PICK1 confers anti-inflammatory effects in acute liver injury via suppressing M1 macrophage polarization

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Abstract

Protein interacting with C kinase 1 (PICK1) is a scaffolding protein mainly implicated in neurological diseases, however, the function of PICK1 in acute liver injury (ALI) remains unknown. Our study found a dramatical decrease in mRNA and protein levels of PICK1 in liver tissues and isolated Kupffer cells (KCs) from the liver in mice with ALI. Furthermore, pretreatment the mice with ALI with FSC-231, a pharmacological inhibitor of PICK1, could significantly augment inflammatory response. Furthermore, *in vitro* studies showed that both lipopolysaccharide (LPS) and interferon gamma (IFN- γ) significantly reduced the expression of PICK1, while IL-4 elevated its expression in RAW 264.7 cells. Additionally, over-expression of PICK1 inhibited the expression of M1 biomarkers by suppressing NF- κ B activity, and enhanced the expression of M2 biomarkers by promoting STAT6 activity. In contrast, knockdown of PICK1 or FSC-231 pretreatment promoted M1 polarization and suppressed M2 polarization. Besides, caveolin-1 was identified as a potential target gene controlled by PICK1 in RAW 264.7 cells. Mechanistic investigation revealed a dual role of PICK1 in regulating macrophage polarization and implied PICK1 as a potential therapeutic target in ALI.

Keywords

Protein interacting with C kinase 1; Acute liver injury; Kupffer cells; RAW 264.7 cells;

macrophage polarization; Caveolin-1.

Abbreviations

PICK1, protein interacting with C kinase 1; ALI, acute liver injury; KCs, Kupffer cells; LPS, lipopolysaccharide; IFN- γ , interferon gamma- γ ; iNOS, inducible nitricoxide synthase; TNF- α , tumor necrosis factor; MCP-1, monocyte chemoattractant protein; Arg-1, arginase.

1. Introduction

Liver injury occurs as a common pathological response to acute liver disease with sudden hepatic dysfunction and inflammation-mediated characteristics. Acute liver injury (ALI) is a severe life-threatening disease with complexity and unpredictability and is derived from specific multiple causes in course and outcome, including hepatitis, drug and toxin-induced liver damage, metabolic syndrome, ischemia and miscellaneous rare causes [1-3]. The progression of acute liver disease have been described to be mainly driven by the activated inflammatory cells, among which both resident and infiltrating macrophages could develop into distinct subpopulations upon the surrounding stimulation [4]. Kupffer cells (KCs) belonged to the mononuclear phagocytic system and served as crucial resident macrophage populations in driving both initiation and progression of hepatic inflammation, through in part by the cross-talk with other immune cells, such as T and B lymphocytes [5, 6]. Lipopolysaccharide (LPS) caused liver damage via acting as hepatotoxin and pro-inflammatory mediators to activate mononuclear cells and macrophages in hepatic diseases [7]. Due to the specific plasticity of macrophages, KCs in a mouse model of LPS-induced ALI could adapt tremendous phenotype switch referred to mainly as M1 macrophages/classically activated macrophages [8], thus defending the host against microbial infection and triggering the perturbative production of pro-inflammatory cytokines and chemokines [9]. In contrast, stimulation of macrophages with IL-4 or IL-13 could induce the generation of anti-inflammatory and restorative M2 macrophages/alternatively activated macrophages [10]. In this regard, inhibiting KCs activation and suppressing M1

macrophage polarization have great significance for ameliorating inflammatory response in ALI disease.

Protein interacting with C kinase 1 (PICK1), a scaffolding protein mainly implicated in neurological diseases, was initially prescribed as a PKC α -binding protein [11]. PICK1 is unique in having both PSD-95/DlgA/ZO-1 (PDZ) domain and Bin–Amphiphysin–Rvs (BAR) domain. Due to the specific structure, PICK1 has been proposed as a candidate to modulate neurological diseases [12], tumors [13], cell junction [14] and cell death [15], in which PICK1 was proved to interact with a wide range of receptors, transporters, and enzymes that contain PDZ binding motifs through the single PDZ domain. Ubiquitously-expressed PICK1 in tissues or organs [11, 16] implied the probable biological function of this molecule outside the nervous system. Importantly, Emerging evidence has recognized PICK1 as a critical anti-inflammatory driver in oxidative stress activated microglia during neurological disorders [17]. In the literature, macrophages and microglia cells shared similar properties of plasticity [18], whereas PICK1 expression in macrophages and the potential physiological function of PICK1 during liver disease remains largely unclear. We thus initially sought to uncover the role of PICK1 in determining macrophage polarization and the mechanisms underlying LPS-induced inflammatory injury.

