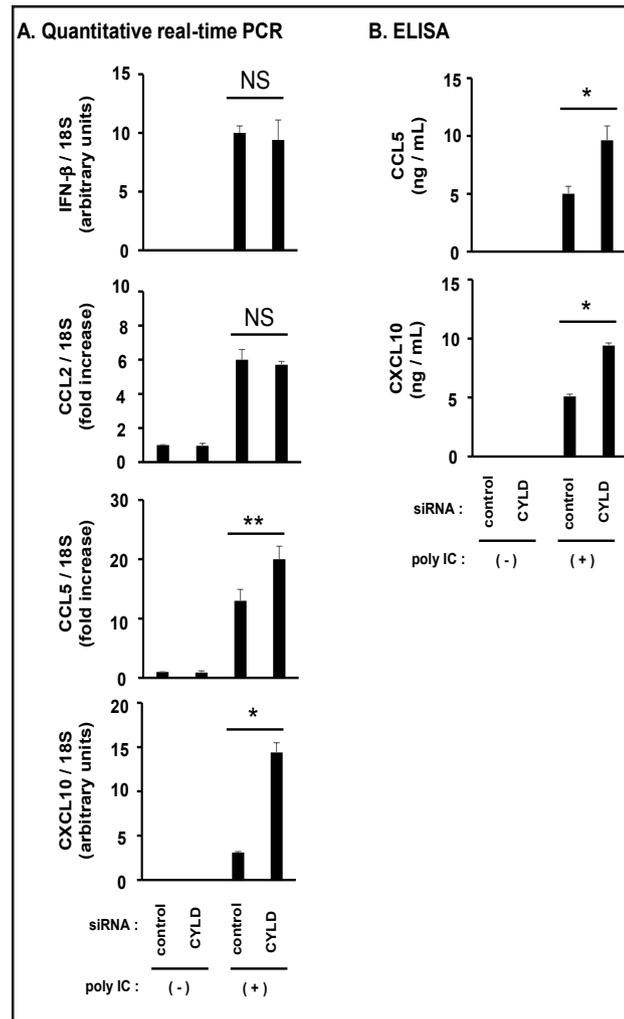


Fig. 1. Effect of small interfering RNAs against CYLD on the expression of IFN- β , CCL2, CCL5, and CXCL10 induced by poly IC in cultured normal human mesangial cells. Cells were transfected with siRNA against CYLD and were incubated for 48 h. Cells were subsequently treated with 30 μ g/mL poly IC for 2 h (for IFN- β) or 16 h (for other molecules). (A) The expression of mRNA for IFN- β , CCL2, CCL5, or CXCL10 mRNA expression was analyzed using quantitative real-time RT-PCR ($n=3$, * $p < 0.01$; ** $p < 0.05$; NS, not significant). (B) Cell-culture-conditioned media were collected, and CCL5 and CXCL10 concentrations were measured by ELISAs ($n=3$, * $p < 0.01$).

