Original Article

CYLD deletion triggers nuclear factor-κB-signaling and increases cell death resistance in murine hepatocytes

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Abstract

AIM: To analyze the role of CYLD for receptor-mediated cell death of murine hepatocytes in acute liver injury models.

METHODS: Hepatocyte cell death in CYLD knockout mice (CYLD<sup>-/-</sup>) was analyzed by application of liver injury models for CD95- (Jo2) and tumor necrosis factor (TNF)-α- [D-GalN/lipopolysaccharide (LPS)] induced apoptosis. Liver injury was assessed by measurement of serum transaminases and histological analysis. Apoptosis induction was quantified by cleaved PARP staining and Western blotting of activated caspases. Nuclear factor (NF)-κB, ERK, Akt and jun amino-terminal kinases signaling were assessed. Primary Hepatocytes were isolated by two step-collagenase perfusion and treated with recombinant TNF-α and with the CD95-ligand Jo2. Cell viability was analyzed by MTT-assay.

RESULTS: Livers of CYLD<sup>-/-</sup> mice showed increased anti-apoptotic NF-κB signaling. In both applied liver injury models CYLD<sup>-/-</sup> mice showed a significantly reduced apoptosis sensitivity. After D-GalN/LPS treatment CYLD<sup>-/-</sup> mice exhibited significantly lower levels of alanine aminotransferase (ALT) (295 U/L vs 859 U/L, P < 0.05) and aspartate aminotransferase (AST) (560 U/L vs 1025 U/L, P < 0.01). After Jo injection CYLD<sup>-/-</sup> mice showed 2-fold lower ALT (50 U/L vs 110 U/L, P < 0.01) and lower AST (250 U/L vs 435 U/L, P < 0.01) serum-levels compared to WT mice. In addition, isolated CYLD<sup>-/-</sup> primary murine hepatocytes (PMH) were less sensitive towards death receptor-mediated apoptosis and showed increased levels of Bcl-2, XIAP, cIAP1/2, survivin and c-FLIP expression upon TNF- and CD95-receptor triggering, respectively. Inhibition of NF-κB activation by the inhibitor of NF-κB phosphorylation inhibitor BAY 11-7085 inhibited the expression of anti-apoptotic proteins and re-sensitized CYLD<sup>-/-</sup> PMH towards TNF- and CD95-receptor mediated cell death.

CONCLUSION: CYLD is a central regulator of apoptotic cell death in murine hepatocytes by controlling NF-κB dependent anti-apoptotic signaling.

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Key words: CYLD; Apoptosis; Nuclear factor-κB; Tumor necrosis factor-α

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necrosis factor-α; CD95; Liver

Core tip: Activation of death receptors, such as CD95 (Fas/APO-1) and tumor necrosis factor (TNF)-R1, is involved in the pathophysiology of acute and chronic liver diseases. Inactivation of the deubiquitinase CYLD is accompanied by increased survival of different cell types. However, the role of CYLD in death receptor-mediated apoptosis of hepatocytes has not been addressed so far. The study showed for the first time that CYLD negative hepatocytes are less sensitive to CD95 and TNF-R-mediated apoptosis, at least in part via triggering nuclear factor-κB signaling leading to induction of anti-apoptotic proteins. Inhibition of CYLD might represent a therapeutic approach to protect hepatocytes from death receptor-mediated apoptosis.

INTRODUCTION

Dysregulation of apoptosis is mechanistically important in the pathogenesis of liver diseases. Hepatocytes can undergo apoptosis via an extrinsic, death receptor-mediated pathway, or alternatively, intracellular stress can activate the intrinsic pathway of apoptosis. Both pathways converge on mitochondrial activation, which is a prerequisite for hepatocyte apoptosis[1]. The integrity of the outer mitochondrial membrane is regulated by the Bcl-2 protein family, which is divided into anti- and pro-apoptotic members[2]. Persistent apoptosis is a feature of chronic liver diseases. Acute liver failure (ALF) is characterized by massive apoptosis and is associated with life threatening consequences[3]. It is one of the most challenging gastrointestinal emergencies encountered in clinical practice and carries a high mortality rate worldwide[4]. Autoimmune hepatitis, viral hepatitis, alcohol consumption and hepatotoxins have been identified as triggers of ALF. Therapeutic approaches for delaying or reversing liver failure apart from orthotopic liver transplantation are rare. Understanding of the mechanisms of hepatocyte survival and cell death pathways would offer potential therapeutic targets.

Targeting ubiquitin related posttranslational modifications of signaling molecules is a novel approach in the treatment of several human diseases[5]. Ubiquitination controls the half-life of proteins, but also acts as modulator of the enzymatic activity or docking of regulatory proteins. The functional outcome of ubiquitination processes is determined by the linkage type of single or polyubiquitin chains: Lysine 48 (K-48)-linked polyubiquitination mainly targets proteins for proteasomal degradation, whereas lysine 63 (K-63)-linked polyubiquitination primarily leads to non-proteasomal modifications such as subcellular localization or protein-protein interactions. Ubiquitination is a dynamic process that can be counterbalanced by deubiquitinating enzymes including the tumor suppressor CYLD[6].