2. Materials and methods

2.1. Animal, mouse model of acute liver injury.

All the 18~22g C57BL/6J mice were provided by the Experimental Animal Center of Anhui Medical University and conducted in accordance with the Guideline of Animal Care and Ethics Committee of Anhui Medical University. Acute liver injury model was generated by intraperitoneal injection of LPS (10 mg/kg, body weight, dissolved in sterile pyrogen-free saline solution). Simultaneously, mice in control group were treated intraperitoneally with the same volume of saline solution. In each experiment, C57BL/6J mice were divided randomly into 5 groups (n= 5) including control group, ALI group, ALI group pretreated with different

concentrations of FSC-231 (5 $\mu\text{mol/L}$, 25 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$) 2 h prior to the LPS injection. After sacrificing the mice, both centrifuged serum and liver tissues were harvested for the further analysis and frozen at -80°C

2.2. Isolation of liver kupffer cells (KCs).

Primary isolated liver KCs were perfused from liver tissue of mice in the above five groups, using the methods as described previously [19]. Briefly, Putting a 20-G catheter through the portal vein, and cutting the inferior vena cava. The liver was perfused with diluted perfusion buffer [PB, dissolve NaCl (103.75 g), KCl (6.25 g) and Hepes (28.70 g) in H_2O (350 ml)], followed by a digestion buffer [$1 \times \text{PBC}$, supplemented with collagenase (type IV; Sigma-Aldrich, USA), Pronase E (Sigma, USA) and 4.76 mmol/L CaCl_2]. The cells and minced tissues were collected in BSA, 25% Percol and 50% Percol (Sigma-Aldrich, USA) were further used to fractionate the cells. Stationary maintaining the cells in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and incubating at 37°C in plastic Dishes for hours. After washing non-adherent cells off, the remained adhered cells, which were considered to be KCs, were collected for further analysis.

2.3. Cell culture and cell treatment.

RAW 264.7 cell lines and THP-1 cell lines were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin. As confirmed by our laboratory, after seeding and culturing the cells up to density of 50%, the macrophages were incubated in medium supplemented with LPS (1 $\mu\text{g/ml}$, Sigma, USA) alone or LPS combined with interferon gamma ($\text{IFN-}\gamma$, 20 ng/ml, Pepro Tech, USA) to orient towards M1 phenotype, while M2 macrophages were induced in medium supplemented with IL-4 (20 ng/mL, PeproTech, USA). Besides, macrophages in FSC-231 groups were

pretreated with different concentrations (5 $\mu\text{mol/L}$, 25 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$) of FSC-231 (Merck, USA) before appropriate polarization. The non-treated RAW 264.7 cells were used as control. The THP-1 cell lines were cultured in medium (1640, Gibco, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin and differentiated into macrophages by using Phorbol-12-myristate-13-acetate (PMA, 100 ng/ml, Sigma, USA). All cells were incubated at 37°C at an atmosphere of 5% before being used in assays.

2.4. Flow cytometry analysis

The transfected M1 macrophages were resuspended in FACS staining buffer containing anti-mouse iNOS and anti-mouse F4/80. PE-conjugated anti-mouse iNOS and APC-conjugated anti-mouse F4/80 were purchased from eBioscienc. As for cells in M2 polarized group, cells was stained with PE-conjugated anti-mouse Arg-1 and purchased from R&D Systems. Additionally, data were acquired on a FACS Calibur flow cytometer (Beckman, USA), and analyzed on FlowJo software (Tree Star, USA). All the experiments were repeated in triplicates.

2.5. ALT/AST activity assay

The ALT and AST levels in serum from C57BL/6J mice were measured using alanine aminotransferase/aminotransferase Assay Kits (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instruction. The absorbance at 510 nm was measured with a micro-plate reader model 680 (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Total RNA extraction and quantitative real-time PCR(Real-time PCR).

Total RNA was extracted from liver tissues, primary KCs and RAW 264.7 cells using TRIzol reagents (Invitrogen, USA), and the extracted RNA was synthesized into first-strand cDNA using Thermoscript Real-time PCR reverse transcription kit (Fermentas, USA), according to the manufacturer's instructions. Subsequently,

analysis for cDNA were performed by using Thermoscript Real-time PCR kits (Fermentas, USA) to evaluate relative gene mRNA expression. The mRNA levels of GAPDH and β -actin were used as internal control. The oligonucleotide primer sequences used for PCR amplification are listed in Table 1.

2.7. Western blot

Whole proteins in mouse liver tissues, KCs and RAW 264.7 cells were lysed by RIPA lysis buffer containing phenylmethylsulfonyl fluoride (Beyotime, China). Protein concentration was measured using enhanced BCA protein assay kit (Boster, China). Equal amounts of protein were separated and electrophoresed by SDS-polyacrylamide gel and transferred onto PVDF membranes (Millipore Corp, USA). After blockade of non-specific protein binding, the membranes were incubated with specific primary antibodies diluted in dilution buffer (Beyotime, China). The Rabbit polyclonal antibody against PICK1, IL-6, TNF- α , p-STAT6, STAT6 (Abcam, USA) were diluted into 1:500, The Rabbit monoclonal antibody against iNOS, p65, p-p65, caveolin-1 (Cell Signaling, USA) were diluted into 1:500. The Mouse monoclonal antibody against β -actin and Rabbit polyclonal antibody against Arg-1 (Santa Cruz, USA) were diluted into 1:1000. The Rabbit polyclonal antibody against p-IkB α , IkB, p-p38, P38, CD86 were diluted into 1:200 (Bioss, China). Incubating overnight, the membranes were washed and incubated with Horseradish peroxidase conjugated secondary anti-rabbit and anti-mouse antibodies (1:10000) for 1h. After extensive washing with TBS/Tween-20 again, the membranes were placed for detection of antigen using the enhanced chemiluminescence system. Proteins were visualized with ECL-chemiluminescent kit (ECL-plus, Thermo Scientific).

2.8. ELISA assay

RAW 264.7 cells (1×10^5 /well) were plated in 24-well culture plates. The IL-6 and IL-10 concentrations in each cell supernatant and mouse serum were harvested and measured with ELISA kit (Hushang Biotechnology, China) according to the

instructions of the manufacturer. The optical density at 450 nm each well was immediately determined by using Thermomax microplate reader (bio-tekEL, USA). All experiments were performed in triplicate.

