Proteomic analysis and identification reveal the anti-inflammatory mechanism of clofazimine on lipopolysaccharide-induced acute lung injury in mice

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Abstract
Background and objective Acute lung injury (ALI)/ acute respiratory distress syndrome (ARDS) was increasingly recognized as one of the most severe acute hyperimmune response of coronavirus disease 2019 (COVID-19). Clofazimine (CFZ) has attracted attention due to its anti-inflammatory property in immune diseases as well as infectious diseases. However, the role and potential molecular mechanism of CFZ in anti-inflammatory responses remain unclear.

Methods We analyze the protein expression profiles of CFZ and LPS from Raw264.7 macrophages using quantitative proteomics. Next, the protective effect of CFZ on LPS-induced inflammatory model is assessed, and its underlying mechanism is validated by molecular biology analysis.

Results LC–MS/MS-based shotgun proteomics analysis identified 4746 (LPS) and 4766 (CFZ) proteins with quantitative information. The key proteins and their critical signal transduction pathways including TLR4/NF-κB/HIF-1α signaling was highlighted, which was involved in multiple inflammatory processes. A further analysis of molecular biology revealed that CFZ could significantly inhibit the proliferation of Raw264.7 macrophages, decrease the levels of TNF-α and IL-1β, alleviate lung histological changes and pulmonary edema, improve the survival rate, and down-regulate TLR4/NF-κB/HIF-1α signaling in LPS model.

Conclusion This study can provide significant insight into the proteomics-guided pharmacological mechanism study of CFZ and suggest potential therapeutic strategies for infectious disease.

Keywords Clofazimine · Acute lung injury · Anti-inflammatory · Lipopolysaccharide · Proteomics · Acute respiratory distress syndrome

Bo Yang, Zhan Gao, Qi-Shuang Li, Xiang-Ye Zhang have contributed equally to this work and share first authorship.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a highly infectious RNA virus, is responsible for coronavirus disease 2019 (COVID-19) [1], which has caused more than 5.8 million deaths (February 2022, Johns Hopkins University Coronavirus Resource Center) in modern history so far. The target of SARS-CoV-2 primarily infects the respiratory tract epithelial cells and activates the immune system, in turn, leading to cytokine storm (CS) and causing fever, cough, breathlessness, etc. [2]. In some cases, 10–20% of patients with COVID-19 might also develop acute lung injury (ALI) or progress to acute respiratory distress syndrome (ARDS) and fatal multi-organ dysfunction (MODS) and even death [3, 4].

Current management of severe COVID-19 focus on symptomatic and supportive treatment [3, 4]. Aside from combination therapy, there is no widely available antiviral therapies for severe pneumonia. Thus, it is in critical need to develop additional therapeutic strategies for COVID-19. Recently, clofazimine (CFZ) has raised some interest, which simultaneously inhibits viral activity and alleviates the symptoms in COVID-19 patients [5]. Previously, it has been used in treating leprosy, and more recently in multidrug-resistant tuberculosis [6, 7]. Accumulated evidence points out that CFZ displays an important immunomodulatory activity and anti-inflammatory property in immune disease, including discoid lupus erythematosus, chronic lymphocytic leukemia, Crohn disease, ulcerative colitis, etc. [5, 8–11]. In addition, it is anti-bacteria by generating reactive oxygen species (ROS), particularly superoxide and hydrogen peroxide (H₂O₂) [12, 13]. Up to now, despite the aforementioned interesting findings in the literature, there is still much to be understood about the role of CFZ in ALI/ARDS.

Lipopolysaccharide (LPS)-induced inflammatory responses are an ideal model closely resembling ALI/ARDS [14–16]. Raw264.7 macrophage, a critical immunological cell, plays a hub role in the pathogenesis of ALI/ARDS [17]. Here, the first proteomic study is presented to assess the effects of CFZ on Raw264.7 macrophage cells and validate the mechanisms involved in the anti-inflammatory effect in LPS-induced ALI/ARDS model. In this study, a rich resource for data mining and guidance for clinical validation is provided. Data analysis and experimental research workflow is detailed in Fig. 1.

Materials and methods

Cell and reagents

Raw264.7 macrophage cell lines were acquired from the Beijing Proteome Research Center, National Center for Protein Sciences (Beijing, China). Fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM), penicillin and streptomycin were purchased from Thermo Fisher Scientific. Lipopolysaccharide (LPS) from Escherichia coli 0111: B4 (in vitro) and O55: B5 (in vivo) were purchased from Sigma-Aldrich. Clofazimine (CFZ, Fig. 2a) was purchased from the Cayman Chemical. Cell Counting Kit-8 (CCK-8) was obtained from Gene-Protein Link. Mouse TNF-α, and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Sigma-Aldrich. COX-2, Aim 2, TLR4, NF-κBp65, p-NF-κBp65 and HIF-1α were purchased from Cell Signaling Technology. All other chemicals were of reagent grade.