The ubiquitin C-terminal hydrolase domain of CYLD allows the enzyme to remove K-63-linked ubiquitin chains, e.g., from signaling molecules involved in the nuclear factor (NF)-κB pathway, the stress-activated protein kinase (SAPK)/Jun amino-terminal kinases (JNK) and Akt pathway[7,8]. Increased NF-κB activation promotes cell survival, at least in part via induction of anti-apoptotic Bcl-2 family members[9] as well as several inhibitor of apoptosis (IAP) proteins[10,11]. A loss of CYLD expression or its function was shown to increase NF-κB signaling in several cell types including hepatocytes[12,13].

Here we show for the first time that livers of CYLD-/- mice are less sensitive to CD95 and TNF-R-mediated apoptosis, at least in part via triggering NF-κB signaling leading to induction of anti-apoptotic proteins such as survivin. Therefore, inhibition of CYLD might represent a therapeutic approach to protect hepatocytes from death receptor-mediated apoptosis.

MATERIALS AND METHODS

Generation and genotyping of CYLD-/- mice

CYLD-/- mice were generated and genotyping was performed as previously described[14]. Animals were bred and housed at the animal facility of the University of Mainz in a standard laboratory animal environment (fresh filtered air, 15 changes per hour; temperature, 21 ± 2 °C; humidity, 50% ± 20%; and 12:12-h light:dark cycle). All experiments were done in accordance with the governmental and institutional guidelines and were performed under the written approval of the state animal care commission (Regierungspräsidium Koblenz, Germany).

Liver injury

Acute liver injury was induced in 8-10 wk old mice by i.p. injection of Jo2 antibody (0.5 µg/g bodyweight, BD Pharmingen, Heidelberg, Germany) or D-galactosamine (D-GalN; 0.75 mg/g bodyweight, Carl Roth, Karlsruhe, Germany) and lipopolysaccharide (LPS; 2.5 µg/g bodyweight, Sigma-Aldrich, Hamburg, Germany). 3 and 5 h after D-GalN/LPS and Jo2 injection, respectively, mice were anaesthetized by i.p. injection of Ketamine/Xylazine (350 mg/kg/55mg/kg bodyweight, Sigma-Aldrich), blood for measurement of serum transaminase levels was collected via cardiac puncture and mice were scarified by cervical dislocation.

Quantitative real-time polymerase chain reaction

Isolation of total RNA and cDNA synthesis was performed as previously described[15]. Quantitative real-time
polymerase chain reaction (q-RT PCR) was performed using primer assay kits (Qiagen, Hilden, Germany). Data acquisition and determination of gene expression was performed using the LightCycler software package (Roche). Each PCR reaction was run in duplicates. mRNA expression was normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase.

**Serum transaminases levels**

Blood was collected by cardiocentesis. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured in the Institute of Clinical and Laboratory Medicine at the University Hospital Mainz by standard procedures.

**Isolation, culturing and treatment of primary murine hepatocytes**

Hepatocytes were isolated by a two-step perfusion technique and cultured as previously described\[3\]. After 24 h, cells were treated with TNF-\(\alpha\) (Biomol, Hamburg, Germany), Jo2 (BD Pharmingen), actinomycin D (Carl Roth), BAY 11-7085 (Enzo-Life-Science, Lörrach, Germany) and YM155 (Selleckchem, Houston, United States). Cell viability was determined using a colorimetric 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

**Analysis of livers and immunohistochemistry**

Initially, livers were assessed visually. To investigate liver architecture and tumor histology, 3 \(\mu\)m thick sections were made from formalin-fixed paraffin-embedded liver tissues and were stained with hematoxylin and eosin (HE). Modified Gomori (Gom) staining was used to assess fibrotic remodeling and architectural distortion. For detection of cleaved poly (ADP-ribose) polymerase (cl. PARP) and RelA frozen liver tissues were sectioned (10 \(\mu\)m) and further proceeded using the NovoLink\textsuperscript{TM} Min Polymer Detection System (Leica Microsystems, Wetzlar, Germany) according to the manufacturer's instructions. For quantification six fields of view per liver section of 5 \(\mu\)m were counted. The primary antibodies anti-cleaved PARP [E51] (Abcam, Cambridge, United Kingdom) and anti-NF-\(\kappa\)B p65 antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) were used.

**Western blotting**

Tissue lysis, protein extraction and preparation of nuclear and cytosolic extracts were performed as previously described\[3,4\]. Protein concentration was determined by Bradford assay (Bio-Rad, Munich, Germany). Lysates were incubated in reaction buffer (25 mmol/L HEPES pH 7.5, 50 mmol/L NaCl, 10\% glycerol, 0.05\% CHAPS, and 5 mmol/L DTT) in the presence of 50 \(\mu\)mol/L caspase-3 fluorogenic substrate (Ac-DEVD-AFC) (Biomol). Assays were performed in black Maxisorb microtiter plates (Nunc, Langenselbold, Germany), generation of free AFC at 37 \(^\circ\)C was measured using a fluorometric plate reader set to an excitation wavelength of 400 nm and emission wavelength of 505 nm.

**Statistical analysis**

For comparison of experimental groups, the nonparametric Mann-Whitney \(U\)-test was applied. Statistical differences of in vitro PMH experiments were determined by standard analysis of variance (ANOVA) following Post-Hoc tests. A \(P\)-value less than 0.05 was considered as significant.