2.9. RNA interference (RNAi)

RNAi was transfected by using Lipofectamine®2000 (Invitrogen, USA), according to the manufacturer's protocol. Small interfering RNA (siRNA) oligonucleotides were purchased from GenePharma (Shanghai, China). RAW264.7 cells were subjected to transfection with siRNA in Opti-MEM (Gibco, USA) upon plating and culturing the cells in antibiotics-free DMEM for 12 h. The culture medium was changed 6 h after transfection, into medium supplemented with different stimuli subsequently. The siRNA sequences were as follows: PICK1-siRNA (sense: 5'-GUCCUUGUCUCUAUAUCGUTT-3' and antisense: 5'-ACGAUUAUAGAGACAAGGACTT-3'). Caveolin-1-siRNA (sense: 5'-AGAUGUGAUUGCAGAACCATT-3' and antisense: 5'-UGGUUCUGCAAUCACAUCUTT-3'). Scrambled siRNA (sense : 5'-UUCUUCGAACGUGUCACGUTT-3' and antisense: 5'-ACGUGACACGUUCGGAGAATT-3'). The efficiency of knockdown was determined by fluorescence microscopy assay and Western blot. All the experiments were conducted in triplicate and repeated three times.

2.10. Recombinant mouse PICK1 plasmid construction.

PICK1 plasmid was synthesized by Genechem (Shanghai, China) and extracted by using Plasmid Extraction Kit (Tiangen Biotech, China). Ectopic expression of PICK1 was acquired by using GV143-PICK1 transfection, empty vector GV143 (CMV-3FLAG-MCS-EGFP-SV40-Neomycin) was used as a control. All constructs were confirmed by DNA sequencing and the constructed plasmid was transfected into RAW264.7 cells by using Lipofectamine®2000. The efficiency of transfection was also assessed by Western blot. The culture medium was also removed and replaced

with different stimuli 6 h after transfection. All the experiments were conducted in triplicate.

2.11. Statistical analysis.

All results were represented as means $\pm$ SD. Statistical significant differences were determined by one-way ANOVA with SPSS17.0 software. For the significance in mRNA or protein levels, mRNA (relative expression) and protein (densitometric values) levels were measured, comparing to respective housekeeping controls. In all cases, statistical significance was considered at values of $P < 0.05$.

3. Results

3.1. Characterization of inflammatory response and PICK1 expression in a mouse model of LPS-induced acute liver injury (ALI).

To identify the expression of PICK1 in ALI, a mouse model of LPS-induced advanced ALI was established. Firstly, we employed saline solution-injected mice as control group. As illustration in Fig.1A and Fig.1B, LPS alone provoked extensive inflammatory response, as indicated by a significant increase in the mRNA levels of IL-6, IL-1 β , monocyte chemoattractant protein (MCP-1), nitric oxide synthase (iNOS) and the protein levels of tumor necrosis factor (TNF- α), IL-6, iNOS, CD86 in liver tissues in a time-dependent manner 2 h upon injection. Therefore, we selected treatment with LPS (10 mg/kg, body weight) for 24 h to establish a mouse model of ALI. Besides, a distinct decrease of PICK1 expression in the progression of ALI was confirmed (Fig.1B), suggesting a potential role of PICK1 during ALI.

3.2. Anti-inflammatory capacity of PICK1 in LPS-induced ALI

As PICK1 expression in liver tissues of ALI group was down-regulated, we further addressed the function of PICK1 in the pathogenesis of ALI. FSC-231, a pharmacological inhibitor of PICK1, could abolish the interaction of functional PDZ domain with other PDZ domain-containing proteins [20]. Firstly, FSC-231 was administrated by intraperitoneal injection to test the hypothesis that it would aggravate LPS-induced injury to the liver. As shown in Fig.2A, liver tissues of LPS-treated mice

exhibited hepatocyte necrosis, irregularly arranged, intercellular spaces dilatation, inflammatory cells infiltration, while liver tissues in control group showed normal lobular architecture with central veins. Furthermore, pretreatment with different concentrations of FSC-231 (5 $\mu\text{mol/L}$, 25 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$) for 2 h before LPS injection resulted in more robust inflammatory responses which enhanced degree of liver injury (Fig.2A). These results were further confirmed by an increase in ALT, AST (Fig.2B) and IL-6 levels in serum (Fig.2C). Moreover, rather lower PICK1 expression, along with extensive inflammatory indicators, occurred in FSC-231 pretreated mice than that in LPS-treated mice (Fig.2D and Fig.2E). Then we isolated and cultured the primary KCs *ex vivo*. As we expected, LPS treatment induced the KCs polarization into an M1 phenotype in LPS-treated mice compared to that in control mice (Fig.2F). More importantly, when PICK1 expression was inhibited by FSC-231 in a concentration-dependent manner, the expressions of M1 biomarkers were gradually increased (Fig.2G). These observations indicate that down-regulation of PICK1 may be involved in the development of ALI, a process induced originally by macrophages.

