The E3 Ubiquitin Ligase c-Cbl Inhibits Microglia-Mediated CNS Inflammation by Regulating PI3K/Akt/NF- κ B Pathway

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SUMMARY

Background: Microglia-mediated inflammation may play an important role in the pathophysiology progression of neurodegenerative diseases, such as Parkinson's disease (PD), but the molecular mechanisms are poorly understood. **Aims:** This study sought to determine whether E3 ubiquitin ligase c-Cbl plays a role in the brain inflammation and to explore the relevant molecular mechanism. **Methods:** After BV2 microglial cells and c-Cbl-deficient mice were treated with lipopolysaccharide (LPS), neuroinflammation and microglial activation were evaluated by immunohistochemistry, ELISA and Western blot. We further investigated the possible mechanism of c-Cbl in regulating microglial activation. **Results:** Here, we showed that the E3 ubiquitin ligase c-Cbl had high expression in brain tissues including substantia nigra pars compacta (SNc), striatum and hippocampus, and it was abundantly expressed in microglia. Systemic LPS administration resulted in more severe microglial activation in CNS and increased expression of brain proinflammatory factors (TNF- α , IL-6, IL-1 β and MCP-1) in c-Cbl knockout mice than wild type mice (WT). Downregulation of c-Cbl expression with c-Cbl siRNA in BV-2 microglial cells demonstrated a more robust increase in the proinflammatory factors release and NF- κ B p65 nuclear translocation than that in control siRNA. Interestingly, Akt phosphorylation induced by LPS was also significantly augmented after c-Cbl knockdown. Moreover, blockade of PI3K/Akt activation by LY294002 significantly reduced inflammation response and NF- κ B p65 nuclear translocation. **Conclusion:** In sum, c-Cbl inhibits expression of LPS-stimulated proinflammatory cytokines and chemokines in microglia. We demonstrate an unprecedented role for c-Cbl in microglia-mediated neuroinflammation involving PI3K/Akt/NF- κ B pathway.

Introduction

Microglia, the resident innate immune cells in the central nervous system (CNS), play an important role in immune surveillance and have been implicated as active contributors to neuron damage in various inflammatory neuropathologies, such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, stroke, and traumatic brain injury [1–3]. Microglia are derived from myeloid cells in the periphery during the embryonic stage. They account for approximately 12% of cells in the brain, and predominate in the gray matter, with the highest density being found in the hippocampus, olfactory telencephalon, basal ganglia, and substantia nigra [4,5]. Under normal CNS homeostasis, microglia typically exist in a resting state with a ramified

morphology and monitor the brain environment. In response to certain cues such as brain injury or immunological stimuli, microglia migrate toward the toxic stimuli and elicit inflammatory responses and undergo dramatic phenotypic changes from resting ramified state to an amoeboid morphology. Paradoxically, these activated cells serve diverse beneficial functions essential to maintaining neuron survival, regulating brain development, involving in neurogenesis, secreting antiinflammatory factors. However, if the neuroinflammatory reaction remains unchecked, the hyperactive cells also lead to disastrous and progressive neuropathologic damage by the excess production of a large array of cytotoxic mediators, including superoxide, nitric oxide (NO), and the proinflammatory cytokines [tumor necrosis factor- α (TNF- α), IL-1 β , IL-6, monocyte chemoattractant protein-1 (MCP-1)] [6]. Therefore,

understanding the regulation of microglial activation is critical to comprehend the inflammatory process extant in the CNS pathology, and provide ideal prospects for targeted antiinflammatory therapy capable of slowing and preventing the progress of neuroinflammatory diseases [7].