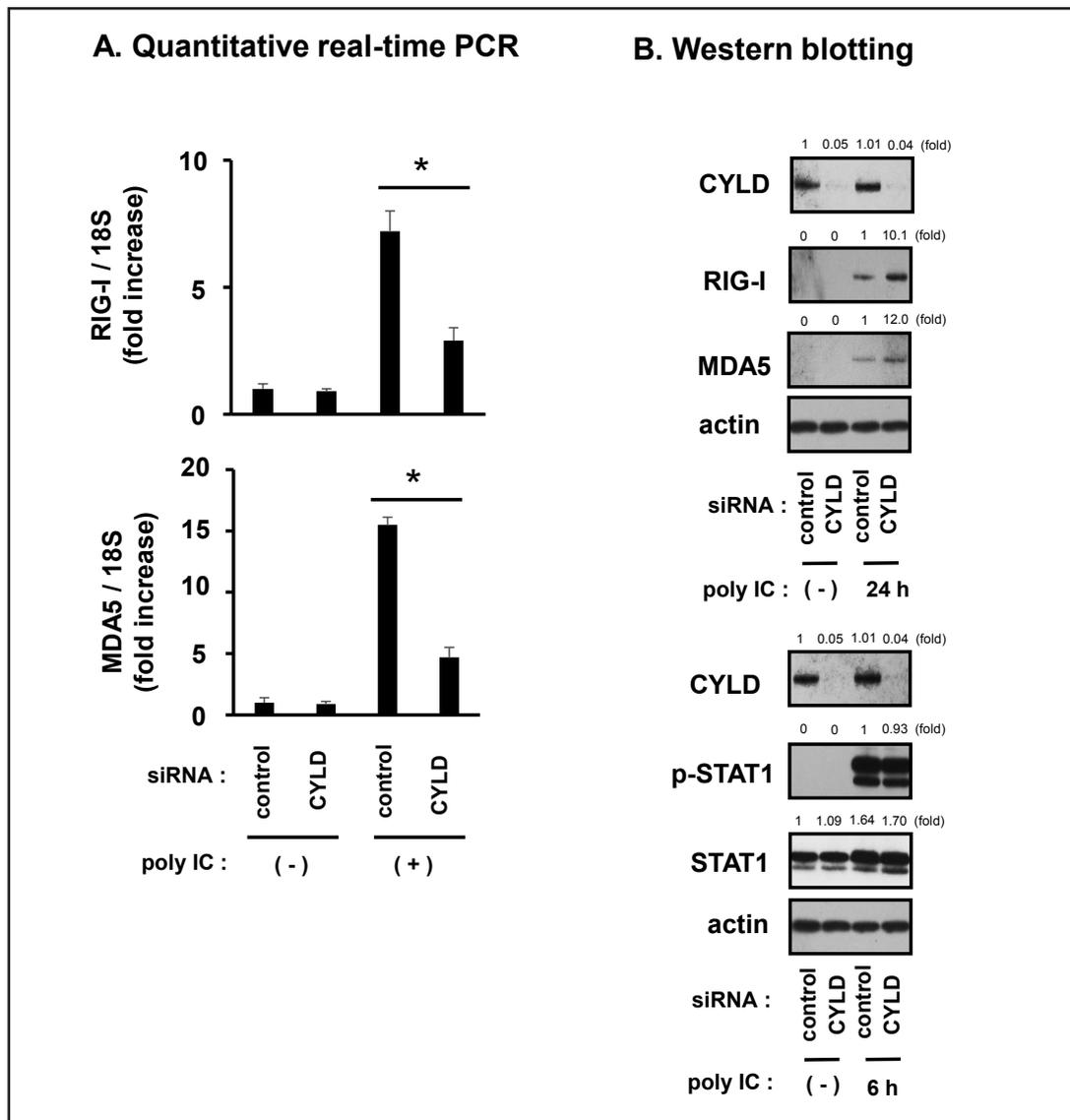
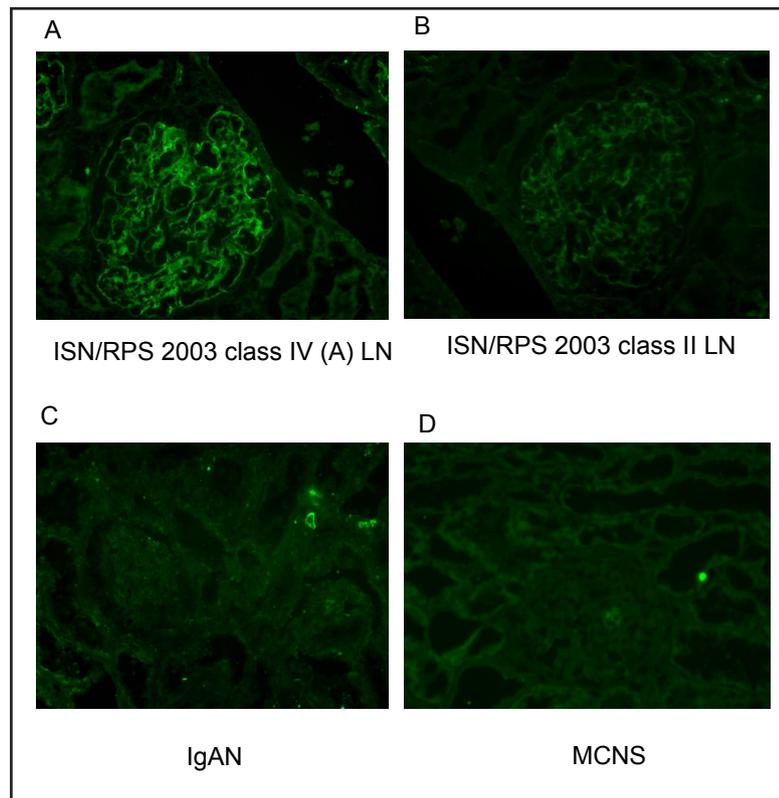