3.3. Change of PICK1 expressions in LPS treated macrophages and M1/M2 macrophages

Given the reduction in PICK1 expression and an increased number of M1 KCs in ALI model, murine RAW 264.7 cell lines were frequently applied to demonstrate the interplay between PICK1 and macrophage activation *in vitro*. To provoke inflammatory response, 1 $\mu\text{g/ml}$ LPS was prepared to stimulate RAW 264.7 cells at the indicated time points to examine the release of pro-inflammatory cytokines. Compared with control group, the expressions of several pro-inflammatory cytokines including TNF- α and IL-6 were dramatically enhanced at 6 h and peaked at 24 h in a time-dependent manner following LPS treatment (Fig.3A and Fig.3B). Simultaneously, PICK1 was notably decreased as early as 6 h post LPS-treatment. Furthermore, in line with data in RAW 264.7 cells, the expression of PICK1 was decreased in PMA-treated THP-1 cell lines after exposure to 1 $\mu\text{g/mL}$ LPS (Fig.3C). According to the aberrant PICK1 expression in activated macrophages, we further

investigated the expression of PICK1 in murine M1/M2 macrophages. Results showed that combination of 1 ug/ml LPS with 20 ng/ml IFN- γ time-dependently increased the mRNA (Fig.3D) and protein levels (Fig.3E) of M1 biomarkers including TNF- α , IL-6, iNOS and CD86 in RAW 264.7 cells within 24 h. Besides, stimulation of RAW 264.7 cells with IL-4 time-dependently induced the up-regulation of Arg-1 and IL-10, biomarkers for M2 macrophages, within 48 h (Fig.3F and Fig.3G). Declined PICK1 expression was also rapidly detected by 6 h after stimulation of LPS and IFN- γ (Fig.3D and Fig.3E). These changes were somewhat larger than that in LPS alone-treated RAW 264.7 cells. Additionally, treatment of RAW 264.7 cells with IL-4 promoted PICK1 production, which was gradually up-regulated 24-48 h in M2 macrophages (Fig.3F and Fig.3G).

3.4. PICK1 suppresses M1 macrophage polarization.

To further gain insight into the significance of PICK1 in macrophage polarization, PICK1 over-expression and PICK knockdown in M1/M2 macrophages were realized by using lipofectamine to transfect plasmid GV143-PICK1 and PICK1-siRNA, respectively. Firstly, GV143-PICK1 and the control vector GV143 were successfully transfected into RAW264.7 cells (Fig.4A). Over-expression of PICK1 significantly suppressed the mRNA levels of M1 biomarkers including IL-6, IL-1 β , IL-12, MCP-1, iNOS, CD86 (Fig.4B), the protein levels of TNF- α , IL-6, iNOS, CD86 (Fig.4C) and the IL-6 level in supernatant (Fig.4D) compared with that in M1 macrophages transfected with the control vector. Additionally, fluorescence microscopy assay and western blot analysis were carried out to measure the efficiency of siRNA transfection. As illustration in Fig.5A and Fig.5B, the applied PICK1 siRNA efficiently inhibited PICK1 expression to 50% of level observed in control cells (Fig.5B). Conversely, PICK1 knockdown significantly increased the mRNA levels of M1 biomarkers including IL-6, IL-1 β , IL-12, MCP-1, iNOS, CD86 (Fig.5C), the protein levels of TNF- α , IL-6, iNOS (Fig.5D) and the IL-6 levels in supernatant (Fig.5E). Moreover, the similar results of Flow cytometry analysis on the frequency of F4/80⁺iNOS⁺ cells

were detected in M1 macrophages transfected with GV143-PICK1 and PICK1-siRNA, respectively (Fig.4E and Fig.5F).

3.5. PICK1 promotes the induction of M2 biomarkers in M2 macrophages.

Furthermore, we determined whether PICK1 influenced M2 macrophage polarization. Results showed that knockdown of PICK1 decreased the expression of M2 biomarkers including Arg-1 (Fig.6A and Fig.6B) and IL-10 level in supernatant (Fig.6C), whereas over-expression of PICK1 increased Arg-1 expression (Fig.6E and Fig.6F) and IL-10 level in supernatant (Fig.6C and Fig.6G). Besides, PICK1 silencing also induced a decrease in the frequency of Arg-1⁺ cells in M2 macrophages.

3.6. Essential role of PDZ domain in the effect of PICK1 on macrophage polarization

To test whether the effect of PICK1 on macrophage polarization was mediated via its PDZ domain, RAW 264.7 cells were treated with FSC-231 at concentrations of 5 μ mol/L, 25 μ mol/L, 50 μ mol/L prior to stimulation. As we expected, FSC-231 pretreatment augmented inflammatory response (Fig.7A and Fig.7B), as indicated by an increase of IL-6, MCP-1, IL-1 β , iNOS and CD86 expression in M1 macrophages, whereas FSC-231 pretreatment reduced Arg-1 expression and STAT6 phosphorylation in M2 macrophages (Fig.7C and Fig.7D). Taken together, the above results suggest that blockage the PDZ domain of PICK1 by FSC-231 at concentration of 50 μ mol/L may repress M1 and drive M2 macrophage polarization.