Cell culture

Raw264.7 cells were grown in culture media (DMEM), supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were cultured at 37 °C in a 5% CO₂ humidified incubator, and grown to 80% confluence before use.

CCK-8 assay

A CCK-8 test kit was used according to the manufacturer’s instructions. Briefly, Raw264.7 cells were inoculated into 96-well plates at a density of 6 × 10³ per well for 24 h. Then, the cells were treated with CFZ and (or) LPS for 24 h. 10% of CCK-8 solution was added into the well and incubated for 1 h at 37 °C away from light. The absorbance was measured at 450 nm by a microplate reader (Bio-Rad) to assess the half-maximal inhibitory concentration (IC₅₀) value and cell proliferation.

Sample preparation for proteomics analysis

First, cells were plated in triplicate wells in six-well plates. After adherence, CFZ (4 μM) or LPS (100 ng/ml) were added to cells and incubated at 37 °C for 0 h, 2 h, 8 h, and 24 h. Then, samples were lysed in lysis buffer supplemented with phosphatase inhibitors and incubated at 95 °C for 15 min. After sonication, the lysates were centrifuged at 16,000 × g at 4 °C for 10 min and the supernatants were collected as whole cell extract. Next, protein concentration was determined by NanoDrop (Thermo Fisher Scientific).
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Fig. 1 The general workflow of quantitative proteomics and molecular biology analysis
Subsequently, samples (100 μg per sample) were digested with trypsin (2 μg per sample) overnight at 37 °C. Then, peptides were eluted, separated and desalted into fractions. Finally, the samples were vacuum-centrifuged to dryness (Thermo Fisher Scientific), and used for liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis.

Subsequently, peptides were analyzed on an Orbitrap Q-Exactive HF Mass Spectrometer (Thermo-Fisher Scientific) operating in the data-dependent acquisition (DDA) mode. The Proteome Discoverer (Thermo Scientific) was used to analyze the raw MS files against the mouse refseq protein database. Enzyme specificity was set to trypsin. The search set none as a fixed modification, while acetylation (Protein N-term), oxidation (M), and carbamidomethylation (C) were considered variable modifications. Charges of precursor ion were limited to +2, +3, and +4. The protein quantification was represented in intensity-based absolute quantification (iBAQ).

Bioinformatics analysis

Proteins with a probability p value < 0.05, fold change > 2 or < 0.5 and false discovery rate (FDR) < 0.01 were chosen as the mostly affected proteins. Then, the expression pattern of six clusters of dynamic proteins were analyzed by Mfuzz (Kumar and Futschik, 2007). Hierarchical cluster and heatmap analysis of differentially expressed proteins were performed using the ward.D's hierarchical clustering and heatmap function in the R package. The volcano plots were visualized in Hiplot analysis with the website (https://hiplot.com.cn/advance). Enrichment analysis using Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), and the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/home.jsp) were available. Correlation analysis was carried out using Pearson's correlation. Enrichment p values in GO, KEGG, and correlation analysis less than 0.05 were considered significantly enriched.

ELISA assay

RAW 264.7 macrophage cells were cultured in six-well plates, and four groups were set including control group, LPS group, CFZ group, LPS + CFZ group. The cells were then treated with freshly prepared Escherichia coli LPS (100 ng/ml) and CFZ (4 μM) for 24 h. Control group was treated with equivalent endotoxin-free PBS. According to manufacturer’s protocol, cell free supernatant was obtained after 24 h incubation, and enzyme-linked immunosorbent assay (ELISA) was employed to determine the levels of TNF-α and IL-1β.

LPS-induced ALI/ARDS model in mice

Six-week-old female C57BL/6 J mice (weighing 20–22 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. They were housed in pathogen-free cages with free access to food and water with a 12-h light/dark schedule at 22 ± 2 °C. All study protocols were accredited by the Institutional Animal Care and Use Committee of National Center for Protein Sciences (Beijing, China) (IACUC-20210702-26MT).