Casitas B-lineage lymphoma (c-Cbl) is a 120 kDa protooncogene product that is comprised of an N-terminal tyrosine kinase-binding (TKB) domain, a RING finger, and C-terminal proline-rich sequences and tyrosine phosphorylation sites. c-Cbl is the founding member of a highly evolutionarily conserved family. In mammals, there are three distinct Cbl homologs (c-Cbl; Cbl-b, and Cbl-c). c-Cbl and Cbl-b are ubiquitously expressed, while Cbl-c expression is restricted in epithelial cells [8]. They are RING finger E3 ubiquitin ligases and are recruited to a series of active receptor tyrosine kinases (RTKs) by interacting with TKB domain, so as to promote RTKs ubiquitination and degradation. Moreover, c-Cbl and Cbl-b both have distinct C-terminal regions that can act as adaptor molecules to mediate interactions with a plethora of proteins in signaling pathway. Intriguingly, c-Cbl is implicated in regulating the functional activity of various immune cells. For example, c-Cbl and Cbl-b are important negative regulators in lymphocyte signaling, and have been identified to be the modulators of cell activation in dendritic cells and macrophage as well. Combined transgenic deletion of Cbl plus Cbl-b in peripheral T lymphocytes (using Lck-Cre) indeed led to a substantially more severe and spontaneous systemic autoimmune/inflammatory disease and dramatic hyper-responsiveness of T cells to antigen stimulation [9]. In c-Cbl-deficient dendritic cells, Toll-like receptor (TLR)-induced expression of pro-inflammation cytokines are increased. A recent study reported that Cbl-b deficiency was associated with infiltration and activation of macrophages in white adipose tissue, and expression of cytokines, such as TNF- α , IL-6, and MCP-1 [10]. However, the function of c-Cbl in the control of brain inflammation is currently unknown. Given c-Cbl broad function in immunity, we hypothesize that c-Cbl might also regulate microglial activation in brain.

In the present study, we investigate the effects of inflammation challenged with LPS *in vitro* and *in vivo* model. To our knowledge, this is the first demonstration that c-Cbl has very abundant expression in CNS compared with other tissues *in vivo*. Moreover, we discover a crucial role for c-Cbl in regulating microglial activation and neuroinflammation *in vivo* induced by LPS. We also provide molecular evidences that these effects appear to be mediated through c-Cbl/PI3K/Akt/ NF- κ B pathway.

Materials and Methods

Reagents and Antibodies

LPS (*Escherichia coli* 0111:B4, L4391) and Dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). LY294002 were purchased from Calbiochem (San Diego, CA, USA). Antibodies used in this study: mouse monoclonal anti-c-Cbl (1:1000, 05-440; Millipore, Temecula, CA, USA); NF- κ B p65 (1:3000, ab32536; Abcam, Cambridge, UK); phosphor-Akt (Ser473) (1:1000, #4060; Cell Signaling Technology, Beverly, MA, USA) and total Akt (1:2000, #2920; Cell Signaling Technology); mouse monoclonal anti- β -Actin (1:5000, A5316;

Sigma-Aldrich); Lamin B1 (1:1000, 12987-1-AP; Proteintech, Chicago, IL, USA); ionized calcium binding adaptor molecule 1 (Iba-1) (1:500, Wako, Osaka, Japan).

Cell Cultures

The immortalized mouse microglial cell line BV-2 was cultured in DMEM/F12 (Gibco, NY, USA) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT, USA), 1% GlutaMax (Gibco), and 1% penicillin/streptomycin (Gibco) at a density not exceeding 5×10^5 cells/mL and maintained in 5% CO₂ at 37°C. Cells were serially passaged when they reached 80% confluence. BV-2 cells were used up to passage 20. Primary microglia cells and primary neuronal cells were cultured as described by Honghong Yao [11] and Yichuan Xiao [12].

Animals

c-Cbl knockout mice were generated as described previously [13,14]. Genotyping was performed using polymerase chain reaction (PCR) analysis of tail DNA. Mice used in this study were bred and maintained in a specific pathogen-free animal facility at Capital Medical University. All the animals were housed under conditions of constant temperature and humidity, with free access to standard laboratory chow and distilled water. All animal procedures were performed according to the protocols approved by the Animal Ethics Committee of Capital Medical University.

Immunohistochemistry

Immunohistochemistry was performed as described previously [15]. Mice were anesthetized and perfused with 0.9% saline followed by 4% paraformaldehyde via intracardiac infusion. Brains were dissected and postfixed in the 4% paraformaldehyde overnight. The brain was transferred to 20–30% sucrose solution separately for tissue cytoprotection. Coronal sections (30 μ m) were cut by a freezing microtome (Leica, Germany) and stored in an antifreeze solution. Sections of SN were collected for immunohistochemistry. Briefly, they were incubated overnight with a rabbit antibody against Iba-1 overnight at 4°C. After rinsing three times with PBS, sections were incubated with a second antibody (1:200) and AB work solution (Vector Laboratories, Burlingame, CA, USA) for 30 min at 37°C. DAB solution was used to visualize the staining. Images of the Iba-1-stained sections were obtained by an Olympus microscope. The mean optical density (OD) value in the SN from each section was determined using the Image Pro Plus software 6.0 (Media Cybernetics, Bethesda, MD, USA). All sections were coded and examined blindly.