Fig. 2. Poly IC-induced expression of RIG-I and MDA5 mRNAs was decreased but the corresponding proteins were increased by CYLD silencing. Cells were transfected with siRNA against CYLD and incubated for 48 h as in Fig. 1. Cells were then treated with 30 μ g/mL poly IC. (A) After 16 h incubation, RNA was extracted from the cells and quantitative real-time RT-PCR was performed (n=3, *p<0.01). (B) After incubation for 6 h (for STAT1 and p-STAT1) or 24 h (for RIG-I and MDA5), the cells were lysed and expression of CYLD, STAT1, p-STAT1, RIG-I, MDA5, and actin was determined by western blotting. The intensity of the bands was quantified using image J software, and normalized with actin.

Discussion

CYLD is a deubiquitinase that removes lysine-63 ubiquitination and negatively regulates RIG-I signaling in innate immunity [5, 6]. Reportedly, CYLD-knockout mice spontaneously develop autoimmune disorders and intestinal inflammation, suggesting that CYLD negatively regulates hyperresponsive inflammation [19, 20]. CYLD has previously reported to physiologically attenuate interstitial inflammatory reactions and tubulointerstitial injury in patients with proteinuric IgAN [8]. Interestingly, CYLD was predominantly expressed in renal tubular epithelial cells but not in glomerular lesions in patients with IgAN [8]. So far, pathophysiological roles of CYLD in MCs in CKD have not been investigated. In this study,

Fig. 3. Immunofluorescence staining of CYLD in renal biopsied specimens obtained from patients with proliferative LN (A, Class IV-G (A) according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification for LN, and B, Class II LN), proteinuric IgAN (C, diffuse proliferative mesangial proliferation with u-prot/cr excess than 2.0), and minimal change nephrotic syndrome (D, served as a non-inflammatory control), respectively. A significant increase in glomerular CYLD immunoreactivity was observed in the proliferative lupus nephritis specimens (A), whereas immunoreactivity was low (B) or negligible in the other specimens (C and D).



we found that substantial amount of CYLD protein was constitutively expressed in cultured normal human MCs. Further, we firstly observed intense mesangial CYLD immunoreactivity of CYLD in biopsy specimens from patients with proliferative LN, whereas only low CYLD levels were expressed in specimens from patients with mesangial proliferative or mild form of IgAN. Accordingly, we previously found that mesangial RIG-I and MDA5 were highly expressed in the renal biopsy specimens of LN but lowly expressed in IgAN, suggesting that TLR3/IFN- β signaling is more prominently activated in LN than in IgAN [14]. Although detailed mechanisms of CYLD regulation remain to be elucidated, CYLD gene expression is induced reportedly by pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α and interleukin-1 β , in several cell types [20]. It is noteworthy that TNF- α has been reportedly highly activated in active lupus patients [21]. Taken together, we may postulate that prominent regional inflammation causes high expression of CYLD in biopsy specimens from proliferative LN, and this is enough for detection by immunofluorescence, but this theory remains to be tested in future studies.

Then, we examined the role of CYLD in representative TLR3/IFN- β signaling pathways, TLR3/IFN- β /RIG-I/CCL5, and TLR3/IFN- β /MDA5/CXCL10, in poly IC-treated MCs [13, 14]. We found that CYLD silencing resulted in enhanced the poly IC-induced expression of CCL5 and CXCL10, whereas induction of IFN- β and p-STAT1 was not affected. Because NF- κ B is involved upstream of IFN- β and p-STAT1 in the above signaling pathways activated by TLR3 in MCs [22], CYLD attenuated CCL5 and CXCL10 independently of NF- κ B and selectively regulated cytokine/chemokine expression downstream of TLR3 signaling. This observation also supports the theory that CYLD regulates the cellular physiology through alternative signaling pathways [7, 20]. Next, we found that poly IC-mediated induction of RIG-I and MDA5 proteins was increased by CYLD silencing, whereas induction of RIG-I and MDA5 mRNAs was decreased unexpectedly. Negative regulation of RIG-I and MDA5 proteins by CYLD is likely due to its deubiquitinase activity. Thus, our present results suggest that CYLD, at least in part, negatively regulates expression of RIG-I and MDA5 proteins post-