Mice were randomly assigned to the following groups (n = 5 per group):

- Control group: PBS was administered intraperitoneally (i.p.) without LPS stimulation.
- LPS group: Mice were stimulated with LPS (20 mg/kg).
- CFZ group: Mice were administered intragastrically with CFZ (25 mg/kg). CFZ was suspended in a 0.05% (w/v) agarose solution.
- LPS + CFZ group. Mice in this group were stimulated with LPS and then treated with CFZ.

All mice were euthanized (i.p.; 80 mg/kg sodium pentobarbital) 8 h after the last challenge. The lung tissues were quickly removed for further analyses.

Histological analysis

Lung tissues were collected, fixed and embedded in paraffin. Subsequently, the tissues were serially sectioned into 4 μm thickness, stained with hematoxylin and eosin (H&E) and then mounted in neutral gum. Lung injury scores were based on the infiltration of neutrophils (0–4), alveolar oedema (0–4), hyperemia or congestion (0–4) and intra-alveolar hemorrhage, necrosis (0–4). The severity of lung injury was scored as: 0 = normal; 1 = minimal (<5%); 2 = mild (<10%); 3 = moderate (15 - 20%); and 4 = severe (>25%).
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Statistical analysis

Data were evaluated using GraphPad Prism 8.3.0 (GraphPad Software, La Jolla, CA). Differences between the groups were performed with Student’s t test or one-way ANOVA method. A p value < 0.05 was set at statistically significant.

Results

In vitro study

Effect of CFZ on CCK-8 assay

First, after 24 h-incubation, the cytotoxic effect of CFZ on Raw264.7 cell lines by CCK-8 assay after treatment was estimated with different doses. The results showed that IC_{50} of CFZ in the Raw264.7 cells was 4.1 μM (Fig. 2b). The non-toxic concentrations (4 μM) were used for the following experiment. Then, after different concentrations (0.05, 0.1, 0.25, 0.5, 1, 10 and 100 μg/mL) of LPS were applied, the viability of cells remained the same after treatment with 100 μg/mL of LPS compared to the control group (Fig. 2c). Therefore, 100 ng/mL was selected as the optimal concentration of LPS for activating the macrophages. Additionally, the results of CCK-8 assay also revealed that LPS promoted the viability of Raw264.7 cells, while CFZ could significantly reduce the proliferation of Raw264.7 cells (Fig. 2d).

A dynamic protein expression landscape of CFZ and LPS

To study the damage of LPS and CFZ on the Raw264.7 cells, shotgun proteomics was utilized to discover the alteration of protein expression patterns. 4746 and 4766 proteins were identified with quantitative information in this study (Fig. 2e). Then, the correlation between LPS and CFZ proteomic data in each timepoint (0 h, 2 h, 8 h, and 24 h) was analyzed by Mfuzz. In total, it is found that six distinct clusters of temporal patterns representing proteins are regulated differently, indicating different expression kinetics. Among the 4746 proteins of LPS, clusters 1 represents up-regulated proteins, clusters 2 and 3 represent down-regulated proteins, whereas clusters 4, 5, and 6 represent proteins displaying a bimodal expression pattern (Fig. 3a, b; Supplementary Table S1). Among the 4766 proteins of CFZ, clusters 1 and 3 represent proteins are down-regulated, clusters 4 and 5 represent proteins are up-regulated, whereas clusters 2 and 6 represent proteins displaying a bimodal expression pattern (Fig. 3c, d; Supplementary Table S2). By overlaying Mouse Genome Informatics (MGI, http://www.informatics.jax.org.

Fig. 3 Temporal profiles of protein expression in each timepoint (0 h, 2 h, 8 h, and 24 h) in Raw264.7 macrophage cells. a Mfuzz clustering identified six distinct temporal patterns of proteins expression of LPS. b The left pie chart shows the number of proteins of LPS in six dynamic expression clusters in (b), the right pie chart shows the number of inflammatory proteins of LPS in six dynamic expression clusters in (b). c Mfuzz clustering identified six distinct temporal patterns of proteins expression of CFZ. d The left pie chart shows the number of proteins of CFZ in six dynamic expression clusters in (d), the right pie chart shows the number of inflammatory proteins of CFZ in six dynamic expression clusters in (d). e Venn diagram shows the opposite expression patterns of 30 inflammatory proteins identified by LPS (green) and CFZ (purple) comparisons. f KEGG pathway analysis of opposite protein clusters

Pulmonary wet-to-dry ratio

The lung tissue from each animal was harvested and placed onto pre-weighed tin foil to measure the wet weight (W). The tissue was then placed in an incubator at 37 °C for 48 h and obtained dry weight (D). The W/D ratio of the lung tissue was calculated.