Enzyme-Link Immunosorbent Assay (ELISA)

Mouse IL-6, IL-1 β , MCP-1, and TNF- α in cell culture supernatants or brain homogenate were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol. Absorbance was determined at 450 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA), with the correction wavelength set at 540 nm. All treatments were completed at least three times and data were expressed as mean pg/mL $\pm$ SEM.

Nitrite Assay

The nitrite concentration in the culture media was used as a measure of NO production. After stimulation, the generation of NO in the cell culture supernatants was determined by measuring nitrite accumulation in the medium using Griess Assay (Promega, Madison, WI, USA) following the manufacturer's protocol. The absorption was measured in an automated plate reader at 540 nm (Bio-Tek Instruments). Nitrite concentrations were calculated using a standard curve with sodium nitrite. All treatments were completed at least three times and data were expressed as mean $\mu\text{M} \pm \text{SEM}$.

Western Blot Analysis

Protein lysates were prepared using RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, EDTA, NaF, Na_3VO_4) (Beyotime, Shanghai, China) with protease inhibitor cocktail and phosphatase inhibitor cocktail (EDTA free; Roche, Germany). Nucleic proteins were prepared as previously described [16]. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins (30–50 μg) were separated by denaturing 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.2 μm nitrocellulose membrane (Millipore). The nitrocellulose membrane was then incubated with primary antibodies and secondary antibodies conjugated to horseradish peroxidase. Chemiluminescence was detected using the enhanced chemiluminescence (ECL) reagent according to the manufacturer's protocol (Appligene Technologies Inc., Beijing, China). The signal was visualized using the FluorChem-HD2 imager and densitometrically quantified using FluorChem software (ProteinSimple, San Jose, CA, USA).

Small Interfering RNA (siRNA) Transfection

siRNAs targeting mouse c-Cbl were designed and synthesized by Oligobio (Beijing, China). Mouse c-Cbl siRNA sequences were as follows: siRNA1, 5'-GCACUGUCUUGUCAAGAUATT-3' (sense) and 5'-UAUCUUGACAAGACAGUGCTT-3' (antisense); siRNA2, 5'-GCGAAACCUGACCAAAUUATT-3' (sense) and 5'-UAAUUUGGUCAGGUUUCGCTT-3' (antisense); siRNA3, 5'-CCAAUUUUAUCCUGAUCUUTT-3' (sense) and 5'-AAGAUCAAGGAUAAUUUGGTT-3' (antisense); siRNA4, 5'-GCAGAAGUUAUCCACAAATT-3' (sense), and 5'-UUUGUGGAUGAACUUCUGCTT-3' (antisense); siRNA transfection was performed using the SiPORTERTM (Millipore) according to the manufacturer's protocol. Fluorescent FAM-labeled negative control siRNAs were used to monitor transfection efficiency by fluorescence microscopy. The knockdown efficiency of siRNA was determined 3 days posttransfection by Western Blot analysis, respectively.

Statistical Analysis

Data were expressed as the mean $\pm$ SEM. Statistical significance was assessed with one-way ANOVA followed by Newman–Keul's *post hoc* test using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Expression Patterns of c-Cbl Protein in Different Tissues in Mice

c-Cbl was originally isolated from a murine leukemia virus as the transforming oncogene [17]. In mammals c-Cbl was ubiquitously expressed. To date, c-Cbl mRNA has been found in spleen, testis, lung, skeletal muscle, thymus, prostate, pancreas, and brain, as well as in a wide variety of cell lines of hematopoietic origin [18]. However, a systematic study of its tissue distribution has not been performed. In this study, we firstly evaluated the protein expression of c-Cbl by Western Blot in mouse tissues. The data showed similar patterns with previous studies in testis and lung with expression being very high. It is barely undetectable in liver and kidney. The c-Cbl protein level in spleen and pancreas was very low. Interestingly, we observed that c-Cbl was highly expressed in CNS, especially in SNc, striatum, and hippocampus (Figure 1A,B).

Based on these results, we further sought to define the expression patterns of c-Cbl in different neural cells in CNS. We analyzed the c-Cbl protein expression level in primary neurons, astrocytes and microglia from mouse brain by Western Blot. As shown in Figure 1C,D, among the three main neural cell types, the expression level of c-Cbl in microglia was the highest compared with that in neurons and astrocytes. This highlights the potential functional role of c-Cbl in microglia which acts as the resident innate immune cells in brain.