transcriptionally. These results are not always consistent with previous study postulating that CYLD negatively regulates RIG-I signaling in 293 EBNA cells infected with Sendai virus [5, 6]. We think the discrepancy may be due to the difference of the cell types or of the stimuli. Although precise mechanisms by which induction of mRNA for RIG-I and MDA5 was decreased by CYLD knockdown is not clear in the present study, we speculate that enhanced target protein expression, at least in part, resulted in reduced mRNA expression in a possible negative feedback system. To clarify this issue, further studies are needed. In this study, RIG-I protein was detected as a single band by western blotting, suggesting that most of the endogenous RIG-I protein may have not been ubiquitinated. To assess RIG-I's as well as MDA5's ubiquitination status in normal human MCs, we treated the cells with proteasome inhibitors. However, this approach was unsuccessful because treatment with proteasome inhibitors was harmful in this cell type, and viability of the cells markedly decreased viability in this cell type (data not shown). To examine the ubiquitination of target molecules, most of the studies have used co-overexpression of constructs for tagged-ubiquitin and another tagged-target molecule in immortalized cell lines [23]. However, such overexpression experiments are impossible in primary culture of MCs, because the number of cells are limited and the transfection of large constructs are harmful for these primary culture cells. Thus, detailed mechanisms by which CYLD negatively regulates RIG-I and MDA5 protein expression have not been clarified by this study, and these mechanisms should be further investigated in future studies.

Because immune and inflammatory reactions downstream of TLR3 signaling are important in host defenses against viral or "pseudoviral" infections in CKD, especially in LN [11, 15], we believe that regional CYLD dysregulation in MCs may lead to inflammatory renal diseases. Thus, CYLD is, at least in part, involved in the pathogenesis of CKD, but this theory remains speculative. Since we found that cross-talk between functional molecules induced by TLR3 activation, such as type I IFN-stimulated genes and pro-inflammatory chemokines/cytokines stimulated by type I IFNs, plays protective or deleterious roles during the pathogenesis of glomerular inflammation in MCs in CKD [17, 18], detailed understanding of interactions among these molecules during immune reactions may lead to the development of new therapeutic strategies for CKD.

Conclusion

CYLD was expressed in normal human MCs and biopsied specimens from patients with proliferative LN. CYLD negatively regulated expression of poly IC-induced RIG-I and MDA5 proteins and their downstream CCL5 and CXCL10 (Fig. 4). CYLD is probably involved in preventing excessive immune and inflammatory reactions in the kidney and thereby in the pathogenesis of CKD. Further studies are needed to confirm these preliminary findings.

Disclosure Statement

The authors have no conflict of interest.

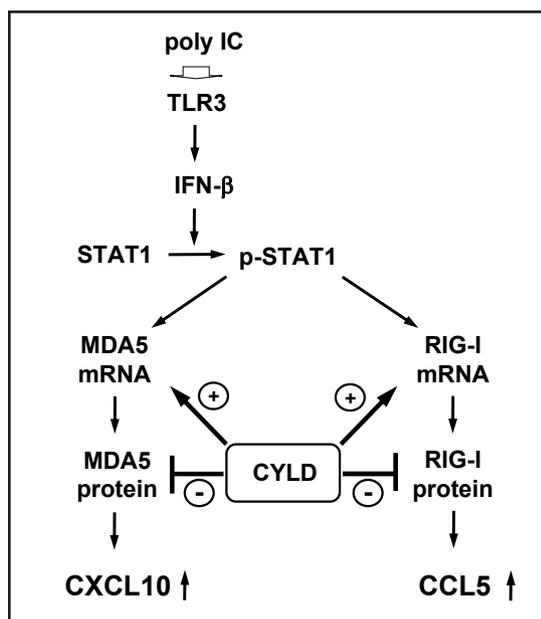


Fig. 4. Proposed roles of CYLD in negatively regulating RIG-I/CCL5 and MDA5/CXCL10 in human mesangial cells treated with poly IC.

Acknowledgements

This work was supported by grants-in-aid for science from the Ministry of Education, Culture, Sports, Science and Technology of Japan (JSPS KAKENHI Grant Number 16K10055 to H. T.) and a grant from the Okinaka Memorial Lifestyle Related Disease Foundation (to T. I.). The authors thank K. Nakata, M. Ono and K. Munakata for their help.

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