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A pro-inflammatory role of deubiquitinating enzyme Cylindromatosis (CYLD) in vascular smooth muscle cells

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Abstract

CYLD, a deubiquitinating enzyme (DUB), is a critical regulator of diverse cellular processes, ranging from proliferation and differentiation to inflammatory responses, via regulating multiple key signaling cascades such as nuclear factor kappa B (NF- κ B) pathway. CYLD has been shown to inhibit vascular lesion formation presumably through suppressing NF- κ B activity in vascular cells. However, herein we report a novel role of CYLD in mediating pro-inflammatory responses in vascular smooth muscle cells (VSMCs) via a mechanism independent of NF- κ B activity. Adenoviral knockdown of Cyld inhibited basal and the tumor necrosis factor alpha (TNF α)-induced mRNA expression of pro-inflammatory cytokines including monocyte chemoattractant protein-1 (Mcp-1), intercellular adhesion molecule (Icam-1) and interleukin-6 (Il-6) in rat adult aortic VSMCs (RASMCs). The CYLD deficiency led to increases in the basal NF- κ B transcriptional activity in RASMCs; however, did not affect the TNF α -induced NF- κ B activity. Intriguingly, the TNF α -induced I κ B phosphorylation was enhanced in the CYLD deficient RASMCs. While knocking down of Cyld decreased slightly the basal expression levels of I κ B α and I κ B β proteins, it did not alter the kinetics of TNF α -induced I κ B protein degradation in RASMCs. These results indicate that CYLD suppresses the basal NF- κ B activity and TNF α -induced I κ B kinase activation without affecting TNF α -induced NF- κ B activity in VSMCs. In addition, knocking down of Cyld suppressed TNF α -induced activation of mitogen activated protein kinases (MAPKs) including extracellular signal-activated kinases (ERK), c-Jun N-terminal kinase (JNK), and p38 in RASMCs. TNF α -induced RASMC migration and monocyte adhesion to RASMCs were inhibited by the Cyld knockdown. Finally, immunochemical staining revealed a dramatic augment of CYLD expression in the injured coronary artery with neointimal hyperplasia. Taken together, our results uncover an unexpected role of CYLD in promoting inflammatory

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responses in VSMCs via a mechanism involving MAPK activation but independent of NF- κ B activity, contributing to the pathogenesis of vascular disease.

Keywords

CYLD; Vascular smooth muscle cells; TNF α ; Inflammation; MAPK; NF- κ B

Introduction

Cardiovascular disease is a leading cause of disability and mortality in the United States, and the majority of cardiovascular disorders results from complications of vascular diseases [1]. While it is still far from a comprehensive understanding of molecular and cellular mechanisms leading to vascular diseases, a preponderance of evidence supports a notion that inflammation plays a critical role in a wide range of vascular complications and dysfunctions [2,3,4,5,6,7]. Vascular inflammation is a complex process involving endothelial dysfunction, leukocyte recruitment, inflammatory activation of VSMCs, and malfunction of inflammatory mediators including both anti-inflammatory and pro-inflammatory cytokines. Of note, the inflammatory activation of VSMCs which exhibit a phenotypic switch characterized by loss of contractility, increased migration, proliferation, apoptosis, and production of extracellular matrix and cytokines, or foam-cell-like transition, plays a key role in vascular lesion formation and disease progression. However, the molecular mechanism of the inflammatory phenotypic switch of VSMCs remains partly understood.