**RESULTS**

**Liver phenotype of CYLD deficient mice**

To explore the function of CYLD in vivo, CYLD-deficient mice were generated as previously described\[6\]. The absence of FL- (full length) CYLD and CYLD splice variants in liver tissues from CYLD deficient mice (CYLD\textsuperscript{-/-}) mice were asserted by Western blotting (Figure 1A, upper panel). Livers from CYLD\textsuperscript{-/-} mice were macroscopically normal. Pathohistological analysis revealed a regular liver architecture. Histological staining of cl. PARP indicated no spontaneous apoptotic liver damage in CYLD\textsuperscript{-/-} mice at the age of 3, 6 and 9 mo (Figure 1A, lower panel). ALT serum level as marker for hepatocyte damage was not different
from WT animals (Figure 1B). Interestingly, CYLD−/− mice showed a significantly higher liver/body weight ratio at the age of 9 mo compared to WT [0.05 < 0.035; P < 0.05; (Figure 1C)], while their body weight was comparable to WT animals (data not shown). To assess proliferation of hepatocytes, BrdU was administered i.p. in 8 wk old animals. Proliferation rates in WT and CYLD−/− mice were not significantly different (data not shown).

D-GalN/LPS- and CD95-induced liver injury is diminished in CYLD−/− mice

CYLD−/− hepatocytes did not show differences in TNF-RI and CD95 surface expression compared to WT (data not shown). To assess a potential impact of the CYLD deletion on liver injury resulting from activation of TNF-α signaling, WT and CYLD−/− mice were injected i.p. with D-GalN and LPS[16]. Triggering of TNF-RI by LPS-induced TNF-α leads to apoptotic liver injury upon D-GalN/LPS treatment, CYLD−/− mice exhibited significantly lower levels of ALT (295 U/L vs 859 U/L, P < 0.05) and AST (560 U/L vs 1025 U/L, P < 0.01, Figure 2A). Activation of caspase-8, -9 and -3 was reduced compared to WT as indicated by less detection of the respective cleaved (cl.) forms (Figure 2B). In line with this, caspase-3 activity assay showed significantly reduced substrate turnover in liver lysates of CYLD−/− mice compared to WT (Figure 2C). Analysis of Bid cleavage revealed slightly lower tBid levels after D-GalN/LPS treatment (Figure 2B). In pooled liver lysates from untreated mice no cl. caspase-3 levels were detectable (data not shown), confirming the results of immunohistological analysis of cl. PARP (Figure 1A, lower panel).

CYLD−/− mice showed significantly less hepatocyte damage compared to WT mice after D-GalN/LPS induced liver damage, which was further confirmed by a significant lower apoptosis rate of CYLD−/− hepatocytes compared to WT as indicated by less cl. PARP positive nuclei (24.8% vs 62.3%, P < 0.001, Figure 2D).

Administration of the agonistic CD95 receptor antibody Jo2 is an established model to induce acute liver injury[17]. To further examine the role of CYLD in acute liver injury, Jo2 was injected i.p. in WT and CYLD−/− mice. Liver injury was assessed by measurement of serum transaminases 3 h after injection: CYLD−/− mice showed 2-fold lower ALT (50 U/L vs 110 U/L, P < 0.01) and lower AST (250 U/L vs 435 U/L, P < 0.01) serum-levels compared to WT mice (Figure 2E). As in the D-GalN/LPS injury model CYLD−/− mice showed less caspase activation and liver cell damage compared to WT (Figure 2F-H).

Increased NF-κB activation in livers and PMH of CYLD−/− mice

NF-κB is known as a crucial decider of life and death[18].
Urbanik T et al. CYLD in liver injury

A

WT          CYLD

AST (U/L)

2800
2400
2000
1600
1200
800
400
0

0          15          30          45          60          75          90          105          120

HS (U/L)

2800
2400
2000
1600
1200
800
400
0

5 h D-GalN 0.75 mg/gbw

/LPS 2.5 µgbw

UL

ALT (U/L)

2800
2400
2000
1600
1200
800
400
0

5 h D-GalN 0.75 mg/gbw

/LPS 2.5 µgbw

UL

Caspase-3 activity (emission 505 nm)

WT          CYLD

B

Caspase-8

Tubulin

cl. Caspase-8/p43

cl. Caspase-8/p18

Tubulin

Caspase-9

cl. Caspase-9

Tubulin

Caspase-3

cl. Caspase-3

Tubulin

Bid

Tubulin

tBid

Tubulin

57 kDa

55 kDa

43 kDa

18 kDa

55 kDa

49 kDa

39 kDa

37 kDa

55 kDa

35 kDa

19 kDa

17 kDa

55 kDa

22 kDa

55 kDa

15 kDa

55 kDa

35 kDa

2000

1600

1200

800

400

0

Caspase-3 activity (emission 505 nm)

WT          CYLD

C

D

WT          CYLD

HE

cl. PARP

HE

cl. PARP

Ratio of cl. PARP positive nuclei (%)

WT          CYLD

15 kDa

55 kDa

22 kDa

55 kDa

19 kDa

55 kDa

17 kDa

55 kDa

2000

1600

1200

800

400

0

Ratio of cl. PARP positive nuclei (%)