Survival rates and weight of mice

Survival rates were studied in another set of experiments: 30 mice were randomly divided into 3 groups [Control group; LPS (60 mg/kg) group; LPS + CFZ (25 mg/kg) group] as described above (n = 10 per group). Mice were weighed every 24 h.

Western blot analysis

Lung tissues were washed three times with ice-cold PBS and lysed in RIPA lysis buffer. Protein concentration was determined using the BCA assay (Thermo Fisher Scientific). Samples were run using 10% sodium dodecyl sulfate—polyacrylamide gels (PAGE) and electro-transferred onto nitrocellulose membranes (Bio-Rad). After blocking in 5% non-fat dry milk in TBST at room temperature for 1 h, the membranes were washed and immunoblotted with primary antibodies COX-2 (1:1000, #12,282), TLR4 (1:1000, #14,358), NF-κBp65 (1:1000, #8242), p-NF-κBp65 (1:1000, #3033), AIM2 (1:1000, #63,660), HIF-1α (1:1000, #14,358), NF-κBp65 (1:1000, #8242), p-NF-κBp65 (1:1000, #3033), AIM2 (1:1000, #63,660), HIF-1α (1:1000, #14,358), NF-κBp65 (1:1000, #8242), p-NF-κBp65 (1:1000, #3033), AIM2 (1:1000, #63,660). Enhanced chemiluminescence reagent (ECL) detection system (Thermo Fisher Scientific) was used to detect immunoreactive bands. All acquired images were calculated by Image J (v1.53e).
obtained from the LPS dataset. (Fig. 4b; Supplementary Table S3). The enrichment of the KEGG analysis of CFZ suggested that cluster 1 is mainly enriched for four time points, 0, 2, 8, and 24 h, whereas it displayed a distinguished pattern between each time point (i.e., three samples per treatment, LPS and CFZ enriched for four time points, 0, 2, 8, and 24 h, p < 0.05; Fig. 4a). To gain better insight into the difference of protein expression profiles of LPS and CFZ (0 h, 2 h, 8 h, and 24 h), we performed the degree of differential proteins in each sample by hierarchical clustering and heatmap. To reveal the molecular mechanism of proteins in each cluster and understand their biological significance, KEGG pathway analysis were performed on differentially expressed proteins in the LPS and CFZ dataset (ANOVA, FDR < 0.01, p < 0.05). The LPS dataset in cluster 1 mainly involves ribosome, spliceosome, RNA transport, biosynthesis of antibiotics, and phagosome. Cluster 2 proteins participate in systemic lupus erythematosus, spliceosome, alcoholism, viral carcinogenesis, and proteasome. Cluster 3 is enriched in herpes simplex infection, viral carcinogenesis, ubiquitin-mediated proteolysis, alcoholism, spliceosome. Intriguingly, the KEGG analysis of CFZ suggest that cluster 1 is mainly enriched in pathways including biosynthesis of antibiotics, spliceosome, RNA transport, carbon metabolism, and metabolic pathways. Cluster 2 is involved in systemic lupus erythematosus, alcoholism, carbon metabolism, biosynthesis of antibiotics, and viral carcinogenesis. Cluster 3 is mainly associated with spliceosome, biosynthesis of antibiotics, metabolic pathways, carbon metabolism, and citrate cycle (TCA cycle). Overall, the CFZ clustering analysis revealed that KEGG pathway has similar biological features to those obtained from the LPS dataset. (Fig. 4b; Supplementary Table S4 and Table S5).

### Hierarchical cluster analysis of the protein identification and functions

Principal component analysis (PCA) results showed that each sample within treatment groups shared a similar expression pattern, whereas it displayed a distinguished pattern between each time point (i.e., three samples per treatment, LPS and CFZ enriched for four time points, 0, 2, 8, and 24 h, p < 0.05; Fig. 4a). To gain better insight into the difference of protein expression profiles of LPS and CFZ (0 h, 2 h, 8 h, and 24 h), we performed the degree of differential proteins in each sample by hierarchical clustering and heatmap. To reveal the molecular mechanism of proteins in each cluster and understand their biological significance, KEGG pathway analysis were performed on differentially expressed proteins in the LPS and CFZ dataset (ANOVA, FDR < 0.01, p < 0.05). The LPS dataset in cluster 1 mainly involves ribosome, spliceosome, RNA transport, biosynthesis of antibiotics, and phagosome. Cluster 2 proteins participate in systemic lupus erythematosus, spliceosome, alcoholism, viral carcinogenesis, and proteasome. Cluster 3 is enriched in herpes simplex infection, viral carcinogenesis, ubiquitin-mediated proteolysis, alcoholism, spliceosome. Intriguingly, the KEGG analysis of CFZ suggest that cluster 1 is mainly enriched in pathways including biosynthesis of antibiotics, spliceosome, RNA transport, carbon metabolism, and metabolic pathways. Cluster 2 is involved in systemic lupus erythematosus, alcoholism, carbon metabolism, biosynthesis of antibiotics, and viral carcinogenesis. Cluster 3 is mainly associated with spliceosome, biosynthesis of antibiotics, metabolic pathways, carbon metabolism, and citrate cycle (TCA cycle). Overall, the CFZ clustering analysis revealed that KEGG pathway has similar biological features to those obtained from the LPS dataset. (Fig. 4b; Supplementary Table S4 and Table S5).