Since ubiquitin (Ub) was discovered in the early 1970's, the protein quality control by the ubiquitin proteasome system (UPS) that involve specific enzyme proteins of Ub-activating enzyme (E1), Ub-conjugating (E2), Ub-ligating (E3), proteasome and deubiquitinating enzymes (DUBs) has emerged as a critical regulatory process in virtually all aspects of cell biology [8,9,10,11,12,13,14,15]. The process of ubiquitination is catalyzed by the sequential actions of E1, E2, and E3. The removal and processing of ubiquitin are mediated by DUBs. Human genome encodes approximately 100 functional DUBs. Relative to the other components of UPS, less is known about the DUBs. Nevertheless, emerging evidence has revealed that DUBs act as crucial regulators in diverse biological processes such as proliferation, migration, apoptosis, and inflammatory responses [10,16,17,18]. Importantly, a potential role of DUBs in the pathogenesis of vascular diseases was highlighted by the inhibitory role of DUB A20 (also called TNF α -induced protein 3 or Tnfaip3) in vascular lesion formation [19,20,21]. We and others have found that other DUBs of ubiquitin carboxyl-terminal hydrolase-L1 (UCH-L1) and CYLD may serve as endogenous inhibitors of vascular lesion formation [22,23,24]. At a molecular level, these DUB-mediated deubiquitination of I κ B kinase γ (IKK γ), I κ B α , or TNF receptor associated factor 2 (TRAF2) that leads to suppression of NF- κ B activity [25,26,27] has been considered to resolve vascular inflammatory responses thus inhibiting vascular injury [19,22,23]. Because the DUB-mediated deubiquitination has been proposed to be non-selective, these findings seem quite predictable. However, several lines of evidence have recently revealed that DUBs are often found as part of large multiprotein complexes that functions to regulate their localization, substrate availability, and activity in cellular processes, and the association of DUBs with substrate adaptors, scaffolds, and inhibitors results in regulatory interactions driving specificity [28]. These results suggest that DUBs might be a unique class of molecules for the determination of biological consequences of diverse signaling cascades at differential pathological settings such as maladaptive vascular remodeling and dysfunction. However, precise role of DUBs in the vasculature remains to be further determined.

In the present study, we explored a role of CYLD in regulating inflammatory responses in primary culture of RASMCs by Cyld gene loss-of-function approach. Our results uncover an unexpected mediator role of CYLD in pro-inflammatory activation of VSMCs via a mechanism independent of NF- κ B activation, highlighting functional significance of CYLD splicing variants in the pathogenesis of vascular disease.

Material and methods

Animals and treatment

Male ICR/sv129 mice at age of 8 weeks were treated subcutaneously with vehicle saline or infused with angiotensin II (Ang II) (Sigma-Aldrich) at a rate of 1.4 μ g/kg/min for 2 weeks by osmotic mini-pumps as previously reported [29]. All of the animal procedures were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at University of South Carolina, USA.

Immunohistochemistry

Hearts were harvested and immunohistochemistry were performed as previously described [30]. Primary antibodies included a goat CYLD polyclonal antibody (Abcam Inc.) and a rabbit tropomyosin I antibody (Abcam Inc.). Cardiomyocytes were stained with anti-tropomyosin I antibody. Nuclei were labeled with 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

Cell culture, adenoviral infection, transfection and NF- κ B reporter assay

RASMCs were isolated from thoracic aorta of adult Sprague-Dawley rats and cultured as previously described [24]. Sub-confluent RASMCs were infected with adenovirus of control scramble shRNA (Ad-cont sh) or Cyld shRNA (Ad-Cyld sh) (Welgen Inc.) in serum free Dulbecco's Modified Eagle's Medium (DMEM) for 48 hours. Transfection and NF- κ B reporter assay were performed as previously described [31].

Cell migration assay

TNF α -induced RASMC migration was assessed by measuring wound closure rate as previously described [32]. Briefly, RASMCs were infected with adenovirus of scramble shRNA or Cyld shRNA in serum free DMEM for 48 hours, and then split into 6-well plates to reach approximately 100% confluence. The monolayer of RASMCs was further scratched with a plastic tip (200 μ l), and followed with or without the treatment of TNF α (5 ng/ml) in serum free DMEM for 24 hours (hrs). The wound area was calculated by tracing along the border of wound using image analysis software (Image ProPlus 6.0), and the percentage wound closure was calculated using the following equation: Wound closure rate = [Wound area (0 hr) – Wound area (24 hr)]/Wound area (0 hr).

Monocyte adhesion assay

Adhesion of monocyte to RASMCs was assessed utilizing fluorescein labeled THP-1 cells as previously described [33].

Quantitative real time reverse transcription-polymerase chain reaction (Q-PCR)

Total RNA purification, reverse transcription (RT) reaction, and Q-PCR were performed as described previously [30]. Primers that were used for Q-PCR are as follows: rat Cyld, forward primer (5'-GCAGTGTAACAGACAAACAGACACA-3') and reverse primer (5'-TTGCCTTTAGCAGAAGGAACT-3'); rat Icam-1, forward primer (5'-AGAAGGACTGCTTGGGGAA-3') and reverse primer (5'-

CCTCTGGCGGTAATAGGTG-3'); rat Mcp-1, forward primer (5'-ATGCAGGTCTCTGTCACG-3') and reverse primer (5'-CTAGTTCTCTGTCATACT-3'); rat Il-6, forward primer (5'-TGTTCTCAGGGAGATCTTGG-3') and reverse primer (5'-TCCAGGTAGAAACGGAATC-3'); rat glyceraldehyde-3-phosphate dehydrogenase (Gapdh), forward primer (5'-ACCACAGTCCATGCCATCAC-3') and reverse primer (5'-TCCACCACCCTGTTGCTGTA-3').