WT          CYLD

0

20

40

60

80
Figure 2  Decreased liver injury in CYLD−/− mice after D-galactosamine/lipopolysaccharide and Jo2 treatment. A: Box-plots of serum transaminases alanine aminotransferase (ALT) (left panel) and aspartate aminotransferase (AST) (right panel) levels 5 h after D-galactosamine (D-GalN)/lipopolysaccharide (LPS) injection, n = 10 vs 10; B: Western blot analysis of caspase and Bid activation in pooled liver lysates from D-GalN/LPS treated WT and CYLD−/− mice; C: Caspase-3 activity assays of pooled liver lysates from D-GalN/LPS treated WT and CYLD−/− mice. Caspase-3 substrate turnover was measured by fluorometric analysis at the indicated time points; D: WT mice showed more single cell necrosis and apoptosis compared to CYLD−/− mice (HE). Representative immunohistological stainings of cl. PARP (Scale bar: 40 µm) and quantification of cl. PARP positive nuclei. Values represent the mean ± SD; E: Box plots of serum transaminases ALT (left panel) and AST (right panel) levels 3 h after Jo2 injection. n = 10 vs 10; F: Western blot analysis of caspase and Bid activation in pooled liver lysates from Jo2 treated WT and CYLD−/− mice. (G) Caspase-3 activity assays of pooled liver lysates from Jo2 treated WT and CYLD−/− mice. Caspase-3 substrate turnover was measured by fluorometric analysis at the indicated time points; H: Liver cell damage was increased in WT compared to CYLD−/− (HE). Representative immunohistological stainings of cl. PARP (Scale bar: 40 µm) and quantification of cl. PARP positive nuclei. Values represent the mean ± SD. *P < 0.05, WT vs CYLD−/−; †P < 0.01, WT vs CYLD−/−; ‡P < 0.01, WT vs CYLD−/−.
Because CYLD acts as a negative regulator in NF-κB signaling, we analyzed livers of CYLD<sup>-/-</sup> mice for altered NF-κB activity. Freshly isolated CYLD<sup>-/-</sup> primary murine hepatocytes (PMH) showed a significantly increased basal NF-κB activation level. P50 activity levels were 1.6-fold and the activity of the p50 dimerization partner RelA was 2.3-fold increased in CYLD<sup>-/-</sup> PMH (Figure 3A). In addition, analysis of the non-canonical NF-κB pathway revealed higher basal p52 and RelB activity levels. We found a 1.3-fold increase of p52 and a 6.7-fold higher basal RelB activity in CYLD<sup>-/-</sup> compared to WT PMH (Figure 3A). Protein analysis of whole liver lysates demonstrated increased expression levels of p100 and p52. P105 and p50 were expressed equally compared to WT. RelB was most profoundly expressed in CYLD<sup>-/-</sup> compared to WT livers (Figure 3B). IκB-α phosphorylation was not substantially increased in CYLD<sup>-/-</sup> but total IκB-α levels were slightly reduced (Figure 3C, upper panel).

Q-RT PCR experiments revealed increased levels of the NF-κB inducible genes survivin: 2.8-fold and Bcl-2: 1.8-fold (P < 0.05). The mRNA expression levels of Bel-x<sub>L</sub>, c-IAP1/2, XIAP and c-Flip were not significantly different compared to WT (Figure 3D, upper panel).

Protein expression analysis confirmed higher expression levels of survivin. Bel-x<sub>L</sub> was slightly lower expressed in CYLD<sup>-/-</sup> compared to WT livers. The expression of other analyzed anti-apoptotic proteins was not significantly different (Figure 3D, lower panel).

After 5 h D-GalN/LPS treatment IκB-α phosphorylation was substantially increased in CYLD<sup>-/-</sup> but IκB-α degradation was not different compared to WT livers (Figure 3C, middle panel). Immunohistochemical RelA stainings revealed highly increased nuclear RelA levels in CYLD<sup>-/-</sup> livers after D-GalN/LPS treatment (Figure 3E, upper panel). Additionally, liver lysates of D-GalN/LPS treated CYLD<sup>-/-</sup> mice showed increased expression levels of the NF-κB precursor proteins p105 and p100. However, only the active subunit p52 showed increased expression levels. RelA and RelB expression levels were increased compared to WT (Figure 3F, left panel).

Following 3 h CD95 triggering in vitro, p-IκB-α levels were increased and total IκB-α levels were slightly reduced in CYLD<sup>-/-</sup> compared to WT mice (Figure 3C, lower panel). Immunohistochemical RelA staining of liver sections substantiated the increased NF-κB activation in CYLD<sup>-/-</sup> livers after Jo2 treatment (Figure 3E, lower panel). Expression levels of NF-κB subunits (RelA, p52 and RelB) were substantially elevated after Jo2 treatment (Figure 3F, right panel).

Further analysis of signaling events relevant for hepatocyte survival showed reduced Akt and slightly reduced JNK activation in untreated livers of CYLD<sup>-/-</sup> mice. The levels of phosphorylated ERK were not different compared to WT (Figure 3G, left panel). We observed considerably lower Akt activation and increased ERK activation in livers of CYLD<sup>-/-</sup> mice after D-GalN/LPS treatment. JNK activation was slightly reduced in CYLD<sup>-/-</sup> livers compared to WT (Figure 3G, middle panel).

Interestingly, we found decreased ERK and JNK activation in CYLD<sup>-/-</sup> mice after Jo2 administration. Akt activation was not different compared to WT (Figure 3G, right panel).