3.7. PICK1 negatively regulates NF- κ B activity in M1 macrophages

Activation of transcription factor NF- κ B, which coordinated the expression of various immune-related genes [21], has always been implicated in LPS-induced liver inflammatory response [22] and macrophage polarization [23]. Blockage of NF- κ B co-activators has been demonstrated to be essential for the suppression of M1 genes [24]. To gain more information about PICK1, therefore, we investigated whether PICK1 had negative regulatory effects on NF- κ B activity during M1 macrophage polarization. Firstly, phospho-p65 and phospho-I κ B α expressions were

visibly increased in LPS and IFN- γ -stimulated RAW264.7 cells. Furthermore, over-expression of PICK1 reduced the phosphorylation of p65 and I κ B α (Fig.8A). In contrast, NF- κ B signaling was highly activated in PICK1-siRNA transfected M1 macrophages compared with that in scramble-siRNA transfected M1 macrophages (Fig.8B). In addition, different concentrations (10 μ mol/L, 50 μ mol/L, 100 μ mol/L) of PDTC, a chemical inhibitor for NF- κ B, was administrated to disrupt NF- κ B signaling in M1 macrophages. Firstly, the protein levels of M1 biomarkers and phospho-p65 were significantly suppressed in the presence of 100 μ mol/L PDTC (Fig.8C). Next, Western blot results showed that PICK1 protein was obviously up-regulated in the presence of PDTC (Fig.8C). In conclusion, these results reveal a feedback loop between PICK1 and p65 in M1 macrophages. Thus PICK1 could negatively regulate M1 macrophage polarization, at least partially by inhibiting NF- κ B activity *in vitro*

3.8. PICK1 positively regulates caveolin-1 expression and the downstream STAT6, P38 activity in M2 macrophages

Considering the association between M2 macrophages and p38 activation, which occurred as the downstream signaling of STAT6 signaling [25], we speculated that one possible explanation for the effects of PICK1 on M2 polarization might be that the prolonged activation of p38 and STAT6 signaling was induced. In the current study, the phosphorylation of p38 and STAT6 were significantly diminished in PICK1-siRNA transfected M2 macrophages (Fig.9A), while increased in PICK1 over-expressed M2 macrophages (Fig.9B). Recent studies have revealed that caveolin-1 not only exhibited anti-inflammatory capacity in murine macrophages through P38 signaling [26], but also regulated the expression of IL-4 mediated STAT6-dependent signaling in asthma [27]. The characteristic of caveolin-1 draws us to suppose that it may be responsible for the anti-inflammatory capacity of PICK1. Hence, to better elucidate the mechanism by which PICK1 mediated p38 and STAT6 activities, we transfected siRNA specifically targeting caveolin-1 into M2

macrophages. A dramatic suppression of caveolin-1 expression was observed in caveolin1-knockdown cells. Of note, knockdown of PICK1 decreased Arg-1 expression and the phosphorylation of p38 and STAT6, whereas knockdown of caveolin-1 partially abrogated the effects of PICK1 on Arg-1 expression and phosphorylation levels of p38 and STAT6 (Fig.9C). Given these results, we conclude the essential role of caveolin-1 for PICK1 in promoting M2 macrophage polarization via interacting with p38 and STAT6 signaling.

4. Discussion

As data accumulated, PICK1 has exhibited various biological functions mainly in neurologic disease [12]. Recent studies [17] revealed that deletion of PICK1 aggravated the pathogenesis of neurodegenerative disease, in which increased oxidative stress, as proved by the decreased expression of excitatory amino acid carrier1 and the defective Rab11 activity, and enhanced microglia activation were induced. Microglia cells were classified as phagocytic cells of myeloid origin [28] and regarded as brain-resident macrophage of central nervous system [29]. They shared similar plasticity with macrophages to suit their functional requirements [30]. Oxidative stress, which was caused by imbalance mechanisms between reactive oxygen species and antioxidant [31], could be produced by macrophages under acute injured conditions [32]. Oxidative stress triggered both tissue homeostasis lesions [33] and robust inflammatory response [34] in alcoholic liver disease [35]. Nevertheless, the regulatory effect of PICK1 on diseases was not restricted to neurological disorders, it also cooperated with Sirt-7 to maintain anti-fibrotic activity of rat cardiac fibroblasts and mouse dermal fibroblasts, as indicated by suppressing inflammatory cell infiltrations and attenuating the expressions of F4/80, CD163, MCP-1 and TNF- α in the myocardium of MI mice [36]. Based on the considered role of activated KCs, the major contributor to inflammation in progressive fibrotic disease [37], we thus designed the present study to uncover the anti-inflammatory and hepatoprotective capacity of PICK1 in immune-mediated liver injury.

LPS-induced liver injury has been widely used as a model to trigger immune response, liver dysfunction and failure [38, 39]. KCs could undergo phenotypic switching to eliminate the invading pathogens. KCs in ALI model exerted hepatic and systemic toxicities by producing chemical mediators and pro-inflammatory cytokines, such as IFN- γ , TNF- α and TLR ligands [40]. We also tested our hypothesis by using RAW 264.7 murine macrophage cell lines, one of the most common models used to estimate *in vitro* study of immune response [41, 42] and macrophage polarization [43].

In the current study, a well-established ALI model, as indicated by increasing the levels of ALT, AST and IL-6 in serum, was adapted to investigate the effect of PICK1 on ALI, especially focus on isolated KCs. We provided the initial evidence that a dramatical decrease of PICK1 expression was occurred in liver tissues and KCs of LPS-treated mice. In line with the data *in vivo*, PICK1 was also significantly down-regulated under inflammatory conditions and macrophage polarization *in vitro*. Interestingly, FSC-231 pretreatment in LPS-treated mice significantly augmented inflammatory response and accelerated deterioration of liver function. Several *in vitro* loss-and gain-of-function studies found that blockage of PICK1 by both PICK1-siRNA transfection and selective FSC-231 pretreatment could not only increase the expression of M1 biomarkers, but also remarkably weaken efficiency of M2 macrophage phenotype changes. It indicates that PICK1 possesses the capacity of forwarding macrophage into an anti-inflammatory phenotype. Moreover, over-expression of PICK1 conferred opposite effects. Additionally, these observations suggest the inhibitory effect of PICK1 on NF- κ B activity via blocking phosphorylation of I κ B α and p65 in M1 macrophages. On the contrary, PICK1 conferred promotive effects on M2 macrophage polarization through P38 and STAT6 signaling as well. Taken together, we conclude the potential role of PICK1 in controlling macrophage polarization, partially through NF- κ B, P38 and STAT6-dependent mechanisms.