### Essential pathways of CFZ in proteome

The distribution of proteins is identified at each time point (0, 2, 8, and 24 h), then they are represented in Venn diagrams (FDR < 0.01, p < 0.05, fold change > 2 or fold change < 0.5; Fig. 5a). Notably, by comparing the expression levels of total proteins, the differentially expressed proteins (DEPs) at each time point (0, 2, 8, and 24 h) were screened (FDR < 0.01, p < 0.05, fold change > 2 or fold change < 0.5; Fig. 5b). Volcano plots is a scatter graph used to highlight the up-regulated, down-regulated proteins and spatial occurrence in large datasets. As shown in Fig. 5c, the great alterations in the Raw264.7 proteome were induced by LPS and CFZ at the 24 h time point, where 1203 (Supplementary Table S6) and 1332 (Supplementary Table S7) proteins showed significantly changed expression compared to controls, respectively.

To extract molecular mechanisms of proteome from the 1332 DEPs of CFZ, gene ontology (GO) and KEGG enrichment analysis (Fig. 6) were used. The top enriched categories of the biological process (BP) were related to generation of precursor metabolites and energy, aerobic respiration, cellular respiration, energy derivation by oxidation of organic compounds, ATP metabolic process, oxidative phosphorylation, carboxylic acid catabolic process, organic acid catabolic process, nucleotide metabolic process, and purine-containing compound metabolic process. The cellular component (CC) terms of the DEPs were mitochondrial matrix, mitochondrial protein-containing complex, mitochondrial inner membrane, organelle inner membrane, oxidoreductase complex, myelin sheath, organellar ribosome, mitochondrial ribosome, inner mitochondrial membrane protein complex, and respiratory chain complex. The molecular function (MF) terms of the DEPs were NAD binding, structural constituent of ribosome, electron transfer activity, isomerase activity, ribonucleoprotein complex binding, iron-sulfur cluster binding, metal cluster binding, ATP hydrolysis activity, primary active transmembrane transporter activity, and ligase activity. The enrichment of the KEGG pathway included carbon metabolism, oxidative phosphorylation, fatty acid metabolism, HIF-1 signaling pathway, and lysosome.

### CFZ inhibits TLR4/NF-κB/HIF-1α pathway and IL-1β and TNF-α release

NF-κB pathway has been defined as a key participant in LPS infections [18, 19]. Meanwhile, hypoxia-inducible factor-1α (HIF-1α) is considered one of endogenous ROS’s core cellular targets, which further regulates the hypoxia signaling in the inflammatory response [20]. To
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Fig. 4 Principal component analysis of temporal proteomic data and their biological functions. **a** PCA analysis of LPS and CFZ protein. **b** Hierarchical cluster and heatmap show significant values of KEGG pathway describing each of clusters.
further correlate CFZ with inflammation on the levels of transduction pathways, key proteins involved in the pathways implicated in TLR4/NF-κB/HIF-1α signaling pathway were listed (Fig. 7a). Among these protein expressions, there were better correlation in TLR4/NF-κB/HIF-1α transduction pathway (Fig. 7b).

Next, TNF-α and IL-1β expression was measured in the culture supernatants of Raw264.7 cells. It can be seen from the results that compared to the control group, the levels of TNF-α and IL-1β were significantly increased with LPS exposure alone. However, when treated with CFZ, the levels of TNF-α and IL-1β in LPS + CFZ group were markedly reduced compared with the LPS group (Fig. 8a).