**CYLD<sup>-/-</sup> PMH are less sensitive towards death receptor triggering**

To confirm reduced apoptosis sensitivity of hepatocytes, we isolated PMH from WT and CYLD<sup>-/-</sup> mice and treated them with increasing concentrations of TNF-α and Jo2. For receptor-mediated cell death induction in vitro, co-treatment with mRNA/protein synthesis inhibitors, like actinomycin D, was necessary as described<sup>19</sup>. In line with the in vitro results, freshly isolated primary hepatocytes of CYLD<sup>-/-</sup> mice were less sensitive towards 24 h TNF-α and Jo2 treatment (Figure 4A and B). To induce apoptosis without the need of mRNA/protein synthesis inhibitors, FasL oligomer (SFL) was applied. Again, CYLD deficient hepatocytes were less sensitive compared to WT hepatocytes (Figure 4C). Western blot analysis of caspase activation clearly demonstrates reduced levels of claspase-8, -9 and -3 in CYLD<sup>-/-</sup> hepatocytes compared to WT after treatment with TNF-α/ActD, Jo2/ActD and SFL for 4 h (Figure 4D).

**Increased expression of NF-κB regulated anti-apoptotic proteins in CYLD<sup>-/-</sup> PMH after death receptor triggering**

To explore the role of NF-κB activation for reduced sensitivity of CYLD negative hepatocytes, we next analyzed the expression of NF-κB regulated anti-apoptotic genes after stimulation with TNF-α and Jo2. TNF-α treatment for up to 1 h showed stronger induction of Bel-2 expression in CYLD<sup>-/-</sup> compared to WT hepatocytes. Moreover, XIAP, survivin and cIAP1/2 were increasingly expressed in CYLD<sup>-/-</sup> hepatocytes, but were not further induced by TNF-α. Interestingly, Bel-x<sub>L</sub> and Mcl-1 expression were decreased in CYLD<sup>-/-</sup> compared to WT hepatocytes (Figure 4E, left panel).

Stimulation with Jo2 led to a pronounced induction of cIAP1/2 in CYLD<sup>-/-</sup> hepatocytes, which we could not observe in WT. XIAP and c-FLIP were increased in CYLD<sup>-/-</sup> hepatocytes. Bel-2 and Bel-x<sub>L</sub> expression levels were higher in WT hepatocytes after stimulation with Jo2 compared to CYLD<sup>-/-</sup> hepatocytes (Figure 4E, right panel), whereas unstimulated CYLD<sup>-/-</sup> PMH exhibited higher basal levels of Bel-2.

**Inhibition of NF-κB blocks expression of anti-apoptotic proteins and re-sensitized PMH towards receptor-mediated cell death**

Next we examined whether inhibition of NF-κB leads to sensitization of PMH towards death receptor-induced cell death. For chemical inhibition we used BAY 11-7085, an irreversible inhibitor of IκB-α phosphorylation<sup>20</sup>. BAY 11-7085 efficiently blocked phosphorylation of IκB-α triggered by TNF-α treatment. Control treated CYLD<sup>-/-</sup> PMH showed strong IκB-α phosphorylation and increased degradation. Pre-incubation of CYLD<sup>-/-</sup>
Urbanik T et al. CYLD in liver injury

A DNA binding activity (OD450)

WT CYLD−/−
p50 0.3 0.2 0.1 0.0
p52 RelA RelB

B rel. m-RNA expression (fold change compared to WT)

WT CYLD−/−
Bcl-2 1.00 1.28 1.30 1.25 1.56 1.29
Bcl-xL 1.00 1.07 1.09 0.90 1.06 0.92
Mcl-1 1.00 0.69 0.90 1.98 3.57 2.07
cIAP1 1.00 0.99 0.74 2.05 1.48 0.20
cIAP2 1.00 1.57 1.78 1.90 2.08 1.81
Survivin 1.00 0.66 0.69 2.45 2.30 1.69
XIAP 1.00 1.57 1.56 1.56 1.56 1.56
c-Flip 1.00 1.25 1.53 1.30 1.13 1.15

C

p-IκB-α rel. m-RNA expression

WT CYLD−/−
5 h D-GalN 0.75 mg/g bw/LPS 2.5 µg/g bw
Bcl-2 1.00 1.25 1.30 1.13 1.15
Bcl-xL 1.00 1.06 1.14 1.22 0.70 0.48
Mcl-1 1.00 1.62 1.57 1.22 0.70 0.48
Tubulin 1.00 1.62 1.57 1.22 0.70 0.48

D

WT CYLD−/−

E

WT CYLD−/−

D-GalN/LPS

RelA

RelA

RelA
hepatocytes with BAY 11-7085 reduced basal and TNF-α-induced expression levels of anti-apoptotic, NF-κB regulated target genes including cIAP1/2, XIAP, survivin, c-FLIP, Bcl-2, Bcl-xL and Mcl-1 (Figure 5A, left panel).