PICK1 has been proved to be a scaffold protein implicated in regulating subcellular localization and surface expression of its PDZ-binding partners [44]. The above results demonstrated that FSC-231 mediated PDZ mutation in PICK1 gene could diminish its anti-inflammatory capacity, indicating a critical role of PDZ domain in mediating protein-protein interactions. Caveolin-1, a major cholesterol- and glycosphingo lipid-rich ampullate protein presented in invaginations of the plasma membrane, has multiple functions, such as regulating cell apoptosis, lipid metabolism and endocytosis in macrophages [45-47]. Accumulated evidence has implied the existent of caveolin-1 in immune cells and its participation in pathogenesis of inflammatory diseases including asthma [27, 48]. Interestingly, Zhao *et al.* revealed that PICK1 functioned as a scaffold protein to accumulate caveolin-1 to the C-terminus of TbetaRI via its PDZ domain and led to an enhanced caveolin-1 expression and lipid raft/caveolae localization at the endogenous protein levels in NMuMG cells [49]. As reported in previous study, caveolin-1 served as a potent immune-regulatory protein in mediating differentiation of monocytes into macrophage. It cooperated with P38 signaling to confer anti-inflammatory capacity in macrophages [26, 50] and also cooperated with STAT6 signaling to modulate IL-4 mediated airway remodeling [51, 52]. However, the correlation between PICK1 and caveolin-1 in macrophage is still obscure, the exact role of caveolin-1 in PICK1-related inflammatory response is worthy of further investigation.

In light of the above illustration, si-caveolin-1 was adapted to explore the involvement of caveolin-1 in PICK1 mediated anti-inflammatory mechanism. As expected, over-expression of PICK1 led to up-regulation of STAT6 and p38 in M2 macrophages. Inversely, down-regulation of these two signaling pathways were observed in PICK1-siRNA transfected M2 macrophages. More importantly, deletion of caveolin-1 in these cells was sufficient to diminish this effect, suggesting the role of caveolin-1 in the regulation of PICK1. However, we still don't know the detailed mechanism of caveolin-1 on PICK1, which may need to further study.

Although our knowledge of PICK1 in this study is limited, these findings provide the first evidence to illustrate the expression and potential role of PICK1 in non-neurological disease, especially in LPS-induced ALI. These findings are in agreement with the illustration by Liu et al, who found that the levels of serum nitrotyrosine were increased in patients with a homozygous mutation in the PICK1 gene [53]. In addition, PICK1 has recently been confirmed to cooperate and colocalize with the small GTPase Rac1 and Rac GEF Kalirin7 in neurons [54-56]. The critical role of Rac1 in transducing intracellular signaling, modulating astrocytic tumors [57, 58] and restraining inflammation [54, 59] attracted us to suppose that RAC1 could also affect the anti-inflammatory capacity of PICK from another point of view. Further investigation of the molecular biological functions of PICK1 are required to shed light on immune mechanisms.

Collectively, the current study suggests that PICK1 suppresses M1 macrophage polarization and promotes M2 macrophage polarization, more importantly, PICK1 may have anti-inflammatory and hepato-protective capacity via suppressing M1 KCs polarization in ALI. Moreover, it also reasonably clarifies a possible link between PICK1 and NF- κ B, STAT6 signaling in macrophage polarization, as well as a possible direct correlation between PICK1 and caveolin-1 in M2 macrophage. Thus our findings lay a foundation for illuminating the regulatory role of PICK1 in macrophage polarization and encourage the therapeutic potential of PICK1 in inflammation-associated liver disease.

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Conflict of interests

The authors declare that there is no financial conflict of interests to publish these results.

Figure Legends

Figure.1. Extensive inflammation and reduced PICK1 expression in a mouse model of LPS-induced acute liver injury (n=5 mice per group). (A) Total RNAs were isolated from liver tissues of saline solution-treated mice and LPS-treated mice at indicated times (2h, 6h, 24h), the mRNA levels of TNF- α , IL-6, IL-1 β , MCP-1, iNOS and PICK1 in liver tissues were assessed by Real-time PCR. (B) The protein levels of TNF- α , IL-6, iNOS, CD86 and PICK1 in liver tissues were assessed by Western blot. The values represent means $\pm$ SD (n = 3). * P < 0.05, ** P < 0.01 for the LPS-treated mice vs saline solution-treated mice. Similar results were conducted in three independent experiments.

Figure.2. Pretreatment of LPS-treated mice with FSC-231 significantly accelerated the deterioration of ALI (n=5 mice per group). (A) Pathological observations of liver tissues stained with hematoxylin and eosin (H&E) staining. Representative views from each group were showed (original magnification, $\times 100$). The scale bar is 100 μ m. (B) Serum ALT, AST levels and (C) serum IL-6 level in each group were measured by ELISA. (D) The mRNA levels of IL-6, IL-1 β , IL-12, iNOS, TNF- α and PICK1 in each group were assessed by Real-time PCR. (E) The protein levels of TNF- α , iNOS, CD86 and PICK1 in each group were assessed by Western blot. (F) Total mRNA levels of IL-1 β , IL-12, MCP-1, iNOS, IL-6 and PICK1 in primary KCs isolated from the mouse liver tissues in each group were measured by Real-time PCR. (G) The protein levels of TNF- α , iNOS and PICK1 in primary KCs were measured by Western blot. The values represent means $\pm$ SD (n = 3). * P < 0.05, ** P < 0.01 for the LPS-treated mice vs saline solution-treated mice, # P < 0.05, ## P < 0.01 for the LPS-treated mice pretreated with different concentrations of FSC-231 vs LPS-treated mice. Similar results were conducted in three independent experiments.