Microglial Activation is Augmented in SNc of c-Cbl Knockout Mice in Response to Systemic LPS Administration

Microglia-mediated neuroinflammation could contribute to the progressive nature of several neurodegenerative diseases, such as PD. This has been most effectively demonstrated in LPS-treated mice model [19,20]. To better understand the role of c-Cbl in brain inflammation, we peripherally injected 5 mg/kg LPS or saline each day to the peritoneal cavity of c-Cbl knockout mice and normal C57BL/6 mice for continuous 3 days. Brains were harvested and dissected 6 h after the last LPS injection. We performed immunohistochemistry with the classic antibody specific for ionized calcium binding adaptor molecule 1 (Iba-1) to assess morphological microglial activation. The results showed that microglia in SNc of the saline-injected mice appeared phenotypically to have small cell bodies with thin, highly ramified processes, consistent with the classic resting morphology. Meanwhile, a large number of activated microglia were observed in the SNc of LPS-treated mice, as detected by intensified Iba-1 staining with activated characteristics of thicker processes and enlarged cell bodies (Figure 2A). As shown in Figure 2B, quantification of expression levels of Iba-1 by measuring optical density of Iba-1 staining confirmed the staining intensity. Microglial morphology was similar in c-Cbl knockout mice and WT mice treated with saline, and there's no significant difference between them by the quantification of Iba-1 intensity. In contrast, a significantly more increase in Iba-1 immunostaining in SNc was observed in c-Cbl knockout mice treated by LPS than that in WT mice. These results indicate that c-Cbl deficiency augments the activation of microglia in mice

Figure 1 Expression patterns of c-Cbl protein in different tissues and neural cells in mice. **(A, B)** Different tissues and different brain regions were collected from 3 months mice, and whole tissue homogenates were prepared for Western Blot. The anti-c-Cbl antibody used recognized a protein band of about 120 kDa consistent with the complete c-Cbl. β -Actin was used as loading control. Quantification of c-Cbl protein bands illustrated relative expression level in different tissues. **(C, D)** Western Blot analysis detected c-Cbl protein expression in three neural cell types (neuron, astrocyte and microglia). They were isolated from new pups of 1 day postpartum and cultured in corresponding medium. Cell lysates were analyzed by Western Blot. Quantification chart showed relative expression of c-Cbl protein in different cell types. Data represented a typical result from three independent experiments.

challenged with LPS, supporting an important role of c-Cbl in the regulation of microglial activation in CNS.

c-Cbl Inhibits Expression of LPS-Stimulated Proinflammatory Cytokines and Chemokines in Microglia

As shown above, the brain had very high c-Cbl expression among the tissues in WT mice. In particular, c-Cbl was predominantly expressed in microglia, the resident immune cells in CNS. Although in the present study, we showed that c-Cbl deficiency increased the response of microglial inflammation challenged with LPS in c-Cbl^{-/-} mice, there were no clues to indicate that c-Cbl could directly regulate microglial inflammation response. To solve this doubt, we performed experiments to determine whether c-Cbl regulated the production of proinflammatory cytokines IL-6, IL-1 β , TNF- α , nitric oxide (NO) and chemokine MCP-1 in brain regions and microglial cell line treated with LPS. To do this, we harvested brain SNc tissue homogenate from c-Cbl knockout mice and normal C57BL/6 mice treated with LPS or saline as described above, and quantified the release of inflammation factors (IL-6, IL-1 β , TNF- α , and MCP-1) and NO using ELISA and Griess assay, respectively. As expected, LPS treatment in c-Cbl knockout mice induced a significant increase in the levels of IL-6, IL-1 β , TNF- α ,

MCP-1, and NO from SNc of mice compared to LPS-treated WT mice (Figure 2C–G).