Stimulation of CYLD+/− PMH with Jo2 for up to 1h did not induce phosphorylation of IkB-α. However, after a short induction of total IkB-α expression after 10 min Jo2 treatment, which is a well known event[21], it degraded only in mock treated CYLD+/− PMH. In contrast, 4 h pre-incubation with BAY 11-7085 led to increased basal IkB-α expression levels and reduced IkB-α degradation after Jo2 treatment. BAY 11-7085 blocked basal expression and/or the induction of the anti-apoptotic proteins survivin, c-FLIP, Bcl-2, Bcl-xL and Mcl-1 after CD95 triggering. Interestingly, XIAP expression was increased after NF-κB inhibition. In line with our results from TNF-α stimulation experiments, BAY 11-7085 was capable of reducing cIAP1/2 levels in untreated CYLD+/− PMH, but did not repress increased expression after CD95 triggering (Figure 5A, right panel).

On the basis of these results we next tried to compare TNF-α and Jo2 triggered cell death induction in WT and CYLD+− PMH in combined treatment with BAY 11-7085. Interestingly, WT PMH showed significantly higher cell death sensitivity towards BAY 11-7085 treatment alone (Figure 5B, left panel). 4 h pre-incubation with BAY
Permanent Eacher WJG December 7, 2014 Volume 20 Issue 45

Urbanik T et al. CYLD in liver injury

Figure 4 Increased resistance and induction of anti-apoptotic genes in CYLD−/− PMH after death receptor triggering. A: Freshly isolated PMH were treated for 24 h with increasing concentrations of tumor necrosis factor (TNF)-α and (B) Jo2 in combination with actinomycin D (ActD) as well as with (C) SuperFasLigand (SFL) as indicated; D: Western blot analysis of caspase activation in WT and CYLD−/− PMH after 4 h TNF-α/ActD, Jo2/ActD and SFL treatment with the indicated concentrations; E: Western blot analysis of WT and CYLD−/− PMH for nuclear factor (NF)-κB dependent gene expression after TNF-α (left panel) and Jo2 (right panel) treatment. bP < 0.01, WT vs CYLD−/−; dP < 0.01, WT vs CYLD−/−.
Urbanik T et al. CYLD in liver injury

**A**

|                | Vehicle | BAY 11-7085 (20 μmol/L) | BAY 11-7085 (20 μmol/L) |
|----------------|---------|--------------------------|--------------------------|
| p-IκB-α        | 1.00    | 0.46 0.37               | 0.44 0.32 0.42          |
| IκB-α          | 1.00    | 0.46 0.37               | 0.44 0.32 0.42          |
| cIAP1/2        | 1.00    | 0.65 0.57               | 0.72 0.72               |
| XIAP           | 1.00    | 1.13 0.96               | 0.84 0.97 0.94          |
| Survivin       | 1.00    | 2.42 2.51               | 1.14 3.12 3.39 2.72    |
| c-Flip         | 1.00    | 2.64 3.50               | 0.82 1.66 0.84          |
| Bcl-2          | 1.00    | 1.54 1.23               | 0.82 1.01 1.20 0.82    |
| Bcl-xL         | 1.00    | 1.66 1.70               | 1.21 1.10 1.15 1.09    |
| Mcl-1          | 1.00    | 1.46 1.36               | 1.59 0.75 0.94 0.63    |
| Tubulin        | 1.00    | 1.69 1.75               | 1.30 1.07 1.07 1.24    |

**B**

WT

|                | Rel. Viability |
|----------------|---------------|
| 0              | 1.00          |
| 0.1            | 0.50          |
| 1              | 0.46          |
| 10             | 0.37          |
| 20             | 0.44          |
| 50             | 0.32          |

CYLD

|                | Rel. Viability |
|----------------|---------------|
| 0              | 0.87          |
| 0.1            | 0.85          |
| 1              | 0.83          |
| 10             | 0.92          |
| 20             | 1.02          |
| 50             | 1.19          |

**C**

|                | Rel. Viability |
|----------------|---------------|
| 0              | 1.55          |
| 0.1            | 0.90          |
| 1              | 0.88          |
| 10             | 0.93          |
| 20             | 0.92          |
| 50             | 0.90          |

**BAY 11-7085 (20 μmol/L)**

|                | Rel. Viability |
|----------------|---------------|
| 0              | 1.00          |
| 0.1            | 0.87          |
| 1              | 0.85          |
| 10             | 0.92          |
| 20             | 1.02          |
| 50             | 1.19          |
11-7085 in a non-toxic concentration (10 μmol/L) significantly sensitized WT PMH towards TNF-α (0.1, 0.5 ng/mL) and Jo2 (5, 50 ng/mL). CYLD<sup>-/-</sup> PMH could only be significantly sensitized for 50 ng/mL Jo2 treatment (Figure 5C, upper panel). Doubling BAY 11-7085 concentration to 20 μmol/L made CYLD<sup>-/-</sup> PMH also significantly sensitive for lower concentrations of the death receptor agonists (Figure 5C, lower panel).