**In vivo study**

**Effect of CFZ on LPS-induced lung parenchyma**

As CFZ treatment significantly improved the pro-inflammatory cytokines, we examined whether this treatment reduced lung injury in mice. Intact structure and clear pulmonary alveoli in both control group and CFZ group of mice were observed. Following LPS-induced injury, the histological examination showed an increase in the areas of inflammatory infiltration, collapse of air alveoli, and pulmonary congestion. However, CFZ markedly attenuated the LPS-induced pathological changes (p < 0.05) (Fig. 8b).

**Effect of CFZ on the W/D ratio**

Lung edema was a predictive marker of the exudative phase of ALI/ARDS [21, 22]. We evaluated it by W/D lung weight ratio. After 8 h of LPS administration, a marked increase was observed in the average wet/dry ratio of lung tissue compared with the control group; however, the LPS + CFZ group, in particular, exhibited a significantly lower level of W/D ratio than the LPS group (p < 0.05) (Fig. 8c).

**Effect of CFZ on survival rate and body weight loss of mice challenged with a lethal dose of LPS**

The therapeutic effect of CFZ was evaluated on mice challenged with the lethal dose of the LPS (Fig. 8d, e). The survival rate of the mice in LPS group was markedly lower than that in the control group, while the survival rate in the LPS + CFZ group was distinctly higher than that in the LPS group during 72 h observation (p < 0.05). These results showed that CFZ could improve the survival rate of LPS-challenged mice (Fig. 8d). The effects of CFZ were further validated on LPS-induced ALI in mice. Our results suggested that on day 3, mice in the normal group increased a considerable weight, compared to day 0. Mice began to lose weight on day 1 and all weight loss on day 3 following LPS injury. Treatment of CFZ effectively improved the loss and presented a slight weight increase on day 3, compared to LPS group (p < 0.05).

**Effect of CFZ on LPS-induced COX-2 and AIM 2 expression**

The activation of COX-2 and AIM 2 were detected by western blot analysis to investigate the mechanism underlying the anti-inflammatory effect of CFZ. As expected, LPS application dramatically increased COX-2 and AIM 2 expression compared with the control group. However, compared with the LPS group, CFZ markedly decreased the protein expression (Fig. 9a).

**Effect of CFZ on LPS-induced TLR4/NF-κB/HIF-1α expression**

The protein expression levels of CFZ on TLR4/NF-κB/ HIF-1α pathway were assessed for further investigating the anti-inflammatory mechanisms of CFZ. As a result (Fig. 9b), the protein levels of three essential proteins, including TLR4, NF-κBp65 and HIF-1α were significantly enhanced by western blot analysis to investigate the mechanism underlying the anti-inflammatory effect of CFZ. As expected, LPS application dramatically increased COX-2 and AIM 2 expression compared with the control group. Conversely, CFZ decreased the expression of these three essential proteins in LPS + CFZ group (p < 0.05).