Western blot

Cell lysates were subjected to immunoblot analysis as described elsewhere [30] using antibodies of anti-phospho-I κ B α (Ser32/36), anti-phospho-ERK, anti-phospho-p38 and anti-phospho-JNK (Cell Signaling Technology), as well as anti-I κ B α , I κ B β , anti-I κ B ϵ , anti-ERK, anti-JNK, anti-p38 and anti- α -tubulin (Santa Cruz Biotechnology).

Statistical analysis

Data are expressed as mean $\pm$ s.d.. Comparison between groups was done by two-sample *t*-test. A *p* value of <0.05 was considered significant. Statistical analysis was performed with Statistical Package for Social Sciences version 13.0 (SPSS, Inc.).

Results

Knocking down of Cyld suppresses TNF α -induced pro-inflammatory cytokine expression in RASMCs

Considering the inhibitory effect of over-expression of CYLD on NF- κ B activity and its downstream inflammatory gene expression in A7r5 VSMCs as well as neointimal formation in rat carotid artery after balloon injury [23], we hypothesized that CYLD deficiency in VSMCs could exaggerate vascular inflammatory responses, thereby leading to progressive vascular lesion formation. To test this hypothesis, we applied Cyld RNA interference (RNAi) approach to determine a pathophysiological significance of CYLD deficiency in the regulation of vascular lesion formation *in vitro* utilizing primarily cultured RASMCs. Adenoviral over-expression of Cyld shRNA dose-dependently inhibited Cyld mRNA expression with an efficacy of $> 80\%$ knockdown of endogenous Cyld mRNA expression at dose of 50 multiplicity of infection (MOI) in RASMCs (Fig. 1A). Adenoviral over-expression of Cyld shRNA (50 MOI) resulted in suppression of $> 90\%$ CYLD protein expression (Fig. 1A). We did not observe any apparent cytotoxic effects of the adenoviral infection in RASMCs (data not shown). Thus, Cyld RNAi approach was established in RASMCs. Surprisingly, the Cyld knockdown suppressed both basal and TNF α -induced expression of Mcp-1, Icam-1, and Il-6 mRNAs in RASMCs (Fig. 1B), suggesting a pro-inflammatory role of CYLD in VSMCs.

Knocking down of Cyld increases basal NF- κ B activity without affecting TNF α -induced NF- κ B activity and inhibits TNF α -induced activation of MAPKs in RASMCs

Because Mcp-1, Icam-1 and Il-6 are well-documented downstream genes of NF- κ B, a key regulator of vascular inflammatory responses and lesion formation [34,35], we examined the effect of CYLD deficiency on NF- κ B signaling in RASMCs. Knocking down of Cyld led to an enhancement of the basal NF- κ B transcriptional activity; however, did not affect TNF α -induced NF- κ B transcriptional activity (Fig. 2A), suggesting that endogenous CYLD negatively regulates constitutive rather than induced NF- κ B activity in VSMCs. Intriguingly, the TNF α -induced I κ B phosphorylation was enhanced in the CYLD deficient RASMCs (Fig. 2B), indicating an inhibitory role of endogenous CYLD in suppressing the induced activation of I κ B upstream kinases such as IKKs that phosphorylate I κ Bs leading to NF- κ B activation [27] in VSMCs. While the CYLD deficiency slightly decreased the basal

expression levels of I κ B α and I κ B β proteins, it did not affect the basal expression of I κ B ϵ in RASMCs (Fig. 2B). In addition, it did not alter the kinetics of TNF α -induced I κ B protein degradation in RASMCs (Fig. 2B). These results reveal that endogenous CYLD constitutively stabilizes the basal I κ B α and I κ B β proteins thereby suppressing NF- κ B activity [27] in VSMCs. Moreover, endogenous CYLD suppresses the activity of kinases for I κ B phosphorylation; however, this suppression probably contributes to the basal I κ B degradation rather than induced I κ B degradation leading to NF- κ B activation in VSMCs.