Microglial cells are the main source of proinflammatory factors in the brain after peripheral LPS injections. It is worthwhile to investigate the role of c-Cbl in microglia *in vitro*. Therefore, further validation of these findings was carried out in BV2 microglial cells transfected with the c-Cbl siRNA oligos (Figure 3A,B). Consistent with the findings, knockdown of c-Cbl in BV2 microglial cells exacerbated LPS-mediated release of proinflammatory factors (IL-6, TNF- α , MCP-1, and NO) compared with that in siRNA mock treatment (Figure 3C–F). Taken together, we validated the role of c-Cbl in LPS-induced microglial inflammation response *in vivo* and *in vitro*. Our findings demonstrated that c-Cbl negatively regulated the activation of microglia.

c-Cbl Knockdown Increases Akt Phosphorylation in BV2 Microglial Cells Challenged with LPS

c-Cbl is an adaptor protein and a member of the RING-type E3 ubiquitin ligase family, which has important roles in several signaling pathways that affect various cellular functions [8,21,22]. Recent research demonstrated that repressing c-Cbl expression resulted in the aberrant activation of PI3K/Akt signaling pathway during the osteogenic differentiation of human adipose-derived

Figure 2 c-Cbl deficiency augments microglial activation in SNc of c-Cbl knockout mice in response to systemic LPS administration. 8-week-old male c-Cbl^{-/-} mice (KO) and c-Cbl^{+/+} mice (WT) were intraperitoneally injected with a single dose of LPS (5 mg/kg) or saline each day for continuous 3 days and maintained under normal conditions. Mice were sacrificed and brain sections that encompassed the entire SNc were collected. **(A)** We performed immunostaining with Iba-1 to visualize the activation of microglia in the SNc of saline or LPS-treated animals. Scale bar = 50 (right) or 500 μm (left). **(B)** Quantitative analysis of Iba-1 expression based on optical density (OD) of Iba-1 staining signal in the SNc illustrated intensity of microglial activation. **(C–G)** Inflammation factors (IL-6, IL-1β, TNF-α, MCP-1, and NO) were measured by ELISA and Griess assay respectively as described in Materials and Methods. Data were expressed as mean ± SEM (n = 3–4 per group). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, compared with the LPS-treated c-Cbl^{+/+} mice. #*P* and #*P* < 0.05, compared with saline group.

mesenchymal stem cells [23]. Previous studies have shown that activation of Akt plays an essential role in LPS-induced microglial activation by stimulating NF-κB activity [24]. We further performed experiments to investigate whether c-Cbl knockdown

affected PI3K/Akt activation in LPS-stimulated BV2 microglial cells. As shown in Figure 4A,B, Akt phosphorylation was significantly enhanced in BV2 microglial cells after LPS treatment. In addition, downregulation of endogenous c-Cbl by siRNA

Figure 3 c-Cbl inhibits expression of LPS-stimulated proinflammatory cytokines and chemokines in BV2 microglial cells. **(A, B)** BV2 microglial cells were treated with four different sequences of siRNA (denoted c-Cbl siRNA1-4) or scrambled siRNA (control siRNA). c-Cbl protein was measured using Western Blot after 72 h of siRNA transfection, and quantification analysis of c-Cbl protein bands was used to assess c-Cbl knockdown. **(C–F)** BV2 microglial cells were treated with c-Cbl siRNA1 or control siRNA for 48 h followed by 10 ng/mL LPS treatment and assessed for inflammation factors production of IL-6, TNF- α , MCP-1, and NO. Data were expressed as mean $\pm$ SEM. ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with c-Cbl siRNA-treated without LPS. # $P < 0.05$, compared with corresponding control group without LPS treatment.

remarkably promoted PI3K/Akt activation after LPS treatment as demonstrated by Western Blot analysis of Akt phosphorylation. Interestingly, c-Cbl knockdown also significantly increased LPS-induced NF- κ B P65 nuclear translocation (Figure 4C,D).

Next, to define whether PI3K/Akt activation was functionally associated with the expression of proinflammatory factors, we performed ELISA with the presence of LY294002 in BV2 microglial cells. PI3K inhibitor LY294002 pretreatment significantly inhibited Akt phosphorylation and partially suppressed NF- κ B P65 nuclear translocation (Figure 4E–H). As presumed, LY294002 pretreatment also substantially attenuated the expression of LPS-stimulated proinflammatory factors (IL-6, TNF- α , MCP-1, and NO) (Figure 4I–L). These results indicate that c-Cbl-mediated PI3K/

Akt/NF- κ B pathway is a pivotal axis during the microglial inflammation activation in response to LPS.