**Discussion**

Today, while most COVID-19 cases are asymptomatic or cause only mild symptoms after SARS-CoV-2 infection, 10–20% of the patients develop severe pneumonia and ARDS [23]. ALI is increasingly recognized as one of the most severe acute hyperimmune responses of COVID-19, which can be manifested as dyspnea and gradually develop into ARDS [24]. Numerous studies have also been reported that several conditions, including infection, trauma, or inflammatory pathways are associated with ALI/ARDS [25, 26]. Hence, obtaining an additional therapeutic strategy of ALI/ARDS remains a significant challenge. Many CFZ-related articles focus on its antibiotic function, like treatment for tuberculosis, leprosy, and Salmonella enterica, but anti-inflammatory effect has not yet been elucidated. With this in mind, this study aims to fill this gap in the literature. Here, our results have demonstrated the anti-inflammatory roles of CFZ in both Raw264.7 cells and the mice model. Proteomics has become the mainstay in systematically studying the expression, function, and interaction of all proteins in cells [27]. This technology can reveal the entire
Fig. 6 KEGG and Gene Ontology biological process analysis of the differentially expressed proteins of CFZ. a Top 50 KEGG pathways with the lowest adjusted p values. b Top ten significantly enriched terms in biological processes (BPs), cellular components (CCs), and molecular functions (MFs).
protein change spectrum under specific biological processes and the mechanisms of drugs on the changes of the whole proteome [28]. The researchers performed a comprehensive proteomic analysis to study the damage of LPS and CFZ on the Raw264.7 cells, and they used bioinformatic interpretation to identify the potential alteration of proteins of LPS and CFZ. As a result, 4746 (LPS) and 4766 (CFZ) proteins were identified and contained quantitative information in this study. In addition, mainly 29 proteins showed a low expression level in clusters 1 and 3 of CFZ and a high expression level in clusters 1 of LPS, overlaying MGI containing primarily inflammatory response proteins. As a result, the expression levels of IRPs including SYK, GSDMD, CELF1, NFκB1, MYD88, AIM 2, PTPN2, STAT5A, IL16, NCF1, PIK3AP1, DDX3X, RIPK1, FCGR1, COX-2, etc. Studies have revealed the potential roles of SYK, PTPN2, STAT5A, IL-16, NCF1, PIK3AP1, and FCGR1 in different inflammatory diseases and autoimmune pathologies [29–35]. Additionally, GSDMD, AIM 2, and COX-2 have recently been identified as a key role in the pathogenesis of sepsis and COVID-19 [36–38]. Cel5 is known as an RNA-binding protein [39]. Hu et al. found that Celf1-deficiency could improve oxidative stress and apoptosis in cardiomyocytes [40]. NFκB1 (p50) is one of the five members of the NF-κB family, which is IkB kinase-like protein [41]. MYD88 is implicated in inflammatory signaling downstream of IL-1R and mammalian TLRs [42]. Recently, a plethora of studies have implicated that massive release of TNF-a, IFN-g, IL-1b, IL-8, MCP-1, and IP-10 seen in ALI/ARDS may probably be linked to NLRP3, which is pivotal involved in TCA cycle, fatty acid metabolism, HIF-1 signaling pathway, and lysosome. The importance of the carbon metabolism, oxidative phosphorylation, TCA cycle, fatty acid metabolism, HIF-1 signaling pathway, and lysosome in physiology and pathophysiology has now been confirmed by many experimental studies in LPS models. For instance, when Raw 264.7 cells were exposed to LPS, several inflammatory reactions could be inhibited by the low doses of carbon monoxide (CO) or endogenous CO [46]. HIF-1α is induced in LPS-activated macrophages, where it is pivotal involved in TCA cycle and glycolytic metabolites as well as the induction of inflammatory response, notably IL-1β. As the mechanisms for CFZ-mediated macrophage protein expression are poorly understood, then key proteins involved in the signaling pathways implicated in TLR4/ NF-κB/HIF-1α signaling pathway were listed in this study according to KEGG pathway analysis. Among these protein expressions, there was better correlation in this signaling pathway. These observations suggest CFZ exerted anti-inflammatory activity via TLR4/NF-κB/HIF-1α pathway in macrophages.

Raw264.7 macrophage, an important immunological cell, plays a major role in regulating pro-inflammatory chemokines, cytokines, and mediators in the pathogenesis of ALI/ARDS [47]. Our experiments demonstrated that LPS promoted the viability of Raw264.7 cells, while CFZ could significantly decrease the proliferation of Raw264.7 macrophages. We further evaluated the anti-inflammatory effect of CFZ in Raw264.7 cells models. It was reported that targeting pro-inflammatory cytokines (such as TNF-α and IL-1β) was one of the most promising treatments against inflammatory response during inflammatory diseases [48].

The above PCA analysis showed that each sample within treatment groups shared a similar expression pattern, whereas displayed a distinguished pattern between each time point. Based on hierarchical clustering and heatmap, the CFZ clustering analysis revealed KEGG pathway with similar biological features to those obtained from the LPS clustering. Our results also showed that CFZ and LPS play a very similar inflammatory and immune function in Raw264.7 cell model.

To ensure the accuracy and reliability of our results, proteins, which had FDR < 0.01, p < 0.05, and fold change > 2 or fold change < 0.5, in each time point (2, 8, and 24 h) of LPS and CFZ compared with 0 h, was considered as significantly expressed proteins. Strikingly, using this cutoff, after 24 h of CFZ exposure in Raw264.7 macrophages, a total of 1332 proteins were significantly changed, including 405 up-regulated and 927 down-regulated. After 24 h of LPS exposure in Raw264.7 macrophages, a total of 1203 proteins were significantly changed, of which 537 were up-regulated and 666 down-regulated. As most of the inflammatory pathological features of Raw264.7 macrophages develop within this time, we focused on the phase (24 h) of the 1332 proteins of CFZ. KEGG pathway analysis showed that DEPs of CFZ were mainly involved in the pathways related to the carbon metabolism, oxidative phosphorylation, TCA cycle, fatty acid metabolism, HIF-1 signaling pathway, and lysosome. The importance of the carbon metabolism, oxidative phosphorylation, TCA cycle, fatty acid metabolism, HIF-1 signaling pathway, and lysosome in physiology and pathophysiology has now been confirmed by many experimental studies in LPS models. For instance, when Raw 264.7 cells were exposed to LPS, several inflammatory reactions could be inhibited by the low doses of carbon monoxide (CO) or endogenous CO [46]. HIF-1α is induced in LPS-activated macrophages, where it is pivotal involved in TCA cycle and glycolytic metabolites as well as the induction of inflammatory response, notably IL-1β. As the mechanisms for CFZ-mediated macrophage protein expression are poorly understood, then key proteins involved in the signaling pathways implicated in TLR4/ NF-κB/HIF-1α signaling pathway were listed in this study according to KEGG pathway analysis. Among these protein expressions, there was better correlation in this signaling pathway. These observations suggest CFZ exerted anti-inflammatory activity via TLR4/NF-κB/HIF-1α pathway in macrophages.