To further dissect the underlying molecular mechanisms of CYLD-mediated pro-inflammatory responses in VSMCs, we determined whether Cyld knockdown affects the TNF α -induced activation of MAPKs including ERK, JNK, and p38, a group of key signaling molecules in the regulation of inflammatory responses [36,37,38,39] in RASMCs. In fact, a critical role of ERK, JNK or p38 in mediating TNF α -induced expression of Mcp-1, Icam-1, or Il-6 has been established in rat or mouse VSMCs [40,41]. Interestingly, not the basal but TNF α -induced phosphorylation/activation of ERK, JNK, and p38 was attenuated by knocking down of Cyld in RASMCs (Fig. 3), indicating a mediator role of endogenous CYLD in inducing activation of MAPKs in VSMCs at a setting of inflammation.

Knocking down of Cyld inhibits TNF α -induced RASMC migration and monocyte adhesion to TNF α -inflamed RASMCs

To establish a functional link between CYLD deficiency and inflammatory responses in VSMCs, we determined effect of Cyld knockdown on TNF α -induced RASMC migration and monocyte adhesion to TNF α -inflamed RASMCs *in vitro* reflecting inflammatory activation of VSMCs [6]. In consistent with the inhibitory effect of Cyld knockdown on inflammatory cytokine expression, Cyld knockdown inhibited TNF α -induced RASMC migration and adhesion of monocyte to TNF α -activated RASMCs (Figs. 4A and B), revealing a critical role of CYLD in pro-inflammatory phenotypic switch of VSMCs leading to vascular lesion formation.

CYLD is up-regulated in the injured coronary artery with neointimal hyperplasia at a setting of sustained Ang II stimulation

To further establish a pathophysiological relevance of the CYLD-mediated pro-inflammatory activation of VSMCs, we examined CYLD expression in injured coronary arteries caused by sustained stimulation of Ang II that exhibits significant pro-inflammatory actions in the vessel wall via inducing the production of inflammatory cytokines and adhesion molecules, recruitment of inflammatory cells, and activation of VSMCs, culminating in vascular lesion formation with adverse clinical outcome [42]. As shown in Fig. 4C, CYLD was dramatically up-regulated in the small intramural coronary arteries (50-100 μ m internal diameter) associated with neointimal hyperplasia that was induced by sustained Ang II infusion for 2 weeks.

Discussion

CYLD was originally identified as a tumor suppressor gene of human familial cylindromatosis (CYLD) [43]. Rapid growing evidence has demonstrated that CYLD plays a critical role in immunity, lipid metabolism, spermatogenesis, osteoclastogenesis, antimicrobial defense, and inflammation predominantly via regulating NF- κ B and JNK pathways [16,17,18]. To date, the majority of characterized CYLD targets are involved in the signaling transduction mediating NF- κ B activation. CYLD has been considered as a negative regulator of IKK complex thereby suppressing NF- κ B-driven pro-inflammatory signaling in diverse organ systems including vasculature [23]. However, our current study demonstrates for the first time that CYLD acts as a positive mediator of pro-inflammatory

activation of VSMCs, potentially contributing to coronary arterial lesion formation. Mechanistically, CYLD mediates the pro-inflammatory phenotypic switch of VSMCs via a mechanism involving MAPK activation but independent of NF- κ B activity.