Figure.3. Expression of PICK1 in murine LPS-treated macrophages and M1/M2 macrophages. (A and B) The expressions of TNF- α , IL-6 and PICK1 in non-treated and LPS-treated RAW 264.7 cells were assessed by Real-time PCR and Western blot. (C) The mRNA levels of TNF- α , IL-6, and the PICK1 expression in LPS-treated

THP-1 cells were assessed by Real-time PCR. (D and E) LPS and IFN- γ stimulated RAW 264.7 cells into M1-phenotype, the expression of M1 biomarkers including TNF- α , IL-6, iNOS, CD86 and the PICK1 expression were determined in RAW 264.7 cells treated with combination of LPS with IFN- γ . (F and G) IL-4 (20 ng/ml) generated RAW 264.7 cells into M2-phenotype, the expression of Arg-1, IL-10 and PICK1 were determined by Real-time PCR and Western blot. The values represent means $\pm$ SD (n = 3). * P < 0.05, ** P < 0.01 vs non-treated cells. Similar results were conducted in three independent experiments.

Figure.4. Effect of PICK1 over-expression on murine M1 macrophage polarization. (A) Efficiency of PICK1 over-expression in LPS-induced RAW 264.7 cells was detected by Western blot. (B) The mRNA levels of M1 biomarkers including IL-6, IL-1 β , IL-12, MCP-1, iNOS, CD86 in PICK1 over-expressed cells were assessed by Real-time PCR. (C) The protein levels of TNF- α , IL-6, iNOS, CD86 and PICK1 in PICK1 over-expressed M1 macrophages were assessed by Western blot. (D) IL-6 levels in the culture supernatants of over-expressed M1 macrophages were determined by ELISA. (E) The frequency of F4/80⁺iNOS⁺ cells in M1 macrophages were assessed by Flow cytometry with PE-A and APC-A staining. The values represent means $\pm$ SD(n = 3). ** P < 0.01 for M1 macrophages vs non-treated cells, ^{##} P < 0.01 for PICK1 over-expressed M1 macrophages vs control M1 macrophages. Similar results were conducted in three independent experiments.

Figure.5. Effect of PICK1-knockdown on murine M1 macrophage polarization. (A) Transfected RAW 264.7 cells were photographed by fluorescence microscope. Representative views were showed (original magnification, $\times 100$) (B) The efficiency of PICK1-knockdown in RAW 264.7 cells was detected by Western blot. (C) The mRNA levels of M1 biomarkers including IL-6, IL-1 β , IL-12, MCP-1, iNOS, CD86 were assessed by Real-time PCR. (D) The protein levels of TNF- α , IL-6, iNOS and PICK1 were assessed by Western blot. (E) The IL-6 levels in supernatants were determined by ELISA. (F) The frequency of F4/80⁺iNOS⁺ cells in M1 macrophages were assessed by Flow cytometry with PE-A and APC-A staining. The values represent means $\pm$ SD (n = 3). ** P < 0.01 for M1 macrophages vs non-treated cells, ^{##}

$P < 0.01$ for PICK1-siRNA transfected M1 macrophages vs scramble-siRNA transfected M1 macrophages. Similar results were conducted in three independent experiments.

Figure.6. Effect of PICK1 on murine M2 macrophage polarization. (A and B) PICK1-knockdown significantly reduced Arg-1 expression in M2 macrophages. (C) PICK1-knockdown significantly reduced IL-10 level in supernatant, which was determined by ELISA. (D) The frequency of Arg-1⁺ cells in M2 macrophages were assessed by Flow cytometry with PE-A staining. The values represent means $\pm$ SD ($n = 3$). (E and F) PICK1 over-expression resulted in an increase of Arg-1 expression in M2 macrophages. (G) PICK1 over-expression increased IL-10 level in supernatant, which was determined by ELISA. $^{**}P < 0.01$ for M2 macrophages vs non-treated cells, $^{##}P < 0.01$ for PICK1-siRNA transfected M2 macrophages vs scramble-siRNA transfected M2 macrophages. $^{###}P < 0.01$ for PICK1 over-expressed M1 macrophages vs control M1 macrophages. Similar results were conducted in three independent experiments.

Figure.7. Effects of FSC-231 on murine macrophage polarization. (A and B) The expressions of M1 biomarkers including IL-6, MCP-1, IL-1 β , iNOS, TNF- α in M1 macrophages following FSC-231 (50 μ mol/L) pretreatment were assessed by Real-time PCR and Western blot. (C) The mRNA level of Arg-1 in M2 macrophages following FSC-231 (50 μ mol/L) pretreatment was assessed by Real-time PCR. (D) The protein levels of Arg-1 and phospho-p38, phospho-STAT6 in M2 macrophages were assessed by Western blot. The values represent means $\pm$ SD ($n = 3$). $^{**}P < 0.01$ for M1/M2 macrophages vs non-treated cells, $^{##}P < 0.01$ for FSC-231 pretreated M1/M2 macrophages vs M1/M2 macrophages. Similar results were conducted in three independent experiments.