Raw264.7 macrophage, an important immunological cell, plays a major role in regulating pro-inflammatory chemokines, cytokines, and mediators in the pathogenesis of ALI/ARDS [47]. Our experiments demonstrated that LPS promoted the viability of Raw264.7 cells, while CFZ could significantly decrease the proliferation of Raw264.7 macrophages. We further evaluated the anti-inflammatory effect of CFZ in Raw264.7 cells models. It was reported that targeting pro-inflammatory cytokines (such as TNF-α and IL-1β) was one of the most promising treatments against inflammatory response during inflammatory diseases [48].

Our study demonstrated that CFZ inhibited TNF-α and IL-1β production in the Raw264.7 cells caused by LPS. Similar to Yoon et al., they also reported that CFZ decreased TNF-α
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Production and boosted IL-1RA secretion after treatment with LPS in macrophages [49].

Animal models can provide highlight ways in the complex pathophysiology of human disease. Therefore, in the present study, LPS model was also applied to examine the effect of CFZ on lung and lethality in mice. In our work, it is demonstrated that CFZ treatment decreased the severity of LPS-induced lung injury by improving the inflammatory infiltration into the lung. Previous studies have shown that pulmonary edema is the hallmark of ALI/ARDS in LPS model [25, 26]. Alveolar epithelial barrier dysfunction in response to inflammatory reaction results in lung edema in ALI/ARDS [50]. Our results also suggest that CFZ significantly attenuated the lung edema. LPS challenge contributed to a 40% survival rate within 72 h, while administration of CFZ improved the survival rate to 80%. Besides, a significant deterioration of weight loss for the first 2 days was observed, followed by weight recovery in mice treatment with CFZ compared to mice receiving LPS. The significance of TLR4/NF-κB/HIF-1α in LPS-induced ALI/ARDS model has been previously reported. Subsequently, we further investigated the anti-inflammatory effect of CFZ on TLR4/NF-κB/HIF-1α
pathway in LPS-induced lung injury mice. COX-2, an evolutionary enzyme, plays a central role in controlling host defense response to viral and bacterial infections [51]. Several lines of evidence demonstrated that COX-2 inhibitor is one of the major targets in treating ALI model induced by LPS in mice [52]. AIM 2, which can be expressed in the cytosol, is a member of innate immune sensors. During the inflammatory response, expression levels of COX-2 and AIM 2 are related to the NF-κB pathway [53]. Recent studies have alluded to the fact that the NF-κB and HIF crosstalk. For instance, macrophages or mice infected with LPS signaled through NF-κB and promoted the inflammatory cytokine to modulate HIF-1α expression. We found that LPS activates the expression of key proteins involved in TLR4/NF-κB/HIF-1α signaling, including the TLR4, NF-κB, and HIF-1α, as well as inflammatory enzyme (COX-2) and the inflammasome (AIM 2), and its stimulation was reversed through CFZ. Taken together, these results demonstrated the strong anti-inflammatory effects of CFZ on LPS-induce lung injury.

**Conclusion**

In summary, this project revealed that CFZ markedly inhibits LPS-challenged inflammatory response in vitro and in vivo. Through performing a systematically
proteomic analysis on Raw264.7 macrophage, the progressive alteration of protein expression of LPS and CFZ dataset has been explored. Our LPS model demonstrated that CFZ decreases the level of pro-inflammatory cytokine, attenuates lung injury and pulmonary edema, and reduces the lethality of septic shock. In particular, our results show that TLR4/NF-κB/HIF-1α is a critical signaling of CFZ-mediated anti-inflammatory responses. Thus, the study of quantitative proteomic data reveals valuable information to understand the anti-inflammatory effects of CFZ and provide potential therapeutic strategies for infectious diseases.

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Declarations
Conflict of interest None of the authors have any conflicts of interest to declare.

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