Our findings are unexpected; however, raise several intriguing issues which need further investigation: Firstly, the signaling function of CYLD is complex and varies among different cell types and pathological settings. Activation of IKK, NF- κ B, ERK, JNK, and p38 induced by Toll-like receptors and TNF receptors is normal in Cyld deficient macrophages; however, activation of ERK and JNK is diminished in Cyld deficient thymocytes [44]. We have demonstrated that loss of Cyld function does not affect NF- κ B activity but enhances JNK activity in renal tubular epithelial cells contributing the tubulointerstitial inflammation of IgA nephropathy [33]. Notably, truncation of deubiquitinating domain of CYLD specifically in myelomonocytic cells suppresses the development of lethal LPS-induced endotoxic shock and the accumulation of thioglycollate-elicited peritoneal macrophages with hyperactivation of NF- κ B [45]. Because the over-expression of exogenous CYLD may cover the endogenous function of CYLD in VSMCs [23], aforementioned, we believe that results obtained by Cyld loss-of-function RNAi approach reflects more precisely the intrinsic function of CYLD in VSMCs. Thus, our data uncovers a novel role of CYLD in mediating pro-inflammatory responses in VSMCs probably via activating MAPKs. Secondly, TNF α exerts either pro- or anti-inflammatory effects in the vasculature [46,47]. In addition, protein ubiquitination cascades have recently been illustrated to play a critical role in TNF receptor signaling and account for spatial and temporal separation of IKK and MAPK signaling cascades thereby determining biological specificity and outcome [48]. Since CYLD has implicated into the deubiquitinating of TRAFs, IKK γ , Bcl3, TGF β -activated kinase (TAK), receptor-interacting protein 1 (RIP1) and other molecules that are critical for TNF receptor-mediated IKK and MAPK signaling [17,18,48], it is possible that CYLD may deubiquitinate the putative molecules coupling with the TNF α -mediated pro-inflammatory responses specifically in VSMCs. Finally, it is worthy to note that CYLD can both positively and negative regulate NF- κ B signaling, depending on the expression of CYLD splicing variants. A splicing variant of CYLD which lacks the TRAF2 and IKK γ binding sites increases basal rather than anti-IgM-induced NF- κ B activity and enhances LPS-induced MAPK p38 activation in B cells [49]. Moreover, this shorter CYLD (sCYLD) enhances NF- κ B activity accompanied by an increased nuclear translocation of the I κ B molecule Bcl-3, along with nuclear p50 and p65 leading to a pro-inflammatory phenotype in dendritic cells [50]. Considering the similarities between these previous finding and ours, such as the increased basal rather induced NF- κ B activity or the enhanced p38 activity in both Cyld deficient VSMCs and Cyld deficient with sCyld overexpressing B or dendritic cells, it is possible that sCYLD is responsible for pro-inflammatory activation of VSMCs whereas full-length CYLD is a negative regulator of inflammatory responses in diverse organ systems including vasculature. Therefore, our results highlight a pathophysiological significance of the splicing variants of CYLD at least in vasculature.

In summary, we have demonstrated that endogenous CYLD is critical for the pro-inflammatory activation of VSMCs via mechanism of activating MAPK independent of NF- κ B. Importantly, our findings also reveal a potential role of CYLD splicing variants in the control of NF- κ B and MAPK pathways leading to biological specificity and outcome of inflamed VSMCs. Further investigation of the precise role of CYLD splicing variants in the vasculature is warranted in future.

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Highlights

- Cyld deficiency suppresses pro-inflammatory phenotypic switch of VSMCs.
- Cyld deficiency increases I κ B phosphorylation without affecting I κ B degradation and NF- κ B activity; however, inhibits MAPK activity in inflamed VSMCs.
- CYLD is up-regulated in the coronary artery with neointimal hyperplasia.

Fig. 1.

Effect of Cyld knockdown on TNF α -induced inflammatory cytokine expression in RASMCs. (A) Efficacy of adenoviral knockdown of Cyld in RASMCs. Upper: Cyld mRNA expression. * $p < 0.05$ vs control (0), $n = 4$. Lower: Results are representative of three independent Western blot analysis of CYLD in infected RASMCs. Cell lysates from human 293 cells transfected with pDEST-HA-hCyld (Addgene) were used as positive (Posi) control. (B) Q-PCR analysis of Mcp-1, Icam-1 and Il-6 mRNA expression. * $p < 0.05$ vs Ad-cont sh (-), $n = 4$.

Fig. 2.

Effect of Cyld knockdown on NF- κ B signaling in RASMCs. (A) RASMCs infected with Ad-cont sh (50 MOI) or Ad-Cyld sh (50 MOI) were treated with or without TNF α (5 ng/ml) for 6 hours. ns; non-significant. (B) RASMCs infected with Ad-cont or Ad-Cyld sh as described in (A) were stimulated with TNF α (5 ng/ml) as indicated. Results are representatives of three independent experiments.

Fig. 3.

Effect of Cyld knockdown on activation of MAPK in RASMCs. Infected RASMCs were treated with TNFα (5 ng/ml) as indicated. Results are representatives of three independent experiments. Left panel is the semi-quantification of ERK, JNK, and p38 activities.

Fig. 4. Effect of Cyld knockdown on monocyte adhesion to RASMCs (A) and RASMC migration (B). Images are representatives of three independent experiments *p<0.05 vs TNF α (-), n=4. (C) Ang II-induced CYLD expression in murine coronary artery of right ventricle. Green indicates cardiomyocytes. Red is CYLD. Blue is nuclei.