Discussion

Microglia with a morphologically activated phenotype are present in CNS tissue from patients with chronic neurodegenerative diseases such as AD, PD, and ALS. Microglial activation is an early event in the development of CNS inflammatory disorders. Microglia play pivotal role in inflammatory responses in brain [1]. Aberrant activation of microglia could consequently cause neuronal damage, specifically PD, AD, and multiple sclerosis, through overproduction of the proinflammatory mediators, such as IL-6,

Figure 4 c-Cbl knockdown increases Akt phosphorylation in BV2 microglial cells challenged with LPS. **(A, B)** BV2 microglial cells were treated with c-Cbl siRNA or control siRNA for 48 h followed by 10 ng/mL LPS treatment over 30 min and assessed for Akt activation by Western Blot. β -Actin was used as loading control. **(C, D)** For detecting NF- κ B P65 nuclear translocation after c-Cbl knockdown, cell lysates were collected after 2 h of 10 ng/mL LPS treatment. LaminB was used as loading control. **(E–L)** PI3K inhibitor LY294002 treated BV2 microglial cells with silencing c-Cbl stimulated by LPS, Western Blot analysis was performed to assess Akt phosphorylation and NF- κ B P65 nuclear translocation. The production of IL-6, TNF- α , MCP-1, and NO were measured by ELISA and Griess assay, respectively. Data were expressed as mean $\pm$ SEM. ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with LPS alone treatment. $\neq$ and # $P < 0.05$, compared with corresponding control group without LPS treatment.

TNF- α , MCP-1, and NO [25]. Data from series of studies demonstrated that the persistent activation of microglia contributed the progression of chronic neurodegeneration, rather than simply being a consequence of the neuropathology, emphasizing the

promise of targeting microglial activation in the treatment of chronic neurodegeneration [3]. So, inhibiting microglial activation is thought to be a promising therapeutic strategy in the treatment of chronic neurodegeneration [7].

Cbl proteins are E3 ubiquitin ligases and have been implicated in regulating the functional activity of various immune cells [26–29]. Previous study showed that Cbl-b is necessary to prevent the inappropriate activation of NF- κ B in response to LPS and polymicrobial sepsis [30]. In addition to Cbl-b, recent study has reported that c-Cbl plays an important role in regulating dendritic cell maturation and negatively regulating the transcription of proinflammatory cytokines, probably by indirectly modulating the stimulatory function of NF- κ B complexes. Cbl proteins are highly expressed in normal lungs in both mice and humans, and the loss of Cbl-b expression accentuates LPS-mediated acute lung inflammation and reduces survival in Cbl-b^{-/-} mice [30]. Interestingly, we find that c-Cbl proteins, another member of Cbl family, are also expressed in brain at comparatively high level, but nothing is known about the function of c-Cbl in brain. In the present study, we identified the functions of E3 ubiquitin ligase c-Cbl as a pivotal regulator of microglial activation in LPS-mediated neuroinflammation in mice. c-Cbl^{-/-} mice showed overactivated microglia morphology and an obvious upregulation of cytokine and chemokine production in mouse brain after systemic LPS administration compared to c-Cbl^{+/+} mice.

To further explore the possible molecular mechanism through which c-Cbl proteins act in microglial inflammation, we performed *in vitro* study and found that downregulation of c-Cbl by siRNA resulted in higher level of PI3K/Akt activation and NF- κ B P65 nuclear translocation in BV2 microglial cells after treatment of LPS. Recent studies indicate that LPS-induced NF- κ B activation is directly regulated by phosphorylation of PI3K/Akt in microglial cells, implying that suppression of PI3K/Akt may be useful to prevent neuroinflammation [24, 31, 32]. Based on these clues, we further confirmed that inhibition of PI3K by LY294002 could effectively reverse high expression level of proinflammatory factors such as IL-6, TNF- α , MCP-1, and NO in BV2 microglial cells

treated with c-Cbl siRNA after LPS stimulation. This result at least indicates that activation of PI3K/Akt plays a relatively pivotal role in microglial activation stimulated by LPS.

In conclusion, our work establishes that E3 ubiquitin ligase c-Cbl is crucial to modulate microglia-mediated neuroinflammation. It is possible that c-Cbl is capable of negatively regulating PI3K/Akt activation upon LPS stimulation in microglial cells. Our data reveal a previously unknown role for c-Cbl in microglia-mediated neuroinflammation. This study elucidated the c-Cbl's role and relevant molecular mechanism mainly by genetic knockout mouse model and siRNA knockdown approach. So, the future study will further confirm the role of c-Cbl in microglial activation through c-Cbl overexpression method. In addition, this study provides evidences for c-Cbl as a novel potential therapeutic target for CNS neuroinflammation-related disorders. Therefore, developing compound that could positively up-regulate endogenous c-Cbl's expression is an interesting future investigation.

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Conflict of Interest

The authors declare no conflict of interest.

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