Figure.8. PICK1 suppressed M1 macrophage polarization via inhibiting NF- κ B activity. (A) The protein levels of phospho-p65 and phospho-I κ B α in plasmid or PICK1 siRNA transfected murine M1 macrophages were assessed by Western blot. The values represent means $\pm$ SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ for M1 macrophages vs non-treated cells, $^{#}P < 0.05$, $^{###}P < 0.01$ for PICK1-siRNA transfected M1

macrophages vs scramble-siRNA transfected M1 macrophages. (B) The protein levels of phospho-p65 and phospho-I κ B α in plasmid or PICK1 siRNA transfected murine M1 macrophages were assessed by Western blot. The values represent means $\pm$ SD (n = 3). * P < 0.05, ** P < 0.01 for M1 macrophages vs non-treated cells, # P < 0.05, ## P < 0.01 for PICK1 over-expressed M1 macrophages vs control M1 macrophages. (C) The effect of PDTC at different concentrations on the protein levels of TNF- α , IL6, CD86, phospho-p65, and PICK1 in M1 macrophages was determined by Western blot. The values represent means $\pm$ SD (n = 3). ** P < 0.01 for M1 macrophages vs non-treated cells, ## P < 0.01 for FSC-231 pretreated M1 macrophages vs M1 macrophages. Similar results were conducted in three independent experiments.

Figure.9. Involvement of caveolin-1 in PICK1-mediated M2 macrophage polarization by interacting with p38 and STAT-6. (A) The protein levels of phospho-p38 and phospho-STAT6 in PICK1 siRNA transfected murine M2 macrophages were assessed by Western blot. The values represent means $\pm$ SD (n = 3). * P < 0.05, ** P < 0.01 for M2 macrophages vs non-treated cells, # P < 0.05, ## P < 0.01 for PICK1-siRNA transfected M2 macrophages vs scramble-siRNA transfected M2 macrophages. (B) The protein levels of phospho-p38 and phospho-STAT6 in PICK1 plasmid transfected murine M1 macrophages were assessed by Western blot. The values represent means $\pm$ SD (n = 3). * P < 0.05, ** P < 0.01 for M2 macrophages vs non-treated cells, # P < 0.05, ## P < 0.01 for PICK1 over-expressed M2 macrophages vs control M2 macrophages. (C) The protein levels of Arg-1 and phospho-STAT-6, phospho-p38 in each group were assessed by Western blot. The values represent means $\pm$ SD (n = 3). ** P < 0.01 for scramble-siRNA transfected M2 macrophages vs non-treated cells, ## P < 0.01 for caveolin-1-siRNA transfected M2 macrophages vs scramble-siRNA transfected M2 macrophages. && P < 0.01 for PICK1-siRNA transfected M2 macrophages vs scramble-siRNA transfected M2 macrophages. Similar results were conducted in three independent experiments.

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Table1. Primer sequences for Real-time PCR

Gene	Forward Primer	Reverse Primer
TNF- α (mouse)	5'-TGTCCCTTTCACTCACTGGC-3'	5'-CATCTTTTGGGGGAGTGCCT-3'
IL-6(mouse)	5'-GAGGATACCACTCCCAACAGACC-3'	5'-AAGTGCATCATCGTTGTTCATACA-3'
IL-1 β (mouse)	5'-CCATGGCACATTCTGTTCAAA-3'	5'-GCCCATCAGAGGCAAGGA-3'
IL-12 (mouse)	5'-CCAAATTACTCCGGACGGTTCAC-3'	5'-CAGACAGAGACGCCATTCCACAT-3'
MCP-1 (mouse)	5'-TCAGCCAGATGCAGTTAACGC-3'	5'-TGATCCTCTTGTAGCTCTCCAGC-3'
iNOS (mouse)	5'-CCCTTCAATGGTTGGTACATGG-3'	5'-ACATTGATCTCCGTGACAGCC-3'
CD86 (mouse)	5'- TTGTGTGTGTTCTGGAAACGGAG -3'	5'-AACTTAGAGGCTGTGTTGCTGGG-3'
IL-10 (mouse)	5'-GCTGCCTGCTCTTACTGACT-3'	5'-CTGGGAAGTGGGTGCAGTTA-3'
Arg-1 (mouse)	5'-GAACACGGCAGTGGCTTTAAC-3'	5'-TGCTTAGCTCTGTCTGCTTTGC-3'
PICK1 (mouse)	5'-GCACACCGCAGCATTGAGA-3'	5'-AGGTACGTGTTCAGGTCCGTCAG-3'
GAPDH(mouse)	5'-GGACCTCATGGCCTACATGG-3'	5'-TAGGGCCTCTCTTGCTCAGT-3'
TNF- α (human)	5'-AAGCCTGTAGCCCATGTTGT-3'	5'-CAGATAGATGCGCTCATACC-3'
IL-6 (human)	5'-CACTCACCTCTTCAGAACGA-3'	5'-CTGTTCTGGAGGTACTCTAGG-3'
PICK1(human)	5'-CCTGCCTCTATATCGTCCAGGTA-3'	5'-ATCGCCAGCTGCTGCCACTGT-3'
β -actin (human)	5'-CCCACACTGTGCCCATCTACG-3'	5'-GCCATCTCTTGCTCGAAGTCC-3'

Figure.1

Figure.2

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