Absence of survivin renders the liver more sensitive to Fas<sup>[22]</sup>. To elucidate the role of one distinct NF-κB regulated anti-apoptotic protein in the increased cell death resistance of CYLD negative hepatocytes, we treated WT and CYLD<sup>-/-</sup> PMH with YM155, an inhibitor of endogenous survivin expression<sup>[23]</sup>. 24 h YM155 treatment alone did not induce caspase-3 activation and did not reduce viability in concentrations up to 50 μmol/L,

Urbanik T <i>et al.</i> CYLD in liver injury

**Figure 5** Nuclear factor-κB and survivin inhibition sensitized primary murine hepatocytes towards receptor-mediated cell death. A: Western blot analysis for nuclear factor (NF)-κB regulated gene expression after tumor necrosis factor (TNF)-R (left panel) and CD95-R (right panel) triggering of CYLD<sup>+</sup> primary murine hepatocytes (PMH) 4 h pre-incubated with BAY 11-7085; B: 24 h treatment of WT and CYLD<sup>-/-</sup> PMH with increasing concentrations of BAY 11-7085 (left panel) and YM155 (right panel); C: WT and CYLD<sup>-/-</sup> PMH were pre-incubated with 10 μmol/L BAY-11 7085 for 4 h. Afterwards PMH were treated for 24 h in combination with TNF-α or Jo2 as indicated (upper panel). Pre-incubation of CYLD<sup>-/-</sup> PMH with 20 μmol/L BAY-11 7085 and following TNF-α or Jo2 treatment (lower panel); D: Western blot analysis for survivin expression in WT and CYLD<sup>-/-</sup> PMH 24 h after treatment with YM155 as indicated (upper panel). WT and CYLD<sup>-/-</sup> PMH were pre-incubated with 10 μmol/L YM155 for 4 h. Afterwards PMH were treated for 24 h in combination with TNF-α or Jo2 as indicated (lower panel). DMSO was used as vehicle. Values represent the mean ± SD. *P < 0.05, CYLD<sup>-/-</sup> vs control group; **P < 0.01, WT vs CYLD<sup>-/-</sup>; ***P < 0.001, WT vs control group; ****P < 0.01, CYLD<sup>-/-</sup> vs control group.
in both \( W^T \) and \( CYLD^- \) PMH (Figure 5B and D, right panel). YM155 caused a remarkable reduction of survivin protein levels in \( W^T \) but not in \( CYLD^- \) PMH (Figure 5D, upper panel). The combined treatment of 10 \( \mu \)mol/L YM155 with TNF-\( \alpha \) or Jo2 reduced cell viability only in \( W^T \) PMH (Figure 5D, lower panel).

**DISCUSSION**

Increased hepatocyte apoptosis via death receptors such as CD95 and TNF-R1 plays a prominent role in liver diseases[26]. Previous reports show that NF-\( \kappa B \) activation counterbalances CD95- and TNF-R1-mediated death pathways[4]. NF-\( \kappa B \) participates in the induction of a wide variety of cellular genes involved in immunity, inflammation and regulation of apoptosis[11,25]. Inhibition of NF-\( \kappa B \) subunits is an interesting approach to manipulate pathophysiological processes but its inactivation can also exert a deleterious role in hepatic diseases[26]. Thus, there is a need for further basic research in understanding cell death and survival pathways to elucidate novel therapeutic targets for liver injury treatment.

The deubiquitinating enzyme CYLD removes K-63-linked polyubiquitin chains from distinct proteins involved in NF-\( \kappa B \) signaling[27,28]. Reduced CYLD expression was shown to increase the survival of several cell types[12,13]. Only little is known about the role of CYLD for cell death sensitivity of hepatocytes. We recently showed that mice lacking exon 7 and 8 of the CYLD gene exhibit an increased sensitivity towards chemical-induced hepatocarcinogenesis and postulated the involvement of an impaired apoptosis machinery promoting carcinogenesis in the liver[13]. In contrast, a conditional knockout of exon 9 of the CYLD gene resulted in massive apoptosis induction in hepatocytes[29]. However, in both studies on liver specific CYLD knockouts the role of the remaining splice variants remains elusive and needs to be further clarified (Figure 6).

In the present study, we show that complete deletion of CYLD protected hepatocytes from TNF-R- and CD95-mediated apoptosis via promoting anti-apoptotic NF-\( \kappa B \) signaling. In contrast to studies on liver specific knockout models[11,29], \( CYLD^- \) livers showed no obvious changes in liver architecture. However, the weight of \( CYLD^- \) mice might be a consequence of an increased life span of \( CYLD^- \) hepatocytes by alterations in survival related signal transduction.

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**Figure 6** Model of CYLD’s role in receptor mediated apoptosis or survival of hepatocytes. Binding of tumor necrosis factor (TNF)-\( \alpha \) to TNF-R1 induces trimerisation and the recruitment of several adaptor proteins, including TRADD, RIP1 and TRAF2 to form the membrane-proximal complex I. K-63 Deubiquitination of RIP1 and TRAF2 by CYLD promotes the conversion of complex I to complex II building the death inducing signaling complex (DISC) leading to induction of apoptosis. K-63 polyubiquitinated RIP1 and TRAF2 facilitate nuclear factor (NF)-\( \kappa B \) activation by the recruitment and activation of IKK and its activating kinase, Tak[44]. Missing CYLD expression or a lack of CYLDs function leads to increased K-63 polyubiquitination of TRAF2 and RIP1 and therewith to a pronounced activation of the IKK complex. Activation of CD95 promotes recruitment of FADD, thereby assembling the DISC. RIP1 and TRAF2 are also known modulators of CD95 signaling[45]. The role of K-63 polyubiquitination and CYLD in the dynamic of CD95 mediated apoptosis and NF-\( \kappa B \) induction is not clear. Known is, that cFLIP is involved in the connection of CD95 induced anti-apoptotic NF-\( \kappa B \) signaling via its ability to activate IKK[44], CYLD can remove K-63 polyubiquitin chains from the IKK regulatory subunit IKK\( \gamma \) thereby inhibiting IKK activation. Increased IKK activation by the absent of CYLD leads to inhibitor of NF-\( \kappa B \) phosphorylation, K-48 polyubiquitination and following proteasomal degradation. Subsequently released NF-\( \kappa B \) subunits can enter the nucleus to promote transcription of anti-apoptotic genes[44].
Accordingly to the known role of CYLD as negative regulator of NF-κB[6], we detected increased NF-κB activity in CYLD⁻/⁻ hepatocytes. We next analyzed CYLD⁺/⁺ livers for expression of NF-κB regulated anti-apoptotic genes. Members of the IAP protein family, such as survivin, were shown to be regulated by NF-κB. Survivin has the capacity to directly bind to and inhibit caspase-3 activation and thus, to suppress cell death[35]. Interestingly, we detected significantly increased expression of survivin in CYLD⁻/⁻ livers and consequently reduced levels of cleaved caspase 3.

CYLD was additionally shown to negatively regulate Akt activation in lung injury[7] as well as JNK activation in melanoma[36,37]. In contrast, CYLD⁻/⁻ livers showed decreased Akt and JNK activation, which points to organ specific functions of CYLD in this context and excludes Akt and JNK activation as potential anti-apoptotic mechanisms in our CYLD knockout model.

To evaluate the function of CYLD in receptor-mediated apoptosis in hepatocytes and liver injury in vivo, we administered CD95-agonistic antibodies. Hepatocytes are acutely sensitive to CD95-induced apoptosis and triggering of CD95 is an established in vivo model to induce ALT[38]. CYLD⁻/⁻ mice clearly showed a decreased apoptosis sensitivity. Analysis of survival related signaling revealed unaltered Akt, slightly reduced ERK expression is very low[39]. In CYLD⁺/⁺ hepatocytes Bcl-2 could be dramatically increased by TNF-α treatment. Triggering of CD95 profoundly induced cIAP1/2 in CYLD⁺/⁺ but not in WT PMH. cIAPs are known to mediate anti-apoptotic effects of NF-κB, e.g., in Jurkat cells[40]. Furthermore, it was shown that cIAP2 prevents apoptosis in rat hepatocytes[41]. Finally, our study included the application of the IkB-α phosphorylation inhibitor BAY 11-7085[33] and YM155 as an inhibitor of survivin expression[23]. BAY 11-7085 was able to re-sensitize CYLD⁺/⁺ PMH towards TNF-α and Jo2-induced cell death without co-treatment with transcription/translation inhibitors. This was accompanied by a reduction of NF-κB regulated anti-apoptotic protein expression and, additionally, by blockage of their induction after TNF-R and CD95 triggering. In addition to the anti-apoptotic NF-κB activity, we exemplarily investigated the involvement of NF-κB regulated survivin expression. Treatment with YM155 led only in WT PMH to remarkable decreased survivin levels. This raises the question about altered survivin protein stability in CYLD⁺/⁺ cells and has to be addressed in future studies. Importantly, in comparison to CYLD+, WT PMH were much more sensitive towards co-treatment with BAY 11-7085/YM155 and TNF-α or Jo2, which underlines the relevance of anti-apoptotic NF-κB signaling for the increased resistance of CYLD⁺ hepatocytes towards death receptor agonists.

Our study demonstrates for the first time that deletion of CYLD increases resistance of murine hepatocytes towards TNF-α and CD95 induced apoptosis and points to a crucial role of increased anti-apoptotic NF-κB signaling following CYLD deletion. Thus, inhibition of CYLD represents a potential approach for the treatment of acute and chronic liver injury triggered by death receptor-induced apoptosis of hepatocytes.

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**Urbanik T et al. CYLD in liver injury**

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17062
COMMENTS

Background

The tumor suppressor gene CYLD, involved in the control of nuclear factor (NF)-κB signaling, was initially identified as mutated in patients suffering from familial cylindromatosis. The product of the CYLD gene contains an ubiquitin C-terminal hydrolase domain allowing it to act as a Deubiquitinating enzyme (DUB) in removing K-63-linked poly-ubiquitin chains from distinct proteins involved in the NF-κB survival signaling pathway.

Research frontiers

Persistent apoptosis is a feature of chronic liver diseases. Acute liver failure is characterized by massive apoptosis and is associated with life threatening consequences. Therapeutic approaches for delaying or reversing liver failure apart from orthotopic liver transplantation are rare. Understanding of the mechanisms of hepatocyte survival and cell death pathways would offer potential therapeutic targets.

Innovations and breakthroughs

The study showed for the first time that CYLD negative hepatocytes are less sensitive to CD95 and TNF-R-induced apoptosis will help to improve our understanding of the mechanisms of acute and chronic liver injury. Inhibition of CYLD might represent a therapeutic approach to protect hepatocytes from death receptor-mediated apoptosis.

Peer review

This study evaluated the CYLD's function in the murine hepatocytes apoptosis network which controlled by NF-κB. The apoptosis related factors including Bcl-2, XIAP, cIAP and survivin were assessed after hepatocyte cell death in CYLD knockout mice. Subsequently, the study speculate CYLD regulate NF-κB dependent anti-apoptotic pathway. An excellent work had been done in this study.

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